

# The Glen Report

2 2 8 2 5 D A V I S D R I V E      S T E R L I N G , V I R G I N I A      2 0 1 6 4



**GLEN RESEARCH**

**VOLUME 22**

**NUMBER 1**

**MAY 2010**

**I N S I D E**

**5C FLUORESCENT PROBES**

**NEW SUPPORTS**

**NEW PHOSPHoramIDITES**

**α-TOCOPHEROL**

**DEPROTECTION 4**

**GLEN-PAK™ PURIFICATION**

## SIMPLE OLIGONUCLEOTIDE MODIFICATION USING CLICK CHEMISTRY

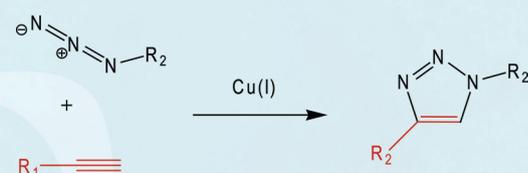
### INTRODUCTION

For several years, Glen Research has been offering a modest selection of products for click chemistry. However, we have had a few concerns about the technology and we have been reluctant to make a more enthusiastic commitment. Our main concern was strictly practical. In the absence of commercially available and click-tested solutions, the performance of the click reactions was quite variable. In fact, we were very concerned that poor performance in customers' hands would lead to the early demise of this very promising technology. We believe that there are simple solutions to this situation and we are now pleased to give this technology our full attention, as discussed below.

#### *baseclick*

Our collaboration with baseclick GmbH has removed our concerns and we now enthusiastically endorse click chemistry. We also expect that it will rapidly become the premiere method for oligonucleotide conjugation. In the accompanying article from baseclick researchers, you will see some representative samples of high efficiency click conjugations. Look to Example 3 to see the results of conjugating an oligonucleotide with biotin at 5 positions. With no purification, the oligonucleotide is essentially 100% pure. The results by conjugating an oligonucleotide at 5 amine positions with biotin NHS ester would be substantially inferior and a difficult purification would be required.

### COPPER(I)-CATALYZED AZIDE-ALKYNE CYCLOADDITION



An additional benefit of click chemistry is that conjugations can be done on solid phase on the synthesis column so that excess expensive azide tags can be recovered prior to oligonucleotide deprotection.

One key ingredient of our collaboration with baseclick is the reagents necessary for successful click reactions. We now offer baseclick grade Cu(I) solutions as well as the Cu(I)-stabilizing ligand required for optimal click condensation reactions.

### SINGLE TO TRIPLE CLICK REACTIONS

More research-oriented examples of baseclick ingenuity can be imagined from the description of click-click and click-click-click conjugations. Using nucleoside alkyne derivatives with and without protecting groups allows up to three separate and independent click reactions to be done, always with the same high efficiency. Try that with a mixture of amino- and thiol-modifiers!

We are delighted to offer the click products described in the following article in collaboration with baseclick GmbH, Tutzing, Germany.

*(Continued on Page 2)*

## THE COPPER(I)-CATALYZED AZIDE-ALKYNE CYCLOADDITION (CUAAC)

Authors: Antonio Manetto, Simon Warncke and Thomas Frischmuth

Address: baseclick GmbH, Bahnhofstrasse 9 – 15, 82327 Tutzing, Germany, email: info@baseclick.eu

Note: All products of baseclick are patent protected and available from Glen Research Corporation, 22825 Davis Drive, Sterling, VA 20164, USA, email: support@glenres.com, in collaboration with baseclick.

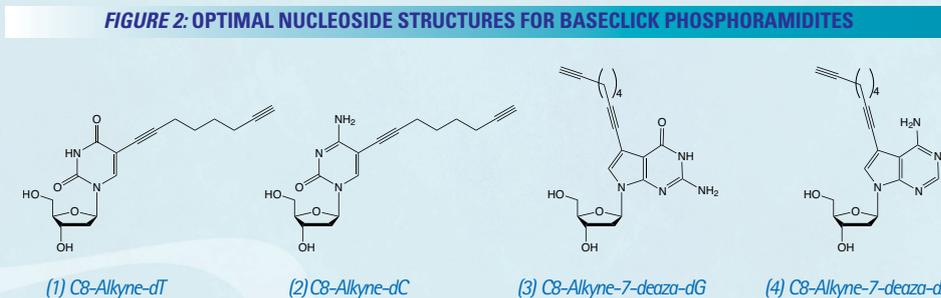
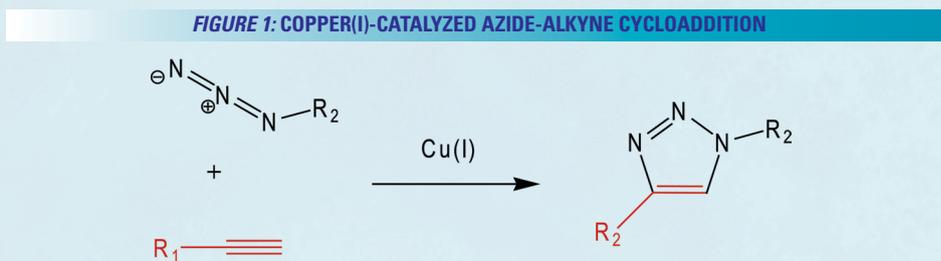
### INTRODUCTION

The copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) is the most prominent example of a group of reactions named click-reactions (Figure 1, Page 2). According to Sharpless's definition, these reactions are characterized by high yields, mild reaction conditions, and by their tolerance of a broad range of functional groups.<sup>1</sup> Typically, the reactions require simple or no workup, or purification of the product. The most important characteristic of the CuAAC reaction is its unique bio-orthogonality, as neither azide nor terminal alkyne functional groups are generally present in natural systems.

The use of this method for DNA modification has been somewhat delayed by the fact that copper ions damage DNA, typically yielding strand breaks.<sup>2</sup> As these problems have now been overcome by the use of copper(I)-stabilizing ligands (e.g., tris(benzyltriazolylmethyl)amine, TBTA<sup>3</sup>), Carell *et al.* and Seela *et al.* discovered that the CuAAC reaction can be used to functionalize alkyne-modified DNA nucleobases with extremely high efficiency.<sup>4</sup>

### BASECLICK PHOSPHoramidites

It has been shown that the 5-position of pyrimidine and the 7-position of 7-deazapurine nucleosides are the ideal positions to introduce functionalities, as these sites lie in the major groove of the



DNA providing steric freedom. In order to enable efficient click-chemistry labelling of alkyne modified oligonucleotides, our nucleosides provide a 5-(octa-1,7-diynyl) side chain. Phosphoramidites of nucleosides 1–4 (Figure 2, Page 2) were shown to be incorporated into DNA oligomers by solid-phase synthesis with excellent coupling efficiency (e.g., 1: > 99 %). Another feature of the octadiynyl side chain is its stabilizing effect on DNA duplexes (e.g., 1:  $T_m$  increase of 1–2 °C).

### CLICK-REACTION ON OLIGONUCLEOTIDES

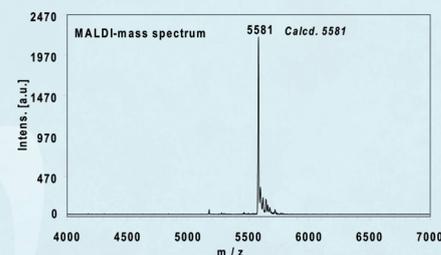
#### Single labelling:

Purified oligonucleotides bearing a single alkyne moiety are usually modified with 2–5 equivalents of the corresponding marker-azide (e.g., fluorescent-dye azides). After the addition of precomplexed Cu(I), complete conversion to the labeled oligo is observed in a time span between 30 min and 4 hours. After a simple precipitation step, labeled oligonucleotides can be recovered in near quantitative yields. On this and the following page, some examples of MALDI-mass spectra measured directly after the click reaction and the precipitation step, without further

purification are presented.

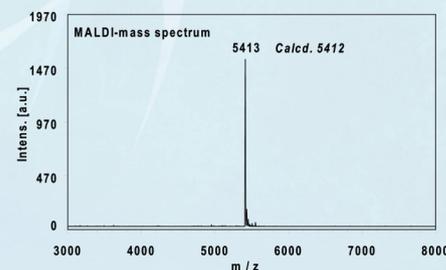
#### Example 1:

16mer, internal alkyne reacted with 2 equivalents Eterneon-(480/635)-Azide, 3 h at 37 °C. Ethanol precipitation with 98% recovery of the labelled oligo. MALDI-mass analysis of the crude product → 100% oligo-dye conjugate.



#### Example 2:

16mer, internal alkyne reacted with 2 equivalents Fluorescein-Azide (FAM-Azide), 3 h at 37 °C. Ethanol precipitation with 99% recovery of the labelled oligo. MALDI-mass analysis of the crude product → 100% oligo-dye conjugate.

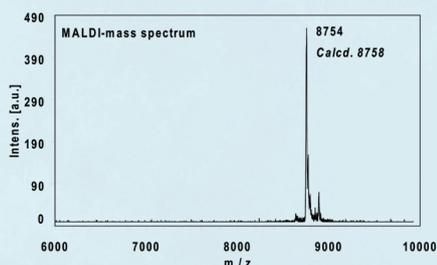


### Multiple labelling:

The Cu(I)-catalyzed Huisgen reaction enables the multiple post synthetic labelling of alkyne modified DNA as well. Complete high-density functionalization of several alkyne moieties can be achieved without the formation of by-products.

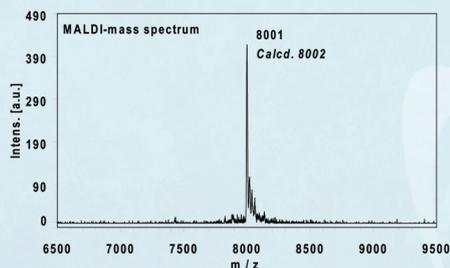
#### Example 3:

22mer, five internal alkynes reacted with 5 equivalents Biotin-Azide, 4 h at 37 °C. Ethanol precipitation with 99% recovery of the labelled oligo. MALDI-mass analysis of the crude product → 100% oligo-dye conjugate.



#### Example 4:

22mer, five internal alkynes reacted with 5 equivalents PEG-Azide, 4 h at 37 °C. Ethanol precipitation with 86% recovery of the labelled oligo. MALDI-mass analysis of the crude product → 100% oligo-dye conjugate.



#### Example 5:

22mer, five internal alkynes reacted with 5 equivalents Eterneon-(350/430)-Azide, 4 h at 37 °C. Ethanol precipitation with 85% recovery of the labelled oligo. MALDI-mass analysis of the crude product → 100% oligo-dye conjugate.

