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Protocol: Large-scale lentivirus production of Cas9, shRNA, sgRNA and ORF clones

This protocol describes production of lentivirus stocks from Cas9, pLKO (shRNA), pXPR (sgRNA) or pLX (ORF) plasmids in T175 flasks.

Materials and reagents needed:

- 293T cells
- Cell maintenance media: DMEM + 10% hiFBS
  - Dulbecco's Modification of Eagle's Medium (Mediatech Cat. #10-013-CV)
  - Heat-Inactivated Fetal Bovine Serum (Sigma Cat. #F2442)
- PBS and Trypsin
- T175 flasks (VWR #29185-308)
- Cell counter
- Pipettes
- Sterile microcentrifuge tubes and 15, 50 and 250 mL conicals
- 500 mL or 1 L sterile bottles (VWR #82051-492, #82051-594)
- Reduced serum medium Opti-MEM (Thermo Fisher Cat. #31985070)
- Packaging Plasmids:
  - Available from gpp-reagents@broadinstitute.org, or
  - Order from Addgene (VSV-G plasmid #12259, psPAX2 plasmid #12260) then amplify with GenScript
- Plasmid DNA
- Viral harvest media: DMEM + 10% FBS + 1% BSA + 1% Penn/Strep
  - Dulbecco's Modification of Eagle's Medium (Mediatech Cat. #10-013-CV)
  - Fetal Bovine Serum (Sigma Cat. #F4135)
  - Microbiology-grade Bovine Serum Albumin (VWR Cat. #14230-738)
  - Penicillin Strepomycin (VWR Cat. #45000-652)
- LT-1 transfection reagent (Mirus Cat. #MIR 2305)

Part 1: 293T cell expansion (1 – 2 weeks before transfection)

A: Maintain a stock of 293T cells:

- Doubling time is ~21 hours
- Visually inspect cell confluency under the microscope to ensure that it

always remains below 80%. If ever over 80-90%, thaw a new vial.

B: Split cells on Mondays, Wednesdays and Fridays:

- Seed in 40 mL of media per T175:
    - 5E6 cells per T175 on Monday and Wednesday
    - 2.5E6 cells per T175 on Friday

C: Seed 293Ts the Friday before transfection:

1. Calculate the number of T175s needed for the following week's transfection: the anticipated volume harvested from one T175 is 60 mL for high-titer vectors (i.e. pLKO, pXPR_003, pLX304) and 40 mL for low-titer vectors (i.e. Cas9, pXPR_023, pLX317).
2. Seed half the number of T175s needed for the following week's transfection, (i.e. 10 T175s for a 20-flask transfection) with 2.5E6 cells in 40 mL of media per T175.

Part 2: Seeding Cells for Transfection

D: Seed 293Ts (Monday 1 PM):

1. Work in small batches (3-4 T175s) so that cells do not get over-trypsinized.
2. Aspirate media, then gently wash with 5-10 mL PBS per T175 and aspirate PBS. If the cells are starting to lift off without trypsinization, the PBS wash will appear cloudy → do not use for virus production.
3. Add 3 mL trypsin per T175 and rock to cover the surface. Allow to trypsinize in the hood for less than 2 min.
4. Quench trypsin with 5 mL of media per T175 and collect cell suspension into a conical.
5. Wash each T175 with 6-8 mL media and collect wash in same conical.
6. Thoroughly mix cell suspension and count.
7. Seed 18E6 cells in 25 mL media per T175 + 2 more T175s than needed, i.e. 22 T175s for a 20-flask transfection.

Part 3: Transfection

E: Prepare reagents (Tuesday AM):

1. Check a T175 for confluency, they should be around 60%.
2. Warm Opti-MEM in water bath, take LT-1 out of 4C and allow to come to room temp, and thaw out packaging plasmids and plasmid DNA.
3. Calculate the amounts of plasmids needed for the transfection + 1 T175 extra (see table below):

Reagent	Amount needed per T175	i.e. for 20-flask (+1 extra) transfection
pDNA	40 ug	840 ug
psPAX2	50 ug	1050 ug
VSV-G	5 ug	105 ug
OptiMEM	50 uL	1050 uL

4. Make a master mix of all four reagents.
5. Divide the master mix total volume by the number of transfection T175s to calculate the volume per flask. Aliquot this volume into sterile microcentrifuge tubes, one for each T175.
6. Aliquot 6 mL of Opti-MEM into a 15 mL conical, one for each T175.

F: Perform transfection(s) (Tuesday AM):

1. Gently pipette 305 uL LT-1 into the first Opti-MEM conical. Carefully invert the conical 5 – 7 times, avoiding bubbles. Let the mixture sit for 3 minutes.
2. Gently pipette the plasmid mix into the LT-1+Opti-MEM conical. Carefully invert the tube 5 – 7 times, avoiding bubbles. Let sit at room temperature for 27-30 minutes.
3. Subsequent transfection conicals can be processed with 3-min intervals to optimize time (see "Quick Virus Production" flow chart below).
4. Change the pipette-aid to slow release.
5. After 27-30 min, gently take out a T175 from the incubator.
6. Slowly drip the plasmid+LT-1+Opti-MEM mixture in a zig-zag or circular motion over the surface of the cells. Make sure to cover as much surface area as possible.
7. Carefully place the T175 back into the incubator without moving the media too much.
8. Note time of transfection completion in order to change the media after 6-8 hrs.

G: Change media (Tuesday PM):

1. 6-8 hours post-transfection, carefully aspirate the media.
2. Gently replace with viral harvest media by pipetting down the top side (not on the cells) of the T175 with a slow dispense speed. Use 60 mL of harvest media per T175 for a high-titer vector and 40 mL of harvest media per T175 for low-titer vector.
3. Gently move the T175 back to incubator. Note the time of the media change.

Part 4: Harvesting and Aliquoting Virus

H: Collect virus 36 hours post-transfection (Thursday AM):

1. Choose appropriately sized aliquots to avoid freeze/thawing the virus and label the tubes.
2. Carefully pipette viral supernatant from each T175 into a 250 mL conical, splitting across multiple conicals if necessary.
3. Spin at 230 g for 1 min to pellet 293T cell debris.
4. Avoiding the cell pellet, pipette off viral supernatant into a new 250 mL conical, or a 500 mL or 1 L sterile bottle. Only fill bottle(s) up to 75%.
5. If multiple bottles are needed, mix the virus by pouring from one bottle to the next, until homogeneous.
6. Aliquot virus into labeled tubes.
7. Freeze at -80°C.
8. Perform standard titration (see **Protocol: Relative Viral Titering with an AlamarBlue Cell Viability Assay**).

Quick Virus Production

Add 305 uL LT-1 to 6 mL Opti-MEM already in 15 mL tube, mix gently by inverting 5-7 times.
Wait 3 min.

Add plasmid mix to LT-1+Opti-MEM tube, mix gently by inverting 5-7 times.
At same time add 305 uL LT-1 to next tube of Opti-MEM.
Wait 3 min

Repeat until all plasmid/LT-1/Opti-MEM mixtures have been made.
Wait 27 min from the addition of the first plasmid mix.

Add first tube of plasmid/LT-1/Opti-MEM mixture over surface of cells dropwise while holding flask horizontally. Be careful not to disrupt cells!
Wait 3 min

Continue adding plasmid/LT-1/Opti-MEM mixture to all flasks.
Wait 6-8 hours.

Change media on cells to viral media.
Wait 36 hours.

Harvest and aliquot.