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# iLenti™ siRNA Expression System

**Cat No: LV300**

# Table of Contents

# Introduction

Small interfering RNA (siRNA), also known as short interfering RNA, is a class of 20-29 nucleotide-long double-stranded RNA molecules that play a variety of roles in biology. siRNAs were initially discovered to be involved in the RNA interference (RNAi) pathway, where they knockdown the expression of specific genes through post-transcription mechanisms (Fire *et al.* 1998). Recent studies demonstrate that siRNAs also act in other RNA related pathways, such as antiviral mechanisms and shaping of chromatin structure in a genome. The complexity of these pathways has only recently been elucidated (Hawkins and Morris 2008).

Mirroring the success of PCR, siRNA is proving to be one of the few groundbreaking technologies that will completely change the landscape of life science research. Since the initial demonstration of its functionalities in mammalian cells, siRNA has been an indispensable tool in functional genomics and is predicted to be an novel form of future medicine (Grünweller and Hartmann 2005).

## Use Of Recombinant Lentiviral Vectors

Vector-based siRNAs are the method of choice for both transient and stable gene knockdown. Due to its high transduction efficiency, lentiviral vector-based siRNA is considered to be the most efficient delivery method available. By incorporating siRNA into lentiviral vectors, specific gene expression can be knocked-down by either plasmid transfection or lentiviral infection for any target cell. Another advantage of using lentiviral vector expressed siRNA is the efficient and stable expression of siRNA in both dividing and non-dividing cells, including even transfection-resistant cells such as primary cells.

## Advantages of iLenti Expression System.

ABM's iLenti expression system is the most advanced lentiviral vector-based siRNA expression system, with better features than our competitor vector-based siRNA expressions systems:

1. iLenti™ vectors are very stable and have the ability to grow in regular competent cells, which eliminates the need for special, stable competent cells.
2. iLenti™ uses convergent promoters to avoid hairpin loop structure design. Thus cloned siRNA will be less likely to form secondary structures, allowing easier plasmid propagation and sequencing.
3. The convergent promoter design also enables longer siRNA designs of 27-29bp oligos, which have been shown to be more efficient than traditional 21mers in specific gene knockdown (Kim et al. 2005).
4. iLenti™ significantly reduces the production cost of oligos, as ~33bp inserts are used instead of the ~60bp oligos used by our competitors .
5. The iLentiTM vector has a unique, single Bbs I restriction enzyme site in the multiple cloning site, which allows efficient, directional cloning of siRNA target sites.
6. iLenti also has an EGFP reporter gene incorporated under the CMV promoter, which allows simultaneous tracking of expressed siRNAs *in vivo*.

# Biosafety

Our iLenti Expression System is comprised of 3rd-generation 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 biosafety. 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 an 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 only three (Gag, Pol, Rev), and their expressions are derived from separate plasmids, each lacking packaging signals. These plasmids share no significant homology to the expression vector, preventing the regeneration 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 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. 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 autoclaving

# Materials

*Table 1. Kit Contents*

| Component (Cat. No.) | Quantity | Kit Catalog No. | | | |
|---|---|---|---|---|---|
| | | LV300 | LV301 | LV310 | LV311 |
| Bbs I Linearized iLenti™-EGFP Vector (LV014) | 2.5µg | ✓ | ✓ | | ✓ |
| Lenti-Combo Packing Mix (LV003) | 100µg | ✓ | ✓ | | ✓ |
| Lentifectin™ (G074) | 1.0ml | ✓ | ✓ | | |
| 293T cell line (LV010) | $1x10^6$ | ✓ | | | |
| Lenti-GFP (LV011) | 5µg | ✓ | | ✓ | |
| Scramble Control (LV015) | 5µg | ✓ | | ✓ | |
| iLenti™ Sequence Primers (LV012); 10µM | 100µl | ✓ | ✓ | ✓ | |

