



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)



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.

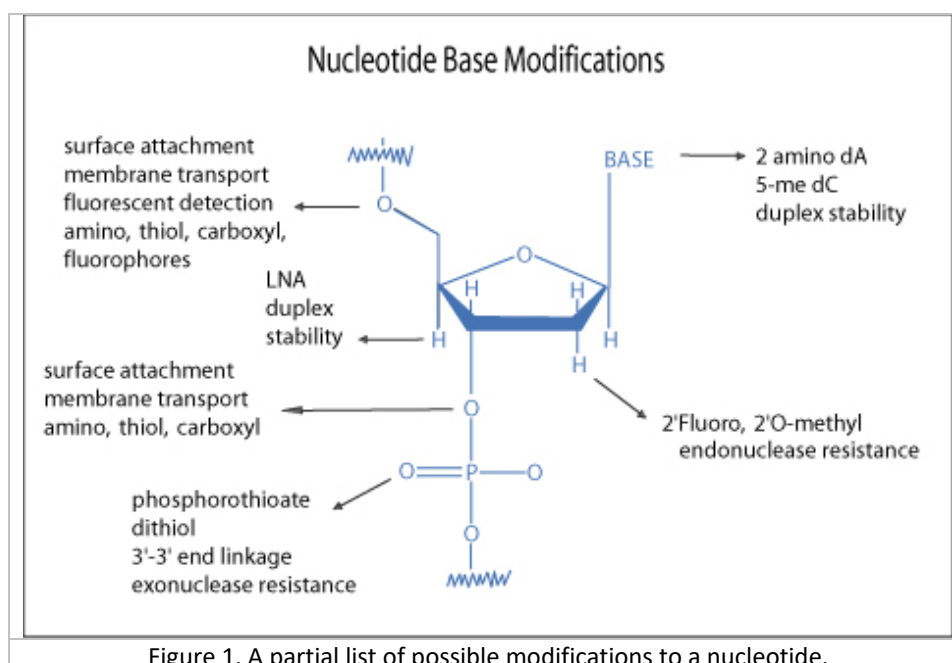
There are several other factors that influence effective functional performance of the designed siRNA; half-life of the siRNA is crucial for sustained activity and in many cases to increase effect for a longer time the dosage (concentration) is increased that leads to toxicity and off target effects. 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 T_m below 21.5 of this region. 2'O methyl base hybridization with RNA has a lower T_m. (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.

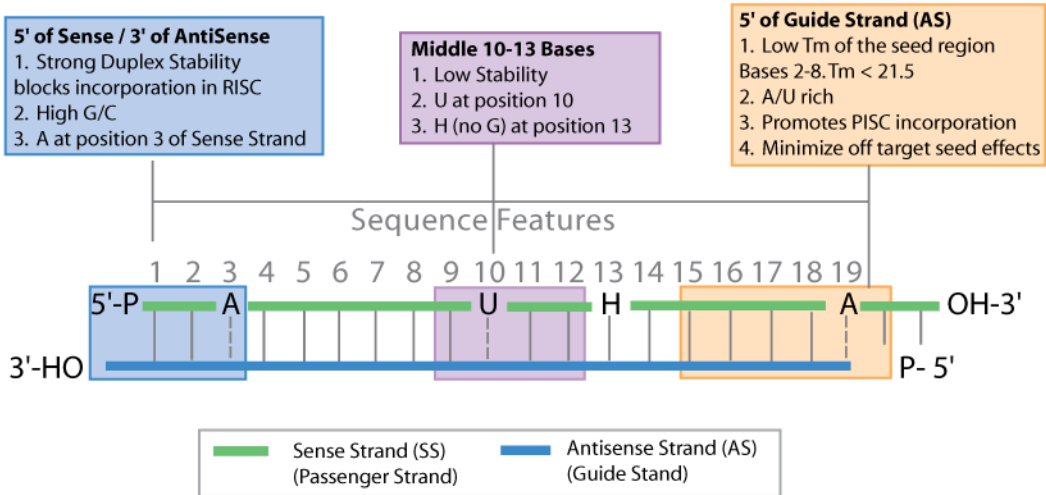
- 3 specific target siRNAs custom synthesized by Gene Link. Each siRNA is supplied annealed and ready to use after reconstitution. Additional charges for synthesis. Visit this [link](#) for pricing information.