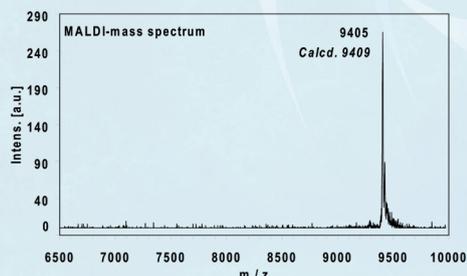
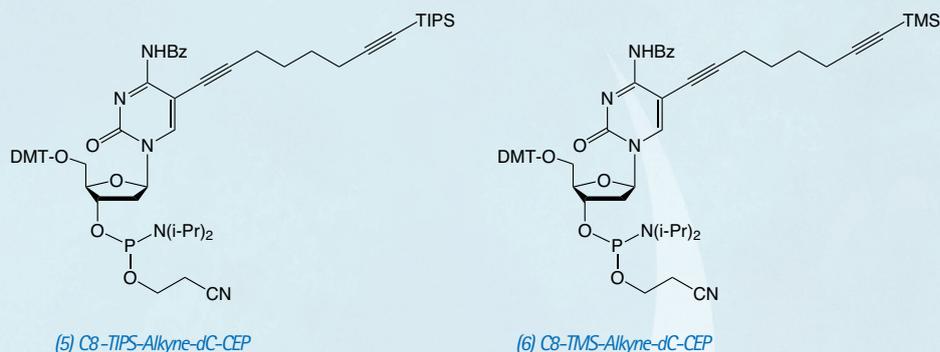


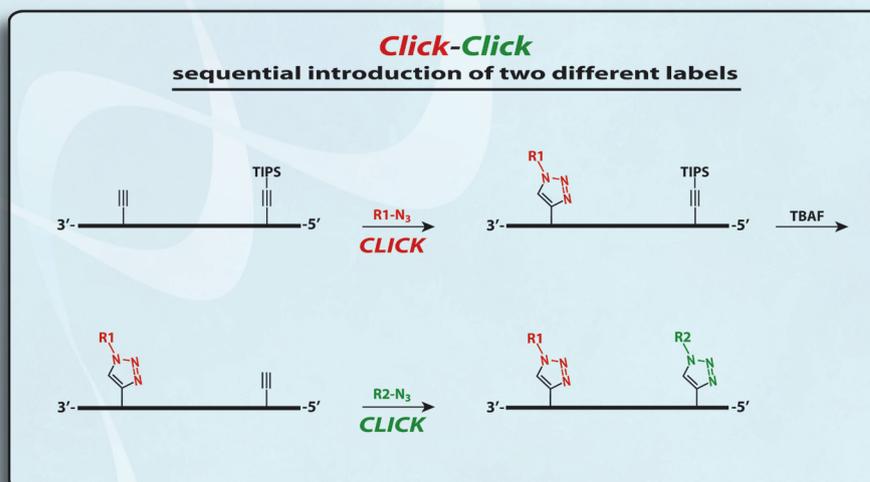
FIGURE 3: BASECLICK PHOSPHORAMIDITES FOR MULTIPLE CLICK REACTIONS



### MULTIPLE SEQUENTIAL LABELLING WITH UP TO THREE DIFFERENT MARKER AZIDES

For the attachment of up to three different labels, phosphoramidites 5 and 6 have been developed. The alkyne groups are protected, respectively, with triisopropylsilyl (TIPS) and trimethylsilyl (TMS) protecting groups.<sup>5</sup>

In order to modify oligonucleotides with two sensitive molecules, nucleosides 1 and 5 are incorporated into DNA strands using standard phosphoramidite chemistry. The first click reaction yields the singly modified oligonucleotide with full retention of the TIPS protecting group. For the second click, the TIPS protecting group is cleaved with tetrabutylammonium fluoride (TBAF) without causing any damage to the DNA. The second click reaction in solution yields the doubly modified oligonucleotides in excellent yields (60–90% over three steps, see picture below).



For the introduction of three different labels, nucleosides 1, 5 and 6 are introduced into oligonucleotides. The first click reaction is performed directly on the resin. The singly modified oligonucleotide is subsequently cleaved from the support under concomitant cleavage of the TMS group and retention of the TIPS protecting group. The second click reaction is performed in solution. Precipitation of the doubly modified oligonucleotide, cleavage of the TIPS group with TBAF, and a subsequent third click reaction in solution furnishes the desired triply modified oligonucleotides in excellent overall yields (see picture on next page).

## CONCLUSION:

In comparison to the common post synthetic labelling methods of oligonucleotides like amine/NHS-ester, thiol/iodoacetamide or maleimide labelling, modification of oligonucleotides with click chemistry is providing by far the highest conjugation efficiency.<sup>6</sup> Single and multiple labelling can be performed with as little as two equivalents of label-azides resulting in complete conversion and high yields of labeled oligo. Alkyne-modified nucleoside phosphoramidites are incorporated into DNA strands during solid-phase synthesis in excellent yields, even stabilizing the DNA-duplexes. In addition, the label-azides used for click functionalization are stable to hydrolysis (in contrast to sensitive NHS esters and maleimides) and excess amounts can be recovered after the click reaction.

The easy-to-use copper(I)-catalyzed azide-alkyne cycloaddition reaction with its outstanding selectivity is in an excellent position to take over as the state-of-the-art methodology to label and modify DNA and nucleic acids in general.

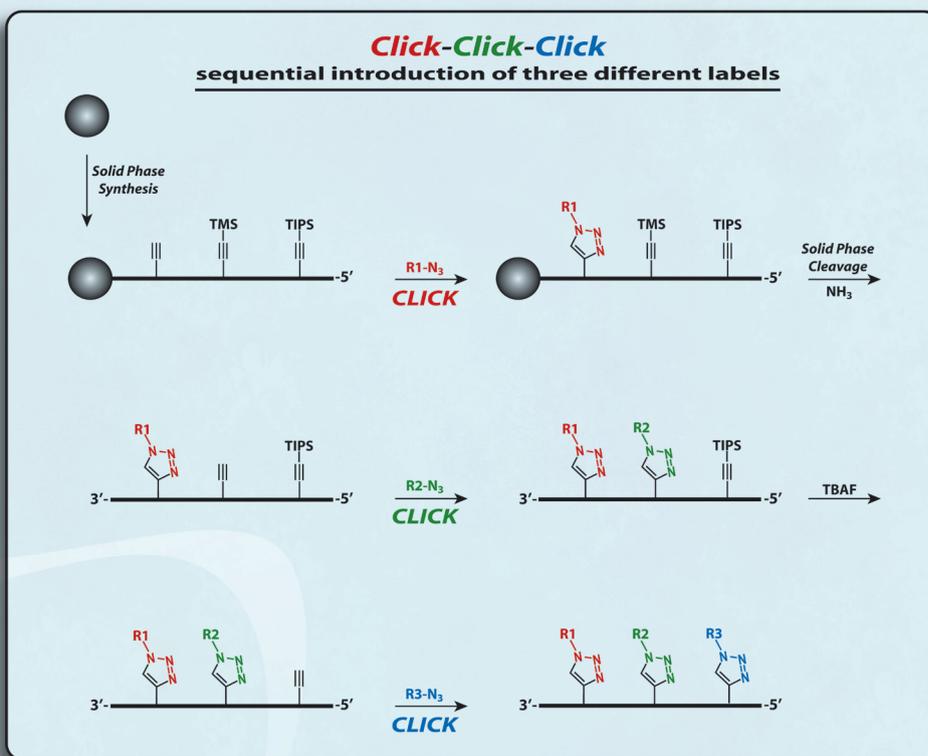
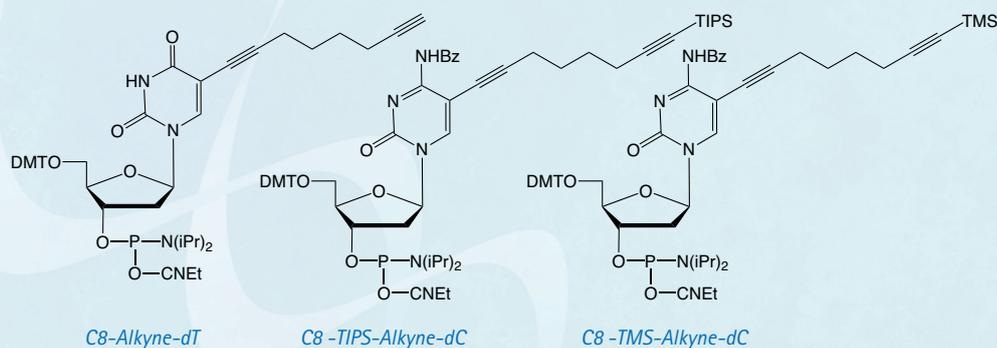


FIGURE 4: BASECLICK PRODUCTS FROM GLEN RESEARCH



## ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
C8-Alkyne-dT-CE Phosphoramidite	10-1540-95	50 µmole	165.00
	10-1540-90	100 µmole	315.00
	10-1540-02	0.25g	900.00
C8-TIPS-Alkyne-dC-CE Phosphoramidite	10-1541-95	50 µmole	295.00
	10-1541-90	100 µmole	575.00
	10-1541-02	0.25g	1275.00
C8-TMS-Alkyne-dC-CE Phosphoramidite	10-1542-95	50 µmole	inquire
	10-1542-90	100 µmole	
	10-1542-02	0.25g	
CuBr (baseclick grade)	50-1000-50	10 x 5mg	200.00
TBTA-Ligand (baseclick grade)	50-1001-10	10mg	100.00
Click-Solution (DMSO/t-BuOH)	50-1002-11	10 x 1.0mL	185.00

## References:

- [1] C.W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* 2002, **67**, 3057-3064; V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem.* 2002, **114**, 2708-2711; *Angew. Chem. Int. Ed.* 2002, **41**, 2596-2599.
- [2] C. J. Burrows, J. G. Muller, *Chem. Rev.* 1998, **98**, 1109 – 1151.
- [3] T. R. Chan, R. Hilgraf, K. B. Sharpless, V. V. Fokin, *Org. Lett.* 2004, **6**, 2853 – 2855.
- [4] J. Gierlich, G. A. Burley, P. M. E. Gramlich, D. M. Hammond, T. Carell, *Org. Lett.* 2006, **8**, 3639-3642. F. Seela, V. R. Sirivolu, *Chem. Biodiversity* 2006, **3**, 509-514.
- [5] P. M. E. Gramlich, S. Warncke, J. Gierlich, T. Carell, *Angew. Chem.* 2008, **120**, 3491–3493; *Angew. Chem. Int. Ed.* 2008, **47**, 3442–3444.
- [6] P. M. E. Gramlich, C. T. Wirges, A. Manetto, T. Carell, *Angew. Chem. Int. Ed.* 2008, **47**, 8350-8358.

## Intellectual property rights:

Baseclick GmbH has filed the following patent applications:

1. WO2006/117161, New labelling strategies for the sensitive detection of analytes
2. WO2008/952775, Click Chemistry for the production of reporter molecules

Baseclick GmbH holds a worldwide license for the research market of the "Click Chemistry" patent from "The Scripps Research Institute":

3. WO03/101972, Copper-catalysed ligation of azides and acetylenes

## tC, tC<sup>o</sup> AND tC<sub>nitro</sub>: TRICYCLIC CYTOSINE PROBES OF DNA AND RNA STRUCTURE

### INTRODUCTION

The sensitivity in quantum yield to immediate surroundings is a common feature for the fluorescent base analogues that were developed - until very recently. This sensitivity normally gives rise to considerable changes in quantum yield (up to 100 times). These changes can successfully be used for monitoring, for example, DNA hybridization/melting, nucleic acid conformational changes and nucleic acid-protein interactions. Glen Research offers important fluorescent base analogues, such as 2-aminopurine<sup>1</sup> and pyrrolo-C<sup>2</sup>, that are sensitive to their microenvironment. Now, with technical and scientific support from ModyBase HB, we have added to our catalog two new fluorescent base analogues, tC and tC<sup>o</sup> (Figure 1), which are virtually insensitive to their microenvironment. These analogues were developed by the researchers that founded ModyBase HB.

