The Glen Report

22825 DAVIS DRIVE • STERLING, VIRGINIA • 20164

GLEN RESEARCH

VOLUME 21

NUMBER 2

OCTOBER 2009

INSIDE

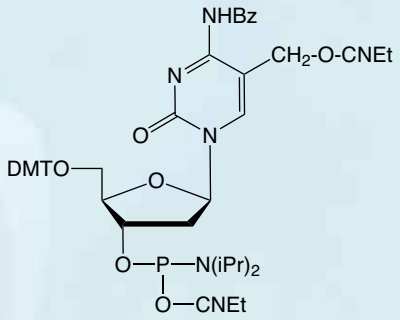
5-HYDROXYMETHYL-DC: A NEW ACTOR IN THE FIELD OF EPIGENETICS

One of the fastest growing fields in biology and cancer research is epigenetics. While the underlying genetic code defines which proteins and gene products are synthesized, it is epigenetic control that defines when and where they are expressed. This dynamic control of gene expression is essential for X chromosome inactivation, embryogenesis, cellular differentiation and appears integral to memory formation and synaptic plasticity.[1,2]

Epigenetic control is generally mediated by methylation of cytidine to 5-methyl-dC in CpG sites and post-translational modification of histones. Methylation of CpG sites near promoters is associated with gene silencing, as is deacetylation of histones. A number of diseases can result when CpG methylation control is lost or when the *de novo* methylation is incorrect, such as Rett, Fragile X, ATR-X, Prader-Willi and Angelman syndromes. In addition, dysregulated methylation patterns are often seen in cancers and this is widely thought to contribute to tumorigenesis.[3]

While a number of mammalian DNA methyltransferases are known in the DNMT family, the enzymes responsible for demethylation in mammals have yet to be identified conclusively since none has shown clear activity *in vitro*.[4] However, recent work by Skirmantas Kriaucionis in the Heintz Lab. demonstrated that 40% of the purported 5-methyl-dC in Purkinje neurons was actually 5-hydroxymethyl-dC.[5] This hydroxymethyl cytidine analog has low affinity for the Methyl-CpG-binding Protein MeCP2, which is a known transcriptional repressor, as well as DNMT1, which

FIGURE 1: **5-HYDROXYMETHYL-DC CE PHOSPHORAMIDITE**



5-Hydroxymethyl-dC

is the maintenance DNA methyltransferase.[6,7] This opens up the possibility that demethylation would be acquired passively over multiple cell cycles if a means could be found to convert 5-methyl-dC to 5-hydroxymethyl-dC. Less than a month after Kriaucionis' publication, the Rao Lab. found an enzyme, TET1, which catalyzes the conversion of 5-methyl-dC to 5-hydroxymethyl-dC *in vitro* and *in vivo*, ushering in a new chapter in the field of epigenetics with 5-hydroxymethyl-dC taking center stage.[8]

Another consideration is the potential for 5-hydroxymethyl-dC to be a bad actor in DNA damage. After all, 5-hydroxymethyl-dU is a product of oxidative damage of thymidine by hydroxyl radicals or ionizing radiation. Similarly, 5-hydroxymethyl-dC may be a damaged form of 5-methyl-dC. However, it is unlikely that the 5-hydroxymethyl-dC detected in neuronal cells is formed by oxidative damage since no other

(Continued on Back Page)

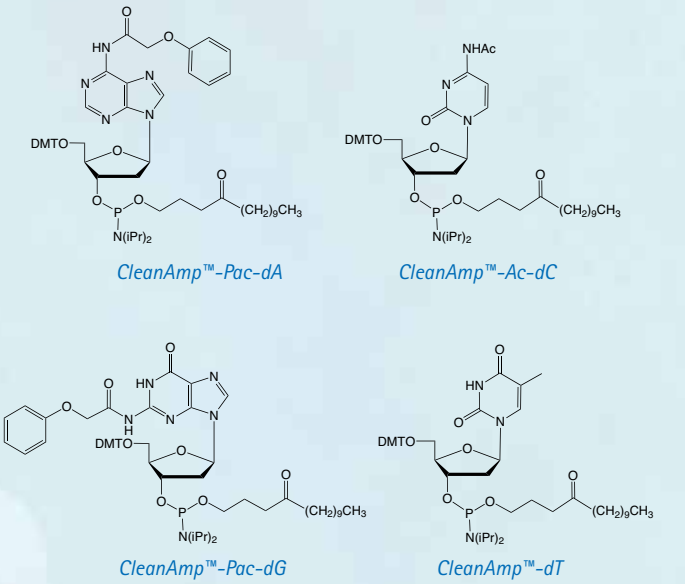
PURIFICATION OF CLEANAMP™ DNA OLIGONUCLEOTIDES (DMT-ON)

In the previous Glen Report 21.1, we were happy to launch CleanAmp™ monomers for the production of Turbo and Precision Primers under license from TriLink BioTechnologies, Inc. Customer feedback has indicated that the purification scheme which we detailed, while successful, is quite tedious, with large volumes of solutions being pushed slowly through Sep-Pak® Cartridges. In the past few months, we have been working to optimize this procedure using Sep-Pak cartridges, as well as our Poly-Pak™ cartridges, representing older OPC-type cartridges, and our Glen-Pak™ cartridges, representing the most up-to-date pH-stable purification cartridges.

Unfortunately, Poly-Pak cartridges gave primers of unacceptable purity but Sep-Pak and Glen-Pak cartridges gave both Turbo and Precision Primers of good quality following a much simplified procedure. The procedure is described below for our Glen-Pak cartridges.

Turbo and Precision Primers were synthesized normally using CleanAmp™ Phosphoramidites (Figure 1) to generate the sequences shown in Figure 2. Crude and purified Turbo and Precision Primers are shown in Figures 3 and 4, respectively.

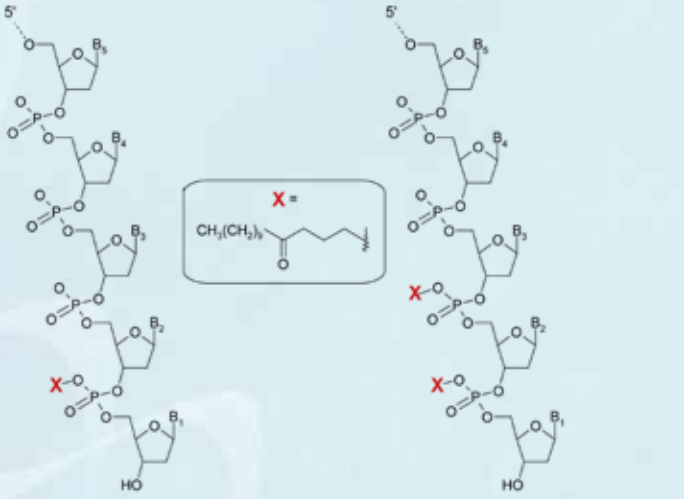
FIGURE 1: STRUCTURES OF CLEANAMP™ PHOSPHORAMIDITES



CleanAmp™-Pac-dA *CleanAmp™-Ac-dC*

CleanAmp™-Pac-dG *CleanAmp™-dT*

FIGURE 2: STRUCTURES OF CLEANAMP™ TURBO AND PRECISION PRIMERS



CleanAmp™ Turbo primers
Single 4-oxo-tetradecyl modification

CleanAmp™ Precision primers
Double 4-oxo-tetradecyl modification

Primer Sequences:

forward: 5'-CAGGAGCTGGACTTTACTGATGC

reverse: 5'-CGGGATATCGACATTTCTGCACC

MATERIALS — *AMOUNT USED*

Glen-Pak™ DNA Purification Cartridge (60-5100-XX, 60-5200-XX)	1
0.05 M Potassium Carbonate in Methanol (60-4600-30)	1mL
Vacuum Manifold or handheld 5 mL syringe	1
HPLC Grade Acetonitrile (ACN)	2mL
2.0 M Triethylammonium Acetate (TEAA) (60-4110-57)	1mL
1.0 M TEAA, prepared from 2.0 M TEAA	1mL
2% Trifluoroacetic acid (TFA) in Water (60-4040-57)	4mL
Deionized Water	1mL
0.1 M TEAA in water	6mL
0.1 M TEAA containing 35% ACN‡	3mL
0.1 M TEAA containing 25% ACN‡,+	3mL
0.1 M TEAA containing 15% ACN+	3mL
DMSO, anhydrous	1mL

For Precision‡ and Turbo+ Primers. Note: Prepare these solutions at a final concentration of 0.1 M TEAA using 2.0 M TEAA. Do not dilute 0.1 M TEAA with Acetonitrile.

PROCEDURE

Sample Preparation

1. Following DNA synthesis using UltraMild monomers on either an UltraMild CPG or US III Universal Support, deprotect the DMT-ON oligonucleotide in 1 mL of 0.05 M potassium carbonate solution for 4 hours at room temperature. Just prior to beginning the purification of the CleanAmp Turbo or Precision primers on the Glen-Pak cartridge, quench the 0.05 M potassium carbonate with 1 mL of 1.0 M TEAA. *Note: Once the purification is begun, avoid interruptions during the protocol; this is especially true when removing the DMT. If the purification cannot be completed the same day, store the oligo in the freezer in the original 0.05 M potassium carbonate solution until ready for purification.*

Cartridge Preparation

1. Rinse the cartridge with 2 mL of acetonitrile followed by 1 mL of 2.0 M TEAA. *The acetonitrile wets the Glen-Pak resin, while the TEAA acts as an ion-pairing reagent to enhance the binding of the DMT-ON oligonucleotide to the resin.*

Purification Procedure.

1. Load the CleanAmp primer in the quenched potassium carbonate/TEAA onto the prepped Glen-Pak cartridge dropwise. Collect the eluent and save in case of a loading failure or error. *During the loading process, the DMT-ON Triesters bind to the resin while the failure sequences do not.*
2. Wash the cartridge with 2 mL of 0.1 M TEAA. *This removes the salts and methanol from the cartridge.*
   a. *Turbo Primers*
   Rinse the cartridge with 3 mL of 25% ACN in 0.1 M TEAA.

