Producing Lentiviruses

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Validated by / Date:

This protocol describes how to produce lentiviruses using HEK293 cells, and is largely based on the protocol of E. Campeau (Campeau E, et al. A Versatile Viral System for Expression and Depletion of Proteins in Mammalian Cells. PLOS one 2009 4(8): e6529) with modifications based on the protocol published by Addgene (https://www.addgene.org/tools/protocols/plko/#E).

This protocol requires a BSL safety level 2 cabinet because it produces active lentiviruses. Be sure to take adequate PPE precautions when working with the lentiviruses. We use double gloving. All pipets and flasks should be bleached before discarding.

The entire protocol takes about a week so plan accordingly.

Related protocols: Counting Cells; Sterile Technique and Subculture

Materials and reagents required:

- HEK 293 cells (ATCC CRL-1573)
- Full Growth Media (GM) (DMEM,10% FBS (v/v), 2 mM L-Glutamine)
- DMEM (Thermo-Fisher # 11960-044) - 500 mL
- Fetal bovine Serum (*heat inactivated*, Biowest # S162H) - 50 mL
- L-Glutamine 2 mM (Corning # 25-005-CI) - 5 mL
- Opti-MEM (Thermo-Fisher #31985-070)
- Lipofectamine 2000 (LP2K) (Thermo-Fisher # 11668027)
- T-75 tissue culture flask (BD #353110)
- Amicon Ultra-15 filters NMW 100 kD (Millipore # UFC910008)
- Bleach
- 96-well plate (Corning, Inc. 353227)
- 500mL Rapid Flow Filter (Thermo-Fisher 566-0020)
- 0.25% Trypsin, 2.21 mM EDTA in HBSS (Corning 25-053-ci)
- Opti-MEM™ Reduced Serum Medium (Thermo-Fisher 31985070)

Plasmids:

- Packaging plasmid, pPAX (Addgene #12260)
- Envelope plasmid, pCMV-VSV-G (Addgene #8454)
- Transfer plasmid (contains your sequences/genes of interest in a lentiviral backbone)

1. **Day 1- Seed Cells (Afternoon)**
    a. Warm GM (full growth medium, see above), in a 37^{o}C water bath along with 0.25% Trypsin.
    b. See Subculture Protocol to harvest HEK293 cells.
    c. Seed 5X10^{6} HEK293 cells in a T75 flask with 10 mL of GM.
        i. Note: For producing larger amounts of virus, this can be scaled to multiple T75 flasks, or, presumably to a larger T175 flask (although we have not yet tried the latter). Whether a larger production scale is needed is only determined after titer, and experience with the particular transfer plasmid being used.
    d. Let the cells attach overnight.

2. **Day 2-Transfection (Afternoon)**
    a. Warm GM and Opti-MEM in a 37^{o}C water bath. Ensure availability of sterile 1.5 mL centrifuge tubes and micropipette tips.
    b. Calculate the amount of each reagent needed. We use a spreadsheet formatted as below.
    c. Prepare the lipofectamine (LP2K) master mix in a sterile 1.5 mL tube, and incubate for 5 min at RT. While this is incubating, prepare the DNA master mix in a separate 1.5 mL tube.
        i. Remember all transfection components (DNA and lipofectamine dilutions) need to be prepared using Opti-MEM Media.
    d. Add the contents of the DNA master mix drop wise to the lipofectamine master mix and incubate for 30 min at RT.

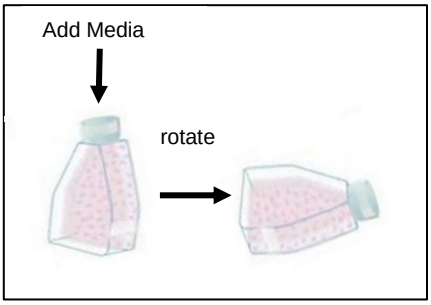
DNA master mix		Amount (ng)	stock conc (ng/uL)	Vol needed
	Plasmid of interest	15 000		
	pPAX	6 000		
	pVSG.G	3 000		
	Opti-MEM (to 750ul)			

Lipofectamine	amount /T75	Num of flasks	Vol needed
LP2K	48		
Opti-MEM to (750ul)	702		

    e. Aspirate the media from the T75 flask and add the contents of your transfection reaction into the flask with a micropipette. Gently rock the flask to disperse the transfection mix across the surface with cells. Let stand for ~ 1 minute.

i. Be gentle; the HEK 293 cells can easily lift during this process.

f. Stand the flask upright so the cap is pointing to the ceiling. Add 10 mL of warm GM slowly to the bottom and gently rotate the flask so the cell monolayer is appropriately positioned.



