The Glen Report

22825 DAVIS DRIVE • STERLING, VIRGINIA • 20164

GLEN RESEARCH

VOLUME 24

NUMBER 2

DECEMBER 2012

INSIDE

CLICK DNA AND RNA LIGATION – A BIOCOMPATIBLE TRIAZOLE LINKAGE

Afaf H. El-Sagheer and Tom Brown
School of Chemistry , University of Southampton
Highfield, Southampton SO17 1BJ , United Kingdom

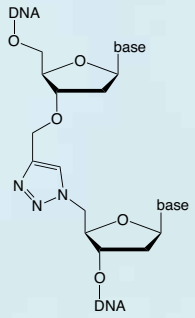
Biochemical strategies that use a combination of synthetic oligonucleotides, thermostable DNA polymerases and DNA ligases can produce large DNA constructs up to 1 megabase in length. Although these ambitious targets are feasible biochemically, comparable technologies for the chemical synthesis of long DNA strands lag far behind. The best available chemical approach is the solid-phase phosphoramidite method, which can be used to assemble DNA strands up to 150 bases in length. Beyond this point deficiencies in the chemistry make it difficult to produce pure DNA.

A possible alternative approach to the chemical synthesis of large DNA strands is to join together synthetic oligonucleotides by chemical methods. Recently click ligation by the copper-catalyzed azide-alkyne (CuAAC) reaction has been shown to facilitate this process, and a biocompatible triazole linkage has been developed that mimics a normal phosphodiester group.[1]

This requires an oligonucleotide with a 5'-azide and another with a 3'-propargyl group. The two oligonucleotides can be joined together by splint mediated ligation to produce a triazole linkage at the ligation site (Figure 1, Page 2). Three or more oligonucleotides can be joined together by this methodology using internal difunctionalized oligonucleotides with 5'-azide and 3'-propargyl groups in the same strand. The alkyne and azide oligonucleotide strands can be prepared by standard protocols and, as it is chemical rather than enzymatic, the chemical ligation reaction is compatible with a wide range of other oligonucleotide modifications.

Click ligation has been employed to synthesize DNA constructs up to 300 bases in length and, when the resulting triazole linkage is placed in a PCR template, various DNA polymerases correctly copy the entire base sequence.[1] *In vitro* transcription through the modified linkage has also been successfully demonstrated.[2]

BIOCOMPATIBLE INTERNUCLEOTIDE TRIAZOLE LINKAGE



Cyclic DNA duplexes with potential therapeutic applications can be made using this methodology and have been shown to be substrates for rolling circle amplification.[1]

This modified triazole linkage has shown *in vivo* biocompatibility; an antibiotic resistance gene containing triazole linkages is functional in *E. coli*[1] and a triazole-containing gene for a (non-essential) fluorescent protein has been expressed.[3]

A recent NMR study of a DNA duplex containing the triazole linkage has provided a rationale to explain the biocompatibility of this linkage.[4]

Click ligation in the RNA field has enabled the synthesis of an enzymatically active hammerhead ribozyme with the triazole linkage located at the substrate cleavage site.[5]

Possible applications of click ligation include assembly of long DNA or RNA strands incorporating unusual sugar or backbone modifications (including chimeric molecules), epigenetic modifications, fluorescent dyes and other reporter groups which might be unstable to enzymatic ligation conditions. In the RNA world, segmental labeling followed by the assembly of large chemically-modified RNA

(Continued on Page 2)

(Continued from the Front Page)

constructs for functional studies is a distinct possibility.

3'-Propargyl-5-Me-dC-CPG, (1) in Figure 2, is used to prepare 3'-propargyl modified oligonucleotides. In collaboration with Tom Brown's group at the University of Southampton, Glen Research has added this product to the catalog of reagents for Click Chemistry.

References:

1. A.H. El-Sagheer, A.P. Sanzone, R. Gao, A. Tavassoli, and T. Brown, *Proc Natl Acad Sci U S A*, 2011, **108**, 11338-43.
2. A.H. el-Sagheer, and T. Brown, *Chem Commun (Camb)*, 2011, **47**, 12057-8.
3. A.P. Sanzone, A.H. El-Sagheer, T. Brown, and A. Tavassoli, *Nucleic Acids Res*, 2012.
4. A. Dallmann, et al., *Chemistry*, 2011, **17**, 14714-7.
5. A.H. El-Sagheer, and T. Brown, *Proc Natl Acad Sci U S A*, 2010, **107**, 15329-34.

PROTOCOLS

Conversion of 5'-dC or 5'-dT to 5'-azido dC or 5'-azido dT for oligonucleotides with an unmodified 3'-end

Oligonucleotides were assembled on the 0.2 or 1.0 µmole scale (trityl-off) with a normal 5'-HO-dC or 5'-HO-dT terminus. To convert the 5'-hydroxyl group to 5'-iodo,[1] the protected oligonucleotide attached to the synthesis column was treated with a 0.5 M solution of methyltriphenoxyphosphonium iodide in DMF (1 mL), which was periodically passed through the column via two 1 mL syringes over 15 min at room temperature. The column was then washed several times with dry DMF. To convert the 5'-iodo to 5'-azido, sodium azide (30 mg) was suspended in dry DMF (1 mL), heated for 10 min at 70 °C then cooled down. The supernatant was taken up into a 1 mL syringe, passed back and forth through the column then left at room temperature overnight (or for 5 hr at 55 °C with periodic mixing). The column was then washed with DMF, acetonitrile and dried by the passage of a stream of argon gas. The resultant 5'-azide oligonucleotide was cleaved from the solid support and deprotected by heating in aqueous ammonia for 5 hr at 55 °C.

5'-Azido oligonucleotides can also be synthesized using the 5'-iodo-dT monomer, (2) in Figure 2, and converting it to 5'-Azido using sodium azide in DMF as described above.

Conversion of 5'-dC or 5'-dT to 5'-azido dC or 5'-azido dT for oligonucleotides with 3'-propargyl-5-Me-dC at the 3'-end

5'-O-(4,4'-Dimethoxytrityl)-3'-O-propargyl-5-methyl-2'-deoxyCytidine on a solid support was packed into a TWIST column and used to assemble the required sequence in the 3'- to 5'-direction (standard phosphoramidite oligonucleotide synthesis) with 5'-iodo dT, 5'-HO-dT or 5'-HO-dC at the 5'-end. The 5'-hydroxyl or iodo groups were then converted to azide using the conditions described above for the synthesis of the 5'-azide oligonucleotides using unmodified solid support.

In the case of the 3'-propargyl-5-Me-dC functionalized resin, the linkage is not very stable to prolonged contact with the azide solution in DMF, therefore some of the oligonucleotide will be released from the resin into solution. To achieve better yield, the DMF solution and washings were evaporated, the residue was dissolved in water and desalted using NAP-25 columns. After evaporating the water under vacuum, the residue can be combined with the washed and dried resin and heated in aqueous ammonia for 5 hr at 55 °C for deprotection.

FIGURE 1: SYNTHESIS AND AMPLIFICATION OF THE BIOCOMPATIBLE TRIAZOLE LINKAGE

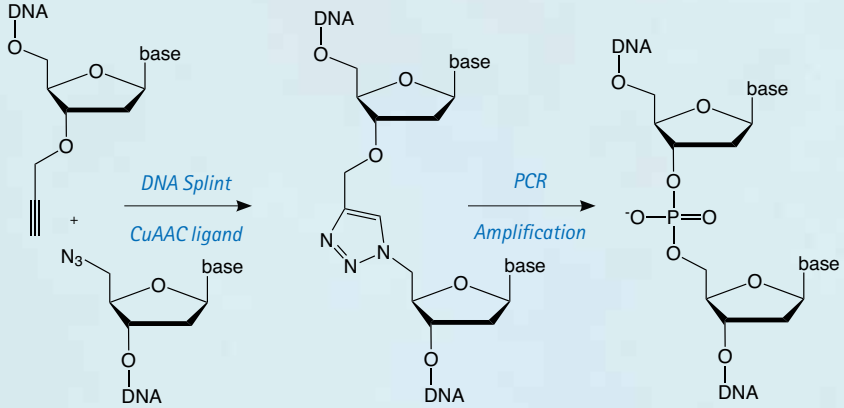
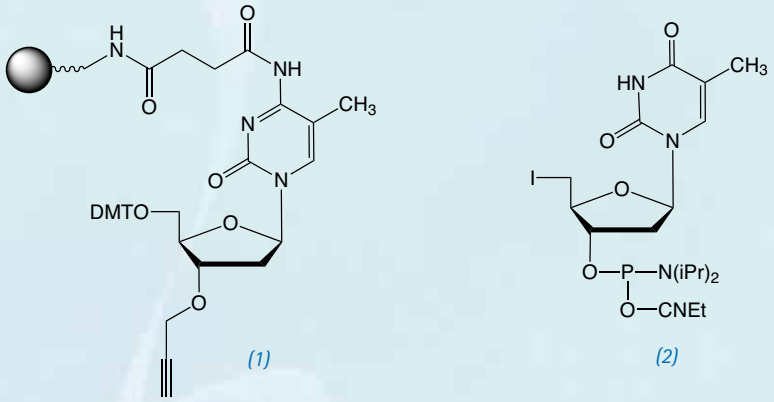


FIGURE 2: STRUCTURE OF 3'-PROPARGYL-5-Me-dC-CPG AND 5'-I-dT-CE PHOSPHORAMIDITE



(1) (2)

Reference:

1. G.P. Miller, and E.T. Kool, *J Org Chem*, 2004, **69**, 2404-2410.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
5'-I-dT-CE Phosphoramidite	10-1931-90	100 µmole	85.00
	10-1931-02	0.25g	295.00
3'-Propargyl-5-Me-dC CPG	20-2982-01	0.1g	180.00
	20-2982-10	1.0g	1500.00
1 µmole columns	20-2982-41	Pack of 4	300.00
0.2 µmole columns	20-2982-42	Pack of 4	150.00
10 µmole column (ABI)	20-2982-13	Pack of 1	750.00
15 µmole column (Expedite)	20-2982-14	Pack of 1	1125.00

NEW PRODUCT - DBCO-dT FOR COPPER-FREE CLICK CHEMISTRY

INTRODUCTION

The copper(I) catalyzed [3+2] azide-alkyne cycloaddition (CuAAC) is the most prominent example of a group of reactions known as click reactions, as shown in Figure 1. Glen Research has been active in supporting Click Chemistry for several years and, in this newsletter, we highlight an excellent example of click ligation to generate a biocompatible internucleotide linkage (Page 1), a simplified kit-based click system (Page 8), and some new products for CuAAC Click Chemistry (Page 5).

