Product Profile

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

Smart FISH Probe Design & Synthesis

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

Modifications
Phosphorothioate linkages
5-Me-dC & 2-amino dA
2'F bases
2'-5' linked Oligos

Gene Link™

Smart FISH Probe Design & Synthesis

Introduction

Probes for fluorescent in situ hybridization (FISH) are derived from repetitive chromosomal regions to paint a chromosome or are directed towards unique gene(s) for identification. These probes are usually developed from clones (BAC and cosmids) and more recently synthesized chemically as oligonucleotides appropriately labeled with fluorescent dyes. The oligo probes can be synthesized as tiles or longer sequences up to 250mer in length by Gene Link.

Gene Link presents various design options for synthesizing 'Smart' FISH probes. Smart FISH probes can be synthesized in a predetermined way to exhibit the features that is desired; for instance to increase nuclease resistance we can substitute standard bases with 2' Fluoro bases. Standard DNA probes are like natural molecules that are prone to degradation under normal conditions, ubiquitous nucleases as 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 design and synthesis of Smart FISH Probes. There are four common features that are desirable and in particular can be improved by using a combination of available nucleic acid modifications that modify the phosphodiester linkages, nucleic acid bases, the sugar moieties and addition of various other functional groups.

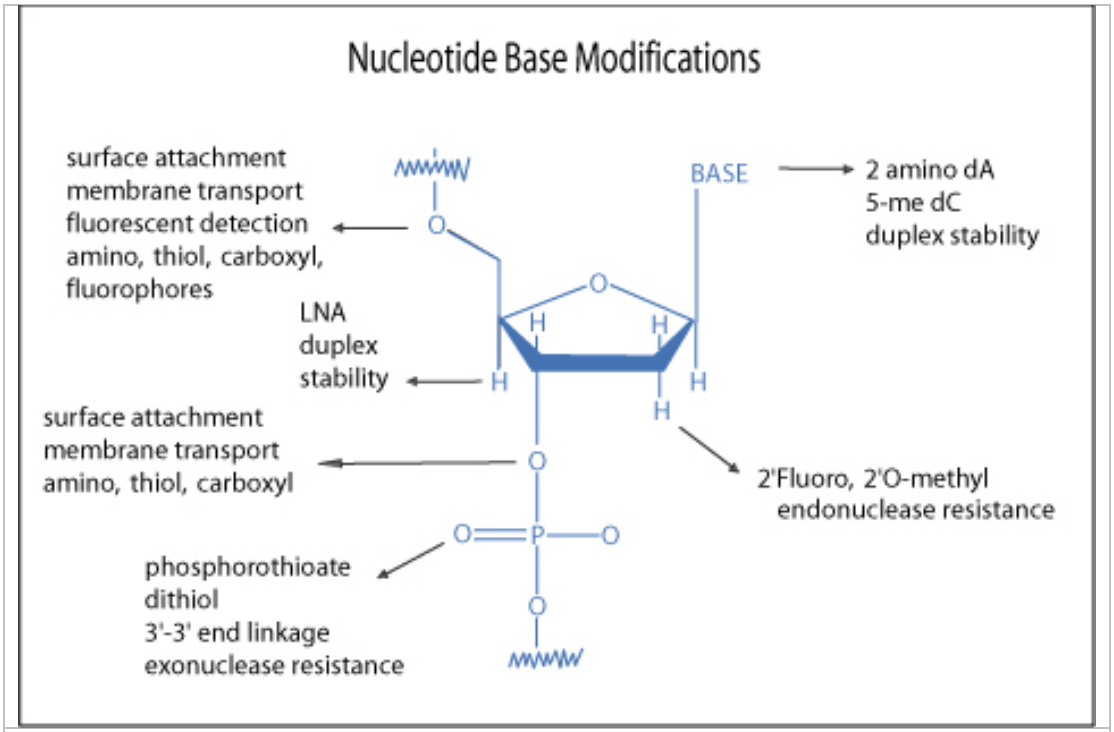


Figure 4. A partial list of possible modifications to a nucleotide.

