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RNA Synthesis Using 2'-*O*-(*Tert*-Butyldimethylsilyl) Protection

Brian S. Sproat

Summary

This chapter enables the reader to carry out the solid-phase synthesis of ribonucleic acid (RNA) using β-cyanoethyl phosphoramidite chemistry combined with *tert*-butyldimethylsilyl protection of the ribose 2'-hydroxyl group. Phosphoramidite monomers are activated with 5-benzylmercapto-1*H*-tetrazole enabling fast and highly efficient coupling to the 5'-hydroxyl group of the support-bound oligonucleotide. On completion of the synthesis, the stepwise deprotection of the nucleobase, phosphate, and ribose protecting groups is carried out using optimized protocols. Subsequently the various high-pressure (performance) liquid chromatography (HPLC) procedures are described enabling the purification and analysis of the RNA. For this purpose anion-exchange and reversed-phase HPLC are used singly or in combination according to the final purity requirement of the RNA.

Key Words: Oligoribonucleotide synthesis; TBDMS; 5-benzylmercapto-1*H*-tetrazole; solid phase; triethylamine tris(hydrofluoride); anion exchange; HPLC; β-cyanoethyl phosphoramidite, chaotropes.

1. Introduction

Until a few years ago the chemical synthesis of ribonucleic acid (RNA) was still very much in its infancy, lagging way behind deoxyribonucleic acid (DNA) technology, with only a few groups worldwide able to synthesize and purify it properly. However, with the very recent intense interest in synthetic RNA, largely triggered by the discovery and utility of small interfering RNAs (siRNAs) ***(1,2)*** but also owing to the development of ribozymes and aptamers for therapeutic and/or diagnostic applications, RNA synthesis has become routine and highly reliable. This boom in the use of synthetic RNA has not only caused specialty reagent manufacturers to improve the quality of their products but has also created competition between suppliers which has subsequently led to reduced consumer cost.

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Described here are reliable procedures to enable the novice to produce good-quality synthetic RNA either manually or machine assisted. The synthesis is performed on solid-phase using standard β-cyanoethyl phosphoramidite chemistry ***(3)*** with *tert*-butyldimethylsilyl (TBDMS) protection of the ribose 2'-hydroxyl group ***(4)***, meaning that the 3'-terminal nucleoside is anchored via a succinyl linkage to an insoluble matrix, generally aminopropyl functionalized controlled pore glass (CPG) or polystyrene, contained in an appropriate reaction vessel. The nucleobases of the phosphoramidites and functionalized supports are protected with the *N-tert*-butylphenoxyacetyl group to enable mild deprotection of the RNA at the end of the synthesis ***(5)***. Reagents are introduced into the vessel for removing protecting groups and enabling chain extension of the RNA, one nucleotide at a time, and excess reagent is simply flushed away with a suitable solvent. The process is cyclical and repeated until the desired length of RNA is obtained. Because there is no intermediate purification, all reactions should be as close to 100% yield as possible. In practice the chain extension reactions have a yield of 98.5–99%. Each cycle comprises a detritylation step that unmasks the 5'-hydroxyl group for chain extension, washing with acetonitrile, a coupling step in which the desired nucleotide as a phosphoramidite building block activated with 5-(benzylmercapto)-1*H*-tetrazole ***(6)*** is added, a capping step that acylates any unreacted 5'-hydroxyl group, an oxidation step that converts the phosphite triester to a phosphate triester, a further capping step that removes any occluded iodine, and finally a washing step with acetonitrile. The use of 5-(benzylmercapto)-1*H*-tetrazole for activation of the sterically hindered 2'-*O*-TBDMS protected phosphoramidites is strongly preferred over conventional 1*H*-tetrazole regarding both speed and coupling efficiency ***(6)***.

Subsequently the fully protected support-bound RNA is deprotected in a stepwise fashion, comprising in the first step cleavage of the linkage to the solid-phase and cleavage of the nucleobase and phosphate protecting groups. In the second step the 2'-*O*-TBDMS groups are cleaved using a special fluoride reagent, namely, triethylamine tris(hydrofluoride) ***(7,8)***. For RNAs longer than about 25 nucleotides it is best to leave the 5'-terminal dimethoxytrityl group attached as it is lipophilic and can be used as a purification aid. Purification is achieved by anion-exchange and/or reversed-phase high-performance liquid chromatography (HPLC) according to the length of the RNA and the purity required.

2. Materials

1. 5'-*O*-Dimethoxytrityl-*N*(pac or tac)-2'-*O*-TBDMS-3'-*O*-(β-cyanoethylphosphoramidites) of A, U, C, and G (Pierce, Milwaukee, WI, or Proligo, Hamburg, Germany). Store dry at –20°C.

