DATA SHEET

Lenti-siRNA Expression Systems

iLenti-siRNA Vector	LV014
iLenti-siRNA-GFP Vector	LV016
Complete iLenti-siRNA Expression Kit	LV300
Complete iLenti-siRNA-GFP Expression Kit	LV301
Partial iLenti-siRNA Expression Kit	LV310
Partial iLenti-siRNA-GFP Expression Kit	LV311
Supplemental Kit for All Lentivirus Systems	LV098
Supplemental Kit for All Lentivirus Systems	LV099

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Biosafety

Our iLenti-siRNA Expression System employs 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 its biosafety.

All Lentiviral Expression Systems provided from 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 & integration into the target cell's genomic DNA.

• Utilization of a RSV promoter upstream of 5'ΔLTR allows efficient Tet-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, all lacking packaging signals. The 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.

Despite the safety features discussed above, it is highly recommended that all 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. oncogenes). For more information about the BL-2 guidelines and lentivirus handling, refer to "Biosafety in Microbiological and Biomedical Laboratories," 5th Edition. This may be downloaded at: www.cdc.gov/biosafety/publications/bmbl5/index.htm

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

• Wear gloves and a lab coat at all times.

• Always work with pseudoviral particles in a Class II culture facility and that all procedures are performed carefully to minimize splashes and aerosols.

• Work surfaces are decontaminated at least once a day and after any spills of viable material.

• All cultures, stocks and other regulated wastes are decontaminated before disposal by an approved decontamination method, like autoclaving.

