Lentiviral generation of stable animal cell lines

Lentiviral transduction enables stable integration of genetic material into dividing and non-dividing animal cells. This makes it a powerful tool for long-term expression of transgenes, genetic perturbation screens, and the generation of stable cell lines, especially in cell types that are difficult to transfect by conventional means.

The viral envelope protein determines host range ("tropism"). VSV-G is the most commonly used envelope protein, conferring broad tropism and high particle stability. Alternative envelopes such as RD114 or GALV may be preferred for hematopoietic or progenitor cells, or to reduce cytotoxicity.

Lentiviral vectors are derived from human immunodeficiency virus (HIV-1) and produced by co-transfection of a packaging cell line with a set of helper plasmids. This protocol uses a second-generation packaging system, in which the *gag*, *pol*, *rev*, and *tat* genes are encoded on a single plasmid. The recombinant envelope protein, typically VSV-G, is provided on a separate plasmid, and the gene of interest is cloned into a third, replication-deficient transfer vector.

Although second-generation vectors are widely used and considered safe when handled under BSL-2+ conditions, their simpler architecture—fewer plasmids and retention of *tat*—introduces a higher theoretical risk of recombination into replication-competent lentivirus compared to third-generation systems. Federal regulations and institutional biosafety policies require that researchers handling lentiviral vectors complete appropriate training, receive supervision, and follow strict decontamination and disposal protocols.

Risk assessment

- **Work with lentiviral vectors (BSL-2+)!**
- **Risk of exposure to replication-deficient virus through aerosols, sharps, skin or mucous membrane contact**
- **Risk of generation and exposure to replication-competent virus and insertional mutagenesis**
- **Work with human-derived material or transgenic cell lines (BSL-2)**

▷ New users must be trained and supervised by a designated experienced lab member for at least two procedures before working independently

▷ All work must be contained in a certified class II biosafety cabinet

▷ DO NOT use sharps or needles without written permission

▷ Wipe down all surfaces with freshly prepared 1% sodium hypochlorite or equivalent virucidal disinfectant (Opti-Cide® 3) before and after work; 70% ethanol is NOT effective to cleanup spills

▷ Transfer containers in secondary containment such as a sealed box or disinfected tray when moving between lab spaces

▷ Wear double gloves, splash goggles, disposable lab coat and sleeves

☐ DO NOT aspirate infectious waste using a vacuum line

☐ Collect liquid waste into a vessel with 20–30% bleach; ensure at least 60 min contact time before sink disposal; empty daily

☐ Discard pipette tips, plates, and any disposable items into 10% bleach or equivalent virucidal disinfectant (Vesphene® III); soak for 30 min before disposal; empty when filled to 80% capacity or after four days

☐ Dispose waste after inactivation as REGULATED MEDICAL WASTE

In the event of SPILL:

▷ **Evacuate the area and allow aerosols to settle for 30 min before re-entry**

▷ **Soak thoroughly with freshly prepared 1% sodium hypochlorite or equivalent virucidal disinfectant (Opti-Cide® 3).**

In the event of EXPOSURE:

▷ **Wash affected area for 15 min**

▷ **Seek medical attention within 1 hour**

Report to (all of):

- **Principal Investigator/Supervisor**
- **Biosafety Officer**

Reviewed: Jul 25, 2025

Procedures

›› *Production of virus particles*

- ☐ 6-well plate
- ☐ Flip-top filter, 0.45 µm, PES
- ☐ Lenti-X™ 293T
- ☐ DMEM + 10% FBS, without antibiotics
- ☐ Culture medium for the transduced cells
- ☐ Transfer plasmid (with transgene)
- ☐ Packaging plasmid
- ☐ Envelope plasmid

(1.) On the day of transfection, replace medium with antibiotic-free DMEM + 10% FBS.

> ***This is why:*** Aminoglycoside antibiotics like streptomycin can bind to the small subunit of the eukaryotic ribosome and thereby slow down viral protein production. Penicillin does not negatively impact viral titer.

