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Generation of Stable Expression Mammalian Cell Lines Using Lentivirus

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[Abstract] Lentiviruses are used very widely to generate stable expression mammalian cell lines. They are used for both gene down-regulation (by using shRNA) or for gene up-regulation (by using ORF of gene of interest). The technique of generating stable cell lines using 3rd generation lentivirus is very robust and it typically takes about 1-2 weeks to get stable expression for most mammalian cell lines. The advantage of using the 3rd generation lentivirus are that are very safe and they are replication incompetent.

Keywords: Lentivirus, Stable cell lines, Gene knockdown, Gene over-expression, Mammalian cell lines, Transduction

[Background] As compared to the short term protein expression resulting from transient transfection, stable cell lines generated using lentivirus exhibit long term protein expression and the system is highly reproducible (LaGory *et al.*, 2015). Below is the outline for generation of stable cell lines using lentivirus (Figure 1). In this protocol, we describe the use of the 3rd generation lentiviral system which uses three different plasmids for generating stable cell lines. First plasmid contains your gene of interest usually flanked by Long Terminal Repeat (LTR) sequences, that are integrated into the host genome. A variety of these plasmids can be obtained from Addgene. Second is the pCMV Delta R8.2 plasmid that encodes all components necessary for packaging the lentivirus viz. HIV-1 Gag, Pol, Tat and Rev. Third and the final plasmid is the pCMV VSVG that encodes the viral envelope protein.

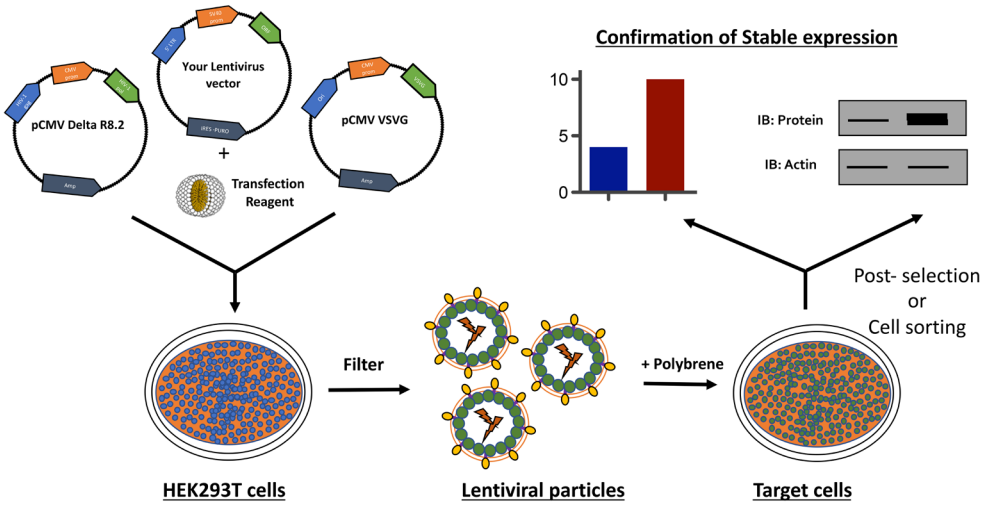

Figure 1. Schematic representation of generation of stable cell lines using lentivirus

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Materials and Reagents

1. 10 cm^2 tissue culture treated plates (Corning, catalog number: CLS430165)
2. Low protein binding 0.45 µm PDVF filters (Millipore, catalog number: SLHV033RS)
3. 10 ml plastic syringes sterile (any brand)
4. Sterile Glass jar
5. Disposable plastic pipettes (5 ml and 10 ml)
6. Sterile 1.5 ml Eppendorf tubes
7. Pasteur pipettes
8. HEK293T (293T) (ATCC, catalog number: CRL-3216)
9. Target cell line (any human cancer cell line)
10. Xtreme gene 9 transfection reagent (Sigma-Aldrich, 636577900)
11. Packaging plasmids: pCMV delta R8.2 (Addgene, catalog number: 12263) and pCMV-VSV-G (Addgene, catalog number: 8454) and your plasmid of interest (test and control plasmid – see Notes)
12. 10% bleach
13. Polybrene solution: 8 mg/ml in water filter sterilized (Hexadimethrine bromide, Sigma, catalog number: H9268), made fresh every time
14. Opti-MEM reduced serum medium (Thermo Fisher, catalog number: 31985062)
15. DMEM complete medium with 10% FBS and pen-strep
16. Selection antibiotic (*e.g.*, Puromycin)
17. Dry ice or liquid nitrogen
18. DMSO
19. Liquid nitrogen

Equipment

1. Cell culture incubators (37 °C, 5% CO_2 and 32 °C, 5% CO_2)
2. Hemocytometer (Standard)
3. Laminar hood BSL-2 type
4. 37 °C water bath
5. Autoclave
6. Micropipettes (1,000 µl, 200 µl)
7. -80 °C freezer

