Strategies for large scale purification of synthetic oligonucleotides

Application Note

Process chromatography

Introduction

Interest in clinical applications for synthetic oligonucleotides, for example Anti-Sense therapy against viral infections and cancer, has lead to a need to produce large amounts of well-defined products. Such products must be efficacious, safe and reproducible and still be produced at a reasonable cost.

Commercially available synthesizers like OligoPilot® II DNA/RNA Synthesizer, with a capacity to produce up to 4 mmoles of oligonucleotide and OligoProcess™ Engineering systems for larger scales up to 100 mmoles, put demands on purification methods suitable for large scale.

A number of small prepacked columns can provide adequate clean-up for some laboratory tests. When selecting methods for large scale synthesis and purification, factors such as reliability and scalablility have a major influence on productivity and consistency of the process.

Solid phase synthesis

Oligonucleotides are synthesized on OligoPilot and OligoProcess by sequential addition of activated amidites to the immobilized sequence from the previous step, after washing out any remaining reactants and removal of the protective Trityl group. Failures in the coupling reactions, for example early termination of the synthesis and duplet or branch formation, result in lost yield of full length oligonucleotide and give a crude mixture of sequences of different length. This leads to a need for purifying the full length oligonucleotide from such failures. (1)

With the above solid phase synthesizers, the coupling efficiency in each step usually exceeds 99% for DNA synthesis and 97% for RNA synthesis, resulting in, for example, 50–60% full length DNA (20 mer) and 25–30% full length RNA (30–35 mer).

Depending on the efficiency of the synthesis method and the wash steps, the amount of failures may vary, but the following purification steps should discriminate between the full length oligonucleotide and such failures.

Standard oligonucleotides have limited stability *in vivo* and are easily degraded by nucleases. By modification of the phosphodiester bridge (Fig. 1), the stability can be improved, which is of considerable interest for clinical applications. Such modifications, however, change the net charge and/or hydrophobicity of the molecule and thereby the chromatographic behaviour. Modification can also result in some additional failures. Sequences with unmodified phosphodiester bridges are common impurities that should be removed during the purification process.

Regulatory requirements for purity of oligonucleotide products for clinical trials are still under investigation, but exceed 90% for an IND (Investigation on New Drug) application. This requirement will probably increase when more experience from ongoing trials is at hand.

18-1116-00

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Important parameters for purification

Depending on the structure and length of the oligonucleotide, irrespective of whether the trityl group is still present or not and on modifications like thiolation or methylation, different protocols may have to be applied. Due to the often rather small differences in size, charge and hydrophobicity between the product and the impurities, high resolution techniques have to be applied.

- **Number of phosphate groups:** The net charge of each sequence depends on three things: the number of phosphate or modified groups, the charge of the bases involved, and the secondary structure of the sequence which may hide charged groups.

  Anion exchange purification under denaturing conditions, i.e. high pH, minimizes the charge effect from the bases, since they have no net charge above their pKs, and abolishes hydrogen bonding and secondary structural effects. The sequences are stretched out and the net charge depends essentially on the number of phosphate groups.

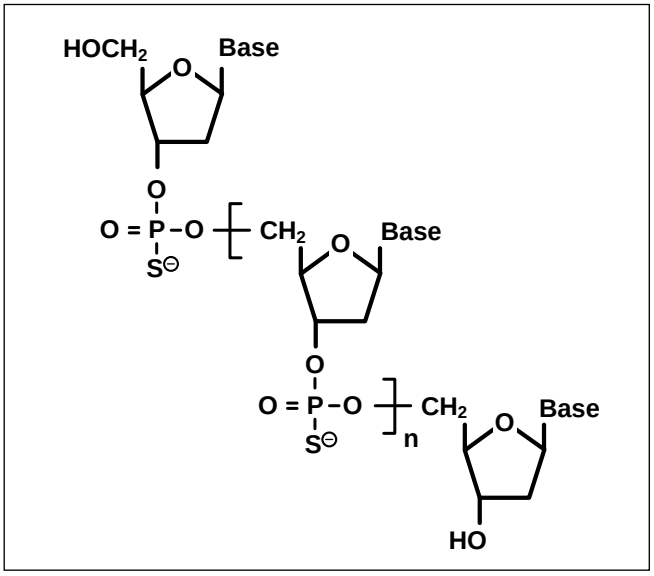

Fig. 1. Chemical structure of phosphorothioate DNA.

- **Trityl ON/OFF:** The trityl group is hydrophobic and tritylated oligonucleotides interact strongly with reversed phase chromatography (RPC) media in ion-pairing mode with trifluoroacetic acid. Detritylated sequences, i.e. failures, will not interact as strongly.

  If the trityl group is left on the oligonucleotide after cleavage from the solid support, this group can be used as a hydrophobic handle to separate it from most failure sequences which have been detritylated during the synthesis. The difference in hydrophobicity decreases with increasing length of the sequence. The bond between the trityl group and the oligonucleotide is unstable under acidic conditions and the separation should preferably be performed under neutral or mildly alkaline conditions for high recovery, thus limiting the use of silica based media which are unstable above pH 8. SOURCE™15RPC, a polymer based RPC medium, may be used at high pH.

- **Thiolation:** When the oxygen in the phosphate groups is exchanged for sulphur (2), the negative charge becomes more polarized and thiolated oligos therefore bind harder to an anion exchanger at most pHs, than a standard phosphodiester oligonucleotide, which elutes earlier in the gradient.

- **Methylation:** When the oxygen is replaced with a methyl group, the negative charge is lost and a more hydrophobic molecule is obtained. This limits the possibilities to separate the product from failures.

Purification strategy

For small scale synthesis, several purification procedures are available, mainly based on three different techniques:

– Desalting
– Reversed phase chromatography
– Anion exchange chromatography

- Desalting separates the oligonucleotides from protective groups and very short (<10 mer) failure sequences. However, it does not separate full length oligonucleotides from shorter, or branched, failure sequences. Recommended media: Sephadex® G-25.

