

Video Article

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

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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.

Video Link

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Introduction

Viral replication fitness is defined as the capacity of a virus to produce infectious progeny in a given environment¹ and is an important contributing factor in determining the prevalence of a virus variant at the population level over time². 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 populations³⁻⁶. 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 culture⁹.

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 recombinants¹⁴, 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 gene¹⁵ 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 results⁹.

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 clones⁶⁻⁸ 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* sequence²³ 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 fitness from slight to significantly lower than the prototype virus. The T242N mutation was previously reported to have a moderate fitness cost²⁴⁻²⁶, similar to the result shown in this study. The fitness cost of the other two mutations had not been reported previously.

Protocol

NOTE: 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

- 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.
 - 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/>).
- Use PCR²⁷ 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 of DNA sample carrying insert region. Add dH₂O to a final volume of 50 µl.
- 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.
- Take 5 µl of the PCR products from the previous step and run agarose gel electrophoresis²⁸.
 - 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 system²⁸.
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.
- 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.
 - 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)

- Use purified PCR products from step 1.1.5 as PCR primers. Use pNL4-3VifA²⁰ as template DNA in one PCR reaction and use pNL4-3VifB²⁰ 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.
- Add 10 U of *DpnI* to 50 µl of the PCR reaction and incubate at 37 °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*.
- Use *DpnI* digested product to transform competent bacterial cells. Use heat-shock reformation 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.
 - 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 O/N.
 - Use QIAprep Spin Miniprep kit to isolate plasmid DNA from the bacterial liquid culture, according to the manufacturer's protocol.

4. Use double restriction digestion²⁹ 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. Digest at least 300 ng of plasmid DNA in a 10 μ l final reaction volume. Select restriction buffers, incubation temperature and incubation time according to the manufacturer's protocol of the selected restriction enzymes. Take 9 μ l 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.
5. Confirm sequence integrity of the recombinant plasmids by Sanger sequencing. While rare, unwanted mutation(s) can be introduced during the PCR reactions.
 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.
 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.
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 μ g of endotoxin-free plasmid DNA for transfection in the following step.

1.3) Introduce Small-scale Mutations Via Site-directed Mutagenesis

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.
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 μ M of forward mutagenic primer, 0.5 μ M of reverse mutagenic primer and 50 ng of chimeric pNL4-3VifB, from step 1.2.6, in a final volume of 50 μ l. Set the thermal cycling parameters to: 98 $^{\circ}$ C for 30 sec, 25 cycles at 98 $^{\circ}$ C for 10 sec, 48 $^{\circ}$ C for 1 min and 72 $^{\circ}$ C for 10 min, followed by 72 $^{\circ}$ C for 10 min.
3. Repeat step 1.2.2 to 1.2.6.

2. Generation of Viral Stock Using Transfection

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 mono-infections, each done in triplicate. For a transfection done in a 6-well plate, about 1.8 ml supernatant is harvested per well. One μ g of plasmid DNA is needed for each transfection done in a 6-well plate.
2. For each well of a 6-well plate, prepare 100 μ l of transfection mixture, e.g., consisting of 1 μ l X-tremeGENE 9 transfection reagent (or comparable product), 1 μ g of plasmid DNA and serum-free DMEM.
 1. Determine the volume of plasmid DNA needed, using 1 μ g plasmid DNA per well. Ensure that the final concentration of the plasmid DNA is at least 50 ng/ μ l.
 2. Determine how much serum-free medium (DMEM) is needed per well using the formula: Total volume of DMEM in μ l = 100 μ l - DNA volume in μ l.
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 $^{\circ}$ C in a 5% CO₂ atmosphere. Seed as many wells as needed (determined in step 2.1).
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.
 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 RT (15 $^{\circ}$ C to 25 $^{\circ}$ C) to allow the formation of transfection complexes.
 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.
 3. Seal plates with plastic wrap.
5. Incubate cultures at 37 $^{\circ}$ C in a 5% CO₂ atmosphere for 48 hr.
6. Use a pipette to carefully collect and transfer supernatant to a 15 ml tube through a 0.22 μ m filter top.
7. Use pipette to transfer 250 μ l or more of the filtered supernatant to 1.8 ml microfuge tubes with rubber gaskets in the lids.
8. Store filtered supernatants at -80 $^{\circ}$ C until use.