This rinses away DMT-Off failures and hydrolyzed Diester DMT-ON sequences.

b. *Precision Primers*

Rinse the cartridge with 3mL of 35% ACN in 0.1 M TEAA. *This rinses away DMT-Off failures and mixed Diester/Triester DMT-ON sequences.*

3. Rinse cartridge with 2 mL 2% TFA dropwise. Repeat. *This removes the DMT. The faint orange color of the DMT cation is often visible at this step.*
4. Rinse the cartridge with 2 mL of 0.1 M TEAA. *This removes residual TFA and neutralizes the resin.*

a. *Turbo Primers*

Rinse the cartridge with 3 mL of 15% ACN in 0.1 M TEAA. *This rinses away Diesters that are produced during detritylation.*

b. *Precision Primers*

Rinse the cartridge with 3 mL of 25% ACN in 0.1 M TEAA. *This rinses away Diesters and mixed Diester/Triesters that are produced during detritylation.*

5. Rinse the cartridge with 2 mL of 0.1 M TEAA. *This removes residual ACN.*
6. Rinse the cartridge with 1 mL of water. *This removes excess TEAA.*
7. Using one syringe volume of air, blow out any residual water.
8. With a clean, dry syringe, elute the oligonucleotide in three fractions of DMSO, collecting each fraction separately. After collecting a fraction, blow the residual DMSO into the collection tube after each aliquot using a syringe full of air.

   1st fraction 300 µL of DMSO; *removes water and remaining failure sequences.*

   2nd fraction 400 µL of DMSO; *elutes the bulk of the oligo*

   3rd fraction 300 µL of DMSO; *elutes the remaining oligo*
9. Determine ODs of each fraction. Generally, fraction 2 is the most pure and contains the majority of the Turbo or Precision CleanAmp primer. If the concentration is low, add a portion of fraction 3 to fraction 2 as necessary to increase the amount of CleanAmp primer. Fraction 1 generally contains excess water that will reduce the long-term stability of the CleanAmp primers.

FIGURE 3: RP HPLC ANALYSIS OF A CLEANAMP™ TURBO PRIMER

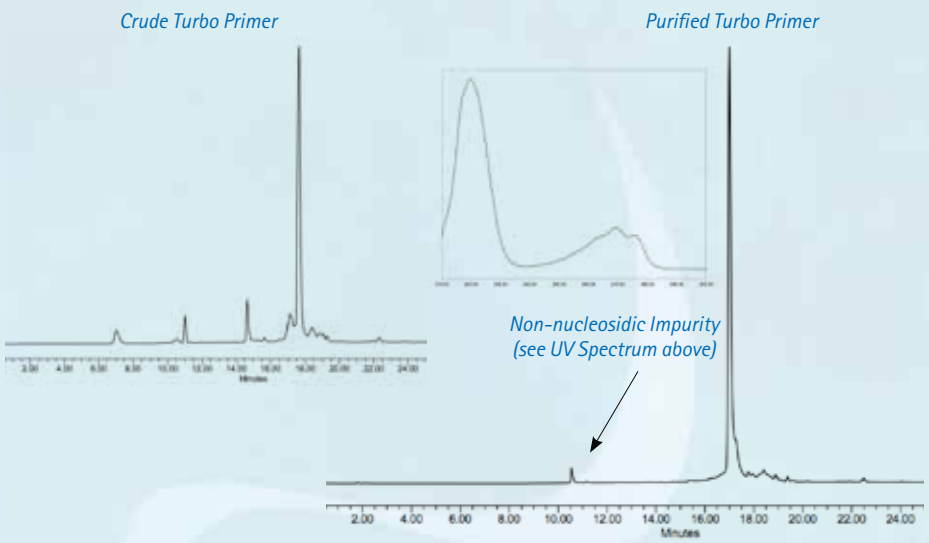
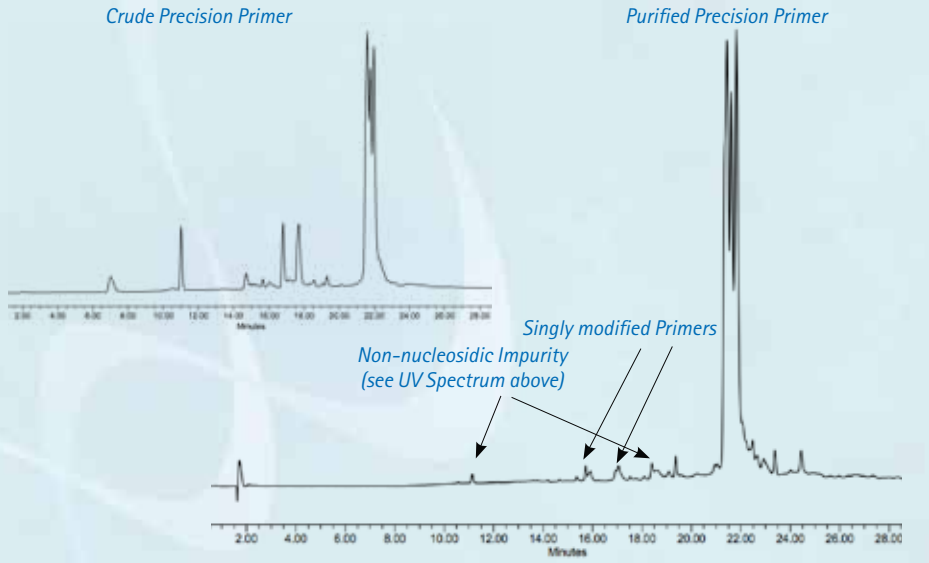


FIGURE 4: RP HPLC ANALYSIS OF A CLEANAMP™ PRECISION PRIMER



ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
CleanAmp™-Pac-dA-CE Phosphoramidite	10-1440-90	100 µmole	100.00
	10-1440-02	0.25g	240.00
	10-1440-05	0.5g	480.00
CleanAmp™-Ac-dC-CE Phosphoramidite	10-1450-90	100 µmole	100.00
	10-1450-02	0.25g	240.00
	10-1450-05	0.5g	480.00
CleanAmp™-Pac-dG-CE Phosphoramidite	10-1460-90	100 µmole	100.00
	10-1460-02	0.25g	240.00
	10-1460-05	0.5g	480.00
CleanAmp™-dT-CE Phosphoramidite	10-1470-90	100 µmole	100.00
	10-1470-02	0.25g	240.00
	10-1470-05	0.5g	480.00

A 3'-CAP FOR IMPROVED TARGET AFFINITY AND SPECIFICITY

Glen Research is pleased to offer a new controlled pore glass support for the synthesis of oligonucleotides. Syntheses on the 3'-Uaq Cap CPG (1) produce oligonucleotides with a 3'-cap. Caps are covalently appended residues that stabilize double helices by binding to termini.[1-3] Caps increase duplex melting points and improve base pairing fidelity by selectively binding to Watson-Crick base pairs. Thus far, only reagents for caps at the 5'-terminus had been available.[1,3] The Uaq support now allows for the routine synthesis of oligonucleotides with a cap at the 3'-terminus.

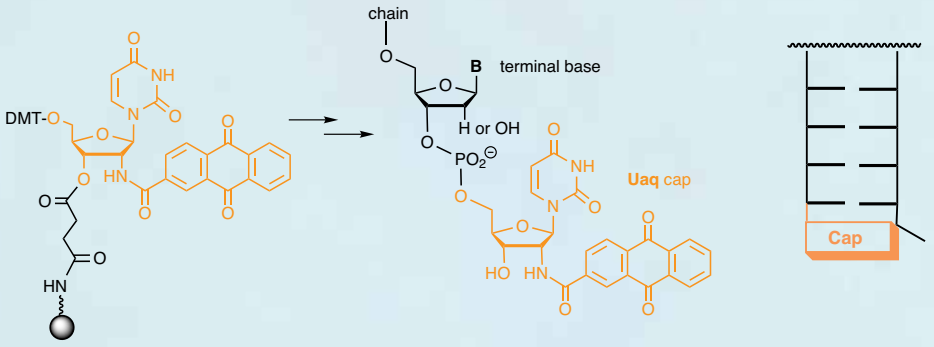
Figure 1 shows the structure of 3'-Uaq Cap CPG (1), the 3'-terminus of an oligonucleotide featuring the Uaq cap (2), and an illustration of a capped duplex (3). Note that the cap does not require a blunt end and will readily bind to target strands with a 5'-overhang.

The 3'-Uaq Cap is also the most effective cap known to date. It increases the melting point (Tm) of DNA:DNA, RNA:RNA, and DNA:RNA hybrid duplexes. For short hybrid duplexes between DNA probes and RNA target strands, the increase is up to 18 °C.[2] Further, appending the cap leads to larger ΔTms between duplexes of perfectly matched sequence and duplexes with a terminal mismatch, thereby improving base pairing fidelity at its terminus. The chart in Figure 2 demonstrates the drop in UV-melting point of the terminal mismatches (stated below each pair of bars) over the perfectly matched control. The sequences are duplexes of a DNA probe with an RNA target strand.[2]

Figure 2 shows clearly the melting point depressions induced by any of the 12 possible terminal mismatches in octamer DNA:RNA duplexes. In the presence of the 3'-Uaq Cap, even quasi-isostable base combinations, such as G:T wobble and A:A mismatch are discriminated against effectively. Similar data sets are available for DNA:DNA and RNA:RNA duplexes.[2] The duplex-stabilizing effect of the 3'-Uaq Cap has also been demonstrated on DNA microarrays.[2]

As shown in Figure 3, the three-dimensional structure of a DNA:DNA duplex with a Uaq cap at either terminus has been solved.[2] This structure confirmed that the anthraquinone residue stacks on the terminal base pairs, and that the

FIGURE 1: **STRUCTURES OF UAQ CPG, OLIGONUCLEOTIDE 3'-TERMINUS, AND CAPPED DUPLEX**



(1) 3'-Uaq Cap CPG *(2) Oligonucleotide with 3' Uaq Terminus* *(3) Illustration of Capped Duplex*

