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Lentiviral transduction of neuronal cells

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Abstract

Here we describe a general method for the construction of a lentivirus vector using a specific example of the construction of a lentivirus containing the luciferase reporter gene under the control of two hypothetical promoters and derived HIV-1 based lentivirus expression vector pLVX-Puro. This method can be used to compare the strength and regulation of different promoters. In this example, the target cells for transduction are human primary fetal astrocytes but the method is applicable to any primary cell culture from the CNS or other tissue and can be used to examine the strength of a particular promoter in different cell types. HIV based lentivirus particles are prepared by transfection of 4 plasmids into 293T cells using the Eugene 6 transfection reagent.

Keywords

Lentiviral vectors; transduction; primary human fetal astrocytes

1. INTRODUCTION

Lentiviruses are a complex genus of retroviruses, which were named from “lente”, which is the Latin word for slow referring to the prototypic slow progressing neurologic disease of sheep caused by maedi/visna virus [1]. They are enveloped particles containing homodimers of the linear single-stranded RNA genome [2]. Lentiviruses resemble γ -retroviral vectors and stably integrate into genome of the host cell, allowing the persistent expression of the gene of interest. However, unlike retroviruses, lentiviruses require active transport of the pre-integration complex across the nuclear pore by the action of the nuclear import machinery of the host cell [3], which allows lentiviruses to infect both dividing and non-dividing cells [4–6].

The first lentiviral vectors were derived from HIV [7], the most extensively studied lentivirus. The incorporation of envelope proteins of other viruses in HIV particles was a significant step for the development of HIV based vectors. Most of HIV derived lentiviruses contain the envelop protein of rhabdovirus family (VSVG) instead of the HIV envelope glycoprotein. This modification not only increase host-range of VSVG pseudotyped lentiviruses but also increases the ability to concentrate VSV-G pseudotyped particles more than 1000-fold by ultracentrifugation. Lentiviral vectors (LVS) have become one of the most widely used vectors for the fundamental biological research. Currently, most lentiviral

vectors are produced using transient transfection of packaging and vector plasmids. Using the 293T cells, which are susceptible to efficient transfection, one routinely obtains titers of $1 \times 10^9 - 1 \times 10^{10}$ IU/ml following transient transfection with the most recent generation of packaging and vector constructs, and then concentrating of the virus particles using ultracentrifugation [3].

Lentiviral vectors have been successfully used to transduce most cell types within the central nervous system (CNS) in vivo, including neurons, astrocytes, adult neuronal stem cells, oligodendrocytes, and astrocytes. Lentivirus are also well suited for the transduction of various stem/progenitor cells, including hematopoietic stem cells (HSC) [5,8,9].

It has been reported that the high level expression of the gene of interest in lentivirus transduction of different cell lines in CNS depends on the strength of the promoter used. If the promoter is active in glia cells, then high level transgene expression in astrocytes can be achieved. The activity of the internal promoter used in lentiviral transduction determines the different expression pattern that have been reported although it is likely that the different cell types in the CNS can be transduced with lentivirus with similar efficiencies [9].

2. MATERIALS

1. Complete DMEM medium: 4 mM L-glutamine; 4.5 g/l D-glucose; 100 U/ml Penicillin-Streptomycin; 10% heat-inactivated FBS. (See Note 1)
2. 293T cells: 293T cells are good for viral production. It is essential that the cells be well maintained and of relatively low passage number. 293T Cells were maintained in a complete DMEM.
3. Primary fetal astrocytes: Purified primary human fetal astrocytes (PHFA) will be prepared as we have previously described [10] using a method based on Cole & de Vellis [11], and Yong & Antel [12]. Briefly, 12–16 week old human fetal brain tissue is obtained from Advanced Biosciences Resources, Inc. (Alameda, CA) and dissected to remove blood vessels and meninges. Tissue is mechanically disrupted and digested with trypsin and DNase I. Cells are passed through a 70 μ m filter to remove debris and pelleted by centrifugation. Mixed primary cultures are grown for 5 d in culture and then astrocytes purified. This is done by briefly trypsinizing cultures to remove non-astrocytic cells and washing the astrocyte monolayers. Culture purity is ascertained by staining for glial fibrillary acidic protein.
4. Plasmids: The purity of the DNA is important for transfection. Plasmids should be prepared using a high quality kit such as that from Qiagen. Plasmid systems are used for the production of lentivirus: PLVX-puro is an HIV-1 based lentiviral expression vector with gene of interest and 3 lentiviral packaging plasmids pMDlg/PRRE(expresses HIV gag/pol), Prsv-REV(express HIV REV) and

¹It is essential that the cells be well maintained and of relatively low passage number. The proper cell density is crucial for high production yield.

pMD2.G(express VSV glycoprotein) (see Note 2). These packaging plasmids can be purchased from Clontech.

