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RNA UPDATE

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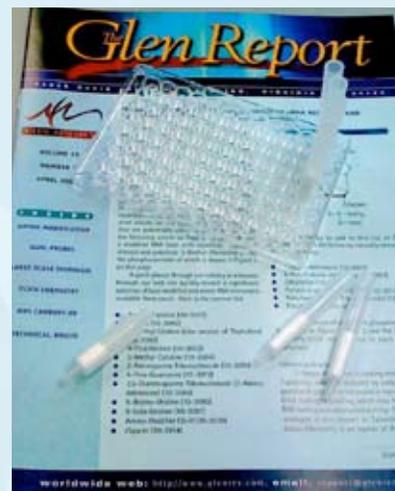
GLEN-PAK™ CARTRIDGES – THE ULTIMATE PURIFICATION CARTRIDGES

Introduction

The use of Oligonucleotide Purification Cartridges has been a popular option for purifying synthetic oligonucleotides based on the DMT-on procedure. These cartridges typically contain polymeric packing materials which are stable to dilute ammonium hydroxide or ammonium hydroxide/methylamine (AMA). The technique relies on the affinity of reverse phase packing materials for the 4,4'-dimethoxytrityl (DMT) group at the 5' terminus of synthetic oligonucleotides. The advantages of these cartridges are clear: speed, inexpensive, no need to evaporate corrosive solutions, product elutes in a small volume of aqueous acetonitrile. However, there is also a major disadvantage of traditional cartridges in that the longest oligos that can be reliably purified by this method are approximately 50mers. In addition, the yield of purified product from traditional cartridges is normally 40-60% of theoretical and the crude oligos need to be loaded in at least two passes. This clearly makes traditional cartridges unwieldy for use in high throughput robotic work stations.

Poly-Pak™

Of the traditional oligonucleotide purification cartridges, our favorite remains Poly-Pak™ cartridges and barrels – and we are in no way biased! By using 3-5 micron polydivinylbenzene packing, we were able to produce cartridges and barrels that use the smallest wash volumes in the market. Also, the product elutes in 0.5mL of aqueous acetonitrile, making evaporation fast and simple. Poly-Pak cartridges have been a staple for oligonucleotide purification for 15 years. Unfortunately, traditional cartridges, including Poly-Paks, have the disadvantage that they require the crude oligo solution to be loaded in two passes. This is clearly unsuitable for the use of these cartridges in high throughput situations.



Glen-Pak™ Cartridges

Fluoro-Pak™

As an example of the next generation of purification cartridges, Glen Research began in 2006 to offer a new cartridge purification system – Fluoro-Pak™ cartridges for the purification of longer oligonucleotides as a complement to the Poly-Pak range. Fluoro-Pak cartridges use the exceptionally high affinity of fluororous-tagged oligonucleotides for fluororous packing materials. In this case, the DMT group at the 5' terminus is adorned with a fluororous ponytail, which allows simple purification of the full-length DMT containing oligonucleotide from the capped failure sequences. The Fluoro-Pak system works just fine for shorter oligos but really excels by offering excellent purification of longer oligos in the 50-100+ range. The system also offers one pass loading of the crude oligonucleotides to the cartridges, making it much more acceptable in high throughput systems. Fluororous DMT protected monomers are required for this system to work and a variety of these are available, including all

(Continued on Page 2)

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four regular DNA monomers and fluorous CPR II, which may be used to purify 5'-phosphorylated oligos. However, a clear downside to this purification technique is the requirement of these special monomers, which add to the cost of purification.

Glen-Pak™

Our latest option is Glen-Pak™ cartridges, which offer the very best of Poly-Pak with the improvements offered by Fluoro-Pak rolled into one – the Ultimate Purification Cartridge. Glen-Pak cartridges:

- Are available in two formulations, one optimized for DNA purification and the other for RNA purification.
- Extend the range of purification of Poly-Pak cartridges to 100+.
- Use the regular DMT group as the affinity tag.
- Are available as cartridges for syringe use.
- Are available as cartridges for use on vacuum manifolds and high-throughput devices.
- Require a single loading of the crude oligonucleotide solution diluted with an aqueous salt solution.
- Can be used for desalting oligonucleotides.
- Have been tested with amino-modifiers, biotin and biotinTEG, and phosphorylation with CPR II.
- Have been tested with most dyes, including Cy dyes, FAM, HEX and TET.
- Demonstrate recoveries of full-length oligonucleotide with yields typically greater than 90%.
- Cost effective.

Comparison

Salient features of all three cartridge types are collected in the Table, which shows clearly the advantages that Glen-Pak cartridges offer over traditional RP cartridges, like Poly-Pak, and even over more sophisticated systems, like Fluoro-Pak.

Examples

1. DNA Purification

Glen-Pak DNA Purification cartridges have been used with a variety of sequences, from short to long (>100mer). They are fully compatible with any DMT-on oligonucleotide, including those with a variety of labels and tags, e.g., 5'-phosphate using CPR II, 5'-amine, 5'-biotin. Although

TABLE: COMPARISON OF AVAILABLE CARTRIDGES

Feature	Poly-Pak™	Fluoro-Pak™	Glen-Pak™
Single loading	-	√√√	√√√
Oligo range to 100 +	√√	√√√	√√√
Desalting cycle available	√√√	-	√√√
Variety of formats available	√	√√	√√√
Yield of available full-length oligo	√	√√√	√√√
Performance with modifiers	√√√	√	√√√
Performance with dyes	√√√	-	√√√
Optimized for RNA	√√	-	√√√
Cost	√√	√	√√√

FIGURE 1: IEX HPLC ANALYSIS OF A CRUDE AND GLEN-PAK PURIFIED 71-MER

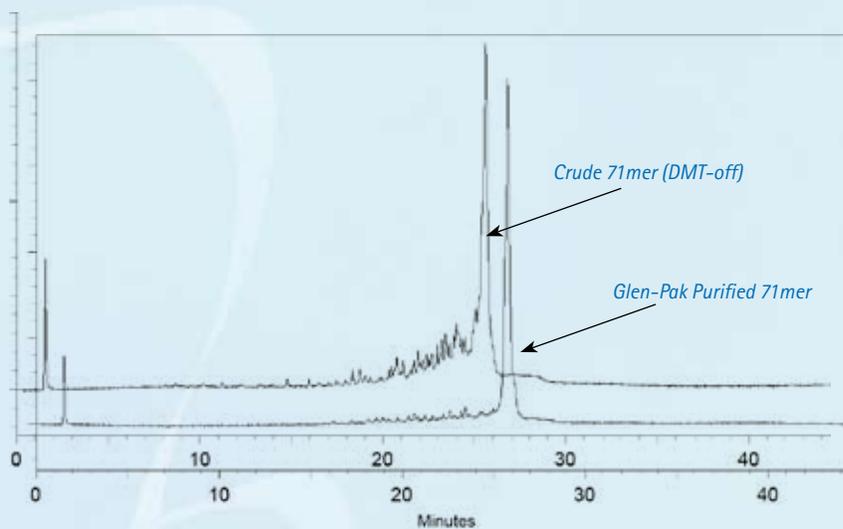
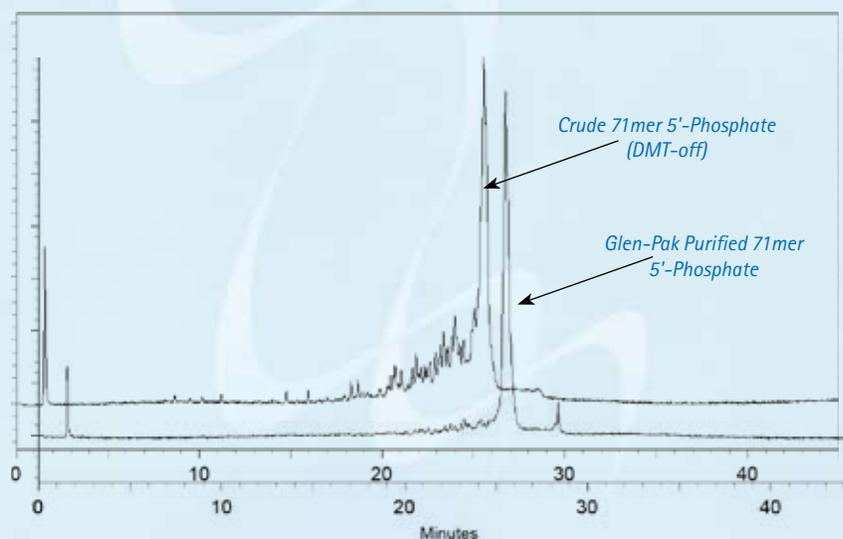


FIGURE 2: IEX HPLC ANALYSIS OF A CRUDE AND PURIFIED 71-MER 5'-PHOSPHORYLATED WITH CPR II



Column: Dionex DNAPac PA200, 250 X 4mm Buffers: A- 10mM NaClO₄, 25mM TRIS-HCl, 20% Acetonitrile, pH 7.4 ; B- 600mM NaClO₄, 25mM TRIS-HCl, 20% Acetonitrile, pH 7.4 Gradient: 0-40% Buffer B at a flow rate of 1 mL/min.

Glen-Pak cartridges are optimized for the capture of DMT groups, they are also compatible with dyes with no DMT group, e.g., Cy dyes, FAM, HEX and TET.

Figure 1 illustrates the purification capability of Glen-Pak DNA Purification cartridges with a mixed base 71mer. A small portion of the crude oligonucleotide was detritylated and run on ion-exchange HPLC to illustrate the large number of failure sequences present. The majority of the crude oligonucleotide was purified on a Glen-Pak DNA Purification cartridge and the product was also run on ion-exchange HPLC. The two chromatograms demonstrate the remarkable enhancement of purity while the recovery of full-length product was >90%.

Figure 2 shows the results of a similar experiment where a 71mer was phosphorylated with CPR II and purified on a Glen-Pak DNA Purification cartridge. Again the recovery of full-length product was >90% and the purity was substantially enhanced.