FIGURE 2: **MELTING POINT DEPRESSIONS FOR THE TERMINAL MISMATCHES SHOWN BELOW**

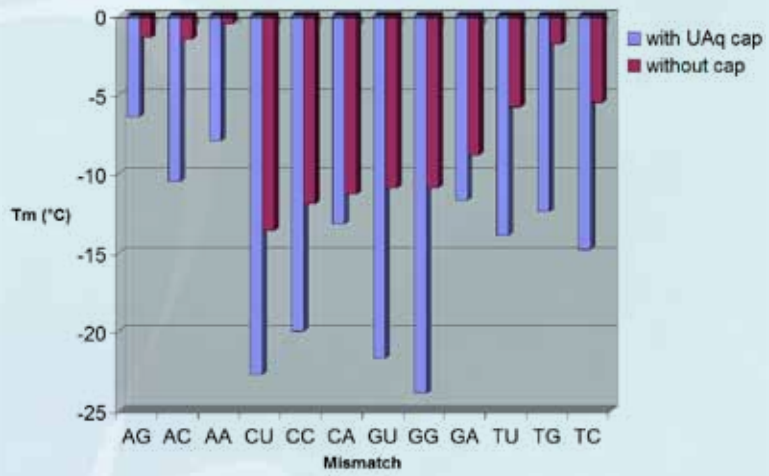
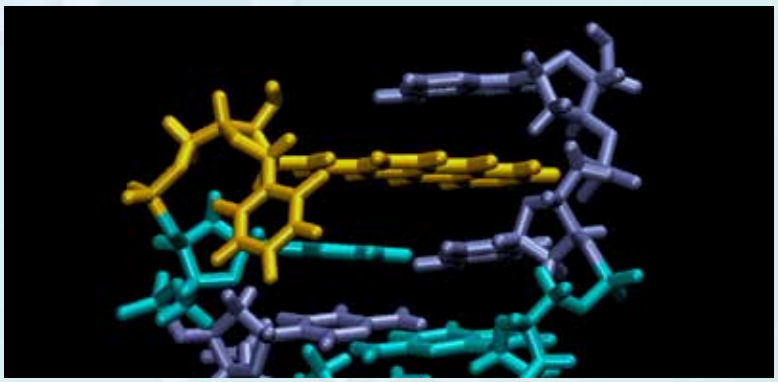


FIGURE 3: **THREE-DIMENSIONAL STRUCTURE OF A DNA DUPLEX**



Three-dimensional structure of a DNA duplex with a Uaq cap (orange) bridging the terminal base pair of a DNA:DNA duplex.[2] Coordinates are from PDB entry 2KK5.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
3'-Uaq Cap CPG	20-2980-01	0.1g	180.00
	20-2980-10	1.0g	1500.00
1 µmole columns	20-2980-41	Pack of 4	300.00
0.2 µmole columns	20-2980-42	Pack of 4	150.00
10 µmole column (ABI)	20-2980-13	Pack of 1	750.00
15 µmole column (Expedite)	20-2980-14	Pack of 1	1125.00

uridine residue acts as a linker, while also engaging in molecular interactions in the minor groove. The precise structural information should help those who wish to employ anthraquinone for photophysical or photochemical studies. Anthraquinones are attractive chromophores for electron transport and electrochemical detection.

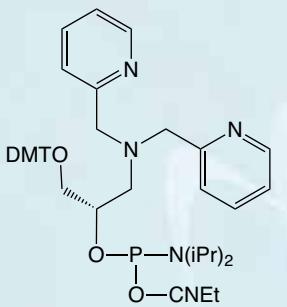
3'-Uaq Cap CPG, available in popular column formats as well as in bulk, is used in the same way as most other supports. Cleavage, if carried out separately, is done in the same way as for other non-modified nucleoside supports. Deprotection can be carried out using ammonium hydroxide at room temperature (to avoid minor degradation at elevated temperatures), AMA at 65°C or potassium carbonate in methanol, as required for deprotection of the nucleobases.

We would like to take this opportunity to thank Clemens Richert for participating in the preparation of this article and for his enthusiasm for cap structures that enhance DNA binding.

References and Notes:

1) 5'-Trimethoxystilbene cap phosphoramidite, Glen Research catalog no. 10-1986, and 5'-pyrene cap phosphoramidite, Glen Research catalog no. 10-1987.
2) A. Patra, C. Richert, High fidelity base pairing at the 3'-terminus. *J. Amer. Chem. Soc.* 2009, **131**, 12671-12681.
3) C. Ahlborn, K. Siegmund, C. Richert, Isostable DNA. *J. Amer. Chem. Soc.* 2007, **129**, 15218-15232.

2,2'-DIPICOLYLAMINE PHOSPHORAMIDITE



2,2'-Dipicolylamine Phosphoramidite

METALLOBASE NUCLEIC ACID MODIFICATION

Exciting new technologies have emerged from the capability to manipulate oligonucleotides using transition metal coordinating organic ligands covalently linked to DNA. Chelate-ligand modified oligonucleotides have been used for *in vivo* siRNA imaging,[1] or sequence selective DNA cleavage.[2] Researchers have prepared metallo-DNA supramolecular assemblies that raise the fascinating possibility of metal-based molecular nano-devices such as molecular scale magnets and wires.[3, 4] Metallobases can be used to precisely control modified DNA through the addition (or removal) of a small amount of selected metal ion, which may lead to astonishing advancements in oligonucleotide research. Several groups have already reported exceptionally stable unnatural metallobase pairs using ligand modified oligonucleotides.[5-8]

Each unique chelation complex exhibits a distinct character regarding affinity and specificity of metal-ion coordination and interaction with the DNA backbone or nucleobases. 2,2'-Dipicolylamine is a versatile metal-coordinating ligand capable of forming complexes with common metal ions including Zn^{2+}, Ni^{2+}, Cu^{2+}, or Ag^{+}.[9] A tremendous advantage of dipicolylamine is complete compatibility with standard DNA synthesis, cleavage and purification protocols. Other chelating ligands may require nonstandard conditions or additional protection and deprotection steps.

Dipicolylamine-metal complexes have been used for the biochemical detection of phosphate[10] and zinc.[11] Kady and Groves used dipicolylamine-Fe^{2+} modified oligonucleotides for the sequence-specific cleavage of a DNA target.[12] Other research groups have proposed using the dipicolylamine ligand for chelation of ^{99m}Tc or ^{188}Re for biomolecular imaging and therapy.[13, 14] Dipicolylamine modified oligonucleotides may also find exciting and innovative uses for surface or nano-particle functionalization, real-time PCR, DNA microarrays, or antisense therapeutics.

We are pleased to offer 2,2'-dipicolylamine phosphoramidite. This product was manufactured and developed by Syntrix Biosystems Inc. Patents Pending. For Research Use Only.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
2,2'-Dipicolylamine Phosphoramidite	10-5801-95	50 µmole	105.00
	10-5801-90	100 µmole	200.00
	10-5801-02	0.25g	625.00

References:

1. Bartlett, D. W.; Su, H.; Hildebrandt, I. J.; Weber, W. A.; Davis, M. E., Impact of tumor-specific targeting on the biodistribution and efficacy of siRNA nanoparticles measured by multimodality in vivo imaging. *Proc Natl Acad Sci U S A* 2007, **104**, (39), 15549-54.
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TC AND TC°: NEW TRICYCLIC FLUORESCENT CYTIDINE ANALOGUES WITH A VERY BRIGHT FUTURE

Glen Research has offered fluorescent nucleosides for a long time: 2-Aminopurine deoxynucleoside and Etheno-dA have been in our catalog for more than 15 years. More recently, we have become interested in the potential for dC analogues to offer interesting fluorescence properties while maintaining excellent base pairing behavior with dG. In partnership with Berry and Associates, we introduced pyrrolo-dC[1], which has intriguing properties that allow its exact position in a duplex to be determined. And, recently, we have pointed out the fluorescence properties of AP-dC (G-Clamp).[2]

Now we are happy to introduce the tricyclic fluorescent nucleoside analogues, 1,3-diaza-2-oxophenothiazine, tC, and 1,3-diaza-2-oxophenoxazine, tC°. Tricyclic dC base analogues have been shown to base pair faithfully with dG with virtually no disruption of the normal duplex structure.[3-5] This means that the stability of the DNA duplex is not compromised as compared to the control regardless of DNA sequence.

1,3-DIAZA-2-OXOPHENOTHIAZINE (TC)

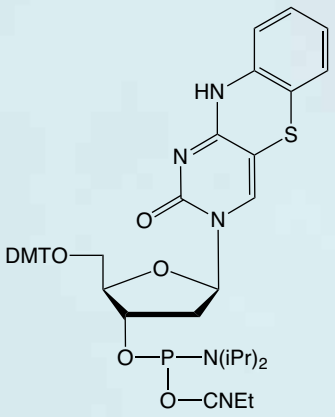
Wilhelmsson and coworkers have demonstrated that tC is unique in the group of fluorescent base analogues that mimic cytosine.[5] As might be predicted, the structure of duplex DNA containing tC retains the native B-form. Melting studies on duplexes containing tC have demonstrated that it base pairs strongly with G and the melting temperatures of duplexes are increased by 2.7° over the equivalent C-containing duplex.

tC is strongly fluorescent with an absorption maximum at 385nm, which is far outside the wavelength of DNA absoption. Its emission maximum is at 500nm.[4]

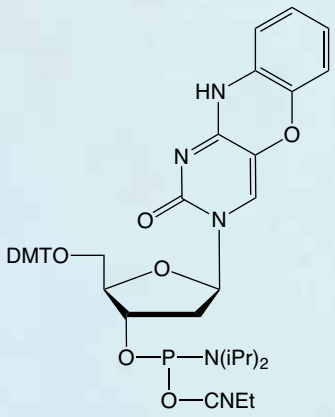
Of great significance is the fact that the fluorescence quantum yield of tC is essentially unchanged between single stranded and double stranded DNA. The average quantum yield was 0.21 for single stranded DNA and 0.19 for duplex DNA. Also, the fluorescence characteristics of tC were not sensitive to neighboring base combinations.[4]

This combination of characteristics make tC one of the most interesting fluorescent bases so far evaluated.

***FIGURE 1:* TRICYCLIC FLUORESCENT CYTIDINE ANALOGUE STRUCTURES**



tC-CE Phosphoramidite (1)



tC°-CE Phosphoramidite (2)

1,3-DIAZA-2-OXOPHENOXAZINE (TC°)

Like its sulfur-containing cousin, tC° is also a very bright fluorophore. It has been reported to be 25-50 times brighter than 2-aminopurine.[3] The reason for this is twofold. First, tC° has a large extinction coefficient with an E_{360} of 9,000 L/mol·cm. Second, unlike other nucleoside analogs, tC° is not severely quenched when in double-stranded DNA. Peter Sandin and colleagues did an exhaustive study of neighboring base effects in single- and double-stranded oligos, determining the quantum yield of fluorescence (QY) for all permutations of bases immediately 5′ and 3′ to tC° when the oligonucleotide is single versus double-stranded.[3] They found that on average, the QY is 0.30 for single-stranded oligos and a quite respectable 0.21 when double-stranded, making tC° the brightest fluorescent nucleoside analogue in double-stranded DNA reported so far.

