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Lentiviral Gene Transduction of Mouse and Human Stem Cells

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Summary

This chapter describes the methods we use to transduce mouse and human hematopoietic stem cells (HSCs) and human embryonic stem cells (hESCs). We provide detailed protocols for producing high-titer lentiviral supernatants by transient transfection and for measuring viral titers. Methods to concentrate viral supernatants to achieve a higher titer are also described. The protocols given here have been used successfully to transduce engrafting mouse and human HSCs as well as progenitor cells. These cells maintained stable transgene expression after engraftment in mice and in vivo differentiation. Human ESCs can also be transduced with a high efficiency, and transgene is expressed stably after hematopoietic differentiation.

Key Words: Lentivirus; hematopoietic stem cells; hematopoietic progenitor cells; embryonic stem cells.

1. Introduction

Hematopoietic stem cells have the unique capability of repopulating the entire hematopoietic system because of their self-renewal and pluripotent differentiation potentials, thus represent an important target for treatment of various blood and immune disorders. Stable gene transfer to these stem cells therefore has great potential to achieve both long-term and short-term therapeutic effects for the treatment of these diseases. Oncoretroviral (also called as gamma-retroviral) and lentiviral vectors represent two major choices for efficient transduction and stable integration of transgenes into hematopoietic stem and progenitor cells ***(1)***. Lentiviral vectors (lentivectors or LVs) offer

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several advantages over traditional gamma-retroviral vectors. LVs efficiently transduce slowly dividing cells, including HSCs, resulting in stable gene transfer and expression ***(2,3)***. Additionally, recently developed self-inactivating (SIN) LVs allow promoter-specific transgene expression ***(2,4)***. The SIN safety modification of LVs, which permanently disables the viral promoter within the viral long-terminal repeat (LTR) after integration, enables transgene expression in the targeted cells to be controlled solely by internal promoters ***(1–7)*** without reducing viral titers ***(2,5)***. High-titer lentivector supernatants ($\geq 10^7$ infectious units per milliliter) can be easily made by transient transfection of commonly used 293 cells or derivatives by $CaPO_4$ precipitation or liposome-mediated methods. In addition to the transducing (template) lentivector that serves as transgene template, two other (helper) plasmids required for viral assembly are used in transfection. One is to express the HIV-1 *gag/pol* gene and the second is to express the VSV-G envelope protein that is good for essentially any vertebrate cell types. The VSV-G pseudo-typed recombinant viruses are much more stable and can be concentrated easily. Upon incubation with target cell in culture or injected in vivo, VSV-G pseudo-typed recombinant viruses will fuse to cell membrane, un-load two copies of RNA templates, reversely transcribe into DNA form, and eventually integrate into cellular chromosomes favorably at a transcribed region. Then integrated transgene and its promoter will be regulated as part of cellular chromosomes.

Since the first human hESCs line was derived in 1998, it has attracted significant attention because of its ability to self-renew in culture and its potential to differentiate into all types of cells in the body, including blood lineages ***(8–10)***. It has been shown that hESCs can be transduced with lentivirus vectors at a high efficiency and that their hematopoietic progeny maintain stable transgene expression ***(11,12)***. Genetically modified human ESCs therefore offer great tools for study of molecular events in early hematopoiesis and provide potentially unlimited sources of HSCs for clinical applications.

In this chapter, we describe the procedures to produce lentiviral supernatants and to transduce both mouse and human hematopoietic stem/progenitor cells. A protocol for transducing human embryonic stem cells (hESCs) is also described.

2. Materials

1. Medium for culturing 293T cells (used to produce lentiviral vectors):
   D-MEM with high glucose (4.5 g/ml), 10% fetal bovine serum (FBS), 1× penicillin-streptomycin.
2. Medium for virus collecting in 293T cells: Essentially any culture medium. We commonly use D-MEM + 1% FBS or any serum-free medium (with insulin + transferrin supplements).

