The Glen Report

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GLEN RESEARCH

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INSIDE

INTRODUCTION

In this issue of *The Glen Report*, we are introducing a selection of new products and methodologies. One would think that capping in oligonucleotide synthesis would have been developed to the nth degree, but incomplete capping continues to be one of the main contributors to problems. We have, therefore, reviewed the capping efficiency of the current capping mixes and have compared this efficiency achieved to that achieved by a new phosphoramidite capping reagent, UniCap Phosphoramidite.

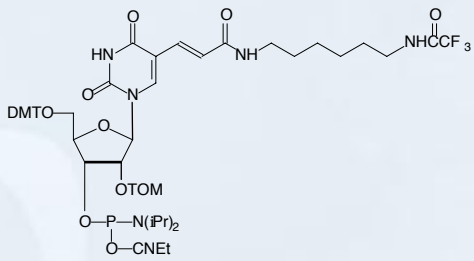
As usual, we are adding many new products to our range of nucleosides available for DNA and RNA research. As siRNA increases in significance, we have increased our focus on RNA research. We are introducing a uridine derivative (Amino-Modifier C6-U Phosphoramidite) for labeling RNA and 6-Thio-G Phosphoramidite for cross-linking to associated RNA or protein molecules. These new phosphoramidites are listed beginning on this page. We have had a long-term interest in 2'-fluoro-RNA monomers and we are happy to be able to add 2'-fluoro-A and 2'-fluoro-G to that family of products. We are also introducing our more popular supports for 3' modification on polystyrene in columns compatible with the Applied Biosystems 3900 synthesizer.

Also, we recently completed an agreement with Biosearch Technologies to begin supplying Black Hole Quencher™ (BHQ) products to the research market. These quenchers complement our current quenchers, Dabcyl and Eclipse™, and cover the complete spectral range offered by our extensive line of fluorescent dyes.

Although molecular biology products are unusual for Glen Research, we have been working recently on preparing unusual triphosphates with specific unique properties. In partnership with Lawler Scientific, LLC, we have used our chemical expertise to produce Internally Quenched Nucleotides (IQNs), which we expect will find broad acceptance in a variety of biological applications.

PRODUCTS FOR siRNA RESEARCH

FIGURE: AMINO-MODIFIER C6-U



Amino-Modifier C6-U

Amino-Modifier C6-U

Amino-Modifier C6-dT was first described[1, 2] in the mid 1980s when interest in labeling oligos was very limited. The original usage was to attach alkaline phosphatase to oligos for diagnostic applications. The molecule was set up perfectly for this kind of use since the linker arm projects into the major groove of double-stranded DNA where there is room for large molecules without disruption of hybridization.[3] Over the years, we have added products to our range based on amino-modifier C6-dT labeled with Biotin, Dabcyl, TAMRA, Fluorescein and, in this issue, BHQ-1™ and BHQ-2™. Now it is time to add the RNA analogue Amino-Modifier C6-U. Initially, we have chosen the popular TOM group[4] for protection of the 2'-hydroxyl. We welcome Amino-Modifier C6-U to this growing structural family and envisage applications in RNA structural studies as well as for labeling siRNA to probe uptake and cellular distribution.

References:

(1) J.L. Ruth, C. Morgan, and A. Pasko, *DNA*, 1985, **4**, 93.

(2) E. Jablonski, E.W. Moomaw, R.H. Tullis, and J.L. Ruth, *Nucleic Acids Res.*, 1986, **14**, 6115.

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UNICAP PHOSPHORAMIDITE, AN ALTERNATIVE TO ACETIC ANHYDRIDE CAPPING

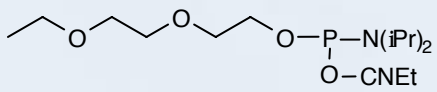
Introduction

The major impurities generated during oligonucleotide synthesis are n-1 and, to a lesser extent, n-2 deletion sequences. These deletion sequences are not homogeneous and result not from a single coupling failure but have been shown to be a statistical distribution of all possible n-1 or n-2 deletions by sequencing[1], mass spectroscopy[2] and hybridization to arrays of all possible complementary deletion strands.[3] Deletion mutations can arise from an incomplete reaction at any of the steps during the synthesis cycle including; incomplete detritylation, incomplete capping of coupling failures or incomplete oxidation of the phosphite triester and subsequent hydrolysis back to the 5'-hydroxyl of the previous base during the next detritylation step. By far the predominant source of these deletions is incomplete capping of coupling failures during each synthesis cycle.

Standard capping is accomplished by acetylation of any remaining unreacted 5'-hydroxyls using a mix of acetic anhydride in THF (Cap A) and a capping activator, either dimethylaminopyridine (DMAP) or N-methylimidazole (MeIm) in THF (Cap B). A weak organic base, either pyridine or lutidine, is added to one of the Cap mixes.

The failure to cap, and the resulting generation of excess n-1 deletion sequences, present particular problems in trityl-on purifications and in the synthesis of long oligonucleotides for gene construction and cloning. Trityl-on purification relies on the increased hydrophobicity of the trityl group which is present only on the last base or monomer unit added and not on the capped failures. Unfortunately the n-1 deletions also possess 5'-trityls which make them elute along with the full-length oligo. These trityl-on deletions can be partially eliminated in HPLC purifications by collecting only the middle of the trityl-on peak since shorter deletion oligos elute on the backside of the peak. This is not possible with cartridge purification techniques, so final oligo purity is directly dependent on the ability to efficiently cap coupling failures. Long oligos are usually purified by denaturing PAGE and n-1 deletions represent the most difficult contaminant to remove, which explains why so many long oligos used for cloning are incorrect.

FIGURE 1: **UNICAP PHOSPHORAMIDITE**



UniCap Phosphoramidite

UniCap Phosphoramidite

In an attempt to improve overall synthesis fidelity, other approaches to capping have been explored. Since the coupling reaction is so efficient, one option is to use a phosphoramidite for capping. This option is the approach used in H-phosphonate chemistry. To that end the phosphoramidite of diethylene glycol monoethyl ether, UniCap, has been synthesized and compared to the standard capping mixes. Each capping mix was first evaluated for its ability to block oligo synthesis. Following a mock coupling using acetonitrile in place of amidite, three additional couplings were performed with the final trityl left on. This is an extreme case and represents a complete coupling failure. Quantification of the trityl-on peak represents the relative amount of capping failure. The results of these experiments conducted in quadruplicate are shown in Table 1.

The results clearly demonstrate that the capping is dependent on the activator in the Cap B solution. MeIm was a less effective catalyst for acetylation with 90% capping efficiency at 10% concentration. Increasing the concentration to 16% increases capping to 97%. UniCap performs substantially better at close to 99% capping, as seen in Table 1. Although DMAP is an extremely efficient catalyst for acetylation, its use has been reported to result in modification of O^6-dG resulting in the formation of a fluorescent adduct.[4] For this reason DMAP has been replaced in most Cap B mixes by MeIm.

Capping with UniCap Phosphoramidite

To use UniCap as a capping amidite on the Expedite 8909 or ABI synthesizers, dilute it to the standard amidite concentration and place the vial in position 5 on the instrument. ABI cycles can be modified by adding coupling steps after the last column coupling step "Column Off", replacing "Base + Tetrazole to Column" (Function 33) with "5 + Tetrazole to Column" (Function 35). For use on the Expedite synthesizer, copy the coupling steps for amidite reservoir 5 and paste them into the coupling section of each of the other amidite cycles. The standard capping steps can be left out of the cycle. Although we have been unable to confirm that acetate capping reverses any O^6-dG modification formed during coupling, regular capping can certainly be left in the cycle if this is a concern.

