

Video Article

A Restriction Enzyme Based Cloning Method to Assess the *In vitro* Replication Capacity of HIV-1 Subtype C Gag-MJ4 Chimeric Viruses

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Abstract

The protective effect of many HLA class I alleles on HIV-1 pathogenesis and disease progression is, in part, attributed to their ability to target conserved portions of the HIV-1 genome that escape with difficulty. Sequence changes attributed to cellular immune pressure arise across the genome during infection, and if found within conserved regions of the genome such as Gag, can affect the ability of the virus to replicate *in vitro*. Transmission of HLA-linked polymorphisms in Gag to HLA-mismatched recipients has been associated with reduced set point viral loads. We hypothesized this may be due to a reduced replication capacity of the virus. Here we present a novel method for assessing the *in vitro* replication of HIV-1 as influenced by the *gag* gene isolated from acute time points from subtype C infected Zambians. This method uses restriction enzyme based cloning to insert the *gag* gene into a common subtype C HIV-1 proviral backbone, MJ4. This makes it more appropriate to the study of subtype C sequences than previous recombination based methods that have assessed the *in vitro* replication of chronically derived *gag-pro* sequences. Nevertheless, the protocol could be readily modified for studies of viruses from other subtypes. Moreover, this protocol details a robust and reproducible method for assessing the replication capacity of the Gag-MJ4 chimeric viruses on a CEM-based T cell line. This method was utilized for the study of Gag-MJ4 chimeric viruses derived from 149 subtype C acutely infected Zambians, and has allowed for the identification of residues in Gag that affect replication. More importantly, the implementation of this technique has facilitated a deeper understanding of how viral replication defines parameters of early HIV-1 pathogenesis such as set point viral load and longitudinal CD4+ T cell decline.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51506/>

Introduction

Determining both the host and viral characteristics that influence HIV-1 pathogenesis and disease progression is paramount for rational vaccine design. The cellular immune response is a key component of the human immune response to HIV-1 infection. Cytotoxic T lymphocytes (CTL) are necessary for the initial control of acute viremia, and allow the host to establish a steady state (set point) viral load^{1,2}. Experimental depletion of these effector cells results in loss of viral control^{3,4}. Despite this, escape mutations arise within the viral genome that subvert CTL recognition of virally infected cells⁵⁻⁹.

Certain HLA alleles have been associated with lower viral loads and slower disease progression including B*57, B*27 and B*81¹⁰⁻¹⁵. Part of the protective benefit of HLA class I alleles can be attributed to the fact that they target functionally constrained regions of the genome such as Gag and select for escape mutations that decrease the ability of the virus to replicate *in vitro*¹⁶⁻²¹. Although escape from the cellular immune system is beneficial to the virus in the context of the selecting HLA class I allele, the effect of these mutations may have differential consequences for the host upon transmission to an HLA-mismatched individual^{22,23}. Therefore, understanding the effects of transmitted HLA-associated escape mutations on viral replication capacity will be important to further our understanding of early HIV-1 pathogenesis.

While much progress has been made to identify and characterize the fitness defects of individual escape mutations associated with specific HLA class I alleles²⁴⁻²⁹, naturally occurring HIV-1 isolates have unique and complex footprints of HLA-associated polymorphisms, likely arising from the HLA-mediated immune pressure of different immunogenetic backgrounds³⁰. In a previous analysis, Goepfert *et al.* showed that an accumulation of HLA-associated mutations in the transmitted Gag sequences derived from 88 acutely infected Zambians was associated with a reduction in set point viral load³¹. This suggested that the transmission of deleterious escape mutations, specifically in Gag, to HLA-mismatched recipients provides a clinical benefit, and may be due to attenuated viral replication. Moving forward, it is imperative to study how complex combinations of Gag polymorphisms within naturally occurring isolates work in concert to define characteristics of the transmitted virus such as replication capacity, and how early replication might in turn affect HIV-1 clinical parameters and late-stage pathogenesis.

Brockman *et al.* first demonstrated a link between the replication capacity of *gag-pro* sequences isolated during chronic stage infection and viral load in both subtype C and B infections³²⁻³⁵. The experimental approach presented in these studies, although appropriate for examining the *in vitro* replication capacity of sequences derived from chronically infected individuals, has several technical caveats and limitations that make studying HIV-1 replicative capacity in subtype C acutely infected individuals difficult. This method relies on the recombination of population based PCR-amplified sequences into the subtype B NL4-3 provirus, which was derived in part from LAV, a laboratory adapted virus stock³⁶. Virus generation was accomplished by co-transfection of a CEM-based T-cell line³⁷ with PCR amplicons and digested delta-*gag-pro* NL4-3 DNA. This method requires the outgrowth of virus over a period of weeks to months, potentially skewing the nature of the recovered virus stock in relation to the viral quasispecies *in vivo*, and therefore altering the measurement of replication capacity *in vitro*. This method is more appropriate for studying chronically infected individuals, where it effectively selects for virus with the highest replicative capacity, and where cloning numerous different viral variants from a large number of chronically infected individuals is quite labor intensive and therefore not feasible. However, within an acutely infected individual, there are generally one to two variants present, and thus eliminating the risk of skewing the nature of the recovered virus stock, through *in vitro* selection pressures, allows for a more accurate assessment of *in vitro* replication capacity. Secondly, this method requires recombining subtype C *gag-pro* sequences into a subtype B derived backbone, and could introduce backbone incompatibility biases into the analysis. Due to these limitations, large numbers of sequences must be analyzed in order to overcome any potential biases introduced.

Here we describe an alternative experimental approach appropriate for studying sequences derived from subtype C acutely infected individuals. We use a restriction enzyme based cloning strategy to introduce the *gag* gene derived from acute infection time points of HIV-1 subtype C infected individuals into the subtype C proviral backbone, MJ4. The use of MJ4 as a common backbone in which to clone *gag* genes is crucial for the analysis of subtype C derived sequences. MJ4 is derived from a primary isolate³⁸, and thus would be less likely to introduce bias due to subtype incompatibility between the backbone and *gag* gene. In addition, the approach of using enzyme based restriction cloning allows for the proviral constructs to be transfected directly into 293T cells, and for the recovery of a clonal virus stock identical to the cloned *gag* sequence.

The method presented below is a high throughput method for assessing the replication capacity of subtype C derived Gag-MJ4 chimeric viruses. Transfection into 293T cells is straightforward and recovery of virus takes only three days. *In vitro* replication capacity is assayed on the same CEM-CCR5 based T-cell line created by Brockman *et al.*³⁷, but using important protocol modifications necessary for the successful replication of subtype C MJ4 chimeric viruses. The use of an appropriate T-cell line rather than PBMCs allows for large numbers of subtype C MJ4-chimeric viruses to be tested with high assay reproducibility. Finally, using a radiolabeled reverse transcriptase assay for quantification of virus in the supernatant is more cost effective than using commercially available p24 ELISA kits. It also gives a higher dynamic range, which was important for detecting both poorly and highly replicating viruses within the same assay and for detecting subtle differences in replication between isolates.

In conclusion, the method presented here has allowed for the in-depth study of the replication capacity of *gag* sequences derived from HIV-1 subtype C acutely infected individuals from Zambia, and as written, could also be expanded to study other subtype C infected populations. A high degree of variation in replication capacities between different Gag isolates was observed. In addition, we were able to show a statistical association between the replication capacity of the transmitted Gag and set point viral load as well as with CD4+ decline over a three-year period³⁹. Such results highlight the importance of studying how transmitted viral characteristics, such as replication capacity, interact with the host immune system to influence pathogenesis during early infection and will be integral for developing effective vaccine interventions as well as treatment.