Copper-free click has some advantages over CuAAC, especially in situations where users do not perform click reactions regularly and are looking for a simplified alternative to CuAAC. Cyclooctyne is the smallest cyclic octyne that can be isolated. Because of the severe deformation of the alkyne from its desired linear geometry, cyclooctynes are highly reactive towards azides without the need for copper catalysis.

COPPER-FREE CLICK

From the variety of cyclooctyne-based copper-free click reagents so far described, we chose to offer compounds based on the dibenzocyclooctyl (DBCO) structure, shown in Figure 2.[1-3] DBCO products exhibit the following desirable properties:

- Simple to use
- Stable in solution on the synthesizer
- Stable to ammonium hydroxide and AMA
- Excellent click performance in 17 hours or less at room temperature

For 5'-modification, we chose to use 5'-DBCO-TEG Phosphoramidite (1), in which the very hydrophobic DBCO moiety is separated from the phosphoramidite and subsequent oligo with a triethyleneglycol (TEG) spacer. In addition, we chose to offer a soluble DBCO-sulfo-NHS ester sodium salt (2) for post-synthesis conjugation reactions with amino-modified oligonucleotides and proteins.

In addition to these DBCO-based products, we now offer DBCO-dT-CE Phosphoramidite (3) for inserting a DBCO group at any position within the oligonucleotide. This type of dT analogue has proved to be popular in the past since the tag is projected into the major groove of duplex DNA where it does not disrupt the DNA duplex while being readily accessible for further reaction.

FIGURE 1: CuAAC REACTION

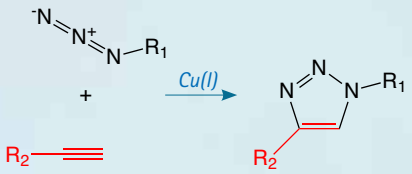
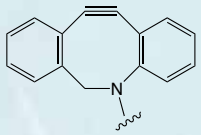
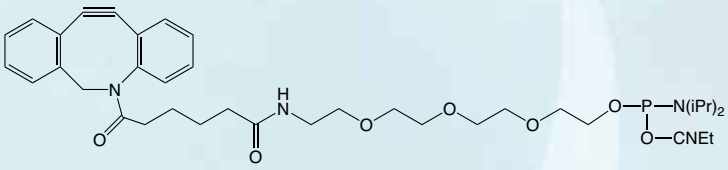


FIGURE 2: DIBENZOCYCLOOCTYL (DBCO)

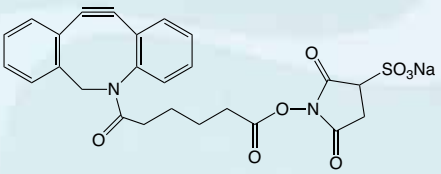


DBCO

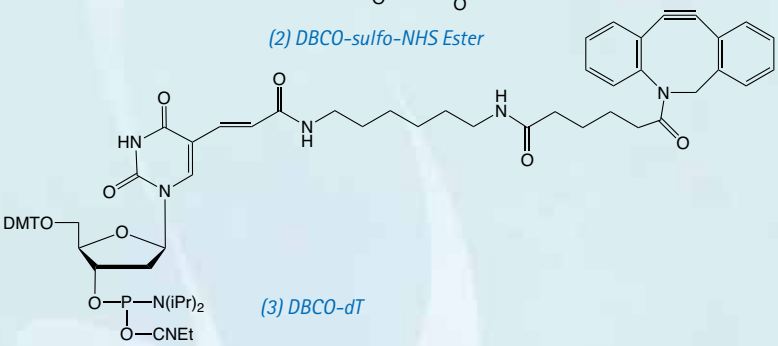
FIGURE 3: PRODUCT STRUCTURES



(1) 5'-DBCO-TEG Phosphoramidite



(2) DBCO-sulfo-NHS Ester



(3) DBCO-dT

SYNTHESIS AND DEPROTECTION

A coupling time of 12 minutes was found to be optimal for DBCO-dT (3). It was found that DBCO-modified oligos were stable to deprotection with ammonium hydroxide for 2 hours at 65°C or overnight at room temperature, which would allow the use of regular phosphoramidites, including dmf-dG but not ibu-dG. Deprotection with AMA for 2 hours at room temperature showed only slight degradation of the cyclooctyne, making the modification compatible with ibu-dG if Ac-dC is used. DBCO-modified oligos are also compatible with UltraMild deprotection conditions.

References:

1. P. van Delft, *et al.*, *Org Lett,* 2010, **12**, 5486-9.
2. P. van Delft, *et al.*, *Synthesis-Stuttgart,* 2011, 2724-2732.
3. I.S. Marks, *et al.*, *Bioconjugate Chemistry,* 2011, **22**, 1259-1263.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
5'-DBCO-TEG Phosphoramidite	10-1941-95	50 µmole	125.00
	10-1941-90	100 µmole	230.00
	10-1941-02	0.25g	775.00
DBCO-sulfo-NHS Ester	50-1941-23	5.2mg	60.00
(Dissolve 5.2mg in 60µL water or DMSO)	50-1941-24	52mg	300.00
DBCO-dT-CE Phosphoramidite	10-1539-95	50 µmole	250.00
	10-1539-90	100 µmole	485.00
	10-1539-02	0.25g	975.00

ANTISENSE TRIMER PHOSPHORAMIDITES - A NEW METHOD FOR MUTAGENESIS SPANNING ENTIRE GENES

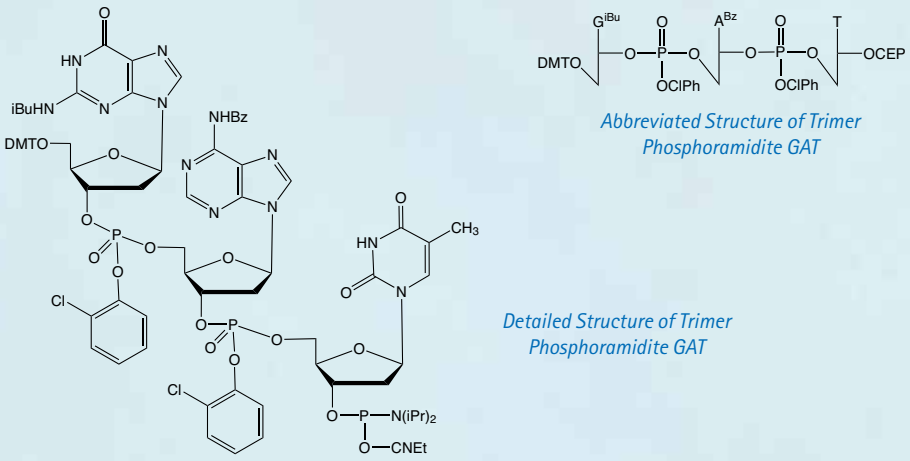
Trimer phosphoramidites, depicted in Figure 1, have proven to be extremely valuable because they allow codon-based mutagenesis, which circumvents the common problems of codon-bias, frame-shift mutations, and the introduction of nonsense or stop codons.[1] This leads to the production of clonal libraries of exceptional diversity with order-of-magnitude increases in amino acid sequence variance while either maintaining a uniform amino acid distribution[2] or one that is biased toward a desired set of amino acids.[3] However, difficulties arise when trying to introduce mutations in multiple distal regions of a gene simultaneously. The synthesis of long oligonucleotides is required, which inevitably leads to lower sequence fidelity due to deletion mutants, depurination events and, to a lesser extent, mutations arising from deamination of cytidine, for example.

An elegant solution to this problem is the use of Antisense Trimer Phosphoramidites. These trimers are the reverse complement of the cannonical 'sense' codons. When these antisense codons are put into the noncoding strand of a template DNA and amplified by PCR, they will code for the sense codon in the opposite strand of DNA. This allows the powerful technique of PCR Assembly[4] to generate not only kilobase-sized genes from short 50mer oligonucleotides, but to simultaneously mutate multiple distal regions of that gene, as shown in Figure 2.

The sense and their corresponding antisense codons are listed in Table 1. Conveniently, many of our existing sense trimers can act as antisense codons. For example, AAC, which codes for asparagine, has the anticodon GTT, which is the sense codon for valine. However, some of the existing trimers, while they can act as an antisense codon, are not good choices for use. For example, TGG, which codes for tryptophan, could be used as an antisense codon for proline because CCA is one of proline's synonymous codons. However, CCA has a relatively low Codon Adaptation Index (CAI) value[5] in *E. coli*, which could limit protein expression in that commonly used organism. For this reason, the anticodon CGG was chosen for optimal expression in *E. coli*, as were the other new antisense codons shown in color in Table 1.

As with our existing trimers, the antisense trimer phosphoramidites are compatible with standard DNA synthesis reagents noting, however, that a special diluent is required, ACN/DCM 1:3 (v/v), to dissolve the phosphoramidite and a 15

FIGURE 1: STRUCTURE OF TRIMER PHOSPHORAMIDITES



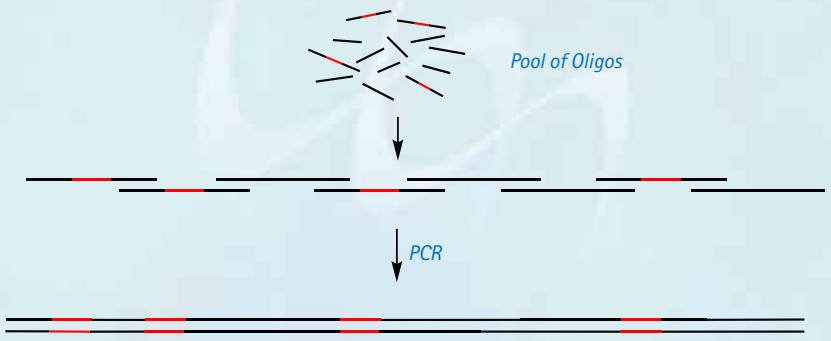
Abbreviated Structure of Trimer Phosphoramidite GAT

Detailed Structure of Trimer Phosphoramidite GAT

TABLE 1: ANTISENSE TRIMER PHOSPHORAMIDITES

Sense codons 5'->3'	*Antisense codons 5'->3'*	*MW*	*RF (Temp)*
AAA (Lys)	TTT	1572.4	1.425
AAC (Asn)	GTT		
ACT (Thr)	GGT		
ATC (Ile)	GAT	1780.5	1.425
ATG (Met)	CAT		
CAG (Gln)	CTG		
CAT (His)	ATG		
CCG (Pro)	CGG	1851.5	1.425
CGT (Arg)	GCG	1851.5	1.425
CTG (Leu)	CAG		
GAA (Glu)	TTC		
GAC (Asp)	ATC		
GCT (Ala)	TGC		
GGT (Gly)	ACC	1863.5	1.425
GTT (Val)	AAC		
TAC (Tyr)	GTA	1780.5	1.425
TCT (Ser)	AGA	1893.5	1.425
TGC (Cys)	GCA	1869.5	1.425
TGG (Trp)	CCA	1863.5	1.425
TTC (Phe)	GAA		

FIGURE 2: SIMULTANEOUS MUTATION OF MULTIPLE DISTAL REGIONS OF GENE



minute coupling time is recommended. To prevent strand scission, oligos containing the antisense trimers should be deprotected in 30% ammonium hydroxide at room temperature for 17 hours, followed by an additional 4 hours at 55 °C to complete the deprotection of the nucleobases.

