Lenti-Easy-His™ Gene Expression System

Cat No: LV100

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Introduction

The Lenti-Easy Expression System allows production of replication-incompetent 3rd generation lentivirus that can stably transduce both dividing and non-dividing mammalian cells with high efficiency (Naldini, 1998 and Dull *et al.*, 1998). Our lentiviral expression vector has been fully optimized for simple manipulations such as subcloning the gene of interest into our pLenti-expression vector, and easy viral DNA production including maxi DNA purification. The vector simply works like any other plasmid. In fact, our vectors are so stable that non-specific recombination or rearrangement in regular DH_5a cells is rarely observed. This is a significant advantage compared to similar lentiviral vectors offered by other companies, which are associated with substantial adversity in subcloning and DNA production. Because of our vector stability, there is no need for special competent cells during transformation.

Our lentiviral vectors are 3rd-generation and are compatible with packaging mixes that support the production of 3rd-generation vectors. Our optimized Lenti-Combo Mix (LV003) supplies all the structural and replication proteins *in-trans* that are required for high-titer lentivirus production in packaging cells (Titers can be obtained up to 10^7IU/ml).

General Information About Lentiviral Vectors

Morphology:

Virions consist of an envelope, a nucleocapsid, a nucleoid, and matrix proteins. The enveloped virions assume a spherical to pleomorphic shape of 80-100nm in diameter. The virion surface is covered with dense inconspicuous spikes of 8 nm in length.

Physical Properties:

Virions have a buoyant density of 1.13-1.18g/cm^3 in sucrose. Virions are sensitive to treatment with heat, detergents, and formaldehyde. The infectivity is not affected by irradiation.

How Lentivirus Works

Once target cells are transduced with recombinant lentivirus, the viral RNA is reverse-transcribed, actively imported into the nucleus (Lewis & Emerman, 1994; Naldini, 1999), and stablely integrated into the host genome (Buchschacher & Wong-Staal, 2000; Luciw,1996). One or two days after the lentiviral genome is integrated into the host genome, you may begin to assay for the transient expression of your recombinant protein or use appropriate selection markers to generate a stable cell line for long-term expression studies.

VSV Envelope Glycoprotein

Most commercial retroviral vectors are limited in gene delivery applications because of their restricted tropisms and generally low titers. For recombinant lentiviral vectors, these limitations are resolved by pseudotyping the vector with the G glycoprotein gene from Vesicular Stomatitis Virus (VSV-G) envelope. The significant advantages associated with the use of VSV-G envelope include:

- allowing production of high titer lentiviral vector
- significantly increasing viral particle stability
- broadening target cell ranges
- generating highly efficient transduction efficiency (Burns *et al.*, 1993; Emi *et al.*, 1991; Yee *et al.*,1987, 1994, 1999)

Packaging Limits

Recombinant lentiviral titers will decrease with increasing insert gene size. The packaging limits for our Lenti-Easy expression system is approximately 5.5 kb; above these limits, little to no virus is produced.

Biosafety

Our Lenti-Easy Expression System implements 3rd-generation of self-inactivating recombinant lentiviral vectors with enhanced biosafety and minimal relation to the wild-type, human HIV-1 virus. The lentiviral particles produced with this system are replication-incompetent and designed with a number of safety features to enhance its biosafety.

Biosafety Features of Lenti-Easy™ System

All Lentiviral Expression Systems provided by ABM Inc. include the following safety features:

- An enhancer deletion in the U3 region of 3'ΔLTR ensures self-inactivation of the lentiviral vector following transduction and integration into genomic DNA of the target cells.
- Utilization of a RSV promoter upstream of 5'ΔLTR allows efficient Tat-independent production of viral RNA.
- The number of lentiviral genes necessary for packaging, replication and transduction is limited to three (Gag/Pol/Rev), and their expression is derived from different plasmids, which all lack packaging signals. These plasmids share no significant homology to the expression vector, preventing the generation of replication-competent virus.
- None of the Gag, Pol, or Rev genes will be present in the packaged viral genome, thus making the mature virus replication-incompetent.

