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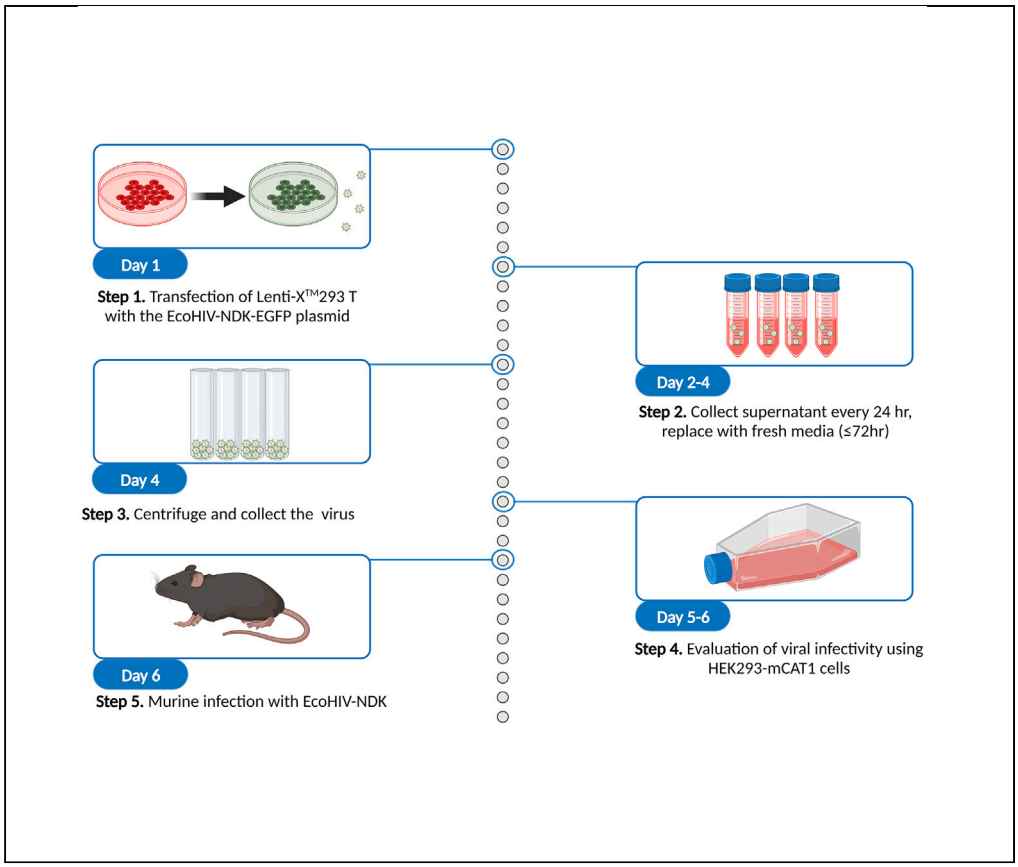
Protocol for optimizing production and quality control of infective EcoHIV virions



EcoHIV is a model of HIV infection that recapitulates aspects of HIV-1 pathology in mice. However, there are limited published protocols to guide EcoHIV virion production. Here, we present a protocol for producing infective EcoHIV virions and essential quality controls. We describe steps for viral purification, titering, and multiple techniques to analyze infection efficacy. This protocol produces high infectivity in C57BL/6 mice which will aid investigators in generating preclinical data.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Hammodah R. Alfar, Daphne N. Pariser, Harry Chanzu, ..., David J. Volsky, Kelly Metcalf-Pate, Sidney W. Whiteheart

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Highlights

Improved EcoHIV preparation for optimal acute and chronic infection

Enhanced infectivity rate with ~100% of mice infected and actively producing EcoHIV

Multiple quality controls to calculate the virus titer and check viral infectivity

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Protocol for optimizing production and quality control of infective EcoHIV virions

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SUMMARY

EcoHIV is a model of HIV infection that recapitulates aspects of HIV-1 pathology in mice. However, there are limited published protocols to guide EcoHIV virion production. Here, we present a protocol for producing infective EcoHIV virions and essential quality controls. We describe steps for viral purification, titering, and multiple techniques to analyze infection efficacy. This protocol produces high infectivity in C57BL/6 mice which will aid investigators in generating preclinical data.

BEFORE YOU BEGIN

1. Investigators should note that when working with EcoHIV, a Material Transfer Agreement (MTA) must be procured from Dr. David Volsky at Icahn School of Medicine at Mount Sinai.
2. This protocol is optimized for C57BL/6J mice.
3. The entire procedure should be performed under sterile conditions in a biosafety cabinet (excluding plasmid amplification and purification).
4. HEK293-mCAT1 cells were kindly provided by Dr. Alan Rein at the National Cancer Institute (NCI).

Institutional permissions

A protocol for the infection of EcoHIV in mice and working with infected mouse cells requires the approval of your local Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee (IBC) or equivalent. All animal work in this protocol was performed according to the protocols approved by the IACUCs and IBCs at the University of Kentucky.

Cell line culture

Timing: 30–60 min (for steps 5 to 7)

It is essential to follow the general guidelines for cell culture and perform all cell cultures in a microbiological safety cabinet using aseptic techniques. This section outlines the general steps for cell culture.

5. Plating frozen cells (Lenti-X™ 293T and HEK293-mCAT1 cells).
   a. Warm the growing media at 37°C for at least 20 min.
   b. Add 20 mL growing media to each 150 × 20 mm tissue culture-treated dish.
   c. Remove frozen cells from the liquid nitrogen tank and warm an aliquot at 37°C for 1 min.
   d. Add 3 × 10^6 cells/mL to the dish and incubate for 24 h at 37°C and 5% CO_2.
   e. Next day, aspirate the media and add 20 mL fresh growing media to remove DMSO in the freezing media and cell debris.

   Optional: If the cells need to be resuspended in a smaller dish and volume, the manufacturer of Lenti-X™ 293T cells (Takara) recommends doing a low-speed spin (100 × *g* for 5 min at RT) and gently resuspending the cell pellets with the growing media to prevent disruption of the cell membrane.

   ⚠ CRITICAL: While freezing the cells should be done slowly (e.g., in Mr. Frosty), thawing the cells must be as quick as possible.

6. Passaging cells (Lenti-X™ 293T cells and HEK293-mCAT1).
   a. Warm the growing media and 0.25% trypsin-EDTA at 37°C for at least 20 min.
   b. Aspirate the media and incubate with 1 mL of 0.25% trypsin-EDTA for 1 min.
   c. Add 4 mL of the growing media to neutralize the 0.25% trypsin-EDTA action and use the pipet to detach any remaining adherent cells.
   d. Transfer the cell suspension into a 15 mL tube and centrifuge at 500 × *g* for 6 min.
   e. Aspirate the media and use 1 mL of fresh growing media to resuspend the cell pellet.
   f. Take 10 μL of the resuspended cells and add 10 μL of 0.4% trypan blue to count the cells and assess viability.
   g. Add 20 mL growing media to 150 × 20 mm tissue culture-treated dishes.
   h. Plate 1 × 10^5 cells/mL by adding the appropriate volume of the resuspended cells to the culture dishes.
7. Freezing cells.
   a. Harvest the cells as described above and adjust the concentration to 6 × 10^6 cells/mL.
   b. Add one volume of 2 X cell freezing media for each volume of the cell suspension (e.g., 500 μL of cell suspension and 500 μL of 2 X cell freezing media).
   c. Aliquot 1 mL into a cryogenic vial.
   d. Place the cryogenic vial in Mr.Frosty and store it at −80°C.

   ⚠ CRITICAL: It is very important to store frozen cells in liquid nitrogen for long-term storage.

