Application Note

Biopharmaceuticals

Oligonucleotide Analysis with Ion-Pair Reversed-Phase Chromatography and Agilent 1260 Infinity II Prime LC

Authors

Ryu, Chae-Young
Korea Agilent Technologies Co., Ltd.

Brian Liau
Agilent Technologies, Inc.

Abstract

Ion-pair reversed-phase chromatography is the most common type of separation used for HPLC analysis of oligonucleotides. The chromatographic resolution can depend on the size and characteristics of the sample, as well as on the chromatographic conditions, choice of column, and mobile phase composition.

This application note demonstrates the use of Agilent AdvanceBio oligonucleotide columns 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 (for example, siRNA or mRNA) or as vital inputs to bioprocesses (such as plasmid DNA).

Depending on the application, oligonucleotides may vary widely in size: siRNA (20 to 24 nt, nucleotides), 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. The type of separation must be chosen carefully to enable reliable detection of impurities, such as prematurely terminated short-mers or host-cell DNA.

Retention times of oligonucleotides will depend on factors such as whether it is DNA or RNA, the presence of secondary structures, and the relative abundance of each oligonucleotide. In general, DNA has a longer retention time than RNA, water solubility of nucleotides is in the order C < G < A < T, and single-stranded oligonucleotides are more soluble than double-stranded ones.

Oligonucleotides are highly hydrophilic molecules that will require ion-pairing agents for analysis with reverse-phase (IPRP) chromatography. IPRP analysis of oligonucleotides typically use positively charged mobile phase additives containing tri- or tetraalkylammonium salts, such as triethylammonium acetate (TEAA), tetrabutylammonium acetate (TBAA), dibutylammonium acetate (DBAA), hexylamamine acetate (HAA), and triethylammonium bicarbonate (TEAB). For LC/MS applications, the volatile weak acid hexafluoroisopropanol is often used as a counterion because it greatly increases chromatographic resolution and ionization efficiency. In general, chromatographic retention time is positively correlated with the chain length of the ion-pairing agent.

Experiment

Reagents

Alkylamine used in the experiment was purchased from Sigma-Aldrich, acetic acid was purchased from JT Baker, acetonitrile was purchased from B&J.

Standard

The following standards were used:

- Oligonucleotide ladder standard: Agilent ssDNA(dT 15, 20, 25, 30, 35, 40 nt) (part number 5190-9029)
- Oligonucleotide resolution standard: Agilent ssRNA (part number 5190-9028)

Mobile phase composition

Ion-pairing agents were mixed with acetic acid in the quantities shown in Table 1. Acetic acid was used to pH-correct each solution to pH 7. Ion-pairing mixtures were prepared in either water or organic solvent to yield mobile phases A and B.

Table 1. Ion-pairing reverse chromatography mobile phase compositions.

Kind	Composition (/L)
100 mM TEAA, pH 7	Acetic acid 6 g, triethylamine 10.12 g
100 mM DBAA, pH 7	Acetic acid 6 g, dibutylamine 12.92 g
100 mM HAA, pH 7	Acetic acid 6 g, hexylamine 10.12 g

Analytical instruments and conditions

Typical analytical conditions used in ion-pairing reverse chromatography are shown in Table 2. Deviations from these conditions are indicated where appropriate in each section of the text.

Table 2. IPRP chromatography conditions.

Parameters	Value
Instrument	1260 Infinity II Prime LC
Column	Agilent AdvanceBio oligonucleotide, 2.1 × 150 mm, 2.7 µm (p/n 653750-702)
Flow Rate	0.6 mL/min
Column Temperature	60 °C
Injection Volume	5 µL
Sampler Temperature	4 °C
Detector	UV 260 nm (G7117B DAD HS, flow cell 60 mm)
Mobile	A) 100 mM Alkylamine acetate, pH 7 B) 100 mM Alkylamine acetate, pH 7 in acetonitrile
Gradient	**Time (min) %A %B** 0 90 10 1 90 10 20 85.5 14.5 20.1 10 90 25 10 90 25.1 90 10 30 90 10

Software

Agilent OpenLab CDS 2.6

Abbreviations

- **HPLC:** High performance liquid chromatography
- **TEAA:** Triethylamine acetate
- **DBAA:** Dibutylamine acetate
- **HAA:** Hexylamine acetate
- **RNA:** Ribonucleic acid
- **DNA:** Deoxyribonucleic acid
- **NMP:** Nucleoside monophosphate
- **dNMP:** Deoxynucleoside monophosphate
- **Rs:** Resolution
- **nt:** Nucleotides (unit)
- **bp:** Base pair

Results and discussion

Comparison of ion pairing reagents

Single-stranded oligonucleotide standards (ssDNA oligo-dT and ssRNA) were analyzed using 100 mM TEAA, DBAA, and HAA mobile phases on the AdvanceBio oligonucleotide column (2.1 x 150 mm, 2.7 µm).

