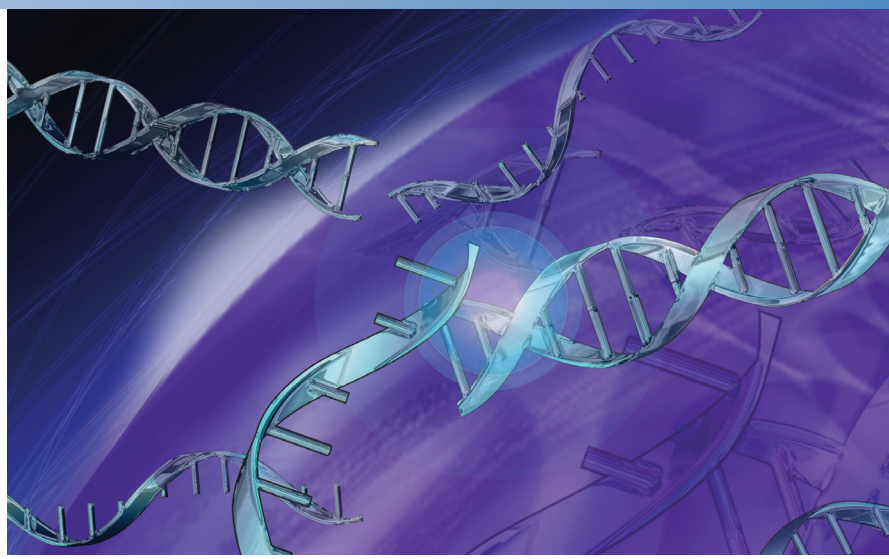
DNAPac RP Column

High Performance Reversed-Phase Chromatography Column for Oligonucleotides and Large Double-Stranded DNA/RNA Analysis

The Thermo Scientific™ DNAPac™ RP is a reversed-phase (RP) liquid chromatography column designed for analysis of oligonucleotides and double-stranded (ds) DNA/RNA fragments using LC/UV or LC/MS. The unique column chemistry provides excellent performance under a broad range of pH, temperature, and mobile phase compositions. In addition, the wide pore size of the resin provides excellent separation of large double-stranded nucleic acids up to 10k base pairs.

Product Highlights

- Designed for ion-pair reversed-phase (IP-RP) separations of both oligonucleotides and double-stranded nucleic acids
- High resolution and high throughput
- Excellent MS compatibility
- Wide operating pH range (0–14) and high temperature stability (up to 100 ºC)



Introduction

Synthetic oligonucleotides are widely used for various applications including DNA amplification and sequencing, in situ hybridization, gene silencing and molecular diagnostics. They have also emerged as promising drug candidates for diseases such as cancer, viral infections, Alzheimer's disease and cardiovascular disorders. Many antisense oligonucleotides, aptamers and small-interfering RNAs (siRNAs) are currently being evaluated in clinical trials. Oligonucleotides used for molecular biology applications and as therapeutic agents require high purity. Therefore quality control of synthetic oligonucleotides is important, and stable, reliable means to accomplish such evaluations are necessary.

High performance liquid chromatography (HPLC) and mass spectrometry (MS) comprise valuable tools for the purity assessment and identification of impurities in oligonucleotide samples. Anion-exchange chromatography and ion-pair reversed phase chromatography (IP-RP) are the most commonly used techniques for LC/UV or LC/fluorescence analysis of oligonucleotides.

Anion exchange (AEX) chromatography provides high resolution separation of oligonucleotides and requires no organic solvent. Although desalting methods may be coupled to AEX LC of nucleic acids, the high salt concentration used in their mobile phases, preclude direct coupling of AEX LC approaches to MS.

IP-RP chromatography with volatile mobile phase components, is compatible with direct LC/MS analysis, and can provide exact mass data for positive identification of oligonucleotide products as well as the impurities that may be problematic for critical applications.

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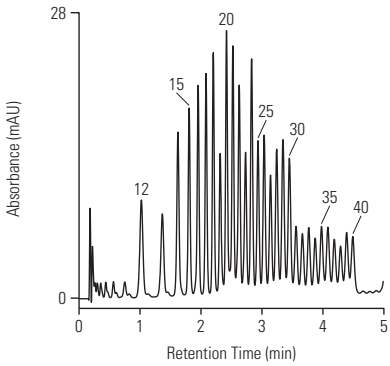
Column Technology

The DNAPac RP column is based on spherical wide-pore 4 µm polymer resin. The phase is highly stable to extremes of pH (0–14), and temperature (5–100 °C), and is fully compatible with mass spectrometry friendly organic solvents and ion-pair reagents such as triethylamine (TEA) and hexylamine (HA). The column may be operated in both HPLC and UHPLC instruments, producing high resolution at high throughput for oligonucleotides. The wide pore size of polymer resin enables efficient separation of large nucleic acids with low carry-over.

Applications

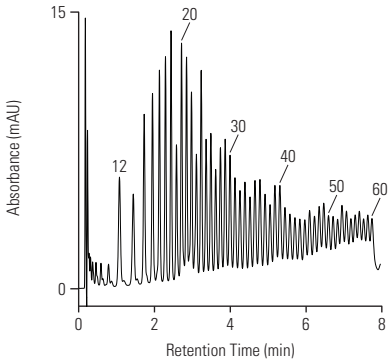
High Resolution and Fast Separation of Poly-Deoxythymidine

The high resolving power of DNAPac RP column is demonstrated by the separation of poly-deoxythymidine oligonucleotides as shown in Figure 1 and 2. Poly-dT 12–40mer is separated at high temperature and high flow rate with a 4 minute gradient using triethylammonium acetate (TEAA) as mobile phase. In addition, up to 60mer poly-dT oligonucleotide can be separated using a 7 minute gradient. The Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System and the Thermo Scientific™ Vanquish™ pumps can generate curved gradients that allow improved resolution of longer oligonucleotides, in shorter time.



