Advancing Nucleic Acid Analysis: Large-Pore Superficially Porous Particles for Enhanced Separations

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Introduction to Oligos

- Why are oligonucleotides being analyzed?
  - Oligonucleotides are being developed as drugs
    - These drugs can be used to modulate gene expression
  - Impurity analysis of said drugs is required for effective products
  - The development of mRNA vaccines (Covid-19)

- What are the challenges of separating oligonucleotides?
  - Complicated structures and sizes
  - A phosphate backbone that reduces retention under RPLC
  - Modifications can range broadly: phosphorothioates, lipids, sugars or nucleobases
  - Sample stability requires suitable handling (nucleases and metals)
  - Hybrid structure formation can be either intended or unwanted

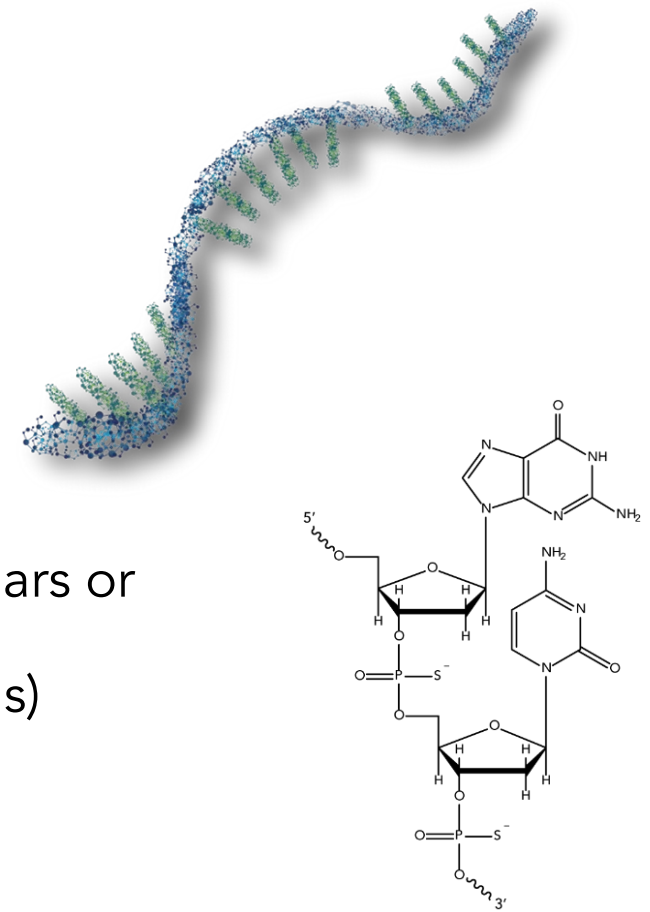


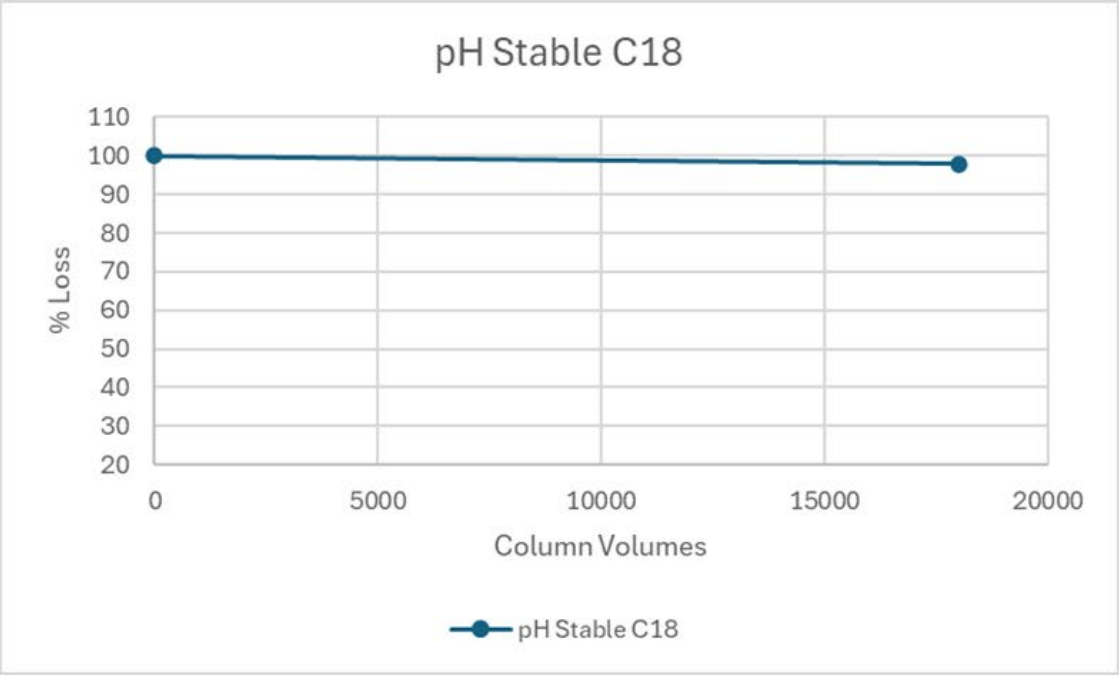
Figure 1: Structure of a 2'-O-modified RNA. (idtdna.com)

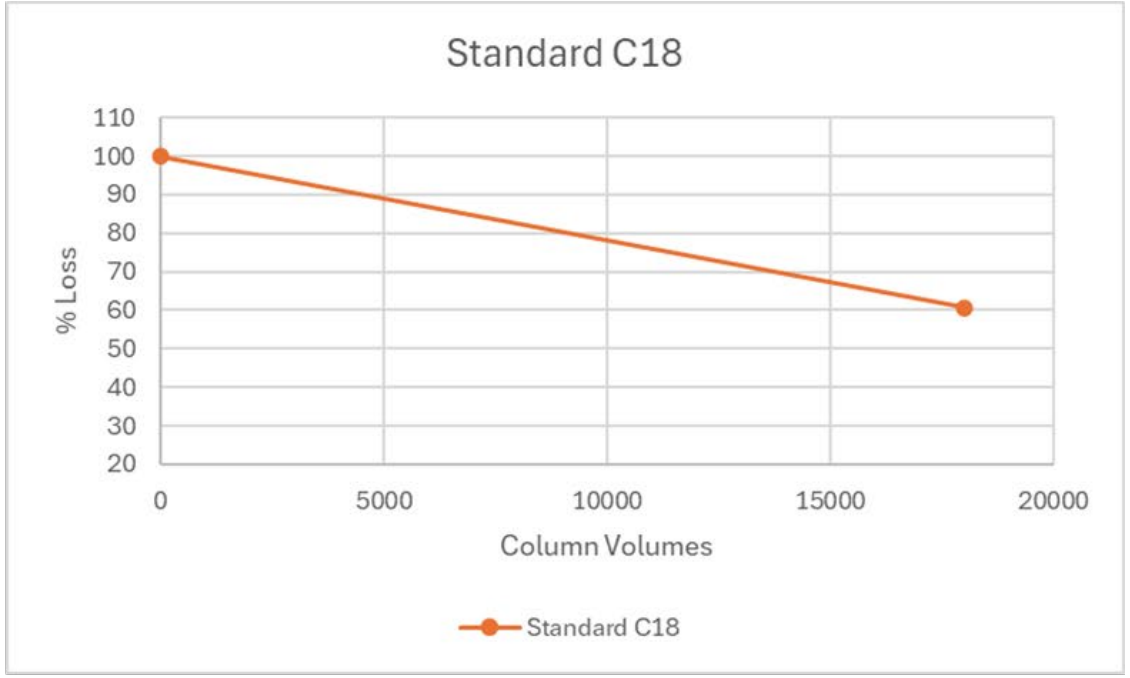
Technical Considerations for Oligonucleotide Separations

- Reversed-phase or ion-pairing RP HPLC methods have largely replaced gel electrophoretic methods for small (<20 nts) and medium size (<60 nts) oligo/poly nucleotides.
- IP-RP methods can be rapid, and with suitable choices can be used with online MS detection for identity and sequence analysis.
- Most IP-RP approaches are at pH 6-11, and often at elevated temperature, especially when complementary hybrids can be formed (internal or intermolecular hybrids).
- Hybrid formation is temperature, ionic strength and solvent dependent, and defined by sequence (GC vs AT or AU), as well as backbone (RNA vs DNA).

Importance of High pH Stability

- At high pHs, native silica will be solubilized unless treated for high pH stability
- The graph measures the result of efficiency (plates) of both a standard silica and the new surface modified silica from AMT after stability testing.
- The pH stable phase maintained good efficiency after the stability while a standard, silica particle bonded phase material, lost efficiency.





