

# **Retroviral GeneSuppressor System**

(version 09/03)

Catalog No.: IMG-1000

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## pSuppressorRetro™ System

### STORAGE CONDITIONS:

Store the vectors at -20°C. Please amplify the plasmid vectors on LB/Amp (50 µg/ml) plate for your use. This product is for personal use only. Not for distribution.

Transfection buffer and CaCl<sub>2</sub> solution can be stored at room temperature or at 4°C up to six months

*For research use only. Not for diagnostic or other commercial applications. Not for use in humans.*

**Disclaimer:** *Production of retroviruses in mammalian cells is covered by several United States patents issued to CellGenesys, Inc. Imgenex will not be responsible for any patent infringements resulting from use of the RetroMax kits or vectors to produce retroviral particles in mammalian cells.*

*Please read the entire manual, especially Pages 19-20 (safe use of retroviruses) before starting any experiment.*

## I. Kit Components and Storage:

The components included in this kit need to be stored at different temperatures.

Reagents (-20°C storage)		Quantity
IMG-1000-1	Linearized pSuppressorRetro (50 ng/μl)----	20 μl
10045P	pCL-Eco-(0.5 μg/ul)-----	10 μl
10046P	pCL-Ampho-(0.5 μg/ul)-----	10 μl
10047P	pCL-10A1-(0.5 μg/ul)-----	10 μl
10048P	pCL-MFG-LacZ-(0.5 μg/ul)-----	10 μl
GSC-1005	Sequencing primer (10 pmol/μl)-----	20 μl
IMG-1000-6	Negative Control Plasmid (100 ng/ μl)-----	20 μl
Reagents (4°C or Room temperature storage)		
10049K	Transfection Buffer (2 x)-----	10ml
10050K	2 M CaCl <sub>2</sub> -----	2ml
GSC-1003	Deionized water-----	2 ml
GSC-1004	Annealing Buffer-----	50 μl

The negative control plasmid (IMG-1000-6) contains a scrambled sequence that does not show significant homology to rat, mouse or human gene sequences. The sequence of the insert in the control plasmid is:

```
5' TCGATCAGTCACGTTAATGGTCGTTtccaagagaAACGACCATTAACGTGACTGATTTTT -3'
   AGTCAGTGAATTACCAGCAAaagttctctTTGCTGGTAATTGCACTGACTAAAAAGATC-5'
```

*All plasmids must be grown in LB medium containing ampicillin.*

### Additional items required (not included in the kit)

293 Cells

G418

Cell culture reagents: DMEM, Fetal bovine serum, penicillin-streptomycin

Trypsin-EDTA

Phosphate buffered saline

Cloning cylinders (for picking up stably transfected colonies)

Polybrene for retroviral infection.

## II. Introduction

The retroviral GeneSuppressor kit combines the advantage of U6 promoter for expression of hairpin siRNA for gene knock down with the efficient delivery of siRNAs into mammalian cells using our RetroMax™ retroviral vectors. Retroviral vectors are very efficient tools for stably introducing genes into dividing cells. They are also useful for delivering genes into cells normally refractory to transfection methods such as lipid-based reagents, calcium-phosphate, and electroporation.

The RetroMax™ retrovirus vector system is based on the pCL vector system developed by Naviaux et al. (1). The vectors used in this system have been designed to maximize recombinant-retrovirus titers in a simple, efficient, and flexible experimental system. The pSuppressorRetro vector has an extended packaging signal ( $\psi^+$ ) and is derived from a safety-modified retrovirus vector in which the gag open reading frame has been stopped by a point mutation, thereby minimizing the opportunity for replication-competent retrovirus production by recombination with packaging genome. The 5'-enhancer of Moloney murine sarcoma virus long terminal repeat (LTR) which is inhibited by E1A has been deleted and fused at the TATA box of the human CMV immediate early region. (1). This results in initiation of viral RNA at or near the +1 position in the R region of the naturally programmed retrovirus RNA (Figure 1). This results in transient-retrovirus titers in the range of  $2\text{-}5 \times 10^6$  CFU/ml when 293 cells are used.

All three members of the RetroMax packaging vectors (pCL-Eco, pCL-Ampho, and pCL-10A1) have been also safety modified by deleting the packaging signal and the 3' LTR enhancer. This makes the RNAs of the helper genome virtually unpackageable. The advantage of these pCL packaging plasmids is a high level of expression of gag, pol, and env proteins with a balanced stoichiometry that is not achieved with either transiently or stably expressed split-genome packaging constructs. Inclusion of these three packaging plasmids in the RetroMax kit allows the choice of

expressing ectotropic, amphotropic, or 10A1 envelopes which leads to greater experimental flexibility.

