High performance RNA oligonucleotide purification using Agilent TOP-RNA

Application Note

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Introduction

Because of the significant progress achieved in the solid phase chemical synthesis of oligonucleotides during the past four decades, synthetic oligonucleotides have become readily available and have fuelled the biotechnology revolution that has irreversibly changed biomedical research and the pharmaceutical industry.

Although a powerful technique, solid phase synthesis has some drawbacks. The main limitation is the need for very high coupling yields in every chain extension step. A consequence of yields of <100% is the accumulation of failure sequences containing deletions. Separation of the full-length product from the shorter failure sequences and especially the n-1 failure sequence is a significant problem.

Trityl-on purification enhances the selectivity between the full length sequence and the truncated failures. Solid phase extraction (SPE) tubes provide a low cost method for this type of purification. Historically, these are capable of performing efficient separations for DNA oligomers of up to 50 bases in length, but have been of limited value for RNA purifications. The reasons for this have been poorly optimized media and the lack of a robust de-silylation procedure that yields de-silylated trityl-on oligos for purification.

Agilent has developed an optimized tube system, trityl-on oligonucleotide purification (TOP), specifically designed to work for RNA oligomers. The method can purify as few as one, or as many as 96 RNA oligonucleotides in less than 20 minutes using a vacuum procedure (requires the Agilent VersaPlate baseplate, part no. 75700001).

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Materials and reagents

- TOP-RNA 100 mg tubes, 96/pk - part no. 7573915C
- Vac Elut 20 manifold - part no. 12234100
- 2 M TRIS Quenching Buffer pH 8.2
- 2 M triethylammonium acetate (TEAA)
- 1-methyl-2-pyrrolidinone, anhydrous (99.5%)
- Triethylamine (≥ 99.5%)
- Triethylamine trihydrofluoride (98%)
- Acetonitrile (HPLC grade)
- Trifluoroacetic acid (99%)
- 2% trifluoroacetic acid in RNase free water
- Ammonium bicarbonate (≥ 99.5%)
- 1 M ammonium bicarbonate:acetonitrile (70:30 v/v)

De-silylation procedure (200 nmol scale preparation)

1. **Cleavage/base deprotection.** The oligomer should be cleaved from the synthesis support and base deprotected using standard methods (typically concentrated aqueous ammonium hydroxide cleavage, then 55 °C, 16 hours in ammonia solution for base deprotection or 1:1 concentrated aqueous ammonium hydroxide: 40 wt % aqueous methylamine at 65 °C for 10 minutes (the RNA cytosine phosphoramidite must be acetyl protected for this method to work successfully).
2. **Drying of oligonucleotide.** The oligonucleotide is then dried either via vacuum/centrifuge concentration or by freeze-drying. (The bulk of the ammonia is removed prior to freeze-drying by bubbling nitrogen through the sample).
3. **De-silylation.** Add 0.25 mL TEA-3HF de-silylation solution (1.5 mL 1-methyl-2-pyrrolidinone, 0.75 mL triethylamine, 1.0 mL triethylamine trihydrofluroride mixed in that order in a plastic tube) to the dried oligoribonucleotide. Shake to dissolve the oligoribonucleotide and place in an oven at 65 °C for 120 to 150 minutes.
4. **Quench.** Remove from the oven and allow to cool. Add 1.75 mL of 2 M TRIS Quenching Buffer at pH 8.2 to the sample. The sample is now ready for purification.

Method

1. **Condition tube.** With the vacuum on (7 in Hg), add 0.5 mL of acetonitrile to the tubes. Ensure a drop rate of approximately 1 to 2 drop/sec. The vacuum may remain on and unadjusted through step 9.
2. **Equilibrate tube.** As soon as possible, after the acetonitrile has flowed through, add 1 mL of 2 M TEAA to the tubes.
3. **Apply sample.** Add pretreated sample. The sample can be added in 1 mL aliquots.
4. **Wash.** Add 1 mL of 90:10 v/v 2 M TEAA: acetonitrile solution.
5. **Wash.** Add 1 mL of RNase free water.
6. **Detritylate.** Add 2 x 1 mL of 2% trifluoroacetic acid in RNase free water.
7. **Rinse.** Add 2 x 1 mL of water (RNase free).
8. **Prepare to collect sample.** Remove the cover, using the vacuum release valve. Place a collection tube in the correct position in the rack and replace the cover.
9. **Elute.** Re-apply the vacuum and add 1 mL of 1 M ammonium bicarbonate:acetonitrile (70:30 v/v) to elute product.

The purification should take approximately 10 to 15 minutes if carried out without any breaks between the additions of the solutions to the tube.

Results

A series of trityl-on RNA oligomer sequences were purified using the above protocol on 100 mg TOP-RNA tubes. A summary of the yield and purity data for the purified products is provided below in Table 1, and HPLC chromatographs in Figures 1, 2, 3 and 4.

Table 1. *Purity and yield values from reversed-phase chromatograms*

Sequence	Crude purity	Final purity	Yield
Figure 1	58%	95%	90%
Figure 2	61%	95%	87%
Figure 3	63%	97%	86%
Figure 4	45%	95%	86%
Figure 5	27%	94%	85%

HPLC Conditions

HPLC analysis of crude and TOP-RNA tube-purified RNA.

