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GLEN RESEARCH

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NEW PRODUCT

Tri-GalNAc CPG - Expanding the Therapeutic Toolbox

N-Acetylgalactosamine (GalNAc) labeling has become a dominant strategy for delivering therapeutic oligonucleotides to hepatocytes. By exploiting the asialogylcoprotein receptor (ASGPR), a highly abundant and rapidly recycling transmembrane protein expressed almost exclusively on hepatocytes, GalNAc conjugates enable selective, efficient uptake of siRNA and antisense oligonucleotides (ASOs) into the liver.[1-2]

The ASGPR recognizes terminal galactose and GalNAc residues on circulating glycoconjugates. In GalNAc-oligonucleotide therapeutics, triantennary GalNAc ligands binds ASGPR with nanomolar affinity, triggering clathrin-mediated endocytosis into hepatocytes. Following acidification of the endosome, the ligand dissociates and the receptor rapidly recycles to the cell surface, while the oligonucleotide payload then proceeds to exert its RNAi or antisense activity (Figure 1).[1-2] ASGPR is estimated to be present at hundreds of thousands to millions of copies per hepatocyte, with a recycling half time of around 15 minutes, making it a uniquely high-capacity gateway for liver-specific drug delivery.[2]

Figure 1. Targeted Delivery of GalNAc-siRNA conjugates[1]

Triantennary GalNAc architectures, in which three GalNAc residues are spatially clustered, exhibit higher avidity for ASGPR than mono- or bivalent constructs. This multivalency translates into order-of-magnitude improvements in *in vivo* potency relative to unconjugated oligonucleotides.[3-5] In one study, a trimer of GalNAC led to a 1,000-fold higher affinity towards ASGPR compared to a dimer; meanwhile a tetramer did not offer a significant improvement over a trimer.[6] With that said, in another study, divalent GalNAc still provided sufficient gene silencing activity.[7] In combination with high levels of modification (e.g. 2'-OMe, 2'-F, LNA, PS linkages, etc.). designed to improve stability, enhanced delivery platforms increase therapeutic indices by requiring lower clinical doses leading to reduced off-target effects.[1]

The clinical success of GalNAc delivery is unmistakable by multiple FDA approvals since 2019, all targeting liver-expressed genes (Table 1). All 10 oligonucleotide therapeutic agents shown in Table 1 employ the triantennary sugar ligand. Together, these approvals firmly establish GalNAc conjugation as the industry standard for hepatocyte-targeted oligonucleotide therapeutics. In addition, there is a rapidly expanding late-stage pipeline using this clinically validated delivery platform.[8]

Table 1. FDA-approved GalNAc-conjugated therapeutic oligonucleotides[2, 9]

Name	Type	Approved for	Year
Givosiran	siRNA	Acute hepatic porphyria	2019
Lumasiran	siRNA	Primary hyperoxaluria type 1	2020
Inclisiran	siRNA	Hypercholesterolemia (PCSK9)	2020
Vutrisiran	siRNA	Hereditary transthyretin (TTR) amyloidosis	2022
Nedosiran	siRNA	Primary hyperoxaluria type 1	2023
Eplontersen	ASO	Hereditary transthyretin (TTR) amyloidosis	2023
Olezarsen	ASO	Familial Chylomicronemia Syndrome	2024
Fitusiran	siRNA	Hemophilia A or B	2025
Donidalorsen	ASO	Hereditary Angioedema	2025
Plozasiran	siRNA	Familial Chylomicronemia Syndrome	2025

While FDA-approved therapies demonstrate the clinical maturity of GalNAc delivery, recent literature continues to drive innovation in GalNAc ligand design, synthesis, and application. These studies broaden the scope of GalNAc technology beyond approved indications and highlight emerging directions in oligonucleotide chemistry, pharmacology, and liver biology.

Foundational mechanistic work reinforces why triantennary GalNAc remains the optimal ligand architecture for ASGPR targeting. Trivalent GalNAc constructs consistently outperform single or double analogs in hepatocyte uptake and potency, even at reduced doses.[5-7] Preclinical programs are actively evaluating triantennary GalNAc-siRNA and GalNAc-ASO conjugates against diverse hepatic targets spanning cardiometabolic, fibrotic, and rare liver diseases.[1-3] These studies focus on optimization of ligand position, dose-response relationships in rodent models, and pharmacodynamic durability enabled by GalNAc-mediated hepatocyte enrichment. Emerging RNAi programs using multivalent GalNAc constructs are in mid-stage preclinical development, including targets in metabolic diseases, genetic disorders, and even viral infections. For example, a preclinical study from Novo Nordisk showed efficient reduction of

glycogen synthase GYS2 using a tetravalent GalNAc-siRNA conjugate in a glycogen storage disease (GSD) mouse model.[8] In addition, Roche developed a trivalent GalNAc LNA-modified ASO for chronic hepatitis B that completed Phase 1 clinical studies, but has since been discontinued.[10-11] While not regulatory approvals, these works provide primary *in vivo* data supporting long-acting silencing, reduced systemic exposure, and favorable safety profiles.[12]

Triantennary GalNAc ligands have also been leveraged outside classical therapeutic silencing. In 2020, a tri-GalNAc-based molecular imaging probe was developed to non-invasively monitor ASGPR expression in a rat model of non-alcohol steatohepatitis.[13] This work demonstrated that GalNAc clusters can serve as functional probes of hepatocyte health and receptor density, with ASGPR downregulation correlating with disease progression. Importantly, this study validated that triantennary GalNAc motifs retain high *in vivo* specificity even when conjugated to non-oligonucleotide payloads, underscoring their versatility as a hepatocyte-targeting platform.