At present, we do not have enough data to accurately assign a Reaction Factor (RF) to the antisense trimers. Until those have been determined, a value of 1.425 is being used which is the average RF of the well-characterized sense trimers. As such, an antisense trimer mix designed to provide 20 amino acids represented equally would yield upon sequencing, some trimers that were over represented (i.e., >5%) and others under represented (<5%) upon sequencing. We will update the RF values when the data become available.

References:

1. C. Neylon, *Nucleic Acids Res*, 2004, **32**, 1448-59.
2. L.R. Krumpe, K.M. Schumacher, J.B. McMahon, L. Makowski, and T. Mori, *BMC Biotechnol*, 2007, **7**, 65-72.
3. F.A. Fellouse, et al., *J Mol Biol*, 2007, **373**, 924-40.
4. W.P. Stemmer, A. Crameri, K.D. Ha, T.M. Brennan, and H.L. Heyneker, *Gene*, 1995, **164**, 49-53.
5. P.M. Sharp, and W.H. Li, *Nucleic Acids Res*, 1987, **15**, 1281-95.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
ACC Trimer Phosphoramidite	13-1011-95	50 µm	350.00
	13-1011-90	100 µm	700.00
AGA Trimer Phosphoramidite	13-1020-95	50 µm	350.00
	13-1020-90	100 µm	700.00
CCA Trimer Phosphoramidite	13-1110-95	50 µm	350.00
	13-1110-90	100 µm	700.00
CGG Trimer Phosphoramidite	13-1122-95	50 µm	350.00
	13-1122-90	100 µm	700.00
GAT Trimer Phosphoramidite	13-1203-95	50 µm	350.00
	13-1203-90	100 µm	700.00
GCA Trimer Phosphoramidite	13-1210-95	50 µm	350.00
	13-1210-90	100 µm	700.00
GCG Trimer Phosphoramidite	13-1212-95	50 µm	350.00
	13-1212-90	100 µm	700.00
GTA Trimer Phosphoramidite	13-1230-95	50 µm	350.00
	13-1230-90	100 µm	700.00
TTT Trimer Phosphoramidite	13-1333-95	50 µm	350.00
	13-1333-90	100 µm	700.00

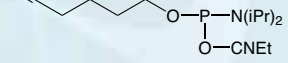
NEW PRODUCT - ALKYNE-MODIFIER SERINOL PHOSPHORAMIDITE

Two of our products for Click Chemistry have proved to be especially popular over the years. For simple 5'-alkyne modification, our 5'-Hexynyl Phosphoramidite (1) is an inexpensive option. However, this product does not allow DMT-On purification. More recently, our 3'-Alkyne-Modifier Serinol CPG (2) has also proved popular as a support for alkyne modification of the 3' terminus.

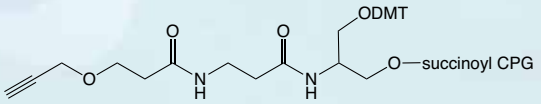
We are now offering Alkyne-Modifier Serinol Phosphoramidite (3), also based on our proprietary serinol backbone, which can be used to modify the 5' terminus or any other position(s) within an oligonucleotide while still being compatible with DMT-On purification. Unlike 5'-Hexynyl Phosphoramidite, which is a viscous oil, Alkyne-Modifier Serinol Phosphoramidite is a solid that also allows easy weighing of the product by our customers prior to use.

Alkyne-Modifier Serinol Phosphoramidite is used with a coupling time of 3 minutes and is compatible with most popular procedures for oligonucleotide deprotection - AMA, 10 minutes/65°C or ammonium hydroxide, 17h/RT or 2h/65°C.

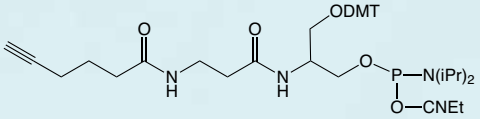
FIGURE 1: **STRUCTURES OF ALKYNE MODIFIERS**



(1) 5'-Hexynyl Phosphoramidite



(2) 3'-Alkyne-Modifier Serinol CPG



(3) Alkyne-Modifier Serinol Phosphoramidite

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
5'-Hexynyl Phosphoramidite	10-1908-90	100 µmole	60.00
	10-1908-02	0.25g	200.00
Alkyne-Modifier Serinol Phosphoramidite	10-1992-95	50 µmole	100.00
	10-1992-90	100 µmole	185.00
	10-1992-02	0.25g	575.00
3'-Alkyne-Modifier Serinol CPG	20-2992-01	0.1g	105.00
	20-2992-10	1.0g	800.00
0.2 µmole columns	20-2992-42	Pack of 4	100.00
1 µmole columns	20-2992-41	Pack of 4	175.00
10 µmole column (ABI)	20-2992-13	Pack of 1	260.00
15 µmole column (Expedite)	20-2992-14	Pack of 1	390.00

INTRODUCTION

MicroRNAs (miRNAs) are short, non-coding double-stranded RNAs approximately 22 nucleotides in length that are estimated to regulate 5300 human genes.[1] Given their importance, several methods have been developed for the detection of miRNAs[2], however, few allow the simultaneous detection of multiple miRNAs. To overcome this analytical deficiency, the Richert group has recently developed an ingenious method[3] to selectively detect miRNAs on microarrays without interference from endogenous pre-mRNAs, mRNAs and other RNA species.

In the method described by Richert and Vogel[3], a short oligonucleotide containing 3'-amino-dT and a 5' reporter molecule is chemically ligated to the microRNA (Figure 1) in a one-step procedure by *in situ* activation of the microRNA. This is specifically achieved by taking advantage of the fact that miRNAs, unlike other RNAs, are 5'-phosphorylated. The reaction is template-directed (and thus sequence specific) and can be performed together with enzymatic 3'-extension/labeling, either in solution or on a support. The short DNA labeling strand may feature one of a variety of different labels, such as a biotin group or a fluorophore.

FIGURE 1: **MicroRNA 5'-LABELED BY CHEMICAL LIGATION TO A 3'-AMINOTERMINAL LABELING STRAND**

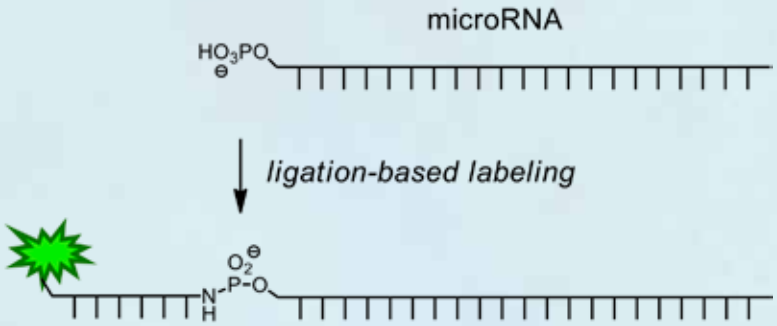
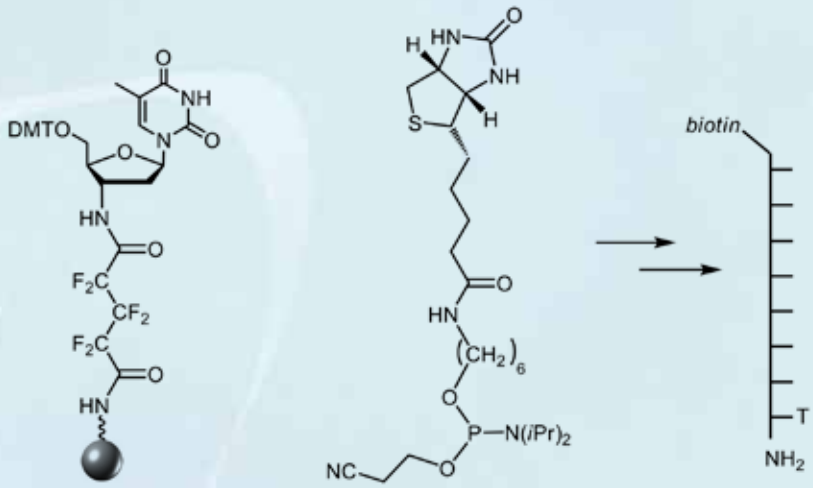


FIGURE 2: **REAGENTS FOR THE SYNTHESIS OF 5'-BIOTINYLATED OLIGONUCLEOTIDES**

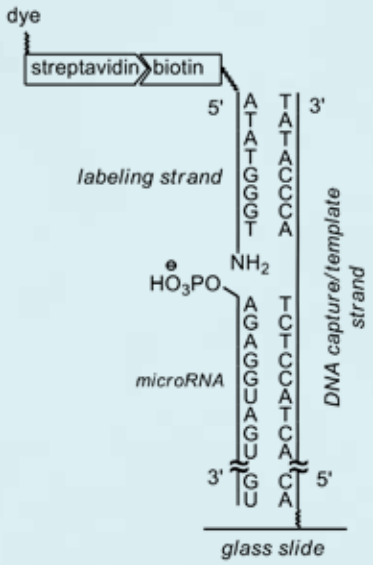


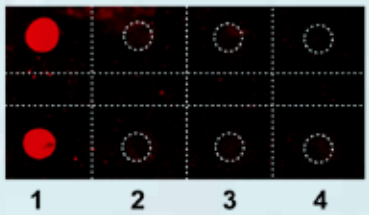
CPG loaded with 3'-aminothymidine

5'-Biotin Phosphoramidite

labeling oligonucleotide with 5'-biotin

FIGURE 3: **RESULT OF ENZYME-FREE 5'-LABELING OF LET7D ON A MICROARRAY**





labeled microRNA (let7d) AGAGGUAGUAGGUUGCAUAGU

capture strands

1	let7d	TTTTTACTATGCAACCTACTACCTCTACCCATATCCA
2	miR17	TTTTTCTACCTGCACTGTAAGCACTTTGACCCATATCCA
3	miR106a	TTTTTCTACCTGCACTGTAAGCACTTTTACCCATATCCA
4	negative control	TTTTTTTGAGTGAGATTGGTAGAAAGTACCCATATCCA

Left: Components on a molecular level. Upper Right: The spot pattern and a fluorescence image of the microarray in pseudocolor. Lower Right: The sequences of the microRNAs in 5' to 3'-direction.

