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Gene Transfer into Pluripotent Stem Cells via Lentiviral Transduction

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Abstract

Recombinant lentiviral vectors are powerful tools to stably manipulate human pluripotent stem cells. They can be used to deliver ectopic genes, shRNAs, miRNAs, or any possible genetic DNA sequence into diving and nondividing cells. Here we describe a general protocol for the production of self-inactivating lentiviral vector particles and their purification to high titers by either ultracentrifugation or ultrafiltration. Next we provide a basic procedure to transduce human pluripotent stem cells and propagate clonal cell lines.

Keywords: Primed pluripotent stem cells, Human pluripotent stem cells, Lentivirus production, Transduction, Selection of cell clones

1 Introduction

Over the past decade, recombinant lentiviral vectors have been established as powerful tools for transgene delivery in the research fields of neuroscience, hematology, developmental biology, stem cell biology, and gene therapy (1). Lentiviruses pseudotyped with the protein of vesicular stomatitis virus VSV-G are perhaps the most versatile retrovirus since they are able to stably transduce almost any mammalian cell type, including dividing and nondividing cells, stem cells, and primary cell cultures (2). In stem cell research lentiviral vector systems are valuable tools to manipulate differentiation processes by transgene expression, to identify a favored phenotype differentiated cell populations by the use of specific promoter/reporter gene constructs (linage tracing) (3, 4) or to localize transplanted differentiated stem cells in a graft after transplantation in vivo (5).

The lentiviral vector system described herein is based on HIV-1. For the production of vector stocks, it is mandatory to avoid the emergence of replication-competent recombinants. Therefore several modifications in the wild-type HIV-1 genome were introduced into the vector system. For biosafety reasons six (*env*, *vif*, *vpr*, *vpu*, *nef*, and *tat*) of the nine HIV-1 genes were deleted from the vector system without impairing its gene-transfer ability. In the third

generation of lentiviral vector systems, attuned towards clinical applications, only *gag*, *pol*, and *rev* genes are still present, using a chimeric 5′ LTR (long terminal repeat) to enable transcription in the absence of Tat (6). In the so-called self-inactivating (SIN) vector design the U3 of 3′ LTR, which is essential for the replication of a wild-type retrovirus, is additionally deleted; thus SIN vectors cannot reconstitute their promoter and are safer than their counterparts with full-length LTRs (7, 8). On the other hand additional viral elements like the Woodchuck Hepatitis virus posttranscriptional regulatory element (WPRE) and central polypurine tract (cPPT) have been added to improve the transcriptional efficiency, which is especially important for the transduction of embryonic stem cells. The WPRE increases the overall levels of transcripts both in producer and target cells, hence increasing titers and transgene expression (9). The cPPT sequence facilitates nuclear translocation of the pre-integration complex and enhances transduction efficiency (10).

Although the latest versions of lentiviral vector system comprise four plasmids and represent the system of choice for clinical gene therapy approaches for most research applications it is adequate and more efficient to use a three-plasmid vector system with an all-purpose packaging plasmid, such as the psPAX2.

For transgene expression the choice of the promoter, which initiates the transcription, is of particular importance since it is known that widely used viral promoters, like the cytomegalovirus (CMV) promoter, can be efficiently silenced in embryonic stem cells (11). Therefore tissue-specific promoters or tetracycline-inducible elements for regulated gene expression are in most cases the promoter of choice (12, 13).

2 Materials

2.1 Cell Culture

2.1.1 Cultivation of HEK-293T Cells

1. 293T human embryonic kidney cell line (*see* **Note 1**).
2. Dulbecco's modified Eagle medium with 4.5 g/l glucose.
3. Glutamine.
4. Pyruvate.
5. Penicillin/streptomycin.
6. Fetal bovine calf serum (FBS).
7. PBS w/o Ca^{2+}/Mg^{2+} (PBS).
8. 0.25 % Trypsin/EDTA in PBS.
9. Cell culture-grade plastic plates, dishes, or flasks.

2.1.2 Feeder-Free Cultivation of Human Pluripotent Stem Cells

1. Matrigel hESC-qualified matrix (*see* **Note 2**) (Corning, Amsterdam, The Netherlands, cat # 354277).

2. mTeSR™1 culture medium (*see* **Note 3**) (STEMCELL Technologies, Vancouver, Canada, cat # 05850).
3. Knockout DMEM/F-12 (Life Technologies, Darmstadt, Germany).
4. Penicillin/streptomycin.
5. Dispase (1 U/ml) (STEMCELL Technologies, Vancouver, Canada, cat # 07923).
6. Cell culture-grade plastic plates, dishes, or flasks (e.g., 6-well plates, Corning/Falcon, cat # 353046).
7. Parafilm M.
8. PBS w/o Ca^{2+}/Mg^{2+} (PBS).
9. ROCK inhibitor, Y-27632 (Selleck Chemicals, Munich, Germany, cat # S1049).

