Lentivirus Production, Lentivirus Transduction, and Sorting Protocol

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Protocol status: Working
We use this protocol and it's working

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ABSTRACT

Lentivirus Production, Lentivirus Transduction, and Sorting Protocol for making the transduced iPSCs and SH-SY5Ys

MATERIALS

Materials and Reagents (Transfection and transduction)

- 0.25% Trypsin-EDTA [Gibco, #25300-054]
- 500 ml PBS [Gibco, #14190-144]
- Lipofectamine 3000 Transfection reagent (Cat. L3000001)
- Polybrene (Cat. TR-1003-G)
- Puromycin
- Transfer Plasmid (A2, Mart1, or LRRK2+)
- Packaging Plasmid (ex. psPAX2)
- Envelope Plasmid (ex. VSV.G)
- 45 µm cell strainer

Reagents (Sorting)

1. DPBS no calcium not magnesium Cat.14190144
2. Trypsin-EDTA (0.25%) Cat. 25200056
3. Knockout SR (Serum Replacement for ESCs/iPSCs) Cat.10828010
4. Sterile Corning Falcon Cell Strainer 70µm
5. Falcon 5mL Round Polystyrene sorting tube with strainer snap cap Cat. 352235

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Transfection

1 Seed HEK293FT cells into 6-well dishes, 1.1e6, 2 ml/well.
: 80% confluence after 20 h.

2 After 20 h, transfect HEK293FT cells using Lipo3000.

a. Tube A: Dilute 7 ul Lipo3000 in 250 ul OPTI-MEM/well, vortex 3s.

b. Tube B: Dilute below in 250 ul OPTI-MEM/well.
1 ug Packaging plasmid psPAX2
1.5 ug Envelope plasmid VSV.G
2 ug Transfer plasmid (ex. pLVX-EF1alpha-IRES-mCherry-HLA-A2)
Then add 6 ul P3000.

c.
Mix Tube A + B. Incubate 10 minutes at room temperature.

d.
Remove 1 ml of media from each well.

e.
Add #c to cells and slowly add Tube A+B mixture. Incubate 6 h.

f.
After 6 hours, remove and replace the medium, 1.5 ml/well. Incubate 18 h.

Virus Packaging

3 (24 h post-transfection) Harvest entire volume of cell supernatant, store @4°C.

4 (Slowly) Replace with 1.5 ml pre-warmed HEK293FT media/well. Incubate 28 h.

5 (52 h post-transfection) Harvest entire volume of cell supernatant and combine with #1 (total 3 ml/well collected).

6 Centrifuge viral supernatant @2000rpm.

7 Filter supernatant through a 0.45um pore size filter.

8 Aliquot 1ml/cryovial and store @-80°C.

Transduction and Selection

9 Fibroblast

1. Seed fibroblast 2e5 cells in a 6-well plate or 24-well plate
2. Next day, Infect the fibroblast with 8 µg/ml Polybrene media, 48 hrs.
!!! Titer to set the ratio of lentivirus to media.
A) 0.5 ml Lentivirus + 1.5 ml media (6 well plate)
B) 50ul Lentivirus + 250ul cell media (24 well plate)
3. Change media with cell media, 48 hrs.
4. Select transduced cells with 2 ug/ml Puromycin media or Flow cytometry for tagged cells.
5. Change media with fresh 2 ug/ml Puromycin media, 24 h.
6. Subculture cells to 60mm dish and expand
7. Freeze and store the cells in LN2.

10 iPSCs

1. Seed iPSCs 3.0e5 cells/well in a 6-well plate-Vitronectin(0.5ug/ml).
2. Next day, replace medium in wells with fresh media and place back into the incubator.
3. Infect the iPSCs, 16 h with 8µg/mL of Polybrene
!!! Titer to set the ratio of lentivirus to the media.
A) 0.5 ml Lentivirus + 1.5 ml iPSC media
B) 1 ml Lentivirus + 1 ml iPSC media
4. Change media with iPSC media.
5. Next day, select transduced cells with antibiotics (2 ug/ml Puromycin media, 48 h) or flow cytometry for tagged cells.
A) Flow core: https://docs.google.com/forms/d/e/1FAIpQLSdEXgZn6EKFfuUuVGj-JruBkCRCNHt6XynndK8rXHNtXPKErA/viewform
6. Collect about 2 to 3e5 cells and plate them in 2(35mm dishes) with RVC
7. Change media next day without RVC
8. Expand and freeze the cells for storage in LN2.

Sorting Protocol

11 1. Aspirate the medium, wash with 2mL DPBS twice

2. Add mL Trypsin or 0.5mM EDTA to the dishes and incubate to lift the cells

A. for iPSCs lift the cells as single cells

3. Add 2ml complete medium to stop trypsinization, and pipette up and down to collect all cells

3. Transfer all cell suspension into a 15ml conical tube, spin down to get the cell pellet 200g for 4min

4. Resuspend each cell pellet in 1ml sorting medium (Add 2% (vol/vol) KnockOut serum replacement to 50 ml of DPBS. Can be stored at 4 °C for 6 weeks.)

To make 50 mL add 1mL of KnockOut serum into 49mL DPBS

5. Prime the cell strainer with 2mL of sorting medium making sure to cover the entire mesh.

6. Discard the sorting medium in the 50mL tube

7. Apply each cell suspension to the center of a cell strainer (pushing through with pipette where necessary, and – with a new tip – pulling off strained cell suspension stuck to the bottom of filter).

8. After straining the cell suspension, add about 50µL of sorting medium to wash the strainer for any left-over cells.

9. Aliquot cell suspension into sorting tubes and put it on ice.

10. Add DAPI (diluted 1:10,000 to make final concentration at 0.1ug/ml) to the strained cell suspension. This helps to distinguish live from dead cells.

0.1µL per 1mL

12 To prep for FACS: for each condition,

•Take 2 culture tubes with 1 mL of growth medium to catch the sorted cells

•Take 3ml extra sorting medium (in case they ask us to dilute the sample) put everything on ice to take to the FACS facility

13 **Sorting Parameters:**

1. Go to the FACS facility at 149, 5th floor, and ring the bell to be let in. Sorting parameters:

(i) Use nozzle 1 (100um)
(ii) mCherry detection (blue channel; ex: 587nm; em: 610)
(iii) Just collect mCherry-positive cells; give them the sushi medium-containing tubes to collect cells
(iv) Tell them you want to try to get >200,000 cells per condition where possible but prioritize getting through as many samples as possible.
(v) Can keep cold while sorting, or sort at RT (either is fine).
(vi) Can let them know how inclusive/restrictive to be when making gates. Threshold parameters include:

- sorting for singlets (cells on diagonal); doublets usually indicate 2 cells stuck together
- getting rid of particles that are likely debris.
- selecting the mCherry intensity threshold