Chapter 8

Lentivirus Production and Purification

Matthew J. Benskey and Fredric P. Manfredsson

Abstract

Lentiviral (LV) vectors offer unique advantages over other gene delivery systems, namely the ability to integrate transgenes into the genome of both dividing and nondividing cells. Detailed herein is a simple protocol for the production LV vectors, describing the triple transfection of an LV transfer vector and LV helper plasmids into HEK-293 cells, and the subsequent purification of virions from the cellular media. The current protocol is versatile, and can be easily modified to fit the specific needs of the researcher in order to produce relatively high-titer LV vectors which can be used to transduce a wide variety of cells both in vitro and in vivo.

Key words Lentivirus, VSV-G, Viral vector, Gene delivery, Gene integration

1 Introduction

Lentivirus (LV) is an enveloped, plus-strand RNAvirus belonging to the family of retroviridae [1]. LV vectors, derived from the human immunodeficiency virus (HIV-1), are powerful genetic tools that offer several advantages over other viral vector systems. Principle amongst the advantages of LV vectors is the ability to integrate genetic material into the genome of dividing and nondividing host cells, both in vitro and in vivo [2, 3]. LV vectors can provide efficient, stable, and long-term gene expression in terminally differentiated cells such as immune cells or neurons [4–6]. Due to the ability of LV vectors to integrate genetic material into the host genome, progeny produced from the infected cell will harbor the same transgene, an attribute that is exploited in applications such as stem cell therapy [7, 8]. Further, LV vectors offer a relatively large carrying capacity (8–9 Kb) and exhibit low immunogenicity, making these vectors extremely useful gene delivery tools.

Progress in the production of LV vectors has led to the development of replication incompetent LV vectors, which can be produced easily with minimal effort. Replication incompetent LV vectors are produced by replacing all viral genes from the LV

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genome with a transgene of interest, leaving only the *cis*-acting elements essential for viral packaging (i.e., long terminal repeats (LTR), tat activation region, Ψ packaging signal, Rev-responsive element, and polypurine tracts). The removed components of the viral genome are supplied in *trans* by separate plasmids encoding the viral genes necessary for packaging and envelope pseudotyping. The transfer vector containing the transgene of interest and the separate helper plasmids are co-transfected into a mammalian cell line, and LV vectors can be harvested and purified from the media. Providing the necessary viral genes in separate plasmids greatly reduces the possibility of mobilization and the production of a replication competent virus. Further, this system also affords the ability to easily mix and match glycoproteins from alternative enveloped viruses in order to optimize the expression levels and tropism of LV vectors specifically to the desired application [9, 10].

Here we describe a simple and efficient protocol for packaging a vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped LV vector. The current protocol describes the triple transfection of a transfer vector and two LV helper plasmids, and the subsequent purification and concentration of LV vectors from cellular media by ultracentrifugation [11]. This protocol is flexible and can be easily scaled up or down in order to meet the needs of the researcher. Utilizing the current protocol we have generated LV-vectors with titers in the range of 2×10^{12} viral genomes (vg)/mL, that are capable of transducing neurons within an adult animal in vivo, as well as difficult-to-transfect cells in vitro (Fig. 1).

