Product Profile

Custom Oligo Synthesis, Aptamers antisense oligos, RNA oligos, chimeric oligos, Fluorescent dye labeled oligos, Molecular Beacons, TaqMan Probes Locked Nucleic Acids (LNA), siRNA,

SmartAptamers™: Applications & Effective Design Options

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

Oligo Types & Modifications
RNA & 2'O-methyl Aptamers
Fluorescent Modifications
Thioaptamers
Propyne dC and dU labeled Oligos
Phosphorothioate Oligos
2'-5' linked Oligos
Methylated Oligos
Biotin & Digoxigenin Aptamers

Applications
Aptamers Detection Probes
Real Time Quantitative Aptamers
Nanotechnology
Molecular Discrimination
Biosensors
Therapeutics
DNA Enzymes

Gene Link™

Smart Designed Aptamers: Applications & Effective Design Options

Introduction

The term aptamer is derived from the Latin ‘aptus’ meaning “to fit” and is based on the strong binding of single stranded oligos to specific targets based on structural conformation. Aptamers are single-stranded RNA or DNA oligonucleotides 15 to 60 base in length that bind with high affinity to specific molecular targets; most aptamers to proteins bind with Kds (equilibrium constant) in the range of 1 pM to 1 nM similar to monoclonal antibodies. These nucleic acid ligands bind to nucleic acid, proteins, small organic compounds, and even entire organisms. Aptamers have many potential uses in intracellular processes studies, medicine and technology (1-7, excellent background and review articles).

In addition to the genetic information encoded by nucleic acids they also function as highly specific affinity ligands by molecular interaction based on the three dimensional folding pattern. The three dimensional complex shape of a single stranded oligonucleotide is primarily due to the base composition led intra-molecular hybridization that initiates folding to a particular molecular shape. This molecular shape assists in binding through shape specific recognition to it targets leading to considerable three dimensional structure stability and thus the high degree of affinity. Natural examples of molecular shape recognition interactions of nucleic acids with proteins are tRNA, ribozymes, DNA binding proteins and DNAzymes (8,9).

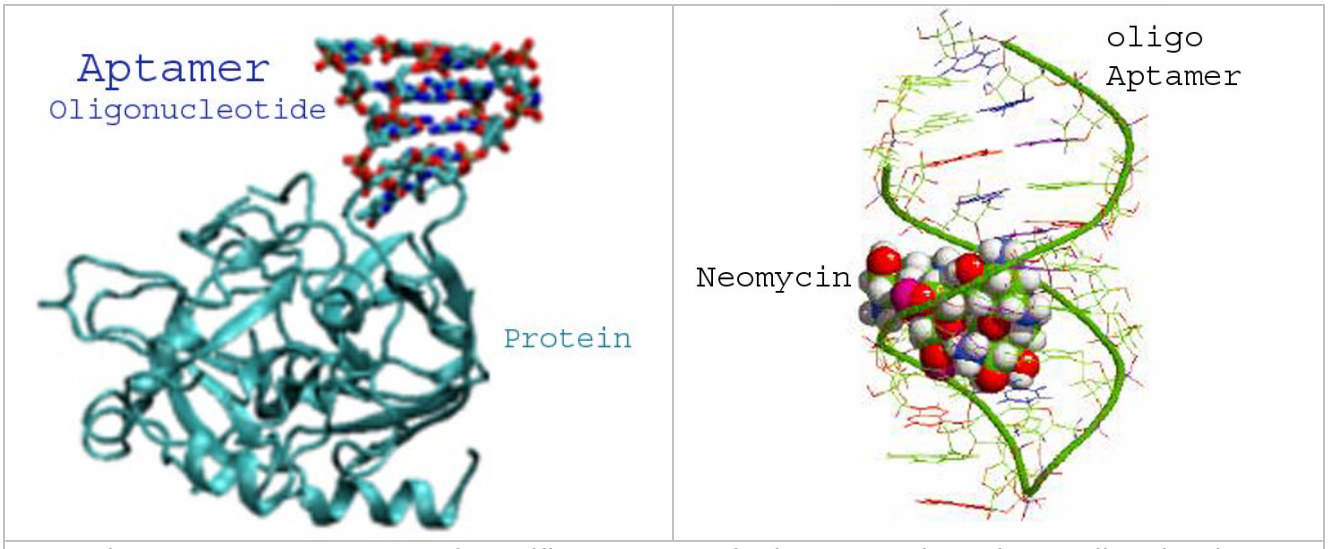


Figure 1. NMR structures of specific aptamers of a large protein and a small molecule

Theoretically it is possible to select aptamers virtually against any molecular target; aptamers have been selected for small molecules, peptides, proteins as well as viruses and bacteria. The aptamers are selected by incubating the target molecule in a large pool of oligonucleotide (usually 40 to 60mers), the pool size of the oligonucleotide is from 10^{10} to 10^{20}. The large pool size of the oligonucleotide ensures the selection and isolation of the specific aptamer. The structural and informational complexity of the oligonucleotide pool and its functional activity is an interesting and active area to develop an algorithm-based development of nucleic acid ligands (10). Aptamers can distinguish between closely related but non-identical members of a protein family, or between different functional or conformational states of the same protein. In a striking example of specificity, an aptamer to the small molecule theophylline (1,3-dimethylxanthine) binds with 10,000-fold lower affinity to caffeine (1,3,7-trimethylxanthine) that differs from theophylline by a single methyl group. The protocol called systematic evolution of ligands by exponential enrichment (SELEX) is generally used with modification and variations for the selection of specific aptamers. Using this process, it is possible to develop new aptamers in as little as two weeks (1-4).

Gene Link routinely synthesizes the random oligonucleotide pools that are used for the initial specific aptamer selection procedure for SELEX. A version of the SELEX protocol is given below.