Importance of High pH Stability

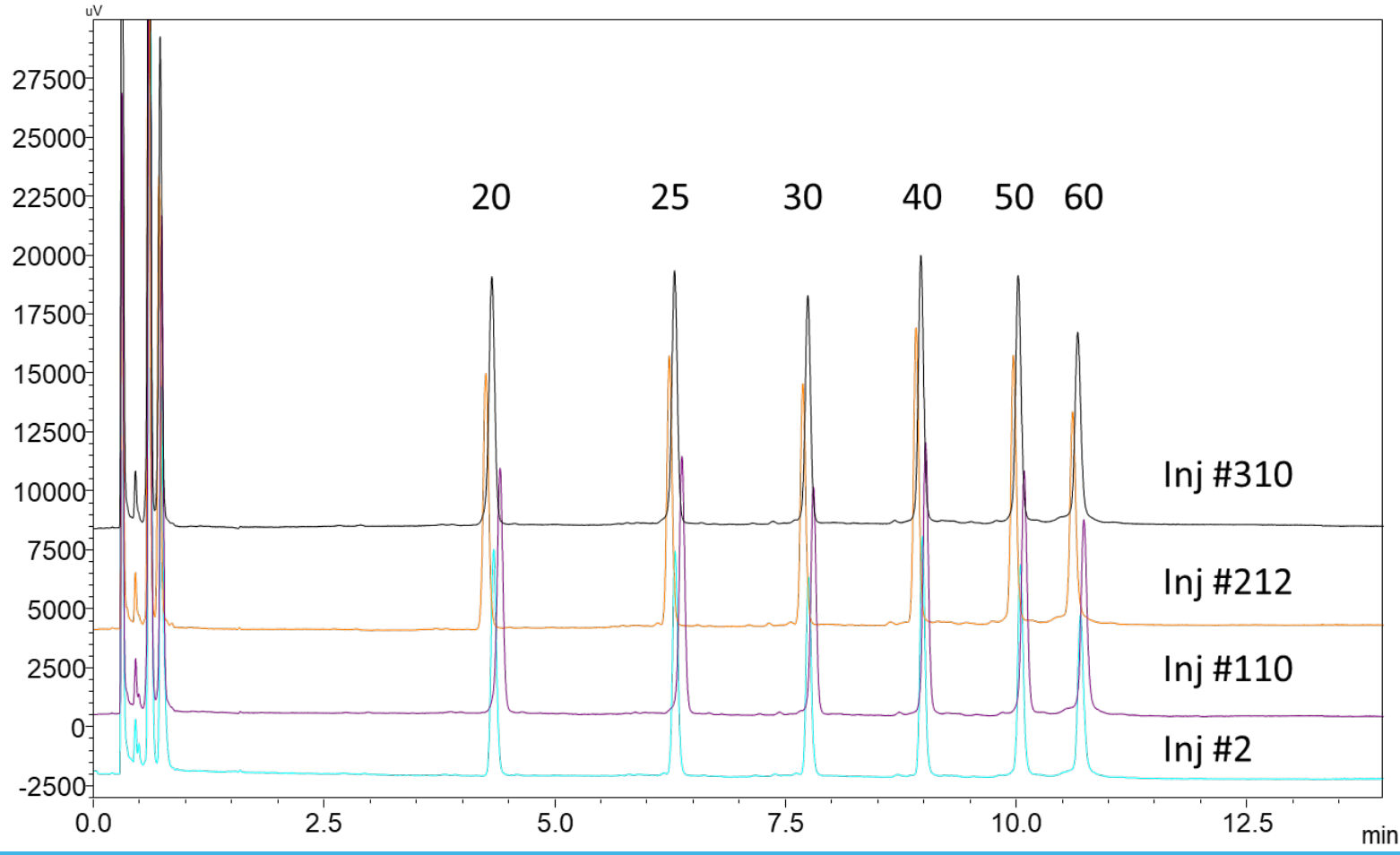
Elevated pH and Temperature Testing of Halo Oligo C18

Columns: 2.1 x 50 mm, Oligo C18
Flow Rate: 0.5 mL/min
Temp: **60°C**
A- 15 mM TEA/50 mM HFIP, **pH 8.9**
B- MeOH
Detection: 260 nm, 10 nm
Sample: 1 µL, 10/60 IDT Standard @ 10ng

Gradient:

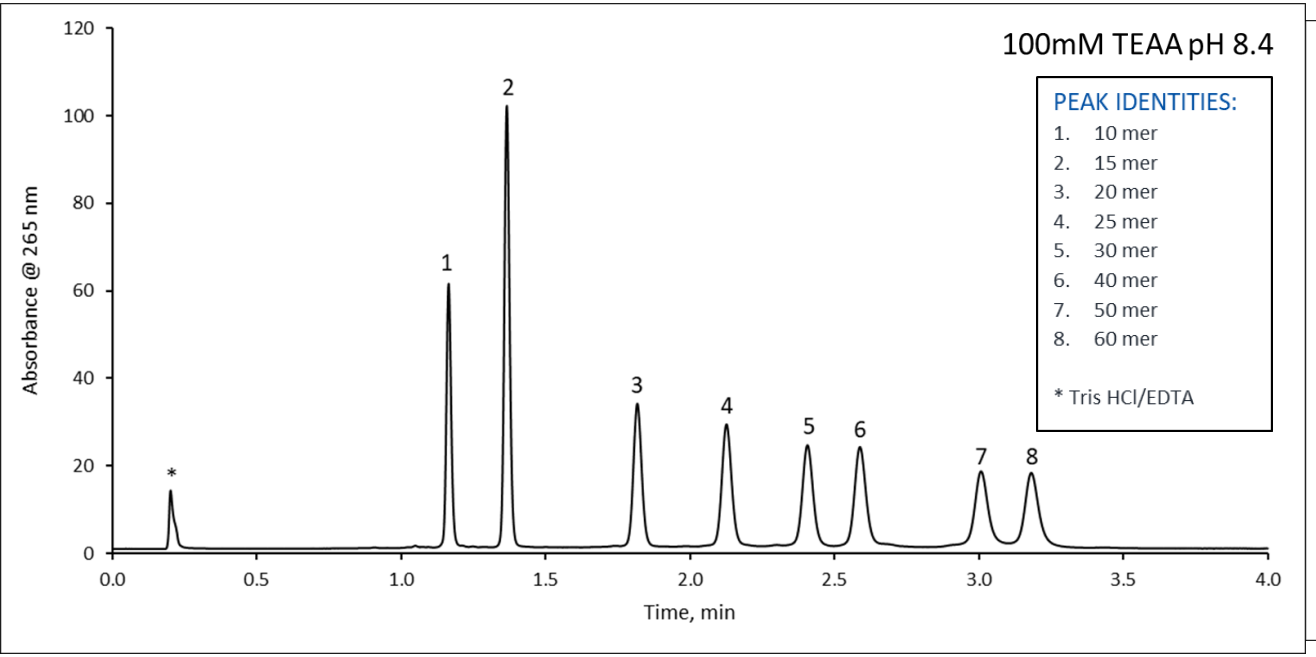
Time	%B
0.0	5.0
14	12
14.1	30
14.4	30
14.5	5
20.0	Stop

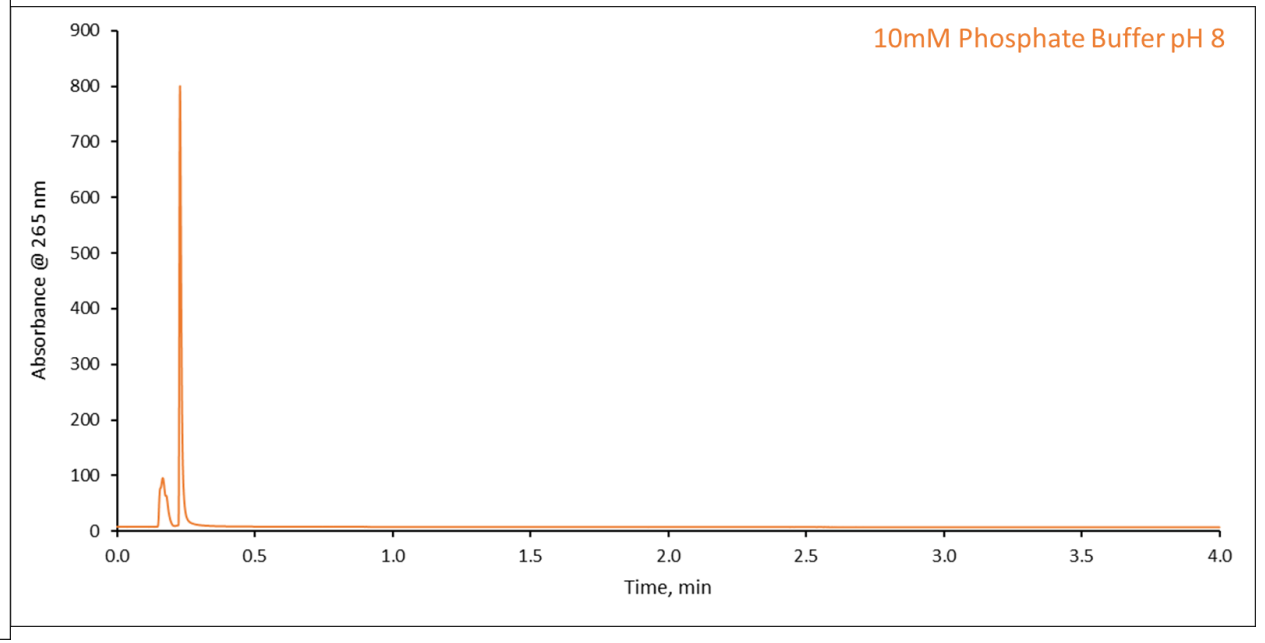
- A stability on the prototype HALO® 1000Å oligo product from AMT
- Over 300 injections, retention of oligos shifts very little
- With mass spec friendly ion pairing conditions (TEA/HFIP)



Why Ion Pairing?

- Contemporary oligonucleotide analysis is frequently conducted using ion pair reversed phase chromatography (IPRP).
- TEA (triethylamine) or TEA-Acetate (TEAA) buffer is a common IPRP modifier, creating favorable IPRP separations.
- Phosphate buffer does not support IPRP, meaning little to no retention.