2. Solid-phase supports, either CPG (Proligo or Pierce) or polystyrene (Amersham Biosciences) functionalized with A, U, C, and G.
3. 5-Benzylmercapto-1*H*-tetrazole (BMT) (emp Biotech, Berlin, Germany).
4. Capping solutions A (fast deprotection) and B (Proligo). *Caution:* Hazardous.
5. Oxidation solution containing iodine (Proligo). *Caution:* Hazardous.
6. Deblock solution composed of 3% trichloroacetic acid in dichloromethane (Proligo). *Caution:* Toxic and corrosive.
7. DNA synthesis-grade acetonitrile containing less than 30 ppm H_2O. *Caution:* Toxic.
8. Assorted 1000 series gas-tight syringes with volumes of 0.5, 1, and 2.5 mL (Hamilton Company; Reno, NV).
9. DNA/RNA synthesizer (Applied Biosystems or Amersham Biosciences) or a glass reaction vessel fitted with a ground glass joint at the top and a fine porosity glass frit and a tap at the bottom.
10. High purity concentrated aqueous ammonium hydroxide. Irritating to eyes and respiratory system. Use in a well-ventilated fume cupboard.
11. Anhydrous 8*M* methylamine in ethanol. Irritating to eyes and respiratory system. Use in a well-ventilated fume cupboard.
12. Anhydrous dimethyl sulfoxide (DMSO) (Fluka, Biotech. grade). Wear gloves.
13. Triethylamine tris(hydrofluoride) (Aldrich). *Hazardous:* wear full protection and use only in a well-ventilated fume cupboard.
14. Anhydrous triethylamine. Irritant; use in a fume cupboard.
15. *N*-Methylpyrrolidone, peptide synthesis grade.
16. Prop-2-yl trimethylsilyl ether prepared according to Jones ***(9)***.
17. Diethyl ether. *Caution:* Fire hazard.
18. Biocompatible HPLC equipment (Amersham Biosciences).
19. Anion-exchange HPLC columns: MonoQ 5/5, Source 15Q 16/10 and/or FineLINE 35 pilot column packed with Source 15Q (Amersham Biosciences).
20. Sodium perchlorate. *Caution:* Toxic and corrosive, wear gloves.
21. Disodium ethylenediaminetetraacetic acid (EDTA).
22. Sterile 1*M* Tris-HCl buffer, pH 7.4.
23. Hi-Prep 26/10 desalting column (Amersham Biosciences).
24. Reversed-phase HPLC columns: Hamilton PRP-1, 7 × 305 mm and XTerra™ RP_8, 4.6 × 250 mm (Waters).
25. HPLC-grade acetonitrile. Toxic.
26. Ammonium bicarbonate.
27. Glacial acetic acid. *Caution:* Corrosive.

3. Methods

The methods outlined below describe: (1) the solid-phase synthesis of oligoribonucleotides, manually or machine aided, (2) the deprotection of the oligoribonucleotides, (3) the purification of oligoribonucleotides, and (4) the analysis of the purified product.

3.1. Solid-Phase Synthesis

The solid-phase synthesis of oligoribonucleotides is described in **Subheadings 3.1.1.** and **3.1.2.** For those people without access to a solid-phase synthesizer, the RNA can be synthesized manually with a minimum of equipment. The various steps involved in each cycle of the synthesis are illustrated in **Fig. 1**.

3.1.1. Manual RNA Synthesis

1. Weigh out the requisite amounts of the 4 monomers required in small vials (*see* **Note 1**) that can be closed with a septum and dry them overnight *in vacuo* over separate containers of phosphorus pentaoxide and potassium hydroxide pellets (*see* **Note 2**). For syntheses in the scale range of 1–3 μmol, it is recommended to use 8–10 equivalents of monomer per coupling relative to the amount of support used. For synthesis scales above 5 μmol a monomer excess of five fold is sufficient.
2. Release the vacuum with dry argon, and fit the monomer bottles with tight-fitting rubber septa.
3. Using a gas-tight syringe, dissolve each monomer in the requisite volume of dry acetonitrile to give a 0.1*M* solution and seal the top with Parafilm. It is not recommended to store the monomer solutions for more than 2–3 d at room temperature.
4. Transfer the requisite amount of CPG carrying the desired 3'-terminal ribonucleoside into the glass reaction vessel (*see* **Note 3**). For a 1-μmol scale synthesis the vessel should have a volume of about 5 mL, whereas for a 10-μmol scale a volume of 20 mL is more appropriate to allow good washing.
5. Add 3% trichloroacetic acid in dichloromethane (deblock solution) to the support using a Pasteur pipet, and let it percolate through. A deep orange color is produced owing to the released dimethoxytrityl cation. Continue to add acid until the effluent is no longer orange.
6. Drain the support using a slight pressure of argon.
7. Wash the CPG batchwise 8–10 times with acetonitrile (best done using a Teflon wash bottle), removing the supernatant each time with argon pressure.
8. Wash the CPG once with very dry acetonitrile, < 30 ppm H_2O, flush away with argon pressure, close the tap, and stopper the vessel.
9. Using two dry gas-tight syringes add the desired monomer as a 0.1*M* solution in acetonitrile and an equal volume of 0.3*M* BMT stock solution in acetonitrile to the CPG, stopper the vessel, and agitate several times during a period of 5 min.
10. During the coupling reaction, **step 9**, clean both syringes thoroughly with acetonitrile and store in a desiccator.
11. Drain the CPG, wash once with acetonitrile, and flush away with argon pressure.
12. Add a few milliliters of capping mixture comprising 1 vol of fast deprotection capping solution A and 1.1 vol of capping solution B (*see* **Note 4**), stopper the vessel, and agitate for 1 min, then drain.