Common Features for Improvement

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

Increased Duplex Stability and Manipulation of Duplex Stability

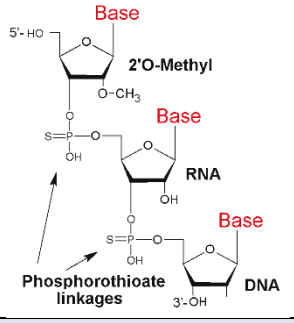
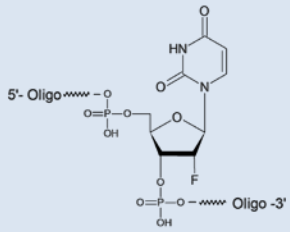
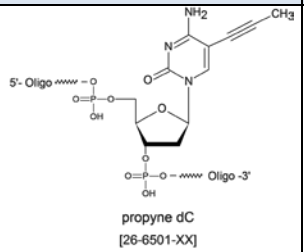
Specific and stable hybridization of the probe to its cognate sequence is the desired outcome of a successful FISH protocol. The melting temperature of the probe dictates to strength of the affinity and thus the stability of the hybridization. There are many nucleic acid modifiers that increase duplex 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.

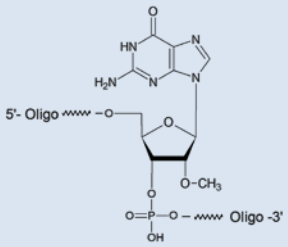
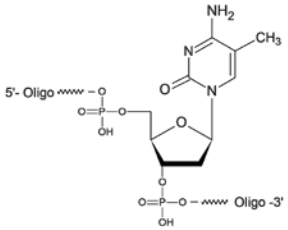
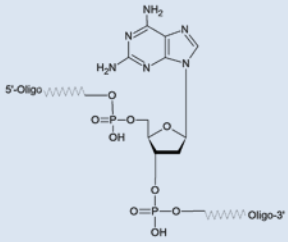
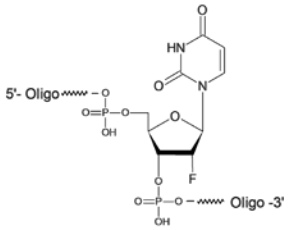
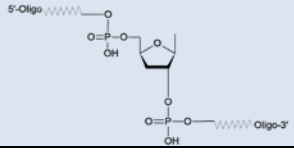
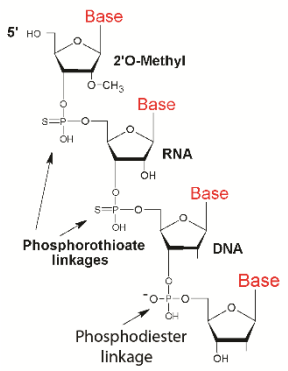
Increased Nuclease Resistance

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.

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.

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		Increased binding affinity to the target sequence and increased stability	Increased nuclease resistance	C-5 propyne analogs of dC and dT
				

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.

Duplex Stabilization

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

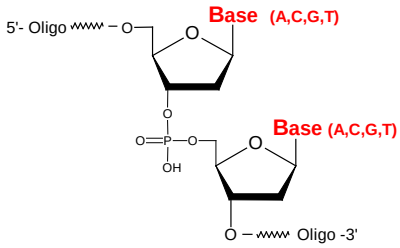
Modifications Increasing Duplex Stability and Nuclease Resistance		
Modification*	**Duplex Stability [Tm Increase]**	**Nuclease Resistance**
Phosphorothioate	Slightly decreased	Increased
2'-OMethyl	Increased	Increased
2'-Fluoro	Increased [1-2° per substitution]	Increased
2-Amino-A	Increased [3.0° per substitution]	No effect
5-Methyl-C	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.		

