

Takara Bio USA, Inc.

Retroviral Gene Transfer and Expression User Manual

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Takara Bio USA, Inc.

1290 Terra Bella Avenue, Mountain View, CA 94043, USA

U.S. Technical Support: techUS@takarabio.com

United States/Canada
800.662.2566

Asia Pacific
+1.650.919.7300

Europe
+33.(0)1.3904.6880

Japan
+81.(0)77.565.6999

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I. Introduction

A. Gene Transfer and Expression Using Recombinant Retroviruses

Recombinant retroviral vectors are highly efficient tools for transferring heritable genetic material into the genome of a broad range of dividing cells. Lentiviral or adenoviral systems are recommended for viral delivery to nondividing cell types. This User Manual supports many Clontech® brand packaging cell lines, retroviral vectors, and retroviral expression systems.

B. Retro-X Retroviral Vectors

Takara Bio offers a choice of the following Retro-X retroviral vectors, vector sets, and systems (Figure 1).

1. Constitutive Retroviral Vectors with Antibiotic Selection

These retroviral vectors, which are derived from the Moloney murine leukemia virus (MMLV), allow you to express your transgene from a constitutive promoter (CMV, SV40, or LTR), and select for stable integration using antibiotics.

a. Q-Series Retroviral Vectors and Vector Sets

- Takara Bio's Q-Series of vectors contain a CMV/MSV hybrid promoter in the 5' LTR to drive high titers during the packaging step. They also contain a self-inactivating 3' LTR to reduce promoter interference and drive high expression of your transgene from the internal CMV promoter. Expression of an antibiotic resistance gene is achieved through ribosome binding to an internal ribosome entry site (IRES).
- The **Retro-X Q Vector Set** (Cat. No. 631516) gives you a choice of Q-Series vector backbones with alternative resistance markers for selection with G418 (pQCXIN), hygromycin (pQCXIH), or puromycin (pQCXIP).

b. L-Series Retroviral Vectors, Vector Sets, and Systems

- **pLNCX2 and pLXSN** (Cat. Nos. 631503 & 631509) are basic retroviral vectors that utilize wild-type LTRs from MMLV. These vectors allow you to express your gene of interest from either a CMV promoter (pLNCX2) or the 5' LTR promoter (pLXSN). Integration of these constructs into the genome of your target cell can be selected for by adding G418 to your culture medium.
- The **Retro-X System** (Cat. No. 631508) includes the L-Series retroviral expression vectors pLNCX2 and pLXSN. Takara Bio's RetroPack™ PT67 packaging cell line is also included in this system.
- The **Pantropic Retroviral Expression System** (Cat. No. 631512) contains vectors designed for use with the included GP2-293 retroviral packaging cell line and pVSV-G envelope vector. The system includes the Retro-X Pantropic Vector Set, which allows you to express your gene of interest from an hsp70 promoter (pLNHX) or a 5' LTR promoter (pLXRN).
- **pLXIN** (Cat. No. 631501) is a bicistronic retroviral vector containing an internal ribosome entry site (IRES). The 5' LTR thus regulates expression of a gene of interest and the neomycin resistance gene on the same bicistronic message.

c. Vectors Optimized for Expression in Stem Cells

The **MSCV (Murine Stem Cell Virus) Retroviral Expression System** (Cat. No. 634401) contains vectors that are optimized for introducing and expressing target genes in pluripotent cell lines, including murine or human hematopoietic, embryonic stem (ES), and embryonal carcinoma (EC) cells. However, they can also be used very effectively with most other mammalian cell lines.

- These vectors contain a specially designed 5' long terminal repeat (LTR) from the murine stem cell PCMV virus. This LTR differs from the MMLV LTR by several point mutations and a deletion that together enhance transcriptional activation and prevent transcriptional suppression in ES and EC cells. As a result, the PCMV LTR drives high-level, constitutive expression of the target gene in stem cells or other mammalian cell lines.
- This system includes the MSCV Vector Set, which gives you a choice of three pMSCV vectors with alternative resistance markers for selection with neomycin, hygromycin, or puromycin.

2. Constitutive Retroviral Vectors with No Selection

a. Retroviral Vector for Simultaneous Expression of Two Genes of Interest

pQCXIX (Cat. No. 631515) lacks a selection marker, but contains two multiple cloning sites, one on each side of an IRES, to allow simultaneous expression of two transgenes.

b. Retroviral Vector for Library Construction

pRetro-Lib (Cat. No. 635002) contains Sfi1A and Sfi1B restriction sites flanked by a stuffer fragment to enable simple cloning of a full-length cDNA library using Takara Bio's **SMART® cDNA Library Construction Kit** (Cat. No. 634901).

3. Constitutive Retroviral Vectors with Fluorescent Selection

These retroviral vectors allow you to monitor your transgene by fusing it with a Living Colors® fluorescent protein to track its location, or express it separately from an IRES to monitor expression.

a. Retroviral Vectors that Provide Monomeric Fusion Tags

pRetroQ-AcGFP1-N1/C1 (Cat. Nos. 632505 & 632506), **pRetroQ-mCherry-N1/C1** (Cat. Nos. 632567 & 632568), and **pRetroQ-DsRed-Monomer-N1/C1** (Cat. Nos. 632507 & 632508) allow you to fuse your gene of interest to monomeric fluorescent proteins, which make ideal fusion tags. These vectors are available in both N- and C-terminal formats.

b. Retroviral Vectors that are Ideal for Reporter Studies

pRetroX-IRES-ZsGreen1 (Cat. No. 632520) and **pRetro-X-IRES-DsRedExpress** (Cat. No. 632521) provide bright fluorescent proteins that are excellent reporters for gene expression, or for measuring transduction/transfection efficiency.

4. Tet-Inducible Retroviral Vectors—Tet-On® 3G & Tet-Express™ Systems

These retroviral vectors provide tightly controlled expression of your transgene using Takara Bio's Tet-On 3G and Tet-Express Inducible Expression Systems.

a. Tet-Inducible Expression Using a Retroviral Vector Set

The **Retro-X Tet-On 3G Inducible Expression System** (Cat. No. 631188) is a tightly-regulated, tetracycline-inducible, retroviral gene expression system using Retro-X-Q Vector technology (pRetroX-Tet3G and the pRetroX-TRE3G) to provide efficient retroviral delivery and inducible expression of your gene of interest. In the presence of doxycycline (Dox), the Tet-On 3G transactivator protein expressed by pRetroX-Tet3G specifically binds and activates high-level transcription from the inducible P_{TRE3GV} promoter in pRetroX-TRE3G, which controls expression of your gene of interest cloned downstream of this promoter.

b. Tet-Inducible Expression Using a Single Retroviral Vector

The **Retro-X Tet-Express Inducible Expression System** (Cat. No. 631190) is a faster, simpler adaptation of the Retro-X Tet-On 3G Inducible Expression System requiring only a single retroviral vector, pRetroX-TRE3G, with your gene of interest cloned downstream of the inducible P_{TRE3GV} promoter. Transduced cells are induced to express your transgene by adding Tet-Express self-transducible protein to the culture medium. This protein specifically binds to and activates the P_{TRE3GV} promoter.

5. Inducible Protein Stabilization/Destabilization—ProteoTuner™ Systems

These vectors allow you to fuse your protein to a destabilization domain (DD) and rapidly stabilize/destabilize the resulting fusion protein by adding/removing Shield1 from your culture medium.

a. Inducible Protein Stabilization/Destabilization Using a Retroviral System

The **Retro-X ProteoTuner Shield System N** (Cat. No. 632171) includes the pRetroX-PTuner Vector, which is designed to express a protein of interest tagged on its N terminus with a mutant FKBP destabilizing domain (DD). If transfected into mammalian cells, it expresses the protein of interest as a fusion protein with the DD.

b. Inducible Protein Stabilization/Destabilization Using a Retroviral System with a Fluorescent Protein Reporter

The **Retro-X ProteoTuner Shield System N (w/ ZsGreen1)** (Cat. No. 632167) includes the pRetroX-PTuner IRES vector, a bicistronic retroviral expression vector containing ZsGreen1 as a reporter to monitor transfection/infection efficiency. The vector is designed to express a protein of interest tagged with a mutant FKBP destabilizing domain (DD) simultaneously with ZsGreen1, a human codon-optimized variant of the *Zoanthus sp.* green fluorescent protein (ZsGreen), from the same transcript in transfected mammalian cells.

6. Retroviral Cell Cycle Reporter Vectors

Fucci cell cycle vectors express fluorescent, ubiquitination-based reporters known as Fucci, which allow you to identify cells in various phases of the cell cycle and visualize cell shape. Retroviral Fucci vectors include **pRetroX-G1-Red** (Cat. No. 631463), which allows you to identify cells in G1 phase—and **pRetroX-SG2M-Cyan**, (Cat. No. 631462), **pRetroX-SG2M-Red** (Cat. No. 631465), and **pRetroX-SG2Mcyto-Red** (Cat. No. 631464), which allow you to identify cells transitioning between S, G2, and M phases.

