Bio-Works

APPLICATION NOTE

Purification of oligonucleotides by anion exchange chromatography

Solid-phase synthesis of oligonucleotides generally give material of rather high purity. However, for many applications, and especially for therapeutics, there is a need for purification to remove incomplete or erroneous sequences. Purification is best achieved by combining several separation techniques. A common combination is hydrophobic interaction chromatography followed by anion exchange chromatography. Anion exchange chromatography (AIEX) is an efficient technique for oligonucleotide purification giving high purity and good yields also in a single step. WorkBeads™ 40Q was used to purify a 20-mer trityl-OFF oligonucleotide released from the solid-phase.

Oligonucleotides

Oligonucleotides are short sequences of deoxyribonucleic acids or ribonucleic acids that are negatively charged due to the phosphate groups in their backbones. The purity of oligonucleotide preparations produced by solid-phase synthesis can be very high. However, purification is often required to remove incomplete or erroneous sequences. There is always a small percentage of sequences where the oligonucleotide has deletions or insertions of elements (*e.g.*, N-1 or N+1, N is the length of the target oligonucleotide). The frequency of failures increases with length of the synthesized oligonucleotide. The N-1 contaminants are usually difficult to separate from the full-length oligonucleotide.

Anion exchange chromatography

Since anion exchange chromatography can separate the negatively charged oligonucleotides based on length, it is common used method for oligonucleotide purification. The negatively charged phosphate groups in the nucleic acid backbone is bound to the resin via positively charged groups on the AIEX resin. Generally, the strength of the binding increases with length of the target. Elution of the adsorbed oligonucleotides can be done by increasing the salt concentration gradient to release the oligonucleotides in the order of increasing length.

WorkBeads 40Q

WorkBeads 40Q resin is a high-capacity strong anion exchange resin derivatized with quaternary amine ligands. WorkBeads 40Q resin demonstrates the property of high-resolution separation while giving low backpressure to facilitate purification in bioprocess columns. The agarose-based resin is manufactured using a proprietary method that results in porous beads with a tight size distribution and exceptional mechanical stability. Agarose based matrices have been successfully used for decades in biotechnology purification, from research to production scale, due to their excellent compatibility with biomolecules including proteins, peptides, nucleic acids and carbohydrates. WorkBeads 40Q is highly suitable for the purification of oligonucleotides giving high purity and good yields.

Table 1. Properties of WorkBeads 40Q.

WorkBeads 40Q	
Average particle size[1] (D_{v50})	45 µm
Ionic group (ligand)	Quaternary amine ($-N^+(CH_3)_3$)
Ionic capacity	180 – 250 µmol/mL
Dynamic binding capacity (DBC), 20-mer oligonucleotide[2]	48.3 mg/mL resin
Max flow rate (20 cm bed height, 5 bar)	600 cm/h
pH stability	2–13

[1] The average particle size of the cumulative volume distribution
[2] Tested in a 6.6 × 100-mm column at 150 cm/h, 4 minutes residence time)

Experimental conditions

Optimization of the binding and elution conditions is essential for obtaining a purification process giving high purity and yield of the full-length oligonucleotide. High resolution separations require high efficiency and good selectivity. This can be achived using a resin with small particle sizes as used in analytical columns. However, this type of resin is not suitable for large-scale purification due to the high backpressure, and the hardware pressure limitation of process scale columns. WorkBeads 40Q has an average particle size of 45 µm which has been designed as a trade-off between high resolution and low pressure.

We have compared gradient elution conditions for a 20-mer phosphodiester oligonucleotide (trityl-OFF) using NaCl in the presence of 20 mM Tris-HCl, pH 8 or 20 mM NaOH, pH 12 (Fig. 1). One mg of crude oligonucleotide preparation (diluted 1:30 with binding buffer after the release from the solid support) was loaded onto the WorkBeads 40Q column. The sample also contained ammonia and benzamide impurities derived from the synthesis step. After washing with binding buffer, a salt gradient was applied for elution.

