

GenScript Adenoviral Vector-based siRNA Protocol

Technical Manual No. 0165

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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. ADENOVIRAL siRNA VECTOR

Adenoviruses are linear, double stranded DNA viruses capable of infecting a majority of human and many nonhuman cell types including mouse, rat, dog, chicken, rabbit, sheep, pig, and nonhuman primates, and infection is not dependent on active host cell division. Adenoviral vectors have two outstanding features: high efficiency gene delivery and high-level gene expression. Due to these attributes, adenoviral vectors can be used for overexpressing siRNAs in a broad range of mammalian cells. The adenoviral vectors developed at GenScript have proved to be high efficient for siRNA expression in mammalian cells [9].

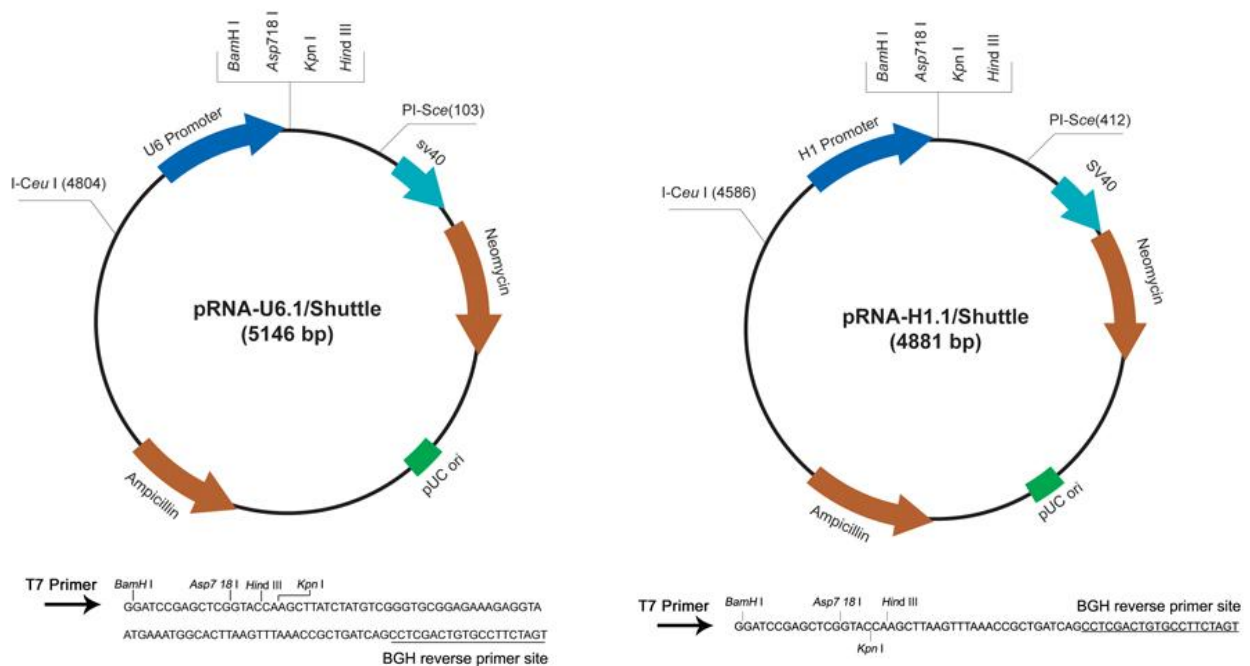
GenScript has developed two kinds (named differently) of adenoviral shuttle siRNA vectors:

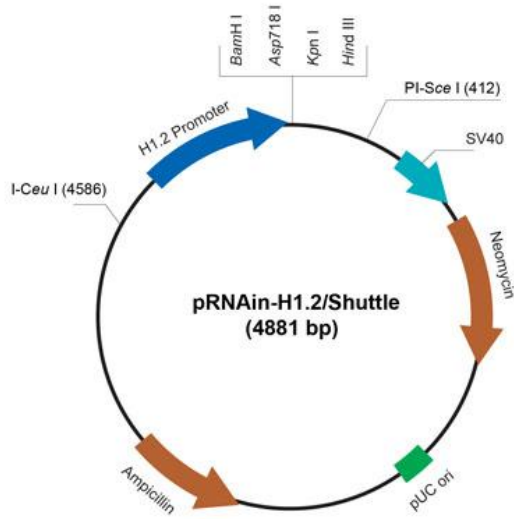
1. **Shuttle vectors.** These adenoviral shuttle vectors are compatible with Clontech Adeno-X™ Expression System 1 (Cat. No. 631513) (<http://orders.clontech.com/AIT/Ecommerce/Clontech/ProductCatalog.aspx?item=492574>). These vectors are: pRNA-U6.1/Shuttle (SD1205), pRNA-H1.1/Shuttle (SD1206), pRNAT-H1.1/Shuttle (SD1216), and pRNAin-H1.2/Shuttle (SD1234), they use U6 or H1 or inducible H1 promoter to drive the siRNA expression.
2. **Adeno vectors.** GenScript adenoviral shuttle vectors are compatible with Stratagene AdEasy Adenoviral (<http://www.stratagene.com/products/displayProduct.aspx?pid=86>) Vector System. These vectors are: pRNA-H1.1/Adeno (SD1209), pRNAT-H1.1/Adeno (SD1219) and pRNATin-H1.2/Adeno (SD1229), they use H1 or inducible H1 promoter to drive the siRNA expression.

These vectors contain elements that allow recombination of the siRNA construct into adenoviral genome. The adenoviral particles are then used to infect mammalian cells to express siRNA in the host cells. U6, H1 promoter (H1.1) and inducible H1 promoter (H1.2) are used to drive the siRNA expression. The H1.2 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), H1.2 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:

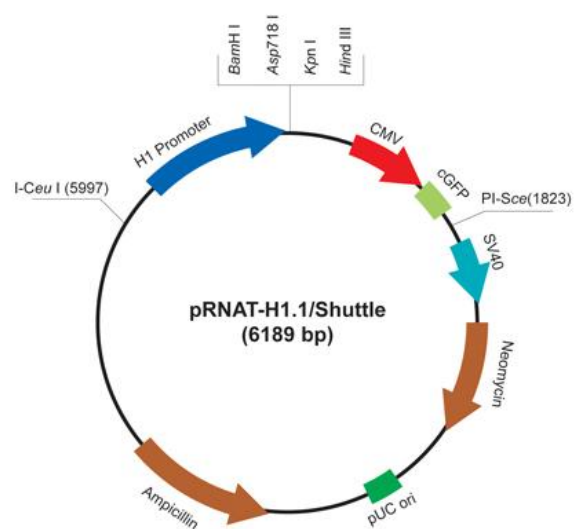
1. Three of the vectors (SD1216, SD1219 and SD1229) carry a GFP (coral GFP) marker under CMV promoter* control for tracking the transfection efficiency and viral infection.
2. siRNA effect can be tested first for siRNA optimization before you produce adenoviral particles and infect target cells. After cloning the siRNA construct into the vectors, you can test the siRNA effect by transfection of the siRNA-containing vectors into the mammalian cells. H1 will drive the siRNA expression.

The circle maps of all these vectors:



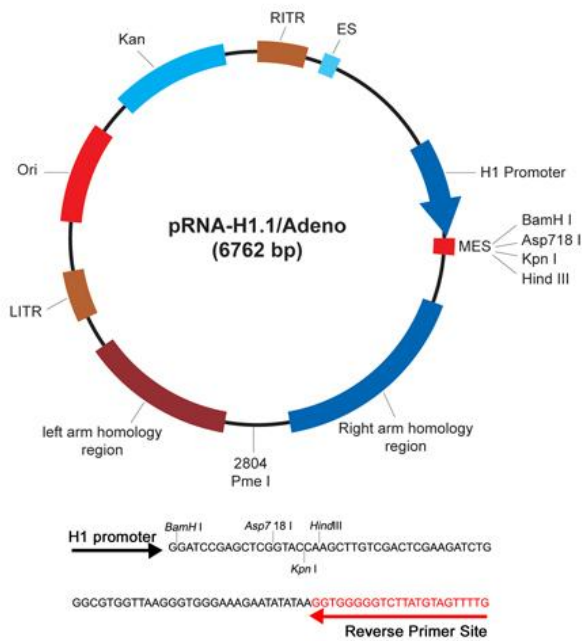


T7 Primer → `GGATCCGAGCTCGGTACCAAGCTTAAGTTTAAACCGCTGATCAGCGCTCGACTGTGCGCTTCTAGT`
 Restriction sites: BamHI, Asp718 I, Kpn I, Hind III
 BGH reverse primer site



