Product Profile

SmartBase™ siRNA Modifications
Guaranteed RNAi Explorer kit with Molecular Probe
RNAi Explorer™ Online siRNA Design Algorithm
Custom siRNA Synthesis

SmartBase™ siRNA:
Design Guide for Enhanced siRNA Performance

Modifications for Increased Cell Permeability, Duplex Stability & Nuclease Resistance

Duplex Stability & Nuclease Resistance Conferring Modifications

Propyne dC and dU
Phosphorothioate linkages
5-Me-dC & 2-amino dA
2'F bases
2'O methyl bases
2'MOE Bases
2'-5' linked Oligos
Methylated Oligos

Cell Delivery/Cell Permeation Modifications

Thiol & Amine
Cholesterol TEG
Spacer 18
Polyethylene Glycol (PEG)
Stearyl & α-tocopherol
GalNAc (N-acetylgalactosamine-C3)
Cell Penetrating Peptides (CPPs)

Gene Link™

SmartBase™ siRNA Modifications

Modifications for Increased Duplex Stability, Nuclease Resistance & Cell Permeation

Introduction

Well designed siRNAs are potent and specific modulators of gene expression and have immense potential in therapeutic gene silencing. Very successful design rules and algorithms have been established based on performance of hundreds of siRNA and miRNAs (miRNAs are naturally occurring and structurally resemble siRNA and use a similar silencing complex). The most important determinant of siRNA function is sequence specific recognition and Watson-Crick base pairing of the target single stranded mRNA sequence facilitated by the RISC complex followed by cleavage of the target mRNA leading to gene silencing. Robust algorithms are available for predictive selection of sequences that are patterned on established design rules (1-4).

Other than specific sequence requirements, effective siRNA functionality requires the following attributes.

1. Effective cell membrane permeation.
2. Targeted delivery.
3. Nuclease resistance confers increased half-life.
4. Sustained gene silencing with increased duplex stability.
5. Minimal off-target seeding and silencing.
6. Low dosage. Minimal Toxicity

siRNAs are conveniently synthesized chemically similar to common primers; synthetic oligos are used ubiquitously for molecular applications from the simplest as amplification primers to the more complex as siRNA and aptamers. In general a well designed oligo to serve as a primer, probe or siRNA will perform using standard bases for hybridization to its cognate sequence(s); but we can make it perform better using modified bases that are specifically developed by nucleic acid chemists to enhance exacting characteristics.

As with most natural molecules oligos are prone to degradation under normal conditions, specifically once introduced in body fluids. Ubiquitous nucleases as well as chemical instability lead to fast degradation with a finite half life.

Gene Link presents various design options for synthesizing effective siRNAs, probes and oligos based on the application. SmartBase™ siRNA can be synthesized in a predetermined way to exhibit the features that is desired; for instance to increase duplex stability 2'-fluoro C and U bases and 5-methyl dC and 2-Amino dA can be substituted, for nuclease resistance the phosphodiester linkages can be selectively substituted with phosphorothioate, selected bases substituted with 2'O methyl bases and for cellular delivery we may add cholesterol-TEG or other lipophilic modifications like stearyl, polyethylene glycol, α-tocopherol for lipid membrane permeability. The siRNA sequence can also be modified with thiol or amine for post synthesis conjugation with Cell Penetrating Peptides (CPP's) that are known to aid transport and facilitate cellular uptake.

The premise of this product guide is to introduce the use of SmartBase™ modifications and go beyond the traditional use of standard DNA and RNA bases for constructing synthetic siRNAs in particular and as well emphasize their use in primers, oligos and probes. SmartBase™ modifications also introduces the molecular biologists to develop a cross-disciplinary synergy of molecular applications to a wealth of nucleic acid chemistry tools available as modified bases to impart specific properties compatible with biological applications and gene expression pathways.

Effective siRNA Design

Several guidelines have been proposed to design effective siRNA (3-5). The strategy for siRNA design is based on our present understanding of the biochemical mechanisms involved in RNA interference and, in particular, structural features that allow the antisense-strand of the siRNA duplex to be more efficiently incorporated into the RNA-induced silencing complex (RISC). The main characteristics are listed below (3).

