# pVPack Vectors

## Instruction Manual

**Catalog #217566, #217567, #217568, #217569, #217570**

Revision C0

**For Research Use Only. Not for use in diagnostic procedures.**

217566-12

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# pVPack Vectors

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# pVPack Vectors

## MATERIALS PROVIDED

| Vectors | Quantity |
|---|---|
| *gag-pol*-expressing vector, (1 µg/µl in TE buffer) | |
| pVPack-GP vector (Catalog #217566) | 20 µg |
| *env*-expressing vectors, (1µg/µl in TE buffer) | |
| pVPack-VSV-G vector (Catalog #217567) | 20 µg |
| pVPack-Ampho vector (Catalog #217568) | 20 µg |
| pVPack-Eco vector (Catalog #217569) | 20 µg |
| pVPack-10A1 vector (Catalog #217570) | 20 µg |
| pFB-Neo-LacZ control vector (1µg/µl in TE buffer, Catalog #240029) | 10 µg |

## STORAGE CONDITIONS

**All components:** –20°C

## ADDITIONAL MATERIALS REQUIRED

Transfection MBS Mammalian Transfection Kit (Agilent Catalog #200388)
5-ml BD Falcon polystyrene round-bottom tubes (BD Biosciences catalog #352054)
HEK293 cells [ATCC, Catalog #CRL-1573] or 293T cells (Stanford University[1])
PBS[§]
Cell growth medium[§]
MBS-containing medium[§]
Chloroquine solution[§]
DEAE-dextran solution[§]
50-ml conical tubes
0.22 µm and 0.45 µm filters
Sterile $H_2O$

## MATERIALS REQUIRED FOR OPTIONAL PROTOCOLS

Viral RNA isolation kit
RNase/DNase-free microcentrifuge tubes
DNase I, RNase-free
10 × DNase I buffer[§]
thermocycler

[§] See *Preparation of Media and Reagents*

Revision C0

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## CMV Promoter

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## IRES Sequence

Use of the translation enhancer of the pVPack vectors is covered by U.S. Patent No. 4,937,190 and is limited to use solely for research purposes. Any other use of the translation enhancer of the pVPack vectors requires a license from WARF. WARF can be reached at P.O. Box 7365 Madison, WI 53707-7365.

# INTRODUCTION

Efficient delivery of markers, genes, or whole libraries into cultured cells is a necessity for many areas of research. The development of tissue culture systems for the production of high titer recombinant retrovirus that are capable of infecting a virtually limitless range of cell types has had a tremendous impact on these fields. With Moloney Murine Leukemia Virus (MMLV)- based vectors, transduction efficiencies of >90% are achievable for most mitotic cell types, and the copy number per cell can be easily controlled by varying the multiplicity of infection. Standard transfection methods typically result in only a small population of transfected cells capable of the uptake and stable integration of vector DNA. Additionally, copy number is unpredictable and often prohibitively high for many applications, such as cDNA library expression screening.

The pVPack vector system comprises a set of 5 vectors that can be used with any MMLV-based retroviral vector to produce viral supernatants that have titers that are consistently $\geq 10^7$ colony forming units (cfu)/ml in transient triple-transfection experiments. The vectors include a *gag-pol*-expressing vector that is cotransfected with the retroviral expression vector together with one of a choice of 4 envelope (*env*)-expressing vectors. The choice of the *env*-expressing vector is based on the range of cell types the user wishes to transduce. With the pVPack vector system, all of the *cis* and *trans* elements required to produce infectious virus are separated onto three plasmids, with minimal or no sequence overlap between the plasmids. This makes the pVPack system much safer than the majority of stable producer cell lines or vector-based systems for which there is a large degree of homology between the packaging vector(s) and the retroviral expression vector. In these latter systems there is a relatively high probability of production of replication-competent retrovirus (RCR) due to homologous recombination between the vectors.[2]

Although the relative speed and simplicity of the transient high titer virus production protocol described in this manual will be attractive for most applications, the *gag-pol* and *env* open reading frames (ORFs) are all followed by an IRES linked to a downstream drug-resistance gene so that these vectors may also be used to produce stable producer lines if desired. The antibiotic-resistance genes used in the *gag-pol* and *env* vectors are different from each other, and from those used in most of the more popular retroviral vectors, so that any *env* vector may be used with the *gag-pol* vector and with a wide range of antibiotic-resistant retroviral vectors to produce triple-stable viral producer lines. The position of the antibiotic-resistant gene as the second ORF in a bicistronic expression cassette, as opposed to its expression from a second cassette on the same plasmid, ensures that expression of the viral packaging proteins is comaintained with the antibiotic-resistant genes by the inclusion of antibiotics in the media.

## Overview of Replication-Defective Virus Gene Transfer Systems

Non-replicating retroviral vectors contain all of the *cis* elements required for transcription of mRNA molecules encoding a gene of interest, and packaging of these transcripts into infectious virus particles (Figure 1). The vectors are typically composed of an *E. coli* plasmid backbone containing a pair of 600 base pair viral **l**ong **t**erminal **r**epeats (LTRs) between which the gene of interest is inserted. The LTR is divided into 3 regions. The U3 region contains the retroviral promoter/enhancer. The U3 region is flanked in the 3´ direction by the R region, which contains the viral polyadenylation signal (pA), followed by the U5 region which, along with R, contains sequences that are critical for reverse transcription. Expression of the viral RNA is initiated within the U3 region of the 5´ LTR and is terminated in the R region of the 3´ LTR. Between the 5´ LTR and the coding sequence for the gene of interest resides an extended version of the viral packaging signal (ψ+),which is required in *cis* for the viral RNA to be packaged into virion particles.

