Lentivirus and Retrovirus Transfection

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[Abstract] 293T and Pheonix cells grow in DMEM + 10% FBS. If you are transfecting other cells, you can use whatever medium those cells normally grow in and change to DMEM + 10% FBS on the day of the transfection. You can change back to “normal” medium 24 h post-transfection. Calcium phosphate transfection of M2182 in RPMI (w/o FBS) was reported to cause cells to die. All transfection experiments in our lab have been done in DMEM + 10% FBS, no transfection in other media has been tested so far.

Materials and Reagents

1. 293T and Pheonix cell line
2. 1x Dulbecco's modified eagle medium (DMEM) (high glucose, liquid) (Life Technologies, Invitrogen™)
3. Fetal bovine serum (FBS) (Hyclone)
4. Na_2HPO_4
5. NaCl
6. TE
7. HEPES (Sigma-Aldrich, catalog number: H7006)
8. Packaging vector PcmvR8.74
9. Envelop vector PMD2VSVG
10. Reporter vector DsRED, GFP or LacZ
11. 2x HBS (see Recipes)
12. 2 M $CaCl_2$ (Covidien, Mallinkrodt™, catalog number: 4160) (see Recipes)

Equipment

1. Tissue culture incubator
2. Tissue culture plates of the appropriate size (Thermo Fisher Scientific)
3. 6 cm or 6 well plates
4. Microscope
5. 0.45 µm filter
6. p1000 pipettor

Procedure

Day 0

Plate 4.5 x 10^6 293T (or Pheonix for retrovirus) on 10 cm plate. Incubate overnight at 37 °C. You can also scale down to 6 cm or 6 well plates.

Note: Alternatively, you can plate the cells the same day as the transfection. See Day 1.

Day 1: Transfection

Note: If you plated cells on the previous day, change medium to DMEM + 10% FBS 2 h prior *to transfection and skip to step 4.*

1. Plate 293T (or Pheonix for retrovirus) at ~50% confluence on 10 cm TC plates. I calculate it roughly from the original plate confluence. *Example*: I will plate 2 x 10 cm plates from 100% confluence 10 cm plate. I have scaled down to 6 cm and 6 well plates. Just keep final confluence of cells at roughly 50%.
2. Incubate plates in 37 °C incubator for ~3 to 5 h to allow cells to attach.
3. Thaw out all reagents to room temp before proceeding with transfection.
4. Once cells attached, prepare DNA mix for transfection. For each 10 cm plate, add the following to 5 ml polypropylene tube:
   a. 10 µg DNA of interested (for retrovirus, 10 µg DNA)
   b. 6.5 µg packaging vector (packaging vector already in Pheonix, so no need to add)
   c. 3.5 µg envelope vector (for retrovirus, 5 µg envelope vector)
   d. 0.1 to 0.2 µg of reporter vector (optional) - GFP, DsRED, or LacZ
   e. Bring mixture up to 437.5 µl with 0.1% TE in H_2O

   If you use different size plates, just scale down. See table below.

   Notes:

   a. You can make a master mix if you have multiple plates of the same transfection. However you need to do each transfection separately for high efficiency.

   b. The ratio of DNA: packaging vector: envelope vector is crucial for max viral titer. The ratio provided here was determined by the Weissman Lab. Please keep to this ratio as much as you can.
5. Vortex the DNA mixes on highest speed setting and add 62.5 µl of 2 M $CaCl_2$. While still vortexing, add 500 µl 2x HBS drop wise to the DNA/$CaCl_2$ mix (roughly 2 drops/sec using p1000 pipettor).
6. Immediately add HBS/DNA solution onto cells. Do this in a gentle, drop wise manner and spread it across cells in medium.

7. In a few minutes you should be able to observe, under microscope, evenly distributed small black particles on top of the cells.
8. Incubate cells in 37 °C incubator overnight.

	2x HBS	2 M $CaCl_2$	DNA mix	DNA: Pack: Env
10 cm plates	500 µl	62.5 µl	437.5 µl	10 µg: 6.5 µg: 3.5 µg
6 cm plates	250 µl	31 µl	219 µl	5 µg: 3.3 µg: 1.8 µg
6 wells plate	83 µl	10.5 µl	73 µl	1.5 µg: 1 µg: 0.6 µg

Day 2: 24 h post-transfection

1. Change medium to 10 ml fresh DMEM 10% FBS. You can generally see the efficiency of your transfection by now if you used a reporter vector. At 0.2 µg of DsRED, I can usually observe >75% transfection efficiency. If you can't see much fluorescent cells at this time, wait until 48 h post-transfection and observe again.

   Note: We have always packaged our viruses in 37 °C incubator although it has been reported that the virus is more stable if incubation is carried out at 32 °C.
2. If you want to titer the virus, plate target cells now for infection the next day. I often plate 3 x 10 cm plates per virus for 3 different titrations.

Day 3: 48 h post-transfection

1. Aliquot supernatant of transfected cells into desired volume and freeze at -80 °C to kill any cells in the supernatant. Alternatively, you can filter the supernatant through a 0.45 µm filter to remove the cells. You can also choose to centrifuge the supernatant first if there are many floating cells. However, you need to either freeze or filter the supernatant before using the virus to completely remove any chances of cell contamination.
2. Store viruses at -80 °C.

   If you are titering or infecting target cells, follow the rest of the protocol.
3. Add 10 µl 1,000x polybrene (1,000x = 5-8 µg/ml) to each 10 cm plate of target cells (10 ml medium). Add 0.1 µl, 1 µl, or 10 µl of virus supernatant to each plate of cells for titering your virus. Incubate at 37 °C overnight.

Day 4: Remove virus supernatant

1. 24 h post-infection, change to fresh medium. Incubate overnight at 37 °C.

Day 5: 24-48 h post-infection

1. Cells are now ready to be assayed for any biochemical event of interest (example: start selection with appropriate antibiotic). The actual reverse transcription and integration take place within 24-36 h, depending on cell growth kinetics.

2. Culture cells as normal.

Recipes

1. 2x HBS for calcium phosphate coprecipitation transfection
   a. Make stock sln of Na_2HPO_4 dibasic (5.25 g in 500 ml H_2O)
   b. Make 2x HBS
      8.0 g NaCl
      6.5 g HEPES
      10 ml Na_2HPO_4 stock solution
   c. Bring volume close to 500 ml. Divide into 3 batches and pH each to 6.95, 7.00, and 7.05. Test each batch using LacZ, DsRed, or GFP to see which pH gives best transfection efficiency.
2. 2 M $CaCl_2$
   a. Add 14.702 g of $CaCl_2 \cdot 2H_2O$ to 50 ml H_2O.
   b. F.W. of $CaCl_2 \cdot 2H_2O$ = 147.02 g. -- > 2 M= (2 mol/L) (147.02 g/mol)
   c. $CaCl_2$ is from Mallinkrodt (catalog number: 4160). It is important you use their $CaCl_2$.