Protocol at a Glance

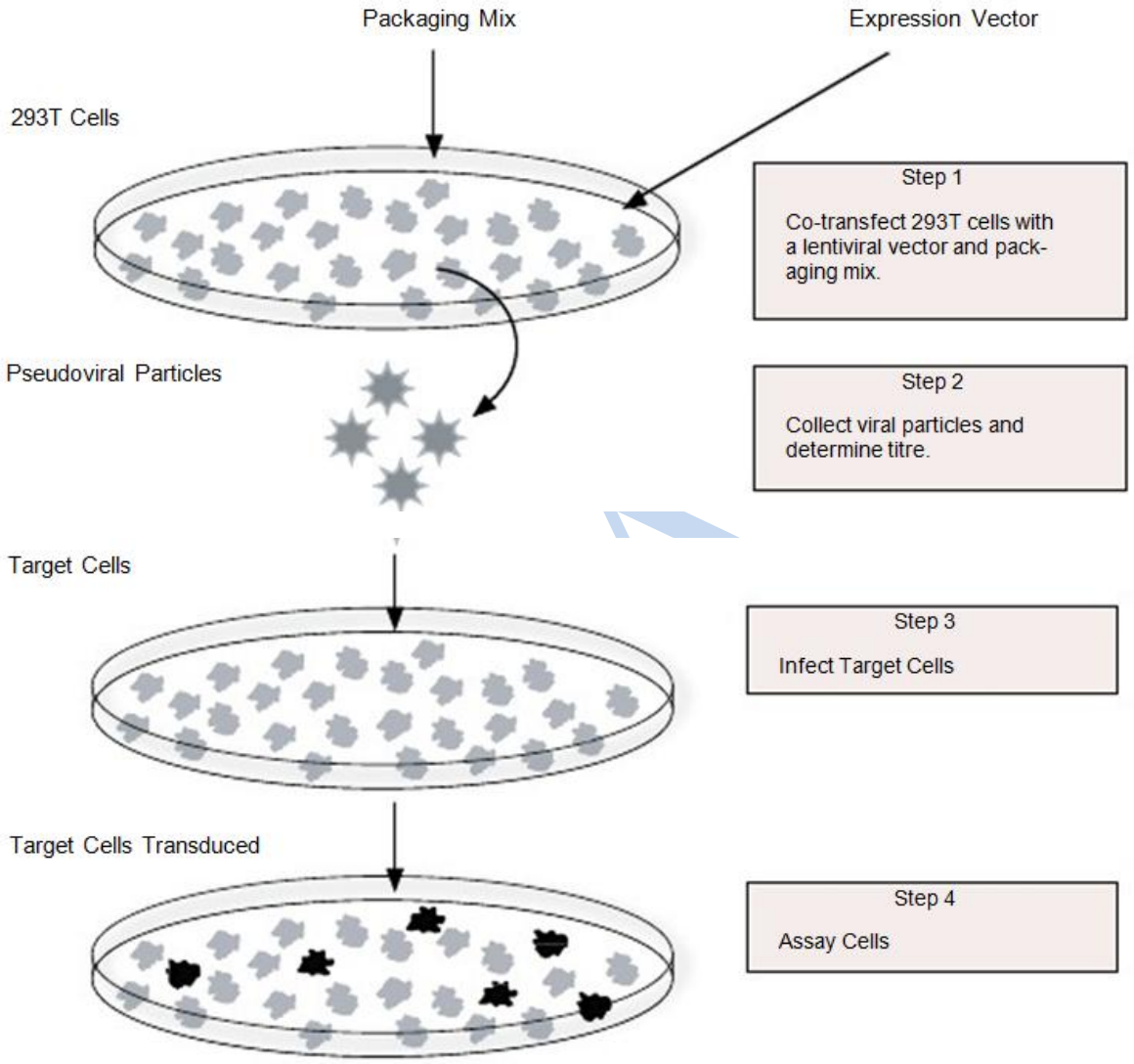
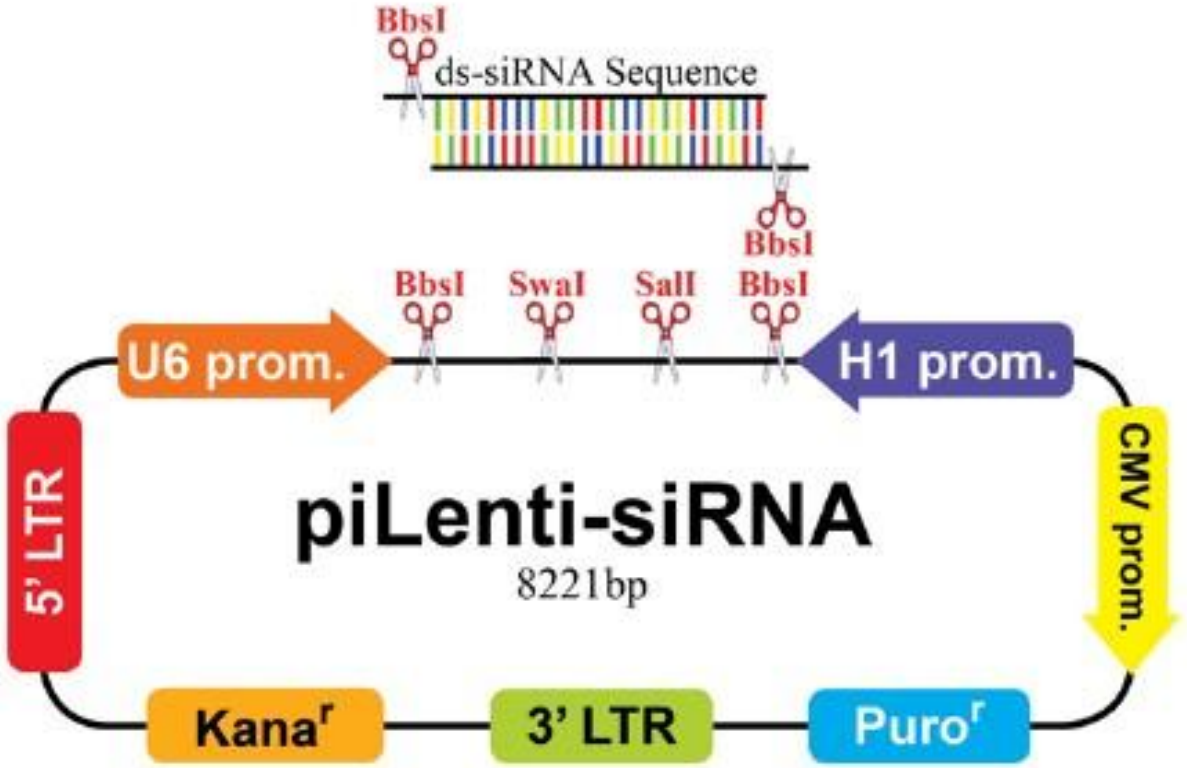


Figure 1: Procedure for Transient Production of Pseudoviral Particles and Transduction of Target Cells.