PROCEDURE

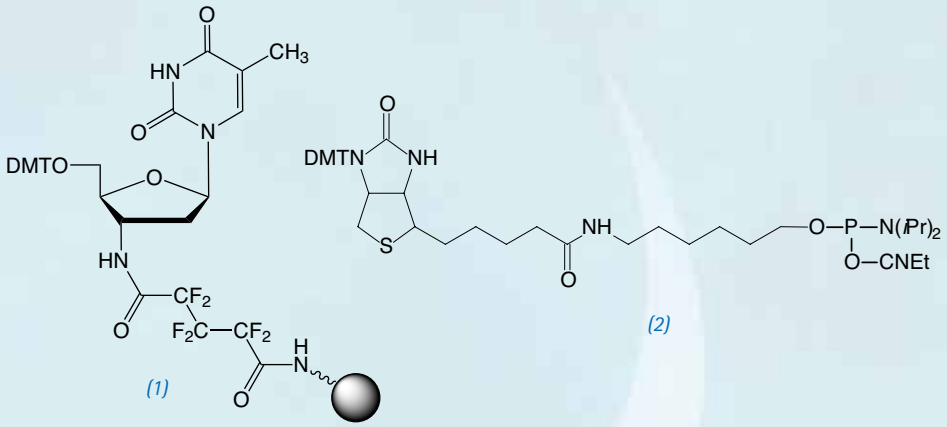
First, a microarray is spotted with capture sequences for the miRNAs of interest. These capture sequences have attached to their 3′ terminus, a short sequence that is complementary to a labeling oligo. The labeling oligo is short – only 8 nucleotides in length – and contains a 5′ label such as biotin or a fluorescent dye, and on the 3′ terminus, a 2′,3′-dideoxy-3′-amino dT (3′-Amino-dT) nucleoside.

When the miRNAs and labeling oligo are hybridized onto the capture sequence, the 5′-phosphate of the miRNA is brought into close proximity to the 3′-amino of the labeling oligo, as shown on the left side of Figure 3. By activating the phosphate *in situ* with 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC), the 3′-amino group of the labeling oligo reacts to form a stable phosphoramidate linkage, chemically ligating the miRNA and the labeling oligo. Because the Tm of the ligated labeling oligo is significantly higher than the non-ligated labeling oligo, the unreacted labeling oligo can easily be washed away, affording a very low background signal, as seen in the pseudocolor image of the microarray in Figure 3.

This procedure makes microarrays an attractive tool for the detection of these medically relevant species. Figure 3 details the result of a microRNA detection assay performed with a biotinylated labeling strand on a microarray. The microRNA let7d was labeled by chemical ligation with subsequent staining of the biotins *via* Cy5-bearing streptavidin. The fluorescence scan shows little background and exquisite[3] discrimination against other RNAs, including closely related microRNAs.

For the differentiation between microRNAs that only differ in a base at the 5′-terminus, false positive signals obtained for enzymatic labeling could be avoided with chemical 5′-labeling. Chemical ligation at the 5′ terminus of miRNA has also been successfully combined with enzymatic 3′-labeling using an on-chip protocol, with reactions performed directly on microarrays. It was found that the dual labeling with two different dyes greatly improves selectivity when detecting closely sequence-related microRNAs and can be used to massively reduce background via subtraction of unmatched signals.

FIGURE 4: **STRUCTURES OF 3′-AMINO-2′,3′-ddT CPG AND 5′-BIOTIN PHOSPHORAMIDITE**



3′-Amino-dT CPG[4], (1) in Figure 4, may also be used to prepare aminoterminal oligonucleotides for other applications, such as chemical primer extension.[5]

OLIGO SYNTHESIS AND DEPROTECTION

To obtain an oligo modified with a 3′-amino-dT residue, the Richert group developed the 3′-amino-dT CPG, (1) in Figure 4.[4] After standard synthesis and labeling of the 5′ terminus with a fluorophore or biotin, the 3′-amino-dT labeled oligo is isolated on deprotection with ammonium hydroxide. Following standard purification protocols using Glen-Pak cartridges or RP HPLC, the labeling strand can be used without special steps or precautions.

Notes:

1. This support is not compatible with modifiers requiring mild or UltraMild deprotection and it is not compatible with UltraFast deprotection with AMA.
2. The cleavage of the hexafluoroglutaroyl linker requires a minimum of 17 hours at 55°C with fresh ammonium hydroxide (28-30%) in water. A small percentage of the linker (~5%) may remain and full cleavage may require up to 30 hours at 55°C.

References:

1. B.P. Lewis, C.B. Burge, and D.P. Bartel, *Cell*, 2005, **120**, 15-20.
2. K.A. Cissell, and S.K. Deo, *Anal Bioanal Chem*, 2009, **394**, 1109-16.
3. H. Vogel, and C. Richert, *ChemBioChem*, 2012, **13**, 1474-82.
4. R. Eisenhuth, and C. Richert, *Journal of Organic Chemistry*, 2008, **74**, 26-37.
5. E. Kervio, A. Hochgesand, U.E. Steiner, and C. Richert, *Proc Natl Acad Sci U S A*, 2010, **107**, 12074-9.

We thank Clemens Richert and Heike Vogel for their help in preparing this article. We also hope that this microarray procedure will develop into an attractive tool for the detection of these medically-relevant miRNA species.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
5′-Biotin Phosphoramidite	10-5950-95	50 µmole	125.00
	10-5950-90	100 µmole	225.00
	10-5950-02	0.25g	650.00
3′-Amino-dT CPG	20-2981-01	0.1g	120.00
	20-2981-10	1.0g	995.00
1 µmole columns	20-2981-41	Pack of 4	200.00
0.2 µmole columns	20-2981-42	Pack of 4	120.00
10 µmole column (ABI)	20-2981-13	Pack of 1	500.00
15 µmole column (Expedite)	20-2981-14	Pack of 1	750.00

ADVANCES IN COPPER(I)-CATALYZED AZIDE-ALKYNE CYCLOADDITION (CUAAC)

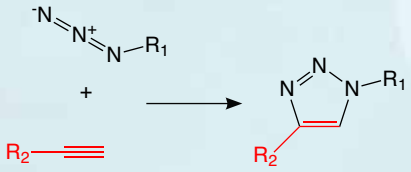
Antonio Manetto, Simon Warncke and Thomas Frischmuth
baseclick GmbH
Bahnhofstrasse 9 – 15
82327 Tutzing, Germany
info@baseclick.eu

Note: All products of baseclick described in this article 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

In 2010, we published an article in The Glen Report, Volume 22, No 1, describing the technology that baseclick has offered for Click Chemistry. In the present article, we review advances since that time and specifically highlight our new Oligo-Click Kits, which are designed to make conventional Click reactions much more user-friendly. At the same time, we position these techniques in comparison to Cu-Free Click.

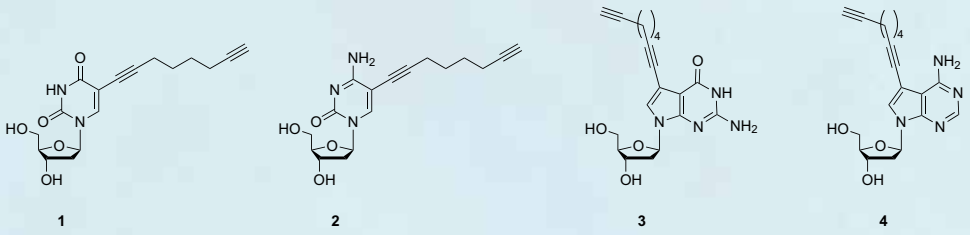
The copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) is the most prominent example of a group of reactions named click-reactions, as shown below.



These reactions are characterized by high yields, mild reaction conditions, and by their tolerance of a broad range of functional groups.[1] 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.[2] As these problems have now been overcome by the use of copper(I)-stabilizing ligands (*e.g.*, tris(benzyltriazolylmethyl)amine, TBTA[3]), 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.[4]

FIGURE 1: STRUCTURES OF IDEAL CLICK NUCLEOSIDES



1 2 3 4

In comparison to the common post synthetic labeling methods of oligonucleotides like amine/NHS-ester, thiol/iodoacetamide or maleimide labeling, modification of oligonucleotides with Click Chemistry is providing by far the highest conjugation efficiency.[6]

Single and multiple labeling can be performed with as little as two equivalents of label-azides resulting in complete conversion and high yields of labeled oligo. In addition, the marker azides used for click functionalization are stable to hydrolysis which allows storage in solution (in contrast to sensitive NHS esters and maleimides). Excess amounts can even be recovered after the click reaction.

BASECLICK AND GLEN 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 labeling of alkyne modified oligonucleotides, our nucleosides provide a 5-(octa-1,7-diynyl) side chain. Phosphoramidites of nucleosides **1**-**4** (Figure 1) 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).

Since alkyne-modified nucleoside phosphoramidites are incorporated into DNA strands during solid-phase synthesis in excellent yields and even stabilize the DNA-duplexes, Glen Research offers the dC and dT analogues, shown in Figure 2 on the following page, under license from baseclick.

CLICK-REACTION ON OLIGONUCLEOTIDES

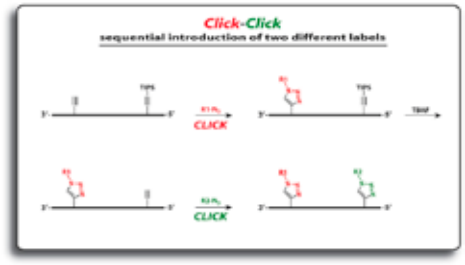
Purified oligonucleotides bearing a single alkyne moiety are usually modified with 2-5 equivalents of the corresponding marker-azide (*e.g.*, fluorescent-dye azides). After the addition of precomplexed Cu(I), complete conversion to the labeled oligo is observed in a time span of between 30 minutes and 4 hours. After a simple precipitation step, labeled oligonucleotides can be recovered in near quantitative yields.

