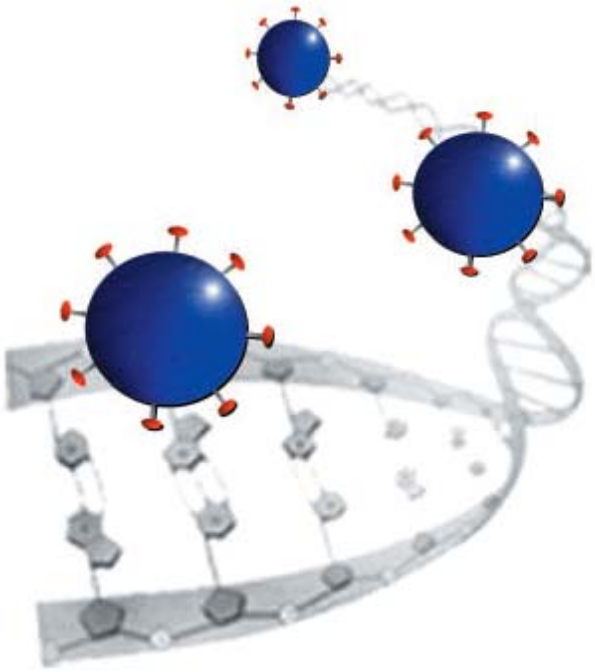
TELEVECTOR™ Lentiviral Vector

Easy-high Production System

Instruction Manual



For Technical Service
Chenhui Road 377/144，Zhangjiang High-Tech Zone
Shanghai 201203 China
0086-21-50271899 (Head Office)
Tel: 0086-15921731086 (Department of Technic Support)
Email: televector@hotmail.com
Fax: 0086-21-50277136 Http:// www.telebio.cn

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TELEVECTOR™ Lentiviral Vector Easy-high Production Kit

Cat. No. L-1109

Cat. No. L-1227

Store at 4℃ (do not freeze before reconstitution)

Store at -20℃ after reconstitution

IMPORTANT:

When you receive this Kit, Please make a small storage of each reagent (A and B), each reagent 100ul per tube, store at -20℃. Defreeze it just 10 minutes before using.

All decontamination steps should be performed using 70% ethanol/1% SDS. Gloves should be worn at all times when handling lentiviral preparations, transfected cells or the combined TELEVECTOR™ reagents. Just remember that although this virus has been significantly modified for biosafety, it derived from HIV and with a VSV pseudotype human cells can be infected even if they are not dividing. That said, the following modifications have been made to prevent viral replication.

Description

TELEVECTOR™ Lentiviral Vector Easy-high Production Kit is a proprietary formulation for the production of lentiviral vector providing the following advantages:

- The ratio of several help plasmids has been optimized, and separated into reagent A and reagent B
- A high transfection reagent for 293T cells has been researched and optimized
- It is not necessary to remove complex or change/add medium after transfection, but complexes maybe removed after 12 hours.