- 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

Effective siRNA Design



SmartBase™ siRNA Modifications

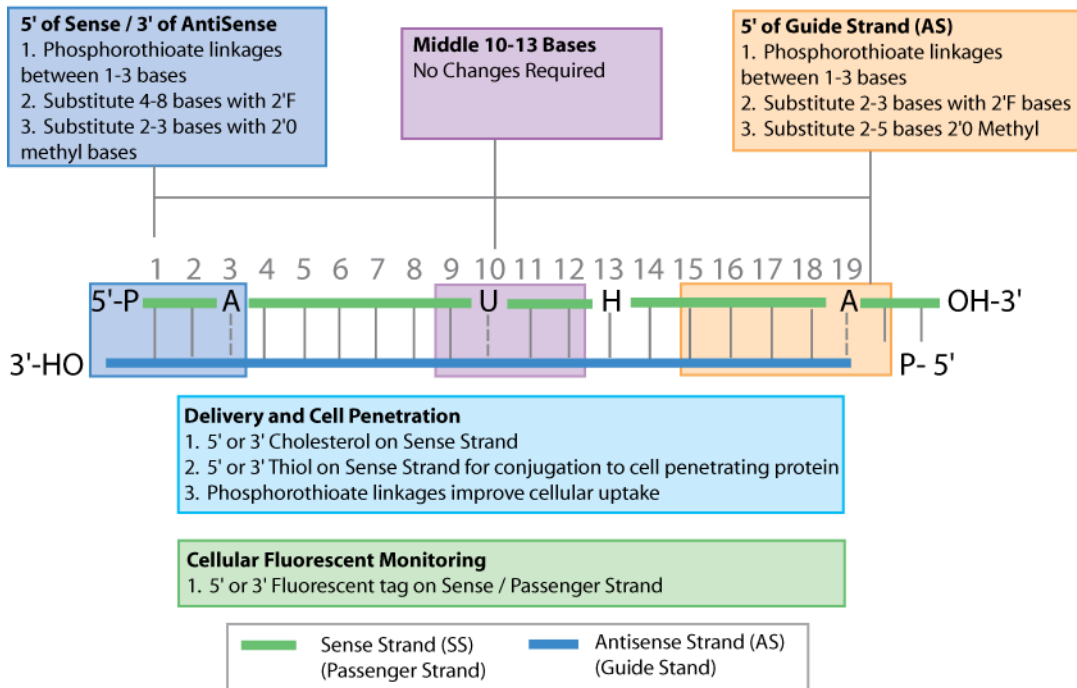


Figure 2. Effective siRNA design features based on sequence requirements and suggested modified bases to impart specific desired characteristics.

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

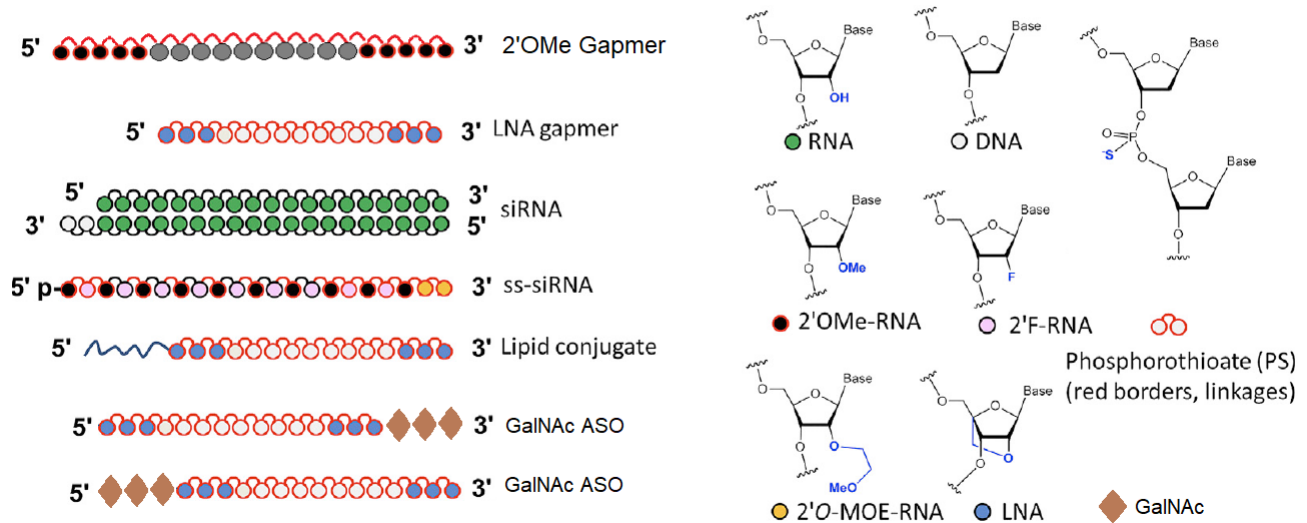
Using these base substitutions, duplex stability and therefore melting temperatures are raised by the approximate amounts shown below.

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

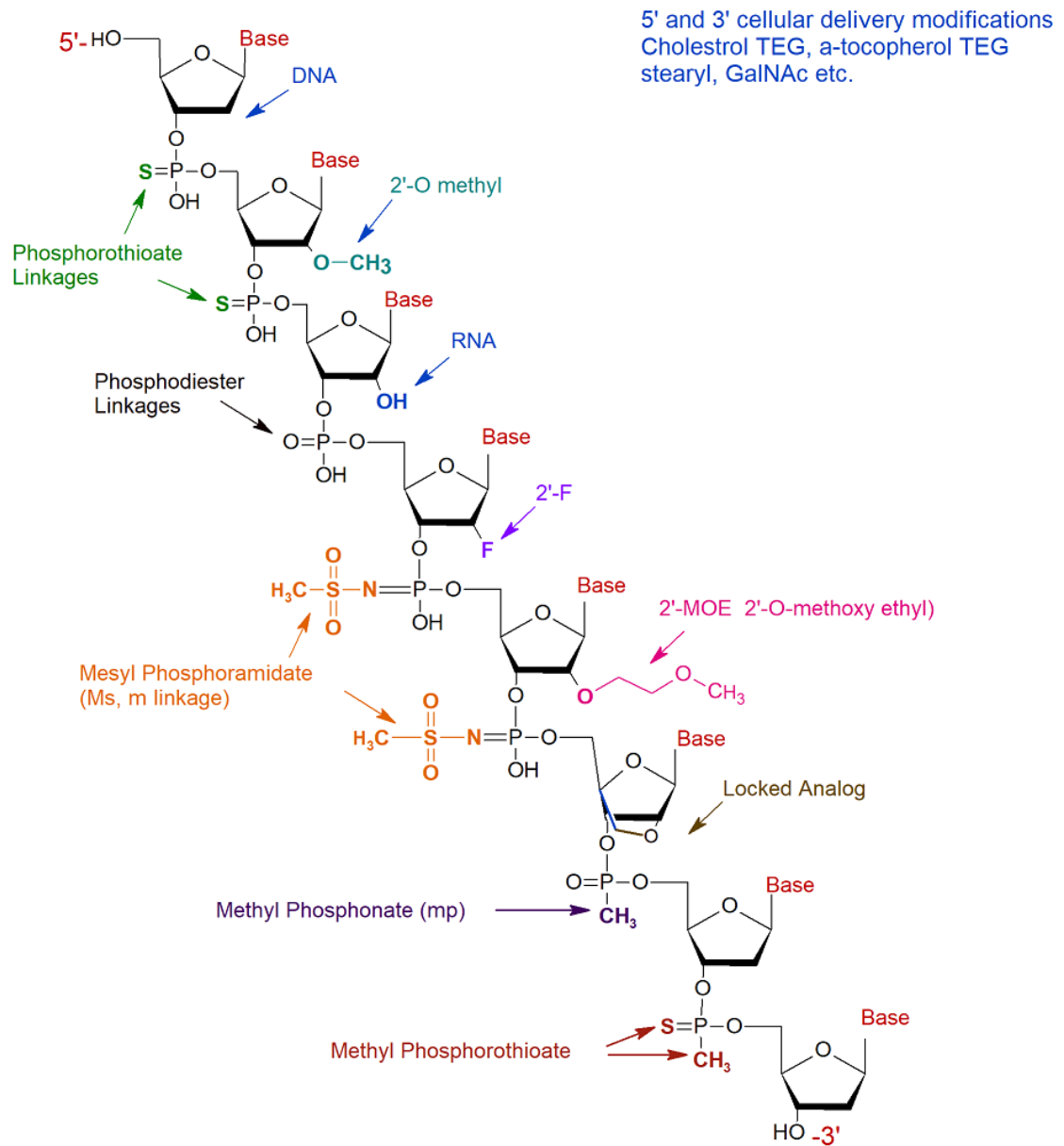
*Selected list of modifications available from Gene Link, Inc. are listed in this table. Visit www.genelink.com for complete offerings.

