Amy Shore/Heather LaMarca 3/08

Lentiviral Propagation

DAY 0:

Plate 293T cells.

- Plate 1.2×10^7 cells/plate in a volume of 25ml/plate in 10%FBS/DMEM
- Seed cells on 6 x 15cm plates.

Incubate at 37°C overnight.

DAY 1:

Transfect 293T cells.

- Three hours prior to transfections, remove media and replace with 25 ml of fresh 10%FBS/DMEM (no antibiotics).

  Cells should be ~60-70% confluent at the time of transfection.

[A.] Prepare Fugene:DNA complex

1. Add the indicated amount of DMEM to a 5ml sterile polypropylene tube.
   Add the indicated amount of Fugene 6 to the media. Do NOT touch the tube with the reagent.
   Gently tap the tube to mix.
   Incubate at RT for 5′.

2. Add the indicated amount of DNA to the Fugene solution.
   Gently tap the tube to mix.

3. Incubate at RT for 45′.

Optimized DNA concentrations:

a) pPRIME vector library
   1733µl DMEM + 54µl Fugene 6 +
   6µg gag/pol + 3µg VSVG + 9µg pPRIME-gene of interest

b) pGIPZ vector library
   1733µl DMEM + 54µl Fugene 6 +
   2.5µg pRC-CMV-RaII + 2.5µg HDM-Tat16 + 2.5µg HDM-HGPM2 +
   2.5µg HDM-VSVG + 8µg pGIPZ-gene of interest

c) pEIZ or pEIT (Welm et al, Cell Stem Cell 2008)
   1713µl DMEM + 72µl Fugene 6 + 4µg pRSV-Rev + 4µg pMDLg-pRRE +
   4µg pCMV-VSVG + 12µg pGIPZ-gene of interest

[B.] Transfect Cells

- Dropwise, add 1800 µl of the Fugene:DNA complex to each plate containing 25ml of 10%FBS/DMEM, evenly distributing it around the well. (use a micropippetor)
- Swirl the wells to ensure even dispersal.

Incubate the cells for 24 h at 37°C.

DAY 2:

- Remove media containing Fugene and replace with 20ml of fresh 10%FBS/DMEM.
- Check fluorescence on microscope for GFP expression.

DAY 3:

- Collect virus-containing supernatant from each plate and transfer to a 50ml conical tube. Store virus at 4°C till ready to ultracentrifuge.
- Add 20ml of fresh 10%FBS/DMEM to each plate.
- Check fluorescence on microscope for GFP expression 48 h post-transfection.

DAY 4:

- Collect virus-containing supernatant from each plate and transfer to a 50ml conical tube. Store virus at 4°C till ready to ultracentrifuge.
- Check fluorescence on microscope for GFP expression 72 h post-transfection.

Note: Virus can be stored for up to 1 week at 4°C without affecting the titer.

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Ultracentrifugation of Lentivirus

Concentrate LV by ultracentrifugation.

Ultracentrifuge: Beckman LE 80K
Rotor: SW28
Tubes: #326823 autoclaved (polyallomer)

1. Pool all viral media into and filter through a 0.45µm HV Durapore membrane (Millipore #SCHVU01RE).

2. Place tubes into rotor holders and place on a scale.
   Slowly add ~37ml of virus/media to each tube and balance. (One 15cm plate should correspond to one ultracentrifuge tube).
   Be sure the tubes are filled to about 1-3mm from the tube, or the tube will collapse while spinning.

3. Cap holders with a dab of grease and connect to the rotor.

4. Spin at 25,000 rpm (~82,000 × g) for 1 hour 45 min at 4°C. (It is not necessary to prechill the rotor.)

5. Carefully pour off supernatant and invert tube on kimwipes to dry (5-10′ at RT).
   There should be a visible pellet.

6. Resuspend each pellet in 125µl of MEC growth media (or growth media for the cells you will be transducing).
   Incubate at 4°C O/N to allow pellets to fully resuspend.

