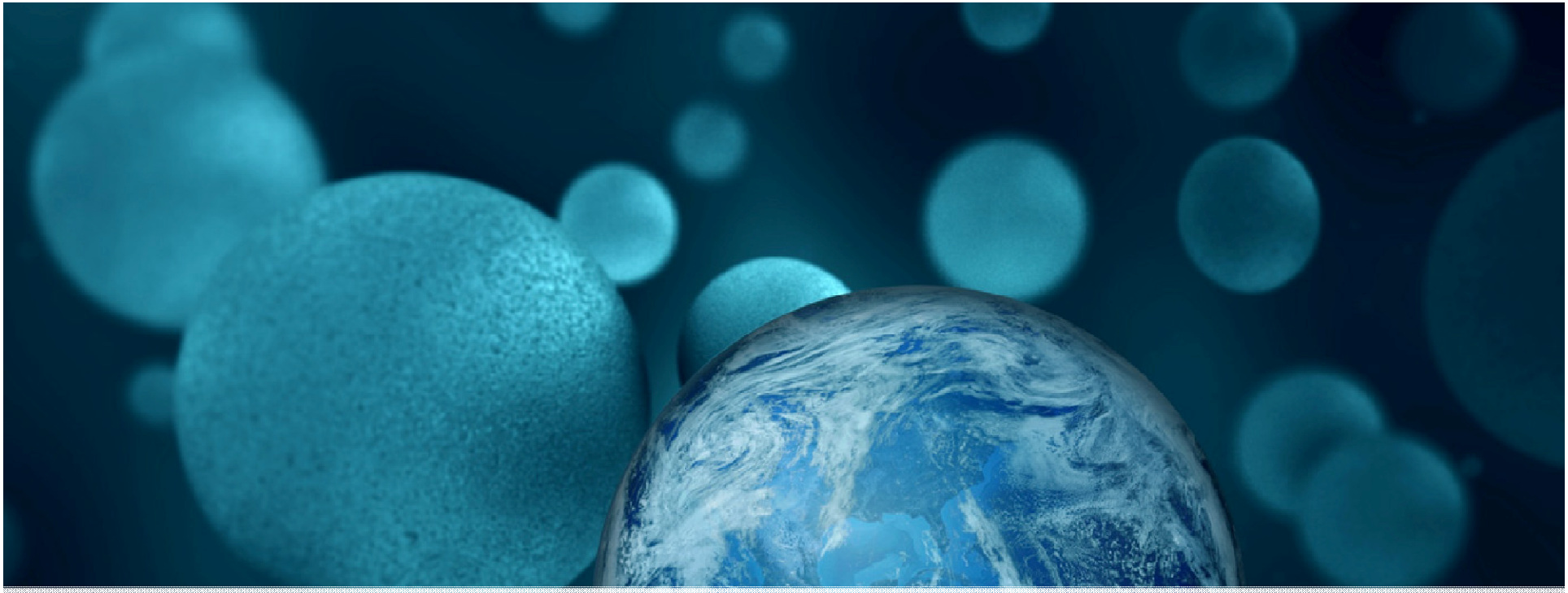


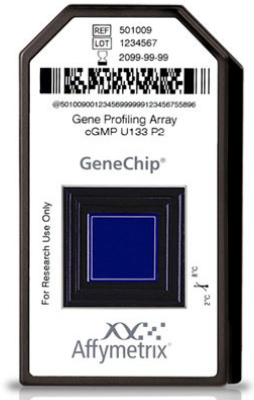
ThermoFisher SCIENTIFIC

High Resolution LC or LC-MS Analysis of Oligonucleotides and Large DNA Fragments Using a New Polymer-Based Reversed Phase Column

5/24/2016

Julia Baek , Jim Thayer, Shanhua Lin, Yizhu Guo, Yoginder Singh, Jessica Wang, Xiaodong Liu, Ilze Birznieks

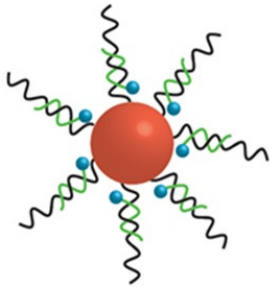
Oligonucleotides as Tools for Molecular Biology Research



- DNA Amplification (PCR)
- DNA Sequencing

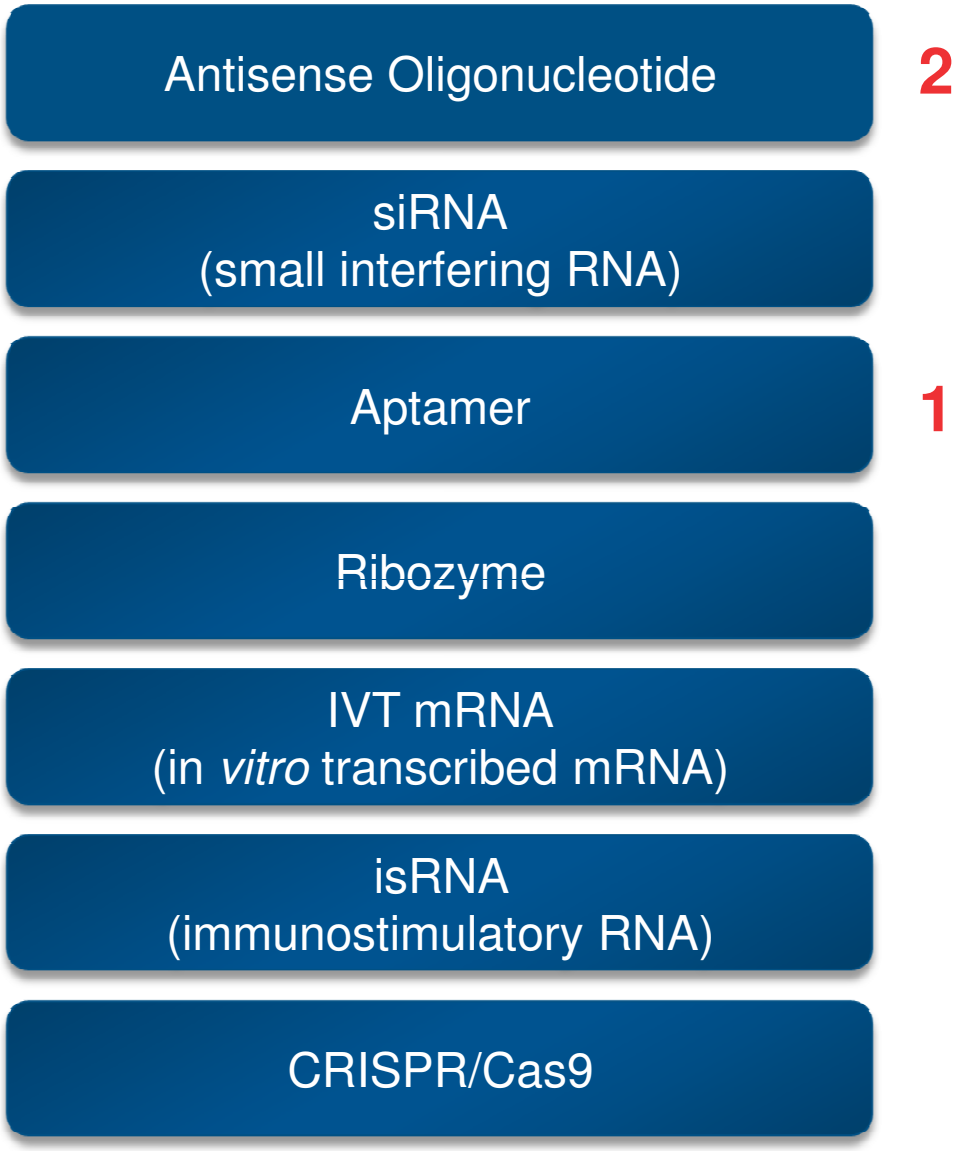
Primers

- Fluorescent *in situ* hybridization (FISH)
- Molecular Diagnostics
- DNA Nanomaterials

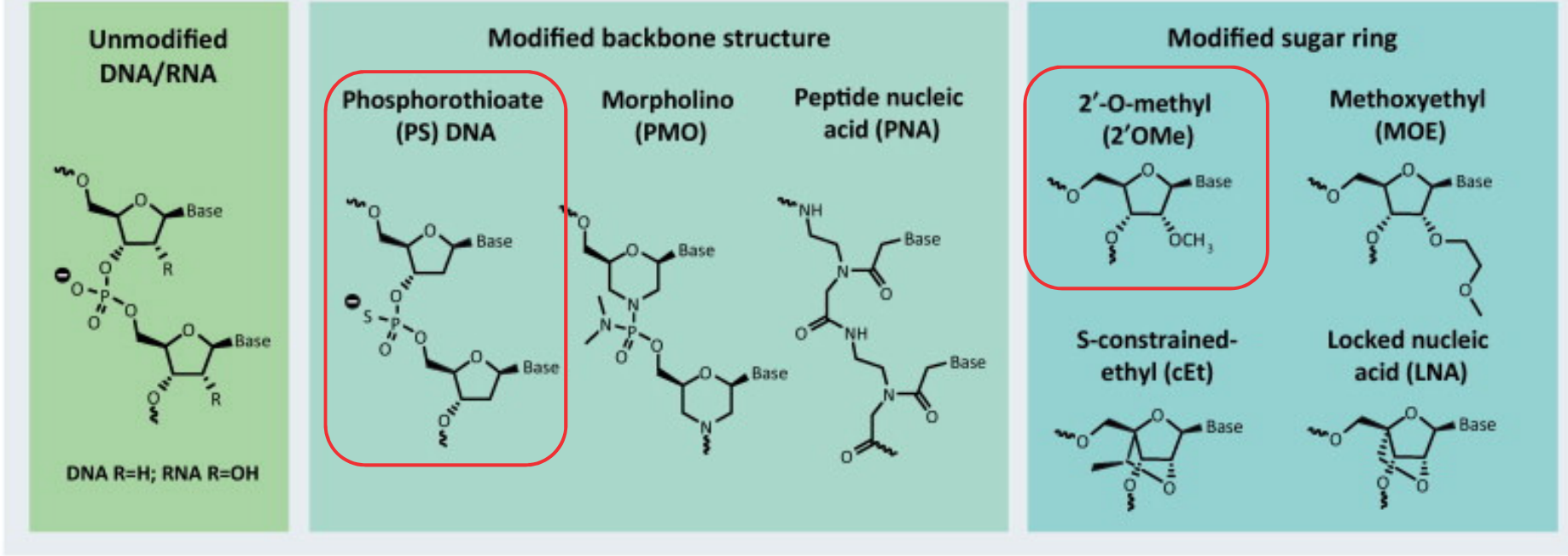


ThermoFisher SCIENTIFIC

Oligonucleotides as Therapeutic Agents



Chemical Modifications of Oligonucleotides



ThermoFisher SCIENTIFIC

Oligonucleotide Impurities

- N-1, N-2, N+1
- PO impurity in PS sample
- Imperfect deprotection (DMT)
- Depurination

HPLC Analysis of Nucleic Acids

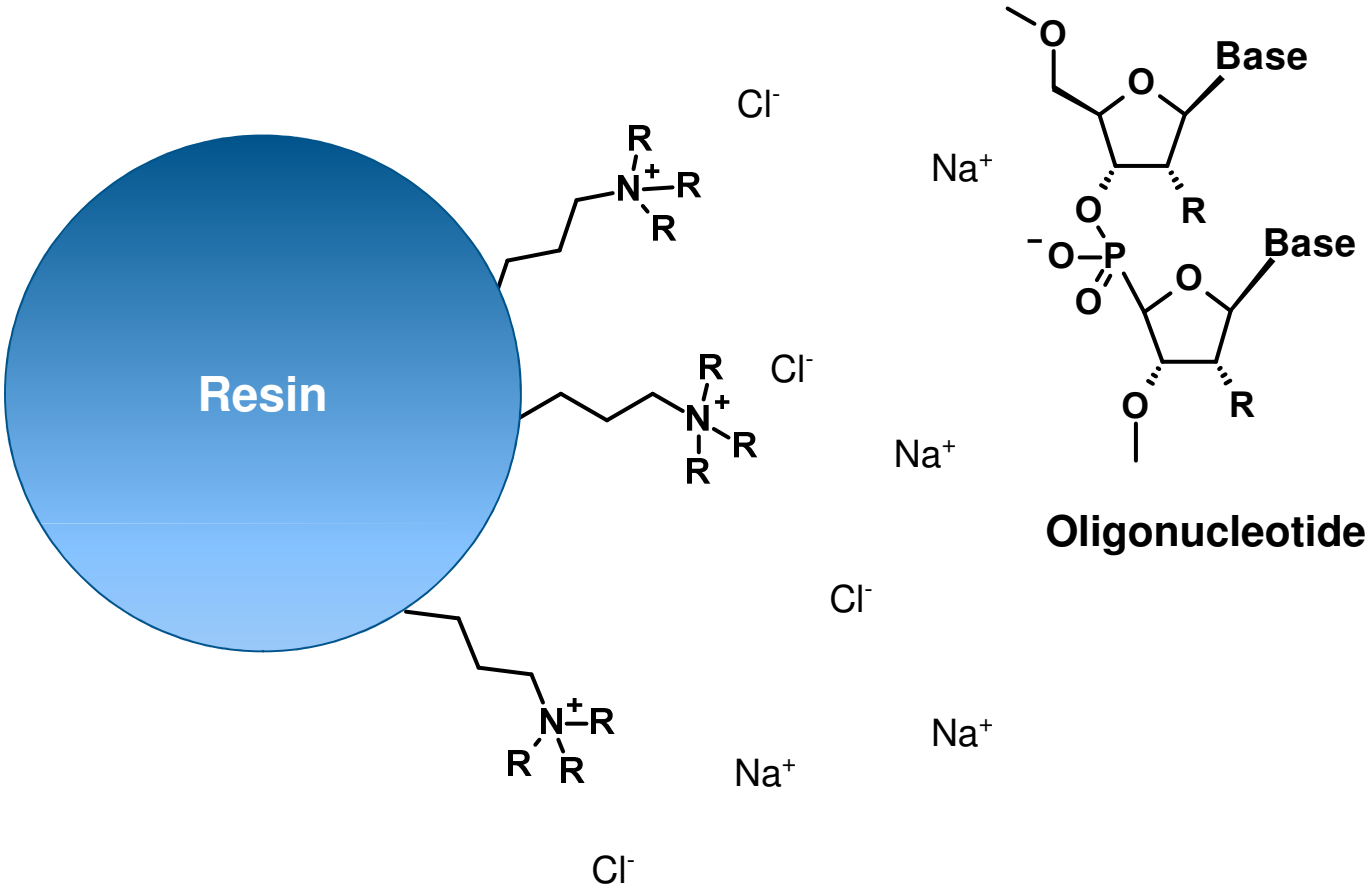
Anion Exchange Chromatography

- Provides high resolution separation oligonucleotides
- Low or no organic solvent
- Cannot be directly coupled to Mass spectrometer

Ion-Pair Reversed Phase Chromatography

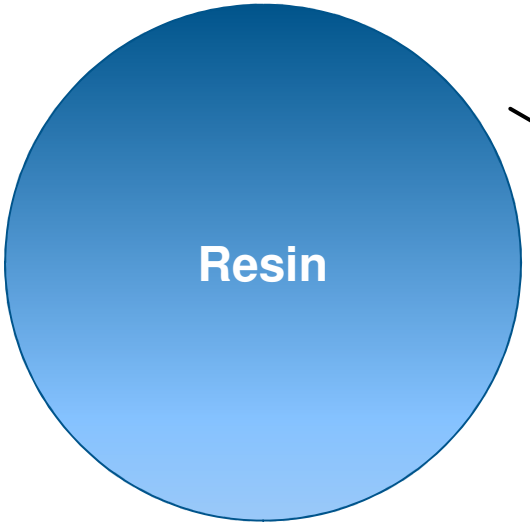
- Provides high resolution separation oligonucleotides
- Can be directly coupled to Mass spectrometer

Anion Exchange Chromatography

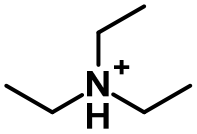


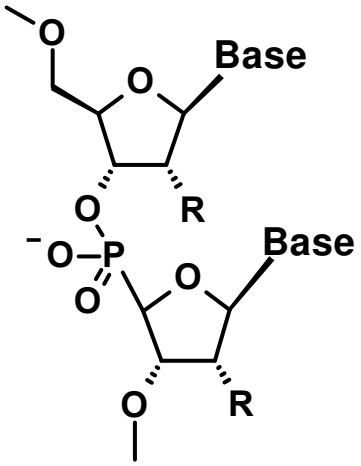
ThermoFisher SCIENTIFIC

Ion-Pair Reversed-Phase Chromatography



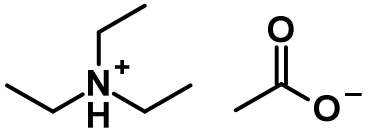
Ion-pairing reagent



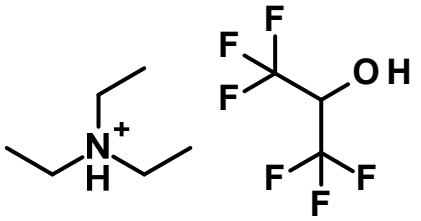


Oligonucleotide

LC/UV Triethylammonium acetate (TEAA)



LC/MS Triethylamine (TEA) + Hexafluoroisopropanol (HFIP)

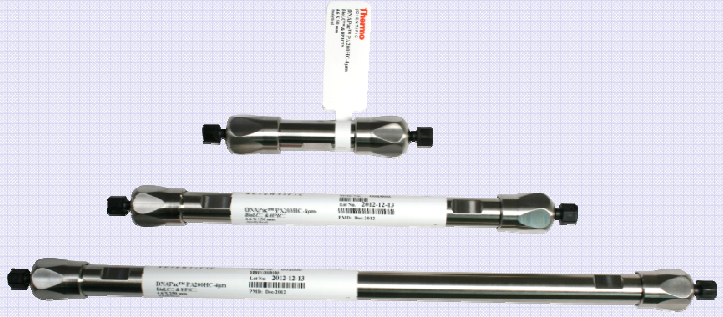


ThermoFisher SCIENTIFIC

HPLC Analysis of Nucleic Acids

Anion Exchange Chromatography

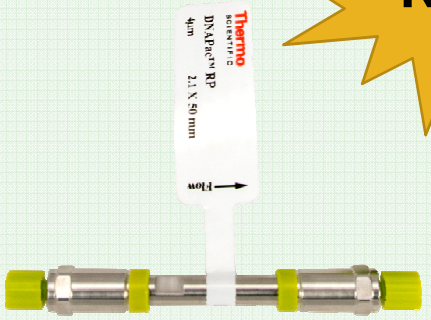
DNAPac PA100
DNAPac PA200
DNAPac PA200 RS
DNASwift SAX-1S



Ion-Pair Reversed Phase Chromatography

DNAPac RP

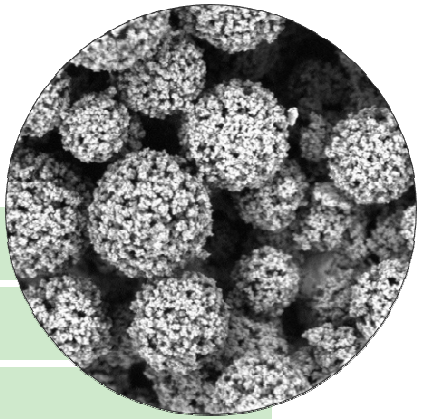
NEW



ThermoFisher SCIENTIFIC

DNAPac RP Column

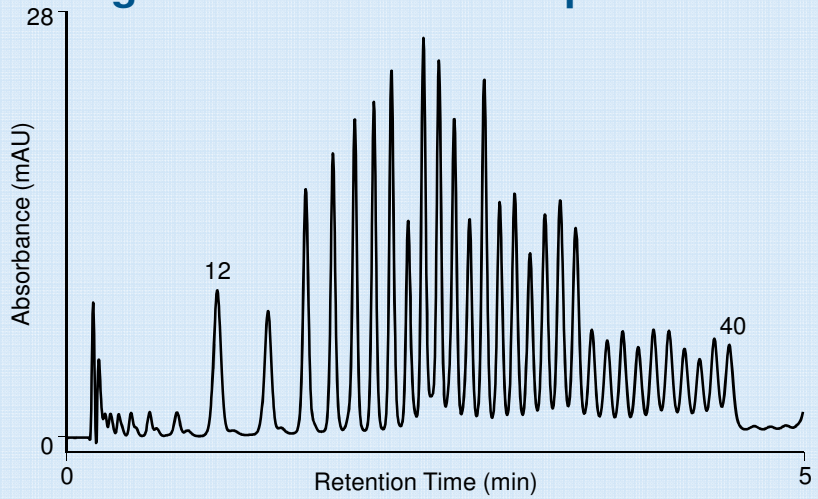
- Reversed phase column designed for separation of oligonucleotides and large DNA/RNA fragments
- High resolution and high efficiency
- Excellent MS compatibility
- Wide operating pH range: 0-14
- High temperature stability: Up to 100 ºC
- High throughput