Biosafety Level II

Despite the consideration of the safety features discussed, it is highly recommended that any manipulation with lentiviral vectors, including viral production and transduction, be performed under Biosafety Level 2 (BL-2). All published BL-2 guidelines with proper waste decontamination should be strictly followed. In addition, exercise extra caution when creating lentivirus carrying potentially harmful or toxic genes (e.g. activated oncogenes).

For more information about the BL-2 guidelines and lentivirus handling, refer to "Biosafety in Microbiological and Biomedical Laboratories," 4th Edition, published by the Center for Disease Control (CDC). This document may be downloaded at the following address:

http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

Other Safety Considerations

It is also important to consult with the health and safety officers at your institution for guidelines regarding the use of lentiviruses, and to always follow standard microbiological practices, which include:

- Wear gloves and lab coat at all times
- Always work with pseudoviral particles in a Class II culture facility
- All procedures are performed carefully to minimize splashes or aerosols
- Work surfaces are decontaminated at least once a day and after any spill of viable material
- All cultures, stocks, and other regulated wastes are decontaminated before disposal by an approved decontamination method such as autoclaving

Materials

Table 1. Kit Contents

Component (Cat. No.)	Quantity	Unit Price	Kit Catalog No.			
			LV100	LV110	LV098	LV099
Lenti-Easy-His Tag Vector (LV009)	10µg	$225	✓	✓		
Lenti-Combo Packing Mix I (LV003)	100µg	$225	✓	✓	✓	
Lentifectin™ (G074)	1.0ml	$135	✓		✓	
293T cell line (LV010)	$1x10^6$	$150	✓			✓
Lenti-GFP (LV011)	10µg	$135	✓			✓
		Kit Price	$595	$395	$295	$235

Additional Materials Required

The following materials and reagents are required but not provided:

- Dulbecco's Modified Eagle's Medium (Invitrogen Cat: 11995)
- Fetal bovine serum (FBS)

