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SYNTHESIS THROUGH INGENUITY

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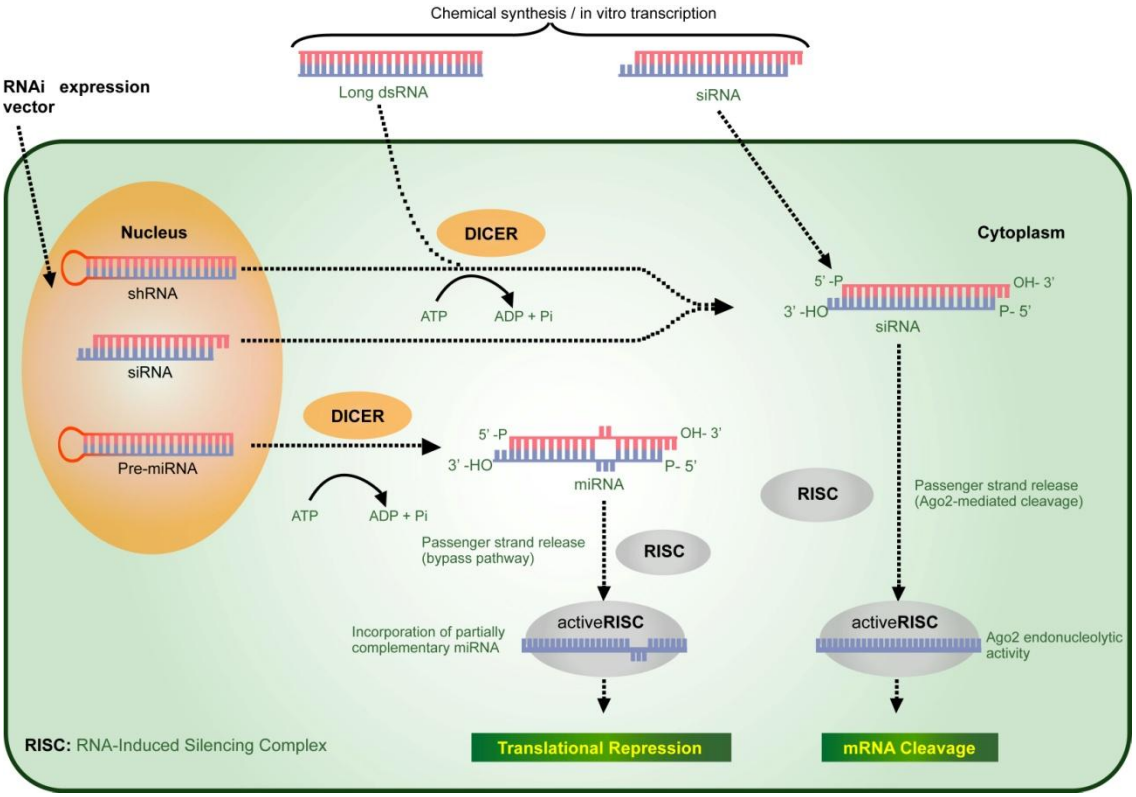
RNA INTERFERENCE



CONTENTS

1. INTRODUCTION

RNA interference (RNAi) or double-stranded RNA (dsRNA) is a system within living cells that helps to control which genes are active and how active they are. siRNAs were first discovered by David Baulcombe's group in Norwich, England, as part of post-transcriptional gene silencing (PTGS) in plants[1] and later independently identified in wide variety of eukaryotic organisms. These dsRNAs are rapidly processed into short RNA duplexes of 21 to 28 nucleotides in length, which then guide the recognition and ultimately the cleavage of complementary single-stranded RNAs, such as messenger RNAs or viral genomic/antigenomic RNA (*Fig. 1*). According to their origin or function, naturally occurring small RNA have been described: short interfering RNAs (siRNA), repeat-associated short interfering RNA (rasiRNA or shRNA) and microRNA (miRNA). RNA interference has many biological functions – it is a vital part of the immune response against viruses and also downregulates gene expression by transcriptional silencing of genes or upregulates promoting by RNA activation. Finally, artificial introduction of long dsRNA or siRNA has been adopted as a tool to inactivate gene expression, both in cultured cells and in living organisms.

A biochemical understanding of the RNAi pathway was crucial to realizing that dsRNAs shorter than 30 base pairs (bp) could be used to trigger an RNAi response in mammals. Tuschl and colleagues showed that transfection of mammalian cells with short RNAs could induce the sequence-specific RNAi pathway, and so overcame the barrier to the use of RNAi as a genetic tool in mammals[2]. The impetus to use siRNAs and other small RNAs in mammalian cells also came from the long-standing view that protein kinase receptor (PKR) activation[3] and similar responses were not effectively triggered by short dsRNAs. Following the initial reports, it took a remarkably short period of time for siRNAs triggers to be adopted as a standard component of the molecular biology toolkit. siRNAs can be introduced into mammalian cells using a variety of standard transfection methods. The strength and duration of the silencing response is determined by several factors: on a population basis, the silencing response is affected mainly by the overall efficiency of transfection, which can be addressed by optimizing conditions. In each cell, silencing depends on the amount of siRNA that is delivered and on the potential of each siRNA to suppress its target, or its potency. Even a relatively impotent siRNA can silence its target provided that sufficient quantities of the siRNA are delivered. However, essentially 'forcing' the system by delivering large amounts of reagent is likely to lead to numerous undesired effects.

