

GenScript siRNA Cassette Protocol



Technical Manual No. 0168

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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), and 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. Here are some of its applications:

- A stable cell line with a specific gene knocked-out can be established, and its phenotype can be studied [2-5].
- A knock-out mouse line can be established using transgenic siRNA method [8].
- siRNA can be put into a vector with an inducible promoter to study its effect.
- siRNA can be delivered by using viral vector [6,7] and used for gene therapy purpose.
- siRNA can be mimicked by chemical molecule and used for drug development.

II. siRNA cassette

siRNA cassette is a PCR product which consists of a promoter (e.g. U6 or H1 promoter) and terminator sequence flanking a DNA insert encoding a hairpin siRNA. After transfected into cells, the DNA insert encoding a hairpin siRNA is expressed from the PCR product, and induces gene specific silencing.

siRNA cassette is the most cost-effective way for identifying siRNA target sequences. By screening multiple siRNA cassettes, you can find the most potent and most specific siRNA target. Here are the key features of GenScript siRNA cassette technology:

1. **The most cost-effective:** Genscript siRNA cassette costs less than half of the corresponding synthetic siRNA oligos.
2. **More stable:** siRNA cassette is delivered as DNA fragment, which is much more stable than RNA.



It is recommended that multiple siRNA cassettes (at least 8 target sequences) be screened for each gene to find the most potent and most specific siRNA target. 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 find out the most potent target by using multiple siRNA cassette screening.
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 identify specific siRNA targets by using multiple siRNA cassette screening.
3. **The experiment is still the gold test stone:** Although we are proud of our siRNA design program, the best design is still not as good as what the experiments can tell you.

III. Product Description

siRNA cassette is a PCR product which consists of a promoter (e.g. U6 or H1 promoter) and terminator sequence flanking a DNA insert encoding a hairpin siRNA.



- Promoter: Options include human U6 or human H1 promoter.
- Stuffed: additional sequences added to the upstream of promoter to facilitate quality control.
- Modified bases (P): To increase the stability of PCR product in transfected cells.
- Term: Poly(T) termination signal.
- *Mlu* I and *Hind* III can be used to subclone the fragment into GenScript pRNA vectors.

Quantity: 20 µg.

Quality Control: The PCR fragment is analyzed by 2% agarose gel electrophoresis. The expected size of products:

- Human U6 promoter siRNA Cassette: 808 bp
- Human H1 promoter siRNA Cassette: 505 bp

Storage: -20 °C after receiving.

Cat No.	SC1003	SC1006
Promoter	Human U6	Human H1



IV. Transfecting mammalian cells.

General considerations before transfection: siRNA cassette is delivered as a lyophilized DNA fragment (20 µg). Before use, add 40 µl of sterilized water into each tube (0.5 µg/µl concentration), vortex it diligently. Incubate the tube at room temperature for **at least 10 min** so that the lyophilized DNA is completely dissolved. The behavior of siRNA cassette in transfection is different from that of plasmid. We recommend using Lipofectamine Plus™ reagent for transfection. The following are important issues to consider before performing the transfection:

- Cell density: The recommended cell density for transfection using Lipofectamine Plus™ is 50-90%. If the cell density is less than 50%, the Lipofectamine Plus™ may have some toxicity on the cells.
- siRNA cassette amount: For 12-well plates, it is recommended to use 0.2 µg as a starting point. siRNA cassette is very potent. As little as 50 ng of siRNA cassette has effect in 12-well plates. To screen most potent siRNA cassette, it is recommended to use less DNA. For other plate size, the DNA amount can be increased proportionally.
- Cell Proliferation: Maintaining healthy cell culture is essential for cell transfection. It is essential to minimize decreased cell growth associated with nonspecific transfection effects and to maintain cell culture under sub-confluent conditions to assure rapid cell division.
- Positive control and negative control: It is always a good idea to include a positive and a negative control in the experiment. Since there are a lot of uncertainties in siRNA experiments, it is recommended to use a positive control to optimize your system. GenScript positive control is siFluc cassette (Cat. No. SC0001 and SC0002), which is targeted to firefly luciferase (pGL-3 control vector from Promega).
- 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.
- Transfection efficiency: High transfection efficiency is essential for achieving siRNA effect using a transient transfection approach. You can use a GFP plasmid as a transfection efficiency control.