- RPC (in ion-pairing mode) can separate *n* from ≥ (*n*-2) failure sequences, but only partly from (*n*-1) and can not discriminate between thiolated oligonucleotides and non-thiolated failures. It may discriminate methylated from non-methylated. Recommended media: SOURCE 15RPC.

- Anion exchange separates *n* from (*n*-1) sequences. Anion exchange can also discriminate between thiolated oligonucleotides and non-thiolated failures. It may also discriminate methylated from non-methylated. Recommended media: Mono Q® (lab scale), SOURCE 15Q, SOURCE 30Q (lab-and large scale production).

In preparative scale there are two main routes to follow:

Trityl OFF

Ion exchange:

- Adsorption to SOURCE 15Q or 30Q
- Salt gradient elution

Trityl ON

1. Ion exchange:
   - Adsorption to SOURCE 15Q or 30Q
   - Detritylation on column
   - Salt gradient elution
2. RPC + Ion exchange
   - Adsorption to SOURCE 15RPC
   - Elution
   - Detritylation
   - Adsorption to SOURCE 15Q
   - Salt gradient elution
3. RPC + Ion exchange
   - Adsorption to SOURCE 15RPC
   - Detritylation on column and elution
   - Adsorption to SOURCE 15Q
   - Salt gradient elution

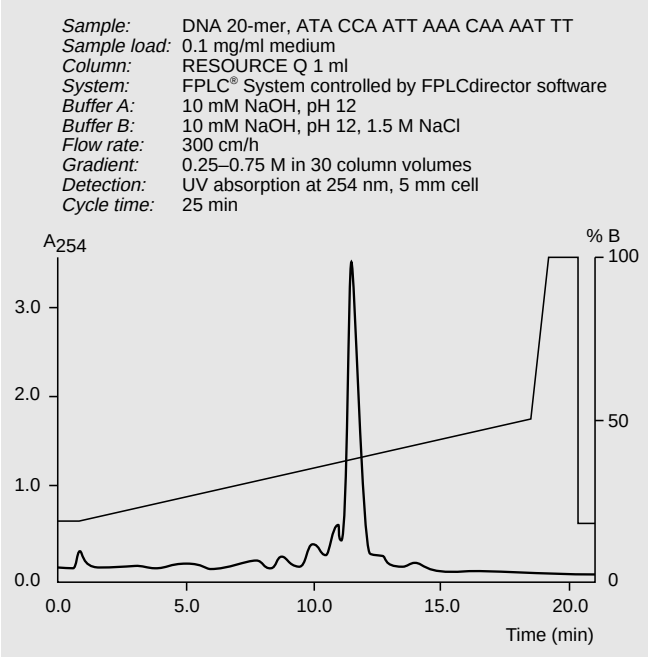

Fig. 2. DNA 20 mer (trityl OFF) / RESOURCE Q 1 ml. 0.1 mg crude oligonucleotide/ml medium. The slope shows the programmed gradient.

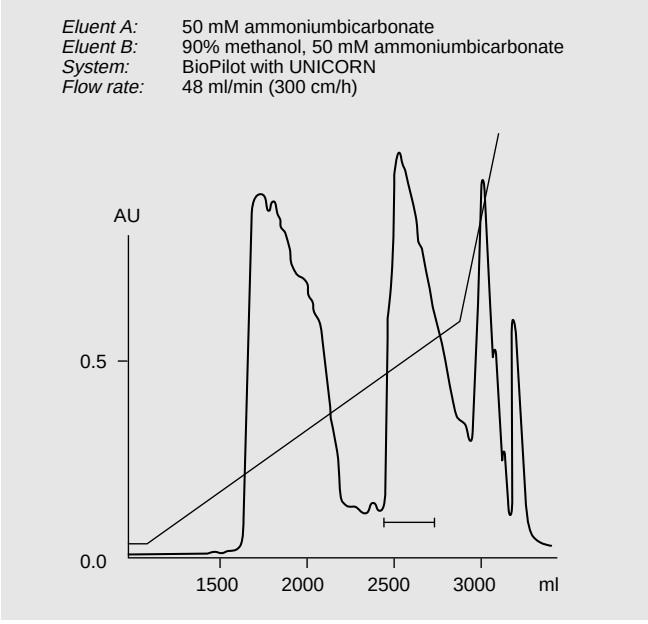

Fig. 3. Trityl ON RNA 37 mer / SOURCE 15RPC. (Work from Ribozyme Pharmaceuticals Inc. USA).

Trityl OFF:

If the protecting trityl group has already been removed, the hydrophobic handle is missing which makes RPC less useful to capture completed sequences.

The differences in negative charge are normally large enough to obtain good separation with anion exchange chromatography, using denaturing conditions (10 mM NaOH, pH 12). Shallow gradients of NaCl are usually needed (Fig 2). Even self-complementary or G-rich sequences that often forms aggregates or hairpin structures have been successfully purified using this technique. RNA is not stable at pH 12 and must be purified using a buffer at neutral pH.

Trityl ON:

If the trityl group is left on the full length oligonucleotide, initial group separation on RPC media followed by detritylation and final purification by anion exchange may be useful. This approach is commonly used for purifying RNA oligonucleotides. Fig. 3 shows the separation of detritylated failures (peak 1) from tritylated full length RNA oligonucleotide (peak 2).

Recent results from our laboratories have shown strong interaction of trityl ON oligonucleotides with SOURCE 15Q and 30Q media resulting in an efficient single-column purification procedure for both phosphodiester and phosporothioate DNA (patent pending, see ref. 6). This procedure involves on-column detritylation after initial step elution of non-tritylated failures and finally separation of detritylated sequences using gradient elution.