Resin:	WorkBeads 40Q
Column:	6.6 × 100 mm, 3.4 mL
Binding buffers:	A. 20 mM Tris-HCl, pH 8 B. 20 mM NaOH, pH 12
Elution buffers:	A. 20 mM Tris-HCl, 1.5 M NaCl, pH 8 B. 20 mM NaOH, 1.5 M NaCl, pH 12
Sample:	1 mL of 1 mg/mL crude preparation of a 20-mer oligonucleotide (ssDNA) (trityl-OFF)
Flow rate:	150 cm/h
Gradient:	1. Step: 20% elution buffer, 10 CV (column volumes) 2. Linear: 20% to 40% or 50%, 20 CV

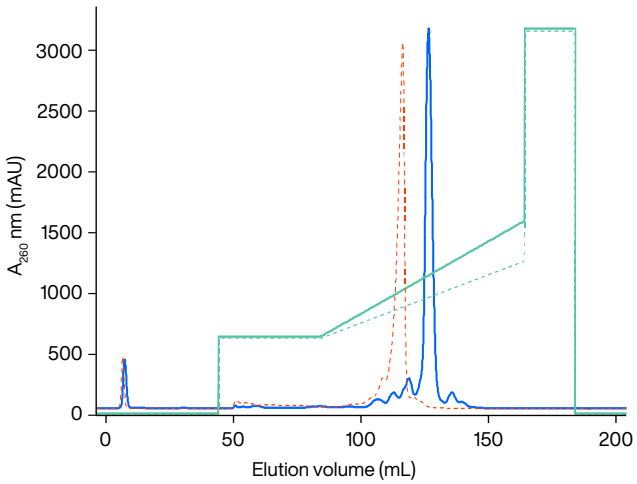

Figure 1. Purification of oligonucleotides on WorkBeads 40Q using 20 mM NaOH, pH 12 (solid blue) or 20 mM Tris-HCl, pH 8 (dotted red). The elution gradients are shown in green (dotted line 20-40% for 20 mM Tris-HCl, pH 8, and solid line 20-50% for 20 mM NaOH, pH 12).

The main peak was collected in 1 mL fractions. Each individual fraction was analyzed for purity on a DNAPac™ PA200 analytical AIEX column (Thermo Fisher Scientific) and for yield by comparing the A_{260} absorbance with the absorbance of the loaded crude oligonucleotide preparation.

The purity, the yield and the level of N-1 impurity in each fraction is visualized in Figure 2. The N-1 impurity is highest at the beginning of the eluted peak. The purity is highest close to the mid-peak area, and the N-1-impurity is decreased in later fractions. Both buffers systems were able to resolve the full-length oligonucleotide from the N-1 accordingly to the DNAPac analyses. In Figure 1 the N-1 oligonucleotide seemed to be slightly better resolved in presence of 20 mM NaOH, pH 12.

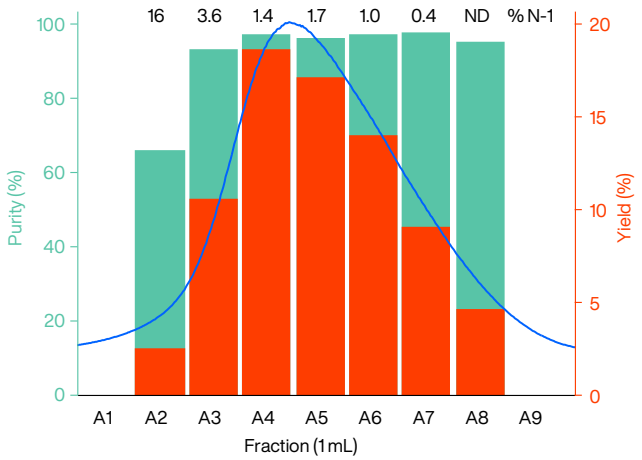

Figure 2. Purity and yield were measured in each individual fraction across the main peak using 20 mM NaOH, pH 12. Contents of N-1-mer are given above the bars.

For optimization, selected 1 mL fractions were combined to assess the effect of pooling on yield and purity, see Fig. 3. A purity of 95.5% with 76.9% yield was obtained with the broadest pooling using NaOH buffer (7 mL), and a purity of 95.6% with 74.2% yield using Tris-buffer (Table 2). Higher purities could be obtained by a more narrow pooling scheme as illustrated in Figure 3.

