

## APPLICATION NOTE

# Optimization of ALEX purification parameters for phosphorothioate oligonucleotides

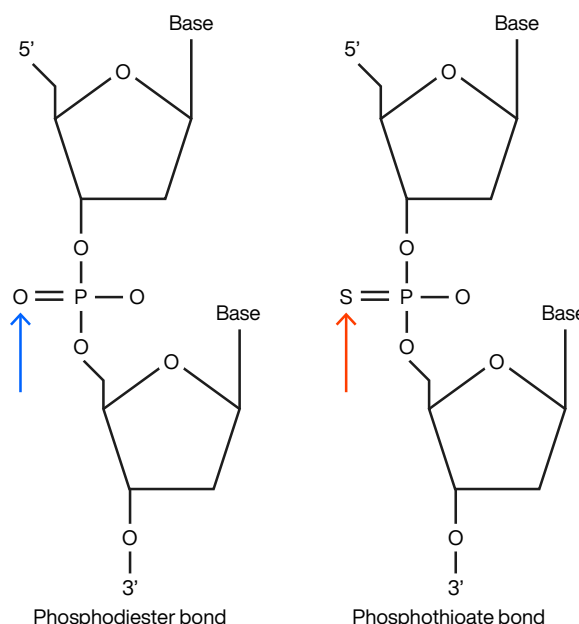
Many therapeutic oligonucleotides, such as antisense oligonucleotides (ASO), have emerged onto the market in recent years. These drugs are highly specific and have both high efficacy and long duration once administered. To increase the *in vivo* stability, modifications in the backbone are commonly employed in which the oxygen atoms are replaced by sulfur atoms, allowing the oligonucleotide to avoid nuclease-mediated degradation. However, the properties of oligonucleotides with phosphorothioate backbone (PS) render them more difficult to purify. In this study we describe important anion exchange chromatography (AEX) parameters that can be optimized in the development of a successful and robust purification setup.

## Introduction

### Oligonucleotides with a phosphorothioate backbone

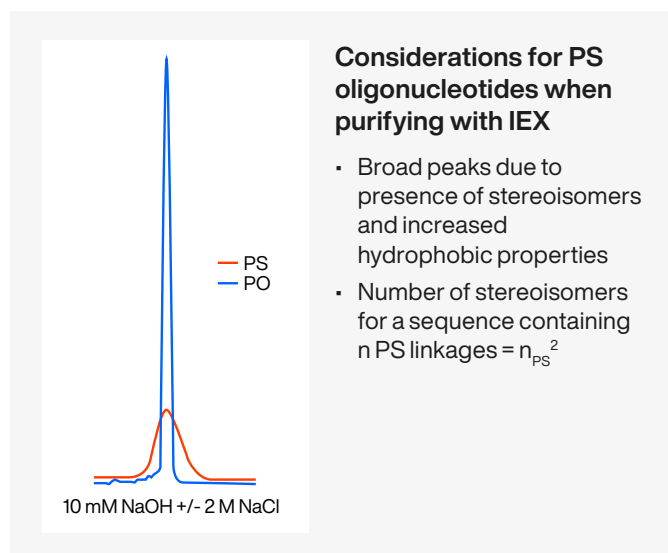
Oligonucleotide (ON)-based therapeutics is an ever-growing market with an increasing number of both FDA approved ON drugs and clinical trials. The growing interest in therapeutic ONs is driven by their high potential to treat diseases. Since ONs are mainly synthetically produced by solid-phase synthesis it is easy to modify them during the synthesis. The most commonly used modification, phosphorothioate (PS) linkages in which the oxygen atom is replaced by a sulfur atom in the backbone (see Fig. 1), increases the *in vivo* stability by reducing the risk of decay by nucleases.

The ON may contain anything from a few to up to 100% PS linkages, resulting in a variety of properties, such as an increase in hydrophobicity, which makes chromatographic purifications more challenging. The introduction of the sulfur atom additionally results in a chiral centre at the phosphorus atom, so that each linkage has two potential configurations and diastereomers are formed. This means that a sequence containing PS modifications gives rise to very complex samples, where the complexity increases with the number of PS linkages (number of stereoisomers =  $n_{PS}^2$ ). The presence of stereoisomers and the increased hydrophobicity results in broad peaks during purification.



