

Packaging and Transduction of Lentiviral Constructs User Manual

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A. Background

The protocols below provide the instructions on how to package, titer, and transduce target cells with Cellecta's lentiviral constructs. Please read the entire user manual before proceeding with your experiment.

B. Required Materials

- Plasmid Lentiviral Construct (Cellecta)
- Packaging plasmid mix (recommended: mix of packaging plasmids, psPAX2 and pMD2.G, provided by AddGene, Cat.#s 12260 and 12259; or custom ordered through Cellecta, pC-Pack2 Packaging Mix, in optimal ratio)
- 293T Cell Line (ATCC. Cat.# CRL-11268™, or Cellecta)
- Dulbecco's Modified Eagle's Medium (D-MEM) (high glucose with sodium pyruvate)
Note: ADD FRESH GLUTAMINE (1X) at the time a sealed bottle of D-MEM is opened, even if the label indicates glutamine has already been added. Glutamine in solution at +4°C has a half-life of 1 – 2 months, so glutamine(+) D-MEM purchased "off-the-shelf" from a supplier is to be regarded as glutamine(-). In our experience, the addition of glutamine increases titer approximately 2-fold.
- Fetal Bovine Serum (recommended: Mediatech, Cat.# MT 35-010-CV)
- D-PBS
- Trypsin-EDTA
- Tissue Culture Plates and Related Tissue Culture Supplies
- Lipofectamine™ Reagent (Invitrogen, Cat. # 18324-111)
- Plus™ Reagent (Invitrogen, Cat. # 11514-015)
- 0.2-0.45 µm PES sterile syringe filters (Nalgene, Cat. # 194-2520)
- Polybrene® (hexadimethrine bromide) (Sigma-Aldrich, Cat.# 107689)
- Puromycin
- Doxycycline

**C. Packaging Protocol for Lentiviral Constructs**

The following protocol describes the generation of ten (10) pseudoviral packaged lentiviral constructs using Invitrogen’s Lipofectamine™ and Plus™ Reagent. Other transfection reagents may be used, but the protocol should be adjusted to fit the manufacturer’s protocol. The yield of recombinant lentiviral particles typically produced under these optimized conditions is $1-10 \times 10^6$ TU/ml. In this protocol, at least 1×10^7 TU of total pseudoviral particles can be produced from each 10-cm plate and then concentrated up to 100-fold using several described methods.

1. Start growing 293T cells in D-MEM medium plus glutamine (see **Required Materials**), supplemented with 10% FBS without antibiotics, 2 to 3 days prior to transfection.

Day 1 – Plate Cells

2. Twenty four (24) hours prior to transfection, plate 4×10^6 293T cells/plate in ten (10) untreated 10-cm plates. Use 10 ml of media per plate. Disperse the cells and ensure even distribution. At the moment of transfection, the cells should have reached ~70%-80% confluency. Increase or decrease the number of 293T cells seeded if optimal confluency is not achieved in 24 hours. Incubate at 37°C in a CO₂ incubator for 24 hours.

Day 2 – Transfection

3. For each construct, mix 20 µl (10 µg) of the packaging plasmid mix with 2 µg (2 µl – 20 µl depending on concentration) of the plasmid construct and then add the plasmid mixture to 1 ml D-MEM medium without serum or antibiotics. Add 20 µl of Plus™ Reagent, mix, and incubate at room temperature for 15 min. in a sterile 5 or 14-ml tube.

