Lentivirus Production

Introduction

This protocol can be used to produce lentivirus from a lentiviral vector transfected into 293T cells using a polyethylenimine (PEI) transfection protocol. This procedure can be modified for alternative packaging cell lines or transfection reagents. Once produced, lentivirus can be used for a variety of downstream applications such as stable-cell line generation.

Workflow Timeline

**Day 0:** Seed 293T packaging cells

**Day 1 (pm):** Transfect packaging cells

**Day 2 (am):** 18 h post-transfection. Remove media, replace with fresh media

**Day 3-4 (am):** Harvest virus

Equipment

* Class II, Type A2 Biological Safety Cabinet
* 0.5–10 µL single channel pipette
* 2–20 µL single channel pipette
* 20–200 µL single channel pipette
* 200–1000 µL single channel pipette
* Ice bucket
* CO2 incubator
* Pipet controller
* Hazardous waste container
* pH meter
* Stir plate
* Magnetic Stir Bar

Reagents

* DMEM high glucose, Corning 10-013-CV
* L-alanyl-L-glutamine (or alternative stable glutamine such as glutaGRO, Corning 25-015-CI)
* Low serum medium such as Opti-MEM or Opti-Pro SFM, Thermo Fisher, 12309019
* 25 mM chloroquine（CQ）
* Polyethylenimine, linear MW 25,000 Da
* Heat-inactivated FBS
* 1x PBS pH 7.4 without calcium or magnesium, Corning 21-040-CV (cations can affect the attachment of adherent cells) （DPBS）
* 0.45 μm polyethersulfone filter, Nalgene, 565-0010
* Microcentrifuge tubes, Neptune 3745.X
* 10 cm tissue culture dish, Corning 430167
* 15 mL conical tubes, VWR 21008-216
* Hydrochloric acid HCl
* Sodium hydroxide NaOH
* 0.22 μm polyethersulfone (PES) filter
* Transfection grade DNAs

Reagent Preparation

1. DMEM Complete (also known as D10): 10% v/v FBS and 4 mM L-alanyl-L-glutamine (or stable alternative, such as glutaGRO)
   * To a 500 mL bottle of DMEM high glucose, add 55 mL of heat-inactivated FBS and 5 mL of glutaGRO. Store at 4 °C.

**\*Pro-Tip\*** Different brands and lots of FBS can promote or inhibit transfection. Test a variety of brands and lots of FBS to find one suitable with your protocols. FBS can be purchased already heat inactivated or it can be inactivated in the lab by heating to 56°C for 30 min.

1. 1 mg/mL polyethylenimine, linear MW 25,000 Da (PEI)
   * Dissolve 100 mg of powder in 100 mL of deionized water. **(1mg/ml)**
   * While stirring, slowly add hydrochloric acid until the solution clears.
   * Check the pH of the solution.
   * Use hydrochloric acid or sodium hydroxide to adjust the pH to 7.0. Typically, the solution will be basic and will need adjustment with hydrochloric acid first.

**\*Pro-Tip\*** The pH of this solution will drift rapidly upon addition of acid or base. Add only a few drops at a time, allow them to mix and recheck the pH to prevent over or undershooting the desired pH.

* + Allow the solution to mix for 10 min and then recheck the pH to ensure that it has not drifted.
  + Filter the solution through a 0.22 μm membrane.
  + Aliquot 500–1000 μL into sterile tubes.
  + Store the tubes at -20 °C or -80 °C.

After thawing, the solution can be stored at 4 °C for up to 2 months.

After 2 months, discard the tube and thaw a new working stock.

* + The optimal mass DNA : mass PEI ratio will need to be empirically determined for each new batch of 1 mg/mL PEI and for each cell line.

Considerations Before You Start

* The health of the packaging cell line is critical for obtaining high viral titer.
* 293T cells should be split 3 times a week:
  + Monday: Plate 1×106 cells in a T75 flask in 15 mL DMEM Complete.
  + Wednesday: Plate 1×106 cells in a T75 flask in 15 mL DMEM Complete.
  + Friday: Plate 8×105 cells in a T75 flask in 15 mL DMEM Complete.
* Do not add pen-strep to the media.
* Use cells that are below passage 15 for viral production.

**Procedure**

1. Seed 293T packaging cells at 3.8×106 cells per plate in DMEM Complete in 10 cm tissue culture plates.
2. Incubate the cells at 37 °C, 5% CO2 for ~20 h.
3. Prepare a mixture of the 3 transfection plasmids into OptiPro SFM:

| **Reagent** | **Amount per 10 cm dish\*** |  |
| --- | --- | --- |
| psPAX2 | 1.3 pmol | 4.3ug |
| pMD2.G | 0.72 pmol | 1.3ug |
| Transfer Plasmid\* | 1.64 pmol | 6.5ug（blastin） |
| OptiPro SFM to total volume | 500 μL |  |

1. \*Plasmid concentrations and ratios should be optimized for each transfer plasmid.

