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Beyond the UV Region — DEACM-dG as a Versatile Tool for Light-Activatable ("Caged") Oligonucleotides

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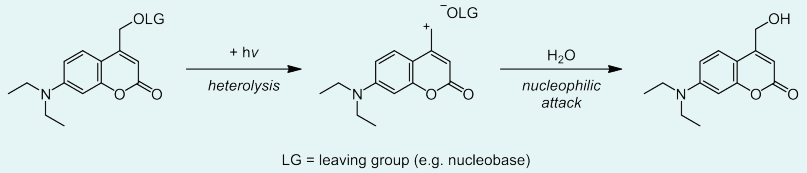
Almost all major processes of life on the molecular and cellular scale are inherently three-dimensionally structured and time-dependent. Investigating these aspects requires tools that allow asking spatiotemporal questions. A very elegant way to do this is to use triggers so that the system under investigation can be prepared in a non-equilibrium state. After triggering, the system reacts in space and time so that we can build models about the 4D behavior. In this domain, "photocaging" has recently gained more and more interest. Technically, this process makes use of what are otherwise known as photolabile protecting groups. However, in this mode of application – as opposed to their use in organic synthesis – it is biochemical activity that is temporarily protected. Thus, light becomes the trigger signal. This is appealing because there are many different and easily available light sources (especially the vast array of high-power LEDs, currently available for low cost). There are many technologies that allow applying light with high precision (optical setups including microscopes and also catheters for an organismic application). Light is a biorthogonal trigger signal, as most cells do not respond to light. In contrast to chemical reagents, light regulation is a non-invasive method and has no cell-damaging effect as long as its wavelength lies beyond the UV region (> 380 nm). This and many more reasons make light an ideal tool for structural or kinetic investigations of biologically active compounds.



Scheme 1: Simplified photolysis mechanism of DEACM with a leaving group (LG) attached to the 4-position.

Early studies only used statistic caging – a method where an oligonucleotide was incubated with unselective, electrophilic precursors of photocages to afford a randomly modified oligonucleotide with a varying number of modifications.[1] However, this approach was of limited use. We[2] and the groups of Deiters[3] and Dmochowski[4] decided to introduce

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photocages in specific positions – preferably the nucleobases to render them temporarily incapable of forming what is probably one of the most important features of oligonucleotides: Watson-Crick-Franklin base pairing. This approach is a very general one: on the one hand it allows the light-control of global secondary structure formation (duplex, quadruplex, stem-loop, …). On the other hand, it is also useful for regulating local, specific interactions such as for example, ligand binding in aptamers or seed region recognition in siRNAs or miRNAs.

Some applications require the incorporation of multiple photocages, while in others one single well positioned modification can already be sufficient to show the desired effect. For example, an entire 21 nt long antimiR for the regulation of angiogenesis in living mice could be blocked by incorporation of six photocages.[5] Ligand binding in an aptamer on the other hand was shown to be successfully modulated with only one single caged nucleotide at the ligand binding site.[6] As another example, position-specific nucleobase caging allowed the investigation of G quadruplex folding kinetics by real-time NMR spectroscopy.[7] Applications of photocaging in oligonucleotides have been reviewed in the literature extensively, and more examples can be found there.[8–10]

One of the earliest and most popular classes of photocages are *o*-nitrobenzyl (ONB) cages, with 1-(2-nitrophenyl) ethyl (NPE) as the most prominent one. Their major drawbacks are their poor photorelease quantum yields and absorption maxima in the UV region, making them arguably suitable for *in vivo* use. Furthermore, the photolysis of ONB cages results in the formation of an *o*-nitrosobenzaldehyde byproduct that can potentially react with biological material to form toxic side products.[11,12]

Coumarin derivatives are widely known as fluorophores and by chemical modification can have absorption maxima between 300 and 700 nm.[13–15] Givens *et al*. discovered their potential as caging groups,[16] and 7-diethylamino substitution was later shown to positively influence the uncaging quantum yields.[17] More importantly, the 7-diethylamino group resulted in a large red-shift of the absorption maximum out of the UV region to 400 nm and has since then been established as a commonly used substituent for coumarins.

The uncaging mechanism of coumarin cages is well understood.[18] After excitation through irradiation, the molecule undergoes heterolytic cleavage of the C-O bond at the caging site, followed by nucleophilic attack and therefore addition of a solvent molecule (Scheme 1). After irradiation of a coumarin-caged nucleobase, the native nucleobase is recovered and no reactive or toxic side products are formed.

Uncaging of 7-(diethylamino)coumarin (DEACM) can be achieved by two different processes. One-photon excitation (1PE) in the region of the absorption maximum (~400 nm) is the simplest way of uncaging. Nowadays, technical progress has led to LEDs that have enough power for efficient and fast uncaging. They offer a simple and cheap solution for most uncaging experiments. In addition to 1PE, two-photon excitation (2PE) can be stimulated with femtosecond pulsed lasers allowing the uncaging of DEACM at 780 nm within the phototherapeutic window (650–900 nm) in which organic tissue has the smallest optical density. Both possibilities were demonstrated in oligonucleotides by uncaging of a DEACM-dT moiety using a 390 nm LED for 1PE and a 780 nm laser for 2PE.[19]

DEACM-dG specifically has been investigated by our group several years ago.[20] This exact derivative is now available from Glen Research. We could show that photocleavage of the DEACM moiety was possible within wavelengths from 365 nm up to even 470 nm and that the uncaging efficiency was 17 times higher than in a nitrophenethyl photocage control. Due to the faster cleavage and higher uncaging wavelengths, it was possible to even selectively uncage DEACM in a mixture of both cages. We later found out, that we could even further drive the uncaging wavelength for DEACM to 505 nm.[21] Also, the faster uncaging of DEACM makes this candidate more suitable for kinetic studies, where the photolysis process has to be faster than the kinetic process one wants to investigate.