2.2 Production, Purification, and Titer Determination of Lentiviral Vector Particles

2.2.1 Production of Lentiviral Vector Particles

1. Plasmids (available from www.Addgene.org) (*see* **Note 4**): Packaging plasmids: e.g., psPAX2 (encoding Gag, Pol, Tat, and Rev proteins) or pMDLgag/polRRE (encoding the HIV-1 Gag and Pol proteins) and pRSVrev (encoding the HIV-1 Rev protein). Envelope plasmid: pMD2G (encoding the VSV G envelope protein). Transfer plasmids: e.g., pRRLSIN.cPPT.PGK-GFP. WPRE containing your GOI (gene of interest), pLenti X1 Puro DEST, or pLenti PGK Hygro DEST (third-generation transfer vectors) (Fig. 1).
2. 293T human embryonic kidney cell line (*see* **Note 1**).
3. Routine 293 cell growth medium consisting of Dulbecco's modified Eagle medium (4.5 g/l glucose), glutamine, pyruvate, and penicillin/streptomycin with 10 % FBS.
4. 293 cell growth medium (Dulbecco's modified Eagle medium (4.5 g/l glucose), glutamine, pyruvate, and penicillin/streptomycin) but supplemented with 2 % FBS (*see* **Note 5**).
5. 2 M $CaCl_2$ solution (*see* **Note 6**).
6. 2× HEPES-buffered saline (HeBS), pH 7.00 (*see* **Note 7**).
7. 0.22 μm nitrocellulose filter.
8. PBS, pH 7.4.
9. Trypsin/EDTA, same as above.
10. 10 cm tissue culture dishes.
11. Vortex.

2.2.2 Purification of Lentiviral Vector Particles by Ultracentrifugation

1. 15 and 50 ml conical centrifuge tubes, sterile.
2. Centrifuge with swinging bucket rotor for 50 ml tubes.
3. 50 ml syringes and 0.45 μm PVDF filters.
4. 20 % sucrose solution.

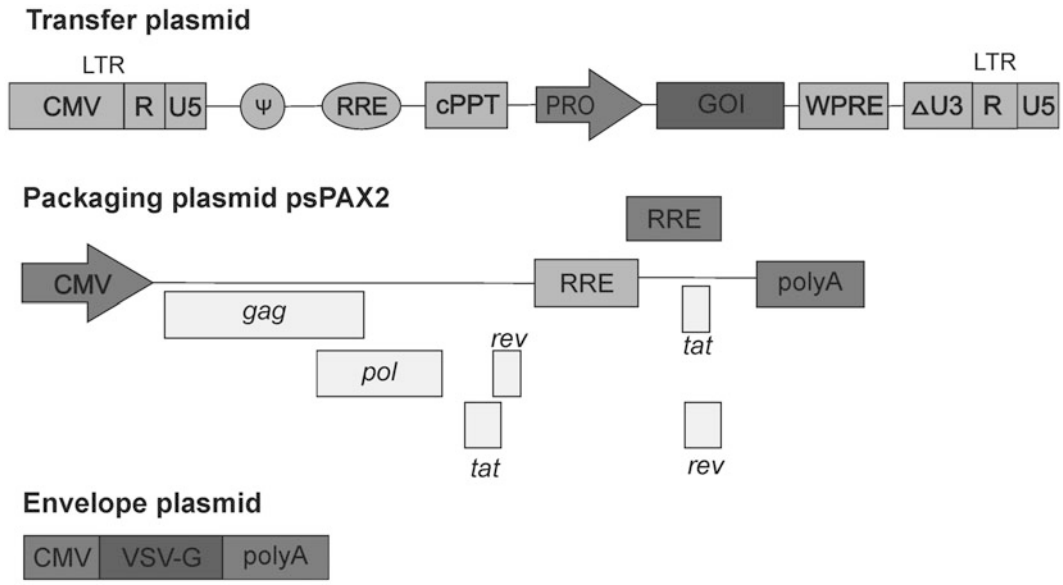

Fig. 1 Scheme of a second-generation HIV-1 vector system. The packaging plasmid psPAX expresses only the structural and regulatory HIV-1 proteins. All accessory lentiviral genes are removed. The transfer plasmid contains a self-inactivating (SIN) deletion in the U3 sequence of the 3′LTR. The Tat-dependent element in the 5′LTR is exchanged with the CMV promoter. The envelope plasmid encodes for the vesicular stomatitis virus glycoprotein, which allows the generation of pseudotyped lentivirus with broad tropism. *Psi* HIV-1 packaging signal, *RRE* rev-responsive element, *cPPT* central polypurine tract, *WPRE* woodchuck hepatitis virus posttranscriptional regulatory element, *LTR* long terminal repeat, *DU3* self-inactivating deletion of the U3 part, *Prom* promoter, *GOI* gene of interest, *Poly A* polyadenylation signal

5. 30 ml ultracentrifugation tubes (e.g., Beckman-Coulter cat # 358126).
6. Ultracentrifuge with swinging bucket rotor (50,000 × *g*, e.g., Beckman-Coulter SW28 rotor).
7. Sterile 1.5 ml microcentrifuge tubes.

2.2.3 Purification of Lentiviral Vector Particles by Ultrafiltration (Alternative Protocol)

1. 15 and 50 ml conical centrifuge tubes, sterile.
2. Centrifuge with swinging bucket rotor for 50 ml tubes.
3. 50 ml syringes and 0.45 μm PVDF filters.
4. Amicon Ultra ultrafiltration cartridges (Merck-Millipore, cat # UFC910096).
5. Sterile 1.5 ml microcentrifuge tubes.

2.2.4 Virus Titration by qPCR

1. Target cell line: e.g., HT-1080 cells (www.attc.org, cat # CCL-121) or human pluripotent stem cells.
2. DMEM medium, same as above.
3. Trypsin/EDTA, same as above.

