# Separation of Mixed-Base Oligonucleotides Using a High-Resolution, Reversed-Phase Chromatography Column

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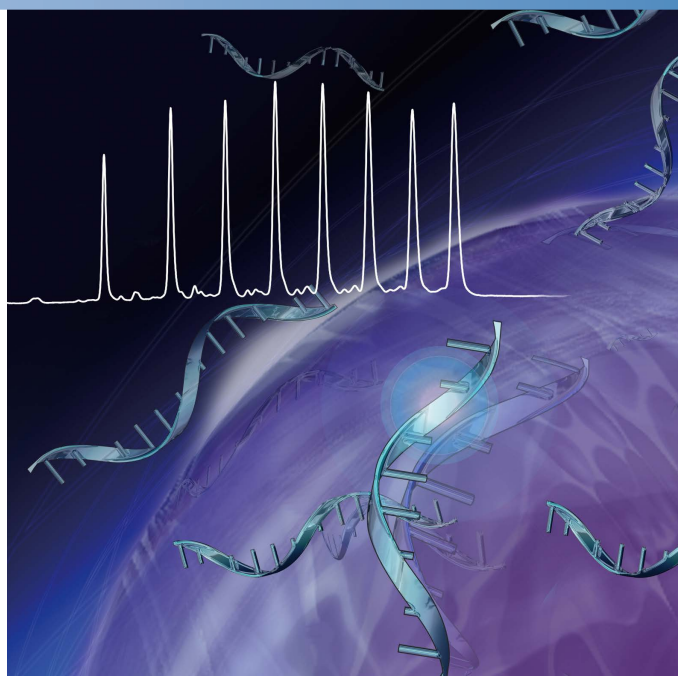
Application Note 21476

**Key Words**
Ion-pair reversed-phase chromatography, DNA, oligonucleotides, DNAPac RP, HPLC

## Goal

To demonstrate the impact of different chromatography conditions to achieve fast and high-resolution separation of mixed-base oligonucleotides (ONs) using a porous reversed-phase chromatography column. Mixed-base ONs were separated using different flow rates, gradient curves, temperatures, and ion-pair reagents.



## Introduction

Synthetic ONs are used extensively in laboratories as primers for polymerase chain reactions (PCR) and DNA sequencing, probes to visualize a specific DNA or RNA, tools to study gene function, and biopharmaceutical drugs for treating various diseases.[1-4] ONs are most often synthesized using solid-phase chemistry, which consists of many sequential coupling reactions. Although the yield of each reaction is high, accumulation of minor reaction failures results in truncated ONs in addition to the target ON. These truncated ONs and other impurities (e.g., deprotection failures) must be removed for most molecular biology applications and to ensure efficacy and safety of therapeutic agents. Analyses of synthetic ONs are most commonly performed using ion-pair reversed-phase chromatography (IP-RP).[5-7] IP-RP utilizes the ionic interaction between the analyte and the ion-pair reagent, which also interacts with the stationary phase via its hydrophobic moiety. IP-RP provides high-resolution separation of failure sequences and can be directly coupled to mass spectrometry for identification of target ON and impurities.

Here we describe the use of the Thermo Scientific™ DNAPac™ RP column for the separation of mixed-base ONs. The DNAPac RP column is a wide-pore, polymer-based reversed-phase column, well suited for the separation of a wide range of ON lengths. The polymeric nature of the DNAPac RP column allows the use of high pH and high temperature conditions, which afford alternative selectivities and control of specific ON resolution. In this study, we examine the influence of flow rate, gradient curve, and temperature and compare two ion-pair reagents on retention and resolution of ONs using the DNAPac RP column.

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## Experimental

### Consumables

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Acetonitrile (Fisher Scientific™ P/N A955-4)
- Triethylammonium acetate (TEAA) 2.0 M (Applied Biosystems™ P/N 400613)
- Hexylamine (HA) (Alfa Aesar™ P/N A15663)
- Acetic acid (Fisher Scientific P/N A507-P500)

### Oligonucleotides

8-combo DNA

- 12mer: $(GACT)_3$
- 16mer: $(GACT)_4$
- 20mer: $(GACT)_5$
- 24mer: $(GACT)_6$
- 28mer: $(GACT)_7$
- 32mer: $(GACT)_8$
- 36mer: $(GACT)_9$
- 40mer: $(GACT)_{10}$

ONs were purchased from Integrated DNA Technologies, Inc.

