Video Article

Production of Lentiviral Vectors for Transducing Cells from the Central Nervous System

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Abstract

Efficient gene delivery in the central nervous system (CNS) is important in studying gene functions, modeling neurological diseases and developing therapeutic approaches. Lentiviral vectors are attractive tools in transduction of neurons and other cell types in CNS as they transduce both dividing and non-dividing cells, support sustained expression of transgenes, and have relatively large packaging capacity and low toxicity [1-3]. Lentiviral vectors have been successfully used in transducing many neural cell types *in vitro* [4-6] and in animals [7-10].

Great efforts have been made to develop lentiviral vectors with improved biosafety and efficiency for gene delivery. The current third generation replication-defective and self-inactivating (SIN) lentiviral vectors are depicted in **Figure 1**. The required elements for vector packaging are split into four plasmids. In the lentiviral transfer plasmid, the U3 region in the 5' long terminal repeat (LTR) is replaced with a strong promoter from another virus. This modification allows the transcription of the vector sequence independent of HIV-1 Tat protein that is normally required for HIV gene expression [11]. The packaging signal (Ψ) is essential for encapsidation and the Rev-responsive element (RRE) is required for producing high titer vectors. The central polypurine tract (cPPT) is important for nuclear import of the vector DNA, a feature required for transducing non-dividing cells [12]. In the 3' LTR, the cis-regulatory sequences are completely removed from the U3 region. This deletion is copied to 5' LTR after reverse transcription, resulting in transcriptional inactivation of both LTRs. Plasmid pMDLg/pRRE contains HIV-1 gag/pol genes, which provide structural proteins and reverse transcriptase. pRSV-Rev encodes Rev which binds to the RRE for efficient RNA export from the nucleus. pCMV-G encodes the vesicular stomatitis virus glycoprotein (VSV-G) that replaces HIV-1 Env. VSV-G expands the tropism of the vectors and allows concentration via ultracentrifugation [13]. All the genes encoding the accessory proteins, including Vif, Vpr, Vpu, and Nef are excluded in the packaging system. The production and manipulation of lentiviral vectors should be carried out according to NIH guidelines for research involving recombinant DNA (http://oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.pdf). An approval from individual Institutional Biological and Chemical Safety Committee may be required before using lentiviral vectors. Lentiviral vectors are commonly produced by cotransfection of 293T cells with lentiviral transfer plasmid and the helper plasmids encoding the proteins required for vector packaging. Many lentiviral transfer plasmids and helper plasmids can be obtained from Addgene, a non-profit plasmid repository (http://www.addgene.org/). Some stable packaging cell lines have been developed, but these systems provide less flexibility and their packaging efficiency generally declines over time [14, 15]. Commercially available transfection kits may support high efficiency of transfection [16], but they can be very expensive for large scale vector preparations. Calcium phosphate precipitation methods provide highly efficient transfection of 293T cells and thus provide a reliable and cost effective approach for lentiviral vector production.

In this protocol, we produce lentiviral vectors by cotransfection of 293T cells with four plasmids based on the calcium phosphate precipitation principle, followed by purification and concentration with ultracentrifugation through a 20% sucrose cushion. The vector titers are determined by fluorescence- activated cell sorting (FACS) analysis or by real time qPCR. The production and titration of lentiviral vectors in this protocol can be finished with 9 days. We provide an example of transducing these vectors into murine neocortical cultures containing both neurons and astrocytes. We demonstrate that lentiviral vectors support high efficiency of transduction and cell type-specific gene expression in primary cultured cells from CNS.

Video Link

The video component of this article can be found at https://www.jove.com/video/4031/

Protocol

1. Packaging of Lentiviral Vectors

Lentiviral vectors are produced by cotransfection of a lentiviral transfer vector and other plasmids required for packaging into 293T cells by calcium phosphate transfection method. We use 10 100-mm tissue culture dishes in this protocol. It can be scaled up or down depending on applications. The 293T cell line is maintained in Dulbecco's modified Eagles medium (DMEM) with high glucose (4500 mg/L), supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin in 37 °C incubator with 5% CO_2.

