Application Note

Biopharmaceuticals

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Analysis of Oligonucleotides with Ion Exchange Chromatography and Agilent Infinity II UHPLC

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Abstract

Although oligonucleotide analysis is frequently conducted using ion-pair reversed-phase chromatography, ion exchange chromatography can be better suited for certain analyses. This application note demonstrates the utility of Agilent Bio SAX columns for oligonucleotide analysis and provides suggested starting points for further method development.

Introduction

With the advent of FDA-approved gene therapies based on mRNA and adeno-associated viral (AAV) vectors, there is growing interest in developing new treatments based on these technologies. The production and characterization of high-quality genetic material is crucial to these efforts, as they may serve as active ingredients (e.g., siRNA or mRNA) or as vital inputs to bioprocesses (e.g., plasmid DNA).

Depending on the application, oligonucleotides may vary widely in size: siRNA (20 to 24 nucleotides (nt)), oligonucleotide aptamers (20 to 80 nt), sgRNA for CRISPR/Cas9 gene editing (~100 bp), and mRNA (1,000 to ≥4,000 nt). The appropriate separation mode for analyzing oligonucleotides often depends on their size and specific chemistry and must be chosen carefully to enable reliable detection of impurities such as prematurely terminated short-mers or host cell DNA.

As oligonucleotides carry a high density of negative charges, anion exchange chromatography can be used for high-resolution separations. Mobile phases are typically buffered using Tris-HCl, and analytes are often eluted using NaCl or $NaClO_4$ salt gradients.

Experimental

Reagents

Trizma base (Tris), sodium chloride, and sodium perchlorate used in the experiment were purchased from Sigma-Aldrich.

Standards used were as follows:

- Agilent Oligonucleotide ladder standard: ssDNA (dT 15, 20, 25, 30, 35, 40 nt) (part number 5190-9029)
- Agilent Oligonucleotide resolution standard: ssRNA (part number 5190-9028)
- Invitrogen dsDNA 100bp ladder (part number 15628019)
- Thermo-Fisher Scientific GeneRuler 1kb dsDNA ladder (part number SM0311)
- Thermo-Fisher Scientific PhiX174 DNA/BsuRI (HaeIII) dsDNA Marker (part number SM0251)

Mobile phase composition was as shown in Table 1. All solutions were adjusted to pH 8 with 1M HCl.

Table 1. Composition of ion exchange chromatography mobile phase.

Solution	Composition (/L)
20 mM Tris-HCl	Tris base 2.42 g
20 mM Tris-HCl + 1.5 M NaCl	Tris base 2.42 g + NaCl 87.66 g
20 mM Tris-HCl + 1.5 M $NaClO_4$	Tris base 2.42 g + $NaClO_4$ 183.66 g

Analytical instruments and conditions

The following instrumentation and chromatographic gradients (Table 2) were used for ion exchange chromatography. Agilent OpenLab CDS 2.6 was used for data acquisition and analysis.

Abbreviations

- **HPLC:** High-performance liquid chromatography
- **UHPLC:** Ultrahigh-performance liquid chromatography
- **SAX:** Strong anion exchange
- **RNA:** Ribonucleic acid
- **DNA:** Deoxyribonucleic acid
- **NMP:** Nucleoside monophosphate
- **dNMP:** Deoxynucleoside monophosphate
- **Rs:** Resolution
- **nt:** Nucleotides (unit)
- **bp:** Base pairs
- **PEEK:** Polyether ether ketone

Table 2. Ion exchange chromatography analysis conditions.

Parameter	Value
Instrument	Agilent 1290 Infinity II Bio UHPLC with high-speed pump
Column	Agilent Bio SAX NP5 PEEK, 4.6 × 250 mm, 5 µm (p/n 5190-2467) Agilent PL-SAX 4000A, 4.6 × 250 mm, 10 µm (p/n PL1551-5103)
Flow Rate	1.0 mL/min
Column Temperature	65 °C
Injection Volume	5 µL
Sampler Temperature	4 °C
Detector	UV 260 nm (G7117B DAD HS, flow cell 60 mm)
NaCl Mobile Phase	A: 20 mM Tris-HCl, pH 8 B: 20 mM Tris-HCl + 1.5 M NaCl, pH 8
$NaClO_4$ Mobile Phase	A: 20 mM Tris-HCl, pH 8 B: 20 mM Tris-HCl + 1.5 M $NaClO_4$, pH 8
NaCl Gradient 1	Time (min) %A %B 0 70 30 20 45 55 25 0 100 25.1 70 30 30 70 30
NaCl Gradient 2	Time (min) %A %B 0 80 20 0.5 80 20 5 55 45 50 30 70
NaCl Gradient 3	Time (min) %A %B 0 50 50 1 50 50 30 30 70
$NaClO_4$ Gradient 1	Time (min) %A %B 0 95 5 0.5 95 5 5 85 15 50 60 40

Results and discussion

Comparison of Agilent Bio SAX and PL-SAX columns

Agilent Bio SAX columns contain nonporous particles, whereas PL-SAX columns are highly porous to increase binding capacity. Situations where mass transfer effects are small favor the use of nonporous columns despite their lower binding capacity, because they can typically sustain higher flow rates at lower backpressures.