When UniCap Phosphoramidite was tested, it was found to be very highly effective at nearly 99% capping efficiency and, in addition, was determined to be stable for at least one week on the synthesizer.

UniCap Phosphoramidite was originally developed for capping in oligo synthesis on the surface of chips. Capping is often omitted in this situation because acetylation by acetic anhydride can change the polarity and surface characteristics of the chips. UniCap provides virtually quantitative capping without changing the polarity of chip surfaces. This reduces the background and increases the contrast of the array fluorescence. We are grateful to Dr. Xiaolian Gao, University of Houston, for providing the following information.

TABLE 1: **CAPPING EFFICIENCIES USING DIFFERENT SOLUTIONS**

Synthesizer	Cap A Solution	Cap B Solution	Cap Efficiency (%)
Expedite	THF/Ac_2O (9:1)	10%MeIm/THF/Pyr (8:1)	90.5 ± 1.9
ABI 394	THF/Pyr/Ac_2O (8:1:1)	10% MeIm/THF	88.8 ± 2.5
ABI 394	THF/Lut/Ac_2O (8:1:1)	10% MeIm/THF	89.1 ± 2.0
ABI 394	THF/Lut/Ac_2O (8:1:1)	16% MeIm/THF	96.6 ± 1.4
ABI 394	THF/Lut/Ac_2O (8:1:1)	6.5% DMAP/THF	99.4 ± 0.3
ALL	UniCap Phosphoramidite		98.6 ± 0.4

Oligonucleotide Microarray Synthesis on Microfluidic Chip

An oligonucleotide microarray containing 3888 sequences, which are selected from human cancer related genes, was synthesized as described previously.[5] One chip synthesis used a regular protocol with acetic anhydride (AC) capping and the other chip used the same protocol except for UniCap Phosphoramidite (PEG) capping.

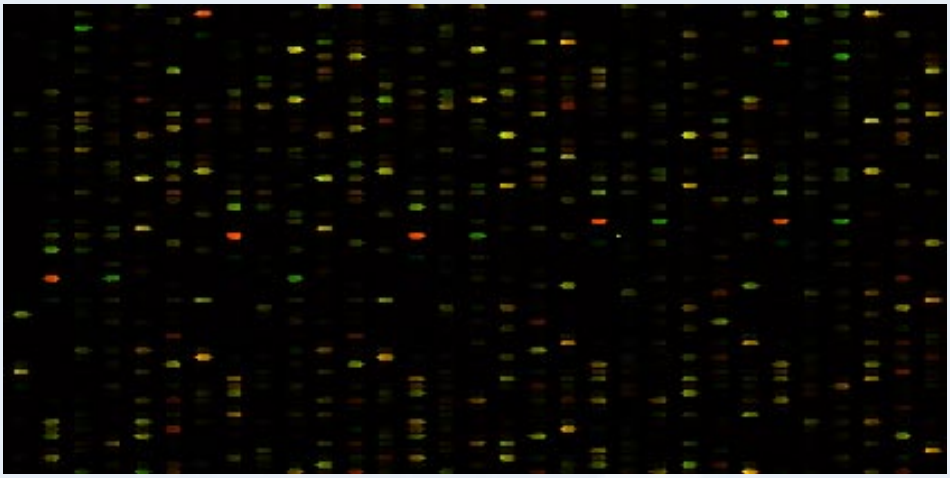
DNA Chip Hybridization Using cDNA Samples

Two cDNA samples were prepared according to procedures described (http://cmgm.stanford.edu/pbrown/protocols/index.html). The universal (univ) and skeletal muscle (sk) total RNA was from Clontech. Fluorescent Cy3 and Cy5 dyes were incorporated using dye-dU for the univ and sk cDNA samples, respectively. The co-hybridization of the cDNA samples to the DNA chip used 6´ SSPE (0.9 M NaCl, 60 mM Na_2HPO_4, 6 mM EDTA, pH 6.8) buffer (80 mL) mixed with 25% formamide at 32 °C for 18 h under micro-flow conditions. The chips were washed briefly with the 6´ SSPE buffer before image scanning on an Axon GenePix 4000B laser scanner. The PMT level was adjusted according to the signal strength observed. The images of the AC and PEG capping DNA chips are shown in Figure 2.

Validating Oligonucleotide Synthesis on a Chip Using Hybridization

The PEG capping was implemented in DNA chip synthesis and the comparison chip was synthesized using regular AC capping. The goals in these experiments were to compare hybridization results when the two DNA chips were treated with cDNA samples labeled with Cy3 (universal total RNA sample) or Cy5 (skeletal muscle total RNA sample) fluorescent dye. The two samples were co-hybridized to the chip and the ratio of Cy3 to Cy5 is shown in color ranging from green (Cy3 > Cy5) to yellow (Cy3 = Cy5) to red (Cy5 > Cy3). The color ratio image comparison of the PEG capping *versus* the AC capping chip is shown in Figure 2. This experiment validates that PEG capping is applicable to DNA microarray synthesis for the improvement of capping efficiency, which is critical for the initial synthesis steps of *in situ* oligonucleotide synthesis on glass surfaces. In addition, the capping reaction time using the PEG capping reagent is several fold shorter than that of AC capping.

AC Capping Chip



PEG Capping Chip

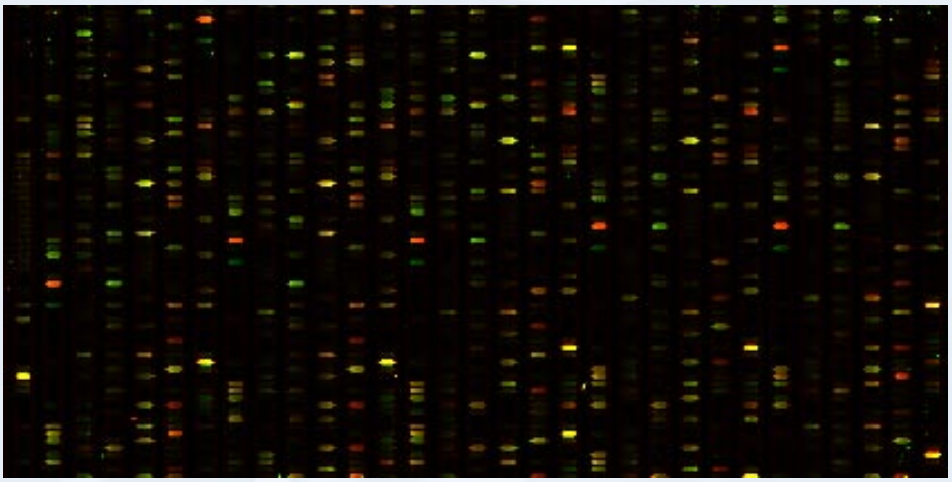


Figure 2: Images of cDNA hybridization to miniCancer Chip
Cy3(green): universal mRNA_cDNA
Cy5 (red): skeletal muscle mRNA_cDNA

The capping reagent was developed by Peilin Yu and Xiaolian Gao, Department of Chemistry, University of Houston. UniCap Phosphoramidite is sold by Glen Research under license from the University of Houston and is intended for research purposes only.