### Sample Handling Equipment

Vial and closures: Polypropylene, 0.3 mL vials (P/N 055428)

### Sample Preparation

Stock solutions were prepared by dissolving the ONs to 200 µM with deionized water (DI). Equivalent amounts of each ON were mixed to prepare 25 µM for each ON. The sample was further diluted five-fold with either DI water or mobile phase A to make 5 µM.

| Separation Conditions | |
|---|---|
| Instrumentation | Thermo Scientific™ Vanquish™ Flex UHPLC system consisting of: |
| | System Base (P/N VF-S01-A) |
| | Quaternary Pump Flex (P/N VF-P20-A) |
| | Split Sampler FT (P/N VF-A10-A) |
| | Column Compartment H (P/N VH-C10-A) |
| | Active Pre-heater (P/N 6732.0110) |
| | Diode Array Detector HL (P/N VH-D10-A) |
| | Thermo Scientific™ LightPipe™ Flow Cell, Standard, 10 mm (P/N 6083.0100) |
| Column(s) | DNAPac RP, 2.1 × 50 mm (P/N 088924) |
| Mobile Phases | |
| Set I<br>Mobile Phase A<br>Mobile Phase B | <br>0.1 M TEAA in water, pH 7.0<br>0.1 M TEAA in water / acetonitrile (75:25 v/v) |
| Set II<br>Mobile Phase A<br>Mobile Phase B | <br>0.1 M HAA in water, pH 7.4<br>0.1 M HAA in water / acetonitrile (50:50 v/v) |
| Gradient | As specified in Figures. |
| Flow Rate | As specified in Figures. |
| Column Temp. | As specified in Figures. |
| UV Detector Wavelength | 260 nm |
| Resolution | $R = 1.18 \times \frac{t_{Ref\,Peak\text{-}t_R}}{W_{50\%RefPeak}+W_{50\%R}}$<br>$t_R$ = *retention time of the current peak*<br>$t_{RefPeak}$ = *retention time of the reference peak for the resolution (the peak after the current peak)*<br>$W_{50\%,R}, W_{50\%,RefPeak}$ = *Widths of the two peaks at 50% of the peak height* |

### Software

Thermo Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System

## Results and Discussion

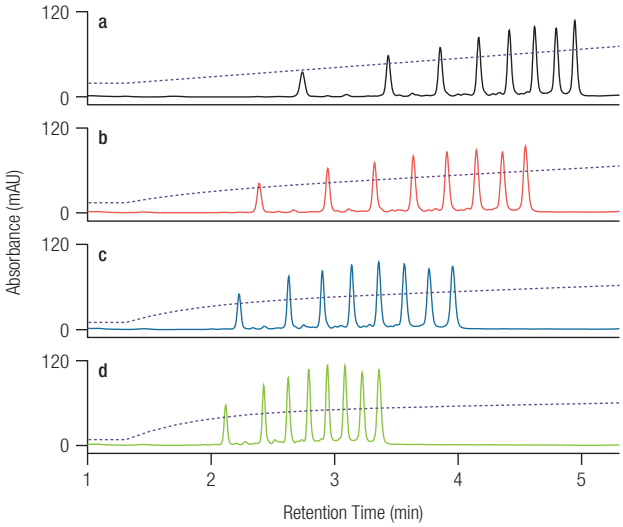
A mixture of eight mixed-base DNA ONs from 12mer to 40mer that differ in the number of GACT units (3–10) was used to study the impact of different chromatographic conditions on mixed-base ONs.

### General Considerations

At pH values between 6 and 8, standard oligonucleotides have one negative charge for each phosphodiester bond. Therefore, a terminally non-phosphorylated ON will harbor a charge equal to one less than the number of bases. The amine on the ion-pair reagent interacts with the negatively charged phosphodiester bond "coating" the ONs with a hydrophobic layer. This enhances the hydrophobic interaction with the stationary phase. Since the number of phosphodiesters, and thus the number of IP reagent molecule interactions, is proportional to the length of the ON, longer ONs elute later at neutral pH values. As the ONs get longer, the percent charge difference between ONs decreases, so resolution between ONs decreases with ON length.