Figures 1 and 2 show analysis of a dsDNA ladder using Bio SAX NP5 PEEK, 4.6 × 250 mm (part number 5190-2467) and PL-SAX 4000A, 4.6 × 250 mm, 10 µm (part number PL1551-5103) columns. The Bio SAX NP5 PEEK column was eluted with NaCl gradient 2 and the PL-SAX 4000A column was eluted with NaCl gradient 3 (Table 2). The Bio SAX column was capable of high-resolution separations up to 10,000 bp and showed excellent performance in the BsuRI enzyme-digested sample (Figure 1C) where each fragment was clearly identified. The PL-SAX column had lower resolution compared to Bio SAX, which could be suitable for purification applications.

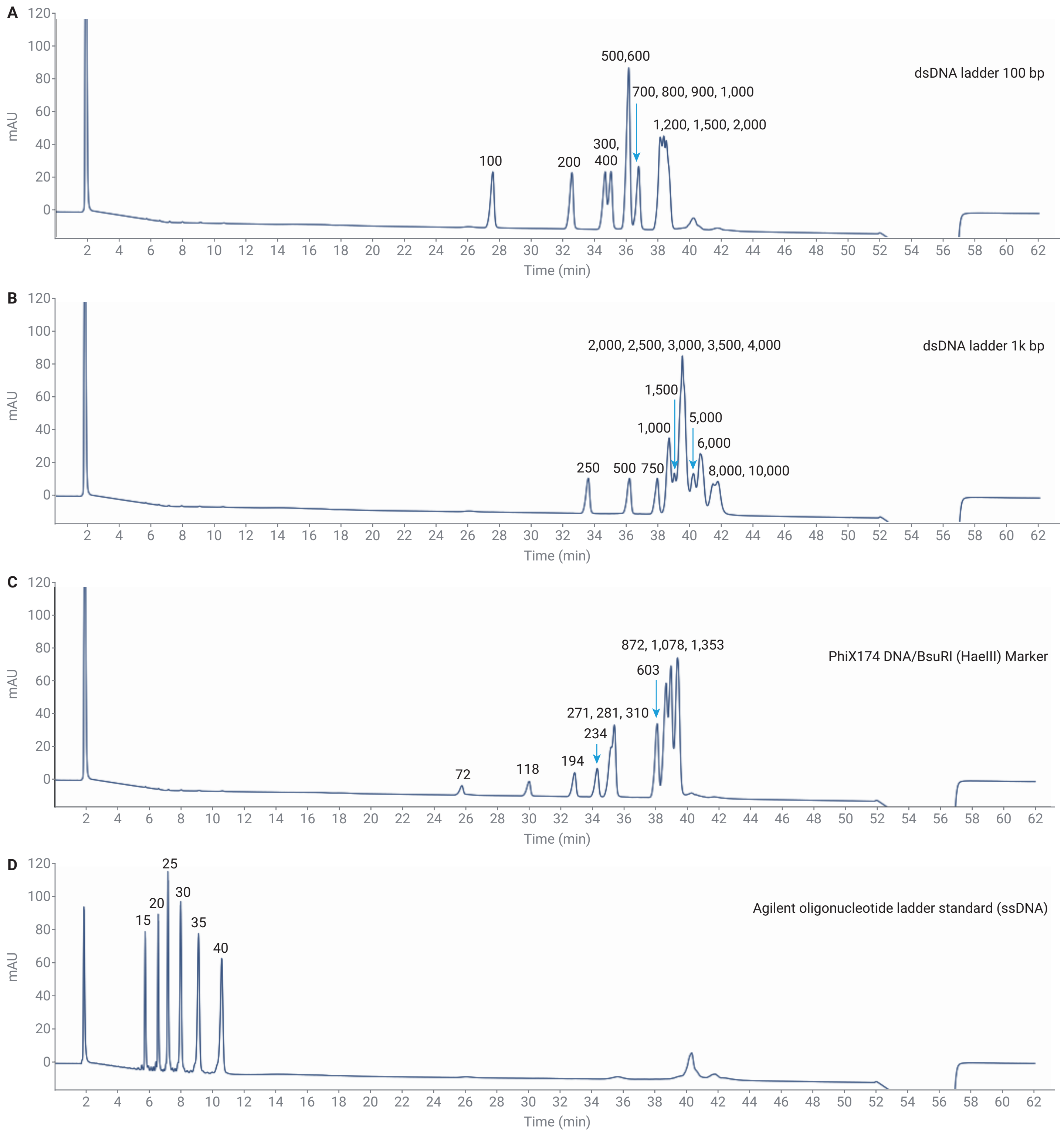

Figure 1. DNA standards separated on an Agilent Bio SAX NP5 PEEK column: (A) dsDNA ladder 100 bp, (B) dsDNA ladder 1k bp, (C) PhiX174 DNA/BsuRI (HaeIII) Marker, and (D) Agilent ssDNA oligonucleotide ladder standard.

