Transient, Helper virus-Free Retroviral Production

The following protocols are adopted from others (Kotani, et al., 1994). We currently use the 293 cell-derived Phoenix amphotropic and ecotropic cell lines (Grignani, et al., 1998) obtained through the American Type Culture Collection, which express the Moloney virus structural genes (*gag, pol, pro* and *env*). Briefly, viral mRNA is transcribed from the retroviral plasmid template within transfected producer cells. The viral mRNA is encapsulated and shed non-lytically into the cell culture media, collected and then added to target cells. Standard biosafety level 2 precautions are used in working with the producer cells and any material derived from them. This is relatively safe material to work with but because the viruses are infectious, heightened awareness of potential dangers is necessary. Sterile, single-use aliquots of 25 mM chloroquine, 2M $CaCl_2$ and 2X BES solution should be stored frozen at –20C for consistency.

1. Producer cells received from the ATCC require expansion and storage as frozen stocks for subsequent use. Plate the contents of the original cell vial in a 100 mm dish in bicarbonate-buffered DMEM with 10% heat-inactivated FBS and 1X penicillin and streptomycin, at 37C in a 5% CO2 incubator. After growing to ~80% confluence, remove by gentle trypsinization (0.05% trypsin versine) and distribute onto 15 new 100 mm plates. Repeating this expansion one or two additional times provides sufficient stocks for an extended period.
2. After reaching ~70-80% confluence freeze back 2 aliquots/plate in 1 ml of heat-inactivated FBS plus 10% DMSO. Slowly freezing the vials overnight at –80C using an isopropyl alcohol-buffered container before long term storage in liquid nitrogen improves subsequent viability.
3. To prepare cells for retroviral production, thaw a freezer vial and place its contents in 25 ml growth media on a 150 mm plate. From this point on, the use of heat-inactivated serum is no longer necessary. After reaching ~80% confluence, trypsinize and inoculate a 100 mm plate for each planned transfection, using 7 X 10^6 cells in 15 ml of media and incubate 16 hr before transfection. Rather than maintaining constant selection pressure on the retroviral structural genes, we typically subculture the contents of a frozen vial for 5 to 8 passages before thawing earlier passages of frozen stock.
1. Just before transfection, replace growth media with a fresh 8 ml and add chloroquine to a final concentration of 25 μM.
5. Bring 25 to 40 μg of retroviral plasmid DNA to a final volume of 875 μl in deionized H_2O using capped, sterile 13 X 75 mm polypropylene tubes. Mix in 125 μl of 2M $CaCl_2$, and then while vortexing at a medium setting rapidly add 1.0 ml of 2X BES solution (50 mM BES (Sigma B-6137), 280 mM NaCl, 1.5

mM Na_2HPO_4; optimal pH ranges between 6.95 to 7.05). Immediately transfer the slightly clouded 2 ml mixture to the producer cell plate by drop-wise addition before returning the plate to a 37C, 5% CO_2 incubator. When performing multiple transfections, we find working one plate at a time is better. This and vortexing prevents large clumps from forming, yielding a finer precipitate and more efficient transfection.

1. After 6 to 8 hr, aspirate the transfection media and add 25 ml of fresh growth media to the plates. Beginning 24 hr after initiating transfection, aspirate growth media and replace with 9 ml of fresh media, then place the plates in a 5% CO_2 incubator set at 32C. Twenty-four hours after this, (that is, 48 hr after starting a producer cell transfection), collect the conditioned media and pass it through a 0.45 μm cellulose acetate syringe tip filter. Either freeze aliquots immediately by immersion in liquid nitrogen before storage at –80C, or use fresh as described below. Add 9 ml of fresh growth media to the producer cells and incubate again at 32C for twelve hours before the next collection. We typically collect two to four supernatants at twelve hour intervals after th first harvest. Unused frozen supernatants are discarded 3-6 months later after autoclaving.

References

1. Kotani, H., P. B. Newton, 3rd, S. Zhang, Y. L. Chiang, E. Otto, L. Weaver, R. M. Blaese, W. F. Anderson, and G. J. McGarrity. (1994) Improved methods of retroviral vector transduction and production for gene therapy. *Human Gene Therapy* **5**(1)**:**19-28.
2. Grignani, F., T. Kinsella, A. Mencarelli, M. Valtieri, D. Riganelli, L. Lanfrancone, C. Peschle, G. P. Nolan, and P. G. Pelicci. (1998) High-efficiency gene transfer and selection of human hematopoietic progenitor cells with a hybrid EBV/retroviral vector expressing the green fluorescence protein. *Cancer Res* **58**(1)**:**14-9.