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Robust and Simple Workflows in Therapeutic Protein Characterization

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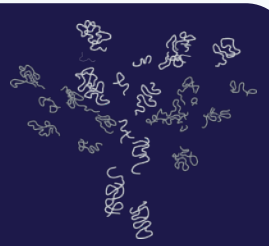
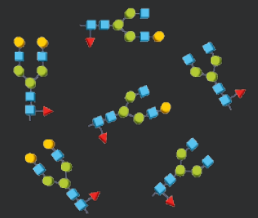
Thermo Fisher Scientific, Germering/Germany

Fulfilling the Needs of Biotherapeutic Characterization

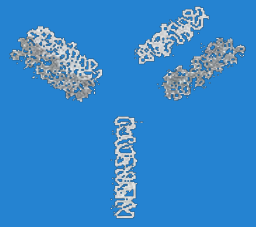
Peptide mapping



Released glycan analysis



Sub-unit analysis



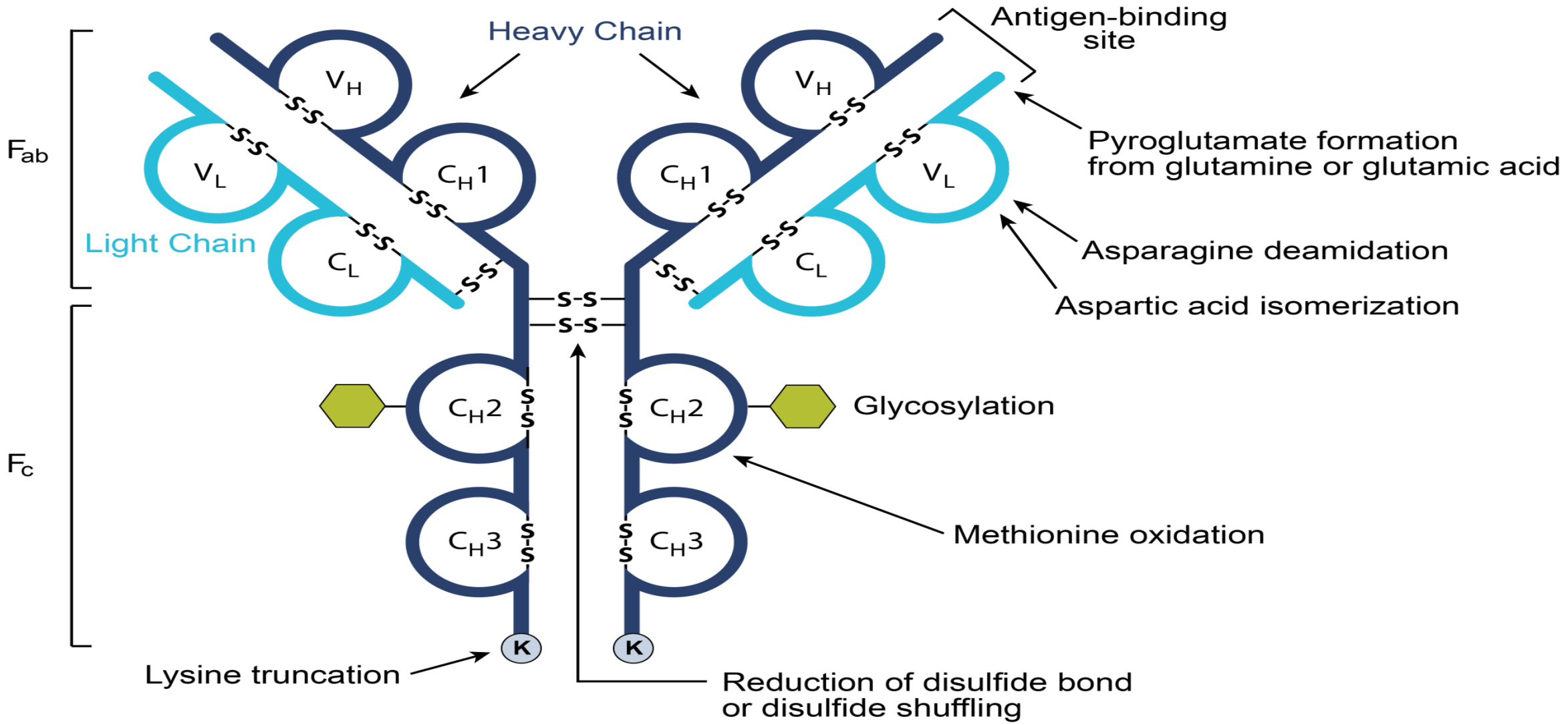
Intact analysis

Charge variant screening

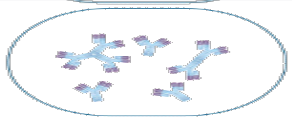
Aggregate screening

Glycan analysis

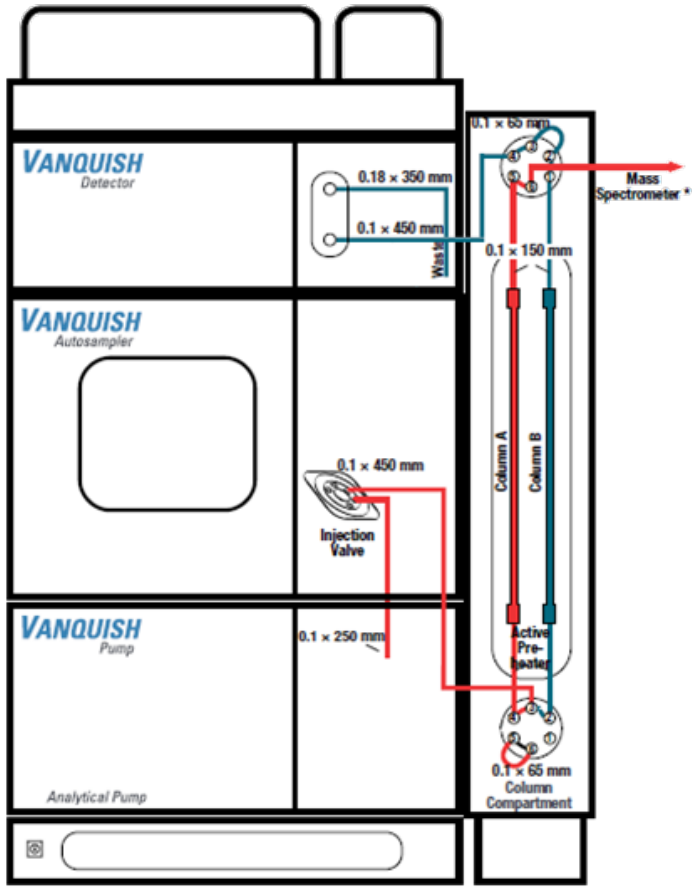
Structure of IgG and Typical Forms of Heterogeneity

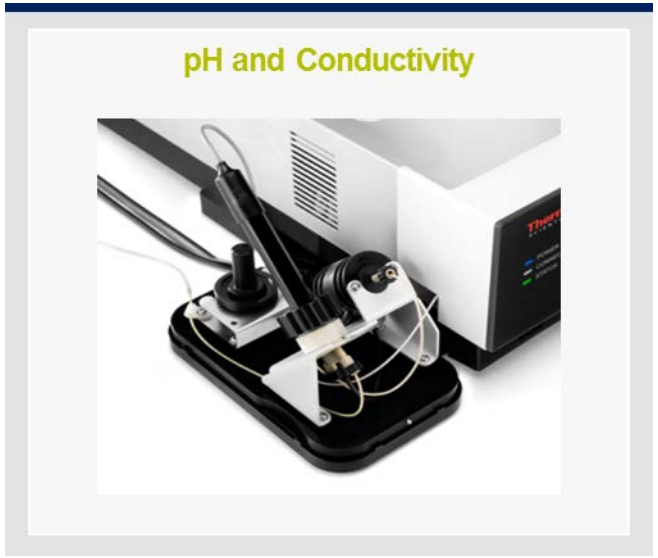


BioColumn Selection Guide

Analysis		Description	Columns and buffers	Detection
Titer		mAb capture, titer and screening	Thermo Scientific™ MAbPac™ Protein A	UV
Aggregate		Routine screening for aggregates and fragments	Thermo Scientific™ MAbPac™ SEC-1	UV and light scattering
Charge heterogeneity		Routine variant profiling including; lysine truncation, deamidation and acylation	Thermo Scientific™ MAbPac™ SCX-10 Thermo Scientific™ MAbPac™ SCX-10 RS Thermo Scientific™ ProPac™ WCX-10 Thermo Scientific™ CX-1 pH Gradient Buffer Kit	UV
Methionine and Tryptophan oxidation		Targeted analysis of methionine and tryptophan oxidation	Thermo Scientific™ MAbPac™ HIC-20 Thermo Scientific™ MAbPac™ HIC-10 Thermo Scientific™ ProPac™ HIC-10	UV
Antibody drug conjugate (ADC)		Drug to Antibody ratios	Thermo Scientific™ MAbPac™ HIC-10 Butyl Thermo Scientific™ MAbPac™ HIC-20 Thermo Scientific™ MAbPac™ HIC-10 Thermo Scientific™ MAbPac™ RP	UV
Antibody drug conjugate (ADC) using MS		Drug to Antibody ratios and intact mass	Thermo Scientific™ MAbPac™ SEC-1 Thermo Scientific™ MAbPac™ RP Thermo Scientific™ Acclaim™ SEC-300	
Intact or fragment mass		Intact, light (LC), heavy chain (HC) and fragment (Fab & Fc) analysis	Thermo Scientific™ MAbPac™ RP	UV and MS
Native mass		Intact native mass analysis	Thermo Scientific™ MAbPac™ SEC-1 Thermo Scientific™ Acclaim™ SEC-300 Thermo Scientific™ MAbPac™ SCX-10 RS	UV and MS

Thermo Scientific™ Vanquish™ UHPLC Platform for Bio-Therapeutic Characterization





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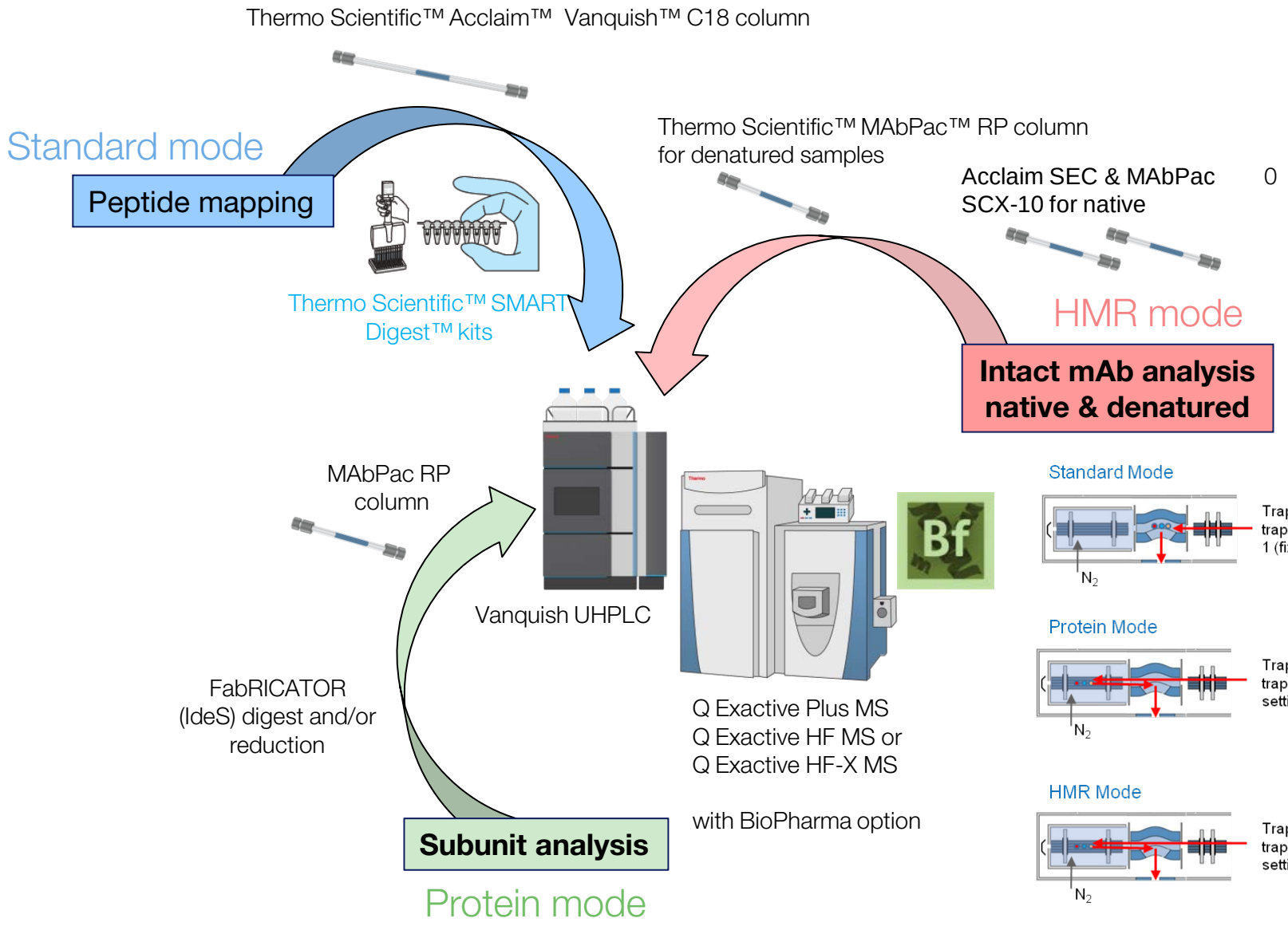
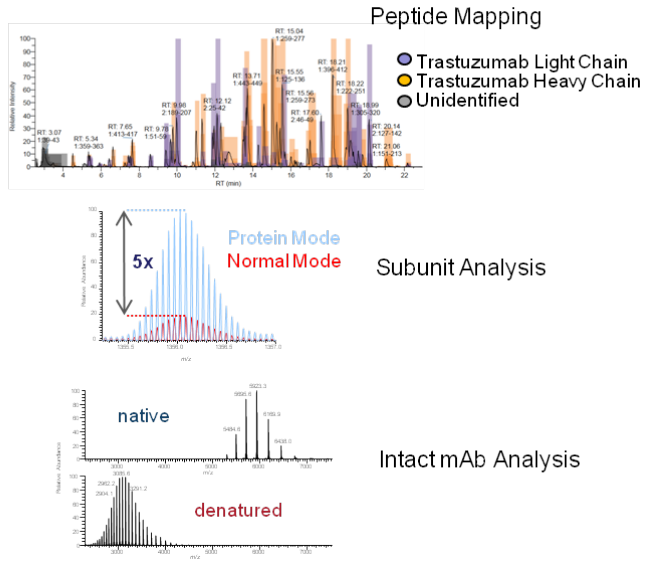
Thermo Scientific™ Q Exactive™ MS BioPharma Option



The Q Exactive Plus and Q Exactive HF mass spectrometers equipped with the BioPharma Option provide three different modes to cover the three major workflows in BioPharma. Coupled to the inert Vanquish UHPLC system and the high resolution BioPharma columns



