GLEN RESEARCH

part of **Maravai LifeSciences**

Deprotection Guide

Deprotect to Completion

Contents

Introduction

This guide offers practical information that will help newcomers to the field of oligo synthesis to understand the various considerations before choosing the optimal deprotection strategy, as well as the variety of options that are available for deprotection. It is not the intent of this guide to provide a comprehensive, fully referenced review of deprotection strategies in oligonucleotide synthesis - they are simply guidelines. For more detailed information, see, for example, the review[1] by Beaucage and Iyer.

Oligo deprotection can be visualized in three parts: cleavage, phosphate deprotection, and base deprotection. Cleavage is removal from the support. Phosphate deprotection is the removal of the cyanoethyl protecting groups from the phosphate backbone. Base deprotection is the removal of the protecting groups on the bases or modifier. There are many considerations when approaching oligo deprotection. However, when reviewing the procedures available to deprotect any oligonucleotide, you must heed the primary consideration: *First, Do No Harm*. You can then proceed with confidence to Deprotect to Completion.

Key Considerations – Deprotection to Completion

1. Do I have very special components in my oligo or not?
2. Am I in a rush or not?
3. Do I have one or many oligos to treat?
4. Do I need/want to purify my oligo after deprotection or not?
5. Does my oligo contain RNA, 2'-OMe-RNA, or 2'-F-RNA linkages?

First, Do No Harm!

Determination of the appropriate deprotection scheme should start with a review of the components of the oligonucleotide to determine if any group is sensitive to base and requires a mild deprotection or if there are any pretreatment requirements. Sensitive components are usually expensive components so it is imperative to follow the procedure we recommend for any individual component. For example, the presence of a dye like TAMRA or HEX will require a different procedure from regular unmodified oligonucleotides. Similarly, an oligo containing a base-labile monomer like 5,6-dihydro-dT will have to be treated according to the procedure that is noted on the product insert (Analytical Report, Certificate of Analysis, or Technical Bulletin). Occasionally, some products require a special pretreatment to prevent unwanted side reactions. For example, amino modifiers use a special diethylamine pretreatment to improve the overall yield of the amino-labelled oligo. If the oligo has several unusual components, you must follow the mildest procedure recommended and, yes, things can get complicated fast.

RNA deprotection is unique because of the necessity to retain the 2' protecting group during cleavage and base deprotection. 2'-OMe-RNA and 2'-F-RNA, however, are virtually identical to DNA during deprotection. But, if a hybrid oligonucleotide contains even a single RNA linkage (with the exception of a 3'-ribonucleoside linkage), the oligo must be treated as RNA. See the appropriate RNA deprotection protocols:

https://www.glenresearch.com/reports/gr19-22

https://www.glenresearch.com/media/productattach/import/application_guides/tb_rna_tom_deprotection_20191015.pdf

Another consideration for potential harm is loss of trityl group during vacuum concentration of the oligo solution prior to purification, which will reduce product yield. During evaporation the heat should be turned off the vacuum concentrator to avoid loss of the DMT group. It should be noted that most DMT-on purification protocols, including Poly-Pak™

and Glen-Pak™, do not require evaporation of the deprotection solution prior to purification.

A unique case for potential harm is an oligonucleotide containing a 5'-amine protected with the MMT protecting group (e.g., 10-1906). In this situation, deprotection should not be carried out at > 37 °C to avoid thermal loss of the MMT group.

Cleavage

On classic synthesizers from Applied Biosystems, the cleavage of the oligo from the synthesis support can be carried out separately on the machine, prior to deprotection. As a result, many researchers still carry out the cleavage reaction separately and so the time required to do this is mentioned at the beginning of each deprotection section. However, most researchers do a one step cleavage/deprotection reaction, which has the advantage of ensuring optimal yields. The only downside to this strategy is the fact that the basic solution at elevated temperatures will dissolve a small amount of silica from CPG and a white insoluble residue will be apparent if the deprotection solution is evaporated to dryness. However, any residual silicate is easily removed by filtration, desalting or any purification procedure.

Deprotect to Completion

The rate-determining step in oligonucleotide synthesis is more than likely the removal of the protecting group on the G base. Ignore this at your peril since, traditionally, one of the most common reasons for poor performance of oligonucleotides is the presence of a small percentage of the G protecting groups remaining in the final product oligonucleotide. Chromatographic methods may miss the presence of the G protecting groups but these are readily revealed by mass spectral analysis. What are the options with attendant pros and cons for oligonucleotide deprotection?