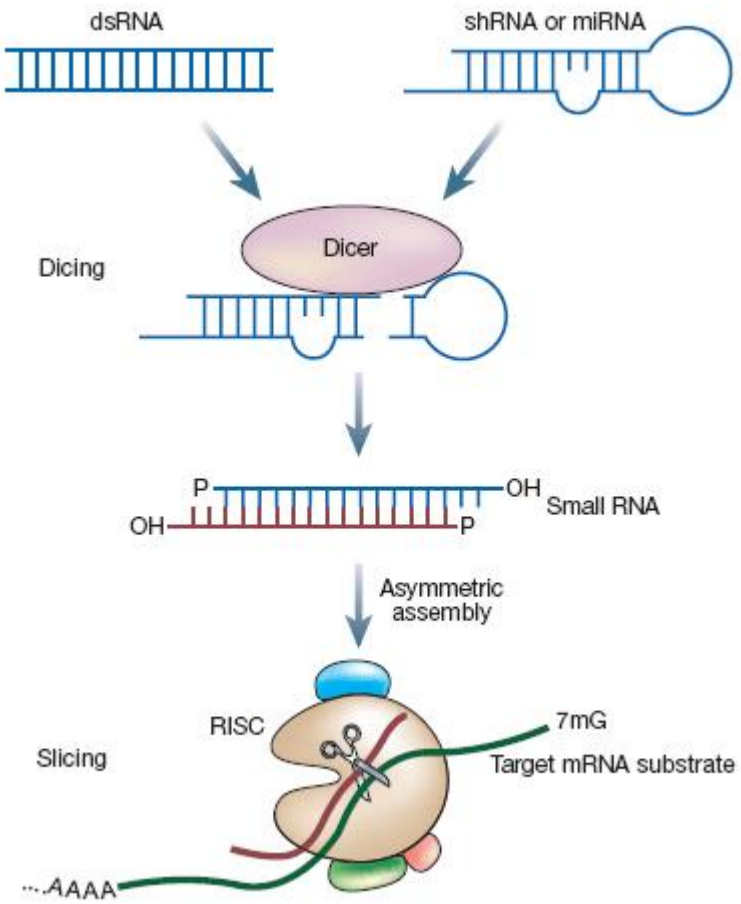


Figure 1. RNA silencing pathways in different organisms. *Long dsRNA and miRNA Precursors are processed to siRNA/miRNA duplexes by the RNase-III-like enzyme Dicer. These short dsRNAs are subsequently unwound and assembled into effector complexes, RISCs, which can direct RNA cleavage, mediate translational repression or induce chromatin modification. S. pombe, C. elegans and mammals carry only one Dicer gene. In D. melanogaster and A. thaliana, specialized Dicer or DLC proteins preferentially process long dsRNA or miRNA precursors. 7mG, 7-methyl guanine; AAAA, poly-adenosine tail; Me, methyl group; P, 5-phosphate (Hannon and Rossi, 2004)*[4].

2. siRNA- A GENE SILENCER

siRNA, also known as silencing RNA, is a class of 20-25 nucleotide-long dsRNA molecules. These siRNA are produced due to the cleavage of dsRNA by the RNase-III-like enzyme Dicer. The siRNA is then separated into single strands and incorporated into the active RNA-induced silencing complex (RISC). After integration into the RISC, siRNAs base-pair to their target mRNA and induce cleavage of the mRNA, thereby preventing it from being used as a translation template[5]. The discovery that small 21mer siRNA, in contrast to longer dsRNA, elicits a very limited unspecific response allowed the use of technology as a tool to assess gene function in mammalian cells. Because of its high efficiency and specificity, RNAi has revolutionized functional genomics and drug discovery.

Therapeutic applications of RNAi are being extensively studied because of their potential for the development of gene-specific medicine6[6,7]. To allow the successful delivery of the RNA duplexes in to mammalian cell lines, different strategies have been developed over the last few years, including chemical synthesis, in vitro transcription or vector based delivery. siRNA have to be highly efficient and as specific to be used with confidence. Many algorithms are now available for the rational design of siRNA molecules in silico giving the researcher a higher chance to perform a successful knockdown.

3. COMPARISION OF GENE SILENCING APPROACHES

Several different molecules that act to inhibit gene expression by sequence specific targeting of mRNAs have been developed in the hope of creating therapeutic agents. The two other major nucleic-acid based gene-silencing molecules are – Chemically modified antisense oligodeoxyribonucleic acids (ODNs) and Ribozymes. The less utilized antisense molecules include PNAs, DNAzymes

ODNs- ODNs are generally ~20 nucleotides in length, their mode of action is by hybridizing to pre-mRNA and mRNA to produce a substrate for ribonuclease H (RNaseH) which specifically degrades the RNA strand of the formed RNA-DNA duplexes. Modification of ODN's in a way to prevent the action of RNaseH they can inhibit the translation of mRNA via steric hinderance. ODNs and modifications can be used to target dsDNA for the inhibition of transcription by the formation of triple helices.

Ribozymes- Ribozymes act to degrade target RNA by binding to through Watson-Crick base pairing and catalyze the hydrolysis of the phosphodiester backbone. There are several different classes of ribozymes of which the 'Hammerhead' ribozymes is well studied. Hammerhead ribozymes forms a unique secondary structure when hybridized to its target mRNA. The hammerhead ribozymes are short enough to be chemically synthesized or can be transcribed from vectors30, allowing for the continuous production of ribozymes within cells.

As compared to the above gene-silencing approaches, studies have suggested that siRNAs are far more potent and longer-lasting than various types of ODN. It is estimated that the half-maximal inhibition levels (IC50) of siRNAs are some 100- to 1,000-fold lower than an optimal phosphorothioate-modified oligodeoxynucleotide directed against the same target. RNA-binding proteins and extensive secondary or tertiary structures within mRNA are suggested to interfere with the hybridization of ODNs to their target RNA molecules. Several groups have investigated whether these variables also affect the efficiency of siRNAs. Most of these studies have found a direct correlation between the efficiency of an ODN and an siRNA relative to the target position on mRNA. Although a systematic and extensive comparison of the gene silencing efficiency mediated by ribozymes and/or DNAzymes and siRNAs has yet to be done, several experiments have indicated that siRNAs are also more effective than ribozymes and DNAzymes. Long hairpin loops that seem to silence gene expression by RNAi are also more potent than hammerhead ribozymes.

TABLE 1. *Comparison of different gene silencing strategies*

Agent	Mode of Action	Outcome
Other drugs	Bind to target protein	Protein inhibition
RNaseH-dependent ODNs	Act by hybridizing to target mRNA	Inhibition of translation of target protein
RNaseH-dependent ODNs	Hybridize to target mRNA	Degradation of mRNA by RnaseH
Ribozymes & DNAzymes	Catalyze cleavage of target mRNA	Degradation of mRNA
siRNA	Hybridize to target mRNA by its antisense strand and guide it into endoribonuclease enzyme complex (RISC)	Degradation of mRNA

4. siRNA DESIGN

In designing siRNA one can choose siRNA target sites in a variety of different organisms based on the following guide lines.