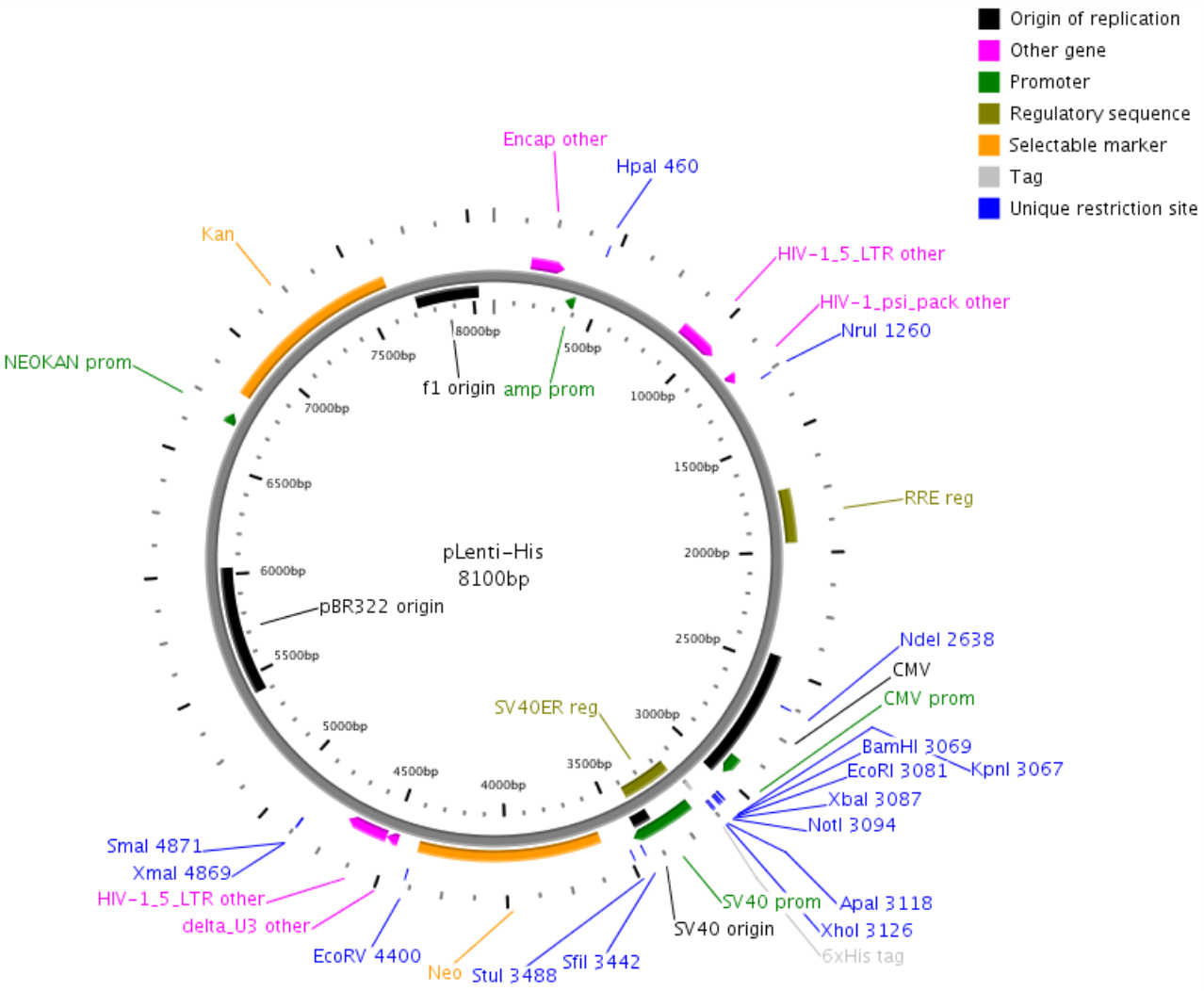
  Note: *serum need not be heat-inactivated.*
- 200 mM L-Glutamine (Sigma Cat. No. G7513)
- Solution of 10,000 units/ml Penicillin G sodium and 10,000 µg/ml Streptomycin sulfate (Sigma Cat. No. P0781)
- Complete Medium: DMEM supplemented with 100 units/ml penicillin G sodium, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS)
- G418 (Cat. No. C020)

  Note: *Make a 10 mg/ml active stock solution by dissolving 1g of powder in approximately 70ml of complete medium without supplements. Filter sterilize and store at 4°C.*
- Puromycin (Cat. No. C021)
- Polybrene (Hexadimethrine Bromide; Sigma Cat. No. H9268)
- Trypsin-EDTA (Trypsin; Sigma Cat. No. T3924)
- Dulbecco's phosphate buffered saline (DPBS; VWR Cat. No. 82020-066)
- Tissue culture plates and flasks

Storage

- 293T cells in liquid nitrogen.
- Lentifectin at 4°C.
- All other components at –20°C.
- Spin briefly to recover contents.
- Avoid repeated freeze-thaw cycles.

Maps & Diagrams



5' G CTA GCG TTT AAA CTT AAG CTT GGT ACC GGA TCC ACT AGT
NheI KpnI BamHI SpeI
GAA TTC TCT AGA GCG GCC GCA CCG CGG AGT ACT GGG CCC
EcoRI XbaI NotI SacII ScaI ApaI
CCT AGG CTC GAG CAT CAT CAC CAT CAC CAT TGA 3'
AvrII XhoI H H H H H H Stop

Figure 1: *pLenti-His Vector Map*

Experimental Procedure

Currently, the most efficient and widely used protocol for producing high-titer lentiviral particles is based on transient co-transfection of a lentiviral construct and structural protein plasmids in the packaging 293T cells. Following co-transfections in packaging cells, the highly-efficient hybrid CMV/5'ΔLTR (or RSV/5'ΔLTR) promoter from the expression construct drives the expression construct transcript containing all the functional elements (i.e., Psi, RRE, and cPPT) required for efficient packaging. The expression construct transcript is then efficiently packaged into VSV-G pseudotyped viral particles along with other structural proteins expressed from separate plasmids (all included in packaging mix). Viral particles can thus transduce both dividing and non-dividing cells through VSV-G protein, which mediates viral entry through lipid binding and plasma membrane fusion (Burns, 1993).