Since DEACM-dG is based on deoxyribose, standard coupling times can be applied using either ETT or BTT as the activator. Also, cleavage and deprotection can be carried out using standard procedures in 33% aqueous NH_3 at room temperature overnight. An advantageous side-effect of the DEACM-modification is its absorption and fluorescence profile, which allows easy monitoring during oligonucleotide synthesis and purification or tracking in cells.

During all steps of sample handling including deprotection, purification, or simple pipetting, ambient light exposure should be kept to a minimum. This can for example be achieved by covering the sample with aluminum foil. However, it is not necessary to work in a darkroom. Also, the use of brown tubes can make handling of photolabile samples easier. Unwanted deprotection can be further minimized by storing all samples at –21 °C in the dark. Elevated temperatures in combination with harsh conditions, e.g. basic deprotection, can lead to accidental uncaging. Therefore, we recommend keeping temperatures moderate (under 40 °C), especially during deprotection, but also purification and further handling. We decided to put our vacuum concentrators in the cold room to prevent deprotection through heat development.

The fastest uncaging of DEACM-dG can be induced by irradiation with a 400 nm LED. The uncaging rate is dependent on the amount of oligonucleotide and the number of cages installed, optical density, and the irradiation power. As a rule of thumb, quantitative uncaging of 100 pmol of an oligonucleotide containing one single cage is well achieved within 2 min of irradiation with a 160 mW LED.[20]

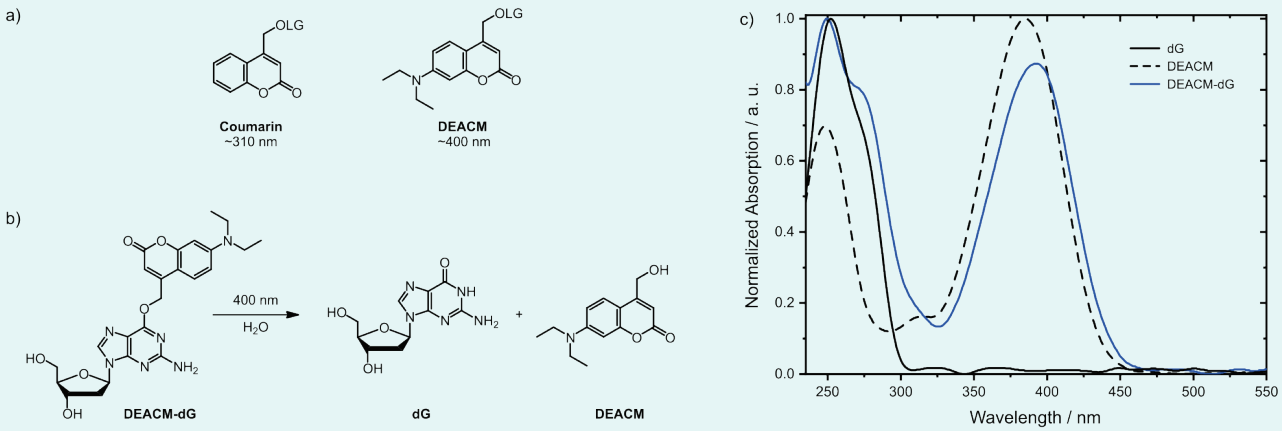

Figure 1: a) Coumarin core and modified DEACM. b) Photolysis of DEACM-dG in aqueous medium. c) UV/vis spectra of dG, DEACM and DEACM-dG in 1x PBS/acetonitrile 95:5.[20]

In conclusion, DEACM-dG offers a variety of possibilities for spatiotemporal control of oligonucleotides. Its fast uncaging properties as well as higher uncaging wavelengths than common ONB cages offer better resolution in kinetic studies and even allow wavelength-selective uncaging in mixtures of both cages with high selectivity. Together with its compatibility with standard deprotection conditions and purification protocols, DEACM-dG will offer new possibilities in structural as well as kinetic studies or gene regulation, controlled by harmless blue light.

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New Product — DEACM Caged-dG

As detailed in the previous article, the photocaging of oligonucleotides is a powerful method for structural and kinetic investigations. For this reason, we introduced NPOM Caged-dT (Figure 1) years ago[1] to give our customers the ability to perform such experiments. The NPOM is an *o*-nitrobenzyl group that is attached to the base pairing face of thymine. Upon irradiation with UV light at 365 nm, the NPOM is readily removed to give native thymine. Oligonucleotides containing NPOM Caged-dT have been successfully used in a whole host of applications including DNAzymes,[2] antisense oligonucleotides,[3] microRNA logic gates[4] and more recently, CRISPR.[5] With the help of Dr. Heckel and his team, we are happy to now provide a second option for synthesizing photocaged oligonucleotides, DEACM Caged-dG (Figure 1). This will give our customers access to another nucleobase as well as a very different photoremovable protecting group.

In our hands, we found DEACM Caged-dG relatively straightforward to use in oligonucleotide synthesis. For insertion, a three minute coupling time was required using tetrazole as the activator. For deprotection, both the coumarin and the 6-position of dG are susceptible to reaction with nucleophiles. For example, treatment with ammonium hydroxide and prolonged heating will convert the guanine to a diaminopurine. For this reason, the phosphoramidite is provided with the UltraMILD protecting group, iPr-Pac, to facilitate UltraMILD synthesis and deprotection. Possibly due to steric hindrance from the DEACM, deprotection of the iPr-Pac group requires more time. For a 7 nt sequence with a single insertion of DEACM-dG, deprotection required three and six hours, respectively, for ammonium hydroxide and potassium carbonate (50 mM in MeOH). For a 12 nt sequence with three insertions, both conditions required overnight treatment. In all cases, the potassium carbonate treatment always gave cleaner chromatograms. Therefore, in general use, an UltraMILD synthesis and potassium carbonate deprotection (50 mM in MeOH) for 6-17 h at room temperature is recommended. Those who prefer a standard synthesis can still obtain relatively good results by using ammonium hydroxide at room temperature overnight (dmf-dG required) or AMA at room temperature for two hours (Ac-dC required) for deprotection. Heat should be avoided, both during deprotection and processing.