3. Determine Infectious Titer of Viral Stocks on Peripheral Blood Mononuclear Cells (PBMCs)

1. Stimulate PBMCs with phytohemagglutinin (PHA). Per one viral stock, seed 2×10^6 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 μ g/ml). Seed PBMCs at 2×10^6 cells/ml. Incubate PBMCs at 37 $^{\circ}$ C in a 5% CO₂ atmosphere for 72 hr.
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 2×10^5 PBMCs/ml in cIMDM.
3. Seed 2×10^4 PHA stimulated PBMCs/well in 100 μ l/well cIMDM in a round bottom 96-well plate.
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.
 1. For example, add 20 μ l of virus stock to 180 μ l media in the 1.5 ml tube. Mix the dilution by pipetting carefully. Then transfer 90 μ l of the diluted stock into 180 μ l media of the first well and mix well by pipetting.

2. Continue dilution series by transferring 90 μ l from the current well to 180 μ l 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.
5. Add 40 μ l 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 °C in a 5% CO₂ atmosphere for 16-24 hr.
6. Carefully remove 100 μ l of supernatant from each well, and replace with 160 μ l of fresh cIMDM to a total volume of 200 μ l/well. Incubate plates at 37 °C with 5% CO₂ atmosphere (day 1).
7. On days 4, 7, 10 and 13 transfer 100 μ l of supernatant from each well to 100 μ l disruption buffer (2% TritonX-100 in PBS), and replace with 100 μ l of fresh cIMDM. Store the supernatants at -20 °C.
 1. Keep sampling and adding fresh cIMDM every three days until the titer stabilizes.
8. Determine the 50% tissue culture infectious dose (TCID₅₀) of the viral stock by p24 ELISA using the day 7 and 13 samples as described in step 4.
 1. If the TCID₅₀ 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 laboratory³⁰. Commercial HIV-1 p24 ELISA plate/kits can also be used, following the manufacturer's protocol.

1. Prior to working with samples, prepare working stocks of primary antibody (rabbit anti-HIV-1 SF2 p24 antiserum).
 1. Thaw p24 antiserum at RT.
 2. Mix 2.5 ml of glycerol with 2 ml of 10% FBS in phosphate buffer saline (PBS).
 3. Add 0.5 ml of antiserum and mix.
 4. Store 1 ml of aliquots at -20 °C.
2. Thaw samples from step 3.7 in a 37 °C incubator.
3. Wash the p24 capture plate 5 times with wash buffer (1x PBS with 0.05% Tween-20).
4. Add 50 μ l/well of sample diluent (1% bovine serum albumin (BSA), 0.2% Tween-20 in RPMI-1640), then add 50 μ l of sample to appropriate wells. Include at least three wells with sample diluent only as mock/negative controls. Incubate for 2 hr at 37 °C or O/N at 4 °C.
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 μ l solution per each sample/control well in a 96-well plate.
 1. For example, to make enough solution for one 96-well plate, add 5 μ l of the primary antibody working stocks to the primary antibody diluent for a final volume of 10 ml.
6. Wash capture plate 5 times with wash buffer.
7. Add 100 μ l of the primary antibody solution to each well. Incubate for 1 h at 37 °C in a 5% CO₂ atmosphere.
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 μ l solution per each sample/control well in a 96-well plate.
 1. For example, to make enough solution for one 96-well plate, first add 1 μ l of the secondary antibody to 99 μ l of the secondary antibody diluent. Then add 70 μ l of first dilution to the secondary antibody diluent for a final volume of 10 ml.
9. Wash capture plate 5 times with wash buffer.
10. Add 100 μ l of the secondary antibody solution to each well. Incubate for 1 hr at 37 °C in a 5% CO₂ atmosphere.
11. Wash plate 5 times with wash buffer.
12. Add 100 μ l of RT TMB substrate. Incubate 30 min at RT in a closed container to protect from light.
13. Add 100 μ l of RT stop solution (1 N H₂SO₄).
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 TCID₅₀ of the viral stock using the Reed-Meunch method³¹.