Standard Separation with Ion Pairing

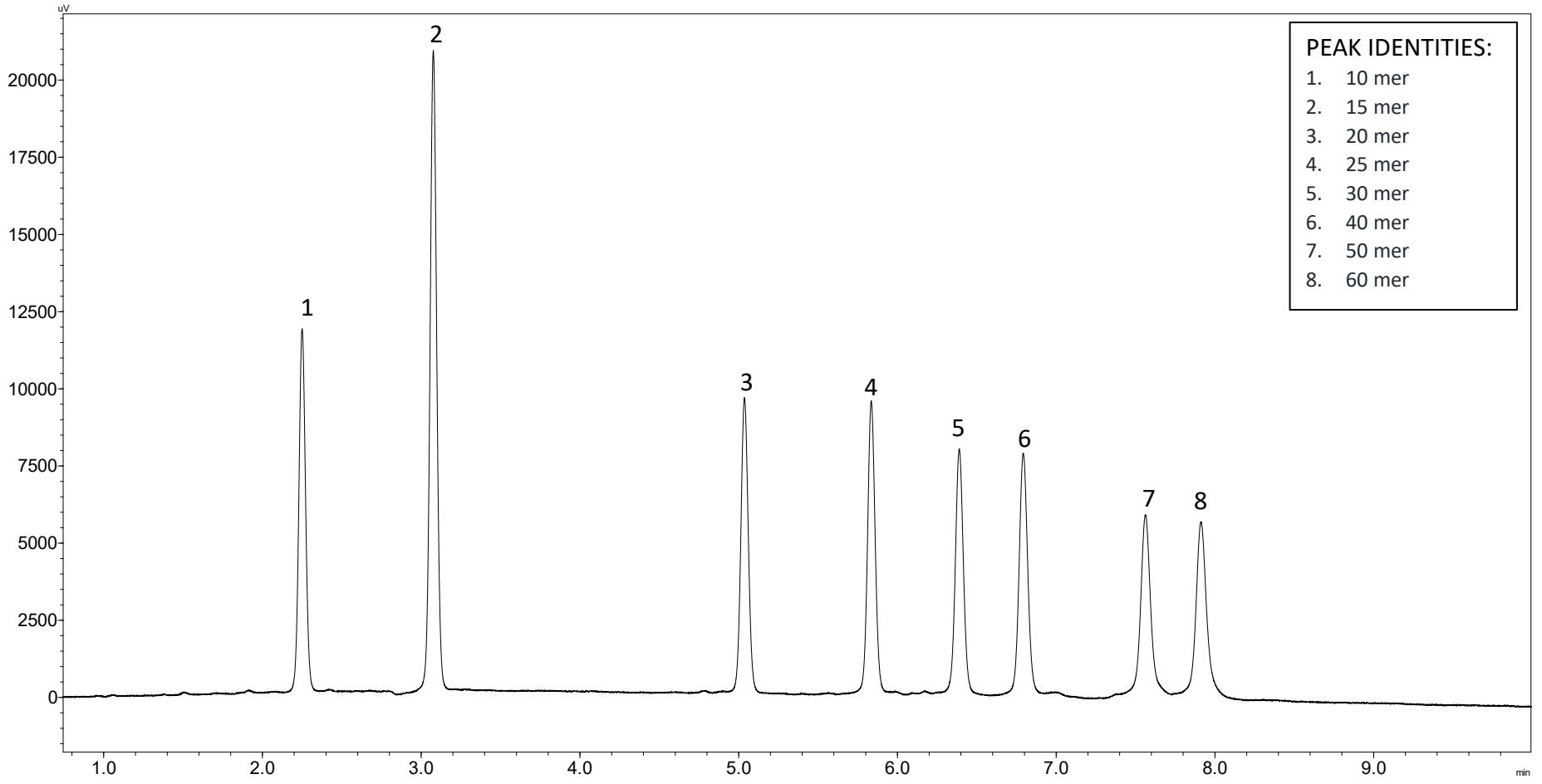
HALO®

Testing Conditions:

Column: HALO 120 Å OLIGO, 2.7 µm, 2.1 x 50 mm
Mobile Phase: A: 100mM TEAA
Adjusted to pH = 8.5
B: ACN
Gradient:

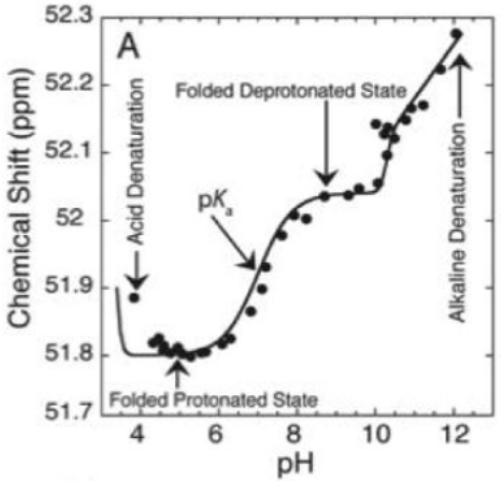
Time	%B
0.0	5
10.0	11
11.0	11
11.5	0
16.5	0

Flow Rate: 0.5 mL/min
Back Pressure: 140 bar
Temperature: 60 °C
Injection: 1.0 µL, 10µg on Column
Sample Solvent: 10mM Tris HCl/1mM EDTA pH=8.0
Wavelength: PDA, 254 nm
Flow Cell: 1 µL
Data Rate: 100 Hz
Response Time: 0.025 sec.
LC System: Shimadzu Nexera X2



Effects of pH on Oligonucleotides

- Five different pHs were used to evaluate how the retention of oligonucleotides differs at the pHs 6 and 9.5
- TEAA was used as the ion pairing reagent for each mobile phase and acetic acid was used to adjust the pH as specified
- The concentration of TEA was maintained at 100mM to ascertain the role that pH has on oligonucleotide retention
- By adjusting pH with acetic acid, we can see, retention modestly decreases as pH increases
- Around a pH of 9, it is suggested that at 60°C oligonucleotides will adopt a folded deprotonated state, reducing retention



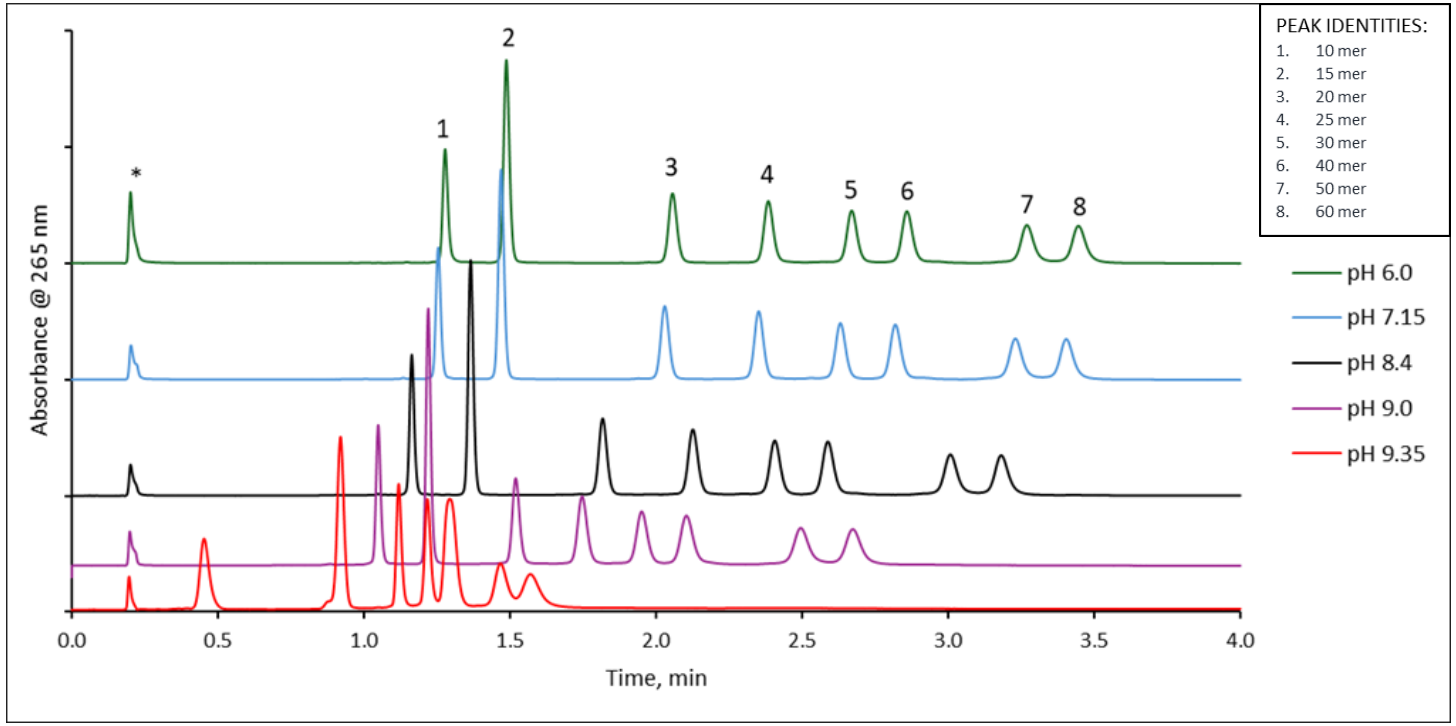
Testing Conditions:

Column: HALO 120 Å OLIGO, 2.7 µm, 2.1 x 50 mm
Mobile Phase: A: Refer to Chromatogram
B: ACN

Gradient:

Time	%B
0.0	5
0.5	7.4
3.5	10.7
3.6	20
4.1	20
4.2	5
9.0	5

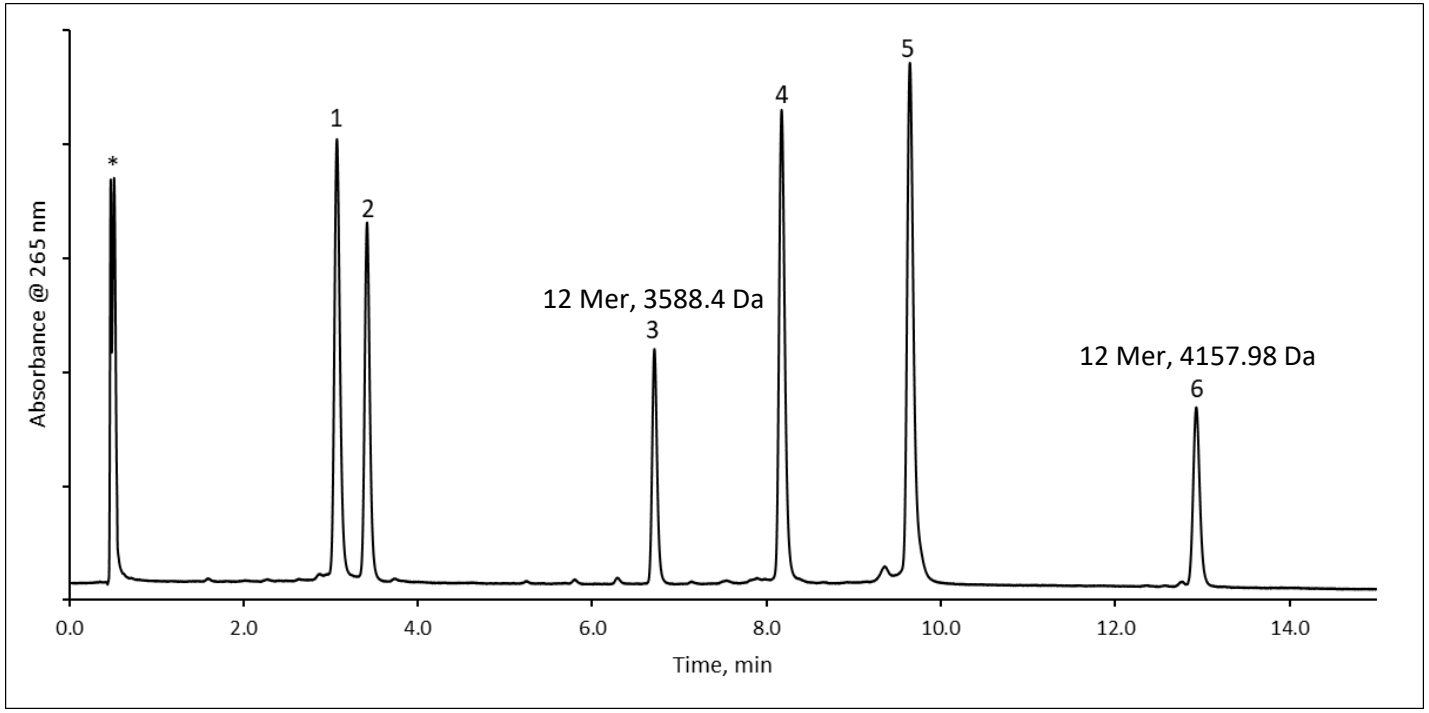
Flow Rate: 0.5 mL/min
Back Pressure: 140 bar
Temperature: 60 °C
Injection: 1.0 µL, 10µg on Column
Sample Solvent: 10mM Tris HCl/1mM EDTA pH=8.0
Wavelength: PDA, 265 nm
Flow Cell: 1 µL
Data Rate: 40 Hz
Response Time: 0.05 sec.
LC System: Shimadzu Nexera X2



Thaplyal, P., & Bevilacqua, P. C. (2014). Experimental approaches for measuring pKa's in RNA and DNA. Methods in enzymology, 549, 189–219. https://doi.org/10.1016/B978-0-12-801122-5.00009-X

Oligomer Composition Matters!

- Oligonucleotide sequences can change retention characteristics if composition differs, but base length stays the same.
- Two oligomers in this mix are the same base length, 12 mer, but are well resolved The difference in retention can be attributed to a difference in sequence which also affects mass.
- The earlier eluting 12 mer oligonucleotide has a mass of 3588 Da compared to the later eluting oligomer which has a mass of 4157 Da.



Testing Conditions:

Column: HALO 120 Å OLIGO C18, 2.7 µm, 2.1 x 100 mm
Part Number: P2A62-602
Mobile Phase: A: 100mM TEAA @ pH 7.0
B: Acetonitrile
Gradient:

Time	%B
0.0	7.5
20.0	15
20.3	60
20.6	60
20.8	7.5

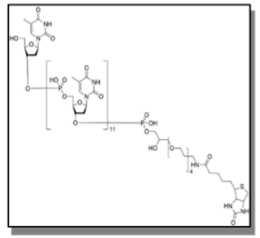
Flow Rate: 0.4 mL/min
Back Pressure: 142 bar
Temperature: 50 °C
Injection: 4 µL of Oligonucleotide Performance Standard Mix, 12-33 NT
P/N: PHR8667-1EA
Sample Solvent: 10mM Tris HCl/ 1mM EDTA
Wavelength: PDA, 265 nm
Flow Cell: 1 µL
Data Rate: 40 Hz
Response Time: 0.05 sec.
LC System: Shimadzu Nexera X2

PEAK IDENTITIES:

1. 20 mer
2. 15 mer
3. 12 mer
4. 25 mer
5. 33 mer
6. 12 mer

Sequence	Mer	Chemical Formula	Molecular Weight	Content nmol/vial
TTT TTT TTT TTT	12	$C_{120}H_{157}N_{24}O_{82}P_{11}$	3588.40	0.8
TTT TTT TTT TTT 3'mod {BtnTg} [1]	12	$C_{142}H_{197}N_{27}O_{92}P_{12}S_1$	4157.98	1.0
AGC TGT ACT TTT TTT TTT TTT TTT T	25	$C_{248}H_{320}N_{64}O_{165}P_{24}$	7580.90	1.0
AGC TGT ACT TTT TTT TTT TTT TTT TTT TTT TTT	33	$C_{328}H_{424}N_{80}O_{221}P_{32}$	10014.40	1.0
TGT GAC CAC GTA GAC TGA CT	20	$C_{195}H_{246}N_{75}O_{118}P_{19}$	6117.04	1.0
TCT CTC TCT CTC TCT	15	$C_{143}H_{189}N_{37}O_{96}P_{14}$	4395.90	1.0

[1] TTT TTT TTT TTT 3'mod {BtnTg} structure below.



ssRNA Under Ion Pairing

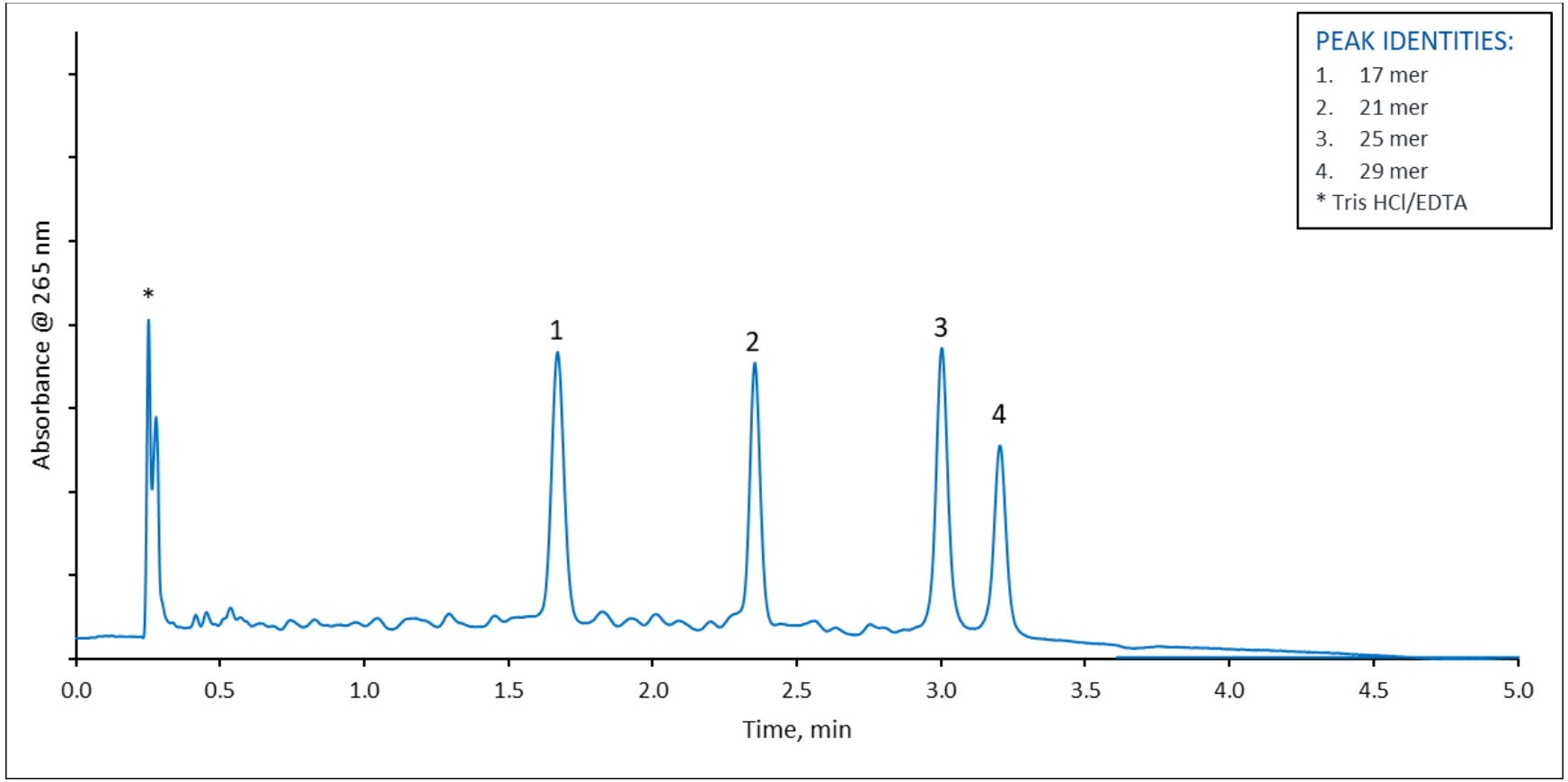
- ssRNA has less retention compared to ssDNA under the same conditions
- ssRNA has a relatively flexible structure that can reduce its hydrophobic interactions
- As a result, ssRNA may not form stable complexes with the ion-pairing agents

