Application Note 20996

High-Resolution Separation of Oligonucleotides on a Pellicular Anion-Exchange Column

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Key Words
DNA, ssDNA, siRNA, antisense, aptamers, nucleic acids, oligonucleotides, oligodeoxynucleotides, therapeutic, diagnostic, DNAPac PA200

Abstract
Multiple oligonucleotide (ON)-based therapeutic models have been developed to date including antisense oligonucleotides (ASOs), aptamer oligonucleotides (AOs) and short interfering RNA oligonucleotides (siRNA). In addition, modifications to oligonucleotide base-, backbone-, and ribose-moieties have all been introduced to limit nuclease-mediated degradation in therapeutic ONs. Mechanisms to resolve oligonucleotides from their modified forms, synthetic failure fragments, and their metabolic products are therefore necessary, although these can be technically challenging to develop. The Thermo Scientific™ DNAPac™ PA200 column offers industry-leading selectivity and resolution for the separation of therapeutic and diagnostic oligonucleotides.

Introduction
DNA expression control is implicated in disease initiation and progression. Several groups have developed nucleic acid approaches to diagnose and treat otherwise “undruggable” diseases. Such efforts include development of antisense oligonucleotides, [1] aptamers, [2] and RNA interference [3] to control expression of genes involved in diseases.

As nucleic acids are susceptible to degradation by several endo- and exo-nucleases, numerous modifications have been applied to enhance their stability and biological half-life. These include 2'-O substitutions on ribose, backbone modifications including phosphorothioate and phosphoro*di*thioate insertion, phosphoramidate chemistries, abasic inverted linkages, “locked” and “unlocked” nucleic acids, and insertion of methylene bridges (between phosphorous and the 5' oxygen to elongate the linkage) [4–13]. In addition, other oligonucleotide therapeutic modalities are under development [14–16]. These nucleic acid modifications are often applied to ASO, aptamer, and siRNA therapeutics leading to a need for methods to characterize the resulting candidate therapeutics.

Chromatographic oligonucleotide analysis is performed for therapeutic and diagnostic applications, as well as in manufacturing and research environments where demonstrated oligonucleotide purity improves applications to regulatory agencies. Oligonucleotide characteristics influencing chromatographic interactions include length, base composition, the presence of coupling failures, and dyes (or “molecular beacons”); in addition residual-protecting groups, employed during single-stranded nucleic acid (ssNA) synthesis, can also have an effect. Each modification may be combined with the others in a given oligonucleotide.

Oligonucleotide assays are typically accomplished by anion-exchange or ion-pair reversed-phase chromatography. Both approaches employ ionic interactions between the analytes, either directly with the stationary phase (i.e. anion exchange) or with the ion-pair reagent, which in turn interacts with the stationary phase. Both approaches enjoy wide popularity as both often deliver good resolution and analysis throughput for

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oligonucleotides [17–20]. This application note will focus on a *pellicular* anion-exchanger designed for high-resolution ON separations.

Because ssNAs harbor both hydrophilic phosphate backbones and more hydrophobic purine and pyrimidine bases, ssNA length-based retention methods must emphasize interactions with the hydrophilic phosphate backbone and minimize interactions with the hydrophobic bases. Conversely, when evaluating ssNA mixtures of similar or identical length, the chromatographic system must minimize phosphate backbone interactions and emphasize hydrophobic base and/or protecting group interactions to resolve the differing components. Haupt and Pingoud [21] reported that anion exchange-based ssNA separations were superior to both direct reversed-phase and ion-pairing reversed phase approaches because more ssNAs were resolved per unit of time with the anion exchange process.

Here, we report the influence of gradient type on oligonucleotide retention and resolution using the DNAPac PA200 strong anion-exchange column. The pellicular nature of the DNAPac substrate provides very narrow peak width, and the monomer employed supports retention control by pH, as well as use of solvent modifiers (e.g., acetonitrile). Changes to oligonucleotide selectivity may also be produced by the use of alternate eluent salts (e.g., Cl^- or ClO_4^-). The DNAPac PA200 column accommodates increased throughput of normal-length single-stranded nucleic acids (e.g., 15–25-mers) on short columns when using curved eluent concentration gradients. We also demonstrate the advantage of a curved gradient in this application.

