Product Profile

Custom Oligo Synthesis, antisense oligos, RNA oligos, chimeric oligos,
Fluorescent dye labeled oligos, Molecular Beacons, TaqMan Probes
siRNA, Aptamers

SmartBase™ Fluorescent Molecular Probes

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

Design Guide for Enhanced Probe Performance

Oligo Types & Modifications

Molecular Beacons
TaqMan® Probes
Aptamers
RNA Probes
Fluorophores & Quenchers
Propyne dC and dU labeled Oligos
Phosphorothioate Oligos
2'-5' linked Oligos
Methylated Oligos

Applications

Real Time Quantitative PCR Analysis (QPCR) Probes
Fluorescent Genotyping
siRNA Gene Knockout Validation
Fluorescent in situ hybridization (FISH) Probes
Allelic Discrimination
Antisense Targeting
SNP Detection
Aptamers Detection Probes

Gene Link™

SmartBase™ Fluorescent Molecular Probes

Design Guide for Enhanced Probe Performance

The use of fluorescent dyes in molecular biology has rapidly transformed from just single dye-labeled primers for fragment analysis to the use of dual-labeled oligos, containing dyes and dark quenchers, as probes for quantitative analysis. Fluorescence based detection utilizing TaqMan, Molecular Beacon and other probes offers a safe and sensitive method for quantitative detection. This also means that molecular biologists have to understand new terms like donors, acceptors, quenchers, FRET, Stokes shift and more importantly the use of modified bases that impart specific enhanced properties. The molecular basis of some of the probe designs are simple and elegant, and thus have led to an exponential use of molecular probes, and consequently, furthering new developments.

Well designed molecular probes are potent and specific tools in determining gene expression, allelic discrimination, drug metabolizing enzymes polymorphism and have immense potential in therapeutic gene expression studies. The most important determinant of molecular probe function is sequence specific recognition and Watson-Crick base pairing to the target. Other than specific sequence requirements, effective molecular probe functionality requires the basic attributes of enhanced duplex stability and increased resistance to nuclease degradation.

Molecular probes 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.

Gene Link presents various design options for synthesizing effective and enhanced performing probes using modified bases. SmartBase™ Fluorescent Molecular Probes 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 and for cellular delivery we may add cholesterol to the synthetic oligonucleotide sequence or modify 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 probes in particular and as well emphasize their use as primers and siRNA. 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.

Gene Link offers synthesis of all different forms of molecular probes. We provide technical service in the design of novel probes and have synthesized numerous combinations of dyes, quenchers, RNA, phosphorothioate, 2'O methyl and chimeric probes.

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.

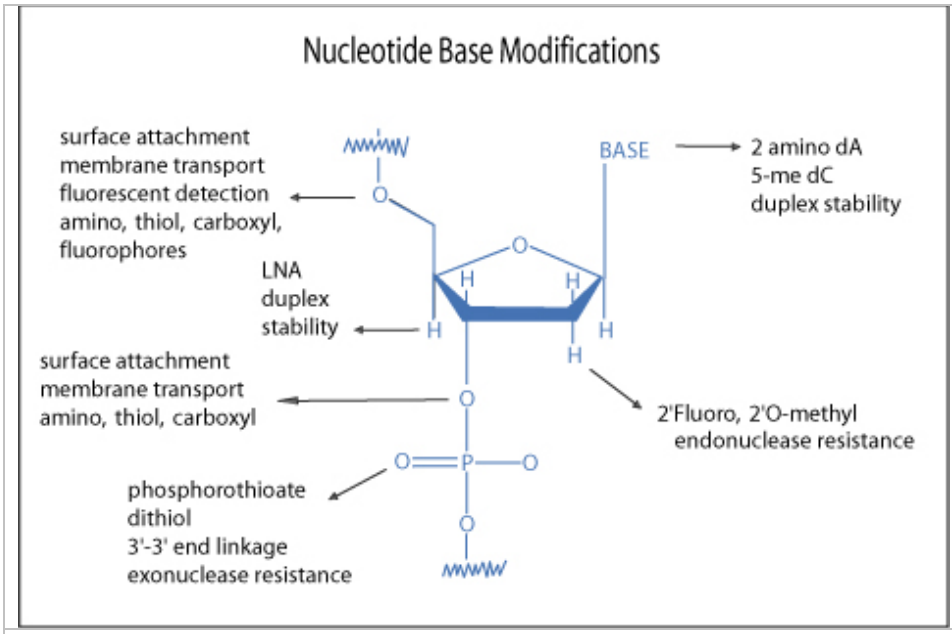


Figure 1. A partial list of possible modifications to a nucleotide.