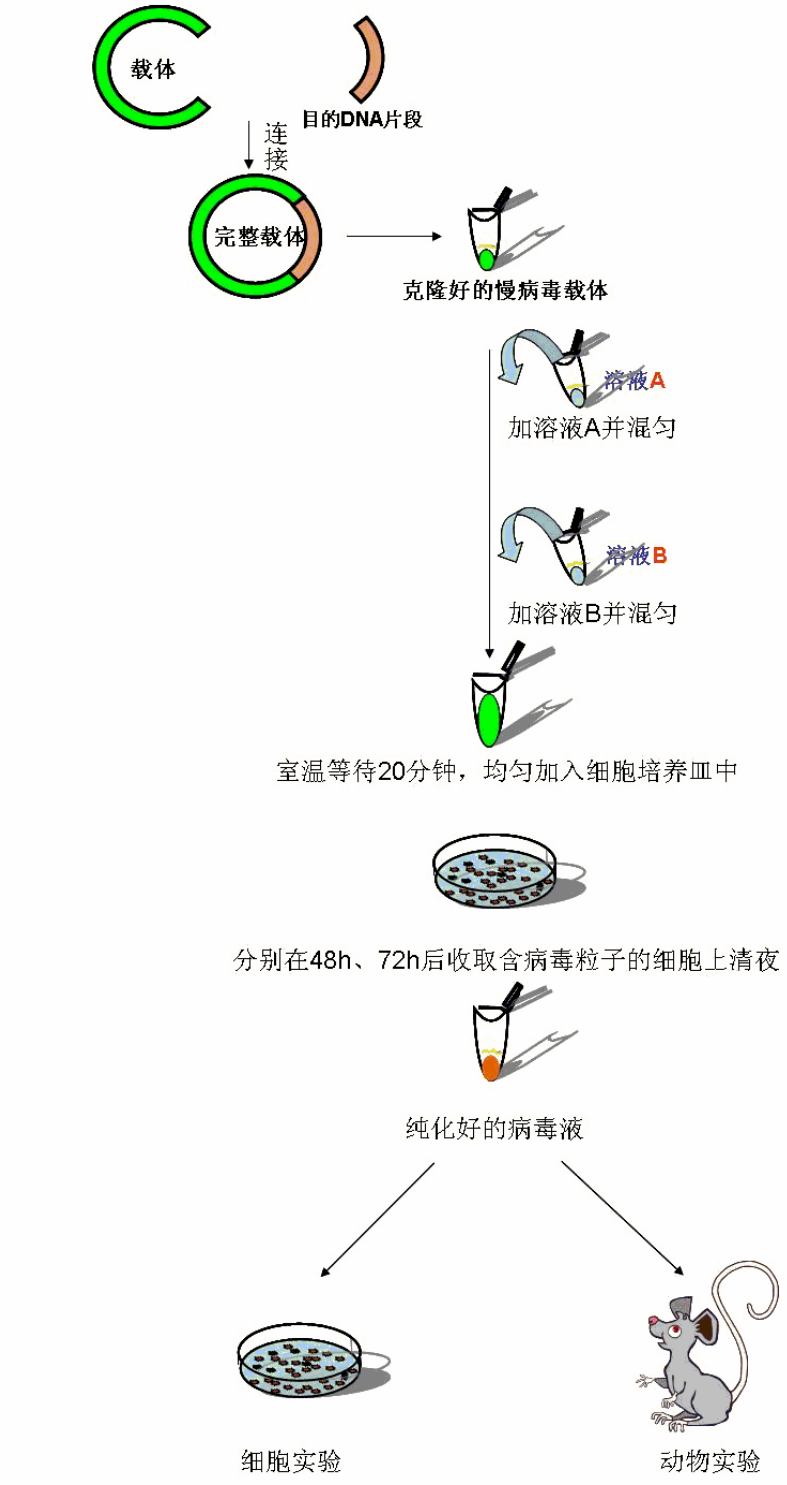
Kit contents

Cat. No.	Control Plasmid	Reagent A	Reagent B	Televector	Packaging cells	Televector Medium	Invitation Letter for Training Course
L-1109	10 ug	1.5 ml	1.5 ml	10 ug	10^7	30ml	1
L-1227	20 ug	3 ml	3 ml	20 ug	10^7	60ml	2

Not: *Education DVD and Invitation Letter for Training Course.Check the information on web page www.telebio.cn*

TELEVECTOR™ Lentiviral Vector Easy-high Production Kit

用Lentiviral vector easy-high Production Kit 生产慢病毒操作流程图



Overview

Introduction

The **TELEVECTOR™** Lentiviral Vector Easy-high Production Kit allows creation of a replication incompetent HIV-1-based lentivirus that is used to deliver and express your gene of interest in either dividing or non-dividing mammalian cells. The major components of the system include:

Televector ： plasmid containing the gene of interest under the control of a choice of promoters, and elements that allow packaging of the construct into virions

Control expression plasmid：EGFP expressing lentivector to optimize virus production and cell transduction.

For more information on expression vectors and the corresponding positive control vectors, refer to the manual for the specific expression or control vector
you are using.

Solution A:

An optimized mix of the third generation packaging plasmids that supply the structural and replication proteins *in trans* that are required to produce the lentivirus and transduction buffering solution. Also containing some reagents that could improve the efficiency of transfection.

Solution B:

An optimized mix of the buffering solution can carry the lentivectors and solution A into 293T cells. Some elements were added and specially improve the efficiency of cell transfection.

Solution C: The special medium for culturing 293T cells transfection process.

The 293T cell line: Which allows production of lentivirus following cotransfection of the expression plasmid and plasmids in the mix of solution A and B.

Advantages of the System

Use of the **TELEVECTOR™** to facilitate lentiviral-based expression of the gene of interest provides the following advantages:

Generates an HIV-1-based lentivirus that effectively transduces both dividing and non-dividing mammalian cells, thus broadening the potential applications beyond those of traditional Moloney Leukemia Virus (MoMLV)-based retroviral systems (Naldini, 1998), Efficiently delivers the gene of interest to mammalian cells in culture or *in vivo*(Dull *et al.*, 1998)

Provides stable, long-term expression of a target gene beyond that offered by traditional adenoviral-based systems (Dull *et al.*, 1998; Naldini *et al.*, 1996), Produces a pseudotyped virus with a broadened host range (Yee *et al.*, 1994) Includes multiple features designed to enhance the biosafety of the system.

Purpose of this Manual

This manual provides an overview of the **TELEVECTOR™** and provides instructions and guidelines to:

1. Co-transfect the Televector-based expression vector in solution A and B of **TELEVECTOR™** into the 293T Cell Line to produce a lentiviral stock.
2. Titer the lentiviral stock.

3. Use the lentiviral stock to transduce your mammalian cell line of choice.
4. Assay for “transient” expression of your recombinant protein or generate a stably transduced cell line, if desired.

For details and instructions to generate your expression vector, refer to the manual for the Televector you are using. For instructions to culture and maintain the 293T producer cell line, refer to the 293T Cell Line manual. These manuals are supplied with the **TELEVECTOR™** Lentiviral Vector Esy-high Production Kits, and are also available by contacting Technical Service for free.

Components of the TELEVECTOR™ Lentiviral Vector Easy-high Production System

The **TELEVECTOR™** Lentiviral Vector Esy-high Production kit facilitates highly efficient, *in vitro* or *in vivo* delivery of a target gene to dividing and non-dividing mammalian cells using a replication-incompetent lentivirus. **TELEVECTOR™** Lentiviral Vector Esy-high Production kit possesses features which enhance its biosafety while allowing high-level gene expression in a wider range of cell types than traditional retroviral systems. The System includes the following major components:

A Televector-based expression vector into which the gene of interest will be cloned. The vector also contains the elements required to allow packaging of the expression construct into virions (*e.g.,* 5’ and 3’ LTRs, Ψ packaging signal). For more information about the Televector expression vectors, refer to the manual for the specific vector you are using.

Solution A contains an optimized mixture of the three packaging plasmids and tranduction element. These plasmids supply the helper functions as well as structural and replication proteins *in trans* required to produce the lentivirus.

Solution B is optimized mix of the buffering solution can carry the lentivectors in solution A into 293T cells.

Solution C is the special medium for 293T in transduction process.

An modified 293T producer cell line that stably expresses the SV40 large T antigen under the control of the human CMV promoter and facilitates optimal production of virus. For more information about the 293T Cell Line, refer to the 293T Cell Line manual. You will cotransfect the Solution A in **TELEVECTOR™** and the Televector vector containing your gene of interest into 293T cells to produce a replication incompetent lentivirus, which will be used to transduce a mammalian cell line of interest.