1 plate	Component
20 µl	Packaging plasmid mix (0.5 µg/µl)
4 µl	Plasmid shRNA Construct (0.5 µg/µl) *
1,000 µl	D-MEM, no FBS, no antibiotics
20 µl	Plus Reagent™
1,044 µl	Total volume

4. Make a master mix, using 30 µl Lipofectamine™ Reagent and 1 ml of D-MEM medium, without serum or antibiotics, per plate. Mix gently.

per plate	11X Master Mix	Component
1,000 µl	11,000 µl	D-MEM, no FBS, no antibiotics
30 µl	330 µl	Lipofectamine™
1,030 µl	11,330 µl	Total volume

5. Add 1 ml of the diluted Lipofectamine™ Reagent (from step 4) to the DNA / Plus™ Reagent complex (from step 3), mix gently by inversion, and incubate at room temperature for 15 min.
6. Add 2 ml of the DNA / Plus™ Reagent / Lipofectamine™ Reagent complex (from step 5) to each 10-cm plate from step 2, and mix complexes with medium by gentle rotation. Take care not to dislodge cells from the plate. Incubate at 37°C in the CO₂ incubator for 24 hours.

Day 3 – DNase Treatment (Optional)

7. At 24 hours post-transfection, replace the medium containing complexes with fresh D-MEM medium supplemented with 10% FBS, DNase I (1 U/ml), and MgCl₂ (4 mM). Continue incubation in the CO₂ incubator at 37°C for 24 hours. Overnight DNase I treatment before harvesting pseudovirus does not negatively affect viral titer or infectivity and helps prevent undesirable carryover of plasmid construct into the pseudovirus prep.

Note: Failure to change the media the day after transfection results in large carryover of plasmid (free and/or Lipofectamine™-bound) in your pseudoviral prep. This may cause problems with

most downstream molecular biology applications, especially whenever there is a PCR step involved.

Day 4 – Collect Pseudoviral Supernatant

- At 48 hours post-transfection, collect all 10 ml of the pseudovirus-containing medium from each plate and filter through a Nalgene 0.2 – 0.45 μm PES filter (a low protein binding filter) to remove debris and floating packaging cells. Failure to filter supernatant could result in carry-over of cells into your pseudoviral prep.

Note: Usually, the peak of pseudovirus production is achieved at 48 hours post-transfection. We recommend collecting the supernatant only once, at 48 hours post-transfection, in order to achieve higher titers. Supernatant can also be collected at 72 hours post-transfection—replace the collected 48-hour supernatant with 10 ml of fresh D-MEM medium supplemented with 10% FBS and continue incubation in the CO_2 incubator at 37°C for 24 hours.

Caution: You are working with infectious pseudoviral particles at this stage. Please follow the recommended guidelines for working with BSL-2 safety class materials (see Section H, Safety Guidelines).

- Proceed to concentration step, or aliquot and store the non-concentrated supernatant at -80°C . Freezing and thawing may result in 10 – 20% loss of pseudoviral titer with each cycle.

Concentrating Pseudovirus (Optional)

Although concentrating pseudovirus is optional, it is recommended if (1) very high titer pseudovirus stock may be needed to achieve desired MOI in hard-to-infect target cells or (2) pseudovirus should be suspended in another media (besides the standard PBS/10%FBS) which is optimal for sensitive target cells. However, because of the additional manipulation of samples, there is the added risk of contamination and loss of pseudovirus.

The following protocol was optimized to concentrate pseudovirus with high recovery. The protocol assumes that pseudoviral supernatant was harvested 48 hours after transfection and filtered as in step 8 above.

- Aliquot pseudoviral supernatant in clear centrifuge tubes.
- Add Polybrene to a final concentration of 5 $\mu\text{g}/\text{ml}$, and incubate for 1 hour at $+4^\circ\text{C}$.
- Centrifuge at 10,000 rpm for 1 hour at $+4^\circ\text{C}$ in a Beckman JA-14 (or JA-10) or equivalent rotor. Mark the tubes to identify the location where the pellet will be. At the end of centrifugation, you may or may not be able to see a pellet—assume it is at the location of the mark.
- Immediately discard the supernatant by aspirating.
- Place the tube on ice, resuspend the (in)visible pellet in PBS/10%FBS, make aliquots, and freeze at -80°C .

Alternatively, you may concentrate pseudovirus by any of the methods below. However, the yield of pseudovirus is superior ($\sim 80\%$ recovery) using Cellecta's protocol above.

