SEC for Ultra-Large Analytes

Column Selection and Method Development for Next Generation Biotherapeutics

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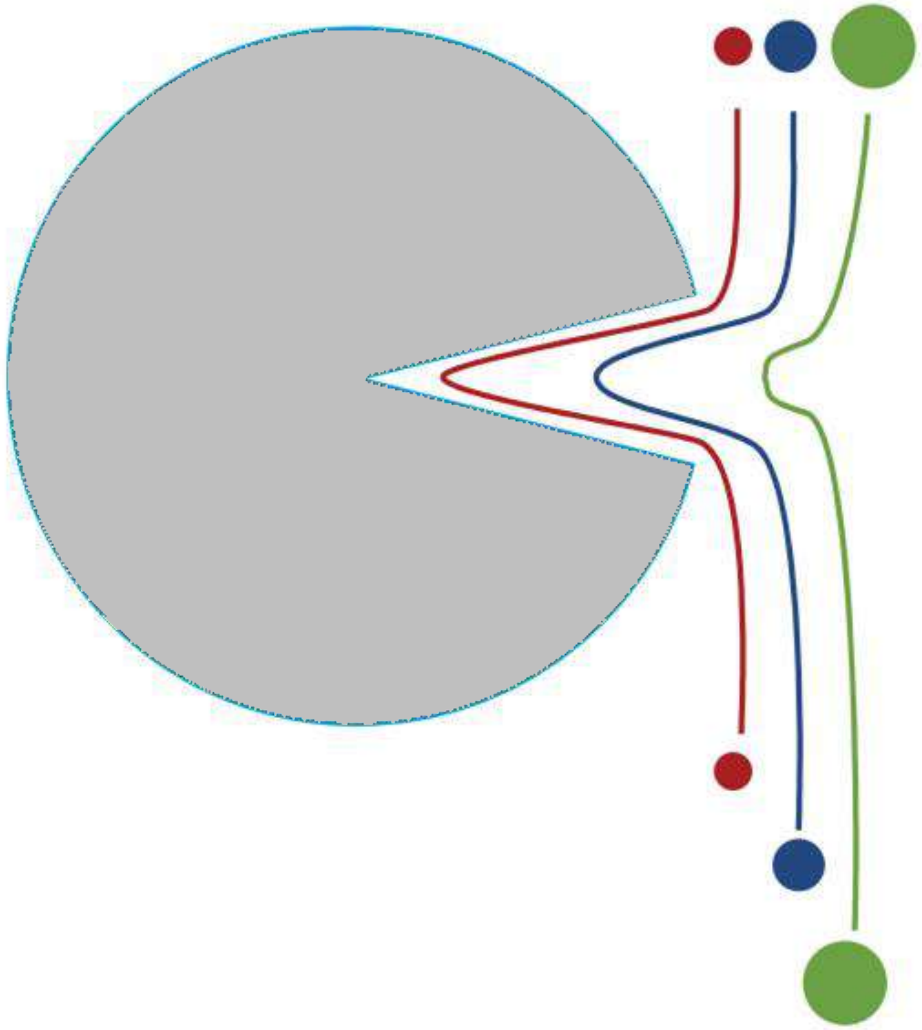
Agilent Technologies

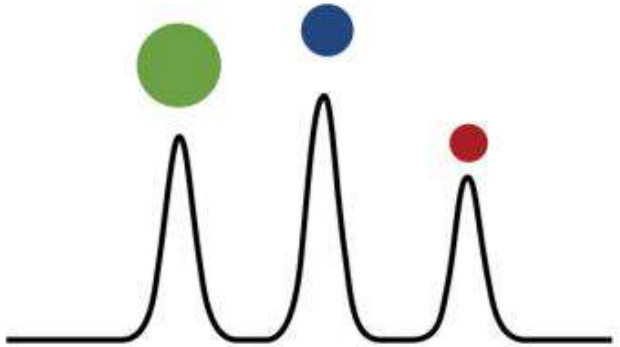
Agilent
Trusted Answers

DE-005324

Size Exclusion Chromatography (SEC)

Separation by size in solution

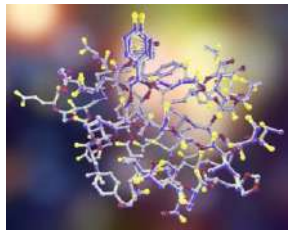




Smaller molecules spend longer in the pores and elute later

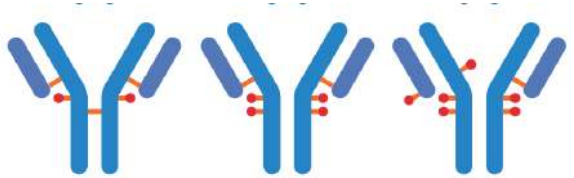
Larger molecules spend less time in the pores and elute sooner

Sample Types & Their Sizes

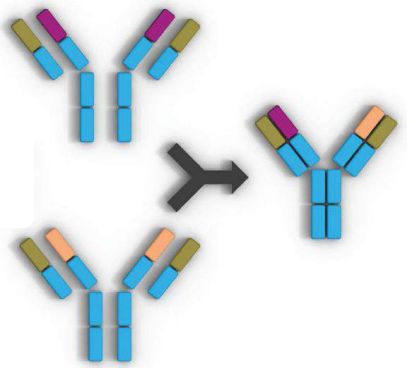


Peptides & Proteins
(Insulin, GLP-1, enzymes…)
~1kDa – 1 MDa

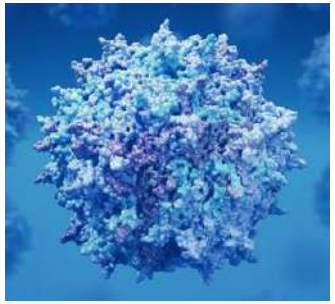
Monoclonal Antibodies
(mAbs)
~150 kDa, 10-12 nm diameter



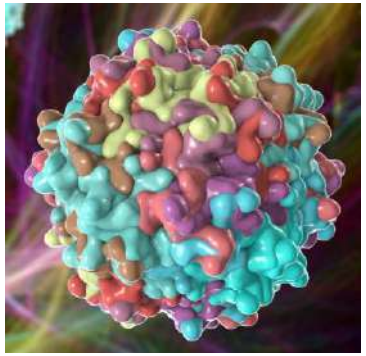
Antibody-Drug Conjugates
(ADCs)
>150 kDa



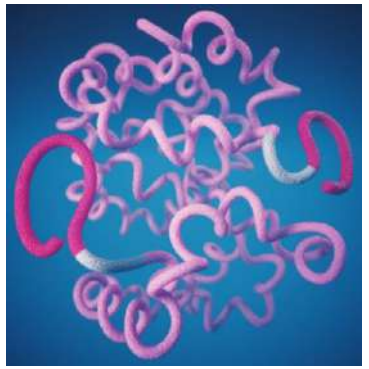
Bispecific Antibodies
~150 kDa



Adeno-Associated Viruses
(AAVs)
3.5-5 MDa, 20-25 nm diameter



Virus-Like Particles (VLPs), Viral
Vectors, Adenovirus (Adv), Lentivirus
1-50 MDa, 20-100+ nm diameter



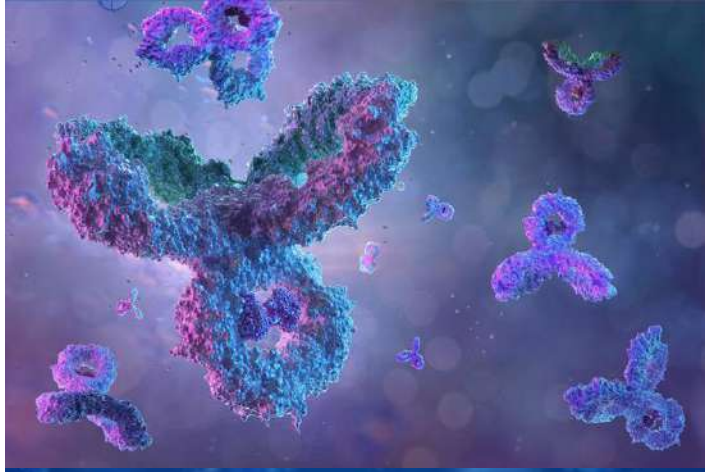
mRNA, DNA
Single or double stranded
20 – 1000s of nt or bp

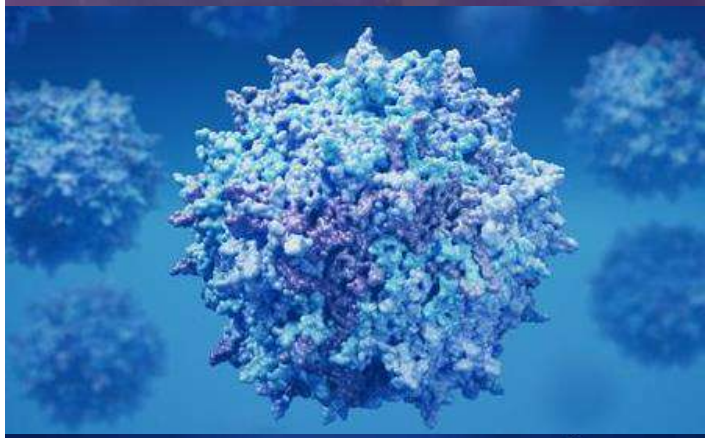
Critical Quality Attribute Analysis via SEC

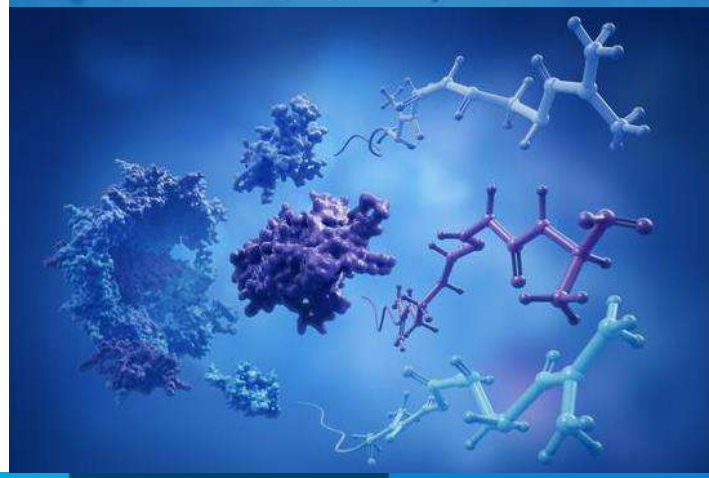
- Aggregation
- Destabilization or partial assembly
- Empty/full capsid analysis
- Molecular weight estimation
- Size distribution

mAbs
~150 kDa
~10-12 nm diameter

AAVs
~3.5-5 MDa
~20-25 nm diameter

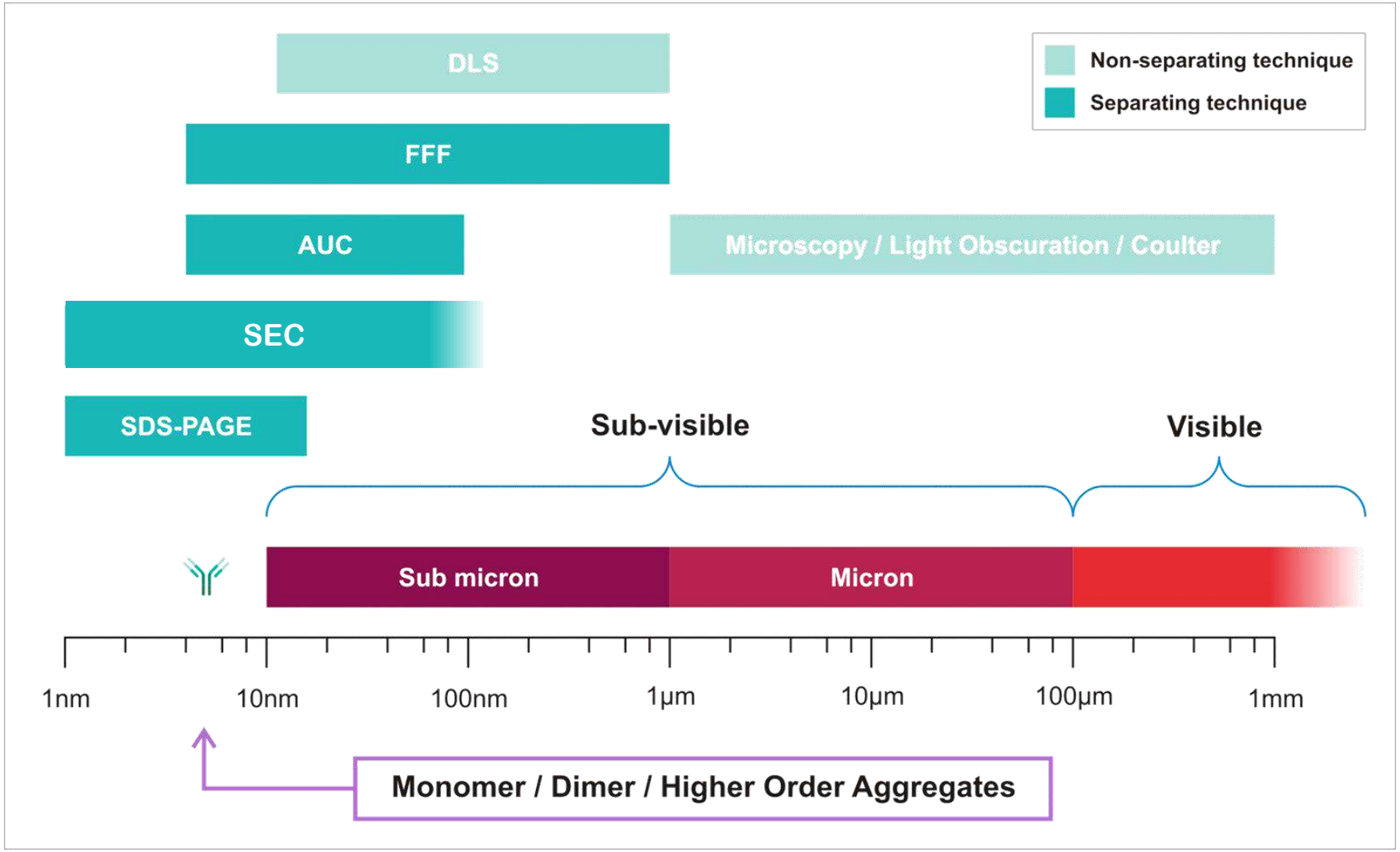






Aggregate Analysis Techniques

How are aggregate and fragments monitored?



Agilent's GPC/SEC Column Portfolio

SDV	POLEFIN	GRAM	PolarSil	PFG
Neutral Polymers PVC Polycarbonate	High Temp GPC Polyethylene Polypropylene	Polyurethanes Starches Cellulosics	Formaldehyde resins	Polyester Nylons PET

SUPREMA	NOVEMA	MCX	PROTEEMA	
Neutral Polymers	Charged Polymers Cationic	Charged Polymers Anionic	Diol, USP L20 Proteins, Peptides	

PLgel	PLgel Olexis	PolarGel	PLMultisolvent	PL HFIPgel
PL Rapide PlusPore PLgel LS PLgel MiniMix Neutral Polymers	High Temp GPC Polyethylene Polypropylene	Polyurethanes Starches Cellulosics	Formaldehyde resins	Polyester Nylons PET

PL aquagel			AdvanceBio SEC	Bio SEC-5
Rapide Aqua Water Soluble Polymers			130-1000 Å Peptides Proteins mAbs Oligos	2000 Å Ultra-large biotherapeutics

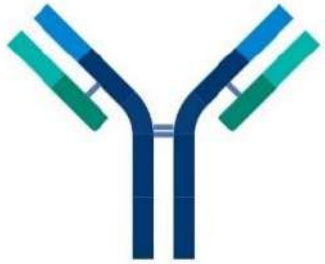
Organic Solvent Soluble Polymers

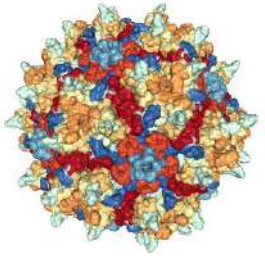
Water Soluble Polymers

Biologics

Agilent

Agilent SEC Portfolio for Aggregate Analysis





AdvanceBio SEC

- Unique hydrophilic coating for minimal secondary interactions
- Large pore volume for maximum separation opportunity
- 2.7 µm particle option for routine analysis, 1.9 µm option for high throughput
- Pore sizes ranging from 120 Å to 1000 Å for peptides through mRNA

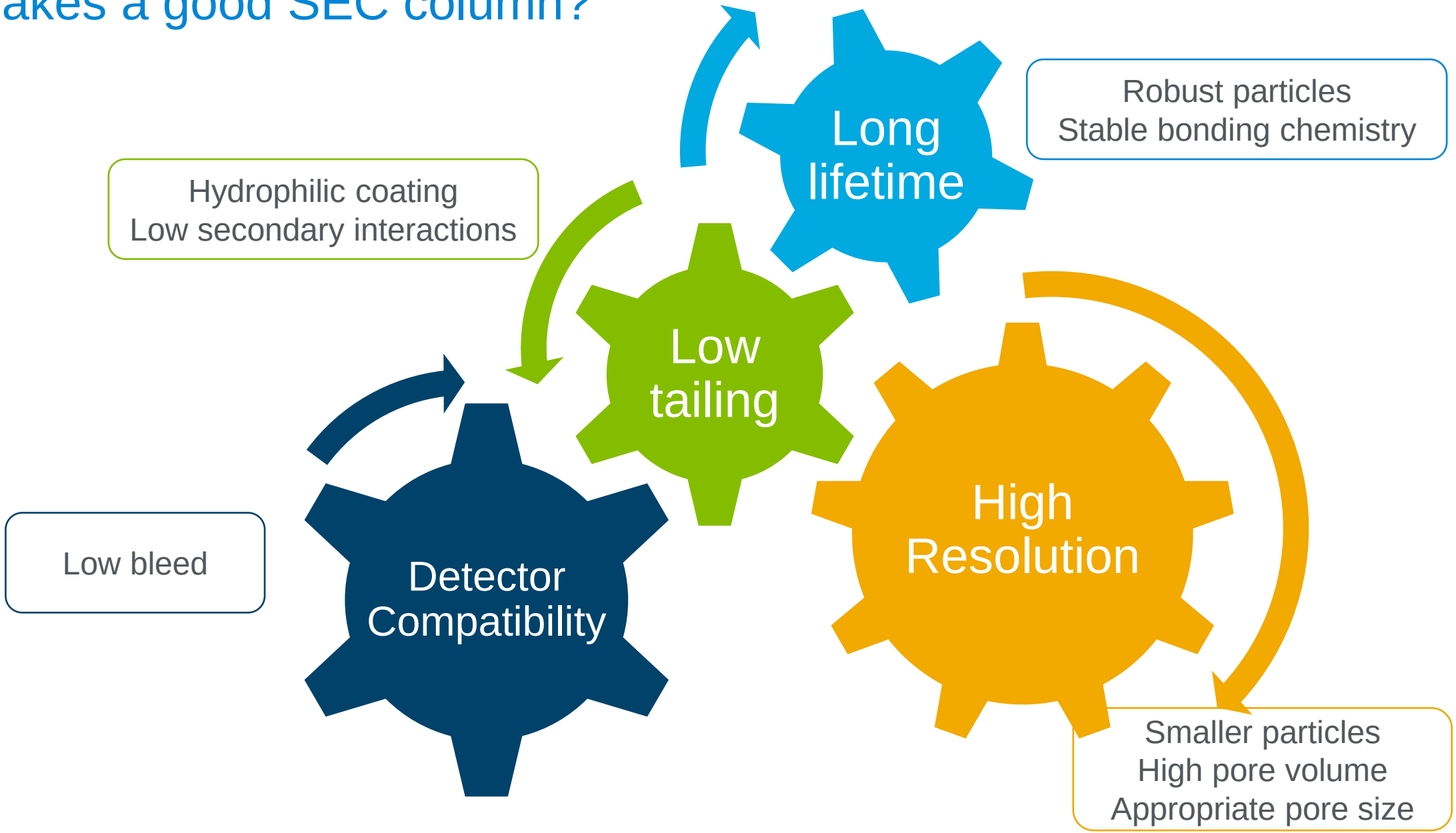
Agilent Bio SEC-5 2000 Å

- For the largest biotherapeutics - VLPs, oligonucleotides

PROTEEMA

- Diol chemistry for USP L20 compliance

What makes a good SEC column?

