Product Profile

Custom Oligo Synthesis, antisense oligos, RNA oligos, chimeric oligos,
Fluorescent dye labeled oligos, Molecular Beacons, siRNA,
2'-F bases; 2'-5' linked Oligos

Smart Oligo & Probe Design

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

Oligo Modifications for Increased Duplex Stability & Nuclease Resistance

Modifications

Propyne dC and dU labeled Oligos
Phosphorothioate Oligos
5-Me-dC & 2-amino dA
2'F bases
2'-5' linked Oligos
Methylated Oligos

Applications

Amplification Primers
Fluorescent Molecular Probes
Molecular Beacons
Taqman Probes
In situ hybridization
RNA Interference
Allelic Discrimination
Antisense Oligos
SNP Detection

Gene Link™

Oligo Modifications for Increased Duplex Stability & Nuclease Resistance

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Oligo Modifications for Increased Duplex Stability & Nuclease Resistance

Introduction

Gene Link presents various design options for synthesizing effective oligo and probes based on the application. Smart oligo and probes can be synthesized in a predetermined way to exhibit the features that is desired; for instance to increase cellular delivery we may add cholesterol to the synthetic oligonucleotide sequence or for nuclease resistance convert the phosphodiester linkages to phosphorothioate as is commonly used for antisense oligonucleotides (ODN's) or to add a short peptide sequence that is known to aid in transport of aptamers to the nucleus.

The premise of this product profile is to go beyond the standard DNA and RNA bases for constructing synthetic primers, oligos and probes and to develop a cross-disciplinary synergy of molecular applications to a wealth of nucleic acid chemistry tools available as modified bases.

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 particular characteristics.

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

The premise of this product guide is to discuss various modifications that are offered by Gene Link that may be used for oligo modification to enhance specific properties based on the application.