## Storage

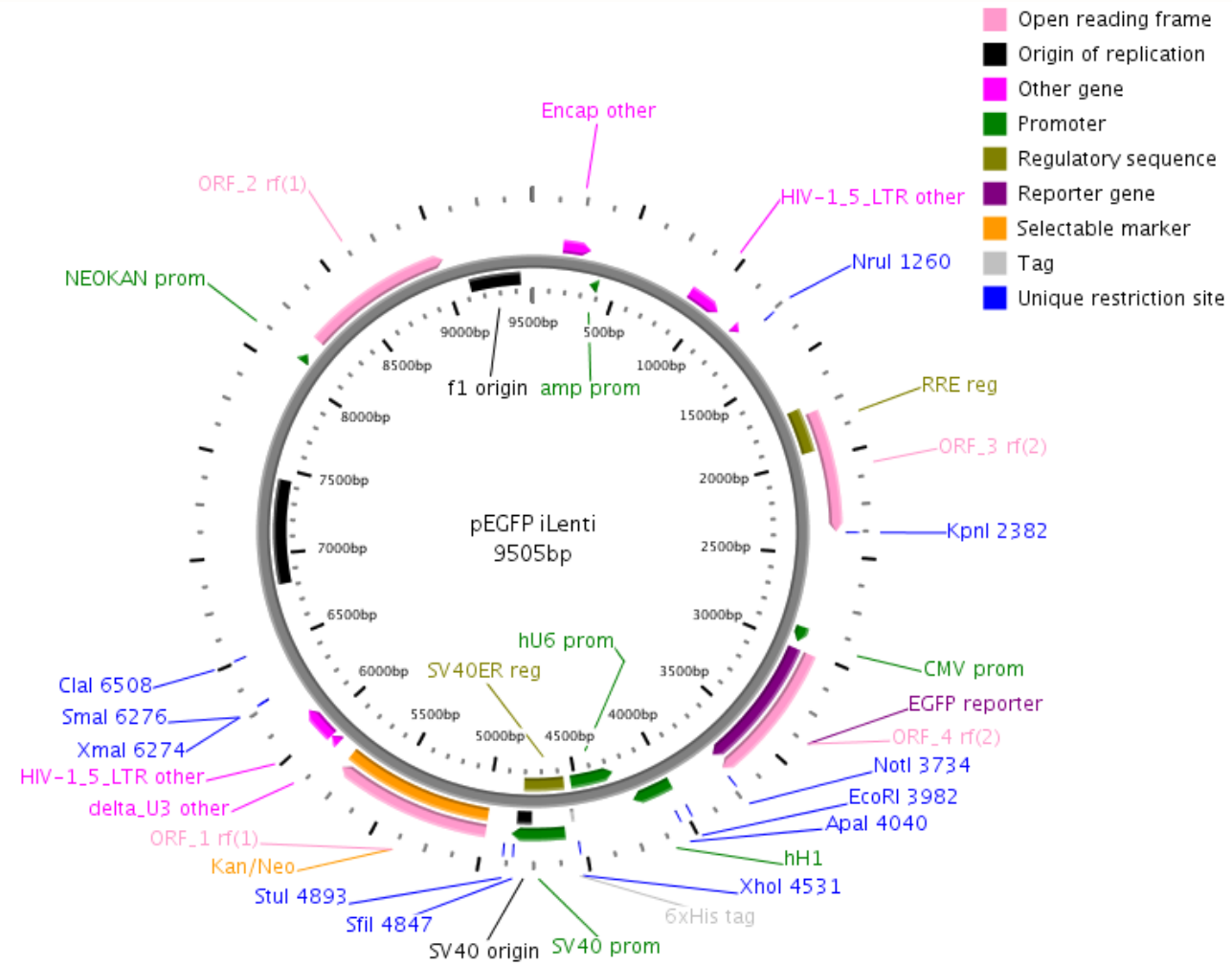
- 293T cells in liquid nitrogen.
- Lentifectin at 4°C.
- All other components at –20°C.
- Spin briefly to recover contents.
- Avoid repeated freeze-thaw cycles.

## Additional Materials Required

The following materials and reagents are required but not provided:

- Dulbecco's Modified Eagle's Medium (Invitrogen: Cat. No. 11995)
- Fetal bovine serum (FBS)
  ***Note:*** *serum need not be heat-inactivated.*
- 200mM L-Glutamine (Sigma: Cat. No. G7513)
- Solution of 10,000units/ml Penicillin G sodium and 10,000µg/ml Streptomycin sulfate (Sigma: Cat. No. P0781)
- Complete Medium: DMEM supplemented with 100units/ml penicillin G sodium, 100µg/ml streptomycin and 10% FBS
- G418 (Cat. No.C020)
  ***Note:*** *Make a 10mg/ml active stock solution by dissolving 1g of powder in approximately 70ml of complete medium without supplements. Filter sterilize and store at 4°C*
- 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
- T4 DNA Ligase (Invitrogen: Cat. No. 15224-025)
- DNA plasmid DNA purification kits (Mini and Maxi)