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

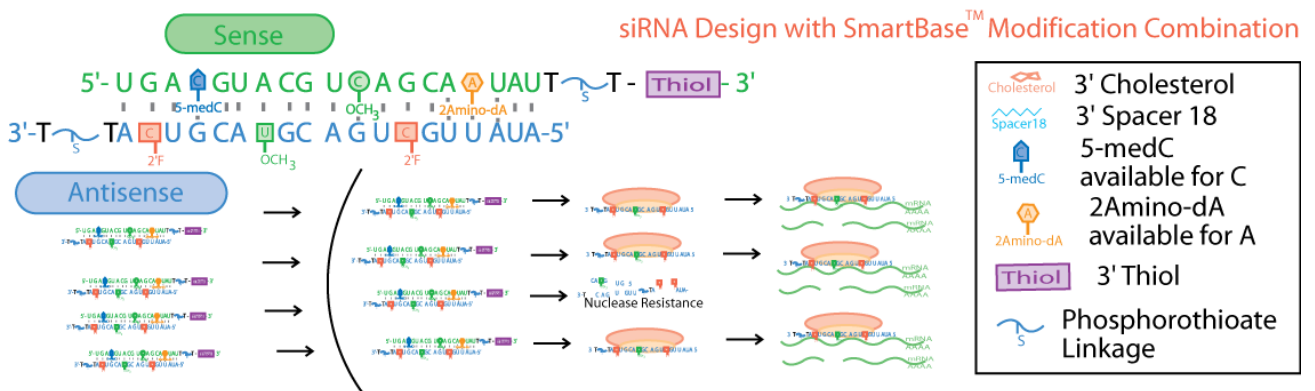
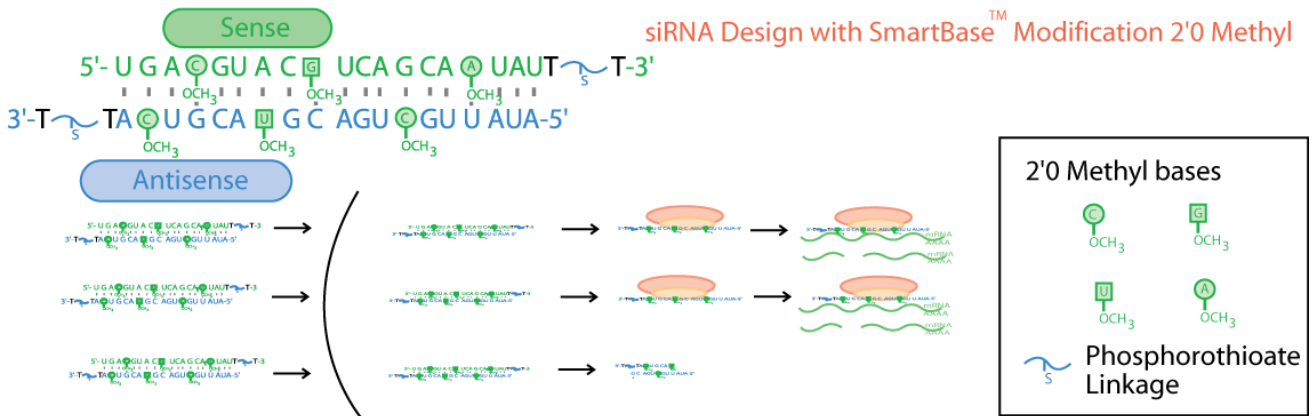
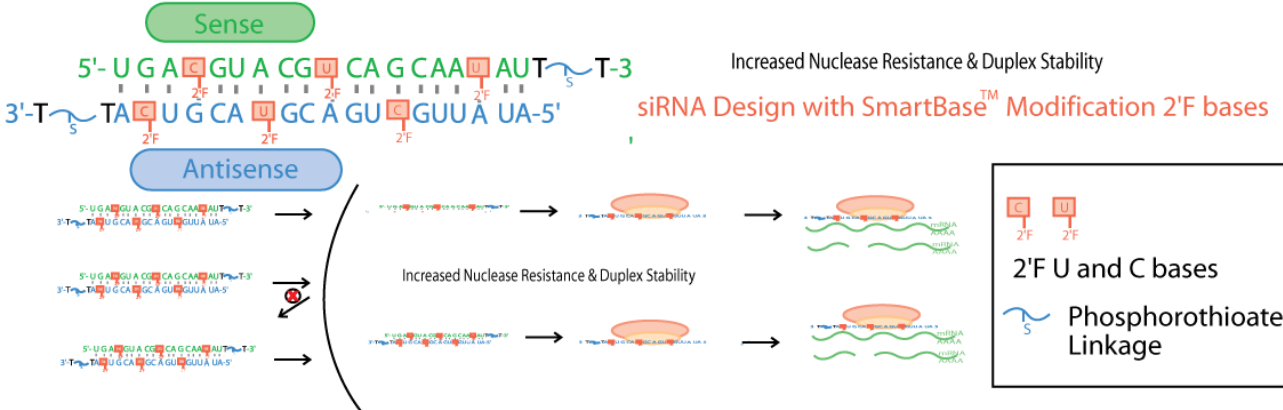
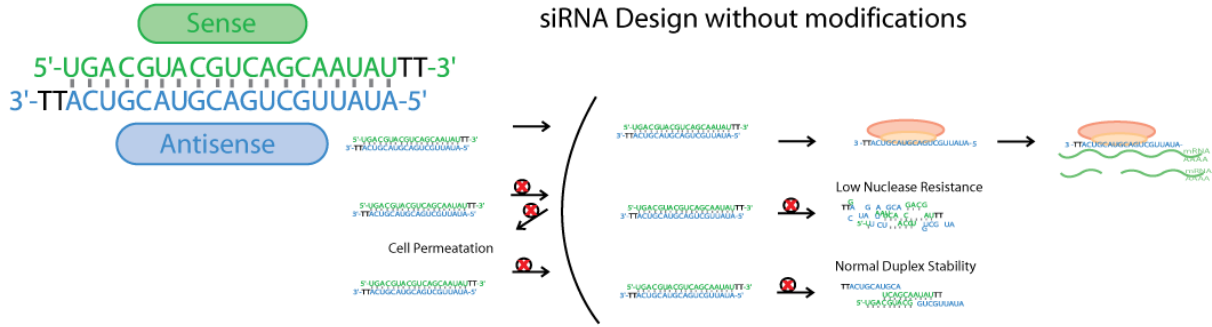