Testing Conditions:

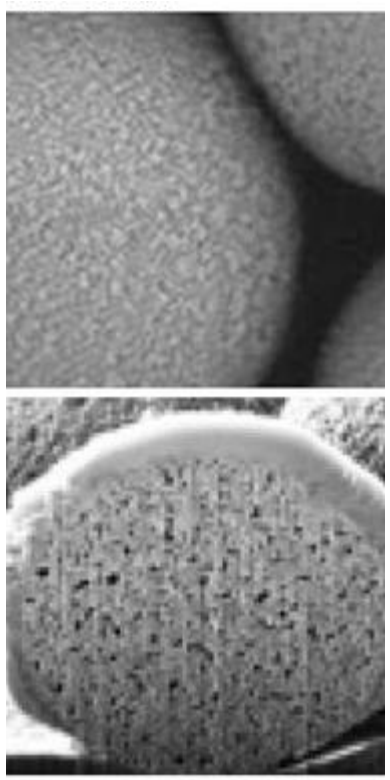
Column: HALO 120 Å OLIGO C18, 2.7 µm, 2.1 x 50 mm
Part Number: P2A62-402
Mobile Phase: A: 100mM TEAA, Adjusted to pH = 8.56
B: ACN
Gradient:

Time	%B
0.0	5
5.0	10
5.3	60
5.6	60
5.7	5
9.0	5

Flow Rate: 0.4 mL/min
Back Pressure: 116 bar
Temperature: 60 °C
Injection: 3.0 µL, 30µg on Column
Sample Solvent: 10mM Tris HCl/1mM EDTA pH=8.0
Wavelength: PDA, 265 nm
Flow Cell: 1 µL
Data Rate: 40 Hz
Response Time: 0.05 sec.
LC System: Shimadzu Nexera X2

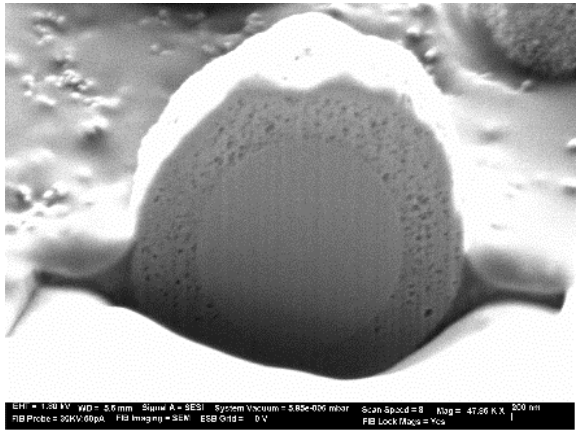


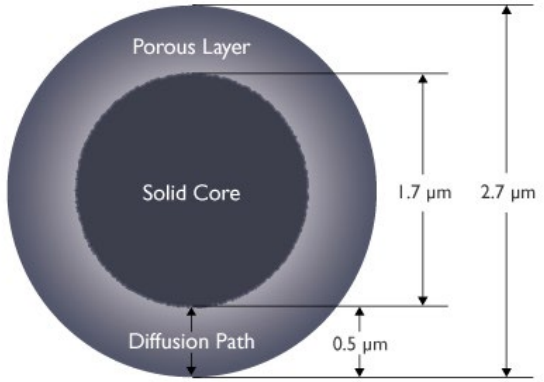
SPP Technology



Fully Porous Particle (FPP)

HALO 90 Å, 2.7 µm





Superficially Porous Particle (SPP)

Brief terminology lesson:

FPP – Fully Porous Particle

TPP- Totally Porous Particle

SPP – Superficially Porous Particle

Terms associated with SPP – Fused-Core®, solid-core, core shell

Effects of SPP Technology

HALO®

Testing Conditions:

Column: HALO 120 Å OLIGO C18, 2.7 µm, 2.1 x 50 mm
Part Number: P2A62-402
Competitor: FPP 120 Å C18, 1.9 µm, 2.1 x 50 mm
Mobile Phase: A: 100mM TEAA, pH 7
B: MeOH
Gradient:

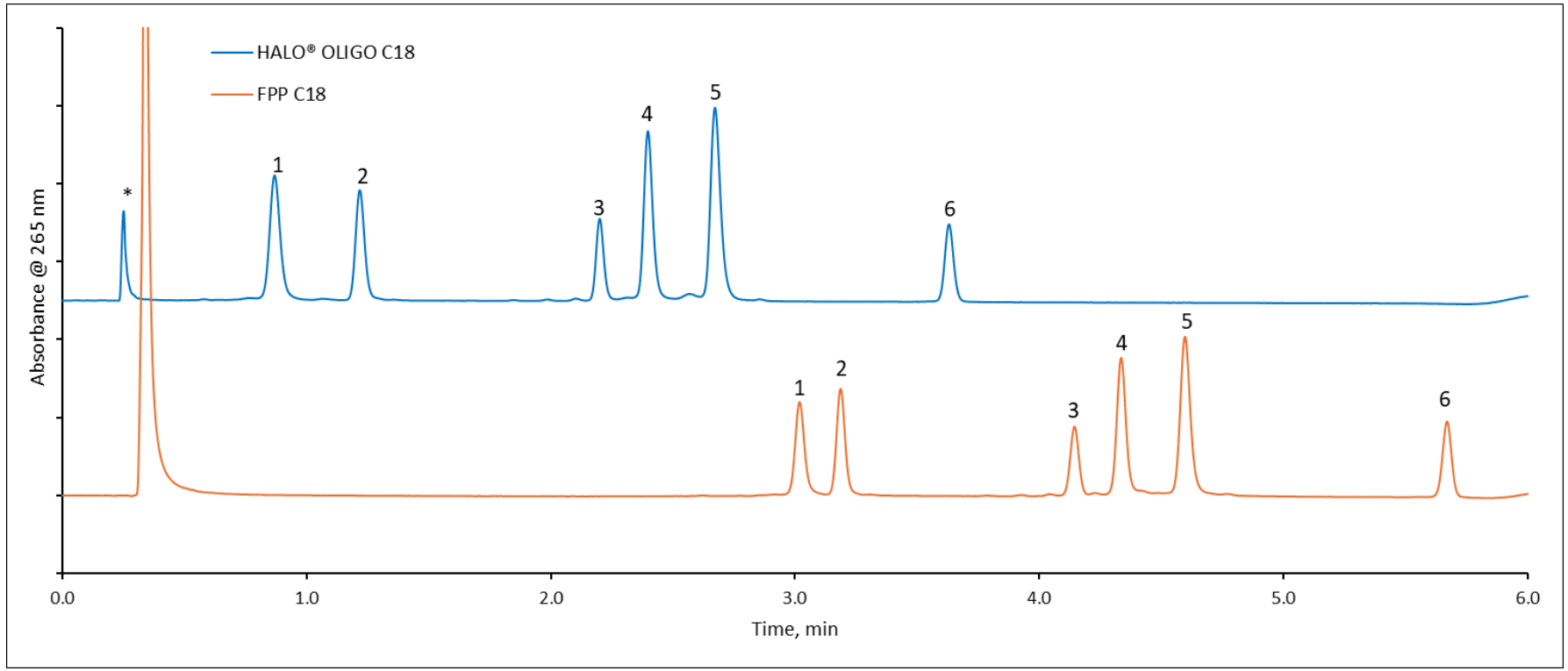
Time	%B
0.0	17
5.0	30
5.3	60
5.6	60
5.8	17

Flow Rate: 0.4 mL/min
Back Pressure: HALO® - 135 bar
FPP - 302 bar
Temperature: 50 °C
Injection: 1.0 µL
Sample Solvent: 10mM Tris HCl/1mM EDTA pH=8.0
Wavelength: PDA, 265 nm
Flow Cell: 1 µL
Data Rate: 40 Hz
Response Time: 0.05 sec.
LC System: Shimadzu Nexera X2

PEAK IDENTITIES:

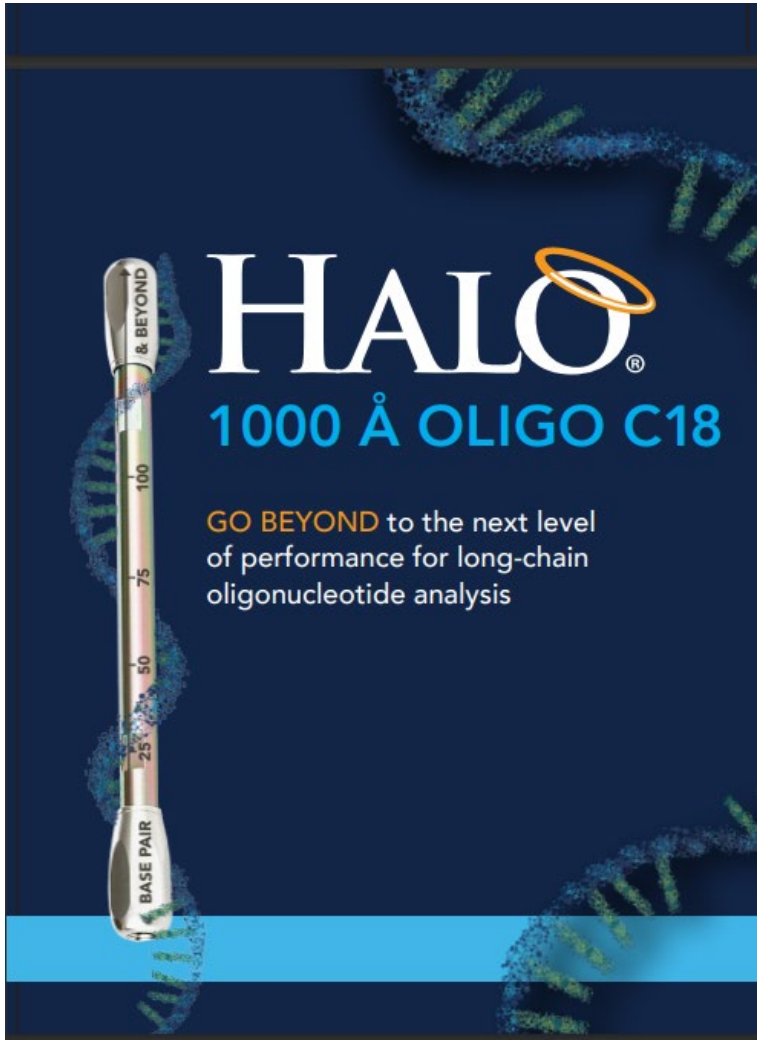
1. 20 mer
2. 15 mer
3. 12 mer
4. 25 mer
5. 33 mer
6. 12 mer

* Tris/EDTA



Coming soon!

