Clontech Laboratories, Inc.

Lenti-X™ Lentiviral Expression System User Manual

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Table of Contents

Table of Figures

Table of Tables

I. Introduction

A. Gene Transfer and Expression Using Recombinant Lentiviruses

Recombinant lentiviral vectors are powerful and efficient tools for transferring heritable genetic material into the genome of virtually any cell type (Ausubel *et al.*, 1995; Coffin *et al.*, 1996). Lentiviruses are perhaps the most versatile of retroviruses since they are able to infect, transduce, and sustain expression in almost any mammalian cell, including dividing and nondividing cells, stem cells, and primary cell cultures. In the Lenti-X systems, high titers of recombinant, replication-incompetent virions are easily generated when a Lenti-X expression vector (Figure 1) containing your gene of interest (GOI) is transfected into the **Lenti-X 293T Cell Line** (Cat. # 632180) using a Lenti-X packaging system. These 4th generation lentiviral packaging systems are available in three different formats:

- The **Lenti-X Packaging Single Shots (VSV-G)** (Cat. #s 631275 & 631276) can generate lentiviral titers that are superior to most other commercially available lentiviral packaging systems. The concerted effects of multiple components in an optimized five-vector plasmid mix, pre-aliquoted and lyophilized with Xfect™ Transfection Reagent, allow Lenti-X 293T cells to produce the highest amounts of safe, replication-incompetent VSV-G-pseudotyped lentivirus (see **www.clontech.com**).
- The **Lenti-X HTX Ecotropic Packaging System** (Cat. # 631251) produces lentivirus pseudotyped with the MLV ecotropic envelope glycoprotein, which allows you to limit transduction to mouse and rat cells.
- The **Lenti-X HTX Packaging System (Integrase Deficient)** (Cat. # 631258) is the only 4th generation lentiviral packaging system that produces integrase-deficient lentiviruses (IDLV), which are gradually lost by dilution in dividing cells (transient expression), but are stable in quiescent cells. Inherently, IDLVs have a greatly reduced risk of causing insertional mutagenesis compared to integrating lentiviruses.

The VSV-G envelope protein works for almost any cell type, while the ecotropic envelope glycoprotein (gp70) from MLV enables mouse and rat cells to be transduced with high efficiency. The Lenti-X HTX Packaging Mix (Integrase Deficient) uses the VSV-G envelope, but produces lentiviral particles deficient for integration into the host cell's genome. Therefore, expression is provided episomally. The lentiviral supernatants produced by the transfected packaging cells can then be used to infect and transduce target cells to express your GOI, fusion protein, or shRNA. Clontech has developed several highly advanced Lenti-X expression systems that provide the broad cellular tropisms of pseudotyped lentivirus; very high titers of safe, nonreplicating virus; and excellent transgene expression levels.

B. Lenti-X Vectors

Clontech offers Lenti-X vectors for many applications (Figure 1). All pLVX vectors possess the requisite HIV-1 LTRs and the lentiviral packaging signal (Ψ) as well as other elements to improve transgene expression, viral titer, and overall vector function.

- **WPRE:** A woodchuck hepatitis virus posttranscriptional regulatory element prevents poly A site readthrough, promotes RNA processing and maturation, and increases nuclear export of RNA (Zufferey *et al.* 1999; Higashimoto *et al.*, 2007). It works in the context of viral genomic transcripts in packaging cells to enhance vector packaging and increase the viral titers. In addition, the WPRE boosts expression of your GOI in transduced target cells by facilitating the maturation of mRNA transcripts produced by the vector's internal promoter (e.g. P_{CMV} or P_{Tight}).

- **cPPT/CTS:** A central polypurine tract/central termination sequence creates a "DNA flap" that increases nuclear importation of the viral genome during target cell infection. The cPPT/CTS element improves vector integration and transduction efficiency (Zennou *et al.*, 2000).
- **RRE:** A Rev response element helps to increase titers by promoting the nuclear export of unspliced viral genomic RNA (Cochrane, *et al.*, 1990).

