

- One step purification of a DNA 20-mer
- Gram scale purification capacity
- Purification under denaturing conditions using aqueous buffers

Abstract

A single step chromatographic method for pilot scale purification of a synthetic DNA 20-mer under denaturing conditions was developed. The method was based on anion exchange chromatography with SOURCE™ 15Q medium. Purification of a 0.15 g crude sample was done on a 30 ml column run on BioPilot® System. This gave a final product with a purity of 96%. Cycle time was 25 minutes, which corresponds to a purification capacity of about 8 g of crude DNA oligomer per 24 hours.

Introduction

Oligonucleotides are widely used as tools in basic research and as reagents in diagnostic kits. They are also increasingly used in therapeutic applications. Although the field of oligonucleotide therapeutics is still in its infancy, available data suggest a tremendous potential for antisense oligonucleotides drugs.

Technology to synthesize gram quantities of oligonucleotides has been one key requirement in this rapidly evolving field.

The commercially available OligoPilot™ DNA/RNA Synthesizer now meets this requirement for large scale, economic synthesis of oligonucleotides. As a result, the focus of attention has turned to the need for an efficient method for purifying synthetic oligonucleotides on a large scale.

Controlled and reproducible drug action puts very high purity requirements on the final oligonucleotide product. Furthermore, the purification procedure itself must have high reliability and productivity.

While a number of small scale oligonucleotide purification procedures (i.e. up to mg level) have been described, reports of gram level purification procedures suitable for therapeutic or diagnostic oligonucleotides are lacking.

In this Application Note, we describe the development of a single step chromatographic purification procedure for a synthetic, unmodified DNA 20-mer. This method, based on BioPilot chromatography system and SOURCE 15Q anion exchange medium, is capable of handling up to 8 grams of DNA per 24 hours. The purification method matches the production capacity of OligoPilot DNA/RNA Synthesizer (400 μmol synthesis scale).

Experimental

Details of chromatographic equipment and conditions are shown in the figures.

Sample loads were calculated from 260 nm absorbance measurements of diluted samples using a conversion factor of 1 AU = 0.033 mg DNA.

Purity was determined by analytical reversed phase chromatography or analytical anion exchange chromatography (see Fig. 5) using the peak integration function in FPLCdirector™ and SMART™ Manager respectively. Purity (in %) was calculated as:

$$\frac{\text{peak area of full length product}}{\text{total peak area}} \times 100$$

Yield, expressed as mg full length product, was determined from analytical chromatography data (see “purity determination” above) and 260 nm absorbance measurements as:

$$\text{purity} \times A_{260} \times 0.033$$

Synthesis

Table 1 describes the conditions for synthesizing the DNA 20-mer. The purity of the crude 20-mer was 53% as determined by analytical reversed phase chromatography. For initial experiments, another 20-mer sequence was used (see Fig. 1).

Table 1. Conditions for synthesis and deprotection of DNA 20-mer.

Instrument:	OligoPilot™ DNA/RNA Synthesizer
Sequence:	ATA CCG ATT AAG CGA AGT TT
Synthesis scale:	400 μmol
Detritylation time:	3.3 min
Phosphoramidite:	1.5 equivalents
Coupling time:	5.0 min
Cycle time:	29 min
Waste/cycle:	726 ml
Coupling efficiency:	>99%
Yield:	1 620 mg

Purification

1. Goals

The requirements for the purification procedure were set as:

- High productivity to match the synthetic capacity of OligoPilot
- More than 95% purity of the final product
- Scaleability
- Simplicity

2. Separation technique

The procedure developed was based on anion exchange chromatography using SOURCE 15Q, a polymer-based medium with a bead size of 15 μm.

Separation of nucleic acids by anion exchange chromatography is mainly based on electrostatic interactions between phosphate groups in the nucleic acid backbone and positively charged groups on the ion exchange matrix ($-N^+(\text{CH}_3)_3$ groups on SOURCE 15Q). Oligonucleotides are adsorbed to the ion exchange matrix and eluted by a salt gradient in order of increasing length. Preparative high performance anion exchange media can thus be used to isolate full-length oligonucleotide product (n) from (n-1) failure sequences. Furthermore, the high pH stability of SOURCE 15Q allows purification under denaturing conditions at pH 12. This is of vital importance to avoid aggregation of self-complementary or G-rich oligonucleotides.

3. Sample pretreatment

A DNA 20-mer was synthesized as described in Table 1. After synthesis, the protecting groups were removed and the reaction mixture was filtered and then evaporated to dryness as described in the OligoPilot User Manual. The oligonucleotide product was then redissolved in an appropriate solution for subsequent chromatography.

4. Loading study

Initial optimization experiments were done with small sample loads on a RESOURCE™ Q 1 ml column (a pre-packed column containing SOURCE 15Q) run on FPLC® System.