Default parameters: (N19)TT

1. Low to medium GC content (30-50%).
2. Absence of internal repeats or palindromes.
3. Presence of an A at position 3 of the sense strand.
4. Presence of A at position 19 of the sense strand.
5. Absence of G or C at position 19 of the sense strand.
6. Presence of U at position 10 of the sense strand.
7. Absence of a G at position 13 of the sense strand.
8. At least 3 A/Us at positions 15-19 of the sense strand.

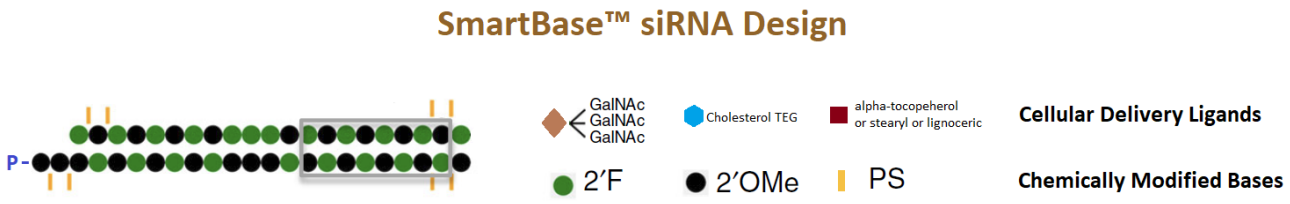
There are several other factors that influence effective functional performance of the designed siRNA; half-life of the siRNA is crucial for sustained activity and in many cases to increase effect for a longer time the dosage (concentration) is increased that leads to toxicity and off target effects. Similarly serum or cellular delivery methods can be improved. A common list of features for improvement is given below and possible sites in the nucleobase that can be modified to impart specific customized properties.

Common Features for Improvement

1. Increased nuclease resistance.
2. Increased duplex stability and manipulation of duplex stability.
3. Cellular delivery.
4. Surface attachment.

Common Modification Sites

1. Phosphodiester linkages.
2. Nucleic acid bases.
3. Sugar moieties.
4. Functional group addition.



Modifications for siRNA & Antisense Oligonucleotides (ASO, ODN) for Enhanced Activity			
Modification Type	**Mod Name**	**Main Features**	**Application**
Phosphate linkage	phosphodiester	Naturally occurring	Steric hindrance & RNase H cleavage
Phosphate linkage	phosphorothioate (PS)	Inexpensive, improved nuclease resistance	Steric hindrance & RNase H cleavage
Sugar modification	LNA & 2'-Fluoro	Increased duplex stability and nuclease resistance. Does not support RNase H cleavage. N	Steric hindrance
	2'-O Me & 2'-O MOE	Increased duplex stability and nuclease resistance. Does not support RNase H cleavage. Inhibits immune stimulation of PS backbone	Steric hindrance
Non-nucleotide & non-lipophilic	GalNAc	Used in combination with other modifications in ASO or siRNA for cellular delivery	Targeted liver delivery
Lipophilic	Cholesterol TEG, Stearyl or α-tocopheryl	Non-toxic lipophilic modifications used in combination with other modifications in ASO or siRNA for cellular delivery	Cellular delivery

SmartBase™ siRNA Modification Increasing Duplex Stability, Nuclease Resistance & Cell Permeation			
Modification*	**Duplex Stability [Tm Increase]**	**Nuclease Resistance**	**Cell Permeation**
Phosphorothioate	Slightly decreased	Increased	Slightly increased
2’-O Methyl	Increased	Increased	No effect
2’-Fluoro A and U	Increased [1-2° per substitution]	Increased	No effect
2-Amino-dA	Increased [3.0° per substitution]	No effect	No effect
5-Methyl-dC	Increased [1.3° per substitution]	No effect	No effect
3’-Cholesterol	No effect	No effect	Increased
3’-PEG	No effect	No effect	Increased
3’-Spacer 18	No effect	No effect	Increased
Selected list of modifications available from Gene Link, Inc. are listed in this table. Visit www.genelink.com for complete offerings.			