> ***Critical:*** From this step forward, perform all steps in a certified class II biosafety cabinet. This includes transfection, medium changes, harvesting, and filtering virus-containing supernatant. Do not perform any viral manipulations on the open bench. If the biosafety cabinet is in the same room, carry culture plates carefully using both hands. If moving between rooms or floors, place the cultures in a leak-proof secondary container lined with absorbent paper, and disinfect the exterior before transport.

(2.) Transfect Lenti-X™ 293T cells at 70% confluency with the following plasmids using chemical transfection with Lipofectamine® 2000 0012. By default, use one well of a 6-well plate per transgene:

		Format	Plates (per well)		
Plasmid		**6-well**		**12-well**	**24-well**
Packaging plasmid	psPAX2	0.22 pmol	1.46 µg	530 ng	300 ng
Envelope plasmid	pMD2.G (VSV-G)	0.12 pmol	0.43 µg	155 ng	90 ng
Transfer plasmid	pLJM1, pLKO.1, etc.	0.28 pmol	1.20 µg	430 ng	240 ng

> ***Hint:*** Plasmid ratios may require optimization depending on vector size, packaging efficiency, or cell line used.

> ***Hint:*** The default production format is the 6-well plate, but 24-well plates can be used for small-scale preparations. 48-well plates are typically reserved for optimization or screening, not virus production.

> ***Critical:*** This SOP describes small-scale virus production (less than 100 mL total supernatant with titers less than 1×10^9 cfu/mL). Larger volumes or higher titers may require additional IBC review or enhanced containment measures.

(3.) *Optional:* After 18 h, replace the medium with 2 mL of the culture medium of the cells to transduce. ⊞ ⏲ 18 h

> ***Note:*** This allows direct use of viral supernatant without purification of the viral particles.

(4.) *Optional:* Supplement the culture medium with 10 mM sodium butyrate to restrict cell growth. ⊞

(5.) Harvest the virus-containing supernatant at 48 h, and optionally at 72 h and 96 h post transfection. Combine batches or store separately as needed. ☾

> ***Critical:*** Handle supernatants with care; even replication-deficient vectors remain infectious until inactivated. Do not remove viral supernatant from the biosafety cabinet unless sealed and disinfected.

> ***This is why:*** To increase viral yield, multiple harvests may be pooled. Lentiviral titers typically peak between 48–72 h, depending on the packaging system and transgene burden.

(6.) *Optional:* Pre-clear the viral supernatant at 500 × *g* for 5 min in a certified aerosol-tight centrifuge. ⊞

> ***Critical:*** Centrifugation must be performed in sealed buckets and followed by immediate disinfection. Skip this step for small-scale preparations or if a certified aerosol-tight centrifuge is not available near the biosafety cabinet.

(7.) Filter the virus-containing supernatant through a sterile 0.45 µm PES flip-top filter inside the biosafety cabinet to remove remaining packaging cells and debris. Do not use needles!

> ***Critical:*** Flip-top filters reduce the risk of aerosol generation and spills, increasing user safety. If using syringe filters, do not force the plunger or push air through the filter as this will generate aerosols, or can cause the filter to pop off.

(8.) Disinfect all tubes and external surfaces of containers with 1% sodium hypochlorite or equivalent virucidal disinfectant (Opti-Cide® 3) before removing them from the biosafety cabinet.

⊞ Optional: *PEG precipitation of viral supernatants*

- ☐ 5 × PEG virus precipitation solution, 100 mL ⟨*R*⟩

(1.) Add 0.25 vol PEG virus precipitation solution per 1.0 vol of viral supernatant.

(2.) Mix well by shaking for 1 min. Incubate with constant rocking at 4 °C for at least 4 h. ⏲ 6 h

> ***Hint:*** Viral particles are stable in PEG-containing solution and can be kept overnight at 4 °C without significant loss in titer. PEG will also serve as cryopreservative. Longer incubation will enhance recovery upon precipitation.

(3.) Spin at 1 600 × *g* for 1 h at 4 °C in a certified aerosol-tight centrifuge. ⏲ 1 h

> ***Critical:*** Centrifugation must be performed in sealed buckets and followed by immediate disinfection. Skip this step for small-scale preparations or if a certified aerosol-tight centrifuge is not available near the biosafety cabinet.

(4.) Carefully remove the supernatant without disturbing the pellet.