In order to generate infectious virus particles that carry the gene of interest, the vector is transfected into tissue culture cells that harbor a source of the viral proteins that are required for virus production. In the past specialized packaging cell lines have been generated. These cell lines contain chromosomally integrated expression cassettes for viral Gag, Pol, and Env proteins, all of which are required in *trans* to make virus. The *gag* gene encodes internal structural proteins, *pol* encodes reverse transcriptase (RT) and integrase, and the *env* gene encodes the viral envelope protein, which resides on the viral surface and facilitates infection of the target cell by direct interaction with cell type-specific receptors; thus the host range of the virus is dictated not by the vector DNA but by the choice of the *env* gene used to construct the packaging cell. Recent advances in transfection technology have allowed the production of high-titer viral supernatants following transient cotransfection of the viral vector together with expression vectors encoding the *gag*, *pol*, and *env* genes. This obviates the need for the production and maintenance of stable packaging cell lines.

Once the viral RNA is encapsidated, virus particles bud off and are released into the cell supernatant. The supernatant of these transiently transfected cells can be collected and used to infect target cells. Upon infection of the target cell, the viral RNA molecule is reverse transcribed and the cDNA of interest, flanked by the LTRs, is integrated into the host DNA. Because the vector itself carries none of the viral proteins, once a target cell is infected the LTR expression cassette is incapable of proceeding through another round of virus production.