Figure 1 shows the optimized separation run at a sample load of 0.1 mg oligonucleotide/ml medium. As the aim of the overall method development was to optimize a method suitable for pilot scale production, a separation time of approximately 15 min was chosen. Although much faster separations (about 4 minutes) are possible with SOURCE 15 media, such speed offers no real advantage in production, where other processing steps are often rate limiting.

Optimized purification

Sample: DNA 20-mer, ATA CCA ATT AAA CAA AAT TT
Sample load: 0.1 mg/ml medium
Column: RESOURCE™ Q 1 ml
System: FPLC® System controlled by FPLCdirector™ software
Buffer A: 10 mM NaOH, pH 12
Buffer B: 10 mM NaOH, pH 12, with 1.5 M NaCl
Flow rate: 300 cm/h
Gradient: 0.25–0.75 M in 30 column volumes
Detection: UV absorption at 254 nm, 5 mm cell
Cycle time: 25 min

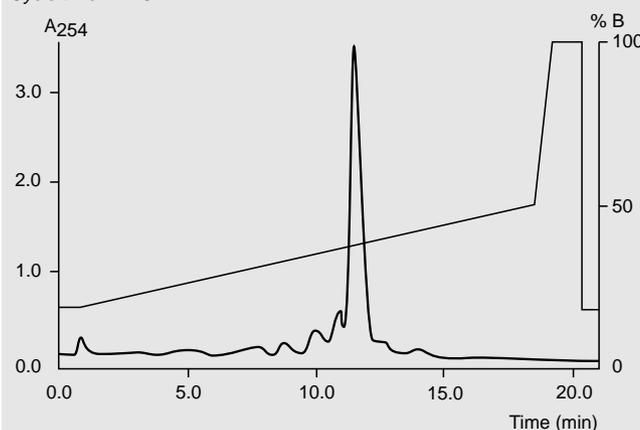


Fig. 1. Optimized purification of a DNA 20-mer using RESOURCE™ Q 1 ml at a sample load of 0.1 mg oligonucleotide/ml medium. The slope shows the programmed gradient.

A study was done to establish the loading capacity. Loadings were varied between 0.1 and 10 mg/ml medium on a RESOURCE Q 6 ml column. At a load of 5 mg/ml medium, the purity and yield were still satisfactory (Table 2). At a load of 10 mg/ml, however, resolution was insufficient (data not shown).

Table 2. Purity and yield with a sample load of 5 mg crude DNA/ml medium on a RESOURCE Q 6 ml column. Purity and yield were calculated from analytical reversed phase chromatography data.

Material	Purity (%)	Yield (mg)
Crude material	53	—
Purified pool	96	14.6

5. Scalability

To determine the scalability of the purification method, a sample load of 1 mg/ml medium was applied to three columns packed with 1, 6 and 30 ml SOURCE 15Q (Fig. 2). These columns have the same bed height (3 cm) but increasing diameters. The method was scaleable as judged by comparing the fine details in the chromatograms and by analysis of collected fractions (data not shown).

6. Scale-up

A sample containing 150 mg of crude 20-mer was purified in a single run on BioPilot chromatography system controlled by UNICORN software (Fig. 3), a combination dedicated for process development and small scale production. A 30 ml column packed with SOURCE 15Q was used. Figure 4 shows the result. Individual fractions were screened by analytical anion exchange to check purity before material was pooled.

Table 3 summarizes this scaled up purification. The yield was 56 mg full-length product, and the purity was 96%. Note that yield and purity are interrelated. Yield could be increased by including adjacent fractions in the pool (Fig. 4) at the cost of a decrease in purity. Conversely, sacrificing yield by excluding the end fractions gave higher purity. Yield could also be increased by pooling and re-running side fractions.

Table 3. Purity and yield with a sample load of 5 mg crude DNA/ml medium on a 30 ml SOURCE 15Q column (see Fig. 4 for details). Purity and yield of pooled material were calculated from analytical anion exchange chromatography data.

Material	Purity (%)	Yield (mg)
Crude material	53	—
Pool 51–57	99	37
Pool 49–61	96	56