Experimental Flow Chart

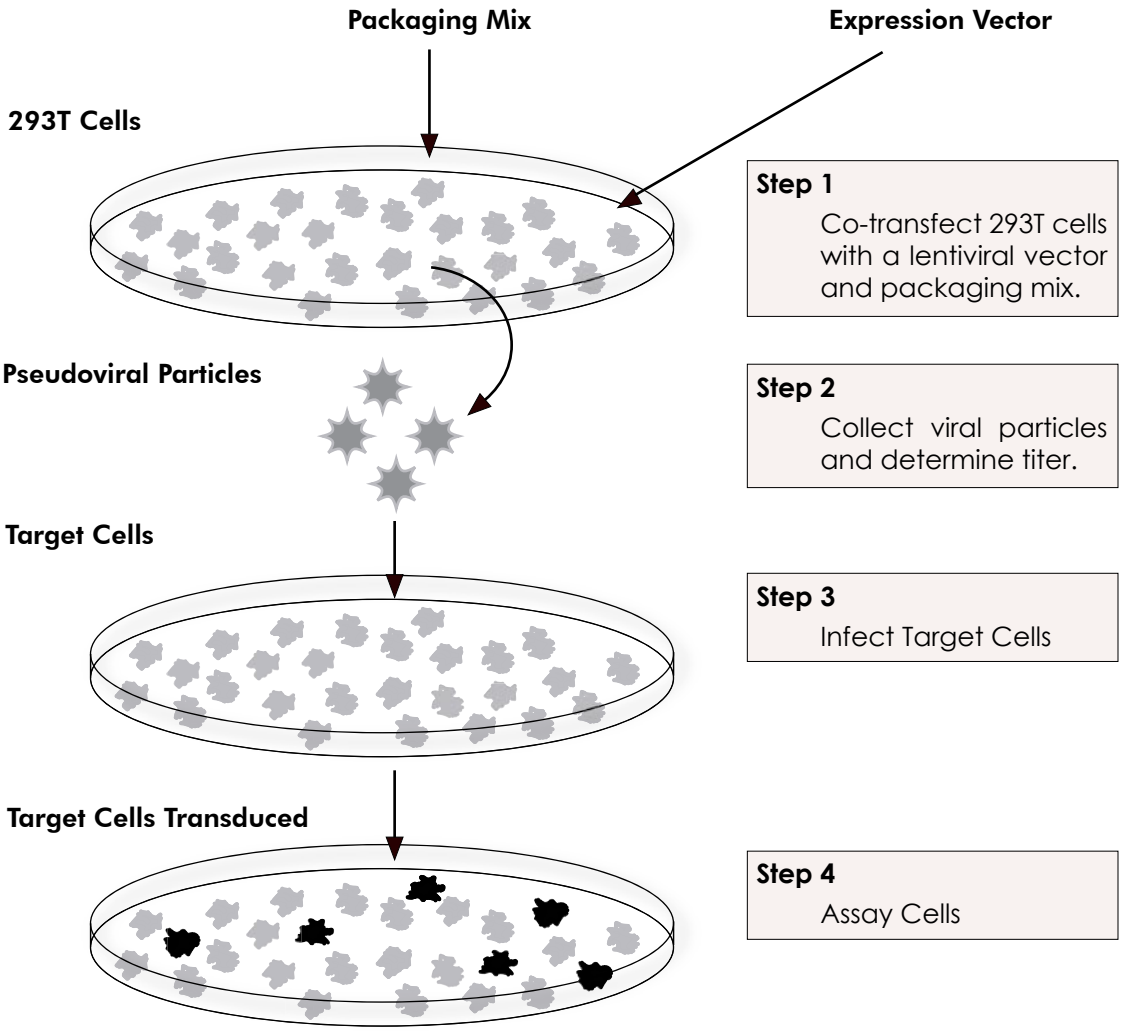


Figure 2: *Procedure for transient production of pseudoviral particles and transduction of effector expression constuct into target cells.*

Protocol

The following protocol has been broken into sections for convenience. However, time should be taken to familiarize oneself with the full procedure before attempting the experiment.

Lenti-Easy Construct Generation

Generate a pLenti expression construct containing your gene of interest. For detailed information on subcloning using the multiple cloning sites available in our lentiviral vectors, refer to a standard molecular cloning manual (Sambrook, J.& Russell, D.W.). Once the expression construct has been produced, perform a Maxi DNA purification for transfection.

Packaging Mix

All plasmids required for the production of recombinant lentivirus are provided in optimized mixtures. We have developed 3 different packaging mixes for the production of recombinant lentiviral particles from different vector generations. Lenti-Combo I (LV003) is used for the production of 2nd and 3rd generation lentiviral particles; Lenti-Combo II (LV019) is used only for the packaging of 3rd generation lentiviral particles; Lenti-Combo III (LV020) is used only for Feline HIV (FIV)-based vectors. All lentiviral vector provided by ABM Inc. are 3rd generation and can be packaged by either Lenti-Combo Mix I or Lenti-Combo Mix II. In general, relatively higher titer can be achieved with Lenti-Combo I, but it is much safer using Lenti-Combo II for production.

293T Cells