   Optional: We recommend passaging the cells at least three times before trying to use them for viral production and avoid cells that have been passaged >15 times.[1]

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial strain		
Subcloning Efficiency™ DH5α Competent Cells	Thermo Fisher Scientific	Cat# 18265017
Experimental models: Cell lines		
Lenti-X™ 293T Cell Line	Takara	Cat# 632180
HEK293-mCAT1	Dr. Alan Rein (NCI)	N/A
Experimental models: Organisms/strains		
Male and female (8–12 weeks old) C57BL/6J mice	Jackson Laboratory	Cat# 000664

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
Plasmids: 1- EcoHIV/NL4/3 2- EcoHIV/NL4/3-EGFP 3- EcoHIV-NDK-EGFP 4- EcoHIV-NDK 5- Gag standard (for RT-qPCR) 6- Spliced vif standard (for RT-qPCR)	Drs. Mary Jane Potash, Jennifer Kelschenbach and David J. Volsky (Icahn School of Medicine at Mount Sinai)	N/A
Chemicals, peptides, and recombinant proteins		
PBS	Quality Biological	Cat# 119-069-131
HindIII HF®	New England Biolabs	Cat# R3104M
Cut smart® buffer 10 X	New England Biolabs	Cat# B7204
Agarose LE	Agarose Products	Cat# AP105
DNA molecular weight ladder (1 kb)	New England Biolabs	Cat# N3232L
Tris base	Fisher Scientific	BP152-10
Boric acid	Fisher Scientific	BP168-1
Na_2EDTA	Sigma-Aldrich	ED2SS-500G
Ethidium bromide solution, MP Biomedicals	Fisher Scientific	Cat# 50-841-663
Fisher Science Education™ Potassium Bicarbonate	Fisher Scientific	Cat# S25475A
Ammonium chloride	Fisher Scientific	Cat# A661-500
Lennox L agar	Invitrogen	Cat# 22700-041
Bacto-yeast extract	VWR	Cat# J850
Bacto-Tryptone	Gibco	Cat# 211699
NaCl	Macron Fine Chemicals	Cat# 7581-06
DMEM, high glucose, HEPES	Thermo Fisher Scientific	Cat# 12430047
Fetal bovine serum (FBS), sterile, USDA approved, suitable for cell culture, liquid	Sigma-Aldrich	Cat# F0926
Trypsin-EDTA (0.25%), phenol red	Thermo Fisher Scientific	Cat# 25200072
Antibiotic antimycotic solution (100 X), stabilized	Sigma-Aldrich	Cat# A5955
Gibco™ L-Glutamine (200 mM)	Fisher Scientific	Cat# 25030081
DMSO	Thermo Fisher Scientific	Cat# D12345
Gibco™ Sodium pyruvate (100 mM)	Fisher Scientific	Cat# 11-360-070
Gibco™ MEM Non-Essential Amino Acids Solution (100 X)	Fisher Scientific	Cat# 11-140-050
PEI MAX - Transfection Grade Linear Polyethyleneimine Hydrochloride (MW 40,000)	Polysciences	Cat# 24765-100
Trypan blue, 0.4%	Thermo Fisher Scientific	Cat# T8154
PEG-8000	Fluka Biochemika	Cat# 81268
Applied Biosystems™ TaqMan™ Fast Virus One Step Master Mix	Fisher Scientific	Cat# 44-444-36
RNAlater	Thermo Fisher Scientific	Cat# AM7020
Zombie Red™ Fixable Viability Kit	BioLegend	Cat# 423109
Ethanol, 200 proof (100%), USP, Decon™ Labs	Fisher Scientific	Cat# 04-355-223
Oligonucleotides		
Gene	Forward and reverse primers	Probe
MLV envelope protein Saini et al.[2]	5′-GGCCAAACCCCGTTCTG-3′ and 5′-ACTTAACAGGTTTGGGCTTGGA-3′	5′-CAGACCAACAGCCACT-3′
EcoNDK Gag Hadas et al.[3]	5′ –TGGGACCACAGGCTACACTAGA–3′ and 5′ –CAGCCAAAACTCTTGCTTTA TGG–3′	5′ –TGATGACAGCATGCCAG GGAGTGG–3′
Spliced vif transcript for EcoNDK Hadas et al.[3]	5′ –AAGAGGCGAGGGGCAGCGA–3′ and 5′ –TCTTTACTTTTCTTCTTGGTA CTACCTTTATG–3′	5′ –AGTA GTAATACAAGA CAATAGTG–3′

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Double-spliced HIV/NDK nef transcript He et al.[4]	5′GAGTGAAAAATCTCTAGCAGTGG CGC-3′ and 5′-GCTGAAGAGGCACA GGTTCCTCAGGTCG-3′	5′GAAGAAGCGGAGACAGC GACGAAAACCTCC3′
Spliced tat transcript for EcoNDK Omeragic et al.[5]	5′CCTAGG ACTGCTTGT AATAAG TGT3′ and 5′GTCGGG TCCCCTCGGGACTGG GAG-3′	5′-AAAGGCTTAGGC ATCTC3′
Software		
BD FACSDiva software	BD Biosciences	https://www.bdbiosciences.com/en-eu
Image Lab software	Bio-Rad	https://www.bio-rad.com/en-us/product/image-lab-software?ID=KRE6P5E8Z
FlowJo 10.8.1	FlowJo	https://www.flowjo.com/
Gen 5 Microplate Reader and Imager Software	Agilent	https://www.biotek.com/products/software-robotics-software/gen5-microplate-reader-and-imager-software/
GraphPad Prism Software 9.3.1	GraphPad Prism Software, Inc.	https://www.graphpad.com/
NIS Elements Software 5.10.01 (Advanced Research)	Nikon	https://www.microscope.healthcare.nikon.com/products/software/nis-elements/nis-elements-advanced-research
Critical commercial assays		
Hispeed plasmid Maxi kit	Qiagen	Cat# 12662
ZeptoMetrix RETROtek HIV-1 p24 Antigen ELISA	ZeptoMetrix	Cat# 0801111
PureLink™ RNA Mini Kit	Thermo Fisher Scientific	Cat# 12183018A
Other		
Heavy-duty orbital incubator/shaker	New Brunswick Scientific	Innova 4200 incubator shaker
Nalgene™ General Long-Term Storage Cryogenic Tubes Cryogenic vial	Thermo Fisher Scientific	Cat# 5000-1020
BD™ Needle 1/2 in. single use, sterile, 27 G (For retro-orbital injection)	BD Biosciences	Cat# 305109
ChemiDoc Imaging System	Bio-Rad	Cat# 12003153
Sterile Petri dish (100 × 15 mm)	VWR	Cat# 25384-302
Sterile Petri dish (35 × 10 mm)	CELLTREAT	Cat# 229635
Owl™ EasyCast™ B2 Mini Gel Electrophoresis Systems	ThermoFisher Scientific	Cat# B2-BP
pluriStrainer Mini 100 µm (Cell Strainer)	pluriSelect	Cat# 43-10100-60
BD 1 mL TB syringes	BD Biosciences	Cat# 309659
NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer	Thermo Fisher Scientific	Cat# 701-058112
Fluorescence plate reader	Agilent	Cytation 5 Cell Imaging Multimode Reader
Countess® II FL Automated Cell Counter	Thermo Fisher Scientific	Cat# AMQAF1000
Countess™ Cell Counting Chamber Slides	Thermo Fisher Scientific	Cat# C10283
Mr. Frosty™ Freezing Container	Thermo Fisher Scientific	Cat# 5100-0001
Water bath	Fisher Scientific	Cat# FSGPD05
Bel-Art™ SP Scienceware™ Cleanware™ Aqua-Clear™ Water Conditioner	Fisher Scientific	Cat# 23-278339
150 × 20 mm tissue culture treated dish, sterile	CELLTREAT Scientific Products	Cat# 229651

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Disposable/round bottom/sterile 17 × 100 mm culture tubes with closures	VWR	Cat# 60818-689
Tabletop centrifuge	Beckman	Avanti J-15R high-performance refrigerated benchtop centrifuge
Beckman ultracentrifuge	Beckman	Optima Ultra XPN-90
SW 28 Ti Swinging-Bucket Aluminum Rotor Package	Beckman	Cat# 342204
Beckman Coulter 38.5 mL, Open-Top Thin wall Ultra-Clear Tube, 25 × 89 mm	Fisher Scientific	Cat# NC9146666
Nunc™ F96 MicroWell™ Black and White Polystyrene Plate	Thermo Fisher Scientific	Cat# 237105
MicroAmp™ Optical 96-Well Reaction Plate with Barcode	Applied Biosystems	Cat# 4306737
MicroAmp™ Optical Adhesive Film	Applied Biosystems	Cat# 4311971
Eclipse Ti2 inverted microscope	Nikon	https://www.microscope.healthcare.nikon.com/products/inverted-microscopes/eclipse-ti2-series
Flow cytometer (BD Symphony A3)	BD Biosciences	https://www.bdbiosciences.com/en-us/products/instruments/flow-cytometers/research-cell-analyzers/bd-facsymphony-a3
Bio-Rad RT-qPCR	Bio-Rad	CFX Opus 96

MATERIALS AND EQUIPMENT

Recipes are provided to a specified final volume, but investigators may choose to prepare different volumes depending on the number of samples processed.