Antisense Oligos, siRNA, Antagomir (anti-miRNA, blockmers) Oligo Modifications



SmartBase™ siRNA Design

siRNA Design without modifications



References

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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.

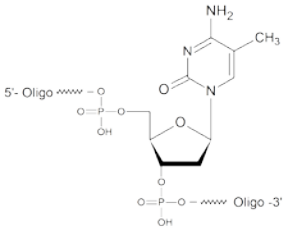
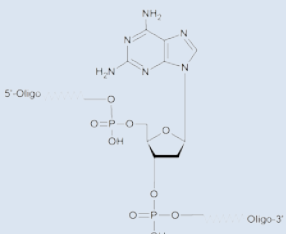
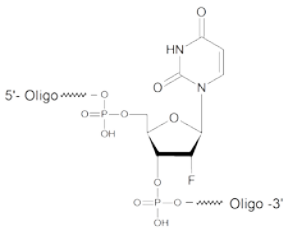
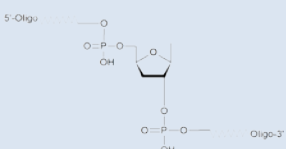
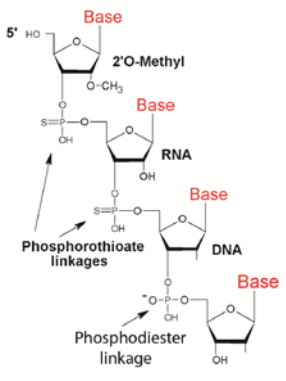
Appendix

SmartBase™ Modifications Summary

Modification*	Molecular Structure	Duplex Stability	Nuclease Resistance	Chemical Characteristics
Phosphorothioate		Hybridizes to the target sequences with lesser affinity than oligos with phosphodiester backbone	Imparts resistance to nuclease degradation	Modification of the phosphodiester bond by replacing one of the non-bridging oxygens by sulfur
Propyne Analogs				
2'-O methyl RNA		Binding similar to DNA bases	Increased	2'-O-methyl at the 2' hydroxyl position
5-me-dC		Increased	Similar to DNA	C-5 methylated dC

RNAi: Smart Base siRNA Design

For research use only. Not for use in diagnostic procedures for clinical purposes.

