High-speed, High-resolution Oligonucleotide Separations Using Small Particle Anion-Exchangers

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Overview

Purpose:
Develop an oligonucleotide (ON) separation column capable of increased throughput with comparable resolution, and improved resolution with comparable throughput, to the Thermo Scientific™ DNAPac™ PA200.

Methods:
• Employ 4 µm resin with a DNAPac PA200 chemistry in PEEK™ lined SST hardware for improved peak shape and high-pressure compatibility.

• Maintain DNAPac PA200 functional film thickness equivalent to that of the DNAPac PA200 to help increase capacity.

Results:
• The new DNAPac PA200 RS (Rapid Separation) column is available in 4.6 × 50, 4.6 × 150 and 4.6 × 250 mm formats.

• The 4.6 × 250 mm format improves oligonucleotide resolution using gradient times comparable to the 4 × 250 mm (8 µm) DNAPac PA200.

• The 4.6 × 150 mm column delivers resolution comparable to the 4 × 250 mm DNAPac PA200, but improves throughput.

• The 4.6 × 50 mm format provides very high throughput where the highest resolution is not needed.

• This phase tolerates pressures up to 690 bar (10,000 psi).

Using the high throughput 4.6 × 50 mm format we have partially resolved up to 51 oligonucleotide components in less than 3 minutes.

Introduction

Pellicular anion-exchange chromatography has provided industry-leading oligonucleotide (ON) resolution since 1990.

The DNAPac PA100 (1990) provided new options for control of ON selectivity, allowing facile separations of closely-related sequences, even of the same lengths.

The DNAPac PA200 (2005) improved those separations and increased throughput as well as column longevity, especially at high pH and temperatures.[1]

Here we describe the new DNAPac PA200 RS. Available in different formats, these improve resolution further for ON separations where the very highest resolution is required;[2-4] improve throughput where good resolution has already been achieved; and greatly improve throughput where high ON resolution is readily available.

This column is best used with chromatographs capable of very high pressures, and harboring very low system volumes.

Methods

Sample Preparation

ON samples were acquired from Integrated DNA Technologies (Coralville, IA). ONs were suspended in deionized (DI) water, typically to 1.0, 1.5 or 6 mg/mL, and diluted as needed.

Liquid Chromatography

Separations were performed on Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS chromatographs, consisting of Biocompatible LPG-3400RS pumps, WPS-3000RS split-loop samplers, TCC-3000RS thermal compartments and VWD3400RS variable wavelength or DAD3000RS diode-array detectors.

Data Analysis

Chromatographic system control, data acquisition and peak integration employed Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System version 6.8.

TABLE 1. Oligonucleotides used

(ª = 2′,5′-linkage, * = PS linkages)