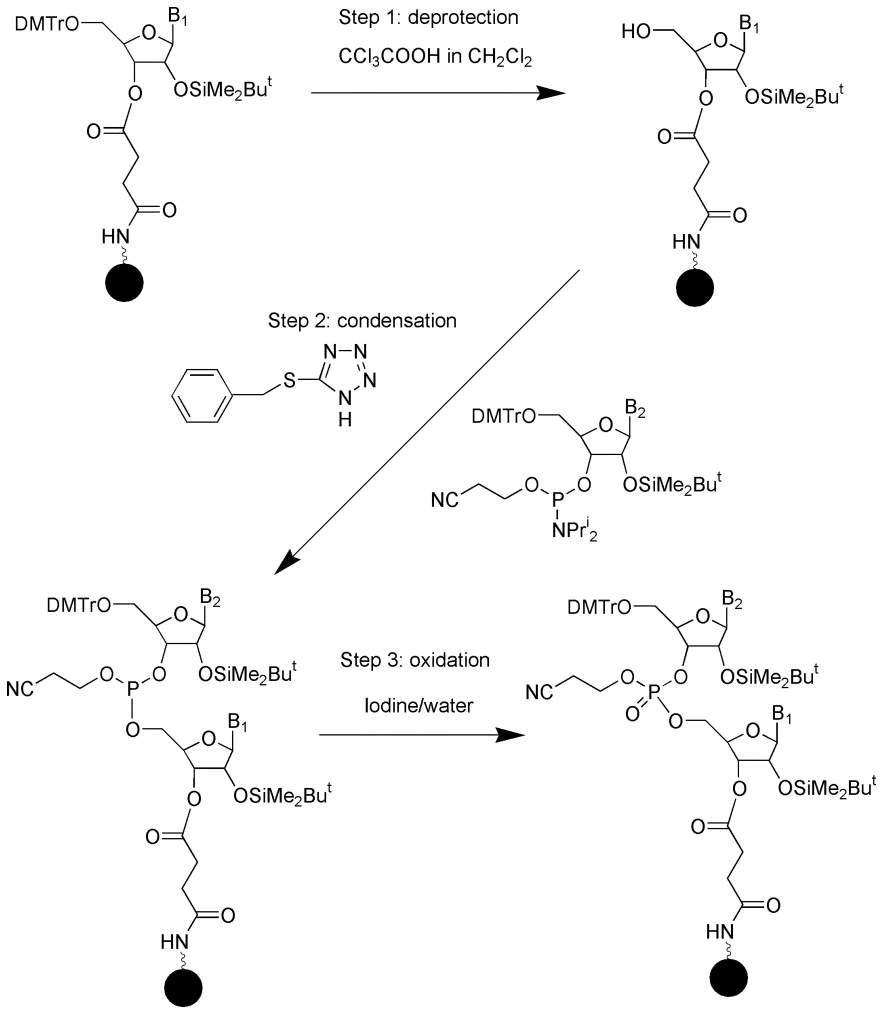

Fig. 1. Scheme illustrating a single cycle of solid-phase RNA synthesis via the phosphoramidite method. The filled-in *black circle* represents the CPG support. B_1 and B_2 represent protected nucleobases, that is, uracil-1-yl, N^4-(4-*t*-butylphenoxyacetyl)cytosine-1-yl, N^2-(4-*t*-butylphenoxyacetyl)guanin-9-yl or N^6-(4-*t*-butylphenoxyacetyl)adenin-9-yl.

13. Wash the CPG once with acetonitrile and flush away with argon pressure.
14. Add a few milliliters of oxidation mixture and allow it to slowly percolate through the CPG for 2 min (*see* **Note 5**). This oxidizes the phosphite triester to a phosphate triester.

15. Drain the CPG and wash once with acetonitrile and drain with argon pressure.
16. Add a few milliliters of fresh capping mixture, agitate for 30 s, and drain using argon pressure.
17. Wash the CPG thoroughly with acetonitrile six times, draining each time in between using argon pressure.
18. Repeat **steps 5–17** as many times as necessary until the desired sequence is reached.
19. For RNAs longer than about 25 nucleotides the final trityl group should be left on as a purification aid. For shorter RNAs remove the final trityl group as in **step 5**, and wash the CPG thoroughly with acetonitrile.
20. Dry the CPG using a stream of argon (*see* **Note 6**).

3.1.2. Automated RNA Synthesis

Follow the instructions for the particular instrument plus the program for the RNA synthesis scale you intend to use. The CPG or polystyrene support is now placed inside a small plastic cartridge. The reagents are available in the correct bottles to fit the various instruments on the market.

3.2. Deprotection

In the first deprotection step the succinate linkage connecting the 3'-terminal nucleoside to the solid support is cleaved, the β-cyanoethyl protecting groups on the internucleotide linkages are removed by β-elimination, and the nucleobase exocyclic amine protecting groups are cleaved. This step is best performed with a 1:1 mixture of concentrated aqueous ammonia and 8*M* ethanolic methylamine, which prevents premature loss of the TBDMS groups, which would otherwise lead to degradation of the RNA under basic conditions. In the second deprotection step the TBDMS groups are removed using triethylamine tris(hydrofluoride) plus an appropriate solvent.

