Application Note

Biopharma/Pharma

Agilent

Trusted Answers

Scalable Purification of a Synthetic Oligonucleotide Using Agilent PL-SAX Columns

Authors

Andrea Angelo P. Tripodi and
Andrew Coffey
Agilent Technologies, Inc.

Abstract

Ion-exchange chromatography (IEX) is an effective method for separating and purifying oligonucleotides based on their charge. Given their negatively charged phosphate backbone, anion-exchange chromatography (AEX) is the preferred approach. While ion-pair reversed-phase chromatography (IP-RP) is commonly used for oligonucleotide analysis, IEX offers distinct advantages for specific applications, providing robustness and compatibility with standard buffers across both analytical and preparative scales. This application note highlights the excellent performance of Agilent PL-SAX columns for synthetic oligonucleotide analysis and suggests an optimal kickoff for exploring alternative method conditions.

Introduction

Synthetic oligonucleotides have gained significant attention in recent years due to their ability to regulate protein expression and enable gene editing. Advances in synthetic methods have led to greater accuracy and improved efficiency in oligonucleotide production. However, the inherent complexity and extensive modifications of these molecules can often result in significant variability in yield and purity. Choosing the appropriate column chemistry for purification depends on factors such as desired final purity, buffer compatibility, and production scale. AEX is widely used for purifying oligonucleotides, from short sequences to large molecules up to 100 mer in length.[1,2] A key advantage of AEX is its compatibility with commonly available buffers, such as tris, phosphate, or ammonium salts, rather than volatile additives such as hexafluoroisopropanol (HFIP), typically required for IP-RP. HFIP is costly and toxic for the environment. In addition, IP-RP often requires the use of organic solvents, like acetonitrile (ACN) or methanol, which are harmful and more expensive than aqueous, salt-based buffers. Moreover, IP-RP leaves the purified oligonucleotide with the residual ion-pair counter ion requiring further cleanup. This makes AEX a highly cost-effective and practical technique for separating oligonucleotides from related impurities.

Experimental

Analytical equipment

An Agilent 1290 Infinity III LC system was composed of the following modules:

- Agilent 1290 Infinity III high-speed pump (G7120A)
- Agilent Infinity III multisampler with sample thermostat (G7167B)
- Agilent 1290 Infinity III multicolumn thermostat (G7116A)
- Agilent 1290 Infinity III diode array detector (G7165A) with a 10 mm InfinityLab Max-Light cartridge cell (G7117-60020)

Preparative equipment

An Agilent 1290 Infinity II preparative LC system was composed of the following modules:

- Agilent 1290 Infinity II preparative binary pump (G7161B)
- Agilent 1260 Infinity II preparative fraction collector (G7157A)
- Agilent 1290 Infinity II preparative column compartment (G7163B)
- Agilent 1260 Infinity II diode array detector (G7165A)

Columns

Analytical columns:

Agilent PL-SAX 1000Å HPLC column, 4.6 × 150 mm, 8 µm (part number PL1551-3802)

Agilent AdvanceBio oligonucleotide, 4.6 × 150 mm, 2.7 µm (part number 653950-702)

Preparative column:

Agilent PL-SAX 1000Å, 25 × 150 mm, 10 µm (part number PL1251-3102)

Sample

The sample to be purified was a crude all-2'-O-methylated 22-mer oligonucleotide provided by a customer.

Sample preparation

The RNA sample was diluted in mobile phase A containing 10 mM Tris HCl pH 8.0. The concentration for the analytical run was 1 mg/mL, and 20 mg/mL for the preparative run.

Software and data processing

Agilent OpenLab software suite, version 2.8.

Reagents and chemicals

All reagents were HPLC grade or higher.

Method parameters

Table 1. Analytical LC parameters for the analysis of the crude sample.

Parameter	Value
Column	Agilent PL-SAX 1000Å, 4.6 × 150 mm, 8 µm
Eluent A	10 mM tris buffer pH 8.0
Eluent B	10 mM tris buffer + 1 M NaCl
Gradient	20 to 80% B in 30 minutes
Flow Rate	1.0 mL/min
Column Temperature	6 °C
Detection (DAD)	260 nm
Injection Volume	10 µL

Table 2. Preparative LC parameters for the purification of the crude sample.