## Maps & Diagrams




5' G TAT GAG ACC ACA AAA ATA GTC TTC GAT ATC ATT TAA ATG
BbsI EcoR V Swa I
TCG ACA GAA GAC AGC TTT TTT TGG TGT TTC GTC CTT TCC 3'
BBs I

***Figure 1:*** *piLenti-EGFP Vector Map*

# Experimental Procedure

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.

## Designing siRNA Target Sequences

Since the initial demonstration of the functionalities of siRNA in mammalian cells, great efforts have been made to develop of criteria for designing effective siRNA target sites for specific gene knockdown. Many free software programs based on bioinformatic analysis and experimental data are available online for quick selection of siRNA target sites for any given target gene. In general, one out of four designed target sites, made using these free algorithms, will give rise to over 70% knockdown of the target gene. It is therefore highly recommended to design at least 4 siRNA oligos for any target gene. For your reference, please visit the following links for siRNA target selection:

http://katahdin.cshl.org:9331/siRNA/RNAi.cgi?type=shRNA

https://rnaidesigner.invitrogen.com/rnaiexpress/

Once a siRNA target sequence is selected, 4 specific additional nucleotides (as seen in the following figure) need to be added to both ends of the designed oligos for subsequent cloning of siRNA constructs into Bbs I linearized iLenti™ vector. We recommend synthesizing PAGE-purified target oligos, although oligos without PAGE purification may also work for subsequent ligations. The designed oligoes do not need to be phosphorylated for the subcloning.

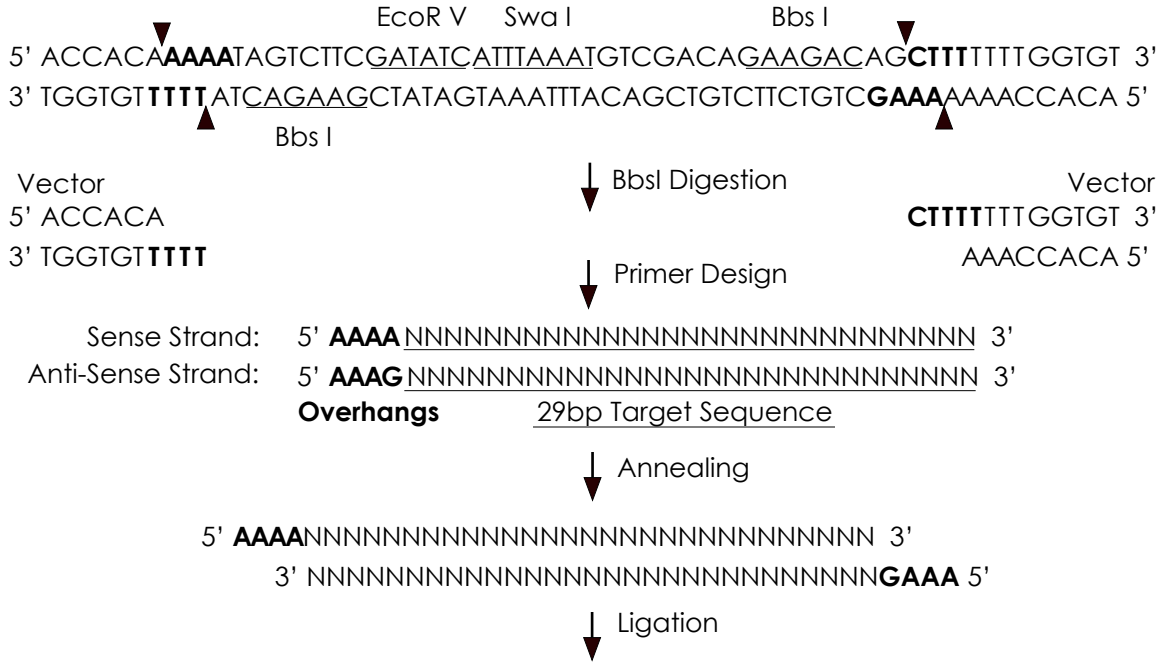



***Figure 1:*** *siRNA target design and subcloning.*

## Subcloning Target Sequences into iLenti™ Vector

1. Resuspend each target oligonucleotide in $ddH_2O$ to a concentration of 100µM.
2. Mix the sense and antisense oligos at a 1:1 ratio, resulting in 50µM of ds oligo (assuming 100% annealing efficiency).
3. Using a PCR thermocycler, perform oligo annealing in the following program:

*Table 1. Thermocycler Conditions*

| Process | Temperature | Time |
|---|---|---|
| Denaturation | 95°C | 30sec |
| Annealing | 72°C | 2min |
| | 37°C | 2min |
| | 25°C | 2min |
| Storage | 0°C (on ice) | variable |

The annealed oligonucleotide is now ready for ligation into iLenti vector. Alternatively, the ds oligonucleotide can be stored at -20°C until needed.

4. Set up the following ligation reaction:

*Table 3. Ligation of siRNA target into iLenti vector*

| Reagent | Volume/Reaction |
|---|---|
| Bbs I linearized vector | 4µl |
| Annealed oligonucleotides | 3µl |
| 5x DNA Ligase Buffer | 2µl |
| T4 DNA Ligase | 1µl |
| **Total Volume** | **10µl** |

Mix gently by pepitting up and down, followed by incubation at room temperature for 1-2 hours.

***Note:*** *Temperatures greater than 25°C may negatively affect ligation efficiency.*

5. Transform 3-5µl of ligation product (above) with 50µl standard DH5a competent cells.

   ***Note:*** *Ensure that bacterial strains are authentic DH5a competent cells. Other strains of competent cells may negatively effect lentivral titer or compromise lentiviral particle production altogether.*
6. Following recovery, plate all cells on LB plates with 50µg/mL Kanamycin and incubate overnight at 37°C.

## Identification of iLenti™ Clones

1. Randomly pick 10 well separated colonies from a LB plate and innoculate the colonies in 5ml of LB Broth with 50µg/mL Kanamycin at 37°C overnight.
2. Extract DNA using a Mini-preparation kit.
3. Perform an EcoR V digestion of mini-prepared DNA to screen for recombinant colony.

   ***Note:*** *The parental iLenti™ vector has two EcoR V sites, one of which is located between the two Bbs I sites. Digestion of parental iLenti™-EGFP plasmid would thus produce two fragments of 1.5kb and 8.0kb in size. Ligation of siRNA target oligoes into the iLenti™ vector will result in the recombinant iLenti™ vector with a single EcoR V site, leading to a linearized vector of 9.5kb after EcoR V digestion. The identified recombinant clones can be sequence-confirmed using the primers that are included in the kit for target siRNA sequence accuracy.*
4. Perform a Maxi DNA preparation of recombinant iLenti clone for either target gene knockdown by direct transfection or for lentivirus production.

## Lentiviral Particle Production

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'LTR (or RSV/5'LTR) 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 separated plasmids (all included in packaging mix). Viral particles can thus be used to transduce both dividing and non-dividing cells through VSV-G protein, which mediates viral entry through lipid binding and plasma membrane fusion (Burns, 1993).

## Lentiviral Particle Production Flow Chart

The following diagram describes the major steps for the production of recombinant lentiviral particles using the iLenti Expression System.