SPECTRAL CHARACTERISTICS

Figure 2 shows the absorption and emission spectra of the oligo 5′-ATC GXT CAT GAT G-3′, where X is tC, as determined in house. The absorption maximum was found to be at 392nm with no shift in wavelength in duplex DNA. The emission maximum was found to be at 506nm. Figure 2 illustrates the melting curves for the tC oligo as well as the control where X = dC. We found that the T_m of this oligo was raised by 1.0° relative to the control oligo containing dC.

Figure 3 shows the absorption and emission spectra of the oligo 5′-ATC GXT CAT GAT G-3′, where X is tC°, as determined in house. The absorption maximum was found to be at 360nm, shifting to 365nm in duplex DNA. The emission maximum was found to be at 465nm. Figure 3 also shows the melting curves for the tC° oligo, as well as the control where X = dC. We found that the T_m of this oligo was raised by 0.5° relative to the control oligo containing dC.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
tC-CE Phosphoramidite	10-1516-95	50 µmole	250.00
	10-1516-90	100 µmole	490.00
	10-1516-02	0.25g	1460.00
tC°-CE Phosphoramidite	10-1517-95	50 µmole	250.00
	10-1517-90	100 µmole	490.00
	10-1517-02	0.25g	1460.00

SYNTHESIS AND DEPROTECTION

tC- and tC°-CE Phosphoramidites couple optimally with a reaction time of 3 minutes. Deprotection can be carried out using ammonium hydroxide at room temperature (to avoid minor degradation at elevated temperatures), AMA at 65°C or potassium carbonate in methanol, as required for deprotection of the nucleobases.

We are happy to offer tC as a very bright and stable probe of DNA structure and tC° as the brightest and most promising internal fluorescent probe for DNA to date. In combination, they offer great potential for the analysis of DNA structure and function. We would also like to thank Marcus Wilhelmsson for his help in preparing this article.

References:

1. D.A. Berry, et al., *Tetrahedron Lett*, 2004, **45**, 2457-2461.
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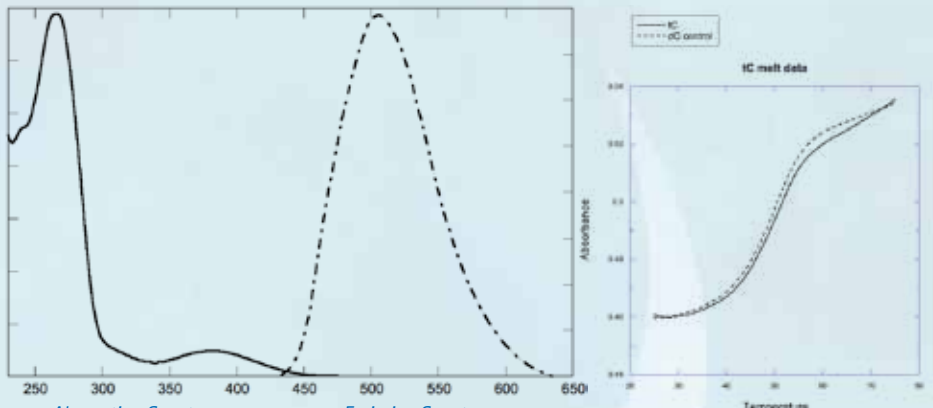
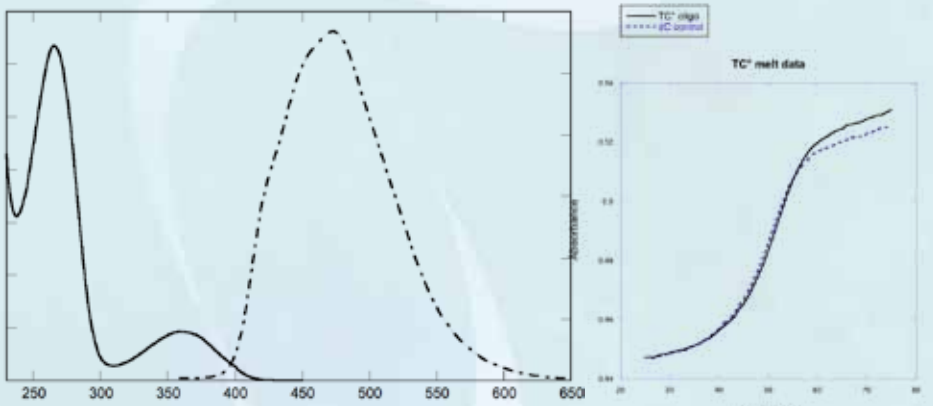
FIGURE 2: **ABSORPTION AND EMISSION SPECTRA OF TC OLIGO AND MELTING CURVES**



Absorption Spectrum *Emission Spectrum* *Melting Curves*

FIGURE 3: **ABSORPTION AND EMISSION SPECTRA OF TC° OLIGO AND MELTING CURVES**

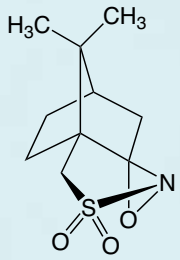


Absorption Spectrum *Emission Spectrum* *Melting Curves*

NON-AQUEOUS OXIDATION FOR PACE CHEMISTRY

Glen Research recently began offering phosphoramidites for the synthesis of phosphonoacetate (PACE) modified oligonucleotides.[1] These PACE modified oligonucleotides offer improved cellular uptake of oligonucleotides in cell culture, enhanced nuclease resistance, and the ability to stimulate RNase activity.[2-4] Synthesis of PACE modified oligonucleotides requires a very mild oxidation reagent to maintain the desired PACE modification and reduce the formation of phosphodiester linkages. For the synthesis of fully PACE modified oligonucleotides, the recommended oxidation reagent is 0.10M (1S)-(+)-(10-camphorsulfonyl)oxaziridine (CSO) in anhydrous acetonitrile.[2] This non-aqueous oxidation reagent prevents the formation of phosphodiester linkages during the oligonucleotide synthesis and is used in place of the standard 0.02M Iodine oxidation solution. In the past, we have recommended commercial sources of CSO but found that they are expensive and the solutions required filtering prior to use on a standard synthesizer. To support the development PACE modified oligonucleotides, we are now offering 0.10M(1S)-(+)-(10-camphorsulfonyl) oxaziridine in anhydrous acetonitrile as the recommended oxidation solution for PACE modified oligonucleotides.

CSO **STRUCTURE**



CSO

References:

1. *The Glen Report*, 2008, **20**, 1-5.
2. D.J. Dellinger, D.M. Sheehan, N.K. Christensen, J.G. Lindberg, and M.H. Caruthers, *J. Amer. Chem. Soc.*, 2003, **125**, 940-950.
3. D. Sheehan, et al., *Nucleic Acids Res.*, 2003, **31**, 4109-4118.
4. C.M. Yamada, D.J. Dellinger, and M.H. Caruthers, *J. Amer. Chem. Soc.*, 2006, **128**, 5251.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
0.10M CSO in Anhydrous Acetonitrile (ABI)	40-4631-52	200mL	140.00
0.10M CSO in Anhydrous Acetonitrile (Expedite)	40-4631-52E	200mL	140.00

NEW PRODUCT - AZOBENZENE PHOSPHORAMIDITE FOR THE INTRODUCTION OF PHOTO-REGULATED

Since the beginning of automatic oligonucleotide synthesis in the late 80's, the possibilities available to researchers have expanded exponentially. Starting from simple primers for sequencing, PCR or cloning, oligos are now used for gene expression modulation[1], as probes for quantitative PCR[2,3,4], and even for some futuristic applications in nanotechnology such as logic-gates[5].

Controlling DNA functions using an external stimulus that could be easily and quantitatively controlled at any given moment and position might be an incredible and interesting way to develop novel applications in cell biology and pharmacology. It could also provide new tools for the analysis of the mechanisms underlying DNA recognition and DNA-mediated bioprocesses.

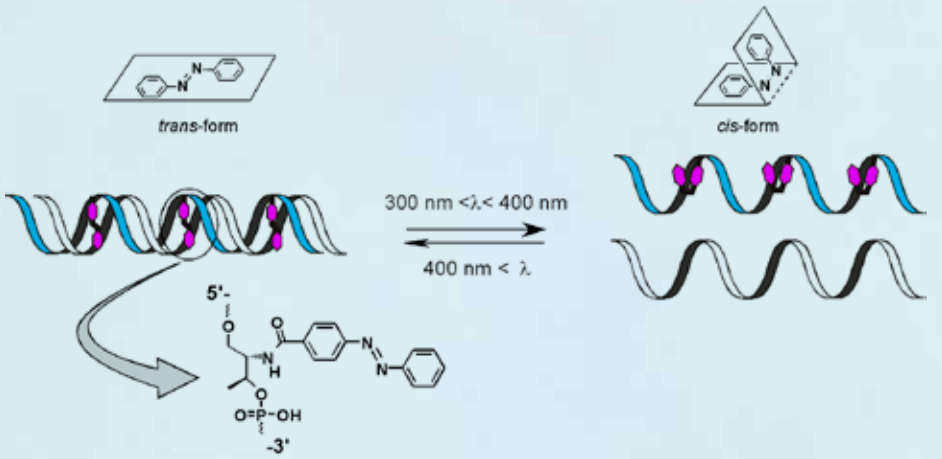
Photo-control, the use of ultraviolet or visible light to control a reaction, has a number of advantages over other external stimuli:

- Light does not introduce contaminants into the reaction system,
- Excitation wavelength can be controlled through the design of the photo-responsive molecule, and
- It is now straightforward to control irradiation time and/or local excitation.

When a photo-responsive molecule is directly attached to DNA as a receptor, photo-regulation of the bioprocess regulated by that DNA molecule could, in principle, be achieved. Such photo-responsive DNA could also be used as a switch in a DNA-based nano-machine.

