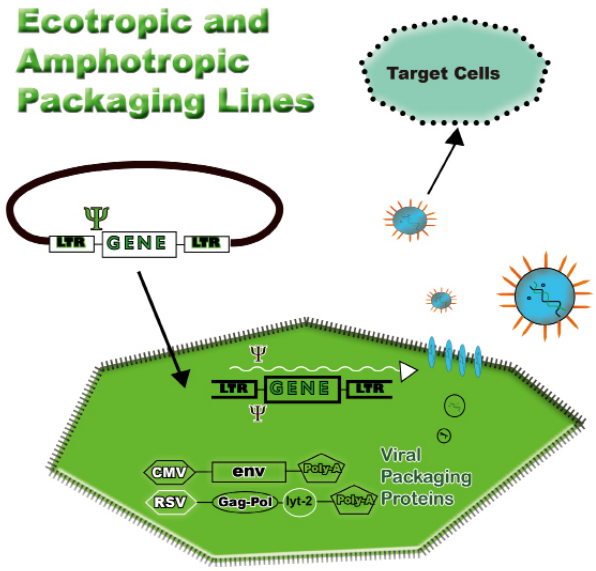
Gryphon™ Retrovirus Expression System, The Gryphon Retroviral system and the pCHAC vectors can be used to infect cells of human or mouse origins.

The Gryphon retroviral system is an upgraded version of the Phoenix™ Retroviral System, previously provided by Orbigen. This system has been used in over 2500 laboratories worldwide for delivery of genes and libraries to cells for biomedical research. An Ecotropic packaging system or an Amphotropic system is currently available for the delivery of genes to most dividing mammalian cell types. The Retrovirus Expression Kit includes: an Ecotropic, an Amphotropic packaging cell line, and two retroviral expression vectors for high-level protein expression in most mammalian cells.

Gryphon retrovirus producer lines, Gryphon Ecotropic and Gryphon Amphotropic, for the generation of helper free ecotropic and amphotropic retroviruses are based on the 293T cell line, which is highly transfectable using either calcium phosphate mediated transfection (see section VI) or lipid-transfection protocols. These retrovirus producer lines were created by placing constructs into 293T cells capable of producing gag-pol and envelope protein for ecotropic and amphotropic viruses. They are also selected for stable growth and accessibility to transfection independently from the previous Phoenix Eco and Ampho packaging cells. The lines offer many advantages over previous stable systems and these have led to their broad application worldwide.



Box 1 | Contents

Cat No. ABP-RVK-10001
10^6 Gryphon™ amphotropic packaging cells
10^6 Gryphon™ ecotropic packaging cells
5 µg pCHAC-MCS-IRES-mWasabi
5 µg pCHAC-mWasabi

Features

1. Virus can be produced in just a few days.

2. By introducing an IRES-CD8 surface marker downstream of the reading frame of the gag-pol construct, CD8 expression is a direct reflection of intracellular gag-pol and the stability of the producer cell population's ability to produce gag-pol can be readily monitored by flow cytometry.

3. To prevent potential recombination, different non-Moloney promoters for gag-pol and envelope were used.

4. Both lines are capable of carrying episomes for long-term stable retrovirus production.

5. Helper virus production in these lines have been extensively tested and found to be helper-virus free.

6. All[ele]ustrious mWasabi is a monomeric green fluorescent protein that can be easily detected using standard GFP filter sets. mWasabi may be used as a direct replacement for EGFP or other GFPs for superior performance, and may be co-imaged with blue and red fluorescent labels without substantial bleed-through.

Box 2 | Storage

- ♦Store the vectors at –20°C.
- ♦Store the medium and Transfection Reagent at 4°C for up to two months.
- ♦Amplify and store aliquots of amplified Gryphon cell lines in liquid nitrogen.