Despite broad clinical adoption, the manufacturing of triantennary GalNAc oligonucleotides remains synthetically demanding. Our existing line up requires sequential coupling of three individual GalNAc monomers (1+1+1 strategy).[14] Generally, monomeric GalNAc addition tends to be more favorable for studies using non-trivalent clusters since this method offers more flexibility. Other commercial approaches required post-synthetic conjugation of a preassembled GalNAc cluster in solution. Both approaches add synthetic complexity, longer coupling times, and lower overall yields, particularly at scale.[4] This highlights the need for a simplified, robust GalNAc solid support to streamline manufacturing and improve reproducibility for discovery.

We are introducing a next generation triantennary GalNAc solid support that delivers the full trivalent GalNAc ligand as a single building block during synthesis (Figure 2, Table 2). Importantly, this solid support employs a triethylene glycol (TEG)-based linker that is structurally distinct from legacy GalNAc linkers used in earlier commercial products. As a result, the support is not covered by existing GalNAc IP estates and does not require licensing, offering a clear freedom-to-operate advantage for both industrial and clinical programs.

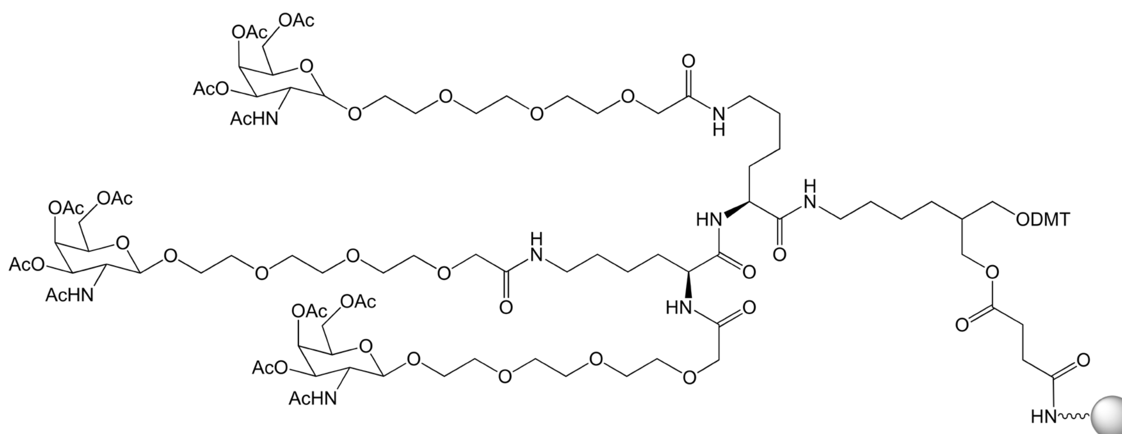

Figure 2. Tri-GalNAc CPG Structure

As GalNAc-conjugated oligonucleotides continue expanding into cardiometabolic, rare disease, and chronic liver indications, the demand for simpler, manufacturing solutions will only increase. By eliminating consecutive coupling steps while preserving the clinically validated triantennary GalNAc architecture, this new solid support represents a practical advance in oligonucleotide process chemistry. It complements recent efforts to improve GalNAc ligand synthesis efficiency and sustainability, while directly addressing bottlenecks faced by discovery and Chemistry, Manufacturing, and Controls teams.

Use of Tri-GalNAc CPG

This solid support is a great option for introducing a trivalent GalNAc modification at the 3'-end of

oligonucleotides, an approach most commonly used for siRNA therapeutics. For 5'-modification, typically preferred for ASOs, the most practical strategy remains the sequential incorporation of monomeric GalNAc phosphoramidites. In contrast, a 5'-triantennary GalNAc ligand phosphoramidite is inherently complex and challenging to handle, making it difficult to reliably maintain and use in solution during oligonucleotide synthesis.

For synthesis using Tri-GalNAc CPG, no changes are needed from the standard method recommended by the synthesizer manufacturer. Deprotection is as required per the nucleobases. The support is compatible with 30% ammonium hydroxide for 2 hours at 65 °C, 17 hours at room temperature, AMA (ammonium hydroxide/40% methylamine 1:1) for 10 minutes at 65 °C and UltraMild deprotection conditions. Extended deprotection in ammonium hydroxide (17 hours at 55 °C) may lead to some degradation.

Table 2. Product Information

Item	Pack Size	Catalog No.
Tri-GalNAc CPG	0.1 g	20-2976-01
	1.0 g	20-2976-10
1 μmol columns	Pack of 4	20-2976-41
0.2 μmol columns	Pack of 4	20-2976-42
10 μmol columns (ABI)	Pack of 1	20-2976-13
15 μmol columns (Expedite)	Pack of 1	20-2976-14
5'-GalNAc C3 Phosphoramidite	100 μmol	10-1974-90
	0.25 g	10-1974-02

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NEW PRODUCT

Spacer Phosphoramidite C6

Spacer phosphoramidites are simple but powerful building blocks in solid-phase oligonucleotide synthesis, used to introduce defined, non-nucleotidic linkers into DNA or RNA sequences. By inserting a spacer at the 5' end, 3' end, or internally, researchers can precisely control the distance between an oligonucleotide and a functional group, surface, or neighboring biomolecule. This spatial control is often critical for maintaining biological activity, reducing steric hindrance, and improving reproducibility in downstream applications.

Among the available options, a six-carbon aliphatic chain (C6) is one of the most widely used. It offers a practical balance between flexibility, length, and synthetic robustness, making it a default choice for many

laboratories. It is a popular linker used for many modifier phosphoramidites. The C6 chain is commonly employed to separate oligonucleotides from fluorophores, quenchers, biotin, or reactive handles such as amines and thiols. In surface-based assays, such as microarrays, biosensors, or bead-based systems, spacers help lift the oligonucleotide away from the surface, improving hybridization efficiency and target accessibility.[1-4]

Spacer phosphoramidites are also essential tools in aptamer research.[5] Aptamers often rely on precise three-dimensional folding to bind their targets, and direct attachment to a surface or label can disrupt this structure.[6] Incorporating a C6 spacer (or longer alternatives when needed) can preserve binding affinity by minimizing structural interference.[7] Beyond aptamers, spacers are routinely used in general assay development and testing, including PCR primer modification, probe design, and proof-of-concept conjugation experiments where consistent spacing improves signal quality and experimental comparability.