	 <p>5-Methyl-dC [26-6413-XX]</p>			
2-Amino dA	 <p>2-Amino dA [26-6525-XX]</p>	Increased	Similar to DNA	2-amino dA
2'-F bases	 <p>2'-F-U</p>	Increased	Substantially increased	Fluoro at the 2' position
2'-5' linked oligos		Increased binding efficiency	Increased	2'-5' phosphodiester linkages and 3' deoxy.
Chimeric		Substantially increased	Substantially increased	Properties based on modifications used.
<p>*Select list of modifications listed in this table. Visit www.genelink.com for complete offerings.</p>				

Phosphorothioate

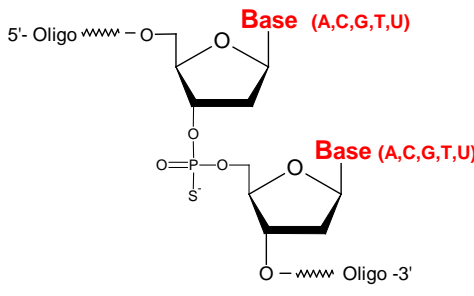
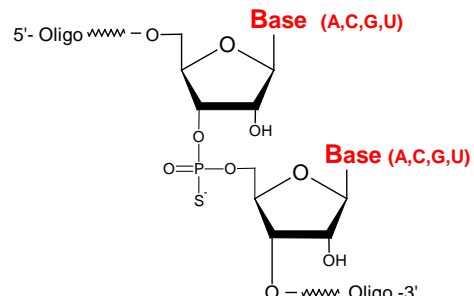
The driving force for the search for novel chemical modification groups compatible with Watson-Crick hybridization of oligonucleotide was based on the observation of the short stability of naturally occurring oligonucleotides with phosphodiester bonds. Oligonucleotides with natural phosphodiester bonds are highly susceptible to rapid degradation by cellular nucleases. Cellular nucleases have endonuclease activity as well such that 3' and 5' end caps are not sufficient to prevent from degradation.

Modifications of the phosphodiester bond by replacing one of the non-bridging oxygen by sulfur imparts resistance to nuclease degradation, but in general hybridize to the target sequences with lesser affinity than the phosphodiester counterpart. The sulfur-substituted oligonucleotides have a phosphorothioate linkage and are termed as **phosphorothioates** or simply as **S-oligo**. Phosphorothioate oligos are synthesized by Gene Link using the Beaucage sulfurizing reagent. The sulfurization reaction is rapid and is performed on automated DNA synthesizers yielding greater than 96% phosphorothioate linkages; the remainders are phosphodiester linkages. Custom phosphorothioate oligonucleotides synthesized by Gene Link can be specified to have all the diester bonds substituted or only some selected diester linkages depending upon the researcher's experimental requirement. Substitution of all diester linkage is recommended to provide greater nuclease resistance.

The most common usage for oligonucleotide phosphorothioates has been in the production of antisense oligodeoxynucleotides destined for use in identifying or modifying gene expression. Now, phosphorothioate linkages are popping up in the RNA world and sulfurizing RNA linkages with reagents like Beaucage Reagent has proved to be much more difficult than DNA linkages. The phosphorothioate (PS) linkage is a not-so-expensive way of increasing the stability of nucleic acids and increasing nuclease resistance of RNA. It has been shown that fully PS oligos can promote the delivery of siRNA in cell culture. This siRNA uptake is sequence-independent and the length seems to vary between 30 and 70 nucleotides depending on the cell line. Even though this method is not yet as efficient as the cationic lipids, it opens the way to possible new methods. Reasons that may explain this are not understood at this time.

A method for the inactivation of micro RNA (miRNA) that may help to elucidate their functions has been described, it uses 2'-OMe-RNA oligonucleotides (23-mers, complementary to a target miRNA) with a cholesteryl group at the 3' terminus and phosphorothioates at positions 1 and 2 at the 5' end and at the last four positions at the 3' end. These oligos are called antagomirs. These molecules promote the cleavage of complementary miRNAs and thus should allow analysis of their function. The role of the PS linkages presumably is the stabilization against degradation in the mouse experiments as it is standard in the antisense field in such in vivo situations. And finally, a recent paper shows that PS does not systematically abolish siRNA activity, opening the way for some potentially less expensive stabilization of such molecules. Incorporation of 2'-OMe (in the sense strand) in combination with PS linkages should confer to siRNA increased resistance to degradation by nucleases, as well as prolonged serum retention. And it is also possible that such easy modification of siRNA may increase the specificity by eliminating sense strand recruitment in the RISC complex and thus reducing a source of off-target effect.

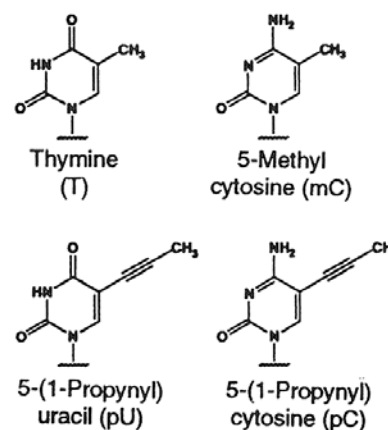


 <p style="text-align: center;">Phosphorothioate Linkage [26-6401-XX]</p>	 <p style="text-align: center;">Phosphorothioate Linkage [26-6401-XX]</p>
<p style="text-align: center;">Chimeric Phosphorothioate Linkages (DNA, RNA, LNA, 2'O Me and other bases)</p>	<p style="text-align: center;">RNA phosphorothioate Linkage</p>

Propyne* Analogs

It has been shown that C-5 propyne analogs of dC and dT when substituted in phosphorothioate oligonucleotide imparts greater inhibition of gene expression due to increased binding affinity to the target mRNA and increased stability (5). Based on the above information antisense oligonucleotide could either be Phosphorothioated at all diester linkages or combined with substitutions of dC and dT by C-5 propyne analogs pdC and pdU.