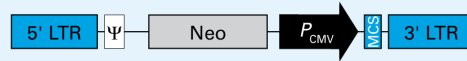
Constitutive promoter with antibiotic selection

Express your transgene from a constitutive promoter (CMV, SV40, or LTR) and select for stable integration using antibiotics.

pQCXIN/pQCXIH/pQCXIP



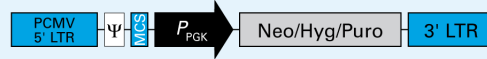
pLNCX2



pLXSN



pMSCVneo/hyg/puro



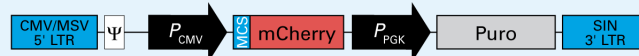
Constitutive promoter with fluorescent selection

Monitor your transgene by fusing it with a fluorescent protein to track its location, or express it separately from an IRES to monitor expression.

pRetroQ-AcGFP1-N1 (C1 version also available)



pRetroQ-mCherry-N1 (C1 version also available)



pRetroX-IRES-ZsGreen1



pRetroX-IRES-DsRedExpress



Constitutive promoter with no selection

Two available multiple cloning sites separated by an IRES, for constitutive coexpression of two separate transgenes.

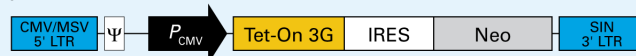
pQCXIX



Inducible expression – Tet-On 3G

Tightly controlled expression of your transgene using Clontech's Tet-On 3G Inducible Expression System.

pRetroX-Tet3G



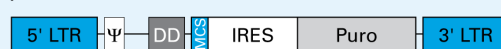
pRetroX-TRE3G



Inducible expression – ProteoTuner

Fuse your protein to a destabilization domain (DD) and rapidly stabilize/destabilize by adding/removing Shield1 from your culture medium.

pRetroX-PTuner



pRetroX-PTuner-IRES



Figure 1. Retroviral vectors for many applications.

C. Retro-X Retroviral Packaging Cell Lines

To produce recombinant retrovirus for target cell infection, retroviral transfer vectors must be transfected into retroviral packaging cells that provide the *gag*, *pol*, and *env* genes *in trans*. Various options are available, and all retroviral packaging cell lines and systems are compatible with all retroviral transfer vectors sold by Takara Bio, including MMLV- and MSCV-based retroviruses. Table 1 summarizes the retroviral packaging cell lines available from Takara Bio and Table 2 lists the host range of retroviruses created with the different types of commonly used envelopes.

- The **Retro-X Universal Packaging System** (Cat. No. 631530) is our premium retroviral packaging system featuring the GP2-293 packaging cell line. All four commonly used envelopes are supplied on separate vectors that you cotransfect with your transfer vector. Envelope choices include VSV-G, eco, amphi, and 10A1 to allow you to choose the tropism that is most appropriate for your target cells (see Table 2).
- The **EcoPack™ 2-293 Cell Line** is no longer available from Takara Bio; this information is provided as a courtesy for those who have previously purchased this cell line. This cell line is an ecotropic HEK 293-based packaging cell line that is also easy to transfect and produces retrovirus with a tropism that limits infection to mouse and rat cells*. The viral envelope protein is integrated and expressed by EcoPack 2-293, and recognizes the ecotropic receptor (mCAT1) found only on the surface of these cell types.

***NOTE:** It is possible to transduce human cells with ecotropic retrovirus provided that the cells are pretreated with **Ecotropic Receptor Booster** (Cat. No. 631471; Section III.J). Ecotropic Receptor Booster consists of a vial of concentrated exosome-like vesicles that are densely coated with the mCAT-1 receptor protein. When the booster vesicles are applied to your cells, they fuse with the plasma membrane, resulting in an increased level of the receptor on the cell surface.

- The **AmphoPack-293 Cell Line** (Cat. No. 631505) can be used to produce recombinant MMLV-based viral particles that infect a broad range of mammalian cells, including human, mouse, and rat cells. AmphoPack 293 is also derived from an HEK 293-based cell line that is easily transfected to produce high viral titers. The viral envelope protein recognizes the amphotropic receptor (Ram-1).
- The **RetroPack PT67 Cell Line** (Cat. No. 631510) is the recommended packaging cell line for creating a stable producer cell line for continuous production of retrovirus. This retroviral packaging system includes an NIH/3T3-based packaging cell line that expresses the 10A1 viral envelope. Virus packaged using RetroPack PT67 cells can be used to infect a broad range of mammalian cells, including human, mouse and rat cell types. The virus is able to enter cells via two different surface molecules, the amphotropic retrovirus receptor (Ram-1) and the GALV receptor.

NOTE: If you wish to make retrovirus via transient transfection, which is the preferred method for most researchers, we recommend the Retro-X Universal Packaging System, or the AmphoPack 293 or EcoPack 2-293 Cell Lines.

- The **Pantropic Retroviral Expression System** (Cat. No. 631512) also features the GP2-293 packaging cell line, which expresses *gag* and *pol* proteins. The envelope protein is supplied on a separate plasmid, pVSV-G, which is cotransfected with your transfer vector. Delivery using a VSV-G envelope is not dependent on a cell surface receptor; instead, it mediates viral entry through lipid binding and plasma membrane fusion. Two transfer expression vectors are included with the Pantropic System, giving you the choice of using a retroviral 5' LTR or the *Drosophila hsp70* promoter to express your gene of interest.

Retroviral Gene Transfer and Expression User Manual

Table 1. Summary of retroviral packaging cell lines available from Takara Bio.

Cell line	GP2-293	AmphoPack-293	EcoPack 2-293*	RetroPack PT67
Cat. Nos.	631530, 631512	631505	N/A	631510,
Cell line origin	HEK 293	HEK 293	HEK 293	NIH/3T3
Tropism	Pantropic, ecotropic, amphotropic, dualtropic	Amphotropic	Ecotropic	Dualtropic
Envelopes	VSV-G, amphi, eco or 10A1	4070A (amphi)	gap70 (eco)	10A1
Target cells	Wide range of mammalian/non-mammalian cells	Wide range of mammalian cells	Mouse, rat cells	Wide range of mammalian cells

*The EcoPack 2-293 cell line is no longer available from Takara Bio. This information is provided as a courtesy to those who have previously purchased this cell line.

Table 2. Tropisms associated with commonly used retroviral envelopes.

Envelope	VSV-G	4070A (Amphi)	gap70 (Eco)	10A1 (Dual)
Tropism	Pantropic	Amphotropic	Ecotropic	Dualtropic
Receptor (target cell)	Unknown**	RAM1 (Pit2)	mCAT-1	GALV (Pit1), RAM1 (Pit2)
Possible target cell types*	Human	+	+	–
	Mouse	+	+	+
	Rat	+	+	+
	Hamster	+	+/-	–
	Cat	+	+	–
	Dog	+	+	–
	Monkey	+	+	–
	Avian	+	–	–
	Fish	+	–	–
Insect	+	–	–	

*This listing of the most commonly transduced target cell types is not intended to be exclusive.

**It appears likely that a gp96 chaperone client is responsible for binding (Bloor et al. 2009)

II. List of Components

Store cell lines at -196°C and all other components at -20°C .

A. Constitutive Retroviral Vectors & Expression Systems

Retro-X Q Vector Set (Cat. No. 631516)

- 20 μg pQCXIN Retroviral Vector (500 ng/ μl) (also sold separately as Cat. No. 631514)
- 20 μg pQCXIH Retroviral Vector (500 ng/ μl)
- 20 μg pQCXIP Retroviral Vector (500 ng/ μl)
- 20 μg pQCLIN Retroviral Vector (500 ng/ μl)
- 100 μl 5' pQC Seq/PCR Primer (20 μM)
- 100 μl 3' pQC Seq/PCR Primer (20 μM)

Other Available Q Series Retroviral Vectors

- 20 μg pQCXIX Retroviral Vector (500 ng/ μl) (Cat. No. 631515)

LRCX Retroviral Vector Set (Cat. No. 631511)

- 20 μg pLNCX2 Retroviral Vector (500 ng/ μl) (also sold separately as Cat. No. 631503)
- 20 μg pLHCX Retroviral Vector (500 ng/ μl)
- 20 μg pLPCX Retroviral Vector (500 ng/ μl)
- 100 μl 5' pLNCX Seq/PCR Primers (20 μM)
- 100 μl 3' pLNCX Seq/PCR Primers (20 μM)

Other Available L Series Retroviral Vectors

- 20 μg pLXIN Retroviral Vector (500 ng/ μl) (Cat. No. 631501)
- 20 μg pRetro-Lib Vector (500 ng/ μl) (Cat. No. 635002)