Column: RESOURCE Q 1 ml, 3 cm bed height
Flow rate: 1.6 ml/min (300 cm/h)
Sample: Trityl-on 25-mer phosphodiester oligonucleotide, in 25% ammonia
Sample load: 214 ODU
Buffer A: 10 mM NaOH
Buffer B: 10 mM NaOH, 2.0 M sodium chloride
Buffer C: 0.2% TFA, cleaving solution. (Contact time 30 min)
Regeneration: 2 M NaCl in 30% 2-propanol
System: FPLC® system controlled by FPLCdirector™

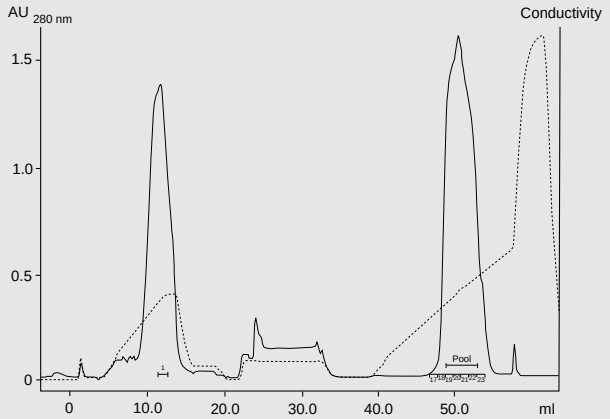

Fig. 4. Phosphodiester DNA 25 mer.

Column: SOURCE 30Q, FineLINE 100, 100 mm i.d. 100 mm (785 ml)
Sample: Trityl-on 25-mer phosphorothioate oligonucleotide, (washed with 2.0 M sodium chloride and subsequently treated with o.4% trifluoroacetic acid before gradient elution)
Sample load: Approximately 2.3 g of full length product
Eluent A: 0.8 M sodium chloride, pH 12
Eluent B: 2.0 M sodium chloride, pH 12
Flow rate: 390 ml/min (300 cm/h)
Gradient: 0–80% B, 25 column volumes
System: BioProcess™ Engineering System

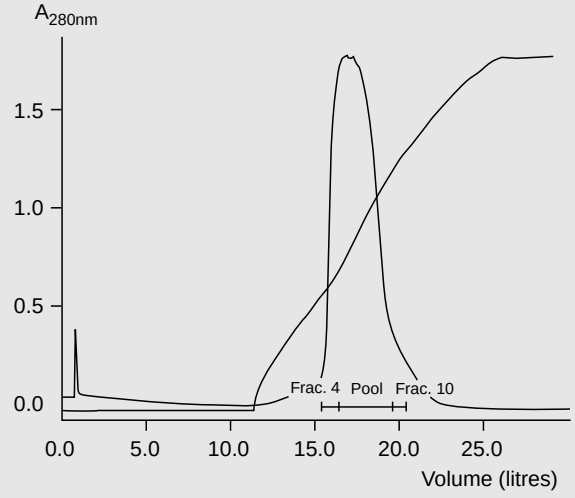

Fig. 5. Phosphorothioate DNA.

Figure 4 shows the separation of phosphodiester DNA on SOURCE 30Q. The non-tritylated failure sequences are removed by an initial linear salt gradient that is automatically stopped at a pre-set UV level. After cleaving the trityl groups from the remaining DNA, it is eluted with a linear gradient, resulting in 99% purity at 92% recovery in our hands.

Figure 5 shows the separation of phosphorothioate DNA on SOURCE 30Q. The tritylated phosphorothioate does not elute even at 3 M salt concentration and all non-tritylated failure sequences are easily washed out with a high salt wash.

After cleaving the trityl groups on column, the remaining phosphorothioate DNA is eluted with a linear gradient. Analytical runs on the product pool and rejected fractions are shown in Fig. 6a–c.

All samples were desalted on NAP 10 Columns.
Capillary: µPAGE (5% T, 5% C), capillary length: 40 cm (J&W Scientific, FISON)
Buffer: Tris-borate and urea buffer, (J&W Scientific, FISON)
Running conditions: 8 kV, 10 s (sample), 16 kV, 30 min (run)
System: Waters Quanta 4000 E (Millipore)
Data collection: FPLCdirector

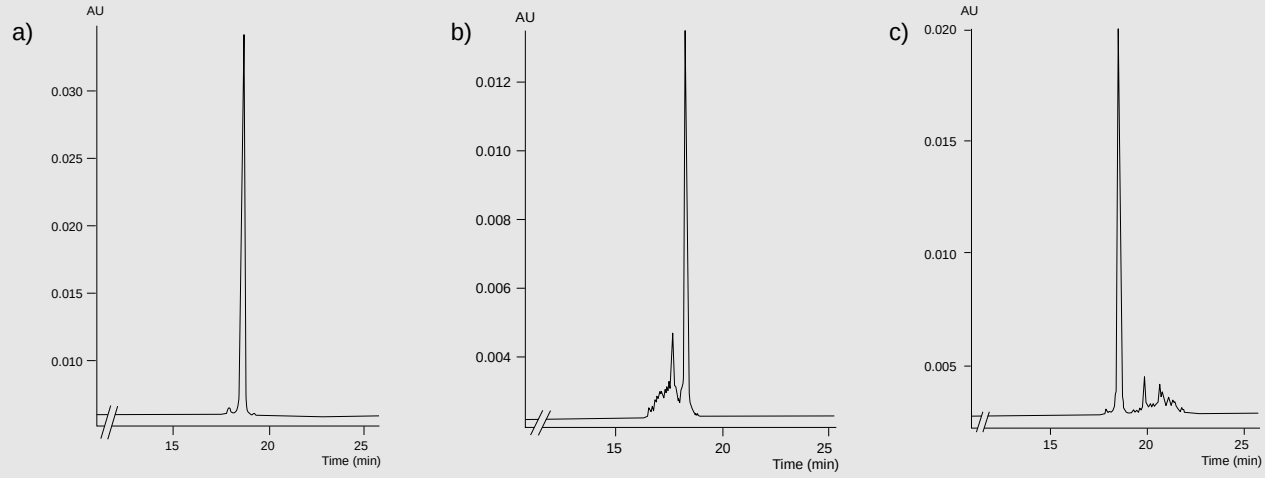

Fig. 6. Analysis by capillary electrophoresis of phosphorothioate DNA fractions.
a) Product pool fraction 5–9, 97% purity at 70% recovery. b) Fraction 4. c) Fraction 10.