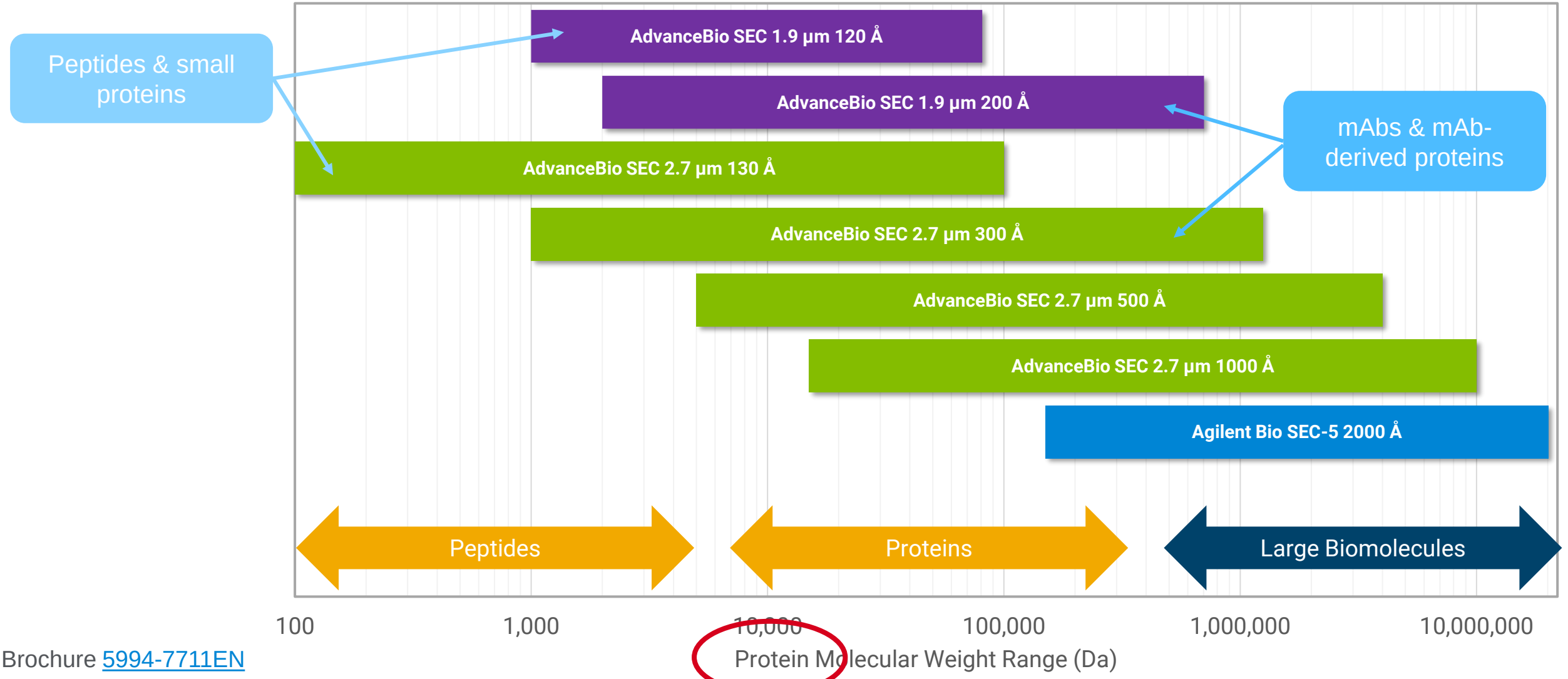


Column Properties for High Resolution

Smaller particles
High pore volume
Appropriate pore size

Choosing an SEC Column for Your Application

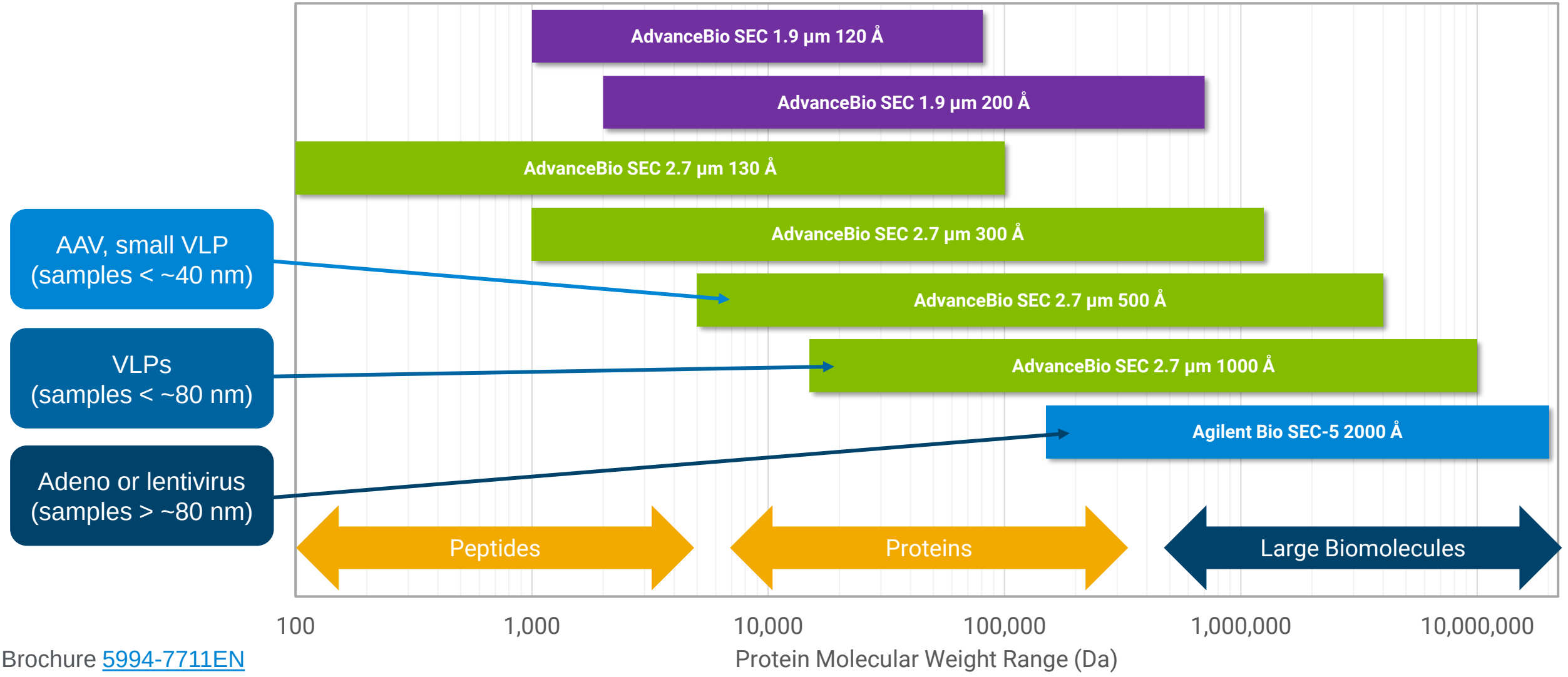
Getting the Right Pore Size



Brochure [5994-7711EN]

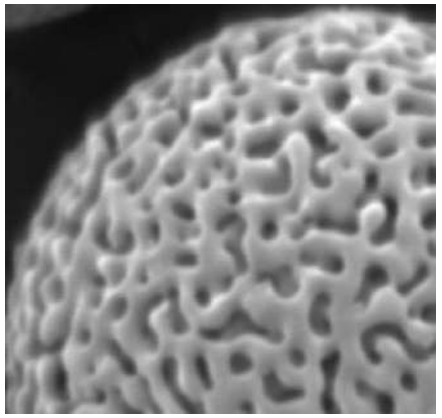
Choosing an SEC Column for Your Application

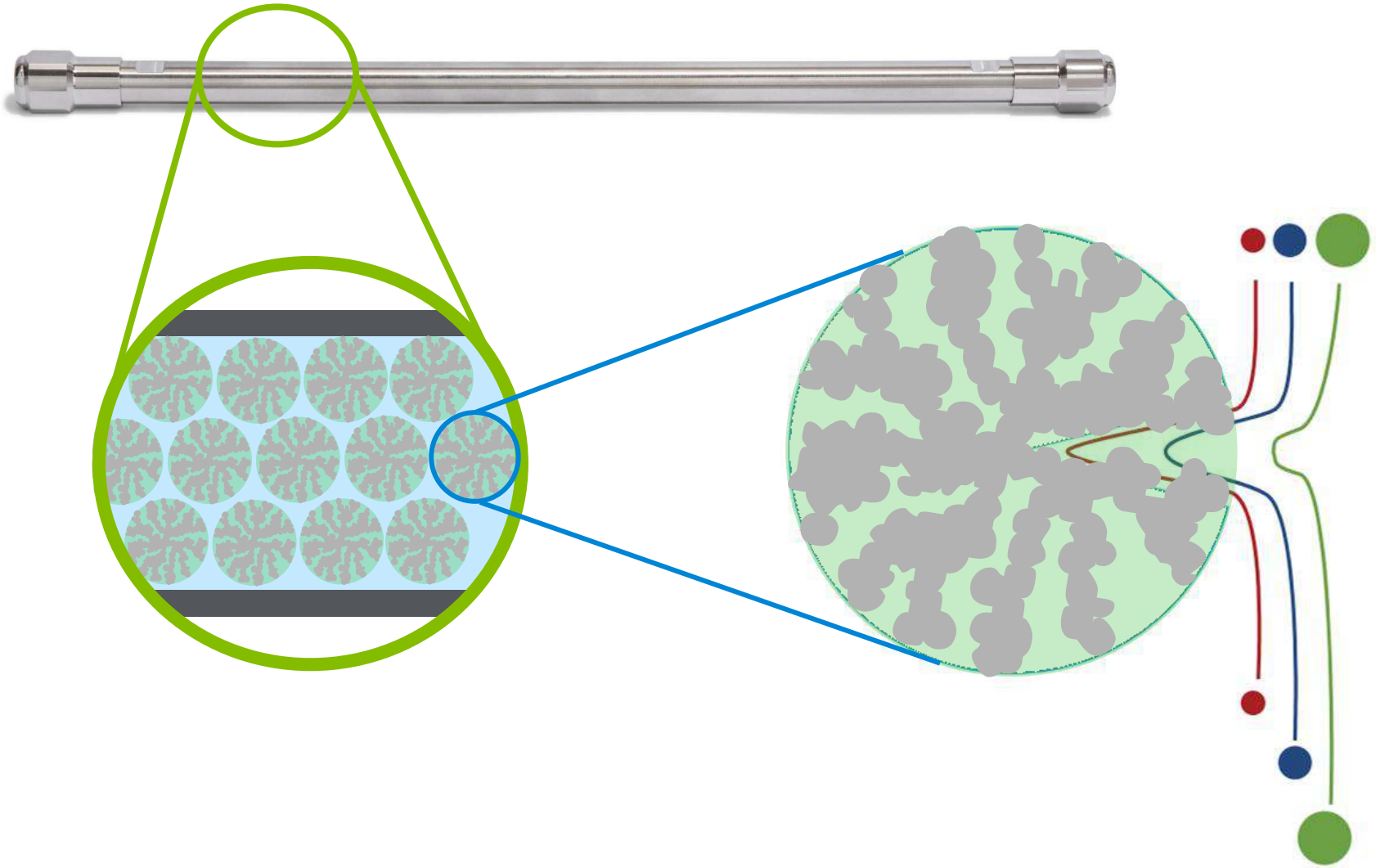
Getting the Right Pore Size



Brochure 5994-7711EN

How to Choose the Right Pore Size



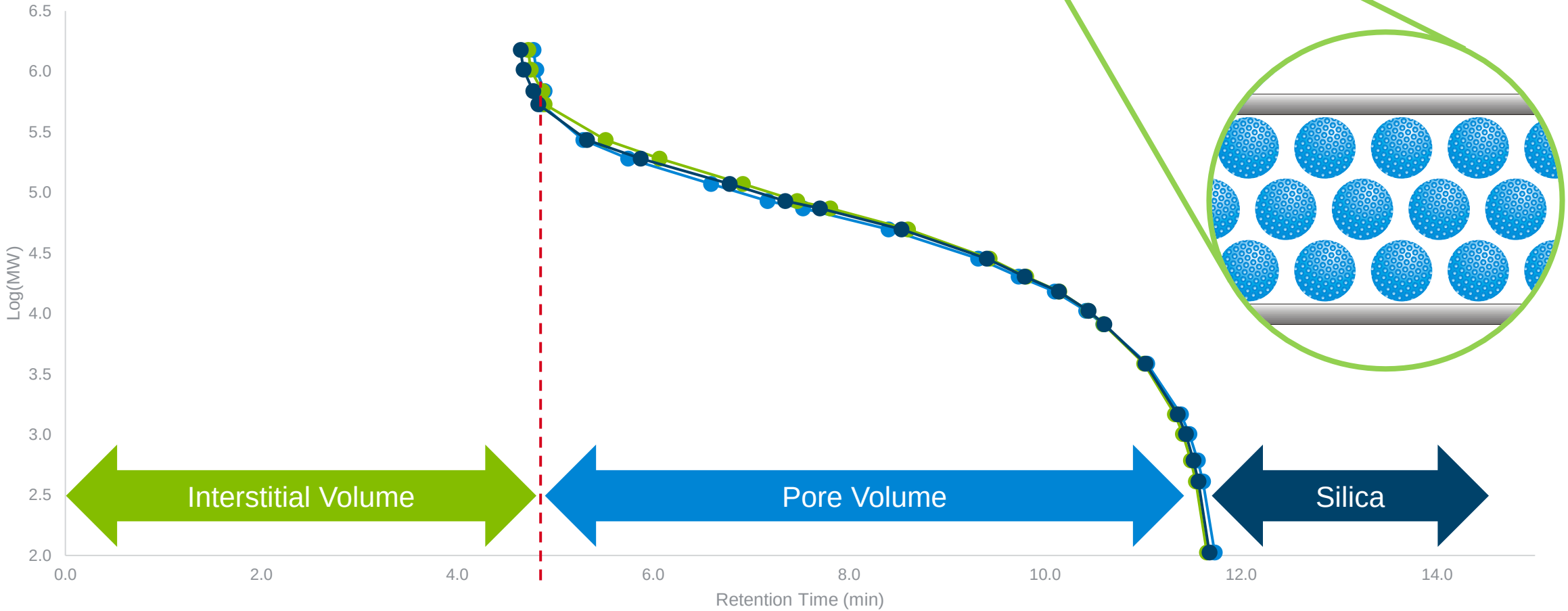


Three approaches:

- Published molecular weight ranges
- Literature examples for similar samples
- Empirically

Composition of an SEC Column & Impact on Resolution

Column Volume ($length \times \pi \times radius^2$)

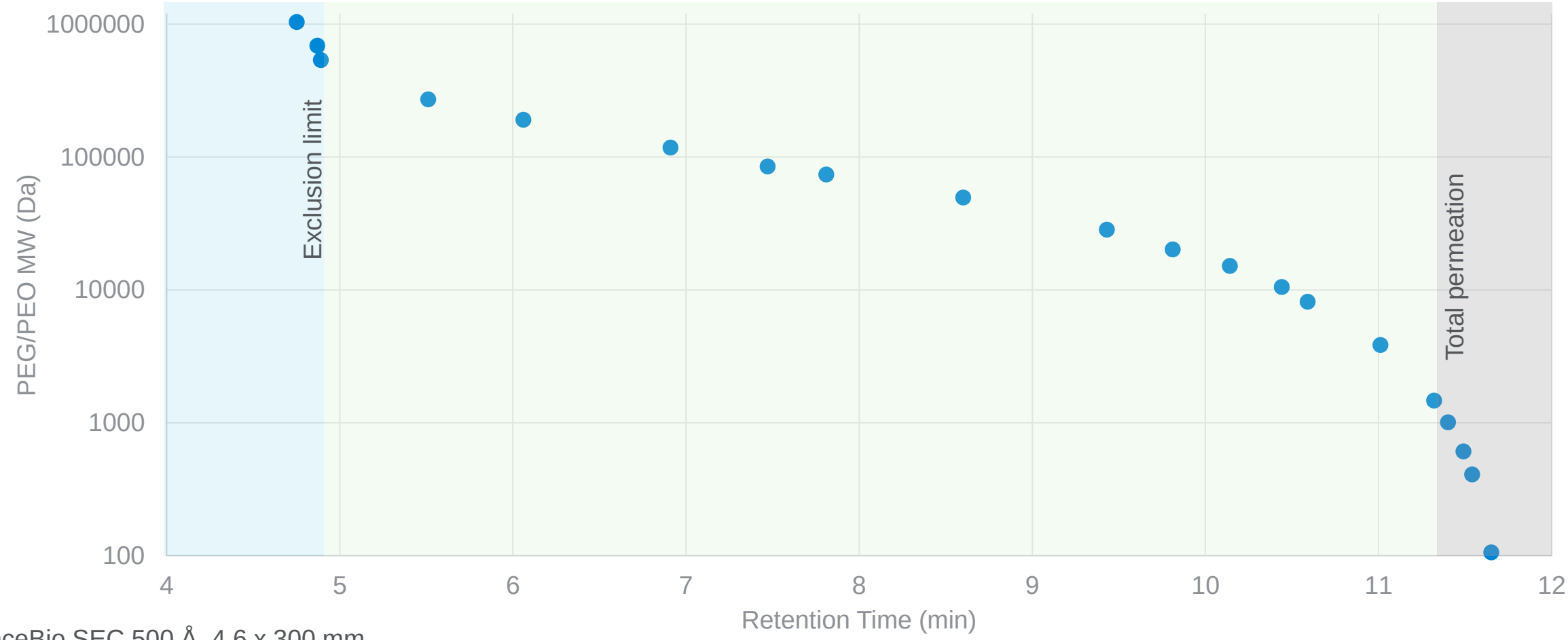


Application Note 5994-7934EN

Agilent

How to Calibrate Larger Pore Columns?