### Curved Gradients

Since early-eluting ONs are typically well resolved and longer (later-eluting) ONs less well resolved, non-linear gradients can help improve resolutions of the longer ONs, provided early eluting ONs are sufficiently separated. The influence of different gradient types on resolution is shown in Figures 1 and 2. Curved gradients asymptotically reduce the slope of the gradient over time. Figure 1 (a, b, c and d) shows the chromatography of eight ONs using gradient curves with increasing initial gradient rates. The gradient curve number is inversely proportional to initial gradient slope and proportional to the ending gradient slope (Figure 2). In addition to increasing the separation between the longer ONs, gradient curves below 5 can be used to reduce the analysis time. Using the same gradient program, gradient curve 2 provides adequate separations in 3.5 minutes, instead of 5 minutes (30% faster, Figure 1d).




| | |
|---|---|
| Column: | **DNAPac RP**, 4 µm |
| Format: | 2.1 × 50 mm |
| Mobile Phase A: | 0.1 M TEAA, pH 7.0 |
| Mobile Phase B: | 0.1 M TEAA in water/acetonitrile (25:75 v/v) |
| Gradient: | Time (min) %A %B |
| | -3.0 85 15 |
| | 0.0 85 15 |
| | 4.0 61 39 |
| | 4.1 10 90 |
| | 6.0 10 90 |
| Gradient Curve: | a) 5 (linear) |
| | b) 4 |
| | c) 3 |
| | d) 2 |
| Flow Rate: | 0.60 mL/min |
| Inj. Volume: | 2 µL |
| Temp.: | 60 °C |
| Detection: | UV (260 nm) |
| Sample: | 8-Combo DNA (5 µM) |

Figure 1. Adjustment of gradient curve.

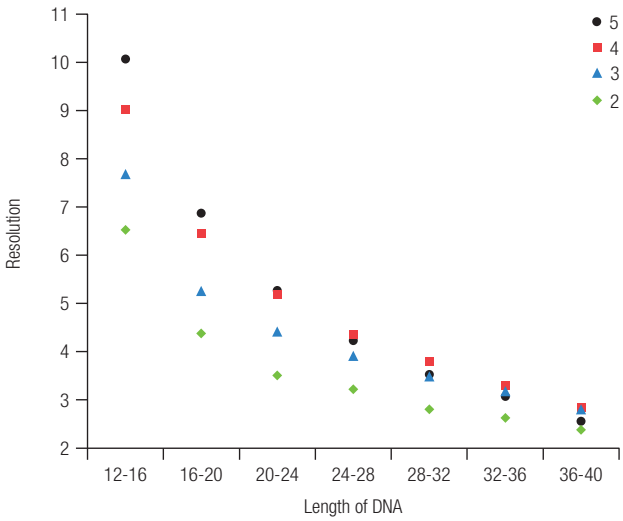
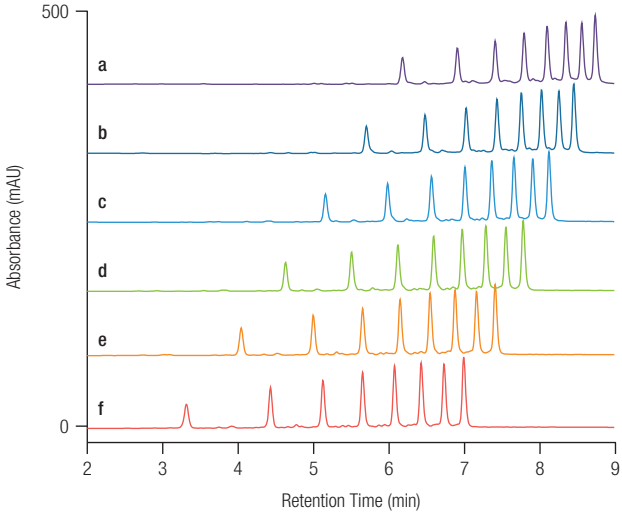



Figure 2. Effect of gradient curve on resolution.