Scalability

In laboratory scale purification, resolution and pure fractions may be obtained by careful peak cutting at the expense of yield, but in production scale the separation must be optimized to give the appropriate purity at maintained yield. Robustness has to be built into the process to give consistent results between runs without constant operator attention. Total process economy has to be taken into consideration already at the design phase. Considerations when scaling up chromatographic processes include media, columns and systems. Procedures should be as straightforward as possible. Critical chromatographic parameters such as bed height, load per ml gel and linear flow rate are kept constant.

Choice of media

SOURCE is a new type of synthetic high performance, preparative, chromatography media, based on a monosized, rigid polystyrene/divinyl benzene polymer matrix. These media have excellent physical and chemical characteristics, allowing high flow rates and consistent performance at both laboratory and process scales.

SOURCE can be used over a wide range of working conditions and has good resistance to cleaning conditions at high pH.

SOURCE media are available in prepacked RESOURCE columns (1,3 & 6 ml) for lab scale and as bulk media for larger column sizes.

Specially designed FineLINE™ columns allow the performance of SOURCE media to be maintained over a large number of cycles without the need for repacking and are well suited for industrial scale purification. Laboratory scale resolution is maintained in the production hall.

Figure 7 shows the purification of a phosphorothioate DNA 20 mer in 1 ml RESOURCE column, 6 ml RESOURCE column and 30 ml FineLINE Pilot 35 column. Purity and yield of pooled material were constant at the different scales, Table 1.

Table 1. Purity and yield of full length phosphorothioate DNA 20 mer at different scales, determined by RPC. Sample load 6.6 mg crude oligonucleotide/ml gel.

Scale	Purity	Productivity/run
1 ml column	95.4 %	2.7 mg
6 ml column	95.9 %	15.4 mg
30 ml column	96.2 %	79.0 mg

Sample: Fully thiolated DNA 20-mer, ATA CCG ATT AAG CGA AGT TT
Sample load: 6.6 mg/ml medium
Column: a) RESOURCE Q, 1 ml (Bed height 3 cm)
b) RESOURCE Q, 6 ml (Bed height 3 cm)
c) SOURCE 15Q, 30 ml column (Bed height 3 cm)
System: a, b) FPLC system controlled by FPLCdirector software
c) BioPilot System with UNICORN control
Buffer A: 10 mM NaOH, pH 12, 0.8 M NaCl
Buffer B: 10 mM NaOH, pH 12, 1.8 M NaCl
Flow rate: 300 cm/h (a = 1.67 ml/min, b = 10 ml/min, c = 48 ml/min)
Gradient: 0–80% B in 32 column volumes
Detection: UV absorption at 280 nm, 2 mm cell
Cycle time: 28 min

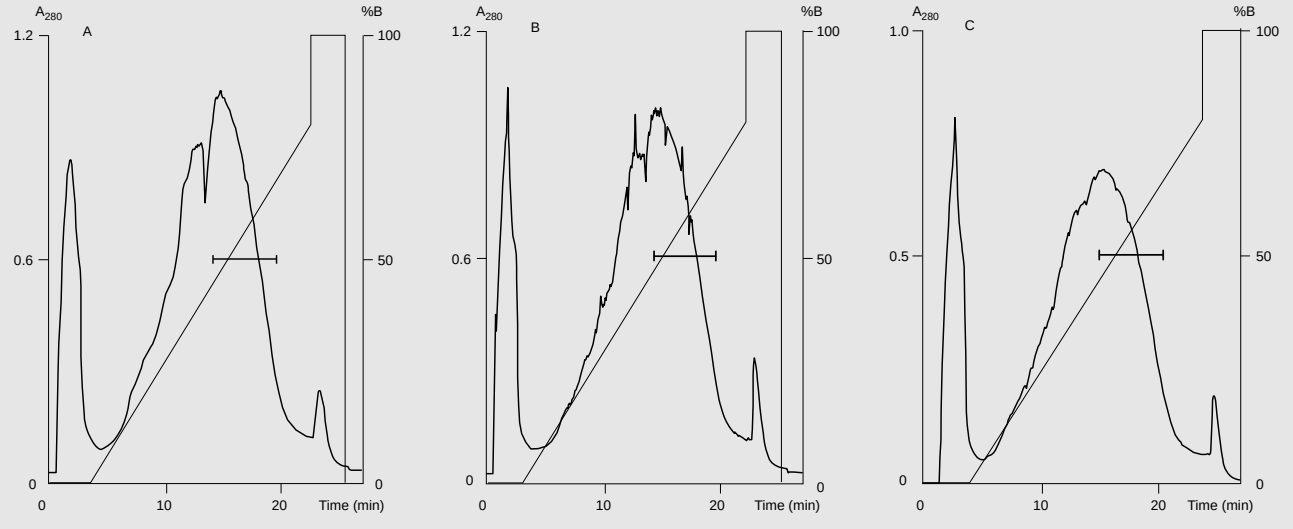

Fig. 7. Scale-up of the optimized purification method for phosphorothioate DNA 20-mer using 1 ml, 6 ml and 30 ml columns packed with SOURCE 15Q. Pooled fractions are indicated by bars. The slope is the programmed gradient.

Analysis of the pooled material is shown in Fig. 8 a-c.

