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INSIDE

DITHIOL SERINOL

APA - ALTERNATIVE TO AMA

CAPPING 5'-AMINO-MODIFIERS

PSEUDOURIDINE UPDATE

PC BIOTIN AND SOMAscan™

REVERSIBLE PHOTO-SWITCHING OF DNA FUNCTION WITH AZOBENZENE-TETHERED DNA

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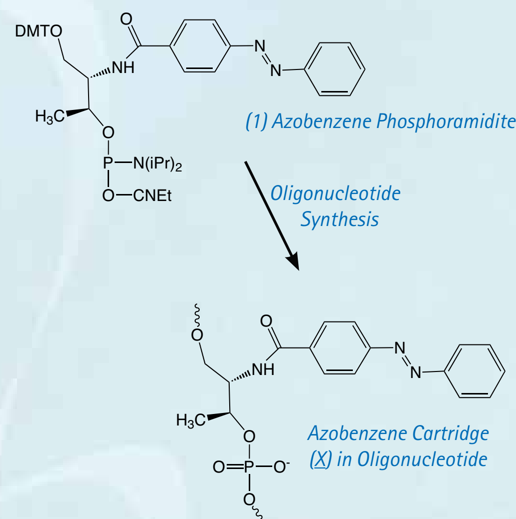
Deoxyribonucleic acids are recognized not only as biomolecules encoding genetic information but also as structural materials in nanotechnology due to their supramolecular properties. The ability to control DNA properties artificially using external stimuli offers great potential for a wide variety of applications. Among external stimuli, light has several advantages: (1) unlike molecular stimuli, light does not contaminate the microenvironment of the reaction system; (2) spatiotemporal control of the reaction is possible; and (3) irradiation wavelengths are tunable by suitable molecular design. In this report, we introduce the photo-responsive DNA that we developed over the past decade.

INTRODUCTION OF AZOBENZENE INTO DNA-LIKE CARTRIDGES

Azobenzene derivatives are the most popular photo-responsive molecules for versatile applications because of their ready availability and chemical stability. A planar *trans* form can be obtained upon photo-irradiation at wavelengths > 400 nm and a nonplanar *cis* form is obtained by photo-irradiation at 300 nm - 400nm. Therefore, azobenzene can reversibly photo-isomerize between *trans* and *cis* forms upon irradiation with the appropriate wavelength of light. On the basis of this property, azobenzene can be used as a photo-switch in a DNA-based nanomachine or a DNA-mediated bioprocess. By synthesizing a cartridge-like unit of azobenzene (Figure 1a),[1] we have attained 1) photo-regulation of the formation and dissociation of a DNA duplex (Figure 1b), and 2) photo-regulation of transcription and translation with photo-responsive T7 promoter (Figure 1c).[2,3]

AZOBENZENE INCORPORATION INTO AN OLIGONUCLEOTIDE



In our methods, we synthesized a phosphoramidite monomer (1) bearing an azobenzene group covalently connected through an amide bond to D-threoninol as the scaffold (above and Figure 1a on Page 2). Use of this monomer allows the simple introduction of an azobenzene cartridge into DNA. To provide the functionality of azobenzene in DNA, the azobenzene cartridge (X) is introduced between base pairs of the DNA. For example, if we want to provide 5'-GCGAGTCG-3' with photo-responsiveness, the X residue is introduced to obtain, e.g., 5'-GCGAXGTCG-3'. The modified DNA strand can still form a duplex with its complementary strand, 3'-CGCTCAGC-5' and all the base pairs are maintained in the duplex. It should be noted that replacing a natural nucleotide with X is not

(Continued on Page 2)

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recommended as it causes destabilization of the duplex.[4] Our NMR analyses revealed that *trans*-azobenzene, which is planar, stabilizes the double-stranded DNA as it intercalates in the duplex and that the non-planar *cis*-azobenzene destabilizes the duplex due to steric hindrance.[4] In order to attain efficient photo-regulation, introduction of multiple azobenzene residues is effective, such as 5′-GCXGAXGTXCG-3′/3′-CGCTCAGC-5′ (right panel of Figure1b). If both strands can be modified, sequence design like 5′-GCXGAXGTXCG-3′/3′-CXGCXTCXAGXC-5′ is particularly effective (left panel of Figure1b). For the introduction of multiple azobenzenes, at least two nucleotides should be inserted between the X residues. Using these design strategies, repetitive and efficient regulation of hybridization and dissociation of the DNA duplex can be promoted by alternating between UV and visible light. In addition to control of DNA duplexes, photo-control of the hybridization of DNA/RNA and RNA/RNA duplexes and triplex DNA can also be achieved by introducing azobenzene as a photo-responsive switch.[5,6]

LIGHT DRIVEN DNA NANO-MACHINE WITH A PHOTO-RESPONSIVE MOLECULAR ENGINE

Recently, DNA has been recognized as a useful material in the field of nanotechnology. From the viewpoint of constructing DNA-based nanomachines, modulating DNA hybridization in response to light provides an interesting application. By using azobenzene modified DNA, we demonstrated a photon-driven DNA nanomachine. We designed a photo-responsive DNAzyme which changes its structure between open and closed forms upon light irradiation in a tweezer-like motion (Figure 2).[7] To make open and closed forms of the DNAzyme, extended azobenzene-modified DNA regions were attached at both the 3′ and 5′ ends of the DNAzyme. In this design, we introduced multiple azobenzenes to increase photo-responsiveness. The DNAzyme with *trans*-azobenzenes was inactive due to being in the closed state caused by hybridization of the extended regions. Upon *cis*-isomerization of the azobenzenes, the extended regions are dissociated and the DNAzyme returned to the active form, inducing RNA cleavage. Alternate irradiation with UV and visible light clearly caused on-off photo-switching of RNA cleavage.

FIGURE 1: AZOBENZENE AMIDITE, REVERSIBLE PHOTO- REGULATION AND PHOTO-SWITCHING

(a) Azobenzene amidite synthesized from D-threoninol for introducing an azobenzene unit into DNA. (b) Reversible photo-regulation of formation and dissociation of the duplex with photo-responsive DNA containing multiple azobenzene units. (c) Reversible photo-switching of transcription by photo-responsive T7 promoter containing two azobenzene units.

PHOTO-REGULATION OF TRANSCRIPTION BY A PHOTO-RESPONSIVE PROMOTER

Artificial control of biofunctions by external stimuli is one of the current hot topics in chemical biology or synthetic biology. Using azobenzene, a photo-triggered gene expression system can be constructed. Isomerization of azobenzenes in the modified T7 promoter allows reversible photo-regulation of the transcription reaction by T7-RNA polymerase (Figure 1c).[8] This regulation is based not on photo-regulated hybridization but on a local structural change of the DNA duplex induced by photo-isomerization of azobenzenes between the *trans* and *cis* forms, allowing control of the DNA-protein interaction.

We have developed an effective photo-regulation design. In this design, the template strand remains intact and two azobenzenes are introduced into the non-template strand of the T7 promotor region. One azobenzene is inserted between -3 and -4 and another is inserted between -9 and -10 in the non-template strand of the T7 promoter region (Figure 1c). The DNA duplex does not dissociate when the azobenzenes are in the *cis* form even though the local structure of the duplex around azobenzene is altered. If the azobenzenes are in the *trans* form, transcription is terminated by inhibition of binding of the polymerase with the promoter region. Upon *cis*-isomerization of azobenzene, transcription is switched on because the unwound promoter region facilitates binding of the polymerase. This change of the binding mode of the polymerase to the photo-responsive promoter allows reversible photo-regulation of the transcription reaction.

In addition to the commercially available azobenzene phosphoramidite, we have also developed several advanced azobenzene derivatives.[9-11] However, most of the photo-regulatory work can be achieved with the current commercial version. Since hybridization is a fundamental supramolecular property of DNA and most of the activity of DNA-based nanomachines is attributed to spontaneous hybridization, these machines can be easily converted to photo-responsive nanomachines just by adding azobenzenes into the sequence. Photo-regulated transcription and subsequent translation, *i.e.*, photo-regulated gene expression, is also possible with a photo-responsive promoter.