References:

(1) J. Temsamani, M. Kubert, and S. Agrawal, *Nucleic Acids Res.*, 1995, **23**, 1841-1844.
(2) K.L. Fearon, J.T. Stults, B.J. Bergot, L.M. Christensen, and A.M. Raible, *Nucleic Acids Res.*, 1995, **23**, 2754-2761.
(3) D.H. Chen, Z.M. Yan, D.L. Cole, and G.S. Srivatsa, *Nucleic Acids Res.*, 1999, **27**, 389-395.
(4) J.S. Eadie and D.S. Davidson, *Nucleic Acids Res.*, 1987, **15**, 8333-49.
(5) X.L. Gao, *et al.*, *Nucleic Acids Res.*, 2001, **29**, 4744-4750.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
UniCap Phosphoramidite	10-4410-02	0.25g	$50.00
	10-4410-05	0.5g	$100.00
	10-4410-10	1.0g	$200.00
	10-4410-20	2.0g	$400.00

(For Expedite vials, add "E" to catalog number.)

EXPANDING OUR REPERTOIRE OF DARK QUENCHERS: BLACK HOLE QUENCHERS

Introduction

Fluorescence Resonance Energy Transfer (FRET) has become one of the most popular tools to assay nucleic acids. This is because FRET lends itself to high throughput automation and is quite sensitive, making it the method of choice for sequence and single nucleotide polymorphism (SNP) analysis. In addition, it is highly useful for probing DNA and RNA structure, dynamics and intermolecular interactions.

The basis of FRET is the dipole-dipole coupling of donor and acceptor molecules in which the energy of the donor in the excited state is transferred to the acceptor molecule. The efficiency of the energy transfer depends upon a variety of factors – the distance between the donor and acceptor molecules and their orientation, the quantum yield of fluorescence of the donor, the extinction coefficient of the acceptor and the spectral overlap between the emission of the donor and the absorbance of the acceptor.[1] In a traditional FRET experiment, both the acceptor and donor molecules are fluorophores, with the 5' terminus labeled with the donor and the 3' with the acceptor. Upon excitation of the donor, the acceptor fluoresces and the donor is quenched. If, however, the donor/acceptor pair is separated by a conformational change or action such as cleavage by a nuclease, the donor fluorescence is unaffected, as shown in Figure 1.

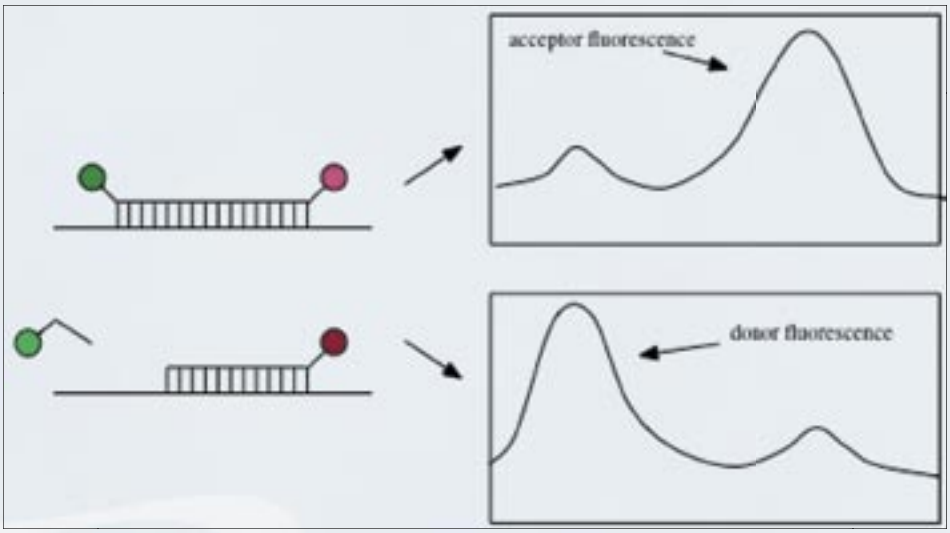


Figure 1: Schematic of FRET with a dual labeled probe before and after action of a nuclease and representative fluorescence spectra.

Dark Quenchers

The quencher need not be a fluorophore, however. A non-fluorescent chromophore can be used that overlaps with the donor's emission (a dark quencher). In such a case, the transferred energy is dissipated as heat.

FRET probes that utilize dark quenchers have a number of advantages over their fluorophore-labeled counterparts. They exhibit lower background fluorescence which leads to a larger signal-to-noise ratio, and, therefore, greater dynamic range.[2] In addition, since there is no secondary fluorescence arising from a dark quencher, multiple fluorophores can be simultaneously spectrally resolved, making dark quencher probes amenable to multiplex assays. But one of the most endearing qualities of a FRET probe designed with a dark quencher

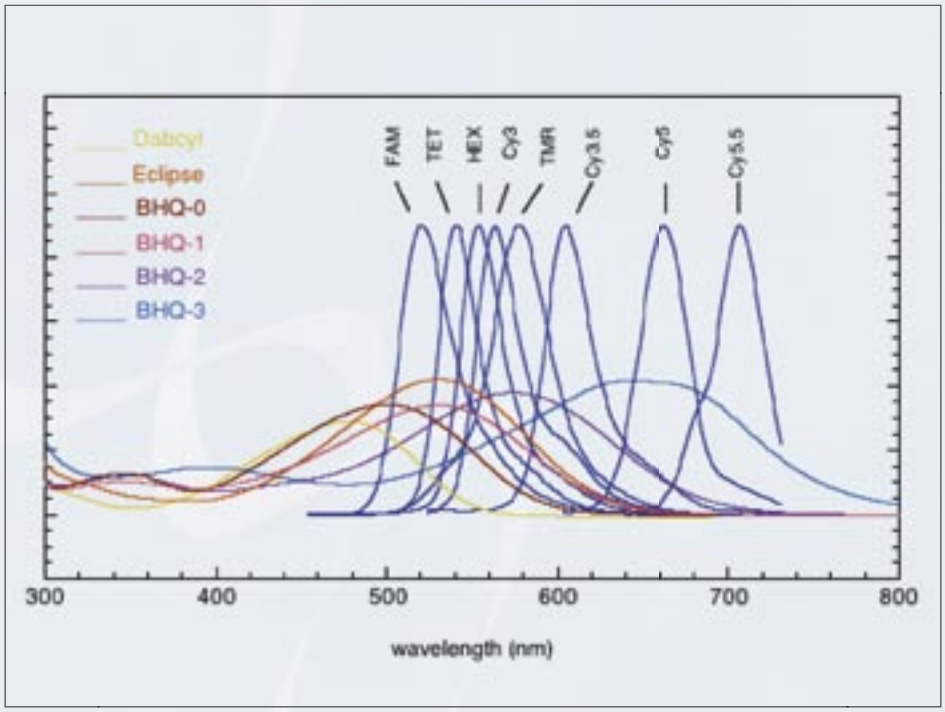


Figure 2: UV/Visible spectra of the variety of dyes and quenchers currently available from Glen Research.

TABLE 1: PHYSICAL PROPERTIES OF BLACK HOLE QUENCHERS

Quencher	λ_{max} (nm)	E_{260} (L/mol·cm)	E_{max} (L/mol·cm)
BHQ-0	493	7,700	34,000
BHQ-1	534	8,000	34,000
BHQ-2	579	8,000	38,000
BHQ-3	672	13,000	42,700

is the ease of synthesis; dark quenchers are generally more robust than their fluorescent counterparts and resist degradation during oligonucleotide deprotection. As a result, the more expensive UltraMild monomers are not required. And as an added bonus, because the failure sequences are non-fluorescent, dark quencher probes are not plagued by high background fluorescence with even unpurified probes.