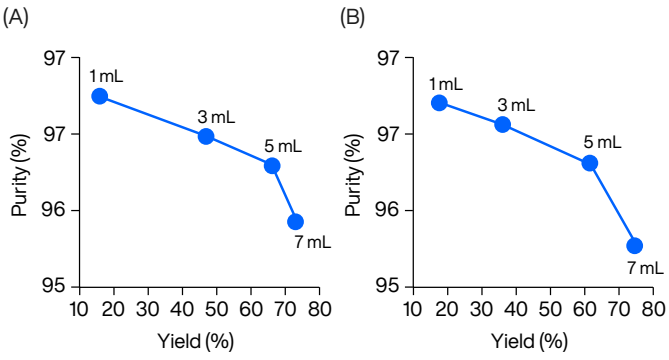

Figure 3. Purity *vs* yield plots for different fraction pools (1 mL, 3 mL, 5 mL and 7 mL) of the eluted main peak using 20 mM NaOH, pH 12 (A) or, 20 mM Tris-HCl, pH 8 (B).

WorkBeads 40Q was compared regarding purity and yield with Capto™ Q ImpRes (Cytiva). WorkBeads 40Q gave both higher purity and better yield then Capto Q ImpRes see Table 2.

Capto Q ImpRes gave for unknown reasons a broader main peak than WorkBeads 40Q, despite that the columns had the same efficiency (measured by acetone injections). Data from a customer (unpublished) also showed similar results with these two resins, when the fraction pooling was selected to get 85% yield giving a purity of 93% for WorkBeads 40Q *vs* 91% for Capto Q ImpRes.

Table 2. Yield and purity. Pooled 1 mL fractions (seven fractions each) of the main peak obtained on WorkBeads 40Q and Capto Q ImpRes were compared.

Resin	Buffer	Purity	Yield
Crude preparation	–	84.5%	100%
WorkBeads 40Q	20 mM Tris-HCl, pH 8	95.5%	76.9%
WorkBeads 40Q	20 mM NaOH, pH 12	95.6%	74.2%
Capto Q ImpRes	20 mM Tris-HCl, pH 8	93.7%	68.9%
Capto Q ImpRes	20 mM NaOH, pH 12	94.4%	64.4%

From the results obtained we can conclude that there is only a minor difference between using denaturing conditions (20 mM NaOH, pH 12) or not (20 mM Tris-HCl, pH 8) for this oligonucleotide preparation. This may depend on lack of secondary structure of this oligonucleotide. It is likely that purification of longer oligonucleotides, which are more prone to form internal structures, would be more impacted by denaturing buffers, chaotrophic salts and potentially organic additives.

Determination of dynamic binding capacity

The dynamic binding capacity (DBC) using the sample described earlier was determined by frontal analysis at 10% breakthrough to be 48.3 mg/mL (feed: 0.1 mg/mL) at a flow rate of 150 cm/h in the presence of 20 mM Tris-HCl, pH 8. Figure 4 shows the breakthrough curve for this oligonucleotide feed on WorkBeads 40Q.

Resin: WorkBeads 40Q
Column: 6.6 × 100 mm, 3.4 mL
Buffer: 20 mM Tris-HCl, pH 8
Sample: 0.1 mg/mL crude preparation of a 20-mer oligonucleotide (ssDNA) (trityl-OFF)
Flow rate: 150 cm/h

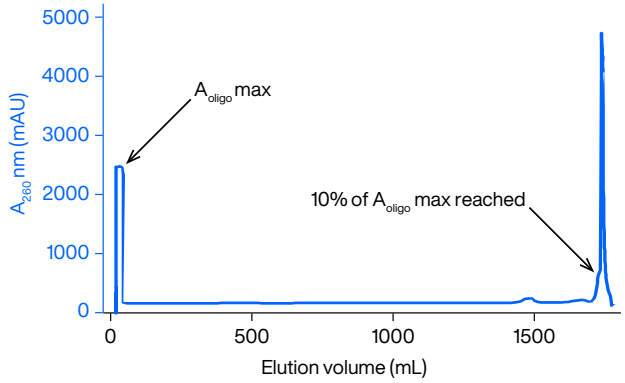

Figure 4. Frontal analysis determination of binding capacity.