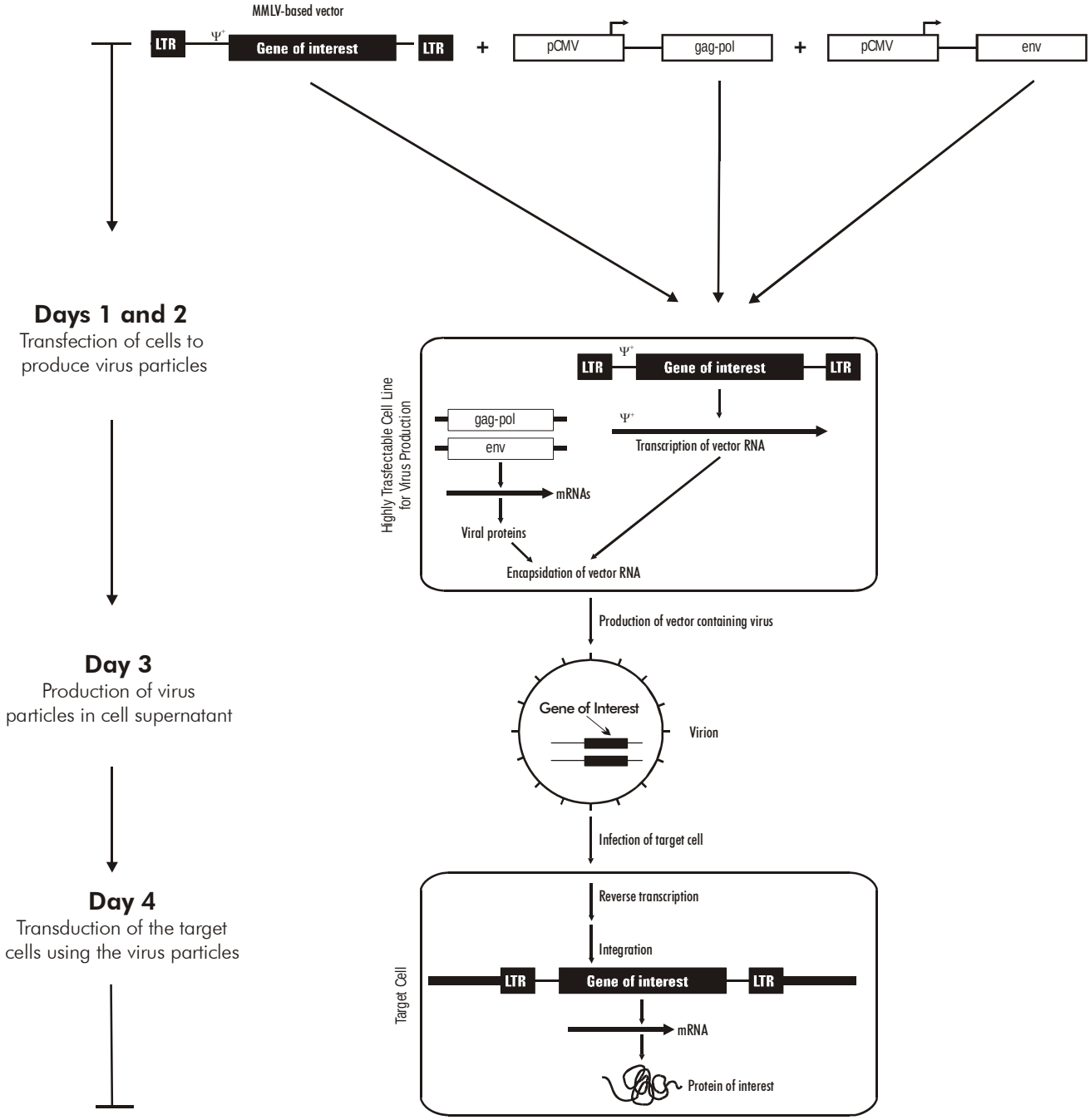


**FIGURE 1** Production of a replication-defective virus

## Description of the Vectors

### Choice of *env*-Expressing Vector

In addition to the *gag-pol* expression vector pVPack-GP, the pVPack vector system offers 4 different *env*-expressing vectors. Which of those 4 is selected depends on the choice of host cell type; see Table I and Miller (1997).[2] The pVPack-Eco vector is the safest vector, providing experiments can be performed in transduced mouse or rat cells; ecotropic virus infects human cells with extremely low efficiency. The amphotropic envelope protein has historically been the protein of choice for infection of human and other mammalian cell lines. More recently the 10A1 envelope protein has been used due to its increased versatility relative to the amphotropic protein. The 10A1 protein recognizes the same cell-surface receptor as the amphotropic envelope protein plus a second receptor, and thus can essentially infect any cell that an amphotropic virus can infect, although in some cases with a higher efficiency. The ecotropic, amphotropic and 10A1 proteins are all natural MMLV variants, and are all relatively labile and thus considered relatively safe compared with other viral systems. The vesicular stomatitis virus G protein (VSV-G) is rapidly becoming the most popular envelope protein. Unlike the other three MMLV-derived envelope proteins which recognize cell surface receptors, VSV-G recognizes a phospholipid that is present on all cell types, and thus can theoretically allow the efficient infection of any mitotic cell.[3] Special precautions must be used when working with this vector (see *Preprotocol Safety Considerations*).

**TABLE I**
**Host Range of Packaging Vectors**

| Target cell | pVPack-Eco | pVPack-Ampho | pVPack-10A1 | pVPack-VSV-G |
|---|---|---|---|---|
| Mouse | + | + | + | + |
| Rat | + | + | + | + |
| Hamster | – | +/– | + | + |
| Rabbit | – | + | not determined | + |
| Mink | – | + | + | + |
| Cow | – | +/– | not determined | + |
| Cat | – | + | + | + |
| Dog | – | + | + | + |
| Monkey | – | + | + | + |
| Human | – | + | + | + |
| Chicken | – | +/– | not determined | + |

\+ indicates good transduction efficiency
+/– indicates highly reduced transduction efficiency
– indicates very poor transduction efficiency

## Vector Features

Figures 2 and 3 illustrates the important features of the vectors in the pVPack system. The expression of both the *gag-pol* elements in the pVPack-GP vector and the envelope elements in the *env*-expressing vectors are driven by the CMV promoter. Each of these vectors also contains an **i**nternal **r**ibosome **e**ntry **s**ite (IRES) linked to a downstream drug-resistance cassette that enables the selection of stable producer lines. The vector pVPack-GP and the *env*-expressing vectors employ different resistance cassettes, *hisD* and puromycin, respectively. Methods for selecting stable producer lines are discussed in Hartman and Mulligan (1988)[4] and Wirth and colleagues (1988).[5]

**Notes** *If a compatible MMLV-based retroviral vector is chosen, all three vectors can be maintained simultaneously. Although stable VSV-G-expressing cells lines have been successfully constructed, in general they are of poor quality due to the toxicity of VSV-G. For the production of VSV-G pseudotyped virus, transient transfection rather than selection of stable cell lines is recommended.*

*The bacterial origin of replication, pUC, and ampicillin resistance cassette are included to permit maintenance and production of the vectors in* E. coli.