The RetroMax system is designed for maximal virus titer in 293 cells. It takes advantages of two properties of 293 cells, i) high level of transfectibility, ii) strong E1A-mediated stimulation of CMV promoter controlled transcription. 293 cells are of nonmurine origin, hence the problems of selective packaging and transfer of VL30 genomes (present in all murine packaging cells) are avoided. Vector supernatants are free of helper virus and are of sufficiently high titer within 2 days of transient transfection in 293 cells to permit infection of more than 50% of dividing target cells in culture.

By introducing a retroviral vector into a cell expressing retroviral proteins, retroviral particles (virions) are shed into the culture medium at the rate of about one infectious particle/cell/day. Retrovirus tropism is determined at three levels. The first is simply a function of viral envelope protein, gp70. The envelope determines which cells the virus will enter.

1. Ecotropic (usually (MoMuLV) mouse and rat cells only (not human).
2. Amphotropic (from 4070A MuLV) most mammalian cells (but not hamster).
3. Gibbon Ape Leukemia virus (GALV) many mammalian cells (including hamster).
4. 10A1 (MuLV) most mammalian cells (including hamster).

The second level of tropism is nuclear translocation and integration. This is defined by structural features of p30CA (but requires the full 160S nucleoprotein pre-integration complex, comprised of all the gag proteins and viral RNA and/or DNA). Naked DNA in the cytoplasm after retrovirus uncoating and reverse transcription is never seen.

The product of the FV-1 locus in murine cells interacts with p30CA, and can reduce the efficiency of translocation and integration (and thus apparent titer) 20-100 fold. Fortunately, the common Moloney-based packaging cells supply a p30CA form (NB tropic) that avoids

this problem. The human equivalent of FV-1 has not yet been identified.

The third level of retrovirus tropism is determined by the transcriptional activity of the LTR (and/or internal promoter) in the transfected cell. In general, the Moloney (and MSV) LTR is active in most mammalian cell types, with the distinct exception of embryonic stem cells and teratocarcinoma cell lines (like F9), in which it is silenced. It is also potentially inhibited by E1A/p300 in 293 cells.

Ping-pong amplification is sometimes used to increase retrovirus vector titers, by coculturing vector-producing ectotropic and amphotropic cell lines. This can increase vector titers 10 fold, but often at a heavy cost:

1. Frequent truncations, deletions, and point mutations may occur in the inserted cDNA.
2. You may generate helper virus if you are not using safety-modified system.

### **III. Physical Properties**

Retrovirus particles are fragile. They are easily inactivated by 0.1% detergent, chloroform, phenol, 1% bleach, 70% ethanol, at 65°C for 30 min, pH <6.5 or >9.0, UV light, and autoclaving.

Simple high-speed centrifugation (100,000g x 90 min) produces enough hydrodynamic shear to strip many virions of their gp70, and thus infectivity (although reverse transcriptase activity is preserved).

Virus can be stored in culture medium (with 10% serum) at -70°C indefinitely. One freeze-thaw cycle reduces the titer about 2-3 fold compared to the fresh virus. The second freeze-thaw drops the titer another 5-10 fold. Aliquot your virus for storage at -70°C.

When filter sterilizing retrovirus, be sure to use non-detergent treated 0.22 or .45 um filters. Any trace of detergent will strip virus envelope and reduce your titers. Filter before freezing, and not after in order to avoid losses due to aggregation.

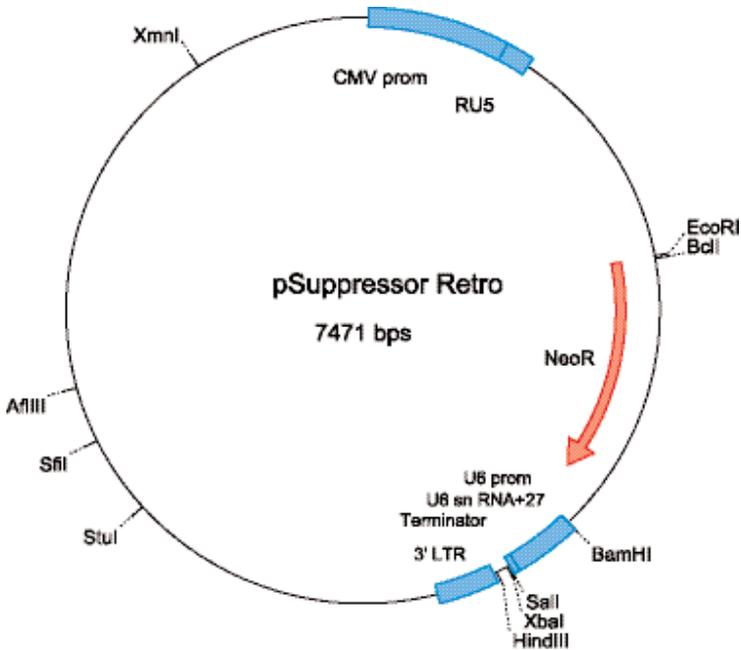
All murine retrovirus vectors produced in either mouse cells (like the NIH 3T3-based packaging cell lines) or primate cells (COS and 293) are rapidly inactivated by human serum complement, with kill

kinetics of 2-3 logs in 5 min at 37°C. Human C1q initiates the cascade by binding p15ETM at the virion surface. This is an antibody-independent process.