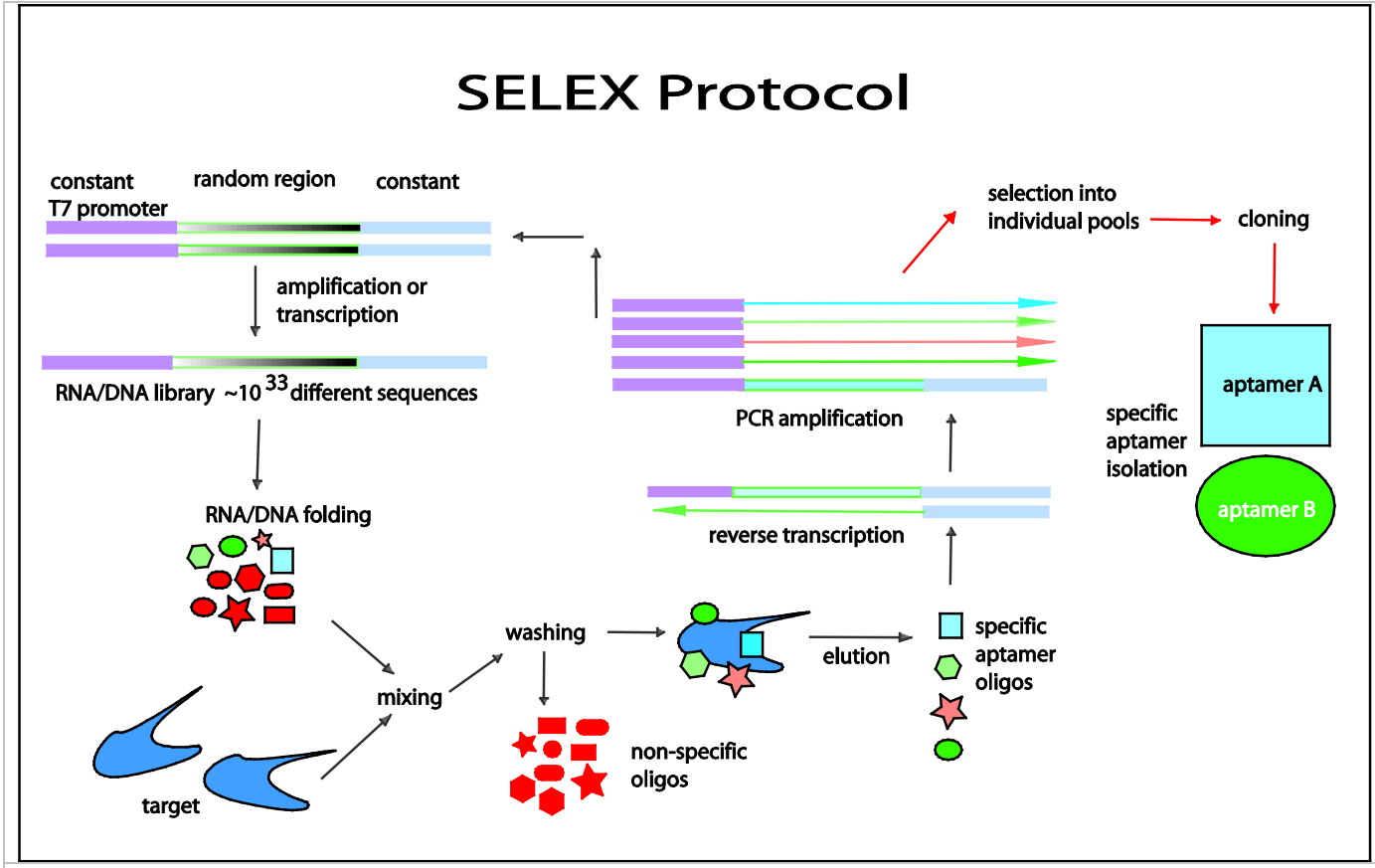


Figure 2. A schematic representation of the procedure called 'systematic evolution of ligands by exponential enrichment' (SELEX) for Aptamer selection.

Smart Aptamers

The structural stability of Aptamers as affinity ligands can be calculated by strict criteria of equilibrium (K_d), kinetic (k_{off}, k_{on}) and thermodynamic (ΔH, ΔS) parameters of bio-molecular interaction. Thus "Smart Aptamers" term was coined for a pool of aptamers with different equilibrium constants (K_d) such that they will be effective in varying concentration range of the target. Kinetic capillary electrophoresis (KCE) has been recently proven to generate smart DNA aptamers with a wide range of predefined values of K_d and high selectivity (11).

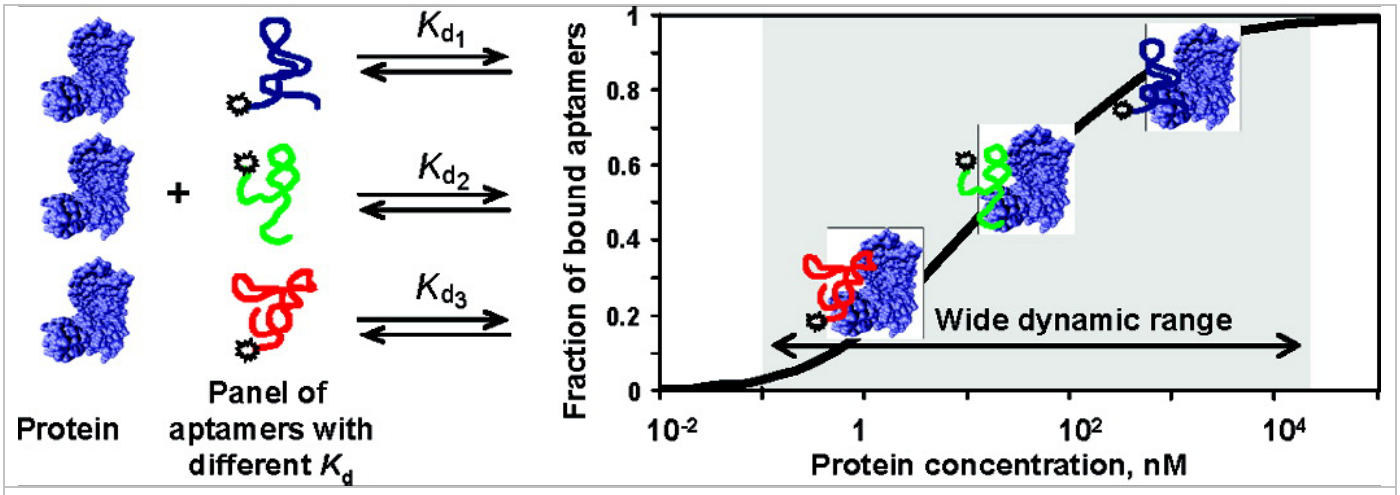


Figure 3. Three Aptamers to the same target with different Kd. Taken from: Smart Aptamers Facilitate Multi-Probe Affinity Analysis of Proteins with Ultra-Wide Dynamic Range of Measured Concentrations. Andrei P. Drabovich, Victor Okhonin, Maxim Berezovski, and Sergey N. Krylov. J. Am. Chem. Soc., 2007, 129 (23), 7260-7261

References

1. Ellington, A.D., and J.W. Szostak. J.W (1990), In vitro selection of RNA molecules that bind specific ligands. Nature 346: 818-822.
2. Tuerk, C. and L. Gold, L. (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 249:505-510.
3. Wilson, D.S., Szostak, J.W. (1999). In vitro selection of functional nucleic acids. Annu Rev Biochem 68, 611–647.
4. The Aptamer Handbook. Edited by S. Klussmann. (2006) WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.
5. S. Tombelli, S., Minunni, M. and M. Mascini. (2005) Analytical applications of aptamers. Biosensors and Bioelectronics 20: 2424-2434.
6. Lee, J.F., Stovall, G.M. and Ellington, A.D. (2006) Aptamer therapeutics advance. Current Opinion in Chemical Biology 10:282–289.
7. Shahid M. Nimjee, Christopher P. Rusconi and Bruce A. Sullenger, B.A (2005) Apaterms: An emerging class of therapeutics. Annu. Rev. Med. 56:555–83. Antidote control of aptamer activity enables safe, tightly controlled therapeutics. Aptamers may prove useful in the treatment of a wide variety of human maladies, including infectious diseases, cancer, and cardiovascular disease.
8. Razvan, N., Shirley Mei, S., Zhongjie Liu, Z. and Yingfu Li. Y. (2004) Engineering DNA aptamers and DNA enzymes with fluorescence-signaling properties. *Pure Appl. Chem.*,76:1547–1561.
9. Breaker, R.R. DNA Enzymes. (1997) Nat Biotechnology. 15(5):427-31.
10. Carothers, J.M., Oestreich, S.C., Davis,J.H. and Jack W. Szostak, J.W. (2004) Informational Complexity and Functional Activity of RNA Structures. J. Am Chem. Soc. 126:5130-5137.
11. Drabovich, A.P., Okhonin, V., Berezovski, M. and Krylov, S.N. (2007) Smart aptamers facilitate multi-probe affinity analysis of proteins with ultra-wide dynamic range of measured concentrations. *J. Am. Chem. Soc.*129: 7260-7261.