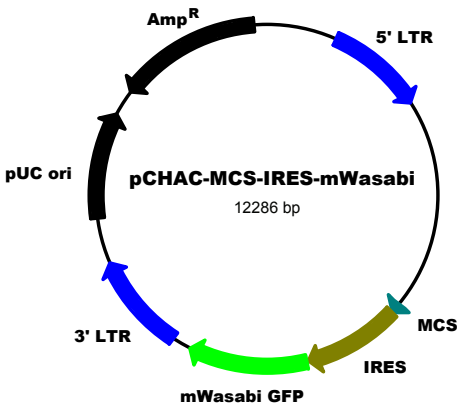
Retrovirus Vectors

Encoding GFP, these Moloney murine leukemia virus based vectors were specifically designed to facilitate the optimization of protein expression in a variety of mammalian cells utilizing FACS analysis. For comprehensive reviews of retrovirus biology, please refer to listed publications (Section V).

pCHAC-MCS-IRES-mWasabi

Catalog No: ABP-PVL-IRES20W
Quantity: 5 µg

1-596 bp	5' LTR
1901-1962 bp	MCS
1981-2556 bp	IRES
2557-3276 bp	mWasabiGFP
3340-3932 bp	3' LTR
4206-4845 bp	pUC Origin
10905-11765 bp	Amp Resistance gene



MCS

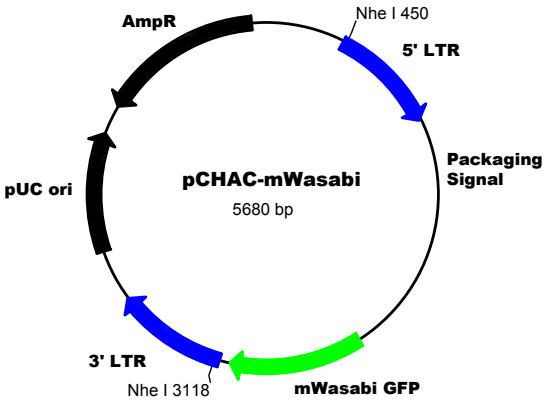
GGATCCATTTAAATTCGAATTCCTGCAGGCCTCGAGGGCCGGCGCGCCGCGGCCGCTACGTA

BamHI BstBI EcoRI SfiI XhoI NgoMI AscI NotI SnaBI

pCHAC-mWasabi

Catalog No: ABP-FP-LWasabi
Quantity: 5 µg

421-1012bp	5' LTR
2322-3032bp	mWasabiGFP
3089-3680bp	3' LTR
3955-4594bp	pUC Origin
4744-5604bp	Amp Resistance gene



Box 3 | Outline of Gryphon Retrovirus Protocol

Day 0 Seed Gryphon cells and grow overnight.
Day 1 Transfect into the appropriate Gryphon cells with retroviral vectors encoding gene of interest.
Day 2 Replace medium if necessary.
Day 3 Harvest virus-containing media, which may be stored for future use or utilized immediately to infect target cells for titer determination or for gene expression.
Day 4-5 For transient expression, harvest the cells and assay for protein of interest. For stable expression, initiate drug selection to isolate cells expressing high level of protein of interest.
Day 9 Refresh medium and continue drug selection.
Day 14 Isolate high expression clones.

I. Passaging Gryphon cells:

To achieve optimal cell conditions, passage cells at 1:4 or 1:5 at 70-80% confluent every 2-3 days. Never let cells reach confluence since this will reduce transfection efficiency in the short term.

Passage of Gryphon cells every few months in Allele's selection medium for packaging Cells (Dulbecco's Modified Eagle's Medium containing 10% heat inactivated fetal bovine serum, 300 µg/ml Hygromycin and 1µg/ml Diphtheria Toxin) for one week (2-3 passages depending on cell density) is recommended. The selection medium should be renewed with each passage. Cells can be analyzed and sorted by fluorescent activated cell scan (FACS) for expression of CD8 (a proxy measure of gag-pol in this cell line) and for surface expression of envelope protein with 83A25 antibody.

I-1. Thawing Gryphon cells:

It is important to freeze multiple vials of each producer cell line after first receiving and expanding them.
This will ensure a ready supply of backup vials to allow for uniform virus production over several years. If the cells are to be carried in selective media, this should not be applied until after the first passage.

1. Remove the vial containing frozen cells from Liquid Nitrogen or shipping box with dry ice.
Thaw rapidly at 37°C by holding the vial gently shaking in the water bath. Take out the vial from the water bath when the frozen cells start to thaw (about 1-2 minutes, the key point is "Not to thaw the cells completely").

2. Immediately add 1 ml Growth Medium (GM: DME containing 10% heat inactivated fetal bovine serum, 100 U/ml Penicillin, 100 U/ml Streptomycin, 2 mM L-Glutamine) to the freezing vial and gently transfer this solution to a 15 ml sterile conical screw cap tube.

3. Add 2 ml of GM and gently mix the tube to allow for osmotic equilibration.

4.Add 10 ml of GM, close the tube, invert several times and spin cells at 500 x g for five (5) minutes.

