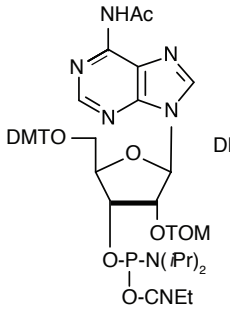
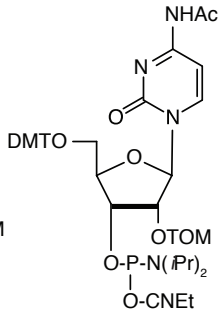
Application Guide

Procedure for the synthesis, deprotection and isolation of RNA using TOM-protected monomers



(1) A-TOM



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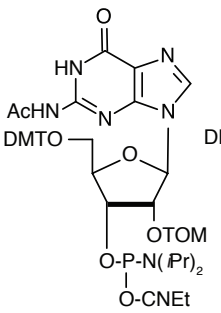

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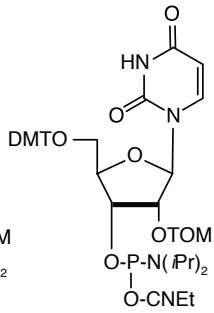
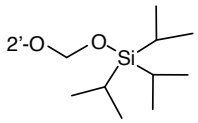


(4) U-TOM



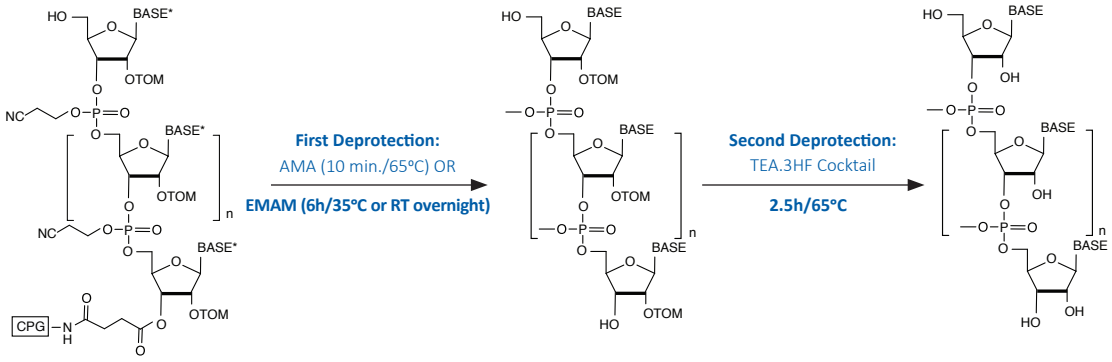
TOM-Protecting-Group™

A. INTRODUCTION

RNA synthesis using monomers containing the 2'-O-TriisopropylsilylOxyMethyl (TOM) group (TOM-Protecting-Group™) is characterized by very high coupling efficiency along with fast, simple deprotection. High coupling efficiency is achieved because the TOM-Protecting-Group exhibits lower steric hindrance than the 2'-O-t-butyldimethylsilyl (tBDMS) group used in our previous RNA monomers. Indeed the TOM-Protecting-Group is similar sterically to the 2'-OMe group and exhibits high efficiency similar to 2'-OMe-RNA monomers.

Fast and reliable deprotection is achieved using ammonium hydroxide/methylamine (AMA) or methylamine in ethanol/water (EMAM). AMA works best for regular oligos while EMAM is optimal for long oligos. A further feature of the TOM-Protecting-Group is that during basic steps it cannot undergo 2' to 3' migration. This migration under basic conditions leads to non-biologically active 2'-5' linkages when using the tBDMS group. These features allow the TOM-Protected monomers to produce longer oligonucleotides and, for this reason, we offer only 1000Å supports. TOM-Protected RNA monomers are also fully compatible with minor bases with 2'-O-tBDMS protection.

The synthesis cycle used with TOM-Protected RNA monomers is identical to previous DNA and RNA cycles, with coupling times as shown below. Cleavage and deprotection conditions are similar to previous RNA techniques and are outlined below, with optimal times being shown in the text overlay.