How Lentivirus Works

Once the lentivirus enters the target cell, the viral RNA is reverse-transcribed, actively imported into the nucleus (Lewis & Emerman, 1994; Naldini, 1999), and stably integrated into the host genome (Buchschacher & Wong-Staal, 2000; Luciw, 1996). After the lentiviral construct has integrated into the genome, you may assay for transient expression of your recombinant protein or use antibiotic selection to generate a stable cell line for long-term expression studies.

VSV Envelope Glycoprotein

Most retroviral vectors are limited in their usefulness as gene delivery vehicles by their restricted

tropism and generally low titers. In the **TELEVECTOR™**, this limitation has been overcome by use of the G glycoprotein gene from Vesicular Stomatitis Virus (VSV-G) as a pseudotyping envelope, thus allowing production of a high titer lentiviral vector with a significantly broadened host cell range (Burns *et al.*, 1993; Emi *et al.*, 1991; Yee *etal.*, 1994).

Biosafety Features of the System

Introduction

The **TELEVECTOR™** is a third-generation system based on lentiviral vectors developed by TELEBIO BIOMEDICAL CO., Ltd. This third-generation lentiviral system includes a significant number of safety features designed to enhance its biosafety and to minimize its relation to the wild-type, human HIV-1 virus. These safety features are discussed below.

Biosafety Features of the TELEVECTOR™ Lentiviral System

The **TELEVECTOR™** System includes the following key safety features:

The Televector expression vector contains a deletion in the 3′ LTR that does not affect generation of the viral genome in the producer cell line, but results in "self-inactivation" of the lentivirus after transduction of the target cell (Yee *et al.*, 1987; Yu *et al.*, 1986; Zufferey *et al.*, 1998). Once integrated into the transduced target cell, the lentiviral genome is no longer capable of producing packageable viral genome.

The number of genes from HIV-1 that are used in the system has been reduced to three (*i.e. gag*, *pol*, and *rev*). The VSV-G gene from Vesicular Stomatitis Virus is used in place of the HIV-1 envelope (Burns *et al.*, 1993; Emi *et al.*, 1991; Yee *et al.*, 1994).

Genes encoding the structural and other components required for packaging the viral genome are separated onto four plasmids. All four plasmids have been engineered not to contain any regions of homology with each other to prevent undesirable recombination events that could lead to the generation of a replication-competent virus (Dull *et al.*, 1998).

Although the three packaging plasmids allow expression *in trans* of proteins required to produce viral progeny (*e.g.* gal, pol, rev, env) in the 293FT producer cell line, none of them contain LTRs or the Ψ packaging sequence. This means that none of the HIV-1 structural genes are actually present in the packaged viral genome, and thus, are never expressed in the transduced target cell. No new replication-competent virus can be produced.

The lentiviral particles produced in this system are replication-incompetent and only carry the gene of interest. No other viral species are produced.

Expression of the *gag* and *pol* genes from packaging plasmids has been rendered Revdependent by virtue of the HIV-1 RRE in the gag/pol mRNA transcript. Addition of the RRE prevents gag and pol expression in the absence of Rev (Dull *et al.*, 1998).

A constitutive promoter (RSV promoter) has been placed upstream of the 5′ LTR in the Televector expression vector to offset the requirement for Tat in the efficient production of viral RNA (Dull *et al.*, 1998).

Biosafety Level 2

Despite the inclusion of the safety features discussed on the previous page, the lentivirus produced with this System can still pose some biohazardous risk since it can transduce primary human cells. For this reason, **we highly recommend that you treat lentiviral stocks generated using this System as Biosafety Level 2 (BL-2) organisms and strictly follow all published BL-2 guidelines with proper waste decontamination.** Furthermore, exercise extra caution when

creating lentivirus carrying potential harmful or toxic genes (*e.g.* activated oncogenes). For more information about the BL-2 guidelines and lentivirus handling, refer to the document, "Biosafety in Microbiological and Biomedical Laboratories," 4th Edition, published by the Centers for Disease Control (CDC). This document may be downloaded at the following address: http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm , and you can find Chinese version on www.telebio.cn. Handle all lentiviruses in compliance with established institutional guidelines. Since safety requirements for use and handling of lentiviruses may vary at
individual institutions, we recommend consulting the health and safety guidelines and/or officers at your institution prior to use of the **TELEVECTOR™** System.

Methods

General Information

Introduction The **TELEVECTOR™** System is designed to help you create a lentivirus to deliver and express a gene of interest in mammalian cells. Although the system has been designed to help you express your recombinant protein of interest in the simplest, most direct fashion, use of the system is geared towards those users who are familiar with the principles of retrovirus biology and retroviral vectors. We highly recommend that users possess a working knowledge of virus production and tissue culture techniques. For more information about these topics, refer to the following published reviews:

Retrovirus biology and the retroviral replication cycle: see Buchschacher and Wong-Staal *(*2000) and Luciw (1996). Retroviral and lentiviral vectors: see Naldini (1999), Naldini (1998), Yee (1999) and Pandya *et al.*, (2001)

Generating Your Televector Expression Construct

To generate a Televector expression construct containing your gene of interest, refer to the manual for the vector you are using for instructions. Once you have created your expression construct, you will isolate plasmid DNA for transfection.

Important: You should verify that your lentiviral plasmid has not undergone aberrant recombination by performing an appropriate restriction enzyme digest. See the vector manual for details.

DNA Isolation Guidelines

Plasmid DNA for transfection into eukaryotic cells must be very clean and free from contamination with phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with DNA complexing, decreasing transfection efficiency. When performing plasmid DNA isolation with commercially available kits from *E. coli* strains (such as STBL3) that are wild type for endonuclease 1 (*end*A1+), ensure that Solution I of the Lysis or Resuspension Buffer contains 10 mM EDTA. EDTA will inactivate the endonuclease and avoid DNA nicking and vector degradation. Alternatively, follow the instructions included the plasmid purification kits for *end*A1+ *E. coli* strains. Do not use mini-prep plasmid DNA for lentivirus production. We recommend preparing lentiviral plasmid DNA using the Maxi-Prep Kit.