5.Remove the supernatant, resuspend cell pellet in GM, and transfer to a 10 cm tissue culture dish.

I-2. Growth and passage of Gryphon cells

Gryphon cells derived from 293 cells are carried in GM and grown in a 37°C incubator containing 5% CO2. To split and passage the cell lines:

1. Gently rinse x 1 with PBS (without Ca2+ or Mg2+).
2. Trypsinize (.05% trypsin/0.53 mM EDTA) until the cells easily detach and can be readily pipetted into a single cell suspension.
3. Trypsinization is quenched with GM prior to subculturing in fresh medium.

It is suggested that the cells not be split at densities more dilute than 1:5 in order to maintain the uniformity of the cells in culture and minimize the outgrowth of clonal variants. The cells should not be allowed to become overconfluent, as this leads to the formation of cell clumps in culture which can cause uneven cell distribution after replating and result in less efficient transfection.

I-3. Freezing Gryphon cells:

To assure viability of the cell line, it is recommended that the cells are frozen prior to confluence.

Gryphon cells are much less adherent than NIH3T3 cell lines and easily detach from the tissue culture dishes after approximately 30 seconds of treatment with trypsin at room temperature.

1. Wash, trypsinize, and quench cells as described in I-2.
2. Centrifuge the cells at 500 x g for 5 min.
3. Remove the media and add 1 ml of freezing solution (Freezing Medium: 90% heat-inactivated fetal calf serum, 10% DMSO) per 10^6 cells.
4. Transfer to a 2 ml cryogenic vial.
5. Place the freezing vial at -70°C overnight and transfer to liquid nitrogen on the following day.

II. Gryphon **retrovirus production**

Important Considerations

♦It is recommended that during initial set up, the user optimize the system by using a retroviral vector expressing an easily detectable marker such as a cell surface protein. During optimization, one should check for transfection frequency of the producer clone and test infection rate of target cells. Tests for transfection and infection frequencies using a GFP based system can be readily measured by FACS respectively. Only when one is satisfied with the transfection conditions and infection rates should one proceed to using vectors with no readily detectable marker. It should be possible to scale up the protocols.

♦The initial plating of the cells may be the most important step in successfully obtaining high retroviral titers. It is extremely important that the cells are not overly clumped and are at the correct density.

♦Unlike NIH3T3-derived cell lines, Gryphon cell lines do not readily form well-spread monolayers. Instead, they tend to clump before confluence, and if the clumping is excessive, the cells will never reach confluence during the 48-72 hour period following transfection. In order to prevent clumping, it is essential that the cells are extremely healthy prior to plating. If they are overconfluent, it may be necessary to split them 1:2 or 1:3 for several passages prior to plating for transfection. In addition, the cells are much less adherent than murine fibroblasts and should be handled very gently when washing and changing medium. For consistency, it is important to count the cells rather than estimating the split.

♦The recommended cell number for transfection below is optimized for pCHAC-mWasabi. Expression of other inserts may be detrimental to the growth of the cells. This effect may be noted by failure of the packaging cell line to reach confluence by 48-72 hours post-transfection. If this occurs, it may be necessary to plate more cells prior to transfection.

♦For example, with constructs expressing either fas or P210bcr/abl, it is necessary to plate 3.0 x 10^6 cells per 60 mm plate 24 hours prior to transfection. In general, the cells should be plated at a density so that they are 95-100% confluent at 24 hours post-transfection.

♦The addition of chloroquine to the medium appears to increase retroviral titer by approximately two fold.

This effect is presumably due to the lysosomal neutralizing activity of the chloroquine. It is extremely important that the length of chloroquine treatment does not exceed 12 hours. Longer periods of chloroquine treatment have a toxic effect on the cells causing a decrease in retroviral titers. The range for chloroquine treatment is 7-12 hours with 9-10 hours of treatment giving the best results. For purposes where achieving maximal retroviral titer is not necessary, such as when comparing the relative titers of different constructs, it may be preferable to omit chloroquine treatment. If chloroquine is not used, it is unnecessary to change the medium prior to transfection. On some occasions, we have obtained slightly improved transfection efficiencies by adding the chloroquine to a 1:1 mixture of 293 conditioned media (obtained from any of the 293-based cell lines) and fresh GM.