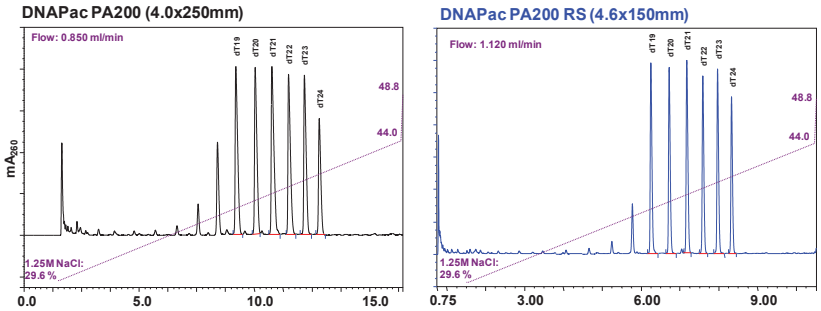
RNA	
Dio-1:	5′ - AUG AAC UUC AGG GUC AGC UUG -3′
Dio-6:	5′ - AUG AAC UUC AªGªG GUC AGC UUG -3′
Dio-9:	5′ - AUG AAC UUC AGG GUCª AGC UUG -3′
eGFP-S:	5′ - AGC UGA* CCC UGA AGU UCA UdCdT -3′
DNA	
dT19-24:	(homopolymer dT 19-24 bases)
dA 12-18:	(homopolymer dA 12-18 bases)
PdA 19-24:	(homopolymer PdA 19-24 bases)
PdA 25-30:	(homopolymer PdA 25-30 bases)
PdA 40-60:	(homopolymer PdA 40-60 bases)
ON34:	5′-TAG GTT CTC TAA CGC TGA CTG ATT GTA GGT GTT C- 3′
ON35:	5′-GTA GGT TCT CTA ACG CTG ACT GAT TGT AGG TTC TC- 3′
ON44:	5′-TGA CTG ATT GTA GGT TCT CTA ACG CTG ACT GAT TGT AGG TTC TC - 3′
ON45:	5′-CTG ACT GAT TGT AGG TTC TCT AAC GCT GAC TGA TTG TAG GTT CTC-3′
ON54:	5′-TCT GTA ACG CTG ACT GAT TGT AGG TTC TCT AAC GCT GAC TGA TTG TAG GTT CTC-3′
ON55:	5′-TTC TGT AAC GCT GAC TGA TTG TAG GTT CTC TAA CGC TGA CTG ATT GTA GGT TCT C-3′

Results

I. Column Performance

Our goal is to deliver a column with resolution similar to, and throughput better than, the DNAPac PA200, in one format; and better resolution and throughput equivalent to the standard DNAPac, in a second format.

FIGURE 1. DNAPac gradient test.
Elution of deoxythymidine ONs (19–24 bases) using 370–550 mm NaCl in 4 column volumes (Tris buffer at pH 8 and 30 °C). Absorbance detection at 260 nm. Linear velocity was 1.12 mm/s



Gradient comparison of standard DNAPac with small particle version shows:

- Reduced retention
- improved Peak Width
- Improved Resolution
- Appropriate selectivity

Column #	Flow (µL/min)	d-PSI (Max)	Asym. (dT23)	t_r (dT23)	PW-dT23 (5%,CV)	Rs (19/20)	Rs (22/23)	Rs (22/23)	T, ratio (22/23)
DP200 SN3265	850	1655	1.51	10.92	0.056	4.8	4.4	4.1	0.94
DNAPac PA200 RS	1120	4736	1.85	7.97	0.048	6.1	5.3	4.8	0.95

In this test this 4.6 × 150 mm DNAPac PA200 RS column delivered 21% higher resolution in 35% less time than this 4 × 250 mm DNAPac PA200.

II. Applications

Most ON separations attempt to resolve related sequences on the basis of length, and the DNAPac performs this function well.

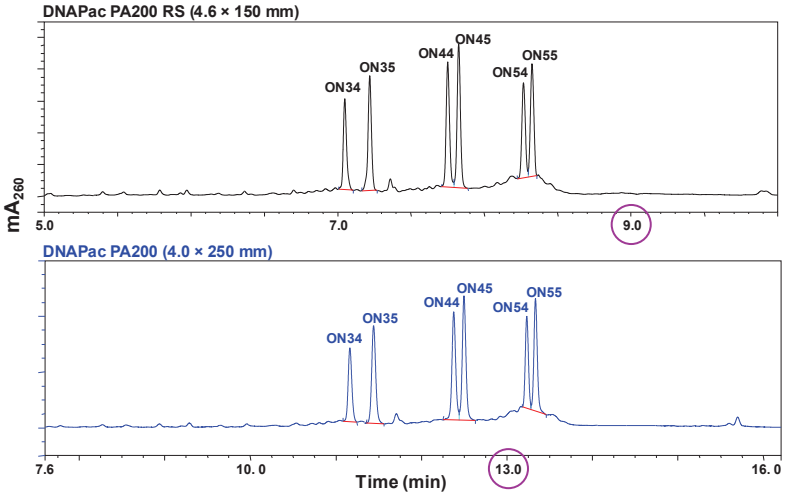
Annealing of single stranded RNAi sequences into duplexes can generate 2´,5´-linkages and phosphorothioate linkages introduce diastereoisomers.

