

Thermo Scientific
DNAPac Family of Columns



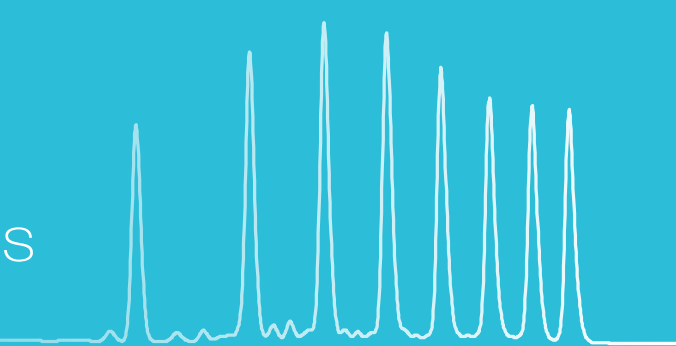
Superior

Oligonucleotide Analysis

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Oligonucleotide Analysis



Nucleic acid analysis is important for characterization of therapeutics, amplification primers or reporters, sequencing libraries and aptamers. Variants can originate from synthetic errors, metabolic processing and chemical degradation. Where oligonucleotide (ON) purity is critical (when used as therapeutics, and in probes to amplify or detect target DNA and RNA by hybridization) chromatographic purity analyses have proved invaluable. In many cases, high resolution HPLC separations are coupled to mass spectrometric analysis to verify the ON structure, and help characterize any impurities.



High Resolution Separations

The Thermo Scientific™ family of columns sets the standard for oligonucleotide purity analysis, fast screening, and purification:

1. **Anion-exchange columns**
 - a. **Thermo Scientific™ DNAPac™ PA200 for ultra-high resolution**
 - b. **Thermo Scientific™ DNASwift™ SAX-1 for mg-scale purification**
2. **Reversed-phase column**
 - a. **DNAPac RP for MS compatibility and dsDNA fragments covering a very wide size range**

The DNAPac PA200 columns support screening of synthetic oligonucleotides for production yield and failure sequences on a routine basis; unit-base resolution of synthetic oligonucleotides has been demonstrated for up to 60 bases and beyond. These columns provide unsurpassed resolution of full length from $n-1$, $n+1$ (and other synthetic failures) not possible with other columns.

The DNASwift column delivers the highest Yield-Purity results for purifications after synthesis. Employing the same selectivity as the DNAPac PA200 columns, the DNASwift is recommended for mg-scale separations of crude mixtures that can be further characterized with the DNAPac PA200 and/or the DNAPac RP products.

The DNAPac RP reversed-phase column is designed for analysis of oligonucleotides and double-stranded (ds) DNA/RNA fragments using LC/UV or LC/MS. The column employs a wide-pore 4 μm polymer resin that provides excellent performance under a broad range of pH, temperature, and mobile phase compositions, and usually employs MS-compatible eluents to streamline LC/MS ON applications. The DNAPac RP column provides excellent separation of long oligonucleotides and large double-stranded nucleic acids.



Properties and Characteristics

	Anion Exchange Columns			Reversed-Phase Column
	DNAPac PA 200	DNAPac PA 200 RS	DNASwift	DNAPac RP
	High resolution anion exchange column	Ultra-high resolution anion exchange UHPLC column	Small scale HPLC purification column	High resolution reversed-phase column
Column Chemistry	55% crosslinked nonporous polymer with quaternary amine functionalized latex MicroBeads		Crosslinked monolithic support with quaternary amine functionalized latex MicroBeads	Hydrophobic wide-pore, polymer resin
Particle Size	8 µm	4 µm	Monolithic support	4 µm
Loading Capacity	~ 14 µeq/mL (anion exchange capacity) ~ 20 µg/mL*	~ 20 µeq/mL (anion exchange capacity) ~ 29 µg/mL*	~ 20 mg/mL* (breakthrough capacity)	~ 29 µg/mL**
pH Range	4–10 (12.5 if [salt] >5× [sodium hydroxide])		6–10 (12.5 if [salt] >5× [sodium hydroxide]) 2–14 for cleaning NOTE: Keep [salt] >5× [sodium hydroxide]	0–14
Pressure Maximum	4,000 psi (276 bar)	10,000 psi	1,500 psi	4,000 psi
Temperature Range	≤35 °C at pH 8.5–12.5 ≤85 °C at pH < 8.5		≤35 °C at pH 8.5–12.5 ≤85 °C at pH < 8.5	<100°C

* Measured using a 20mer oligonucleotide

** Measured using a 25mer oligonucleotide

Applications

Separation	DNAPac Anion Exchange	DNAPac Ion-Pair Reversed Phase
DNAPac Ion-Pair Reversed Phase X failure sequence	Excellent	Excellent
PS to a PO[†]	Excellent	Good
Diastereoisomer separation	Good	Possible
2'-5' linkage isomers	Possible	Possible
Antisense from sense	Good	Good
Double-stranded from single-stranded	Good	Good
ON extraction from tissue	Good	No
Metabolite identification	Best first dimension in 2D LC/MS	Best second dimension in 2D LC/MS