Murine retroviruses are heat labile. They have an infectious half-life of only 6 hrs in culture medium at 4°C.

#### IV. Vector

The retroviral siRNA plasmid included in the kit has been modified from pCLNCX vector. The double-stranded oligonucleotide coding for the siRNA can be cloned into the *Sal* I and *Xba* I sites of the vector. This vector contains a neomycin resistant gene (NeoR) under the control of 5'-LTR, which is useful for generation of permanent mammalian cell lines using G418 in the culture medium.



**Figure 1.** A schematic diagram of pSuppressorRetro retroviral siRNA plasmid.

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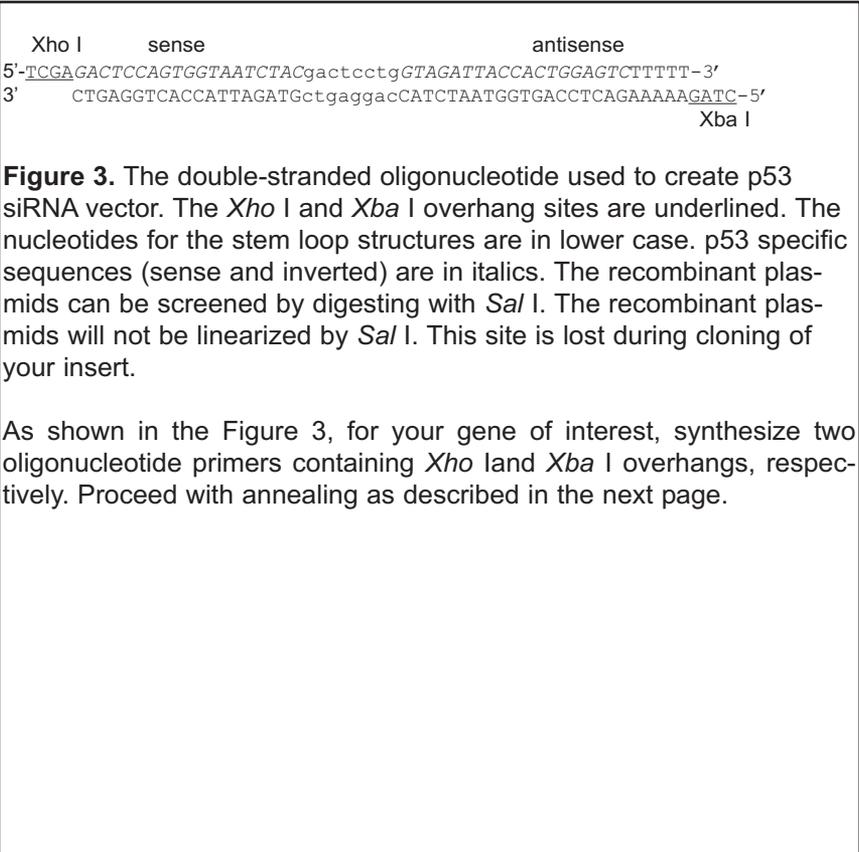
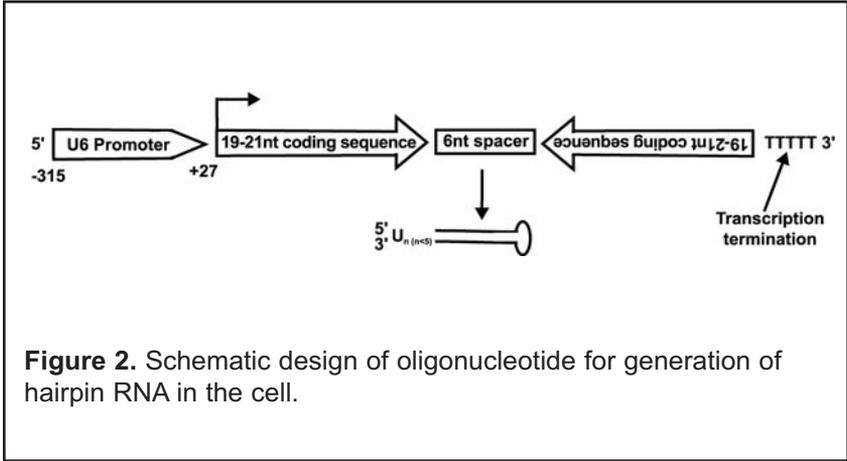
**Detailed protocol- Cloning the target siRNA of interest into pSuppressor cassette.****a) Preparation of vector and insert for cloning:**