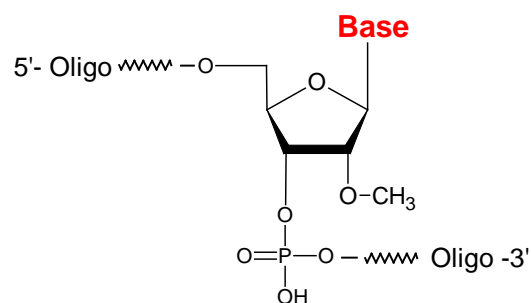
The use of propyne analogs is covered by patents and licensing agreements. The sale of propyne-modified oligos is for research use only. See license agreement*



2'-O-methyl RNA oligonucleotides

RNA oligos are susceptible to degradation to the same extent as native RNA extracted from various sources. An attractive alternate to prevent degradation from nucleases is the use of 2'-O- methyl RNA bases, when specific 2'OH is not required. The 2'-O- methyl oligonucleotides confer considerable nuclease resistance and are similar in hydrogen bonding properties to RNA/RNA than the lower RNA/DNA binding property (7). The coupling efficiency of 2'-O- methyl phosphoramidite is also higher than the RNA monomers resulting in higher yield of full-length oligos.

Gene Link also offers custom synthesis of RNA and DNA chimeric oligos with investigator specified ribo or deoxy bases or 2'-O-methyl bases.

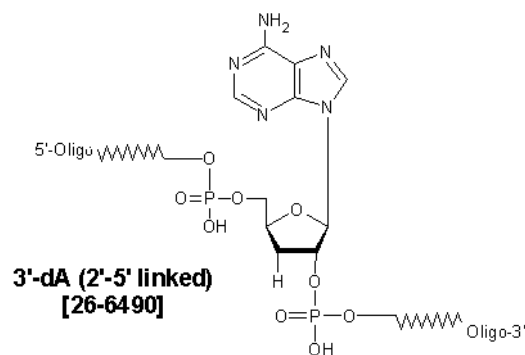


2' O-Methyl Base
[27-6410-XX]

The chimeric oligos can also be synthesized with the regular phosphodiester bonds or substituted with phosphorothioate linkages. The combination of 2'-O- methyl RNA bases with phosphorothioate internucleotide linkages imparts these oligos greater nuclease resistance, which is particularly useful for antisense studies. Custom phosphorothioate oligonucleotides synthesized by Gene Link can be specified to have all the diester bonds substituted or only some selected diester linkages depending upon the researcher's experimental requirement. Substitution of all diester linkage is recommended to provide greater nuclease resistance.

2'-5' Linked Oligonucleotides

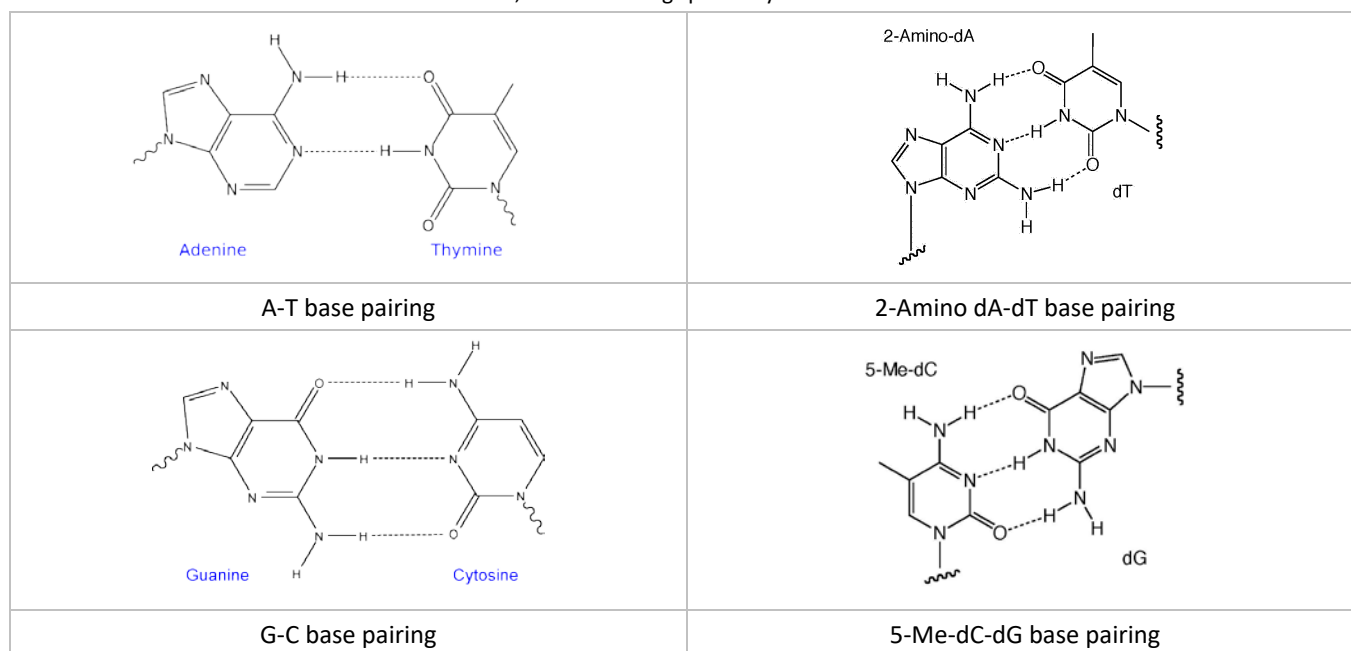
Cellular DNA and RNA are made up of ribo- and 2'-deoxyribonucleic acids linked together via 3'-5' phosphodiester linkages and by far comprise the bulk of polynucleic acids found in cells. Much less common are oligonucleotides which have 2'-5' linkages. However, a unique feature of 2'-5' linked oligonucleotides is their ability to bind selectively to complementary RNA (12-13). These features suggest a number of interesting uses for 2'-5' linked oligos such as their use as RNA specific probes or in antisense oligos.