With Larger Standards

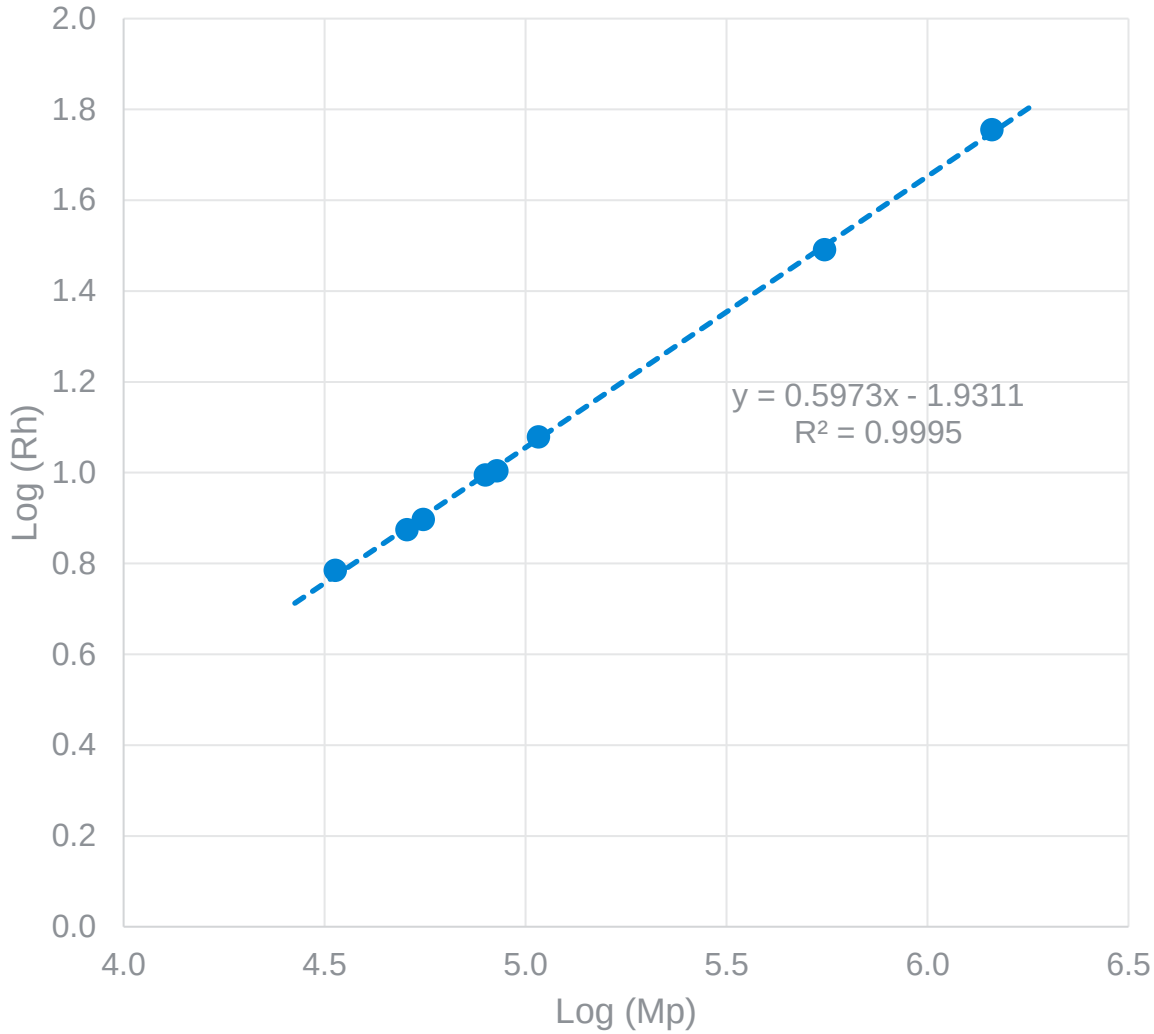


AdvanceBio SEC 500 Å, 4.6 x 300 mm
EasiVial PEG/PEO Standards
Flow rate 0.35 mL/min

Translating Separation Range to Analyte Size

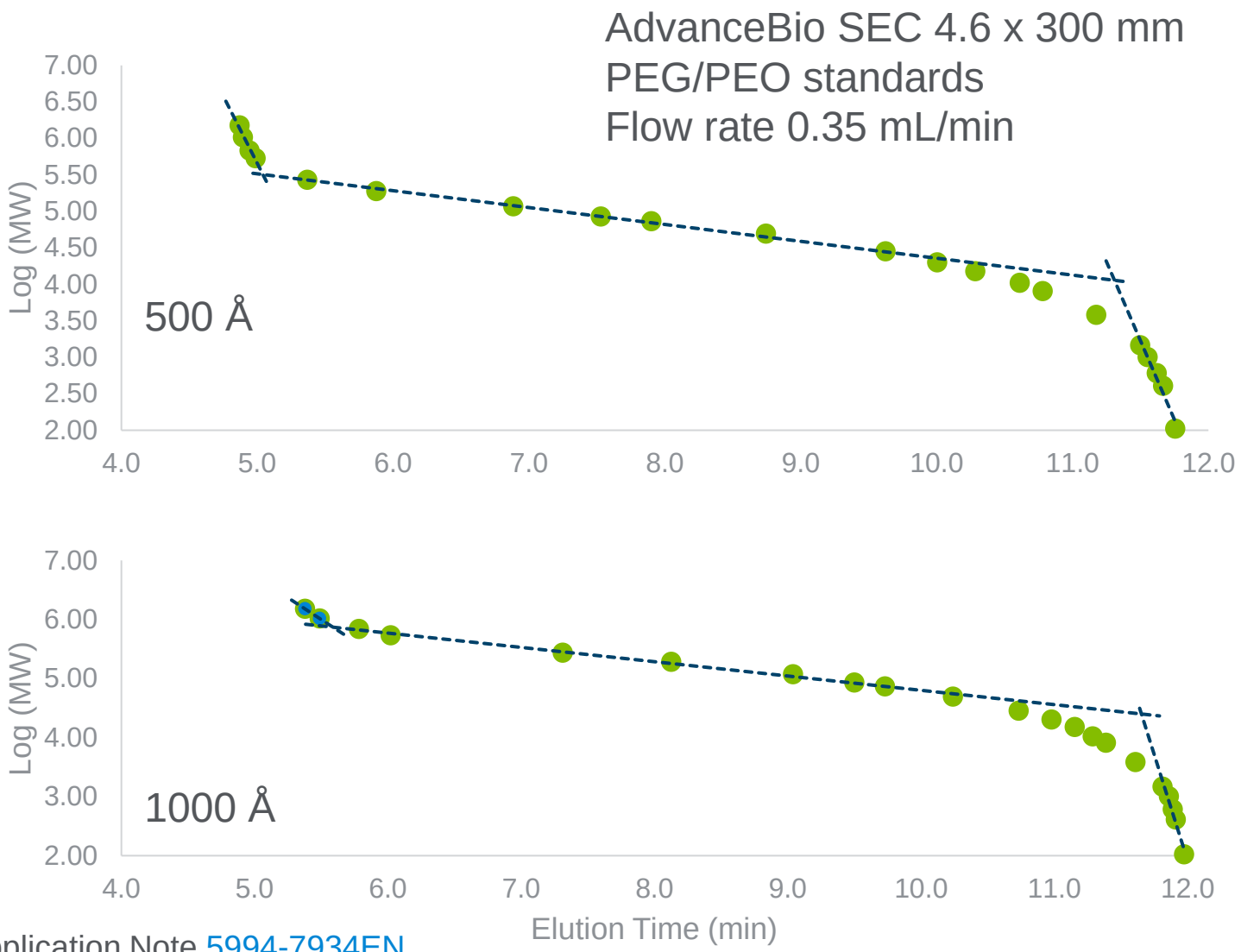
Triple detection was used to measure the hydrodynamic radius (Rh) of each polymer standard

Mp	Rh (nm)
1444836	57.0
554248	31.0
107606	12.0
84769	10.1
79353	9.9
55590	7.9
50680	7.5
33582	6.1



Application Note 5994-7934EN

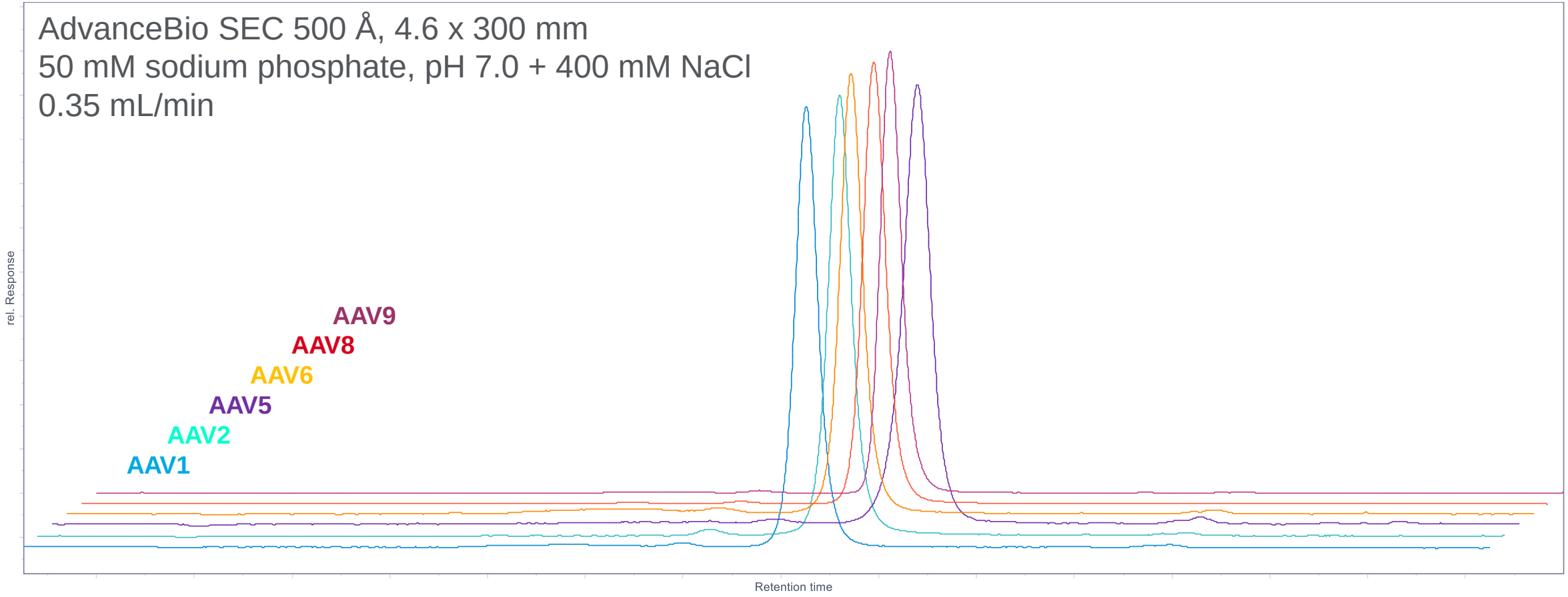
Defining Sample Size Ranges



	500 Å	1000 Å
Exclusion Limit		
Time	5.04	5.59
Log(MW)	5.50	5.87
MW	319500	739500
Rh (nm)	22.7	37.5
Analyte Diameter (Å)	455	751
Total Permeation Point		
Time	11.31	11.65
Log(MW)	4.05	4.40
MW	11250	25000
Rh (nm)	3.1	5.0
Analyte Diameter (Å)	62	99

Application Note 5994-7934EN

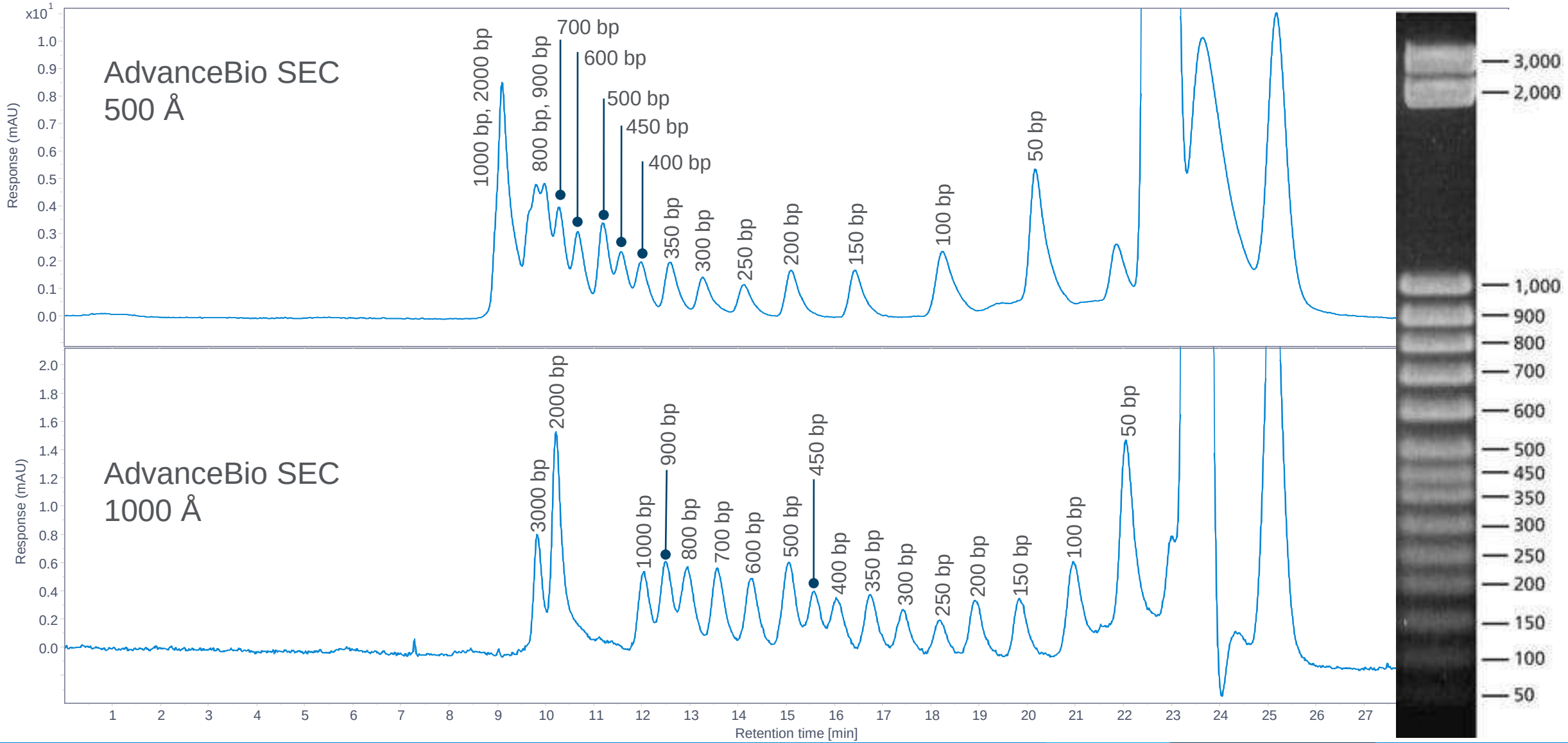
AAV Serotypes – Platform Method Potential