**Figure 1.** Phosphodiester linkage (PO) vs. phosphorothioate (PS) linkage.

See Figure 2 for the different elution behavior of a 100% PS DNA oligonucleotide compared to a non-modified DNA oligonucleotide (PO oligonucleotide) on an anion exchange chromatography (AEX) resin using the same experimental running conditions



**Figure 2.** Peak broadening of a 20 nt DNA ON due to PS linkages.

## Purification strategies

Both ion-pair chromatography (IPC)/reversed-phase chromatography (RPC) and AEX are commonly used for oligonucleotide purifications. IPC can provide a good single-step purification for non-problematic oligonucleotides as well as for initial research purification. In a process environment, and with phosphorothioates or difficult-to-purify phosphodiesteres, it is beneficial to also include an AEX step in the setup. Uncharged synthesis byproducts that might form complexes with oligonucleotides will normally fall through without retention on anion exchange chromatography. It is therefore an excellent first purification step and can be followed by reversed phase for final clean-up and polish.

IEX is often the preferred choice at larger scales since it generally has higher loading capacities, is scalable and can tolerate harsh conditions for regeneration.

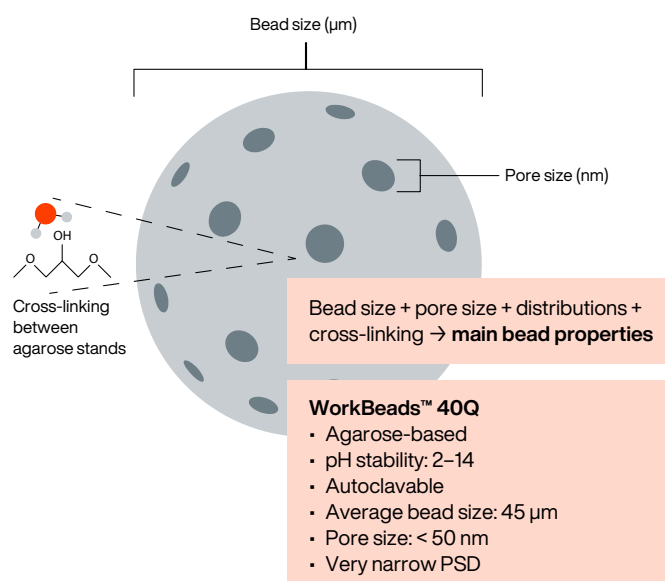
## Chaotropic factors

Synthetic PS oligonucleotides can form many complexes promoted by e.g. hydrophobic interactions, such as secondary structures (hairpins), or multimeric complexes. High sample concentration can increase hydrophobic complex formation. To reduce the risk of these complex formations, organic solvents are commonly added to the mobile phases (acetonitrile, ethanol or isopropanol). These are chaotropic, i.e. they have complex-breaking abilities. Organic solvents both increase solubility of the phosphorothioates and inhibit non-specific hydrophobic interactions and thereby strengthen the ionic separation.

Other factors with chaotropic abilities are high pH, elevated temperature and some elution salts.

## Study design

High resolution separations require high efficiency and good selectivity. To achieve this, optimization is required of both running conditions and resins. The optimal resin should have beads small enough to achieve good separation and rigid enough to be used in scaled-up processes. It is equally important to choose a bead with a pore size optimal for the target molecule, and that exhibits a narrow pore size distribution (PSD) to achieve uniform mass transport, two factors that ultimately lead to greater purity. WorkBeads 40Q is an AEX resin exhibiting all these important properties and is therefore ideal for use in oligonucleotide purifications (Fig. 3).



**Figure 3.** Illustration of the agarose-based WorkBeads 40Q bead. Bead size + pore size + distributions + cross-linking  $\rightarrow$  main bead properties.

In this study we have investigated the influence of different chaotropic parameters for the purification of a DNA ON with fully phosphorothioate backbones using WorkBeads 40Q. The 20 nucleotides (nt) long ON represents a typical antisense oligonucleotide and was thus chosen for this study. At neutral to slightly basic pH, oligonucleotides generally elute as broad peaks and are poorly resolved, which can be partially mitigated by increasing the pH to 12. This is however only possible for DNA-based ONs and not for RNA-based ONs due to stability issues. The chosen buffer system was 10 mM sodium hydroxide (NaOH).

