Preparative Anion Exchange Chromatography for the Purification of DNA Oligonucleotides

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Abstract

DNA oligonucleotides obtained by solid-phase synthesis contain incomplete or erroneous sequences that require removal for the advancement of these molecules as therapeutics. DNA oligonucleotides have a high degree of negative charge and therefore can be separated according to length with anion exchange chromatography. Using crude preparations of 20- and 21-mer oligonucleotides, we demonstrate the efficacy of the Bio-Rad Nuvia HP-Q Resin with a bead size of 50 µm for DNA oligonucleotides. Evaluated key variables during method development include pH, flow rate, dynamic binding capacity, gradient slope, and scalability. Both small- and large-scale experiments with a 5 ml and 25 ml column, respectively, gave excellent yields in the range of 90% while maintaining the high purity (>97%) of the full-length products.

Introduction

Oligonucleotides play a fundamental role in gene regulation and are considered a promising novel class of biopharmaceuticals (Catani et al. 2020). In 1998, the first oligonucleotide (ON) drug was approved by the U.S. Food and Drug Administration, leading to a growing interest in the development of oligonucleotide-based therapeutics. Oligonucleotide-based drug platforms (Roberts et al. 2020) comprise many types of nucleic acids for specific gene modulation, including antisense oligonucleotides (ASO), short interfering RNA (siRNA), micro RNA (miRNA), aptamers, and messenger RNA (mRNA). ASOs, which are short (15–25 bases), single-stranded DNA oligonucleotides designed antisense to target RNA (Vanhinsbergh 2020), represent an important therapeutic subclass. They are usually manufactured by cyclic solid-phase synthesis based on phosphoramidite chemistry. Although individual cycling efficiency is high (>98%), a low percentage of the oligonucleotide chain does not grow to the desired full-length product, leading to overall product yield reduction and many structurally similar impurities (Enmark et al. 2020). ON-failed sequences are typically caused by the deletion or addition of one or more nucleotides called shortmers (n – x) or longmers (n + x). Other impurities, which are comprehensively discussed by El Zahar and colleagues (El Zahar et al. 2018), may be produced by failures during deprotection and detritylation or are caused by depurination, deamination, and adduct formation.

To increase biostability and the therapeutic efficiency of ONs, they are commonly chemically modified at multiple sites: phosphodiester backbone, ribose sugar, and the heterocyclic bases. Phosphorothioation is the most common chemical modification of ASOs and many ASOs are fully phosphorothioated to be protected from in vivo nuclease degradation.

It is anticipated that ONs will gain importance as therapeutic agents, and their production requires a highly efficient downstream processing strategy after synthesis. Chromatography holds a key position in this workflow and ON purification is usually achieved by a combination of several techniques. A very successful purification approach for the large-scale production of ASOs is based on the combination of hydrophobic interaction chromatography (HIC) and anion exchange chromatography (AEX) (Gronke 2020). HIC is usually applied for the initial purification of DMT (dimethoxytrityl)-on material while AEX is performed on the HIC eluate after detritylation as a second polishing step (Andrews et al. 2021). AEX mainly separates ASOs based on charge differences, leading to a length-based elution order, permitting the removal of key impurities exhibiting deletion sequences. However, with AEX is it challenging to separate full-length products from n – 1 contaminants and requires the careful evaluation of a given AEX resin for its applicability in a preparative ON purification process.

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Here, we present the characterization of the high-performance strong anion exchange resin Nuvia HP-Q for the purification of single-stranded DNA oligonucleotides. Developed for the industrial downstream purification of large biomolecules at fast flow rates, Nuvia HP-Q Resin has technical characteristics such as bead size, pH stability, and flow characteristics that make the resin a promising candidate for ON purification. Key variables we evaluated include pH, flow rate, dynamic binding capacity, gradient slope, and scalability.

Materials and Methods*

Oligonucleotides

We used four custom-made (Sigma Aldrich) desalted, lyophilized single-stranded DNA oligonucleotides (3 x 20-mer and 1 x 21-mer) with phosphodiester backbones (Table 1).

Table 1. DNA oligonucleotides used in this study. Name, length, and sequence are listed.

Oligo Name	Length (nucleotides)	Sequence (5'–3')
Oligo 1 (ON 1)	20	ATA CCG ATT AAG CGA AGT TT
Oligo 2 (ON 2)	21	ATA CCG ATT AAG CGA AGT TTT
Oligo 3 (ON 3)	20	TAT GGC TAA TTC GCT TCA TC
Oligo 4 (ON 4)	20	GCC CAA GCT GGC ATC CGT CA

ONs 1–4 were used during method development while ONs 1 and 2 were also used for large-scale purification experiments. For chromatography, dried ONs were dissolved in buffers prepared using nuclease-free water.

Chromatography

Equipment and resins used for chromatography experiments (Table 2) were from Bio-Rad Laboratories, Inc. Chromatography was monitored at 260 and 280 nm and performed at room temperature using an NGC 10 Medium Pressure Chromatography System. The chromatography buffers were thoroughly degassed and filtered through 0.45 µm membranes. The four-tier chromatography system was configured with a sample pump including a sample inlet valve, two buffer inlet valves, a column switching valve, a buffer blending valve, a multi-wavelength UV detector (5 mm path length), a pH and conductivity meter, an outlet valve, and a fraction collector with a benchtop Peltier cooling option. Chromatography operating conditions are presented in Figure 1. Samples from analytical and preparative runs were stored at 4°C and pH was not adjusted prior to analysis by electrophoresis.