With the ssDNA oligo-dT ladder, although the main 15 to 40 nt peaks were clearly separated using TEAA (Figure 1A), short-mer contaminants were not well resolved. However, DBAA and HAA were able to clearly resolve short-mers such as the 16 to 19 nt peaks. HAA showed a superior resolution of 3.9 between the 19 and 20 nt peaks, compared to a resolution of 3.2 when using DBAA (Figures 1B and 1C).

The ssRNA resolution standard showed that ssRNA retained poorly using TEAA (Figure 2A), but resolved well using DBAA and HAA. As before, chromatographic resolution was superior with HAA than with DBAA. The resolution of 20 and 21 nt peaks was 2.3 when using DBAA and 2.8 when using HAA (Figures 2B and 2C).

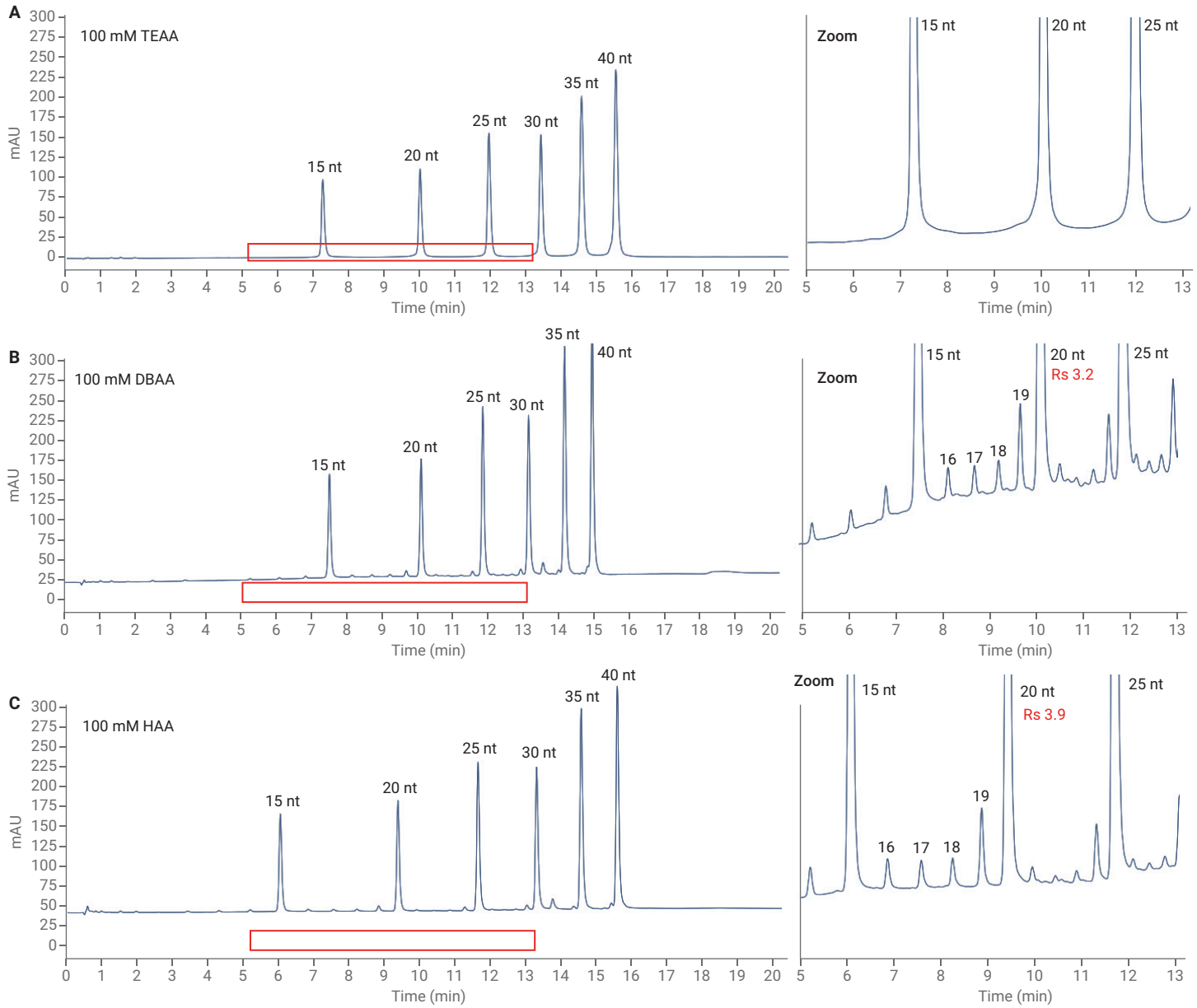


Figure 1. Resolution of ssDNA oligo-dT ladder using 100 mM TEAA, DBAA, or HAA. (A) 100 mM TEAA, 10 to 14.5 %B in 19 min, (B) 100 mM DBAA, 26 to 46 %B in 19 min, (C) 100 mM HAA, 30 to 45 %B in 19 min.