## Screening conditions

The following screening parameters were investigated:

- Concentrations of acetonitrile (0–20%)
- Effect of elution salts (NaCl vs. NaBr vs. NaClO<sub>4</sub>)
- Temperature effect (22°C vs. 40°C)

Analytical scale was applied, where only 20 µg of oligonucleotide sample was injected onto the resin. The output values of the screening parameters were yield, peak width at half peak height and conductivity at peak maximum. Based on the optimal conditions acquired, purification conditions were developed for larger sample load purifications.

## Organic modifiers

As stated above, organic modifiers are needed for optimal resolution for PS oligonucleotides, and the most commonly used is acetonitrile. In Figure 4 the effect of different acetonitrile concentrations on the elution pattern is seen when purifying the 20 nt PS ON and while keeping other parameters constant.

To study the yield in the presence of acetonitrile, areas of the eluted peaks were compared to the peak area when bypassing the column (the same amount of sample was loaded). There is a positive correlation between the concentration of acetonitrile and the yield (Fig. 4B) where 20% of acetonitrile is required to obtain maximum yield without portions of the oligonucleotide being retained on the resin. As can be seen from the peak shape, the ON is eluted in lower volume (Fig. 4A, C), i.e. the purification has higher efficiency, and at lower conductivity (Fig. 4A, C) when a higher concentration of acetonitrile is used. This clearly indicates the presence of complex formation and/or non-specific hydrophobic interactions due to the very hydrophobic nature of the PS oligonucleotide.

## Good to know

- DNA is stable under alkaline conditions, but not under acidic conditions due to depurination (loss of purine bases from DNA)
- RNA is not stable at high pH due to potential base-catalyzed RNA hydrolysis in the backbone since there is a hydroxyl group on the 2' position that may be deprotonated
- Formation of hydrophobic complexes and non-specific interactions are factors that complicate the chromatographic separation using IEX
- Chaotropic abilities is bond/complex breaking abilities
- Components and conditions used in ON purifications with chaotropic effect:
  - High pH
  - Organic solvents
  - Elution salts (NaBr and NaClO<sub>4</sub>)
  - Elevated temperatures
- The negative charge of PS ON is more polarized resulting in tighter binding to an AIEX resin compared to unmodified ONs

## Elution salts

Some elution salts are more chaotropic than others, see Figure 5 which shows the Hofmeister series. NaClO<sub>4</sub> and NaBr are recommended when separating PS oligonucleotides due to their chaotropic behavior. Chaotropic salts reduce the polarity of water and therefore exhibit a lower 'salting-out' effect on hydrophobic molecules. Using such salts ensures maximum solubility during elution. In general, a more chaotropic salt gives shorter retention times and narrower peaks.

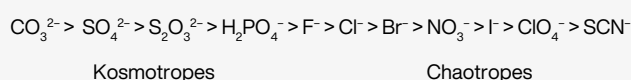


Figure 5. Hofmeister series.

