DNAPac RP Columns

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Thermo SCIENTIFIC

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Product Manual

for

DNAPac RP Analytical and Guard Columns

DNAPac RP, 4 µm, 3.0 × 100 mm (P/N: 088919)
DNAPac RP, 4 µm, 3.0 × 50 mm (P/N: 088920)
DNAPac RP, 4 µm, 3.0 × 10 mm Guard Cartridges 2/pk (P/N: 088921)

DNAPac RP, 4 µm, 2.1 × 100 mm (P/N: 088923)
DNAPac RP, 4 µm, 2.1 × 50 mm (P/N: 088924)
DNAPac RP, 4 µm, 2.1 × 10 mm Guard Cartridges 2/pk (P/N: 088925)

Revision History:
Revision 01, March, 2016, Original Publication.

Safety and Special Notices

Make sure you follow the precautionary statements presented in this guide. The safety and other special notices appear in boxes.

Safety and special notices include the following:

 **SAFETY**	***Indicates a potentially hazardous situation which, if not avoided, could result in death or serious injury.***
 **WARNING**	***Indicates a potentially hazardous situation which, if not avoided, could result in damage to equipment.***
 **CAUTION**	***Indicates a potentially hazardous situation which, if not avoided, may result in minor or moderate injury. Also used to identify a situation or practice that may seriously damage the instrument, but will not cause injury.***
 **NOTE**	***Indicates information of general interest.***
IMPORTANT	***Highlights information necessary to prevent damage to software, loss of data, or invalid test results; or might contain information that is critical for optimal performance of the system.***
Tip	***Highlights helpful information that can make a task easier.***

Contents

1. Introduction

1.1 Introduction to the DNAPac RP Column

DNAPac RP is a reversed phase (RP) column specifically designed for separation of single- and double-stranded nucleic acids. The stationary phase is designed to employ mobile phases compatible with mass spectrometry, such as acetonitrile, methanol and hexafluoroisopropanol (HFIP). The DNAPac RP is based on a wide-pore, 4 µm polymer particles that are stable at extremes of pH (0 – 14) and temperature (up to 100 °C). Particles are inherently hydrophobic so there is no bonded phase, such as alkyl ligand required for silica-based reversed-phase separations. The large pore size enables efficient separation of small to large oligonucleotides.

1.2 DNAPac RP Operating Limits and Specifications

1.2.1 Operating Conditions

Parameter	Recommendation
Flow Rate Range	The following flow rates are recommended when running at 60 °C. 0.4– 1.0 mL/min for the 3.0 mm I.D. columns 0.2 –0.6 mL/min for the 2.1 mm I.D. columns
Long Term Storage Solution	Water / Acetonitrile (50:50 v/v)
Common Mobile Phases	**LC/UV** Mobile phase A: 100 mM TEAA Mobile phase B: 100 mM TEAA in Water / Acetonitrile (75:25 v/v) **LC/MS** Mobile phase A: 15 mM TEA, 400 mM HFIP Mobile phase B: 15 mM TEA, 400 mM HFIP / Methanol (50:50 v/v)
	TEAA: triethylammonium acetate TEA: triethylamine HFIP: hexafluoroisopropanol
Solvents Compatibility	Compatible with 100% acetonitrile, isopropanol, and methanol
Temperature Range	Up to 100 °C
Pressure Limit	4,000 psi
pH Range	0 – 14

NOTE

Do not expose the column to following organic solvents: tetrahydrofuran, dioxane, or methylene chloride.

NOTE

Assistance is available for any problem during the shipment or operation of Thermo Scientific columns at techsupport.ccs@thermofisher.com

1.2.2 Physical Characteristics

Substrate:	Proprietary wide-pore poly(styrene-divinylbenzene) particles
Particle size:	4 µm

1.3 Formats of the DNAPac RP Columns

Currently, DNAPac RP columns are available in 3.0 mm and 2.1 mm diameter formats.

Product Description	Part Number
DNAPac RP, 4µm, Analytical column 3.0 × 100 mm	088919
DNAPac RP, 4µm, Analytical column 3.0 × 50 mm	088920
DNAPac RP, 4µm, Guard cartridge 3.0 × 10 mm*	088921
DNAPac RP, 4µm, Analytical column 2.1 × 100 mm	088923
DNAPac RP, 4µm, Analytical column 2.1 × 50 mm	088924
DNAPac RP, 4µm, Guard cartridge 2.1 × 10 mm*	088925

*Guard cartridges require a guard cartridge holder (P/N 069580).

2. Getting Started; Step-By-Step Procedure

Thermo Fisher Scientific recommends that you perform an efficiency test on your DNAPac RP column before use. The purpose of column performance validation is to ensure no damage has occurred during shipping. Steps 1 – 5 below outline the necessary process to validate system operation. Test the column using the conditions described on the Quality Assurance (QA) report enclosed in the column box. Repeat the test periodically to track the column performance over time. Note that slight variations may be found on two different HPLC systems due to system electronic, hardware, plumbing, operating environment, reagent quality, column conditioning, and operator technique.

2.1 Step 1 – Visually inspect the column

Report any visible damage upon receiving the column to Thermo Fisher Scientific immediately. Depending upon the nature of the damage, we may request that you return the damaged column back to us for a replacement column.

2.2 Step 2 – Prepare mobile phases

To obtain reliable, consistent and accurate results, use HPLC/MS grade solvents and Type 1 reagent grade water with a specific resistance of 18.2 megohm-cm or greater filtered through a 0.22 µm filter.

QAR test report mobile phase: 0.1 M TEAA / Acetonitrile (93:7 v/v)	
0.1 M TEAA, pH 7.0	In a clean 1 L bottle equipped with a stir bar, add 50.65 g of 2.0 M TEAA and 950.0 g of DI water (18.2 megohm-cm). Stir for 2 minutes.
0.1 M TEAA / Acetonitrile (93:7 v/v)	In a clean 500 mL bottle equipped with a stir bar, add 465.30 g of 0.1 M TEAA and 27.50 g of acetonitrile. Stir for 2 minutes.

2.3 Step 3 – Set up the LC system

Use a standard LC system equipped with a LC pump, a column oven, a UV detector (260 nm) and an autosampler. It is highly recommended that the system be optimized for low dead volume; usage of small internal diameter tubing with Viper™ connectors (such as 100 µm) and a proper detector flow cell (such as the 2.5 µL semi-micro flow cell) is required for best results. The system should be thoroughly primed before use. In addition, temperature gradients across the column will degrade peak shape. Insert an eluent preheater between the pump or injection valve and the column.

2.4 Step 4 – Condition the column

Install the column and preheat the column oven. Slowly ramp up the flow rate to 0.4 mL/min for 3.0 mm ID column and 0.2 mL/min for 2.1 mm ID column. If possible, set the flow ramps up and down to 0.5 mL/min^2 or less. Equilibrate the column with mobile phase specified in Quality Assurance Report (QAR) for 10 minutes. Run a blank run before the column performance test.

2.5 Step 5 – Verify the performance of the column

Compare the result of your test with that provided in the QAR (isocratic) test report.

NOTE

Due to various reasons, such as difference of LC systems, mobile phases, etc, you may observe somewhat different separation from that in the report.

NOTE

Acetonitrile may evaporate over time causing the retention time to shift to a longer time. Pressurizing the mobile phase bottle with 3-5 psi nitrogen will help reduce evaporation of acetonitrile. Otherwise, prepare fresh buffer when shift in retention time is observed.

2.6 Step 6 – Real sample analysis

Once the column performance is satisfactorily confirmed in Step 1-5, the column is ready for your sample analysis. Equilibrate the column with the desired mobile phase before sample analysis. Run 1~2 black runs with the desired mobile phase and gradient before running the sample.

NOTE

It is recommended that the column performance test be executed periodically to monitor the condition of the column.

3. Column Care

3.1 Column storage

The column can be stored in the mobile phase for short-term storage. For long-term storage (more than 5 days), it is recommended to store the column in water / acetonitrile (50:50 v/v). If high pH or low pH mobile phases were used, wash the column thoroughly with the storage solution before storage.

3.2 Operating pH range: pH 0 to 14

3.3 Operating temperature limit: up to 100 °C

This polymer column is stable at high temperature up to 110 °C. The recommended operating temperature for nucleic acid separation is between 30 °C to 95 °C. For native analysis of double-stranded nucleic acids, keep the temperature below the melting point (T_m).

3.4 Pressure limit: 4,000 psi

The column pressure should not exceed 4,000 psi. The back pressure of the column is strongly correlated to the column ID, length, column temperature, flow rate and type of organic solvent used for mobile phase.

3.5 Flow rate

Maximum flow rate will depend on the column ID, length, column temperature and type of organic solvent used for mobile phase. Below is the recommended flow rate at 60 °C.

3.0 mm I.D. columns	0.4– 1.0 mL/min
2.1 mm I.D. columns	0.2 –0.6 mL/min

3.6 Column washing procedure

To elute very hydrophobic nucleic acid samples, increase the mobile phase organic content up 90% (such as 90% acetonitrile) and wash the column for 15~30 minutes at 0.2 mL/min for 2.1 mm ID columns and 0.4 mL/min for 3.0 mm ID columns at 60 °C.

4. Considerations in Method Development

4.1 Mobile Phase

The ion pairing agent, counter ion, and pH are important for nucleic acid analysis using reversed phase chromatography. The most commonly used ion pairing agent is triethylamine (TEA). However hexylamine (HA) has shown higher resolution for some applications. The pH of the mobile phase affects the charge state of oligonucleotides, and thus column selectivity. Therefore adjusting the pH may provide higher resolution due to the selectivity change. For mass spectrometric assays, addition of hexafluoroisopropanol (HFIP) supports increased nucleic acid ionization which increases the signal intensity. The concentration of HFIP may be optimized for highest signal intensity.

4.1.1 Mobile Phase Recommendations

Mobile phase sets 1 or 2 (below) are recommended for LC/UV analysis. For LC/MS analysis, use sets 3 or 4. Further optimization of the concentration of ion pairing agent and pH may improve resolution and/or signal intensity in mass spectrometer. To limit adduction by sodium, or potassium, addition of 10µM ETDA may be helpful.

LC/UV Analysis

Mobile Phase Set 1	
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)

Mobile Phase Set 2	
Mobile phase A	25 mM HAA, pH 8.5
Mobile phase B	25 mM HAA, pH 8.5 in Water / Acetonitrile (50:50 v/v)

LC/MS Analysis

Mobile Phase Set 3	
Mobile phase A	15 mM TEA, 400 mM HFIP, pH 7.9
Mobile phase B	15 mM TEA, 400 mM HFIP, pH 7.9 / Methanol (50:50 v/v)

Mobile Phase Set 4	
Mobile phase A	35 mM TEA, 40 mM HFIP, pH 9.9
Mobile phase B	35 mM TEA, 40 mM HFIP, pH 9.9 / Methanol (75:25 v/v)

4.1.2 Mobile Phase Preparation

Like any HPLC application detected by UV, the mobile phase should have a low UV background. Make sure the salt, water and the organic solvent do not absorb UV and free of UV absorbing impurities.