T7 Primer → `GGATCCGAGCTCGGTACCAAGCTTAAGTTTAAACCGCTGATCAGCGCTCGACTGTGCCITCTAAA`
 Restriction sites: BamHI, Asp718 I, Kpn I, Hind III
 BGH reverse primer site

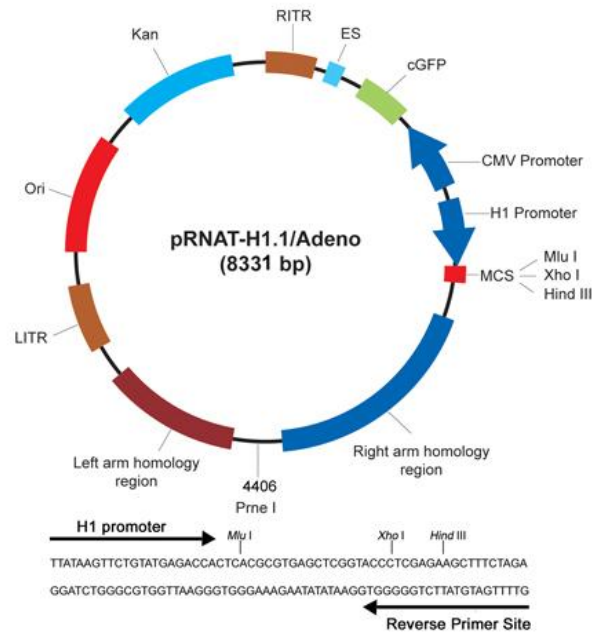
SD1234: pRNAin-H1.2/Shuttle



H1 promoter → `GGATCCGAGCTCGGTACCAAGCTTGTGCGACTCGAAGATCTG`
 Restriction sites: BamHI, Asp718 I, Kpn I, Hind III
 Reverse Primer Site: `GGCGTGGTTAAGGTTGGGAAAGAATATATAAGTGGGGTCTTATGATTTTG`

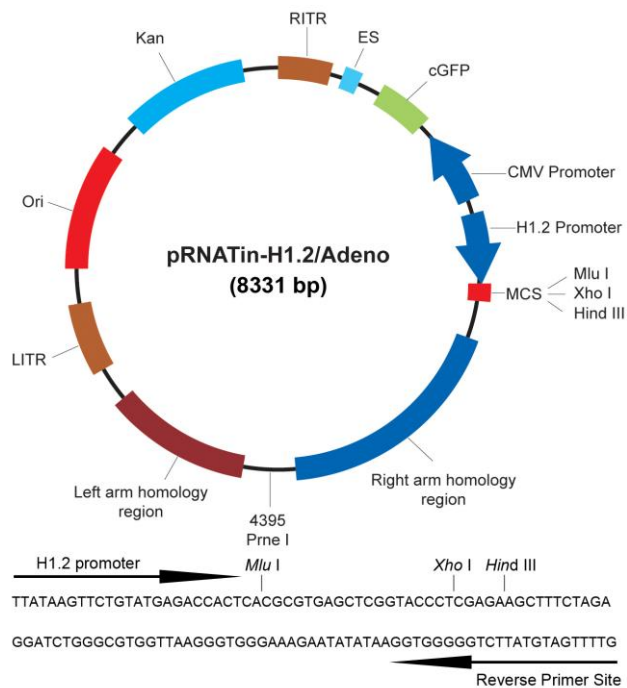
SD1209: pRNA-H1.1/Adeno

SD1216: pRNAT-H1.1/Shuttle



H1 promoter → `TTATAAGTTCTGTATGAGACCACTCACGCGTGAGCTCGGTACCCGAGAAGCTTTCTAGA`
 Restriction sites: Mlu I, Xho I, Hind III
 Reverse Primer Site: `GGATCTGGCGTGGTTAAGGTTGGGAAAGAATATATAAGTGGGGTCTTATGATTTTG`

SD1219: pRNAT-H1.1/Adeno



SD1229: pRNATin-H1.2/Adeno

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 doxycycline can be used as an inducer reagent. The concentration for tetracycline recommended is 1 µg/ml to 5 µg/ml, and the concentration for doxycycline 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

Cat. No. SD1205, SD1206, SD1216, SD1234, SD1209, SD1219 and SD1229.

