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L-DNA: Applications and the Recognition of Mirror Image Nucleic Acids

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When you think about DNA, do you consider its stereochemistry? You should! DNA is a chiral molecule, and consequently, has non-superimposable mirror images (enantiomers). The source of chirality in DNA is the deoxyribose sugar, which contains three chirality centers (Figure 1). On one "hand", native DNA found in all living organisms consists exclusively of D-deoxyribose sugars (D-DNA) and forms right-handed helices. On the other hand, its enantiomer, L-DNA, consists of L-deoxyribose sugars and forms left-handed helices. L-DNA is not found in nature, but can be prepared synthetically in the laboratory, with the earliest syntheses of L-oligonucleotides dating back to the 1970s.[1] As enantiomers, D-DNA and L-DNA are physically indistinguishable, except for their opposed optical activity, making them identical from a design perspective (i.e. same hybridization rates, duplex thermal stability, etc.). Importantly, L-DNA is orthogonal to the stereospecific environment of native biology, which has evolved to recognize D-nucleic acids. As a result, L-DNA is highly resistant to nuclease degradation and off-target interactions with other cellular macromolecules.[2-3] These favorable properties, along with the recent commercialization of L-DNA phosphoramidite building blocks, has catalyzed the development of many promising L-DNA based biotechnologies.[4]

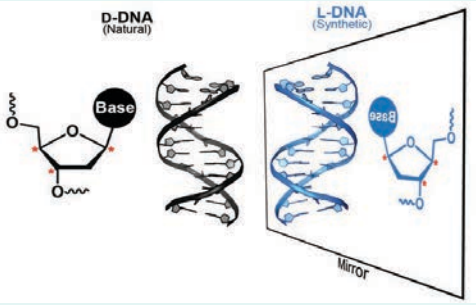

Figure 1. The two enantiomers of DNA. Chirality centers are indicated with an asterisk.

One of the first[5-6], and still common, uses of L-DNA is in the form of aptamers. To date, L-DNA aptamers (also called Spiegelmers) have been evolved to bind a variety of targets, including small molecules, peptides, and proteins.[7] Our laboratory has recently pioneered the use of L-DNA aptamers to bind native D-RNA structures.[8] When applied *in vivo*, Spiegelmers retain a high affinity for their targets, while being nontoxic and have very low immunogenic potential.[9] Given these desirable

Continued on Page 2

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Continued from Front Page 1

properties, it is not surprising that several Speigelmer therapeutics are in various stages of clinical development.[10]

Another powerful application of L-DNA is in the construction of bio-orthogonal molecular sensors. For example, L-DNA-based molecular beacons, which are not substrates for native polymerases, can be used for optical monitoring of PCR reactions, a method referred to as "adaptive PCR".[11] L-DNA molecular beacon probes have also been employed for measuring intracellular temperatures.[12] DNA-based sensors for achiral analytes can be mirrored without a loss in activity. This property was exploited to generate nuclease-resistant L-DNAzyme sensors for monitoring metal ion concentrations in living cells.[13-14] In the case of chiral analytes, use of both enantiomers of the corresponding nucleic acid sensor enables rapid screening of enantiomeric purity.[15]

Watson–Crick base pairing between complementary strands is stereospecific, and thus, L-DNA is incapable of hybridizing to native DNA and RNA.[3, 16] While this property excludes the direct use of L-DNA as antisense agents, it ensures that sensors and other biotechnologies constructed from L-DNA have minimal off-target hybridization, an important consideration for analytical applications. Indeed, one of the earliest applications of L-DNA was the development of a universal microarray platform that employed L-DNA capture strands as a means to reduce cross-hybridization of nucleic acid samples with different regions of the array.[3] The stereospecificity of hybridization can also be exploited to create internal controls during sample analysis.[17]

Although D-DNA and L-DNA do not interact directly, sequence information can still be transferred between the two enantiomers via strand-displacement strategies.[18-20] For example, our laboratory showed that DNA enantiomers can be sequence-specifically interfaced via toehold-mediated strand-displacement from peptide nucleic acid (PNA), a process referred to as "heterochiral" strand displacement (Figure 2).[18] Because PNA is achiral, and hybridizes equally well to both D-DNA and L-DNA, it functions as the intermediary, allowing a strand of D-DNA to displace a strand of L-DNA (and vice versa) in a sequence-specific manner. In principle, heterochiral strand displacement

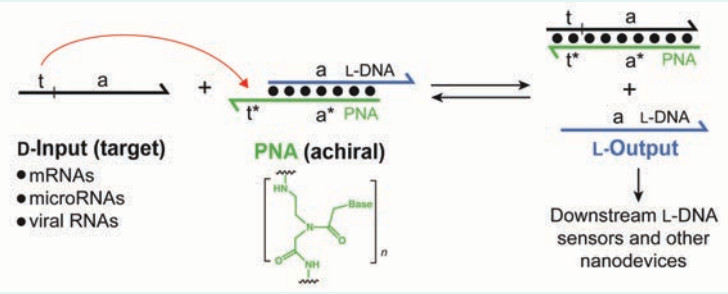

Figure 2. Schematic illustration of the heterochiral toehold-mediated strand displacement reaction. Nucleic acids are depicted as lines with half arrows denoting the 3' end and an asterisk indicating complementarity between sequence domains. The single-stranded toehold domain (t*) resides on the achiral PNA strand in the L-DNA/PNA heteroduplex, allowing a D-Input strand bind (via t and t*) and displace the incumbent L-Output strand.

allows for any D-nucleic acid target (DNA or RNA) to be interfaced with sensors and nanodevices composed of bioorthogonal L-DNA. For example, we used this approach to interface microRNAs with L-DNA-based logic circuits and catalytic amplifiers in vitro[18, 21-22], and with an L-RNA-based fluorescent biosensor in living cells.[23] Chimeric strands of both D-DNA and L-DNA also enable native nucleic acids to be interfaced with L-DNA, and have been employed for various biosensing purposes.[24-25]

It is clear from the examples discussed herein (and elsewhere[4]) that L-DNA provides a powerful opportunity to develop nucleic acid-based technologies having capabilities not possible using only the native stereoisomer. As more researchers step "Through the Looking-Glass", and use of mirror image DNA becomes more routine, completely new and exciting applications will become possible.