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

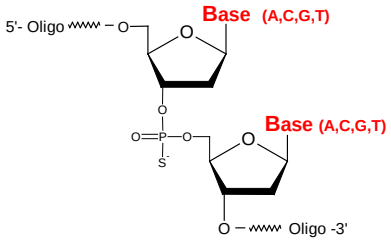
The sulfur-substituted oligonucleotides have a phosphorothioate linkage and are termed as **phosphorothioates** or simply as **S-oligo.** Phosphorothioate oligos are synthesized by Gene Link using the Beaucage (4) sulfurizing reagent. The sulfurization reaction is rapid and is performed on automated 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.



Phosphodiester Linkage

[26-6400-XX]

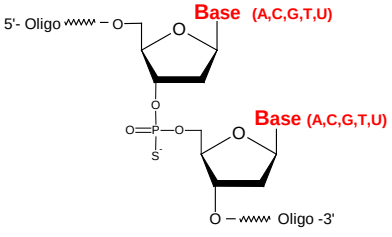
Phosphodiester Linkage



Phosphorothioate Linkage

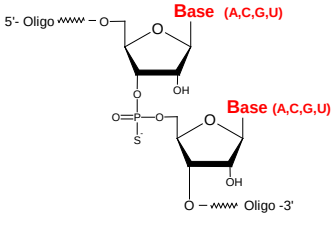
[26-6401-XX]

Phosphorothioate Linkage (PS)



Phosphorothioate Linkage

[26-6401-XX]



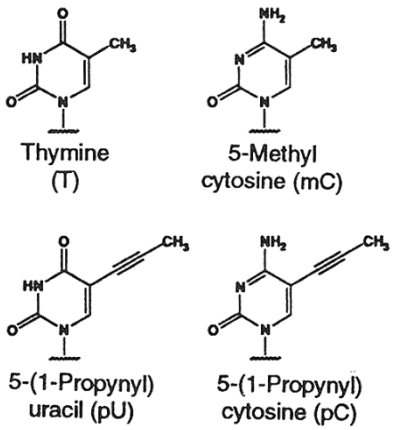
Phosphorothioate Linkage

[26-6401-XX]

Propyne* Analogs

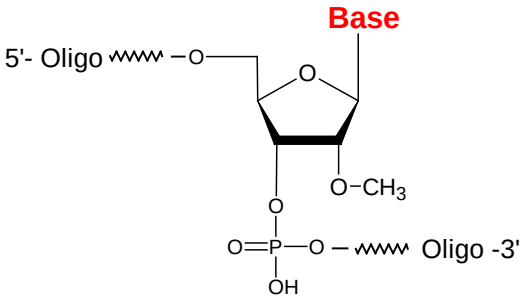
It has been shown that C-5 propyne analogs of dC and dT when substituted in phosphorothioate oligonucleotide imparts greater inhibition of gene expression due to increased binding affinity to the target mRNA and increased stability (5). Based on the above information antisense oligonucleotide could either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



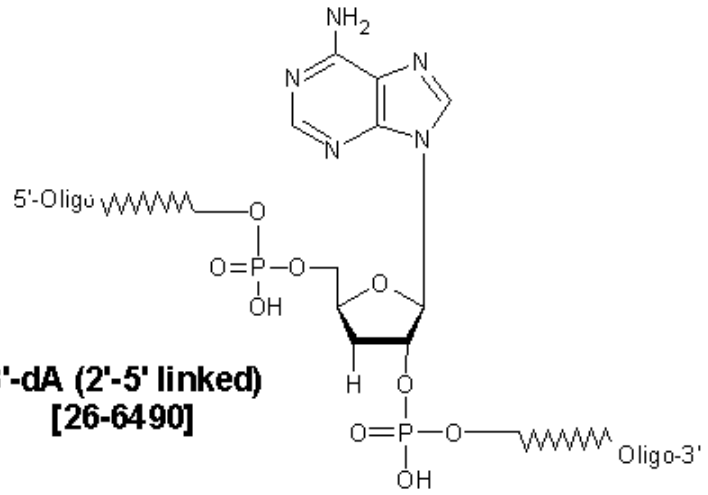
2' O-Methyl Base

[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.