3. Medium for culturing primary human hematopoietic cells:
   QBSF-60 serum-free medium with L-glutamine (Quality Biological, Inc., Gaithersburg, MD) (*see* **Note 1**); gentamycin, 20 ng/ml (human) thrombopoietin; 100 ng/ml human SCF or KIT ligand, 50–100 ng/ml (human) FLT3 ligand (collectively called TSF).
4. Medium for culturing primary mouse hematopoietic stem/progenitor cells: QBSF-58 serum-free medium with L-glutamine (Quality Biological, Inc., Gaithersburg, MD; *see* **Note 1**), gentamycin, 100 ng/ml mouse KIT ligand as well as 20 ng/ml (human) thrombopoietin, 50–100 ng/ml (human) FLT ligand (*see* **Note 2**).
5. Medium for culturing hESCs:
   KNOCKOUT™ D-MEM or DMEM/F12, 20% knockout serum replacement, 2 mM L-glutamine, 0.1 mM non-essential amino acids (all from Invitrogen, Carlsbad, CA), 0.1 mM of β-mercaptoethanol, and 4 ng/ml of bFGF (Pepro Tech, Rocky Hill, NJ or other sources).
6. 1x phosphate-buffered saline (PBS) Ca^{+2} and Mg^{+2} free.
7. 0.05% Trypsin–ethylenediaminetetraacetic acid (EDTA).
8. Red blood cell (RBC) lysis buffer: 8.3 g NH_4Cl, 1.0 g $KHCO_3$, 1 mM EDTA in 1 l solution.
9. Lineage depletion kit for mouse cells (StemCell Technologies, Vancouver, Canada).
10. CD34-positive selection kit (Miltenyi Biotec, Bergisch Gladbach, Germany)
11. Matrigel™ matrix (Becton Dickinson, Bedford, MA): Matrix for human ESC culture.
12. Poly-D-lysine (500–550 KDa polymers, BD 35-40210): to make 293T cells more adherent.
13. Polybrene (as an attachment factor to enhance virus-cell fusion): dissolve hexadimethrine bromide (Sigma, St. Louis, MO, Cat. No. H9268) in water to make final concentration to 8 mg/ml. Sterilize solution by 0.22-μm filter.
14. Sterile polystyrene 5-ml tube (Falcon 2054).
15. Stericup™ (Millipore, Billerica, MA) or other sterilization filtration units, pore size 0.22 and 0.45 μm.
16. Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA). The unit with a filter of 100,000 MWCO is an easy way to concentrate retro- and lentivectors by up to 100- to 200-fold.
17. Regents for 293T transfection: basically you may use any type of liposomes you might have tried before. We commonly use lipofectamine 2000 (Invitrogen), which is insensitive to serum in media. However, the classic $CaPO_4$ method is also fine, albeit validation of the solution and practice is often required to ensure high and consistent efficiency. The following is a recipe for two solutions required for the classic $CaPO_4$ method.
18. 2 M $CaCl_2$ solution: dissolve 22.2 g of $CaCl_2$ in water to final volume of 100 ml. Sterilize solution by 0.22-μm filter. Store at 4ºC.

19. Hank's balanced salt solution (2×): dissolve 16.4 g of NaCl, 11.9 g of HEPES acid, and 0.21 g of Na_2HPO_4 in 800 ml of water, adjust pH value to 7.05 with 5N NaOH solution, add water to a final volume of 1 l. Sterilize solution by 0.22-μm filter. Store at 4ºC.

3. Methods

3.1. Production of Lentiviral Supernatant

The protocol described here utilizes co-transfection of 293T cells with three plasmids: the lentiviral vector coding for the viral genome that contains the transgene, CMVΔR8.91 expressing the required three lentiviral (HIV-1) proteins ***(5)***, and MD.G expressing the VSV-G envelope proteins ***(6)***.

