Oligonucleotide Analysis: Practical Techniques and Method Development Optimization for HPLC

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LC Columns and Consumables Technical Support
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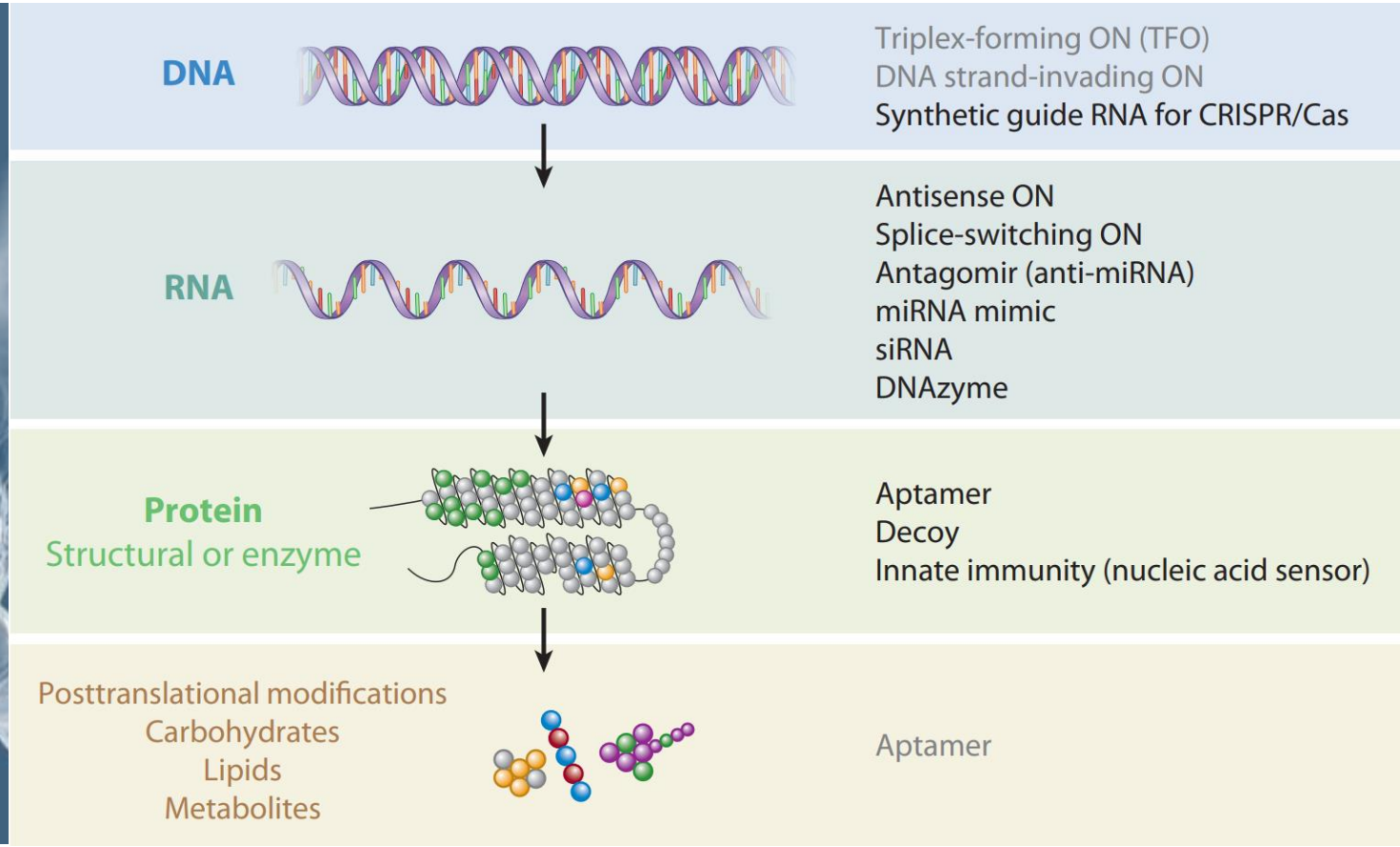
What Are Oligonucleotide Therapeutic Types?

Oligonucleotides are being increasingly developed as therapeutics against a wide range of disease conditions

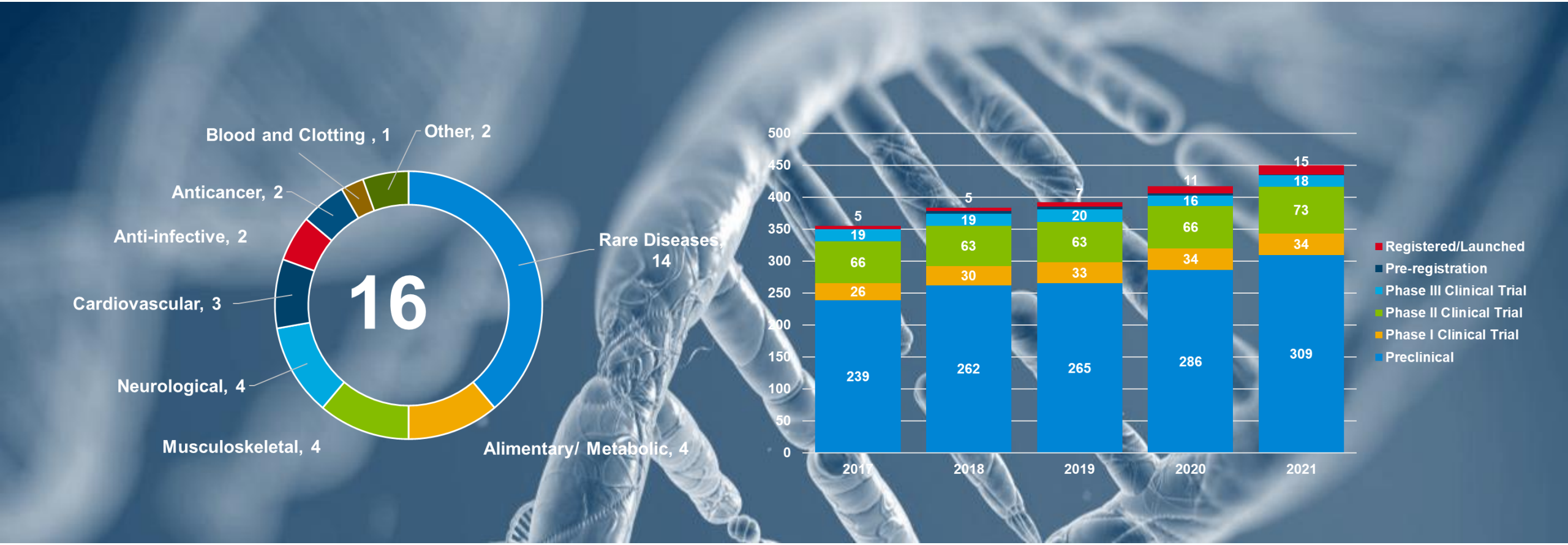
Why do people study/synthesize/analyze oligonucleotides?

- **Inactivating or modifying genes as potential therapeutics**
  - **siRNA**
  - **Guide RNA (CRISPR gene editing)**
  - **Aptamers**
- **Moving genetic info (cloning)**
- **Reading genetic info (genetic testing)**
- **Comparing genetic info (forensics/DNA profiling)**

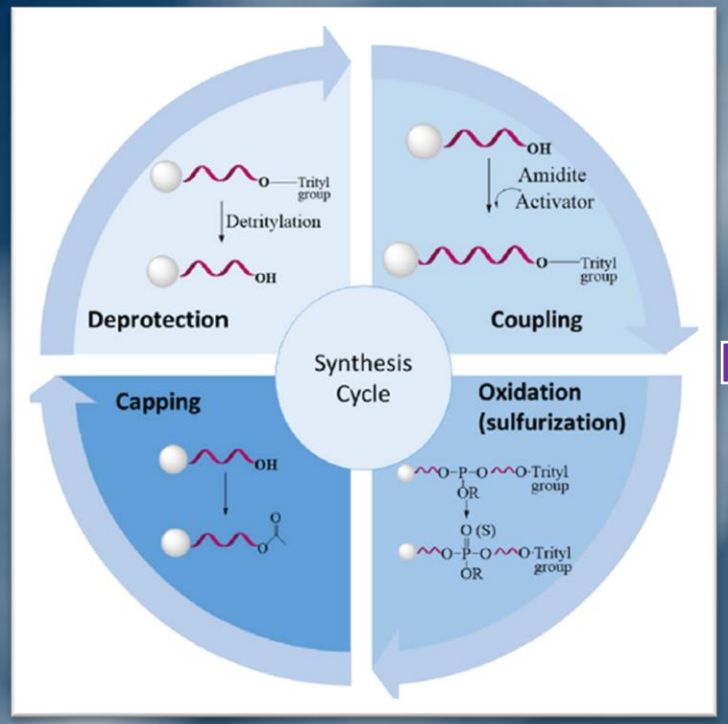


Smith and Zain. (2019) Annual Review of Pharmacology and Toxicology. Vol. 59:605-630.

What Are the Approved Synthetic Oligonucleotide Drug Disease Indications?



What Are the Manufacturing Challenges Associated with Synthetic Oligos?



- The manufacturing batch consists of both the target and failed closely related sequences
- Irregularities in the manufacturing process leads to the formation of the common impurities.

Shortmers (N-1) – oligonucleotides missing one or more nucleotides

Longmers (N+1) – oligonucleotides that include more than the intended number of nucleotides

Lack of protecting groups (derivatives of existing functional groups that decrease reactivity and increase stability)

Other product-related species – phosphodiester analogs, depurinated sequences, partially deprotected sequences, and aggregated sequences

Most short oligonucleotides are synthesized by solid phase phosphoramidite chemistry using a four-step cyclic process of the growing oligomer.

What Does Agilent Offer for Synthetic Oligonucleotide Characterization?

Reliable and robust tools for analytical characterization and preparative separation

- Identity – molecular weight and sequencing (enzymatic or chemical digestion + LC/MS/MS)
- Higher order structures – melting temp (UV); counterions (ICP/MS)
- PI/charge variants (IEX)

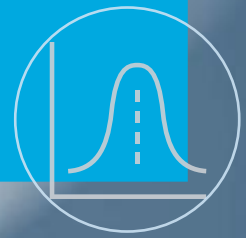
Identity, structure, characterization

- Purity assay (CE/UV, LC, LC/MS, UV, FTIR)
- Product-related impurities (LC, LC/MS, LC/MS/MS)
- Process-related impurities
  - Residual solvents (GC)
  - Elemental analysis (ICP/MS)
- Raw material identification
  - Molecular spectroscopy
  - Raman spectroscopy

Purity and impurities analysis

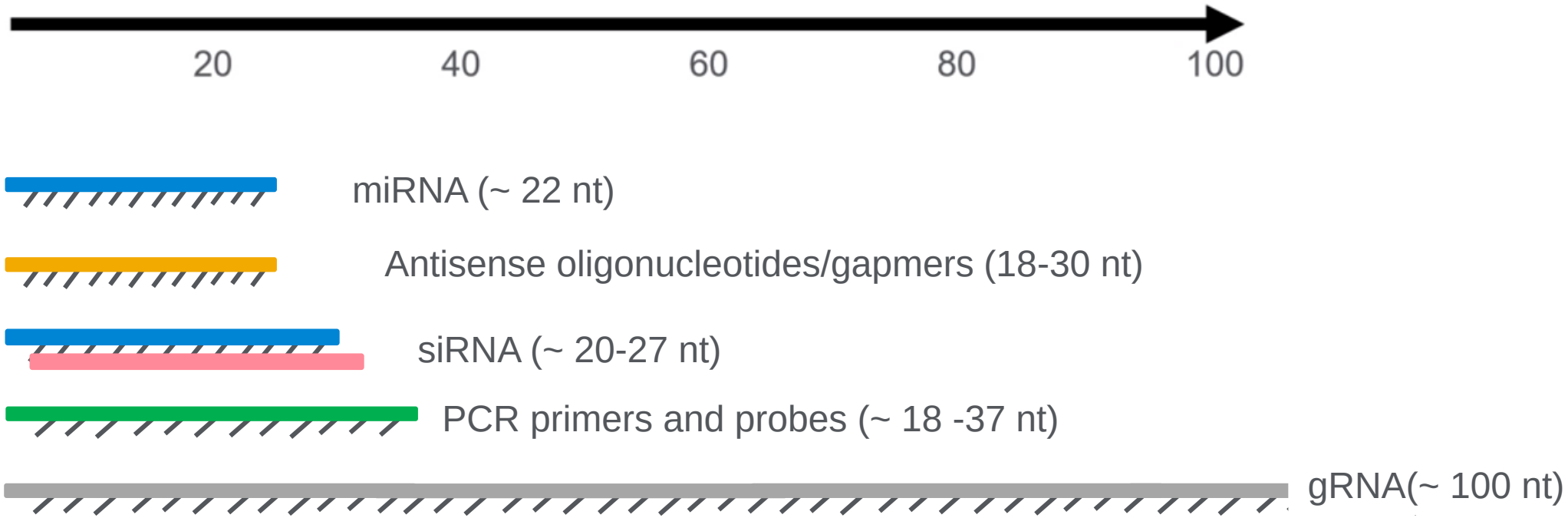
- Multiple prep scale formats
  - Analytical, semiprep, prep LC systems
  - Scale up fraction collection, options of crude OGNs
- Multiple detector-based prep formats (LC/UV; LC/MS)

Purification



Columns, Consumables, Accessories, Compliant SW, Services & Support

Oligonucleotides Represent a Diverse Set of Therapeutics



Oligonucleotides come in many shapes and sizes…

…the linear length of 1,000 nt mRNA would be ~300 nm.

They are often engineered to reduce RNase degradation.
They may also incorporate conjugates or polymer spacers.

Oligonucleotides Represent a Diverse Set of Therapeutics

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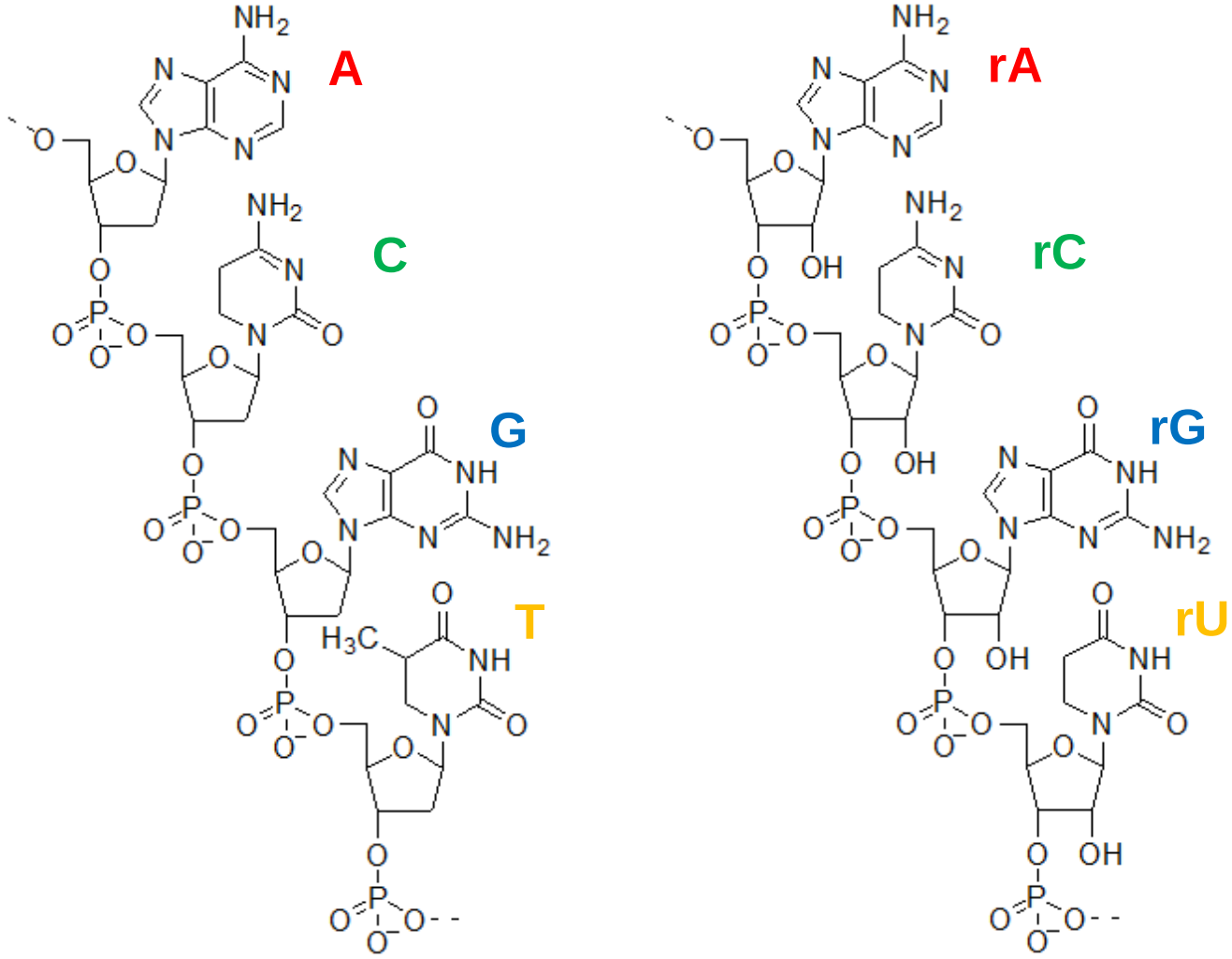
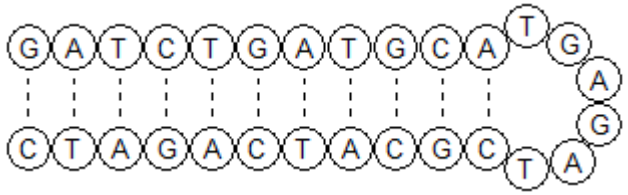
…the linear length of 1,000 nt mRNA would be ~300 nm.

They are often engineered to reduce RNase degradation.
They may also incorporate conjugates or polymer spacers.