SmartBase™ siRNA Pathway Interactions

SmartBase™ modifications go beyond the traditional use of RNA bases for constructing synthetic siRNAs to specifically increase duplex stability, nuclease resistance and cell permeation. SmartBase™ can be incorporated based on the following observations

- 2'- OH is not required for siRNA to enter the RNAi pathway.
- The major groove of the A-form helix is required for RNAi.
- Guide strand 5' end (Antisense) has a seed region of 2-8 bases that should be A-U rich. Modified siRNAs that stabilize A–U base-pair interactions can induce RNAi.
- Modified siRNAs enter into the RNAi pathway in vitro.
- Phosphorothioate increases stability but with slight decreased or no effect on silencing.
- Add 3'-thiol for conjugation to cell penetrating peptides (CPPs).

SmartBase™ siRNA Recommended Modifications

- Alternating 2'-F bases and 2'OMe bases in siRNA enhances duplex stability and are more resistant to RNase degradation.
- Use a few 2'OMe bases in the seed region of the guide strand to decrease the Tm below 21.5 of this region. 2'O methyl base hybridization with RNA has a lower TM. (5' end of guide or antisense strand has a seed region of 2-8 bases that should be A-U rich). Modified siRNAs that stabilize A–U base-pair interactions can induce RNAi.
- Phosphorothioate linkages confer oligonucleotides resistance to nuclease degradation.
- Incorporate 2'-F bases, 5-me dC or 2-amino dA preferentially at the 5' end of the sense strand to block incorporation of the sense strand in to the RISC.
- 2'F U and C substituted siRNA are more resistant to RNase degradation.
- 3' Cholesterol modification helps in cellular uptake. Alternates are PEG and long chain spacers.
- 5' or 3' GalNAc C3 modification for targeted liver delivery.

SmartBase™ siRNA and ASO Design Service

Gene Link offers SmartBase™ siRNA and ASO design services for a fee. You simply give us the accession number or sequence for the gene to down regulate. Gene Link designs siRNA's using published criteria's and proprietary algorithms. Gene Link guarantees a minimum of 70% silencing of your gene with at least one of the siRNA supplied. Three target siRNA will be supplied.

SmartBase™ siRNA and ASO design service is only available for Human and Mouse Reference Sequence (RefSeq) Accession Numbers.

- 3 specific target siRNAs custom synthesized by Gene Link. Each siRNA is supplied annealed and ready to use after reconstitution. Additional charges for synthesis. Visit this link for pricing information.

- Guarantee: Gene Link guarantees a minimum of 70% silencing of your gene with at least one of the siRNA supplied. Gene Link will synthesize another set of 3 siRNA if evidence is provided of none of the provided siRNA was functional in achieving a minimum of 70% silencing.

- Let Gene Link design and supply the custom RNAi Explorer™ and you simply knock out a gene!

Contact Gene Link technical services for expert help in designing custom SmartBase™ siRNAs
support@genelink.com