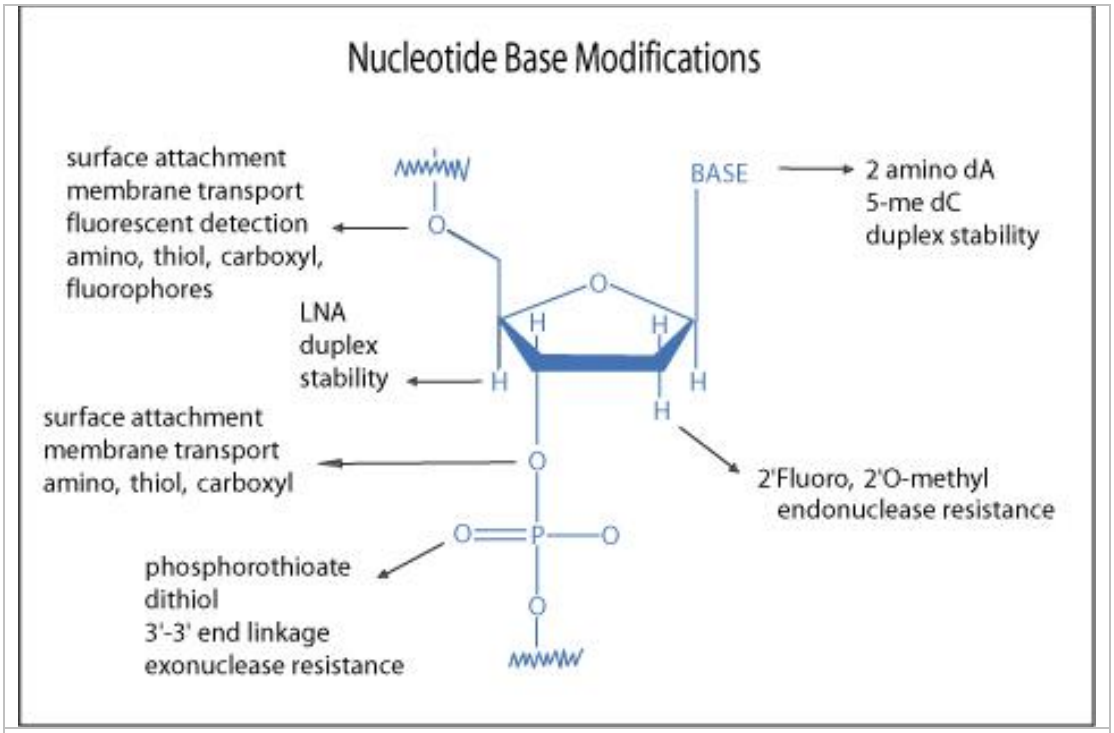
Common Features for Improvement

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

Common Modification Sites

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

Careful selection of modifications and verifying their performance 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 the recommendations will provide a good start.



A partial list of possible modifications to a nucleotide.

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

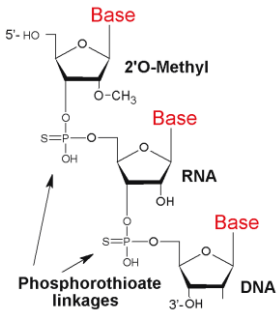
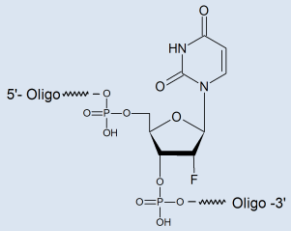
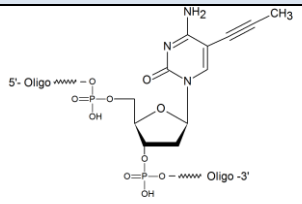
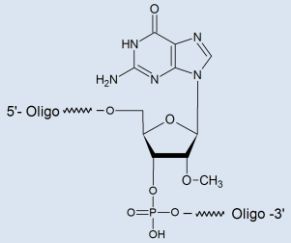
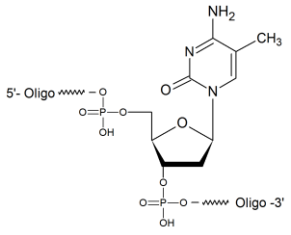
Gene Link offers an extensive array of modifications to accomplish duplex stability and nuclease resistance to synthetic oligos. We have the ability to synthesize complex combinations of modifications, chimeric oligos and fluorescent probes. In addition to the synthesis of these modified oligos, we routinely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

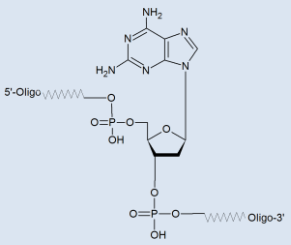
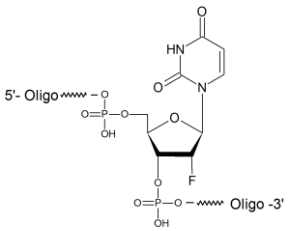
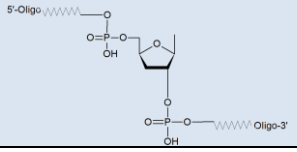
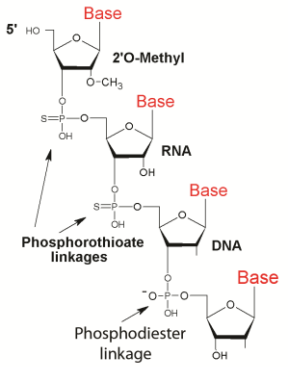
Duplex Stabilization

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.		

Modifications

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'-F-U	Increased binding affinity to the target sequence and increased stability	Increased nuclease resistance	C-5 propyne analogs of dC and dT
	 propyne dC [26-6501-XX]			
2'-O methyl RNA	 2' O-Methyl G [27-6410-XX]	Binding similar to DNA bases	Increased	2'O-methyl at the 2' hydroxyl position
5-me-dC	 5-Methyl-dC [26-6413-XX]	Increased	Similar to DNA	C-5 methylated dC

2-Amino dA	 **2-Amino dA** [26-6525-XX]	Increased	Similar to DNA	2-amino dA
2'-F bases	 2'-F-U	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.
*Select list of modifications listed in this table. Visit www.genelink.com for complete offerings.				