3.2.1. Deprotection of Base Labile Protecting Groups

1. Transfer the argon-dried support to a screw-top vial or small Duran bottle equipped with a tight-fitting screw top.
2. Add a 1:1 mixture of concentrated aqueous ammonia and 8*M* ethanolic methylamine. For a 0.2–1-μmol scale synthesis, use a volume of 2 mL, otherwise use 2 mL per μmol of support (*see* **Note 7**). Close the vial or bottle tightly and seal further with Parafilm.
3. Place the vial or bottle in a preheated oven at 65°C for 30 min for small vials or 40 min for larger bottles, which take longer to reach the desired temperature.
4. Allow the vial/bottle to cool completely before opening. This can be speeded up by cooling it in an ice bath.
5. Remove the supernatant and wash the support several times with a few milliliters of ethanol/sterile water (1:1 v/v).

6. Dry the supernatant and combined washings in a Falcon tube in a SpeedVac or, for larger volumes, evaporate to dryness on a rotary evaporator. Do not use water bath temperatures above 30°C for trityl-on material.
7. Dry the residue once by evaporation of absolute ethanol.

3.2.2. Desilylation of Trityl-Off RNA

1. In a Falcon tube dissolve the trityl-off residue obtained as described in **Subheading 3.2.1.** in a 1:1 mixture of dry DMSO and triethylamine tris(hydrofluoride) ***(10)***, using 600 µL per µmol and sonicate briefly. If the oligoribonucleotide has been dried down in a glass flask, dissolve it in the required volume of dry DMSO by gently warming the flask with a hair dryer, then transfer the solution to a Falcon tube and add an equal volume of the fluoride reagent in a well-ventilated fume cupboard.
2. Close the tube and seal with Parafilm and place in a preheated oven at 65°C for 2.5 h.
3. Cool the tube to room temperature.
4. Quench the reaction by addition of 2 vol of isopropyl trimethylsilyl ether ***(9)***, close, and shake vigorously at intervals during 10 min. A precipitate appears.
5. Carefully open the tube and add 5 vol of diethyl ether; close and agitate.
6. Collect the precipitate by centrifugation at 2200*g* at 4°C for 5 min.
7. Remove the supernatant by careful decantation.
8. Resuspend the pellet in ether, close the tube, agitate, and collect the precipitate by centrifugation.
9. Repeat **steps 7** and **8**.
10. Dry the pellet carefully *in vacuo.*

3.2.3. Desilylation of Trityl-On RNA

1. In a Falcon tube dissolve the trityl-on residue, as obtained in **Subheading 3.2.1.**, in 600 µL per µmol of a freshly prepared solution of *N*-methylpyrrolidone/triethylamine/triethylamine tris(hydrofluoride) (6:3:4 by vol) ***(11)***. For material that has been dried down in a glass flask, dissolve the residue in the minimum volume of dry DMSO, transfer the solution to a Falcon tube, and add the freshly prepared desilylation solution.
2. Perform **steps 2–8** as described in **Subheading 3.2.2.**
3. Dry the RNA pellet very briefly with an argon stream and then dissolve it immediately in sterile 0.1*M* aqueous ammonium bicarbonate ready for immediate purification by reversed-phase HPLC.

3.3. Purification

This section is devoted to the anion-exchange HPLC purification of fully deprotected RNA using gradients of sodium perchlorate ***(11)*** or lithium perchlorate ***(12)*** (*see* **Note 8**) as chaotropes, the reversed-phase HPLC purification of trityl-on RNA, detritylation, and desalting.

3.3.1. Anion-Exchange HPLC Purification

Anion-exchange HPLC is recommended as the first or only purification step for oligoribonucleotides shorter than about 25 nucleotides. It generally results in a purity of 95–98%. It is recommended to use a gradient of sodium perchlorate in sterile water/acetonitrile (9:1 v/v) containing 50 m*M* Tris-HCl buffer, pH 7.6, and 50 μ*M* EDTA. The purpose of the EDTA is to complex traces of heavy metals that could otherwise lead to cleavage and degradation of the RNA. Source 15Q 16/10 columns are recommended for purification of 1-μmol scale syntheses with a flow rate of 5 mL per min. For syntheses in the 10- to 100-μmol scale, these are best purified using a FineLINE Pilot 35 column packed with Source 15Q and eluted at 20 mL per min (*see* **Note 9**). The low-salt or A buffer contains 10 m*M* sodium perchlorate and the high-salt or B buffer contains 600 m*M* sodium perchlorate. A gradient from 0–60% B during 40 min gives good resolution. When not in use store the columns in 20% ethanol in sterile water to prevent microbial growth. Prior to use flush the column with several column volumes of sterile water before equilibrating the column with buffer B followed by buffer A. The desired product peak is the late-eluting major component. This material is then desalted as described in **Subheading 3.3.4.** A typical trace of an anion-exchange HPLC purification is shown in **Fig. 2**. In this example the oligomer is a 21-mer synthesized manually on a 20-μmol scale and purified on Source 15Q packed in a FineLINE Pilot 35 column. As can be seen, the failure peaks are very small compared to the product peak, which elutes at 24–27 min.

3.3.2. Reversed-Phase HPLC Purification of Trityl-On RNA

The effect of the highly lipophilic dimethoxytrityl group is to profoundly retard the full-length product when purified on a reversed-phase HPLC column. Although the best separation of failure peaks from the desired trityl-on product peak is obtained using aqueous triethylammonium acetate/acetonitrile buffers, for ease of salt removal and minimal damage to the RNA, the use of ammonium bicarbonate is preferred.