Smart Designed Aptamers by Gene Link

Gene Link presents various design options for synthesizing effective aptamers based on the application. Smart Designed Aptamers 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 aptamer oligonucleotide sequence or add a short peptide sequence that is known to aid in transport of the aptamer to the nucleus.

Aptamers have received widespread recognition as an alternate to the use of antibody-based protocols. Aptamers are very stable as compared to antibodies, are chemically stable more stable, can withstand more harsh environmental conditions and can be boiled or frozen without loss of activity. Production methods are less demanding and are synthetically produced. The production can be easily scaled up to kilogram quantities for therapeutic applications with minimal lot to lot variations. As with most natural molecules these 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 aptamer modification based on application. 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.

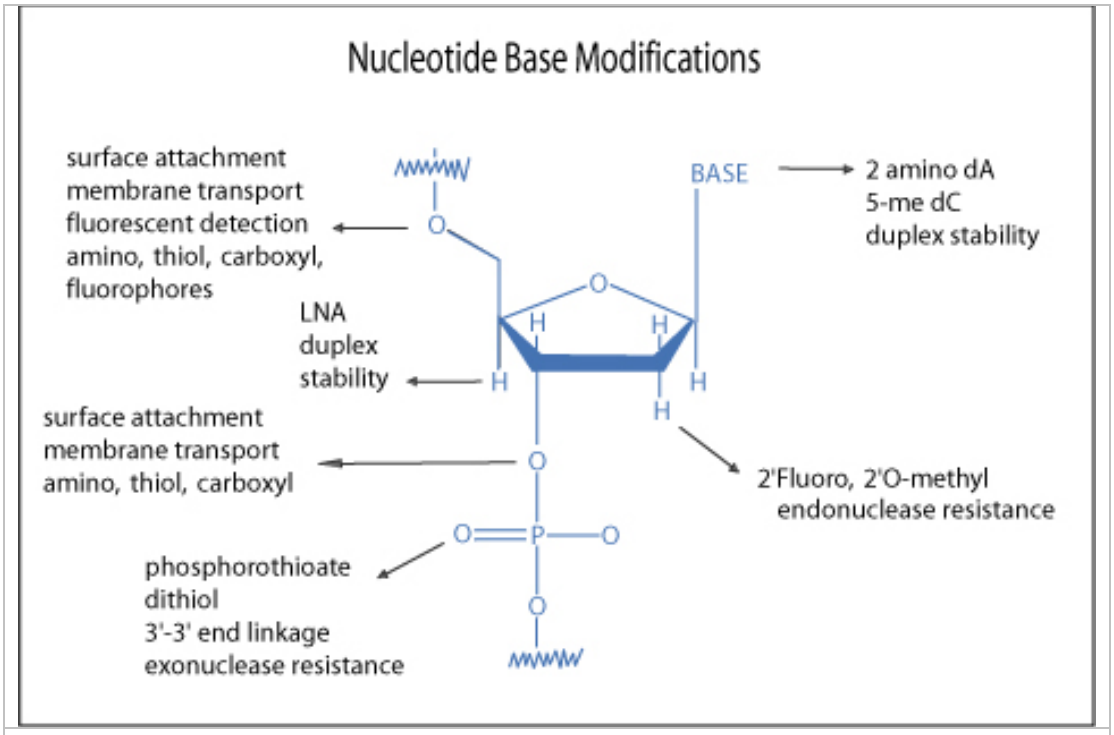


Figure 4. A partial list of possible modifications to a nucleotide.

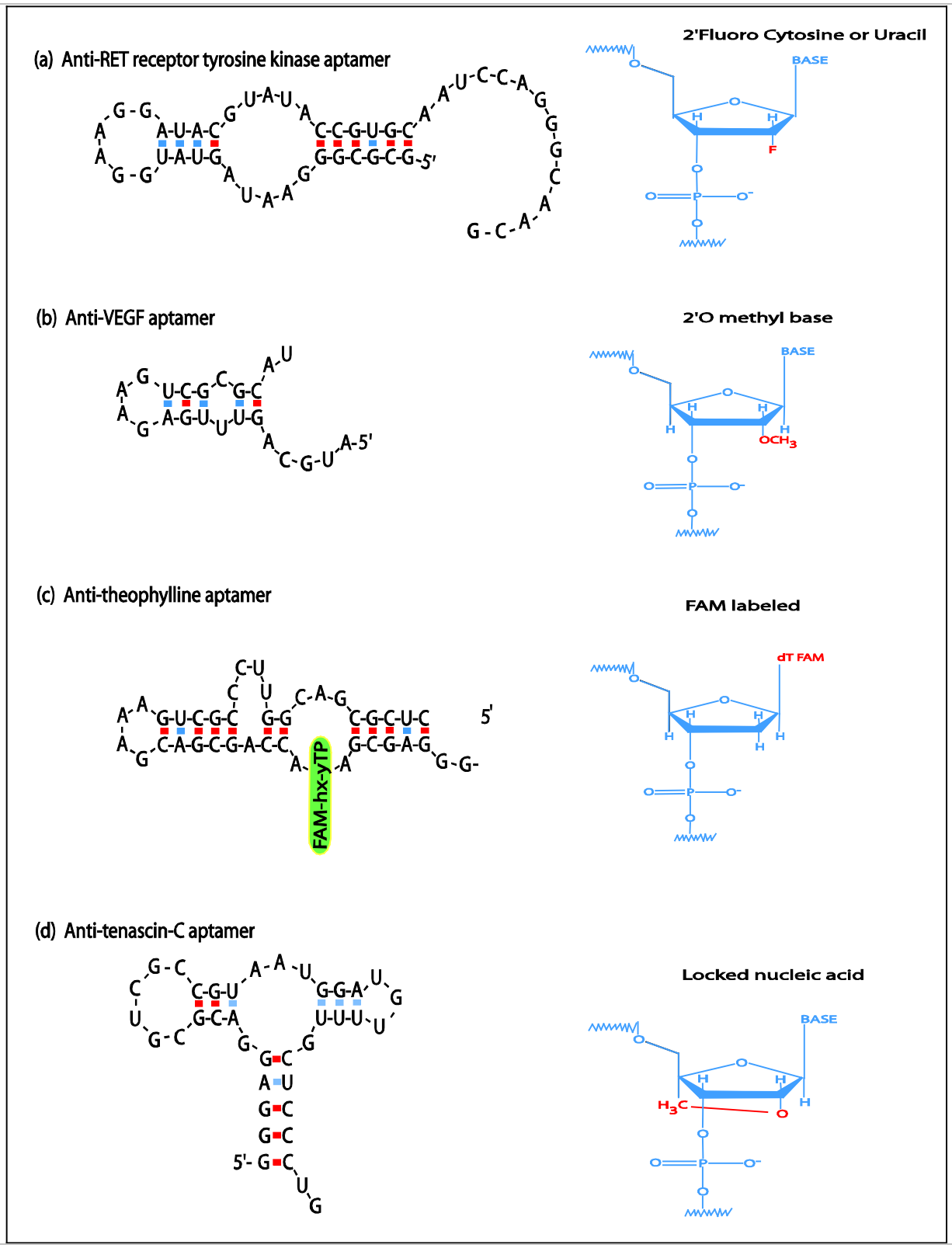


Figure 5. Examples of different Aptamers with modifications. For details see Lee, J.F., Stovall, G.M. and Ellington, A.D. (2006) Aptamer therapeutics advance. Current Opinion in Chemical Biology 10:282–289.