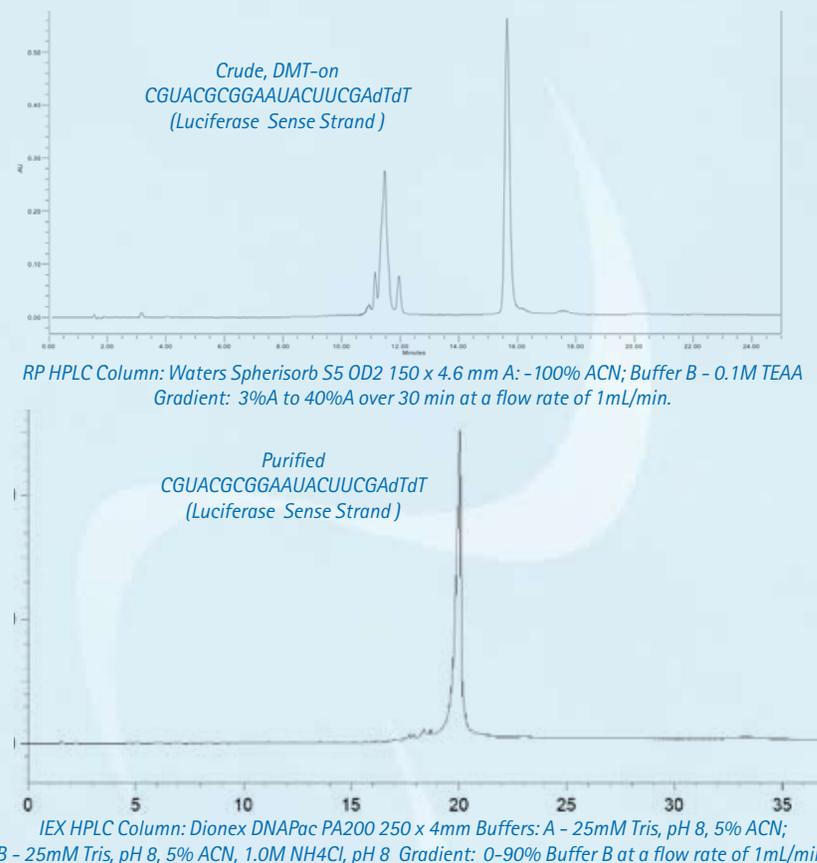
2. RNA Purification

The use of Glen-Pak RNA Purification cartridges is illustrated in the Technical Brief on Page 5. Following DMT-on RNA synthesis and regular base deprotection with your favorite basic mix, the silyl protecting groups are removed with triethylamine trihydrofluoride containing dimethylsulfoxide and triethylamine. This mixture is diluted with the RNA Quenching Buffer and applied directly to Glen-Pak RNA Purification cartridges to complete the purification step. Figure 3 demonstrates purification of an RNA 22mer using a Glen-Pak RNA Purification cartridge.

Conclusion

DMT-on purification of oligonucleotides using traditional cartridges, exemplified by Poly-Pak cartridges, has served the oligo synthesis community well for many years. However, two major deficiencies in the traditional cartridges quickly became apparent: a single loading process did not work, making them problematical for high throughput robotics systems; and the range of successful purification was restricted to around 50mers. The Fluoro-Pak purification system, as applied to oligonucleotide purification, solved both of these problems but at a higher cost. The required fluoros DMT monomers must be available to be

FIGURE 3: RP HPLC OF A CRUDE RNA 22MER AND IEX HPLC ANALYSIS OF A GLEN-PAK PURIFIED RNA 22MER



added in the final synthesis cycle.

Glen-Pak DNA and RNA cartridges have advantages over Poly-Pak cartridges in that a single loading of the diluted crude deprotection solution is all that is necessary. Also, the range of purification has been extended to 100+ using DMT-on oligos. Glen-Pak cartridges have similar performance to Fluoro-Pak cartridges but

without the need for the fluoros DMT group at the 5' terminus, so the cost is lower. In addition, Glen-Pak cartridges allow purification of virtually the complete range of dyes and modifiers.

Poly-Pak™ and Glen-Pak™ are trademarks of Glen Research Corporation. Fluoro-Pak™ is a trademark of Berry & Associates, Inc.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
DNA Purification			
Glen-Pak™ DNA Purification Cartridge (For use in vacuum manifolds and high-throughput devices)	60-5100-10	Pack of 10	80.00
	60-5100-30	Pack of 30	200.00
	60-5100-96	Pack of 96	475.00
Glen-Pak™ DNA Purification Cartridge (For use with disposable syringes)	60-5200-01	each	8.00
	60-5200-10	Pack of 10	80.00
RNA Purification			
Glen-Pak™ RNA Purification Cartridge (For use in vacuum manifolds and high-throughput devices)	60-6100-10	Pack of 10	95.00
	60-6100-30	Pack of 30	225.00
	60-6100-96	Pack of 96	575.00
RNA Quenching Buffer	60-4120-82	250mL	80.00
	60-4120-80	1L	200.00

INTRODUCTION

RNA synthesis has come a long way since the first RNA monomers were introduced in the 1980s. From a research-oriented technique carried out by groups of dedicated scientists, RNA synthesis is finally becoming part of the mainstream. Probably the most significant reason for this change has been the development of siRNA and other short RNA sequences with significant biological activity. Nevertheless, RNA synthesis still worries some researchers so much that they are reluctant to carry out their own synthesis. With this article, we demonstrate that RNA synthesis has advanced to a level where it can be approached with very few worries and success is guaranteed. In the following article, we demonstrate that cost need not be a major obstacle and in a third article about RNA synthesis, Richard Pon updates us on the use of supports containing the Q-Linker for faster and more complete cleavage of RNA sequences from synthesis supports.

Synthesis and deprotection of RNA is considered to be more complex and time consuming than DNA synthesis. However, with the use of the acetyl protecting group available on all of our Cytidine RNA products, the time required for deprotection is greatly reduced when using the UltraFAST deprotection strategy. In this update, we discuss some of the significant improvements that have been made in RNA synthesis, deprotection, and purification that have dramatically reduced the time necessary to prepare functional RNA. This update focuses on TBDMS protected RNA. However, the procedure can equally be applied to TOM-protected RNA.

MATERIALS

1. Acetyl protected¹ C RNA monomer (Ac-C CE phosphoramidite, 10-3015) and support (Ac-C-RNA CPG, 20-3315), if needed.
2. Sturdy 2mL centrifuge tube or sealable glass vial for carrying out deprotection. When using methylamine, vials which use black rubber O-rings for sealing should not be used.
3. Ethanolic methylamine/Aqueous Methylamine² (EMAM) 10M Methylamine in ethanol/water (1:1). Mixture of 33% Methylamine in ethanol/ 41% Methylamine in water (1:1). (Fluka 65590 and Fluka 65580) Alternatively, use 50:50 ammonium hydroxide/40% aqueous methylamine (AMA).³
4. Triethylamine trihydrofluoride⁴⁻⁶ (Aldrich 34,464-8 or equivalent).
5. DMSO: Dimethylsulfoxide, anhydrous, 99.9% (Aldrich 27,685-5 or equivalent).
6. TEA: Triethylamine, puriss. p.a. ≥ 99.5% (GC) (Fluka 90340 or equivalent).
7. RNase free water (Fisher BP 2484100 or equivalent).
8. RNase free, sterile tubes and pipets.

SAFETY

1. The procedures described in the bulletin should be performed by technically qualified individuals.
2. Methylamine solutions are under pressure and can rupture containers. Use safety glasses when handling hot vials containing methylamine solutions.

RNA SYNTHESIS

1. Synthesize RNA using 5-ethylthio-1*H*-tetrazole (ETT) or 5-benzylthio-1*H*-tetrazole (BTT) (Glen Research 30-3140 or Glen Research 30-3170, respectively) as activator.
2. Use 6 minute coupling time for RNA monomers with ETT or a 3 minute coupling time for RNA monomers with BTT.

RNA CLEAVAGE AND DEPROTECTION

1. Remove the columns from the synthesizer and thoroughly air-dry the support in the columns or dry in a stream of argon gas.
2. Connect a clean syringe to the luer fitting of the column (VWR 53548-000, Norm-Ject 1mL sterile plastic syringe). Avoid the use of syringes that have a rubber plunger.
3. With a second syringe, take up 1mL of EMAM into a plastic syringe. Connect the second syringe to the other luer fitting on the column and gently pass the solution carefully through the column 4-5 times.
4. Allow the column to stand at room temperature with the solution in full contact with the CPG for 20 minutes.
5. Transfer the solution to a clean screw cap vial. Rinse the column with 0.5mL of EMAM and combine solutions for a total volume of around 1.5mL.
6. Deprotect at 65°C for 10 minutes to remove the exocyclic amine protecting groups. Dry down the oligo in a speed-vac.

DMT-OFF RNA DEPROTECTION

Removal of the 2' Protecting Group

1. Fully redissolve the oligo in 100µL anhydrous DMSO. If necessary, heat the oligo at 65°C for about 5 minutes to get it into solution.
2. Add 125µL of triethylamine trihydrofluoride, mix well and heat to 65°C for 30-90 minutes, depending on the length of the oligo. Cool in freezer briefly.

Desalting by precipitation

1. Add 25µL of 3M Sodium Acetate in RNase free water, filtered. Mix well by vortexing for 15 seconds.
2. Add 1mL butanol. Mix well by vortexing for 30 seconds.
3. Cool at -70°C for 30 minutes. (-20°C has also worked.)
4. Centrifuge for 10 minutes at 12,500rpm.
5. Decant butanol using sterile pipet tip.
6. Rinse with 0.75mL ethanol, twice.
7. Dry under high vacuum in a speed-vac to remove traces of butanol.