Incorporation of spacer phosphoramidites at the 3' terminus serves an additional, specialized function as a polymerase block.[8] Because spacers lack a 3'-hydroxyl group, their presence prevents enzymatic extension by DNA or RNA polymerases. This property is widely exploited in applications such as blocked primers, control oligonucleotides, sequencing adapters, and surface-immobilized probes, where unintended primer extension must be avoided.

While C6 is the workhorse, other spacer lengths and chemistries are valuable in specific contexts. Shorter linkers like C3 spacers are useful when minimal separation is required, whereas longer alkyl (e.g. C12) or hydrophilic linkers, such as triethylene glycol (TEG)-based spacers, provide additional reach while maintaining a flexible backbone. Spacer length can also be tuned during oligonucleotide synthesis by incorporating multiple spacer phosphoramidites sequentially. For example, two C3 spacers added consecutively will produce a linker with a comparable overall length to a single C6 spacer. However, because each phosphoramidite coupling cycle introduces a phosphate linkage, the resulting structure is not strictly identical to a continuous alkyl chain of the same nominal length. These intervening phosphate groups slightly alter the chemical composition and flexibility of the spacer region, which may be relevant in applications where precise linker properties are important.

Alkyl spacers such as C6 or C12 are chemically simple, synthetically robust, and compatible with standard phosphoramidite chemistry, but their hydrophobic character can promote surface adsorption or intermolecular interactions in certain assay environments.[9] Flexible hydrophilic spacers based on polyethylene glycol (PEG) or TEG introduce ether linkages that increase polarity, flexibility, and water solubility. These glycol-based chains are particularly useful in surface-based assays and conjugation strategies where minimizing nonspecific binding is critical.[10] PEG/TEG spacers reduce hydrophobic interactions with polymeric surfaces, proteins, or nanoparticles. They often improve probe accessibility by creating a more solvated and dynamic interface. In biosensor and microarray applications, an enhanced hydration layer can translate into lower background signal and improved hybridization efficiency compared to solely aliphatic linkers. Structurally, the rotational freedom around C-O bonds make PEG/TEG spacers more conformationally dynamic and can provide greater reach in solution despite similar nominal lengths.[11]

The choice between aliphatic and glycol-based spacers is often guided by the balance between hydrophobicity, flexibility, surface chemistry, and assay stringency. Together, the multiple spacer options allow fine control over oligonucleotide presentation in applications ranging from aptamer immobilization to nanoparticle conjugation and diagnostic probe development.

Expanding our catalog offerings with the addition of Spacer Phosphoramidite C6 provides our customers another option for fine control over molecular architecture, enabling more reliable design, testing, and optimization across a wide range of research and diagnostic applications (Figure 1, Table 1).

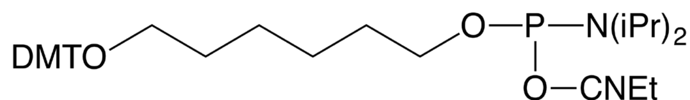

Figure 1. Spacer Phosphoramidite C6

Use of Spacer Phosphoramidite C6

This phosphoramidite does not require any changes from the standard method recommended by your synthesizer manufacturer. It is compatible with standard and UltraMild deprotection conditions.

Table 1. New Product Information

Item	Pack Size	Catalog No.
Spacer Phosphoramidite C6	100 mol	10-1966-90
	0.25g	10-1966-02

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APPLICATION NOTE

An Update on Triazole Linkages for Oligonucleotide Ligation

The efficient synthesis of long oligonucleotides is essential for applications across biotechnology, therapeutics, and molecular biology. Traditional phosphoramidite solid-phase synthesis struggles above ~150 bases due to declining yields and difficulty in purification. Click ligation, specifically via copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC), enables chemical joining of shorter fragments to build longer constructs.[1] The resulting 1,2,3-triazole linkage closely mimics the natural phosphodiester bond, offering chemical stability and biological compatibility. CuAAC selectively combines a terminal alkyne and an azide to yield a 1,4-disubstituted triazole ring under mild, aqueous, and biocompatible conditions (Figure 1).

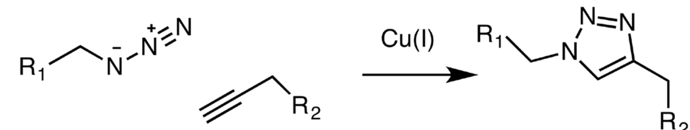

Figure 1. Triazole ring formation

We previously discussed sustainability efforts applied to oligonucleotide synthesis.[2] Click ligation improves atom efficiency in oligonucleotide synthesis by enabling highly selective and quantitative bond formation without generating significant byproducts. Unlike traditional ligation and coupling methods that often require activating agents, excess reagents, or protecting groups, click reactions proceed under mild conditions with near-stoichiometric yields. This contributes to a greener and more sustainable workflow by reducing chemical waste, solvent consumption, and energy input.

Oligonucleotide ligation through splint-mediated CuAAC has successfully prepared single-stranded or cyclic

DNA constructs up to 300 nucleotides. Introducing a ligation junction with a triazole linkage enables both PCR amplification and *in vitro* transcription across the junction, with multiple polymerases able to accurately read through the non-natural backbone. Such constructs were compatible with rolling-circle amplification and were also functional in primary biological systems, including expression of genes producing antibiotic resistance or fluorescent proteins in *E. coli*.