- Ultracentrifugation at 50,000 x g for 90 min at $+4^\circ\text{C}$
- Sucrose cushion ultracentrifugation
- PEG precipitation, followed by low speed centrifugation

D. Pseudoviral Titer Estimation

Most of Cellecta's lentiviral vectors express both RFP and Puromycin Resistance markers. Titers are calculated based on the percentage of either RFP-positive or Puromycin-resistant cells.

We recommend that you estimate the titer of the pseudovirus-containing supernatant before proceeding with transduction experiments for the following reasons:

- To ensure that pseudoviral stock is viable
- To determine the amount of pseudoviral particles (transduction units (TU)) necessary to achieve the desirable percentage of transduced target cells (multiplicity of infection (MOI))
- To control the number of copies of integrated pseudoviral constructs per target cell

To check pseudoviral titer, we recommend choosing a cell line appropriate for your experimental system. Most of the commonly used mammalian cell lines can be effectively transduced by lentiviral constructs. Relative titers can vary up to 50-fold depending on the chosen cell line.

Check Toxicity of Polybrene®

Polybrene is a polycation that neutralizes charge interactions to increase binding between the pseudoviral envelope and the cellular membrane. The optimal concentration of Polybrene depends on cell type and may need to be empirically determined. Excessive exposure to Polybrene can be toxic to some cells.

Before conducting the titer estimation experiment, we recommended performing a Polybrene toxicity titration in target cells. Grow cells in complete culture medium with a range of Polybrene concentrations (0 µg/ml, 2.5 µg/ml, 5 µg/ml) for 24 hours, and then replace old medium with Polybrene-free complete culture medium. Grow cells for an additional 48 hours, and then check toxicity by counting viable cells. For your experiments, use the highest concentration of Polybrene that results in less than 10% cell toxicity compared to no Polybrene (typically, 5 µg/ml is recommended). For some cell types, you cannot use Polybrene.

Transduction Protocol For Titering in HEK293 cells.

Please read the entire protocol before beginning your experiment. For other plate formats, the volumes should be adjusted depending on the growth area of the well or plate.

Day 1.

1. Quickly thaw the pseudoviral particles in a water bath at 37°C. Transfer the thawed particles to a laminar flow hood, gently mix by rotation, inversion, or gentle vortexing, and keep on ice.

Caution: Only open the tube containing the pseudoviral particles in the laminar flow hood.

Note: Unused pseudoviral stock may be refrozen at -80°C, but it will result in a loss of about 10 – 20% in titer.

2. Trypsinize and resuspend cells to a density of 1×10^5 cells/ml in D-MEM supplemented with 10% FBS and 5 µg/ml Polybrene. Aliquot 1 ml/well in a 12-well plate and add 0 µl, 1 µl, 3.3 µl, 10 µl, 33 µl, and 100 µl pseudoviral stock, prepared by serial dilution, to six different wells. Mix and return cells to CO₂ incubator. Grow cells under standard conditions for 24 hours.

Day 2.

3. At 24 hours post-transduction, replace media with fresh D-MEM supplemented with 10% FBS and without Polybrene. Return cells to CO₂ incubator, and grow under standard conditions for 24 hours.

Day 3. (48 hours after transduction)

Fluorescence-based titering:

Transduced cells express RFP reporter protein from an internal UbiC promoter. By calculating the percentage of RFP-positive cells using flow cytometry, you can determine the copy number of integrated lentiviral constructs (multiplicity of infection (MOI)) in infected cells. You may also visualize the cells for RFP fluorescence by microscopy, but the results will be inaccurate due to lower detection sensitivity compared to flow cytometry. Detach cells from the plate by trypsin treatment, centrifuge, resuspend in 1X D-PBS at approximately 10×10^6 cells/ml, and determine the percentage of transduced (RFP-positive) cells by flow cytometry. In order to set up a gate for counting RFP-positive cells, first analyze the background (RFP) level of control non-transduced cells (0 μ l of pseudoviral stock). Based on the percentage of transduced cells and the volume of pseudoviral stock used, calculate the multiplicity of infection (MOI) and original concentration of infective pseudoviral particles in the pseudoviral stock (transduction units) per ml (TU/ml).