BbsI
ds-siRNA Sequence
BbsI
BbsI
SwaI
SalI
BbsI
U6 prom.
H1 prom.
5' LTR
CMV prom.
piLenti-siRNA
8221bp
Kana^r
3' LTR
Puro^r

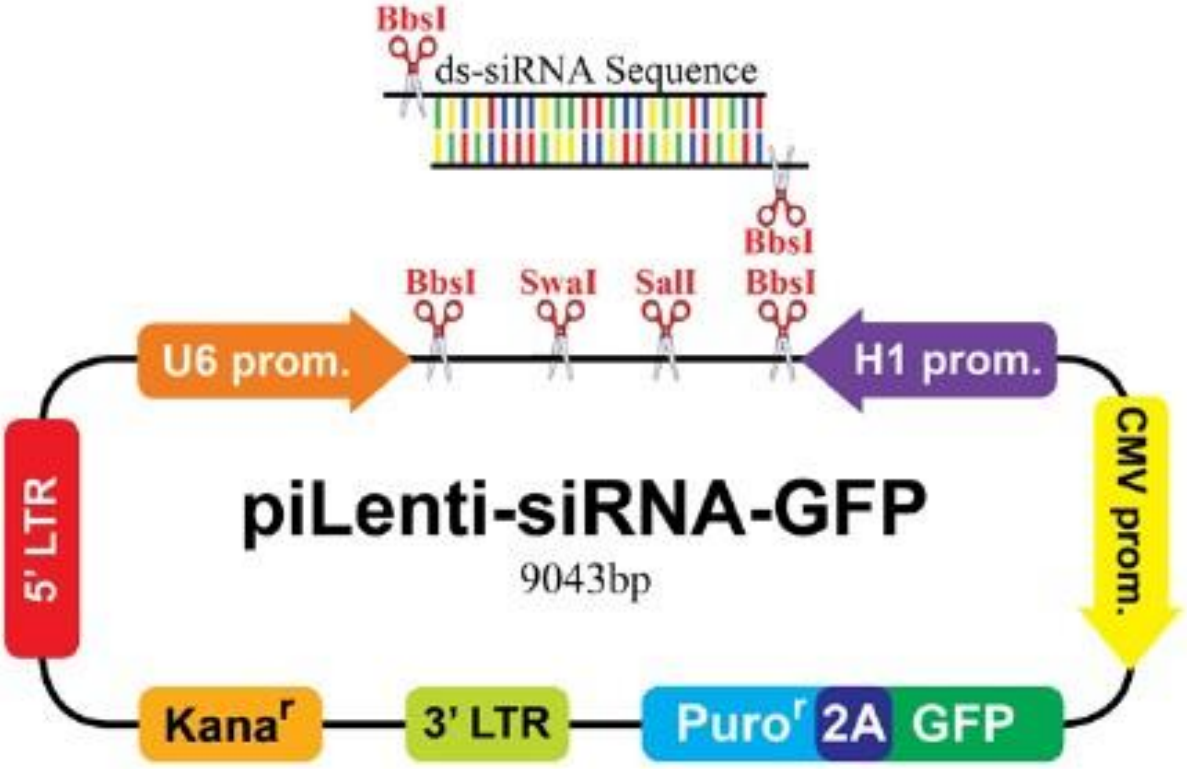

BbsI
ds-siRNA Sequence
BbsI
BbsI
SwaI
SalI
BbsI
U6 prom.
H1 prom.
5' LTR
CMV prom.
piLenti-siRNA-GFP
9043bp
Kana^r
3' LTR
Puro^r
2A
GFP

Figure 2: Map of iLenti-siRNA & iLenti-siRNA-GFP

Lenti-siRNA Expression System

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 biological processes. 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 the 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 functionality in mammalian cells, siRNA has been an indispensable tool in functional genomics and is predicted to be a novel form of future medicine (Grünweller and Hartmann 2005).

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

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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 iLenti™ vector has a unique, single BbsI restriction enzyme site in the multiple cloning site, which allows efficient, directional cloning of siRNA target sites.

