

**ThermoFisher**  
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# Unlocking Biomolecular Secrets : Comprehensive Biopharmaceutical Characterization Workflows

Dr Rowan Moore, Thermo Fisher Scientific, UK

The world leader in serving science



## 1. **Biotherapeutic analytical challenges**

- Unlocking their secrets

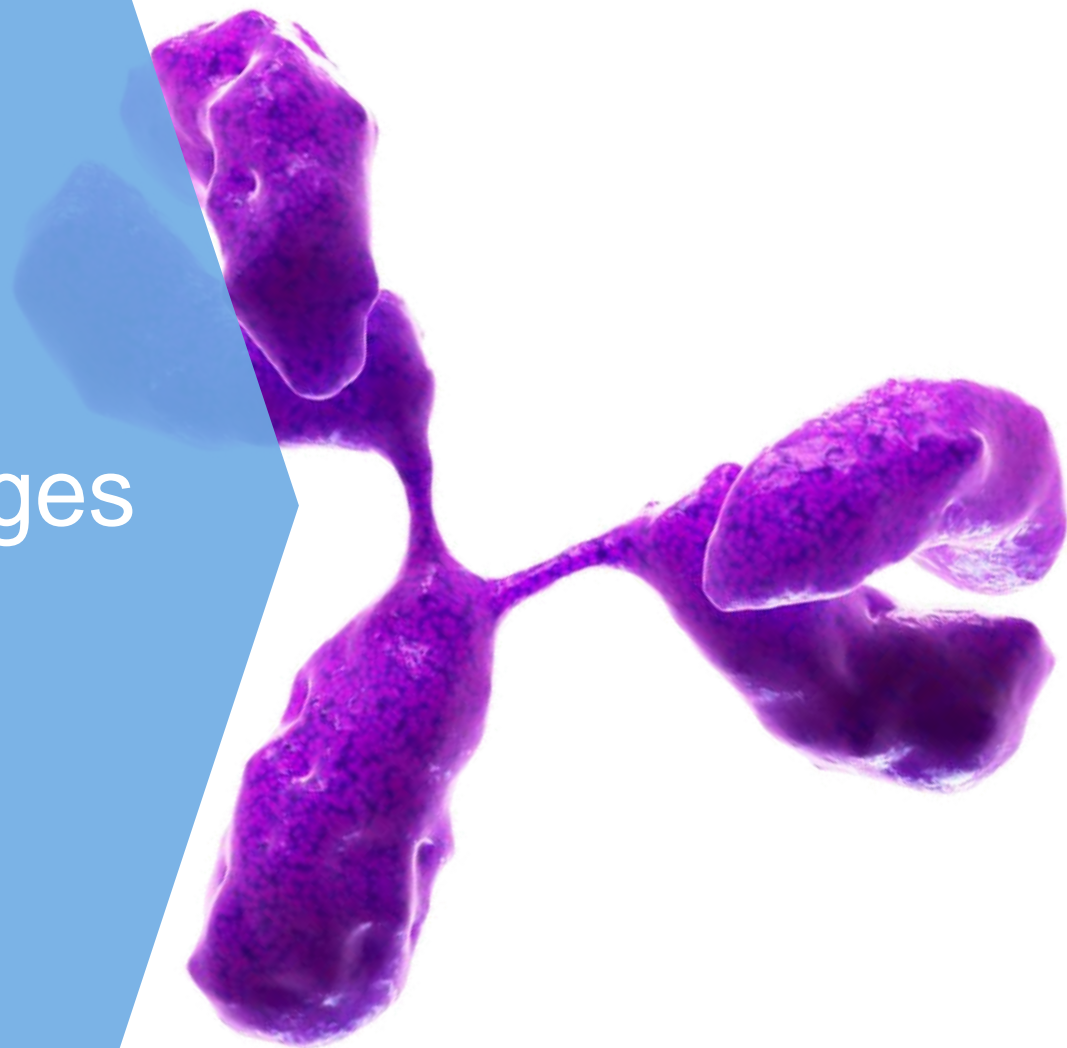
## 2. **Biopharma toolkit**

- From vial to file, what tools do we need?

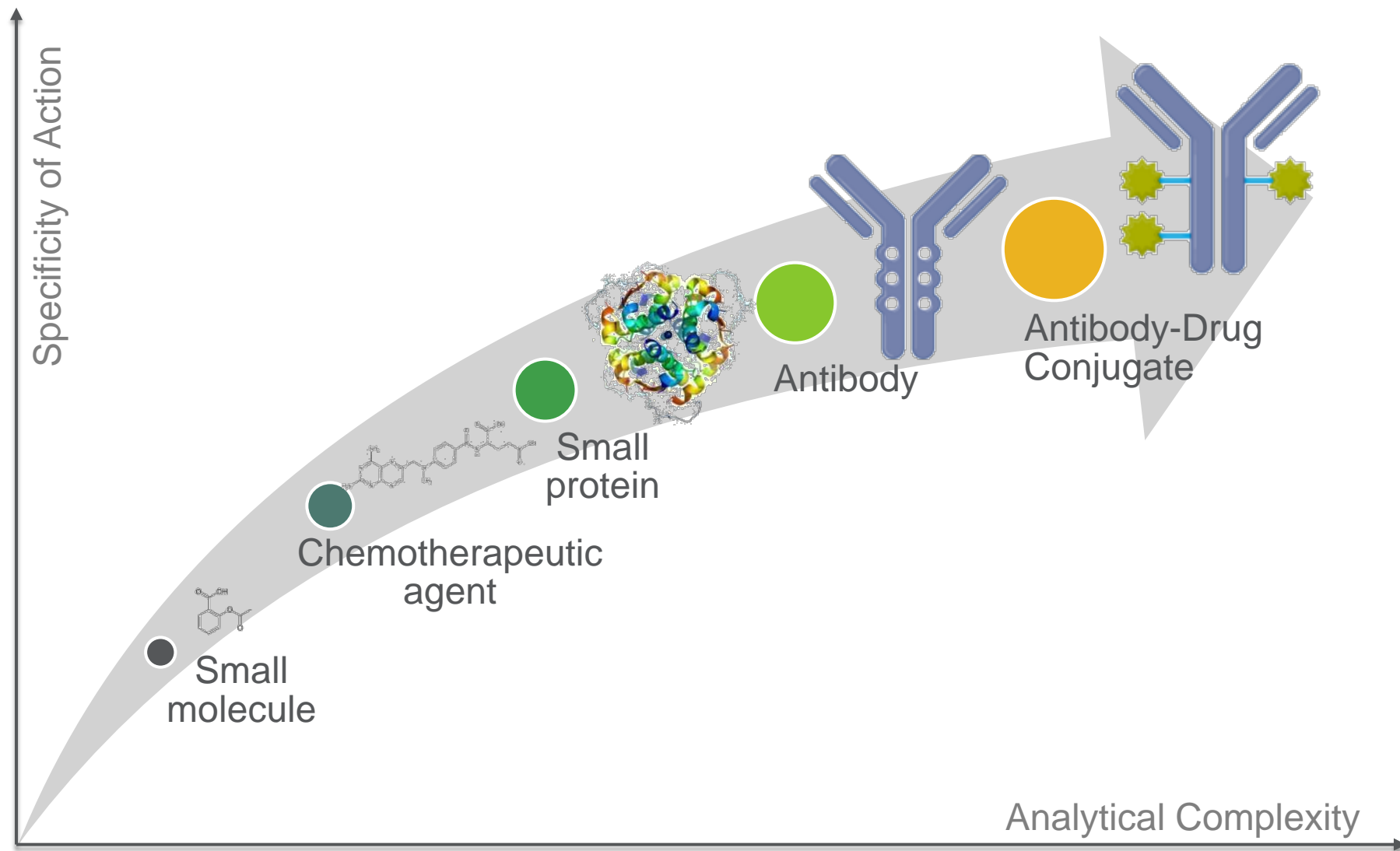
## 3. **Biopharma workflows**

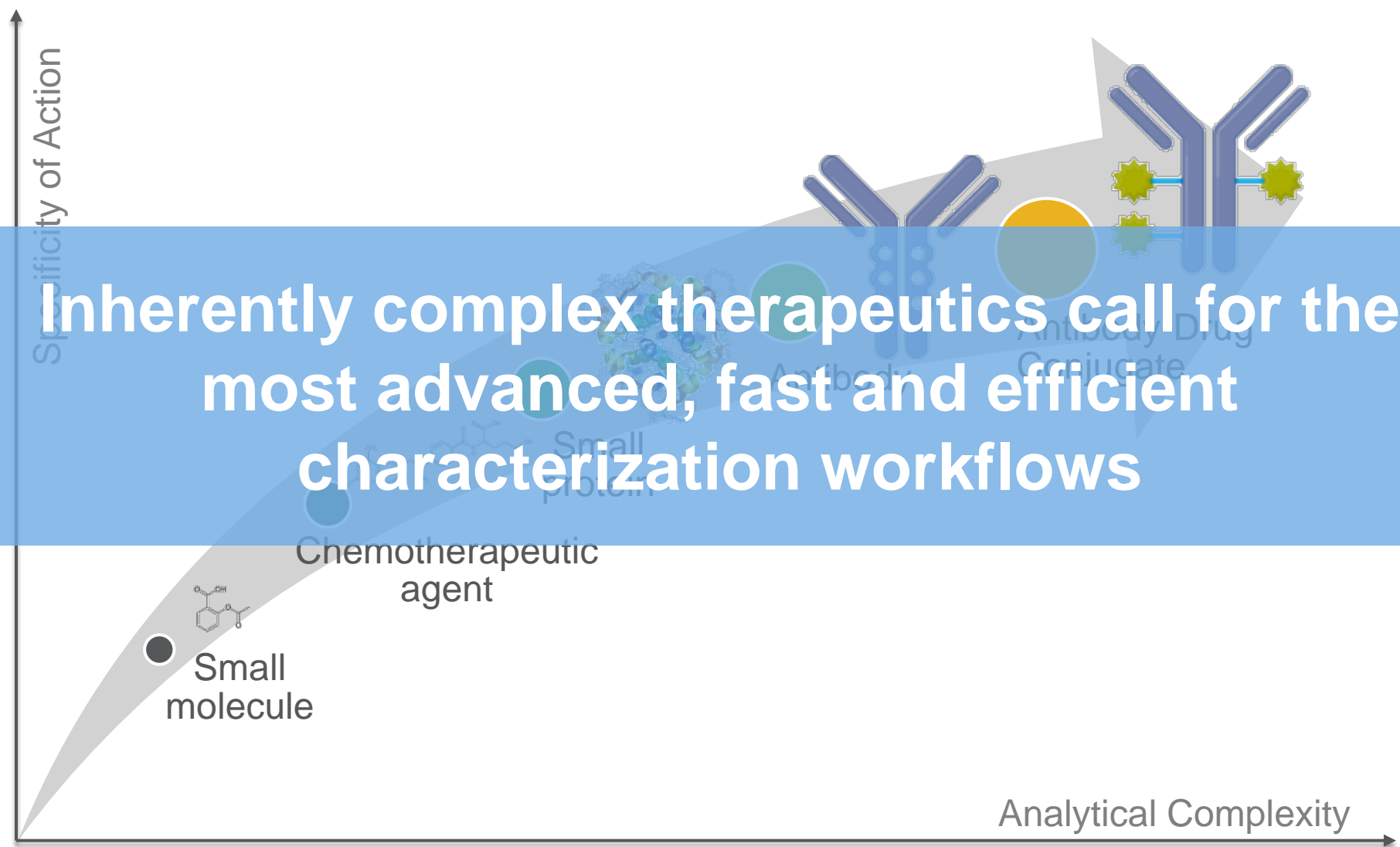
- 5 fundamental workflows
- Focus on peptide mapping & intact