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

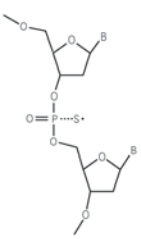
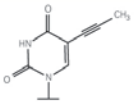
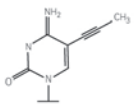
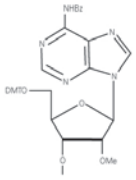
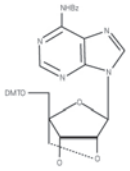
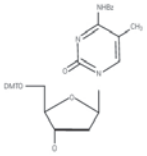
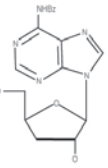
1. Phosphodiester linkages.
2. Nucleic acid bases.
3. Sugar moieties.
4. Functional group addition.

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 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.

Increased Duplex Stability and Manipulation of Duplex Stability

The folding of single stranded nucleic acids in a particular shape leads to its interaction with the target molecule. Successful interactions are those where the nucleic acid ligand –Aptamer binding to the target leads to overall stability of the structural conformation of the specific aptamer. There will be a pool of such aptamers for a particular target molecule. This pool of specific aptamers differs in their dissociation constant and this is directly related to its three dimensional shape. The shape is due to its sequence and in turn to the intra-molecular hybridization. Manipulation of this sequence to increase the duplex stability or in some cases to decrease the duplex stability in certain loop structure will lead to aptamers 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.

Oligo Modifications							
Modification	**Phosphorothioate**	**Propyne analogs**	**2'-O-methyl RNA**	**Locked Nucleic Acids**	**5-Me-dC**	**2'-5' Linked Oligonucleotides**	**Chimeric Linkages**
Molecular Structure	 2'-Deoxy thioate	 5-(1-Propynyl) uracil (pU)  5-(1-Propynyl) cytosine (pC)	 2'-O-methyl A	 dA LNA	 5-Me-dC	 3' dA (2'-5' linked)	Structure varies
Chemical Characteristics	Modification of the phosphodiester bond by replacing one of the non-bridging oxygens by sulfur	C-5 propyne analogs of dC and dT	2'-O-methyl at the 2' hydroxyl position	Bicyclic nucleic acid where a ribonucleoside is linked between the 2'-oxygen and the 4'-carbon atoms with a methylene unit	C-5 methylated dC	2'-5' linked phosphodiester linkage, 3' deoxy bases	Mixed phosphorothioate and phosphodiester linkages and modifications
Duplex Stability	Hybridizes to the target sequences with lesser affinity than oligos with phosphodiester backbone	Increased binding affinity to the target mRNA and increased stability	Binding similar to DNA	Highest thermal stability of all available modifications	Increased	Increased binding efficiency to RNA	Increased
Nuclease Resistance	Imparts resistance to nuclease degradation	Increased nuclease resistance	Increased	Increased	Similar to DNA	Increased	Increased

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 phosphorothioate linkages, 2'O methyl, propyne bases etc.

Cellular Delivery

Cellular delivery is an important functional aspect for therapeutic aptamers in addition to duplex stability and nuclease resistance. Polyethylene glycol, cholesterol, and various peptide structures have been used to attach to aptamers to facilitate cellular delivery or even targeting to the nucleus. Photo-cleavable linkers have been used that can be cleaved under controlled conditions.

Surface Attachment

Developments in nanotechnology, biosensors and similar applications for nanoscale detection require facile surface attachment chemistries. Oligonucleotides have been attached to various solid surfaces for hybridization-based applications. The classic protocol is the attachment of oligo dT to cellulose for the isolation of poly A+ RNA. Microarray based protocols also require oligonucleotide and DNA attachment. Various functional group modifications are commonly available for such applications; examples are amino, thiol and carboxy for covalent attachment, and biotin for affinity-based attachment. Recent applications are focused on gold coated surfaces.

Different chemistries and approaches have been used for attachment of oligonucleotides to peptides. These chemistries have been extended for aptamer (oligonucleotide) attachment. Figure 6 shows a schematic illustration of three different conjugation schemes for linking the delivery peptide to molecular beacons. (A) The streptavidin± biotin linkage in which a molecular beacon is modified by introducing a biotin-dT to the quencher arm of the stem through a carbon-12 spacer. The biotin-modified peptides are linked to the modified molecular beacon through a streptavidin molecule, which has four biotin-binding sites. (B) The thiol-maleimide linkage in which the quencher arm of the molecular beacon stem is modified by adding a thiol group which can react with a maleimide group placed to the C terminus of the peptide to form a direct, stable linkage. (C) The cleavable disulfide bridge in which the peptide is modified by adding a cysteine residue at the C terminus which forms a disulfide bridge with the thiol-modified molecular beacon. This disulfide bridge design allows the peptide to be cleaved from the molecular beacon by the reducing environment of the cytoplasm.

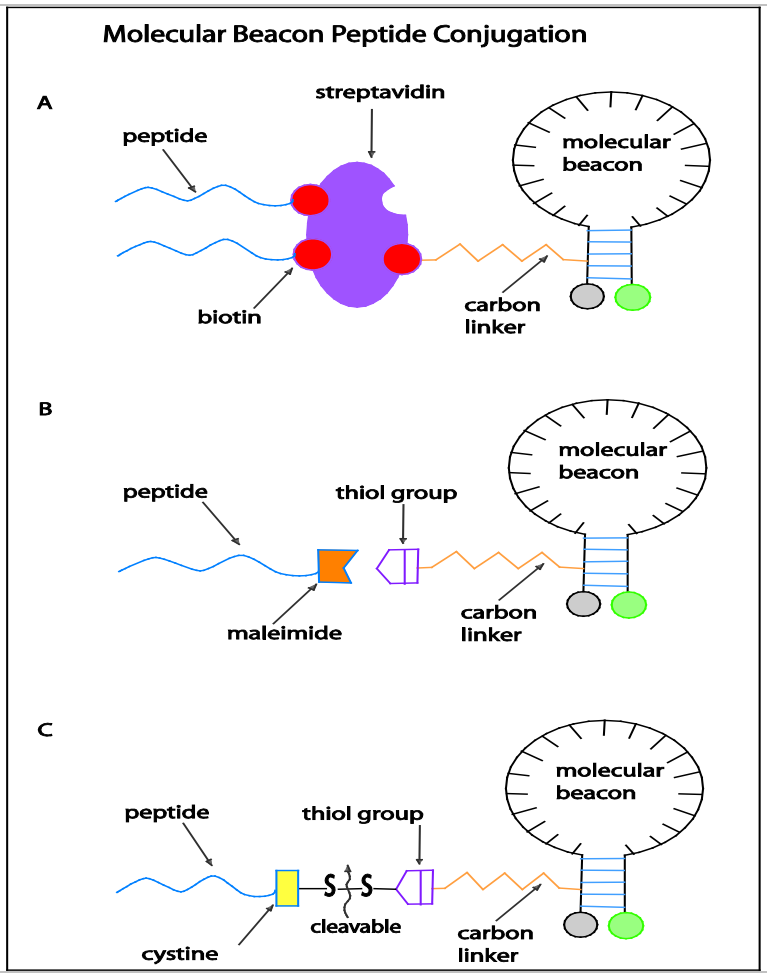


Figure 6. Molecular beacon attachment chemistries to peptides. See text for details. Taken from: Peptide-linked molecular beacons for efficient delivery and rapid mRNA detection in living cells. Nitin Nitin, Philip J. Santangelo, Gloria Kim, Shuming Nie and Gang Bao. Nucleic Acids Research, 2004, Vol. 32, No. 6 e58. Figure used with permission.