HALO1000Å OLIGO C18



Greater Peak Capacity for Larger Pore SPP: TEA/HFIP Acetonitrile

Conditions:

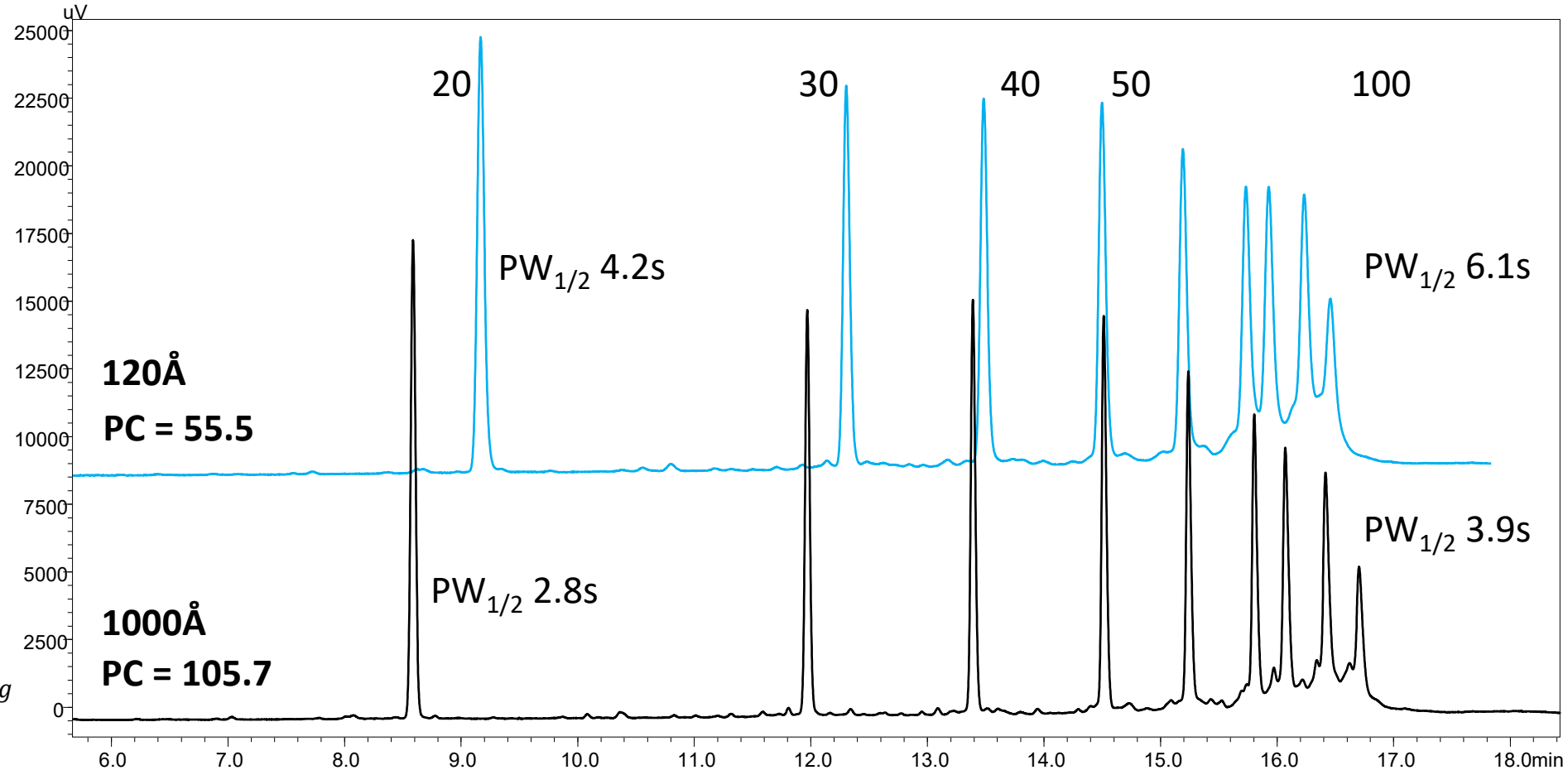
Columns: 2.1 x 100 mm, Inert HW
Flow Rate: 0.5 mL/min
Temp: 60°C
A- 15 mM TEA/50 mM HFIP, pH 8.9
B- Acetonitrile

Gradient:	Time	%
	0.0	1.5
	20	6.5
	21	15
	22	15
	22.5	1.5
	30	Stop

Detection: 260 nm, 10 nm
Sample: 1 µL, 20/100 IDT @ 10ng

$$PC = \Delta(Rt_{100} - Rt_{20})/4\sigma_{avg}$$

(0.25% AcN/min)



Resolution of Longer Oligonucleotides

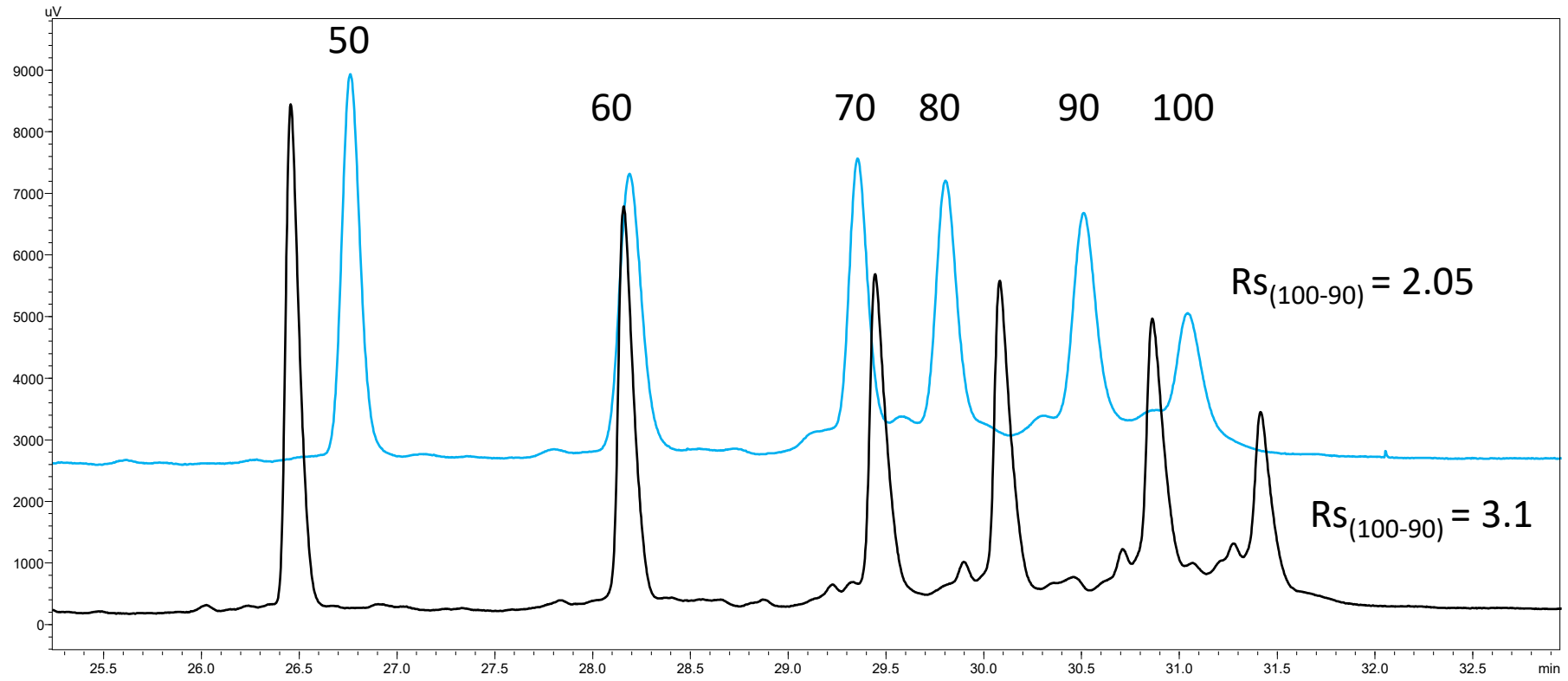
(0.125% AcN/min)

Conditions:

Columns: 2.1 x 100 mm, Inert HW
Flow Rate: 0.5 mL/min
Temp: 60°C
A- 15 mM TEA/50 mM HFIP, pH 8.9
B- Acetonitrile