Regular Deprotection

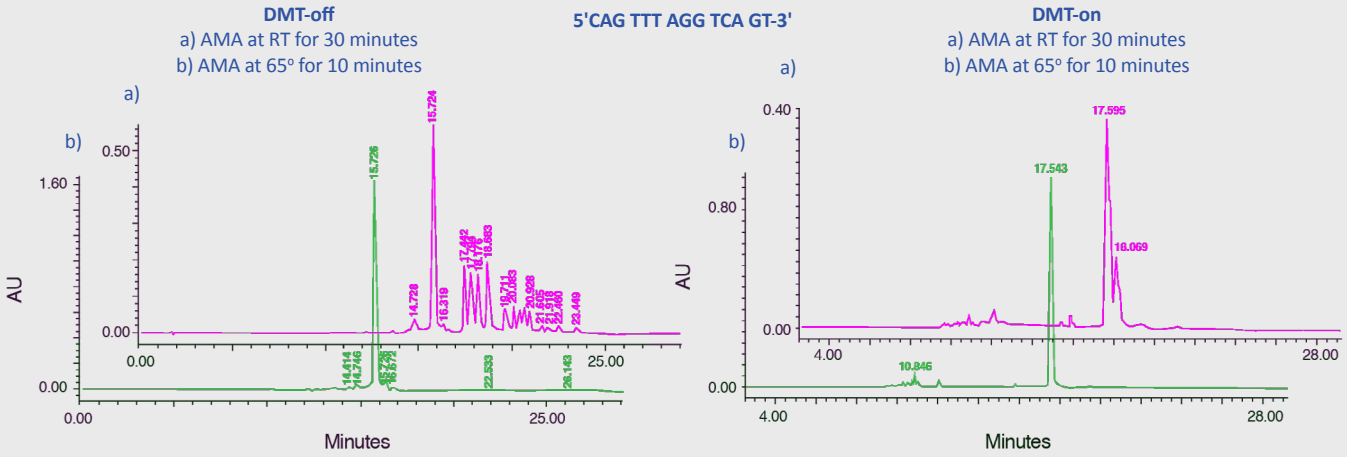
The cleavage reaction with concentrated ammonium hydroxide (28% to 33% NH_3 in water), if carried out separately, is normally considered to be 1 hour at room temperature. Deprotection using ammonium hydroxide is the most traditional method and dates back to the earliest days of oligonucleotide synthesis. One of the critical issues when using ammonium hydroxide, which is water saturated with ammonia gas, is to keep the solution fresh. We aliquot and store ammonium hydroxide in the refrigerator in portions appropriate for use in 1 week. Using an old bottle of ammonium hydroxide is false economy since the resulting oligos are not going to be completely deprotected.

Table 6, Page 9 shows the various times and temperatures appropriate for deprotection with FRESH ammonium hydroxide.

Table 1

dG Protection	Temperature	Time
iBu-dG	RT	36 h
	55 °C	16 h
	65 °C	8 h
dmf-dG , Ac-dG	RT	16 h
	55 °C	4 h
	65 °C	2 h
iPr-Pac-dG	RT	2 h
	55 °C	0.5 h

Figure 1: RP HPLC of an Oligo Partially and Fully Deprotected, DMT-off and DMT-on



Deprotection Conditions
Partial Deprotection: AMA at RT for 30 min.
Full Deprotection: AMA at 65° C for 10 min.
RP HPLC Conditions
Column: Waters X-Bridge C18, 250 x 4mm; Buffers: A - ACN; B - 0.1M TEAA , pH 7.
DMT-off Gradient: 3-15% B over 15 min. DMT-on Gradient: 3-40% B over 15 min. Flow rate: 1mL/min.

UltraFAST Deprotection

Using the UltraFAST procedure, cleavage of the oligonucleotide from the support is performed using AMA[2] which is a 1:1 mixture (v/v) of aqueous ammonium hydroxide and aqueous methylamine. If carried out separately, it is accomplished in 5 minutes at room temperature.

UltraFAST deprotection allows 5-10 minute deprotection of oligonucleotides using AMA. It is important to note that the UltraFAST system requires acetyl (Ac) protected dC to avoid base modification at the C base if Bz-dC is used. The three other monomers remain unchanged and the system works equally well with iBu-, Ac-, or dmf-dG, the last being our preferred dG phosphoramidite.

The deprotection step is carried out at 65 °C for 5 minutes. Deprotection can also be carried out at lower temperatures as shown in Table 2. In all cases, no base modification has been observed.

Figure 1 illustrates the differences in RP HPLC between partially and fully deprotected oligos, DMT-off and DMT-on.

Table 2

dG Protection	Temperature	Time
iBu-dG, dmf-dG or Ac-dG	RT	120 min.
	37 °C	30 min.
	55 °C	10 min.
	65 °C	5 min.

Note: UltraFAST system requires acetyl (Ac) protected dC to avoid base modification at the C base.

UltraMILD Deprotection

Cleavage is not carried out separately when using UltraMILD techniques. Since many of our nucleosides and dye products are not stable to deprotection with ammonium hydroxide or AMA, the procedure to deprotect the labelled oligonucleotide must be changed.