4. 6-well tissue culture plates.
5. PBS, same as above.
6. Real-time PCR machine (Applied Biosystems, ABI ViiA7 PCR System, or equivalent).
7. Genomic DNA extraction kit (Macherey-Nagel, NucleoSpin Tissue, Düren, Germany).
8. 2× Reaction buffer (ThermoScientific, ABsolute qPCR Low ROX Mix, cat # AB-1319/A).
9. 96-well optical reaction plate (Applied Biosystems, cat # 4306737).
10. Optical caps (Applied Biosystems, cat # N801-0935).
11. Probe and primers for quantification of a common HIV-1-based lentiviral vector DNA sequence (primer: FPLV2, RPLV2; probe: LV2 (FAM/TAMRA labeled)).
    Probe and primer for quantification of the human actin beta genomic sequence (primer fw hActb, primer rv hActb, probe hActb (Yakima Yellow labeled)) (*see* **Note 8**).

2.3 Lentiviral Transduction of Human Pluripotent Stem Cells

1. Matrigel hESC-qualified matrix (*see* **Note 2**).
2. Purified lentiviral particles (*see* Sections 2.2 and 3.2).
3. mTeSR™1 culture medium (*see* **Note 3**).
4. Knockout DMEM/F-12.
5. Cell culture-grade plastic plates, dishes, or flasks.
6. Polybrene (stock solution 10 mg/ml in water).
7. ROCK inhibitor, Y-27632.
8. Antibiotics.
9. Dispase, trypsin/EDTA (same as above), or a nonenzymatic passaging solution.

3 Methods

3.1 Cell Cultivation

3.1.1 Cultivation of HEK-293T Cells

The cultivation of the human embryonic kidney cell line HEK-293T (*see* **Note 1**) is performed following standard cell culture procedures. In the following the cultivation is only briefly described.

1. The culture should never overgrow.
2. Remove the old medium from a confluent culture and wash twice with PBS to remove dead cells and cell debris.
3. Add trypsin/EDTA (TE) to the culture and incubate the cells for 3–5 min in the incubator (37 °C, 5 % CO_2).

4. To stop the trypsin reaction, add routine 293 cultivation medium (twice the volume of TE) to the well (Dulbecco's modified Eagle medium (4.5 g/l glucose), glutamine, pyruvate, and penicillin/streptomycin with 10 % FBS).
5. Transfer the cells into a conical tube and count the cells.
6. Seed 5×10^5 cells per 75 cm^2 flask and cultivate them until they reach ideal confluency for passaging. Normally, these cultures will be ready for passaging after 6–7 days. Perform a medium change at day 3 and 5 to ensure good growth conditions.

3.1.2 Feeder-Free Cultivation of Human Pluripotent Stem Cells

The coating of the cell culture plastic with Matrigel is performed according to the supplier's instructions. The following points briefly describe the coating procedure:

Preparation of Matrigel-Coated 6-Well Plates

1. To coat the cell culture plastic thaw one aliquot of the hESC-qualified Matrigel (*see* **Note 2**) slowly on ice (~1–2 h), and dilute it in a conical 50 ml tube that contains the required volume of cold knockout DMEM/F-12. Ideally, keep the conical tube during whole preparation time on ice.
2. Add the required volume of diluted Matrigel (from the conical tube) to the cell culture plastic and ensure that the whole surface is covered with a liquid film. The prepared plates should be sealed with parafilm to avoid evaporation and can be stored for up to 7 days at 4 °C in the fridge (*see* **Note 10**).
3. To ensure polymerization of the Matrigel, cell culture plates must be kept for 30–60 min at room temperature (*see* **Note 11**).
4. The remaining supernatant has to be removed prior to passaging of cells. Ensure that the well is not drying out by adding a sufficient amount of the required cultivation medium to cover the whole surface of the cavity. Now the cell culture plastic can be used for cultivation of human pluripotent stem cell (PSC) either in cell clusters or single cells.

Enzymatic Passaging of Human Pluripotent Stem Cells Using Dispase

1. When the PSC colony size is appropriate for passaging (ideally 70–90 % confluency) aspirate the medium from the cavity and wash it once with knockout DMEM/F-12 to remove dead cells and cell debris (2 ml per well of a 6-well plate).
2. Add 1 ml dispase (1 U/ml) to each well of a 6-well plate and incubate the plate for 7–10 min in the incubator (37 °C, 5 % CO_2) (*see* **Note 12**).
3. Wash twice with 1.5–2 ml knockout DMEM/F-12. After removing the knockout DMEM/F-12 add 1 ml mTeSR™1 medium (*see* **Note 13**) per well.
4. Detach the colonies with a cell scraper or a 1 ml pipette tip by washing/scraping and collect the cell clusters with a wide bore cell safer tip. During this step take care not to disrupt or

dissociate the colonies into very small fragments or even single cells because it will decrease the passaging efficiency (*see* **Note 14**). Optionally, the wells can be washed with additional medium to collect the remaining cell clusters.

5. Transfer the fragments into a conical 15 ml tube by using a wide bore cell safer tip, adjust to the required amount of mTeSR™1 culture medium, and seed the fragments onto the freshly prepared Matrigel-coated 6-well plate. Routinely, the cells can be passaged every 5–7 days with a ratio of 1:3 to 1:10 depending on the respective human PSC line. To increase the cell reattachment rate you may optionally adjust the mTeSR™1 culture medium to 10 μM ROCK inhibitor (Y-27632) for the first 24 h (*see* **Note 15**).
6. Perform a daily medium change until next passaging.