1. Seed 293T cells at 30-40% confluence to 10 100-mm tissue culture dishes (3 x 10^6 cells/dish) in culture medium. Return the cells to incubator.
2. After 20-24 h culture, check the cell density. The cells should be about 80% confluence at the time of transfection.
3. Prepare a 50-ml tube. Add 4.4 ml TE79/10 (1 mM TrisHCl, 0.1 mM EDTA, pH 7.9) minus the total volume of the following plasmid DNA. Add 100 µg lentiviral transfer plasmid (**Figure 1**), 58 µg pMDLg/pRRE, 31 µg pCMV-G, 25 µg pRSV-Rev, 600 µl 2M $CaCl_2$. Gently Mix.
4. Prepare another 50-ml tube. Add 5 ml 2x HBS (0.05 M HEPES, 0.28 M NaCl, 1.5 mM Na_2HPO_4, pH 7.12).
5. Take the DNA-$CaCl_2$ mixture by 10-ml pipette and add to the tube containing 2 x HBS, dropwise while vortexing the tube.
6. Keep the precipitation reaction at room temperature (RT) for 30 min.
7. Remove the culture dishes from incubator. Mix the precipitation reaction well by vortexing. Add 1 ml of suspension to each 100-mm dish containing cells. The suspension must be added slowly, dropwise while gently swirling the medium in the dish. Return these dishes to the incubator and leave for 5 h.
8. Remove the medium from the culture. Add 6 ml fresh culture medium containing 6 mM sodium butyrate to each dish. Return the cultures to incubator. After overnight culture, if there is a fluorescent reporter in the construct, check reporter gene expression under fluorescent microscope. Usually, over 80% of the cells express the reporter gene if it is driven by a ubiquitous promoter (e.g. CMV promoter).
9. Two days (40-44 h) after transfection, collect supernatant from 10 dishes into 2 50-ml tubes (about 30 ml each tube). Freeze the supernatant in -80 °C freezer or go to next step.

2. Concentration and Purification of the Vectors

1. Centrifuge the freshly collected or thawed supernatant at 900 g (about 2000 rpm) for 10 min to remove any cell debris in the supernatant.
2. Attach a 60-ml syringe to a 0.2-µm SFCA syringe filter. Transfer supernatant from 50-ml tube to the syringe. Filter the supernatant into a polyallomer centrifuge tube.
3. Take 5 ml 20% sucrose (prepared in PBS) up in a 5-ml pipette. Insert the pipette to the bottom of the centrifuge tube containing supernatant. Slowly add the sucrose solution under the vector supernatant. Repeat these steps for supernatant from another tube.
4. Centrifuge the supernatant at 11000 rpm and 4 °C for 4 h with Beckman SW28 swing rotor.
5. Remove the supernatant. Add 150 µl 4% Lactose (prepared in PBS) to each centrifuge tube. Resuspend the pellets.
6. Transfer the concentrated vector from all centrifuge tubes to a 1.5-ml tube. Leave the tube on ice for 15 min.
7. Mix the vector suspension by pipetting. Spin with microcentrifuge at full speed (about 16000 g) for 1 min.
8. Transfer supernatant to a new 1.5-ml tube. Divide the final sample into 20 µl aliquots and stored them in -80 °C freezer.

3. Titration of the Vectors

1. Seed 5 x 10^4/well of HT1080 cells in 12-well plate in 1 ml DMEM medium supplemented with 10% FBS.
2. After overnight culture, count cells from one well and score the cell number.
3. Make 5-fold serial dilution (1:5, 1: 25; 1:125; and 1:625) of the concentrated vector with culture medium. Add l µl of each diluted vector to separate wells. The samples may be duplicated to increase accuracy.
4. Add 1 µl 4 mg/ml Polybrene (Hexadimethrine Bromide) in each well containing vector and into a well without vector. Mix by gently shaking the plate. Return to incubator for 48 h.
5. Remove medium from the cell culture wells. Wash each well with PBS. Add 250 µl 1x trypsine-EDTA solution to the cells. When the cells are detached (3-5 min), add 1 ml culture medium. Resuspend the cells by pipetting. Transfer cell suspension to 1.5 ml centrifuge tubes.
6. Centrifuge at 900 g for 6 min. For vectors with a fluorescent reporter gene (e.g. GFP), go to step 3.7 for FACS analysis. For vectors without a reporter, go to step 3.8 for real time qPCR.
7. For vectors containing a fluorescent reporter gene, remove the supernatant and resuspend the pellet with 300 µl of 3.7% formaldehyde in PBS. Determine the percentage of the reporter positive cells by FACS analysis. The titer will be represented as transduction units per milliliter concentrated vector (TU/ml).

$$\text{Titer} = \frac{\text{cell number x \% of reporter}^{+}\text{ cells x dilution factor}}{\text{vector volume (ml) x 100}}$$

For example, if 1 x 10^5 cells was transduced with 1/25 µl (0.04 µl) vector and 30% cells are reporter positive, the titer will be:

$$\frac{1 \times 10^5 \times 30 \times 25}{100} = 7.5 \times 10^5/\mu l = 7.5 \times 10^8/ml$$

Only use the dilutions fall in a linear relationship between the percentage of positive cells and the amount of vector added to calculate titer. The final titer should be an average of the titers obtained from transductions of at least 2 different amounts of the vector.