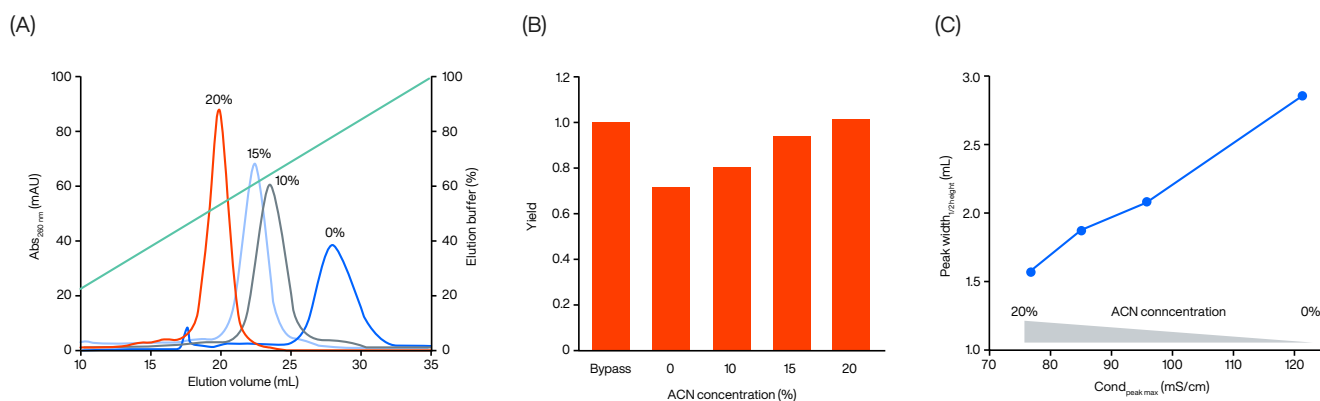


Figure 4. Effect of acetonitrile on PS oligonucleotides. (A) Eluted ON peak at 0% (dark blue), 10% (grey), 15% (light blue) and 20% (red) acetonitrile. Green line represents concentration of elution buffer. (B) Plot visualizing yield obtained at different acetonitrile concentrations. Loaded amount (feed) is the first bar which represent 100% yield. Yield was estimated by integration of UV peaks. (C) Peak width at half height vs. conductivity at peak maximum using different acetonitrile concentrations.

In the following experiments buffers containing 20% acetonitrile were used. Under those conditions, varying the elution salt showed no effect on the yield (Fig. 6B). The chaotropic level of ions, as given in the Hofmeister series above, is also reflected in the elution pattern as shown in Figure 6A and 6C. The highest efficiency was obtained using the more chaotropic salt  $\text{NaClO}_4$  (Fig. 6A, C). Again, this demonstrates the high degree of hydrophobicity of the PS oligonucleotide.

Phosphorothioates and some difficult phosphodiester oligonucleotides often require both chaotropic salts and organic solvents to obtain maximal results.

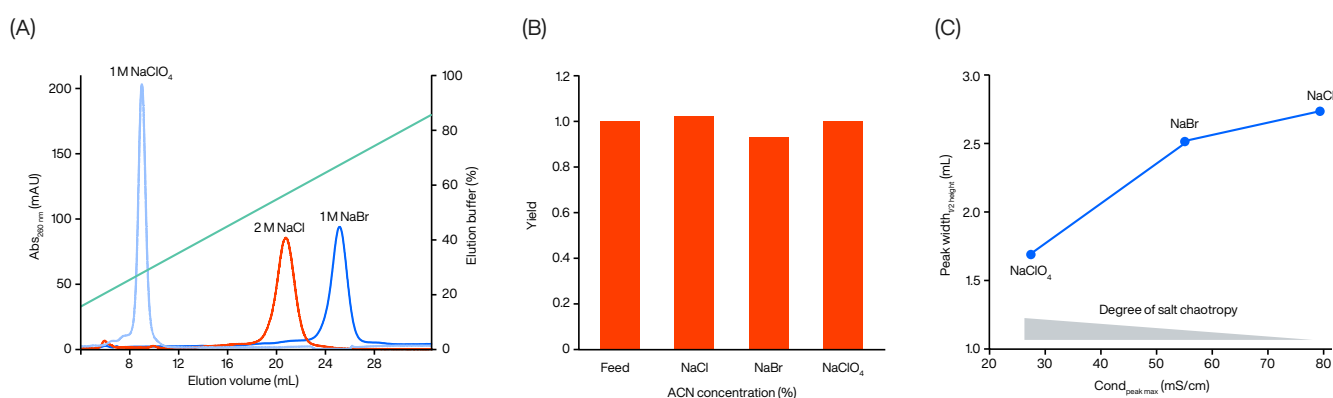
### Temperature effects

Increasing the target temperature had no additional significant effect when both acetonitrile and  $\text{NaClO}_4$  were already included in the buffers. However, it is known that longer oligonucleotides are more prone to form internal structures and it is therefore more beneficial for such ONs to use elevated temperatures to break those structures.