The human 293T cell line is widely used for optimal lentivirus production (Naldini *et al*., 1996). The health of 293T cells at the time of transfection is a critical factor for the success of lentivirus production. The use of "unhealthy" cells will negatively affect the transfection efficiency, resulting in low titer lentiviral stocks. For optimal lentivirus production, follow the guidelines below to culture 293T cells before use in transfection:

- Ensure cell viability is greater than 90%.
- Subculture and maintain cells in complete medium containing 0.1mM MEM, Non-Essential Amino Acids, 4mM L-Glutamine, 1mM sodium pyruvate,
- 500µg/ml Geneticin® and 10% FBS.
- Do not allow cells to overgrow before passaging.
- Use cells that have been subcultured for less than 16 passages.

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Positive Control

We recommend including a positive control vector such as Lenti-EGFP in your co-transfection experiment to generate a control lentiviral stock that can be used to help you optimize expression conditions in your target cells.

Transfection Conditions

We produce lentiviral stocks in 293T cells using the following optimized transfection conditions. The amount of lentivirus produced using these recommended conditions (10ml of virus at a titer of at least $1x10^5$ transducing units (TU)/ml) is generally sufficient to transduce at least $1x10^6$ cells at a multiplicity of infection (MOI) of 1. For example, 10 wells of cells plated at $1x10^5$ cells/well in 6-well plates could each be transduced with 1ml of a $1x10^5$ TU/ml virus stock to achieve an MOI of 1.