Analysis and purification

1. Analyze using Dionex PA-200 or equivalent with a sodium perchlorate gradient at 50-60°C.
2. Trityl-on RNA oligos can be purified using our Glen-Pak RNA purification columns, as described in the following sections.

GLEN-PAK™ RNA CARTRIDGE PURIFICATION

MATERIALS

1. Glen-Pak RNA Quenching Buffer
2. Glen-Pak RNA Purification Cartridge
3. HPLC Grade Acetonitrile
4. 2.0M Triethylamine Acetate (TEAA) (pH7)
5. 10% Acetonitrile, 90% 2M TEAA, pH 7
6. 2% Trifluoroacetic Acid (TFA)/Water
7. 1M ammonium bicarbonate/30% Acetonitrile
8. RNase free water (Fisher BP 2484100 or equivalent)
9. RNase free, sterile tubes and pipets

DMT-ON RNA DEPROTECTION

Removal of the 2' Protecting Group

1. Fully dissolve the RNA oligonucleotide in 115µL DMSO. If necessary, heat the oligo at 65°C for about 5 minutes to get it into solution.
2. Add 60µL of TEA to the DMSO/oligo solution and mix gently.
3. Add 75µL of triethylamine trihydrofluoride and heat the mixture at 65°C for 2.5 hours.

RNA PURIFICATION PROCEDURE

1. Immediately before cartridge purification is to begin, cool the 2' deprotection sample and add 1.75mL of Glen-Pak RNA Quenching Buffer to the deprotected RNA solution. Mix well and go immediately to step 2.
2. Place the desired number of cartridges into the female luer ports of the manifold and collection tubes (if desired) in the rack below the output guides.
3. Turn on the vacuum and adjust the pressure to ~7 mm Hg using the vacuum control valve (if no control valve is available on your manifold, target a flow rate of about 1-2 drops per second). Condition the cartridge using 0.5mL of Acetonitrile followed by 1.0mL 2M TEAA.
4. Apply the RNA Quenching Buffer mixture to the cartridge in 1.0mL aliquots. (Collect the eluent and save in case of loading failure or error).
5. Wash the cartridge with 1.0mL of 10% Acetonitrile, 90% 2M TEAA, pH 7.0.
6. Wash the cartridge with 1.0mL of RNase Free water.
7. Rinse the cartridge with 2 x 1.0mL of 2% TFA.
8. Wash the cartridge with 2 x 1.0mL of deionized water.
9. Place the appropriate receptacle (96 deep-well plate or sample tube) into the manifold and elute the purified oligo using 1 x 1.0mL 1M ammonium bicarbonate/30% Acetonitrile.

RNA SOLUTIONS

A. TEA.3HF De-silylation Solution:

1. 115µL DMSO: Dimethylsulfoxide, anhydrous, 99.9% (e.g., Aldrich 27,685-5)
2. 60µL TEA: Triethylamine, puriss. p.a. ≥ 99.5% (GC) (e.g., Fluka 90340)
3. 75µL TEA.3HF: Triethylamine trihydrofluoride, 98% (e.g., Aldrich 34,464-8)

B. 10% Acetonitrile, 90% 2M TEAA, pH 7.0 (100mL):

1. 10mL HPLC grade Acetonitrile
2. 90mL TEAA, pH 7.0

C. 1M ammonium bicarbonate/30% Acetonitrile (33mL):

1. 1.82 g Ammonium Bicarbonate
2. 23.1mL RNase Free water
3. 9.9 mL HPLC grade Acetonitrile

SUMMARY

A novice user can obtain good yields of functional RNA following these methods. Note, RNA can form secondary structures that can interfere with the analysis and purification. The use of Sodium Perchlorate buffer and heat should denature most oligoribonucleotides. For alternative protocols and methods optimized for your application, please contact our technical support group.

References:

- 1a. M.P. Reddy, N.B. Hanna, and F. Farooqui, *Tetrahedron Letters*, 1994, **35**, 4311-4314.
- 1b. M.P. Reddy, F. Farooqui, and N.B. Hanna, *Tetrahedron Letters*, 1995, **36**, 8929-8932.
2. S. Pitsch, et al., *Helv Chim Acta*, 2001, **84**, 3773-3795.
3. F. Wincott, et al., *Nucleic Acids Res.*, 1995, **23**, 2677-2684.
4. E. Westman and R. Stromberg, *Nucleic Acids Res.*, 1994, **22**, 2430-2431.
5. B. Sproat, et al., *Nucleosides and Nucleotides*, 1995, **14**, 255-273.
6. D. Gasparutto, et al., *Nucleic Acids Res.*, 1992, **20**, 5159-5166.

IMPROVED RNA SYNTHESIS USING THE Q-LINKER FOR FASTER PRODUCT CLEAVAGE FROM SOLID-PHASE SUPPORTS

Application note prepared by Richard T. Pon, June 11, 2007

Introduction

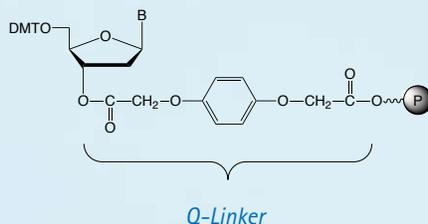
The first nucleoside residue of an oligonucleotide sequence is always attached to the solid-phase support through a dicarboxylic acid linker arm. Traditionally, this linker has been succinic acid. However, the need for faster oligonucleotide processing led to the introduction of hydroquinone-*O,O'*-diacetic acid ("Q-Linker") as a superior replacement. With the Q-Linker, all the steps required for synthesis and deprotection remain unchanged except for the time required to release the oligonucleotide product from the support. With oligodeoxyribonucleotides, the time required for the ammonium hydroxide cleavage step was reduced from 60 minutes to only 2 minutes, thus eliminating the need for multiple NH₄OH treatment steps and significantly increasing sample throughput. With ultra-fast deprotection using AMA (1:1 ammonium hydroxide and aqueous methylamine) the cleavage time is reduced to only a few seconds. In this case, the throughput gained using the Q-Linker is not as beneficial since AMA also cleaves the traditional succinyl linkers much faster (~ 5 minutes). However, we have found that this is not the case with RNA synthesis and, as described below, the Q-Linker provides significant benefits to RNA syntheses, even when using ultra-fast deprotection.

Application to RNA synthesis

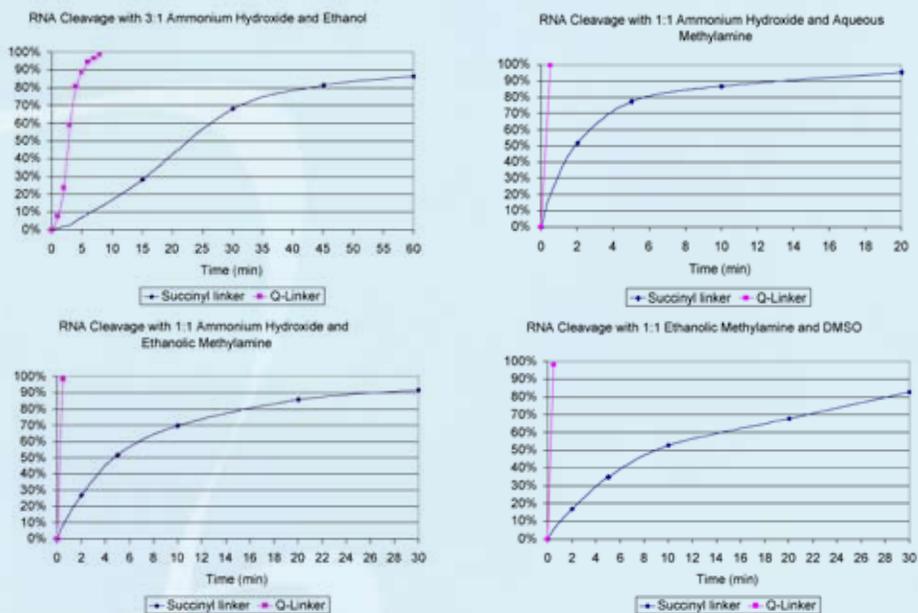
Recent discoveries of novel properties for short RNA sequences, such as siRNAs and miRNAs, have greatly increased the demand for chemically synthesized oligoribonucleotides. While the synthesis of oligoribonucleotides is very similar to oligodeoxyribonucleotides, the deprotection and work-up conditions are quite different. In particular, modified cleavage and base deprotection conditions are required to minimize loss of 2'-*O*-protecting groups and the subsequent chain cleavage which occurs; and to improve the solubility of the partially protected intermediates. The 2'-*O*-protecting group also slows the cleavage from the support through steric hindrance.

Therefore, reagents containing organic modifiers, such as ethanol or dimethyl sulfoxide (DMSO), are required. These eliminate chain cleavage and improved the solubility of long RNA products.

FIGURE: STRUCTURE OF Q-LINKER SUPPORT



FIGURES 1-4: RATES OF HYDROLYSIS OF OLIGOS FROM Q-LINKER AND SUCCINATE SUPPORTS



However, these organic modifiers further decrease the rate of linker hydrolysis and cleavage times of 30-60 minutes at room temperature are required to recover most of the oligoribonucleotide product. Thus, cleavage of an RNA sequence is more than 15 times longer than cleavage of a DNA sequence, when ethanolic methylamine is used instead of aqueous methylamine in the AMA reagent. If the cleavage step is combined with the base deprotection step at elevated temperatures, then the products become contaminated with dissolved silica and separation of the partially deprotected oligoribonucleotide from the heterogeneous mixture containing the residual support is more time consuming and loss of product may also occur.

However, ultra-fast cleavage times can be restored by using the Q-Linker, as shown in Figures 1-4. In particular, ethanolic methylamine reagents only require cleavage times of less than 2 minutes for

complete product recovery. The cleavage rate for our preferred RNA deprotection reagent (AMA with ethanolic methylamine) for both the succinyl and Q-Linker is shown below. Such fast cleavage at room temperature enables simplified sample processing (no multiple treatment and wait steps), greater sample throughput, reduced handling of volatile and noxious reagents, reduced risk

of incomplete base deprotection (through accidental evaporation of methylamine or ammonia), and greater product recovery and quality. Most importantly, no other changes to the synthesis or deprotection procedures are required. The Q-Linker is compatible with all of the remaining steps in common RNA synthesis protocols.