The pSuppressor vector DNA (provided in the kit) has been digested with *Sal* I and *Xba* I to generate compatible ends for cloning. To ensure that the correct restriction fragment has been cloned, add compatible restriction sites: *Xho* I at the 5' end and *Xba* I at the 3' end of the primer sequences. *Xho* I restriction site is compatible with *Sal* I site and will allow cloning into *Sal* I site. However, after cloning into *Sal* I site, both *Sal* I and *Xho* I sites are lost. This strategy allows quick screening of the recombinant plasmids. The recombinant plasmids containing the inserts will not be linearized when digested with *Sal* I, where as the wild type of plasmid DNA will be linearized with *Sal* I.

**b) Design of Oligonucleotide inserts:**

The most critical part of the construction of the siRNA expression plasmid is the selection of the target region of the gene, which is currently a trial-and-error process. However, Elbashir et al, 2002 have provided a guideline that may work 80% of the time. According to them for synthesis of synthetic siRNA, a target region may be selected preferably 50 to 100 nt downstream of the start codon. 5' or 3' untranslated region and region close to the start codon should be avoided as these may be richer in regulatory protein binding sites. The ideal sequence for synthetic siRNA should be 5'-AA(N19)UU, where N is any nucleotide in the mRNA sequence and should be approximately 50% G-C content. However, for designing oligonucleotides for the expression vector, AA and UU dimer in the sequence is not needed.

For the expression vector, siRNA will be designed to produce hairpin RNAs, in which both strands of an siRNA duplex would be included within a single RNA molecule. The individual motif will be 19-21 nt long and corresponds to the coding region of the gene of interest. However, Paddison and Hannon, 2002 have suggested use of 18-28 nucleotides. The two motifs that form the inverted repeat are separated by a spacer of 4-9 nt (to form the hairpin loop) (Figure 3). The transcriptional termination signal for 5 Ts is added at the 3' end of the inverted repeat. Avoid 4-5 continuous stretches of "A" and "T" in your gene sequence, which may act as internal terminator. The insert can be prepared by synthesizing and annealing of two complementary oligonucleotides. An example of p53 siRNA sequence is given in Figure 3.



**c) Primer annealing (siRNA insert):**

1. Add the following components to a sterile microfuge tube.

Forward primer:	1 $\mu$ l	(1 $\mu$ g)
Reverse primer:	1 $\mu$ l	(1 $\mu$ g)
Annealing buffer	2 $\mu$ l	
Deionized water:	<u>16<math>\mu</math>l</u>	
	20 $\mu$ l	

2. Incubate at 95°C for 10 minutes.

3. Gradually cool the tube to room temperature.

The annealed siRNA insert can be used immediately or stored at -20°C until needed.

**d) Ligation of vector and insert:**

1. Linearized vector (completely digested with *Sa*I and *Xba*I) that has been provided in the kit should be used. Set up a ligation reaction as described below. A negative control ligation should be done without insert.

Vector DNA ( 50 ng/ $\mu$ l)	1 $\mu$ l
Insert DNA (100 ng/ $\mu$ l)	1 $\mu$ l
T4 DNA ligase	1 $\mu$ l
10X ligase buffer	2 $\mu$ l
Deionized water	<u>15 <math>\mu</math>l</u>
Final volume	20 $\mu$ l

2. Incubate the mixture at 16°C overnight.

3. Following the ligation reaction, transform the ligated plasmid DNA (10  $\mu$ l of the ligation mixture) into 90  $\mu$ l of competent cells of an appropriate host strain (DH5 $\alpha$ ). You may also use a transformation protocol routinely used in your laboratory. Plate on LB plates containing 100  $\mu$ g/ml of ampicillin.

**Note: We suggest to use competent cells with a minimum transformation efficiency of  $1 \times 10^9$  per microgram of DNA.**

**Note:** In order to monitor the efficiency of the ligation and transformation steps, as a negative control, competent cells should also be transformed with cut vector DNA that has been ligated in the absence of an insert. Check the presence of insert by digesting with *Sal* I restriction enzyme. As mentioned earlier, the recombinant plasmids containing inserts will not be linearized by *Sal* I. **Before transfecting the cells with the construct, always confirm presence of the correct insert by sequencing with the primer provided in the kit.**

If you have difficulty in sequencing the insert, For example: If the sequence stops at the loop, use a sequencing reaction that will allow to read through secondary structures. Addition of up to 4 % DMSO in the reaction may help. Please refer to paper published by D.C. Ducat F.J. Herrera and S.J. Triezenberg. *BioTechniques* 34:1140-1144 (June 2003) in order to overcome sequencing problems where secondary structures are involved.