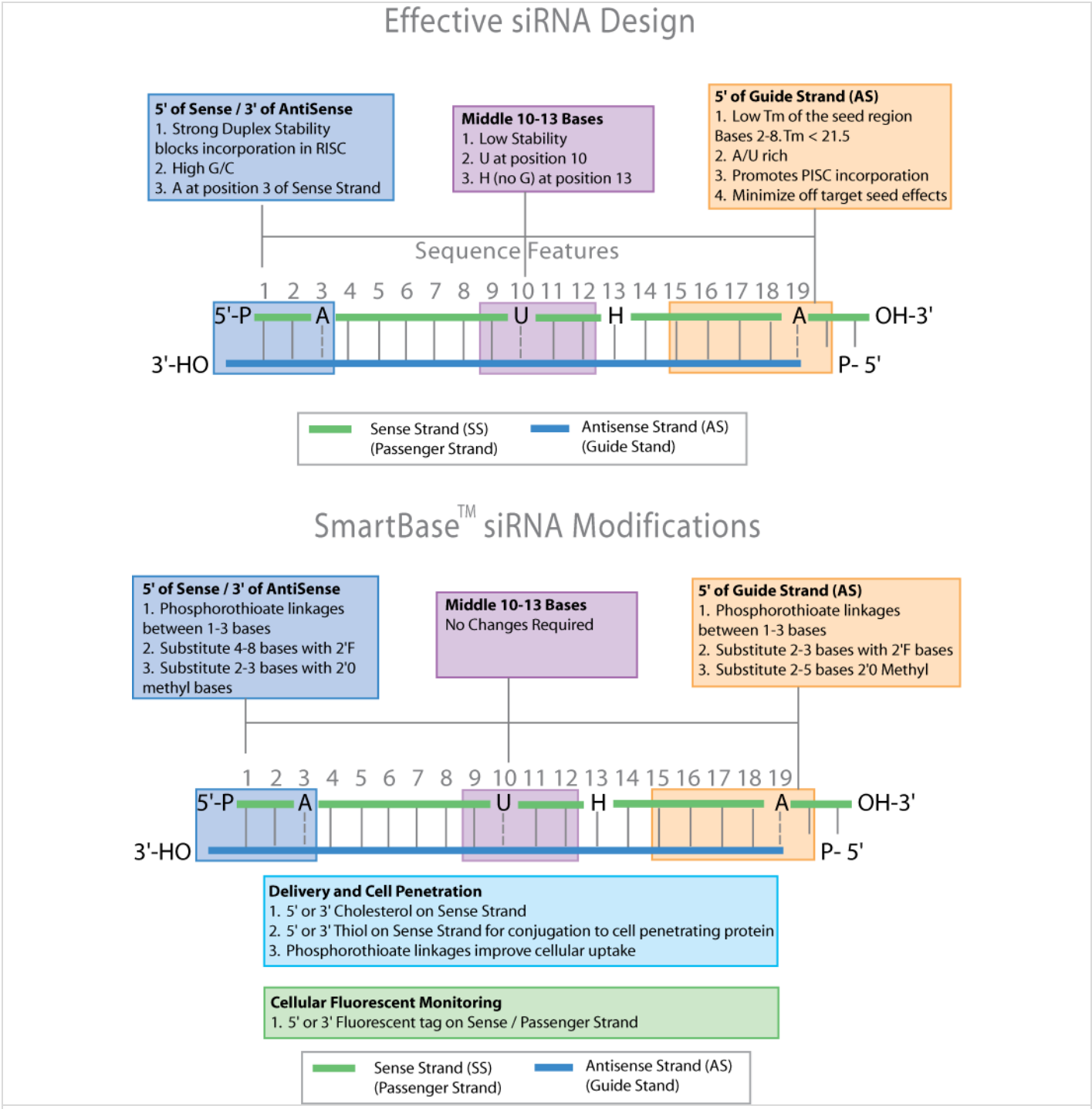


Figure 2. Effective siRNA design features based on sequence requirements and suggested modified bases to impart specific desired characteristics.

Increased Duplex Stability and Manipulation of Duplex Stability

Specific and stable hybridization of the oligo to its cognate sequence is the desired outcome of a successful experimental protocol. The melting temperature of the oligo dictates the strength of the affinity and thus the stability of the hybridization. Manipulation of the oligo sequence to increase the duplex stability or in some cases to decrease the duplex stability in certain loop structure will lead to oligos with increased affinity for the target molecule. There are many nucleic acid modifiers that increase duplex stability, examples are 5-methyl dC, 2-amino dA, locked nucleic acids etc.

A summary is presented in the table below. Gene Link does not presently offer LNA substituted oligo synthesis due to licensing issues and as such LNA base modifications are not included in this guide.