Professor Hiroyuki Asanuma and his group at the department of Molecular Design and Engineering of the Graduate School of Engineering of the Nagoya University (Japan) have developed an efficient method to achieve this goal. They have attached azobenzene to DNA and made it photo-responsive[6,7]. Azobenzene is a typical photo-responsive molecule that isomerizes from its planar *trans*-form to the non-planar *cis*-form after UV-light irradiation with a wavelength between 300 nm and 400 nm (λ_{max} is around 330 nm). Interestingly, the system reverts from the *cis*-form to the *trans*-form after further irradiation with visible light (wavelength over 400 nm). This process is completely

FIGURE 1: **PHOTO-REGULATION OF THE FORMATION AND DISSOCIATION OF DNA DUPLEXES WITH AZOBENZENE LABELED OLIGONUCLEOTIDES**



reversible, and the azobenzene group does not decompose or induce undesirable side reactions even on repeated *trans-cis* isomerization.

By introducing azobenzenes into DNA through D-threoninol as a linker, Asanuma and co-workers succeeded in achieving photo-regulation of:

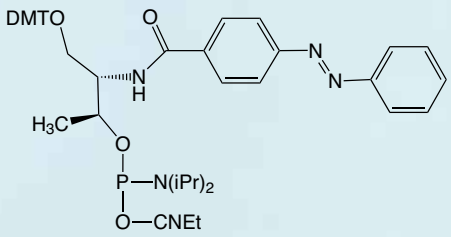
- Formation and dissociation of a DNA duplex[8,9] and
- Transcription by T7-RNA polymerase reaction[10,11,12].

PHOTO-REGULATION OF DNA HYBRIDIZATION

For photo-regulation of DNA hybridization or DNA transcription as effected by T7-RNA polymerase, the azobenzene [X] residues must be inserted between the base pairs of the template DNA duplex. In other words, the X residue is introduced as a bulge adduct. For example, to make the sequence 5'-GCGAGTCG-3' photo-responsive, the X residue must be incorporated in the sequence, without any base replacement to obtain 5'-GCGAXGTCG-3'. This sequence's complementary strand, 3'-CGCTCAGC-5', remains composed only of natural nucleotides. As long as the X residue is additionally inserted, there are no limitations with regard to the position of its introduction. X can equally be inserted at the center of a sequence or in the vicinity of its 5'- or 3'-terminus.

The introduction of several X residues increases the ΔT_m created by the photo-induced isomerization. For example, with the sequence described in the previous

AZOBENZENE PHOSPHORAMIDITE



Azobenzene Phosphoramidite

paragraph with one X residue in the middle, the ΔT_m between the *trans* [more stable] form and the *cis* form is 14.3 °C and with 2 residues as in 5'-G CXG AGT XCG-3' the ΔT_m between *cis* and *trans* is 21.5 °C.

In order to photo-regulate the hybridization of relatively long duplexes efficiently, introduction of multiple azobenzenes is recommended. In this case as well, X residues should be additionally introduced without replacing any bases. A photo-responsive DNA sequence is designed as 5'-$(NNX)_n$-NN-3'. If chemical modification is allowed to both strands, azobenzenes can be symmetrically introduced to both strands (i.e., 5'-$(NNX)_n$-NN-3'/3'-$(NXN_n$-XN-5') to further raise the photoregulatory efficiency[13].

PHOTO-SWITCHING OF T7 RNA POLYMERASE TRANSCRIPTION

This application was described in detail in a Nature Protocols paper[12]. In brief, adding azobenzene groups to the T7 promoter allows transcription by T7-RNA polymerase to be reversibly photo-regulated by the *trans–cis* isomerization of the labels. Transcription is suppressed

by visible-light irradiation, which triggers the formation of *trans*-azobenzene, and is recovered by UV-light irradiation (formation of *cis*-azobenzene), as shown in Figure 2. For effective photo-regulation, two X residues are inserted between –3 and –4 and between –9 and –10 in the non-template strand of the T7 promoter region (see Figure 3 From Nature Protocols[12] - erratum). The template strand is kept intact.

Introduction of the X labeled promoter into gene construct for effective photo-regulation of gene expression *in vitro* was described in Liang, et al.[14]

FIGURE 2: PHOTO-SWITCHING OF THE TRANSCRIPTION REACTION BY T7-RNA POLYMERASE WITH PHOTO-RESPONSIVE T7-PROMOTER.

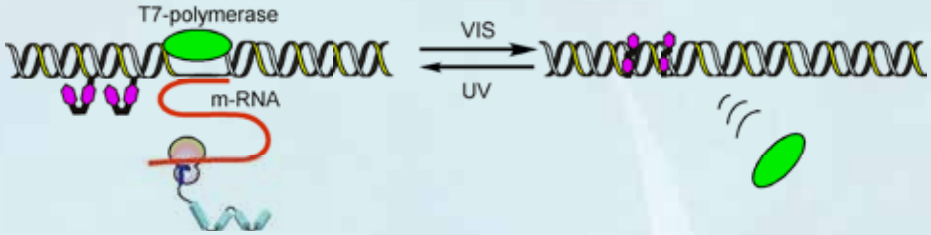


Image from Asanuma web site – http://www.nubio.nagoya-u.ac.jp/seigyo1/english/kenkyu.html

FIGURE 3: SEQUENCE OF THE PHOTO-RESPONSIVE T7-PROMOTER. X REPRESENTS THE AZOBENZENE RESIDUE.

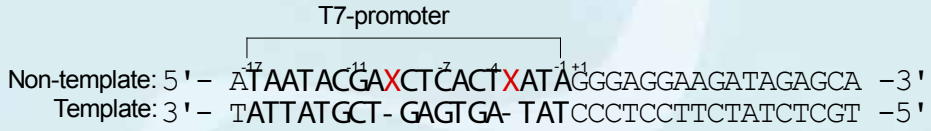


PHOTO-CONTROLLABLE INHIBITORS (PCI'S)

Oligonucleotides containing azobenzene modifiers have also been used in order to control the activity of some enzymes. Tan's group[15] has linked an azobenzene-containing sequence to a Thrombin-binding aptamer via a Spacer-18 residue. This molecule contains 3 parts: an inhibitory part, that is the Thrombin-aptamer, a regulatory part, that is a complementary oligo designed to bind to the aptamer part and contains azobenzenes and a regulatory part, and a linker – in this case Spacer 18. Depending on light exposure, the regulatory part will hybridize to the aptamer – blocking the inhibition – or not – releasing the inhibitory activity.

Using the properties of the photoisomerization, they have been able to optimize a probe whose inhibitory effect can be inhibited or restored using photo-exposition. Possible applications of the technology are also described in this paper.

SYNTHESIS OF OLIGONUCLEOTIDES CONTAINING AZOBENZENE RESIDUES

The synthesis of azobenzene-containing oligos can be performed without any modification using a coupling time of 10 minutes for the coupling of Azobenzene Phosphoramidite. The oligos can be deprotected using standard procedures. The Nature Protocols paper[12] described methods for purification of the labelled oligos using HPLC, PAGE or Poly-Pak cartridges.

For the measurement of the concentration by measure of the absorption, the use of an extinction coefficient of 4,100 $M^{-1}.cm^{-1}$ at 260 nm is recommended.

For UV irradiation, a hand-held, conventional UV illuminator can be used. Although the time required for *cis* isomerization depends on the intensity of the UV light, 5 min irradiation will usually be enough to achieve a steady state, irrespective of the light source. More information about irradiation using a Xenon-lamp and filter is available in the Nature Protocols paper[12].

Oligonucleotides containing azobenzene are easily prepared using a preferred coupling time of 10 minutes. They are deprotected according to the conditions required to deprotect the nucleobases. AMA at 65 °C or UltraMild chemistry can be used. However, deprotection with ammonium hydroxide has to be carried out at room temperature to avoid degradation. For example, no degradation was observed after 17 hours at room temperature using ammonium hydroxide.

Obviously, *cis* and *trans* isomers exist for azobenzene analogues, which may complicate HPLC analysis of the monomer and the oligos produced. Using a simple oligonucleotide, 5'-azobenzene-T6, we illustrate the potential for isomerism. In Figure 4, the HPLC shows two peaks for the simple 5'-azobenzene-T6, the earlier eluting and minor peak being the *cis* isomer and the later eluting peak being the *trans* isomer. The first peak in the chromatogram is a T6 standard added as the internal standard. On irradiation at 330nm, the ratio changes as the trans isomer converts to the cis isomer. Figure 5 shows the UV spectra of the pure *cis* and *trans* isomers.

We are happy to introduce this new and interesting product to our repertoire of modifiers. We would also like to thank Professor Asanuma for his help in preparing this article. In Japan, this product is only available through our distributor Nihon Techno Service Co., Ltd (http://www.ntsbio.com/).

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
Azobenzene Phosphoramidite	10-5800-95	50 µmole	105.00
	10-5800-90	100 µmole	200.00
	10-5800-02	0.25g	550.00

References:

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FIGURE 4: **PHOTO-SWITCHING OF THE RATIO OF CIS AND TRANS AZOBENZENE ISOMERS BY UV LIGHT.**