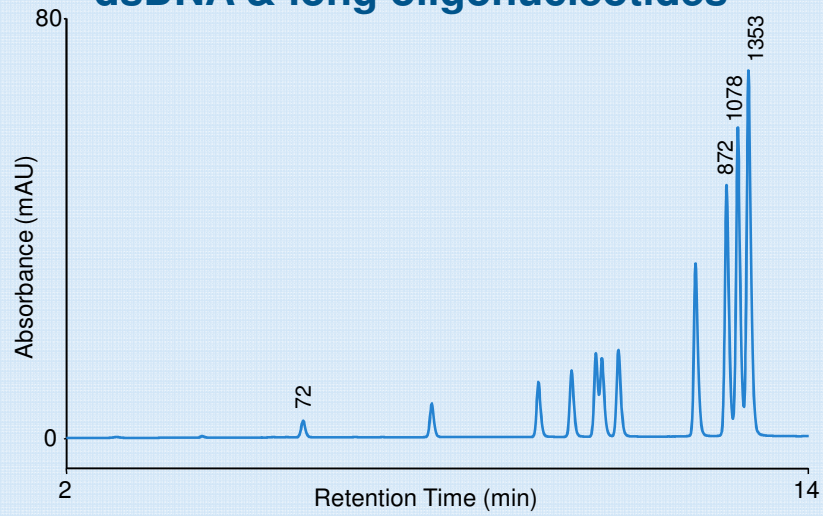
Substrate	**Poly(styrene-divinylbenzene) particles**
Particle Size	4 µm
Pore Size	1,000~2,000 Å
Column Formats	3.0 × 100 mm 3.0 × 50 mm 2.1 × 100 mm 2.1 × 50 mm

Applications

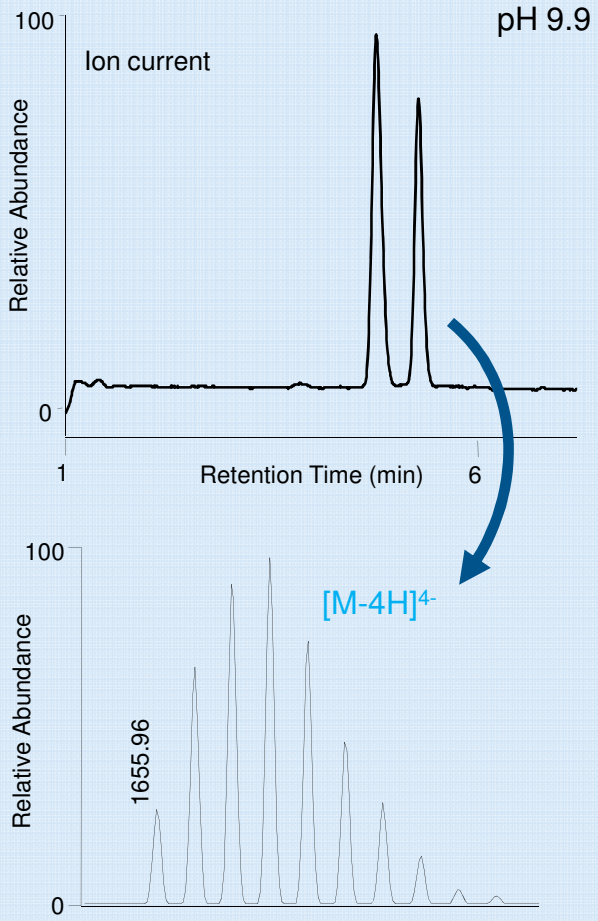
High resolution / Fast separation



dsDNA & long oligonucleotides

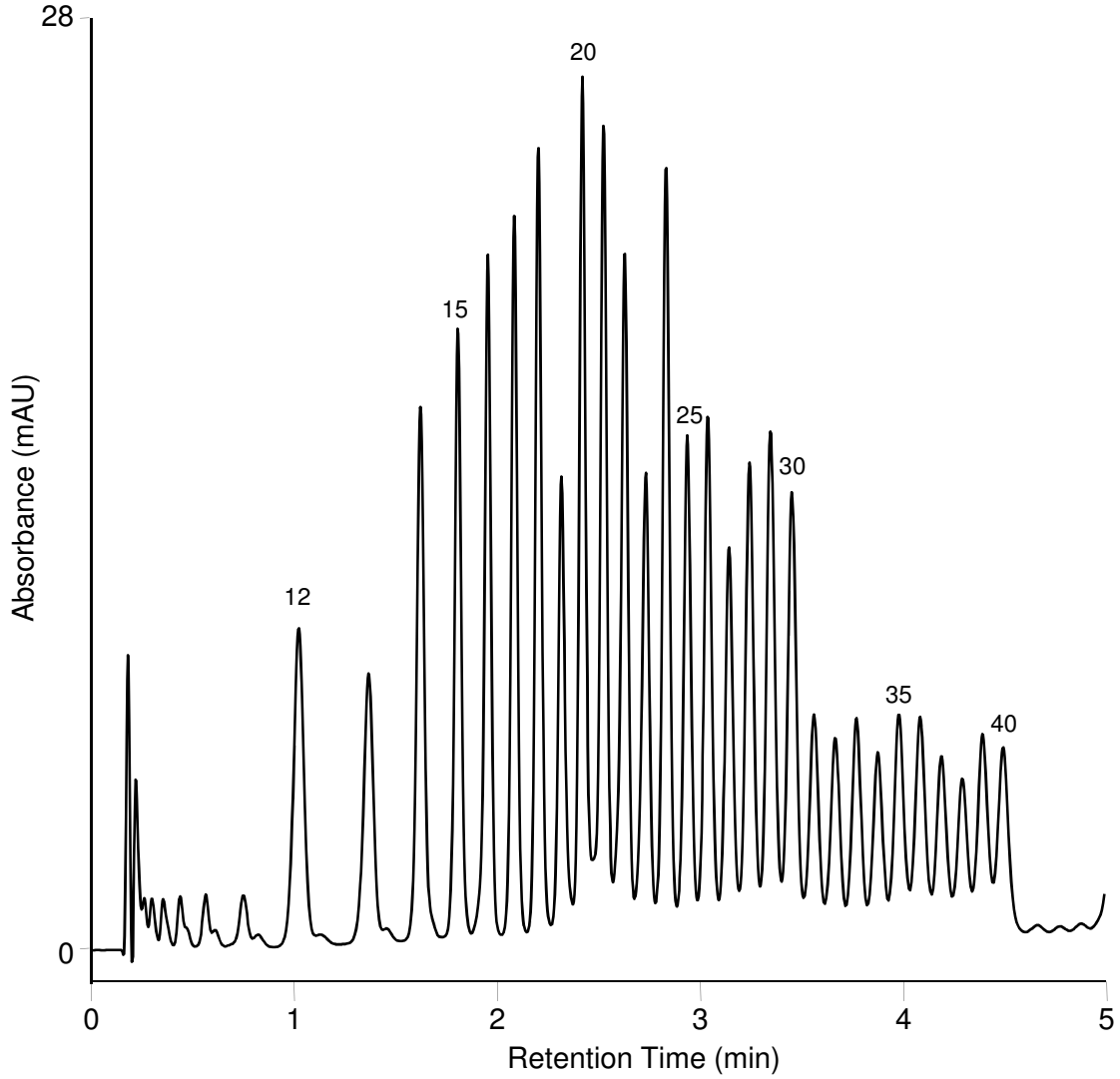


MS compatible



ThermoFisher SCIENTIFIC

Fast Sparation of 12-40mer Deoxythymidines (dTs)



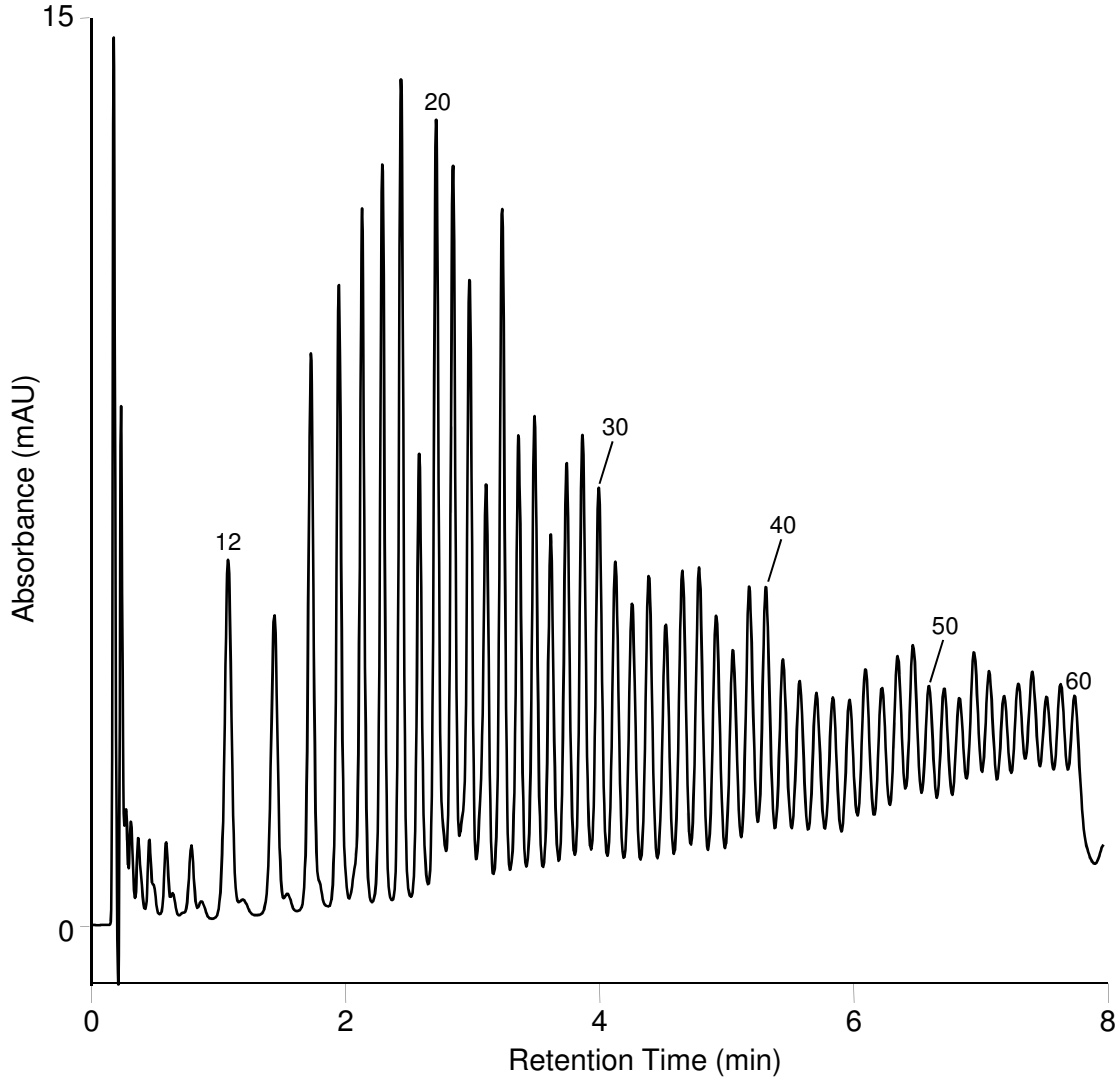
Column:	DNAPac RP, 4 µm
Format:	2.1 ×50 mm
Mobile phase A:	0.1 M TEAA, pH 7.0
Mobile phase B:	0.1 M TEAA in Water / Acetonitrile (75:25 v/v)

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 ºC
Detection:	UV (260 nm)
Sample:	poly dT12-40 (1 A/mL)
Peak label:	Length of DNA

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)

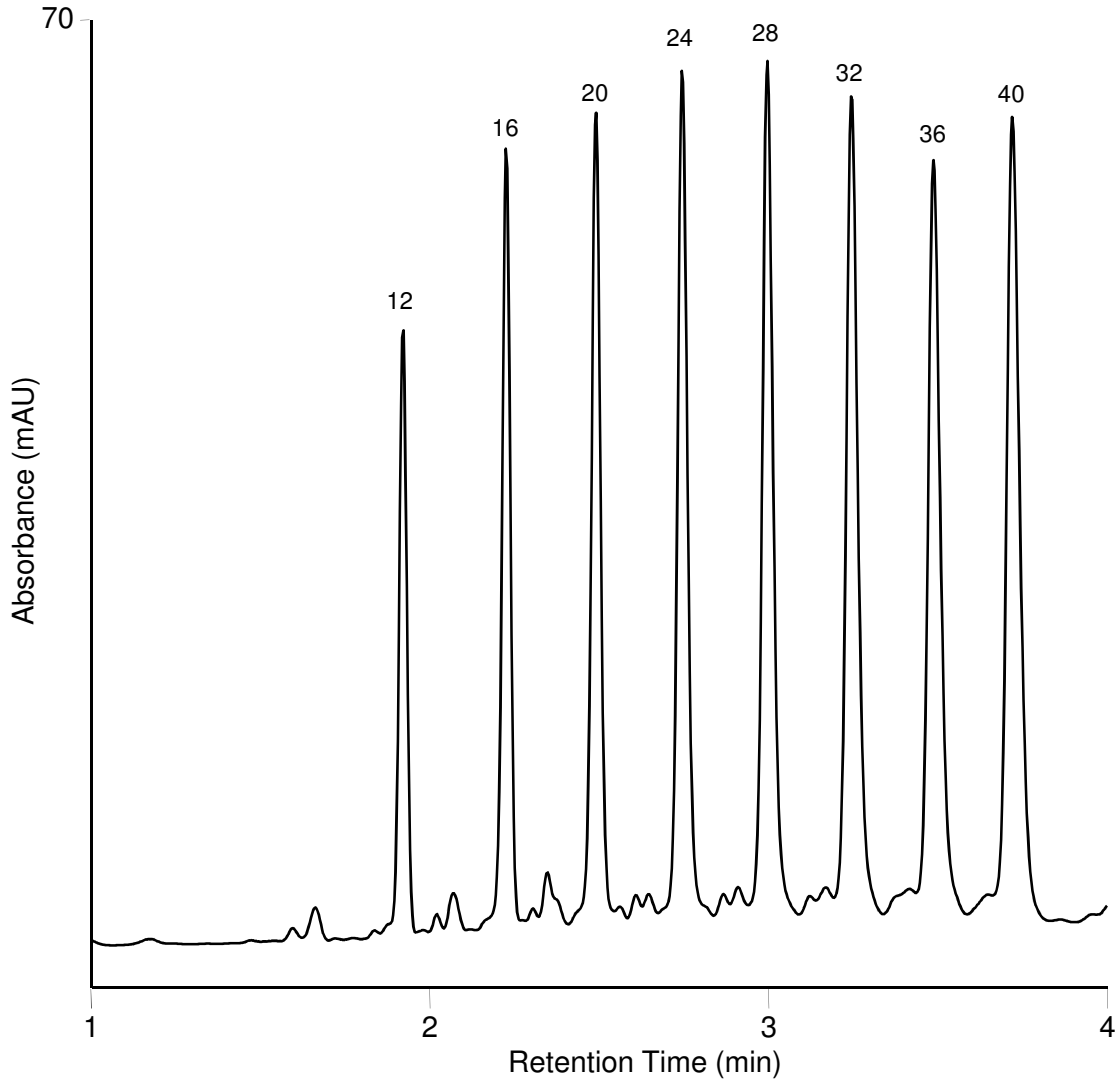
Gradient:

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 ºC
Detection:	UV (260 nm)
Sample:	poly dT12-60 (0.5 A/mL)
Peak label:	Length of DNA

ThermoFisher SCIENTIFIC

LC/UV Analysis of Mixed-Base DNA



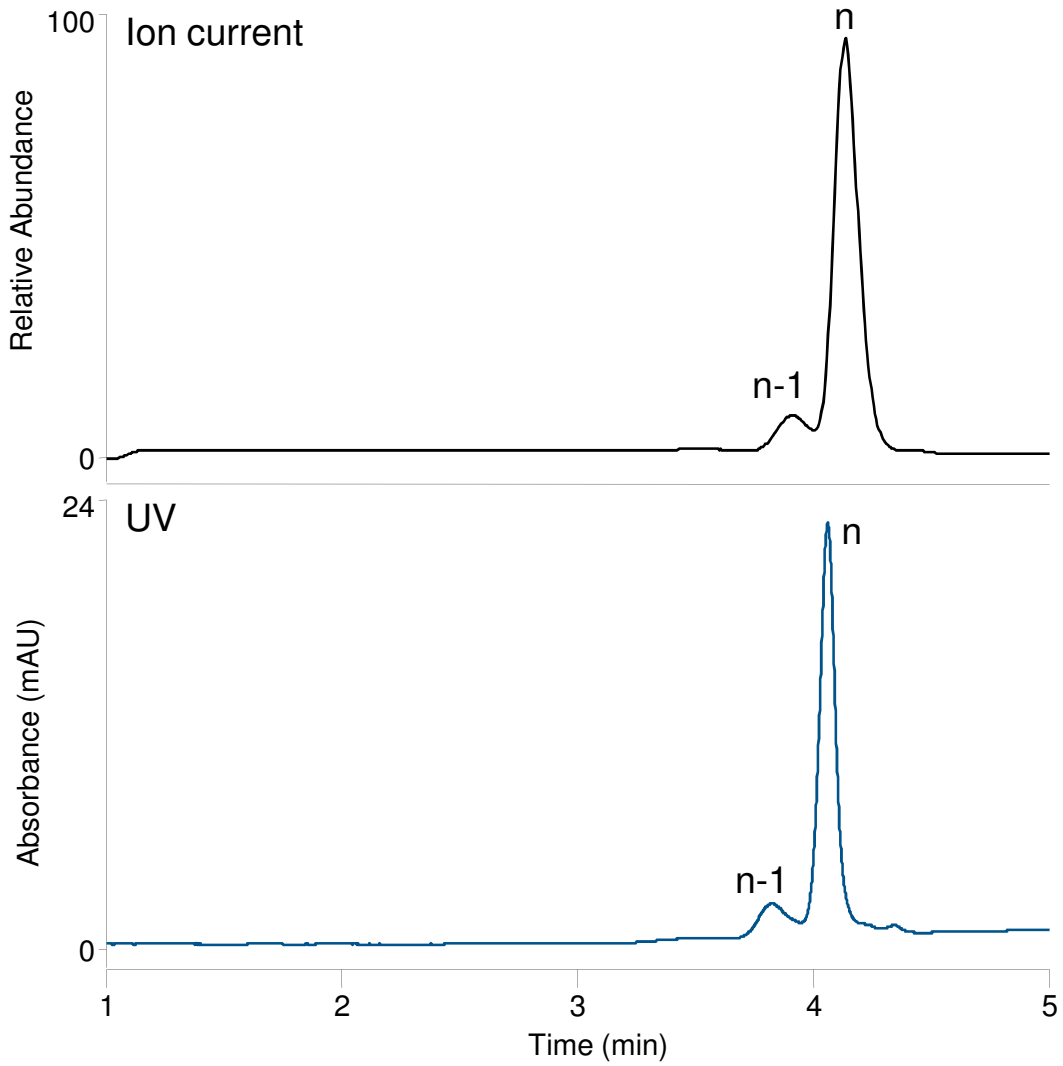
Column:	DNAPac RP, 4 µm
Format:	2.1 ×50 mm
Mobile phase A:	0.1 M TEAA, pH 7.0
Mobile phase B:	0.1 M TEAA in Water / Acetonitrile (75:25 v/v)

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
Temperature:	80 ºC
Detection:	UV (260 nm)
Sample:	8-Combo DNA (5 µM)*
Peak label:	Length of DNA

LC/MS Analysis of n and n-1 Sequences



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)