Vectors with Fluorescent Protein Reporters

- 20 μg pRetroQ-AcGFP1-N1 Vector (500 ng/ μl) (Cat. No. 632505)
- 20 μg pRetroQ-AcGFP1-C1 Vector (500 ng/ μl) (Cat. No. 632506)
- 20 μg pRetroQ-DsRed Monomer-N1 Vector (500 ng/ μl) (Cat. No. 632507)
- 20 μg pRetroQ-DsRed Monomer-C1 Vector (500 ng/ μl) (Cat. No. 632508)
- 10 μg pRetroQ-mCherry-N1 Vector (500 ng/ μl) (Cat. No. 632568)
- 10 μg pRetroQ-mCherry-C1 Vector (500 ng/ μl) (Cat. No. 632567)
- 20 μg pRetroX-IRES-DsredExpress Vector (500 ng/ μl) (Cat. No. 632521)
- 20 μg pRetroX-IRES-ZsGreen1 Vector (500 ng/ μl) (Cat. No. 632520)

Retro-X System (Cat. No. 631508)

- 1 ml RetroPack PT67 Cell Line (2×10^6 cells/ml) (also sold separately as Cat. No. 631510)
- 20 μg pLNCX2 Retroviral Vector (500 ng/ μl) (also sold separately as Cat. No. 631503)
- 20 μg pLXSN Retroviral Vector (500 ng/ μl) (also sold separately as Cat. No. 631509)
- 20 μg pLAPSN Retroviral Vector (500 ng/ μl)
- 100 μl pLNCX Seq/PCR Primers (20 μM)
- 100 μl pLXSN Seq/PCR Primers (20 μM)

MSCV Retroviral Expression System (Cat. No. 634401)

- 1 each MSCV Vector Set (Cat. No. 631461; not sold separately)
 - 20 μl pMSVneo Retroviral Vector (500 ng/ μl)
 - 20 μl pMSVhyg Retroviral Vector (500 ng/ μl)
 - 20 μl pMSVpuro Retroviral Vector (500 ng/ μl)
 - 100 μl 5' MSCV Seq/PCR Primer (20 μM)
 - 100 μl 3' MSCV Seq/PCR Primer (20 μM)
- 1 ml RetroPack PT67 Cell Line (2×10^6 cells/ml) (also sold separately as Cat. No. 631510)

B. Tet-Inducible Expression Systems

Retro-X Tet-On 3G Inducible Expression System (Cat. No. 631188)

- 1 each Retro-X Tet-On 3G Vector Set (Cat. No. 631192; not sold separately)
 - 20 µl pRetroX-TRE3G Vector (500 ng/µl)
 - 20 µl pRetroX-TRE3G-Luc Control Vector (500 ng /µl)
 - 20 µl pRetroX-Tet3G Vector (500 ng /µl)
- 1 each Retro-X Universal Packaging Vector Set (Cat. No. 631457; not sold separately)
 - 20 µl p10A1 Vector (500 ng/µl)
 - 20 µl pAmpho Vector (500 ng /µl)
 - 20 µl pEco Vector (500 ng /µl)
 - 20 µl pVSV-G Vector (500 ng /µl)
 - 20 µl pQCLIN Retroviral Vector (500 ng /µl))
- 1 ml GP2-293 Packaging Cell Line (2 x 10⁶ cells/ml) (Cat. No. 631458; not sold separately)
- 100 rxns Xfect™ Transfection Reagent (Cat. No. 631317)
- 50 ml Tet System Approved FBS, US-Sourced (Cat. No. 631105)

Retro-X Tet-Express Inducible Expression System (Cat. No. 631190)

- 1 each Retro-X Tet-Express Vector Set (Cat. No. 631194; not sold separately)
 - 20 µl pRetroX-TRE3G Vector (500 ng/µl)
 - 20 µl pRetroX-TRE3G-Luc Control Vector (500 ng /µl)
- 1 each Retro-X Universal Packaging Vector Set (Cat. No. 631457; not sold separately)
 - 20 µl p10A1 Vector (500 ng/µl)
 - 20 µl pAmpho Vector (500 ng /µl)
 - 20 µl pEco Vector (500 ng /µl)
 - 20 µl pVSV-G Vector (500 ng /µl)
 - 20 µl pQCLIN Retroviral Vector (500 ng /µl))
- 1 ml GP2-293 Packaging Cell Line (2 x 10⁶ cells/ml) (Cat. No. 631458; not sold separately)
- 100 rxns Xfect Transfection Reagent (Cat. No. 631317)
- 50 ml Tet System Approved FBS, US-Sourced (Cat. No. 631105)
- 25 rxns Tet-Express (Cat. No. 631177)

C. Retroviral Protein Stabilization/Destabilization Systems

Retro-X ProteoTuner Shield System N (Cat. No. 632171)

- 20 µg pRetroX-PTuner Vector (Cat. No. 632169; not sold separately)
- 60 µl Shield1 (60 µl) (Cat. No. 631037)

Retro-X ProteoTuner Shield System N (w/ ZsGreen1) (Cat. No. 632167)

- 20 µg pRetroX-PTuner IRES Vector (Cat. No. 631035; not sold separately)
- 60 µl Shield1 (60 µl) (Cat. No. 631037)

D. Retroviral Cell Cycle Reporter Vectors

Retroviral Fucci Cell Cycle Vectors

- 10 µg pRetroX-G1-Red Vector (500 ng/µl) (Cat. No. 631463)
- 10 µg pRetroX-SG2M-Cyan Vector (500 ng/µl) (Cat. No. 631462)
- 10 µg pRetroX-SG2M-Red Vector (500 ng/µl) (Cat. No. 631465)
- 10 µg pRetroX-SG2Mcyto-Red Vector (500 ng/µl) (Cat. No. 631464)

E. Retroviral Packaging Systems and Cell Lines

Retro-X Universal Packaging System (Cat. No. 631530)

- 1 each Retro-X Universal Packaging Vector Set (Cat. No. 631457; not sold separately)
 - 20 µl p10A1 Vector (500 ng/µl)
 - 20 µl pAmpho Vector (500 ng /µl)
 - 20 µl pEco Vector (500 ng /µl)
 - 20 µl pVSV-G Vector (500 ng /µl)
 - 20 µl pQCLIN Retroviral Vector (500 ng /µl)
- 1 ml GP2-293 Packaging Cell Line (2 x 10⁶ cells/ml) (Cat. No. 631458; not sold separately)

Pantropic Retroviral Expression System (Cat. No. 631512)

- 1 each Retro-X Pantropic Vector Set (Cat. No. 631460; not sold separately)
 - 20 µl pVSV-G Vector (500 ng /µl)
 - 20 µl pLNHX Vector (500 ng µl)
 - 20 µl pLXRN Vector (500 ng /µl)
 - 20 µl pLLRN Vector (500 ng /µl)
- 1 ml GP2-293 Packaging Cell Line (2 x 10⁶ cells/ml) (Cat. No. 631458; not sold separately)

RetroPack PT67 Cell Line (Cat. No. 631510)

- 1 ml RetroPack PT67 Cell Line (2 x 10⁶ cells/ml)

AmphoPack-293 Cell Line (Cat. No. 631505)

- 1 ml AmphoPack 293 Cell Line (2 x 10⁶ cells/ml)

III. Additional Materials Required

A. Mammalian Cell Culture Supplies

- Packaging Cell Line growth medium: 90% Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g/L), 10% Fetal Bovine Serum; 4 mM L-glutamine, and 3.7 g/L sodium bicarbonate (Sigma-Aldrich Co., No. D5796). Add 1 mM sodium pyruvate.
- Cell growth medium and supplies specific for your target cells
- Sodium pyruvate solution, 100 mM, sterile filtered (Sigma-Aldrich Co., Cat. No. S8636), for supplementing cell culture media
- Penicillin/streptomycin solution of 10,000 units/ml penicillin G sodium and 10,000 µg/ml streptomycin sulfate (100X; Sigma-Aldrich Co., Cat. No. P0781)
- Trypsin-EDTA (Trypsin; Sigma-Aldrich Co., Cat. No. T3924)
- Dulbecco's phosphate buffered saline (DPBS; Sigma-Aldrich Co., Cat. No. D8662)
- L-glutamine solution, 200 mM, sterile filtered (Sigma-Aldrich Co., Cat. No. G7513) [Optional]
- Cell Freezing Medium, with or without DMSO (Sigma-Aldrich Co., Cat. No. C6164 or No. C6039)
- Tissue culture plates (100 mm) for packaging cell transfections; other plates and flasks as required
- 15 ml polystyrene conical centrifuge tube (for the CalPhos™ transfection protocol; e.g., BD Biosciences, Cat. No. 352099)
- Sterile microfuge tubes (1.5 ml) for use in titrating virus stocks; and cryovials for freezing virus stocks
- Crystal violet (Sigma-Aldrich Co., Cat. No. C3886), 1% solution prepared in ethanol, for staining colonies of transduced cells in the virus titration protocol (see Section VIII.B)
- Cloning cylinders (PGC Scientific, No. CORN31666, -31668, or -316610), for isolating clones of stable transductants (recommended only for RetroPack PT67; see Section VII.D)

B. Transfection Reagents

We recommend using either Xfect Transfection Reagent or CalPhos Mammalian Transfection Reagent for transfection of the retroviral transfer vector into Takara Bio's packaging cell lines.