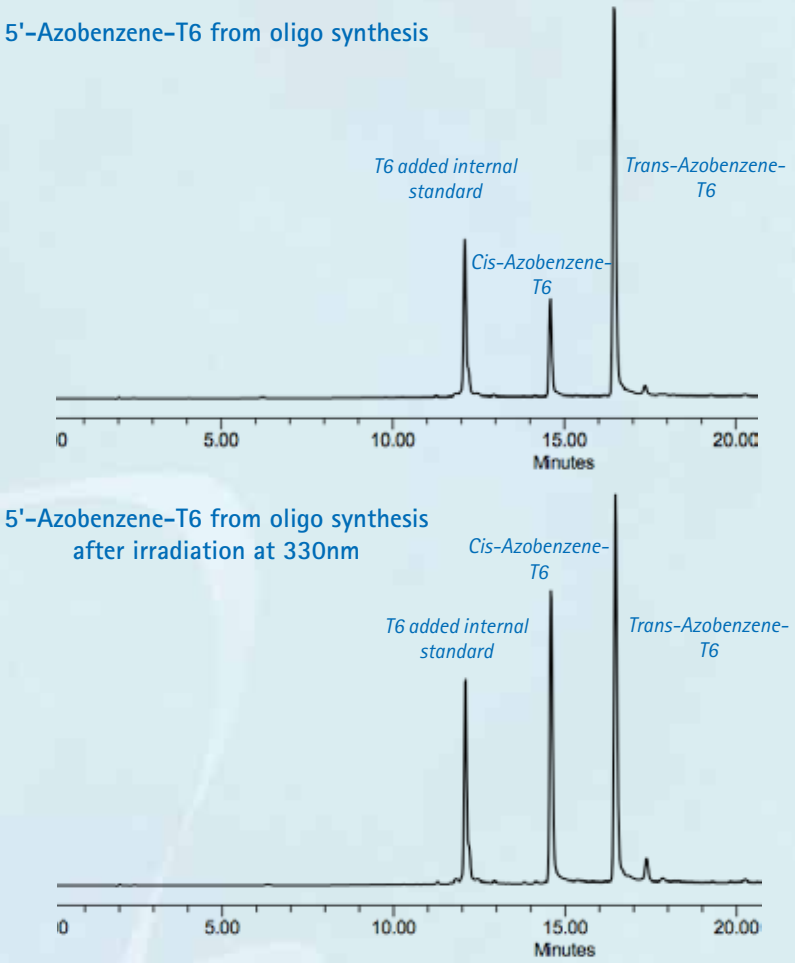
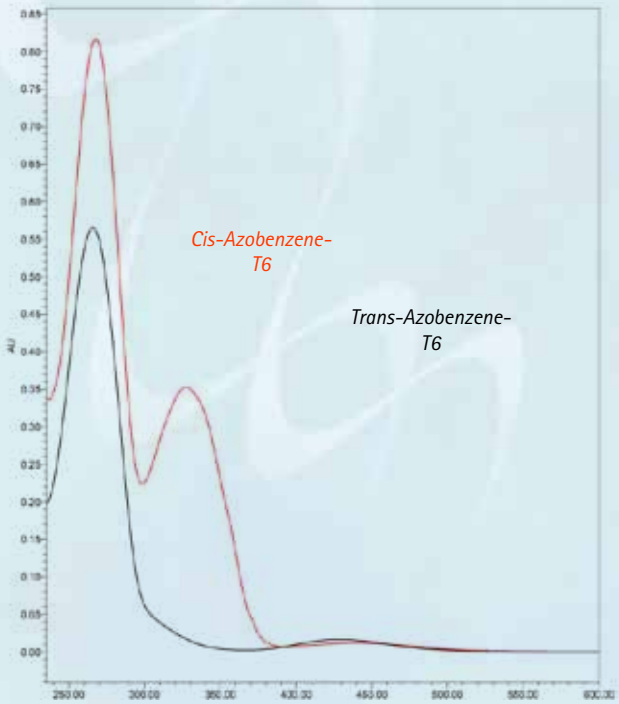


FIGURE 5: **UV VISIBLE SPECTRA OF CIS AND TRANS AZOBENZENE -T6**



DEPROTECTION - VOLUME 3 - DYE-CONTAINING OLIGONUCLEOTIDES

In the previous two articles in this series on Deprotection, we focused on DNA and RNA deprotection. Our first priority in deprotection is to "Deprotect to Completion" by removing 100% of the protecting groups on the nucleobases, while following the mandate to "Do No Harm". Dyes tend to have the unfortunate properties of being quite sensitive to the basic conditions of oligonucleotide deprotection while being expensive. The "Do No Harm" stricture is doubly important when deprotecting dye-labelled oligonucleotides. To make matters worse, many dye labelled oligonucleotides also contain a quencher molecule that may also be base sensitive. This combination of properties is guaranteed to lead to confusion and possibly decomposed, worthless oligos may result if incompatible deprotection conditions are used.

In this article, we have generated a Table which we hope will remove some of the challenges from the deprotection of dye-labelled oligonucleotides. We have focused on a variety of methods for oligonucleotide deprotection:

A: 30% NH4OH 17 hours at 55 °C; sufficient to deprotect all standard bases, A/C/G/T
B: 30% NH4OH 17 hours at room temperature; sufficient to deprotect A, C and dmf-dG
C: 30% NH4OH 2 hours at 65 °C; sufficient to deprotect A, C and dmf-dG
D: 30% NH4OH 2 hours at room temperature; sufficient to deprotect only UltraMild monomers, Pac-dA, Ac-dC, ipr-Pac-dG when UltraMild Cap A is used.
E: 50 mM Potassium Carbonate in Methanol for 4 hours at room temperature; sufficient to deprotect only UltraMild monomers, Pac-dA, Ac-dC, ipr-Pac-dG when UltraMild Cap A is used.
F: Tert-Butylamine/water 1:3 (v/v) 6 hours at 60 °C; sufficient to deprotect A, C and dmf-dG.

***VOLUME 3:* DYES - DEPROTECT TO COMPLETION**

1) Even with oligos containing sensitive dyes, the nucleobases must be fully deprotected for full functionality.
2) Will the dye-labelled oligo survive my preferred deprotection scheme?
3) If not, which deprotection scheme will fit best with my equipment and purification strategy?

G: 30% Ammonium Hydroxide/40% Methylamine 1:1 (v/v) 10 minutes at 65 °C; sufficient to deprotect all standard bases, however, Ac-dC must be used.

The Table illustrates the conditions suitable for deprotecting oligos containing one or two of the dyes listed. We will continue to update this Table on our web site.

Note: JOE has not been tested with Condition F.
$ Denotes an acceptable, but not preferred method.

***TABLE:* DEPROTECTION CONDITIONS SUITABLE FOR POPULAR DYES AND QUENCHERS**

	FAM	*TET*	*HEX*	*JOE*	*TAMRA*	*Yakima Yellow*	*Cy3/ DyLight 547*	*Cy5/ DyLight 647*	*Dabcyl*	*Eclipse*	*BHQ-1*	*BHQ-2*	*BHQ-3*
FAM	A..G	A..G	B, D, E,G	A..E, G	E, F	A..D, F, G	B..E, G	D, E	A..E, G	A..G	$A, B..E, $F,G	A..E	$B, D
TET	A..G	A..G	B, D, E, G	A..E, G	E, F	A..D, F, G	B..E, G	D, E	A..E, G	A..G	$A, B..E, $F,G	A..E	$B, D
HEX	B, D, E, G	B, D, E, G	B, D, E, G	B, D, E, G	E	B, D, G	B, D, E, G	D, E	B, D, E, G	B, D, E, G	B, D, E, G	B, D, E	$B, D
JOE	A..E, G	A..E, G	B, D, E,	A..E, G	E	A..D, G,	B..E, G	D, E	A..E, G	A..E, G	$A, B..D	A..E	$B, D
TAMRA	E, F	E, F	E	E	E, F	F	E	E	E	E, F	E, $F	E	Incompatible
Yakima Yellow	A..D, F, G	A..D, F, G	B, D, G	A..D, G	F	A..D, F, G	B, C, D, G	D	A..D, G	A..D, F, G	$A, B, C, D, $F,G	A..D	$B, D
Cy3/DyLight 547	B..E, G	B..E, G	B, D, E, G	B..E, G	E	B, C, D, G	B..E, G	D, E	B..E, G	B..E, G	B..E, G	B..E	$B, D
Cy5/DyLight 647	D, E	D, E	D, E	D, E	E	D	D, E	D, E	D, E	D, E	D, E	D, E	D
Dabcyl	A..E, G	A..E, G	B, D, E, G	A..E, G	E	A..D, G	B..E, G	D, E	A..E, G	A..E, G	$A, B..E, G	A..E	$B, D
Eclipse	A..G	A..G	B, D, E, G	A..E, G	E, F	A..D, F, G	B..E, G	D, E	A..E, G	A..G	$A, B..E, $F,G	A..E	$B, D
BHQ-1	$A, B..E, $F,G	$A, B..E, $F,G	B, D, E, G	$A, B, C, D	E, $F	$A, B, C, D, $F,G	B..E, G	D, E	$A, B..E, G	$A, B..E, $F,G	$A, B..E, $F,G	$A, B..E	$B, D
BHQ-2	A..E	A..E	B, D, E	A..E	E	A..D	B..E	D, E	A..E	A..E	$A, B..E	A..E	$B, D
BHQ-3	$B, D	$B, D	$B, D	$B, D	Incompatible	$B, D	$B, D	D	$B, D	$B, D	$B, D	$B, D	$B, D

NEW PRODUCTS - GLEN UNYSUPPORT™ FRITS

GLEN UNYSUPPORT

Glen UnySupport™ is a version of UnyLinker™, shown in Figure 1, that was developed at Isis Pharmaceuticals, and is preferred for high throughput oligonucleotide synthesis. Glen UnySupport is compatible with most deprotection strategies from gas phase deprotection with methylamine to UltraMild deprotection with potassium carbonate in methanol. We supply Glen UnySupport under license from Isis Pharmaceuticals.

Glen UnySupport is fast becoming one of our most popular universal supports since it is compatible with such a wide range of deprotection strategies and conditions, as shown in Table 1.

For instance, Universal Support III (Figure 1) is an excellent universal support for DNA *and* RNA synthesis and for oligos that require UltraMild conditions because of the presence of sensitive dyes or minor bases. However, since efficient cleavage is dependent upon the cyanoethyl phosphotriester remaining intact until treatment with 2 M NH_3 in methanol, it is not compatible with the popular DEA post-synthesis treatment to reduce N3 alkylation by acrylonitrile nor is it compatible with gas phase deprotection conditions.

This is not the case with the Glen UnySupport. We have now established conditions for UltraMild deprotection using both 50 mM Potassium Carbonate in methanol and tert-Butylamine/Water, UltraFast deprotection using AMA, and Gas Phase deprotection using Methylamine. While US III continues to be the universal support of choice for RNA synthesis, in most cases, Glen UnySupport performs as well using a wide variety of popular deprotection cocktails. In Table 1, we have summarized the current cleavage conditions necessary to completely eliminate the Glen UnySupport from the 3' terminal base.

GLEN UNYSUPPORT FRITS

Universal Supports are the supports of choice for high throughput synthesis since they remove the need for individual wells or columns to contain a unique support. In the past, supports have been added to synthesis plates by slurrying in a dense (and usually toxic) liquid and dispensing conventionally. An alternative approach would be to embed the support into frits suitable for inserting into standard columns or plates.