## The pVPack-GP Vector

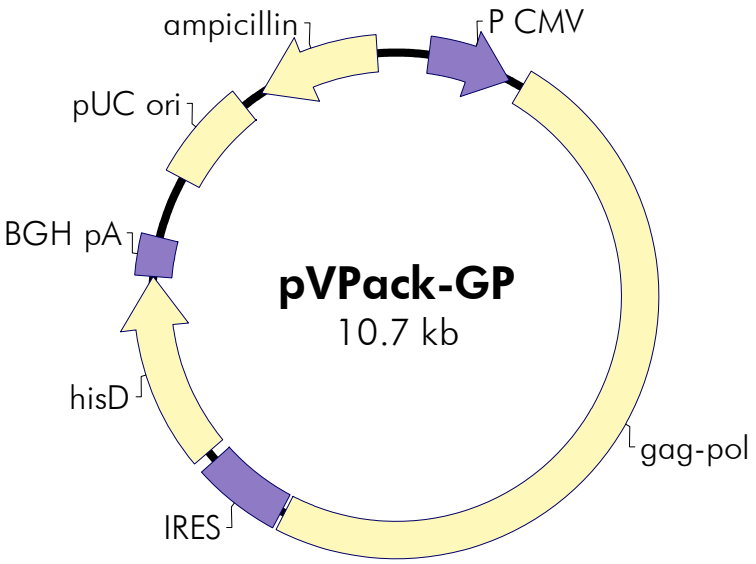


**FIGURE 2** Map of the pVPack-GP vector.

## The *Env*-Expressing Vectors

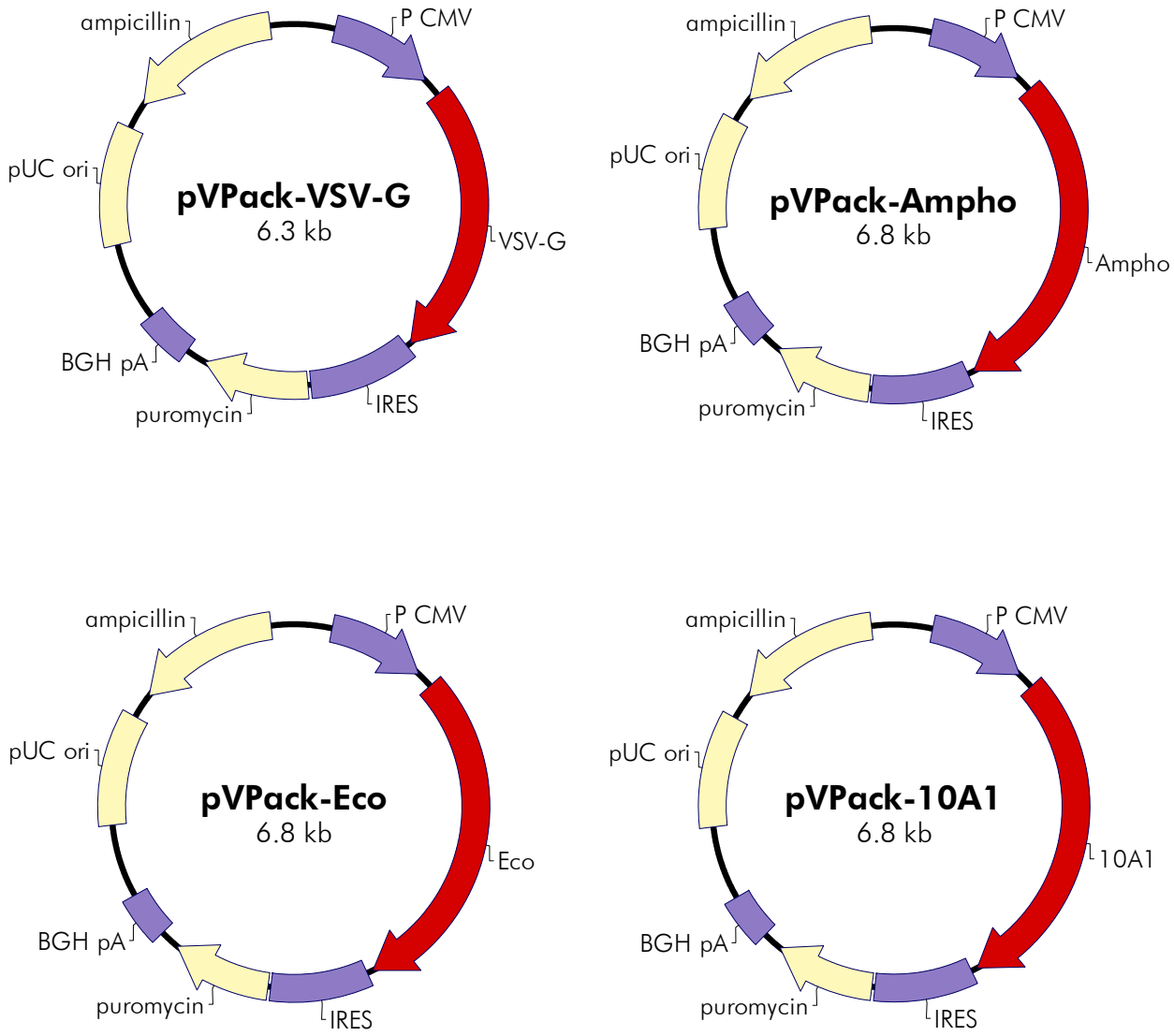


**FIGURE 3** Maps of the *env*-expressing vectors, pVPack-VSV-G, pVPack-Ampho, pVPack-Eco, and pVPack-10A1. (See ***Notes*** in the *Vector Features* section above regarding the production of triple-stable producer lines when using pVPack-VSV-G).

## Choice of Expression Vector

Any MMLV-based retroviral vector for gene delivery and expression can be used with the pVPack vector system to produce high-titer retroviral stocks. The pFB and pCFB retroviral vectors and ViraPort retroviral cDNA libraries are compatible with the pVPack system. They contain the elements necessary for virion packaging: a bacterial origin of replication and ampicillin-resistance gene from pBR322, an extended MMLV packaging signal (ψ+), and a multiple cloning site (MCS) that is located between the MMLV 5´ and 3´ long terminal repeat sequences (LTRs).

## pFB-Neo-LacZ, pFB-hrGFP, and pFB-Luc Control Vectors

The pFB-Neo-LacZ plasmid vector provided with the kit contains a bicistronic transcript; the β-galactosidase gene is expressed from the first open reading frame, and is followed by the neomycin-resistance marker downstream from an IRES. The vector may be used as an expression control, and can also be used to determine viral titer by FACS, *in situ* staining with X-gal, or G418-resistant colony formation.

Also available for use as a positive control is the vector pFB-hrGFP (Aglient Catalog #240027), which contains coding sequence for the humanized green fluorescent protein from a novel marine organism. The hrGFP-expressing vector can be used to determine the transfection efficiency of the packaging cell line and to determine viral titer by FACS.

The pFB-Luc control* (also available separately) allows a qualitative assessment of the efficiency with which the target cell type is transduced by retrovirus. Direct comparisons between the cell lines based on luciferase activity should be made with caution however, as differences in luciferase activity may be due to cell type-dependent differences in luciferase expression rather than differences in transduction efficiencies.

* Available as pFB-Luc plasmid vector (Agilent Catalog #240030).