Increased Nuclease Resistance

As with most natural molecules synthetic DNA and RNA oligos are prone to degradation under normal conditions, specifically once introduced in body fluids. Ubiquitous nucleases as well as chemical instability lead to fast degradation with a finite half life. Nucleic acids are degraded rapidly once introduced in bodily fluids, RNA are more susceptible to degradation under normal laboratory conditions particularly due to RNase contamination. Special precautions must be taken to prevent RNA degradation. Nuclease resistant modifications can be introduced chemically in oligonucleotides that still retain its molecular structure and its shape based molecular interaction. These modifications are almost indispensable and have been used intensely in antisense applications. Also nucleic acids with mirror image chemistry have been developed that evade natural nucleases completely. Examples are converting the normal phosphodiester linkages to phosphorothioate or phosphorodithioate linkages, 2'O methyl, propyne bases etc.

Antisense oligonucleotides refer to short, synthetic oligonucleotide that are complementary in sequence and upon specific hybridization to its cognate gene product induces inhibition of gene expression. Oligonucleotides, as short as 15 mer have the required specificity to inhibit gene expression of a particular gene by annealing to the cellular mRNA (1,2). The mechanism of gene expression inhibition is based on two properties; the first is the physical blocking of the translation process by the presence of the short double stranded region, secondly the presence of the RNA-DNA duplex is susceptible to cellular RNase H activity. RNase H cleaves the RNA-DNA duplex region of the mRNA thus preventing the faithful translation of the mRNA (3).

The stability of the RNA-DNA duplex in terms of hybridization and half-life is crucial to successful gene inhibition. Vigorous research activity in the area of nucleic acid chemistry has been devoted in developing novel base analogs that are resistant to degradation and that possess strong hybridization properties. This product profile aims at listing some analogs that meet the above criteria and are amenable to be synthesized by currently available standard DNA synthesis chemistry. This includes the classical phosphorothioate linkages (4), propyne analogs (5) and 2'Fluoro bases.

RNA interference studies have shown the effectiveness of short interfering RNA (siRNA) in gene silencing. siRNA technology is now extensively recognized as a powerful tool for the specific suppression of gene expression and is presently being used by researchers in a wide range of disciplines for the assessment of gene function. These are generally 21mer double stranded RNA. Active research to render the siRNA more stable to degradation and to increase the duplex stability has led to the use of modified bases. 2'O methyl and/or 2'Fluoro bases are an attractive substitute together with phosphorothioate linkages to impart greater duplex stability and resistance to nuclease degradation

Gene Link offers an extensive array of modifications to accomplish duplex stability and nuclease resistance to synthetic oligos. We can synthesize complex combinations of modifications, chimeric oligos and fluorescent probes. In addition to the synthesis of these modified oligos, we routinely assist customers in the design of the oligos that are particularly suited to their application.