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 led 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.

SmartBase™ 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.			

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.

SmartBase™ Design of Fluorescent Molecular Probes

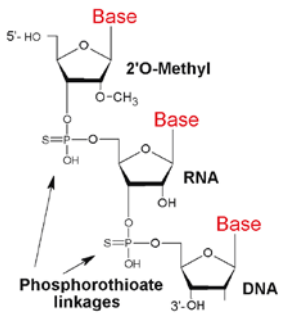
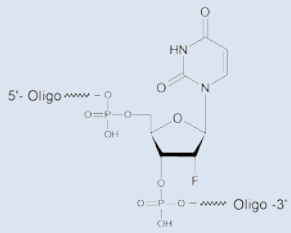
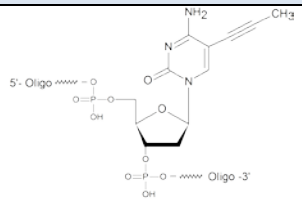
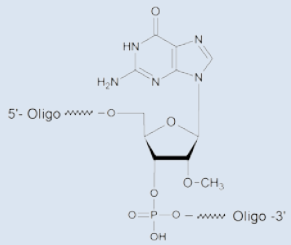
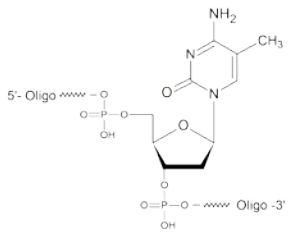
1. G-C content between 20% and 80% and avoid stretches of more than 3 G or C bases.
2. Do not substitute modified bases in the 4-5 bases at the 5' and 3' end of probes.
3. Several internal bases can be substituted with 2' F bases, 5me dC and 2 Amino dA to increase duplex stability.
4. Fluorescent dyes with high extinction coefficient should be selected.
5. 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.

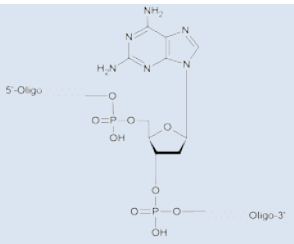
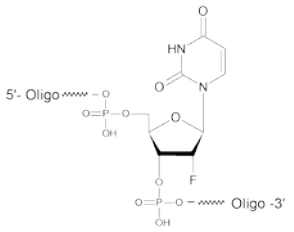
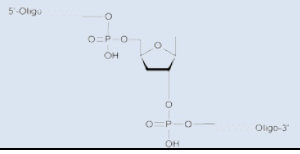
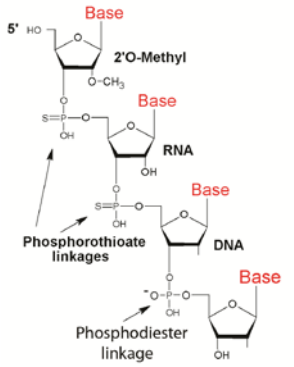
SmartBase™ **Design Guidelines for FISH Probes**

1. Design multiple 24 to 30mer probes. Avoid stretches of more than 3 G or C bases.
2. 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.
3. Several internal bases can be substituted with 5me dC and 2 Amino dA to further increase duplex stability.
4. 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.
4. Multiple dye sites should be spaced apart by 10 or more bases.

Careful selection of modifications and verifying the performance of modified probes 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.