Emerging Applications, Future Trends, and Considerations

Recent research has significantly expanded the applications and capabilities of triazole-linked oligonucleotide ligations. One notable advancement is ClickSeq, a next-generation sequencing (NGS) method that uses 3'-azido-terminated cDNAs ligated via CuAAC to sequencing adaptors bearing a 5'-hexynyl.[3] This technology was used to generate a cDNA library for RNAseq. This method demonstrates that triazole-linked oligonucleotides are compatible with high-throughput sequencing workflows, amplifiable by PCR, and retain functional integrity. Structural and biophysical analyses, such as NMR studies of triazole-containing duplexes, further support the utility of this approach.[4] These analyses show that the triazole linkage closely resembles the geometry of native phosphodiester bonds, which helps explain why polymerases are often able to faithfully replicate or transcribe across them.

The modularity of CuAAC chemistry also enables multi-ligation strategies, where successive click reactions assemble large or branched DNA constructs, peptide-oligonucleotide conjugates, and other complex biomolecules. For example, ten oligonucleotides functionalized with 5'-azide and 3'-alkyne groups were successfully ligated in a single reaction to assemble the gene encoding the green fluorescent protein iLOV.[3] This work provided compelling evidence that DNA containing a triazole backbone at a ligation site could not only be replicated in cells but could also interface seamlessly with the full central dogma, from replication to protein production. This validated the chemical triazole linkage as not just a structural mimic but a functional equivalent to the native phosphodiester bond in biological systems. These advances open new doors for synthetic biology, especially in the design of long, chemically defined nucleic acids and functionalized bioconjugates.

As this technology continues to evolve, emerging innovations enable researchers to assemble highly complex oligonucleotide architectures, including branched or cyclic structures, DNA-peptide hybrids, and long constructs that were previously inaccessible using phosphoramidite chemistry alone. However, despite its growing impact, CuAAC-based ligation faces practical challenges. Chief among them is copper toxicity, particularly relevant in therapeutic or live-cell applications. This has driven the development of stabilizing ligands like THPTA and water-soluble copper complexes to limit cytotoxicity, as well as the rise of copper-free click reactions such as strain-promoted azide-alkyne cycloaddition (SPAAC). These methods retain the modularity and specificity of CuAAC without requiring metal catalysts, potentially expanding their compatibility with living systems.

Relevant Products

We offer reagents to enable all combinations of modifications at either terminus (Figure 2, Table 1). When we initially reviewed this topic in 2012, we highlighted our 3'-Propargyl-5-Me-dC CPG and 5'-I-dT-CE Phosphoramidite to install a 3'-alkyne and 5'-azide, respectively.[1] Is the other direction possible? While we have introduced a 3'-Azido solid support (20-2999), it won't create a triazole backbone since the azide is part of the serinol linker. A recent paper may provide a

work around for this. A 3'-amino terminal oligonucleotide was reacted with a 5'-phosphate azidodeoxythymidine (AZT) to form a phosphoramidate linkage and a 3'-azide terminus using EDC under aqueous conditions.[5, 6] We offer several 5'-alkyne products, such as our 5'-Hexynyl Phosphoramidite. The extended branch on the triazole linkage produced is still compatible with PCR amplification, as the ClickSeq technique found common DNA polymerases, including Klenow and OneTaq, that could read through the bulky triazole linkage.[3] We also offer THPTA for copper-mediated click reactions.

To incorporate an azide into an oligonucleotide to make the unnatural triazole linkage, a post-synthesis reaction is required. Azide phosphoramidites are unstable due to the Staudinger reaction with the P(III) center and are not commercially available.

Triazole-linked ligation is more than just a chemical workaround. It is a foundational technology for building scalable, functional, and biologically compatible nucleic acid systems. Our reagents support modular assembly of complex oligonucleotide architectures, enabling new possibilities in synthetic biology and nucleic acid engineering.

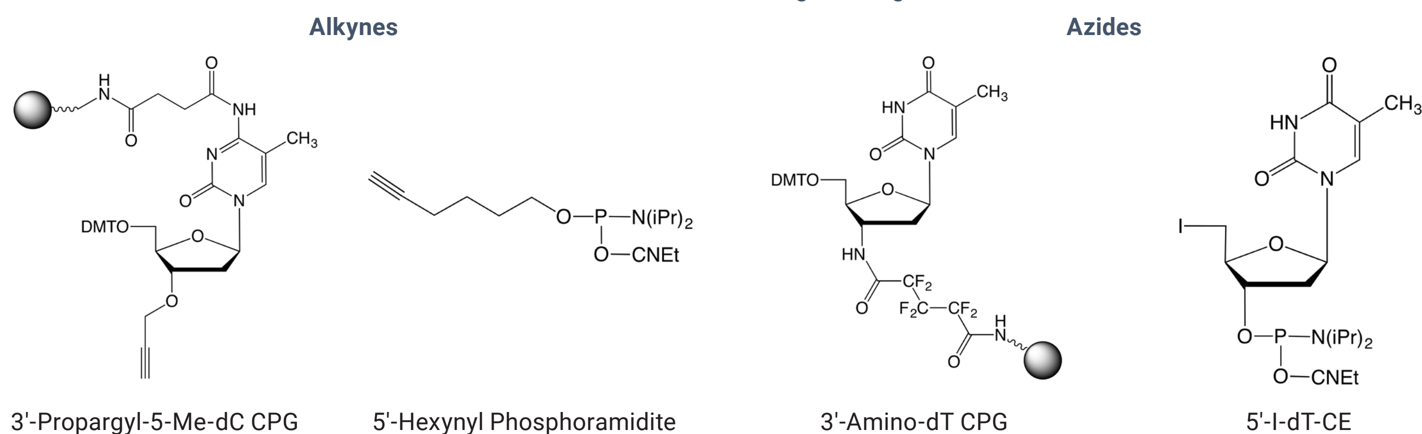

Figure 2. Reagents suitable to triazole linkage formation

Table 1. Relevant Products

Modification	Product	Catalog No.
Alkyne	3'-Propargyl-5-Me-dC CPG	20-2982
	5'-Hexynyl Phosphoramidite	10-1908
Azide	3'-Amino-dT CPG	20-2981
	5'-I-dT-CE Phosphoramidite	10-1931
Ligand	THPTA Ligand	50-1004