Protocol

1. Amplification of the HIV-1 *gag* Gene from Infected, Frozen Plasma

1. Extract viral RNA from 140 μ l thawed HIV-1 infected plasma using an extraction kit.
 1. When feasible, immediately proceed to cDNA synthesis after RNA extraction as unfrozen viral RNA yields the best amplification results. If possible, set up PCR master mix for first-round DNA amplification and store at 4 $^{\circ}$ C prior to viral RNA extraction.
2. Reverse-transcribe cDNA from RNA and amplify first-round DNA products using reverse-transcriptase and a thermostable DNA polymerase in a one-step RT-PCR.
 1. Take RNA out of -80 $^{\circ}$ C freezer (if freezing was necessary) and briefly thaw at room temperature then place in cold block. Transfer 5 μ l of RNA to each PCR tube containing 45 μ l of master mix to give a final volume of 50 μ l. Immediately place remaining RNA samples into -80 $^{\circ}$ C freezer.
 2. After enzyme is added to the first-round PCR master mix (**Table 1A**), aliquot 45 μ l into each thin-walled PCR amplification tube for the number of reactions desired. Make sure to use PCR tubes with individual caps and not strip caps to ensure minimal cross-contamination between samples. Place PCR tubes in a cold block to protect the temperature sensitive RT enzyme and RNA template. Note: Samples should be run in triplicate in order to adequately sample the viral quasispecies. It is also important to utilize a product with a high fidelity DNA polymerase enzyme in order to minimize PCR-introduced misincorporation. Please refer to the reagents list for the recommended product.
 3. After RNA template has been added to all reaction tubes, transfer PCR tubes to a thermocycler for amplification using the cycler program described in **Table 1B**. Note: The product of this amplification will be used in a second-round nested PCR.
3. Perform a nested second-round PCR amplification, using 1 μ l of the first round PCR amplification (1.2) as the DNA template.
 1. Aliquot 49 μ l of second-round PCR master mix (**Table 2A**) to each thin-walled PCR tube for the number of reactions desired. Transfer 1 μ l from the first-round PCR amplification to each reaction tube to give a final volume of 50 μ l. Note: This will serve as template DNA for the second-round amplification. It is also important to utilize a DNA polymerase with very high fidelity and proofreading capabilities in order to minimize PCR-introduced misincorporation. Please refer to the reagents list for the recommended product.
 2. Transfer second-round PCR reaction mix to thermocycler and run program described in **Table 2B**. Note: After completion of the program, the products of this reaction will be run on an agarose gel to confirm the production of product.

4. Add 3 μ l of 5x loading dye to 5 μ l of the 50 μ l second-round reaction volume and run at 120 V on a 1% agarose-TAE gel containing a UV-fluorescent DNA stain until bands are resolved. Visualize 1.6 kb bands on a blue light illuminator (see **Figure 1A**).
5. Run the remaining 45 μ l reaction volume on a 1% agarose-TAE gel containing a DNA stain, and excise the appropriate bands with a clean razor blade.
 1. Extract DNA from the gel slice using a gel purification kit, elute in nuclease-free water, combine three positive reactions per individual, and freeze product at -20 °C for subsequent use.

2. Preparing *gag* Amplicons for Cloning by Introduction of the Necessary Restriction Sites

1. Amplify the Long Terminal Repeat (LTR)/5' UTR portion of the MJ4 plasmid (see **Table 3**).
2. Visualize 1.3 kb PCR products on illuminator, excise positive amplicons, gel purify and freeze as previously described (1.4,1.5; see **Figure 1B**).
3. Create fused MJ4 LTR-*gag* amplicons via "splice-overlap-extension" PCR (see **Table 4**).
4. Visualize, excise, purify, and freeze the 3.2 kb amplicons as in 1.4,1.5 (see **Figure 1C**).

3. Cloning Amplified *gag* Genes into the MJ4, Subtype C, Infectious Molecular Clone

1. Digest 1.5 μ g of MJ4 plasmid and 1.5 μ g of purified LTR-*gag* PCR product with the BclI (methylation sensitive) restriction endonuclease for 1.5 hr at 50 °C (see **Table 5A**).
2. Add 1 μ l of NgoMIV to the digestion reaction and incubate at 37 °C for 1 hr.
3. Add 5x loading dye to restriction digest reactions and slowly electrophorese the total volume on a 1% agarose-TAE gel containing a DNA gel stain for 1-2 hr at 100 V. Visualize, excise, and purify indicated bands for cloning as previously described (see **Figure 2**).
4. Prepare ligation reactions using purified LTR-*gag* insert and MJ4 vector DNA at a 3:1 insert to vector ratio. Incubate ligation reactions overnight at 4 °C (see **Table 5B**).
5. Transform JM109 chemically competent bacteria with ligation products and spread on LB agar plates with 100 μ g/ml ampicillin and grow at 30 °C for 22+ hr.
 1. Thaw JM109 competent cells on ice for 15 min. Label 1.5 ml microcentrifuge tubes and chill on ice.
 2. Aliquot 50-100 μ l of JM109 cells to pre-chilled 1.5 ml microcentrifuge tubes. Add 2.5-5 μ l of ligation reaction to JM109 cells, flicking tube lightly to mix and immediately returning to ice. Incubate competent cells with ligation product on ice for 30 min.
 3. Heat shock 1.5 ml microcentrifuge tubes containing JM109 competent cells and ligation reaction in a 42 °C heat block for 45 sec and return to ice for at least 3 min.
 4. Add 50 μ l of SOC media to each microcentrifuge tube and plate the entire transformation reaction onto room temperature LB-agar plates supplemented with 100 μ g/ml of ampicillin.
 5. Transfer plates to a 30 °C incubator and leave for 20 hr, or until colonies are clearly formed.
6. Pick isolated colonies and grow in 4 ml of LB with 100 μ g/ml ampicillin at 30 °C for 22+ hr.
7. Spin down cultures at 3,200 x g for 15 min, pour off broth, and extract plasmid DNA.
8. To confirm cloning fidelity, cut miniprep DNA with NgoMIV and HpaI restriction enzymes in a double digest at 37 °C for 2 hr. Analyze on 1% agarose-TAE gel (see **Table 5C**).
9. Sequence the LTR-*gag* insert region of each plasmid using the following primers: GagInnerF1, GagF2, Rev1, Rev3, and GagR6 (**Table 6**) to confirm sequence identity.
10. Compare the cloned sequences obtained with the population sequences derived from the initial purified amplicon from 1.5.1. Note: It is important to ultimately assess the replication capacity of two independent clones in order to ensure that any *in vitro* replication phenotypes are not due to backbone errors introduced during the cloning process.

4. Generation and Titering of Replication Competent Gag-MJ4 Chimeric Viruses

1. Generate replication competent virus by transfecting 1.5 μ g of the chimeric MJ4 plasmid miniprep DNA into 293T cells using a 4:1 ratio of Fugene HD as described by Prince *et al*³⁹.
2. Titer harvested virus stocks on TZM-bl cells as described below and in Prince *et al*³⁹.
 1. Plate TZM-bl cells 24 hr before infection with virus in order to have a 30-40% confluent cell monolayer on the following day. This can usually be achieved by adding 5×10^4 cells in a total volume of 800 μ l per well in a 24-well plate.
 2. After adding cells to the well, gently move the 24-well plate forward and back, then side to side in order to efficiently distribute cells. Never swirl – this will result in cells accumulating in the center of the plate.
 3. The following day, prepare 1% FBS in DMEM; this will be used to dilute DEAE-Dextran and to dilute virus stocks. Stock DEAE-Dextran is 10 mg/ml or 125x, and a final concentration of 80 μ g/ml or 1x is desired.
 4. Take out virus stock to be tested from -80 °C freezer and place on shaker to thaw at room temperature.
 5. Using a multichannel pipette, serially dilute virus stocks in a round-bottomed tissue culture treated 96-well plate with lid. This is a 3-fold dilution protocol. See **Table 7** for the dilution scheme.
 6. Remove media from seeded TZM-bl 24-well plates using a vacuum aspirator making sure not to disturb cell monolayer. Only remove the media from one 24-well plate at a time so that cells do not dry out.
 7. Add 150 μ l of the 1x DEAE-Dextran + 1% FBS in DMEM mixture to each well using a 1,000 μ l pipette. Do not use repeat pipette as this disrupts the monolayer.
 8. Add 150 μ l of each virus dilution to the appropriate well. Add virus to the center of the well and swirl gently to evenly distribute virus across the cell monolayer. Incubate infected cells for 2 hr in a tissue culture incubator at 37 °C and 5% CO₂.
 9. After the 2 hr incubation, add 0.5 ml 10% FBS in DMEM to each well and return plates to incubator for an additional 48 hr.