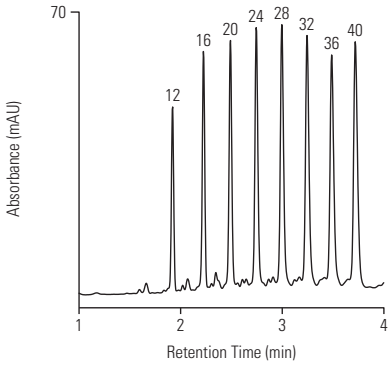
Column:	**DNAPac RP**, 4 µm		
Format:	2.1 × 50 mm		
Mobile Phase A:	0.1 M TEAA, pH 7.0		
Mobile Phase B:	0.1 M TEAA in water / acetonitrile (75:25 v/v)		
Gradient:	Time (min)	%A	%B
	-2.0	76	24
	0.0	76	24
	4.0	59	41
	4.1	10	90
	5.0	10	90
Gradient Curve:	3		
Flow Rate:	0.80 mL/min		
Inj. Volume:	4 µL		
Temp.:	80 °C		
Detection:	UV (260 nm)		
Sample:	poly dT12-40 (1 A/mL)		
Peak Label:	Length of DNA		

Figure 1. Fast separation of 12-40mer deoxythymidines (dTs).



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	76	24
	0.0	76	24
	7.0	57	43
	7.1	10	90
	5.0	10	90
Gradient Curve:	3		
Flow Rate:	0.80 mL/min		
Inj. Volume:	10 µL		
Temp.:	80 °C		
Detection:	UV (260 nm)		
Sample:	poly dT12-60 (0.5 A/mL)		
Peak Label:	Length of DNA		

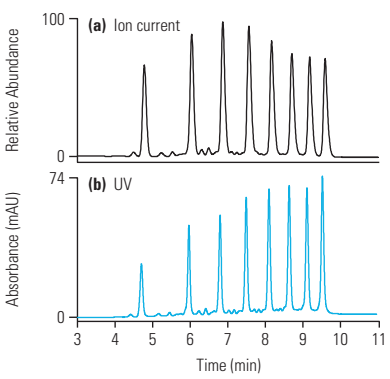
Figure 2. High resolution separation of 12-60mer dTs.



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:	0.80 mL/min		
Inj. Volume:	2 µL		
Temp.:	80 °C		
Detection:	UV (260 nm)		
Sample:	8-Combo DNA (5 µM)*		
Peak Label:	Length of DNA		

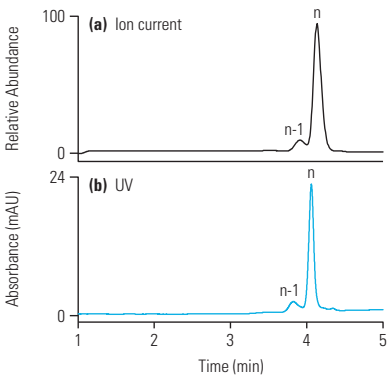
*Sequence of 8-Combo DNA
GACTGACTGACT - **12**
GACTGACTGACTGACT - **16**
GACTGACTGACTGACTGACT - **20**
GACTGACTGACTGACTGACTGACT - **24**
GACTGACTGACTGACTGACTGACTGACT -**28**
GACTGACTGACTGACTGACTGACTGACTGACT -**32**
GACTGACTGACTGACTGACTGACTGACTGACTGACT -**36**
GACTGACTGACTGACTGACTGACTGACTGACTGACTGACT -**40**

Figure 3. LC/UV analysis of mixed-base DNA.

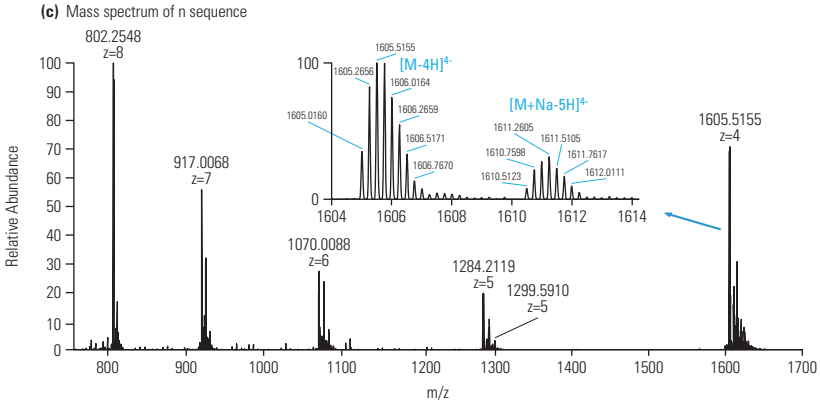


Column:	**DNAPac RP**, 4 µm		
Format:	2.1 × 50 mm		
Mobile Phase A:	15 mM TEA, 400 mM HFIP, pH 7.9		
Mobile Phase B:	15 mM TEA, 400 mM HFIP in water / methanol (50:50 v/v)		
Gradient:	Time (min)	%A	%B
	0.0	85	15
	8.0	51	49
	8.1	10	90
	10.0	10	90
	10.1	85	15
	16.0	85	15
Gradient curve:	3		
Flow Rate:	0.25		
Inj. Volume:	2 µL		
Temp.:	60 °C		
Detection:	UV (260 nm)		
	MS (Negative-ion mode)		
Mass Spec:	Q Exactive Plus		
Sample:	8-Combo DNA (5 µM)*		

Figure 4. LC/MS analysis of mixed-base DNA.