We often recommend using the UltraMILD monomers (Pac-dA, Ac-dC and iPr- Pac-dG) and deprotection with potassium carbonate in methanol. In this way, some of these very sensitive oligonucleotides can be conveniently isolated. If capping is carried out using Cap A containing phenoxyacetic anhydride, it is possible to deprotect UltraMILD oligonucleotides in 4 hours at RT with 0.05 M potassium carbonate in methanol or 2 hours at RT with ammonium hydroxide. Alternatively, using the regular Cap A containing acetic anhydride, it is necessary to deprotect overnight at room temperature to remove any Ac-dG formed during the capping step. For TAMRA containing oligonucleotides, an alternative deprotection[3] may be carried out using t-Butylamine/methanol/water (1:1:2) overnight at 55 °C. Another option that we have found to be excellent uses t-Butylamine/water (1:3) for 6 hours at 60 °C. In this case, the regular protecting groups on the monomers may be used. An even milder approach has been described as "Ultra- UltraMild".[4] In this technique, Q-supports[5] are combined with UltraMild monomers to allow extremely gentle deprotection. After completion of the synthesis, the solid support is dried and treated overnight at 55 °C with a solution containing 10% (v/v) diisopropylamine (iPr_2NH) in 0.25 M ß-mercaptoethanol in MeOH.

Summary

Successful oligonucleotide cleavage and deprotection require consideration of the deprotection conditions for each product and some products may require pretreatment or special deprotection conditions. Each synthesis should be reviewed to ensure the products have compatible deprotection conditions. Special deprotection requirements can be found on our Analytical Reports, Certificates of Analysis, Technical Bulletins, and website: www.glenresearch.com.

References:

1. S.L. Beaucage and R.P. Iyer, *Tetrahedron*, 1992, **48**, 2223-2311.
2. M.P. Reddy, N.B. Hanna, and F. Farooqui, *Nucleos Nucleot*, 1997, **16**, 1589-1598.
3. B. Mullah and A. Andrus, *Tetrahedron Lett*, 1997, **38**, 5751-5754.
4. L.C.J. Gillet, J. Alzeer, and O.D. Scharer, *Nucleic Acid Res*, 2005, **33**, 1961-1969.
5. R.T. Pon, and S.Y. Yu, *Nucleic Acids Res*, 1997, **25**, 3629-3635.

RNA Deprotection

Key Considerations – RNA Deprotection

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

Introduction

In the previous section of this guide on deprotection we focused on the absolute necessity to *Deprotect to Completion* while following our mandate to *Do No Harm*. In this section, 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:

https://www.glenresearch.com/media/productattach/t/b/tb_rna_tbdms_deprotection_20190712.pdf

https://www.glenresearch.com/media/productattach/import/application_guides/tb_rna_tom_deprotection_20191015.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 (>75 mer) 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 3.

Table 3: RNA and DNA Cleavage

	Temperature	Time
RNA		
AMA	RT	20 min.
NH_4OH/EtOH (3:1)	RT	2 h
DNA		
AMA	RT	10 min.
NH_4OH/EtOH (3:1)	RT	1 h

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 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 previously[3] 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 4 shows the temperatures and times for regular RNA and UltraMild RNA deprotection.

Table 4: Deprotection Conditions

	Temperature	Time
AMA	65 °C	10 min.
NH_4OH/EtOH (3:1)	RT	4 h

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 1.75 mL 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 2 mL 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 1.0 mL RNase free 1 M 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.

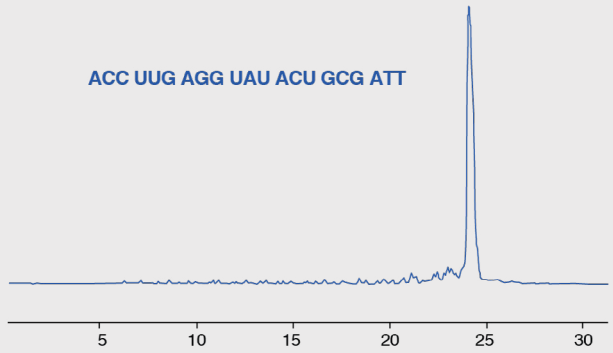
RNA Analysis

Accurate analysis of Glen-Pak purified RNA by Ion Exchange HPLC using more traditional buffer systems can be hampered by formation of secondary structures. The use of a sodium perchlorate buffer system as well as heat should denature most oligoribonucleotides. Figure 2 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 2 also shows the results of an ESI MS analysis of a Glen- Pak purified 21-mer siRNA.

