

The Glen Report

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GLEN RESEARCH

VOLUME 21

NUMBER 1

MAY 2009

I N S I D E

NEW LABELLING REAGENTS

DEPROTECTION 2 - RNA

CYCLO-DA

NEW SUPPORTS

NEW PHOSPHoramidites

FLUORESCENT MONOMERS

HOT START PCR UPDATE: CLEANAMP™ PRIMERS

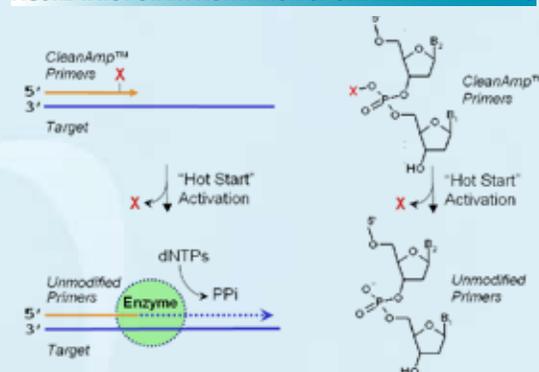
By Jonathan Shum, Joyclyn Yee, Elena Hidalgo Ashrafi, and Natasha Paul, Ph.D.; TriLink BioTechnologies

The Polymerase Chain Reaction (PCR) is a commonplace molecular biological method to amplify a DNA target of interest. Although PCR is a powerful technique, its sensitivity and reproducibility are often plagued by off-target amplifications. These include primer dimer and mis-priming products caused by the hybridization of primers to one another or to non-specific regions on the template. Such off-target amplifications lower the efficiency of PCR by effectively sequestering PCR substrates, such as the primers, the DNA polymerase, and the dNTPs, from amplifying the desired template. At lower template concentrations, the problem of primer dimer formation and mis-priming is exacerbated as less template is available causing an increase in non-desired primer hybridization and extension.(1) Therefore, it is critical to substantially decrease or even eliminate off target amplification, especially when available template is limited.

Attempts have been made to alleviate the problem of primer dimer formation by the use of Hot Start technologies, which include physical separation of reaction components, inhibition of the DNA polymerase, and the use of accessory proteins. Many of the specialized DNA polymerase compositions can add significant cost to the reaction by the need for extensive manipulations, such as in the preparation of the DNA polymerase. In contrast, primers, which have CleanAmp™ thermolabile protecting groups, represent a simple approach to Hot Start activation in PCR. These modifications can be easily introduced to any primer sequence using standard solid phase oligonucleotide synthesis protocols. In addition, CleanAmp™ modifications are compatible with many commonly used DNA polymerases.

The introduction of thermolabile CleanAmp™ modifications into PCR primers allows for greater control of primer hybridization and extension during PCR. The thermolabile primer modifications prevent DNA polymerase extension at lower less-

FIGURE 1: HOT START ACTIVATION OF CLEANAMP™ PRIMERS



discriminating temperatures of reaction set-up and manipulation but also display the flexibility to allow intended extension after activation at higher temperatures (Figure 1).

Furthermore, by using either the slow-releasing Precision primers or the faster-releasing Turbo Primers, the rate of formation of unmodified primer can be controlled to suit your reaction needs. Herein, we investigate the utility of the CleanAmp™ Primer modifications in standard PCR protocols and in more advanced techniques such as multiplex PCR, one-step RT-PCR, and fast cycling PCR.

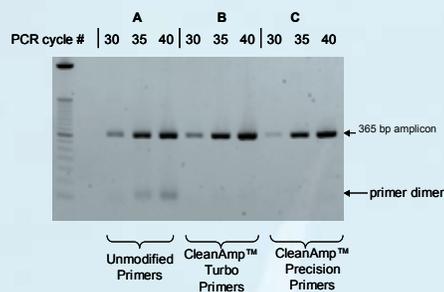
GENERAL APPLICATIONS

CleanAmp™ Turbo and Precision Primers significantly lower, if not eliminate, off target amplification

Primer dimer formation has been found to be problematic in the amplification of a region of the HIV-1 tat genomic DNA.(1) In these studies, the reaction progression was monitored by removing aliquots after 30, 35, and 40 thermal cycles (Figure 2). Amplifications using unmodified PCR primers were found to be prone to robust primer dimer formation, which competes with the formation

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PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂), Primers (0.5 μM), dNTPs (0.2 mM), 5 copies HIV-1 gDNA, Taq DNA polymerase (1.25U), 50 μL. **Thermal cycling conditions:** 95°C (10 min); [95°C (40 sec), 56°C (30 sec), 72°C (1 min)]40X, 72°C (7 min).

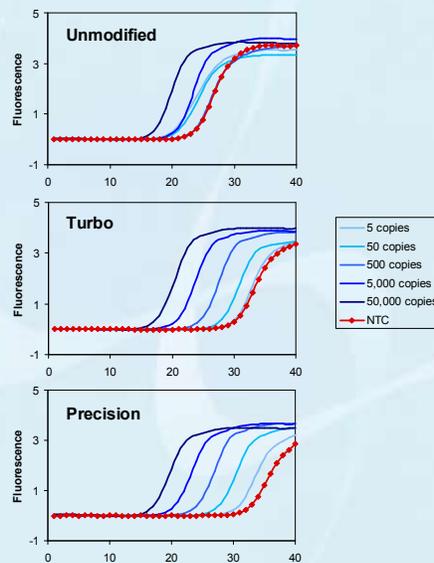
Figure 2: Endpoint PCR evaluation of CleanAmp™ Primers in a primer/template system prone to primer dimer. Aliquots of reaction were removed at 30, 35, and 40 cycles.

of the desired 365 bp amplicon (Figure 2A). By contrast, the introduction of Turbo Primers significantly reduced primer dimer formation and promoted an even greater target yield as compared to the unmodified primers (Figure 2B). For Turbo Primers, only a slight amount of primer dimer is seen after 40 cycles. In the same system, the use of Precision Primers yielded only the desired amplicon, with no detectable primer dimer formation. However, while the slower release of the Precision protecting groups significantly reduced primer dimer formation, robust target amplification was slightly delayed at thirty thermal cycles but fully recovered after forty thermal cycles (Figure 2C). These studies demonstrated that Turbo Primers were able to efficiently form the desired amplicon, while significantly reducing primer dimer formation. Precision Primers were found to have the greatest utility when pure amplicon formation is required.

Mis-priming can also be a significant hindrance to the fidelity and efficiency of amplification of the desired target. In comparison to unmodified primers, Turbo Primers reduce much of the mis-priming products, with Precision Primers providing the greatest benefit. This reduction in off-target amplification is evident over a wide range of input template concentrations, with improved amplicon yield also being much greater than with unmodified primers.

CleanAmp™ Primers provide amplification specificity over a large range of template concentrations.

Detection of a target at low concentrations is another difficulty encountered in PCR. Often, at low template concentrations, off-target amplifications compete with the desired amplification, complicating real-time PCR detection of the desired amplicon formation. CleanAmp™ Primers have been found to successfully amplify the correct amplicon at 10–100-fold lower template concentration as compared to unmodified primers. In Figure 3, the lower limit of detection of a 533 base pair amplicon from Lambda genomic DNA was explored over a range of input template concentrations using SYBR Green detection in real-time PCR. For the unmodified primers, the range of detection started above 500 copies. Because the amplification curve coincided with the no template control (NTC) curve at 500 copies or less, it would



PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂), Precision CleanAmp™ Primers (0.5 μM), SYBR Green (.15X), ROX (30nM), dNTPs (0.2 mM), 0–50,000 copies Lambda gDNA, 1.25 U Taq DNA polymerase, 25 μL; reactions performed in duplicate. **Thermal cycling conditions:** 95°C (10 min); [95°C (40 sec), 57°C (30 sec), 72°C (1 min)] 40X.

Figure 3: SYBR Green real-time PCR assay where 0–50,000 copies of Lambda gDNA were assayed using unmodified, Turbo and Precision Primers.

be impossible to differentiate between desired amplicon formation and other off target formation.

The use of Turbo Primers provides at least a ten-fold increase in detection, as

the 50 copy concentration is distinguishable from the NTC curve. Precision Primers ultimately provide the greatest level of detection, detecting as low as 5 copies. This increased limit of detection using Precision Primers is indicative of their utility in a number of high-sensitivity downstream applications, such as single molecule detection.

CleanAmp™ Primers outperform other Hot Start Technologies

Since CleanAmp™ Primers provide significant benefit relative to reactions with unmodified primers, experiments were then performed to compare the performance of CleanAmp™ Primers to that of other Hot Start technologies. In these studies, the performance of unmodified primers with one of a series of Hot Start DNA polymerases, such as a chemically modified version of Taq (2), was compared to the performance of CleanAmp™ Primers with unmodified Taq DNA polymerase. Amplicon was formed with equal or much lower yield than reactions that employed unmodified Taq DNA polymerase with Precision Primers (Figure 4A, B). Moreover, Turbo Primers and unmodified Taq DNA polymerase gave the greatest benefit, as the amplicon yield was much higher than each of the Hot Start polymerases examined. These findings are significant, as they indicate that CleanAmp™

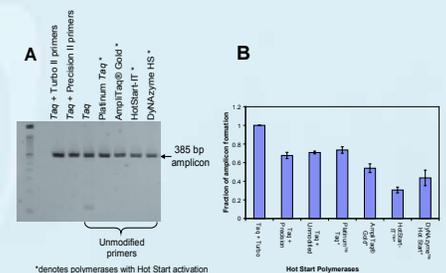
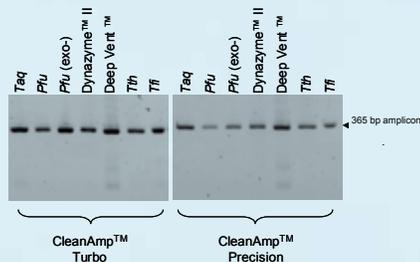


Figure 4: Comparison of CleanAmp™ Primers to other commercially available Hot Start DNA polymerases. A. Endpoint PCR analysis of amplification reactions containing 5 copies of HIV-1 genomic DNA. B. Graphical representation of the relative amplicon yield, normalized to reactions containing Taq DNA polymerase plus CleanAmp™ Turbo Primers.

Primers and unmodified Taq DNA polymerase can be employed without compromising amplicon yield, while efficiently reducing primer dimer formation and mis-priming.

CleanAmp™ Primers are compatible with other DNA polymerases

Taq DNA polymerase was used as a point of reference to determine whether Precision and Turbo CleanAmp™ Primers could be employed with other DNA polymerases in endpoint PCR experiments. In the endpoint reactions, seven DNA polymerases devoid of Hot Start activation were evaluated for their ability to robustly form the desired 365 bp amplicon (Fig. 5). Each of the DNA polymerases examined was able to support efficient amplification of the DNA target. For all cases, with the exception of Deep Vent™ and Tfi polymerase, the units of DNA polymerase were kept constant. Overall,



PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂), Primers (0.4 μM), dNTPs (0.2mM), 5 copies HIV-1 gDNA, Taq DNA polymerase (1.25U), Pfu (2.5U), Pfu(exo-) (2.5U), Dynzyme™ (1.2U), Deep Vent™ (2.5U), Tth (2.5U), Tfi(20U), 50 μL. Thermal cycling conditions: 95°C (10 min); [95°C (40 sec), 56°C (30 sec), 72°C (1 min)] 35X, 72°C (7 min).

Figure 5: Evaluation of the performance of CleanAmp™ Primers in amplification reactions with a variety of thermostable DNA polymerases.

CleanAmp™ Primers are versatile as they can be used with other thermostable DNA polymerases in endpoint PCR. This compatibility gives great potential for the implementation of CleanAmp™ Primers in a number of applications.

MULTIPLEX PCR APPLICATIONS

One promising application of PCR is the ability to amplify multiple targets in a single reaction. This approach, known as multiplex PCR, employs a distinct primer pair for each amplicon of interest. This application has been an essential tool for many different medical diagnostic and scientific applications, such as viral screens(3), where PCR based assays have proven to be more sensitive and less time consuming than traditional cell culture tests(4).