References:

1. H. Asanuma, *et al., Angew. Chem. Int. Ed.* 2001, **40**, 2671–2673.
2. H. Asanuma, *et al., Nat. Protoc.* 2007, **2**, 203–212.
3. Y.Kamiya and H. Asanuma, *Acc. Chem. Res.* 2014, **47**, 1663–1672
4. X.G. Liang, *et al., J. Am. Chem. Soc.* 2003, **125**, 16408–16415.
5. H. Ito, *et al., Org. Biomol. Chem.* 2010, **8**, 5519–5524.
6. X.G. Liang, *et al., J. Am. Chem. Soc.* 2002, **124**, 1877–1883.
7. M. Zhou, *et al., Angew. Chem., Int. Ed.* 2010, **49**, 2167–2170.
8. M. Liu, *et al., J. Am. Chem. Soc.* 2003, **128**, 1009–1015.
9. H. Nishioka, *et al., Chem. Commun.* 2007, 4354–4356.
10. T. Fujii, *et al., , Chem. Eur. J.* 2009, **15,** 10092–10102.
11. H. Nishioka, *et al. Angew. Chem., Int. Ed.* 2012, **51**, 1165–1168.

FIGURE 2: PHOTO-RESPONSIVE DNAzyme

Photo-responsive DNAzyme that can perform tweezer-like motion by light-irradiation and achieve on-off cleavage of RNA.

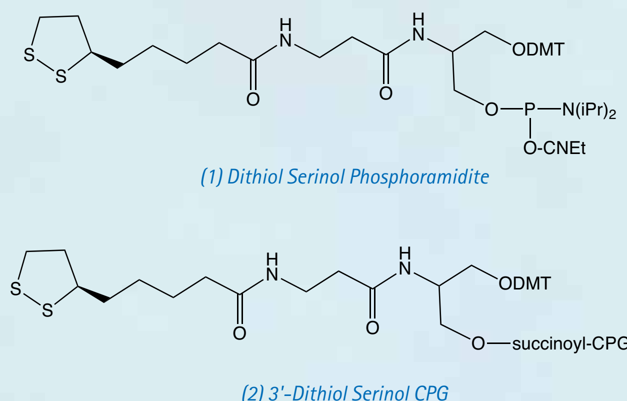
ORDERING INFORMATION

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

NEW PRODUCTS - DITHIOL SERINOL PHOSPHORAMIDITE AND 3'- DITHIOL SERINOL CPG

Gold nanoparticles (AuNPs) have become versatile tools for manufacturing biological sensors based on colorimetric, fluorescent, electrochemical, and other detection techniques.[1,2] AuNPs are of particular interest due to their ease of preparation and the diverse options for their functionalization.[1,3] For example, aptamers in conjunction with AuNPs have been used for efficient target recognition by using the colorimetric change as the aptamer structure is modified when the target binds.[2] For recent reviews on AuNPs, colloidal gold, and other nanomaterials, see Saha et al[1] and Wang et al[2].

FIGURE 1: STRUCTURES OF DITHIOL SERINOL PHOSPHORAMIDITE AND CPG



(1) Dithiol Serinol Phosphoramidite

(2) 3'-Dithiol Serinol CPG

An essential component of the preparation of functionalized AuNPs is the method for attaching ligands to form activated nanoparticles. Due to the strong affinity of thiols and disulfides to gold surfaces and the ready availability of oligonucleotides functionalized with thiol groups at the 3' or 5' terminus, thiol-modified oligos have been used extensively for the preparation of oligonucleotide functionalized AuNPs.[4]

However, Au-S bonds are susceptible to cleavage in the presence of other thiols or at elevated temperatures.[5] Consequently, polythiols, such as a dithio-steroid or trebler-thiol-modified oligos, provide a higher level of stability over simple alkylthiol ligands.[6,7] The trebler-thiol-modified oligos containing three alkylthiol ligands per molecule exhibited enhanced stability in AuNPs relative to dithiol-labelled oligos.

The introduction of dithiol phosphoramidite (DTPA) offered a straightforward approach to the synthesis of oligo functionalized AuNPs. DTPA exhibited a combination of ease of oligo synthesis with the ability to add multiple dithiol-ligands and ultimately generate very stable AuNPs. Indeed, multiply DTPA-modified oligos provided AuNPs with stability equivalent to or greater than those prepared with trebler-thiol-modified oligos.

DTPA has proved to be a popular product that Glen Research began offering in 2003.[5] However, effective January 1, 2015, Glen Research has discontinued DTPA and it is now available directly from FRIZ Biochem in Germany.

Our research into multiply thiol-containing structures led us to the readily available lipoic acid (thioctic acid). Lipoic acid has already been used extensively for labelling gold nanoparticles and surfaces.[8-11] Combining lipoic acid and our patented serinol backbone, Glen Research is pleased to offer our Dithiol Serinol Phosphoramidite (1) and the related 3'-Dithiol Serinol CPG (2). This unique architecture incorporates a linker that moves the bulky dithiol away from the phosphate backbone. The longer spacer arm of Dithiol Serinol also allows multiple consecutive incorporations of the modifier without the need for intermediate spacer phosphoramidite additions to achieve optimal stepwise coupling efficiency.

Use of Dithiol Serinol

Our initial experiments have confirmed that Dithiol Serinol Phosphoramidite can be easily incorporated into oligonucleotides using standard procedures of synthesis and deprotection. We have confirmed that Dithiol Serinol Phosphoramidite can be added several times consecutively in high yield without the need for a spacer between each addition.

Dithiol Serinol has been used by several groups experienced in work with AuNPs. Our collaborators have confirmed the ease of use and purification of multiply dithiol-labelled oligos. Their feedback also indicates that oligos produced using this new modifier give coverage of gold surfaces similar to equivalent multiply thiol-modified oligos.

References:

1. K. Saha, S.S. Agasti, C. Kim, X. Li, and V.M. Rotello, *Chemical Reviews*, 2012, **112**, 2739-2779.
2. H. Wang, R. Yang, L. Yang, and W. Tan, *ACS Nano*, 2009, **3**, 2451-2460.
3. M. Giersig, and P. Mulvaney, *Langmuir*, 1993, **9**, 3408-3413.
4. C.A. Mirkin, R.L. Letsinger, R.C. Mucic, and J.J. Storhoff, *Nature*, 1996, **382**, 607-9.
5. http://www.glenresearch.com//GlenReports/GR16-12.html
6. R.L. Letsinger, R. Elghanian, G. Viswanadham, and C.A. Mirkin, *Bioconjugate Chemistry*, 2000, **11**, 289-291.
7. Z. Li, R. Jin, C.A. Mirkin, and R.L. Letsinger, *Nucleic Acids Res*, 2002, **30**, 1558-62.
8. J.A. Dougan, C. Karlsson, W.E. Smith, and D. Graham, *Nucleic Acids Research*, 2007, **35**, 3668-3675.
9. J. Sharma, *et al.*, *J Am Chem Soc*, 2008, **130**, 7820-1.
10. J.A. Dougan, A.K. Reid, and D. Graham, *Tetrahedron Letters*, 2010, **51**, 5787-5790.
11. S. Perez-Rentero, S. Grijalvo, G. Penuelas, C. Fabrega, and R. Eritja, *Molecules*, 2014, **19**, 10495-523.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
Dithiol Serinol Phosphoramidite	10-1991-95	50 µmole	120.00
	10-1991-90	100 µmole	215.00
	10-1991-02	0.25g	585.00
3'-Dithiol Serinol CPG	20-2991-01	0.1g	120.00
	20-2991-10	1.0g	995.00
0.2 µmole columns	20-2991-42	Pack of 4	120.00
1 µmole columns	20-2991-41	Pack of 4	200.00
10 µmole column (ABI)	20-2991-13	Pack of 1	300.00
15 µmole column (Expedite)	20-2991-14	Pack of 1	450.00

TECHNICAL BRIEF - APA: AN ALTERNATIVE TO AMA DEPROTECTION

Back in December of 1993 in Glen Report 6.2, we reported the excellent results obtained when deprotecting oligonucleotides using AMA – a 1:1 solution of 30% ammonium hydroxide and 40% aqueous methylamine. AMA has a number of advantages over standard ammonium hydroxide deprotection, the primary advantage being the speed at which an oligo can be fully deprotected. Only 10 minutes at 65 °C is required to fully deprotect an oligo synthesized with standard protecting groups in a dry heat block and a mere 5 minutes in a 65 °C water bath.