Luria Broth (LB) Media		
Reagent	Final concentration	Amount
NaCl	1%	5 *g*
Bacto™ Tryptone	1%	5 *g*
Yeast Extract	0.5%	2.5 *g*
Distilled H_2O	NA	Up to 500 mL

Note: Autoclave and store at RT until use.

Ampicillin resistance LB agar plates		
Reagent	Final concentration	Amount
LB media	NA	500 mL
Agar	1.5%	7.5 *g*
Ampicillin	100 µg/mL	500 µL of 100 mg/mL stock of Ampicillin

Note: Store at 4°C until use, preferably not exposed to light and upside down to prevent condensation of water on the agar.

Bacterial transformation		
Reagent	Final concentration	Amount
DH5α *E. coli*	NA	50 µL
EcoHIV plasmid (100 ng/ µL)	~2 ng/µL	1 µL

Note: Prepare immediately before use.

Bacterial Amplification		
Reagent	Final concentration	Amount
LB media	NA	1 L
Ampicillin	100 µg/mL	1 mL of 100 mg/mL stock of Ampicillin

Note: Autoclave and store at RT until use without ampicillin, which should be added before use.

Tris-Borate-EDTA buffer (TBE), (10 X)		
Reagent	Final concentration	Amount
Tris base	891.5 mM	108 *g*
Boric acid	889.5 mM	55 *g*
Na_2EDTA	25 mM	9.3 *g*
Distilled H_2O	NA	Up to 1 L

Note: Store at RT until use.

1% Agarose gel		
Reagent	Final concentration	Amount
1 X TBE buffer	1 X	100 mL
Agarose	1%	1 *g*
Ethidium Bromide	0.006%	6 µL

Note: Wrap the gel with a wet paper towel and plastic wrap. Store at 4°C until use.

Optional: If the use of Ethidium Bromide is limited, SYBR™ Safe DNA Gel Stain can be used according to the manufacturer's instructions.

Note: Add Ethidium Bromide after melting the agarose solution (in a microwave) and just before pouring the agarose into the Owl™ EasyCast™ B2 Mini Gel Electrophoresis Systems.

Note: Ethidium Bromide is carcinogenic, and the gel should be discarded according to each institution's regulations.

DNA agarose gel-loading dye (10 X)		
Reagent	Final concentration	Amount
Na_2EDTA	10 mM	37.2 mg
SDS	0.5%	50 mg
Bromophenol blue	0.25%	25 mg
Xylene cyanol	0.25%	25 mg
Glycerol	39%	3.9 mL
Distilled H_2O	NA	Up to 10 mL

Note: Aliquot and store at -20°C until use.

Lenti-X™ 293T and HEK293-mCAT1 cell's growing media		
Reagent	Final concentration	Amount
Dulbecco's Modified Eagle Medium (DMEM)	N/A	430 mL
Heat Inactivated Fetal Bovine Serum (FBS)	10%	50 mL
Sodium pyruvate (100 mM)	1 mM	5 mL
Gibco™ L-Glutamine (200 mM)	2 mM	5 mL
Gibco™ MEM Non-Essential Amino Acids Solution (100 X)	1 X	5 mL
Antibiotic Antimycotic Solution (100 X)	1 X	5 mL

Note: Store at 4°C for 1 month. To heat-inactivate FBS, incubate for 30 min at 56°C in a water bath.

Optional: The media can be filtered using a 0.2 μm filtration unit.

40% PEG-8000		
Reagent	Final concentration	Amount
PBS pH 7.4	N/A	Up to 50 mL
NaCl	1.2 M	3.5 *g*
PEG-8000	40%	20 *g*

Note: Store at 4°C for 1 month.

2 X cell freezing media		
Reagent	Final concentration	Amount
Growing media (see above)	1 X	30 mL
Heat inactivated Fetal Bovine Sera (FBS)	30%	15 mL
DMSO	10%	5 mL

Note: Store at 4°C for 1 month protected from light.

Ammonium Chloride Potassium (ACK) buffer		
Reagent	Final concentration	Amount
NH_4Cl	155 mM	8.29 *g*
$KHCO_3$	10 mM	1 *g*
Na_2EDTA	~100 mM	37 mg
Distilled H_2O	NA	Up to 1 L then adjust the pH to 7.2 with HCl

Note: Store at 4°C for 1 month.

STEP-BY-STEP METHOD DETAILS

EcoHIV plasmid amplification and purification

Timing: 3 days (for steps 1 to 3)

This section provides detailed steps for making agar plates, bacteria transformation and plasmid amplification. Furthermore, critical steps are provided, which can help researchers to troubleshoot their experiments.

⚠ CRITICAL: Before starting, ensure you have ample plasmid for the production of the EcoHIV. All of the EcoHIV plasmids used in this paper carry ampicillin resistance. The full plasmid maps can be requested from Drs. Mary Potash, David J. Volsky, or Jennifer Kelschenbach.

Note: There are companies that will do virus production, but because EcoHIV is replication-competent, this will limit the number of potential vendors.

1. Ampicillin-containing LB agar plates.
   Make the ampicillin-containing LB agar plates as follows:
   a. Make 500 mL of LB media.
   b. Add 7.5 *g* agar (15 *g*/L is the standard ratio).

c. Mix, but the agar will not dissolve.
d. Autoclave on liquid small cycle (20 min exposure).
e. Let the agar cool until it is warm but not hot to touch.
f. Add ampicillin (100 μg/mL, final).
g. Pour into plates approximately 25 mL/plate.
h. Let plates sit capped to solidify. This step can be done in a sterile hood if needed.
i. Once agar is solidified, cap the plates and store at 4°C until use, preferably not exposed to light and upside down to prevent water accumulation on the agar.

2. Bacterial transformation.

Note: The EcoHIV plasmids can be transformed into DH5α E. coli.

a. Clean the lab bench with 70% ethanol, and set the water bath at 42°C.
b. Thaw a 50 μL aliquot of DH5α E. coli on ice.
c. Add 1 μL of the plasmid (100 ng/μL of the plasmid should be enough) into the DH5α E. coli and keep it on ice for 30 min.
d. Heat shock the bacteria at 42°C for 1.5 min.

CRITICAL: Do not exceed 1.5 min as you might kill the bacteria.

e. Keep the cells on ice for 2 min.
f. Add the bacteria to the agar plate and spread them.
g. Incubate the plate upside down at 37°C overnight (maximum for 16 h).

3. Bacterial amplification and plasmid purification.
a. Next day, in the morning, pick one colony and add it to 4 mL autoclaved LB media supplemented with 100 μg/mL ampicillin in a sterile culture tube.
b. Incubate at 37°C with shaking at 250 rpm for 8 h.
c. Transfer the 4 mL autoclaved LB media containing the colony into 1 L autoclaved LB media with 100 μg/mL ampicillin and shake at 250 rpm at 37°C overnight.

Optional: Keep the closure loose on the flask to enhance aeration and bacterial growth.

Note: Next day, the LB media should be turbid.

d. Harvest the bacteria by centrifugation at 3,000 *g* for 30 min at 4°C.
e. We highly recommend using the Hispeed plasmid Maxi kit according to the manufacturer's instructions to isolate EcoHIV plasmids. (https://www.qiagen.com/us/resources/resourcedetail?id=52d67294-f2d7-4181-a532-7ea147b28b36&lang=en).
f. Measure the plasmid concentration using Nanodrop. Usually, 1 L of LB growth media yields ~700 μg–1 mg of the EcoHIV plasmids.