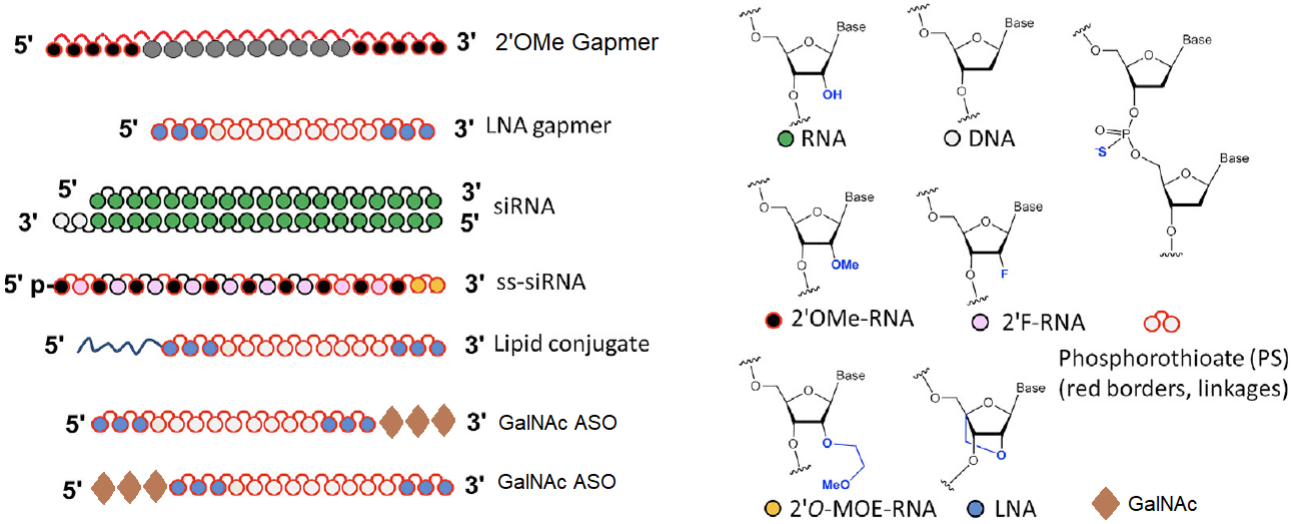
Duplex Stabilization

Using these base substitutions, duplex stability and therefore melting temperatures are raised by the approximate amounts shown below.

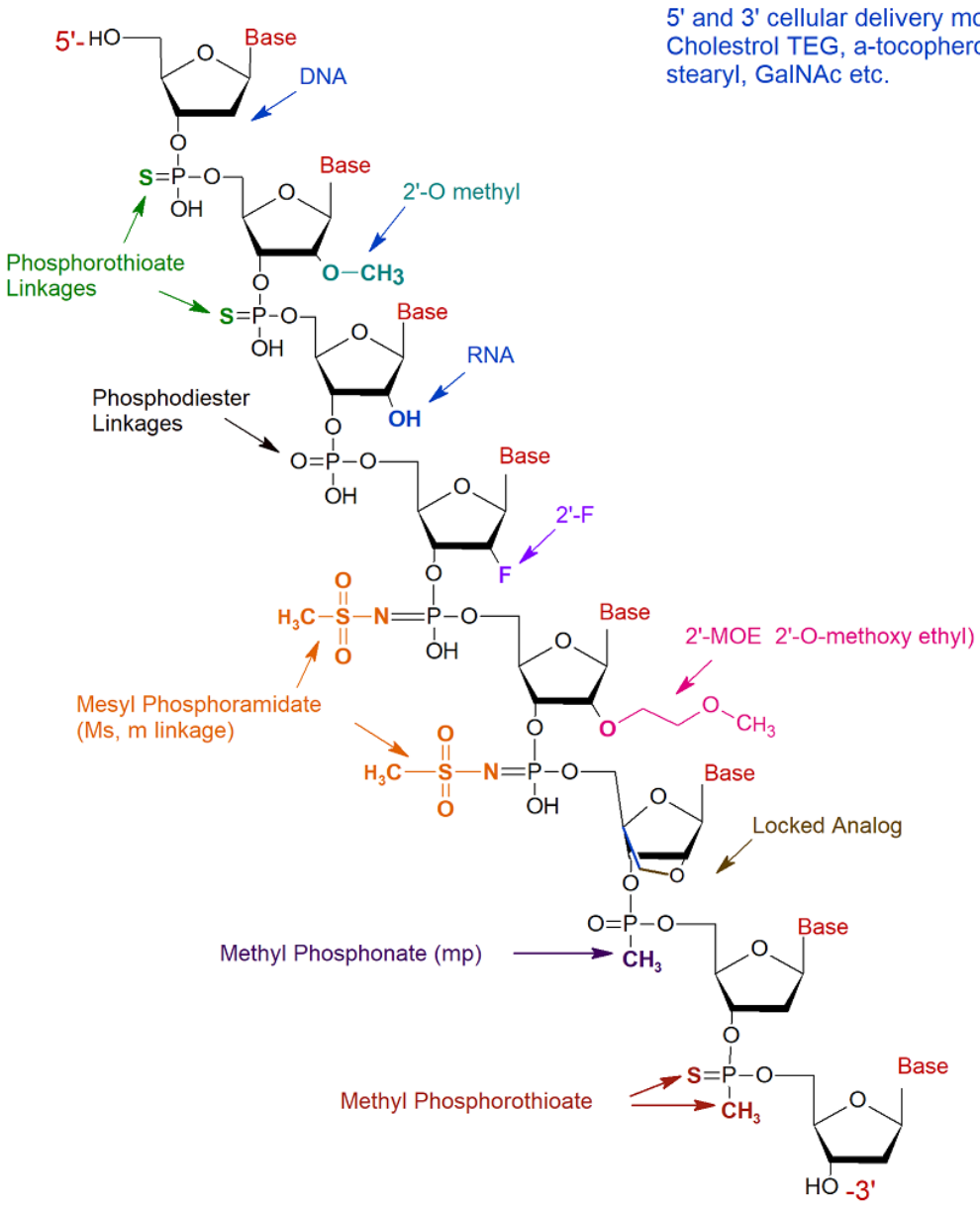
Modifications Increasing Duplex Stability and Nuclease Resistance		
Modification*	**Duplex Stability [Tm Increase]**	**Nuclease Resistance**
Phosphorothioate	Slightly decreased	Increased
2'-OMethyl	Increased	Increased
2'-Fluoro	Increased [1-2° per substitution]	Increased
2-Amino-dA	Increased [3.0° per substitution]	No effect
5-Methyl-dC	Increased [1.3° per substitution]	No effect
C-5 propynyl-C	Increased [2.8° per substitution]	Increased
C-5 propynyl-U	Increased [1.7° per substitution]	Increased
*Selected list of modifications available from Gene Link, Inc. are listed in this table. Visit www.genelink.com for complete offerings.		