$$\text{MOI} = \text{viral integrants/cell}$$

$$\text{Titer} = \text{MOI} \times \text{cells at infection} \times \text{ml pseudovirus used} = \text{TU/ml}$$

Example: if 10 μ l of pseudovirus used to infect 1×10^5 cells resulted in 20% RFP-positive cells, the titer is $0.23 \times 100,000 / 0.010 = 2.3 \times 10^6$ TU/ml

*To convert % of infected cells to MOI, refer to the table below:

% transduced cells:	10	20	30	40	50	60	70	80	90	>90*
MOI:	0.1	0.23	0.36	0.51	0.7	0.93	1.22	1.64	2.3	>2.5*

† MOI cannot be reliably calculated if % of transduced cells is >90%.

Note: Depending on cell type, you may need to wait 72 hours after transduction before estimating titer by RFP fluorescence.

Puromycin-based titering:

At 48 hours after infection, split cells into two samples. Grow one sample with antibiotic selection, and one sample without (**NOTE:** Before performing the following experiment, we recommend to first determine the optimal concentration of antibiotic using the **Puromycin Kill Curve** protocol below). After 48 hours of growth in puromycin, count the number of viable cells in the selected and unselected samples. The ratio of selected/unselected viable cells gives the percentage of infected cells. Then, calculate relative pseudoviral titer as previously described. Please note that the titer determined by Puromycin selection may differ from the titer determined by counting RFP-positive cells using flow cytometry, and it also depends on cell type and selection conditions.

Puromycin Kill Curve

In order to generate a purely transduced population of cells, it is important to determine the minimum amount of puromycin required to kill untransduced cells. This can be done empirically by generating a kill curve, as follows:

Trypsinize and resuspend cells to a density of 1×10^5 cells/ml in growth media, aliquot 1 ml per well in a 12-well plate, and add puromycin at 0 μ g/ml, 0.5 μ g/ml, 1 μ g/ml, 2 μ g/ml, 5 μ g/ml, and 10 μ g/ml in six different wells. Mix and return cells to incubator. Grow cells under standard conditions for 48 hours.

For puromycin, the minimum antibiotic concentration to use is the lowest concentration that kills 100% of cells in 48 hours.

E. Transduction of Target Cells

General considerations:

- The transduction efficiency of the lentiviral expression construct varies significantly for different cells and experimental conditions, including pseudovirus concentration, exposure time to pseudovirus, and growth area of cells. To determine the concentration of pseudoviral particles required to provide the desired multiplicity of infection (MOI) for your target cells, you should titer the viral stock in the same target cells used in the experiment.
- Cellecta's Lentiviral vectors contain a deletion in the 3'LTR that leads to self-inactivation of the lentiviral vector after reverse transcription and integration into genomic DNA. Although more than one copy of a lentiviral construct may be integrated into the genome of a single cell, the lentiviral construct cannot produce infectious viral particles. However, despite these safety features, please remember that you are working with transducible pseudoviral particles. Although the particles are replication-incompetent, they are infection-competent, so the expression cassette that they carry will infect, integrate, and express in any mammalian cell. Please follow the recommended guidelines for working with Biosafety Level 2 (BSL-2) materials.

Transduction Protocol (HEK293).

Please read the entire protocol before beginning your experiment. For other plate formats and other cell types, the volumes, cell number, and media should be adjusted depending on the growth area of the well or plate and the growth characteristics of the cell type.

Day 1.

1. Quickly thaw the pseudoviral particles in a water bath at 37°C. Transfer the thawed particles to a laminar flow hood, gently mix by rotation, inversion, or gentle vortexing, and keep on ice.