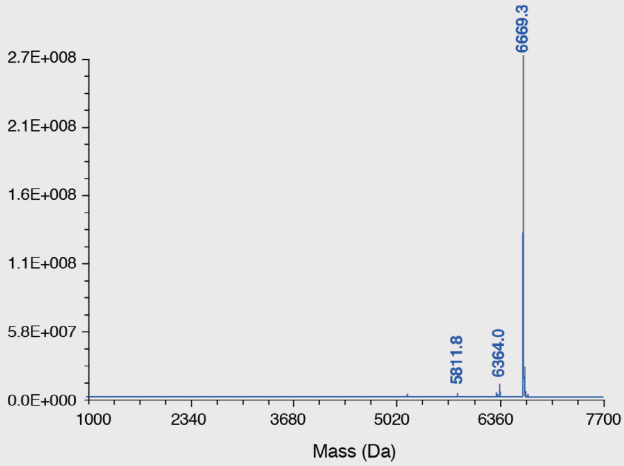
Further details for both DMT-on and DMT-off 2' RNA deprotection methods, suggested reagents, Glen-Pak purification and downstream processing can be found in our Glen Report 19.2 (*www.glenresearch.com/reports/gr19-21*) and the technical bulletins above.

Figure 2: Ion Exchange HPLC and ESI MS of Synthesized siRNA



Column: Dionex DNAPac PA200, 250 X 4 mm; Buffers: A- 10 mM $NaClO_4$, 25 mM TRIS-HCl, 20% Acetonitrile, pH 7.4;

B- 600 mM $NaClO_4$, 25 mM TRIS-HCl, 20% Acetonitrile, pH 7.4; Gradient: 0-10% Buffer B over 30 minutes; Flow 1 mL/min.



Target Mass(Da), 6670.1; Observed Mass(Da), 6669.3; Mass error: -0.8 Da (-0.012%), %Purity (Estimate) 93.55%,

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 inhouse 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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8. E. Westman, and R. Stromberg, *Nucleic Acids Res.*, 1994, **22**, 2430-2431.
9. B. Sproat, et al., *Nucleosides and Nucleotides*, 1995, **14**, 255-273.
10. D. Gasparutto, et al., *Nucleic Acids Res.*, 1992, **20**, 5159-66.

Dye-Containing Oligonucleotides

Key Considerations – Deprotection of Dye-Labelled Oligonucleotides

1. Even with oligos containing sensitive dyes, the nucleobases must be fully deprotected for full functionality.
2. Will the dye-labelled oligo survive my preferred deprotection scheme?
3. If not, which deprotection scheme will fit best with my equipment and purification strategy?

In the previous two sections, we focused on DNA and RNA deprotection. Our first priority in deprotection is to *Deprotect to Completion* by removing 100% of the protecting groups on the nucleobases, while following the mandate to *Do No Harm*. Dyes tend to have the unfortunate properties of being quite sensitive to the basic conditions of oligonucleotide deprotection while being expensive. The *Do No Harm* stricture is doubly important when deprotecting dye-labelled oligonucleotides. To make matters worse, many dye-labelled oligonucleotides also contain a quencher molecule that may also be base sensitive. This combination of properties is guaranteed to lead to confusion and possibly decomposed, worthless oligos may result if incompatible deprotection conditions are used.

We have generated a table which we hope will remove some of the challenges from the deprotection of dye-labelled oligonucleotides. The table illustrates the conditions suitable for deprotecting oligos containing a variety of dye-dye and dye-quencher combinations.

We have focused on a variety of methods for oligonucleotide deprotection:

A. 30% NH_4OH 17 hours at 55 °C; sufficient to deprotect all standard bases, A/C/G/T

B. 30% NH_4OH 17 hours at room temperature; sufficient to deprotect A, C, and dmf-dG

C. 30% NH_4OH 2 hours at 65 °C; sufficient to deprotect A, C, and dmf-dG

D. 30% NH_4OH 2 hours at room temperature; sufficient to deprotect only UltraMild monomers, Pac-dA, Ac-dC, iPr-Pac-dG when UltraMild Cap A is used.

E. 50 mM potassium carbonate in methanol for 4 hours at room temperature; sufficient to deprotect only UltraMild monomers, Pac-dA, Ac-dC, iPr-Pac-dG when UltraMild Cap A is used.

F. Tert-Butylamine/water 1:3 (v/v) 6 hours at 60 °C; sufficient to deprotect A, C and dmf-dG.

G. 30% ammonium hydroxide/40% methylamine 1:1 (v/v) 10 minutes at 65 °C; sufficient to deprotect all standard bases, however, Ac-dC must be used.

[$] Denotes an acceptable, but not preferred method.