Column:	**DNAPac RP**, 4 µm		
Format:	2.1 × 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 µL		
Temp.:	60 °C		
Detection:	UV (260 nm)		
	MS (Negative-ion mode)		
Mass Spec:	Q Exactive Plus		
Sample:	21mer DNA GATTGTAGGTTCTCTAACGCT		



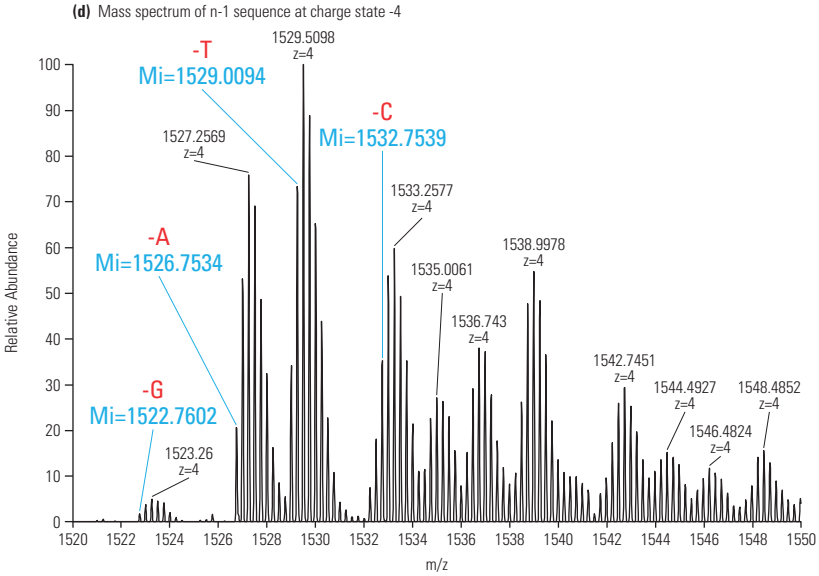


Figure 5. LC/MS analysis of n and n-1 sequences.

LC/UV and LC/MS analysis of mixed-base DNAs

Synthetic oligonucleotide molecules are used as PCR primers, aptamers, library adaptors for genomic studies and therapeutic agents. High purity oligonucleotides in these applications are required. Similar to anion exchange chromatography, ion-pair reversed phase chromatography provides fast, high resolution separation of full-length oligonucleotides from their failure sequences and other impurities. High resolution mass spectrometers such as the Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer provide accurate mass information of nucleic acids ensuring high confidence in nucleic acid analyses.

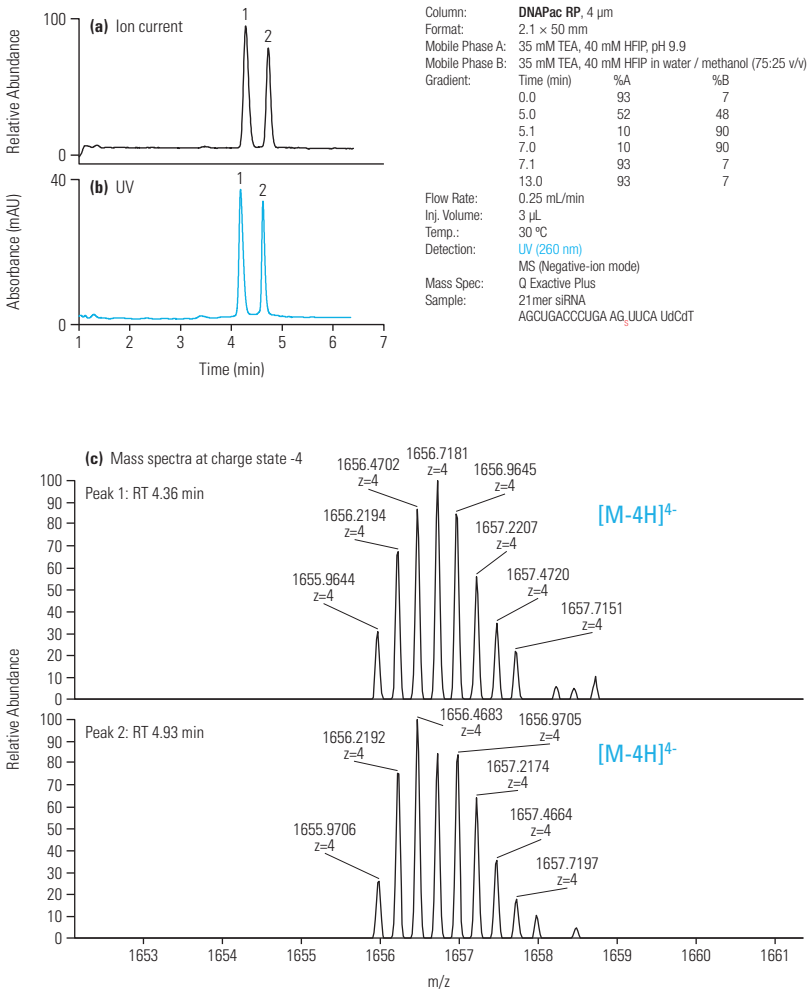
Separation of mixed-base DNA using UV detection and TEAA mobile phase at high flow rate is shown as Figure 3. The same sample separated using mass spectrometer compatible mobile phase at lower flow rate is shown in Figure 4. Accurate masses of these eight mixed-base DNA molecules were measured.