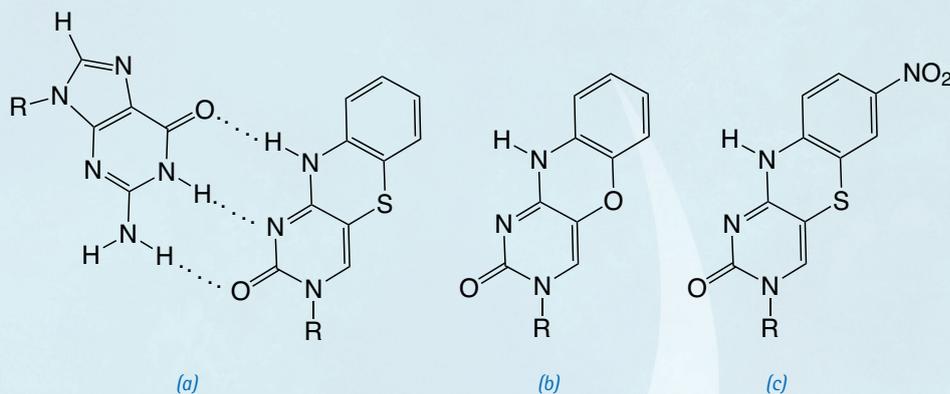
### PHYSICAL PROPERTIES OF tC-FAMILY

In contrast to previous fluorescent base analogues, the tC products have been shown to be virtually insensitive to their immediate surroundings in single- and double stranded (tC) and double stranded systems (tC<sup>o</sup>), respectively.<sup>3-5</sup> It has been shown in extensive investigations that these fluorophores work excellently as analogues of cytosine and only give minor perturbations to the overall structure of the nucleic acids they are incorporated into.<sup>4-7</sup> For example, they leave duplex DNA in the natural B-form and increase the T<sub>m</sub> of 10-mers by on average 3 °C.<sup>4-6</sup>

The spectral properties of tC ( $\lambda_{exc}=395$  nm,  $\lambda_{em}=505$  nm) and tC<sup>o</sup> ( $\lambda_{exc}=365$  nm,  $\lambda_{em}=455$  nm) have been comprehensively investigated (Figure 2).<sup>4-5</sup> Interestingly and surprisingly, both tC and tC<sup>o</sup> have, as mentioned above, virtually insensitive quantum yields in double stranded DNA ranging from 0.16 to 0.21 and 0.17 to 0.27 (Figure 3, Page 6), respectively.<sup>4-5</sup> Furthermore, there is basically no change when going from single- ( $\phi_f=0.17-0.24$ ; Figure 3, Page 6), to double-stranded systems containing tC.<sup>4</sup> Moreover, the fluorophores display single lifetimes (tC<sub>duplex</sub> ≈ 6.3 ns, tC<sub>ss</sub> ≈ 5.7 ns, tC<sup>o</sup><sub>duplex</sub> ≈ 4.1 ns) under the conditions mentioned.<sup>4-5</sup>

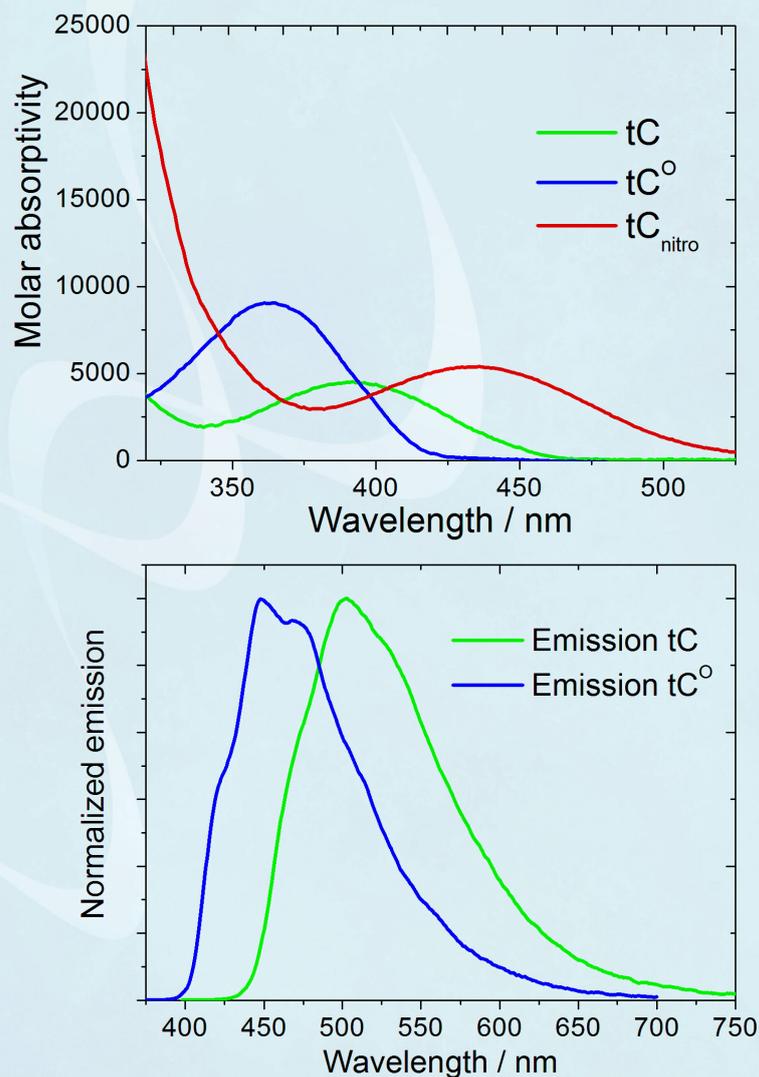
These properties of tC and tC<sup>o</sup> in

FIGURE 1: TRICYCLIC CYTOSINE ANALOGUE STRUCTURES



Tricyclic cytosine analogues: (a) guanine base-paired with tC, (b) tC<sup>o</sup>, and (c) base analogue FRET-acceptor tC<sub>nitro</sub>. R = (deoxy)ribose.

FIGURE 2: SPECTRAL PROPERTIES OF tC, tC<sup>o</sup> AND tC<sub>nitro</sub>



Absorption and fluorescence properties of tC, tC<sup>o</sup> and tC<sub>nitro</sub> (tC<sub>nitro</sub> is virtually non-fluorescent under normal conditions).

combination with the high control of their orientation and position within the nucleic acid base stack make them unique among fluorescent base analogues and particularly well-suited for fluorescence resonance energy transfer (FRET) and fluorescence anisotropy experiments. As will be described in detail below, these properties are vital in accurately determining the Förster distance ( $R_0$ ) and, thus, also for the precision of distance ( $R_{DA}$ ) estimates using FRET. Moreover, a single lifetime and bright emission, in combination with a firm stacking and a low rate of base-flipping, are crucial properties for detailed studies of the local dynamics of nucleic acids using fluorescence anisotropy.<sup>5-6</sup>

### tC FRET-PAIRS

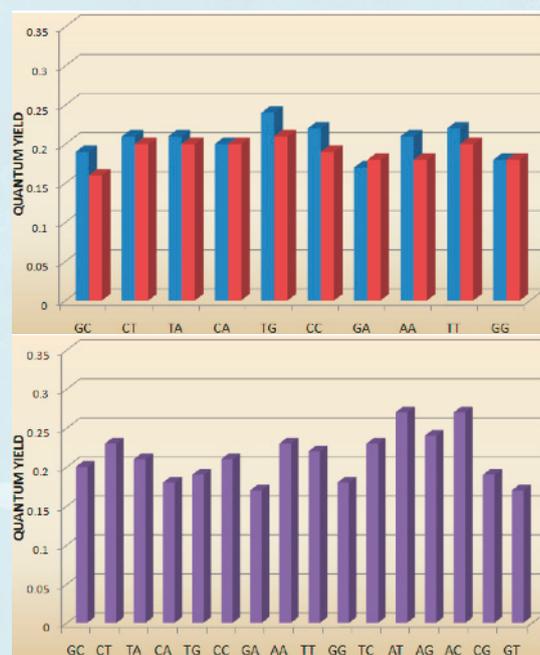
In what may be the largest step forward in the development of fluorescent base analogues since 2-aminopurine was presented in the late 60s,<sup>1</sup> the base analogue tC<sub>nitro</sub> (Figure 1) was presented as a FRET-acceptor in 2009 together with tC<sup>0</sup> (or tC) as the donor molecule.<sup>8</sup> This constitutes the first ever description of a nucleobase FRET-pair. Thorough examination of the photophysical properties (Figure 2), including spectral resolution into distinct transition dipole moments, has also been performed to allow future detailed nucleic acid structure determinations using FRET.<sup>9</sup>

As discussed above, tC<sup>0</sup> (and tC) have several properties that make them ideally suited as FRET-donors in nucleic acid systems:

- 1) a high and virtually unaffected quantum yield ( $\phi_D$ );
- 2) stable spectral envelopes ( $J_{DA}$ );
- 3) a high control of orientation and position within the nucleic acid ( $\kappa^2$ );
- 4) give minimal perturbation to the structure of the nucleic acid; and
- 5) provide the possibility to have the probe close to the site of examination.

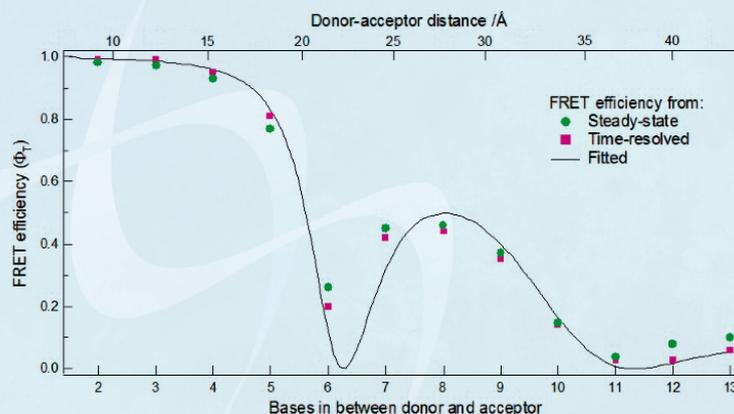
The first three properties are important in order to have a high control of the Förster distance ( $R_0=0.211(J_{DA}\kappa^2n^{-4}\phi_D)^{1/6}$  in Å), which in turn enables very accurate distance determinations ( $R_{DA}$ , distance between the donor and acceptor) using FRET. This novel FRET-pair provides a unique tool for investigations of nucleic acid containing systems. It also makes it possible to study nucleic acid structure and to monitor

FIGURE 3: QUANTUM YIELD VARIATION OF tC AND tC<sup>0</sup> IN SINGLE AND DOUBLE STRANDED DNA



Fluorescence quantum yield of tC in DNA single- (blue) and double-stranded (red) systems (top) and of tC<sup>0</sup> in DNA double-stranded systems (bottom). Letters denote bases surrounding tC (5'...X(tC<sup>0</sup>)Y...-3').

FIGURE 4: EFFICIENCY OF ENERGY TRANSFER FOR THE FRET PAIR tC<sup>0</sup>-tC<sub>nitro</sub>



Efficiency of energy transfer for the base analogue FRET-pair tC<sup>0</sup>-tC<sub>nitro</sub> estimated using decreases in tC<sup>0</sup> emission (green circles) and tC<sup>0</sup> average lifetimes (purple squares) as the two analogues are separated by 2 to 13 bases in a DNA duplex.

structural changes with a very high level of detail both in distance and orientation over more than one turn of the DNA helix (see Figure 4).<sup>8-9</sup>

### tC-FAMILY AS STRUCTURAL PROBES

In addition to the interesting properties of tC and tC<sup>0</sup> already mentioned, these fluorophores are on average the brightest fluorescent base analogues when

incorporated into duplex DNA.<sup>5</sup> With a molar absorptivity of 9000 M<sup>-1</sup> cm<sup>-1</sup>, the average brightness of tC<sup>0</sup> is almost double that of tC. Furthermore, compared to other fluorescent base analogues that are highly quenched inside DNA, tC<sup>0</sup> has a brightness that is up to 50 times higher on average in dsDNA. With a quantum yield change reaching approximately a factor of 2 between single and double strands and no influence on T<sub>m</sub> for wisely chosen neighboring bases,

this high brightness makes  $tC^0$  interesting not only in duplex FRET and fluorescence anisotropy measurements but also highly attractive as a probe of nucleic acid secondary structure changes.<sup>10</sup>

The  $tC$ -family has already been utilized in several biophysical, biochemical and biological investigations. In a recent anisotropy study, it was shown that  $tC$  and  $tC^0$  work excellently as probes for directly monitoring motion of nucleic acid helical structures, rather than a combination of motion of the overall structure and the probe itself, as is the case for most of the currently available fluorescent base analogues.<sup>5</sup> Furthermore,  $tC$  has been utilized as a FRET-donor in pair with rhodamine in a PNA-DNA-hybrid,<sup>3</sup> and in pair with Alexa-555 in a study of conformational dynamics of DNA polymerase.<sup>11</sup>

In other polymerase studies, it was shown that the 5'-triphosphates of  $tC$  and  $tC^0$  are efficiently incorporated into DNA by the Klenow fragment although with increased frequency of mutations.<sup>12-13</sup>

In addition to these DNA polymerization investigations, the ribonucleotide form of  $tC$  has been synthesized and tested as a substrate for T7 RNA polymerase.<sup>14</sup> Interestingly, it was found in this study that T7 RNA polymerase does not misincorporate  $tC$  and it was shown that the ribonucleotide form of  $tC$  could replace normal cytosine in a ~800 nucleotide RNA, enabling straightforward fluorescent labelling of long RNAs.