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 counter part.
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.

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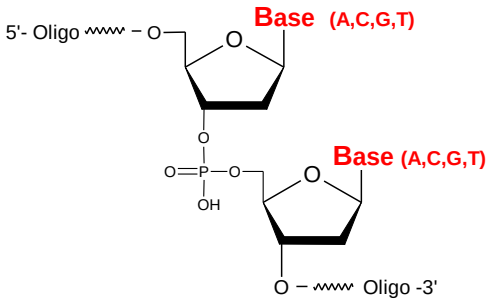
<td> 

Phosphodiester Linkage
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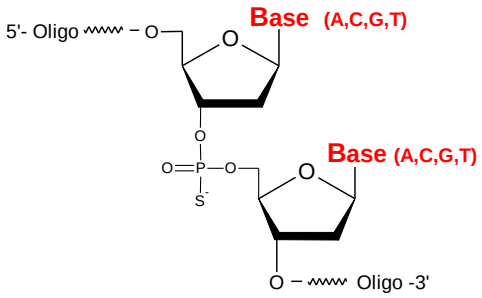
<td> 

Phosphorothioate Linkage
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<td>Phosphodiester Linkage</td>
<td>Phosphorothioate Linkage (PS)</td>
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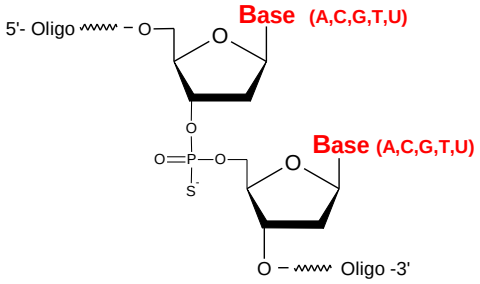
<td> 

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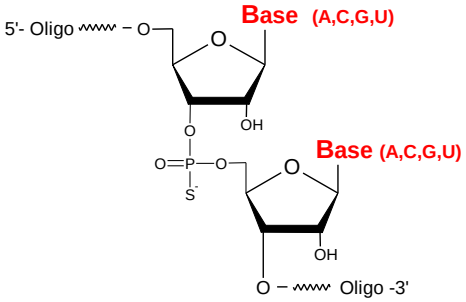
<td> 

Phosphorothioate Linkage
[26-6401-XX]</td>
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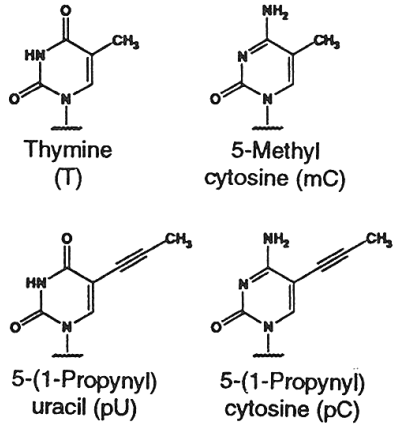
<tr>
<td>Chimeric Phosphorothioate Linkages
(DNA,RNA, LNA, 2'O Me and other bases)</td>
<td>RNA phosphorothioate Linkage</td>
</tr>
</table>

Propyne* Analogs

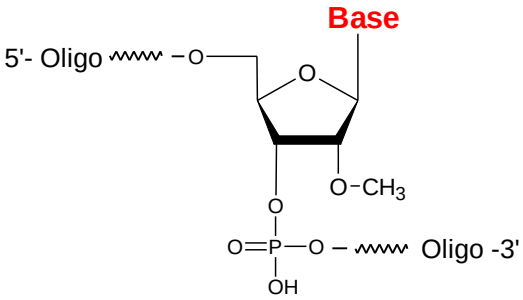
It has been shown that C-5 propyne analogs of dC and dT when substituted in phosphorothioate oligonucleotide imparts greater inhibition of gene expression due to increased binding affinity to the target mRNA and increased stability (5). Based on the above information antisense oligonucleotide could either 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.