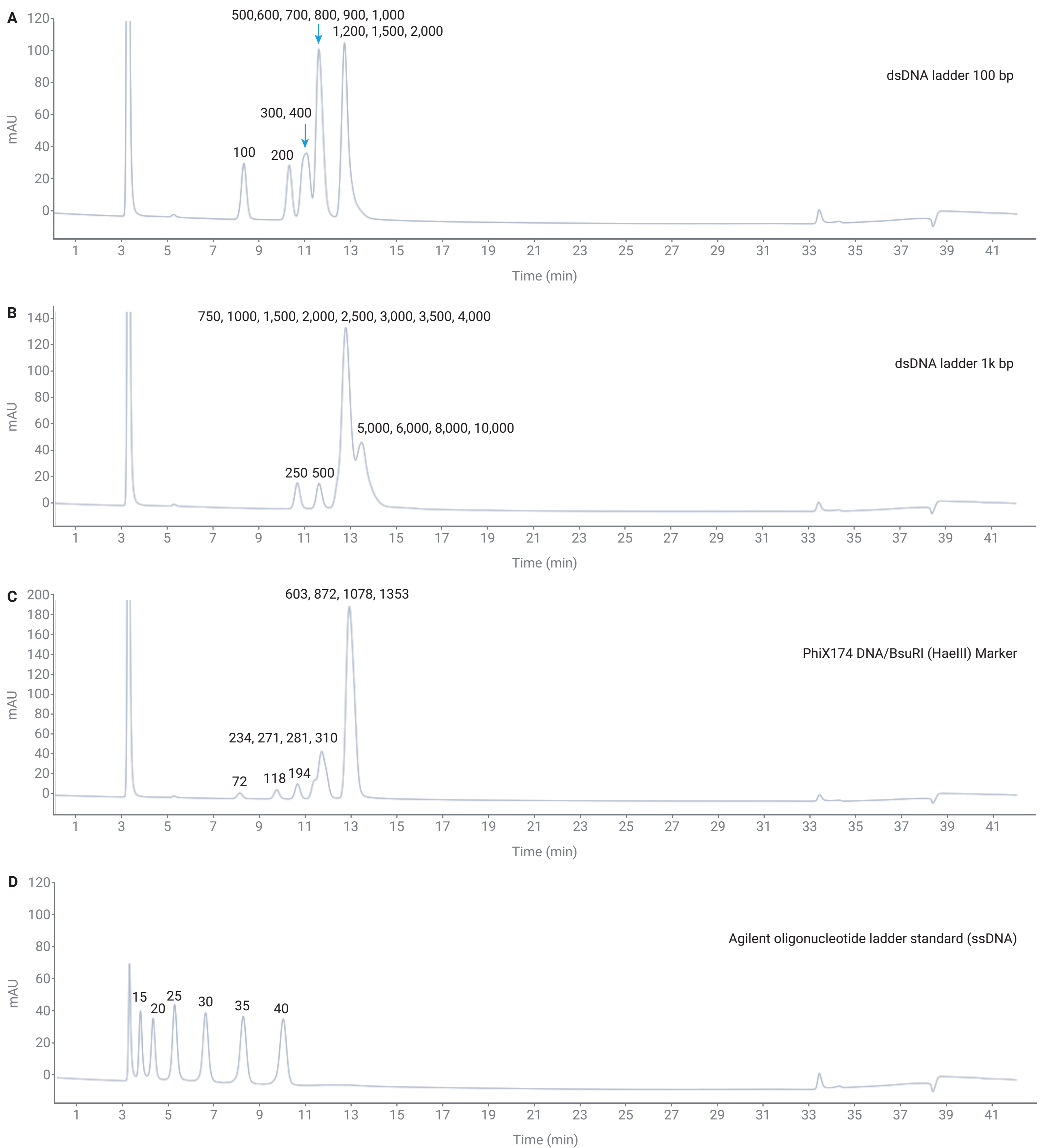

Figure 2. DNA standards separated on an Agilent PL-SAX column: (A) dsDNA ladder 100 bp, (B) dsDNA ladder 1k bp, (C) PhiX174 DNA/BsuRI (HaeIII) Marker, (D) Agilent ssDNA oligonucleotide ladder standard.

Salt gradient elution with sodium perchlorate

Sodium chloride (NaCl) and sodium perchlorate ($NaClO_4$) are often used as eluting salts for oligonucleotide analysis. $NaClO_4$ has more than twice the elution strength of NaCl at the same concentration and has high hydrophobicity compared to NaCl, which is advantageous for oligonucleotide analysis in conjunction with hydrophobic moieties such as fluorescent markers. On the other hand, the selectivity of ion exchange chromatography analysis using NaCl can be modified with the addition of 10 to 30% acetonitrile. As these eluting salts are either corrosive or oxidizing, a chemically inert system such as the Agilent 1290 Infinity II Bio UHPLC is strongly recommended for anion exchange chromatography analysis.

Figure 3 shows separation of DNA standards using a Bio SAX NP5 column and $NaClO_4$ as the eluting salt using $NaClO_4$ Gradient 1 (Table 2). When compared to separations carried out using NaCl as the eluting salt (Figure 1), it was noted that analytes generally eluted at lower concentrations of $NaClO_4$ than NaCl. In addition, $NaClO_4$ appeared to improve chromatographic resolution for some analytes (e.g., 8,000 and 10,000 bp dsDNA (Figure 3B) and Agilent oligonucleotide ladder standard (Figure 3D)), while compromising the separation of others (e.g., BsuRI enzyme digested sample, Figure 3C). This indicates that $NaClO_4$ alters the specificity of separation, in addition to enhancing eluting power.