Transfection Procedure

1. One day before transfection (Day 1), plate 293T cells in a 10cm tissue culture plate so that they will be 90-95% confluent on the day of transfection (i.e. $5x10^6$ cells in 10ml of growth medium containing serum). As a general rule, one 15cm culture dish at 95% confluence can be subcultured into 5x 10cm dishes; whereas one 10cm dishes at 95% confluence can be subcultured into 2x 10cm dishes.
2. On the day of transfection (Day 2), set up the transfection mix as follows:
   a. In a sterile 15ml culture tube, dilute 10mg of Lenti-Combo Mix and 10µg of pLenti expression plasmid DNA in 1.0ml of medium without serum. Mix gently.
   b. In a separate sterile 15ml tube, dilute 80µl of Lentifectin (mix gently before use) in 1.0ml of medium without serum. Mix gently and incubate for 5 minutes at room temperature.
   c. After the 5 minute incubation, combine the diluted DNA with the diluted Lentifectin. Mix gently.
   d. Incubate for 20 minutes at room temperature to allow the Lentifectin/ DNA complexes to form.
   e. Add 4.5ml serum-free medium to the complexes followed by gentle mixing.
   f. Remove the medium from the cells, and then add Lentifectin/DNA complexes carefully to culture dishes without dislodging cells. Incubate the cells for 5-8 hours at 37°C in a humidified 5% CO_2 incubator.

      Note: *293T cells are poorly adhesive to most culture dishes. It is always recommended to add or change medium against the wall of culture dishes to avoid dislodging cells.*
   h. Add 0.65ml serum to each transfected culture dish and return the dishes to incubator. Incubate overnight.

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Transfection Procedure

3. The following day (Day 3), remove the medium containing the Lentifectin/ DNA complexes and replace with 10ml complete culture medium. Incubate at 37°C in a humidified 5% CO_2 incubator.

   Note: Expression of the VSVG glycoprotein can cause 293T cells to fuse, resulting in the appearance of large, multinucleated cells known as syncytia. This morphological change is normal and does not affect production of the lentivirus.

4. Harvest virus-containing supernatants 48-72 hours post-transfection (Day 4-5) by collecting medium into to a 15ml sterile, capped, conical tube.

   Caution: Remember that you are now working with infectious virus at this stage. Follow the recommended guidelines for working with BL-2 organisms (see page 3 for more information).

5. Centrifuge supernatants at 3000rpm for 15 minutes at +4°C to pellet debris.

   Optional: Filter the viral supernatant through 0.45µm PVDF syringe filter (Millipore, Cat. No. SLHVR25LS).

6. Aliquot viral supernatants into cryovials in 1.0ml portions and store viral stocks at -80°C. Proceed to titering your lentiviral stock (page 10).

If you plan to use your lentiviral construct for *in vivo* applications, we recommend filtering your viral supernatant through a sterile, 0.45ml low protein binding filter after the low-speed centrifugation step to remove any remaining cellular debris. The viral supernatant can be concentrated using the protocols discussed in the following section if higher titer virus is required.

Concentrating Virus

There are several protocols that have been established to concentrate VSV-G pseudotyped lentiviruses without significantly affecting their ability to transduce target cells. These include ultracentrifugation (Yee, 1999), filter-based ion exchange chromatography (Lenti-Pure Cat. No. LV998), and size exclusion chromatography (Speedy Lentivirus Purification Cat. No. LV999).

Long-Term Storage

Viral stocks stored at -80°C should be stable for at least one year. Repeated freezing and thawing will result in loss of viral titer. Based on our in-house data, each freeze-thaw will lead to a 25% loss of viral titer.

Viral Titer Assays

It is useful to titer the viral supernatant before proceeding with transduction experiments for the following reasons:

- To ensure that viral stock is viable
- To determine the percentage of target cells that can be transduced with the pseudoviral stock
- To control the number of copies of integrated viral constructs per target cell

The commonly used protocol for measuring relative titers uses a positive control expression plasmid (i.e. GFP mixed with expression construct) as an internal control at a ratio of 1:100 and is packaged into pseudoviral particles. In an alternative approach, the GFP control plasmid can be packaged separately but in parallel with your construct, as an external control. In this scenario, the control plasmid can be used to check and optimize the transfection/packaging steps (see transfection procedure). Recently, other in vitro protocols including qRCR and HIV p24 protein-based ELISA have been developed for quick assays.

To determine the relative viral titer, transduce a target cell line such as MDA-MB-468 in the presence of Polybrene (2µg/ml) for 12-16 hours, and then count the number of cells expressing GFP either by fluorescence microscopy or by FACS.

