# GenScript Retroviral Vector-based siRNA Protocol

**Technical Manual No. 0176** **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.

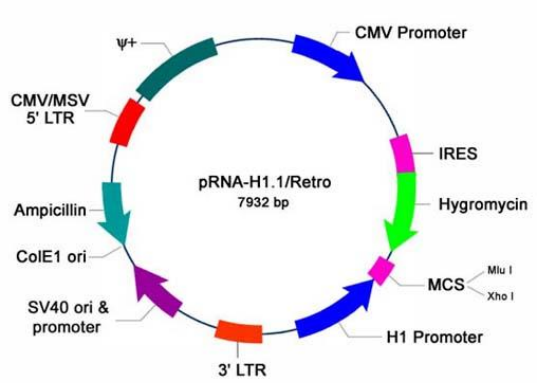
It is recommended that at least 3 vector-based siRNA should be prepared for each gene to find the most potent and most specific siRNA. Here are the reasons:

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 experiment 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.

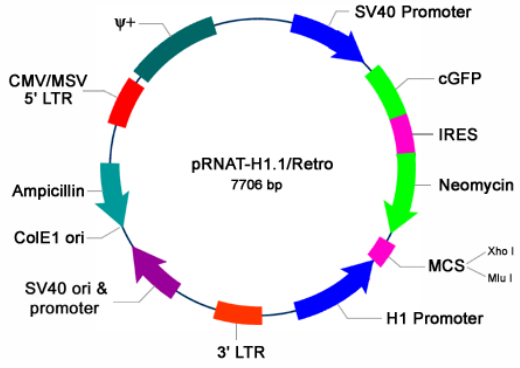
## III. Retroviral siRNA Vector

Retroviruses are single-stranded RNA viruses that can infect a number of organisms, including humans and many other mammals. Following infection, the virus genome is reversely transcribed into double-stranded DNA, which is integrated into the host chromosome via the integration mechanisms. The ability of retroviruses to infect target cells as well as the stable replication of retroviruses and high-level gene expression make them very attractive as gene transfer vectors. The developed retroviral vectors have proved highly efficient for gene delivery and achieved stable long-term expression of the transgenes.

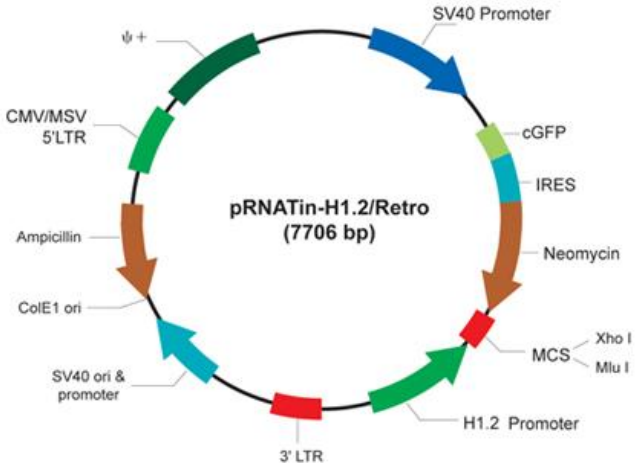
GenScript has developed three Retroviral Vectors for siRNA expression, these vectors are compatible with Clontech Retro-X Expression System (http://www.bdbiosciences.com) for high level and long-term siRNA expression. These three vectors are: pRNA-H1.1/Retro (SD1241), pRNAT-H1.1/Retro (SD1255, coming soon) and pRNATin-H1.2/Retro (SD1256, coming soon) with the circle maps shown below.