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 oxygen 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]			
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 counterpart. *This can be minimized by the use of 2’F bases 2’-5’ linked oligos as described in the section below.*

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 (4) 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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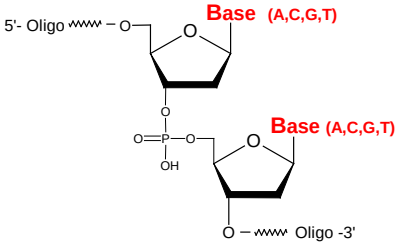
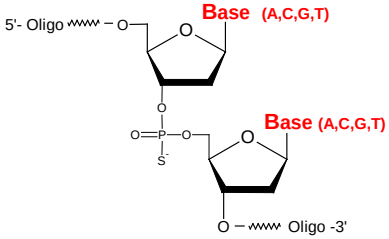
<td> 

Phosphodiester Linkage
[26-6400-XX]</td>
<td> 

Phosphorothioate Linkage
[26-6401-XX]</td>
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<tr>
<td>Phosphodiester Linkage</td>
<td>Phosphorothioate Linkage (PS)</td>
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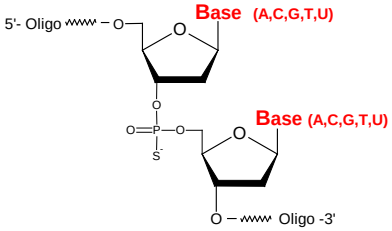
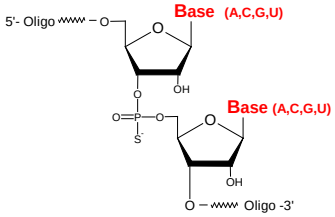
<td> 

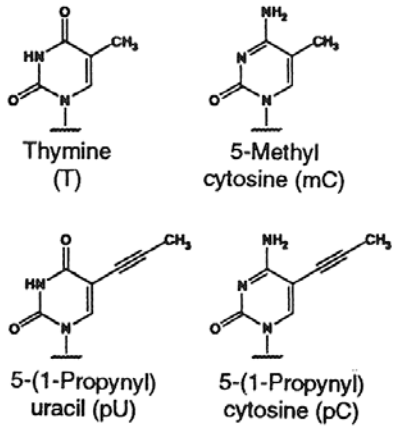
Phosphorothioate Linkage
[26-6401-XX]</td>
<td> 

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

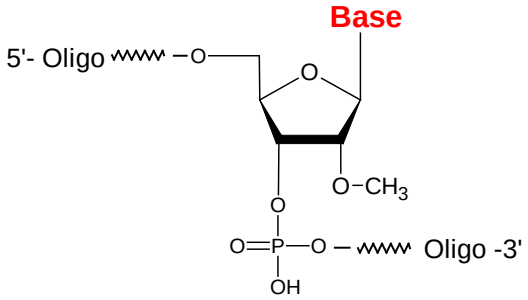
Propyne* Analogs

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



2' O-Methyl Base

[27-6410-XX]

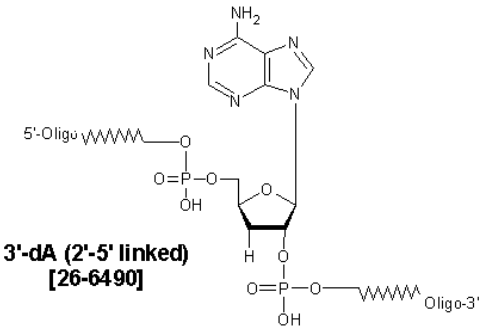
RNA oligos are susceptible to degradation to the same extent as native RNA extracted from various sources. An attractive alternate to prevent degradation from nucleases is the use of 2’-O- methyl RNA bases, when specific 2’OH is not required. The 2’-O- methyl oligonucleotides confer considerable nuclease 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.

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.

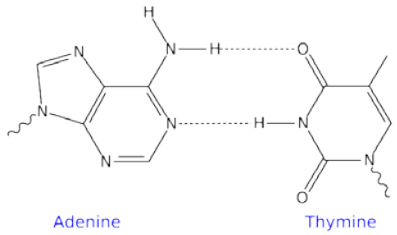
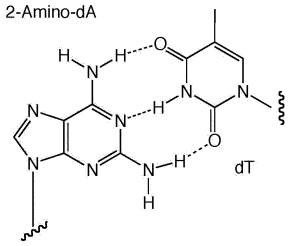
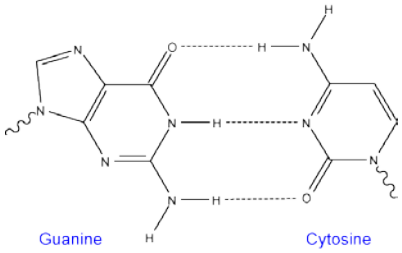
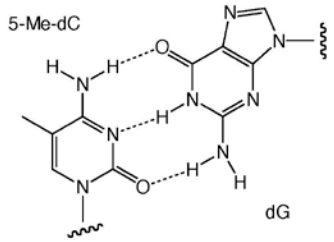