Resolution of these forms may be critical for Therapeutic ON developments. These isomeric separations are much more difficult.

FIGURE 2. Length-based oligonucleotide resolution. Oligonucleotides composed of 34, 35, 44, 45, 54 and 55 bases were injected and eluted with a gradient of 350-675 m*M* NaCl over 4 column volumes in Tris buffered eluent at pH 8 and 30 °C. Linear velocity: 1.12 mm/sec.

Top Panel: DNAPac PA200 RS 4.6 × 150 mm

Bottom Panel: Standard DNAPac PA200 4.0 × 250 mm

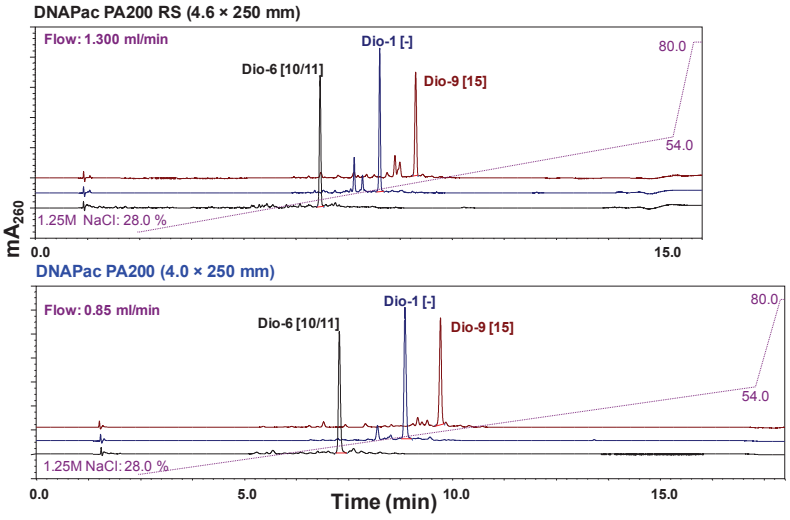


The RS column resolves all pairs in much less time, while also improving detection sensitivity.

FIGURE 3. Linkage isomer separations. Resolution of three 21-base identical sequence ONs with 2´,5´-linkages in different positions. Conditions as in Figure 2, 4.6 × 250 mm RS column pressure was 8800psi.

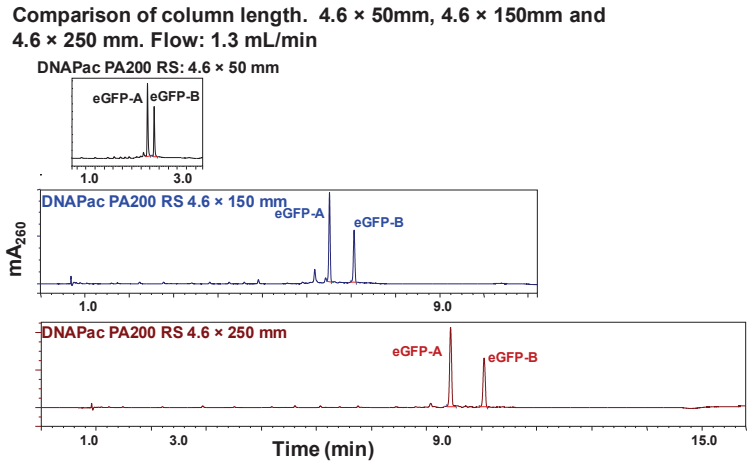
Top Panel: DNAPac PA200 RS 4.6 × 250 mm

Bottom Panel: Standard DNAPac PA200 4.0 × 250 mm



The 4.6 × 250 mm RS column provided better throughput and resolution.

