GenScript Vector-based siRNA Protocol (Lenti-viral)

Technical Manual No. 0175 **Version 04032007**

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I. Introduction:

RNAi (RNA interference) is a phenomenon that small double-stranded RNA (referred as small interfering RNA or siRNA) can knock down the expression of its corresponding gene. RNAi has been observed in plants, *C.elegans* and Drosophila long time ago. It was until recently that RNAi was discovered to work in mammalian system [1].

Small interfering RNA (siRNA) is 19-22 nt double-stranded RNA. It works by cleaving and destroying its cognate RNA. siRNA first assembles into RNA-induced silencing complexes (RISCs), where it then activates the complex by unwinding its RNA strands. The unwound RNA strands subsequently guide the complex to the complementary RNA molecules, where the complex cleaves and destroys the cognate RNA, which results in RNAi phenomenon. RNAi has evolved into a powerful tool to study gene functions.

II. Vector-based siRNA

Using DNA vector-based siRNA technology, a small DNA insert (about 70 bp) encoding a short hairpin RNA targeting the gene of interest is cloned into a commercially available vector. The insert-containing vector can be transfected into the cell, and it expresses the short hairpin RNA. The hairpin RNA is rapidly processed by the cellular machinery into 19-22 nt double stranded RNA (siRNA).

Key features of vector-based siRNA:

1. **More stable and easier to handle**: Vector-based siRNA is delivered as plasmids, which is more stable and easier to handle than synthetic siRNA.
2. **Stable cell line can be established**: Vector based siRNA allows you to obtain a stable cell line, and observe long-term effects of RNAi [2-5].
3. **Viral siRNA can be prepared** [6,7]: Viral siRNA can be used to infect primary cell lines and used for gene therapy purpose.
4. **Inducible system can be established**: Vector based siRNA allows you to establish an inducible system by using a vector with an inducible promoter.
5. **A knock-out mouse line can be established using transgenic siRNA method** [8].
6. **Unlimited supply**: once a DNA construct is made, you will have unlimited supply of siRNA.

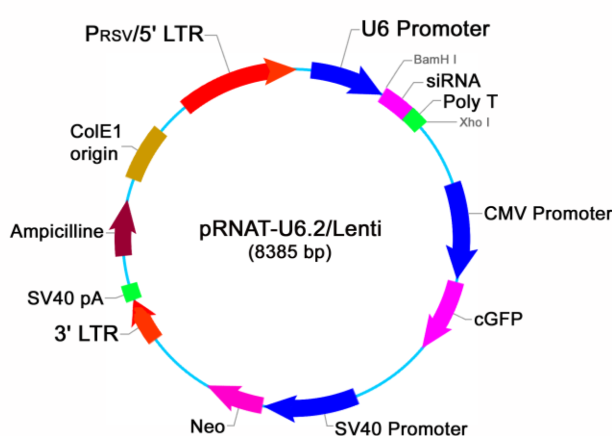
It is recommended that at least 3 vector-based siRNA should be prepared for each gene to find the most potent and most specific siRNA. Here are the reasons:

1. **Not all siRNA target sequences are equally potent:** Because of secondary structure and other factors, some target sequences are more potent than others. It is better to test at least three vector-siRNA constructs to find the most potent one.
2. **Not all siRNA silencing effects are gene-specific:** It has been reported that some siRNA silencing effects are not gene-specific because of various reasons. It is better to validate your experiments results using three vector-based siRNA constructs.
3. **Results from synthetic siRNA or siRNA cassette cannot be completely transferred to vector-based siRNA construct**: Vector-based siRNA is different from synthetic siRNA oligos or siRNA cassette. Although the results from synthetic siRNA oligos or siRNA cassette can suggest the most potent siRNA targets, the results cannot be completely duplicated in vector-based siRNA for unknown reasons.
4. **The experiment is still the gold test stone:** Although we are proud of our vector-based siRNA design program, the best design is still not as good as what the experiments can tell you.