Recommended columns for trityl-on purification are the Hamilton PRP-1, 7 × 305 mm for a few μmol scale syntheses or a 21.5- × 250-mm column for 10–20-μmol scale purifications (*see* **Note 10**). Buffer A is 0.1*M* ammonium bicarbonate prepared in sterile water and buffer B is 0.1 *M* aqueous ammonium bicarbonate/acetonitrile (1:1 v/v). Recommended gradient is 0–90% B during 40 min. The failure peaks elute early and are well separated from the desired trityl-on product peak, which elutes last. Collect the product fraction in a polypropylene Falcon tube and dry down on a SpeedVac. Residual ammonium bicarbonate is then removed by lyophilization of the product, which is now ready for detritylation. A typical trace of a trityl-on RNA purification by

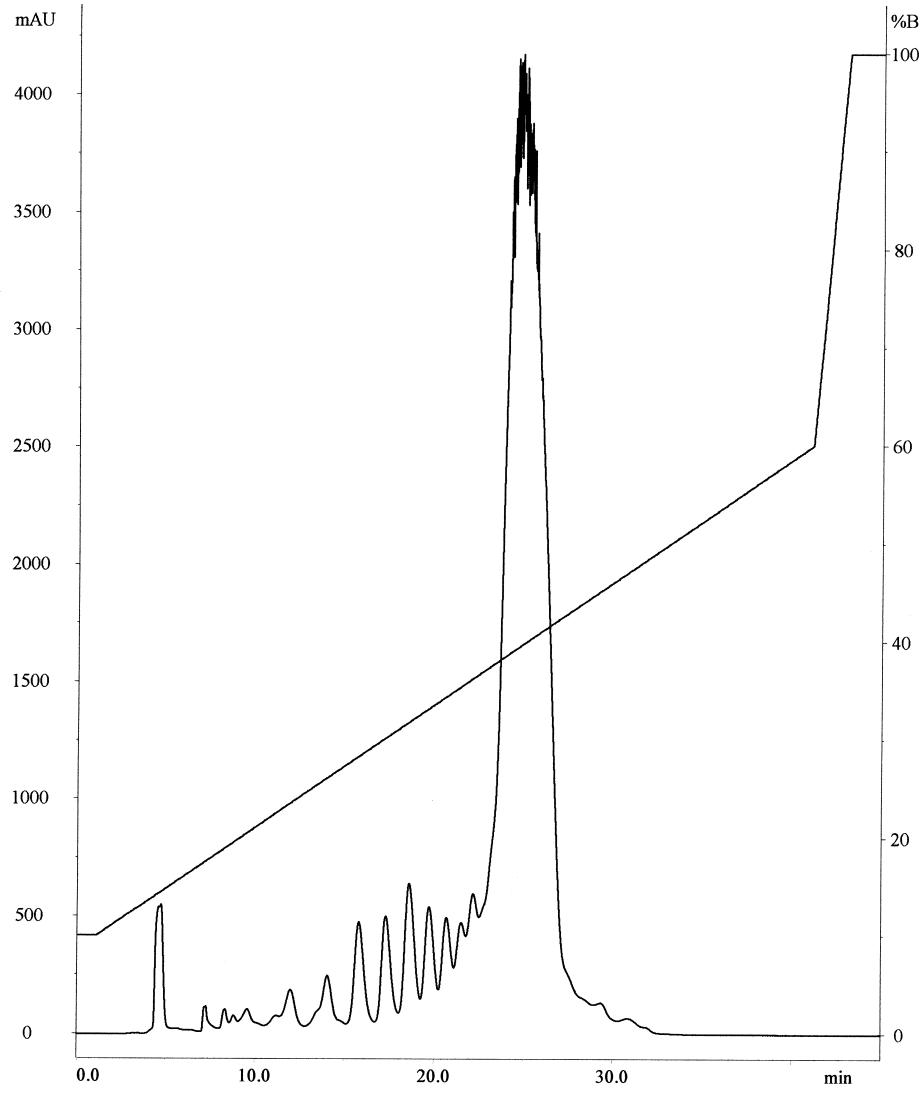

Fig. 2. A preparative anion-exchange HPLC trace of a 21-mer oligoribonucleotide synthesized manually on a 20-μmol scale and purified on Source 15Q packed in a FineLINE Pilot 35 column. Absorbance was monitored at 280 nm.

reversed-phase HPLC is shown in **Fig. 3**. The example shows a trityl-on 58-mer oligoribonucleotide synthesized by machine on a 1-μmol scale and purified on a 7- × 305-mm Hamilton PRP-1 column. The desired product peak elutes at 20–22 min well separated from the trityl-off failure sequences (at 7–12 min).