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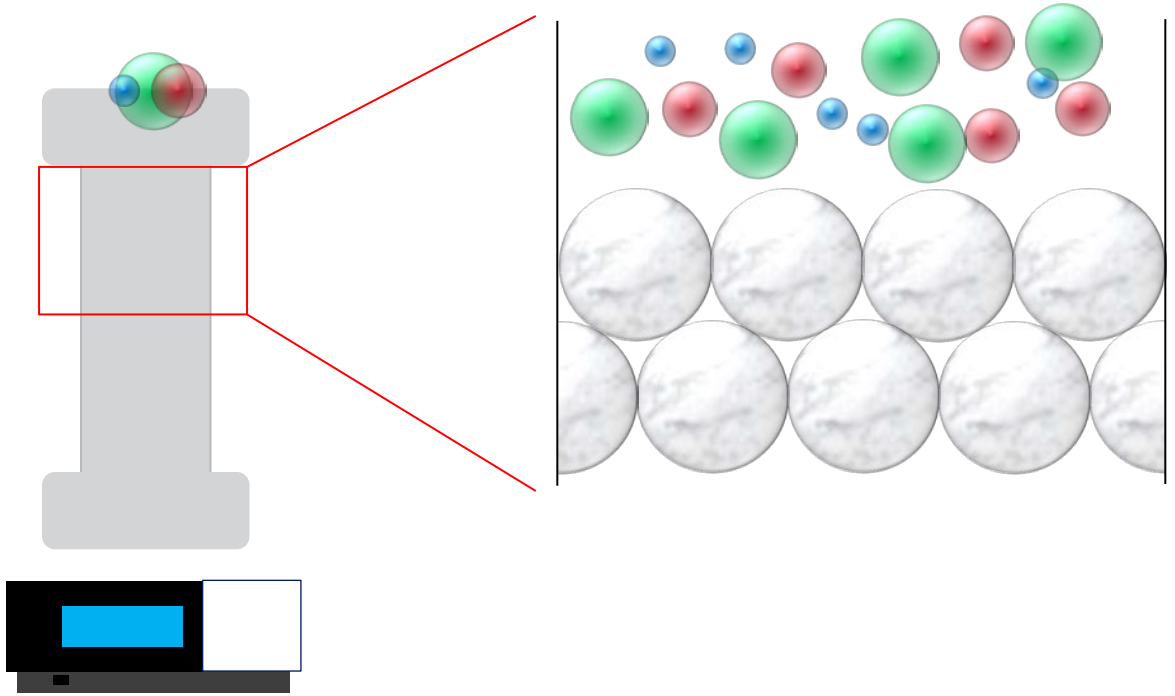
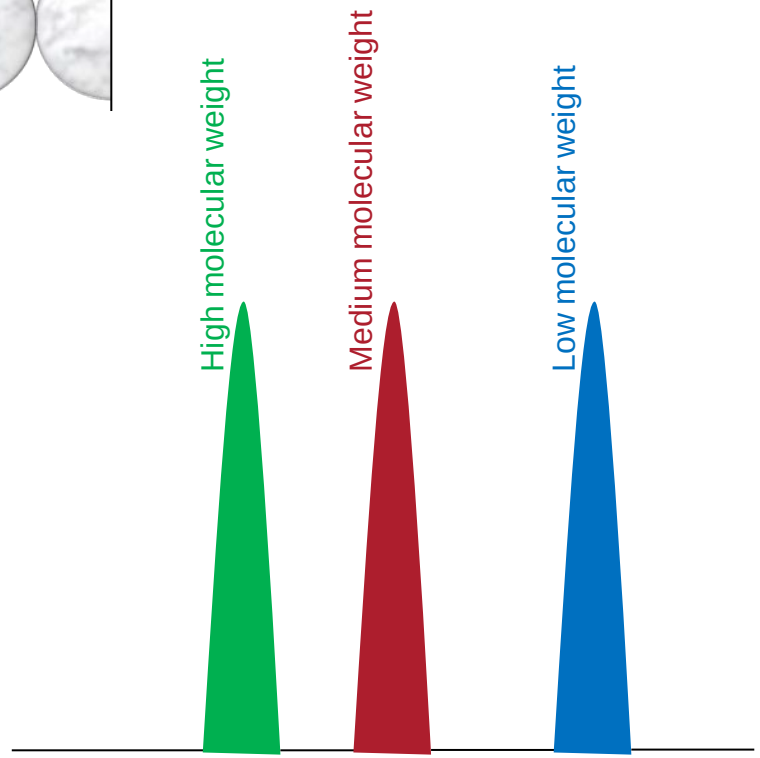
Aggregation Analysis

- Typically using size exclusion chromatography (SEC)
- MAbPac SEC-1
  - Silica, 5 µm, 300Å





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Column Formats Versus Target Applications

Formats	Target application	Why is it important
7.8 × 300 mm	Highest resolution separation of mAb and their aggregates.	Accurate quantification of mAb aggregates. Used in the batch QC release assay
4.0 × 300 mm 4.0 × 150 mm	High resolution separation of mAb and their aggregates	The 4.0 × 300 mm column enables baseline separation of mAb monomer and dimer, required ¼ of sample comparing to the 7.8 × 300 mm column
2.1 × 300 mm 2.1 × 150 mm	Designed for SEC-MS application	Low flow rate and low sample loading makes this format perfect for MS detection

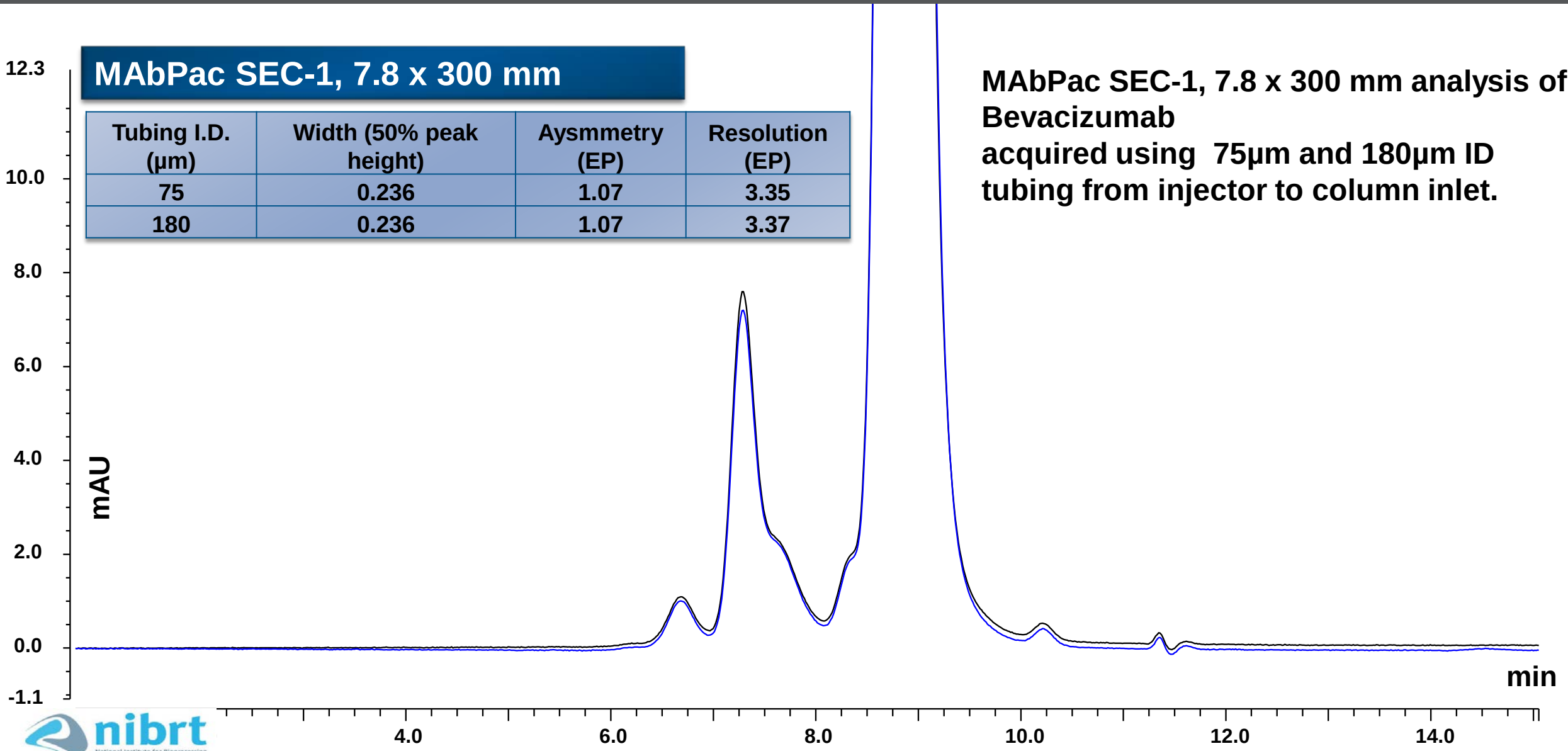
Column ID (mm)	2.1	4.0	7.8
Flow rate (µL/min)	50-75	200-300	760-1,000
UV flow cell	Micro (180 nL)	Semi-micro (2.5 µL)	Analytical (11 µL)
Tubing ID (µm)	50	75	150-250
Sample loop size (Pull loop WPS) (µL)	1	5	20

Effect of Pre-Column Tubing

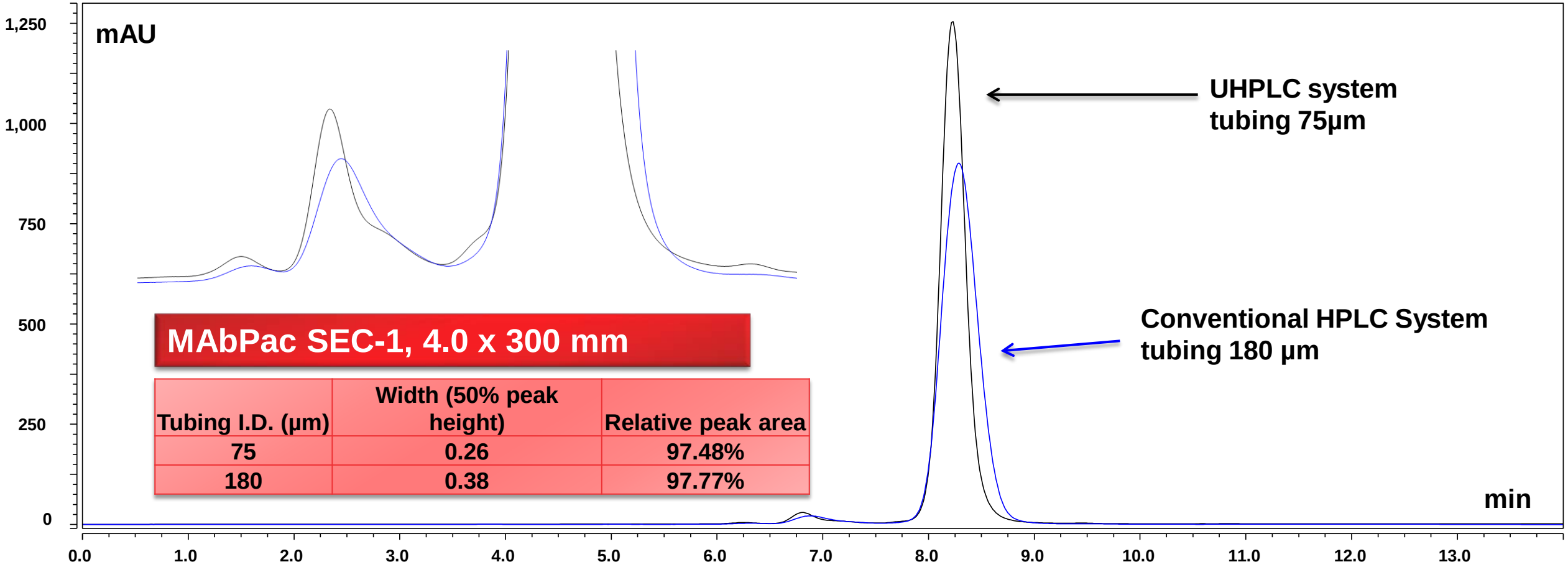
MAbPac SEC-1, 7.8 x 300 mm

Tubing I.D. (µm)	Width (50% peak height)	Aysmmetry (EP)	Resolution (EP)
75	0.236	1.07	3.35
180	0.236	1.07	3.37

MAbPac SEC-1, 7.8 x 300 mm analysis of Bevacizumab acquired using 75µm and 180µm ID tubing from injector to column inlet.



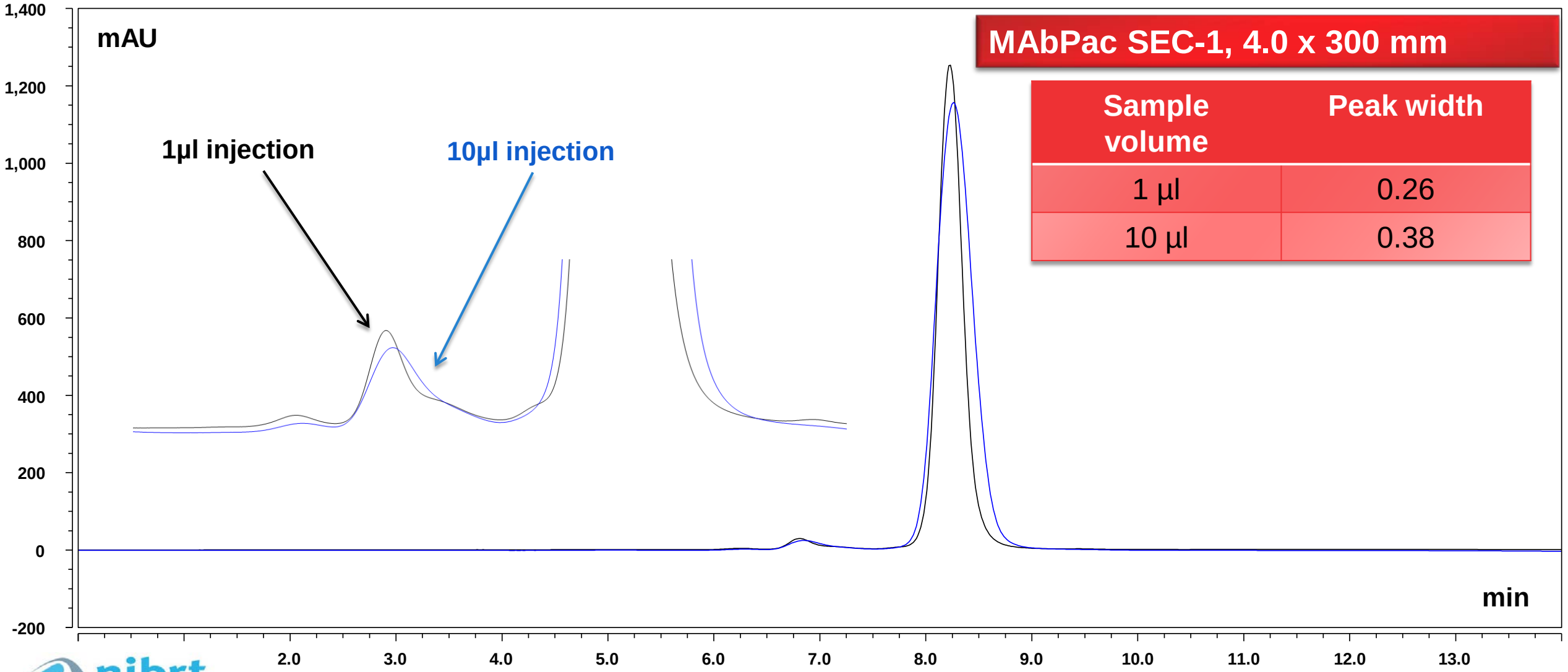
Effect of Pre-Column Tubing: Column 4mm



Tubing I.D. (µm)	Width (50% peak height)	Relative peak area
75	0.26	97.48%
180	0.38	97.77%