Caution: Only open the tube containing the pseudoviral particles in the laminar flow hood.

Note: Unused pseudoviral stock may be refrozen at -80°C, but it will result in a loss of about 10 – 20% in titer.

2. Trypsinize and resuspend cells to a density of 1×10^5 cells/ml in D-MEM supplemented with 10% FBS and 5 µg/ml Polybrene. Aliquot 1 ml/well in a 12-well plate and add an appropriate amount of pseudoviral stock to each well. For one well (mock well control), do not add pseudovirus. Mix and return cells to CO₂ incubator. Grow cells under standard conditions for 24 hours.

Note: For shRNA constructs, we recommend starting with an MOI of about 1 (ratio of pseudovirus to cells). In some cases, a higher MOI (or multiple integrants per cell) may be needed to reach an optimal knockdown efficiency.

Day 2.

3. At 24 hours post-transduction, replace media with fresh D-MEM supplemented with 10% FBS and without Polybrene. Return cells to CO₂ incubator, and grow under standard conditions for 24 hours.

Day 3.

4. By day 3, the *culture may* be confluent (depending on cell type). The cells can be assayed for RFP expression at this point. Split the cells 1:4 to 1:8, depending on the type of cells, and incubate for 48 hours in complete medium containing Puromycin at 1 µg/ml (or the concentration determined empirically by the Puromycin Kill Curve in Section D above).



Day 5. (NON tet-regulated constructs)

5. Change media to remove dead cells, dilute culture as needed to keep cells from confluence, and incubate 24-48 hours in complete medium with Puromycin.

Day 5. (tet-regulated constructs)

6. Change media to remove dead cells, dilute culture as needed to keep cells from confluence, split culture into 2 separate samples, and incubate 48 hours in complete medium with Puromycin, with (one sample) or without (the other sample) 0.1 ug/ml dox.

Day 6 or 7. (NON tet-regulated constructs)

The infected target cells can now be analyzed using an appropriate assay. If prolonged shRNA expression is required, it is recommend that cells are kept under optimal growth conditions until harvesting.

Day 7. (tet-regulated constructs)

The infected target cells can now be analyzed using an appropriate assay. If a prolonged shRNA expression is required, it is recommend that cells are kept under optimal growth conditions and fed fresh media containing 0.1 ug/ml dox every 48 hours until harvesting.

Knockdown efficiency

To determine knockdown percentage by RT-PCR, it is important to avoid contamination of non-infected cells and to isolate CYTOPLASMIC, not total RNA. Please contact Cellecta if you need additional information.

F. Troubleshooting

Low Pseudoviral Titer (<10⁶ TU/ml in supernatant)

1. Poor transfection efficiency

Problem: 293T Cells have too high or too low density

Solution: Plate fewer or more cells in order to have about 70%-80% confluency at transfection stage.

Problem: Plasmid DNA/Lipofectamine™/Plus Reagent™ ratios are incorrect

Solution: Optimize the ratios using the guidelines provided in the Lipofectamine™ protocol.

2. Inefficient production of the pseudovirus

Problem: 293T Cells are of poor quality

Solutions:

- Optimize growth conditions, check growth medium, and don't grow 293T cells for more than 20 passages.
- Check for mycoplasma contamination.
- Do not overgrow the cells (do not allow the cells to reach more than 90% confluency in order to keep the culture continuously in logarithmic growth phase).

Problem: Pseudoviral supernatant harvested too early or too late

Solution: Harvest supernatant 48 hours after transfection.

Inefficient Transduction of Packaged Lentiviral Constructs

1. Poor infection efficiency

Problem: Target cells have too high or too low density

Solution: Plate fewer or more cells in order to have about 50% confluency at infection stage.

Problem: Target cell line may be difficult to transduce

Solutions:

- Use a higher concentration of pseudoviral particles.
- Optimize the transduction protocol and use HEK293 cell line as positive control cells.
- Perform "Spinoculation" to improve transduction efficiency (email tech@cellecta.com for protocol).
- Check to see if Polybrene was added at 5 µg/ml.