# 1. Biotherapeutic Analytical Challenges



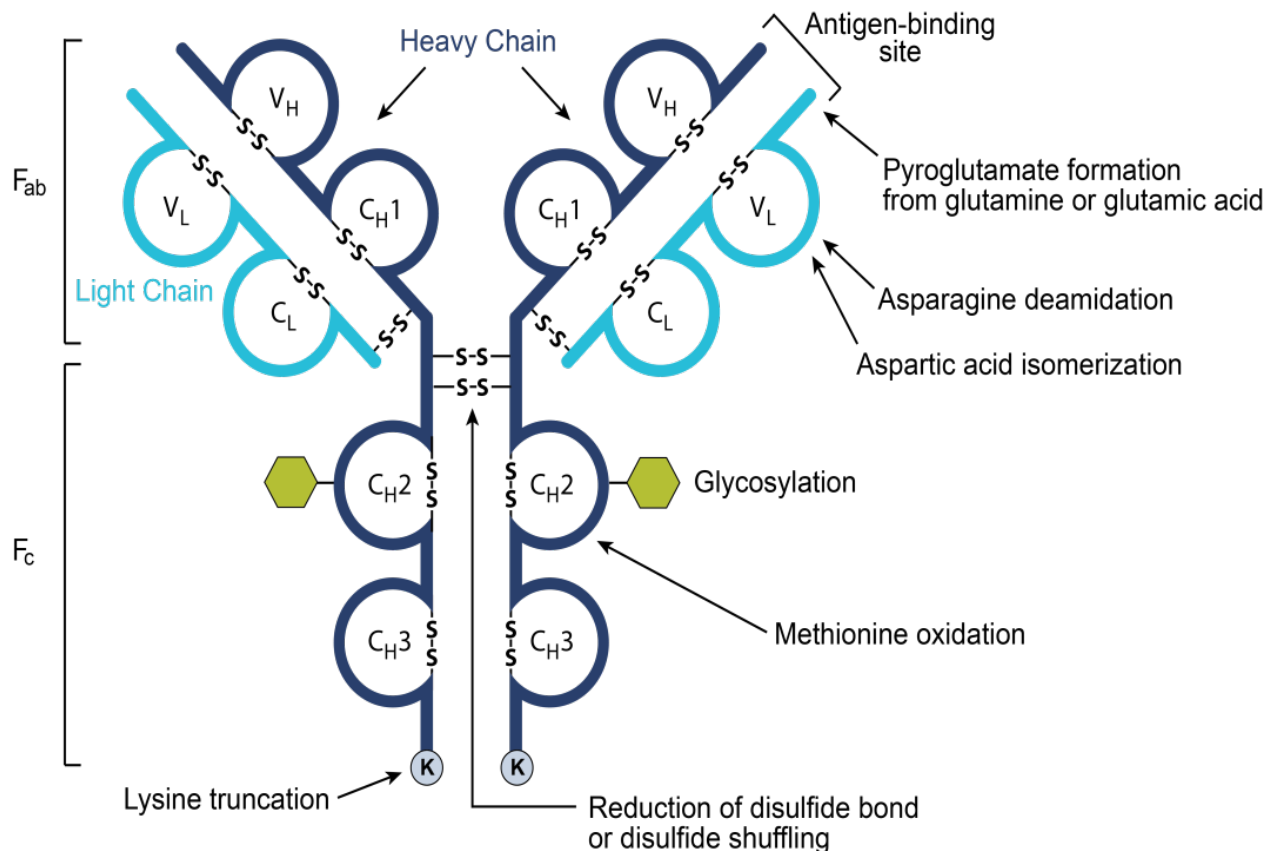
# Biotherapeutics - The Evolution of Analytical Challenges





- Biologically produced, heterogeneous
- Consistent , controlled biomanufacturing
- Monitoring of critical quality attributes (CQAs)
- Routine CQA measurements require the right tools:
  - Sample preparation and reagents
  - UHPLC - separations
  - Mass spectrometry & other methods of detection
  - Intuitive workflow-driven software

# Structure and Heterogeneity



# Structure and Heterogeneity

## CQAs

Deamidation

Oxidation

Disulfide bond scrambling

Sequence truncation

Change in glycosylation

Low abundance host cell proteins

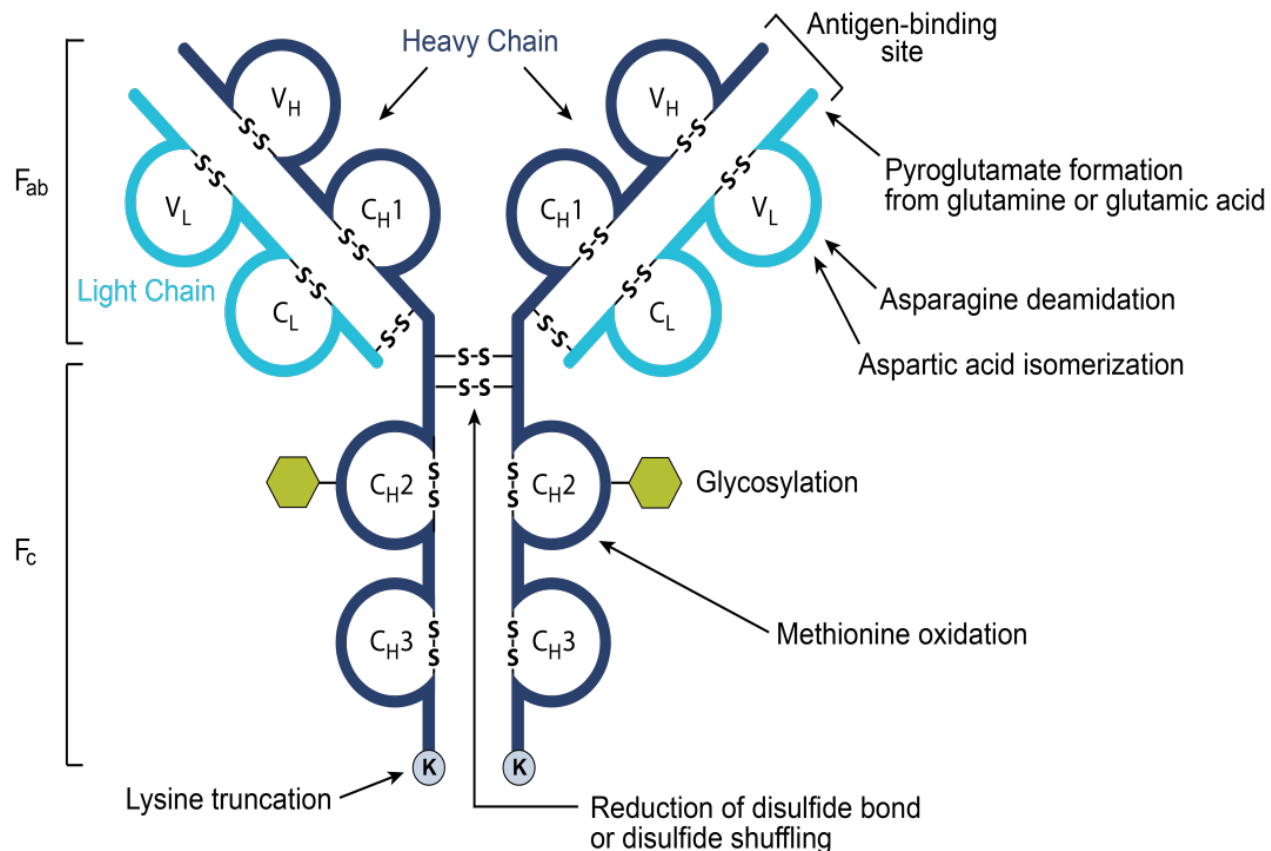
Low abundance sequence variants

Aggregation, protein complexes

Drug : Antibody Ratio

Linker measurements

Bound vs unbound drug and more!