## Effect of Temperature

The impact of temperature on retention time and resolution (Figures 3 and 4) was also investigated. Samples were separated at different temperatures using the same gradient program (linear, curve 5). As the temperature increased, ON retention decreased. Since viscosity of the mobile phase decreases with increasing temperature, the pressure also decreases (data not shown). Therefore, ON separation at higher temperatures requires less organic solvent and can employ higher flow rates without exceeding the maximum recommended column pressure. Also, higher ON resolution is observed with increasing temperatures (Figure 4). A change from 30 °C to 80 °C produced more than 40% improvement in resolution. For longer ONs, especially those with internal hydrogen bonds, higher temperatures can minimize those interactions and improve separations between the ON target and its impurities.




| | | | |
|---|---|---|---|
| Column: | **DNAPac RP**, 4 µm | | |
| Format: | 2.1 × 50 mm | | |
| Mobile Phase A: | 0.1 M TEAA, pH 7.0 | | |
| Mobile Phase B: | 0.1 M TEAA in water/acetonitrile (25:75 v/v) | | |
| Gradient: | Time (min) | %A | %B |
| | -3.0 | 90 | 10 |
| | 0.0 | 90 | 10 |
| | 8.0 | 54 | 46 |
| | 8.1 | 10 | 90 |
| | 10.0 | 10 | 90 |
| Flow Rate: | 0.40 mL/min | | |
| Inj. Volume: | 2 µL | | |
| Temp.: | a) 30 °C | | |
| | b) 40 °C | | |
| | c) 50 °C | | |
| | d) 60 °C | | |
| | e) 70 °C | | |
| | f) 80 °C | | |
| Detection: | UV (260 nm) | | |
| Sample: | 8-Combo DNA (5 µM) | | |

Figure 3. Separation of oligonucleotides at different temperatures.

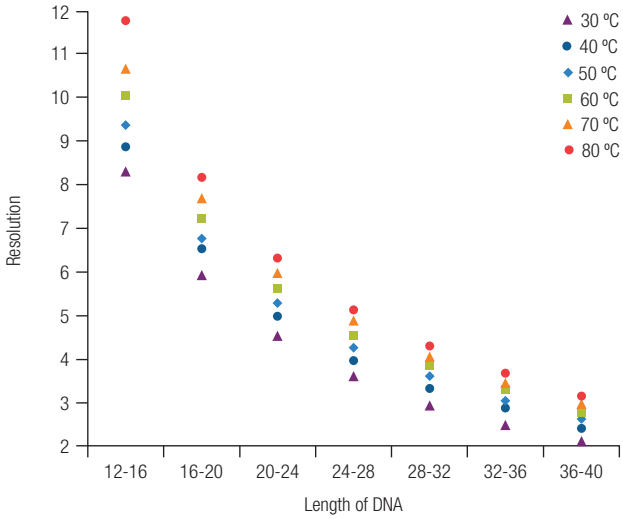



Figure 4. Effect of temperature on resolution.

## Effect of Flow Rate

In Figures 5 and 6, the relationship between flow rate and resolution is depicted. The flow rates were varied while the gradient volume was kept constant. A flow rate of 0.2 mL/min produced the best resolution for these ONs. As the flow rate increased, decreased resolution was observed. However, at 0.8 mL/min, the separation of up to 40mer mixed-base DNA can be achieved in 3.5 minutes, as opposed to 0.2 mL/min completing in ~13 minutes, which is a 3.7-fold improvement. For more challenging separations, we suggest the use of a 0.2 mL/min flow rate, and for separations where target ONs are well resolved, we suggest up to 0.8 mL/min and temperatures at or above 60 °C.