General Information of Transfection reagent A and B

The three plasmids are provided in an optimized mixture (**reagent A**) to facilitate viral packaging of your Televector expression vector following cotransfection into 293T producer cells. The amount of the packaging mix and transfection reagent supplied in the solutionA and B, Lentiviral Expression kit is sufficient to perform 5 cotransfections in 10 cm plates. To use the Transfection reagent A and B, resuspend the contents of A and B, and divided into 1ml/bottle stocks.

293T Cell Line

The human 293T Cell Line is supplied with the **TELEVECTOR™** Lentiviral Expression kits to facilitate optimal lentivirus production (Naldini *et al.*, 1996). The health of your 293T cells at the time of transfection has a critical effect on the success of lentivirus production. Use of “unhealthy” cells will negatively affect the transfection efficiency, resulting in production of a low titer lentiviral stock. For optimal lentivirus production (*i.e.* producing lentiviral stocks with

the expected titers), follow the guidelines below to culture 293T cells before use in transfection:

Make sure that cells are healthy and greater than 90% viable.

Subculture and maintain cells in DMEM and 10% fetal bovine serum (GIBCOL , PAA or Hyclone) that is not heatinactivated.

Do not allow cells to over grow before passaging.

Use cells that have been subcultured for less than 16 passages.

Preparation of plasmid and 293T cells

Culture vessel	Cells for production	Medium	TeleVector	Reagent A	Reagent B	Viral TU/ production	Kit for Production Times
12-well	$1*10^5$	1 ml	1.0 ug	50 ul	50 ul	$5*10^6$	30
6-well	$4*10^5$	2 ml	2.0 ug	100 ul	100 ul	$1*10^7$	15
6cm Dish	$2*10^6$	5 ml	4.0 ug	200 ul	200 ul	$3*10^7$	8
10cm Dish	$8*10^6$	15ml	10 ug	500 ul	500 ul	$1*10^8$	3
15cm Dish	$1*10^7$	16 ml	22.5 ug	750 ul	750 ul	$2.5*10^8$	2

Positive Control

We recommend including a positive control vector in your experiment to generate a control lentiviral stock that may be used to help you optimize expression conditions in your mammalian cell line of interest

Each Televector expression kit includes a positive control vector(containing EGFP for fluorescent detection) for use as an expression control. For more information about the positive control vector supplied with each kit, refer to the appropriate expression vector manual.

Solution C reagent supplied with the kit is aproprietary, specific medium is need to replace normal culture medium in the process of transfection, add solution C in transfected cells offers the following advantages:

Provides the highest transfection efficiency in 293T cells

Medium change or addition following transfection can be removed after 8 hours without loss of cell viability.

Standard Protocol

If you producing lentivirus for the first time using the **TELEVECTOR™** System and 293T cells, you should perform to familiar with the growth characteristics of 293T cells

Transfection procedure

In this procedure, solution B are added to solution A containing the Lentivectors.

Producing Lentivirus in 293T Cells

Introduction Before you can create a stably transduced cell line expressing your gene of interest, you will first need to produce a lentiviral stock (containing the packaged Televector expression construct) by cotransfecting the optimized packaging plasmid mix and your Televector expression construct into the 293T Cell Line. The following section provides protocols and

instructions to generate a lentiviral stock.

Recommended Transfection Conditions

We produce lentiviral stocks in 293FT cells using the following **optimized** transfection conditions below. The amount of lentivirus produced using these recommended conditions (10 ml of virus at a titer of at least 1 x 10^5 transducing units (TU)/ml) is generally sufficient to transduce at least 1 x 10^6 cells at a multiplicity of infection (MOI) = 1. For example, 10 wells of cells plated at 1 x 10^5 cells/well in 6-well plates could each be transduced with 1 ml of a 1 x 10^5 TU/ml virus stock to achieve an MOI of 1.

Condition Amount

Tissue culture plate size 10 cm (one per lentiviral construct)

Number of 293T cells to transfect 6 x 10^6 cells

Amount of solutionA and B Mix 500μl separately

Amount of Televector expression plasmid 25μg

Note: You may produce lentiviral stocks using other tissue culture formats, but keep in mind that optimization will be necessary to obtain the expected titers.

Culture vessel	Cells for production	Medium	TeleVector	Reagent A	Reagent B	Viral TU/ production	Kit for Production Times
12-well	1*10^5	1 ml	1.0 ug	50 ul	50 ul	5*10^6	60
6-well	4*10^5	2 ml	2.0 ug	100 ul	100 ul	1*10^7	30
6cm Dish	2*10^6	5 ml	4.0 ug	200 ul	200 ul	3*10^7	15
10cm Dish	8*10^6	15ml	10 ug	500 ul	500 ul	1*10^8	6
15cm Dish	1*10^7	16 ml	22.5 ug	750 ul	750 ul	2.5*10^8	4

Materials Needed

You should have the following materials before beginning:

Solution A and B (supplied with the kit)

Televector expression vector containing your gene of interest (0.1-3.0 μg/μl in sterile water or TE, pH 8.0)

Televector control vector containing EGFP (supplied with the kit) resuspended in sterile water to a concentration of 1 μg/μl)

Solution C 10ml/plate, the medium was replaced 25 min before mix of A and B was added.

293T cells cultured in the appropriate medium (*i.e.* D-MEM containing 10% FBS)

Sterile, 10 cm tissue culture plates (one each for the lentiviral construct, positive control, and negative control)

Sterile, tissue culture supplies

50 ml sterile, capped, conical tubes

Vortex can be worked in super clean plate.

