Lentivirus Production

Material

- PEI stock solution (1 µg/µl):
    1. Dissolve Polyethyleneimine (Polysciences PN 23966) by swirling using de-ionized H_2O heated to 80°C and using ~90% of volume required to reach a final concentration of 1 g/l (1 µg/µl).
    2. Cool to room temperature.
    3. Set pH to 7.0 by adding HCl.
    4. Add de-ionized H2O to reach final concentration of 1 µg/µl.
    5. Filter using a 0.22 µm membrane and divide into aliquots. Store in -20°C freezer.
    6. Thaw in 37 °C water bath until precipitates have fully dissolved.
    7. Store thawed aliquot in fridge (4°C)
    8. Re-dissolve precipitates by incubating at 37 °C before use if needed.
- 15 ml and 50ml falcons
- T_{175} Cell culture Flasks
- Complete DMEM (DMEM with Glutamax + 10%FBS + Penicillin (100U/ml) + Streptomycin (100ug/ml))
- DNeasy blood and tissue kit (Qiagen AB, Sollentuna, Sweden)
- Plasmids;
    1. transfer plasmid,
    2. pMD2G (envelope)
    3. pRSV (REV)
    4. pMDL (GAG/POL)

Day -4 or-3

- Make media, warm in water bath
- Seed cells on Thursday (0.9 x 10^6) or Friday (1.8 x 10^6) before (25 ml media). This will yield approx. 20 million cells per T175. Seed 8-9 flasks for a 6 virus batch.
- Wash with 10ml PBS (on the roof, then submerge carefully)
- 2ml trypsin, 5 min in incubator, hit on the side
- 10ml media, spin (400g, 5 min)
- resuspend in 5mls, count, (10ul Trypan Blue + 80ul PBS +10ul cell susp, i.e. 1:10 dil)
- add cells to flasks, plus 10-20ml media

Day 0: Seeding of cells (Approximately 24 hours before transfection)

1. Warm media in water bath
2. Wash with 10ml PBS (on the roof, then submerge carefully)
3. 2ml trypsin, 5 min in incubator, hit on the side
4. Add 5-7 ml medium to each flask to neutralize trypsin.
5. Rinse flasks and transfer cells to a (or two) 50 ml tube.

6. Spin down at 400 x g, for 5 min at 20°C. (NOTE: Pressing start saves settings)
7. Resuspend cells in appropriate volume (about 20mls).
8. Count
9. Add 20 ml media to each flask (DMEM Glutamax + 10 % FBS + Penicillin 100 U/ml + Streptomycin 100 U/ml)
10. Plate 12.5 million cells per T175 flask. Need 12 for 6 virus batch.
11. Add the calculated cell suspension to the flask with the media.
    - Note: To keep cells going, put out 1.5 million cells in a T175. These can go until Thursday

PLASMID	AMOUNT NEEDED PER BATCH
pMD2G (envelope)	5,5ug
pRSV (pack. 1, REV)	3,9ug
pMDL/RRE(pack 2, GAG/POL)	7,5ug

TRANSFER PLASMID SIZE	AMOUNT OF PLASMID	PEI
8 KB	17 ug	102 ul
9 KB	19 ug	108 ul
10 KB	21 ug	114 ul
11 KB	23 ug	120 ul
12 KB	25 ug	126 ul
13 KB	27 ug	132 ul
14 KB	29 ug	138 ul
15 KB	31 ug	144 ul

Day 1: Transfection of cells

1. Check to see how confluent cells are (should preferably be 75 – 90 %).
2. Make sure you have all calculations done prior to starting, including the volume of PEI to be used (102 – 132 ul)
3. Mix packaging vectors (pMDL, psRev and pMD2G) and Transfer vector in a 50 ml tube with serum free media (DMEM+P/S) or PBS, so that the total volume (including PEI) becomes 3.6 ml.
4. Add PEI and mix by vortexing for a few seconds.
5. Incubate the DNA/PEI mix at RT for 15 min.
6. Change media in flasks and add 16.2ml fresh complete media.
7. Add 1.8 ml transfection mix to each T175 flask, mix gently over cells by tilting the flask.

Day 3: Harvest (45 hours after transfection)

NOTE: Start using Virkon and double gloves. Rinse everything in Virkon when used (flasks, pipettes etc.). When finished, turn off incubator and turn on UV.

1. Check the cells under the microscope.
2. Collect media into 50 ml tubes.
3. Spin the supernatant at 800 x g 10 min +4°C
4. While tubes are in the centrifuge, get together in hood the requirements for the next step. Also, rinse the now empty culture flasks with Virkon and discard.

    - Beckman centrifuge tubes (Label!)
    - Spare tube if required to balance
    - 45 µm filters
    - 50 ml syringes
    - Centrifuge canisters
    - Beaker of Virkon

5. Remove 50 ml tubes from centrifuge, and in the hood, pour the supernatant into open syringe with attached filter over the labelled Beckman UC tube. After each virus/50ml tube, change the syringe and filter. Ensuring that the discarded syringe and filter are placed and rinsed in Virkon.
6. Put Beckam UC tubes into their chambers using forceps.
7. To avoid collapsing the tubes, fill up sample tubes with media. Fill up counterweight tubes with DPBS.
8. Balance the weight of the tubes carefully, down to 5 ug difference.
9. Clean everything in the hood thoroughly. Any plastics that have been used must be rinsed with Virkon before discarding. Spray pipettes, racks, pipette gun etc. with ethanol. Clean hood surfaces with Virkon first, then with ethanol.
10. Turn off the hood fully and then put the UV on.

Ultracentrifuge:

11. Put down rotor, spin it (you don't have to screw).
12. Put down <u>all 6 chambers</u> into centrifuge (you do not have to weigh the empty ones).
13. Check settings of centrifuge:

19500G 2.00hrs 4°C Enter to save
Ensure Acceleration is set to max
Ensure Deceleration is set to max

14. Wait until centrifuge reaches full speed before leaving.
15. Set alarm for when centrifugation is done, don't let the tubes stand longer.
16. Deceleration of centrifuge takes approx. 5 min. After it reaches 3000G, you can release the vacuum to increase the speed of deceleration.
17. Take out Beckman UC tubes using forceps, and pour out liquid into Virkon.
18. Place tuned upside down on paper to dry, be careful to remove all liquid, also from edges and walls.

19. Take a bottle of cold PBS form freezer and resuspend pellet in 80 ul (75 – 100 ul is ok, some people pipette up and down)
20. Seal tubes with Parafilm, vortex and put into fridge, leave them there for at least 2 hours – but preferably overnight.
22. Aliquot virus (10ul/tube) and store -80°C.