SAFETY

HFIP is toxic, and can damage your eyes! Handle HFIP in a ventilated hood. Avoid exposure to the vapor.

NOTE

Whenever applicable, degas the aqueous component and solvent component separately before mixing them together. Excessive purging or degassing of mobile phases should be avoided because it may result in changing mobile phase composition.

NOTE

Acetonitrile may evaporate over time causing the retention time to shift to a longer time. Pressurizing the mobile phase bottle with 3-5 psi of nitrogen reduces the evaporation of acetonitrile. Otherwise, prepare fresh buffer when shift in retention time is observed.

4.1.3 Mobile Phase Set 1

4.1.3.1 Mobile Phase A (1 L)

0.1 M TEAA, pH 7.0

To a 1-L HPLC mobile phase bottle equipped with a magnetic stir bar add the following and stir for 2 minutes.

	Weight (g)	Volume (mL)
DI water	950.00	950.00
2.0 M TEAA*	50.65	50.00

*2.0 M TEAA (Fisher Scientific P/N 400613)

4.1.3.2 Mobile Phase B (1 L)

0.1 M TEAA in Water / Acetonitrile (75:25 v/v)

To a 1-L HPLC mobile phase bottle equipped with a magnetic stir bar add the following and stir for 2 minutes.

	Weight (g)	Volume (mL)
DI water	700.00	700.00
2.0 M TEAA	50.65	50.00
Acetonitrile	196.50	250.00

4.1.4 Mobile Phase Set 2

4.1.4.1 Mobile Phase A (1 L)

25 mM HAA, pH 8.5

	Weight (g)	Volume (mL)
DI water	995.25	995.25
HA	2.53	3.29
Acetic acid	1.50	1.43

1. To a 1-L HPLC mobile phase bottle equipped with a magnetic stir bar add DI water, HA and acetic acid and stir for 2 minutes.
2. Measure the pH of the solution while stirring.
3. Adjust the pH to 8.5 with HA or acetic acid.

4. Stir for 2 minutes.

4.1.4.2 Mobile Phase B (1 L)

25 mM HAA, pH 8.5 / Acetonitrile (50:50 v/v)

	Weight (g)	Volume (mL)
DI water	495.25	495.25
HA	2.53	3.29
Acetic acid	1.50	1.43
Acetonitrile	393.00	500.00

1. To a 1-L beaker equipped with a magnetic stir bar add DI water, HA and acetic acid.
2. Measure the pH of the solution while stirring.
3. Adjust the pH to 8.5 with HA or acetic acid.
4. Add acetonitrile.
5. Stir for 2 minutes.
6. Transfer to a 1-L HPLC mobile phase bottle.

4.1.5 Mobile Phase Set 3

4.1.5.1 Mobile Phase A (1 L)

15 mM TEA, 400 mM HFIP pH 7.9

To a 1-L HPLC mobile phase bottle equipped with a magnetic stir bar add the following and stir for 2 minutes.

	Weight (g)	Volume (mL)
DI water	955.79	955.79
TEA	1.52	2.09
HFIP	67.22	42.12

4.1.5.2 Mobile Phase B (1 L)

15 mM TEA, 400 mM HFIP pH 7.9 / Methanol (50:50 v/v)

To a 1-L HPLC mobile phase bottle equipped with a magnetic stir bar add the following and stir for 2 minutes.

	Weight (g)	Volume (mL)
DI water	455.79	455.79
TEA	1.52	2.09
HFIP	67.22	42.12
Methanol	395.90	500.00

4.1.6 Mobile Phase Set 4

4.1.6.1 Mobile Phase A (1 L)

35 mM TEA, 40 mM HFIP pH 9.9

To a 1-L HPLC mobile phase bottle equipped with a magnetic stir bar add the following and stir for 2 minutes.

	Weight (g)	Volume (mL)
DI water	990.91	990.91
TEA	3.54	4.88
HFIP	6.72	4.21

4.1.6.2 Mobile Phase B (1 L)

35 mM TEA, 40 mM HFIP pH 9.9 / Methanol (75:25 v/v)

To a 1-L HPLC mobile phase bottle equipped with a magnetic stir bar add the following and stir for 2 minutes.

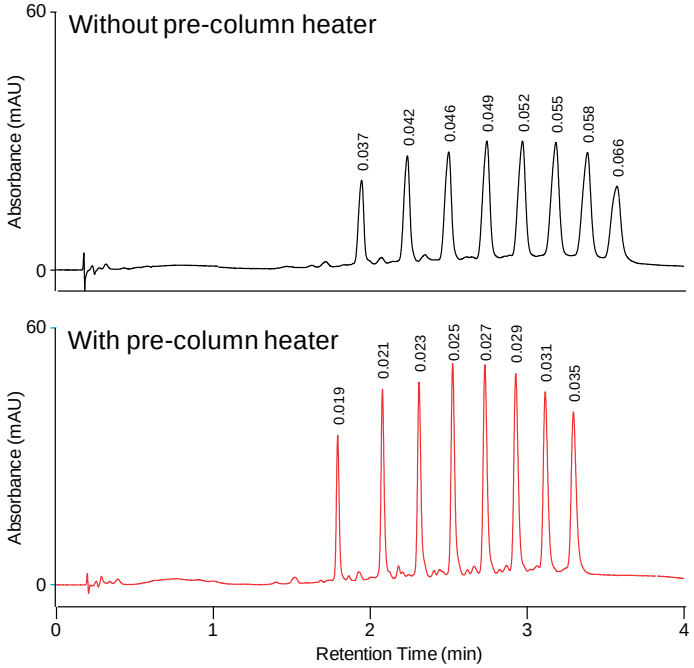
	Weight (g)	Volume (mL)
DI water	740.91	740.91
TEA	3.54	4.88
HFIP	6.72	4.21
Methanol	197.95	250.00

4.2 Pre-Column Heater

Temperature gradients across the column may induce band broadening. Thermo Scientific highly recommends use of a pre-column heater. Sharper peaks and higher resolution are observed when a pre-column heater is installed (Figure 1).

Product Description	Part Number
11 µL Pre-Column Heater	6723.0252
Active Pre-Heater (For Vanquish Systems)	6732.0110

Figure 1. Effect of pre-column heater



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	88	12
0.0	88	12
3.0	64	36
3.1	10	90
5.0	10	90

Gradient curve: 3

Temperature: 60 °C
Flow rate: 0.80 mL/min
Inj. volume: 2 µL
Detection: UV (260 nm)
Sample: 8-Combo DNA (5 µM)*
Peak label: PWHH

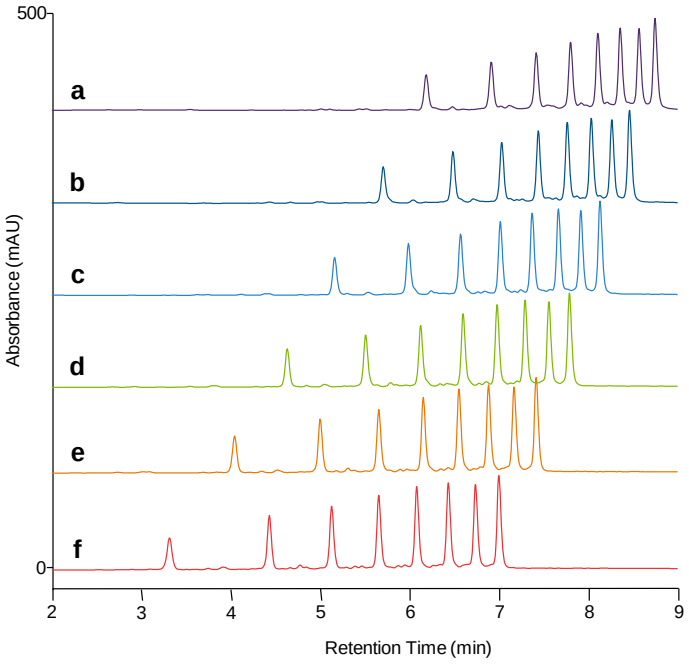
*Sequence of 8-Combo DNA

```
GACTGACTGACT - 12
GACTGACTGACTGACT - 16
GACTGACTGACTGACTGACT - 20
GACTGACTGACTGACTGACTGACT - 24
GACTGACTGACTGACTGACTGACTGACT -28
GACTGACTGACTGACTGACTGACTGACTGACT -32
GACTGACTGACTGACTGACTGACTGACTGACTGACT -36
GACTGACTGACTGACTGACTGACTGACTGACTGACTGACT -40
```

4.3 Temperature

Analysis temperature alters retention time and resolution as shown in Figure 2. Higher temperatures reduce retention and increases resolution (Figure 2g). Since the mobile phase viscosity decreases with increasing temperature, the pressure also decreases. Therefore, ON separation at higher temperature requires less organic solvent and supports higher flow rates, increasing throughput. For longer ONs, or those with intra or inter-strand hydrogen bonds, higher temperatures help break the hydrogen bonds, and improve ON resolution.

Figure 2. Effect of temperature on retention time and resolution



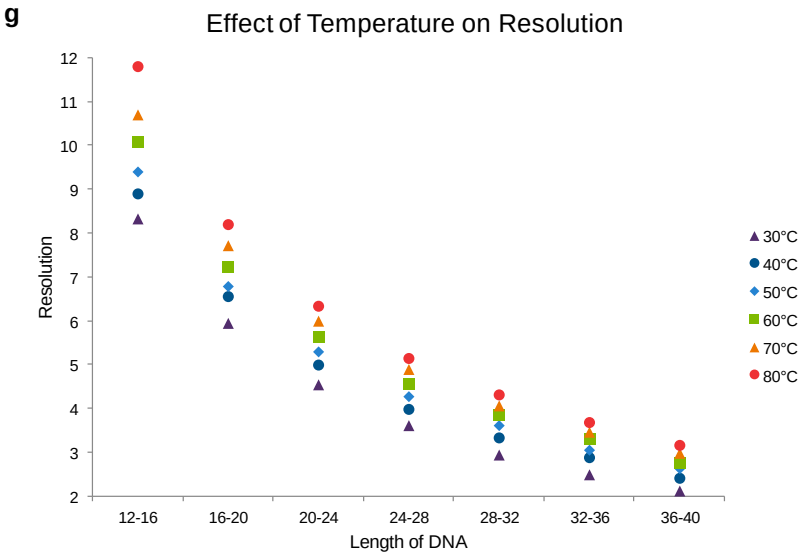
Column: DNAPac RP, 4 µm
Format: 2.1 × 50 mm
Mobile phase A: 0.1 M TEAA, pH 7.0
Mobile phase B: 0.1 M TEAA in Water / Acetonitrile (25:75 v/v)
Gradient:

Time (min)	%A	%B
-3.0	90	10
0.0	90	10
8.0	54	46
8.1	10	90
10.0	10	90

Flow rate: 0.40 mL/min
Inj. volume: 2 µL

Temperature:
a) 30 °C
b) 40 °C
c) 50 °C
d) 60 °C
e) 70 °C
f) 80 °C

Detection: UV (260 nm)
Sample: 8-Combo DNA (5 µM)*

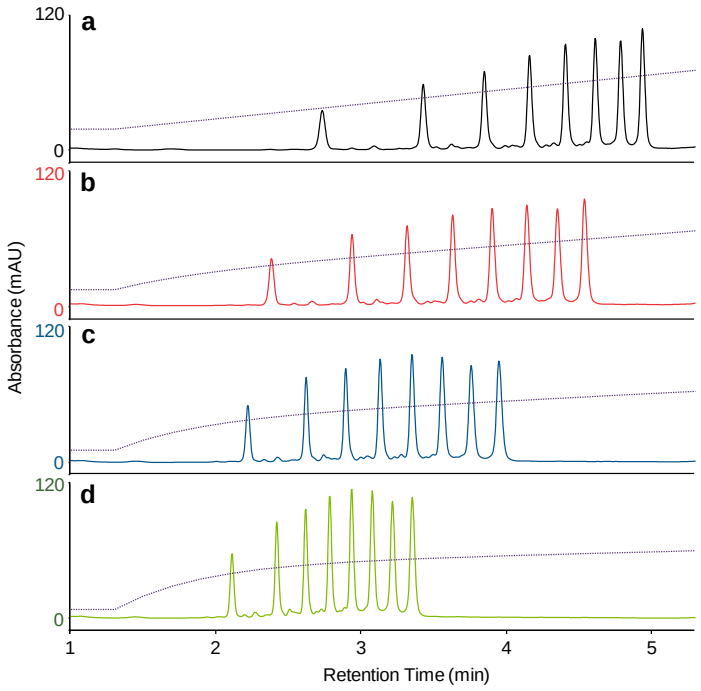


4.4 Gradient and Flow Rate

Thermo Fisher recommends initial tests employ a survey gradient (e.g., 10 to 90% B) and using the data generated to optimize the concentration range for optimal resolution and throughput. When subsequent runs with narrow gradients result in unresolved early-eluting components, revise the gradient to start with a lower concentration of organic solvent.

When analyzing mixtures of ONs having disparate lengths, throughput may be improved by programming curved gradients. In Figure 3, a mixture of 12, 16, 20, 24, 28, 32, 36 and 40mer DNAs with mixed base composition are separated using a linear gradient and convex gradients (see Figure 1 for the sequences). These convex gradients employ high initial concentration slopes followed by continually decreasing slopes as time progresses. This maintains complete resolution at the beginning of the separation, where resolution is very high, then improves resolution between later-eluting ONs as the gradient progresses. However, Figure 3e shows that excessively curved gradients can result in loss of resolution of late-eluting ONs.

Figure 3. Gradient curve adjustment

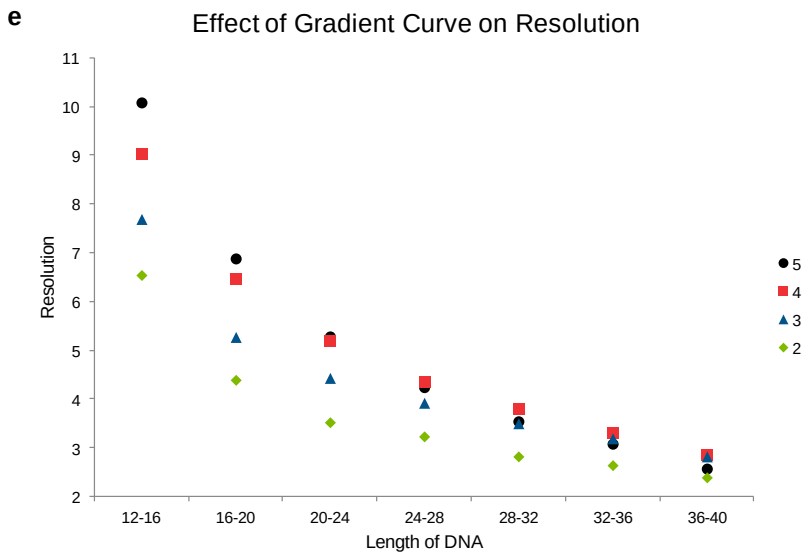


Column: DNAPac RP, 4 µm
Format: 2.1 × 50 mm
Mobile phase A: 0.1 M TEAA, pH 7.0
Mobile phase B: 0.1 M TEAA in Water / Acetonitrile (25:75 v/v)
Gradient:

Time (min)	%A	%B
-3.0	85	15
0.0	85	15
4.0	61	39
4.1	10	90
6.0	10	90

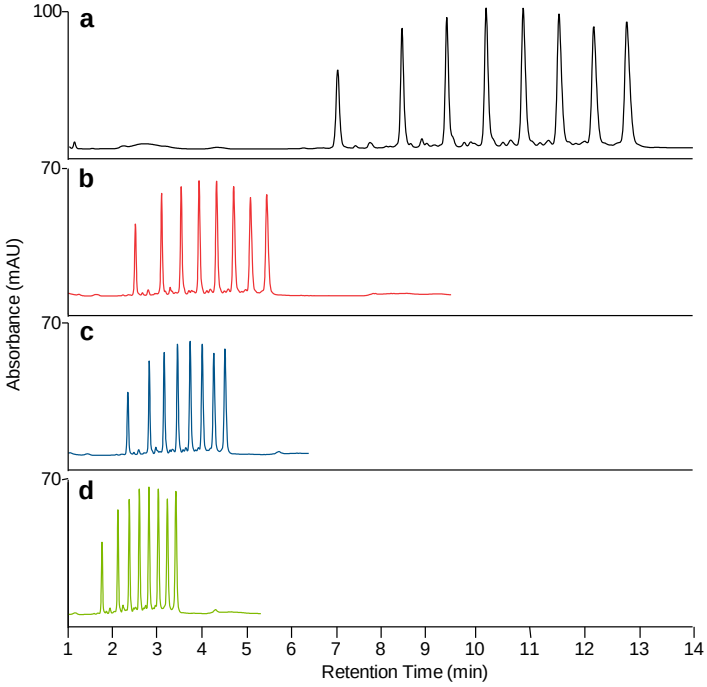
Gradient curve: a) 5 (linear)
b) 4
c) 3
d) 2

Flow rate: 0.60 mL/min
Inj. volume: 2 µL
Temperature: 60 °C
Detection: UV (260 nm)
Sample: 8-Combo DNA (5 µM)*

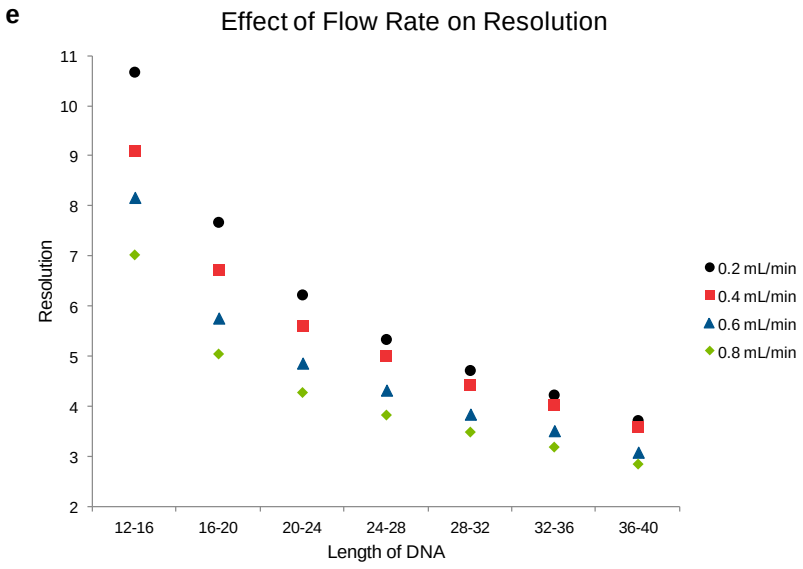


Flow rate affects the resolution and separation time. When comparing flow rates from 0.2 mL/min to 0.8 mL/min (Figure 4), the highest resolution is achieved at 0.2 mL/min (Figure 4e). As the flow rate increases, resolution decreases. However, at 0.8 mL/min, the separation of ONs between 12 and 40 bases can be achieved in 3.5 minutes (cf. 13 minutes for 0.2 mL/min), producing a 3.7 fold improvement in throughput. For more challenging separations, employ lower flow rates, and for separations where target ONs are well resolved, employ higher flows to optimize throughput. But consider that higher flows will induce greater pressures, therefore Thermo Fisher recommends running higher flow rates at higher temperature.

Figure 4. Separation of oligonucleotides at different flow rates



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:	a) 15 to 37%B in 12 min. b) 15 to 37%B in 6 min. c) 15 to 37%B in 4 min. d) 15 to 37%B in 3 min.
Gradient curve:	3
Flow rate:	a) 0.20 b) 0.40 c) 0.60 d) 0.80
Inj. volume:	2 µL
Temperature:	60 °C
Detection:	UV (260 nm)
Sample:	8-Combo DNA (5 µM)*

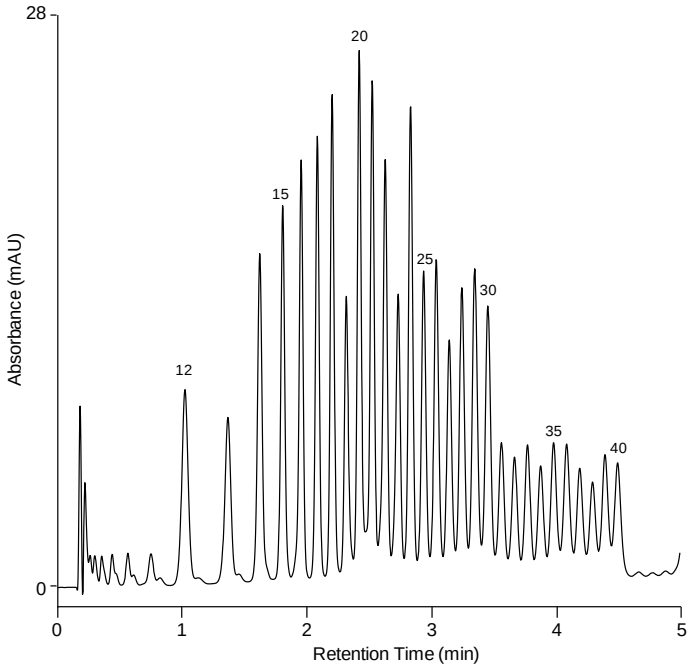


5. Applications

5.1 Separation of polydeoxythymidine oligonucleotides

The DNAPac RP column is designed for high resolution separation of single-stranded nucleic acids. In Figure 5, synthetic poly deoxythymidine [pd(T)$_{19-40}$] ONs are separated using TEAA and acetonitrile mobile phase. All the dT molecules are separated in 4 minutes using a curve 3 gradient. A sample that includes longer pdT molecules (up to dT$_{60}$) was separated with a 7-minute, curve 3 gradient (Figure 6).