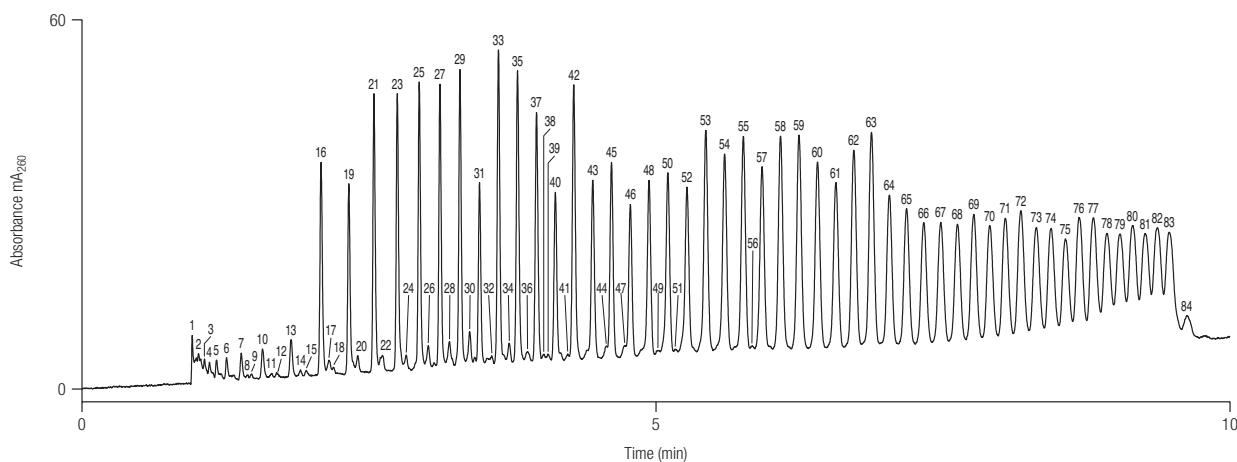
† PO: Phosphodiester; PS: Phosphorothioate

ANION EXCHANGE SEPARATION OF OLIGONUCLEOTIDES

Ultra-high Resolution Separation of Oligonucleotides

The primary separation mechanism with anion ion-exchange chromatography of oligonucleotides is charge, and that is directly related to length. At pH < 8, each nucleotide adds one negative charge to the ON, so each additional nucleotide increases the overall charge on the molecule and consequently increases anion-exchange retention. However, as the eluent pH increase from 8 to ~11.5, the tautomeric oxygen on each G and T (U for RNA) becomes an oxyanion, thereby increasing the charge further, in proportion to the T(U) + G content.

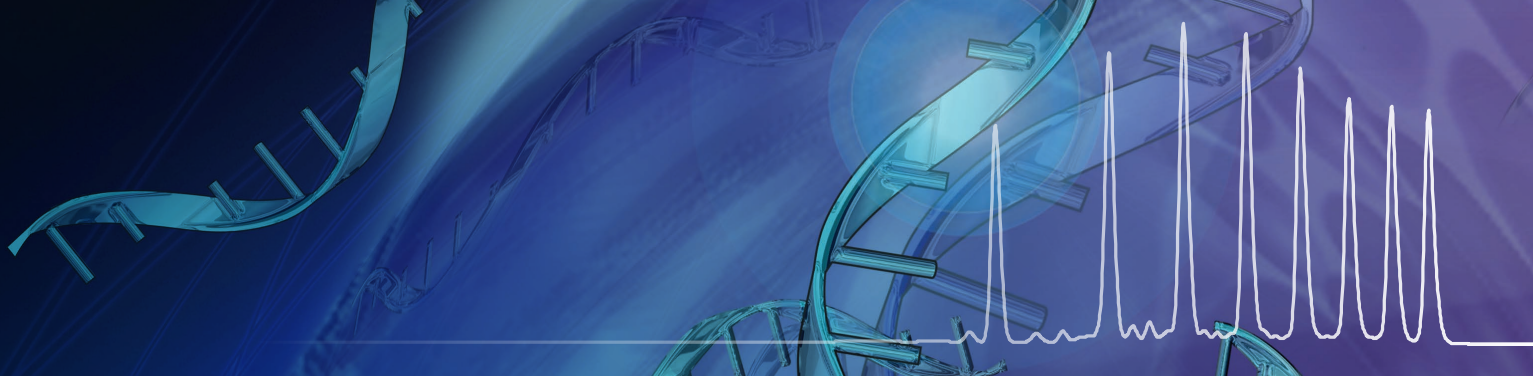
The high resolving power of DNAPac PA200 RS column is demonstrated below by the separation of a poly-dT 12-61mer sample. All 49 full-length synthetic ONs (with eight failure sequences eluting early between 1.0 and 2.0 minutes), are resolved. The chromatogram also reveals separation of 26 ONs that are incompletely deprotected or otherwise modified, and which therefore do not co-elute with, but between, the fully deprotected deoxythymidine ONs in the 8.4 minute gradient employed for this chromatogram.



Column: **DNAPac PA200 RS**, 4 μ m
Format: 4.6 \times 150 mm
Mobile Phase A: 40 mM Tris, pH 8.0
Mobile Phase B: 40 mM Tris, pH 8.0, 1.0 M NaCl
Gradient:

Time (min)	%A	%B
-10.0	59	41
0.0	59	41
8.4	35	65
8.5	20	80

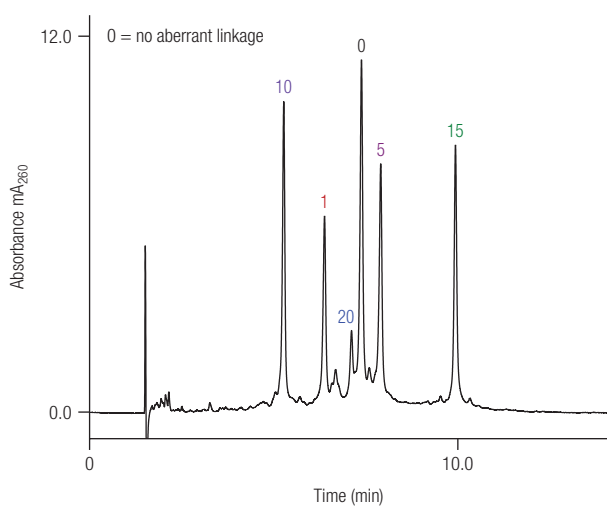
Gradient Curve: 3
Flow Rate: 1.0 mL/min
Inj. Volume: 15 μ L
Temp.: 30 $^{\circ}$ C
Detection: UV (260 nm)
Sample: poly dT12-60 (0.4 A/mL)