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Beta L-DNA Phosphoramidites

We are incredibly grateful to Prof. Sczepanski for an updated review on the multiple uses of L-DNA and a closer look at some of the work coming from his lab at Texas A&M University. Glen Research offers four beta-L-DNA phosphoramidites (Figure 1). Check out our previous article from when we introduced these monomers for more information.[1]

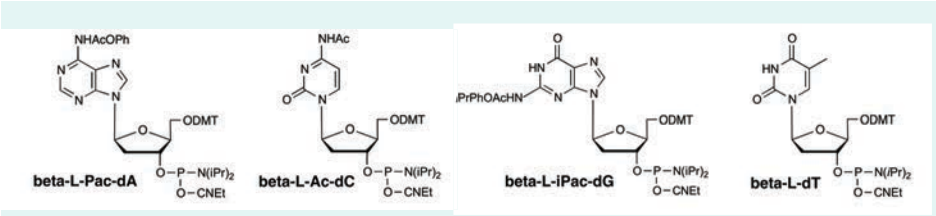

Figure 1. Structures of L-DNA Phosphoramidites

References

1. *The Glen Report*, 2019, **31.2**, 4-6.

Item	Pack Size	Catalog No.
Beta-L-Pac-dA-CE Phosphoramidite	0.25 g	10-2101-05
	0.5 g	10-2101-10
	1.0 g	10-2101-02
Beta-L-Ac-dC-CE Phosphoramidite	0.25 g	10-2115-05
	0.5 g	10-2115-10
	1.0 g	10-2115-02
Beta-L-iPr-Pac-dG-CE Phosphoramidite	0.25 g	10-2121-05
	0.5 g	10-2121-10
	1.0 g	10-2121-02
Beta-L-dT-CE Phosphoramidite	0.25 g	10-2130-05
	0.5 g	10-2130-10
	1.0 g	10-2130-02

Fluorescent Base Analogues Offer Unique Features for Oligonucleotide Drug Development

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The rapid emergence of oligonucleotide-based therapeutics, such as small interfering RNA (siRNA), antisense oligonucleotides (ASOs), and therapeutic messenger RNA (mRNA), is transforming the strategies of the pharma industry and opening exciting new avenues of research.[1] To support this development, new and innovative ways of introducing fluorescent entities (fluorophores) to these types of modalities are needed. Drug development projects are particularly reliant on using fluorophores that do not perturb drug-target interactions or affect affinities towards, for instance, lipid membranes or proteins, to deliver results that are representative of the performance of the corresponding unmodified drug.

Glen Research has a long-standing tradition of providing a range of fluorescent base analogues (FBAs), which can be used as integrated and nature-mimicking alternatives to bulky, amphiphilic fluorophores such as ATTO-, Alexa-, or cyanine dyes.[2] Glen Research's FBA product line includes the tricyclic cytosines tC and tC^{O} (Figure 1), which are well-known for their excellent fluorescent properties inside RNA and/or DNA, as well as for maintaining canonical base-pairing and secondary structures of the corresponding duplexes.[3-6]

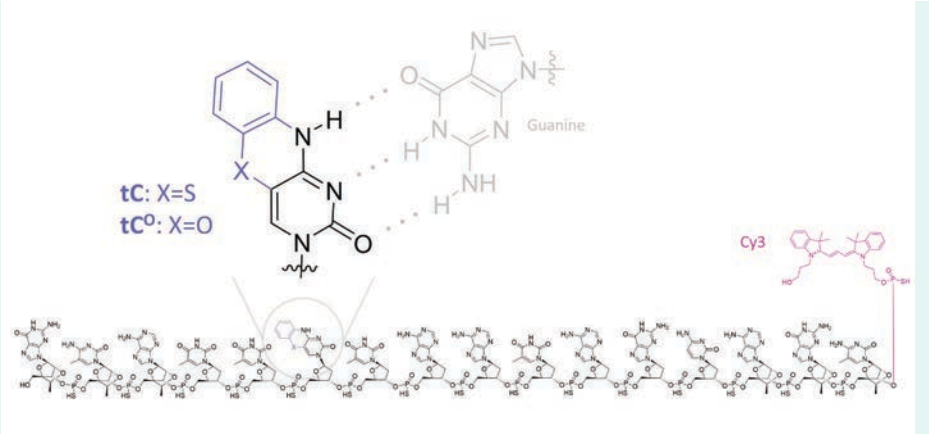

Figure 1. Fluorescent cytosine analogues tC or tC^{O} inside the 16 nt ASO; the comparably small modification required to render the ASO fluorescent using these FBAs is shown in blue. For comparison, the conventional end-labelling approach, here using a Cy3 fluorophore (magenta), is also shown.

Table 1. Melting temperatures (ΔT_m) and gene knockdown efficiencies (ΔpEC_{50}) of the tC- and tC^{O}-containing ASOs.

ASO	5'–3' Sequence (X = FBA[a])	Type of FBA	ΔTm (°C)[b]	ΔpEC50 (log(M))[c]
U	**GCA**TTCTAATAGC**AGC**	-	0 ± 0.5	0 ± 0.3
tC-1	**GCA**TTCTAATAG**X****AGC**	tC	-1.8 ± 0.5	0.2 ± 0.4
tC-2	**GCA**TT**X**TAATAG**X****AGC**	tC	-0.8 ± 0.7	0.3 ± 0.4
tC-2^{w}	**G****X****A**TTCTAATAG**X****AGC**	tC	-5.3 ± 0.8	0.3 ± 0.4
tC-3	**G****X****A**TT**X**TAATAG**X****AGC**	tC	-3.7 ± 0.5	0.3 ± 0.4
tC^{O}-1	**GCA**TTCTAATAG**X****AGC**	tC^{O}	-1.6 ± 0.4	0.2 ± 0.4
tC^{O}-2	**GCA**TT**X**TAATAG**X****AGC**	tC^{O}	0.9 ± 0.4	0.1 ± 0.4

[a] All FBA nucleosides have deoxyribose sugars. [b] ΔTm for binding to the complementary RNA was determined relative to the unlabeled ASO (U: Tm = 65.5 °C, pEC50 = 5.7) and measured in 10 mM phosphate buffer (pH 7.4) containing 1.0 mM EDTA and 100 mM added salt.
[c] Knockdown efficiencies were evaluated using RT-qPCR in HEK 293 cells.