Figure 5. Fast separation of 12-40mer deoxythymidines (dTs)

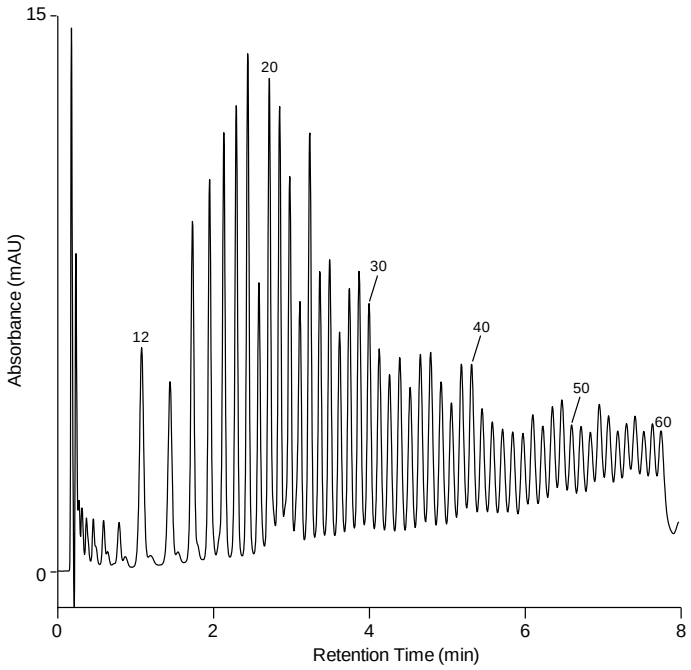


Column:	DNAPac RP, 4 µm
Format:	2.1 × 50 mm
Mobile phase A:	0.1 M TEAA, pH 7.0
Mobile phase B:	0.1 M TEAA in Water / Acetonitrile (75:25 v/v)
Gradient:	

Time (min)	%A	%B
-2.0	76	24
0.0	76	24
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
Temperature:	80 °C
Detection:	UV (260 nm)
Sample:	poly dT12-40 (1 A/mL)
Peak label:	Length of DNA

Figure 6. 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:	

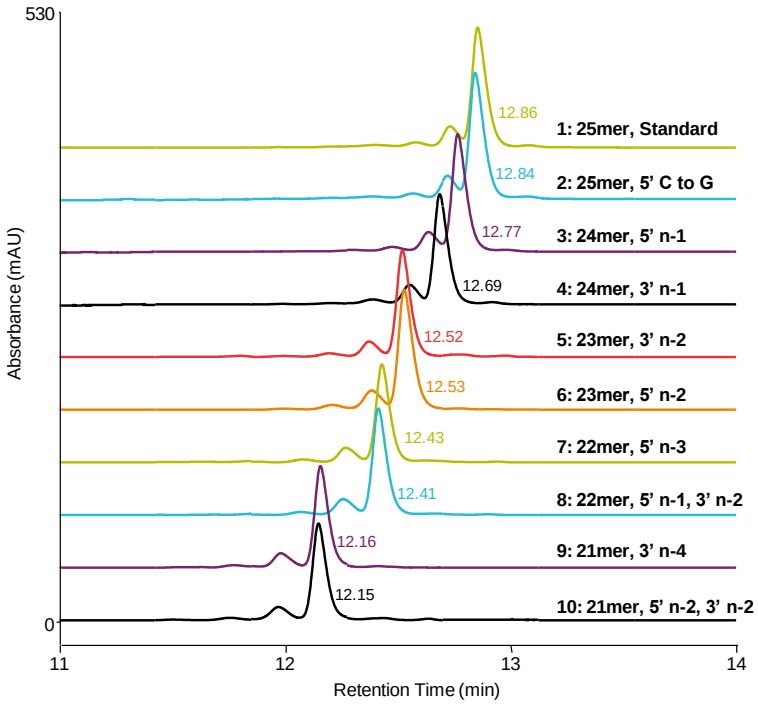
Time (min)	%A	%B
-2.0	76	24
0.0	76	24
7.0	57	43
7.1	10	90
5.0	10	90

Gradient curve:	3
Flow rate:	0.80 mL/min
Inj. volume:	10 µL
Temperature:	80 °C
Detection:	UV (260 nm)
Sample:	poly dT12-60 (0.5 A/mL)
Peak label:	Length of DNA

5.2 Separation of mixed-base DNAs

A series of mixed-base DNAs was analyzed using the HAA mobile phase (see section 4.1.1; set 2; Figure 7). Retention was affected primarily by the ON length. Here, all the ONs of differing length were resolved. Therefore separations of n-1 and n-2 impurities are readily achieved with the DNAPac RP column.

Figure 7. Separation of mixed-base DNAs



Column:	DNAPac RP, 4 µm
Format:	2.1 × 50 mm
Mobile phase A:	25 mM HAA, pH 8.5
Mobile phase B:	25 mM HAA, pH 8.5 / Acetonitrile (50:50 v/v)

Gradient:

Time (min)	%A	%B
-0.1	80	20
0.0	80	20
15.0	20	80
15.1	5	95
17.0	5	95
17.1	80	20
22.0	80	20

Gradient curve:	5
Temperature:	60 °C
Flow rate:	0.25 mL/min
Inj. volume:	3 µL
Detection:	UV (260 nm)
Sample:	Mixed-base DNAs*
Peak label:	Retention time (on the right side of the main peak)

