Biopharma Application Note

HAMILTON

Achieving High-Resolution Separation of Oligonucleotides with Novel PRP-Z2 HPLC Columns

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Introduction

Oligonucleotide therapeutics and advanced nucleic acid tools have rapidly transitioned from niche research reagents to key components of commercial drug development pipelines. Consequently, contract development and manufacturing organizations (CDMOs) and institutional research cores face increasing demand for sophisticated chromatographic support, including high-resolution impurity profiling, length ladder characterization, and robust system suitability testing across a broad range of oligonucleotide sizes and chemical modifications.

These expanding requirements place significant stress on conventional reversed-phase LC platforms. Silica-based stationary phases, which dominate small-molecule separations, are inherently limited by pH and temperature instability. Furthermore, silica phases are prone to hydrolytic degradation and progressive loss of efficiency under typical oligonucleotide separation conditions (>60°C and ion-pairing agents). The exposure of residual silanols to the separation conditions, leaves active metal sites from degraded silica, which can introduce secondary interactions with the anionic phosphate backbone of oligonucleotides, leading to peak tailing, variable recoveries, and difficulties in quantifying low-level impurities.

Additionally, many widely adopted oligonucleotide methods rely on high concentrations of ion-pairing reagents such as triethylammonium acetate (TEAA) or hexylammonium acetate (HAA) during gradient elution. While effective for generating retention and resolution, these high salt loads complicate integration with mass spectrometry (MS), increase the burden of fraction cleanup, and accelerate fouling of chromatographic systems.

Polymeric reversed-phase packings based on crosslinked polystyrene–divinylbenzene (PS–DVB) offer a promising solution by providing a silica-free, pH-independent matrix with exceptional chemical and thermal stability. However, for widespread adoption in oligonucleotide workflows, these materials must demonstrate not only robustness but also consistent, high-efficiency separations across a range of sequence lengths and mobile phase systems.

In this work, we evaluate a PS–DVB-based reversed-phase material (PRP-Z2) for the separation of model oligonucleotide systems: a deoxycytidine homopolymer ladder ($d(C)_{12}$–$d(C)_{18}$), a 10–60 nucleotide (nt) ladder, and a 20–100 nt ladder. Using TEAA- and HAA-based ion-pair systems at elevated temperatures (60, 80 and 85°C) and column formats ranging from 50 × 2.1 mm to 150 × 4.6 mm, we assess chromatographic performance, robustness, and scalability. Emphasis is placed on the ability of the polymeric phase to support organic-rich gradients and moderate ion-pairing conditions, thereby reducing non-volatile salt exposure while maintaining resolution and peak shape.

Discussion

Resolution and Peak Shape Across Oligonucleotide Lengths

Adequate separation of the $d(C)_{12}$–$d(C)_{18}$ homopolymer ladder using 5 µm PRP-Z2 columns which utilized TEAA/ACN gradients at both 60 and 80°C, achieved baseline resolution of all components with symmetric, well-defined peaks (Figure 1).

System suitability and column performance verification of oligo workflows is generally achieved through the analysis of this type of short homopolymer. The ability of PRP-Z2 to maintain consistent reproducible resolution of closely spaced oligomers across multiple column dimensions (50 × 2.1 mm, 100 × 4.1 mm, 150 × 4.6 mm) serves as a litmus test that enables its use as a material that can be used as qualification procedures of both regulated and research environments.

Analysis of the broader oligo ladders, which can span from 10–60 nt and 20–100 nt, offer a more challenging environment for the assessment of a stationary phase's efficiency and resolution. Under TEAA-based conditions, the 10–60 nt ladder exhibited a clear, length-dependent elution pattern (Figure 2), with sufficient resolution to support both qualitative ladder identification and quantitative assessment of varied length species within a sample.