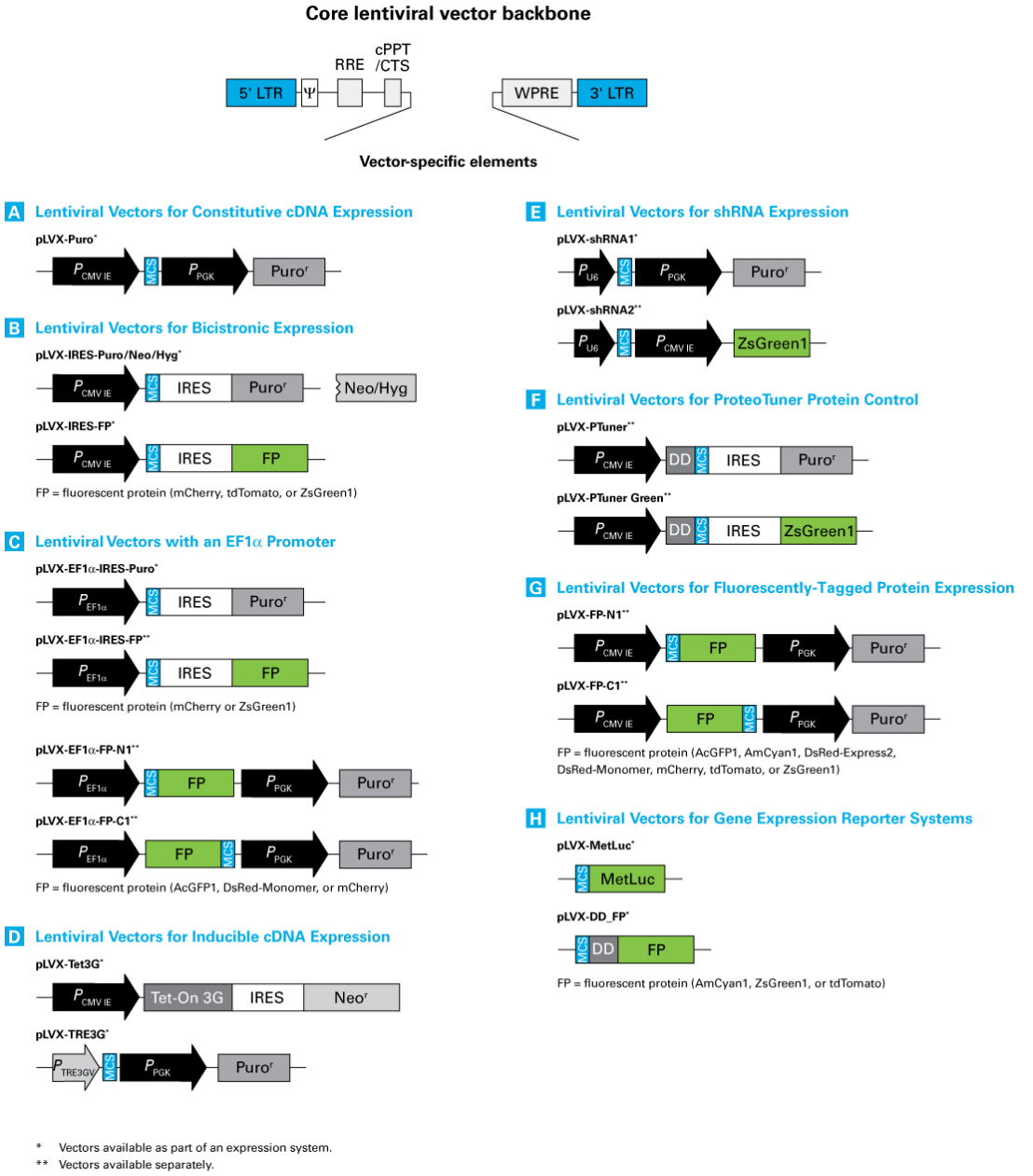


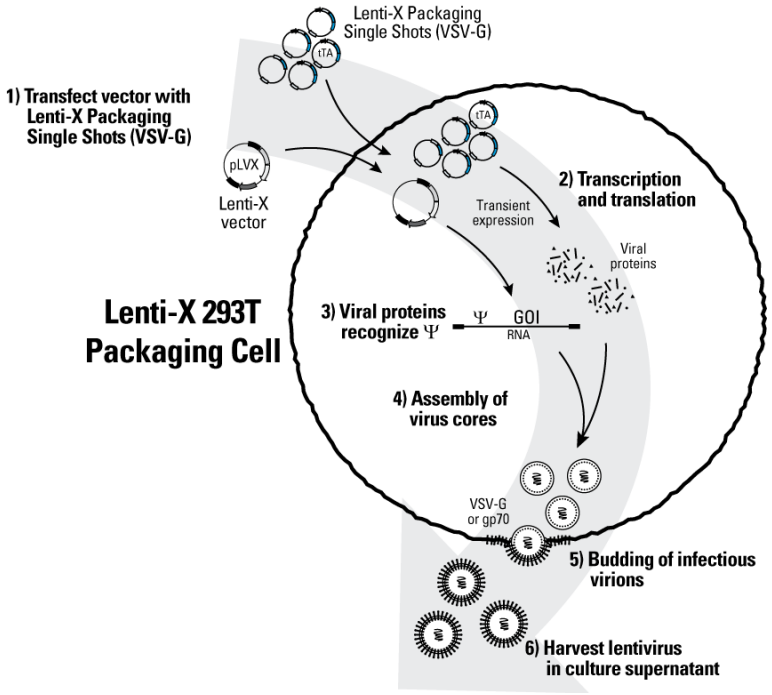
Figure 1. Lentiviral vectors for many applications. Lenti-X vectors contain the LTRs, packaging signal (Ψ), Rev response element (RRE), and central polypurine tract/central termination sequence (cPPT/CTS) from HIV-1; and include a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE). See text for descriptions. All vectors are designed to be used with the **Lenti-X packaging systems** and the **Lenti-X 293T Cell Line**, which together produce very high titers of pseudotyped lentivirus for transducing virtually any cell type. DD: degradation domain; IRES: internal ribosome entry sequence; FP: fluorescent protein; MCS: multiple cloning site; MetLuc: *Metridia luciferase*; $P_{CMV\ IE}$: cytomegalovirus immediate early promoter/enhancer; P_{PGK}: phosphoglucokinase promoter; P_{Tight}: the modified Tet-responsive promoter; P_{U6}: human U6 snRNA promoter (RNA Pol III).

C. Lenti-X Packaging Systems

To produce recombinant lentivirus for target cell infection, Lenti-X plasmid vectors must be co-transfected into Lenti-X 293T cells using a Lenti-X packaging system, in order to assemble your vector and accompanying GOI into infectious virions (Figure 2).