♦It is important that the pH of the Hepes be adjusted to 7.05 (within .05 units). Although, we generally add the HBS to the DNA/CaCl2 solution by bubbling, equivalent results can be obtained by adding the HBS to the DNA/CaCl2 solution and immediately inverting or vortexing the tube. The HBS/DNA/CaCl2 solution should be added to the cells within 1-2 minutes of preparation. It is not only unnecessary to wait for the formation of a visible precipitate, but waiting this long (15-30 minutes) may have a detrimental effect on transfection efficiency and subsequent retroviral titers. In addition, the presence and/or amount of precipitate that one visualizes following transfection is not a reliable indicator of transfection success.

♦We have used DNA prepared by both cesium chloride gradients and several commercial kits and have not found significant differences among titers generated with retroviral plasmids derived from the different preparation methods. It is unnecessary to perform additional phenol or precipitation steps prior to using the DNA. Up to a point, transfection efficiency and retroviral titers increase with increasing amounts of input DNA. The benefit of increasing the amount of input DNA must be weighed against our findings that this appears to have a direct toxic effect to the cell lines. If it is found that the amount of DNA is toxic to the cell line, it may be necessary to decrease the amount of input DNA or increase the number of plated cells. In some experiments, we have introduced up to 15 µg of DNA to a 60 mm plate during transfection. Incubator CO2 concentrations, which are outside the range of 4.5%-5.5%, may adversely affect transfection efficiency.

Day 0: Preparation of Gryphon Retrovirus Producer cells for transfection.

At 18-24 hours prior to transfection, seed Gryphon cells at 1.5-2 x 10^6 cells per 60 mm plate in GM. After adding cells gently shake forward and backward, then side to side, 3-4 times to distribute cells evenly about the plate. Set plates in 37°C incubator. Subconfluent cells (60-70% are best suited for transfection and potentially generate the highest viral titer. Transfection efficiency of 50-60% should be achieved.

Day 1: Transfection

Transfect subconfluent Gryphon cells using either a DNA shuttle (catalog no. ABP-TC-ATR125U), conventional cationic lipids, or calcium phosphate protocols (see section VI). Applied about 5-10 µg of plasmid DNA (pCHAC-MCS-IRES-mWasabi or pCHAC-mWasabi) per 60 mm dish. Including chloroquine (25 mM chloroquine stock solution prepared in either PBS or GM and filtered through a 0.2 µM filter and stored at -20°C) at 25 µM during calcium phosphate transfection have been found to yield higher viral titer (2 folds). Transfections of Gryphon cells with cationic lipids have been found to work well. After transfection, cells should be incubated at 32°C for greater virus stability and thus, higher yield. A lower level of viral production will occur at 37°C.

Day 2: 24 hours post-transfection

Because cells exhibit significant lethality if treated with chloroquine for more than 24 hours, overnight media should be replaced with 3 ml of fresh GM. If transfection was performed with cationic lipid, follow the manufacturer's instruction.

Day 3: Harvest at 48 hours post-transfection

Harvest supernatant from transfected Gryphon cells into 15 ml tubes and centrifuge at 2000 x g for 5 minutes to pellet cell debris. The virus stock can be frozen at -80°C for future use although the titer drops by one-half for each freeze-thaw cycle and thus, the virus stock should be stored in many small aliquots. Allele Biotech does not recommend the use of ultracentrifugation to concentrate the retrovirus stock since hydrodynamic force generated during centrifugation can remove most of the viral glycoprotein and thus, the infectivity of virus particles. Transfection efficiency of transfected Gryphon cells should be determined and optimized since this parameter is the primary determinant of high virus yield. If the gene of interest is expressed from Allele's retroviral vector pCHAC-MCS-IRES-mWasabi, the transfection efficiency can be easily monitored under fluorescent microscopy.

III. Determination of virus titer

III-1. Adherent cells

Day 0: Preparation of NIH3T3 cells for Gryphon retrovirus infection.

Split NIH3T3 cells at 5x10^5 per 35 mm plate.

Day 1: Infecting cells with retroviral supernatant.

Perform a serial dilution of your virus stock (e.g. 10 fold dilution to 10-5) with standard fibroblast medium (SFM: DME containing 10% heat inactivated donor bovine serum, 100 U/ml Penicillin, 100 U/ml Streptomycin, 2 mM L-Glutamine) supplemented with 15 µg/ml of polybrene (the stock concentration is 4 mg/ml dissolved in PBS and subsequently filtered through a 0.2 µM filter and stored at either 4°C or 20°C). Infect NIH3T3 cells on 35 mm plate with 1 ml of diluted stock for 12-24 hours at 37°C.