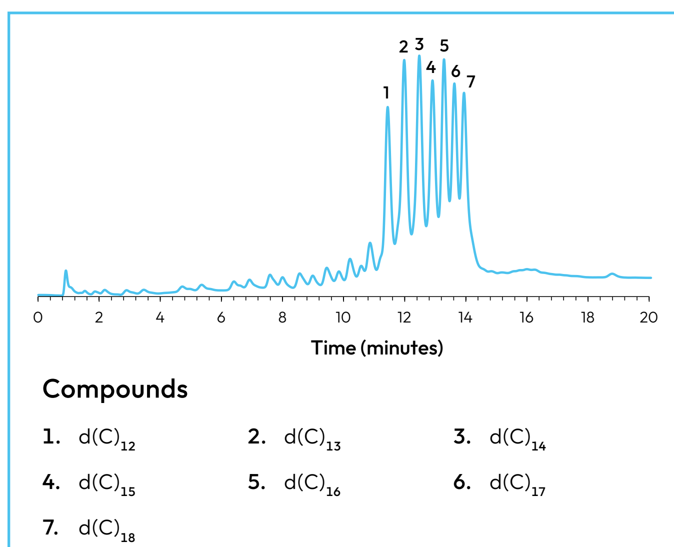

Figure 1: Separation of a deoxycytidine homopolymer ladder ($d(C)_{12}$–$d(C)_{18}$) on a PRP-Z2.

Chromatographic Conditions for Figure 1

Specifications	
Packing Material	PRP-Z2, 5 μm
Part Number	79641
Chromatographic Conditions	
Gradient	8–15% B 0.0–10 min 15–19% B 10.01–14 min
Temperature	60°C
Injection Volume	5 μL
Detection	UV at 260 nm
Dimensions	100 x 2.1 mm
Eluent A	100 mM TEAA pH = 8.5
Eluent B	CH_3CN
Flow Rate	0.6 mL/min

In the HAA/ACN system at 80°C, the 20–100 nt ladder was effectively resolved on a 5 μm, 150 × 4.6 mm PRP-Z2 column (Figure 3), demonstrating that the polymeric phase provides adequate mass transfer and pore accessibility even for longer oligonucleotide sequences under high-temperature, high-organic gradients (Figure 4).

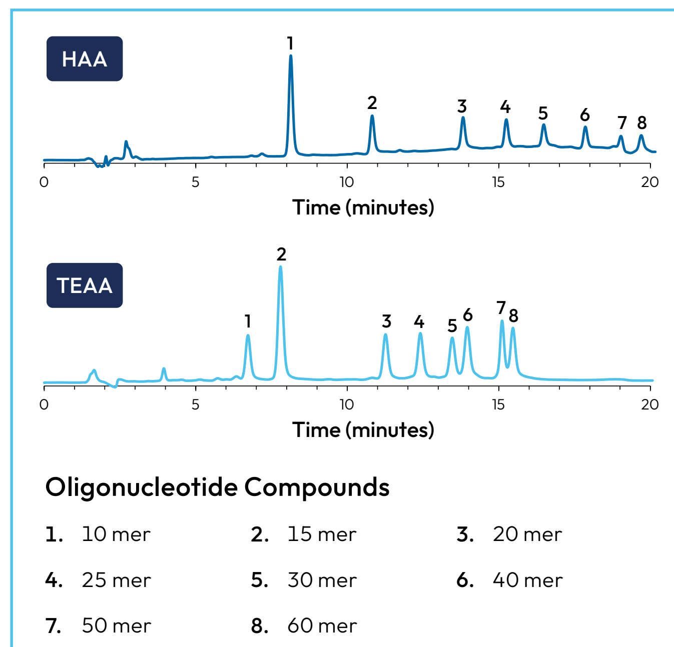

Figure 2: The effect of ion-pairing reagents on the separation of oligonucleotide ladder (10-60 nt) under the same exact chromatographic conditions. HAA (top) and TEAA (bottom).