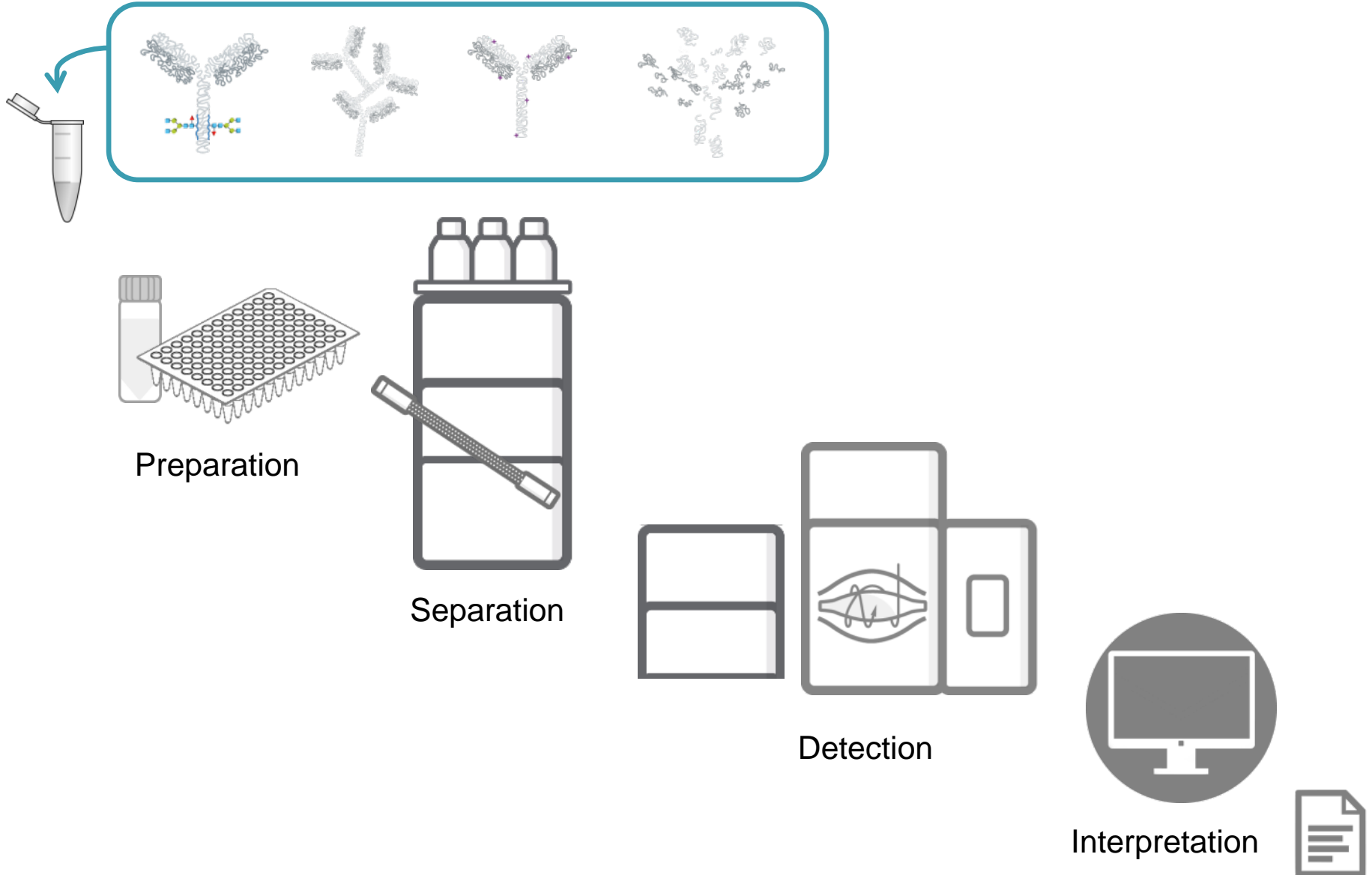




## 2. BioPharma Toolkit

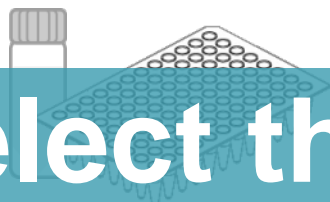


# Vial to File Workflows

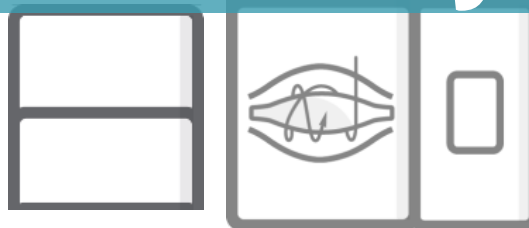




Select the most appropriate and advanced tools to stay ahead



Separation



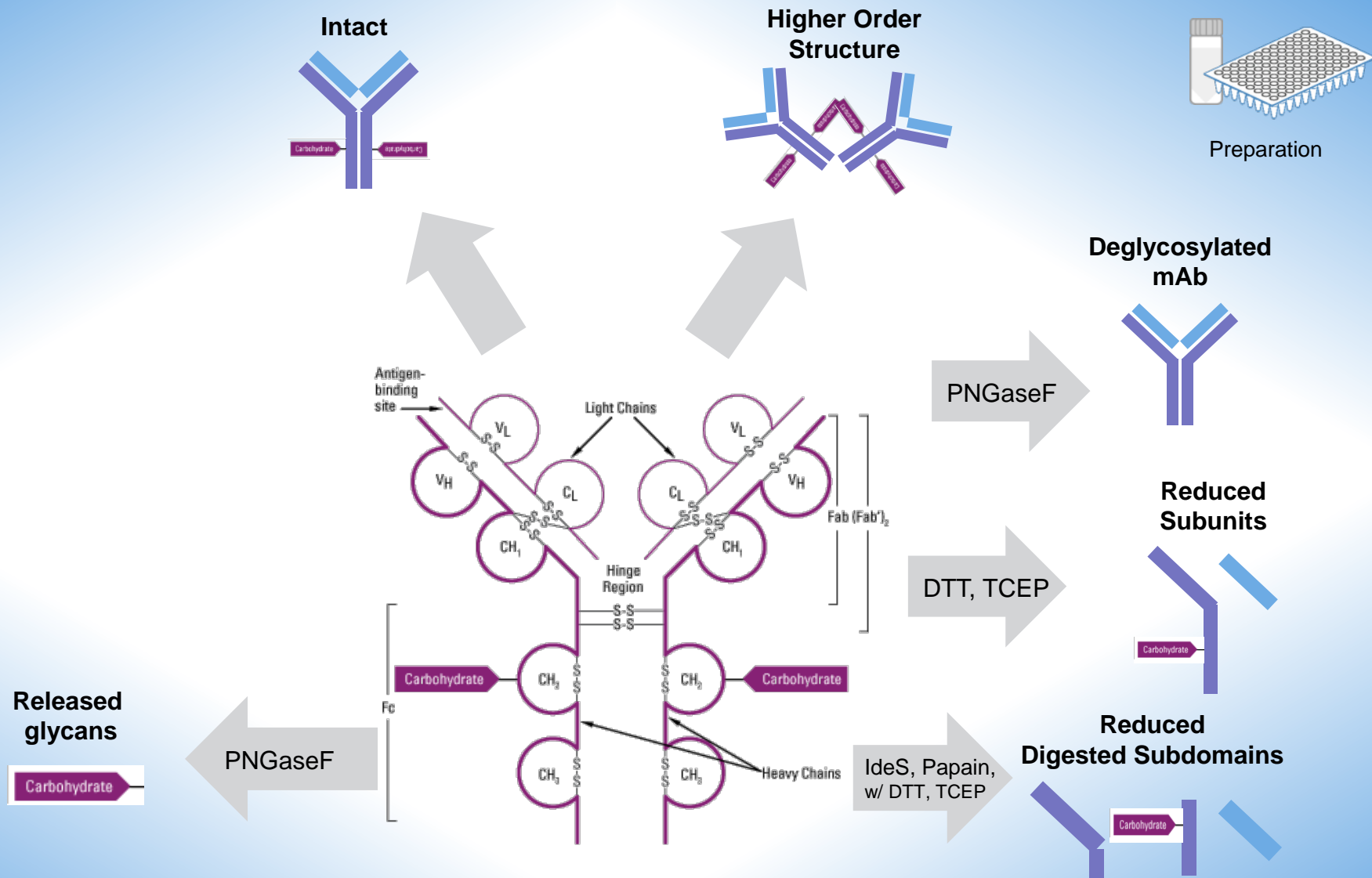
Detection



Interpretation

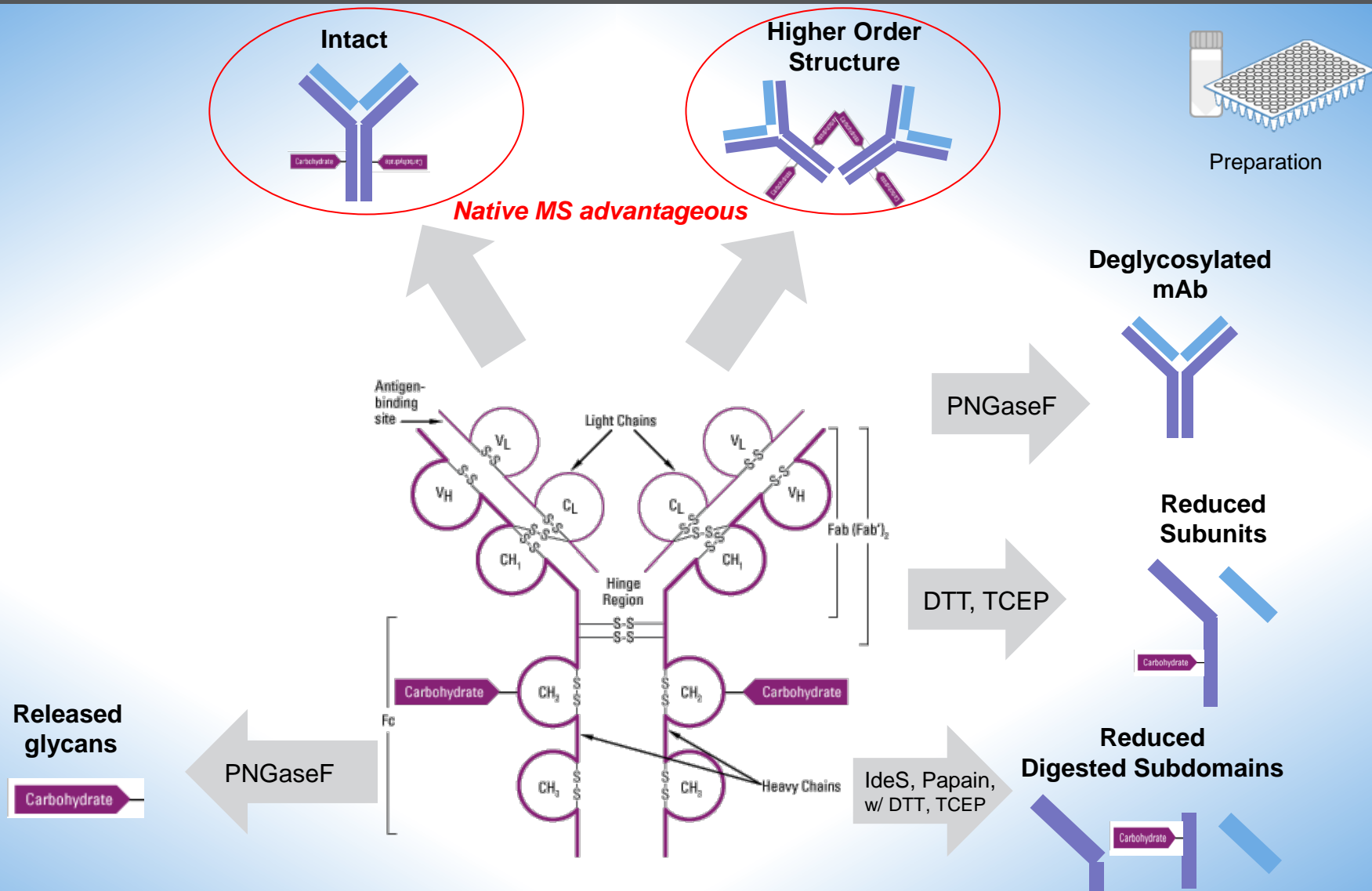


# Sample Preparation

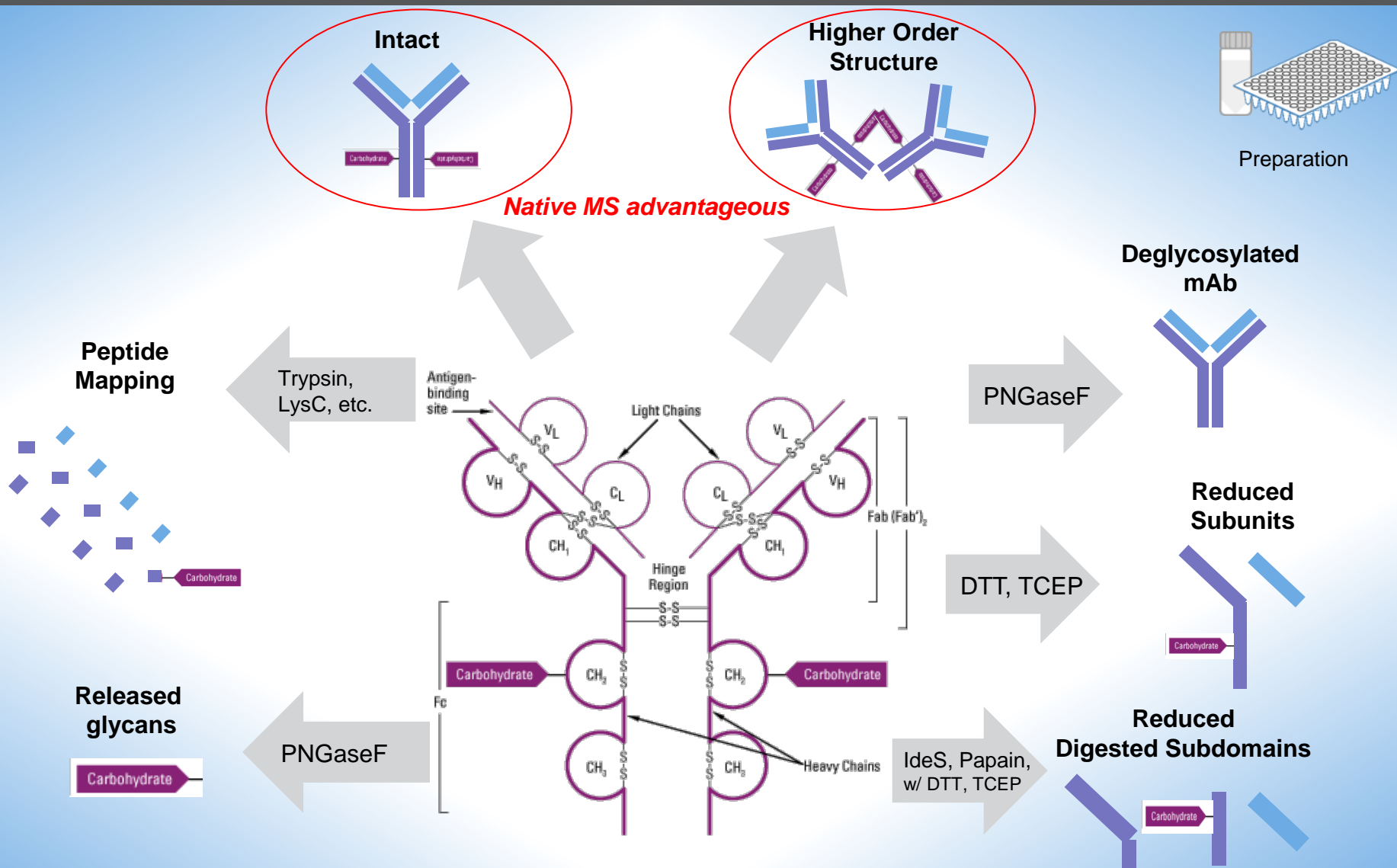




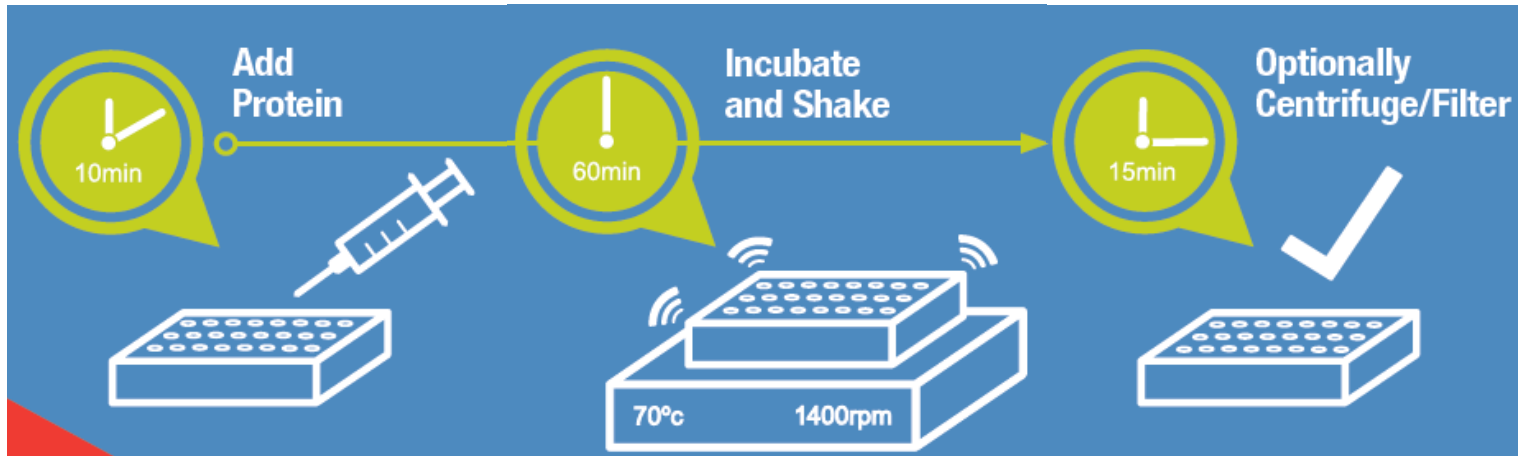
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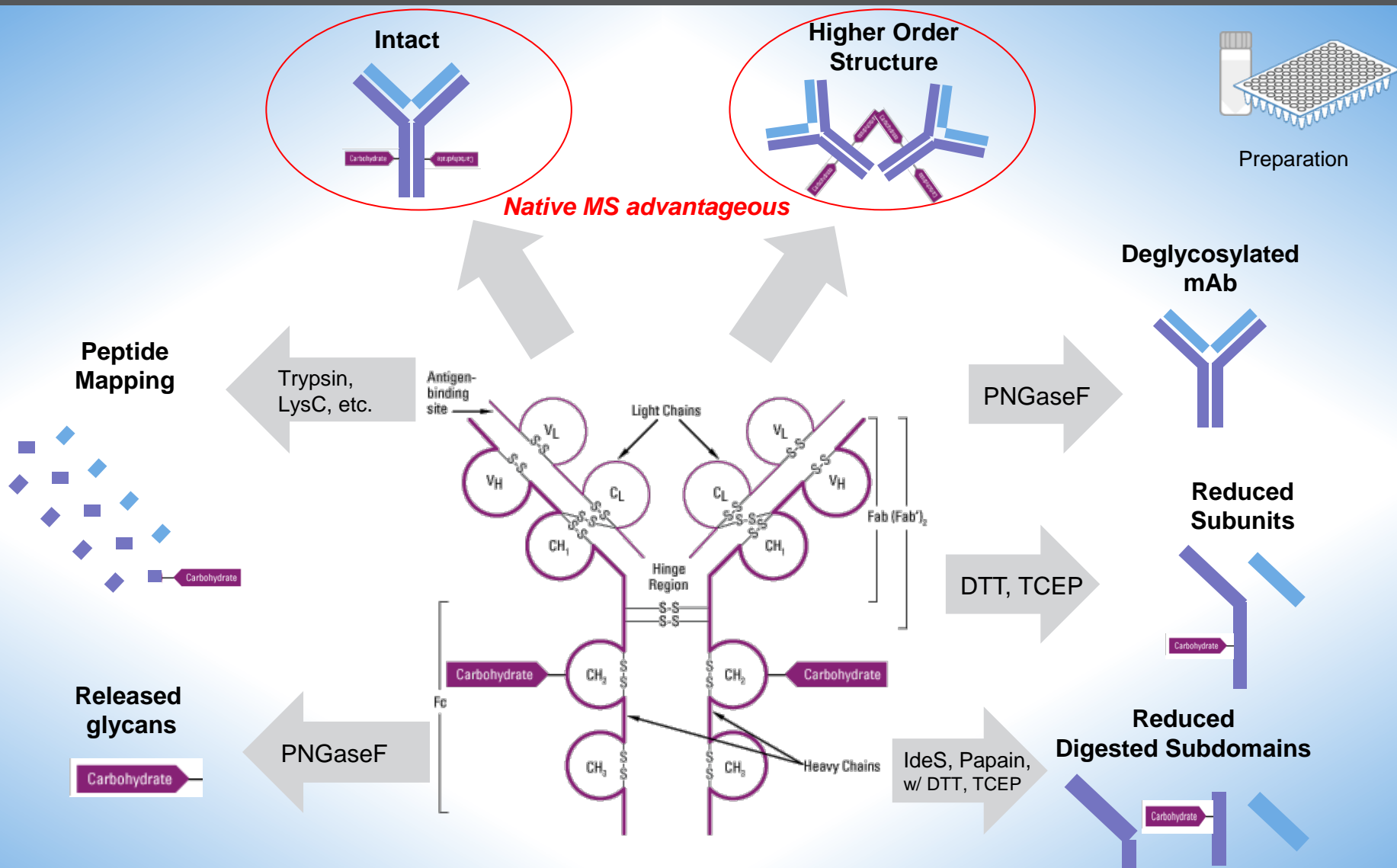
# Sample Preparation



# Thermo Scientific™ SMART™ Digest Kits



# Sample Preparation





# Sample Separation

## Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC System

New generation  
biocompatible UHPLC  
(quaternary pump, 1000 bar)



## Thermo Scientific™ Vanquish™ Flex Binary UHPLC System

New generation  
biocompatible UHPLC  
(binary pump, 1000 bar)



**NEW**

## Thermo Scientific™ Vanquish™ Horizon UHPLC System

New generation  
biocompatible high-end  
UHPLC system  
(binary pump, 1500 bar)



Separation

## Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC System

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## Thermo Scientific™ Vanquish™ Horizon UHPLC System

New generation  
biocompatible high-end  
UHPLC system  
(binary pump, 1500 bar)



Separation

# Cutting-edge fit-for-purpose UHPLC

NEW

# Vanquish: Built for Biopharma

- Tool-free fluidics
- Compact footprint
- Intuitive fluidic layout
- Maintenance-free valves
- Easy switching of modules



# Vanquish: Built for Biopharma

- Tool-free fluidics
- Compact footprint
- Intuitive fluidic layout
- Maintenance-free valves
- Easy switching of modules



- Precise flow rate and gradient delivery
- Ultra low pump pressure ripple
- Thermo Scientific™ LightPipe™ Flow Cell
- Accurate temperature control
- SmartInject



# Vanquish: Built for Biopharma

- Tool-free fluidics
- Compact footprint
- Intuitive fluidic layout
- Maintenance-free valves
- Easy switching of modules

- Customizable gradient delay volume
- Multiple heating modes
- Large sample capacity
- Broad flow rate and temperature range
- 4 x detectors and simple MS integration
- Supports long column capacity & all major chemistries



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- Precise flow rate and gradient delivery
- Ultra low pump pressure ripple
- Thermo Scientific™ LightPipe™ Flow Cell
- Accurate temperature control
- SmartInject

- Barcode reader
- Dry-air sample cooling
- Dosage precision
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) Software

## Simplicity

- Tool-free fluidics
- Compact footprint
- Intuitive fluidic layout
- Maintenance-free design
- Easy switching of modules

## Performance

- Precise flow rate and gradient delivery
- Ultra low pump pressure (1000 psi)
- SmartInject™ LightPipe™ Flow Cell
- Accurate temperature control
- SmartInject