10. After 48 hr, cells should be 100% confluent. Because MJ4 is a replication competent virus, there will be some cell death, but this is to be expected.
11. Remove media from each well and add 400 μ l/well of fixing solution (see **Table 8A** for recipe). Fix only one plate at a time. Add fixing solution using a 1,000 μ l pipette, and let sit for 5 min at room temperature.
12. Remove fixing solution and wash 3x with PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$. Use a squirt bottle to gently add PBS to the side of the well to avoid disrupting the monolayer. After the third wash, blot plate dry on absorbent paper.
13. Add 400 μ l/well of staining solution (see **Table 8B** for recipe) to each well of the 24-well plate, and incubate at 37 °C for at least 2 hr. Longer incubation times are acceptable, but incubation times should be kept consistent between titrating experiments.
14. Wash 2x with PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$ and score for infection, or add PBS and store at 4 °C for scoring later. Plates can be kept at 4 °C for up to four days, as long as the monolayer is kept hydrated.
15. To score for infection, use a permanent marker to divide wells of the 24-well plate into quadrants. Count all blue cells within a field of view, using a total magnification of 200X, once in each of the four quadrants of a well.
16. Compute Infectious Units per μ l as follows: $[(\# \text{ blue cells}/4) \times 67] / (\mu\text{l virus added}) = \text{IU}/\mu\text{l}$. Refer to **Table 8C** for volume in μ l of virus added to each well. Average several wells together; the numbers should be relatively similar for an accurate titration.

5. Preparation of Culture Media and Propagation of GXR25 Cells for *in vitro* Replication Assay

1. Prepare complete RPMI (cRPMI) for propagation of GXR25 cells. NOTE: It is extremely important to culture GXR25 cells at least 4 months prior to planned replication experiments (see **Table 9**).
2. Split cells 1:10 twice per week and maintain cell density between 1×10^5 to 2×10^6 cells/ml.

6. *In vitro* Replication of Gag-MJ4 Chimeras in GXR25 (CEM-CCR5-GFP) Cells

1. The day before the infection, split cells to a concentration between $2\text{-}3 \times 10^5$ cells/ml so that they are in logarithmic growth on the day of the infection.
2. On the day of infection, remove virus stocks from -80 °C freezer and thaw. Calculate the volume of virus needed from each stock based on the IU/ μ l titer from TZM-bl cell assay in order to infect 5×10^5 GXR25 cells at a multiplicity of infection of 0.05, using the formula:
Volume of virus for infection (μ l) = $1 \mu\text{l}/\text{X IU} \times 0.05 \times 500,000$
NOTE: We have chosen an MOI of 0.05 as this results in a logarithmic growth phase for all of the viruses that we have tested. It is important to conduct initial experiments in order to determine the optimal MOI to capture logarithmic growth for the virus to be tested.
3. Dilute virus in cRPMI to a volume of 100 μ l (in order to achieve a 0.05 MOI) and add into a well of a V-Bottom tissue culture treated 96-well plate. Note: Include both a positive (MJ4 WT infected) and a negative (mock infected cells) control in each set of replication experiments. Set up the plate such that every other column is blank to limit cross-contamination between wells. The positive control is imperative, as it will be used later as a standard to normalize the replication slopes, which are a measure of the replication rate of experimental replicates.
4. Count GXR25 cells using an automated cell counter. Calculate the number of cells needed in total for all infections ($5 \times 10^5 \times \# \text{ infections}$). Aliquot the required volume into a 50 ml conical tube and centrifuge to pellet cells. Always calculate for 25% more infections than needed.
5. Aspirate media carefully and resuspend in cRPMI at a concentration of 5×10^5 cells/100 μ l.
6. Pipette cells into a sterile trough and mix thoroughly. Using a multi-channel pipette, add 100 μ l of cells into each well of the 96-well plate containing the diluted virus. Mix thoroughly.
7. Add 2 μ l of 5 mg/ml (100x) solution of polybrene to each well and mix cells thoroughly.
8. Incubate at 37 °C in tissue culture incubator with 5% CO_2 for 3 hr.
9. In order to wash infected cells, centrifuge 96-well V-bottom plate to pellet cells. Then carefully remove 150 μ l of the medium and replace with fresh 150 μ l of cRPMI without disturbing the cell pellet. Repeat 2 more times (including centrifugation) to sufficiently wash cells.
10. After the last centrifugation and addition of 150 μ l cRPMI, resuspend the cell pellet with a multichannel pipette and add the entire cell/virus mixture (approximately 200 μ l) from each well independently to 800 μ l of cRPMI in a well of a 24-well tissue culture plate.
11. Place plate in 5% CO_2 tissue culture incubator at 37 °C.
12. Every two days, remove 100 μ l of supernatant from surface of culture well and transfer to a 96-well U-bottom plate and store frozen at -80 °C until running virus quantitation assay. NOTE: Careful arrangement of supernatants in 96-well plates will allow for multi-channel pipette transfer of culture supernatants during virion quantification via a radiolabeled reverse transcriptase (RT) assay. Every plate should contain samples from an infection standard in order to normalize inter-plate variation in the RT assay readout.
13. After removing each 100 μ l sample, split cells 1:2 by thoroughly resuspending cells and removing half of the remaining volume (450 μ l). Restore original volume (1 ml) by adding 550 μ l of fresh cRPMI to each well.

7. Analysis of Reverse Transcriptase (RT) in Cell Culture Supernatants

Protocol adapted from Ostrowski *et al*⁴⁰.

1. Set up biological safety hood in a BSL-3 facility for use with radioactive materials.
 1. Place absorbent paper in hood and replace aspirator for aspirator designated for radioactive use. Note: All radioactive waste must be properly disposed of with accordance to safety regulations.
2. Add 1-2 μ l of 10 mCi/ml of [α -³³P] dTTP and 4 μ l of 1 M dithiothreitol (DTT) to each 1 ml aliquot of RT master mix (see **Table 10** and Ostrowski *et al*⁴⁰).
3. Carefully mix each 1.5 ml microcentrifuge tube of RT master mix, DTT, and [α -³³P] dTTP with a 1,000 μ l pipette and transfer to a sterile trough.

4. Dispense 25 μ l of labeled RT mix into each well of 96-well thin-walled PCR plate. Note: Include space for a positive control (MJ4 WT infection) and negative control (Mock infection).
5. Add 5 μ l of each supernatant to the PCR plate containing the RT master mix.
6. Seal PCR plate with adhesive foil cover and incubate for 2 hr at 37 °C in a thermocycler. For safety reasons, rinse pipette tips with Amphyl and dispose of tips in small container containing Amphyl waste, which will later be disposed of in radioactive waste.
7. After incubation, make small holes in the foil cover using a 200 μ l multi-channel pipette. Note: If pipette tips touch liquid in well, replace tips before moving to the next column or row.
8. Mix samples 5x and transfer 5 μ l of each sample to the DE-81 paper. Air dry 10 min.
9. Wash blots 5x with 1x SSC (sodium chloride, sodium citrate), and then 2x with 90% ethanol at 5 min per wash. Allow to air dry.
 1. Place each blot in a separate washing container, such as a sandwich storage box, add enough wash buffer (1x SSC or 90% EtOH) to cover blot, and shake for 5 min.
 2. Pour off wash buffer into separate container and repeat. Note: The first 3 washes are considered radioactive and should be disposed of properly. The last 2 washes can be disposed of regularly.
10. Once dry, carefully wrap blot in Saran wrap and expose to a phosphoscreen in a tightly sealed cassette overnight at room temperature.
11. Analyze phosphoscreens with a phosphorimager and quantify radioactive transcripts.
12. Draw a circle around each radioactive signal using OptiQuant software. Graph the Digital Light Unit (DLU) values to generate replication curves.
13. In order to generate replication capacity scores, DLU values were \log_{10} -transformed and slopes were calculated using the day 2, 4, and 6 time points. Replication slopes are then divided by the slope of WT MJ4 in order to generate replication capacity scores. It is important to normalize based on the MJ4 WT values obtained from the same RT plate to reduce intra-assay variability.

Representative Results

In order to properly execute this protocol, which creates a proviral plasmid capable of assembling fully functional, infectious Gag-MJ4 chimeras, great care must be taken to generate the appropriate PCR amplicons. Determining whether the PCR has generated the appropriately sized *gag* amplicon is crucial. Products should be within 100 base pairs (bp) of the approximately 1,700 bp amplicon depicted in **Figure 1A**. The exact length of this fragment will vary depending on the *gag* gene under study. Next, the 5' long terminal repeat (LTR) portion of the MJ4 molecular clone must be amplified and spliced to the *gag* amplicon in order to make it suitable for subsequent cloning. The MJ4 LTR amplicon should be 1,474 bp in length. **Figure 1B** shows a representative gel image for which correct band sizes are indicated. After the splice-overlap-extension PCR⁴¹, combined LTR-*gag* products should be approximately 3,200 bp in length, as depicted in **Figure 1C**.