ASOs, which induce their pharmacological effect by first binding to their target RNA sequence via Watson-Crick base pairing, typically comprise several different types of base and/or backbone modifications and consequently behave differently than pure DNA and RNA. To investigate the potential of using FBAs to label oligonucleotide drugs, the Wilhelmsson lab recently explored the impact on RNA affinity, therapeutic efficacy, secondary structure, photophysical properties, and imageability upon incorporating tC and tC^{O} (along with two other FBAs) at different positions in a 16mer ASO (Table 1).[7]

It was found that neither tC^{O} nor tC, even when incorporated at three positions, affect the therapeutic efficacy of the ASO, as evidenced by the unchanged effective concentration for gene knockdown (ΔpEC_{50}, Table 1). Similar non-perturbing properties have been demonstrated in a recent account on tC^{O} inside mRNA, where it was shown that, in contrast to Cy5-labeling, substituting native cytosines for tC^{O} does not impair the protein expression of the mRNA.[8] Furthermore, the effect on the ASOs' affinity towards its target RNA is small to non-existent (ΔT_m, Table 1), with only a slight destabilization noted upon substituting one of the six flanking bases. The study also employed circular dichroism to show that the ASO:RNA duplex, as expected, adopts an A-form type duplex and that neither tC- nor tC^{O} incorporation affects the secondary structure, which is crucial considering the mechanism of action for this class of therapeutics.

Knowing the photophysical properties of fluorophores in their oligomer environment is important for accurate interpretation of fluorescence-based data; this was therefore

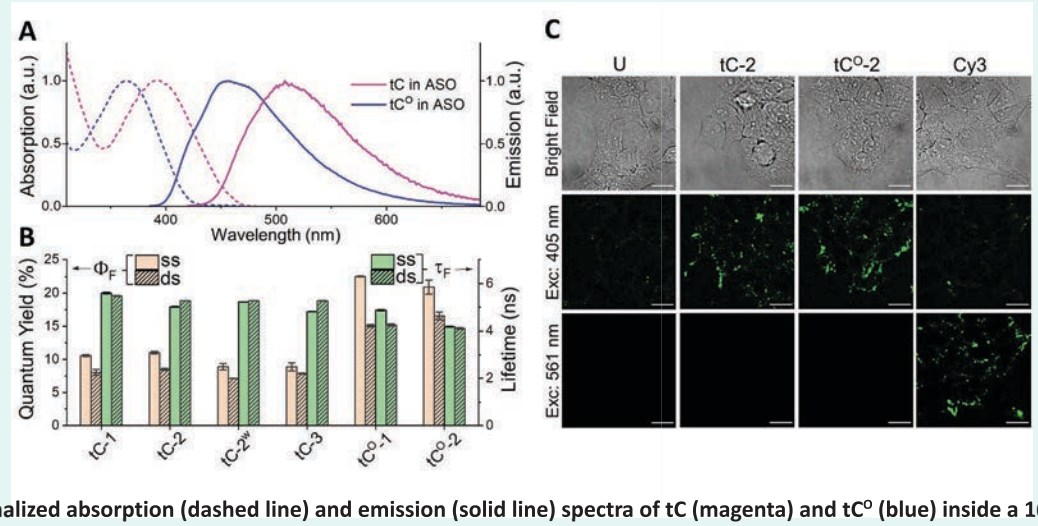

Figure 2. (A) Normalized absorption (dashed line) and emission (solid line) spectra of tC (magenta) and tC^O (blue) inside a 16 nt ASO. (B) Fluorescence quantum yield and lifetime of tC and tC^O inside the ASO as a single strand (ss, plain bars) and bound to the complementary RNA (ds, striped bars). Measurements were performed at room temperature in a 10 mM phosphate buffer (pH 7.4) containing 1.0 mM EDTA and 100 mM added salt. (C) Confocal microscopy images of live HEK 293T cells exposed to 3 µM labeled ASO for 24 h.

investigated for tC and tC^O using steady-state and time-resolved spectroscopy (Figure 2).

The absorption and emission spectra of tC and tC^O inside the ASO (Figure 2A) are similar to those reported inside DNA[4,5] and RNA[3], and both FBAs are highly fluorescent, with an average quantum yield of 10% and 22% for tC and tC^O, respectively (Figure 2B). The average fluorescence lifetimes are 5.2 ns and 4.6 ns for tC and tC^O, respectively (Figure 2B), and exhibit bi- or mono-exponential decays. Overall, the photophysical properties of the FBAs are largely independent of substitution pattern in the investigated ASOs, and binding to RNA has a minor impact on these parameters. These robust characteristics greatly simplify interpretation when undertaking quantitative studies on, for instance, uptake in cells, which make both tC and tC^O excellent choices for labeling ASOs. To further demonstrate the applicability of FBAs as fluorescent probes, the labeled ASOs were added to live cells and imaged using confocal microscopy (Figure 2C). The images clearly show that the FBA-ASOs are readily detected inside the cells using a conventional microscopy setup and that comparable results to the end-labelled Cy3-ASO can be achieved.

We conclude that the tC- and tC^O phosphoramidite building blocks provided by Glen Research are bright and robust fluorophores that are highly suitable for fluorescence spectroscopy and microscopy investigations of therapeutic oligomers. The proven track record of these FBAs for studying DNA and RNA secondary structures using FRET methodologies[9-11] also offers a unique potential for in-depth structure and dynamics investigations of ASOs and siRNA.