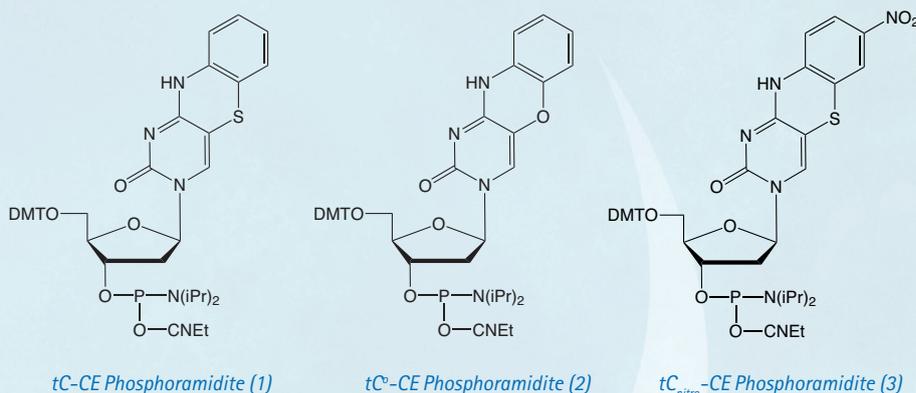
Finally, it has been established that the change in quantum yield of  $tC^0$  between single and double stranded systems can be utilized for the detection of individual melting processes of complex nucleic acid structures.<sup>10</sup>

## CONCLUSION

Previously, the use of the  $tC$ -family of molecules was possible only because  $tC/tC^0/tC_{nitro}$  were synthesized in the labs of individual researchers. Glen Research is proud to offer these products for sale and now make it possible for the whole research society to use these interesting probes.

We thank Marcus Wilhelmsson, Department of Chemical and Biological Engineering/Physical Chemistry, Chalmers University of Technology, Göteborg, Sweden for his immense help in preparing this article.

FIGURE 5: TRICYCLIC FLUORESCENT CYTIDINE ANALOGUE PHOSPHORAMIDITES



## References:

1. Ward, D.C., Reich, E., Stryer, L. *Journal of Biological Chemistry*, 1969, **244**, 1228-1237.
2. Berry, D.A., Jung, K.Y., Wise, D.S., Sercel, A.D., Pearson, W.H., Mackie, H., Randolph, J.B., Somers, R.L. *Tetrahedron Letters*, 2004, **45**, 2457-2461.
3. Wilhelmsson, L. M., Holmén, A., Lincoln, P., Nielsen, P. E., Nordén, B. *Journal of the American Chemical Society*, 2001, **123**, 2434-2435.
4. Sandin, P., Wilhelmsson, L.M., Lincoln, P., Powers, V.E.C., Brown, T., Albinsson, B. *Nucleic Acids Research*, 2005, **33**, 5019-5025.
5. Sandin, P., Börjesson, K., Li, H., Mårtensson, J., Brown, T., Wilhelmsson, L.M., Albinsson, B. *Nucleic Acids Research*, 2008, **36**, 157-167.
6. Engman, K.C., Sandin, P., Osborne, S., Brown, T., Billeter, M., Lincoln, P., Nordén, B., Albinsson, B., Wilhelmsson, L.M. *Nucleic Acids Research*, 2004, **32**, 5057-5095.
7. Lin, K., Jones, R.J., Matteucci, M. *Journal of the American Chemical Society*, 1995, **117**, 3873-3874.
8. Börjesson, K., Preus, S., El-Sagheer, A.H., Brown, T., Albinsson, B., Wilhelmsson, L.M. *Journal of the American Chemical Society*, 2009, **131**, 4288-4293.
9. Preus, S., Börjesson, K., Kilså, K., Albinsson, B., Wilhelmsson, L.M. *Journal of Physical Chemistry B*, 2010, **114**(2), 1050-1056.
10. Börjesson, K., Sandin, P., Wilhelmsson, L.M. *Biophysical Chemistry*, 2009, **139**, 24-28.
11. Stengel, G., Gill, J.P., Sandin, P., Wilhelmsson, L.M., Albinsson, B., Nordén, B., Millar, D. *Biochemistry*, 2007, **46**(43), 12289-12297.
12. Sandin, P., Stengel, G., Ljungdahl, T., Börjesson, K., Macao, B., Wilhelmsson L.M. *Nucleic Acids Research*, 2009, **37**(12), 3924-3933.
13. Stengel, G., Purse, B.W., Wilhelmsson, L.M., Urban, M., Kuchta, R.D. *Biochemistry*, 2009, **48**(31), 7547-7555.
14. Stengel, G., Urban, M., Purse, B.W., Kuchta, R.D. *Analytical Chemistry*, 2010, **82**, 1082-1089.

These products are offered under license from Isis Pharmaceuticals, Inc. and in collaboration with ModyBase HB.

## ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
$tC$ -CE Phosphoramidite	10-1516-95	50 $\mu$ mole	250.00
	10-1516-90	100 $\mu$ mole	490.00
	10-1516-02	0.25g	1460.00
$tC^0$ -CE Phosphoramidite	10-1517-95	50 $\mu$ mole	250.00
	10-1517-90	100 $\mu$ mole	490.00
	10-1517-02	0.25g	1460.00
$tC_{nitro}$ -CE Phosphoramidite	10-1518-95	50 $\mu$ mole	inquire
	10-1518-90	100 $\mu$ mole	
	10-1518-02	0.25g	

## SACCHARIN 1-METHYLIMIDAZOLE - AN ACTIVATOR FOR DNA AND RNA SYNTHESIS

Glen Research offers a number of activators for oligonucleotide synthesis and, for some applications, the choice of activator can make a significant difference in overall performance (purity and yields). Our newest offering is saccharin 1-methylimidazole (SMI) (Figure 1). SMI is considered a general-purpose activator for DNA and RNA synthesis. We also currently offer 1H-tetrazole, 5-(ethylthio)-1H-tetrazole (ETT), 5-(benzylthio)-1H-tetrazole (BTT), and 4,5-dicyanoimidazole (DCI).

The classic activator, 1H-tetrazole, performs well in DNA synthesis and can be used in RNA synthesis provided the coupling times ( $\geq 12$  minutes) are adjusted accordingly. Solubility limits, long RNA coupling times, as well as regulatory changes, make 1H-tetrazole a less than ideal general-purpose activator.

In previous studies, it was found that activators that were better proton donors (more acidic) or more nucleophilic, improved the overall rate of reaction.<sup>1,2</sup> As ETT and BTT are indeed more acidic than 1H-tetrazole, they are better activators and have been found to reduce the coupling time for RNA synthesis. A risk with acidic activators ( $pK_a < 4.9$ ) is that they have the potential to detritylate the incoming phosphoramidite during long coupling times. This can lead to dimer phosphoramidite formation, causing a dimer coupling that increases the  $n+1$  peaks in the final product and reduces overall product yields. Dimer addition can be minimized by using a shorter coupling time that still meets the required coupling efficiency.

Activators that are more nucleophilic and less acidic, such as DCI, can reduce the amount of dimer addition and improve the overall yields. This improvement can be significant in the synthesis of long oligos but especially for larger scale oligo synthesis where extended coupling times are less important than overall yield.

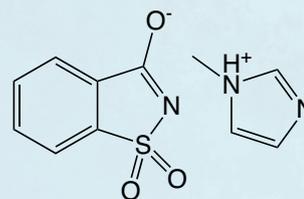
The risk with nucleophilic activators is that they can cause branching from secondary amines. Branching is the initiation of a second oligonucleotide sequence from a secondary site on an oligonucleotide, commonly the secondary amines as found on unprotected biotin, N-Ethyl-dC, and N6-methyl-dA. For SMI, branching occurs at a level similar or slightly less than 4,5-DCI. We have found that branching can occur with all activators.

SMI performs very well for DNA and RNA synthesis. (See results in the table below.) We have evaluated SMI in RNA synthesis using TBDMS phosphoramidites using 3 minute, 6 minute, and 12 minute coupling times. Results indicate that a 6 minute coupling time for SMI outperforms ETT at a 6 minute coupling time. Longer coupling times for SMI can realize even further improvements in RNA coupling efficiencies.

Activator	Type	Coupling	CE (%)
SMI	TBDMS RNA	3 min.	97.3
SMI	TBDMS RNA	6 min.	97.8
SMI	TBDMS RNA	12 min.	97.8
ETT	TBDMS RNA	6 min.	97.1
SMI	DNA	30 sec.	99.6
TET	DNA	30 sec.	99.5

The resulting DMT-ON chromatograms using DNA monomers and tetrazole or SMI with standard 30 second couplings are shown in Figure 2. Similarly, DMT-ON chromatograms using TBDMS RNA

FIGURE 1: STRUCTURE OF SMI



Saccharin 1-methylimidazole (SMI)

monomers and ETT or SMI with 6 minute couplings are shown in Figure 3.

Saccharin 1-methylimidazole is a useful general purpose activator for DNA and RNA synthesis with excellent performance characteristics.

SMI is sold under license from Avecia Biotechnology Inc.

### References:

1. B.H. Dahl, J. Nielsen, and O. Dahl, *Nucleic Acids Res.*, 1987, **15**, 1729-43.
2. S. Berner, K. Muhlegger, and H. Seliger, *Nucleic Acids Res.*, 1989, **17**, 853-64.