- **Highest Titers:** Lenti-X packaging systems contain plasmid mixtures that provide the necessary viral packaging components in specific, optimized ratios. When your vector and packaging components are delivered into Lenti-X 293T cells, the Pol (RT & IN), Tat, Rev, and Gag lentiviral proteins are expressed together with an envelope that determines the viral particle's tropism (see "Choice of Viral Envelopes and Integration Properties" below). (Wu *et al.*, 2000). The recombinant viral vector is then replicated and assembled into complete, pseudotyped virus particles (Figure 2). The packaging mix includes an expression vector for the Tet-Off® transcriptional activator (tTA) and uses Tet transactivation to produce very high expression levels of specific viral proteins (Gossen & Bujard, 1992). This optimized expression strategy, combined with high-efficiency transfection, produces very high virus titers that can be as much as 25–50 times higher than other commercially available systems. Lenti-X supernatants can very often be used to infect target cells directly without prior concentration.
- **Highest Safety:** For added biosafety, the genes that express the viral packaging proteins have been split onto different plasmids to prevent the collective inclusion of these coding sequences into viral particles during the packaging process. The lack of sequence homology between the packaging mix plasmids and our Lenti-X Vectors also prevents transfer via homologous recombination. This split-gene, trans-expression strategy effectively prevents the production of replication-competent lentivirus, e.g. the viruses cannot replicate autonomously in target cells.
- **Choice of Viral Envelopes and Integration Properties:**
  1. **VSV-G envelope:** The Lenti-X Packaging Single Shots (VSV-G) include all of the lentiviral elements listed previously but also also includes the envelope glycoprotein from the vesicular stomatitis virus (VSV-G).Virions pseudotyped with this envelope can infect both mammalian and non-mammalian cells (Burns *et al.*, 1993), and unlike other viral envelope proteins, VSV-G mediates viral entry through lipid binding and plasma membrane fusion (Emi *et al.*, 1991). VSV-G pseudotyped lentivirus is also very stable and can be concentrated to very high titers by centrifugation.
  2. **Ecotropic envelope:** The Lenti-X HTX Ecotropic Packaging Mix includes all of the lentiviral elements listed previously but also also includes the ecotropic envelope (gp70). Virions pseudotyped with this envelope recognize the mCAT-1 receptor that is expressed primarily on both mouse and rat cells.
  3. **VSV-G envelope (Integrase Deficient):** The Lenti-X HTX Packaging Mix (Integrase Deficient) includes all of lentiviral elements listed previously, including the VSV-G envelope, but also contain a mutation in the sequence encoding the viral integrase. Transduction of target cells produced with this packaging mix leads to the formation of circular lentiviral episomes capable of transient expression of a gene of interest (Banasik & McCray, 2010). Also, because this form of lentivirus is episomal with an integration profile similar to that of naked DNA, it dissipates with each cell division.

- **Overview of Lentivirus Production:**



Step 1: Transfection of a Lenti-X vector with Lenti-X Packaging Single Shots (VSV-G).

Step 2: Resulting production of the corresponding recombinant lentiviral genomic RNA transcript and viral packaging proteins. A vector in the packaging mix encodes the Tet-Off transactivator (tTA), which produces extra-high expression of specific packaging proteins via Tet-Off transactivation.

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 ecotropic/gp70 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. The use of multiple plasmids with which to express the viral proteins 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 2. Lentivirus production with the Lenti-X Packaging Single Shots and Lenti-X 293T cells.

II. Additional Materials Required

A. Cell Lines for Lentivirus Packaging and Titration

- **Lenti-X 293T Cell Line** (Cat. # 632180): This is an HEK 293T-derived cell line optimized for Lenti-X virus production. To obtain high-titer supernatants of infectious lentivirus, transfect your Lenti-X vector into Lenti-X 293T cells using a Lenti-X packaging system. The transfected cells will consistently produce very high titers of pseudotyped lentivirus.
- **HT-1080 cell line:** American Type Culture Collection HT-1080 (ATCC No. CCL-121) [Recommended]. This cell line is easily transduced by recombinant lentiviruses and is frequently used for lentiviral titration. Alternatively, virus stocks can be titrated with the Lenti-X qRT-PCR Titration Kit (Cat. # 631235) or the Lenti-X p24 Rapid Titer Kit (Cat. # 632200). Or, you can save time and test the quality of your lentiviral supernatant in 10 minutes using Lenti-X GoStix (Cat. #s 631243 & 631244).

B. Mammalian Cell Culture Supplies

- **Lenti-X 293T Cell Line growth medium:** 90% Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g/L), 4 mM L-glutamine, and 3.7 g/L sodium bicarbonate (Sigma-Aldrich Co., No. D5796); and 10% *tetracycline-free* fetal bovine serum. *Add 1 mM sodium pyruvate.*
- **HT-1080 growth medium:** 90% Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g/L), 4 mM L-glutamine, and 3.7 g/L sodium bicarbonate (Sigma-Aldrich Co., No. D5796); and 10% fetal bovine serum. *Add 1 mM sodium pyruvate.*

- **Tetracycline-free fetal bovine serum** (FBS; see important information below). *We strongly recommend* using **Tet System Approved FBS** (Cat. #s 631101, 631105, 631106 & 631107) for all packaging cell transfections and for culturing target cells when using a Lenti-X Tet-One™ or Tet-On® 3G Inducible Expression System.
- Cell growth medium and supplies specific for your target cells
- Sodium pyruvate solution, 100 mM, sterile filtered (Sigma-Aldrich Co., 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., No. P0781)
- Trypsin-EDTA (Trypsin; Sigma-Aldrich Co., No. T3924)
- Dulbecco's phosphate buffered saline (DPBS; Sigma-Aldrich Co., No. D8662)
- L-glutamine solution, 200 mM, sterile filtered (Sigma-Aldrich Co., No. G7513) [Optional]
- Cell Freezing Medium, with or without DMSO (Sigma-Aldrich Co., No. C6164 or No. C6039)
- Tissue culture plates (100 mm) for packaging cell transfections; other plates and flasks as required
- Polystyrene culture tubes, 12 x 75 mm (e.g., BD Falcon No. 352054), for packaging cell transfections.
- Sterile microfuge tubes (1.5 ml) for use in titrating virus stocks; and cryovials for freezing virus stocks.
- Crystal violet (Sigma-Aldrich Co., No. C3886), 1% solution prepared in ethanol, for staining colonies of transduced cells in the virus titration protocol (Section VII.B).
- Cloning cylinders (PGC Scientific, No. CORN31666, -31668, or -316610), for isolating clones of stable transductants.