1. For each viral stock, plate MDA-MB-468 cells one day prior to viral infection in a 24-well plate at a density of 0.6 – 1×10^5 cells per well. Add 1ml of complete D-MEM medium (with serum and antibiotics) and incubate cells at 37°C with 5% CO_2 overnight.

   Note: *It is possible to use bigger culture dishes for transduction, especially when a large number of cells is required for FACS analysis. In this case, the amount of cells should be adjusted depending on the growth area of the well/plate.*

2. Prepare complete D-MEM medium plus 10% FBS with Polybrene to a final concentration of 2µg/ml.

   Note: *Polybrene is a polycation that neutralizes charge interactions to increase binding between the pseudoviral capsid and the cellular membrane. The optimal concentration of Polybrene depends on cell type and may need to be empirically determined (usually in the range of 1–8µg/ml). Excessive exposure to Polybrene (>12 hr) can be toxic to some cells.*

3. Remove culture medium and replace with 0.5ml of complete DMEM medium with 10% serum and Polybrene (from Step 2). For each viral stock, use three wells. Infect MDA-MB-468 cells by adding 1ul of viral stock into the first well (dilution factor of 500), 10µl of viral stock into the second well (dilution factor of 50), and 100µl of viral stock into the third well (dilution factor of 5). For mock well controls, add 0.5ml of DMEM medium with Polybrene (from Step 2). Incubate cells at 37°C with 5% CO_2 overnight.
4. Remove culture medium and replace with 1ml of complete DMEM medium (without Polybrene). Incubate the cells at 37°C with 5% CO_2 overnight.
5. The following day, split the cells 1:3 to 1:5 if necessary, depending on the growth rate of cells. Incubate in complete D-MEM for an additional 24-48 hours.

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Viral Titer Assays

6. Count the fraction of fluorescent cells by FACS analysis. You may also count the GFP positive cells under a fluorescent microscope, but the results may be less accurate due to inconsistencies in counting methods. Use an average of the fraction of GFP+ cells in 5 – 10 random fields to estimate the overall percentage of GFP+cells on the plate. Multiply the number of infected cells by 1.5×10^5 (in this example, the expected number of MDA-MB-468 cells on the plate at the moment of infection) and by the corresponding dilution factor, then divide by 0.5ml to determine the relative titer of the virus in the supernatant.

Alternative Methods

The viral titer can also be estimated by real time PCR using ABM's Lentiviral qPCR Titer Kit (Cat. No. LV500) or p24-based ELISA titer kit (Cat. No. LV501).

General Considerations For Transduction

The following information should be considered before one attempts target cell transduction:

- The transduction efficiency of target cells varies significantly under different experimental conditions, including virus concentration, exposure time to virus, and growth area of cells. To determine the viral concentration required to provide the desired multiplicity of infection (MOI) for your target cells, perform several transductions with different concentrations of viral particles containing GFP control plasmid. Results from these test transductions should be used to determine an optimal concentration that yields the highest percentage of infected cells based on GFP expression.
- Recombinant gene expression can be measured directly 48 – 72 hours after transduction ("transient transduction"), but selecting stably transduced cells will require additional time after transduction. The decision to use "transiently transduced" cells or selected stable cells will depend on the nature of your target cells, biological assay, and transduction efficiency. For efficient transducable cells (e.g., 293, HT1080, HeLa, MDA-MB-468 cells, etc), most biological assays can be performed following transient transduction. However, for "difficult-to-transduce" cells, it is desirable to select the clones that stably expresses the Lentivector construct for experimental assays.

Transduction Procedure

The following protocol provides general guidelines as a starting point for determining optimal conditions for your target cell transduction.