Protocol of the TELEVECTOR™ Lentiviral Vector Easy-high Production System

1. The day before transfection (Day 1), plate 293T cells ($6*10^6$)in a 10 cm tissue culture plate, 24 hours later, they will be70-80% confluent on the time of transfection, In general, one plate per virus.
2. Add 25 μg Televector to 500 μl Reagent A and mix by tapping gently, and transfer to 50ml tube, vortex machine was prepared before solution B was added. The following steps are done, one plate at a time.
3. Vortexing mixture of Televector and reagent A and adding 500 μl Reagent B drop-wise to mixwell When finished, continue to bubble for 12-15 seconds. Leave the mixture30 minutes in the hood at room temperature.
4. Take plate of 293T out of the incubator (plate remains in incubator for long as possible), Change the old medium with solution C, 25 minutes after incubation at 37°C, add the mixture (prepared in step3) drop-wise all over the plate. Gently swirl plate from front to back, and return immediately to incubator.
5. 8 hours later, add 10 ml warm growth medium onto plate and place in incubator.
6. 24 and 48 hours after transfection, harvest viral supernatant individually and spin 2000 rpm, 7 min at 4°C in a 50ml tube.
7. Filter viral supernatant through 0.45 μm filter. Add filtered supernatant to an ultracentrifuge tube. Balance tubes with additional media.
8. Spin tubes at 80000g, 90 min or 72000g, 2.5 hr. 4°C. Decant liquid and leave tube upside down on vortex for 1 min. Aspirate remaining media being careful not to touch bottom of tube.
9. Add 200 μl cold PBS or special medium which you will use for culturing special cells, then leave tube at 4°C for 30 min with no shaking.
10. To resuspend, hold tube at angle and pipet fluid over pellet 20 times
11. Aliquot or use virus. Virus should be aliquoted stored at -80℃. There should be no change in titer with freezing concentrated virus. Avoid multiple freeze-thaws.
12. The supernatant which containing the virus could infect the cells. Concentrated virus could do the experiment with animals.

Titering Your Lentiviral Stock

Introduction

Before proceeding to transduction and expression experiments, we highly recommend determining the titer of your lentiviral stock. While this procedure is not required for some applications, it is necessary if:

You wish to control the number of integrated copies of the lentivirus

You wish to generate reproducible expression results

Guidelines and protocols are provided in this section to titer your lentiviral stock.

Note: If you are using Televector-EGFP Expression Control Vector to produce a lentiviral stock, refer to the user manual for titer methods using fluorescent detection. Remember that you will be working with media containing infectious virus. Follow the recommended Federal and

institutional guidelines for working with BL-2 organisms.

Perform all manipulations within a certified biosafety cabinet.

Treat media containing virus with bleach.

Treat used pipettes, pipette tips, and other tissue culture supplies with bleach and dispose of as biohazardous waste.

Wear gloves, a laboratory coat, and safety glasses or goggles when handling viral stocks and media containing virus.

Experimental Outline

To determine the titer of lentiviral stocks, you will:

1. Prepare 10-fold serial dilutions of your lentiviral stocks.
2. Transduce the different dilutions of lentivirus in the presence of the polycation Polybrene into a mammalian cell line (Hela is recommended).
3. Select for stably transduced cells using Blasticidin.
4. Stain and count the number of Blasticidin-resistant colonies in each dilution.

Factors Affecting Viral Titer

A number of factors can influence viral titers including:

The size of your gene of interest- Titers will decrease as the size of the insert increases. We have determined that virus titer drops approximately 2-fold for each kb over 4 kb of insert size. If you wish to produce lentivirus with an insert of > 4 kb, you will need to concentrate the virus to obtain a suitable titer. The size of the wild-type HIV genome is approximately 10 kb. Since the size of the elements required for expression from Televector total approximately 4-4.4 kb, the size of your insert should not exceed 5.6 kb.

The characteristics of the cell line used for tittering- We strongly recommend the Hela cells as the “gold standard” for reproducibly titering lentivirus. However, other cell lines may be used. In general, these cells should be an adherent, non-migratory cell line, and exhibit a doubling time in the range of 18-25 hours.

The age of your lentiviral stock- Viral titers may decrease with long-term (>1 year) storage at -80℃. If your lentiviral stock has been stored for longer than 6 months, we recommend titering your lentiviral stock prior to use.

Number of freeze/thaw cycles- Viral titers can decrease as much as 10% with each freeze/thaw cycle.

Improper storage of your lentiviral stock- Lentiviral stocks should be stored at -80℃ in cryovials.

Selecting a Cell Line for Titering

We strongly recommend the Hela cells as the“gold standard”for reproducibly titering lentivirus. However, you may wish to use the same mammalian cell line to titer your lentiviral stocksas you will use to perform your expression studies (*e.g.* if you are performing expression studies in a dividing cell line or a non-primary cell line). If you have more than one lentiviral construct, we recommend that you titer all of the lentiviral constructs using the same mammalian cell line. For more information on cells for titering, see **Factors Affecting Viral Titer**.

Using Polybrene During Transduction

Lentivirus transduction may be enhanced if cells are transduced in the presence of hexadimethrine bromide (Polybrene). For best results, we recommend performing transduction in the presence of Polybrene. Note however, that some cells are sensitive to Polybrene (*e.g.* primary neurons).

Before performing any transduction experiments, you may want to test your cell line for sensitivity to Polybrene at a range of 0-10 µg/ml. If your cells are sensitive to Polybrene (*e.g.* exhibit toxicity or phenotypic changes), do not add Polybrene during transduction. In this case, cells should still be successfully transduced with your lentivirus.

