DNA Transfection for production of recombinant lentiviruses

Lung-Ji Chang, 1998

BES-$CaCl_2$ transfection of HeLa, 293T, or TE671 cells in 6-well plates

Cultured Cells

General Notes:

The cells need fresh medium immediately before the transfection. When 293 cells are used, be careful when removing the medium since the cells come off easily. On the day before the transfection, the wells of 6-well plates are seeded with 6-7 x 10^5 cells per well. By the time of the transfection, the cells will have reached 95-100% confluency. The addition of DNA to the cells has to be done carefully. Dropwise additions are important. If added too fast, the strong local pH changes in the well will result in reduced titer.

1. Split cells 17-20 hours before transfection at 90-100% confluency, in a 6-well plate with 6 x 10^5 cells per well in 2 ml DMEM with 10% FBS.

2. Replace with fresh medium (DMEM + 10% FBS) on cells no more than 1 hour before transfection. This can be done during DNA $CaPO_4$ precipitate incubation time.

DNA Transfections

1. Clean a test tube rack and pipetman with 70% ethanol.

2. Combine BRL sterile ddH_2O (90 μl per transfection) and 2.5 M $CaCl_2$ (Mallinckrodt, 10 μl per transfection) in 15 ml polystyrene tubes (polypropylene reduces the efficiency of the transfection). Mix by gentle vortexing.

3. To each tube, add:

   A) 15 μg helper construct (pHP). May use 10 μg, but virus titer will not be as high with this amount.
   B) 3 μg VSV-G plasmid (envelope plasmid). The production of VSV-G is toxic to the cells, so do not use more than 6 μg.
   C) 10 μg reporter plasmid (pTV).
   D) 0.5 μg Tat plasmid pCEP4tat. Although Tat is made by the transfection plasmid, extra Tat aids in virus production.
   E) Optional: A reporter DNA or control DNA (0.1 μg per transfection of human growth hormone plasmid or 1 μg eGFP plasmid; we routinely use eGFP expression as an indicator).

4. To each tube, add 100 μl of 2X BES saline (pH 6.96). Add 2-3 drops at a time, mixing with each addition of 2X BES. Be sure that the pH of the 2X BES is correct!! A fine precipitate will form immediately after mixing. Incubate at room temperature for 15-60 minutes, depending on the turbidity of the mix.

5. Shake the DNA mix in each tube thoroughly, and add the DNA mix to each well (one tube per well). Swirl the plate as the mix is added to prevent local alterations in pH.

6. Incubate the plates overnight at 37°C under **3% CO_2**.

7. The next morning, remove the DNA-medium mix. Wash the cells once with 1 ml of fresh medium, if necessary, and then replace with 2 ml fresh medium. Incubate the plates overnight again at 37°C under 5% CO_2.

8. Virus is secreted into the medium. Harvest the virus over a period of three days, once or twice daily.

Improved TE671 transfection protocol

GeneJammer transfection of TE671 cells for the production of recombinant lentiviral vectors

General Notes:

1. We prefer TE671 to HEK (293) cells for its easy handling.

2. Due to the difficulty of transfecting TE671 cells by many laboratories and the requirement of large quantities of plasmid DNA, we have tested a series of different transfection methods, comparing TE671 and HEK cells, and established the following improved TE671 transfection protocol. This protocol is reproducible and requires only small amounts of plasmid DNA. However, the peak production time is narrowed down to 24-48 hr, in contrast to 48-60 hr observed with the calcium phosphate protocol.

3. The GeneJammer protocol works well for TE671 but not for HEK cells. For HEK transfection, calcium phosphate method is still the best method.

4. It is critical to follow each step exactly as instructed below. We routinely obtain 10^6 infectious units per ml with this protocol. Transfection efficiency can be markedly reduced by slight deviations of this protocol (e.g. cells should be used 16-18 hr after splitting).

Step-by-step GeneJammer Transfection of TE671 Cells:

1. Split cells at 90% confluency (6-7 x 10^5 per well of a 6-well dish) **16-18 hours** before transfection.

2. Prepare master packaging DNA mix (at final concentration 1 μg/μl) as following:
   - Lentiviral packaging DNA mix (for each well): pHP (gag-pol) 1.8 μg, pHEFVSV-G 0.3 μg, pCEP4tat, 0.2 μg, and pHEFeGFP, 0.2 μg; the pHEFeGFP DNA is included for easy assessment of transfection efficiency.
   - Transducing vector DNA (per well): 1 μg of pTY (lentiviral SIN vector).
   - In an eppendorf tube, mix 2.5 μl of the packaging DNA and 1 μl of the pTY DNA.

3. To a 5 ml polystyrene tube, pipette 100 μl of serum-free DMEM, and dispense 5 μl of GeneJammer solution **into the center** of the 100 μl DMEM, and mix by gently shaking immediately. The mixture is kept at room temperature for **5-10 min**.

4. Transfer the 3.5 μl of DNA mix to the center of the DMEM-GeneJammer solution, and shake the tube gently by hand immediately. The mixture is kept at room temperature for **5-10 min**.

5. While waiting, feed the cells with fresh 900 μl per well of complete DMEM (with 10% FBS).

6. Transfer the DNA-GeneJammer mix to the well dropwise with constant shaking, and incubate the cell plates at 37°C under 3% CO_2 for 5 hr.

7. Add an additional 1 ml of complete DMEM (with 10% FBS) and continue at 37°C under 3% CO_2 overnight.

8. The next morning, remove the DNA-media mix; wash the cells once with 1 ml medium if necessary, and change to fresh medium (1-2 ml). Under a fluorescent microscope, the bright GFP expression should be observed at this time. The virus production peaks at 48 hr after DNA-GeneJammer addition. Virus production is markedly decreased after 60 hr.

Lentiviral (HIV) Vector Titration on TE671 (for β-galactosidase reporter gene)

1. Split TE671 cells at about 90% confluency and seed into a 24-well plate (6 x 10^4 cells per well).

2. For lentivirus titration, place different volumes of virus stock (typically 2-20 μl for a titer of 10^4-10^5) in polybrene medium (4-8 μg polybrene per ml of medium) at minimal volume (200-300 μl per well) into the wells. Volume should be just enough to cover all of the cells. Incubate overnight at 37°C under 5% CO_2.

3. The next morning, add 0.5 ml of growth medium (DMEM + 10% FBS) directly to the infected culture and incubate for 24 hours (NOTE: Incubation time can be up to 48 hours from the time the virus was added).

4. For nLacZ β-Gal assay, wash the cells twice with PBS. Fix the cells at room temperature for **exactly 5 minutes** using:

   A) 9.65 ml PBS
   B) 1% formaldehyde (270 μl of a 37.6% solution)
   C) 0.2% glutaraldehyde (80 μl of a 25% solution)

   Add enough fixation solution to just cover the cells.

5. Wash the cells three times with PBS.

6. Prepare staining solution by mixing the following (adjust the volume to fit your need):

   A) 9.58 ml ddH_2O (you can adjust the pH with Tris buffer to 8.0-8.5 to reduce background on other cell types)
   B) 4 mM K-ferrocyanide (100 μl of a 0.4 M stock solution)
   C) 4 mM K-ferricyanide (100 μl of a 0.4 M stock solution)
   D) 2 mM MgCl2 (20 μl of a 1 M stock solution)
   E) 0.4 mg/ml X-Gal (200 μl of 20 mg/ml in DMF stock solution). NOTE: Store all stock solutions at -20°C.

   Add enough staining solution to just cover the cells, and incubate overnight at 37°C under 5% CO_2.

7. The next day, count the number of blue nucleated cells using an inverted microscope.