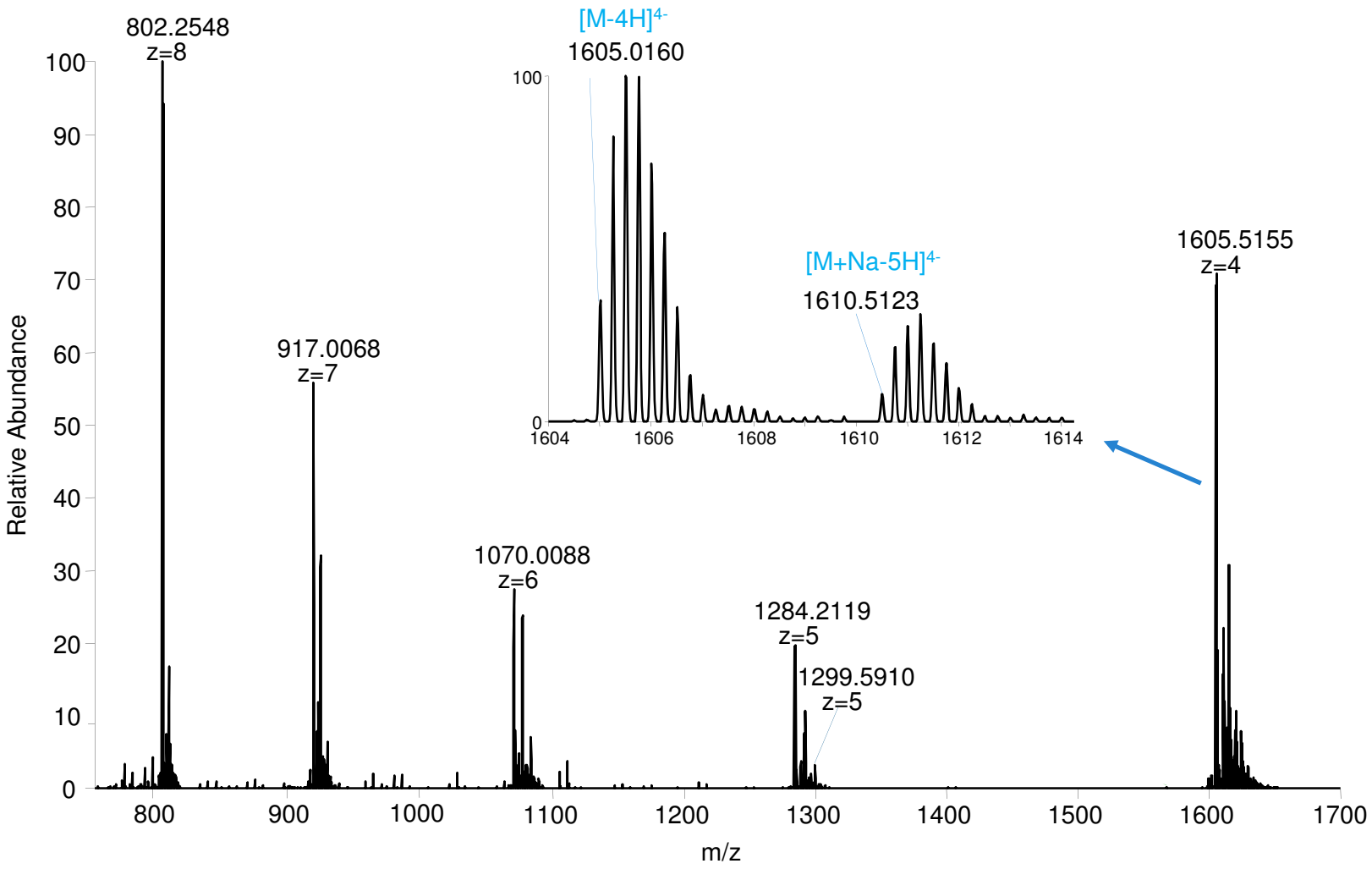
Gradient:

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 ºC
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

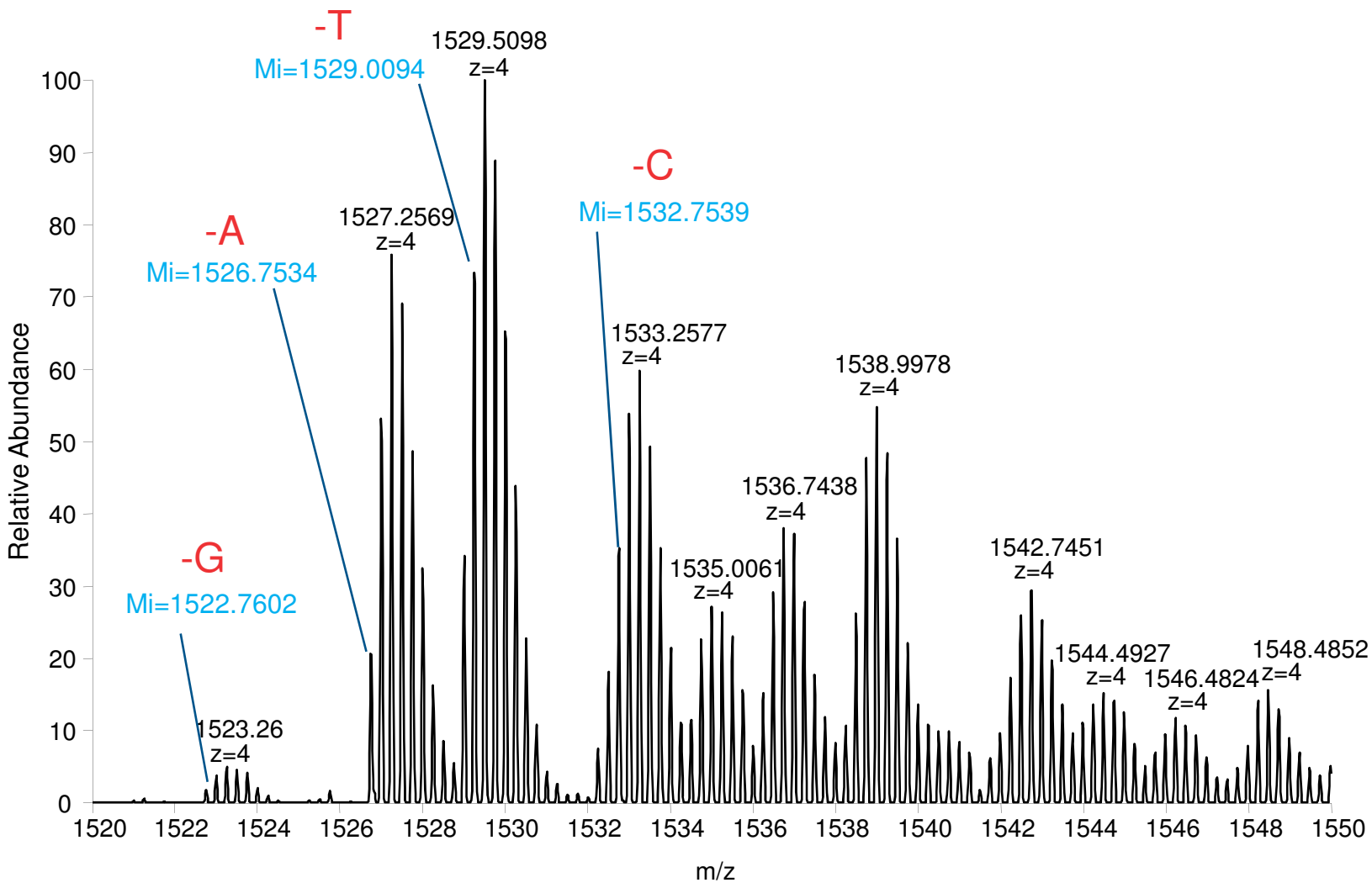
ThermoFisher SCIENTIFIC

LC/MS Analysis of n and n-1 Sequences: Mass Spectrum of n Sequence



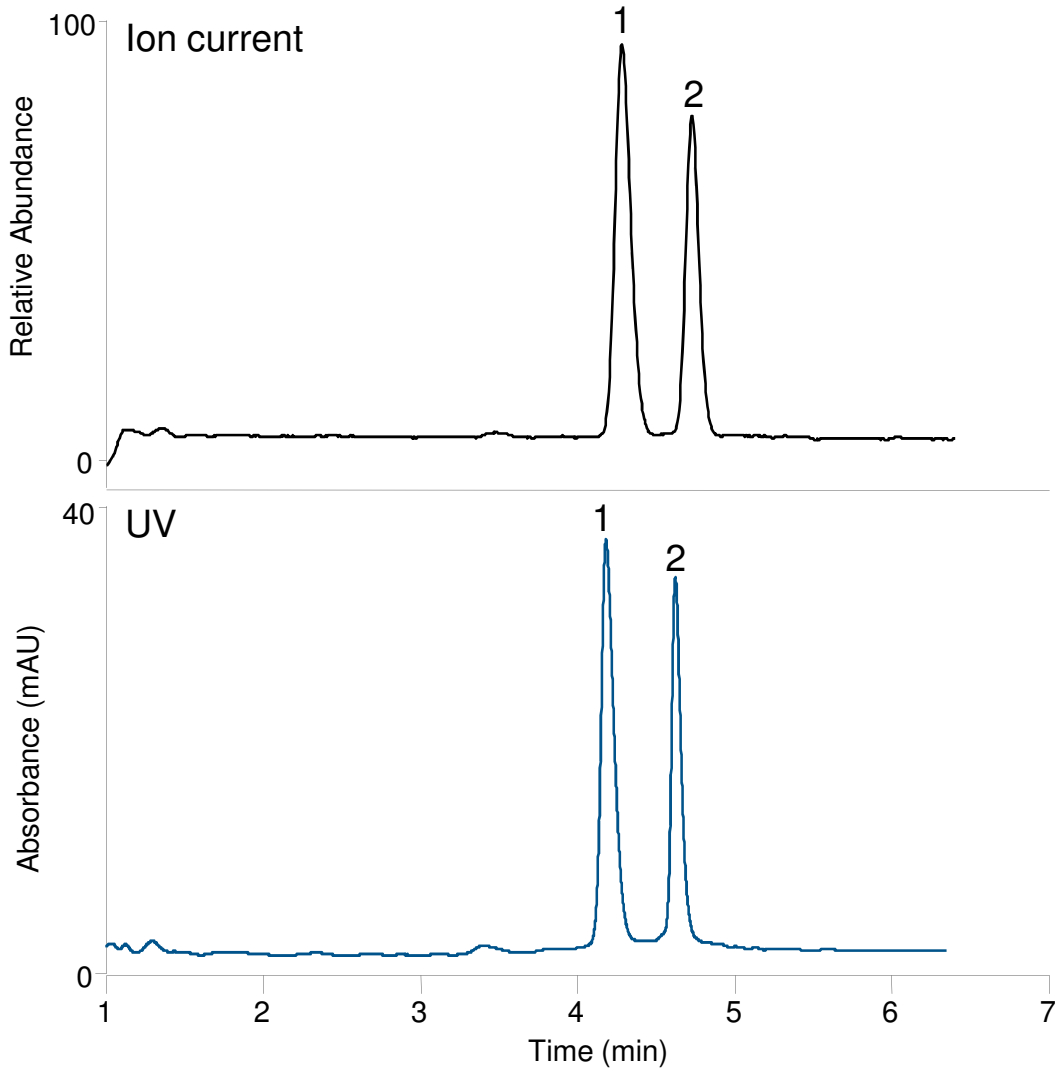
ThermoFisher SCIENTIFIC

LC/MS Analysis of n and n-1 Sequences: Mass Spectrum of n-1 Sequence



ThermoFisher SCIENTIFIC

LC/MS Analysis of Phosphorothioate Modified siRNA

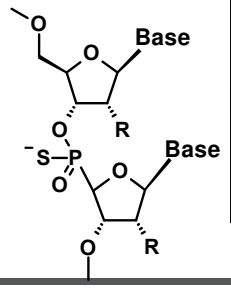


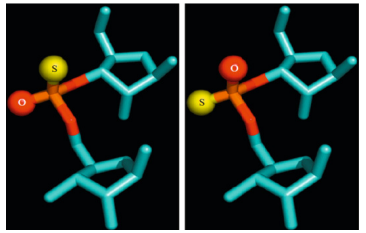
Column:	DNAPac RP, 4 µm
Format:	2.1 ×50 mm
Mobile phase A:	35 mM TEA, 40 mM HFIP, **pH 9.9**
Mobile phase B:	35 mM TEA, 40 mM HFIP in Water / Methanol (75:25 v/v)

Gradient:

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

Temperature:	30 ºC
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

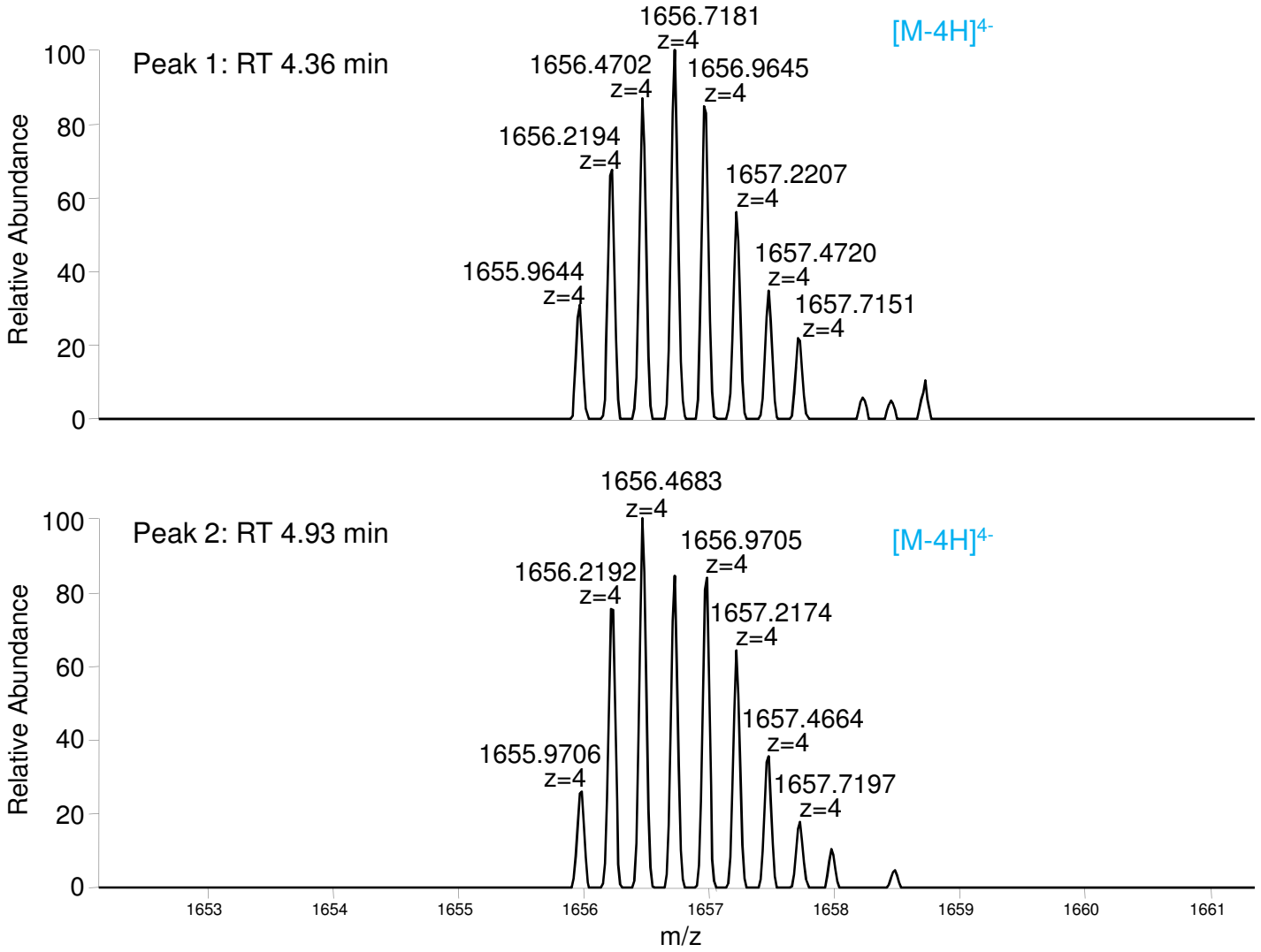




Rp **Sp**

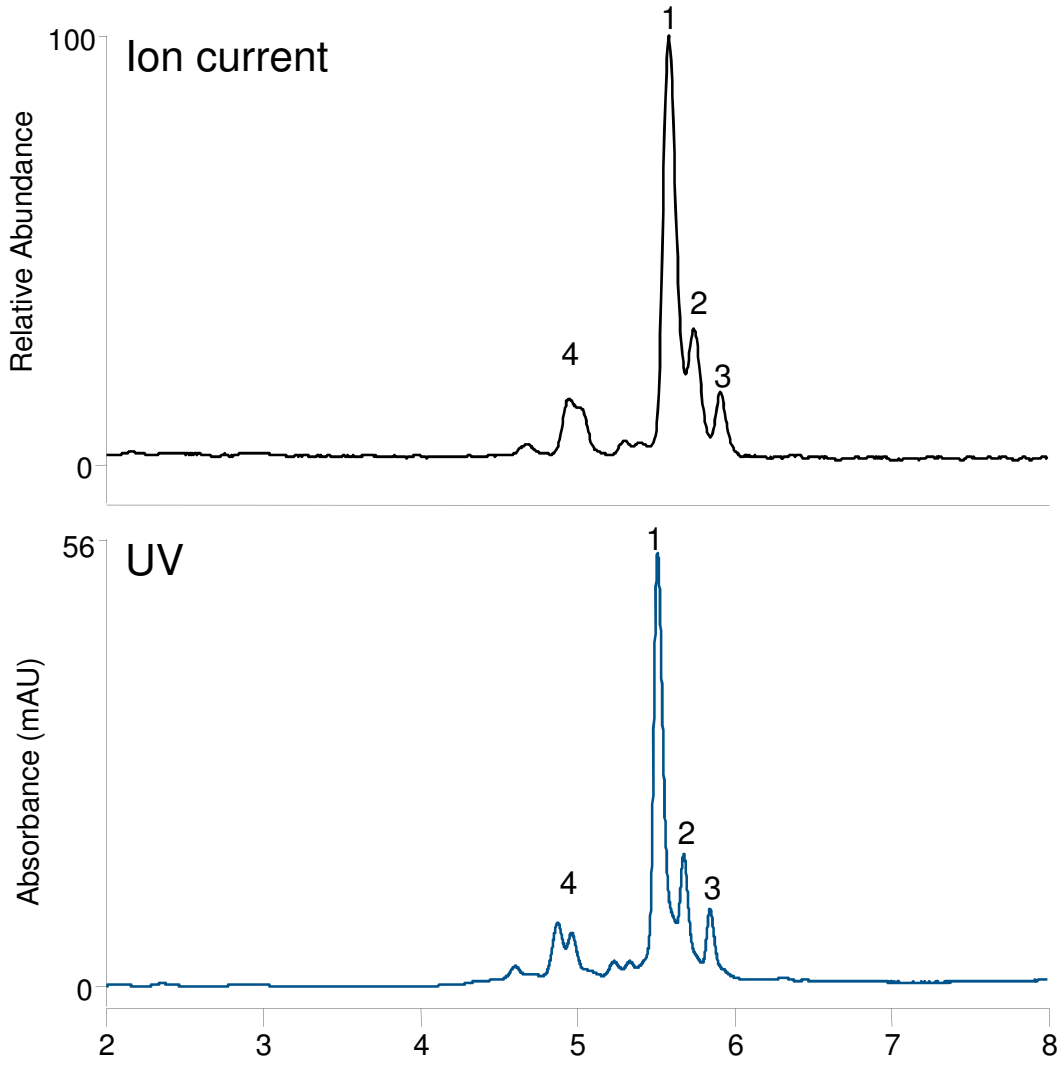
ThermoFisher SCIENTIFIC

LC/MS Analysis of Phosphorothioate Modified siRNA



ThermoFisher SCIENTIFIC

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)

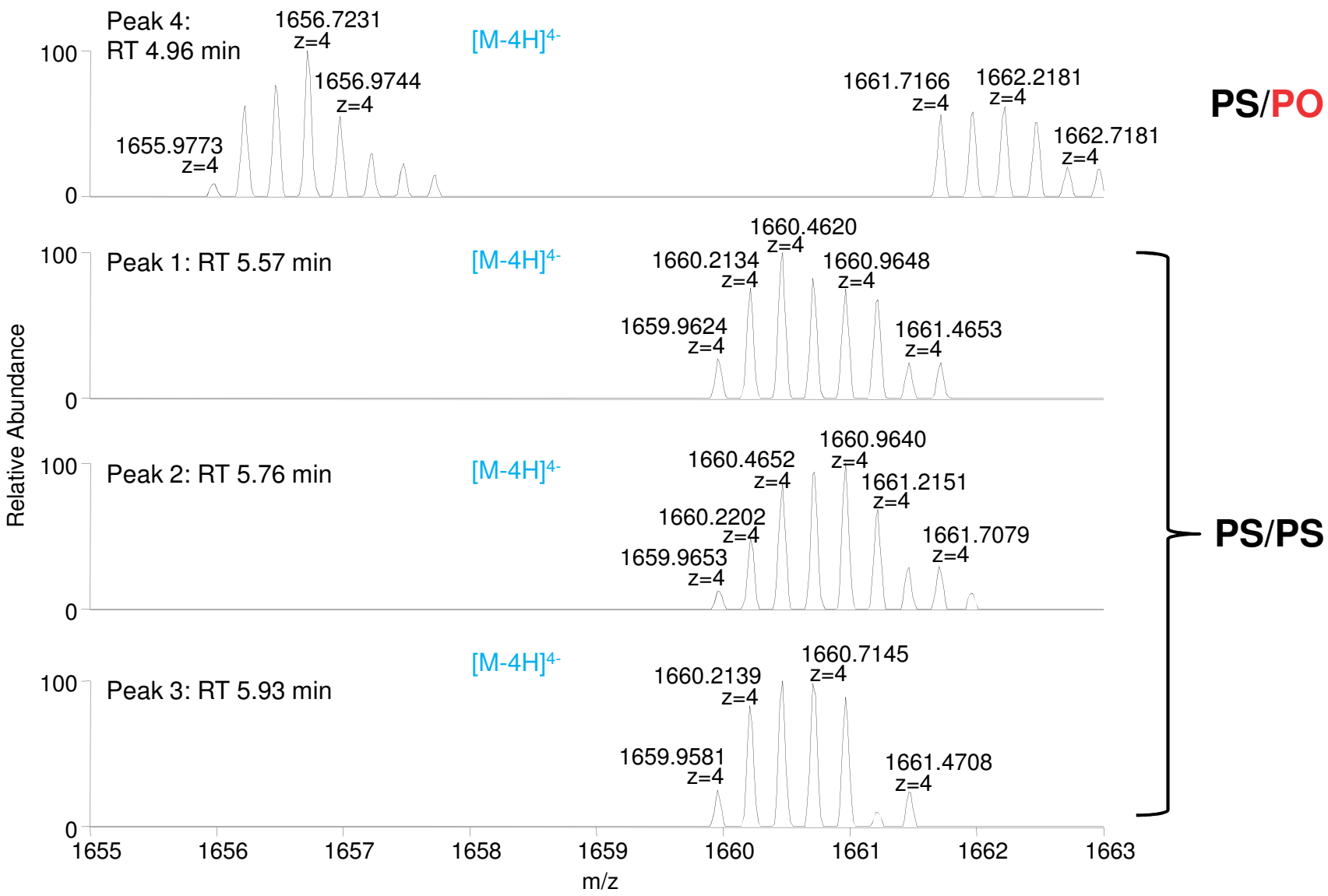
Gradient:

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

Temperature:	30 ºC
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 AGCUGACCCUGAAGUUCAU$_S$d$_S$dT

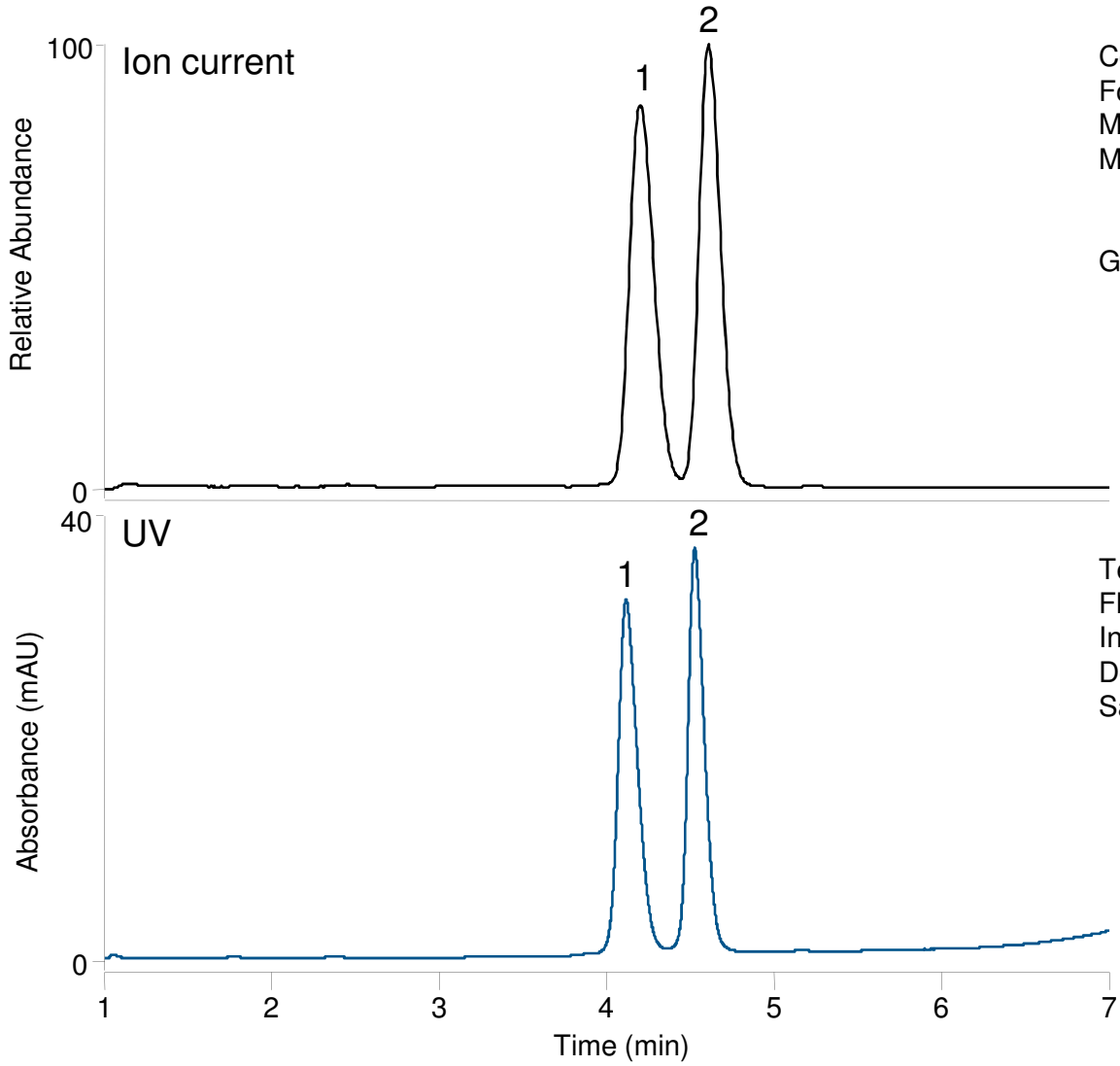
ThermoFisher SCIENTIFIC

LC/MS Analysis of Phosphorothioate Modified siRNA



ThermoFisher SCIENTIFIC

Analysis of CpG methylation

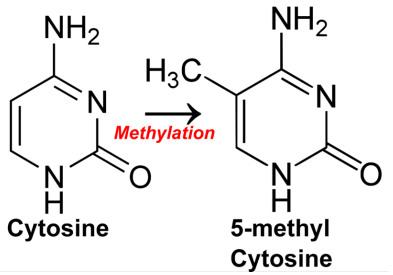


Column:	DNAPac RP, 4 µm
Format:	2.1 ×50 mm
Mobile phase A:	15 mM TEA, 400 mM HFIP, pH 7.6
Mobile phase B:	15 mM TEA, 400 mM HFIP in Water / Methanol (50:50 v/v)

Gradient:

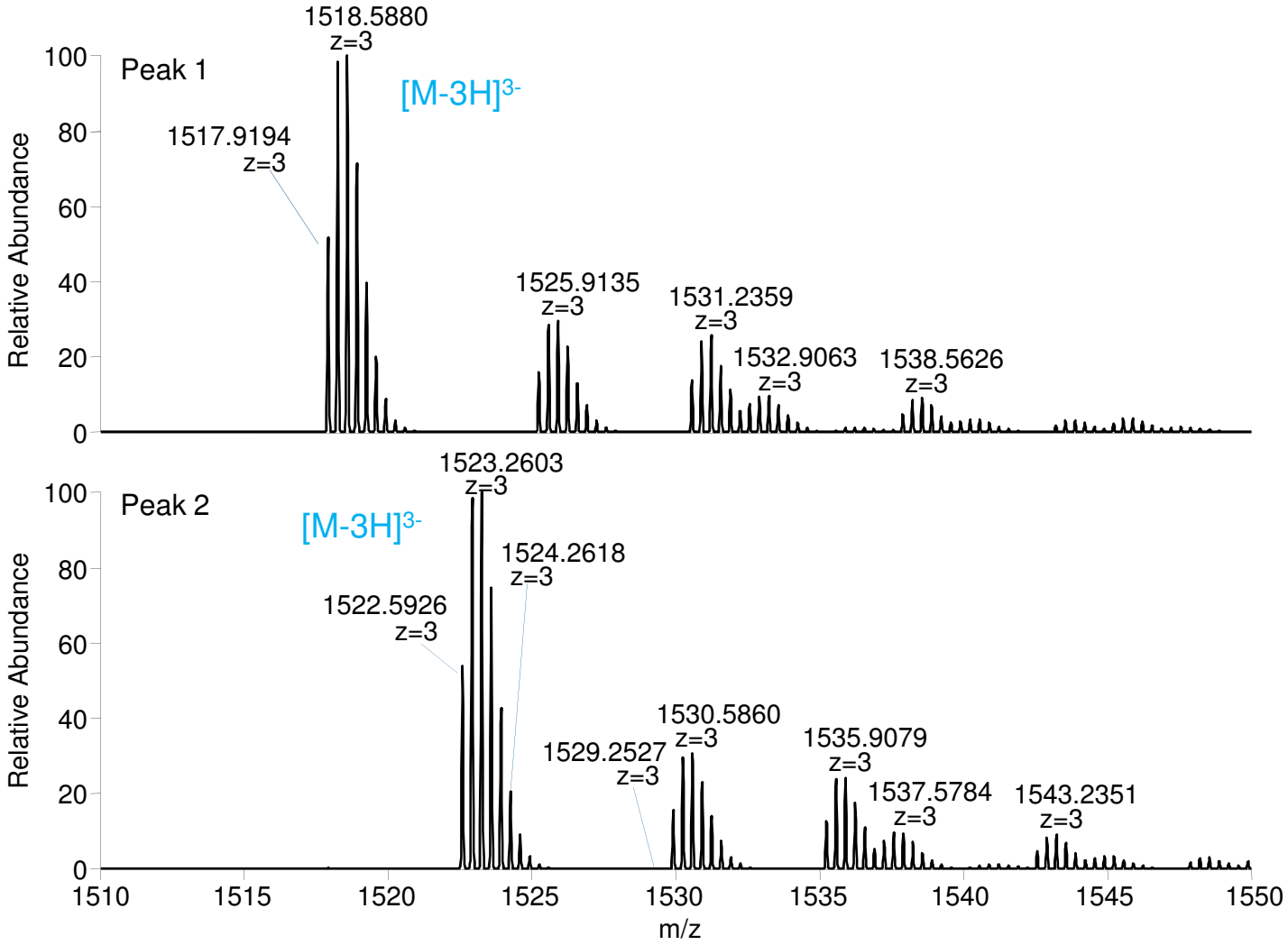
Time (min)	%A	%B
0.0	75	25
4.0	56	44
4.1	10	90
6.0	10	90
6.1	75	25
11.0	75	25

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



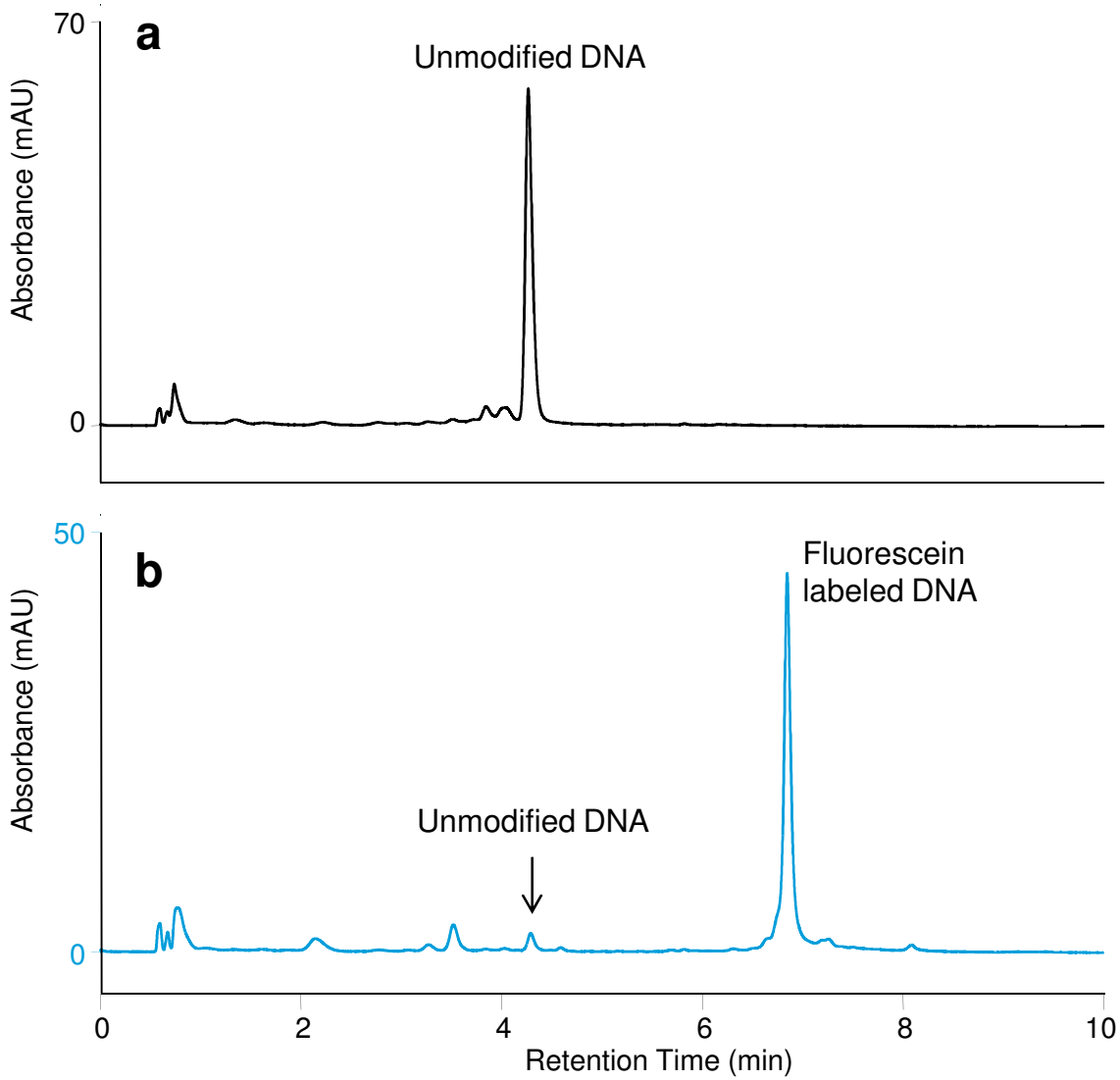
ThermoFisher SCIENTIFIC

Analysis of CpG methylation: Mass spectra



ThermoFisher SCIENTIFIC

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)

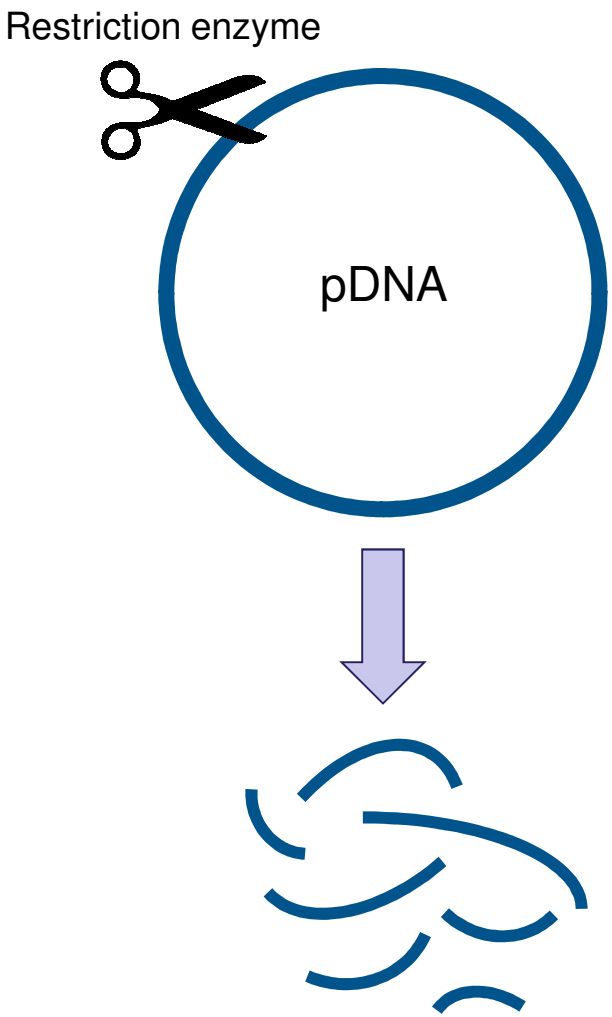
Gradient:

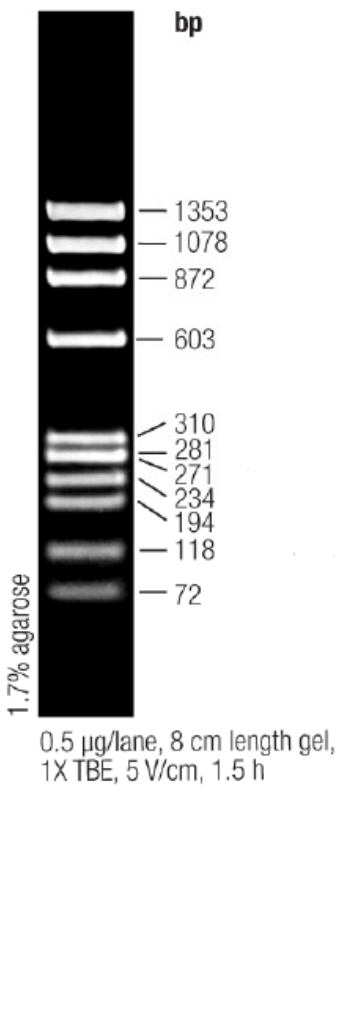
Time (min)	%A	%B
0.0	75	25
10.0	0	100
12.0	0	100
12.1	75	25
16.0	75	25

Flow rate:	0.60 mL/min
Inj. volume:	2.5 µL
Temperature:	60 ºC
Detection:	UV (260 nm)
Sample:	a. 20mer DNA (60 µg/mL) b. Fluorescein labeled 20mer DNA (60 µg/mL)

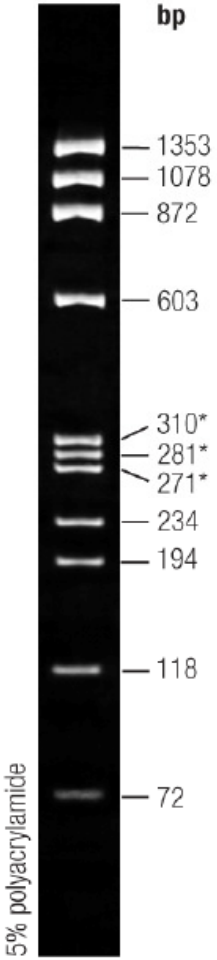
ThermoFisher SCIENTIFIC

Separation of Restriction Enzyme Digests of pDNAs: Gel





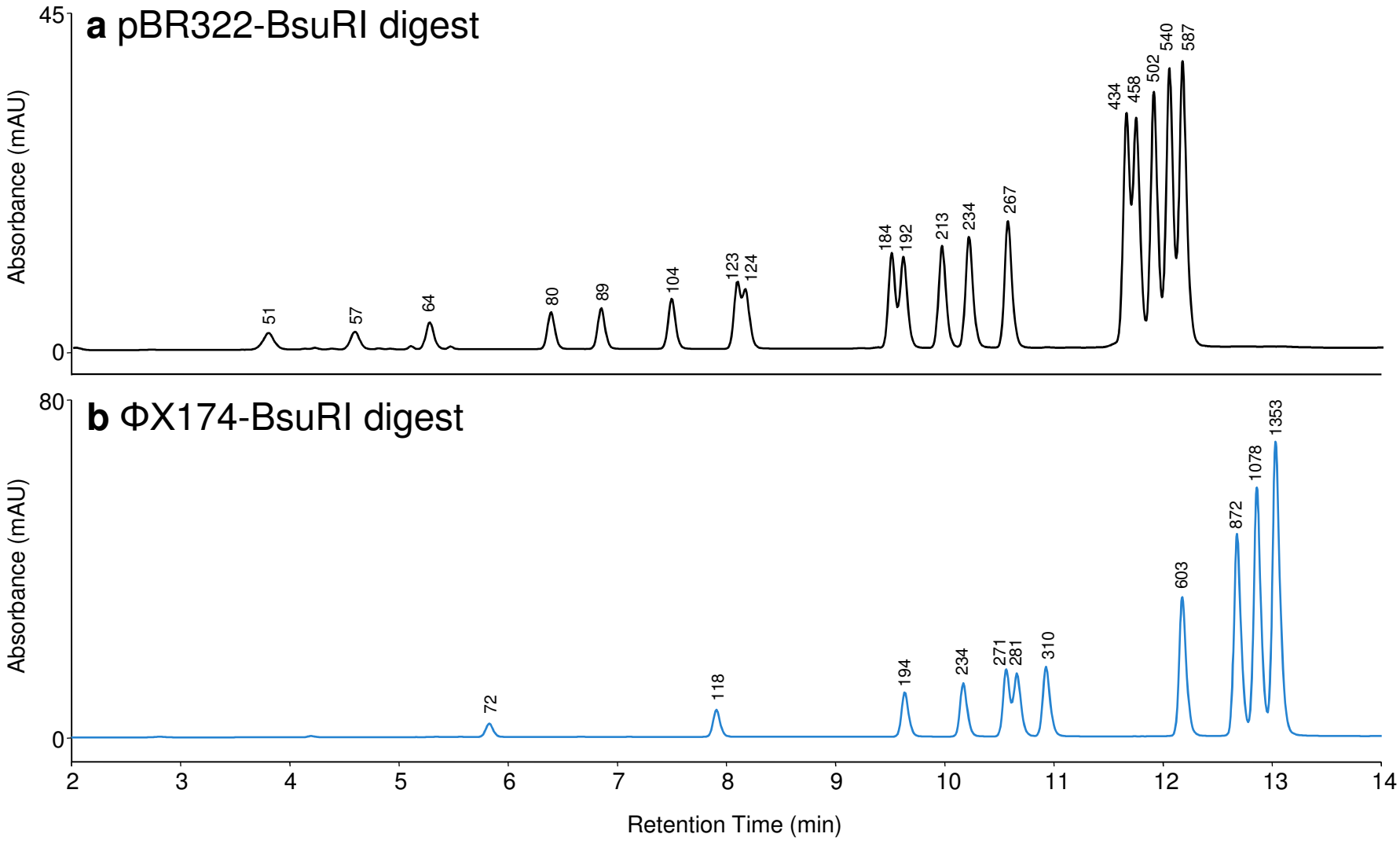
0.5 µg/lane, 8 cm length gel,
1X TBE, 5 V/cm, 1.5 h