5. Penicillin-Streptomycin (100X) solution of 10,000 units/ml Penicillin G sodium and 10000 µg/ml streptomycin sulfate.
6. Tryple express (GIBCO). (See Note 3)
7. Dulbecco's phosphate buffered saline (DPBS).
8. High-efficiency Transfection reagent (e.g., Fugene 6, Promega).
9. High-efficiency Plasmid Maxiprep Kit (e.g., Qiagen).
10. Cell freezing medium with 5% DMSO, 20% FBS.
11. Cryovials for freezing virus stocks

3. METHODS

3.1. Lentiviral transfection and production

1. Plate 4×10^6 293T cells in a T-75 flask 24 hours before transfection. It is important that cells be well maintained and of relatively low passage number.
2. Transfect cells with a total of 8 µg DNA

In a 15 ml polystyrene culture tube, mix the following

- 500 µl optimum medium
- 3 µl/µg DNA Fugene 6 reagent

In another 15 ml polystyrene culture tube prepare the DNA mix

- 100 µl optimum
- 3 µg of pLVX-puro vector
- 1.5 µg VSVG
- 1.2 µg RSV-REV
- 2.3 µg pMDL g/p RRE

3. Incubate the two tubes 5 min separately.
4. Add the content of tube number 2 to tube number 1 and incubate the mixture for additional 25 min. (See Note 4)
5. By the end of the above incubation time, prepare in 50 ml falcon tube 5.6 ml of DMEM and add the Fugene DNA mix directly to this medium and mix well. Take 293T cells out of the incubator and remove the medium from the cells and wash the

²The purity of the DNA for transfection is very critical as is the establishment of the optimal ratio of packaging, envelope and vector plasmids and the amount of Fugene 6 transfection reagent.

³Tryple™ Express is a special formulation of Trypsin/EDTA optimized for the culture of neurons.

⁴Prolonged incubation of DNA and Fugene 6 mix decreases the transfection efficiency.

cells once with PBS and add transfection mixture to the cells distribute evenly by swirling the flask. Return the plates immediately to incubator.

6. After 4 hours, add an additional 4 ml fresh medium to the flask and incubate overnight.
7. The next day remove the medium and wash the cells 2X with PBS, and add 10 ml of fresh medium containing 6 µg/ml Polybrene.
8. After 48 hours from the first transfection, harvest the lentivirus containing supernatants and refeed the cells with new fresh medium.
9. Centrifuge lentivirus containing supernatants 10 min at 787 g at 4°C in 50 ml tube to remove debris and filter viral supernatant through 0.45 µm filter and keep it at 4°C for one day. (See Note 5).
10. On the next day, collect the second round of the supernatant from the cells and process it in the same way as the first supernatant.
11. Combine the two supernatant together and concentrate by ultracentrifugation using SW-28 rotor at 140,000 g for 90 min at 4°C.
12. Aspirate the supernatant carefully and resuspend pellet in DMEM and leave tube at 4°C overnight with gently shaking.
13. Centrifuge the viral suspension for 5 min at 7580 g, 4°C and carefully remove the supernatant that contain the concentrated viral particles.
14. Virus particles should be aliquoted, flash frozen in liquid nitrogen and stored at -80°C. Avoid multiple freezing and thawing which leads to reduction on vector titers. (See Note 6)

3.2 Titration of lentivirus

We determine viral titer by infecting primary astrocytes with serial dilution of concentrated virus. One day after transduction, the cells were harvested and luciferase activity of measured as indication of the efficiency of infection.

Transduction of primary fetal astrocytes

1. One day before transduction, plate primary astrocytes (in duplicate) in 6 well plate at cell density of 6×10^5 cell/ well
2. On the next day, aspirate the medium and wash the cell once with PBS and incubate the cell with 2 ml of viral particles containing DMEM in the presence of 6 µg/ml polybrene.
3. Incubate the cells overnight.