In Figure 5, a 21mer mixed-base DNA and its n-1 failure sequence is separated. The MS data confirmed that the main peak is the desired product. At charge state -4, monoisotopic m/z for the 21 mer DNA is at 1605.0160 yielding a mass accuracy of 1.87 ppm (Figure 5c). The base composition of the n-1 peak was identified by MS (Figure 5d). The masses of failure sequences with missing guanine or adenine or thymine or cytosine were detected. This data demonstrates the use of the DNAPac RP column and the Q Exactive Mass Spectrometer for the separation and identification of the product oligonucleotide and failure sequences.

LC/MS analysis of siRNA molecules

Small interfering RNAs (siRNAs) are dsRNA molecules that specifically target messenger RNA (mRNA) for degradation resulting in suppression of transcript expression. Synthetic siRNAs have become important tools to study gene function and as potential therapeutic agents for various diseases. The backbone of single-stranded nucleic acids is often modified to increase in vivo stability. A common modification in DNA and RNA is incorporation of phosphorothioate (PS) linkages. Similarly an RNA-specific modification is 2'-O-methylation on ribose. The PS linkage introduces a chiral center at phosphorus in addition to the chiral centers in D-ribose of the nucleic acid. Therefore PS modified linkages produce diastereoisomer pairs at each PS linkage. Using a high pH mobile phase, the DNAPac RP column separated the Rp and Rs phosphorothioate diastereoisomers. Since the DNAPac RP column is directly compatible with MS, LC/MS analysis reveals identical high resolution masses for the resolved isomers. Sense siRNA strands harboring this linkage were evaluated in Figures 6, 7 and 8.

Figure 6 shows the separation of a sense strand that has one PS linkage incorporated at the 14th base. The two possible diastereoisomers were baseline separated on the DNAPac RP column. Both peaks exhibit identical high-resolution mass, indicating these molecules to be diastereoisomers rather than failure sequences or other impurities.

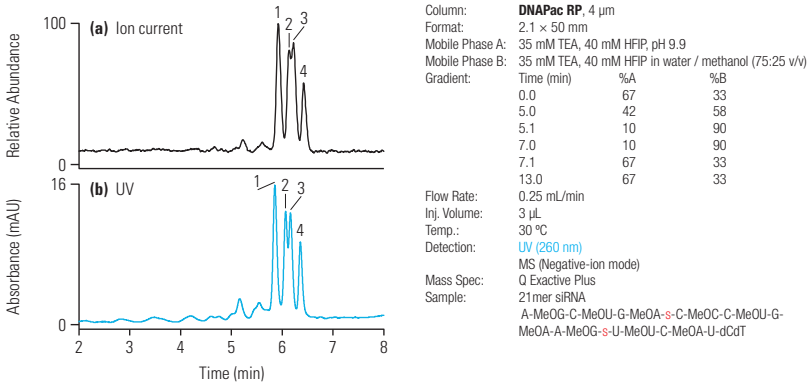


Column: **DNAPac RP**, 4 µm
Format: 2.1 × 50 mm
Mobile Phase A: 35 mM TEA, 40 mM HFIP, pH 9.9
Mobile Phase B: 35 mM TEA, 40 mM HFIP in water / methanol (75:25 v/v)

Gradient:	Time (min)	%A	%B
	0.0	93	7
	5.0	52	48
	5.1	10	90
	7.0	10	90
	7.1	93	7
	13.0	93	7

Flow Rate: 0.25 mL/min
Inj. Volume: 3 µL
Temp.: 30 °C
Detection: UV (260 nm)
MS (Negative-ion mode)
Mass Spec: Q Exactive Plus
Sample: 21mer siRNA
AGCUGACCCUGA AG$_s$UUCA UdCdT

Figure 6. LC/MS analysis of phosphorothioate modified siRNA.



Column: **DNAPac RP**, 4 µm
Format: 2.1 × 50 mm
Mobile Phase A: 35 mM TEA, 40 mM HFIP, pH 9.9
Mobile Phase B: 35 mM TEA, 40 mM HFIP in water / methanol (75:25 v/v)

Gradient:	Time (min)	%A	%B
	0.0	67	33
	5.0	42	58
	5.1	10	90
	7.0	10	90
	7.1	67	33
	13.0	67	33

Flow Rate: 0.25 mL/min
Inj. Volume: 3 µL
Temp.: 30 °C
Detection: UV (260 nm)
MS (Negative-ion mode)
Mass Spec: Q Exactive Plus
Sample: 21mer siRNA
A-MeOG-C-MeOU-G-MeOA-s-C-MeOC-C-MeOU-G-MeOA-A-MeOG-s-U-MeOU-C-MeOA-U-dCdT