Experimental Details

Consumables	Part Number
Deionized (DI) water, 18.2 MΩ-cm	
Tris base, Fisher Scientific	BP152-500
Sodium chloride, 99.5% purity for analysis, Fisher Scientific	S640-500
Hydrochloric acid, Optima™, Fisher Chemical	A466-500
Deoxythymidine oligonucleotide samples (purchased from Midland Certified Reagent Co. Inc.)	
$dT_{12\text{-}18}$	D-1218
$dT_{19\text{-}24}$	D-1924
$dT_{25\text{-}30}$	D-2530
$dT_{31\text{-}40}$	D-3140
$dT_{41\text{-}60}$	D-4160
Thermo Scientific™ Chromacol™ polypropylene screw top vial, 300 µL	03-PPSV
Thermo Scientific Chromacol 8 mm screw cap, open top, black, silicone/red PTFE seal	8-SC-ST15

Sample Handling Equipment	Part Number
Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC Analytical LC system consisting of:	
SRD-3000 solvent degas system	5035.9230
DGP-3600RS dual ternary gradient pump	5040.0066
TCC-3000RS thermal compartment	5730.0000
WPS-3000TBRS autosampler	5841.0020
DAD-3000RS absorbance detector	5082.0020
Biocompatible (PEEK™) 13 µL analytical flow cell	6082.0400
Thermo Scientific™ Nalgene™ nylon 0.2 µm filter unit	0974026A

Buffer and Sample Preparation
Tris buffer, 2.0 M (pH 8.0): Dissolve 242.28 ±0.05 g Tris base in 742.7 ±0.1 g DI water. Bring to pH 8.0 with 112.7 g concentrated HCl and verify pH using a calibrated pH meter. Filter the solution through 0.2 µm pore membrane filter (d=1.097).
Sodium chloride, 5.0 M: Dissolve 584.4 ± 0.1 g sodium chloride in 1766.0 ±0.1 g of DI water. Filter the solution through 0.2 µm pore membrane filter (d=1.175).
Tris buffer, 40 mM (pH 8): Dilute 21.94 ±0.05 g of 2.0 M Tris buffer pH 8.0 into 980.0 ±0.05 g DI water. Mix well and transfer into chromatographic eluent A reservoir.

1.0 M NaCl, 0.04 M Tris (pH 8.0): Dilute 21.94 ± 0.05 g 2.0 M Tris buffer and 235.0 ± 0.05 g 5 M NaCl into 780 g DI water. Mix well and transfer into chromatographic eluent B reservoir.

Dilute each 25 unit (25 A_{260}) dry sample into 1.25 mL DI water to prepare 20A/mL stocks. Transfer 8.0 µL of each stock into a sample vial, and add 392.0 µL D.I. water. Cap the vial(s), mix well, and place vial(s) in autosampler rack. In this application, 15 µL injections were employed.

Note: Store the dissolved standards at -20 °C

Separation Conditions for Figure 1		Part Number
Column:	DNAPac PA200 (8 µm, 4.0 × 250 mm)	063000
Mobile Phase:	A: 40 mM Tris, pH 8	
	B: 40 mM Tris, pH 8, 1.0 M NaCl	
Flow Rate:	1.0 mL/min	
Initial Pressure:	Approximately 140 bar (2000 psi)	
Temp.:	30 °C	
Sample Amount:	15 µL	
Diode-Array Detector:	260 nm	

Time (min)	% A	% B	Gradient Curve
-3	71.0	29.0	5 (linear)
0	71.0	29.0	5
12.56	44.0	56.0	5
12.6	20.0	80.0	5
13.4	71.0	29.0	5