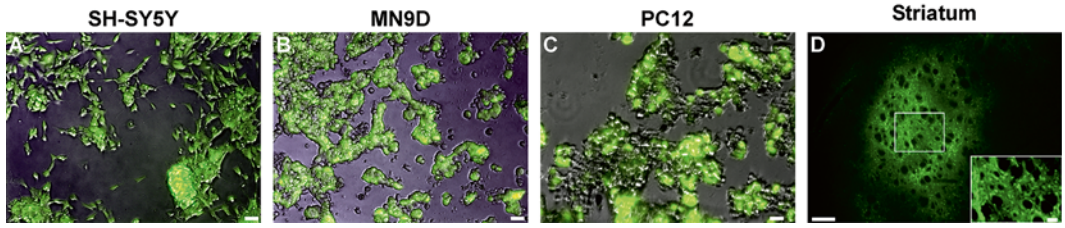

Fig. 1 GFP expression following transduction with a VSV-G pseudotyped LV vector. LV vectors produced using the current protocol efficiently transduces cells in vitro (**a**–**c**) and in vivo (**d**). SH-SY5Y (**a**), MN9D (**b**), or PC12 (**c**) cells were plated and grown to approximately 50 % confluency. Cells were transduced with the VSV-G pseudotyped LV vector expressing GFP under the control of the cytomegalovirus/chicken β-actin hybrid promoter at a multiplicity of infection (MOI) of 100. GFP expression was visualized 48 h post-transduction. To demonstrate LV vector transduction in vivo, adult male rats were injected with 2 μL of the VSV-G pseudotyped LV vector expressing GFP under the control of the cytomegalovirus/chicken β-actin hybrid promoter (2.07×10^{12} vg/mL) into the striatum. Animals were sacrificed 4 weeks post-transduction, and native GFP fluorescence was visualized in the striatum (**d**). The *inset in panel* (**d**) shows a higher magnification image of the area within the *box* in panel (**d**). Scale bar in panel (**a**)–(**c**) represents 50 μm. Scale bar in panel (**d**) and inset represent 200 μm and 50 μm, respectively

2 Materials

2.1 Plasmids and Cells

1. Human Embryonic Kidney 293 T (HEK-293 T) Cells (ATCC, Manassas, VA, USA).
2. pNHP packaging vector (Addgene, Cambridge, MA, USA; *see* **Notes 1** and **2**).
3. pHEF-VSVG (Addgene, Cambridge, MA, USA; *see* **Note 3**).
4. pFIN transfer vector (Addgene, Cambridge, MA, USA; *see* **Note 4**).

2.2 Reagents and Supplies

1. Penicillin/Streptomycin (Pen/Strep; 10,000 units/mL).
2. Fetal bovine serum (FBS).
3. TrypLE Express 1× dissociation reagent (Thermo Fisher Scientific, Grand Island, NY, USA).
4. 25 kDa linear Polyethylenimine (PEI) (Polysciences, Warrington, PA, USA).
5. Dulbecco's modified Eagle media (DMEM) containing high glucose, glutamine, and sodium pyruvate.
6. 1× Dulbecco's phosphate-buffered saline (DPBS) containing calcium and magnesium.
7. 0.22 μm Stericup filter unit (EMD Millipore, Billerica, MA, USA).
8. 1.5 M NaCl: Add 87.66 g of NaCl to 800 mL of dH_2O, stir until completely dissolved. Adjust to 1 L with dH_2O and filter sterilize with a 0.22 μm filter unit.
9. 0.45 μm Stericup-HV filter units (EMD Millipore, Billerica, MA, USA).
10. Centricon Plus-70 Centrifugal filter units (EMD Millipore, Billerica, MA, USA).
11. 38.5 mL conical thinwall polyallomer ultracentrifuge tubes or equivalent (Beckman Coulter, Indianapolis, IN, USA).
12. T175 Culture flasks.
13. SW32Ti rotor (Beckman Coulter, Indianapolis, IN, USA) or equivalent.
14. Beckman Coulter L-100XP Ultracentrifuge (Beckman Coulter, Indianapolis, IN, USA) or equivalent.
15. Sorvall RC 6 superspeed centrifuge (Thermo Fisher Scientific, Grand Island, NY, USA) or equivalent.
16. Real-time PCR system: Applied biosystems 7500 (Thermo Fisher Scientific, Grand Island, NY, USA) or equivalent.

17. Lenti-X-qRT-PCR kit (Clonetech, Mountain View, CA, USA).
18. Standard tissue culture equipment.

3 Methods

3.1 Prepare Reagents

1. Prepare complete HEK-293 T media (DMEM with 10 % FBS and 1 % Pen/strep). Add 100 mL of FBS and 10 mL of Pen/strep to 890 mL of DMEM (*see* **Note 5**).
2. Prepare PEI transfection reagent. Heat 200 mL of dH_2O to 80 °C in a beaker on a stirring hotplate. Add 80.75 mg of PEI to the beaker and stir with a stir bar. Stir at 80 °C until the PEI is completely dissolved. Adjust pH to 8.0 with hydrochloric acid. Adjust to 250 mL with dH_2O. Filter sterilize with a 0.22 μm filter unit (*see* **Note 6**).
3. Prepare the required amount of plasmidDNA (for each respective plasmid) using a standard plasmid DNA preparation method. DNA must be endotoxin free.