Gradient:	Time	%
	0.0	1.5
	40	6.5
	41	15
	42	15
	43	1.5
	50	Stop

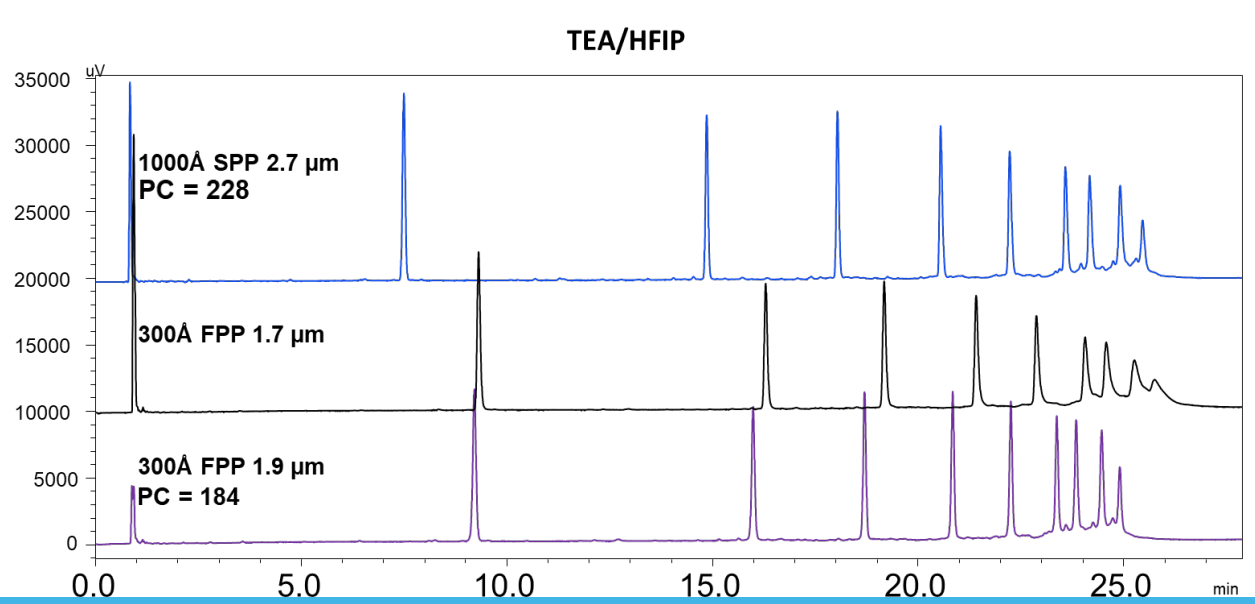
Detection: 260 nm, 10 nm
Sample: 1 µL, 20/100 IDT @ 10ng

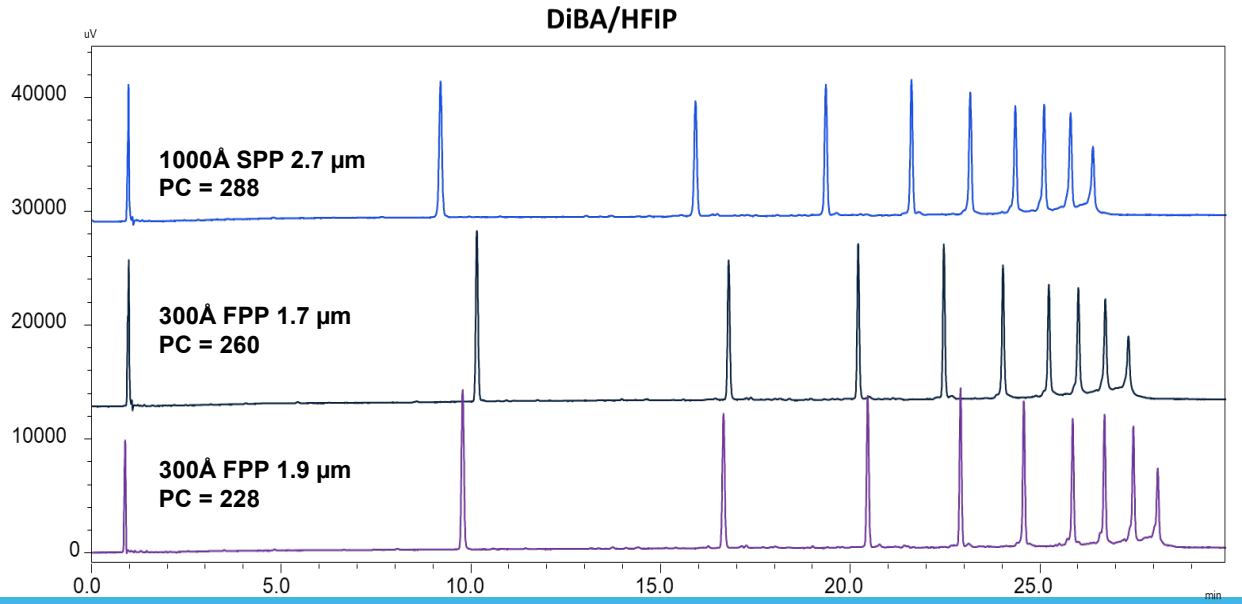


- Adjusting gradient for closer matching of Rt across this sample yields little effect on Rs or PC

Comparing SPP Technologies

- Separations of oligonucleotides are compared on the 1000Å material to commercially available wide pore (300Å) sub-2 µm particle silica materials. The prototype 1000Å SPP exhibited modest improvements in peak widths in TEA/HFIP, and greater gradient range difference between the 20 and 100 base oligonucleotide, contributing to the higher PC.
- Peak shapes improved for larger oligonucleotides using all of the columns, (particularly for the 1.7 µm material), by use of the more hydrophobic DiBA/HFIP mobile phase condition.
- Peak capacities were larger on the 1000Å SPP material, due to both lower PW @ 50% and larger gradient span of the separation.

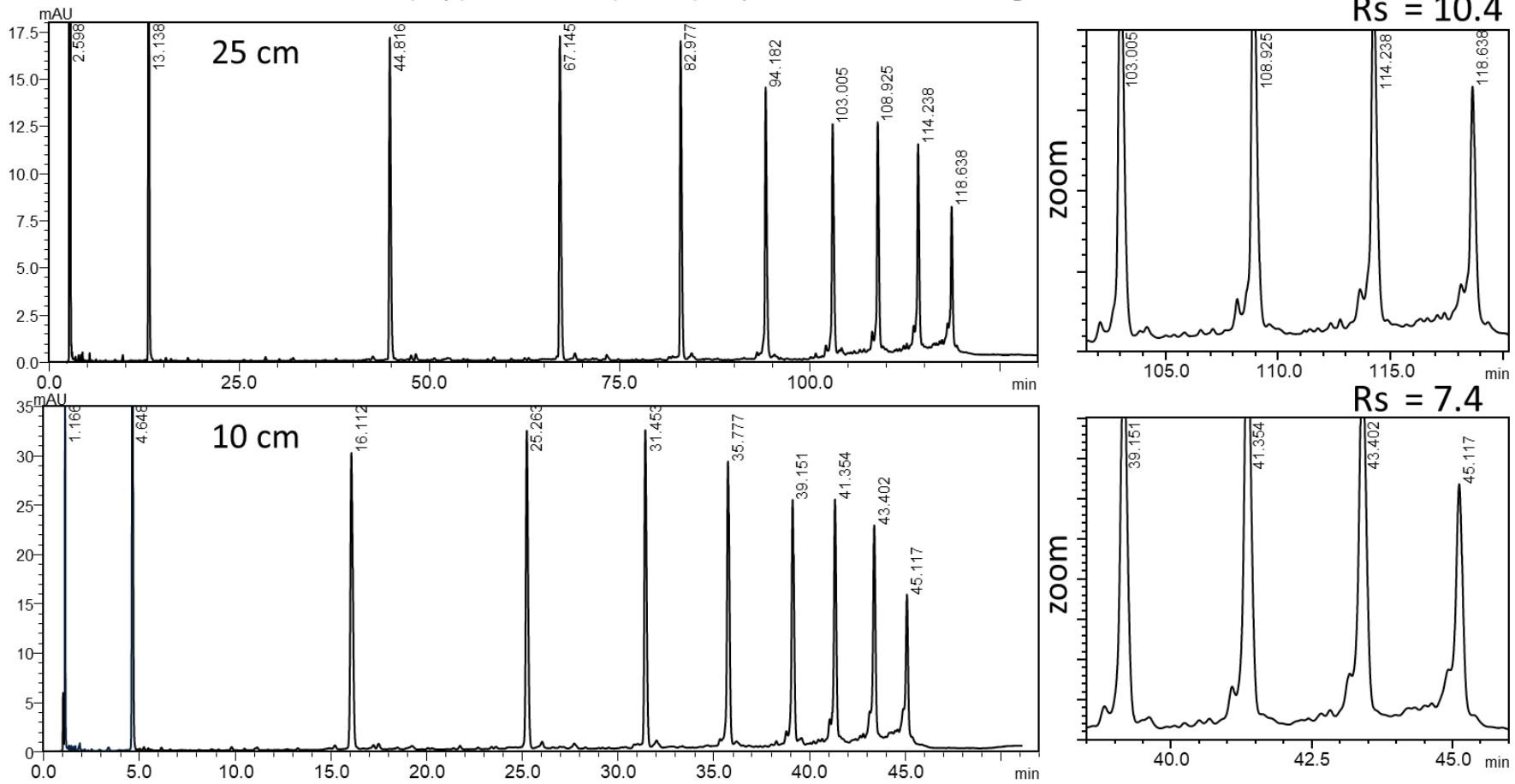




Utility for Resolution of Longer Chain Nucleic Acids

- Resolution of the oligonucleotide mixture on a longer column (25 cm) using a shallow gradient, resolving out to 100 nts.
- Resolution scales close to the √L for column length using the gradient rate scaled to L (150 min to 60 min) for comparing the 25 and 10 cm columns (across the range increase by 1.34; expected 1.58).