The only caveat is that acetyl-protected dC must be used (10-1015) to prevent transamination. If the standard benzoyl-dC is used (10-1010), there is a small, but significant, amount of N-methyl-dC produced. In addition, some care must be taken to ensure that any labels or minor bases are compatible with methylamine. For example, most fluorescein analogs show sensitivity to AMA, leading to the production of a non-fluorescent impurity.[1]

Interestingly, despite being such a strong nucleophile, methylamine is actually more gentle on certain monomers. For example, RNA monomers, which are susceptible to loss of the 2'-O-TBDMS/TOM protecting groups in ammonium hydroxide, are much more cleanly deprotected in AMA.

In addition, AMA has the added benefit of acting as a scavenger of acrylonitrile produced *in situ* upon the removal of the cyanoethyl protecting groups, thereby suppressing N3-cyanoethylation of thymidine.

However, methylamine does have a downside, in that it is used as a reagent in the illicit production of methamphetamine.[2] As a result, it is a DEA-regulated substance and in California additional regulatory hurdles have been implemented on the purchase and use of methylamine solutions and salts, making it difficult to procure quickly or easily.[3]

As a result, we investigated alternative alkylamines that are not under the DEA's list of regulated chemicals for our Californian customers. The best candidate we found was propylamine. Propylamine is a liquid at room temperature, boiling at 48 °C, and is miscible with water. However, we found that adding it directly to 30% ammonium hydroxide led to significant degassing of ammonia from the solution. To avoid this, water was added to the solution yielding the final formulation of APA as 30% ammonium hydroxide/propylamine/water 2:1:1 (v/v/v).

When evaluating the APA solution, the two criteria for its success were: 1) a reasonably short cleavage time off a standard succinyl-lcaa support; and 2) a short deprotection time without unforeseen side-reactions.

When investigating the increased cleavage rate for our Glen UnySupport FC, we determined that the standard succinyl support required a 10 minute cleavage time in AMA at room temperature for quantitative cleavage.[4] The rate of cleavage of a standard succinyl support was found to be significantly slower when the APA solution was used. To obtain 97% cleavage at 20 °C, 1.5 hours was required when using APA. However, 90% of the oligo was fully cleaved in 1 hour at 20 °C (and most labs are kept above 20 °C). At 65 °C, less than 10 minutes was required.

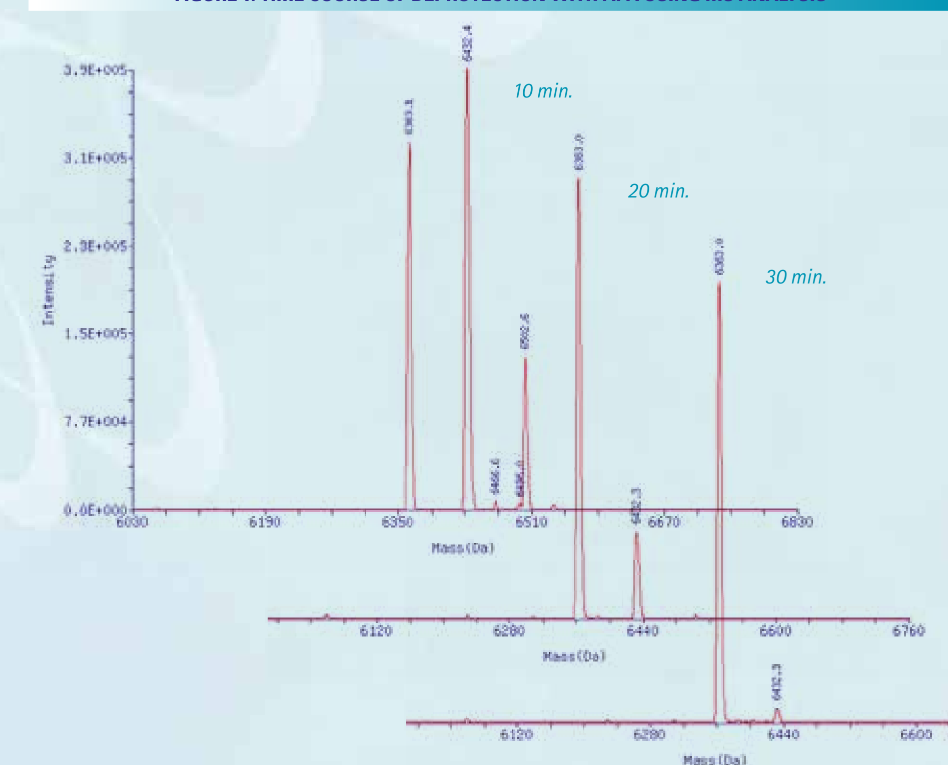
To determine the length of time required for the APA solution to completely deprotect an oligo, a mixed-base 20mer was synthesized using standard phosphoramidites: Bz-dA, Ac-dC, iBu-dG and dT. The CPG was split and subjected to 10, 20 and 30 minutes at 65 °C in APA. The crude oligos were analyzed by electrospray MS and the deconvolved spectra are shown in Figure 1. At the 30 minute mark, the oligo was 97% deprotected, with some dG residues still retaining the isobutyryl protecting group, as shown by the +70 Da peak. Given these data, we recommend 45 minutes at 65 °C to ensure the oligo is completely deprotected. As with AMA, the APA is seen to suppress cyanoethylation with no +53 Da peaks observed.

Given these results, the APA deprotection solution has been shown to be a good substitute for AMA, albeit with slower kinetics. We hope that this information may be of assistance to our customers looking for alternative deprotection solutions that are free of methylamine.

References:

1. TECHNICAL BRIEF - SIDE REACTION OF FLUORESCEIN DURING DEPROTECTION WITH METHYLAMINE, http://www.glenresearch.com//GlenReports/GR25-13.html
2. Vince Gilligan et al., Breaking Bad, ep 5.05, "Dead Freight" 2012
3. See: http://ag.ca.gov/bne/pdfs/laws03.pdf
4. It should be noted that the FC Linker afforded quantitative cleavage using AMA in 2 minutes. See: TECHNICAL BRIEF - GLEN UNYSUPPORT NOW AVAILABLE WITH FAST CLEAVAGE, http://www.glenresearch.com//GlenReports/GR25-28.html