⁵To filter the virus supernatant use filter made of cellulose acetate or low protein binding filters. It has been reported that filters with Nitrocellulose inactivate or destroy lentivirus particles by binding to the envelope.

⁶Aliquot and store your virus stock in such way that multiple freezing-thaw step will be avoided. Frequent freezing and thaw step lead to significant loss of the titer the stock.

4. Next day remove the medium and replace with fresh medium and incubate the cells for 24 h before measuring promoter activity.

CONCLUSION

In this protocol, we described a simple and reproducible method of lentiviral vector production in 293 cells using the four plasmids system based on the Fugene 6 transfection method. Using this method of transfection significantly reduced the amount of DNA needed for transfection compared to the traditional method of calcium phosphate precipitation. The Fugene 6 transfection reagent used in the protocol can also be left on the cells for 24 hours or longer without having a toxic effect. Finally, we recommend the use of a transfer plasmid with a reporter gene like GFP that significantly facilitates the determination of the transduction efficiency and viral titer.

References

1. Pfeifer A, Verma IM. Gene Therapy: Promises and problems. *Annual Review of Genomics and Human Genetics*. Annu Rev Genomics Hum Genet. 2001; 2:177–211. [PubMed: 11701648]
2. Denning W, Das S, Guo S, et al. Optimization of the Transductional Efficiency of Lentiviral Vectors: Effect of Sera and Polycations. *Mol Biotechnol*. 2013 In Press.
3. Mátrai J, Chuah MK, VandenDriessche T. Recent Advances in Lentiviral vector Development and Application. *Mol Ther*. 2010; 18:477–490. [PubMed: 20087315]
4. Sakuma T, Barry MA, Ikeda Y. Lentiviral vectors: basic to translation. *Biochem J*. 2012; 443:603–618. [PubMed: 22507128]
5. van Hooijdonk LW, Ichwan M, Dijkmans TF, et al. Lentivirus-mediated transgene delivery to the hippocampus reveals sub-field specific differences in expression. *BMC Neuroscience*. 2009; 10:2. [PubMed: 19144149]
6. Naldini L, Blomer U, Gage FH, et al. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brain injected with a lentiviral vector. *Proc Natl Acad Sci USA*. 1996; 93:11382–11388. [PubMed: 8876144]
7. Naldini L, Blomer U, Gallay P, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*. 1996; 272:263–267. [PubMed: 8602510]
8. Logan AC, Lutzko C, Kohn B. Advances in lentiviral vector design for gene-modification of hematopoietic stem cells. *Curr Opin Biotech*. 2002; 13:429–436. [PubMed: 12459333]
9. Varma NR, Janic B, Ali MM, et al. Lentiviral Based Gene transduction and promoter studies in Human Hematopoietic Stem Cells (hHSCs). *J Stem Cells Regen Med*. 2011; 7:41–53. [PubMed: 21743782]
10. Radhakrishnan S, Otte J, Enam S, et al. JC Virus-induced changes in cellular gene expression in primary human astrocytes. *J Virol*. 2003; 77:10637–10644.
11. Cole, R.; de Vellis, J. Astrocyte and oligodendrocyte culture. In: Fedoroff, S.; Richardson, A., editors. *Protocols for Neural Cell culture*. Humana press; Totowa, NJ: 1997.
12. Yong, VW.; Antel, JP. Culture of glial cells from human brain biopsies. In: Fedoroff, S.; Richardson, A., editors. *Protocol for Neural Cell culture*. Humana press; Totowa, NJ: 1997.

Figure 1. Production of transducing lentivirus

Diagram illustrating the transduction of the target cells using the packaging plasmids and transfer plasmid. The transfer plasmid contains the gene of interest (in this case luciferase driven by promoter A or B), the psi packaging element (ψ), a polypurine tract DNA sequence (PPT) to increase the transduction efficiency and cis-acting transcriptional regulatory elements, rev response element (RRE), the woodchuck hepatitis post-transcriptional regulatory element (WPRE) to improve the transgene expression and the Puromycin resistance gene (Pur) driven by the phosphoglycerate kinase promoter (PGKP). The REV protein, which is important for the expression of gag and pol, is provided in trans with a separate plasmid. The viral stocks produced by the transfected cells can be used to transduce parallel cultures of target cells and compare the strength and regulation of promoters A and B.