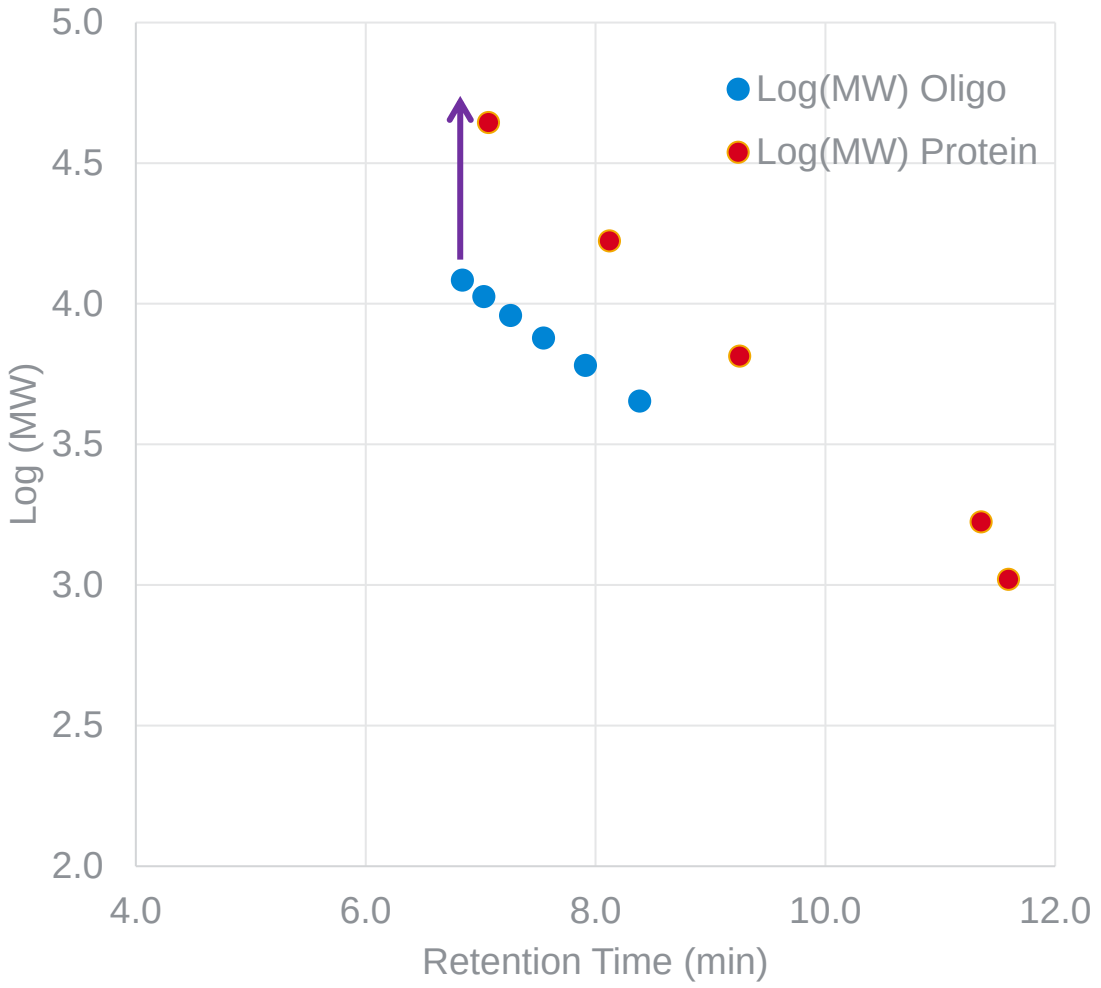
Oligonucleotide Synthesis

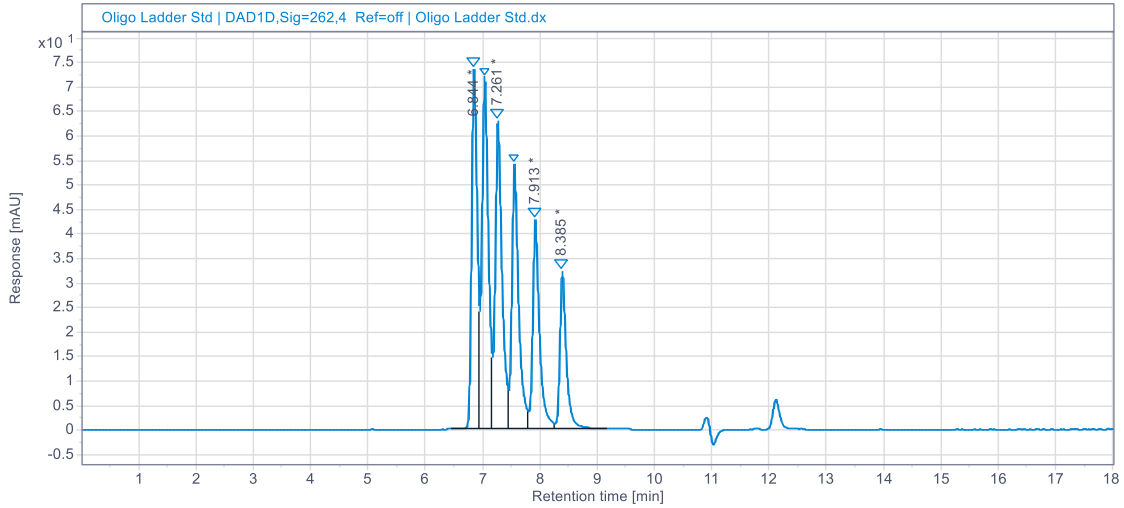


Additional Considerations



Importance of Stationary Phase Pore Size

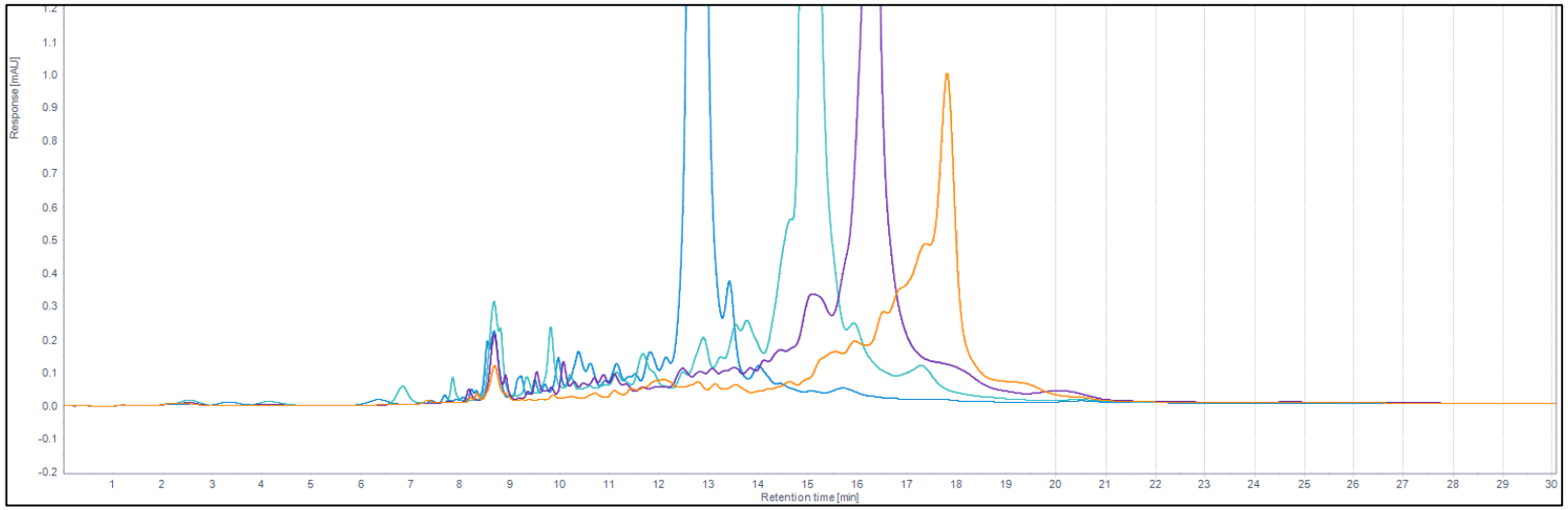




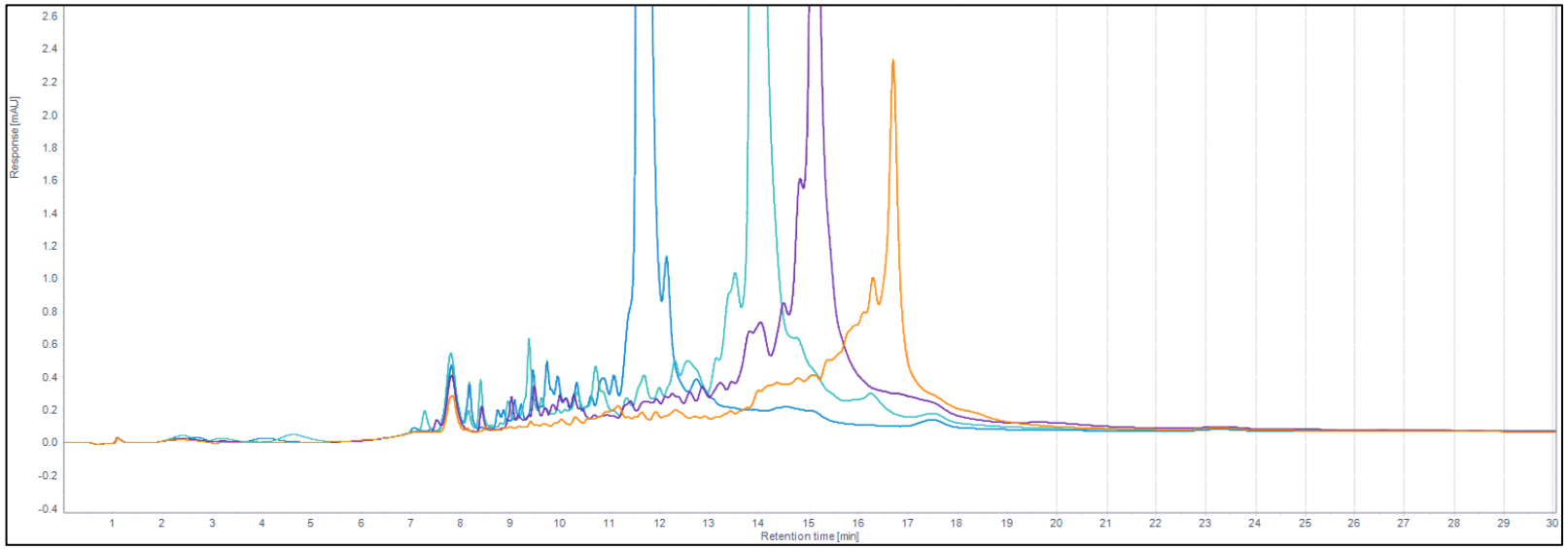
Size exclusion chromatogram of oligonucleotide ladder standard containing dT_{15}, dT_{20}, dT_{25}, dT_{30}, dT_{35}, dT_{40} (part number 5190-9029).

dT_{40} has a MW of around 12.1 kDa, but elutes at a time that corresponds to a globular protein of around 50 to 60 kDa.

25 mer, 50 mer, 75 mer, 100 mer



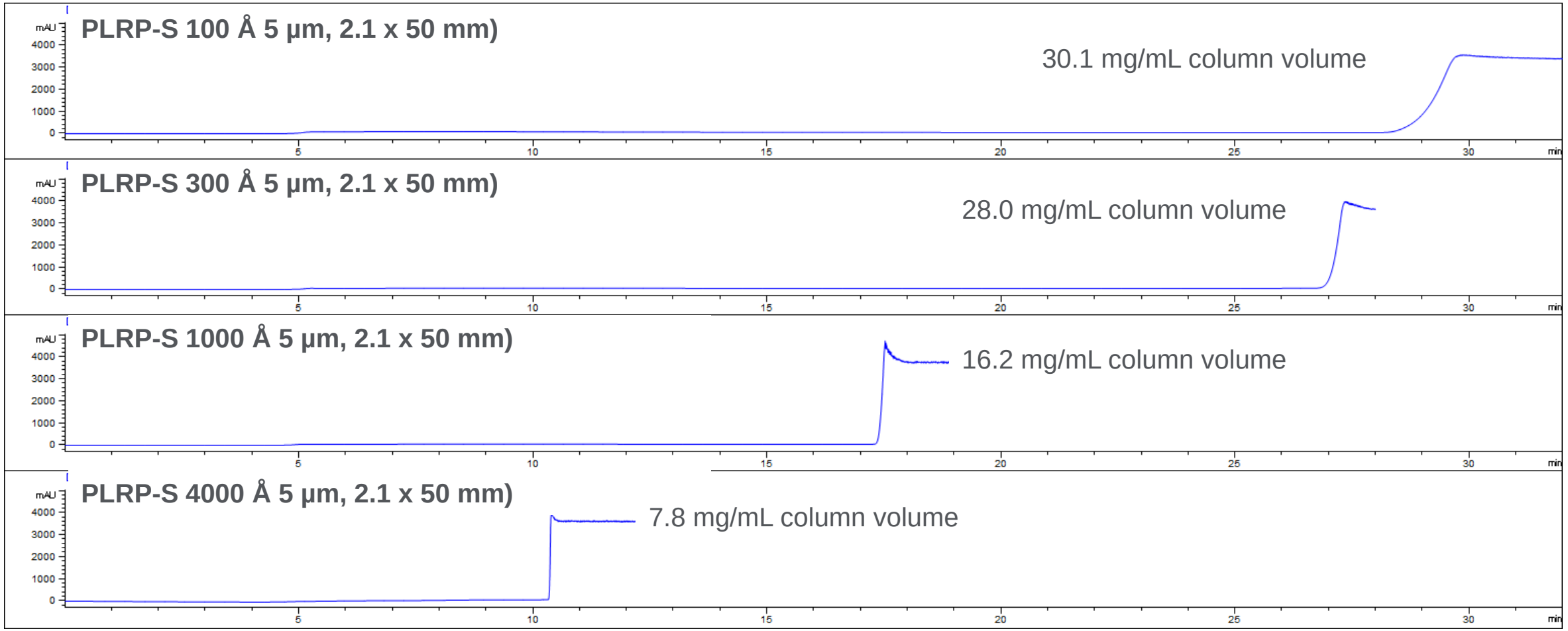
PLRP-S 100 Å



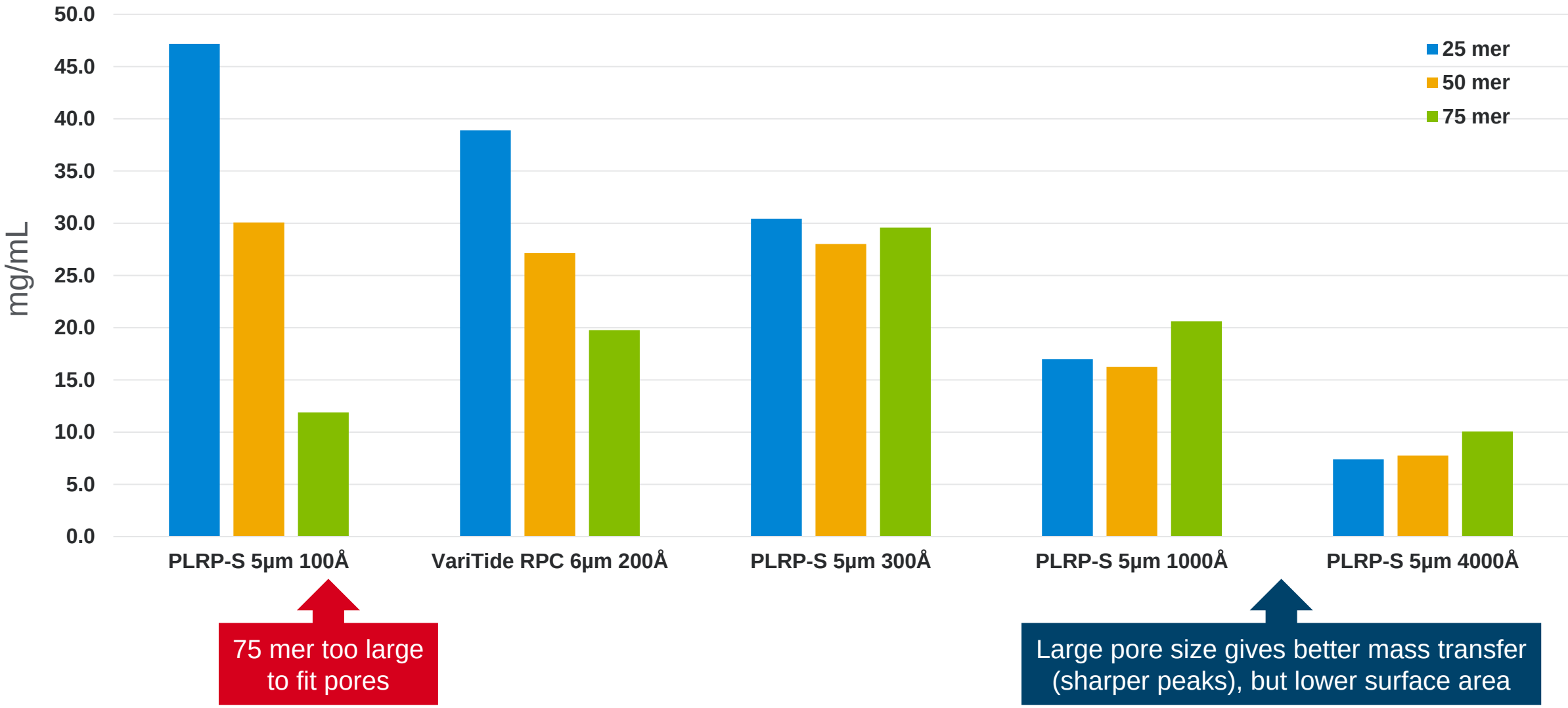
PLRP-S 1000 Å

Pore Size vs. Surface Area

Effect on dynamic binding capacity (50 mer, 1 mg/mL)



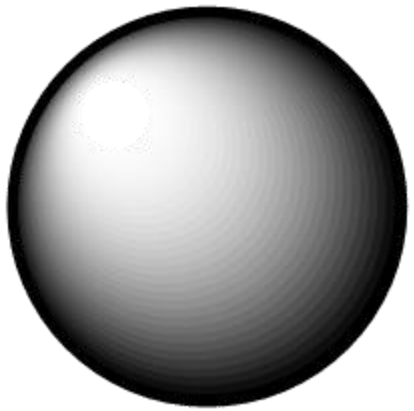
Effect of Pores Size on Oligonucleotide Binding Capacity



Stationary Phase Considerations

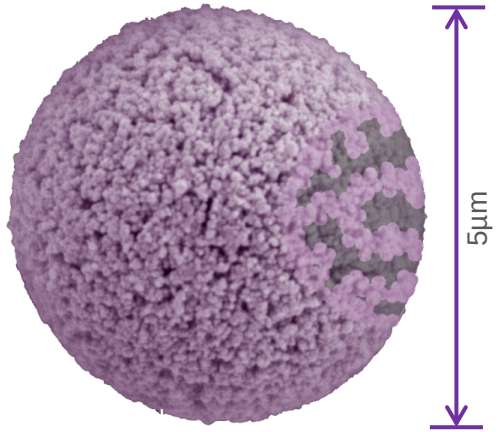
Strong anion exchange

Agilent Bio SAX NP5
5 µm, nonporous



Other particle sizes:
1.7 µm, 3 µm 10 µm

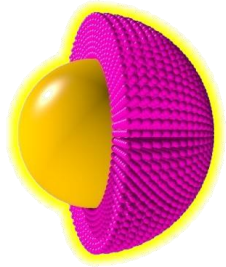
PL-SAX 1000 Å
Pore size 1000 Å



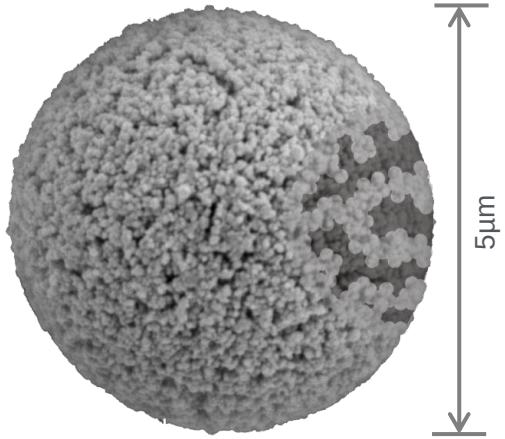
Other particle sizes:
10 µm, 30 µm
Other pore sizes:
4000 Å

Reversed phase

AdvanceBio Oligonucleotide
2.7µm, pore size 120Å



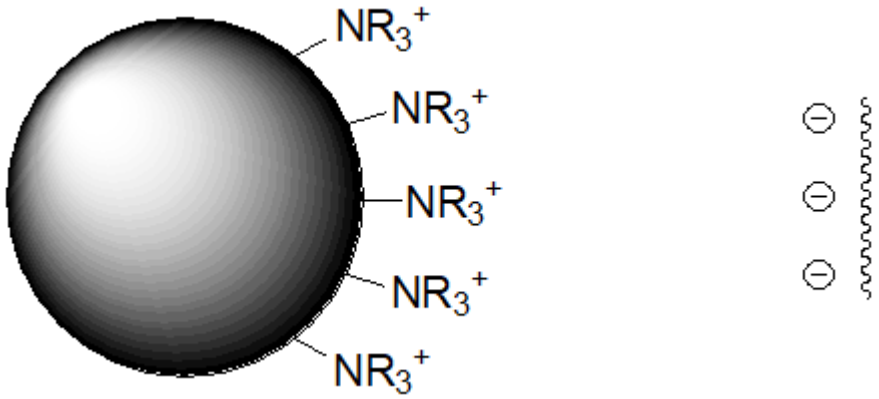
PLRP-S 1000 Å
Pore size 1000 Å



Other particle sizes:
10 µm, 15 to 20 µm, 30 µm
Other pore sizes:
100 Å, 300 Å, 4000 Å

HPLC: Anion Exchange of Oligonucleotides

Anion exchange chromatography

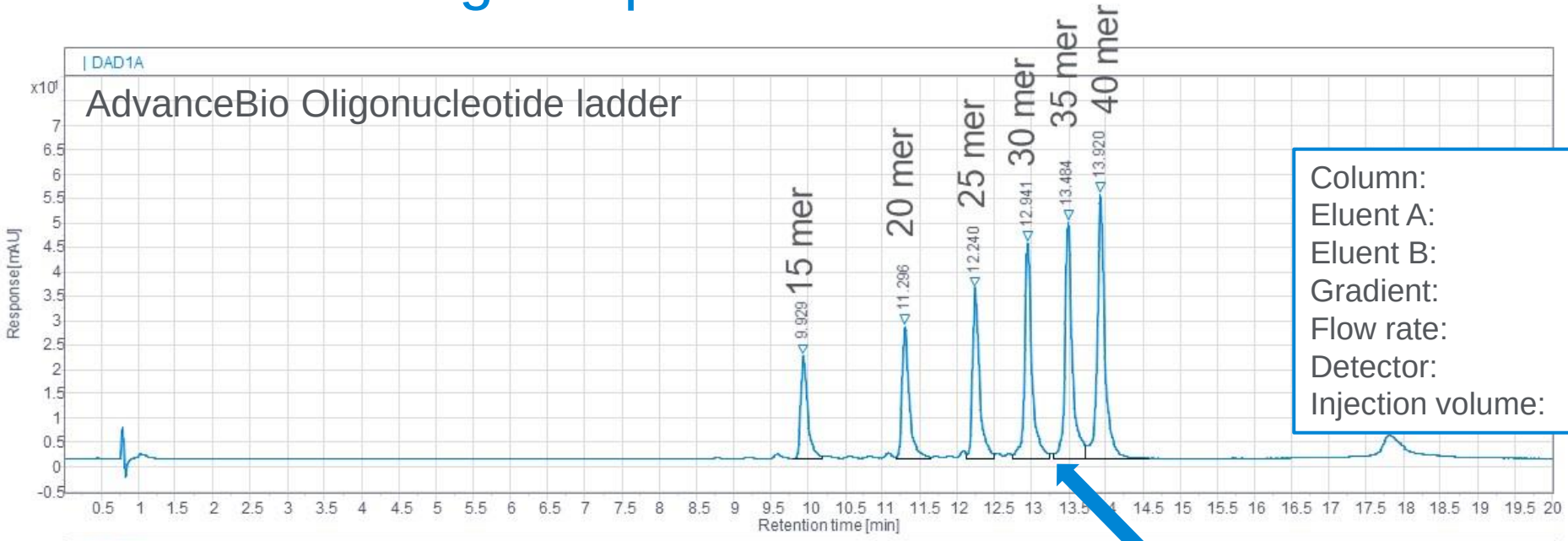


Variables :