3.2 Production, Purification, and Titer Determination of Lentiviral Vector Particles

3.2.1 Production of Lentiviral Vector Particles

1. Cultivate 293T cells in DMEM medium on 10 cm tissue culture dishes in a 37 °C humidified incubator with a 5 % CO_2 atmosphere.
2. The day before the transfection, seed the required number of dishes at 2.5 million cells per dish (10 cm). Incubate overnight in a 37 °C humidified incubator with a 5 % CO_2 atmosphere. On the following day, co-transfect the cells by calcium phosphate precipitation according to the following recipes (Fig. 2).
3. For one 10 cm dish mix 5 μg of envelope plasmid, 10 μg of packaging plasmid, and 12 μg of vector plasmid containing your gene of interest (GOI) and adjust to 438 μl with the water in a 15 ml tube (*see* **Note 9**) (Fig. 1).
4. Add 62 μl of 2 M $CaCl_2$.
5. Mix well and add this mix dropwise and slowly (one drop every second) to 500 μl of 2× HeBS, while vortexing with top speed.

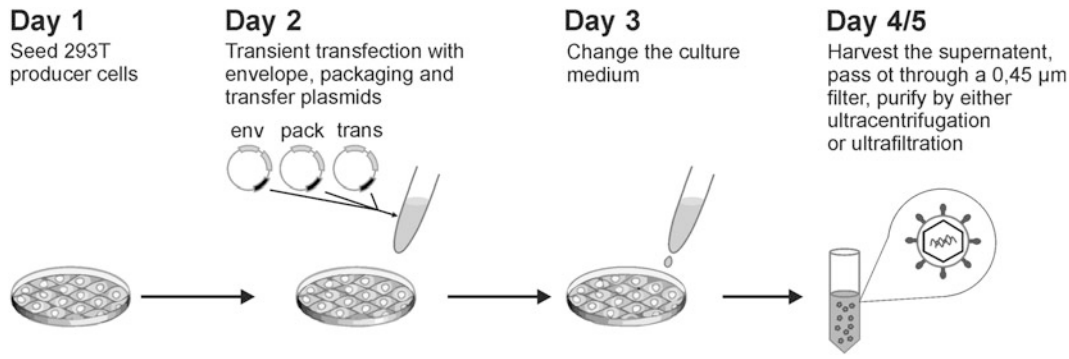

Fig. 2 Schematic presentation of the production of lentivirus vector particles. Details of the production procedure are outlined in Section 3.2

6. Let stand still for 20 min (not longer than 30 min) on the bench.
7. Just prior to transfection of 293T cells, remove the medium and gently add fresh 293 DMEM medium.
8. Add the precipitate slowly dropwise on the cell monolayer. Shake (not stir) the plate gently (*see* **Note 16**).
9. Incubate the dishes overnight in a 37 °C humidified incubator with a 5 % CO_2 atmosphere.
10. On the next day discard the medium after gentle, but firm, stirring (to eliminate as much precipitate as possible), and replace with 10 ml of fresh and pre-warmed medium supplemented with 10 % or 2 % FBS (*see* **Note 17**) (Fig. 2).
11. Discard the medium after gentle, but firm, stirring (to eliminate as much precipitate as possible) and replace with 10 ml of new medium containing 10 mM sodium butyrate.
12. On day 4, harvest the supernatant and replace with 10 ml DMEM medium supplemented with 10 % or 2 % FBS (Fig. 2).
13. Collect the supernatant in a 50 ml tube and pellet detached 293T cell at 500 × *g* for 5 min at +4 °C.
14. Transfer the supernatant to a new 50 ml tube and filter the fluid through 0.45 μm PVDF filter to remove remaining cell debris. Store the virus preparation at +4 °C (*see* **Note 18**) (Fig. 2).
15. Additional lentiviral particles can be harvested after 24 h later by repeating the procedure.
16. For determination of the biological titer seed 1×10^5 target cells on 24-well plates.

3.2.2 Lentivirus Concentration by Ultracentrifugation

1. For the concentration use 30 ml conical tubes (e.g., Beckman-Coulter cat # 358126,) with an SW 28 rotor in an ultracentrifuge. Sterilize the centrifuge tubes through incubation with 70 % ethanol for 5 min. Remove the ethanol, and place the tubes into a laminar flow hood with UV light on for 30 min for drying.
2. Fill 4 ml of 20 % sucrose on the bottom of the tube. Pour the purified virus solution from Section 3.2.1 very slowly on the surface of the sucrose cushion until the tube is completely filled. Spin at 25,000 rpm (82,700 × *g*) for 2 h at +4 °C.
3. Carefully remove the tubes from the rotor, pour off the supernatant, and leave tubes on a paper towel in an inverted position for 10 min to allow the residual liquid to drip away from the pellet.
4. Place the conical tube in a 50 ml Falcon tube and quickly add 30–100 ml of PBS on the pellet (not always visible) (*see* **Note 19**).
5. Close the Falcon tube. Incubate the tubes at +4 °C for 2 h. Vortex very gently every 20 min.

6. Spin the tubes at 500 × *g* for 1 min to collect the virus-containing liquid.
7. The concentrated virus solution can be stored at −80 °C for at least 1 year.

3.2.3 Lentivirus Concentration by Ultrafiltration

1. If no ultracentrifuge is available lentiviral vector particles can be concentrated by ultrafiltration (e.g., Merck-Millipore, Amicon Ultra Ultracel 100 K, cat # UFC910096).
2. Add 15 ml H_2O to the filter cartridge according to the manufacturer's manual and spin at 3,000 × *g* for 5 min.
3. To sterilize the filter cartridge add 15 ml 70 % ethanol and incubate for 2 min (*see* **Note 20**).
4. Discard the ethanol from the reservoir, dry the filter by centrifugation at 3,000 × *g* for 5 min, and remove the flow through.
5. Pipette 15 ml of the purified virus solution from Section 3.2.1 on the filter cartridge and centrifuge at 3,000 × *g* at +4 °C for approx. 20 min. The volume of the concentrated virus solution should be between 160 and 200 µl.
6. The concentrated virus solution can be stored at −80 °C for at least 1 year.