3'-dA (2'-5' linked)
[26-6490]

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'-F-RNA Monomer

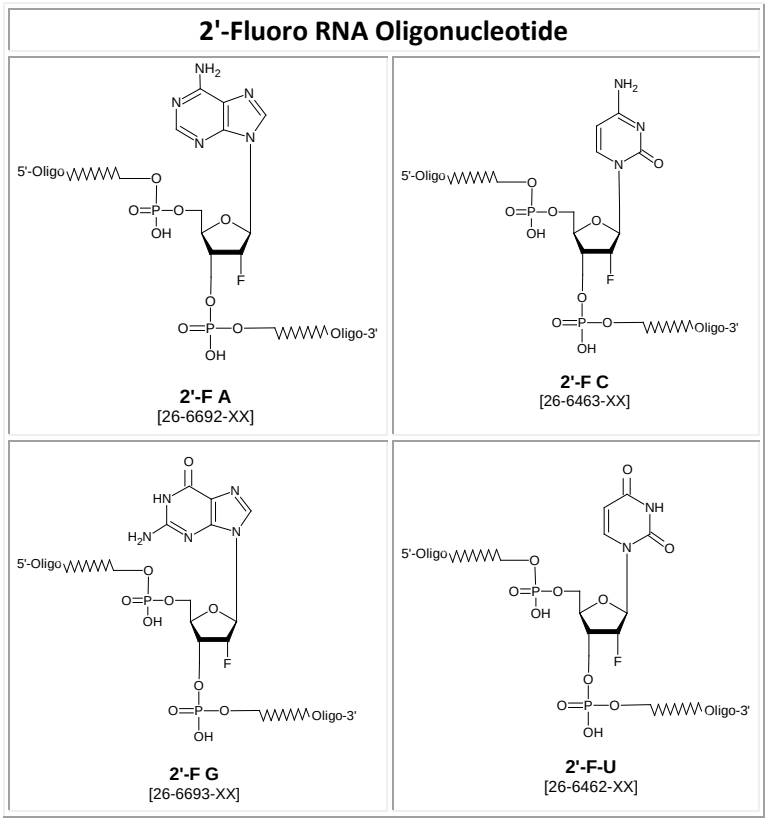
2'-Deoxy-2'-fluoro-nucleosides adopt an RNA-type sugar conformation, presumably due to the high electronegativity of fluorine. Because of this sugar conformation, RNA duplexes (A-form) are generally more thermodynamically stable than DNA duplexes (B-form). As expected, the addition of 2'-F-RNA residues 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 (1).

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 (2). 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 (3). 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.



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Appendix

Fluorescence Resonance Energy Transfer (FRET)

Resonance energy transfer, often known as fluorescence resonance energy transfer (FRET) or Förster energy transfer. It is the radiation-less transfer of excitation energy from a donor to an acceptor. An important consequence of this transfer is that there is no emission of light by the donor. The acceptor may or may not be fluorescent. FRET is a distance-dependent interaction where the energy transfer occurs typically over a distance of 1-10 nm. The distance dependent nature of FRET is highlighted by the fact that it is proportional to the inverse sixth power of the intermolecular separation. The fact that FRET typically occurs in the 1-10 nm region means that these separation distances are comparable with the dimensions of biological macromolecules. This means that FRET can be a valuable tool in studying proximity events in biological systems. One helical turn is ~3.4nm and comprises 10 nucleotides. A nucleotide is thus ~0.34 nm or 3.4Å (1 Å= 0.1nm). Thus, to observe FRET and quenching the donor and acceptor should not be placed more than 30 nucleotides away.
FRET varies based on the degree of spectral overlap of the donor and acceptor. That is to say, the degree to which the emission band of the donor and the absorption band of the acceptor overlap. This is called the "spectral overlap" or sometimes the "Förster overlap integral". This describes the amount of overlap where resonance can occur, i.e. where the donor and acceptor have the same frequencies.