3.1.1. Transfection of 293T Cells Using Lipofectamine 2000

1. Coat tissue culture plates or dishes with poly-D-lysine (50–100 μg/ml) for 60 min at room temperature, then wash twice with PBS (*see* **Note 3**).
2. (Day 0) Plate 293T cells using 293T medium, see **Table 1** for the cell number needed (*see* **Note 4**).
3. (Day 1) Change 293T medium to virus collecting medium (*see* **Note 5**). Transfect 293T cells with the three plasmids (*see* **Table 2**) using lipofectamine 2000 following manufacturer's instruction (*see* **Note 6**): (for a 6-well plate or for a 150-mm dish, underlined)
    a) 4 or 24 μg DNA + 0.15 or 1.2 ml of OPTI-MEM (Falcon 2054 polystyrene 5-ml tube).
    b) 6 or 36 μl of lipofectamine 2000 + 0.15 or 1.2 ml of OPTI-MEM, incubate for 5 min at RT (in polystyrene 5-ml tube, Falcon 2054).
    c) Mix the diluted DNA with diluted lipofectamine 2000; incubate for 20 min at room temperature.
    d) Add the DNA–lipid complexes dropwise into the media.
    e) After 6 h of incubation, change the medium to fresh virus collecting medium (*see* **Notes 7** and **8**).

Table 1
Cell Numbers Needed for Plating 293T Cells in Virus Production

Plate	Cell number per well, × 10^6	Media volume (ml)
35 mm = one well in 6-well plate	0.5	2
60 mm	1	4
100 mm	4	8
150 mm	≥6	≥12

Table 2
Amount of Plasmids DNA Needed for Transfection

Plates	35 mm	150 mm
Total plasmid DNA:	≤4 μg	24 μg
1. LV-transducing vector	1	9
2. CMVΔR8.91 (expressing three required HIV proteins)	2	12
3. MD.G (expressing the VSV-G envelope proteins)	0.5	3

4. (Day 2–4) Harvest viral supernatant (*see* **Note 9**). Centrifuge the collected supernatant at 2500 x *g* for 10 min to get rid of cells/debris. If it is critical to eliminate cell contamination, pass the supernatant through 0.45 um filter units. The virus can be used immediately or stored for later use (*see* **Note 10**).

3.1.2. Transfection by $CaPO_4$ Method

1. Plate 293T cells as described in the lipofectamine 2000 method (*see* **Subheading 3.1.1.**).
2. When cell density reaches approximately 70–80%, make transfection cocktails according to **Table 2** (DNA amounts) and **Table 3** in 5- or 15-ml conical tube. Add water, $CaCl_2$, and DNA in the tube first. The quantity of water is based on the total volume of other ingredients.
3. When adding the Hank's balanced salt solution (HBSS), bubble air through the cocktail with pipette for about 10–20 times until the solution becomes slightly cloudy.
4. Let the mixed cocktail stand in room temperature for 10–20 min.
5. Drop cocktail directly onto media currently on the cells. Swirl media on plates once.
6. After 6 h of incubation, change the medium to fresh virus-collecting medium (*see* **Note 8**).
7. Collect viral supernatant the same way as described in **Subheading 3.1.1**.

Table 3
Transfection Cocktails for $CaPO_4$ Method

Plate size (mm)	$CaCl_2$ (μl)	Total DNA (μg)	2× HBS	Total final volume
35	31	4–5	250 μl	500 μl
60	62	8–10	500 μl	1 ml
100	124	15–20	1 ml	2 ml
150	372	45–60	2.5 ml	5.0 ml