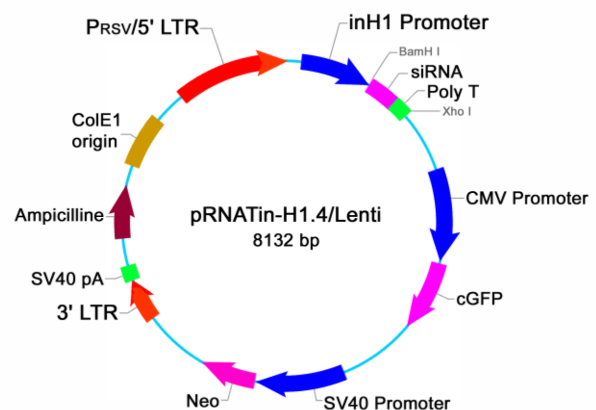
III. Lenti siRNA Vector

Lentiviruses, a subclass of retrovirus, can infect both dividing and nondividing cells due to their ability to get through the intact membrane of the nucleus of the target cells. After infection, the viral genome will be integrated into the target genome and viral genes will be expressed. Several recombinant lentiviruses have been developed and used as efficient vectors for gene delivery and expression. Like other retroviral vectors, lentiviral vectors are capable of producing high virus titers and inducing high-level gene expression in target cells. One special feature of lentiviral vectors is that they can be used for nondividing or terminally differentiated cells such as neurons, macrophages, hematopoietic stem cells, retinal photoreceptors, muscle cells and liver cell, etc.

Human immunodeficiency virus (HIV)-derived lentiviral vector system is the most used lentiviral vector for *in vitro* or *in vivo* delivery of a target gene or siRNA to dividing and non-dividing mammalian cells using a replication-incompetent lentivirus. GenScript developed two Lenti siRNA Vectors that are compatible with Invitrogen ViraPower™ Lentiviral_Expression System (https://catalog.invitrogen.com/index.cfm?fuseaction=viewCatalog.viewProductDetails&productDescription=900) for high level and long-term siRNA expression. These two vectors are: pRNAT-U6.2/Lenti (SD1259) and pRNATin-H1.4/Lenti (SD1260) with the circle maps shown below.



SD1259: pRNAT-U6.2/Lenti



SD1260: pRNATin-H1.4/Lenti

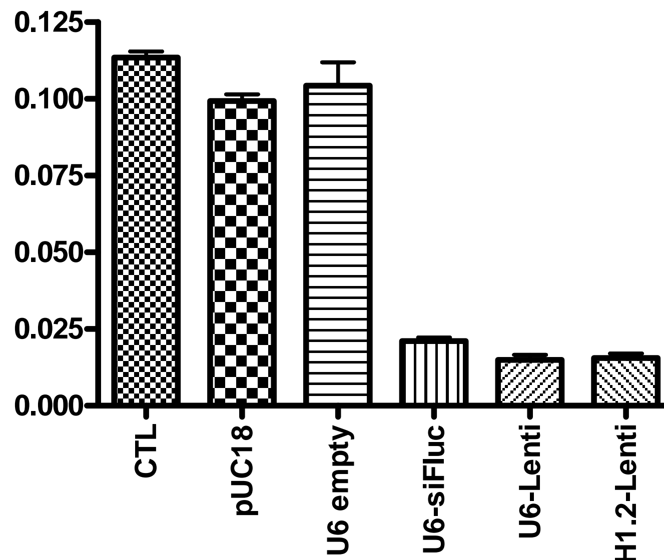
These vectors contain elements that allow packaging of the siRNA construct into lentiviral particles. The lentiviral particles are then used to infect both dividing and non-dividing mammalian cells to express siRNA in the host cells. inH1.4 and U6 promoters are used to drive the siRNA expression. The inH1.4 promoter is an engineered inducible H1 promoter containing a tetracycline operator (TetO1). The tetracycline operator itself has no effect on expression, in the absence of the tetracycline repressor (TetR), inH1.4 just behaves like H1. When the tetracycline repressor (TetR) is present, it effectively binds the TetO1 and blocks transcription. In the presence of tetracycline or doxycycline, the inducer binds TetR and causes the TetR protein to release the TetO1 site, and derepresses the transcription from H1 promoter. These vectors are designed for mammalian transfection. These vectors also have the following features:

1. They carry a neomycin resistance gene that can be used for establishing stable cell line.
2. They carry a GFP (coral GFP) marker under CMV promoter to track the transfection efficiency and viral infection.
3. siRNA effect can be tested first for siRNA optimization before you produce lentiviral particles and infect target cells. After cloning the siRNA construct into the vectors, you can test the siRNA effect by transfection of the siRNA-containing vectors into the mammalian cells. U6 or H1 will drive the siRNA expression.

Important points for consideration before using Inducible vector:

1. H1.4 promoter only works as an inducible promoter in the cells containing tetracycline repressor (TetR). In the cells without TetR, H1.4 behaves as a regular H1 promoter.
2. For inducible experiments, the serum is very critical. As FBS from most vendors contains Tetracycline itself, it is very critical to use Tetracycline free FBS. We recommend to use Clontech Tet approved FBS (Clontech, Cat#631101).
3. Either tetracycline or doxcycline can be used as an inducer reagent. The concentration for tetracycline recommended is 1 μg/ml to 5 μg/ml, and the concentration for doxcycline recommended is 1 μg/ml.
4. Tetracycline or doxycycline should be added to the medium right after transfection. The induction time recommended is at least 48 hours.