MAbPac SEC-1, 4.0 x 300 mm, analysis of Bevacizumab using Vanquish UHPLC chromatograms acquired using 75µm and 180µm ID tubing from injector to column inlet

Injection Volume – Effect on SEC (4 mm)

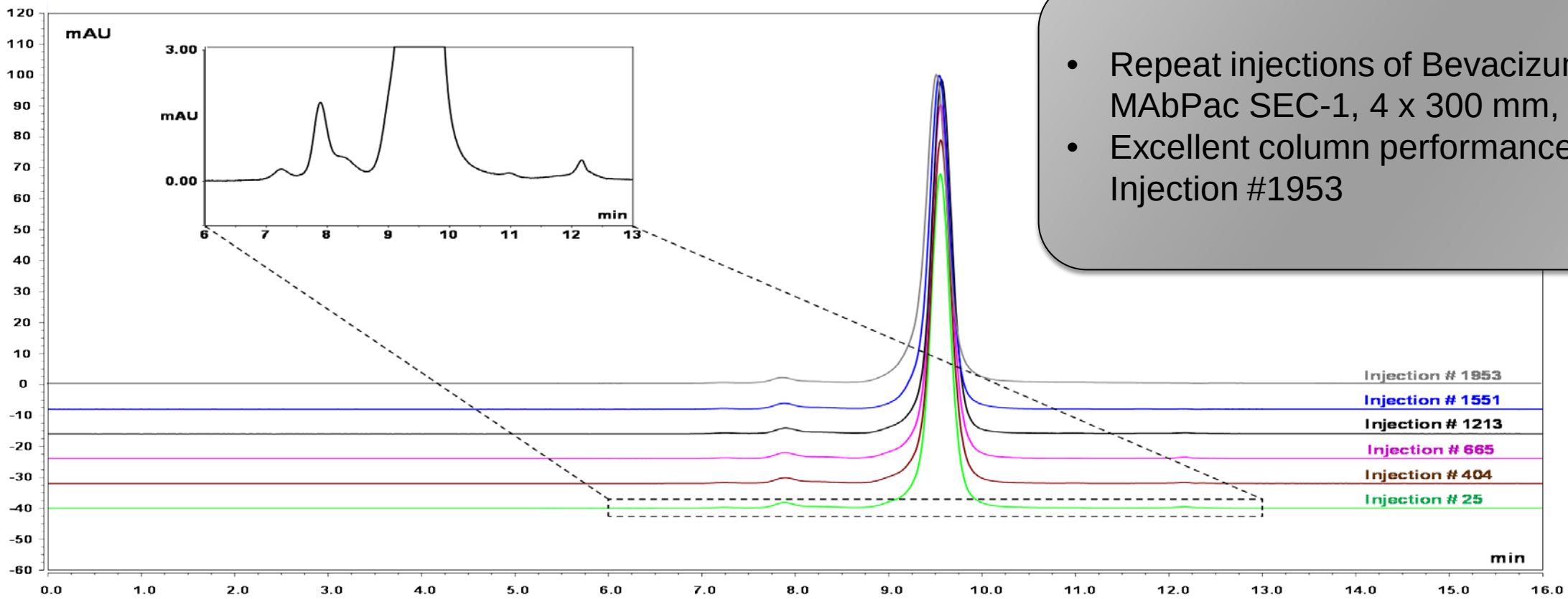


MAbPac SEC-1, 4.0 x 300 mm

Sample volume	Peak width
1 µl	0.26
10 µl	0.38

Increasing sample injection volume results in sample dispersion

Column Lifetime Stability Evaluation



- Repeat injections of Bevacizumab on MAbPac SEC-1, 4 x 300 mm, 5µm
- Excellent column performance up to Injection #1953

'Inject until failure' study performed on MAbPac SEC-1 column performed on Vanquish Flex quaternary with DAD Light Pipe detector using repeat injections of Bevacizumab, selectivity and reproducibility maintained for >1950 injections!

nibrt National Institute for Bioprocessing Research and Training

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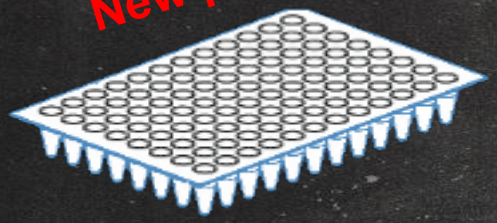
Peptide Mapping: Our Workflow Solution

New product

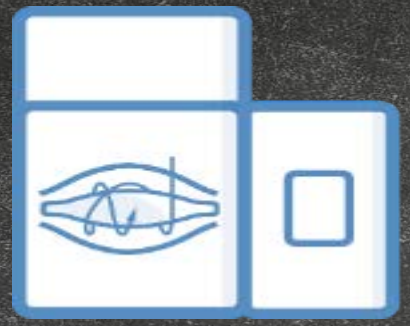


Thermo Scientific™ SMART Digest™ offer extremely reproducible and rapid protein digestion

New product

Thermo Scientific™ Vanquish™ UHPLC is engineered for high resolution, reproducible peptide separations

Thermo Scientific™ Acclaim™ 120 C18 column is the perfect column choice to ensure sharp peaks during peptide mapping



Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometers are the gold standard for accurate mass measurement

Brand new product

Thermo Scientific™ BioPharma Finder™ software is the perfect software tool for peptide identification and sequence mapping

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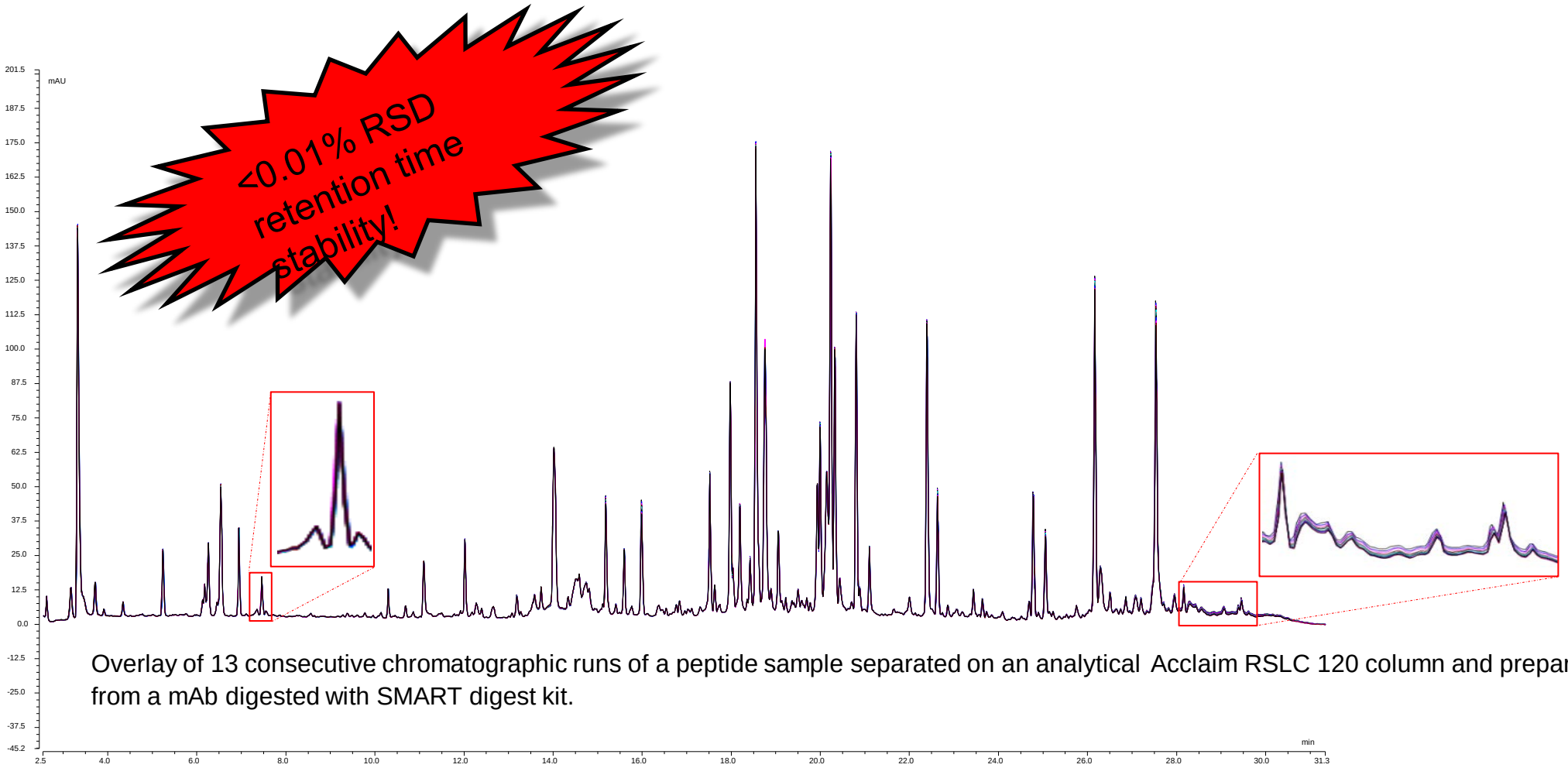
Vanquish UHPLC System: Retention Time Reproducibility

Retention time repeatability of a peptide separation



Overlay of 13 consecutive chromatographic runs of a peptide sample separated on an analytical Acclaim RSLC 120 column and prepared from a mAb digested with SMART digest kit.

Retention time repeatability

peak #	RT (min)	RSD (%)
3	3.315	0.082
9	5.231	0.065
14	6.532	0.017
15	6.937	0.023
19	10.290	0.021
23	12.013	0.012
31	14.011	0.013
39	15.177	0.012
42	15.589	0.010
51	17.511	**0.007**
55	17.969	0.011
61	18.546	0.010
83	20.798	0.010
85	21.095	0.012
87	22.386	**0.009**
96	24.774	0.012
103	26.155	**0.009**
106	26.155	**0.009**
109	27.529	0.010

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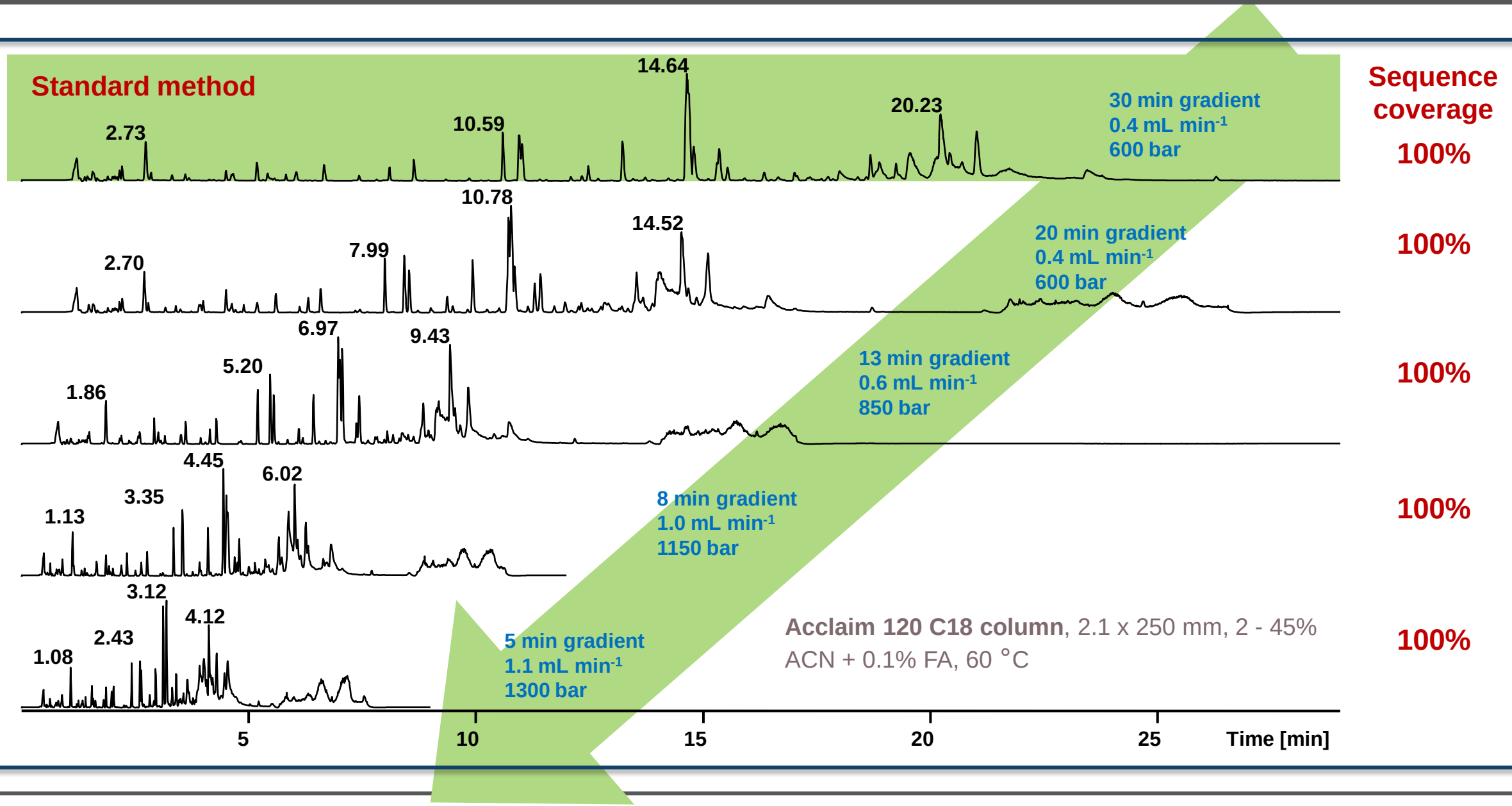
Rituximab Analysis with Reducing Gradient



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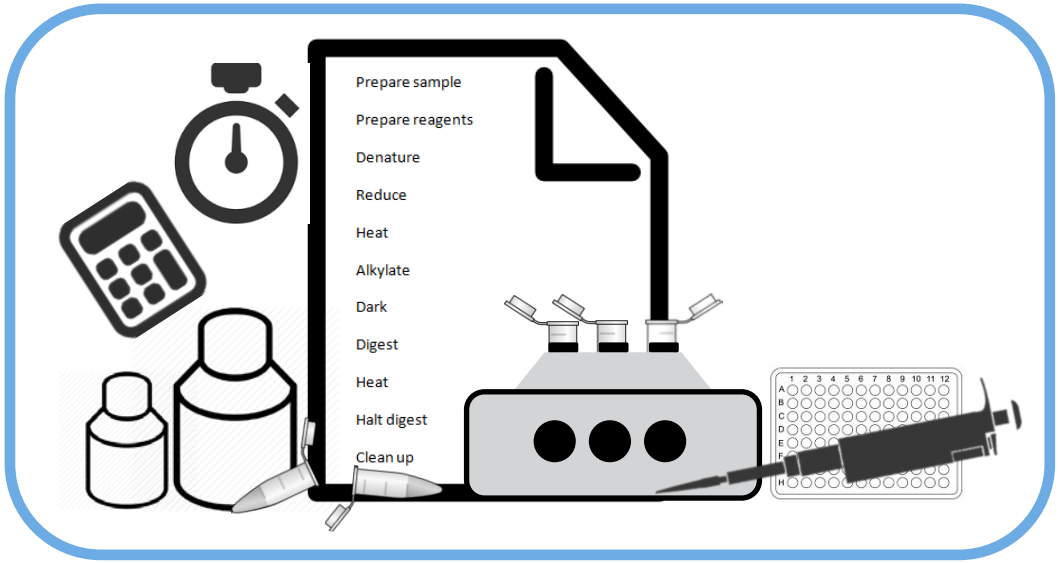
What is the Problem with Protein Digestion?