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

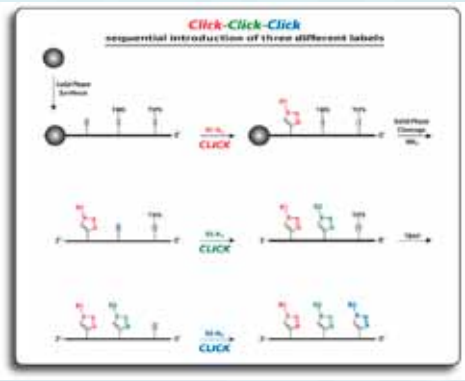
MULTIPLE SEQUENTIAL LABELING WITH UP TO THREE DIFFERENT MARKER AZIDES

For the attachment of up to three different labels, phosphoramidites **with** the alkyne groups protected with triisopropylsilyl (TIPS) and trimethylsilyl (TMS) protecting groups have been developed.[5]

In order to modify oligonucleotides with two sensitive molecules, two alkyne nucleosides, one with no alkyne protection and the second with TIPS protection, 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).



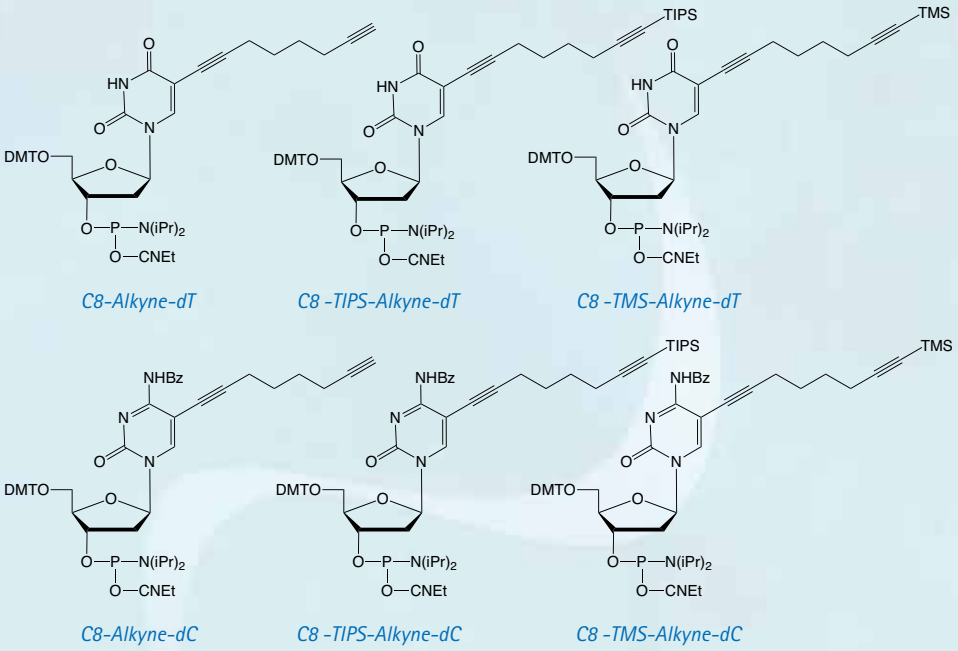
For the introduction of three different labels, three alkyne nucleosides, one with no alkyne protection, the second with TIPS protection, and the third with TMS protection, are introduced into oligonucleotides. The first click reaction is performed directly on the resin. The singly modified oligonucleotide is subsequently cleaved from the support with 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.



RECENT ADVANCES

CuAAC requires the direct use of Cu(I) such as cuprous bromide (CuBr) or a source for Cu(I) such as the combination of Cu(II) salts and a reducing agent (e.g., CuSO4 and sodium ascorbate). The presence of a Cu(I)-stabilizing ligand, such as TBTA, increases the efficacy and decreases the reaction time of the CuAAC. For optimal reaction results, solutions must be freshly prepared and eventually degassed prior to use. Solubility of the ligand TBTA in diluted aqueous solutions may be an issue as well. Although this is not burdensome for regular use, occasional users can find the process troublesome.

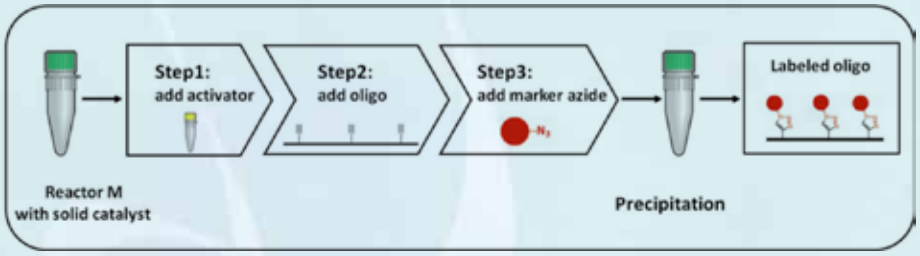
FIGURE 2: **STRUCTURES OF DC AND DT CLICK PHOSPHORAMIDITES**



C8-Alkyne-dT *C8 -TIPS-Alkyne-dT* *C8 -TMS-Alkyne-dT*

C8-Alkyne-dC *C8 -TIPS-Alkyne-dC* *C8 -TMS-Alkyne-dC*

FIGURE 3: **PROCEDURE FOR LABELING WITH OLIGO-CLICK KIT**



Click Chemistry labeling of oligonucleotides with the new Oligo-Click Kits: a simple reaction procedure and minimal hands-on time make oligolabeling even more reliable

To overcome these limitations, baseclick now offers a simple solution: the Oligo-Click Kits. These kits contain an air-stable, insoluble Cu(I) source in pellet form in a pre-loaded and ready-to-use vial. Within the kit, the TBTA ligand is replaced by an activator which is compatible with both aqueous and organic solvents. This innovative combination of catalyst and ligand/activator results in a much easier labeling work-flow including only three simple steps. The preparation of the oligonucleotide labeling via CuAAC now requires only a minimal hands-on time of a few minutes or even less and can be carried out in air without any additional precautions (Figure 3).

The CuAAC reaction time depends on many factors such as label size, oligonucleotide sequence, number of alkynes and other modifications within the sequence, reaction temperature, concentration of the oligonucleotide, as well as the azide in the reaction mixture. Typically, a complete conversion of a single labeled oligonucleotide using the Oligo-Click Kit is achieved in less than 1 hour when operating at 45 °C with an alkyne / azide ratio of 1:2. Lower reaction temperatures (e.g., room temperature or even 4 °C) can be efficiently applied as well in combination with longer reaction times (4 hour and overnight, respectively). The efficacy of the CuAAC reaction remains very high and in some cases even superior to the efficacy of the CuBr / TBTA system.

Labeling of oligonucleotides containing more than two alkynes normally requires the use of a larger amount of azide – up to an alkyne / azide ratio of 1:25 – as reported in the table within the kit user manual.

After the reaction, the labeling mixture is simply transferred into a new vial and

the solid catalyst is discarded. No filtration is needed during this step due to the size of the catalyst pellets. Further processing of the reaction may include a precipitation step (e.g., ethanol precipitation), which removes excess of label-azide, activator and eventually organic solvents used to dissolve the azide. Thus, labeled oligonucleotides can be recovered in near quantitative yields.

AVAILABLE OLIGO-CLICK KITS

Oligo-Click Kits are now available in two different sizes from baseclick:

- *Oligo-Click S (optimal for labeling of up to 10 nmol single and double alkyne oligonucleotide)*
- *Oligo-Click M (optimal for labeling of up to 100 nmol single or multi alkyne oligonucleotide)*

The Oligo-Click Reload series (S and M) provides one vial containing the activator (yellow-capped vial) along with the reactor (green-capped vial) containing the catalyst in pellet form.

Azides are available in red-capped vials within the following Oligo-Click kits:

- *Oligo-Click 488 for kits containing FAM-azide*
- *Oligo-Click 555 for kits containing TAMRA-azide*
- *Oligo-Click Biotin for kits containing Biotin-azide*

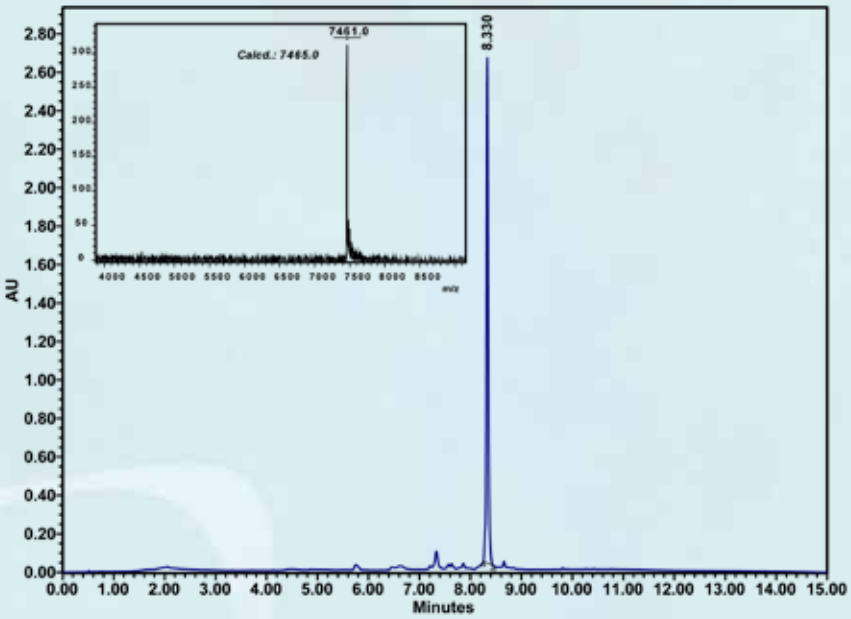
Figure 4 shows an example of RP-HPLC and MALDI-mass spectrum measured directly after the click reaction. In this case, an oligonucleotide containing two internal alkynes was labeled with ATTO425-azide using the Oligo-Click M Kit followed by a simple ethanol precipitation step without further purification.

The results show the outstanding efficiency of the Oligo-Click Kit and of CuAAC in general.

COMPARISON WITH CU-FREE CLICK

It is possible to construct highly strained ring systems containing alkyne groups that will allow Click reactions to occur without the need for a copper catalyst. However, these Cu-free Click reagents tend to be simple 5'-modifiers or dT derivatives.

FIGURE 4: **HPLC AND MALDI ANALYSIS OF OLIGO LABELED WITH 2 ATTO DYES USING OLIGO-CLICK KIT**



18mer containing two internal alkynes reacted with 2.5 equivalents of ATTO425-Azide (MW = 602 g/mol), 4 h at 45 °C. MALDI-mass analysis of the crude product -> 100% oligo-dye conjugate (Calcd. 7465; Found 7461).