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SYNTHESIS AND DEPROTECTION USING TOM-PROTECTED RNA

B. SYNTHESIS MATERIALS:

1. All three of the protected TOM RNA amidites and their corresponding synthesis columns use the acetyl protecting group on the base.[1,2,3] This allows for use of ammonium hydroxide/methylamine (AMA)[1,2] OR ethanolic methylamine (EMAM)[3] in the base deprotection step.

Ac-A RNA CPG, 20-3304;	A-TOM-CE Phosphoramidite, 10-3004
Ac-C RNA CPG, 20-3315;	C-TOM-CE Phosphoramidite, 10-3014
Ac-G RNA CPG, 20-3324;	G-TOM-CE Phosphoramidite, 10-3024
U-RNA CPG, 20-3330;	U-TOM-CE Phosphoramidite, 10-3034

C. DEPROTECTION MATERIALS USED:

1. Sturdy 2mL centrifuge tube or sealable glass vial for carrying out deprotection. When using methylamine, vials which use black rubber O-rings as seals should not be used.
2. Ammonium hydroxide/methylamine (AMA) - ammonium hydroxide/40% aqueous methylamine (Aldrich 42,646-6, or equivalent) (1:1). OR ethanolic methylamine/aqueous methylamine (EMAM) - 10M methylamine in ethanol/41% methylamine in water (1:1). Fluka 65590 and Fluka 65580 or equivalents, respectively. Keep the reagents tightly sealed in the refrigerator. Make up only about 1 weeks supply of AMA or EMAM at a time to avoid loss of the methylamine during use.
3. Triethylamine trihydrofluoride (TEA.3HF) (Aldrich 34,464-8 or equivalent).
4. DMSO: Dimethylsulfoxide, anhydrous, 99.9% (Aldrich 27,685-5 or equivalent).
5. TEA: Triethylamine, puriss. p.a. ≥ 99.5% (GC) (Sigma, BioUltra 90340 or equivalent).
6. RNase free water (Fisher BP 2484100 or equivalent).
7. RNase free, sterile pipettes and polypropylene screw cap tubes.

D. SAFETY:

1. Technically qualified individuals should perform the procedures described in this bulletin.
2. Methylamine based solutions are under pressure and can rupture containers. Use safety glasses when handling hot vials containing methylamine and ammonium hydroxide solutions.

E. RNA SYNTHESIS:

1. Synthesize RNA using 5-ethylthio-1H-tetrazole (ETT) or 5-benzylthio-1H-tetrazole (BTT) (Glen Research 30-3140 or 30-3170, respectively) as activator.
2. Use 6 minute coupling time for RNA monomers with ETT or a 3 minute coupling time when using BTT.
3. As always, consider the desired downstream processing, DMT-on or off status and possible purification paths before moving to the cleavage and deprotection steps below.

F. CLEAVAGE FROM THE SUPPORT AND REMOVAL OF THE BASE AND PHOSPHATE PROTECTING GROUPS:

1. Cleavage options:
   a. Syringe cleavage:
      i. Remove the synthesis column from the synthesizer and thoroughly air-dry the support in the columns or dry in a stream of argon gas.
      ii. Connect a clean syringe to the luer fitting of the column (VWR 53548-000, Norm-Ject 1mL sterile plastic syringe). Avoid the use of syringes that have a rubber plunger.
      iii. With a second syringe, take up 1mL of the appropriate deprotection solution for the amidites used (see below) into a plastic syringe. Connect the second syringe to the other luer fitting on the column and gently pass the solution carefully through the column 4-5 times.
      iv. Allow the column to stand at room temperature with the solution in full contact with the CPG for 20 minutes.
      v. Transfer the solution to a clean, polypropylene screw cap vial. Rinse the column with 0.5mL of deprotection solution and combine with first portion for a total volume of around 1.5mL.
   b. Support removal and incubation:
      i. Remove the synthesis column from the synthesizer and thoroughly air-dry the support in the columns or dry in a stream of argon gas.
      ii. Open the synthesis column and pour the support into a sealable polypropylene vial.
      iii. Add 1.5mL of the appropriate deprotection solution and incubate for the proper duration to both cleave and deprotect the oligo (see below).
2. Deprotection of oligos using TOM RNA amidites (A-TOM, C-TOM, G-TOM, U-TOM):
   a. Seal the deprotection vial containing 1.5mL of AMA solution and oligoribonucleotide solution for 10 minutes at 65°C OR 1.5mL of EMAM solution and oligoribonucleotide solution for 6 hours at 35°C or overnight at room temperature.