Application Notes 5994-7509EN and 5994-7918EN

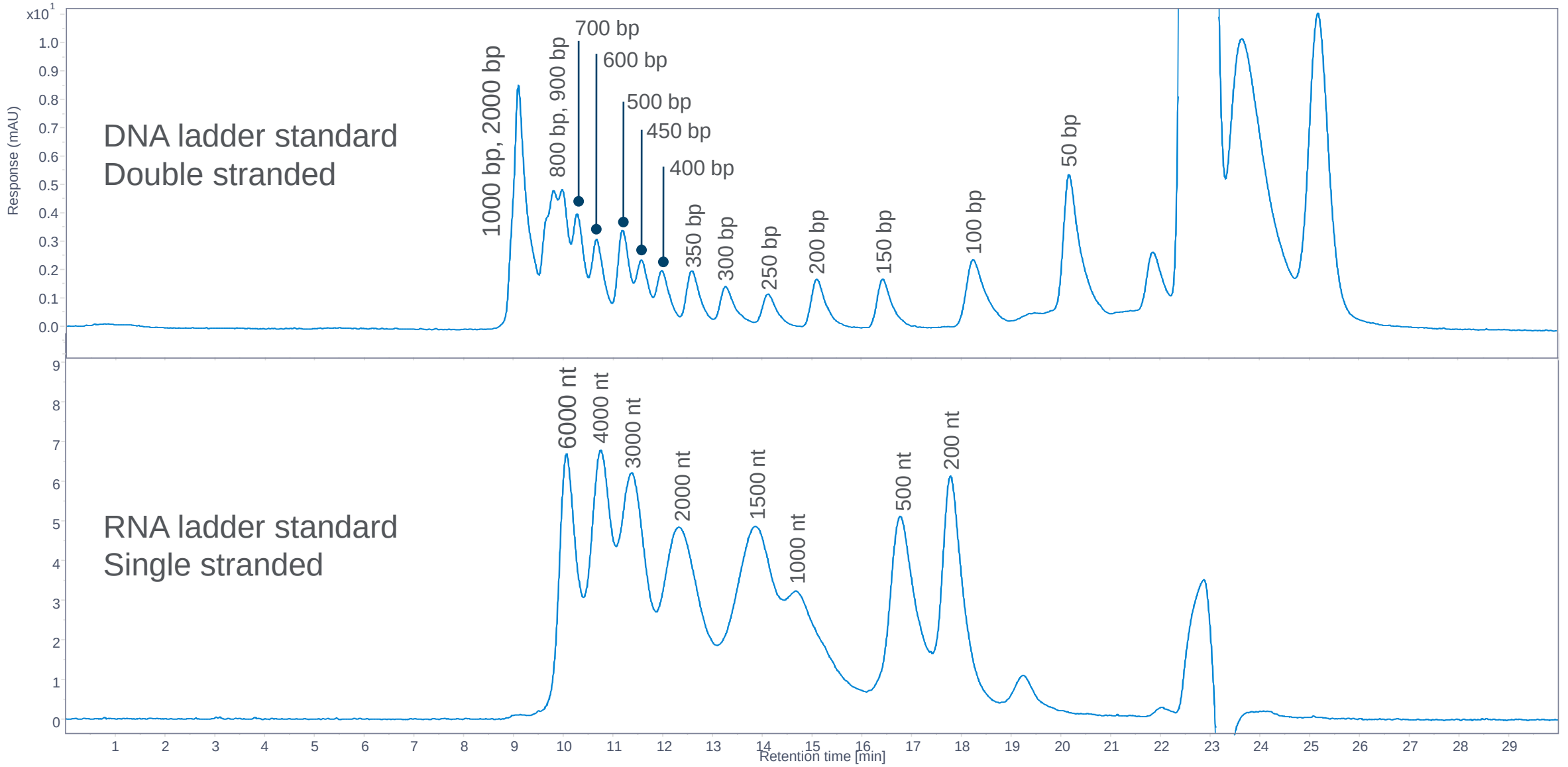
Size Exclusion Separations of Double Strand DNA

AdvanceBio SEC 4.6 x 300 mm
100 bp DNA standard
Mobile phase: 2X PBS
Flow rate 0.175 mL/min



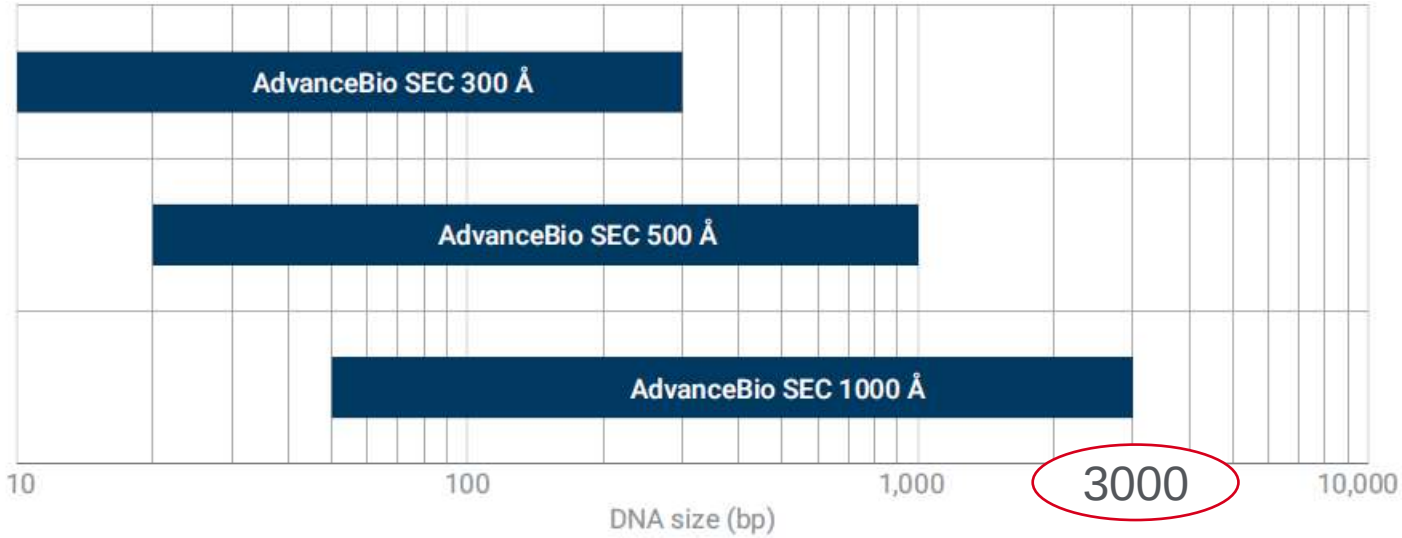
Single vs Double Strand Oligonucleotides