0.5 µg/lane, 20 cm length gel,
1X TAE, 8 V/cm, 3 h

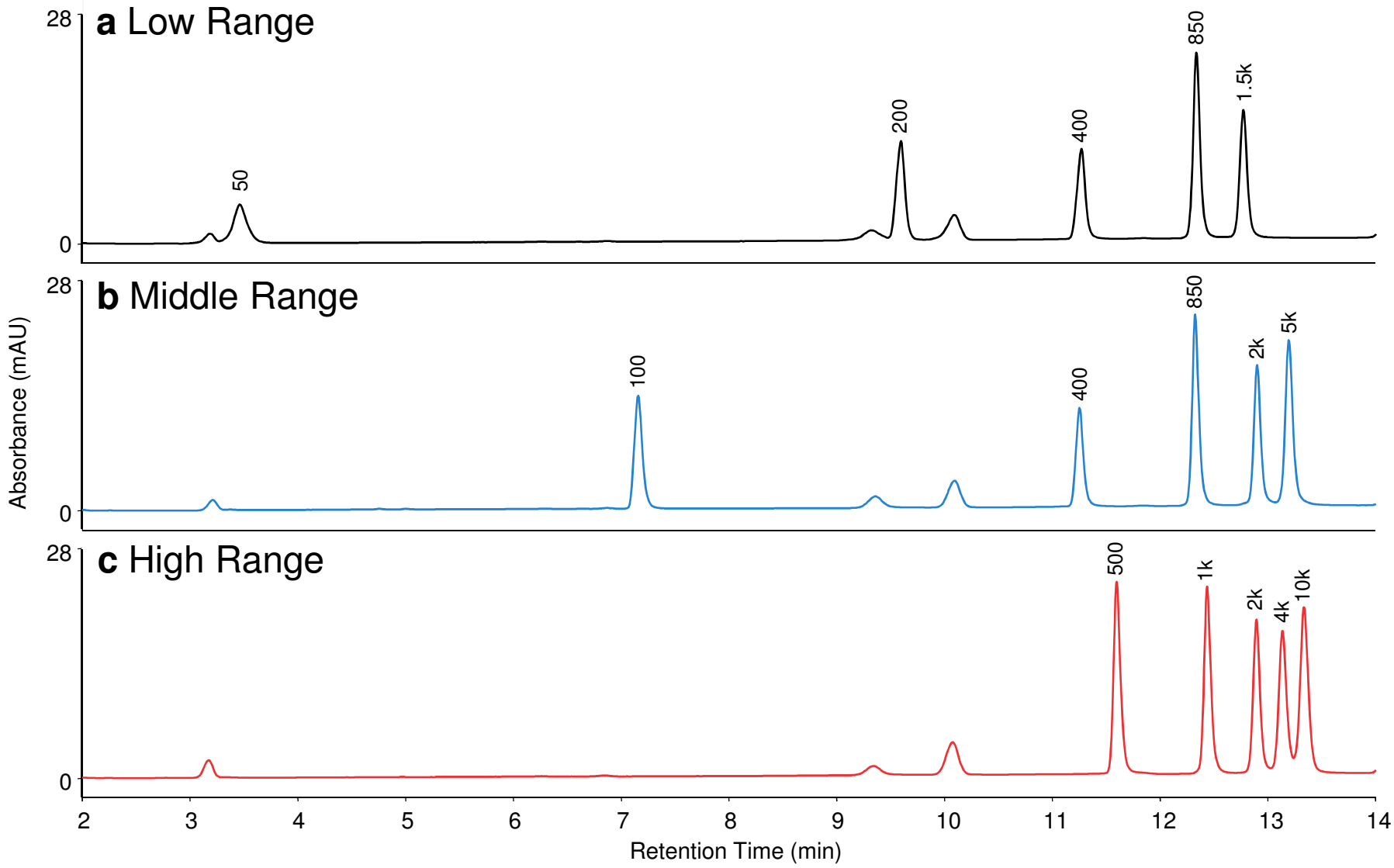
ThermoFisher SCIENTIFIC

Separation of Restriction Enzyme Digests of pDNAs: HPLC



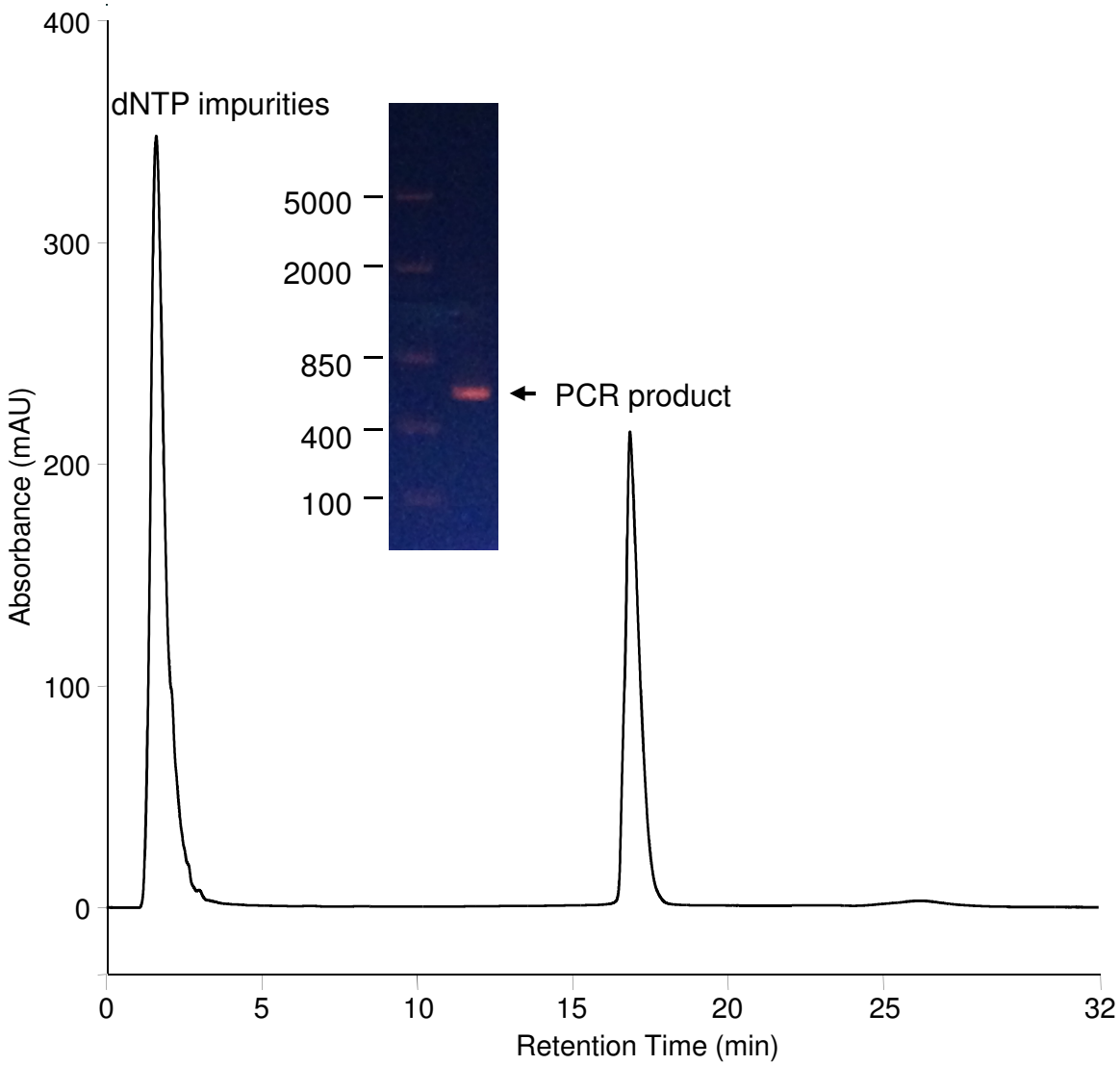
ThermoFisher SCIENTIFIC

Separation of Thermo Scientific™ FastRuler™ DNA ladders



ThermoFisher SCIENTIFIC

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)

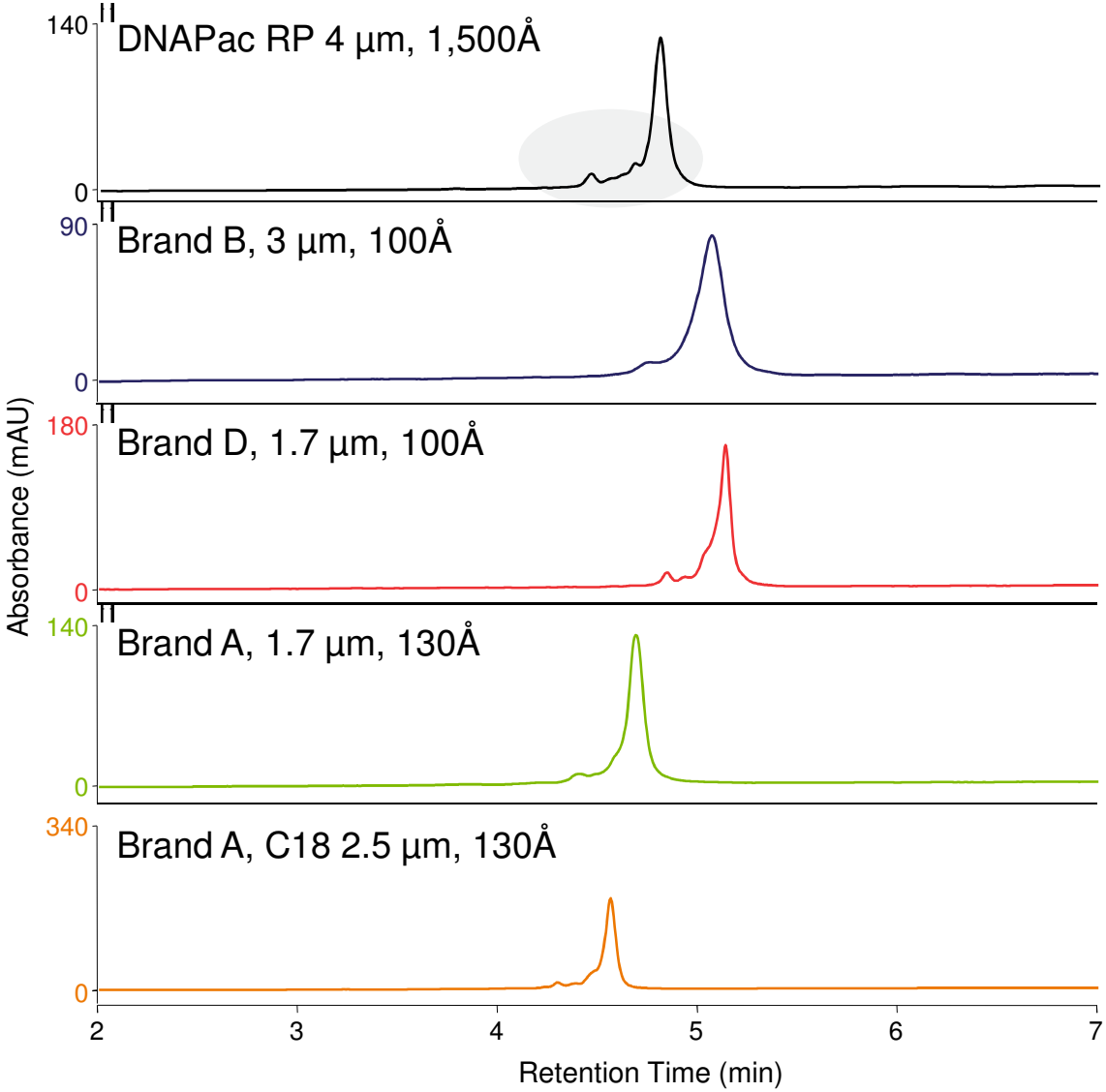
Gradient:

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

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

ThermoFisher SCIENTIFIC

41mer RNA: Comparison with Competitors

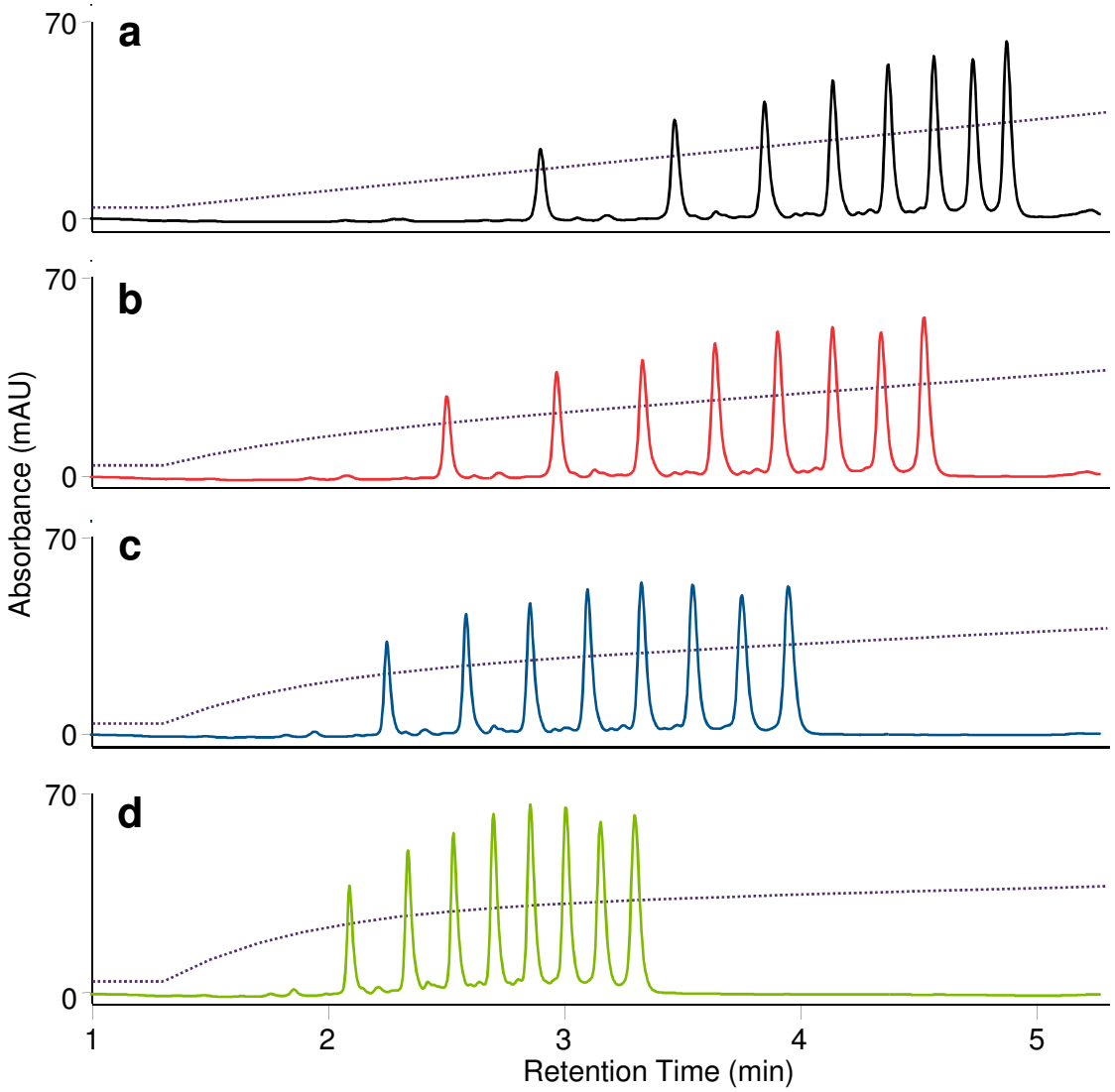


Format:	2.1 × 50 mm
Mobile phase A:	0.1 M TEAA, pH 7.0
Mobile phase B:	0.1 M TEAA in Water / Acetonitrile (90:10 v/v)
Gradient:	Gradient slope: 1.73% B/min Gradient time: 7.5 min
Temperature:	45 ºC
Flow rate:	0.80 mL/min
Inj. volume:	1 µL
Detection:	UV (260 nm)
Sample:	41mer RNA

Method Development

- Pre-Column Heater
- Gradient Curve
- Temperature
- Flow Rate
- Ion-Pair Reagent

Adjustment of Gradient Curve



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)

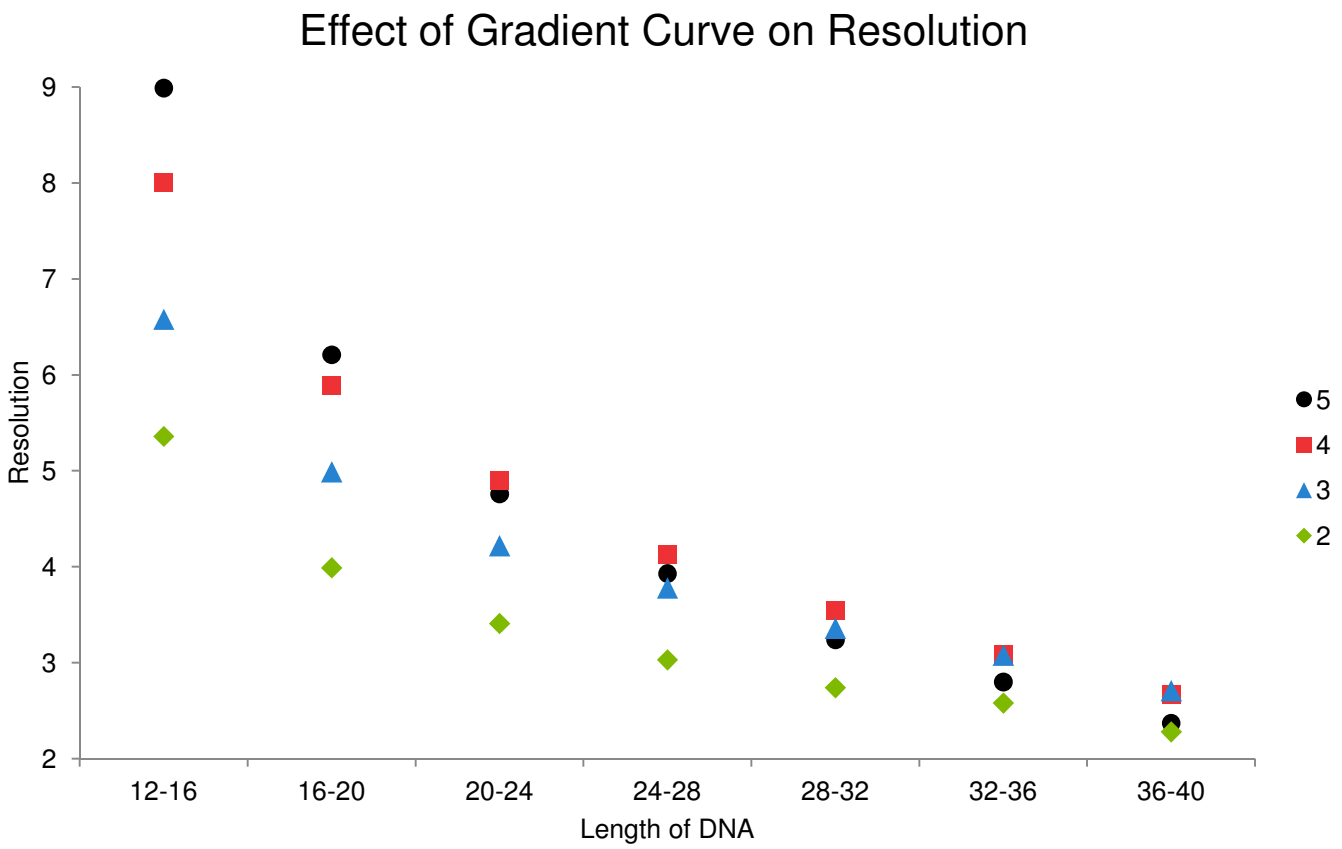
Gradient:

Time (min)	%A	%B
-3.0	88	12
0.0	88	12
4.0	62	38
4.1	10	90
6.0	10	90

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

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

Effect of Gradient Curve on Resolution



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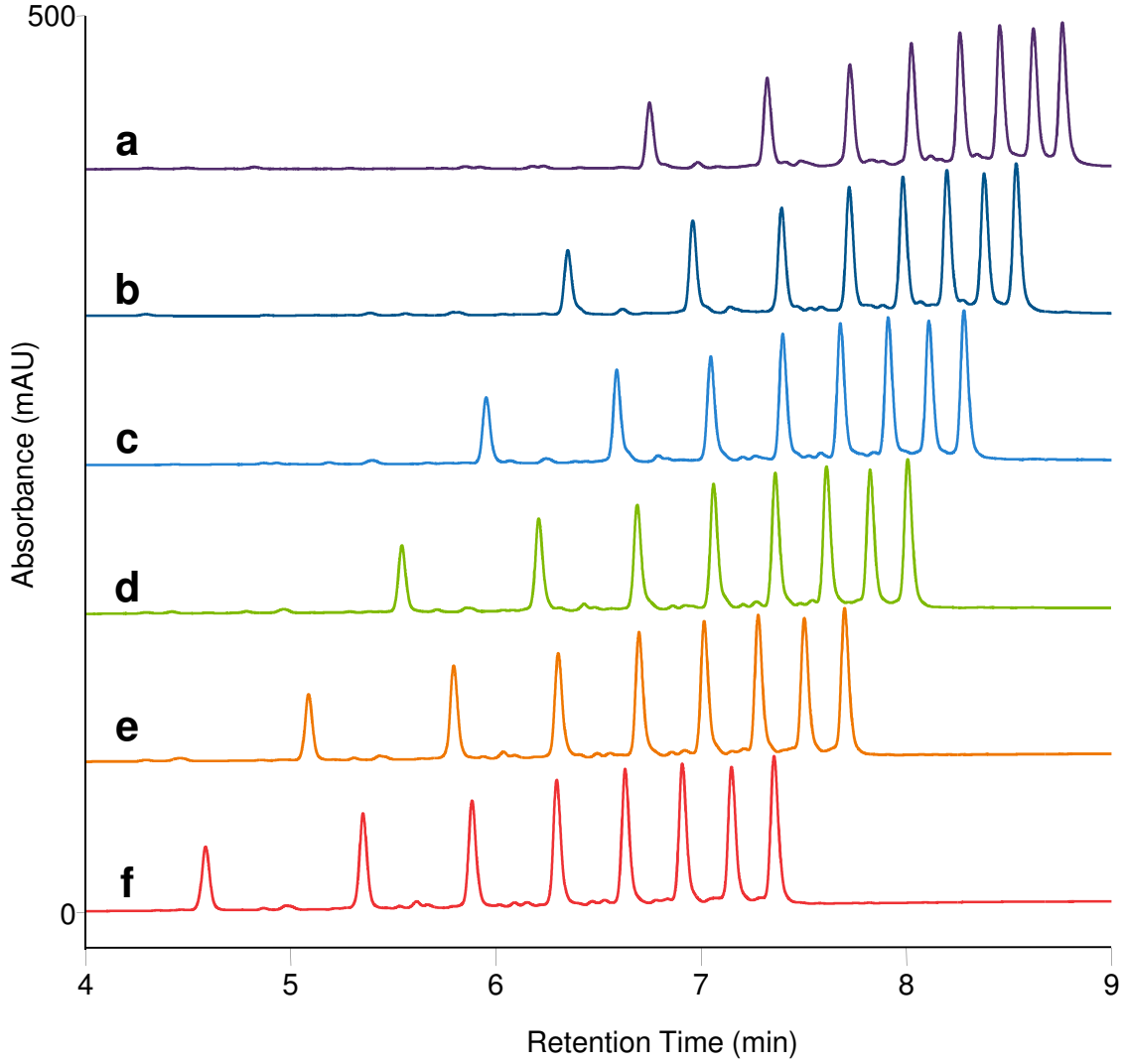
Separation of Oligonucleotides at Different Temperatures



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)

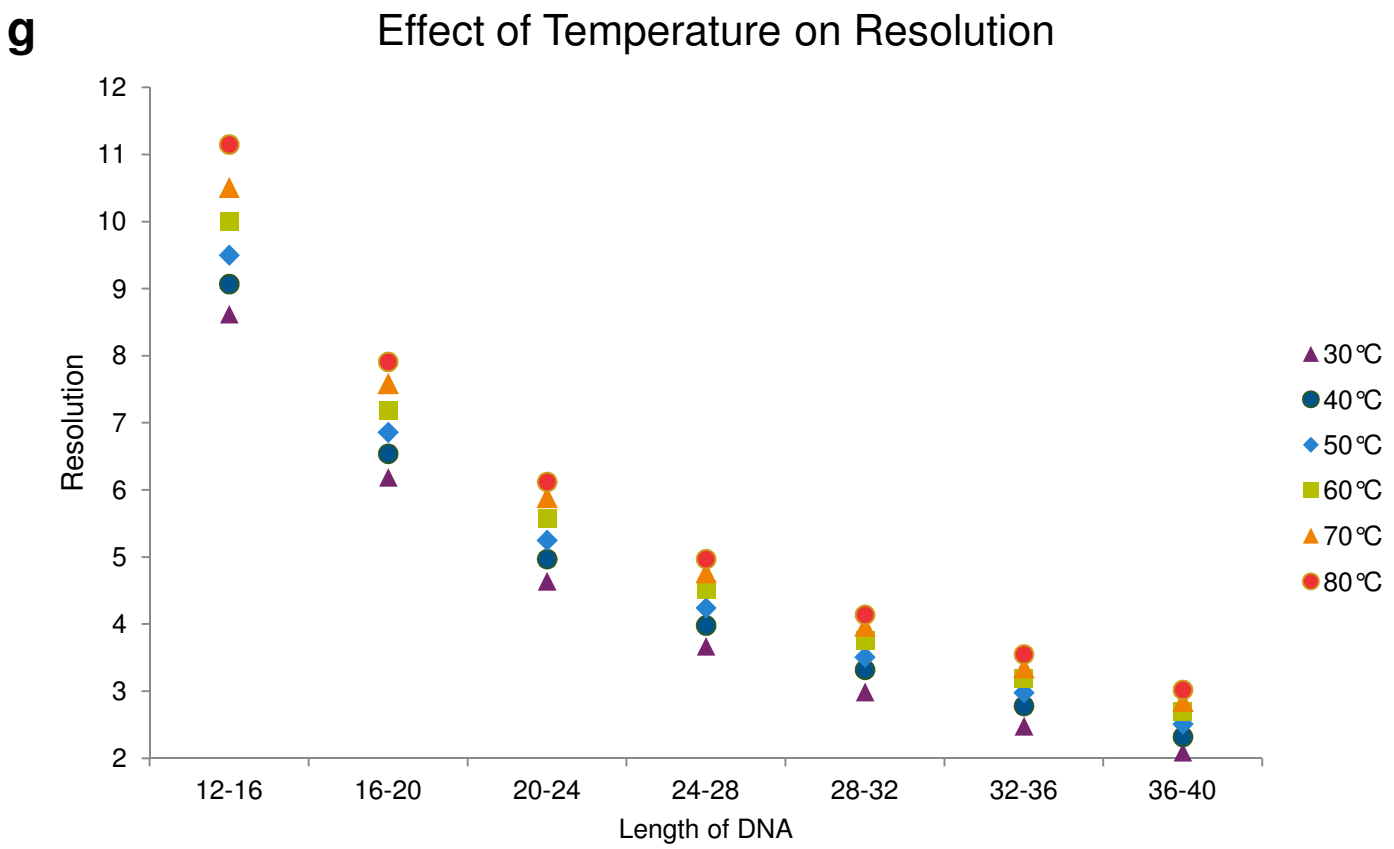
Gradient:

Time (min)	%A	%B
-3.0	94	6
0.0	94	6
8.0	54	46
8.1	10	90
10.0	10	90

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

Flow rate:	0.40 mL/min
Inj. volume:	2 µL
Detection:	UV (260 nm)
Sample:	8-Combo DNA (5 µM)*

Effect of Temperature on Resolution



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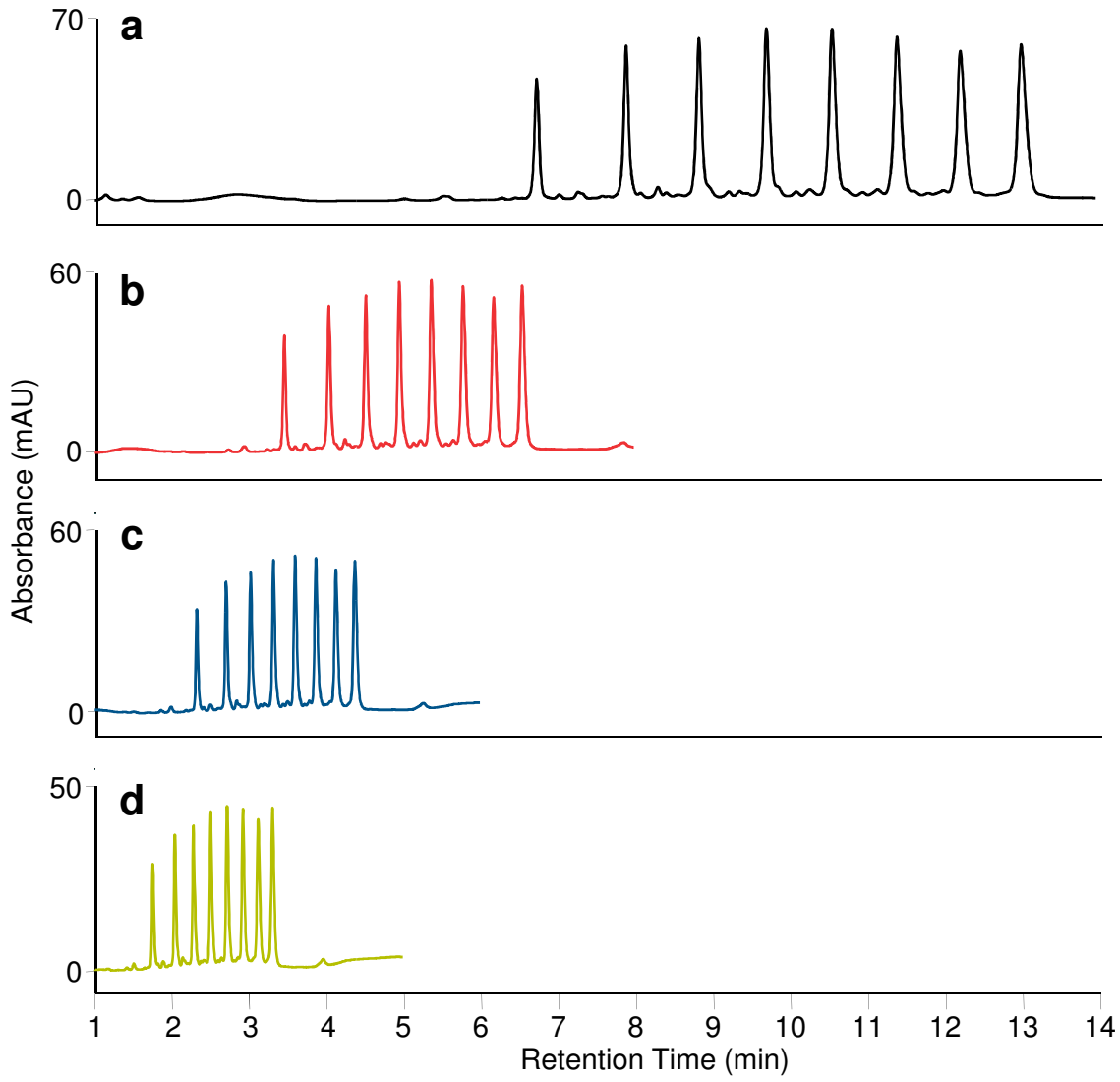
Separation of Oligonucleotides at Different Flow Rates



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) 12 to 36%B in 12 min. b) 12 to 36%B in 6 min. c) 12 to 36%B in 4 min. d) 12 to 36%B in 3 min.
Gradient curve:	3
Temperature:	60 ºC
Flow rate:	a) 0.20 b) 0.40 c) 0.60 d) 0.80
Inj. volume:	2 µL
Detection:	UV (260 nm)
Sample:	8-Combo DNA (5 µM)*

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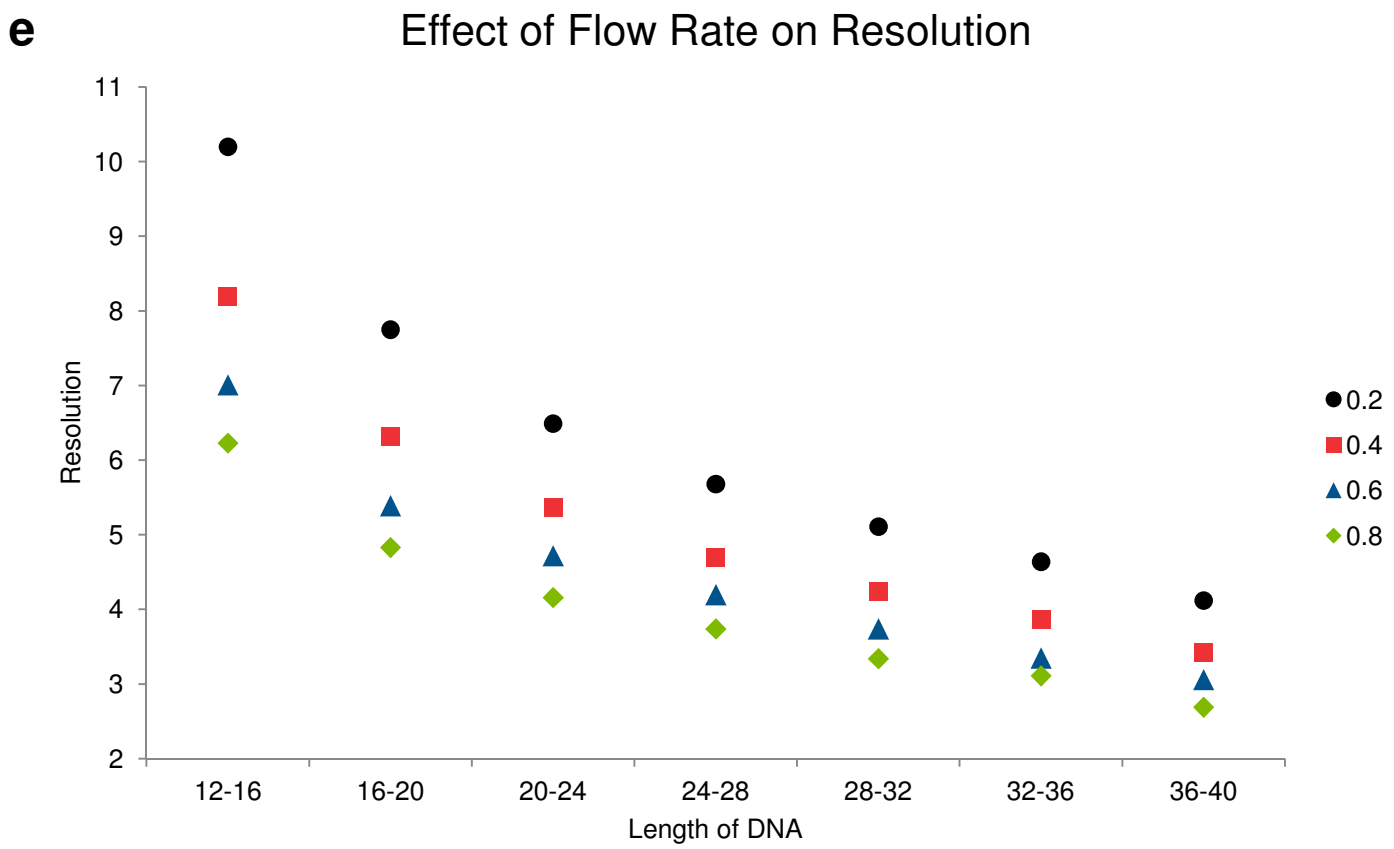
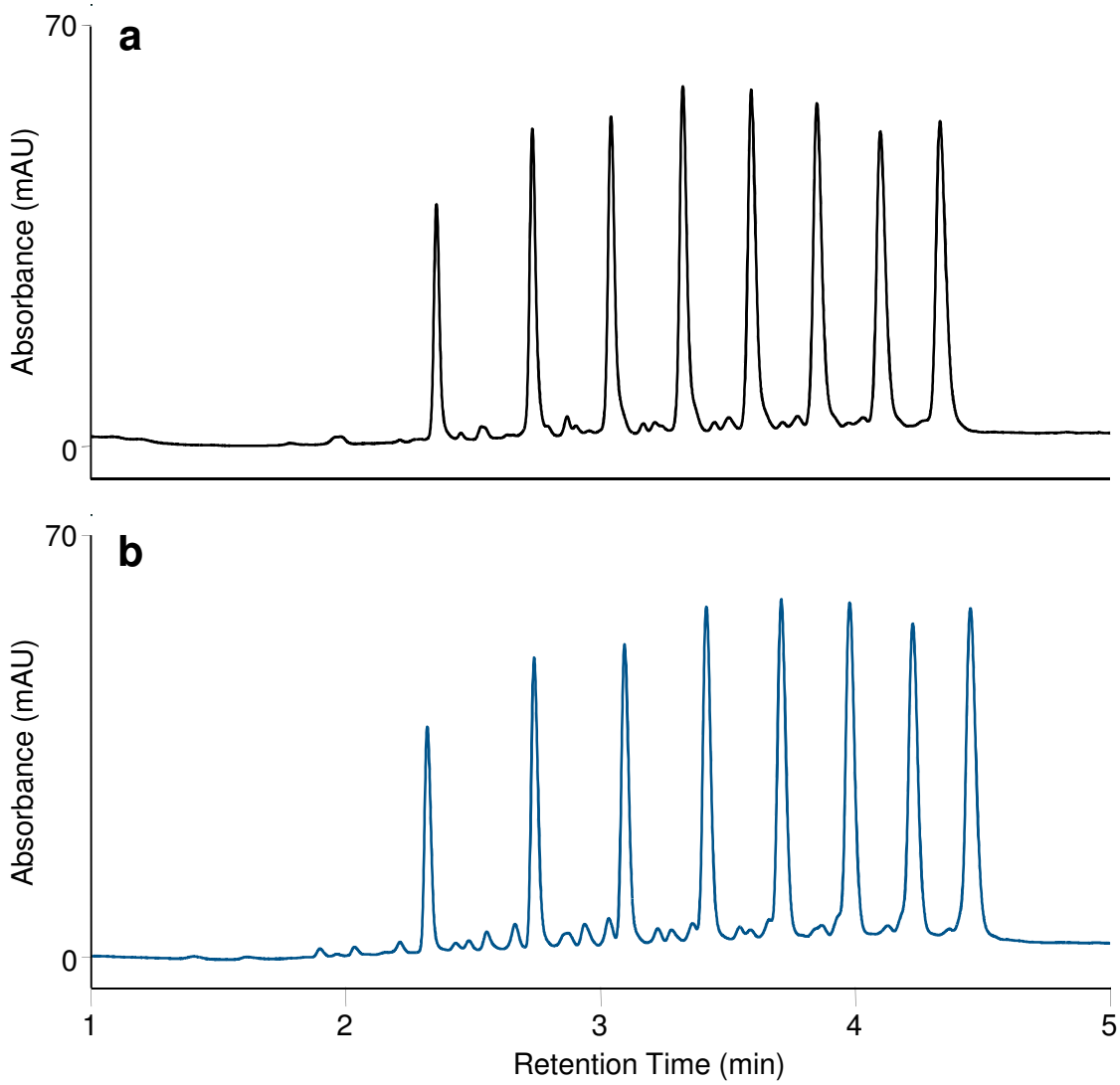
Effect of Flow Rate on Resolution



Comparison of TEA and HA as Ion-Pairing Reagents



Column: DNAPac RP, 4 µm
Format: 2.1 ×50 mm

Mobile phases
a)
Mobile phase A: 0.1 M TEAA, pH 7.0
Mobile phase B: 0.1 M TEAA in Water / Acetonitrile (75:25 v/v)
b)
Mobile phase A: 0.1 M HA, pH 7.4
Mobile phase B: 0.1 M HA in Water / Acetonitrile (50:50 v/v)

Gradient:

a)

Time (min)	%A	%B
-2.0	92	8
0.0	92	8
3.0	68	32
3.1	10	90
5.0	10	90

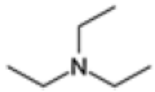
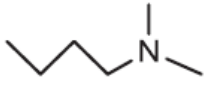
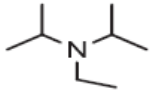
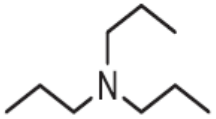
b)

Time (min)	%A	%B
-2.0	78	22
0.0	78	22
3.0	36	64
3.1	10	90
5.0	10	90

Gradient curve: 3
Temperature: 80 ºC
Flow rate: 0.80 mL/min
Inj. volume: 2 µL
Detection: UV (260 nm)
Sample: 8-Combo DNA (5 µM)*