Aptamers specific for different proteins such as thrombin, HIV tat protein and C-reactive protein, have been coupled to optical, piezoelectric and electrochemical transduction, for the development of a new generation of biosensors. The immobilization of the aptamer on a solid support must avoid any steric hindrance or constraint which could prevent the folding of the aptamer in the correct conformation. For these reasons, several immobilization protocols have been studied and optimized. Aptamers were modified with a biotin molecule or with a thiol group in order to immobilize them onto a streptavidin layer or directly onto the gold surface of the transducers, respectively. Moreover, different tails have been also attached to the 5' end of the aptamers to avoid any steric hindrance due to the vicinity of the oligonucleotide to the solid surface.

Developments in nanotechnology and biosensors instruments make the use of aptamers and DNA enzymes an attractive alternate to existing protocols using antibodies or other methods; this is an area of active research and commercial development. The signal of aptamer binding to target or the catalytic activity of DNA enzymes can be transduced using fluorescent dyes. Gene Link presents an array of fluorescent dyes and quenchers together with modification for duplex stability and nuclease resistance.

Modifications

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.

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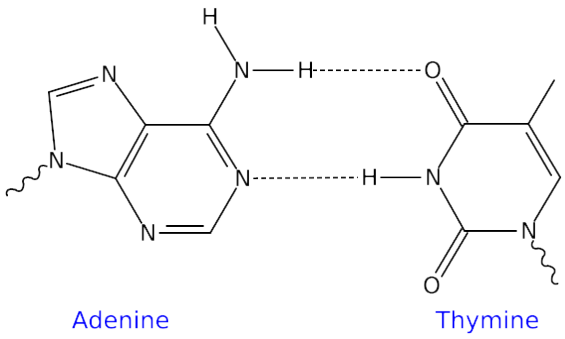
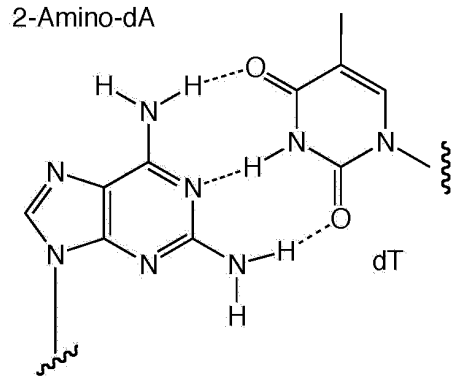
<tr><td> 
</td><td> 
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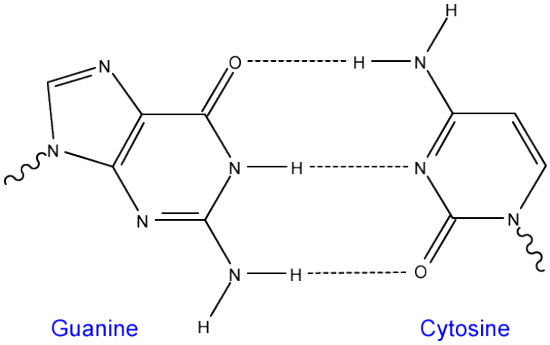
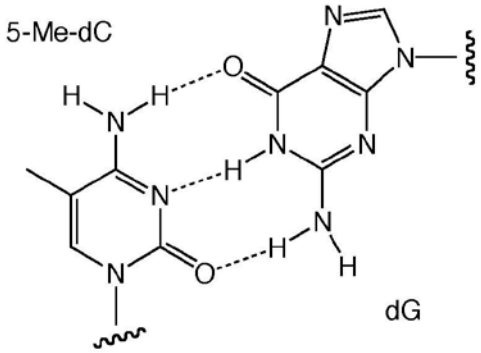
<tr><td> 
</td><td> 
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<tr><td>G-C base pairing</td><td>5-Me-dC-dG base pairing</td></tr>
</table>

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°.

Duplex Stabilization

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

2-Amino-A	3.0° per substitution
5-Methyl-C	1.3° per substitution
C-5 propynyl-C	2.8° per substitution
C-5 propynyl-U	1.7° per substitution

While these modifications would also have a desirable effect on antisense oligonucleotides, the increased costs associated with most of them may limit their use. However, primers are less cost-sensitive because of the smaller scale, so the effects of the modified bases may be more generally useable. Potential improvements would include: the ability to use shorter oligos when sequence information is incomplete; higher melting temperatures, which should minimize the frequency of mutations; and enhanced binding, which should break any secondary structure in the target.

2'-F-RNA Monomer

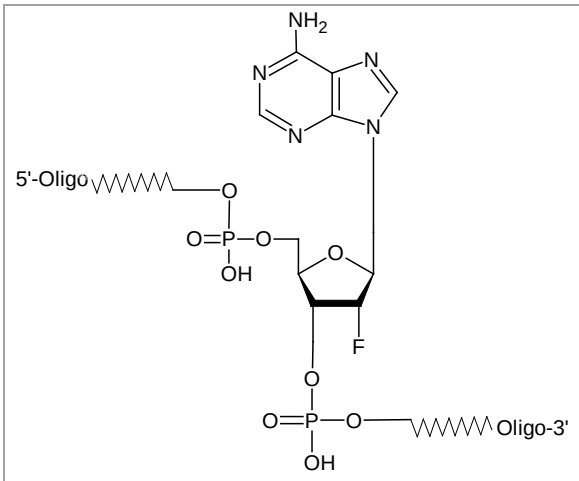
2'-Deoxy-2'-fluoro-nucleosides adopt an RNA-type sugar conformation, presumably due to the high electronegativity of fluorine. Because of this sugar conformation, RNA duplexes (A-form) are generally more thermodynamically stable than DNA duplexes (B-form). As expected, the addition of 2'-F-RNA residues 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 Pharmaceuticals1 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.

2'-Fluoro RNA Oligonucleotide



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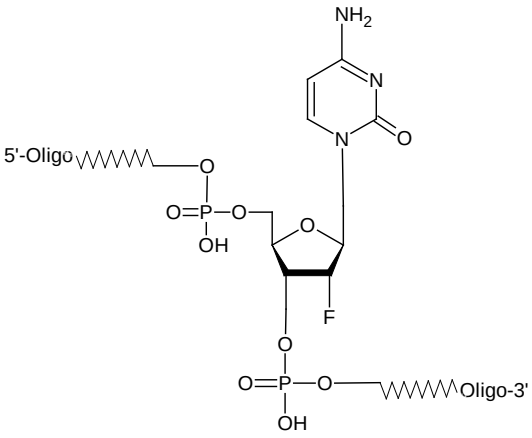
[26-6692-XX]