AdvanceBio SEC 500 Å 4.6 x 300 mm
Mobile phase: 2X PBS
Flow rate 0.175 mL/min

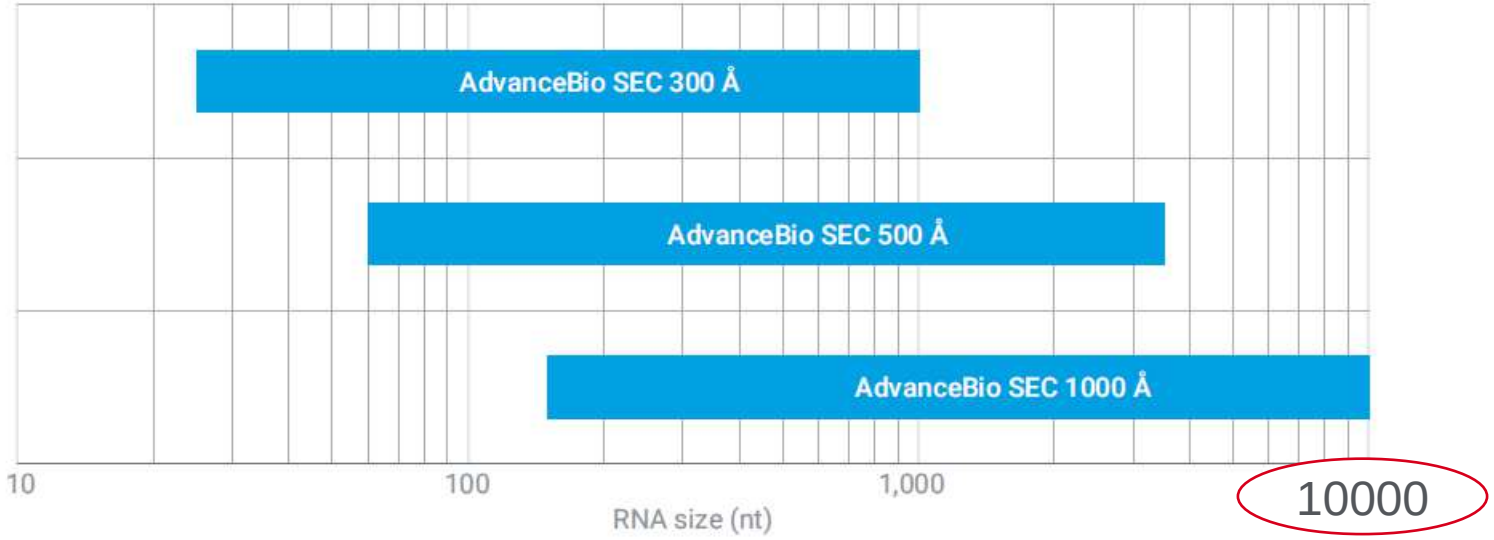


Pore Size Selection for Oligonucleotides

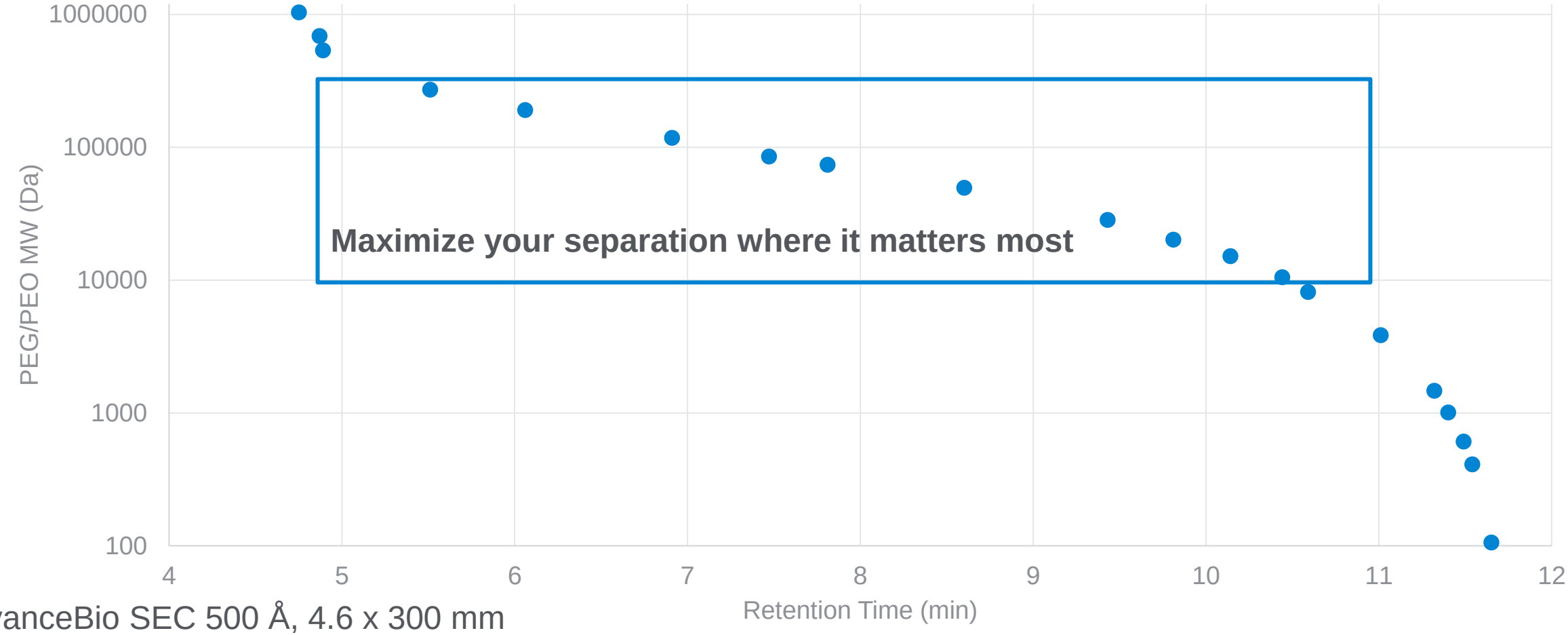
Doubled Stranded Oligonucleotides



Single Stranded Oligonucleotides

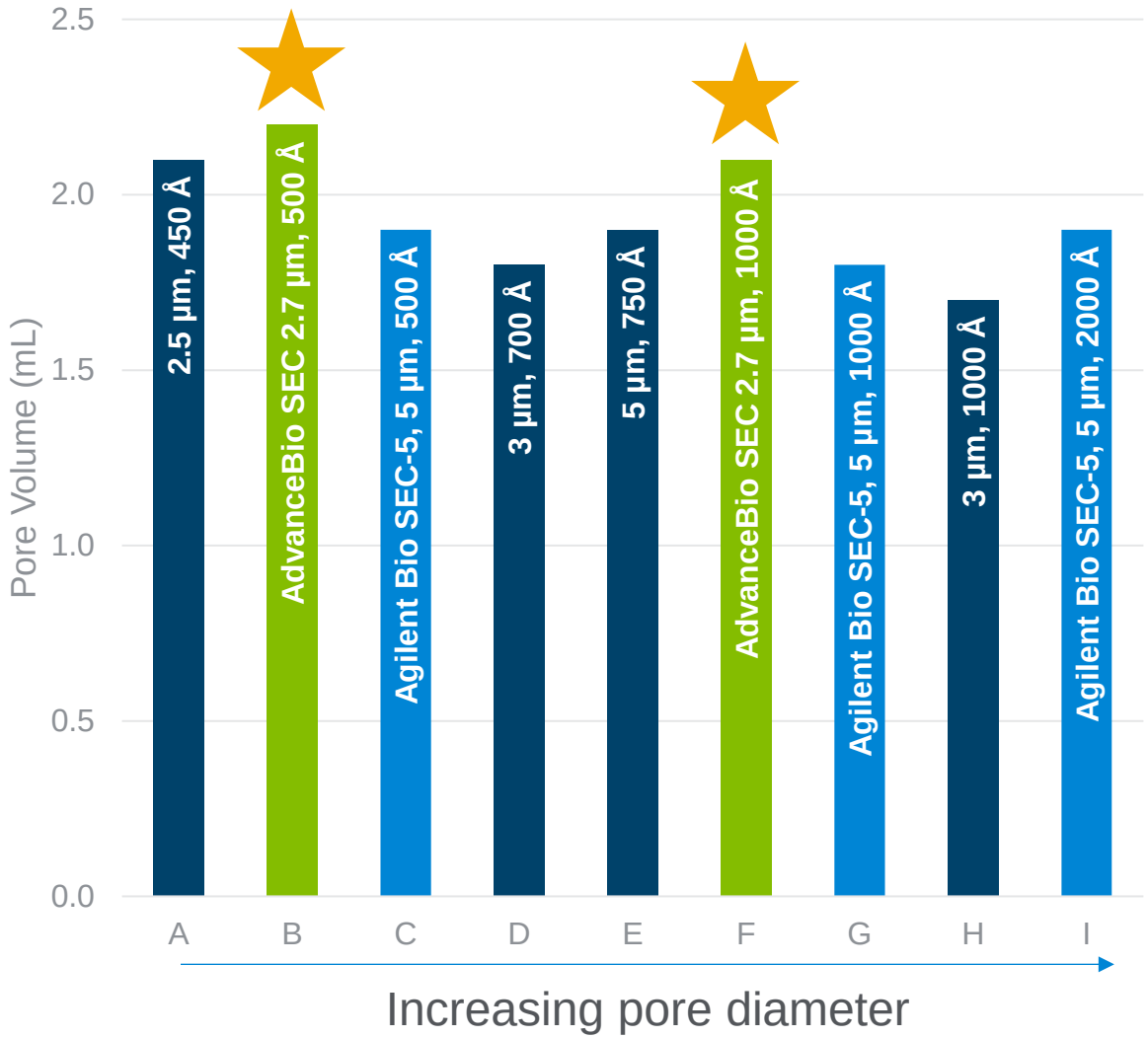


AdvanceBio SEC 500Å PEG/PEO calibration curve



AdvanceBio SEC 500 Å, 4.6 x 300 mm
PEG/PEO Standards
Flow rate 0.35 mL/min

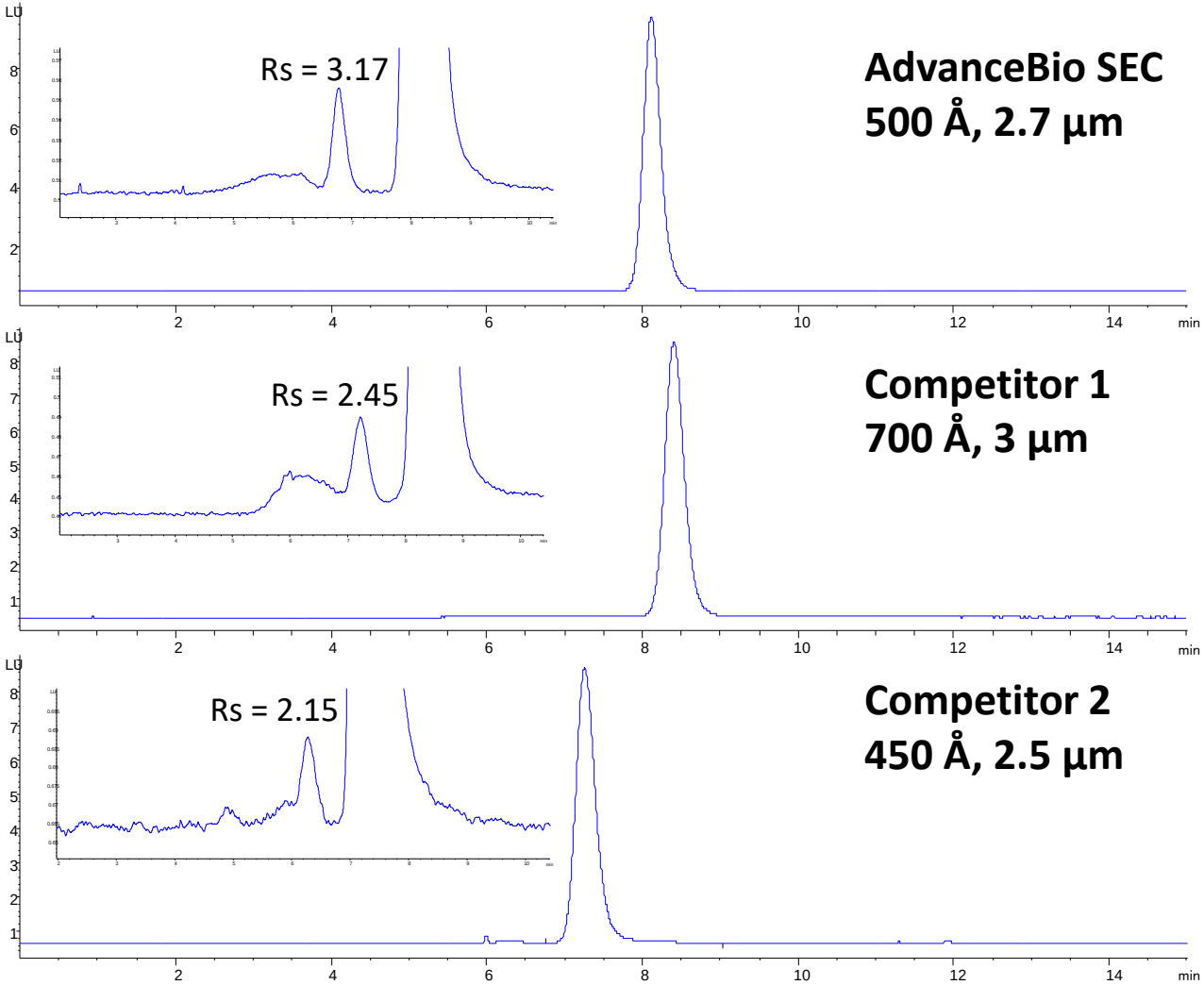
Pore Volume & Linear Region Slope



Application Note 5994-7934EN

High Resolution Viral Vector Separations

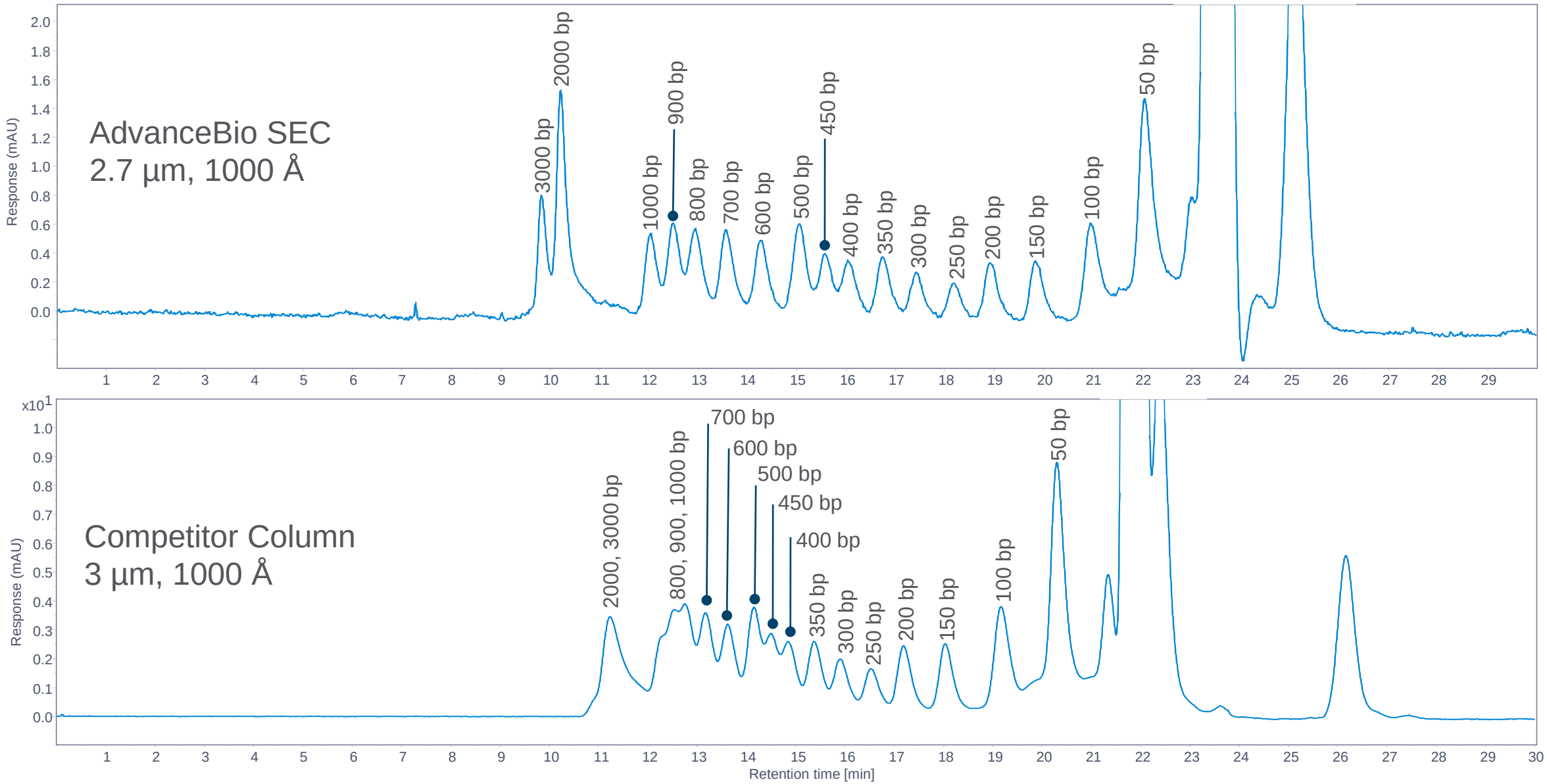
a) AAV-9



Application Note 5994-7918EN

Two 1000 Å Columns with Different Pore Volume

100 bp DNA standard
Mobile phase: 2X PBS
Flow rate 0.175 mL/min

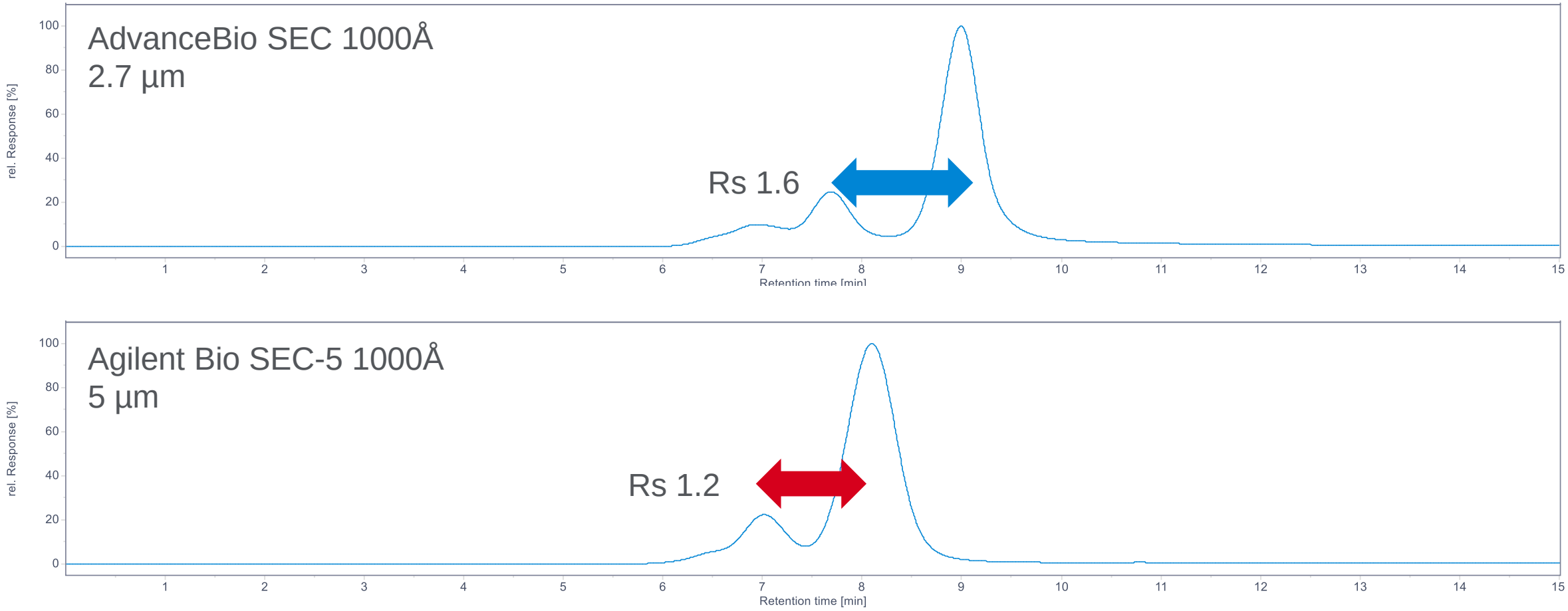


Column Properties for High Resolution

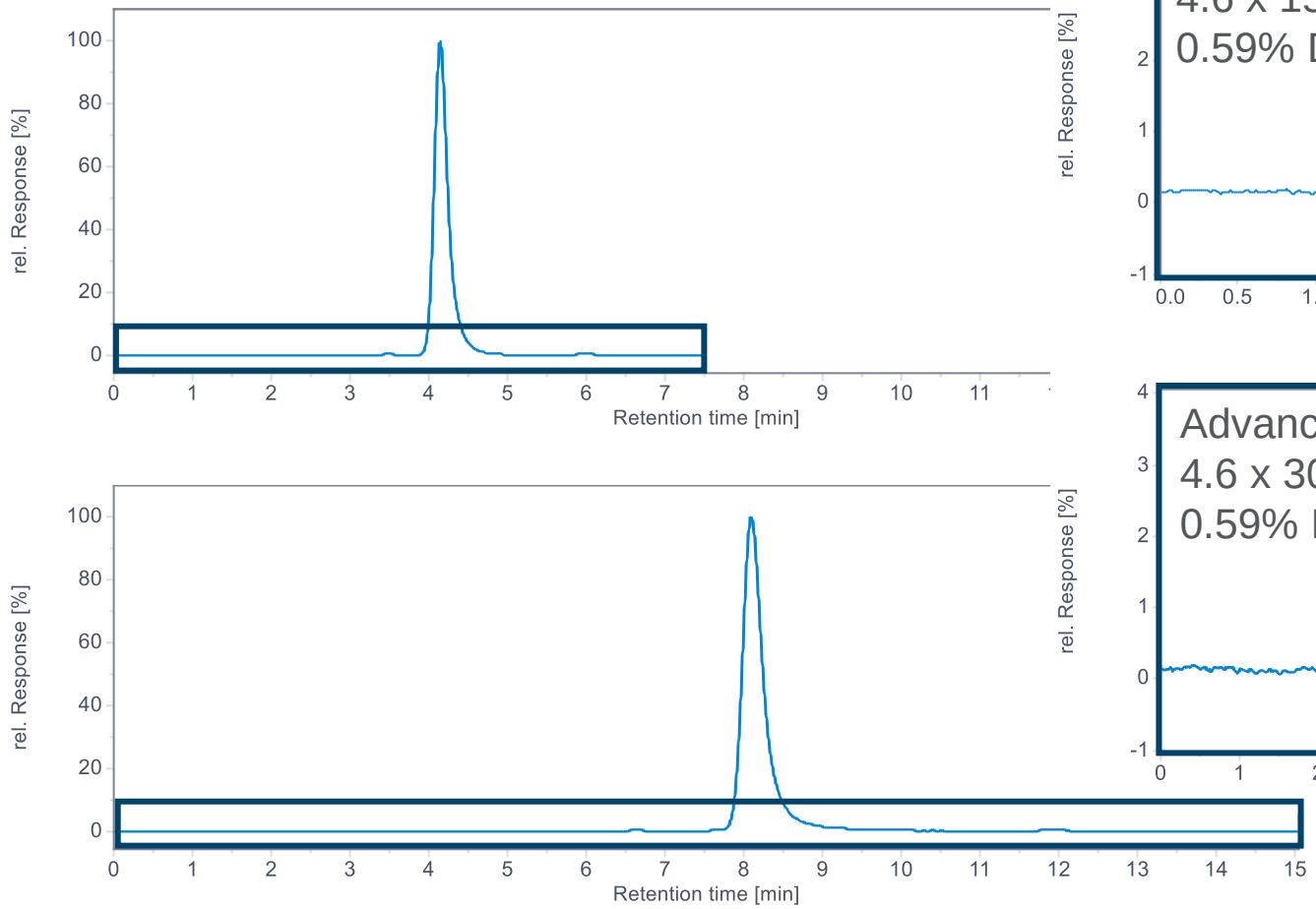
Smaller particles
High pore volume
Appropriate pore size

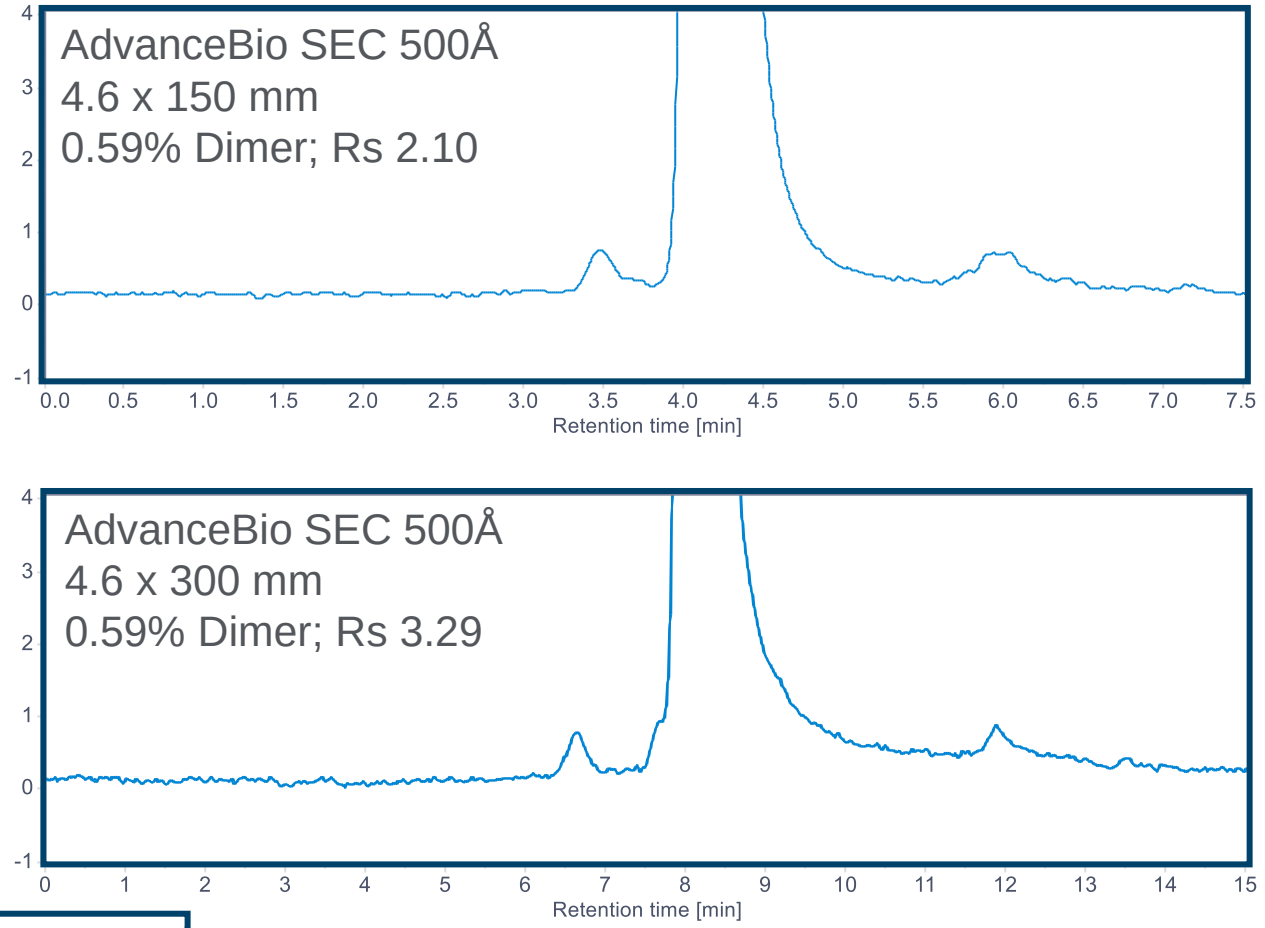
Impact of Particle Size

AdvanceBio SEC 1000Å vs. Agilent Bio SEC-5 1000Å – HPV-16



Higher Throughput Using Shorter Columns





Application Notes 5994-7509EN and 5994-7918EN

Method Conditions for High Resolution

How else can we improve resolution for very large analytes?

Flow rate!

