GenScript Vector-based siRNA Protocol

Technical Manual No. 0169 **Version 04032007**

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:

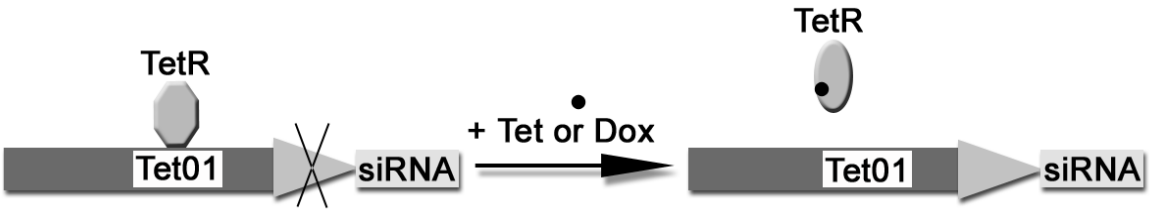
1. **More stable and easier to handle**: Vector-based siRNA is delivered as plasmids, which is more stable and easier to handle than synthetic siRNA.
2. **Stable cell line can be established**: Vector based siRNA allows you to obtain a stable cell line, and observe long-term effects of RNAi [2-5].
3. **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. Inducible siRNA Vector

pRNATin-H1.2 series vector is a siRNA inducible expression vector. The H1.2 promoter is an engineered inducible H1 promoter containing a tetracycline operator (TetO1). The tetracycline operator itself has no effect on expression. 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. pRNATin-H1.2 series vector is designed for mammalian transfection. It carries a neomycin resistance gene which can be used for establishing stable cell line and a GFP (coral GFP) marker under CMV promoter control to track the transfection efficiency.



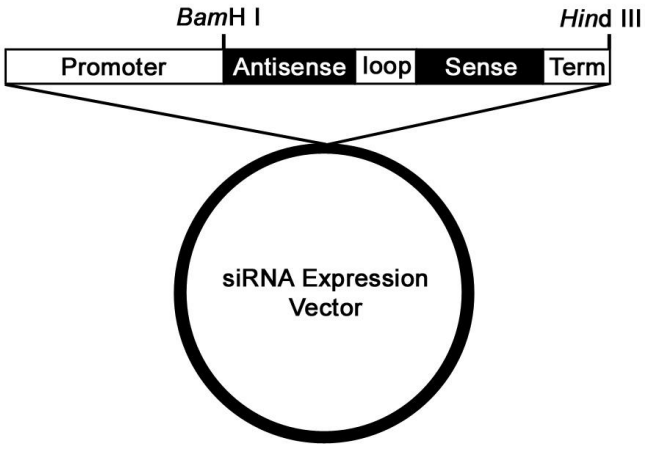
Important points for consideration before using Inducible vector:

1. H1.2 promoter only works as an inducible promoter in the cells containing tetracycline repressor (TetR). In the cells without TetR, H1.2 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.

IV. Product Description

Service Cat. No.: SD1100.

Description: A small DNA insert (about 70-80 bp) encoding a short hairpin RNA targeting the gene of interest is cloned into a siRNA expression vector. The insert-containing vector can be transfected into the cell, and expresses the short hairpin RNA.



- Promoter: Options include human U6 or H1 promoter.
- Vector resistance: Options include neomycin, hygromycin, and zeocin.
- Term: Poly(T) termination signal.
- *Bam*H I and *Hin*d III is used to clone the DNA insert into GenScript pRNA vectors.

Quantity: 10 μg (MiniPrep) or 100 μg (MaxiPrep).

Quality Control: The insert is sequencing verified, and trace data is provided together with the plasmid.

Storage: -20 °C after receiving.

Positive Controls:

Target Gene	**Firefly Luciferase**					**Renilla Luciferase**			
Cat. No.	SD1501	SD1502	SD1503	SD1504	SD1523	SD1601	SD1602	SD1603	SD1604

Negative Controls:

Cat. No.	SD1801	SD1802

GenScript siRNA Expression Vectors:

- Plasmidic Vector

Cat. No.	**SD1201**	**SD1202**	**SD1207**	**SD1203**	**SD1204**	**SD1208**
Promoter	Human U6	Human U6	Human U6	Human H1	Human H1	Human H1
Resistance	Neomycin	Hygromycin	Zeocin	Neomycin	Hygromycin	Zeocin

Cat. No.	**SD1211**	**SD1212**		**SD1213**	**SD1214**	
Promoter	Human U6	Human U6		Human H1	Human H1	
Resistance	Neomycin	Hygromycin		Neomycin	Hygromycin	
Marker	cGFP	cGFP		cGFP	cGFP	

- Inducible siRNA Vectors:

Cat. No.	SD1223	SD1224	SD1220
Promoter	H1.2	H1.2	H1.2
Marker	cGFP	cGFP	-

- Adenoviral siRNA Shuttle Vectors:

Cat. No.	SD1205	SD1206	SD1216
Promoter	Human U6	Human H1	Human H1
Marker	-	-	cGFP

- Custom Adenoviral siRNA Construct
- Custom Retroviral siRNA Construct

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 *Hin*d III 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 Hind III. 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 competent DH5a cells (Invitrogen, Cat. 18258-012). 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 *Hin*d III. 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. Transfecting mammalian cells.