We also found that the DEACM group is relatively stable to ambient lighting. We placed aliquots of the yellow phosphoramidite solid in the lab exposed to standard fluorescent lighting during the day and subsequently analyzed solutions of them by reverse phase HPLC. We found that the purity of the phosphoramidite was reduced by only 0.4 % per day over a three day period. Even though DEACM can be released with visible light, we will fortunately not have to work in the dark. We would like to thank Dr. Heckel and his team for their excellent overview of DEACM-dG, their scientific input as well as their review of this article.

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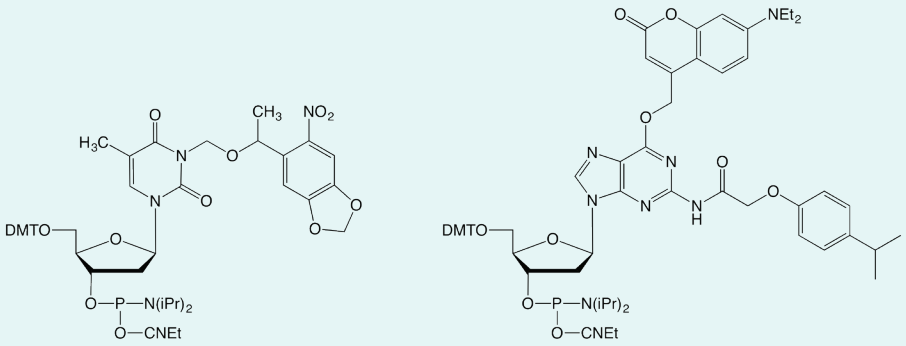

Figure 1. Photocaged Phosphoramidites

Item	Pack Size	Catalog No.
NPOM Caged-dT-CE Phosphoramidite	50 µmol	10-1534-95
	100 µmol	10-1534-90
	0.25 g	10-1534-02
DEACM Caged-dG-CE Phosphoramidite	50 µmol	10-1533-95
	100 µmol	10-1533-90
	0.25 g	10-1533-02

Application Note: Phosphorodithioates in Oligonucleotide Therapeutics

Phosphorothioates are probably the most popular modifications used in oligonucleotide therapeutics. It involves the substitution of a sulfur atom for a non-bridged oxygen at a phosphate. This modification does not significantly hinder duplex formation, retains the ability of the oligonucleotide duplexes to promote RNase H activity and enhances general nuclease stability. This last property has played key roles in modulating the *in vivo* half-lives of several approved oligonucleotide therapeutics and many more candidates in earlier clinical stages.

This modification also has disadvantages, and the most notable of these is that phosphorothioates, introduced via the standard phosphoramidite method, give additional stereocenters. Each phosphorothioate substitution can be either an "Sp" or "Rp" conformation (Figure 1A). As such, a fully phosphorothioate 20 nt antisense oligonucleotide will be a mixture of more than half a million (2^{19}) different molecules. To avoid and/or reduce this type of complexity, one workaround is to use achiral phosphorodithioates instead, where both non-bridged oxygen atoms are replaced with sulfurs. Phosphorodithioates share many of the desirable properties of phosphorothioates without the additional undesired stereocenters.[1, 2] In addition, phosphorodithiotes are even more stable to nucleases than their phosphorothioate counterparts.

Phosphorodithioates were first developed over thirty years ago. The first oligonucleotide syntheses incorporated dinucleotide phosphoramidites with the phosphorodithioate already in place, and later investigations developed thiophosphoramidites that could be used in place of standard phosphoramidites. One sulfur would come from the thiophosphoramidite while the other would be obtained via the sulfurization reagent (Figure 1A). Several versions of these thiophosphoramidites have been described, and the most popular of these employs pyrrolidinyl thiophosphoramidites where the thiol is protected with a beta-(benzoylmercapto)ethyl group (Figure 1).[3] After synthesis is complete, basic deprotection conditions will first remove the benzoyl group and then subsequently eliminate ethylene sulfide to give the desired product (Figure 1B).

Glen Research introduced DNA thiophosphoramidites in 2008[4] and subsequently 2'-OMe thiophosphoramidites in 2015[5] (Figure 2). Since this latter introduction, there have been several studies published on the use of these and other closely related building blocks. Recently, Abeydeera *et al.* highlighted the utility of phosphorodithioates for optimizing aptamers post-SELEX.[6] The researchers synthesized a complete series of singly phosphorodithioate-modified sequences of a $VEGF_{165}$ RNA aptamer and evaluated them for binding to $VEGF_{165}$. Although most of these sequences behaved similarly to the native unmodified aptamer, there were two particular sequences that were very different, each with a single G phosphorodithioate substitution. These sequences had binding constants that were approximately one-thousand-fold enhanced relative to that of the control aptamer, a very impressive improvement in performance.

This type of investigation was also applied to an α-thrombin RNA aptamer, and similar results were observed. In this case, one U phosphorodithioate substitution also enhanced binding affinity by approximately one-thousand-fold relative to that of the unmodified α-thrombin aptamer control. In addition to elevated binding constants, these sequences exhibited relatively unchanged secondary structure, maintained binding specificity and as expected, showed enhanced serum stability.