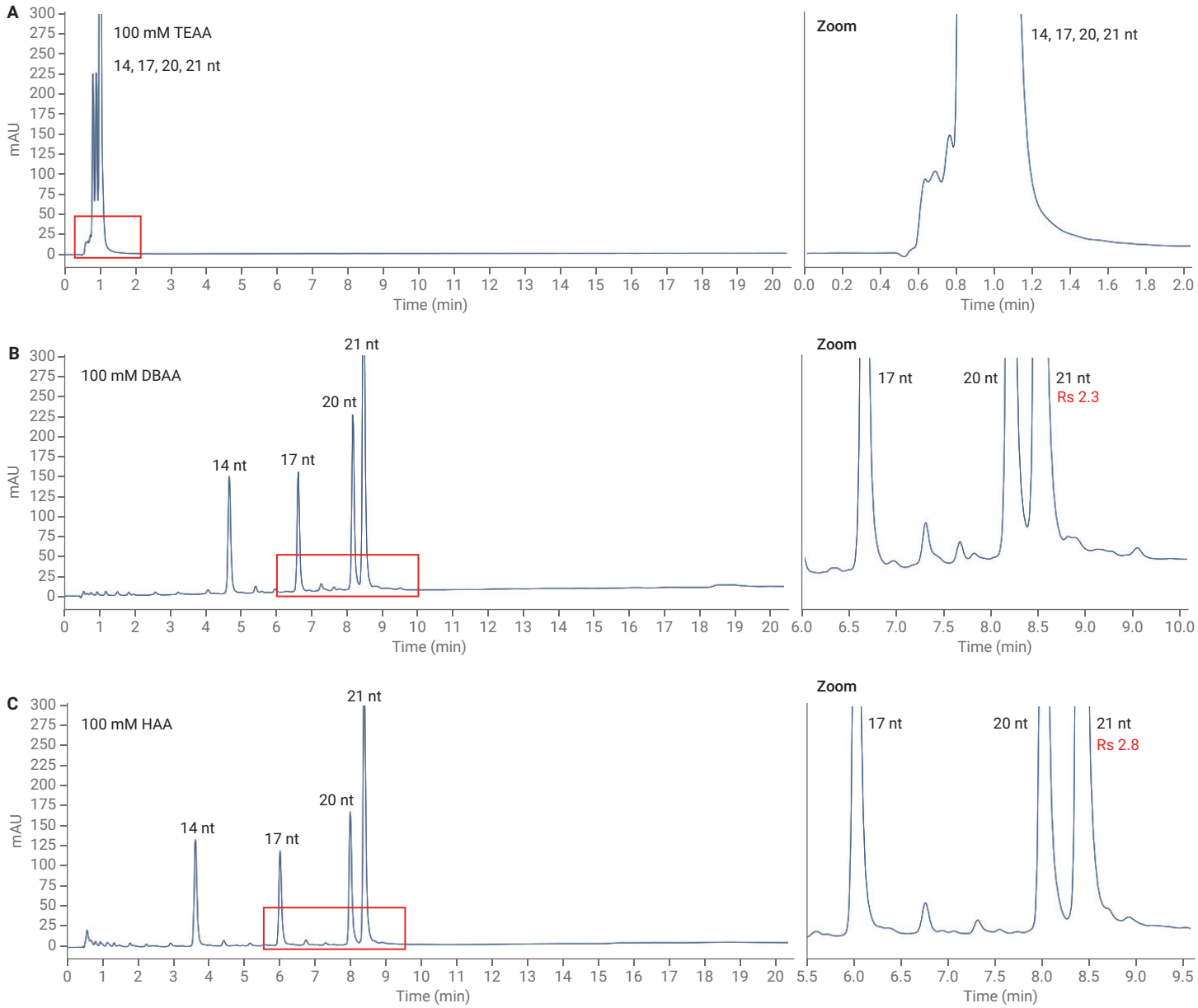


Figure 2. Resolution of ssRNA resolution standard using 100 mM TEAA, DBAA, or HAA. (A) 100 mM TEAA, 10 to 14.5 %B in 19 min, (B) 100 mM DBAA, 26 to 46 %B in 19 min, (C) 100 mM HAA, 30 to 45 %B in 19 min.