#### **e) Plasmid DNA preparation:**

Any standard protocol, which provides high-quality DNA can be used to prepare plasmid DNA for transfection. Most of the time plasmid DNA using Qiagen mini plasmid DNA kit is sufficient for transfection.

## V. Outline of RetroMax Procedure

- |        |   |
|--------|---|
| Day 0  | Seed 293 cells and grow overnight.  |
| Day 1  | Transfect with retroviral vector containing gene of interest and an appropriate packaging vector.   |
| Day 2  | Replace medium.   |
| Day 3  | Harvest virus-containing supernatant. Virus may be stored at -70°C at this stage. Infect target cells, either for titer determination or for gene expression. |
| Day 4  | Split infected target cells and grow for selecting stable virus-producing cell lines. For transient expression experiments, you may harvest the cells now.    |
| Day 5  | Start selection by replacing the medium with G418 containing medium.  |
| Day 9  | Change medium and continue selection.   |
| Day 14 | Count antibiotic resistant colonies and calculate titer.  |

*Note: If you are using retroviral expression system for the first time, we strongly recommend using the LacZ control plasmid included in the kit. The  $\beta$ -galactosidase expression can be monitored using  $\beta$ -gal staining kit (Cat.#10053K) or any other standard protocol.*

## VI. Detailed Protocol

### a). Transfection

#### One Day Before Transfection

1. Seed  $1 \times 10^6$  293 cells in 6 cm tissue culture plates. This should yield a cell density of about 30% confluency on the day of the experiment. You may also use 10 cm plates, and seed  $2 \times 10^6$  cells if you would like to scale up.
2. Incubate overnight at 37°C in DMEM supplemented with 10% fetal bovine serum, pen-strep(1%).

#### Transfection of 293 cells with retroviral vector

##### Day 1

1. Add 0.25 ml for 6 cm tissue culture plates (0.5 ml for 10 cm plates) of RetroMax transfection buffer (previously tested for optimum transfection efficiency) to the required number of sterile 15 ml polypropylene tubes (Falcon 2059). Lipofectamine may have certain advantages in reproducibility, but this has not been tested extensively by us for overall virus titers.
2. Add the following to each required 1.5 ml sterile Eppendorf tube:
  - 30  $\mu$ l of 2 M  $\text{CaCl}_2$  (included in the kit) and 220  $\mu$ l sterile distilled water (60  $\mu$ l of 2 M  $\text{CaCl}_2$  and 440  $\mu$ l of dist. water for 10 cm plate)
  - 10  $\mu$ g pCL-Eco, pCL-Ampho, or pCL-10A1.
  - Add 10  $\mu$ g pSuppressorRetro Vector containing the siRNA construct in 0.25 ml of 0.25 M  $\text{CaCl}_2$ . Mix by vortexing. (If you are transfecting 10 cm plates using 10 ml of medium, use 15-20  $\mu$ g of each plasmid approximately 30-40  $\mu$ g total DNA in 0.5 ml of 0.25 M  $\text{CaCl}_2$ . Mix by vortexing.)
3. Add the DNA/ $\text{CaCl}_2$  mix dropwise to the transfection buffer tubes while lightly vortexing. Incubate at room temperature for 20 min (this step may be omitted). Add the DNA/ $\text{CaCl}_2$  mix dropwise to 293 cells in 4 ml DMEM-10%fetal calf serum, seeded the day before on 6 cm TC plates. Place in humidified  $\text{CO}_2$  incubator for 3-4 hrs. (Longer times may result in cells coming off the plates. However, we have left the cells in the transfection medium

overnight without loss of cells).

4. Carefully aspirate medium. Add 2 ml of warm PBS-15% Glycerol (no serum) for 2 min. This step is optional. In some cases it may increase the transfection efficiency by two-fold.

5. Aspirate PBS/ glycerol medium. Carefully add 4 ml DMEM-10% fetal calf serum along the side of the dish. (10 ml for 10 cm plates). Incubate for 12-18 hrs.