In another aptamer study, Amero *et al.* employed an approach similar to that of what was used for the $VEGF_{165}$ and α-thrombin to further improve a previously characterized and partially optimized aptamer.[7] In this case, the target was the receptor tyrosine kinase, AXL, which is overexpressed in ovarian cancer. By binding to this receptor, the aptamer would silence the activity of AXL and lead to decreased ovarian cancer tumor growth. *In vitro* studies showed that two particular phosphorodithioate aptamers, one with a single dA phosphorodithioate and the other with a single dG phosphorodithioate, were quite effective relative to controls. Subsequently, PEG-conjugated versions of these two aptamers were found to significantly inhibit ovarian cancer tumor weight in mice.

In a third publication, the use of phosphorodithioates in LNA/DNA antisense

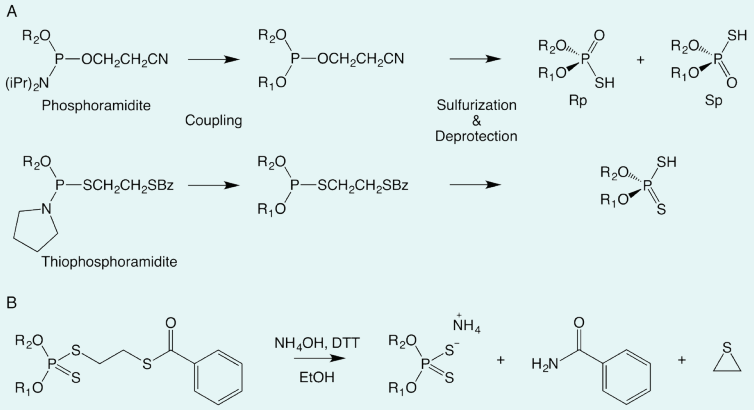

Figure 1. Phosphorothioate and phosphorodithioate chemistry.
A: Top, phosphorothioate synthesis; bottom, phosphorodithioate synthesis.
B: Deprotection of phosphorodithioate linkage.

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oligonucleotides was evaluated.[8] Bleicher *et al.* synthesized LNA thiophosphoramidites and then used these, along with the commercially available DNA versions, to synthesize several series of RNase H activating gapmers (LNA-DNA-LNA) and steric blocking mixmers (alternating LNA/DNA). These oligonucleotides were fully phosphorothioated with up to seven phosphorodithioate substitutions. In general, phosphorodithioates were well tolerated in the gapmers, particularly in the LNA flanking regions. The phosphorodithioate substitutions did not significantly alter melting temperatures, enhanced serum stability, improved cellular uptake and most importantly, enhanced target reduction in both primary rat hepatocytes as well as mice. For the mixmers, phosphorodithioate substitutions were compatible but sequence dependent. In a set of exon-skipping experiments, DNA phosphorodithioate linkages were generally more beneficial than LNA phosphorodithioate linkages.

As demonstrated in these recent investigations, phosphorodithioates continue to be a compelling alternative or complement to phosphorothioates for therapeutic applications. For those who would like to explore DNA and 2'-OMe phosphorodithioate-containing backbones, the required phosphoramidites and the methods for their use are readily available from Glen Research.

We would like to thank Xianbin Yang for reviewing this document and for his many helpful suggestions.

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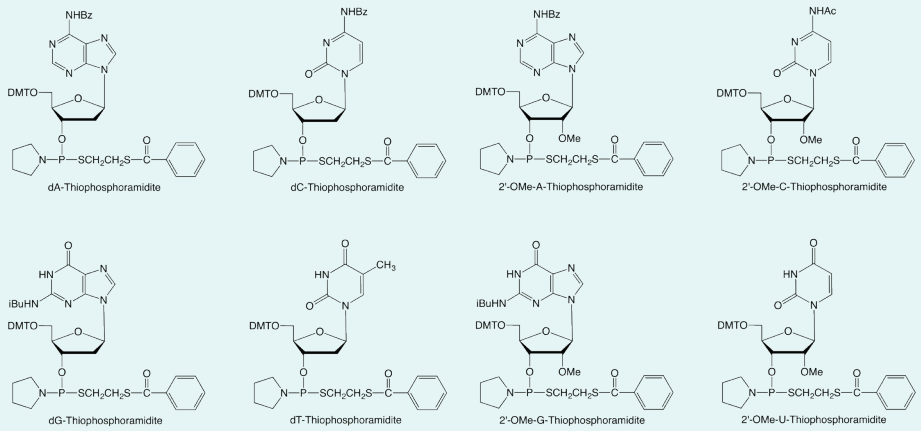

Figure 2. DNA and 2'-OMe Thiophosphoramidites

Item	Pack Size	Catalog No.
dA-Thiophosphoramidite	100 µmol	10-1700-90
	0.25 g	10-1700-02
dC-Thiophosphoramidite	100 µmol	10-1710-90
	0.25 g	10-1710-02
dG-Thiophosphoramidite	100 µmol	10-1720-90
	0.25 g	10-1720-02
dT-Thiophosphoramidite	100 µmol	10-1730-90
	0.25 g	10-1730-02
2'-OMe-A-Thiophosphoramidite	100 µmol	10-3170-90
	0.25 g	10-3170-02
2'-OMe-C-Thiophosphoramidite	100 µmol	10-3171-90
	0.25 g	10-3171-02
2'-OMe-G-Thiophosphoramidite	100 µmol	10-3172-90
	0.25 g	10-3172-02
2'-OMe-U-Thiophosphoramidite	100 µmol	10-3173-90
	0.25 g	10-3173-02

New Product — Ac-dC-5’-CE Phosphoramidite

In recent years, the need for 5’-3’ direction synthesis reagents has increased significantly. Whether researchers are looking for enhanced nuclease resistance, trying to incorporate a special modification like a dideoxynucleotide or simply needing to synthesize oligonucleotides on a surface that can be subsequently extended with polymerases,[1, 2] they are constantly looking for a wider variety of reverse synthesis reagents that give them more options to accomplish what they need. One option that users of our DNA reverse synthesis reagents do not have is AMA deprotection.