Cat. No.	Transfection Reagent
631317	Xfect Transfection Reagent (100 rxns)
631318	Xfect Transfection Reagent (300 rxns)
631312	CalPhos Mammalian Transfection Reagent (700 rxns)

C. Retrovirus Titration

For accurate and consistent transductions, we highly recommend titrating your retroviral stocks. The **Retro-X qRT-PCR Titration Kit** (Cat. No. 631453) provides a fast and simple qRT-PCR-based titration method. The kit determines viral RNA genome content using qRT-PCR and TB Green® technologies, and titrates virus stocks in ~4 hr. This kit is compatible with all of Takara Bio's retroviral vectors except the MSCV vectors.

D. Retrovirus Concentration

Use **Retro-X Concentrator** (Cat. Nos. 631455 & 631456) to increase your available titer up to 100-fold without ultracentrifugation. Concentrated virus allows you to infect target cells at higher MOIs without making more virus or transfecting additional packaging cells.

E. Transduction Enhancers

Use Polybrene (hexadimethrine bromide; Sigma-Aldrich, No. H9268), Lenti-X™ Accelerator (see below), or RetroNectin® reagent (see below).

- Lenti-X Accelerator is a magnetic bead-based technology designed to accelerate lentiviral and retroviral transduction experiments; visit www.takarabio.com for details.
- RetroNectin is a multivalent molecule that simultaneously binds virus particles and cell surface proteins, maximizing cell-virus contact. RetroNectin, in particular, is recommended for increasing the transduction efficiency of suspension cells and stem cells; visit www.takarabio.com for details.

Cat. No.	Transduction Enhancer	Size
631256	Lenti-X Accelerator	400 µl
631257	Lenti-X Accelerator	1,000 µl
631254	Lenti-X Accelerator Starter Kit	each
T110A	RetroNectin Precoated Dish	10 dishes
T100B	RetroNectin Recombinant Human Fibronectin Fragment	2.5 mg
T100A	RetroNectin Recombinant Human Fibronectin Fragment	0.5 mg

F. Antibiotics for Selecting Transduced Cells

Table 3. Recommended antibiotic concentrations for selecting & maintaining stable cell lines.

		Recommended concentration (µg/ml)	
Cat. No.	Antibiotic	Selecting colonies*	Maintenance
631308	G418 (5 g)	100–800	200
631307	G418 (1 g)		
631306	Puromycin (100 mg)	0.25–10	0.25
631305	Puromycin (25 mg)		
631309	Hygromycin B (1 g)	50–400	100

*When selecting for single colonies, the appropriate dose must be determined empirically for your specific cell line. Test a dosage range using dishes of untransfected cells and choose the dose that kills all of the cells in 3–5 days. If all the cells die in less than 24 hr, you should use a lower dose.

G. Competent *E. coli* Cells Suitable for Retroviral Vector Manipulations

Retroviral and lentiviral vector possess long terminal repeats that can make them unstable in some *E. coli* strains. For manipulations of these vectors we recommend Stellar™ Competent Cells

<u>Cat. No.</u>	<u>Product</u>	<u>Size</u>
636763	Stellar Competent Cells	10 x 100 µl
636766	Stellar Competent Cells	50 x 100 µl
636765	Stellar Electrocompetent Cells	10 transformations

H. Plasmid Purification (Transfection-Grade)

<u>Cat. No.</u>	<u>Product</u>	<u>Size</u>
740412.10	NucleoBond Xtra Midi Plus	10 preps
740416.10	NucleoBond Xtra Maxi Plus	10 preps
740422.10	NucleoBond Xtra Midi EF Plus	10 preps
740426.10	NucleoBond Xtra Maxi EF Plus	10 preps

I. In-Fusion® HD Cloning Plus

In-Fusion is a revolutionary technology that greatly simplifies cloning.

For more information, visit <https://www.takarabio.com/products/cloning/in-fusion-cloning/in-fusion-hd-cloning-plus>

<u>Cat. No.</u>	<u>In-Fusion Cloning Kit</u>
638909	In-Fusion HD Cloning Plus (10 rxns)
638910	In-Fusion HD Cloning Plus (50 rxns)
638911	In-Fusion HD Cloning Plus (100 rxns)

J. Ecotropic Receptor Booster

Ecotropic Receptor Booster (Cat. No. 631471) can temporarily increase the efficiency of transduction when using an ecotropic retrovirus or lentivirus—and can even be used to transduce human cells with these viruses.

IV. Safety Guidelines for Working with Retroviruses

The protocols in this User Manual require the production, handling, and storage of infectious retrovirus. It is imperative to fully understand the potential hazards of, and necessary precautions for, the laboratory use of retroviruses.

The National Institute of Health and Center for Disease Control have designated retroviruses such as Moloney murine leukemia virus (MMLV) as Level 2 organisms. This requires the maintenance of a Biosafety Level 2 facility for work involving this virus and others like it. MMLV does not naturally infect human cells; however, virus packaged from the MMLV-based vectors described here is capable of infecting human cells. The viral supernatants produced by these retroviral systems could, depending on your retroviral insert, contain potentially hazardous recombinant virus. Similar vectors have been approved for human gene therapy trials, attesting to their potential ability to express genes *in vivo*.

IMPORTANT: For these reasons, due caution must be exercised in the production and handling of any recombinant retrovirus. **The user is strongly advised not to create retroviruses capable of expressing known oncogenes in amphotropic or polytropic host range viruses.**

For more information on Biosafety Level 2 agents and practices, download the following reference:

Biosafety in Microbiological and Biomedical Laboratories (BMBL), Fifth Edition (February 2007) HHS Pub. No. (CDC) 93-8395. U.S. Department of Health and Human Services Centers for Disease Control and Prevention and NIH.

Available on the web at <https://www.cdc.gov/labs/BMBL.html>

Biosafety Level 2: The following information is a brief description of Biosafety Level 2. ***It is neither detailed nor complete.*** Details of the practices, safety equipment, and facilities that combine to produce a Biosafety Level 2 are available in the above publication. If possible, observe and learn the practices described below from someone who has experience working with retroviruses.

Summary of Biosafety Level 2:

1. Practices:

- Standard microbiological practices
- Limited access to work area
- Biohazard warning signs posted
- Minimize production of aerosols
- Decontaminate potentially infectious wastes before disposal
- Use precautions with sharps (e.g., syringes, blades)
- Biosafety manual defining any needed waste decontamination or medical surveillance policies

• **Safety equipment:**

- Biological Safety Cabinet, preferably a Class II BSC/laminar flow hood (with a HEPA microfilter) used for all manipulations of agents that cause splashes or aerosols of infectious materials; exhaust air is unrecirculated
- PPE: protective laboratory coats, gloves, face protection as needed

• **Facilities:**

- Autoclave available for waste decontamination
- Chemical disinfectants available for spills

V. Plasmid Vector Manipulations

A. General Molecular Biology Techniques

These protocols contain only general information for propagating plasmid vectors and for preparing your customized expression construct in a Retro-X Vector. For users requiring more information on standard molecular biology practices and cloning techniques, we recommend the following laboratory references:

- *Current Protocols in Molecular Biology*, ed. by F. M. Ausubel *et al.* (1995, John Wiley & Sons, NY).
- *Molecular Cloning: A Laboratory Manual, Fourth Edition*, ed. by Green, M. R. & Sambrook, J. (2012, Cold Spring Harbor Laboratory Press, NY).

B. Plasmid Vector Propagation & Construction of Your Customized Retro-X Vector

1. To ensure that you have a renewable source of plasmid DNA, transform each of the plasmid vectors provided in this kit into a *E. coli* host strain suitable for viral vectors, such as **Stellar Electrocompetent Cells** (Cat. No. 636765). Consult the Vector Information Packet provided with each Retro-X vector for further DNA propagation details.
2. To purify plasmid DNA for cloning purposes, use a suitable **NucleoBond or NucleoSpin Kit**. See www.takarabio.com for available kits and options.
3. Using standard cloning techniques, insert your coding sequence into the vector's multiple cloning site (MCS). Consult the Vector Information Packet provided with each Retro-X vector for additional cloning details. You can also use the **In-Fusion HD Cloning Plus** system (Section III.I) which allows PCR products to be easily cloned into any linearized vector.