6) iLenti™ also has a GFP reporter gene incorporated under the CMV promoter, which allows simultaneous tracking of expressed siRNAs in vivo.

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Lenti-siRNA Expression System

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 siRNA Lentiviruses Work

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.

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VSV Glycoprotein Envelope

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

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

Packaging Limits

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

Materials

Scientists at abm have successfully developed a comprehensive product line for siRNA expression, and reagents for packaging viral particles. In addition, ready-to-use lentiviral particles are also available for immediate transduction of any target cells as in shown in Table I.

Table I. Lenti-siRNA Vector and Kits

Component	Cat. #	Quantity	Kit Cat. No. LV300	LV301	LV310	LV311	LV098	LV099
iLenti-siRNA Vector	LV014	10ug	✓		✓			
iLenti-siRNA-GFP Vector	LV016	10ug		✓		✓		
Packaging MixLV003		100ul	✓	✓	✓	✓	✓	
Lentifectin™	G074	1.0ml	✓	✓			✓	
293T Cells	LV010	1x10^6	✓	✓				✓
Lenti-GFP Vector	LV011-a	10ug	✓	✓				✓
Sequencing Primer	LV012	100ul	✓	✓	✓	✓		

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) (Cat. No. TM999-500) Note: does not need to 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 sulphate (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)
- Puromycin (Cat. No. C021)
- Polybrene (Hexadimethrine Bromide; Cat. No. G062)
- 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 and avoid repeated freeze-thaw cycles.

Protocol

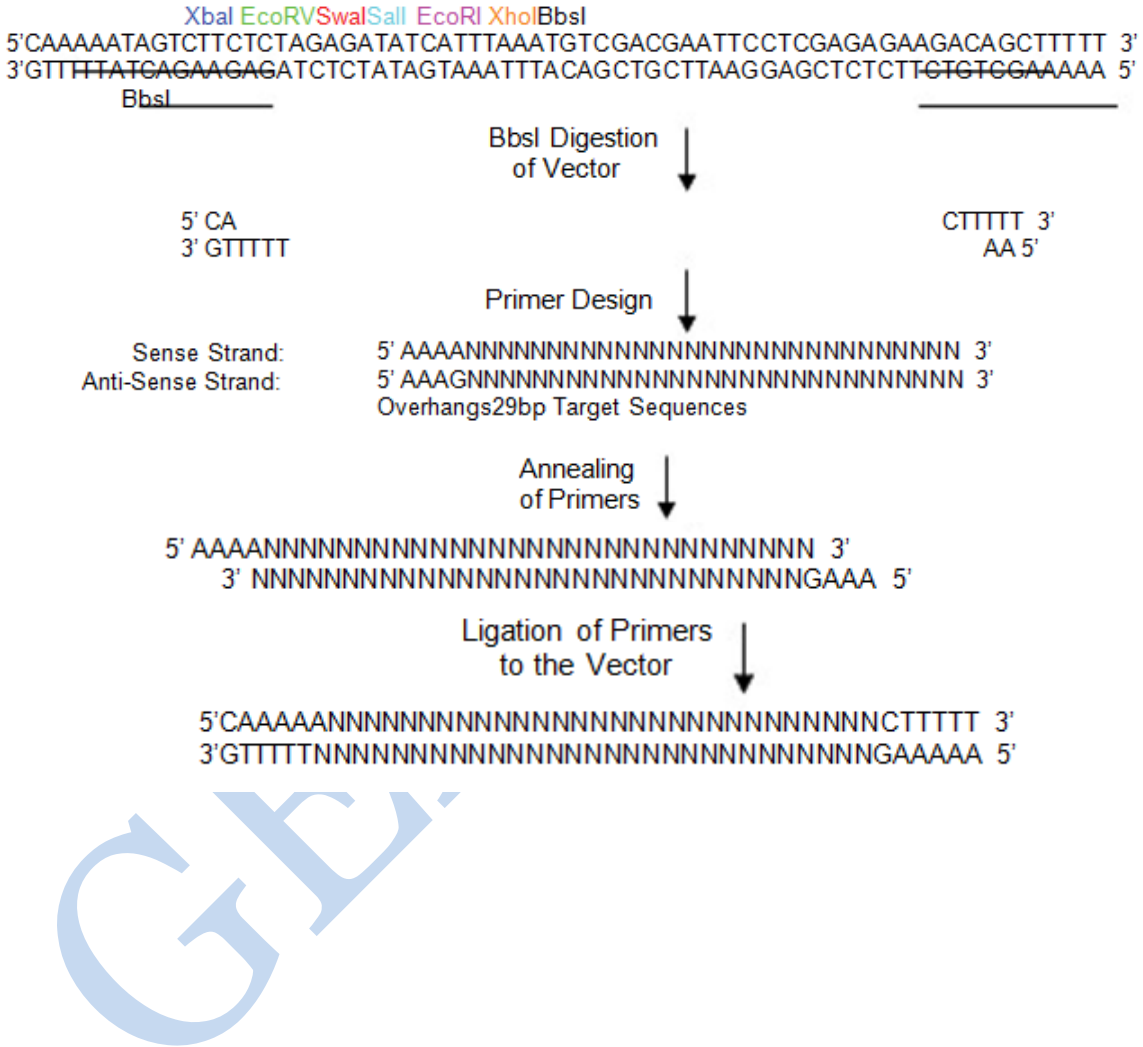
NOTE: The following protocol is broken into sections for convenience. However, time should be taken to read through the full procedure before attempting.