FIGURE 2: RP HPLC OF DMT-ON DNA

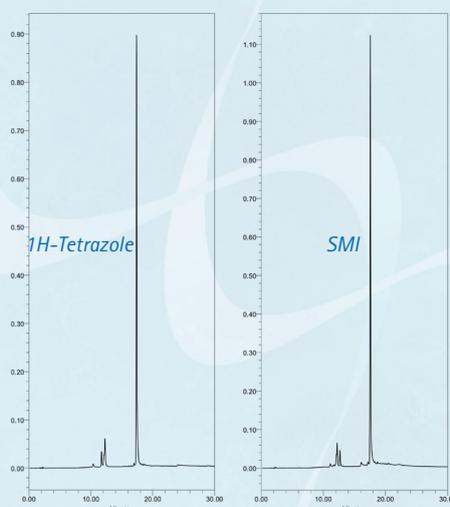
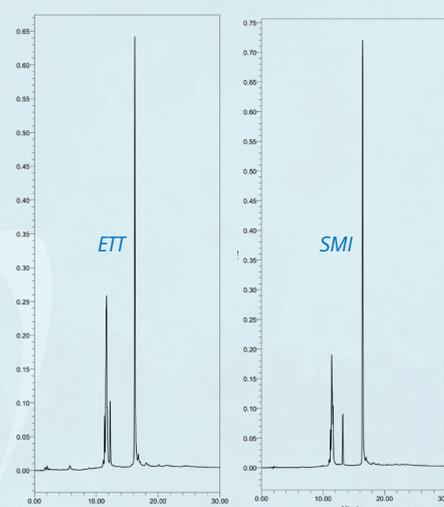


FIGURE 3: RP HPLC OF DMT-ON RNA



### ORDERING INFORMATION

Item	Catalog No.	Pack	Price (\$)
Saccharin 1-Methylimidazole (SMI) (Dissolve 1g in 31mL anhydrous acetonitrile for a 0.2M solution)	30-3080-10	1g	35.00
	30-3080-20	2g	60.00
	30-3080-25	25g	500.00
0.2M Saccharin 1-Methylimidazole (SMI) in Acetonitrile (Applied Biosystems)	30-3180-45	45mL	40.00
	30-3180-52	200mL	100.00
	30-3180-57	450mL	200.00
	30-3180-62	2L	760.00
	30-3182-52	200mL	100.00
	30-3180-57	450mL	200.00

(Expedite)

## NEW PRODUCT - A DG AMINO-MODIFIER WITH IMPROVED HYBRIDIZATION CHARACTERISTICS

In 2006, we introduced Amino-Modifier C6 dG (1) to complete our repertoire of amino-modified base analogs. However, we found that the placement of the linker at the C8 position led to a significant destabilization of the duplex, with the  $T_m$  dropping by 3 °C with a single incorporation. While the analog was still functional and did base pair specifically with dC, the drop in the duplex stability meant it was not a transparent substitution for dG in a sequence.

While working on an unrelated project investigating base analogs that could be used for  $T_m$  leveling (such as N-Ethyl-dC), we found the melting temperature of the C-G base pair to be remarkably insensitive to modifications at the N2 position of dG. For this reason, we decided to place the C6 alkylamine at the N2 position and test the analogue (2) in melting experiments.

The results were quite positive. The sequence 5'-ATC XCT CAT GAT G-3' was synthesized where X = N2-Amino-Modifier C6 dG (2). After deprotection and then trityl-on purification on a Glen-Pak cartridge, the melting curve was determined when annealed to a perfectly matched reverse complement, as well as against a G/A mismatch. These results were compared to a dG control (Figure 2). The melting temperatures of the N2-Amino-Modifier C6 dG and the Control dG oligos were almost identical, with the N2-Amino-Modifier C6 dG having just a slightly higher  $T_m$  than the dG control, as shown in Figure 2. The specificity toward dC by the N2-Amino-Modifier C6 dG is maintained, with the  $\Delta T_m$  ( $T_m$  (match) -  $T_m$  (mismatch)) being 15 °C for the N2-Amino-Modifier C6 dG and 14 °C for the dG control.

As with all the trifluoroacetyl-protected amino-modifiers, we recommend deprotection in AMA to reduce side reactions that can lead to capping of the amine. To maintain conjugation efficiency for amino-modified oligos deprotected in AMA, we recommend desalting the oligo to convert it to a non-nucleophilic salt, such as  $\text{Na}^+$  or  $\text{TEAH}^+$ , prior to conjugation with NHS esters or equivalents.

We are happy to provide this new N2-Amino-Modifier C6 dG to the research community.

FIGURE 1: TRICYCLIC FLUORESCENT CYTIDINE ANALOGUE STRUCTURES

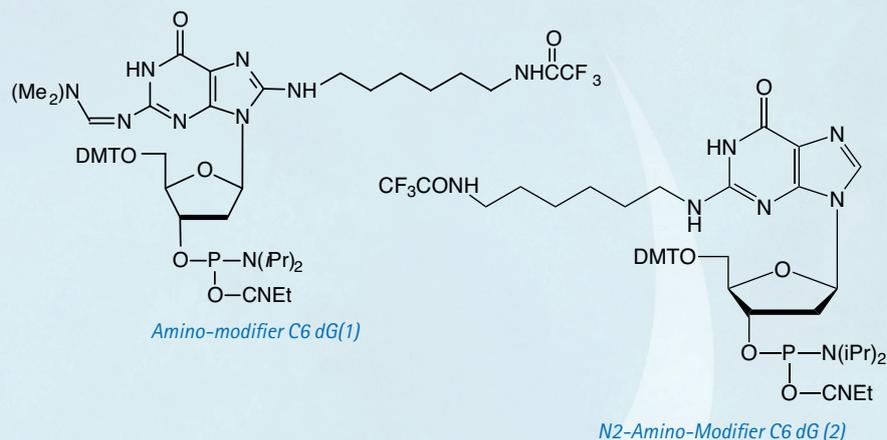


FIGURE 2: MELTING CURVES FOR THE AMINO-MODIFIED DG PROBES

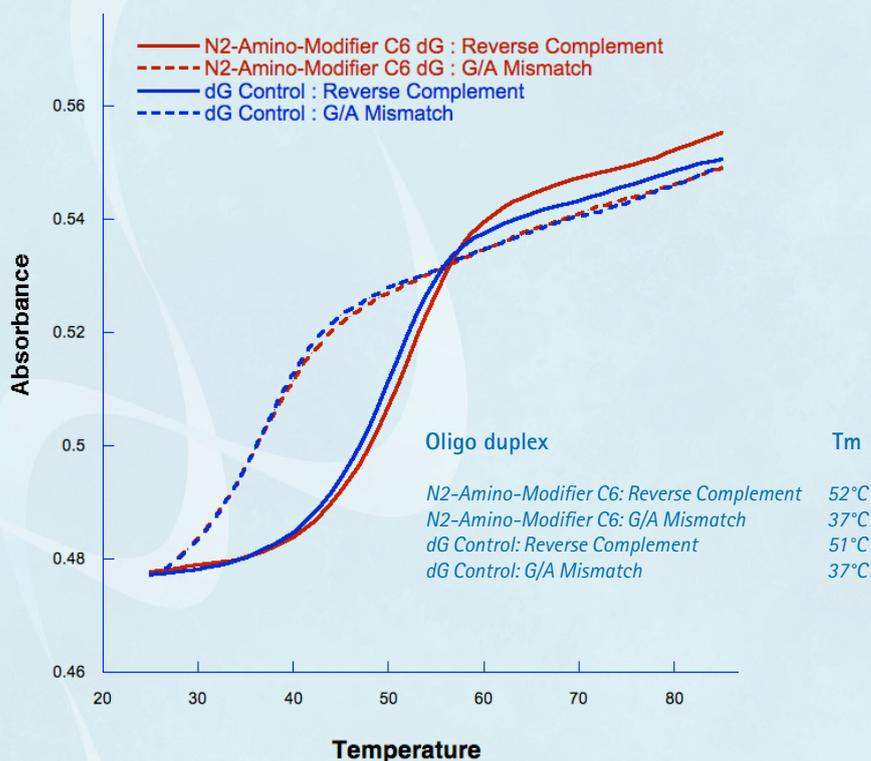


Figure 2: The melting curves for the amino-modified dG probe 5'-ATC XCT CAT GAT G-3', where X = N2-Amino-Modifier C6 dG, when annealed to the perfectly matching reverse complement 5'-CAT CAT GAG CGA T-3' and the G/A mismatch 5'-CAT CAT GAG AGA T-3' at 1.25  $\mu\text{M}$  in 0.1 M Tris-HCl pH 7. These are compared to the dG control probe with the sequence 5'-ATC GCT CAT GAT G-3'.

### ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
N2-Amino-Modifier C6 dG	10-1529-95	50 $\mu\text{mole}$	240.00
	10-1529-90	100 $\mu\text{mole}$	480.00
	10-1529-02	0.25g	1100.00

## NEW PRODUCTS – $\alpha$ -TOCOPHEROL-TEG PHOSPHORAMIDITE AND 3'-THIOL-MODIFIER 6 S-S CPG

### $\alpha$ -TOCOPHEROL-TEG PHOSPHORAMIDITE

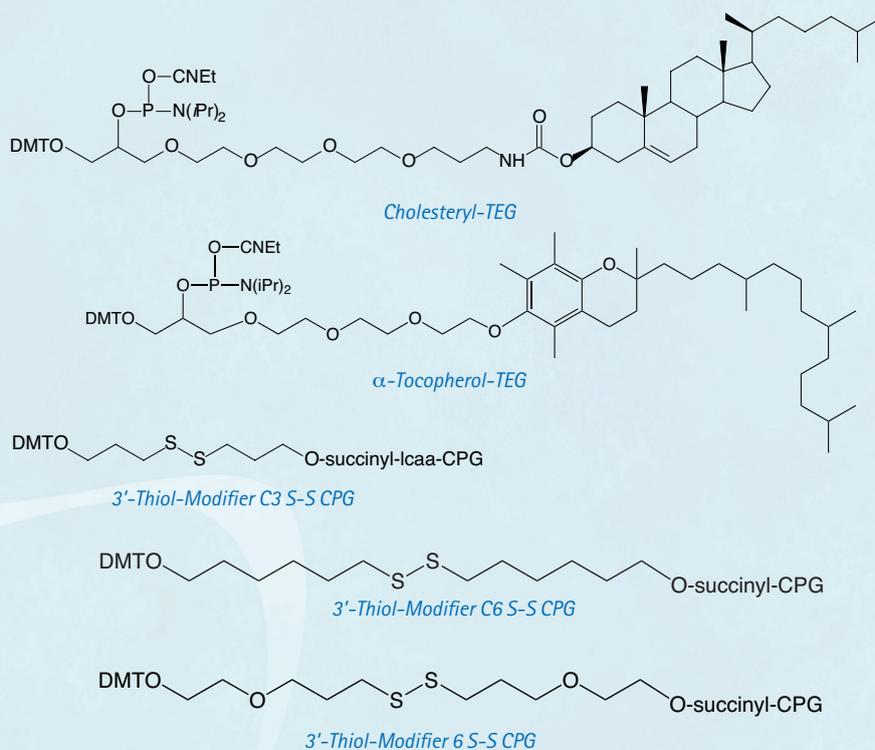
One of the fastest growing segments of the Glen Research product line is our cholesteryl products for adding a lipophilic molecule to oligonucleotides. Cholesteryl modification of oligonucleotides, usually antisense or siRNA, allows them to be delivered more efficiently to the targeted cells. Cholesterol is a very "sticky" molecule with some inherent problems for oligonucleotide synthesis, purification, and transport in biological fluids. Cholesterol is also routinely isolated from animal sources and, as such, is not ideal for therapeutic development. Purely synthetic cholesterol is now available commercially but at a high cost.

Vitamins would be considered to be virtually the ideal carriers for therapeutic oligonucleotides since they are recognized and used by target cells but are not produced by these cells. Vitamin E is both lipophilic and non-toxic even at high doses so would be an excellent candidate as a lipophilic carrier for oligonucleotides. Therefore, as an addition to our cholesteryl product line, we would like to offer simple  $\alpha$ -tocopheryl labelling.  $\alpha$ -Tocopherol is better known as Vitamin E. Even though vitamin E is generally isolated from plant sources, totally synthetic  $\alpha$ -tocopherol is commercially available and is racemic at its three chiral centers.

The use of  $\alpha$ -tocopherol as a lipophilic carrier for ribozymes has been described.<sup>1</sup> In this publication, a 5'-DL- $\alpha$ -Tocopheryl phosphoramidite was used as a lipophilic capture tag with a cleavable linker as a tool for the purification of modified hammerhead ribozymes. In a more recent report,<sup>2</sup>  $\alpha$ -tocopherol was used as a lipophilic carrier for siRNA for efficient delivery to the liver. In this work, the phosphoramidite was prepared by simple phosphitylation of  $\alpha$ -tocopherol.