# PREPROTOCOL SAFETY CONSIDERATIONS

**Note** *The safety guidelines presented in this section are not intended to replace the BSL 2+ safety procedures already in place at your facility. The information set forth below is intended as an additional resource and to supplement existing protocols in your laboratory.*

## Working with Retrovirus

The host range of a retrovirus is determined not by the vector DNA but by the specific *env* gene used to construct the packaging cell line. Viruses produced from amphotropic or polytropic packaging lines are capable of infecting human cells. Prior to use of the pVPack vector system, or of any retroviral packaging system, We strongly recommend that all users become thoroughly familiar with the safety considerations concerning the production and handling of retrovirus. For a description of laboratory biosafety level criteria, consult the Centers for Disease Control Office of Health and Safety Web site *http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s3.htm*. For most applications, production and use of retroviral vectors fall within NIH Biosafety Level 2 criteria. The University of California, San Diego (UCSD) has an active retrovirus research group, the UCSD Vector Development Lab, with experience in safety practices for use with MMLV-derived vectors. The information they have can be found at *http://medicine.ucsd.edu/gt/MoMuLV.html*. Most potential hazards can be avoided by applying good tissue culture technique. The use of gloves and disposable lab coats when working with virus is strongly recommended. When pipetting virus-containing supernatants and transferring plates or flasks to and from the laminar flow hood, the production of aerosols should be avoided. Wherever possible, all pipets and plasticware should be disinfected for 15 minutes with 70% ethanol, 10% bleach, or other disinfectant recommended by the UCSD Vector Development Lab prior to removal from the laminar flow hood. It is strongly recommended that, wherever possible prior to removal from the laminar flow hood, disposable plasticware should be UV irradiated for 20 minutes, enclosed and taped into a red biohazard bag inside the laminar flow hood, enclosed into a second bag outside the hood and autoclaved. Any vector containing a potentially toxic or oncogenic insert should be handled with BSL-2+ precautions, particularly if pseudotyped with the VSV-G protein. All cDNA expression libraries (for example ViraPort retroviral cDNA libraries) are expected to have potently oncogenic inserts at some frequency, regardless of the state of the tissue from which the cDNA was derived. For more information regarding BSL-2+ practices, consult the UCSD Environmental Health and Safety Web site *http://www-ehs.ucsd.edu/ADENO.HTM*.

### Undesired Production of Replication-Competent Retrovirus

Despite the fact that these packaging vectors have been designed for minimal overlap with the majority of MMLV-based vectors in order to greatly reduce the probability of production of replication-competent retrovirus (RCR), there is nevertheless a low risk of RCR production. Furthermore, the use of target cell lines harboring endogenous retrovirus capable of encapsidating vector proviral RNA can result in the undesirable spread of vector-derived virus. There are a number of published protocols describing assays for detection of RCR based on mobilization of a provirus containing a detectable marker ("marker rescue") or by direct detection of reverse transcriptase activity in tissue culture supernatants. It is strongly recommended that all cell lines to be used for viral production or infection be first tested for the presence of endogenous retrovirus using one of the assays described.[6]

## PROTOCOL FOR RETROVIRUS PRODUCTION

**Note** ***Prior to production of virus, users should be thoroughly familiar with the suggestions and Web sites described in the section* Preprotocol Safety Considerations*. All virus work should be performed in a designated virus work area. All cell lines to be used for production of or infection by retrovirus should first be tested for the presence of endogenous retrovirus. See* Undesired Production of Replication-Competent Retrovirus *above*.**

Although a variety of protocols and cell lines may be successfully used with these vectors, the following protocol for the production of viral supernatants is recommended. This protocol consistently results in the production of viral titers $\geq 10^7$ colony forming units (cfu)/ml when transducing NIH3T3 cells with a pFB-derived vector. The protocol employs a calcium phosphate precipitation of the vector DNA and is based on the Transfection MBS Mammalian transfection kit, modified according to Pear and colleagues.[7] Although excellent results may be obtained using 293 cells, we recommend the use of the 293 cell derivative 293T, which has been shown to transfect with a significantly greater efficiency.[7]

**Note** *The steps performed in this section,* Protocol for Retrovirus Production, *need to be carried out under sterile conditions in a laminar flow hood.*

## Day 1: Preparing for Production of Virus by Transfection

### 293T Host Cell Preparation

Split 293T cells at 2.5-3.0 × $10^6$ cells per 60-mm tissue culture plate in growth medium (See *Preparation of Media and Reagents*) 24 hours before the transfection and incubate at 37°C until needed.

***Note*** *To achieve optimal titers, it is important that the 293T cells are healthy and growing exponentially. Cells should be passaged at high density, and ideally passaged no more than 20 times (no more than approximately 2 months); it is thus prudent to initially prepare a large number of frozen vials of the cells while they are at a low passage and healthy. Care should be taken to avoid clumping of the cells during passaging and plating for transfection.*

### Plasmid DNA Preparation

DNA preparations of high purity should be used for the transfections.

1. Pipette the following into a clean 1.5-ml microcentrifuge tube; prepare one tube for each transfection to be carried out.
   - 3 µg an MMLV-based retroviral plasmid containing the gene of interest
   - 3 µg pVPack-GP (*gag-pol*-expressing vector)
   - 3 µg of one of the four *env*-expressing vectors (pVPack-Eco, pVPack-Ampho, pVPack-VSV-G, pVPack-10A1)

2. **(Optional)** Prepare the **positive control vector sample** by pipetting the following into a clean 1.5-ml microcentrifuge tube.
   - **3 µg pFB-Neo-LacZ or pFB-hrGFP**
   - 3 µg pVPack-GP (*gag-pol*-expressing vector)
   - 3 µg of one of the four *env*-expressing vectors (pVPack-Eco, pVPack-Ampho, pVPack-VSV-G, pVPack-10A1)

3. To each of the tubes containing the mixed vector DNA, add 1 ml 100% (v/v) ethanol and 0.1 × volume 3 M sodium acetate to the DNA mixture; mix by inverting the tube, incubate at -80°C for 30 minutes. Collect the DNA pellet by centrifugation at 12,000 × *g* for 10 minutes at 4°C. Aspirate and discard the supernatant. Add 1 ml 70% (v/v) ethanol to the tube, vortex briefly, and collect the DNA pellet by centrifugation at 12,000 × *g* for 5 minutes at 4°C. Remove and discard the supernatant; close the cap of the tube. Store wet pellets at 4°C overnight.