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TECHNICAL NOTE

Limits of DMT-ON Purification

The 4,4'-dimethoxytrityl protecting group, usually referred to as DMT or just trityl, serves several functions in oligonucleotide synthesis. First and foremost, the DMT is an effective protecting group that ensures only one monomer is added per synthesis cycle. The DMT, typically attached to the 5'-OH, is relatively stable during storage or on a synthesizer and can be rapidly removed with acid to allow the next monomer addition to occur. The DMT also allows convenient, stepwise monitoring of a synthesis due to the bright orange color of the DMT cation that is released at the beginning of each synthesis cycle. A less obvious use of the DMT, and the one we would like to highlight, would be its role as a purification handle.

When the oligonucleotide synthesis is complete, there will usually be a DMT on the 5'-end. While one can remove it with an extra deblock cycle of acid via the synthesizer, the DMT can instead be retained for reverse phase (RP) purification. Oligonucleotides are polyanionic and relatively polar. A single DMT can dramatically change the polarity of the resulting oligonucleotide such that the full length DMT-ON sequence can be readily separated from all shorter DMT-OFF truncated sequences. After purification, the DMT can be removed to give desired purified product.

These purifications are typically performed via two approaches, RP-HPLC or Glen-Pak™ purification, and they have been reviewed or discussed in earlier reports.[1-4] Briefly, RP-HPLC is a multi-step process where there are pre- and post-purification procedures while the Glen-Pak is essentially a single procedure where everything is done on the column. RP-HPLC is the high-resolution option that also provides simultaneous analysis. In contrast, disposable Glen-Pak cartridges (Table 1) have notable advantages in terms of speed, simplicity, and throughput. Both approaches are extensively used.

While DMT-ON purification is relatively effective, there are a few instances where it is more challenging. One area where DMT-ON purification is less effective is when there are other modifications present that also affect hydrophobicity. It can be as simple as the placement of a fluorophore on the 3'-end rather than the 5'-end. In such a scenario, every truncated sequence will be attached to a hydrophobic fluorophore, and the full-length sequence will have both a DMT and a fluorophore.

A second area where DMT-ON purification will be less effective is for very long sequences. In general, longer sequences are already more difficult to purify because there are a lot more shorter failure sequences that need to be removed. For DMT-ON purification specifically, increasing oligonucleotide length also decreases resolution. While a single DMT changes the hydrophobicity of a 10 nt sequence significantly, the same effect is much more muted for a 150 nt sequence. For longer sequences, much shorter failures will be readily removed while failures that are close to full-length may still be present to some extent. Due to this, there have been efforts to increase the hydrophobicity of the DMT-ON product.[5]

Finally, DMT-ON purification is limited in its ability to purify away deletion or addition mutants. The former arises due to either insufficient capping, deblocking or other factors while the latter typically arises from more than one addition in a coupling step. Both result in DMT-ON oligonucleotides that are very similar to that of the desired product. Deletion and addition mutants elute slightly later and earlier, respectively, relative to the main peak and are readily co-purified.

To illustrate how complex a DMT-ON purification can be, a test sequence was synthesized and analyzed. Ten additions of dT-CE Phosphoramidite were added to a dye-CPG column. The synthesis was performed DMT-ON, and deprotection was carried out with concentrated ammonium hydroxide. Subsequently, the deprotection solution was diluted with water and analyzed by RP-HPLC (Figure 1). When monitoring the absorbance based on the absorbance maximum wavelength of the dye, one is able to see exactly ten peaks that match the number of dT additions that were made. T_1-dye and T_2-dye eluted later than the full-length, DMT-ON material while T_{3-9}-dye eluted earlier than the full length-material. In addition to these ten peaks, there is also a small shoulder behind the main peak. That should be DMT-T_9-dye. For reference purposes, DMT-ON T_6 and DMT-OFF T_6 have retention times of 15.5 and 10.7 min, respectively, using the same

elution gradient.

In this example, a Glen-Pak purification will not be effective. Based on the retention time of DMT-ON T_6, a Glen-Pak will isolate all of T_{1-4}-dye, DMT-T_9-dye, and DMT-T_{10}-dye. It is also likely that at least portions of the remaining fragments will also be co-eluted. RP-HPLC would fare much better, but even then, it might still be difficult to completely exclude the DMT-T_9-dye and T_3-dye fragments. This is of course a very short oligonucleotide with one additional hydrophobic modification. Longer sequences with an additional quencher or multiple phosphorothioate linkages would be even more tricky.

While DMT-ON purification has its share of limitations, it continues to be a very effective and popular method. In cases where the oligonucleotides are more challenging to purify, optimization may be required and multiple modes of purification may have to be leveraged.