A. Designing siRNA Target Sequences

Since the initial demonstration of the functionality of siRNA in mammalian cells, great efforts have been made to develop a set of criteria for designing effective target sites for specific gene knockdown. Many free software programs based on bioinformatic analysis and experimental data are available for quick selection of siRNA target sites for any given 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. Therefore, it is highly recommended to design at least four siRNA oligos for any target gene. For reference, you can visit the following links for help with siRNA target selection:

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

Once an siRNA target sequence is selected, four specific additional nucleotides, as seen below, need to be added to the ends of the designed oligos for subsequent cloning of siRNA constructs into BbsI linearized iLenti vector. We recommend synthesizing PAGE-purified target oligos, although oligos without PAGE purification may also work for subsequent ligations. The designed oligos do not need to be phosphorylated for subcloning.



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Protocol

B. 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 II. Thermocycler Conditions

Process	Temperature	Time
Denaturation	95°C	30 seconds
Annealing	72°C	2 minutes
Annealing	37°C	2 minutes
Annealing	25°C	2 minutes
Storage	0°C (On Ice)	Variable

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

4. Set up the following ligation reaction:

Table III. Ligation of siRNA Target into the iLenti Vector

Reagent	Volume
BbsI Linearized Vector	4ul
Annealed Oligonucleotides	3ul
5x DNA Ligase Buffer	2ul
T4 DNA Ligase	1ul
Total Volume	10ul

Mix gently by pipetting 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 lentiviral titre 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.

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 EcoRI digestion of mini-prepared DNA to screen for recombinant colony. Note: The parental iLenti vector has two EcoRI sites, one of which is located between the two BbsI sites. Digestion of parental iLenti-siRNA-GFP plasmid would thus produce two fragments of 1.6kb and 7.4kb in size (the iLenti-siRNA plasmid resulting in fragments of 0.8kb and 7.4kb). Ligation of siRNA target oligos into the iLenti vector will result in an iLenti-siRNA-GFP vector with a single EcoRI site, leading to a linearized vector of 9.0kb (8.2kb for iLenti-siRNA) after EcoRI digestion . The identified recombinant clones can be sequence-confirmed using the primers that are included in the kit for target siRNA sequence accuracy. Alternatively, if your insert happens to contain an EcoRI site the same principle can be applied with a SalI digestion, with parental having bands of 1.8kb and 7.3kb and iLenti -siRNA-GFP vector with insert being linearized 9.0kb.

4. Perform a Maxi DNA preparation of recombinant iLenti clone for either target gene knockdown by direct transfection or for lentivirus production.

D. Lentiviral Particle Production

Currently, the most efficient and widely used protocol for producing high-titre 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 separate 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).

E. 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 titre 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.

F. Transfection Procedure

High titre 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 titre 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.