**SD1241: pRNA-H1.1/Retro**




**SD1255: pRNAT-H1.1/Retro**




**SD1256: pRNATin-H1.2/Retro**

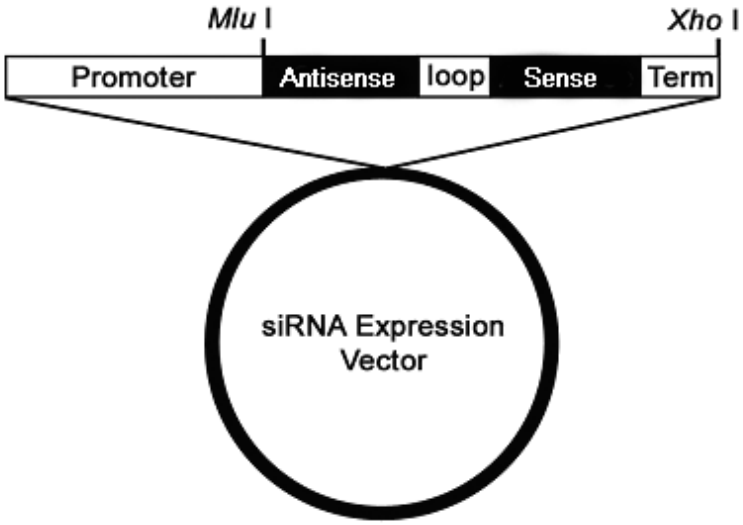
These vectors contain elements that allow packaging of the siRNA construct into retroviral particles. The retroviral particles are then used to infect mammalian cells to express siRNA in the host cells. H1 promoter (H1.1) and inducible H1 promoter (H1.2) are used to drive the siRNA expression. The H1.2 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. They carry a neomycin or hygromycin resistance gene that can be used for establishing stable cell line.
2. Two of the vectors (SD1255 and SD1256) carry a GFP (coral GFP) marker for tracking the transfection efficiency and viral infection.
3. siRNA effect can be tested first for siRNA optimization before you produce retroviral 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.

## IV. Product Description

**Cat. No.:** SD1241, SD1255 and SD1256.
**Description:** The GenScript Retroviral siRNA Vectors are derived from Murino sarcoma virus. 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 Mlu I and Xho I sites. H1 or inducible H1 promoter is used for siRNA expression. Promoter: Options include H1 or inducible H1 promoter.

- Vector resistance: hygromycin or neomycin.
- Term: Poly(T) termination signal.
- coral GFP (cGFP) marker to track the transfection efficiency.
- *Mlu* I and *Xho* I are 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:**

- Retroviral siRNA Expression Vectors

| Cat. No. | SD1241 | SD1255 | SD1256 |
|---|---|---|---|
| **Promoter** | H1 | H1 | inducible H1 |
| **Resistance** | Hygromycin | 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). 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.
2. Order two oligos with cohesive *Xho* I and *Mlu* I sites. The oligos must be PAGE purified oligos. Dissolve the oligos in water to 1 µg/µl concentration.
3. 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
4. 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.
5. Cut the vector with Mlu I and Xho I. Run 1% agarose gel and purify the vector.
6. Ligate the vector with the insert using T4 ligase (the molar ratio of insert to vector is 3:1).
7. Transform the ligation mixture into competent DH5a cells (Invitrogen, Cat. 18258-012). Plate it on LB-amp plates.
8. Choose at least 15 clones and grow them. Prepare MiniPreps from culture using GenScript MiniPrep kit.
9. Cut the plasmids with *Xho* I and *Mlu* I. Run 3% gel to check whether the plasmids have the insert and select positive clones.

10. Sequence the positive clones to verify the sequence of the insert.

## VI. Producing Retroviral Particles and Infecting Mammalian Cells.

To deliver the siRNA expression system into mammalian cells using GenScript Retroviral vectors, you also need to order the following reagents from Clontech (http://www.bdbiosciences.com): RetroPack PT67 Cell Line: #K1060-D.

Described below are the general steps to express your siRNA in the host cell line. For more details, please folllow Clontech Retro-X™ System Manual to produce retroviral particles and infect host mammalian cells.

