User Bulletin No. 53

Models 380A/380B/381A/391 EP DNA Synthesizers

December 1, 1989 (updated 06/2002)

SUBJECT: RNA Synthesis With DMT-Cyanoethyl RNA Phosphoramidites

Introduction

RNA synthesis chemistry has been recently refined to use DMT protected cyanoethyl RNA phosphoramidites on your Applied Biosystems DNA synthesizer. Oligoribonucleotides of high purity and yield have been obtained on the models 380A, 380B, 381A and 391 EP AB oligonucleotide synthesizers. All the necessary cycles, end procedures, and cleavage/deprotection protocols, originally described in Applied Biosystems User Bulletin Number 47 (April 1988), have been revised and are included in this User Bulletin.

The potential utility of defined sequences of oligoribonucleotides in biological experiments has long been recognized. The ability to explore transcription and translation with synthetic constructs of mRNA, tRNA, and rRNA is a powerful prospect. Recently, interest has been heightened by the Nobel prize winning discovery of RNA sequences with catalytic activity[1], RNA with unusual conformations[2], and recombinant RNA technology[3]. Possible chemotherapeutic applications of synthetic RNA are especially exciting and distinct from the use of native double-stranded RNA and interferons as antitumor agents[4]. Historically, these various investigations have been hampered by the unavailability of synthetic RNA. Now, with commercially available RNA monomers and supports, coupled with optimized synthesis cycles on your AB synthesizer, RNA synthesis is as available as DNA synthesis.

Applied Biosystems

Reagents

The ancillary reagents for RNA synthesis are the same as for DNA synthesis:

Reagents	AB Part No.	Bottle Position
Tetrazole/acetonitrile	400233 or 400606	9
	(45 ml) (180 ml)	
Acetic anhydride/lutidinefIHF	400234 or 400607	11
	(45 ml) (180 ml)	
NMI/THF	400777 or 400785	12
	(45 ml) (180 ml)	
Trichloroacetic acid/DCM	400236	14
Iodine/water/pyridinefFHF	400753	15
Acetonitrile	400262	18 (13,16)

IMPORTANT To conduct either RNA or DNA synthesis requires only the changing of the appropriate monomers and support and selecting the appropriate cycle. The above ancillary reagents remain on the instrument and are used at the same bottle positions for RNA and DNA syntheses. Consumption of each reagent per cycle is virtually the same for RNA and DNA.

RNA Monomers

As with DNA monomers, the RNA phosphoramidites are diluted with dry acetonitrile to a concentration of 0.1 molar. The acetonitrile solution stability of the RNA monomers is comparable to DNA monomers, which have a useful lifetime on the synthesizer of about 14 days.

Synthesis Cycles

The RNA synthesis cycles in User Bulletin Number 47 (April 1988) were designed for 5' MMT methylphosphoramidite monomers. Now that 5' DMT cyanoethylphosphoramidite monomers are available, creating an RNA cycle for any scale or AB synthesizer is extremely simple. *When using DMT **cyanoethyl**phosphoramidite RNA monomers, simply increase the coupling wait time of the appropriate scale DNA cycle to 600 seconds to create your RNA cycle*.

The primary difference from the DNA cycles is the longer wait time for coupling. This is a consequence of the steric hindrance conferred on the reactive 3' phosphoramidite group by the neighboring 2' O-silyl group. RNA cycle times are about 10 minutes longer than DNA cycles. Average yields per cycle have been demonstrated to be nearly as high as DNA synthesis, 98%, as measured by trityl UV quantitation. The high average yield per cycle in the synthesis of long oligoribonucleotides was also demonstrated through the synthesis of a 43-mer[5], and a 77-mer[6], and synthesis at the 10 μmol scale[7] on the Models 381A and 380B DNA synthesizers.

Cleavage, Deprotection and Purification

The relatively fragile nature of RNA to pH extremes and enzymatic degradation imposes more stringent techniques in deprotection, analysis, purification, and handling[6]. The following protocols describe the conditions and cautions for generating purified synthetic oligoribonucleotides.

After synthesis is complete, the oligoribonucleotide is ready for deprotection. Although RNA syntheses can be conducted either Trityl On or Trityl Off, we recommend that you synthesize all your oligoribonucleotides Trityl Off. Trityl On purifications (HPLC or OPC) require 2'-hydroxyl deprotection *prior* to acidic removal of the trityl group and are not currently recommended. Exposing fully deprotected RNA to acidic conditions (detritylation) enhances the potential for 2' to 3' phosphate migration. RNA syntheses conducted Trityl Off eliminate phosphate migration, since the 2' protecting group is not removed until after detritylation.