Effect of column temperature on Bio SAX separations

As in other forms of chromatography, mass transfer, mobile phase viscosity, column retention, and the eluting power of salts in ion exchange chromatography are all temperature dependent. In addition, oligonucleotide duplexes may be observed in single-stranded or double-stranded form depending on column temperature. Figure 4 shows that increased temperature improves the retention and separation of DNA and RNA standards on the Bio SAX column. Increasing temperature has a similar effect on PL-SAX separations (Figure 5).

Effect of column temperature on PL-SAX separations

Just as Bio SAX columns are primarily used to determine the purity of oligonucleotides, PL-SAX columns are best suited for purification purposes. The fractions that are obtained through ion exchange chromatography can be easily purified further by isopropanol precipitation and used for a variety of purposes. PL-SAX columns are available in preparative sizes or as bulk resins, making them ideal for fractionating large quantities of material.

The results in Figure 5 illustrate the chromatographic performance of the PL-SAX 4000A column at different temperatures. As with Bio SAX columns, higher temperatures result in longer retention times. Since oligonucleotides several thousand base pairs in size can be analyzed, these columns may also be fully used to isolate and purify large oligonucleotides such as mRNA.

Salt gradient optimization

Salt gradients may be optimized to enhance chromatographic resolution. Figure 6 shows separation of an Agilent ssDNA oligonucleotide standard on a Bio SAX NP5 column with different salt gradients. 19 nt and 39 nt short-mers were better resolved from 20 nt and 40 nt oligonucleotides when using a 0.25%/min salt gradient compared to 1%/min.

Effect of particle size on Bio SAX separations

Bio SAX columns are available in 1.7 µm, 3 µm, 5 µm, and 10 µm, of which 4.6 × 250 mm PEEK columns are available in NP5 and NP10. Figure 7 shows the results of a comparison test between Bio SAX PEEK NP5 (5 µm) and NP10 (10 µm).

Using NaCl Gradient 1 (Table 2), the 10 µm column was found to have higher separating power than the 5 µm column. Although this is a somewhat counterintuitive result, smaller particle sizes do not always result in better separations as flow rate, temperature, and gradient conditions that work well for larger particle sizes are not always optimal for smaller particle size columns.