Table 2. Overview of chromatography columns used for DNA oligonucleotide purification and fraction analysis, including buffer and operating conditions.

Column/Resin Type	Resin Type, Bead Size	Column Dimensions, Pack Type	Application	Buffer A	Buffer B	Flow Rate, cm/hr
Foresight Nuvia HP-Q (5 ml; catalog #12007021)	Strong AEX, 50 µm	8 x 100 mm, prepacked	Method development	25 mM arginine-NaOH, pH 11.7	25 mM arginine-NaOH, 2 M NaCl, pH 11.7	120–240
Nuvia HP-Q (25 ml; catalog #12006693)	Strong AEX, 50 µm	25 x 100 mm, self-packed into Econo Alpha Column (#12009461)	Large-scale purification	25 mM arginine-NaOH, pH 11.7	25 mM arginine-NaOH, 2 M NaCl, pH 11.7	100–120
ENrich Q (1 ml; catalog #7800001)	Strong AEX, 10 µm	5 x 50 mm, prepacked	Fraction analysis	25 mM arginine-NaOH, pH 11.7	25 mM arginine-NaOH, 2 M NaCl, pH 11.7	300

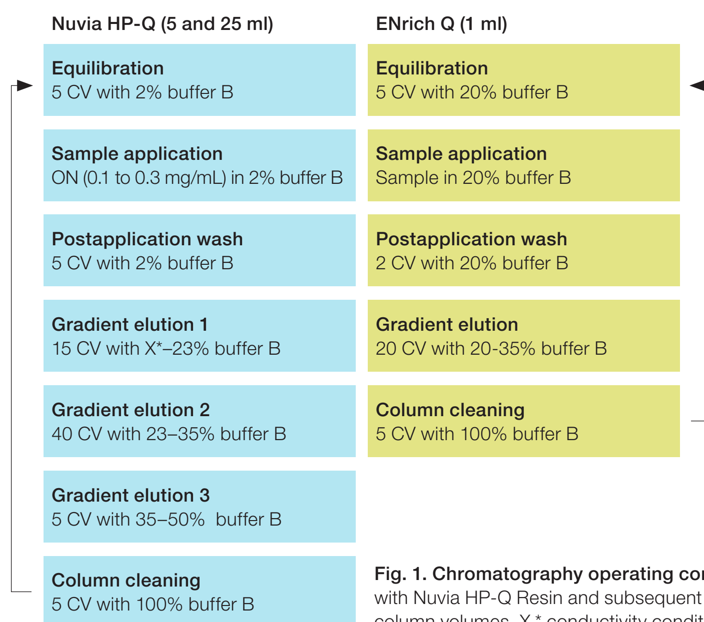

Fig. 1. Chromatography operating conditions. Proposed phases for the preparative purification of DNA oligonucleotides (ONs) with Nuvia HP-Q Resin and subsequent fraction analysis with analytical anion exchange chromatography (Enrich Q Resin). CV, column volumes. X,* conductivity conditions at elution start are ON-specific and are optimized individually.

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Electrophoresis

The quality of the chromatographic purification experiments in general and ON length–based purity were monitored by 15% midsize Criterion TBE-Urea Polyacrylamide Gels available with different well numbers (12 wells, #3450091; 18 wells, #3450092; 26 wells, #3450093) in conjunction with 1x Tris/Boric Acid/EDTA (TBE) running buffer (#1610770). Samples for analysis (typical concentration range: 20–200 ng/µl) were dissolved 1:1 in 2x TBE urea sample buffer (Alfa Aesar, #J60186), which consists of 90 mM Tris base, 90 mM boric acid, 2 mM EDTA, 12% Ficol Solution, 7 M urea, 0.03% bromophenol blue, and 0.03% xylene cyanole. Samples were heated at 70°C for 4 min and the preferred loading volume was 10 µl. After sample loading, gels were run at constant mA until the bromophenol blue front reached two-thirds of the gel length (for example, 30 min at 10 mA and 30–40 min at 20 mA). After the run, the gels were washed 2x for 2 min in water and stained in the dark for 30 min with 1x polyacrylamide gel electrophoresis (PAGE) GelRed Nucleic Acid Gel Stain (Biotium, Inc., #41003) dissolved in electrophoresis buffer or water. The stained gel was washed again in the dark with water for 2x for 2 min and imaged with the GelDoc Go Gel Imaging System (#12009077) using the preset acquisition settings for ethidium bromide. Gel analysis was performed with ImageLab software (version 6.1.0).

Analytical Assays

For rapid ON quantification, 2 µl of sample were applied to a NanoQuant Plate (Tecan Trading AG, #30033939) and absorbance at 260 nm was measured with an Infinite 200 Plate Reader (Tecan, #30016056).

The fraction of the full-length target ON in contaminated samples or side fractions obtained after chromatographic purification were measured after further separation with a high-resolution analytical AEX column (ENrich Q 5 x 50 mm, #7800001). The analytical AEX column can separate the full-length target ON from its contaminants (n – x and n + x). Peak integration and quantification were done with ChromLab Software (version 6.1.27).

* Materials, instruments, and software from Bio-Rad Laboratories, unless otherwise noted.