Chromatographic Conditions for Figure 2

Specifications	
Packing Material	PRP-Z2, 5 μm
Part Number	79641
Chromatographic Conditions	
Gradient	10–24% B 0.0–20.0 min
Temperature	80°C
Injection Volume	5 μL
Detection	UV at 260 nm
Dimensions	50 x 4.1 mm
Eluent A	100 mM pH = 7 HAA & TEAA
Eluent B	A + CH_3CN
Flow Rate	0.20 mL/min

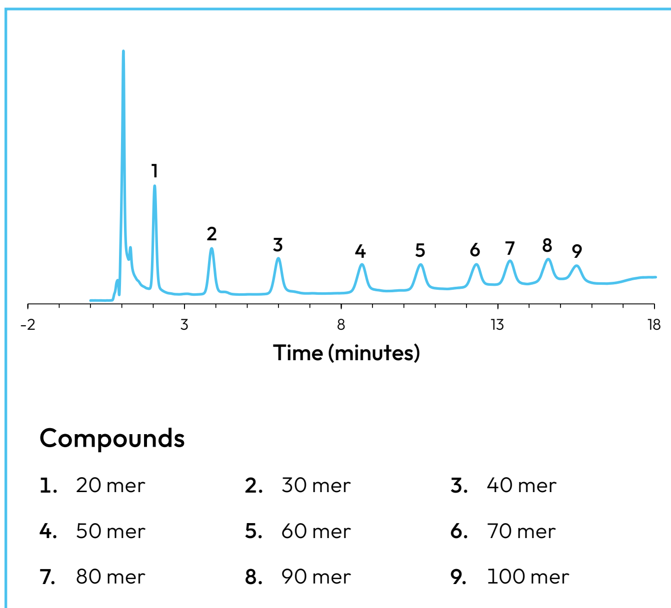

Figure 3: Oligonucleotide ladder (20–100 nt) separated on a PRP-Z2.

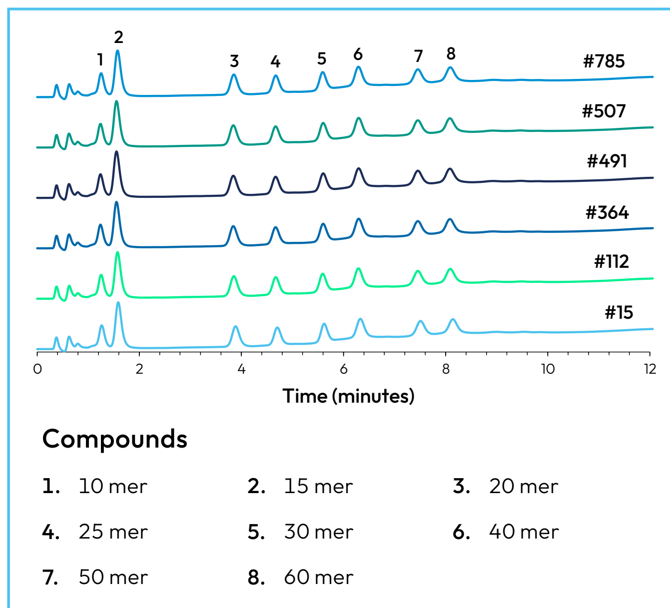

Figure 4: Oligonucleotide ladder (10–60 nt) separated on a PRP-Z2.

Chromatographic Conditions for Figure 3

Specifications	
Packing Material	PRP-Z2, 7 µm
Part Number	79929
Chromatographic Conditions	
Gradient	30–90% B 0.0–20.0 min
Temperature	80°C
Injection Volume	5 µL
Detection	UV at 280 nm
Dimensions	150 x 4.6 mm
Eluent A	100 mM HAA pH = 7
Eluent B	CH_3CN
Flow Rate	2.0 mL/min

Chromatographic Conditions for Figure 4

Specifications	
Packing Material	PRP-Z2, 5 µm
Part Number	79925
Chromatographic Conditions	
Gradient	10–24% B 0.0–20.0 min
Temperature	85°C
Injection Volume	5 µL
Detection	UV at 260 nm
Dimensions	50 x 4.1 mm
Eluent A	100 mM TEAA pH = 9.2
Eluent B	50% A + CH_3CN
Flow Rate	0.50 mL/min

Similarly, the PRP-Z2 was tested against random length oligonucleotides ranging from 12–33 with multiple 12 mers of differing lipophilicity. In this separation, all six species were isolated with baseline resolution in under 10 minutes utilizing the alkaline conditions TEAA 100 mM/ACN pH 8.5 and 60°C (Figure 5). Unlike traditional silica-based separations, the resin was once again stable under more aggressive elution conditions.