Procedure

A. Preparation of lentivirus in HEK293T cells
   1. **Day 0:** Seed about 2-3 million HEK293T cells in a 10 cm^2 plate.

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2. **Day 1:** HEK293T cells should be about 70-80% confluent. If for some reason they seem less you may wait for another 8-10 h.
3. Prepare transfection mix using the following proportions in a sterile 1.5 ml Eppendorf tube as per manufacturer's instructions:

Opti-MEM medium	1 ml
pCMV delta R8.2	2 µg
pCMV-VSV-G	0.5 µg
Target plasmid	1.5 µg
Xtreme gene 9	12 µl (1:3, DNA:Xtreme Gene, Ratio)

   Once you add all the five components in a 1.5 ml Eppendorf tube, mix the solution by pipetting or inverting. Do not vortex. Incubate the mix for about 20 min at room temperature.
4. Aspirate the medium from HEK293T cells using Pasteur pipettes and add about 9 ml fresh complete DMEM medium.
5. After 20 min incubation of the transfection mix, add the mix to the HEK293T using a 200 µl micropipette. Make sure to add the mix drop-by-drop to cover the complete area of the plate.
6. Incubate the plate in a 37 °C cell culture incubator for about 48 h.
7. **Day 3:** Using a 10 ml disposable sterile pipette, collect the supernatant/media off the HEK293T plates in a sterile 15 ml tube. Add another fresh 10 ml DMEM media to the plate and move it back to the incubator. Transfer the collected supernatant into a 10 ml syringe and then filter it through a 0.45 µm PVDF filter inside the laminar hood. This step will result in collection of filtered viral supernatant without any residual HEK293T cells in it.
   Note: To ensure proper disposal of all the equipment that has come in contact with the virus, all the pipettes, tips, syringes and filters used here must be washed with 10% bleach for about 10 min before finally discarding them in a biohazard bin.
8. If you do not have your target cells ready or if you plan to use the lentivirus later, you can snap freeze the viral supernatant using dry ice or liquid nitrogen and then store it at -80 °C. The virus is usually stable for several months at this temperature.
9. **Day 4:** If desired, another round of 10 ml lentiviral supernatant can be collected from still growing HEK293T cell plates, similar to Day 3. Before discarding the HEK293T cell plates in the biohazard bin, they should be treated with 10% bleach for about 5-10 min.

Pro-tip: The probability of high viral titer can be estimated from the amount of HEK293T cell death, i.e., the more the cell death, the more is the probability of collection of virus of high titer.

B. Preparation of target cells

1. **Day 2** (if you want to do this in parallel with the virus preparation, Recommended)**:** Seed about 0.5-1 x 10^6 target cells each in two 10 cm^2 tissue culture plates. One plate is where you will add the viral supernatant, *i.e.*, the lentivirus and the other will be your killing control where you will not add any lentivirus.
2. **Day 3:** The target cells should be around 40%-50% confluent prior to transduction, *i.e.*, addition

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of virus. If your lentivirus is in the freezer, make sure you thaw it in a 37 °C water bath before adding it to the cells. Once the cells are ready, prepare a polybrene medium (target cell medium) mix. The final concentration of polybrene should be 8 µg/ml. Prepare 10x polybrene medium mix by adding 2 ml medium with 20 µl of 8 mg/ml Polybrene. Aspirate the medium in the target cells using Pasteur pipette and add 1 ml polybrene medium mix and let it incubate for about 1-2 min.

Note: It's recommended that polybrene solution is made fresh each time OR you can store aliquots of it at -20 °C. Thaw and use an aliquot each time. Do not re-freeze.

3. Using a disposable 10 ml pipette, add 9 ml lentivirus to the target cells. Add plain medium to the killing control plate.
4. **Day 4:** Repeat the same steps as Day 3, to do a second round of transduction to ensure all the cells get the lentivirus. See the note in Step A7.
5. **Day 5:** Add the selection antibiotic at the killing concentration (*e.g.*, Puromycin 1-2 µg/ml) to both plates. See Notes to determine the killing concentration of the selection antibiotic. Typically, fresh media with antibiotic is added every two days until all the cells in the killing control plate are dead.
6. **Days 8-10:** Before you do any further experiments, make sure to cryo-freeze about 1/4th portion of cells in 10% DMSO with DMEM in liquid nitrogen. Passage the rest of the cells for RNA/protein isolation, in order to validate for stable expression of protein of interest.

Notes

1. **Generation of kill curve:** Prior to setting out to generate stable cells using lentivirus with a selection maker in any cell line, it is always a good idea to generate a kill curve. A quick google search would tell you a rough range of the concentration of antibiotic to be used. Generally, at least 3-4 different concentrations are tested to ensure complete killing. For example, for puromycin the following concentrations would be tested 1 µg/ml, 2 µg/ml, 3 µg/ml and 4 µg/ml for 48-72 h.
2. **Test and control plasmid:** A proper control plasmid should be used along with the test plasmid. For example, if doing an shRNA-based knockdown then you should include shControl plasmid that has non-targeting sequence instead of the shRNA or if you ORF-based over-expression that the empty vector should be used as a control.

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Competing interests

The authors declare no conflicts of interest or competing interests.

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