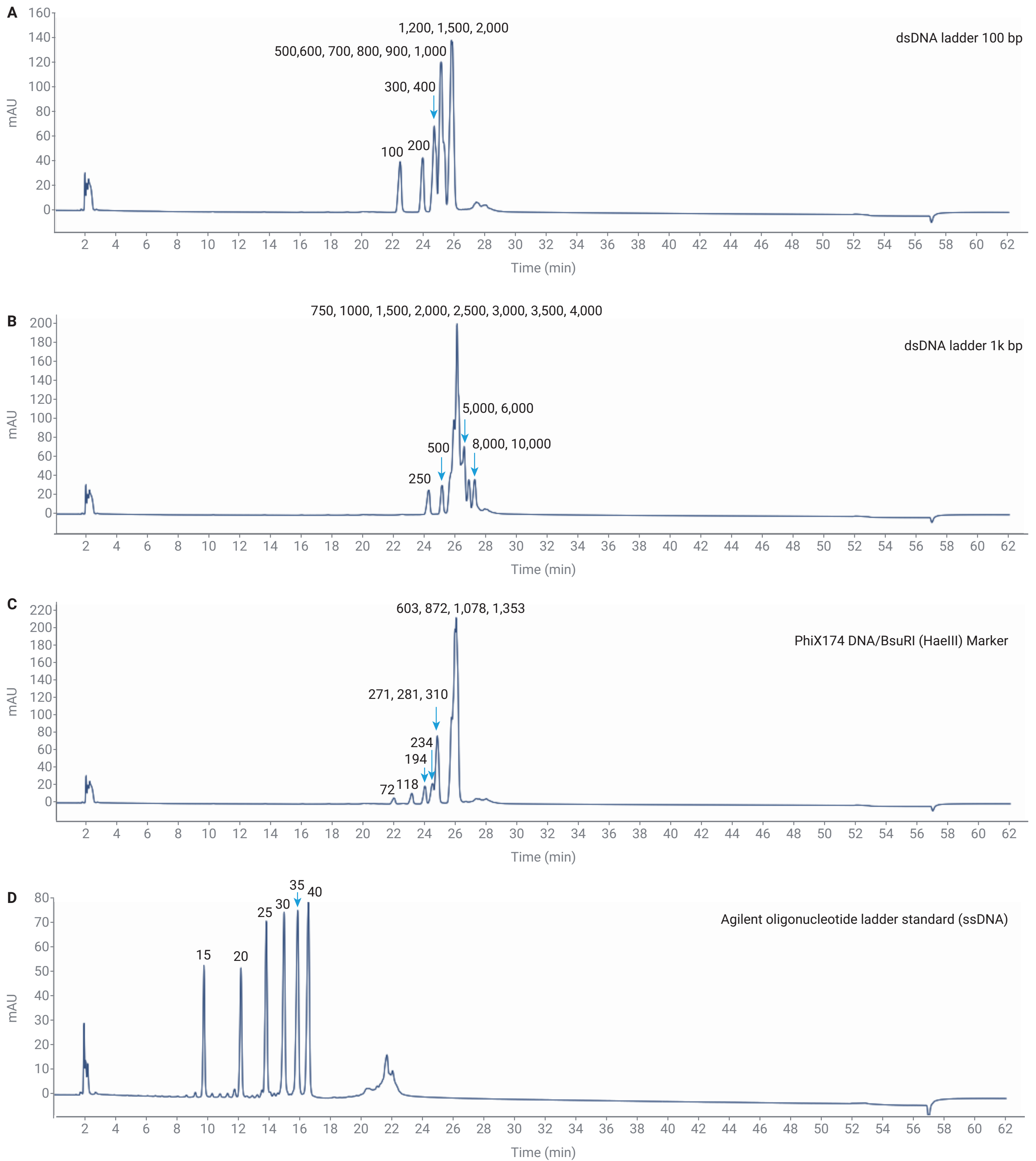

Figure 3. DNA standards separated on an Agilent Bio SAX NP5 PEEK column using a $NaClO_4$ gradient: (A) dsDNA ladder 100 bp, (B) dsDNA ladder 1k bp, (C) PhiX174 DNA/BsuRI (HaeIII) Marker, and (D) Agilent ssDNA oligonucleotide ladder standard.

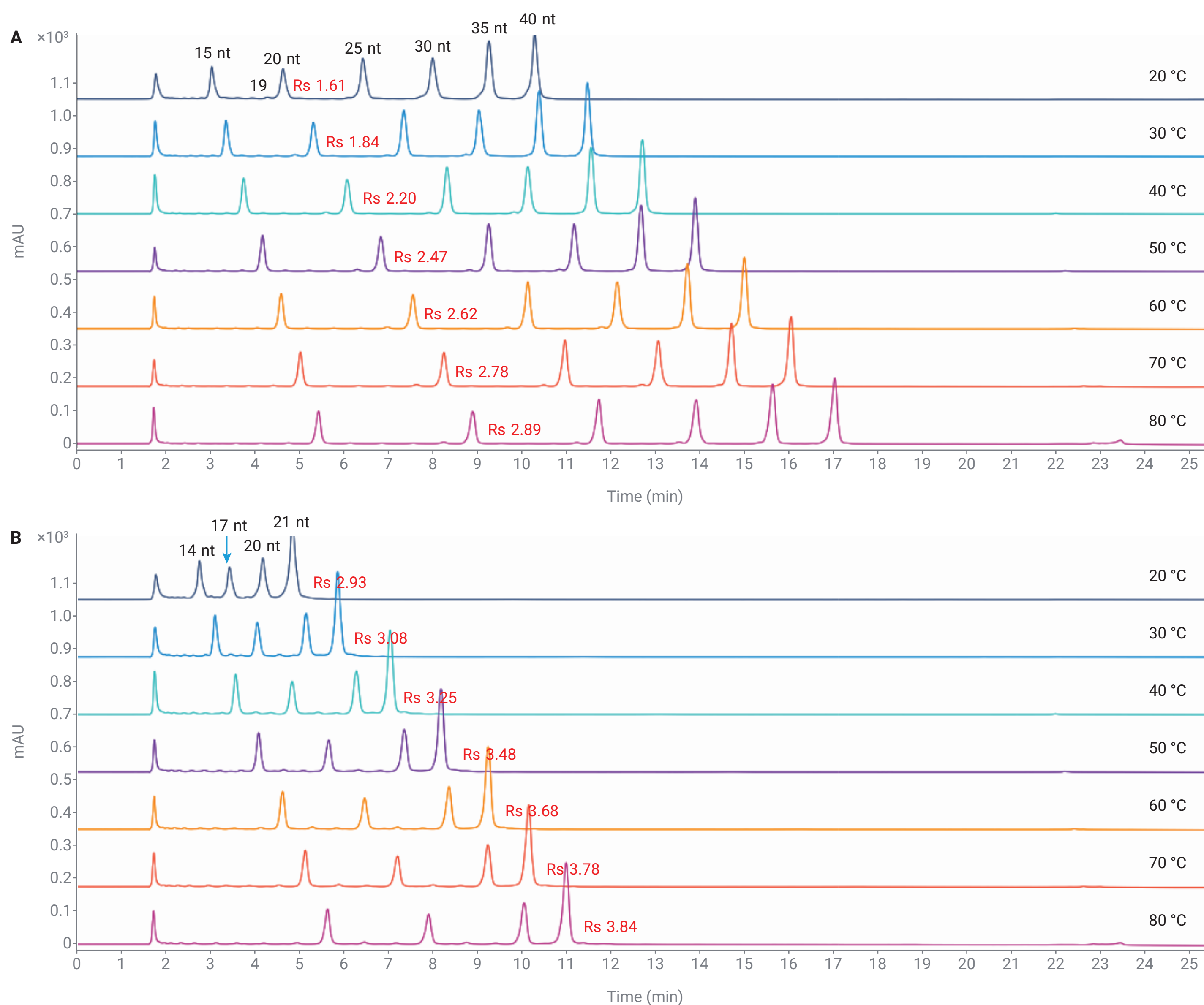

Figure 4. Separation of (A) Agilent ssDNA oligonucleotide standard, and (B) Agilent RNA resolution standard with increasing temperature.

