



Retrovirus-mediated Gene Transfer Manual

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Retrogen, Inc.

Retrovirus-mediated Gene
Transfer Manual

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Retrovirus-mediated Gene Transfer

Retrovirus-mediated gene transfer is by far the most efficient method to express your gene(s) of interest in cells of various lineages, in cells from most mammalian species and in some other eukaryotic species of interest. Without exception, retrovirus-mediated gene transfer allows one to assay genes in cells with, for example, unique growth characteristics, since retrovirus infection and expression levels can be normalized across various cell lines. Normalization of gene transfer and expression across various cell lines by other vehicles, e.g., lipid-mediated or calcium phosphate-mediated transfection may never be achieved.

Current generation retroviral vectors are easy to use and express genes-of-interest at high levels. These vectors, when used with highly transfectable packaging cell lines, produce high titer viral preparations from transient transfections. High titer viral stocks can be used to infect hard to transfect cells of interest. The high efficiency of trans-infection allows genes-of-interest to be assayed en-bulk in either unselected or drug-selected cells.

Retrogen offers retroviral vectors of efficient design. We have eliminated all sequences unnecessary for retrovirus vector replication and gene-of-interest expression. Our vectors use a CMV enhancer-promoter hybrid to produce high levels of retrovector genome in packaging cells. A modified Moloney Murine Leukemia Virus (MoMuLV) LTR is used to express the gene-of-interest in the recombinant provirus. The MoMuLV LTR has been shown to give high levels of recombinant gene expression in cells of diverse lineages. We offer two retrovector kits. The pRT-X kit contains all the components necessary for the production of high titer retroviral particles. The pRT-X vector does not contain a marker for drug selection. The pRT-X kit includes the lacZ (pRT-Z) or the luciferase gene (pRT-L) cloned into pRT-X for use as positive controls. The pRT-XNeo is derived from pRT and contains a Kozak consensus motif-optimized neomycin phosphotransferase gene driven by an internal minimal SV40 early promoter. pRT-XNeo contains the same multiple cloning site sequence as pRT-X for ease of gene-of-interest transfer between vectors. pRT-XNeo transduced cells can be selected with G418 in high yield. Gene of interest expression is via the MoMuLV LTR for expression in a variety of cell lines.



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Retrogen Retroviral Vector Kit Components

I. Retrogen Retrovector Kit common components

Each retrovector kit contains the following components:

1. pPkg Retroviral packaging vector expressing the MoMuLV gag and pol genes
2. pEco Vector expressing the ecotropic envelope for infection of mouse and rat cells
3. pAmpho Vector expressing the amphotropic envelope gene for infection of cells of most species, except hamster
4. 4 each Syringes, 10 ml luer lock, sterile
5. 4 each Syringe Filters, 25mm 0.45 micron, sterile, surfactant-free
6. 1 ml Polybrene solution (1000X) sterile
7. 1 each Manual for producing retroviral particles and transducing cell lines in vitro

II.pRT-X Retroviral Kit

1. pRT-X: retrovector of minimal required sequences containing mcs
2. pRT-Z: vector expresses lacZ gene
3. pRT-L: vector expresses luciferase gene

III.pRT-X Neo Retroviral Kit

1. pRT-XNeo retrovector containing a mcs and internal SV40 early promoter neomycin phosphotransferase gene for stable selection of transduced cells
2. pRT-ZNeo vector expressing the lacZ gene
3. pRT-LNeo vector expressing the luciferase gene
4. 1 ml G418 (100 mg/ml) sterile solution for drug selection of transduced cells



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Safety and Handling of Retroviral Preparations

Current generation retroviral vectors used with highly transfectable packaging cell lines produce high titer viral preparations. The high efficiency of gene transfer by trans-infection requires precautions for the protection of the user and co-workers. Your safety officer should be notified and consulted concerning site-specific requirements for working with retroviruses. A good reference publication is Biosafety in Microbiological and Biomedical Laboratories, Third Edition (May 1993) HHS Pub. #(CDC) 93-8395. U.S. Dept. of Health and Human Services, PHS, CDC, NIH. See also <http://www4.od.nih.gov/oba/guidelines.html> for NIH guidelines to biomedical research.

Retroviral vectors offered by Retrogen produce **replication defective** retroviral genomes. To produce a replication-competent retroviral genome three recombination events are required between the vector of interest, the gag-pol regions of the packaging cell line and the envelope gene provided by a co-transfected plasmid. This has not yet occurred since the introduction of the spilt-genome packaging cell lines (1,2).