1. Clone your siRNA construct into **GenScript vectors** between Mlu I and Xho I sites to generate Retro expression vector. You can use either H1 promoter or inducible H1 promoter for your siRNA expression.
2. Transfect the RetroPack PT67 Cell Line with the Retro expression vector to produce Retroviral particles.
3. Collect supernatant containing retroviral particles and determine the titer.
4. Infect 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. References

1. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411: 494-498.
2. Yu JY, DeRuiter SL, Turner DL. (2002) RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. Proc Natl Acad Sci U S A. 99(9):6047-6052.
3. Brummelkamp, T.R., Bernards, R., and Agami, R. (2002) A system for stable expression of short interfering RNAs in mammalian cells. Science 296: 550-553.
4. Jacque, J.-M., Triques, K., and Stevenson, M. (2002) Modulation of HIV-1 replication by RNA interference. Nature 418: 435-438.
5. Sui, G., Soohoo, C., Affar, E.B., Gay, F., Shi, Y., Forrester, W.C., and Shi, Y. (2002) A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc. Natl. Acad. Sci. USA 99(8): 5515-5520.
6. Shen C, Buck AK, Liu X, Winkler M, and Reske SN. (2003) Gene silencing by adenovirus-delivered siRNA. FEBS Lett 539(1-3):111-114.
7. Barton GM, and Medzhitov R. (2002) Retroviral delivery of small interfering RNA into primary cells. Proc Natl Acad Sci U S A 99(23):14943-14945.
8. Kunach T, Gish G, Lickert H, Jones N, Pawson T, and Rossant J. (2003) Transgenic RNA interference in ES cell-derived embryos recapitulates a genetic null phenotype. Nature Biotechnology 21:559-561.
9. Czauderna F, Santel A, Hinz M, Fechtner M, Durieux B, Fisch G, Leenders F, Arnold W, Giese K, Klippel A, Kaufmann J. (2003) Inducible shRNA expression for application in a prostate cancer mouse model. Nucleic Acids Res. Nov 1;31(21):e127
10. Matsukura S, Jones PA, Takai D. (2003) Establishment of conditional vectors for hairpin siRNA knockdowns. Nucleic Acids Res. Aug 1;31(15):e77
11. van de Wetering M, Oving I, Muncan V, Pon Fong MT, Brantjes H, van Leenen D, Holstege FC, Brummelkamp TR, Agami R, Clevers H. (2003) Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. EMBO Rep. Jun;4(6):609-15.

**Limited Use Label License**

This product is the subject of the following patents: U.S. Patent No. 6,573,099 and foreign counterparts co-owned by Benitec Australia Ltd. Not-for-profit and academic entities who purchase this product receive the non-transferable right to use the product and its components in non-Commercial Purpose internal research.

All commercial and for-profit purchasers using this product for a Commercial Purpose are required to execute a commercial research license with Benitec by contacting Benitec at licensing@benitec.com . Entities wishing to use ddRNAi as a therapeutic agent or as a method to treat/prevent human disease also should contact Benitec at licensing@benitec.com .

Commercial Purpose means any activity by a party for tangible consideration and may include, but is not limited to, (i) use of the product in manufacturing; (ii) use of the product to provide services, information or data for a compensatory fee; (iii) use of the product for therapeutic, diagnostic or prophylactic purposes; (iv) use of the product in drug discovery or target validation and/or (v) resale of the product or its components whether or not the product is resold for use in research.

If purchaser is not willing to accept the limitations of this label license, seller is willing to accept the return of the product with a full refund. For terms and additional information relating to a commercial research license, or for licensing for any human therapeutic purpose, contact Benitec by email at licensing@benitec.com .

* Limited Use Label License: This product is covered by United States Patent Applications No. 60/505,677, owned by Genscript Corporation. The purchase of this product conveys to the buyer the limited, non-exclusive, non-transferable right (without the right to resell, repackage, or further sublicense) under these patent rights to perform the siRNA synthesis methods claimed in the patent application for research and development purposes solely in conjunction with this product.

* Limited Use Label License: The use of CMV promoter is covered under U. S. Patent No. 5,168,062 and 5,385,839 owned and licensed by the University of Iowa Research Foundation and is sold for research use only. Commercial users must obtain a license to these patents directly from the University of Iowa Research Foundation (UIRF), 214 Technology Innovation Center, Iowa City, Iowa 52242. For further information, please contact the Associate Director of UIRF, at 319-335-4546.

---

**GenScript USA Inc.**
860 Centennial Ave., Piscataway, NJ 08854
Tel: 1-732-885-9188
Fax: 732-210-0262, 732-885-5878
Email: info@genscript.com
Web: http://www.Genscript.com