Glen Research offers a variety of supports containing the Q-Linker under license from University Technologies International. We are also planning to add Q-supports optimized for RNA synthesis to our products in the near future.

Contact Information

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NEW LINES OF MONOMERS - HT FOR HIGH THROUGHPUT AND LC FOR LOW COST RNA

HT Monomers

Recently, we have been working on monomers with a new quality designation destined for high throughput and large-scale synthesis customers. These customers normally require high quality materials produced under the guidelines of a validated quality system while still being priced aggressively. We are now happy to introduce the Glen Research HT line of monomers for DNA, RNA and 2'-OMe-RNA synthesis. These products include the usual Glen Research certification and guarantees but they are only available in larger packs or in bulk. As the market evolves, we would expect to expand this line to encompass other bases, modifiers and supports. HT monomers are not subject to regular discounts and a separate HT discount schedule will be set up for our customers. For these products, please request a quote.

The Table covers the monomers that are now available in the HT program along with some necessary ordering requirements.

LC RNA Monomers

We are also introducing a new line of RNA monomers - LC for Low Cost RNA. These monomers are of the highest quality but, in addition, they have been selected as part of a set which will allow simple deprotection, as described in the previous page, and which can be offered at attractive prices.

The minimum order quantity for these LC RNA monomers is 20g total per order in any combination, packaged in 1g vials. Please request a quote.

TABLE: HT PRODUCTS AND ORDERING REQUIREMENTS

Product	Minimum Amount per Order	Notes
DNA Monomers (Bz-dA, Bz-dC, Ac-dC ibu-dG, dmf-dG, T)	400g total per order	1. Packaged as 5g bottles (or greater) or in bulk. 2. Products may be combined to 400g total.
RNA Monomers (Bz-A, Ac-C Ac-G, U, 2'-TBDMS)	40g total per order	1. Packaged as 2g vials (or greater) or in bulk. 2. Products may be combined to 40g total.
2'-OMe-RNA Monomers (Bz-A, Ac-C iBu-G, U)	40g total per order	1. Packaged as 2g vials (or greater) or in bulk. 2. Products may be combined to 40g total.

Item	Catalog No.	Pack
HT DNA Phosphoramidites		
dA-CE Phosphoramidite	10-1000-5HT	5.0g
dC-CE Phosphoramidite	10-1010-5HT	5.0g
Ac-dC-CE Phosphoramidite	10-1015-5HT	5.0g
dG-CE Phosphoramidite	10-1020-5HT	5.0g
dmf-dG-CE Phosphoramidite	10-1029-5HT	5.0g
dT-CE Phosphoramidite	10-1030-5HT	5.0g
HT RNA Phosphoramidites		
Bz-A-CE Phosphoramidite	10-3003-2HT	2.0g
Ac-C-CE Phosphoramidite	10-3015-2HT	2.0g
Ac-G-CE Phosphoramidite	10-3025-2HT	2.0g
U-CE Phosphoramidite	10-3030-2HT	2.0g
HT 2'-OMe-RNA Phosphoramidites		
2'-OMe-Bz-A-CE Phosphoramidite	10-3100-2HT	2.0g
2'-OMe-Ac-C-CE Phosphoramidite	10-3115-2HT	2.0g
2'-OMe-iBu-G-CE Phosphoramidite	10-3120-2HT	2.0g
2'-OMe-U-CE Phosphoramidite	10-3130-2HT	2.0g
LC RNA Monomers		
Bz-A-CE Phosphoramidite	10-3003-10	1.0g
Ac-C-CE Phosphoramidite	10-3015-10	1.0g
Ac-G-CE Phosphoramidite	10-3025-10	1.0g
U-CE Phosphoramidite	10-3030-10	1.0g

AP-DC IS COMPATIBLE WITH PCR AND IS HIGHLY FLUORESCENT

When Mark Matteucci's group at Gilead first described the cytosine analog AP-dC (Figure 1), they called it "G-clamp" because of its phenomenally high and specific affinity for guanosine. The G-clamp base modification recognizes both the Watson-Crick and Hoogsteen faces of the complementary guanine within a helix, leading to a large increase in duplex stability (Figure 2).¹ Binding studies demonstrated that a single G-clamp substitution within an antisense oligonucleotide (ON) dramatically enhanced helical thermal stability and mismatch discrimination. Oligonucleotides containing G-clamp have been evaluated for sequence-context dependence, activity mismatch, sensitivity, RNase-H cleavage, and hybridization kinetics in antisense experiments.

While Matteucci's aim was to improve antisense activity,² there are two other properties of this remarkable cytidine analog that have not been widely examined. First, our work demonstrates that the AP-dC can successfully be used in primers. Second, the AP-dC has been observed to be a highly fluorescent nucleoside analog.

AP-dC in PCR

While much work has been done with AP-dC, the behavior of AP-dC-modified oligos in PCR amplification experiments was unknown. For this report, we examined the ability of oligonucleotides containing AP-dC to function as PCR primers and determined if Taq polymerase would incorporate dG opposite AP-dC in the template.

For this purpose, we synthesized several chimeric oligonucleotides that contained two parts: a universal sequence of 15 bases followed by a specific sequence of 20 bases targeting 3 *S. cerevisiae* ORFs (OAR1/YKL055c). The universal sequence contained standard nucleotides and the specific sequence included some AP-dC bases. The universal tags can then be used for sequencing the amplified fragments. The sequences are displayed in Table 1; positions of AP-dC are marked **C**.

These primers were used to amplify *S. cerevisiae* genomic DNA using standard Taq DNA polymerase.

The results displayed in Figure 3 correspond to an annealing temperature of 60°C and a magnesium concentration of 2 mM and 35 cycles. The lane named R-L corresponds to the PCR performed

FIGURE 1: DC, AP-DC AND AP-DC-CE PHOSPHoramidite

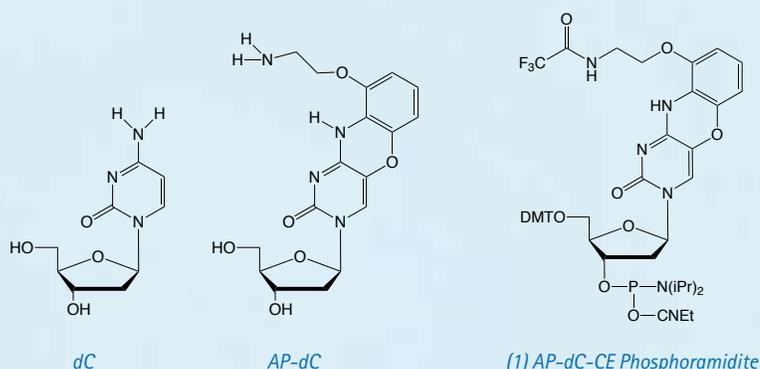


FIGURE 2: G - C AND G - AP-C BASE PAIRING

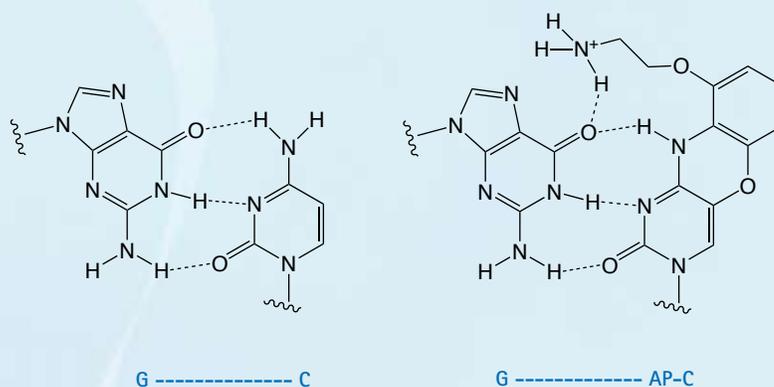


TABLE 1: PRIMER SEQUENCES

PRIMER NAME	SEQUENCE
YKL055c.0.L	CGACGCCCCGCTGATA GAAAGTATAGAGCGCACC
YKL055c.0.Lm1	CGACGCCCCGCTGATA GAAAGTATAGAGCGCACC C
YKL055c.0.Lm2	CGACGCCCCGCTGATA GAAAGTATAGAG C AC C CGC
YKL055c.0.Lm3	CGACGCCCCGCTGATA GAAAGTATAGAG C GCACC C CGC
YKL055c.0.Lm4	CGACGCCCCGCTGATA GAAAGTATAGAG C GCAC C CGC
YKL055c.0.Lm5	CGACGCCCCGCTGATA GAAAGTATAGAG C GC C ACC C CGC
YKL055c.0.R	GTCCGGGAGCCATG CCAATGTTCTCTCCAGCAT
YKL055c.0.Rm1	GTCCGGGAGCCATG CCAATGTTCTCTCCAG C AT
YKL055c.0.Rm2	GTCCGGGAGCCATG CCAATGTTCTCT C AG C AT
YKL055c.0.Rm3	GTCCGGGAGCCATG CCAATGTTCTCT CC AGCAT
YKL055c.0.Rm4	GTCCGGGAGCCATG CCAATGTT C CTCCAGCAT
primer Tag1	GTCCGGGAGCCATG
primer Tag2	CGACGCCCCGCTGATA
specific <i>S. cer.</i>	GAA AGT ATA GAG CGC ACC GC
specific <i>S. cer.</i>	CCA ATG GTT CTC TCC AGC AT

with control primers with no modification and is used as a positive control. R and L correspond to the non-modified primers.

