Producing Lentivirus in 293T Cells with Polyjet

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Abstract

Lentiviruses were produced by reverse transfection of suspended 293T cells using 20 ug lentiviral vector, 10 ug pVSV-G, 20 ug pCMV-dR8.91, and 100 µl Polyjet (SignaGen) or Lipofectamine 2000 (Life Technologies) in 15 cm dishes. The 3 ml plasmids–Polyjet complex and 1.5 ml 293T cell suspension were mixed in 50 ml centrifuge tubes and shaken for half hour before transferred to dishes. Virus harvested 48 and 72 h after transfection was combined, concentrated 50-100-fold by centrifugation at 26,000 rpm for 90 minutes, and suspended in culture medium. 500-2,000 µl of concentrated virus was used to infect 1 x 10(6) cells in 500 µl culture medium in the presence of 4 µg/ml polybrene (Sigma-Aldrich) followed by gentle pipetting 25 times and shaking for 10 minutes in the hood.

Introduction

Production of high titer of lentivirus is the key for ectopic gene expression, RNAi gene knockdown or library screen, CRISPR library screen, and functional analysis. Polyjet and Lipofectamine 2000 are good reagents for transfection of 293T cells to produce lentivirus. Polyjet may be better for transfection of 293T cells to produce lentivirus.

To avoid contamination, the freezing vials should be immerged in 70% alcohol for 2-3 min to thaw 293T cells. Plasmids should be free of contamination.

You can judge the cells you need by calculating the culture surface area and confluence of cells. After transfection, cells should be 90% confluence. Do not need to count cells. Scale up cells and plasmids by ~2.8 X for 15 cm dishes from 10 cm dishes.

Reagents

1. HEK-293T cells
2. Polyjet (SignaGen)
3. deta8.9 plasmid
4. VSVG plasmid
5. Lentiviral vectors
6. Fresh IMDM, DMEM, or Opti-MEM
7. 50 ml centrifuge tubes

8. 10 cm or 15 cm dishes
9. Warm 0.25% trypsin/1mM EDTA
10. Beckman ultraclear tubes (344058, Vol: 38.5 ml)
11. Fetal bovine serum
12. Pipeter and tips (1 ul-1000ul)
13. Cell culture facilities such as Pipet-Aid and serum pipets.
14. 70% Alcohol
15. Penicillin/Streptomycin Mixture
16. Phosphate Buffered Saline
17. Bleach
18. Polybrene
19. Optimal: Puromycin or Neomycin
20. optimal: 0.45 µm or equivalent PVDF filter

Equipment

1. Bench top centrifuge
2. Shaker
3. biosafety hood,
4. Ultracentrifuge: Sorvall or Beckman
5. Swing rotor: SW28 (or equivalent)
6. 5% CO2 incubator
7. Inverted Phase contrast microscope

Procedure

1. On Day 1 afternoon or evening, prepare plasmids-Polyjet complexes for each transfection sample as follows: a. In a sterile 50 ml tube, dilute 7 µg delat8.9 (or delta-NRF) (20 µg per 15 cm) helper construct, 3.5 ug pVSVg (10ug/15 cm), and 7 µg (20ug) of Lenti expression vector (17.5 µg total or 50 ug total) in 0.5 ml (1.5 ml) of

fresh IMDM, DMEM, or Opti-MEM without serum. Mix gently.

b. In a separate sterile 15 (50) ml tube, mix Polyjet gently before use, then dilute 36 µl in 0.5 ml (100 ul in 1.5 ml for 15 cm dish) of fresh IMDM, DMEM, or Opti-MEM without serum. Mix gently and incubate for 5 minutes at room temperature.

c. Prepare the fresh IMDM with 10% FBS. Remove medium of 293T cells and wash by adding 10-20 ml PBS.

d. After the 5 min incubation, combine the diluted plasmids with the diluted Polyjet. Mix gently by inverting 6-8 times.

e. Incubate for 20 minutes at room temperature to allow the plasmids-Polyjet complexes to form. Mix by shaking.

2a. While plasmids-polyjet complexes are forming, trypsinize 293T and spin to remove trypsin and EDTA. Resuspend the cells (0.5 ml for 10 cm dish; 1.5 ml for 15 cm dish) in IMDM or DMEM containing serum. Cell counting is unnecessary if you can judge the cells you need by calculating the culture surface area and cell density. Do not include antibiotics in the medium.