FIGURE 1: TIME COURSE OF DEPROTECTION WITH APA USING MS ANALYSIS



PHOTOCLEAVABLE BIOTIN LINKER FOR USE IN SOMAscan™

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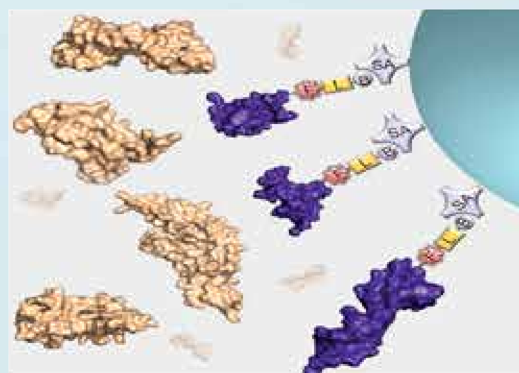
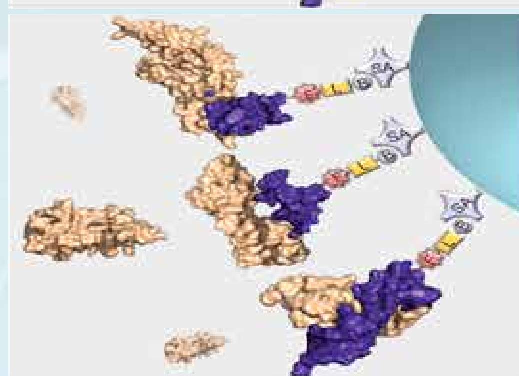
Interrogation of the human proteome in a highly multiplexed and efficient manner remains a coveted and challenging goal in biology. SomaLogic has implemented a new aptamer-based proteomic technology for biomarker discovery capable of simultaneously measuring thousands of proteins from small sample volumes (65 µL of serum or plasma). Our current assay allows us to measure >1000 proteins with very low limits of detection (300 fM median), 7 logs of overall dynamic range, and 5% average coefficient of variation[1,2]. This technology is enabled by a new generation of aptamers that contain chemically modified nucleotides, which greatly expand the physicochemical diversity of the large randomized nucleic acid libraries from which the aptamers are selected. Proteins in complex matrices such as plasma and serum are measured with a process that transforms a signature of protein concentrations into a corresponding DNA aptamer concentration signature, which is then quantified with a DNA microarray. In essence, this assay takes advantage of the dual nature of aptamers as both folded binding entities with defined shapes and unique sequences recognizable by specific complementary hybridization probes (see Figure 1).

For more than two decades, there has been growing interest in proteomic biomarker screening technologies[1,3]. Though several technologies have been applied to this effort with some limitations[4,5], recent work by SomaLogic has demonstrated the use of slow off-rate modified aptamer (SOMAmer®) reagents to enable multiplexed screening of thousands of serum or plasma proteins. Using the SOMAscan™ assay, we are able to measure >1000 proteins and are working towards a 3000-plex. The use of the photocleavable biotin (PCB) reagent[6,7] as a capture/release agent in our assay (see Figure 1) has enabled assay development and implementation for the use of these highly specific and diverse set of reagents. Integration of the PCB reagent and a suitable fluorophore (i.e. cyanine-3) onto the termini of SOMAmer reagents with suitable modified nucleotide positions[2,8,9] (see Figure 2, Page 8) affords these measurements through binding of cognate proteins and subsequent quantification on a DNA microarray. Further work is underway to expand content and implement the larger 3000-plex with appropriate assay improvements and validation.

FIGURE 1: **MULTIPLEXED SOMAmer AFFINITY ASSAY**

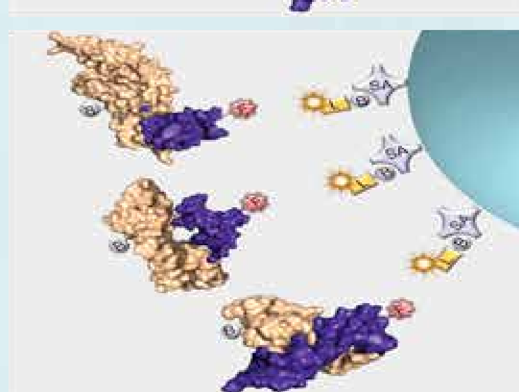


(a) SOMAmers labeled with a fluorophore (F), photocleavable linker (L), and biotin (B) are immobilized on streptavidin (SA)-coated beads and incubated with samples containing a complex mixure of proteins (e.g., plasma).

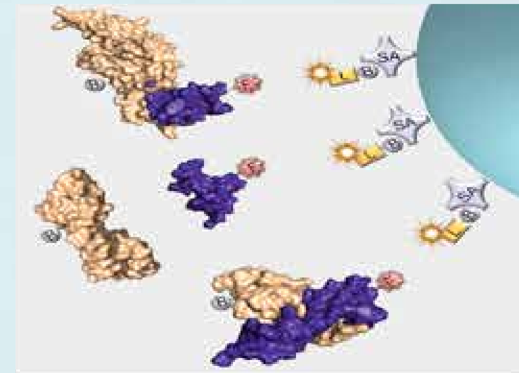


(b) Cognate (top and bottom) and noncognate (middle) SOMAmer–target protein complexes form on the beads.

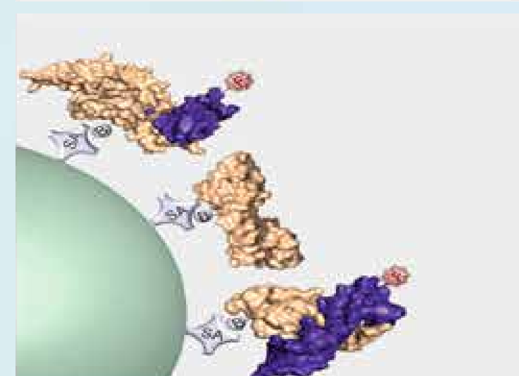
(c) The beads are washed removing the unbound proteins and the proteins are tagged with biotin.



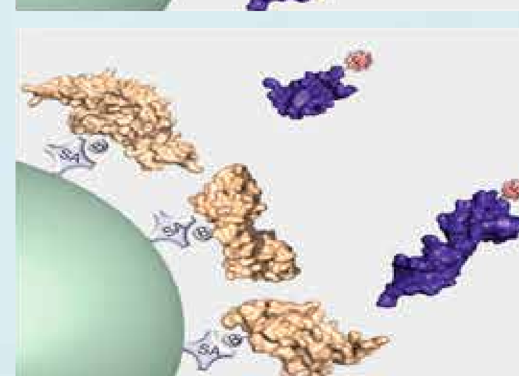
(d) SOMAmer–protein complexes are released from the beads by photocleavage of the linker with UV light.



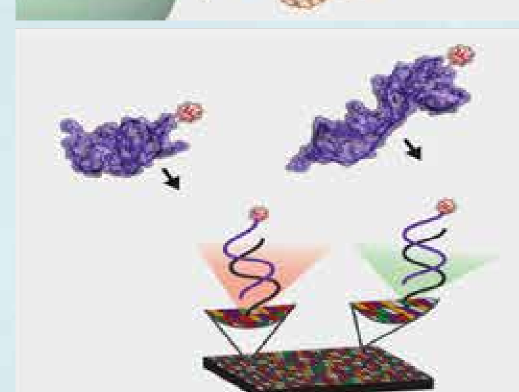
(e) Incubation in a buffer containing a polyanionic competitor selectively disrupts nonspecific interactions.



(f) SOMAmer–protein complexes are recaptured on a second set of streptavidin-coated beads through biotin-tagged proteins followed by additional washing steps that facilitate further removal of nonspecifically bound SOMAmers.



(g) SOMAmers are released from the beads in a denaturing buffer.