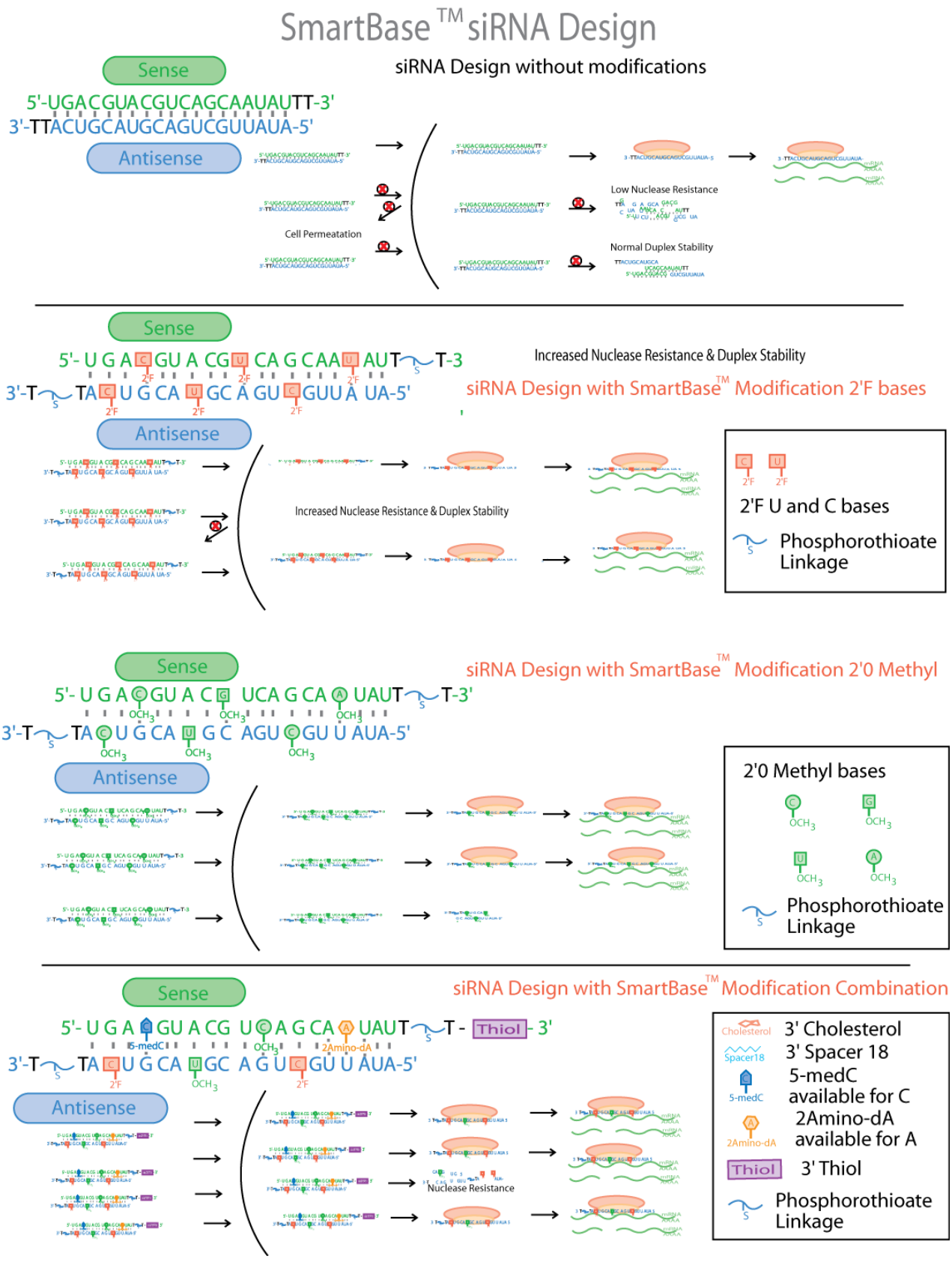
Careful selection of modifications and verifying the performance of modified siRNA is required; the guidelines presented are based on documented physical and chemical properties of the modifications. Design rules may have to be established empirically for very specific or novel assay settings but following these recommendations will provide a good start.