Aberrant RNA Linkage Isomer Separations

Processing and regulation of metabolic pathways by duplexed micro RNAs (miRNA) induced widespread interest in RNA interference (RNAi) as a potential therapeutic model. Unlike DNA, RNA harbors 2'-hydroxyl residues that render RNA significantly more reactive. Under conditions used for annealing of complementary synthetic RNA (after release and purification), RNA can experience phosphoryl-migration, producing 2', 5'-linkages or strand scission. The 2', 5'-linkages can inhibit nuclease and polymerase activities, so they may contribute to 'off-target' effects.

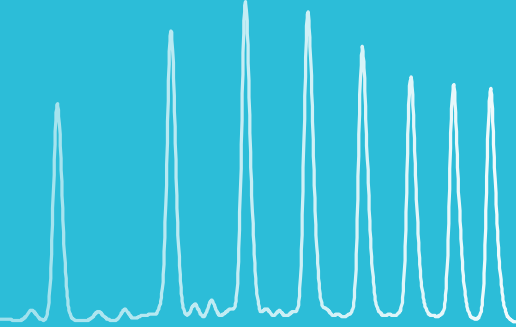
RNAs with these modified linkages have identical masses and sequence as the unmodified 3',5'-linked RNAs, therefore cannot be distinguished by direct mass spectrometry. High resolution DNAPac PA200 RS column separations can resolve many of these aberrant RNA linkage isomers. In the figure below, a mixture of ONs with aberrant linkages at different positions is separated from the RNA that does not contain an aberrant linkage.



Column: **DNAPac PA200 RS**, 4 μm
 Format: 4.6 × 250 mm
 Mobile Phase A: 40 mM AMP[†], pH 9.5
 Mobile Phase B: 40 mM AMP[†], pH 9.5, 1.25 M NaCl
 Gradient:

Time (min)	%A	%B
-10.0	51	49
0.0	51	49
19.5	30	70
20.5	20	80

Flow Rate: 1.0 mL/min
 Inj. Volume: 8 μL
 Temp.: 30 °C
 Detection: UV (260 nm)
 Sample: 21mer RNA with aberrant 2',5'-linkages at positions indicated in the figure (50 μM of each oligonucleotide)
 † AMP: 2-amino-2-methyl-1-propanol



Analysis of siRNA Duplex at Different Temperatures

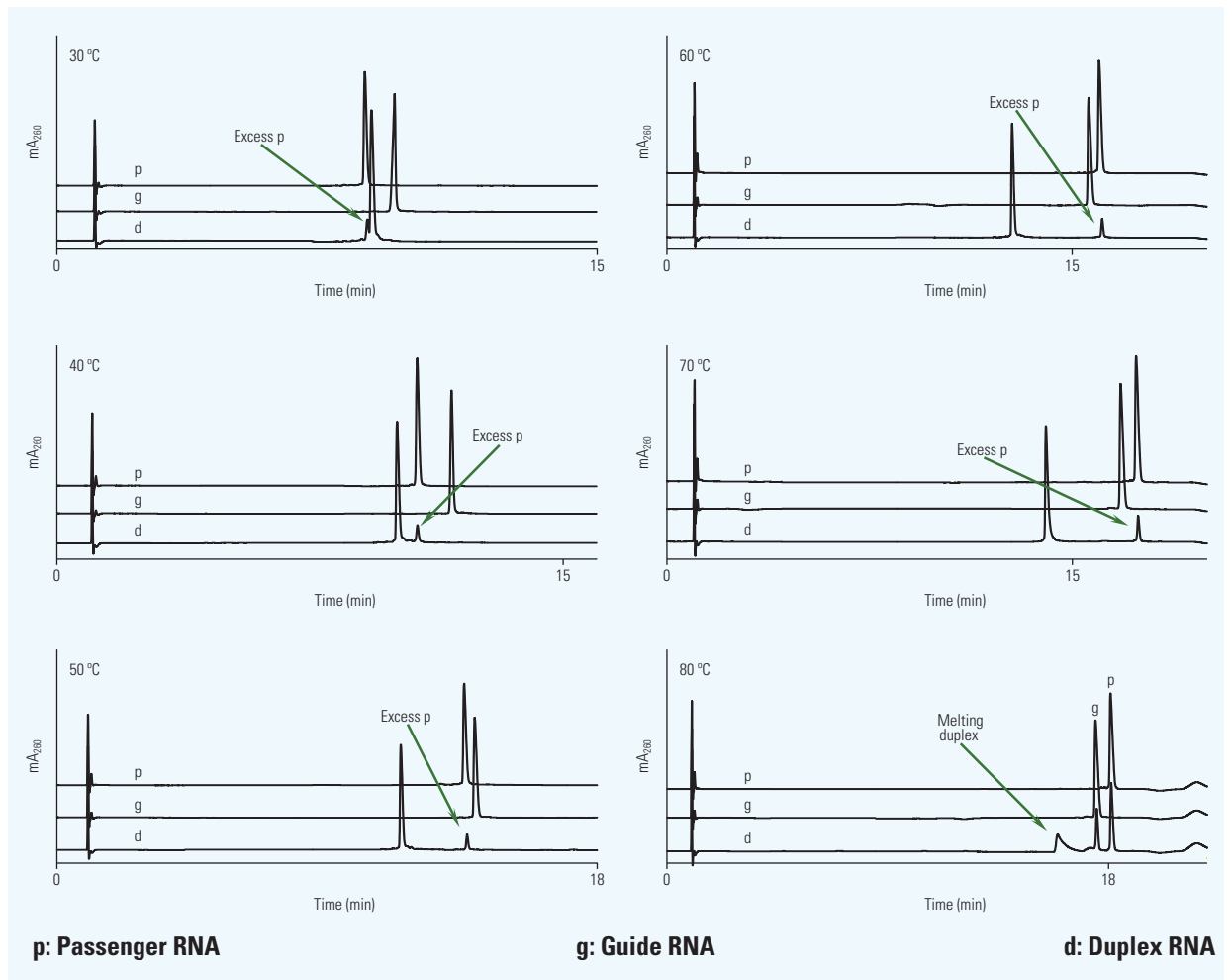
Short interfering RNA (siRNA, short double-stranded RNA molecules) can specifically accelerate degradation of target gene transcripts. These are considered therapeutic candidates and are prepared by annealing synthesized guide strand and passenger strand. Analysis of siRNAs at different temperatures provides information on impurities, stoichiometry and the melting temperature of the siRNA duplex. The DNAPac PA200 column can resolve the guide and passenger strands from the duplex product, and the ss- vs ds- selectivity can be modified with temperature alterations, as shown in the figure below.