Effect of siFluc on Firefly Luciferase



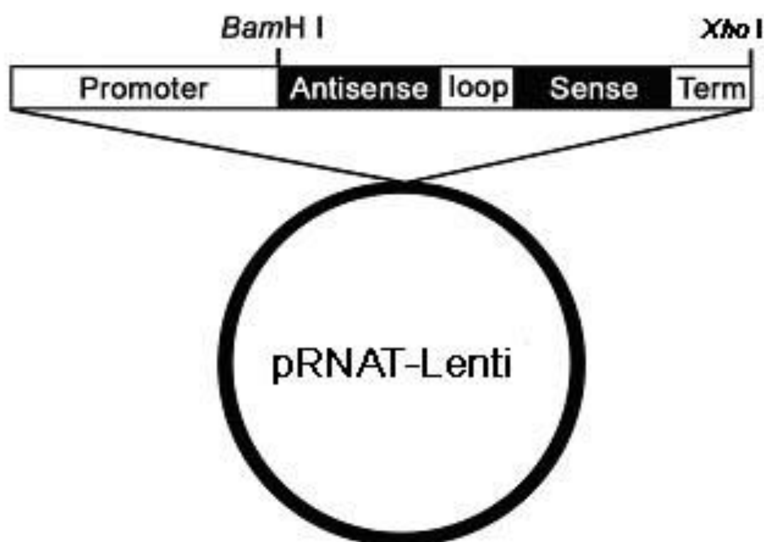
Effect of siFluc on Firefly Luciferase. All the activities are normalized by Renilla Luciferase activity. The activities are from:

- Control: HEK293 cells transfected with pGL3-control (0.16 µg) and pRL-TK (0.16 µg).
- pUC18: HEK293 cells transfected with pGL3-control (0.16 µg), pRL-TK (0.16 µg), and 1.6 µg of pUC18.
- U6-empty: HEK293 cells transfected with pGL3-control (0.16 µg), pRL-TK (0.16 µg), and 1.6 µg of pRNA-U6.1/Neo vector (Cat. SD1201).
- U6-siFLuc: HEK293 cells transfected with pGL3-control (0.16 µg), pRL-TK (0.16 µg), and 1.6 µg of pRNA-U6-Neo-siFLuc (Cat. SD1501).
- U6-Lenti: HEK293 cells transfected with pGL3-control (0.16 µg), pRL-TK (0.16 µg), and 1.6 µg of pRNAT-U6/Lenti-siFluc.
- H1.2-Lenti: HEK293 cells transfected with pGL3-control (0.16 µg), pRL-TK (0.16 µg), and 1.6 µg of pRNATin-H1.2/Lenti-siFluc.

IV. Product Description

Cat. No.: SD1259 and SD1260.

Description: A small DNA insert (about 70-80 bp) encoding a short hairpin RNA targeting the gene of interest can be cloned into these vectors between BamH I and Xho I sites.



- Promoter: Options include human U6 or inducible H1 promoter.
- Vector resistance: neomycin.
- Term: Poly(T) termination signal.
- coral GFP (cGFP) marker under CMV promoter control to track the transfection efficiency.
- *Bam*H I and *Xho* I is used to clone the DNA insert into GenScript pRNA vectors.

Quantity: 10 µg.
Quality Control: The insert can be sequencing verified, and trace data is provided together with the plasmid.
Storage: -20 °C after receiving.

GenScript siRNA Expression Vectors:

- Lenti siRNA Expression Vector

Cat. No.	SD1259	SD1260
Promoter	Human U6	inducible H1
Resistance	Neomycin	Neomycin
Marker	cGFP	cGFP