> ***Hint:*** Pellet size is not well correlated with virus yield since serum proteins and cellular debris will often co-precipitate.

(5.) Thoroughly resuspend the viral pellet in 0.05–0.10 vol PBS or serum-free culture medium without antibiotics by pipetting up and down. Incubate for 10 min.

> ***Critical:*** Avoid generating bubbles which may inactivate the virus.

(6.) *Optional:* Transfer to a microcentrifuge tube and spin at full speed for 3 min to remove cellular debris. ⊞

[SKC91]

⊞ Optional: *Concentration of viral supernatants by centrifugation*

(1.) If an ultracentrifuge is not available, place the viral supernatant on a 0.2 vol 50% sucrose cushion.

(2.) Concentrate depending on the available instrumentation,

- In a high-speed centrifuge at 10 000–20 000 × *g* for 240 min, or
- In an ultracentrifuge at 90 000 × *g* for 90 min.

(3.) Carefully remove the supernatant without disturbing the pellet. Resuspend as desired.

[JHW+15]

›› *Storage of lentiviral particles*

(1.) Store filtered viral supernatants at 4 °C for up to 24 h, or transfer in 1 mL aliquots to −80 °C storage.

> ***Critical:*** Use screw-cap polypropylene vials for storage. DO NOT submerge the vials into liquid nitrogen! Label the secondary container clearly with "***BIOHAZARD: Infectious Material*** (***Lentivirus***)".

> ***Quality assurance:*** Freezing and thawing reduces viral infectivity by 20%. However, storing the supernatant at 4 °C for more than two days reduces infectivity by up to 40–60%. Avoid repeated freeze–thaw cycles.

> ***Hint:*** For larger-scale applications, virus can be concentrated by ultracentrifugation or PEG precipitation (PEG-it™ Virus Precipitation Solution). Consult IBC guidance before concentrating or scaling up production!

›› *Transduction of host cells*

- ☐ Culture medium for the transduced cells
- ☐ 10 g/L Hexadimethrine bromide, 100 mL ⟨*R*⟩

(1.) *Optional:* Determine the viral titer by ELISA, qPCR, or semi-quantitative lateral flow assays.

Hint: Single-use lateral flow sticks are preferred if plate readers are not certified for use with infectious material.

(2.) Seed cells to 25% confluency at the time of transduction.

Hint: Some cell lines, such as 293T or HeLa, tolerate lentiviral transduction when freshly seeded while more sensitive cell lines may require from adherence and recovery prior to infection.

(3.) Supplement 1 mL culture medium with 20 µL 10 g/L Polybrene® to a concentration of 0.2 g/L for a 20 × stock.

Hint: You will need 500 µL of this stock solution to prepare the dilution series to test transduction conditions.

This is why: Polybrene® is a polycationic homopolymer that enhances viral uptake by reducing charge repulsion between the virus and the cell membrane. It is added to a final concentration of 2–10 µg/mL without pre-incubation.

(4.) Prepare a dilution series of the viral supernatant in Polybrene®-supplemented medium. Apply to target cells to determine optimal transduction conditions. For a 6-well plate:

Dilution	**Neat**	**1:10**	**1:25**	**1:50**	**1:100**	**1:250**
Viral supernatant	1 500 µL	150 µL	60 µL	30 µL	15 µL	6 µL
Culture medium	0 µL	1 350 µL	1 440 µL	1 470 µL	1 485 µL	1 494 µL
Culture medium + 0.2 g/L Polybrene®	75 µL	75 µL	75 µL	75 µL	75 µL	75 µL

This is why: Titration allows to estimate the virus amount required for the desired transduction efficiency. This is especially important for pooled screens to achieve single-copy integration—usually at a multiplicity of infection (MOI) of 0.1 (usually in the range of a 1:20 to 1:200 dilution), or if viral toxicity is a concern. Where multiple copies are acceptable, a MOI of 1.0 is used.

Hint: For a new virus preparation or when transducing a new cell line for the first time, a dilution series is strongly recommended to avoid over- or under-transduction.

(5.) The next day, replace the medium with fresh culture medium.

Safety: Do not aspirate viral medium using vacuum lines. Collect liquid waste into a flask containing bleach and incubate for at least 60 min before disposal!