There are many other applications and consequences arising from these equations. For example if the donor and acceptor are the same molecular species it is still possible to observe FRET. This is called homo-transfer and could be thought of as energy migration. The obvious conclusion from this is that the observed fluorescence would not be changed. However, at high concentration of dye it is possible to observe concentration quenching. In this case the transfer of the energy does not result in emission, and the explanation for this is that the transfer is occurring at less than a critical distance and some of the dyes are acting as energy sinks (Source: Amersham Biosciences Website).

TaqMan

TaqMan (also known as Fluorogenic 5’ nuclease assay) probes contain two dyes, a reporter dye (e.g. 6-FAM) at the 5’ end and a 3’ acceptor dye, usually TAMRA. Recent designs substitute the 3’ TAMRA fluorescent acceptor quencher dye with non-fluorescent quencher, e.g. Black Hole Quencher. The proximity of the quencher to the reporter in an intact probe allows the quencher to suppress, or “quench” the fluorescence signal of the reporter dye through FRET. If the target of interest is present, these probes specifically anneal between the forward and reverse primer sites. During the reaction, the 5’ to 3’ nucleolytic activity of Taq polymerase cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target. The probe fragments are displaced from the target, separating the reporter dye from the quencher dye and thus resulting in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. Because increase in fluorescence signal is detected only if the target sequence is complementary to the probe, nonspecific amplification is not detected.

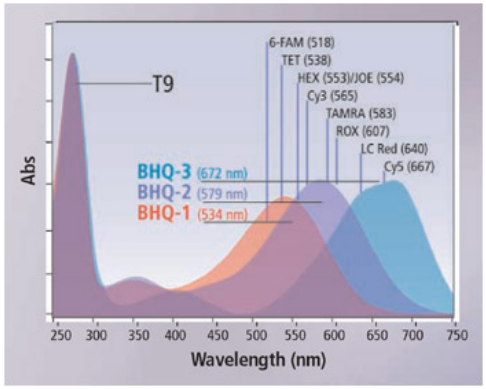
Quenching

Reduction in the expected fluorescence emission intensity is termed quenching. Generally, it would be an impediment if the emission intensity were reduced. The phenomenon of quenching forms the basis of the mode of action of certain molecular probes; the designed and controlled fluorescence based on hybridization to the target sequence.

Natural quenching occurs due to ‘fading’ after repeated cycles of excitation and relaxation. The decrease in the ability of further excitation of a proportion of molecules is termed as photobleaching. Photobleaching is due to

structural damage of molecules due to absorption of high energy photons. Some dyes are much more sensitive than other to photobleaching, for example, fluorescein photobleaches very easily. Often the rate of decomposition is proportional to the intensity of illumination. So a simple practical way to overcome this is to reduce the incident radiation. It is sometimes possible to introduce antioxidants, such as phenylalanine or azide, to reduce bleaching. Quenching is also observed when the concentration of the dye is too high and the overall brightness decreases. This is 'self quenching'. It is observed that multiple labeling of an oligo with the same dye does not always lead to an increase in fluorescence.

Placing a molecule that absorbs light in close proximity to the fluorophore can induce quenching. Quenching is distance-dependent, quite similar to Fluorescence Resonance Energy Transfer (FRET), and it can be assumed that the energy transfer typically occurs over a distance of 1-10 nm. The quenching effect is exhibited by fluorescent as well non-fluorescent molecules. A non-fluorescent ("dark") quencher is the basis of the design of Molecular Beacons. This molecule could be non-fluorescent and acts as an energy sink, and is termed a quencher. In other instances, this molecule could itself be a dye with overlapping absorption and emission spectra; in such cases, energy is transferred from one to the other without any emission of light energy. This is termed as resonance energy transfer. Generally, the term 'quencher' is used for non-fluorescent molecules in probe design, and 'double dye' or 'dual dye' used for probes with two dyes having spectral overlap.



