UC Irvine
UC Irvine Previously Published Works

Title
Production of Replication-Defective Retrovirus by Transient Transfection of 293T cells

Permalink
https://escholarship.org/uc/item/8s6645gh

Journal
Journal of Visualized Experiments

ISSN
1940-087X

Authors
Gavrilescu, L Cristina
Van Etten, Richard A

Publication Date
2007-12-04

DOI
10.3791/550

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Video Article

Production of Replication-Defective Retrovirus by Transient Transfection of 293T cells

L Cristina Gavrilescu, Richard A Van Etten
Molecular Oncology Research Institute, Tufts-NEMC

Correspondence to: L Cristina Gavrilescu at cgavrilescu@tufts-nemc.org

URL: http://www.jove.com/index/Details.stp?ID=550
DOI: 10.3791/550
Citation: Gavrilescu L.C., Van Etten R.A. (2007). Production of Replication-Defective Retrovirus by Transient Transfection of 293T cells. JoVE. 10. http://www.jove.com/index/Details.stp?ID=550, doi: 10.3791/550

Abstract

Our lab studies human myeloproliferative diseases induced by such oncogenes as Bcr-Abl or growth factor receptor-derived oncogenes (ZNF198-FGFR1, Bcr-PDGFRa, etc.). We are able to model and study a human-like disease in our mouse model, by transplanting bone marrow cells previously infected with a retrovirus expressing the oncogene of interest. Replication-defective retrovirus encoding a human oncogene and a marker (GFP, RFP, antibiotic resistance gene, etc.) is produced by a transient transfection protocol using 293T cells, a human renal epithelial cell line transformed by the adenovirus E1A gene product. 293 cells have the unusual property of being highly transfectable by calcium phosphate ($CaPO_4$), with up to 50-80% transfection efficiency readily attainable. Here, we co-transfect 293 cells with a retroviral vector expressing the oncogene of interest and a plasmid that expresses the gag-pol-env packaging functions, such as the single-genome packaging constructs kat or pCL, in this case the EcoPak plasmid. The initial transfection is further improved by use of chloroquine. Stocks of ecotropic virus, collected as culture supernatant 48 hrs. post-transfection, can be stored at -80°C and used for infection of cell-lines in view of transformation and in vitro studies, or primary cells such as mouse bone marrow cells, that can then be used for transplant in our mouse model.

Protocol

1. The evening before transformation, plate 293T cells at3-5x10^6 cells/6 cm tissue cultureplate.
   Note: We use 293T cells for their ease of transfection and efficacyas virus producing cells. These cells are deficient in packaging the virus unless a helper plasmid is introduced.
2. The first morning, gently remove medium and replace with 4 ml 293T cell med containing 25 mM Chloroquine. Put in incubator for 1 hr.
   Note: the plate should be about 80% confluent.
3. Meanwhile, prepare virus-making mixture for 2 plates at a time, in 5 ml tubes:
   1. 2 x 10 mg retroviral plasmid construct
   2. 2 x 5 mg packaging construct (EcoPak),
   3. 2 x 62 ml CaCl
   4. complete to 1 ml with sterile ddH_2O
      Note: While the retroviral plasmid encodes the oncogene of interest and a marker, EcoPak encodes gag-pol-env. Together with the 293T cells, they will produce an ecotropic retrovirus(RV) specific for mouse cells, but not infective for human cells. Both Chloroquine and $CaCl_2$ have been shown to increase transfection efficiency.
4. Add 1ml of 2x sterileHBS drop-wise to the mixture, while gently vortexing the tube.
5. Immediately add this solution to 2 plates, 1 ml each, gently, drop by drop.
6. Put in incubator for 7 to 11 hrs.
7. In the evening (7 to 11 hrs. later), gently remove medium, and very gently replace with 5 ml fresh 293T medium. Put in incubator until noon, the next day.
   Note: this step is important, as you need to take off the chloroquine from the cells. If kept for extended periods of time, chloroquine is toxic. The solution in the plates looks fuzzy, due to a very fine, dust-like precipitation of the transfection mixture.
   Note: It is important to do all media changes with extreme gentleness, as the cells have been sensitized by the chloroquine, and can easily detach from the plate.
8. Next day, 18 to 24 hrs. before retrieving the virus (around noon), gently remove medium, and very gently replace with 3 ml fresh 293T medium. Replace in incubator O/N.
9. The third morning (18 to 24 hrs. later), gently suck up the medium form the plates with a 10 ml syringe fitted with an 18 G needle. This supernatant contains the retrovirus.
10. Change the needle to a 45 mm syringe filter, invert syringe several times to mix the solution well, pass supernatant through filter, and aliquot in cryotubes
    Note: Alternatively, if you are making 3+ plates of virus, pool all supernatants of the same virus into a 50 ml conical tube. Mix well, then aliquot supernatants containing the retrovirus in cryotubes by filtering.
11. The tubes containing the virus-full supernatants are kept in -80°C until used.