Our traditional offering of dC has been a benzoyl-protected one, which, like all other benzoyl-dC protected phosphoramidites, is incompatible with any deprotection method that involves methylamine. Instead of cleaving the benzoyl group off of dC, the methylamine can instead displace benzamide to give N4-methyl-dC.[3] In the case of AMA deprotection, this undesired conversion is 5-10 %. In the case of gaseous methylamine deprotection, it can be much more.

To give our customers the option of using methylamine for deprotection, we are now offering reverse Ac-dC phosphoramidite as well (Figure 1). It can be used in exactly the same way as the reverse Bz-dC phosphoramidite. For both coupling and deprotection, no changes are needed from standard methods recommended by the synthesizer manufacturer.

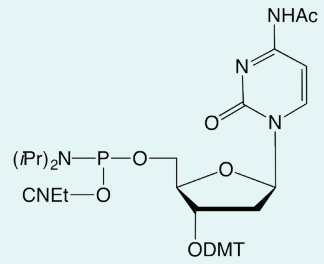

Figure 1. Ac-dC-5’-CE Phosphoramidite

Item	Pack Size	Catalog No.
dC-5’-CE Phosphoramidite	0.25 g	10-0101-02
	0.5 g	10-0101-05
	1.0 g	10-0101-10
Ac-dC-5’-CE Phosphoramidite	0.25 g	10-5101-02
	0.5 g	10-5101-05
	1.0 g	10-5101-10

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Purification of RNA Oligonucleotides (DMT-ON) using Glen-Pak DNA Purification Cartridges

Glen Research provides efficient and inexpensive purification cartridges for DNA and RNA oligonucleotides that can purify up to 60mer and 150mer oligonucleotides using the RNA and DNA cartridges, respectively. The purification mechanism for these cartridges relies on the DMT-ON purification concept, where the desired full-length oligonucleotide with the hydrophobic DMT group sticks to the cartridge’s resin while the shorter, less hydrophobic failures elute out of the cartridges during the initial purification steps.[1,2]

The standard format of the DNA and RNA cartridges is the 1 umol scale version (Figure 1). Physically, they share the same dimensions (65 x 10mm) and can be used as single columns or in a 96-well plate format. What makes them different is the resin type and the amount of resin present, 150mg in the DNA cartridge versus 100mg in the RNA cartridge.

In recent years, we have heard anecdotally from customers that the DNA Glen Pak is also suitable for RNA. The DNA and RNA columns were developed separately, and we had not considered using the DNA version for RNA purification. As such, we decided to give it a try.

We tested the purification of RNA oligonucleotides of a couple of different lengths using our standard Glen-Pak RNA cartridge (60-6100) and its DNA equivalent

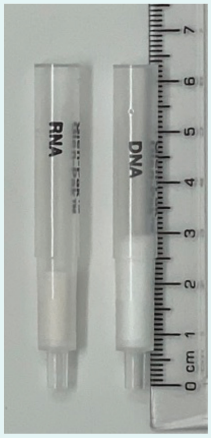
Table 1. 21mer RNA oligonucleotide purification by Glen-Pak cartridges

Cartridge	Cat. No.	*Crude Purity	**Final Purity	Yield
Glen-Pak RNA	60-6100	76.7%	98.5%	66.5%
Glen-Pak DNA	60-5100		97.5%	63.5%

*prior loading to the Glen-Pak cartridge
**eluted from the Glen-Pak cartridge

Glen-Pak RNA Purification cartridge:

- Column 65 x 10mm
- Bed height 14-18mm
- Resin is off-white



Glen-Pak DNA Purification cartridge

- Column 65 x 10mm
- Bed height 22-25mm
- Resin is white

Figure 1. RNA cartridge versus DNA cartridge

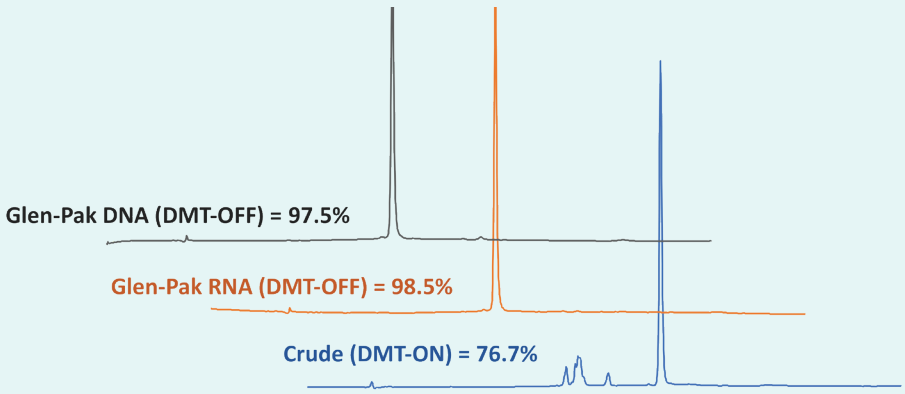


Figure 2. HPLC chromatograms for crude and cartridge-purified RNA. Data correspond to 21mer RNA oligonucleotide (5'- GGCUCCCCUCAACAACUUCTT-3') before and after purification with either a Glen-Pak DNA or RNA cartridge.

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

(60-5100). The RNA oligonucleotides were synthesized in-house, and all the steps prior to the purification step (RNA synthesis, base deprotection, and 2' deprotection) followed our TBDMS-Protected RNA guide and Glen-Pak cartridge purification guide (p26). RNA oligonucleotides were divided into two equal portions and loaded separately into the DNA and RNA cartridges. The RNA protocol (p26) was applied to both cartridges.[2]

The results were quite impressive. For a crude 21mer RNA oligonucleotide of 76.7% purity, the Glen-Pak DNA cartridge produced 97.5% purity compared to 98.5% for the Glen-Pak RNA cartridge. The obtained yields were also comparable at 63.5% and 66.5%, respectively. We also repeated these tests for 56mer RNA oligonucleotides and obtained similar results. Based on this data, we believe the Glen-Pak DNA cartridge is effective for any RNA sequence up to at least 56mer.