Quencher Spectral Data		
Quencher	**Absorbance max (nm)**	**Quenching Range (nm)**
Dabcyl	453	380-520 nm
BHQ-1	534	480-580 nm
BHQ-2	579	559-650 nm
BBQ-650	650	550-750 nm
BHQ-3	672	620-730 nm

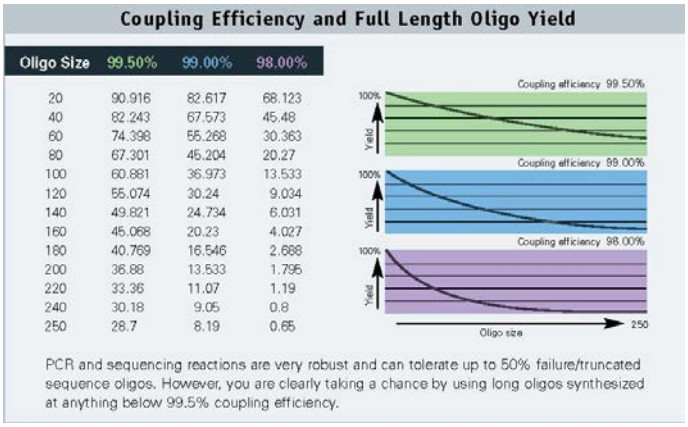
Fluorophore Spectral Data & Quencher Selection Guide

Fluorophore Name	Excitation Max, nm	Emission Max, nm	Extinction coefficient*	Color**	Quencher
AFDye-350 NHS	346	445	19,000	Blue	Dabcyl λ (max) = 453 nm Range = 380-530 nm
AFDye-405 NHS	402	424	33,000		
PBlue-455 NHS	410	455	46,000		
MBlue-460 NHS	362	459	20,000	Blue-Green	
AFDye-488 NHS	494	517	73,000	Yellow-Green	BHQ-1 λ **(max)** = 534 nm Range = 480-580 nm
FAM	**495**	**520**	**75,850**		
TET	**521**	**536**	**99,000**		
AFDye-430 NHS	430	539	15,000		
Cal Fluor Gold 540	552	543	81,100	Yellow	
JOE	**520**	**548**	**75,000**		
Yakima Yellow	**531**	**549**	**83,800**		
AFDye-532 NHS	530	555	81,000		
HEX	**535**	**556**	**98,000**		
Cal Orange 560	**537**	**558**	**81,000**		
Cy3	**550**	**570**	**150,000**	Yellow-Orange	BHQ-2 λ (max) = 579 nm Range = 550-650 nm
AFDye-555 NHS	555	572	155,000		
TAMRA	**555**	**576**	**65,000**		
CAL Fluor Red 590	**569**	**591**	**79,000**		
Redmond Red	579	595	52,300		
Cy3.5	**581**	**596**	**150,000**		
ROX NHS	575	602	82,000	Orange	
AFDye-568 NHS	578	602	88,000		
Cal Red 610	**590**	**610**	**108,000**		
TXRed-616 NHS	589	616	69,000		
AFDye-594 NHS	590	617	92,000	Orange-Red	
CAL Fluor Red 635	616	637	112,000	Red	BBQ-650 λ (max) = 650nm Range = 550-750 nm
LC Red 640 NHS	625	640	110,000		
AFDye-647 NHS	649	671	270,000		
Cy5	**649**	**670**	**250,000**		
Cy5.5	**675**	**694**	**190,000**		
AFDye-680 NHS	678	701	185,000	Near-IR region. Human vision is insensitive to light beyond ~650 nm; it is not possible to view near-IR fluorescent dyes.	
Cy7 NHS	750	773	199,000		
IR 750 NHS	756	776	260,000		
Cy7.5 NHS	788	808	223000		

* Extinction coefficient at λ (max) in cm–1M–1. ** Typical emission color seen through the eyepiece of a conventional fluorescence microscope with appropriate filters.

Yield

Chemical synthesis of oligo starts from the 3’ direction and proceeds to the 5’. All automated synthesis use a solid support that is attached with the first base (nucleoside), for fluorescent probes the 3’ usually is the quencher moiety that is attached to the solid support. The scale of synthesis determines the nanomole (nmol) of first base/quencher or fluorophore. The overall crude nmol yield of full-length oligo depends on the average coupling efficiency of each base and on the number of couplings, as the oligo length increases the overall yield of full length product decreases. At Gene Link we maintain a coupling efficiency of 98-99%. Yields of full-length fluorescent probes vary considerably based on the type of fluorophore and quencher. Most fluorescent dyes that are used directly for coupling on automated DNA synthesizers have a coupling efficiency above 85%, whereas the dyes that are conjugated based on NHS chemistry (succinimidyl ester) is a post synthesis conjugation to amino labeled probes. Gene Link minimum yield guarantees are based on the synthesis, type of dye and purification of your oligo.