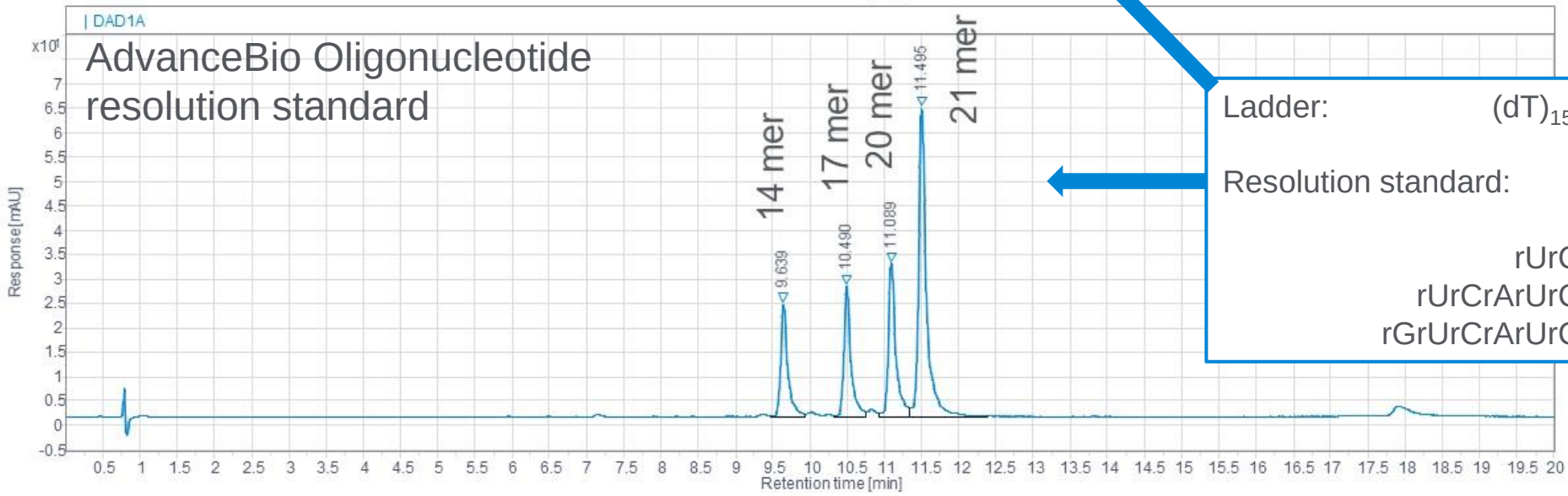
- Buffer type
- Buffer concentration
- Buffer pH
- Salt

- Temperature
- Organic modifiers

Anion Exchange Separation of Standards



Column:	Bio SAX NP5 (4.6 x 50 mm PEEK)
Eluent A:	20 mM Tris, pH 8.0
Eluent B:	1 M NaCl in Eluent A
Gradient:	0 to 100% B in 20 min
Flow rate:	0.5 mL/min
Detector:	UV, 260 nm
Injection volume:	5 µL (reconstituted in 1 mL)



Ladder: $(dT)_{15}$, $(dT)_{20}$, $(dT)_{25}$, $(dT)_{30}$, $(dT)_{35}$, $(dT)_{40}$

Resolution standard:

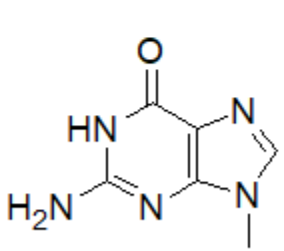
rCrArCrUrGrArArUrArCrCrArArU
rUrCrArCrArCrUrGrArArUrArCrCrArArU
rUrCrArUrCrArCrArCrUrGrArArUrArCrCrArArU
rGrUrCrArUrCrArCrArCrUrGrArArUrArCrCrArArU

HPLC: Anion Exchange of Oligonucleotides

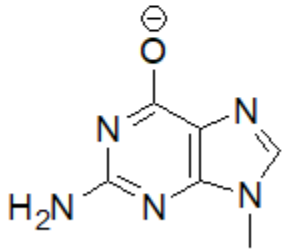
Control of selectivity

Thymine, pH 7

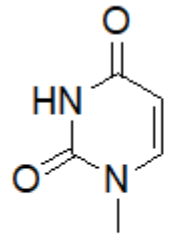
Thymine, pH 11



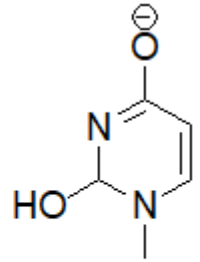
Guanine, pH 7



Guanine, pH 11

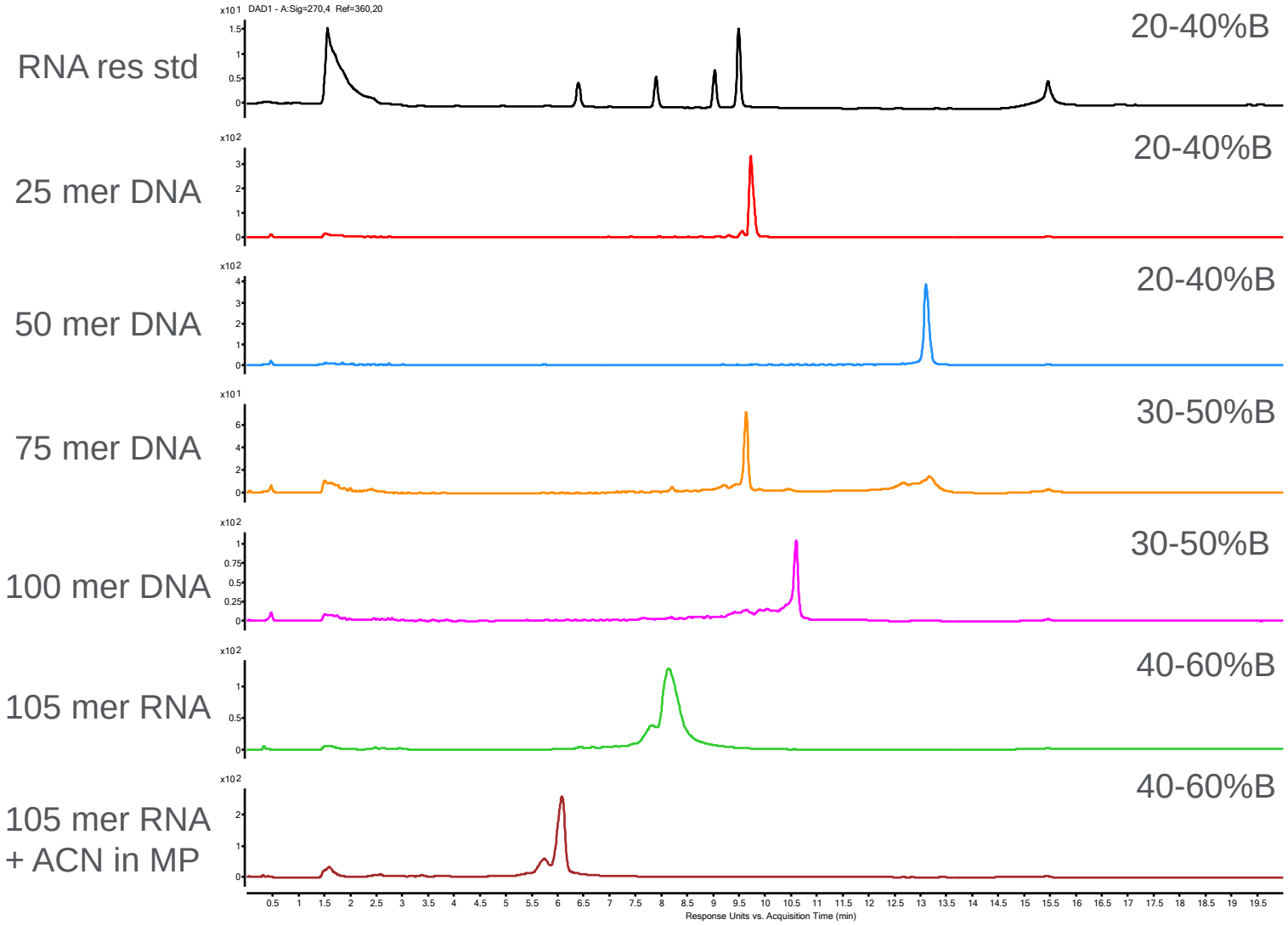


Uracil, pH 7



Uracil, pH 11

Oligo Separation with the Agilent Bio SAX (Nonporous)



Solvent A: 10 mM Tris, pH 8.0 in water

Solvent B: 2 M NaCl in solvent A

Column: Bio SAX 5 µm, 4.6 x 50 mm (p/n 5190-2468)

Column temperature: 80 °C

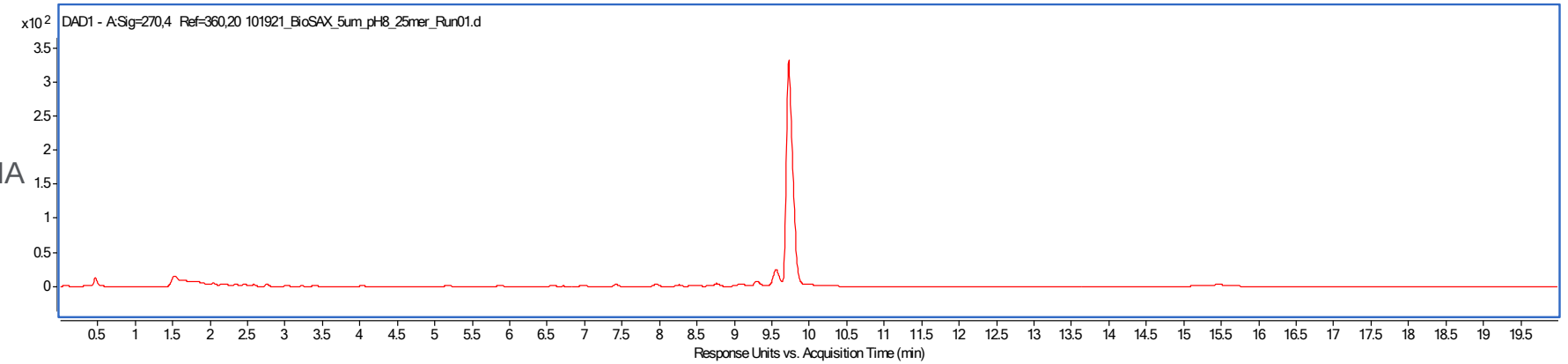
Flow rate: 1 mL/min

1 mg/mL samples: 1 µL injection

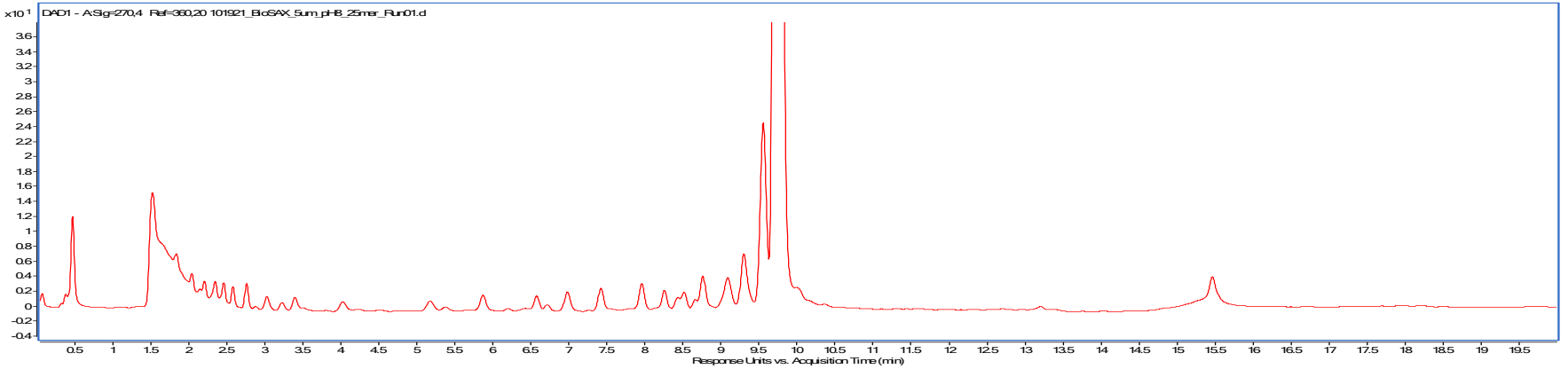
10 min gradients

Bio SAX Provides High-resolution UV Analysis of Oligonucleotides

25 mer DNA



Zoom in



Solvent A: 10 mM Tris, pH 8.0 in water

Solvent B: 2 M NaCl in solvent A

Column: Bio SAX 5 µm, 4.6 x 50 mm (p/n 5190-2468)

Column temperature: 80 °C

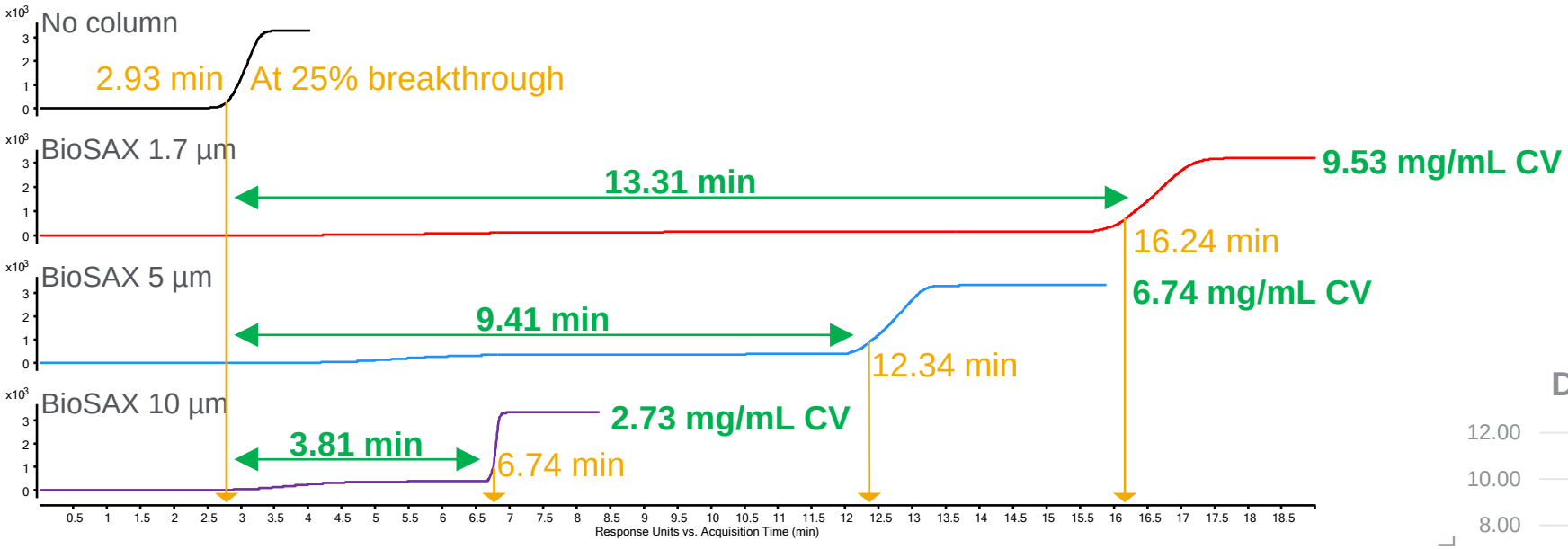
Flow rate: 1 mL/min

1 mg/mL samples: 1 µL injection

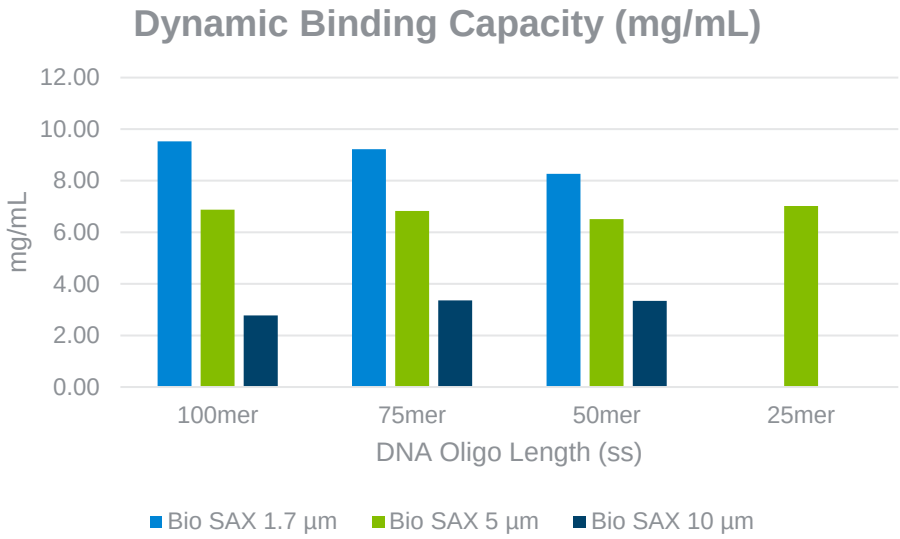
10 min gradients: 20-40% B

DBC for BioSAX 4.6 x 50 mm Columns with 100 mer DNA

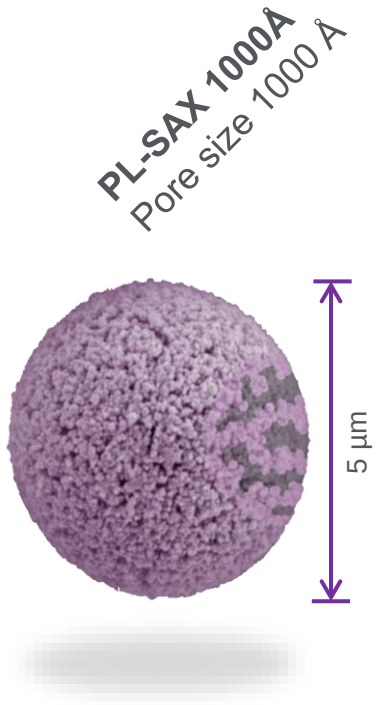
BioSAX 4.6 x 50 mm columns with 100 mer DNA

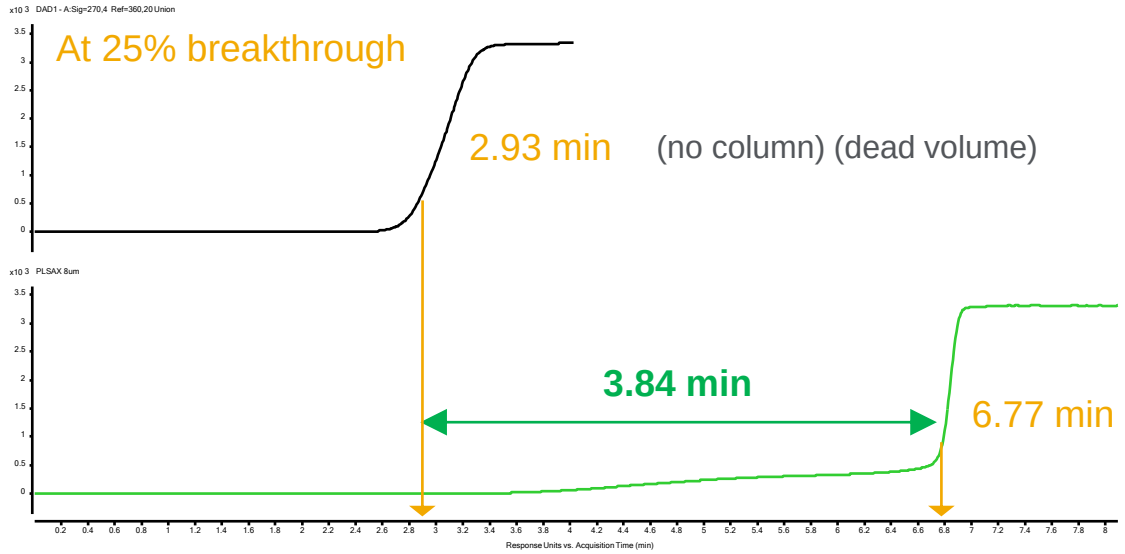


DNA concentration: 100 mer – 1189 ng/uL
75 mer – 884.7 ng/uL
50 mer – 1386.1 ng/uL
25 mer – 1253 ng/uL
Flow rate: 0.5 mL/min
Column: Bio SAX 4.6 x 50 mm (1.7, 5, 10 µm)



PL-SAX totally porous polymeric particles with 100mer DNA





13.2 mg/mL CV

(**PL-SAX**, 2.1 x 50 mm column, 8 µm)

Other particle sizes:
8 µm, 10 µm, 30 µm
Other pore sizes:
4000 Å

DNA concentration: 100 mer to 1189 ng/µL
Column: PL-SAX 1000 Å, 8 µm, 2.1 x 50 mm
Flow rate: 0.5 mL/min

Column id (mm) x 50 mm	Total Binding Capacity (mg)	5% of Binding Capacity (mg)	25 mer DNA 5% Binding Capacity (nmoles)	50 mer DNA 5% Binding Capacity (nmoles)	100 mer DNA 5% Binding Capacity (nmoles)
2.1	2.24	0.11	14.55	7.24	3.68
4.6	10.96	0.55	71.03	35.35	17.95
7.5	29.17	1.46	189.1	94.1	47.8
25	323.9	16.20	2,100	1,045	530.7
50	1,296	64.79	8,401	4,181	2,123
100	5,184	259.2	33,607	16,725	8,493

The Agilent HPLC Portfolio from Analytical to Preparative

Manual systems up to automated method development and autoscale preparative LC/MSD solutions

Scaling Up Your Run with PL-SAX 1000 Å

Column id	Analytical	Semipreparative	Preparative
2.1 mm	0.1 – 0.2 mL/min		
4.6 mm	0.5 – 1.0 mL/min		
7.5 mm	1.3 – 2.7 mL/min		
25 mm		14.7 – 29.5 mL/min	
50 mm			58.8 – 120 mL/min
100 mm			240 – 480 mL/min

1220/1260/1290 Infinity II analytical-scale LC purification system [0.1 mL/min – 10 mL/min]