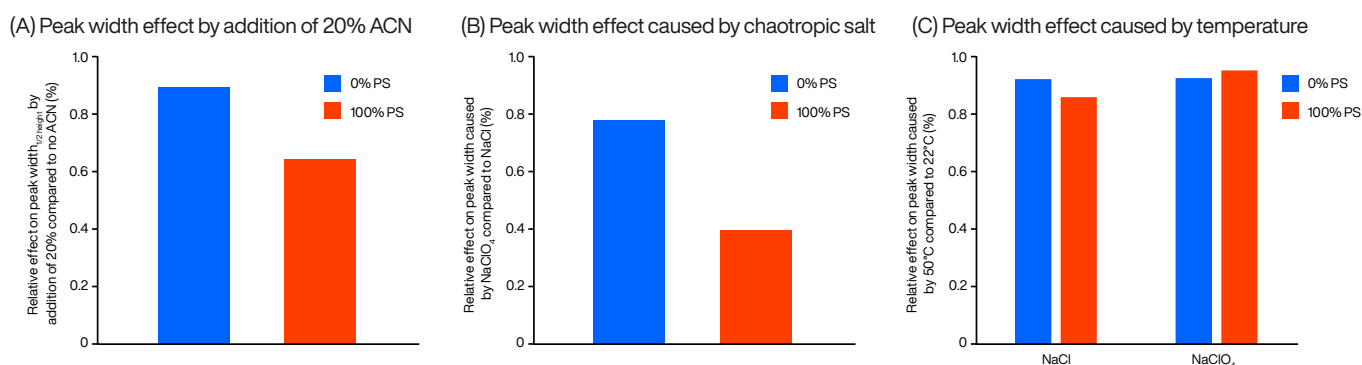
## PO oligonucleotides vs. PS oligonucleotides

To demonstrate the effect of an unmodified oligonucleotide (PO ON) compared to a PS oligonucleotide on the peak width during elution, we subjected both oligonucleotides to the chaotropic conditions and measured the effect on them (value with chaotropic substance/value without chaotropic substance).

When no chaotropic substance is added, the eluted oligonucleotide peak was almost five times broader for the PS oligonucleotide compared to the unmodified PO oligonucleotide (Fig. 1). When 20% acetonitrile is included in the buffers, the PO oligonucleotide peak width is only reduced 10% vs. an almost 40% reduction for the PS oligonucleotide (Fig. 7A). Addition of the next chaotropic substance,  $\text{NaClO}_4$  again had the most profound effect on the PS oligonucleotide (60% compared to 20% reduction of peak width: Fig. 7B). Elevated temperature had minor to no effect when acetonitrile was present regardless of which elution salt was used (Fig. 7C). Here we clearly see the different behaviors in the presence of the chaotropic substances based on the nature of the oligonucleotide and this highlights the importance of optimizing the running conditions for the modified ON.



**Figure 6.** Effect of elution salts on PS oligonucleotides. (A) Eluted ON peak using 2 M NaCl (red), 1 M NaBr (dark blue), or 1 M  $\text{NaClO}_4$  (light blue). Green line represents concentration of elution buffer. (B) Plot visualizing the yield obtained using different elution salts. Loaded amount (feed) is the first bar which represent 100% yield. (C) Peak width at half height vs. conductivity at peak maximum using different elution salts.



**Figure 7.** Effect of chaotropic conditions for PO ON vs. PS ON. (A) The effect of addition of 20% acetonitrile compared to 0% acetonitrile in the buffers regarding peak width. Red bar represents PS ON (value at 20%/value at 0% ACN). Blue bar represents PO ON using same calculations. (B) The effect of addition of 1 M  $\text{NaClO}_4$  compared to 2 M NaCl in the buffers regarding peak width. (C) Peak width effect by elevated temperature (40°C vs. 22°C) using NaCl or  $\text{NaClO}_4$ .

**Purification parameters important for PS ONs**

- Higher ACN → narrower peak/earlier elution
- Chaotropic salt → narrower peak/earlier elution
- ACN and chaotropic salts inhibits hydrophobic interactions/binds weaker to resin
- Increased temperatures have less significant effect on peak width and promote later retentions
- Increased temperature promotes ionic interactions

**20% ACN  
1 M NaClO<sub>4</sub>  
22°C**

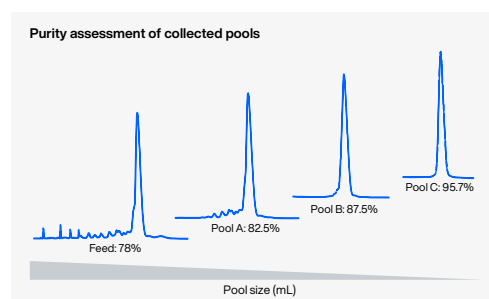
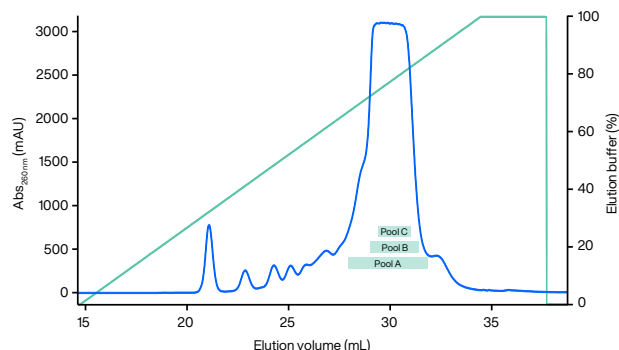
**Purification of PS oligonucleotides**