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New Product — 5’-Biotin II Phosphoramidite

Biotin-based assays have been widely applied in nucleic acids research since the strong affinity between biotin and streptavidin was discovered in the 1970s.[1] The protein-ligand contact is one of the strongest noncovalent interactions, with a K_d of 10^{-15} M. A biotin label is versatile and is often used to enrich proteins that bind to a specific DNA. Alternatively, biotin offers precise detection, amplification, and/or immobilization of DNA substrates. In addition, the orthogonality of the biotin-streptavidin interaction allows researchers to use other labels in their experiments, without having to worry about cross-reactions.

Glen Research’s first biotin product was released in 1991, and our collection has grown substantially since then. Our newest product, 5’-Biotin II phosphoramidite (Figure 1), complements our traditional version I. These two 5’-biotin phosphoramidites differ slightly in terms of the linker, an all-carbon versus an ethylene glycol linker (Figure 1A-B). For those familiar with our amino-modifier offerings, the linkers in versions I and II are derived from amino-modifier C6 and amino-modifier 5, respectively (Figure 1C-D). The addition of 5’-Biotin II phosphoramidite allows researchers accustomed to using the amino-modifier 5 linker to continue to do so in their experiments. Moreover, this new version matches the standard 5’-biotin structure used by major oligonucleotide synthesis providers.

The 5’-biotin phosphoramidites offer several advantages to customers:

- Benefits from on-column automated incorporation, as opposed to post-synthetic conjugations, such as click chemistry or NHS ester couplings.
- Contains a DMT-group that enables reverse phase (RP) cartridge, such as Glen-Paks, and HPLC purification techniques.
- CAUTION: Even though this structure contains a DMT group, the 5’-Biotin II can only be added once to the 5’-terminus of an oligonucleotide. The DMT group in this structure protects one of the urea nitrogens in the biotin structure and does not enable oligonucleotide elongation, like a normal DMT-O would.

Recent Applications

With the surge of public awareness in disease detection, point-of-care (POC) diagnostic testing is becoming essential to the rapid analysis of patient analytes as it facilitates better diagnosis, monitoring, and management. POC diagnosis based on nucleic acid testing typically relies on nucleic acid amplification and detection in a single device. While PCR is a powerful tool and meets requirements of diagnostic tools, such as specificity, sensitivity, and rapidity, it requires numerous experimental steps, skilled technicians, and costly materials. Low-cost tools are of tremendous interest and paper microfluidic devices offer a promising platform to translate isothermal amplification tests to POC diagnostics.[2,3]

The 5’-Biotin II structure was incorporated into oligonucleotides for paper-based devices to detect disease-associated DNA.

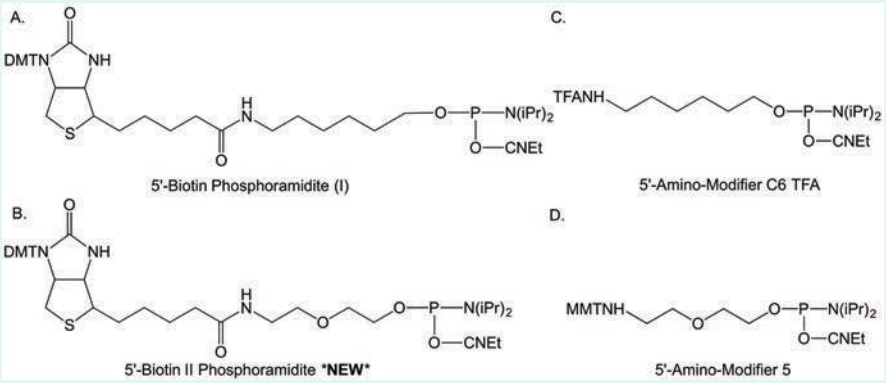

Figure 1. Structures of (A) 5’-Biotin (I), (B) 5’-Biotin II Phosphoramidite, (C) 5’-Amino Modifier C6 TFA, and (D) 5’-Amino Modifier 5.

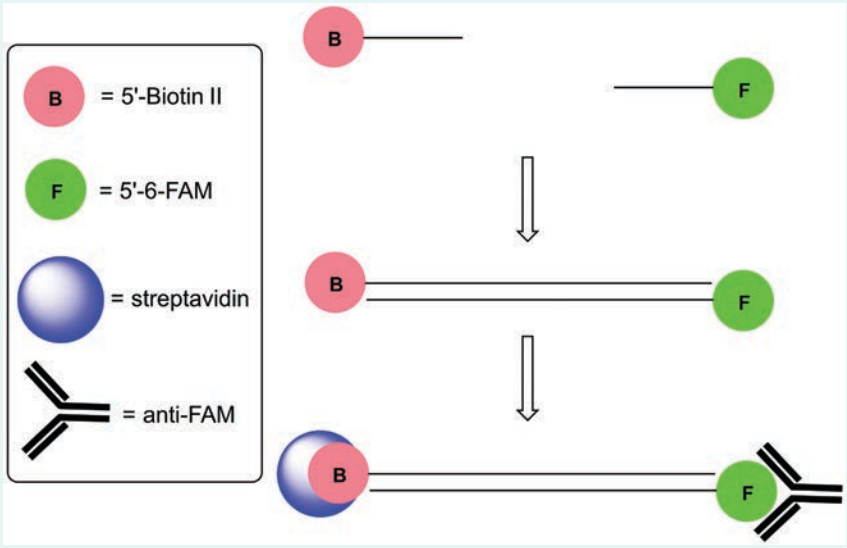

Figure 2. Workflow of point-of-care diagnostics using 5’-biotinylated reverse primers.