Over the years, we have developed a clear preference for the use of ethyleneglycol spacers for separating tags from oligonucleotides. These mixed polarity spacers allow the tag to be readily detected without interfering with oligonucleotide function. We are therefore happy to introduce  $\alpha$ -Tocopherol-TEG Phosphoramidite for simple labelling of DNA and RNA oligonucleotides.  $\alpha$ -Tocopheryl

FIGURE 1: STRUCTURES OF  $\alpha$ -TOCOPHEROL-TEG PHOSPHORAMIDITE AND 3'-THIOL-MODIFIER 6 S-S CPG



oligonucleotides can be deprotected using standard procedures.

#### References:

1. B.S. Sproat, T. Rupp, N. Menhardt, D. Keane, and B. Beijer, *Nucleic Acids Res.*, 1999, **27**, 1950-1955.
2. K. Nishina, et al., *Mol Ther*, 2008, **16**, 734-40.

### 3'-THIOL-MODIFIER 6 S-S CPG

During 2008, Glen Research introduced 3'-Thiol-Modifier C6 S-S CPG (20-2936) to supplement the C3 version (20-2933) and the Thiol-Modifier C6 S-S Phosphoramidite (10-1936), which can easily and efficiently be used to produce 3'-thiols. This seemed like an easy addition to our product line

but the reality was quite different. Many batches of 3'-Thiol-Modifier C6 S-S CPG have been produced and rejected for a variety of performance reasons. Even batches that have met our quality criteria failed to perform as well as the C3 version. We suspect that the C6 linker is too hydrophobic to perform at the highest level. Our colleagues at Berry & Associates have come up with a simple change to the structure of this linker that has had a huge impact on performance. The substitution of an O for CH<sub>2</sub> in the linker has elevated the performance of this new version (20-2938) to the highest level that we would expect. We are pleased to offer this product as a direct replacement for 3'-Thiol-Modifier C6 S-S CPG (20-2936).

#### ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)	
$\alpha$ -Tocopherol-TEG Phosphoramidite	10-1977-95	50 $\mu$ mole	160.00	
	10-1977-90	100 $\mu$ mole	300.00	
	10-1977-02	0.25g	575.00	
3'-Thiol-Modifier 6 S-S CPG	20-2938-01	0.1g	85.00	
	20-2938-10	1.0g	600.00	
	0.2 $\mu$ mole columns	20-2938-42	Pack of 4	75.00
	1 $\mu$ mole columns	20-2938-41	Pack of 4	125.00
	10 $\mu$ mole column (ABI)	20-2938-13	Pack of 1	225.00
15 $\mu$ mole column (Expedite)	20-2938-14	Pack of 1	350.00	

## NEW PRODUCTS - 8-OXO-G CLAMP AND 5'-AMINO-MODIFIER TEG CE PHOSPHORAMIDITES

### 8-OXO-G CLAMP

8-oxo-dG is a key mutagenic base lesion that is linked to oxidative stress in cells and UV irradiation. Recently, the lab of Shigeki Sasaki from Kyushu University in Japan developed a novel means of detecting this lesion in DNA.<sup>1</sup> It is based upon a very simple change in the structure of AP-dC (1), commonly known as 'G-Clamp', which is known for its large stabilizing effect upon duplex DNA when base-paired with guanosine.<sup>2</sup>

When a benzylcarbamoyl analogue (2) was synthesized, the Sasaki lab found that when incorporated into an oligo, it exhibited similar fluorescence to AP-dC. However, when base-paired against the 8-oxo-dG, its fluorescence was severely quenched. Rather remarkably, however, when base paired with dG or any of the other bases, A, C or T, there was no change in fluorescence – making it a specific probe for 8-oxo-dG.

While it is doubtful that the 8-Oxo-G Clamp could be sensitive enough to probe genomic DNA where the percentage of the 8-oxo-dG lesions is very small, we envisage that the 8-Oxo-G Clamp will find application in the study of DNA repair enzyme kinetics and may be very helpful in developing high-throughput fluorescence-based screening assays in the search for small-molecule inhibitors of DNA repair enzymes.

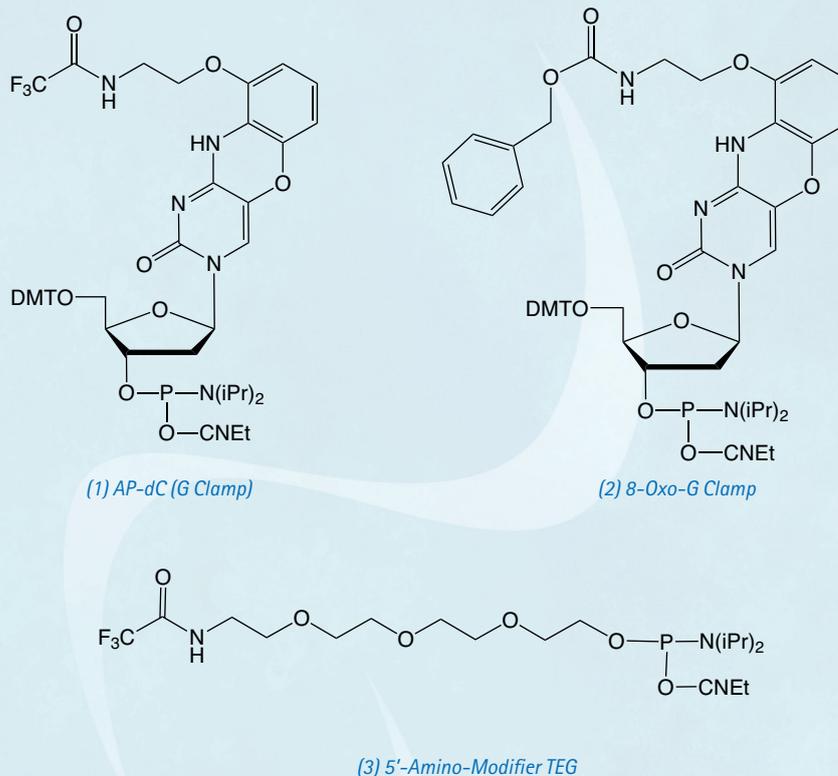
#### References:

1. T. Nasr, et al., *Bioorg Med Chem Lett*, 2009, **19**, 727-30.
2. K.Y. Lin, and M.D. Matteucci, *J Amer Chem Soc*, 1998, **120**, 8531-8532.

### 5'-AMINO-MODIFIER TEG

The use of amino-modified oligos is ubiquitous in the field of DNA and RNA research. They provide a facile means to spot oligos on microarrays and label oligos with a variety of haptens, fluorophores and proteins, such as HRP, for enzymatic detection methodologies. A critical feature of these amino-modified oligos is the linker used to couple the amine to the oligonucleotide. The linker should be long enough to allow efficient conjugation of more bulky, sterically hindered labels and yet remain hydrophilic. The hydrophilicity is an important factor for two reasons – first, it limits non-specific interactions

FIGURE 1: STRUCTURES OF AP-DC, 8-OXO-G CLAMP AND 5'-AMINO-MODIFIER TEG



with hydrophobic surfaces, protein pockets or haptens, and second, it will remain well solvated in water, thereby remaining, on average, further extended into the surrounding aqueous solution. Toward this end, we are pleased to introduce a new hydrophilic amino-modifier, 5'-Amino-Modifier TEG CE Phosphoramidite (3).

This amino-modifier, a triethylene glycol ethylamine derivative, is 12 atoms in length and fully soluble in aqueous media. The trifluoroacetyl protecting group on the amine is completely removed under standard deprotection procedures. We are happy to add this new product to our growing line of 5'-amino modifiers.

#### ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
8-Oxo-G Clamp-CE Phosphoramidite	10-1515-95	50 $\mu$ mole	260.00
	10-1515-90	100 $\mu$ mole	500.00
	10-1515-02	0.25g	1220.00
5'-Amino-Modifier TEG	10-1917-90	100 $\mu$ mole	115.00
	10-1917-02	0.25g	500.00

Back in the 1990s, deprotection of DNA oligos was carried out using ammonium hydroxide overnight at 55 °C. The only option to increase the speed was to raise the deprotection temperature to 80 °C (and even above!) with the time being halved for every 10 °C the temperature was increased. But in those days, the most common application for oligos was as sequencing primers so a small percentage of unprotected ibu-dG was never noticed. The first attempt to increase the speed of deprotection was the introduction<sup>1</sup> of dmf-dG (and dmf-dA) as "Fastphoramidites" since dmf-dG is deprotected at about twice the rate of ibu-dG. In our view, there was no downside to the adoption of dmf-dG but dmf-dA proved to be rather too labile for routine use and was discontinued. However, ammonium hydroxide was still the only deprotection method at this time.

Although ammonium hydroxide is still immensely popular for deprotection of DNA oligos, the advent of high throughput synthesis, labile bases and fluorescent tags has led to the adoption of a variety of newer procedures. In this article, Deprotection - Volume 4, we will describe some of the most popular deprotection procedures and will note when they may be most applicable.

As usual, when reviewing the variety of procedures available to deprotect any modified or unmodified oligonucleotide, you must heed the primary consideration: *First, Do No Harm*. You can then proceed with confidence to *Deprotect to Completion*.

### FIRST, DO NO HARM!

As we have stated in the past, determination of the appropriate deprotection scheme should start with a review of the components of the oligonucleotide to ascertain if any group is sensitive to base and requires a mild deprotection or if there are any pretreatment requirements. Sensitive products are defined as such on the Analytical Report, Certificate of Analysis, or Technical Bulletin. Occasionally, some products require a special pretreatment to prevent unwanted side reactions. If the oligo has several unusual components, you must follow the mildest procedure recommended. As you might expect, some highly modified oligos can become VERY challenging.

### ULTRAFast DEPROTECTION

The use of dmf-dG to speed up deprotection with ammonium hydroxide was only an incremental improvement in speed. However, UltraFast deprotection quickly became a commercial reality with the introduction<sup>2</sup> of deprotection using ammonium hydroxide/methylamine (AMA).

By adding an equal volume of 40% aqueous methylamine solution to ammonium hydroxide to form AMA, it is possible to speed up the deprotection of oligonucleotides enormously.<sup>2</sup> Deprotection can be completed in 5 minutes at 65 °C, thereby allowing oligonucleotides to be delivered to customers on the same day of manufacture. The only change required in the synthesis strategy is the substitution of Ac-dC for Bz-dC to avoid transamination of dC by displacement of benzamide by methylamine to form the mutant N4-Me-dC.<sup>3</sup> This modification is well tolerated and probably codes perfectly as dC in any case. However, as with dmf-dG described above, we see no downside to the use of Ac-dC and recommend it at all times. UltraFast deprotection has found favor with groups processing many oligonucleotides where the decreased processing time, and, therefore, cost savings, becomes highly significant.

Options for UltraFast deprotection, where the removal of the dG protecting group is the rate determining step, are shown in Table 1.

Table 1

dG Protection	Temperature	Time
ibu-dG, dmf-dG or Ac-dG	RT	120 min.
	37°C	30 min.
	55°C	10 min.
	65°C	5 min.

*Note:* UltraFAST system requires acetyl (Ac) protected dC to avoid base modification at the C base.

The consequences of fast but incomplete deprotection are illustrated in Figure 1 on the following page. RP HPLC traces show the location of incompletely deprotected oligonucleotides relative to the main component in DMT-ON and DMT-Off situations.

As an aside, we have found that AMA deprotection is also the optimal procedure for RNA deprotection.

### VOLUME 4: DEPROTECT TO COMPLETION WITHOUT USING AMMONIUM HYDROXIDE

- 1) *Do I need to do my deprotection fast?*  
Use UltraFast Deprotection.
- 2) *Do I have special components in my oligos and need to treat them gently?*  
Use one of the Mild Deprotection procedures.
- 3) *Do I have many oligos to treat in parallel?*  
Consider the use of Gas Phase.