- Lengthy multi-step protocols
- Process-induced PTMs
- Reproducibility
- Throughput/Speed
- Method development ease and transfer



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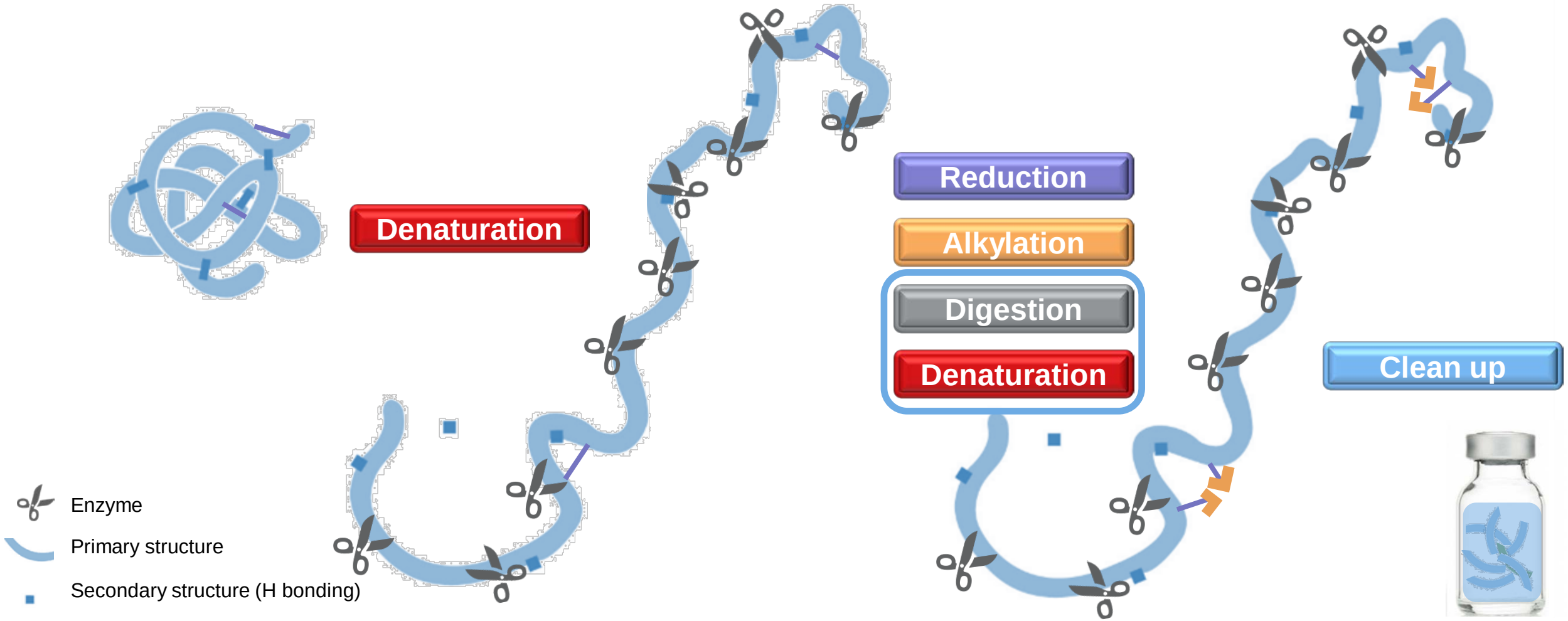
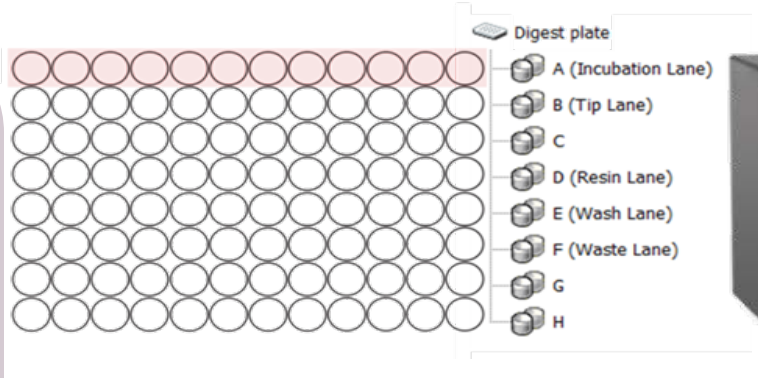
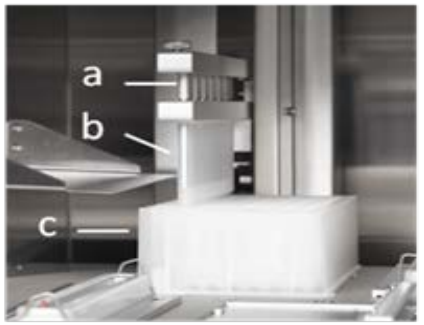
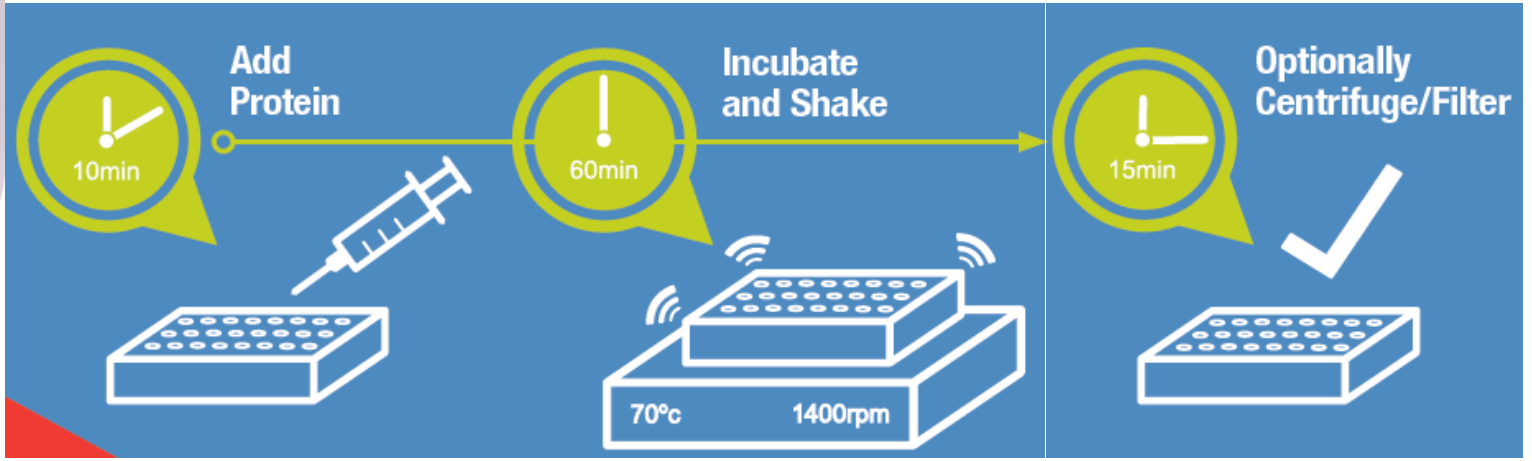
The Fundamental Five…or is that Two?



SMART Digest Kits







Denaturation

Digestion

Clean up

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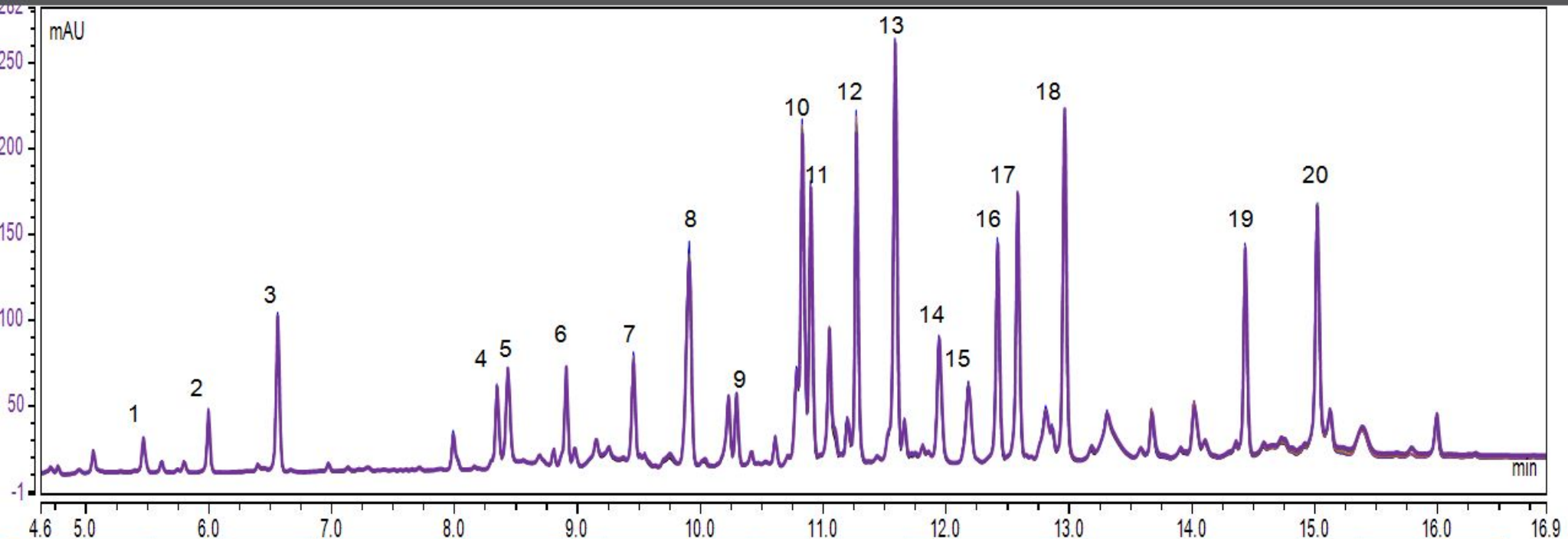
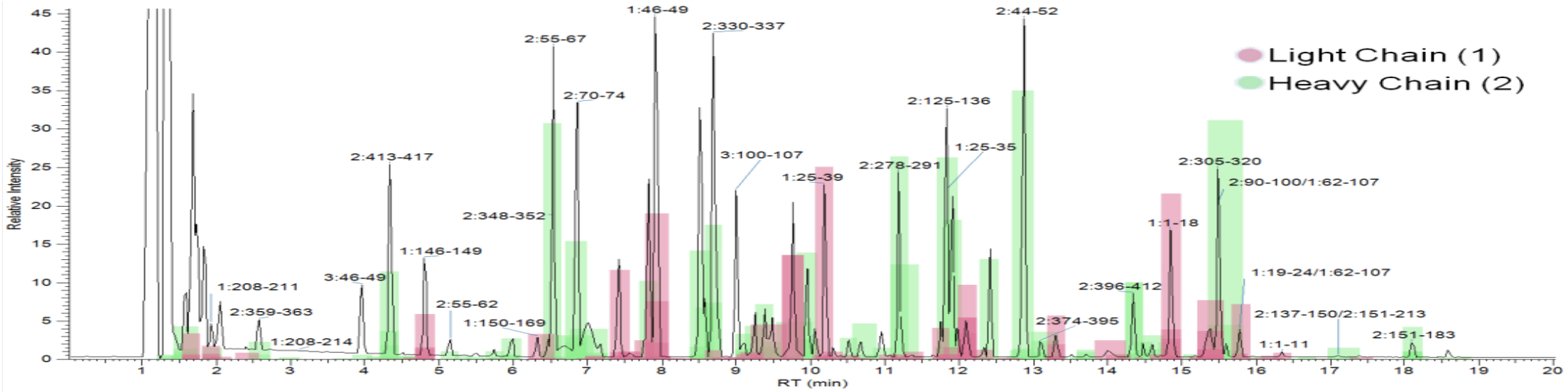
Five Different Rituximab Digests by Five Different Seminar Attendees



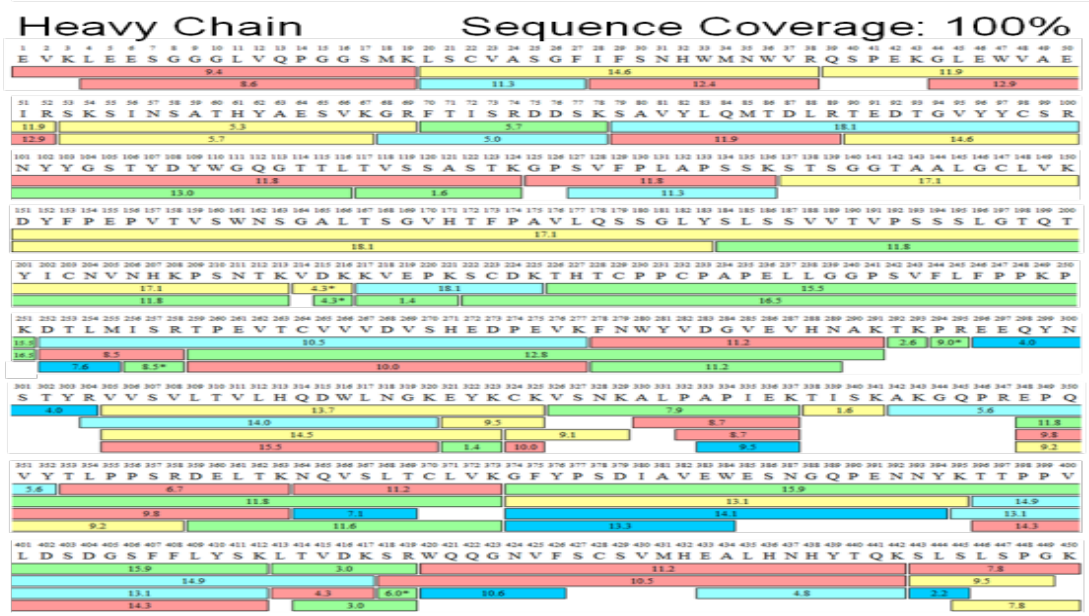
Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8	Peak 9	Peak 10
2.54	2.41	1.89	3.39	3.53	2.16	4.41	2.10	2.10	3.65
Peak 11	**Peak 12**	**Peak 13**	**Peak 14**	**Peak 15**	**Peak 16**	**Peak 17**	**Peak 18**	**Peak 19**	**Peak 20**
1.96	3.51	3.72	2.26	2.91	1.97	3.28	2.62	3.16	1.20

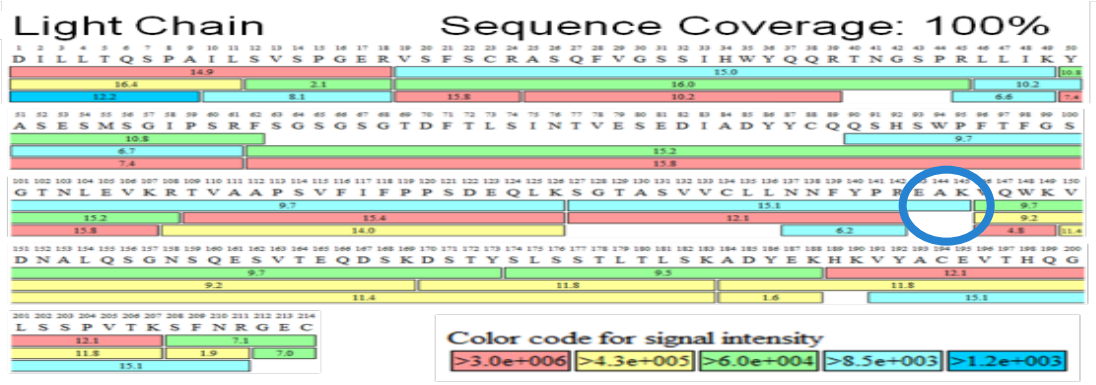
Sequence Coverage Map from Infliximab Using the Magnetic SMART Digest