Once the *gag* gene has been made suitable for cloning by fusion to the 5' LTR from MJ4, which contains the necessary NgoMIV restriction site, both vector and *gag* insert must be digested with NgoMIV and BclI restriction enzymes and excised from an agarose gel after electrophoretic separation. It is imperative to excise the appropriate vector and insert bands. A representative gel is shown in **Figure 2**. The vector portion of the MJ4 plasmid should be approximately 10,000 bp in length, while the LTR-*gag* insert should remain at approximately 3,000 bp in length, as the restriction sites are located at the extreme ends of the amplicon. Any significant decrease in size will indicate an additional cut-site within the *gag* gene under study.

Following ligation of the two fragments, bacterial transformation, and isolation of plasmid DNA, the Gag-MJ4 chimeras must be checked for appropriate size by performing a double digest with NgoMIV and HpaI restriction enzymes. Full-length Gag-MJ4 chimeras that have not incurred any deletion events during bacterial replication should have a restriction pattern similar to that depicted in **Figure 3**, with two bands of approximately 8,700 and 4,300 bp.

An important distinction of this protocol compared to previous approaches, is the use of the HIV-1 subtype C infectious molecular clone, MJ4, rather than the more common laboratory-adapted NL4-3 virus. However, the approaches described in the previous section could be modified for cloning of subtype B *gag* sequences into pNL4-3.

An optimization of the multiplicity of infection (MOI) for use in subsequent experiments was performed in order to select the ideal MOI that showed logarithmic growth of a majority of the viruses tested. **Figure 4** depicts representative replication curves from three different MOIs (0.01, 0.05, and 0.25) for MJ4 (**Figure 4A**) as well as for NL4.3 (**Figure 4B**). MJ4 replicates much less efficiently in GXR25 cells than NL4-3, which is important to take into account, as an MOI appropriate for NL4-3 replication would likely be too low to detect efficient MJ4 replication. As seen in **Figure 4A**, an MOI of 0.05 as opposed to 0.01 or 0.25, was the ideal choice, because logarithmic growth was observed between days 2-6 for MJ4. For the lower MOI, 0.01, day 6 DLU values are barely detectable, and we anticipated the generation of Gag-MJ4 chimeric viruses that replicated lower than MJ4. Therefore this MOI would not capture the growth of the more attenuated Gag-MJ4 chimeras, which may also be the most biologically critical. Additionally, an MOI of 0.25 was not ideal, because the rapid kinetics of viral replication killed a substantial amount of cells even by day 4. This causes the replication curve to plateau, and calculating a slope based on a curve such as this would underestimate the replication capacity. Based on curves generated for NL4-3, even at an MOI of 0.01, available cell targets have been noticeably exhausted by day 6 post infection. In conclusion, an MOI of 0.05 was found to be optimal for a large panel of Gag-MJ4 chimeric viruses, all of which had diverse Gag sequences and varying degrees of replication.

A total of 149 Gag-MJ4 chimeric viruses derived from acute subtype C Gag sequences have been tested for *in vitro* replication using this assay. The normalized RC values ranged from 0.01 to over 3.5 with some viruses replicating more than 100 times more efficiently than wild-type MJ4. **Figure 5** shows the replication curves from nine representative Gag-MJ4 chimeric viruses, with wild-type MJ4 depicted in red, and demonstrates the wide range of replication capacities observed. Thus, the sequence diversity within the *gag* gene alone can drastically impact the ability of the virus to replicate *in vitro*. While this is representative of Gag-MJ4 chimeric viruses derived from acutely infected Zambians, other subtype C sequences have not been extensively tested, and may exhibit different replication kinetics. Therefore, great care must be taken to optimize the MOI to suit the specific replication of the viruses of a particular study, because there can be a wide range in the levels of replication between different HIV-1 backbones and Gag isolates.

One of the advantages of using a T-cell line such as the GXR25 cell line, that supports the replication of MJ4, is the level of reproducibility observed relative to replication experiments using stimulated peripheral blood mononuclear cells as targets. In initial optimization experiments, MJ4 wild type exhibited an intra-assay variability of 8.7%, and different clones of the same Gag-MJ4 chimeric virus exhibited variability in replication of 8.5%. Because different master mixes and phosphoscreen exposures may give DLU values that differ slightly in magnitude, intra-assay variability can further be controlled by running the same virus standard (in our case wild-type MJ4) in each RT assay plate. **Figure 6** graphs the DLU values derived from the same MJ4 infection, quantified in eight different RT plates. Normalizing to a virus that is common to all RT assays can help to mitigate potential error induced by these slight changes in signal magnitude between assays.

Inter-assay variability was also tested and replicates repeated on different days were highly correlated. **Figure 7** plots the normalized RC score values from two independent experiments performed approximately one year apart. A high degree of correlation with the absence of major outliers ($R^2 = 0.873$) was observed between the two independent replicates.

Although highly correlated, there is some variability in the overall magnitude of replication kinetics between the two independent experiments. This can be attributed in part to the difference in passage numbers between the GXR25 cells stocks used in each experiment. In general, GXR25 cells stocks that have been passaged for a period of time greater than 6 months tend to support more efficient replication of Gag-MJ4 chimeras. Therefore, it is advisable to assess replication capacity among groups of chimeras within a one-month time frame. When the previous steps are followed closely, this assay is capable of producing highly robust and reproducible results, which are applicable to a wide range of studies.