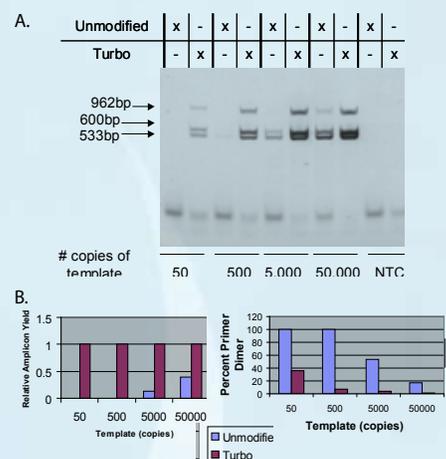
Although multiplex PCR has many

advantages, there are inherent problems that inhibit robust amplification. One major factor is competing, undesired off-target amplifications such as primer dimer formation (5), which limits the number of possible targets that can be detected per reaction, due to the increase in the number of unique primer sequences in the reaction, which in turn increases the probability of primer dimer formation. Another challenge of multiplex PCR is the preferential amplification of certain targets (6). Therefore the design of multiple primer pairs that are both specific for a target of interest and exhibit a low level of off-target amplification can be a challenge. Coupled with this decreased flexibility in primer design, individual primer pair concentrations must be optimized, such that amplification efficiencies of all targets are similar (7). This is a time consuming process, which has a low probability of success, should off-target amplicon formation dominate the reaction. Below, the ability of CleanAmp™ Primers to improve the specificity of amplicon formation for all targets in multiplex PCR is evaluated. The ability of CleanAmp™ Primers to reduce other competing off-target amplification, in single target reactions was applied to a multiplex PCR assay, where amplicon yield and PCR efficiency are extremely sensitive to primer dimer formation. Findings revealed minimal optimization of the design and concentration of the CleanAmp™ Primers with CleanAmp™ Turbo Primers, improving with reduced primer dimer formation.

Multiplexed target amplification at low template concentrations

When individual assays are combined into a single, multiplexed PCR assay, often template concentration must be increased to compensate for inefficient amplification. However, in clinical settings, where template sample is limited, increasing template concentration is not an option.

In these cases where greater sensitivity is necessary, Turbo Primers have demonstrated much promise. When compared to unmodified primers, amplicon formation in a triplex reaction was detected at a hundred-fold lower input of template when using Turbo Primers (Figure 6A), with Turbo Primers efficiently detecting 50 copies of Lambda genomic DNA, where unmodified primers could only detect 5,000



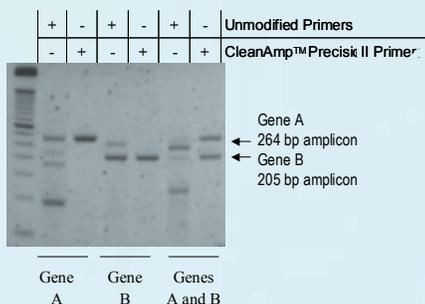
PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂), Primers (0.5 μM), 0.2 mM dNTPs, 50,000 copies Lambda gDNA, 1.25 U Taq DNA polymerase, 50 μL. Thermal cycling conditions: 95°C (10 min); [95°C (40 sec), 56°C (30 sec), 72°C (2 min)] 35X, 72°C (7 min).

Figure 6: Multiplex reactions over a wide range of template amounts. A. Performance of Turbo and unmodified primers with a varying amount of template. B. Quantitative analysis of amplicon yield and primer dimer formation for experiment performed in Figure 6A. For each template concentration, amplicon yield was normalized to Turbo.

copies. Furthermore, the longer targets appeared to amplify less efficiently than the shorter targets when using unmodified primers, with the longest 962 bp amplicon not forming until 50,000 copies of template were employed. On the other hand, at all template concentrations examined, Turbo Primers amplified all three targets with similar efficiency. Furthermore the use of CleanAmp™ Primers improved PCR performance by reducing primer dimer formation (Figure 6B). In summary, efficient amplification by Turbo Primers is less restricted by target size limitations, all three amplicons being formed over a broad range of input template concentrations at increased sensitivity.

CleanAmp™ Turbo Primers outperform in real-time multiplex PCR

To confirm that the range of detection was also reproducible in quantitative real-time PCR, a duplex reaction using Taqman® probe detection was performed. Much like the endpoint experiments, at low template concentrations, the detection of amplification is much more sensitive using CleanAmp™ Turbo Primers. In this duplex reaction, the difference in C_q between unmodified and Turbo Primers increased as



Reaction conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂), Primers (0.5 μM), polydT primer (1 μM), 0.16 mM dNTPs, 0–0.25 mg Human brain total RNA, 25 U reverse transcriptase, 0.3 U Taq DNA polymerase, 25 mL.

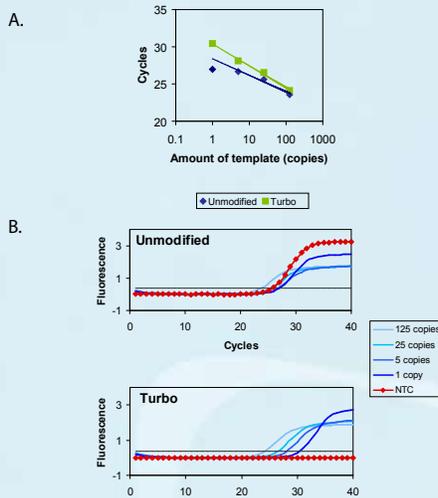
Figure 9: Evaluation of CleanAmp™ Primers in one-step reverse-transcription PCR in Human Liver Total RNA (Clontech) in singleplex and duplex. For the genes of interest (Gene A and Gene B), the PCR primers were unmodified or contained CleanAmp™ Precision modifications. Reverse transcription utilized a polydT18 primer. Reactions contained Taq DNA polymerase and M-MLV Reverse Transcriptase.

product formation in one-step RT-PCR protocols. Non-Hot Start DNA polymerases, such as Taq DNA polymerase, and standard reverse transcriptases, such as M-MLV reverse transcriptase, are recommended. There is no need to use more expensive modified DNA polymerases or RT enzymes. Furthermore, poly dT18 or random primers can be employed during the cDNA synthesis steps as opposed to a gene-specific primer. This universal priming method allows for multiplex one-step RT-PCR reactions. This is also a key advantage of CleanAmp™ Primers because other one-step RT-PCR kits do not recommend the use of poly dT18 or random primers. In summary, CleanAmp™ Precision Primers are a significant improvement over unmodified primers when used in one-step RT PCR.

FAST PCR THERMAL CYCLING APPLICATIONS

In PCR applications such as diagnostics or medical testing, efforts have been directed at reducing the duration of the PCR amplification step, allowing for faster results and greater throughput. However, these faster cycling PCR protocols also have some drawbacks including a decrease in sensitivity and a loss in reproducibility (18). One suggested solution to this decrease in sensitivity is to increase primer concentration. The main complication encountered with this solution is that an increase in primer

concentration often leads to higher off-target amplification, which can increase the false positive rate in real-time experiments, especially with SYBR Green detection (18). Therefore, prevention of off-target



PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂), Primers (1.5 μM), 0.8 mM dNTPs, 0–125 copies HIV gDNA, 3.75 U Taq DNA polymerase, 25 μL 1:2000 SYBR Green™ I. Thermal cycling conditions: [95°C (10 sec), 66°C (30 sec)]40X

Figure 10: (A) Amplification plots of CleanAmp™ Turbo Primers and unmodified primers in a primer/template system prone to primer dimer formation over a range of template concentrations. (B) Corresponding standard curve comparing CleanAmp™ Turbo Primers and unmodified primers.

amplification at high primer concentration could lead ultimately to an increase in sensitivity when using fast cycling PCR. Herein, the utility of CleanAmp™ Turbo Primers in preventing off-target amplification at high primer concentration using fast cycling PCR protocols will be demonstrated.

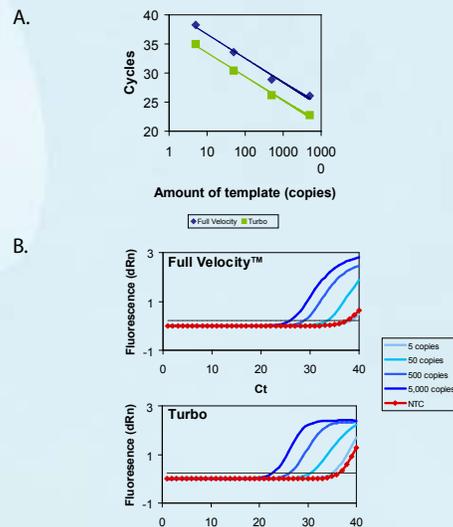
CleanAmp™ Turbo Primers decrease off-target amplification in fast-cycling PCR

Turbo Primers were evaluated for their ability to reduce off-target amplification and to improve the sensitivity of real-time SYBR Green assays using a typical fast cycling protocol (Figure 10). When unmodified primers were employed, interpretation of the amplification plots was complicated by strong amplification of the NTC sample. Although 125 copies of template DNA was accurately detected, the NTC sample amplified before each of the subsequent lower template concentrations. In comparison, the use of CleanAmp™ Turbo Primers allowed for detection down to a single copy. The NTC amplification curve

did not cross the threshold line (Cq) during the 40 thermal cycles of the experiment. In addition, when the Cq values were plotted in standard curves (Figure 10B), CleanAmp™ Turbo Primers exhibited greater linearity compared to unmodified primers. This characteristic is most likely related to the suppression of primer dimer formation when CleanAmp™ Turbo Primers are used and demonstrates Turbo Primer's increased specificity over a large range of template concentrations.

CleanAmp™ Turbo Primers out perform other fast-cycling technologies

Next, CleanAmp™ Turbo Primers were compared with Full Velocity™ SYBR Green QPCR Mastermix (Stratagene/Agilent), a commercially available kit which is formulated specifically for fast cycling PCR. The experiment involved amplification of a 245 base pair target from Lambda genomic DNA. To accurately compare the two products in the same experiment, a 5 minute initial denaturation time necessary for the Full Velocity™ Mastermix was used



PCR conditions (Turbo Primers): 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂), Primers (0.3 μM), 0.8 mM dNTPs, 0–10,000 copies Lambda gDNA, 3.75 U Taq DNA polymerase, 25 μL 1:2000 SYBR Green™ I.

PCR conditions (Unmodified Primers): 1X Full Velocity™ MasterMix (Stratagene/Agilent), Primers (0.15 μM). Thermal cycling conditions: 95°C (5 min) [95°C (10 sec), 66°C (30 sec)] 40X.

Figure 11: (A) Amplification plots of CleanAmp™ Turbo Primers and Full Velocity™ in a primer/template system prone to primer dimer formation over a range of template concentrations. (B) Corresponding standard curve comparing CleanAmp™ Turbo Primers and Full Velocity™.

with these cycling conditions. We found that Turbo Primers had slightly greater sensitivity than the Full Velocity™ Mastermix.

As depicted in Figure 11A, the NTC curve overlapped with the 5 copy sample with Full Velocity™ Mastermix. However, when Turbo Primers were employed, 5 copies of template amplified before the NTC curve. These findings are again likely due to a lower level of off-target amplification by Turbo Primers. When the experimental results were plotted in a standard curve, both approaches displayed good linearity over the range of concentrations evaluated. Although the efficiencies were similar for each technology, reactions that employed Turbo Primers had lower Cq's. This allowed for even greater speed of amplicon detection in a fast cycling protocol.

SUMMARY

In summation, CleanAmp™ Primers allow for greater control of primer hybridization and extension during PCR. Over a broad range of applications CleanAmp™ Primers reduce or eliminate off-target amplification. Greater amplicon yield is also achieved, due to improvement in specificity and sensitivity. It has been demonstrated that CleanAmp™ Primers outperform other technologies in multiple applications.

Which CleanAmp™ Primer is best for my application?

TURBO PRIMERS PRECISION PRIMERS

Fast cycling	Standard cycling
Multiplex PCR	One-step reverse-transcription PCR
Improves amplicon yield	Improved specificity and limit of detection
Reduces mis-priming/primer dimer formation	Greatest reduction in mis-priming/primer dimer formation

MONOMER SUPPLY AND SUPPORT

Glen Research is delighted to be able to support the CleanAmp™ technology by making the CleanAmp™ monomers available for sale and by supporting the synthesis of Turbo and Precision Primers. This is being done under license from TriLink BioTechnologies, Inc.. The following article describes the synthesis and purification of CleanAmp™ Primers.