5. Establish Viral Growth Kinetics

5.1) Monoinfection

1. Seed 3 x 10⁵ PHA-stimulated PBMC/well in 48-well plates in a total volume of 500 μ l/well. Keep the culture plates at 37 °C in a 5% CO₂ atmosphere until inoculation.
2. For each virus, prepare an inoculum containing 6,000 IU in 2 ml of cIMDM.
3. Inoculate wells in triplicate by adding 500 μ l 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.
4. Aliquot 200 μ l of the remaining inoculum to each of two 96-well plates for RNA isolation, one of which is saved as a backup.
5. Incubate cultures at 37 °C in a 5% CO₂ atmosphere for 16-24 hr.
6. Wash cultures 16-24 hr after inoculation.

1. Remove and discard 750 μ l of culture supernatant.
 2. Add 750 μ l of fresh cIMDM. Wrap plates in plastic wrap and spin for 10 minutes at 300 x g. Remove and discard 750 μ l supernatant.
 3. Add 750 μ l of fresh cIMDM. Incubate at 37 °C with a 5% CO₂ atmosphere (day 1).
7. Sample cultures daily from day 2 to day 7.
1. Transfer 500 μ l of culture supernatant to a 1.8 ml centrifuge tube. Spin for 1 min at 3,000 x g.
 2. Transfer 200 μ l 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 °C until RNA isolation.
 3. Add 500 μ l fresh cIMDM to each culture. Incubate at 37 °C in a 5% CO₂ atmosphere.
 4. Discard cultures into Wescodyne at the end of the experiment.
 5. Isolate RNA from 200 μ l 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 QIAextractor.
 6. Store RNA samples at -80 °C until cDNA synthesis.

5.2) cDNA Synthesis (Reverse Transcription)

1. For each RNA sample, add 1.2 nmol of dNTP and 1.2 pmol of cDNA synthesis primer, (5'-GTTGATCCTTTAGGTATCTTCCACAGC-3', HXB2 nucleotide 7968 to 7995) to 10 μ l of viral RNA. Add water to a final volume of 14 μ l. Flick the tube to mix and spin briefly to collect liquid at the bottom of the tube.
2. Incubate mixture for 5 min at 65 °C, then hold at 4 °C until the master mix is prepared.
3. Prepare master mix using 5x first-strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂), 5 mM DTT, 120 U of SuperScriptIII and 240 U of Rnase inhibitor. Add water to final volume of 10 μ l.
4. Add 10 μ l of master mix to RNA mixture, flick to mix and spin to collect.
5. Incubate mixture for 90 min at 50 °C to allow synthesis of cDNA. Incubate for 15 min at 70 °C to inactivate reverse transcriptase. Hold at 4 °C as needed.
6. Add 2 U of Rnase H, flick to mix, and then spin to collect.
7. Incubate 20 min at 37 °C. Store cDNA at -20 °C.

5.3) cDNA Quantitation Using qPCR System

1. Prepare a standard dilution series. Do 10-fold serial dilution, from 3 x 10⁶ copies/ μ l down to 30 copies/ μ l, of pNL4-3VifA. Use distilled water for dilutions. Prepare the standard dilution series fresh before use or prepare small batches and keep at -20 °C. Do not freeze-thaw the standards more than three times.
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.
3. For each qPCR reaction, use 12.5 μ l of qPCR master mix, 0.2 μ M probe, 0.8 μ M each of the forward and reverse primers and 1 μ l of cDNA or the standard dilution series or water/buffer (for the negative control well). Add water to a final volume of 25 μ l. The qPCR probe is light sensitive. Keep it in closed container.
 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').
4. Set PCR cycling parameters to 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. Consult the manufacturer's support document for operation of the qPCR machine.
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

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.
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, see²⁰.