Results and Discussion

Due to the high complexity of contaminants obtained after the chemical synthesis of single-stranded oligonucleotides, a careful analysis and optimization of critical variables in AEX purification is necessary (Deshmukh et al. 2000). Deshmukh and colleagues describe an excellent framework for this task and provide universal findings, which can be used to shorten the characterization process for resins considered for application in this specific workflow. Based on this process, our characterization and optimization strategy was restricted to the effect of pH, flow rate, dynamic binding capacity, and gradient slope accompanied by an analysis strategy based on two complementary techniques: denaturing PAGE and analytical AEX.

Optimization of Media, pH, and Flow Rate

Single-stranded DNA oligonucleotides are relatively stable molecules at slightly alkaline conditions and are negatively charged at physiological pH and above. AEX chromatography separates ONs by their chain length and high-resolution separations can be obtained for up to 40-mers, as demonstrated with the Bio-Rad UNO Q Resin with a continuous-bed matrix (Bio-Rad Laboratories 1998). Resin selectivity in AEX is influenced by a variety of parameters, including bead size, buffer composition (pH, molarity, ion type), column dimensions, gradient slope, flow rate, temperature, and organic modifiers. Usually, bead size for resins applied in ON purification are in the range of 20–45 µm and successful ON purification schemes using AEX have been reported at different pH values (7.5–8, 9.0–9.5, 12), indicating the importance of pH optimization during method development. High pH values provide denaturing conditions and eliminate the negative effects of the ONs' secondary structure and self-assembly by hydrogen bonding. On the other hand, a few AEX resins do not show long-term stability at elevated pH levels and the selection of a buffer system from pH 8–10 is more appropriate. Although higher separation temperatures (>70°C) and the addition of organic modifiers like methanol might be beneficial for ON purification, these measures add additional cost to a commercial production process and are usually not considered further.

In early experiments, two strong AEX resins (Macro-Prep 25 Q and Nuvia HP-Q) were considered for ON purification since they are designed with an optimal bead size to achieve both laboratory- and process-scale purification of biomolecules at high flow rates without being limited by column pressure. The Macro-Prep 25 Q material has the advantage of a smaller bead size (25 µm vs. 50 µm), increasing peak resolution, but has limited alkaline stability of pH_{max} = 10.

As test samples, ON 1 and 2, which differ by a single nucleotide in length, were mixed at a ratio of about 1:1 and analyzed with both resins at each of two different pH values. Figure 2 shows the performance of Macro-Prep 25 Q Resin at pH 8 and 9.5 and Nuvia HP-Q Resin at pH 9.5 and 11.7. With the Macro-Prep 25 Q Resin, no distinct separation of the two ONs was obtained at either pH. The Nuvia HP-Q Resin runs were successful at pH 11.7, showing a clear separation of the two ONs, but at pH 9.5 a profile with largely overlapping ONs was recorded.

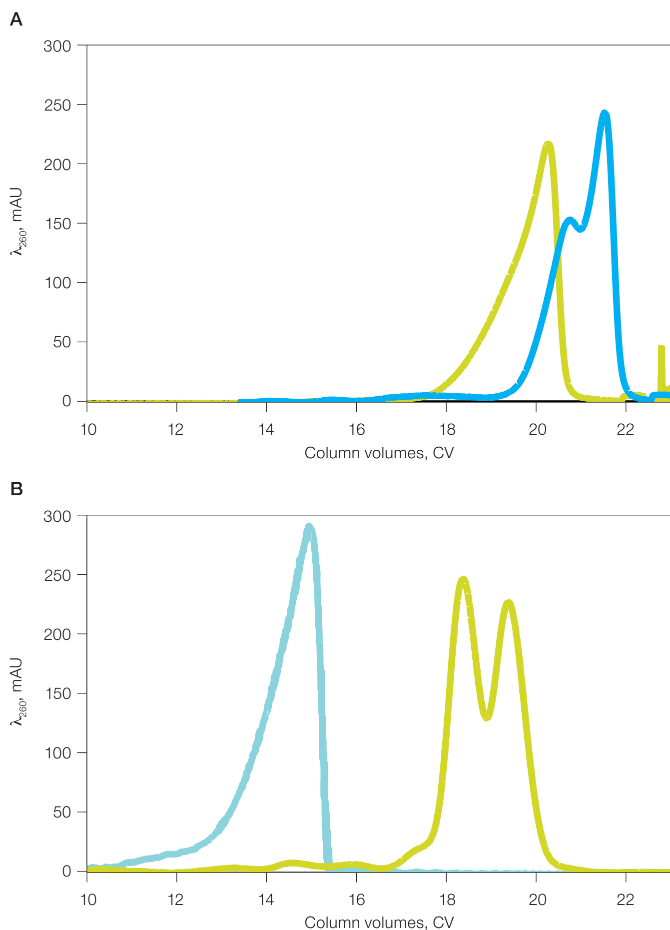

Fig. 2. pH-dependent resin performance testing. Optimization was done at a flow rate of 120 cm/hr with a 1:1 mixture of ON 1 and 2. **A**, Macro-Prep 25 Q Resin at pH 8, (—) and 9.5, (—); elution conditions: 20–35% buffer B (20 CV). **B**, Nuvia HP-Q Resin at pH 9.5, (—) and 11.7, (—); elution conditions: 20–40% buffer B (20 CV). pH 8 and 9.5 buffers are Tris-based (25 mM) instead of arginine-NaOH (see Table 2). Column volume was 5 ml.