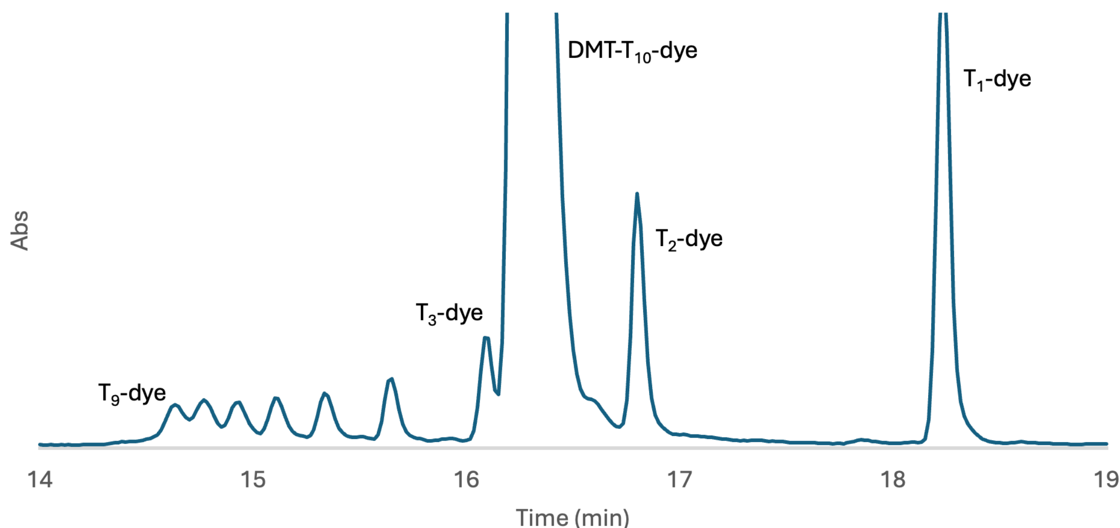

Figure 1. Chromatogram of unpurified T_{10}-dye. Column, RP-18; visible detection at the absorbance maximum wavelength of the dye; mobile phase, acetonitrile/triethylammonium acetate 0.1 M, pH 7.0.

Table 1. Glen-Pak products

Item	Pack Size	Catalog No.
Glen-Pak™ 50 mg Purification Cartridge	Pk/96	60-5000-96
Glen-Pak™ 150 mg Purification Cartridge	Pk/10	60-5100-10
	Pk/30	60-5100-30
	Pk/96	60-5100-96
Glen-Pak™ 150 mg Purification Cartridge (for use with disposable syringes)	Pk/1	60-5200-01
	Pk/10	60-5200-10
Glen-Pak™ 3 g Purification Cartridge	Pk/1	60-5300-01
	Pk/5	60-5300-05
	Pk/10	60-5300-10
	Pk/20	60-5300-20
Glen-Pak™ 30 mg 96-Well Plate	Pk/1	60-5400-01
Glen-Pak™ 3 mg 384-Well Plate	Pk/1	60-5500-01
	Pk/10	60-5500-10

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1.0 M Triethylammonium Acetate for RP-HPLC

HPLC, short for High Performance Liquid Chromatography, is a versatile and powerful separation technique that has been around for over 60 years.[1] It is a standard method of analysis in any analytical chemistry environment and commonly used to purify complex mixtures of materials. Briefly, HPLC involves pushing liquid (mobile phase) containing a sample of interest (analyte) through a column of very small particles (stationary phase) under very high pressures. As the sample moves through the column, it interacts with the particles in it, and that interaction results in them being slowed down relative to the flow of the liquid. Different constituents in the sample will be slowed down to a different degree, separating the constituents, and that eventually results in them being eluted off the column at different times, which is referred to as the retention time. When paired with downstream photodiode array detectors and mass spectrometers, each of the materials eluting off of the column can be analyzed and quantified simultaneously. Modern HPLC instruments control pressure, flow rate and temperature to give consistent and reliable results. They can also be equipped with auto-injectors and fraction collectors for a completely automated analysis and purification process.

At Glen Research, HPLC is used extensively. Most analyses are performed under reverse phase conditions (RP-HPLC). That typically includes a C18 (octadecylsilyl) column and a mobile phase consisting of a mixture of acetonitrile and aqueous 0.1 M triethylammonium acetate (pH 7). For a raw material like DBCO C6 acid, a precursor to several products in our click chemistry lineup, we verify 1) identity based on the retention time and the UV spectrum and 2) purity based on the area under the peaks. In this case, the retention time (Figure 1A) and UV spectrum (Figure 1B) are consistent with historical data, and the purity is excellent as there are no other peaks present (Figure 1A).

Phosphoramidites can also be analyzed similarly even though they are relatively water sensitive. Both identity and purity analyses are performed, and this information is included on our quality reports. For dA-CE Phosphoramidite, we include these results in a table. We also include two chromatograms showing the UV absorbance at 254 nm versus elution time. The first chromatogram consists of the sample lot (Figure 2A) while the second is the same sample spiked with a verified standard (Figure 2B) to confirm the identity of the material. In this case, the identity of the phosphoramidite was confirmed, and purity was excellent. It should be noted that most nucleoside phosphoramidites are synthesized as a mixture of two diastereomers, and that is why there are two peaks present. This was discussed earlier.[2]

Finally, most oligonucleotide synthesis tests are analyzed by RP-HPLC. This verifies that the functional tests produce the correct product and also allow for the calculation of coupling efficiency. Again, for dA-CE Phosphoramidite (Figure 3), a short oligonucleotide is synthesized with multiple insertions of dA. The final DMT (4,4'-dimethoxytrityl) is retained such that the full-length oligonucleotide is much more hydrophobic than any shorter DMT-OFF failures and is eluted much later. By comparing the areas of the curve between the desired product and the shorter failures, the average coupling efficiency can be calculated. At first glance, it appears there are notable amounts of shorter failures. In fact, the peak at about 12 minutes corresponds to N-methylbenzamide. Oligonucleotide deprotection releases several protecting group byproducts, but for this synthesis, N-methylbenzamide is the only one that absorbs UV. Excluding the N-methylbenzamide peak, one can see that coupling of the phosphoramidite was highly efficient.