V. Cloning siRNA insert into GenScript pRNA vectors.

1. siRNA Design: GenScript has developed a proprietary algorithm for designing a siRNA target and building the insert. To find the target sequence and build siRNA insert, please use GenScript's siRNA design center siRNA Target Finder and siRNA Construct Builder (http://www.genscript.com/rnai.html).
2. Custom vector-based siRNA constructs: GenScript provides custom vector-based siRNA at a very competitive price. Alternatively, you can use the following protocol to make the vector-based siRNA constructs yourself.
3. Order two oligos with cohesive *Bam*H I and *Xho* I sites. The oligos must be PAGE purified oligos. Dissolve the oligos in water to 1 µg/µl concentration.
4. Anneal the two oligos. Prepare a 20 µl annealing reaction in the following way:
   1 µl top-strand oligo
   1 µl bottom-strand oligo
   1 µl 20 x SSC (Sigma, Cat. S6639)
   17 µl water
5. Heat the mixture to 95 ^{o}C for 10 min. Take it out and put it at room temperature for one hour. Dilute the mixture to a final concentration of 40 ng/µl.
6. Cut the vector with BamH I and Xho I. Run 1% agarose gel and purify the vector.
7. Ligate the vector with the insert using T4 ligase (the molar ratio of insert to vector is 3:1).
8. Transform the ligation mixture into One Shot Stbl3 competent cells (Invitrogen, Cat. C7373-03). Plate it on LB-amp plates.
9. Choose at least 15 clones and grow them. Prepare MiniPreps from culture using GenScript MiniPrep kit.
10. Cut the plasmids with *Bam*H I and *Xho* I. Run 3% gel to check whether the plasmids have the insert and select positive clones.
11. Sequence the positive clones to verify the sequence of the insert.

VI. Producing Lentiviral Particles and Infecting Mammalian Cells.

To deliver the siRNA expression system to dividing and non-dividing mammalian cells using GenScript Lentiviral vectors, you also need to order the following reagents from Invitrogen (https://catalog.invitrogen.com):
ViraPower™ Packaging Mix, Catalog No.: K4975-00.
293FT Cell Line: R700-07.
Described below are the general steps to express your siRNA in the host cell line. For more details, please folllow Invitrogen ViraPower™ Lentiviral Expression System Manual to produce lentiviral particles and infect host mammalian cells.

1. Clone your siRNA construct into **GenScript vectors** between BamH I and Xho I sites to generate Lenti expression vector. You can use either U6 promoter or inducible H1 promoter for your siRNA expression.
2. Cotransfect the 293FT packaging cell line with the Lenti expression vector and the ViraPower™ Packaging Mix to produce Lentiviral particles.
3. Collect supernatant containing Lentiviral particles and determine the titer.
4. Transduce your host mammalian cells by adding the viral supernatant. If necessary, use neomycin resistant gene to select for stably transduced cells.
5. Use appropriate methods to analyze siRNA silencing effect.

VII. Establishing a Stable Cell Line Expressing cGFP and siRNA.

A stable cell line expressing cGFP and siRNA can be established by using ViraPower™ Lentiviral Expression System (Invitrogen) together with GenScript pRNAT-U6.1/Lenti-siRNA construct.

1. Producing Lentivirus:
    a. One day before transfection, plate 293FT cells (Invitrogen) at 5 x 10^6 cells per 10 cm plate.
    b. Do transfection with Lipofectamine 2000 reagent (invitrongen). Replace the medium with 5ml of Opti-MEM I without serum. Prepare DNA-Lipofectamine 2000 complex with 9 µg of packaging mix and 3µg of pRNAT-U6.1/Lenti-siRNA in 3ml of Opti-MEM I and add the complex to the plate.
    c. On the next day, replace the medium containing the complex with complete culture medium containing serum and antibiotics.
    d. Harvest the medium containing virus particles after 72 hour posttransfection. Spin down the medium to remove cell debris and save the supernatant.

2. Determining the Lentiviral titer:
    a. The day before transduction, plate cells at 30-50% confluence in 12-well plate and incubate cells overnight at 37°C.
    b. On the day of transduction, prepare 10-fold serial dilutions of viral stocks in complete culture medium ranging 1X to 10^6X.
    c. Remove culture media from the cells and add 1ml of the dilutions directly to the cells.
    d. If desired, add Polybrene® to each well to a final concentration of 6µg/ml, and incubate at 37°C overnight.
    e. The following day, replace the media containing virus with fresh complete culture media.
    f. After 3-4 days of transduction, observe cGFP expression under fluorescent microscope and count cGFP positive cells and estimate the titer.

3. Lentivirus transduction and selection:
    a. One day before transduction, plate 293H cells at 1 x 10^5 cells per well in a 12-well plate.
    b. The next day, mix 0.5 ml (or proper volume) of the lentiviral stock with 0.5 ml of complete medium and add to each well.
    c. Add polybrene to each well to a final concentration of 6µg/ml.
    d. The next day, replace the medium containing letiviruses with complete culture medium.
    e. After 3 days of transduction, replace the medium with complete medium containing 500µg/ml of G418 for selection.
    f. Replace medium containing G418 every 3-4days for 3 weeks.
    g. Identify green fluorescence positive colonies under a fluorescence microscope and transfer each colony to a 35 mm culture dish.

VIII. References

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