The catalog of alkyne derivatives for CuAAC is substantially greater. At this point, no Cu-free options are available for attaching more than one type of label or tag to oligonucleotides.

Moreover, the amount of Cu ions during the CuAAC using the Oligo-Click Kits generally does not exceed 100 ng/µL (colorimetric complexation detection). This value drops to 50 ng/µL after a simple precipitation step (e.g., EtOH-precipitation) and it is further reduced to 5-10 ng/µL after RP HPLC purification. Gel purification or ion-exchange filtrations abate the Cu ions content below the detection limit of 0.01 ng/µL.

CONCLUSION

The easy-to-use copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) 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. With baseclick's addition of the Oligo-Click Kits many factors have been radically improved such as the stability of the reagents, their solubility and the comfortable handling of CuAAC, thus providing a powerful labeling tool for chemistry and biology labs.

References:

1. C.W. Tornoe, 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 labeling strategies for the sensitive detection of analytes

   The following patents have been granted:
   EP 1877415
   US 8,129,315

2. WO2008/952775, Click Chemistry for the production of reporter molecules

3. Baseclick GmbH holds a worldwide license for the research market of the "Click Chemistry" patent from "The Scripps Research Institute":
   WO03/101972, Copper-catalysed ligation of azides and acetylenes

GLEN RESEARCH OFFERINGS

Glen Research has collaborated with baseclick for several years on this product line. We have been offering the dC and dT analogues, shown in Figure 2 on Page 9, for simple Click conjugations as well as sequential labeling with up to three separate azides.

We are delighted to be able to offer Oligo-Click Kits to our research customers. Our most popular scale of synthesis is 200 nmoles, so we are offering Oligo-Kit M. This kit has sufficient reagents for conjugating up to nine alkyne-containing oligonucleotides on a 100 nmole scale or a single oligonucleotide on a 1 µmole scale. The user must supply the azide and a solvent such as DMSO for dissolving the azide.

We are also offering kits for biotin, fluorescein and TAMRA labeling. Each kit has sufficient reagents for conjugating up to seven alkyne-containing oligonucleotides on a 100 nmole scale or a single oligonucleotide on a 1 µmole scale. Each kit contains all of the ingredients necessary, including the azide and DMSO solvent.

Our experience with these kits indicates that they will be of enormous help to customers carrying out click conjugation reactions on an occasional basis.

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	270.00
	10-1542-90	100 µmole	525.00
	10-1542-02	0.25g	1275.00
C8-Alkyne-dC-CE Phosphoramidite	10-1543-95	50 µmole	225.00
	10-1543-90	100 µmole	435.00
	10-1543-02	0.25g	1125.00
C8-TIPS-Alkyne-dT-CE Phosphoramidite	10-1544-95	50 µmole	220.00
	10-1544-90	100 µmole	425.00
	10-1544-02	0.25g	1020.00
C8-TMS-Alkyne-dT-CE Phosphoramidite	10-1545-95	50 µmole	205.00
	10-1545-90	100 µmole	395.00
	10-1545-02	0.25g	1050.00
baseclick Oligo-Click-M-Reload (Sufficient for up to 9 conjugations at the 100 nmole scale. Does not include azide or solvent)	50-2100-01	each	120.00
baseclick Oligo-Click-M-Biotin (Sufficient for up to 7 conjugations at the 100 nmole scale. Includes azide and solvent.)	50-2101-01	each	200.00
baseclick Oligo-Click-M-Fluorescein (Sufficient for up to 7 conjugations at the 100 nmole scale. Includes azide and solvent.)	50-2102-01	each	240.00
baseclick Oligo-Click-M-TAMRA (Sufficient for up to 7 conjugations at the 100 nmole scale. Includes azide and solvent.)	50-2103-01	each	270.00

TECHNICAL BRIEF: FRETmatrix – A METHOD FOR SIMULATION AND ANALYSIS OF FRET IN OLIGOS

Søren Preus
Department of Chemistry
University of Copenhagen
Copenhagen, DK-2100, Denmark

Marcus Wilhelmsson
Department of Chemical and Biological Engineering/Physical Chemistry
Chalmers University of Technology
Kemivägen 10, SE-412 96 Göteborg, Sweden

The recent introduction of nucleobase analogues tC, tC^{o} and tC$_{nitro}$ (Figure 1) into the arsenal of commercial FRET probes has provided researchers with a highly useful alternative to traditional, external dyes when studying the structure, dynamics and function of nucleic acids. Since not only the interpair-distance but also the probe orientation play an important role in the energy transfer process, FRET between rigidly attached nucleobase analogues (base-base FRET) can be exploited to obtain both positional and orientational information about nucleic acid geometries without complications associated with linker flexibility. The design and interpretation of base-base FRET experiments has up until now been challenging to a large majority of researchers.

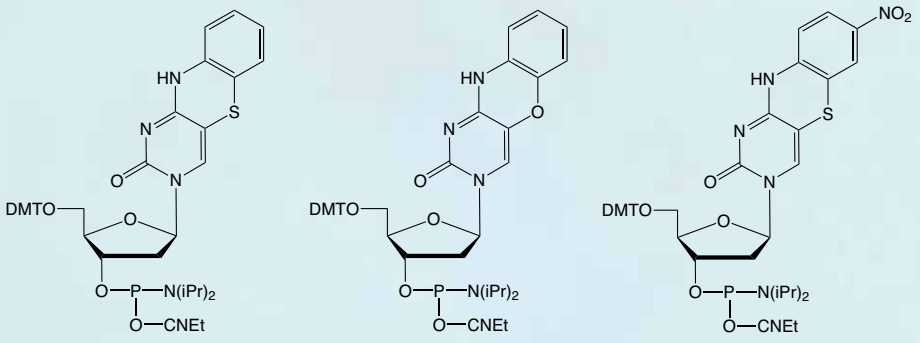
Addressing this issue, Wilhelmsson and co-workers[1] have developed a freely downloadable software package (FRETmatrix) divided into two parts:

(1) the design and (2) the analysis of base-base FRET experiments.

One part of the software simulates theoretical FRET efficiencies between probes positioned in any 3D nucleic acid structure. To significantly facilitate the design and identify optimal positions of the FRET-probes in oligonucleotides of base-base FRET experiments, the software is used to predict theoretical signal changes between all possible donor-acceptor positions in a nucleic acid sequence, e.g., upon binding of a protein or some other structural change (Figure 2).

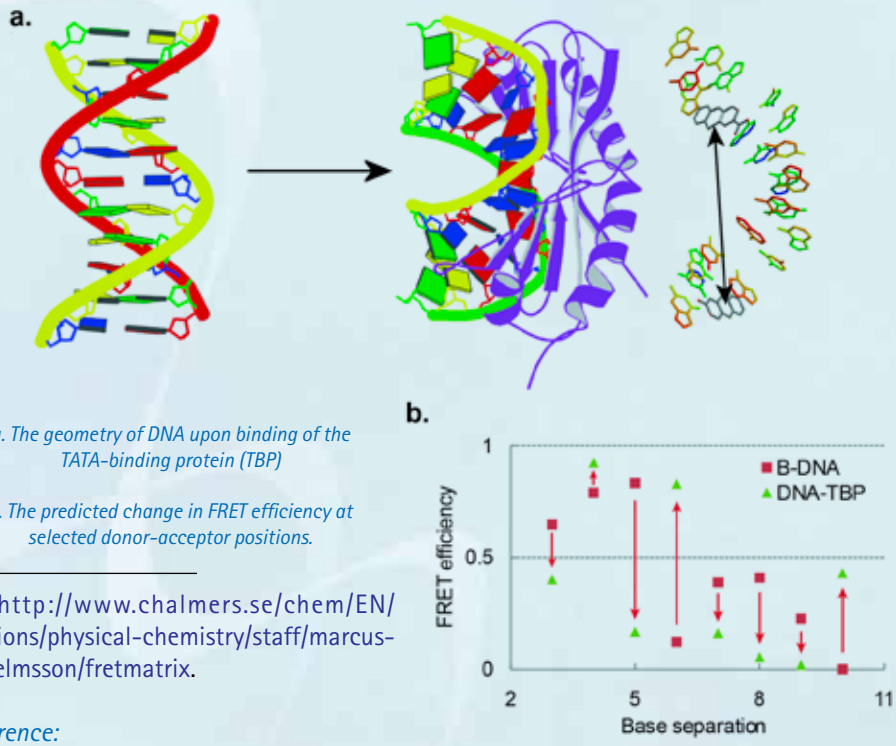
To analyse base-base FRET experiments quantitatively, multiple time-resolved fluorescence decays can be analyzed simultaneously in the second part of the freeware. This novel, rigorous analysis may provide insight into global and/or local 3D structural features, such as the geometry of kinked DNA or the position, orientation and dynamics at specific base positions. The MATLAB-based software is freely available from the following link:

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



tC-CE Phosphoramidite (1) *tC^{o}-CE Phosphoramidite (2)* *tC$_{nitro}$-CE Phosphoramidite (3)*

FIGURE 2: **EXAMPLE OF A BASE-BASE FRET EXPERIMENT SIMULATED USING FRETmatrix**



a. The geometry of DNA upon binding of the TATA-binding protein (TBP)

b. The predicted change in FRET efficiency at selected donor-acceptor positions.

http://www.chalmers.se/chem/EN/divisions/physical-chemistry/staff/marcus-wilhelmsson/fretmatrix.

Reference:

1. S. Preus, K. Kilsa, F.A. Miannay, B. Albinsson, and L.M. Wilhelmsson, *Nucleic Acids Res,* 2012.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
tC-CE Phosphoramidite	10-1516-95	50 µmole	250.00
	10-1516-90	100 µmole	490.00
	10-1516-02	0.25g	1460.00
tC^{o}-CE Phosphoramidite	10-1517-95	50 µmole	250.00
	10-1517-90	100 µmole	490.00
	10-1517-02	0.25g	1460.00
tC$_{nitro}$-CE Phosphoramidite	10-1518-95	50 µmole	265.00
	10-1518-90	100 µmole	520.00
	10-1518-02	0.25g	1460.00

Glen Research offers a selection of 5'-Amino-Modifiers but which one is appropriate for which application? In this Technical Brief, we detail the pros and cons of each and attempt to direct our customers to the optimal solution. Our most popular 5'-Amino-Modifiers are the C6 versions and this article will focus exclusively on these products but the arguments apply equally well to C3, C12 and hydrophilic TEG spacers.