NOTE: Depending on the Retro-X vector selected, your GOI sequence (cDNA or gene fragment) may require an ATG initiation codon. In such cases, addition of a Kozak consensus ribosome binding site (Kozak, 1987) may improve expression levels, but this is generally not required. **However, the fragment or cDNA must not contain a polyadenylation signal.** The insertion of such sequences between viral LTRs can cause premature cleavage and polyadenylation during transcription of the viral genome. This interferes with the production of viable recombinant virions (Coffin *et al.*, 1997).

4. Perform a transfection-grade midi- or maxi-scale plasmid DNA preparation for each plasmid that will be transfected into the packaging cells. For guaranteed transfection-grade plasmid DNA, we recommend using **NucleoBond Xtra Midi Plus or Maxi Plus Kits** (Section III.H). For rapid production of endotoxin-free, transfection-grade plasmid DNA, use **NucleoBond Xtra Midi EF Plus or Maxi EF Plus Kits** (Section III.H). Make sure to perform a diagnostic digest to verify the integrity of the recombinant transfer vector after propagation.

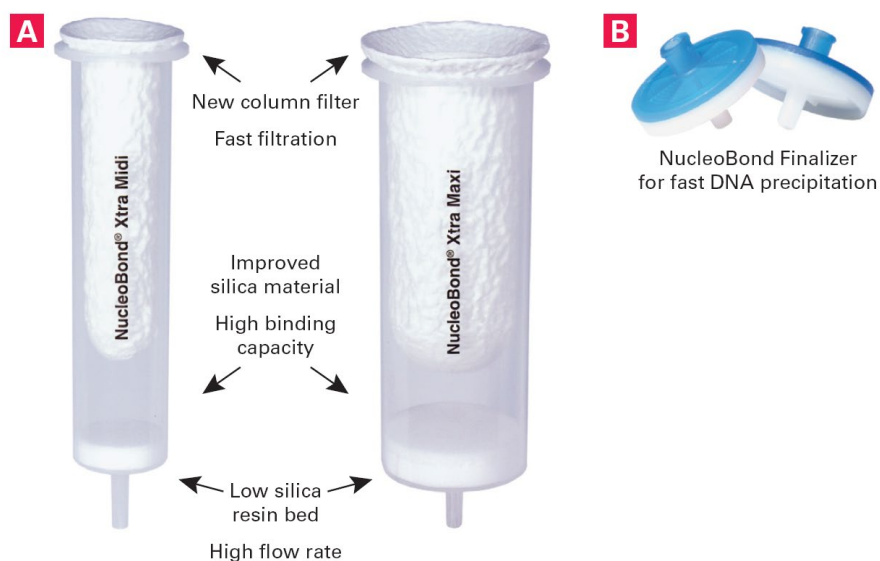


Figure 2. Advanced features of NucleoBond Xtra Maxi and Midi Columns and NucleoBond Finalizer. NucleoBond Xtra columns contain a high-flow column filter that minimizes clogging and clears debris from cell lysates during column loading. An improved silica resin provides high DNA-binding capacity, and a wide column diameter keeps the resin bed low for maximum flow rates (**Panel A**). The NucleoBond Finalizer system speeds preparation and increases purity by capturing precipitated DNA on a syringe filter where it can be easily washed and eluted (**Panel B**).

VI. Working with Retroviral Packaging Cell Lines

A. General Cell Culture and Retrovirus Information

The protocols in this User Manual provide only general guidelines for Retrovirus use and mammalian cell culture techniques. Perform all steps involving cell culture using sterile technique in a Biosafety Level 2 tissue culture hood that has been approved for use with retroviruses. For users requiring more information on retroviruses and mammalian cell culture, we recommend the following general references:

- *Retroviruses*, ed. by J. M. Coffin, S. H. Hughes & H. E. Varmus (1997, Cold Spring Harbor Laboratory Press, NY)
- *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications, 6th Edition*, ed. by R. I. Freshney (2010, Wiley-Liss, NY)
- *Current Protocols in Molecular Biology*, ed. by F. M. Ausubel, *et al.* (1995, Wiley & Sons)

B. Protocol: Reviving Retroviral Packaging Cell Lines from Frozen Stocks

NOTE: This protocol can be completed in 1 hour.

Frozen cells should be cultured immediately upon receipt, or as soon as possible thereafter. If culturing is significantly delayed after receipt, decreased cell viability may result. If cell viability from thawed stocks is low, grow cells for a longer period of time at higher initial density for the first passage.

For HEK 293-based cell lines, we recommend using collagen-coated plates or flasks for efficient culturing of frozen stocks. Vessels coated with compounds other than collagen may also provide suitable growth substrates (e.g. poly-L-lysine), but only collagen has been tested at Takara Bio. Once recovered, the cells may be cultured directly on tissue culture plastic. However, if adherence is poor, we recommend using only collagen-coated vessels.

To prevent osmotic shock and maximize cell survival, perform the following:

1. Warm ~25 ml of complete culture medium in a 37°C water bath. See Section III.A for medium composition.
2. Thaw the vial of cells rapidly in a 37°C water bath with gentle agitation. Immediately upon thawing, wipe the outside of the vial with 70% ethanol. All of the operations from this point on should be carried out in a laminar flow tissue culture hood under strict aseptic conditions. Unscrew the top of the vial slowly and, using a pipet, transfer the contents of the vial to a 15 ml conical centrifuge tube containing 1 ml of prewarmed medium. Mix gently.
3. Slowly add an additional 4 ml of fresh, prewarmed medium to the tube and mix gently.
4. Add an additional 5 ml of prewarmed medium to the tube, mix gently. Centrifuge at 100g for 5 min, carefully aspirate the supernatant, and GENTLY resuspend the cells in complete medium. (This method removes the cryopreservative and can be beneficial when resuspending in small volumes. However, be sure to treat the cells gently to prevent damaging fragile cell membranes.)
5. Mix the cell suspension thoroughly and add to a suitable culture vessel. Gently rock or swirl the dish/flask to distribute the cells evenly over the growth surface and place it in a 37°C humidified incubator (5–10% CO₂ as appropriate) for 24 hr.
6. The next day, examine the cells under a microscope. If the cells are well-attached and confluent, they can be passaged for use. If the majority of cells are not well-attached, continue culturing for another 24 hr. Complete attachment of newly thawed cultures of HEK 293-based cell lines, may require up to 48 hr.
7. Once the culture has been started and the cells are growing normally prepare frozen aliquots to provide a renewable source of cells.

C. Protocol: Freezing Retroviral Packaging Cell Line Stocks

To ensure a renewable source of packaging cells, we recommend expanding and freezing multiple aliquots, soon after thawing or at the earliest passages, according to the following protocol:

1. Expand your cells to multiple 10 cm dishes or T75 flasks.
2. Trypsinize and pool all of the cells, then count the cells using a hemocytometer.
3. Centrifuge the cells at 100g for 5 min. Aspirate the supernatant.
4. Resuspend the pellet at a density of at least $1-2 \times 10^6$ cells/ml in freezing medium. Freezing medium can be purchased from Sigma (Cat. Nos. C6164 & C6039), or use 70–90% FBS, 0–20% medium (without selective antibiotics), and 10% DMSO.
5. Dispense 1 ml aliquots into sterile cryovials and freeze slowly (1°C per min). For this purpose, you can place the vials in Nalgene cryo-containers (Nalgene, Cat. No. 5100-001) and freeze at –80°C overnight. Alternatively, place vials in a thick-walled styrofoam container at –20°C for 1–2 hr. Transfer to –80°C and freeze overnight.
6. The next day, remove the vials from the cryo-containers or styrofoam containers, and place in liquid nitrogen storage or an ultra-low temperature freezer (–150°C) for storage.
7. Two or more weeks later, plate a vial of frozen cells to confirm viability.

D. Packaging Cell Line Maintenance and Passaging

For optimal results, expand your packaging cell line and freeze multiple aliquots to use as master cell stocks as described in Section C. Then create working stocks from the master cell stocks, periodically replacing your working stock from a fresh master. Working stocks that are passaged for extensive periods of time may show reduced performance.

Cells should be plated at 10^6 cells per 100 mm dish and split every 2–3 days when they reach 70–80% confluence. Do not allow your cells to become overconfluent.

- The doubling time for GP2-293, EcoPack 2-293*, and Amphopack-293 cell lines is 24–36 hr.
- The RetroPack PT67 Cell Line has a very short doubling time of <16 hr.

***NOTE:** The EcoPack 2-293 cell line is no longer available from Takara Bio. This information is provided as a courtesy to those who have previously purchased this cell line.