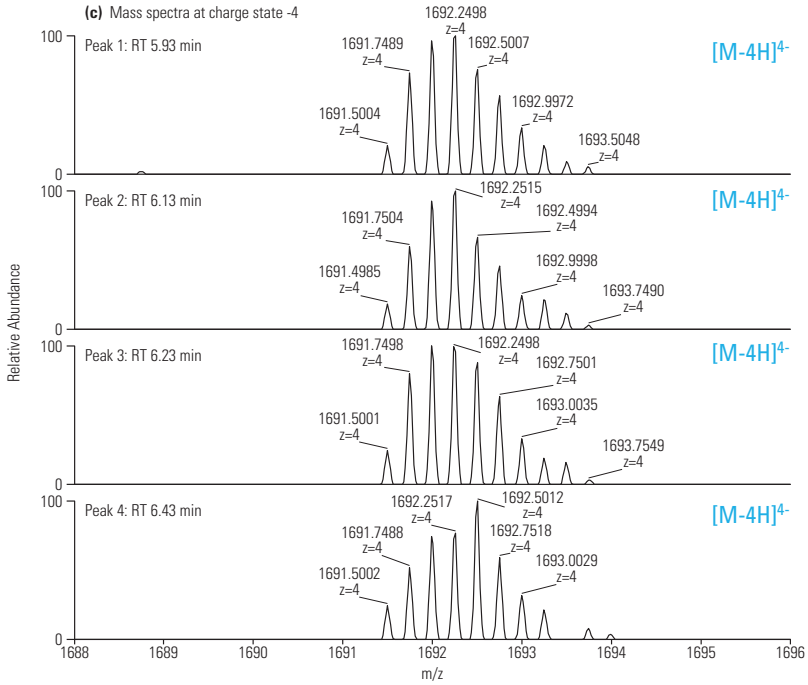


Figure 7. LC/MS analysis of phosphorothioate and 2'-O-Methyl modified siRNA.

In Figure 7, sense strand of the siRNA was 2'-O-methylated on alternate bases and contains PS linkages at the 6th and 14th bases. The UV trace and the ion current traces show partial separation of all four possible PS diastereoisomers (Figure 7b). The high resolution MS data again reveal identical masses for all four peaks confirming these molecules to be diastereoisomers (Figure 7c).

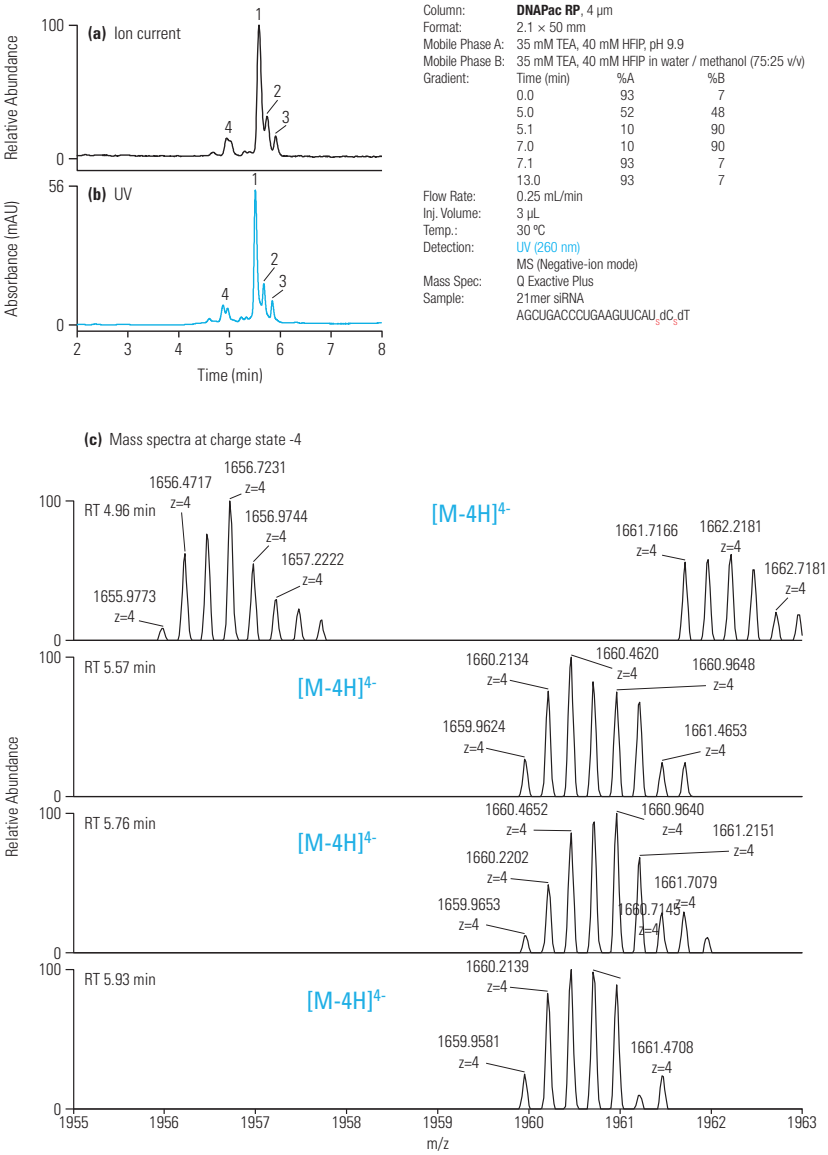


Figure 8. LC/MS analysis of phosphorothioate modified siRNA.

In Figure 8, the sense strand is modified with two PS linkages at the 19th and the 20th bases. In this case, three of the four possible diastereoisomers were chromatographically resolved (Figure 8b). In addition to the diastereoisomer peaks, an impurity which contains a single PS linkage (PO) was detected. The mass difference between peak 4 and all three of the other peaks is 16 Da, corresponding to the mass difference between the oxygen and sulfur (Figure 8c).