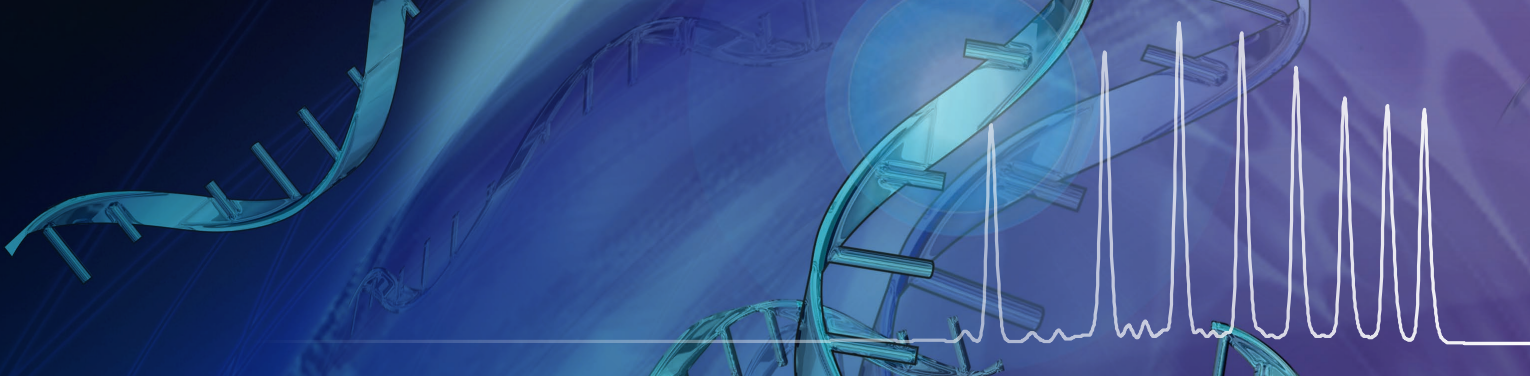
Column: **DNAPac PA200**, 8 μ m
 Format: 2 \times 250 mm
 Mobile Phase A: Water
 Mobile Phase B: 0.4 M Tris, 1.25 M NaCl, pH 7.0
 Mobile Phase C: 0.4 M Tris pH 7.0
 Gradient:

Time	%A	%B	%C
0.0	69	26	5
17.2	39	56	5
17.7	25	70	5
18.7	25	70	5
19.2	69	26	5
23.7	69	26	5

Flow Rate: 0.3 mL/min
 Inj. Volume: 14 μ L
 Detection: UV (260 nm)
 Sample: Guide: AGCUGACCCUGAAGUJUCAIdCdT
 Passenger: AUGAACUUCAGGGUCAGCUdTG

Taken from Chromatography Today, 4 **2011**, 28-32.
 Articles and News on chromatography techniques, applications and products
 – see more at: <https://www.chromatographytoday.com>



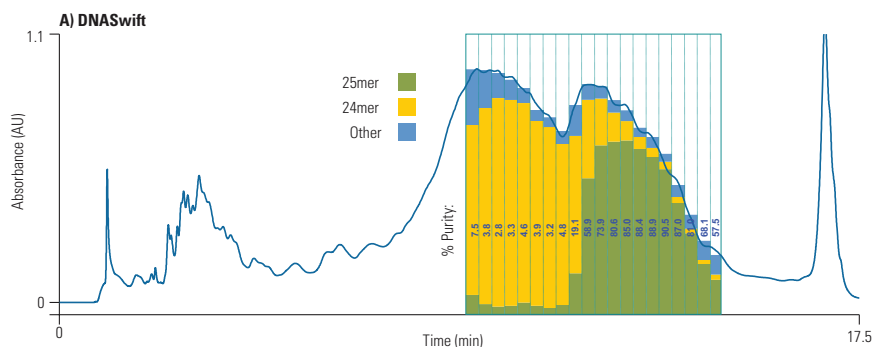


Sample Purification and Desalting

The DNASwift column is specifically designed for mg scale ON purification. Below figure compares the purification of a mixture of full length and n-1 DNA sample on a DNASwift column (Panel A) and a bead based Mono Q™ column (Panel B). 8.25 mg of oligonucleotide sample was loaded to each column and the fraction was set to start approximately half way through the gradient.