VII. Retrovirus Production using Packaging Cell Lines

A. General Considerations

1. Optimizing Retroviral Titer

To obtain the highest titers from Retro-X Systems, adhere strictly to the following protocols, especially with respect to:

- Culture size and volume
- DNA amounts
- DNA quality (use transfection-grade plasmid DNA)

2. Required Materials & Precautions

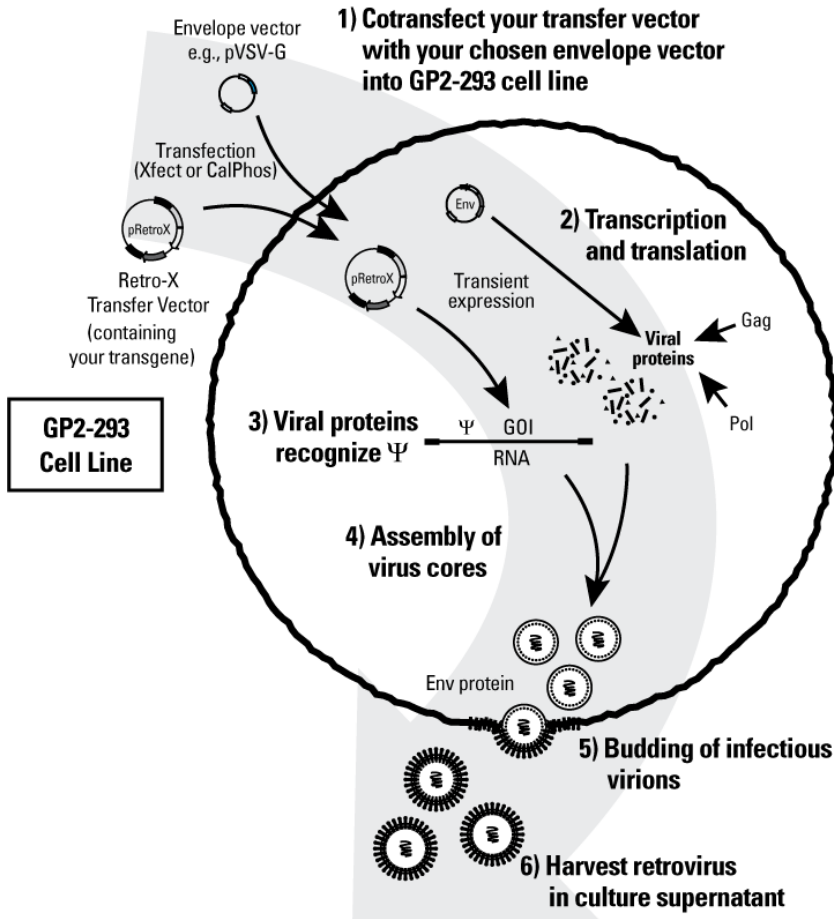
For optimal results, we recommend using:

- Xfect Transfection Reagent or CalPhos Mammalian Transfection Reagent (Section III.B)
- 100-mm culture plates
- Transfection-grade plasmid DNA (e.g., purified using NucleoBond Xtra Maxi Plus; see Section V.B)
- 15 ml polystyrene conical centrifuge tube (for the CalPhos transfection protocol; see Section III.A)

IMPORTANT: Perform all steps in a sterile tissue culture hood. Retrovirus requires the use of a Biosafety Level 2 facility. Recombinant retroviruses pseudotyped with VSV-G, Ampho, or 10A1 are capable of infecting human cells. Know and use appropriate safety precautions (see Section IV).

3. Protocol Summary

A typical protocol involves transient transfection of a retroviral transfer vector that contains a gene of interest into a cell line that expresses some or all of the viral proteins essential for creating a functional retrovirus. The retrovirus supernatant is then harvested 48 hr later. Figure 3 shows a typical order of events when using the Retro-X Universal Packaging System.



Step 1: Cotransfect GP2-293 cells with the Retro-X vector containing your gene of interest (GOI) and an envelope plasmid such as pVSV-G

Step 2: Resulting production of the corresponding recombinant retroviral genome and viral packaging proteins. GP2-293 Cells express gag and pol from genomic locations

Step 3: Recognition of the packaging sequence (Ψ) on the recombinant viral RNA genome by the packaging proteins.

Step 4: Resulting assembly of viral cores, which are transported to the cell membrane.

Step 5: Cores are then enveloped by cellular membrane containing aggregated VSV-G or other envelope proteins. Mature, infectious virions then bud from the cell.

Step 6: Infectious virions are collected in the medium.

NOTE: Although the virions are infectious, they lack several critical genes required for their subsequent replication and production in target cells. Separating the viral proteins and supplying them *in trans* adds a strong measure of safety to virus production, since several low-frequency recombination events would need to occur in order to regenerate a replication-competent viral genome.

Figure 3. Retrovirus production using the Retro-X Universal Packaging System

B. Protocol: Packaging Retroviral Vectors Using Xfect Transfection Reagent

NOTE: This protocol is applicable to all packaging cell types and can be completed in 2–4 days.

1. Approximately 24 hr before transfection, seed 4–5 x 10⁶ cells/100 mm plate, in 10 ml of growth medium. Make sure that the cells are plated evenly. Incubate at 37°C, 5% CO₂ overnight. Continue to incubate the cells until you are ready to add the transfection mixture in Step 7. The cells should be 80–90% confluent at the time of transfection.
2. Thoroughly vortex Xfect Polymer.
3. In a microcentrifuge tube, dilute your retroviral plasmid DNA with Xfect Reaction Buffer to a final volume of 600 µl. Use the following amounts of DNA for the indicated cell lines:
 - **GP2-293:** 15 µg retroviral plasmid + 15 µg envelope plasmid (e.g., pVSV-G)
 - **AmphoPack-293, EcoPack2-293*, and RetroPack PT67:** 15 µg retroviral plasmid

NOTES:

- Always add your plasmid(s) to the Xfect Reaction Buffer before adding Xfect Polymer.
- At least 50% of the solution must consist of Xfect Reaction Buffer.
- *The EcoPack 2-293 cell line is no longer available from Takara Bio. This information is provided as a courtesy to those who have previously purchased this cell line.

4. Mix well by vortexing for 5 sec at high speed.
5. Add the following amounts of Xfect Polymer (for the indicated cell lines) to the diluted retroviral plasmid DNA and mix well by vortexing for 10 sec at high speed.
 - **GP2-293:** 9 µl Xfect Polymer
 - **AmphoPack-293, EcoPack 2-293*, and RetroPack PT67:** 4.5 µl Xfect Polymer

NOTES:

- Always keep the ratio of Xfect Polymer:DNA the same. Use 0.3 µl of Xfect Polymer per 1 µg of plasmid DNA.
- *The EcoPack 2-293 cell line is no longer available from Takara Bio. This information is provided as a courtesy to those who have previously purchased this cell line.

6. Incubate DNA-Xfect mixture for 10 min at room temperature to allow nanoparticle complexes to form.
7. Add the entire 600 µl of DNA-Xfect solution (Step 6) dropwise to the cell culture medium from Step 1. Rock the plate gently back and forth to mix.

NOTE: It is not necessary to remove serum from your cell culture medium. It is normal for the medium to change color slightly upon addition of the DNA-Xfect solution.

8. Incubate the plate at 37°C, 5% CO₂.
9. After 4 hr to overnight, replace the transfection medium with 10 ml fresh complete growth medium and incubate at 37°C for an additional 24–48 hr. Virus titers will generally be highest 48 hr after the start of transfection. *Caution: discarded medium contains infectious retrovirus.*
10. Harvest the retroviral supernatants and pool similar stocks, if desired. *Caution: supernatants contain infectious retrovirus.* Centrifuge briefly (500g for 10 min) or filter through a 0.45 µm filter to remove cellular debris.

NOTE: The filter used should be made of cellulose acetate, or polysulfonate (low protein binding), instead of nitrocellulose. Nitrocellulose binds proteins present in the membrane of retrovirus and destroys the virus.

11. Verify virus production by titrating the virus stock (see Section VIII), then use the virus to transduce target cells, or aliquot and store at -80°C . If smaller volumes are required for transduction, Retro-X Concentrator (Section III.D) can be used.

NOTE: Titers can drop as much as 2–4 fold with each freeze-thaw cycle.

C. Packaging Protocol: Using CalPhos Mammalian Transfection Reagent

NOTE: This protocol is applicable to all packaging cell types and can be completed in 2–4 days.

1. Approximately 24 hr before transfection, seed $4\text{--}5 \times 10^6$ cells/100 mm plate, in 10 ml of growth medium. Make sure that the cells are plated evenly. Incubate at 37°C , 5% CO_2 overnight. The cells should be 80–90% confluent at the time of transfection.
2. In a 15 ml conical centrifuge tube combine your retroviral plasmid DNA with 155 μl 2M Calcium Solution. Use the following amounts of DNA for the indicated cell lines:
 - **GP2-293:** 13 μg retroviral plasmid + 13 μg envelope plasmid (e.g., pVSV-G)
 - **AmphoPack-293, EcoPack 2-293*, RetroPack PT67:** 15 μg retroviral plasmid

***NOTE:** The EcoPack 2-293 cell line is no longer available from Takara Bio. This information is provided as a courtesy to those who have previously purchased this cell line.