The data obtained from this evaluation are very encouraging, and researchers can potentially stock and maintain just the DNA cartridges for both RNA and DNA oligonucleotide purification needs. Also, it should be noted that although we only tested the 150mg cartridge, we believe the other DNA Glen-Pak formats should work fine with RNA. Customers should be able to purify larger and smaller scales of syntheses on the 3g and 50mg cartridges, respectively, by scaling the RNA purification protocol accordingly.

References

1. *The Glen Report*, 2007, **19.2**, 1-3.
2. *The Glen Report*, 2011, **23.2**, 8-9.

New Product — Palmitate Phosphoramidite

Synthetic oligonucleotides such as antisense oligonucleotides (ASO), aptamers, miRNA, and siRNA have all found their place in therapeutic development. Challenges when using nucleic acids in therapy include targeted delivery, nuclease activity, and membrane permeability. In recent years, scientists have frequently used distinct modifications to improve such biological characteristics of therapeutic oligonucleotides.

It is common to employ sugar and/or backbone modifications, such as locked nucleic acids and phosphorothioate (PS) linkages, to evade nuclease activity and improve biodistribution. ASOs tend to accumulate in the liver, kidney, and spleen after systemic administration. Effective mRNA knockdown in other tissues often requires high dosing.[2] Therefore, enhancing the potency of the ASO in other types of tissues through targeted delivery is desirable for certain therapeutic applications. Glen Research has supported these efforts by offering stearyl, cholesterol, α-tocopherol, and GalNAc phosphoramidites (Figure 1).[1]

GalNAc-oligonucleotide conjugates rely on a glycoprotein receptor for cell permeability and directs therapeutic agents to liver cells. Through an alternative mechanism, hydrophobic modifications, like fatty acids and cholesterol, improve cellular uptake and activity in multiple tissues. We are happy to offer our customers a new modification for these efforts: Palmitate Phosphoramidite.

Palmitic acid is a saturated long-chain fatty acid with a 16-carbon backbone. It is naturally found in palm oil and is the most common fatty acid found in the human body. The palmitate phosphoramidite consists of the C16 chain connected to a C6 linker via a traditional amide bond.

Palmitate-Oligonucleotide Conjugates

Palmitate-modified oligonucleotides offer enhanced ASO activity in skeletal and cardiac muscles compared to the unconjugated ASO.[3] The greater potency of palmitate-ASOs was attributed to increased plasma circulation and exposure to extrahepatic tissue. The palmitate modification afforded higher affinity to plasma proteins, especially albumin, and lipoproteins (i.e. HDL and LDL). The lipid conjugates also resulted in less excretion of ASO, allowing more exposure to different tissues. A mechanism for palmitate-facilitated ASO circulation to target cells was proposed a few years ago. The palmitate-ASO yielded association with albumin in the bloodstream, which rapidly distributed the ASO across the vascular endothelium to the interstitium of heart and skeletal muscle.[3]

In another study, a palmitate moiety was employed in a tricyclo-DNA (tcDNA) ASO with and without a phosphorothioate backbone and evaluated for its effectiveness against Duchenne muscular dystrophy (DMD).[4] DMD is caused by a genetic mutation in the *dmd* gene.

Therefore, nucleic acids lend an attractive method for treating muscular dystrophy. Many ASOs developed for treating neuromuscular diseases induce splice switching or skipping (Figure 2).

Mutations in the *dmd* gene yield partially or fully inactivated dystrophin protein, leaving muscle tissue more susceptible to degeneration, weakness, and injury. While the loss of dystrophin activity is experienced in all muscle tissue, the disease is life threatening due to degeneration of skeletal and cardiac muscle. Analysis of biodistribution confirmed the palmitate moiety enhanced skeletal muscle accumulation of fully PS-tcDNA-ASOs and fully PO-tcDNA-ASOs 6- and 28-fold, respectively, relative to unconjugated ASO.[4] Dystrophin production was also significantly restored in skeletal muscles after treatment with the palmitoyl-ASOs. Perhaps even more impressive, levels of dystrophin increased in neuromuscular tissue, owing to the unique capacity of tcDNA to cross the blood-brain barrier.[4]

Palmitate conjugates offer enhanced cellular uptake and delivery to extrahepatic tissues. Recent studies using palmitoyl-

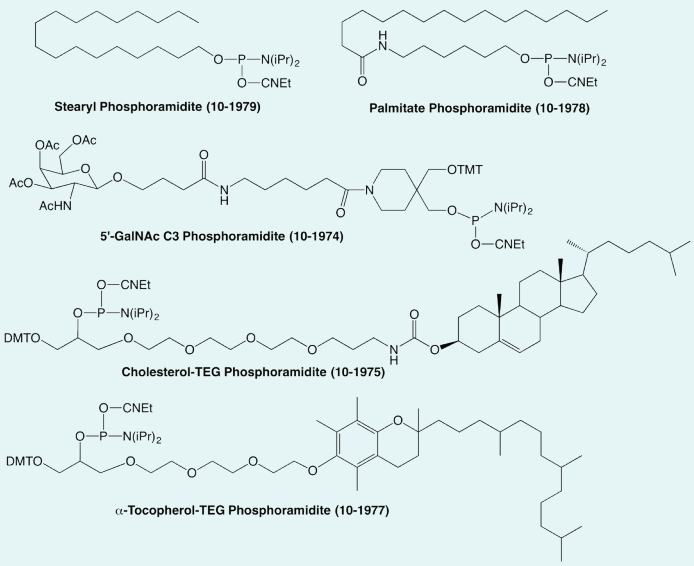


Figure 1. Modifiers that enhance biological characteristics of therapeutic oligonucleotides