2'-F C
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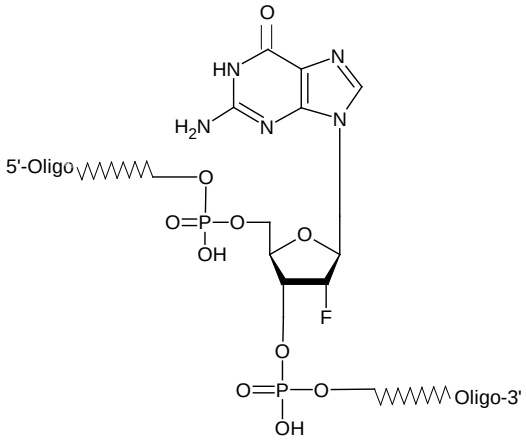
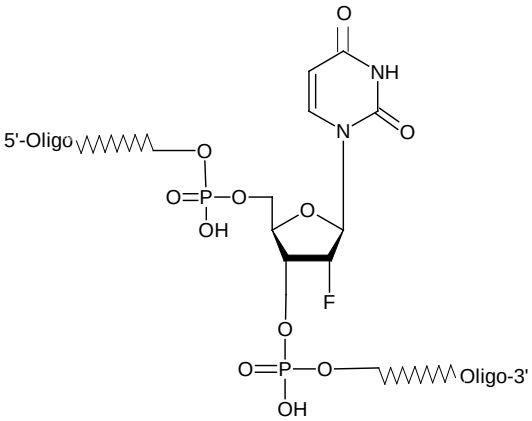


2'-F G
[26-6693-XX]



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

Recent work (4) has 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. In a study (5) of 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 (1) 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.

References

1. Taken from Glen Research Website. www.glenres.com.
2. Kawasaki. A.M., et al., Journal of Medicinal Chemistry, 1993, 36, 831-841.
3. Khati, M., Schuman, M. Ibrahim, J., Sattentau, Q., Gordon, S. and W. James, J Virol, 2003, 77, 12692-8.
4. Layzer, J.M., McCaffrey, A.P., Tanner, A.K., Huang, Z., Kay, M.A. and Sullenger, B.A. (2004) RNA 10:766-71.
5. Yazbeck, D.R. Min, K.L. and Damha, M.J. (2002) Nucleic Acids Res., 30: 3015-25.

Phosphorothioate Linkages

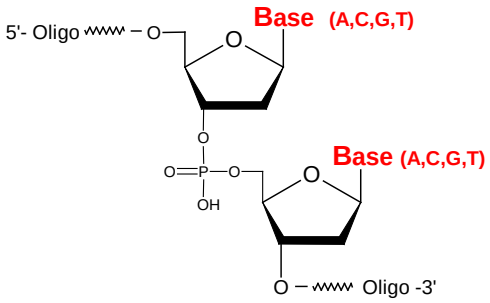
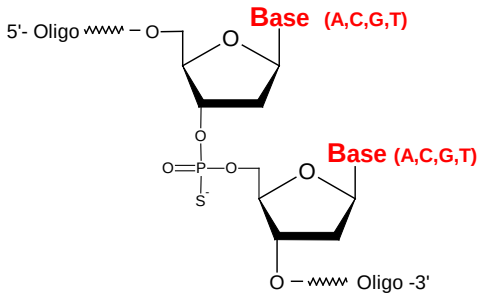
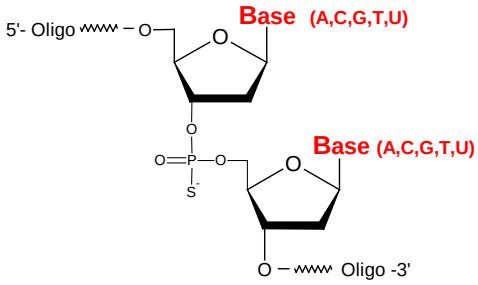
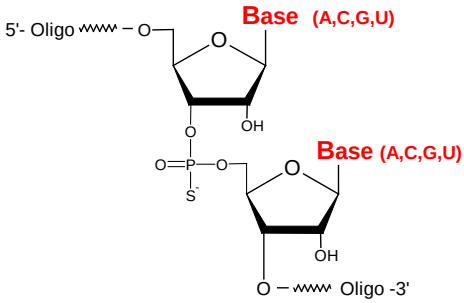
The driving force for the search for novel chemical modification groups compatible with Watson-Crick hybridization of oligonucleotide was based on the observation of the short stability of naturally occurring oligonucleotides with phosphodiester bonds. Oligonucleotides with natural phosphodiester bonds 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 LNA and 2'-5' linked oligos as described later sections.*

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 (1) sulfurizing reagent. The sulfurization reaction is rapid and is performed on automated DNA synthesizers yielding greater than 96% phosphorothioate linkages; the remainders are phosphodiester linkages. Custom phosphorothioate oligonucleotides synthesized by Gene Link can be specified to have all the diester bonds substituted or only some selected diester linkages depending upon the researcher's experimental requirement. Substitution of all diester linkage is recommended to provide greater nuclease resistance.

The most common usage for oligonucleotide phosphorothioates has been in the production of antisense oligodeoxynucleotides destined for use in identifying or modifying gene expression. Now, phosphorothioate linkages are being used for RNA world and sulfurizing RNA linkages with reagents like Beaucage Reagent has proved to be much more difficult than DNA linkages. The phosphorothioate (PS) linkage is a not-so-expensive way of increasing the stability of nucleic acids and increasing nuclease resistance of RNA. Now, it has been shown (2) that fully PS oligos can promote the delivery of siRNA in cell culture. This siRNA uptake is sequence-independent, and the length seems to vary between 30 and 70 nucleotides depending on the cell line. Even though this method is not yet as efficient as the cationic lipids, it opens the way to possible new methods. Reasons that may explain this are not understood at this time.

Another paper (3) describes a method for the inactivation of micro RNA (miRNA) that may help to elucidate their functions. It uses 2'-OMe-RNA oligonucleotides (23-mers, complementary to a target miRNA) with a cholesteryl group at the 3´terminus and phosphorothioates at positions 1 and 2 at the 5´end and at the last four positions at the 3´end. These oligos are called antagomirs. These molecules promote the cleavage of complementary miRNAs and thus should allow analysis of their function. The role of the PS linkages presumably is the stabilization against degradation in the mouse experiments as it is standard in the antisense field in such in vivo situations. And finally, a recent paper (4) shows that PS does not systematically abolish siRNA activity, opening the way for some potentially less expensive stabilization of such molecules. Incorporation of 2'-OMe (in the sense strand) in combination with PS linkages should confer to siRNA increased resistance to degradation by nucleases, as well as prolonged serum retention. And it is also possible that such easy modification of siRNA may increase the specificity by eliminating sense strand recruitment in the RISC complex and thus reducing a source of off-target effect.

 Phosphodiester Linkage [26-6400-XX]	 Phosphorothioate Linkage [26-6401-XX]
Phosphodiester Linkage	Phosphorothioate Linkage (PS)
 Phosphorothioate Linkage [26-6401-XX]	 Phosphorothioate Linkage [26-6401-XX]
Chimeric Phosphorothioate Linkages (DNA,RNA, LNA, 2'O Me and other bases)	RNA phosphorothioate Linkage

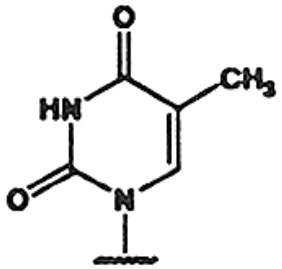
References

1. R.P. Iyer, W. Egan, J.B. Regan, and S.L. Beaucage, J. Amer. Chem. Soc., 1990, 112, 1253-1254.
2. M. Overhoff and G. Sczakiel, EMBO Rep, 2005, 6, 1176-81.
3. J. Krutzfeldt, et al., Nature, 2005, 438, 685-9.
4. B.A. Kraynack and B.F. Baker, RNA, 2006, 12, 163-76.
5. B.J. Bergot and W. Egan, Journal of Chromatography, 1992, 599, 35-42.