*Sequence of mixed-base DNA

```
1:  CTGCTTGTAGGTTCTCTAACGCTGA - 25
2:  GTGCTTGTAGGTTCTCTAACGCTGA - 25
3:   TGCTTGTAGGTTCTCTAACGCTGA - 24
4:  CTGCTTGTAGGTTCTCTAACGCTG  - 24
5:    GCTTGTAGGTTCTCTAACGCTGA - 23
6:  CTGCTTGTAGGTTCTCTAACGCT   - 23
7:     CTTGTAGGTTCTCTAACGCTGA - 22
8:   TGCTTGTAGGTTCTCTAACGCT   - 22
9:  CTGCTTGTAGGTTCTCTAACG     - 21
10:    TTGTAGGTTCTCTAACGCTGA - 21
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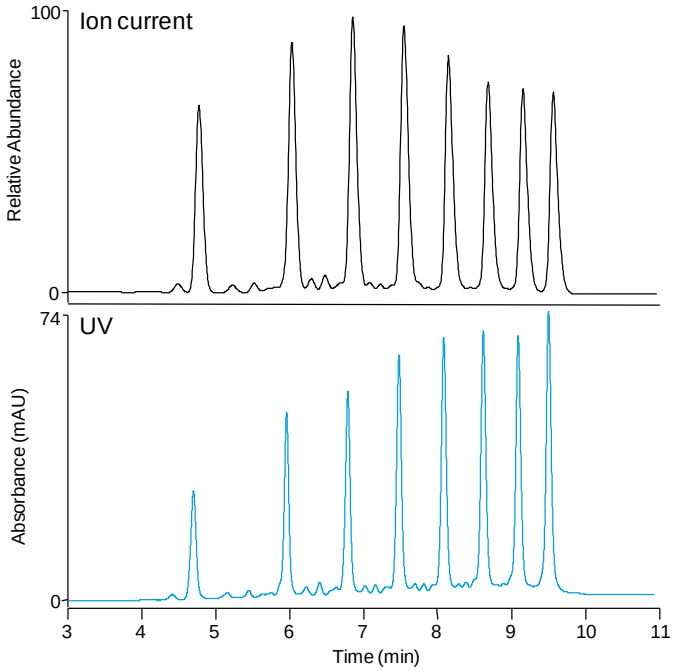
5.3 LC/MS analysis of mixed-base DNAs

High resolution mass spectrometers such as the Thermo Scientific™ Q-Exactive™ Hybrid Quadrupole-Orbitrap™ provide accurate mass data for nucleic acids, ensuring high confidence confirmation of structures in nucleic acid analyses. Mixed-base DNA LC/MS using MS-compatible mobile phases is shown in Figure 8. Accurate masses of these eight mixed-base DNA molecules confirmed their identity.

In Figure 9a, a 21mer mixed-base DNA and its n-1 failure sequence is separated in the UV and ion current traces. The MS data confirms the main peak to be the expected product. At charge state -4, monoisotopic m/z for the 21mer DNA is at 1605.0160 and the mass accuracy is 1.87 ppm (Figure 9b). The masses of failure sequences with missing guanine, cytosine and thymine were detected (Figure 9c). This data demonstrates the use of DNAPac RP LC/MS with the Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer for the separation and identification of the product oligonucleotide and failure sequences.

Figure 8. LC/MS analysis of mixed-base DNA



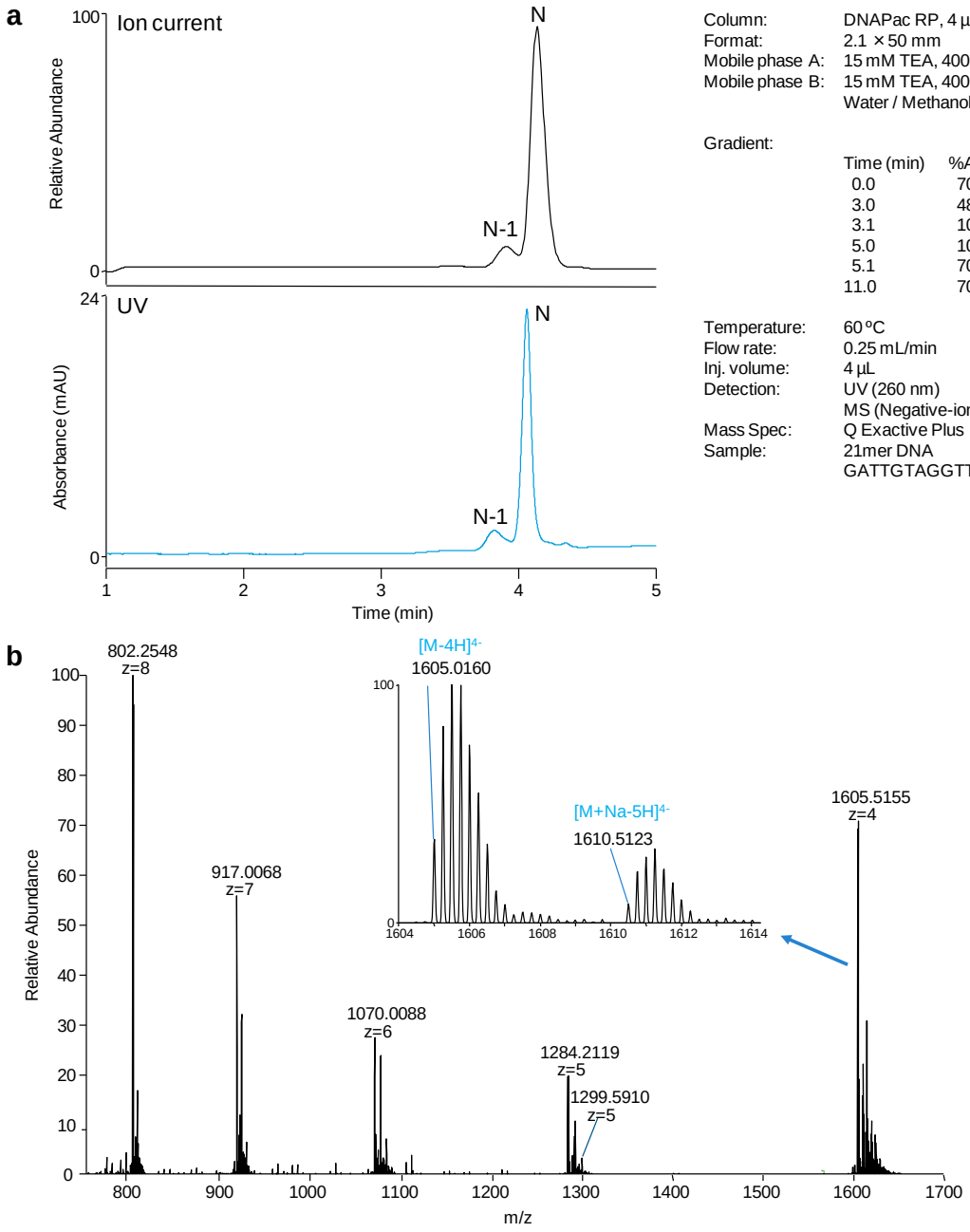
Column:	DNAPac RP, 4 µm
Format:	2.1 × 50 mm
Mobile phase A:	15 mM TEA, 400 mM HFIP, pH 7.9
Mobile phase B:	15 mM TEA, 400 mM HFIP in Water / Methanol (50:50 v/v)

Gradient:

Time (min)	%A	%B
0.0	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
Temperature:	60 °C
Flow rate:	0.25
Inj. volume:	2 µL
Detection:	UV (260 nm) MS (Negative-ion mode)
Mass Spec:	Q Exactive Plus
Sample:	8-Combo DNA (5 µM)*

Figure 9. LC/MS analysis of n and n-1 sequences (a) Ion current and UV traces (b) mass spectrum of n sequence (c) mass spectrum of n-1 sequence at charge state, -4

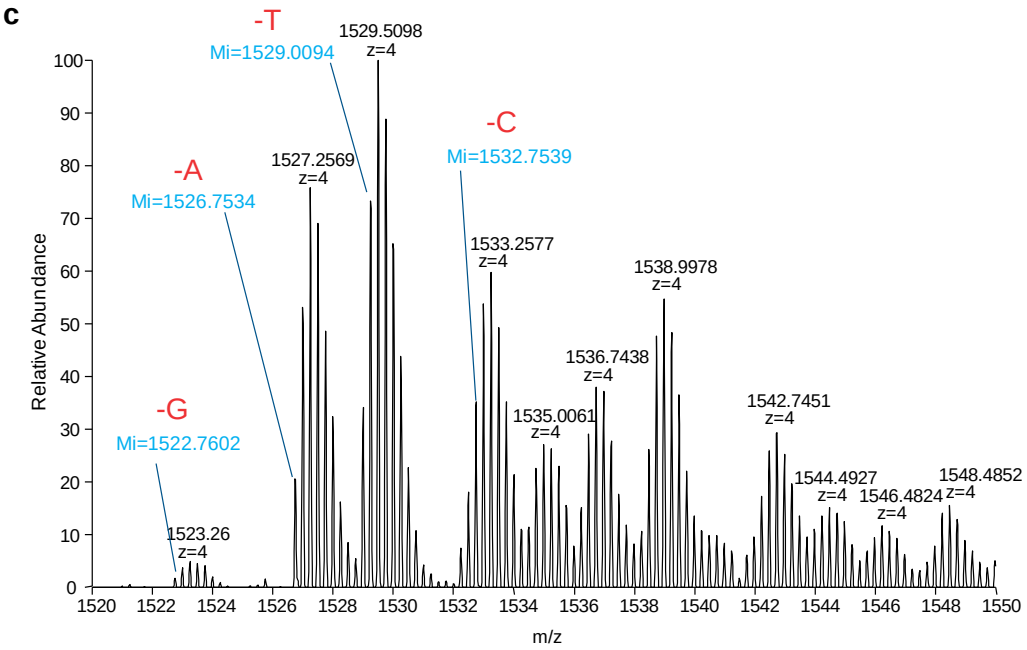


Column:	DNAPac RP, 4 µm
Format:	2.1 × 50 mm
Mobile phase A:	15 mM TEA, 400 mM HFIP, pH 7.9
Mobile phase B:	15 mM TEA, 400 mM HFIP in Water / Methanol (50:50 v/v)

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

Temperature:	60 °C
Flow rate:	0.25 mL/min
Inj. volume:	4 µL
Detection:	UV (260 nm) MS (Negative-ion mode)
Mass Spec:	Q Exactive Plus
Sample:	21mer DNA GATTGTAGGTTCTCTAACGCT


c
-T
Mi=1529.0094
1529.5098
z=4
-C
Mi=1532.7539
-A
Mi=1526.7534
1527.2569
z=4
1533.2577
z=4
1538.9978
z=4
1536.7438
z=4
-G
Mi=1522.7602
1535.0061
z=4
1542.7451
z=4
1544.4927
z=4
1546.4824
z=4
1548.4852
z=4
1523.26
z=4
Relative Abundance
m/z
100
90
80
70
60
50
40
30
20
10
0
1520
1522
1524
1526
1528
1530
1532
1534
1536
1538
1540
1542
1544
1546
1548
1550

5.4 LC/MS analysis of siRNA molecules

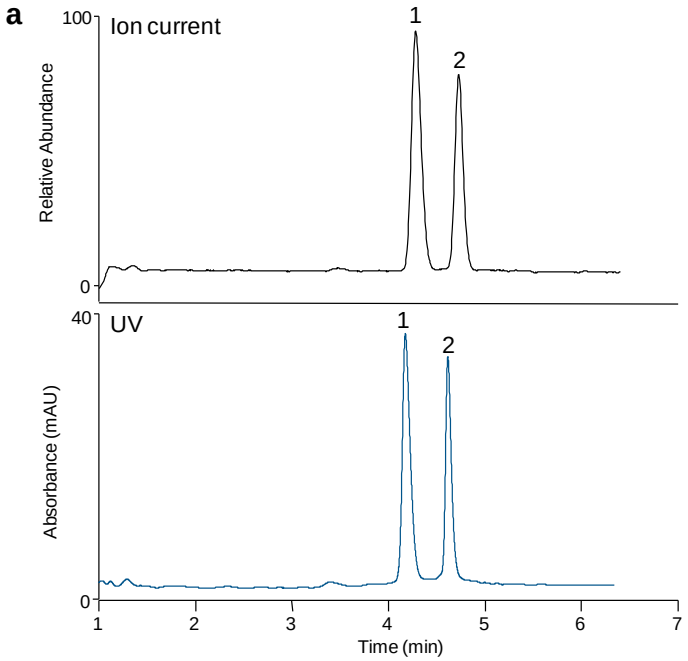
Small interfering RNA (siRNA) describes a process where a dsRNA molecule specifically targets a messenger RNA (mRNA) for degradation resulting in suppression of gene expression. Synthetic siRNAs allow studies of gene function and may serve as therapeutic agents for otherwise undruggable diseases. The backbone of RNA molecules is often modified to increase in vivo ON stability. Two of the most common modifications employed for RNA molecules are phosphorothioate (PS) linkages and 2'-O-methylation on the ribose. The PS linkage introduces a chiral center at phosphorus. In concert with other chiral centers, these produce diastereoisomers at each PS linkage. Using a high pH mobile phase, the DNAPac RP column separated Rp and Rs diastereoisomers. Since the DNAPac RP is directly compatible with MS, an LC/MS study revealed identical high resolution masses for the resolved isomers. Sense siRNA strands harboring this linkage were evaluated in Figures 10, 11 and 12.