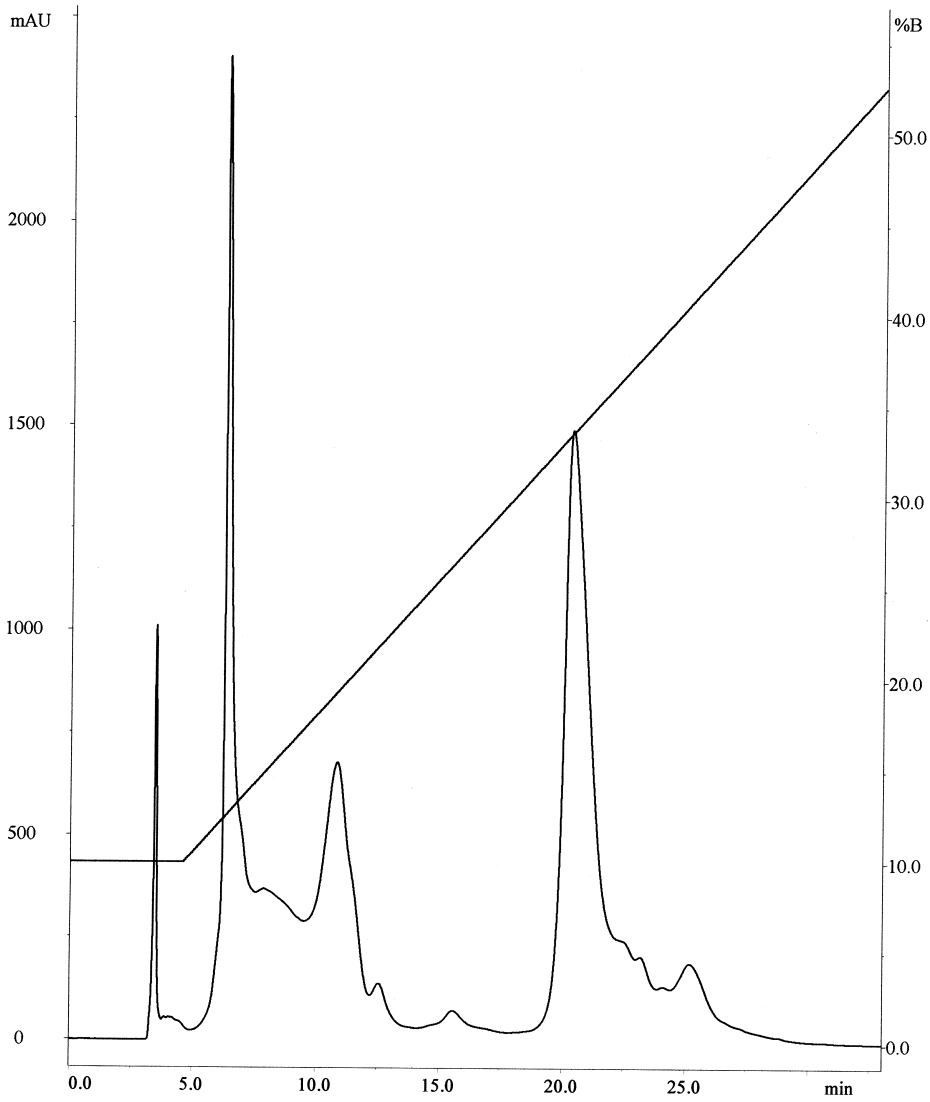

Fig. 3. Reversed-phase HPLC trace of a trityl-on 58-mer oligoribonucleotide purified on a Hamilton PRP-1 column. Absorbance was monitored at 295 nm.

3.3.3. Detritylation of Trityl-On RNA

1. Dissolve the purified trityl-on RNA as obtained from **Subheading 3.3.2.** in 3% sterile aqueous acetic acid (200 µL per µmol) and keep 45 min at room temperature. The pH should be approx 3.5.