2-Amino-dA & 5-Me-dC

The underlying principle of genetic molecular interaction is Watson and Crick base pairing. Consistent efforts have been expended to introduce different modifications to the bases to increase duplex stability in turn making the hybridization stronger. Two such modifications are discussed below that can be easily substituted in almost all primer, oligo, probe and antisense oligonucleotide design.

As shown in Figure below, A-T base pairs have two hydrogen bonds whereas G-C base pairs have three hydrogen bonds. The simplest approach to improving primers would be to substitute A sites with 2-amino-A which forms three hydrogen bonds with T on hybridization. 2-Amino-A also destabilizes A-G wobble mismatches, thus increasing specificity.



2-Amino dA & 5-Methyl dC Duplex Stability and Nuclease Resistance		
Modification*	Duplex Stability [T _m Increase]	Nuclease Resistance
2-Amino-dA	Increased [3.0° per substitution]	No effect
5-Methyl-dC	Increased [1.3° per substitution]	No effect
<p>*Selected list of modifications available from Gene Link, Inc. are listed in this table. Visit www.genelink.com for complete offerings.</p>		

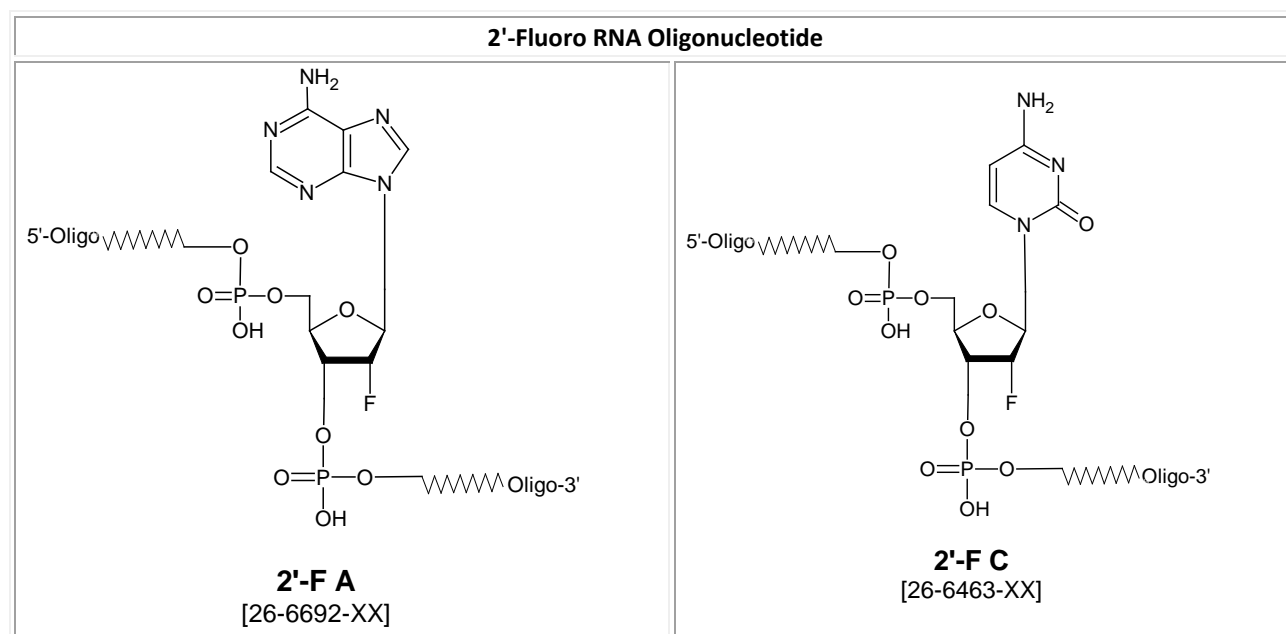
2'-F-RNA Monomer

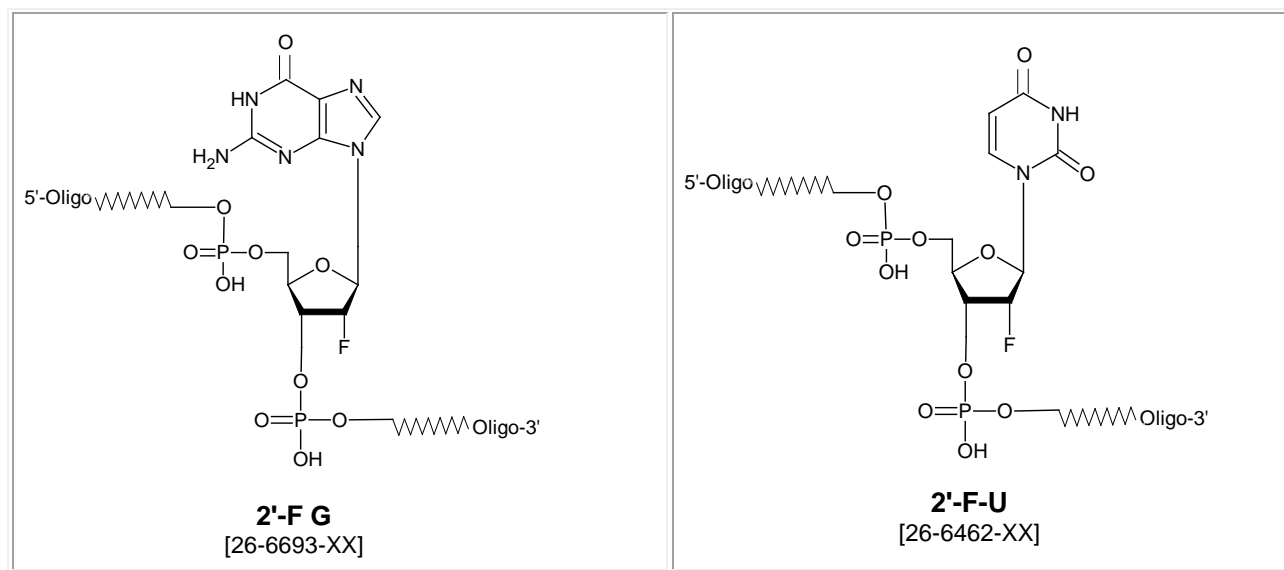
2'-Deoxy-2'-fluoro-nucleosides adopt an RNA-type sugar conformation, presumably due to the high electronegativity of fluorine. Because of this sugar conformation, RNA duplexes (A-form) are generally more thermodynamically stable than DNA duplexes (B-form). As expected, the addition of 2'-F-RNA residues to oligodeoxynucleotides progressively increases the thermal stability of their duplexes with RNA. The stabilization is additive at approximately 2° per residue. This compares favorably with 2'-OMe-RNA at around 1.5° and RNA at 1.1° per residue. In the meantime, base pair specificity remains intact.