A protocol based on Lipofectamine Plus™ from Invitrogen for 293-H cells:

- Purchase Lipofectamine Plus™ reagent from Invitrogen. Lipofectamine™ Reagent (Invitrogen Cat. 11514-015) and Plus™ Reagent (Invitrogen Cat. 18324-012)
- Plate 293-H cells (Invitrogen Cat. 11631017) the day before transfection so that they are 50-90% 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 50-90% is very important. If the cell density is less than 50%, the transfection may have toxicity on the cells.
- Pre-complex the DNA with the Plus™ Reagent: dilute siRNA cassette with Opti-MEM (Invitrogen Cat. 31985062) from Invitrogen and mix. Mix Plus™ Reagent before use, add to DNA, mix again, and incubate at room temperature (RT) for 15 min. It is very important to dilute DNA into medium and mix before adding Plus™ Reagent, or DNA may precipitate. For 12-well plate, 0.2 µg of DNA should be diluted with 50 µl Opti-MEM and mix, and 5 µl Plus™ reagent is added later.
- Dilute Lipofectamine™ Reagent into Opti-MEM medium in a second tube and mix. For 12-well plate, 2 µl of Lipofectamine™ is added to 50 µl Opti-MEM medium.
- Combine pre-complexed DNA (from Step 3) and diluted lipofectamine™ Reagent (from step 4); mix and incubate for 15 min at RT. While complexes are forming, replace medium on the cells with transfection medium (Opti-MEM).
- Add the DNA-Plus™ Lipofectamine™ Reagent complexes to each well containing fresh medium on cells (from Step 5). Mix complexes into the medium gently; incubate at 37 °C at 5% CO₂ for 3 h.
- After 3 h incubation, add the complete medium (containing serum) to increase the medium volume to the normal volume.
- Perform assays on the cells 24-48 h after the start of transfection.

**Table 1.** Suggested starting amounts of reagents for transfection in different culture vessels:

	DNA (µg)	Plus™ Reagent (µl)	Medium for dilution (µl)	Lipofectamine™ Reagent (µl)	Transfection Medium (ml)	Transfection Volume (ml)
Protocol Step	Step 3	Step 3	Step 3 & 4	Step 4	Step 5	Step 6
96 well	0.05	1	10	0.5	0.05	0.07
24 well	0.1	4	25	1	0.2	0.250
12 well	0.2	5	50	2	0.4	0.5
6 well	0.4	6	100	4	0.8	1.0
60 mm	0.8	8	250	12	2	2.5
100 mm	1.6	20	750	30	5	6.5

Table 2. Transfection optimization suggested ranges for different culture vessels:

Vessel	Surface area (cm ²)	DNA (µg)	Plus™ Reagent (µl)	Lipofectamine™ Reagent (µl)
96 well	0.3	0.02-0.2	0.2-2	0.08-0.8
48 well	0.7	0.04-0.4	0.4-4.0	0.18-1.6
24 well	2	0.04-0.8	1-10	0.5-4
12 well	4	0.04-1.6	2-10	1-8
6 well	10	0.08-3.2	3-20	2.5-20
60 mm	20	0.16-6.4	5-20	5-40
100 mm	56	0.32-12.8	15-50	15-120
150 mm	140	0.64-25.6	35-100	34-280
T25	25	0.16-6.4	6-25	6-50
T75	75	0.32-20	18-75	18-150

A brief protocol for siFLuc cassette (Cat. No. SC0001 or SC0002) transfection:

1. To use siFLuc cassette, pGL-3 control vector (Promega, Cat. #E1741) and pRL-TK vector (Promega, Cat. #E2241) need to be purchased from Promega.
2. To observe the silencing effect of siLuc, four sets of transfection are needed: a. pGL-3 control and pRL-TK vector alone; b. pGL-3 control, pRL-TK, and siFLuc plasmid (Cat. No. SD1501); c. pGL-3 control, pRL-TK, and an empty pRNA vector (Cat. No. SD1201); d. pGL-3 control, pRL-TK, and siFLuc cassette.
3. For cell transfection, 12-well plates can be used. For 293-H cell from Invitrogen, 300,000 cells can be seeded the day before transfection.
4. The amount of siLuc cassette used for transfection should be about 2 fold higher than that of pGL-3 control plasmid. For 293-H, 0.16 µg of pGL-3 control and 0.16 µg pRL-TK vector are used, 0.05 µg to 0.8 µg of siFLuc cassette are used for each well.
5. The plasmid can be transfected into mammalian cells using Lipofectamine Plus™ following the protocol.
6. The Firefly and Renilla luciferase activities can be measured using Dual Luciferase assay kit from Promega (Cat. #E1910) after 36 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 cassette is about 70% (Figure 1).

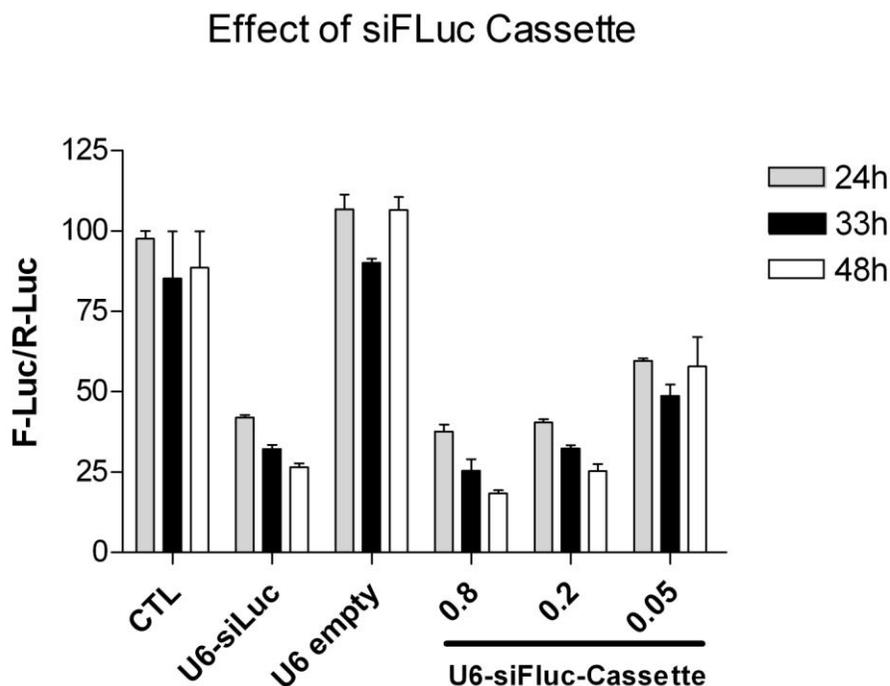


Figure 1.

- CTL: 0.16 μ g of pGL3-control and 0.16 μ g of pRL-TK plasmid
- U6-siFLuc: pGL3-control (0.16 μ g), pRL-TK (0.16 μ g), and 1.6 μ g of pRNA-U6.1/Neo/siLuc (Cat. No. SD1501).
- U6-empty: pGL3-control (0.16 μ g), pRL-TK (0.16 μ g), and 1.6 μ g of pRNA-U6.1/Neo empty vector (Cat. No. SD1201).
- U6-siFLuc cassette: pGL3-control (0.16 μ g), pRL-TK (0.16 μ g), plus 0.8 μ g, 0.2 μ g, or 0.05 μ g of U6-siFLuc cassette (Cat. No. SC0001).

V. References

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GenScript Corporation
120 Centennial Ave., Piscataway, NJ 08854
Tel: 732-885-9188, 732-885-9688
Fax: 732-210-0262, 732-885-5878
Email: info@genscript.com
Web: <http://www.Genscript.com>