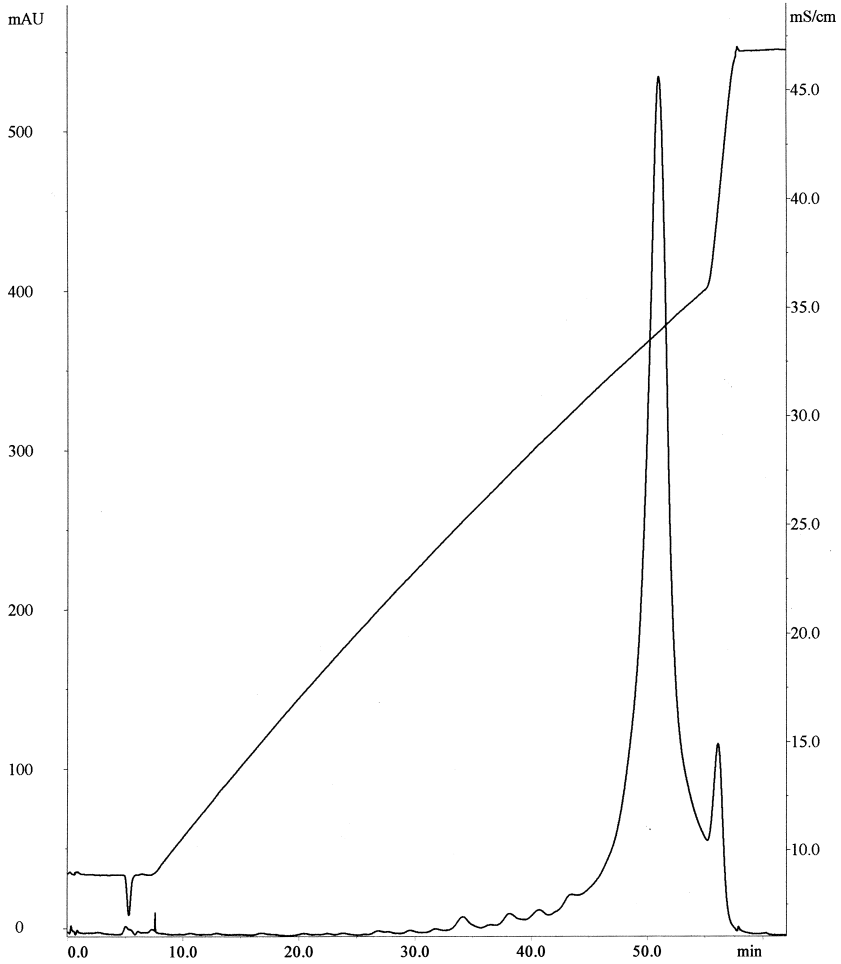

Fig. 4. Anion-exchange HPLC trace showing the purification of a detritylated 58-mer oligoribonucleotide on a Source 15Q 16/10 column. The compound was initially purified trityl-on by reversed-phase HPLC. Absorbance was monitored at 295 nm.

2. Neutralize by careful addition of solid ammonium bicarbonate until evolution of carbon dioxide ceases. The pH will now be approx 7.8.
3. Repurify the product by anion-exchange HPLC as outlined in **Subheading 3.3.1.**, which in addition converts the product into the sodium form.
4. Prior to use the product must be desalted (*see* **Subheading 3.3.4.**).
   Fig. 4 shows the trace of the anion-exchange HPLC purification of the 58-mer oligoribonucleotide that was initially purified trityl-on by reversed-phase HPLC (*see* **Fig. 3**). The peak was collected from 49 to 52 min.

3.3.4. Desalting by HPLC

For oligonucleotides purified by anion-exchange HPLC it is necessary to remove the excess salt, buffer, and EDTA from the RNA regardless of the intended application. This is readily achieved using a desalting column such as the Hi-Prep 26/10, which is filled with Sephadex G-25. The sample should be loaded in a volume not greater than 15 mL, but preferably less than 10 mL, to obtain a complete separation between the first eluting RNA and the salt peak that comes after it. The column is stored in and eluted with 20% ethanol in sterile water; this prevents microbial growth. Monitoring of the column effluent by ultraviolet light and conductivity avoids contamination of the RNA caused by incompletely removed salt.

The salt-free RNA in aqueous ethanol is first concentrated in a SpeedVac and finally lyophilized to obtain the pure RNA in its sodium form as a fluffy white solid.

3.4. Analysis of the Purified RNA

Generally the purity of the final product is checked by analytical anion-exchange HPLC using a MonoQ 5/5 column and buffers according to **Subheading 3.3.1.** Alternatives are capillary gel electrophoresis and polyacrylamide gel electrophoresis, both of which operate under denaturing conditions. These methods are not described here.

RNA destined for structural studies, such as nuclear magnetic resonance spectroscopy (NMR) or X-ray crystallography, is also best checked in addition by analytical reversed-phase HPLC on a high-resolution column such as the 5-μ*M* XTerra RP_8, 4.6 × 250 mm. In this case a gradient from 0–25% acetonitrile in aqueous ammonium bicarbonate will suffice, combined with a flow rate of 1 mL/min^{-1}. As an example an analytical reversed-phase HPLC trace of the double HPLC-purified 58-mer oligoribonucleotide is illustrated in **Fig. 5**.

As an absolute check on product authenticity, the molecular weight of the RNA should be determined by mass spectroscopy, either electrospray ionization or matrix-assisted laser desorption ionization ***(13)*** (*see* **Note 11**). This is a must when modified nucleotides are incorporated. In addition, the RNA can also be sequenced by standard methods to check the absolute order of the monomeric units within the sequence. Here the reader is advised to refer to the appropriate literature as these methods are outside the scope of this chapter.

4. Notes

1. The most suitable vials for this purpose are those amber glass bottles that are used by suppliers of DNA and RNA phosphoramidites and fit directly on ABI synthesizers.