3. Add sterile H_2O to a final volume of 1,250 μl .
4. While vortexing at moderate speed, add 1,250 μl 2X HEPES-Buffered Saline (HBS) dropwise.
5. Incubate each DNA-CalPhos mixture for 5 min at room temperature.
6. Add the entire 2.5 ml of DNA-CalPhos precipitate dropwise to the cell culture medium from Step 1. Rock the plate gently back and forth to mix.
7. Incubate the plate at 37°C , 5% CO_2 .
8. After 6 hr to overnight, replace the transfection medium with 10 ml fresh complete growth medium and incubate at 37°C , 5% CO_2 for an additional 24–48 hr. Virus titers will generally be highest 48 hr after the start of transfection. *Caution: discarded medium contains infectious retrovirus.*
9. Harvest the retroviral supernatants and pool similar stocks, if desired. *Caution: supernatants contain infectious retrovirus.* Centrifuge briefly (500g for 10 min) or filter through a 0.45 μm filter to remove cellular debris.

NOTE: The filter used should be made of cellulose acetate, or polysulfonate (low protein binding), instead of nitrocellulose. Nitrocellulose binds proteins present in the membrane of retrovirus and destroys the virus.

10. Verify virus production by titrating the virus stock (see Section VIII), then use the virus to transduce target cells, or aliquot and store at -80°C . If smaller volumes are required for transduction, Retro-X Concentrator (Section III.D) can be used.

NOTE: Titers can drop as much as 2–4 fold with each freeze-thaw cycle.

D. Creating a RetroPack PT67 Stable Virus-Producing Cell Line

If your retroviral vector contains a selection marker, you may select for stable clones producing high-titer virus.

NOTE: This protocol is recommended only for RetroPack PT67. For 293-based packaging cells, we recommend virus production via transient transfection (Sections B or C).

1. Follow the protocols to transfect your transfer vector (Section VII.B.1–7 or Section VII.C. 1–6).
2. Incubate the plate overnight at 37°C , 5% CO_2 .

3. Culture cells for 7 days in medium supplemented with the appropriate antibiotic (see Section III.F)
4. Isolate at least 20 large healthy clones using cloning cylinders (Section III.A). Transfer the clones to individual wells of a culture plate and continue to expand them.
5. Titer the supernatant of each clone (e.g., using the Retro-X qRT-PCR Titration Kit; see Section VIII).
6. Choose at least 2 clones exhibiting the highest titer. We recommend using $>5 \times 10^5$ IFU/ml as a threshold for screening clones.

VIII. Determining Retroviral Titer

A. Introduction

To produce consistent transduction results using a known multiplicity of infection (MOI), it is necessary to titrate your retrovirus stocks. Freshly harvested virus stocks can be titrated immediately, or frozen in aliquots at -80°C and then titrated. Note that each freeze-thaw cycle can reduce the functional titer of the virus stock by up to 2–4 fold.

Titrations are important for determining the relative virus content of stocks prepared from different vectors, and for:

- Confirming the viability of virus stocks
- Determining the optimal transduction conditions
- Adjusting the MOI to control the viral copy number of transduced cells
- Determining the maximum number of cells that can be infected by a virus stock

Titration can be accomplished using different methods, depending on the presence of a selectable or fluorescent marker:

- **qRT-PCR.** Takara Bio offers a convenient **Retro-X qRT-PCR Titration Kit** (see Section III. C) for rapid titration of retroviral supernatants. It employs One-Step qRT-PCR and TB Green chemistry in a fast 4 hr protocol that can be used with any retroviral vector, regardless of the marker involved—and is beneficial for comparing the titers of different vectors and for titrating freshly harvested virus stocks. Since this titration kit does not rely on antibiotic selection, all particles, regardless of genome sequence or infectivity, can be quantified and compared.

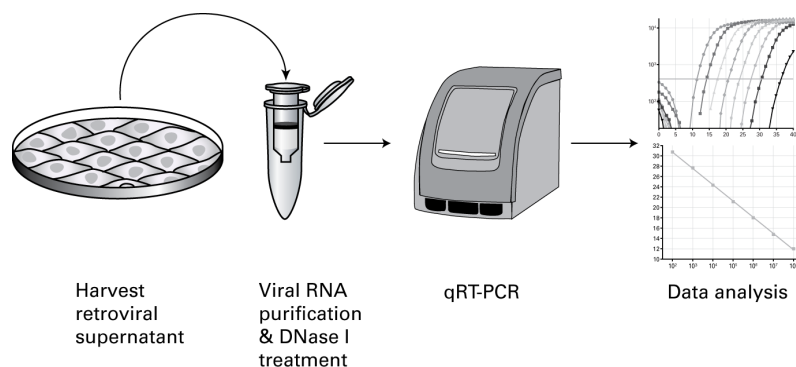


Figure 4. Schematic for titering retrovirus supernatants with the Retro-X qRT-PCR Titration Kit.

- **Flow cytometry.** For Retro-X vectors containing a fluorescent marker, cells can be transduced using the protocol in Section VIII.B, followed by counting the cells ~24–48 hr later using

fluorescence and flow cytometry. Titers determined in this manner are generally higher than those determined by antibiotic selection.

- **Antibiotic selection.** For Retro-X vectors that contain a selectable marker, cells are infected with serial dilutions of the virus stock and then selected for stable transductants using the appropriate antibiotic. Titers are calculated from the number of drug-resistant colonies that develop after selection is completed (Section VIII.B).

B. Protocol: Determining Viral Titer Using Antibiotic Selection

NOTE: This protocol can be completed in 7–14 days.

1. Plate HT-1080 cells (or NIH/3T3 if you are using ecotropic virus) in one 6-well plate the day before performing the titration infections. Plate 2×10^5 cells/well, in 2 ml of medium. Reserve at least one well for a “no infection” control.

NOTE: You can use other cell lines to determine viral titer, but HT-1080 cells are widely accepted as the standard target cell for titrating retrovirus (NIH/3T3 cells for ecotropic virus) because these cells are transduced very efficiently. Note that the same virus preparation can yield different “apparent” titers in different cells lines due to host cell factors that can produce very different transduction efficiencies and hence different titer measurements. Thus, it is important to use the same cell line when comparing titers across experiments.

2. Prepare 20 ml of complete medium and add 60 μ l of 4 mg/ml Polybrene. This concentration of Polybrene (12 μ g/ml) will be eventually diluted 3-fold for a final concentration of 4 μ g/ml during transduction.

NOTE: Polybrene is a polycation that reduces charge repulsion between the virus and the cellular membrane. The optimum final concentration of Polybrene may be determined empirically but generally falls within a range of 2–12 μ g/ml. Excessive exposure to Polybrene (>24 hr) can be toxic to cells.

3. Prepare filtered viral supernatant from the transfected Retro-X packaging cells (Section VII). This is your virus stock.
4. Prepare six 10-fold serial dilutions of the virus stock as follows:
 - a. Add 1.35 ml of medium containing Polybrene (from Step 2) to each of six sterile and numbered 1.5 ml microfuge tubes.
 - b. Add 150 μ l of the virus stock (from Step 3) to Tube 1. Mix gently.
 - c. Transfer 150 μ l from Tube 1 to Tube 2 and mix. Continue making serial dilutions by transferring 150 μ l from each successive dilution into the next prepared tube.
5. Infect the HT-1080 cells by adding 1 ml from each of the five least concentrated viral dilutions (Step 4) to the appropriately labeled wells. The final Polybrene concentration will be 4 μ g/ml in ~3 ml. Centrifuge the cultures to improve transduction efficiency*.

***Culture Centrifugation During Infection Increases Transduction Efficiency**

Centrifuging the plate at 1,200g for 60–90 min at 32°C can significantly increase infection efficiency. A room temperature centrifuge is acceptable if a 32°C unit is not available.

6. After infecting for 8–24 hours, remove the supernatants and begin antibiotic selection using the concentration of antibiotic that is optimal for your cell line (Section III. F). ***Caution: discarded medium contains infectious retrovirus.***

7. Allow drug-resistant colonies to form for 7–14 days. Change media every 2 days, or add fresh antibiotic every fourth day to maintain selection pressure. Stain the colonies with 1% crystal violet solution (in 10% ethanol), and count.
8. The titer of the virus stock corresponds to the number of colonies generated by the least concentrated dilution, multiplied by the dilution factor. For example, the presence of 4 colonies in the 10^6 dilution would represent a titer of 4×10^6 colony forming units.

IX. Protocol: Concentrating Virus Using Retro-X Concentrator

Retro-X Concentrator (Section III.D) is a reagent that allows fast, simple, and highly efficient concentration of any retroviral stock, without using ultracentrifugation. In the simple protocol, retroviral supernatant is mixed with the Retro-X Concentrator reagent, incubated for a short period, and centrifuged in a standard centrifuge.