Scale-up purification

In process-scale purifications the aim is usually to maximize the yield, while reaching a pre-defined purity. Careful selection of parts of the eluted peak is thus important.

To investigate scale-up conditions, sample load of 80% of the resins DBC, *i.e.*, 132 mg of the oligonucleotide preparation was loaded to the column. The crude oligonucleotide preparation from the solid support synthesis in presence of a high concentration of ammonia and benzamide without further adjustment of the feed was loaded straight onto the column (the sample was not diluted).

Resin: WorkBeads 40Q
Column: 6.6 × 100 mm, 3.4 mL
Binding buffer: 20 mM Tris-HCl, pH 8
Elution buffer: 20 mM Tris-HCl, 1.5 M NaCl pH 8
Sample: 4.4 mL of 30 mg/mL crude preparation from a 20-mer oligonucleotide (ssDNA) (Trityl-OFF)
Flow rate: 150 cm/h
Gradient: Linear, 0% to 40%, 40 CV

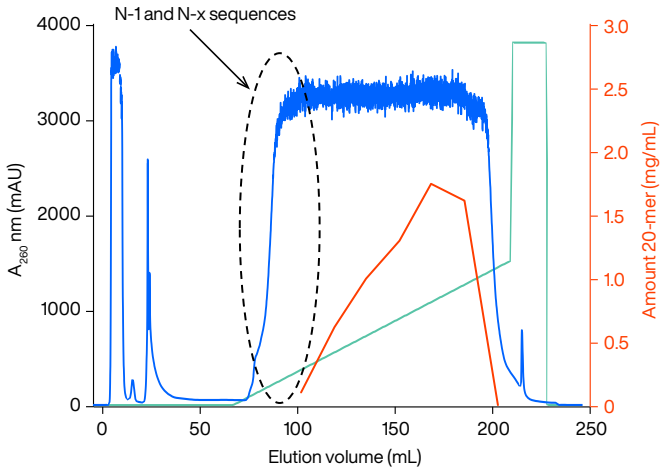

Figure 5. Purification of 20-mer oligonucleotide at 80% DBC on WorkBeads 40Q. UV absorbance (blue line), elution gradient (green line) and amount of full-length oligonucleotide recovered in 1 mL fractions (red line) are shown.

Figure 5 shows a typical recovery shape of the full-length oligonucleotide for a process purification run with a sample load corresponding to 80% of DBC. The absorbance was measured at 260 nm off-line for all fractions to allow calculation of yields (as was done in the low-sample loading experiments described earlier). In the beginning of the elution gradient the N-x species are eluted, and the full-length oligonucleotide starts to elute later in the gradient. The histogram in the chromatogram in Figure 6 shows the purity in individual fractions. This demonstrates the separation of the full-length oligonucleotide.

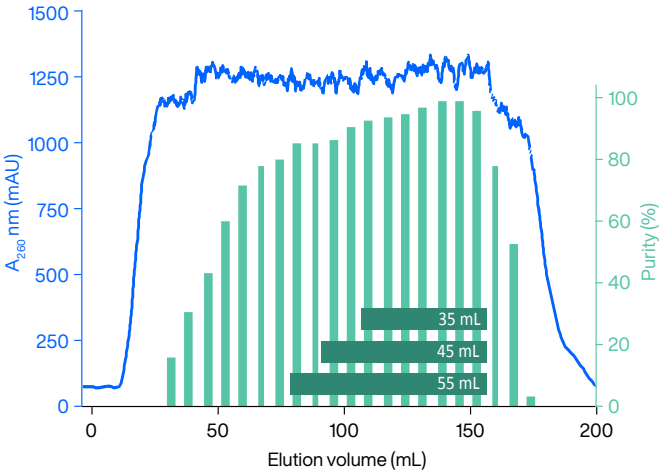

Figure 6. Eluted oligonucleotide peak after loading an amount of crude oligonucleotide corresponding to 80% of the DBC. Purity of full-length oligonucleotide (green bars) was measured in individual fractions. Pools (1–3) of collected fractions are visualized in the chromatogram.