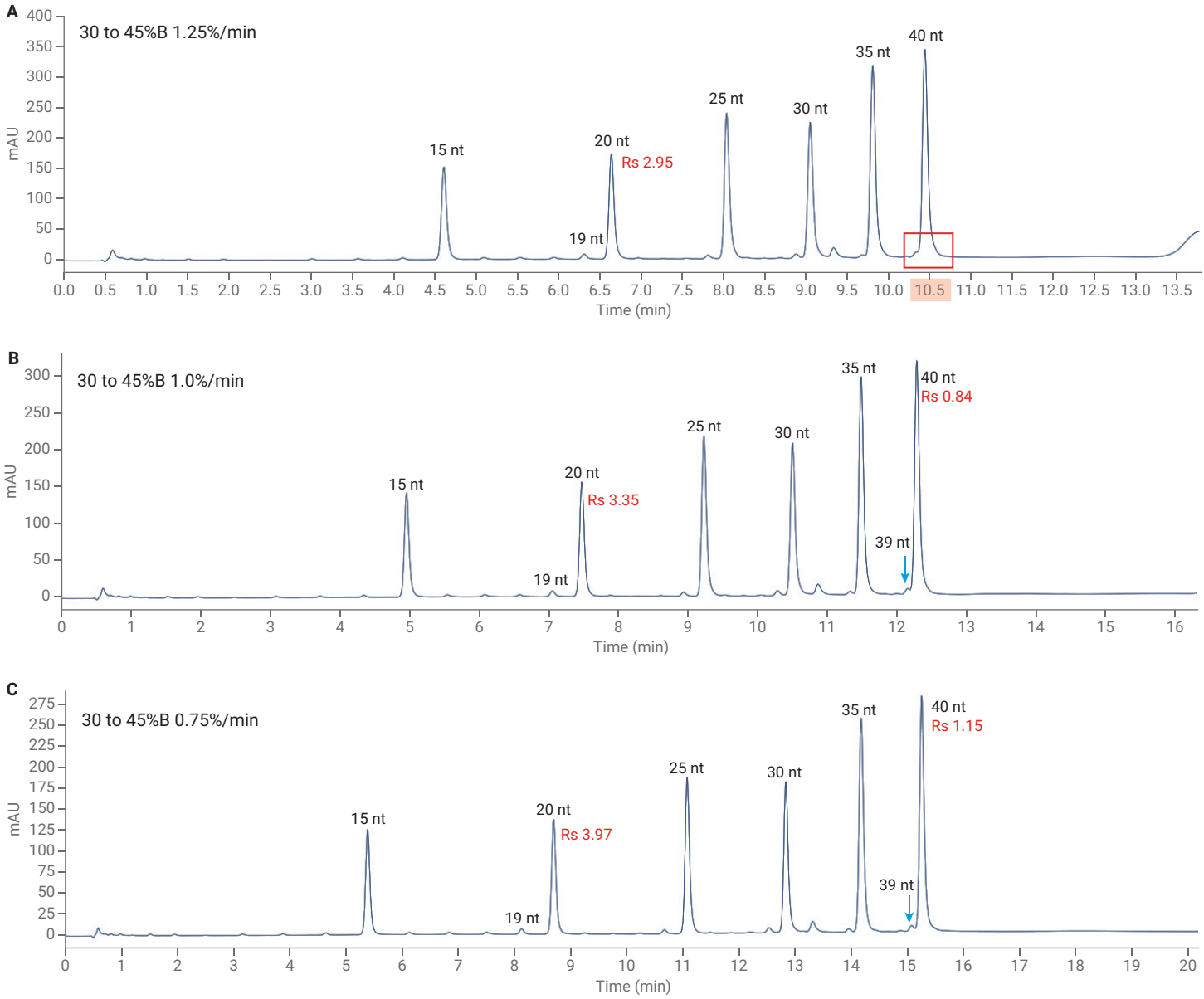
Optimization of gradient elution

The ssDNA oligo-dT and ssRNA resolution standards were analyzed on the AdvanceBio oligonucleotide column using HAA ion-pairing agents with different elution gradients. Changes in mobile phase B were varied between 0.25 and 1.25 %/min.

Considering the 40 nt ssDNA peak and its n-1 short-mer, we can see that chromatographic separation improved as the elution gradient was slowed from 1.25 to 0.25 %/min. Specifically, two peaks were not resolved at 1.25 %/min, but were separated with a resolution of 2.1 at 0.25 %/min.

It was found that improvements in peak resolution far outweighed increases in peak width due to longer elution times, but there was a decrease in peak height to about 40% of the value seen under 0.25 %/min conditions, compared to 1.25 %/min. The elution gradient can therefore be optimized to fit sensitivity, analysis time, and peak resolution requirements.

ssRNA resolution standards showed a similar pattern of increasing resolution (1.97 to 4.26) with shallower elution gradients, as shown in Figure 4.