Generate large amount of DNA:

Vector-based siRNA constructs are delivered as a lyophilized plasmid, and it is can be stored forever at –20 ^{o}C. You can handle the constructs in the same as you handle DNA plasmid. Before use, add 20 µl water to dissolve it. Please vortex diligently and make sure the DNA is dissolved. If needed, incubate at 50 ^{o}C for 10 min. You can use it directly if you only need limited DNA. For large amounts of DNA, please take 2 µl of the solution and transform competent DH5a or TOP10 cells to make a MaxiPrep using Qiagen MaxiPrep kit.

General considerations before transfection:

A variety of protocols such as lipofection and electroporation have been used successfully to transfect vector-based siRNA constructs into mammalian cells. The transfection procedures are identical to those used for DNA plasmid transfection. The choice of transfection procedures will depend on the mammalian cell line used. In general, we recommend using Lipofectamine™ 2000 or LipofectaminTM Plus from Invitrogen. The information and protocol for Lipofectamine™ 2000 can be found using this link: http://www.lifetech.com/content.cfm?pageid=93. The information and protocol for Lipofectamine™ Plus can be found using this link: http://www.invitrogen.com/content/sfs/manuals/18324.pdf.

The following are important issues before performing the transfection:

a. **Cell density:** The recommended cell density for transfection using Lipofectamine™ 2000 is 90-95%. If the cell density is less than 90%, the Lipofectamine™ 2000 may have some toxicity on the cells.
b. **Vector-based siRNA construct amount:** For 12-well plates, it is recommended to use 1.6 µg as a starting point. To screen most potent siRNA target, it is recommended to use less DNA. For other plate size, the DNA amount can be adjusted proportionally.
c. **Cell Proliferation:** Maintaining healthy cell culture is essential for cell transfection. It is essential to minimize decreased cell growth associated with nonspecific transfection effects.
d. **Positive control and negative control:** It is always a good idea to include a positive and a negative control in the experiment. Since there is a lot of uncertainty in siRNA experiments, it is recommended to use a positive control to optimize your system. GenScript offers both siFluc, siRluc and si-cGFP controls. These siRNA constructs can serve as either positive controls (in luciferase or GFP assay) or negative controls (in other assays).
e. **Time:** The optimal time after transfection for analyzing siRNA effects has to be determined empirically by testing a range of incubation time. The time can vary from 24 to 96 hrs depending on the cells used and the experimental targets tested.
f. **Transfection efficiency:** High transfection efficiency is essential for achieving siRNA effect using a transient transfection approach. GFP plasmid can be used as a transfection efficiency control.

A protocol based on Lipofectamine™ 2000 from Invitrogen for 12-well plates

1. Purchase Lipofectamine™ 2000 reagent (Cat#11668-027) from Invitrogen
2. Plate the cells the day before transfection so that they are 90-95% confluent on the day of transfection. At the time of plating and diluting transfection, avoid antibiotics - this helps cell growth and allows transfection without rinsing the cells. The cell density of 90-95% is very important. If the cell density is less than 90%, transfection may have toxicity on the cells.
3. Dilute vector-based siRNA plasmid with 100 µl of Opti-MEM (Cat# 31985062) from Invitrogen and mix gently.
4. Dilute Lipofectamine™ 2000 Reagent into 100 µl of Opti-MEM medium in a second tube; mix gently and incubate at room temperature for 5 min.
5. Combine diluted DNA (from Step 3) and diluted Lipofectamine™ 2000 Reagent (from step 4); mix and incubate at RT for 20 min.
6. Add 200 µl of DNA-Lipofectamine™ 2000 Reagent complexes to each well. Mix gently by rocking the plate back and forth.
7. Perform assays on the cells 24-48 h after the start of transfection. It is not necessary to remove the complexes or change the medium; however, growth medium may be replaced after 4-6 hours without loss of transfection activity.
8. Optimizing transfection: The suggested starting points are listed in Table 1. To obtain the highest transfection efficiency and low non-specific effects, optimize transfection conditions by varying DNA and Lipofectamine™ 2000 concentrations, and cell density. Make sure that cells are greater than 90% confluent and vary DNA (µg): Lipofectamine™ 2000 (µl) ratios from 1:0.5 to 1:5.