The obvious pH-dependency of peak shape and resolution was further evaluated with three 20-mer oligonucleotides that differ significantly in nucleotide sequence or composition (see Methods section). ONs 1, 3, and 4 were chromatographed individually at pH 9.5 and 11.7 and the results are presented in Figure 3. Peak fronting is obvious at pH 9.5 for all three samples, but high quality profiles were obtained at pH 11.7. Similar observations have been published by Deshmukh et al., who reported that ON separations with pH conditions between 11 and 12 show dramatic improvement in peak resolution.

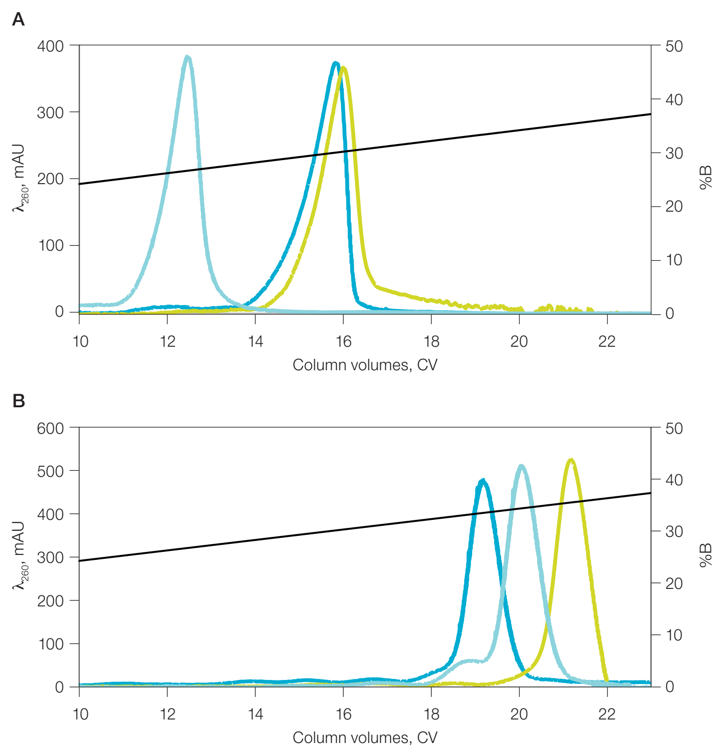

Fig. 3. Influence of eluent pH on retention time. Separation profiles of three different 20-mer oligonucleotides (ON 1, —; ON 3, —; and ON 4, —) at **A**, pH 9.5 and **B**, 11.7 on a 5 ml Nuvia HP-Q Column. The increase in eluent pH results in a retention shift toward higher conductivity conditions and less peak fronting.

The effect of flow rate in preparative AEX on both productivity and peak purity is diametrical and needs to be balanced to serve both needs; it is also probably sample dependent. The 5 ml Nuvia HP-Q Column was tested at two flow rates (120 and 240 cm/hr) and the corresponding results for a 1:1 mixture of ONs 1 and 2 are shown in Figure 4. As expected, the lower flow rate shows a superior resolution. Although separation quality at 240 cm/hr showed an increase of the overlapping zone, this flow rate might still be sufficient for the removal of short and longmers at an acceptable yield. Reported flow rate values from competitor resins with smaller bead sizes are in the range of 150–220 cm/hr. In our case, the experiments for dynamic binding and large-scale purification were all conducted at 100–120 cm/hr, because separation quality and thus improved yield were higher valued than gain in process speed.

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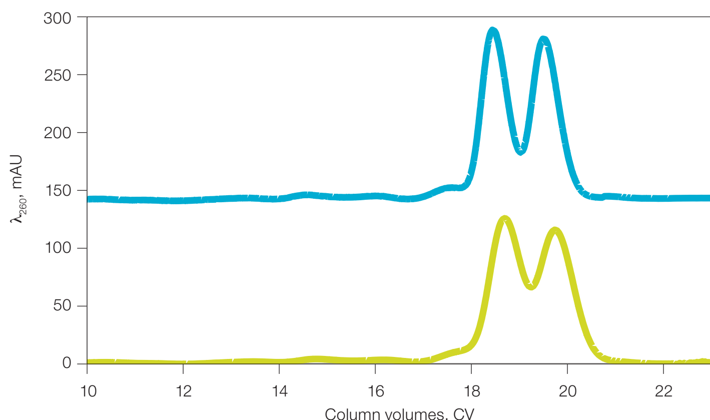

Fig. 4. Influence of flow rate on separation quality. Separation profile of a 1:1 mixture of ON 1 and 2 using a 5 ml Nuvia HP-Q Resin at pH 11.7 with two different flow rates: 120 cm/hr (—) and 240 cm/hr (—). Sample: 1:1 mix of ON 1 and 2.

Dynamic Binding Capacity of Nuvia HP-Q

To assess the dynamic binding capacity of the Nuvia HP-Q Resin, 60 mg of lyophilized ON 1 was first dissolved in 275 ml binding buffer (25 mM arginine-NaOH, 40 mM NaCl, pH 11.7), resulting in a concentration of 0.18 µg/µl at 260 nm. This solution was then applied to determine the dynamic binding capacity at 10% breakthrough with a flow rate of 120 cm/hr. Figure 5 shows the breakthrough curve for a 1 ml Nuvia HP-Q Column with a measured dynamic binding capacity of about 20 mg crude ON 1 per ml of resin.

