Application Note

Biologics and Biosimilars

Agilent

Trusted Answers

# High Resolution Separations of Oligonucleotides using PL-SAX Strong Anion-Exchange HPLC Columns


**Authors**

J. Massi and L. Lloyd

Agilent Technologies, Inc.


## Introduction

Oligonucleotides are synthesized on solid phase supports using an automated procedure of sequential addition of one nucleotide residue. At the end of the synthesis the oligonucleotide remains attached to the support matrix with the nitrogen and oxygen protecting groups left intact. The cleavage from the solid support matrix removes the protecting groups with the exception of the dimethoxytrityl (DMT) protecting group attached to the 5'-O-oligonucleotide terminus. This DMT protecting group can be used to separate based on hydrophobicity and then following deprotection, removal of the DMT group, anion-exchange HPLC can be performed to further resolve the oligonucleotide product from failure sequences.

## Materials and reagents

### Figure 1

| Parameter | Value |
|---|---|
| Column | Agilent PL-SAX 1000Å, 8 µm, 50 × 4.6 mm (p/n PL1551-1802) |
| Eluent A | 7/93 v/v acetonitrile/0.1 M TEAA, pH 8.5 |
| Eluent B | 7/93 v/v acetonitrile/0.1 M TEAA, 1 M ammonium chloride pH 8.5 |
| Gradient | 0 to 40% B in 10 min, 40 to 70% B in 14 min, 70 to 100% B in 25 min |
| Flow Rate | 1.5 mL/min |
| Temperature | 60 °C |

### Figure 2

| Parameter | Value |
|---|---|
| Column | Agilent PL-SAX 1000Å, 8 µm, 150 × 4.6 mm (p/n PL1551-3802) |
| Eluent A | 7/93 v/v acetonitrile/0.1 M TEAA, pH 7.0 |
| Eluent B | 7/93 v/v acetonitrile/0.1 M TEAA, 3.24 M ammonium acetate pH 7.0 |
| Gradient | 0% B for 2 min, 0 to 100% B in 20 min |
| Flow Rate | 1.5 mL/min |
| Temperature | 60 °C |

### Figure 3

| Parameter | Value |
|---|---|
| Column | Agilent PL-SAX 1000Å, 8 µm, 50 × 4.6 mm (p/n PL1551-1802) |
| Eluent A | 1 M sodium hydroxide |
| Eluent B | 1 M sodium hydroxide, 2 M sodium chloride |
| Gradient | 75 to 100% B for 25 min, 0 to 100% B in 20 min |
| Linear Velocity | 360 cm/h |

## Results and discussion

High-resolution separations of oligonucleotides can be achieved using the PL-SAX strong anion-exchanger with separations of n-1 from n being achievable. Figure 1 shows the separation of a poly-T-oligonucleotide size standard spiked with a 10mer, 15mer, 30mer, and 50mer (main peaks). Acetonitrile is added to the eluent to suppress the formation of secondary structures that would have a detrimental effect on the separation/resolution. With the gradient used here baseline separation of the n-1 from n is easily obtained up to the 15mer, but as the size increases so the resolution decreases.

With larger oligonucleotides, mass transfer becomes more difficult, resulting in less efficient separation, but it can be improved by working at elevated temperature. The PL-SAX 1000Å material has good open pore morphology. When this is used at elevated temperature large oligonucleotides can be chromatographed, as shown in Figure 2.

For oligonucleotide therapy, chemical modifications are made to the phosphodiester oligonucleotide to increase its *in vivo* resistance to nuclease degradation. One such modification is thiolation to produce the phosphorothioate analog of the oligonucleotide. The purification challenge is then not only to purify from the failure sequences but also to separate the complete and incomplete thiolated forms. For this separation, purification under denaturing conditions to avoid aggregation is required. The

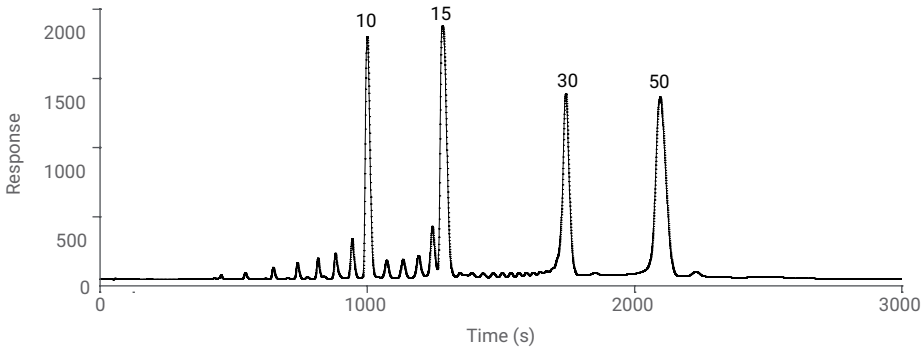


**Figure 1.** High resolution separation of a poly-T-oligonucleotide size standard spiked with a 10mer, 15mer, 30mer and 50mer (main peaks).