Collectively, these results demonstrate that a single PS–DVB stationary phase can address a wide array of oligonucleotide applications—ranging from short system suitability standards to longer process intermediates and final products—eliminating the need to frequently change column chemistries or operating modes.

Reduced Dependence on Extreme Ion Pair Conditions

An important practical outcome of these evaluations remains that satisfactory retention and resolution were achieved with PRP-Z2 under organic-rich gradients without resorting to organic phase dilution with mobile phase A ion-pair reagents. The inherently hydrophobic PS–DVB surface provides strong interactions with ion-paired oligonucleotides, thereby eliminating the need for elevated salts in the mobile phase.

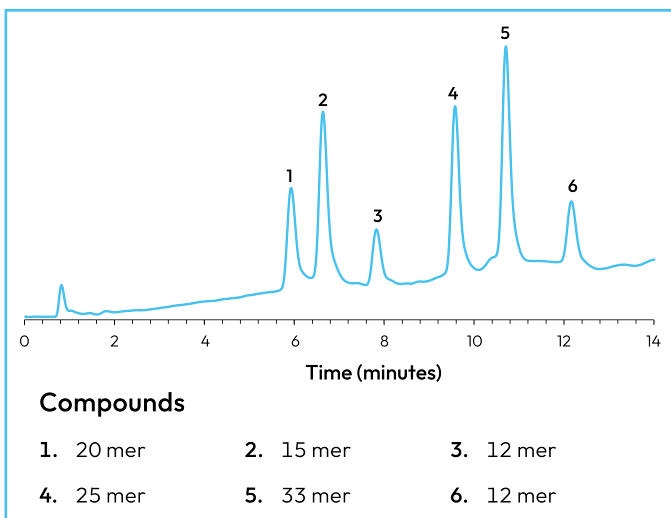

Figure 5: Oligonucleotide Performance Mix (12 – 33 nt) separated on a PRP-Z2.

Chromatographic Conditions for Figure 5

Specifications	
Packing Material	PRP-Z2, 5 μm
Part Number	79641
Chromatographic Conditions	
Gradient	8–15% B 0.0–10.0 min 15–19% B 10.01–14.0 min
Temperature	60°C
Injection Volume	1 μL
Detection	UV at 260 nm
Dimensions	100 x 2.1 mm
Eluent A	100 mM TEAA pH= 8.5
Eluent B	CH_3CN
Flow Rate	0.60 mL/min

For laboratories managing large numbers of oligonucleotide samples, the implications of this shift can be pronounced:

- Reduced non-volatile in the methodology reduces challenges associated with solvent evaporation, desalting, and preparation for subsequent analyses or processing steps.
- Decreased MS maintenance intervals can be achieved through the use of decreased ion-pair reagents.
- Method transfer is generally more robust when performance does not depend on fine control of high salt concentrations, which can vary between instruments, facilities, or suppliers.

Chromatograms from both the 10–60 nt and 20–100 nt ladder experiments (Figures 2 and 3) illustrate that high resolution can be maintained while operating with moderate ion-pair levels and increased organic content.

This combination aligns more closely with operational priorities: stable methods, predictable instrumentation performance, and minimized cleaning and maintenance overhead for the busy chromatographic system.

Thermal and Chemical Robustness Under Realistic Conditions

All separations in this study were conducted at elevated temperatures (60, 80 or 85°C), reflecting standard practice in oligonucleotide chromatography to disrupt secondary structures and improve peak shape. Under these conditions and in the presence of TEAA or HAA at 100 mM levels, PRP-Z2 maintained consistent retention behavior and efficiency throughout the experiments.

The robustness of PRP-Z2 can be attributed to the PS–DVB matrix, which is resistant to hydrolytic breakdown at alkaline pH and temperature extremes, and to the absence of silanol groups that often contribute to secondary interactions with oligonucleotide phosphate backbones. For long-running, high-throughput environments, column lifetimes and method stability under aggressive alkaline conditions have a direct impact on overall cost and maintenance scheduling. A stationary phase that tolerates elevated temperatures and ion-pair reagents reduces downtime and troubleshooting, thereby increasing profit margins.