Table I. Suggested starting amounts of reagents for transfection in different culture vessels:

Culture vessel	Surface Area per Well (cm^2)	Relative Surface Area (vs. 24-well)	Volume of Plating Medium	DNA (µg) and Dilution Volume (µl)	Lipofectamine™ 2000 (µl)
96 well	0.3	0.2	100 µl	0.2 µg in 25 µl	0.5 µl in 25 µl
24 well	2	1	500 µl	0.8 µg in 50 µl	2.0 µl in 50 µl
12 well	4	2	1 ml	1.6 µg in 100 µl	4.0 µl in 100 µl
6 well	10	5	2 ml	4.0 µg in 250 µl	10 µl in 250 µl
35 mm	10	5	2 ml	4.0 µg in 250 µl	10 µl in 250 µl
60 mm	20	10	5 ml	8.0 µg in 0.5 ml	20 µl in 0.5 ml
100 mm	60	30	15 ml	24 µg in 1.5 ml	60 µl in 1.5 ml

A brief protocol for siFLuc construct transfection (Figure 1).

1. To use siFLuc constructs (Cat. SD1501, SD1502, SD1503, or SD1504), pGL-3 control vector (Promega, Cat. #E1741) and pRL-TK vector (Promega, Cat. #E2241) need to be purchased from Promega.
2. To observe the effect of siFLuc, three sets of transfections are needed: a. pGL-control and pRL-TK vector alone; b. pGL-3 control, pRL-TK, and siFLuc; c. pGL-3 control, pRL-TK, and an empty pRNA vector.
3. For cell transfection, 12-well plates can be used. For 293-F Cells SFM adapted (Cat.# 11625-019) from Invitrogen, 200,000 cell can be seeded the day before transfection.
4. The amount of siFLuc plasmid used for transfection should be 10-30 fold higher than that of pGL-3 control plasmid. For 293-F, 0.16 µg of pGL-3 control and 0.16 µg pRL-TK vector were used, 1.6 µg of siLuc construct or empty vector are used for each well.
5. The plasmid can be transfected into mammalian cells using Lipofectamine™-2000 following the protocol.
6. The Firefly and Renilla luciferase activities can be measured using Dual Luciferase assay kit from Promega (Cat. #E1910) after 24 hrs of transfection.
7. The activities of Firefly luciferase need to be normalized using Renilla luciferase activity.
8. Typical inhibition of Firefly luciferase by siFLuc construct is about 80% (See Figure 1).

A brief protocol for inducible siFLuc construct transfection (Figure 2).

1. To use inducible siFLuc constructs (Cat. SD1523), pGL-3 control vector (Promega, Cat. #E1741) and pRL-TK vector (Promega, Cat. #E2241) need to be purchased from Promega.
2. To observe the inducible effect of siFLuc, four sets of transfections are needed: a. pGL-control and pRL-TK vector, and pUC18; b. pGL-3 control, pRL-TK, and pRNA-U6.1/Neo; c. pGL-3 control, pRL-TK, and pRNA-U6.1/Neo /siFLuc; d. pGL-3 control, pRL-TK, and pRNAT-H1.2/Neo/siFluc.
3. For cell transfection, 12-well plates can be used. For T-Rex-293 cells (Cat.# R710-07) from Invitrogen, 200,000 cell can be seeded the day before transfection. The FBS should be Tet system approved FBS (Clontech, Cat#631101).
4. The amount of siFLuc plasmid used for transfection should be 10-30 fold higher than that of pGL-3 control plasmid. For T–Rex-293 Cells, 0.16 µg of pGL-3 control and 0.16 µg pRL-TK vector were used, 1.6 µg of siLuc construct or empty vector are used for each well.
5. The plasmid can be transfected into mammalian cells using Lipofectamine™ 2000 following the protocol. Tetracyline or doxycycline is added into the medium after the transfection.
6. The Firefly and Renilla luciferase activities can be measured using Dual Luciferase assay kit from Promega (Cat. #E1910) after 48 hrs of transfection and induction by tetracycline or doxcycline.
7. The activities of Firefly luciferase need to be normalized using Renilla luciferase activity.
8. Typical inhibition of Firefly luciferase by siFLuc construct is about 80% (See Figure 1).

VII. Selecting antibiotic-resistant transfected cells

There are two major benefits for selecting antibiotic-resistant transfected cells:

1. For cells which are very difficult to be transfected or have very low transfection efficiency, using antibiotic selection will kill the cells that were not transfected with the siRNA expression vector. This will be able to reduce the background when analyzing the knockdown effect of siRNA.
2. By using the antibiotic selection, a stable cell line can be established. The stable cell line can be maintained and assessed for reduction of target gene expression for a long-term period.

pRNA series vectors have three kinds of antibiotics resistant genes: neomycin, hygromycin, and zeomycin. Depending on your need, you can choose the appropriate resistant marker for establishing the stable cell line. The following are general procedures for selecting antibiotic-resistant cells.