Table 5: Deprotection Conditions Suitable for Popular Dyes and Quenchers

	AP593	AP639	BHQ-1	BHQ-2 BBQ-650	BHQ-3	Cyanine 3	Cyanine 5	Fluorescein	HEX	JOE Dabcyl	TAMRA	TET Eclipse	Yakima Yellow
AP593	$A, B, C, G	$A, B, C, G	$A, B, C, G	$A, B, C	$B	B, C, G	⊗	$A, B, C, $G	B, G	$A, B, C, G	⊗	$A, B, C, G	$A, B, C
AP639	$A, B, C, G	A, B, C, F, G	$A, B, C, $F, G	A, B, C	$B	B, C, G	⊗	A, B, C, F, $G	B, G	A, B, C, G	F	A, B, C, F, G	A, B, C, F
BHQ-1	$A, B, C, G	$A, B, C, $F, G	$A, B..E, $F, G	$A, B..E	$B, D	B..E, G	D, E	$A, B, C, $F, $G	B, D, E, G	$A, B..E, G	E, $F	$A, B..E, $F, G	$A, B, C, D, $F
BHQ-2 BBQ-650	$A, B, C	A, B, C	$A, B..E	A..E	$B, D	B..E	D, E	A..E	B, D, E	A..E	E	A..E	A..D
BHQ-3	$B	$B	$B, D	$B, D	$B, D	$B, D	D	$B, D	$B, D	$B, D	⊗	$B, D	$B, D
Cyanine 3	B, C, G	B, C, G	B..E, G	B..E	$B, D	B..E, G	D, E	B..E, $G	B, D, E, G	B..E, G	E	B..E, G	B, C, D
Cyanine 5	⊗	⊗	D, E	D,E	D	D, E	D, E	D, E	D, E	D, E	E	D, E	D
Fluorescein	$A, B, C, $G	A, B, C, F, $G	$A, B..E, $F, $G	A..E	$B, D	B..E, $G	D, E	A..F, $G	B, D, E, $G	A..E, $G	E, F	A..F, $G	A..D, F
HEX	B, G	B, G	B, D, E, G	B, D, E	$B, D	B, D, E, G	D, E	B, D, E, $G	B, D, E, G	B, D, E, G	E	B, D, E, G	B, D
JOE Dabcyl	$A, B, C, G	A, B, C, G	$A, B..E, G	A..E	$B, D	B..E, G	D, E	A..E, $G	B, D, E, G	A..E, G	E	A..E, G	A..D
TAMRA	⊗	F	E, $F	E	⊗	E	E	E, F	E	E	E, F	E, F	F
TET Eclipse	$A, B, C, G	A, B, C, F, G	$A, B..E, $F, G	A..E	$B, D	B..E, G	D, E	A..F, $G	B, D, E, G	A..E, G	E, F	A..G	A..D, F
Yakima Yellow	$A, B, C	A, B, C, F	$A, B, C, D, $F	A..D	$B, D	B, C, D	D	A..D, F	B, D	A..D	F	A..D, F	A..D, F

⊗ = Incompatible $ = Denotes an acceptable, but not preferred method.

Notes:

1. JOE (5'-Dichloro-dimethoxy-Fluorescein Phosphoramidite) has not been tested with Condition F.
2. FAM is compatible with deprotection in AMA without any degradation if the oligo is first treated with 30% NH_4OH to remove the pivaloyl protecting groups from the FAM and then the 40% methylamine is added to complete the deprotection of the bases.

Alternatives to Ammonium Hydroxide

Introduction

Back in the 1990s, deprotection of DNA oligos was carried out using ammonium hydroxide overnight at 55 °C. The only option to increase the speed was to raise the deprotection temperature to 80 °C (and even above!) with the time being halved for every 10 °C the temperature was increased. But in those days, the most common application for oligos was as sequencing primers so a small percentage of unprotected ibu-dG was never noticed. The first attempt to increase the speed of deprotection was the introduction[1] of dmf-dG (and dmf-dA) as "Fastphoramidites" since dmf-dG is deprotected at about twice the rate of iBu-dG. In our view, there was no downside to the adoption of dmf-dG but dmf-dA proved to be rather too labile for routine use and was discontinued. However, ammonium hydroxide was still the only deprotection method at this time.

Although ammonium hydroxide is still immensely popular for deprotection of DNA oligos, the advent of high throughput synthesis, labile bases and fluorescent tags has led to the adoption of a variety of newer procedures. In this section we will describe some of the most popular deprotection procedures and will note when they may be most applicable.

As usual, when reviewing the variety of procedures available to deprotect any modified or unmodified oligonucleotide, you must heed the primary consideration: *First, Do No Harm*. You can then proceed with confidence to *Deprotect to Completion*.

First, Do No Harm!