LmX and RmX indicate the use of various primers with one or more modifications (see Table 1 for sequence and modification

positions.) Briefly, we see that when using a pair of primers containing AP-dC in one of the two primers, (R-LmX or L-RmX), we have amplification comparable to the control with no AP-dC except when using the most heavily modified primers in R-Lm4 (no amplification) and R-Lm5 (low amplification).

When modifications are present in both primers, we see a good amplification in primers containing the lowest number of total modifications (Figure 3 – lower panel). The results presented in Figure 3 are representative of many other combinations.

Based on these results we would conclude that it would be safe to limit:

- the number of AP-dC residues to two per primer;
- the total number of AP-dC residues to three per primer pair.

However, we did not test enough situations to rule out that more AP-dC modifications could result in amplification with other thermostable polymerases or in other particular conditions.

Finally the results of PCR with Lm1 primer indicated that modification of the 3'-position with AP-dC is permitted for DNA amplification (R-Lm1 or Rm1-Lm1).

Several of the amplicons were extracted and sequenced using sequencing primers complementary to the universal tag using an ABI sequencer and Big Dye chemistry. The sequence alignments (data not shown) indicated that it was possible to obtain full length sequences covering the entire primer region and that no base modification was detected at positions opposite to any of the AP-dC. In each sequencing, we observed a dG opposite the AP-dC. Also, no particular sequencing problems were observed and the peaks corresponding to AP-dC positions had similar intensity compared to the control with dC at the same positions.

Substitution of AP-dC for dC can raise the T_m of shorter PCR primers or probes enough to amplify or probe in a region where it is normally difficult to obtain stable sequences due to length restrictions. We also look forward to its use in the development of short, easily prepared oligonucleotides for use in highly specific single nucleotide polymorphism (SNP) and other *in vitro* diagnostic assays.

FIGURE 3: PCR PRODUCTS WITH VARIOUS PRIMER COMBINATIONS

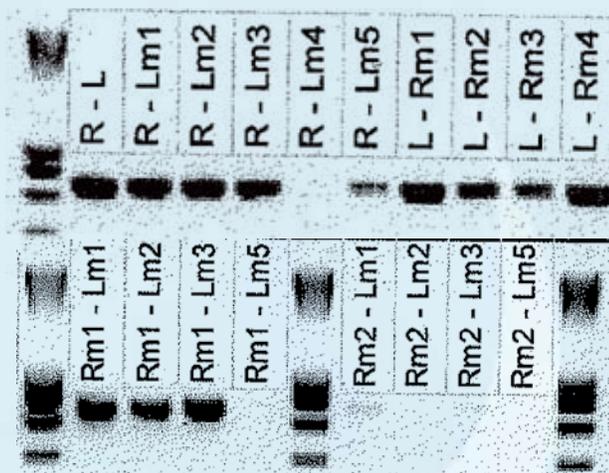
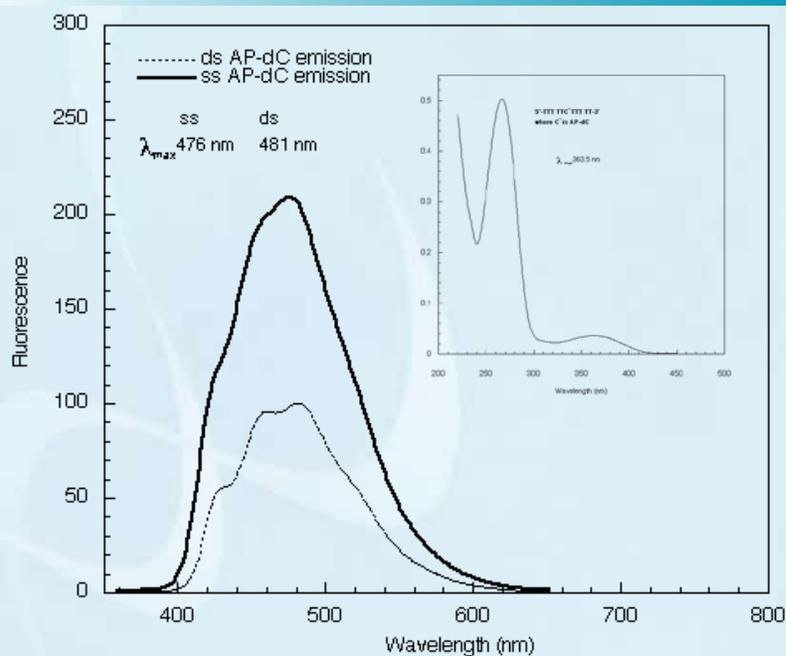


FIGURE 4: FLUORESCENCE SPECTRA OF AP-dC WITH ABSORBANCE SPECTRUM INSERT



Fluorescence Properties of AP-dC

With an extinction coefficient of approximately $10,500 \text{ M}^{-1}$ and a quantum yield of fluorescence of 0.2, AP-dC is 2-3 times as bright as our popular Pyrrolo-dC analog. In addition, AP-dC exhibits a Stokes' shift greater than 100 nm. As with most fluorescent base analogs, it is substantially quenched upon forming a

duplex. The quantum yield drops to 0.1 while gaining significant structure in the emission spectrum (Figure 4), making it an ideal probe of DNA structure.

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ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
AP-dC-CE Phosphoramidite (G-Clamp)	10-1097-95	50 μ mole	230.00
	10-1097-90	100 μ mole	460.00
	10-1097-02	0.25g	1175.00

PYRROLIDINE CE PHOSPHORAMIDITE: A NEW BASE EXCISION REPAIR INHIBITOR

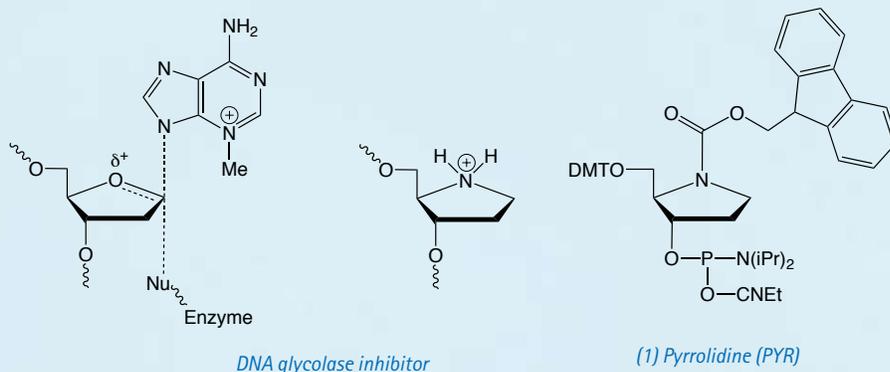
DNA is constantly under attack. Alkylating agents, ionizing radiation and oxidative stress can induce base modification or strand scission that, left unchecked, can lead to the development of cancer. Thankfully, our cells are equipped with DNA damage-monitoring and repair enzymes to correct the damage via base excision repair (BER) or nucleotide excision repair (NER). Ironically, though, the up-regulation of these repair enzymes in cancerous cells frequently causes the development of drug resistance against chemotherapeutic reagents.

One of the most studied repair mechanisms is probably the base excision DNA repair pathway. In this pathway, DNA glycosylases recognize the damaged bases and catalyze their excision through hydrolysis of the *N*-glycosidic bond. Attempts to understand the structural basis for DNA damage recognition by DNA glycosylases have been hampered by the short-lived association of these enzymes with their DNA substrates. To overcome this problem, the design and synthesis of inhibitors that form stable complexes with DNA glycosylases are essential. Complexes can then be studied biochemically and structurally.

Toward this end, the Verdine group at Harvard synthesized a pyrrolidine analog that mimics the charged transition state of the enzyme-substrate complex, as shown in Figure 1. When incorporated into double-stranded DNA, they found the pyrrolidine analog (PYR), introduced as the phosphoramidite (1), forms an extremely stable complex with the DNA glycosylase AlkA, exhibiting a dissociation constant in the pM range and potently inhibited the reaction catalyzed by the enzyme.¹

Later, the same group in collaboration with international researchers investigated the interaction of this inhibitor with a variety of additional DNA glycosylases. With the exception of uracil DNA glycosylase, all the glycosylases tested bind specifically to PYR-containing oligonucleotides² – providing an elegant means by which to study a broad range of DNA glycosylases and BER proteins. (Of course, we would be remiss not to mention Vern Schramm when discussing enzymatic transition state analogs and would recommend his excellent review of the subject.³) One might be tempted to think that any abasic analog, such as our dSpacer, (abasic furan – 10-1914), might also bind to AlkA. However, the charge is clearly

FIGURE 1: A GENERALIZED TRANSITION STATE FOR A DNA GLYCOSYLASE AND ITS INHIBITOR PYR



necessary as shown by Verdine's work.¹ When the dSpacer was incorporated rather than the pyrrolidine, the K_d was estimated to be 10,000-fold lower.

In any event, it is important to note that pyrrolidine is not directly comparable to our existing abasic phosphoramidite (dR precursor – 10-1924) or dSpacer as the new product will introduce a charge residue. Since the paper that Takeshita et al. published in 1987⁴, the dSpacer/abasic furan (which was introduced a few years later) has been extensively used to study, for example, the fidelity of polymerases when the enzyme encounters an abasic site. The paper published by Hogg et al. in 2006 and several citations in this paper⁵ are good examples of such research. Other information and comparison of various building blocks for the incorporation of abasic lesions can be found in a paper written by Shigenori Iwai.⁶

Given the importance of analogues involved in base lesions and their repair hold for Glen Research, one might wonder why we haven't made the Pyrrolidine CE Phosphoramidite ((1) in Figure 1) available before now. It was difficult to provide this analogue because the chemistry used by the Verdine group¹ was complex and not really well suited for production purposes. With the development of an alternative synthetic

route, we are happy to provide this new product to the research community studying DNA repair.