Gene Link offers synthesis of all different forms of molecular primers and probes. We provide technical service in the design of novel probes and synthesize numerous combinations of dyes, quenchers, RNA, phosphorothioate, 2'O methyl, ultramodfied and chimeric probes.

The table below lists the yields for common dyes for use in TaqMan probes with Tamra or BHQ at 3' end and Molecular Beacons with universal fluorescence quencher dabcyl or BHQ at 3’ end. Gene Link considers gel purification to be the best method of purification and essential for optimum performance of fluorescent dye labeled oligonucleotides.

Fluorescent Probes PAGE Purified Yield*			
Dye/Quencher	**50 nmol scale Yield**	**200 nmol scale Yield**	**1 µmol scale Yield**
	PAGE Purified*	PAGE Purified*	PAGE Purified*
5’ 6-Fam/3’-Tamra, BHQ1 or Dabcyl	10 nmol	25 nmol	60 nmol
5’-Hex, Tet, Cy3 or Cy5/BHQ2	5 nmol	15 nmol	40 nmol
5' -Cy3.5, Cy5.5,/quencher	5 nmol	12 nmol	25 nmol
AFDyes and other NHS Dyes/quencher	2 nmol	5 nmol	16 nmol
*Approximate yield for dye and quencher listed. Lower yield with additional modifications. ** Purified yield is for gel purified (PAGE, polyacrylamide gel electrophoresis). Inquire for higher scale of synthesis			

All Gene Link custom oligo products including, molecular probes, RNA and siRNA includes a datasheet that contains the exact nmols, µg, A_{260} units(OD Units) and other physical data. This data is important for reconstituting the product. All fluorescent probes are shipped in amber tubes to prevent exposure to light and minimize photobleaching. Gene Link guarantees the stability of oligos for 1 year and fluorescent molecular probes for 6 months if reconstituted and stored appropriately as detailed below.

In our experience unmodified oligos are stable for numerous years if reconstituted and stored properly. Avoid multiple freeze thaws; do not exceed 6-10 freeze thaw cycles. If the same oligo is intended to be used repeatedly then it is prudent to make a numerous aliquots of the stock solution and frozen.

Reconstitution & Storage
Gene Link oligos are supplied lyophilized. These are stable at room temperature for an extended period of time. The oligonucleotide should preferably be frozen upon receipt. TE buffer (10mM Tris, 1mM EDTA, pH 7.5) is recommended for dissolving the oligonucleotides; EDTA inhibits the activity of the nucleases.

Preferred TE Buffer Reconstitution & Storage pH for Fluorescent Probes	
6-FAM, HEX, TET, ROX, and TAMRA	TE Buffer pH 7.5 or 8.0
Cy3, Cy3.5, Cy5, and Cy5.5	TE Buffer pH 7.0 or 7.5
Cy dyes rapidly degrade in acidic pH	