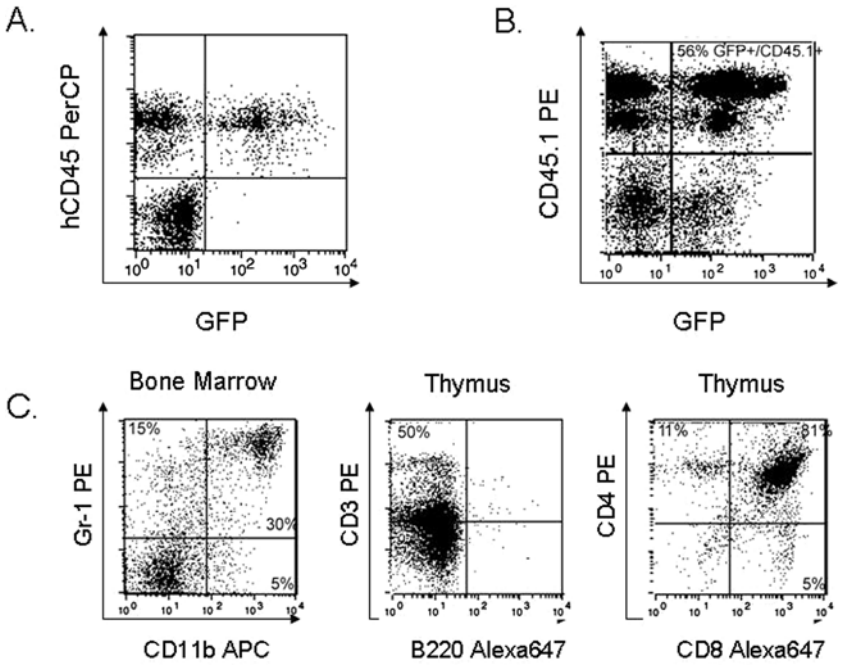

Fig. 1. (A) Human cord blood CD34+ cells were transplanted into sub-lethally irradiated NOD/SCID mice 48 h after transduction with lentivirus expressing GFP. After 8 weeks, bone marrow cells from the transplanted NOD/SCID were analyzed by using FACS for GFP transgene expression in human hematopoietic cells expressing the human (h) CD45 marker. (B) Mouse bone marrow lineage-depleted cells from CD45.1 background mice were transduced with GFP-expressing lentivirus and transplanted into lethally irradiated CD45.2 mice. Analysis of recipients' bone marrow 8 weeks after transplant showed 56% of GFP+ donor (CD45.1+) cells. (C) Further analysis of transduced donor cells (GFP+CD45.1+) cells in the bone marrow and thymus with markers of various lineages.

3.2. Concentrating VSV-G Pseudo-Typed Lentiviral Supernatants (Optional) (see Note 11)

1. Add 15 ml of viral supernatant to the top portion of Amicon filter and centrifuge at 2,500 x *g* for 15 min at 4ºC (*see* **Note 12**).
2. Aspirate the flow-through from the tube and add more supernatant, centrifuge at the same condition.
3. Repeat the process until the desired fold of concentration is reached. Use pipettes or pipette tips to take out the supernatant from the upper part of the centrifugal device. Use the virus immediately or store it (*see* **Note 10**).

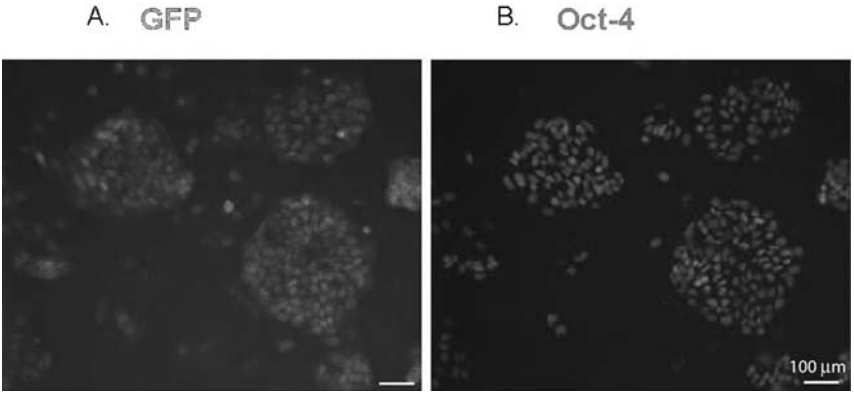

Fig. 2. Human ES cell line H1 transduced with GFP-expressing lentivirus maintained at the undifferentiated state. After selection, the transduced H1 human ES cells (G-GFP) for expanded for years in culture. To illustrate the undifferentiated state, cultured G-GFP human embryonic stem (ES) cells as undifferentiated colonies were fixed and permeabilized before staining for Oct-4 by a specific monoclonal antibody. The fluorescence of GFP signal (A) and Oct-4 staining after a secondary reagent (red, in B) were recorded from the same field.