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ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
CleanAmp™-Pac-dA-CE Phosphoramidite	10-1440-90	100 µmole	100.00
	10-1440-02	0.25g	240.00
	10-1440-05	0.5g	480.00
CleanAmp™-Ac-dC-CE Phosphoramidite	10-1450-90	100 µmole	100.00
	10-1450-02	0.25g	240.00
	10-1450-05	0.5g	480.00
CleanAmp™-Pac-dG-CE Phosphoramidite	10-1460-90	100 µmole	100.00
	10-1460-02	0.25g	240.00
	10-1460-05	0.5g	480.00
CleanAmp™-dT-CE Phosphoramidite	10-1470-90	100 µmole	100.00
	10-1470-02	0.25g	240.00
	10-1470-05	0.5g	480.00

PROCEDURE FOR SYNTHESIS OF CLEANAMP™ PRIMERS

REAGENTS

CleanAmp™ DNA phosphoramidites

UltraMild DNA phosphoramidites

Glen UnySupport

Acetonitrile, ≤ 20 ppm water

Dichloromethane

Activated Molecular Sieves

UltraMild Cap A

Cap B with N-Methyl Imidazole

HPLC Grade Water

0.05M Potassium Carbonate in Methanol

1M TEAA, pH 7.2

2M TEAA, pH 7 - dilute to concentration

2% TFA in water

DMSO anhydrous

Sep-Pak® Cartridges (Part # 20515)

VENDOR

TriLink BioTech

Glen Research

Glen Research

Glen Research

Glen Research

Fisher Scientific

TriLink BioTech

Glen Research

Glen Research

Fisher Scientific

Glen Research

TriLink BioTech

Glen Research

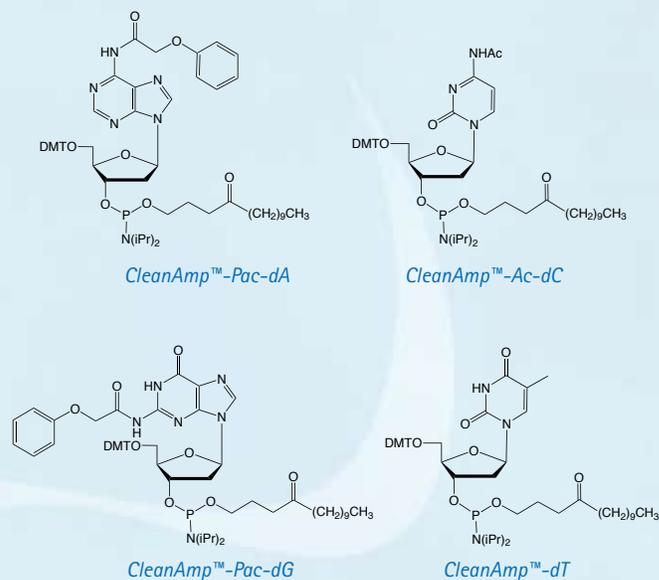
TriLink BioTech

Glen Research

Fisher Scientific

Waters

FIGURE 1: STRUCTURES OF CLEANAMP™ PHOSPHORAMIDITES



PROCEDURE:

A. Synthesis of CleanAmp™ oligonucleotides on solid support at 1 μmole scale :

1. Prepare solutions of each fast UltraMild phosphoramidite in anhydrous acetonitrile (ACN) using manufacturer's instructions. We recommend using a concentration of 0.067M.
2. Prepare 0.067 M solutions of each CleanAmp™ phosphoramidite (except for CleanAmp™-dG) in dry acetonitrile in a standard DNA synthesizer bottle. Prepare 0.067 M solution of CleanAmp™-dG phosphoramidite monomer in mixture of dry dichloromethane (DCM):ACN (1:1) in a standard DNA synthesizer bottle.

To properly dissolve a 100 μmole bottle of a CleanAmp™ monomer to obtain a 0.067 M solution, use 1.50 mL of the appropriate solvent.

3. Add activated molecular sieves (20 beads/mL) to each amidite bottle, flush with dry argon gas, recap the bottle, seal using parafilm and keep it overnight at room temperature before use.
4. Purchase, or manually fill, a 1 μmole synthesis column with Glen UnySupport.
5. Load the DNA synthesizer with the reagents listed in the Reagents table in the appropriate port according to manufacturer's instructions.
 - a. Load the amidites. Use a spare port for the CleanAmp™ monomer(s).
 - b. Load the ancillary reagents. It is critical that the appropriate capping reagents are used for fast deprotecting monomers. "Cap A" must be the phenoxyacetyl anhydride version of the reagent. DO NOT USE ACETIC ANHYDRIDE.
6. Follow an automated synthesis protocol for fast deprotecting phosphoramidite monomers as recommended by the instrument manufacturer with the following exception: increase the

coupling time for CleanAmp™ phosphoramidites to ten minutes.

7. At the last coupling cycle, leave the DMT group "On".
8. When synthesis is completed, dry the column using argon flow.
9. It is recommended to proceed with the cleavage/deprotection of CleanAmp™ oligonucleotide immediately after completion of the synthesis, otherwise keep the dry column at -20°C.

B. Cleavage/Deprotection of CleanAmp™ oligonucleotides:

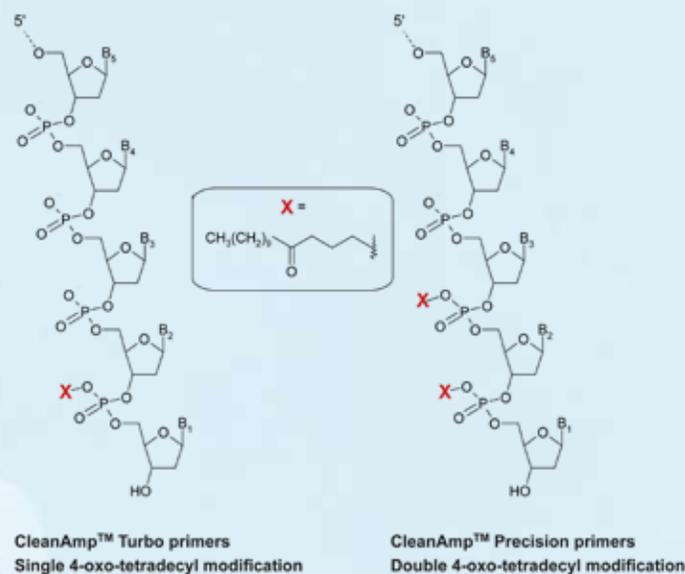
1. Transfer support (with oligonucleotide) from the column to a screw cap 8 mL glass vial.
2. Add 6 mL of 0.05M potassium carbonate (K₂CO₃) in MeOH.
3. Place the vial on a rotary mixer for 20 hrs at room temperature.
4. Let support settle and transfer supernatant solution into a 50 mL conical tube.
5. Wash support with 2 mL of 1 M TEAA and add to the deprotection solution.
6. Measure a total crude yield using UV spectrophotometer.
7. Process the solution immediately using Sep-Pak isolation/purification, otherwise keep the solution at -80°C to -20°C.

C. Sep-Pak® isolation and purification procedure:

1. Fit a 60 mL syringe to the appropriate Sep-Pak cartridge part # 20515.
2. Equilibrate cartridge with 10 mL of acetonitrile, followed by 10 mL of 50% acetonitrile in 0.1M TEAA and finally by 15 mL of 0.025M TEAA.
3. Prepare sample for loading:
 - a. Add 2 mL of 1 M TEAA to the sample and pipette the solution into 5 mL syringe fitted with a luer lock and a 0.44 micron

- filter disc. Filter the solution into a 50 mL conical tube.
- Rinse the deprotection vessel with another 2 mL of water, passing that through the filter and into the tube with the sample also.
 - Dilute the solution to 50 mL with 1 M TEAA.
- Load the solution onto cartridge with a flow rate of 1–2 mL/min using an appropriately sized syringe. Collect and read the absorbance of the flow-through to ensure the oligo is bound to cartridge.
 - Rinse the cartridge with 20 mL of water.
 - Pass 10 mL of 2% TFA through the cartridge over 3 minutes. Observe the appearance of an orange colored band.
 - Immediately rinse the Sep-Pak with 20 mL HPLC water.
 - Pass 10 mL of 1 M TEAA through the cartridge over 5 minutes to neutralize acid.
 - Wash Sep-Pak with a purification buffer:
 - For CleanAmp™ Turbo Oligos, apply 15 mL of 15% ACN, 0.1M TEAA to cartridge. Collect rinse.
 - For CleanAmp™ Precision Oligos, apply 15 mL of 25% ACN, 0.1M TEAA to cartridge. Collect rinse.
 - Rinse cartridge with 20 mL of water. Collect rinse.
 - Elute samples using DMSO.
 - Attach a 3mL syringe to the cartridge.
 - Add 200 μ L DMSO to syringe and elute fraction 1 into a microtube.
 - Remove syringe from cartridge, pull out plunger, reattach the syringe to the cartridge, and push air into same graduated tube to completely remove all the DMSO.
 - Add 200 μ L DMSO to syringe and elute into a fresh microtube (fraction 2) using plunger. Add another 200 μ L to DMSO to syringe and elute into the same graduated tube for a total of 400 μ L DMSO in fraction 2.
 - Add 200 μ L DMSO to syringe and elute into a fresh microtube (fraction 3).
 - Cap, vortex and spin down fractions. Read fraction 2 only. If yield is less than 10 OD₂₆₀ units, read the absorbance of fraction 3. Add just enough of the material in fraction 3 to the material in fraction 2 to achieve 10 OD₂₆₀ units.
 - Determine molar concentration of purified oligonucleotide in DMSO solution using the absorbance reading and the calculated extinction coefficient. If necessary, dilute with DMSO to obtain a 0.2 mM solution.
 - Analyze purified CleanAmp™ oligonucleotides by RP-HPLC to ensure that no more than 1–2% of unmodified primer is in the purified sample.
 - Store the oligonucleotide at 4°C or less. Although it is very stable at room temperature in the DMSO solution, long term storage is improved at lower temperatures.

FIGURE 2: STRUCTURES OF CLEANAMP™ TURBO AND PRECISION PRIMERS



Primer sequences:

forward: 5'-CAGGAGCTGGACTTTACTGATGC

reverse: 5'-CGGGATATCGACATTCTGCACC

HPLC ANALYSIS OF CLEANAMP™ PRIMERS

- Analyze a 0.2 OD₂₆₀ unit sample of the isolated material by reverse phase HPLC to determine the level of contamination by oligonucleotides not protected by the CleanAmp™ moiety.
- Method:
 - Column: Waters μ Bondapak 8 mm RP cartridge (WAT 027324)
 - Buffer A: 100 mM TEAA; Buffer B: ACN
 - Gradient: 0–100% B over 40 minutes
 - Flow Rate: 1 mL/minute
 - Observe at 260 nm.
- Determine the mobility of the unprotected oligonucleotide by heating another 0.2 OD₂₆₀ unit sample for 40 minutes at 95°C, which will completely remove the CleanAmp™ modification from the oligonucleotide.
- Turbo should have less than 1% of the fully deprotected oligonucleotide while Precision should contain less than 2% of that contaminant. Precision should have less than 20% of the singly modified material, which elutes between the unmodified product and the doubly modified Precision oligonucleotide.
- Representative chromatograms of the CleanAmp™ primer sequences shown in Figure 2 are shown in Figures 3 and 4 on the following page.