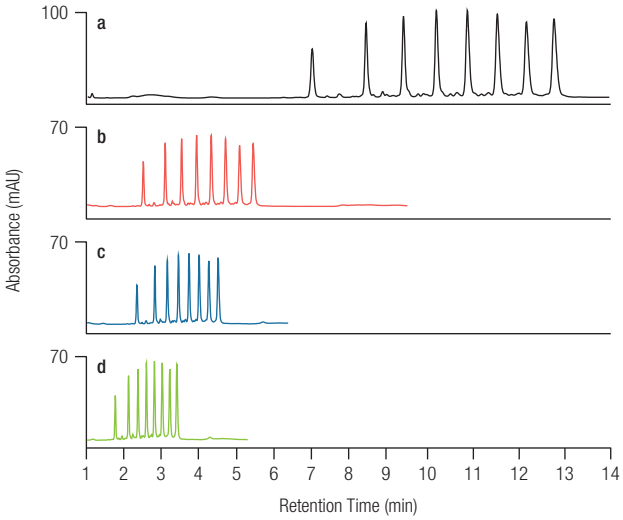



Figure 5. Separation of oligonucleotides at different flow rates.

| | |
|---|---|
| Column: | **DNAPac RP**, 4 µm |
| Format: | 2.1 × 50 mm |
| Mobile Phase A: | 0.1 M TEAA, pH 7.0 |
| Mobile Phase B: | 0.1 M TEAA in water/acetonitrile (75:25 v/v) |
| Gradient: | a) 15 to 37%B in 12 min<br>b) 15 to 37%B in 6 min<br>c) 15 to 37%B in 4 min<br>d) 15 to 37%B in 3 min |
| Gradient Curve: | 3 |
| Flow Rate: | a) 0.20<br>b) 0.40<br>c) 0.60<br>d) 0.80 |
| Inj. Volume: | 2 µL |
| Temp.: | 60 ºC |
| Detection: | UV (260 nm) |
| Sample: | 8-Combo DNA (5 µM) |

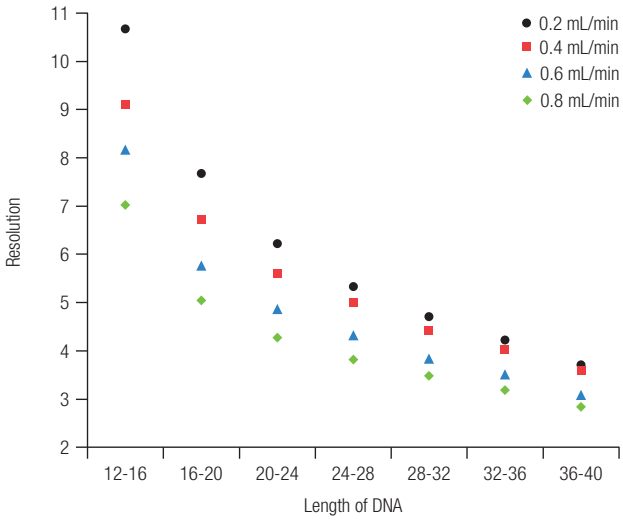



Figure 6. Effect of flow rate on resolution.

### Effect of Ion-Pair Reagent

The type of ion-pair reagent has been reported to affect the separation of ONs.[8] Here triethylamine (TEA) and hexylamine (HA) as ion-pair reagents were compared (Figure 7). Using a 3 minute gradient, the resolution obtained using HA was 18–23% higher for the short oligonucleotides and 4–11% higher for the longer oligonucleotides. Both steeper acetonitrile gradients (2% acetonitrile/min for TEA vs 7% acetonitrile/min for HA) and higher solvent concentrations (initial solvent concentrations of 2% with TEA and 11% with HA) are required for HA mobile phase since HA is more hydrophobic and produces greater interactions between the ONs and the stationary phase.

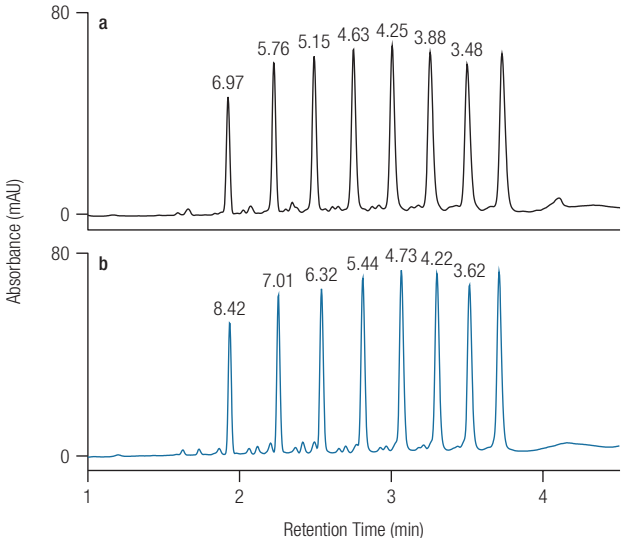



Figure 7. Comparison of TEA and HA as ion-pair reagents.