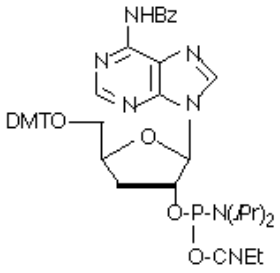
2' O-Methyl Base

[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.

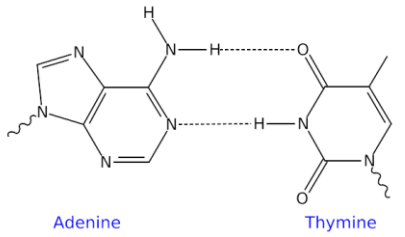


3'-dA-CE Phosphoramidite

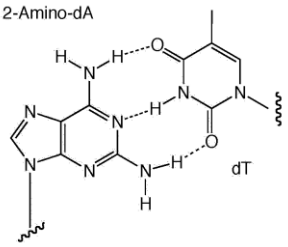
2-Amino-dA & 5-Me-dC

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.

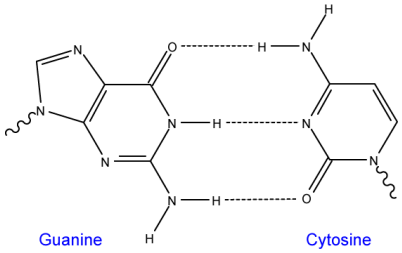
As shown in Figure below, A-T base pairs have two hydrogen bonds whereas G-C base pairs have three hydrogen bonds. The simplest approach to improving primers would be to substitute A sites with 2-amino-A which forms three hydrogen bonds with T on hybridization. 2-Amino-A also destabilizes A-G wobble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



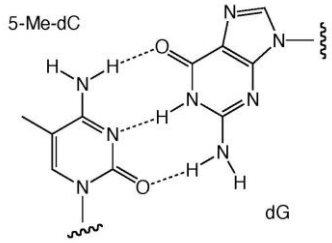
A-T base pairing



2-Amino dA-dT base pairing



G-C base pairing



5-Me-dC-dG base pairing

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

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.

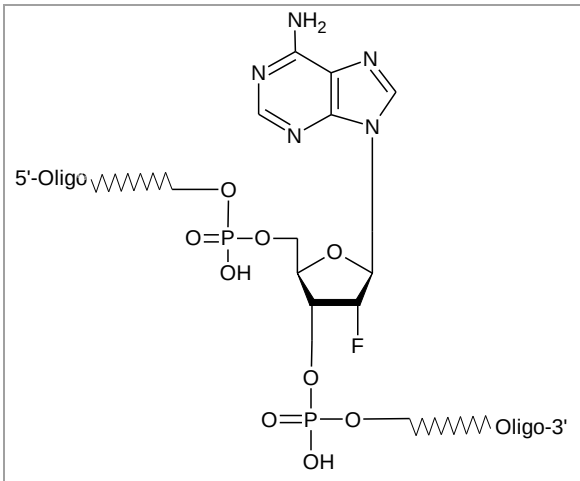
2'-F-RNA phosphodiester linkages are not nuclease resistant, although the corresponding phosphorothioate linkages are highly resistant. Researchers usually design antisense oligonucleotides to form duplexes with RNA, which are then substrates for RNase H. Uniformly modified 2'-F-RNA/RNA duplexes are 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.