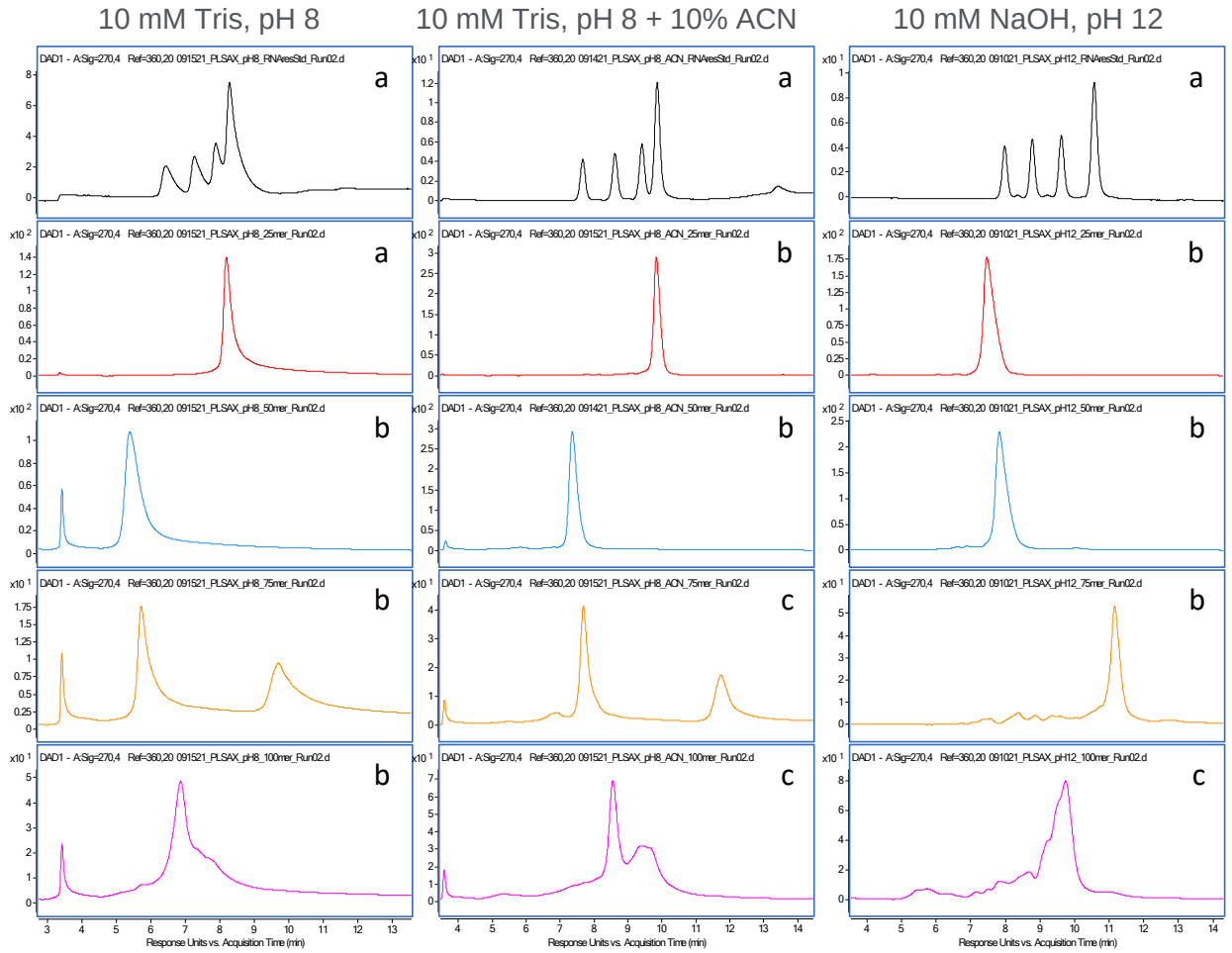
1260 Infinity II preparative LC system [1 mL/min – 50 mL/min]

1290 Infinity II preparative LC system [1 mL/min – 50 mL/min]

1290 Infinity II preparative LC system [4 mL/min – 200 mL/min]

Mobile Phase Selection

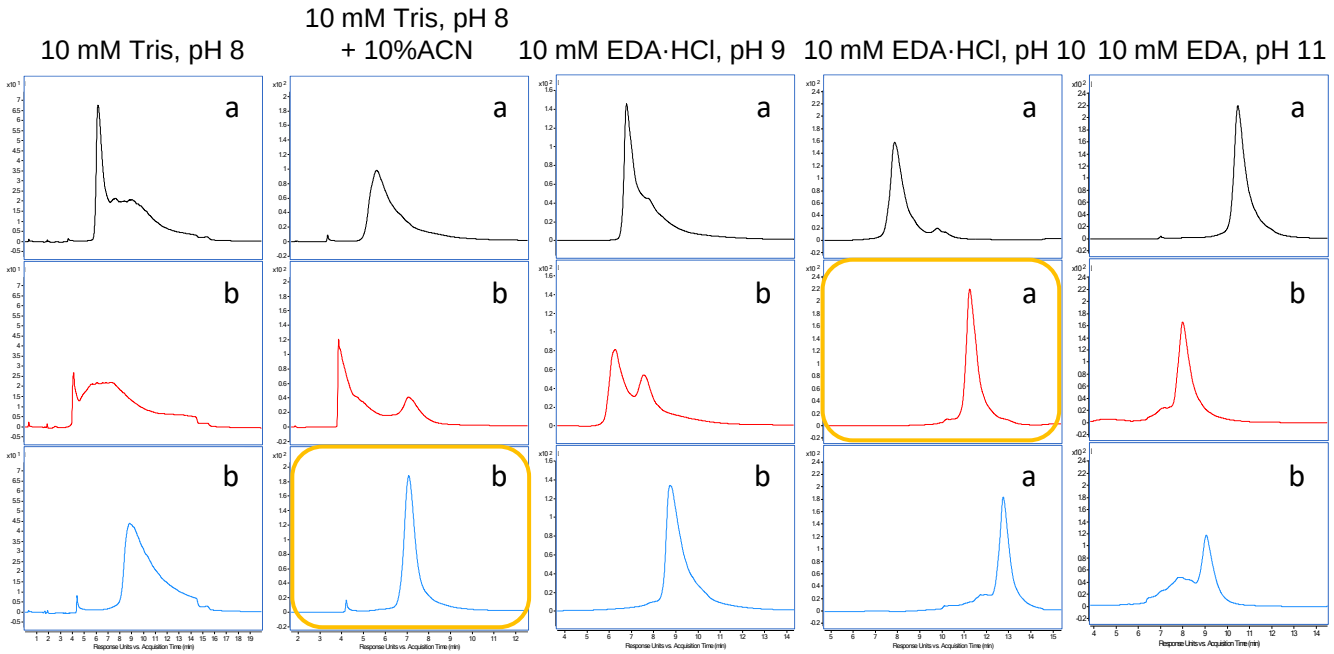
Mobile Phase pH, Temp and organic modifiers for AEX Oligonucleotide Separation



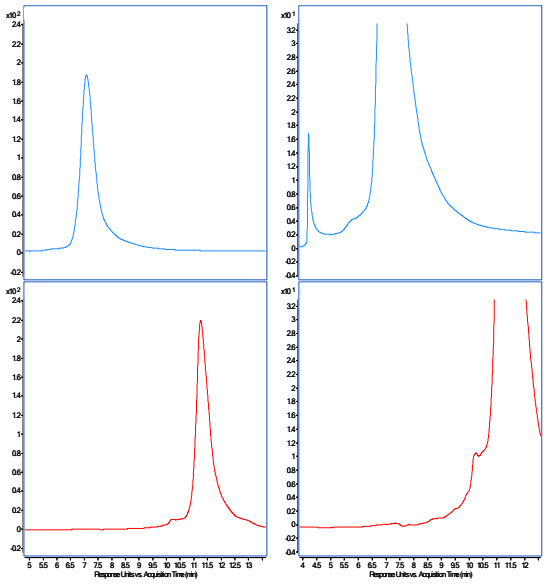
Column: PL-SAX 1000 Å, 5 µm, 2.1 x 50 mm (p/n PL1951-1502)
LC: Agilent 1290 Infinity II bio LC system
Flow rate: 0.5 mL/min
Injection volume: 1 µL
Autosampler temperature: 10 °C

Mobile phase B: 2 M NaCl in mobile phase A
LC gradient and temperature:
a. 10 to 30%B in 10 min, 30 °C
b. 20 to 40%B in 10 min, 30 °C
c. 25 to 45%B in 10 min, 30 °C

Organic Solvent and Temperature's Effect on gRNA Separation



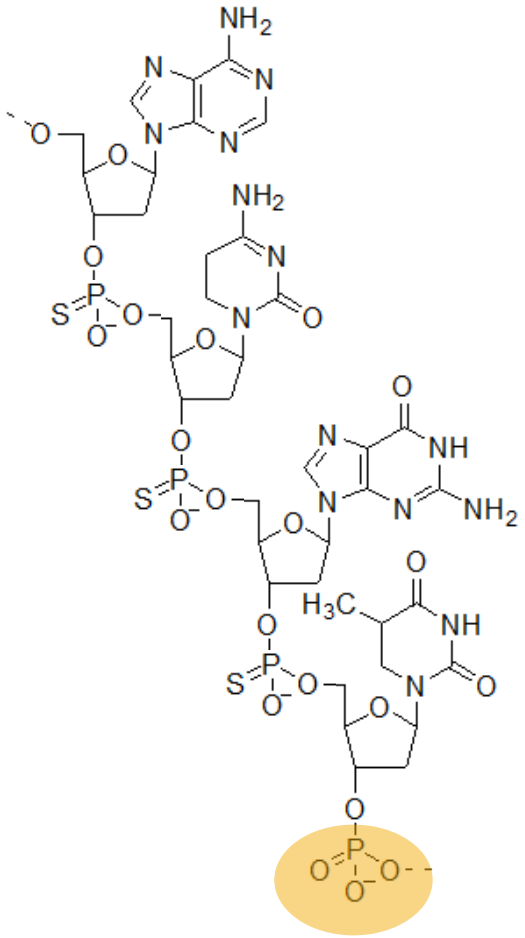
Zoom in



Sample:
sgRNA (105 bases)

Mobile phase B: 2 M NaCl in mobile phase A
LC gradient and temperature:
a. 20 to 40%B in 10 min
b. 30 to 50%B in 10 min

Incomplete Backbone Phosphorothioation



5990-8297EN: High Resolution Separations of Oligonucleotides using PL-SAX Strong Anion Exchange HPLC Columns

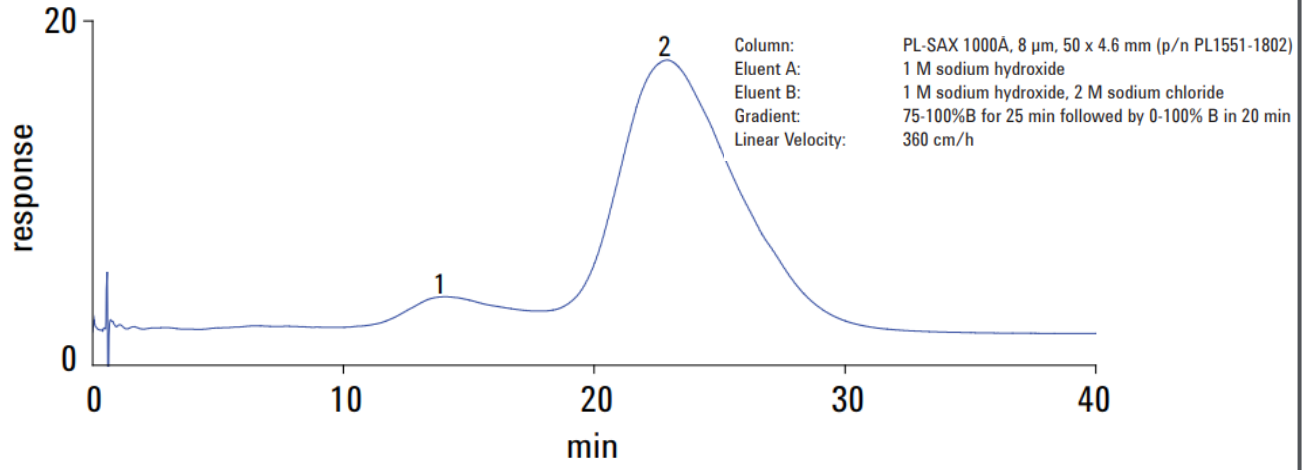


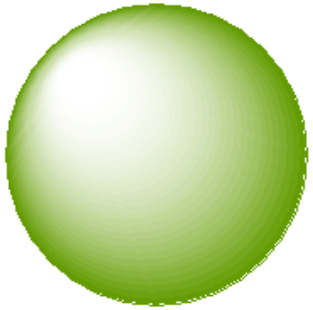
Figure 3. Separation of residual phosphodiester (1) and a phosphorothioate (2) 20mer oligonucleotide.

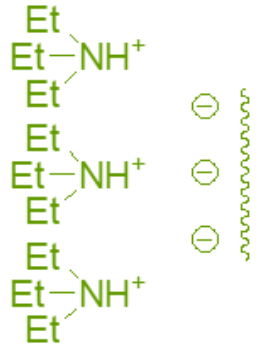
IEX of Oligonucleotides

Variables to consider :

• Buffer type	Suitable buffers include phosphate and tris
• Buffer concentration	Buffer concentration is typically ~20 mM
• Buffer pH	pH 7 or 8 (depending on buffer); higher pH for selectivity changes
• Salt	Sodium chloride is good; sodium perchlorate has been suggested
	Volatile buffers/salts can also be used
• Temperature	Elevated temperature to improve peak shape (longer retention?)
• Organic modifiers	Some secondary interactions with certain stationary phases: addition of acetonitrile (up to 10 – 15% may be beneficial)

Ion Pair RP of Oligonucleotides





Common variables to consider:

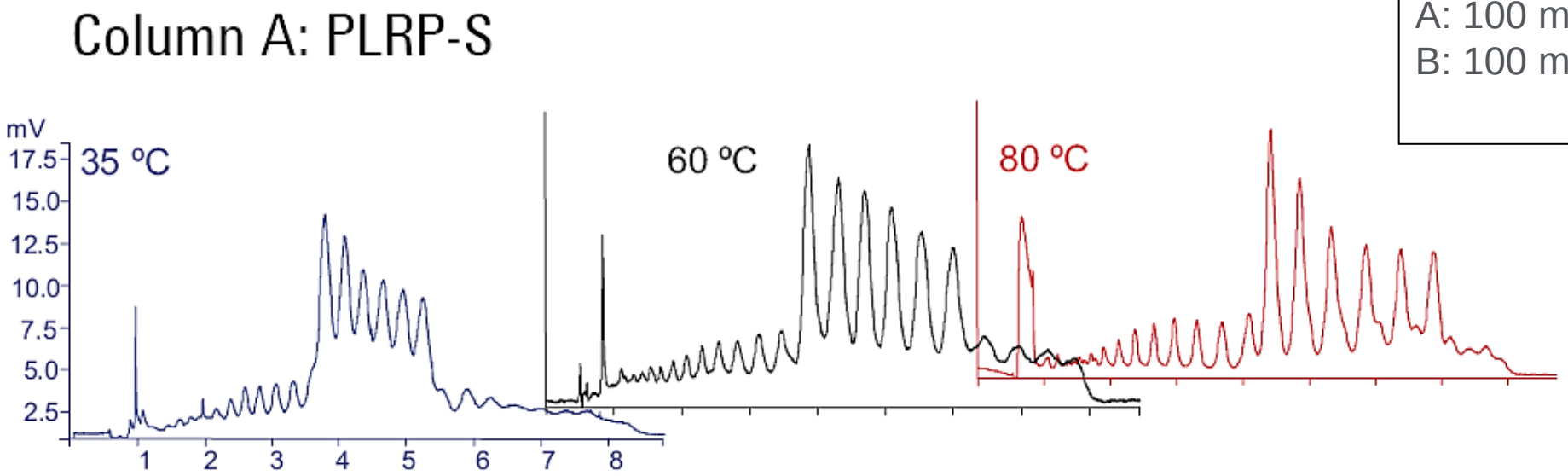
- Method choice, for example:
  - 100 mM TEAA/water-acetonitrile gradient — UV method
  - TEA + HFIP/water-methanol gradient — MS method
- Ion pair selection
  - Alternative ion pair reagents, pH and concentrations
- Temperature
  - 50 to 60°C commonly used

Major challenges: molecule related (coeluting impurities); column lifetime issues

Temperature

Poly dT 19-24 ladder

PLRP-S 50 x 4.6 mm, 3 µm, 100 Å
5%B per minute over 6 mins
A: 100 mM TEAA
B: 100 mM TEAA 75:25 water:MeCN

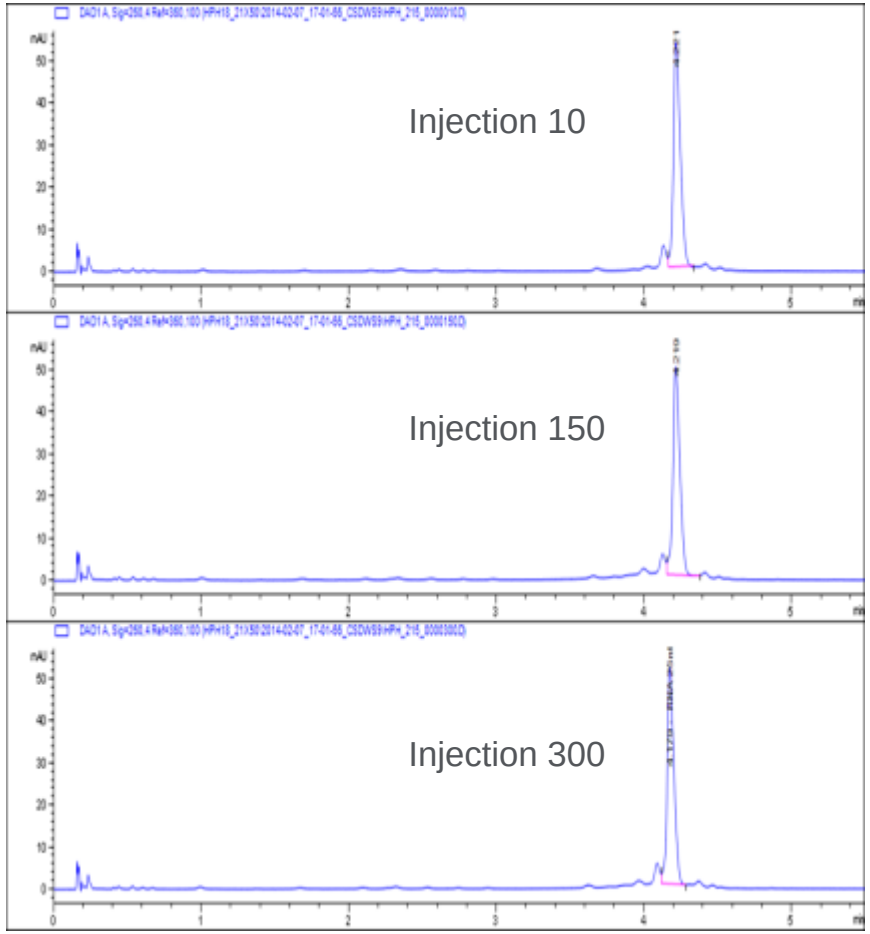


Sharper peaks are usually obtained by running at elevated temperatures to denature the oligos

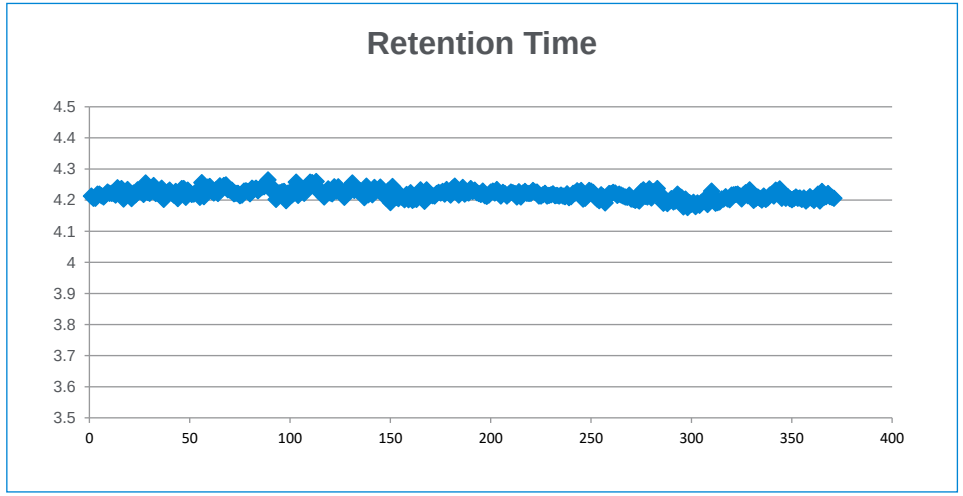
https://www.agilent.com/cs/library/applications/5990-7765EN.pdf