Based on the optimal parameters obtained during this screening study, PS ON purifications were set up to assess purity and yields. Analytical AIEX (DNAPac™ PA200, ThermoFisher Scientific) was used for the purity assessments. The yield was determined by calculations done on off-line measurements of absorbance at 260 nm of elution pools that were then compared to the amount of loaded crude oligonucleotide before purification.

**Loading at semi-preparative scale**

4 mg PS ON crude (78.2% purity) was loaded onto GoBio™ Mini Q 1 mL (AIEX) using the optimized conditions described above and eluted with 20 column volumes (CV) elution buffer. Fractions were collected for purity assessment. The crude oligonucleotide preparation from the solid phase synthesis in the presence of a high concentration of ammonia and benzamide was loaded onto the resin without any pre-treatment. As can be seen in Figure 8, there are many impurities eluting prior to the target ON/main peak, but there are still many nearby failure sequences in the beginning of the main peak. Analyzed pools of increasing sizes (green boxes in chromatogram) demonstrated this (see highlighted grey box). For the pools visualized in the chromatogram, the purities ranged from 82.5% to 87.5% to 95.7% depending on the size of the pool (grey box; Fig. 8). In the purest fractions at the end of the peak >99% purity could be reached.

Resin: GoBio Mini Q, 1 mL  
 Sample: 1 mL of 110AU/mL crude preparation from a 20-mer PS oligonucleotide (trityl-off)  
 Binding buffer: 10 mM NaOH, 20% ACN  
 Elution buffer: 10 mM NaOH, 20% ACN, 0.4 M NaClO<sub>4</sub>  
 Gradient: 0–100% elution buffer in 20 CV  
 Flow rate: 0.5 mL/min (2 minutes residence time (RT))



**Figure 8.** PS ON elution pattern using AIEX. 4 mg of PS ON crude was purified on GoBio Mini Q, 1 mL and eluted in 20 CV. Elution buffer gradient (green) and UV trace at 260 nm (blue) are shown. Green boxes represent collected pools and analytical chromatograms are shown in grey box.

**Loading at preparative scale**

A preparative scale purification was performed in which 20 mg PS ON crude/mL resin was loaded onto a packed 6.6 × 50 mm column. These mass over-loading conditions mimic the situation at production scale where a maximum yield at a set purity should be met. In this study the set target purity was 95%. Detection at 295 nm was included to reduce the signal for visualization of separation and to mediate more accurate fraction pooling (Fig. 9).

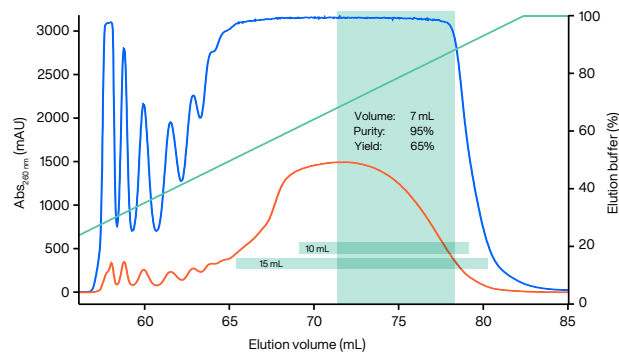
The pre-peaks and the beginning of the plateau, i.e. species eluting prior to the target ON represents n-x and incompletely phosphorothioated ONs. The 95% pure pool collected (highlighted in the chromatogram) had a yield of 65%. Also, larger pools were collected for which higher yields were obtained but at the expense of the purity (see Table 1).