## Biopharma

## Versatility

- Customizable gradient delay volume
- Multiple heating modes
- Large sample capacity
- Broad flow rate and temperature range
- 4 x detectors and simple MS integration
- Supports long column capacity & all major chemistries

## Confidence

- Barcode reader
- Dry-air sample cooling
- Dosage precision
- Thermo Scientific™ Chromatography Data System (CDS) Software

# Integration into the Biopharma Laboratory

- AppsLab library
- Columns, Prep and Thermo Scientific™ Viper™ finger-tight connections
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) Software
- Fully integrated mass spectrometry solutions
- Seamless method transfer





# Bio LC Column Chemistries

## Proteins / Peptides

### Thermo Scientific™ ProPac™ Columns

ProPac Ion Exchange

ProPac HIC-10

ProPac IMAC

ProPac WCX-10

### Thermo Scientific™ ProSwift™ / PepSwift™ Columns

ProSwift Ion Exchange

ProSwift Rev Phase

ProSwift Con A

PepSwift Rev Phase

### Thermo Scientific™ Acclaim™ & Accucore™ Columns

BioBasic

Acclaim PepMap

Acclaim 300

Acclaim 120

Thermo Scientific™ MSPac™ DS-10

## MAbs

### Thermo Scientific™ MAbPac™ Columns

MAbPac SCX-10

MAbPac SEC

MAbPac Protein A

pH Buffers

MAbPac HIC-10, HIC-20, HIC-Butyl

MAbPac RP

## Glycans

### Thermo Scientific™ GlycanPac™ & Accucore™ Columns

GlycanPac AXH-1/AXR-1

Accucore Amide HILIC

## Nucleic Acids

### Thermo Scientific™ DNAPac™ Column

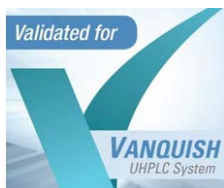
DNAPac 100, 200

DNASwift

DNAPac RP



Separation



# Bio LC Column Chemistries

## Proteins / Peptides

### Thermo Scientific™ ProPac™ Columns

ProPac Ion Exchange

ProPac HIC-10

ProPac IMAC

ProPac WCX-10

### Thermo Scientific™ ProSwift™ / PepSwift™ Columns

ProSwift Ion Exchange

ProSwift Rev Phase

ProSwift Con A

PepSwift Rev Phase

### Thermo Scientific™ Acclaim™ & Accucore™ Columns

BioBasic

Acclaim PepMap

Acclaim 300

Acclaim 120

Thermo Scientific™ MSPac™ DS-10

## MAbs

### Thermo Scientific™ MAbPac™ Columns

MAbPac SCX-10

MAbPac SEC

MAbPac Protein A

pH Buffers

MAbPac HIC-10, HIC-20, HIC-Butyl

MAbPac RP

NEW

## Glycans

### Thermo Scientific™ GlycanPac™ & Accucore™ Columns

GlycanPac AXH-1/AXR-1

Accucore Amide HILIC



Separation

## Nucleic Acids

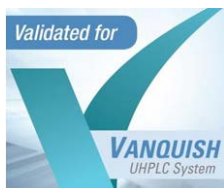
### Thermo Scientific™ DNAPac™ Column

DNAPac 100, 200

DNASwift

DNAPac RP

NEW



# Bio LC Column Chemistries

Proteins / Peptides

MAbs

Glycans

Thermo Scientific™  
ProPac™  
Columns

Thermo Scientific™  
GlycanPac™

Thermo Scientific™  
Accucore™

Thermo Scientific™  
GlycanPac™ &  
Accucore™  
Columns



Separation

## Thermo Scientific™ CX-1 pH Gradient Buffers

Ideal for charge variant separations

Easy, fast, reproducible pH gradients

ProPac Ion  
Exchange

ProPac  
HIC-10

ProPac  
IMAC

ProPac  
WCX-10

GlycanPac  
1-1/AXR-1

Accucore  
HILIC

Organic Acids

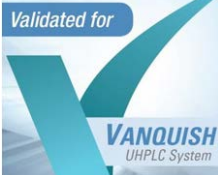
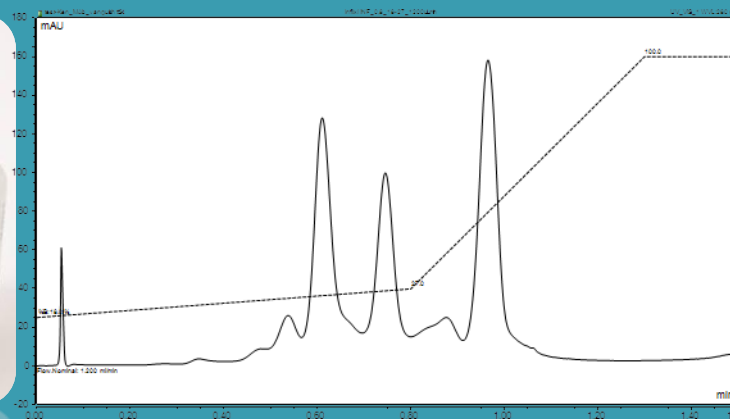
Thermo Scientific™  
GlycanPac™ Column