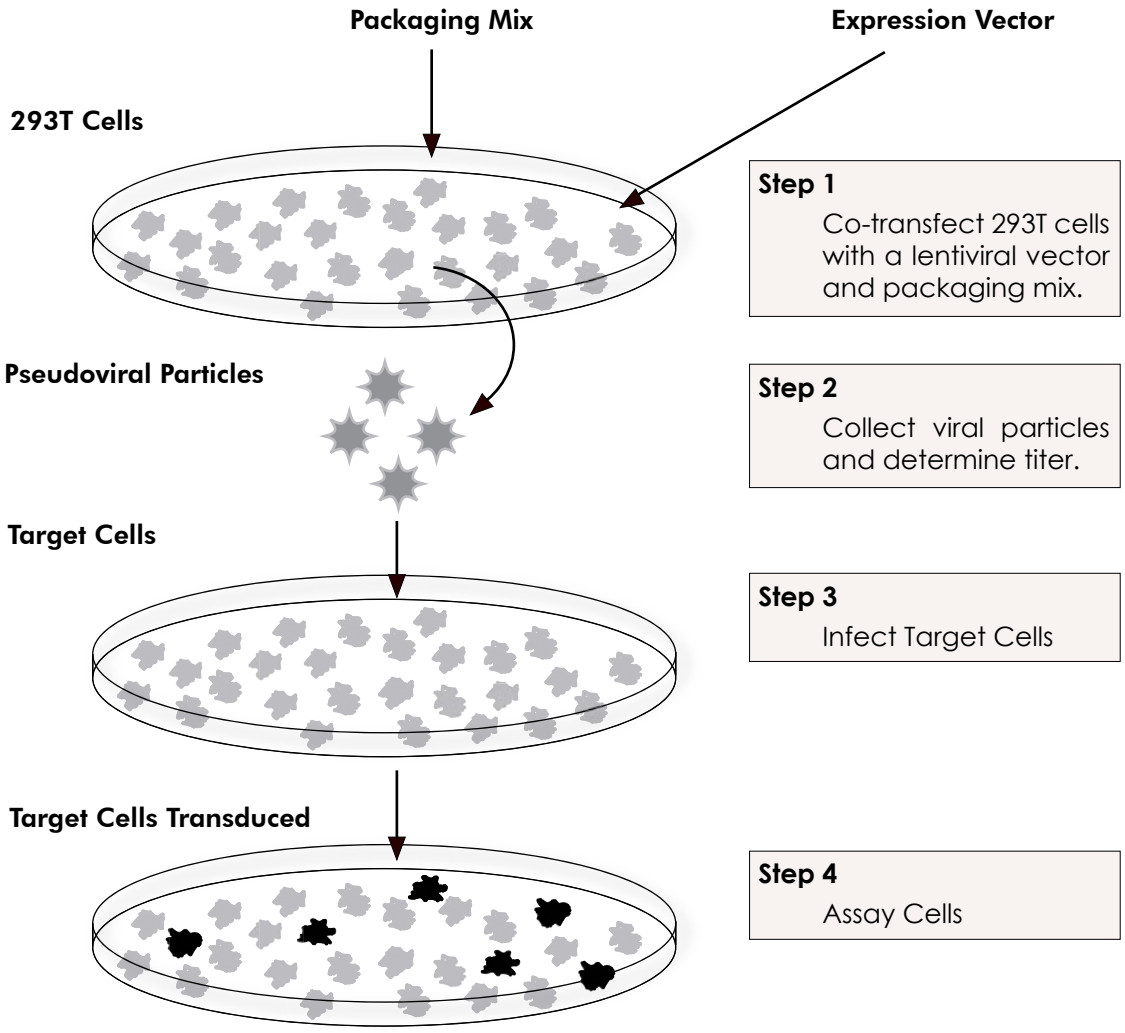



***Figure 2:*** *Procedure for transient production of pseudoviral particles and target cell transduction of effector expression constuct into target*

## 293T Cells

The 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:

- Make sure that cells possess greater than 90% viability.
- 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.

## Transfection Conditions

High titer lentiviral stock can be produced 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) = 10. 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 10.

## Transfection Procedure

1. One day before transfection (Day 1), plate 293T cells in a 10cm tissue culture plate so that they will be ~90% 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 2 x 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 10µg of Lenti-Combo Mix and 10µg of iLenti 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. 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.

*(section continues)*

## Transfection Procedure

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.*

g. Add 0.65ml serum to each transfected culture dish and return the dishes to incubator. Incubate overnight.

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 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 4 for more information).*

5. Centrifuge supernatants at 3,000 rpm for 15 minutes at +4°C to pellet debris.

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

7. Aliquot viral supernatants into cryovials in 1.0ml portions and store viral stocks at -80°C. Proceed to Viral Titer Assays in the following section.

If you plan to use your lentiviral construct for *in vivo* applications, we recommend filtering your viral supernatant through a sterile, 0.45m 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 next section if higher titer lentivirus 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 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 pseudo-viral stock
- To control the number of copies of integrated viral constructs per target cell

The commonly used protocol for measuring relative titers is to infected target cells like MDA-MB-468 for the expression of EGFP following gene transduction. Recently, other *in vitro* protocols including qRCR (Lenti qPCR titer kit, Cat. No. LV500) and HIV p24 protein-based ELISA (Lenti p24 titer kit, Cat. No. LV501) have been developed for quick assays.