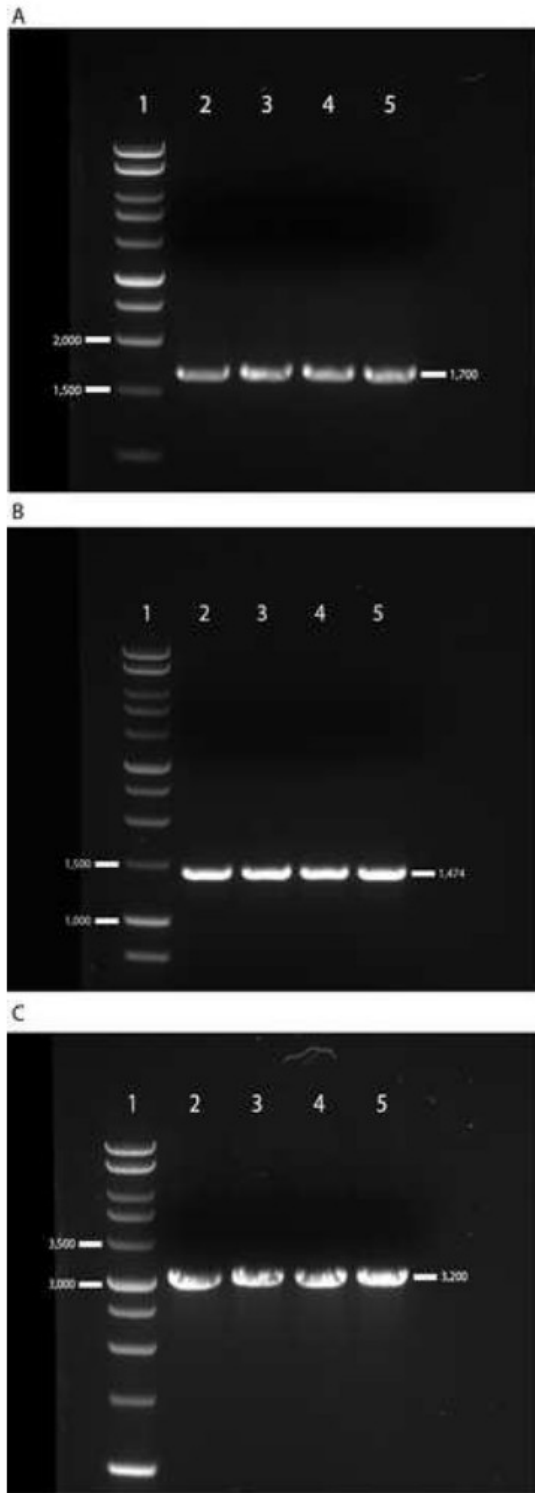


Figure 1. Representative gel images depicting electrophoretic separation of PCR products. For all PCR products, 5 μ l of each 50 μ l reaction was mixed with 3 μ l of 5x loading dye, loaded into a 1% agarose-TAE gel supplemented with 1X SYBR-safe DNA gel stain, and separated by electrophoresis at 120 V for 45 min. The Promega 1 kb DNA ladder (Lane 1) was used to approximate amplicon sizes. **A)** The *gag* gene was amplified from viral RNA using a nested PCR approach. Due to insertions and deletions, the *gag* amplicon may vary from 1,600-1,700 bp in length and appears slightly above the 1,500 bp DNA ladder marker. Lanes 2-5 depict successful *gag* gene amplification. **B)** The 5' LTR of MJ4 was amplified from the wild-type MJ4 plasmid and visualized via electrophoretic separation. Lanes 2-5 depict successful amplification of the 1,474 bp LTR product, which appears slightly below the 1,500 bp DNA ladder marker. **C)** The 5' LTR derived from wild-type MJ4 and the *gag* gene amplified from patient plasma are fused together via splice-overlap-extension PCR and visualized via electrophoretic separation. Lanes 2-5 depict successfully fused amplicons that are approximately 3,200 bp in size, and which appear slightly above the 3,000 bp DNA ladder marker.

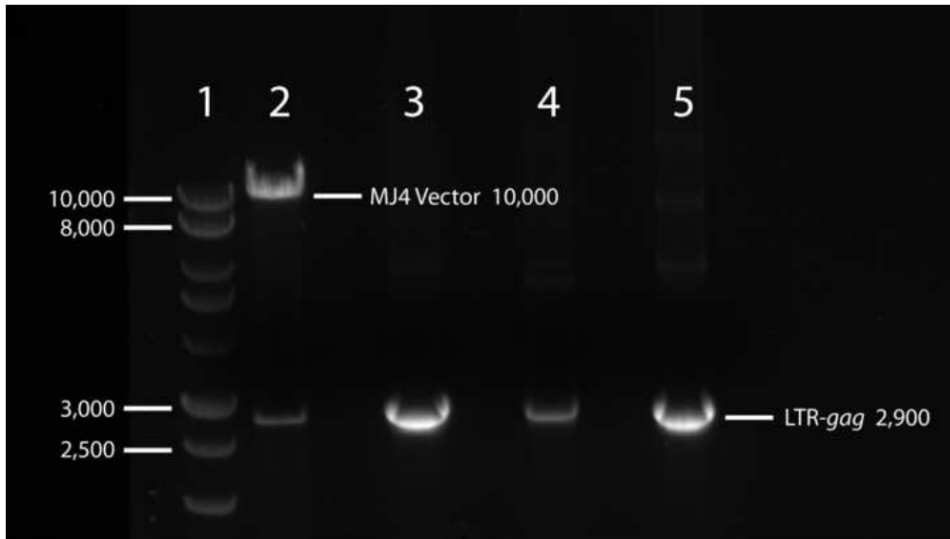


Figure 2. Representative gel image depicting electrophoretic separation of restriction digests for cloning patient gag genes into MJ4. Wild-type MJ4 plasmid and LTR-gag fusion products were digested with BclI for 1.5 hr at 50 °C and NgoMIV for 1 hr at 37 °C. Vector and insert fragments were visualized via electrophoretic separation on a 1% agarose-TAE gel supplemented with 1X SYBR-safe DNA gel stain at 100 V for 2 hr and using a blue light illuminator in order to reduce UV-induced DNA damage. The vector and insert fragments suitable for subsequent cloning steps appear at approximately 10,000 bp and 2,900 bp respectively.

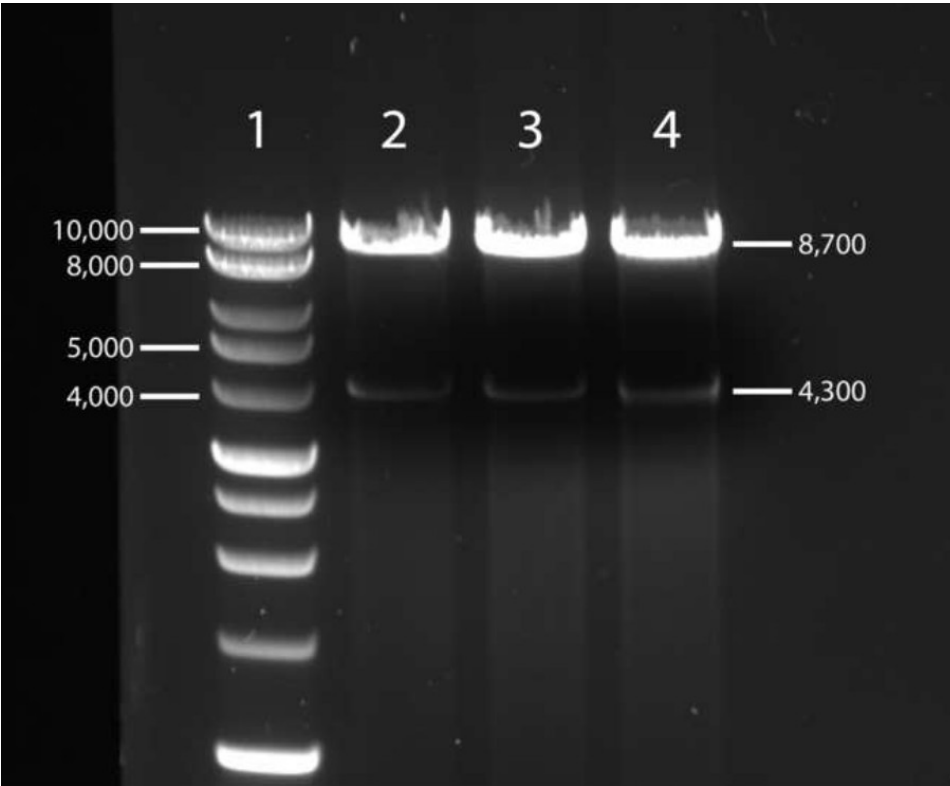


Figure 3. Representative gel image depicting electrophoretic separation of restriction digests of purified Gag-MJ4 chimera plasmid DNA. Purified Gag-MJ4 chimera plasmid DNA was double-digested with NgoMIV and HpaI restriction enzymes for 2 hr at 37 °C. Restriction digests were visualized via electrophoretic separation on a 1% agarose-TAE gel supplemented with 1X SYBR-safe DNA gel stain at 120 V for 45 min. Plasmids without large deletions will resolve to two distinct bands at approximately 8,700 and 4,300 bp.