6. Growth Competition Assay

1. Seed 3 x 10⁵ PHA-stimulated PBMCs (or 1 x 10⁵ CEMx174 cells) in 500 μ l total volume per well in a 48 well flat-bottomed plate.
2. Keep plate in 37 °C in a 5% CO₂ atmosphere until inoculation.
3. For each virus, prepare 3 ml of inoculum containing 6,000 IU.
4. Transfer 1.5 ml of each viral inoculum to a sterile tube to create the dual infection inoculum. Add 500 μ l of the dual inoculum (1,500 IU) to 3 x 10⁵ cells in a 48-well plate. The final culture volume is 1 ml/well. Aliquot 200 μ l of the inoculum to two 96-well plates for RNA isolation; save one plate as a back up.
5. Incubate inoculated cells at 37 °C with a 5% CO₂ atmosphere for 16-24 hr.
6. Wash cultures 16-24 hr after inoculation.
 1. Remove and discard 750 μ l of culture supernatant.
 2. Add 750 μ l of fresh cIMDM. Wrap plates in plastic wrap and spin for 10 min at 300 x g. Remove and discard 750 μ l supernatant.

3. Add 750 μ l of fresh cIMDM. Incubate at 37 °C with a 5% CO₂ atmosphere (day 1) .
7. Select sampling times to include at least 3 time points within the exponential growth phase observed in step 5.4.1.
 1. For each sampling, follow step 5.1.7.
8. Perform cDNA synthesis as described in section 5.2.
9. Determine the viral variant ratio using qPCR.
 1. Prepare a standard serial dilution series in triplicate, diluting 10-fold in each step from 3×10^6 copies/ μ l to 30 copies/ μ l of pNL4-3VifA.
 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.
 3. For each qPCR reaction, use 12.5 μ l of qPCR Master Mix, 0.2 μ M probe, 0.8 μ M each of the forward and reverse primers and 1 μ l of cDNA or the standard dilution series or water/buffer (for the negative control wells). Add water to a final volume of 25 μ l. The qPCR probe is light sensitive, keep it in closed container.
 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.
 4. Set PCR cycling parameters to 50 °C for 2 min, then 95 °C for 10 min, and then 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. Consult the manufacturer's support document for operation of the qPCR machine.
 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. 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, see²⁰.
10. Determine viral ratios using chromatogram peak-heights
 1. PCR amplify HIV-1 *vif* fragments containing the VifAB sequence tag using VifFwd (5'-GAAAGAGACTGGCATTGGGTCAGGG-3'; HXB2 positions 5266–5291) and VifRev primers (5'-GTCTTCTGGGGCTTGTCCATCTGTCC-3'; HXB2 positions 5579–5553).
 1. For each PCR reaction, use 1 μ l of cDNA, 1x NH₄ buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 2.5 U of Taq Polymerase, and 0.45 μ M of each primer. Add water to a final volume of 50 μ l.
 2. Set PCR cycling parameters to 3 cycles at 94 °C for 1 min, 55 °C for 1 min, and 70 °C for 1 min, followed by 34 cycles at 94 °C for 15 sec, 58 °C for 30 sec, and 70 °C for 1 min, and then hold at 4 °C.
 2. Purify PCR products using the QIAquick PCR purification kit according to the manufacturer's protocol.
 3. Submit purified PCR products to a DNA sequencing service provider for Sanger sequencing.
 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
 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. 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 hr. We estimated the 50% tissue culture infectious dose (TCID₅₀) of each viral stock by the Reed-Muench method³¹. The TCID₅₀ of the prototype and mutant viruses ranged from 10⁴ to 10⁵ 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 mono-infection; 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**).