A 5'-biotinylated reverse primer and a FAM-labeled forward primer were used in a paper-and-plastic device coupled to an isothermal recombinase polymerase amplification (RPA) reaction to detect malarial DNA.[3] In this system, the RPA reaction produced a primary product labeled with a biotin tag from the reverse primer. The 5'-FAM forward probe recognized the primary product, resulting in a new secondary probe labeled with both biotin and FAM. The secondary product was detected using lateral flow strips with streptavidin to capture the product on the test line and gold nanoparticles functionalized with anti-FAM to yield a color change in the presence of the target nucleic acid sequence (Figure 2). A similar fluorescence-based detection system used 5'-Biotin II labeled reverse primers to amplify and detect *Chlamydia trachomatis*.[4]

Not only is nucleic acid detection applicable to the field of diagnostics, but it can also be valuable in elucidating the role of certain nucleic acid sequences. Long noncoding RNAs (lncRNAs) play important roles in cellular development, chromatin structure, and gene regulation.[5] Cross-linking and immunoprecipitation (CLIP) methods are typically used to study direct RNA-protein interactions *in vivo*, but they have limited utility in identifying new protein partners for a specific lncRNA. A novel technique called RNA-antisense purification coupled with mass spectrometry (RAP-MS) used UV-light to cross-link zero distance interacting RNA and protein partners followed by capture of the 5'-biotinylated RNA through hybridization on streptavidin beads. This method was used to understand the mechanism of *Xist* lncRNA in female mammals, where one X chromosome is transcriptionally silenced. A new protein partner of *Xist* lncRNA was identified: a large multidomain transcriptional coregulator protein (SHARP), which activated histone deacetylase HDAC3 and excluded Pol II across the X chromosome.

Recently, the strong streptavidin-biotin interaction was used to immobilize nucleic acid substrates. This was particularly helpful in the study of nitrogen mustard-induced interstrand cross-link (ICL) bypass by translesion DNA synthesis (TLS) polymerases. TLS is the bypass of a lesion during DNA replication and TLS polymerases play important roles in DNA damage tolerance. Bypass of a lesion avoids fork stalling and collapse, which can lead to cell death. This process must be tightly regulated as TLS is a major source of DNA damage-induced mutagenesis. Bezalel-Buch *et al.* immobilized a 93-mer ICL oligonucleotide substrate with terminal 5'-Biotin II on streptavidin beads and found the Rev1-Polζ complex faithfully inserted dCMP opposite the dG-ICL, yielding a full-length extension product, while other TLS polymerases inefficiently bypassed the induced ICL.[6] This work has significant implications for disease-associated cells harboring Polζ mutations and their hypersensitivity to ICL-forming agents.

In another study, the 5'-Biotin II structure was used to reduce noise in precise editing through preassembly of CRISPR ribonucleoproteins (RNPs) by S1m, an RNA aptamer with a strong affinity for streptavidin. In this method, the conjugation of S1m to a sgRNA allowed for complexation between streptavidin-S1m-sgRNA-cas9 to form the RNP, termed S1mplex. In the presence of 5'-biotinylated single stranded donor template, accurate homology-directed repair (HDR) was induced at target sequences, confirmed by deep sequencing.[7]

The versatility of 5'-Biotin II phosphoramidite makes it an excellent addition to our product catalog. As always, we ensure this product meets our high-quality requirements and is optimized for your use. A 2-minute coupling time is recommended for 5'-Biotin II Phosphoramidite. 5'-Biotin II is slow to detritylate. If the final DMT-group is to be removed on the synthesizer, we recommend a second deblocking step. If the final DMT-group is to be left on for purification, treat the biotinylated oligonucleotide with TFA solution for 10 minutes. Again, only a single biotin can be incorporated at the 5'-end using this product.

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Item	Pack Size	Catalog No.
5'-Biotin Phosphoramidite	50 µmol	10-5950-95
	100 µmol	10-5950-90
	0.25 g	10-5950-02
5'-Biotin II Phosphoramidite ***NEW***	50 µmol	10-1954-95
	100 µmol	10-1954-90
	0.25 g	10-1954-02

New Products — Dye-dT Phosphoramidites

Introduction

Fluorescent oligonucleotide probes have a long-standing history in detecting and visualizing nucleic acids. Fluorophore labels are integral to multiplex reactions, fluorescent microscopy, PCR, and many other applications. With the addition of new internal fluorophore-dT phosphoramidites, including JOE-dT, Quasar® 570-dT, and Quasar® 670-dT, Glen Research is excited to expand the current line of products (Figure 1). Each fluorophore possesses unique spectroscopic characteristics (Table 1).[1]

Quasar 570 and 670 fluorophores are derivatives of cyanine 3 and 5, respectively. Cyanine dyes consist of two heteroaromatic rings that are separated by a polymethine chain, and due to resonance, these have a positive charge dispersed across the two nitrogens (Figure 2). The length of the conjugated chain determines the distinct properties of the cyanine fluorophores. Although we offer other cyanine products, the structures differ slightly, as linker lengths and substituents on the aromatic rings vary (Figure 3).

The NHS esters have a methyl group on the aromatic quaternary nitrogen, a C6 linker and SO_3 substituents on the aromatic rings. The cyanine 3 and 5 phosphoramidites have symmetrical propanol linkers at the heterocyclic nitrogens. Lastly, the Quasars have an ethyl ammonium and an extended linker off the C5 position of the thymine nucleobase. As expected, and despite some subtle differences, the Cyanine 3 and Cyanine 5 products exhibit the same spectroscopic characteristics.

Historically, the earliest versions of FRET probes used an internal TAMRA, either by TAMRA NHS ester (50-5910) or TAMRA-dT (10-1057), as a quencher of the 5'-fluorophore.[1] Now, internal fluorophore labels are more popular for use as probes in many applications. Internal fluorophores can either be incorporated using a fluorophore-dT amidite, a nonnucleoside phosphoramidite, or an amino modifier-dT labeled with a dye NHS ester. Detritylation and extension at the OMMT group (Figure 3) may allow