4. Alternatively, the viral supernatant can be concentrated by ultracentrifugation 20,000 × *g* at 4°C for 90 min. The viral pellet can be re-suspended in PBS at 4°C overnight.

3.3. Measuring Viral Titers Based on Transgene Expression

1. (Day 0) Plate 1×10^5 of 293T cells per well in 6-well plate.
2. (Day 1) Replace the culture medium with serial diluted (1:10, 1:100, 1:1000, dilute with culture medium) viral supernatants in each well with 2 ml of medium. Add polybrene to final concentration of 8 µg/ml.
3. (Day 3 or later) Analyze the cells by using FACS based on the cell-surface marker expression or fluorescent protein (e.g., GFP). Otherwise, you have to monitor the integrated vector at DNA level, because measuring the titer by measuring RNA levels in viral supernatants or transduced cells is problematic.
4. Calculating infectious or transduction units (TU): If 10% of cells are GFP positive with 0.02 ml (1/100) of original viral supernatants, the titer is: 10% × cell number at Day 1($\sim 2 \times 10^5$)/0.02 ml = 1×10^6 TU/ml (*see* **Note 13**).

3.4. Transduction of Hematopoietic Stem Cells

The protocols described here have been successfully transduced mouse bone marrow lineage-depleted cells, or human CD34+ cells from bone marrow,

G-CSF-mobilized peripheral blood or cord blood. The method for transducing mouse and human hematopoietic stem/progenitor cells are the same except that the different culture media (*see* **Subheading 2**) were used. Two alternative methods are described below.

3.4.1. A Simple and Quick Method

1. Count cell numbers and prepare cell cultures lasting for 48 h.
2. Plate the freshly isolated or thawed cells in 12-well plates with the appropriate QBSF medium with growth factors TSF. Cells were plated at a density of 1–2 million per well with 1–2 ml of medium. Add concentrated (>100-fold) lentivirus ($\geq 10^8$ TU/ml) and co-culture the cells with virus in the presence of 8 μg/ml polybrene.
3. After 48 h of co-culture, the cells can be harvested, washed, and used for further functional analysis. The essence of this method is to transduce HSCs quickly and preserve the engraftment activities.

3.4.2. An Enhancement (Spin-Inoculation) Method

1. One day before transduction, isolate cells or thaw the cryopreserved human CD34+ cells and culture them (10^6 cells/ml) overnight in QBSF media supplemented with appropriate growth factors (*see* **Subheading 2.4**).
2. The next day, harvest the cells and re-suspend them in a sterile 5-ml tube (Falcon 2054) with lentivirus-containing media; add polybrene to final concentration of 8 μg/ml. Incubate for 15–20 min. Tighten up the cap and centrifuge at 2,000 × *g* for 3 h using a bench-top centrifuge at room temperature.
3. After centrifugation, add an equal volume of growth factor-containing media to the tubes to dilute the viruses and polybrene. Re-suspend the cells in a pellet, loosen the cap of the tube, and culture overnight in a tissue culture incubator. If necessary, repeat transduction again.
4. Spin down the cells and transfer the cells with media-containing growth factors to tissue culture plates. Culture the cells for 1–2 more days.
5. The cells are now ready for either FACS analysis or subsequent in vitro and in vivo studies (**Fig. 1**). The essence of this method is to increase transduction efficiency (even when the viral titer is relatively low or un-concentrated) and to monitor transgene expression, such as GFP, at the end of transduction (3–4 days after the start of transduction).

3.5. Transduction of hESCs

3.5.1. Making Matrigel Plates for hESC Transduction

1. Thaw frozen Matrigel vial (10 ml) in 4°C overnight (*see* **Note 14**). Injecting 10-ml medium by a sterile syringe will reduce the viscosity significantly. Aliquot extra into chilled tubes and for long-term storage at –80°C.

2. Dilute Matrigel further (1:15) with cold medium and plate it into 6-well plate with 1 ml per well. The final dilution is 1:30.
3. Leave the plate in room temperature for 1 h. The plates are now ready to use or can be stored at 4°C for 1 week. Aspirate excess Matrigel and wash once before use.