1) **Finding the sequences (21nt) in the target mRNA that start with AA dinucleotide.**

- Beginning with the AUG start codon of your transcript, scan for AA dinucleotide sequences. Record each AA and the 3' adjacent 19 nucleotides as potential siRNA target sites.

- This strategy for choosing siRNA target sites is based on the observation by Elbashir et al. that siRNAs with 3' overhanging UU dinucleotides are the most effective. This is also compatible with using RNA pol III to transcribe hairpin siRNAs because RNA pol III terminates transcription at 4-6 nucleotide poly(T) tracts creating RNA molecules with a short poly(U) tail.

2) **Select 2-4 target sequences**

Choose target sites from among the sequences identified in Step 1 based on the following guidelines:

- siRNAs with 30-50% GC content are more active than those with a higher G/C content.

- Since a 4-6 nucleotide poly(T) tract acts as a termination signal for RNA pol III, avoid stretches of > 4 T's or A's in the target sequence when designing sequences to be expressed from an RNA pol III promoter.

- Since some regions of mRNA may be either highly structured or bound by regulatory proteins, we generally select siRNA target sites at different positions along the length of the gene sequence. We have not seen any correlation between the position of target sites on the mRNA and siRNA potency.

- Compare the potential target sites to the appropriate genome database (human, mouse, rat, etc.) and eliminate from consideration any target sequences with more than 16-17 contiguous base pairs of homology to other coding sequences. We suggest using BLAST, which can be found on the NCBI server at: www.ncbi.nlm.nih.gov/BLAST.

5. METHODS FOR SYNTHESIZING siRNA

Several strategies for synthesizing siRNA- mediated gene silencing have been developed each of them possessing specific advantages and disadvantages which includes-

5.1 Chemical synthesis

5.2 Invitro siRNA synthesis

5.3 Endogenous expression

- Expression plasmids
- Viral vectors

5.1 CHEMICAL SYNTHESIS

Synthesis, purification and annealing of siRNA are by industrial chemical processes which are increasingly becoming popular, where the method is rapid and purity is generally high. Chemical synthesis is the most direct means of generating siRNAs and has several advantages, including precise control of the amount and purity of siRNA, ease in characterization and scale-up, and ease in chemical modifications for enhanced stability and target specificity[8].

The chemical synthesis of siRNA requires several steps including the generation of two homologous strands, annealing of the strands, addition of chemical entities to increase stability, and ensuring that 2-nt overhangs are present. The siRNA duplex requires a 3′-hydroxyl group and a 5′-phosphate group for functional activity[9]**.** Unlike DNA, RNA possesses an additional hydroxyl group at the 2′ position of each ribose building block, which destabilizes RNA under the basic conditions generally present in DNA synthesis reactions. Hence, the most difficult step in RNA synthesis is the simultaneous protection of the 5′- and 2′-hydroxyl groups during solid-phase chemistry.

One drawback of using chemically synthesized siRNA is that the most effective target sequence is unpredictable since gene silencing efficiency may vary depending on the segments of the transcripts that are targeted. For example, Holen *et al*., 2005[10] observed that only a few siRNAs resulted in a significant reduction of human tissue factor (HTF) expression after targeting its mRNA with several siRNAs synthesized against different sites of the same mRNA. Another potential problem inherent in chemically synthesized siRNA is variability in transfection efficiency, especially in difficult-to-transfect cells. To circumvent these limitations, expression vectors currently in use employ siRNA or shRNA expression cassettes that resemble pre-

miRNAs and undergo processing by Dicer. Like synthetic siRNAs, they are designed to pair perfectly with the target mRNA to induce RNAi. These shRNAs are designed for either transient or long-term gene silencing and can be produced from plasmid or viral expression vectors.

5.2 *IN VITRO* siRNA SYNTHESIS

In vitro siRNA synthesis is an alternative approach and relies upon the T7 phage polymerase. This polymerase produces individual siRNA sense and antisense strands that once annealed form siRNAs. Extra nucleotides required by the T7 promoters are removed by RNase digestion and cleaning steps. Otherwise, recombinant Rnase-III can be used to cleave long dsRNAs to produce multiple siRNAs. Although technically easy, this approach presents the drawback of the generation of non-specific siRNAs. siRNAs can be produced by polymerase-III promoter-based DNA plasmids or expression cassettes. These constructs produce small inverted repeats, separated by a spacer of three to nine nucleotides, termed short hairpin RNAs (shRNAs), which are processed by Dicer into siRNAs. Transcription begins at a specific initiation sequence, determined by the promoter used. In addition to a defined initiation sequence, the U6 polymerase-III promoter terminates with TTTT or TTTTT. The products are shRNAs that contain a series of uridines at the 3' end, a feature that seems to favor RNAi. In vitro transcribed siRNA are most cost effective and, can be made more quickly as well as are just as effective as chemically synthesized siRNAs.

5.3 ENDOGENOUS EXPRESSION

Expression Plasmid

Expression plasmids and viral vectors are being employed in applied RNAi research. The reason being expression vectors allow continuous production of siRNAs in cells which relates to sustained depletion of the protein encoded by the targeted mRNA. With respect to viral vectors the transfection efficiency of specific type of cells; postmitotic cells can be greatly increased, and also they are more effective in obtaining sustained expression.

shRNA, siRNA, and miRNA can be produced from plasmid vectors containing promoters that are dependent on either RNA polymerase (Pol) II or Pol III. Among them, Pol III promoters are used most frequently because it is possible to express small RNAs that carry the structural feature of siRNA. *Fig 2* is a schematic representation of different strategies used to create expression cassettes using RNA polymerase promoters for generation of siRNA, shRNA, and miRNA.