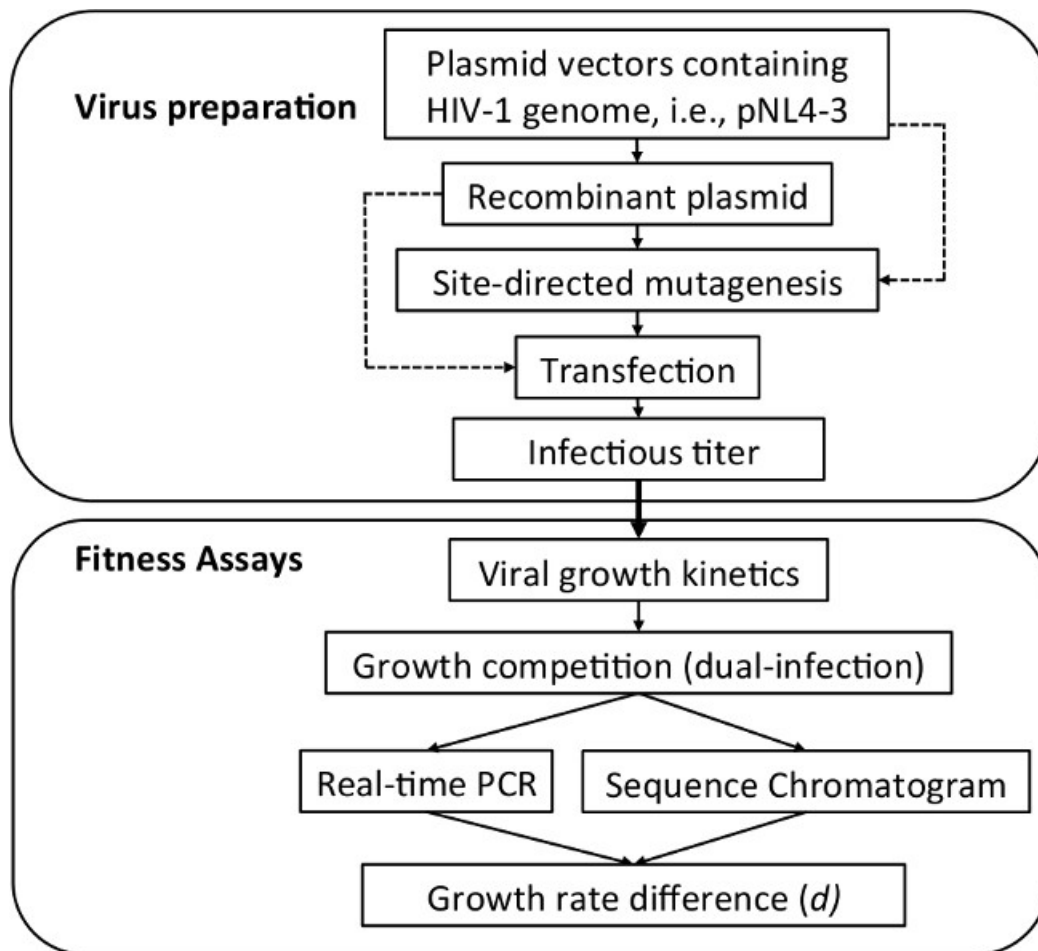


Figure 1: Flow diagram of the protocols presented in this paper. The Virus Preparation protocols concern construction of HIV-1 recombinant clones and generation of viral stocks. The Fitness Assays protocols are for establishing viral growth kinetics and determining relative viral fitness. Dashed lines 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.