As we have stated in the past, determination of the appropriate deprotection scheme should start with a review of the components of the oligonucleotide to ascertain if any group is sensitive to base and requires a mild deprotection or if there are any pretreatment requirements. Sensitive products are defined as such on the Analytical Report, Certificate of Analysis, or Technical Bulletin. Occasionally, some products require a special pretreatment to prevent unwanted side reactions. If the oligo has several unusual components, you must follow the mildest procedure recommended. As you might expect, some highly modified oligos can become VERY challenging.

UltraFast Deprotection

The use of dmf-dG to speed up deprotection with ammonium hydroxide was only an incremental improvement in speed.

However, UltraFast deprotection quickly became a commercial reality with the introduction[2] of deprotection using ammonium hydroxide/methylamine (AMA).

By adding an equal volume of 40% aqueous methylamine solution to ammonium hydroxide to form AMA, it is possible to speed up the deprotection of oligonucleotides enormously.[2] Deprotection can be completed in 5 minutes at 65 °C, thereby allowing oligonucleotides to be delivered to customers on the same day of manufacture. The only change required in the synthesis strategy is the substitution of Ac-dC for Bz-dC to avoid transamination of dC by displacement of benzamide by methylamine to form the mutant N4-Me-dC.[3] This modification is well tolerated and probably codes perfectly as dC in any case. However, as with dmf-dG described above, we see no downside to the use of Ac-dC and recommend it at all times. UltraFast deprotection has found favor with groups processing many oligonucleotides where the decreased processing time, and, therefore, cost savings, becomes highly significant.

Options for UltraFast deprotection, where the removal of the dG protecting group is the rate determining step, are shown in Table 6.

Table 6

dG Protection	Temperature	Time
iBu-dG, dmf-dG or Ac-dG	RT	120 min.
	37 °C	30 min.
	55 °C	10 min.
	65 °C	5 min.

Note: UltraFAST system requires acetyl (Ac) protected dC to avoid base modification at the C base.

The consequences of fast but incomplete deprotection are illustrated in Figure 3 on the following page. RP HPLC traces show the location of incompletely deprotected oligonucleotides relative to the main component in DMT-on and DMT-off situations.

As an aside, we have found that AMA deprotection is also the optimal procedure for RNA deprotection.

Key Considerations – Deprotection of Dye-Labelled Oligonucleotides

1. Do I need to do my deprotection fast? *Use UltraFast Deprotection.*
2. Do I have special components in my oligos and need to treat them gently? *Use one of the Mild Deprotection procedures.*
3. Do I have many oligos to treat in parallel? *Consider the use of Gas Phase.*

Mild Deprotection

Deprotection using sodium hydroxide in aqueous alcoholic solvents is a very mild (and fast) alternative to ammonium hydroxide. For a mild deprotection scheme, you can deprotect DNA oligos with 0.4 M sodium hydroxide in methanol/water (4:1).

For example, we recommend this method for oligos containing acridine. This technique is necessary for oligos where esters are hydrolyzed to carboxylates, such as Carboxy-dT and EDTA-dT, where deprotection with amine-containing reagents would lead to undesired amide formation. You can also deprotect DNA oligos in a few minutes at 80 °C with no concern about vials popping since the mixture contains no volatile gas. The resulting deprotected oligo can be isolated by precipitation or by dilution with water followed by desalting or DMT-on purification. In all cases, the oligos are isolated as desirable sodium salts. The downside is that oligos cannot be isolated simply by evaporation and a desalting step is mandatory.

UltraMild Deprotection

Many years ago, we were confronted with the reality that some DNA bases that we wanted to introduce for DNA damage and repair studies simply were not compatible with ammonium hydroxide deprotection. Although deprotection with ammonium hydroxide at room temperature did allow oligos containing these bases to be isolated, it was not optimal and clearly a new deprotection scheme was needed.

In the early 1990s, we were prompted to look at an alternative DNA protecting group, acetoxymethylbenzoyl (AMB), which could be removed using potassium carbonate in methanol.[4] Unfortunately, the AMB-protected monomers proved to be too unstable to store for long periods. But we found that the use of a combination of Pac-dA, Ac-dC and iPr-Pac-dG allowed complete deprotection with potassium carbonate in methanol at room temperature for four hours as long as capping was carried out using phenoxyacetic anhydride rather than acetic anhydride. (With UltraMild reagents, ammonium hydroxide at room temperature for two hours was also effective.) If acetic anhydride was used, a small amount of transamidation occurred at dG residues and overnight treatment with potassium carbonate or ammonium hydroxide was required to deprotect formed Ac-dG residues.