Tetracycline-Free Fetal Bovine Serum (FBS) for Packaging Cell and Target Cell Culture

- Many lots of bovine sera are contaminated with tetracycline (Tc) or its derivatives, which can affect basal expression or inducibility in Tet Expression Systems (Figure 3). It is critical that the FBS used for cell culture not interfere with Tet-responsive expression.
- The Lenti-X packaging systems utilize Tet-Off transactivation to drive high-level expression of specific viral packaging proteins. The presence of Tc contaminants in FBS will reduce expression of these important components and will negatively affect viral titers. *Therefore, 293T cells that host the Lenti-X packaging systems must be cultured in medium containing Tc-free FBS.*
- Tc-contaminants in FBS will also significantly diminish the performance of the Tet-One and Tet-On 3G Systems in target cells.
- These problems can be eliminated by using a **Tet System Approved FBS** (Cat. #s 631101, 631105, 631106 & 631107) from Clontech. These sera have been functionally tested in our Tet Systems and found to be free of contaminating Tc activity.

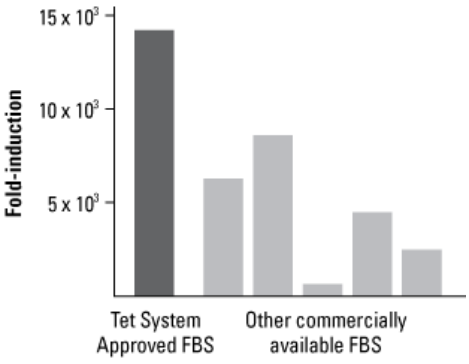


Figure 3. Tetracycline activity in bovine sera. The CHO-AA8-Luc Tet-Off Control Cell Line was grown in media prepared with different lots of FBS. Average uninduced expression level = 0.21 RLU (n=21, S.D = 0.07); maximum expression levels varied from 123 to 3,176 RLU.

C. Lentivirus Titer Determination

For accurate and consistent transductions, we highly recommend titrating your lentiviral stocks. The **Lenti-X qRT-PCR Titration Kit** provides a fast and simple qRT-PCR-based titration method. The kit determines viral RNA genome content using qRT-PCR and SYBR® technologies, and titrates virus stocks in ~4 hr. The **Lenti-X p24 Rapid Titer Kit** uses ELISA to specifically measure the amount of p24 capsid protein present in your viral supernatant, and then correlates the level of p24 directly to virus titer. Alternatively, you can use **Lenti-X GoStix™** to test the quality of your viral supernatant in just 10 minutes. The GoStix detect lentiviral p24 in only 20 µl, and can be used to determine whether virus production is within a usable range or for selecting the best time to harvest your virus.

Cat. #	Lentiviral Titration Technology
632200	Lenti-X p24 Rapid Titer Kit (96 rxns)
631235	Lenti-X qRT-PCR Titration Kit (200 rxns)
631243	Lenti-X GoStix (20 tests)
631244	Lenti-X GoStix (50 tests)

D. Lentivirus Purification

Virus purification enables you to remove cellular contaminants that could otherwise adversely affect your transduction experiments. The **Lenti-X Maxi Purification Kits** produce outstanding yields of highly purified virus from crude supernatants. The gravity column-based protocol is fast, simple, and effective, and produces virus that is fully intact and fully functional.

Cat. #	Lentiviral Purification Kit
631233	Lenti-X Maxi Purification Kit (2 preps)
631234	Lenti-X Maxi Purification Kit (5 preps)
631245	Lenti-X Maxi Purification Kit (with Rack)

E. Lentivirus Concentration

Use Lenti-X Concentrator to easily 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—see **www.clontech.com** for details.

Cat. #	Concentrator
631231	Lenti-X Concentrator (100 ml)
631232	Lenti-X Concentrator (500 ml)

F. Antibiotics for Selecting Stable Cell Lines

		Recommended Concentration (µg/ml)	
Cat. #	**Antibiotic**	**Selecting Colonies[1]**	**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

[1] 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. Transduction Enhancers

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

- Lenti-X Accelerator is a magnetic bead-based technology designed to accelerate lentiviral and retroviral transduction experiments; visit **www.clontech.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; see **www.clontech.com** for details.

Cat. #	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

H. Xfect Transfection Reagent

Xfect Transfection Reagent provides high transfection efficiency and low cytotoxicity for most commonly used cell types.

Cat. #	Transfection Reagent
631317	Xfect Transfection Reagent (100 rxns)
631318	Xfect Transfection Reagent (300 rxns)

I. In-Fusion® HD Cloning System

In-Fusion is a revolutionary technology that permits highly efficient, seamless, and directional cloning. For more information, visit www.clontech.com/infusion

Cat. #	In-Fusion Cloning Kit
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. Stellar™ Competent Cells

Stellar Competent Cells are recommended by Clontech for cloning of lentiviral and retroviral vectors. Propagation of vectors containing repeat sequences such as viral LTRs using other strains of *E.coli* may result in plasmid rearrangements. Stellar Competent Cells are sold separately and provided with all In-Fusion HD Cloning Systems.

Cat. #	Competent Cells
636763	Stellar Competent Cells (10 x 100 µl)
636766	Stellar Competent Cells (50 x 100 µl)

III. Safety Guidelines for Working with Lentiviruses

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

The National Institute of Health and Center for Disease Control have designated recombinant lentiviruses as Level 2 organisms. This requires the maintenance of a Biosafety Level 2 facility for work involving this virus and others like it. The pseudotyped lentiviruses packaged from the HIV-1-based vectors described here are capable of infecting human cells. The viral supernatants produced by these lentiviral systems could, depending on your 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 lentivirus. **The user is strongly advised not to create pseudotyped lentiviruses capable of expressing known oncogenes.**

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 **http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm**

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 lentiviruses.

