Retrovirus Production and Infection

Required Solutions

Polybrene
Stock is 10,000x in medium stored at 4°C (40 mg/ml) and user stock is 100x.
Final concentration is 4 µg/ml.

Medium for Phoenix cells:
DMEM, 10% FBS, 1% Non-essential amino acids, 1% Pen-Strep, 1% Glutamate
Store medium at 4°C, warm to 37°C before use.

2.5 M $CaCl_2$
Filter sterilize and store aliquots at -20°C

2x HBS
50 mM HEPES pH 7.05
10 mM KCl
12 mM Dextrose
280 mM NaCl
1.5 mM Na_2HPO_4 (FW 141.96)
The final pH of the solution should be 7.05 +/- 0.05. Filter through a 0.2 μm filter, aliquot, and store at -20°C. Try to avoid multiple freeze/thaw cycles. To thaw, warm to room temperature and invert or vortex the tube to achieve uniform mixing. Although it is unclear why this occurs, the ability of the 2x HBS solution to produce working $CaPO_4$ precipitates deteriorates after 6 months to one year, even when the 2x HBS solution is stored at -20°C.

Bleach
All materials that have been in contact with the retrovirus must be bleached thoroughly before discarding in the biohazard waste.

Virus production

Day 0 Plate 2.5 x 10^6 Phoenix packaging cells in 9 ml medium/10 cm dish in the afternoon. **Note:** It is very important to have good single cells suspensions (trypsinize well) and to evenly distribute the cells.

Day 1 Transfect cells with 20 μg DNA (±24 hrs after plating), using $CaPO_4$ precipitation. **Note:** At the moment of transfection the cell density should be ± 40-50% such that the cells will be about 90% confluent at Day 3 and completely confluent at Day 4.

1. In a 2 ml eppendorf tube mix:
   - 50 μl of 2.5 M $CaCl_2$
   - 20 μg DNA (Qiagen prep purified)
   - MQ up to total of 500 μl
2. While vortexing the tube, slowly add 500 μl 2x HBS drop by drop.

   Note: if more of the same transfection: mix in 15 ml tube (4 max).
3. Add the 1 ml mix drop by drop to the cells in medium and evenly distribute by swirling the plate.
4. Place cells back in incubator.

Day 2 Change medium 5-20 hours after transfection (precipitate is very fine). Late in the afternoon or in evening replace medium with 9 ml of fresh medium. **Note:** Phoenix cells detach easily, be careful with all medium changes.

Day 3 Collect first supernatant (T1) late in the afternoon (this is 48 hrs after transfection and not later than 24 hrs after changing medium):

1. Remove virus-containing medium and set aside for a moment to supply the packaging cells with 9 mls of fresh medium (1-2 plates at a time).
2. Filter the virus-containing medium through a 0.45 μm filter and immediately use for infection.

Day 4 Collect second supernatant (T2) in the morning.
Collect third supernatant (T3) in the evening (≥ 8 hrs after T2).

Optional: After collecting the last supernatant, trypsinize cells, plate in 2 x 15 cm dishes in medium containing 1.8 μg/ml puromycin. This provides you with information about the transfection efficiency (when using puro vectors) and enables you to collect large amounts of virus from stably producing cells (does not always work because of toxicity transduced gene).

Retroviral Infection

Day 0 Plate cells at a density such that they are growing well for the entire duration of infection and will be confluent at the end of infection (Day 3), about 40% confluent.

For single round of infection:

Day 1

I. If target cells grow in same medium as packaging cells:

Add 4 μg/ml Polybrene to virus (from 100 x stock), remove medium and cover cells with virus + Polybrene:

12-well plate:	300-500 μl/well
6-well plate:	750-1000 μl/well
T25/5 cm dish:	1.5 ml
10 cm dish:	4.5 ml

II. If target cells grow in slightly different medium/FBS percentage (e.g. Phoenix in DMEM and 10% FBS, and BJ in DMEM:199 (4:1), 15% FBS + pyruvate):

1. Add FBS+medium components to get medium composition of target cells (e.g. for BJ add 1/4 of 199 containing pyruvate, 15% FBS, P/S and Glutamate and add 5% extra FBS).
2. Add 4 μg/ml Polybrene and replace medium from target cells with the virus.

III. If target cells grow in different medium as packaging cells (not in DMEM):

Perform the infection using viral supernatant in Phoenix cell medium that is only supplemented with polybrene and not with the medium of the target cells. This means that the infection efficiency will drop because the cells are not growing as optimal as they could be, but this method is better than using virus in other medium.

Day 1, 6-8 hrs later:

Add medium (the type in which the target cells grow) to the virus incubations to dilute the Polybrene which is toxic at concentrations higher than 2 μg/ml.

e.g to: 500 μl of virus, add 750 μl of medium
1 ml of virus, add 1.5 ml medium
1.5 ml of virus, add 2 ml medium
4.5 ml of virus, add 6 ml medium

Grow cells like this for 48 hrs after start of infection (until Day 3) before splitting or starting antibiotic selection.

For 2 rounds of infection:

1. Start with the first infection on the morning of Day 1 as described above.

2. In the morning of Day 2, remove the virus containing medium of the first infection and repeat the infection as described above
3. Leave the cells for 24 hrs, until Day 3.

For 3 rounds of infection:

1. Start with the first infection in the late afternoon/evening of Day 1 by removing medium from target cells and adding virus + Polybrene, leave overnight, do not dilute polybrene.
2. The next morning remove virus and replace by new virus + Polybrene.
3. In evening do not remove virus of second infection, but dilute it by adding an equal amount of new virus without polybrene.

For 4 rounds of infection:

1. Start with the first infection on the afternoon/evening of Day 1 as described above.
2. In the morning of Day 2, remove the virus containing medium of the first infection and repeat the infection as described above.
3. In the evening of Day 2, remove the virus containing medium of the first infection and repeat the infection.
4. In the morning of Day 3 repeat the infection and discard the Phoenix cells.
5. In the evening of Day 3, change the medium of the infected cells.
6. 24 hours later, start antibiotic selection. For puromyocin, select for 3 days. For hygromyocin, select for 7 days.

Note: this schedule follows the schedule of viral supernatant isolation, so fresh virus can be used. This will result in the highest MOI possible.
If an ever higher MOI is necessary (e.g. when using poorly infecting cells) or when combinations of different viruses need to be transduced: superinfect by re-plating the cells on Day 3 and repeating the infection procedure with the same or other virus.
Do not use this triple infection protocol for cells that do not grow well in DMEM (III) , better use the double infection protocol and superinfect if necessary.