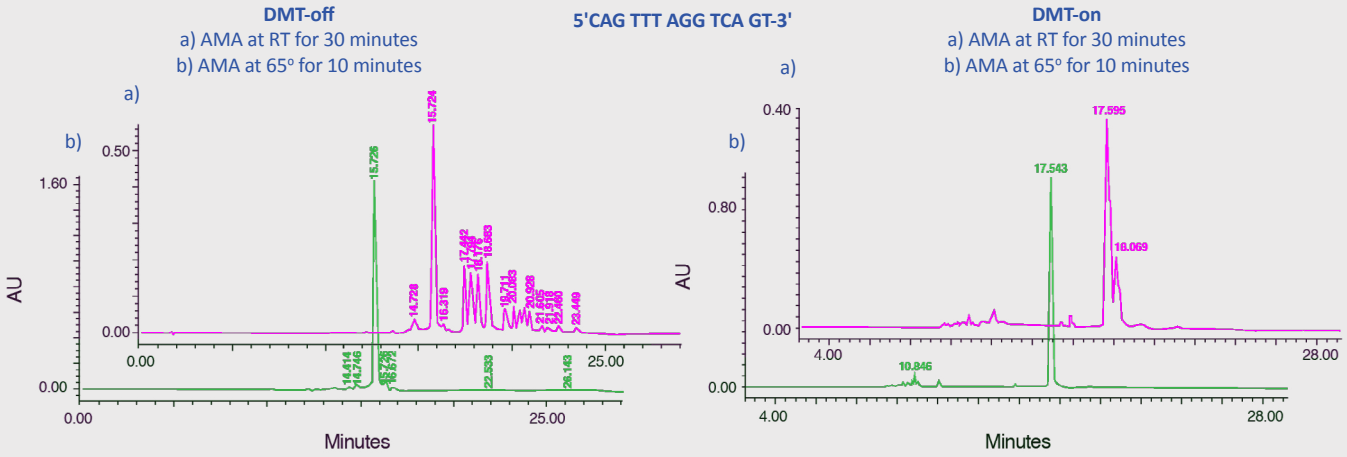
Ultra UltraMild

An even milder deprotection scheme has been described[5] for the synthesis of highly base labile nucleoside adducts. In this UltraMild variation, Q-supports must be used since the succinate linkages of normal supports are virtually untouched under the deprotection conditions. Normal yields are achieved with Q-supports. The reagent for this Ultra UltraMild deprotection procedure is 10% diisopropylamine/0.25 M ß-mercaptoethanol in methanol overnight at 55 °C. This is a method which has not been tested in very many facilities but it is surely worthy of consideration when challenged with the preparation of oligos with very labile bases.

t-Butylamine

TAMRA-containing oligonucleotides remain popular as single and dual labelled probes. Unfortunately, the stability of TAMRA to the conditions of oligonucleotide deprotection

Figure 3: RP HPLC of an Oligo Partially and Fully Deprotected, DMT-off and DMT-on



Deprotection Conditions
Partial Deprotection: AMA at RT for 30 min.
Full Deprotection: AMA at 65° C for 10 min.
RP HPLC Conditions
Column: Waters X-Bridge C18, 250 x 4mm; Buffers: A - ACN; B - 0.1M TEAA , pH 7.
DMT-off Gradient: 3-15% B over 15 min. DMT-on Gradient: 3-40% B over 15 min. Flow rate: 1mL/min.

is really marginal. In the past, we have recommended the use of UltraMild monomers and deprotection and this procedure does indeed work well. An alternative approach has been described[6] using t-Butylamine/methanol/water, which does allow the use of regular monomers. We have evaluated a simpler t-Butylamine/ water (1:3) mix (4 hours at 60 °C), described by Biosearch Technologies, and, in model studies, this generates TAMRA-oligos with the highest purity and with negligible degradation detected.

Gas Phase

Although gas phase deprotection does require specialist equipment, this technique is excellent for high throughput synthesis. Columns and plates can be placed in the reactor without concern for cross-contamination since the product oligos will remain adsorbed to the synthesis support. This is doubly advantageous since the product can be eluted from the columns and plates in such a way that the organic debris can be removed. Also, using anhydrous ammonia gas and using UltraMild monomers, the cleavage and deprotection processes can be completed in less than 1 hour.[7] However, methylamine gas has proved to be more popular for routine synthesis and is in common use in our industry. Please note that deprotection times and temperatures vary with the equipment and number of columns and will need to be optimized.

Summary

Oligonucleotide deprotection has come a long way since the early days when ammonium hydroxide was the only option. Now a variety of procedures are available to fit a variety of circumstances. Each synthesis should be reviewed to ensure that the deprotection conditions are compatible with the components of the oligo. Special deprotection requirements can be found on our website: *www.glenresearch.com*.

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On-Column Deprotection of Oligonucleotides in Organic Solvents

Key Considerations – On-Column Deprotection

1. When should I use on-column deprotection?
   When using high throughput synthesis.
2. Do I need special monomers?
   On-column deprotection requires the use of Ac-dC.
3. Do I need to desalt after on-column deprotection?
   No, the organic byproducts remain on the synthesis column.

