



Optimization parameters for anion exchange analysis of oligonucleotides

The development of oligonucleotide therapeutics is on the rise since the first approval in 1998. The 15-25 nucleotide long single or double-strands are designed to interact with a specific RNA sequence and thus modulate mRNA splicing or stability^[1]. In order to do so, the sequence and length of oligonucleotide therapeutics are essential. However, due to errors in the oligonucleotide synthesis process, nucleotides may either be missing (N-x) or are attached in excess (N+x). The addition or lack of a nucleotide goes along with an additional or missing phosphate/phosphorothioate changing the overall negative charge of the molecule, which makes anion-exchange a method to analyze N+/-x impurities.

This application note demonstrates how to optimize a (U)HPLC method for oligonucleotide analysis by modifying the buffer composition and temperature. This helps adjusting the method to the need of individual oligonucleotides and results in a universal method serving as a starting point for in-depth method optimization. A single-stranded oligonucleotide was employed as an exemplary sample and optimization was performed on a TSKgel® DNA-NPR anion exchange column. With its small, non-porous particles with a weak anion exchange ligand, it is well suited for fast nucleic acid analysis.

Material and Methods

Column: TSKgel DNA-NPR (4.6 mm ID x 7.5 cm L)
 UHPLC: Thermofisher Dionex Ultimate 3000 UHPLC system
 Mobile phase: A: 10 mM NaOH pH 12 or 20 mM Tris-HCl pH 8 as indicated
 B: 10 mM NaOH pH 12, 2 M NaCl or 0.8 M NaBr as indicated
 Gradient: 0 min (0% B) 3 min (0% B) 16 min (100% B)
 Flow rate: 0.5 mL/min
 Detection: UV @ 260 nm
 Injection vol.: 5 µL
 Temperature: 25 °C if not stated differently
 Sample: ssDNA full phosphorothioate

Results

The separation of an oligonucleotide sample was tested at pH 8 and 12 (*Figure 1*). At the more basic pH of 12, the major product as well as shorter impurities (eluting earlier) and longer ones (eluting later) were separated. In contrast, these sample components were hardly separated at lower pH. This is explained by reduced ionization of the oligonucleotide at the given pH of 8. Further experiments were, hence, conducted at pH 12.

Figure 2 shows the analysis of an oligonucleotide at room temperature (25 °C) and 60 °C. For both conditions, the major product, shortmers, and longmers were separated. No difference in retention was observed for the analyzed oligonucleotide.

Figure 1. Influence of pH on the analysis of oligonucleotides by AEX.

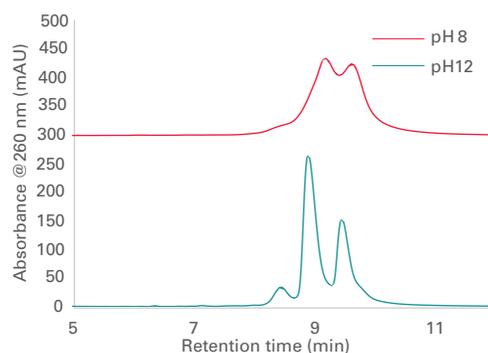


Figure 2. Influence of the temperature on the characterization of oligonucleotides by AEX.

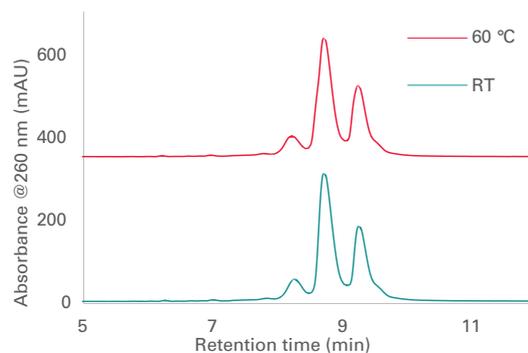
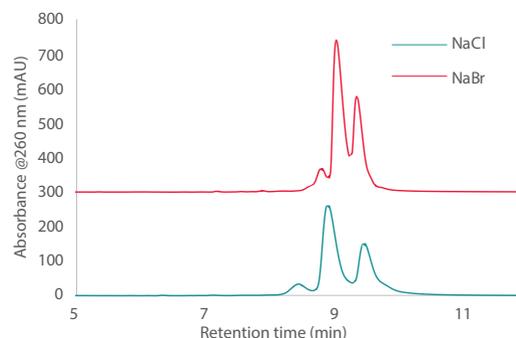


Figure 3. Influence of the type of salt on analysis of oligonucleotides by AEX.