Table 1: Binary gradient for Figure 1

Time (min)	% A	% B	Gradient Curve
-3	71.0	29.0	5 (linear)
0	71.0	29.0	5
12.56	44.0	54.0	3 (concave)
12.6	20.0	80.0	5
13.4	71.0	29.0	5

Table 2: Binary gradient for Figure 2

Data Processing	
Software:	Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System

Results

At pH values between 6 and 8 (a common range for analytical starting conditions), oligonucleotides harbor one negative charge for each phosphodiester bond. Hence, a terminally non-phosphorylated ON will harbor a charge equal to one less than the number of bases. At constant pH, a linear gradient of salt will elute oligonucleotides in order of increasing charge (and therefore increasing length). Since the relative charge *difference* between ONs decreases with increasing ON length, the separation (resolution) between such ONs also decreases.

An example of this is shown in Figure 1, where a collection of deoxythymidine oligonucleotides, including components of 12 to 60 nucleotides long, were mixed to demonstrate the range of ON lengths resolved by this column. In this figure, the first five labeled (minor) peaks represent “failure” sequences where incomplete coupling between dT additions results in shorter than expected ONs. Since the smallest length prepared was 12 nucleotides long, the first five peaks represent ONs of up to 11 bases, and thus 6–10 negative charges. In addition to these numbered peaks, eleven other components were resolved that represent other forms of ON synthetic failures, such as incomplete group (e.g. Trityl-) deprotection. Further analysis by coupling to MS and HRAM-MS instruments may support and extend this characterization [18–19]. In all, at least 67 components were partially resolved in 14.5 minutes.

Many HPLC instrument vendors support use of curved gradients that start at one gradient slope and asymptotically approach a zero slope over time. Because ONs of increasing length are less-well resolved with linear gradients, curved gradients that *reduce* slope over time (concave gradients) provide a means to improve the resolution of increasingly longer ONs. Figure 2 employs a concave gradient that modifies only a single command in the gradient program (designation of curve 3 instead of curve 5). This chromatogram reveals 56 length variants, and 12 structural variants, having a better overall resolution, especially for the longer ONs. In this case the final salt concentration was reduced by 20 mM (from 560 to 540 mM NaCl) and the peak spacing was much closer to uniform, delivering resolution values between 1.0 and 5.4. In many cases, this would be sufficient to characterize peak purity for ONs up to this length.