Smaller particles
High pore volume
Appropriate pore size

Diffusion of Large Molecules & Impact on Resolution

- Large molecules diffuse *very* slowly
- Lower flow rates allow more time for analytes to diffuse in and out of pore structure
- Diffusion slow enough that band broadening is negligible
- Flow rate choice is a practical balance of resolution versus throughput

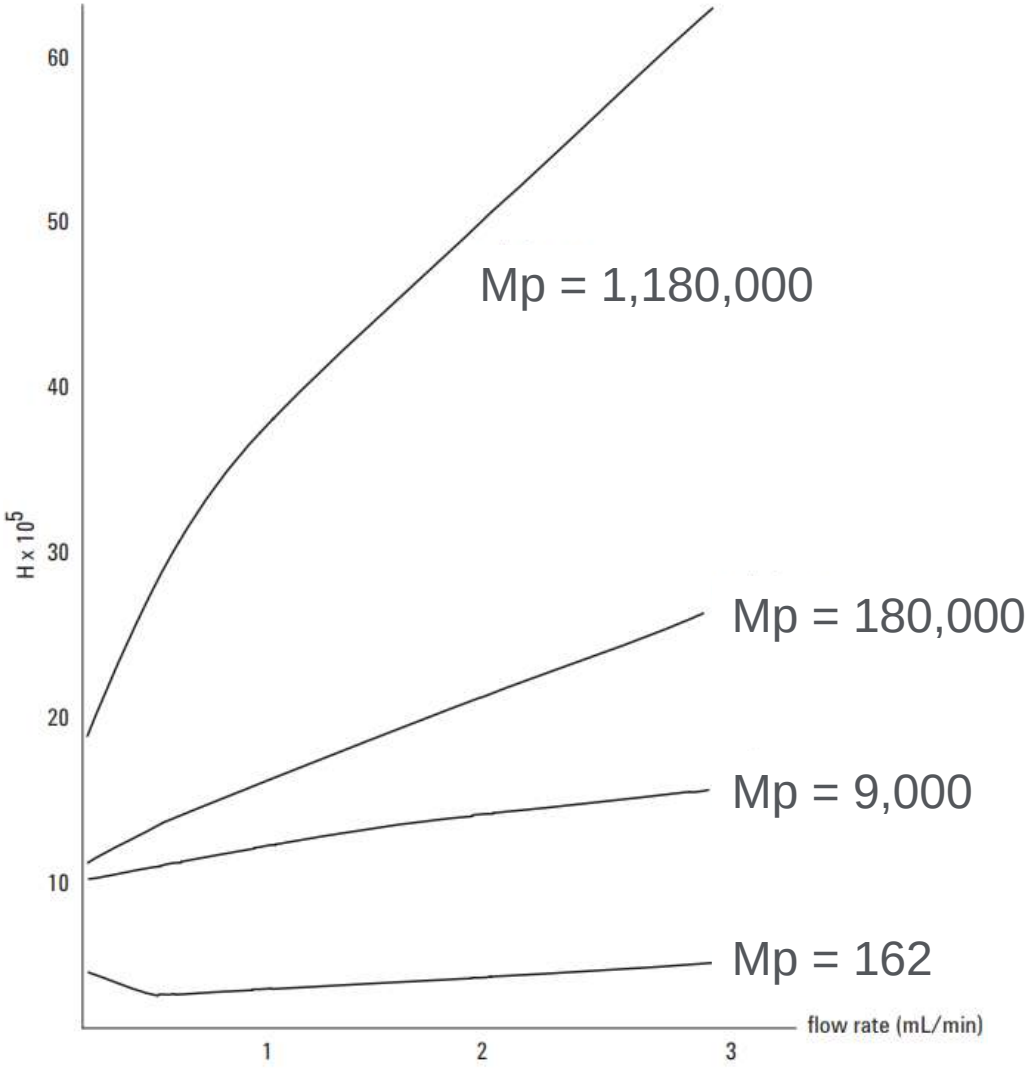


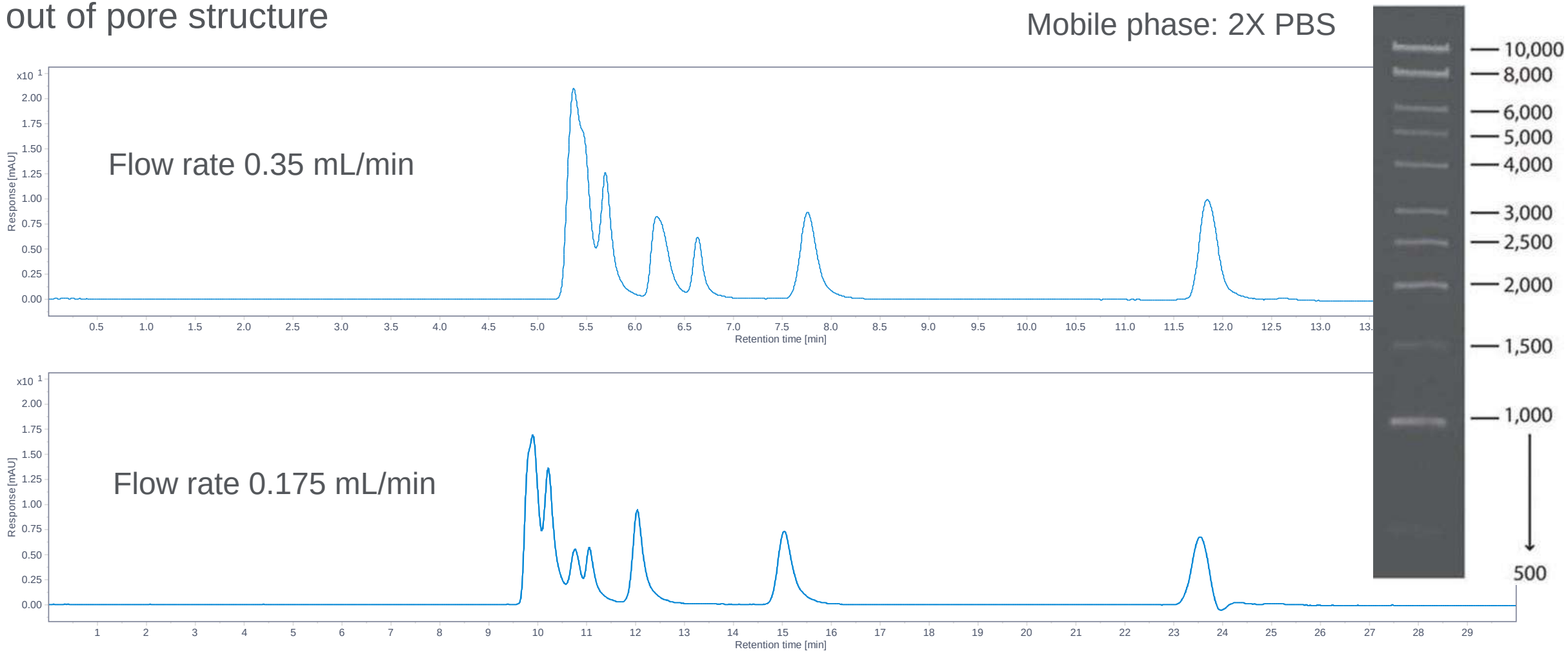
Figure 1. Van Deemter plots obtained using a PLgel MIXED-B column with probes having a range of molecular weight

Technical overview SI-01745

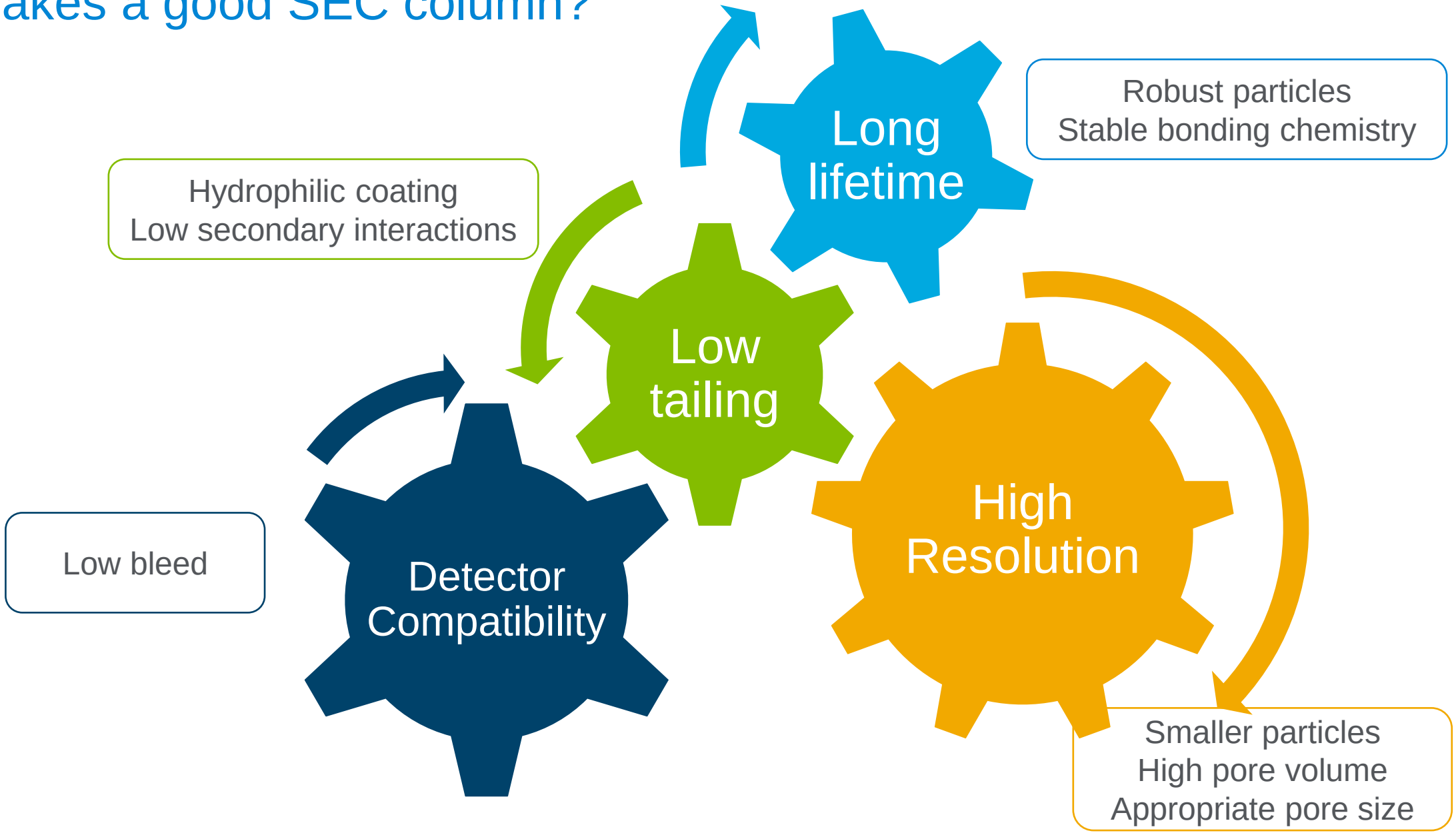
Slower Flow Rates Improve Resolution of Large Analytes

Slower flow allows more time for analytes to diffuse in and out of pore structure

AdvanceBio SEC 1000 Å, 4.6 x 300 mm
1 kb DNA standard
Mobile phase: 2X PBS



What makes a good SEC column?

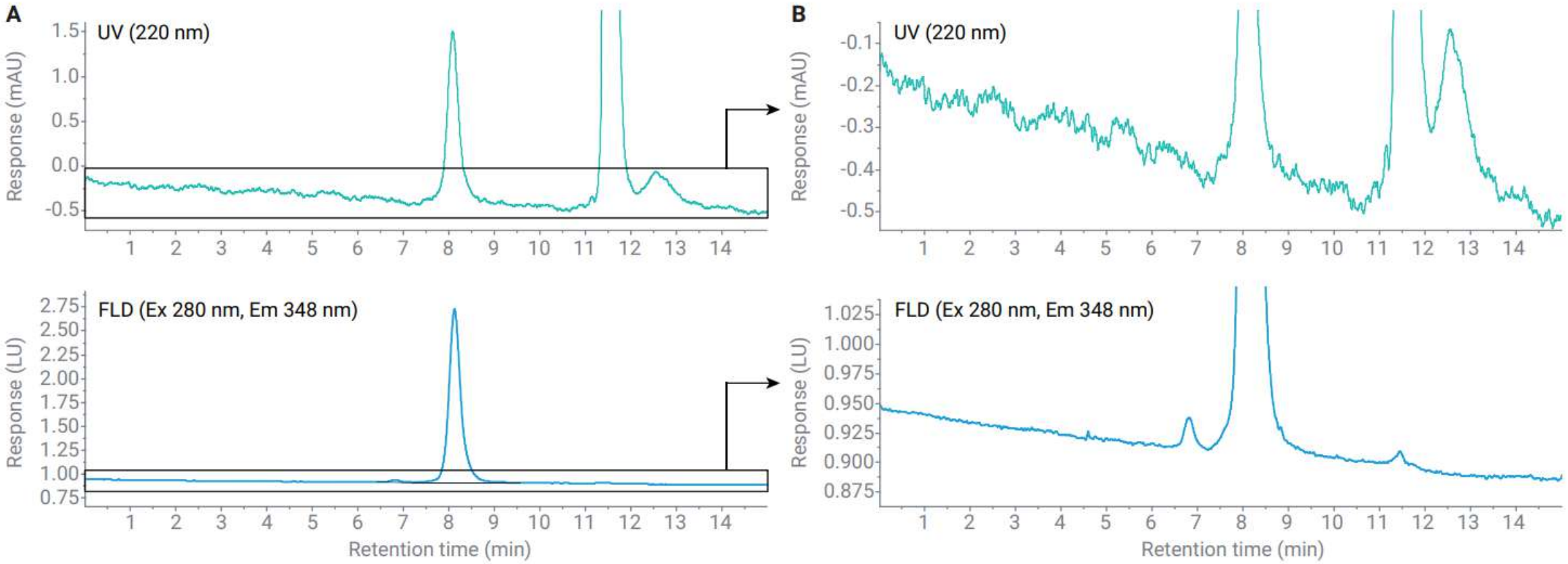


Detector-Related Considerations

- Detector selection
- Wavelength selection
- Low bleed columns
- Mobile phase preparation
- Column dimensions
- Flow rate

Detector Selection for Low Concentration Biotherapeutics

Fluorescence is more sensitive for AAVs



Column Dimension Selection

- Conventional flow rates with equivalent linear velocity:
  - 7.8 mm id – 1.0 mL/min
  - 4.6 mm id – 0.35 mL/min
- At a lower flow rate, sample spends more time in the detector flow cell, leading to higher sensitivity
- MALS methods often compensate the other direction – 7.8 mm id to accommodate larger injection volumes