FIGURE 3: RP HPLC ANALYSIS OF A CLEANAMP™ TORBO PRIMER

Reverse Phase HPLC Analysis:
Forward Primer - CleanAmp™ Turbo (single 4-oxo-tetradecyl modified)

RP3-HPLC : WATERS uBondapak C18, 10 µm, 125 Å, 300x3.9 mm
 Buffer A : 100 mM TEAA pH = 7-7.5 Buffer B : Acetonitrile
 Gradient : 0-100% B over 40 min Flow Rate : 1 mL/min Temperature : RT
 Injection : 30 µL

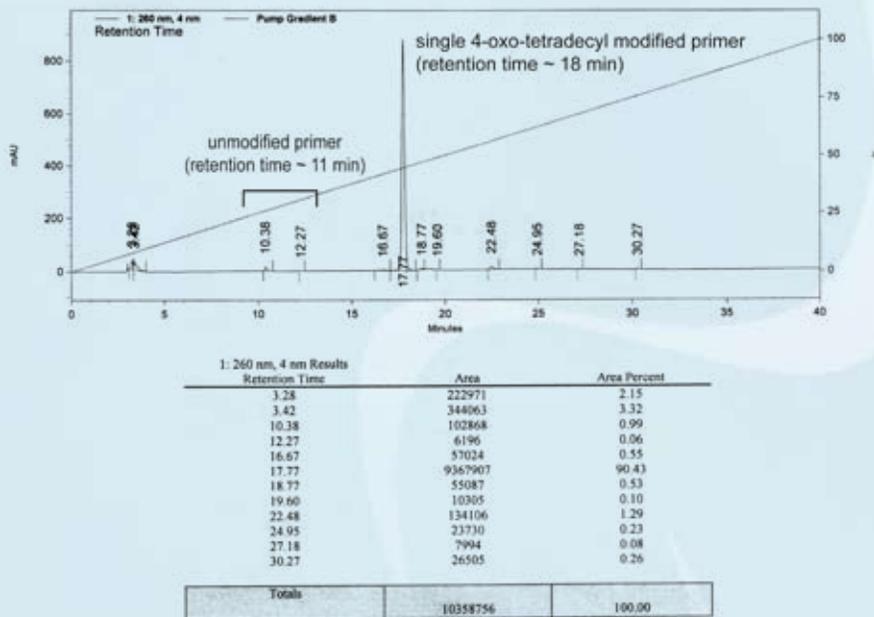
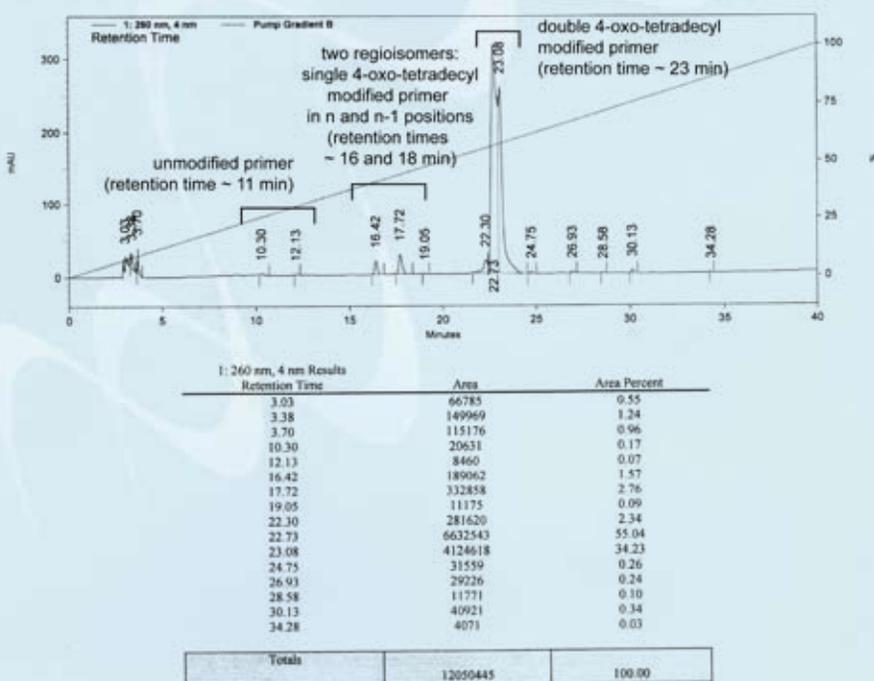


FIGURE 4: RP HPLC ANALYSIS OF A CLEANAMP™ PRECISION PRIMER

Reverse Phase HPLC Analysis:
Forward Primer - CleanAmp™ Precision (double 4-oxo-tetradecyl modified)

RP3-HPLC : WATERS uBondapak C18, 10 µm, 125 Å, 300x3.9 mm
 Buffer A : 100 mM TEAA pH = 7-7.5 Buffer B : Acetonitrile
 Gradient : 0-100% B over 40 min Flow Rate : 1 mL/min Temperature : RT
 Injection : 30 µL



NEW PRODUCT - (5'S)-5',8-CYCLO-DA CE PHOSPHORAMIDITE

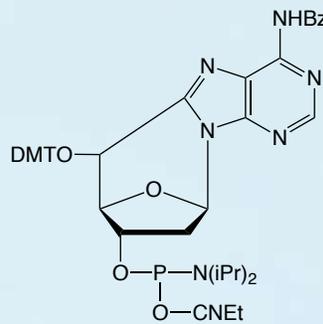
Cellular DNA damage is predominantly due to the action of the short wavelength ultraviolet (UV) component of sunlight on skin cells and the result of oxidation by free radicals generated during routine metabolic activities. Such interactions with individual nucleotides can lead to mutations that are linked to carcinogenesis, neurodegeneration and general aging. These are compelling reasons indeed for investigation.

Nucleotide excision repair (NER) is the principle mechanism for repair of DNA damage induced by sunlight and is also involved in the repair of free radical induced damage. The formation of thymine dimer is the most common DNA lesion from the UV component of sunlight. Thymine dimer phosphoramidite is available from Glen Research, Catalog No.: 11-1330. The NER mechanism is likely to be invoked for repair of lesions that significantly distort the DNA helical structure. The most common studies of oxidative DNA damage have revolved around the damaged nucleosides 8-oxo-dG and thymidine glycol, both of which are available as phosphoramidite derivatives, Catalog Nos.: 10-1028 and 10-1096, respectively. Oxidative DNA damage is more likely to be repaired by base excision repair (BER). Indeed, BER has been shown to be the main repair pathway for the lesions 8-oxo-dG and thymidine glycol.^{1,2}

A further mode of oxidative damage is radiation-induced damage of DNA, which has been shown to lead to bridged cyclonucleosides. The purines, cyclo-dA and cyclo-dG, are predominantly formed, although the cyclo pyrimidines have also been detected. Cyclo-dA is formed when a free radical is induced at the C5' of the deoxyribose sugar by attack by hydroxyl radicals, photolysis or other ionizing processes. The C5' free radical inserts into the C8-N7 double bond of the adjacent adenine residue to form 5',8-cyclo-dA as either a 5'R or 5'S diastereomer. Of the two diastereomers, the (5'S) cyclo-dA appears to be the most cytotoxic.³ Cyclo-dA predominates over cyclo-dG in DNA damage. In a manner analogous to thymine dimer, cyclo purines cause significant distortion of the regular DNA helix and these lesions are repaired not by BER but by NER.^{4,5}

The NER proteins target base lesions which severely distort the helix, such as cyclo-dA and thymidine dimer. In the absence of repair by NER proteins, cyclo-

FIGURE 1: STRUCTURE OF 5',8-CYCLO-DA CE PHOSPHORAMIDITE



5',8-Cyclo-dA

dA is a strong block to both transcription and replication. Interestingly, there are low fidelity polymerases in the Y family that allow the genome to be copied even in the presence of bulky base lesions.⁶ These polymerases are responsible for the build up of tolerance for anticancer cis-platin drugs that crosslink neighboring guanosine residues. Over time, the tumor is enriched in cells that overproduce these polymerases, allowing its survival.⁷ Marietta and Brooks found that cyclo-dA and thymine dimer are strong but incomplete blocks to Pol II transcription in NER-deficient human cell lines.⁸ These transcripts, however, often contained multiple nucleotide deletions. Their results were consistent with the current models of transcription-coupled NER in which Cockayne syndrome proteins are recruited at the Pol II stall site, resulting in nucleotide excision repair of the lesion.⁹

Cyclo-dA is doubly intriguing since it contains both damaged base and damaged sugar residues and, as such, should have a considerable biological impact.¹⁰ It is clear that in-depth analysis of the conformational changes caused by the cyclo-dA lesion and their biological effects requires the availability of Cyclo-dA-CE Phosphoramidite, which we introduce in this article, Catalog No.: 10-1098.

(5'S)-5',8-Cyclo-dA-CE Phosphoramidite, Figure 1, can be used in oligonucleotide synthesis with only minor

changes to the standard protocol. A three minute coupling time for cyclo-dA has been shown to be optimal. However, the subsequent phosphoramidite coupling time has to be increased to six minutes since the 5'-OH once formed is a secondary hydroxyl and is conformationally restricted. Unusually for damaged bases, cleavage and deprotection of oligonucleotides containing cyclo-dA can be achieved using standard procedures.

We are indebted to Didier Gasparutto, CEA - CEN Grenoble, for kindly reviewing and commenting on this article.

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ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
5',8-Cyclo-dA CE Phosphoramidite	10-1098-95	50 μ mole	950.00
	10-1098-90	100 μ mole	1850.00
	10-1098-02	0.25g	5350.00

Introduction

This article is an edited and updated version of the original published in Glen Report 14.1 (2001). Our conclusion at that time was that the Abasic Phosphoramidite that we introduced then offered the best combination of price and functionality for the generation of abasic sites. However, steric hindrance always ensured that low coupling efficiency was observed with this monomer and the subsequent chemistry to form the abasic site was challenging. Consequently, we decided to review the situation.

Background

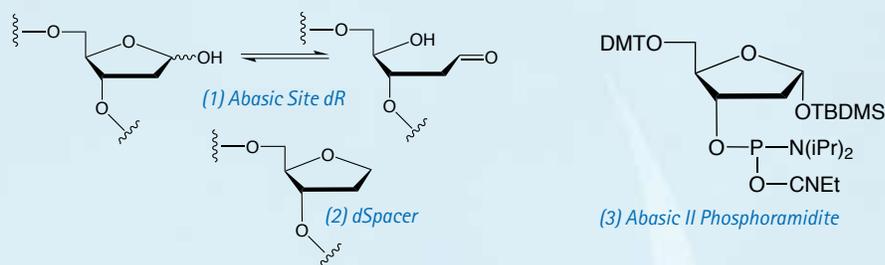
Hydrolysis of nucleoside residues in DNA occurs naturally to generate abasic sites. Most commonly, dA sites are hydrolyzed causing depurination and leading to abasic residues. Although this process is slow under physiological conditions, the reaction is faster at lower pH and especially if the bases are already oxidatively damaged. Damaged bases are also removed enzymatically by the action of DNA N-glycosylases (Base Excision Repair). The abasic residue (dR) exists predominantly in the cyclic form and the structure is shown (1) in Figure 1. The poor stability, especially in basic medium, is caused by the tendency to beta-elimination, leading to chain scission with formation of the 3'-phosphate and 5'-OH.

Because of the instability of the abasic residue, it has not been simple to prepare this variant by chemical synthesis. However, some excellent results have been generated using the stable dSpacer tetrahydrofuran analogue.¹⁻⁴ This variant (2) is missing the 1'-OH of dR and is stable during oligo synthesis, purification and storage.

It is possible to produce the genuine abasic site enzymatically using N-uracil glycosylase to remove uracil base from a 2'-deoxyuridine residue. A potentially very useful chemical method was described by Rayner.⁵ In this method, the abasic site is protected with a photolabile 2-nitrobenzyl group during oligonucleotide synthesis and purification. The 2-nitrobenzyl group is then eliminated by photolysis to produce the abasic site. A similar strategy has been used that incorporates a 2-nitrophenylethyl⁶ photolabile group. As always, there is the concern of thymine dimer formation during photolysis.

Other methods^{7,8} have been used to generate abasic sites but, in all cases to date, the synthesis of the monomer is fairly challenging and, in our opinion, the subsequent chemistry to generate the abasic site is hardly routine.

FIGURE 1: ABASIC STRUCTURES



The chemical method described by Francis Johnson's group⁹ allows the generation of abasic sites in double and single stranded oligonucleotides using very mild specific conditions and with very low probability of side reactions. However, the product exhibits low coupling efficiency due to steric hindrance of the phosphoramidite by the surrounding silyl protecting groups. The post-synthesis chemistry is also fairly challenging.

Christian Leumann's recent photochemical route⁶ prompted us to reexamine a much earlier silyl protected monomer.¹⁰ This product has the advantage of simplicity and the phosphoramidite monomer, Abasic II Phosphoramidite (3) can be prepared at reasonable cost.

Synthesis

The reactivity of Abasic II Phosphoramidite is similar to regular DNA phosphoramidites and a 6 minute coupling reaction gives excellent coupling efficiency. The single silyl protecting group is acid labile but we have shown that it survives 20 synthesis cycles using TCA as the deblocking reagent. The silyl group is removed simply post-synthesis using aqueous acetic acid. The solution is then neutralized with buffer and the oligonucleotide containing abasic sites is desalted on a NAP column in the storage buffer of choice.

Oligonucleotide Stability

With the availability of oligonucleotides containing abasic sites, detailed stability information is now available.⁹ The abasic site is stable almost indefinitely in 0.2M triethylammonium acetate buffer (pH6) at 5°C or less. However, the site is less stable at room temperature (half-life of around 30days) and quite unstable at 55° (half-life

of about 7 hours). Interestingly, the abasic site is largely degraded during evaporation to dryness.