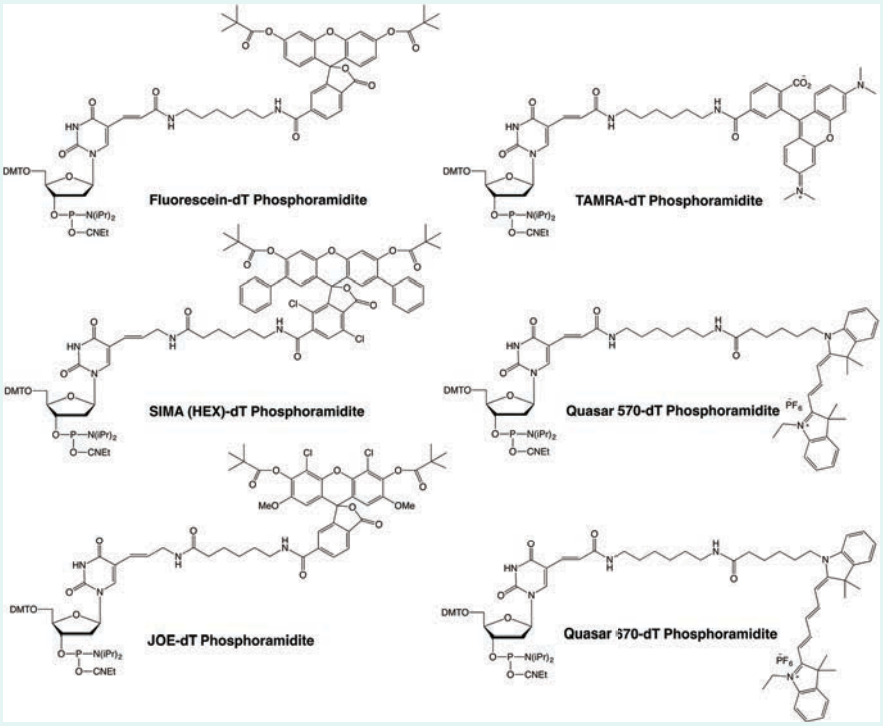

Figure 1. Internal Fluorophore-dT Phosphoramidites

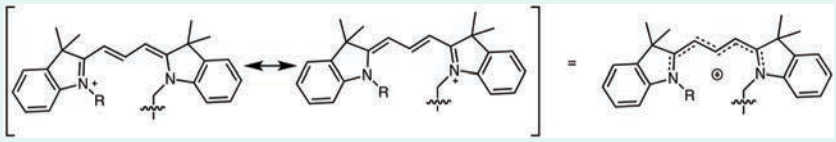

Figure 2. Cyanine resonance structures

Table 1. Fluorophore spectroscopic properties

Fluorophore	Cat. No.	Imax Abs	Imax Em	Application(s)
Fluorescein-dT	10-1056	495 nm	520 nm	Flow cytometry, FISH, and molecular beacons
SIMA (HEX)-dT	10-5945	535 nm	550 nm	Multiplex reactions
JOE-dT	10-5936	525 nm	548 nm	Multiplex reactions
TAMRA-dT	10-1057	550 nm	575 nm	Imaging, flow cytometry, and ELISA
Quasar® 570-dT (Cyanine 3-dT)	10-5953	552 nm	570 nm	Imaging, flow cytometry, and genomic applications
Quasar® 670-dT (Cyanine 5-dT)	10-5955	643 nm	667 nm	Imaging, flow cytometry, and genomic applications

non-nucleoside cyanine phosphoramidites to be used internally, but this method is less commonly employed, as base pairing and stacking in a duplex may be disrupted. For these reasons, the preferred method for labelling oligonucleotides with cyanine dyes site-specifically was cyanine NHS ester coupling with amino-modifier C6 dT.[2]

Fluorophore-dT phosphoramidites offer multiple benefits to customers:

- Internal labels yield the same oligo as an amino modifier dT and dye NHS ester, without requiring post-synthetic reactions.
- A monomer can be inserted as a replacement for a dT residue. Linkers and dyes do not affect the ability of the labelled thymidine to form base pairs.[3]
- They are compatible with DMT-ON purification.

Applications Using Internal Fluorophores

Multiplex assays are now a standard in efficient, high-throughput genetic analysis. About a decade ago, a six-channel system to simultaneously analyze mutations in the human CFTR (cystic fibrosis) gene was established by Prof. Tom Brown from the University of Oxford.[4] Cystic fibrosis (CF) is an inherited life-threatening disorder that damages the lungs and digestive system. Scientists have found more than 1,700 different CF-causing mutations in the CFTR gene.

Six spectroscopically distinct fluorophores were incorporated internally to oligonucleotides probes in a six-color PCR-based HyBeacon system.[4] At the time, the range of fluorophore phosphoramidites was unavailable, so probes were synthesized primarily using dye NHS esters and amino modifiers. HyBeacon systems are currently being developed for the rapid diagnosis of bacterial infections and genetically related diseases, as well as for forensic applications.[5]

Fluorescent nucleic acid probes that do not fluoresce when the probe fails to recognize its target nucleic acid are important for microscopic analysis of intracellular nucleic acids, as they enable reliable, labor saving, and real-time fluorescence observation. More recently, this was achieved using oligonucleotides containing fluorescent dyes with a hybridization-dependent fluorescence response called ECHO (exciton-controlled hybridization-sensitive fluorescent oligonucleotide) probes.[6] Thiazole orange (TO), an asymmetric derivative of cyanine, was doubly incorporated internally at a dT position in an oligonucleotide. Alone, ECHO probes were effective for sequence-specific visualization of single-stranded nucleic acids like mRNA. Adapting ECHO probes for fluorescence *in-situ* hybridization (FISH) techniques proved to be highly reproducible, stringent, and compatible with other fluorescent cellular labeling techniques. ECHO-FISH was used to detect and image intracellular RNA targets in live cells. In order to better understand spatiotemporal correlations between gene expression and interactions between nucleic acids in live cells, the authors used other cyanine derivatized fluorophores to create a series of new ECHO probes of different colors.

Internal fluorophores are also useful in oligonucleotide substrates that do not bear 5'- and 3'- ends, such as circular DNA.