TRITYL-ON PURIFICATION

4-Monomethoxytrityl (MMT)

If you wish to purify a 5'-amino-modified oligonucleotide, the MMT group is a good choice since the oligo can be easily purified by reverse phase techniques. The MMT group is then removed post purification with aqueous acid. Also, the MMT group can be removed by extended deblocking on the synthesizer, allowing a solid-phase conjugation of a tag containing an activated carboxylic acid. However, the tag must be stable to the subsequent conditions of cleavage and deprotection.

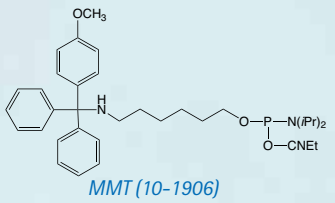
The main problem we have found with this protecting group is that it has a tendency to stay on when it should be removed and fall off when it should stay on. Three key steps can be followed to avoid this problem:

- *Do not remove the MMT group on the synthesis column unless you plan to conjugate the amine while the oligo is still on the support.*
- *Do not dry down the solution of MMT-on oligo if you plan to do MMT-on purification without first adding a non-volatile base, such as TRIS base, to avoid MMT loss.*
- *Do not remove the MMT group during cartridge purification since it will predominantly reattach. Rather, remove the MMT with aqueous acetic acid after purification. Using 20% glacial acetic acid/80% water allows extraction of MMT-OH using ethyl acetate before drying the solution. Or dry the oligo and desalt by your preferred method.*

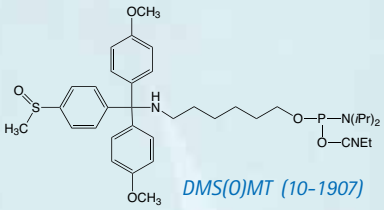
4,4'-Dimethoxy-4''-methylsulfonyl-trityl (DMS(O)MT)

The DMS(O)MT protecting group is designed to be an improvement over MMT while retaining MMT's useful characteristics. The DMS(O)MT group is much more reliable in routine use than MMT and is especially useful for trityl-on purification techniques. Unlike MMT, the DMS(O)MT protecting group can be removed using aqueous TFA on the reverse phase purification cartridge without the need for later removal in solution. Although a substantial improvement over MMT in most ways, the following two key points should be remembered:

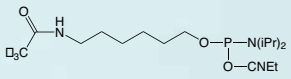
FIGURE 1: STRUCTURES OF 5'-AMINO-MODIFIERS C6



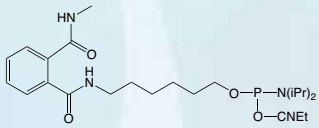
MMT (10-1906)



DMS(O)MT (10-1907)



TFA (10-1916)



PDA (10-1947)

- *Do not remove the DMS(O)MT group on the synthesis column unless you plan to conjugate the amine while the oligo is still on the support.*
- *Do not dry down the solution of DMS(O)MT-on oligo if you plan to do DMS(O)MT-on purification without first adding a non-volatile base, such as TRIS base, to avoid DMS(O)MT loss.*

NO PURIFICATION NECESSARY

Trifluoroacetyl (TFA)

The base-labile TFA group is used when oligo purification prior to conjugation is not necessary. Since only the amino-modified oligo is full-length, the conjugation reaction will select for the full-length oligo. However, there is one key point to be concerned about when using this product. Since an alkylamine is quickly formed on deprotection, it is possible for side reactions to occur which will reduce the amount of amine available for the desired conjugation reaction. This can be minimized by taking steps to avoid two side reactions:

- **Cyanoethylation**

Treat the newly synthesized oligo with 10% diethylamine (DEA) in acetonitrile while still on the support. A simple 5 minute treatment with 1 mL of 10% DEA in acetonitrile, followed by a rinse with acetonitrile will remove all acrylonitrile.

- **Transamidation**

The oligo is then cleaved and deprotected using AMA (ammonium hydroxide/40% methylamine, 1:1 v/v) in UltraFast conditions which minimizes transamidation.

Phthalic acid diamide (PDA)

We are excited about the potential of 5'-PDA-Amino-Modifiers, which were developed by Stefan Pitsch along with Stefan Berger of ReseaChem in Switzerland, and introduced by Glen Research in the spring of 2012. In contrast to the other protected amino modifiers which are viscous oils, the analogous PDA protected compounds are granular powders. This important property of these compounds allows straightforward handling, storage and aliquoting and leads to a significant increase in stability. This property alone makes 5'-PDA-amino-modifiers the first choice for high throughput DNA modification but their structure also allows a significant cost advantage over their oily counterparts.

Again two key points should be noted:

- *Oligonucleotides containing PDA-Amino-Modified oligonucleotides must be treated with aqueous methylamine or AMA for complete deprotection.*
- *If ammonium hydroxide must be used, reaction at 55°C will yield around 80% of the deprotected amino group, even with extended deprotection times.*

TABLE 1: COMPARISON OF 5'-AMINO-MODIFIERS C6

	DMT-ON Purification	No Purification Needed	On-Column Conjugation	Granular Powder	Viscous Oil	Lowest Price
MMT (10-1906)	✔	✘	✔	✘	✔	✔-
DMS(O)MT (10-1907)	✔	✘	✔	✘	✔	✔-
TFA (10-1916)	✘	✔	✘	✘	✔	✔
PDA (10-1947)	✘	✔	✘	✔	✘	✔✔

Technical Bulletins:

http://www.glenresearch.com/Technical/TB_MMT_Amino_Modifiers.pdf
http://www.glenresearch.com/Technical/TB_DMSOMT.pdf
http://www.glenresearch.com//Technical/TFA-Amino-Modifiers.pdf
http://www.glenresearch.com//Technical/TB_avoidaminealkylation.html

TECHNICAL BRIEF - IMPROVED CONDITIONS FOR DEPROTECTING 5-FORMYL-DC

5-Formyl-dC appears to be a crucial intermediate in the demethylation pathway of 5-methyl-dC where it is recognized and excised by Thymidine-DNA glycosylase base-excision repair enzymes, leading to the insertion of a non-methylated deoxycytidine.[1]

While 5-Formyl-dC (1) has been a popular product, its use is complicated by a side reaction which occurs when deprotecting the oligo in ammonia or methylamine solutions. Rather than attacking the carbonyl of the acetyl protecting group, the nucleophile attacks the carbon, displacing acetate as a leaving group. Now vicinal to the second acetyl protecting group, it quickly attacks the carbonyl, leading to the formation of a substituted N-acetamide, rather than the desired glycol. The proposed mechanism is shown in Figure 1.

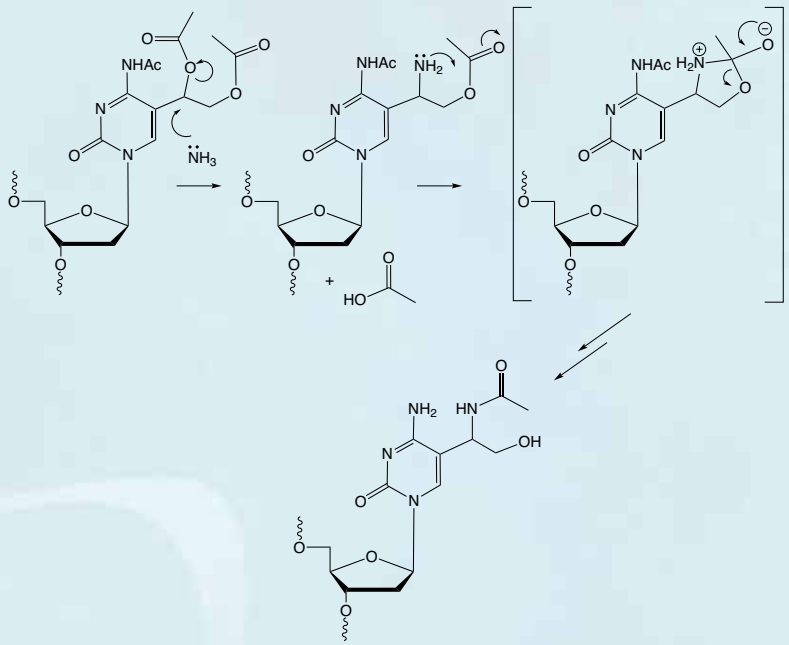
One solution to this problem is to use hydroxide as the nucleophile rather than ammonia or methylamine. This way, even if the acetate displacement occurs, it is a transparent substitution that still affords the desired 5-glycolyl-deoxycytidine, which, upon oxidation with sodium periodate, yields the desired 5-formyl-dC. Typically, 0.4 M NaOH in MeOH/water 4:1 (v/v) for 17 hours at room temperature is used to deprotect the oligo.

However, difficulties can arise. The first thing to consider is the choice of protecting groups for the dG and dC bases. Quite surprisingly, dimethylformamidine (dmf), a popular protecting group for guanosine, is remarkably resistant to the sodium hydroxide solution. While an isobutyryl-protected dG is cleanly deprotected in 17 hours, the dmf group requires over 72 hours at room temperature.

In addition, if benzoyl-protected dC is used, nucleophilic displacement of benzamide can occur due to hydroxide attack of the C4 carbon, leading to deamination and a dC to dU mutation. This can be avoided if acetyl-protected dC (Ac-dC) is used during synthesis.

A final concern is that the yield of oligos can be highly variable when using 0.4 M NaOH in MeOH/water for deprotection. The CPG will tend to fuse to a solid cake in the bottom of the vial of larger-scale syntheses and the oligo will tend to precipitate onto the CPG. However, we found that by first briefly sonicating the vial to break up the CPG, pipetting off the supernatant and then rinsing the CPG with water, will consistently give good oligo yields.

FIGURE 1: PROPOSED MECHANISM FOR IMPURITY FORMATION WITH AMMONIUM HYDROXIDE

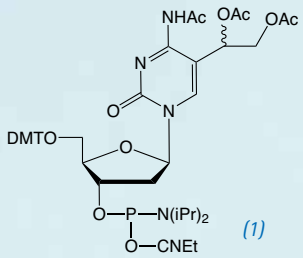


Note, the use of sodium hydroxide necessitates a desalting step. This can be accomplished, while simultaneously purifying the oligo, by using a Glen-Pak™ purification cartridge. An optimized protocol (for a 1 µmole synthesis scale or smaller) is given below. With this procedure, multiple incorporations of the formyl-dC can be introduced into an oligo cleanly.