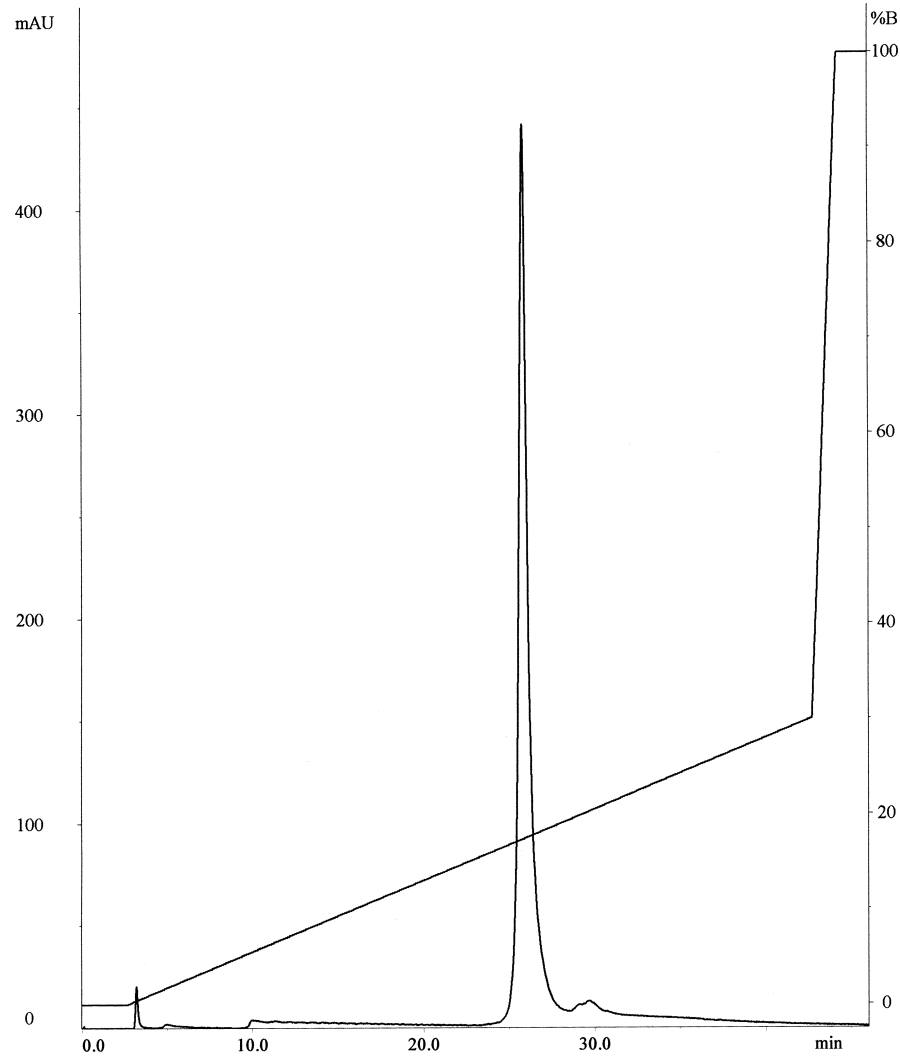

Fig. 5. Analytical reversed-phase HPLC trace of double HPLC purified 58-mer oligoribonucleotide run on a 5-μ*M* XTerra RP_8, 4.6 × 250-mm column. Absorbance was monitored at 260 nm.

2. It is essential to allow monomers to reach room temperature before opening and weighing out material; otherwise condensation will occur leading to eventual degradation. The bottle contents should be put back under argon before sealing and storing again at –20°C. The drying step ensures the removal of any residual moisture that could otherwise impair the coupling reaction.

3. Suitable glass reaction vessels in a variety of sizes can be made by any laboratory glassblower following the drawing in the literature ***(14)***. The vessel is basically a cylindrical tube, fitted with a porous glass frit at the bottom that is connected to a narrow internal-diameter glass tube closed off by a tap. The top end of the vessel is equipped with a B14 ground glass joint that can be closed with a simple B14 stopper.
4. The capping mixture is best made up in a dry-stoppered flask in a volume sufficient for one day's usage.
5. Because the standard oxidation mixture is only 16 m*M* in iodine, that is, 16 μmol per mL, use enough mixture for larger scale syntheses to ensure that there is an ample excess of reagent. For a 20-μmol scale synthesis, use 5 mL of the oxidation mixture. Incomplete oxidation will result in cleavage at the phosphite triester bond during the subsequent detritylation step, hence leading to a reduced yield of full-length product.
6. CPG bearing trityl-off protected RNA can be stored cold and dry ready for deprotection at an appropriate time; however, CPG-bearing trityl-on RNA must be deprotected and purified immediately on completion of the synthesis.
7. Always work in a well-ventilated fume cupboard when working with ammonia and methylamine. Avoid too great an air space in the vial or bottle, otherwise most of the ammonia and methylamine will end up in the vapor phase. In the worst case this could lead to an incomplete deprotection.
8. Oligoribonucleotides that contain 4 or more consecutive Gs are notoriously difficult to purify by anion-exchange HPLC because they form tetraplexes and higher aggregates in solution. Such RNAs are best purified using a lithium perchlorate gradient because these structures do not form if lithium ions are present instead of sodium or potassium ions.
9. To avoid product shoot through owing to the ionic strength of the applied sample solution being too high, it is advisable to apply the crude RNA sample to the column dissolved in a volume of 10–50 mL of 50 m*M* Tris-HCl buffer, pH 7.4, using a 10- or 50-mL super loop. As an alternative, desalt the sample prior to purification.
10. As an alternative for larger scale separations, a FineLINE Pilot 35 column packed with Source 15RPC can be used.
11. RNA samples as sodium salts are not suitable for mass spectrometry. Mass spectrometry samples are best prepared as ammonium salts. This can be done in several ways. One way is to do anion-exchange HPLC using ammonium sulfate for elution, followed by desalting on a small NAP cartridge. A second way is to take a small aliquot of the RNA in its sodium form and exchange the sodium ions with ammonium ions by using ammonium form Dowex 50 resin. A third way is to purify a small sample of RNA by reversed-phase HPLC using the aqueous ammonium bicarbonate/acetonitrile system followed by lyophilization of the residual salt. Once in the ammonium form the RNA should not in contact with glass surfaces again, otherwise sodium and potassium ions will be picked up that will degrade the quality of the mass spectra.

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