For the chemists in charge of oligo synthesis, the coupling time of the PYR phosphoramidite should ideally be extended to 5 minutes (tested with 1-*H* Tetrazole as activator) and the deprotection procedure is standard. We have also carried out accelerated stability studies to confirm that the PYR phosphoramidite is stable even though it contains an Fmoc protecting group. Fortunately, the stability of the PYR phosphoramidite was found to be more than acceptable.

We hope that this addition to our catalog will be helpful to researchers in the field of DNA base excision repair.

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ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
Pyrrolidine-CE Phosphoramidite (PYR)	10-1915-95	50 μ mole	190.00
	10-1915-90	100 μ mole	380.00
	10-1915-02	0.25g	1085.00

NEW PRODUCTS - 2,6-DIAMINOPURINE-TOM AND FMOC-AMINO-MODIFIER C6 DT AMIDITES

2,6-Diaminopurine-TOM

We are happy to provide 2,6-Diaminopurine ribonucleoside (2-Amino-A) TOM-CE Phosphoramidite with a new protection scheme optimized for methylamine deprotection of RNA oligos with TOM-protecting groups. Under standard TOM deprotection conditions (10M MeNH₂ in H₂O/EtOH, RT), our previous diacetyl protected monomer showed significantly different rates of deprotection for the N2-acetyl and N6-acetyl protecting groups. The optimized protection scheme using N2-methoxyacetyl and N6-isobutyryl protecting groups was reported¹ by Porcher and Pitsch.

The following stabilities were determined for diacetyl protected diaminopurines:

N2,N6-diacetyl diaminopurine:
 $t_{1/2}$ (N6) < 2 min and $t_{1/2}$ (N2) = 30 min

The new protecting scheme with the more labile N2-methoxyacetyl and the more resistant N6-isobutyryl protecting groups showed the following deprotection rates:

N2-methoxyacetyl, N6-isobutyryl diaminopurine:
 $t_{1/2}$ (N6 and N2) = 3 min

By contrast, under the same conditions, N2-acetylguanosine, which is the most resistant to deprotection of the TOM-protected monomers, showed a similar deprotection rate:

N2-acetylguanosine: $t_{1/2}$ = 4 min

Conclusion

The new protection scheme for 2,6-Diaminopurine ribonucleoside is virtually ideal for RNA synthesis and leads to simultaneous deprotection of both protecting groups of the diaminopurine, and the stability of the new monomer is comparable to the stability of N2-acetylated guanosine.

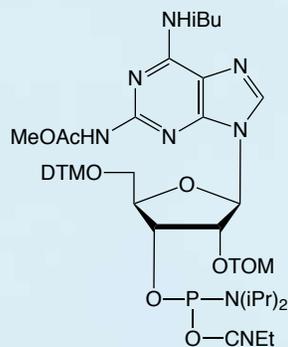
Reference:

1. S. Porcher and S. Pitsch, *Helv Chim Acta*, 2005, **88**, 2683-2704.

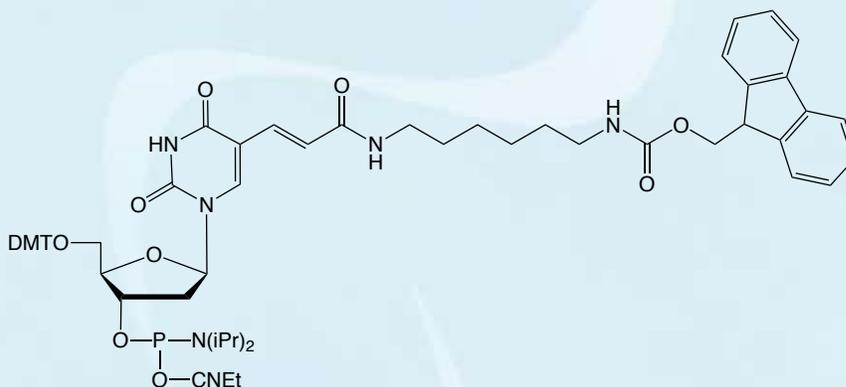
Fmoc-Amino-Modifier C6 dT

Amino-Modifier C6 dT is one of our oldest and best accepted products, and has become virtually a standard in oligonucleotide modification and labelling. Over the years, we have introduced dA, dC, dG and U variants and these products have also found favor as vehicles for highly specific labelling of oligonucleotides. However, these products are unable to address one

FIGURE: STRUCTURES OF 2,6-DIAMINOPURINE-TOM AND FMOC-AMINO-MODIFIER C6 DT



2,6-Diaminopurine-TOM



Fmoc-Amino-Modifier C6 dT

need which our customers have requested - on-column labelling. In all of these cases, the amino group is protected with the trusty trifluoroacetate (TFA) protecting group which is intended to be removed during the standard oligonucleotide deprotection. Try as we might, we have never found conditions which allow the TFA group to be removed while the oligonucleotide remains attached to the support. Our initial attempts to solve this problem by using a 9-fluorenylmethoxycarbonyl (Fmoc) protecting group were not encouraging. However, a simple adjustment to the procedure used to remove the Fmoc protecting group allows the product to function properly. The problem

was caused by alkylation of the amino group by acrylonitrile formed on elimination of the cyanoethyl phosphate protection groups. By using a two step procedure, the first to remove the cyanoethyl protection groups and flush the formed acrylonitrile from the synthesis column using 1% diisopropylamine in acetonitrile, and the second to remove the Fmoc group using 10% piperidine in DMF. The amino group so formed on the column can be reacted with a variety of activated esters. The one caveat is that the added tag must be stable to the basic conditions of the oligonucleotide cleavage and deprotection.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
2,6-Diaminopurine-TOM-CE Phosphoramidite (2-Amino-A)	10-3085-95	50 µmole	212.50
	10-3085-90	100 µmole	425.00
	10-3085-02	0.25g	975.00
Fmoc-Amino-Modifier C6 dT-CE Phosphoramidite	10-1536-90	100 µmole	180.00
	10-1536-02	0.25g	360.00

VERSATILE NEW REAGENTS – PYRENE-DU AND PERYLENE-DU

Pyrene has intrigued researchers ever since Förster and Kasper described its ability to form 'excited state dimers', later to be known as excimers.¹ They observed that while dilute solutions of pyrene fluoresce violet, at higher concentrations the fluorescence shifts 100 nm and becomes blue. This unstructured, long-wavelength emission arises from the formation of a charge-transfer complex between a pyrene in the excited state and another pyrene in the ground state. Since this complex must form within the lifetime of the excited state, the two pyrene molecules must be in close proximity – an ideal situation for developing DNA probes. By having two pyrene-labeled DNA probes, one with the pyrene on the 5' terminus and the other on the 3', the two pyrenes could be brought together by hybridizing in tandem to the target sequence, leading to excimer formation.² This technique has been used recently to probe mRNA in cells, taking advantage of the relatively long fluorescence life time of the pyrene excimer.³

An interesting development came when Korshun attached the pyrene to the 5 position of deoxyuridine through a triple bond (Figure 1).⁴ By doing so, the pyrene is electronically coupled to the deoxyuridine base as shown by the redshifting of the pyrene absorbance by more than 50 nm compared to the unsubstituted 1-ethynylpyrene.⁵ This electronic coupling of the base and the pyrene makes the fluorescence of the pyrene sensitive to the base pairing of the dU portion of the molecule, allowing the discrimination between perfect and one base mismatched targets.⁶ This coupling also allows photoinduced charge transfer to occur upon excitation of the pyrene, essentially 'injecting' electrons into the duplex.⁷ The only downside is a substantial reduction of the Stokes' shift of the excimer's fluorescence compared to the monomer, dropping from *circa* 100 to 14 nm, as shown in Figure 2A on the Back Page.

To complement the Pyrene-dU, we are also introducing its longer wavelength cousin, Perylene-dU (Figure 1). The Perylene-dU is another fluorescent polycyclic aromatic hydrocarbon that can form excimers. As with the Pyrene-dU, the Perylene analog has been shown to be sensitive to its hybridization state.⁸ When used in tandem, perylene and pyrene have been shown to form exciplexes⁹ giving rise to a broad range of fluorescence

FIGURE 1: STRUCTURES OF PYRENE-DU AND PERYLENE-DU PHOSPHoramidITES

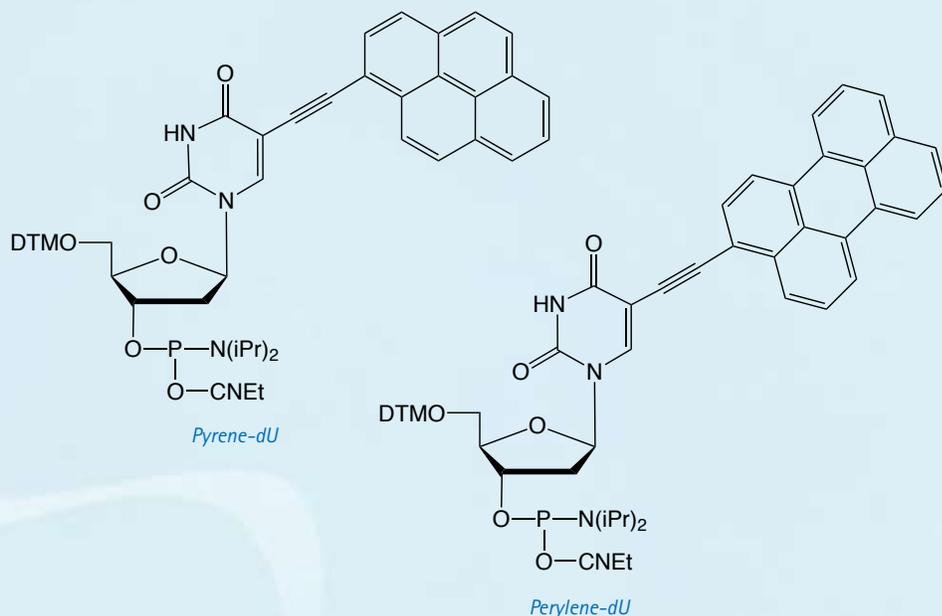


TABLE: ABSORPTION AND EMISSION DATA OF PYRENE-DU AND PERYLENE-DU

	λ (abs)	λ (emission)	λ (emission excimer)
Pyrene-dU	402 nm	472 nm	486 nm
Perylene-dU	473 nm	490 nm	Not Determined

emission depending upon the sequence and spacing between the fluorophores.