Retrovirus production and infection of cells should be performed at **Biosafety Level 2**. Work should be performed in a certified laminar flow hood with HEPA filters. Use sterile technique. Treat all potential virus-containing liquid stocks with bleach to a final concentration of **2-5%** for at least 5 minutes before removing from the hood and discarding. Discard spent plastic labware into biohazard bags before removing from the hood and autoclave before disposing. Spent glassware should be disinfected by, e.g., immersing in a 2-5% bleach solution before removing from the hood and prior to cleaning. Finally, clean the work area with a 70% solution of ethanol or isopropanol when through.



High Titer Retroviral Particle Preparations

- I. Components to Culture Packaging Cells & Produce Retroviral Particles
 - A. A vehicle for transfecting the 293 gag-pol cells.
Lipid-mediated and calcium phosphate-mediated transfection work well with these cells.
 - B. 293 gag-pol cells
These are derivatives of the HEK (Human Embryonic Kidney) cell line 293. The cells do not stick to the plate very well, so be gentle. As the number of passages out of liqN2 increases, the cells adhere even less and start to bunch-up on top of each other. Dump them and thaw a new vial.
 - C. DMEM High Glucose + 10% FBS
 - D. Trypsin solution to detach cells from plate.
 - E. 10 cm tissue culture grade plates. Flasks do not work as well, since the monolayer needs to be accessed.
 - F. Plasmid DNA (pDNA)
Qiagen quality is the best. Silica-purified miniprep DNA will work. Re-solubilize pDNA in 10 mM TRIS, pH 8 + 0.1 mM EDTA at ≥ 1 mg/ml. 1X TE is okay.
 - G. 10 cc sterile syringes (luer lock-type preferred) + sterile 18G needles (if available).
One set for each retroviral vector.
 - H. 0.45 or 0.22 micron, low protein-binding, surfactant-free syringe filters. One for each retroviral vector.
 - I. Freezer vials (1.5 ml) for aliquoting and storing retroviral stocks (optional)
 - J. Polybrene (Hexadimethrine Bromide, Sigma #H9268)
 - K. RPMI-1640 + 10% calf serum
 - L. Selection drug. For G418 dissolve in ddH₂O at 50 mg/ml active drug. Sterile filter and aliquot. 1-2 ml aliquots work well. Store at -20°C.



II. Generating Recombinant Retroviral Particles by Transient Transfection

DAY 1

1. Split and plate 293 gag-pol cells at $1-2 \times 10^6$ cells per 10-cm plate. Grow cells in DMEM high glucose + 10% FBS. Culture overnight.

DAY 2

1. Transfect 293 gag-pol cells with 10 ug of retroviral vector pDNA + 10 ug of envelope vector pDNA.
2. After the appropriate time, aspirate off the transfection mix and add 10-ml fresh DMEM-10% FBS. Culture overnight.

DAY 3

1. Change the medium, but only replate 4-5 ml of DMEM-10% FBS. (This is to keep the virus as concentrated as possible). The medium change is required to remove a growth inhibitory factor produced by 293 cells upon transfection. Culture overnight.

Note: *Virus-containing culture supernatant can be recovered on Day 4. In our hands, higher viral titers are recovered on day 5, 3 days after transfection. For viral harvest on **day 5**, change the medium, but replate 10 ml of DMEM-10% FBS.*

DAY 4

1. Harvest the virus by removing the supernatant with the 10-cc syringe*. Filter out any debris and packaging cells aspirated using the syringe filter. Dispense into a sterile culture tube.

***Note:** To filter virus-containing supernatants we aspirate the supernatants with the syringe-filter on. This abrogates the need for an 18G needle. The aspiration must be done in one continuous "pull" or the filter will clog. Discard the filter tip before dispensing the supernatant.

1B. *For viral harvest on **day 5**, aspirate supernatant and add 4-5 ml of fresh DMEM-10% FBS.*

2. Use the viral stock immediately or aliquot and freeze at -80°C .



DAY 5

1. Harvest the virus as described for Day 4.
2. Use immediately or aliquot and freeze.

***NOTE:** *The viral particle contains RNA so it is very labile. Filtering the virus after it has been frozen reduces titers at least 2-fold. It is difficult to determine when the cells are producing maximal virus. Virus-containing supernatants can be harvested daily, but do not pool the supernatants. The limitation of the packaging cell is the transient transfection from which pDNA is eventually lost. Also, after day 5 the culture is over grown and will start to peel off the tissue culture plate.*

III. Determining Viral Titer

NIH 3T3 cells have been the standard for determining retroviral titer (1). They are highly receptive to infection by ecotropic and amphotropic retroviral particles. They are very sensitive to the drug selection agents currently in use (G418, hygromycin, zeocin, histidinol, puromycin, and blasticidin). Their growth rate is moderate; i.e., they do not grow to confluence during the course of the assay. They grow as adherent cells. Therefore, after drug selection, remaining colonies can be easily counted for calculation of the retroviral titer.