LC/MS analysis of CpG methylation and 5'-phosphorylation

Many housekeeping and other frequently expressed genes contain a CpG dinucleotide islands in their promoter regions. These are methylated at the C-5 carbon of cytosine by methyltransferases. Methylation of CpG sequences in the promoter regions suppresses the expression of the gene and aberrant methylation has been implicated in the development and progression of cancer. Therefore, detection of CpG methylation is important for epigenetics studies and cancer research. LC/MS analysis of CpG methylation can be performed using the DNAPac RP column and high resolution mass spectrometer. In Figure 9, an unmodified oligonucleotide and a CpG methylated oligonucleotide of identical sequence are well resolved on the DNAPac RP column. Figure 9c shows the -3 charge state of unmodified CpG oligonucleotide at m/z 1517.92 and the -3 charge state of methylated CpG oligonucleotide at m/z 1522.59. The delta mass between methylated peak and unmodified peak corresponds to one methyl group mass. In addition to methylation, DNAPac RP column can effectively resolve difference in one phosphate group on the 5' end of the oligonucleotide (Figure 10). The MS result confirms the phosphate group difference in the two peaks (Figure 10c).

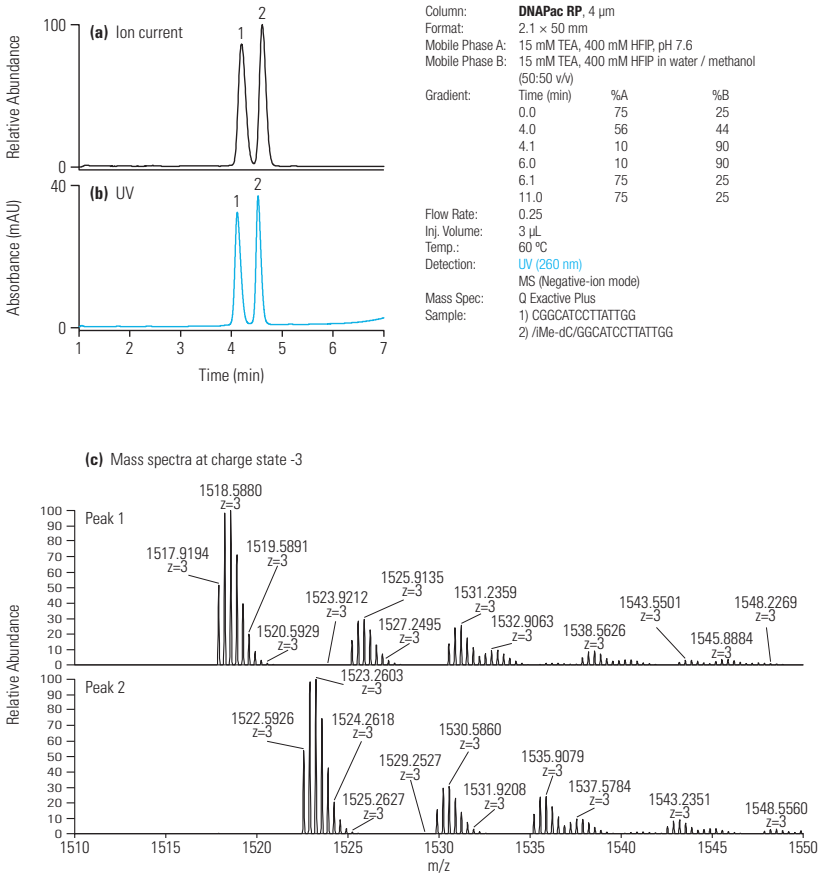


Figure 9. Analysis of CpG methylation.

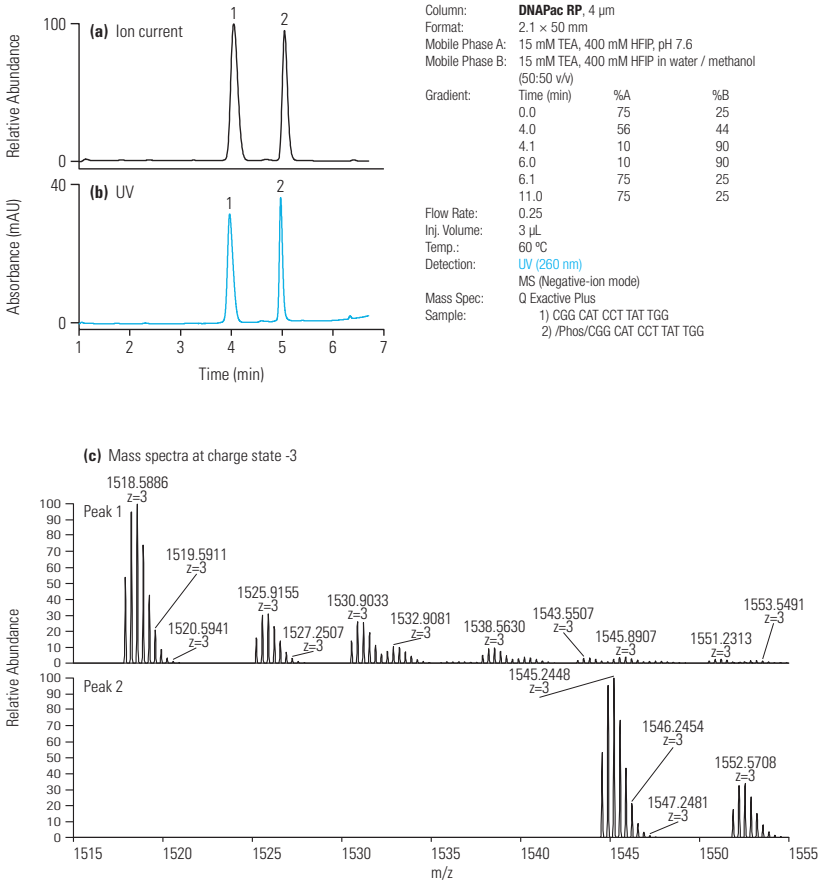
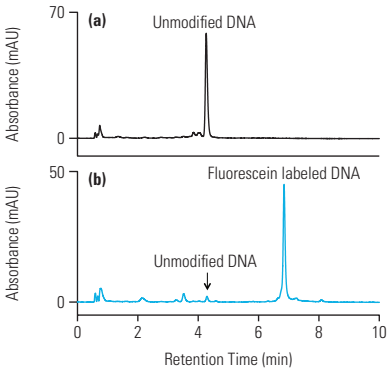