## Day 2: Transfecting Cells

**Note** *The procedure on Day 2 will take a minimum of 10 hours to complete.*

### Adding the MBS-Containing Medium to the Cells

1. Inspect the host cells that were split the day before; they should be approximately 80% confluent. [If cells are significantly less than 80% confluent, viral supernatants may be harvested 72 hours post-transfection rather than 48 hours.]

2. Prepare the MBS-containing medium (see *Preparation of Media and Reagents*). This must be done immediately prior to the transfection. For each 60-mm tissue culture plate, 4 ml of MBS-containing medium must be prepared.

3. Add 4 ml of MBS-containing medium to each 60-mm plate and return the plates to the 37°C incubator. This must be done 20-30 minutes before the addition of the DNA suspension.

### Adding the DNA Suspension to the Cells

1. Remove the microcentrifuge tubes containing the wet DNA pellets (including the pFB-Neo-LacZ or pFB-hrGFP–containing pellet if the control transfection is to be carried out) from storage at 4°C and transfer them to the laminar flow hood.

2. Resuspend each DNA pellet in 450 µl sterile $H_2O$ and transfer the liquid to separate 5-ml BD Falcon polystyrene round-bottom tubes.

3. To each resuspended DNA pellet add 50 µl of Solution I and 500 µl Solution II from the Transfection MBS Mammalian Transfection Kit.

4. **Gently** resuspend any precipitate in the DNA suspension by pipetting the suspension up and down with a pipettor set at 500 µl. The DNA suspension should appear clear to opaque. Allow the DNA suspension to sit at room temperature for 10 minutes.

5. Remove the 60-mm plates to be transfected from the incubator and add the DNA suspension onto the plates in a dropwise fashion, swirling gently to prevent the cells from being lifted from the plate and to distribute the DNA suspension evenly.

   **Note** *From this point on, it should be assumed that infectious virus is present in the supernatant of the transfected cells. Gloves and disposable lab coats should be worn while working with the virus. We recommend that gloved hands be sprayed intermittently with ethanol. When pipetting medium supernatant and transferring plates to and from the laminar flow hood, aerosols should be avoided. In case of spills, follow the procedures recommended in the UCSD Vector Development Lab Web site* http://medicine.ucsd.edu/gt/MoMuLV.html. *All dirty pipets and plasticware should be disposed of as described in the section* Preprotocol Safety Considerations.

6. Return the tissue culture plates to the 37°C incubator.

7. After incubating for 3 hours, remove the medium from the plates and replace it with 4 ml of growth medium supplemented with 25 µM chloroquine (see *Preparation of Media and Reagents)*. Return the plates to the 37°C incubator.

8. After incubating for an additional 6–7 hours, remove the growth medium containing 25 µM chloroquine and replace with 4 ml growth medium—no chloroquine.

## Day 3: Preparing for the Transduction

1. Remove growth medium from 293T plates and replace with 3.0 ml of fresh growth medium. Return the plates to the 37°C incubator.

   **Note** *If virus is to be harvested 72 hours post-transfection rather than 48 hours, steps 2 and 3 should be carried out on Day 4.*

2. Split the target cells, seeding $1 \times 10^5$ cells per well for 6-well plates and $2 \times 10^4$ cells per well for 24-well plates. This seeding density may vary with the cell line; 20–30% confluency is desirable.

3. Return the plates to the 37°C incubator overnight.

## Day 4: Transducing the Target Cells

**Note** *If virus is to be harvested 72 hours post-transfection rather than 48 hours, all steps from the Day 4 section should be performed on Day 5.*

1. Remove the virus-producing 293T cells from the incubator.

2. Collect the virus-containing supernatant from the first plate and filter it through a 0.45 μm filter into a sterile 50-ml conical tube.

   **Note** *If desired, the supernatant can be snap frozen on dry ice or liquid nitrogen and stored at -80°C at this stage. WARNING: Freeze-thawing virus one time typically results in a 2-fold loss in titer. Subsequent freeze-thaw cycles result in less than a 2-fold loss per cycle of the remaining infectious virus.*

3. Dilute viral supernatants as desired in growth medium.

4. Add DEAE-dextran solution to the diluted viral supernatants to a final concentration of 10 μg/ml (1:1000 dilution of the 10 mg/ml DEAE-dextran stock. See *Preparation of Media and Reagents*).

   **Note** *If starting from a frozen supernatant stock, thaw rapidly in a 37°C water bath, minimizing the time the supernatant is at 37°C before the addition of the DEAE-dextran.*

5. Remove the plates containing the target cells from the incubator.

6. Remove and discard the medium from the wells of the target cell plates.

7. Add DEAE-dextran plus virus to the wells containing the target cells: 1.0 ml per well for 6-well plates and 200 μl per well for 24-well plates.

8. Return the plates to the 37°C incubator for 3 hours.

9. After the 3 hour incubation, add growth medium to the wells: 1.0 ml per well for 6-well plates and 200 μl per well for 24-well plates.