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

IV. 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 Lenti-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* ed. by J. Sambrook *et al.* (2001, Cold Spring Harbor Laboratory Press, NY).

B. Plasmid Vector Propagation & Construction of Your Customized Lenti-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 an *E. coli* host strain suitable for viral vectors, such as **Stellar Electrocompetent Cells** (Section II.J). Consult the Vector Information Packet provided with each Lenti-X vector for further DNA propagation details.

2. To purify plasmid DNA for cloning purposes, use a suitable **NucleoBond** or **NucleoSpin** Kit. See **www.clontech.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 Lenti-X vector for additional cloning details. You can also use the **In-Fusion HD Cloning System** (Section II.I) which allows PCR products to be easily cloned into any linearized vector.

   NOTE: Depending on the Lenti-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 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 (Figure 4; Cat. #s 740412.10 and 740416.10). For rapid production of endotoxin-free, transfection-grade plasmid DNA, use **NucleoBond Xtra Midi EF Plus** or **Maxi EF Plus Kits** (Cat. #s 740422.10 and 740426.10; see Figure 4).

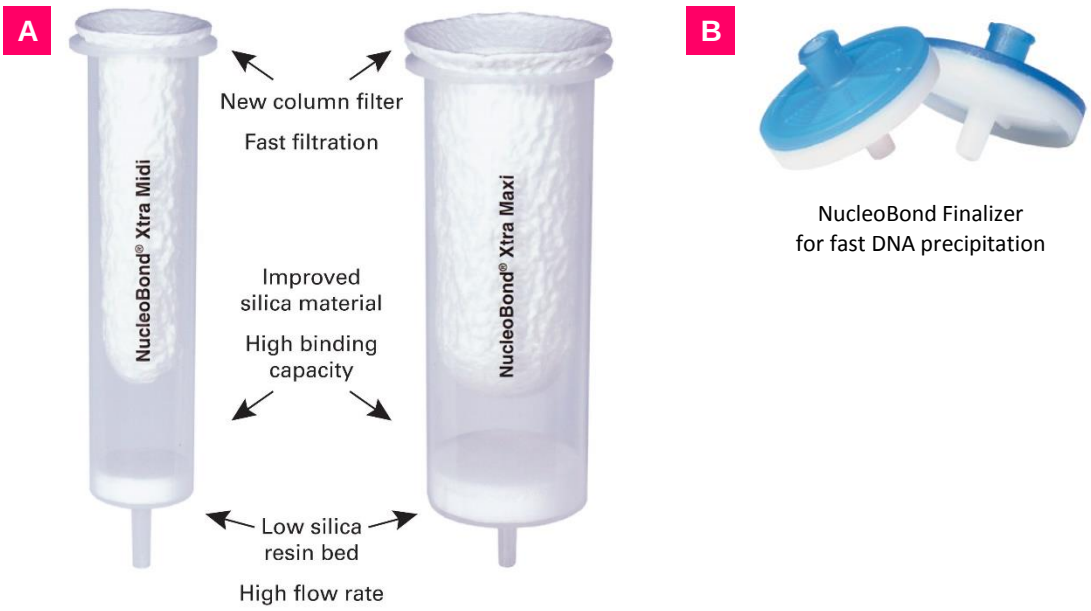


Figure 4. Advanced features of NucleoBond Xtra Maxi and Midi Columns and NucleoBond Finalizer. NucleoBond Xtra columns contain a high-flow column filter that minimizes clogging and clears debris from cell lysates during column loading. An improved silica resin provides high DNA-binding capacity, and 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**).

V. Cell Culture Guidelines

A. General Cell Culture and Lentivirus Information

The protocols in this User Manual provide only general guidelines for lentivirus 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 lentiviruses. For users requiring more information on lentiviruses, 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*, 5th Edition, ed. by R. I. Freshney (2005, Wiley-Liss, NY)
- *Current Protocols in Molecular Biology*, ed. by F. M. Ausubel, *et al.* (1995, Wiley & Sons)

B. Protocol: Starting Lenti-X 293T Cell Line Cultures from Frozen Stock

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. 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 Clontech. 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 II.B for medium composition.

   NOTE: Be sure to use Tet System Approved Fetal Bovine Serum (Cat. #s 631101, 631105, 631106 & 631107) when using these cells with a Lenti-X Packaging System.

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 pre-warmed medium. Mix gently.
3. Slowly add an additional 4 ml of fresh, pre-warmed medium to the tube and mix gently.
4. Add an additional 5 ml of pre-warmed medium to the tube, mix gently. Centrifuge at 100 x g 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 most of them 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, you should prepare frozen aliquots to provide a renewable source of cells. Consult the Lenti-X 293T Cell Line Protocol-at-a-Glance (PT4058-2) for a cell freezing protocol.

VI. Producing Lentivirus from Lenti-X Vectors

Our 4th generation lentiviral packaging systems are available in three different formats, as described in Section I.A. Use the **Lenti-X Packaging Single Shots (VSV-G) t**o produce VSV-G-pseudotyped lentivirus, which readily infects virtually all types of cells. To obtain non-integrating VSV-G-pseudotyped lentivirus, use the **Lenti-X HTX Packaging System (Integrase Deficient).** The **Lenti-X HTX Ecotropic Packaging System** produces lentivirus pseudotyped with the MLV ecotropic envelope glycoprotein, which allows you to limit transduction to mouse and rat cells. (Follow the Protocol-At-A-Glance for each of these systems, which can be found by searching at **www.clontech.com/manuals**).