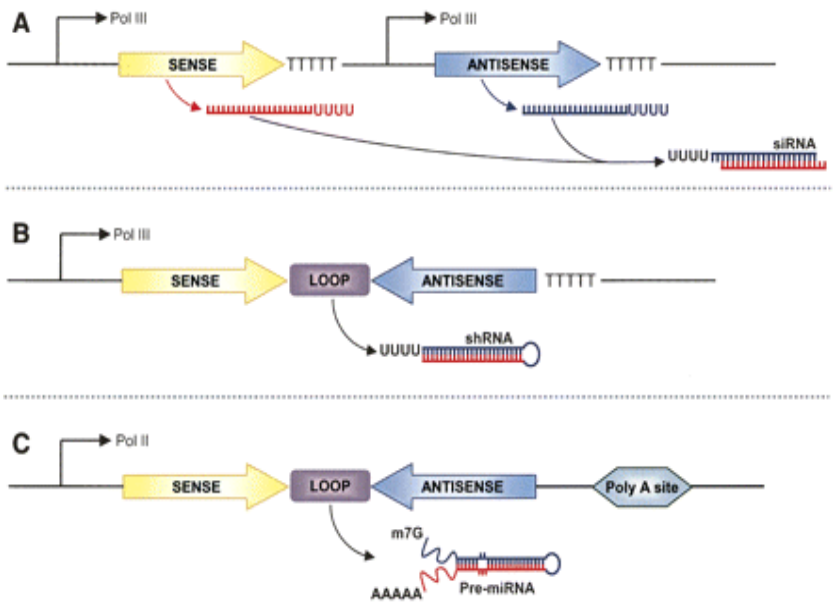


Figure 2. Schematic representation of expression cassettes using RNA polymerase promoters for generation of small-interfering RNAs. *(A) Tandem-type promoters express sense and antisense strands individually. After transcription, both strands hybridize forming a duplex siRNA. (B) Short hairpin RNAs (shRNA) are expressed as a single transcript separated by a short loop of 4–10 nucleotides. The transcripts form a hairpin structure that can be processed by Dicer into functional siRNAs. (C) Expression of imperfect duplex hairpin structures that is based on pre-microRNA (pre-miRNA) structures. pre-miRNAs are processed by Dicer into a mature miRNA, which can direct gene silencing (Daniel De Paula M. Vitória L.B. Bentley, and Ram I. Mahato)*[11].

The activities of these promoters vary from cell type to cell type. To optimize shRNA expression, it is beneficial to create expression vectors with at least two different promoters and transfect them into the cells being targeted for gene knockdown. Construction of shRNA expression vectors poses two serious technical challenges. First, it is difficult to sequence constructs that

contain a hairpin region, probably because of the tight palindromic structure. Second, 20%–40% of constructs get mutated within the hairpin region.

Viral vectors

The introduction of siRNA expression plasmids into cells often requires electroporation, microinjection, or complex formation with synthetic carriers (lipids, polymers, or peptides). While most rapidly dividing cell lines are easily transfected using shRNA expression plasmids, these plasmid vectors are not easily transfected into primary cells, stem cells, and non dividing cells. In the absence of cell division, the siRNA expression plasmids cannot be introduced into the nucleus, where the DNA is transcribed. To overcome this limitation, different viral vectors encoding shRNA including retroviral, adenoviral, and adeno-associated viral (AAV) are being developed. Typically, these vectors use a Pol III promoter, such as U6, H1, or transfer RNA promoters.

Retroviral vectors have been reported to mediate an efficient and stable siRNA expression[12,13]. Unlike Moloney murine leukemia virus (MoMLV), lentiviral vectors efficiently integrate into the genome of non dividing cells, such as pancreatic islets, hematopoietic stem cells, or terminally differentiated cells. A lentiviral vector encoding shRNA has been shown to effectively silence GFP, BCL-2, and Interleukin (IL) 12 receptor (CD25) genes[14]. Lentiviral vectors encoding shRNA have also been shown to inhibit HIV-1 infection in hematopoietic stem cells and human CD4+ T-cells[15].

In terms of high throughput applications, vector based strategies enjoy the advantages of much lower cost and ability to regenerate. The advantage of the vector based siRNA is the capability of removing those cells that are not transfected with the plasmids by selecting the transfected cells with antibiotic resistance genes. Virus vectors also enable the delivery of siRNA expression cassettes in to cells with high transfection efficiency.

TABLE 2. *Comparative account of all the above-mentioned siRNA producing methods, with all their pros and cons are summarized.*

	Chemical Synthesis	*In vitro* Transcription	RNase III Digestion of dsRNA	siRNA Expression Vectors	siRNA Expression Cassette	Viral Vectors
Total preparation/ synthesis time	4-15 days	one day + DNA oligo	one day + transcription template preparation time	5+ days + DNA oligo	~6hrs+ DNA oligo	9-40 days
Validation of siRNA sequences	Required	Required	Not required	Required	Required	Required
Labelling siRNA for analyzing uptake or localization	Yes	Yes	Yes	No	No	No
Transfection efficiency	Good	Good	Good	Fair	Fair	Very good
Shelf life(for long term studies)	No	No	No	Yes with selection	No	Retrovirus
Cost of synthesis	High	Moderate	Low	Moderate	Moderate	High

6. CUSTOM siRNA SYNTHESIS

Custom siRNA Synthesis service will make siRNA according to a sequence user supply, or a sequence determined by their design tool are preloaded in 96-well plates; where one can choose from a range of scales (0.1 nmol, 0.25 nmol, and 1 nmol), and can tag siRNA with a dye on either end of the sense strand (available dyes include the Alexa Fluor family of dyes, fluorescein, rhodamine, Cy3, and Cy5). Fluorescent-labeled siRNA are popular as transfection efficiency controls as they provide a rapid visual evaluation. The fluorescence may be detected by either fluorescence microscopy or flow cytometry. The fluorescent signal localizes to the nucleus as an unmistakable signal of efficient uptake, while the maximum fluorescence correlates to the optimal siRNA uptake conditions.

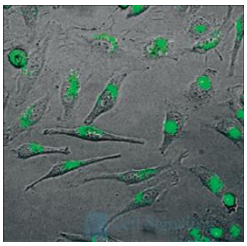


FIGURE 3. *Fluorescent detection of Signal Silence® Control siRNA (Fluorescein Conjugate) in living HeLa cells 24 hours post-transfection, demonstrating nearly 100% transfection efficiency*

Labels that attach to the backbone or bases of the siRNA are also available, such as amino linkers, thio linkers, and biotin, dabcyl, and phosphate modifications.