Improved Protocol for Deprotection and DMT-On Glen-Pak Purification of Formyl-dC

1) Make a fresh solution of 0.4 M NaOH in MeOH/H2O 4:1 (v/v).
2) Treat the column with 3 mL of 10%DEA in ACN for 2 minutes, pushing the solution back and forth occasionally. Rinse with ACN and dry the CPG.
3) Transfer the CPG to a vial and add 1 mL of 0.4 M NaOH in MeOH/H2O 4:1 (v/v).
4) Allow to react for 17 hours at room temperature.
5) Briefly sonicate the vial to break up the CPG.
6) Pipette off the supernatant and transfer to a clean vial. Rinse the CPG with 250 µL of water and combine with the cleaved oligo.

FIGURE 2: STRUCTURE OF 5-FORMYL-dC



(1)

7) Dilute to 10 mL with 100 mg/mL NaCl in water.
8) Load onto a prepped Glen-Pak cartridge (60-5200-xx) fitted with a 10 mL syringe and purify using the standard protocol but beginning with the Salt Wash Step – i.e., the 2 mL rinse with 100 mg/mL NaCl containing 5% ACN.
9) Dry the purified oligo and dissolve it in 250 µL of water. Chill at 4 °C.
10) Make a fresh solution of 50 mM $NaIO_4$ (~~26.75~~ 2.7mg per 250 uL water)
11) Add 250 µL of 50 mM $NaIO_4$ to the chilled oligo solution, briefly vortex and return it to the refrigerator. Keep at 4 °C for 30 minutes.
12) Add 3 mL 0.1 M TEAA and desalt on a prepped Glen-Pak cartridge.

Reference:

1. A. Maiti, and A.C. Drohat, *J Biol Chem*, 2011, **286**, 35334-8.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
5-Formyl-dC-CE Phosphoramidite	10-1514-95	50 µmole	610.00
	10-1514-90	100 µmole	1200.00
	10-1514-02	0.25g	3225.00

NEW PRODUCTS: UNIVERSAL HYBRIDCPG™ SOLID SUPPORTS

Marc L. Rothstein and Dianne M. Rothstein
Prime Synthesis, Inc.
2 New Road, Suite 126
Aston, PA 19014

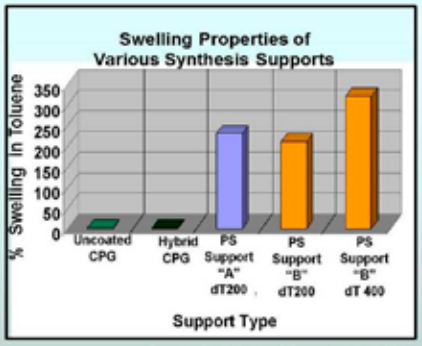
Controlled Pore Glass (CPG) has been widely used as a support for the solid phase synthesis of oligonucleotides. It provides a unique combination of uniform pore size, dimensional stability in synthesis solvents and a pore network structure that facilitates rapid mass transfer during a synthesis. Pore diameters of 500 to 2000 Angstroms are commonly used for this application.

To avoid steric hindrances, the larger pore sizes are used for synthesizing longer oligos. However, since the surface area of CPG is inversely related to the pore size and the density of silanol attachment groups are a function of surface area, large pore CPG has less ligand loading capacity. Additionally, not all silanol attachment points are evenly distributed, with some being too crowded together to be useful for full-length oligo growth. For this reason, maximum loading densities are not used for high purity synthesis. Thus, due principally to steric considerations, oligo length and purity is limited by pore size and loading density. A further limitation of CPG is that some reagents are corrosive to the glass substrate and can cause inconvenient silica precipitates during the oligo work-up or even complete failure in the case of reagents such as acidic fluorinated deblocking solutions.

Porous polystyrene beads have also been popular for oligo synthesis. Highly crosslinked polystyrene is used for micro-scale synthesis. This type of polystyrene is preferred for use in pre-packed synthesis columns, since it does not swell upon exposure to synthesis solvents. The combination of high crosslinking with a relatively broad pore size distribution limits useful ligand loading densities to about 30 µmoles/g. For large scale oligosynthesis, polystyrene of lower crosslinking levels can be used with ligand loadings up to 200-400 µmoles/g. Although these supports can swell up to 6 times their dry volume in synthesis solvents, they can be used in large scale synthesizers by allowing room in the reactor column for this expansion. They have become popular for the synthesis of oligos up to about a 24-mer. However, at these loadings, synthesis of longer oligos is not feasible due to backpressure increases in the reactor bed caused by additional expansion of the polystyrene to accommodate the growing oligo product volume.

FIGURE 1: EFFECT OF SUPPORT SWELLING ON COLUMN LOADING (SYNTHESIS SCALE)

A



B

Support Type	Nucleoside Loading (µmol/g)	MAX 1.2 ml Column Loading (µmol)	MAX 6.3 ml Column Loading (µmol)
Uncoated CPG (1000 Å)	60	22	110
Hybrid CPG (1000 Å)	192	88	440
PS Support "A"	200	50	250
PS Support "B"	400	58.6	308

Figure 1: Effect of Support Swelling on Column Loading (Synthesis Scale)

A. Both uncoated and hybrid CPG have ZERO % swelling in synthesis solvents while polystyrene supports can swell up to 3.5 times in the same conditions.

B. Swelling limitations on column loading are significant in polystyrene based supports: Increased nucleoside loading capacity of non-swelling Hybrid CPG can translate into significant increases in column loading.

TABLE 1: LARGE SCALE SYNTHESIS DATA FROM IN-HOUSE AND FIELD TESTING

Synthesis & Support Properties	Uncoated CPG *	Hybrid CPG*	Uncoated CPG	Hybrid CPG	Hybrid CPG	Hybrid CPG
Sequence	A	A	B	B	B	B
Pore Size (Å)	1000	1500	1000	1000	1360	1780
Support loading (umol/g)	35	90	34	141	137	122
Synthesis Scale (umol)	65	144	45	238	211	223
UV Purity (%)	70	75	73	83.6	83.6	83.0
Total FLP (OD's, A_{260})	5,275	19,285	5250	27,354	28,027	27,826
FLP (OD's/umol)	81	134	117	115	133	125

*Data courtesy of NOXXON Pharma, AG
Sequence A: 5' AGU GAA GCC GUG GCU CUG CG-DdT-3' (L-RNA)
Sequence B: 5' ATA CCG ATT AAG CGA AGT TT-3' (DNA)

Table 1 demonstrates the high performance of a large pore HybridCPG support for some example syntheses made on a large-scale synthesizer (GE Healthcare AKTA 100). Data for two sequences are shown. Note the relative independence of Hybrid CPG pore size to ligand loading. Thus, while it is important to establish a minimum pore size in optimizing the synthesis conditions, the tradeoff of loading capacity is not seen as the pore size is further increased.

HybridCPG™ consists of CPG particles conformally coated with a very thin crosslinked polymer film based on polystyrene. The molecular structure of the polymer coating is designed to have a very high density of evenly distributed attachment points for optimum oligo synthesis. In this way, the pore size - loading density trade-off is minimized and the chemical resistance to glass-aggressive reagents is greatly improved. Although the nano-scale coating is subject to swelling, the rigid pore structure of the CPG substrate accommodates this and, at the same time, maintains a uniform pore space for the oligo synthesis. Thus, HybridCPG exhibits no bulk swelling in the synthesis solvents and allows much higher ligand loadings for a given pore size compared to conventional CPG. As illustrated in Figure 1, the combination of HybridCPG's high ligand loading capacity and freedom from bulk swelling results in much larger column capacities than either conventional CPG or swellable polystyrenes.

Prime Synthesis has partnered with Glen Research to begin offering HybridCPG™ to the research market. We are initially offering two popular universal supports, Glen UnySupport™ and US III. Both supports have a loading of around 75 µmoles/g and support oligo synthesis in the 30-40mer length range. The structures of these supports are shown in Figure 2, Back Page.

The high synthesis performance on these supports is demonstrated by the RP HPLC of a 40mer prepared on US III HybridCPG, as illustrated in Figure 3, Back Page.

HybridCPG™ is a trademark of Prime Synthesis, Inc. Glen UnySupport™ is a trademark of Glen Research Corporation.

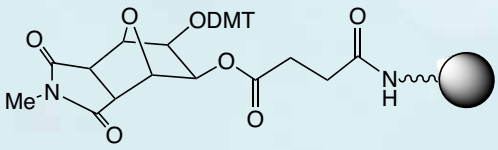
(Continued on Back Page)

GLEN RESEARCH

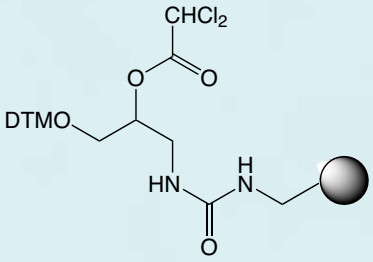
22825 DAVIS DRIVE
STERLING, VIRGINIA
20164

(Continued from Page 15)

FIGURE 2: STRUCTURES OF UNIVERSAL SUPPORTS

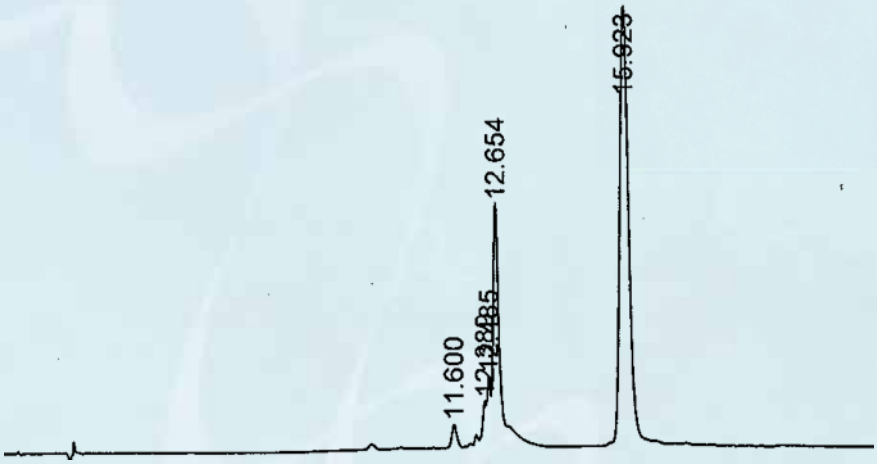


Glen UnySupport



US III

FIGURE 3: RP HPLC OF A MIXED BASE 40MER PREPARED ON US III HYBRIDCPG



ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
Bulk Support			
US III	28-5010-01	0.1g	16.00
(1500Å HybridCPG)	28-5010-02	0.25g	35.00
	28-5010-10	1.0g	125.00
Glen UnySupport	28-5040-01	0.1g	16.00
(1500Å HybridCPG)	28-5040-02	0.25g	35.00
	28-5040-10	1.0g	125.00