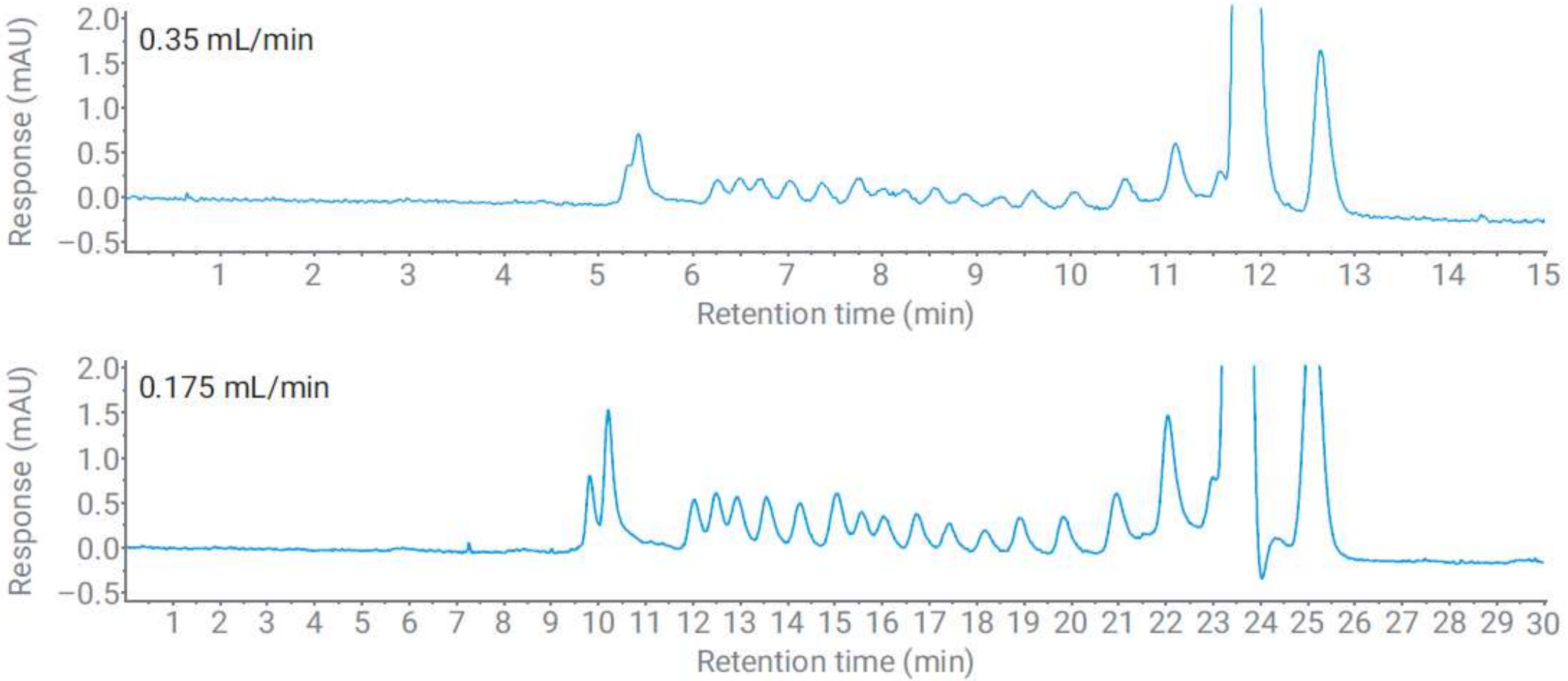
7.8 mm id – larger injection volume

4.6 mm id – smaller injection volume, higher UV/FLD sensitivity

300 mm length – higher resolution

150 mm length – higher throughput

Lower Flow Rate Can Improve Sensitivity

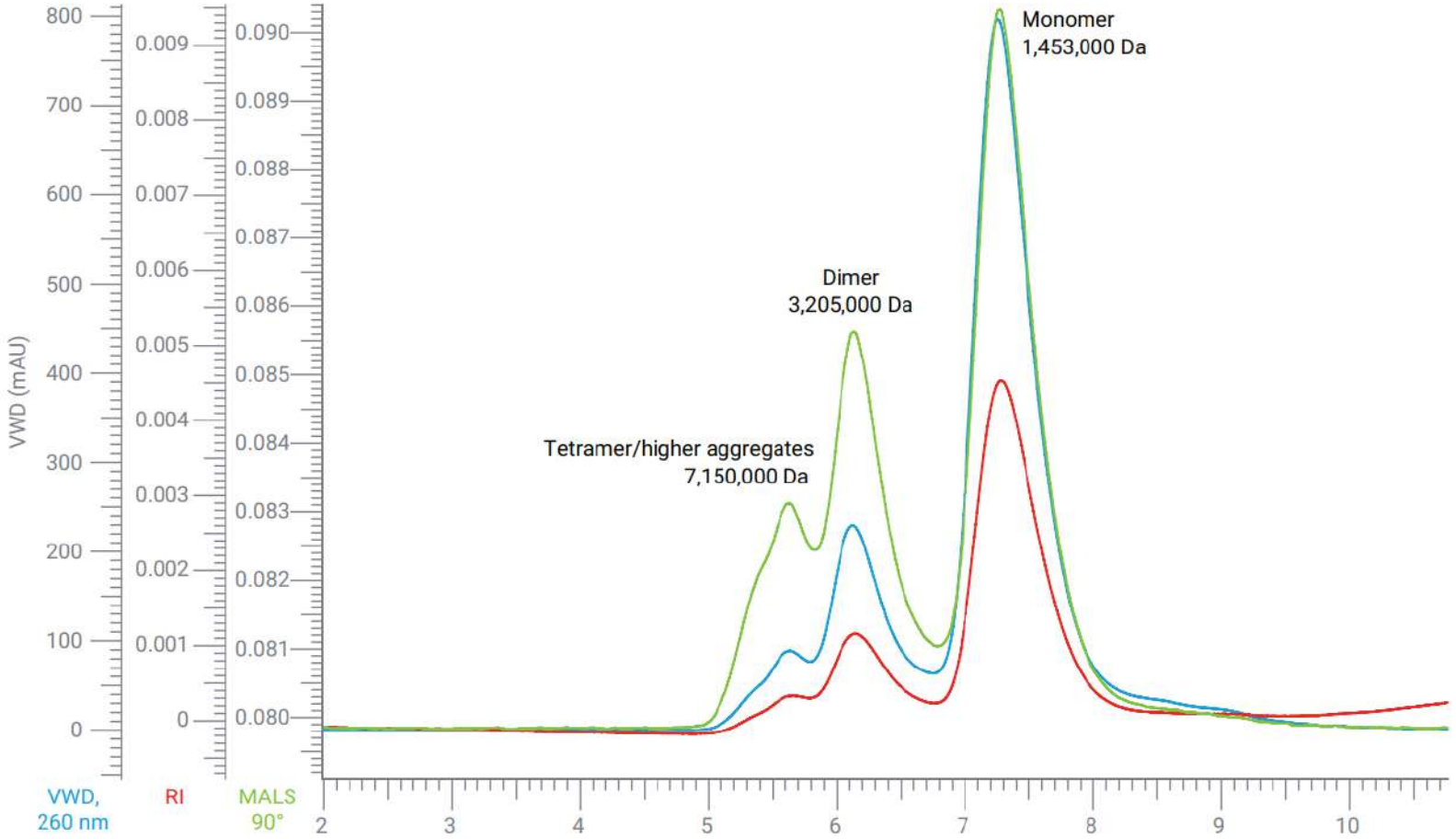


SEC-MALS Aggregate Analysis of mRNA

Key to light scattering is VERY clean background

- Low bleed columns
- Triple-filtered mobile phase

- 4.5 kb mRNA
- AdvanceBio SEC 2.7µm, 1000 Å, 7.8x300 mm
- Flow rate 0.6 mL/min
- Column temp 30 °C
- 150 mM phosphate buffer, pH 7



Application note [5994-7745EN]

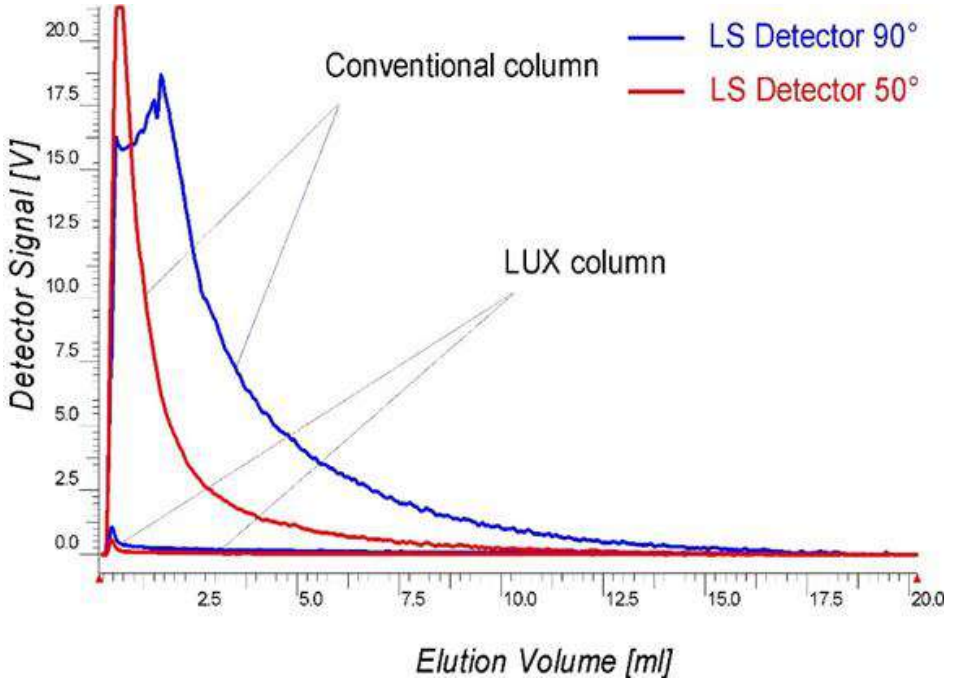
Background Noise Affects Sensitive Light Scattering Detectors

- The users can save themselves time by using columns that have been specially prepared for use with LS detectors

- Light scattering-ready PROTEEMA columns can be identified from the part number as they have LS at the end of the part number and are sometimes referred to as “Lux” columns, e.g.:
  - PROTEEMA **Lux**, 8 x 300 mm, 5 µm, 1000 Å p/n PRA0830051E3**LS**
- AdvanceBio SEC 500 Å and 1000 Å are ready for MALS use without extra conditioning

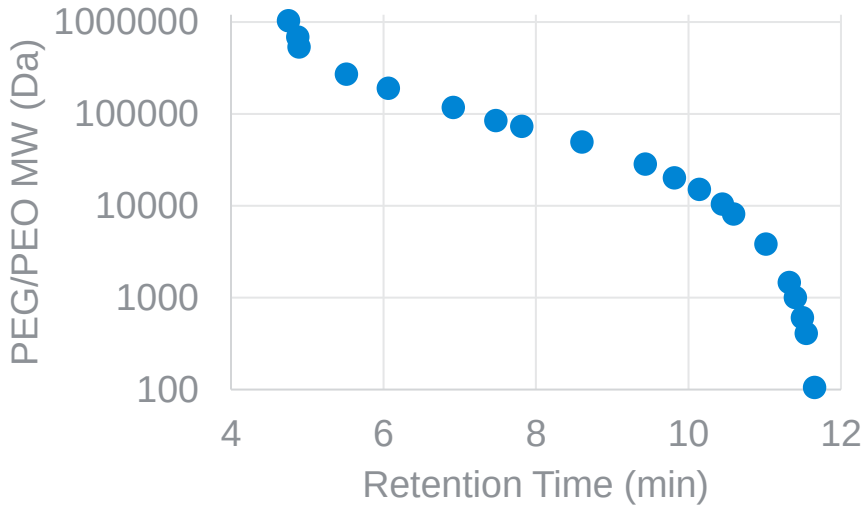
Detector noise caused from the bleeding of conventional columns

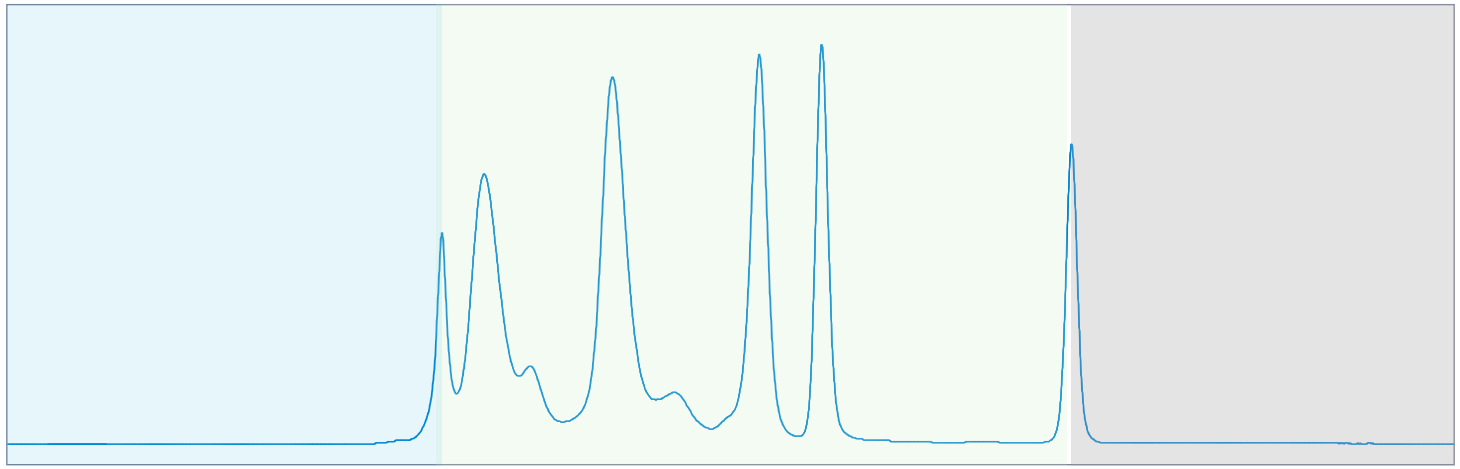
Special prepared LS or LUX columns shows a significant reduction of noise



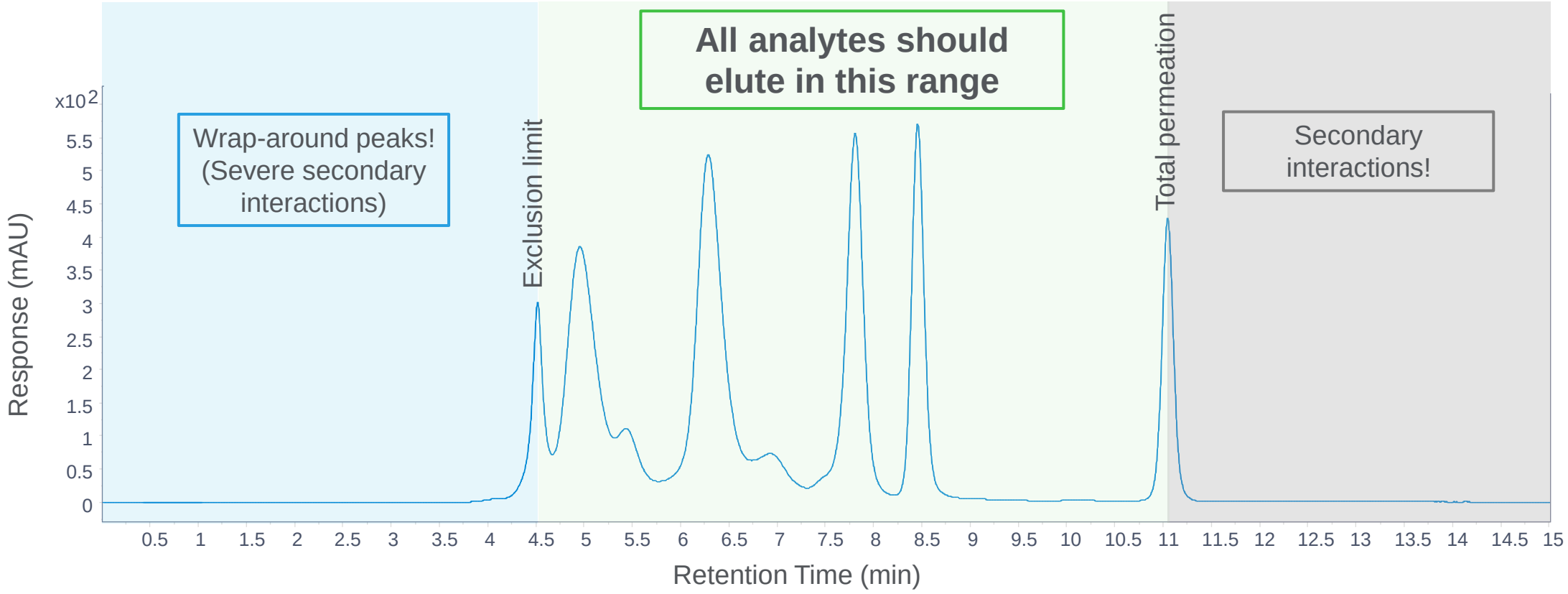
Best Practice – Use of Standards

- During method development
  - Sample(s) that mark the exclusion and total permeation points to define working range of that individual column
- At the beginning and end of each sample sequence
  - Monitor column performance over time
- Applies to all SEC separations, not just with wider pore columns



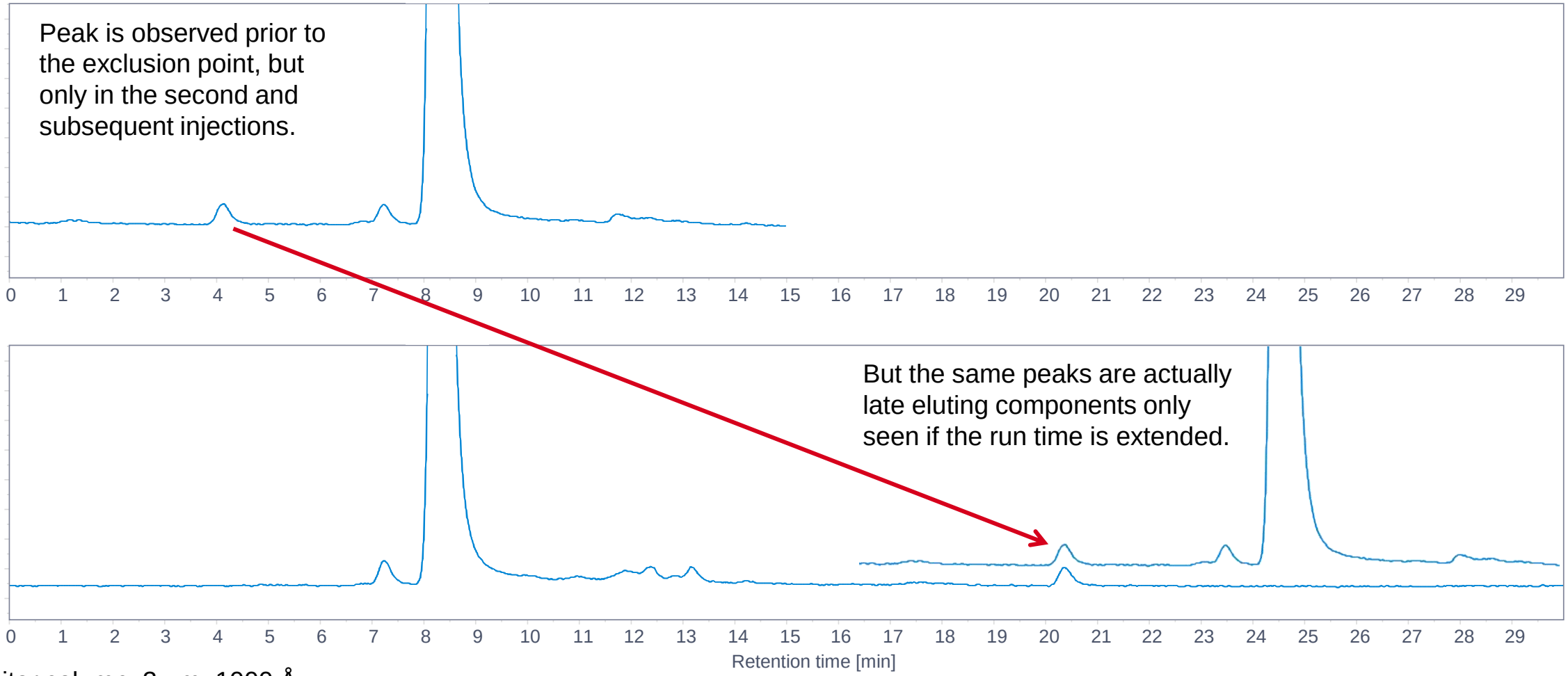


Best Practice – Use of Standards to Define Separation Window



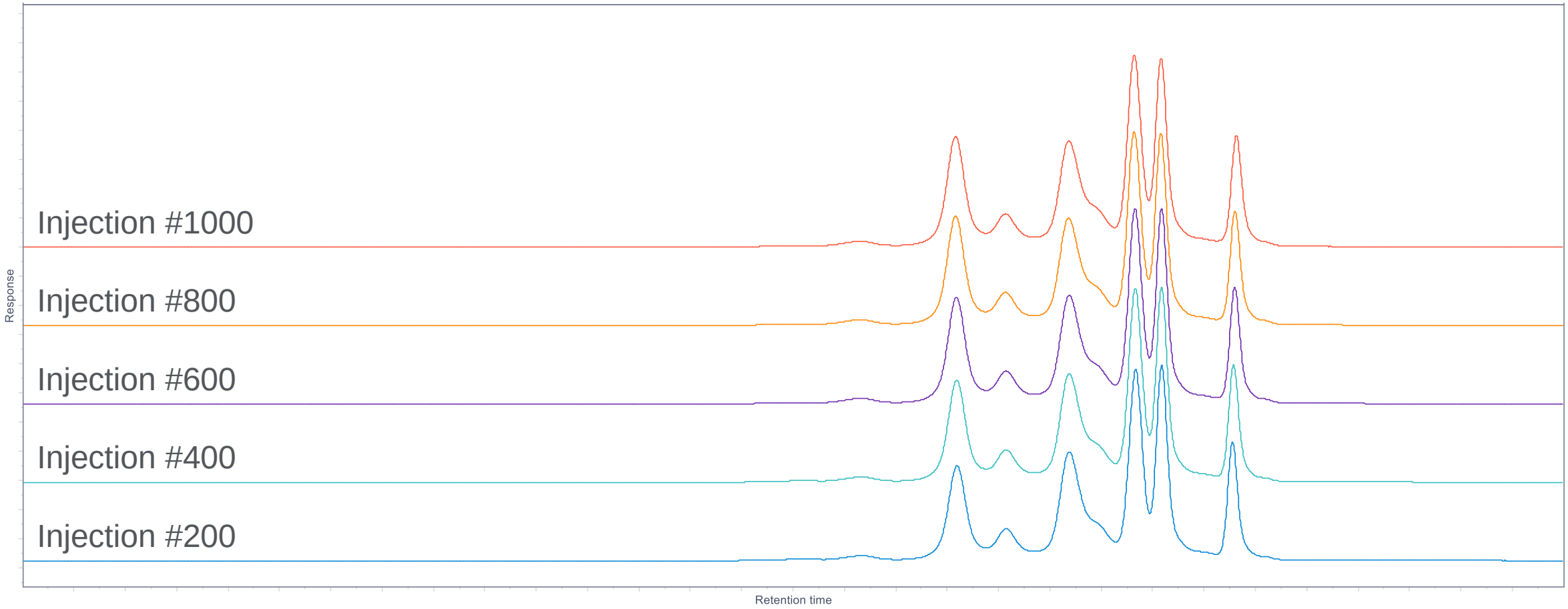
When Peaks Elute Before the Exclusion Limit