1. Plate target cells in a 24-well plate 24 hours prior to viral infection at a density of 0.5×10^5 cells per well. Add 0.5 ml of complete optimal medium (with serum and antibiotics) and incubate cells at 37°C with 5% CO_2 overnight.
   Note: *It is possible to use other plate formats for transduction. In this case, the amount of cells should be adjusted depending on the growth area of the well/plate.*
2. Prepare a mixture of complete medium with Polybrene at a final concentration of 2µg/ml. Remove media from plate wells and replace with 0.5 ml of this Polybrene/media mixture per well (for 24-well plate).
3. Infect target cells by adding several different amounts of viral stock (example: 1µl, 5µl , 10µl, and 100µl of virus). In addition, include a transduction well with GFP positive control virus and other appropriate positive and negative control viral constructs. Incubate cells at 37°C with 5% CO_2 overnight.
4. Remove the culture medium and replace with 1ml of complete medium. Incubate the cells at 37°C with 5% CO_2 overnight.
5. The following day, split the cells 1:3 to 1:5, depending on the growth rate of your target cells, and continue incubating for 48 hours in complete DMEM.
6. The infected target cells can be either analyzed for transient expression or selected for stable expression using appropriate selection markers (G418).

Troubleshooting Guide

Generating Lentiviral Stock

Problem	Possible Cause	Solution
No viral particles.	iLenti plasmid DNA modified (e.g. acetylation or methylation.)	Re-transform plasmidinto an authentic DH5a
Low viral titer.	Low transfection efficiency: • Poor quality DNA ; • Low 293T cell viability; • Transfection media containing antibiotics and serum.	• Use DNA purified from Midi or Maxi; • Use 293T cells under passage 16; • Reduce transfection antibiotics or serum efficiency.
	Plasmid DNA:Lentifectin ratio incorrect.	Optimize DNA:Lentifectin ratio.
	Insufficient DNA used for transfection.	Use 5-10mg of expression vector and 10-15mg of packaging mix.
	293T cell density too low.	Optimal cell density at 90-95%.
	Viral supernatant harvested too early.	Optimal viral titers can be collected 48-72 hours post-transfection.
	Viral supernatant subject to multiple freeze/thaw.	Each freeze/thaw lose 25% of the titer. Make aliquots for long-term storage.
	Gene of interest toxic to cells.	Use an inducible system.
	Gene insert over 5kb.	Viral titer decrease as the size of insert increases. Maximum insert size is 5.5kb.
	Polybrene not used during transduction.	Transduce cells in the presence of Polybrene.

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Troubleshooting Guide

Target Cell Transduction

Problem	Possible Cause	Solution
No transgene expression.	Promoter silencing.	Lentiviral vector may integrate into a chromosomal region that silences the CMV promoter. Screen multiple antibiotic-resistant clones and select the one with the highest expression levels. Subclone your gene of interest under a cellular promoter that is not subject to silencing.
	Viral stocks stored incorrectly.	Aliquot and store at –80°C. Avoid freeze/thaw.
	Low transduction efficiency: Polybrene not used.	Use a GFP lentiviral vector to check the efficiency of transduction of target cells.
	Target cells not transducible with lentiviral vectors.	Transduce target cells in the presence of Polybrene.
	MOI too low.	Use higher MOI.
	Antibiotic concentration too high.	Determine antibiotic sensitivity of target cells by performing a killing curve. Use minimum antibiotic concentration required.
	Cells harvested too early for assay.	Perform expression assay 72 hours post-transduction to allow the accumulation of expressed protein.
	Gene of interest is toxic	Use an inducible promoter

(section continues)

Troubleshooting Guide

Target Cell Transduction

Problem	Possible Cause	Solution
Cytotoxic effects of target cells.	Large volume of viral supernatant used for transduction.	Use less viral supernatant; dilute viral supernatant further with fresh medium (1:3 to 1:5) for transduction.
	Polybrene concentration too high.	Use less or omit Polybrene during transduction.
	Antibiotic concentration too high.	Use minimum antibiotics for effective selection.
	Gene of interest toxic to cells.	Try a different cell line or inducible promoter.

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