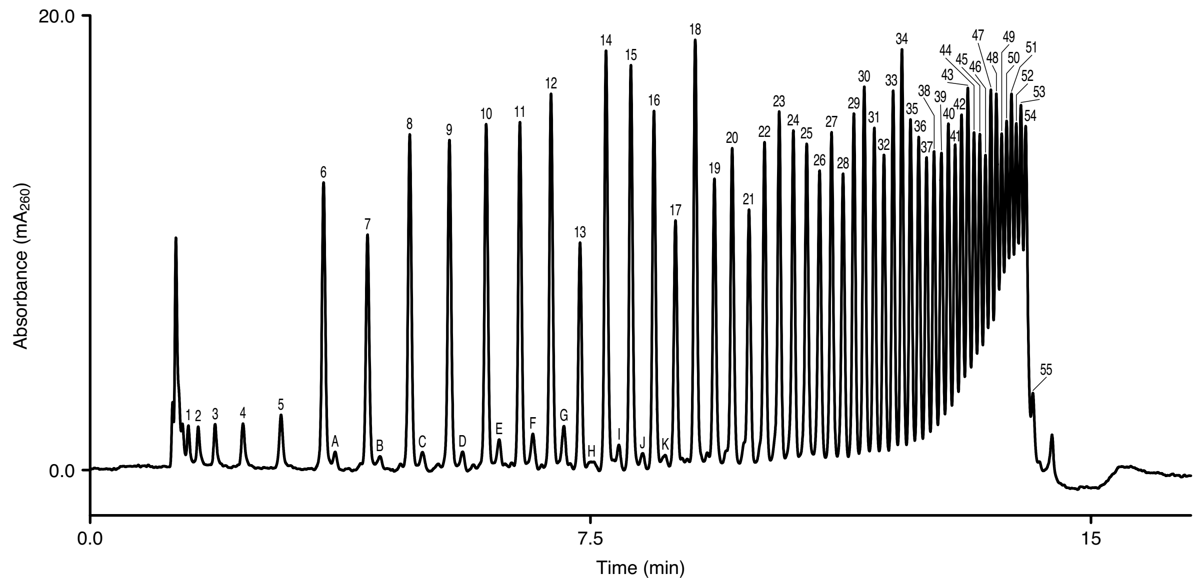

Figure 1: Separation of deoxythymidine oligodeoxynucleotides in a 4.0 x 250 mm DNAPac PA200 column using a 290–560 mM gradient in four column volumes at 1.0 mL/min. Fifty-five oligonucleotide length variants and eleven structural variants were resolved during this four column volume (12.6 minute) gradient

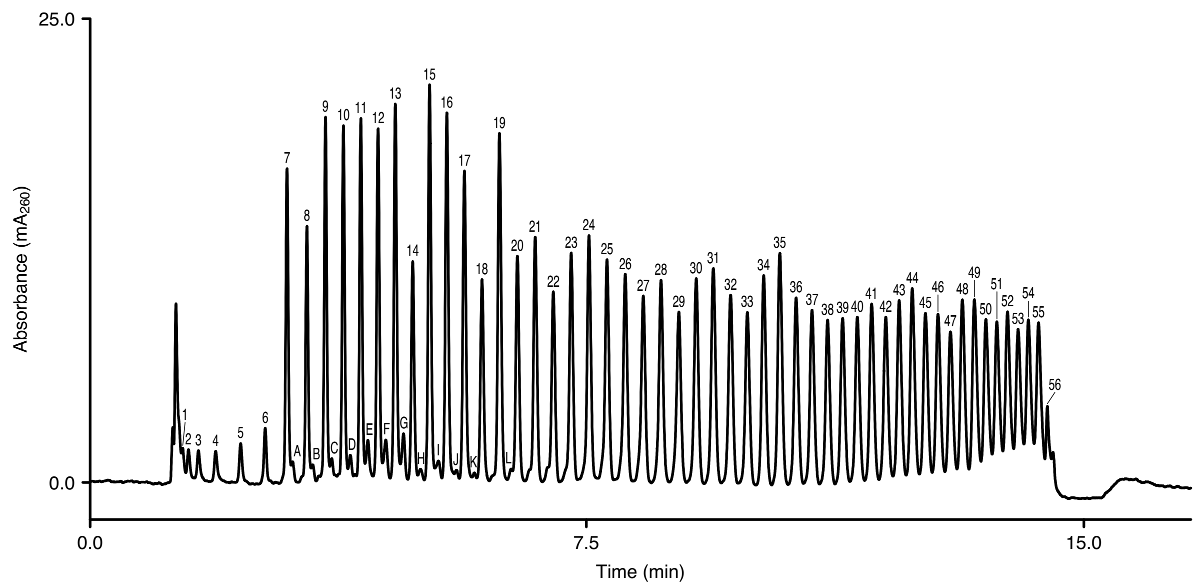

Figure 2: Separation of deoxythymidine oligodeoxynucleotides in a 4.0 x 250 mm DNAPac PA200 column using a 290–540 mM *curved* gradient in four column volumes at 1.0 mL/min. Fifty-six oligonculeotide length variants and twelve structural variants were resolved during this four column volume (12.56 minute) gradient

Conclusion

The Dionex DNAPac PA200 column provides high-efficiency separations for nucleic acids used in therapeutic and diagnostic applications.

- The DNAPac PA200 column provides separation of oligonucleotides over 60 nucleotides long with average separation speeds between 4 and 5 bases per minute over a 14.5 minute range.
- The DNAPac PA200 column offers industry-leading resolution and selectivity for both short and long oligonucleotides.

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