1. Before doing antibiotic-resistant selection, perform a transient assay to check the effects of your siRNA constructs.
2. Following the transfection procedures as outlined in Section V to perform the cell transfection.
3. After 24 hours of transfection, lift the cells from plates using Trypsin-EDTA. Then add G-418, hygromycin, or zeocin to the medium for selection.
4. The concentration for G-418, hygromycin, or zeocin can be optimized from the range 50-1500 μg/ml. 100 μg/ml will be a good start point.
5. Examine the dishes for viable cells every 2 days. Identify the lowest G-418, hygromycin, or zeocin concentration that begins to give massive cell death for wild-type cells in approximately 7-9 days, and kills all wild-type cells within 2 weeks. Using this concentration to select cells containing pRNA plasmid after selection.
6. You can select a mixture of resistant cells or single colony depending on your need.
7. If the target gene is essential for cell survival, a stable cell line may not be obtained.

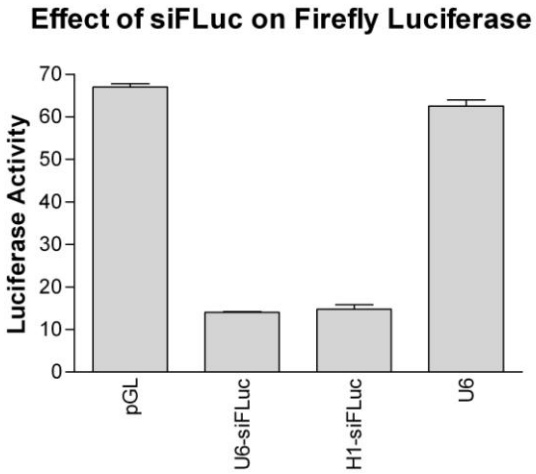


Figure 1. All the activities are normalized by Renilla Luciferase activity. The activities come from:

- pGL: HEK293 cells transfected with pGL3-control (0.16 μg) and pRL-TK (0.16 μg).
- U6-siLuc: HEK293 cells transfected with pGL3-control (0.16 μg), pRL-TK (0.16 μg), and 1.6 μg of pRNA-U6.1/Neo/siFLuc (Cat. SD1501).
- H1-siLuc: HEK293 cells transfected with pGL3-control (0.16 μg), pRL-TK (0.16 μg), and 1.6 μg of pRNA-H1.1/Neo/siFLuc (Cat. SD1502).

- U6: HEK293 cells transfected with pGL3-control (0.16 μg), pRL-TK (0.16 μg), and 1.6 μg of pRNA-U6.1/Neo empty vector (Cat. SD1201).

Fig. 2. Effects of Inducible siRNA on Firefly Luciferase

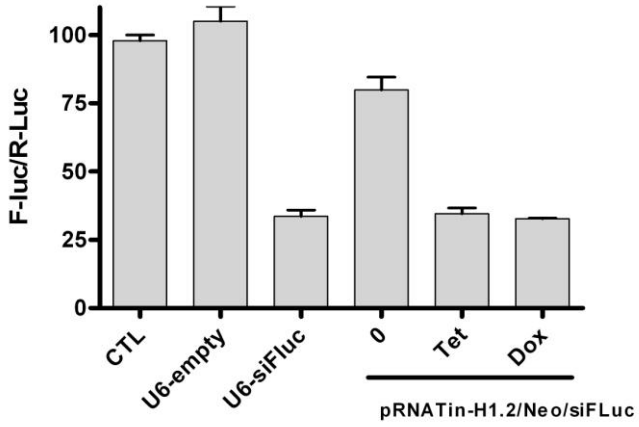


Figure 2. All the activities are normalized by Renilla Luciferase activity. The activities come from:

- CTL: T-Rex-293 cells transfected with pGL3-control (0.16 μg) and pRL-TK (0.16 μg), and 1.6 μg of pUC18.
- U6-empty: T-Rex-293 cells transfected with pGL3-control (0.16 μg), pRL-TK (0.16 μg), and 1.6 μg of pRNA-U6.1/Neo empty vector (Cat. SD1201).
- U6-siLuc: T-Rex-293 cells transfected with pGL3-control (0.16 μg), pRL-TK (0.16 μg), and 1.6 μg of pRNA-U6.1/Neo/siFLuc (Cat. SD1501).
- pRNATin-H1.2/Neo/siFluc: T-Rex-293 cells transfected with pGL3-control (0.16 μg), pRL-TK (0.16 μg), and 1.6 μg of pRNATin-H1.2/Neo/siFLuc (Cat. SD1523). After transfection, the cells are incubated in the absence of Tetracycline or Doxycycline, or 5 μg/ml of Tetracycline, or 1 μg/ml of doxycycline for 48 hours.

VIII. References

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