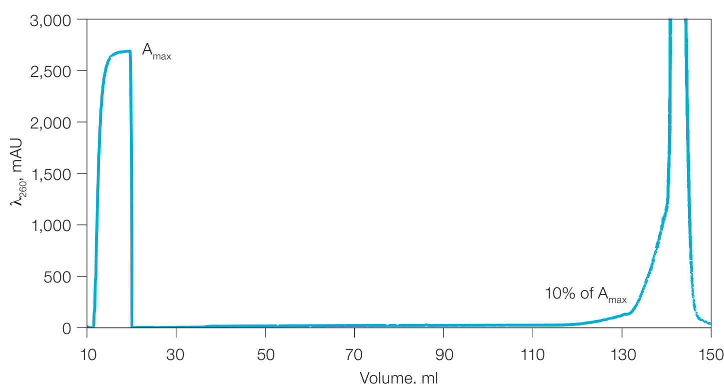

Fig. 5. Dynamic binding capacity of Nuvia HP-Q Resin at 10% breakthrough. Using ON 1 (0.18 mg/ml), binding capacity was determined to be 20 mg oligonucleotide raw material per ml resin. Flow rate: 120 cm/hr.

Analysis Strategy for Determination of Fraction Pooling, Yield, and Purity

Fraction pooling, yield, and purity calculations require a proper analysis strategy. After ON purification, fractions that are significantly contaminated with n – x and n + x species must be identified and excluded from the fraction pooling process to obtain the full-length ON at a desired purity. Analytical methods for successful fraction purity analysis include capillary electrophoresis, high performance liquid chromatography (HPLC), AEX, or size exclusion chromatography (SEC) columns, and denaturing electrophoresis with polyacrylamide gels containing 7 M urea. Here, denaturing PAGE was chosen because up to 26 fractions can be analyzed on a single gel and the results are obtained within 2.5 hours. It is important to mention that samples considered for electrophoresis should be adjusted with water or chromatography buffer to obtain similar conductivity conditions across all samples. This adjustment step can be omitted by extending the sample entry step at 10 mA constant/gel from 30 to 50 min.

After fraction screening with denaturing PAGE, it is recommended to generate a minimum of three fraction pools for further analysis, for example, pool A, ON with n – x contaminants; pool B, ON, highly enriched full-length product; pool C, ON with n + x contaminants.

The second component of our analysis strategy was a chromatography-based quantitative analysis of fraction pools A and C. Using an analytical 1 ml ENrich Q Column, we calculated the ON material lost during the preparative purification process. Analysis of pool B was necessary for assessing product purity.

To understand the nature of and to quantify contaminating species present in ON 1 and 2 raw material and to test the performance of our gel analysis system (15% TBE-urea PAGE), a 1:1 mixture of both ONs (each 2 mg) was generated and purified with a 5 ml Nuvia HP-Q Column (Figure 6A). The chromatogram was divided into seven sections (A–G) and both individual and pooled fractions were analyzed with denaturing PAGE (Figure 6B).

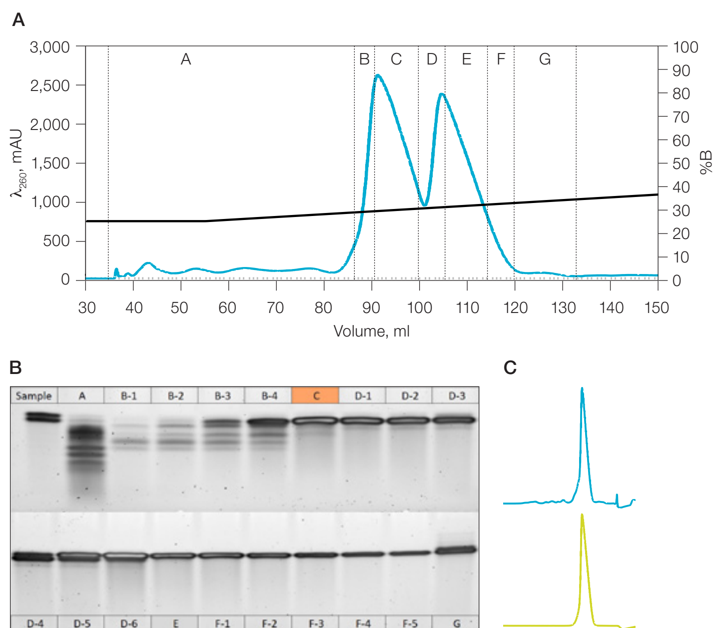

Fig. 6. Separation of individual ONs from contaminating species to assess analysis strategy. A, Separation of each 2 mg ON 1 and ON 2 with a 5 ml Nuvia HP-Q Column at pH 11.7 and 120 cm/hr. Section A: fraction pool, elution volume 34–85 ml, concentrated with a 1 ml Nuvia HP-Q Column; section B: covers four individual 1 ml fractions: B1–B4, elution volume 86–89 ml; section C: fraction pool, elution volume 90–98 ml; section D: covers six individual 1 ml fractions: D1–D6, elution volume 99–104 ml; section E: fraction pool, elution volume 105–113 ml; section F: covers five individual 1 ml fractions: F1–F5, elution volume 114–118 ml; section G: fraction pool, elution volume 119–132 ml. **B**, fractions from sections A–G were further analyzed with denaturing PAGE. **C**, purity comparison of enriched ON 1 (—, fraction pool, Section C) and ON 1 raw material (—) by analytical AEX.