Day 2-3: Assay the expression of gene of interest at 24-48 hours post-infection.

The actual reverse transcription and integration take place within 24-36 hours post-infection, depending on cell growth kinetics. Expression can be observed as early as 24 hours and reaching a maximum at 48 hours. Thereafter continued retroviral expression might drop over a period of weeks to months, depending on the cell line, the site of integration, and the relative toxicity of insert.

III-2. Suspension cells

Day 0: Preparation of Jurkat cells for Gryphon retrovirus infection.

Culture Jurkat cells at log phase growth, approximately 5x10^5 cells/ml.

Day 1: Infecting cells with retroviral supernatant.

Pellet Jurkat cells by a brief centrifugation at 500 x g for 10 min. Resuspend 5 x 10^5 cells in 1 ml of fresh media containing diluted retroviral supernatant (>100 folds) and incubate for 12-24 hours at 37°C.

IV. Infection of target cells.

Efficiency of retrovirus infection is specific to each cell type and can vary greatly.

IV-1. Infection of adherent fibroblasts

Day 0. Preparation of NIH3T3 cells for retrovirus infection.

Approximately 12-18 hours prior to infection, plate 5 x 10^5 NIH3T3 cells in SFM (standard fibroblast medium) on a 100 mm plate.

Day 1. Infecting cells with retroviral supernatant.

Prepare a 3 ml infection cocktail consisting of retroviral-containing supernatant, polybrene at a final concentration of 4 µg/ml, and SFM. Remove the SFM from the NIH3T3 cells and add the 3 ml infection cocktail to the cells and place into incubator for 3 hours. Remove plate, add 7 ml of SFM to the cells, and continue incubation.

Day 3. Harvest (stain, drug select, etc.) the infected cells at 48 hours post infection.

IV-2A. Infection of non-adherent cells.

The conditions described are for infecting 60 mm plates.

Day 0. Preparation of non-adherent cells for retrovirus infection.

Prepare an infection cocktail consisting of the medium in which the target cells are grown, retroviral supernatant and Polybrene (2 µg/ml) such that the total volume is 3 ml.

Day 1. Infecting cells with retroviral supernatant.

Centrifuge exponentially growing target cells at 500 x g for 5 minutes. Remove supernatant and resuspend the cells in the infection cocktail at a concentration of 10^5-10^6 cells per ml and add to 60 mm plate. Spin infect (spinoculate) cells for 90 min at 30°C at 2500 rpm. Remove virus supernatant and resuspend cells in 3 ml of appropriate media for growth of target cells. Incubate at 37°C.

Day 4. Refresh media.

Allow the cells to grow for 72 hours before drug selection or other assays, such as flow cytometry for the presence of mWasabi green fluorescent protein.

When working with non-adherent cells, one can add the retroviral supernatant directly to the cells or co- cultivating the non-adherent cells with the retroviral producer cells. The advantage of the latter is that there is ongoing retroviral production; however, this must be weighed against the disadvantage of harvesting producer cells together with the target cells. Although we have not tried, it may be possible to minimize this problem by irradiating or mitomycin C treating the producer cells prior to co-cultivation. In general, we have obtained higher infection frequencies by co-cultivation. For many non-adherent cells, achieving an optimal infection requires growth in the appropriate medium. Because 293 cells and their derivatives (i.e., Gryphon cells) appear to tolerate many different medium bases and serum types, it is possible to alter the medium at 24 hours post transfection so that the resulting retroviral supernatant will be harvested in the appropriate growth medium. When infecting with supernatants derived from 293 cells or derivatives, it should be remembered that these cells may provide a different cytokine/growth factor milieu than the NIH3T3-derived producer cells. A careful analysis of factor production by these cells has not been performed.

IV-2B. Infection of non-adherent cells by co-cultivation with retroviral producing cells.

Conditions are described for 60 mm plates.

Day 0. Preparation of Gryphon cells for retrovirus infection.

Transfect the Gryphon cells as described previously.

Day 1. Infecting cells with retroviral supernatant.

Twenty four hours post-transfection, prepare a 3 ml infection cocktail consisting of (i) Polybrene at a final concentration of 2 µg/ml, (ii) 1 ml of fresh or freshly thawed retroviral supernatant, and (iii) the non adherent cells at a density of 10^5-10^6 cells per ml in the appropriate media for normal growth of the target cells. Remove the medium from the cells and gently add the infection cocktail to the cells (Add the cocktail to the side of the plate rather than directly to the cells). Follows spinoculation procedures described above. Return the cells to the incubator.