Problem: Wrong amount of Polybrene added during infection stage

Solution: If Polybrene is toxic to the target cells, optimize Polybrene concentration in the range of 0 – 5 µg/ml.

Problem: Loss of pseudoviral titer during storage

Solution: Ensure storage of aliquoted packaged lentiviral constructs at –80°C. Each freeze-thaw cycle causes reduction of the titer by 10 – 20%. Use a fresh stock for transduction.

Problem: The assay is performed too early

Solution: Normally, the maximal expression of integrated provirus is expected to develop by 48-72 hours after infection. However, some cells exhibit delayed expression. Try the assay at a later time, such as 96 hours.

2. Transduction affects target cell viability

Problem: Polybrene is toxic for target cells

Solution: Optimize the concentration and exposure time to Polybrene during the transduction step. For some sensitive cells, Polybrene should not be used.

Problem: Pseudovirus-containing conditioned media is toxic to target cells.

Solution: Concentrate and resuspend the pseudovirus in target cell growth media.

Problem: Pseudovirus itself is toxic to target cells (Polybrene and/or conditioned media is not toxic).

Solutions:

- Decrease the incubation time of pseudovirus with target cells.
- Perform two sequential transductions, with short incubation times.

3. No expression of RFP or Puro^R (or shRNAs) in target cells

Problem: The Pol II (e.g. UbiC, EF1, PGK, CMV) or Pol III (H1 or U6) promoter is not functional in target cells.

Solutions:

- Change the target cells.
- Replace the ineffective promoter(s) with another.
- Contact Cellecta at tech@cellecta.com to have the shRNA cloned in another vector with different promoter(s).



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G. Technical Support

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For more information about Cellecta's products and services, please visit our web site at <http://www.cellecta.com>.

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H. Safety Guidelines

The HIV-based lentivector system is designed to maximize its biosafety features, which include:

- A deletion in the enhancer of the U3 region of 3'ΔLTR ensures self-inactivation of the lentiviral construct after transduction and integration into genomic DNA of the target cells.
- The RSV promoter upstream of 5'LTR in the lentivector allows efficient Tat-independent production of viral RNA, reducing the number of genes from HIV-1 that are used in this system.
- Number of lentiviral genes necessary for packaging, replication and transduction is reduced to three (gag, pol, rev). The corresponding proteins are expressed from different plasmids lacking packaging signals and share no significant homology to any of the expression lentivectors, pVSV-G expression vector, or any other vector to prevent generation of recombinant replication-competent pseudovirus.
- None of the HIV-1 genes (gag, pol, rev) will be present in the packaged pseudoviral genome, as they are expressed from packaging plasmids lacking packaging signal—therefore, the lentiviral particles generated are replication-incompetent.
- Pseudoviral particles will carry only a copy of your expression construct.

Despite the above safety features, use of HIV-based vectors falls within NIH Biosafety Level 2 criteria due to the potential biohazard risk of possible recombination with endogenous viral sequences to form self-replicating pseudovirus or the possibility of insertional mutagenesis. For a description of laboratory biosafety level criteria, consult the Centers for Disease Control Office of Health and Safety Web site at:

<http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s3.htm>

It is also important to check with the health and safety guidelines at your institution regarding the use of lentiviruses and follow standard microbiological practices, which include:

- Wear gloves and lab coat at all times when conducting the procedure.
- Always work with pseudoviral particles in a Class II laminar flow hood.
- All procedures are performed carefully to minimize the creation of splashes or aerosols.
- Work surfaces are decontaminated at least once a day and after any spill of viable material.
- All cultures, stocks, and other regulated wastes are decontaminated before disposal by an approved decontamination method such as autoclaving. Materials to be decontaminated outside of the immediate laboratory area are to be placed in a durable, leakproof, properly marked (biohazard, infectious waste) container and sealed for transportation from the laboratory.

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