A)



B)





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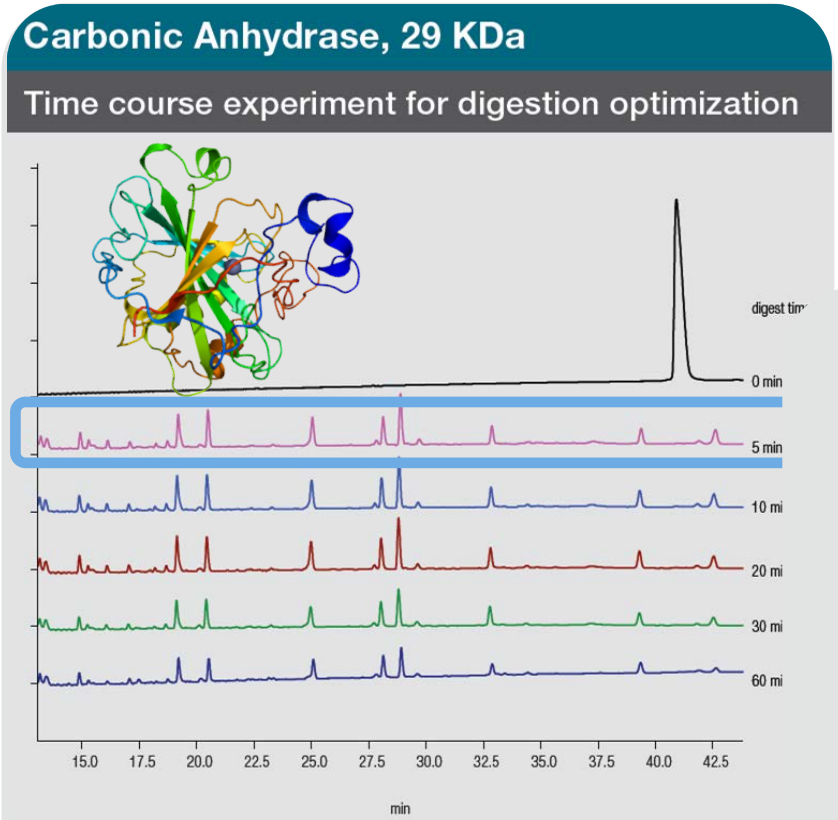
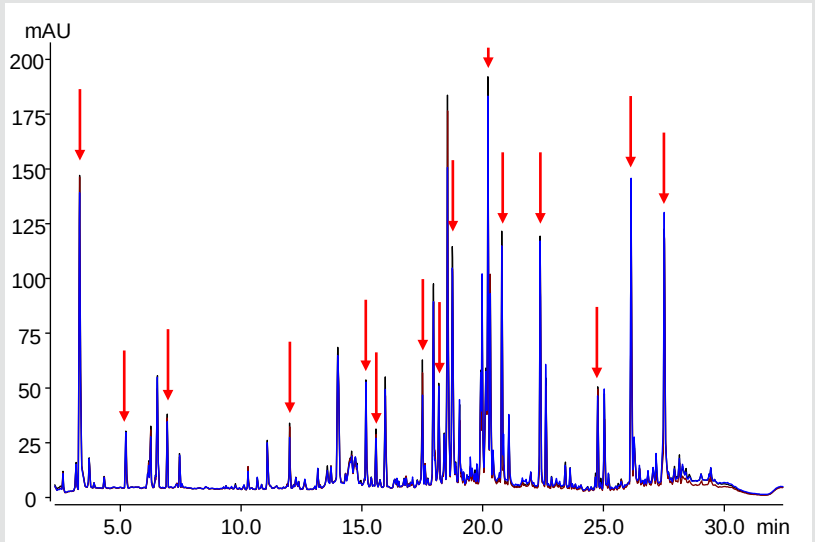
Three Reasons to Change to SMART Digestion



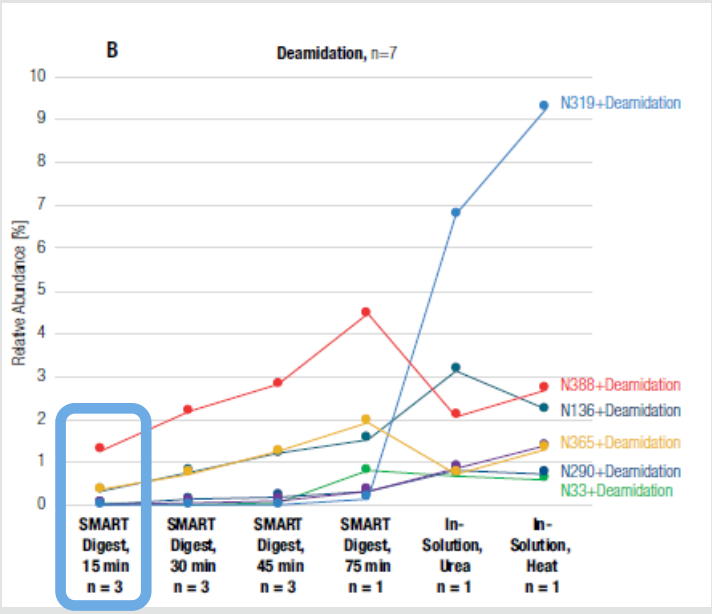
Fast and flexible method development

Automation!



RSDs: RT 0.024 %; Peak Area 2.82 % (n=5 users, based on 15 peaks)

Standardized reproducibility



Reduced complexity

APPLICATION NOTE

SMART Digest Compared to Classic In-Solution Digestion of Rituximab for In-Depth Peptide Mapping Characterization

No. 1159

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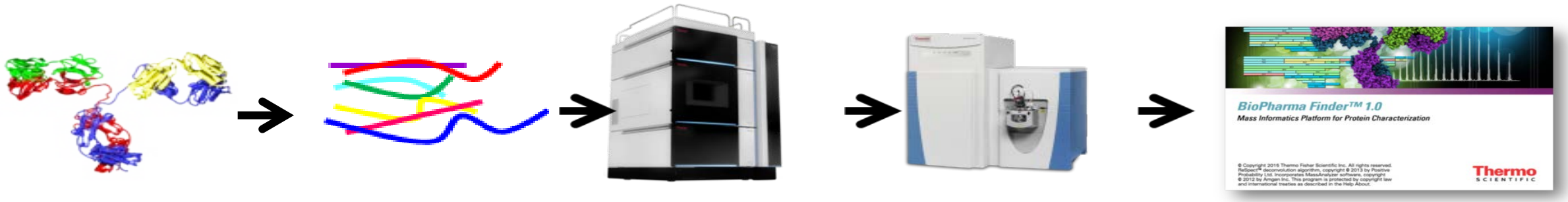
Overview of the Peptide Mapping Workflow



60 min 20 min 10 min

- **Combining all the components of this peptide mapping work flow a complete analysis can be done in less than 90 minutes.**
- **Can be set up and verified by MS very quickly.**
- **A relatively inexperienced analyst can obtain reproducible results from UV with simple sample preparation and walk up UHPLC analysis.**

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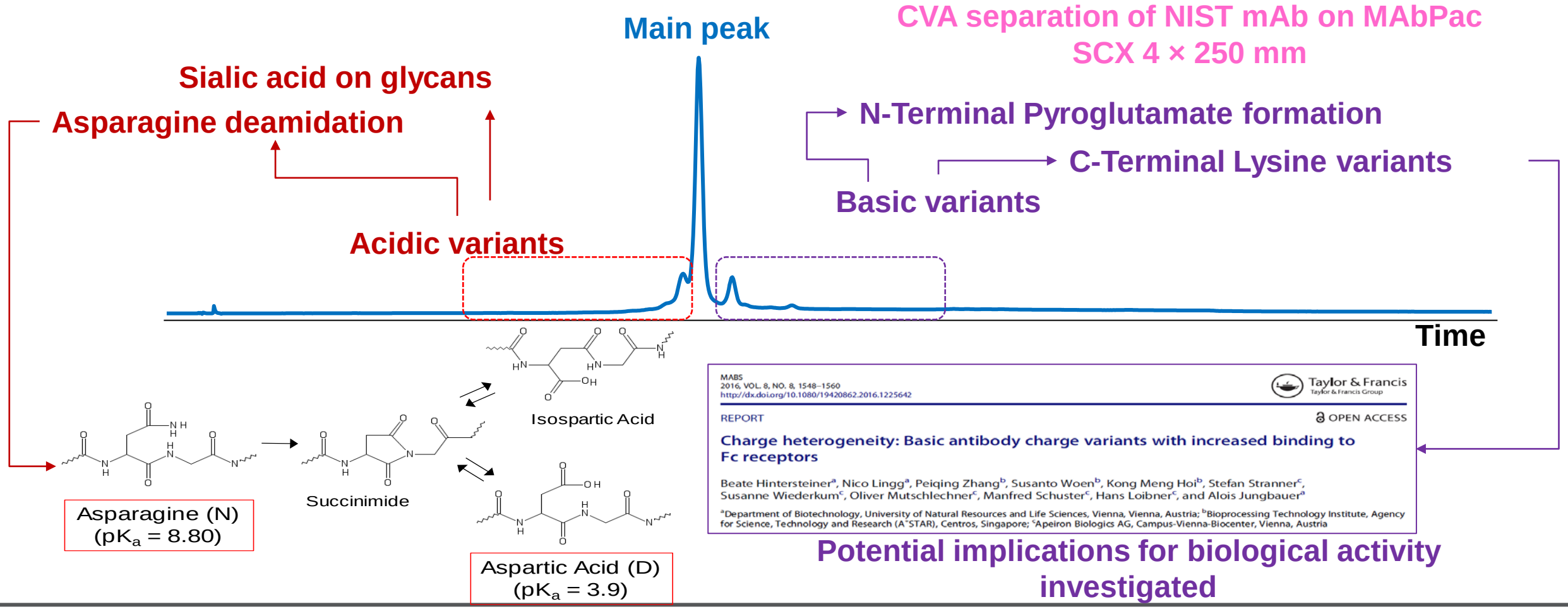
Sources of Charge Variation on MAbs

Considering the complexity of industrial bioprocessing and the number of conditions a mAb is exposed to, certain amino acids can become modified which in turn modifies the charge of the protein.



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2016, VOL. 8, NO. 8, 1548–1560
http://dx.doi.org/10.1080/19420862.2016.1225642

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REPORT

OPEN ACCESS

Charge heterogeneity: Basic antibody charge variants with increased binding to Fc receptors

Beate Hintersteiner[a], Nico Lingg[a], Peiqing Zhang[b], Susanto Woen[b], Kong Meng Hoi[b], Stefan Stranner[c], Susanne Wiederkum[c], Oliver Mutschlechner[c], Manfred Schuster[c], Hans Loibner[c], and Alois Jungbauer[a]

[a]Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, Vienna, Austria; [b]Bioprocessing Technology Institute, Agency for Science, Technology and Research (A*STAR), Centros, Singapore; [c]Apeiron Biologics AG, Campus-Vienna-Biocenter, Vienna, Austria

Potential implications for biological activity investigated

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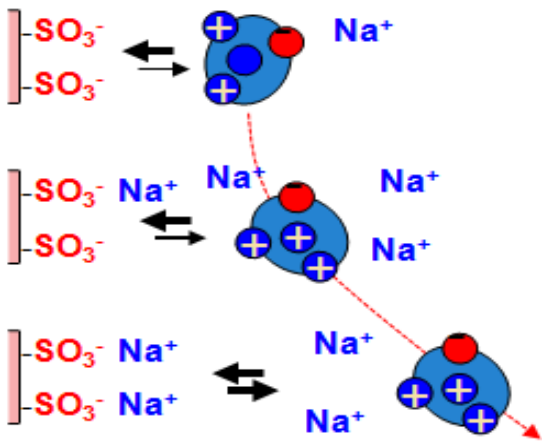
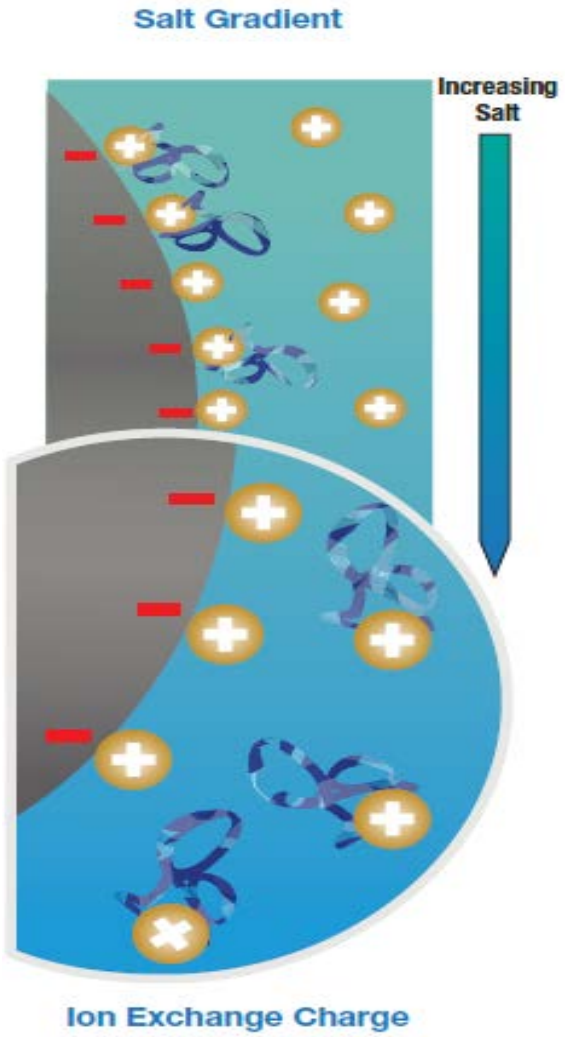
MAb Charge Variant Analysis by CEX Salt Elution

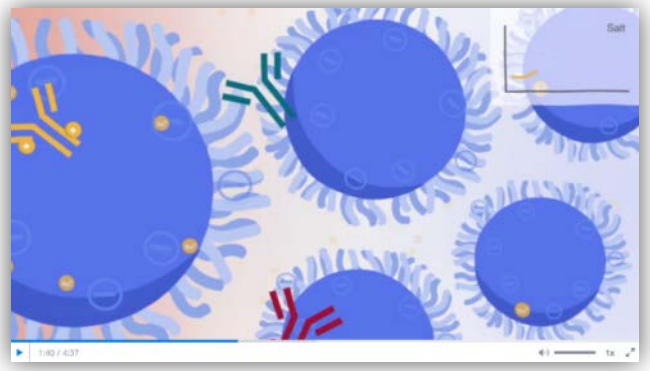
Cation exchange of MAb
Elution with competing sodium ions from an NaCl gradient



Cation exchange



- Competition for ion exchange sites between the MAb and Na^+ ions
- This interaction happens all the way through the column.
- The longer the column the better the resolution
- Surface exchange on a pelicular resin for high resolution