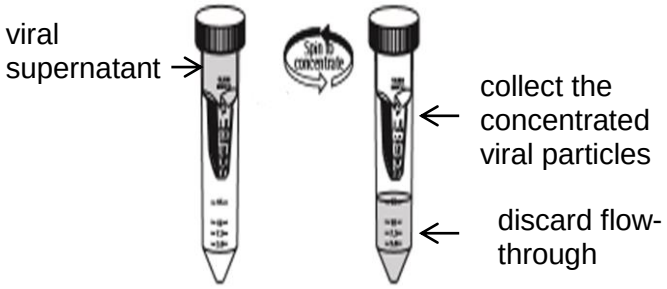
Bottom, Adherent cells

3. **Day 3-Change media (afternoon).**
   a. Warm GM in a 37ºC water bath.
   b. Aspirate the transfection reagent-containing media carefully. This can be done by aspirating the media from the surface of the cell monolayer. The less movement during aspiration the better. (see picture to right)
   c. Gently replace with 10 mL of warm GM.
   d. Return cells to the incubator.

Bottom, Aspirate medium without touching cells

4. **Day 4-Harvest Viral Supernatant (48 hr post-transfection).**
   a. Warm GM in a 37ºC water bath.
   b. Now, the T75 contains active virus particles. Use appropriate PPE.
   c. Carefully transfer the virus-containing GM from the T75 flask into a sterile 50mL conical tube and store at 4°C.
   d. Replace with 10mL of GM carefully as before.
   e. Return cells to the incubator.

5. **Day 5-Harvest and Process Viral Supernatant (72 hr post-transfection).**
   a. Carefully transfer the GM from the T75 flask into the same 50 mL tube as from Step 4.
      i. You can now discard the HEK 293 cell culture appropriately (add ~10 mL of 10% bleach to the flask before discarding).
   b. Centrifuge pooled supernatant at 1000xG for 5 min to pellet any cell debris (RT is fine).
   c. Collect viral supernatant under sterile conditions and transfer into a new sterile 50mL tube. Be careful not to disturb the cell pellet.



   d. Concentrate the viral supernatant using an Amicon Ultra-15 centrifugal filter under sterile environment (see picture below).
      i. Add 10mL of the viral supernatant to each filter tube and centrifuge at 5,000xG (fixed-angle rotor) at 4°C for 20 minutes.

ii. Check that the final volume is less than 500 uL. If not, centrifuge in additional 5 min increments until this goal is achieved.

e. Collect the concentrated viral particles from each filter unit and pool it into a sterile 1.5 mL centrifuge tube.
f. Make 50 uL aliquots in sterile 1.5 mL centrifuge tubes and store at -80°C.

6. **Determine viral titer.**
    a. In most cases the lentivirus will contain a fluorescent reporter. This protocol assumes so. If it does not, a plaque assay is needed (e.g. Crystal Violet staining following puromycin selected).

    b. **Day 1-Seed and transduce cells (Lentivirus containing supernatant will be added while seeding)**
        i. Seed your cell line of interest into 14 wells of a 96-well plate at a density of 5,000 cells per well in GM (cell line dependent formulation—see Subculture Protocol). Use two internal rows of the plate.
        ii. Add lentiviral supernatant to each well with the following dilution series: 1:25K, 1:10K, 1:5K, 1:2500K, 1:1250K, 1:625, 0. Repeat this serial dilution twice (in each of the rows).
        iii. Return cells to the incubator and wait for 48 hrs.

    c. **Day 3-Quantify Titer.**
        i. Observe cell fluorescence using an appropriate fluorescence microscope.
        ii. Quantify and record for each condition the % of fluorescence positive cells and the signal-to-noise ratio. The former requires comparing the fluorescence image to a phase contrast image. The latter requires a digital image and can be estimated by pixel intensity within a transduced cell relative to an area without cells.
        iii. Simultaneously, observe cells in phase contrast and note if there is abnormal morphology or excessive cell death (characteristics are cell type dependent.