6.1 Our custom siRNA services

Bio-Synthesis utilizes many different antisense and RNAi technologies to achieve a robust knockdown of selected genes and help you to understand complex biological systems. Several strategies for synthesizing siRNA-medicated gene silencing have been developed in our laboratory.

- Synthetic based siRNA
  We provide custom synthesis of sequence-specific siRNA for direct transfection, as well as the routine synthesis of shRNA oligos, ~60 bases long purified DNA oligos to be cloned to form shRNA constructs . Our read-to-use siRNA is the optimal siRNA purification grade for efficient gene silencing at an affordable cost. Each siRNA duplex undergoes strignent quality control including MALDI-TOP mass spectrometry analysis. Read-to-use grade siRNA is economicially priced allowing the use of highly pure siRNA in all routine RNAi experiemtns, For very sensitive applications, custom synthesis of HPLC-purified siRNA is also available.
- In vivtro siRNA synthesis
- Endogenous expression
  - Expression plasmids
  - Viral vectors.

6.2 Looking for convenience

Bio-Synthesis has provide siRNA database that contains siRNA targets against all known mRNA sequence throughout a variety of organisms. The database has also been subdivided into folders for siRNA again Kinases, phosphatases, Transcription Factors and Disease genes in order to provide a total solution for your RNAi research needs. All siRNA targets in the database are linked to BSI's web-based siRNA cloning tool that allows users to chose from a wide variety of vectors and also search for specific repeat patterns in complete genomes.

Our web-based siRNA design tool incorporates secondary structure into its target site evaluation. The design program allows you to look at the secondary structure of the siRNA target site, then choose to order siRNAs that pinpoint areas on the secondary structure that would lead to more effective silencing. The secondary structure view of the mRNA target sites including the number of bonds and the local free energy improves the selection of highly efficient siRNA.

6.3 PRODUCT PROFILE AND PRICING

TABLE 3. *Price quotes for siRNA oligo synthesis (From Biotech Desk)*

siRNA synthesis		
siRNA sequence	**Guaranteed nmol**	**Price (USD)**
OPC	10	180.578
	20	216.694
	50	314.092
	100	491.575
HPLC	10	185.594
	20	357.144
	50	565.812
	100	802.571
PAGE	30	475.523

Antisense modification			
Modification	**Antisense modifications**		
	0.05 umole	0.2 umole	1.0 umole
Phosphorothioate Bond	1.504	2.00	5.517
RNA	8.025	10.032	18.057
2' O-Methyl RNA	11.035	12.035	24.077

TABLE 4. *Custom vector development (From Sigma Aldrich)*

MISSION® Luciferase shRNA Control Vector

Product no	**Price**
SHC007	428.773

The MISSION Luciferase shRNA Control Vector is a 7,091 base pair lentivirus plasmid vector that contains an shRNA sequence targeting luciferase from *Photinus pyralis.* The Luciferase shRNA Control Vector is useful as a positive knockdown control in experiments using cell lines expressing firefly luciferase.

7. siRNA TRANSFECTION AND DELIVERING OF siRNA IN TO CELLS

Several different transfection methods which are being used extensively for introducing plasmids or oligodeoxynucleotides in to cells can be employed for introducing siRNA in to cells. It has become apparent there is no single transfection method which can be applied efficiently to all cell types under all experimental conditions. Therefore it is important to optimize transfection conditions so that maximum gene silencing is achieved.

The application of synthetic siRNAs is restricted by both low-to-moderate transfection efficiency and the short term persistence of transient gene expression. A single transfection of siRNA may not provide a sufficient window of functional depletion for proteins with long half-lives.

Following are the transfection parameters that have been shown to effect transfection and gene silencing efficacy-

- Cell culture conditions.

- Cell density and medium composition- For postmitotic cells such as neurons, cell densities in the range of 200 to 500 cells per mm^2 of culture surface work well (O. Milavet and M. P. Mattson, unpublished data).
- Amount and type of transfection agent.
- Quality and amount of siRNA.
- Duration of time the cells are exposed to siRNA- the longer the exposure time, the higher the efficiency rate.

The two common approaches for siRNA delivery are lipid-mediated transfection and viral-mediated transduction. Determining which one of these approaches to use depends on the cell type being studied and whether transient or stable knockdown is desired. The most popular application, transient transfection of unmodified siRNAs or modified Stealth™ RNAi duplexes (from Invitrogen), uses cationic lipid-based reagents because they are suitable for delivering molecules across a diverse range of commonly used cell lines. For cell types not amenable to lipid-mediated transfection, electroporation and nucleofaction techniques have been used. However these require the use of large amount of siRNA ~1µM in final solution, which can be associated with greater incidence of off target effects[16] and also causes massive cell death which should also be taken into consideration[17].

Conjugation with lipids may enhance siRNA uptake via receptor-mediated endocytosis or by an increased membrane permeability of the otherwise negatively charged RNA. Conjugation of nucleic acids with cholesterol has been demonstrated to enhance cellular uptake in cell culture and hepatic deposition after systemic administration[18]. This is because cholesterol conjugation increases the hydrophobicity and cellular association of nucleic acids. Lorenz et al. (2004)[19] have conjugated siRNAs with cholesterol derivatives like lithocholic and lauric acids at the 5′ end of the sense strand. Cholesterol conjugation was shown to increase the cellular uptake of siRNAs in human liver cells without use of any transfection reagent. Incubation of cells during 4 h with 50 nM of siRNAs with a modified sense strand down-regulated β-galactosidase expression to a higher extent than siRNAs with a modified antisense strand or two modified strands.

Viral vectors are often employed, adenoviral vectors work well for transient delivery in many cell types; however, for some difficult cell lines, such as non-dividing cells and for stable siRNA expression, lentiviral vectors are the best delivery method. For some cell types that are not amenable to lipid based transfections.

Other methods that have proven effective for transfecting siRNAs into cultured cells include electroporation. Calcium phosphate-mediated transfection has been used successfully by several laboratories[20]. The most commonly used and effective transfection method for short-term suppression of gene expression is to incorporate siRNAs into liposomes.