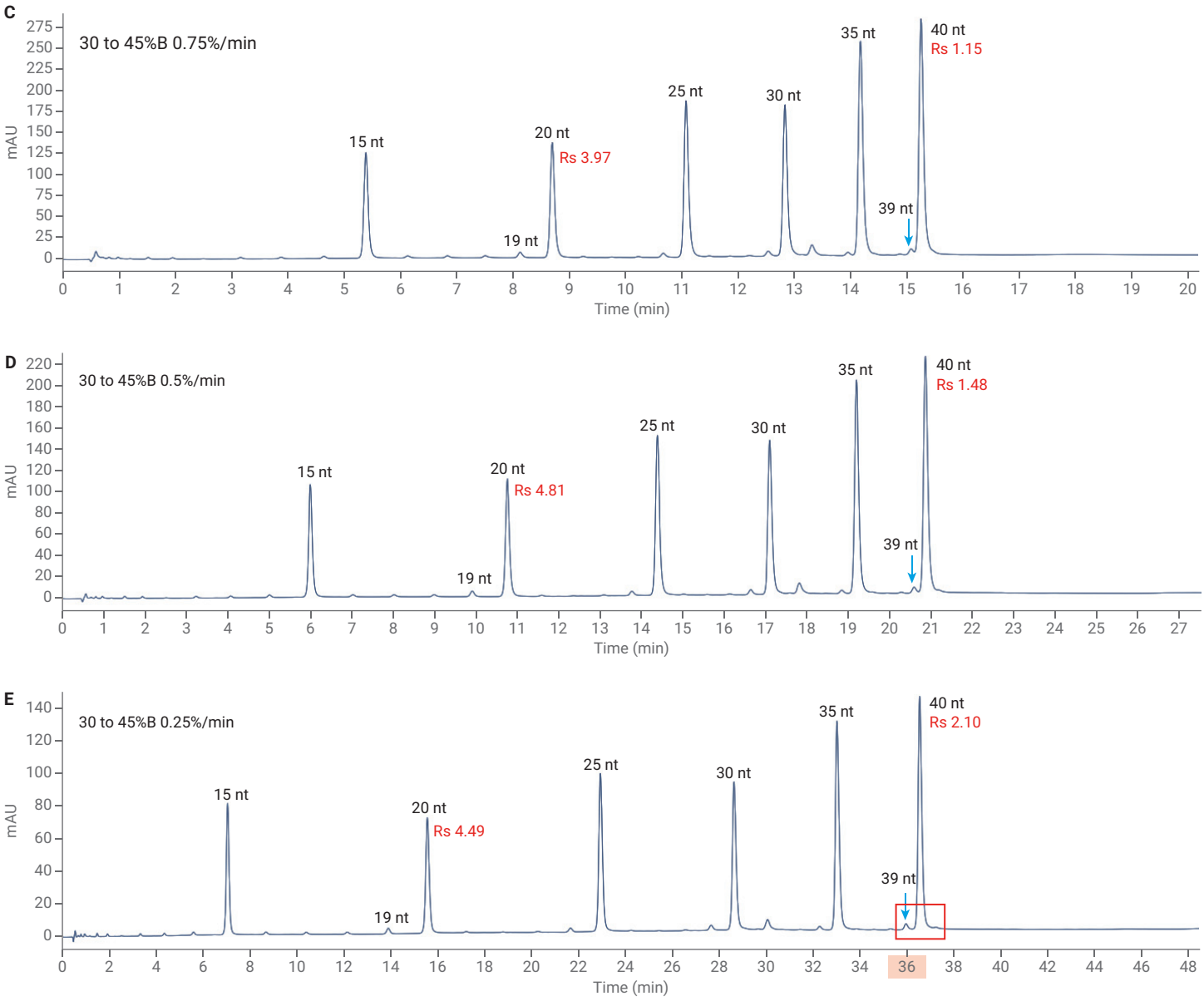