“Wrap around” peaks indicate severe secondary interactions



Competitor column, 3 µm, 1000 Å

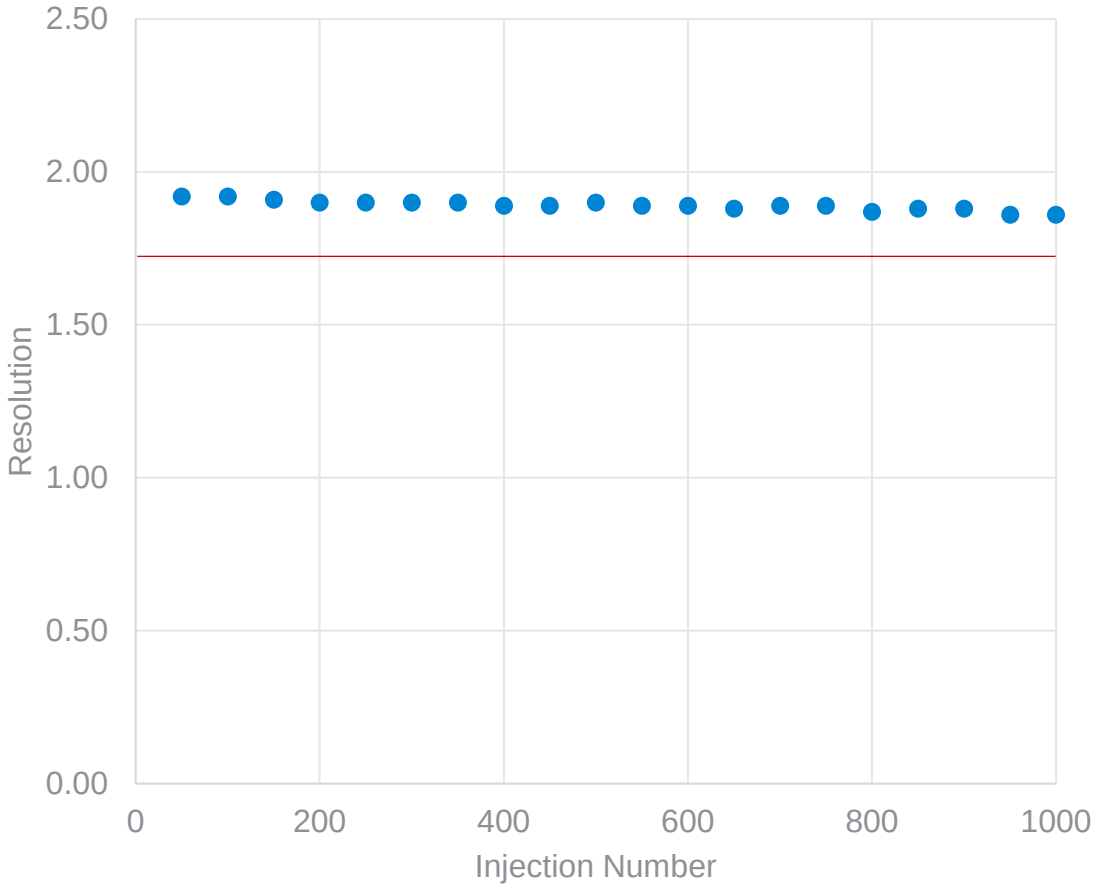
Best Practice – Use of Standards to Monitor Performance over Time



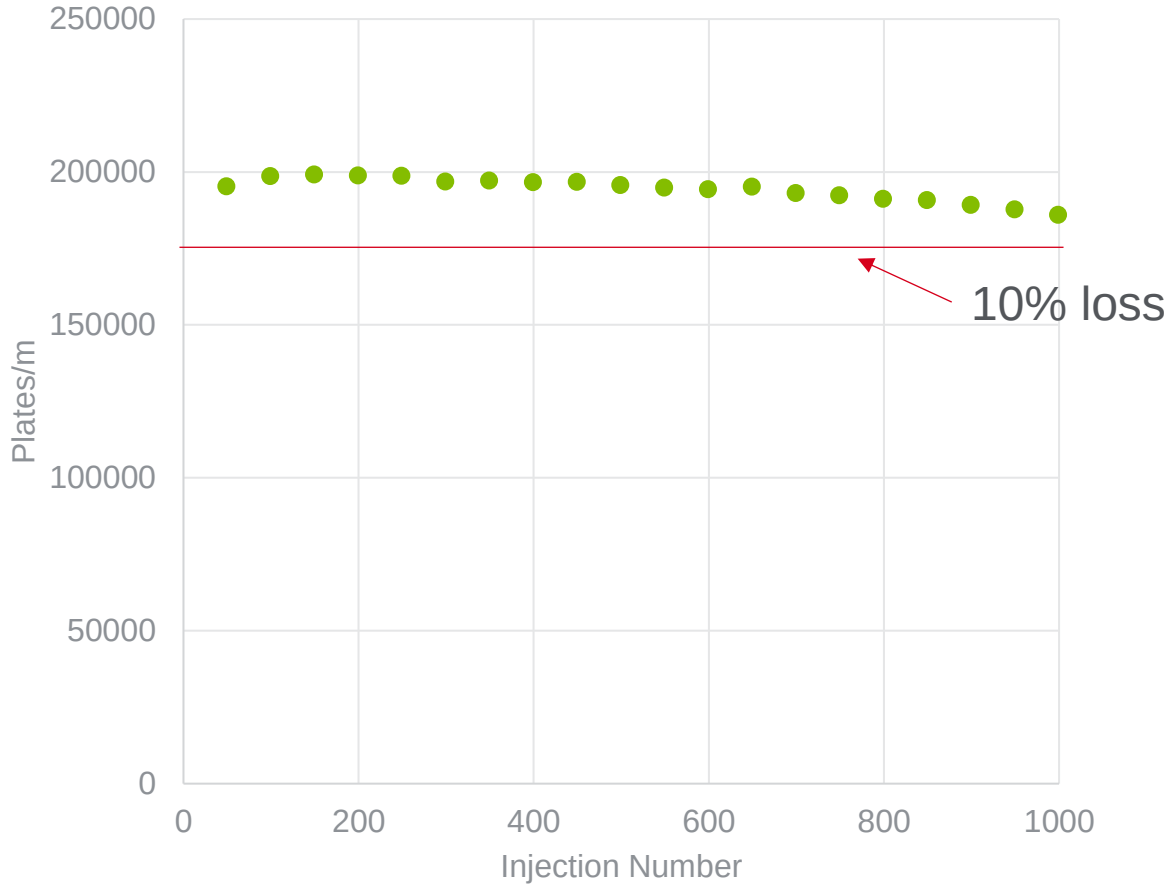
Pick a property to monitor – resolution, tailing, retention time shift, efficiency…

Recognize When Columns Need to Be Replaced

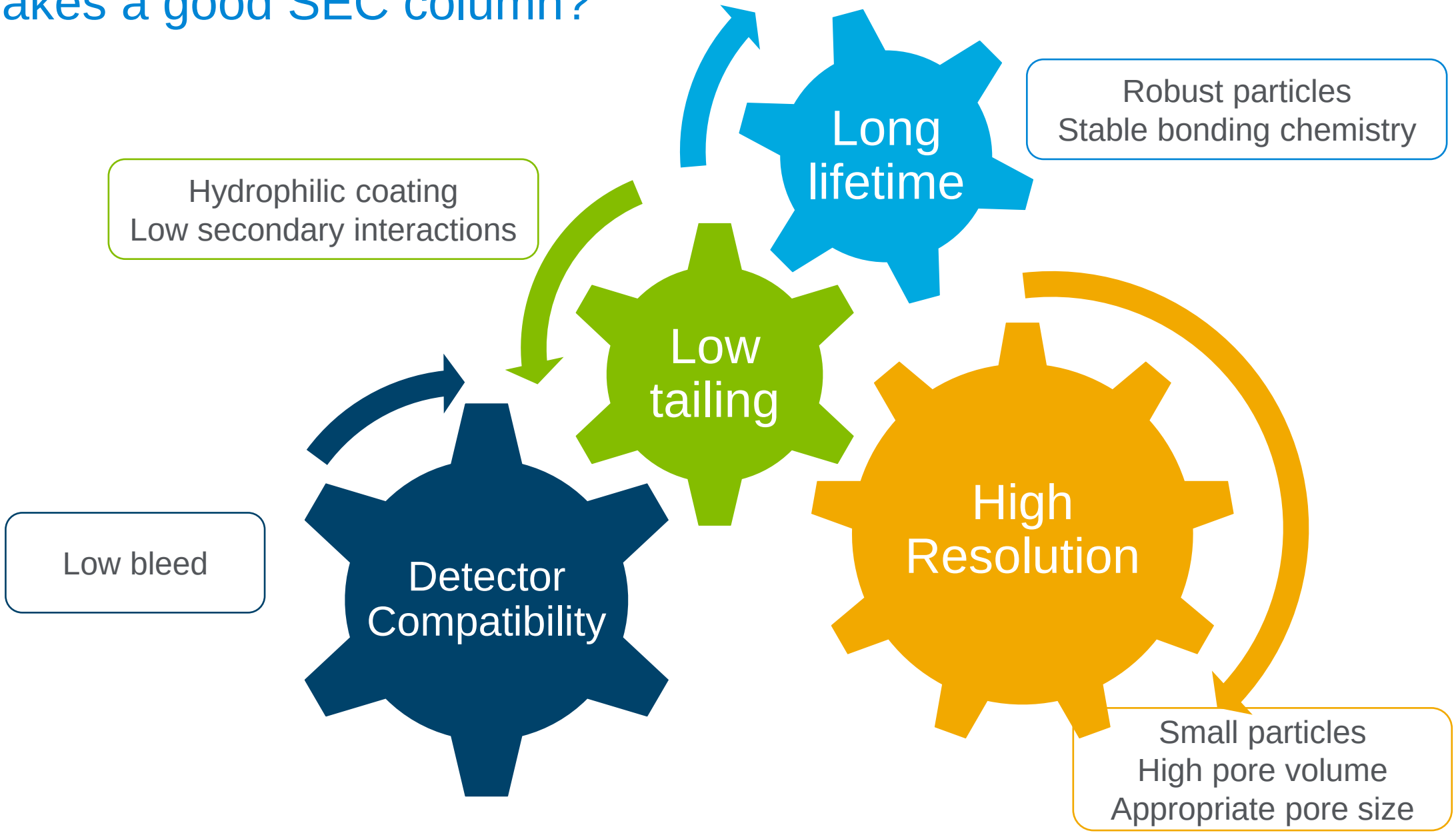
Resolution Thyroglobulin Monomer/Dimer



Uridine plates/m



What makes a good SEC column?



Agilent's GPC/SEC Column Portfolio

SDV	POLEFIN	GRAM	PolarSil	PFG
Neutral Polymers PVC Polycarbonate	High Temp GPC Polyethylene Polypropylene	Polyurethanes Starches Cellulosics	Formaldehyde resins	Polyester Nylons PET

SUPREMA	NOVEMA	MCX	PROTEEMA	
Neutral Polymers	Charged Polymers Cationic	Charged Polymers Anionic	Diol, USP L20 Proteins, Peptides	

PLgel	PLgel Olexis	PolarGel	PLMultisolvent	PL HFIPgel
PL Rapide **PlusPore** **PLgel LS** **PLgel MiniMix** Neutral Polymers	High Temp GPC Polyethylene Polypropylene	Polyurethanes Starches Cellulosics	Formaldehyde resins	Polyester Nylons PET

PL aquagel			AdvanceBio SEC	Bio SEC-5
Rapide Aqua Waters Soluble Polymers			130-1000 Å Peptides Proteins mAbs Oligos	2000 Å Ultra-large biotherapeutics

Organic Solvent Soluble Polymers | **Water Soluble Polymers** | **Biologics**

Agilent Biomolecule LC Columns Portfolio

Protein Therapeutics										Oligonucleotides		Capsid Therapeutics			
Titer Determination	**Aggregate Analysis**	**Intact Purity & PTM Analysis**		**Peptide Mapping & PTMs**	**Charge Variant Analysis**	**Glycan Analysis**	**Surfactant Analysis**	**Amino Acid / Cell Culture Media Analysis**		**Purification & Impurity Analysis**		**Aggregation**	**Empty/Full**	**Capsid Identity**	**Surfactant Analysis**
Affinity	Size Exclusion	Reversed Phase > 150 Å	HIC	Reversed Phase < 150 Å	Ion Exchange	HILIC	Reversed Phase > 150 Å	Reversed Phase < 150 Å	HILIC	Reversed Phase	Ion Exchange	Size Exclusion	Anion Exchange	Reversed Phase	Reversed Phase > 150 Å
Bio-Monolith rProtein A	AdvanceBio SEC 1.9 µm	PLRP-S 1000Å 5 µm	AdvanceBio HIC	AdvanceBio EC-C18	Bio mAb / Bio IEX 5 µm	AdvanceBio Amide HILIC	AdvanceBio Surfactant Profiling	AdvanceBio Amino Acid Analysis	AdvanceBio MS Spent Media	*AdvanceBio Oligo	*PL-SAX	AdvanceBio SEC 2.7 µm 500 & 1000Å	Bio SAX	ZORBAX RRHD 300Å, 1.8 µm	AdvanceBio Surfactant Profiling
Bio-Monolith Protein A	AdvanceBio SEC	PLRP-S*		AdvanceBio Peptide Mapping	Bio mAb (WCX)	AdvanceBio Glycan Mapping		ZORBAX Eclipse AAA 3.5 µm		*PLRP-S	Bio SAX	Bio SEC-5 2000 Å	Bio SAX	AdvanceBio Peptide Mapping	
Bio-Monolith Protein G	*Bio SEC-5	AdvanceBio RP mAb 450Å		AdvanceBio Peptide Plus	Bio IEX (SAX, WAX, SCX, WCX)						*Bio SAX				
	*Bio SEC-3	ZORBAX RRHD 300Å, 1.8 µm		ZORBAX RRHD 300Å, 1.8 µm	*PL SCX, SAX			**Biomolecule Sample Preparation**							
	PROTEEMA	*ZORBAX 300SB 3.5, 5 & 7 µm			Bio-Monolith (QA, DEAE, SO3)			Desalting & Buffer Exchange	Released N-Glycan						
		Poroshell 300 5 µm						AdvanceBio Spin	AdvanceBio Gly-X						
									AdvanceBio Sialic Acid			*Scalable to prep	Stainless steel (SS) column hardware	Solid PEEK or PEEK lined SS bioinert column hardware	

Two Key Resources

Product user guides:

www.agilent.com/chem/bioguides

Agilent AdvanceBio SEC Columns

Size exclusion columns for analysis of biomolecules

Operating parameters

Parameter	Value
Mobile Phase Compatibility	Aqueous buffers with high and low salt can be used. Mixtures of water and organic solvent can be used **with careful attention to solubility of buffer components and system pressure**.
pH Stability	2 to 8.5
Operating Temperature	20 to 30 °C (recommended), 80 °C (maximum)
Maximum Pressure	400 bar (5,800 psi) for 2.7 µm columns 620 bar (9,000 psi) for 1.9 µm columns
Recommended Flow Rates	0.1 to 2.0 mL/min for 7.8 mm id columns 0.1 to 0.7 mL/min for 4.6 mm id columns 0.05 to 0.1 mL/min for 2.1 mm id columns For two columns in series, lower flow rates may be necessary to ensure that maximum pressure is not exceeded.
Injection Volume	1 to 10 µL (recommended) Maximum 1% column volume

Note: Working at extremes of the operating parameters may reduce column lifetime.

SEC Frequently Asked Questions

www.agilent.com/chem/advancebio-sec-faqs

- \+ What is SEC?
- \+ What can I separate by SEC?
- \+ What is an SEC column?
- \+ What makes a good SEC column?