One of the first reported 'dark quenchers' was the azobenzene dye Dabcyl.[3] With a broad absorbance centered around 478 nm, Dabcyl was ideal for quenching dyes by FRET that fluoresce in the blue to green region, such as EDANS. However, its spectral overlap with one of the most prevalent dyes, fluorescein, was not optimal. So, in 2002, Glen Research added the Eclipse™ Quencher from Epoch Biosciences to its product line. With a maximum absorbance at 522 nm, it was ideally suited to quench fluorescein and did so at 96% efficiency.[4]

Black Hole Quencher Dyes

In keeping with the growing popularity of red and near-infrared dyes, we are expanding our dark quencher line further. We are, therefore, happy to provide in collaboration with Biosearch Technologies, the Black Hole Quencher™ dyes (BHQs), whose physical properties are detailed in Table 1. The BHQ dyes are robust dark quenchers that very nicely complement our existing product line. They are compatible with ammonium hydroxide deprotection, exhibit excellent coupling efficiencies, have large extinction coefficients and are completely non-fluorescent. Their absorbances are well-tuned to quench a variety of popular fluorophores – even those far into the red, such as Cy3 and Cy5 (Figure 2).

However, FRET is not the only means by which a fluorophore can be quenched. Another mechanism is static quenching due to the formation of a non-fluorescent ground-state complex. The complex is stabilized by induced-dipole and hydrophobic interactions and its formation is characterized by a decrease in the monomeric dye absorption band and an increase in a blue-shifted, non-fluorescent band. Static quenching is utilized in Molecular Beacons, in which a

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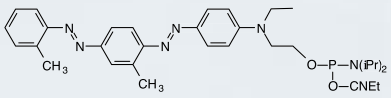
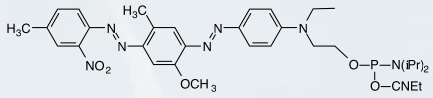
FIGURE 3: BHQ STRUCTURES

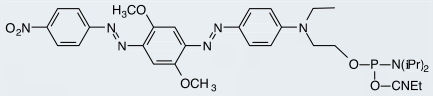
5'-BHQ Phosphoramidites



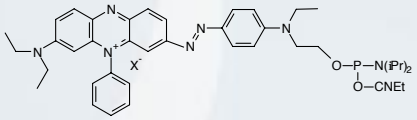
5'-BHQ-0



5'-BHQ-1

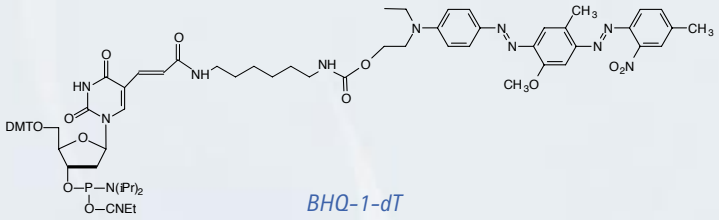


5'-BHQ-2

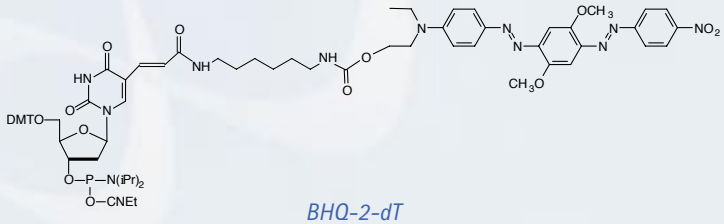


5'-BHQ-3

BHQ-dT Phosphoramidites

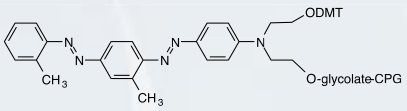


BHQ-1-dT

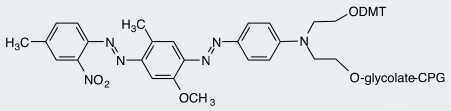


BHQ-2-dT

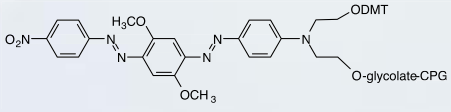
3'-BHQ CPG



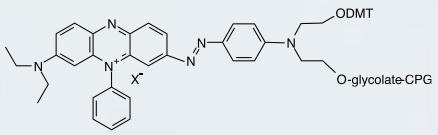
3'-BHQ-0



3'-BHQ-1



3'-BHQ-2



3'-BHQ-3

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dark quencher is held in close proximity to the fluorophore in a hairpin stem.[5] The dark quencher most typically used in a Molecular Beacon is Dabcyl. Because the quenching does not involve FRET, there is little, if any, dependence upon donor-acceptor spectral overlap. However, it appears that not all dark quenchers are made equal. In a comprehensive paper by Marras, Kramer and Tyagi,[6] the ability of BHQ-1 and BHQ-2 to quench 22 different fluorophores was evaluated. For shorter wavelength fluorophores such as fluorescein, the quenching efficiency was roughly the same as Dabcyl (91% – 93%). However, for dyes emitting in the far red, such as Cy5, the BHQ dyes were far superior – quenching the Cy5 with 96% efficiency, compared to 84% with Dabcyl. This may reflect the BHQ's ability to form stable, non-fluorescent complexes which can be a plus even in FRET probes. Indeed, recent work suggests that these non-fluorescent complexes will form even in the absence of a hairpin stem structure used by Molecular Beacons.[7]

"Black Hole Quencher", "BHQ-0", "BHQ-1", "BHQ-2" and "BHQ-3" are trademarks of Biosearch Technologies, Inc., Novato, CA. The BHQ dye technology is the subject of pending patents and is licensed and sold under agreement with Biosearch Technologies, Inc.. Products incorporating the BHQ dye moiety are sold exclusively for R&D use by the end-user. They may not be used for clinical or diagnostic purposes and they may not be re-sold, distributed or re-packaged.

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(1) T. Forster, *Ann Phys. (Leipzig)*, 1948, **2**, 55-75.
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(3) C.T. Chen, H. Wagner, and W.C. Still, *Science*, 1998, **279**, 851-3.
(4) E.A. Lukhtanov, M. Metcalf, and M.W. Reed, *American Biotechnology Laboratory*, 2001, September.
(5) S. Tyagi and F.R. Kramer, *Nature Biotechnology*, 1996, **14**, 303-308.
(6) S.A.E. Marras, F.R. Kramer, and S. Tyagi, *Nucleic Acids Res.*, 2002, **30**, E122.
(7) M.K. Johansson, H. Fidder, D. Dick, and R.M. Cook, *J Am Chem Soc*, 2002, **124**, 6950-6956.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
5'-BHQ-0 Phosphoramidite	10-5930-95	50 µmole	100.00
	10-5930-90	100 µmole	200.00
	10-5930-02	0.25g	700.00
5'-BHQ-1 Phosphoramidite	10-5931-95	50 µmole	100.00
	10-5931-90	100 µmole	200.00
	10-5931-02	0.25g	700.00
5'-BHQ-2 Phosphoramidite	10-5932-95	50 µmole	100.00
	10-5932-90	100 µmole	200.00
	10-5932-02	0.25g	700.00
5'-BHQ-3 Phosphoramidite	10-5933-95	50 µmole	150.00
	10-5933-90	100 µmole	300.00
	10-5933-02	0.25g	950.00
BHQ-1-dT	10-5941-95	50 µmole	150.00
	10-5941-90	100 µmole	300.00
	10-5941-02	0.25g	925.00
BHQ-2-dT	10-5942-95	50 µmole	150.00
	10-5942-90	100 µmole	300.00
	10-5942-02	0.25g	925.00
3'-BHQ-0 CPG	20-5930-01	0.1g	190.00
	20-5930-10	1.0g	1500.00
1 µmole columns	20-5930-41	Pack of 4	300.00
0.2 µmole columns	20-5930-42	Pack of 4	80.00
10 µmole column (ABI)	20-5930-13	Pack of 1	575.00
15 µmole column (Expedite)	20-5930-14	Pack of 1	825.00
3'-BHQ-1 CPG	20-5931-01	0.1g	190.00
	20-5931-10	1.0g	1500.00
1 µmole columns	20-5931-41	Pack of 4	300.00
0.2 µmole columns	20-5931-42	Pack of 4	80.00
10 µmole column (ABI)	20-5931-13	Pack of 1	575.00
15 µmole column (Expedite)	20-5931-14	Pack of 1	825.00
3'-BHQ-2 CPG	20-5932-01	0.1g	190.00
	20-5932-10	1.0g	1500.00
1 µmole columns	20-5932-41	Pack of 4	300.00
0.2 µmole columns	20-5932-42	Pack of 4	80.00
10 µmole column (ABI)	20-5932-13	Pack of 1	575.00
15 µmole column (Expedite)	20-5932-14	Pack of 1	825.00
3'-BHQ-3 CPG	20-5933-01	0.1g	190.00
	20-5933-10	1.0g	1500.00
1 µmole columns	20-5933-41	Pack of 4	300.00
0.2 µmole columns	20-5933-42	Pack of 4	80.00
10 µmole column (ABI)	20-5933-13	Pack of 1	575.00
15 µmole column (Expedite)	20-5933-14	Pack of 1	825.00