TABLE 5. *Recommended RNAi delivery methods*

Cell Type	Transient expression (<7 days)	Transient expression (>7 days)	Stable expression
Fast growing Adherent cells (A549, Hela)	Lipid transfection of siRNA or Stealth™ RNAi	siRNA or Stealth™ RNAi Lipid transfection of RNAi vectors or Adenoviral delivery	Lipid transfection of RNAi vectors or Lentiviral delivery
Fast growing suspension cells (THP-1)	Lipid transfection or electroporation of siRNA or Stealth™ RNAi	Lipid transfection of RNAi vectors or Adenoviral delivery	Lipid transfection or electroporation of RNAi vectors or Lentiviral delivery
Primary cells			Lentiviral delivery
Non-dividing cells			Lentiviral delivery

TABLE 6. *Targeted Delivery vehicles being investigated for commercialization*

Delivery vehicle	Disease	Advantages	Companies	Reference
Lipid nano particles		Modified lipids designed to change under biological pH	sirna	Chemical and engineering news November 13, 2006
Conjugated to fusion proteins made of antibodies and protamine	HIV	Antibodies target HIV + cells protamine deliver	-	Liberman, 2005
Aptamer-siRNA conjugates	Prostrate cancer bclX & HER2	Aptamer targets and facilitates delivery. Does not activate non specific inflammatory responses	-	Chu et al, 2006

TABLE 7. *Selecting transfection reagents and viral delivery methods (From Invitrogen)*

Products (Invitrogen)	Key Advantages
Lipofectamine™ RNAiMAX Transfection Reagent	Specially designed and manufactured for deliver of dsRNA (siRNA or Stealth™ RNAi) Superior efficiencies allow low concentrations of siRNA to be used Mild cytotoxicity profile facilitates optimization Wide range of compatibility with diverse cell lines Optimized protocols are available for many common cell lines.
Lipofectamine™ 2000 Transfection Reagent	Specifically designed for optimal expression when delivering plasmids, including shRNA and miR RNAi vectors Robust co-transfection of vectors and synthetics (siRNA or Stealth™ RNAi duplexes).
Oligofectamine™ Transfection Reagent	Expressly formulated for delivery of antisense oligos Dependable delivery of siRNA.
BLOCK-iT™ Adenoviral RNAi Expression System	Ideal system for long-term transient expression of RNAi vectors in difficult-to-transfect cell lines.

In vivo delivery of siRNA can be enhanced by exogenous application of synthetic siRNA via gene therapy approach that relies on the endogenous expression of siRNA from plasmid or viral vectors. Delivery of oligos for gene therapy has provided much needed vital information that can help in standardization of delivery vehicles for siRNA. The relatively few reports indicate a lack of effective *in vivo* delivery methodology, especially for RNAi mediated down regulation of the specific gene targets in the animal disease models[21]. Delivering siRNA *in vivo* to animal tissues is a complicated process and involves using physical, chemical, biological approaches or a combination of all.

8. METHODS TO ACHIEVE HIGH TRANSFECTION EFFICIENCY

Transfection efficiency describes the percentage of cells that have received the RNAi duplex or expression plasmid. Typically, researchers strive to achieve the highest levels of transfection efficiency possible. This objective is particularly important for RNAi applications because non-transfected cells will continue to express the gene targeted for knockdown, thus contributing to background expression levels.

For many disease models, the most desirable cell types to use are primary cultures. However, these cannot be transfected adequately with commercially available transfection reagents. A powerful alternative to cationic lipid-mediated transfection is viral delivery of vectors expressing RNAi sequences. This option is best for delivery to hard-to-transfect, primary, and no dividing cells. Viral delivery can also be used to create stable cell lines with inducible RNAi expression or to express RNAi sequences with tissue-specific promoters.

9. QUANTIFYING DOWN REGULATION

RNAi down regulates a gene function without actually interacting with the gene. The subtle action is by mRNA degradation. Thus the degree of RNA interference achieved is directly proportional to the level of mature mRNA and the translated proteins. The options are:

1. Measurements of target protein (enzyme) activity. This option is suitable if a robust assay is available or has been in prior use. The assay would vary by the nature of the protein product.
2. Measurement of target mRNA level. This is the preferred method as it directly quantifies the level of mRNA. Quantitative PCR is very effective in measuring relative amount of target sequence. This can be achieved simply by SYBR green or by the use of TaqMan or Molecular Beacons.

10. CHEMICAL MODIFICATION AVAILABLE FOR siRNA

siRNA stability for prolonging the duration of gene silencing and to further dissect its mechanism in human cells, various chemically modified nucleotides were incorporated into siRNAs to study whether specific modifications increased or decreased the efficacy and persistence of RNAi *in vivo*.

10.1 TYPES OF MODIFICATIONS

Modifications of RNA at the 2′-position of the ribose ring have been shown to increase siRNA stability against endonucleases and reduce immune response activation. These modifications include 2′-O-methyl (2′-OMe), 2′-deoxy-2′-fluoro modifications, and locked nucleic acid

Fluoro and methyl linkages

The siRNA motif consisting of 2′-OMe and 2′-fluoro nucleotides has enhanced plasma stability and increased in vivo potency. The 2′-OMe sugar modification retains the canonical right-handed A-form helical geometry, which is required for siRNA activity. This modification has also been shown to increase the nuclease resistance of ODNs (oligo deoxy nucleotide) and siRNA duplexes[22]. The effect of 2′-OMe modification has been found to be dependent on both position and extent of incorporation

The effects of 2′-OH modifications on RNAi were studied by replacing uridine and cytidine in the antisense strand of siRNA by 2′-fluoro-uridine (2′-FU) and 2′-fluoro-cytidine (2′-FC), respectively. These modifications increased the siRNA stability upon exposure to HeLa cell extracts, without losing gene silencing activity. On the other hand, modifying the 2′-OH to a bulky methyl group to create 2′-OMe nucleotides in the sense or antisense strand greatly diminished EGFP gene silencing, whereas double-stranded 2′-OMe-modified siRNAs completely abolished RNAi. One reasonable explanation is that the methyl group, as a bulky group, may severely limit the interactions among siRNA, target mRNA, and the RNAi machinery that are required to successfully mediate gene silencing.