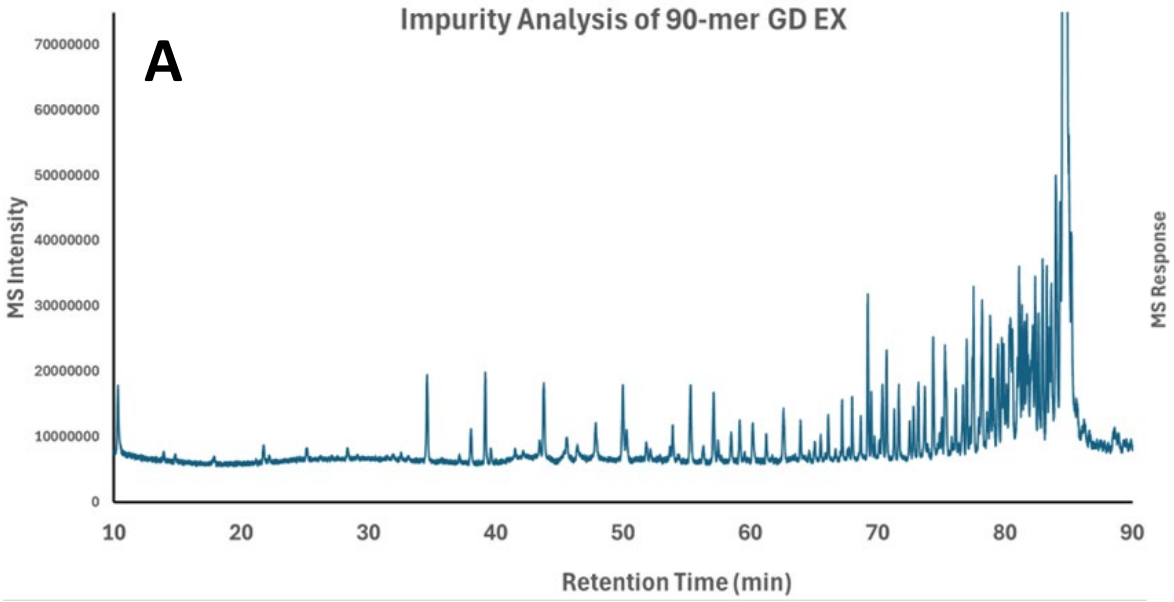
Conditions: 2.1 mm ID columns; 60˚C; Mobile Phase: A – 10 mM DiBA/100 mM HFIP/5% MeOH/5% AcN; B – A at 20% AcN; 15-75%B in 150 min (top) or 60 min (lower); 2 µL, 20/100 IDT @ 20ng

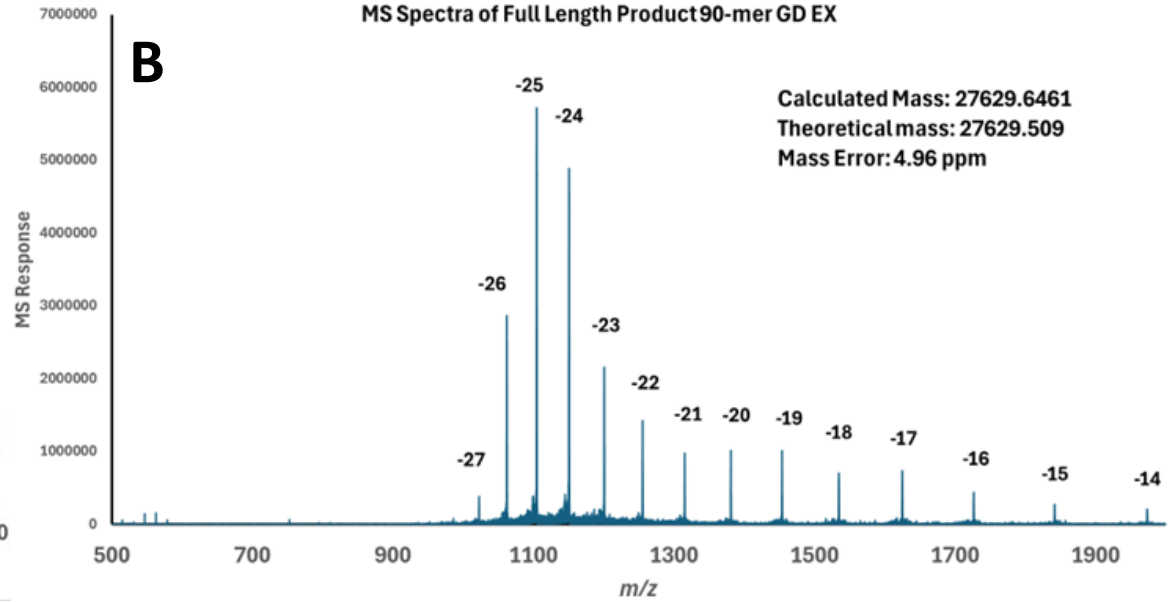


Utility for Resolution of Longer Chain Nucleic Acids – Impurity Analysis

An impurity profile a 90-mer (GD EX) on a 15 cm column by LC/MS, identifying truncates and other impurities (Panel A). The MS spectrum for the full length 90-mer (Panel B). Various truncated species are resolved, including the (n-1) 89 nt 3'-end failure.

Conditions: 2.1 x 150 mm, 1000Å prototype; 0.2 mL/min; 60°C; Mobile Phases:
A- 10 mM DiBA/100 mM HFIP/5% MeOH/5% AcN; B – A at 20% AcN; 0-32%B in 120 min.



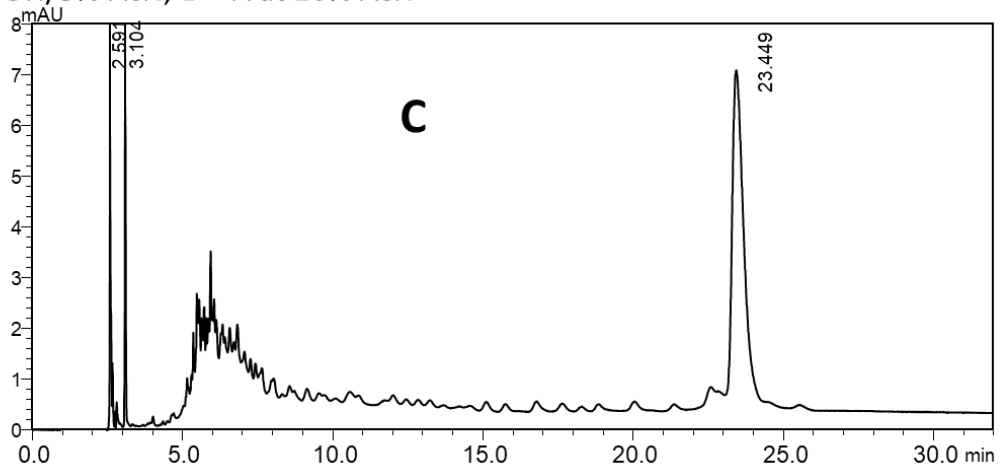


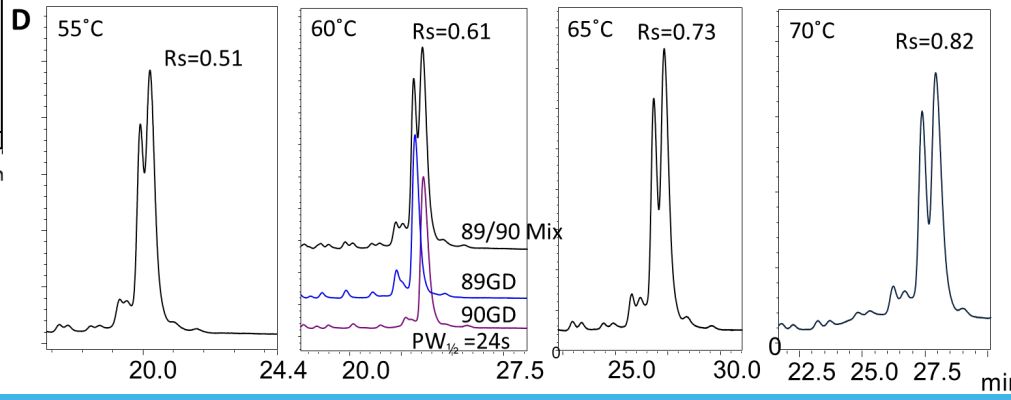
Utility for Resolution of Longer Chain Nucleic Acids – Impurity Analysis

Resolution the GD EX 90-mer and synthesis impurities on a 25 cm column of the 1000Å SPP material (Panel C), with a shallow gradient (0.015% AcN/min). A synthetic 89-mer (-G at 3'-end of GD EX) was obtained. Separation of the 89/90-mer pair is difficult, even with a shallow gradient (0.015% AcN/min). Resolution and retention is surprisingly dependent on temperature, as shown below in Panel D.

Conditions: 2.1 x 250 mm,1000Å; A - 0.2 mL/min; 60°C; Mobile Phase:
A- 10 mM DiBA/100 mM HFIP/5% MeOH/5% AcN; B – A at 20% AcN

Gradient:	Time	%B
	0.0	40.0
	1.0	40
	2.0	64
	30.0	67
	31.0	100
	33.0	100
	34.0	40.0
	40.0	STOP





Summary

- HALO® Oligo C18 is an elevated pH stable column for HPLC, UHPLC, and LC-MS separations. Conditions favorable for oligonucleotide separations are well-tolerated with this hybrid SPP particle for both small and larger pore size column packing materials.
- A novel hybrid silica wide pore SPP particle, exhibiting the efficiency and speed benefits in oligonucleotide separations previously shown today.
- Both gradient and isocratic analysis of column efficiencies demonstrate benefits for separations using wider pore SPP packed columns.
- Large synthetic ssDNA analyses exhibit very high resolution.

Questions?

Photo by Jamie Street on Unsplash

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