## Day 2

1. Aspirate medium and add fresh medium in the morning. This step helps dilute out a cytostatic factor that is produced by the transfected cells (both 293 and COS make it; untransfected cells do not). This factor is present even if virus is not. Transfection with any plasmid DNA stimulates its production. It may be TGF $\beta$  (Naviaux et al., personal communication), but we have not tested this. Since this factor is produced by 293 and COS (primate cells) and inhibits cell cycle progression in murine NIH 3T3 cells, it cannot be just an interferon (which are species-specific). If this step is omitted, you cannot use more than about 100  $\mu$ l of virus-containing medium to infect cells in 4 ml of medium (<2% by volume) if you want to stay in the linear portion of the dilution curves for the determination of virus titers. If more is used, say about 1 ml of supernatant, target cells (at least NIH 3T3 cells react this way) arrest in cell cycle, accumulate perinuclear refractile granules, and cannot be infected with retrovirus because they are not dividing, i.e., more supernatant yields less infection. Replacing the medium on day 2, as indicated here, reduces this problem.

## **Determining the Viral titer**

### Day 3 (24 hrs after addition of fresh medium).

1. Filter sterilize (0.45 mm syringe filters are convenient) the virus-containing supernatant to remove any cells in suspension. The virus can now be used directly, or stored at -70°C until needed.

2. Infect the desired target cells with 1  $\mu$ l to 4 ml of 293 supernatant in 8  $\mu$ g/ml Polybrene. The amount of supernatant you use depends on whether you are titering virus or want to infect the maximum number of target cells possible. Remember that for titer-

ing, you must dilute the transfected supernatant at least 50 fold to stay in the linear part of the dilution curve. If you just want the

maximum number of cells infected, then as little as a 2-fold dilution (equal volume mix) with the medium of the intended target cells is usually enough to prevent significant cell cycle inhibition. **Do not forget the polybrene. Omission of polybrene will drop your apparent titers 100-1000 fold.**

3. For titering, prepare a serial dilutions (four 10-fold dilutions) of vector supernatant in order to be sure that you are in the linear part of the titration curve (ie, out of the Poisson region). Infections for accurate titering must be done at effective MOI 0.1.

Target cells must be growing exponentially and only 30-50% confluent for maximum infection efficiencies.

4. Total virus-cell **contact time should be a minimum of 12-24 hrs.** This is because cycling cells are continuously entering and exiting the window of infectability. Even though the infective half life of the murine retrovirus particle is just 6-8 hrs at 37°C the rate of new cells entering the window is greater for the first 24 hrs, so longer contact times means more infected target cells.

5. Always test your titers on a standard control cell line (we use NIH 3T3) in parallel with infections of other desired target cells. Intrinsic infectability of many target cells can vary widely from 0.01-100% of the titers on NIH 3T3 cells.

- Virus titers on NIH 3T3 cells for empty RetroMax vectors are typically  $2-3 \times 10^6$  CFU/ml for ecotropic virus and  $1 \times 10^6$  for amphotropic virus, assuming a typical 293 transfection efficiency of 30-50%.

- When titering virus on NIH 3T3 cells, infect  $2 \times 10^5$  cells on a 6 cm plate (in 4 ml medium), overnight (16 hrs) with 1, 3, and 10  $\mu$ l of pCL vector supernatant. You will need larger volumes for lower titer vectors, or cells that are more refractory to infection than NIH 3T3.

- If virus stock is limiting: the most efficient use can be made by using 0.5-1 ml volumes to serially infect target cells in 6 cm plates (or 2-3 ml in 10 cm plates), and adding fresh virus every 4-6 hrs for 3-4 infection cycles. Continuous exposure to virus for about 24 hr is necessary in order to ensure that all cells have cycled through their receptivity window (S-G2) for retroviral infection. Be sure to add polybrene to 8  $\mu$ g/ml.

6. Check your transfection efficiency by drawing a 1 cm square on the bottom of the plate of transfected 293 cells. Scrape

harvest all the cells outside of this square (if desired) for RNA or protein analysis (CAT assays, ONPG-LacZ, Westerns, Northern, Hirts, etc.) Fix and stain the transfected cells remaining inside the 1 cm square with X-Gal to determine the transfection efficiency (TXE). Typical transfection efficiencies are 30-50% in this subline of 293 cells. The same DNA and reagents will give TXEs of 2-15% on COS cells.

## **Selection for stable virus-producing cell lines**

### Day 4 (12-24 hrs after infection)

1. If using a vector that confers G418 resistance, split the infected target cells at various dilutions (1:20 to 1:200) into 10 cm TC plates. A 1:20 dilution is about 105 NIH 3T3 cells. If 0.1% of the cells were infected, you will get about 100 colonies after 8-12 days of selection, and your Neo titer (in CFU/ml) can be calculated by a regression line using your two dilutions.

### When infecting primary cells:

Accurate titers cannot be obtained when infecting primary fibroblasts, bone marrow or tumor cells because these cell types display density-dependent growth and typically have low plating efficiencies of 0.1%. This means that if 1000-10,000 cells are plated, only 1-10 colonies will actually clone out, even if they are all infected and G418-resistant. Therefore when infecting these cells, do not split them more than they will tolerate and only if they are >80% confluent (this is usually only a 1:2 to 1:4 dilution).