Column Stability

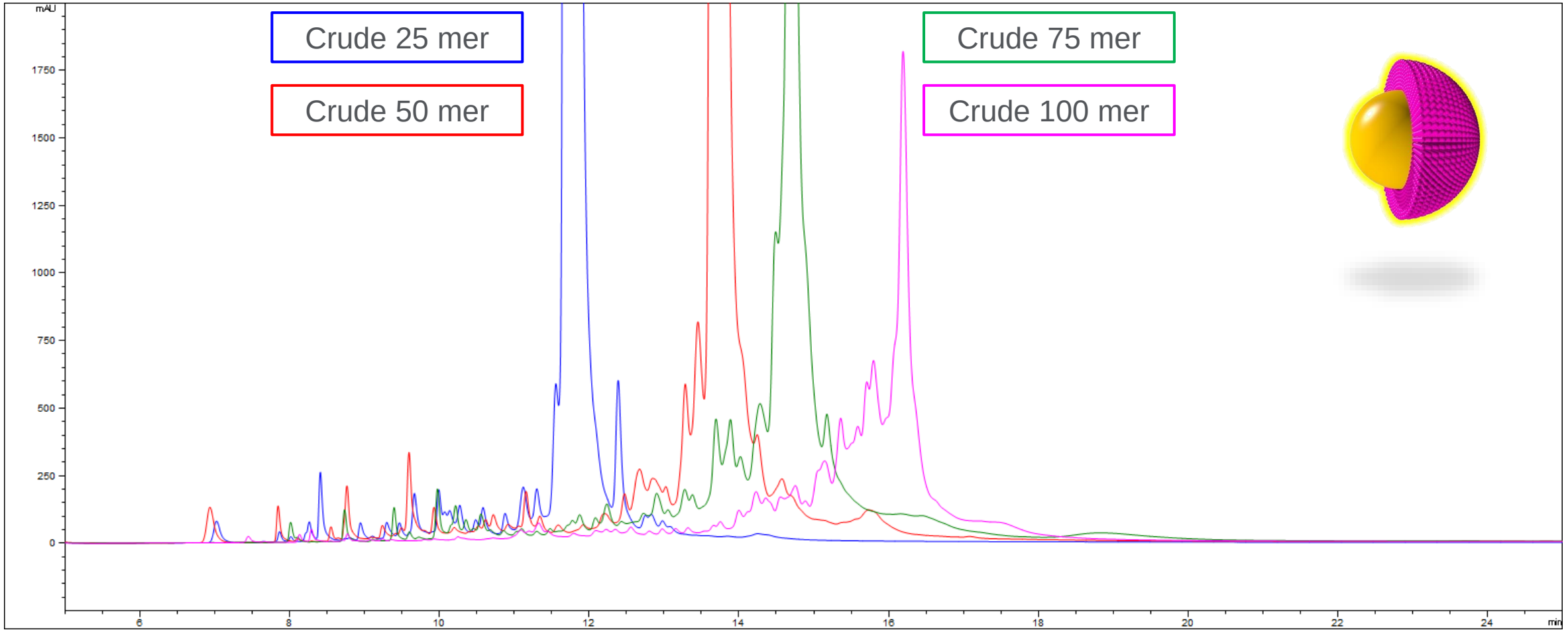
The AdvanceBio Oligonucleotide column shows stability over ~300 injections using TEAA mobile phase – excellent for this application



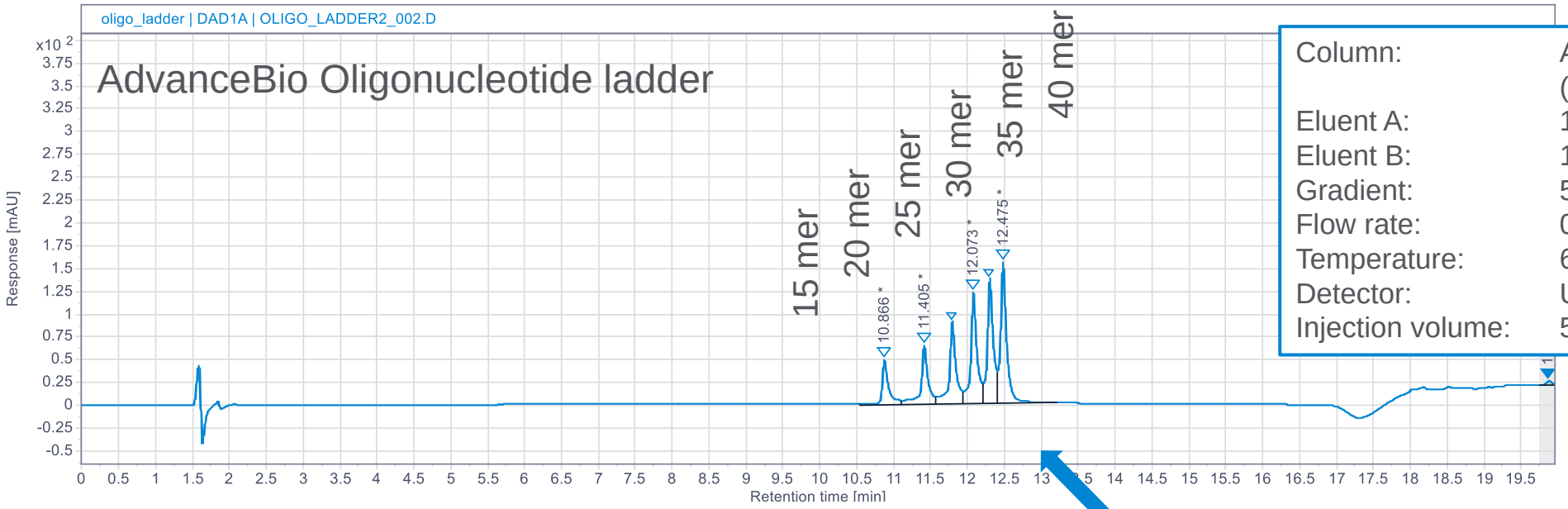
Column: AdvanceBio Oligonucleotide, 2.1 x 50 mm
Mobile phase A: 100 mM TEAA in water
Mobile phase B: 100 mM TEAA in acetonitrile
Flow rate: 0.69 mL/min
Gradient: 7% B to 11%B in 5 min
11% B to 80%B in 5.01 min
Hold at 80%B for 5.50 min
80% B to 7%B in 5.56 min
Total run time: 8.5 min
Sample: 25 mer DNA
Injection: 1 µL of 0.5 mg/mL
Temperature: 65 °C
Detection: UV at 260 nm



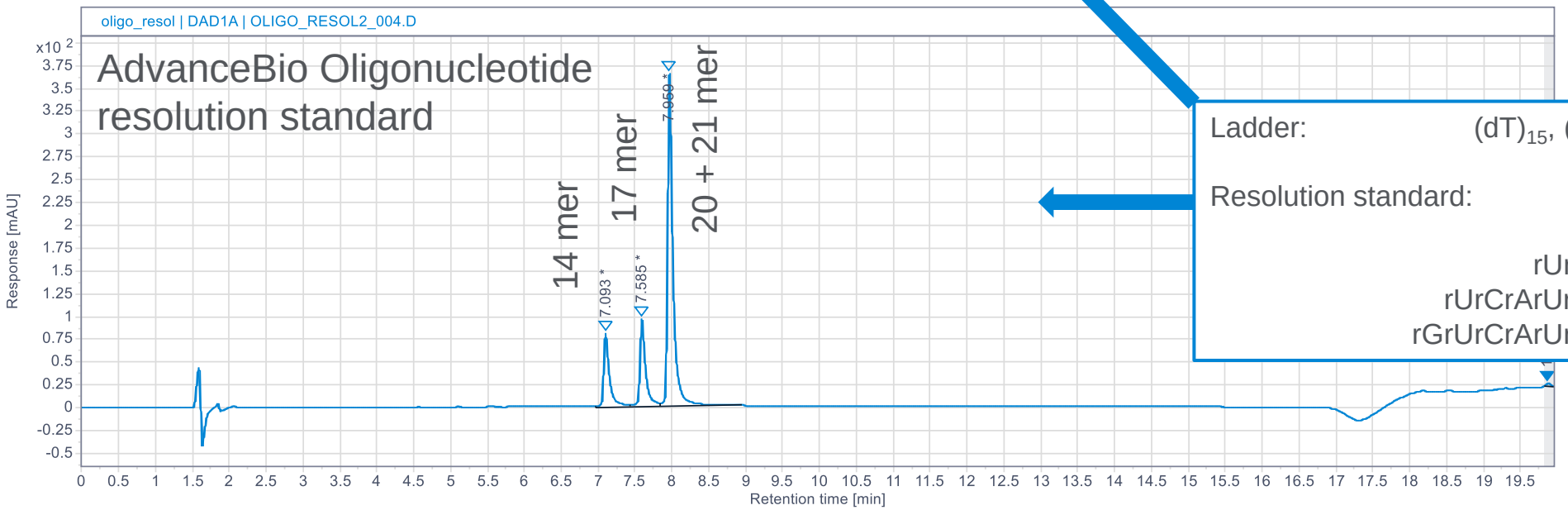
Benefits of Superficially Porous Particles



Ion-pair Reversed Phase Separation of Standards



Column:	AdvanceBio Oligonucleotide 2.7 µm (2.1 x 150 mm)
Eluent A:	100 mM TEAA
Eluent B:	100 mM TEAA in 9:1 ACN
Gradient:	5 to 20%B in 15 min
Flow rate:	0.21 mL/min
Temperature:	65 °C
Detector:	UV, 260 nm
Injection volume:	5 µL (reconstituted in 1 mL)

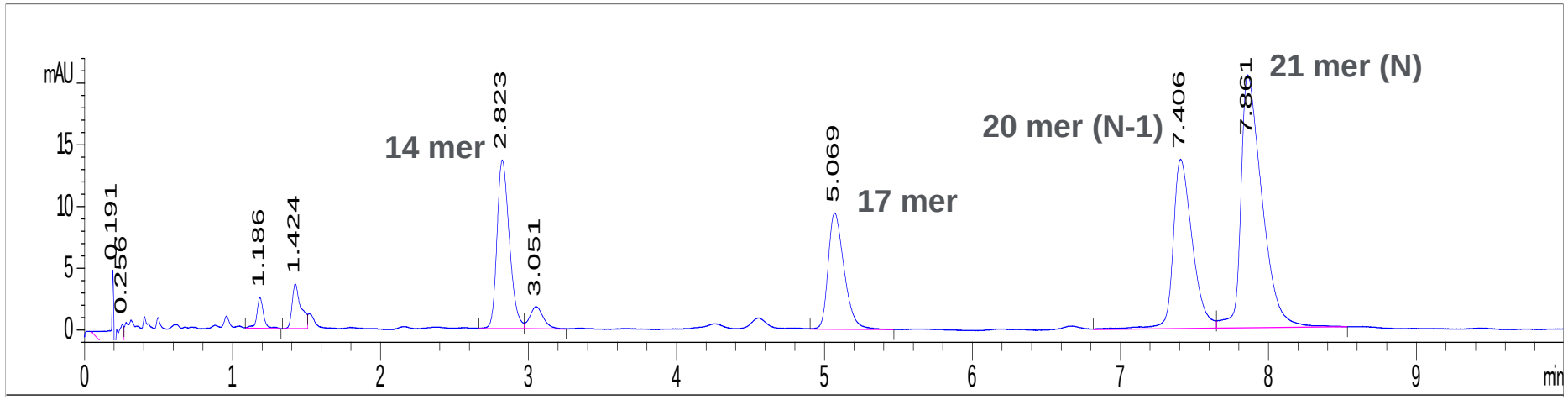


Ladder: $(dT)_{15}$, $(dT)_{20}$, $(dT)_{25}$, $(dT)_{30}$, $(dT)_{35}$, $(dT)_{40}$

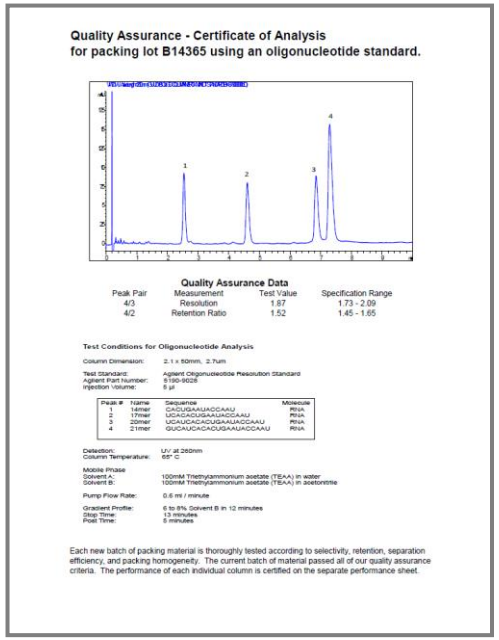
Resolution standard:

rCrArCrUrGrArArUrArCrCrArArU
rUrCrArCrArCrUrGrArArUrArCrCrArArU
rUrCrArUrCrArCrArCrUrGrArArUrArCrCrArArU
rGrUrCrArUrCrArCrArCrUrGrArArUrArCrCrArArU

Optimized Gradient for RNA Resolution Standard



Column:	AdvanceBio Oligonucleotide, 2.1 x 50 mm
Mobile phase A:	100 mM TEAA in water
Mobile phase B:	100 mM TEAA in acetonitrile
Gradient:	6 to 8%B in 10 min
Stop time:	11 min
Post run:	5 min
Flow rate:	0.6 mL/min
Sample:	Agilent Oligonucleotide resolution standard
	14 mer, 17 mer, 20 mer, 21 mer RNA
Temperature:	65 °C
Injection:	5 µL
Detection:	UV at 260 nm



Quality Assurance - Certificate of Analysis
for packing lot B14365 using an oligonucleotide standard.

Quality Assurance Data

Peak Pair	Measurement	Test Value	Specification Range
4/3	Resolution	1.87	1.73 - 2.09
4/2	Retention Ratio	1.52	1.45 - 1.65

Test Conditions for Oligonucleotide Analysis

Peak #	Name	Sequence	Molecule
1	14mer	GACUGAAUACCAAU	RNA
2	17mer	UCACAUGAAUACCAAU	RNA
3	20mer	UCAUCACACUGAAUACCAAU	RNA
4	21mer	GUCAUCACACUGAAUACCAAU	RNA

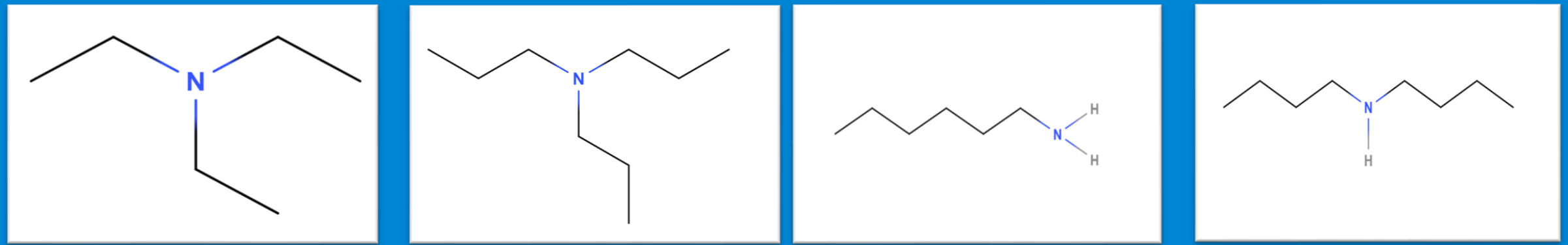
Each new batch of packing material is thoroughly tested according to selectivity, retention, separation efficiency, and packing homogeneity. The current batch of material passed all of our quality assurance criteria. The performance of each individual column is certified on the separate performance sheet.

Options for Oligonucleotide LC Separations

Technique	Oligos	Comment
Ion pair reversed phase	Trityl-on	Limited resolution of impurities, but good purification technique
Ion-exchange	Trityl-off, deprotected	Good resolution of shorter oligos, not MS-compatible
Ion pair reversed phase	Trityl-off, deprotected	Good resolution of impurities and compatible with MS. Requires high performance columns. Mobile phases: TEAA: UV detection HFIP-TEA: MS detection

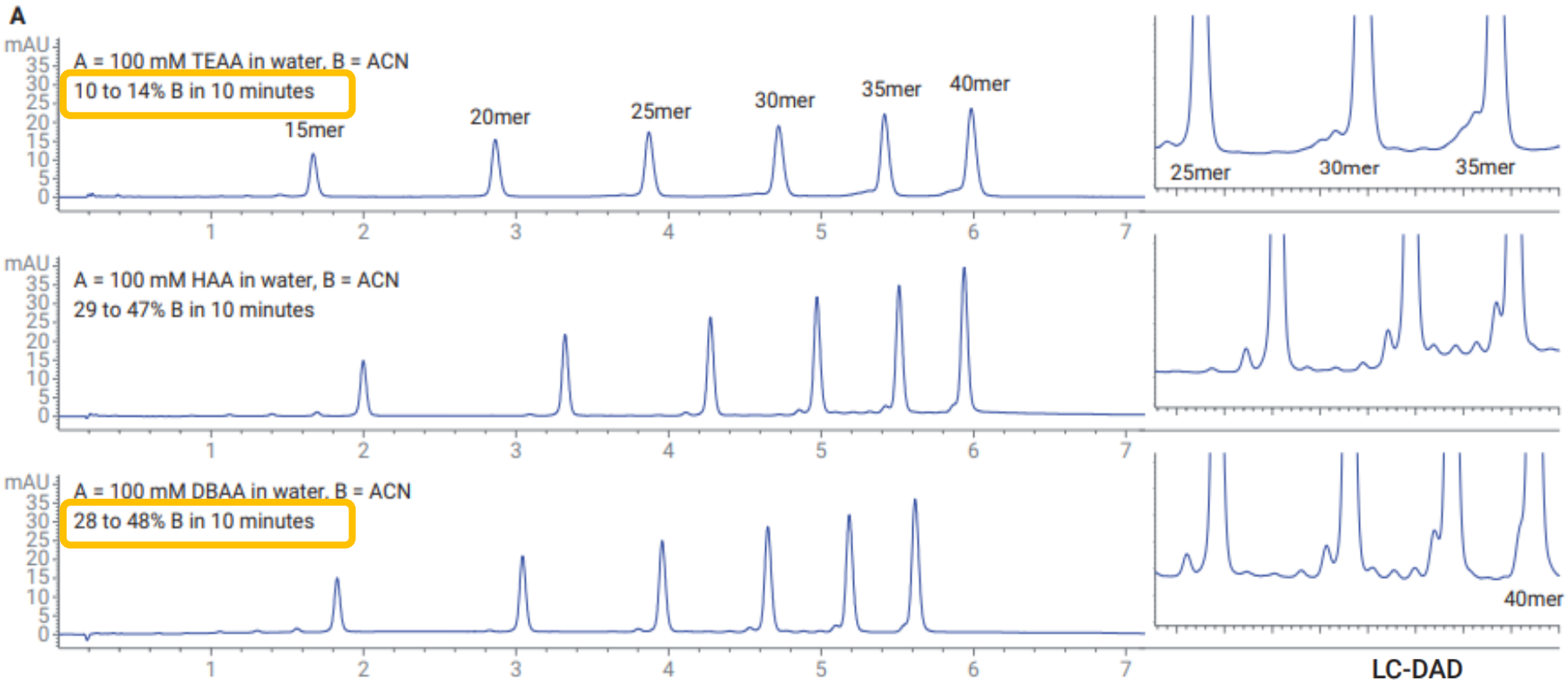
Agilent AdvanceBio Oligonucleotide columns are designed for ion pair reversed-phase separation of the trityl-off, deprotected oligos using either TEAA or TEA-HFIP mobile phases

Selecting an Ion-Pairing Agent



[Evaluation of Different Ion Pairing Reagents for LC/UV and LC/MS Analysis of Oligonucleotides (agilent.com)]

Effect of Hydrophobicity on Oligonucleotide Resolution and Retention



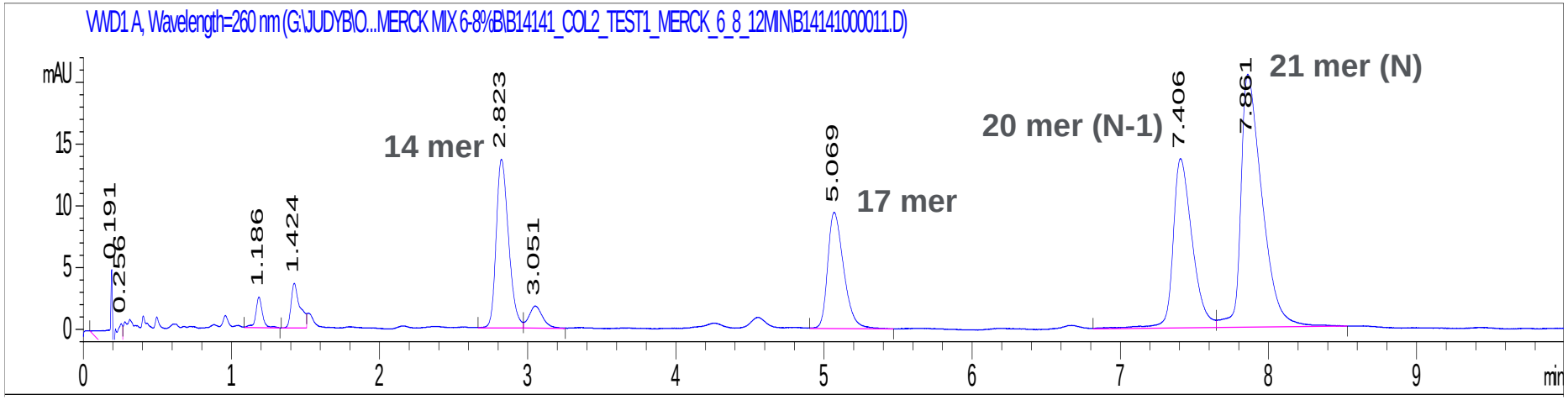
LC-DAD

Parameter	Value
Column	Agilent AdvanceBio oligonucleotide, 2.1 × 50 mm, 2.7 µm (p/n 659750-702)
Mobile Phase A and B	See *Results and discussion* section
Gradient	See *Results and discussion* section
Flow Rate	0.6 mL/min
Column Temperature	65 °C
Detection (DAD)	260/4 nm (reference 355/20 nm) Peak width >0.025 min (10 Hz)
Injection	2 µL (needle wash flush port, 3 seconds, methanol)
Injector Temperature	12 °C

Evaluation of Different Ion Pairing Reagents for LC/UV and LC/MS Analysis of Oligonucleotides (agilent.com)