2'-FLUORO-RNA MONOMERS

2'-Fluoro-RNA

The RNA analogue, 2'-deoxy-2'-fluoro-RNA (2'-F-RNA), has always been intriguing to us and we have offered the C and U analogues for some time. Unfortunately, the A and G analogues have not been available commercially due to their extremely complex chemical syntheses.[1] We are happy to report that all four analogues are now available following production by a novel and elegant enzymatic transformation.

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.[1] 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[1] concluded that oligonucleotides hybridized to a target RNA oligonucleotide in the following order of increasing stability: DNA < RNA < 2'-OMe-RNA < 2'-F-RNA. With an RNA target, melting temperature (T_m) was enhanced relative to an antisense DNA oligonucleotide by 1°C per residue for RNA, 1.3 °C for 2'-OMe-RNA, and 1.8 °C for 2'-F-RNA. The stability enhancement for 2'-F-RNA hybridizing to an RNA target was additive for each 2'-F-RNA residue and slightly cooperative – *i.e.*, the ΔT_m per substitution increases as more 2'-F-RNA residues are incorporated into the oligonucleotide. This has led to the use of 2'-F-RNA in aptamers since the resulting aptamers are not only more resistant to nucleases compared to 2'-OH RNA aptamers, but also bind ligands with higher affinities.[2] 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. Interestingly, 2'-F-RNA can be used quite effectively in siRNA applications.

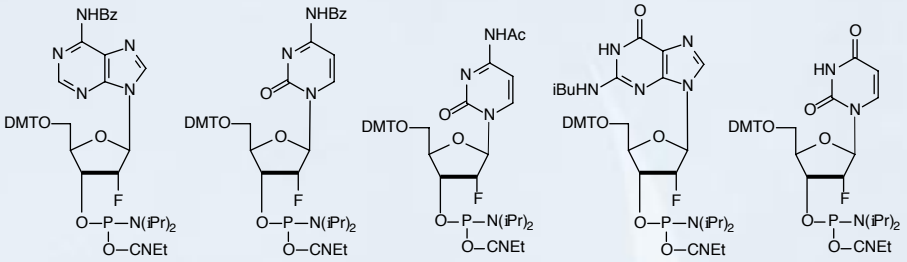
Recent work by Layzer et al., demonstrated that siRNA synthesized with 2'-F pyrimidines showed greatly increased stability in human plasma compared to 2'-OH siRNA.[3] They were functional in cell culture and *in vivo* using BALB/c mice transfected with pGL3 luciferase. Interestingly, though the 2'-F 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[4] 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 T_m of 18-mer oligonucleotides containing rA and/or 2'-F-A to oligo-T18 and found that the homopolymer of 2'-F-A enhanced binding by 0.5° per residue relative to rA. However, chimeras of 2'-F-RNA and rA were unpredictable in their melting behavior and some actually lowered the duplex T_m.

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

With a full set of monomers now available, we predict applications for 2'-F-RNA in ribozymes, siRNA and structural DNA research. By making all four monomers available, we hope to open up a full spectrum of research applications for 2'-F-RNA.

FIGURE 1: 2'-FLUORO-RNA MONOMERS



References:

(1) A.M. Kawasaki, et al., *Journal of Medicinal Chemistry*, 1993, **36**, 831-841.

(2) M. Khati, M. Schuman, J. Ibrahim, Q. Sattentau, S. Gordon, and W. James, *J Virol*, 2003, **77**, 12692-8.

(3) J.M. Layzer, A.P. McCaffrey, A.K. Tanner, Z. Huang, M.A. Kay, and B.A. Sullenger, *RNA*, 2004, **10**, 766-71.

(4) D.R. Yazbeck, K.L. Min, and M.J. Damha, *Nucleic Acids Res.*, 2002, **30**, 3015-25.

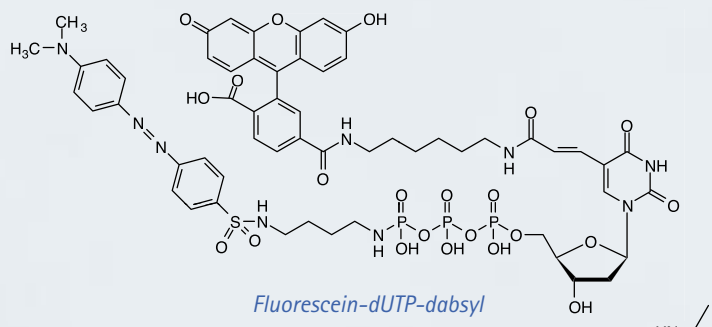
ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
2'-F-A-CE Phosphoramidite	10-3400-90	100 µmole	235.00
	10-3400-02	0.25g	650.00
2'-F-C-CE Phosphoramidite	10-3410-90	100 µmole	165.00
	10-3410-02	0.25g	500.00
2'-F-Ac-C-CE Phosphoramidite	10-3415-90	100 µmole	165.00
	10-3415-02	0.25g	500.00
2'-F-G-CE Phosphoramidite	10-3420-90	100 µmole	235.00
	10-3420-02	0.25g	650.00
2'-F-U-CE Phosphoramidite	10-3430-90	100 µmole	165.00
	10-3430-02	0.25g	500.00

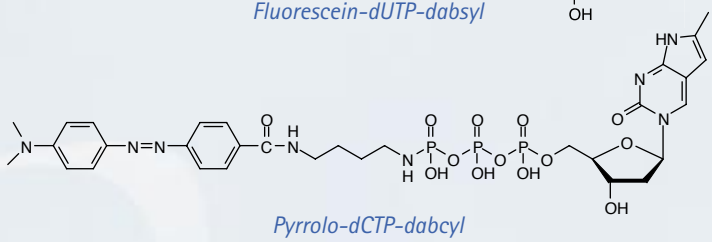
INTERNALLY QUENCHED NUCLEOTIDE FLUORESCENT REPORTERS

Joseph F. Lawler, Jr.
Lawler Scientific, LLC

FIGURE 1: STRUCTURES OF INTERNALLY QUENCHED NUCLEOTIDES (IQNs)



Fluorescein-dUTP-dabsyl



Pyrrolo-dCTP-dabcyl

Introduction

Several methods have been developed for enzymatic fluorescent labeling of nucleic acids. A dNTP analog can be used to incorporate a fluorophore by PCR, nick translation or random priming, either directly into DNA[1] or indirectly via a hapten such as biotin.[2] Though high incorporation efficiencies have been reported,[3] all of these approaches require the separation of unincorporated label prior to downstream applications.