VII. Determining Lentiviral Titer

A. Introduction

To produce consistent transduction results using a known multiplicity of infection (MOI), it is necessary to titrate your Lenti-X virus 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. Titer values will depend heavily on the cell type and method used for titration, so there may be significant differences between titers determined in cells typically used for titration (e.g. HT-1080) and the number of target cells that are ultimately transduced. However, 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:

- **Instant Lentivirus Test.** You can assess the quality of your lentivirus stock in 10 minutes with Clontech's **Lenti-X GoStix** (Cat. #s 631243 & 631244). The GoStix detect lentiviral p24 in only 20 µl, and can be used to determine whether virus production is within a usable range or for selecting the best time to harvest your virus. A 3 prep sample is supplied for free with many of Clontech's Lenti-X systems.

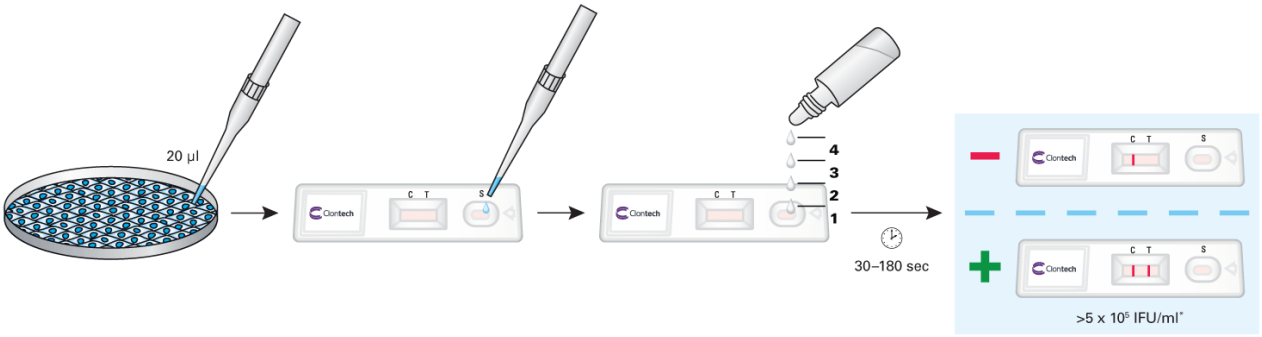


Figure 5. The Lenti-X GoStix protocol takes only 10 minutes.

- **qRT-PCR.** Clontech offers a convenient **Lenti-X qRT-PCR Titration Kit** (Cat. # 631235) for rapid titration of lentiviral supernatants. It employs One-Step qRT-PCR and SYBR Green chemistry in a 4 hr protocol that can be used with any lentiviral vector, regardless of the marker involved, and is beneficial for comparing the titers of different vectors and for titrating freshly harvested virus stocks.
- **p24 ELISA. The Lenti-X p24 Rapid Titer Kit** (Cat. # 632200) uses ELISA to specifically measure the amount of p24 capsid protein present in your viral supernatant and then correlates the level of p24 to virus titer. The assay requires ~4 hr to complete.

NOTE: Lenti-X titration kits can be used with any HIV-1 based lentiviral vector.

- **Flow cytometry.** For Lenti-X vectors containing a fluorescent marker, cells can be transduced using the protocol in Section 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 Lenti-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.

B. Protocol: Determining Viral Titer Using Antibiotic Selection

1. Plate HT-1080 cells (or another cell line) in one 6-well plate the day before performing the titration infections. Plate 2 x 10^5 cells/well, in 2 ml of medium. Reserve at least one well for a "no infection" control.

   NOTE: This procedure is not recommended for cells transduced with the Lenti-X HTX Packaging Mix (Integrase Deficient) due to the potential for selection of rare integration events.

2. Prepare 20 ml of complete medium and add 60 µl of 4 mg/ml Polybrene. This concentration of Polybrene (12 µg/ml) will be eventually diluted 3-fold for a final concentration of 4 µg/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 µg/ml. Excessive exposure to Polybrene (>24 hr) can be toxic to cells.

3. Prepare cleared viral supernatant from the transfected Lenti-X 293T packaging cells (Section VI). 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 µl of the virus stock (from Step 3) to tube 1. Mix gently.
   c. Transfer 150 µl from tube 1 to tube 2 and mix. Continue making serial dilutions by transferring 150 µl from each successive dilution into the next prepared tube.
5. Infect the HT-1080 cells by adding 1 ml from each of the 5 least concentrated viral dilutions (Step 4) to the appropriately labeled wells. The final Polybrene concentration will be 4 µg/ml in ~3 ml. Centrifuge the cultures to improve transduction efficiency*.
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 (Appendix A). Caution: discarded medium contains infectious lentivirus.
7. Allow drug-resistant colonies to form for 7–14 days. 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 x 10^6 colony forming units.

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

Centrifuging the plate at 1,200 x g 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.

C. Protocol: Alternative Titration Methods

- The **Lenti-X qRT-PCR Titration Kit** directly quantitates the viral genomes in your virus stock, which is much faster and more versatile than antibiotic selection. Since it does not rely on antibiotic selection, all particles, regardless of genome sequence or infectivity, can be quantitated. Functional titers do not yield accurate measures of virion concentration, and are subject to the infection and transduction efficiencies of the cell line being used for titration.
- The **Lenti-X p24 Rapid Titer Kit** employs a straightforward ELISA of the HIV-1 p24 capsid protein to measure lentiviral titer. p24 content is typically correlated to the number of infectious units of the same stock, or another stock for which both p24 and infectious unit values are known.
- You may also determine viral titer by infecting HT-1080 cells with serially diluted viral supernatants produced using a control vector containing an easily detectable reporter gene (e.g. *LacZ*, luciferase, or a fluorescent protein). Test virus infection on both HT-1080 cells and your target cells. Infecting your target cell line will give you a rough, but rapid, estimation of infection success. You can use other cell lines to determine viral titer, but HT-1080 cells are widely accepted as the standard target cell for titrating lentivirus because these cells are transduced very efficiently. Note that the same virus preparation can yield different "apparent" titers on different cells lines due to host cell factors that can produce very different transduction efficiencies.
- Some variations of the drug-resistance colony assay employ either a shorter selection period (3 days; Byun *et al.*, 1996); recently-infected target cells (Tafuro *et al.*, 1996; Miyao *et al.*, 1995); or in situ PCR (PRINS; Claudio *et al.*, 2001), but achieve similar results.
- Other methods for the direct quantitation of virus particles include slot blots (Nelson *et al.*, 1998; Murdoch, *et al.*, 1997; Onodera *et al.*, 1997) and PCR applied to viral supernatants (Quinn & Trevor, 1997; Morgan *et al.*, 1990). Reverse transcriptase activity has also been used for titration (Goff *et al.*, 1981).