Preparing and Storing Polybrene

Follow the instructions below to prepare Polybrene (Sigma, Catalog no. H9268):

1. Prepare a 6 mg/ml stock solution in deionized, sterile water.
2. Filter-sterilize and dispense 1 ml aliquots into sterile microcentrifuge tubes.
3. The working stock may be stored at +4° C for up to 2 weeks. Store at -20° C for long-term storage (up to 1 year). Do not freeze/thaw the stock solution more than 3 times as this may result in loss of activity.

Materials Needed

To determine the titer of your lentiviral construct, you should have the following materials before beginning:

Your Televector lentiviral stock (store at -80° C until use)
Adherent mammalian cell line of choice
Complete culture medium for your cell line
6 mg/ml Polybrene, if desired
6-well tissue culture plates
10 cm tissue culture plates
G418 antibiotics
Crystal violet (Sigma, Catalog no. C3886; prepare a 1% crystal violet solution in 10% ethanol)
Phosphate-Buffered Saline (PBS; Invitrogen, Catalog no. 10010-023)

Transduction and Titering Procedure

Follow the procedure below to determine the titer of your lentiviral stock using the mammalian cell line of choice. You will use **at least** one 6-well plate for every lentiviral stock to be titered (one mock well plus five dilutions).

Note: If you are using Televector-EGFP, refer to the user manual for the vector for the FACS titering protocol.

1. The day before transduction (Day 1), trypsinize and count the cells, plating them in a 6-well plate such that they will be 30-50% confluent at the time of transduction. Incubate cells at 37 ℃ overnight in a humidified 5% CO_2 incubator. **Example:** When using Hela cells, we usually plate 2 x 10^5 cells per well in a 6-well plate.
2. On the day of transduction (Day 2), thaw your lentiviral stock and prepare 10-fold serial dilutions ranging from 10^{-2} to 10^{-6}. For each dilution, dilute the lentiviral stock into complete culture medium to a final volume of 1 ml. **DO NOT** vortex.

   Note: You may prepare a wider range of serial dilutions (10^{-2} to 10^{-8}), if desired.
3. Remove the culture medium from the cells. Mix each dilution gently by inversion and add to one well of cells (total volume = 1 ml).
4. Add Polybrene (if desired) to each well to a final concentration of 6 µg/ml. Swirl the plate gently to mix. Incubate at 37° C overnight in a humidified 5% CO_2 incubator.
5. The following day (Day 3), remove the media containing virus and replace with 2 ml of complete culture medium. Incubate at 37° C overnight in a humidified 5% CO_2 incubator.
6. The following day (Day 4), treat cells as follows:

For G418 selection, remove the medium and replace with complete culture medium containing the appropriate amount of G418 to select for stably transduced cells.

7. Replace medium with fresh medium containing antibiotic every 3-4 days.
8. After 10-12 days of selection (day 14-16), you should see no live cells in the mock well and discrete antibiotic-resistant colonies in one or more of the dilution wells. Remove the medium and wash the cells twice with PBS.
9. Add crystal violet solution (1 ml for 6-well dish; 5 ml for 10 cm plate) and incubate for 10 minutes at room temperature.
10. Remove the crystal violet stain and wash the cells with PBS. Repeat wash.
11. Count the blue-stained colonies and determine the titer of your lentiviral stock.

What You Should See

When titering Televector lentiviral stocks using Hela cells, we generally obtain titers ranging from 1-5 x 10^5 (for unconcentrated virus) up to 1 x 10^9 (for concentrated virus) transducing units (TU)/ml.

Example of Expected Results

In this experiment, a Televector-*lacZ* lentiviral stock was generated using the protocol, and was concentrated by ultracentrifugation. Hela cells were transduced with 10-fold serial dilutions of the lentiviral supernatant (10^{-2} to 10^{-6} dilutions) or untransduced (mock) following the protocol. At 48 hours post-transduction, the cells were placed under G418 selection (10μg/ml). After 10 days of selection, the cells were stained with crystal violet, and colonies were counted.

In the plate, the colony counts were:

Mock: no colonies

10^{-2} dilution: confluent; undeterminable

10^{-3} dilution: confluent; undeterminable

10^{-4} dilution: confluent; undeterminable

10^{-5} dilution: 46

10^{-6} dilution: 5

Thus, the titer of this concentrated lentiviral stock is 4.8 x 10^6 TU/ml (*i.e.* average of 4.6 x 10^5 and 5 x 10^6).

Next Steps It is important to note that user experience, the nature of the gene, and vector backbone may affect virus titer. If the titer of your unconcentrated virus is suitable (*i.e.* 1 × 10^5 TU/ml or higher), proceed to **Transduction of Cells With Lentivirus**. If the titer of your concentrated lentiviral stock is less than 1 × 10^5 TU/ml, we recommend producing a new lentiviral stock., for more tips and guidelines to optimize your viral yield.

Titration of vectors in Hela cells by FACS

This method can only be used to titer stocks of vectors that carry a transgene that is easily monitored by FACS (such as GFP, or any living colors, or any membrane protein that can be detected by flow cytometry), and whose expression is governed by a promoter that is active in Hela cells (tissue-specific promoter-containing vector must be functionally assayed in specific cells, and titered by RT-PCR in Hela cells. We describe here the titration of a PGK-GFP vector.

On day 0 seed Hela cells at 100kC per well in MW6 plate in DMEM 10% FCS, 1% glutamine and antibiotics.

On day 1 put in three independent wells 500, 50 or 5µl of the vector suspension (either pure from unconcentrated supernatants or diluted if it comes from a concentrated stock).

On day 2 remove the supernatant and replace by 2 ml of fresh DMEM.

On day 4 to 5 wash the cells with 2 ml of PBS, detach them with 250 µl of Trypsine-EDTA for 1 minute at 37℃, add 250 µl of 2% formaldehyde in PBS (to fix the cells, inactivate the trypsin and the vector particles), resuspend thoroughly, and analyze them for GFP expression by FACS.