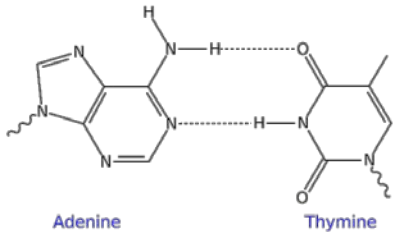
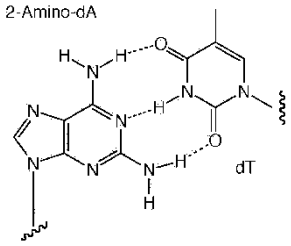


3'-dA (2'-5' linked)
[26-6490]

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.

<table>
<tr><td> 
</td><td> 
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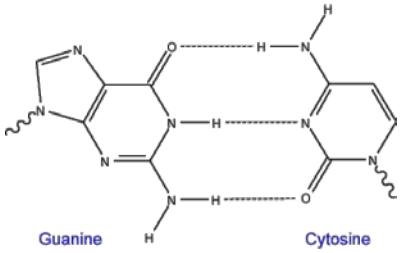
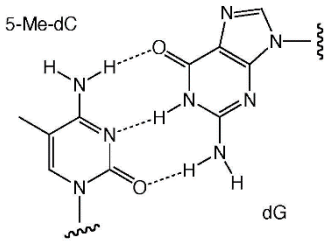
<tr><td>A-T base pairing</td><td>2-Amino dA-dT base pairing</td></tr>
<tr><td> 
</td><td> 
</td></tr>
<tr><td>G-C base pairing</td><td>5-Me-dC-dG base pairing</td></tr>
</table>

2'-F-RNA Monomer

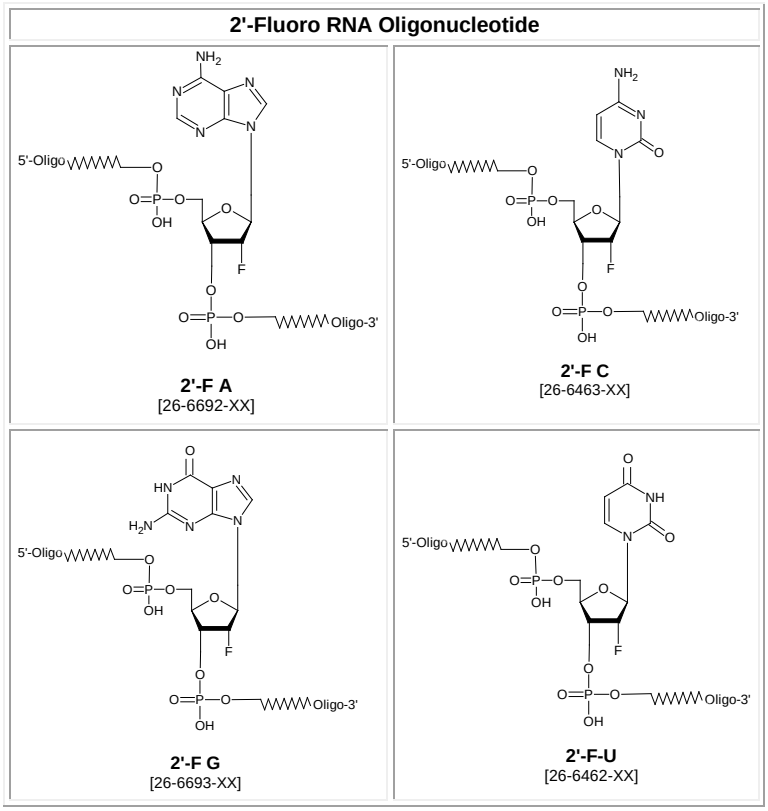
2'-Deoxy-2'-fluoro-nucleosides adopt an RNA-type sugar conformation, presumably due to the high electronegativity of fluorine. Because of this sugar conformation, RNA duplexes (A-form) are generally more thermodynamically stable than DNA duplexes (B-form). As expected, the addition of 2'-F-RNA residues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



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.
5. Multiple dye sites should be spaced apart by 10 or more bases.
6. 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.

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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.

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