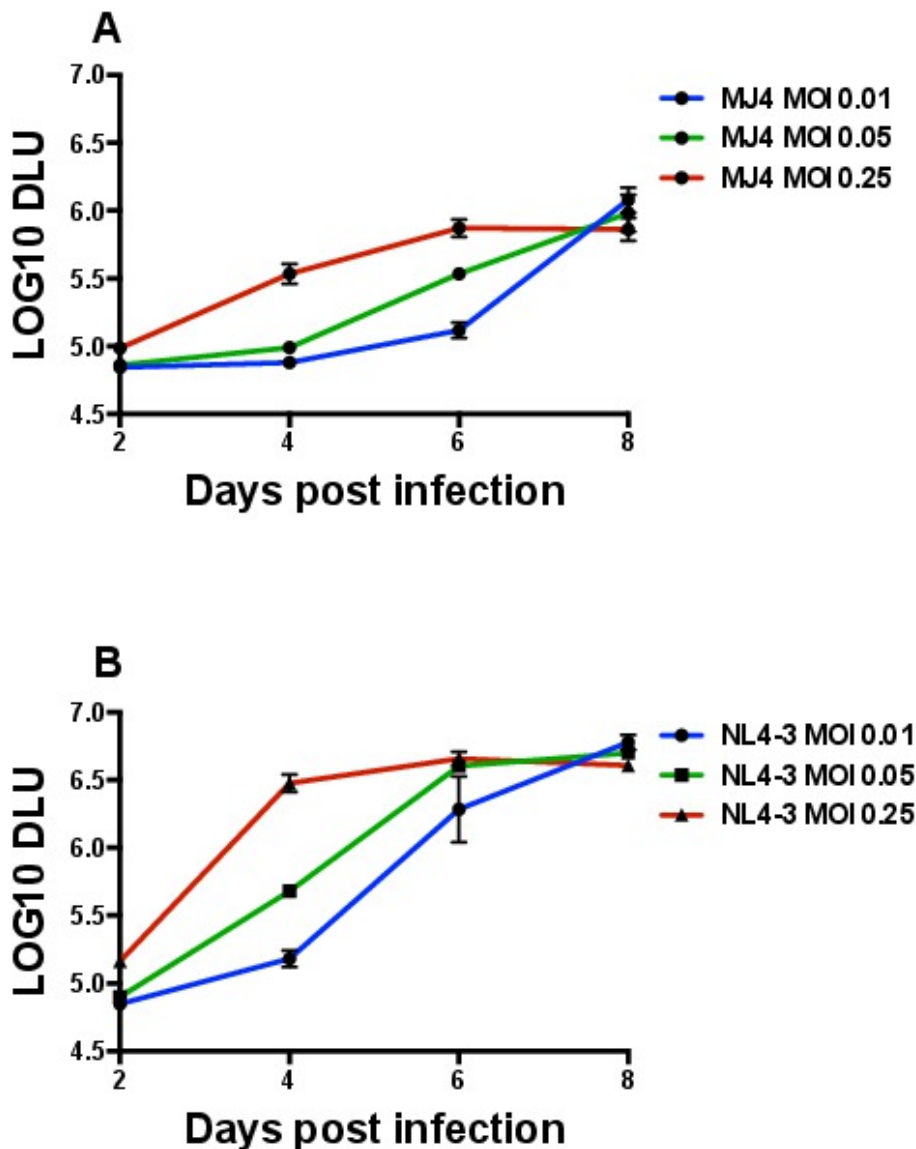


Figure 4. Replication of MJ4 and NL4-3 isolates of HIV-1 in the GXR25 cell line at different multiplicities of infection (MOI). 5×10^5 GXR25 cells were infected as described in the method protocol with 5-fold increasing MOI of each virus stock. Supernatants were collected on days 2, 4, 6, and 8 post infection and virion production was quantified via a radiolabeled reverse transcriptase assay. Infections were run in triplicate and error bars denote the standard deviation for the three replicates. **(A)** MJ4 **(B)** NL4-3.

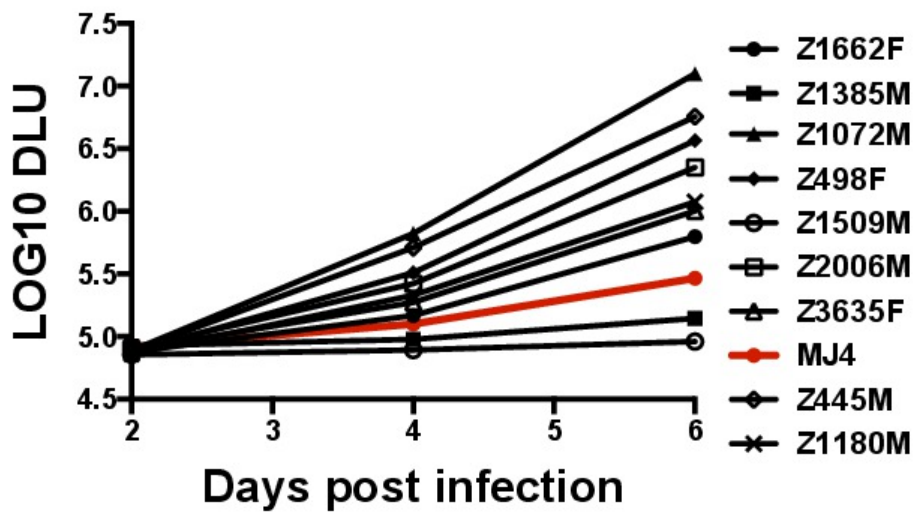


Figure 5. Representative range of replication for different Gag-MJ4 chimeras. As described in the method protocol, 5×10^5 GXR25 cells were infected with wild-type MJ4 or Gag-MJ4 chimeras at an MOI of 0.05, supernatants were collected at two day intervals post infection, and virion production quantified by a radiolabeled RT assay. Insertion of various subtype C derived *gag* genes can have a dramatic impact on the replication capacity of MJ4. Wild-type MJ4 replication is denoted in red.

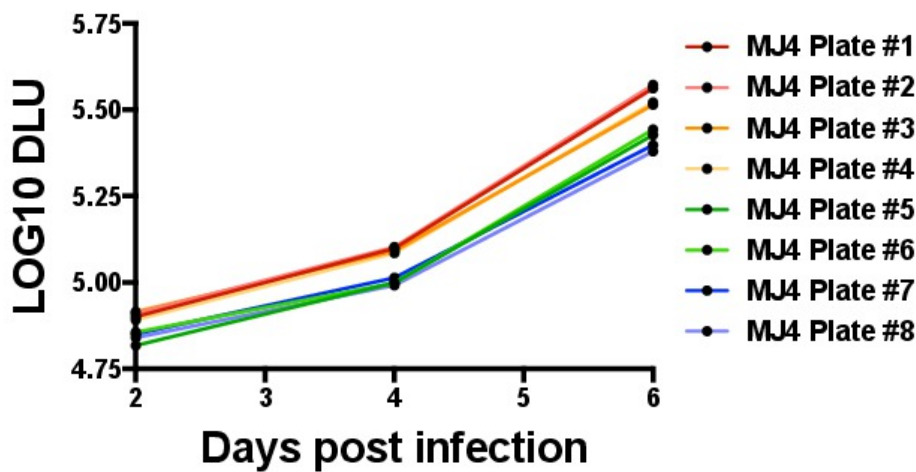


Figure 6. Comparison of intra-assay variation in the radiolabeled reverse transcriptase (RT) quantification assay. The graph depicts inherent intra-assay variability by plotting the DLU values of the same supernatants from a single wild-type MJ4 infection in eight different RT assay plates. The variation in curves reflects the slight changes in signal magnitude between plates, which can be corrected for by running a standard on each RT plate, which can be subsequently used to normalize the slopes of Gag-MJ4 chimeras assayed on the same plate.

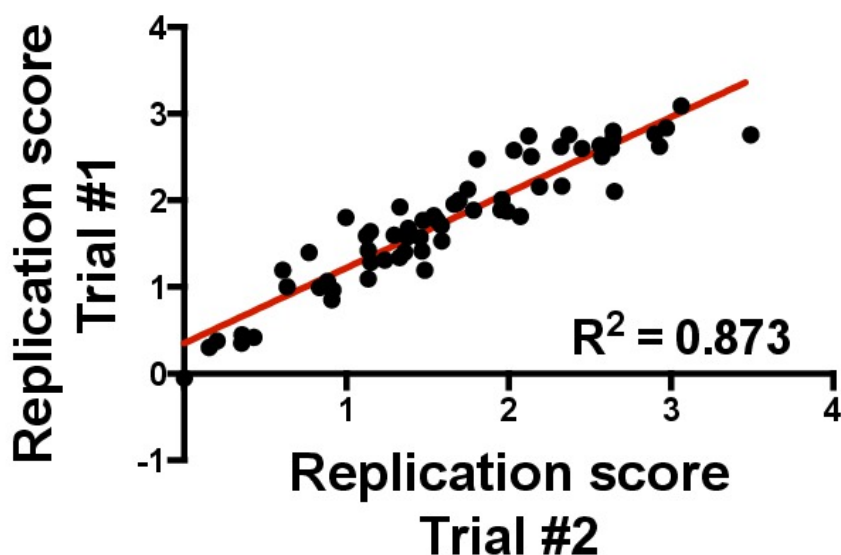


Figure 7. Reproducibility of the replication assay over time in the GXR25 cell line. The same Gag-MJ4 chimeric viruses were used to infect GXR25 cells in two independent experiments performed approximately one year apart. Replication scores were generated by calculating the slope of log-transformed DLU values and normalizing that slope to wild-type MJ4. Gag-MJ4 chimeras that replicate more efficiently than wild-type MJ4 have replication scores greater than 1, and those that replicate less efficiently than wild-type MJ4 have replication scores less than 1. The two independent measurements are strongly correlated ($R^2 = 0.87$, linear regression) and highlight the reproducibility of assays performed at different times and with cells at different passages.