SmartBase™ siRNA & ASO Modifications



Antisense Oligos, siRNA, Antagomir (anti-miRNA, blockmers) Oligo Modifications





References

1. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391:806-811.
2. Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411:494-498.
3. Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W. & Khvorova, A. 2004. Rational siRNA design for RNA interference. Nat. Biotechnol. 22:326–330.
4. Amarzguioui, M., Lundberg, P., Cantin, E., Hagstrom, J., Behlke, M. & Rossi, J. 2006. Rational design and in vitro and in vivo delivery of Dicer substrate siRNA. Nature Protocols. 1:508-517.
5. Naito. Y., Yoshimura, J., Morishita, S. and Ui-Tei, K. 2009. siDirect 2.0: updated software for designing functional siRNA with reduced seed-dependent off-target effect. *BMC Bioinformatics* 10:392.
6. Wang, H., Ghosh, A., Baigude, H., Yang, C-S., Qiu, L., Xia, X., Zhou, H., Rana, T.M. and Xu, Z. 2008. Therapeutic Gene Silencing Delivered by a Chemically Modified Small Interfering RNA against Mutant SOD1 Slows Amyotrophic Lateral Sclerosis Progression. J. Biol. Chem. 283: 15845-5852.
7. Jackson, A. L., Burchard, J., Schelter, J., Chau, B.N., Cleary, M., Lim, L. and Linsey, P.S. (2006) Widespread siRNA ‘‘off-target’’ transcript silencing mediated by seed region sequence complementarity.RNA 12:1179–1187.
8. Bramsen, J.B., Laursen, M.B., Nielsen, A.F., et al. 2009. A large-scale chemical modification screen identifies design rules to generate siRNAs with high activity, high stability and low toxicity. Nucleic Acids Res. 37:2867–2881.
9. Vaish, N., Chen, F., Seth, S. et al. 2011. Improved specificity of gene silencing by siRNAs containing unlocked nucleobase analogs. Nucleic Acids Res. 39:1823–1832.

*RNAi and siRNA
RNA interference (RNAi) is a specific and sequence dependent targeted gene silencing activity. RNAi acts by post transcriptional degradation of mRNA by small interfering RNAs (siRNA’s) of the same sequence. The silencing approaches 100% and has to be empirically determined and optimized. Not every siRNA can effectively down regulate a gene. The process of RNA interference varies by individual siRNA while some do not exhibit any interference at all.

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