The 2' silyl oligoribonucleotides are relatively stable compounds. They are resistant to RNases and may be stored for extended times as a dry pellet or in cold, aqueous, neutral solutions. In the final step, the silyl deprotection reagent (tetrabutylammonium fluoride in tetrahydrofuran) is very selective and mild, preserving the integrity of the RNA[8-10].

The reagent for cleavage from the support and base deprotection (bottle position number 10) is a 3:1 mixture of concentrated ammonia:ethanol. The inclusion of ethanol in the ammonia is necessary to adequately solubilize the more lipophilic 2' O-silyl oligoribonucleotide and to minimize desilylation. Cleavage from the support with the ammonia:ethanol (3:1) is slower than cleavage with ammonia only. Therefore, the cleavage time has been doubled from previously described procedures. The post-RNA synthesis procedures for cleavage from the support and deprotection are described below.

The post-synthesis protocol for cleavage/deprotection of RNA oligos, originally described in Applied Biosystems User Bulletin Number 47, has been improved and simplified. As with DNA, deprotecting the *cyanoethyl*phosphoramidite derived RNA oligonucleotide does not require thiophenol.

For 380A and 380B Users: Support cleavage may be conducted on the 380B and the 380A with minor alterations to the standard DNA synthesis end procedures. To achieve complete cleavage of the RNA oligo, simply double the four ammonia/ethanol wait times to 1800 seconds in the standard DNA end procedure. Store this new end procedure for future RNA synthe-

ses. Upon completion of the end procedure, remove and cap the collection vial, then heat at 55° C for 8-16 hours for complete base deprotection.

For 391 and 381A Users:

For deprotection off the synthesizer, ammonia treatment may be implemented through the double-syringe method.

1. At completion of the synthesis, dry the synthesis column with 60 seconds of reverse flush (function 2), then remove the column from the instrument.
2. Load a fresh syringe (with luer tip not a locking tip) with a 2 mL, solution of 3:1 concentrated ammonia (Baker Part No. 9721-01): ethanol (Aldrich Part No. 27,764-9).
3. Mount another, empty syringe, with plunger fully inserted, into one end of the synthesis column. Mount the ammonia/ethanol loaded syringe into the other end of the column.
4. Holding a syringe in each hand, *gently* inject the reagent through the column to the empty syringe and return several times.
5. Let stand for 2 hours at ambient temperature.
6. Withdraw the ammonia/ethanol solution into one syringe and eject the solution into a screw-cap vial.
7. Heat at 55° C for 8-16 hours for complete base deprotection.

2'-Hydroxyl Deprotection:

1. Dry the ammonia/ethanol solution of crude RNA oligo (DMT OFF), after heating 8-16 hours at 55° C.
2. Add 10 microliters (per ODU of crude RNA oligo) of 1 molar tetrabutylammonium fluoride/THF (Aldrich Part No. 21,614-3). Vortex thoroughly for at least one minute, then let stand for 6-24 hours at room temperature.
3. Add an equivalent volume of 0.1M TEAA, mix, and evaporate. The pellet is substantial and appears as a viscous, semi-solid.
4. Add 1 ml of 0.1M TEAA , mix and apply to an OPC cartridge which has been prewashed with 5 ml acetonitrile, then 5 ml 2M TEAA.
5. Load the RNA oligo, recycling it through the cartridge twice at 1-2 drops per second.
6. Wash the OPC cartridge with 5 ml 0.1M TEAA, then 10 ml deionized water.
7. Elute the desalted RNA oligo (up to 50 ODU) with 1 ml 50% acetonitrile.

IMPORTANT This OPC procedure replaces a long and troublesome Sephadex column for removal of the tetrabutylammonium salts, which complicate further analysis and purification.

The RNA oligo is then ready for PAGE or HPLC purification/analysis. RNase degradation is not usually a severe problem, except when handling less than 0.1 ODU, although general RNA cautions should be considered. When the oligoribonucleotide is completely deprotected, it is labile to RNase degradation. RNase contamination is ubiquitous, necessitating sterile conditions. Procedures for preventing RNase degradation can be found in several sources.[6]

Conclusion

Oligoribonucleotides are now available from your AB DNA synthesizer. The Ogilvie chemistry, using 2' silyl protected phosphoramidite monomers, has been demonstrated on all Applied Biosystems DNA synthesizers. The efficiency of RNA synthesis is nearly equivalent to DNA synthesis as judged by trityl yields, PAGE and HPLC. However, it should be realized that subtle chemistry factors influencing the level of biological activity of synthetic oligoribonucleotides, relative to native RNA fragments, have not been exhaustively investigated. Applied Biosystems developed the first fully defined package for RNA synthesis: reagents, monomers, cycles, and procedures. This User Bulletin provides improvements in the methodology to ensure the reliable production of pure oligoribonucleotides.

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