Absorption and emission data for Pyrene-dU and Perylene-dU are collected in the Table and the emission spectrum for Perylene-dU with the absorption spectrum as an insert is shown in Figure 2B on the Back Page.

We are happy to introduce these interesting new products to our repertoire of unusual bases.

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(Continued on Back Page)

ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
Pyrene-dU-CE Phosphoramidite	10-1590-95	50 μ mole	105.00
	10-1590-90	100 μ mole	210.00
	10-1590-02	0.25g	550.00
Perylene-dU-CE Phosphoramidite	10-1591-95	50 μ mole	150.00
	10-1591-90	100 μ mole	300.00
	10-1591-02	0.25g	720.00

1H-Tetrazole

One of the first activators described for phosphoramidite chemistry was 1H-Tetrazole and this has been the mainstay since its introduction for use with DNA synthesis. Other products have been introduced that have advantages over 1H-tetrazole, however none has been truly a universal replacement for 1H-Tetrazole. Nevertheless, the sun may be setting on 1H-Tetrazole as the activator of choice for DNA and RNA synthesis and this note discusses the properties 1H-Tetrazole and its inherent weaknesses as an activator. Several other choices for activator are also described.

The study of the mechanism of 1H-Tetrazole activation by Dahl¹, Berner², as well as others, led to the proposal of a two step reaction of tetrazole with phosphoramidites. First, tetrazole protonates the diisopropylamino group of the phosphoramidite and then displaces diisopropylamine by nucleophilic substitution to form the active species, the tetrazolide intermediate. Subsequent nucleophilic substitution of the tetrazolide with the 5'-hydroxyl of the growing oligonucleotide forms the new phosphotriester linkage. Thus, a better proton donor and/or a better nucleophile to generate the active intermediate should increase the rate of reaction.

Indeed, alternative tetrazole activators such as ethylthiotetrazole and benzyl thiotetrazole have lower pKa, as shown in the Table, and do improve the rate of reaction. Conversely, DCI also improves the reaction rate presumably because it is a better nucleophile.

Some of 1H-Tetrazole's other properties make it a less than ideal general-purpose activator:

- Limited solubility of 1H-Tetrazole (33.3g/L in acetonitrile) leads to precipitation in transit during the winter months, requiring the solution to be warmed prior to use.
- Similarly, limited solubility leads to precipitation and clogging of the tiny nozzles used in high throughput synthesizers.
- 1H-Tetrazole's performance in activating sterically hindered phosphoramidites, like RNA monomers, is not optimal.

FIGURE: STRUCTURE OF ACTIVATORS

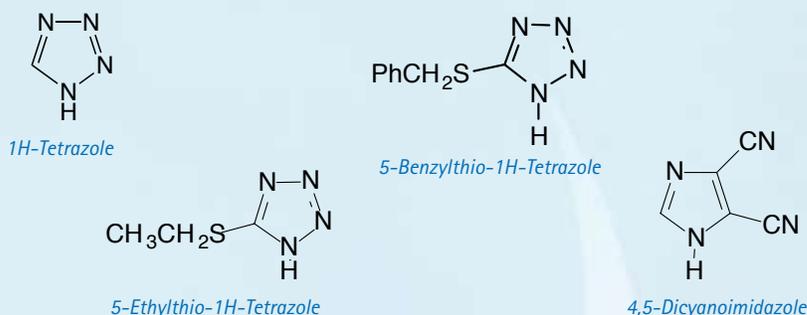


TABLE: PHYSICAL PROPERTIES OF ACTIVATORS

Activator	pKa	Solubility in Acetonitrile
1H-Tetrazole	4.9	0.50M
5-Ethylthio-1H-Tetrazole	4.3	0.75M
5-Benzylthio-1H-Tetrazole	4.1	0.44M
4,5-Dicyanoimidazole	5.2	1.2M
Acetic Acid	4.8	N/A

For standard DNA synthesis, none of this is reason enough to supplant 1H-Tetrazole as the activator of choice. However, the classification of the powder, but NOT the solution, as an explosive, may jeopardize reliable supply of 1H-Tetrazole.

Alternative Activators

5-Ethylthio-1H-tetrazole (ETT) became popular in the 1990s as the preferred activator for RNA synthesis.³⁻⁵ ETT is also much more soluble than tetrazole and this attribute certainly has contributed to its more general popularity.

The renewed interest in RNA synthesis due to the growth of siRNA technology has led us to evaluate 5-benzylthio-1H-tetrazole (BTT), which was described several years ago as an ideal activator for RNA synthesis using TOM-protected RNA phosphoramidites^{6,7} and recently for TBDMS-protected monomers.⁸ For instance, BTT allows the synthesis of RNA using 2'-TBDMS protected monomers on an AB3900 synthesizer with coupling time around 3 minutes compared to 10-15 minutes with tetrazole. However, although BTT has been widely used for RNA synthesis, it must be remembered that BTT is more acidic than ETT.

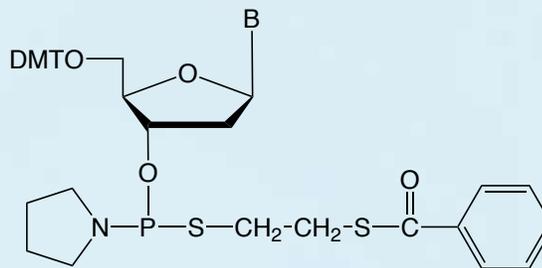
A recent study has revealed a major drawback to the acidity of tetrazole-related activators for large scale synthesis.⁹ This study revealed that tetrazole is sufficiently acidic to deprotect, to a small extent, the

trityl group in the monomer solution, leading to a small amount of dimer formation. Coupling of the dimer phosphoramidite leads to the presence of longer oligos (n+1) in the crude product mixture. The conclusion from this study is the more acidic the activator, the higher the risk of double addition and formation of oligos longer than expected. Since these impurities are all trityl-ON at the end of the synthesis, they represent a complication in purification schemes.

An alternative to the tetrazole-based activators is 4,5-dicyanoimidazole (DCI)¹⁰ that is less acidic but is a much more nucleophilic activator. DCI is even more soluble in acetonitrile (up to 1.2M solution in acetonitrile). The biggest difference between DCI and tetrazole manifests itself at larger scales that allow the use of a lower excess of monomers relative to tetrazole activators. For example, a 34mer oligoribonucleotide, including 2'-fluoropyrimidine residues, was prepared on a 1 mmole scale with 2 equivalents of monomer using 0.45M tetrazole, 0.45M tetrazole + 0.1M N-methylimidazole (NMI), or 1M DCI as activator. No full-length product was detected with tetrazole activation, while a low yield (13%) of product was observed with the activator containing NMI. With DCI, the full-length product was observed in 54% yield. Our studies with DCI show that 0.25M is the optimal concentration for routine small-scale synthesis (< 15

INTRODUCING THIOPHOSPHORAMIDITES

FIGURE 1: GENERIC STRUCTURE AND DESCRIPTION OF THE FOUR THIOPHOSPHORAMIDITES



1. dA-Thiophosphoramidite

5'-Dimethoxytrityl-N-benzoyl-2'-deoxy-Adenosine, 3'-[(β-thiobenzoylethyl)-(1-pyrrolidinyl)]-thiophosphoramidite, M.W.: 955.09

2. dC-Thiophosphoramidite

5'-Dimethoxytrityl-N-benzoyl-2'-deoxy-Cytidine, 3'-[(β-thiobenzoylethyl)-(1-pyrrolidinyl)]-thiophosphoramidite, M.W.: 931.07

3. dG-Thiophosphoramidite

5'-Dimethoxytrityl-N-isobutyryl-2'-deoxy-Guanosine, 3'-[(β-thiobenzoylethyl)-(1-pyrrolidinyl)]-thiophosphoramidite, M.W.: 937.07

4. dT-Thiophosphoramidite

5'-Dimethoxytrityl-2'-deoxy-Thymidine, 3'-[(β-thiobenzoylethyl)-(1-pyrrolidinyl)]-thiophosphoramidite, M.W.: 841.97

μmoles), using normal synthesis cycles. We are therefore providing solutions at that concentration but we also offer the raw material so that researchers can prepare more or less concentrated solutions should they desire.

Like ETT, DCI has proved popular for high throughput synthesizers since it also does not tend to crystallize and block the fine outlet nozzles.

Conclusion

For a general purpose activator and for the synthesis of short oligos in small to medium scale, we recommend ETT or BTT. ETT has the added advantage of being more soluble in acetonitrile than 1*H*-tetrazole (up to 0.75M versus 0.50M solution in acetonitrile). ETT and BTT are more acidic than 1*H*-Tetrazole and retain its nucleophilic properties. For RNA synthesis, BTT seems to be the best choice as of today. For long oligos and for synthesis at larger scales (>15 μmoles), we would suggest using DCI.

Additional activator alternatives also include pyridinium trifluoroacetate, saccharin methylimidazolide (SMI), BTT with methylimidazole, and DCI with methylimidazole. Glen Research is currently reviewing these alternatives to identify the best general multipurpose activator suitable for DNA, RNA, array synthesizers, and large scale synthesis.

Please contact Technical Support at 800-327-GLEN or at support@glenres.com, for assistance in selecting the best activator for your application.

References:

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Thiophosphoramidites are activated modified deoxyribonucleotides which effect the substitution of both internucleotide nonbridging oxygen atoms with sulfur. The resultant oligo has an achiral internucleotide phosphorodithioate (PS2) linkage. The structure of a thiophosphoramidite is shown in Figure 1.