7. Combine all virus into 1 tube.
   Dispense into 25µl aliquots and freeze at -80°C.

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Lentivirus Titering

DAY 0:

Plate 293T cells.

- Plate 7.0×10^4 cells/well in a volume of 500µl/well in 10%FBS/DMEM on a 24-well plate.
  - 1 well → count cells prior to transduction
  - 1 well → untransduced control
  - 6 wells for each virus → serial dilutions

Incubate at 37°C overnight.

DAY 1:

Transduce 293T cells.
Cells should be ~60% confluent at the time of transduction.

1. Count the number of cells in 1 well.

2. Make 10-fold serial dilutions ranging from 10^{-2} to 10^{-7}. (Depending on the concentration of your virus, you may need to expand this range, ie: 10^{-2} to 10^{-12}.)
   Dilute the virus in 10%FBS/DMEM:
   2.5µl stock virus + 247.5µl media → 10^{-2}
   25µl 10^{-2} virus + 225µl media → 10^{-3}
   25µl 10^{-3} virus + 225µl media → 10^{-4}
   25µl 10^{-4} virus + 225µl media → 10^{-5}
   25µl 10^{-5} virus + 225µl media → 10^{-6}
   25µl 10^{-6} virus + 225µl media → 10^{-7}

3. Aspirate media from 293T cells.
   Add 200µl of diluted virus to each corresponding well. Be sure to leave a well untransduced.
   Incubate cells at 37°C O/N.

DAY 2: 24 hours post-transduction

4. Aspirate media containing virus and replace with fresh10%FBS/DMEM.
   Incubate cells at 37°C for an additional 48 hours.

DAY 4: 72 hours post-transduction

5. Visualize GFP expression under a microscope. GFP expression should range from 100% and 0%.
   Pick the wells that have about 5-20% GFP+ cells for titering by flow cytometry.
   - trypsinize cells, resuspend in 300µl of HBSS+, filter and analyze

6. Calculate titer: use numbers from samples that were between 5-20% GFP+ to ensure that you are within the linear range.

$$T = (P \times N)/(D \times V)$$

T = titer (TU/ml)
P = % GFP+ cells (ie: N = 0.2 for 20% GFP+ cells)
N = number of cells at the time of transduction
D = dilution factor (ie: $10^{-3} = 0.001$)
V = volume of viral inoculum (0.2 ml)

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Lentiviral Transduction of Primary MECs

Lentiviral transductions are performed on freshly isolated primary mammary epithelial cells that have been dissociated into single cells. MECs may also be transduced with lentivirus following a hour adenovirus transduction (such as Ad.Cre).

- **Transduce cells in ultra low-attachment 24-well plates (Corning #3473).**
- **Transduce up to 2×10^6 cells in 800µl of media per well.**
- **Dilute the virus in MEC growth media.**

1. Quick thaw virus at 37°C.

2. Transduce with LV at an MOI of 5-10**.**
   - Add the indicated amount of virus to a total inoculum volume of 800µl.
   - Resuspend cells in 800µl of virus (diluted in growth media).
   - Transfer cells/virus to 1 well of a low attachment 24-well plate.

3. Incubate cells with virus for overnight at 37°C.

4. Check GFP expression for efficient viral transduction. About 20-30% of the cells should be GFP+.

5. Transfer cells/virus to a 15ml conical tube. Bring the volume up to 15ml with wash buffer.
   Centrifuge at 300 × g for 5′ at RT to remove any unbound virus.

6. Wash cells with 15ml of wash buffer.
   Centrifuge at 300 × g for 5′ at RT.
   Resuspend each pellet in the appropriate media.

<u>Note</u>: After the overnight incubation, cells resemble organoids, as they like to clump together. GFP+ cells are usually found in large clumps rather than single cells.

<u>To calculate how much virus you need</u>:

(Number of Cells)×(MOI)/Titer (pfu/ml) = ____ ml of virus