### MILD DEPROTECTION

Deprotection using sodium hydroxide in aqueous alcoholic solvents is a very mild (and fast) alternative to ammonium hydroxide. For a mild deprotection scheme, you can deprotect DNA oligos with 0.4M sodium hydroxide in methanol/water (4:1). For example, we recommend this method for oligos containing acridine. This technique is necessary for oligos where esters are hydrolyzed to carboxylates, such as Carboxy-dT and EDTA-dT, where deprotection with amine-containing reagents would lead to undesired amide formation. You can also deprotect DNA oligos in a few minutes at 80 °C with no concern about vials popping since the mixture contains no volatile gas. The resulting deprotected oligo can be isolated by precipitation or by dilution with water followed by desalting or DMT-ON purification. In all cases, the oligos are isolated as desirable sodium salts. The downside is that oligos cannot be isolated simply by evaporation and a desalting step is mandatory.

### ULTRAMILD DEPROTECTION

Many years ago, we were confronted with the reality that some DNA bases that we wanted to introduce for DNA damage and repair studies simply were not compatible with ammonium hydroxide deprotection. Although deprotection with ammonium hydroxide at room temperature did allow oligos containing these bases to be isolated, it was not optimal and clearly a new deprotection scheme was needed.

In the early 1990s, we were prompted to look at an alternative DNA protecting group, acetoxymethylbenzoyl (AMB), which could be removed using potassium carbonate in methanol.<sup>4</sup> Unfortunately, the AMB-protected monomers proved to be too unstable to store for long periods. But we found that the use of a combination of Pac-

dA, Ac-dC and iPr-Pac-dG allowed complete deprotection with potassium carbonate in methanol at room temperature for four hours as long as capping was carried out using phenoxyacetic anhydride rather than acetic anhydride. (With UltraMild reagents, ammonium hydroxide at room temperature for two hours was also effective.) If acetic anhydride was used, a small amount of transamidation occurred at dG residues and overnight treatment with potassium carbonate or ammonium hydroxide was required to deprotect formed Ac-dG residues.

### ULTRA ULTRAMILD

An even milder deprotection scheme has been described<sup>5</sup> for the synthesis of highly base labile nucleoside adducts. In this UltraMild variation, Q-supports must be used since the succinate linkages of normal supports are virtually untouched under the deprotection conditions. Normal yields are achieved with Q-supports. The reagent for this Ultra UltraMild deprotection procedure is 10% diisopropylamine/0.25M  $\beta$ -mercaptoethanol in methanol overnight at 55 °C. This is a method which has not been tested in very many facilities but it is surely worthy of consideration when challenged with the preparation of oligos with very labile bases.

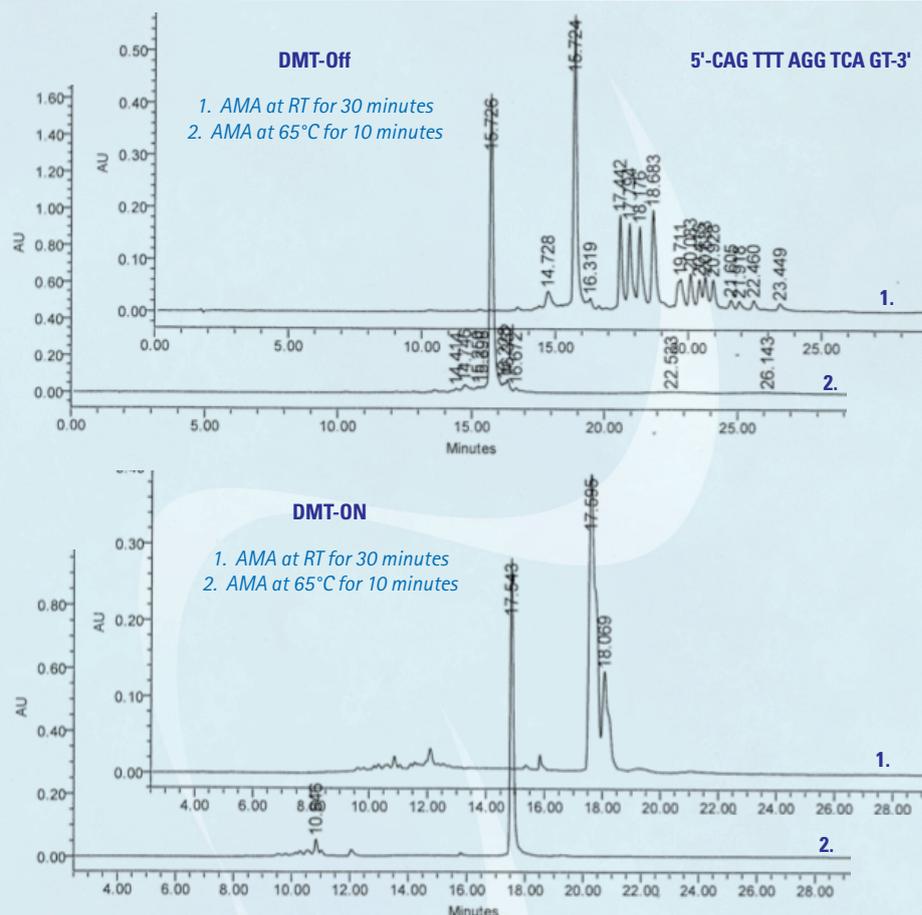
### T-BUTYLAMINE

TAMRA-containing oligonucleotides remain popular as single and dual labelled probes. Unfortunately, the stability of TAMRA to the conditions of oligonucleotide deprotection is really marginal. In the past, we have recommended the use of UltraMild monomers and deprotection and this procedure does indeed work well. An alternative approach has been described<sup>6</sup> using t-butylamine/methanol/water, which does allow the use of regular monomers. We have evaluated a simpler t-butylamine/water (1:3) mix (4 hours at 60 °C), described by Biosearch Technologies, and, in model studies, this generates TAMRA-oligos with the highest purity and with negligible degradation detected.

### GAS PHASE

Although gas phase deprotection does require specialist equipment, this

FIGURE 1: RP HPLC OF AN OLIGO 1. PARTIALLY AND 2. FULLY DEPROTECTED, DMT-OFF AND DMT-ON



### RP HPLC Conditions

Column: Waters X-Bridge C18, 250 x 4mm; Buffers: A - ACN; B - 0.1M TEAA, pH 7.

DMT-Off Gradient: 3-15% B over 15 min. DMT-ON Gradient: 3-40% B over 15 min. Flow rate: 1ml/min.

technique is excellent for high throughput synthesis. Columns and plates can be placed in the reactor without concern for cross contamination since the product oligos will remain adsorbed to the synthesis support. This is doubly advantageous since the product can be eluted from the columns and plates in such a way that the organic debris can be removed. Also, using anhydrous ammonia gas and using UltraMild monomers, the cleavage and deprotection processes can be completed in less than 1 hour.<sup>7</sup> However, methylamine gas has proved to be more popular for routine synthesis and is in common use in our industry. Please note that deprotection times and temperatures vary with the equipment and number of columns and will need to be optimized.

### SUMMARY

Oligonucleotide deprotection has come a long way since the early days when

ammonium hydroxide was the only option. Now a variety of procedures are available to fit a variety of circumstances. Each synthesis should be reviewed to ensure that the deprotection conditions are compatible with the components of the oligo. Special deprotection requirements can be found on our website: <http://www.glenresearch.com>.

### References:

1. H. Vu, et al., *Tetrahedron Lett.*, 1990, **31**, 7269-7272.
2. M.P. Reddy, N.B. Hanna, and F. Farooqui, *Nucleos Nucleot*, 1997, **16**, 1589-1598.
3. M.P. Reddy, N.B. Hanna, and F. Farooqui, *Tetrahedron Letters*, 1994, **35**, 4311-4314.
4. W.H.A. Kuijpers, E. Kuylyeheskiely, J.H. Vanboom, and C.A.A. Vanboeckel, *Nucleic Acids Res.*, 1993, **21**, 3493-3500.
5. L.C.J. Gillet, J. Alzeer, and O.D. Scharer, *Nucleic Acid Res.*, 2005, **33**, 1961-1969.
6. B. Mullah, and A. Andrus, *Tetrahedron Lett.*, 1997, **38**, 5751-5754.
7. J.H. Boal, et al., *Nucleic Acids Res.*, 1996, **24**, 3115-3117.

## TECHNICAL BRIEF - DEPURINATION

Depurination (cleavage of the glycosidic bond connecting the purine base to the sugar) is a term that is not mentioned very much in the context of regular oligonucleotide synthesis - probably because the optimized processes used on modern synthesizers do not really induce depurination. Depurination is more likely to occur in the base protected monomers used for oligonucleotide synthesis than in the nucleosides themselves due to the electron withdrawing effect of acyl protecting groups which then destabilizes the glycosidic bonds. Ribonucleosides with their additional 2'-OH groups are less susceptible than deoxynucleosides. At Glen Research, we are concerned about depurination (and associated depyrimidination) when dealing with unusual modified nucleosides that are more susceptible to this degradative process.

The consequence of depurination during oligonucleotide synthesis is the loss of the purine base to form an internucleotide linkage containing the abasic sugar at that position. This site is stable during further synthesis cycles but, on deprotection with basic reagents, the oligonucleotide is cleaved at that position leading to two shorter fragments. The fragment towards the 5' terminus still contains the DMT group often used for DMT-ON purification. Following such purification, the depurinated fragments remain in the product as oligonucleotides truncated towards the 3' terminus. Clearly, this is not a desirable outcome.

### FORMAMIDINE PROTECTION

One key innovation in protecting groups for oligonucleotide synthesis was the formamidine (Figure 1) protecting group.<sup>1-3</sup> Acyl protecting groups are electron withdrawing and destabilize the glycosidic bond. Formamidines are electron donating and stabilize the glycosidic bond. Formamidines first appeared in commercial DNA synthesis with the introduction<sup>4</sup> of the dimethylformamidine (dmf) group in dmf-dG (and dmf-dA) as "Fastphoramidites". The adoption of dmf-dG was immediate but dmf-dA proved to be rather too labile for routine use and was discontinued.

It is worthy of note that the formamidine family is more stabilizing with increasing length of the alkyl groups. At the same time, formamidine groups become more resistant to deprotection with increasing length of the

alkyl groups. So dibutylformamidine (dbf) is significantly more stabilizing than dmf but dbf deprotects much slower than dmf. Using formamidine protecting groups, a variety of minor bases have become amenable to use in oligonucleotide synthesis. For example, the iso-bases, iso-dC and iso-dG, are both very susceptible to acidic cleavage and are only usable today because of formamidine protecting groups.

### DEBLOCKING ACIDS

Many years ago, in an effort to mimic the conditions of synthesis of long oligos or those produced on a large scale, we conducted experiments where oligonucleotides growing on CPG were subjected to long wait periods in the presence of a deprotection solution containing trichloroacetic acid (TCA). We duly detected depurination at dA sites but not at a very high level. However, when we used a deprotection solution containing dichloroacetic acid (DCA), we were unable to detect any depurination. It was clear that in routine oligonucleotide synthesis, even of long oligos, on a regular column-based synthesizer, depurination is not a very significant event.

A recent report<sup>5</sup> has reminded us that depurination may not be significant in normal synthesis but it is still a problem in certain circumstances. Indeed, LeProust and collaborators have shown that depurination is the limiting factor in the ability to synthesize long oligos (up to 150mers) on Agilent's DNA microarray synthesis platform - even using DCA as the detritylating reagent. The authors note that the difference between a flow-through synthesis on the porous, 3-dimensional surface of CPG (where the support surface area is large and the reagent volume is small) and the synthesis on the flat, non-porous surface of a silicon wafer (where the surface area is low and surrounded by a high volume reagent chamber) is profound. In this case, it was shown that the detritylation conditions were not a significant factor in the level of depurination, which was a little unexpected. Depurination had to be

### ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
dbf-dA-CE Phosphoramidite	10-1500-90	100 $\mu$ mole	155.00
	10-1500-02	0.25g	400.00

FIGURE 1: FORMAMIDINE STRUCTURE

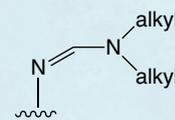
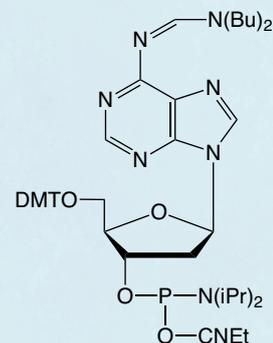


FIGURE 2: STRUCTURE OF DBF-DA



dbf-dA-CE Phosphoramidite (1)

controlled by adjusting the fluidics of the flow cell and by quenching the detritylation solution with oxidizer solution.