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The two components of the ON sample mixture appeared as two individual bands on the gel image, indicating the high resolution of the 15% TBE-urea gel system and its suitability to separate a full-length ON from its n – x and n + x contaminants (as can be seen in fractions B – 3 and B – 4 for the n – x species). Fraction pool C contains highly enriched ON 1, while fractions D1–D6 visualize the transition from ON 1 to ON 2 according to the Nuvia HP-Q chromatogram (Figure 6A). Very pure ON 2 product can be found in fractions E and F1–F5, while in fraction pool G, ON 2 is contaminated by a clearly visible 22-mer.

The purity of fraction pool C, which contains highly enriched ON 1, was analyzed with a 1 ml ENrich Q Column and compared to unpurified material. As can be seen in Figure 6C, pool C appears as a very homogeneous peak without any of the by-products observed in the unpurified sample.

The data obtained from this small-scale ON purification suggest that the proposed analysis strategy for fraction pooling and product quality analysis based on denaturing PAGE and analytical AEX chromatography can be very well integrated into large-scale purification experiments for single ONs.

Purification of ON 1 Using a 5 ml Column

Lyophilized, unpurified ON 1 was dissolved in binding buffer to reach a concentration of ~0.1 mg/ml at 260 nm and 724 ml of sample solution was applied to purification with a 5 ml Nuvia HP-Q Column at 120 cm/hr. The sample solution contained about 56 mg full-length ON 1. The corresponding chromatogram is displayed in Figure 7. Due to the high concentration of ON 1 in the eluate, the detection at 260 nm reached its limits in combination with a 0.5 cm flow cell.

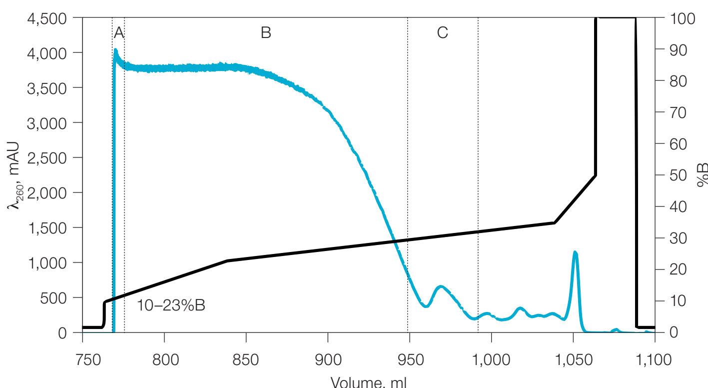

Fig. 7. ON 1 purification with a 5 ml column. Purification of about 72 mg ON 1 raw material with a 5 ml Nuvia HP-Q Column at pH 11.7 and 120 cm/hr. Fraction pool B contains highly enriched ON 1 with a purity of ≥96% at a process yield of 90%.

Fractions containing n – x or n + x species in the presence of full-length ON 1 were easily identified with denaturing PAGE and pooled afterward, as described above (data not shown). Pool A had a volume of 6 ml, pool B with purified ON 1 had 172 ml, and pool C had 40 ml. The three samples, together with the starting material and a 1:1 mixture of ON 1 and ON 2, were applied to denaturing PAGE (Figure 8) and assessed for purity. Additionally, fraction pools A, B, and C were analyzed with a 1 ml ENrich Q Column and quantified for ON 1 content (data not shown).

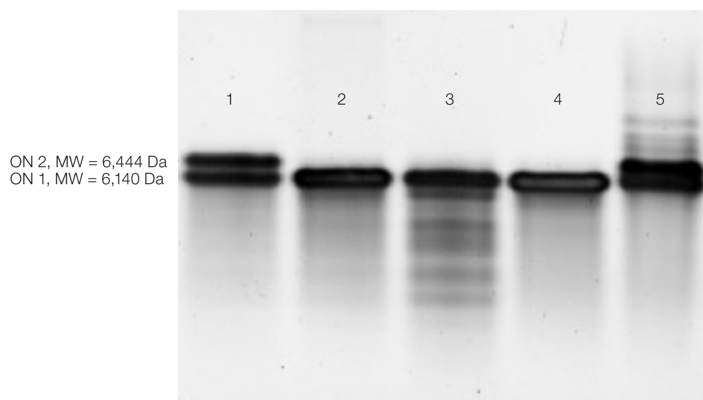

Fig. 8. Denaturing PAGE analysis of fraction pools obtained after the purification of ON 1. Fractions were obtained by AEX chromatography with a 5 ml Nuvia HP-Q Column. Lane 1, 1:1 mixture of ON 1 (6,140 Da) and ON 2 (6,444 Da); lane 2, unpurified ON 1; lane 3, pool A; lane 4, purified ON 1, pool B; lane 5, pool C.

The evaluation of the various datasets revealed an ON 1 purity of ≥96% and the yield of the preparative chromatographic run was calculated to be ~90%. A higher purity level can be easily obtained by changing the pooling strategy based on denaturing PAGE results by excluding additional fractions, either pre– or post–main peak.