Structural Characteristics

Melting behavior of oligonucleotides containing the abasic site was examined⁹ and it was found to behave like a complete mismatch opposite the 4 natural bases, with characteristics almost identical to those of dSpacer, which has been used extensively as a model abasic site. Other physical characteristics of oligonucleotides containing abasic sites have been examined, as well as their implication in DNA damage and repair.^{11,12} Recently, Leumann has examined the stability of DNA abasic sites with equivalent RNA abasic sites.⁶

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ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
Abasic II Phosphoramidite	10-1927-95	50 µmole	80.00
	10-1927-90	100 µmole	150.00
	10-1927-02	0.25g	475.00

INTRODUCTION

In the previous article in this series on Deprotection (Glen Report 20.2), we focused on the absolute necessity to "Deprotect to Completion" while following our mandate to "Do No Harm". In this article, we focus our attention on RNA Deprotection. Again, this is not a comprehensive review of the topic. Rather, we are attempting to offer a unified deprotection strategy that is simple to follow for newcomers to the mysterious art of RNA synthesis, while producing pure, active RNA oligos with the minimum of fuss. Where appropriate, we will mention other suitable techniques but by reference only. In the meantime, our detailed technical bulletins for RNA deprotection have also been updated and can be found on our web site by following these links:

TBDMS-Protected RNA:
http://www.glenresearch.com/Technical/TB_TBDMS.pdf
 TOM-Protected RNA:
http://www.glenresearch.com/Technical/TB_TOM.pdf

Deprotection of RNA and chimeric DNA/RNA oligonucleotides is unique due to the requirement to retain the 2' protecting group during cleavage, phosphate deprotection, and base deprotection. Only after the oligo is cleaved from the support, the cyanoethyl groups removed from the backbone, and bases fully deprotected, can you complete the 2' deprotection step to yield fully functional RNA. However, we must focus on our mandate - First, Do No Harm.

FIRST, DO NO HARM

As with DNA, the modifiers or dyes present in an RNA oligonucleotide will largely dictate the types of RNA phosphoramidites required and thus, the deprotection conditions. For your consideration, we offer three types of RNA monomers, which we will describe briefly below:

TOM-Protected RNA Phosphoramidites

These monomers exhibit high coupling efficiency and are especially useful in high throughput situations since they perform better in situations where moisture control is not perfect. The high coupling efficiency allows very long RNA oligos (>75mer) to be prepared. These monomers are compatible with high speed deprotection techniques using methylamine.

TBDMS-Protected RNA Phosphoramidites

These are our workhorse monomers with an excellent cost / performance ratio. They are also compatible with high speed deprotection techniques using methylamine.

UltraMild RNA Phosphoramidites

Many minor RNA monomers, modifiers and dyes are not compatible with aggressive deprotection techniques and these UltraMild monomers will allow much milder deprotection conditions.

Any downstream purification requirements will also impact the proper handling of the RNA throughout the deprotection process. For example, DMT-on purification, e.g., Glen-Pak RNA, has become increasingly popular for the purification of RNA oligos, especially siRNA, so all deprotection schemes must leave the DMT group intact to allow purification to take place.

Cleavage

For RNA oligos we do not routinely use a separate cleavage step. By exposing the support to the full deprotection conditions, we feel that maximum yield of product in solution is achieved. Any dissolved silica will be lost in the further deprotection steps required for RNA oligos. Nevertheless, we show the recommended cleavage times for various deprotection solutions for both DNA and RNA supports in Table 1.

Table 1 - RNA and DNA Cleavage

	Temperature	Time
RNA		
AMA	RT	20min.
NH4OH/EtOH (3:1)	RT	2h
DNA		
AMA	RT	10min.
NH4OH/EtOH (3:1)	RT	1h

DEPROTECT TO COMPLETION

Base Deprotection

In this article, we will focus on regular base deprotection using ammonium hydroxide/methylamine (AMA) at elevated temperature, which we have shown to be optimal for both TOM-protected and TBDMS-protected RNA, and UltraMild deprotection using ammonium hydroxide/ethanol (3:1) at room temperature for oligos

VOLUME 2: RNA - DEPROTECT TO COMPLETION

- 1) Do I have very special components in my oligo or not? TOM/TBDMS vs UltraMild
- 2) Do I have one or many oligos to treat? TOM vs TBDMS
- 3) Do I need/want to purify my oligo after deprotection or not? Precipitation vs Glen-Pak

containing base labile groups. It must be stressed that all of these schemes require the use of Ac-protected C monomers.^{1,2} We have previously³ recommended ethanolic methylamine/aqueous methylamine (1:1) (EMAM) for deprotecting TOM-protected RNA. This deprotection scheme is preferred for long RNA oligos but is not necessary for regular RNA oligos. Tables 2 shows the temperatures and times for regular RNA and UltraMild RNA deprotection.

Table 2 - Deprotection Conditions

	Temperature	Time
AMA	65°C	10min.
NH4OH/EtOH (3:1)	RT	4h

After deprotection as above, decant the supernatant liquid from the support and evaporate to dryness. If the DMT protection has been retained for purification purposes, the solution should be evaporated using a stream of nitrogen or compressed air to avoid any loss of the DMT group. *Sterile, RNase-free conditions must be maintained from this point onwards.*

2' Deprotection

In the past, we have described several schemes for removing the silyl protecting groups from the 2'-hydroxyl group. *t*-Butylammonium fluoride in THF (TBAF) has been used extensively for this purpose,⁴ as has neat triethylamine trihydrofluoride (TEA.3HF). TEA.3HF based cocktails have become much more commonly used and are compatible with both precipitation and cartridge-based downstream processing methodologies.⁵⁻⁷ Various additives such as triethylamine (TEA) buffer the neat TEA.3HF used in the original methods, which tended to both remove DMT and depurinate dA sites in chimeric oligos. These cocktails also function well with all three types of RNA monomers available in the Glen Research catalog. In addition, it must be noted that TBAF is not compatible with the Glen-Pak RNA purification process.

Butanol Precipitation

To complete the 2' deprotection for a DMT-off RNA, fully re-dissolve the oligo in anhydrous DMSO. Remember to avoid glass and use a sterile or RNase free, polypropylene, o-ring capped tube for this reaction. If necessary, heat the oligo at 65°C for about 5 minutes to get the oligo fully into solution. Add TEA.3HF, mix well and heat to 65°C for 2.5 hours. Cool the solution and desalt the oligo via butanol precipitation.

DMT-on Purification

The 2' deprotection of DMT-On RNA is slightly different due to the addition of TEA to the cocktail, thus aiding in retention of the DMT. The deprotected RNA is then quenched and immediately purified on a Glen-Pak RNA cartridge.

Fully re-dissolve the RNA in anhydrous DMSO and if necessary, heat the oligo at 65°C for about 5 minutes to get the oligo fully into solution. Add TEA to the DMSO/RNA solution and mix gently. Follow this with TEA.3HF, mix well and heat to 65°C for 2.5 hours. Instead of quenching the cocktail by the addition of butanol, add 1.75mL of RNA Quenching Buffer (60-4120-XX) to the reaction. The sample is now ready for Glen-Pak RNA cartridge purification.

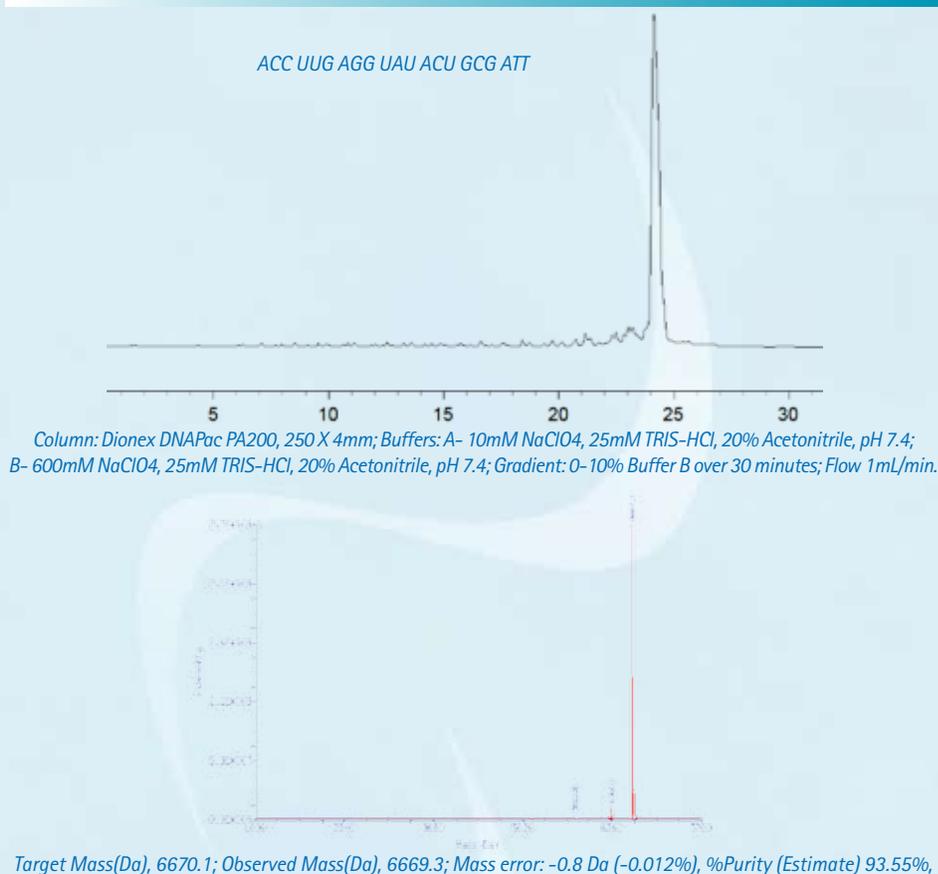
Glen-Pak Purification

Glen-Pak RNA purification cartridges can purify from 40 nmole to 1.0 μmole scale syntheses in one load and are supplied in two formats; one for vacuum manifolds and another for use with a disposable syringe. Glen-Pak purified RNA oligos routinely show purities of between 90 and 95% and yields in the 50 to 80 OD range for a 1.0 micromole synthesis. Once the 2' deprotection solution is quenched, immediately load the 2mL solution on a properly prepared Glen-Pak RNA cartridge and follow the Glen-Pak RNA procedure. At the end of the procedure, the oligo is eluted in 1.0mL RNase Free 1M ammonium bicarbonate/30% Acetonitrile and lyophilized to dryness. Ammonium bicarbonate is a volatile salt, but a second drying step from RNase free water may be required to remove excess bicarbonate.

RNA Analysis

Accurate analysis of Glen-Pak purified RNA by Ion Exchange HPLC using more traditional buffer systems can be hampered

FIGURE 1: ION EXCHANGE HPLC AND ESI MS OF SYNTHESIZED siRNA



by formation of secondary structures. The use of a sodium perchlorate buffer system as well as heat should denature most oligoribonucleotides. Figure 1 shows the Ion Exchange HPLC analysis of a Glen-Pak purified, 21-mer siRNA.

Another way to avoid secondary structure issues and obtain proper identity determination of oligoribonucleotides during purity analysis is Electrospray Mass Spectroscopy (ESI MS). Figure 1 also shows the results of an ESI MS analysis of a Glen-Pak purified 21-mer siRNA.

Further details for both DMT-On and DMT-Off 2' RNA deprotection methods, suggested reagents, Glen-Pak Purification and downstream processing can be found in our Glen Report 19.2 (<http://www.glenresearch.com/GlenReports/GR19-22.html>) and the technical bulletins above.

SUMMARY

RNA synthesis is much more challenging than DNA synthesis but, as these notes indicate, it is not prohibitive. The procedures described above can be used for generating siRNA oligos of high purity on any in-

house synthesizer, as demonstrated by the chromatographic and mass spec data. There is no need to go to a specialist custom oligo service for these oligos. However, the synthesis of RNA oligos >50mer in length remains challenging, but not from the aspect of synthesis, deprotection and purification. The challenge comes from the secondary structure exhibited by RNA, which makes analysis and purity determination very difficult. As interest in modified and labelled RNA oligos continues to increase, we expect to see increasing use of UltraMild techniques.

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NEW PRODUCTS - HIGH LOAD GLEN UNYSUPPORT, CPR II CPG

Glen UnySupport is part of the family of universal supports, first described in 1994¹, where the cleavage from the support is followed by deprotection and dephosphorylation.² Usually in this type of support, dephosphorylation is the slowest step. These universal supports are mostly compatible with deprotection using anhydrous methylamine gas. Glen UnySupport is based on a molecule which is "conformationally preorganized" to accelerate the dephosphorylation reaction.^{3,4} By using a rigid bicyclic molecule on the support (1) in Figure 1, the rate of elimination is markedly faster, as shown in Table 1. It is worth noting that Glen UnySupport is also compatible with UltraMild deprotection using potassium carbonate in methanol.

The phenyl version, developed at Isis Pharmaceuticals as UnyLinker™, is available from several companies for large scale oligo synthesis. Glen UnySupport is the methyl version, which is preferred for high throughput oligonucleotide synthesis since methylamine rather than aniline is formed on deprotection.