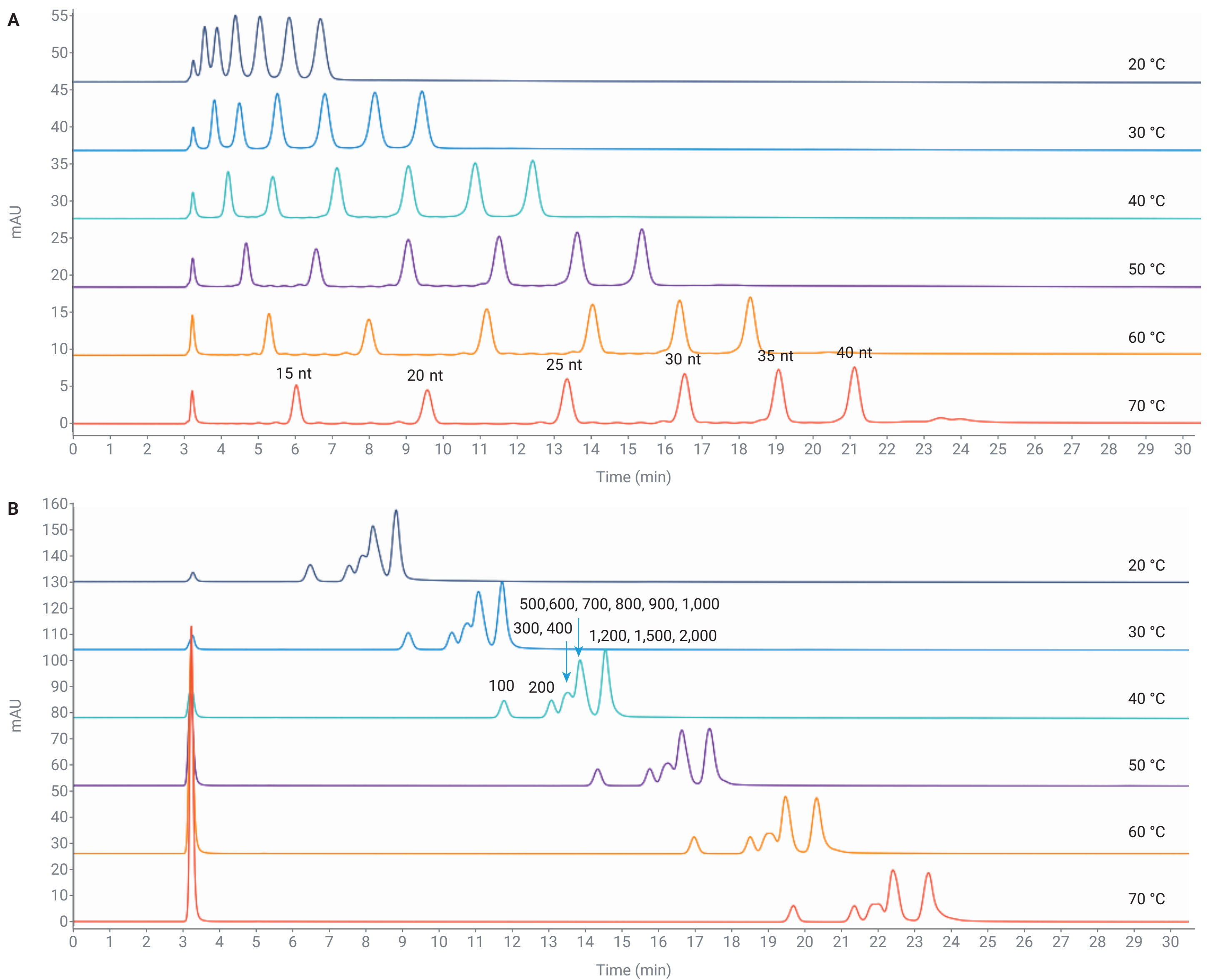

Figure 5A,B. Chromatogram of oligonucleotides separated on an Agilent PL-SAX 4000A column using a 20 mM Tris-HCl mobile phase and eluted with a salt gradient of 600 to 975 mM NaCl over 30 minutes. (A) Agilent oligonucleotide ladder standard (ssDNA), (B) dsDNA ladder 100 bp.