粗略的比例：per p10（1:2:3）

或者（15 ug:9 ug:6 ug(5:3:2)）

|  |  |
| --- | --- |
| DMEM | 750 μL |
| Transfer Plasmid | 10 μg |
| psPAX2 | 6.67μg |
| pMD2.G | 3.33 μg |

**\*Pro-Tip\*** Endotoxins can inhibit transfection; therefore, plasmid DNA purification should include an endotoxin removal step. For high quality plasmid DNA, the plasmids should also be propagated in an endonuclease negative *E. coli* strain such as NEB stable.

1. Make a mixture of a total of 500 μL PEI-OptiPro SFM with enough PEI such that the ratio of μg DNA:μg PEI is 1:3 (1000 μL total per 10 cm dish).

Using transfer plasmid pHAGE TRE dCas9-KRAB (**total μg of plasmid DNA 27.8 μg**), this would be 83.4 μL of 1 mg/mL PEI in 416.6 μL of OptiPro SFM per 10 cm dish.

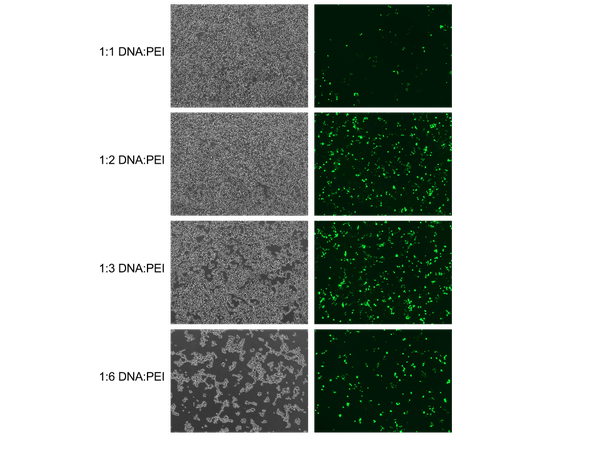
**\*Pro-Tip\*** There can be batch to batch variation when making the PEI working stock, therefore the ratio of μg DNA:μg PEI needs to be empirically determined. Once a batch of PEI is prepared, transfect cells with a fluorescent plasmid using a variety of ratios. Check the cells 1-2 days after transfection to determine what ratio gives the highest percentage of GFP positive cells.

* + Refer to the table below for a possible range of ratios to test:

| **Ratio of DNA:PEI** | **μg of DNA** | **μL of 1 mg/mL PEI** |
| --- | --- | --- |
| 1:1 | 18.9 | 18.9 |
| 1:2 | 18.9 | 37.8 |
| 1:3 | 18.9 | 56.7 |
| 1:4 | 18.9 | 75.6 |
| 1:5 | 18.9 | 94.5 |
| 1:6 | 18.9 | 113.4 |

1. Gently add the diluted PEI mixture to the diluted DNA mixture. Add the diluted PEI dropwise while gently flicking the diluted DNA tube. Incubate the mixture 12–15 min at RT.
2. During the incubation, add 10 mL of DMEM Complete to a 15 mL conical and an appropriate amount of 25 mM chloroquine to the conical so that the final concentration in the dish (once transfection mixes are added) will be 25 uM of chloroquine.
3. After the incubation, add the DNA:PEI-Max mix to the conical containing the media and chloroquine and mix well.
4. Gently aspirate the media out of the previously seeded 10 cm plate.
5. Slowly pipette the transfection mix onto the 10 cm plate, being careful not to disturb the cells.
6. Incubate the cells for **18 h,** or until the following morning.
7. The following morning, carefully aspirate the media. Replace the media with 10 mL of DMEM Complete or OptiPro SFM.
8. Incubate the cells.
9. Virus can be harvested at **48, 72, and 96 h** post transfection in individual harvests or a combined harvest where all the individual harvests are pooled. If pooling harvests, transfer the harvested media to a polypropylene storage tube and store at 4 °C between harvest.
10. Centrifuge the viral supernatant at 2100 rcf for 5 minutes to pellet any packaging cells that were collected during harvesting.
11. Filter supernatant through a 0.45 μm PES filter.
12. The viral supernatant can be stored at 4 °C for several hours but should be aliquoted and snap frozen in liquid nitrogen and stored at -80 °C as soon as possible to avoid loss of titer.

Sample Data



**Figure 1:** 293T cells were transfected with the GFP-expression plasmid [pRosetta](https://www.addgene.org/59700/) using μg total DNA to μg PEI ratios of 1:1, 1:2, 1:3 and 1:6. The 1:2 and 1:3 total DNA:PEI μg ratios provided high transfection efficiencies as measured by the highest proportion of GFP positive cells without limiting cell growth. Left panels: bright field images; right panels: GFP channel images.

取两只无菌的离心管，一只加入500μL Opti-MEM和32μL Lipofectamine 3000，用枪轻轻吹打混匀；另一只加入500μL Opti-MEM和10μg表达目的序列的慢病毒载体质粒（自行提供）、6.67μg psPAX2，3.33μg pMD2.G，及40μL P3000试剂，用枪轻轻吹打混匀。将两管液体混合，再次用枪轻轻吹打混匀，不可Vortex或离心，室温孵育10-15 min。将混合液均匀滴加到提前换液的细胞培养基中，轻轻晃匀，置于培养箱中培，后续无需在数小时后更换培养液。