VIII. Transducing Target Cells with Lenti-X Viruses

A. Protocol: Transducing Target Cells with Lenti-X Viruses

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. This protocol can be used as a starting point for determining the optimal transduction conditions for your target cells. Refer to Appendix B for additional references and alternative infection methods. For cells that are difficult to transduce or that might be sensitive to Polybrene, **Lenti-X Accelerator** (Cat. # 631254) or **RetroNectin Reagent** (Takara Bio Inc, Cat. #s T100A & T100B) can be used to greatly improve speed and transduction efficiency.

1. Plate target cells in their complete growth medium, 12–18 hr before transduction.
2. Thaw aliquots of your cleared and titrated lentiviral stock, or use cleared virus stock freshly prepared from packaging cells (Section VI). 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 lentiviral 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. See Information Box below.
5. Add viral supernatant to the cells and transduce for 8–24 hr. Centrifuge the cultures to improve infection efficiency (see Section VII.B). 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 lentivirus*.
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.

Using Untitrated Lenti-X Virus Stocks and Supernatants

Lenti-X packaging systems are capable of producing very high virus titers. Using excessive amounts of virus can be detrimental to target cell performance and viability. If you have not determined the titer of your virus stock, perform transduction experiments using several different fold-dilutions to test a range of MOIs. At Clontech, our scientists can often transduce an entire 100 mm dish of target cells using 10–100 µl of unconcentrated Lenti-X supernatant.

NOTE: Due to the episomal nature of the vector, when using the Lenti-X Packaging Mix (Integrase Deficient), the duration of transgene expression will depend on the division rate of your target cells (Figure 6).

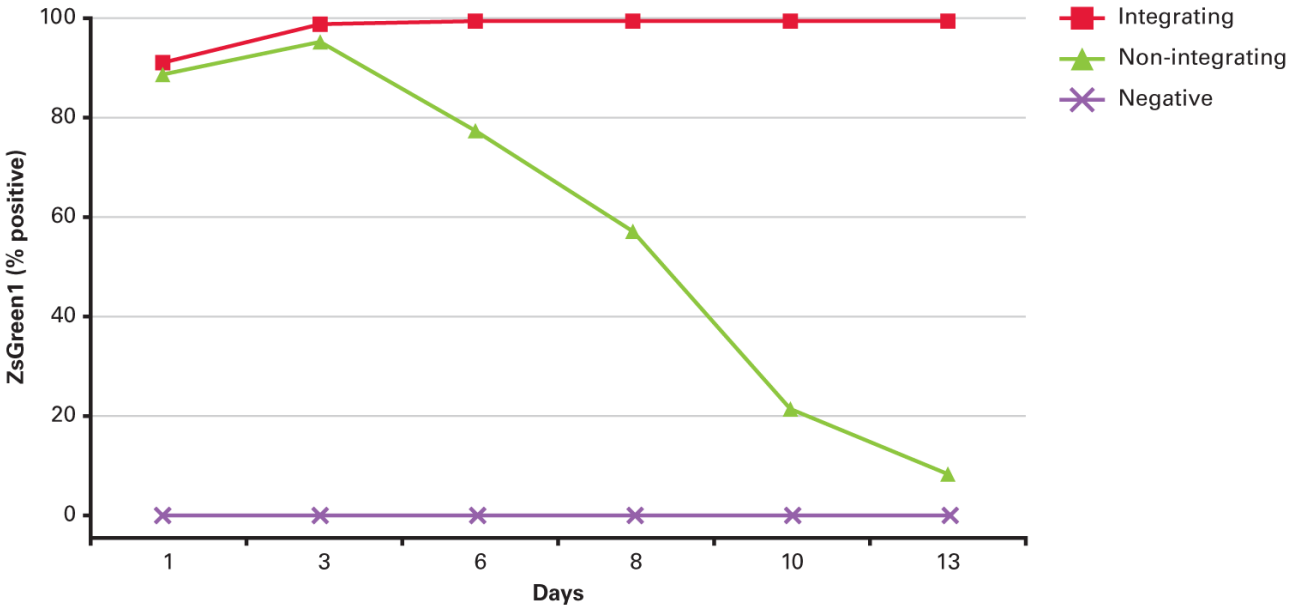


Figure 6. Lenti-X HTX Packaging Mix (Integrase Deficient) transgene expression profile. Integrating and non-integrating lentiviral particles containing a vector expressing ZsGreen1 were each produced in Lenti-X 293T cells using the respective Lenti-X HTX and Lenti-X HTX (Integrase Deficient) packaging mixes. Virus was harvested at 48 hr post-transfection and used to infect HT1080 cells at equivalent MOIs. The percentages of ZsGreen1-positive cells were determined by flow cytometry on the days indicated. Equivalent expression levels were achieved by both transductions up to Day 3, when the cells transduced with the integrase-deficient lentivirus began to demonstrate a decrease in the number of positive cells. The number of positive cells returned to near background levels by Day 13.