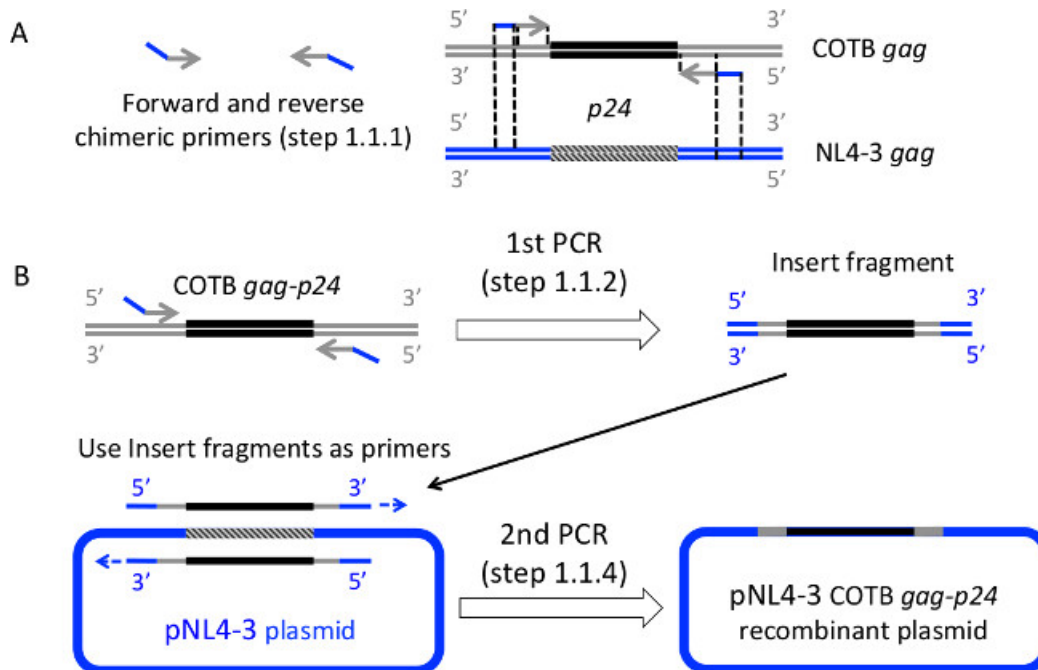


Figure 2: Construction of HIV-1 NL4-3 COTB Gag-p24 recombinant molecular clones using overlap extension PCR. (A) Design the chimeric primers. The 5' halves of the primers contain the NL4-3 vector sequence and the 3' halves contain the ends of the insert sequence. (B) Chimeric primers are used to amplify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