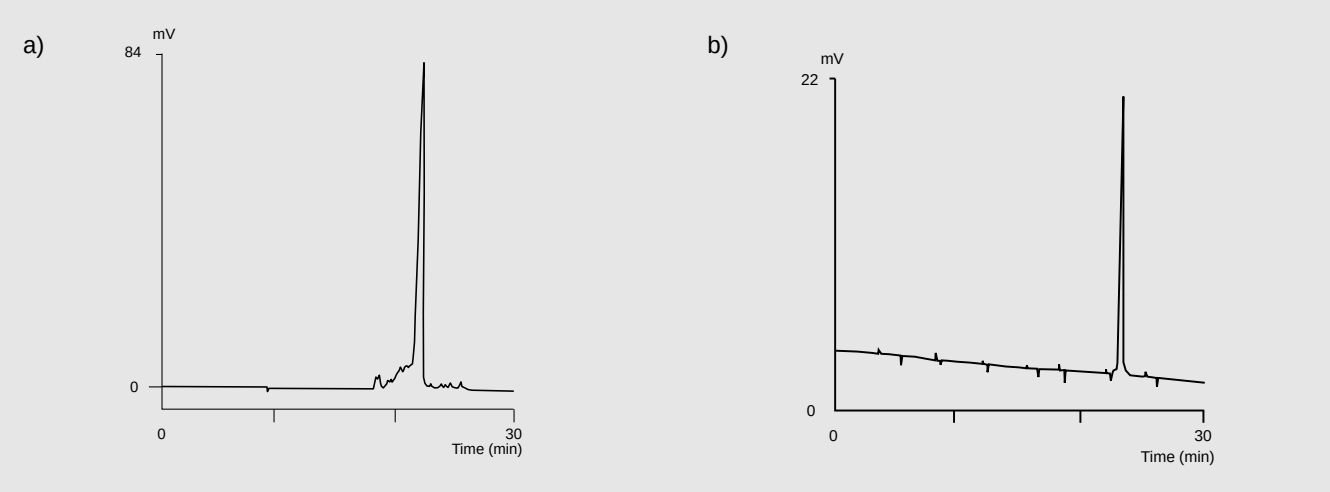

Fig 8 a. Capillary electrophoresis. Analysis of phosphorothioate DNA 20-mer. a) Crude material. b) Pool from 7c.

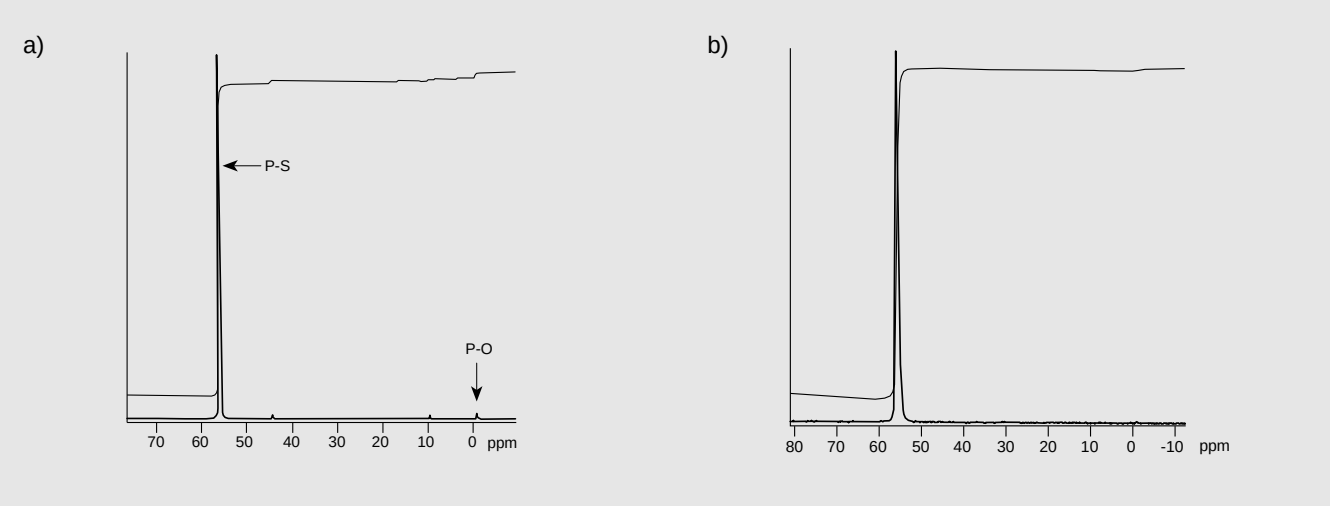

Fig. 8 b. P-NMR-analysis of phosphorothioate DNA 20-mer. a) Crude material. b) Pool from 7c (desalted).

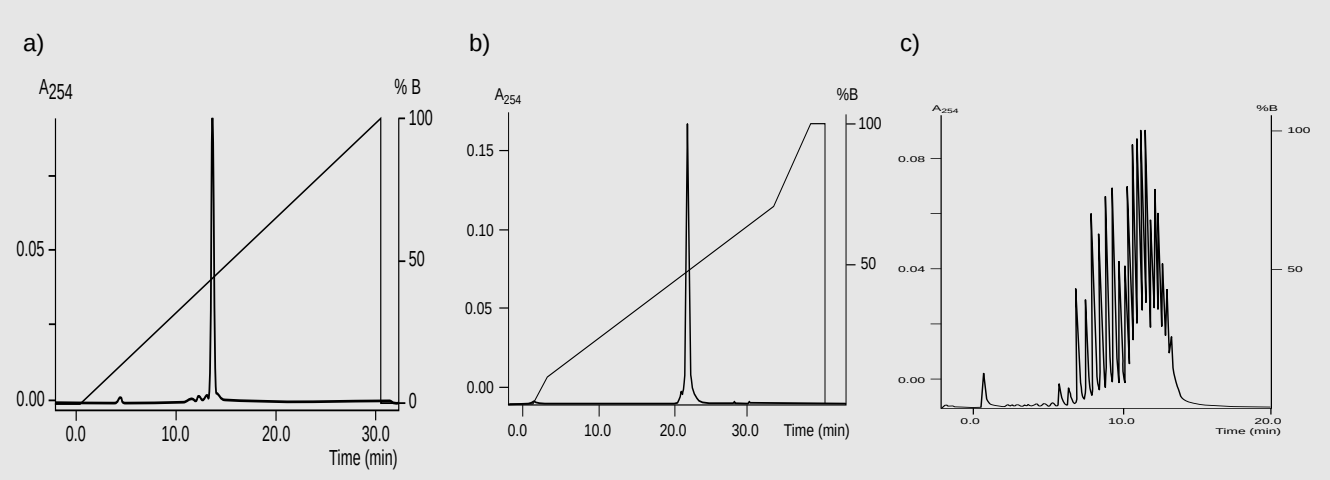

Fig. 8 c. RPC and an ion exchange analysis of phosphorothioate DNA 20-mer.
a) Pool from 7c PepRPC®. b) Pool from 7c Mini Q. c) Mixture of DNA 12–30 mer Mini Q.