In addition to the 500Å CPG, 1000Å CPG and Polystyrene supports introduced in 2008, we now offer Glen UnySupport on a high load CPG suitable for use in larger scale synthesis. Glen UnySupport is sold under license from Isis Pharmaceuticals.

References:

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INTELLECTUAL PROPERTY

This product is covered by US Patent 7,202,264 owned by Isis Pharmaceuticals, Inc..

CPR II CPG

3'-Phosphate CPG, Catalog No.: 20-2900, (1) in Figure 2, has proved to be a popular and successful product for the preparation of oligonucleotide-3'-phosphates. However, the sulfonyl ethyl group in this molecule is susceptible to β -elimination, precluding the ability to

FIGURE 1: UNIVERSAL SUPPORT STRUCTURES

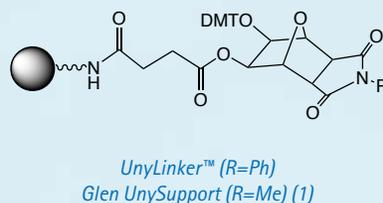


TABLE 1: ELIMINATION CONDITIONS - GLEN UNYSUPPORT (1)

Reagent	Conditions
Ammonium hydroxide	80°C/2h 55°C/8h
Ammonium hydroxide/ 40% Methylamine (AMA)	80°C/0.5h 65°C/1h 55°C/2h
Potassium carbonate in Anhydrous Methanol	RT/17h

FIGURE 2: STRUCTURES OF 3'-PHOSPHATE SUPPORTS

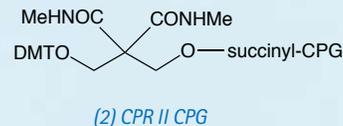
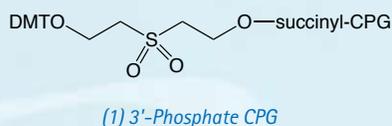
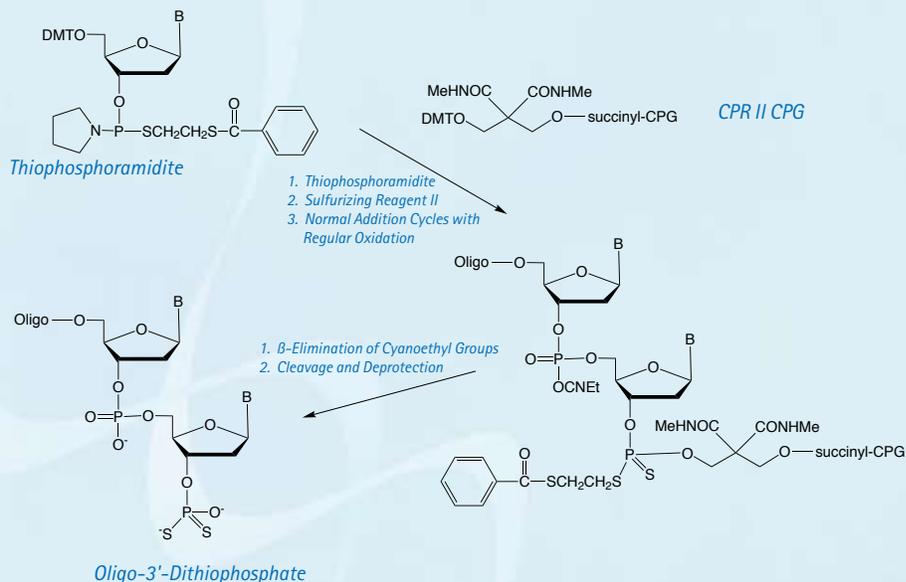


FIGURE 3: USE OF CPR II CPG IN THE PREPARATION OF A 3'-DITHIOPHOSPHATE



conduct base-mediated reactions on the solid support. For example, many researchers treat synthesis supports with a hindered base (e.g., diethylamine, diisopropylethylamine, or DBU) post-synthesis to eliminate and remove the cyanoethyl phosphate groups. In this way, the acrylonitrile formed *in situ* is removed from the support and is not available to alkylate dT residues at the N3 position in the oligos. Since the sulfonyl ethyl group in 3'-Phosphate CPG is also susceptible to β -elimination leading to oligo cleavage, this technique is not compatible with 3'-phosphate CPG.

A more critical example where 3'-phosphate CPG is not compatible is the preparation of 3'-dithiophosphate oligos.

These are formed using a phosphate CPG and a thiophosphoramidite in the first cycle. The dithioate is formed by sulfurization using Sulfurizing Reagent II. The synthesis of the oligo is then completed using regular cyanoethyl phosphoramidites. Sulfur is much more susceptible to alkylation by acrylonitrile and regular deprotection would lead to significant alkylation of the 3'-dithioate. Using CPR II CPG, (2) in Figure 2, which is base labile but does not support β -elimination, the cyanoethyl groups can be removed from the oligo prior to cleavage and base deprotection. This process is illustrated in Figure 3.

We are happy to offer CPR II CPG, Catalog No.: 20-2903, to complement our offering of 3'-Phosphate CPG.

NEW PRODUCTS – CLICK CHEMISTRY, DMF-DG-5'-CE PHOSPHoramidite

Click chemistry has gained such great fame that it now has its own entry in Wikipedia. This conjugation technique using 1,3-dipolar cyclo addition between an azide and a terminal alkyne was first discovered by Rolf Huisgen's group at the University of Munich in the late 50's.¹ Later, the term "Click Chemistry" was introduced for the simple, efficient labeling of biomolecules such as DNA, RNA or peptides by Sharpless, whose significant contribution was the addition of copper iodide as a catalyst to dramatically increase the rate of cycloaddition of the azide to the alkyne.² In general terms, this cycloaddition can be summarized as shown in Figure 1.

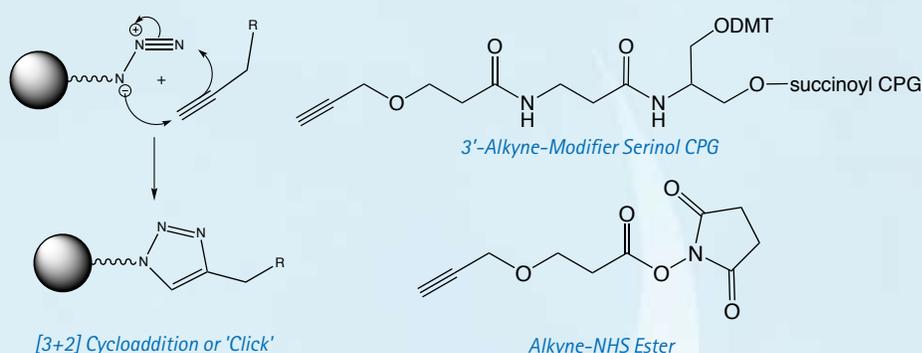
In previous Glen Reports [GR19.1 (2007) and GR20.1 (2008)], Glen Research introduced 5'-Hexynyl Phosphoramidite [10-1908], Azidobutyrate NHS Ester [50-1904], and 5'-Bromohexyl Phosphoramidite [10-1946], for use in cycloaddition reactions. With this brief article, we introduce a new set of products for this application.

NHS esters are common and robust reagents for labeling DNA, RNA and other biomolecules. The popularity of our Azidobutyrate-NHS ester has demonstrated this fact yet again. Consequently, we now introduce Alkyne NHS ester [50-1905] to allow the functionalization of an amino moiety in a variety of molecules, including DNA and RNA oligonucleotides as well as peptides or proteins. In DNA or RNA this could be at the 5'-terminus, 3'-terminus, or intramolecular for oligos modified using any of our amino modifiers. This allows researchers to use a variety of products from the Click Chemistry library that are capable of reacting with NHS esters.

After customer requests to offer additional choices for Click Chemistry, we are pleased to offer a synthesis support for labelling the 3' terminus of oligonucleotides with an alkyne group for use in Click Chemistry. This builds upon our 1,3-diol product portfolio with the serinol backbone introduced in our Glen Report in 2008 (GR20.2).

We expect that these additions to our Click product portfolio will become popular tools. Our commitment to Click Chemistry remains strong and we look forward to further additions in future Glen Reports.

FIGURE 1: STRUCTURE OF NEW PRODUCTS FOR CLICK CHEMISTRY



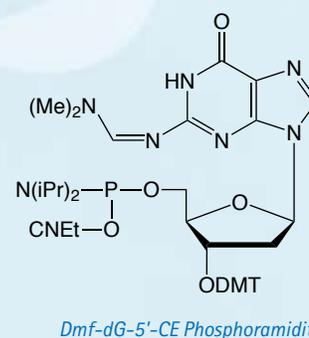
References:

1. R. Huisgen, *Angew Chem Int Ed*, 1963, **2**, 565-598.
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DMF-DG-5'-CE PHOSPHoramidite

We are supplementing our range of products for 5'→3' synthesis by adding dmf-dG-5'-CE Phosphoramidite. Using the dimethylformamide (dmf) protecting group is a clear improvement over the use of isobutryl (ibu) for dG since dmf is removed around 4X as quickly as ibu. This is especially important for the synthesis of oligos

FIGURE 2: STRUCTURE OF DMF-DG-5' AMIDITE



containing labile minor bases or fluorescent tags. Dmf-dG-5'-CE Phosphoramidite (10-9201) is offered at the same price as the original ibu-dG product.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
High Load Glen UnySupport	25-5040-01	0.1g	15.00
	25-5040-02	0.25g	30.00
	25-5040-10	1.0g	115.00
3'-CPR II CPG	20-2903-01	0.1g	70.00
	20-2903-10	1.0g	480.00
	20-2903-42	Pack of 4	60.00
	20-2903-41	Pack of 4	100.00
	20-2903-13	Pack of 1	180.00
	20-2903-14	Pack of 1	280.00
Alkyne-NHS Ester (Dissolve 2.3mg in 60µL of DMSO)	50-1905-23	2.3mg	60.00
	50-1905-24	23mg	300.00
3'-Alkyne-Modifier Serinol CPG	20-2992-01	0.1g	105.00
	20-2992-10	1.0g	800.00
	20-2992-42	Pack of 4	100.00
	20-2992-41	Pack of 4	175.00
	20-2992-13	Pack of 1	260.00
	20-2992-14	Pack of 1	390.00
Dmf-dG-5'-CE Phosphoramidite	10-9201-02	0.25g	75.00
	10-9201-05	0.5g	150.00
	10-9201-10	1.0g	300.00

Chemical modification of the backbone of synthetic oligonucleotides is commonly used for a variety of reasons: to increase the stability of the phosphodiester bond; to adjust duplex stability; to change the conformation of DNA or RNA; or to increase cellular uptake. Some of these modifications include phosphorothioates (Glen Report 18.1, and supplement in 2006), phosphorodithioates (Glen Report 20.1, 2008), and phosphonoacetates (Glen Report 20.2, 2008). Modification of the sugar moiety is commonly used for increasing nuclease resistance or increasing affinity of the oligo to its complementary target. The number of sugar modifications is increasing with the availability of more commercial monomers with non-natural sugars. The first commercially available sugar modification [introduced in the Glen catalogue in 1991] was the 2'-OMe analogue¹, which improves the stability of DNA and RNA to nuclease degradation and increases the T_m of the target duplex.

More recently, locked nucleic acid (LNA) – also named bridged nucleic acid (BNA) in Japan – became quite popular for introducing modifications, for example, in probes and siRNA.^{2,3,4,5} Unfortunately, Glen Research is no longer able to offer LNA monomers and they are now only available directly from Exiqon.

A search for a suitable alternative that could offer some of the same properties as the LNA monomers led us to consider the currently available 2'-fluoro RNA monomers. First of all, the introduction of 2'-F-RNA residues into synthetic oligonucleotides increases the T_m of the duplex by about 1 to 2 °C per incorporation.⁶ Also oligonucleotides containing 2'-F residues are resistant to enzymatic degradation⁷ and siRNAs modified with 2'-F RNA are more resistant to enzymatic degradation than unmodified siRNAs, both *in vitro* and *in vivo*.⁸ It is also known⁹ that 2'-F nucleotides prefer a C3'-endo/north conformation while LNA nucleotides contain a covalent linkage that restricts pseudorotation of the ribose to the C3'-endo conformation.^{4,10}

Oligonucleotides containing 2'-F RNA residues have been used as antisense^{11,12} and as siRNA^{8,13} oligonucleotides. RNA oligonucleotides can even be completely substituted with alternating 2' modifications (2'-OMe and 2'-F) and retain the ability to silence gene expression in human cells,

even exhibiting up to a 500 fold increase in potency relative to native siRNA.¹⁴

Also 2'-F nucleotides proved to be active and beneficial in aptamers¹⁵ as well as in antagomirs¹⁶. These synthetic oligos are able to silence micro-RNAs *in vitro* and *in vivo*.