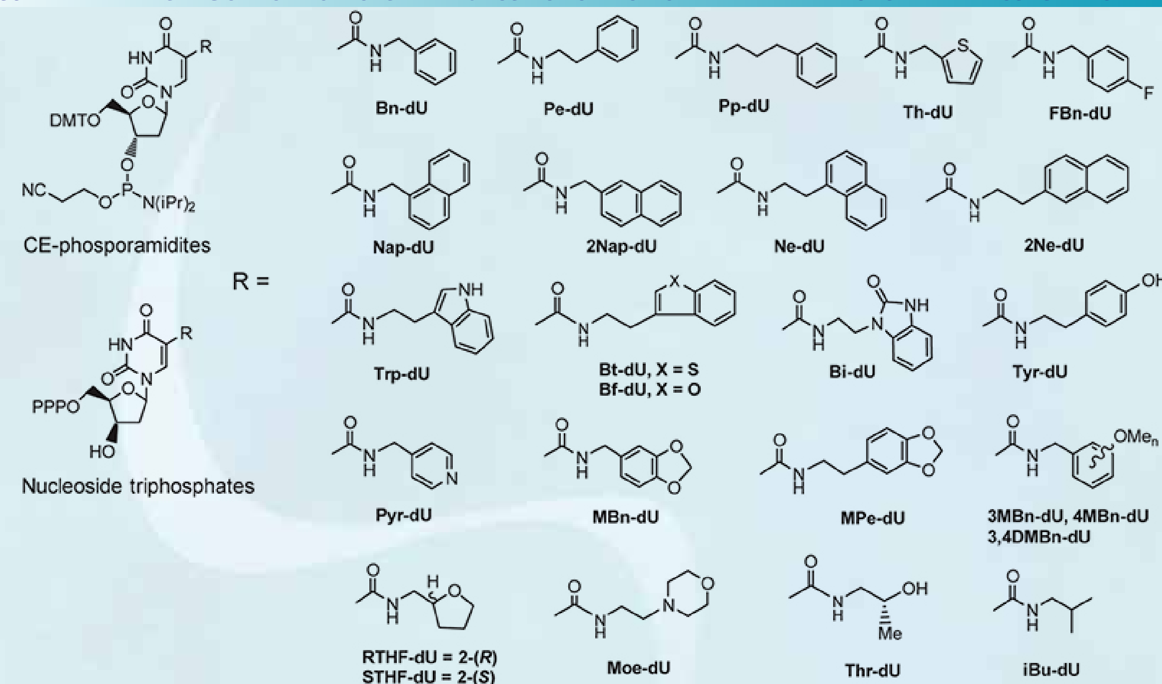
(h) SOMAmers are hybridized to complementary sequences on a microarray chip and quantified by fluorescence. Fluorescence intensity is related to protein amount in the original sample.

(Adapted from Rohloff et al, Molecular Therapy – Nucleic Acids, 2014, 3, e201)

References:

1) Gold *et al*, (2010), Aptamer-based multiplexed proteomic technology for biomarker discovery, *Nature Precedings*
2) Rohloff *et al*, (2014), Nucleic Acid Ligands With Protein-like Side Chains: Modified Aptamers and Their Use as Diagnostic and Therapeutic Agents, *Molecular Therapy – Nucleic Acid*, **3**, e201
3) Zichi, D. *et al*, (2008), Proteomics and diagnostics: Let's get specific, again. *Curr. Opin. Chem. Biol.* **12**, 78-85
4) Knezevic *et al*, (2001), Proteomic profiling of the cancer microenvironment by antibody arrays, *Proteomics*, **1**, 1271–1278
5) Gregorich and Ge,(2014), Top-down proteomics in health and disease: Challenges and opportunities, *Proteomics*, **14**, 1195–1210
6) Olejnik *et al*, (1996), Photocleavable biotin phosphoramidite for 5'-end-labeling, affinity purification and phosphorylation of synthetic oligonucleotides, *Nucleic Acids Research*, **24:2**, 361-366
7) Olejnik *et al*, (1999), Photocleavable peptide-DNA conjugates: synthesis and applications to DNA analysis using MALDI-MS, *Nucleic Acids Research*, **27:33**, 4626-4631
8) Vaught, J. D. *et al*, (2010), Expanding the chemistry of DNA for in vitro selection, *J. Am. Chem. Soc.* **132**, 4141-4151
9) Rohloff *et al*, (2015), Practical Synthesis of Cytidine-5-Carboxamide-Modified Nucleotide Reagents, *Nucleosides, Nucleotides and Nucleic Acids*, **34:3**, 180-198

FIGURE 2: **PARTIAL LISTING OF MODIFICATIONS AT THE 5-POSITION OF DEOXYURIDINE AVAILABLE FOR SELEX AND POST-SELEX OPTIMIZATION**



Side chain abbreviations: Bn, benzyl; Pe, 2-phenylethyl; Pp, 3-phenylpropyl; Th, 2-thiophenylmethyl; FBn, 4-fluorobenzyl; Nap, 1-naphthylmethyl; 2Nap, 2-naphthylmethyl; Ne, 1-naphthyl-2-ethyl; 2Ne, 2-naphthyl-2-ethyl; Trp, 3-indole-2-ethyl; Bt, 3-benzothiophenyl-2-ethyl; Bf, 3-benzofuranyl-2-ethyl; Bi, 1-benzimidazol-2-ethyl; Tyr, 4-hydroxyphenyl-2-ethyl; Pyr, 4-pyridinylmethyl; MBn, 3,4-methylenedioxybenzyl; MPe, 3,4-methylenedioxyphenyl-2-ethyl; 3MBn, 3-methoxybenzyl; 4MBn, 4-methoxybenzyl; 3,4MBn, 3,4-dimethoxybenzyl; RTHF, R-tetrahydrofuranylmethyl; STHF, S-tetrahydrofuranylmethyl; Moe, morpholino-2-ethyl; Thr, R-2-hydroxypropyl; iBu, iso-butyl

(Adapted from Rohloff et al, Molecular Therapy – Nucleic Acids, 2014, 3, e201)

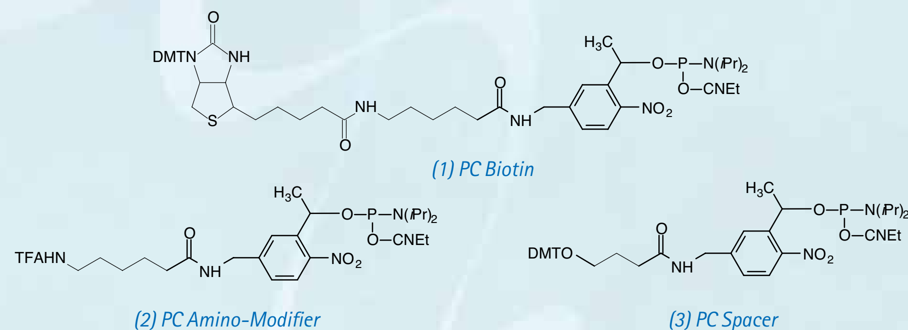
PC MODIFIERS

Glen Research introduced PC Biotin and related PC modifiers in 2001. We are delighted to be able to publish this article illustrating the use of PC Biotin (1) in aptamer development at SomaLogic, Inc. We especially thank Jeff Carter for providing us with this article.

Our range of PC phosphoramidites is shown in Figure 3. PC Amino-Modifier (2) is useful for conjugating an NHS ester labelled tag post oligo synthesis, while PC Spacer (3) allows tags to be added to oligonucleotides as phosphoramidites.

Glen Research offers PC Biotin, PC Amino-Modifier and PC Spacer products in association with AmberGen, Inc. and Link Technologies, Ltd. For a commercial application license, please contact AmberGen, Inc., +617-975-0680, http://www.ambergen.com/.

FIGURE 3: **STRUCTURES OF PHOTO CLEAVABLE PHOSPHORAMIDITES**



(1) PC Biotin

(2) PC Amino-Modifier

(3) PC Spacer

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
PC Biotin Phosphoramidite	10-4950-95	50 μmole	145.00
	10-4950-90	100 μmole	280.00
	10-4950-02	0.25g	675.00
PC Amino-Modifier Phosphoramidite	10-4906-90	100 μmole	135.00
	10-4906-02	0.25g	395.00
PC Spacer Phosphoramidite	10-4913-90	100 μmole	135.00
	10-4913-02	0.25g	395.00

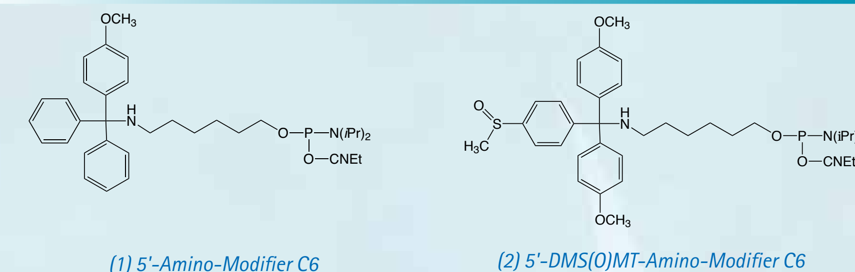
TECHNICAL BRIEF - CAPPING AND TRITYL-PROTECTED AMINO-MODIFIERS

Two of our most popular products are the trityl-protected amino-modifiers, 10-1906 (1) and 10-1907 (2) in Figure 1. These products are particularly useful for two reasons. First, the trityl protecting group on the amine allows for simple reverse-phase purification by HPLC or cartridge to yield a clean, full-length product. Second, the trityl protecting group can be removed from the amine on the DNA synthesizer, which allows the amine to be labelled while the oligo is still bound to the CPG. This eliminates the need for any tedious desalting steps to remove unconjugated NHS ester label. However, we recently determined these products can undergo an unexpected side-reaction that leads to capping of the amine.