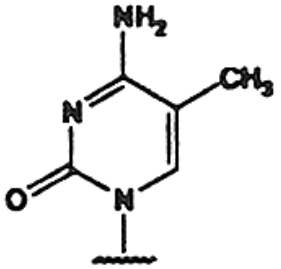
Propyne Analogs

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.

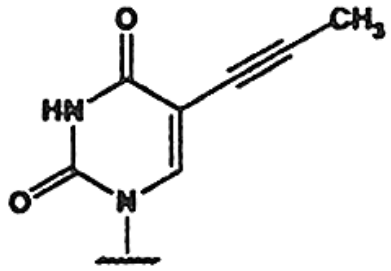
The use of propyne analogs is covered by patents and licensing agreements. The sale of propyne-modified oligos is for research use only. See license agreement*



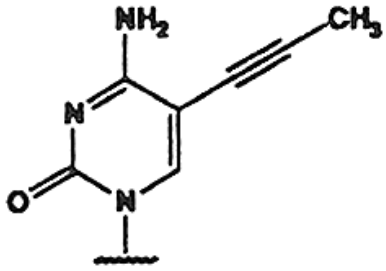
Thymine
(T)



5-Methyl
cytosine (mC)



5-(1-Propynyl)
uracil (pU)

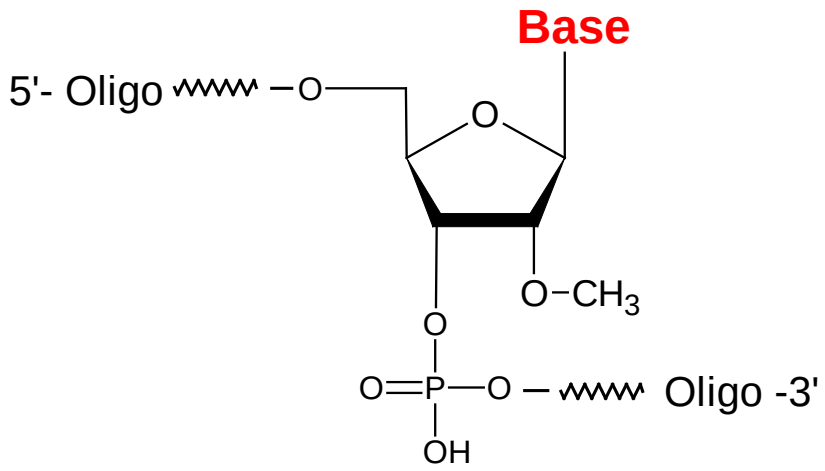


5-(1-Propynyl)
cytosine (pC)

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.

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.

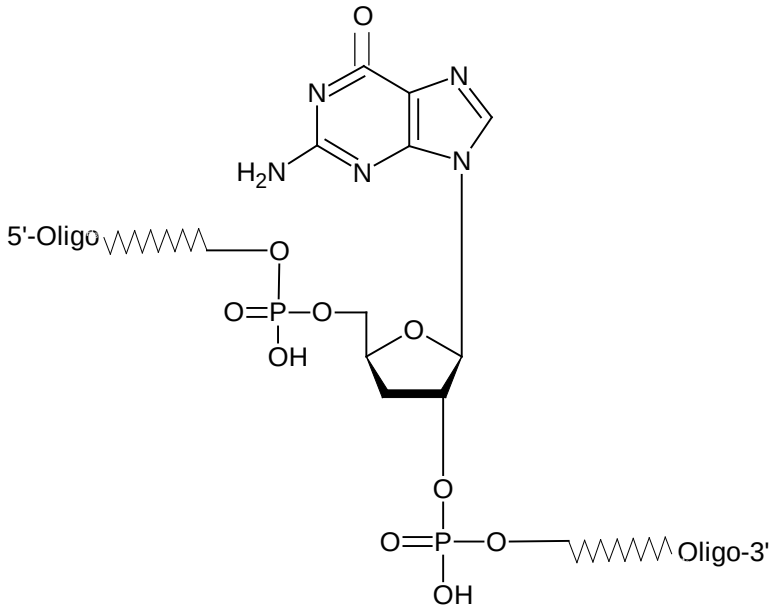


2' O-Methyl Base

[27-6410-XX]

2'-5' Linked Oligonucleotides

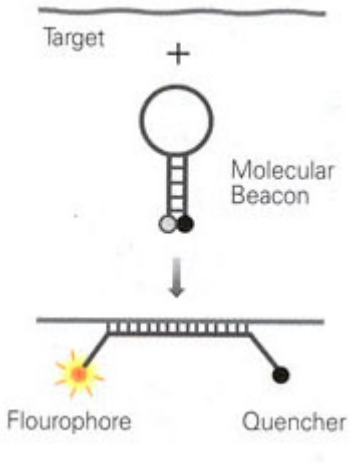
Cellular DNA and RNA are made up of ribo- and 2'-deoxyribonucleic acids linked together via 3'-5' phosphodiester linkages and by far comprise the bulk of polynucleic acids found in cells. Much less common are oligonucleotides which have 2'-5' linkages. However, a unique feature of 2'-5' linked oligonucleotides 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'-dG (2'-5' linked)
[26-6492-XX]

Molecular Beacons

Molecular beacons are oligonucleotide probes that can report the presence of specific nucleic acids in homogenous solutions (Tyagi S, Kramer FR. Molecular beacons: probes that fluoresce upon hybridization, Nature Biotechnology 1996; 14: 303-308.) They are useful in situations where it is either not possible or desirable to isolate the probe-target hybrids from an excess of the hybridization probes, such as in real time monitoring of polymerase chain reactions in sealed tubes or in detection of RNAs within living cells. Molecular beacons are hairpin shaped molecules with an internally quenched fluorophore whose fluorescence is restored when they bind to a target nucleic acid (Figure 1). They are designed in such a way that the loop portion of the molecule is a probe sequence complementary to a target nucleic acid molecule. The stem is formed by the annealing of complementary arm sequences on the ends of the probe sequence. A fluorescent moiety is attached to the end of one arm and a quenching moiety is attached to the end of the other arm. The stem keeps these two moieties in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by energy transfer. Since the quencher moiety is a non-fluorescent chromophore and emits the energy that it receives from the fluorophore as heat, the probe is unable to fluoresce. When the probe encounters a target molecule, it forms a hybrid that is longer and more stable than the stem and its rigidity and length preclude the simultaneous existence of the stem hybrid. Thus, the molecular beacon undergoes a spontaneous conformational reorganization that forces the stem apart, and causes the fluorophore and the quencher to move away from each other, leading to the restoration of fluorescence.



Operation of molecular beacons: On their own, these molecules are non-fluorescent, because the stem hybrid keeps the fluorophore close to the quencher. When the probe sequence in the loop hybridizes to its target, forming a rigid double helix, a conformational reorganization occurs that separates the quencher from the fluorophore, restoring fluorescence.

In order to detect multiple targets in the same solution, molecular beacons can be made in many different colors utilizing a broad range of fluorophores (Tyagi S, Bratu DP, Kramer FR. Multicolor molecular beacons for allele discrimination, Nature Biotechnology 1998; 16: 49-53.) DABCYL, a non-fluorescent chromophore, serves as the universal quencher for any fluorophore in molecular beacons. Owing to their stem, the recognition of targets by molecular beacons is so specific that single-nucleotide differences can be readily detected.