We now offer high density polyethylene (HDPE) frits with embedded Glen UnySupport for high throughput applications and to allow customers to make their own inexpensive columns. The frits contain 40 nanomoles of Glen UnySupport and fit directly into empty MerMade and AB3900 columns. They can also be used on ABI 394 and Expedite instruments when fitted into inexpensive female-female luer adapters. We are also offering the female-female luers required for use of the Glen UnySupport Frits on Expedite and ABI 394 instruments. To assemble the column, simply place the frit in the luer fitting and seat it in the center using an allen wrench or equivalent tool (be careful not to puncture the frit). The luers are re-useable and can be purchased in packs of 10 (Cat. No. 20-0060-00).

***FIGURE 1:* UNIVERSAL SUPPORT STRUCTURES**

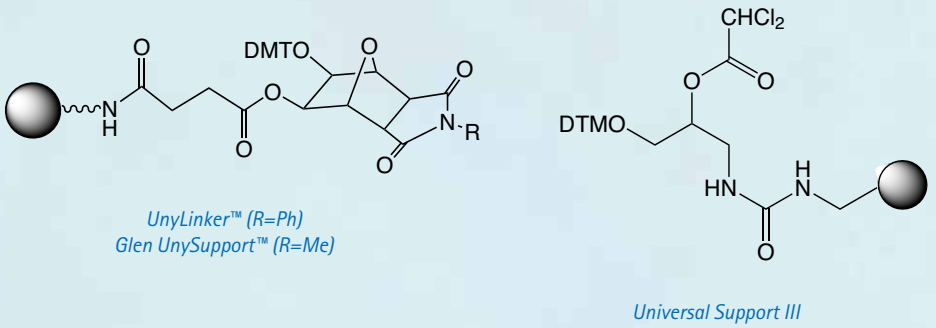


***TABLE 1:* ELIMINATION CONDITIONS - GLEN UNYSUPPORT**

Reagent	Time	Temperature
Standard		
Ammonium Hydroxide	2 hours	80 °C
	8 hours	55 °C
UltraFast		
Ammonium Hydroxide/	0.5 hours	80 °C
40% Methylamine (AMA)	1 hours	65 °C
	2 hours	55 °C
Gas Phase		
Methylamine Gas	0.5 hours	65 °C (30 psi)
UltraMild		
Potassium Carbonate in Anhydrous Methanol	17 hours	Room Temperature
t-Butylamine/Water (1:3) v/v	4 hours	60 °C

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
Glen UnySupport 500Å 40 nmole frits	20-5440-95	Pack of 96	150.00
Female-Female Luer Adapter	20-0060-00	Pack of 10	20.00
Glen-Pak™ DNA Cartridge 3g	60-5300-01	Pack of 1	150.00
Glen-Pak™ DNA 30mg 96-Well Plate	60-5400-01	Pack of 1	475.00

NEW PRODUCTS: GLEN-PAK™ DNA CARTRIDGE 3G AND DNA 30MG 96-WELL PLATES

Since their launch in 2007, the Glen-Pak™ cartridges have been extremely well received. So well, in fact, that some of our customers have reported that they are omitting HPLC and PAGE purifications!

While the existing Glen-Pak DNA and RNA formats are capable of use in 96 well formats from 40 nmole through 1.0 µmole scale, many customers have asked for both a larger scale option (10-20 µmole) to be used in either one time or pre-purification DNA applications, as well as an integrated smaller scale (10 – 50 nmole) DNA purification filter plate that can be used in more standardized, robotic high throughput settings. We are happy to introduce two new products to meet these demands.

GLEN-PAK™ CARTRIDGE 3G:

The Glen-Pak DNA Cartridge 3g is a 20 mL syringe-type tube compatible with luer-type vacuum manifolds. This cartridge enables DMT-ON oligo purification on a scale up to 20 µmoles, as shown in Figure 1. These cartridges may also be used in a pre-purification step before proceeding to HPLC purification. The injection of such a pre-purified product usually results in better separation, as a higher concentration of full-length product is injected on the preparative HPLC column. Assuming the DMT is removed during Glen-Pak processing, the second purification step would ideally be ion-exchange purification, but the option to elute the product DMT-ON is also available if second step RP-HPLC is more desirable.

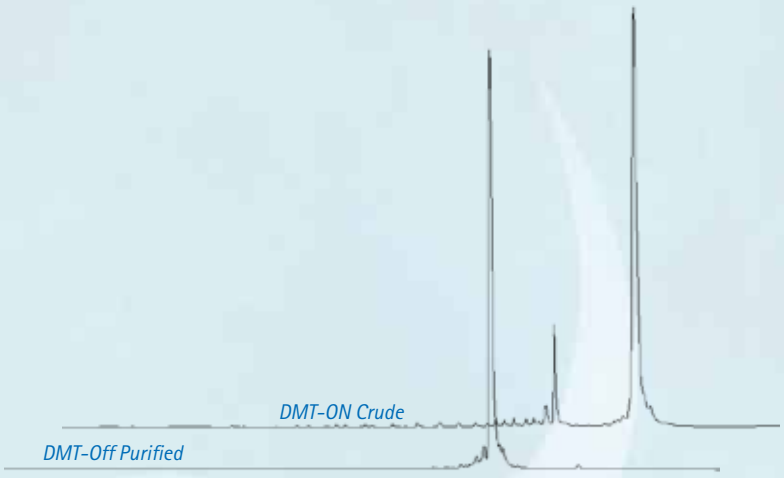
The Glen-Pak 3g cartridge utilizes the same reagent set and basic protocol as the standard Glen-Pak cartridge but with a 10 fold linear increase in volume for each step. A full protocol for use, including suggestions for oligonucleotide work up, is located in the latest version of the Glen-Pak purification booklet on our website:

http://www.glenresearch.com/Technical/Glen_Pak_2-1.pdf

GLEN-PAK™ 30MG 96-WELL PLATES:

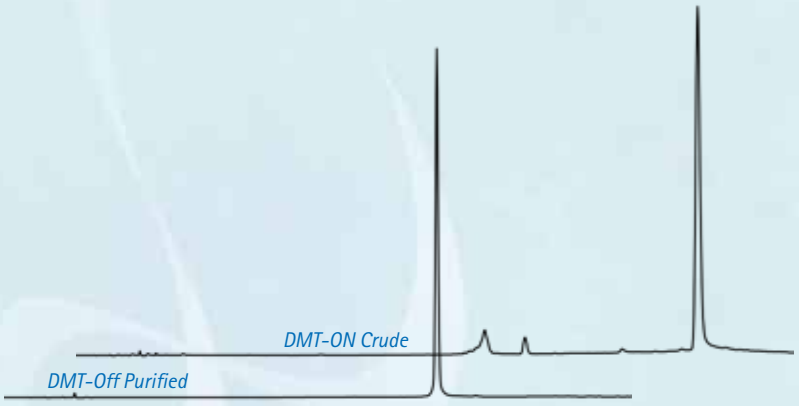
The Glen-Pak DNA 30mg 96-well plate is designed for those customers looking for true high throughput, small scale, DMT-ON purification of oligos via either a stand-alone manifold or with their currently installed SPE robotics-based vacuum systems. Each well in the standardized 96-well filter plate is filled with 30mg of Glen-Pak DNA purification resin, which is enough to purify up to a 50 nmole scale synthesis, as shown in Figure 2.

FIGURE 1: IEX HPLC OF 20 MICROMOLE SYNTHESIS PURIFIED ON GLEN-PAK 3G - CRUDE AND PURIFIED



IEX HPLC Column: Dionex DNAPac PA200 250 x 4mm Buffers: A - 25mM Tris, pH 8, 5% ACN; B - 25mM Tris, pH 8, 5% ACN, 1.0M NH_4Cl, pH 8 Gradient: 0-90% Buffer B at a flow rate of 1mL/min.

FIGURE 2: RP HPLC OF 50 NANOMOLE SYNTHESIS PURIFIED ON GLEN-PAK 96-WELL PLATE - CRUDE AND PURIFIED



RP HPLC Column: Waters Spherisorb 5.0µm ODS2 4.6 x 150mm Buffers: A – 100% Acetonitrile; B – 0.1M TEAA; Gradient: 3-40% Buffer A at a flow rate of 1mL/min.

For stand-alone use, the 96-well plate can be coupled to a vacuum source, a collar-based manifold and a waste tray for loading or rinse steps. The standard sample load and rinse volumes are 500 µL with final elution volumes of 250 µL, which are collected into a 96-well deep well plate. These volumes are only suggestions based on our experience and could be lower with specific methods development. The plate is also compatible with previously installed 96-well filter plate and SPE manifold systems.

The advantages of using the 96-well format include:

- Lower cost processing (less labor and unattended operation if using a robot).
- Less processing time per oligo when using a whole plate.
- Less solvent usage for every purification step.
- Less final elution volume to dry in preparation for analysis or use.

Both products offer the same benefits that are expected from the standard Glen-Pak DNA Cartridges:

- No need to dry down a deprotected oligo prior to purification.
- High affinity for DMT-ON fragments.
- Lack of length restrictions that usually hamper more classical SPE purification methods.

With the advent of gene synthesis and the requirement of longer oligonucleotides for mutagenesis, we thought it would be useful to our customers to review the chemistry of synthesizing long oligonucleotides (>75 nucleotides) and to provide some suggestions for their successful production.

All of the DNA synthesis reagents have an impact on good quality DNA synthesis, as are all the steps in the synthesis cycle – coupling, capping, oxidation and detritylation. However, for the successful synthesis of long oligos, some factors are absolutely critical, as noted below.

COUPLING STEP

Fundamental to the successful synthesis of longer oligos is the need to maintain as high a coupling efficiency as possible. While an average coupling efficiency of 98.0% would theoretically yield 68% full-length product for a 20mer, it would only yield 13% full-length product for a 100mer. So, it is imperative to maintain high coupling efficiency throughout the synthesis. One of the main obstacles to this is the presence of moisture, which lowers coupling efficiency in two ways:

1. Water reacts with the activated tetrazolide of the incoming monomer instead of the 5'-hydroxyl of the support, thereby scavenging excess monomer and lowering the coupling efficiency.
2. Water catalyzes the conversion of the phosphoramidite to the phosphonate as the phosphoramidite sits on the synthesizer, thereby lowering the concentration of phosphoramidite itself.

It is of little surprise, then, that during the humid summer months low coupling efficiency can be problematical. As such, steps must be taken to reduce the water content of all the reagents involved in the coupling step – the acetonitrile (ACN) on the synthesizer, the ACN used to dilute the phosphoramidites, the activator solution (1H-tetrazole, DCI, ETT or others), and even the argon or helium used on the synthesizer, which should be dried with an in-line drying filter before reaching the synthesizer. And of course, the phosphoramidites themselves should be dry.