2'-F-RNA phosphodiester linkages are not nuclease resistant, although the corresponding phosphorothioate linkages are highly resistant. Researchers usually design antisense oligonucleotides to form duplexes with RNA, which are then substrates for RNase H. Uniformly modified 2'-F-RNA/RNA duplexes are not substrates for RNase H. However, it is straightforward to prepare chimeric 2'-F-RNA/DNA phosphorothioate oligonucleotides which exhibit enhanced binding to the RNA target, are substrates for RNase H, and are highly nuclease resistant.

Fluorine has an interesting combination of properties, combining electro negativity similar to a hydroxyl group with size between oxygen and a hydrogen atom. This combination leads to the ring of a 2'-F-ribonucleoside adopting a C3'-endo conformation and the resulting 2'-F-RNA oligonucleotide adopts an A-form helix on hybridization to a target. Indeed, circular dichroism (CD) spectra of 2'-F-RNA/RNA duplexes indicate that they are A-form and that the sugars have all adopted the C3'-endo pucker. An important difference between RNA and 2'-F-RNA is that a hydroxyl group is a hydrogen bond donor while fluorine is a weak acceptor.

In studying antisense oligonucleotides, a group at Isis Pharmaceuticals concluded that oligonucleotides hybridized to a target RNA oligonucleotide in the following order of increasing stability: DNA < RNA < 2'-OMe-RNA < 2'-F-RNA. With an RNA target, melting temperature (T_m) was enhanced relative to an antisense DNA oligonucleotide by 1°C per residue for RNA, 1.3 °C for 2'-OMe-RNA, and 1.8 °C for 2'-F-RNA. The stability enhancement for 2'-F-RNA hybridizing to an RNA target was additive for each 2'-F-RNA residue and slightly cooperative – i.e., the ΔT_m per substitution increases as more 2'-F-RNA residues are incorporated into the oligonucleotide. This has led to the use of 2'-F-RNA in aptamers since the resulting aptamers are not only more resistant to nucleases compared to 2'-OH RNA aptamers, but also bind ligands with higher affinities. The use, however, of 2'-F-RNA in antisense applications is limited since the 2'-F-RNA exhibits little enhanced nuclease resistance compared to DNA and its hybrid duplex does not activate RNase-H.





It has been demonstrated that siRNA synthesized with 2'-F pyrimidines showed greatly increased stability in human plasma compared to 2'-OH siRNA. They were functional in cell culture and in vivo using BALB/c mice transfected with pGL3 luciferase. Interestingly, though the 2'-F siRNA was significantly more stable than 2'-OH siRNA, they were only slightly more inhibitory over time in cell culture than 2'-OH siRNA; in vivo, their activities were practically the same. The authors note that these results may depend upon the siRNA delivery methodology.

Less has been reported on the stability of duplexes between 2'-F-RNA and DNA. The cleavage of RNA/DNA duplexes by RNase H, 2'-F-Adenosine (2'-F-A) oligonucleotides and chimeras containing 2'-F-A and rA were used to evaluate the ability of the modified RNA strand to promote varying levels of RNase-H activity. The authors measured the T_m of 18-mer oligonucleotides containing rA and/or 2'-F-A to oligo-T18 and found that the homopolymer of 2'-F-A enhanced binding by 0.5° per residue relative to rA. However, chimeras of 2'-F-RNA and rA were unpredictable in their melting behavior and some actually lowered the duplex T_m .

Glen Research melting experiments of duplexes containing 2'-F-RNA supported these results. They found that a single substitution of 2'-F-RNA in a mixed base DNA/DNA dodecamer increased the T_m by 1.2 °C. However, further substitutions with two or four 2'-F-RNA residues led to a drop in the T_m by 1.3 °C. Interestingly, a fully substituted 2'-F-RNA/DNA duplex does exhibit higher stability, with the T_m being increased by 0.5° per incorporation.

Pyrimidine Analogues

C-5 methyl pyrimidine nucleosides are known to stabilize duplexes relative to the non-methylated bases. Therefore, enhanced binding can be achieved using 5-methyl-dC in place of dC, duplex melting temperature being increased by 1.3°. Improved stacking in this case is believed to be brought about by elimination of water molecules from the duplex. 2,6-Diaminopurine 2'-deoxyriboside (2-amino-dA) forms an additional hydrogen bond with Thymidine, thereby leading to duplex stabilization with a melting temperature increase of 3°.

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