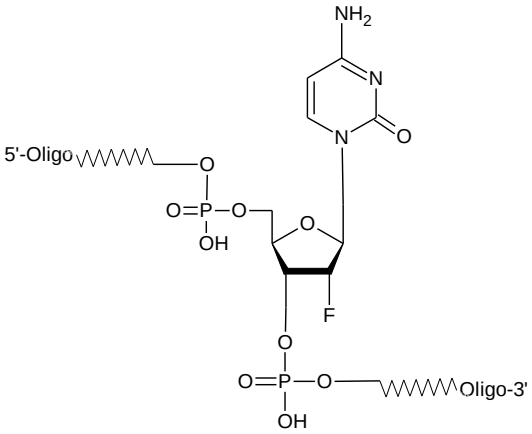
Fluorine has an interesting combination of properties, combining electronegativity similar to a hydroxyl group with size between an oxygen and a hydrogen atom. This combination leads to the ring of a 2'-F-ribonucleoside adopting a C3'-endo conformation and the resulting 2'-F-RNA oligonucleotide adopts 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 DTm 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.

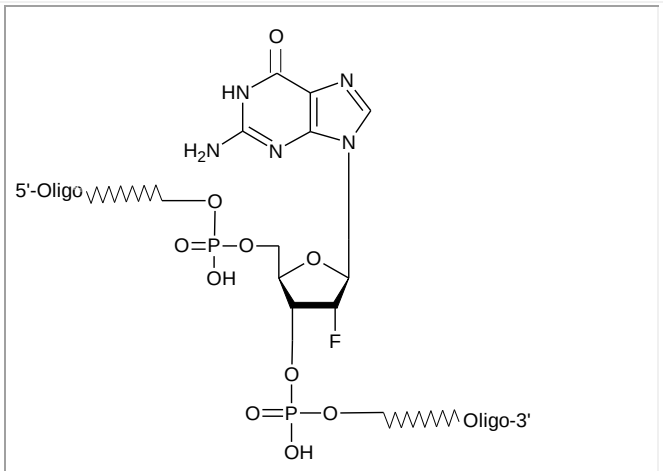
2'-Fluoro RNA Oligonucleotide



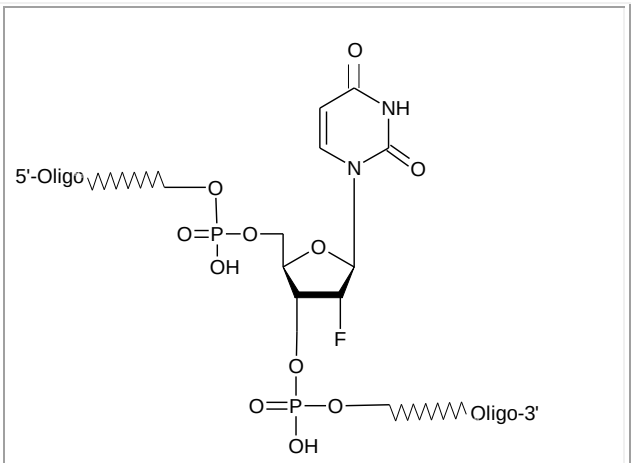
2'-F A
[26-6692-XX]



2'-F C
[26-6463-XX]



2'-F G
[26-6693-XX]



2'-F-U
[26-6462-XX]

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 Tm 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 Tm.
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 Tm by 1.2 °C. However, further substitutions with two or four 2'-F-RNA residues led to a drop in the Tm by 1.3 °C. Interestingly, a fully substituted 2'-F-RNA/DNA duplex does exhibit higher stability, with the Tm 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°.

References:

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Applications & Recommended Modifications

The following guideline represents the wide application of modified base use for almost all oligo and probe design and use. Gene Link technical service offers advice on oligo design and use of modifications based on application.