A customer shared with us an observation that his oligo had an unusual +190 Da peak as seen by mass spectrometry (MS). This mass corresponds to the addition of t-Bu-phenoxyacetyl to the amine and an UltraMild Cap A mix, which contains t-Bu-phenoxyacetic anhydride, was indeed used on the synthesizer. The amino-modifier in question was the 5'-DMS(O)MT-Amino-Modifier C6 (2). The trityl on this amino-modifier, 4,4'-dimethoxy-4"-methylsulfonyl-trityl, is the most acid-labile used in any of the amino-modifiers due to the exceptional stability of the DMS(O)MT trityl cation. This lability, perhaps, facilitated the phenoxyacetylation of the amine during the capping step. While we had not received reports of acetylation of the amino-modifier protected with the DMS(O)MT, there was the possibility of some unexpected chemistry occurring when phenoxyacetic anhydride was used as the capping reagent during synthesis.

To investigate this possibility, the sequence 5'-X-TTT TTT-3', where X is the DMS(O)MT Amino-Modifier C6, was synthesized with the capping steps in the synthesis cycle disabled. The CPG was split, capping one portion off the synthesizer with standard Cap A/B, which uses acetic anhydride, and the other with UltraMild Cap A/B which uses phenoxyacetic anhydride. The samples were then cleaved from the support in ammonium hydroxide and analyzed by RP HPLC. If capping had occurred, the Trityl-On peaks would be reduced and capped-amine T6 peaks would arise. As seen in Figure 2, the chromatogram of the oligo capped with the phenoxyacetic anhydride Cap A shows a sharp peak that elutes around the 15 minute mark with failures and the trityl-on amino-T6 eluting at 12 and 19 minutes, respectively. However, surprisingly, a new peak arose with the acetic anhydride-capped oligo at 12.5 minutes that corresponds to the acetyl-capped amine-T6.

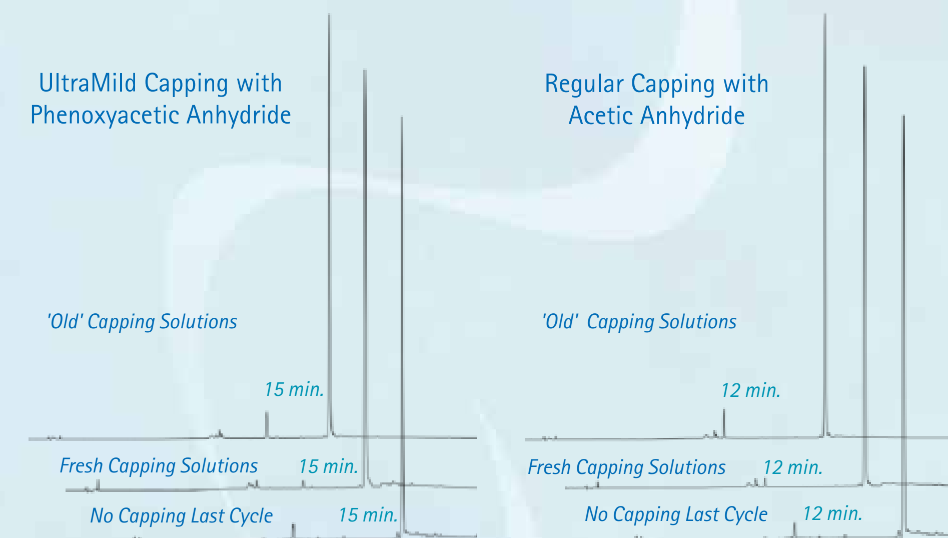
FIGURE 1: **STRUCTURES OF CROSSLINKING PRODUCTS**



(1) 5'-Amino-Modifier C6

(2) 5'-DMS(O)MT-Amino-Modifier C6

FIGURE 2: **RP HPLC ANALYSIS - 5'-DMS(O)MT-AMINO-MODIFIER OLIGOS CAPPED AS SHOWN**



So, the experiment was repeated, this time using fresh, unopened bottles of both the standard Cap A and the UltraMild Cap A and Cap B which contains 16% methyimidazole. As seen in Figure 2, the respective acetyl and phenoxyacetyl capped species are still present, however at a much lower amount.

Our hypothesis to explain these data is that the old capping reagents become more and more acidic over time as the acetic- and phenoxyacetic anhydride hydrolyzes. This in turn results in the loss of the trityl group during capping and gives rise to N-acetyl (+42 Da) and N-phenoxyacetyl (+190 Da) species. Our results indicate that this is a more significant issue with 5'-DMS(O)MT-Amino-Modifier C6 (2), due to its greater lability to acid compared to 5'-Amino-Modifier C6 (1).

However, this side-reaction can be avoided by simply maintaining fresh capping reagents on the synthesizer. It is tempting to pull specialty capping reagents, e.g., UltraMild Cap A, off the synthesizer to be stored until the next time a synthesis that requires UltraMild Cap A comes up. However, these data show that this procedure is not without risk of side reactions. The results also confirm that an even simpler approach is to omit the capping step in the final cycle when using 5'-modifiers that terminate the oligo synthesis.

Reference:

1. http://www.glenresearch.com/GlenReports/GR19-14.html

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
5'-Amino-Modifier C6	10-1906-90	100 μmole	60.00
	10-1906-02	0.25g	200.00
5'-DMS(O)MT-Amino-Modifier C6	10-1907-90	100 μmole	60.00
	10-1907-02	0.25g	200.00

TECHNICAL BRIEF - DBCO-DT - AN UNUSUAL CASE OF IODINE SENSITIVITY

The Dibenzocyclooctyne (DBCO) family of products allows "click" conjugation to an azide simply and cleanly without requiring any copper salts or chelators, making it very convenient - and especially useful for *in vivo* conjugation reactions. After our successful launch of the 5'-DBCO-TEG Phosphoramidite and NHS ester, we later added to our portfolio DBCO-dT (1) which allows internal modification of an oligo with DBCO using a 5-substituted thymidine analog.

Recently, however, we received information from a customer in Europe, Microsynth AG, indicating that full-length production of a poly-dT 63-mer with 3 incorporations of the DBCO-dT could not be obtained in good yield. According to their mass spec data, it appeared that the DBCO had been cleaved off the linker. When the same batch of DBCO-dT was retested here, the coupling efficiency was essentially quantitative and the 12-mer test oligo with 3 incorporations of the DBCO-dT looked excellent, as shown in Figure 2a.

Since the performance of this batch of DBCO-dT looked good, as a courtesy, we offered to synthesize the customer's longer sequence. However, when analyzed by RP HPLC, the resulting oligonucleotide looked very impure and mass spec analysis confirmed that the DBCO had been substantially cleaved off the linker.

Conceptually, this was unexpected since amide linkages are resistant to hydrolysis, which implied that DBCO-dT is sensitive to one or more of the synthesis reagents and that the repeated exposure during the synthesis of long oligos led to cleavage of the DBCO. To test this hypothesis, CPG from the 12-mer synthesis (Figure 2a) was subjected to treatment with standard DNA synthesis oxidizer, 0.02 M Iodine, for 5 minutes at room temperature. This exposure is equivalent to roughly 20 synthesis cycles. As shown in Figure 2b, the resulting degradation was quite dramatic.