Figure 10 shows the separation of a sense strand that has one phosphorothioate linkage incorporated at the 14th base. The two possible diastereoisomers are 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.

In Figure 11, the sense strand of the siRNA is 2'-O-methylated on alternate bases and contains phosphorothioate linkages at the 6th and 14th bases. The UV trace and the ion current traces show partial separation of all four phosphorothioate diastereoisomers. The high resolution MS data again reveal identical masses for all four peaks confirming these molecules to be diastereoisomers.

In Figure 12, the sense strand is modified with two phosphorothioate linkages at the 19th and the 20th bases. In this case, three of the four possible diastereoisomers were chromatographically resolved. In addition to the diastereoisomer peaks, an impurity which contains a single phosphorothioate linkage was detected. The mass difference between peak 1 and 4 was 16 Da, corresponding to the mass difference corresponding to the replacement of a sulfur with an oxygen on one of phosphorothioate positions.

Figure 10. LC/MS analysis of phosphorothioate modified siRNA (a) Ion current and UV traces (b) mass spectra



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

Temperature:	30 °C
Flow rate:	0.25 mL/min
Inj. volume:	3 µL
Detection:	UV (260 nm)
	MS (Negative-ion mode)
Mass Spec:	Q Exactive Plus
Sample:	21mer siRNA
	AGCUGACCCUGAAG$_S$UUCAUdCdT

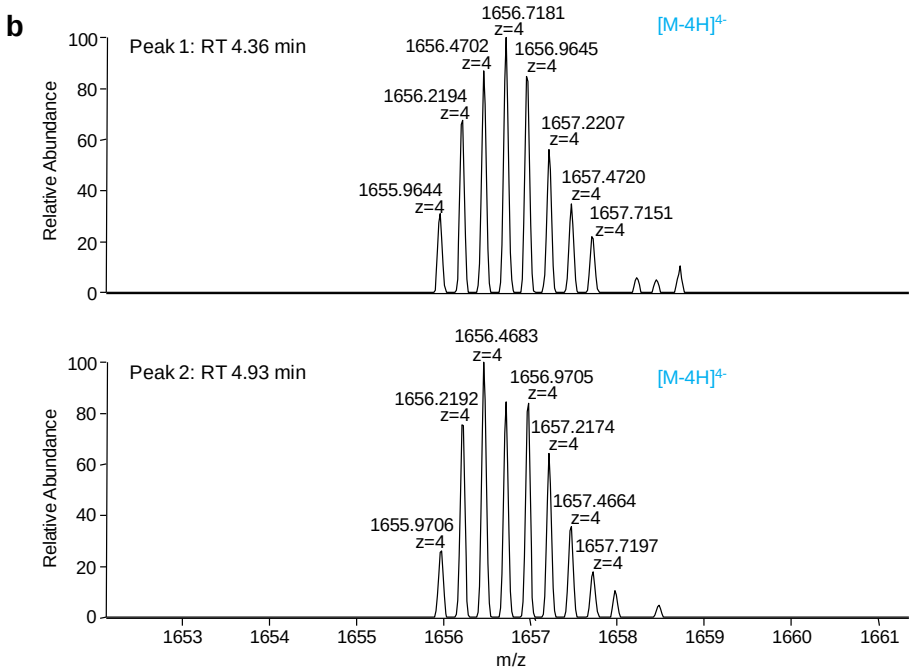
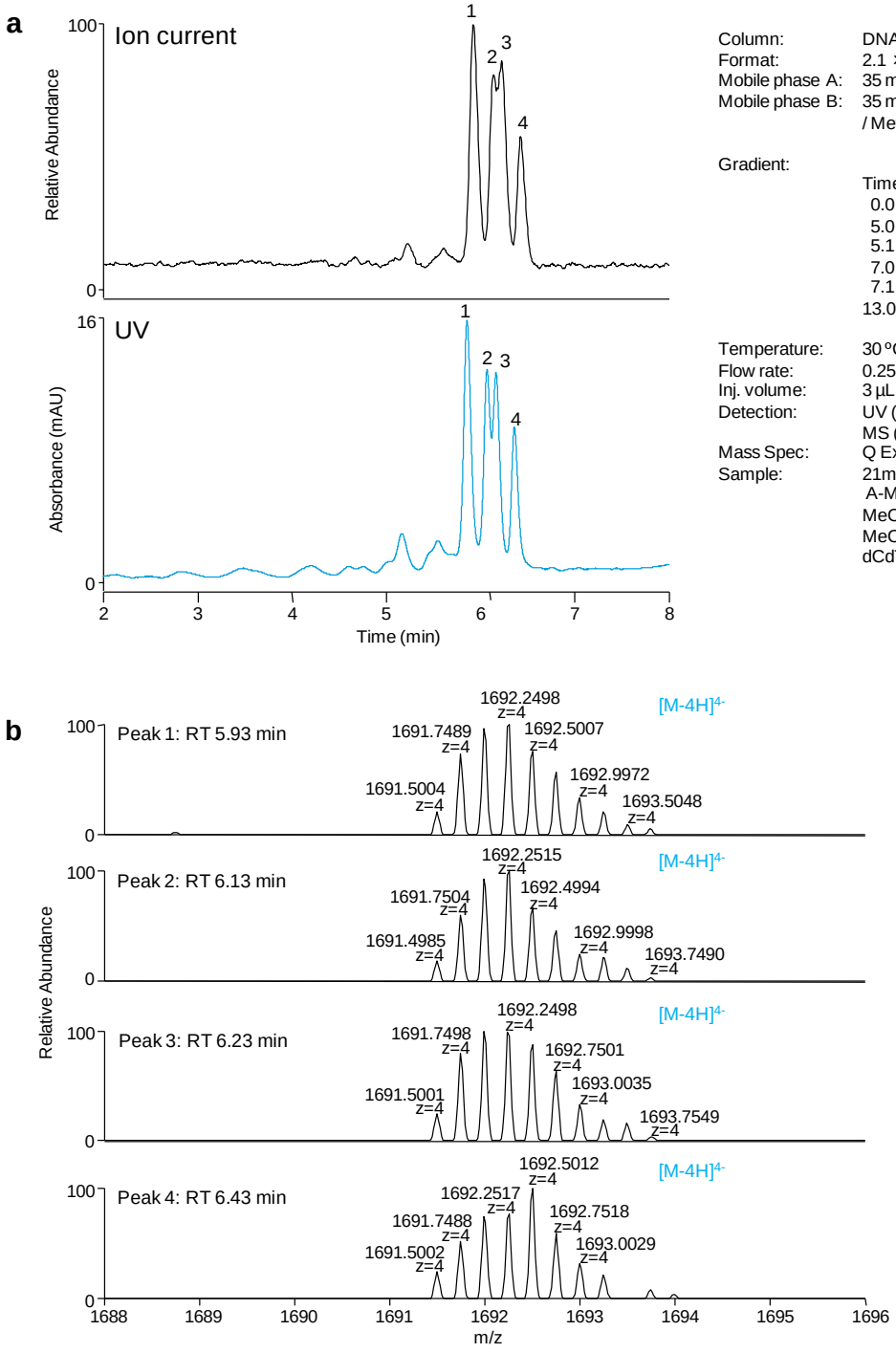


Figure 11. LC/MS analysis of phosphorothioate and 2'-O-methyl modified siRNA (a) Ion current and UV traces (b) mass spectra



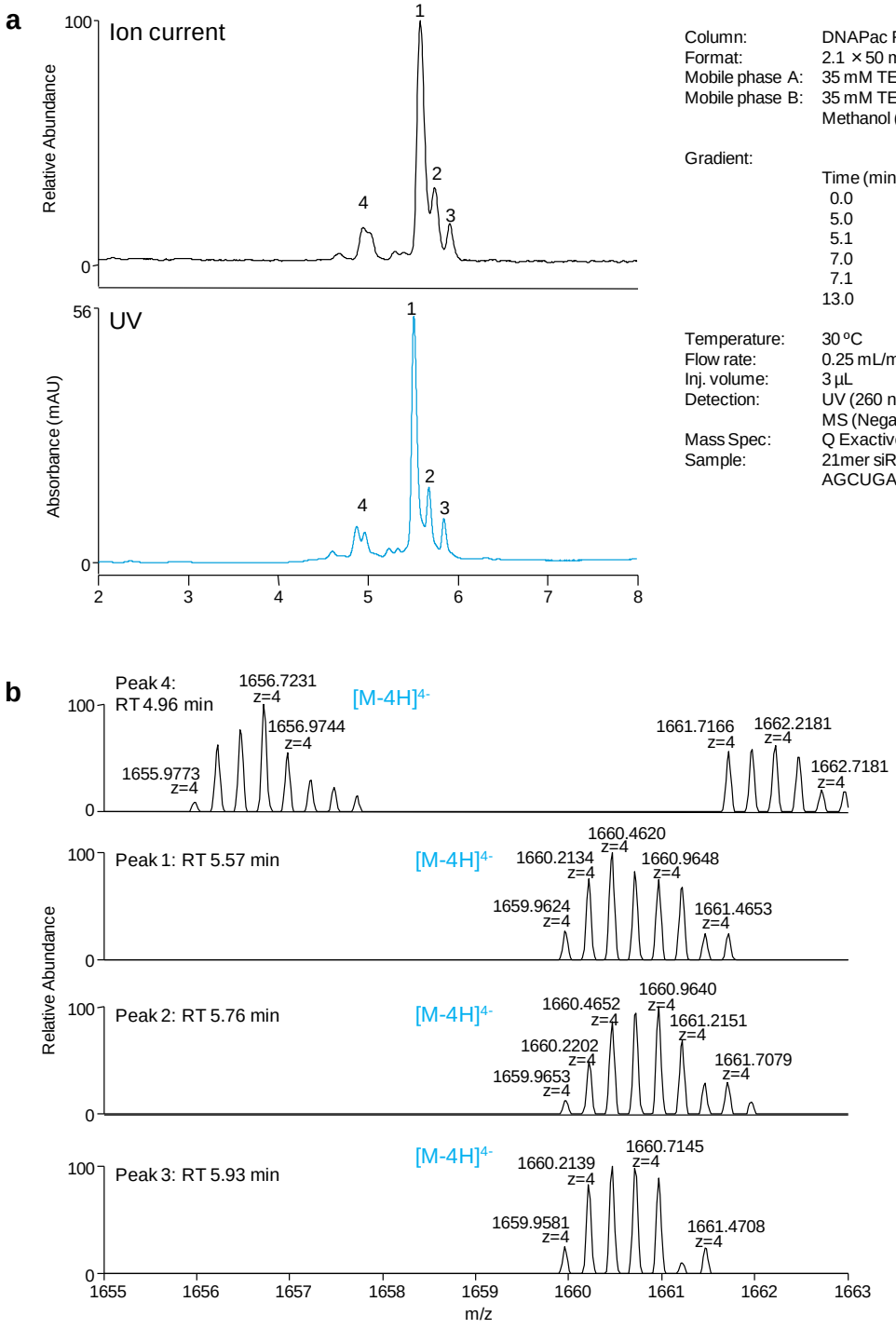
Column:	DNAPac RP, 4 µm
Format:	2.1 × 50 mm
Mobile phase A:	35 mM TEA, 40 mM HFIP, pH 9.9
Mobile phase B:	35 mM TEA, 40 mM HFIP in Water / Methanol (75:25 v/v)

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

Temperature:	30 °C
Flow rate:	0.25 mL/min
Inj. volume:	3 µL
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

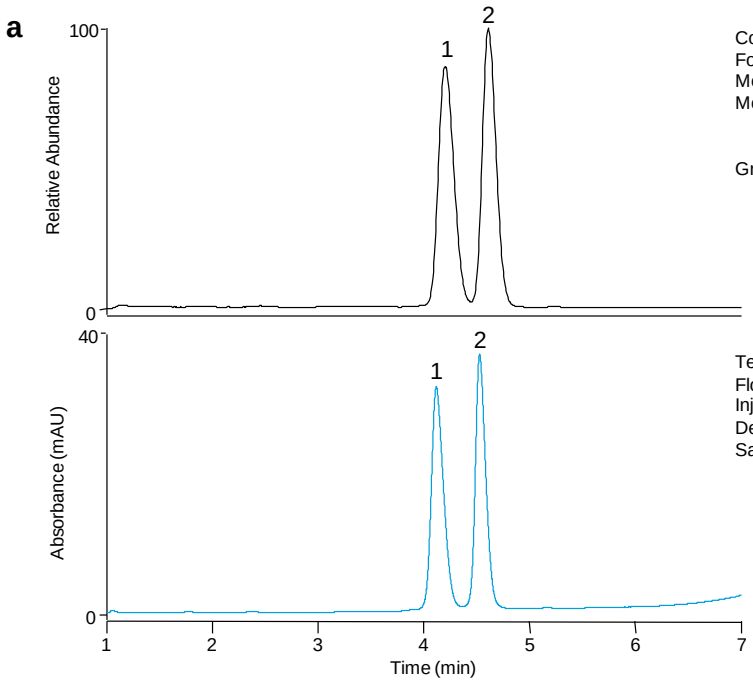
Figure 12. LC/MS analysis of phosphorothioate modified siRNA (a) Ion current and UV traces (b) mass spectra



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

Certain CpG dinucleotides are methylated at the C-5 carbon of cytosine by methyltransferases. This methylation of CpG sequences in promoter regions suppresses gene expression 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 by LC/MS using the DNAPac RP column with mass spectrometry. In Figure 13, an unmodified and a methylated CpG-containing oligonucleotide are well resolved on the DNAPac RP column. Figure 13b shows the -3 charge spectra of unmodified CpG oligonucleotide at m/z 1517.9194 and the -3 charge state of methylated CpG oligonucleotide at m/z 1522.5926.The mass difference between methylated unmodified peaks correspond to the mass of one methyl group. In addition to methylation the, DNAPac RP can effectively resolve 5'-phosphorylated from 5'-dephosphorylated oligonucleotides (Figure 14a). The MS result confirms the phosphate group difference in the two peaks to within 6 ppm of the expected 79.9691 (Figure 14b).

Figure 13. Analysis of CpG methylation (a) Ion current and UV traces (b) mass spectra