For researchers trying to determine if their source of depurination is reagent, fluidics or protocol-based, we are happy to turn the clock back more than two decades and offer a depurination-resistant dA monomer. Based on projected pricing, this is unlikely ever to replace the regular Bz-dA monomer but we hope that dibutylformamidine-protected dA (dbf-dA-CE Phosphoramidite) (1) will prove useful in this basic research. Dbf-dA is compatible with UltraFast deprotection and is deprotected by ammonium hydroxide at 55 °C overnight.

### References:

1. B.C. Froehler, and M.D. Matteucci, *Nucleic Acids Res*, 1983, **11**, 8031-6.
2. L.J. McBride, R. Kierzek, S.L. Beaucage, and M.H. Caruthers, *J. Amer. Chem. Soc.*, 1986, **108**, 2040.
3. Z. Tocik, L. Arnold, and J. Smrt, *Nucleic Acids Res.*, 1987, **18**, 193.
4. H. Vu, *et al.*, *Tetrahedron Lett.*, 1990, **31**, 7269-7272.
5. E.M. LeProust, *et al.*, *Nucleic Acids Res.*, 2010, **38**, 2522-2540.

## GLEN-PAK™ PRODUCT UPDATE: NEW PROTOCOLS FOR DESALTING AND PURIFICATION

We continue to develop new protocols for our popular Glen-Pak™ product line. While DMT-ON purification is the Glen-Pak's obvious strength, our Technical Support team routinely receives questions about other applications using the cartridges.

In this article, we introduce protocols for desalting of crude, DMT-Off DNA and RNA, DMT-ON purification of phosphorothioate oligos, as well as a method for purification of 5' labelled Cy5 and Cy5.5 oligos to separate them from their degradation products created during base deprotection requiring ammonium hydroxide.

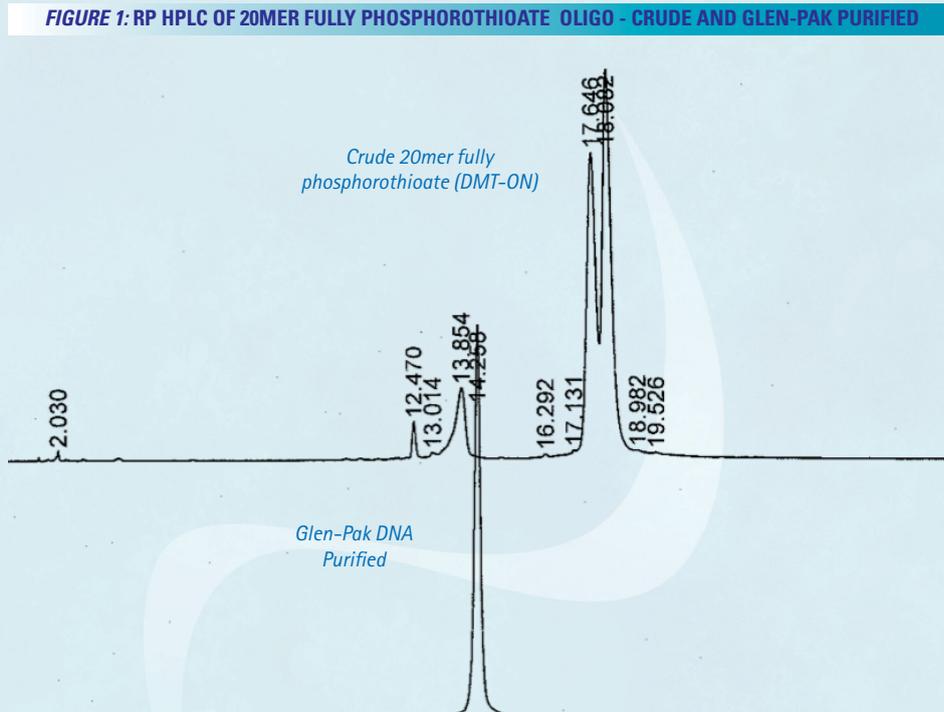
Step by step versions of these new protocols can be viewed in our latest update of the Glen-Pak User Guide found in the purification section of our website or by following this URL: [http://www.glenresearch.com/Technical/GlenPak\\_User\\_Guide.pdf](http://www.glenresearch.com/Technical/GlenPak_User_Guide.pdf)

### DESALTING OF DNA AND RNA ON THE GLEN-PAK DNA CARTRIDGE:

One of the most common questions we have received since the launch of Glen-Pak cartridges is whether or not they can be used for simple desalting of DMT-Off oligos. While our original protocol in this area was less than ideal and the more recent version of our Glen-Pak User Guide only offered options for desalting of DNA post PAGE or HPLC purification, we are now pleased to offer protocols for the desalting of DMT-Off DNA and RNA. Of special note is that the methods use the Glen-Pak DNA cartridge for desalting of BOTH types of oligos. This will allow our customers currently using more than one cartridge platform for downstream processing to harmonize to only one column type for both DNA and RNA DMT-Off desalting.

#### For DMT-Off DNA:

- Reconstitute a fully deprotected, DMT-Off, dried down oligonucleotide in 2.0mL 0.1M TEAA.
- Load the solution on a properly prepared Glen-Pak DNA cartridge.
- Rinse with 2.0mL 0.1M TEAA.
- Rinse with 2.0mL water.
- Elute in 10% Acetonitrile in water.



#### For DMT-Off RNA:

- Conduct standard base deprotection and 2' de-silylation in DMSO and TEA.3HF as recommended in our current TBDMS and TOM Technical bulletins.
- Quench the reaction with 1.75mL RNA Quenching Buffer (60-4120-xx).

Load the resultant 2mL directly on a properly prepared Glen-Pak DNA cartridge

- Rinse with 2.0mL 0.1M TEAA (Fresh 2.0M TEAA diluted in RNase free water).
- Rinse with 2.0mL RNase free water.
- Elute the desalted product in 10% Acetonitrile in RNase free water instead of the standard 30% Acetonitrile/Ammonium Bicarbonate solution currently recommended for DMT-ON purification of RNA.

In both cases described above, the elution buffer containing 10% Acetonitrile allows for full removal of the DMT-Off oligo, while leaving behind organics, such as benzamide, on the column matrix.

### GLEN-PAK PHOSPHOROTHIOATE DNA PURIFICATION:

The Glen-Pak DNA cartridge may also be used to purify phosphorothioates

(S-Oligos) with only some minor changes to the existing DMT-ON method for standard oligonucleotides. The extra hydrophobicity conferred by the addition of sulfur to the oligonucleotide backbone requires both a higher concentration of acetonitrile in the standard salt wash step (12%) to remove the DMT-Off failure products as well as a stronger acid (4% TFA, 60-4042-57) for efficient removal of the DMT prior to elution (See Figure 1).

### GLEN-PAK DNA PURIFICATION OF CY5/5.5 OLIGOS CONTAINING DYE DEGRADATION PRODUCTS:

We routinely recommend the use of UltraMild phosphoramidites when synthesizing oligonucleotides containing Cy5 and Cy5.5, as the dye is very sensitive to the ammonium hydroxide and heat normally utilized in deprotection. However, other sequence/chemistry requirements sometimes dictate the use of concentrated ammonium hydroxide at room temperature that leads to the production of a yellow chromophore, which is the degradation product of Cy5 and Cy5.5. One can tell significant degradation has occurred when the solution goes from a clear blue color to turquoise or green.

A twenty base oligonucleotide containing a 5' Cy5 was synthesized MMT-Off and deprotected for 17 hours at 55 °C in



**GLEN RESEARCH**

**22825 DAVIS DRIVE  
STERLING, VIRGINIA  
20164**

PRESORTED STANDARD

US POSTAGE

PAID

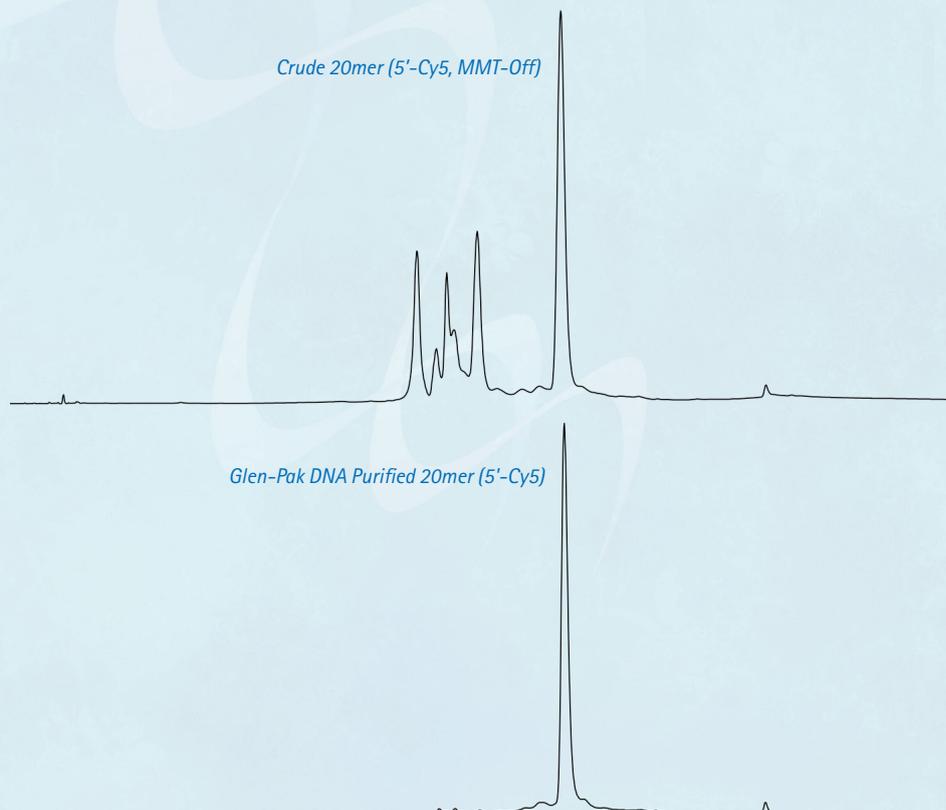
RESTON VA

PERMIT NO 536

*(Continued from Page 15)*

concentrated ammonium hydroxide. These conditions were chosen to generate an oligonucleotide that was partially degraded. The deprotected oligonucleotide was dried down, reconstituted in 2.0mL 0.1M TEAA and loaded on a properly prepared Glen-Pak DNA cartridge. This was followed by rinses with 2mL 0.1M TEAA, 2mL 16% Acetonitrile/0.1M TEAA and an elution from the cartridge in 50% Acetonitrile/Water containing 0.5% Ammonium Hydroxide. As seen in Figure 2, the protocol removes failure sequences AND separates the Cy5 degradation product from the intact dye, so the eluted oligo is again a clear blue color. The accompanying spectra for each of the product peaks confirm removal of the degraded Cy5 dye.

**FIGURE 2: RP HPLC OF 5'-CY5 20MER - CRUDE AND GLEN-PAK PURIFIED**



*Glen-Pak is a trademark of Glen Research Corporation*