3.2 Transfection and Viral Purification

1. Seed the T175 flask with HEK-293 T cells. Grow HEK-293 T cells in a T75 starter flask prior to seeding the T175. Once the T75 starter flask is at 90–95 % confluency aspirate media from the flask and gently rinse with sterile PBS. Incubate cells with 4 mL of dissociation reagent (e.g., TrypLE Express) at 37 °C for 5 min. Add 6 mL of media containing serum to deactivate the dissociation reagent, and triturate with 25–30 full strokes of a serological pipette to create a single cell suspension.
2. Determine cell number per mL using a hemocytometer.
3. Calculate the amount of the single cell suspension needed to seed the T175 flask with 3×10^7 HEK-293 T cells. Add cells to flask and bring the total volume of the flask to 28 mL with warm (37 °C), complete HEK-293 T media.
4. Incubate T175 Flask at 37 °C with 5 % CO_2 overnight or until 80–90 % confluency is reached.
5. Prepare transfection mixture. To create the complete transfection mixture, two separate solutions are first prepared, a DNA solution and a PEI solution.
6. Prepare the DNA solution. In a 50 mL conical add 21.3 μg pNHP, 8 μg pHEF-VSVG, 7.2×10^{11} copies of the pFIN transfer vector (*see* **Note 7**), and 148.75 μL of 1.5 M NaCl. Bring total volume of DNA solution to 1.44 mL with dH_2O and mix well.
7. Prepare PEI solution. In a separate 50 mL conical add 460.59 μL PEI and 148.75 μL 1.5 M NaCl to 1.04 mL dH_2O (*see* **Note 8**). Mix well.

8. Prepare the complete transfection solution by adding the PEI solution dropwise to the DNA solution. Vortex vigorously for 1–2 min (*see* **Note 9**).
9. Incubate the complete transfection solution at room temperature for 20 min. The transfection solution should turn cloudy.
10. Remove all media from the 80–90 % confluent T175 flask. Mix the complete transfection solution with 28 mL of complete HEK-293 T media in a graduated cylinder. Gently add the transfection-media mixture to the T175 flask and be sure all cells are covered.
11. Incubate the T175 flask at 37 °C and 5 % CO_2.
12. Collect the supernatant containing virus (*see* **Note 10**). The supernatant is collected at 24 h post-transfection and again at 40 h post-transfection. At the 24-h time point, collect the viral media in a 50 mL polypropylene conical. Replace the media with 28 mL of warm (37 °C) complete HEK-293 T media.
13. Spin the collected viral media at 783 × *g* in the RC 6 (or equivalent) centrifuge for 5 min to pellet cell debris. Collect the supernatant and store in a fresh tube at 4 °C.
14. At the 40 h time point, again collect the media and remove debris by centrifugation as in **steps 12** and **13**.
15. Pool all viral media from the 24- and 40-h time points. Filter the viral media through the 0.45 μm Stericup-HV filter.
16. Split the viral media into two thinwall polyallomer ultracentrifuge tubes (*see* **Notes 11** and **12**).
17. Carefully load the filled ultracentrifugation tubes into the SW32Ti rotor and spin at 80,000 × *g* for 2 h at 4 °C.
18. Remove the supernatant from the viral pellet by carefully aspirating the media from the ultracentrifugation tube (*see* **Note 13**).
19. After removing the supernatant resuspend the viral pellet by gently overlaying the pellet with 10 μL of sterile DPBS in the conical (*see* **Note 14**).
20. Seal the conical tube with parafilm and store at 4 °C overnight.
21. After overnight incubation, place the sealed conical on ice. Gently shake the conical on an orbital rotating shaker for 2 h [11].
22. Aliquot the resuspended virus into working aliquots and store at −80 °C (*see* **Notes 15**).
23. Utilize one of your aliquots from **step 22** and titer your viral prep using the Lenti-X titering kit following the manufacturer's instructions.