Molecular Beacon Example Sequence

Fluorophore at 5' end; 5'-GCGAGCTAGGAAACACCAAAGATGATATTTGCTCGC -3'-Quencher

```
5'  GCGAGCTAGGAAACACCA
    ||||||              ]
3'  CGCTCGTTTATAGTAGAA
```

STEM AT 1 IS 6 BP LONG. LOOP = 24.

The vertical lines at the 5' and 3' ends identify the arm sequences that are complementary.

The length of the probe sequence (10-40 nt) is chosen in such a way that the probe target hybrid is stable in the conditions of the assay. The stem sequence (5-7 nt) is chosen to ensure that the two arms hybridize to each other but not to the probe sequence. Folding of the designed sequence with the help of a computer program can indicate whether the intended stem-and-loop conformation will occur. The computer program can also predict the melting temperature of the stem.

Molecular Beacon Design

The PCR primers themselves should have been optimized in a regular PCR to see that it performs well. Assuming the melting temperature of the primers are ~55 degrees.

There are two independent features to control in the design of the MB probe. The stem and the target loop sequence. Design the probe sequence and see that there is minimal secondary structures, loop formation and dimers and the TM is ~5 degree higher than the PCR primer annealing temp. For a good guideline keep it at ~60 degrees. Add the stem sequence of 5-7 bp. The TM of the stem itself will be ~ 60-70 degrees. You are done!.

General guidelines for MB design at Gene Link are as follows:

1. Design regular 18-24mer PCR primers for amplification with a TM around 55^0C. The optimal amplified fragment should be between 100-300 bp. Perform PCR, optimize conditions. Should get good clean amplification product visible on ethidium bromide stained gels.
2. Design target probe sequence with a TM ~8-10 degrees higher than the PCR primers annealing temp. Example 60-65 degrees. The probe should be designed near the center of the amplified fragment avoiding stretches of strong secondary structure. [Taqman probes are designed ~ 5-10 bases near the primer of the same strand]
3. Add the stem 5- 7 bp stem sequence with a GC content of 70-80%. Avoid a G at the 5' end next to the fluorophore. G's seem to have a quenching effect. Hairpin Stem TM should be 7-10 degrees higher than the PCR annealing temperature. Example 65-70 degrees.
Caution: See that by adding the stem you have not created secondary structures with the loop sequence. Try variation of stem sequence to avoid secondary structure with the loop sequence.

The Hairpin stem TM is based on free energy stabilization and folding, the following is a good guideline.

GC rich stem
5 bp = 55^0C- 60^0C
6 bp = 60^0C-65^0C
7 bp = 65^0C-70^0C

Quenching

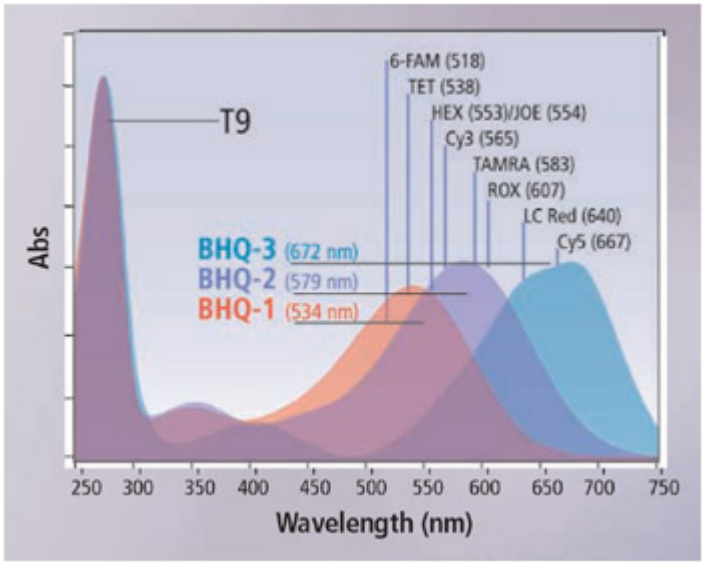
Reduction in the expected fluorescence emission is termed as quenching. Generally, it would be an impediment if the emission were reduced. The phenomenon of quenching forms the basis of the mode of action of molecular probes; the designed and controlled fluorescence based on hybridization to the target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

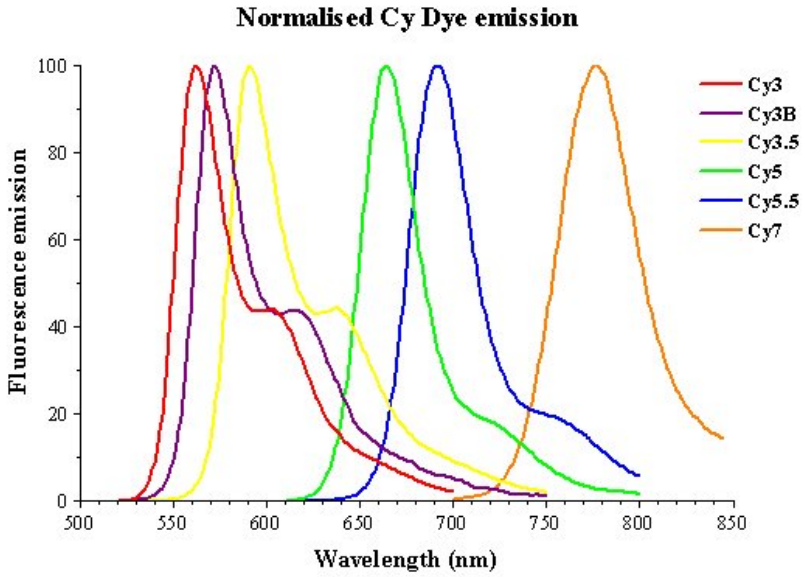
Natural quenching occurs due to 'fading' after repeated cycles of excitation and relaxation. The decrease in the ability of further excitation of a proportion of molecules is termed as photobleaching. Some dyes are much more sensitive than other to photobleaching, for example fluorescein photobleaches 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 FRET, and can be assumed that the energy transfer typically occur over a distance of 1-10 nm. The quenching effect is exhibited by fluorescent as well non-fluorescent molecules. A non-fluorescent quencher is the basis of the design of Molecular Beacons. This molecule could be non-fluorescent and acts as energy sink, and termed as a quencher. In other instances this molecule could itself be a dye with overlapping spectral absorption and emission spectra, in such cases energy is transferred from one to another 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 with 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


6-FAM (518)
TET (538)
HEX (553)/JOE (554)
Cy3 (565)
TAMRA (583)
ROX (607)
LC Red (640)
Cy5 (667)
T9
BHQ-3 (672 nm)
BHQ-2 (579 nm)
BHQ-1 (534 nm)
Abs
250 300 350 400 450 500 550 600 650 700 750
Wavelength (nm)


Normalised Cy Dye emission
Fluorescence emission
100
80
60
40
20
0
500 550 600 650 700 750 800 850
Wavelength (nm)
Cy3
Cy3B
Cy3.5
Cy5
Cy5.5
Cy7

Fluorophore Spectral Data & Quencher Selection Guide

Fluorophore Name	Excitation Max, nm	Emission Max, nm	EC*	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		

* EC, 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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