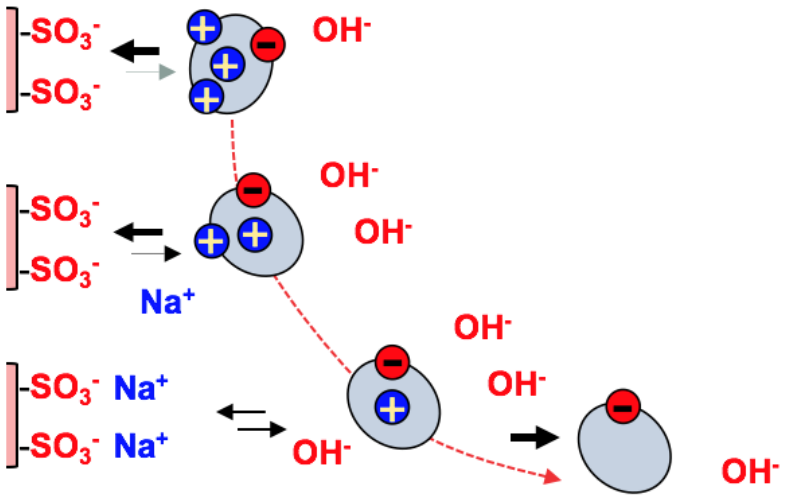


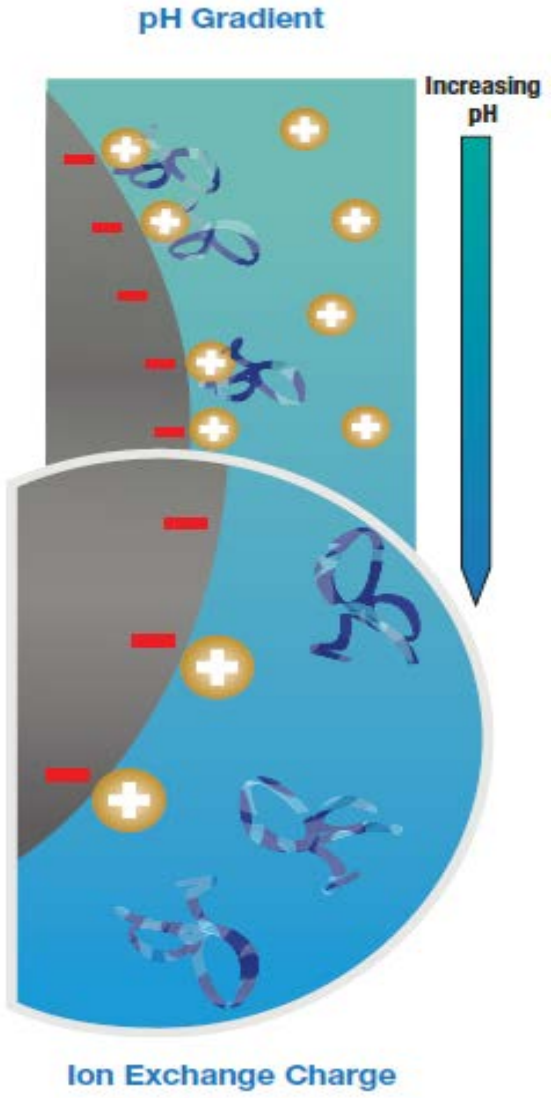
http://bit.ly/ChargeVariants

MAb Charge Variant Analysis by CEX pH Gradient Elution

pH gradient elution

- Based on pI of protein
- Loss of retention with progressing pH gradient, depending on pI
- "Single" binding event, trapping at pH < pI (for CEX)

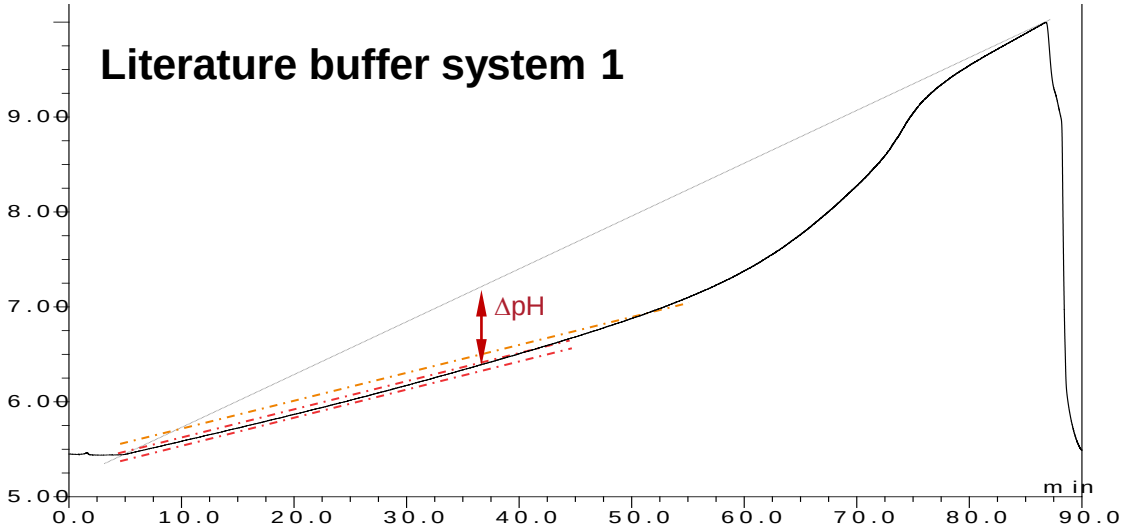


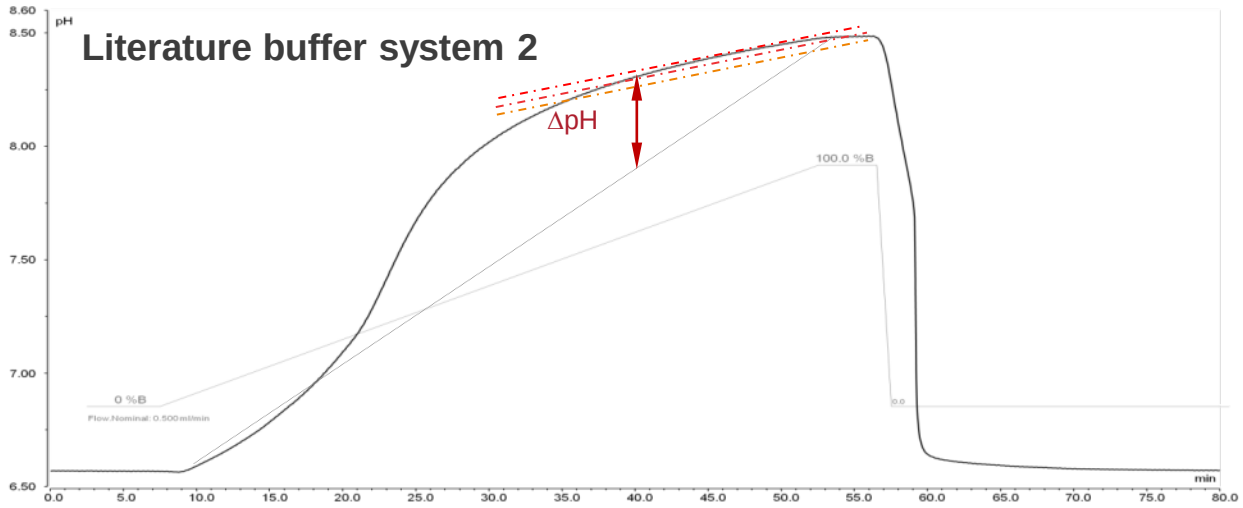


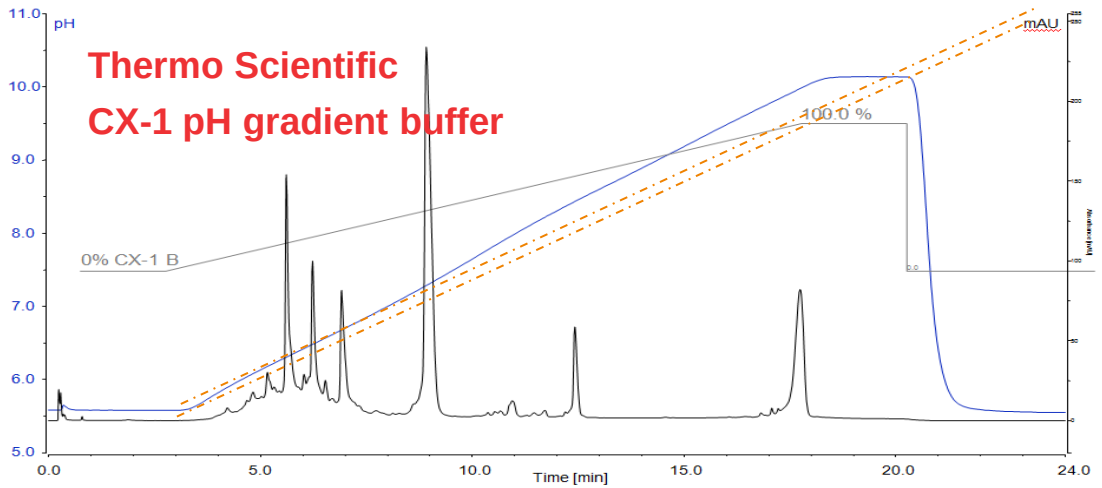
Isoelectric Focusing on a cation exchange column

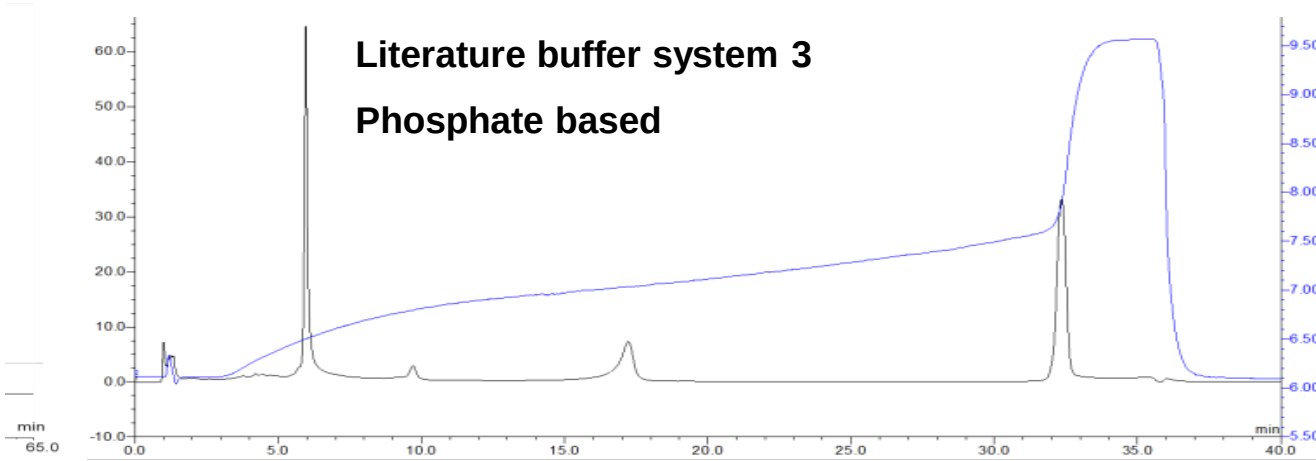
- MAb binds to cation exchange sites on the column.
- A gradient of increasing pH is applied.
- MAb is released from the exchange site when the net charge on the mAb is neutral.
- This interaction happens once, then the mAb runs through the rest of the column.
- Column length has little effect on the resolution.
- This is a concentrating technique.
- Surface exchange on a pelicular resin for high resolution and low buffering capacity effects

Comparison of pH Gradient Buffer Systems









MAbPac SCX-10 (5 µm) 4x50 mm

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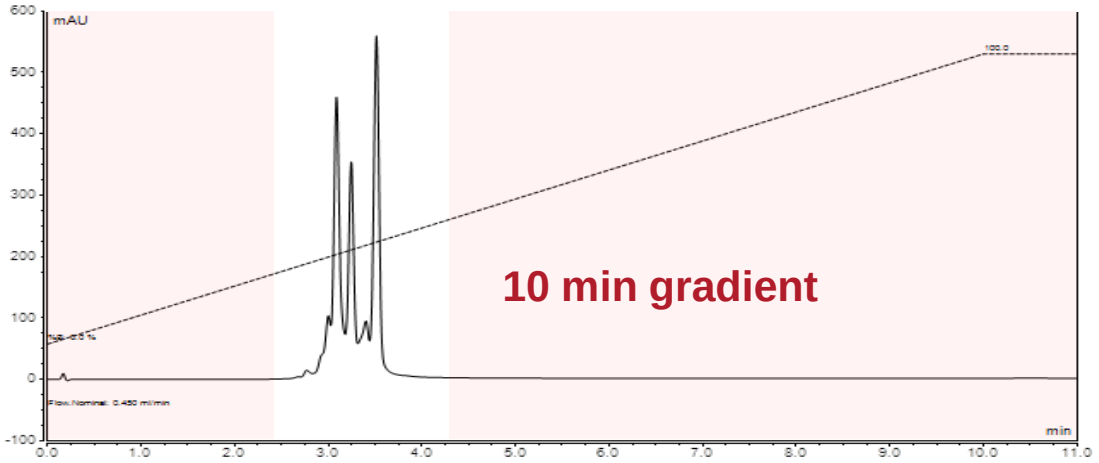
Advantages

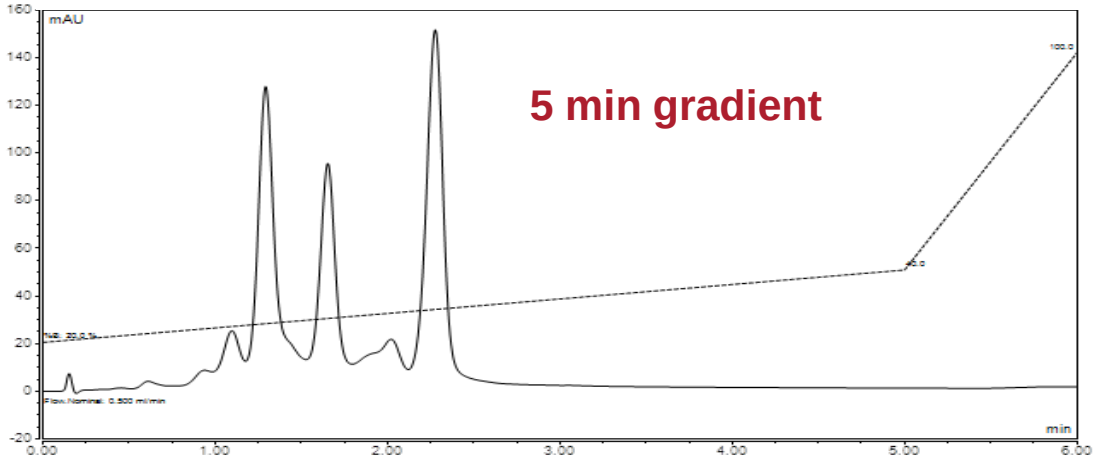
- **Platform** method ⇨ Single method for wide range of mAbs
- Reduced method development and method transfer times
- Outperforms any other charge variant technology
- Less effect from column variability
- Transferability of method from development to QC

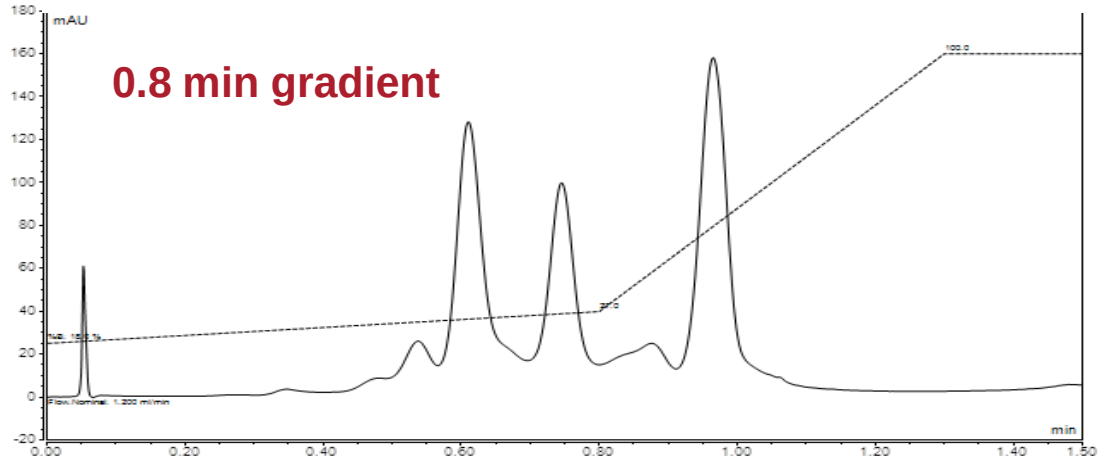
- Dilute buffers 10-fold with DI water
- A linear pH gradient (pH 5.6 - 10.2) is generated by running a linear pump gradient from 100% Buffer A to 100% Buffer B
- Generic, fast & high-resolution!