EcoHIV plasmid quality check

Timing: 4 h (for steps 4 to 5)

Before proceeding with EcoHIV production, it is critical to check the plasmid integrity and quality.

This section describes detailed steps to confirm the quality of the produced EcoHIV plasmids from the above section.

EcoHIV plasmids have many Hind III restriction sites that can generate a diagnostic restriction digest. Using Hind III, EcoHIV-NDK is cut into 6 fragments with the following sizes: 1086, 1172, 1786, 2686,

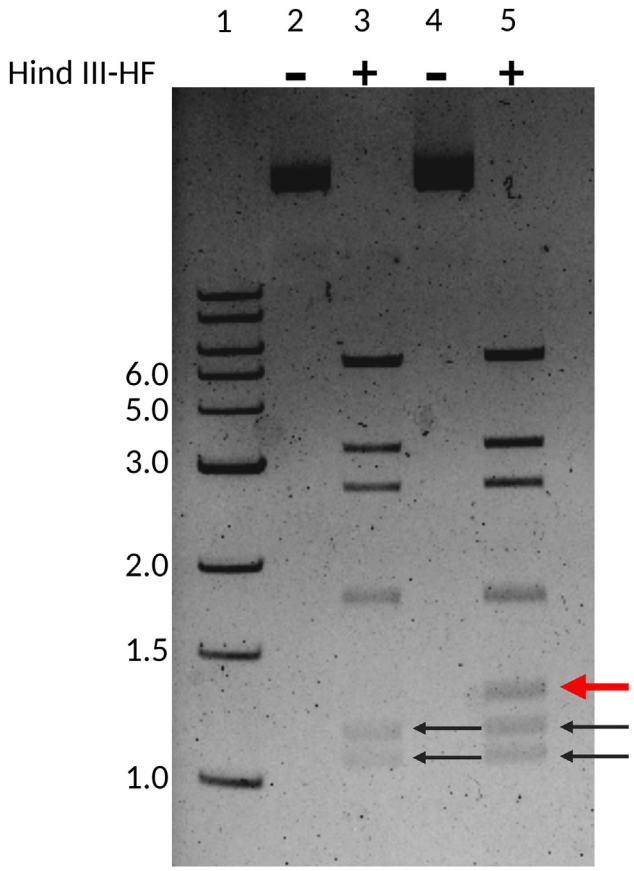

Figure 1. Diagnostic restriction digestions of EcoHIV-NDK and EcoHIV-NDK-EGFP plasmids

Mw ladder (lane 1). EcoHIV-NDK (lanes 2 and 3) and EcoHIV-NDK-EGFP (lanes 4 and 5) plasmids were either cut or not with Hind III-HF. While cutting EcoHIV-NDK plasmid results in 6 fragments (lane 3), cutting of EcoHIV-NDK-EGFP results in 7 fragments (lane 5). The black arrows indicate the shortest two fragments of both plasmids (1086 and 1172 bp). The bold red arrow refers to the extra fragment (1308 bp) that makes the difference between the two plasmids as a result of EGFP fragment insertion.

3235 and 5353 bp and EcoHIV-NDK-EGFP is cut into 7 fragments (1086, 1172,1308, 1786, 2686, 3235 and 5353 bp) (Figure 1).

4. Making 1% agarose gel.
   a. Add 100 mL of 1 X TBE buffer into an Erlenmeyer flask.
   b. Add 1 *g* agarose.
   c. Close the top of the Erlenmeyer flask with a paper towel.
   d. Microwave for 55 s to melt the agarose.
   e. Swirl the Erlenmeyer flask to mix the agarose.
   f. Microwave again for 15 s.
   g. Add 6 µL Ethidium Bromide and mix well.
   h. Pour the solution into the Owl™ EasyCast™ B2 Mini Gel Electrophoresis Systems, insert the appropriate gel comb, and let it set to solidify at RT.
5. Diagnostic restriction digest of the EcoHIV plasmids using Hind III-HF.
   a. For each plasmid label two Eppendorf tubes as (cut and no cut).
   b. Add 16 µL distilled H_2O to each tube.
   c. Add 2 µL of smart cut 10 X buffer to each tube.
   d. Add 1 µL of (160 ng/ µL) of the plasmid of interest to each tube.
   e. Add 1 µL of Hind III-HF to the "cut" tube and 1 µL distilled H_2O to the "uncut" tube.

f. Incubate at 37°C for 1.5 h.
g. Add 2.2 μL of 10 X DNA loading dye and load 10 μL into the 1% agarose gel.
h. Run the gel at 120 V for 1.5 h using 1 x TBE as the running buffer.
i. Image the gel using Ethidium Bromide filter using the ChemiDoc imaging system.

EcoHIV production

Timing: 5 days (for steps 6 to 15)

This section outlines the necessary steps for producing EcoHIV viruses and critical steps that should be followed to have a high viral titer.

6. Day 1: Plate 6×10^6 Lenti-X™ 293T cells in 150 mm × 20 mm tissue culture treated dish in 20 mL growing media without antibiotics.

⚠ CRITICAL: Cell viability must be >85% to get good viral titers.

⚠ CRITICAL: Cells should be 60–70% confluent before transfection.

Optional: We recommend passaging the cells at least three times before trying to use them for viral production and avoid cells that have been passaged >15 times.[1]

7. Day 2: For each plate, prepare 12 mL of DMEM with 18 μL of 40,000 PEI MAX (linear) (stock concentration: 5 mg/mL) and 30 μg of EcoHIV plasmid.

Note: We recommend a 1:3 ratio of PEI to plasmid for optimum EcoHIV production.

8. Vortex for 1 min then mix by inversion for 1 h at RT.
9. Remove the media from the plates with a sterile pipet and add 12 mL of the transfection media in a dropwise manner.

⚠ CRITICAL: Add the transfection media dropwise against the side wall of the dish and not directly on the cells to prevent cell detachment.

10. After 6 h, add 13 mL of growing media without antibiotics to the plate in a dropwise manner.

Note: If you are generating EcoHIV-NDK-EGFP, the transfected cells will be slightly green, and the intensity will increase after 24 h.

11. Collect the supernatant after 24 h.
12. Spin at 3,000 × *g* for 15 min at 4°C.
13. Store the supernatant for viral concentration at 4°C.

Note: EcoHIV is not sensitive to cold inactivation as we have tested the possibility of cold inactivation of the viral preparation after storing at 4°C (Data not shown).

14. Add 20 mL of growing media without antibiotics to the cells and incubate for 24 h.
15. Repeat steps 11–14 twice.

EcoHIV concentration

Timing: variable

Note: There are two methods to concentrate EcoHIV; ultracentrifugation and Polyethylene Glycol (PEG) precipitation. Both yield similar viral infectivity; however, a significant difference between them is the purity of the viral preparations. If an ultracentrifuge is available, it should be used to generate a viral batch with high purity especially if used with a sucrose pad or sucrose gradient. PEG precipitation can be used if the appropriate ultracentrifuges are not available. We will describe both methods here and show the ultracentrifugation method results as it is very commonly used, trickiest to do, and a pure virus preparation is needed for most experiments.

16. Ultracentrifugation to concentrate the viral preparations.

Note: Different viruses can tolerate centrifugation up to a certain g force without losing infectivity. For EcoHIV, we found that it can be centrifuged up to 35,300 × *g* for 3 h without losing infectivity (Data not shown).

To concentrate the EcoHIV with the ultracentrifuge:

a. Place SW28 rotor in the ultracentrifuge and cool it to 4°C before starting the run.
b. Add up to 35 mL of the media from infected cells into ultra-clear centrifuge tubes.

Optional: To increase the purity of the viral preparations:

i. Filter the supernatant through a 0.2 μm filtration unit.
ii. Use a sucrose pad (20% sucrose) or a sucrose gradient (20–70% sucrose) and add the filtered supernatant as described in step b.