A)

Reagent	Volume for 1x reaction (μl)
2x Reaction Mix (Invitrogen)	25
Nuclease-free H ₂ O	17
Forward primer GOF (20 μM concentration)	1
Reverse primer VifOR (20 μM concentration)	1
SuperScript III One-step Enzyme Mix	1
RNA template	5
Total volume	50

B)

Number of Cycles	Time (hr:min:sec)	Temperature (°C)
1	1:00:00	50
1	2:00	94
10	0:15	94
	0:30	56
	5:00	68
40	0:15	94
	0:30	56
	5:00 + 5 sec/cycle	68
1	12:00	68
1	∞	4
		END

Table 1. A) Master mix and B) thermocycler conditions for first-round gag amplification. *Note: GOF primer sequence: 5'-ATTTGACTAGCGGAGGCTAGAA-3'. VifOR primer sequence: 5'-TTCTACGGAGACTCCATGACCC-3'.

A)

Reagent	Volume for 1x reaction (μl)
Nuclease-free H ₂ O	35.5
5x Phusion HF Buffer	10
dNTPs (40 mM deoxynucleotides)	1
Forward primer GagInnerF1 (20 μM concentration)	1
Reverse primer BclIDegRev2 (20 μM concentration)	1
Phusion Hot Start II Polymerase	0.5
First-round PCR as template	1
Total volume	50

B)

Number of Cycles	Time (hr:min:sec)	Temperature (°C)
1	0:30	98
29	0:10	98
	0:30	53
	1:00	72
1	10:00	72
1	∞	4
		END

Table 2. A) Master mix and **B)** thermocycler conditions for nested second-round *gag* amplification. *Note: GagInnerF1 primer sequence: 5'-AGGCTAGAAGGAGAGAGATG-3'. BclIDegRev2 primer sequence: 5'-AGTATTTGATCATAYTGYYTYACTTTR-3'.

A)

Reagent	Volume for 1x reaction (μl)
Nuclease-free H ₂ O	35.5
5x Phusion HF Buffer	10
dNTPs (40 mM deoxynucleotides)	1
Forward primer MJ4For1b (20 μM concentration)	1
Reverse primer MJ4Rev (20 μM concentration)	1
Phusion Hot Start II Polymerase	0.5
MJ4 plasmid as template (10 ng/ul)	1
Total volume	50

B)

Number of Cycles	Time (hr:min:sec)	Temperature (°C)
1	0:30	98
29	0:10	98
	0:30	58
	0:45	72
1	10:00	72
1	∞	4
		END

Table 3. A) Master mix and **B)** thermocycler conditions for 5' MJ4 LTR amplification. *Note: MJ4For1b primer sequence: 5'-CGAAATCGGCAAAATCCC-3'. MJ4Rev primer sequence: 5'-CCCATCTCTCTCCTTCTAGC-3'.

A)

Reagent	Volume for 1x reaction (µl)
Nuclease-free H ₂ O	34.5
5x Phusion HF Buffer	10
dNTPs (40 mM deoxynucleotides)	1
Forward primer MJ4For1b (20 µM concentration)	1
Reverse primer BclIRev (20 µM concentration)	1
MJ4 LTR 1.3 kb amplicon (Gel purified, ~50 ng)	1
Phusion Hot Start II Polymerase	0.5
Gag amplicon (Gel purified, ~100 ng)	1
Total volume	50

B)

Number of Cycles	Time (hr:min:sec)	Temperature (°C)
1	0:30	98
29	0:10	98
	0:30	58
	1:30	72
1	10:00	72
1	∞	4
		END

Table 4. A) Master mix and B) thermocycler conditions for splice-overlap-extension PCR to generate LTR-gag inserts. *Note: BclIRev primer sequence: 5'-TCTATAAGTATTTGATCATACTGTCTT-3'

A)

Reagent	Volume for 1x reaction (µl)	Incubation Time (hr)	Incubation Temperature (°C)
1.5 µg of 3 kb LTR-gag amplicon or MJ4 plasmid	x µl for 1.5 µg		
NEB CutSmart Buffer (previously NEB Buffer #4)	2		
BclI restriction enzyme	1		
Nuclease-free H ₂ O	x		
Total volume	19	1.5	50
NgoMIV restriction enzyme	1		
Total volume	20	1	37

B)

Reagent	Volume for 1x reaction (µl)	Incubation Time (hr)	Incubation Temperature (°C)
50 ng cut MJ4 plasmid vector	x µl for 50 ng		
45 ng cut LTR-gag insert (3:1 ratio)	x µl for 45 ng		
Roche 10x ligase buffer	2		
Roche T4 DNA ligase (5 U/µl)	1		
Nuclease-free H ₂ O	x		
Total volume	20	18+ (overnight)	4

C)

Reagent	Volume for 1x reaction (µl)	Incubation Time (hr)	Incubation Temperature (°C)
450 ng of Gag-MJ4 plasmid	x µl for 450 ng		
NEB CutSmart Buffer (previously NEB Buffer #4)	2		
NgoMIV restriction enzyme	0.5		
HpaI restriction enzyme	0.5		
Nuclease-free H ₂ O	x		
Total volume	20	2	37

Table 5. A) Restriction digest master mix and **B)** ligation reaction for generation of Gag-MJ4 chimeric provirus. **C)** Diagnostic restriction digest to ensure Gag-MJ4 cloning fidelity.

Primer Name	Nucleotide Sequence (5' - 3')
GagInnerF1	AGGCTAGAAGGAGAGAGATG
GagF2	GGGACATCAAGCAGCCAT
For3	CTAGGAAAAAGGGCTGTTGGAAATG
GagR6	CTGTATCATCTGCTCCTG
Rev3	GACAGGGCTATACATTCTTACTAT
Rev1	AATTTTCCAGCTCCCTGCTTGCCCA

Table 6. List of sequencing primers necessary to confirm 5' LTR and *gag* sequence identity of Gag-MJ4 chimeric provirus.

Well A	8 µl virus + 232 µl 1% FBS in DMEM
Well B	80 µl from Well A + 160 µl 1% FBS in DMEM
Well C	80 µl from Well B + 160 µl 1% FBS in DMEM
Well D	80 µl from Well C + 160 µl 1% FBS in DMEM
Well E	80 µl from Well D + 160 µl 1% FBS in DMEM
Well F	80 µl from Well E + 160 µl 1% FBS in DMEM

Table 7. Dilution scheme (3-fold) for titrating infectious viruses on TZM-bl cells.

A)

Reagent	Volume
PBS without Ca ²⁺ or Mg ²⁺	500 ml
Glutaraldehyde	4 ml
Formaldehyde	11 ml

*Note: Store at 4 °C.

B)

Reagent	Volume
PBS without Ca ²⁺ or Mg ²⁺	4.75 ml
Potassium ferricyanide (0.2 M)	100 µl
Potassium ferrocyanide (0.2 M)	100 µl
Magnesium chloride (1 M)	20 µl
X-gal (50 mg/ml)	40 µl

*Note: Make fresh and store away from light until use.

C.

Original Dilution well	A	B	C	D	E	F
Volume of virus (μ l) added to the wells of a 24-well plate row	5	1.6667	0.5556	0.1852	0.06173	0.02057

Table 8. A) Staining and **B)** fixing solutions for titering of Gag-MJ4 chimeric viruses on the TZM-bl indicator cell line. **C)** The volume of virus added per well for calculating infectious units/ μ l.

Reagent	Volume
Fetal bovine serum (FBS), defined	55 ml
Penicillin, Streptomycin, Glutamine (100x)	6 ml
HEPES buffer (1 M)	6 ml

Table 9. Recipe for complete RPMI medium for propagation of GXR25 cells.

Reagent	Volume (ml)
Nuclease-free H ₂ O	419.5
Tris-Cl, pH 7.8 (1 M)	30
Potassium chloride (1 M)	37.5
Magnesium chloride (1 M)	2.5
Nonidet P-40 (10%)	5
EDTA (0.5 M)	1.02
Polyadenylic acid, potassium salt (2 mg/ml)	1.25
Oligo-dT primer (25 μ g/ml)	3.25
Total volume	500

Table 10. Recipe for radiolabeled reverse-transcriptase assay master mix. *Note: Store as 1 ml aliquots at -20 °C.