8. For vectors without a fluorescent reporter gene, extract genomic DNA from HT1080 cells using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. Amplify vector sequence in genomic DNA using ABI Prism 7000 Sequence Detection System (Applied Biosystems) with primers (in HIV-1 PBS/psi region [17]) 5'-CCGTTGTCAGGCAACGTG-3' and 5'-AGCTGACAGGTGGTGGCAAT-3', and TaqMan probe 5'-FAM-AGCTCTCTCGACGCAGGACTCGGC-TAMRA-3'. Albumin gene that is a single copy gene in the genome (2 copies/cell) was also amplified with primers 5'-TGAAACATACGTTCCCAAAGAGTTT-3' and 5'-CTCTCCTTCTCAGAAAGTGTGCATAT-3', and probe 5'-FAM-TGCTGAAACATTCACCTTCCATGCAGA-TAMRA-3' as an internal control. Determine the copy numbers of vector and albumin by PCR in 96-well plate according to the manufacture's instruction with the following program: 50 °C for 2 min, 95 °C for 10 min, and 35 cycles of 95 °C for 15 sec and 60 °C for 2 min. Ten-fold serial dilutions of plasmids of known concentration (represented as copy number)

containing the template sequences should also be amplified to create a standard curve for quantification of unknown samples. The titer will be represented as integration units per milliliter concentrated vector (IU/ml).

$$\text{Titer} = \frac{\text{cell number x copy number of vector x 2 x dilution factor}}{\text{copy number of albumin x vector volume (ml)}}$$

4. Transduction of Neocortical Cultures

Neocortical cultures containing both neurons and glia are prepared from mouse cortices using a two-step plating procedure as previously described [18]. Neocortices obtained from fetal mice at 14-16 days gestation are plated onto a previously established glial monolayer in MEM supplemented with 10% FBS, 20 mM glucose and 2 mM glutamine in 24-well tissue culture plate.

1. After 5 days *in vitro*, add 10 μM cytosine arabinoside (Ara-C) in neocortical culture to inhibit non-neuronal cell division. Continue culture the cells for 2 days.
2. Warm culture medium in 37 °C water bath for 5-10 min. Replace Ara-C containing medium with fresh culture medium (500 μl/well).
3. Add vector with desired MOI (multiplicity of infection; the ratio of the number of vector particles to the number of target cells) to the culture. Continue culture for 24 h. We use MOI of 1-10 (usually 5) in primary cortical cultures.
4. Replace the culture medium with fresh medium. Continue culture. If there is a reporter gene in the vector construct, check cells under fluorescent microscope 2 days after transduction. Reporter gene expression will be visible in neurons 2-7 after transduction, depending on the vector design and the dose used.

5. Representative Results

The titers of lentiviral vectors produced with this protocol range 10^8-10^{10} IU/ml, which are suitable for transduction of a variety of cell types from CNS both *in vitro* and *in vivo*. **Table 1** and **figure 2** show a representative result using the vectors produced by this protocol. We transduced murine neocortical cultures with lentiviral vectors expressing green fluorescent protein (GFP) controlled by synapsin (SYN) promoter or glial fibrillary acidic protein (GFAP) promoter. Seven days after transduction, we performed immunostaining to label neurons and astrocytes with anti-NeuN and anti-GFAP antibodies, respectively. As shown in **table 1** and **Fig. 2A**, after transduction with the vector carrying the synapsin promoter, over 90% of neurons ($NeuN^+$ cells) express GFP and no astrocytes ($GFAP^+$ cells) express this reporter gene. When GFAP promoter is used in the vector construct (**Fig. 2B**), about 80% of astrocytes ($GFAP^+$ cells) express GFP; all GFP^+ cells are astrocytes as confirmed by colocalization with GFAP and the absence of GFP expression in NeuN-labeled cells. These results demonstrate that lentiviral vectors are very efficient to deliver transgenes to cells from CNS and cell-specific gene expression can be achieved when appropriate promoters are used.