Each collected fraction was evaluated on a DNAPac PA200 column for purity and yield. This allows calculation of yield and purity for every combination of fractions from these two columns. Panel C, a plot of the Yield vs Purity curves, reveals the DNASwift to deliver higher yields at all purity levels, and better purity at all yield levels.

For other examples, see *J. Chromatogr. B* 878 **2010** 933–941, J.R. Thayer et al.

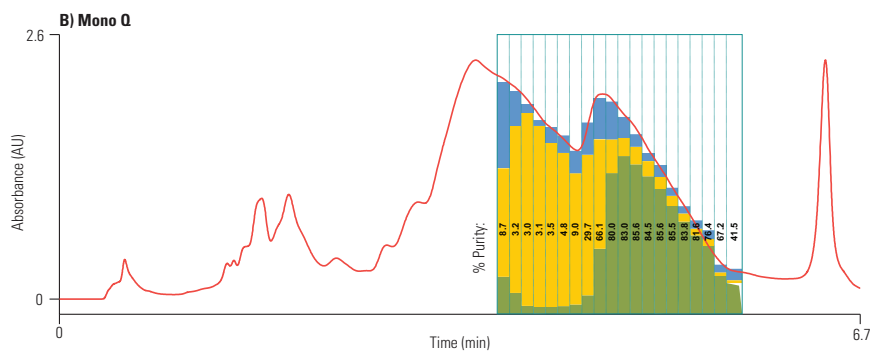


Column: **DNASwift**
 Format: 5 × 150 mm
 Mobile Phase A: Water
 Mobile Phase B: 0.2 M NaOH
 Mobile Phase C: 0.2 M Tris, 0.2 M AMP¹, 0.2 M Diisopropylamine, pH 7.2
 Mobile Phase D: 1.25 M NaCl
 Gradient:

Time (min)	%A	%B	%C	%D
0.00	73.6	12.1	7.9	6.4
0.01	73.6	12.1	7.9	6.4
1.00	32.0	12.1	7.9	48.0
15.01	16.0	12.1	7.9	64.0
15.51	0.0	12.1	7.9	80.0
16.50	0.0	12.1	7.9	80.0
17.00	73.6	12.1	7.9	6.4

Flow Rate: 1.77 mL/min
 Inj. Volume: 1 mL
 Temp.: 30 °C
 Detection: UV (295 nm)
 Sample: Mixture of full length DNA (45%) and n-1 DNA (55%)
 Full length: CTGATTGTAGGTTCTCTAACGCTGG
 n-1: CTGATTGTAGGTTCTCTAACGCTG

¹ AMP: 2-amino-2-methyl-1-propanol

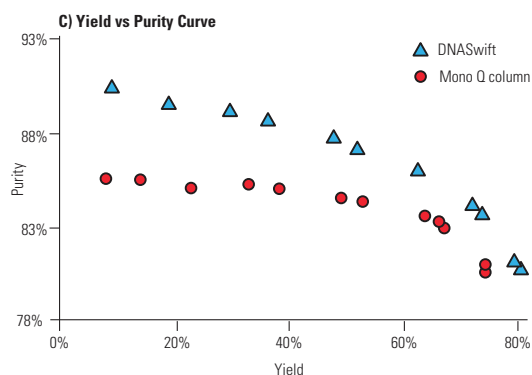


Column: **Mono Q**
 Format: 5 × 50 mm
 Mobile Phase A: Water
 Mobile Phase B: 0.2 M NaOH
 Mobile Phase C: 0.2 M Tris, 0.2 M AMP¹, 0.2 M Diisopropylamine, pH 7.2
 Mobile Phase D: 1.25 M NaCl
 Gradient:

Time (min)	%A	%B	%C	%D
0.00	74.5	12.1	7.9	5.5
0.01	74.5	12.1	7.9	5.5
0.39	38.7	12.1	7.9	41.3
5.95	25.0	12.1	7.9	55.0
6.15	0.0	12.1	7.9	80.0
6.55	0.0	12.1	7.9	80.0
6.74	74.5	12.1	7.9	5.5

Flow Rate: 1.77 mL/min
 Inj. Volume: 1 mL
 Temp.: 30 °C
 Detection: UV (295 nm)
 Sample: Mixture of full length DNA (45%) and n-1 DNA (55%)
 Full length: CTGATTGTAGGTTCTCTAACGCTGG
 n-1: CTGATTGTAGGTTCTCTAACGCTG

¹ AMP: 2-amino-2-methyl-1-propanol

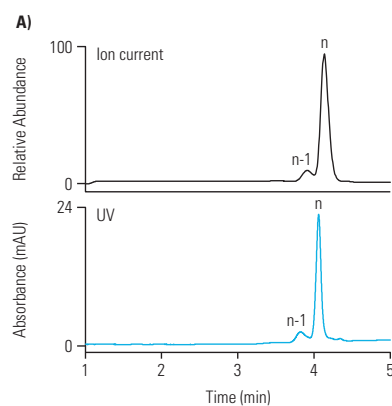


ION-PAIR REVERSED-PHASE SEPARATION OF OLIGONUCLEOTIDES

LC/MS Analysis of Failure Sequences

Ion-pair reversed-phase chromatography with volatile or MS-compatible mobile phases, can be coupled to MS systems and simplify positive identification of oligonucleotide products as well as the impurities that may be present. Below is the separation of a 21mer DNA and its n-1 failure sequence using a short 3 minute gradient using the DNAPac RP column (Panel A). Data from this same separation obtained with a high resolution Thermo Scientific™