Discussion

Four critical points will assure the success of a good viral stock:

1. The 293T producing cells have to be very healthy, meaning they have been split on a very regular schedule, were never overgrown, and are plated at the optimized density of 3.5-5 million per 6 cm tissue culture plate, so that they reach a density of 80% of the plate on the morning of the transfection.

Copyright © 2007 Journal of Visualized Experiments

2. Addition of chloroquine improves transfection efficiency and subsequent virus titer, about 3- to 5-fold, by stabilizing cell lysozomes and increasing the fraction of DNA that reaches the nucleus. Sodium butyrate (another lysosome stabilizer) has also been used for this purpose. Chloroquine can be omitted, in which case changing the medium 7-11 hrs. post-transfection at this point is not required.
3. The quality of DNA is very important. The preferred method of purification of both the vector and packaging plasmid DNAs is twice purified by CsCl-ethidium bromide buoyant density centrifugation. We like the concentration of the DNA to be at least 1 mg/ml, and the OD 260/280 ratio should be between 1.75-1.90. Qiagen-purified DNA will also work, although, in the hands of the authors, the results are not as good. It is important that the combined volume of DNA solutions be small (< 50 ul total per final ml) to avoid adverse effects of Tris and EDTA (TE) on the calcium phosphate precipitate.
4. The choice of the retroviral vector and host range of the packaging construct are important. For stable expression in murine hematopoietic stem cells, a vector based on the myeloproliferative sarcoma virus is preferred. A common vector backbone is the MIG RI, containing an MPSV long terminal repeat sequence, a multiple cloning site for introduction of an oncogene, and a downstream internal ribosome entry site linked to the gene for enhanced green fluorescent protein (eGFP). For transduction of mouse HSC, virus with an ecotropic host range is optimal, but such stocks are unstable at physiologic temperature and cannot be concentrated by centrifugation.

Some check-points: If desired, once harvested, the 293 cells can be used to make protein lysates for immunoblotting to check the expression of proteins encoded by the retroviral vector (e.g., the TK). We also use a LacZ encoding control plasmid in separate plate at the time of transfection (no packaging vector needed) to check for transfection efficinecy by beta-gal assay when we collect the supernatant of the other plates. Note: this will test the cells and reagents, but not the quality of the plasmids used for virus making.

Before using any virus, the titer needs to be determined. This is critical in order to match titers of different virus stocks in the same experiment, and to ensure efficient transduction of hematopoietic stem cells.

References

1. DuBridge, R. B., Tang, P., Hsia, H. C., Leong, P. M., Miller, J. H., and Calos, M. P. (1987). Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Mol Cell Biol 7, 379-387.

2. Cherry, S. R., Biniszkiewicz, D., van Parijs, L., Baltimore, D., and Jaenisch, R. (2000). Retroviral expression in embryonic stem cells and hematopoietic stem cells. Mol Cell Biol 20, 7419-7426.

3. Finer, M. H., Dull, T. J., Qin, L., Farson, D., and Roberts, M. (1994). kat: A high-efficiency retroviral transduction system for primary human T lymphocytes. Blood 83, 43-50.

4. Naviaux, R. K., Costanzi, E., Haas, M., and Verma, I. M. (1996). The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. J Virol 70, 5701-5705.

5. Pear, W. S., Miller, J. P., Xu, L., Pui, J. C., Soffer, B., Quackenbush, R. C., Pendergast, A. M., Bronson, R., Aster, J. C., Scott, M. L., and Baltimore, D. (1998). Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. Blood 92, 3780-3792.

6. Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. (1993). Production of high-titer helper-free retroviruses by transient transfection. Proc Natl Acad Sci USA 90, 8392-8396.

7. Stocking, C., Kollek, R., Bergholz, U., and Ostertag, W. (1985). Long terminal repeat sequences impart hematopoietic transformation properties to the myeloproliferative sarcoma virus. Proc Natl Acad Sci USA 82, 5746-5750.

8. Van Etten, R. A. (2001). Models of chronic myeloid leukemia. Curr Oncol Rep 3, 228-237.

9. Van Etten, R. A. (2002). Studying the pathogenesis of BCR-ABL+ leukemia in mice. Oncogene 21, 8643-8651.

Copyright © 2007 Journal of Visualized Experiments