IX. 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 *E. coli* host strains	Use **Stellar Competent Cells** (Cat. # 636763) to produce high DNA yields and to minimize the potential for DNA rearrangements.
B. Lenti-X 293T Packaging Cells		
Poor viability upon thawing	Improper thawing techniques	Use thawing procedure in Section V.B, and/or consult the Lenti-X 293T Cell Line Protocol-at-a-Glance
	Incorrect culture medium	Use DMEM with additives listed in Section II.B. Use 10% Tet System Approved FBS (Tc-free).
	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 II.B. Use 10% Tet System Approved FBS (Tc-free).
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 Lenti-X 293T cells.
C. Virus Production		
Poor transfection efficiency (as determined by GOI or marker expression in the Lenti-X 293T 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 50–80% confluency. See Section VI.
	Transfection is toxic to cells	Use the optimized conditions provided in Section VI.
	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)	Serum in medium contains tetracycline contaminants	Use **Tet System Approved FBS** (Cat. Nos. 631101, 631105, 631106 & 631107) in the 293T culture medium.
	Poor transfection efficiency	See above section. Concentrate the virus using centrifugation (see Appendix A) or use the **Lenti-X Concentrator** (Cat. Nos. 631231 & 631232) to increase your available titer up to 100-fold without ultracentrifugation.
	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 9.7 kb from the end of the 5'-LTR to the end of the 3'-LTR.)	Concentrate the virus (see Appendix A) for large vectors or reduce size of the insert.
	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.

Problem	Possible Explanation	Solution
D. Transduction of Target Cells		
Poor transduction efficiency	Low titer	See Section C or use the Lenti-X Concentrator (Section II.E) to increase your available titer up to 100-fold without ultracentrifugation.
	Transduction protocol not optimized	See Appendix B for references to help with optimizing transduction protocols.
	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 or purify your virus prior to transduction using the **Lenti-X Maxi Purification Kit** (Cat. #s 631233 & 631234).
		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; or, purify your virus prior to transduction using the **Lenti-X Maxi Purification Kit** (Cat. #s 631233 & 631234).
Low expression of GOI	Low transduction efficiency	See Section D. Poor Transduction efficiency 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 purifying your virus prior to transduction using the **Lenti-X Maxi Purification Kit** (Cat. #s 631233 & 631234).

X. References

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Appendix A: Additional Protocols

A. Protocol: Titrating Antibiotics for Selecting Stable Cell Lines

Prior to using the antibiotics G418 (Cat. # 631308) and/or puromycin (Cat. # 631306) to select cells that have been either singly- or doubly-transduced with Lenti-X lentiviruses, it is necessary to titrate each selection agent to determine the optimal concentration for your target cell line. With HeLa cells, for example, we have found 400 µg/ml G418 and 1.0 µg/ml puromycin to be optimal.

Table 1. Recommended Concentrations for Selection Antibiotics (µg/ml)

		Recommended Concentration (µg/ml)	
Cat. #	**Antibiotic**	**Selecting Colonies[1]**	**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

[1] 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.

- For selecting stable transformants with G418 and hygromycin B, use the lowest concentration that results in massive cell death in ~5 days and kills all the cells within two weeks.
- Puromycin selection occurs more rapidly; use a concentration that will kill all cells within 3–4 days.
- Lot-to-lot variations in potency exist for all selection drugs, so each new lot of antibiotic should be titrated.

1. For each antibiotic to be tested, plate 2 x 10^5 cells in each well of a 6-well plate containing 3 ml of the appropriate complete medium plus increasing concentrations of G418 (0, 50, 100, 200, 400, and 800 µg/ml). For puromycin, add the drug at 0, 1.0, 2.5, 5.0, 7.5, and 10.0 µg/ml.
2. For G418, incubate the cells for 5–10 days or until all cells are dead. Examine the dishes for viable cells every two days. Replace the selective medium every four days (or more often if necessary), until the optimal concentration is determined.
3. For puromycin, incubate the cells 4–7 days. Replace medium after 2 days to remove dead cells.

B. Protocol: Concentrating Virus Using Ultracentrifugation

NOTE: Lenti-X Concentrator (Cat. #s 631231 & 631232) is a very cost-effective reagent that allows fast, simple, and highly efficient concentration of any lentiviral stock, without using ultracentrifugation. In the simple protocol, lentiviral supernatant is mixed with the Lenti-X Concentrator reagent, incubated for a short period, and spun in a standard centrifuge.

This ultracentrifugation protocol should be used for VSV-G-enveloped virions only (Burns *et al*, 1994).

1. Remove cell debris and aggregated virus by low speed centrifugation (500 x g) for 10 min at 4°C.
2. Pellet the virus at 50,000 x g for 90 min at 4°C. Remove the supernatant.
3. Resuspend the virus to 0.5–1% of the original volume in TNE (50 mM Tris-HCl [pH 7.8], 130 mM NaCl, 1 mM EDTA), and incubate overnight at 4°C.

   NOTE: If desired, perform a second round of ultracentrifugation (Steps 1–2).
4. Determine the viral titers of pre- and post-concentrated viral supernatants.
5. Transduce target cells.

Appendix B: Additional Viral Infection Methods

These references are provided for fine-tuning your transduction protocols so that you may improve your transduction efficiency in target cells. This list is not a comprehensive list, but many of these protocols will work for a wide range of cell types. You must determine which methods work best for your targets and certain methods may have additive effects. For optimization experiments, we recommend using one of our Lenti-X Fluorescent Vectors to express a Living Colors Fluorescent Protein, which simplifies the detection and quantitation of lentiviral gene transfer efficiency.

A. Transduction of cells at 32°C. Decreasing temperature increases viral half-life during transduction.

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D. Precipitation, during transduction, facilitates greater contact between the target cells and virions

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F. Addition of fibronectin: adhesion domains within fibronectin allow binding to both target cells and virions to facilitate colocalization

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