Different schemes for pooling of fractions across the peak (Fig. 6) were analyzed for purity, yield and for the content of N-1 impurity (Table 3). The volume of the pools were 35, 45 and 55 mL.

Table 3. Effect on yield and purity of different pooling after the high-load run.

Pool	Purity (%)	N-1 (%)	Yield (%)
Pool 1 (55 mL)	94.5	2.1	82.2
Pool 2 (45 mL)	95,2	1.8	69.9
Pool 3 (35 mL)	96.3	1.6	54.3

Desalting

After ion exchange chromatography there is often a need to remove the salt used for elution before further downstream process steps, *e.g.*, lyophilization. Chromatographic desalting is a fast and efficient tool for removal of salt or unwanted low-molecular weight impurities, or for buffer exchange. In this technique the large size-difference between the oligonucleotide and salt is utilized. The large oligonucleotide molecules are eluted in the void volume (they do not enter inside the resin particles), whereas salt molecules are eluted close to the total volume of the column (they pass through most of the porous resin particle inner volume and are thus delayed). We applied the eluted oligonucleotide preparation (0.85 mg) to a desalting column (GoBio™ Mini Dsalt 5 mL) to demonstrate the ease and efficiency of desalting. The sample load (1.5 mL) corresponds to about 1/3 of the column volume. The salt was completely removed from the oligonucleotide, as shown by the non-overlapping UV absorbance trace for the oligonucleotide detection and the conductivity trace for salt detection (Fig. 7).

Column: GoBio Mini Dsalt, 5 mL
Buffer: 20 mM Tris-HCl, pH 8
Sample: 1.5 mL of 0.57 mg/mL AIEX-purified 20-mer
Flow rate: 5 mL/min

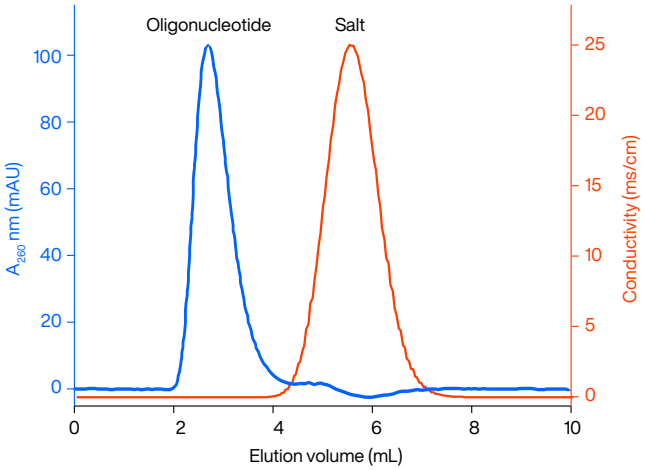

Figure 7. Desalting of 20-mer oligonucleotide. Chromatogram showing the separation of the oligonucleotide, UV trace (blue) and salt, conductivity (red).

Conclusion

WorkBeads 40Q is a high-performance resin excellent for oligonucleotide purifications. The rigidity of this 45 µm-bead resin, allows efficient purifications at process scales. The high dynamic binding capacity reduces the overall process cost by decreasing the process time and volumes of solvents needed. WorkBeads 40Q gave both higher purity and yield than Capto Q ImpRes.

WorkBeads 40Q resin can also be used to purify other types of nucleic acids, *e.g.*, modified deoxyribose nucleic acids and ribonucleic acids, phosphorothioates, phosphoro-diamidate morpholino oligomers, siRNAs, miRNAs, mRNAs, crRNAs/sgRNAs.

Ordering information

Visit www. bio-works.com for information regarding all WorkBeads resins.

Orders: sales@bio-works.com or contact your local distributor.

For more information about local distributor and products visit www.bio-works.com or contact us at info@bio-works.com

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