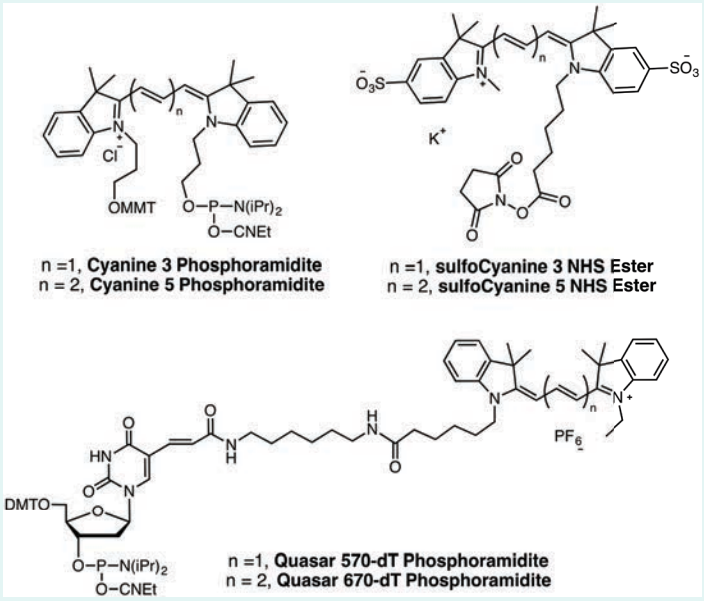

Figure 3. Cyanine products

DNA topology and topoisomerases play important roles in many biological processes, including DNA replication, recombination, and transcription. Internal fluorophore and quencher labeled oligonucleotides were inserted into a circular DNA, which was then converted into a supercoiled form via the addition of DNA gyrase in the presence of ATP. The DNA was relaxed by the addition of Topoisomerase 1.[7] The commonly employed Förster resonance energy transfer (FRET) was used to study the transition between supercoiling and topoisomerase activity on the circular DNA template. This DNA molecule has many potential applications, including the screening of small molecule inhibitors targeting topoisomerases and gyrases, which may have implications in both anti-cancer and antibacterial therapeutics.[7]

Synthesis and Deprotection

As with our other fluorophore-dT phosphoramidites, we recommend a 10 min coupling during oligonucleotide synthesis.

For Quasar® 670-dT in particular, the use of UltraMILD monomers is preferred. (Catalog Numbers: dA: 10-1601-xx, dC: 10-1015-xx, dG: 10-1621-xx, dT: 10-1030-xx). To avoid any exchange of the iPr-Pac group on the dG with acetyl, use the UltraMild Cap Mix A

Continued on Page 10

Continued from Front Page 9

(40-4210-xx/ 40-4212-xx). 0.02M Iodine for oxidation is recommended.

Each fluorophore is unique and sensitive to certain deprotection conditions. We have found the best deprotection conditions of the fluorophore-dT phosphoramidites compare well with their 5'-phosphoramidite counterparts (see Table 2).

Intellectual Property

Quasar® products are subject to certain intellectual property rights owned or controlled by Biosearch Technologies, Inc. and its affiliates. Products are sold for research use only.

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Table 2: Recommended Deprotection Conditions

Phosphoramidite	Recommended Deprotection Condition(s)
JOE-dT	Use Ammonium Hydroxide and deprotect as required by nucleobases. If AMA is used, a small amount of a non-fluorescent impurity will be formed. To eliminate this impurity, first deprotect with ammonium hydroxide for 30 minutes at room temperature, add an equal volume of 40% methylamine, and then complete the deprotection as required by the nucleobases- e.g. 10 minutes at 65°C or 2 hours at room temperature for standard bases. Extended deprotection in ammonium hydroxide for 17 h at 55 °C also yields acceptable results.
Quasar® 570-dT	If UltraMILD reagents were used, deprotect in 0.05M Potassium Carbonate in Methanol for 4 hours at room temperature, OR for 2 hours at room temperature in 30% Ammonium Hydroxide. If standard bases were used, deprotect for 24 hours at room temperature.
Quasar® 670-dT	If UltraMILD reagents were used, deprotect in 0.05M Potassium Carbonate in Methanol for 4 hours at room temperature, OR for 2 hours at room temperature in 30% Ammonium Hydroxide. If standard bases were used, deprotection in Ammonium Hydroxide at room temperature for 24 hours will provide acceptable yields. However, the oligonucleotide will require additional purification.

Item	Pack Size	Catalog No.
JOE-dT Phosphoramidite	50 µmol	10-5936-95
	100 µmol	10-5936-90
	0.25 g	10-5936-02
Quasar® 570-dT Phosphoramidite	50 µmol	10-5953-95
	100 µmol	10-5953-90
	0.25 g	10-5953-02
Quasar® 670-dT Phosphoramidite	50 µmol	10-5955-95
	100 µmol	10-5955-90
	0.25 g	10-5955-02

New Product — 2.0M Hexylammonium Acetate, HPLC Grade, pH=7

Reversed-phase high performance liquid chromatography (RP-HPLC) is a popular method of analyzing and purifying oligonucleotides for both DMT-ON and DMT-OFF preparations.[1, 2] For the former, the hydrophobicity of the DMT group is leveraged to separate DMT-OFF truncated sequences/failures away from what should be desired, full-length product. For DMT-OFF syntheses, an ion pairing agent interacts with the charged oligonucleotide backbone to facilitate resolution of sequences based on length. For both applications, a gradient of acetonitrile in 100 mM triethylammonium acetate buffer (pH 7) will generally work well. We offer a neutral 2.0 M solution of triethylammonium acetate (TEAA) (60-4110) that can be conveniently diluted 20-fold for exactly these purposes, and many of our customers appreciate the convenience of not having to make this buffer themselves.

In recent years, there have been reports of using hexylammonium acetate (HAA) for DMT-OFF purifications.[3, 4] HAA gives much higher resolving power for both polythymidine sequences as well as sequences of mixed base composition. Although hexylamine and triethylamine share the same chemical formula (Figure 1), the longer alkyl chain of hexylamine interacts more strongly with typical reversed-phase

resins, making the ion pairing process more effective. To give our customers more options in terms of analyzing and purifying oligonucleotides, we are adding a 2.0 M HAA buffer to our offerings.

Like our TEAA buffer, a 20-fold dilution of the 2.0 M HAA will give a 100 mM working solution. For purification, some modifications in workflow may be necessary. The enhanced ion pairing of HAA results in much stronger retention of oligonucleotides that require higher gradients of acetonitrile to elute. A product fraction obtained using TEAA that has 11 % acetonitrile in it might have 40 % acetonitrile if purified with HAA. Also, HAA is a less volatile buffer because the boiling point of hexylamine is notably higher than that of triethylamine (131 versus 89 ºC).