DNAPac 100, 200

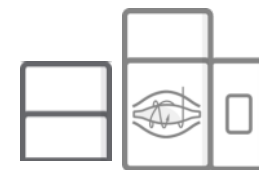
DNASwift

DNAPac RP

NEW



# Sample Detection



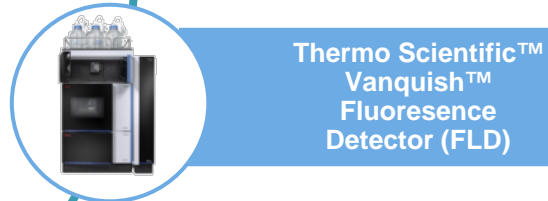
Detection



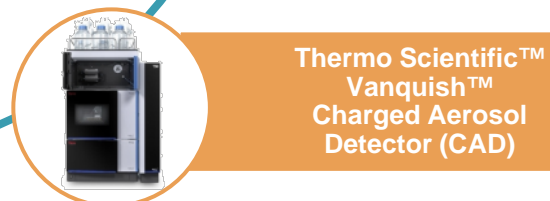
Thermo Scientific™  
Vanquish™ Diode  
Array Detector (DAD)



Thermo Scientific™  
Vanquish™ Variable  
Wavelength Detector  
(VWD)

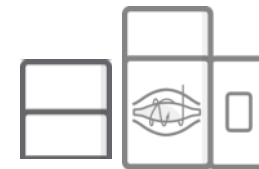


Thermo Scientific™  
Vanquish™  
Fluorescence  
Detector (FLD)



Thermo Scientific™  
Vanquish™  
Charged Aerosol  
Detector (CAD)

- 4 modular detector options
- Can be used discretely or in series
  1. DAD with LightPipe technology outstanding signal to noise performance and linearity
  2. VWD supporting a wide operating range
  3. FLD with high detection precision
  4. CAD offers a sensitive, universal detection with a near uniform response



Detection



Thermo Scientific™  
Vanquish™ Diode  
Array Detector (DAD)



Thermo Scientific™  
Vanquish™ Variable  
Wavelength Detector  
(VWD)



Thermo Scientific™  
Vanquish™  
Fluorescence  
Detector (FLD)



Thermo Scientific™  
Vanquish™  
Charged Aerosol  
Detector (CAD)

- 4 modular detector options
- Can be used discretely or in series

## Sensitivity, linearity, and the flexibility to switch

1. DAD with LightPipe technology cuts on-line signal noise for performance and linearity
2. VWD supporting a wide operating range
3. FLD with high detection precision
4. CAD offers a sensitive, universal detection with a near uniform response



# MS Tools for Major Biopharma Characterization Workflows



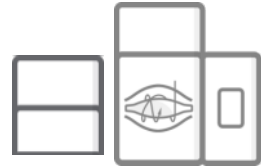
Dedicated connection kit



SII for Xcalibur



Chromeleon

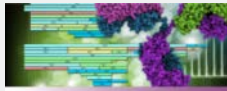


Detection

## Thermo Scientific™ Q Exactive™ MS Family



For all the routine characterization workflows: intact/top-down, bottom-up, glycan, etc. qualitative and quantitative.



BioPharma Finder™ 1.0  
Mass Informatics Platform for Protein Characterization



ProSightPC™ 3.0  
Software by Protein Technologies

## Thermo Scientific™ Exactive™ Plus EMR MS



Native MS to analyze large protein complex while preserving non-covalent interactions: antibody-antigen complex, ADCs, antibody mixtures, etc.



BioPharma Finder™ 1.0  
Mass Informatics Platform for Protein Characterization

## Thermo Scientific™ Orbitrap Fusion™ Tribrid™ MS Family



From routine to the most challenging tasks. The most advanced technology that provides the most comprehensive solutions.



BioPharma Finder™ 1.0 SPI  
Mass Informatics Platform for Protein Characterization



ProSightPC™ 3.0  
Software by Protein Technologies



# MS Tools for Major Biopharma Characterization Workflows



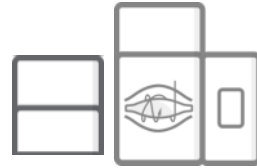
Dedicated connection kit



SII for Xcalibur



Chromeleon



Detection

NEW

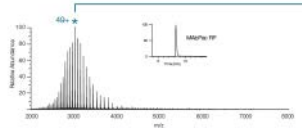


Q Exactive BioPharma

1. Denatured and native MS intact analysis with the new High Mass Range (HMR) mode
2. Subunit and top/middle-down analysis with Protein mode
3. Peptide mapping with Standard mode

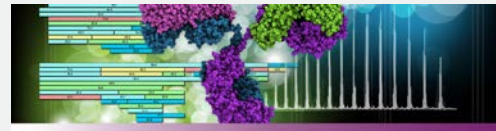
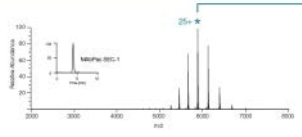
## Denaturing

Conventional denaturing conditions, including mobile phase containing organic and acidic pH, result in protein unfolding and increased protonation. Denaturing MS is compatible with reverse phase chromatography, which allows sensitive, salt-free conditions for identifying intact proteins at lower m/z ranges.



## Native

Native MS conditions using volatile salt-based buffers at physiological pH retain compact or native conformations resulting in lower charge states. Native MS is compatible with size exclusion chromatography for observation of intact proteins at high m/z ranges. This strategy reduces mass interferences in complex protein spectra by increasing peak capacity in m/z space.



BioPharma Finder™ 1.0 SP1  
Mass Informatics Platform for Protein Characterization

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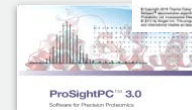
## Thermo Scientific™ Orbitrap Fusion™ Tribrid™ MS Family



From routine to the most challenging tasks. The most advanced technology that provides the most comprehensive solutions.



BioPharma Finder™ 1.0 SP1  
Mass Informatics Platform for Protein Characterization



ProSightPC™ 3.0  
Software for Protein Research



# MS Tools for Major Biopharma Characterization Workflows



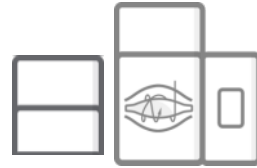
Dedicated connection kit



SII for Xcalibur



Chromeleon



Detection

NEW

Thermo Scientific™  
Orbitrap Fusion™ Tribrid™  
MS Family

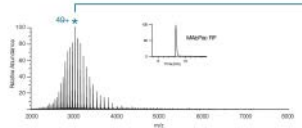
## Simple, stress-free MS connection

1. Denatured and native MS intact analysis with the new High Mass Range (HMR) mode
2. Subunit and top/middle-down analysis with Protein mode
3. Peptide mapping with Standard mode

From routine to the most challenging tasks. The most advanced technology that provides the most comprehensive solutions.

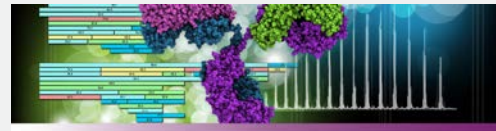
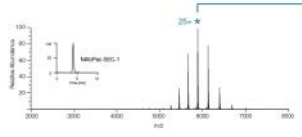
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BioPharma Finder™ 1.0 SP1  
Mass Informatics Platform for Protein Characterization

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BioPharma Finder™ 1.0 SP1  
Mass Informatics Platform for Protein Characterization

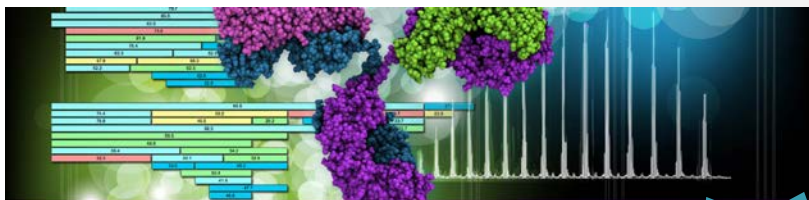


ProSightPC™ 3.0  
Software for Protein Research



## Thermo Scientific™ BioPharma Finder™ Software

- Intact protein analysis & peptide mapping in **one package**
- Orbitrap™ & IT-based instruments
- Protein Sequence Manager



**BioPharma Finder™ 1.0 SP1**  
*Mass Informatics Platform for Protein Characterization*

NEW

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Probability Ltd. Incorporates MassAnalyzer software, copyright  
© 2012 by Amgen Inc. This program is protected by copyright law  
and international treaties as described in the Help About.

**Thermo**  
SCIENTIFIC

## Chromeleon CDS

- Increase lab productivity via eWorkflows
- Control all of your lab, including LC/IC/GC/MS & >300 3<sup>rd</sup> party modules
- Operate in a regulated environment
- Connect your lab



**ThermoFisher**  
SCIENTIFIC



## Thermo Scientific™ BioPharma Finder™ Software

- Intact protein analysis & peptide mapping in **one package**
- Orbitrap™ & IT-based instruments
- Protein Sequence Manager



**BioPharma Finder™ 1.0 SP1**  
*Mass Informatics Platform for Protein Characterization*



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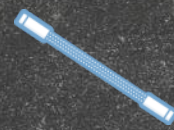
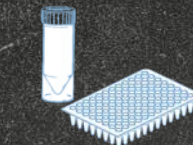
**Thermo**  
SCIENTIFIC

## Chromeleon CDS

- Increase lab productivity via eWorkflows
- Control all of your lab, including LC/IC/GC/MS & >300 3<sup>rd</sup> party modules
- Operate in a regulated environment
- Connect your lab




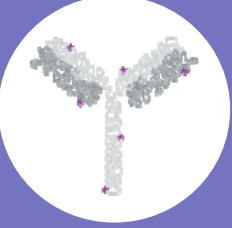
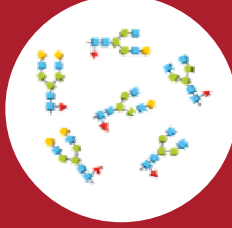