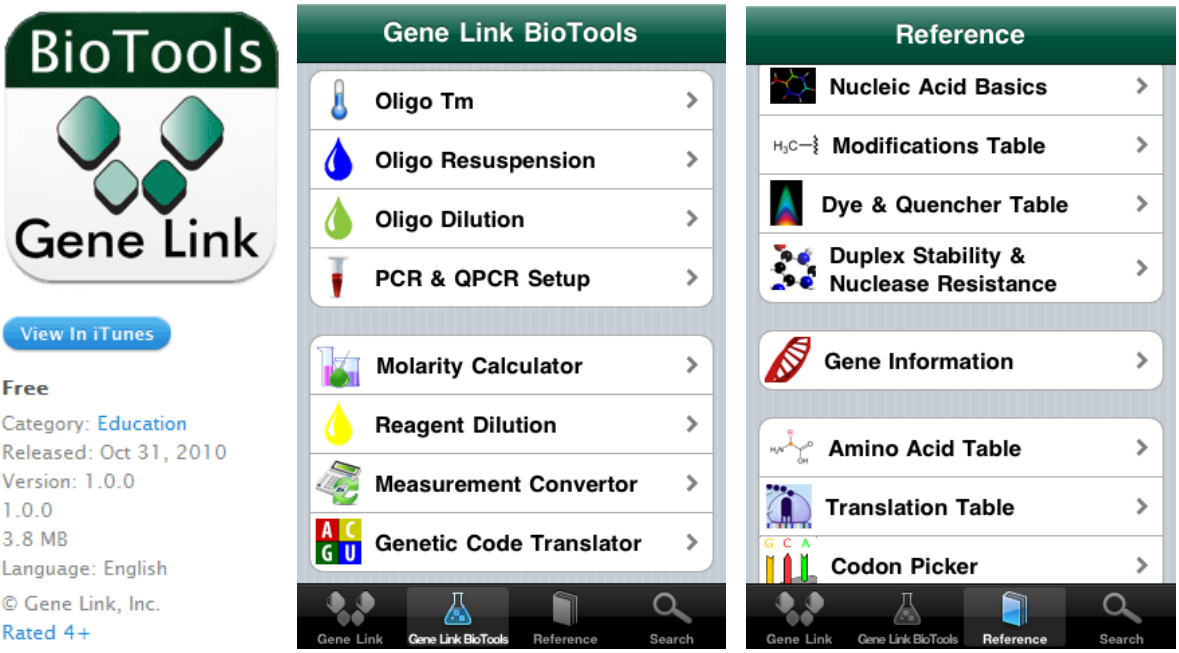
Further dilution can be made in TE buffer. After reconstitution store the stock solution at -80°C or -20°C. Fluorescently labeled oligos should be stored in light-free conditions.

Preparation of Stock Solution of 100 pmols/µl [100µM]
Gene Link provides the exact amount of nmols of each oligo supplied on the tube and on the Oligo Report. Multiply the 'nmol' amount by 10 to arrive at the volume of TE to be added.
Example: 45.10nmols x 10 = 451µl
Dissolve the oligo in 451µl to get 100pmols/µl stock solution.
Use as required.
Dilute 10 fold to prepare a 10pmols/µl [10µM]. Use as required.

For optimal long-term storage, it is recommended that the oligonucleotides should be stored dry at -20°C in the dark. If numerous experiments are planned using the same oligonucleotide, prepare aliquots, dry them and store the aliquots at -20°C.

Stability
Gene Link unmodified oligos are stable for 1 year and fluorescent probes for 6 months if reconstituted and stored appropriately as recommended by Gene Link. The stability is usually several years and can be increased several folds by instituting proper handling conditions, avoiding exposure to light and multiple freeze thaws

BioTools application from Gene Link for iPhone/iPod/iPad
BioTools: An Array of Genetic Tools



The BioTools app also has advanced modules for setup of Polymerase Chain Reaction (PCR) and Quantitative Real Time PCR (Q-PCR).

The main focus of this app is to have a handy source of calculation modules and quick reference sections for designing and executing experiments involving PCR and Q-PCR.

BioTools	**Reference**
1. Oligo Tm: A robust oligo melting temperature calculation module using three methods; it also calculates other physical attributes. 2. Oligo Resuspension 5. Oligo Dilution 6. PCR & QPCR: Convenient calculator for multiple reaction setup for PCR, TaqMan QPCR and Molecular Beacon QPCR setup. Includes stock solution information and cycling profiles 7. Molarity Calculator 8. Reagent Dilution 9. Measurement Convertor: A convenient selection of calculators to convert length, area, mass, temperature and volume units. 10. Genetic Code Translator: Enter DNA sequence to see coding pattern.	A selection of topics, relevant to life scientists for quick access to basic information. This section includes the following sections and sub sections. 1. Nucleic Acid Basics 2. Modifications Table: A list of common modifications with molecular structure and basic properties. 3. Dye & Quencher Table: A convenient list of fluorophores and quencher matching the emission max. 4. Duplex Stability & Nuclease Resistance 5. Gene Information: Simply enter the accession number and retrieve detailed gene information from NCBI database, 6. Amino Acid Table: Molecular structure and detailed physical properties of all amino acids. 7. Translation Table: Genetic code for all amino acids. 8. Codon Picker: Select codon sequence and see the corresponding amino acid and detailed information.

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