Hint: If cytotoxicity is observed, or if your downstream application requires removal of Polybrene® or residual virus, change the medium already after 6–12 h.

(6.) The next day (or the day after), split the cells 1:3 to 1:5 depending on cell type, and proceed with selection or analysis as appropriate. For antibiotic selection, refer to 0019.

Critical: Maintain cells in a subconfluent state throughout antibiotic selection to outgrow successfully transduced clones.

Critical: For biosafety reasons, all transduced cells must be cultured in a designated incubator for at least ***three to five additional passages,*** typically over two to three weeks, to allow clearance of any potentially infectious particles.

Analyses

- ○ Calculate the multiplicity of infection (MOI) by quantifying the percentage of transduced cells.

  > ***This is why:*** If your construct encodes a fluorescent marker such as GFP, measure transduction efficiency by flow cytometry or fluorescence microscopy. For constructs with antibiotic resistance, count the number of surviving colonies after selection.

  > ***This is why:*** The MOI is calculated as $-\log(1-r)$ where r is the fraction of positive cells. For example, 70% positive cells corresponds to an MOI of approximately 1.2, while 25% of positive cells correspond to an MOI of 0.3.

- ○ Test for replication-competent lentivirus (RCL) if required by your IBC or when handling large volumes or high-titer preps.

  > ***This is why:*** Assays for RCL involve passaging transduced cells and testing for p24 antigen or reverse transcriptase activity over time. These are typically outsourced or conducted in specialized facilities.

Troubleshooting

PEG precipitation of viral supernatants

In Step 1:

– No turbidity and no or small precipitate

  ◦ If the virus was collected in serum-free or low-serum medium, add sterile-filtered BSA to a final concentration of 3%.

Recipes

PEG virus precipitation solution, pH 7.0, 5×

Amount	Ingredient	Stock	Final
10 mL	Phosphate-buffered saline (PBS), pH 7.4 R0037	10×	1×
50 g	Polyethylene glycol 8000 (PEG 8000) [25322-68-3]		50%
20 mL	NaCl R0046	5 M	1.5 M
To 100 mL	Water, reagent-grade		

Adjust pH to 7.2 with sodium hydroxide if necessary. Filter sterilize through a 0.22 µm filter. Dispense into 10 mL aliquots. Store at 4 °C.

5× PEG virus precipitation solution

10% PEG 8000, 0.3 M NaCl, pH 7.0

[At 1× dilution]

Date: Sign: R0195

Hexadimethrine bromide (Polybrene®), 10 g/L

Amount	Ingredient	Stock	Final
1 g	Hexadimethrine bromide [28728-55-4]	374.20 g/mol	10 g/L
To 100 mL	Water, reagent-grade		

Filter sterilize through a 0.22 µm PES membrane. Dispense into 500 µL aliquots. Stable for 2 years at −20 °C. ***Note:*** Discard after thawing.

10 g/L Hexadimethrine bromide (Polybrene®)

WARNING

Expiry: Sign: R0134

List of references

W. Jiang, R. Hua, M. Wei, C. Li, Z. Qiu, X. Yang, and C. Zhang, *Sci. Rep.* **5** 13875 (2015).

D. Sanyal, G. Kudesia, and G. Corbitt, *J. Med. Microbiol.* **35**(5), 291—293 (1991).

Change log

2023-03-09	Benjamin C. Buchmuller	Adaptation as SOP.
2025-04-07	Benjamin C. Buchmuller	Revised abstract to clarify packaging system and tropism; expanded transduction procedure; added comprehensive biosafety guidance including spill response, waste handling, and infectious material clearance. Updated dilution series table and MOI analysis. Reorganized production steps and annotated media changes.
2025-07-18	Benjamin C. Buchmuller	Added reference to lateral flow assay to determine viral titer; corrected seeding density for transduction from 5×10^4 to 3×10^5; corrected Polybrene® stock concentration from 0.1 g/L to 0.2 g/L.
2025-07-25	Benjamin C. Buchmuller	Added low-speed PEG 8000 concentration protocol adapted from the Xin Chen lab (Johns Hopkins University) and the Functional Genomics Core (MD Anderson Cancer Center); Added high-speed concentration protocols from the cited references.