⚠ CRITICAL: It is critical to add the supernatant slowly on the top of sucrose to prevent mixing the layers together.

c. Make sure the tubes are properly balanced.
d. Spin at 35,300 × *g* using the acceleration of 7 and brake of 7 (slow acceleration and slow brake) for 3 h.
e. After centrifugation, bring the viral preps to a tissue culture hood and discard the media via aspiration or by carefully and quickly dumping the media from the tube into the appropriate waste container containing bleach.
f. Add 350 μL of cold PBS to each tube and swirl gently to re-suspend the virus pellet (100-fold concentration; the viral pellet might not be visible).

Note: As mentioned above, EcoHIV is not sensitive to cold inactivation as we have tested the possibility of cold inactivation of the viral preparation after storing at 4°C (Data not shown).

g. Place the tubes on a rocker for 10 min at 4°C.
h. Pool the collected virus suspensions together and put them on the rocker again at 4°C for 5 min to mix well.
i. Aliquot the virus preparations (100 μL) into cryogenic vials.

Note: As recommended by the gene vector and virus core of Wu Tsai Neurosciences Institute at Stanford University, do not use polystyrene tubes to store the virus (use the cryogenic vials). Also, do not introduce air bubbles while you are suspending the viral pellet to keep the viral particles intact.

j. Snap-freeze the tubes in a methanol/dry ice bath or with liquid nitrogen.
k. Store at −80°C.
l. Next day, thaw an aliquot to calculate the virus titer.

⚠ CRITICAL: It is critical to calculate the virus titer using an aliquot that has been subjected to one freeze/thaw cycle so the virus titer can be assumed to be the same for the other frozen aliquots once they are thawed and ready to use.

⚠ CRITICAL: EcoHIV can tolerate one freeze/thaw cycle. Do not exceed more than one freeze/thaw cycle.

17. PEG-8000 precipitation to concentrate the viral preparations.

Note: This method is affordable, easy to perform in most research institutions and yields more viable viral particles. Its main limitation is the lack of specificity to precipitate only viral particles. This method will precipitate much of the soluble macromolecular material in the media, which can complicate experiments. To use this method, follow these steps:

a. Prepare 40% PEG-8000 solution (1.2 M NaCl in PBS pH 7.4), then filter sterilize in a biological safety cabinet.
b. Mix the collected supernatant (after the 3,000 × *g* spin) with the PEG-8000 solution as 4:1 (20 mL of the supernatant with 5 mL of the PEG solution) and incubate at 4°C overnight.
c. Next day centrifuge at 1,500 × *g* for 30 min at 4°C and discard the supernatant without disturbing the pellet.
d. Spin the pellet tube again at 1,500 × *g* for 1 min at 4°C and discard the supernatant.
e. Resuspend the pellet in 200 μL sterile PBS if the starting volume was 20 mL media (100-fold concentration).

Note: As mentioned above, do not use polystyrene tubes to store the virus (use the cryogenic vials). Also, do not introduce air bubbles while you are suspending the viral pellet to keep the viral particles intact.

Viral preparations quality controls

Timing: 2–3 days (for steps 18 to 26)

It is essential to calculate the viral titer and check the quality of the prepared viral batches as they may vary between different viruses and batches. It is worth noting that disrupted viruses are still detectable by the p24 ELISA. Therefore, it is crucial to check the infectivity and quality of the newly made virus preparations.

18. Evaluation of viral infectivity using HEK293-mCAT1 cells.

Note: For the following assays using the HEK293-mCAT1 cells, HEK293 that do not express mCAT1 cells can be used as a negative control.

EcoHIV utilizes the mCAT-1 receptor to enter cells.[6,7,8] To check the infectivity of the viral preparation and to determine the viral titer, use HEK293-mCAT1 cells (Kindly provided by Dr. Alan Rein, NIH/NCI). Currently, there are two fluorescent versions of the EcoHIV expressing EGFP (EcoHIV-EGFP NL4/3 and EcoHIV-EGFP NDK).[7,9] To utilize this method, one of these EcoHIV versions is recommended. Below is a summary of the infectivity assay.

a. Split HEK293-mCAT1 cells into a 24-well plate (plate A) with 1 × 10^5 cells/well (1 mL final volume) for next-day infection.
b. The next day, in a fresh 24-well plate (plate B), add 450 μL/well growing media.
c. Add 50 μL virus to the first well of plate B, mix thoroughly, and then serially transfer 50 μL into the remaining wells (10-fold dilutions).

d. Aspirate and discard the media from plate A.
e. Transfer 400 μL from the first well of plate B into the first well of plate A and then continue to transfer the media from the other wells of plate B to plate A in this fashion.

Note: Make sure to include a non-infected cell control to determine the background.

f. Incubate the cells for 24–48 h at 37°C and 5% CO_2.
g. Analyze the infectivity of the viral preparation.
After 24–48 h, four options can be utilized to evaluate the infectivity of the viral preparations depending on the availability of resources:
i. Analysis by Flow Cytometry.
ii. Analysis by Fluorescence Plate Reader.
iii. Analysis by Fluorescence Microscopy.
iv. Analysis by RT-qPCR.

19. Analysis by Flow Cytometry.

Note: This method is the most accurate, quantitative, and sensitive way to calculate the viral titer and to check the quality of the viral prep while using EGFP-containing constructs of the EcoHIV. The availability of flow cytometry is the major limitation of this method. To use this method:

a. Aspirate the media from the HEK293-mCAT1 cells and add 100 μL of 0.25% trypsin-EDTA and incubate for 5 min.
b. Add 400 μL of the growing media to neutralize the 0.25% trypsin-EDTA.
c. Transfer the detached cells into 1.5 mL Eppendorf tubes and spin at 500 × *g* for 5 min.
d. Repeat steps a-c.
e. Resuspend the cells in 1 mL PBS and add 1:1000 Zombie Red™ Fixable Viability dye.
f. Incubate at RT for 10 min in the dark and spin again at 500 × *g* for 5 min.
g. Resuspend the cells in 2% paraformaldehyde (PFA) and incubate at RT for 30 min.
h. Wash cells with 500 μL PBS to remove residual PFA and spin at 500 × *g* for 5 min.
i. Repeat step h one more time and resuspend the cells in 200 μL PBS.

20. Gating Strategy for Flow Cytometry.
a. Use forward and side scatters to define the cell population.
b. Use the forward scatter area and height for doublets exclusion.
c. Gate on the population that is negative for Zombie Red™ (live cells).
d. Use the non-infected cells to determine the proper laser voltage to have a minimum background.
e. Acquire at least 10,000 events and check the percentage of cells that express EGFP (Figure 2).
f. Calculate the viral titer (Transducing Units/mL) using the following formula:

TU = (% of transduced cells X dilution factor X 10^5)/well volume (mL).

21. Analysis by Fluorescence Plate Reader.

This method is sensitive and gives quantitative data regarding viral infectivity as well, but the viral titer cannot be calculated, because single infection events cannot be scored. In addition, the percentage of cells infected with the virus cannot be calculated. To use this method, follow the same steps as mentioned in the flow cytometry section (above) except there is no need to add the viability dye, but the cells should be resuspended in phenol red-free media or PBS as phenol red gives a false positive signal in the fluorescence plate reader (Figure 3).

⚠ CRITICAL: This experiment is useful only to check if the virus batch is infective or not. The virus titer cannot be calculated using this method.