Column:	DNAPac RP, 4 µm
Format:	2.1 × 50 mm
Mobile phase A:	15 mM TEA, 400 mM HFIP, pH 7.6
Mobile phase B:	15 mM TEA, 400 mM HFIP in Water / Methanol (50:50 v/v)

Gradient:

Time (min)	%A	%B
0.0	75	25
4.0	56	44
4.1	10	90
6.0	10	90
6.1	75	25
11.0	75	25

Temperature:	60 °C
Flow rate:	0.25
Inj. volume:	3 µL
Detection:	UV (260 nm)
Sample:	1) CGGCATCCTTATTGG 2) /iMe-dC/GGCATCCTTATTGG

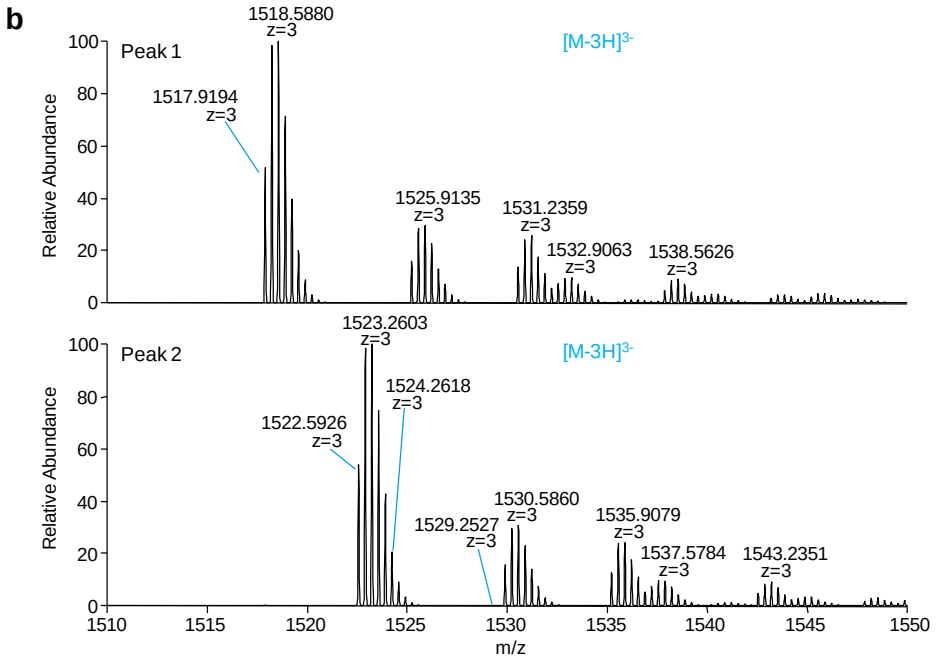
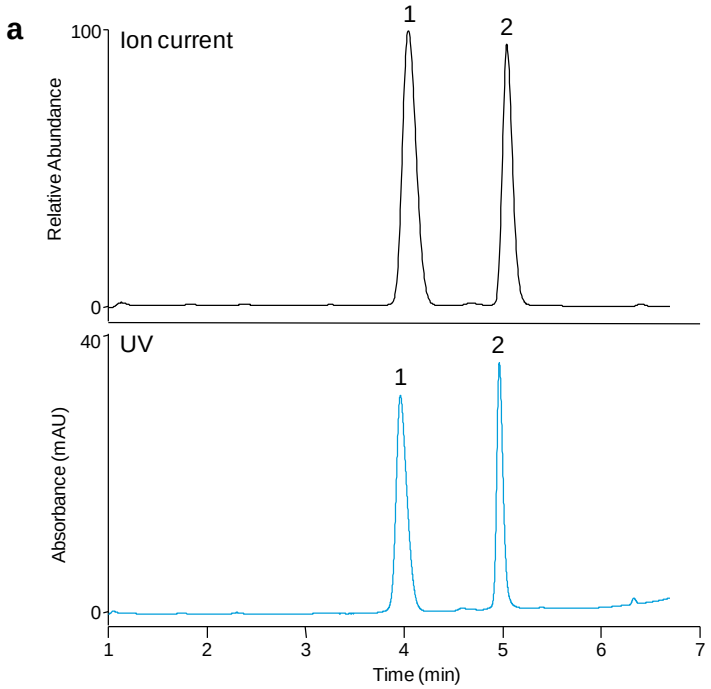


Figure 14. Analysis of 5'-phosphorylation (a) Ion current and UV traces (b) mass spectra



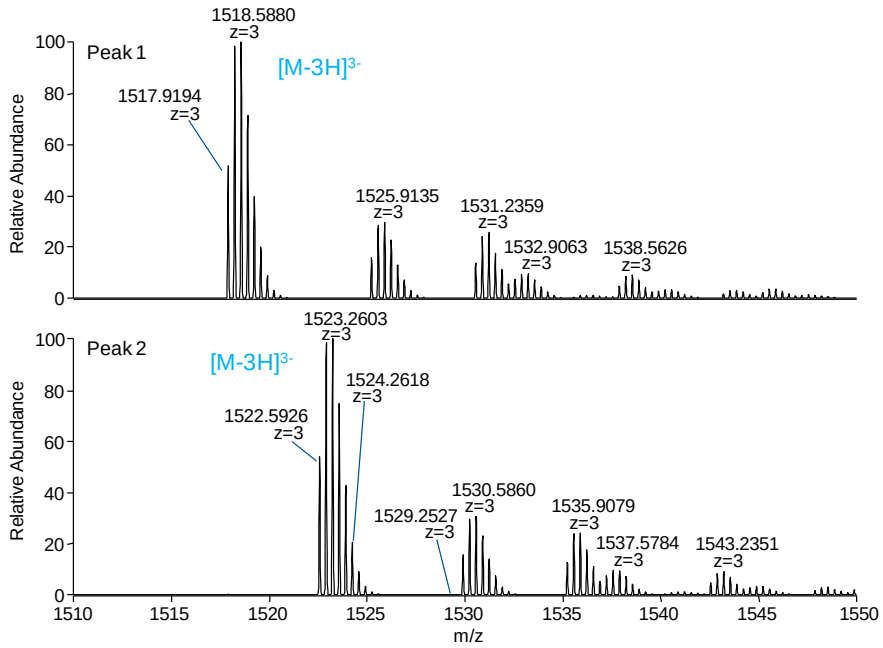
Column:	DNAPac RP, 4 µm
Format:	2.1 × 50 mm
Mobile phase A:	15 mM TEA, 400 mM HFIP, pH 7.6
Mobile phase B:	15 mM TEA, 400 mM HFIP in Water / Methanol (50:50 v/v)

Gradient:

Time (min)	%A	%B
0.0	75	25
4.0	56	44
4.1	10	90
6.0	10	90
6.1	75	25
11.0	75	25

Temperature:	60 °C
Flow rate:	0.25
Inj. volume:	3 µL
Detection:	UV (260 nm)
Sample:	1) CGG CAT CCT TAT TGG 2) /Phos/CGG CAT CCT TAT TGG

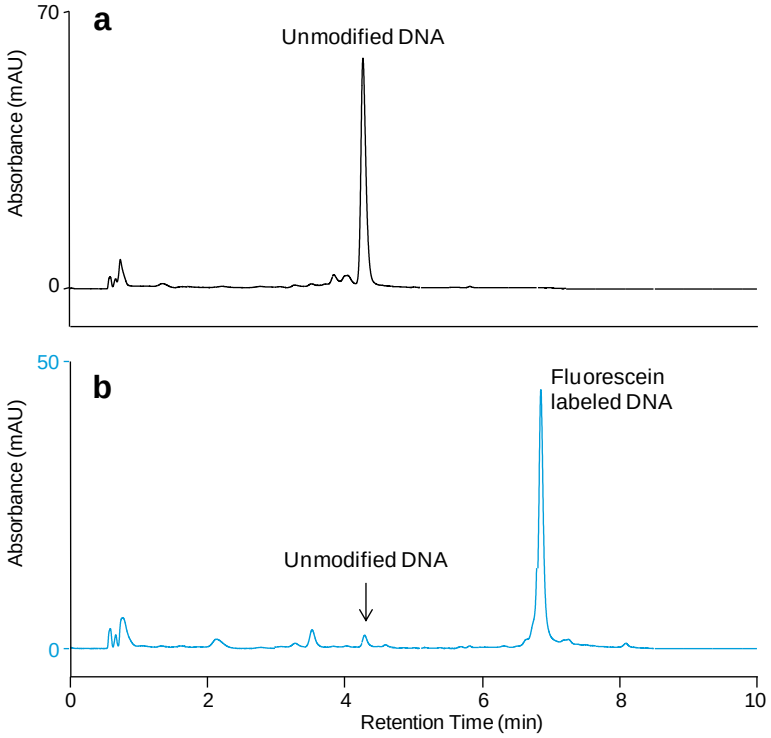
b


Peak 1
1518.5880
z=3
[M-3H]3-
1517.9194
z=3
1525.9135
z=3
1531.2359
z=3
1532.9063
z=3
1538.5626
z=3
Relative Abundance
Peak 2
1523.2603
z=3
[M-3H]3-
1524.2618
z=3
1522.5926
z=3
1530.5860
z=3
1529.2527
z=3
1535.9079
z=3
1537.5784
z=3
1543.2351
z=3
Relative Abundance
m/z

5.6 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. Fluorophores are hydrophobic, so their attachment increases ON hydrophobicity, and thus retention by IP-RP of the oligonucleotide. Figure 15 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 15b).

Figure 15. 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 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
Temperature:	60 °C
Detection:	UV (260 nm)
Sample:	a. 20mer DNA (60 µg/mL) b. Fluorescein labeled 20mer DNA (60 µg/mL)

5.7 Separation of double-stranded oligonucleotides

Purification and analysis of large double-stranded DNA fragments is important for DNA cloning, preparation of sequencing libraries and PCR product purity analysis. Large dsDNA fragments are often purified by agarose or acrylamide gel electrophoresis. However, extraction of the dsDNA from gels requires manual excision of the target size (or size range) from the gel, maceration of the gel and separation of the DNA from the gel fragments. This labor-intensive and time consuming step produces relatively low DNA yields, which depend on the amount of DNA excised. IP-RPLC dsDNA purification can provide higher and more reproducible yields and can be readily automated. The wide-pore resin in the DNAPac RP column produces resolution sufficient for purification of dsDNA from 100 up to 10,000 bp.

Figure 16 depicts the resolution of DNA fragments generated from plasmids digested by the restriction endonuclease BsuRI (HaeIII). Fragments ranging from 67 bp to 1353 bp are separated on the DNAPac RP 2.1x100 mm column in 15 minutes.

Figure 16. Separation of large dsDNA samples

Column:	DNAPac RP, 4 µm	Flow rate:	0.40 mL/min
Format:	2.1 × 100 mm	Inj. volume:	5 µL
Mobile phase A:	0.1 M TEAA, pH 7.0	Temperature:	55 °C
Mobile phase B:	0.1 M TEAA, pH 7.0 in Water / Acetonitrile (75:25 v/v)	Detection:	UV (260 nm)
		Sample:	a. pBR322-BsuRI digest (100 µg/mL)
Gradient:			b. ΦX174-BsuRI digest (100 µg/mL)
		Peak label:	base pairs

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

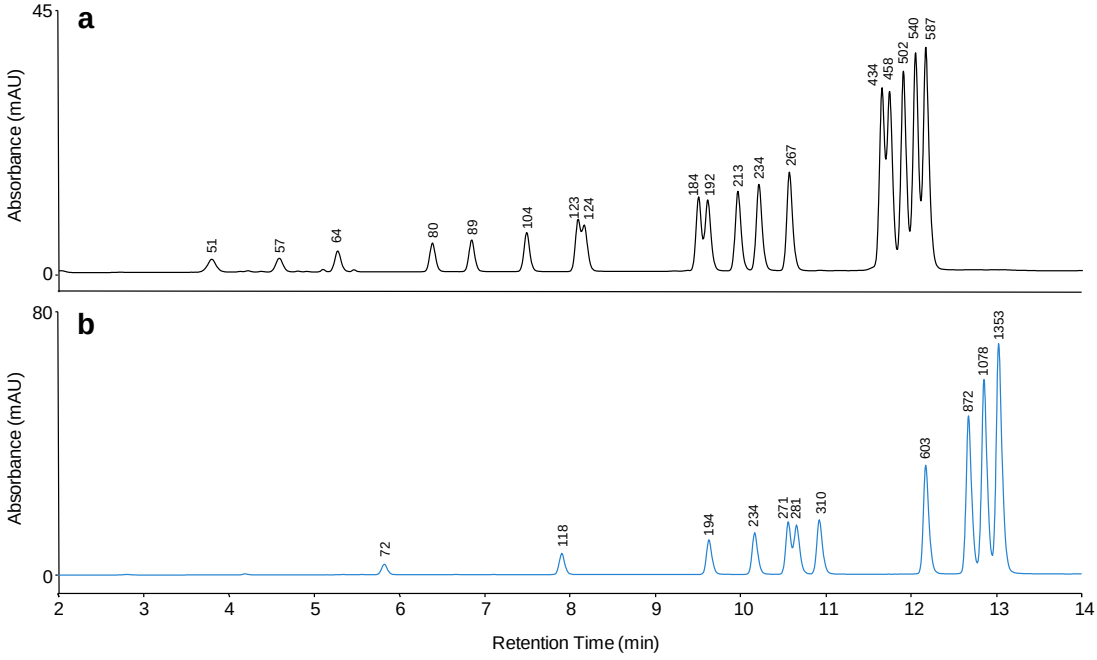


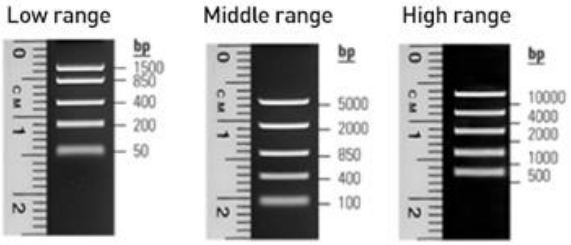
Figure 17 shows the separation of Thermo Scientific™ FastRuler™ DNA ladders. All the DNA fragments were separated based on size by both agarose gel electrophoresis, and IP-RPLC on a DNAPac RP column. These separations demonstrate the use of DNAPac RP column for the separation of dsDNA fragments ranging from 50 to 10,000 base pairs.