4 Notes

1. The current protocol utilizes the second-generation LV packaging system in which all necessary genes are separated into three distinct plasmids (the transfer vector, envelope plasmid and packaging plasmid). A third-generation packaging system is also available, in which the necessary genes are further separated into four distinct plasmids (transfer vector, envelope, and two packaging plasmids). Although the third generation system offers increased biosafety, it is also more cumbersome due to the increased number of plasmids. However, the current protocol is amenable to third generation packaging systems.
2. The pNHP packaging helper plasmid encodes the following genes: Gag (encodes the capsid, nucleocapsid and matrix structural proteins), Pol (encodes reverse transcriptase, protease and integrase proteins), Tat (encodes protein for transactivation of transcription from viral LTR), Rev (encodes protein that mediates export of viral RNAs from nucleus), as well as vpr (mediates cell cycle arrest and assists in nuclear import) and vpu (enhances virion production and degradation of CD4 receptor in the host cell).
3. pHEF-VSVG encodes the envelope glycoproteins derived from the VSV. LV vectors pseudotyped with VSV-G exhibit a broad host cell range; however, the tropism, titers, and expression of levels LV vectors can be altered by pseudotyping vectors with glycoproteins from alternative enveloped viruses [9, 10].
4. The pFIN LV transfer vector contains a green fluorescent protein (GFP) gene followed by the woodchuck posttranscriptional response element driven by the EF1α promoter. This expression cassette is flanked by HIV-1 LTRs. The pFIN vector also contains components from the LV genome absolutely necessary for viral packaging. If desired, the promoter and transgene can be easily replaced with a promoter or transgene of interest using standard cloning methods.
5. All work should be done in a culture hood using sterile technique.
6. Very little HCl is required to bring the PEI solution to a pH of 8.0. If too much HCl is added, use NaOH to bring the pH back to 8.0. Aliquot the PEI in 12 mL aliquots and store at −80 °C for future use.
7. The size (in nucleotide bases) of the genetic material to be packaged in the lentivirus vector will alter the size of the pFIN plasmid and the corresponding weight of DNA needed for

transfection. To maintain accuracy, determine the molecular weight of the pFIN plasmid containing your genetic material of interest and then calculate the total amount (in nanograms) necessary to transfect 7.2×10^{11} copies of the pFIN plasmid. This can be done easily using an online DNA molecular weight calculator.

8. PEI must be completely homogenous prior to use. If using a previously frozen aliquot, completely thaw at room temperature. Once thawed, heat solution to 55 °C to ensure all PEI is in solution. Let cool to room temperature before use.
9. Adding the PEI dropwise to the DNA mixture prevents the rapid precipitation of DNA and ensures a homogenous DNA/PEI solution. It is helpful to place the DNA mix on a stir plate and gently stir the DNA mixture while adding the PEI mixture dropwise.
10. The quality of the transfection can be determined by viewing the cells under a fluorescent microscope. Alternatively if the GFP transgene was removed, transfection efficiency can be determined by observing the VSV-G mediated fusion of HEK-293 T cells into multinucleated cell syncytia [11].
11. If making larger preparations of lentivirus (using more flasks or larger flasks) it is necessary to concentrate the viral media prior to ultracentrifugation. This can be done using a column concentrator and centrifugation (e.g., Centricon-70 ultra centrifugation column [11]) or using tangential flow filtration [12].
12. Be sure to fill the ultracentrifugation tubes to the required level according to the manufacturer's instructions or the tube will collapse during centrifugation.
13. To remove the final few mL of media from the tube, tilt the tube at a 45° angle and continue to aspirate the media as it flows down the side of the tube away from the pellet. Do not disturb the pellet at the bottom of the tube.
14. Due to the inherently "sticky" nature of the viral envelope and exposed glycoproteins, it is necessary to use siliconized pipette tips and tubes when handling the virus. For a detailed protocol on siliconizing *see* Chapter 14.
15. LV cannot be refrozen after thawing; as such it is best practice to aliquot the virus into small working aliquots.

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