22. Analysis by Fluorescence Microscopy.

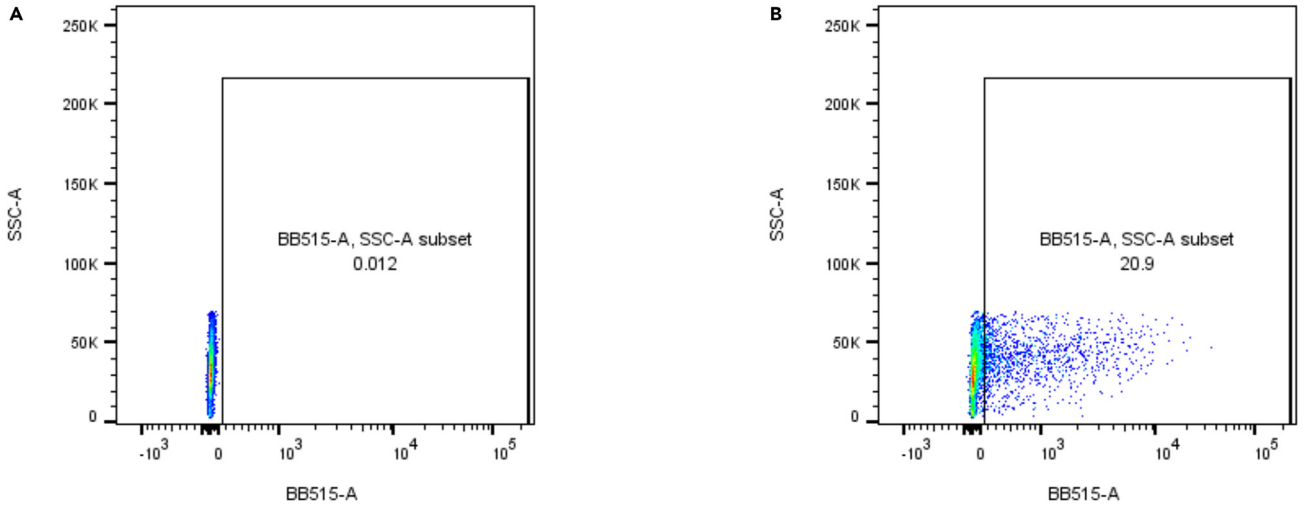

Figure 2. HEK293-mCAT1 cells are infected with EcoHIV-NDK-EGFP and, after 24 h of transduction, analyzed by flow cytometry

HEK293-mCAT1 cells were infected for 24 h with 1:100 diluted EcoHIV-NDK-EGFP to calculate the virus titer after concentration and one freeze/thaw cycle. The cells were harvested as described under the "Analysis by Flow Cytometry" section and stained with Zombie Red™ Fixable Viability Kit to exclude dead cells. The cells were analyzed using BD Symphony A3, with 10,000 events acquired. This experiment demonstrates a successful concentration process of the EcoHIV-EGFP and the production of a highly infectious virus. The virus titer calculated by this method is $\sim 5.2 \times 10^6$ TU/mL.

Note: Infectivity of the HEK293-mCAT1 cells can be measured using a fluorescence microscope such as the EVOS cell imaging system (Thermofisher) or Nikon ECLIPSE Ti2. This method can be used to calculate viral titer based on how many cells express EGFP. To utilize this method:

a. Use the Nikon ECLIPSE Ti2 or EVOS cell imaging system to titer the virus, choose the first well that has a countable number of cells (between 50–100 cells), then count the number of EGFP-positive cells (Figure 4).
b. The virus titer can be calculated using the following formula:
[(Avg count x dilution factor)/ (volume in mL)] = particle titer per mL.

23. Analysis by RT-qPCR.

RT-qPCR is the method of choice to check the viral infectivity but not the viral titer, especially when the investigator does not have access to fluorescent forms of the virus. To use this method:

a. Harvest the cells after 24 h of HEK293-mCAT1 infection as described in the flow cytometry section (above) except there is no need to add the viability dye.
b. Harvest the cellular RNA (described below) and perform RT-qPCR to detect the different spliced genes and *gag* of the virus inside of the HEK293-mCAT1 (Figure 5).

Note: It is critical to wash the cells well to remove any unbound viruses. As a negative control, HEK293 cells can be used to calculate the background.

⚠ CRITICAL: This experiment is useful only to check if the virus batch is infective or not. The virus titer cannot be calculated using this method.

24. Cellular and viral RNA isolation.
To confirm that all necessary genes are expressed in the viral preparations, and to have a positive control for the RT-qPCR reactions, viral RNA can be isolated and analyzed with RT-qPCR.

Note: Viral RNA can be isolated from a 100-fold concentrated aliquot and 1 ng of the viral RNA can be used as a positive control for RT-qPCR reactions.

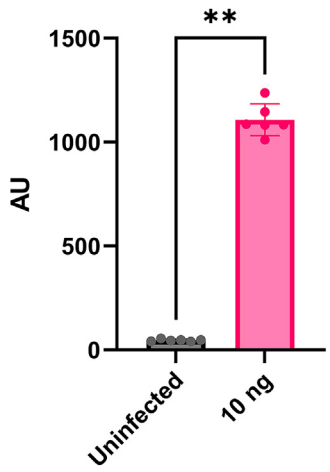

Figure 3. HEK293-mCAT1 cells are infected with EcoHIV-NDK-EGFP and, after 24 h of infection, analyzed by a fluorescence plate reader (Cytation 5 Cell Imaging Multimode Reader)

HEK293-mCAT1 cells were infected with 10 ng of intact p24 EcoHIV-NDK-EGFP for 24 h. The cells were harvested as described under the "Analysis by Flow Cytometry" section without the use of Zombie Red™ Fixable Viability Kit and read on a fluorescence plate reader. AU: Arbitrary Units (n = 6). The data is represented as mean ± SEM with statistical significance indicated as **p < 0.005.

Note: The viral RNA can be isolated with the PureLink™ RNA Mini kit which can be used to isolate both viral and splenic RNA. The kit can be used according to the manufacturer's instructions summarized below:

a. Follow steps a-d from the steps mentioned in the flow cytometry section (above).
b. Add 600 μL lysis buffer (included with the kit) supplemented with 1% β-mercaptoethanol to the cell pellet.

Note: For viral RNA isolation, add 600 μL lysis buffer supplemented with 1% β-mercaptoethanol for each 100 μL of the concentrated viral solution, and vortex well.

c. Vortex well to lyse the cells.

Note: After lysing the cells, the solution becomes viscous because of the DNA, and the viscosity depends on how many cells were lysed.

d. To shear the DNA, pass the lysate 10 X through an 18 G syringe needle.
e. Add one volume of 70% ethanol to each volume of the cell homogenate.
f. Vortex to mix and disperse any precipitate.
g. Transfer the 700 μL to the spin cartridge (with the collection tube).
h. Centrifuge at 12,000 × *g* in a tabletop centrifuge for 15 s at RT. Discard the flow-through and reinsert the spin cartridge into the same collection tube.
i. Add 700 μL Wash Buffer I to the spin cartridge.
j. Centrifuge at 12,000 × *g* for 15 s at RT, discard the flow-through and the collection tube and place the spin cartridge into a new collection tube.
k. Add 500 μL Wash Buffer II with ethanol to the spin cartridge.
l. Centrifuge at 12,000 × *g* for 15 s at RT and discard the flow-through.
m. Repeat steps k-l once.
n. Centrifuge the spin cartridge at 12,000 × *g* for 2 min to dry the membrane with bound RNA.
o. Discard the collection tube and insert the spin cartridge into a recovery tube.
p. Add 50 μL RNase-free water to the center of the spin cartridge.

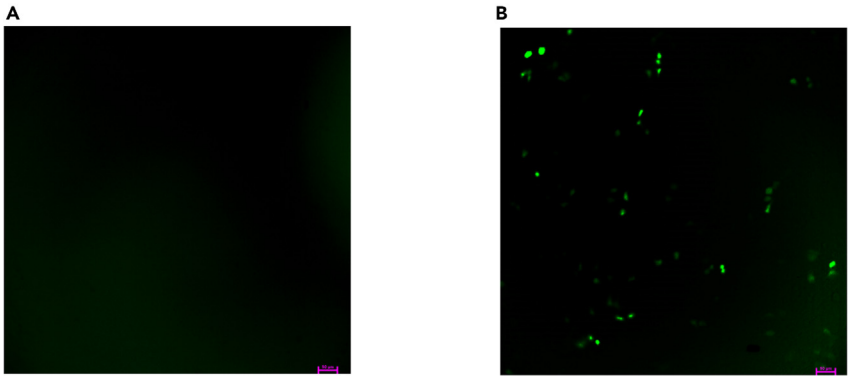

Figure 4. HEK293-mCAT1 cells are infected with EcoHIV-NDK-EGFP and, after 24 h of transduction, analyzed by epifluorescence microscopy

HEK293-mCAT1 cells were either (A) Uninfected or (B) Infected with 10 ng of intact p24 EcoHIV-NDK-EGFP for 24 h and analyzed by epifluorescence microscopy (Nikon Eclipse Ti2). The virus titer can be calculated by counting the green cells in the field. Scale bar 50 μm.

q. Incubate at RT for 1 min.
r. Centrifuge the spin cartridge for 2 min at 12,000 × *g* at RT to elute the RNA from the membrane into the recovery tube.
s. Measure the RNA concentration with Nanodrop.
t. Store the purified RNA at −80°C.