**Note** *For expression studies, allow at least two days between target cell infection and cell harvest. To assess transduction efficiency using pFB-Neo-LacZ or pFB-hrGFP transduced cells see* Determination of Target Cell Transduction Efficiency Using the pFB-Neo-LacZ or pFB-hrGFP Control Vectors, *below.*

# DETERMINATION OF TARGET CELL TRANSDUCTION EFFICIENCY USING THE pFB-NEO-LACZ OR pFB-HRGFP CONTROL VECTORS

The pFB-Neo-LacZ control vector provided can be used for determining the efficiency with which the chosen target cell is transduced, and also allows a quantitative assessment of viral promoter strength. Titer determination using the β-galactosidase gene may be carried out by fixing and staining cells with X-gal using a suitable β-galactosidase staining kit and determining the number of blue cells as a percentage of the total number of visible cells in a field by light microscopy. Alternatively, β-galactosidase titers may be determined by Fluorescence Activated Cell Sorting (FACS)[8] using the fluorescent substrate CMFDG (Molecular Probes, Eugene, OR). Titers may also be determined with this vector by G418-resistant colony formation from populations of cells infected with various dilutions of viral supernatant. For a quantitative determination of promoter strength in the target cell of choice, lysates from transduced cells may be assayed for β-galactosidase enzyme activity using a suitable β-galactosidase staining kit.

As an alternative, the vector pFB-hrGFP (Agilent Catalog #240027) can be used for titer determination by FACS, and can also be used for a consistent qualitative assessment of transfection efficiency of producer cells. The vector contains the coding sequence for a humanized green fluorescent protein from a novel marine organism.

VSV-G pseudotyped pFB-hrGFP has been tested in NIH3T3, COS-7, CHO, 293, and HeLa cells, and consistently gives titers $\geq 10^7$ cfu/ml. Similarly, pFB-Neo-LacZ gives titers on the order of $10^6$ cfu/ml by visual inspection of *in situ* stained cells.

# PROTOCOL FOR TITER DETERMINATION BY QUANTITATIVE OR ENDPOINT RT-PCR

Many retroviral vectors contain antibiotic resistance markers or other readily detectable markers that allow titer determination based on measuring the number of transduced cells for a series of supernatant dilutions. For viral vectors that carry no such markers, such as the pFB/pCFB vectors of the ViraPort system, RNA copy number may be measured directly in RNA isolated from viral supernatants using QRT-PCR. Follow the reagent manufacturer's guidelines for performing QRT-PCR reactions.

Alternatively, if QRT-PCR techniques are not available, endpoint RT-PCR may be used. Using this approach, serially diluted test RNAs are compared with RNA isolated from a reference virus of known titer (for example, a viral stock produced from the G418-resistant virus pFB-Neo) and approximate titers are determined by visual comparison of the signal intensities of bands in ethidium bromide-containing agarose gels.

Prior to titering ViraPort or other viral supernatants using RT-PCR, prepare a dilution series of RNA isolated from the reference viral supernatant and verify that the RT-PCR assay produces a quantitative response in the required range of input amounts. A typical dilution series includes 5-fold or 10-fold dilutions, made over a range of at least 3 orders of magnitude. Depending on the reagent system used, PCR cycling conditions may need to be modified from the manufacturer's recommended conditions (e.g. reduced cycle number), to allow quantitative detection.

## Choice of Reference Virus

Any MMLV-based viral vector with a detectable marker may be used. Viral supernatant from the reference should be produced in sufficient quantity to allow reasonably accurate titer determination by transduction (for example, by antibiotic-resistant colony formation or FACS) and at the same time leaving enough supernatant such that numerous aliquots may be frozen away for RNA isolation. The amount of viral supernatant and viral RNA required depends on how many times the titering experiment will be done. Although freeze-thawing of the viral stocks will reduce the infectious titer of the virus, no loss of RT-PCR signal with a single freeze-thaw cycle of virus or up to three freeze-thaw cycles of purified RNA has been observed. Thus, when comparing titers between test and reference virus, the relative freeze-thaw cycles of the two stocks should be taken into account.

## RNA Isolation

There are a number of commercially available kits for purification of viral RNA from cell culture supernatants, most of which should yield RNA of sufficient quality and quantity to perform consistently accurate titer determinations. Because the yields may vary from kit to kit, it is recommend that a set of pilot experiments be performed using reference virus RNA to determine the optimal amount of RNA to use in the RT-PCR reaction.

**Note** *RNA is very susceptible to degradation by RNases, present on any surface touched with bare hands. Use appropriate precautions to minimize RNA degradation.*

## DNase I Treatment

The purified viral RNA will contain a low level of contaminating retroviral plasmid carried over from the 293T cell transfection.

1. Treat the RNA sample (volume determined previously in pilot experiments) with DNase I by adding the appropriate volume of 10× DNase I buffer (see *Preparation of Media and Reagents*) and 2 U DNase I, RNase-free directly to the sample, and incubating at 37°C for 30 minutes.

   **Note** *Due to the high sensitivity of the assay, it is prudent from this point on to use sterile, cotton-plugged pipette tips, and to use pipettors that have not been exposed to MMLV-derived plasmids. Wherever possible, carry out the rest of the procedure in a work area where MMLV-based vectors are not used.*

2. Following the DNase I treatment, add sufficient EDTA to the reaction to chelate the $MgCl_2$. Inactivate the DNase I by incubating the tube at 75°C for 10 minutes.