Description: A small DNA insert (about 70-80 bp) encoding a short hairpin RNA targeting the gene of interest is cloned into a shuttle siRNA expression vector at the multiple cloning site (MCS). The insert-containing vector can be transfected into the cell, and Promoter: Options include human U6 or H1 promoter.

- Term: Poly(T) termination signal.
- Coral GFP (cGFP) marker to track the transfection efficiency and viral infection.
- MCS 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.

GenScript Adenoviral siRNA Expression Vectors:

Cat. No.	SD1205	SD1206	SD1216	SD1234	SD1209	SD1219	SD1229
Promoter	Human U6	Human H1	Human H1	Human inH1	Human H1	Human H1	Human inH1
Marker	-	-	cGFP	-	-	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 appropriate restriction 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 °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 appropriate restriction enzymes. 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. For SD1209, SD1219 and Sd1229, transform the ligation mixture into One Shot Stbl3 competent cells (Invitrogen, Cat. C7373-03) and plate it on LB-kan plates (Kanamycin at 25 µg/ml).
9. Choose at least 15 clones and grow them. Prepare MiniPreps from culture using GenScript MiniPrep kit.
10. Cut the plasmids with appropriate restriction enzymes. 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 Adenoviral Particles and Infecting Mammalian Cells.

A. Using shuttle vectors that are compatible with Clontech Adeno-X expression system:

To deliver the siRNA expression system into mammalian cells using GenScript shuttle vectors, you also need to order BD Adeno-X Expression System 1 (Cat. No. 631513) from BD Biosciences Clontech.

A brief procedure to express your siRNA in the host cell line. For more details, please follow Clontech Retro-X™ System Manual to produce adenoviral particles and infect host cells.

1. Clone your siRNA construct into **GenScript shuttle vectors** using appropriate restriction enzymes. You can use U6 promoter, H1 promoter or inducible H1 promoter for your siRNA expression.
2. Digest shuttle vectors with *PI-Sce I* and *I-Ceu I*, ligate the fragment containing the siRNA to the predigested Viral DNA.
3. Transform *E. coli* strain DH5 α to select for and amplify recombinant clones.
4. Transfect a low-passage HEK 293 cell line with the recombinant adenoviral DNA and harvest recombinant adenovirus 4-7 days later.
5. Infect your host cells by adding the viral supernatant.
6. Use appropriate methods to analyze siRNA silencing effect.

B. Using adeno vectors that are compatible with Stratagene AdEasy Adenoviral Vector System:

To deliver the siRNA expression system into mammalian cells using GenScript adeno vectors, you also need to order Stratagene pAdEasy-1 vector (Catalog#240005), BJ5183 competent cells (Catalog#200154), AD-293 cells (Catalog#240085) and MBS Mammalian Transfection Kit (Catalog#200388).

A brief procedure to express your siRNA in the host cell line. For more details, please follow Stratagene AdEasy Adenoviral Vector System Manual to produce adenoviral particles and infect host cells.

1. Clone your siRNA construct into **GenScript adeno vectors** using appropriate restriction enzymes. You can use either H1 promoter or inducible H1 promoter for your siRNA expression.
2. Contransform the resultant plasmid into *E. coli* strain BJ5183 with the adenoviral backbone plasmid pAdEasy-1. Recombinant adenoviral plasmids are selected on kanamycin and confirmed by restriction digest.
3. Transfect AD-293 cells with the recombinant adenoviral plasmid and harvest recombinant adenovirus a few days later.
4. Infect your host cells by adding the viral supernatant.
5. Use appropriate methods to analyze siRNA silencing effect.

VII. References

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