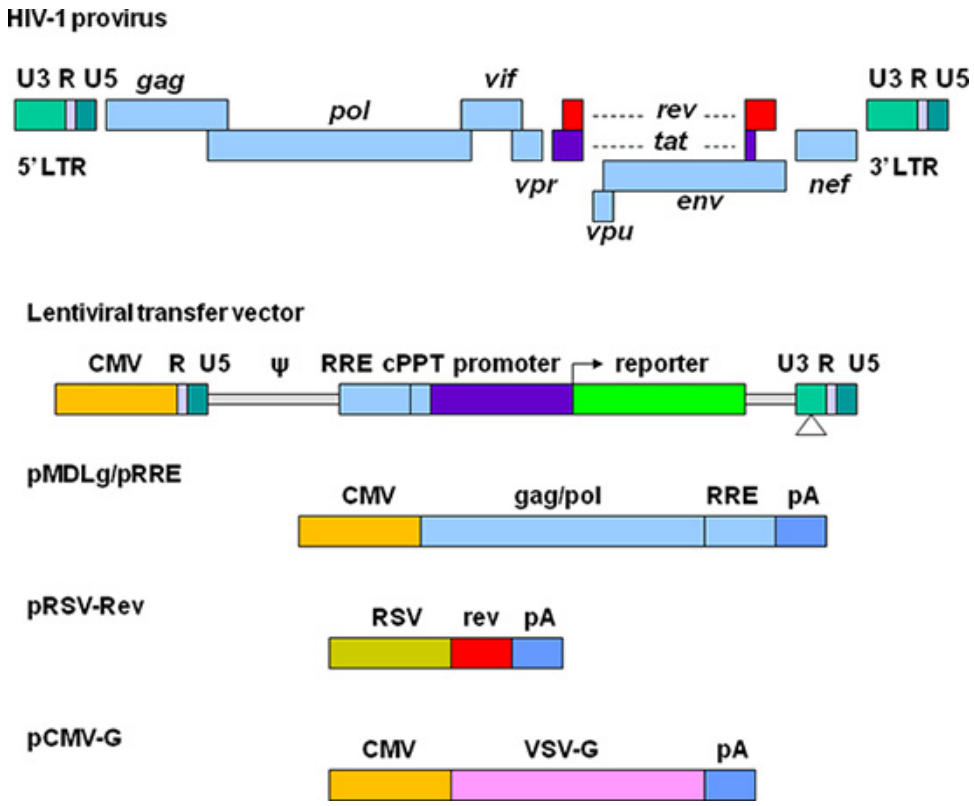

Figure 1. Schematic representation of HIV-based lentiviral vectors and the packaging plasmids. The HIV-1 provirus is shown at the top. The elements for vector production are separated into four different plasmids. The lentiviral transfer plasmid contains a hybrid 5' LTR in which the U3 region is replaced with the cytomegalovirus (CMV) promoter, the packaging signal (ψ), the RRE sequence, the central polypurine tract (cPPT), a gene of interest (e.g. a fluorescent reporter) along with a promoter of choice, and the 3' LTR in which the *cis* regulatory sequences are completely removed from the U3 region. pMDLg/pRRE contains the gag and pol genes and RRE sequence from HIV-1 under the control of the CMV promoter. pRSV-Rev contains the coding sequence of Rev driven by the RSV promoter. pCMV-G contains the VSV-G protein gene under the control of the CMV promoter. PA indicates the polyadenylation signal from human β-globin gene.

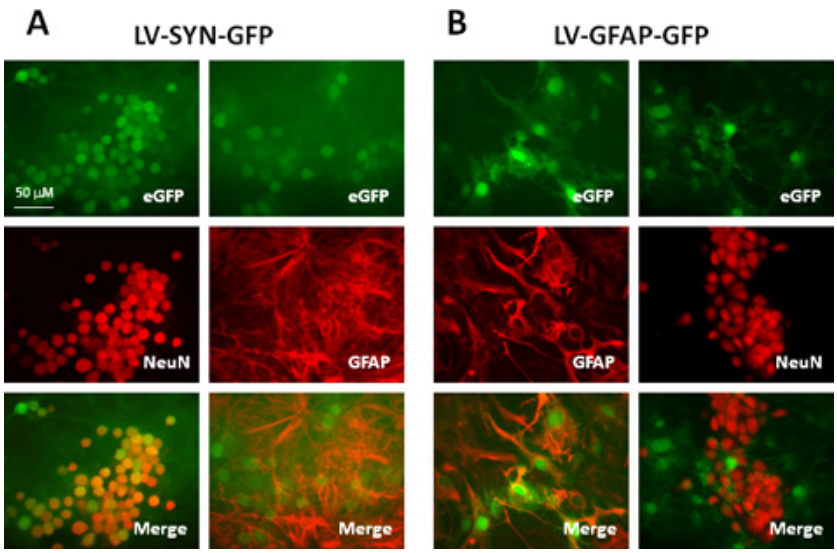

Figure 2. Expression of reporter genes in mouse neocortical mixed culture transduced with lentiviral vectors carrying cell type-specific promoters. The cultures were transduced with LV-SYN-GFP (A) or LV-GFAP-GFP vectors (B) at a MOI of 5. Seven days after transduction, the cells were immunostained with anti-NeuN or anti-GFAP antibody. Upper panels show GFP fluorescence, middle panels show immunostaining and lower panels are merged images (GFP: green; NeuN or GFAP: red).