Lawler Scientific, LLC has developed a series of reagents called Internally Quenched Nucleotides or IQNs. These reagents consist of a nucleoside triphosphate with a fluorescent reporter attached to the nucleobase and a quencher moiety attached to the gamma-phosphate. The nucleotides remain non-fluorescent until the quencher is enzymatically separated from the parent nucleotide. Since the IQNs are non-fluorescent until incorporated into a nucleic acid, they should not give rise to the background fluorescence signals commonly observed when DNA labeled by standard means is inadequately purified.

Nucleic Acid Labeling

The first generation IQN consists of a fluorescein-dUTP with a dabsyl quencher linked to the gamma phosphate (see Figure 1). The first generation molecules were developed to address the cDNA labeling application described above. Fluorescein and dabsyl were selected because of their superior optical properties and because the photophysics governing their interaction is well described in the literature.[4] In addition, this IQN is soluble and stable in aqueous solution.

Molecular modeling of the fluorescein and dabsyl moieties of the IQN predicts that these moieties will be well within effective quenching distances. Consistent with this prediction is the observation that fluorescence emission of this first generation molecule is >98% quenched (see Figure 2). Chemical fragmentation of the triphosphate backbone restores fluorescence emission as does hydrolysis by snake venom phosphodiesterase (SVP). As shown in the accompanying Figure 3, SVP rapidly hydrolyzes the IQN with a concomitant increase in fluorescence intensity at 520nm.

Reverse transcriptases are commonly used in cDNA labeling protocols. An oligonucleotide primer extension assay was performed using Avian Myeloblastosis Virus (AMV) reverse transcriptase (RT). AMV RT correctly incorporated the IQN opposite dA

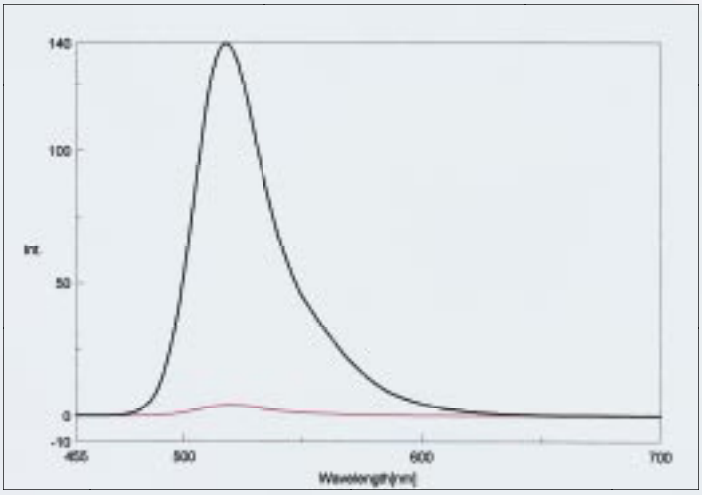


Figure 2: The fluorescence spectrum of equimolar amounts of Fluorescein-dUTP (black) and Fluorescein-dUTP-dabsyl (red) were recorded at an excitation wavelength of 440nm.

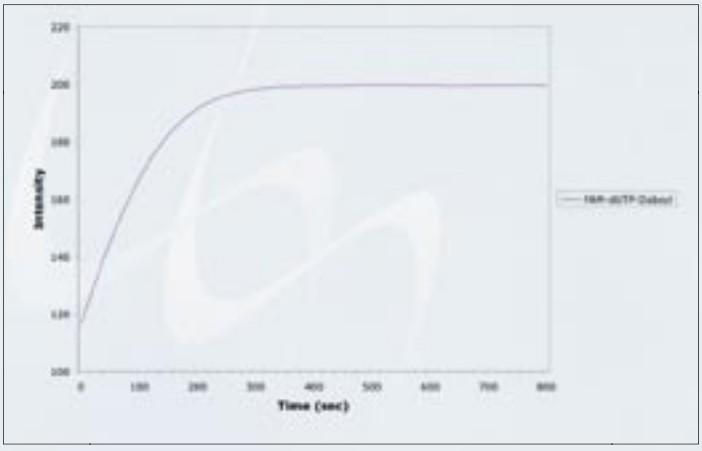


Figure 3: The hydrolysis of fluorescein-dUTP-dabsyl by snake venom phosphodiesterase. Note: The background fluorescence was essentially zero prior to the addition of the SVP.

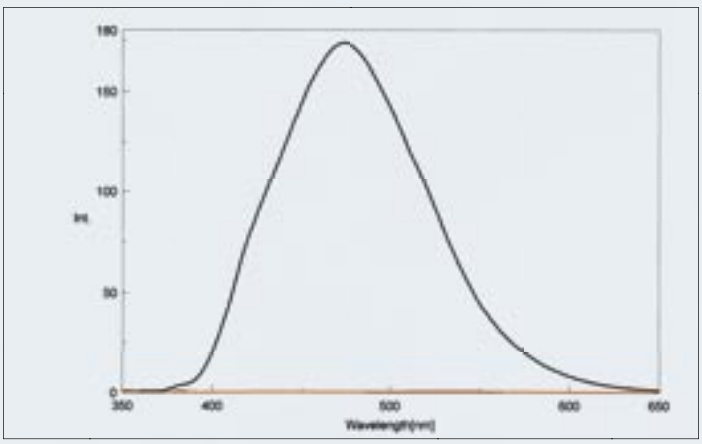


Figure 4: The fluorescence spectrum of equimolar amounts of Pyrrolo-dCTP (black) and Pyrrolo-dCTP-dabcyl (red) were recorded at an excitation wavelength of 340nm.

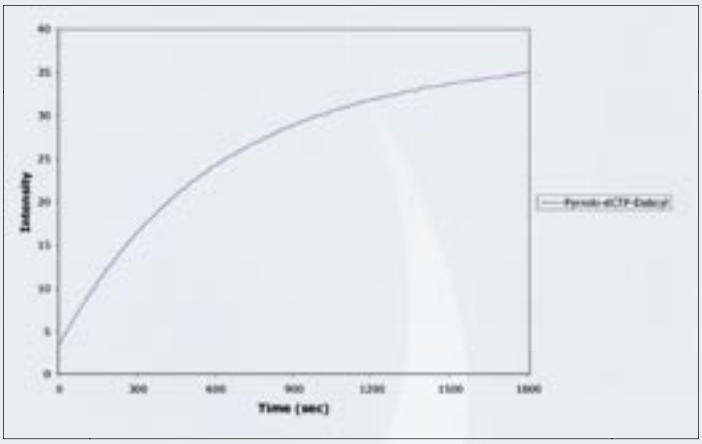


Figure 5: The hydrolysis of pyrrolo-dCTP-dabcyl by snake venom phosphodiesterase.

residues in the template strand. In a second set of experiments, this IQN was added to an AMV RT mediated cDNA synthesis reaction. The addition of the IQN resulted in label incorporation in the resulting 351nt cDNA. cDNA synthesis reactions can therefore be "doped" with IQNs to achieve the desired amount of label incorporation.