3.2.4 Titer Determination of Lentiviral Vector Particles

1. For the determination of the biological titer, seed 10^5 HT-1080 cells or 10^6 human PSCs per well on a 6-well plate.
2. After 24-h cultivation trypsinize 1 well and count the number of cells.
3. Aspirate the medium of the wells and add 2 ml fresh medium. Transduce the cells typically with 1:100, 1:1,000, and 1:10,000 dilutions of the concentrated virus per well.
4. Cultivate the cells overnight in a 37 °C humidified incubator with a 5 % CO_2 atmosphere.
5. Aspirate the culture medium and add 5 ml DMEM medium or 3 ml mTeSR™1 (for human PSCs).
6. Incubate the cells for additional 2 days in a 37 °C humidified incubator with a 5 % CO_2 atmosphere.
7. Trypsinize the cells and extract target cell DNA from each individual well plate using a genomic DNA extraction kit following the manufacturer's recommendations. Elute the DNA in 100 µl elution buffer.
8. DNA samples from the transduced cells are measured in triplicates. Prepare qPCR master mix for the desired number of samples as follows:

Reaction buffer ABsolute qPCR	
Low ROX Mix 2×	5
Primer fw LV	0.5 × *n*
Primer rev LV	0.5 × *n*
Primer fw hActb	0.5 × *n*
Primer rev hActb	0.5 × *n*
Probe LV2 (10 μM, FAM labled)	0.2 × *n*
Probe hActb (10 μM, Yakima Yellow labled)	0.2 × *n*
H_2O	0.1 × *n*
Total	7.5 × *n*

n = number of reactions

9. Pipette 7.5 μl of the master mix per well on the 96-well optical reaction plate.
10. Add 2.5 μl template solution (genomic DNA of transduced cells) per well.
11. Seal the wells with optical caps, stripes, or foils, vortex the plate gently, spin down the liquid, and put the plate into the qPCR machine.
12. The amplification is performed with the following program: 95 °C for 15 min, 95 °C for 20 s, 60 °C for 2 min.
13. For the duplex Taqman assay the FAM and Yakima Yellow fluorescence is detected by the qPCR device.
14. Data analysis: Vector copy numbers in HT-1080 cell or human PSCs are normalized to human actin beta gene copies and presented as proviral copies per genome equivalent. Calculate titers (IP = infective particles per ml) according to the following formula:

    $IP/ml = (C \times N \times D \times 1{,}000)/V$ (C = proviral copies per genome, N = number of cells at time of transduction, D = dilution of vector preparation, V = volume of diluted vector added in each well for transduction).
15. To determine an accurate titer calculate mean values from at least two vector dilutions.

3.3 Lentiviral Transduction of Human Pluripotent Stem Cells

3.3.1 Lentiviral Transduction of Human PSC Colonies

1. Passage the human PSCs as clumps onto a Matrigel-coated 6-well plate as described in Section 3.1.2. Cultivate the cells in mTeSR™1 medium until the colonies are recovered from the last passaging procedure and reached a good size for transduction. In our hands the best results were obtained when the colonies were approximately 3–4 days prior to the next passaging (Fig. 3).

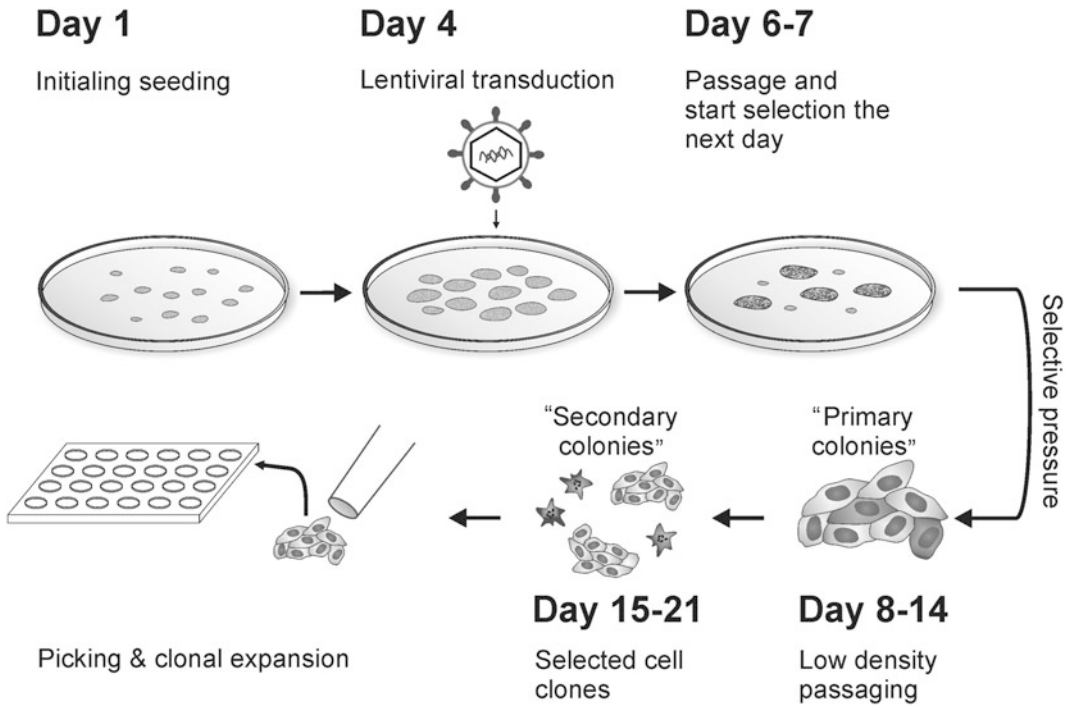

Fig. 3 Schematic presentation of the transduction and selection of pluripotent stem cells. Details of the procedure are outlined in Section 3.3