Day 3. Transfer target cells to fresh medium.

Forty eight hours post-infection, gently remove the medium, which will contain many non-adherent cells, and transfer to a conical tube. Centrifuge for 5 min at 500 x g. Remove the supernatant and gently resuspend the cell pellet in a freshly prepared infection cocktail as described previously. With extreme care to avoid disruption of the adherent cells, add the infection cocktail containing non-adherent cells to the wall of the plate rather than directly to the cells. Return the plate to the incubator.

Day 4. Transfer target cells to growth medium.

Seventy hours post-transfection, remove the non-adherent cells from the dish by gently pipetting. Centrifuge the cells (5 min, 500 x g) and resuspend in the appropriate media for normal growth of the target cells. Allow the cells to grow for an additional 24-48 hours before drug selection or other assays.

At this point, it is important that disturbance of the packaging cells is minimized. Use extreme care when removing the non-adherent cells from the packaging cells and do not wash the plate at this step. Also, it is not necessary to remove all of the non-adherent cells at this step since the purpose of this step is to add fresh medium and retroviral supernatant without losing non-adherent cells. Sufficient residual media remains on the plate to maintain the cells during the short centrifugation step described above. With longer centrifugation times, return the culture plates to the incubator.

The plate may be washed at this step; however, extreme care should be used so that the adherent cells do not detach. At this step, one is trying to achieve maximal removal of non-adherent cells. With this procedure, contamination by packaging cells is often less than 10%.

Transfection:

Cells are prepared and transfected generally as you would with a typical expression plasmid transfection.

Most commercial transfection reagents may be used with these cells. Use 5-10 µg plasmid DNA per well of a 6-well plate as a starting point.

Using AvantGene:

* All Volumes are for each well of 6-well plate:

1. Plate cells approximately 24 hours prior to transfection at a cell density of 20-40% confluence in complete medium (with serum and antibiotics if required).

2. Mix 10 µl AvantGene reagent to 40 µl serum free, antibiotics-free medium, incubate 5-10 min at room temperature.

3. Add 50 µl DNA Diluent to 5-10 µg DNA in 5-10µl of TE or water, incubate 1-5 min at room temperature. Do not incubate longer than 5 min.

4. While waiting, change cell medium to 1.5-2 ml of serumfree and antibiotics-free medium.

5. Mix diluted transfection reagent from Step 2 with DNA solution from Step 3, incubate at room temperature for 5-10 min.

6. Add the transfection mixture from step 5 dropwise to cells.

7. After 2-4 hours incubation under appropriate conditions in an incubator, add 1.5 ml serum-containing normal medium.

Important Note

Before initiating retrovirus experiments, Allele Biotech recommends that the user follows the NIH guidelines regarding retroviral production and transduction in a Biosafety Level 2 (BL2) facility. The viral supernatants produced by these methods, depending upon your retroviral insert, may contain potentially hazardous recombinant virus. The user of these systems must exercise due caution in the production, use, and storage of recombinant virions, especially those with amphotropic and polytropic host ranges. This consideration should be applied to all genes expressed as amphotropic and polytropic pseudotyped retroviral vectors. Appropriate NIH and regional guidelines should be followed in the use of these recombinant retrovirus production systems.

Box 4 | References

♦Heemskerk, M. H. M., Blom, B., Nolan, G., Stegmann, A. P. A., Bakker, A. Q., Weijer, K., Res, P. C. M. & Spits, H. (1997) J. Exp. Med. 186, 1597-1602

♦Kinsella, T.M. and Nolan, G.P. (1996) Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. Human Gene Therapy. 7:1405-1413.

♦Pear, W., Scott, M., and Nolan, G.P. (1997) Generation of high titer, helper-free retroviruses by transient transfection. In Methods in Molecular Medicine: Gene Therapy Protocols, (P. Robbins, ed.), Humana Press, Totowa, NJ), pp. 41-57.

♦Mulligan, R.C. (1993) The basic science of gene therapy. Science 260, 926-932.

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

Gentaur Molecular Products
Voortstraat 49
1910 Kampenhout, BELGIUM

Tel 0032 16 58 90 45 | Fax 0032 16 50 90 45
www.gentaur-worldwide.com
info@gentaur.com