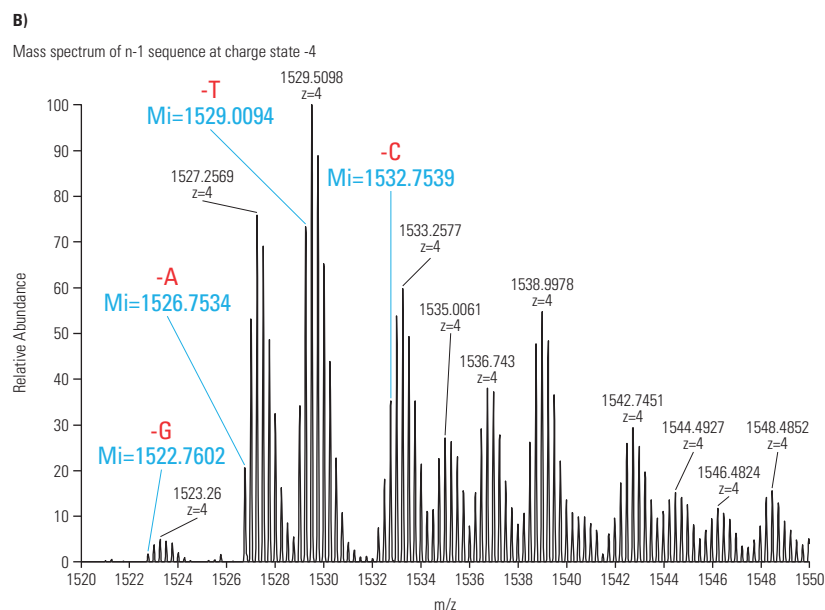
Q Exactive™ Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer revealed loss of each of the four bases in the n-1 peak as follows (Panel B). In this spectrum, mass envelopes corresponding to loss of G (m/z 1522.7602), loss of A (1526.7534), loss of T (1529.0094) and loss of C (1532.7539) all appear in the peak labeled "n-1" in the base peak chromatogram in figure "A". Hence, this peak includes synthetic failures for each of the four bases.

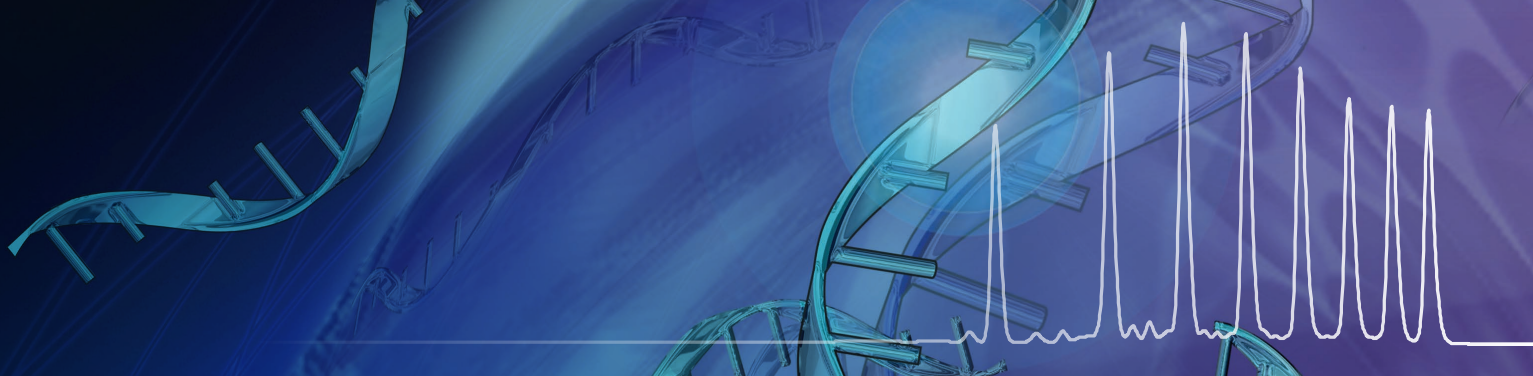


Column: **DNAPac RP**, 4 μ m
 Format: 2.1 \times 50 mm
 Mobile Phase A: 15 mM TEA, 400 mM HFIP, pH 7.9
 Mobile Phase B: 15 mM TEA, 400 mM HFIP in water / methanol (50:50 v/v)
 Gradient:

Time (min)	%A	%B
0.0	70	30
3.0	48	53
3.1	10	90
5.0	10	90
5.1	70	30
11.0	70	30

Flow Rate: 0.25 mL/min
 Inj. Volume: 4 μ L
 Temp.: 60 $^{\circ}$ C
 Detection: **UV (260 nm)**
 MS (Negative-ion mode)
 Q Exactive Plus
 Sample: 21mer DNA GATTGTAGGTTCTCTAACGCT