2. Thaw the concentrated lentiviral solution (produced according to Section 3.2) on ice.
3. For 1 well of a 6-well plate add the volume of the concentrated lentiviral solution with the desired amount of IP, move the plate to equally distribute the lentiviral solution, and add 1 ml mTeSR™1 medium. To increase the transduction efficiency 5–10 μg/ml polybrene (1:1,000–2,000 dilution from the stock solution) can be added.
4. Place the cells back into the incubator (37 °C, 5 % CO_2) for 4–6 h. Thereafter, add one additional milliliter of mTeSR™1 medium and cultivate the cells overnight in an incubator (*see* **Note 21**).
5. If a second, optional transduction round should be performed, repeat the points 2 to 4 24–48 h after the first transduction.
6. One day after the last transduction wash the cells twice with 2 ml knockout DMEM/F-12 per well and add fresh mTeSR™1 medium for the further cultivation. Perform daily medium changes with mTeSR™1 until the culture is ready for passaging. Begin the antibiotic selection after this passaging.

3.3.2 Lentiviral Transduction of Single Cells

Human PSCs can be passaged with different methods into single cells. For this purpose enzymatic passaging (e.g., with TE) or nonenzymatic passaging solutions (e.g., gentle cell dissociation reagent from Stem Cell Technologies) can be used.

1. Wash the cells once (or twice) with PBS.
2. Dissociate the human PSCs either enzymatically or nonenzymatically into single cells. If TE is used incubation for 7–10 min at 37 °C is recommended. Stop the trypsin reaction by adding a medium supplemented with 10 % FBS, if your pluripotent cell line accepts this procedure. Alternatively, use knockout DMEM/F-12. If a nonenzymatic passaging solution is used please follow the manufacturer's instructions.
3. Collect the cells in a conical tube and break up potential remaining clusters by pipetting the cell solution up and down.
4. Centrifuge for 3–5 min at 400–700 × *g* and remove the supernatant completely.
5. Add the desired amount of mTeSR™1 medium, adjust to 10 μM Y-27632, and count the cells.
6. Seed 50,000–100,000 cells/cm^2 (*see* **Note 22**) on a 12-well or 6-well plate freshly coated with Matrigel in mTeSR™1 medium containing 10 μM Y-27632 to allow reattachment of dissociated cells. Culture for 24 h in the incubator (37 °C, 5 % CO_2).
7. At the next day thaw the concentrated lentiviral solution (produced as described in Section 3.2) on ice.
8. For transduction remove the old medium and add the desired volume of the concentrated lentiviral solution (containing the desired IP) into the well. Distribute the lentiviral solution equally by moving the plate and add 1 ml mTeSR™1 medium. Identically to the transduction of hPSC colonies the efficiency can be increased by addition of 5–10 μg/ml polybrene (1:1,000–2,000 dilution from the stock solution).
9. Place the cells for 4–6 h into the incubator (37 °C, 5 % CO_2); thereafter add one additional milliliter of mTeSR™1 medium and cultivate the cells overnight (*see* **Note 21**).
10. Optionally, a second transduction can be performed 24–48 h later by repeating points 7 to 9.
11. After the last transduction round the cells should be washed twice with 1–2 ml knockout DMEM/F-12 per well. Further cultivation is performed typically with mTeSR™1 medium. Therefore, perform daily medium changes until the culture is ready for passaging. Passage the cells thereafter as clusters as described above (Section 3.1.2).
12. Start with the antibiotic selection after this passaging procedure.

3.4 Selection, Propagation, and Clonal Derivation of Transduced Cells

The selection can be initiated already 1–2 days after the last transduction round but from our experience we recommend to split the cells once prior to selection. In addition, a kill curve should be performed for every human PSC line to determine the ideal concentration of the selective antibiotics. In the case of a fluorescent reporter positive cells may be purified by fluorescence-activated cell sorting (FACS). The following paragraph describes a general protocol for the selection via an antibiotic resistance gene.

3.4.1 Determination of the Ideal Antibiotic Concentration

1. Passage the human PSCs as described in Section 3.1.2. For a kill curve minimally 4–6 different concentrations should be tested for the specific human PSC line. Classical antibiotics for the selection are blasticidin, hygromycin, neomycin (G418), puromycin, and zeocin.
2. Begin with the treatment 2–3 days after passaging and perform daily media changes with mTeSR™1 containing the different concentrations of the selective antibiotics.
3. Check the cells under the microscope and monitor the selection progress.
4. Choose the concentration, at which the human PSCs show clear signs of a toxic effect of the selective antibiotic after 2–3 days. After this time point cell death should clearly occur. In our hands, such an ideal concentration will increase the number of positive selectable clusters/colonies after lentiviral transduction, especially if colonies were used for transduction.