Parameter	Value
Column	Agilent PL-SAX 1000Å, 25 × 150 mm, 10 µm
Eluent A	10 mM tris buffer pH 8.0
Eluent B	10 mM tris buffer + 1 M NaCl
Gradient	20 to 100% B in 27 minutes
Flow Rate	29.5 mL/min
Column Temperature	25 °C
Detection (DAD)	260 nm
Injection Volume	300 and 800 µL

Table 3. Analytical LC parameters for fraction re-analysis.

Parameter	Value
Column	Agilent AdvanceBio oligonucleotide, 4.6 × 150 mm, 2.7 µm
Eluent A	100 mL 1 M TEAA pH 8.65 + 900 mL water
Eluent B	100 mL 1 M TEAA pH 8.65 + 900 mL ACN
Gradient	5 to 22% B in 20 minutes
Flow Rate	1.0 mL/min
Column Temperature	60 °C
Detection (DAD)	260/4 nm (reference 360/20 nm) peak width > 0.025 min (10 Hz)
Injection Volume	5.0 µL

Results and discussion

The separation and purification of a 22-mer synthetic oligonucleotide can be easily achieved using a PL-SAX column. The fractions that are obtained through IEX can be easily analyzed by IP-RP using 0.1 M TEAA with an AdvanceBio oligonucleotide column. The results demonstrate the performance of the PL-SAX 1000Å, 4.6 mm id, 8 µm for the analysis of the crude sample before purification (Figure 1). An analytical PL-SAX 1000Å, 4.6 × 150 mm, 8 µm column was used, containing stationary phase media with the same specifications as the preparative 150 × 25 mm column.

An initial scouting gradient was used, from 20 to 80% B over 30 minutes, to ensure that adequate separation of the main peak from the impurities was achieved (Table 1).

A second gradient from 40 to 80% B over 20 minutes, using the same 2% B per minute gradient did not offer any improvement. Integration of this analytical separation suggests that the purity of the crude sample was approximately 85%. For the preparative scale separation, the same mobile phase composition was used, allowing for the column to be flushed with up to 1 M NaCl at the end of each run. This can help to ensure that no later-eluting impurities are allowed to build up on the column. The flow rate was scaled up linearly from 1.0 mL/min on the 4.6 mm id column to 29.5 mL/min on the 25 mm id column. Two runs were made (see Tables 1 and 2 for conditions). The first run used an injection volume of 300 µL of 20 mg/mL crude oligonucleotide solution (equating to 6 mg injected) to ensure that the detector was not saturated, and that the separation efficiency could be determined (Figure 2). After demonstrating an equal separation performance to the one seen on the analytical column, a larger 800 µL injection was made (equating to 16 mg injected), as shown in Figure 3. Notice that the main peak has now saturated the detector. There is also a slight shift in retention time due to the amount of material loaded onto the column.