3.5.2. Culturing hESCs on Matrigel Plates

1. Split the hESCs from feeder plates using 0.05% Trypsin/EDTA or 1 mg/ml Collagenase IV solution by following standard protocols.
2. Aspirate the medium from the Matrigel plate and wash the wells once with PBS.
3. Plate the cells onto Matrigel plates with 2.5 ml of the conditioned medium (CM) with growth factors. Change CM everyday.

3.5.3. Transduction of hESCs

1. When hESCs reach 60–80% confluence, add concentrated lentiviral supernatant ($\sim 10^8$ TU) into 2 ml of CM in the presence of 4–8 μg/ml of polybrene.
2. Incubate the hESCs with viral supernatant for 4–6 h in tissue culture incubator.
3. Aspirate the virus-containing medium and add 2.5 ml of fresh CM.
4. When cells reach confluence on Matrigel plates, transfer them to normal feeder plates for expansion.
5. Cells can be analyzed 3 days after transduction for transgene expression (**Fig. 2**).

4. Notes

1. We have good experience with the media from Quality Biological, Inc.; however, you can also use other serum-free culture media you prefer.
2. Human FLT ligand and thrombopoietin are active for both human and mouse cells, and a higher concentration (100 and 20 ng/ml, respectively) is preferred. Human and mouse SCF (or Kit ligand) do not interact cross the species well (10- to 1,000-fold less efficient); thus, species-specific Kit ligand is important. Although lentivectors can transduce quiescent cells, adding growth factors during the transduction (and in prior culture before the transduction step) will significantly activate cells and enhance transduction efficiency.
3. 293T cells are loosely adherent cells. They will easily detach from the bottom of plates when confluent. Coating the plates with poly-D-lysine is recommended. When coating the plates, add enough solution to cover the bottom of the plates. If not used immediately, coated plates can be stored in cold room for weeks. Poly-D-lysine solution can be re-used up to three times.
4. The numbers given in **Table** 2 are for your reference. You can titrate cell numbers to obtain the ideal density for optimal efficiency. We found that 70% confluence is ideal start point for transfection.
5. Depending on the cell type that is going to be transduced with, different media specific for culturing that cell type can be used to collect viruses (if no virus

concentration is planed). In our experience, the viral supernatant collected in the medium described here is easy to be concentrated following the protocol provided in this chapter; the concentrated virus (>100-fold) can then be diluted in any preferred medium for transduction, which often gives better results.

6. The protocol described here is modified from lipofectamine's protocol. We found that lipofectamine 2000 (serum-insensitive) gives consistent transfection efficiency. You can, however, use your favorite transfection methods for 293T cells.
7. Serum-free medium with insulin-transferrin is fine, too. Collection media containing a higher percentage of serum (>5%) are hard to be concentrated efficiently.
8. If you start the transfection late in the day, you can skip the first medium change. Collect the supernatant the next day.
9. Collect the supernatant at day 4 only if the cells are still healthy and attached to the plate. The titer may be significantly lower.
10. If the viral supernatant is to be used within 3–5 days, it can be stored at 4ºC. If longer term storage is desired, the supernatant should be aliquoted and stored at –80ºC. Avoid repeated freeze and thaw process, which will significantly reduce the titer.
11. For most viral constructs, the titer of the collected supernatant before concentration is around 10^6–10^7 TU/ml, which is adequate for many experiments. If a significant higher titer is required or the titer of a specific construct is consistently low, then concentrate viral supernatants.
12. Depending on the concentration of the virus as well as the composition of the collecting medium, the centrifugation time may vary from 10 to 20 min; Start with 10 min to see how much more you should centrifuge.
13. This calculation is based on the assumption that the 293T cells would double the cell number overnight after initial plating. Alternatively, an extra well of cells can be plated on day 0, and the cells can be harvested and counted at the time of adding virus to get more accurate number.
14. It is important to keep the Matrigel solutions cold throughout the whole process until it is plated on the plate; higher temperature will solidify the gel prematurely and reduce the quality of the plates.

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