To determine the relative viral titer, transduce MDA-MB-468 cells in the presence of Polybrene (2mg/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 - $1\times10^5$ cells per well. Add 1µl of complete DMEM 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 to be plated should be adjusted depending on the growth area of the well/plate.*

2. Prepare complete DMEM medium plus 10% FBS with Polybrene to a final concentration of 2mg/ml.

   *Note: Polybrene is a polycation that neutralizes charge interactions to increase binding between the pseudo-viral 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 1-8mg/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 1ml 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. On 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.
6. Count the fraction of fluorescent cells by FACS analysis. You may also count the EGFP 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 $1.5\times10^5$ (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 Lenti qPCR Titer Kit (Cat. No. LV500 ) or p24-based ELISA titer kit (Cat. No. LV501).

## Guidelines for 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 iLentiviral particles. Results from these test transductions should be used to determine an optimal concentration that yields the highest percentage of infected cells based on EGFP expression.

• Specific gene expression knockdown 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, gene knockdown efficiency as well as transduction efficiency. For efficient transducable cells (e.g., 293, HT1080, HeLa, MDA-MB-468 cells, etc), gene knockdown can be assayed by qPCR and/or Western Blot following transient transduction. However, for "difficult-to-transduce" cells, it is desirable to select the clones that stably expresses the iLentivector construct for experimental assays.

## Target Cell Transduction

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 $0.5x10^5$ cells per well. Add 0.5ml 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 to be plated 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 0.5ml 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). Make sure to include a scramble control of iLentiviral transduction for your experiments.

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 it 1:3 to 1:5, depending on the growth rate of your target cells, and continue incubation for 48 hours in complete DMEM.

6. The infected target cells can be analyzed for transient expression, or selected for stable expression using appropriate selection markers (Cat. No. G418) at minimum concentration as determined by the killing curve.

# Troubleshooting Guide

**subcloning of siRNA Target Sequence into iLenti Vector**

| Problem | Possible Cause | Solution |
|---|---|---|
| No colonies after transformation. | Incorrect antibiotic used; Antibiotic concentration too high. | Use Kanamycin at 50µg/ml of LB agar medium. |
| | Poor transformation efficiency. | Check transformation efficiency using a control plasmid with Kanamycin selection marker. |
| Plasmid much smaller. | DNA rearrangement. | Make sure to inoculate mini culture for less than 24 hours. |

**Generating Lentiviral Stock**

| Problem | Possible Cause | Solution |
|---|---|---|
| No viral particles. | iLenti plasmid DNA modified (e.g. acetylation and/or methylation) during production. | Re-transform plasmid into an DH5a bacteria strain. |
| Low viral titer. | Low transfection efficiency: Poor quality DNA; Low 293T cell viability; Transfection media containing antibiotics or serum. | Use DNA purified from Midi or Maxi; Use 293T cells under passage 16; Use fresh media without antibiotics or serum for transfection. |
| | Plasmid DNA:Lentifectin ratio incorrect. | Optimize DNA:Lentifectin ratio. |
| | Insufficient DNA used for transfection. | Use 5-10µg of expression vector, 10-15µg of packaging mix. |
| | 293T cell density too low. | Optimal cell density at ~90% |
| | 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. |

*(section continues)*

# Troubleshooting Guide

**Generating Lentiviral Stock**

| Problem | Possible Cause | Solution |
|---|---|---|
| Low viral titer. | Gene of interest toxic to cells. | Use an inducible system. |
| | Gene insert larger than 5.0kb. | 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. |

**Target Cell Transduction**

| Problem | Possible Cause | Solution |
|---|---|---|
| No transgene expression. | Promoter silencing. | Lentiviral vector may integrate into a chromosomal region that silences the U6/H1 promoter. Screen multiple antibiotic-resistant clones and select the one with the highest expression levels. |
| | Viral stocks stored incorrectly. | Aliquot and store at –80°C; Avoid freeze/thaw. |
| Low transduction efficiency. | Polybrene not used. | Transduce target cells in the presence of Polybrene. |
| | Target cells not transducible. | Check EGFP expression under microscope for transduction efficiency. |
| | 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. |

*(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 is toxic to cells | Try a different cell line or inducible promoter. |

## Legal

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