Introduction

The deprotection of oligonucleotides, especially for high throughput syntheses, can be the rate-limiting step during the production of oligos and is often difficult to automate due to issues with liquid handling. To streamline the deprotection process, gas phase deprotection using ammonia or methylamine gas is often employed.[1] After the removal of the protecting groups is complete, the oligo is conveniently eluted directly in water or the buffer of choice. However, the equipment necessary to safely handle a pressurized, corrosive gas is expensive and the additional cost is not worthwhile for many smaller production facilities and research labs.

On-Column Deprotection

An alternative method that incorporates much of the convenience of gas-phase deprotection but still utilizes low-cost and simple equipment is on-Column deprotection. In this case, the nucleophilic amine used to remove the protecting groups is dissolved in a non-polar solvent, such as toluene, in which the deprotected oligonucleotide is insoluble. After the deprotection of the oligonucleotide is complete, the column is rinsed, allowed to briefly dry and the oligo, still bound to the support, is eluted in the aqueous buffer of choice, as described by Kempe.[2] A similar strategy was used by Damha for the deprotection of RNA on glass slides.[3] Based upon a protocol used to deprotect TC RNA monomers,[4] we have developed a procedure for the deprotection of standard DNA as shown in the following procedure.

In Figure 4, we show the results of an oligonucleotide synthesis that was split, with half being deprotected in standard aqueous AMA and the other half in an EDA/toluene solution. Both product oligos had the same molecular weight as determined by electrospray mass spectrometry. We also found there was no drop in yield from the on-Column deprotection compared with the standard aqueous deprotection.

When oligos of the same length but different molecular weights were synthesized on Glen UnySupport frits and deprotected in the same EDA solution, we found there was no indication of any cross-contamination of oligos between the frits by mass spec analysis. This means that an entire 96 well plate can be conveniently deprotected in a single vessel. It should be noted, however, that the Glen UnySupport required 2 hours at 65 °C to be fully eliminated from the 3' terminus of the oligo in the EDA solution.

We have found that this method is compatible with PS supports as well as CPG supports. However, if there are hydrophobic labels on the product oligo, e.g., DMT or cyanine (Cy) dyes, some oligo (10-20%) may be retained on a PS support. In this case, we recommend eluting the oligo in buffer containing 10-20% acetonitrile.

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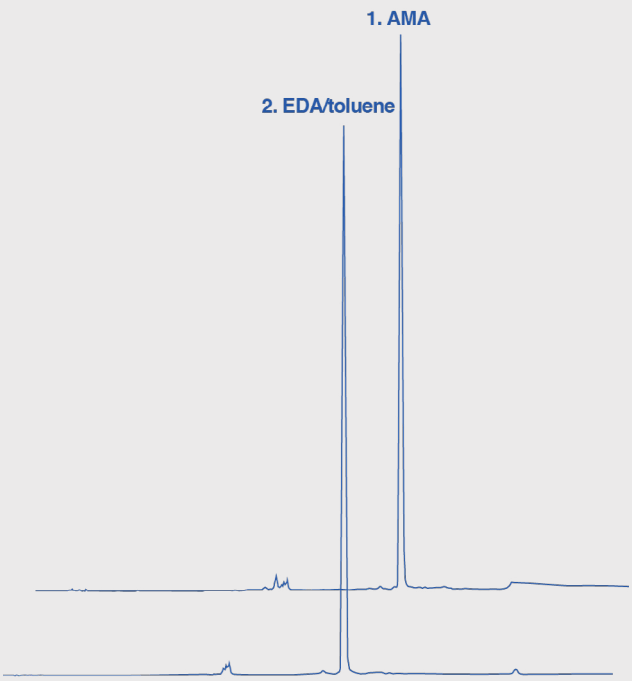
Figure 4: RP HPLC of an Oligo Deprotected With 1. AMA and 2. EDA/toluene



Procedure : Deprotection Using EDA/toluene (1:1)

1. After the synthesis is complete, treat the support with 10% diethylamine in acetonitrile, slowly pushing the solution through the column to waste over a 3-5 minute period. This will remove the cyanoethyl protecting groups from the phosphate backbone. This initial treatment is critical to the success of the protocol.
2. Rinse the column with acetonitrile.
3. Briefly dry the CPG under vacuum.
4. Treat the column with ethylenediamine (EDA)/toluene solution 1:1 (v/v), pulling the EDA solution into the column so that the support is completely wettened. Use approximately 500 µL per µmole for small-scale syntheses.
5. Let the solution sit over the support for 2 hours at room temperature. Apply vacuum and remove the deprotection solution.
6. Rinse the column with toluene (3x).
7. Briefly dry the support under vacuum.
8. Elute the oligo from the support in aqueous buffer of choice.

Notes:

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