BROAD INSTITUTE | GENETIC PERTURBATION PLATFORM

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

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

Material needed:

-293T cells
-Plasmid DNA
-Packaging Plasmids: order from Addgene (VSV-G plasmid # 12259, psPAX2 plasmid # 12260) then amplify with GenScript.
-Reduced serum medium Opti-MEM (Thermo Fisher Cat.# 31985070)
-Cell maintenance/seeding media: DMEM + 10% iFBS
    Dulbecco's Modification of Eagle's Medium; Mediatech Cat.# 10-013-CV
    Heat-Inactivated Fetal Bovine Serum; Sigma Cat.# F2442
-Virus Production Media: DMEM +10% FBS + 1% BSA
    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 +P/S
-LT-1 transfection reagent (Mirus Cat.# MIR 2305)

293T cell expansion: 1 – 2 weeks before transfection:

1. Maintain a stock of 293T cells, doubling time is ≈21 hours, cells must remain below 80% confluent.
2. Split cells on Mondays, Wednesdays and Fridays. Re-seed 5E6 cells per T175 on Monday and Wednesday, 2.5E6 cells per T175 on Friday in 40 ml of media/flask.
3. Visually inspect cell confluency under the microscope to insure that it remains below 80%. If over 80-90%, thaw a new vial.

Friday before transfection:

1. Calculate the number of flasks needed, the anticipated volume harvested from one flask is 60ml for high-titer viruses (pLKO, pXPR_003, pLX304) and 40ml for low-titer viruses (pXPR_023, pLX317).
2. Seed 2.5E6 cells/T175 in 40mL media at 1:00 PM. Seed 20 – 25 flasks for a 40-flask infection.

Day before transfection (Monday):

1. Gently remove media and wash with 5-10 ml PBS. If the cells are starting to lift without trypsinization, the PBS wash will appear cloudy, do not use for virus production. Add 3 ml trypsin per T175 and rock to cover the surface, trypsinize in the hood for less than 2 min. Work in small batches (3-4 flasks) so that cells do not get over-trypsinized.
2. At 1pm, seed 18E6 cells in 25 mL media in 2 more flasks than needed.

Day of transfection (Tuesday):

1. Check T175 for confluency, they should be around 60%. Warm Opti-MEM, LT-1, and thaw out plasmids.

2. Calculate amount of plasmids needed for transfection. Make a mix of plasmids for # target flasks +1

Reagent	Amount needed per T175
pDNA	40 ug
psPAX2	50 ug
VSV-G	5 ug
OptiMEM	50 uL

1. Aliquot a separate tube of DNA for each T175.
2. Aliquot 6 mL OPTI-MEM into 15 ml falcon tube for the number of flasks to be transfected.
3. Add 305 uL LT-1 into the first Opti-MEM aliquot. Invert the tube 5 – 7 times carefully to avoid bubbles. Let the mixture sit for 3 minutes.
4. Add the DNA plasmid mix to the first Opti-MEM mixture, invert 5 – 7 times carefully to avoid bubbles. Let sit at room temperature for 30 minutes.
5. Subsequent transfection tubes can be processed with 3-min intervals to optimize time (see “Quick Virus Production” flow chart).
6. Take out a flask of 293T cells from the incubator. Pipette the Opti-MEM mixture and carefully drip onto the cells, making sure to cover as much of the surface of the flask as possible.

7. It is helpful to put the pipet-aid on slow release and to drip in a zig-zag or circular motion over the surface of the cells.
8. Carefully place the flask back into the incubator without moving the media too much.
9. Note time of transfection completion in order to change the media change after 6-8 hrs.
10. Six to eight hours later, carefully aspirate transfection media and gently replace with virus production media by pipetting down top-side (no cells) with slow pipette-aid dispense speed. Use 60 ml of media per T175 for high-titer virus, and 40 ml of media for low titer virus. Gently move flask back to incubator. Note time of media change.

Day of collection and aliquoting (early AM Thursday):

Collect virus 36 hours post-addition of virus production media (8 – 9 AM).

1. Label storage boxes and tubes (example 1.25, 5, 10, 25 mL) with virus information.
2. Carefully remove supernatant from each T175 and pool into multiple 250 mL conical bottles.
3. Spin at 1000 RPM for 1 min to pellet cell debris.
4. Pipette supernatant into 1L sterile bottles. Fill at 75%. If multiple bottles, mix bottles by pouring from one to the next several times until homogeneous.
5. Aliquot into tubes.
6. Freeze at -80°C.
7. Perform standard titration (see Viral tittering protocol with Alamar Blue)

Quick Virus Production

Add 305 uL LT-1 to 6 mL OptiMEM already in 15ml tube, mix gently by inverting 6 times

Wait 3 min

Add DNA mixture to LT-1 mixture, mix gently by inverting 6 times
At same time add LT-1 to next tube of OptiMEM

Wait 3 min

Repeat until all DNA/LT-1 mixtures have been made
Start adding DNA/LT-1 mixture to cells 30 minutes after adding DNA. Add dropwise over surface of cells while holding flask horizontally. Be careful not to disrupt cells!

Wait 3 min

Continue adding DNA/LT-1 mixture to all flasks

Wait 6-8 hours

Change media on cells to viral media

Wait 36 hours

Harvest and aliquot