Resolution of N/N-1 Oligonucleotides

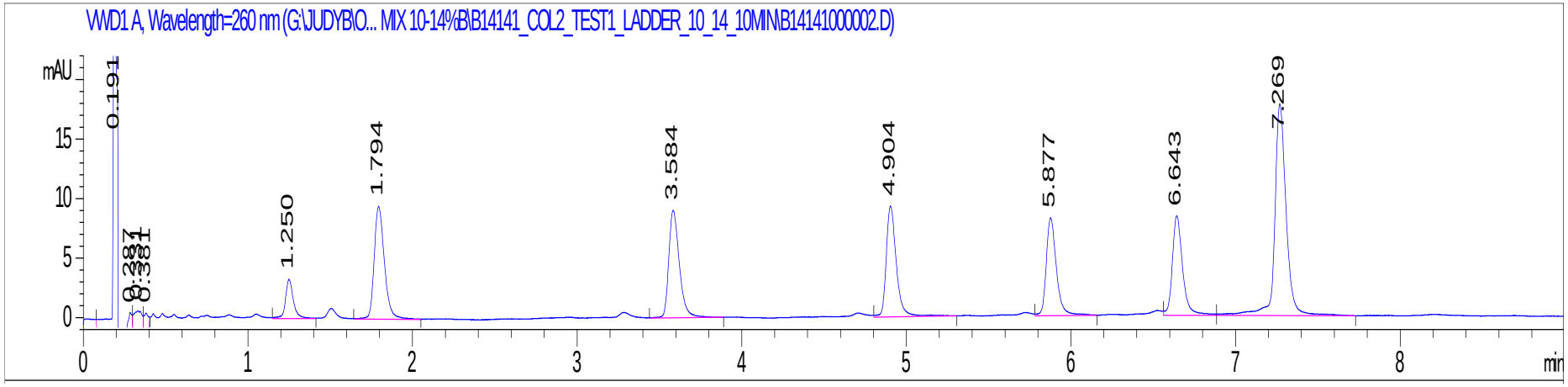
The AdvanceBio Oligonucleotide column gives sharp peaks and high resolution for N/N-1 oligonucleotides



Column:	AdvanceBio Oligonucleotide, 2.1 x 50 mm (p/n 659750-702)
Mobile phase A:	100 mM TEAA in water
Mobile phase B:	100 mM TEAA in acetonitrile
Gradient:	10 to 14%B in 10 min
Stop time:	11 min
Post run:	5 min
Flow rate:	0.6 mL/min
Sample:	Agilent Oligonucleotide resolution standard (p/n 5190-9028) 14 mer, 17 mer, 20 mer, 21 mer RNA
Temperature:	65 °C
Injection:	10 µL
Detection:	UV at 260 nm

Separation of Oligonucleotide Ladder

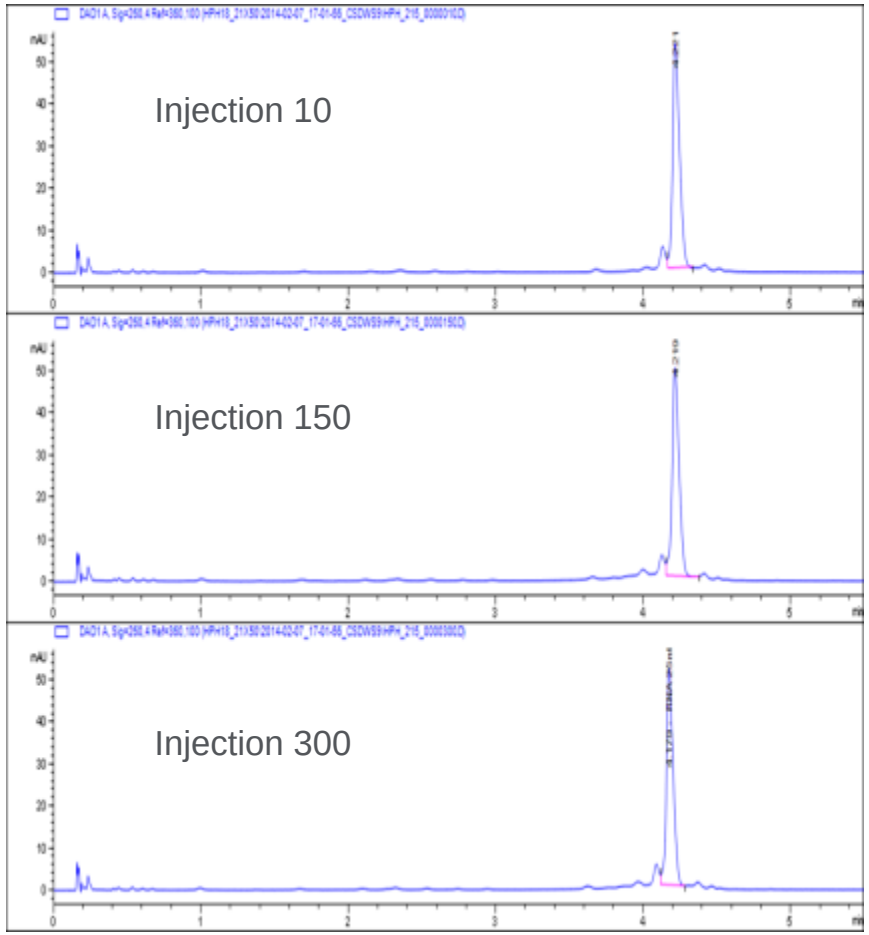
The AdvanceBio Oligonucleotide column gives sharp peaks and good selectivity for a 15 to 40 mer oligonucleotide ladder



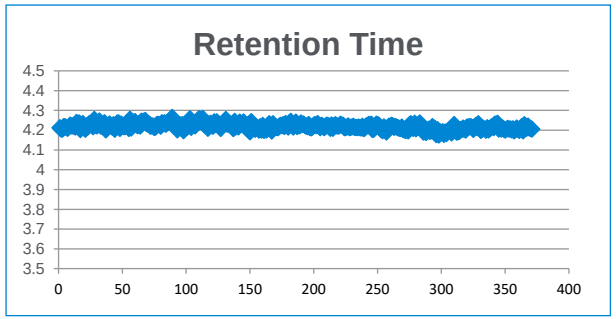
Column:	AdvanceBio Oligonucleotide, 2.1 x 50 mm (p/n 659750-702**)**
Mobile phase A:	100 mM TEAA in water
Mobile phase B:	100 mM TEAA in acetonitrile
Gradient:	6 to 8%B in 12 min
Stop time:	13 min
Post run:	5 min
Flow rate:	0.6 mL/min
Sample:	Agilent Oligonucleotide ladder standard (p/n 5190-9029) 15 mer, 20 mer, 25 mer, 30 mer, 35 mer, 40 mer DNA
Temperature:	65 °C
Injection:	0.5 µL
Detection:	UV at 260 nm

Column Stability

The AdvanceBio Oligonucleotide column shows stability over ~300 injections using TEAA mobile phase (excellent for this method)

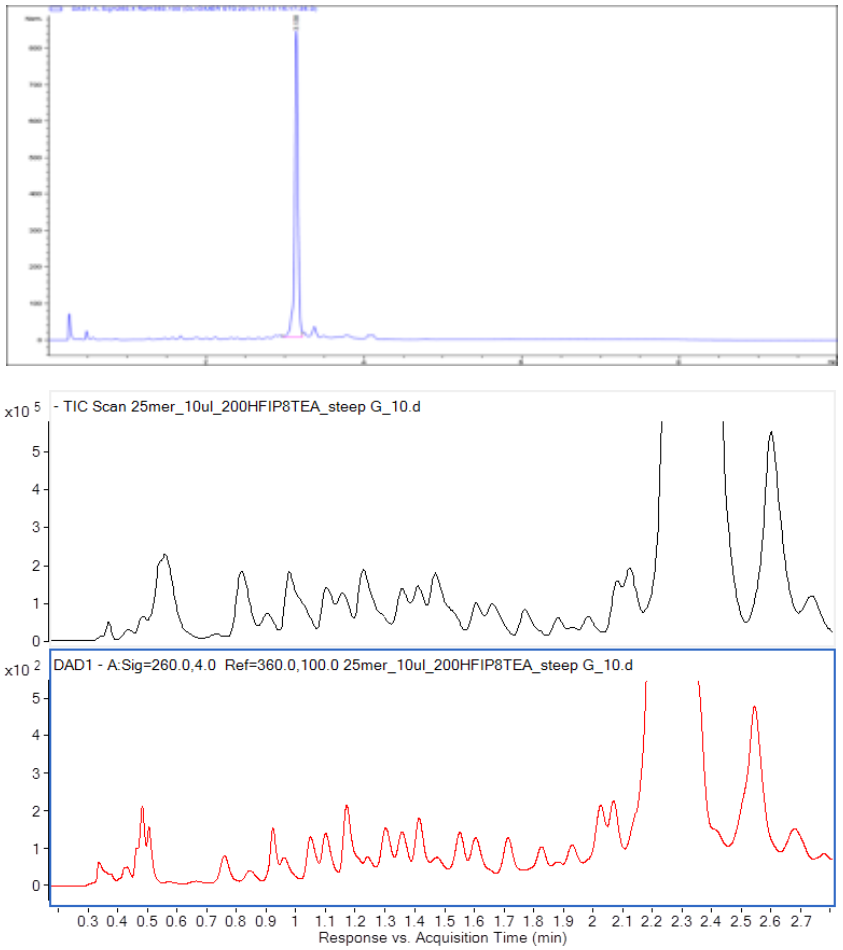


Column:	AdvanceBio Oligonucleotide, 2.1 x 50 mm (p/n 659750-702)
Mobile phase A:	100 mM TEAA in water
Mobile phase B:	100 mM TEAA in acetonitrile
Flow rate:	0.69 mL/min
Gradient:	7%B to 11%B in 5 min 11%B to 80%B in 5.01 min Hold at 80%B for 5.50 min 80%B to 7%B in 5.56 min
Total run time:	8.5 min
Sample:	25 mer DNA
Injection:	1 µL of 0.5mg/mL
Temperature:	65 °C
Detection:	UV at 260 nm



MS Compatibility

The AdvanceBio Oligonucleotide column gives high chromatographic resolution and MS sensitivity using HFIP-TEA mobile phase



Column:	AdvanceBio Oligonucleotide, 2.1 x 50 mm (p/n 659750-702)
Mobile phase A:	HFIP:TEA (400 mM:15mM) in water
Mobile phase B:	MeOH:mobile phase A (50:50)
Flow rate:	0.4 mL/min
Gradient:	30 to 40%B in 0.5 min; 40-70%B in 5 min
Sample:	25 mer DNA
Temperature:	65 °C
Detection:	UV at 260 nm
Detection:	MS
Min range:	400 m/z
Max range:	1,700 m/z
Scan rate:	3.00 spectra/s
Ion polarity:	-ve
VCap:	3,500
Nozzle voltage:	1,000 V
Fragmentor:	200

Features, Advantages and Benefits

Feature	Advantages		Benefits
Poroshell particle	High efficiency	High resolution	Improved reliability of results
0.5 µm porous layer	Good retention and loading capacity		
100 Å pore diameter	Good retention and peak shape		
C18 chemistry	Good selectivity for oligonucleotides		
Oligonucleotide standards	Ensured performance for N/N-1 oligonucleotides		
HPH surface	High pH stability	Long column lifetime	Reduce costs
2 µm inlet frit	Does not plug easily		
2.7 µm particle diameter	Reasonable back pressure	Compatibility with HPLC and UHPLC	Increases flexibility
SS hardware	600 bar pressure limit		

Economic Benefits

Advantage	Benefit	Economic Benefit
High resolution	Improves reliability of results	Improved reliability reduces the likelihood of incorrect or incomplete results and the need for rework. This reduces risk and costs
Long column lifetime	Reduce costs	Reduced costs increase laboratory profitability
Compatibility with HPLC and UHPLC	Increases flexibility	Increased flexibility allows more efficient use of existing laboratory resources, thus reducing costs

Ordering Details

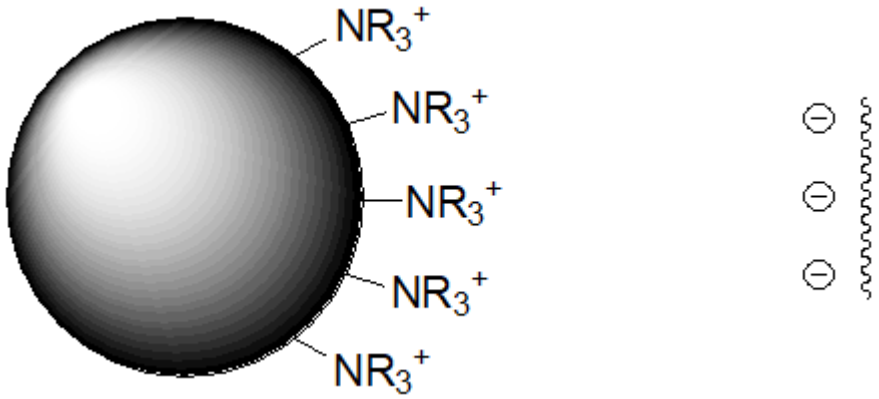
Description	Part Number
AdvanceBio Oligonucleotide 2.1 x 50 mm, 2.7 µm	659750-702
AdvanceBio Oligonucleotide 2.1 x 100 mm, 2.7 µm	655750-702
AdvanceBio Oligonucleotide 2.1 x 150 mm, 2.7 µm	653750-702
AdvanceBio Oligonucleotide 2.1 mm Fast Guard	821725-921
AdvanceBio Oligonucleotide 4.6 x 50 mm, 2.7 µm	659950-702
AdvanceBio Oligonucleotide 4.6 x 100 mm, 2.7 µm	655950-702
AdvanceBio Oligonucleotide 4.6 x 150 mm, 2.7 µm	653950-702
AdvanceBio Oligonucleotide 4.6 mm Fast Guard	820750-921
Oligonucleotides resolution standard	5190-9028
Oligonucleotides ladder standard	5190-9029

Bonded Phase	Pore Size	Temp. Limits	pH Range	End Capped
C18	100 Å	65 °C	3.0 - 11.0	Double

Anion Exchange

HPLC: IEX of Oligonucleotides

Anion exchange chromatography

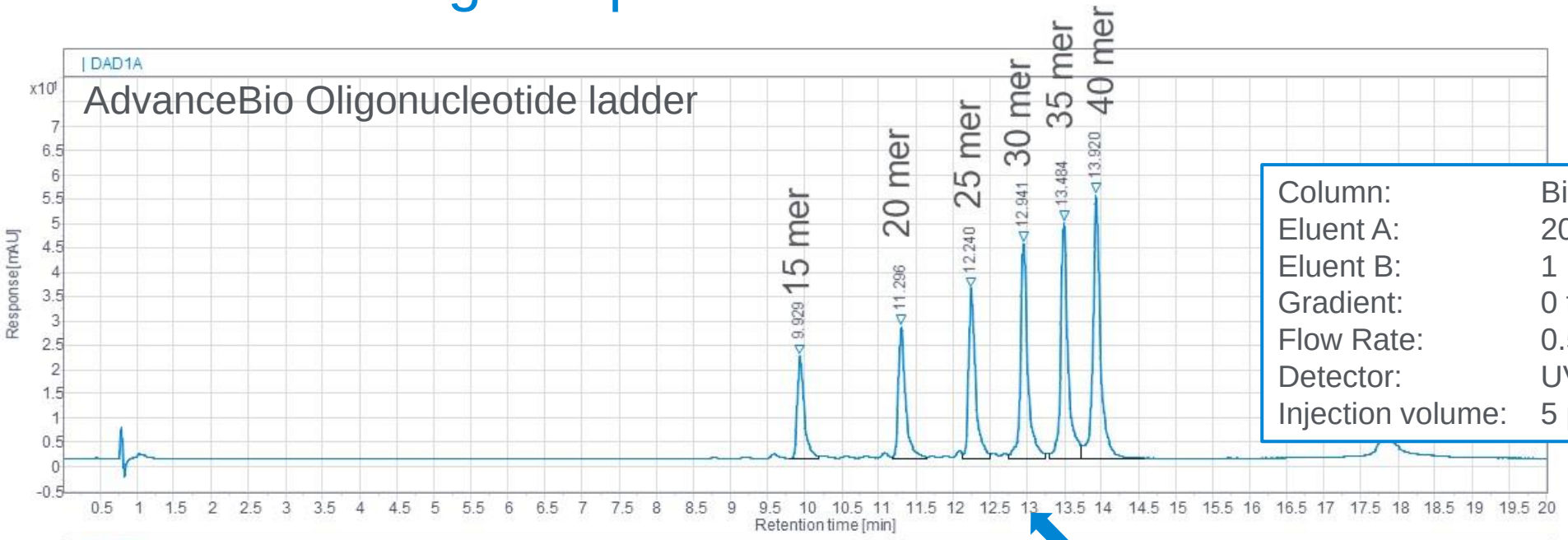


Variables:

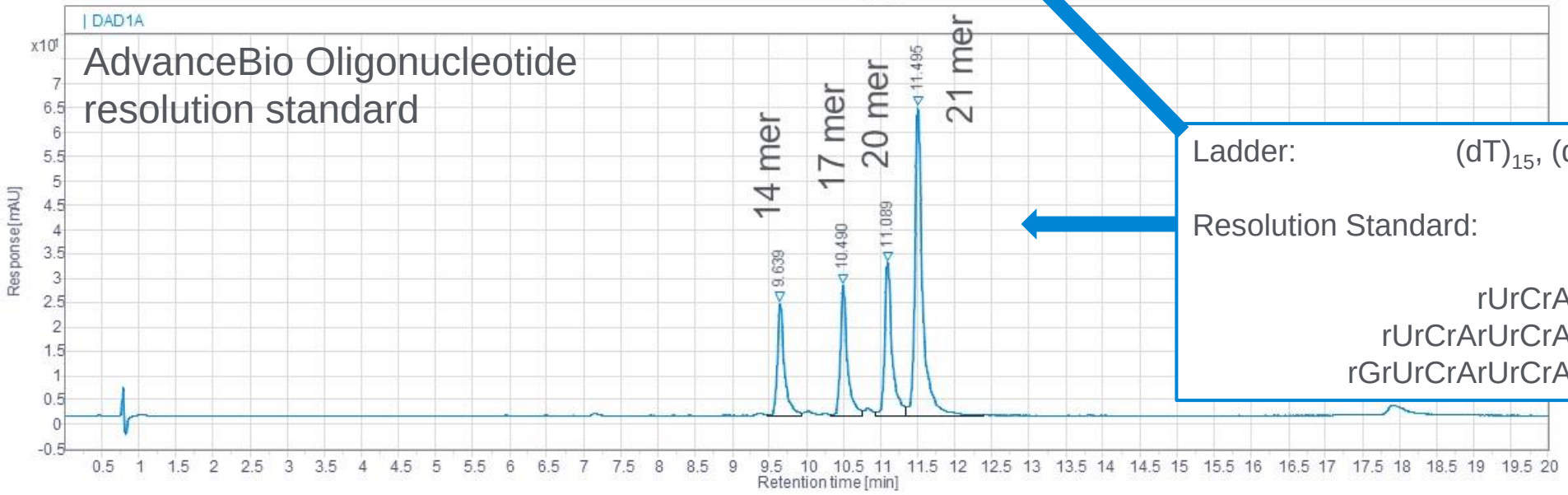
- Buffer type
- Buffer concentration
- Buffer pH
- Salt

- Temperature
- Organic modifiers

Anion Exchange Separation of Standards



Column:	Bio SAX NP5 (4.6 x 50 mm PEEK)
Eluent A:	20 mM Tris, pH 8.0
Eluent B:	1 M NaCl in eluent A
Gradient:	0 to 100%B in 20 min
Flow Rate:	0.5 mL/min
Detector:	UV, 260 nm
Injection volume:	5 µL (reconstituted in 1 mL)

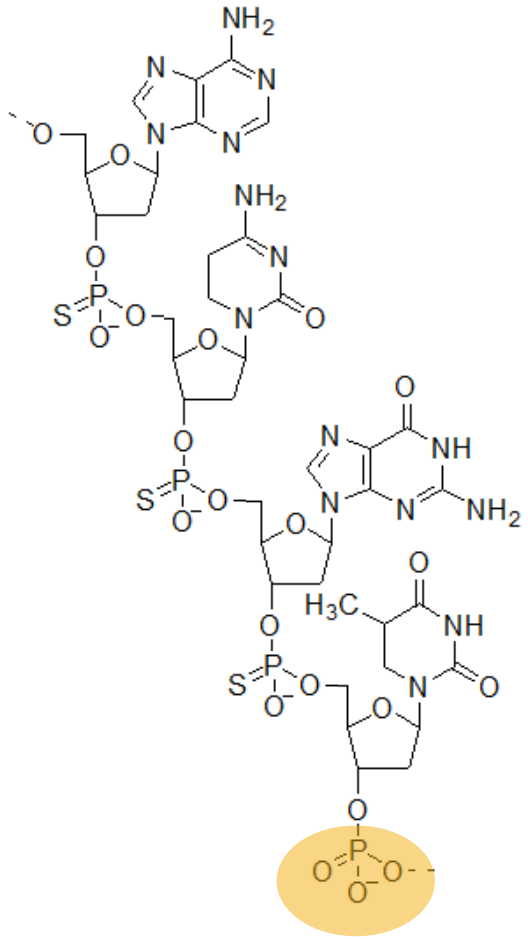


Ladder: $(dT)_{15}$, $(dT)_{20}$, $(dT)_{25}$, $(dT)_{30}$, $(dT)_{35}$, $(dT)_{40}$

Resolution Standard:

rCrArCrUrGrArArUrArCrCrArArU
rUrCrArCrArCrUrGrArArUrArCrCrArArU
rUrCrArUrCrArCrArCrUrGrArArUrArCrCrArArU
rGrUrCrArUrCrArCrArCrUrGrArArUrArCrCrArArU

CQA: Incomplete Backbone Phosphorothioation



5990-8297EN: High Resolution Separations of Oligonucleotides using PL-SAX Strong Anion Exchange HPLC Columns

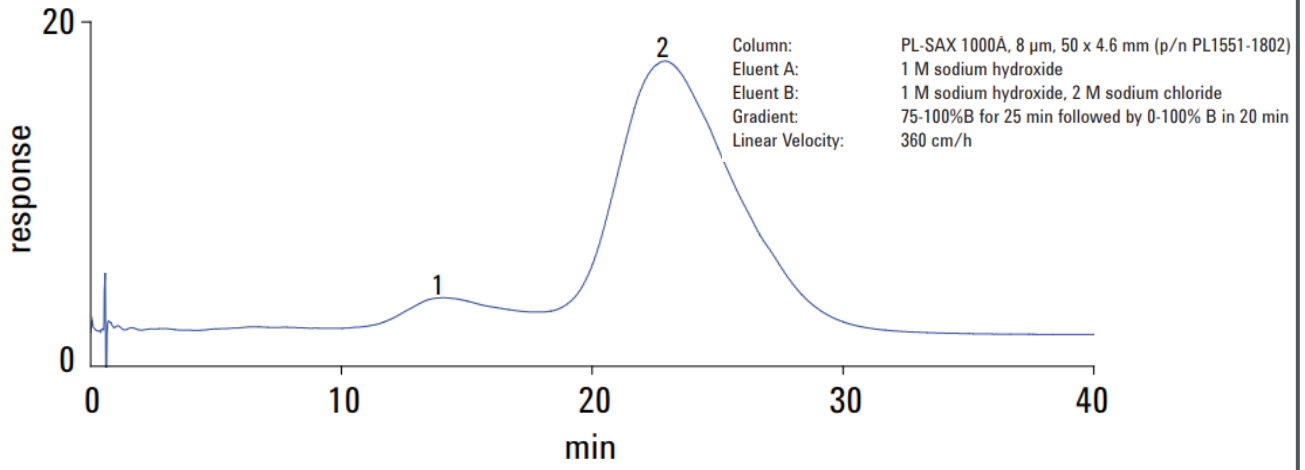
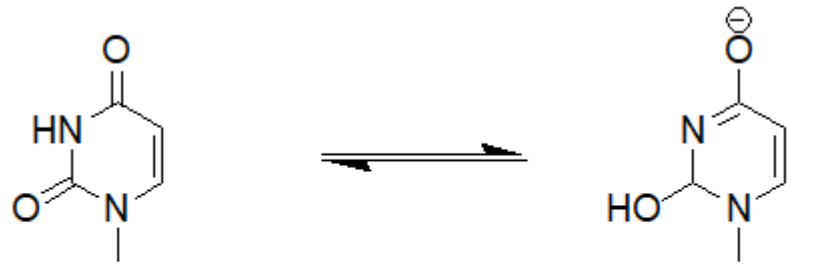


Figure 3. Separation of residual phosphodiester (1) and a phosphorothioate (2) 20mer oligonucleotide.