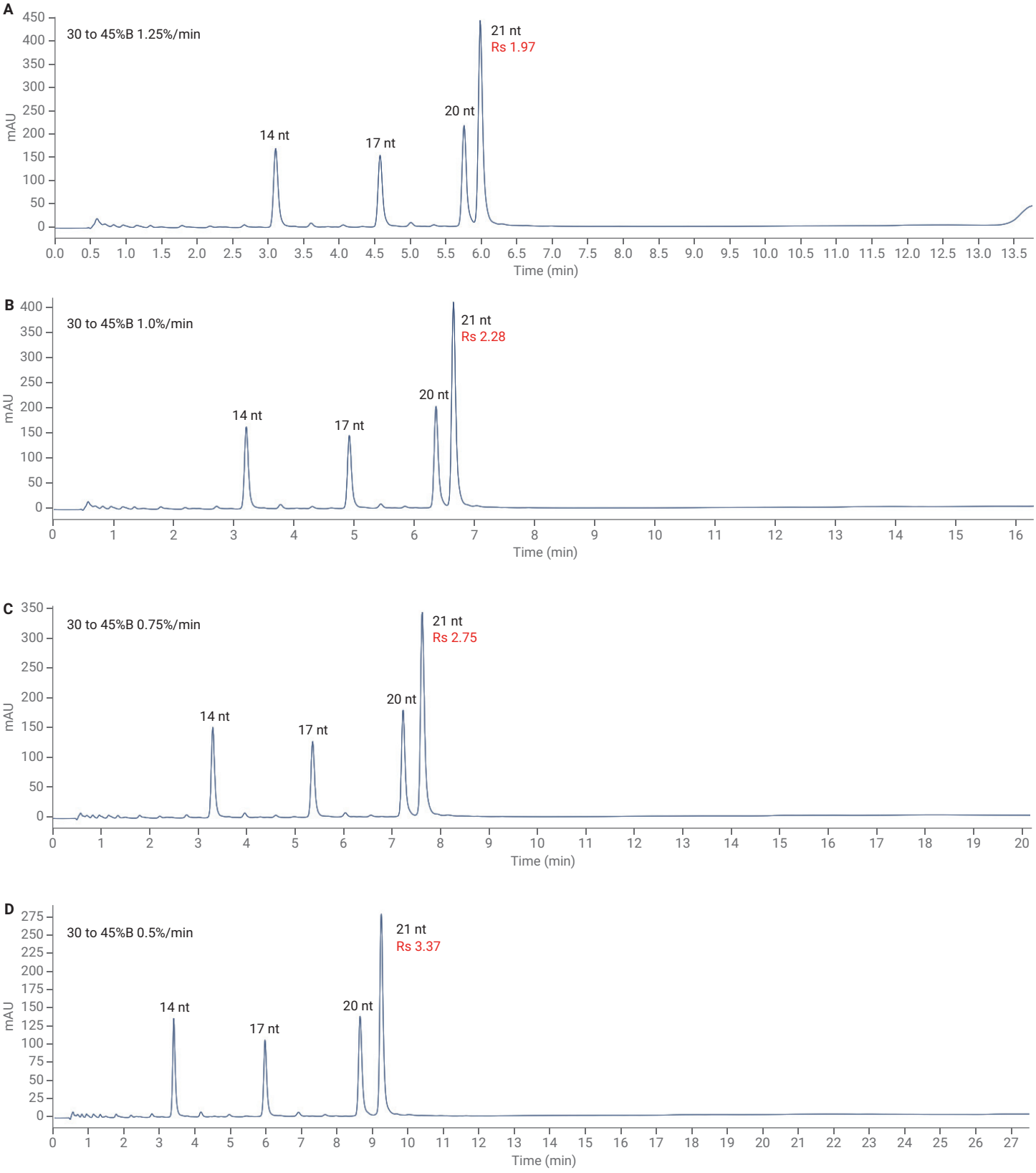


