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

A. INTRODUCTION

Cleavage, deprotection and purification of synthetic oligoribonucleotides, although routine, are complex processes. No two labs seem to carry out the procedures the same way and there are many routes to successful completion of the tasks.
In the procedures here, we assume that cleavage and deprotection will occur off the synthesizer and that our TBDMS protected RNA monomers are used to manufacture the oligoribonucleotide. Glen Research offers both UltraFast and UltraMild versions of our TBDMS protected phosphoramidites. The UltraMild option allows for the use of other minor bases and/or sensitive labels that may not be amenable to the downstream processing steps for UltraFast monomers. Regular deprotection with ammonium hydroxide/ethanol can be used for all monomers, with the conditions being determined by the protecting group on the G monomer. UltraFast deprotection with AMA requires acetyl protected RNA C monomer and is compatible with virtually any other protecting groups for A and G as well as Glen-Pak RNA cartridge purification. AMA works best for regular oligos while EMAM is optimal for long oligos. We have described a convenient method for removal of the silyl protecting groups using triethylamine trihydrofluride. While tetrabutylammonium fluoride (TBAF) solution also works well, it exhibits variable performance due to variations in water content, and it is not compatible with downstream, DMT-on, Glen-Pak purification. Precipitation, desalting, Glen-Pak RNA cartridge and HPLC purification can accomplish isolation of oligoribonucleotides. Gel electrophoresis is normally used for purification of RNA but ion exchange HPLC may also be used. Details of these techniques are not provided here. Please note that a separate technical bulletin details the procedures for use and deprotection of our TOM-protected monomers.

B. MATERIALS:

1. For UltraFast deprotection of TBDMS RNA, acetyl protected C monomer (Ac-C, 10-3015) and support (Ac-C-RNA CPG, 20-3315) must be used. These are the ideal configurations for use with the Glen-Pak RNA cartridge.
2. The remaining complement of monomers and supports used in the UltraFast configuration are as follows:

   Bz-A RNA CPG, 20-3303;
   Bz-A-CE phosphoramidite, 10-3003
   Ac-G RNA CPG, 20-3424;
   Ac-G-CE phosphoramidite, 10-3025
   U-RNA CPG, 20-3430;
   U-CE phosphoramidite, 10-3030
3. For synthesis of RNAs requiring UltraMild deprotection, use the following TBDMS protected monomers and supports:

   Pac-A-RNA-CPG, 20-3300;
   Pac-A-CE Phosphoramidite, 10-3000
   Ac-C-RNA-CPG, 20-3315;
   Ac-C-CE Phosphoramidite, 10-3015
   iPr-Pac-G-RNA-CPG; 20-3321;
   iPr-Pac-G-CE Phosphoramidite, 10-3021
   U-RNA CPG, 20-3430;
   U-CE phosphoramidite, 10-3030
4. 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.
5. Base deprotection solutions:
   a. Ethanolic ammonium hydroxide- Prepare a solution by mixing 1 volume of ethanol with 3 volumes of ammonium hydroxide. Keep the reagents tightly sealed in the refrigerator. Make up only about 1 weeks supply at a time to avoid loss of the ammonia during use. OR

B. MATERIALS (Con nued):

b. Ammonium hydroxide/methylamine (AMA)[1,2] – Prepare as solution by mixing 1 volume of ammonium hydroxide with 1 volume of 40% aqueous methylamine (Aldrich 42,646-6, or equivalent). Keep the reagents tightly sealed in the refrigerator. Make up only about 1 weeks supply of AMA at a time to avoid loss of the methylamine during use. OR

c. Ethanolic methylamine/aqueous methylamine[3] (EMAM) -*33%* methylamine in ethanol/water (1:1). Mixture of 33% methylamine in ethanol/ 41% methylamine in water (1:1).(Fluka 65590 and Fluka 65580).

C. Triethylamine trihydrofluoride (TEA.3HF) (Aldrich 34,464-8 or equivalent).

D. DMSO: Dimethylsulfoxide, anhydrous, 99.9% (Aldrich 27,685-5 or equivalent).

E. TEA: Triethylamine, puriss. p.a. ≥ 99.5% (GC) (Sigma, BioUltra 90340 or equivalent).

F. RNase free water (Fisher BP 2484100 or equivalent).

G. RNase free, sterile pipettes and polypropylene screw cap tubes.

C. SAFETY:

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

D. 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. When using the UltraMild TBDMS RNA amidites (Pac-A, Ac-C, iPr-Pac-G, U), remember to utilize UltraMild Cap Mix A (40-4210-xx/ 40-4212-xx) to avoid any exchange of the iPr-Pac group on the G with acetyl.
4. 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.

E. RNA CLEAVAGE AND DEPROTECTION:

1. Cleavage options:
   a. Syringe cleavage (may be used if using AMA or EMAM):
      i. NOTE: Use AMA if you are planning to purify the oligo using the Glen-Pak RNA cartridge.
      ii. Remove the synthesis column from the synthesizer and thoroughly air-dry the support in the columns or dry in a stream of argon gas.
      iii. 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.
      iv. 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.
      v. Allow the column to stand at room temperature with the solution in full contact with the CPG for 20 minutes.
      vi. 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 (may be used with all three deprotection solutions):
      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 UltraMild TBDMS RNA amidites (Pac-A, Ac-C, iPr-Pac-G, U)
   a. Seal the deprotection vial containing 1.5mL of ethanolic ammonium hydroxide and oligoribonucleotide solution for 4-17 hours 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. Deprotection of oligos using UltraFAST TBDMS RNA amidites (Bz-A, Ac-C, Ac-G, U)
    a. Seal the deprotection vial containing 1.5mL of AMA and oligoribonucleotide solution, heat for 10 minutes at 65°C.
    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.
4. 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.

F. 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[5-7] and are compatible with both cartridge and precipitation based downstream processing methodologies. 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 desalting.
    d. Please see our Glen Report 19-2 for the 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)

G. 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.

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