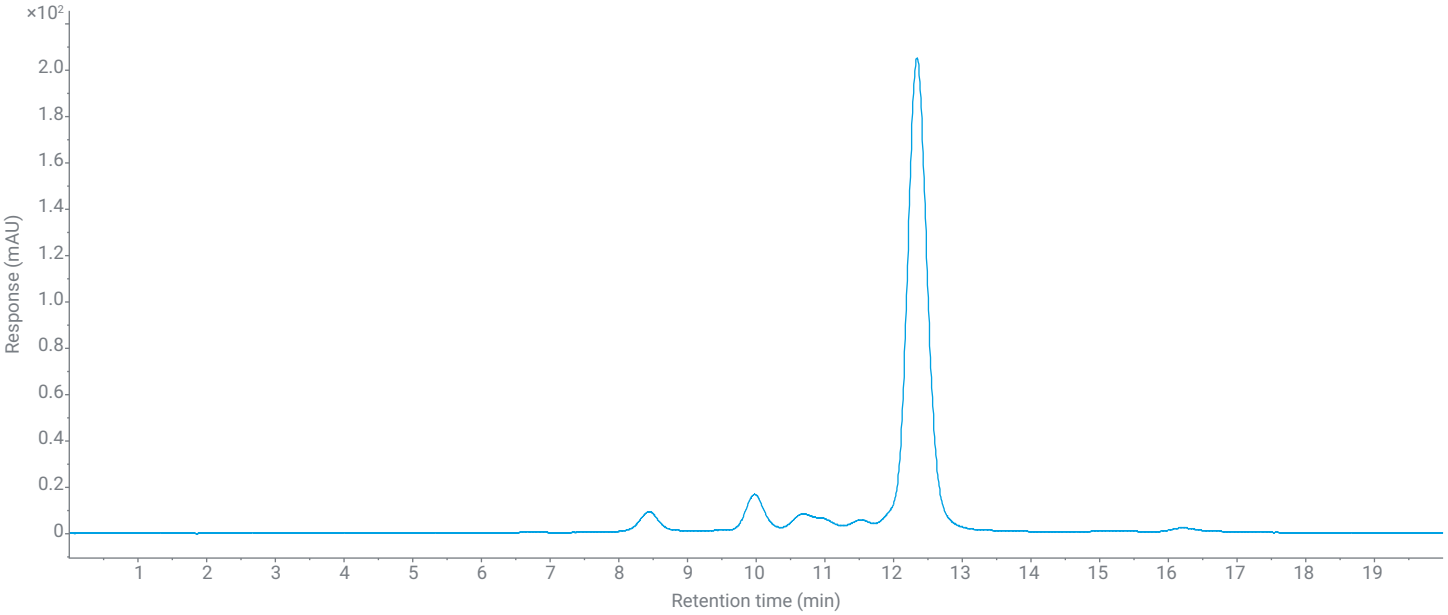

Figure 1. Analytical LC/UV analysis of the 22-mer crude synthetic oligonucleotide. LC gradient and temperature 40 to 80% B, 0 to 20 minutes (2% B per minute) at 60 °C (Table 1).

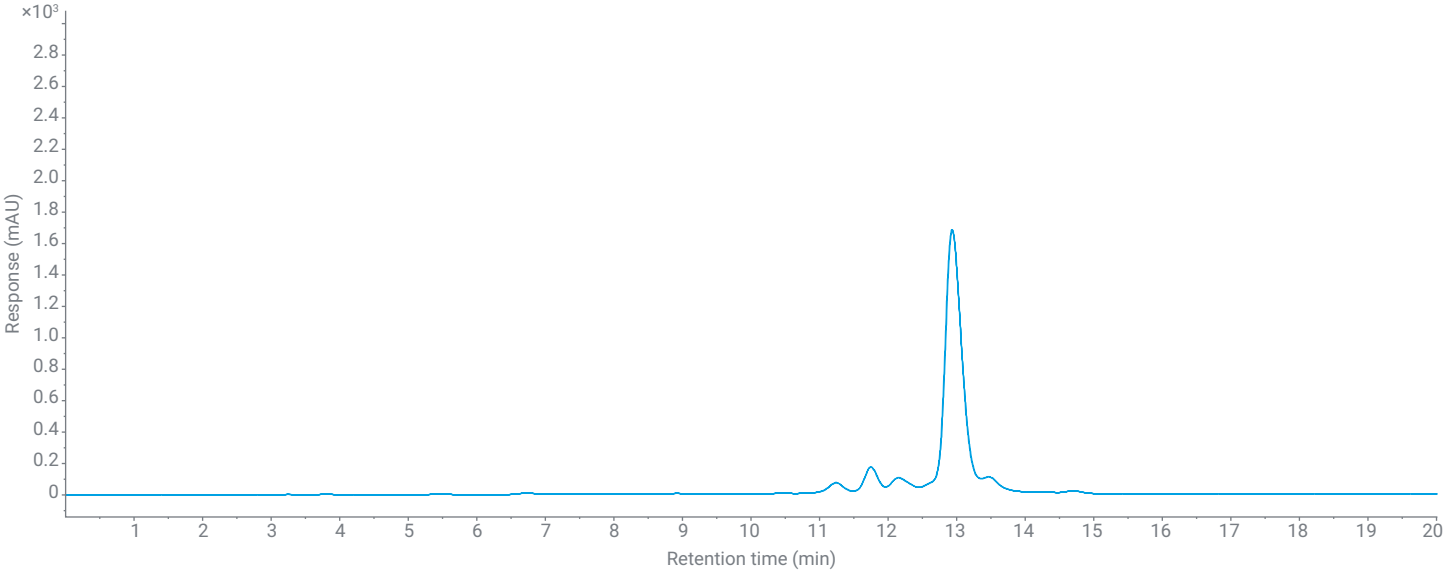

Figure 2. Preparative LC/UV separation of the 22-mer crude synthetic oligonucleotide, 300 µL injection (20 mg/mL). LC gradient and temperature 20 to 100% B, 0 to 27 minutes; 25 °C (Table 2).