	Buffer A	Buffer B
pH	5.6	10.2
Form	Liquid	Liquid
Concentrate	10X	10X
Shipping condition	Room Temp	Room Temp
Storage condition	4 ~ 8 ˚C	4 ~ 8 ˚C

Infliximab – Vanquish System Ultra-fast Gradients







3 step method development

1. **Full gradient run**
   10 minutes 0→100% B
2. **Adjusted pH window**
   20→40% B in 5 minutes
3. **Flow and shape optimized**
   18→27% B in 0.8 minutes

Resolution and number of charge variants maintained in sub-minute gradients

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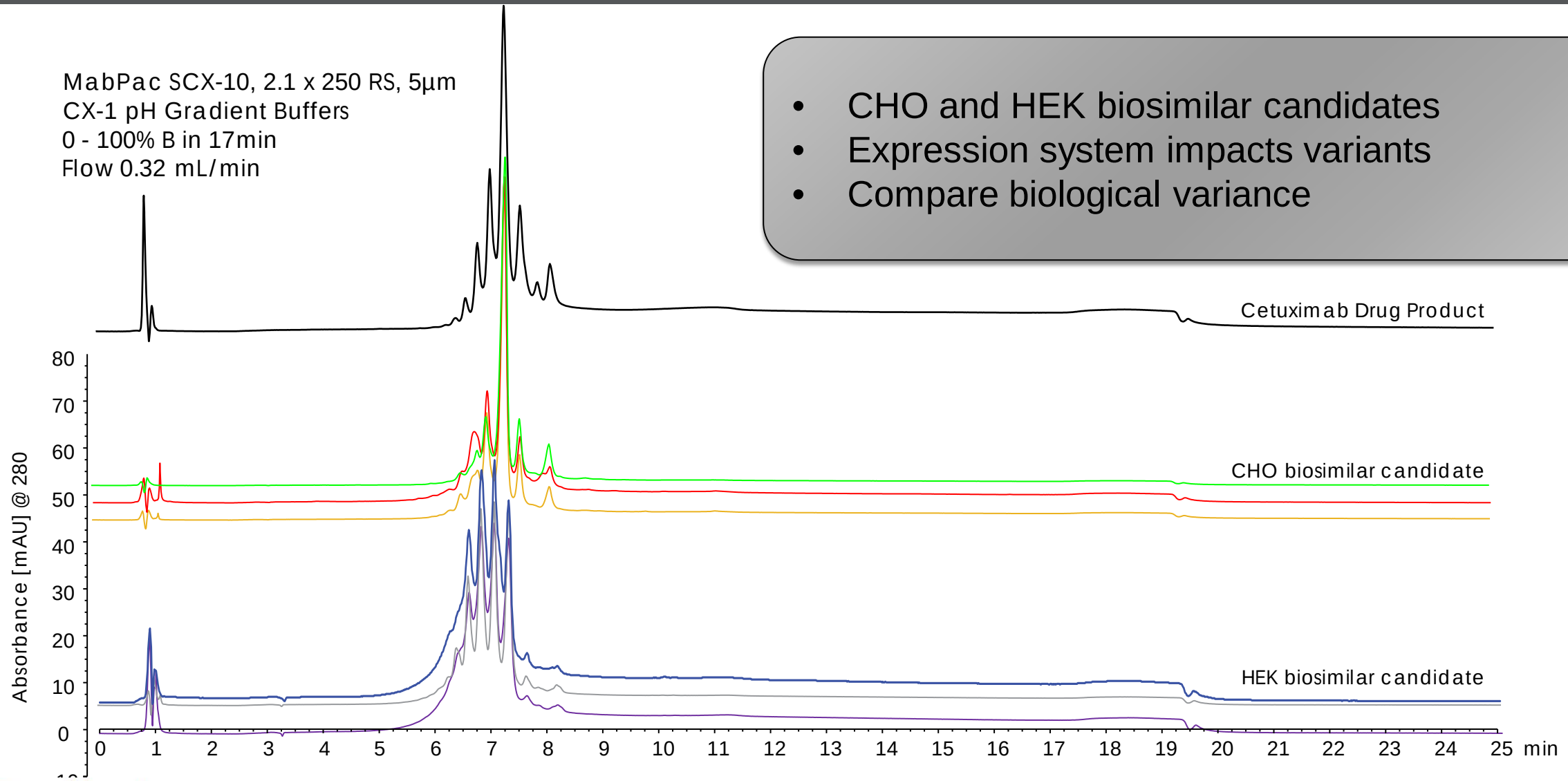
Different Charge Variants



- CHO and HEK biosimilar candidates
- Expression system impacts variants
- Compare biological variance

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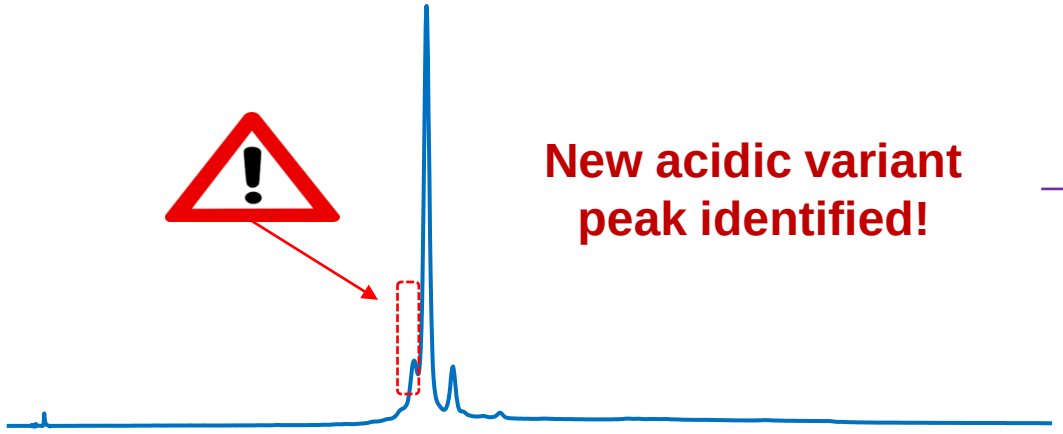
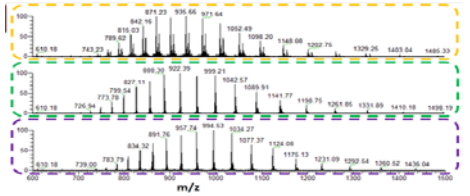
Something Different in the CVA Pattern – Multistep Strategy

Differences in CVA patterns – Peak areas or appearance of new peaks, require investigation to determine root cause analysis.

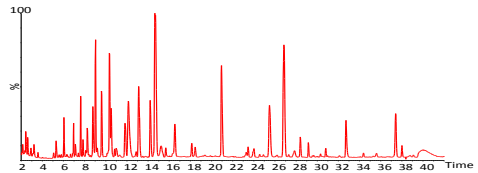


New acidic variant peak identified!

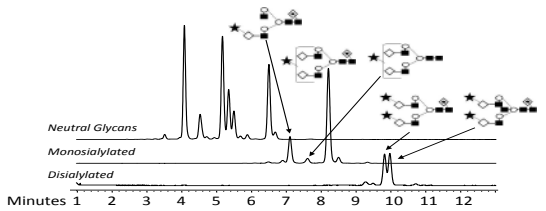
Preparative separation



Middle down analysis



LC-MS peptide map



Glycan analysis

Multi-step analytical strategy required to identify the induced posttranslational modification, location within primary sequence and potential on activity

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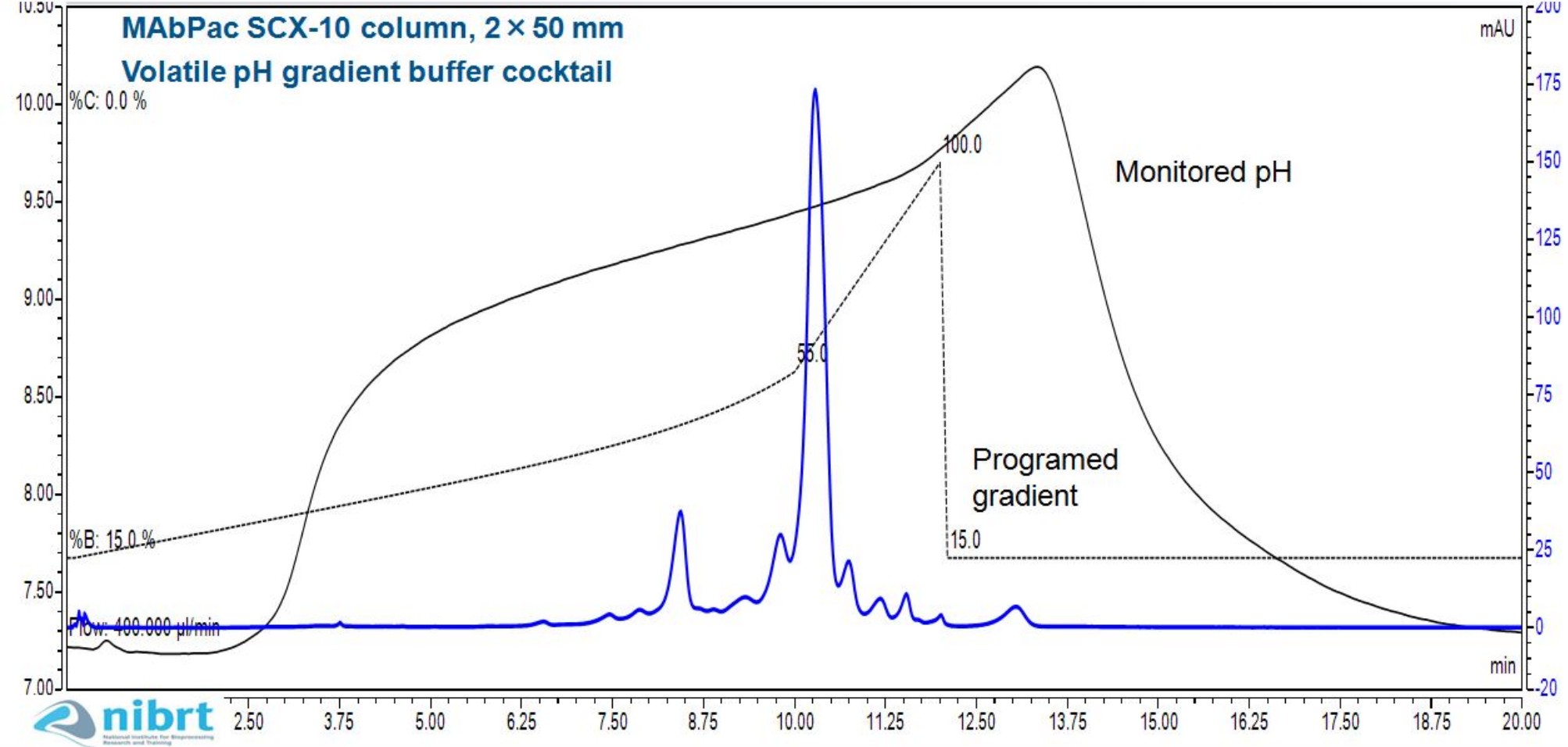
Trastuzumab Elution with MS Friendly Eluents



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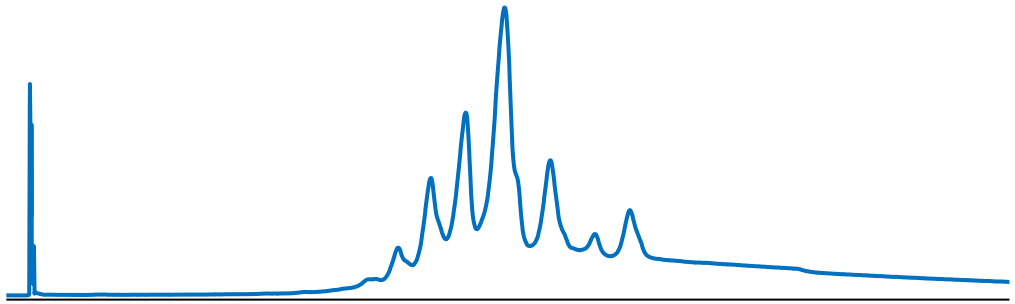
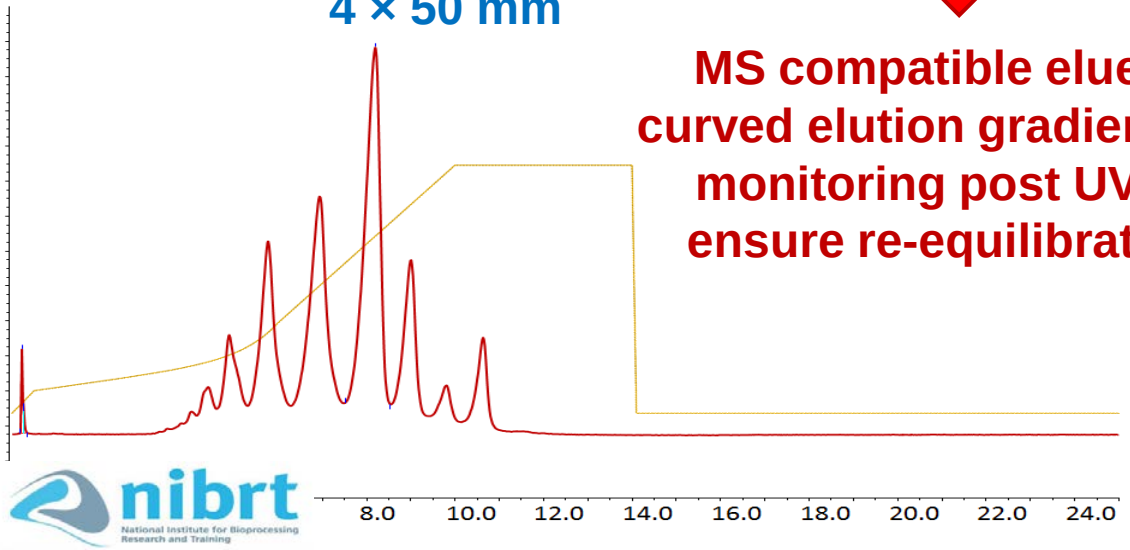
MS Friendly Eluents Required – Direct coupling of CVA to MS

Matching performance of offline CVA-UV and online CVA-MS – Combination of MS friendly eluents and non-linear gradient curves to generate linear pH elution gradients