Long Term Column Stability and Method Robustness

To rigorously evaluate the long-term performance and reproducibility of PRP-Z2. The phase was placed in an extended longevity study under aggressive chromatographic conditions. The 100 mM TEAA/ACN gradient at pH 9.2 was heated to 85°C for 789 consecutive chromatographic runs. The analysis of this assay provides critical insights into column equilibration behavior, extended stability, and method robustness, all key considerations for high-throughput analysis for regulated environments.

Throughout the experiment, eight oligomers (10mer through 60mer) were consistently identified. The results confirmed remarkably stable retention times: 10mer (1.142 min), 15mer (1.483 min), 20mer (3.858 min), 25mer (4.700 min), 30mer (5.625 min), 40mer (6.317 min), 50mer (7.492 min), and 60mer (8.117 min). The retention pattern confirms the length-dependent separation mechanism and reproducible interaction between the PS–DVB stationary phase and ion-paired oligonucleotides.

The result from this statistical analysis demonstrates that PRP-Z2 maintains excellent chromatographic efficiency and reproducibility over extended use, with minimal drift in retention, peak shape, or column performance under the most challenging of chromatographic conditions (Figure 4).

Longevity Statistics of Variation under 85°C, and TEAA 100 mM pH 9.2 conditions

Longevity Statistics	
Retention time coefficient of variation (CV)	1.55%
Peak height CV	7.36%
Peak width CV	2.53%
Plate count CV	3.36%

Additionally, 12 spiked samples were analyzed within this study, revealing a spike recovery percentage of 95–102% for all injected oligomers. This experiment confirms the method's capability to both detect and quantify concentration differences, while supporting its utility for impurity profiling and quantitative analysis.

This 789-injection longevity study provides compelling evidence for long-term column stability and method robustness of PRP-Z2 in the face of overly aggressive eluents and elevated temperature conditions. The rapid equilibration extended stable performance window, and outstanding precision metrics truly validate PRP-Z2 as a reliable platform for high-throughput regulated workflows where consistency, reproducibility, and minimal maintenance are paramount.

Scalability and Method Standardization

The ability to obtain comparable separation behavior on PRP-Z2 across multiple column geometries and particle sizes is particularly advantageous in organizations that must coordinate development, scale-up, and routine QC. The d(C) ladder and oligonucleotide ladder separations performed on narrow-bore (2.1 mm i.d.) and larger-bore (4.6 mm i.d.) columns showed consistent elution order, peak shapes, and relative resolution (Figures 1–3), indicating that PRP-Z2 is amenable to straight-forward scaling of flow rate and sample loading.

This scalability, combined with the demonstrated long-term stability and robustness, positions PRP-Z2 as an ideal platform for method development, transfer, and routine application across multiple sites and instrumentation configurations.

Conclusion

The PS–DVB-based reversed-phase stationary phase, PRP-Z2 was evaluated for the separation of model oligonucleotide systems including a $d(C)_{12}$–$d(C)_{18}$ ladder, a 10–60 nt ladder, and a 20–100 nt ladder. Across multiple column dimensions and particle sizes, utilizing TEAA and HAA ion-pair gradients with temperatures at 60, 80, and 85°C, PRP-Z2 consistently delivered high efficiency, length-dependent resolution with excellent peak shape.

A key finding from this study includes high resolution separations which can be achieved using organic-rich gradients and moderate ion-pair concentrations, thereby reducing non-volatile salt load on the chromatographic system and collected fractions. The combination of thermal and chemical robustness reduced dependence on extreme salt conditions, and consistent performance across column geometries supports the use of PRP-Z2 as a platform stationary phase for oligonucleotide workflows.

Furthermore, a 789-injection longevity study demonstrated outstanding long-term column stability and method robustness, with precision metrics (RT CV 1.55%, peak width CV 2.53%, plate count CV 3.36%) that exceeded expectations under the most aggressive of chromatographic conditions. This performance translates into more robust and scalable methods, simplified maintenance, improved compatibility with LC–MS-based analyses and reduced total cost of ownership for high-throughput laboratories. PRP-Z2 represents a scientifically sound and operationally practical solution for modern oligonucleotide analysis, supporting both research and regulated applications with confidence.

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