The IQN technology may extend microarray and real-time PCR techniques but it would do so in different ways. Microarray hybridization experiments may be made more time- and cost-efficient by eliminating the fluid handling steps associated with the cDNA labeling and purification processes. Real-time PCR based experiments may also be made more time- and cost-efficient by eliminating the empiric and costly process of designing and testing fluorescent quantification probes.

Real time PCR

Lawler Scientific, LLC also recognized the potential utility of these reagents for monitoring nucleic acid amplification reactions in real time. Several probe based methods have been developed for quantifying PCR. While effective, these methods require the design and synthesis of an oligonucleotide for each amplicon of interest. Since the use of IQNs is independent of the amplicon chosen, IQNs can serve as an "off the shelf" reagent for monitoring the progress of a PCR.

Thermophilic DNA polymerases tend to be more stringent than reverse transcriptases with respect to substrate specificity. Using an extensive body of structure activity relationship data, Lawler Scientific, LLC developed a less extensively decorated IQN for use in real-time PCR. A second generation IQN was prepared using pyrrolo-dC, an intrinsically fluorescent nucleobase developed at Glen Research,[5] as a fluorophore and dabcyl as a quencher (see Figure 1).

Again, the fluorescence emission of pyrrolo-dCTP-dabcyl is >98% quenched (Figure 4) and hydrolysis by SVP leads to the return of fluorescence (Figure 5). The absorbance and emission spectra of pyrrolo-dC allow it to be detected in the presence of other nucleobases and nucleic acids. In addition, pyrrolo-dC is known[5] to be incorporated opposite dG positions by *Taq* DNA polymerase.

Other Applications

Lawler Scientific, LLC is commercializing several additional IQNs with varying fluorophore and quencher pairs. It is anticipated that these molecules will be useful in multiple-color hybridization reactions, SNP analysis, and polymerase detection.

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References:

(1) H. Yu, J. Chao, D. Patek, R. Mujumdar, S. Mujumdar, and A.S. Waggoner, *Nucleic Acids Res.*, 1994, **22**, 3226-32.
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(3) T. Tasara, et al., *Nucleic Acids Res.*, 2003, **31**, 2636-46.
(4) S.A.E. Marras, F.R. Kramer, and S. Tyagi, *Nucleic Acids Res.*, 2002, **30**, E122.
(5) D.A. Berry, et al., *Tetrahedron Lett*, 2004, **45**, 2457-2461.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
Fluorescein-dUTP-dabsyl 1mM, 25nmoles, 10mM Tris, 1mM EDTA	88-1056-01	25 µL	$270.00
Pyrrolo-dCTP-dabcyl 1mM, 25nmoles, 10mM Tris, 1mM EDTA	88-1017-01	25 µL	$270.00

COLUMNS

AB 3900 and MerMade Columns

We are happy to announce the availability of some of our more popular minor base supports on polystyrene and in columns fully compatible with the Applied Biosystems 3900 synthesizer. These include our popular Universal Support II, which will allow oligos to be produced on the 3900 with ANY base at the 3' terminus. At the same time, we are offering 1 µmole columns for the 3900 instrument.

By popular demand, we are also adding our two universal supports in CPG columns for use in 96 well synthesizers, such as the MerMade, that are adapted to use the column format.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
AB 3900			
US II PS			
200 nmole columns	26-5110-52	Pack of 10	100.00
40 nmole columns	26-5110-55	Pack of 10	100.00
3'-Phosphate PS			
200 nmole columns	26-2900-52	Pack of 10	150.00
40 nmole columns	26-2900-55	Pack of 10	150.00
3'-PT-Amino-Modifier C6 PS			
200 nmole columns	26-2956-52	Pack of 10	220.00
40 nmole columns	26-2956-55	Pack of 10	220.00
3'-(6-FAM) PS			
200 nmole columns	26-2961-52	Pack of 10	300.00
40 nmole columns	26-2961-55	Pack of 10	300.00
3'-Dabcyl PS			
200 nmole columns	26-5912-52	Pack of 10	300.00
40 nmole columns	26-5912-55	Pack of 10	300.00
3'-TAMRA PS			
200 nmole columns	26-5910-52	Pack of 10	300.00
40 nmole columns	26-5910-55	Pack of 10	300.00
3'-BiotinTEG PS			
200 nmole columns	26-2955-52	Pack of 10	300.00
40 nmole columns	26-2955-55	Pack of 10	300.00
dA PS 1 µmole columns	26-2600-61	Pack of 200	927.00
dC PS 1 µmole columns	26-2610-61	Pack of 200	927.00
dmf-dG PS 1 µmole columns	26-2629-61	Pack of 200	927.00
dT PS 1 µmole columns	26-2630-61	Pack of 200	927.00
96 Well Format			
Universal Support 1000			
200 nmole columns	20-5001-92	Pack of 96	250.00
40 nmole columns	20-5001-95	Pack of 96	250.00
Universal Support II			
200 nmole columns	20-5110-92	Pack of 96	250.00
40 nmole columns	20-5110-95	Pack of 96	250.00

(Continued from Front Page)

Amino-Modifier C6-U (cont.)

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(4) S. Pitsch, P.A. Weiss, L. Jenny, A. Stutz, and X.L. Wu, *Helv Chim Acta*, 2001, **84**, 3773-3795.

6-Thio-G

Nucleotides with sulfur substituting for oxygen at the 4-position of pyrimidines and the 6-position of purines have proved to be very useful in molecular biology. In particular, the sulfur is active photochemically and available to photo-crosslink to an adjacent molecule, allowing study of internucleotide and nucleotide-protein interactions. Because the sulfur is at a position actively used for hydrogen bond interactions, the distance to the complementary target is short and cross-linking occurs specifically, allowing internucleotide interactions to be explicitly defined. Sulfur analogues of 2'-deoxynucleosides have been available as phosphoramidites for a long time and 4-thiouridine (4-thio-U) in the RNA series has been available also. We now add 6-thioguanosine (6-thio-G) to the tools available for studying RNA-RNA and RNA-protein interactions by offering 6-thio-G phosphoramidite for incorporation into oligoribonucleotides.

There have been several reports[1-3] in the literature describing 6-thio-G phosphoramidite but it is only recently that the demand for minor RNA phosphoramidites has made this feasible as a product. It is easy to envisage applications for this product in ribozyme and siRNA applications, as well as in RNA-protein interactions.

The removal of the silyl protecting group without interfering with the sulfur is critical, so we have used the more traditional t-butyldimethylsilyl protecting group on the 2'-hydroxyl. This is removed[1] cleanly by triethylamine trihydrofluoride in DMSO but t-butylammonium fluoride (TBAF) leads to degradation of the thio-nucleotide analogue and should not be used.

References:

(1) C.J. Adams, J.B. Murray, M.A. Farrow, J.R.P. Arnold, and P.G. Stockley, *Tetrahedron Lett.*, 1995, **36**, 5421-5424.