**Table 1.** Purities and yields shown for collected pools.

Volume (mL)	Yield (%)	Purity (%)
7	65	95
10	85	92
15	93	85



Resin: WorkBeads 40Q  
 Sample: 9 mL of 110AU/mL crude preparation from a 20-mer PS oligonucleotide (trityl-off)  
 Column: 6.6 × 50 mm  
 Binding buffer: 10 mM NaOH, 20% ACN  
 Elution buffer: 10 mM NaOH, 20% ACN, 0.4 M NaClO<sub>4</sub>  
 Gradient: 0–100% elution buffer in 20 CV  
 Flow rate: 0.45 mL/min (4 minutes RT)

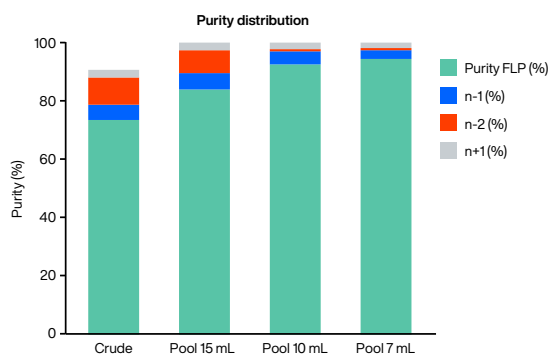


**Figure 9.** PS ON elution pattern on AIEX. 20 mg PS ON crude was purified per mL WorkBeads 40Q resin. Elution buffer gradient (green) and UV traces at 260 nm (blue) and 295 nm (orange) are shown. Green boxes represent collected pools.

To further investigate the identity of the impurities, analyses of the pools were conducted with RPC to increase the resolution between the species. In Figure 10, the distributions for the resolved ON species full length product (FLP), n-1, n-2 and n+1 are visualized. Since the crude contains additional shorter failure species, this bar does not reach 100%, as the others do where only these 4 species were identified. There is a clear inverse correlation between reduced amounts of individual impurities and collected volume size. If higher purities are required, a downstream orthogonal polishing IPC step is recommended.

Column\*: C18-SPS 150-THE (4.6 × 250 mm)  
 Samples: Crude and pooled fractions  
 Sample volumes: 2–4 µl  
 Mobile phase A: 0.1 M TEAA, 5% ACN, pH 7.2  
 Mobile phase B: 0.1 M TEAA, 75% ACN, pH 7.2  
 Flow rate: 1 mL/min

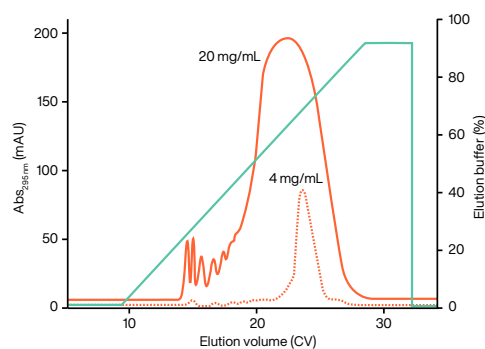
\* Chromatorex



**Figure 10.** PS ON purity distribution within the crude, and each pool, for the resolved species: FLP (green), n-2 (blue), n-1 (red) and n+1 (grey).

## Loadability conclusion

In analytical chromatography, maximal separation is desired in order to identify individual species without too much focus on the yield, whereas maximum yield at a preset purity is often the aim of preparative chromatography. Semi-preparative scale is the transition step between the two phases and important for scaling up. In Figure 11, a comparison between the semi-preparative (4 mg PS-crude/mL resin) and preparative (20 mg PS-crude/mL resin) loading can be seen. Up to 40 mg PS crude/mL WorkBeads 40Q resin can be loaded without any breakthrough.



**Figure 11.** Comparison of different sample loading. Detection at 295 nm.

## Concluding remarks

The increased production of PS oligonucleotide drugs places high demands on downstream purification setups to be robust and to generate high purity end products. It is essential to optimize the purification parameters for these more difficult to purify molecules. The agarose-based AIEX WorkBeads 40Q resin which has optimal properties for such molecules is an excellent first step in a purification setup in which high purities can be obtained at different loading scales. The addition of chaotropic additives significantly improved the separation of impurities that elute close to the PS oligonucleotide target on the AIEX resin and a purity of 95% at a yield of 65% could be obtained at preparative loading scales.

- Longer phosphodiester chains, and especially phosphorothioates will form complexes and are prone to purification difficulties
- Short phosphodiester oligonucleotides are relatively easy to purify

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