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 rule, one 15cm culture dish at 95% confluence can be subcultured into five 10cm dishes; while one 10cm dish at 95% confluence can be subcultured to two 10cm dishes.

2. On the day of transfection (Day 2), set up the transfection mix:

a. In a sterile 15ml culture tube, dilute 15µg 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 minutes of 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 gently 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.

g. Add 0.65ml serum to each transfected culture dish and return the dishes to the 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 VSV 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 48-72hrs 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 titre your lentiviral stock (page 10). Note: If you plan to use your lentiviral construct for in vivo applications, we recommend filtering your viral supernatant through a sterile, 0.45µm 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 titre virus is required.

G. Concentrating Virus

There are many 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 (Ultra-Pure Cat. No. LV998), and size exclusion chromatography (Speedy Lentivirus Purification Cat. No. LV999). However, we would strongly recommend using abm's Ultra-Pure Lentivirus Purification Kit (Cat. No. LV998) for iLenti-siRNA vector concentration and purification based on our in-house testing data.

H. Long Term Storage

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

I. Viral Titre Assays

It is useful to titre the viral supernatant before proceeding with the 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 viral constructs per target cell.

The commonly used protocol for measuring relative viral titres 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 pseudo-viral 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 titre, transduce a target cell line, like MDA-MB-468, in the presence of Polybrene (2µg/ml) for 12-16 hrs, and then count the number of cells expressing GFP either by fluorescence microscopy or 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 0.6-1x10^5 cells per well. Add 1ml 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 needed for FACS analysis. In this case, the amount of cells should be adjusted based on the growth area of the dish.

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 last well (dilution factor of 5). For controls, add 0.5ml of DMEM medium with Polybrene (from Step 2). Incubate 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 DMEM for an additional 24-48 hours.

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 $1.5 \times 10_5$ (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 titre of the virus in the supernatant. Alternatively, the viral titre can be estimated by real time PCR using ABM's Lentiviral qPCR Titre Kit (Cat. No. LV500) or p24-based ELISA titre kit (Cat. No. LV501).

J. Transduction Procedure

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.

- 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 express the lentivector construct for experimental assays.

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

1. Plate target cells in a 24-well plate 24 hours prior to viral infection at a density of $0.5\times10_5$ 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 media with Polybrene at a concentration of 2µg/ml. Remove media from the wells and replace with 0.5ml of the 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 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 (puromycin) at a minimum concentration as determined by a killing curve.

Problem	Possible Cause	Solution
No Viral Particles	Lenti-III-GFP DNA modified (e.g. acetylation or methylation).	Re-transform plasmid into an authetic DH5a.
Low Viral Titre	Low transfection efficiency: -poor quality DNA. -low 293T viability. -transfection media containing antibiotics and serum.	-Purify DNA with an endotox-in-free Maxi colomn. -Use 293T cells under passage 16. -Reduce transfection antibiotic or serum.
	Low transfection efficiency.	Optimize calcium transfection procedure.
	Insufficient DNA used for transfection.	Use 30-50ug of expression vector and 30-50ug of packaging mix.
	293T cell density too low.	Optimal cell density is 90-95%.
	Viral supernatant harvested too early.	Optimal viral titres can be collected 48-72 hours post-transfection.
	Viral supernatant subjected to multiple freeze-thaw cycles.	Each freeze/thaw cycle can lose 25% of the titre. Make aliquots for long-term storage.
	Polybrene not used during transduction.	Transduce cells in the presence of polybrene.
No Transgene Expression	Promoter silencing.	Lentiviral vector may integrate into a chromosomal region that silences the promoter. Screen multiple antibiotic-resistant clones and select the one with the highest expresssion levels.
	MOI too low.	Use maximum MOI for cell transduction. This is very critical for Tet-expression vectors.
	Viral stocks stored incorrectly.	Aliquot and store at -80°C. Avoid freeze/thaw cycles.
	Target cells not transducible with lentiviral vectors.	Transduce target cells in the presence of polybrene.
	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 3-4 days post-transduction and induction to allow the accumulation of expressed protein.
Cytoxic Effects of Target Cells	Large volume of viral supernatant used for transduction.	Dilute viral supernatant 1:2 to 1:3 during 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.

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