HPLC: IEX of Oligonucleotides

Control of selectivity

Thymine, pH 7 Thymine, pH 11



Uracil, pH 7 Uracil, pH 11

Guanine, pH 7 Guanine, pH 11

HPLC: IEX of Oligonucleotides

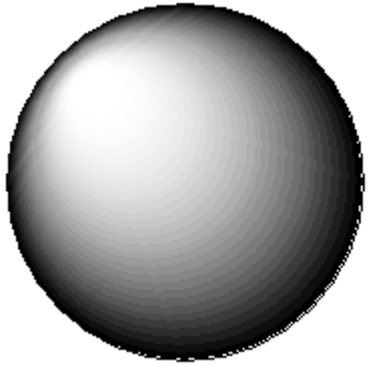
Variables:

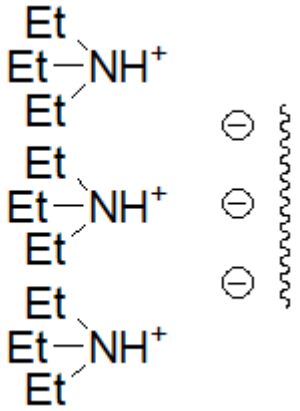
• Buffer type	Suitable buffers include phosphate and Tris
• Buffer concentration	Buffer concentration typically ~20 mM
• Buffer pH	pH 7 or 8 (depending on buffer); higher for selectivity changes
• Salt	Sodium chloride is good; sodium perchlorate has been suggested
• Temperature	Elevated temperature to improve peak shape (longer retention?)
• Organic modifiers	Some secondary interactions with certain stationary phases: addition of acetonitrile (up to 10 to 15% may be beneficial)

Ion Pair Reversed Phase

HPLC: IP-RPC of Oligonucleotides

“Ion-pair” chromatography





Variables:

- Ion-pair type
- Ion-pair concentration
- Ion-pair pH
- Temperature
- Organic modifier

Oligonucleotide Analysis – Ion Pair RP Chromatography

100 mM TEAA/water-acetonitrile gradient — UV method

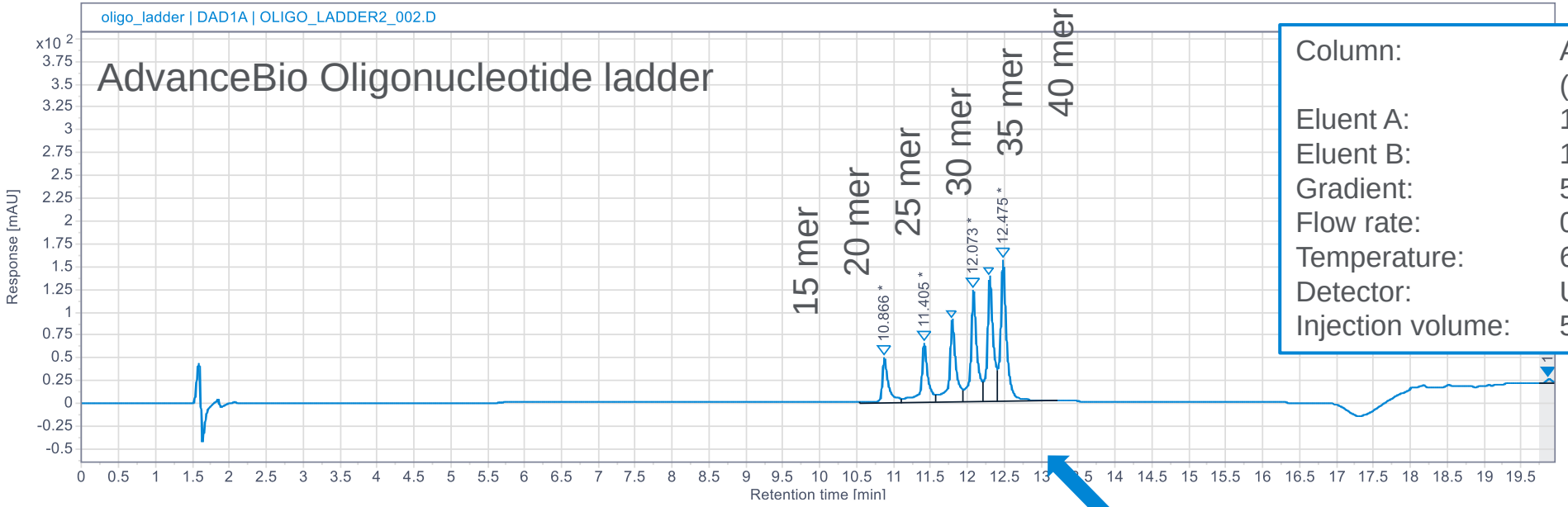
TEA + HFIP/water-methanol gradient — MS method

50 to 60°C commonly used

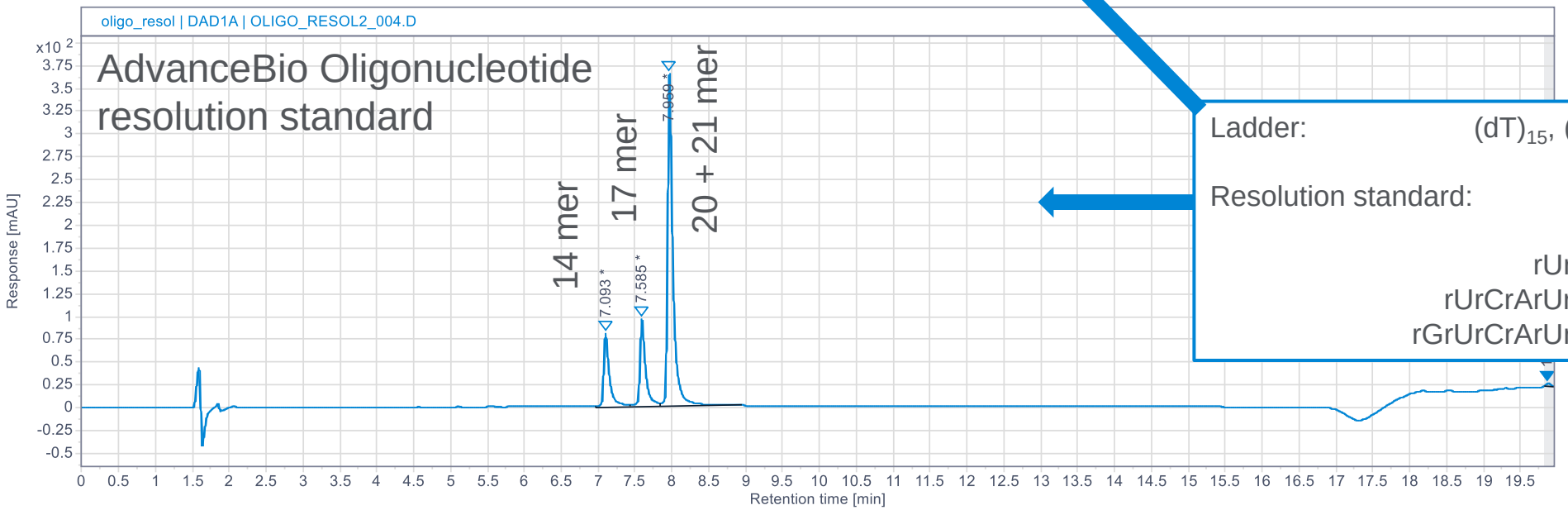
Now: alternative ion-pair reagents and concentrations

Major challenges: molecule-related (coeluting impurities); column lifetime issues

Ion-Pair Reversed Phase Separation of Standards



Column:	AdvanceBio Oligonucleotide 2.7µm (2.1 x 150 mm)
Eluent A:	100 mM TEAA
Eluent B:	100 mM TEAA in 9:1 ACN
Gradient:	5 to 20%B in 15 min
Flow rate:	0.21 mL/min
Temperature:	65 °C
Detector:	UV, 260 nm
Injection volume:	5 µL (reconstituted in 1 mL)

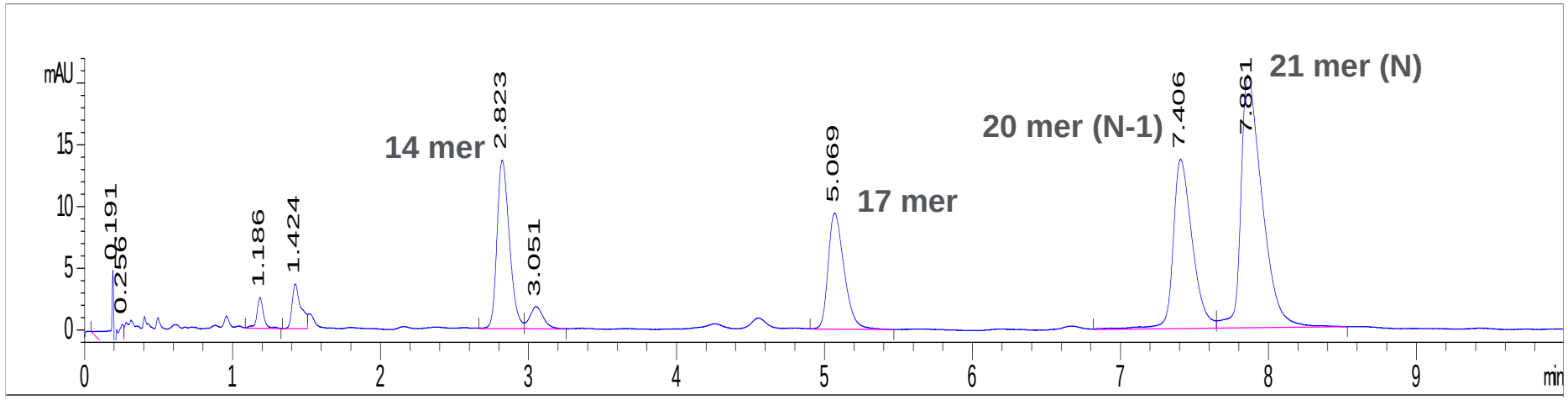


Ladder: $(dT)_{15}$, $(dT)_{20}$, $(dT)_{25}$, $(dT)_{30}$, $(dT)_{35}$, $(dT)_{40}$

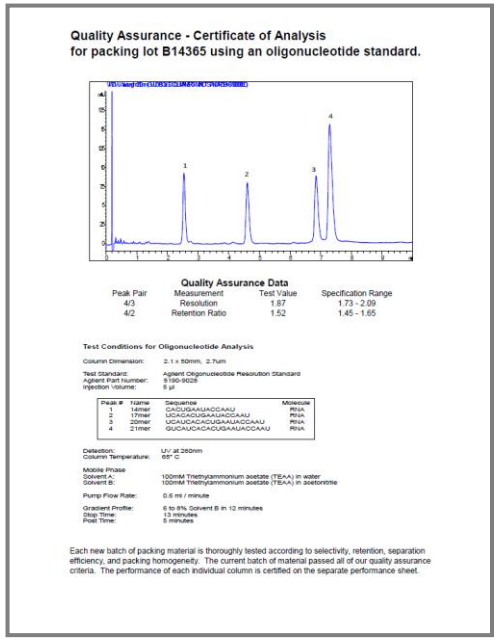
Resolution standard:

rCrArCrUrGrArArUrArCrCrArArU
rUrCrArCrArCrUrGrArArUrArCrCrArArU
rUrCrArUrCrArCrArCrUrGrArArUrArCrCrArArU
rGrUrCrArUrCrArCrArCrUrGrArArUrArCrCrArArU

Optimized Gradient for Resolution Standard



Column:	AdvanceBio Oligonucleotide, 2.1 x 50 mm
Mobile phase A:	100 mM TEAA in water
Mobile phase B:	100 mM TEAA in acetonitrile
Gradient:	6 to 8%B in 10 min
Stop time:	11 min
Post run:	5 min
Flow rate:	0.6 mL/min
Sample:	Agilent Oligonucleotide resolution standard
	14 mer, 17 mer, 20 mer, 21 mer RNA
Temperature:	65 °C
Injection:	5 µL
Detection:	UV at 260 nm



Quality Assurance - Certificate of Analysis
for packing lot B14365 using an oligonucleotide standard.

Quality Assurance Data

Peak Pair	Measurement	Test Value	Specification Range
4/3	Resolution	1.87	1.73 - 2.09
4/2	Retention Ratio	1.52	1.45 - 1.65

Test Conditions for Oligonucleotide Analysis

Column Dimension: 2.1 x 50mm, 2.7um

Test Standard: Agilent Oligonucleotide Resolution Standard
Agilent Part Number: 6190-9028
Injection Volume: 5 µl

Peak #	Name	Sequence	Molecule
1	14mer	GACUGAAUACCAAU	RNA
2	17mer	UCACACUGAAUACCAAU	RNA
3	20mer	UCAUCACACUGAAUACCAAU	RNA
4	21mer	GUCAUCACACUGAAUACCAAU	RNA

Detection: UV at 260nm
Column Temperature: 65° C

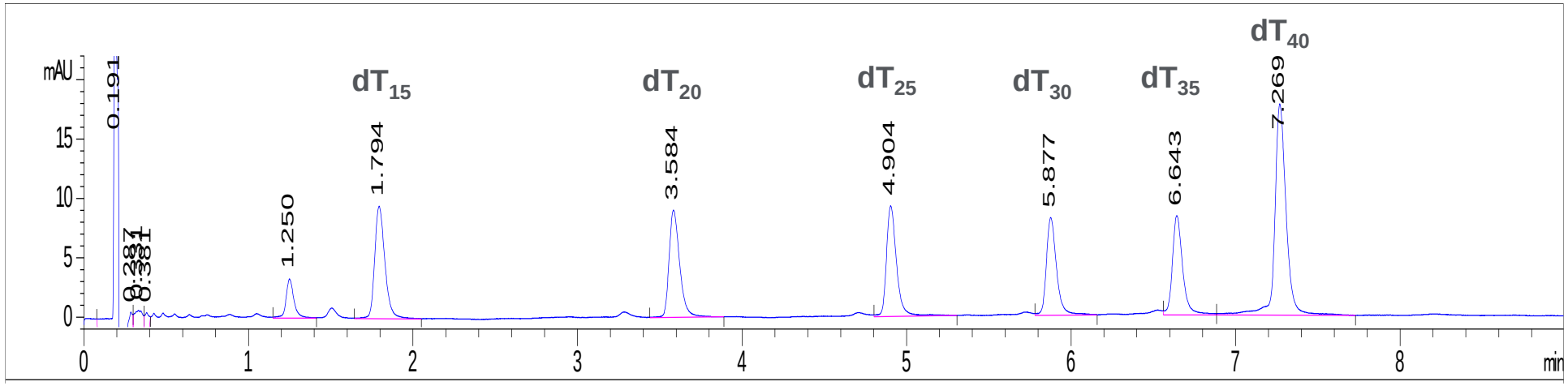
Mobile Phase
Solvent A: 100mM Triethylammonium acetate (TEAA) in water
Solvent B: 100mM Triethylammonium acetate (TEAA) in acetonitrile

Pump Flow Rate: 0.6 ml / minute

Gradient Profile: 6 to 8% Solvent B in 12 minutes
Stop Time: 13 minutes
Post Time: 5 minutes

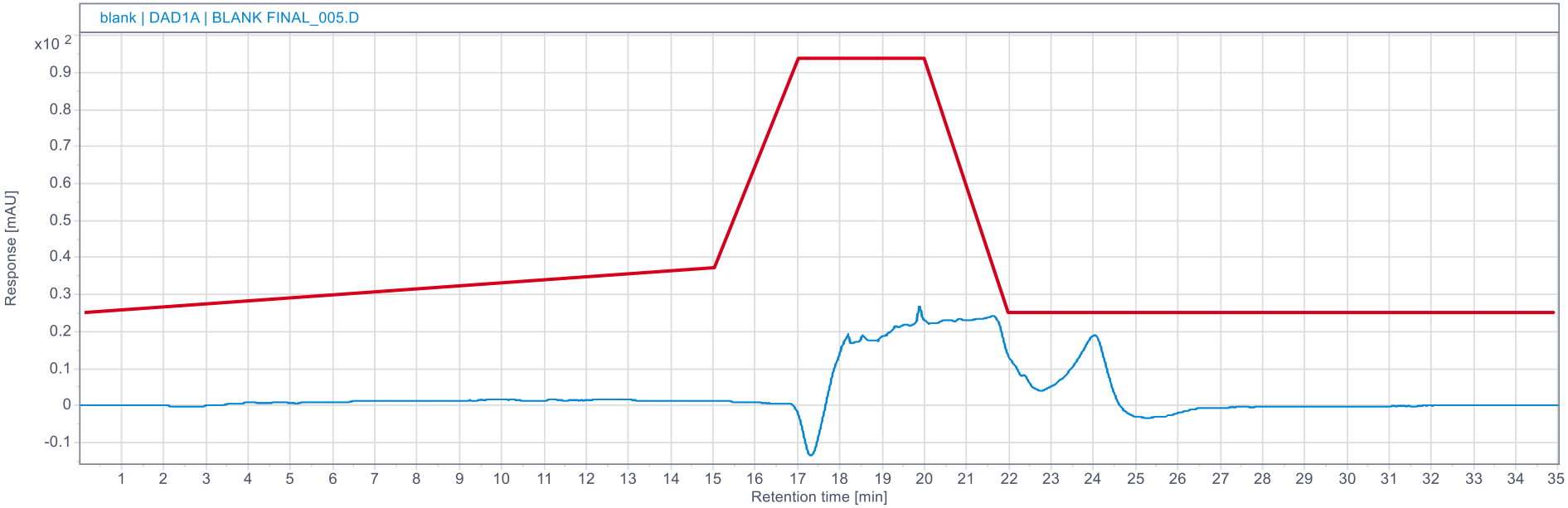
Each new batch of packing material is thoroughly tested according to selectivity, retention, separation efficiency, and packing homogeneity. The current batch of material passed all of our quality assurance criteria. The performance of each individual column is certified on the separate performance sheet.

Optimized Gradient for Ladder Standard



Column:	AdvanceBio Oligonucleotide, 2.1 x 50 mm
Mobile phase A:	100 mM TEAA in water
Mobile phase B:	100 mM TEAA in acetonitrile
Gradient:	10 to 14%B in 12 min
Stop time:	13 min
Post run:	5 min
Flow rate:	0.6 mL/min
Sample:	Agilent Oligonucleotide ladder standard 15 mer, 20 mer, 25 mer, 30 mer, 35 mer, 40 mer DNA
Temperature:	65 °C
Injection:	0.5 µL
Detection:	UV at 260 nm

Blank Gradient



Time	%A	%B
0	95%	5%
15	80%	20%
17	10%	90%
20	10%	90%
22	95%	5%
35	95%	5%

Note

TEAA prepared as 1 M concentrated stock solution (1 mol acetic acid in 900 mL water plus 1 mol TEA; made up to 1L).