In each of these three examples, as well as the data in the DMT-ON purification article in this same issue,[3] there is one common reagent, triethylammonium acetate

buffer. The buffer serves several purposes. First and foremost, it maintains the pH at 7.0. Any fluctuations in pH will affect performance, and the buffer ensures that is not an issue. The buffer can also act as an ion pairing agent where it interacts with both the column resin and the negatively charged oligonucleotide backbone to further retain material on the column based on sequence length. Finally, it acts as a volatile buffer. Triethylamine and acetic acid have boiling points of 89 and 118 °C Celsius, respectively, and can be conveniently removed under vacuum in the same way as for acetonitrile and water, the other two liquids typically present.

For many years, Glen Research has been offering triethylammonium acetate buffer as a 2.0 M solution for customers to dilute as needed for their purifications. To give users more options and flexibility, we are now adding a 1.0 M solution (Table 1). This is half the concentration of our traditional offering and represents a 10x solution of the commonly used concentration of 0.1 M. Both concentrated buffers continue to be HPLC grade products that are suitable and convenient for oligonucleotide-related work.

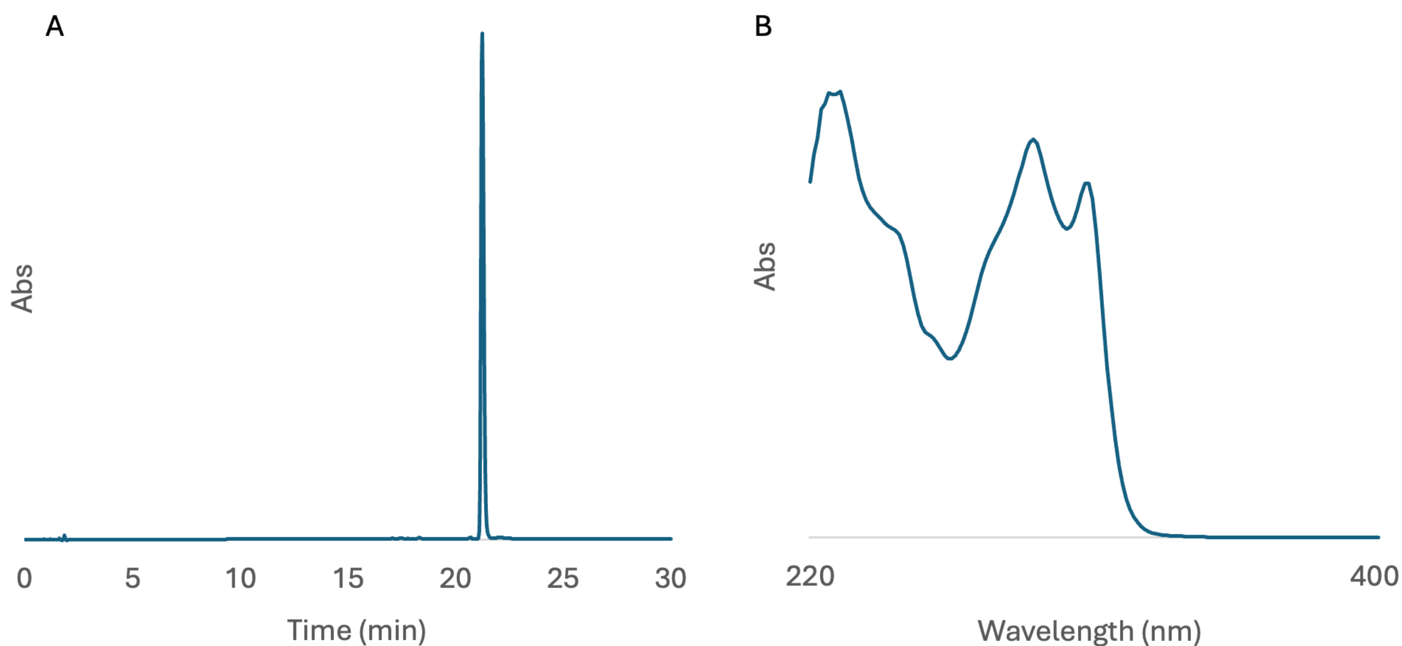
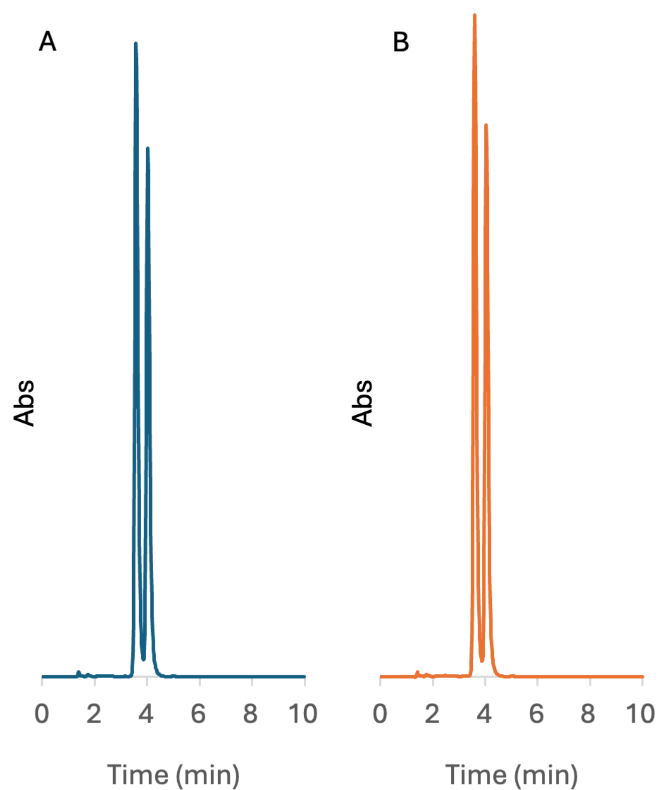


Figure 1. RP-HPLC analysis of DBCO C6 acid. Column, RP-18; detection, UV; mobile phase, acetonitrile/triethylammonium acetate 0.1 M, pH 7.0 (3-40% gradient). A, chromatogram @254 nm; B, UV spectrum at time of 21.2 min.