To illustrate some of these differences, both buffers were used to analyze a mixture of crude, DMT-OFF T_{10}, T_{15}, T_{20}, T_{25}, T_{30}, T_{35} and T_{40} sequences (Figure 2). The HPLC gradients were set up such that the first peak and the last peak eluted at approximately the same time for both analyses. We were able to confirm that the HAA buffer separation indeed gives higher resolution separation for poly-T sequences. Many of the peaks are notably sharper and narrower. This is probably because the gradient of acetonitrile in TEAA (9-11 %) was much shallower than that in HAA (30-40 %).

It should be noted that we offer both reversed-phase-like cartridges and gel filtration cartridges to facilitate DMT-OFF HPLC purification. A Glen-Pak™ cartridge can remove shorter failure sequences prior to HPLC and allow the HPLC process to focus on the removal of other oligonucleotide impurities such as deletion mutants. Likewise, a Glen Gel-Pak™ can be used to remove the HAA from product fractions post-HPLC. Solutions that are diluted to have less than 20 % acetonitrile can generally be applied directly to the Glen Gel-Pak. Otherwise, evaporation of the samples and re-solvation in water would first be necessary.

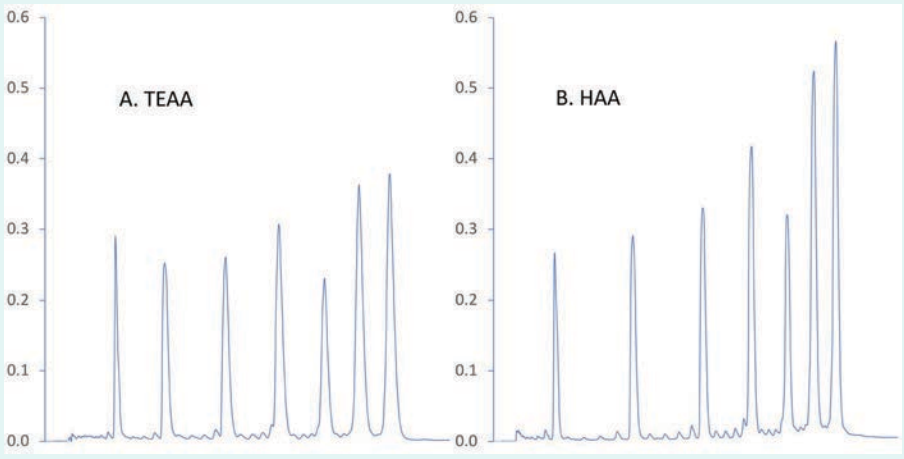

Figure 2. RP-HPLC of poly-T sequences. Buffers: A. 100 mM TEAA/acetonitrile (4:1) in 100 mM TEAA (45-55 %); B. acetonitrile in 100 mM HAA (30-40 %). Sample, a mixture of crude, DMT-OFF T_{10}, T_{15}, T_{20}, T_{25}, T_{30}, T_{35} and T_{40}; flow rate, 0.5 mL/min; column, 3.5 µm, 3.0x150 mm C18; temperature, 60 ºC; monitoring, A254. Both chromatograms share the same time profile.

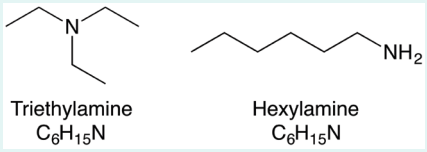

Figure 1. Triethylamine versus hexylamine.

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Item	Pack Size	Catalog No.
2.0M Triethylamine Acetate, HPLC grade, pH=7	200 mL	60-4110-52
	450 mL	60-4110-57
	960 mL	60-4110-60
	2000 mL	60-4110-62
2.0M Hexylammonium Acetate, HPLC grade, pH=7	200 mL	60-4210-52
	450 mL	60-4210-57

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Technical Snippets

Do you offer a Vacuum Manifold for the Glen-Pak™ Cartridges?

No, we only sell oligonucleotide synthesis reagents and do not offer instruments/equipment. Manifolds for the cartridges are readily available from most laboratory supply vendors. One should search for Solid Phase Extraction (SPE) manifolds for available options, and most vacuum manifolds will work with the Glen-Pak Columns. We recommend a glass block unit to observe the process. It is important to note that our Glen-Pak Cartridges (DNA and RNA) have a male standard Luer fitting designed to connect to female Luer fittings. Therefore, manifolds with a female standard Luer fitting are necessary. A standard SPE manifold is a chamber that includes a vacuum port, vacuum gauge, collection rack and 16-24 individual miniature stopcocks for each of the female Luer fitting ports.

Products:

Glen-Pak™ DNA purification cartridge	(60-5100)
Glen-Pak™ RNA purification cartridge	(60-6100)
Glen-Pak™ 50 mg DNA purification cartridge	(60-5000)
Glen-Pak™ DNA cartridge 3g	(60-5300)

Do you offer TBDMS-protected RNA supports?

No, our RNA supports are actually protected with 2'-OAc. Why not 2'-silyl groups? A specific 2'-silyl group is unnecessary at this position from a process perspective. The 2'-OAc groups are more versatile and can be used for 2'-TBDMS RNA, 2'-TOM RNA, or a chimera. The acetate is removed during cleavage from the support to yield a 2'-OH. After subsequent 2'-deprotection, the oligo is fully 2'-OH RNA.

If a 2'-TBDMS group is required for any reason at the 3'-end of your oligo, this can still be accomplished using our universal support, US III PS (26-5010), with our 2'-TBDMS RNA phosphoramidites.

Products:

Pac-A-RNA-CPG	(20-3300)
Bz-A-RNA-CPG	(20-3303)
Ac-A-RNA CPG	(20-3304)
Ac-C-RNA-CPG	(20-3315)
iPr-Pac-G-RNA-CPG	(20-3321)
Ac-G-RNA-CPG	(20-3324)
U-RNA-CPG	(20-3330)