Figure 3. Optimization of elution gradient used to separate ssDNA oligo-dT standard. (A) 30 to 45 %B, 1.25%/min, (B) 30 to 45 %B, 1.0%/min, (C) 30 to 45 %B, 0.75%/min, (D) 30 to 45 %B, 0.5%/min, (E) 30 to 45 %B, 0.25%/min.


A
30 to 45%B 1.25%/min
21 nt
Rs 1.97
20 nt
14 nt
17 nt
mAU
Time (min)
B
30 to 45%B 1.0%/min
21 nt
Rs 2.28
20 nt
14 nt
17 nt
mAU
Time (min)
C
30 to 45%B 0.75%/min
21 nt
Rs 2.75
20 nt
14 nt
17 nt
mAU
Time (min)
D
30 to 45%B 0.5%/min
21 nt
Rs 3.37
20 nt
14 nt
17 nt
mAU
Time (min)

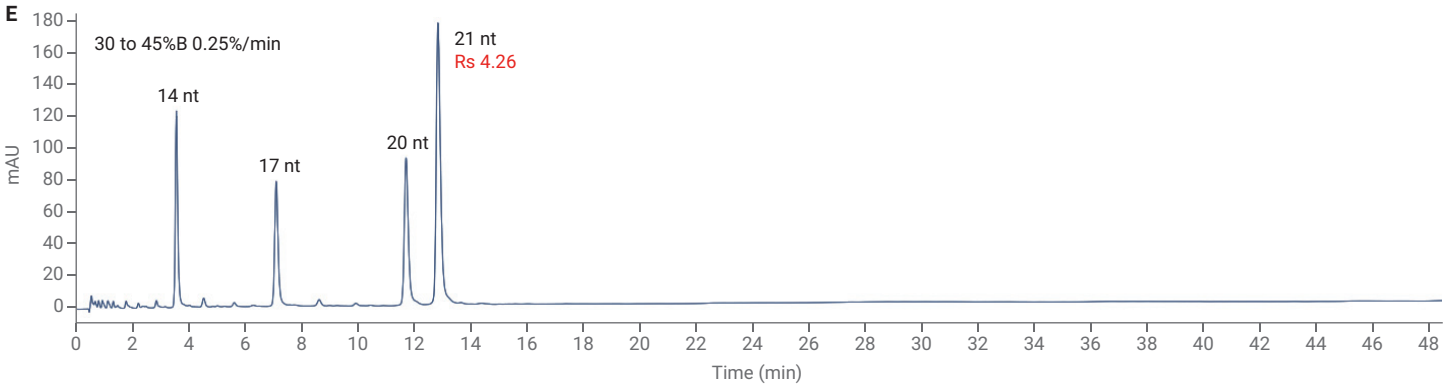


Figure 4. Optimization of elution gradient used to separate ssRNA resolution standard. (A) 30 to 45 %B, 1.25%/min, (B) 30 to 45 %B, 1.0%/min, (C) 30 to 45 %B, 0.75%/min, (D) 30 to 45 %B, 0.5%/min, (E) 30 to 45 %B, 0.25%/min.

Optimization of column temperature

The denaturation temperature of ssDNA and ssRNA depends largely on their GC percentages. Because column temperature can affect the extent of secondary structure in an oligonucleotide sample, the oven temperature should be set differently for the purpose of analysis. In some cases, reversed-phase columns may be unstable at alkaline pH under high temperatures, which must be considered during method development.

The data below demonstrate how chromatographic resolution changes for ssDNA oligo-dT and ssRNA standards with increasing column temperature when using an 100 mM HAA ion-pairing agent. Interestingly, ssRNA retention time decreases without appreciable improvement in chromatographic resolution with increasing temperature. On the other hand, ssDNA retention time is maximal at 40 °C, and separation improves with increasing temperature. This could be because deoxyribonucleotides are more nonpolar than ribonucleotides, hence they are more strongly affected by mass transfer limitations. Therefore, ssDNA oligonucleotides show a stronger temperature-dependent increase in separation compared to ssRNA.

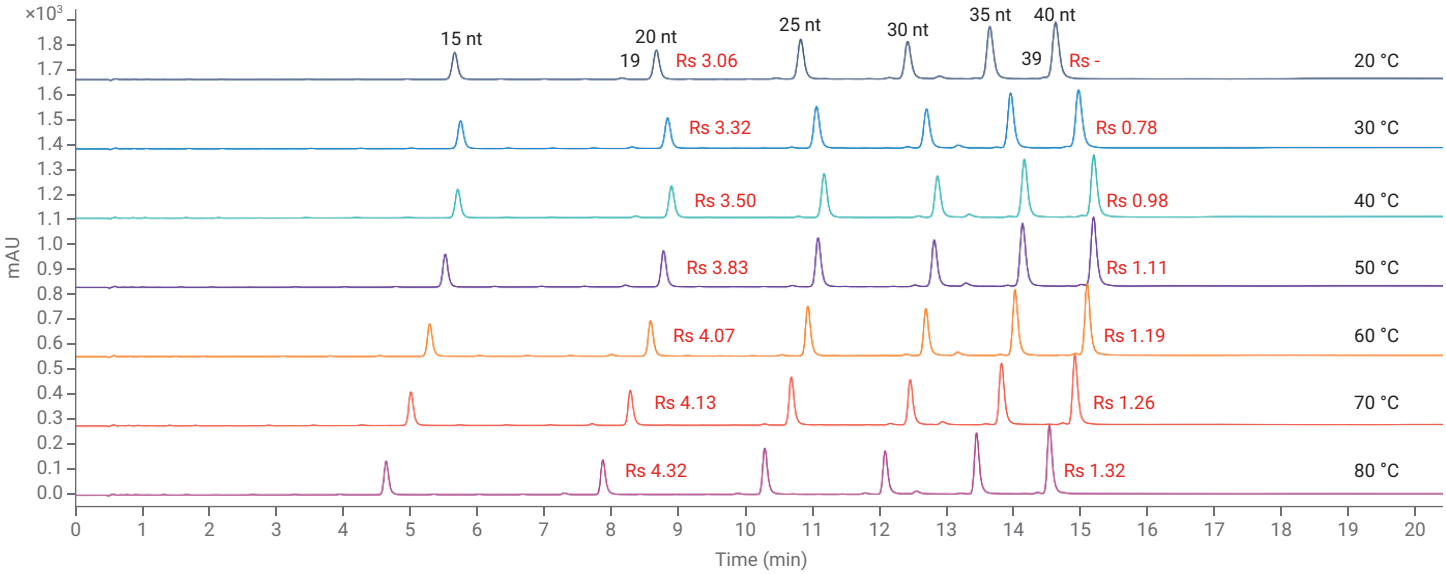


Figure 5. Oligonucleotides column temperature-specific DNA ladder overlaid chromatogram.