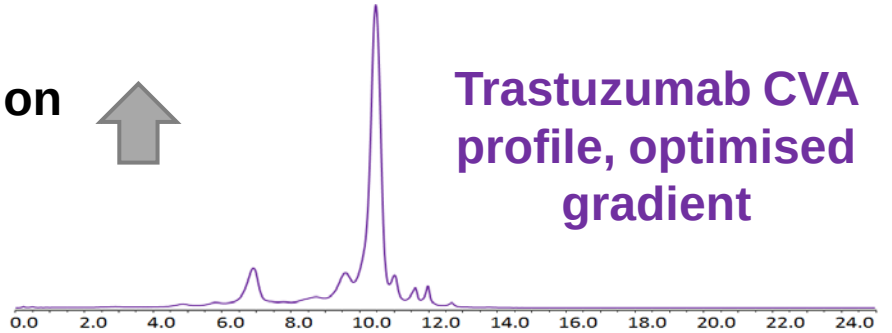


Cetuximab CVA analysis, CX-1 pH gradient buffers on MAbPac SCX 10 4 × 50 mm

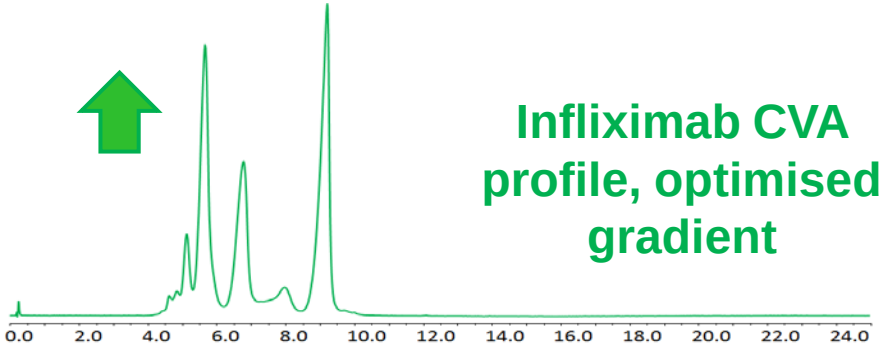
MS compatible eluent, curved elution gradient, pH monitoring post UV to ensure re-equilibration



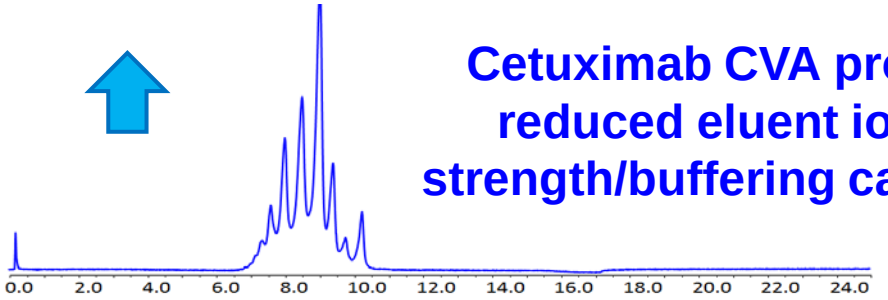
MS hyphenation ready



Trastuzumab CVA profile, optimised gradient



Infliximab CVA profile, optimised gradient



Cetuximab CVA profile, reduced eluent ionic strength/buffering capacity

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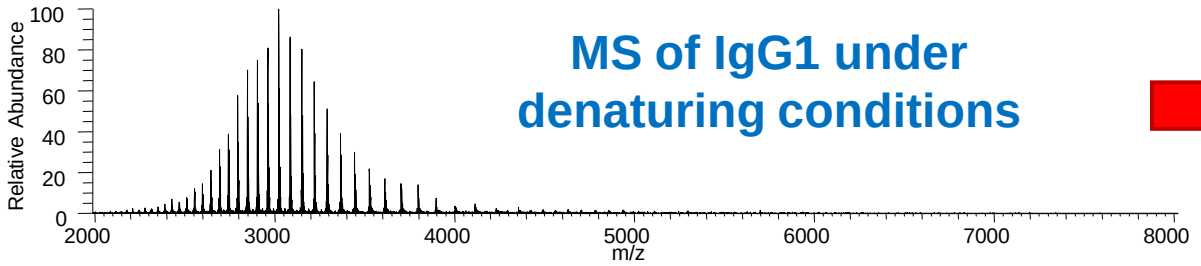
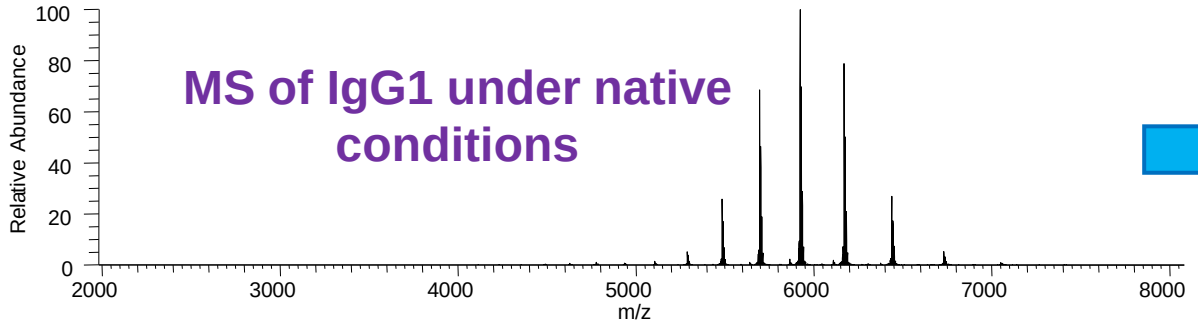
Coupling to High Resolution Native MS



MS of IgG1 under denaturing conditions

Reversed phase chromatography or other separation modes containing organic solvents



MS of IgG1 under native conditions

Traditional protein chromatography: SEC, now also CEX, AEX

- MS analysis of mAbs under native conditions is powerful, reflection of surface charge present in solution
- Greater spatial spectral resolution in native MS spectra
- Requires optimisation of source parameters: temperature, gas flow, in source CID for desolvation

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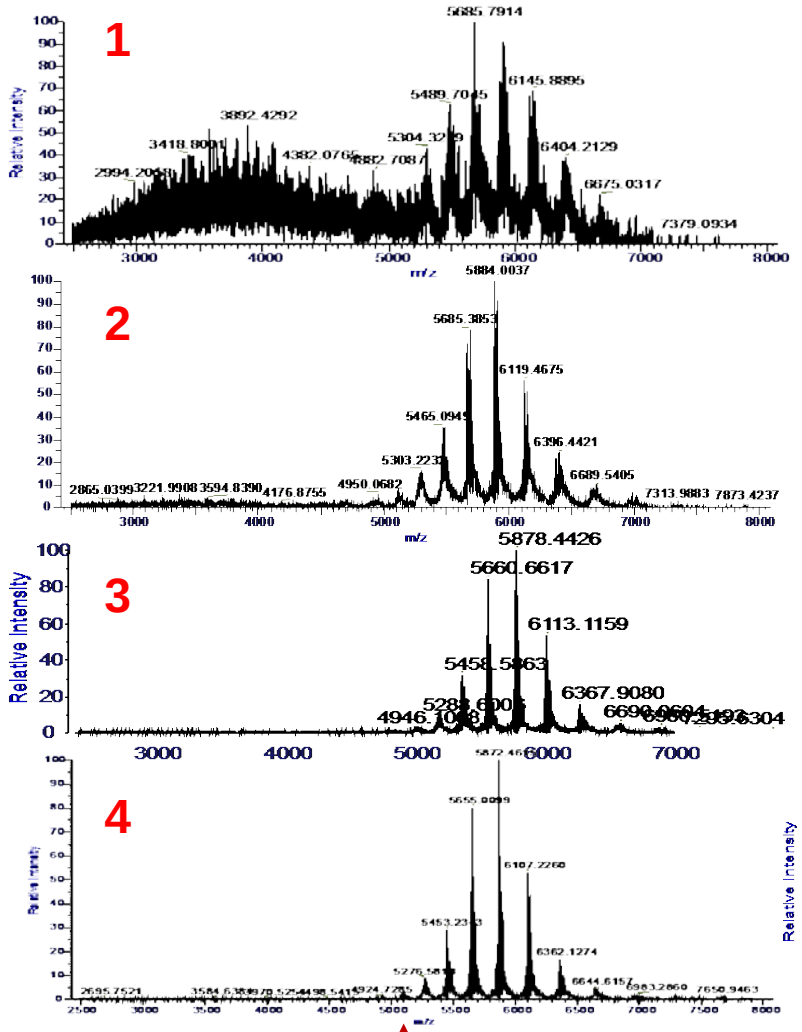
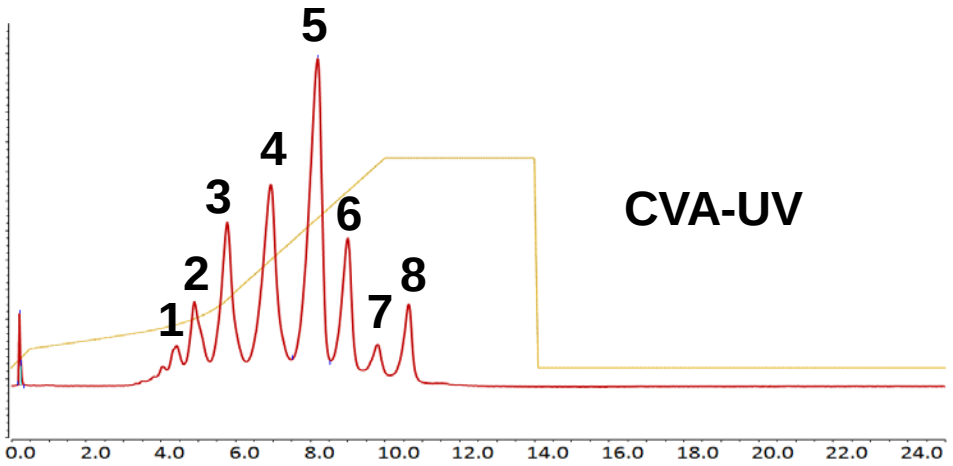
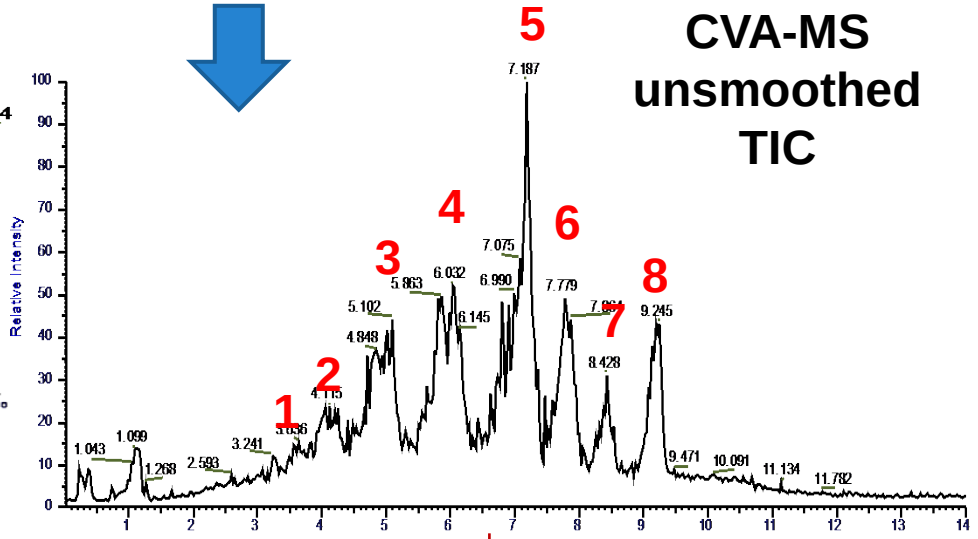
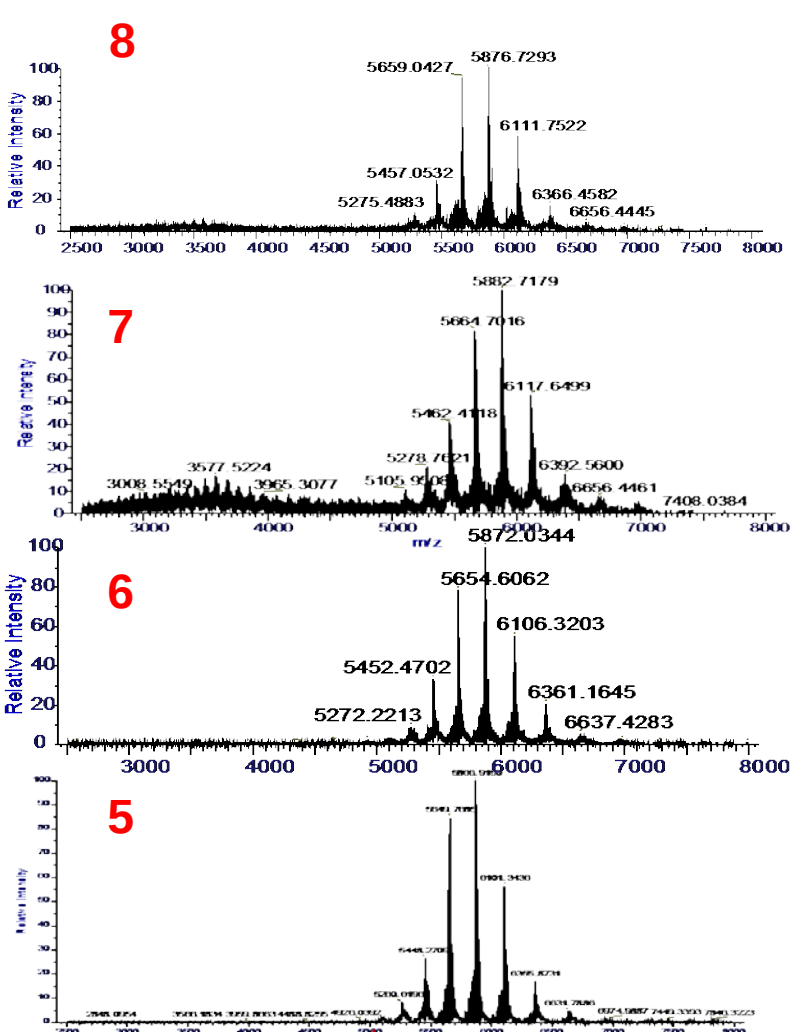
Native CVA-MS of Cetuximab

MAbPac SCX using developed pH gradient elution









Conclusions

Game changing automation of protein digestion for ultimate precision and reproducibility in a robust peptide mapping workflow;

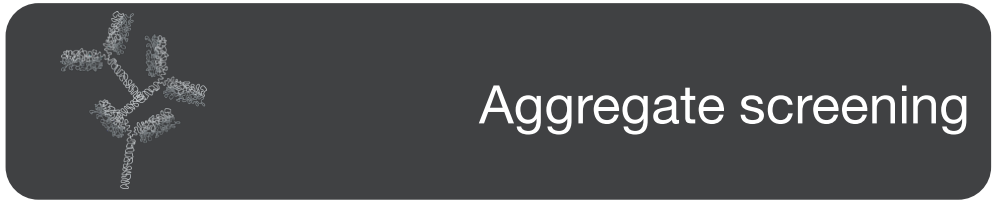
pH gradient elution of proteins for CVA has several advantages including; global applicability, increased speed, fast method development, high loading capacity, easy method transfer, new native MS compatibility;

Inert UHPLC with a sensitive high resolution mass spectrometer enables several critical workflows.

Industry specific Thermo Scientific™ Biopharma Finder™ software;

New workflows enabling characterization of several attributes in one injection provides ease of use and time saving – peptide mapping and CVA/MS.

Native MS now has SEC and ion exchange.



Any Questions?

Do you have additional questions or do you want to talk to an expert from Thermo Fisher Scientific?

Please send an E-Mail to analyze.eu@thermofisher.com and we will get back to you.