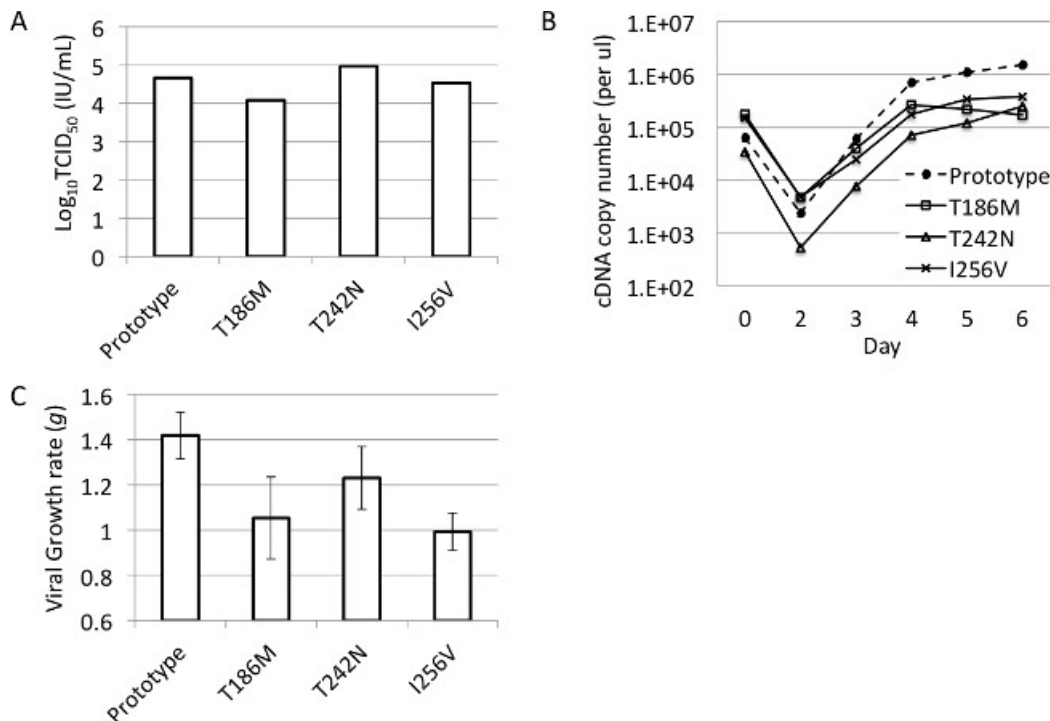


Figure 3: Viral growth characteristics in PBMCs. (A) Log₁₀ TCID₅₀ of viral stocks. (B) Viral growth kinetics in mono-infections begun at an MOI = 0.005. (C) Viral growth rates over six days, including the exponential growth phase (days 2-4) derived from panel (B). The values shown represent the average of three replicates from one experiment. The error bars represent 95% confidence intervals.

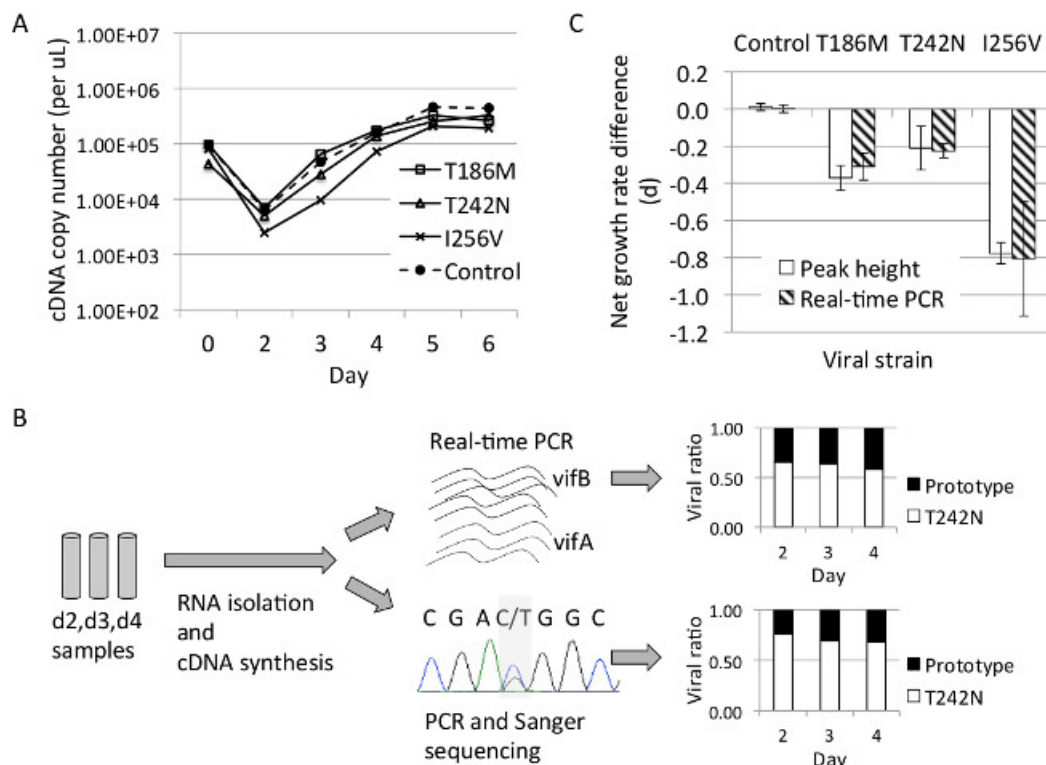


Figure 4: Viral growth and relative fitness determinations. (A) Viral growth in dually infected PBMC cultures. (B) Ratios of T242N and prototype viruses determined using RT-qPCR or the sequencing peak-height method. (C) Net viral growth rate differences (d) between the mutant and the prototype viruses 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 fitness²⁰. 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 kb³⁴. 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 phase⁹. 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 MOIs⁹. 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 excess⁹.

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

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 (see²⁰). 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 bases³⁷, 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 used 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.

Disclosures

The authors declare that they have no competing financial interests. The opinions expressed herein are those of the authors and should not be construed as official or representing the views of the US Department of Defense or the Department of the Army.

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