(2) Y. Wang, E. Lattmann, and Q. Zheng, *Nucleos Nucleot Nucleic Acids*, 2003, **22**, 1247-1249.
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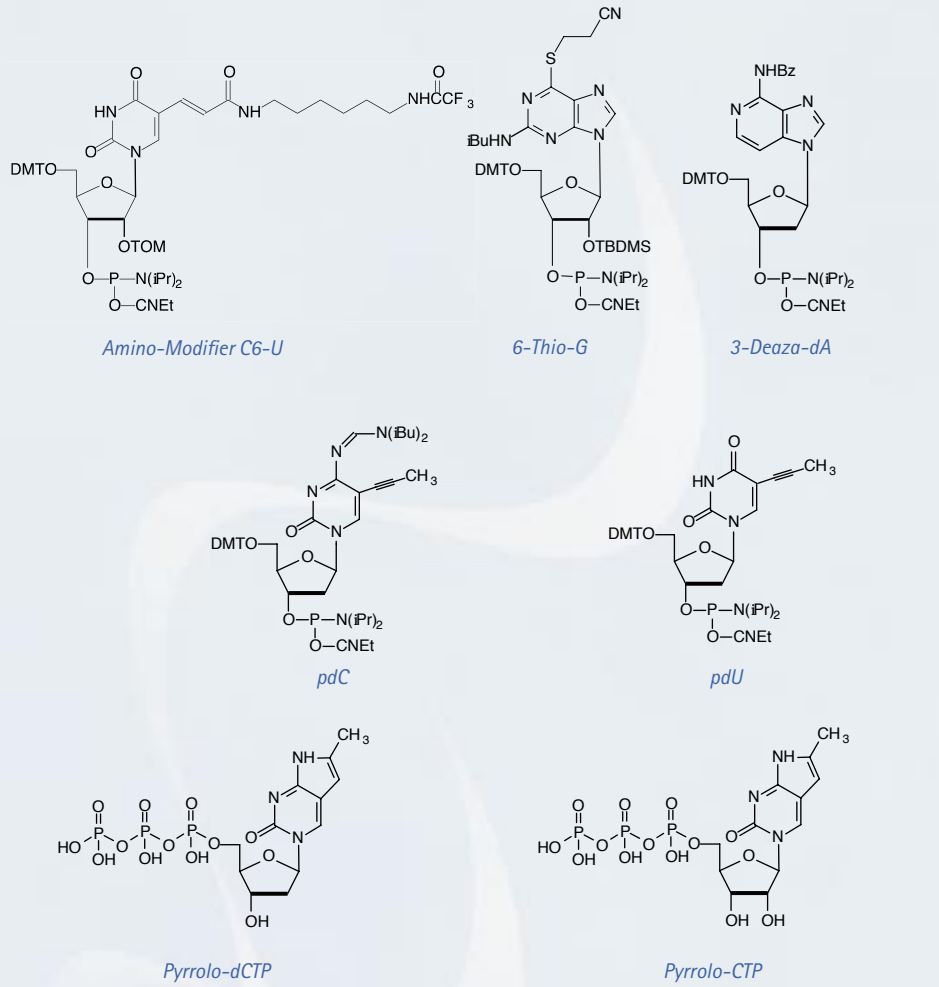
3-Deaza-dA

Modified base analogues of 2'-deoxynucleosides are readily available for probing interactions in the major groove of duplex DNA. However, there are far fewer analogues available to investigate interactions in the minor groove. The standard nucleobases have an unshared pair of electrons that project into the minor groove of duplex DNA. In the case of the purines, this is the nitrogen at N^3 and, for the pyrimidines, it is the keto group at C^2. Enzymes that interact with DNA, polymerases, reverse transcriptases, restriction enzymes, etc., may use a hydrogen bond donating group to contact the hydrogen bond acceptor in the minor groove. 3-Deaza-2'-deoxyadenosine is very interesting in that it maintains the ability for regular Watson-Crick hydrogen bonding to T but is lacking the electron pair at the 3-position normally provided by N^3. A very interesting recent publication from the Benner group describes[1] using 3-deaza-2'-deoxyadenosine to probe minor groove contacts by polymerases and reverse transcriptases in the context of biological evolution.

An earlier paper[2] discussed the thermodynamic stability of oligonucleotides containing 3-deaza-2'-deoxyadenosine. Surprisingly, substitution of 3-deaza-2'-deoxyadenosine for 2'-deoxyadensine lowered duplex stability substantially by around 4° per insertion. The authors surmised that this was due to the higher pKa (6.80) of 3-deaza-2'-deoxyadenosine in comparison to 2'-deoxyadenosine (3.62), which allowed protonation of the base, and the loss of stabilizing hydration of the minor groove electron pair.

We have had a long-term interest in supplying the phosphoramidite of 3-deaza-2'-deoxyadenosine, which was very challenging[3,4] to prepare in quantity. We are delighted to offer this phosphoramidite as a result of the perseverance of our colleagues at Berry and Associates, Inc.

FIGURE 1: NEW MONOMERS



References:
(1) C.L. Hendrickson, K.G. Devine, and S.A. Benner, *Nucleic Acids Res.*, 2004, **32**, 2241-50.
(2) C. Lever, X. Li, R. Cosstick, S. Ebel, and T. Brown, *Nucleic Acids Res.*, 1993, **21**, 1743-1746.
(3) R. Cosstick, X. Li, D.K. Tuli, D.M. Williams, B.A. Connolly, and P.C. Newman, *Nucleic Acids Res.*, 1990, **18**, 4771.
(4) F. Seela, T. Grein, and S. Samnick, *Helvetica Chimica Acta*, 1992, **75**, 1639-1650.

C-5 propynyl Pyrimidine Analogues

Substitution of C-5 propynyl-dC (pdC) for dC and C-5 propynyl-dU (pdU) for dT[1] are effective strategies to enhance base pairing. This increase in hybridization efficiency is due to the hydrophobic nature of the groups at the C-5 position which helps to exclude water molecules from the duplex.

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

C-5 propynyl-C	2.8° per substitution
C-5 propynyl-U	1.7° per substitution

While these modifications have found most applications in antisense oligonucleotides, their ability to enhance binding while maintaining specificity will also prove useful in the synthesis of high affinity probes.

At the time of writing, we are completing an agreement with Isis Pharmaceuticals, Inc. to recommence the supply of these two valuable products. We believe these will be a welcome addition to the selection of modified bases that affect hybridization.

References:
(1) B.C. Froehler, S. Wadwani, T.J. Terhorst, and S.R. Gerrard, *Tetrahedron Lett.*, 1992, **33**, 5307-5310.

(Continued from Previous Page)

Pyrrolo-CTP

Pyrrolo-dC is a fluorescent nucleoside that codes as dC and base pairs efficiently with dG. We have published a preliminary report[1] on the chemistry of pyrrolo-dC and further details of the chemistry and biology are currently in press. Preliminary evidence indicates that pyrrolo-dC triphosphate is an excellent substrate for Taq, Pfu and Vent polymerases and is incorporated specifically opposite dG. Pyrrolo-dCTP has been available for some time and is in use in biological assays. We are now introducing pyrrolo-CTP, the ribonucleoside triphosphate. We anticipate that the addition of a fluorescent ribonucleotide with fluorescence exquisitely sensitive to its environment would be of great interest for RNA structural research.

The pyrrolo-C project is a joint development by Berry and Associates (http://www.berryassoc.com) and Glen Research.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
Amino-Modifier C6-U Phosphoramidite	10-3039-95	50 µm	360.00
	10-3039-90	100 µm	720.00
	10-3039-02	0.25g	1475.00
6-Thio-G-CE Phosphoramidite	10-3072-95	50 µm	250.00
	10-3072-90	100 µm	500.00
	10-3072-02	0.25g	1200.00
3-Deaza-dA-CE Phosphoramidite	10-1088-95	50 µm	177.50
	10-1088-90	100 µm	355.00
	10-1088-02	0.25g	975.00
Pyrrolo-dCTP 10mM	81-1017-01	100 µL	$150.00
Pyrrolo-CTP 10mM	81-3017-01	100 µL	$270.00

Patents covering this modified fluorescent base and its uses are currently pending.

References:

(1) D.A. Berry, et al., *Tetrahedron Lett*, 2004, **45**, 2457-2461.

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