1. Transfer clarified supernatant (Section VII.B, Step 10, or Section VII.C, Step 9) to a sterile container and combine 1 volume of Retro-X Concentrator with 3 volumes of clarified supernatant. Mix by gentle inversion. Larger volumes may be accommodated through the use of larger (i.e., 250 ml or 500 ml) centrifuge tubes. It is recommended to start with at least 10 ml or more of viral supernatant if the viral titer is expected or known to be low ($\sim 10^5$ IFU/ml).

NOTE: For easy calculation of the amount of Retro-X Concentrator to use, simply measure the amount of viral supernatant to be concentrated, divide by 3 and add the resulting amount of Retro-X Concentrator to your viral supernatant.

2. Incubate mixture overnight at 4°C .
3. Centrifuge sample at 1,500g for 45 minutes at 4°C . After centrifugation, an off-white pellet will be visible.
4. Carefully remove supernatant, taking care not to disturb the pellet. Residual supernatant can be removed with either a pipette tip or by brief centrifugation at 1,500g.
5. Gently resuspend the pellet in 1/10 to 1/100th of the original volume using complete DMEM, PBS, or TNE. The pellet can be somewhat sticky at first, but will go into suspension quickly. Remove cell debris and aggregated virus by low speed centrifugation (500g) for 10 min at 4°C .
6. Titrate sample or store at -70°C in single-use aliquots.

EXPECTED RESULTS: For VSV-G and ecotropic pseudotyped virus, this technique can concentrate up to 100-fold; for amphotropic and dualtropic virus, the expected maximum concentration level is 50-fold.

X. Transducing Target Cells with Retro-X Viruses

NOTE: This protocol can be completed in 2–3 days.

The following protocol is a general method for transducing adherent cell lines, such as HT-1080 or HeLa, using Polybrene. Polybrene is a polycation that reduces charge repulsion between the virus and the cellular membrane. The optimum final concentration of Polybrene may be determined empirically but generally falls within a range of 2–12 µg/ml. However, excessive exposure to Polybrene (>24 hr) can be toxic to cells.

For cells that are difficult to transduce or sensitive to Polybrene, **Lenti-X Accelerator** or **RetroNectin Reagent** can be used to greatly improve speed and transduction efficiency (Section III.E). The Lenti-X Accelerator has the added benefit of providing a much faster 30 min transduction and is equally effective for retroviral and lentiviral transduction. For more information, visit www.takarabio.com

1. Plate target cells in their complete growth medium, 12–18 hr before transduction.
2. Thaw aliquots of your cleared and titrated retroviral stock, or use cleared virus stock freshly prepared from packaging cells (Section VII.B, Step 10, or Section VII.C, Step 9)). Mix gently, but do not vortex. Note that each freeze-thaw cycle will decrease titer by ~2–4-fold.
3. Adjust the volume of medium in the target cell cultures to accommodate the addition of virus and Polybrene. Use sufficient Polybrene to obtain the desired final concentration during the transduction step (e.g., 4 µg/ml).
4. Dilute the retroviral stock with medium to obtain the desired MOI. If titer values are unknown, use serial dilutions of the virus stock or supernatant such that the total volume of virus represents no more than 1/3 the final volume of culture medium used for transduction.
5. Add viral supernatant to the cells and transduce for 8–24 hr. Centrifuge the cultures to improve infection efficiency (see Section VIII.B, Step 5). If you are concerned that exposure to either the Polybrene or to the viral supernatant (which contains medium conditioned by the packaging cells) may adversely affect your target cells, limit the transduction to 6–8 hr.
6. Remove and discard the virus-containing transduction medium and replace it with fresh growth medium. **Caution: discarded medium contains infectious retrovirus.**
7. Continue to incubate the cells for 24–48 hr to allow your gene product to accumulate in the target cells.
8. Harvest the cells for analysis or proceed with selection using the appropriate antibiotic.

NOTE: To determine the efficiency of transduction, you can subject a small subpopulation of cells to antibiotic treatment and harvest the remaining cells for analysis. The cells should be used as soon as possible, but not earlier than 24 hr after transduction.

XI. Troubleshooting Guide

Problem	Possible explanation	Solution
A. Vector cloning		
Plasmid is difficult to grow or clone	Some viral vectors may undergo rearrangements between the 5' and 3' LTRs when propagated in less-than-optimal <i>E. coli</i> host strains	Use Stellar Competent Cells (Cat. No. 636763) to produce high DNA yields and to minimize the potential for DNA rearrangements.
B. Packaging cells		
Poor viability upon thawing	Improper thawing techniques	Use thawing procedure in Section VI.B.
	Incorrect culture medium	Use DMEM with additives listed in Section III.A.
	Improper tissue culture plasticware	Use collagen I-coated plates to aid cell adherence during initial seeding.
Slow growth	Incorrect culture medium	Use DMEM with additives listed in Section III.A.
Cells do not attach to plate	Improper tissue culture plasticware	Use collagen I-coated plates to aid cell adherence during initial seeding.
Cells appear morphologically different	Passage of cell culture is too high (old cells)	Thaw/purchase new aliquot of packaging cells.
C. Virus production		
Poor transfection efficiency (as determined by GOI or marker expression in the packaging cell line)	Cells plated too densely	Plate 4–5 x 10 ⁶ cells/100 mm plate, or fewer if the cells divide rapidly. Use at 80–90% confluency. See Section VII.
	Transfection is toxic to cells	Use the optimized conditions in Section VII.
	Cells harvested or analyzed too soon after transfection	Wait 48 hr after transfection for maximal expression of GOI or marker to determine efficiency.
Low titers (<10 ⁵ cfu/ml)	Poor transfection efficiency	See above Section. Concentrate the virus using the Retro-X Concentrator (Section III.D) to increase your available titer up to 100-fold without ultracentrifugation.
	Poor quality plasmid	Use NucleoBond Xtra (Section III.H) to purify your plasmid.
	Virus was harvested too early	Harvest virus 48–72 hr after the start of transfection.
	Vector is too large.	The limit for efficient packaging function is 8.3 kb from the end of the 5'-LTR to the end of the 3'-LTR.
	Polybrene is missing or at suboptimal concentration	Add Polybrene (4 µg/ml) during transduction or optimize the concentration (2–12 µg/ml)
	Virus was exposed to multiple freeze-thaw cycles	Each cycle reduces titer by approximately 2–4 fold. Limit the number of freeze-thaws.
	Suboptimal selection procedure during titration	Perform an antibiotic kill curve on the cell line prior to using it for titration.

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Problem	Possible explanation	Solution
D. Transduction of target cells		
Poor transduction efficiency	Low titer	See Section C or use the Retro-X Concentrator (III.E) to increase your available titer up to 100-fold without ultracentrifugation.
	Low viability of target cells during transduction	Optimize culture conditions for target cells prior to infection
		Packaging cell line-conditioned media may affect cell growth; dilute viral supernatant or shorten exposure time to viral supernatant. Consider using RetroNectin Reagent and the RetroNectin-Bound Virus transduction protocol
		Excessive exposure to Polybrene: optimize amount (titrate) or shorten exposure time to viral supernatant.
	Viral supernatant contains transduction inhibitors	Use RetroNectin Reagent or RetroNectin-coated plates in the RetroNectin-Bound Virus transduction protocol, which allows virions to bind the RetroNectin substratum and be washed free of inhibitors prior to target cell infection.
Division rate of cells not sufficient to permit transduction	Stimulate cells through passage to a lower density or addition of a mitogen, or switch to a lentiviral format such as the Lenti-X System, which can transduce nondividing or slowly dividing cells.	
Low expression of GOI	Low transduction efficiency	See Section D above.
	Promoter may be weak or possibly inactivated in target cells	Insert a tissue-specific promoter for GOI expression.
	Poor target cell viability	Check growth parameters.
Infection is toxic to target cells	MOI too high (i.e. too much virus used)	Dilute virus stock; titrate the virus.
	Polybrene toxicity	Reduce or optimize Polybrene concentration; reduce infection time.
	Packaging cell supernatant or medium is toxic to cells	Dilute virus stock using target cell culture medium; harvest virus from packaging cells using target cell medium. Consider using RetroNectin Reagent and the RetroNectin-Bound Virus transduction protocol
E. Establishment of stable cell lines		
Untransduced cells do not die at the high antibiotic concentration established via titration in Section III.F	The cells have not been recently passaged, so they remain well-attached to the plate surface even when they are dead.	To determine the appropriate antibiotic concentration, use cells that have been split within the last 2–3 days.
	You have achieved 100% transduction efficiency.	
There are no surviving cells after transduction followed by selection	The antibiotic concentration which caused massive cell death when determining the appropriate dose via titration could be too high.	Use a lower antibiotic concentration for selection of stably transfected cell clones.
Poor cell viability	Cells were not properly frozen.	See Section VI.C.
	Cells were not properly thawed.	See Section VI.B.

XII. References

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