Discussion

Due to the length and technical nature of this protocol, there are several steps that are critical for both the successful construction of chimeric Gag-MJ4 plasmids as well as for quantification of viral replication capacity. Although the restriction enzyme based cloning strategy for the introduction of foreign *gag* genes into MJ4 outlined in this protocol has numerous advantages over previously used recombination based methods, the protocol can be technically challenging if critical steps are not followed precisely.

First, it is absolutely essential to use MJ4 plasmid DNA that has been generated in a competent bacterial strain lacking the *dcm* and *dam* DNA methylases. This is necessary as the enzymatic activity of the *Bcl*I restriction endonuclease, which is used to clone *gag* genes into the MJ4 backbone, is *dam/dcm* methylation sensitive. The JM110 and SCS110 (an *endA* negative JM110 derivative) *E. coli* strains are suitable for generating unmethylated MJ4 plasmid DNA. Additionally, for the excision of vector and insert bands, it is highly recommended that a blue light illuminator be used for visualization. This will reduce UV wavelength-dependent DNA damage and drastically increase cloning efficiency. If a blue light illuminator is unavailable, cloning efficiency can be maximized by visualizing DNA with SYBR Safe DNA gel stain instead of ethidium bromide and minimizing UV exposure time.

Finally, molecular cloning with large (>10kb) and/or retroviral plasmids such as MJ4 has traditionally been difficult for a variety of reasons. Large plasmids reduce transformation efficiency of competent bacterial strains⁴² while retroviral inserts, which contain long terminal repeat (LTR) sequences, reduce stability of the plasmid and compromise replication fidelity of the plasmid DNA within the bacterial host leading to deletions of the retroviral genome⁴³. Bacteria transformed with MJ4 or Gag-MJ4 plasmid products must be grown at 30 °C rather than the traditional 37 °C for most protocols. Recovery steps after heat shock transformation, growth of transformed bacteria on agar plates, and growth of bacterial colonies in liquid culture should all be performed at 30 °C. This lower temperature reduces the growth rate of the bacteria and thus helps to ensure replication fidelity of the MJ4 plasmid. Additionally, replication of the MJ4 plasmid is more stable when using the JM109 *E. coli* strain over the DH5 α strain, in our hands. Due to unstable nature of the plasmid, purified plasmid products should always be checked for correct plasmid size by restriction enzyme digestion; here, a double digest with the *Ngo*MIV and *Hpa*I restriction endonucleases at 37 °C for 2 hr.

Once successful generation of chimeric Gag-MJ4 plasmids has been accomplished, virus is generated via transfection of 293T cells, titered on an indicator cell line, TZM-bl cells, and replication capacity is measured using a CEM-based T cell line. The CEM-based GXR25 cell line used for these replication studies is one of the few established T cell lines able to support entry and replication of CCR5-tropic strains of HIV-1. This has been achieved by retroviral transduction to allow stable expression of human CCR5³⁷. This cell line naturally expresses CXCR4 and will support replication of CXCR4-tropic HIV-1, such as the laboratory-adapted strain NL4-3. However, in order to support efficient replication of CCR5-tropic strains, such as MJ4, the GXR25 cells must be propagated for no less than 4 months prior to infection. Properly passaged cultures can support

replication even after passaging for up to 1 year. Careful monitoring of CCR5-tropic replication throughout passaging is essential for successful experiments.

As with any technique, there are limitations to the protocol that must be considered. Due to the location of restriction sites in the MJ4 plasmid as well as the availability of conserved restriction sites in naturally occurring HIV-1 isolates, the 3' distal restriction site, BclI, is located 137 nucleotides from the *gag* stop codon. Although this generates a chimeric *protease* gene, this region is 96.5% conserved in this cohort, and we did not observe an abundance of dead or defective chimeric viruses.

One of the advantages of using the MJ4 subtype C infectious molecular clone with subtype C derived sequences is that it reduces the risk of suboptimal gene pairing between *gag* genes and backbone vectors of different subtypes. However, a certain amount of within-clade diversity exists as evidenced by the clustering of HIV-1 sequences by country or region even when found within the same subtype³¹. This could contribute to suboptimal pairing between the *gag* genes derived from acutely infected Zambians and the MJ4 infectious molecular clone backbone, which was derived from a chronically infected individual from Botswana³⁸. However, a majority of the analyzed constructs produced infectious progeny virus. As different HIV-1 subtype C infectious molecular clones become more widely available, it will be important to further validate this system by cloning in these HIV-1 clade C *gag* genes into other backbones in order to ensure that there is a minimal bias introduced due to backbone incompatibilities.

The GXR25 cell line is a unique cell line, specifically due to its ability to support both CXCR4 and CCR5-tropic strains and its HIV-1 inducible GFP reporter³⁷. However, some limitations exist and should be carefully considered before using this cell line for experiments adapted from this protocol. The GXR25 cell line does not appear to support entry of a majority of subtype C or A, CCR5-tropic, primary isolates we have tested. Additionally, the parent CEM cell line from which the GXR25 cell line was derived exhibits high levels of cyclophilin A, up to 2 to 4-fold higher expression than the Jurkat cell line⁴⁴. Due to high levels of cyclophilin A, the replication defect normally associated with the canonical HLA-B*57 associated escape mutation, T242N, which is attributed to a decreased ability of capsid to bind cyclophilin A, cannot be easily detected in this particular cell line²⁵. Thus the CEM-based GXR25 cell line is not ideal for studying replication defects associated with mutations in the HIV-1 capsid cyclophilin-binding loop.

Finally, while this protocol is ideal for analyzing the replication capacity of *gag* sequences derived from acute time points, modifications must be made in order to study the replication capacity of *gag* genes derived from chronically infected individuals. This protocol involves the amplification of population sequences from acute time points (median 45 days post estimated date of infection) when viral diversity is limited. The *gag* gene from each chimera is then sequenced and compared to the initial population PCR amplicon to ensure cloning fidelity. Due to limited sequence diversity at acute time points, cloning from population PCR products is possible. However, sequence diversity exists within the viral quasispecies of a chronically infected individual; therefore, in order to accurately assess the replication capacity of the chronic quasispecies, single genome amplification must be employed to capture several representative variants. Each of these variants must then be assayed for *in vitro* replication.

This technique has several broader applications, which stem from its advantages over existing methods. Since this process results in a clonal replication competent plasmid, it is simple to use constructs for additional mutagenesis studies, which can help to elucidate the contributions of specific residues to viral replication. Furthermore, by cloning in *gag* genes from longitudinal time points, one can assess the evolution of viral replication capacity over time and how these changes may affect pathogenesis in an HIV-1 infected individual.

This technique can be modified in order to expand its utility for different applications. Additional HIV-1 viral proteins can be engineered into the MJ4 plasmid in order to assess their effects on viral replication or their interactions with host proteins. This can be accomplished by engineering additional restriction sites at desired regions in the genome through the introduction of silent nucleotide changes. However, special consideration must be taken when engineering novel restriction sites into the 3' half of the HIV-1 genome as many accessory proteins are encoded in alternate reading frames, and silent changes in one protein could lead to amino acid substitutions in another. In this instance, restriction site independent cloning methods such as those discussed by Dudley *et al.*⁴⁵ may overcome this limitation. Viral sequences derived from different populations may have varying ranges of replication capacities, and the MOI can be adjusted accordingly in order to capture the majority of viral isolates within their logarithmic phase of growth. Finally, the GXR25 cell line has been stably transfected with GFP under an LTR-driven, Tat-inducible promoter, and viral spread can be measured as a function of GFP positive cells via flow cytometry³⁷ as an alternative to the RT assay described here or a traditional p24 ELISA.

In conclusion, this protocol provides an efficient and powerful technique for assessing HIV-1 viral replication as conferred by the *gag* gene, which encodes a conserved structural protein necessary for proper virion formation, budding, maturation, and disassembly⁴⁶⁻⁴⁸. Furthermore, this technique generates a replication competent clonal plasmid, which is ideal for mutagenesis studies and, thus, provides a method for elucidating the specific amino acid determinants of viral fitness. Studies such as these are imperative to enhance the understanding of how immune-driven viral evolution affects pathogenesis and disease progression in HIV-1 infected individuals.

Disclosures

The authors have nothing to disclose.

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