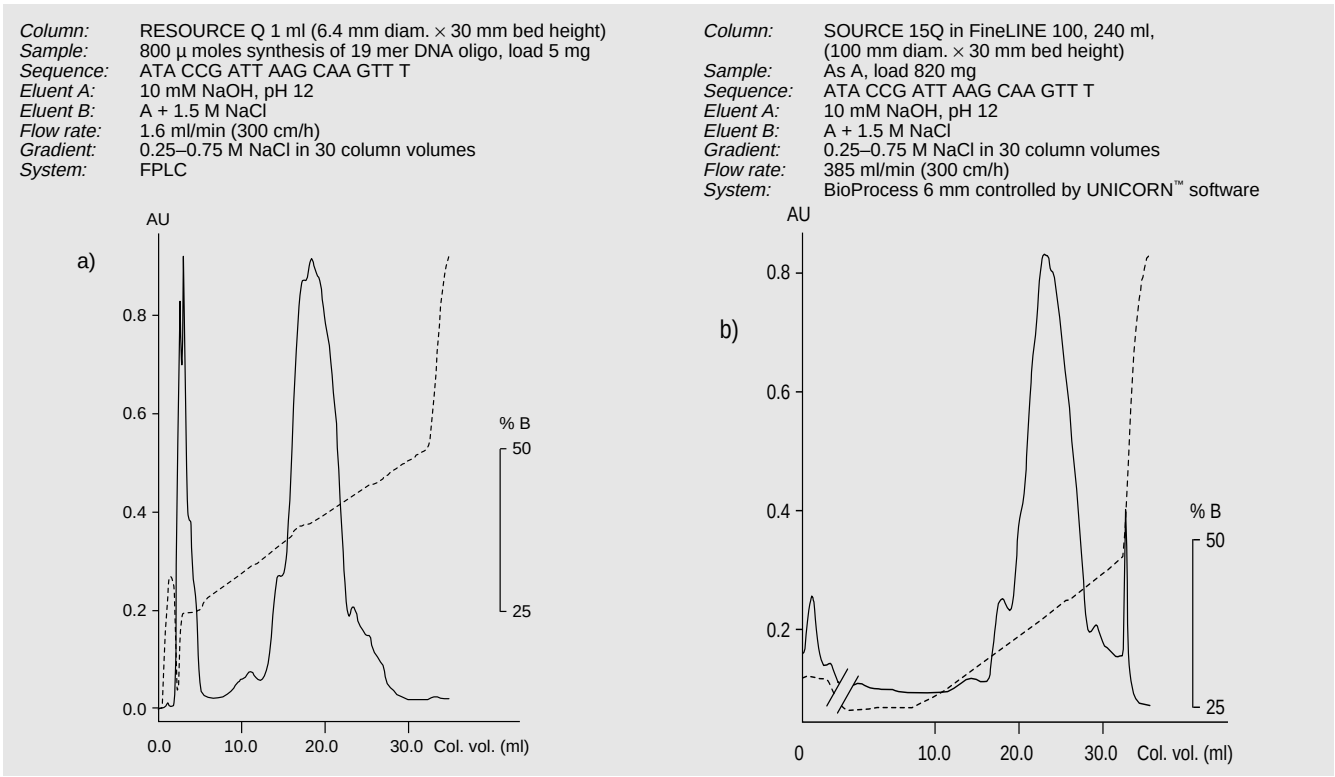

Fig. 9. Scale up from 1 ml to 240 ml column.

Figure 9 shows the purification of a DNA 19 mer oligonucleotide in 1 ml RESOURCE column and 240 ml FineLINE 100 column. The separations are optimized to give maximum yield at a purity of >95%, giving overloaded chromatograms; realistic for a production situation. In this case the sample load was 5 mg/ml medium. The yield of full length oligonucleotide was around 80%.

The gradient was also cut after sample application and curtailed earlier in the production run which makes the chromatogram look slightly different.

Capacity/loadability

In analytical chromatography, the separation is optimized to give as much information as possible about the contents of the sample and on the purity of single components, see Fig. 8. In preparative scale, the aim is to obtain as much product as possible at a predefined purity from each run, i.e. the sample load is increased to give high throughput without unnecessary loss of yield due to peak cutting.

Figure 10 shows a loading experiment with phosphorothioate DNA 20 mer: 0.66–6.6 mg/ml gel. For further details see Application Note 18-1102-62 (5).