## Selection of RT-PCR Primers

Choose a PCR primer pair that will yield a relatively short amplicon. For the pFB- and pCFB-derived vectors, a primer pair useful for this diagnostic PCR is provided in the table below. This primer pair anneals immediately downstream of the splice donor in the ψ region and amplifies a 259-bp PCR product derived only from the unspliced viral RNA and is therefore suitable for diagnostic PCR for detection of most MMLV-based vectors. (Full sequences of the pFB and pCFB vectors are available at http://www.genomics.agilent.com/vectorMapsAndSequence.jsp.)

| Primer | Sequence | Position in pFB | Position in pCFB |
|---|---|---|---|
| Sense Primer | 5′ GTCTGTCCGATTGTCTAGTGT 3′ | 846–866 | 1021–1041 |
| Antisense Primer | 5′ AGGTTCTCGTCTCCTACCAGA 3′ | 1105–1085 | 1260–1280 |

# TROUBLESHOOTING

| Observation | Solution(s) |
|---|---|
| Low titer | Transfection efficiency is key to production of high titer virus. Early passage 293T stocks should be used; cells should be passaged at high density, and thoroughly trypsinized to avoid clumping. Cells should be transfected at 80% confluency. DNA preparations of high purity should be used. Freeze-thawing of MBS and chloroquine stocks should be minimized. 293T cells are weakly adherent, thus all media changes should be performed with extreme care. If transfection efficiency is suspect, pilot experiments using a readily assayable reporter should be carried out (for example, using *B*-Galactosidase staining). High titer virus production requires a minimum transfection efficiency of 30%. |
| No RT-PCR product after optional titering using QRT-PCR or RT-PCR | If the reporter pilot experiments described above indicate adequate transfection efficiency yet no RT-PCR product is observed, investigate potential problems with the RNA isolation/DNase treatment/RT-PCR steps. Primer quality may be assessed by performing PCR reactions using the retroviral plasmid as template. Ensure that DNase I is thoroughly inactivated prior to first strand cDNA synthesis, by setting up PCR reactions in which the primer/template mix is pre-incubated with heat-inactivated DNase I. It is important to add EDTA to the DNase I reaction prior to heat inactivation, since RNA is degraded at high temperature in some $MgCl_2$-containing buffers. RT-PCR pilot experiments should be performed using RNA isolated from the reference viral supernatants... |
| Background RT-PCR product in reverse transcriptase–minus reactions or in reactions with no RNA template | The appearance of appropriate size PCR products in reactions containing no RNA template indicates the presence of contaminating template in the primers, reagents or pipettors. Sterile, cotton-plugged pipette tips and pipettors that have not been exposed to the target plasmid should be used. Wherever possible, carry out the RT-PCR reaction in a work area where MMLV-based vectors are not used. If the problem persists, the procedure should be repeated with fresh primers and reagents. If there are no background PCR products in the control, but there are products in the reverse transcriptase-minus, template-containing reactions, it is likely due to incomplete DNase I digestion of the RNA prep. In this case, the amount of DNase I or DNase I reaction time should be increased. |
| Poor Transduction Efficiency | Transduction efficiency will vary from cell line to cell line even with VSV-G and the other polytropic envelope proteins. If it is clear that adequate 293T transfection efficiencies are achieved and virus is produced, yet the target cell of choice is poorly transduced, it is prudent to test the transduction efficiency on NIH3T3 cells if the vector has a readily detectable marker. If the vector does not have an assayable marker, vector-specific PCR of mass-infected cells may be performed to verify transduction. All four of the envelope-expressing plasmids consistently give rise to titers in excess of $10^7$ cfu/ml on NIH3T3 cells using the vector, pFB-hrGFP. Conversely, the transduction efficiency for a given target cell line may be tested with reporter vectors such as pFB-Neo-LacZ or pFB-hrGFP. The latter vector will give a rapid, qualitative indication of the ability of a cell line to be transduced; the former will allow titer determination based on X-gal staining or G418-resistant colony-formation. In the event that transduction of the target cell is poor, concentration of VSV-G pseudotyped virus and infection at high MOI is recommended. |

## PREPARATION OF MEDIA AND REAGENTS

### Stock Chloroquine Solution

***Note*** *Chloroquine is toxic and should be opened in a fume hood only*

1.29 g of chloroquine diphosphate [$C_{18}H_{26}ClN_3 \cdot 2H_3PO_4$] (25 mM final concentration)

Add 100 ml of 1 × PBS, dissolve the solid chloroquine. Filter sterilize and store in aliquots at –20°C. Discard aliquots that are older than one month. Dilute 1:1000 into media for use during the transfection procedure.

### PBS

137 mM NaCl
2.6 mM KCl
10 mM $Na_2HPO_4$
1.8 mM $KH_2PO_4$
Adjust the pH to 7.4 with HCl

### Growth Medium

DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum [FBS], 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine.

### MBS-Containing Medium

***Note*** *Chloroquine solution is toxic and should be opened in the laminar flow hood*

Add stock chloroquine solution to DMEM containing 7% (v/v) modified bovine serum (from the Transfection MBS Mammalian Transfection Kit) to a final concentration of 25 μM. Filter sterilize. Prepare just before use and keep at 37°C until required.

### Growth Medium (supplemented with 25 μM chloroquine)

***Note*** *Chloroquine solution is toxic and should be opened in the laminar flow hood*

Prepare growth medium as above. Add chloroquine from stock chloroquine solution to a final concentration of 25 μM. Filter sterilize. Prepare just before use and keep at 37°C until required.

### DEAE-Dextran Solution

1 g DEAE-dextran [diethylaminoethyl-dextran, approx. mol. wt. 500,000], (10 mg/ml final concentration)

Add 100 ml of high purity water, dissolve the DEAE-dextran, filter sterilize into a sterile container and keep sterile until required.

### 10× DNase I Buffer

40 mM Tris-HCl, pH 7.9
10 mM NaCl
6 mM $MgCl_2$
1 mM $CaCl_2$

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## MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.chem.agilent.com/en-US/search/library/Pages/MSDSSearch.aspx*. MSDS documents are not included with product shipments.