Application	Recomended Modifications
Antisense Gene Target	• Oligonucleotides containing 2'-OMe-nucleotides (2'-OMe-RNA) forms more stable hybrids with complementary RNA strands than equivalent DNA and RNA sequences. • Phosphorothioate linkages confer oligonucleotides resistance to nuclease degradation • 3′ Cholesterol modification helps in cellular uptake.
RNA Interference (siRNA)	• Alternating 2'-F bases and 2'OMe bases siRNA enhances duplex stability and are more resistant to RNase degradation. • 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.
Real-Time PCR probes, QPCR	• Appropriately substituted 2'Amino dA and 5'Me dC bases imparts greater specificity with higher Tm. Substituting 4-6 bases increases the Tm by 6-10 degrees. These probes enhance duplex stability and thus shorter probes can be synthesized. • All types of fluorescent dyes and backbone modifications can be performed. • 5 methyl dC behave similar to LNA bases in imparting duplex stability.
SNP Genotyping, Allelic Discrimination	• Appropriately substituted 2-Amino dA and 5-Me dC bases imparts greater specificity with higher Tm. Substituting 4-6 bases increases the Tm by 6-10 degrees. These primers and probes enhance duplex stability and thus shorter primers and probes can be synthesized. • All types of fluorescent dyes and backbone modifications can be performed. • All types of fluorescent dyes and backbone modifications can be performed. • 5 methyl C behave similar to LNA bases in imparting duplex stability.
Fluorescent in situ Hybridization Probes (FISH)	• Design multiple 24 to 30mer probes. Avoid stretches of more than 3 G or C bases. • To impart exonuclease resistance substitute 3-4 bases at the 5' and 3' end with 2'F bases. The 2' F bases imparts resistance to exonuclease degradation and increases duplex stability by 4-6 degrees. • Several internal bases can be substituted with 5me dC and 2 Amino dA to further increase duplex stability. • 5 methyl C behave similar to LNA bases in imparting duplex stability. • Affinity ligands such as Digoxigenin or Biotin or fluorescent dye e.g Cy3, Cy5 or any other can be labeled at the 3' and 5' end. Multiple internal sites can also be labeled with affinity ligands or fluorescent dyes to increase sensitivity.

	• Multiple dye sites should be spaced apart by 10 or more bases. The above guidelines are for all initial FISH probe design. Design rules may have to be established empirically for very specific or novel assay settings, but following the above recommendations will provide a good start.
PCR Amplification Primers	• To impart exonuclease resistance substitute 3-4 bases at the 5' and 3' end with 2'F bases. The 2' F bases imparts resistance to exonuclease degradation and increases duplex stability by 4-6 degrees. • Several internal bases can be substituted with 5me dC and 2 Amino dA to further increase duplex stability. • 5 methyl C behave similar to LNA bases in imparting duplex stability.

Ordering Information

Modified Bases & Linkages	
Product	**Catalog No.**
Phosphorothioates	26-6401-XX
Propyne dC or propyne dU*	26-6408-XX
Propyne dC or propyne dC	26-6501-XX
2'O methyl bases	27-6410-XX
2'-5' linked bases	26-6414-XX
5-Me-dC	26-6413-XX
2-Amino dA	26-6525-XX
Spacer 9	26-6440-XX
2'F A	26-6692-XX
2'F C	26-6463-XX
2'F G	26-6693-XX
2'F U	26-6462-XX
Spacer 9	26-6440-XX
2'O-Me-5-Me-C	26-6508-XX
Chimeric Linkage	26-6420-XX
3' dA (2'-5' linked)	26-6490-XX
3' dC (2'-5' linked)	26-6491-XX
3' dG (2'-5' linked)	26-6492-XX
3' dT (2'-5' linked)	26-6493-XX
XX in catalog number is replaced by the specific scale of synthesis	

Fluorophore*	Color	Absorbance max (nm)	Emission max (nm)
Dabcyl	(Quencher)	453	
BHQ-1, BHQ-2, BHQ-3	(Quencher)	534, 579, 672	
6-FAM (Fluorescein)	Green	494	525
TET	Orange	521	536
HEX	Pink	535	556
Cy 3	Red	552	570
Cy 5	Violet	643	667
Tetramethylrhodamine	Rose	565	580
Alexa Dye Series	Varies	Varies	Varies
Please see our complete list at www.genelink.com or call at 1-800-436-3546			

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