Figure 10. Analysis of 5'-phosphorylation.

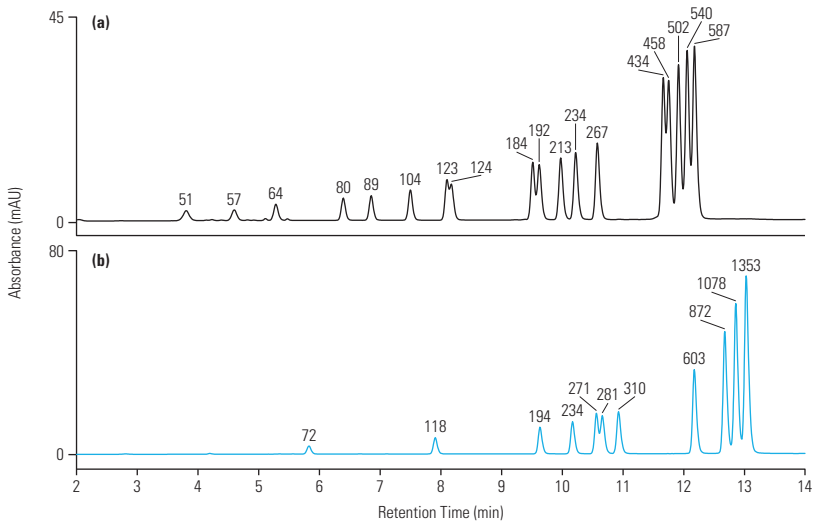


Column:	**DNAPac RP**, 4 µm
Format:	2.1 × 100 mm
Mobile Phase A:	0.1 M TEAA, pH 7.0
Mobile Phase B:	0.1 M TEAA in water / acetonitrile (80:20 v/v)

Gradient:	Time (min)	%A	%B
	0.0	75	25
	10.0	0	100
	12.0	0	100
	12.1	75	25
	16.0	75	25

Flow Rate:	0.60 mL/min
Inj. Volume:	2.5 µL
Temp.:	60 °C
Detection:	UV (260 nm)
Sample:	a. 20mer DNA (60 µg/mL)
	b. Fluorescein labeled 20mer DNA (60 µg/mL)

Figure 11. Separation of fluorescein-labeled DNA.



Column:	**DNAPac RP**, 4 µm
Format:	2.1 × 100 mm
Mobile Phase A:	0.1 M TEAA, pH 7.0
Mobile Phase B:	0.1 M TEAA, pH 7.0 in water / acetonitrile (75:25 v/v)

Gradient:	Time (min)	%A	%B
	-8.0	64	36
	0.0	64	36
	12.0	31	69
	12.1	5	95
	15.0	5	95

Flow Rate:	0.40 mL/min
Inj. Volume:	5 µL
Temp.:	55 °C
Detection:	UV (260 nm)
Sample:	a. pBR322-BsuRI digest (100 µg/mL)
	b. ΦX174-BsuRI digest (100 µg/mL)
Peak Label:	base pairs

Figure 12. Separation of restriction enzyme digests of plasmid DNAs.

Separation of fluorescent dye-labeled DNA

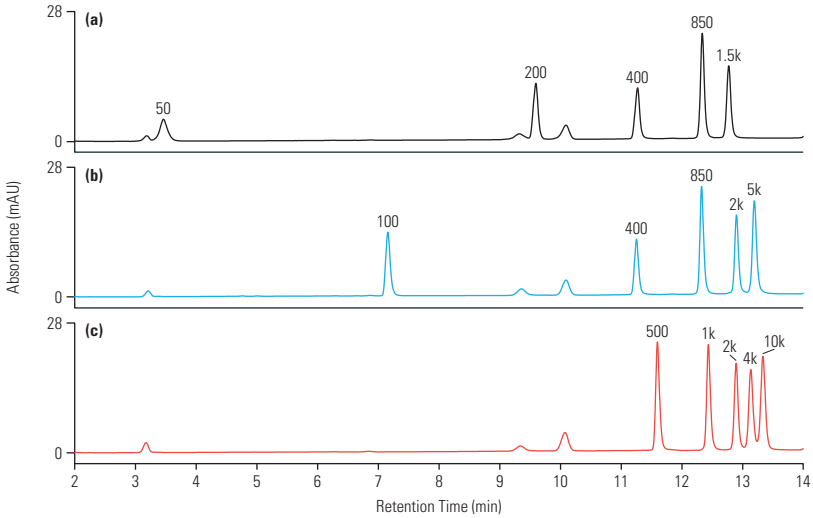
Fluorescent dye-labeled oligonucleotides are used in many applications such as DNA sequencing, PCR, DNA microarrays, and in situ hybridization. In most cases, fluorophore attachment increases the hydrophobicity, and thus retention by IP-RP of the oligonucleotide. Figure 11 illustrates the separation of a fluorescein labeled oligonucleotide and its unmodified form on a DNAPac RP column. The unmodified oligonucleotide and other impurities were separated from the main dye-labeled oligonucleotide peak (Figure 11b).