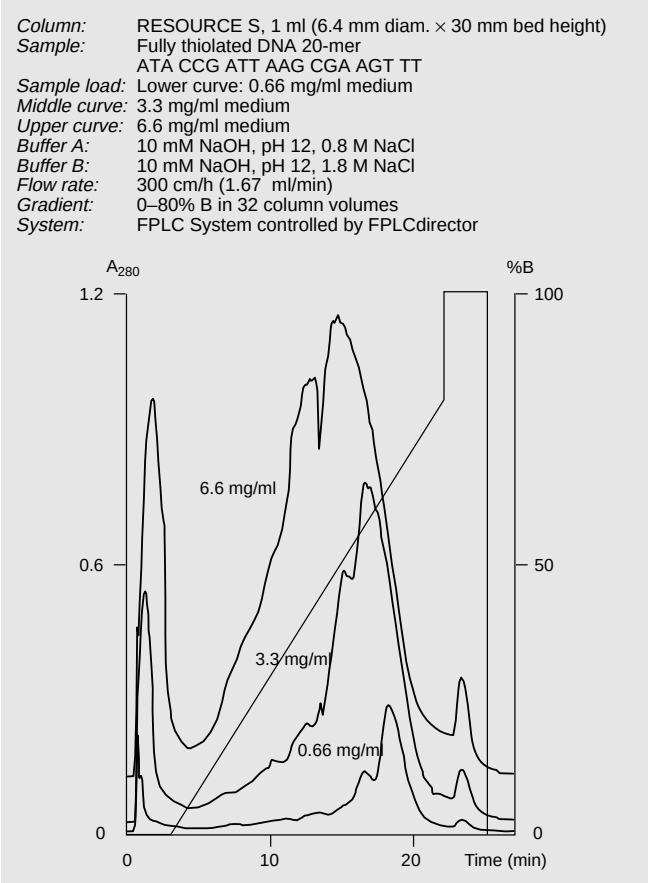

Fig. 10. Comparison of different sample loading.

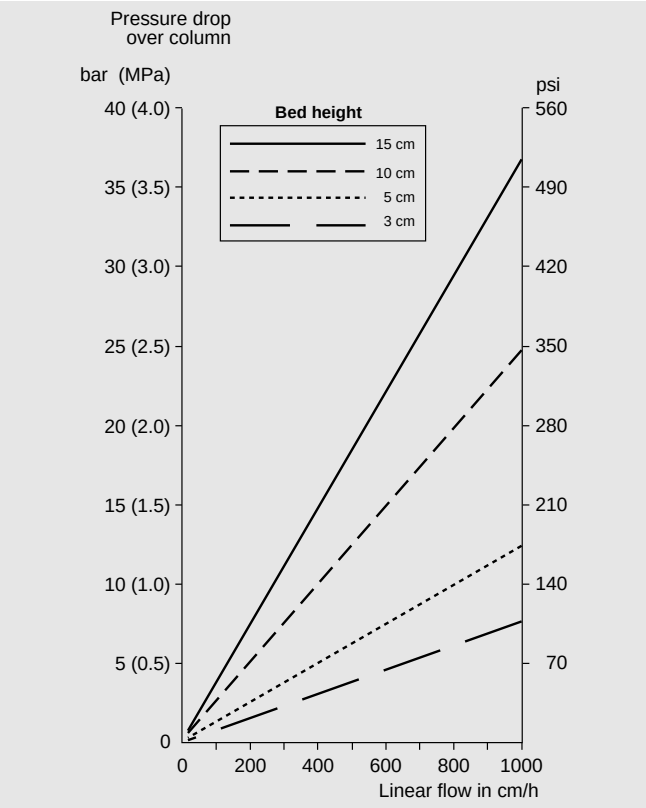

Fig. 11. FineLINE 100 /SOURCE 15Q. Back pressure at different bed heights.

Column: SOURCE 30Q, 4 separate batches, 7.5 mm i.d. × 50 mm (2.2 ml)
Sample: Mixture of gammabind T2 (protein G) and lactoglobulin
Sample load: 0.3 mg/ml bed volume
Eluent A: 20 mM BIS-TRIS PROPANE, pH 7.0
Eluent B: 0.35 M sodium chloride, BIS-TRIS PROPANE, pH 6.8
Flow rate: 2.2 ml/min (300 cm/h)
Gradient: 0–100% B, 20 column volumes

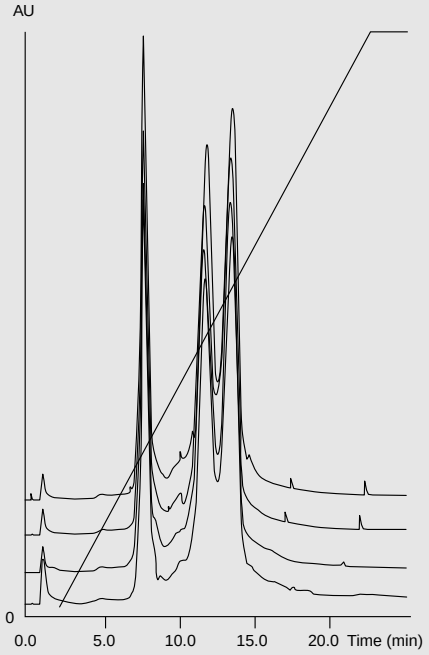

Fig. 12. Reproducibility of SOURCE media.

Pressure/flow rate

Through the unique particle size uniformity of SOURCE media, very high column efficiency and very high flow rates can be obtained at reasonable back-pressures. For example, SOURCE 30 in FineLINE Columns will give more than 11 000 plates per metre at back-pressures typical for less efficient 50 µm polydisperse media. Fast separation can be achieved without the need for high pressure systems. Convenient cycle times of about 20 minutes are achieved at moderate back-pressures (0.6 MPa, 6 bar, 84 psi). This considerably decreases the investment costs for large scale purification systems.

Batch-to-batch reproducibility

Batch reproducibility is here demonstrated by the separation of model proteins on four consecutive batches of SOURCE 30Q. The chromatograms are essentially identical. Similar results have been demonstrated with SOURCE 15Q. See Data File code no: 18-1107-12.

Cleaning and sanitization

A very important issue in large scale chromatography is efficient cleaning protocols between runs to ensure unaltered separation performance, long media life and to avoid any risk of cross-over contamination between runs. These procedures often make use of harsh conditions, such as extremes of pH, to break remaining interactions between the media and components from the sample. There is also a risk of microbial contamination so sanitization protocols may have to be included. This calls for a high chemical stability of media and columns as well as hygienic design. SOURCE media can withstand most extreme cleaning protocols.