# 3. Biopharma Workflows

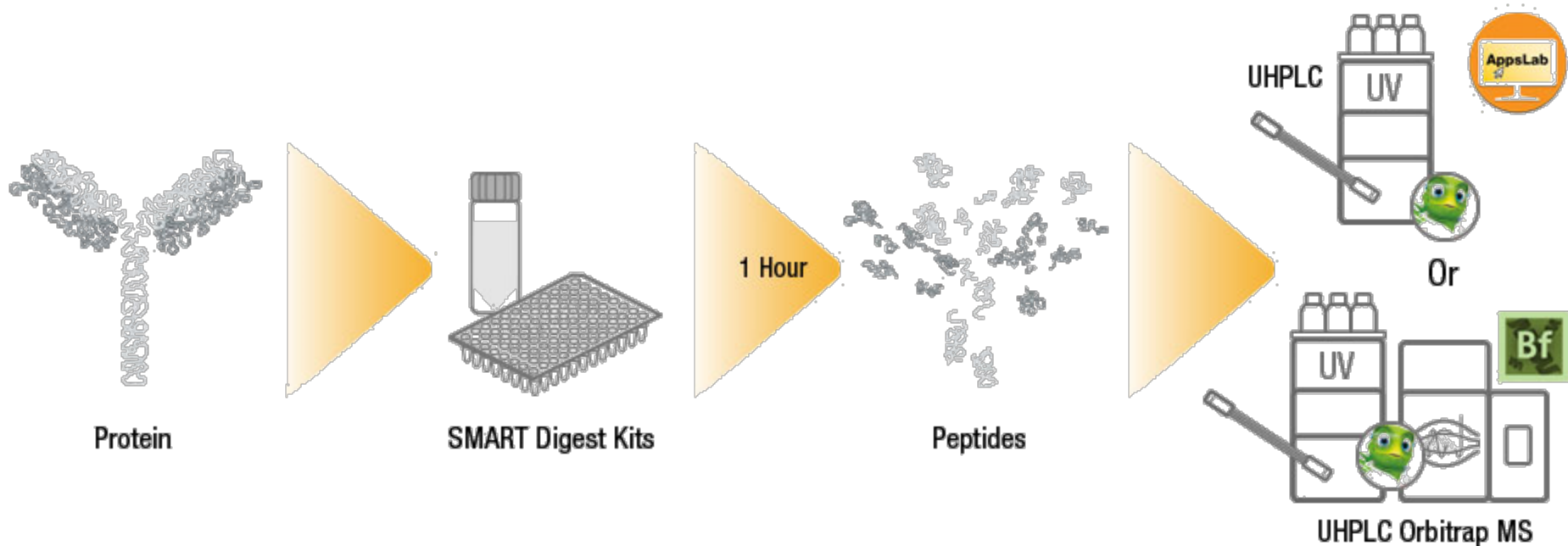




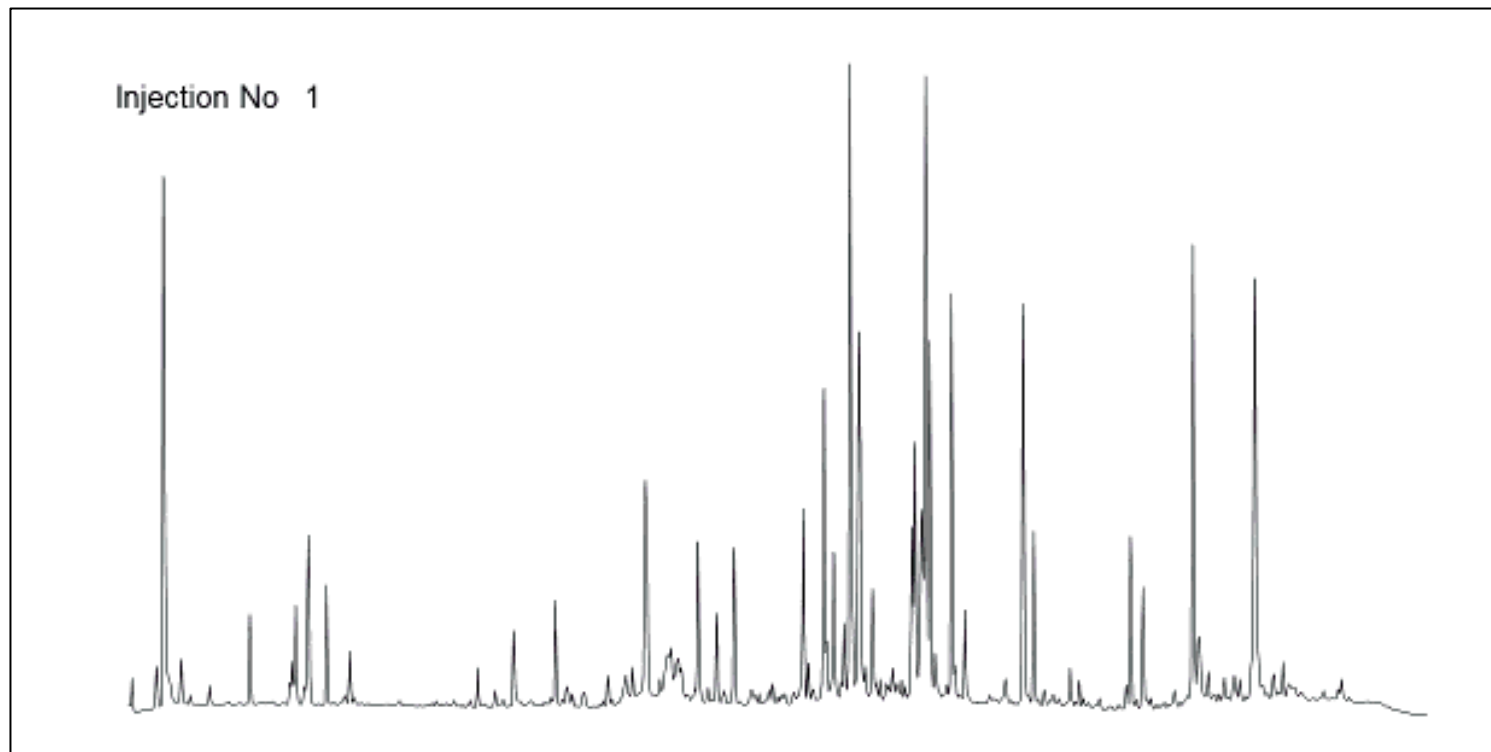
# 5 Fundamental Workflows

				
<b>Peptide mapping</b>	<b>Intact &amp; subunit analysis</b>	<b>Aggregation screening</b>	<b>Charge variant screening</b>	<b>Glycan analysis</b>
RP	RP HIC	SEC	IEX	HILIC Mixed Mode
Confirm sequence	Check purity of the antibody	Check monomer vs Aggregates	Check charge variation within antibody sample	Check glycan presence and structure
UV and UV-MS	UV and UV-MS	UV and UV-MS	UV, FLD	FLD, FLD-MS, CAD
Analytical reproducibility Peak capacity	Biocompatible, gradient and thermostating capabilities	High salt condition, biocompatible, low dispersion, reproducibility	Biocompatible selectivity	Gradient capabilities

# Peptide Mapping Workflow

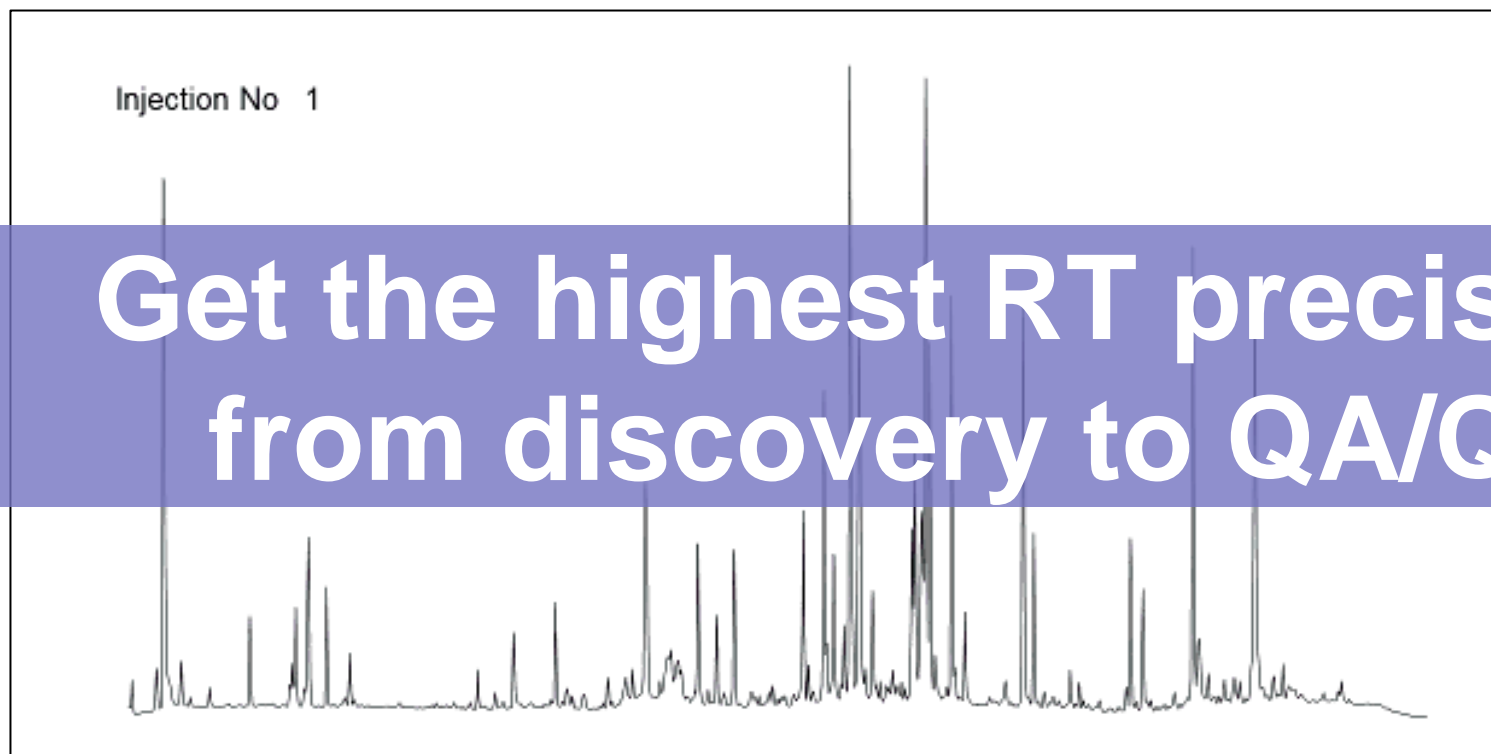


# Peptide Mapping - Reproducibly



Retention time Reproducibility		
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	<b>0.007</b>
55	17.969	0.011
61	18.546	0.010
83	20.798	0.010
85	21.095	0.012
87	22.386	<b>0.009</b>
96	24.774	0.012
103	26.155	<b>0.009</b>
106	26.155	<b>0.009</b>
109	27.529	0.010

Overlay of **13 consecutive chromatographic runs** of a peptide sample separated on an analytical Acclaim™ 120 C18 column and prepared from a mAb digested with SMART™ Digest Kit.