Locked nucleic acid

Locked nucleic acid (LNA) is a family of conformationally locked nucleotide analogs that displays unprecedented hybridization affinity towards complementary DNA and RNA. LNA may be used to increase the functional half-life of siRNA *in vivo* by two different mechanisms:

(1) Enhancing the resistance of the constituent RNA strands against degradation by ssRNases, and
(2) Stabilizing the siRNA duplex structure that is crucial for silencing activity

Stability of siRNA can be enhanced by conjugating LNA at the 3′ ends of the sense strand of siRNA. For instance Introduction of LNA modifications at the 3′ overhangs in either one or both strands of siRNA against firefly luciferase revealed no loss of silencing effect of siRNA in cultured cells. Additionally, one LNA at the 5′ end of the sense strand was shown to be fully compatible with silencing activity, while an LNA at the 5′ end of the antisense strand dramatically impaired the silencing effect. Since the strand that displays the weakest binding energy at its closing 5′ base pair is incorporated preferentially into the RISC[23], the conjugation of LNA at the 5′ sense strand might be used to alter strand bias in favor of incorporation of the antisense

Phosphonoacetates

Recently Caruther's[29] et. al. have described ODN's that have a phosphonoacetate (PACE) group in the internucleotide linkages. It is reported that these modifications confer fairly unique properties on the ODN's rendering permeable to a large variety of cell lines, all without the need of any transfection reagent. These ODN's have great promise in a wide array of research applications and have been shown to be very active in siRNA duplexes and also accelerate the initial rate of cleavage by RNase H-1 when incorporated with phosphorothioates. But most newsworthy is the observation that they possess an unique and improved enhancement in penetration of a large variety of cell lines.

Modification	Gene silencing	Cell system
Sense strand 5′ or 3′ termini		
Aminolinker	++++	HeLa, HeLa extract
Puromycin, biotin	++++	HeLa
Fluorescein	++++	HeLa
Antisense strand 3′ terminus		
Aminolinker	++++	HeLa, HeLa extract
Puromycin, biotin	++++	HeLa
Fluorescein	+++	HaCaT
Fluorescein, Alexa488	-	HeLa 63
Inverted 2′-deoxy abasic cap	++++	HeLa
Antisense strand 5′ terminus		
Aminolinker		HeLa, HeLa extract
Fluorescein	++++	HeLa
Inverted 2′-deoxy abasic cap	-	HeLa

TABLE 8. *Scale of the silencing effect as compared with the efficiency of unmodified siRNA duplex: –, modification rendering the duplex inactive; +, 20–40%; ++, 40–60%; +++, 60–80%; ++++, >80% of efficiency of unmodified duplex. siRNA, small interfering RNA.*

11. CONTROLS USED FOR siRNA EXPERIMENT

11.1 POSITIVE CONTROL

siRNA delivery efficiency can vary between the cell types used and the delivery method used. All delivery methods can produce a negative effect on cell viability. A well-characterized positive control siRNA is an ideal reagent for establishing parameters that result in successful siRNA delivery without affecting cell viability. An siRNA that targets a housekeeping gene ensures that the target gene is expressed in all cell types at a level that does not fluctuate with cell cycle. An abundantly expressed gene allows easy and accurate assay of its mRNA and protein levels. However, silencing of the gene should not affect either the cell phenotype or its viability. Three popular target genes that meet these criteria are cyclophilin B (also known as PPIB), glyceraldehyde-3-phosphate dehydrogenase (GAPD), and Lamin.

The ideal positive control siRNA is highly functional, as the level of silencing it produces needs to be correlated with the efficiency of delivery siRNA. The silencing level and cell viability of the positive control sample allows confirmation that the siRNA delivery process in each experiment is successful and non-toxic. Lower than expected silencing of the target gene indicates less than optimal delivery, but still allows normalization of the data to allow comparison with other experiments.

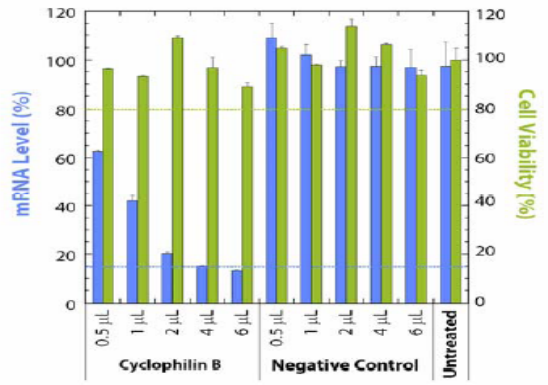


FIGURE 4. *Using a positive control siRNA to optimize transfection efficiency- CyclophilinB mRNA level was measured in Hela cells 24hr after transfection with siRNA targeting cyclophilinB (From Dharmacon).*

11.2 NEGATIVE CONTROL

Negative control siRNAs are designed to have no known target in the cells being used. They are important for distinguishing sequence-specific silencing from non-specific effects in the RNAi experiment. Samples that are treated with a negative control siRNA are analyzed similarly

to samples that are treated with the siRNA targeting your test gene. Neither the mRNA nor protein level of the experimental gene should be affected by the negative control siRNA

There are a variety of negative control siRNAs commercially available. The most common group of negative control siRNAs are designed to have no known mRNA targets in the cells used, and are described as non-targeting siRNAs.

TABLE 9. *Different types of controls used for RNAi experiments*

Type of control	Recommended use
Transfection control	Calculate and monitor transfection efficiency with fluorescence
Negative controls	Non-specific or scrambled controls used to measure knock down levels vs. background
Positive controls	RNAi reagents known to achieve high levels of knockdown used to measure delivery and optimize experimental condition
Untransfected control	Measure normal gene expression level and phenotype
Toxicity controls	Calculate and monitor transfection toxicity
Downstream controls	Measure mRNA or protein levels downstream
Interferon controls	qRT-PCR primer sets to detect induction of the interferon response

12. siRNA LIBRARIES AND siRNA SETS AVAILABLE IN THE MARKET

The concept of down-regulating a single gene by siRNA can be exploited by high-throughput technologies to facilitate large scale genomic studies. This can be done in a microtiter-based format or by designing libraries of siRNA or shRNA molecules. Libraries may be engineered based on validated siRNA sequences or by using randomly produced sequences and a biochemical or cellular assay to identify positive elements. If multiple iterations are used, the siRNA or shRNA may be rescued, re-introduced and identified by sequencing. This strategy may be effectively used to identify drug targets or new components of signaling pathways

To generate siRNA library one critical factor to consider is the design of siRNA sequence. Several studies of currently available information for effective siRNA have been carried out which have resulted in different siRNA design tools that based on partially overlapping criteria[24,25,26]. siRNA vendors have also made their own siRNA design software available on the web. siDirect (http://design.RNAi.jp/) is a web-based online software system for computing highly effective siRNA sequences with maximum target-specificity for mammalian RNAi. siRNA libraries have been synthesized by integration of 19-mer fully randomized DNA sequences in to different siRNA encoding vectors[27]. Such vectors should have theoretical complexity of 2.75 x 10^{11} in order to encode all the permutations of siRNA.