The applied elution scheme worked very well for ON 1, but we assume that elution segment 1 needs to be adjusted for other types of ONs, especially for n-mers smaller than 20, because elution segment 1 starts with a NaCl concentration of 200 mM (10% B) to concentrate n – x species at a comparable volume as used for ON 1. A more universal approach, such as gradient elution from 40 to 800 mM salt over 50 column volumes, is a good starting point, but was not verified in practice and depends on the nature and amount of ON to be purified.

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Separating Purified ON 2 “Contaminated” with Purified ON 1

The separation quality of n – 1 from n was analyzed in more detail with a defined artificial sample (ON 1:ON 2 ratio of 1:15) by using purified ONs. Purified ON 1 (3 mg) was spiked into 45 mg purified ON 2. Additionally, elution gradient segment 1 was changed from 10–23% B to 2–23% B to evaluate the distribution width of ON 1 (here, our contaminant) by applying a smoother transition of conductivity conditions after the sample application step. The corresponding chromatogram is displayed in Figure 9 and the first 18 ml of the elution peak were analyzed with denaturing PAGE (Figure 10).

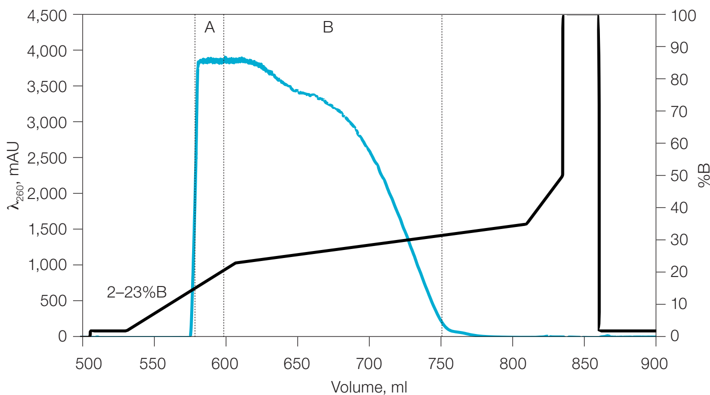


Fig. 9. Separation of purified ON 2 spiked with purified ON 1. Purification of an artificial mixture of both purified ON 1 and ON 2 (ratio 1:15) with a 5 ml Nuvia HP-Q Column.

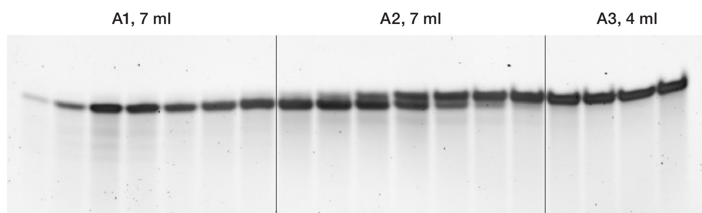
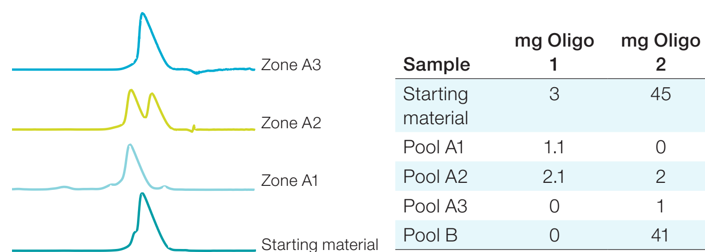


Fig. 10. Analysis of the first eluent peak with denaturing PAGE. Denaturing PAGE analysis of 18 individual fractions of section A from purified ON 2 spiked with ON 1.

The first seven 1 ml fractions contained highly enriched ON 1 (pool A1), the next seven 1 ml fractions (pool A2) contained mixtures where both ONs were present at different ratios, and the final 4 ml (pool A3) was dominated by ON 2. Fraction pools A1–A3 were further analyzed with ENrich Resin and compared against the artificial sample (Figure 11). Data analysis revealed 97% and 93% ON 2 purity and yield, respectively. Finally, Figure 12 shows an overview gel with all relevant samples under investigation.



Sample	mg Oligo 1	mg Oligo 2
Starting material	3	45
Pool A1	1.1	0
Pool A2	2.1	2
Pool A3	0	1
Pool B	0	41

Fig. 11. Analytical AEX chromatography for the characterization of different samples obtained after Nuvia HP-Q purification (see Figure 9) of an artificial ON sample (ON 1:ON 2 ratio = 1:15). The shoulder to the left of the main peak for the starting material is the ON 1 “contamination” in the sample.

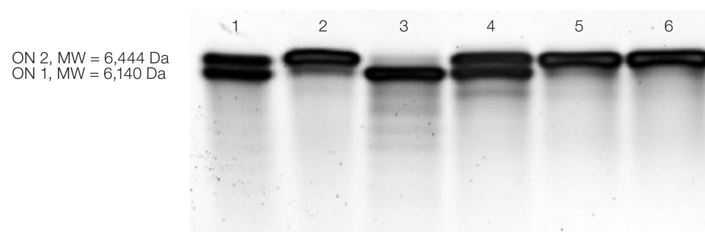


Fig. 12. Assessment of ON 2 purity and yield. Denaturing PAGE analysis of fraction pools obtained after the purification of an artificial ON sample (ON 1:ON 2 ratio = 1:15) by AEX chromatography with a 5 ml Nuvia HP-Q Column. Lane 1, 1:1 mixture of ON 1 and ON 2; lane 2, sample with ON 1:ON 2 ratio = 1:15; lane 3, pool A1; lane 4, pool A2; lane 5, pool A3; lane 6, pool B.