The most simple and effective first measures to increase coupling efficiency are to use truly anhydrous ACN on the synthesizer (10-15 ppm water content or lower), use fresh phosphoramidites during synthesis, and to dissolve the phosphoramidites while maintaining an anhydrous atmosphere. We recommend purchasing septum-sealed bottles of ACN (e.g., 40-4050-45) and to use a fresh bottle when putting new monomers on the synthesizer. The technique we suggest to dissolve a phosphoramidite under anhydrous conditions is described in Table 1.

Some synthesizers are more susceptible to humidity than others. In extreme situations, customers have gone so far as to make a 'tent' of non-static plastic sheeting around the synthesizer and placed a dehumidifer inside. The increase in coupling efficiency was dramatic.

A final note on humidity and synthesizers: if the synthesizer has been sitting idle for a while, it generally takes some time before it is fully dried out. Generally, the first oligos synthesized are not of stellar quality. However, as the lines in the machine dry and become truly anhydrous, the coupling efficiency will rise - especially after a fresh set of phosphoramidites is installed.

CAPPING STEPS

While it may not be readily apparent, it is critical to maintain a high capping efficiency when synthesizing long oligos. The reasons are two-fold:

1. There is the practical concern of post-synthesis purification. When the capping efficiency is low, deletion mutants begin to build up to very significant levels. These give rise to the 'n-1' deletion mutant, which is actually a population of n-1mers, with the missing base scattered throughout the sequence. This impurity is very difficult to remove as it has a DMT group just as the full-length oligo.
2. It seems that having more efficient capping helps dry the support for the next coupling step. This is quite apparent with the Expedite 8909 synthesizers, which use a CAP/OX/CAP cycle.

Certain synthesizers are less efficient at capping than others. Our in-house work indicates that an ABI 394 caps total failures (generated by injecting ACN instead of monomer solution) with about a 97% efficiency, while an Expedite 8909 only caps at 90% efficiency in these tests. Part of this disparity is due to the concentration of N-methylimidazole in their respective Cap B mixes. For an ABI, it is normally a16% solution, whereas it is 10% for an Expedite. Indeed, we found that when 10% N-methylimidazole mix is used on the ABI 394, the capping efficiency drops to 89%. As a result, we recommend increasing the delivery of the Cap A/B mix on Expedites by 50% (going from 8 to 12 pulses) as well as increasing the time interval by 50% (going from 15 to 22 seconds).

The most efficient capping reagent though is 6.5% DMAP solution used for Cap B. When this is used on an ABI 394, the capping efficiency jumps to > 99%. While early work suggested that capping with DMAP could lead to a fluorescent adduct (ABI Nucleic Acid Research News, 7, Oct 20, 1988), we have never observed this side-reaction.

SUPPORTS

The supports themselves are critical to maintaining a high coupling efficiency throughout the sequence. It is for that reason that we report the recommended synthesis length of every batch of support.

TABLE 1: MOISTURE CONTROL WHILE DISSOLVING PHOSPHORAMIDITES

1. *Fill the syringe with argon and insert the needle through the ACN diluent bottle septum while keeping the crimp on the diluent bottle. Invert the diluent bottle and then inject portions of the argon into the diluent bottle, releasing when the backpressure becomes apparent so that ACN begins to fill the syringe. Repeat until the ACN has displaced all the argon in the syringe.*
2. *Inject the ACN into the monomer bottle. Use the same displacement technique in reverse, pushing the ACN into the phosphoramidite vial while it is still crimped and releasing the plunger with the needle in the headspace to allow argon to backfill the syringe.*
3. *After repeating the push-release displacement a few times, the ACN will be transferred to the monomer vial without a significant build up of pressure.*

This number reflects the drop-off point of the CPG and indicates when the pores of the suport are beginning to become essentially 'clogged' with nascent DNA strands. This leads to a drop in the coupling efficiency because the reagents are unable to diffuse quickly enough to pass through the nascent DNA strands before the next step in the synthesis cycle. For very long oligos (>100), we generally recommend a 2000 Å support. The difficulty of using 2000 Å CPGs is that they are quite friable, which can lead to 3' base deletions, and have very low loadings (10 - 20 µmoles/g), which means it is generally not possible to synthesize oligos at a 1 umole scale. However, polystyrene (PS) supports are generally also good for long oligonucleotide synthesis and can be a worthy alternative. Indeed, it has been argued that it is easier to make the hydrophobic PS anhydrous prior to the coupling step.

SIDE REACTIONS

Depurination

Even if the coupling efficiency is high and maintained throughout the length of the sequence, there are a number of side reactions that can lead to poor quality oligos. The most prominent of these is depurination. Trichloroacetic acid (TCA), which is the standard acid used in deblock solutions, is quite strong, with a pK_a of approximately 0.7. Detritylation using TCA is quite fast and, for this reason, it is the standard deblocking reagent on most DNA synthesizers. However, TCA is strong enough to protonate the N7 nitrogen of adenosine and guanosine, which can lead to depurination and the formation of abasic sites. Upon deprotection, the abasic sites cleave, leading to the production of DMT-ON species truncated at the 3'-terminus, greatly complicating down-stream purification.

To limit depurination, there are two strategies:

1. Use monomers that are resistant to depurination. The dimethylformamidine protecting group, commonly known as dmf, is electron donating. As such, it quite effectively protects the guanosine from depurination. The difficulty is that the dmf group is not stable enough on adenosine to be used. While more stable alternatives, such as di-n-butylformamidine, have been described, they are rather expensive and difficult to synthesize.
2. Rather than use a more expensive depurination-resistant dA, the alternative is to use a deblocking agent with a higher pK_a. The best choice is dichloroacetic acid (DCA). DCA has a pK_a of 1.5. When using 3% DCA as the deblocking solution, we have not been able to induce depurination in a standard column synthesizer. (Note: When synthesizing on other platforms, such as on a chip, this may not be the case.) The drawback of using DCA is that the rate of detritylation is much slower. To some extent, this is compensated by the use of 3% v/v DCA/DCM, which is actually 4.5% w/v and 1.5 X as concentrated as 3% w/v TCA/DCM. Regardless, we recommend at least doubling the delivery of deblock when going from TCA to DCA since incomplete DMT removal will also lead to deletion mutations. With the increased delivery of DCA, we have not observed any appreciable depurination.

GG Dimer addition

The activators used in DNA synthesis are mild organic acids. They protonate the nitrogen of the phosphoramidite, leading to the highly reactive tetrazolide intermediate. However, because these activators are acidic, they can remove a small percentage of the 5'-DMT from the dG phosphoramidite during the coupling step, which, in turn, can react with activated dG phosphoramidite. This leads to the formation of a GG dimer and its subsequent incorporation in the sequence. Over the large number of couplings in the synthesis of long oligonucleotides, this can lead to a significant n+1 peak. As with the n-1 impurity, this too is DMT-ON and difficult to separate from the full-length oligo. The reason that GG dimer addition is seen rather than AA or TT is that guanosine detritylates faster than the other bases and hence leads to more dimer formation. To minimize this side reaction, strongly acidic activators such as BTT which has a pK_a of 4.1, and ETT (pK_a 4.3) should be avoided. Probably the best activator is DCI. While a strong activator, its pK_a is 5.2 – even less acidic than tetrazole. DCI is a much better nucleophile than the tetrazole derivatives and this compensates for its lower acidity.

N3 Cyanoethylation

Alkylation of the N-3 position on thymidine can occur from the reaction of acrylonitrile (which is produced *in situ* when the cyanoethyl protecting group is eliminated) with thymidine during ammonia deprotection. It is especially noticeable when synthesizing very long oligonucleotides. The result is in an impurity that runs on reverse phase HPLC like an n+1 peak. It is easily recognized by mass spectrometry as a +53 Da species. This side reaction can be minimized by using a larger volume of ammonia when cleaving the oligo or using AMA, since methylamine is better at scavenging acrylonitrile. However, this side reaction can be eliminated completely by treating the column after the synthesis is completed with a solution of 10% diethylamine (DEA) in acetonitrile prior to cleavage in ammonia. Typically, the column if fitted with a syringe and a few milliliters of the DEA solution are pushed through slowly over a 5 minute period. Alternatively, the synthesizer can be set up with the DEA solution on one of the additional ports. In this way, the DEA rinse can be automated by writing a custom end procedure.

PURIFICATION

Purification difficulties can not be considered a side reaction *per se*, but we would be remiss not to mention that the purification of long oligos can pose unique problems. Traditional "OPC-type" purification cartridges do not purify long oligos very well since the DMT group is swamped by the large number of charged phosphate groups in the backbone. Recent improvements have included high affinity fluorous purification of long oligos and specially designed packing with a high affinity for DMT such as Glen-Pak™ DNA cartridges.

However, the longer the oligo, the greater is the probability of the formation of unusual secondary structures. We have found, even for cartridges like Glen-Pak cartridges, that these sequences are problematic and give poor yields. However, by heating the crude DMT-ON oligo in the loading buffer to >65 °C just prior to loading on to the Glen-Pak cartridge, the yield of purified oligo increased dramatically, as did the purity of the oligonucleotide.

(Continued from Front Page)

damaged nucleosides, e.g., 8-oxo-dG, were detected in these cells.[5] Nevertheless, 5-hydroxymethyl-dC has been detected in bacteria[9] and it is certainly feasible that it could still be a product of oxidative damage in mammalian cells.

With a growing audience for all aspects of 5-hydroxymethyl-dC activity, it seems useful to offer this nucleoside as a phosphoramidite. The protection scheme described by Sowers[9] has performed well for oligonucleotide synthesis in our hands. With benzoyl protection of the 5-hydroxymethyl-dC residue, this monomer is not compatible with methylamine deprotection or UltraMild chemistry. However, deprotection is clean and complete with ammonium hydroxide at ~~65°C overnight~~ 75°C for 17 hours.

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ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
5-Hydroxymethyl-dC-CE Phosphoramidite	10-1062-95	50 µmole	335.00
	10-1062-90	100 µmole	650.00
	10-1062-02	0.25g	1675.00

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22825 DAVIS DRIVE
STERLING, VIRGINIA
20164