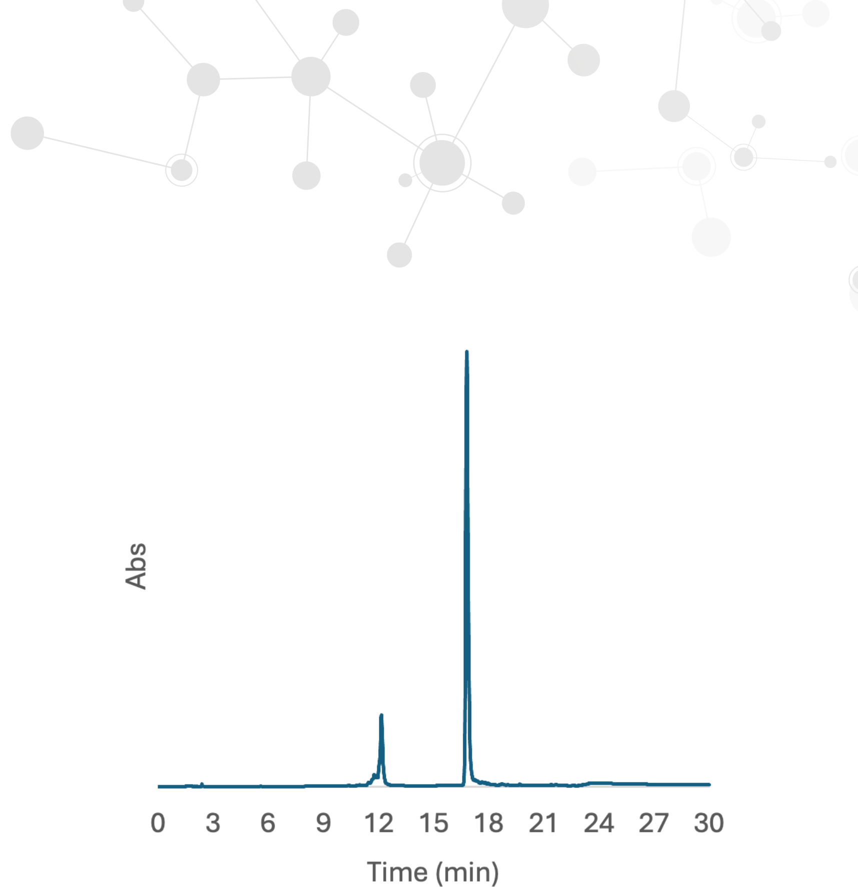


Figure 2. RP-HPLC analysis of dA-CE Phosphoramidite. Column, RP-18; detection, UV at 254 nm; mobile phase, acetonitrile/triethylammonium acetate 0.1 M, pH 7.0 (80/20). A, sample; B, sample spiked with standard.

Figure 3. RP-HPLC analysis of dA-CE Phosphoramidite incorporation. Column, RP-18; detection, UV at 254 nm; mobile phase, acetonitrile/triethylammonium acetate 0.1 M, pH 7.0 (3-40% gradient).

Table 1. Triethylammonium acetate buffers

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
1.0M Triethylamine Acetate, HPLC grade, pH=7	200 mL	60-4111-52
	450 mL	60-4111-57
	960 mL	60-4111-60
	2000 mL	60-4111-62

References

1. L.R. Snyder, Anal Chem, 2000, **72**, 412A-420A.
2. *The Glen Report*, 2020, **32.1**, 12.
3. *The Glen Report*, 2026, **37.1**, 10-11.

Notes

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Technical Snippets

Why is my oligonucleotide precipitate a jelly-like consistency?

The root cause is likely a result of a physical aggregation rather than a chemical issue. There are certain situations where the risk of observing a jelly-like consistency is high. For example, a very high oligonucleotide concentration (>1-5 mg/mL) could lead to a thick consistency. A GC-rich or self-complementary sequence can also have a higher tendency to form a jelly-like precipitate. In some cases, residual amines, salts, and protecting-group byproducts, or dissolved silica from the deprotection step can exacerbate the problem.

The precipitation method may also contribute to this phenomenon. Precipitating with isopropanol leads to more rapid collapse of the oligonucleotide, which can trap water and lead to a soft, squishy pellet. In contrast, ethanol is slower at precipitating nucleic acids and lowers the risk of forming a jelly-like precipitate during desalting.

An oligonucleotide that is stuck in a jelly-like pellet can typically be recovered by heating for a few minutes and/or diluting it 5-10x in water or buffer. A 70% ethanol wash or desalting using Glen Gel-Pak may also help if the root cause is from residual contaminants or the use of isopropanol.

How does one transfer CPG from a synthesis column to a vial for subsequent cleavage and deprotection?

For Twist columns with threaded caps, this is very straightforward. For Expedite, MerMade or Dr Oligo columns, it involves a bit more work. While there is always the option of slicing the column open with a razor, sliding out a frit is probably the better approach. If one taps a syringe needle on a bench, they will get a hook that can fish one of the frits out of the column. Any other equivalent tool will also suffice. Sometimes, the frits may break, and that is perfectly fine. Some random bits of frit in the CPG will not affect downstream processes.

Relevant products:

Empty Synthesis Columns, 1um Expedite Styles (20-0021-01)

Empty Synthesis Columns, 40nm, 0.2um Expedite Style (20-0021-02)

Empty MerMade Columns (200nm and 1μm) (20-0050-02)

Empty MerMade Columns (50nm) (20-0050-05)

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