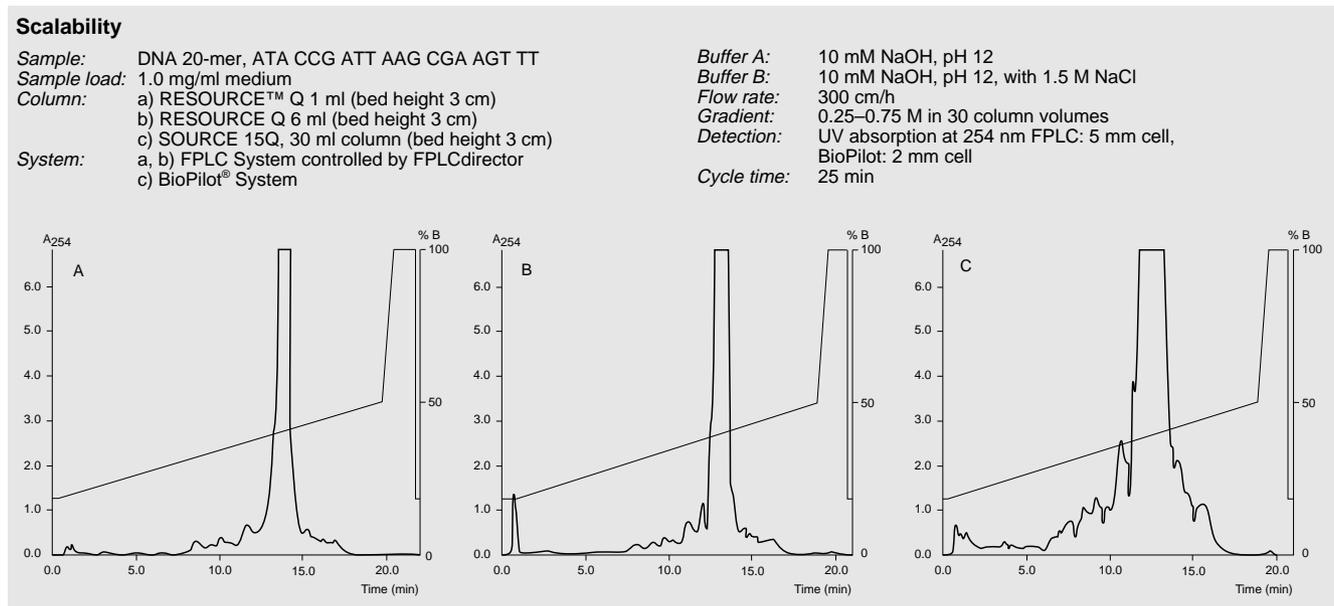


Fig. 2. Scalability of the optimized purification method for a DNA 20-mer, using 1 ml, 6 ml and 30 ml columns with SOURCE™ 15Q. The slopes show the programmed gradient.



Fig. 3. BioPilot® System with UNICORN™ control.

7. Analysis of purified material

Pooled material (49–61) from the 150 mg load experiment (Fig. 4) was analysed by reversed phase chromatography and anion exchange chromatography on a non-porous medium (Fig. 5 A and B). A control experiment verified that a mixture of DNA oligomers containing 12–30 nucleotide units was completely resolved by the analytical anion exchange method (Fig. 5 C).

Purification on BioPilot System

Sample: DNA 20-mer, ATA CCG ATT AAG CGA AGT TT
Sample load: 5 mg/ml medium
Column: BioPilot 35/100, 30 ml (bed height 3 cm) packed with SOURCE 15Q
System: BioPilot System with UNICORN control
Buffer A: 10 mM NaOH, pH 12
Buffer B: 10 mM NaOH, pH 12, with 1.5 M NaCl
Flow rate: 300 cm/h
Gradient: 0.25-0.75 M in 30 column volumes
Detection: UV absorption at 254 nm, 2 mm cell. Conductivity (sloping curve)
Cycle time: 25 min

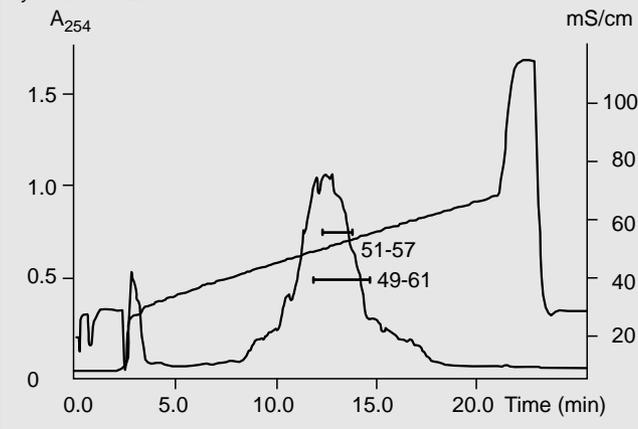


Fig. 4. Purification of 0.15 g DNA 20-mer using SOURCE 15Q and BioPilot System.