Other Ion-Pairing Reagents

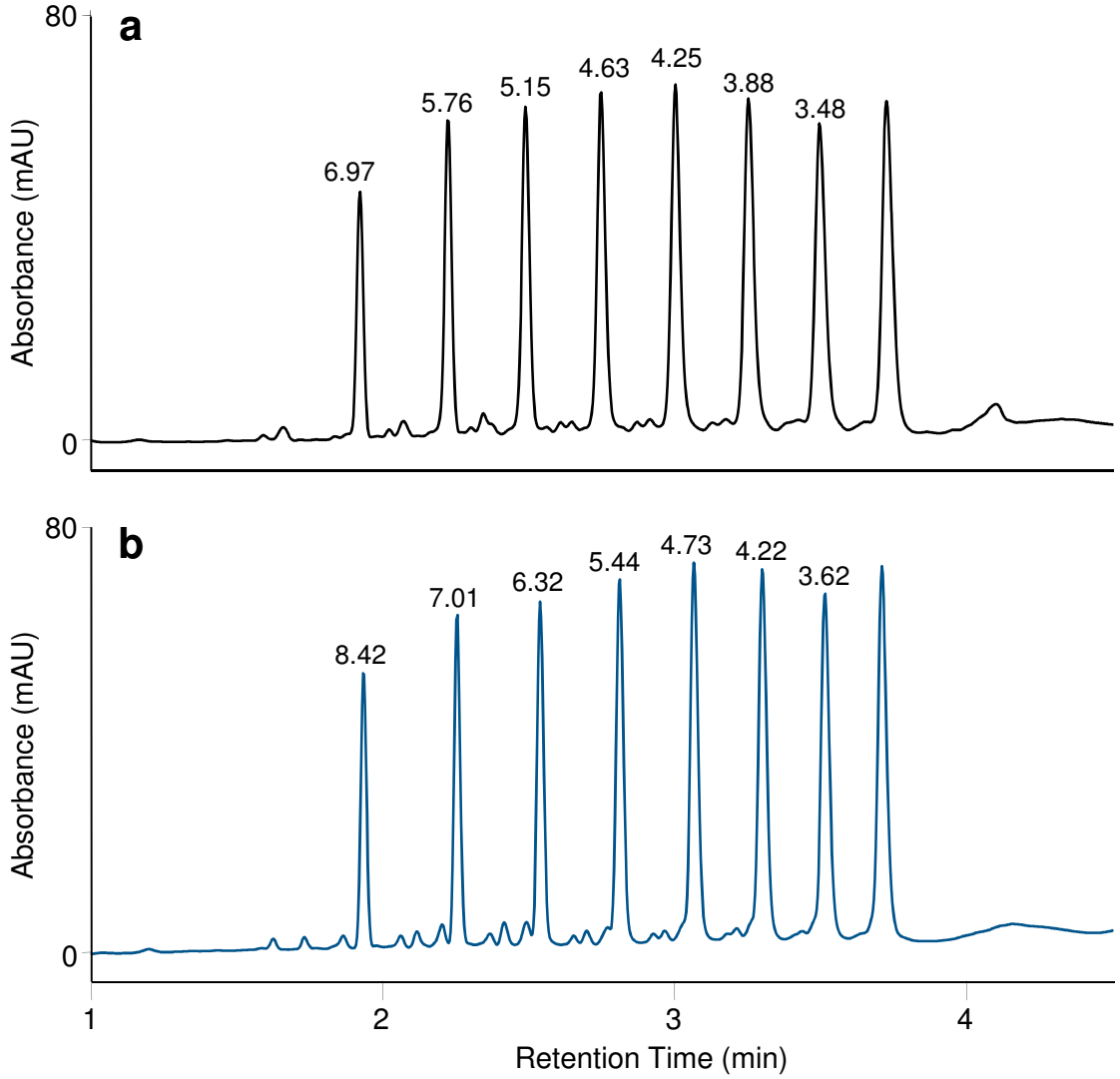
Table 2. Physicochemical properties of the six ion-pairing (IP) reagents

IP reagent	TEA	HA	DBA	DMBA	DIPEA	TPA
MW (g/mol)[a]	101.19	101.19	129.24	101.19	129.24	143.27
Boiling point (°C)[a]	88.8	131-132	159	93.3	127	155-158
Chemical structure						
Water solubility (g/L) (25 °C)	73.7[b]	12[b]	3.5[b]	41.8[b]	3.9[b]	0.748[b]
Log Pow	1.15[a]	1.9[a]	2.06[a]	1.70[a]	2.35[b]	2.68[a]
pKa (25 °C)	10.78[b]	10.64[b]	11.39[b]	10.19[b]	11.4[b]	10.65[b]

[a]The values were obtained from Sigma-Aldrich.
[b]The values were obtained from the U.S. National Library of Medicine database.

Rapid Commun. Mass Spectrom. 2014, 28, 339–350.

Comparison of TEA and HA as Ion-Pairing Reagents



Column:	DNAPac RP, 4 µm
Format:	2.1 ×50 mm
Mobile phases	
a)	
Mobile phase A:	0.1 M TEAA, pH 7.0
Mobile phase B:	0.1 M TEAA in Water / Acetonitrile (75:25 v/v)
b)	
Mobile phase A:	0.1 M HA, pH 7.4
Mobile phase B:	0.1 M HA in Water / Acetonitrile (50:50 v/v)
Gradient:	a) 8 to 32%B in 3 min. b) 23 to 63.5%B in 3 min.
Gradient curve:	3
Flow rate:	0.80 mL/min
Inj. volume:	2 µL
Temperature:	80 ºC
Detection:	UV (260 nm)
Sample:	8-Combo DNA (5 µM)*
Peak label:	Resolution (ep)

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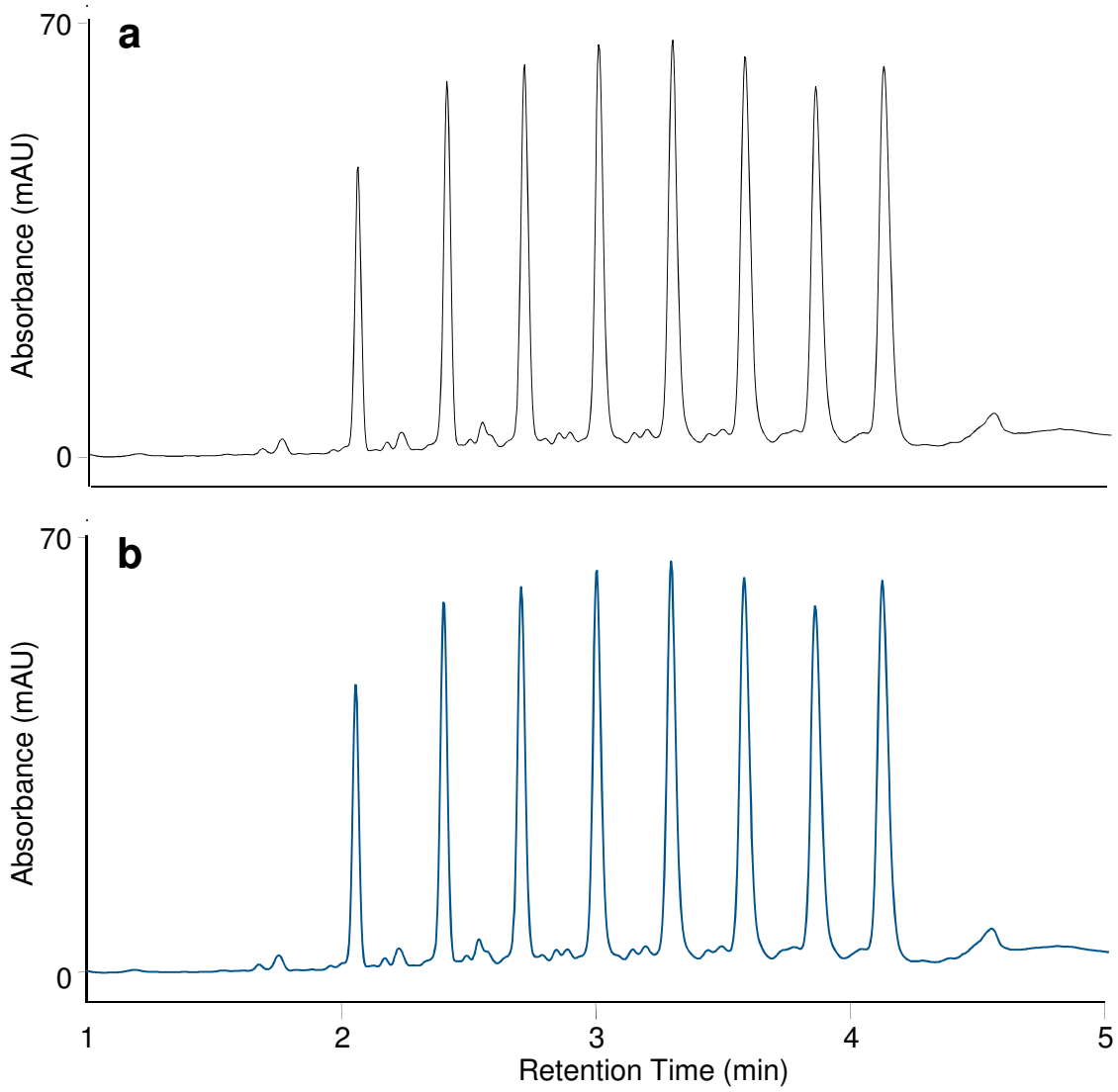
Effect of Oligonucleotide Diluent



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:	0.60
Inj. volume:	2 µL
Temperature:	60 ºC
Detection:	UV (260 nm)
Sample:	8-Combo DNA (5 µM)*
Sample diluent:	a) Water b) Mobile phase A

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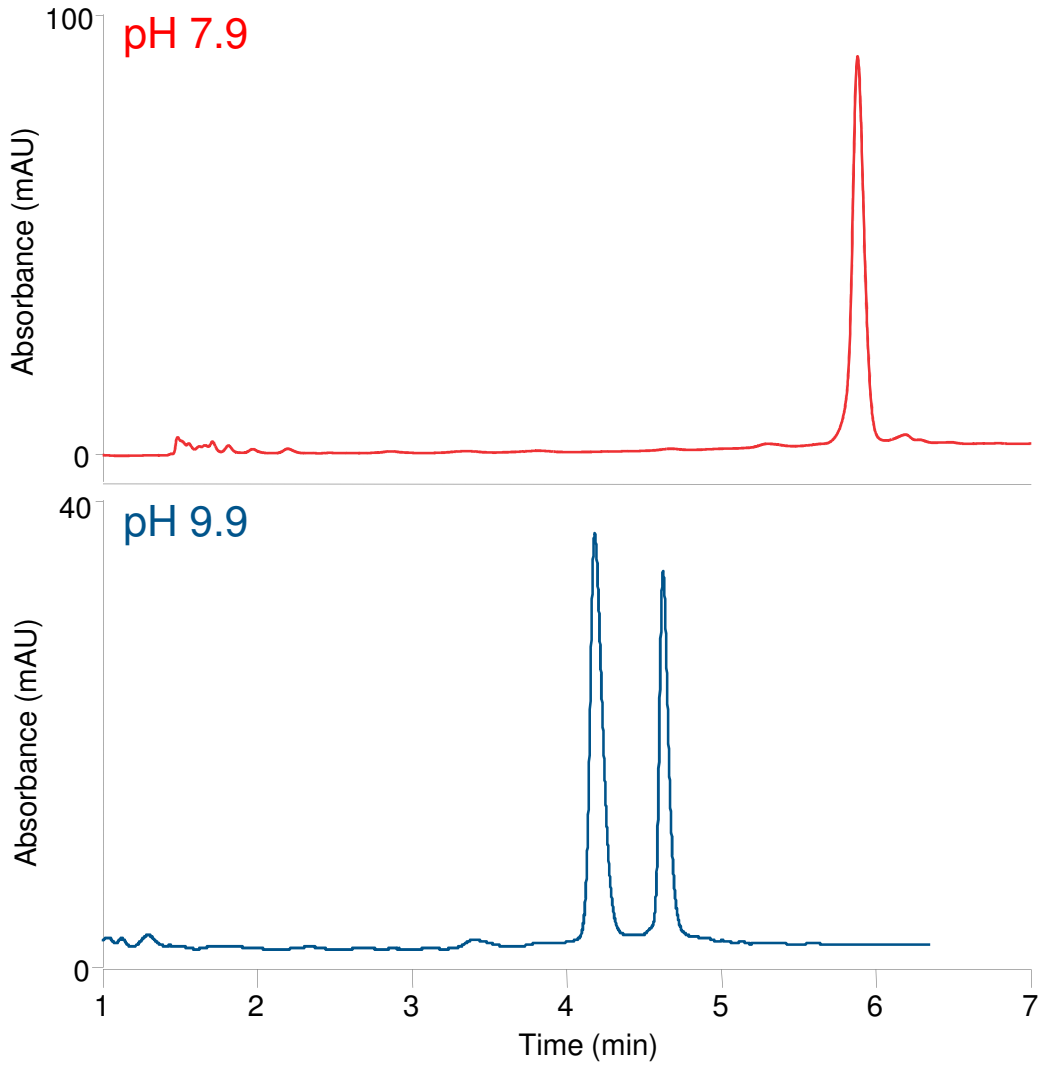
Separation of Diastereoisomers of siRNA at Different pHs

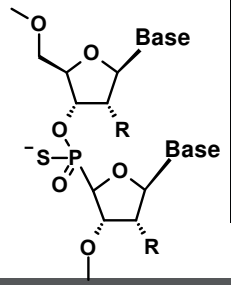


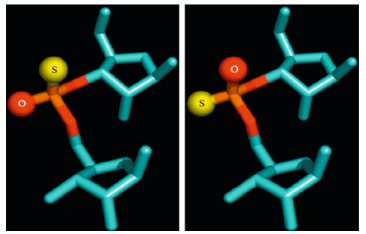
Column:	DNAPac RP, 4 µm
Format:	2.1 × 50 mm
Mobile phase A:	15 mM TEA, 400 mM HFIP, **pH 7.9**
Mobile phase B:	15 mM TEA, 400 mM HFIP in Water / Methanol (75:25 v/v)

Gradient:

Time (min)	%A	%B
0.0	65	35
5.0	42	58
5.1	10	90
7.0	10	90
7.1	65	35
13.0	65	35

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





Rp **Sp**

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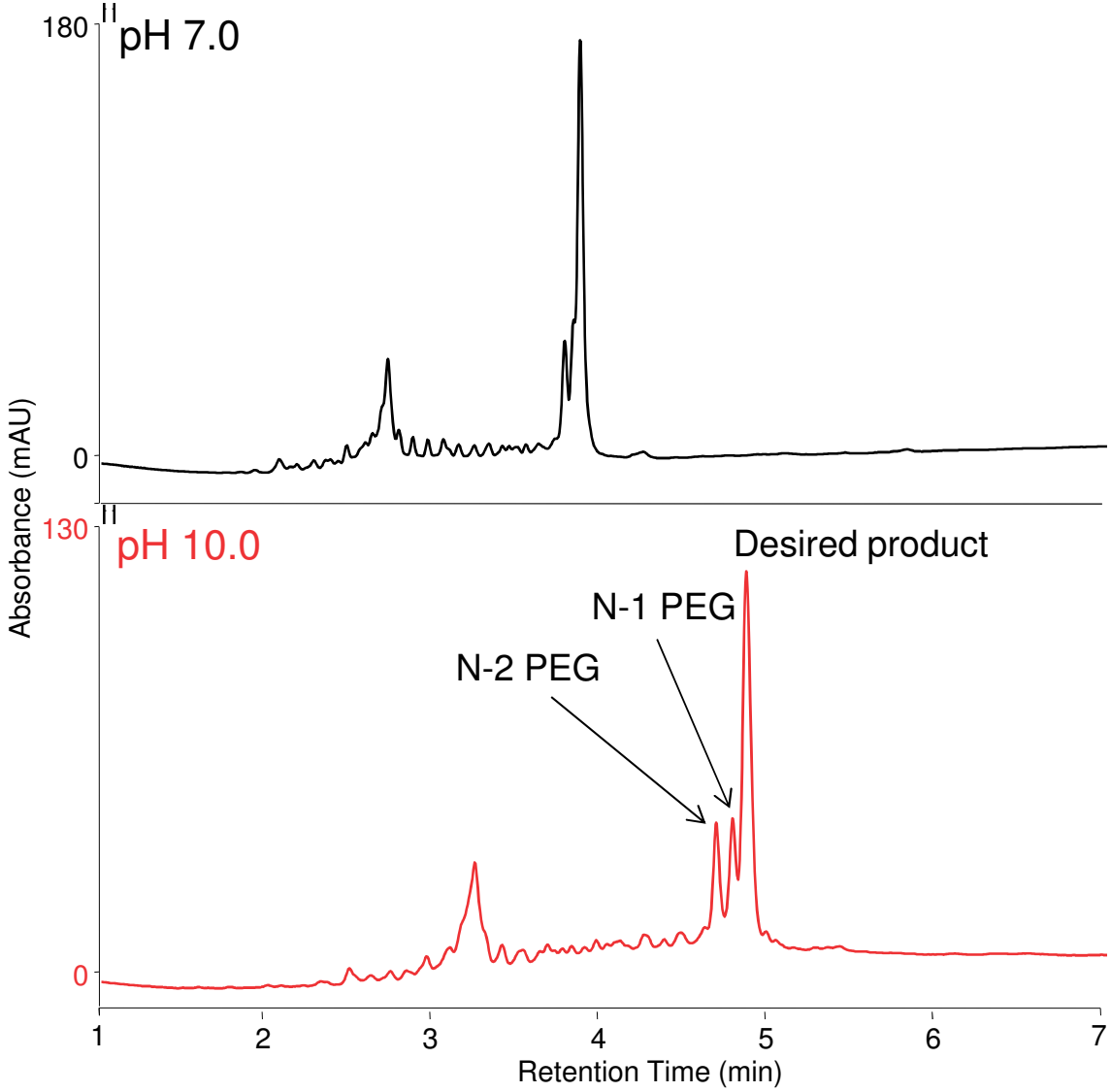
Separation of Mobility Modifier DNA at Different pHs



Column:	DNAPac RP
Format:	2.1 ×50 mm
a)	
Mobile phase A:	0.1 M TEAA, pH 7.0
Mobile phase B:	0.1 M TEAA in Water / Acetonitrile (90:10 v/v)
b)	
Mobile phase A:	0.05 M TEAA + 0.05% TEA, pH 10.0
Mobile phase B:	0.05 M TEAA + 0.05% TEA in Water / Acetonitrile (50:50 v/v)
Gradient:	a) 5-35% B in 7.5 minutes b) 5-40% B in 7.5 minutes
Temperature:	45 ºC
Flow rate:	0.80 mL/min
Inj. volume:	2 µL
Detection:	UV (260 nm)
Sample:	Sequencing primer labeled with fluorescein and contains at least 10 units of mobility modifier (ethylene oxide)

DNAPac PA200 RS vs DNAPac RP

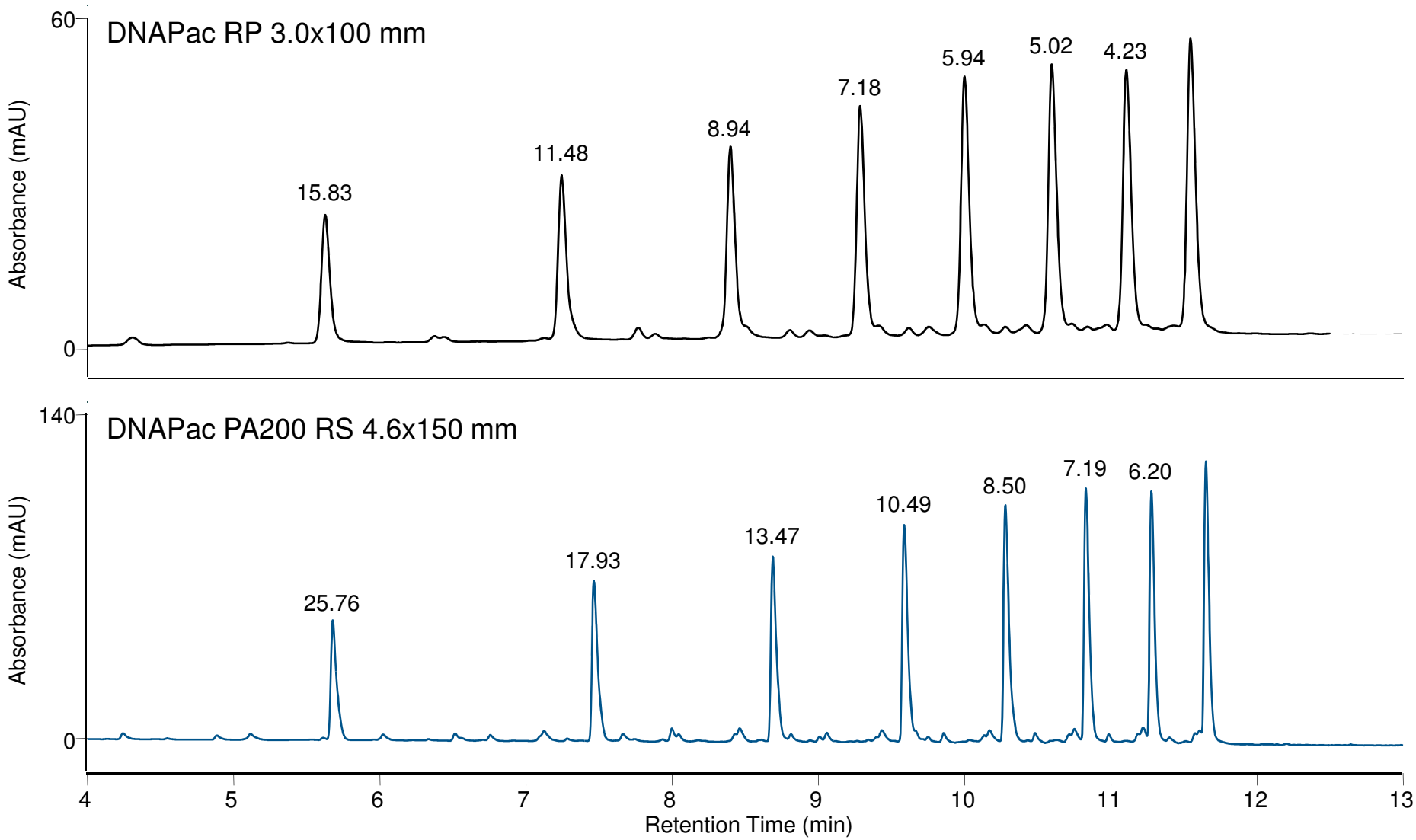
DNAPac PA200 RS
Anion Exchange Chromatography