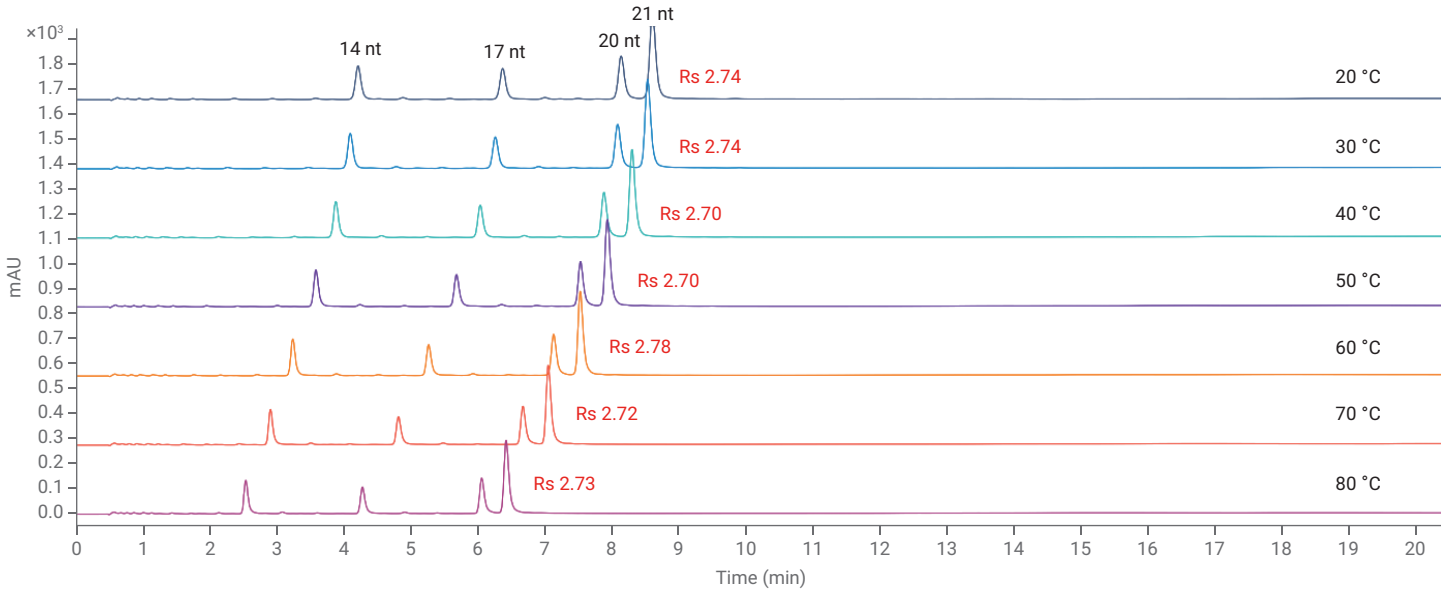


Figure 6. Oligonucleotides column temperature-specific RNA resolution standard overlaid chromatogram.

As shown in Table 3, ssRNA demonstrates a much larger decrease in retention time with increasing temperature compared to ssDNA, even when considering molecules of equal length. This can potentially be used to analyze RNA-DNA duplexes efficiently.

Table 3. Oligonucleotides column temperature-specific DNA 20 nt and RNA 20 nt retention time comparison.

Temperature (°C)	DNA 20 nt Retention Time (min)	RNA 20 nt Retention Time (min)	Difference (min)
20	8.660	8.133	0.527
30	8.827	8.081	0.746
40	8.884	7.877	1.007
50	8.767	7.527	1.240
60	8.574	7.125	1.449
70	8.273	6.668	1.605
80	7.864	6.053	1.811

Conclusion

Using the Agilent 1260 Infinity II Prime LC, we showed how differences in ion-pairing agents and chromatography conditions could influence the separation of ssDNA and ssRNA standards, ranging from 14 to 40 nt. In general, the use of ion-pairing agents with longer chain lengths and slower elution gradients led to superior separations. However, ssDNA and ssRNA behaved differently in response to increasing column temperature. While ssDNA resolution was improved, ssRNA resolution was unchanged. Additionally, ssRNA retention time showed much larger decreases with increasing temperature than ssDNA.

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