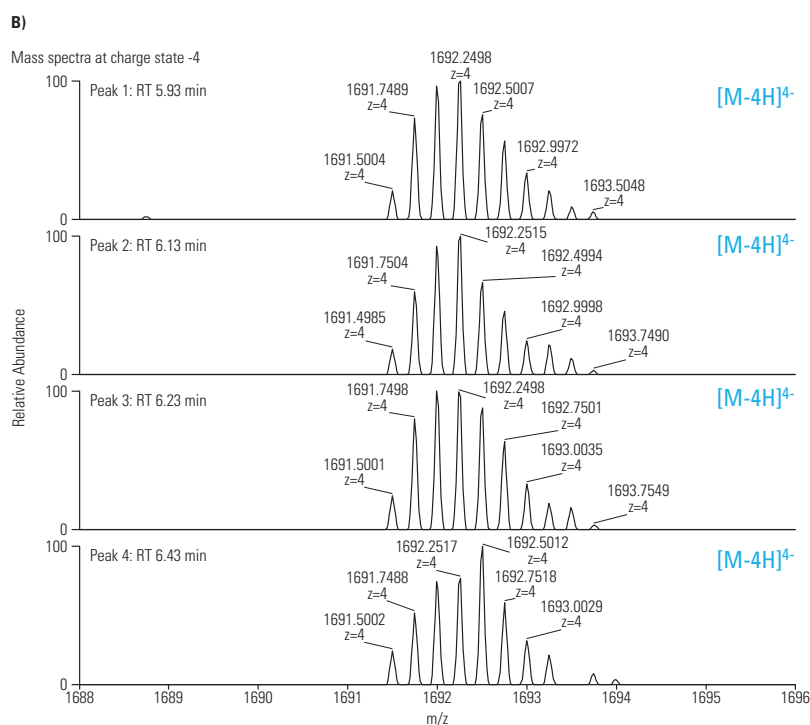
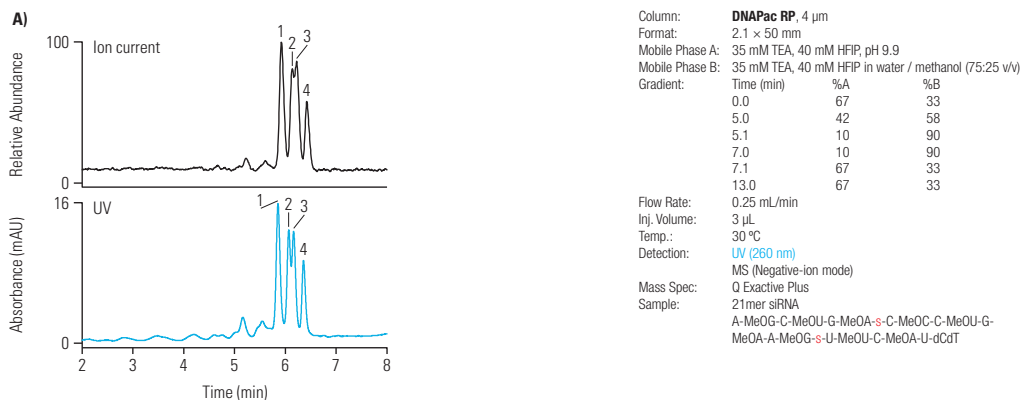




LC/MS Analysis of Phosphorothioate on 2'-O-Methyl Modified siRNAs

Therapeutic and diagnostic nucleic acids are often modified to increase in vivo stability. Ribose 2'-O-methylation is often applied to siRNAs. Another common modification in both DNA and RNA is incorporation of phosphorothioate (PS) linkages. The PS linkage introduces a chiral center at phosphorus. In addition to the other chiral centers in D-ribose, the PS linkage creates diastereoisomer pairs in

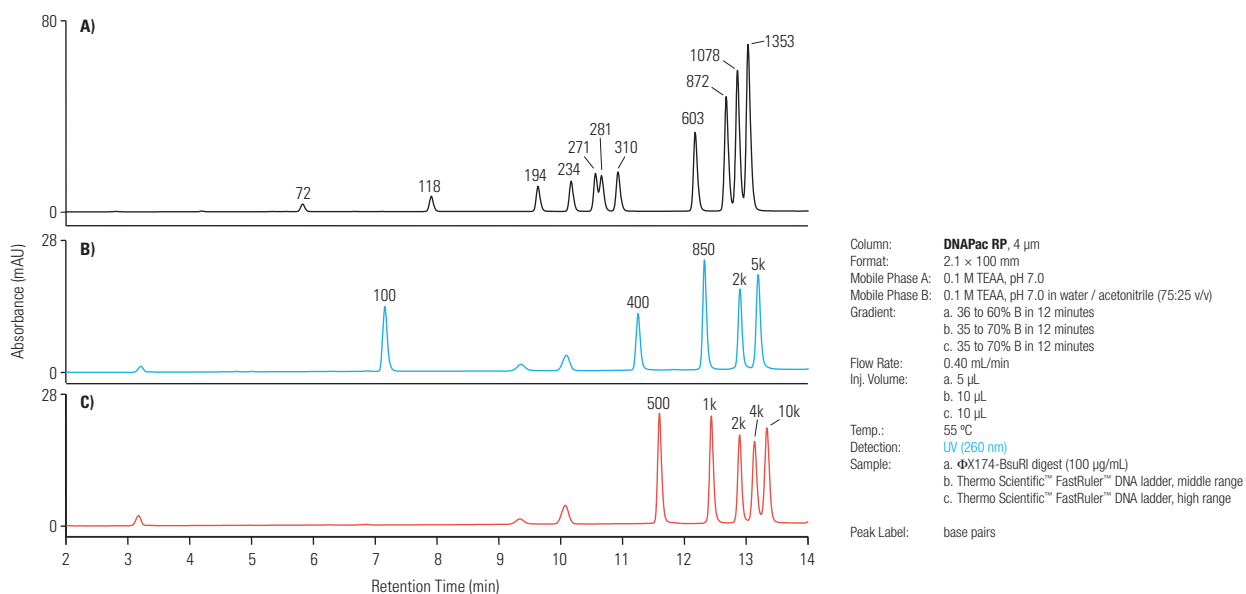
the nucleic acids. The chromatogram below shows separation of a phosphorothioate diastereoisomers in a 2'-O-methyl modified siRNA sense strand. All four phosphorothioate diastereoisomers are observed using a high pH eluent system. High resolution MS data revealed masses within 2 ppm of calculated value for all four peaks confirming these molecules to be diastereoisomers, as shown below.