- Provides *ultra*-high resolution separation oligonucleotides
- Low or no organic solvent
- Cannot be directly coupled to Mass spectrometer

DNAPac RP
Ion-Pair Reversed Phase Chromatography

- Provides high resolution separation oligonucleotides
- Can be directly coupled to Mass spectrometer
- Excellent separation of long oligonucleotides and large dsDNA fragments

ssDNA: 8-Combo



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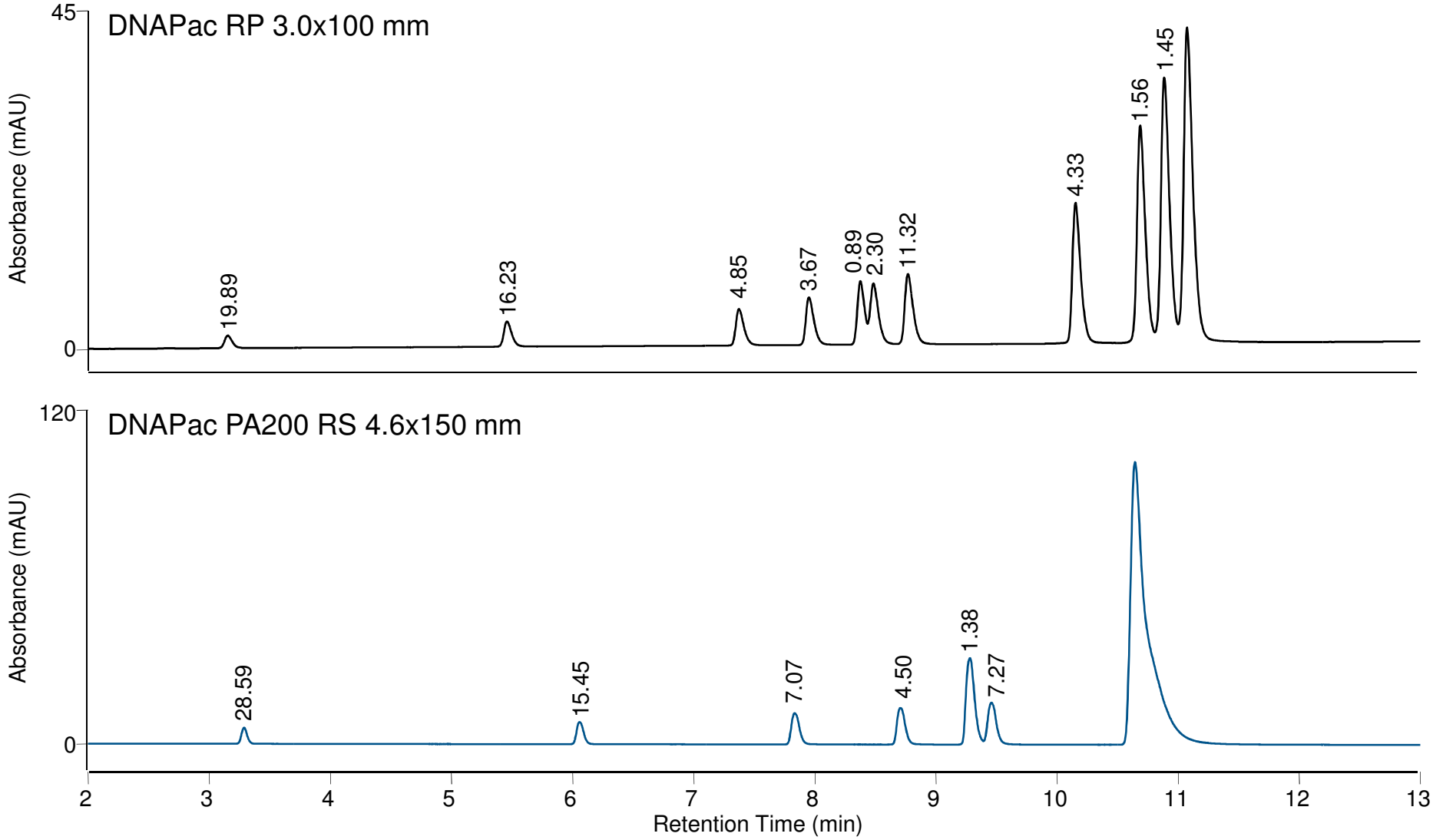
dsDNA: ΦX174-BsuRI digest



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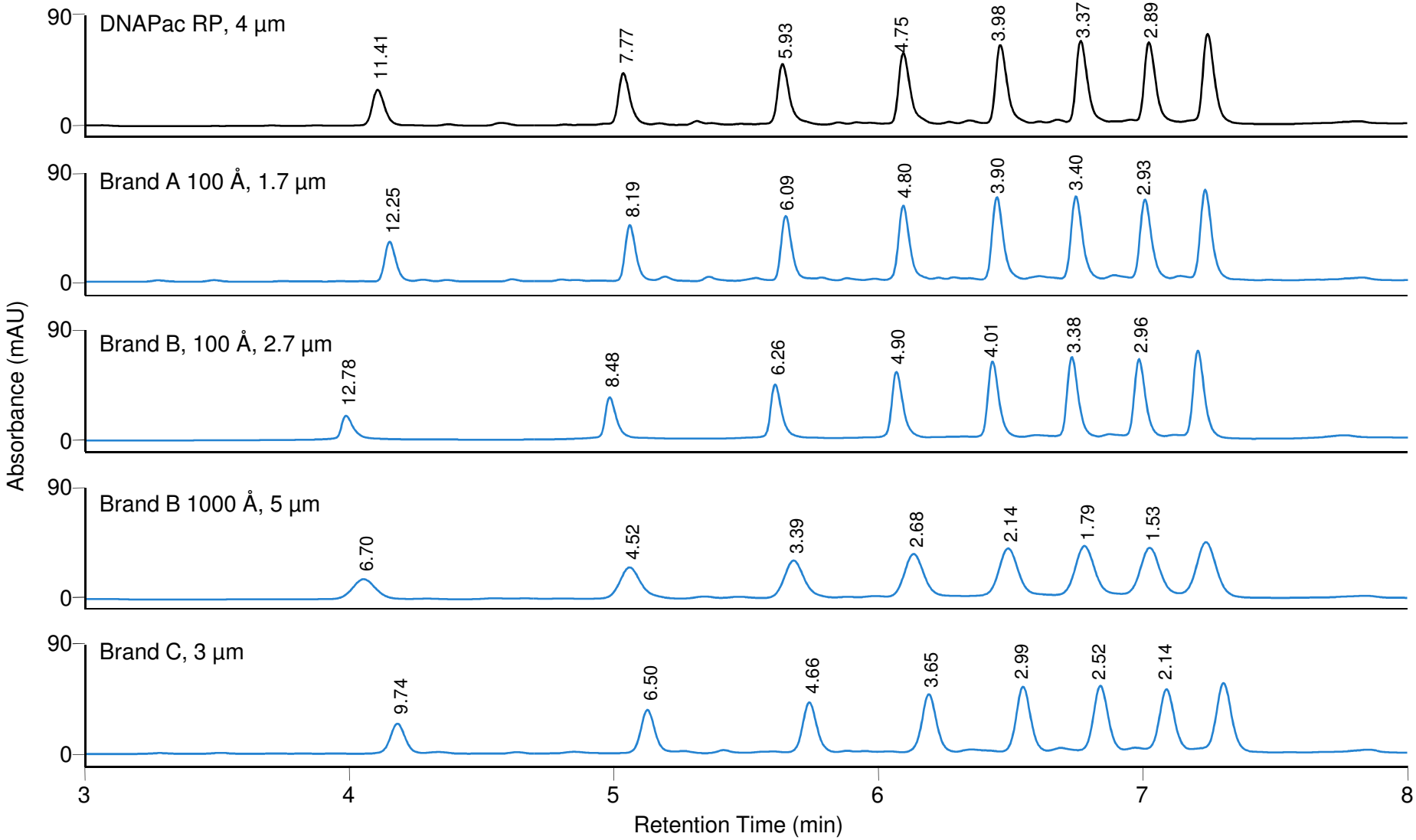
DNAPac RP vs Competitors: ssDNA



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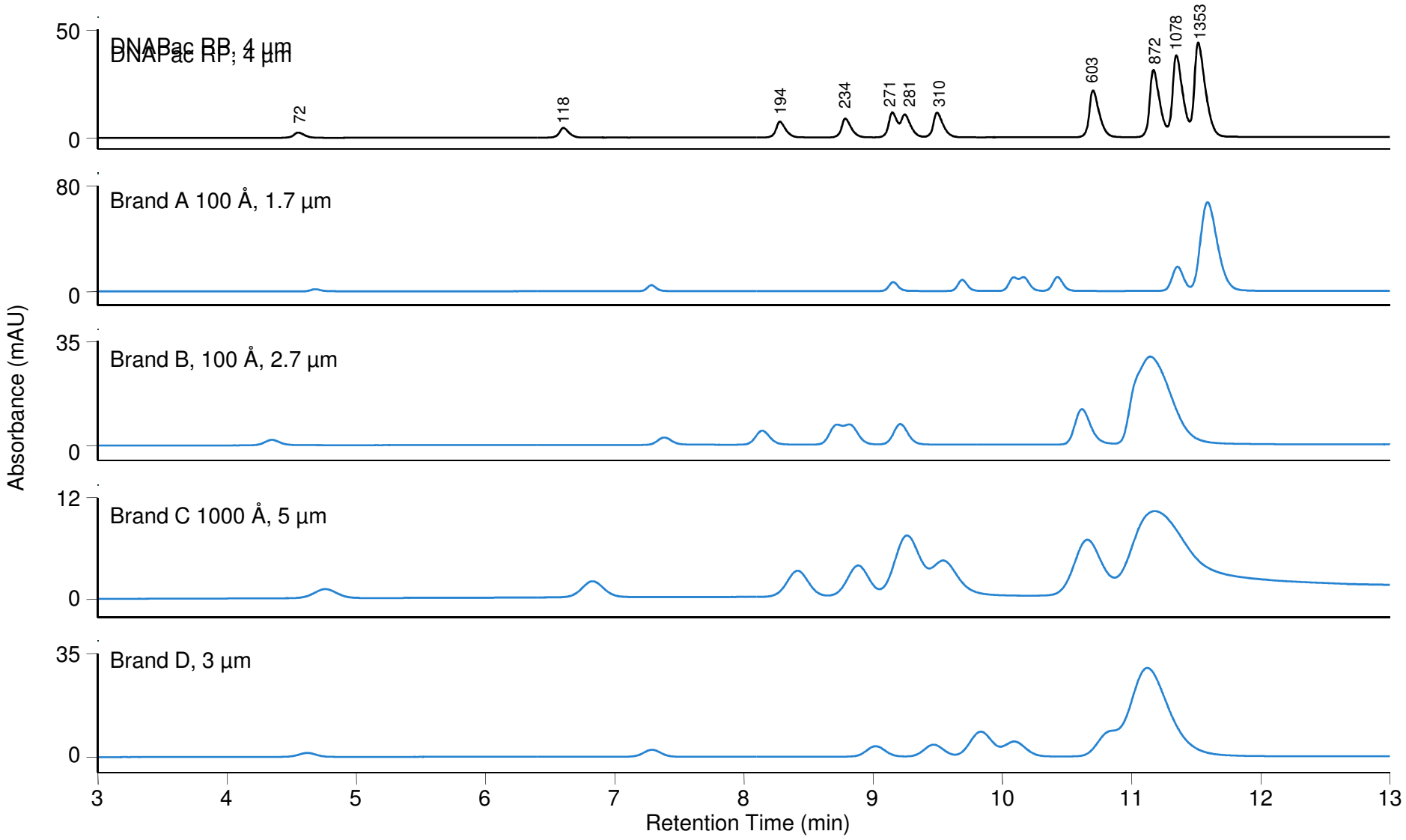
DNAPac RP vs Competitors: dsDNA



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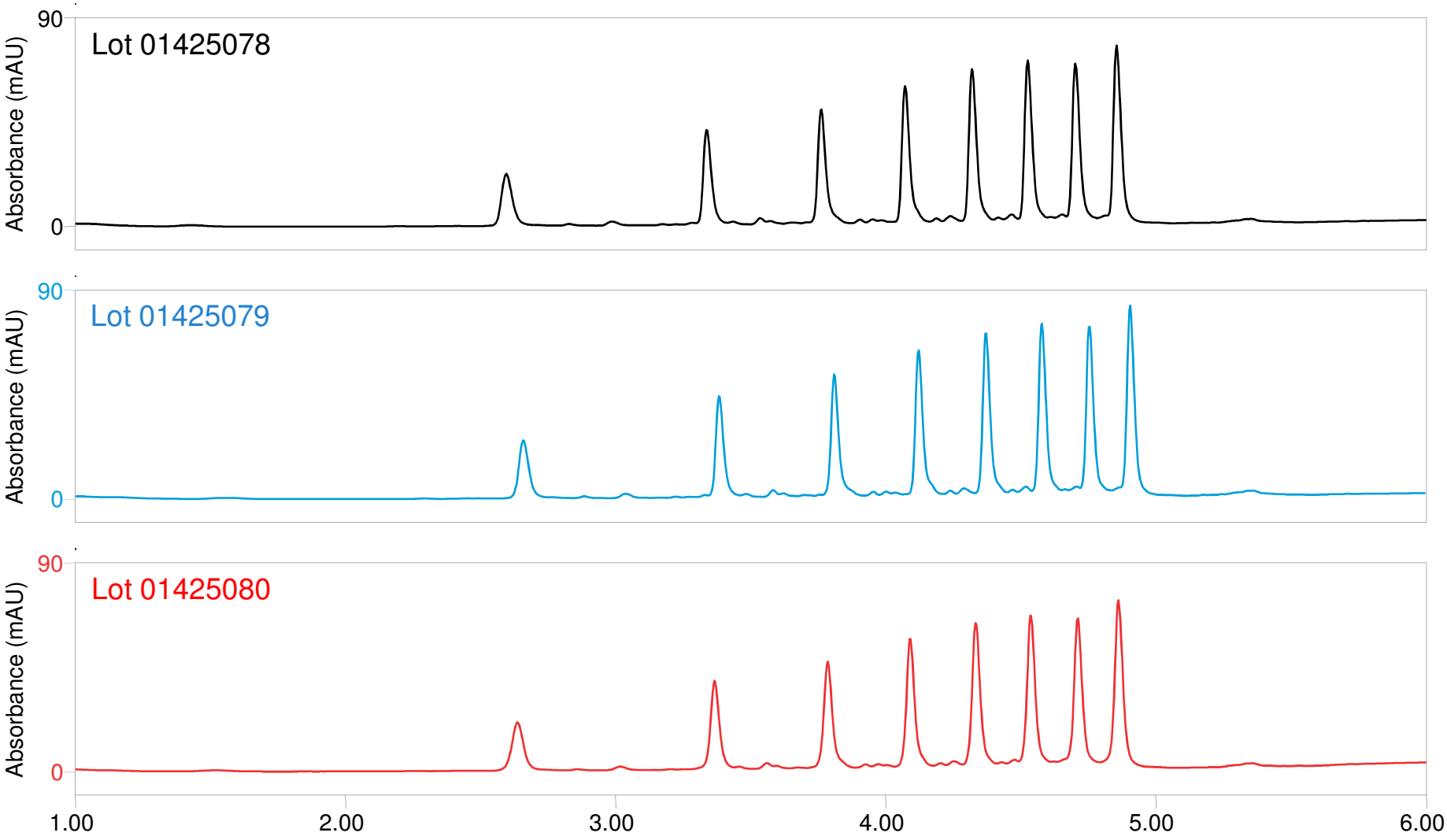
Lot-to-Lot Variability: ssDNA



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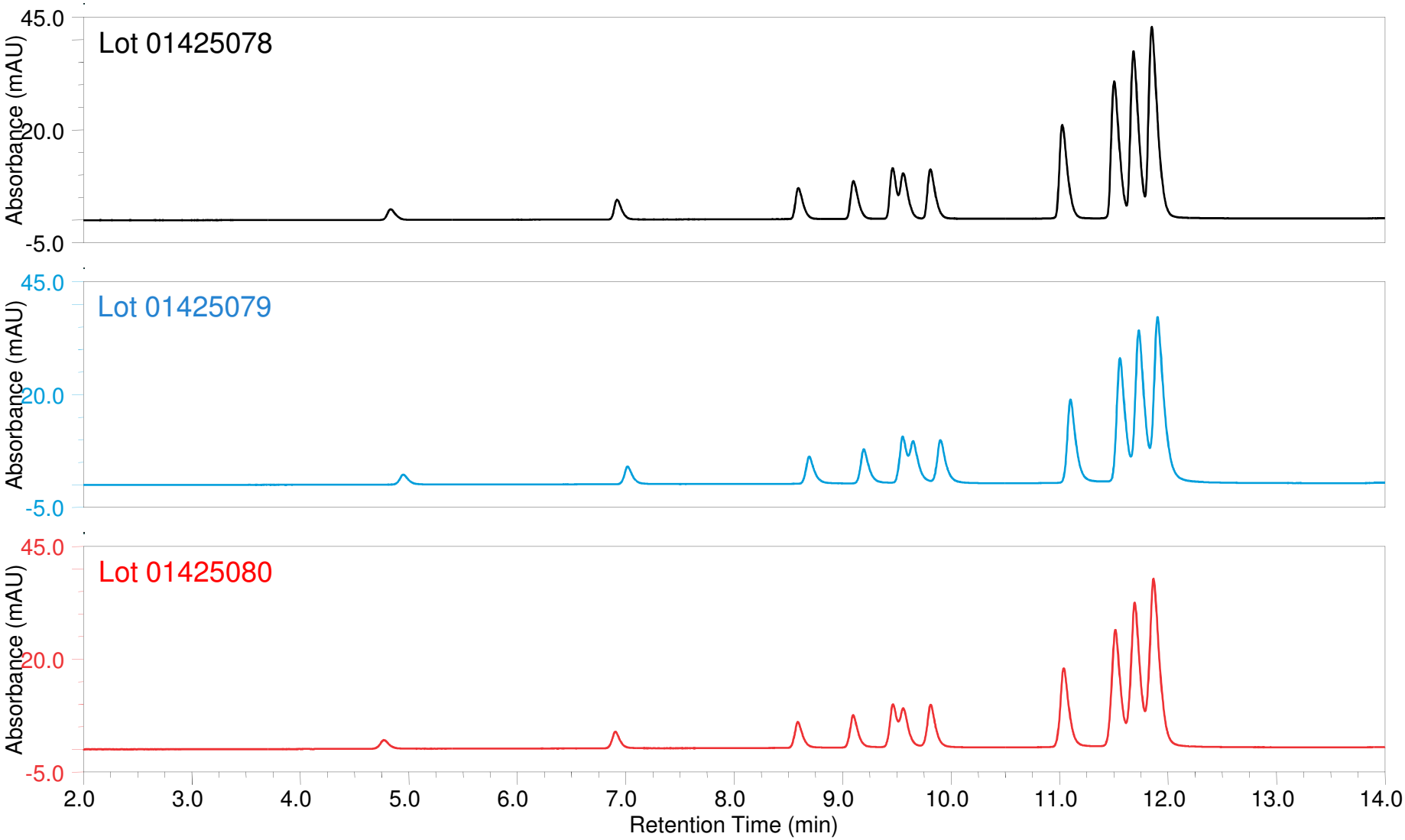
Lot-to-Lot Variability: dsDNA



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Summary

- DNAPac RP is a polymer based reversed phase column
- DNAPac RP provides high resolution separation of single-stranded oligonucleotides
- LC/MS analysis of oligonucleotides can be achieved by using DNAPac RP column coupled with Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer
- Wide-pore size of DNAPac RP column provides high resolution separation of large double-stranded DNA/RNA fragments
- DNAPac RP can be used with high pH mobile phase and at high temperature which often provides alternative selectivity and higher resolution for more challenging samples
- DNAPac RP has minimal lot-to-lot variability

Thank You!