The change in elution strategy for segment 1 seems to be beneficial, since a significant portion of ON 1 was collected as pure fractions in comparison to the dataset presented previously, although the width of the contamination zone A2 with both ONs present is similar to the results reported above for ON 1 purification with the 5ml column.

Purification of ON 1 Using a 25 ml Column

For large-scale purification of ON 1, an Econo Alpha Glass Column (25 x 100 mm) was used to pack a 25 ml Nuvia HP-Q Column at a flow rate of 120 cm/hr and later operated at 100 cm/hr. Lyophilized, unpurified ON 1 (new batch) was dissolved in binding buffer to reach concentration of ~0.23 mg/ml at 260 nm and 1,740 ml of sample solution (400 mg raw material) was applied to a fivefold scale-up purification run by using the optimized gradient slope described for the spiked experiments above. In comparison to the 5 ml column (8 x 100 mm) used for method optimization, the bed height of the Nuvia HP-Q Resin in the preparative Econo Alpha Column is about 50% shorter, while its diameter is increased by a factor of three. The corresponding AEX chromatogram is displayed in Figure 13 and fractions were analyzed with denaturing PAGE to identify the distribution of ON 1 across a 1,500 ml elution volume.

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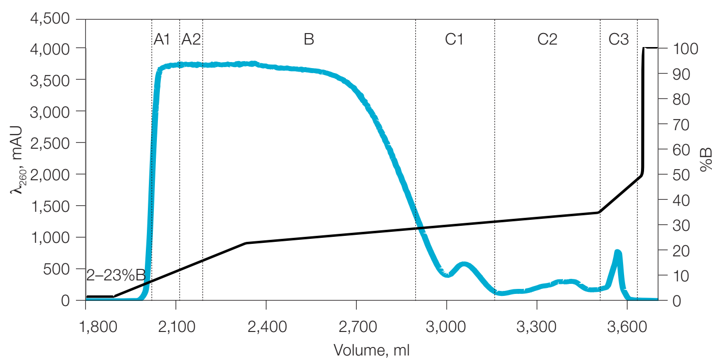

Fig. 13. Large-scale purification of ON 1. About 400 mg raw ON 1 was purified using a 25 ml Nuvia HP-Q Column at pH 11.7 and a flow rate of 100 cm/h. Fraction pool B contains highly enriched ON 1 with a purity of 97% at a process yield of 90%.

Six fraction pools (A1, A2, B, C1, C2, and C3) were generated for further yield and purity analysis based on analytical AEX chromatography (data not shown) and denaturing PAGE (Figure 14). Pool B contains highly purified ON 1, while in pools A2 and C1, ON 1 is contaminated with either n – x or n + x oligo species. Fraction pools A1, C2, and C3 are solely composed of contaminating species without ON 1. Data analysis of fraction pools A2, B, and C1 revealed an ON 1 content of 25 mg, 297 mg, and 6 mg, respectively. In summary, the total yield for ON 1 at a purity of > 97% is ~90% and very comparable to the results obtained by the small-scale analytical 5 ml HP-Q Column. Together, the data suggest a successful scale-up strategy.

ON 1, MW = 6,140 Da

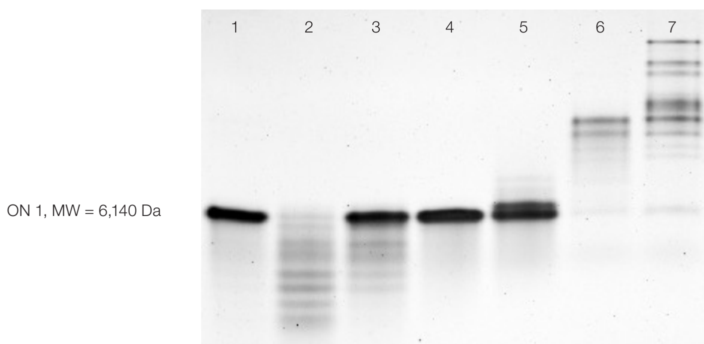

Fig. 14. Denaturing PAGE analysis of fraction pools obtained after large-scale purification of ON 1. Fraction pools were obtained by AEX chromatography with a 25 ml Nuvia HP-Q Column. Lane 1, starting material; lane 2, pool A1; lane 3, pool A2; lane 4, pool B (purified ON 1); lane 5, pool C1; lane 6, pool C2; lane 7, pool C3.

Concluding Remarks

The production of pure single-stranded DNA oligonucleotides requires efficient, scalable, and cost-effective chromatographic purification methods applied after chemical synthesis. Key impurities in crude samples are length based and are best removed by AEX chromatography. Here, we show that Nuvia HP-Q Resin is well-suited for ON purification, as demonstrated with two crude ON species (20-mer and 21-mer) with phosphodiester backbones. Key process parameters like flow rate, gradient slope, buffer pH, and dynamic binding capacity were explored with a prepacked 5 ml column and the resulting protocol optimized for yield was successfully applied to a self-packed 25 ml column. Similar yield (~90%) and purity (~97%) data were obtained with both column sizes and strongly indicate the scalability of the method, although not tested at pilot scale. We also suggest that Nuvia HP-Q is a promising resin for the efficient purification of fully thioated oligonucleotides used in clinical trials.

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