An achiral dithio linkage is produced by the coupling of the appropriate thiophosphoramidite and the subsequent sulfurization step using Beaucage Reagent. The reaction is shown in Figure 2.

Background

Initially, PS2-oligos were investigated as potential antisense compounds and exhibited the ability to interfere with the expression of erbB-2 mRNA associated with breast cancer, to inhibit HIV-1 reverse transcription activity, and to induce B-cell proliferation and differentiation.^{1,2} The HIV-1 inhibition of HIV-1 reverse transcriptase is dependent on the number of dithioate linkages and the length of the dithioate oligo.^{1,3} A comparative analysis with phosphoromonothioate equivalents indicates that dithioate oligos are much better inhibitors and are able to inhibit potently with relatively short oligomer length.^{1,3} Inhibition of HIV-1 reverse transcriptase by PS2-oligos appears to be a general phenomenon as all of the

nucleotide base sequences examined inhibit its activity.

What are the attributes and applications for phosphorodithioate oligonucleotides (PS2-oligos)?

PS2-oligos have potentially useful characteristics such as: 1) high binding affinities to proteins and cell surfaces; 2) are nuclease resistant and therefore stable in biological preparations; 3) are easily prepared; and 4) as thioaptamers show excellent specificity to proteins.⁴ Significantly, the PS2-oligos, in contrast to the monothio phosphate oligos (S-oligos), are achiral about the dithiophosphate center, so problems associated with diastereomers are completely avoided.

Protein specificity and affinity

Oligos with high proportions of phosphorothio and dithio linkages appear to lose some of their specificity and are "stickier" toward proteins in general than oligos with normal phosphate esters, an effect often attributed to non-specific interactions. This can be quite important since the recognition of nucleic acid sequences by proteins involves specific side chain and backbone interactions with both the nucleic acid bases as well as the phosphate ester backbone. One can take advantage of this "stickiness" to enhance

the affinity of PS2-oligos for a protein target but the total number of PS2 linkages must be optimized to decrease non-specific binding to the protein target and only enhance the specific favorable interactions with the specific proteins.

Aptamers

PS2 analogues have been successfully used as aptamers for a variety of protein targets including activated protein 1 (AP-1) as well as transcription factor NF- κ B for which the PS2-oligos demonstrated 150 picomolar Kd with a dissociation time >12 hours. PS2-oligos demonstrate up to 300x greater binding affinity for proteins than oligos without PS2 linkages with no loss of aptamer specificity. For HIV-1 RT, dithioates bind 28 (vs. monothioate) or 600 (normal backbone) times more tightly than the normal aptamer oligonucleotide or the S-analogue. For aptamer applications, it is recommended that no more than six dithio linkages be included in any PS2-oligo in order to minimize non-specific protein interactions.

PS2 oligos have been used in a variety of assays, instruments and technologies offering a wide field of use. They have shown utility in gel shift assays, nanoparticle technologies, microarrays, BioPlex based applications as well as in diagnostic applications.^{5,6}

Attributes of PS2-oligos

The Tm of PS2-oligos is lower than the equivalent PS-oligo or the normal phosphate ester, with the degree of Tm depression paralleling the percent phosphorodithioate composition of the oligomer. It is in the range of 0.5 ~ 1.5 °C per dithio linkage.

Toxicity has not been observed in cell culture assays with PS2-oligos.^{1,3}

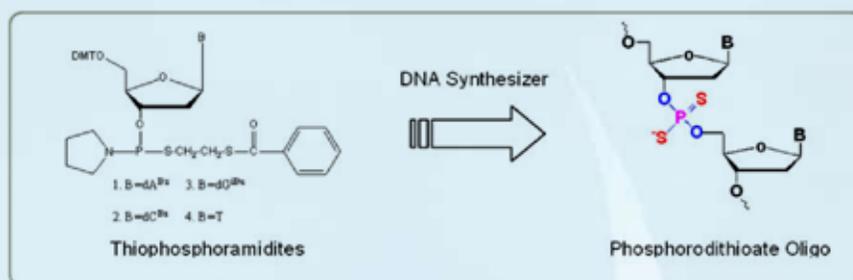
Phosphorodithioate and phosphoromonothioate anions preferentially bind to Cd²⁺ and Mn²⁺ while phosphate ester anions preferentially bind to Mg²⁺ which may provide a mechanism to demonstrate protein binding domains for specific oligo sequences.^{7,8}

Synthesis

Thiophosphoramidites can be used on a variety of commercial synthesizers with only slight changes in protocol.

Extended coupling times and sulfurization times are recommended to

FIGURE 2. SYNTHESIS OF PHOSPHORODITHIOATE OLIGOS USING THIOPHOSPHORAMIDITE MONOMERS



ensure optimum coupling efficiency and complete sulfurization. Coupling times of six minutes and sulfurization times of six minutes have been used successfully with the Expedite synthesizer. Caution: if sulfurization is incomplete, a proportion of PS linkages will be present along with dithio linkages.

Monomer concentrations are typically 0.05M or higher. Monomers are dissolved in anhydrous acetonitrile with a small proportion of dichloromethane. Typical coupling efficiencies exceed 90%. The monomers have a lifetime of two days on the synthesizer.

Cleavage conditions for PS2-oligos are equivalent to normal phosphate ester oligos and are amenable to various known cleavage cocktails and conditions.⁹⁻¹¹

Purification of PS2-oligos

PS2-oligos have been purified by PAGE, RP-HPLC, and IEX-HPLC.¹¹⁻¹³

IEX-HPLC at pH 8 is the preferred method. Thiocyanate (SCN⁻) as the eluting ion can be used for oligos with a high proportion of PS2 linkages. PS2-oligos are separated on the basis of charge and the number of sulfur substitutions. Purities of oligos (2-30 mers) of greater than 95% are routinely achieved. Highly purified PS2-oligos with less than 50% PS2 linkages have been obtained. The retention times of PS2-oligos on a MonoQ column are in direct proportion to the number of PS2 linkages. The average retention time increase is 2.3 minutes per sulfur addition on MonoQ 5/5 columns.¹⁴

PAGE allows for excellent pure preparations but the technique suffers from manual gel excision and typically low yields.

RP-HPLC is used with the DMT-on method but is not a method of choice because of the hydrophobicity of the PS2-

oligo. In addition, it can be difficult to remove the DMT-on PS-oligo impurities (desulfuration products) using RP-HPLC. Crude PS2-oligo chromatograms are routinely complex and identifying the full length oligo is not always obvious with UV detection.

Characterization

PS2-oligos can be confirmed by MALDI-TOF MS and ³¹P-NMR. Resonances of PS2 linkage (112 ppm), PS linkage (58 ppm), phosphate (0 ppm) in ³¹P-NMR spectra are well resolved.^{15,16}

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FIGURE 2A: FLUORESCENCE SPECTRUM OF PYRENE-DU WITH ABSORBANCE SPECTRUM INSERT

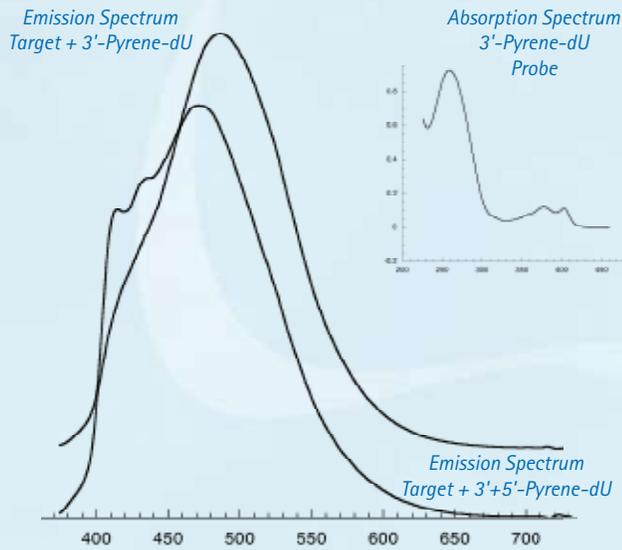


FIGURE 2B: FLUORESCENCE SPECTRUM OF PERYLENE-DU WITH ABSORBANCE SPECTRUM INSERT

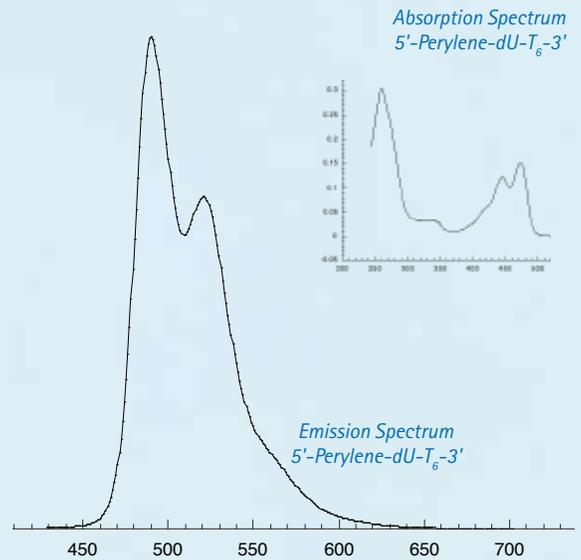


Figure 2A: Emission of 5'-AGC ATG AAT CAT CGA CTC AAG GT-Pyrene-dU-3' when annealed to the target sequence 5'-GTT AGT CGT ACG TAC GCT AGA TCC GAA ACC TTG AGT CGA TGA TTC ATG CT-3'. Upon addition of 5'-Pyrene-dU-CG GAT CTA GCG TAC GTA CGA CTA AC-3', there is a reduction in the pyrene emission at 414 nm and a redshift of the emission to 486 nm, indicating the formation of the excimer. Inset is the 3'-Pyrene-dU probe absorbance spectrum. Figure 2B: Emission spectrum of 5'-Perylene-dU-T₆-3'. Inset is the 5'-Perylene-dU-T₆-3' absorbance spectrum.



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