3.4.2 Selection of Transduced Cell Clones

1. Passage the human PSCs transduced with the lentiviral vector particles as clusters with dispase as described under Section 3.1.2 and schematically shown in Fig. 3.
2. Begin the selection procedure 1 day after passaging with the defined concentration of the selective antibiotic (*see* Section 3.4.1) in mTeSR™1 medium.
3. Perform daily media changes with selective medium (mTeSR™1 containing the desired concentration of the selective antibiotic).
4. Typically, after 3–4 days first small colonies/clusters (or parts of a larger colony) are visible, which show no signs of a toxic effect from the selection. These small colonies will increase in size during the further cultivation (*see* **Note 23**).
5. Cultivate the transduced cell colonies until they reach a sufficient size to passage them. Ideally, the first clones should not be picked before day 7 of selection (*see* **Note 24**).

3.4.3 Clonal Expansion of Transduced hPSCs

Stably transduced cell colonies can be directly picked once they have reached a sufficient size. However, these primary colonies might have been derived from more than one transduced cell. To

minimize the risk of mixed clonal identity, it is recommended to passage primary colonies once more. Ideally, these primary colonies should be passaged into single cells in low density so that newly arising secondary colonies will be formed from a single-cell clone. However, this might not be feasible for all human PSC lines and should be experimentally tested for each PSC line. Thus, this chapter describes the procedure starting with primary colonies.

1. Once primary colonies (cell clones) have reached a passaging-ready size they are passaged in the presence of the selective antibiotic as described in Section 3.2.1 onto a Matrigel-coated 6-well plate. The ideal passage ratio depends on the obtained number of colonies. Use a high passage ratio so that only a few small clusters evenly distribute in a well (*see* **Note 23**) (Fig. 3).
2. Cultivate these clusters until they grow up to a size ideal for passaging in the presence of antibiotics in the culture medium. This takes usually 7–10 days. Under microscopical inspection using an inversed cell culture microscope mark colonies from the underside of the well with a permanent marker. Mark only colonies that leave sufficient space to neighboring colonies without direct cell contacts.
3. Each of these marked colonies can be transferred into a new well of a 24- or 48-well plate by manual picking. At the day of picking prepare the required number of wells of a 24- or 48-well plate and coat them with Matrigel. To ensure that the picked colonies have a sufficient amount of mTeSR™1 medium add approx. 0.5–0.75 ml per well.
4. Wash the wells once with knockout DMEM/F-12 to remove dead cells and cell debris (2 ml per well of a 6-well plate).
5. Add dispase (1 U/ml, 1 ml into each well of a 6-well plate) and incubate the plate for 7–10 min in the incubator (37 °C, 5 % CO_2) (*see* **Note 12**).
6. Wash twice with 1.5–2 ml knockout DMEM/F-12, remove the knockout DMEM/F-12 completely, and add 1.5–2 ml mTeSR™1 medium per well of a 6-well plate where colonies should be picked from.
7. Place the plate under the microscope and search for marked colonies. Center one colony in the field of vision. Remove the lid of the plate and use a sterile 100 µl tip (on a pipette) or a sterile cannula to cut the colony into smaller clumps under optical inspection. Collect the fragments by scraping with the 100 µl tip of the pipette over the bottom and aspirate the clumps.
8. Transfer the clumps of one colony into 1 well of the coated 24- or 48-well plate. Change the tip after each picked colony. Take care not to aspirate floating cell debris/fragments during the

picking process as this could lead to mixed clonal identity (Fig. 3).

9. After the picking place the 24- or 48-well plate containing the primary clones into the incubator (37 °C, 5 % CO_2).
10. Analyze the picking efficiency the next day under the microscope and cultivate the clones until they are ready for passaging. This takes usually 7–10 days.
11. Propagate the cell clones and immediately freeze stocks when the cell mass is appropriate.
12. For each clone a quantification of the number of lentiviral integrations, a characterization of pluripotency (minimal expression analysis and staining of key pluripotency genes or a teratoma assay), and ideally a karyotype analysis are recommended.

4 Notes

1. The HEK293T human embryonic kidney cell line is available from ATCC (cat # CRL-11268). There are also several commercial cell lines available which were selected for high virus titer production.

293 FT Cell Line (Cat. # R700-07)	Life Technologies
Lenti-X™ 293T Cell Line (Cat. # 632180)	Clontech
293LTV Cell Line (Cat. # LTV-100)	Cell Biolabs

2. The hESC-qualified Matrigel should be stored in aliquots at −80 °C following the manufacturer's instructions. After coating, the supernatant still contains matrix proteins that have not yet polymerized; thus, Matrigel may be reused but it has to be tested for each human PSC line separately. The used Matrigel can be stored at 4 °C for up to 7 days and could be used for coating just like fresh Matrigel.
3. After preparing mTeSR™1 medium it can be stored for up to 14 days at 4 °C. The required mTeSR™1 for each day should be warmed up as aliquot to ensure that the residual medium is always stored at 4 °C. To increase the shelf life it can be stored upon preparation in aliquots at −20 °C for up to 6 months and thawed in appropriate amounts.
4. The specified lentiviral transfer plasmids are only examples. Various plasmids for different applications are available from Addgene (www.addgene.org) or from commercial suppliers.