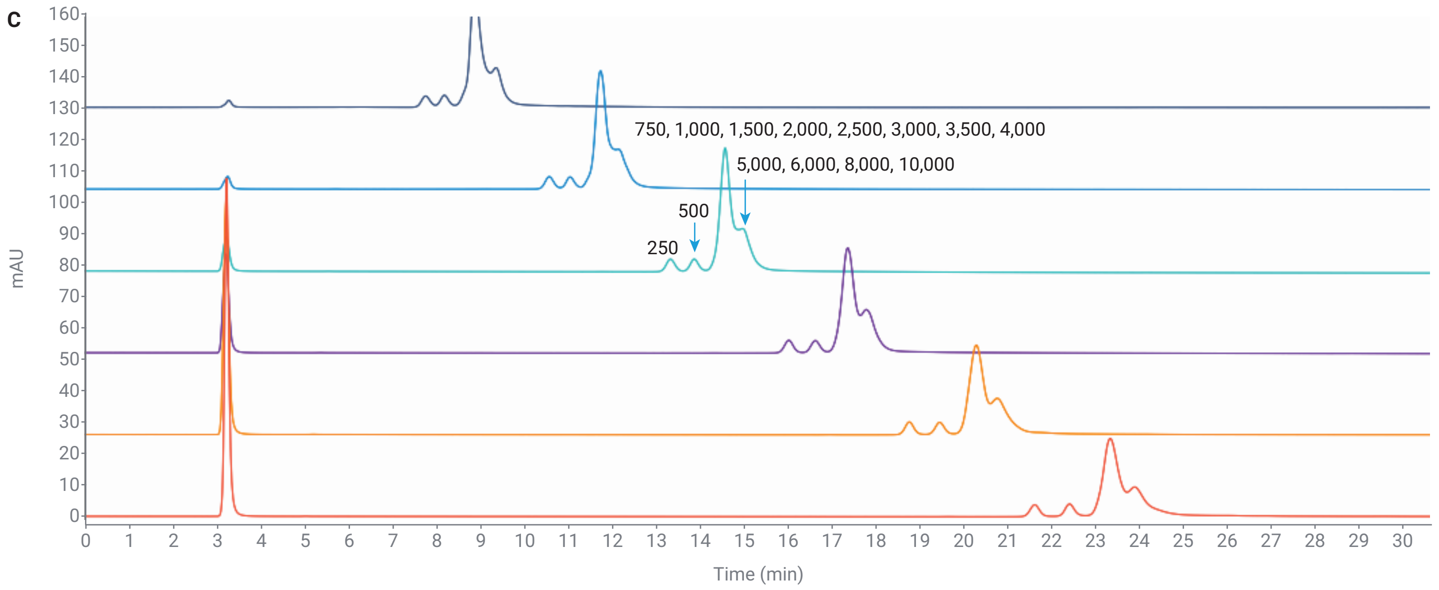

Figure 5C. Chromatogram of dsDNA ladder 1k bp oligonucleotides separated on an Agilent PL-SAX 4000A column using a 20 mM Tris-HCl mobile phase and eluted with a salt gradient of 600 to 975 mM NaCl over 30 minutes.

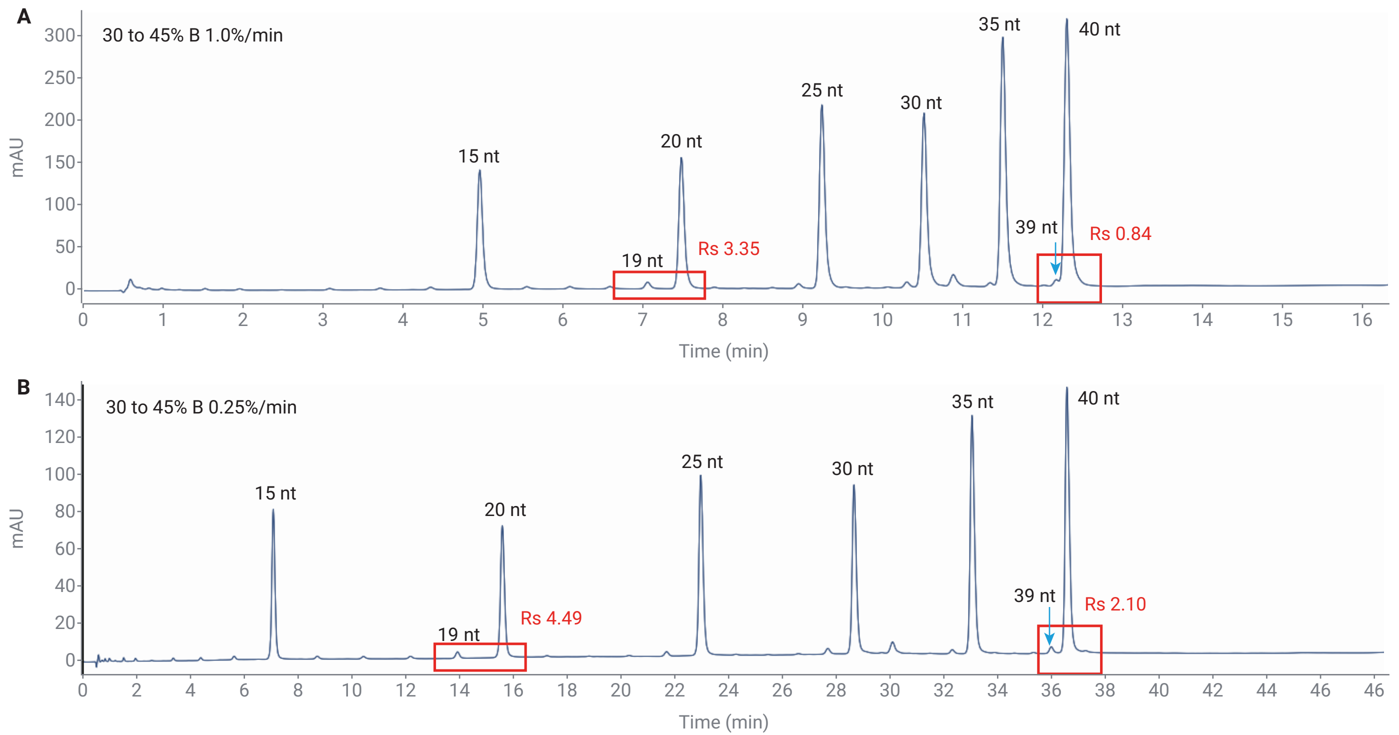

Figure 6. Optimization of salt gradient elution of Agilent ssDNA oligonucleotide standard.