One of the biggest advantages of 2'-F RNA is that the monomers are commercially available at a significant savings over LNA monomers. We are delighted to be able to offer 2'-F-RNA monomers at prices which have been significantly lowered.

While LNA monomers and oligos are protected by several patents filed by Danish and Japanese groups, our customers should also be aware that a license may be required from Isis Pharmaceuticals, Inc. to incorporate 2'-F modified nucleotides into oligonucleotides as claimed in US Patent Numbers 5,670,633; 6,005,087; 6,531,584 and foreign equivalents.

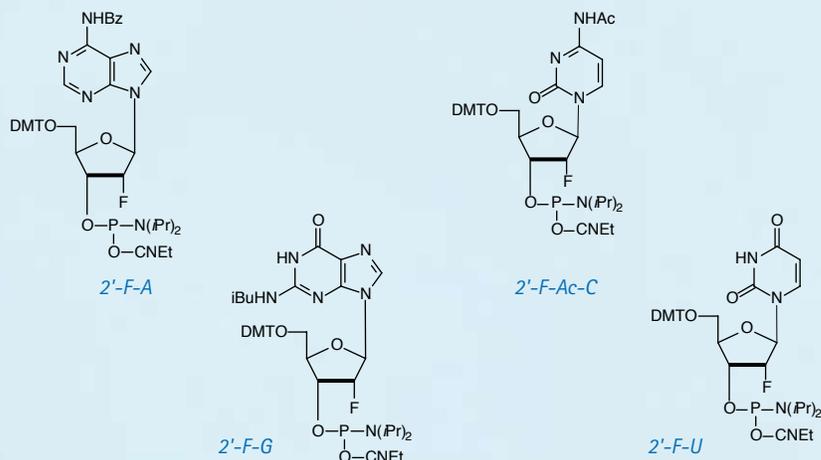
References:

1. B.S. Sproat, A.I. Lamond, B. Beijer, P. Neuner, and U. Ryder, *Nucleic Acids Res.*, 1989, **17**, 3373.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
2'-F-A-CE Phosphoramidite	10-3400-90	100 µmole	50.00
	10-3400-02	0.25g	125.00
2'-F-Ac-C-CE Phosphoramidite	10-3415-02	0.25g	50.00
	10-3415-05	0.5g	100.00
2'-F-G-CE Phosphoramidite	10-3420-90	100 µmole	50.00
	10-3420-02	0.25g	125.00
2'-F-U-CE Phosphoramidite	10-3430-02	0.25g	50.00
	10-3430-05	0.5g	100.00

FIGURE 1: STRUCTURES OF 2'-F-RNA MONOMERS



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TECHNICAL BRIEF – AVOIDING TRITYL LOSS WHEN PROCESSING SYNTHETIC OLIGONUCLEOTIDES

A major issue with the processing of oligonucleotides, especially for large-scale synthesis, is the loss of the 5'-trityl group when drying down an oligonucleotide in preparation for a trityl-on purification or down-stream processing. Even on a small scale, oligonucleotides can lose 5'-trityl protection upon drying – but the amount of trityl loss can be variable.

Addition of Tris Base to Amino-Modifiers

This trityl loss can be especially prominent for trityl-protected amino-modifiers, such as 5'-Amino-Modifier C6 (10-1906) and the latest version, 5'-DMS(O)MT Amino-Modifier C6 (10-1907). The chromatograms for a 5'-DMS(O)MT Amino-Modified oligo before and after drying, shown in Figure 1, demonstrate the complete loss of the trityl protecting group on drying.

One observation we noted was that drying for extended periods of time, or the use of very high vacuum when drying down the oligonucleotides, resulted in greater amounts of trityl loss. This led us to the hypothesis that high vacuum was driving the equilibrium from a protonated volatile base as a counterion toward the nucleic acid with loss of either ammonia or methylamine gas, as shown in Figure 2.

If this model is indeed correct, a simple test would be to dry the oligo down in the presence of varying amounts of a non-volatile base such as Tris. The results of these experiments, shown in Figure 3, indicate that the addition of Tris prevented the loss of the 5'-trityl in a concentration-dependent manner. Addition of 45mg of Tris base/mL of crude oligo solution prevented any loss of the trityl group.

Thermal Stability of Trityl-Amino-Modifiers

The data noted above prompted us to revisit our recommended deprotection conditions for both the 5'-DMS(O)MT Amino-Modifier C6 and the MMT-protected 5'-Amino-Modifier C6. For these products, we have always recommended deprotection at 40°C or lower to avoid reported thermally-induced trityl loss. We deprotected trityl-amino-modified oligos in three ways: room temperature ammonium hydroxide, ammonium hydroxide for 17 hr at 55°C, and finally deprotection in AMA at 65°C for 10 minutes. HPLC analysis revealed that there was no indication of any loss

FIGURE 1: RP HPLC OF OLIGOS

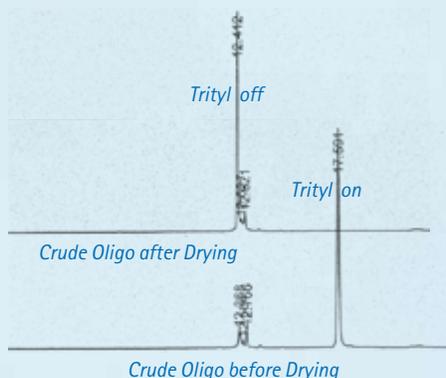


FIGURE 2: DETRITYLATION ON DRYING

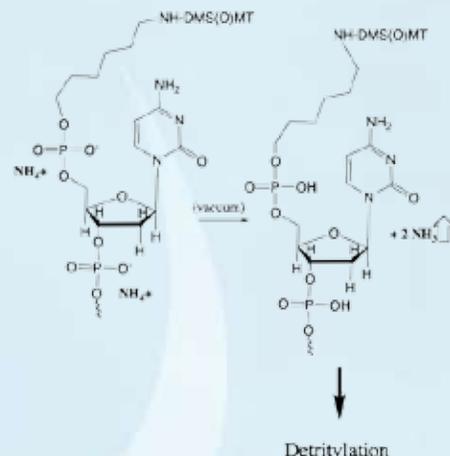
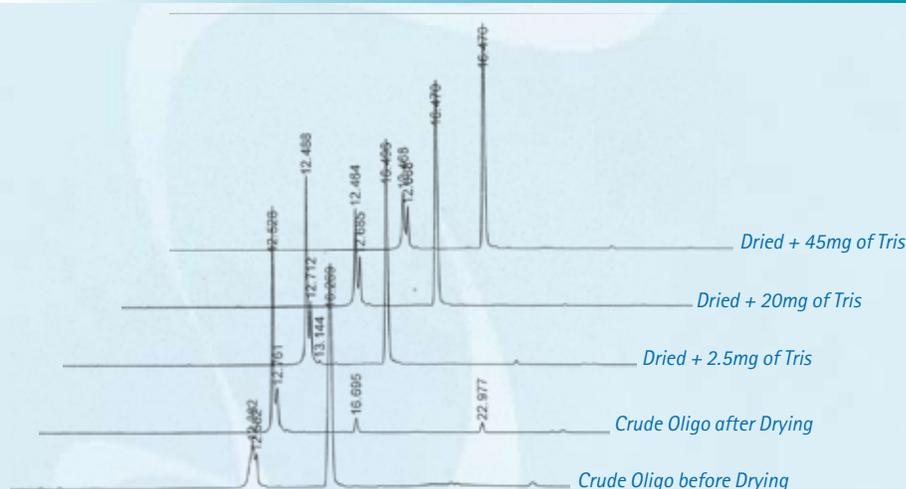


FIGURE 3: EFFECT OF ADDING TRIS BASE TO PREVENT TRITYL LOSS DURING CONCENTRATION



of the trityl protecting group during any of the deprotection conditions at elevated temperatures. We are now able to remove the recommendation that these amino-modified oligos should be deprotected at 40°C or lower.

Salt-Free Retention of Trityl During Drying

The addition of large amounts of salt can be problematic for downstream applications. A good example of this would be the synthesis of a 5'-amino-modified RNA in which the 5'-trityl is maintained for trityl-on purification. While the addition of the Tris would maintain the trityl, its presence would severely inhibit the removal of the 2'-silyl protecting groups in either TEA.3HF or TBAF. Therefore, we tested the possibility of converting the oligonucleotide to the nonvolatile sodium salt to maintain the trityl upon drying. The following desalting protocol was used on the model oligo 5'-DMS(O)MT Amino-Modifier T12:

- 1) Deprotect the oligo in NH_4OH or AMA.
- 2) Add 45 mg Tris base/mL of deprotection solution.
- 3) Desalt on a Glen-Pak DNA cartridge.
 - i) Condition cartridge with 0.5 mL ACN, followed by 1 mL 2 M TEAA.
 - ii) Dilute with 1 mL water and load oligo drop-wise onto Glen-Pak DNA Cartridge.
 - iii) Rinse with 2 mL distilled water.
 - iv) Rinse 2x with 2 mL 0.5 M aqueous sodium hydroxide.
 - v) Rinse with 2 mL distilled water.
 - vi) Elute with 1 mL 75% ACN/water and dry down.

HPLC analysis revealed that the crude amino-modified oligo without drying, the Glen-Pak purified oligo converted to the sodium salt prior to drying, and finally the eluted and dried oligo were identical in purity and that the trityl group was maintained throughout the process.

FLUORESCIN DERIVATIVES

A triumvirate of fluorescein derivatives has dominated fluorescent labelling of oligonucleotides over the last decade. Fluorescein, hexachlorofluorescein and tetrachlorofluorescein, better known as FAM, HEX and TET, have been in the forefront of DNA analysis in the fields of sequencing and genetic analysis using instruments with multicolor detection. However, HEX has proved to be the weakest performer of the three due to its instability to the basic conditions of oligonucleotide deprotection. HEX-labelled oligonucleotides must be treated with care to avoid some level of degradation. Moreover, the mechanism of degradation involves the loss of chlorine residues from the ring systems leading to products that are fluorescent at lower wavelengths, even covering the wavelength of TET.

In the previous Glen Report, we introduced a new fluorescein phosphoramidite, 5'- dichloro-dimethoxy fluorescein, better known as JOE™ in the world of multicolor sequencing. This fluorescein derivative exhibits excellent stability to a range of deprotection conditions. In addition, its fluorescence is much less pH sensitive than fluorescein itself.

Now, we are happy to introduce further fluorescein-based products: SIMA (HEX) (1) and SIMA (HEX)-dT (2) in Figure 1, which exhibit virtually identical absorbance and emission spectra as HEX.

SIMA (HEX) Deprotection and Stability

Dichloro-diphenyl-fluorescein, SIMA (HEX), is much more stable to basic deprotection conditions than HEX and oligonucleotides can be deprotected using ammonium hydroxide at elevated temperatures and even ammonium hydroxide/methylamine (AMA) at room temperature or 65°C for 10 minutes. The increased stability of SIMA (HEX) over HEX is illustrated in Figure 2. The HEX-labelled oligo deprotected with ammonium hydroxide at 55°C overnight shows considerable degradation, while the SIMA (HEX) equivalent shows no decomposition. Deprotection with AMA at 65°C for 10 minutes led to around 10% decomposition of HEX while SIMA (HEX) showed no signs of degradation. Similar levels of degradation of HEX