25. RT-qPCR of the isolated RNA.

This process can be done in one step using the Applied Biosystems™ TaqMan™ Fast Virus 1-Step Master Mix as follows:

a. In a MicroAmp™ Optical 96-Well Reaction plate, add 5 μL of the Applied Biosystems™ TaqMan™ Fast Virus 1-Step Master Mix.
b. Add 500 ng of cellular RNA or 1 ng of viral RNA.
c. Add 2 μL of the primer/probe mix.
d. Make the volume up to 20 μL by adding RNase-free water.
e. Cover the plate with a MicroAmp™ Optical Adhesive Film.
f. Centrifuge the plate briefly (~1 min) to mix the reagents together.
g. Place the plate in the appropriate qPCR machine. Set the PCR program as follows:

PCR cycling conditions

Steps	Temperature	Time	Cycles
Reverse transcription	50°C	5 min	1
RT inactivation/initial denaturation	95°C	20 s	1
Denature	95°C	3 s	40
Anneal/extend	60°C	30 s	

26. Quantification of the RT-qPCR experiments.

At the end of RT-qPCR experiments, the Cycle threshold (Ct) of the reaction indicates how abundant the RNA of interest is in the sample. There are two ways to normalize the Ct values:

a. Using housekeeping genes such as GAPDH in which the expression of gag can be calculated relative to GAPDH expression.

⚠ CRITICAL: Human GAPDH primers have to be used for the HEK293-mCAT1 cells and murine GAPDH primers have to be used for the mice samples.

b. Using plasmid standards with known copy numbers of each viral gene such as standards for *gag* and spliced *vif*. These standards are available upon request from Drs. Mary Potash, David J. Volsky, or Jennifer Kelschenbach (Figures 5 and 7).

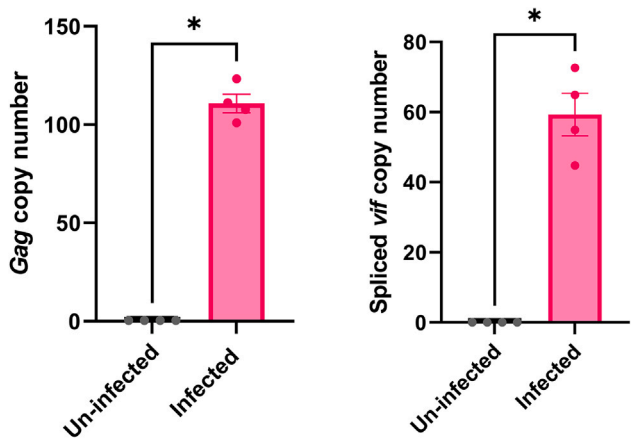

Figure 5. HEK293-mCAT1 cells are infected with EcoHIV-NDK-EGFP for 24 h and the RNA was isolated for RT-qPCR

HEK293-mCAT1 cells were infected with 10 ng of intact p24 EcoHIV-NDK-EGFP for 24 h and the RNA was isolated as described under the "Cellular and viral RNA isolation" section. For each reaction, 500 ng RNA were used. The copy numbers for each gag and the spliced vif were calculated using standards with known copy numbers (n = 4). The data is represented as mean ± SEM with statistical significance indicated as *p < 0.05.

Quantification of intact virus in viral preparations using p24 ELISA (lysed and un-lysed viral particles)

HIV-1 is an enveloped virus, and the presence of the envelope is essential for effective viral infection. EcoHIV differs from HIV-1 by having a Murine Leukemia Virus (MLV) envelope, which enables it to infect only murine cells.[6] Therefore, the presence of an intact viral envelope is essential for infection.

A major limitation of the p24 ELISA is the presence of free p24 in the sample, which can confound attempts to measure intact viruses. However, the number of intact viral particles can be determined by comparing the amount of p24 following detergent lysis of the viral preparations and subtracting the amount of free p24 in the unlysed sample.

Note: We recommend doing a serial dilution between 10^4 and 10^6 of the concentrated viral preparations before doing the p24 ELISA assay to be within the linear range of the standard curve.

Note: To calculate the concentration of free p24, dilute the viral preparations in PBS only and avoid using any detergent. To calculate the total concentration of p24 (free and inside the viral particle), lyse the viral preparations with the assay diluent buffer included in the p24 ELISA kit or with 1% Triton™X-100 and vortex the sample (Figure 6).

Note: We recommend performing this method to calculate the viral titer if a non-EGFP version of the virus is being used.

Murine infection with EcoHIV

As the final application of the prepared EcoHIV is to inject it into mice or rats, a pilot experiment should be used to detect the gag and the spliced variants of nef, vif or tat in the spleen, lymph nodes, peritoneal macrophages or liver of the infected mice.[6] In our hands, retroorbital injections of 10 μg of intact p24 (250 μL of 40 μg/mL of intact p24) produced acute and chronic infection in mice and the results are consistent (Figure 7). For the EcoHIV-NDK-EGFP, we found that 10 μg of intact p24 are equivalent to 6.25×10^5 TU were enough to induce infection in mice (Data not shown).

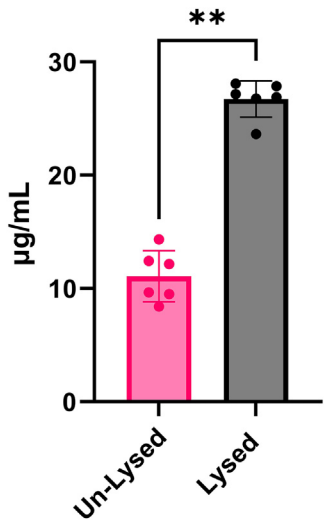

Figure 6. p24 ELISA of the lysed and un-lysed concentrated EcoHIV-NDK

The supernatant of transfected Lenti-X™ 293T cells was concentrated at 35,300 × *g* for 3 h at 4°C. The concentrated virus was then either diluted first in 1% Triton™ X-100 and vortexed to lyse the viral particles or diluted with PBS (for the un-lysed). The difference between the lysed and un-lysed concentrations resembles the concentration of p24 from the intact viral particles (n = 6). The data is represented as mean ± SEM with statistical significance indicated as **p < 0.005.

27. Spleen RNA isolation (to check the viral replication capability).
    For the spleen tissue, isolate the RNA from a fresh spleen sample, or incubate the tissue with RNAlater at 4°C overnight for next-day RNA isolation. Storing the spleen in the RNAlater can increase the RNA yield.

    To isolate RNA from the spleen:
    a. Isolate the mouse spleen and keep it in 300 µL of RNAlater solution on ice.
    b. Move the spleen into a small dish that has 300 µL cold PBS and use the plunger of sterile 1 mL syringes to release the splenocytes gently.
    c. Place a mini-strainer (100 µm) on a 15 mL tube and prime it with 300 µL RNAase-free PBS.
    d. Transfer the splenocytes from step b to the strainer and add 700 µL PBS.
    e. Spin at 500 × *g* at RT for 7 min.
    f. Discard the supernatant and add 5 mL of cold ACK buffer to lyse the RBCs. Keep on ice for 2 min, then spin again as in step e.

    Note: There should be a visible brown pellet after the spin.

    g. Add 600 µL of the lysis buffer provided in the PureLink™ RNA Mini kit to lyse the cell pellet.
    h. Follow the steps as described in the cellular and viral RNA isolation section (steps b-t).
    i. Perform RT-qPCR on the purified RNA (500 ng of RNA) and measure either one of the following spliced genes: spliced *vif*, spliced *tat* or spliced *nef* as they are metrics of *de novo* virus production (Figure 7).