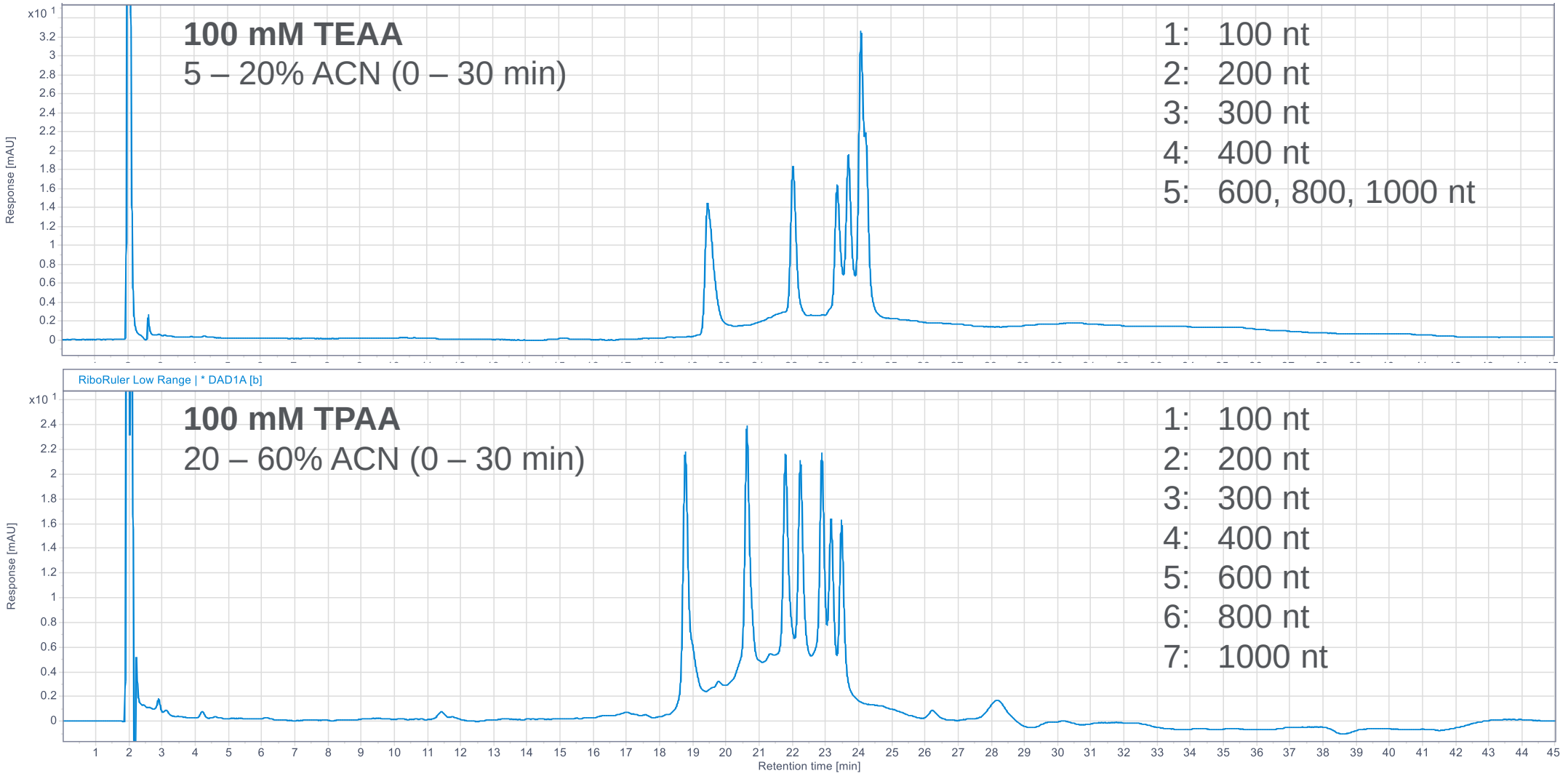
Mobile phase A: 100 mL TEAA stock solution + 900 mL water

Mobile phase B: 100 mL TEAA stock solution + 900 mL ACN

Blank baseline can be dependent on quality of ion-pair reagents and also age of mobile phase.

Ion-Pair Comparisons

PLRP-S 4000 Å for Higher MW RNA

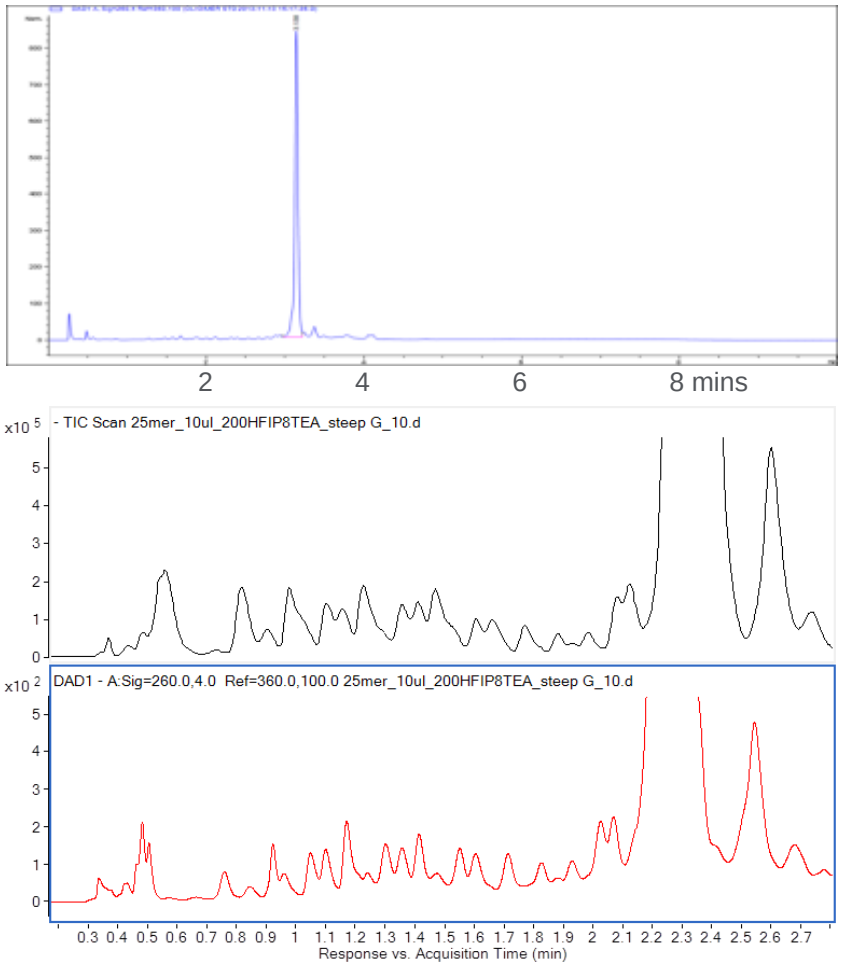


Ion Pair RP + MS Detection

For mass verification and impurity analysis

MS Compatibility

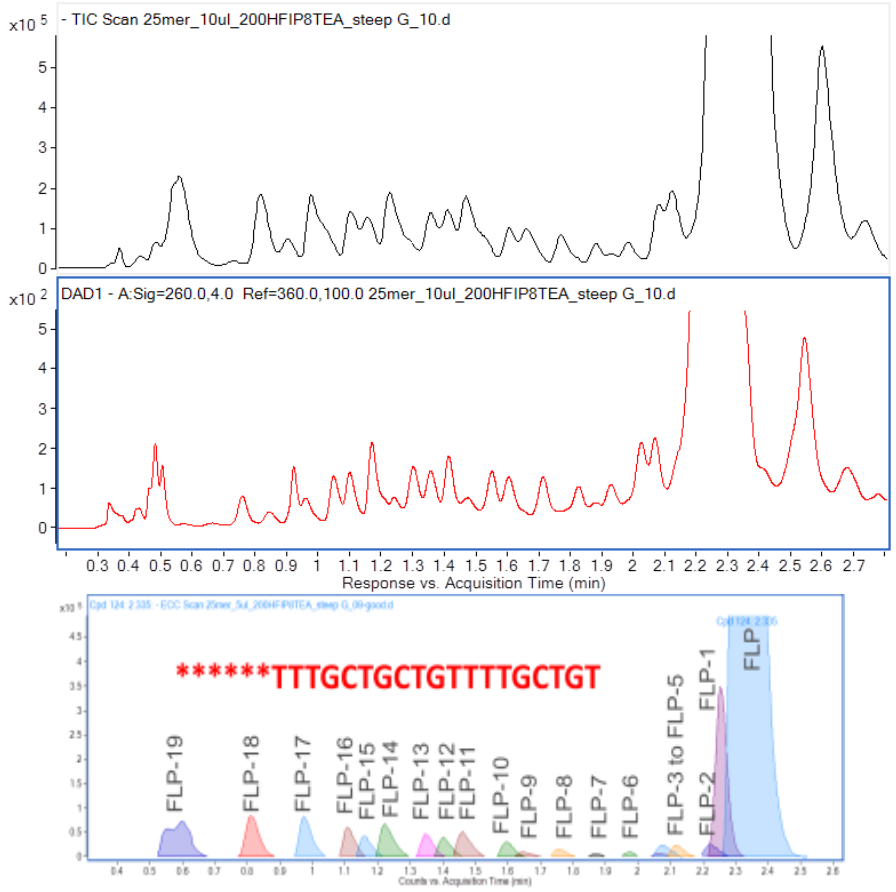
AdvanceBio Oligonucleotide column gives high chromatographic resolution and MS sensitivity using HFIP-TEA mobile phase



Column:	AdvanceBio Oligonucleotide 2.7 µm 2.1 x 50 mm
Mobile phase A:	HFIP:TEA (200 mM:8 mM) in water
Mobile phase B:	MeOH:mobile phase A (50:50)
Flow rate:	0.4 mL/min
Gradient:	30 to 40%B in 0.5 min; 40 to 70%B in 5 min
Sample:	25 mer DNA
Temperature:	65 °C
Detection:	UV at 260 nm
Detection:	MS
Min range:	400 m/z
Max range:	1,700 m/z
Scan rate:	3.00 spectra/s
Ion polarity:	-ve
VCap:	3,500
Nozzle voltage:	1,000 V
Fragmentor:	200

MS Compatibility

AdvanceBio Oligonucleotide column with accurate mass MS provides characterization of the oligonucleotide structures and sequences

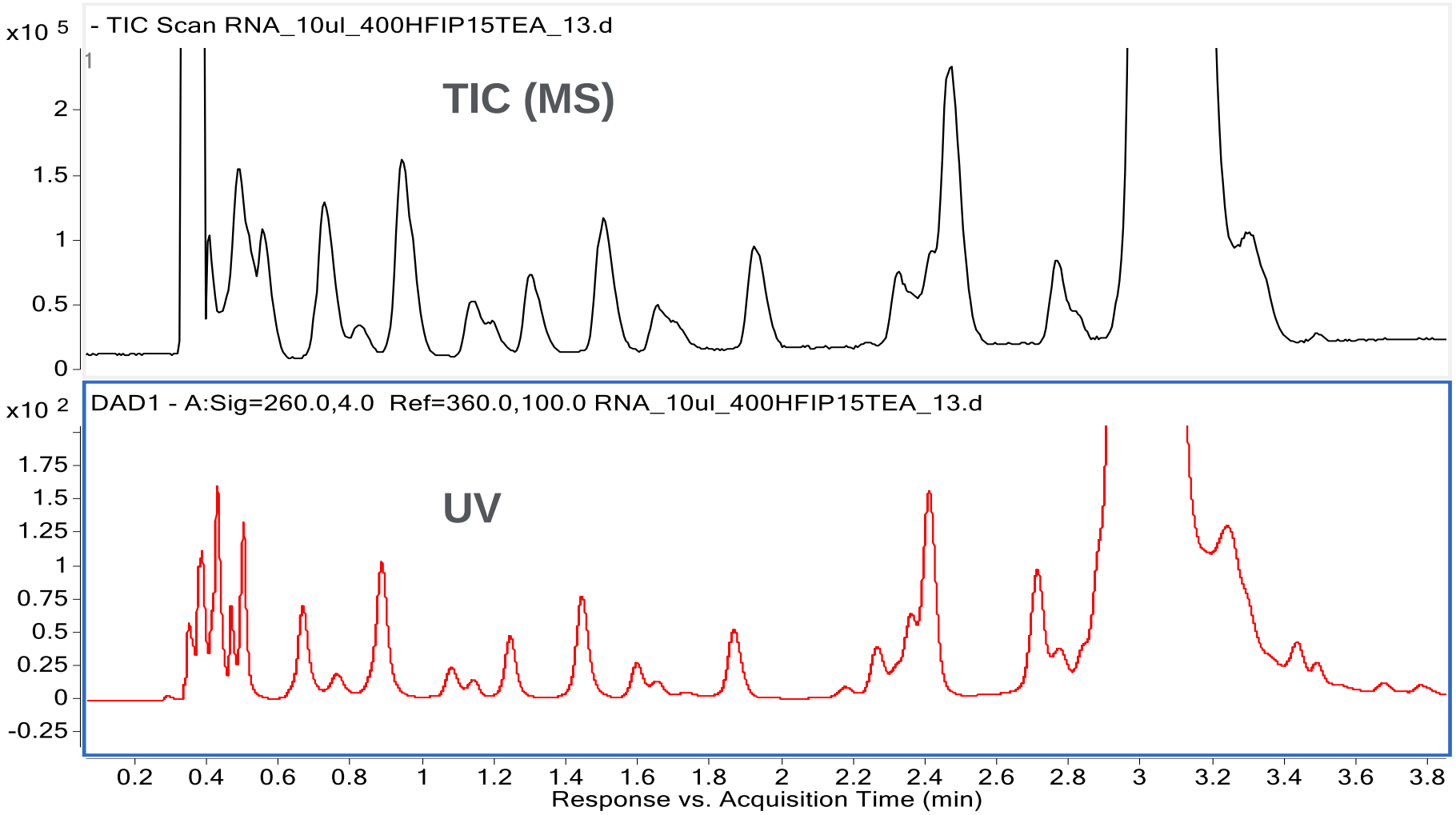


HFIP:TEA (200 mM:8 mM) in water

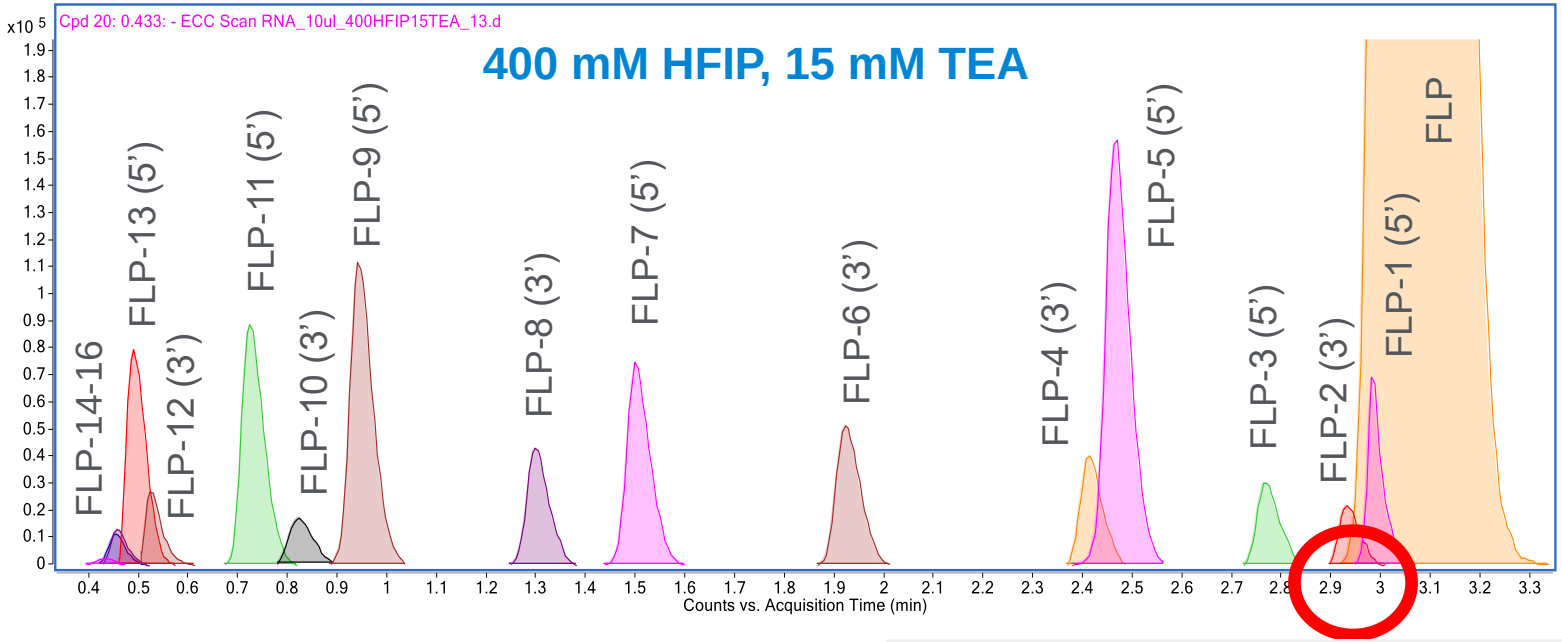
Peak	Response	%
FLP	5089897	44.33%
FLP-1	1656225	14.42%
FLP-2	304129	2.65%
FLP-3	303848	2.65%
FLP-4	218243	1.90%
FLP-5	113062	0.98%
FLP-6	104555	0.91%
FLP-7	110327	0.96%
FLP-8	134341	1.17%
FLP-9	134080	1.17%
FLP-10	186947	1.63%
FLP-11	358833	3.12%
FLP-12	251690	2.19%
FLP-13	272844	2.38%
FLP-14	416306	3.63%
FLP-15	238205	2.07%
FLP-16	304333	2.65%
FLP-17	403038	3.51%
FLP-18	459344	4.00%
FLP-19	422518	3.68%
Sum	11482765	100%

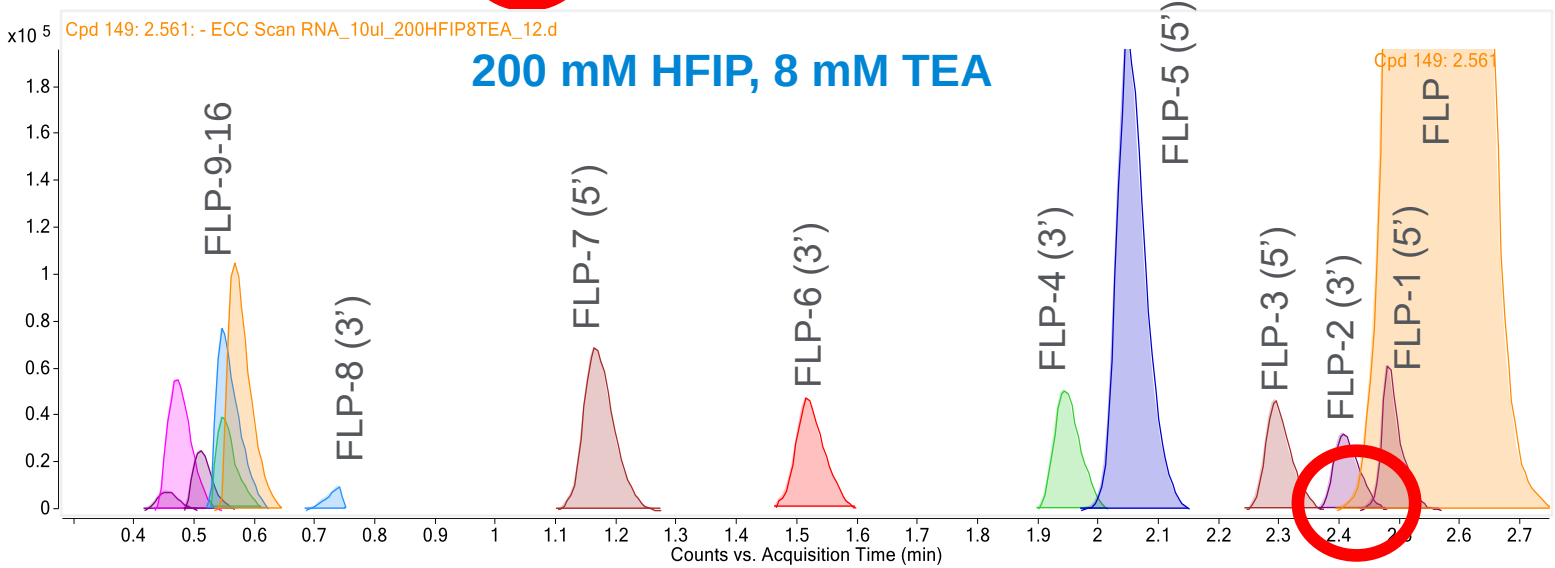
RNA Sense with 2’-OMe (19 nt)

HFIP:TEA (400 mM:15 mM) in water



EIC for Two LC Conditions





Biochromatography

Biomolecules come in different shapes and sizes, with different surface characteristics…

… so do Agilent biocolumns

Resources for Support

Technical support www.agilent.com/chem/techsupport

Agilent product catalogs www.agilent.com/en/promotions/catalog

- InfinityLab Supplies catalog (5991-8031EN)

Resource page www.agilent.com/chem/agilentresources

- Quick reference guides
- Catalogs, column user guides
- Online selection tools, how-to videos

Agilent University http://www.agilent.com/crosslab/university

YouTube – Agilent Channel

Your local FSE and specialists

Agilent service contracts

Contact Agilent Chemistries and Supplies Technical Support

Available in the U.S. and Canada, 8am to 5pm all time zones

Web chat: Product pages of Agilent.com

1-800-227-9770 option 3, option 3:

- Option 1 – GC and GC/MS columns and supplies
  gc-column-support@agilent.com
- Option 2 – for LC and LC/MS columns and supplies
  lc-column-support@agilent.com
- Option 3 – for sample preparation, filtration, and QuEChERS
  spp-support@agilent.com
- Option 4 – for spectroscopy supplies
  spectro-supplies-support@agilent.com
- Option 5 – for standards
  chem-standards-support@agilent.com
- Option 6 for ProZyme products
  pzi.info@agilent.com

Thank you!

Questions?

mohit.patel@agilent.com

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Trusted Answers