12.1 CUSTOM LIBRARIES OF siRNA AND siRNA SETS IN THE MARKET

TABLE 10. *siRNA libraries for use in mammalian cells*

Company	Species	Coverage	Reagent description
Synthetic siRNA libraries*			
Ambion	Human, mouse and rat	Genome-wide	21 nt with 3′ overhangs; unmodified RNA
Dharmacon	Human, mouse and rat	Genome-wide	21 nt with 3′ overhangs; unmodified RNA
Qiagen	Human	Genome-wide	21 nt with 3′ overhangs; unmodified RNA
Invitrogen	Human	Kinase genes	25 nt with blunt ends; modified backbone
Vector-based shRNA libraries			
Open Biosystems	Human, mouse	Genome-wide	shRNAs with miR backbone in retroviral vectors, sold as bacterial stocks; second-generation design by the Hannon and Elledge laboratories
Open Biosystems, Sigma- Aldrich	Human, mouse	Genome-wide	shRNAs in lentiviral vectors; second-generation design by the RNAi Consortium

TABLE 11. *Different siRNA sets available in the market*

siRNA sets available in the market	Description
SARS siARRAY Gene Set	Library of short interfering RNA (siRNA) duplexes targeted against multiple regions of the coronavirus that is believed to cause SARS
Human Validated Kinase siRNA Set	Functionally validated siRNA set targeting over 500 kinase genes. This unique siRNA set comprises siRNAs that have been verified by real-time RT-PCR to provide at least 70% target gene knockdown
Human Druggable Genome siRNA Set	siRNA set directed against human druggable genes
Human Phosphatase siRNA Set	siRNA set directed against phosphatase and phosphatase-associated genes
Human GPCR siRNA Set	siRNA set directed against human GPCRs
Human Epigenetics siRNA Set	siRNA set directed against human epigenetics genes

13. APPLICATIONS OF RNAi TECHNOLOGY

13.1 RNAi in drug discovery and disease therapy

RNAi has begun to produce a paradigm shift in the process of drug discovery. With the large-scale screening approaches, RNAi can winnow lists of potential drug targets so that efforts can

be focused on the most promising candidates. Moreover, since the first description of RNAi in mammalian cells, there have been numerous studies aimed towards using RNAi to treat disease. The strong appeal of RNAi in therapeutics is the potency and specificity with which gene expression can be inhibited. The possible targets for various diseases range from oncogenes to growth factors and single nucleotide polymorphisms (SNP).

There is also potential for using RNAi for the treatment of viral diseases such as those caused by the hepatitis C virus (HCV) and the human immunodeficiency virus (HIV). Despite the excitement and some early proofs of principle in the literature, there are important issues and concerns about the therapeutic application of this technology, including difficulties with delivery and uncertainty about potential toxicity. However, proposals for clinical trials using either synthetic siRNAs or viral-vector delivered shRNAs have been put forward — although none has yet been approved.

13.2 RNAi for genetic diseases

A promising lead towards using RNAi for the treatment of genetic diseases has been provided by preliminary studies that demonstrate how SNPs in mutant allele transcripts can be used as selective targets for RNAi. Finding an siRNA that is highly selective for a particular SNP is a challenge, but has been accomplished by systematic analyses of siRNAs in which the polymorphic nucleotide is complementary to the mid-region of the siRNA. In certain examples, the siRNAs direct selective degradation of only the mutant transcripts, leaving the wild-type transcripts intact despite only a single mismatch. Another example of siRNAs targeting an SNP was recently reported in studies of amyotrophic lateral sclerosis (ALS) caused by mutations in the Cu, Zn superoxide dismutase (*SOD1*) gene. Because the wild type SOD1 performs important functions, it is important to selectively eliminate expression of only the mutant allelic transcript. Many SOD1 mutations are single-nucleotide changes. Ding *et al.,*[28] achieved selective degradation of a mutant *SOD1* allele, thereby providing a potential therapeutic application for the treatment of ALS.

Disease-causing polyglutamine proteins encoded by CAG-repeat containing transcripts are found in several neurological diseases such as Huntington's disease. These proteins are especially challenging targets for RNAi because CAG repeats are common to many normal transcripts as well, and the repeats themselves cannot be selectively targeted by siRNAs. But with the recent finding that delivery of siRNAs and viral vectors expressing siRNAs to diseased regions of the brain is technically feasible, coupled with selective targeting of SNPs in the mutant transcripts, the promise of clinical use of RNAi for the treatment of degenerative, neurological diseases should be realized.

14. FUTURE PERSPECTIVES

In a remarkably short time since its discovery in model organisms, the RNAi pathway has emerged as a powerful tool for the study of gene function in mammals. As our understanding of the under-lying biology and biochemistry of this conserved gene-regulatory mechanism

improves, so does our ability to exploit RNAi as an experimental tool. With the use of RNAi in whole animals increasing, we anticipate growing enthusiasm for the use of RNAi triggers in therapy. Despite considerable hurdles to overcome, it seems likely that RNAi will find a place alongside more conventional approaches in the treatment of diseases, although it is unclear how long we will have to wait to witness the first RNAi-based drug.

The future studies of siRNA will pertain to it being investigated for many more applications of human, animal and plant therapeutics. The elucidation of mechanisms underlying its intracellular and intercellular trafficking will lead to discovery of techniques with high transfection efficiency of non-viral vectors. Since non-viral vectors are preferable from biosafety angle more studies of pharmacokinetics and cellular uptake of siRNA will help in designing and optimizing their formulation.

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