With other nucleoside analogs with sensitivity to iodine, we have achieved good results using an alternative oxidizer, (1S)-(+)-(10-Camphorsulfonyl)-oxaziridine (CSO) (2).

So, when the 63-mer was re-synthesized using 0.5 M CSO in acetonitrile (40-4632-xx) and a 3 minute oxidation time, the hoped-for improvement was dramatic. Figure 3 shows the deconvolved electrospray MS data

***FIGURE 1:* STRUCTURES OF DBCO-DT AND CSO**

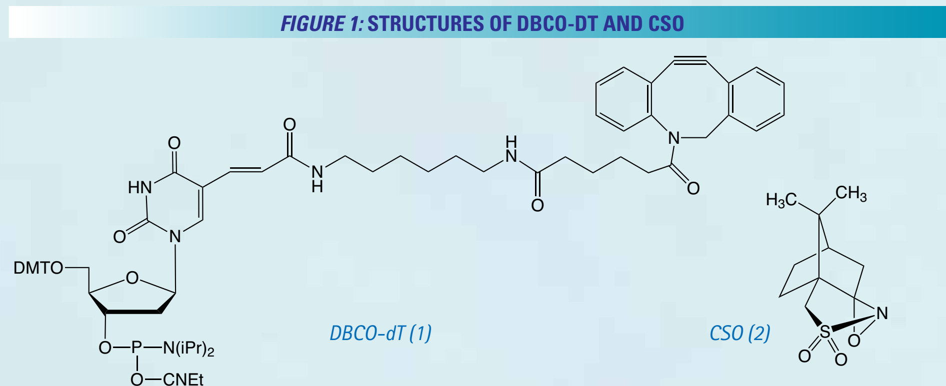


***FIGURE 2:* RP HPLC OF TEST 12-MER CONTAINING THREE DBCO-DT ADDITIONS**

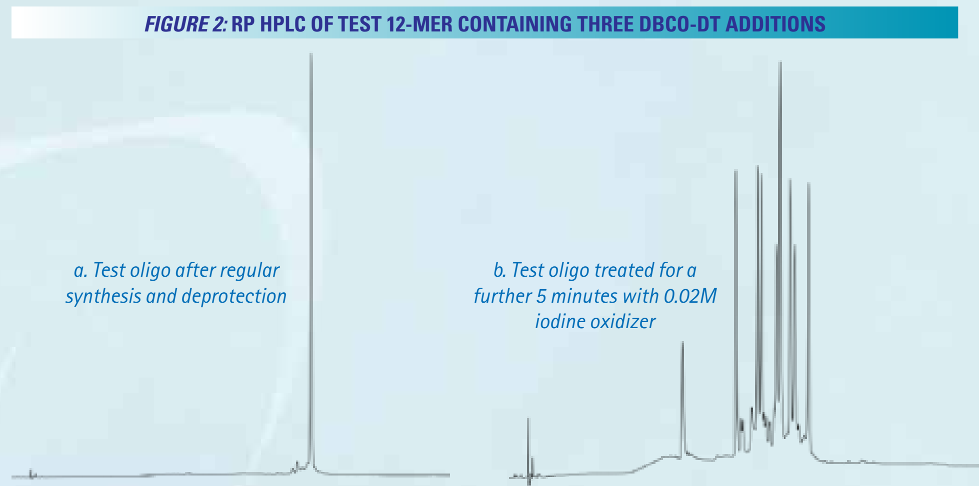
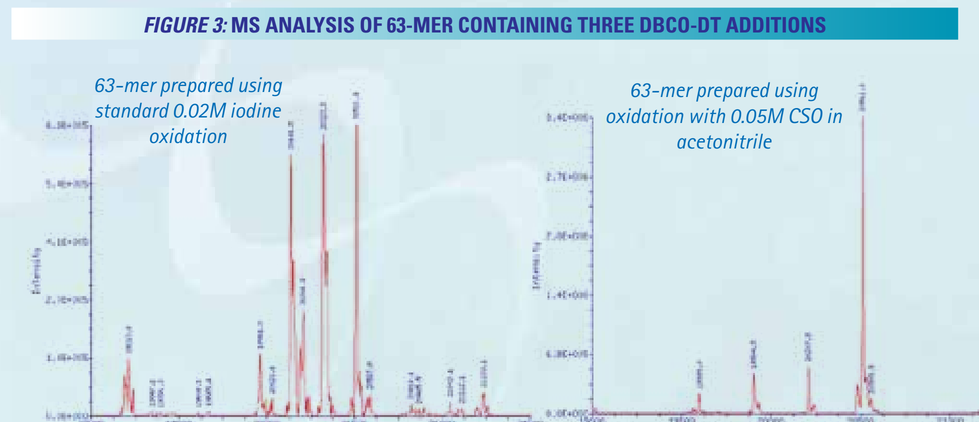


***FIGURE 3:* MS ANALYSIS OF 63-MER CONTAINING THREE DBCO-DT ADDITIONS**



for the same sequence synthesized using standard 0.02 M Iodine versus 0.5 M CSO with the target mass being 20,511 Da. It is clear that the DBCO moiety is being cleaved off when exposed to iodine-based oxidizers. What appears to have occurred during oxidation with iodine is the formation of an N-iodo amide, making the amide linkage unstable. During deprotection, the DBCO is cleaved off, leaving a hexamido linker present. (The splitting of the -DBCO peaks is 14 Da, indicating the formation of both the amide and N-methylamide linkers which results from the oligo being deprotected in AMA). The lower molecular weight peaks associated with the CSO-oxidized oligo are deletion mutants (-1, -2 and -3 dTs), which suggests the oxidation time of 3 minutes should have been increased slightly for an oligo of this length.

As a result of these data, we now recommend that synthesis of oligos containing DBCO-dT be completed using 0.5 M CSO oxidizer. Acceptable results can be achieved with iodine oxidation if DBCO-dT is subjected to no more than 8-10 cycles. We thank Microsynth AG for bringing this issue to our attention.

TECHNICAL BRIEF - PSEUDOURIDINE: A NEW PERSPECTIVE ON FUNCTION AND ACTIVITY

Pseudouridine (Ψ), a simple glycosidic N1 to C5 isomer of uridine, is one of the most prevalent of the modified ribonucleotides. It is found in a variety of non-coding RNAs - ribosomal RNA (rRNA), transfer RNA (tRNA), and small nuclear RNA (snRNA) associated with spliceosomes. However, recent papers suggest it may also function in coding messenger RNA (mRNA).

A seminal paper to suggest this expanded role for pseudouridine in coding mRNA was by Karijolich and Yu who found that replacing U with Ψ in the stop codon UAA allowed read-through by the ribosome and suppression of the translation termination *in vitro* using a synthetic mRNA transcript.[1] To further their argument, they then used the cell's own pseudouridylation machinery, the H/ACA RNAs, to convert *in vivo* a stop codon introduced into the CUP1 gene. The gene product of CUP1 infers tolerance to copper sulfate in the culture media by the chelation of Cu^{2+}. By plating the cells in media containing $CuSO_4$, they had a convenient reporter system to monitor suppression of translation termination.

By mutating a naturally occurring H/ACA RNA guide sequence, they were able to specifically target the U of the stop codon UAA, which they introduced in the CUP1 gene, and convert it to ΨAA. They found that the transformed *S. cerevisiae* only survived in the high Cu^{2+} media when the guide RNA specifically targeted the UAA codon for pseudouridylation.

The authors went on to look at the suppression of termination of the stop codons UAA, UAG and UGA in plasmids containing the TRM4 gene. Again, site-specific pseudouridylation, using guide RNA strands targeting the stop codons introduced in the TRM4 gene, led to observable TRM4 protein production. When the resulting TRM4 protein was purified and analyzed by mass spectrometry, it was found that the translation of the ΨAA and ΨAG codons led to the incorporation of serine and threonine in roughly equal frequency for ΨAA and predominantly serine for ΨAG. Whereas ΨGA led to the incorporation of predominantly tyrosine (with some phenylalanine observed).