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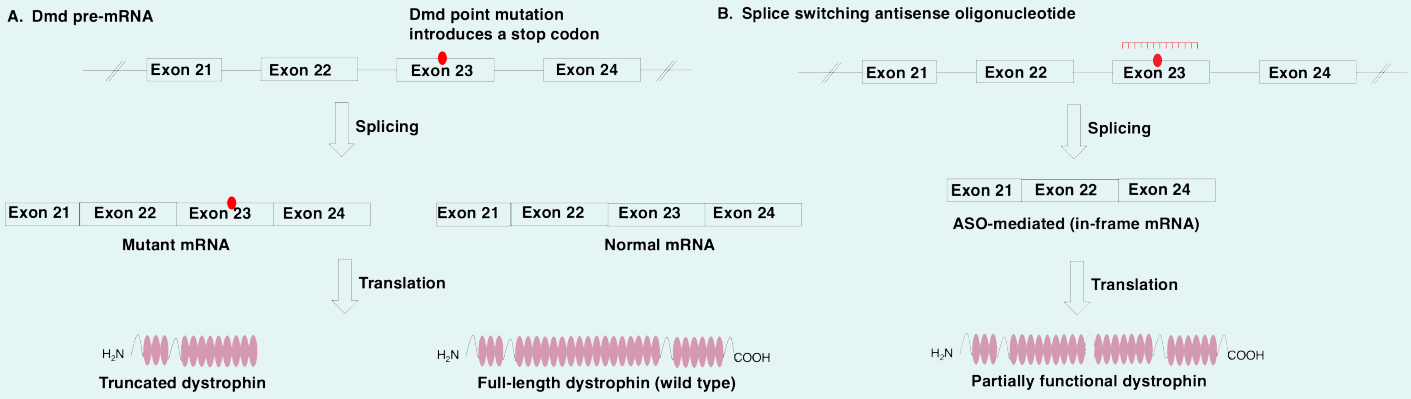

Figure 2. (A) Mechanism of pre-mRNA splicing in dystrophin gene. (B). Mechanism of splice switching antisense oligonucleotides.

oligonucleotides suggest promising outcomes as the use of therapeutic oligonucleotides continues to rise.

Recommended Protocols for Palmitate Phosphoramidite

Much like the 5'-stearyl phosphoramidite is not fully soluble in acetonitrile, palmitate phosphoramidite must be dissolved in a mixture of acetonitrile/dichloromethane (1:3). A 6-minute coupling time is recommended. For deprotection and cleavage, no changes are necessary aside from standard methods as required by nucleobases.

References

1. *The Glen Report*, 2012, **24.1**, 10.
2. S.K. Pandey, *et al.*, *J Pharmacol Exp Ther*, 2015, **355**, 329-40.
3. A.E. Chappell, *et al.*, *Nucleic Acids Res*, 2020, **48**, 4382-4395.
4. K. Relizani, *et al.*, *Nucleic Acids Res*, 2022, **50**, 17-34.

Item	Pack Size	Catalog No.
Palmitate Phosphoramidite	50 µmol	10-1978-95
	100 µmol	10-1978-90
	0.25 g	10-1978-02

New Product — Universal-CE Phosphoramidite

Cleavable linkers have been used in a wide range of applications and have been discussed in a recent Glen Report.[1] We offer a range of linkers compatible with multiple cleavage methods, including enzymatic, hydrolysis, photolabile, and disulfide.

Among the cleavable linker products that can yield a strand break when incorporated in an oligonucleotide internally are PC linker and CPR (Chemical Phosphorylation Reagent). When incorporated, the cleavage of these two products leaves behind 5'- and 3'-phosphates.[2,3] We are introducing Universal-CE phosphoramidite (10-5000), a new cleavable linker that yields two different end moieties, one bearing a 3'-OH and one bearing a 5'-amino modifier with a C3 linker, upon cleavage. The process of the cleavage reaction is illustrated below in Figure 1 alongside those of PC Linker and CPR.

No phosphoramidite products in our current offerings directly yield a 3'-OH upon cleavage. Oligonucleotides with 3'-OH after cleavage may be crucial for certain downstream applications, such as primer extension or ligation assays. A few years ago, Universal-CE was used to remove truncated probe sequences from the full-length probes on *in situ* synthesized arrays.[4] The process involved inverting the orientation of the probe in such a way that only the full-length probe was immobilized. Failure sequences incapable of probe inversion were removed from the system upon cleavage.

Based on the cleavage products, Universal-CE can also be used as a 5'-modification to yield an oligonucleotide with a 5'-amino modifier C3. The end product would be identical to if one used 5'-amino modifier C3-TFA (10-1923) (Figure 2). TFA amino modifiers are commonly used when oligonucleotide purification prior to conjugation is not required. In addition to not being compatible with reverse phase purification, modifiers with TFA groups are more susceptible to degradation during summer shipping.[5] Universal-CE is a great substitute without the limitations

of 5'-amino modifier C3-TFA. Universal-CE is a powder and easier to handle than an oil. When used as an amino modifier, Universal-CE requires less harsh conditions for deprotection. To release the amine, ammonium hydroxide overnight at 55 °C or 48 hours at room temperature or AMA, 10 minutes at 65 °C is sufficient.

Universal-CE is based on the same chemistry as Glen UnySupport. Many of our customers are very familiar with UnySupport, which is compatible with multiple standard deprotection and cleavage conditions. Automated synthesis on a universal support has allowed unique modification at the 3'-end of an oligonucleotide. Universal-CE phosphoramidite could also be used to modify a hydroxyl- or amine-containing surface. This would create a universal-like support, capable of oligonucleotide synthesis and subsequent cleavage.

Synthesis & Deprotection

For coupling, no changes are needed from standard method recommended by synthesizer manufacturer.