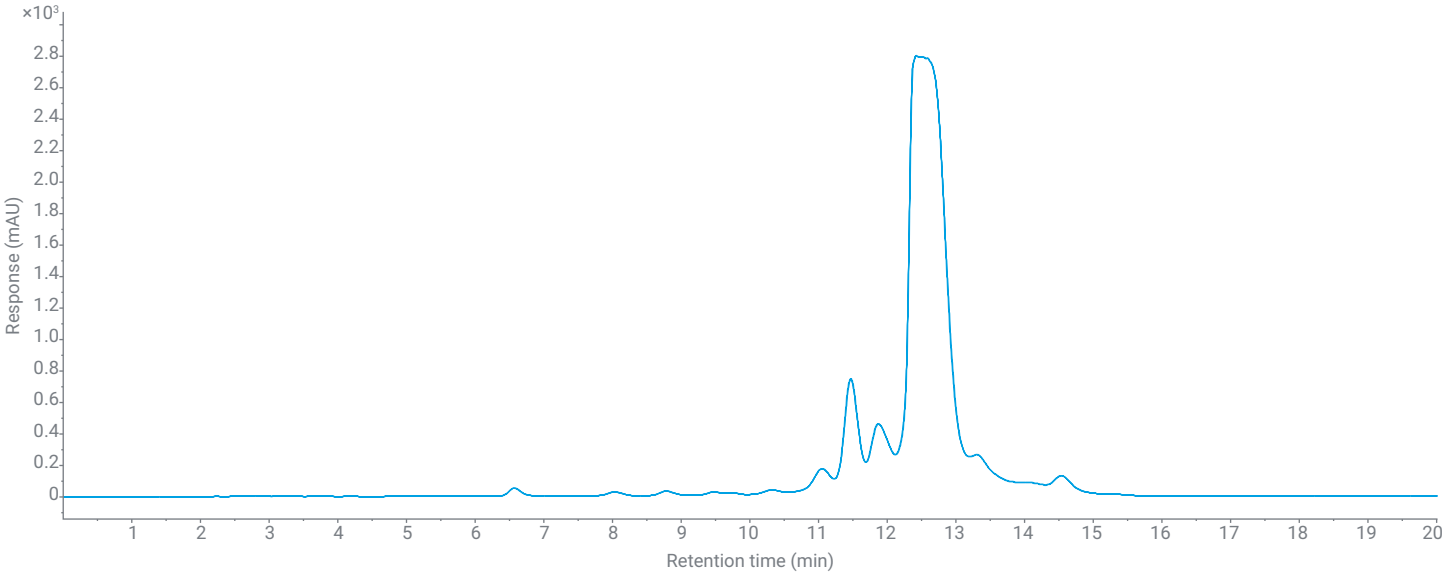

Figure 3. Preparative LC/UV separation of the 22-mer crude synthetic oligonucleotide, 800 µL injection (20 mg/mL). LC gradient and temperature 20 to 100% B, 0 to 27 minutes; 25 °C (Table 2).

The dynamic binding capacity for the PL-SAX 8 µm 1000Å is approximately 12 mg per mL of column volume. A 150 × 25 mm column has a volume of 74 mL and so an overall capacity (to saturation) of about 880 mg. A 16 mg injection therefore equates to almost 2% of the binding capacity, which is an ideal amount to ensure there is minimal risk of overloading the column causing impurities to coelute with the main peak. Of course, it is possible to further optimize the separation to maximize the amount of crude sample that can be injected per run. The fraction collector was set to conservatively collect 1.5 mL volume fractions. This was to ensure that closely eluting impurities that may partially coelute with the main peak could be removed if necessary. After collecting 16 fractions as shown in Figure 4, each fraction was analyzed by IP-RP (see Table 3 for the method).