The lab of Venki Ramakrishnan went on to crystalize the 30S ribosomal subunit docked to the tRNA(ser), which presents the anticodon AGI bound to the ΨAG codon. Intriguingly, the structure showed

***FIGURE 1:* STRUCTURES OF URIDINE AND PSEUDOURIDINE MONOMERS**

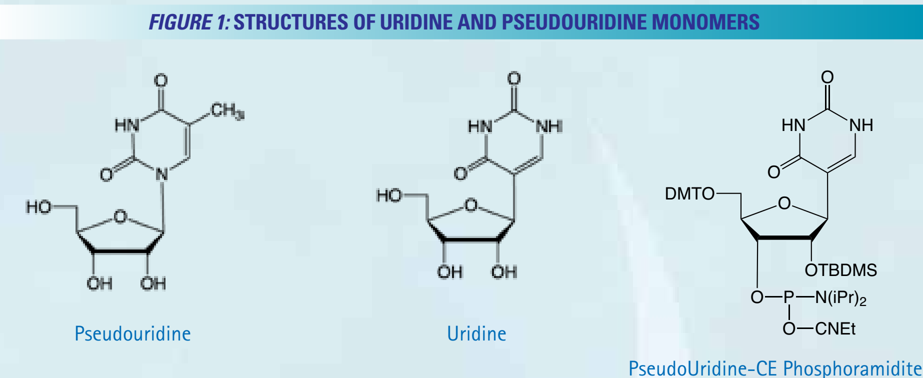
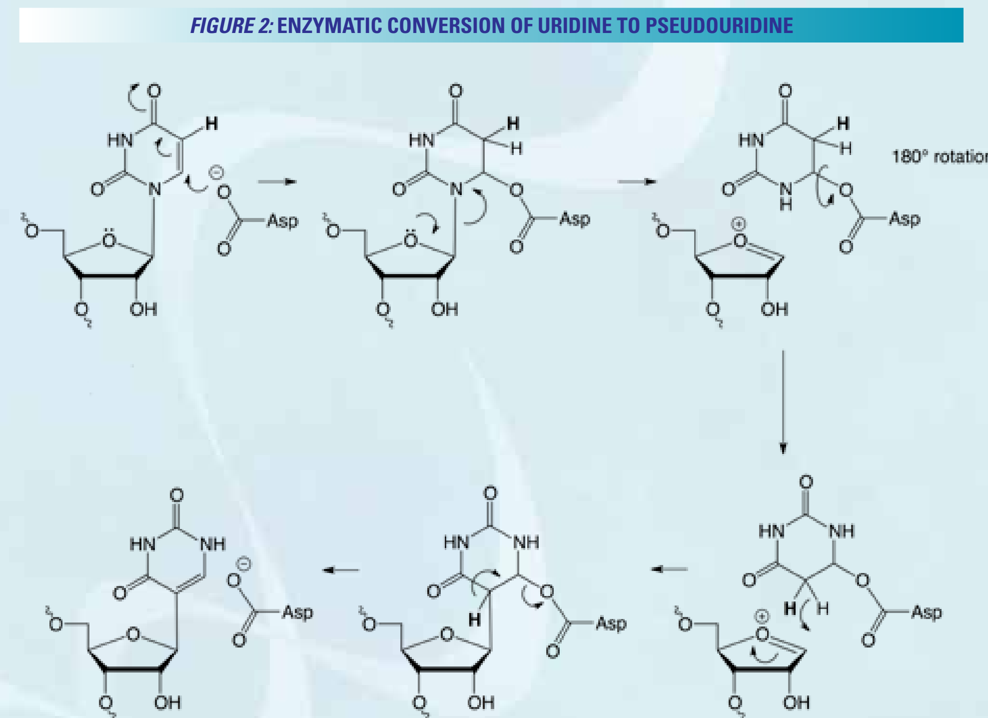


***FIGURE 2:* ENZYMATIC CONVERSION OF URIDINE TO PSEUDOURIDINE**



Adapted from the mechanism determined for the interaction of pseudouridine synthase I with 5-fluorouracil-tRNA[1]

1. X. Gu, Y. Liu, and D.V. Santi, *Proc Natl Acad Sci U S A*, 1999, **96**, 14270-5.

that the N1 imino proton of pseudouridine does not form any hydrogen bonds and the canonical Ψ-A Watson-Crick base pair was observed. However, the pseudouridine seems to exert a subtle but profound effect upon the anticodon stem loop structure, leading to a purine-purine A-G Hoogsteen base pair with the adenosine of the ΨAG codon in an unusual *syn* conformation.[2]

However tantalizing the results of Karijolich and Yu, there was still no demonstration of naturally occurring pseudouridylation of mRNA in cells. This soon changed with a second seminal paper on pseudouridine that was recently published by the Gilbert lab at MIT, who used next-generation sequencing to demonstrate that pseudouridylation of mRNAs occurred naturally in both human and yeast cells.[3]

The Gilbert lab utilized techniques developed by Bakin and Ofengand[4], who had found that pseudouridine would specifically and irreversibly react with N-Cyclohexyl-N'-(ß-[N-methylmorpholino]ethyl) carbodiimide p-toluenesulfonate (CMC). After reacting the RNA with CMC and treating with sodium carbonate to remove non-specific reactions with G and U bases, the resulting carbodiimide-Ψ adduct blocked reverse transcription. By comparing the +CMC transcripts with -CMC controls to correct for Ψ-independent transcription

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stops, the locations of pseudouridine could be determined in RNA (a sequencing procedure termed Pseudo-seq).

After confirming the correct Ψ-calling in non-coding RNA, they then used poly-dT cellulose beads to pull-down poly-A+ transcripts to look for pseudouridylation in mRNA. Remarkably, they found hundreds - conservatively, 260 pseudouridylated sites - in over 238 mRNA coding transcripts. Not only that, they also found that the pseudouridylation was regulated depending upon environmental conditions. As the yeast growth went from exponential (log phase) to stationary (as the nutrients were depleted in the media), pseudouridylation was found to be upregulated for some mRNA and down-regulated for others, indicating pseudouridylation was responsive to environmental stress. Not unexpectedly, pseudouridylation sites of rRNA changed little.

The authors then looked at the sites of pseudouridylation in mRNA to see if they corresponded to H/ACA RNA guide sequences and found that most were not. Therefore, they turned their attention to the family of pseudouridine synthases in yeast (PUS1-9) that do not require guide RNA sequences. When PUS deletion strains were grown and pseudouridylation of mRNAs was analyzed for the different ΔPUS strains, PUS1 was found to be responsible for most of the mRNA pseudouridylation. However, PUS2-4, PUS6-7 and PUS9 all had unique mRNA targets.

Knowing that pseudouridine synthases are conserved in all eukaryotes, HeLa cells were analyzed by Pseudo-seq during normal growth and serum-starvation conditions. Their conservative estimate suggested that 89 human mRNAs were pseudouridylated, some in response to growth-state conditions. As a number of diseases are associated with mutations in PUS genes, the authors raised the possibility that misregulation of pseudouridylation in mRNAs may contribute to the diseased state.

These results taken together hint at a second layer of functions and activities of pseudouridine in not only non-coding RNA but coding RNA as well.

We envision continuing interest in PseudoUridine CE Phosphoramidite as researchers continue to investigate termination suppression in premature termination codons, potential alternative codon coding, and the function of conditional pseudouridylation of mRNA.

References:

1. J. Karijolich, and Y.T. Yu, *Nature*, 2011, **474**, 395-8.
2. I.S. Fernandez, et al., *Nature*, 2013, **500**, 107-10.
3. T.M. Carlile, et al., *Nature*, 2014, **515**, 143-6.
4. A. Bakin, and J. Ofengand, *Biochemistry*, 1993, **32**, 9754-62.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
PseudoUridine-CE Phosphoramidite	10-3055-95	50 µmole	175.00
	10-3055-90	100 µmole	350.00
	10-3055-02	0.25g	995.00