For complete cleavage, deprotect using Ammonium Hydroxide:MethylAmine (AMA) 1:1 for 1 hour at 65 °C or Ammonium Hydroxide for 8 hours at 55 °C. For sensitive minor bases or dyes, use 50 mM Potassium Carbonate in Methanol for 17 hours at room temperature or with Tert-Butylamine/water 1:3 (v/v) for 4 hours at 60 °C.

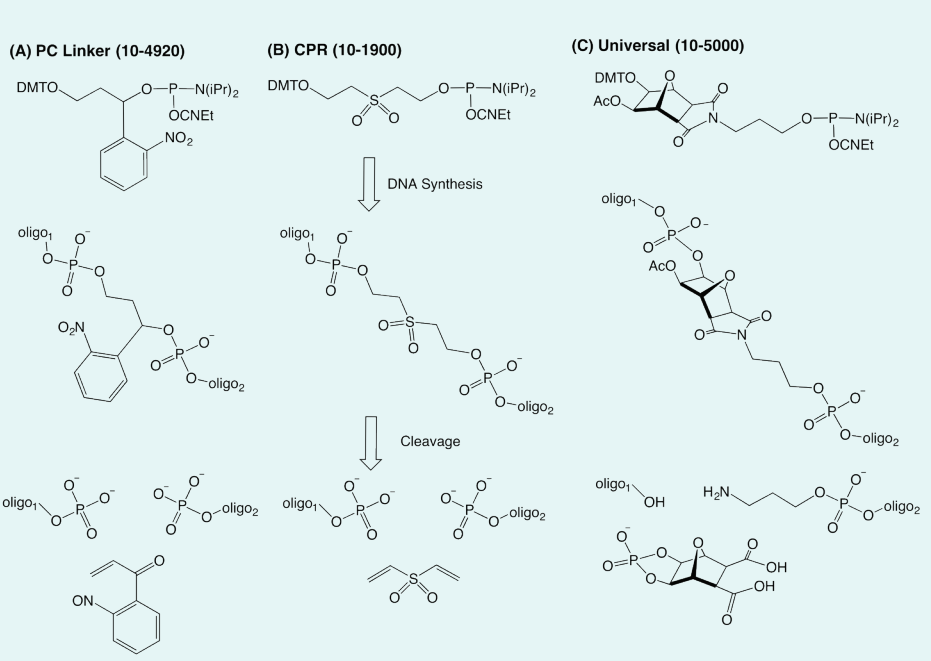

Figure 1. Cleavage mechanisms of several products.

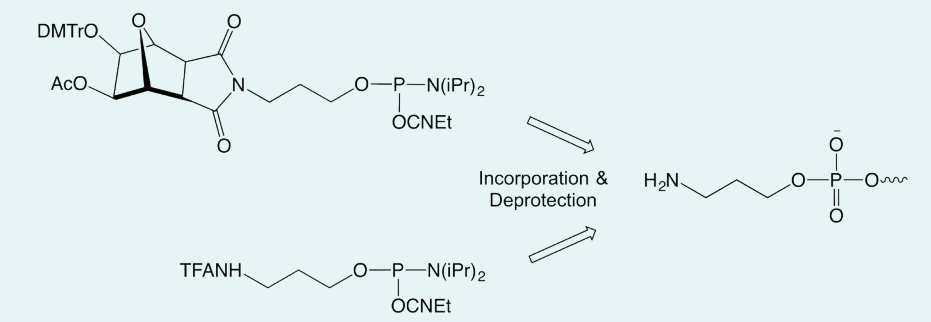

Figure 2. Universal-CE as an amino modifier C3.

Item	Pack Size	Catalog No.
Universal-CE Phosphoramidite	100 µmol	10-5000-90
	0.25 g	10-5000-02

References

1. *The Glen Report*, 2021, **33.1**, 6-8.
2. *The Glen Report*, 2003, **16.2**, 8-9.
3. *The Glen Report*, 2011, **23.1**, 10-11.
4. W. Zhou, G. McGall, and V. Singh: February 2017, United States Patent, Centrillion Technology Holdings Corporation, PCT/US2016/047488.
5. *The Glen Report*, 2013, **25.1**, 11-12.

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Technical Snippets

What does the F.W. mean on a product webpage/How do I calculate the M.W. of my oligo?

The F.W. of a product is the unit formula weight once it has been incorporated into an oligonucleotide after deprotection. The F.W. accounts for the loss of any protecting groups, including the β-cyanoethyl group, and the transition of the P(III) phosphoramidite to a P(V) phosphate diester. The F.W. should be used when determining the molecular weight of an oligo.

To calculate an oligonucleotide's molecular weight, one should use equation **1**. The loss of 61.96 is accounting for the loss of a phosphate group. The FW of each product is provided on the product webpage, the certificate of analysis provided upon purchase, and the physical data section of our Product Catalog.

Sum of Unit F.W. – 61.96 + DMT (if applicable) (1)

Some standard unit free weights are listed below:

dA = 313.21 dC = 289.18 dT = 304.20 dG = 329.21 DMT = 302.40

Products:

- Non-nucleosidic and nucleoside phosphoramidites
- Solid supports excluding universal and oligo-affinity supports
- NHS esters

What if I store Glen Research's reagents at temperatures lower than the recommended temperature, are they still safe and active to use?

Yes, as a general rule of thumb, lower storage temperatures are not harmful. In fact, the chemicals will be more stable. Basically, a cooler temperature slows down any undesired decomposition reaction that may occur otherwise in warmer storage temperatures. For example, it is perfectly fine storing something in the freezer (-10 to-30°C) instead of the recommended refrigerated storage (maximum of 2-8°C). It should be noted that oligonucleotide synthesis reagents stored in the cold should always be equilibrated back to room temperature prior to use.

Products: All phosphoramidites and solid supports.