Validation support

Regulatory authorities demand complete documentation as part of validation requirements. A lot of this information may be supplied by the vendor thus saving time and resources. At Amersham Biosciences, we are committed to supplying such information about our products whether they are media, columns or systems. Regulatory Support Files (RSFs), including product specifications, chemical and chromatographic stability and toxicological information are available for all BioProcess Media as well as for some other media. (SOURCE media are classed as BioProcess Media which means amongst other things, that this documentation is available.) Validation Support Packages for columns, control systems and equipment are also available and may considerably decrease the time required to obtain approval to start clinical trials or to proceed to the next stage. Amersham Biosciences also offers validation support such as Installation Qualification and Operational Qualification Packages, education and consulting through our Fast Trak® services, to further shorten the time to market approval.

Conclusions

Traditional group separation methods for routine purification at small scale are often impractical at large scale and do not fulfil the demands for purity and/or recovery. The introduction of SOURCE media for both RPC and anion exchange, with a wide pH working range, high resolving power and excellent scalability gives a new dimension to oligonucleotide purification in both laboratory and process scale.

The outstanding performance of SOURCE media together with matching columns and equipment ensures good total process economy at both laboratory and production scale.
There are many issues affecting the choice of media and equipment. Not only is the actual chromatographic performance under the conditions used important, but also reliability and documentation are serious considerations. Technical and regulatory support as well as timely delivery, change control and manufacturing plans are important issues when selecting your vendor, see Fig. 13.

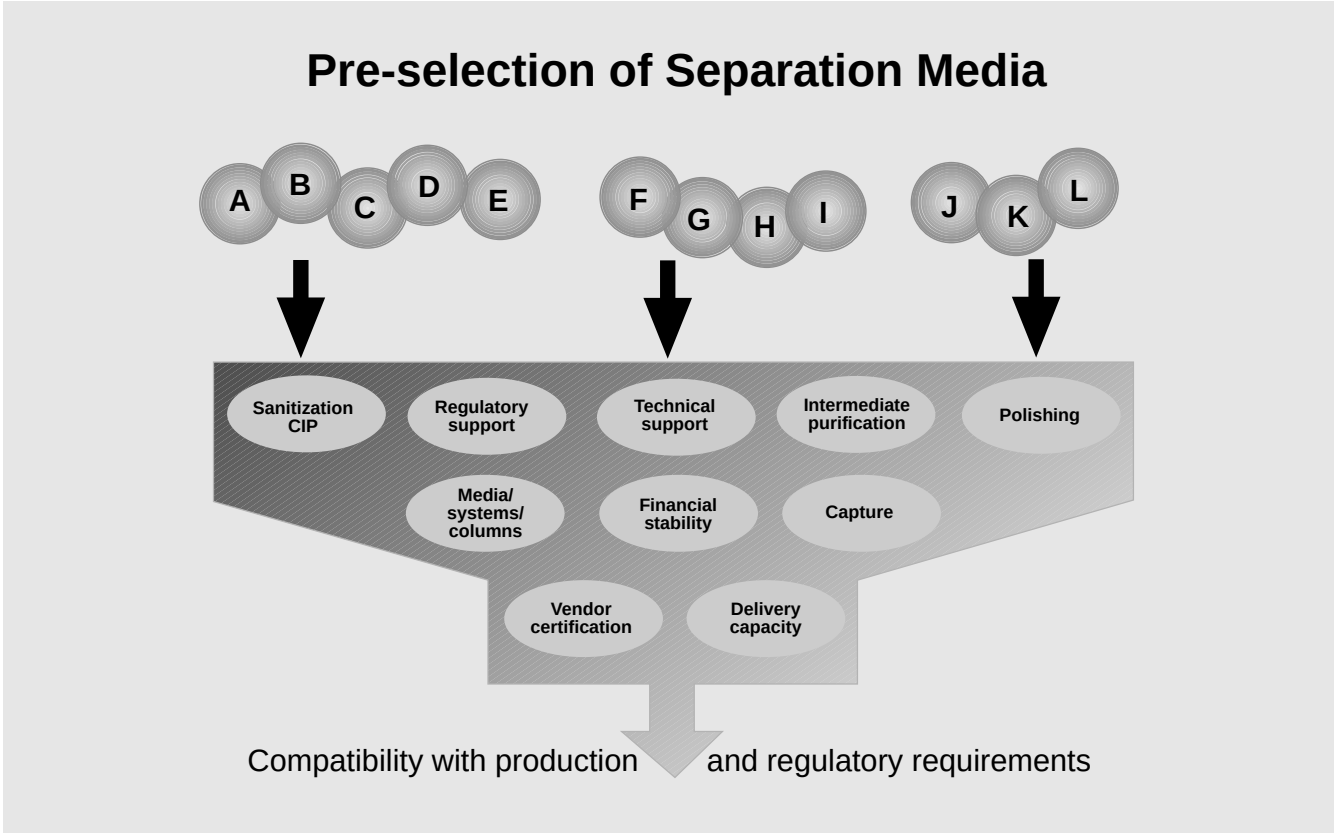

Fig. 13. Vendor related criteria used to select candidate media for process development.

References

1. OligoPilot® II DNA/RNA Synthesizer User Manual 56-1159-01 Ed AA
2. Beaucage, S.L. and Caruthers, M.H., *Tet. Let. 22, 1859–1862 (1981)*
3. Downstream 15 & 16
4. Application Note 18-1102-61
5. Application Note 18-1102-62

6 Johansson, Hans J., Svensson M. Poster presented at Nucleic Acid-Based Therapeutics, June 19–20 1995, San Diego, USA.

Product information

The following products from Amersham Biosciences are mentioned in this Application Note. For further information, please contact your nearest Amersham Biosciences office.

OligoPilot® II DNA/RNA Synthesizer
OligoProcess™ Engineering Systems
Special amidites for oligonucleotide synthesis
RESOURCE® RPC prepacked columns
SOURCE™ RPC media
Sephadex®G-25
RESOURCE® 15Q prepacked columns
SOURCE™15Q media
SOURCE™30Q media
FineLINE™ Pilot 35 column
FineLINE™ 100 column
FPLC® System
BioPilot® System
Fast Trak® Purification Services (Consulting, Education, Validation)

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