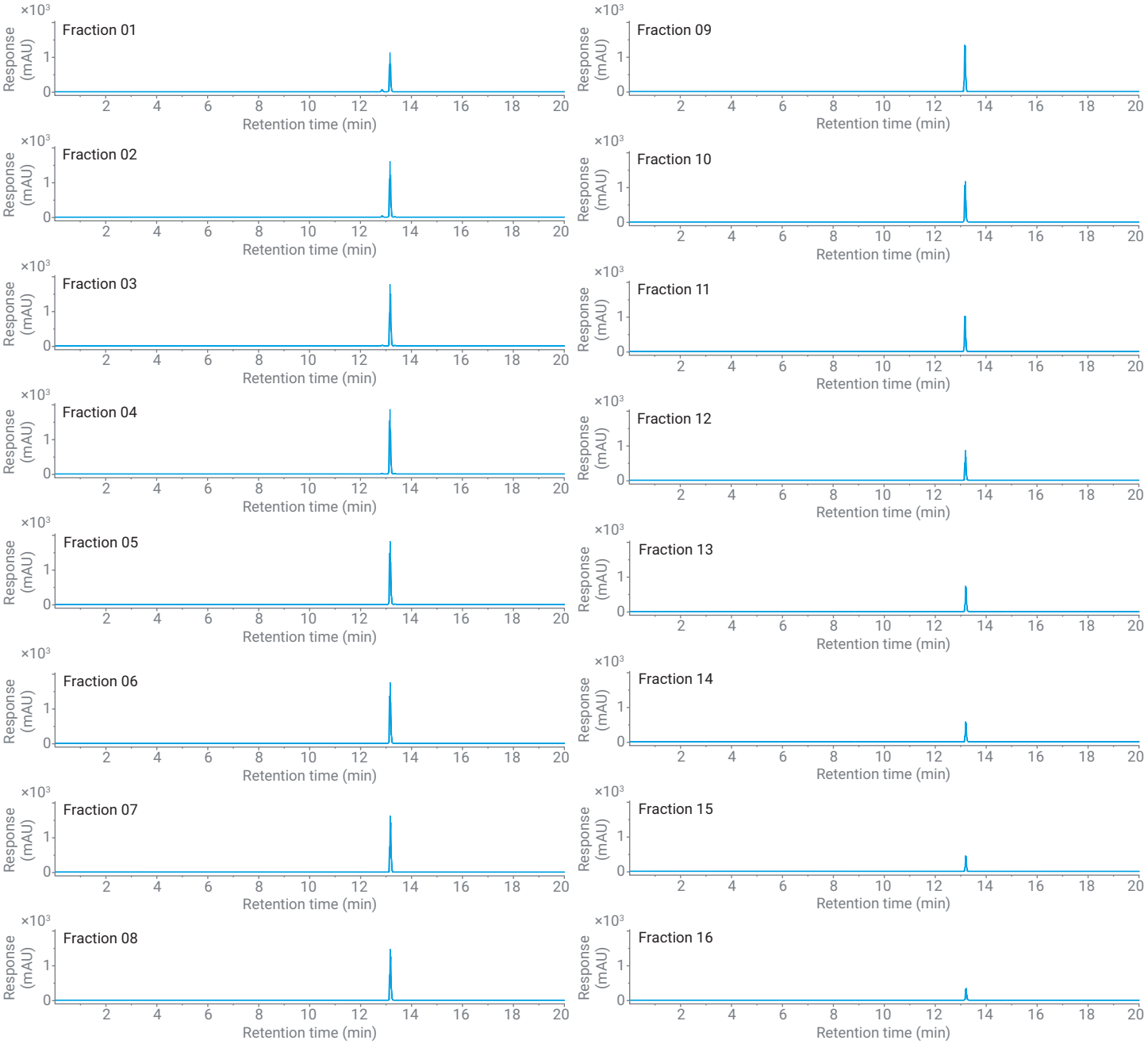

Figure 4. Analytical LC-UV chromatograms (260 nm) showing the main fractions collected from the fraction collector.

By assessing the yield and purity of each fraction it is possible to calculate which fractions should be combined to give the overall best yield and purity (Table 4).

Figure 5 shows an offset overlay of the chromatogram of each fraction. The first two or three fractions contain a small amount of an earlier eluting component as well as the main peak.

Table 4. Peak area and product purity of fractions for the main peak by IP-RP.