b. Cool the sealed vial and open cautiously. The use of sterile conditions from this point forward is essential.

c. If support was included in the deprotection: Remove the supernatant using a sterile pipette and transfer to a new, sterile tube. This tube should be polypropylene (not glass) in order to allow for 2' deprotection in the next step. Rinse the support with 2 x 0.25mL RNase free water and add to supernatant.

3. Evaporate the combined solution to dryness:

   a. NOTE: If you are doing this with a DMT-on oligonucleotide in preparation for future purification via Glen-Pak or other method, it is preferable to use a stream of nitrogen or vacuum manifold apparatus with no heat to dry the cleaved and deprotected product. This will help maintain the DMT group on the RNA prior to 2' deprotection.

   b. Some speedvac units have been used successfully, but we do not recommend heating the oligo during the dry down process, as it may also remove the DMT group.

G. 2' SILYL GROUP REMOVAL STRATEGIES: OUT WITH THE OLD IN WITH THE NEW.

Tetrabutylammonium fluoride (TBAF), while still a valid 2' silyl group removal method,[4] is not compatible with the Glen-Pak RNA purification cartridge. Triethylamine trihydroflouride (TEA.3HF) based cocktails have become much more commonly used and are compatible with both cartridge and precipitation based downstream processing methodologies.[5-7] Various additives such as triethylamine (TEA) have altered the pH from original methods using neat TEA.3HF, 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.

1. DMT-off RNA 2' deprotection (DMSO, TEA.3HF) and desalting by precipitation:

   a. Fully re-dissolve the oligo in 100µL anhydrous DMSO. If necessary, heat the oligo at 65°C for about 5 minutes to get it into solution.

   b. Add 125µL of TEA.3HF, mix well and heat to 65°C for 2.5 hours.

   c. Cool in freezer briefly prior to lyophilizing and desalting.

   d. Please see our Glen Report 19-2 for the preferred desalting protocol (https://www.glenresearch.com/reports/gr19-22)

2. DMT-on RNA 2' deprotection (DMSO, TEA, TEA.3HF) followed by Glen-Pak RNA cartridge purification:

   a. Fully dissolve the RNA oligonucleotide in 115µL DMSO. If necessary, heat the oligo at 65°C for about 5 minutes to get it into solution.

   b. Add 60µL of TEA to the DMSO/ oligo solution and mix gently.

   c. Add 75µL of TEA.3HF and heat the mixture at 65°C for 2.5 hours.

   d. Immediately before cartridge purification is to begin, cool the 2' deprotection solution and add 1.75mL of Glen-Pak RNA Quenching Buffer (Glen Research 60-4120) to the deprotected RNA solution. Mix well and immediately load the oligo on the Glen-Pak RNA purification cartridge.

   e. Please see our Glen Report 19-2 for the Glen-Pak RNA protocol (https://www.glenresearch.com/reports/gr19-22)

H. ANALYSIS VIA HPLC:

1. Analyze using Dionex PA-200 or equivalent with a sodium perchlorate gradient at 50-60°C.

2. RNA can form secondary structures that tend to interfere with HPLC analysis and purification. The use of sodium perchlorate buffer and heat should denature most oligoribonucleotides to enable efficient chromatography. For alternative protocols and methods that suit your application, please contact our technical support group.

REFERENCES:

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TOM-RNA phosphoramidites are supplied under agreement with QIAGEN. RNA synthesis using the TOM-Protecting-Group is covered by US Patent No. 5,986,084.

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