A representative FACS analysis of Hela cells used for titration of GFP-coding LV.

Hela cells (10^5) were incubated with various volumes of a supernatant containing a LV expressing GFP under the control of the human PGK promoter (pRRLSIN.cPPT.PGK.GFP.WPRE (Follenzi, 2000)) as described in text. After 4 days, cells were detached, fixed and analyzed by FACS for GFP fluorescence (x axis, 4-decade log scale, FL1) versus number of cells (y axis, linear scale). The percentage of GFP-expressing cells was measured by placing a marker discriminating between GFP-negative (mean of fluorescence intensity 3-4) and GFP-positive cells (mean of fluorescence intensity 200).

In a typical titration experiment, only dilutions yielding to 1 to 20% of GFP-positive should be considered for titer calculations. Below 1%, the FACS may not be accurate enough to reliably determine the number of GFP-positive cells. Above 20%, the chance for each GFP-positive target cell to be transduced twice significantly increases, resulting in underestimation of the number of transducing particles. Once chosen the appropriate dilution, apply the following math:

Titer (Hela-transducing units / ml) = 100000 (target Hela cells) x (% of GFP-positive cells/100) / volume of supernatant (in ml).

Transduction and Analysis

Introduction Once you have generated a lentiviral stock with a suitable titer, you are ready to transduce the lentiviral construct into the mammalian cell line of choice and assay for expression of your recombinant protein. Guidelines are provided below. Your lentiviral construct contains a deletion in the 3'LTR that leads to self-inactivation of the lentivirus after transduction into mammalian cells. Once integrated into the genome, the lentivirus can no longer produce packageable virus.

Transient vs Stable Expression

After transducing your lentiviral construct into the mammalian cell line of choice, you may assay for expression of your gene of interest in the following ways:

Pool a heterogeneous population of cells and test for expression directly after transduction (*i.e.* "transient" expression). Note that you must wait for a minimum of 48-72 hours after transduction before harvesting your cells to allow expressed protein to accumulate in transduced cells.

Select for stably transduced cells using Neomycin, as appropriate. This requires a minimum of 10-12 days after transduction, but allows generation of clonal cell lines that stably express the gene of interest.

Note: We have observed stable expression of a target gene for at least 6 weeks following transduction and selection.

Determining Antibiotic Sensitivity for Your Cell Line

If you wish to select for stably transduced cells, you must first determine the minimum concentration of G418, as appropriate, required to kill your untransduced mammalian cell line (*i.e.* perform a kill curve experiment). If you titered your lentiviral construct in the same mammalian cell line that you are using to perform your stable expression experiment, then you may use the same concentration of G418 for selection that you used for titering.

Multiplicity of Infection (MOI)

To obtain optimal expression of your gene of interest, you will need to transducer the lentiviral construct into your mammalian cell line of choice using a suitable MOI. MOI is defined as the number of virus particles per cell and generally correlates with the number of integration events and as a result, expression of your gene of interest. Typically, expression levels increase linearly as the MOI increases.

Determining the Optimal MOI

A number of factors can influence optimal MOI including the nature of your mammalian cell line (*e.g.* non-dividing vs. dividing cell type; see **Recommendation**), its transduction efficiency, your application of interest, and the nature of your gene of interest. If you are transducing your lentiviral construct into the mammalian cell line of choice for the first time, we recommend using a range of MOI (*e.g.* 0, 0.5, 1, 2, 5, 10) to determine the MOI required to obtain the optimal expression of your protein for your application. In general, we have found that 80-90% of the cells in an actively dividing cell line (*e.g.*Hela) express a target gene when transduced at an MOI of ~1. Some nondividing cell types transduce lentiviral constructs less efficiently. For example, only about 50% of the cells in a culture of Hela express a target gene when transduced at an MOI of ~1. If you are transducing your lentiviral construct into a non-dividing cell type, you may need to increase the MOI (*e.g.* MOI = 10) to achieve optimal expression levels for your recombinant

protein.

Positive Control

Control lentiviral vectors expressing EGFP are available for optimization (see your vector manual for information). If you have generated a lentiviral stock of a *EGFP* expression control (*e.g.* TelevectorEGFP), we recommend using the stock to help you determine the optimal MOI for your particular cell line and application. Once you have transduced the control lentivirus you're your mammalian cell line of choice, the gene encoding EGFP will be constitutively expressed and can be easily assayed(refer to the expression vector or expression control vector manual for assay methods).Viral supernatants are generated by harvesting spent media containing virus from the 293T producer cells. Spent media lacks nutrients and may contain some toxic metabolic waste products. If you are using a large volume of viral supernatant to transduce your mammalian cell line (*e.g.* 1 ml of viral supernatant per well in a 6-well plate), note that growth characteristics or morphology of the cells may be affected during transduction. These effects are generally alleviated after transduction when the media is replaced with fresh, complete media.

Materials Needed

You will need the following materials before beginning:

Your titered lentiviral stock (store at -80° C until use)
Mammalian cell line of choice
Complete culture medium for your cell line
6 mg/ml Polybrene , if desired
Appropriately sized tissue culture plates for your application
G418, as appropriate (if selecting for stably transduced cells)

Transduction Procedure

Follow the procedure below to transduce the mammalian cell line of choice with your lentiviral construct.

Reminder: If you are performing Neomycin selection, remember that cells should not be confluent at the time of selection. Plate your cells accordingly.

1. Plate cells in complete media as appropriate for your application.
2. On the day of transduction (Day 1), thaw your lentiviral stock, and if necessary, dilute the appropriate amount of virus into fresh complete medium to obtain a suitable MOI. Keep the total volume of medium containing virus as low as possible to maximize transduction efficiency. **DO NOT** vortex.
3. Remove the culture medium from the cells. Mix the medium containing virus gently by pipetting and add to the cells.
4. Add Polybrene (if desired) to a final concentration up to 10 μg/ml. Swirl the plate gently to mix. Incubate at 37° C in a humidified 5% CO_2 incubator overnight.

