

Lentivirus Protocol:

DAY 1

CELL GROWTH:

HEK293T (from CORE-T. Low passage, high viability cells. Cells must be very healthy for good transfection and virus production)

DMEM+10% FBS

150cm² flask

1) Grow HEK293T cells in DMEM+10% serum. Thaw cells, wash off media, add ~10-15 million cells to 20mL to 150mm² flask

DAY 2

Trypsin

PBS

DMEM+10% FBS

100x20mm plates

1) Plate 2-3 million cells in 10mL DMEM+10% serum per 100mm petri dish and grow overnight. Transfect at 60-70% confluency. 2 dishes per transfection.

----To remove cells from 150cm² flask: Aspirate + discard media, wash in 8-10mL PBS, aspirate + discard PBS, add 2mL Trypsin (just enough to cover the cells), incubate 2-3 minutes, knock cells loose. Add 10mL 10% DMEM (to a concentration of ~2million cells/mL) which also deactivates Trypsin. Plate cells or freeze down at 10-15 million cells/2mL. Final volume in 150cm² flask is 20mL

DAY 3

TRANSFECTION:

H₂O (autoclaved and .22 filter sterilized)

20 µg of DNA per transfection

-3 µg Rev, 5 µg gp, 1.5 µg VSV G make up 9.5 µg, the rest is transfer vector

2M CaCl₂

2xHBS

50mM Chloroquine

1) Check the confluency of the cells. 80-90%.

2) Prepare DNA for transfection, ALL REAGENTS AT ROOM TEMPERATURE

Rev	3µg	1.8µg/µL	1.6µL	(totals are for Abhik's DNA)
gp	5µg	2.3µg/µL	2.17µL	
VSV G	1.5µg	.5µg/µL	3µL	
Transfer vector	10.5µg			
TOTAL	20µg		6.77µL	

Make up the volume with H₂O (autoclaved, .22 µm filtered sterilized) up to a final volume of 438 µL

Add 62 µL 2M CaCl₂

Add 500 µL 2xHBS slowly, mix by bubbling from the bottom of the tube.

Incubate in the hood at room temperature for 30 minutes, the DNA should precipitate

3) 5 minutes prior to transfection, Add 6 µL of 50mM Chloroquine into the 10mL of plated media (to a concentration of 25µM). Don't leave Chloroquine on the cells for more than 24 hours due to cytotoxicity.

4) Pipette up and down and carefully add (don't add too quickly or you'll wash the cells off the plate!) the 1mL of precipitated DNA to cells and incubate overnight **in 3% CO₂ incubator** (10-12 up to 24 hours from adding chloroquine. 24 Hours has been done and is fine. I did 22 and it seemed a little bit long...).

DAY 4

DMEM+5% FBS

1M HEPES

0.5M NaBu

- 1) Warm 10mL per dish of DMEM + 5% FBS + 10mM HEPES + 10mM Sodium Butyrate to 37°C. [for me, 39mL of DMEM + 5% FBS + 800 µL 0.5M Sodium Butyrate + 400 µL of .22µm filtered 1M HEPES]
- 2) Check cells under fluorescent microscope for %transfection. Take pictures.
- 3) Aspirate/discard the transfection media
- 4) Add 10mL of the warmed media to each 100mm² plate. Use the 25mL stripette, it's end is wider so you're less likely to wash the cells off.
- 5) Incubate 6-10 hours (in 10% CO₂ if possible). Don't go over 10 hours because Sodium Butyrate is toxic to cells
- 6) Aspirate/discard the media, add 10mL of DMEM + 5% FBS + 10mM HEPES [39.6mL 5%DMEM + 400µL of .22µm filtered 1M HEPES]
- 7) 12 hours or so later (at convenience), take 8 mL of media, filter through .45µm filter and store in 4°C ice bucket. Add 8-8.25mL of DMEM + 5% FBS + 10mM HEPES [39.6mL 5%DMEM + 400µL of .22µm filtered 1M HEPES]

DAY 5

DMEM+5% FBS
1M HEPES

- 1) 12 hours or so later, Aspirate 8mL of media, filter through .45µm filter and store in 4°C ice bucket. Add 8-8.25mL of DMEM + 5% FBS + 10mM HEPES [39.6mL 5%DMEM + 400µL of .22µm filtered 1M HEPES]
- 2) repeat 12 hours or so later (at convenience)
- 3) Expose ultracentrifuge tubes and holders to UV for ~1 hour.

DAY 6

TRANSDUCTION

DMEM+5% FBS
1M HEPES

17mL Ultracentrifuge tubes

Cells to be infected, conditioned media and fresh media (RAMOS and RPMI + 7% FBS)

8mg/mL polybrene

6-well plate

- 1) 12 hours or so later, Aspirate 8mL of media, filter through .45µm filter and store in 4°C ice bucket. Add 8-8.25mL of DMEM + 5% FBS + 10mM HEPES [39.6mL 5%DMEM + 400µL of .22µm filtered 1M HEPES]
- 2) Fill 17mL ultracentrifuge tube with 16mL of the media and spin at 70,000 x g average RCF (23,500 rpm at 4°C) for 2.5-3 hours. Repeat until all media is spun down into a single tube. 2 spins per induced plate (8mL*4 collections/16mL=2 spins). Between spins, aspirate/discard media carefully by tilting the tube and not touching the bottom of the tube with the Pasteur pipette.
- 3) Resuspend in 1mL total of 37°C RPMI, let sit in the hood for 30 minutes to dissolve the virus pellet.
- 4) Add 5µL of 8mg/mL polybrene to 1,000,000 cells in 1mL conditioned media in one well of a 6-well plate. One protocol says that 8µg/mL final concentration of polybrene. We use 20µg/mL final concentration, 2.5 times that amount.
- 5) Pipette up and down (no bubbles!) and add the 1mL of virus to transduce

DAY 7

Conditioned and fresh media for the cells to be infected (in my case RPMI + 7% FBS)

- 1) After 24 hours, change media. 1 mL new media, 1mL conditioned media.

DAY 8

- 1) Check transduction efficiency ~12 hours later

Luciferase activity assay: lysed over 30 minutes, use luciferase assay plates (BioVision luciferase reporter assay kit)

RECIPIES

0.5M Na-But stock solution: 1.0g Butyric acid sodium salt in 16.1mL of ddH₂O. Adjust pH to 7.0 and volume to 18.1 and .22μm filter sterilize

2x HBS: 0.8g NaCl, 0.04g Na₂HPO₄•7H₂O, 1.2g HEPES in 90mL H₂O. Adjust pH to 7.050 and adjust volume to 100mL. .22μm filter and store in 5mL aliquots up to a year at -20°C

2M CaCl: 5.9g of CaCl₂•2H₂O in 20mL H₂O, .22μm filter sterilize, store in -20°C

50mM Chloroquine: 52mg of chloroquine diphosphate in 2mL of H₂O, .22μm filter sterilize, store in foil-wrapped at -20°C

Polybrene: 800μg/mL polybrene in distilled water, filter sterilized, store at -20°C

1M HEPES: 11.9g of HEPES in 50mL H₂O, filter in .22μm, wrap in aluminum foil to protect from light.