Purity analysis

A) Reversed phase chromatography

Sample: Pool 49–61 from SOURCE 15Q run (see Fig. 4)
Column: PepRPC HR 5/5 (1 ml)
System: FPLC System, controlled by FPLCdirector software
Eluent A: 100 mM triethylammonium acetate pH 7, 10% acetonitrile
Eluent B: 100 mM triethylammonium acetate pH 7, 30% acetonitrile
Gradient: 0–100% B in 30 column volumes
Flow rate: 300 cm/h
Detection: UV absorption at 254 nm, 5 mm cell

B) Anion exchange chromatography

Sample: Pool 49–61 from SOURCE 15Q run (see Fig. 4)
Column: Mini Q™ PC 3.2/3
System: SMART™ System
Buffer A: 50 mM triethyl ammonium acetate
Buffer B: 50 mM triethyl ammonium acetate, 60% acetonitrile
Gradient: 0–10% B in 5 min, 10–70% B in 30 min
Flow rate: 200 µl/min
Detection: 254 nm

C) Anion exchange chromatography

Sample: Mixture of synthetic DNA (dA), 12–30 mers
 Other conditions as in B)

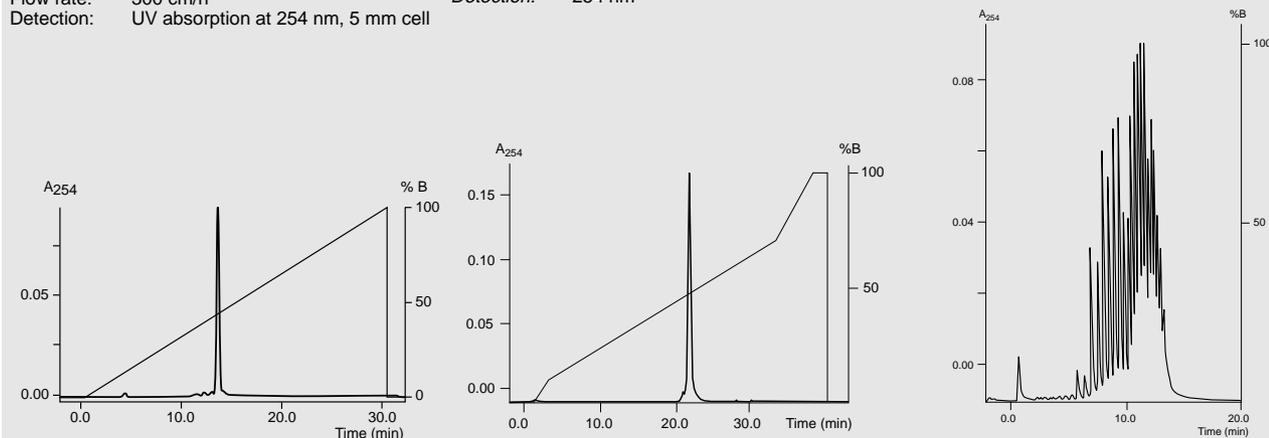


Fig. 5. A and B) Purity analysis of pooled fractions from 0.15 g scale purification of DNA 20-mer (see Fig. 4 for details). C) Analytical separation of a mixture of DNA 12–30 mers.

This method is thus suitable for discriminating between (n) and (n-1) sequences. The purity determinations in this study are based on UV absorbance measurements, and do not necessarily reflect mass relations. Purity determinations of purified material using the anion exchange method and reversed phase chromatography differed by less than 5%.

Discussion

Purity and recovery of the final product are key issues when producing oligonucleotides intended for therapeutic use. Furthermore, such methods must be robust, reproducible and based on reliable materials. In this work, SOURCE 15Q, a small bead diameter (15 µm) rigid anion exchange medium was used to develop a single step purification process for a DNA 20-mer. This approach offers high resolution, good sample loads and recoveries and, therefore, high throughput.

The developed method can be scaled up further. A 300 ml column would have the capacity to purify 1.5 g crude material per cycle, which would lead to a productivity of 32 g of purified DNA oligomer per 24 hours (assuming continuous operation).

Ordering information

Chromatography media and columns

Item	Code No.
RESOURCE Q 1ml (pre-packed column with SOURCE 15Q)	17-1177-01
RESOURCE Q 6 ml (pre-packed column with SOURCE 15Q)	17-1179-01
SOURCE 15Q, 50 ml	17-0947-01
PepRPC® HR 5/5 (pre-packed 1 ml column)	17-0532-01
Mini Q™ PC 3.2/3 (pre-packed 240 µl column)	17-0686-01

For details of other sizes of columns pre-packed with SOURCE 15Q or other sizes of SOURCE media packs, please contact your local Amersham Biosciences office.

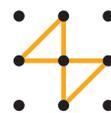
Literature

Item	Code No.
Ion exchange chromatography — Principles and Methods	18-1022-19
Application Note: Pilot scale purification of phosphorothioate DNA oligonucleotides	18-1102-62

Further information — synthesis and chromatography systems

Details of the following systems are given in publications available from your local Amersham Biosciences sales office:

OligoPilot DNA/RNA Synthesizer
BioPilot System with UNICORN control
FPLC System
SMART System



Amersham
Biosciences