EXPECTED OUTCOMES

EcoHIV is an exceptionally powerful translational tool with numerous clinical applications, including the identification or improvement of vaccines and therapeutics. Following this protocol, investigators can generate infective EcoHIV virions at high titer, which productively infect C57BL/6 mice acutely and chronically. This protocol provides methods for concentrating virions via ultracentrifugation and PEG-

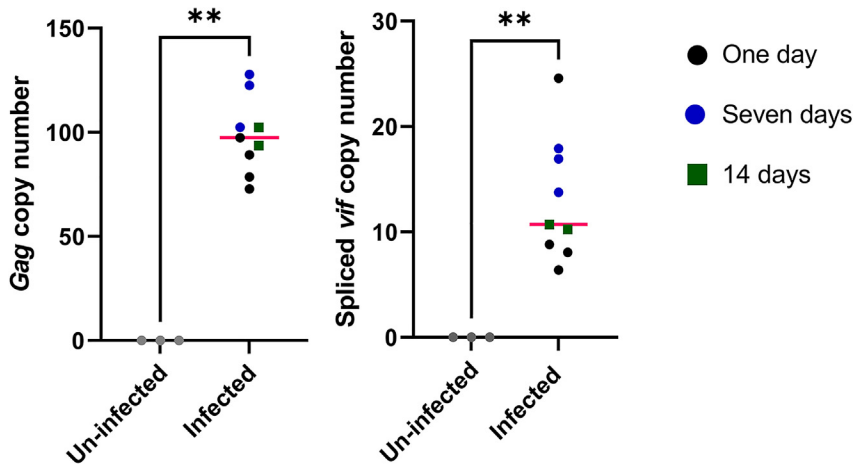

Figure 7. Splenocytes of acutely and chronically infected mice with the EcoHIV-NDK are actively infected with the virus

8–10 weeks old male and female C57BL/6 mice were injected with 10 μg of intact p24 and harvested either after one, seven, or 14 days. The spleen RNA was isolated, and 500 ng were used for the RT-qPCR reactions. The presence of the gag and spliced vif indicates *de novo* virus synthesis in the splenocytes. The copy numbers for the gag and the spliced vif were calculated using standards with known copy numbers. n ≥ 8.

8000 precipitation. Using the described methods to calculate viral titers and evaluate infectivity, investigators will get a more exact viral concentration measurement, which is superior to a simple p24 ELISA.

This protocol combines different assays that can be performed as quality control checks of the prepared viral batch based on the resource's availability. If all of the suggested resources are available, we strongly encourage using the flow cytometry method to calculate the virus titer as it is the best quantitative method described here.

QUANTIFICATION AND STATISTICAL ANALYSIS

For each experiment, statistical significance was determined using the Mann-Whitney test. The data is represented as mean ± SEM with statistical significance indicated in the figure as *$p < 0.05$ and **$p < 0.005$.

LIMITATIONS

To generate EcoHIV and efficiently infect mice with the virus there are numerous processes involved. Importantly, EcoHIV-NDK has been documented to be more infective than EcoHIV, therefore it is suggested to perform the protocol above with the EcoHIV-NDK plasmid.[3]

Investigators using the previous method have reported variability in infection.[6] With this protocol, the variability was minimal in which all of the EcoHIV-NDK injected mice were actively infected and produced viral particles.

The location of the injection site should be considered carefully. We had more success with *i.v.* injections via the orbital plexus than with standard *i.p.* injections.

The viral dosage, age of the mice, the health status of the mice at the start of the protocol, and the volume of the inoculum should all be considered prior to the start of the protocol and should be consistent between groups.

TROUBLESHOOTING

Problem 1

The Lenti-X™ cells and HEK293-mCAT-1 are growing slowly after thawing.

Potential solution 1

Check the cell viability after thawing. If the cell viability is <85%, plate the cells in a smaller dish.

> *Optional:* If needed, EasySep™ Dead cell removal (Annexin V) Kit (Cat# 17899) can be used to enrich viable cells.

Problem 2

No colonies are obtained after plating the bacteria on the agar plate.

Potential solution 2

- Make sure to use ampicillin-containing agar plates and not any other antibiotic-resistant plates.
- Use DH5α *E. coli* cells to amplify the plasmid. We have tried XL1 blue competent cells and they produced very small colonies.
- Make sure to heat shock the bacteria for 1.5 min only, do not exceed as you might kill the bacteria.

Problem 3

Low bacterial yield after overnight incubation in LB media or low plasmid yield.

Potential solution 3

- Make sure to leave enough space in the flask for oxygen for the bacteria. For example, use a 5 L flask to grow bacteria in 1 L of LB media.
- Shake the bacteria at 250 rpm. Do not shake at low speed as this leads to lower growth.

Problem 4

Low transfection rate or the cells detached from the plate after transfection.

Potential solution 4

- Use 1:3 ratio (Plasmid: PEI) as the standard ratio for transfection. High levels of plasmid and PEI can be toxic to the cells. We highly recommend using PEI MAX Transfection Grade Linear Polyethyleneimine Hydrochloride (MW 40,000).
- If a fluorescence microscope is available and the GFP version of the virus plasmid is being used, the transfection efficiency can be checked by using a fluorescence microscope and observing how many green cells are present in the plate.
- In our hands, 30 μg of the plasmid works and yields high transfection rates. However, in the literature, other researchers used 10 μg or 20 μg of the plasmid and had successful transfection and viral production for mice and rats infection.[10]

Problem 5

Low virus titers or the virus is not actively replicating in mice.

Potential solution 5

- Check the quality of the virus-producing cells. It is recommended to passage the frozen cells 3 times before attempting to use them for viral production.[1]
- Calculate the virus titer from an aliquot that has been subjected to one freeze/thaw cycle in order to inject the correct dose.
- The viability of the virus-producing cells should be >85%.
- Lenti-X™ 293T should be used as they give the highest virus titers.
- Do not vortex or generate air bubbles during viral preparations.
- Do not exceed the recommended ultracentrifugation speed (35,300 × *g*).

- Do not exceed more than one thaw-freeze cycle of the viral aliquot.
- If none of the above helps, we highly recommend titering and injecting the virus directly after the concentration step (without freezing).

Problem 6

None of the viral spliced transcripts (spliced *vif*, spliced *tat* or spliced *nef*) can be detected in the splenocytes of infected mice with RT-qPCR.

Potential solution 6

- Check the settings of the RT-qPCR machine and the quality of the RNA using Nanodrop.
- Use the viral RNA as a positive control for the spliced genes and the plasmid as a negative control.
- Check the sequence of the ordered primers and make sure they matched the one you requested.
- If none of the above helps, then most probably you have a low virus titer that cannot overcome the mice's immune system. Therefore, a higher virus titer is required.

Problem 7

The virus batch does not infect the HEK293-mCAT1 cells even after >24 h.

Potential solution 7

- You might have a low virus titer that is not enough for infection. Therefore, try to use a smaller volume of PBS to resuspend the virus pellet.
- Ensure that you did not switch the labels of Lenti-X™ 293T and HEK293-mCAT1 as they look similar under the microscope.
- Wait up to 72 h to see if you get any green cells. Low virus titer needs a longer time to see green cells. If you do not see green cells after 72 h then you have a defective virus.

Problem 8

No viral pellet is seen after ultracentrifugation.

Potential solution 8

In most cases, no viral pellet is seen during EcoHIV concentration. Hence, proceed with the protocol and check the infectivity of the viral batch.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sidney W. Whiteheart (whitehe@uky.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate a dataset or code.

ACKNOWLEDGMENTS

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The graphical abstract was created with BioRender.com.

AUTHOR CONTRIBUTIONS

H.R.A. and S.W.W. conceived, designed, and performed all of the experiments and analyzed the data. W.C., D.J.V., J.K., and M.J.P. contributed reagents and materials in addition to reviewing the manuscript. J.L., K.S.P., D.M.C., S.J., and H.C. reviewed the manuscript and helped in some parts of the experiments. D.N.P. and K.M.P. reviewed the manuscript and put some of the text together.

DECLARATION OF INTERESTS

M.J.P. and D.J.V. have a patent for EcoHIV.

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