DAY 1

1. Use a 6-well plates. Plate at 5×10^4 NIH 3T3 cells per well. Culture overnight.

DAY 2

1. Set up 10-fold dilutions of virus in sterile polystyrene tubes. Start with 0.2 ml virus stock + 1.8 ml DMEM-10% FBS. Take 0.2 ml of the 1st dilution and add to 1.8 ml medium. Continue this dilution regimen as shown below. Aspirate the culture medium from the 6-well plate and add 1 ml of the virus dilution to its respective well. Add 10 μ l of the 800 μ g/ml polybrene stock for a final concentration of 8 μ g/ml. Gently swirl the 6-well plate to mix. Let the infection proceed for 6 to 24 hr at 37°C. After infection, aspirate the virus+polybrene containing medium and add fresh DMEM-10% FBS. Culture for the time required for the cells to undergo at least 1 population doubling. The virus dilutions asterisked (*) are used for infection. Discard the two highest concentrations.



| <u>Well #</u> | <u>Virus stock</u> | <u>DMEM-10% FBS</u> | <u>[virus stock]final</u> |
|---------------|-----------------------------|---------------------|--|
| 1.* | 0 ml | 2 ml | 0 % (negative control) |
| 2. | 0.2 ml 100% stock | 1.8 ml | 10% |
| 3. | 0.2 ml 10% stock | 1.8 ml | 1% (10^1) |
| 4.* | 0.2 ml 1% stock | 1.8 ml | 0.1% (10^{-1}) |
| 5.* | 0.2 ml 0.1% stock | 1.8 ml | 0.01% (10^{-2}) |
| 6.* | 0.2 ml 0.01% stock | 1.8 ml | 0.001% (10^{-3}) |
| 7.* | 0.2 ml 0.001% stock | 1.8 ml | 0.0001% (10^{-4}) |
| 8.* | 0.2 ml 0.0001% stock | 1.8 ml | 0.00001% (10^{-5}) |
| 9. | 0.2 ml 0.00001% stock | 1.8 ml | 0.000001% (10^{-6}) |

***Note:** Cells may not all tolerate 8 ug/ml polybrene overnight. Some cells like the murine P815 mastocytoma cells and the murine A20 B-cell lymphoma can only tolerate 4 ug/ml polybrene overnight.*

DAY 4+

1. Add G418 at 400 ug/ml in RMPI-1640+10% calf serum. If using DMEM or FBS, the G418 concentrate will need to be increased to 600 ug/ml. Do not include pen-strep in the selection medium. The combination of G418 + P/S is toxic even for cells expressing neomycin phosphotransferase. The titer determined with pen-strep in the growth medium does not represent the true potential of that virus stock. Let the cells select for 3 to 10 days. Change the medium every 4-5 days. The G418 concentration may be halved versus the initial selection concentration.

DAY 7+

1. Count the number of surviving colonies after all the cells in well #1 are dead and detached from the plate.. If, for example, there are 65 colonies at the 10^{-4} dilution you have a 65×10^4 or 6.5×10^5 DRU/ml virus stock. For G418 we use GRU (G418-resistant units)/ml.



V.SHORTCUT PROCEDURE TO MAKING STABLE, ADHERENT CELLS

The shortcut procedure to create stable adherent cells saves you about 1-2 week's of time. Remember that this does not reflect the "true" titer of the virus stock because the accepted read-out is 3T3 cells. Some cells, especially human cells, do not have as many viral receptors for transduction efficiency. One integration or one viral genome per cell is typically what people want using the retrovirus-mediated gene transfer method. Therefore, cultures that show close to 100% transduction may have multiple integrations. Titrate your virus stock on your target cells as done above with the NIH 3T3 cells. After selection, the well that shows 25-50% transduction irrespective of the amount of virus stock used is typically sufficient for use. Wells with lower transduction percentages require extra time to attain useful numbers for assay. Lower transduction percentages may reflect a more clonal population of cells.

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