Separation of Large Double-stranded DNA Fragments

Purification of large double-stranded DNA fragments is required for DNA cloning and PCR product collection. Traditionally, these dsDNA fragments are purified by agarose gel electrophoresis, and that requires manual excision of the target DNA band from the gel followed by extraction of the product. This laborious, time consuming step generally produces relatively low (50–70%) yield. HPLC delivers more reliable, higher yields and is readily automated. The wide-pore resin in the DNAPac RP column can deliver resolution comparable to gels in similar times, and without the added excision and extraction steps.

The figure below depicts the resolution of DNA fragments generated from restriction enzyme activity on a viral DNA and DNA ladders. The figure shows separation of fragments ranging from 72 bp to 10k bp on the DNAPac RP 2.1 × 100 mm column in 15 minutes.



ORDERING INFORMATION

DNAPac PA200 Column

Particle Size (µm)	Format	Length (mm)	2.0 mm ID	4.0 mm ID	9.0 mm ID	22.0 mm ID
8	Standard Analytical Column	250	063425	063000	063421	088781
5	Guard Column	50	063423	062998	063419	088780

DNAPac PA200 RS, High Resolution Analytical Column

Particle Size (µm)	Format	Length (mm)	4.6 mm ID
4	UHPLC Column	50	082508
		150	082509
		250	082510

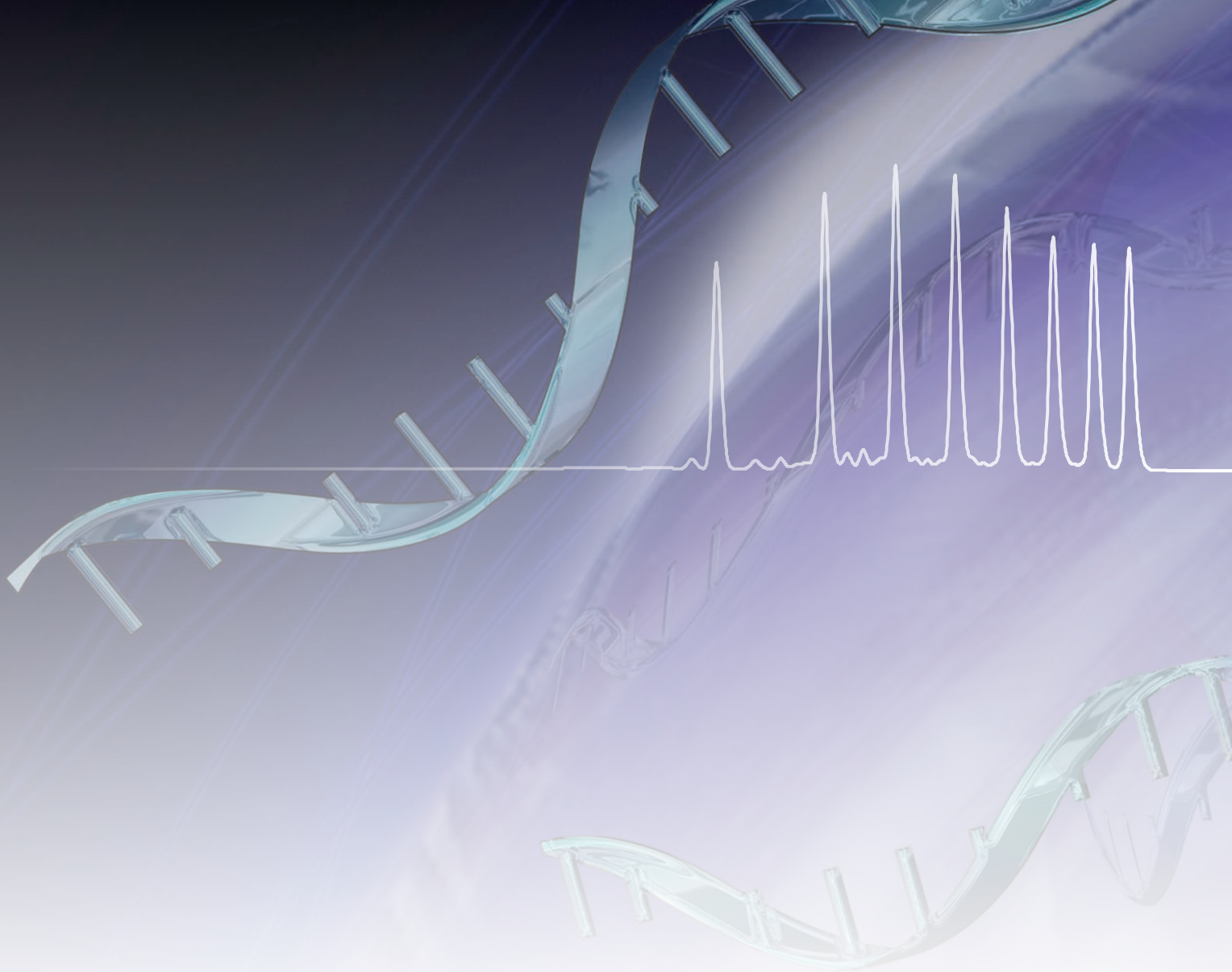
DNAPac RP Column

Particle Size (µm)	Format	Length (mm)	2.1 mm ID	3.0 mm ID
4	Analytical Column	100	088923	088919
		50	088924	088920
		Guard Cartridges (2/pkg)	10	088925
Guard Cartridge Holder			069580	069580

DNASwift SAX-1S Column

Particle Size (µm)	Format	Length (mm)	5.0 mm ID
Monolith	HPLC Column	150	066766





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