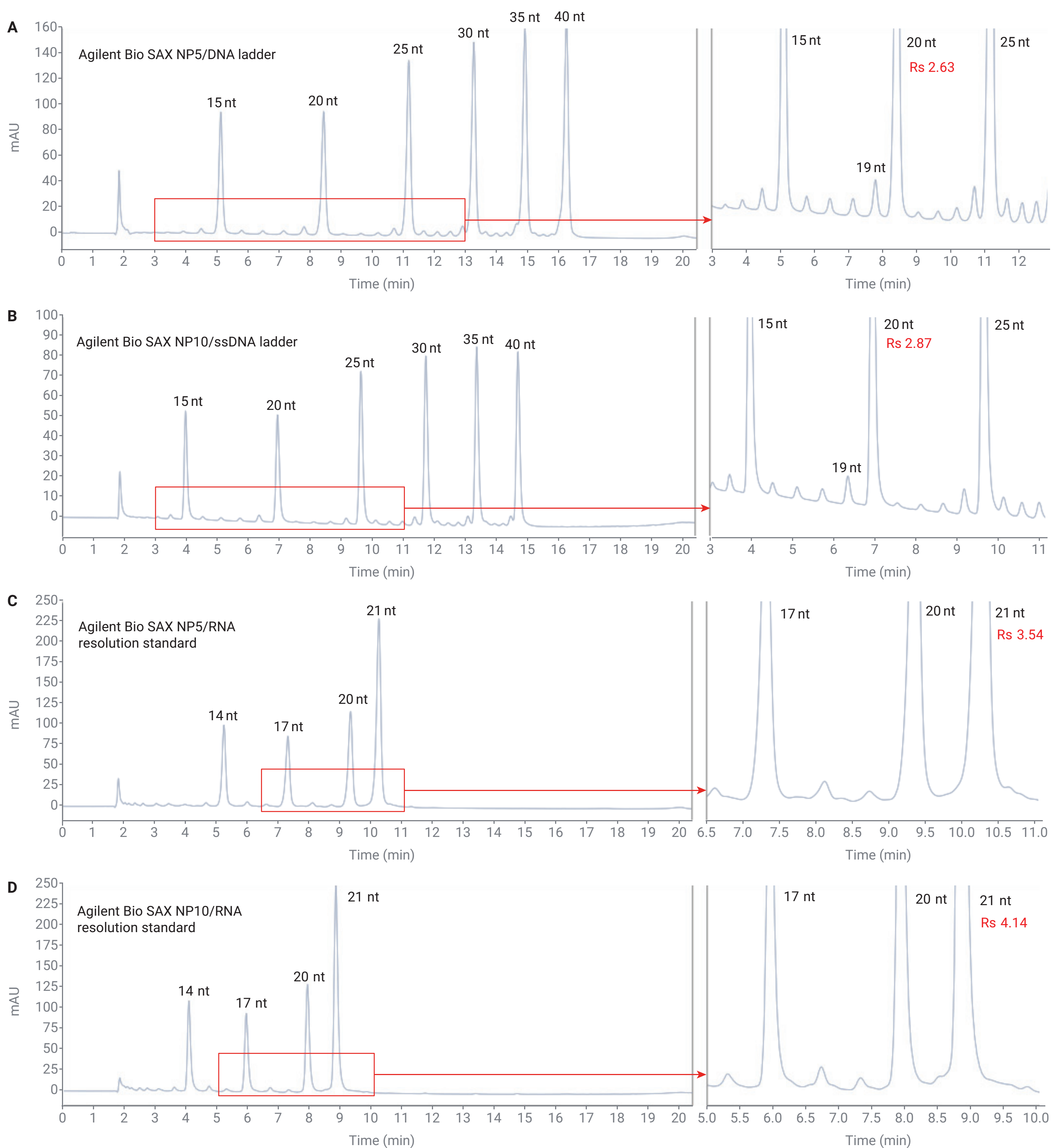

Figure 7. ssDNA and RNA resolution standards separated on Agilent Bio SAX NP5 and NP10 columns.

Conclusion

This experiment has demonstrated that the Agilent 1290 Infinity II Bio UHPLC is able to perform high-resolution ion exchange chromatographic separations of DNA and RNA oligonucleotides over a wide range of sizes without suffering the effects of corrosion or oxidative damage from eluting salts. Agilent Bio SAX columns enabled analysis of up to 10,000 bp dsDNA ladders, while maintaining single-nucleotide resolution for 15 to 40 nt ssDNA ladders. Bio SAX columns also effectively separated RNA ladders of 14 to 21 nt. Highly porous PL-SAX columns have higher loading capacities than nonporous Bio SAX columns but displayed a lower resolution that would nevertheless be adequate for purification purposes.

This application note demonstrates the versatility of ion exchange chromatography for oligonucleotide analysis and purification processes and should provide a good starting point for further method optimization.

References

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