5. DMEM supplemented with 2 % FBS for virus concentration with Amicon Ultra ultrafiltration cartridges (Merck-Millipore).
6. 2 M $CaCl_2$: Dissolve 7.35 g of $CaCl_2 \times 2H_2O$ (Sigma-Aldrich cat # C5080) in 25 ml H_2O. Sterilize the solution by filtration with 0.22 μm nitrocellulose filter. Store in 2 ml aliquots at −20 °C.
7. 2 × HeBS (HEPES-buffered saline): Dissolve 16.36 g of NaCl (Sigma-Aldrich cat # S7653; 0.28 M final), 11.9 g of HEPES (Sigma-Aldrich cat # H7523; 0.05 M final), and 0.213 g of Na_2HPO_4, anhydrous (Sigma-Aldrich cat # S7907; 1.5 mM final) into 800 ml H_2O. Adjust pH to 7.0 with 10 N NaOH. It is very important to adjust to the correct pH. The precipitate will not form below 6.95. Above 7.05 the precipitate will be coarse and transfection efficiency will be low. Add H_2O to 1,000 ml, and perform the final pH adjustment. Filter sterilize through a 0.22 mm nitrocellulose. Store at −20 °C in 50 ml aliquots. Once thawed, the HeBS solution can be kept at +4 °C for several weeks.
8. The primer (FPLV2, ACCTGAAAGCGAAAGGGAAAC; RPLV2 CACCCATCTCTCTCCTTCTAGCC) and probe (LV2, 5′-FAM AGCTCTCTCGACGCAGGACTCGGC-BHQ1) sequences for detecting the copy number of the lentiviral genome were selected according to (14). Primer and probe sequences for the quantification of the human actin beta: FPhActb tcgtcgtcgacaacggct; RPhActb agagaaagcgcccttgcct; probe YY 5′-catgtgcaaggccggcttcgc-BHQ1. If no duplex assay capable for qPCR device for the parallel detection of green and yellow fluorescence dyes is available the TaqMan assay for the copy number quantification can be split into two separate reactions/wells using FAM-labeled probes.
9. If a four plasmid-based lentiviral vector system is used, 10 μg packaging plasmid (e.g., psPAX2), 7 μg pMDLgag/polRRE (encoding the HIV-1 Gag and Pol proteins), and 3 μg pRSVrev (encoding for Rev) are necessary.
10. The time for storing Matrigel can be prolonged to up to 10 days, which had in our hands no negative effects. Generally, it is recommended to prepare only the amount of Matrigel that is required within the next 7–10 days.
11. If a faster polymerization is required the cell culture plastics containing the cold Matrigel solution can be incubated for 15–20 min in the incubator (37 °C, 5 % CO_2) to polymerize quicker. Thereafter, the surface should be washed once with knockout DMEM/F-12 (room temperature) prior to seeding the passaged human PSC clusters.
12. The ideal incubation time depends on the cell line and the size of the colonies and has to be determined individually.

Generally, inspect the wells after around 7 min of incubation and look whether the colony rim lifted up (brighter rim of the colony under a phase-contrast microscope). Ideally the colony rim should be lifted up but not the core of the colony.

13. To minimize costs the cells can also be scraped and collected in 1 ml knockout DMEM/F12 instead of mTeSR™1 medium. In this case after collecting the clusters an additional centrifugation step (3 min at 300 × *g*) is required. The supernatant must be removed and the required amount of mTeSR™1 medium can be carefully added (take care not to break up the clusters too much). Now the human PSC cluster can be distributed into the new Matrigel-coated wells in the desired density.
14. If the fragments got too small cell survival will be quite low indicated by floating dead cells and cell debris on the next day. To avoid this, scrapping should be reduced to max 2–3 times over a given surface area. Collect the clusters with a wide bore cell safer tip to reduce steric stress. Still attached clusters can be washed away with 1 ml of mTeSR™1 or knockout DMEM/F12 medium.
15. Generally, the optimal passaging ratio should be determined individually for each human PSC line. The addition of ROCK inhibitor Y-27632 may result in a higher split ratio. However, in our experience prolonged passaging with Y-27632 resulted in a worsening morphology over time compared to passaging without Y-27632.
16. At this point, there should be no visible precipitation because it is too fine. If a precipitation is visible, then it is too coarse and your transfection will most likely be less efficient.
17. For the alternative concentration protocol by ultrafiltration, a reduction of the FBS supplementation to 2 % is recommended for an optimal filtration result. The FBS reduction might slightly reduce the virus titer.
18. The lentivirus stocks can be stored at +4 °C for 1–4 days without significant titer loss before they are used for transduction of target cells or concentration. For longer storage stocks must be kept at −80 °C.
19. Do not leave the pellet dry for more than 5 min or it may result in a significant titer decrease.
20. Longer ethanol incubation may negatively affect the constancy of the filter cartridge material.
21. Alternatively, the cells can be washed twice with 2 ml knockout DMEM/F-12 after the transduction time of 4–6 h. The cultivation afterwards is typically performed with mTeSR™1 medium. This procedure is preferable if a second transduction round will be performed within the next 24–48 h.

22. The ideal cell number per cm^2 must be empirically determined and depends on the reattachment rate of the used human PSC line.
23. It may happen that some of the primary colonies will die during further selection. This is likely caused by two independent phenomena. First, if a primary colony is mixed with transduced and non-transduced cells, later cells will start to deteriorate causing a disintegration of the whole colony. Second, PSCs are known for epigenetic silencing of transgenes especially of ectopic viral DNA sequences such as viral promoters or other regulatory elements. Silencing takes place very rapidly and may cause loss of resistance against the selection. Thus, our recommendation is to passage transduced cells once before selection to increase the likelihood that the transduced cells are able to form a clonal cell cluster and to keep up the selective pressure by adding antibiotics to the culture medium. However, it might be useful to reduce the antibiotic concentration after 1 week of selection.
24. Cell clones may be passaged under selective pressure. Residual non-transduced cells are additionally destroyed and ongoing selection reduces the risk of transgene loss/epigenetic silencing. However, the reattachment rate after the first passaging is lower compared to passaging without selection pressure. Generally, the minimal selection time should be around 14–21 days. In our hands no negative effects were detectable upon permanent culture in the presence of the selective antibiotic.

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