If you are selecting primary cells in G418, you will need to trypsinize and concentrate the cells by replating on sequentially smaller dishes until sufficient G418-resistant cells have grown out that you can begin expanding the infected pool of cells. This process can take 2 weeks. Effective titers for a particular primary cell type and vector will be a constant percentage of the titer observed on NIH 3T3 cells.

If using vectors that do not confer antibiotic resistance (like LacZ or GFP), simply change the medium today. Primary bone marrow cells should always be infected by cocultivation of autochthonous stromal cells and virus producer cells in the presence of IL3 (or WEHI-conditioned medium) and GM-CSF (a potent stromal cell

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growth factor). Never select them in G418.

### Day 5 (2 days after infection)

1. Begin selection of cells infected with virus vectors conferring antibiotic resistance by adding 100  $\mu$ l of a 100X stock to a 10 cm dish containing 10 ml of medium.

The correct concentration of G418 (or any antibiotic) varies widely for different cell types. You must determine the concentration empirically. For NIH 3T3 cells this is 400-1000  $\mu$ g/ml (active) G418. For other cell types, the right concentration is that which results no observable death at day 2 and about-30-50% on day 4. Complete G418 selection is usually achieved in 7-10 days.

2. If using a virus vector that does not contain a selectable marker (e.g., pCL-LacZ, MFG-GM-CSF, GFP), or if you would like a rapid assessment of gene expression in the infected target cells (for vectors expressing CAT, Luciferase, GFP, or LacZ), this can be tested today: B-gal staining of fixed cells *in situ*, (you can calculate the LacZ titer of your virus from this); CAT, Luciferase, or ONPG assays are done from cell lysates).

3. Because of the natural kinetics of retroviral infection, integration, and expression, no selection pressure (antibiotics) or assessment of gene expression should be made until 48 hrs after infection, i.e., if cells are infected on day 3, gene expression cannot be accurately tested until day 5.

### Day 9 (4 days after starting selection).

1. Add fresh medium (and antibiotic) to cells under selection.

2. If infected cells were primary fibroblasts or primary tumor cells, you may need to increase the cell density (that has fallen due to the death of uninfected cells under selection) by one of two methods, in order to avoid cell death due to densities falling below that tolerated by your particular primary cell type: concentrate the infected cells by trypsinization and plating on a smaller dish, or add uninfected primary cells (of the same type) to bring the density up to 50%, and continue selection. You must let the added (non-G418 resistant) cells attach to the plates for 3-4 hrs before adding G418 again.

3. Most primary cells will not grow as isolated clones because

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of density-dependent growth requirements. Attempts to pick clones frequently result in the loss of all infected cells.

### Day 14 (10-13 days after infection)

1. Count the antibiotic resistant colonies, and calculate the titer (e.g., Neo titer) in your virus supernatants.

Example: Let us say you count 125 G418-resistant colonies on a 10 cm plate. If you infected ( $5 \times 10^5$ ) NIH 3T3 cells with 1 ml of virus supernatant, then split out the infected cells 1:20, your calculated titer is  $125 \times 1000 \times 20 = 2.5 \times 10^6$  CFU/ml

Note: Many cDNAs of interest may have either cytostatic or cytotoxic effects on infected cells, so that stable colony formation under G418 selection does not actually reflect the true number of cells initially infected. Only growing cells make colonies.

## **VII. Maximizing Retrovirus Titers**

1. The principal determinant of retrovirus titer is the abundance of packagable RNA, and not the abundance of viral proteins. Viral proteins are typically made in 20 fold stoichiometric excess. In fact, too much gp85 env can actually lower your titers because of impaired glycoprotein processing and assembly.

2. The RetroMax (pCL) system generates the highest abundance of packagable viral RNA of any known transient system by exploiting the power of the CMV IE enhancer-promoter in E1A-expressing 293 cells. The natural enhancer of the unmodified MuLV LTR is inhibited by E1A-p300 in 293 cells, so attempts to use non-pCL retroviral vectors in 293 cells will yield 20-50 fold lower titers, even with the same transfection efficiencies.

3. If you are studying cDNAs that do not have cytostatic or cytotoxic phenotypes, it may be possible to generate higher titer virus using traditional retrovirus packaging cells. This process takes 2 months (instead of 2 days for pCL). The highest titers are always obtained from stably infected (not transfected), cloned (not pooled) packaging cell lines. This is because transfected sequences are often inactivated by methylation, and because provirus integration position effects can influence gene expression from the same retrovirus vector in different clones of infected cells can vary over a 100 fold range (i.e., integration into heterochromatic regions of the genome gives poor expression, while integration into euchromatin regions gives high expression).