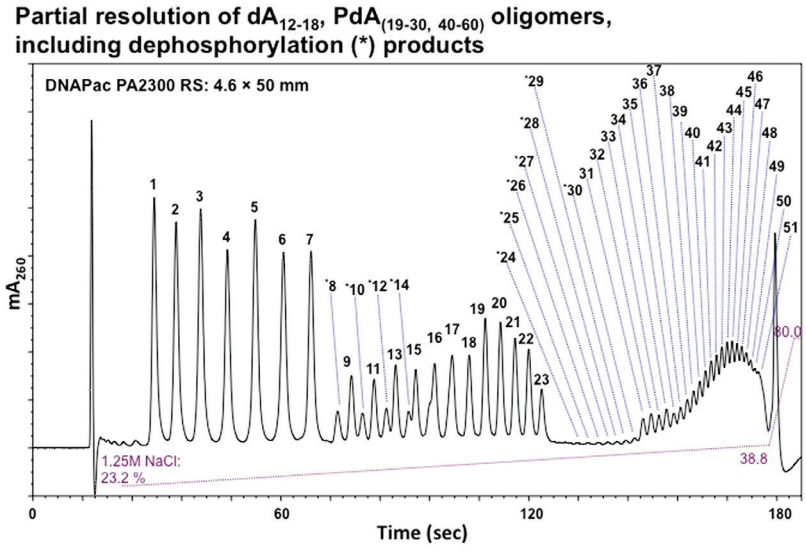
FIGURE 4. Phosphorothioate diastereoisomer separations. The DNAPac PA200 RS resolves a 21-base ON (eGFP-S) on DNAPac PA200 RS in three lengths; 50, 150 and 250 mm. Conditions as in Figure 2.



Resolution of PS diastereoisomers is complete in10.5 min (250 mm), 7.5 min (150 mm) and 2.6 min (50 mm) using the RS column. Resolution on the short column was > 4 in < 3 minutes.

FIGURE 5. Fast ON separations: 51 peaks in < 3 minutes. The 4.6 × 50 mm format demonstrates fast separations, with partial resolution of PdA and dA homopolymers between 12 and 60 bases.

Conditions: DNAPac PA200 RS 4.6 × 50 mm, 290-485mM NaCl in 4 column volumes, 30 °C, pH 8, Flow: 1.3 mL/min.



III. Column ruggedness

Many columns designed for oligonucleotide analysis exhibit degradation within a relatively few sample injections. Since the DNAPac PA200 RS operates at elevated pressures (up to 10,000 psi, 690 bar), we evaluated stability with a column longevity test.

We repeated the PS diastereoisomer separation for 400 cycles. The retention RSDs for isomers “A” and “B” were 0.14% and 0.12%, and the asymmetry RSDs were 0.79% and 0.98% respectively. These values indicate excellent stability, both for the column and the new BioRS biocompatible UHPLC system.

Conclusions

Characteristics of the DNAPac PA200 RS (4 µm):

- 150 mm format: Speed and Resolution > 8 µm DNAPac PA200
- 250 mm format: Speed ≈, Resolution >> 8 µm DNAPac PA200
- 50 mm format: Speed >>, Resolution < 8 µm DNAPac PA200
- All standard DNAPac 200 applications are accelerated
- Difficult isomeric ON separations confirmed and improved
- Reliability and ruggedness are demonstrated

References

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2. Thayer, J.R., S. Rao, N. Puri, C.A. Burnett, and M. Young. 2007. *Identification of aberrant 2´-5´ RNA linkage isomers by pellicular anion exchange chromatography.* Analytical Biochemistry 361: 132-139.
3. Thayer. J.R., N. Puri, C. Burnett, M. Hail and S. Rao. 2010. *Identification of RNA linkage isomers by anion exchange purification with electrospray ionization mass spectrometry of automatically desalted phosphodiesterase-II digests.* Analytical Biochemistry 399: 110-117.
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