Note: If you are transducing cells with undiluted viral stock and are concerned about possible toxicity or growth effects caused by overnight incubation, it is possible to incubate cells for as little as 4 hours prior to changing medium.

5. The following day (Day 2), remove the medium containing virus and replace with fresh, complete culture medium. Incubate at 37° C in a humidified 5% CO_2 incubator overnight.
6. The following day (Day 3), perform one of the following: Harvest the cells and assay for expression of your recombinant protein if you are performing transient expression experiments. Remove the medium and replace with fresh, complete mediumcontaining the

appropriate amount of G418, as appropriate to select for stably transduced cells. Proceed to Step 7.

7. Replace medium with fresh medium containing antibiotic every 3-4 days until antibiotic-resistant colonies can be identified (generally 10-12 days after selection).
8. Pick at least 5 antibiotic-resistant colonies (see **Note** below) and expand each clone to assay for expression of the recombinant protein. Integration of the lentivirus into the genome is random.

Note：Depending upon the influence of the surrounding genomic sequences at the integration site, you may see varying levels of recombinant protein expression from different antibioticresistant clones. We recommend testing at least 5 antibiotic-resistant clones and selecting the clone that provides the optimal expression of your recombinant protein for further studies.

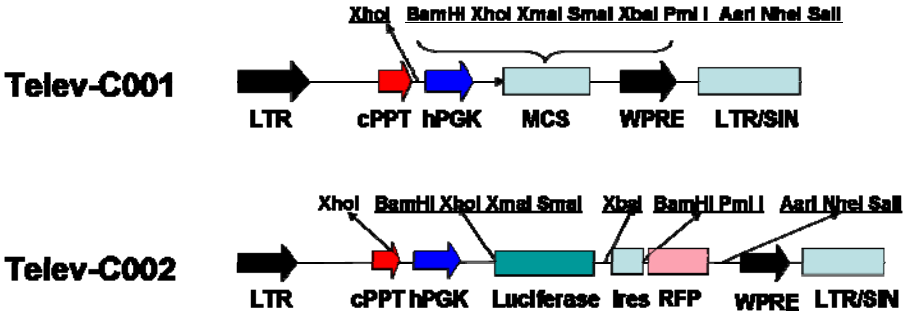
Detecting Recombinant Protein

You may use any method of choice to detect your recombinant protein of interest including RT PCR, functional analysis, immunofluorescence, or western blot.

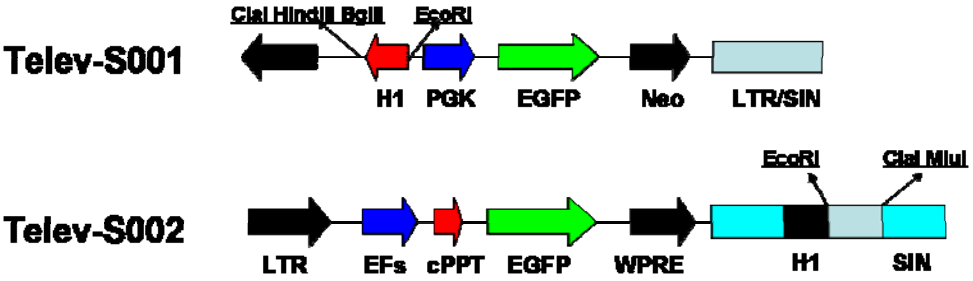
Televector Products

We have several general lentiviral vector for gene insertion. You may choice a Televetor for cloning, and select the interest gene.

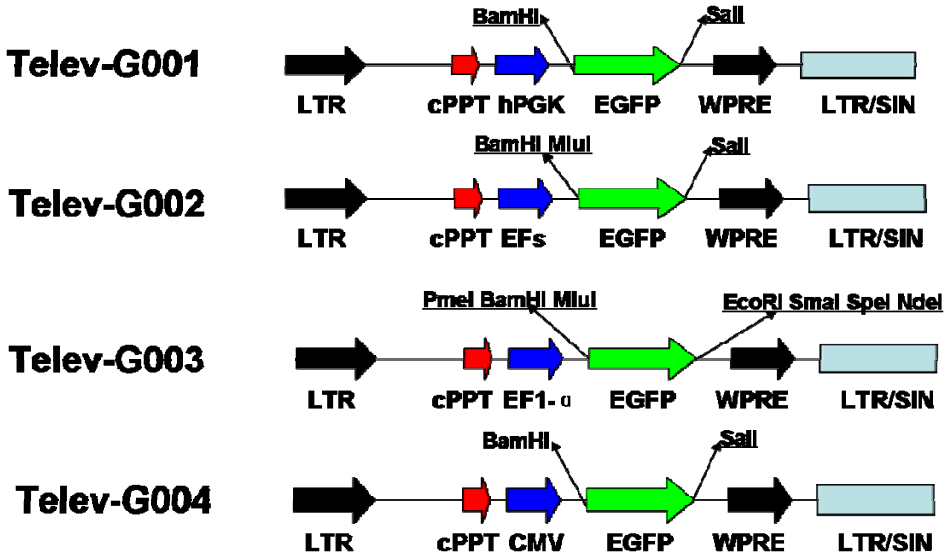
General Televector



Televector for siRNA cloning



Televector expressing EGFP



Contact Us

上海比昂生物医药科技有限公司
SHANGHAI TELEBIO BIOMEDICAL Co., Ltd.

Chenhui Road 377/144，Zhangjiang High-Tech Zone Shanghai 201203 China

Tel: 0086-21-50271899
0086-15921731086

Fax: 0086-21-50277136

Email: vector@telebio.cn
telebio@163.com

Http:// www.telebio.cn