| | |
|---|---|
| Column: | **DNAPac RP**, 4 µm |
| Format: | 2.1 × 50 mm |
| Mobile Phases & Gradient | |
| a) | |
| Mobile Phase A: | 0.1 M TEAA, pH 7.0 |
| Mobile Phase B: | 0.1 M TEAA in water/acetonitrile (75:25 v/v) |
| Gradient: | 8 to 32% B in 3 min |
| b) | |
| Mobile Phase A: | 0.1 M HAA, pH 7.4 |
| Mobile Phase B: | 0.1 M HAA in water/acetonitrile (50:50 v/v) |
| Gradient: | 23 to 63.5% B in 3 min |
| Gradient Curve: | 3 |
| Flow Rate: | 0.80 mL/min |
| Inj. Volume: | 2 µL |
| Temp.: | 80 °C |
| Detection: | UV (260 nm) |
| Sample: | 8-Combo DNA (5 µM) |
| Peak Label: | Resolution (ep) |

### Effect of Oligonucleotide Diluent

In Figure 8, the effect of sample diluents was examined. For some chromatographic systems, diluents must be carefully considered to support optimal peak shape and resolution. In this system, significant impact on peak shape or resolution was not observed when the ON diluent was mobile phase A or DI water.

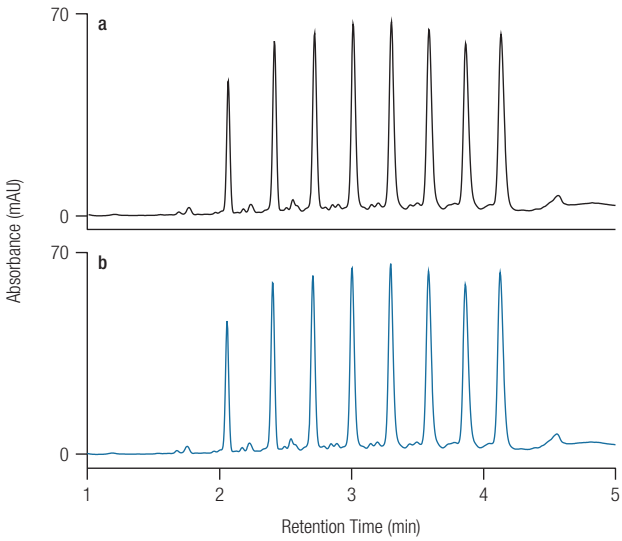



Figure 8. Effect of oligonucleotide diluent.

| | | | |
|---|---|---|---|
| Column: | **DNAPac RP,** 4 µm | | |
| Format: | 2.1 × 50 mm | | |
| Mobile Phase A: | 0.1 M TEAA, pH 7.0 | | |
| Mobile Phase B: | 0.1 M TEAA in water/acetonitrile (75:25 v/v) | | |
| Gradient: | Time (min) | %A | %B |
| | -2.0 | 92 | 8 |
| | 0.0 | 92 | 8 |
| | 3.0 | 68 | 32 |
| | 3.1 | 10 | 90 |
| | 5.0 | 10 | 90 |
| Gradient Curve: | 3 | | |
| Flow Rate: | 0.60 | | |
| Inj. Volume: | 2 µL | | |
| Temp.: | 60 °C | | |
| Detection: | UV (260 nm) | | |
| Sample: | 8-Combo DNA (5 µM) | | |
| Sample diluent: | a) Water | | |
| | b) Mobile phase A | | |

## Conclusion

- The DNAPac RP column delivers fast, high-resolution separation of mixed-base ONs on a stable polymeric stationary phase.
- Gradient curve, temperature, and flow rate can be adjusted to improve resolution and/or reduce analysis time.

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