Vector	GFP^+ cells in neurons	GFP^+ in astrocytes
LV-SYN-GFP	92.2±7.3	0

LV-GFAP-GFP	0	78.3±11.5

Table 1. Comparison of GFP expression in murine neocortical cultures transduced with lentiviral vectors carrying different promoters[a].
[a]Murine neocortical cultures (5 x 10^5/well in 24-well plate) were transduced with LV-SYN-GFP or LV-GFAP-GFP at an MOI of 5. Seven days after transduction, cultures were fixed and immunostained for NeuN or GFAP. The number of GFP and NeuN/GFAP expressing cells were counted in images from 10 fields per experimental condition. The values represent the percentage of neurons ($NeuN^+$ cells) or astrocytes ($GFAP^+$ cells) that also expressed the GFP reporter gene. The values shown are means ± SD from three independent experiments.

Discussion

In this protocol, we have shown the production of lentiviral vectors and application of these vectors in neocortical cultures. We demonstrated efficient and cell type-specific transduction with the vectors produced by these methods. When the synapsin promoter is used, GFP expression is strictly neuron specific. When the GFAP promoter is used, GFP expression is exclusively in astrocytes. If no cell type-specific expression is required, a ubiquitous promoter may be used. We found both ubiqutin and phosphoglycerate kinase (PGK) promoters can drive high level gene expression in neocortical cultures [6]. Gene expression driven by lentiviral vectors can be customized for specific levels of expression or cell types by choice of promoters (e.g., ubiquitous or cell type-specific) or by using different envelope proteins or vector pseudotypes to target specific tissues or applications. For example, pseudotyping with rabies G protein or a fusion of VSV-G and rabies protein supports retrograde axonal transport [19,20]. The transient transfection protocol allows vector packaging with different envelope proteins.

Lentiviral vectors are commonly concentrated by ultracentrifugation without purification steps. These unpurified vectors are suitable for many cell types. However, primary neuronal cultures are sensitive to contaminants from producer cells, resulting in batch to batch variation for cytotoxicity. The 20% sucrose cushion purification step makes the vectors consistently non-toxic in primary neurons. We recommend using purified vectors for transduction of primary neurons and injection into animals. If larger scale or higher purity of vector preparations are required, other purification techniques, such as affinity chromatography [21] and anion exchange membrane chromatography [22] may be used. As long-term transduction is generally required in primary neuronal cultures, special caution should be taken to minimize contamination during preparation of the vectors. Passing the vector supernatant with 0.2 μM filter and using autoclavable polyallomer centrifuge tubes during concentration of the vector will serve this purpose. Bottle-top filters may be used if a large volume of supernatant needs to be filtered. Sodium butyrate has been reported to stimulate the activity of promoters [23]. Addition of sodium butyrate to the culture medium after transfection of the producer cells can increase the vector titer more than 10 folds [24]. Polybrene (hexadimethrine bromide) has been widely used for gene transfer protocols to increase the efficiency of retroviral gene transfer by neutralizing the negative charges, thereby facilitating vector-cell interaction [25]. In our hands, polybrene is toxic to neurons in neocortical cultures. Therefore, polybrene should be avoided in transducing primary neuronal cultures. If there is a fluorescent reporter in the vector, it is convenient to determine the vector titer by FACS analysis. When no reporter is available or a reporter gene is driven by a tissue specific promoter, qPCR for detecting integration of the vector in target cells should be a better choice as the integration of vector is independent of transgene expression. The titer of specific vectors determined by FACS analysis will be lower than that of qPCR as not all integrated copies of the vector are functional. Vector titers can also be determined by measuring HIV p24 protein by ELESA or vector genomic RNA by qRT-PCR in vector preparations. However, these methods are less accurate due to the significant number of defective viral particles unavoidably generated during the packaging process and the functional particles that do not successfully transduce target cells [26]. The vectors made by this protocol have been used successfully in both *in vitro* and *in vivo* in rodent brain [27]. Investigators should test in their systems individually, as vector-mediated gene expression is not necessarily identical in cell cultures and *in vivo* systems even in the same cell type [6,28]. Lentiviral vectors have been widely used for overexpressing or knocking down genes of interest in a variety of cell-types in CNS. Our protocol should be helpful for neuroscience investigators to develop lentiviral vectors in their research applications.

Disclosures

No conflicts of interest declared.

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