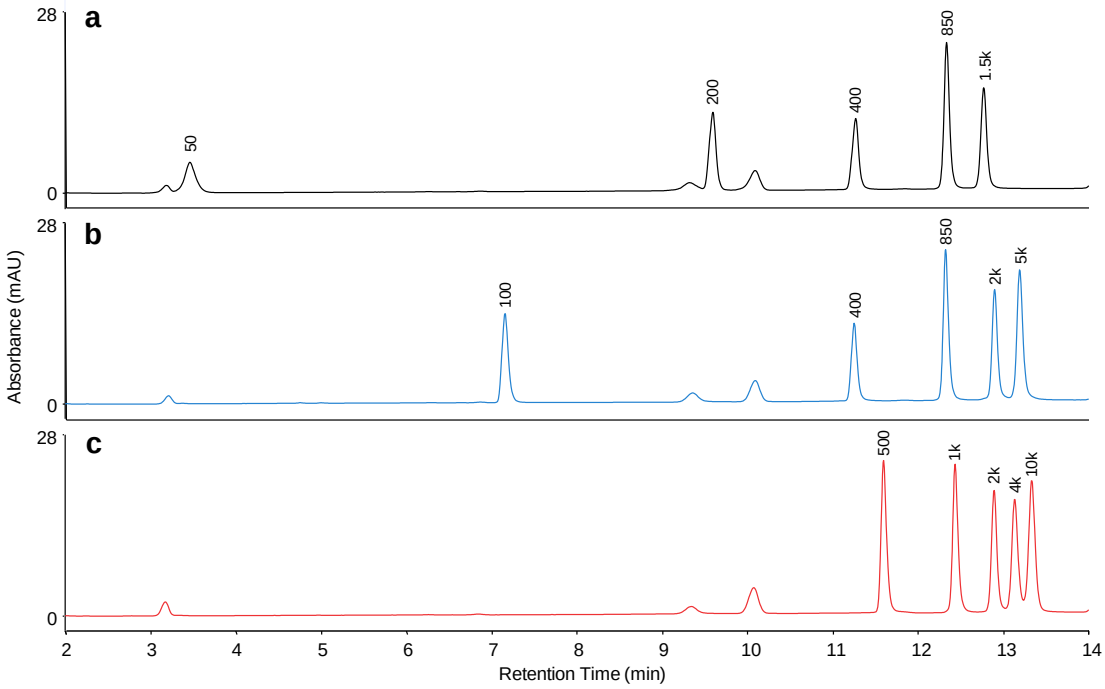
Figure 17. Separation of Thermo Scientific™ FastRuler™ DNA ladders

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	35
0.0	65	35
12.0	30	70
12.1	10	90
15.0	10	90

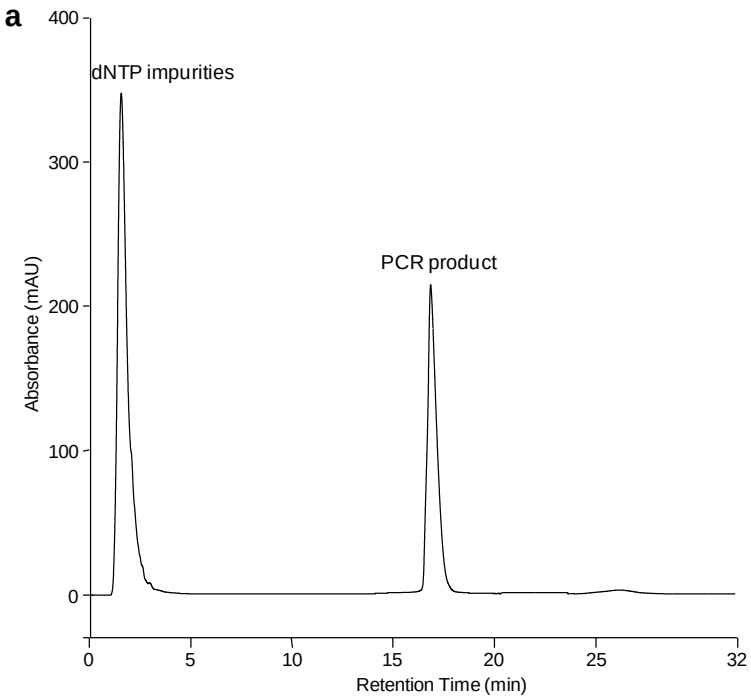
Flow rate:	0.40 mL/min
Inj. volume:	10 µL
Temperature:	55 °C
Detection:	UV (260 nm)
Sample:	DNA ladders a. Low range b. Middle range c. High range
Peak label:	Base pairs





In Figure 18, a PCR product was purified on a DNAPac RP 2.1 x 100 mm column. The primary peak was harvested using an UltiMate™ WPS-3000TBFC Well Plate Autosampler configured for automated Fraction Collection. The collected PCR product purity is confirmed by 1.2% Agarose gel electrophoresis (Figure 14b).

Figure 18. Purification of a PCR product

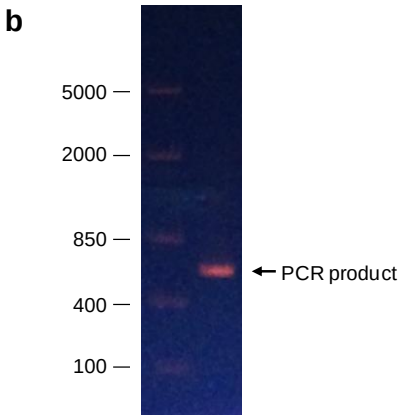


Column:	DNAPac RP, 4 µm
Format:	2.1 × 100 mm
Mobile phase A:	0.1 M TEAA, pH 7.0
Mobile phase B:	0.1 M TEAA in Water / Acetonitrile (75:25 v/v)

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

Temperature:	50 °C
Flow rate:	0.25
Inj. volume:	50 µL
Detection:	UV (260 nm)
Sample:	PCR product



6. Frequently Asked Questions

6.1 What factors do I need to consider for developing high throughput method using DNAPac RP

Column format, flow rate, column operating temperature, gradient slope and gradient curve should be considered (see Section 4). DNAPac RP is a polymer column which is stable at high temperature and pH. Higher resolution may be observed at elevated temperature, higher pH or both of these conditions.

6.2 What is the recommended operating temperature for DNAPac RP?

For single-stranded nucleic acids, 30 ºC to 95 ºC is recommended. For native analysis of double-stranded nucleic acids, maintain a temperature below the melting point (T_m). Note that retention decreases with increasing temperature (see section 4.3).

6.3 What is the maximum recommended flow rate?

Maximum flow rate will depend on the column ID, length, operating temperature and organic solvent used in the mobile phases. See section 3.5 for recommended flow rates at 60°C. Do not exceed maximum pressure.

6.4 What is the recommended equilibration time?

Equilibration with 10 column volumes of initial mobile phase prior to sample injection produced reproducible retention of single-stranded oligonucleotides. However, 20 column volumes were required to produce stable retention for short double-stranded nucleic acids.

6.5 What should I do if the peaks are broad?

Peak broadening may occur with excessive pre- and post-column volume. Reduce all unnecessary volume by minimizing tubing lengths between the autosampler and column and between the column and detector.

6.6 What should I do if I see carryover?

Carry over is the appearance of unexpected peaks in a chromatogram which arise from prior sample injections. When these occur, the *system* may retain some sample (incorrect fitting connections) or components from a previous sample may not have eluted under the eluent condition used. Wash the system and the column with 90% acetonitrile at pH 6-7, re-equilibrate the column, and re-run the chromatogram. Several blank runs are recommended before sample analysis after the 90% acetonitrile wash.

Another factor that may cause carryover is a contaminated autosampler needle. If your autosampler does not wash the needle after injection, you may observe carryover. Change the autosampler settings so that the needle is washed after sample injection.