FIGURE 1: STRUCTURES OF SIMA (HEX) AND SIMA-DT PHOSPHoramidites

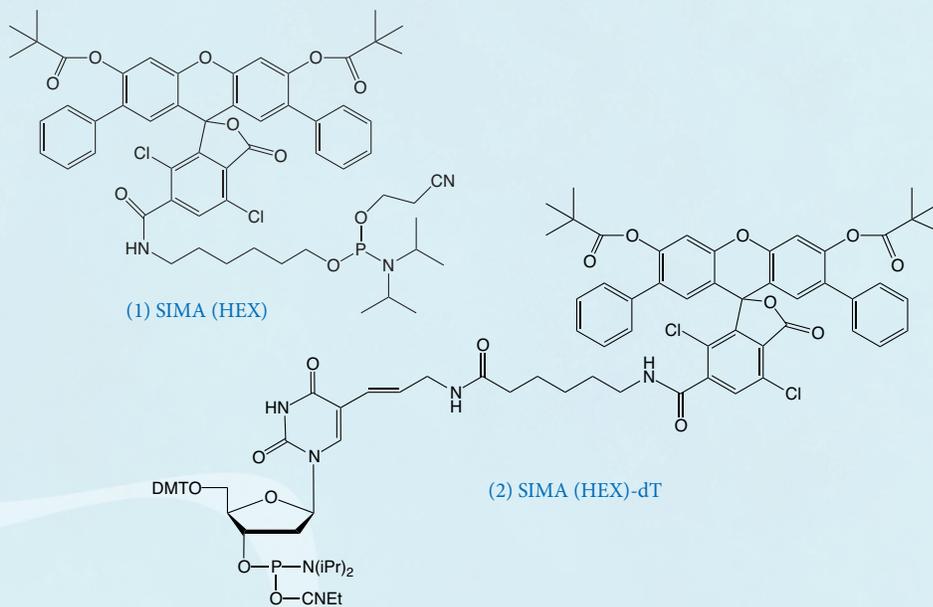


FIGURE 2: HPLC OF HEX AND SIMA (HEX) OLIGOS DEPROTECTED AT ROOM TEMP AND 55°C OVERNIGHT

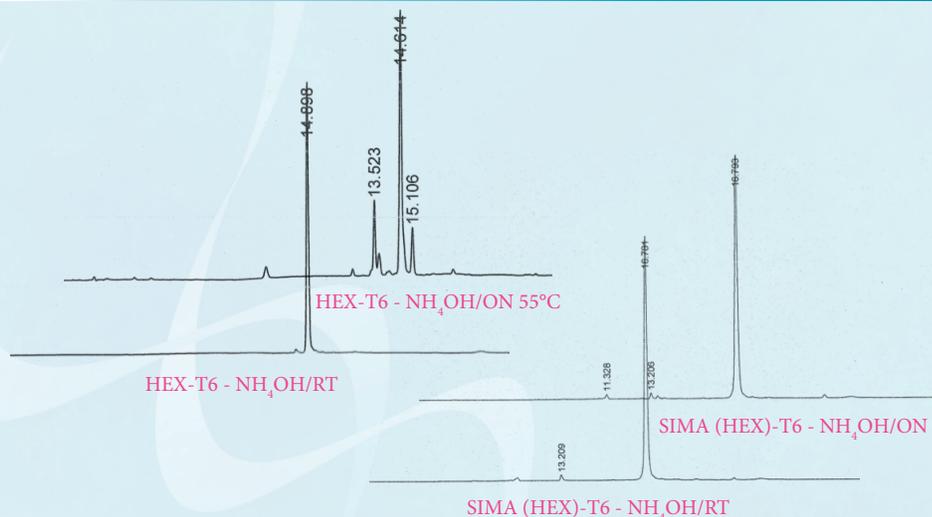
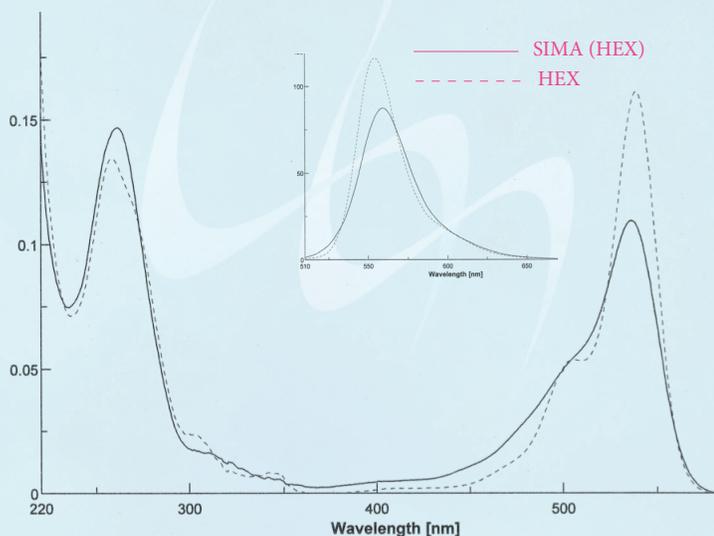


FIGURE 3: ABSORPTION AND EMISSION (INSET) SPECTRA OF SIMA (HEX)-T6 AND HEX-T6



and stability of SIMA (HEX) were found during deprotection with AMA at room temperature for 2 hours.

SIMA (HEX) Spectral Characteristics

SIMA (HEX) exhibits absorption and emission maxima that are virtually identical to HEX, as shown in Figure 3. SIMA absorption maximum was 3 nm blue-shifted compared to HEX at pH 7. The absorbance is broader, so the extinction coefficient is smaller than that of HEX, but when exciting at 500 nm where the absorbance was normalized, the emission was still 90% of HEX and the emission was red-shifted by 5 nm.

The extinction coefficients of SIMA (HEX) determined at pH 7.0 are:

E260: 34,000

E536: 64,000

As an added bonus, SIMA (HEX) can be offered at significantly lower prices than HEX itself.

A second SIMA (HEX) product, SIMA (HEX)-dT, can be used to introduce SIMA (HEX) in the synthetic oligonucleotide sequence, usually as a replacement for the native dT linkage. Again, this product is fully compatible with deprotection schemes using ammonium hydroxide at elevated temperatures or AMA at room temperature and 65°C.

CYANINE DERIVATIVES

Cyanine dyes are highly fluorescent molecules that have become very popular in biological imaging. Several versions of cyanine dyes are available commercially with the most popular being the CyDye™ series from GE Healthcare. Cy3, Cy3.5, Cy5 and Cy5.5 phosphoramidites are already offered for sale by Glen Research under license from GE Healthcare. Commercial applications for Cy-labelled oligonucleotides are also covered by GE Healthcare's suite of patents. Consequently, a rapidly expanding market segment has opened up for generic, unpatented cyanine dyes that do not have the constraints of the licensing and royalty fees associated with Cy dyes.

DyLight™ DY547 and DY647

We are happy to begin offering two DyLight™ dyes, DY547 and DY647, as phosphoramidite alternatives to Cy3 and Cy5. The structures of these two products

FIGURE 4: STRUCTURES OF DYLIGHT DY547 AND DY647 PHOSPHORAMIDITES

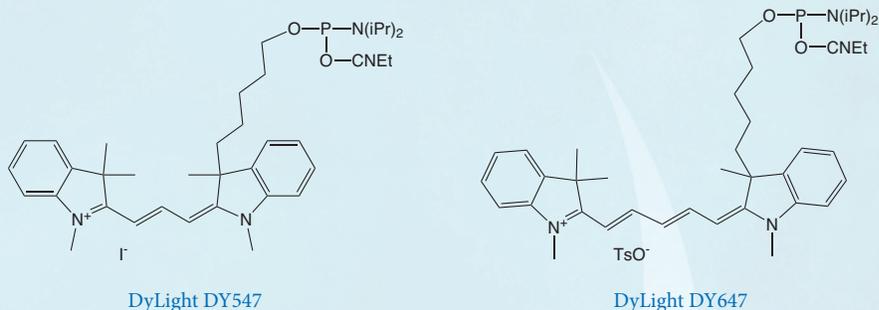


FIGURE 5: COMPARISON OF ABSORPTION SPECTRA OF CY3 AND DY547 AND CY5 AND DY647 OLIGOS

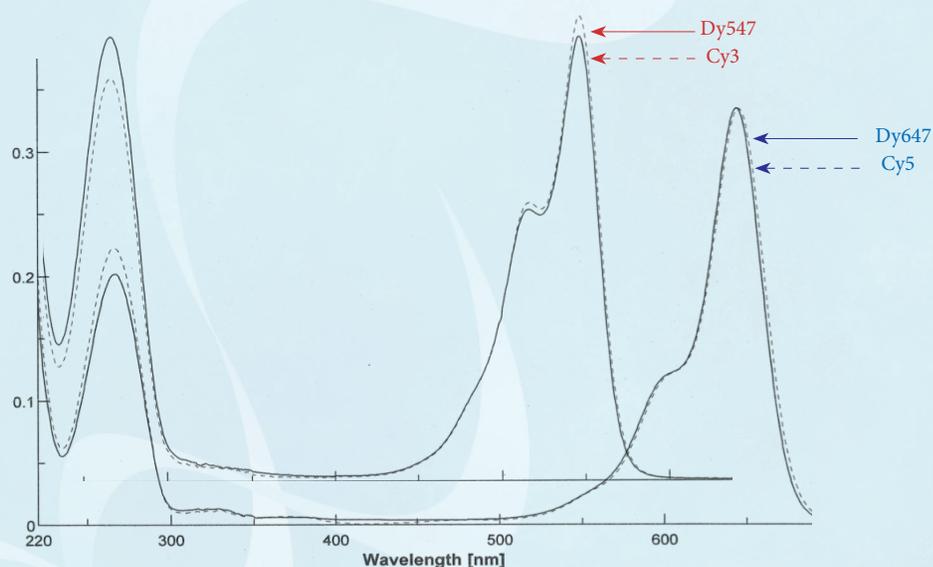
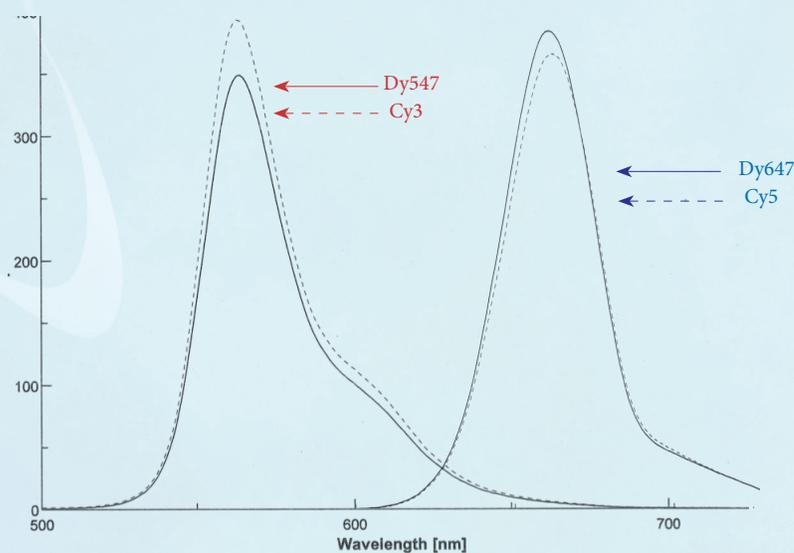


FIGURE 6: COMPARISON OF EMISSION SPECTRA OF CY3 AND DY547 AND CY5 AND DY647 OLIGOS



(Continued from Page 19)

are shown in Figure 4. The performance of these two dyes is similar to the equivalent Cy dyes and the absorption and emission spectra are virtually identical. This is demonstrated in Figure 5, which shows the absorption spectra of Cy3 and DY547 oligos and the equivalent spectra for Cy5 and DY 647. Similarly, Figure 6 shows the fluorescence emission spectra of Cy3 and DY547 oligos and the equivalent spectra for Cy5 and DY 647.

DY547 and DY647 Spectral Characteristics

A comparison of the emission or absorbance spectra of the oligos shown in Figures 5 and 6 demonstrates that the spectra are virtually identical for Cy3 and DY547. Similarly, Cy5 and DY 647 were virtually identical. When oligos containing Dy547 were excited at 488 nm, the quantum yield (QY) was 12% greater than oligos containing Cy3. When oligos containing Dy647 were excited at 580 nm, the QY was 5% greater and the emission was blue-shifted by only 1 nm compared to Cy5.

INTELLECTUAL PROPERTY

SIMA (HEX) and the DyLight™ dyes, DY547 and DY647, are not covered by patents and can be used for any purpose, including use by oligo synthesis companies and in vitro diagnostic development

companies without need for a separate license.

DyLight is a trademark of Thermo Fisher Scientific Inc. and its subsidiaries. CyDye is a trademark of GE Healthcare.

ORDERING INFORMATION

<i>Item</i>	<i>Catalog No.</i>	<i>Pack</i>	<i>Price(\$)</i>
SIMA (HEX) Phosphoramidite	10-5905-95	50 µmole	90.00
	10-5905-90	100 µmole	175.00
	10-5905-02	0.25g	400.00
SIMA (HEX)-dT Phosphoramidite	10-5945-95	50 µmole	345.00
	10-5945-90	100 µmole	675.00
	10-5945-02	0.25g	995.00
DyLight DY547 Phosphoramidite	10-5917-95	50 µmole	175.00
	10-5917-90	100 µmole	335.00
	10-5917-02	0.25g	995.00
DyLight DY647 Phosphoramidite	10-5918-95	50 µmole	175.00
	10-5918-90	100 µmole	335.00
	10-5918-02	0.25g	995.00



GLEN RESEARCH

**22825 DAVIS DRIVE
STERLING, VIRGINIA
20164**

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