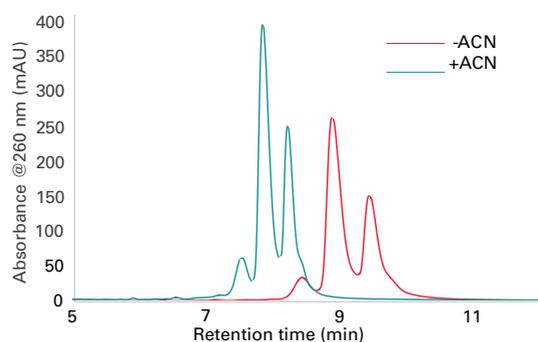


As higher temperature resolves secondary structures when present or -as in our example- have no negative effect, a universal method would still be performed at 60 °C.

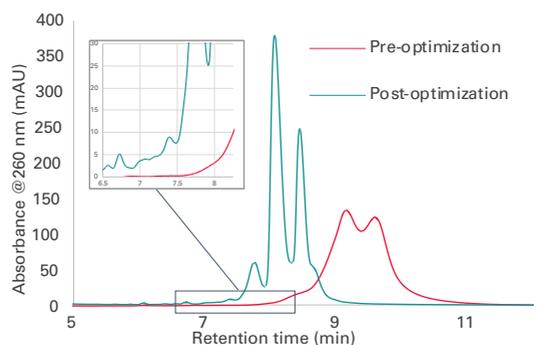
The effect of sodium chloride and the less ionic and more soluble sodium bromide on oligonucleotide separation was tested. Due to a better elution, a lower concentration for NaBr (0.8 M) was employed, allowing for evaluation of selectivity differences. Both salt types separated the major product from smaller and longer impurities. While sodium chloride resulted in broader peaks, sodium bromide led to sharper peaks but reduced resolution (*Figure 3*). Further gradient optimization could potentially lead to a better separation with both salts.

The addition of 5 % acetonitrile decreased the retention time and led to peak-sharpening to both mobile phases (NaCl) (*Figure 4* when eluting with NaCl). This is due to decreased hydrophobic interactions between phosphorothioates and the stationary phase in the presence of organic modifiers.

➤ **Figure 4.** Influence of 5 % acetonitrile (ACN) on analysis of oligonucleotides by AEX.



➤ **Figure 5.** Result of optimization process.



Conclusion

We investigated how various conditions influence oligonucleotide analysis by anion-exchange chromatography. By modifying the buffer conditions (i.e., salt type, organic modifier addition, and temperature), we developed a universal method, which is a good starting point for oligonucleotide method development (A: 10 mM NaOH, 5% ACN, pH12, B: 2M NaCl, 5% ACN, *Figure 5*).

The TSKgel® DNA-NPR anion exchange column has proven ideal for this application as it is stable at high pH and high temperatures, which is required for oligonucleotide analyses to prevent formation of secondary structures. In addition, the non-porous base beads offer fast mass transfer and consequently an analysis in less than 15 min.

Reference

[1] Dhuri K *et al.* (2020) Antisense Oligonucleotides: An Emerging Area in Drug Discovery and Development. *J Clin Med.* 2020 Jun; 9(6): 2004. doi: [10.3390/jcm9062004](https://doi.org/10.3390/jcm9062004)

➤ **Table 1.** Featured product..

P/N	Description	Dimension
0018249	TSKgel DNA-NPR	4.6 mm ID x 7.5 cm L

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