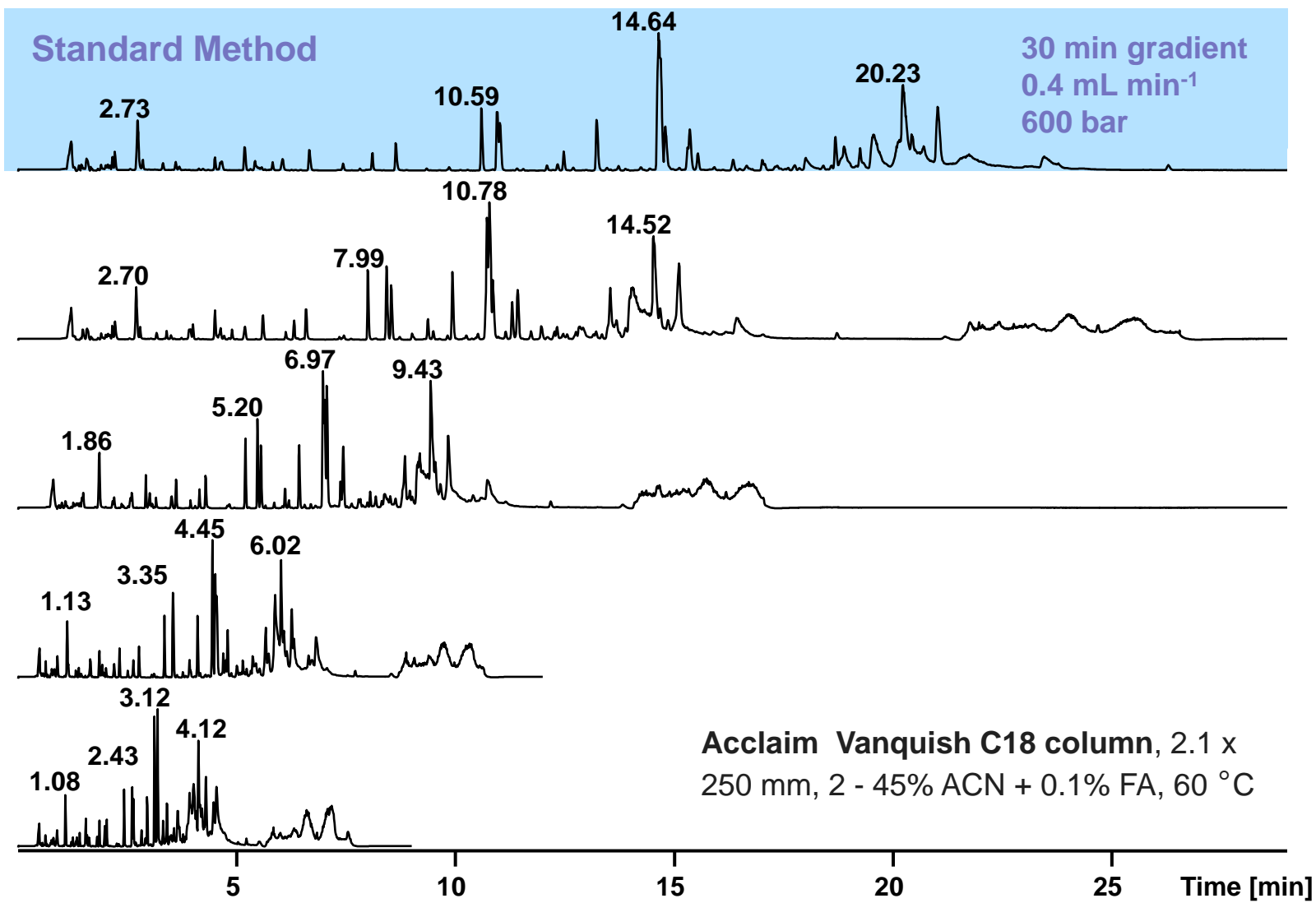


## Retention time Reproducibility

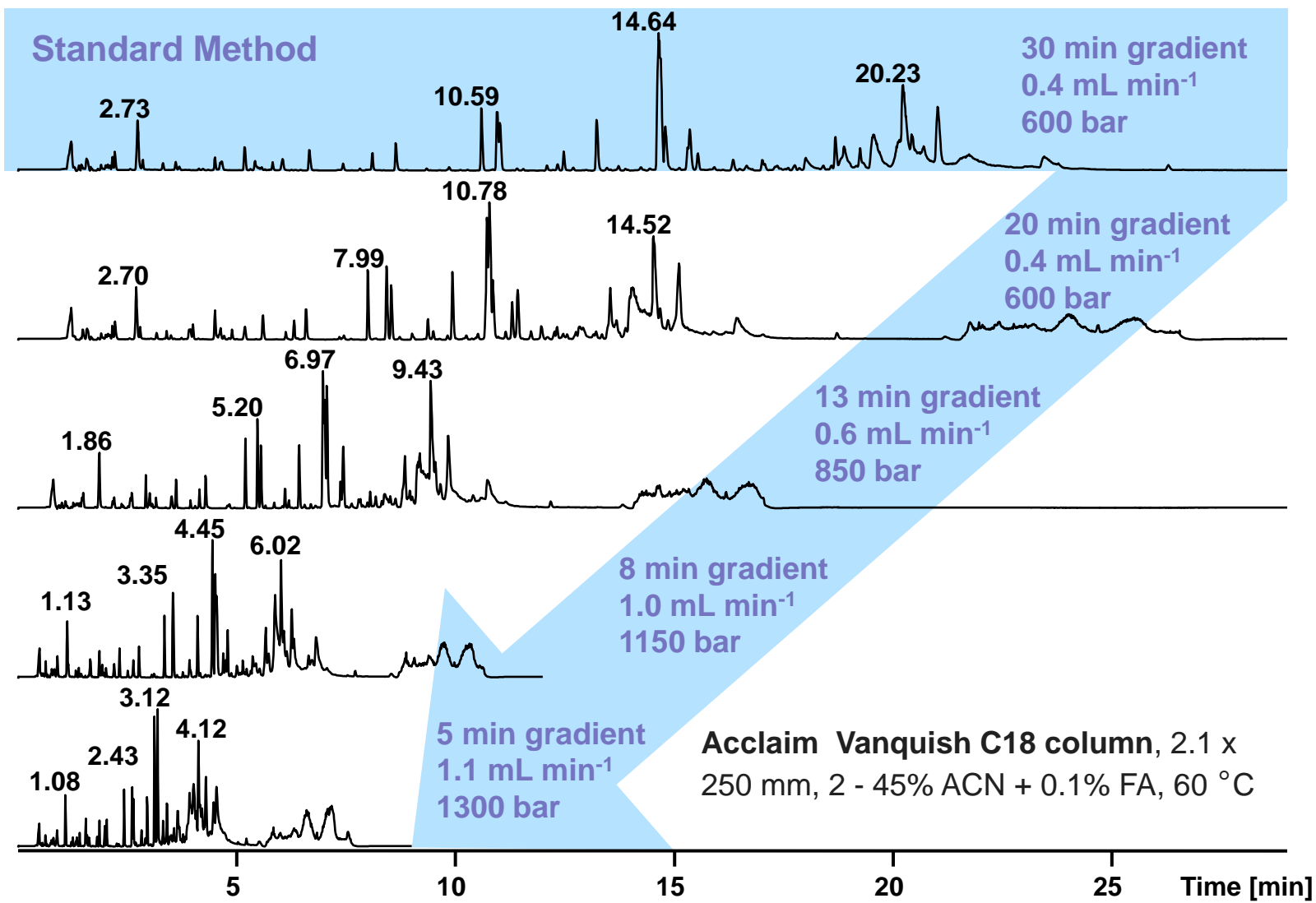
peak #	RT (min)	RSD (%)
3	3.315	0.082
9	5.231	0.065
14	6.532	0.017
15	6.937	0.023
17	10.290	0.021
23	12.713	0.012
31	14.011	0.013
39	15.177	0.012
42	15.589	0.010
51	17.511	<b>0.007</b>
55	17.969	0.011
61	18.546	0.010
83	20.798	0.010
85	21.095	0.012
87	22.386	<b>0.009</b>
96	24.774	0.012
103	26.155	<b>0.009</b>
106	26.155	<b>0.009</b>
109	27.529	0.010

Overlay of **13 consecutive chromatographic runs** of a peptide sample separated on an analytical Acclaim™ 120 C18 column and prepared from a mAb digested with SMART™ Digest Kit.