2b. Mix cells with plasmids-polyjet complexes, lay down the tube, fix it, and shake for 30 minutes on the shaker.

3. Add 6 ml (14ml for 15 cm dish) of growth medium DMEM or IMDM containing serum and Pen/Strep to a 10 cm tissue culture dish.
4. Add 1.54-2 ml (4.6-5.0 ml for 15 cm dish) of the 293T-plasmids-Polyjet complexes suspension to the plate containing media. Mix gently by rocking the plate back and forth for 1 min in the hood. Incubate cells overnight at 37°C in a humidified 5% CO_2 incubator.
5. The next day (Day 2) morning, remove the medium containing the remaining plasmids-Polyjet complexes and replace gently with 10 ml (25ml) complete culture medium with antibiotics (1% P/S). Incubate cells overnight at 37°C in a humidified 5% CO_2 incubator. Note: The VSVG causes 293T cells to fuse, resulting in the

appearance of large, multinucleated syncytia. The percentage of syncytia can be used as marker of transfection efficiency. This morphological change is normal and does not affect production of the lentivirus. One day after successful transfection, 293T cells should be a little rounded up without significant proliferation.

6. Harvest virus-containing supernatants separately at 48, 72, and 96 h hours post-transfection (Day 3-5) by transferring medium into a 50 ml sterile, capped, conical tube. You can put virus supernatants on ice in cold room for 1-2 days. Minimal differences in viral yield are observed whether supernatants are collected 48 or 72 hours post-transfection. Caution: You are working with infectious virus. Follow BL-2 guidelines. All materials contacting virus should be treated with bleach or 70% Alcohol.
7. Centrifuge supernatants at 3000 rpm for 10 minutes at +4°C to pellet debris.
8. Optional: Filter the bottom viral supernatants through a Millex-HV 0.45 µm or equivalent PVDF filter to remove the contamination of 293T. Generally, I use PVDF syringe filters and 20-30 ml syringes. If not filtered, at least 5 ml of bottom viral supernatants must be left in the tube to prevent 293T contamination. Filtration may reduce the titer of lentivirus.
9. Transfer supernatant to ultraclear tubes in buckets without disturb the pellet. Concentrate by centrifugation in SW28 (or equivalent) at 26,000 rpm, 90 min, 4°C (with 38 ml in Beckman ultraclear tubes (344058, brought to 0.5 cm to top of tubes). The inner surface of the buckets, caps, and ultraclear tubes must be cleaned by 70% alcohol and dried. Tip and aspirate supernatatant, leaving 0.5 ml over the pellet, for 80X virus concentration. Suspend by gentle triteration 100 times without bubbles, then leave on ice at 4°C for 4-12 h, and resuspend by gentle triteration again. Concentrated virus can be used directly to infect cells for high infection efficiency, or

can be stored in cryo-vial aliquots at -80°C. Multiple freeze-thaw cycles should be avoided to prevent loss of lentivirus. Clean tubes, pipets, dishes, and surface with bleach. Clean buckets with 70% alcohol.

10. Store viral stocks at -80°C. If the virus is not filtered by 0.45 um filter, one freeze-thaw cycle may be necessary to kill contaminated 293T cells.

11. For infection of suspended cells, the cells should be dissociated to a single cell suspension. For small attached cells, we also recommend to infect cells in suspension. For lentivirus such as LKO lentivirus mediated shRNA knockdown, virus (pool of 48h and 72h after transfection) from 100mm dish can infect 5 x 105 cells. Using small volume of medium for infection. Mix virus with cells by triteration for 20-30 times with 1000µl tip and by shaking for 10 min and incubate overnight. Polybrene (3-4 ug/ml, depends on cell type) can enhance the infection efficiency. Polybrene stock solution should be aliquoted and stored at -20°C. Storage of polybrene at 4°C will cause degradation and produce toxic products to cells.

12. Selection should be started at 48 h after infection. Puromycin (1.4-2.5 ug/ml) selection needs 2-3 days, and neomycin (50-300 ug/ml) selection needs >5-7 days.

13. For shRNA-mediated knockdown, we generally isolate RNA to check the knockdown level by real time PCR on day 3-5 after infection. Western blot should be performed on day 4-7 after infection.

Timing

It will take 2 hours for transfection; 5 days for lentivirus collection and concentration.

Anticipated Results

This protocol will produce high titer of lentivirus for functional analysis.

References

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