4. In deciding whether to go through the trouble of selecting and characterizing clones of packaging cells or simply preparing virus by the rapid pCL system, one must consider the intended applications. If you need a rapid test for the stable expression properties of a battery of mutant cDNAs that you have prepared, the pCL system is often adequate, or in the case of cytostatic and cytotoxic cDNAs, it is often the only way to produce usable amounts of virus.

Sometimes producing the virus (with a toxic or static cDNA) in cells from a different species can overcome the titer problems that result from cell growth inhibition.

If on the other hand, if you plan to use the virus produced as a reagent that you can go back to many times over the next few years, then you need to pick clones of stable packaging cells.

5. pCL vectors reproducibly produce titers of  $0.5-5 \times 10^6$  CFU/ml with good transfections, independent of phenotype and size (less than 4 kb) of the cDNA.

6. Typical retrovirus titers from cloned packaging cells are  $10^4-10^6$  CFU/ml (sometimes you can get a titer of  $10^7$ , depending profoundly on the size and toxic properties of the cDNA expressed in these mouse fibroblast cell lines. cDNAs that are 2-4 kb long lead to modest reductions in titer because of packaging constraints. cDNAs larger than 4 kb are subject to frequent spontaneous deletions and truncations during retroviral reverse transcription, and show large reductions in virus titers, and frequent non-expressing clones.

### **Scaling Up:**

1. Transfect 10 cm plates of 293 cells with 30-40  $\mu$ g of pCL vector containing your gene of interest in 1 ml of  $\text{CaCl}_2$ -HBS.

2. Replace the medium on day 2.

3. Harvest and replace the medium every 24 hrs on days 3, 4, and 5. This should give you 30 ml of virus supernatant from each transfected plate. The titers in supernatants harvested on days 3 and 4 are equivalent. We suspect that day 5 will be almost the same, but we have not specifically tested day 5 supernatants yet.

## VIII. The Safe Use of Murine Retrovirus Vectors and Safety Precautions

-Replication competent retroviruses (RCR) are called helper virus, or simply "Helper".

-They require 3 trans- (gag, pol, and env), and 7 major, cis-active control elements (U3, R, U5, PBS, SD, y, and SA) in order to replicate.

-The most common retrovirus vectors are based on the Moloney Murine Leukemia Virus (MoMuLV). Vectors encode only the 7 cis elements.

-These vectors are defective and can not replicate without picking up 7.1 kb of sequence by homologous recombination with a helper genome (while simultaneously deleting your cDNA). Modern vectors are now "safety modified" by including a stop mutation early in "gag" (or a frame-shift) that prevents gag translation and limits the sequence window available for productive recombination with helper genomes.

### Safety-Modified Vectors

- 1)LXSN
- 2)LNCX
- 3)MFG
- 4)pCLXSN
- 5)pCLNCX
- 6)pCLNRX
- 7)pCLNDX

### First-generation vectors

- 1) N2
- 2)BAG
- 3)pMSV-Neo
- 4)LNL-SLX
- 5)LNL-SLX

Packaging cells supply the nine processed proteins encoded by gag, pol, and env (p15MA, p12, p30CA, p10NC, p14PRO, p85RT, p40IN, gp70SU, and p15ETM) necessary for virion assembly.

Modern packaging cells are safety-modified by dividing the gag-pol genes, and the env gene on two separate plasmids. These two plasmids are serially transfected (not cotransfected) into NIH 3T3 cells. The resulting safety modifications yield the modern split genome packaging cells.

Current evidence suggests that in order to initiate a pathogenic infection in primates with amphotropic murine retroviral vectors, three requirements must be met:

1. The infected host must be immunocompromised.
2. The vector preparation must be contained with helper virus.
3. Direct body fluid contact, e.g., intravenous inoculation is required for transfer.

**HOWEVER, FOR SAFE USE OF THE RETROMAX SYSTEM, THE USER IS STRONGLY ADVISED TO FOLLOW THE FOLLOWING GUIDELINES:**

1. According to NIH guidelines all retroviral production and transduction work must be done in a Biosafety Level 2 (BL2) facility.
2. Work in laminar flow, HEPA filtered hoods that receive annual maintenance and recertification.
3. Use sterile technique (flaming is not necessary and not recommended because of convection disturbances to airflow patterns).
4. Aspirate all liquid waste into flasks containing 5-10% (v/v) of a microbicidal agent.
5. Discard spent plasticware in biohazard bags and autoclave before discarding.
6. Dispose spent glassware in detergent containers for cleaning and autoclaving.
7. Clean all surfaces with 70% ethanol at the end of the work.
8. Switch on the UV light.

## **IX. References:**

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