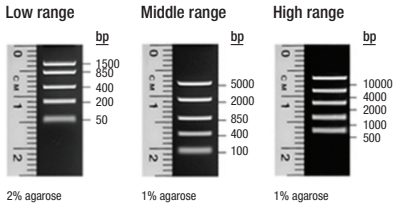
Separation of large double-stranded DNA fragments

Purification and analysis of large double-stranded DNA fragments is an important step for DNA cloning and PCR. Traditionally, large dsDNA fragments have been purified by agarose or acrylamide gels. This process requires gel electrophoresis, manual excision of the target size range from the gel and extraction of the DNA from the excised gel. This laborious and time consuming step generally produces relatively low DNA yield. HPLC provides more reliable and higher yields and can be readily automated. The wide-pore resin in the DNAPac RP column can produce resolution comparable to many DNA gels in similar times.

Figure 12 depicts the resolution of DNA fragments generated from restriction enzymes. Fragments ranging from 51 bp to 1353 bp have been separated on the DNAPac RP 2.1 × 100 mm column in 15 minute chromatograms.

Figure 13 shows the separation of Thermo Scientific™ FastRuler™ DNA ladders. All the DNA fragments were separated based on size. This demonstrates the use of DNAPac RP column for the separation of dsDNA fragments ranging from 50 to 10,000 base pairs.

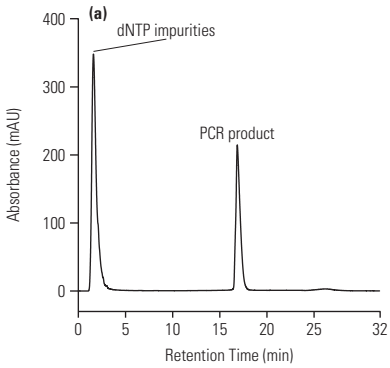




Column:	**DNAPac RP**, 4 µm		
Format:	2.1 × 100 mm		
Mobile Phase A:	0.1 M TEAA, pH 7.0		
Mobile Phase B:	0.1 M TEAA, pH 7.0 in water / acetonitrile (75:25 v/v)		
Gradient:	Time (min)	%A	%B
	-8.0	65	36
	0.0	65	36
	12.0	30	70
	12.1	10	90
	15.0	10	90
Flow Rate:	0.40 mL/min		
Inj. Volume:	10 µL		
Temp.:	55 °C		
Detection:	UV (260 nm)		
Sample:	DNA ladders		
	a. Low range		
	b. Middle range		
	c. High range		
Peak Label:	Base pairs		

Figure 13. Separation of Thermo Scientific™ FastRuler™ DNA ladders.

In Figure 14a, a PCR product (~750 bp) was purified by DNAPac RP column. Fraction collection of the main peak using the UltiMate WPS-3000TBFC Well Plate Autosampler with Fraction Collector resulted in pure PCR product which was confirmed by gel electrophoresis (Figure 14b).



Column:	**DNAPac RP**, 4 µm		
Format:	2.1 × 100 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
	0.0	56	44
	20.0	32	68
	20.1	10	90
	22.0	10	90
	22.1	56	44
	32.0	56	44
Flow Rate:	0.25		
Inj. Volume:	50 µL		
Temp.:	50 °C		
Detection:	UV (260 nm)		
Sample:	PCR product		

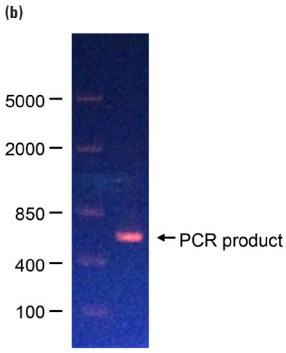


Figure 14. Purification of a PCR product.

Physical Data

Substrate	Poly(styrene-divinylbenzene)
Particle size	4 µm
Pore size	Proprietary wide pore
Column housing	Stainless steel

Operational Specifications

Dimension (mm)	Recommended Flow Rate (mL/min)	Pressure Limit (psi)	Temperature (°C)	pH Range
3.0 mm	0.4–1.0	4,000	≤ 100	0–14
2.1 mm	0.2–0.6	4,000	≤ 100	0–14

Ordering Information

Description	Particle Size	Part Number
DNAPac RP, Analytical, 3.0 × 100 mm	4 µm	088919
DNAPac RP, Analytical, 3.0 × 50 mm	4 µm	088920
DNAPac RP, Guard Cartridges, 3.0 × 10 mm (2/pk)	4 µm	088921
DNAPac RP, Analytical, 2.1 × 100 mm	4 µm	088923
DNAPac RP, Analytical, 2.1 × 50 mm	4 µm	088924
DNAPac RP, Guard Cartridges, 2.1 × 10 mm (2/pk)	4 µm	088925
Guard Cartridge Holder	–	069580

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