**Small scale Lentivirus Production and Infection**

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Rationale: This protocol can be used for production and transduction of lentiviral sgRNA, shRNA and protein overexpression in conjunction with generation 2 and generation 3 lentivirus plasmids.

**Materials:**

BSL-2+ facility cell culture lab

Addgene plasmids - PsPax2 (#12260), VSV-G (#12259), lentiviral vector

Polyethyleneimine (PEI, Polysciences) 1mg/ml stock

HEK 293T cells

Polyethylene glycol (PEG) 8000

Polybrene (10mg/ml)

4X lentivirus concentrator solution

Ultracentrifuge and compatible tubes

**Make lentivirus**

Plate 293T cells at 40% confluency in a 6 well tissue plate submerged under 2ml medium per well

After 6h, most cells will have attached

**Day 0**

Prepare DNA mix for transfection:

Add the following to 100µl Optimem per well for transfection:

|  |  |
| --- | --- |
| 1µg | PsPAX2 1µg helper plasmid (Addgene ##12260) |
| 0.5µg | VSV-G / pMD2.g (Addgene #12259) |
| 1µg | Lentivirus vector (see below) |

Add PEI (from a 1mg/ml stock) to this mixture solution at ratio 5:1 w/w (PEI:DNA).

Example, 12.5µg PEI for 2.5µg DNA mix

Mix DNA mix gently and incubate for 20 min at RT

Add the mix to the cells dropwise

**Day 1 (16 hours later)**

Check for cell viability; at this time, >70% of the cells should be transfected and virus is already being produced and is being released into the supernatant

**Day 2**

48h after transfection, collect the culture supernatant in a BSL-2+ facility; centrifuge in an enclosed rotor and remove supernatant with care. This is “Day-2 virus”.

Carefully add an additional 2ml complete DMEM medium into each well without splashing or disturbing the monolayer

Bleach all tips and pipettes used to collect the virus

**Day 3**

72h after transfection, collect the culture supernatant in BSL-2+ facility as before. This is “Day-3 virus.” Day-2 and Day-3 virus are then pooled; Day-2 titre is lower than Day-3.

The pooled virus (~4ml) is transferred into a 15ml tube and centrifuged at 250Xg for 5 min

* The pellet represents cell debris as well as 293T cells that can contaminate the target cell line to be infected with the virus; care should be taken when aspirating the virus supernatant. Filtration can decrease viral titre and is not required.

Prepare 0.5ml aliquots of the lentivirus and freeze at -80°C

**Lentivirus Infection**

Thaw a 0.5ml virus aliquot in a 37°C water bath, flicking tube gently to facilitate gentle thaw.

Add 1µl, 10mg/ml Polybrene

Transfer virus mixture to the medium covering 1 well of a 6 well plate containing the target cell line. Polybrene will become diluted in the cell medium to a final concentration of 4µg/ml.

48hr postinfection, cells are ready for analysis or selection.

**Concentrating the virus**

Rationale: To achieve 100% infection and/or if you have low titers or do not care about precise multiplicity of infection, it is beneficial to concentrate the lentivirus.

**4×Lentivirus Concentrator Solution**

Dissolve 80g PEG-8000 and 14.0g NaCl in 80ml MilliQ water; add 20ml, 10X PBS (pH7.4). Mix with gentle stirring, heating gently only if necessary, until the solids are dissolved then adjust pH to 7.0~7.2; adjust the final volume to 200ml. Sterilize by passage through a 0.2μM filter. The concentrations of PEG-8000 and NaCl in the stock solution are 40% (w/v) and 1.2M, respectively. Store at 4°C.

**Virus concentration protocol**

* Carefully transfer the virus supernatant into a new 50 ml tube
* Add 1 volume of concentrator solution to 3 volumes of virus supernatant (eg. 1ml concentrator solution for 3ml virus)
* Mix by gentle shaking for ~20 sec then incubate with constant rocking at least 4 hours at 4°C
  + Overnight rotation or rocking will enhance recovery
* Spin down at 1600Xg for 60 min at 4°C
* Carefully remove supernatant without disturbing the pellet
  + ​​Pellet size does not necessarily correlate with virus yield
* Thoroughly resuspend the viral pellet in PBS or desired medium using 1/10~1/20 of the original volume by gentle pipetting using a 1ml Pipetman
* Aliquot and store at -80°C until use

**Alternative Centrifugation- based Virus concentration method**:

In case of low transduction efficiency, consider ultracentrifugation as follows:

1. 72h after transfection, collect the virus containing supernatant in a BSL-2+ facility (take only Day 3 supernatant)
2. Spin down at 250Xg for 5 min at RT
3. Transfer the precleared supernatant to ultracentrifuge tubes and pellet at 90,000Xg for 90 minutes at 4°C
4. Remove the supernatant and leave a little less than 1 mL in the tube. Use a 1 mL pipette to recover the remaining pellet which may be difficult to see.
5. Make aliquots of 0.2 mL concentrated virus and freeze at -80˚C.