Fraction	Main Peak Area	Impurity Area	Total Area	Main Peak Area Percentage
F01	1,267.9	151.8	1,419.7	89%
F02	1,909.1	131.4	2,040.5	94%
F03	2,142.3	109.9	2,252.2	95%
F04	2,243.4	89.1	2,332.5	96%
F05	2,194.8	75.1	2,269.9	97%
F06	2,082.3	67.5	2,149.8	97%
F07	1,925.2	59.7	1,984.9	97%
F08	1,732.8	49.4	1,782.1	97%
F09	1,546.4	45.7	1,592.1	97%
F10	1,353.4	41.9	1,395.3	97%
F11	1,159.1	38.8	1,197.9	97%
F12	977.6	45.0	1,022.6	96%
F13	803.5	40.9	844.4	95%
F14	635.8	32.8	668.5	95%
F15	489.9	28.1	517.9	95%
F16	373.0	27.9	400.9	93%
	22,836.5	**1,034.9**	**23,871.4**	**96%**

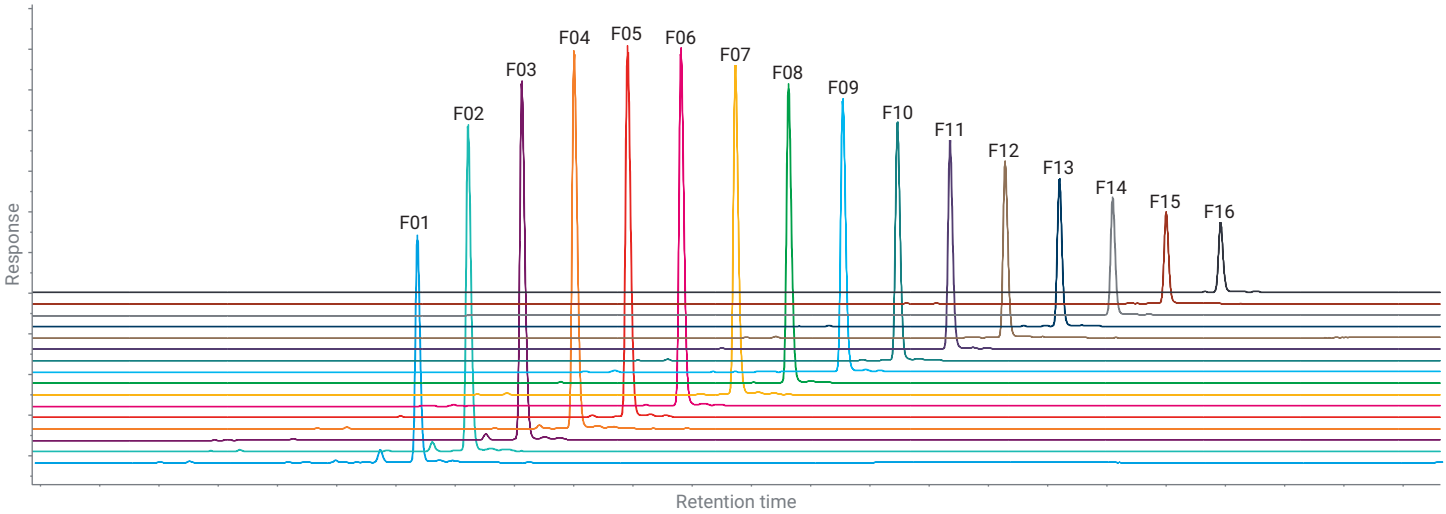

Figure 5. Offset, overlaid chromatograms of Figure 4 (UV 260 nm) of the IP-RP analysis of sixteen fractions. See Table 2 for LC gradient and temperature.

Conclusion

This application note demonstrates optimized method conditions for the purification of an RNA sample using anion-exchange liquid chromatography (AEX). Moreover, a fast and easy scale-up method from 4.6 mm id analytical to preparative scale was successfully achieved using a 25 mm id Agilent PL-SAX column, enabling higher sample loading and helping you to meet your yield requirements using aqueous buffer or nonvolatile conditions. Finally, PL-SAX is also available as bulk resins, making them ideal for fractionating large quantities of material.

References

1. Hsiao, J.; Apffel, A.; Turner, M. Optimizing Separation of Oligonucleotides with Anion-Exchange Chromatography, *Agilent Technologies application note*, 5994-4753EN, **2022**.
2. Massi, J.; Lloyd, L. High Resolution Separations of Oligonucleotides using PL-SAX Strong Anion-Exchange HPLC Columns, *Agilent Technologies application note*, 5990-8297EN, **2021**.
3. Tripodi, A. A. P.; Coffey, A. Superficially Porous Columns for Semi-Preparative Purification of s Oligonucleotides, *Agilent Technologies application note*, 5994-7478EN, **2024**.

www.agilent.com

DE-006667

Printed in the USA, May 22, 2025
5994-8381EN

Agilent
Trusted Answers