Column:	Agilent PLRP-S 5 µm, 4.6 x 150 mm (part no. PL1111-3500)
Mobile phase A:	100 mM TEAA, pH 7
Mobile phase B:	Acetonitrile
Gradient:	A/B (95:20) from 0 to 20 minutes
Flow rate:	1 mL/min
Detection:	UV @ 256 nm, temperature ambient for 21mer sequences, 60 °C for 63mer

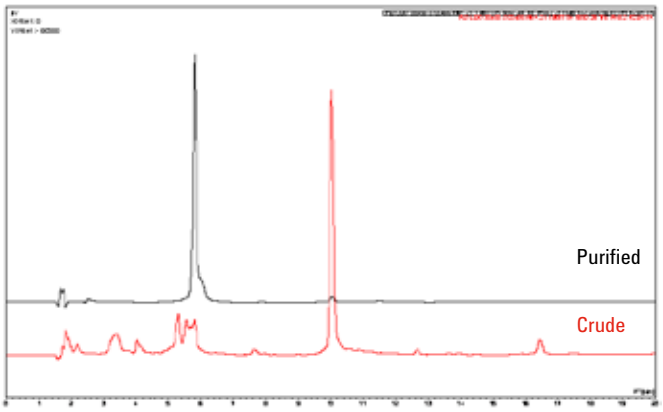

Figure 1. *Purification of a 21mer RNA sequence - 5′(DMT) UUG UAC UAC UUU CUG ACG CUU 3′*

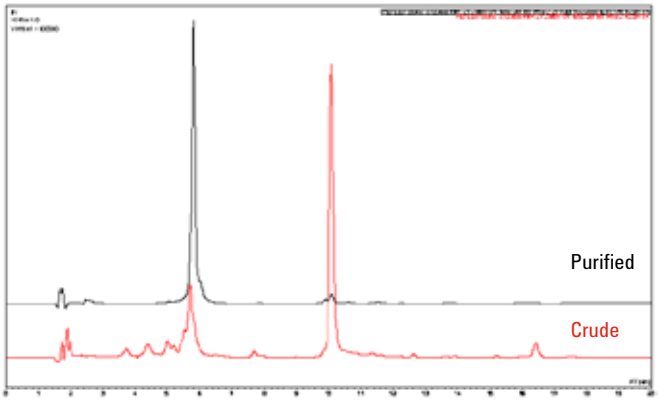

Figure 2. *Purification of a 21mer RNA sequence - 5′(DMT) UCC UUU GUC UAU CUC CGU UCA 3′*

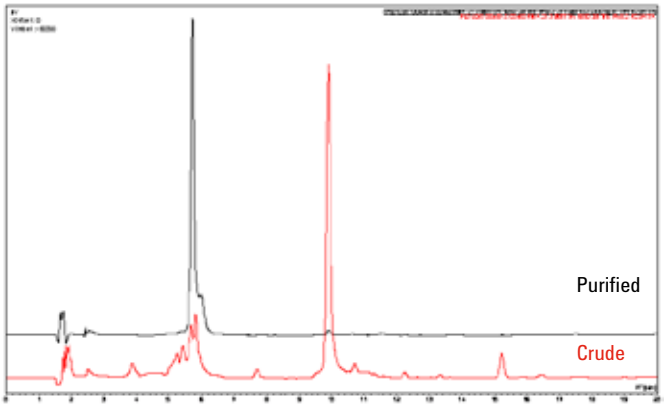

Figure 3. *Purification of a 21mer RNA sequence - 5′(DMT) CUC AAC AUU CAU UGC UGU CGG 3′*

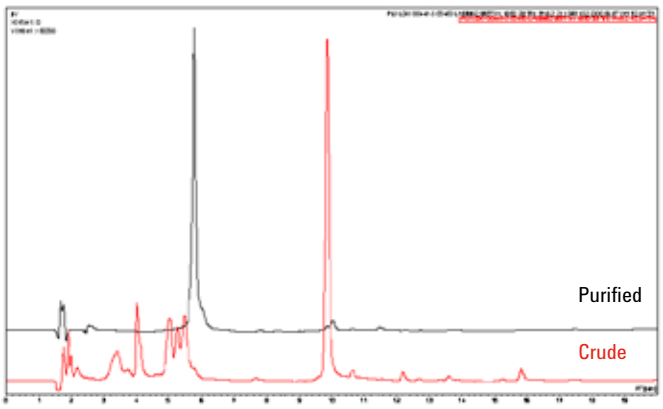

Figure 4. *Purification of a 21mer RNA sequence - 5′(DMT) ACU CGG CUU CCU CCU CUU 3′*

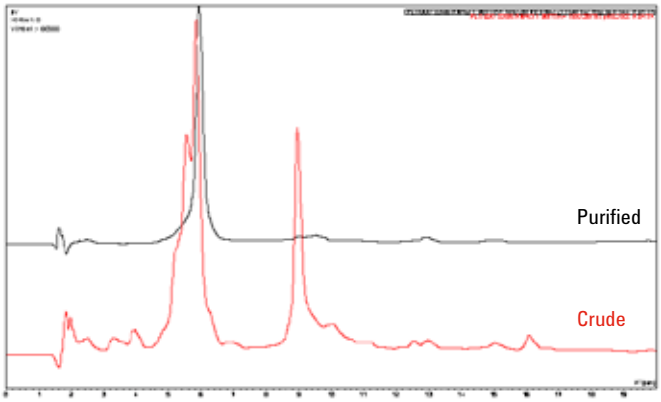

Figure 5. *Purification of a 63mer RNA sequence - 5′(DMT) UCC UUU GUG UAU CUC CUC CGU UCA CUC AAC AUU CAU UGC UCU CGG ACU CGG CUU CCU CCU CCU CUU 3′*

Conclusion

This protocol, combined with the TOP-RNA high-affinity sorbent, 96-format, and optimized reagents, provides a simple, high throughput process for effective purification of both long and short chain RNA oligonucleotides. Both high yields (> 85%) and high purity were achieved (> 90%).

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