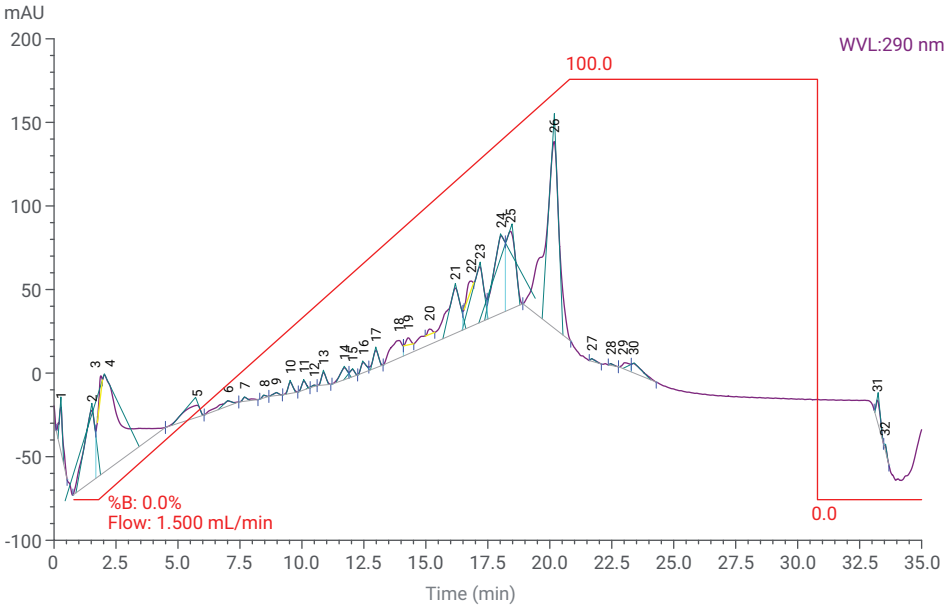


**Figure 2.** Purification of a large, 91mer, synthetic oligonucleotide.

use of high pH also suppresses the charge effects of the bases. With a strong ion-exchange functionality on a chemically stable polymeric particle, anion-exchange chromatography can be performed at pH 12 to 13. Figure 3 shows the separation of complete and incomplete thiolated forms of a typical 20mer oligonucleotide using sodium hydroxide as the eluent.

## Conclusion

Polymeric strong anion-exchange materials, such as the PL-SAX, are ideal for anion-exchange analysis and purification of oligonucleotides, due to the material being both thermally and chemically stable. By working at elevated temperature, enhanced mass transfer increases the resolution for longer oligonucleotides. Denaturing conditions, pH 12 and above, can be used to avoid aggregation of modified oligonucleotides, self-complementary and GC-rich oligonucleotides during analysis and purification. The high pH also suppresses ionization of the individual bases, working above their pH to enable resolution of phosphodiester and phosphothioate forms of the oligonucleotide.

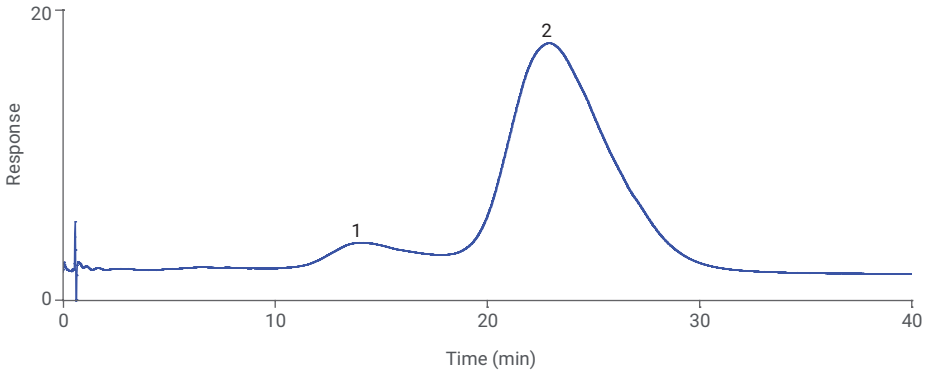


**Figure 3.** Separation of residual phosphodiester (1) and a phosphorothioate (2) 20mer oligonucleotide.

**www.agilent.com/chem**

DE44257.4713888889


Printed in the USA, March 15, 2021
5990-8297EN

Agilent
Trusted Answers