# Fast Peptide Mapping of Rituximab

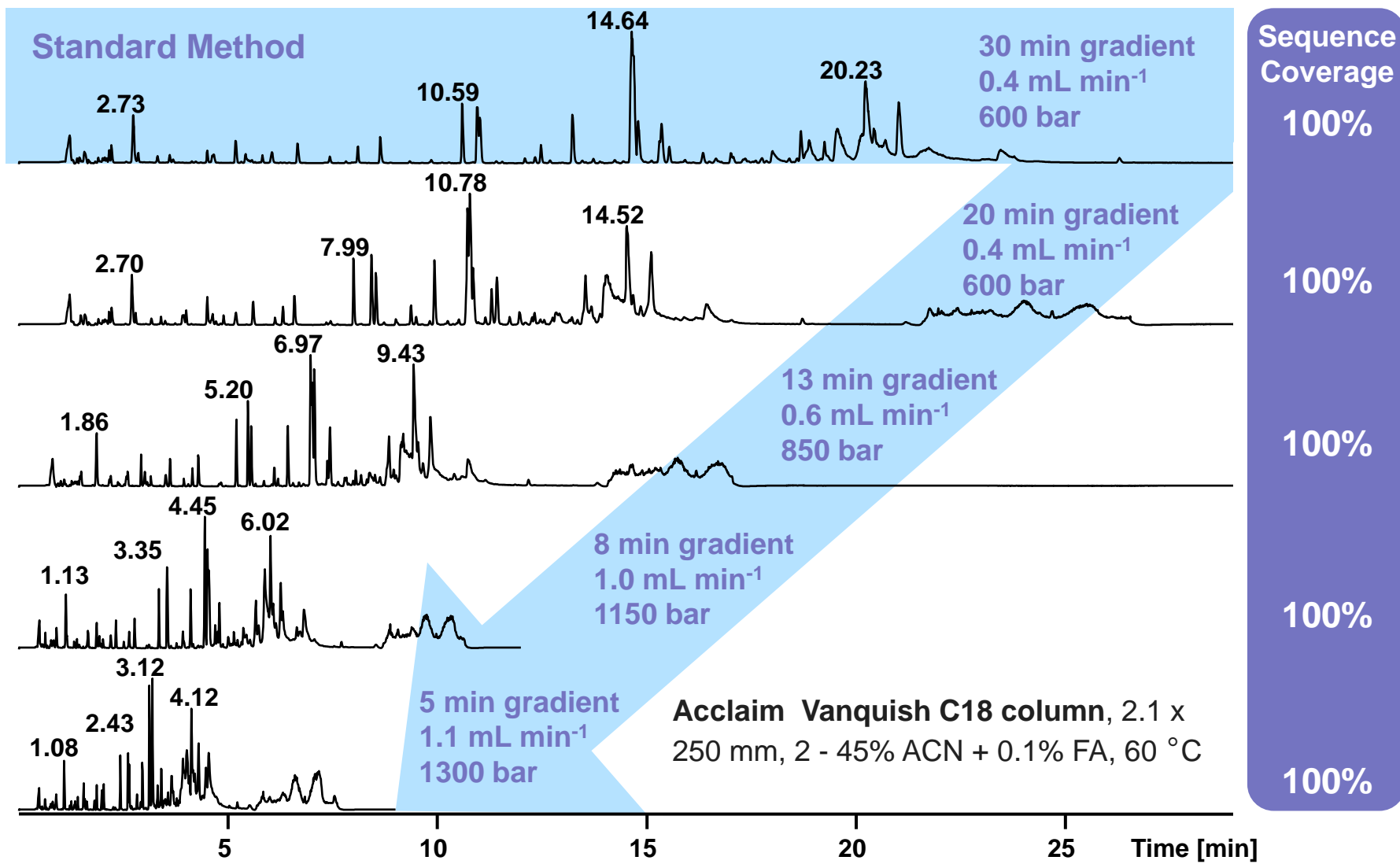


# Fast Peptide Mapping of Rituximab

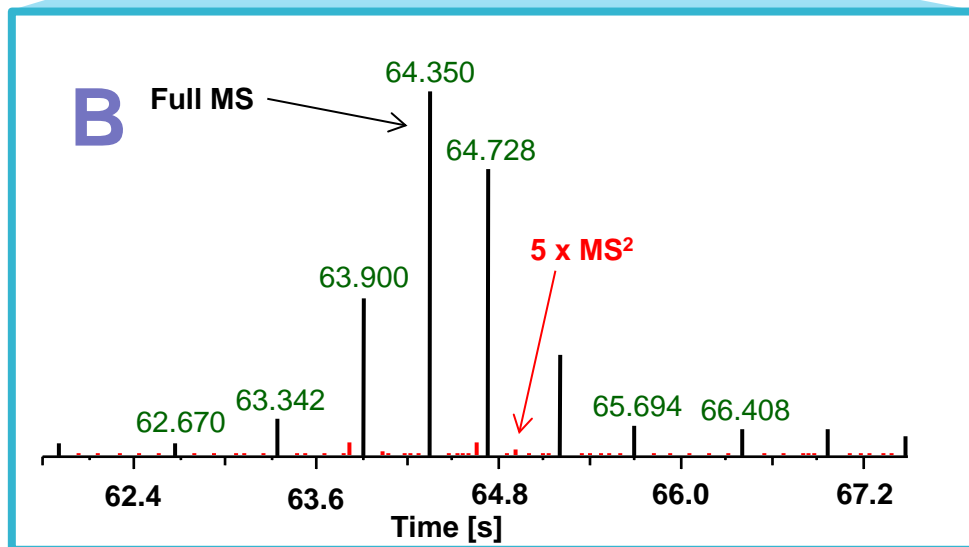
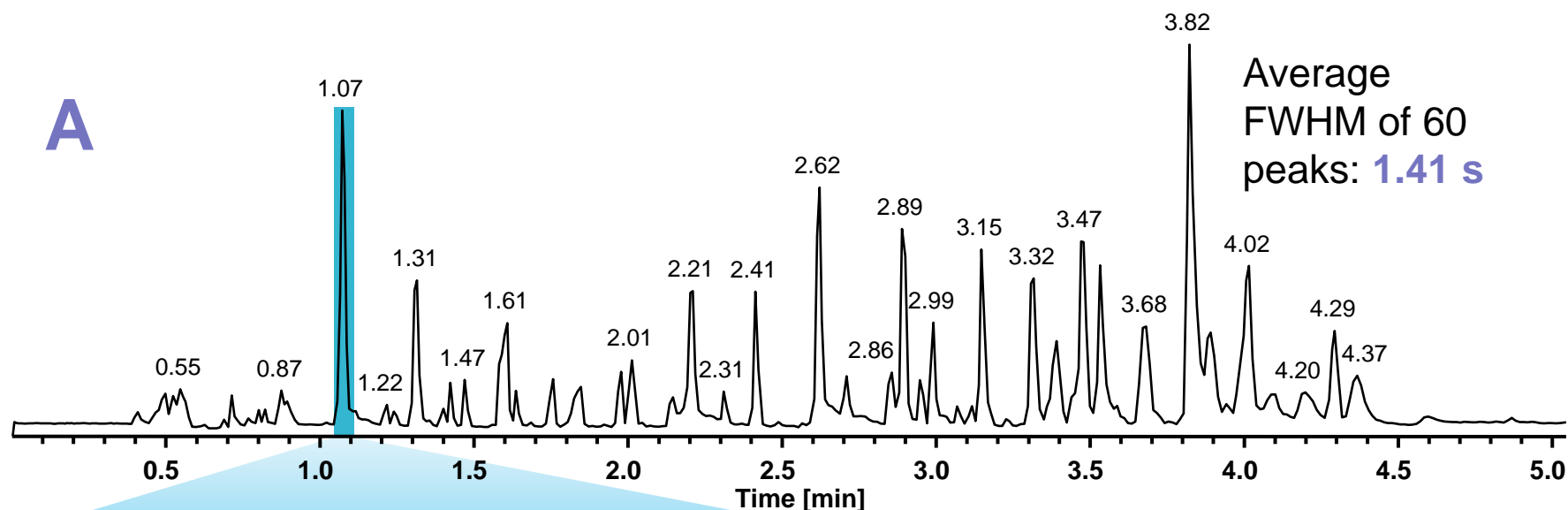




# Fast Peptide Mapping of Rituximab



# MS Data Point Distribution in Fast UHPLC Separations



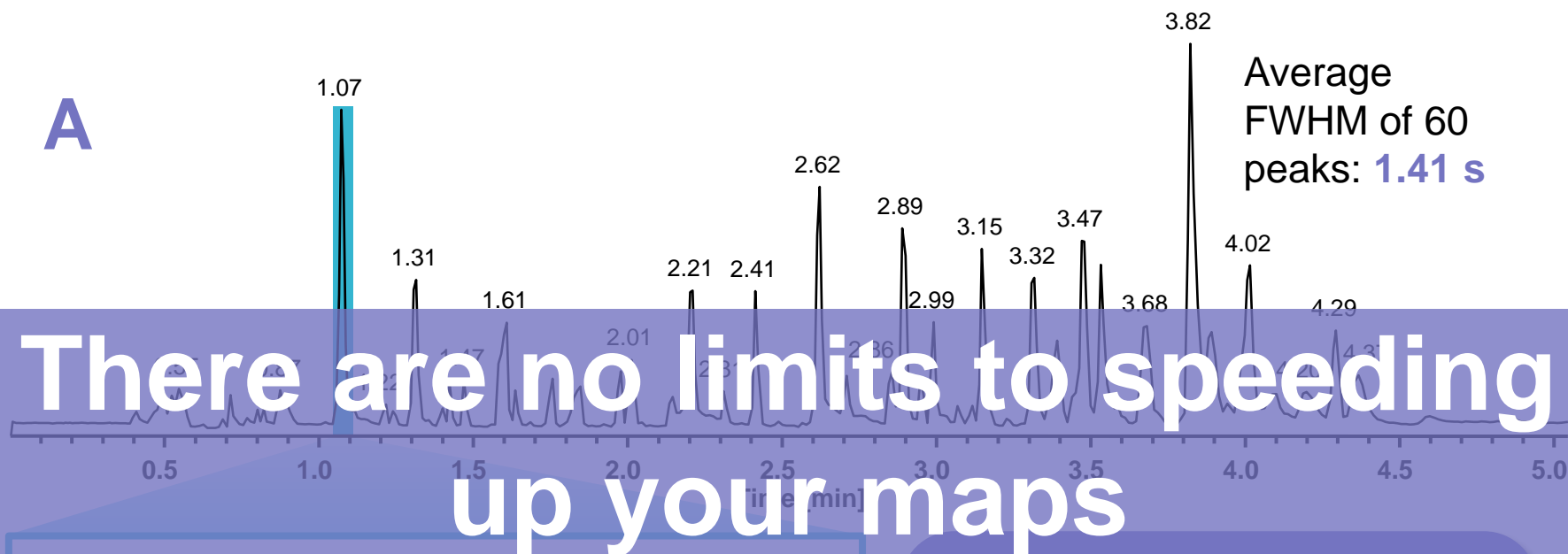
6 Full MS scans  
5 x Top5 MS<sup>2</sup> scans  
 $\Sigma=31$  scans over a peak  
with 0.9 s FWHM

**A)** Total ion chromatogram of a five minute gradient separation of Denosumab.

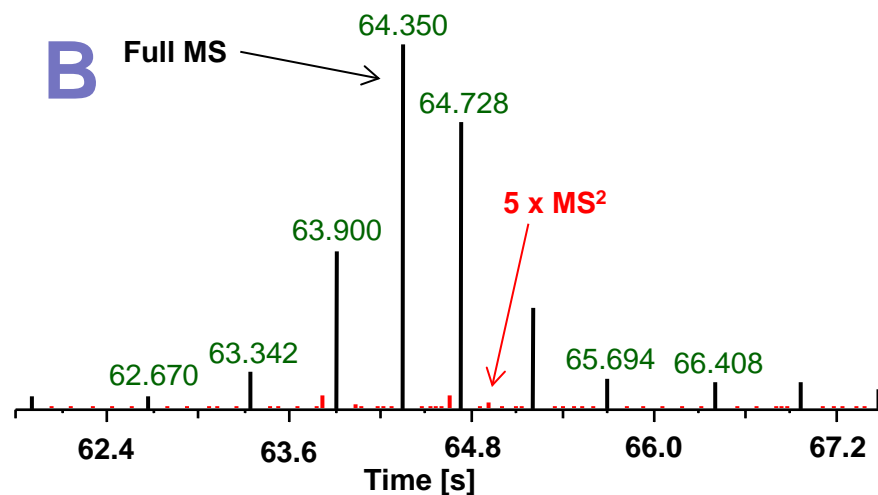
**B)** Data point distribution for a Full MS / ddMS<sup>2</sup> Top5 method during a representative chromatographic peak.

# MS Data Point Distribution in Fast UHPLC Separations

A



B



6 Full MS scans  
5 x Top5 MS<sup>2</sup> scans  
 $\Sigma=31$  scans over a peak  
with 0.9 s FWHM

A) Total ion chromatogram of a five minute gradient separation of Denosumab.

B) Data point distribution for a Full MS / ddMS<sup>2</sup> Top5 method during a representative chromatographic peak.

# Native & Denatured ADC Analysis



Antibody-Drug Conjugate (ADC)



HIC UHPLC  
RP/SEC

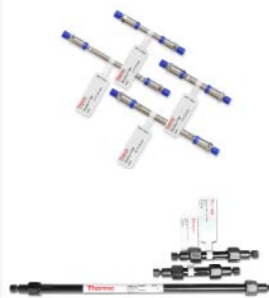
Or



HIC UHPLC Orbitrap MS

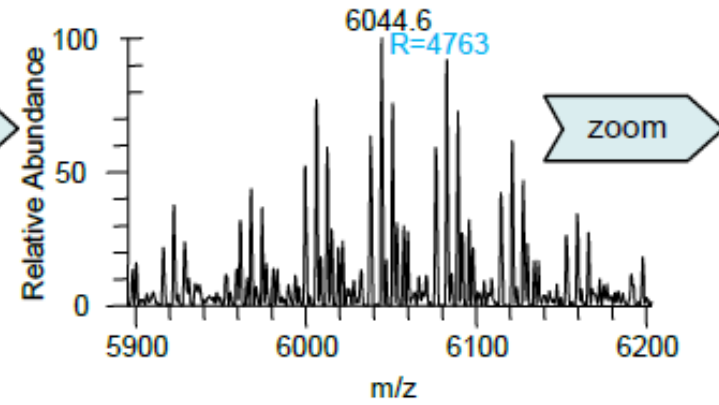
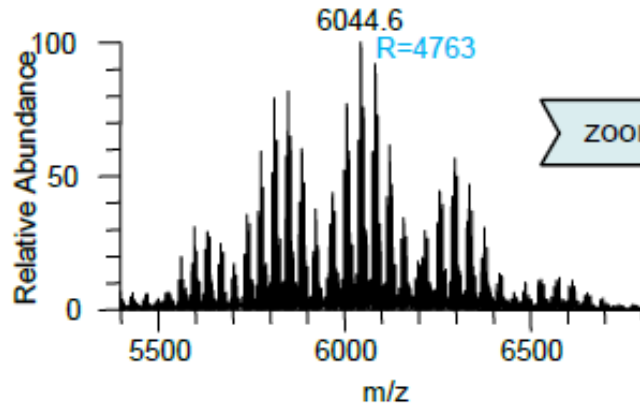
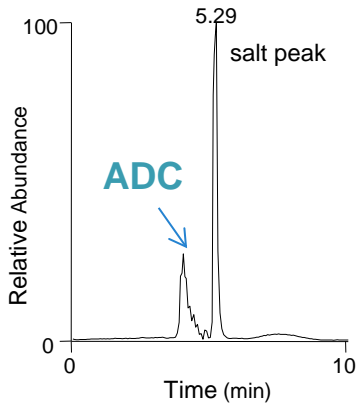
**Denatured ADC** → MAbPac  
RP with 0.1 % formic acid in  
water/acetonitrile

**Native ADC** → MAbPac  
SEC with isocratic 50-100  
mM ammonium acetate



# Trastuzumab-Emtansine ADC

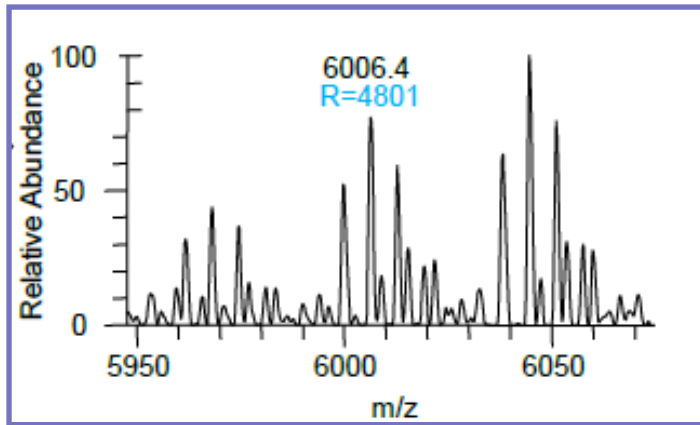
## Native- SEC





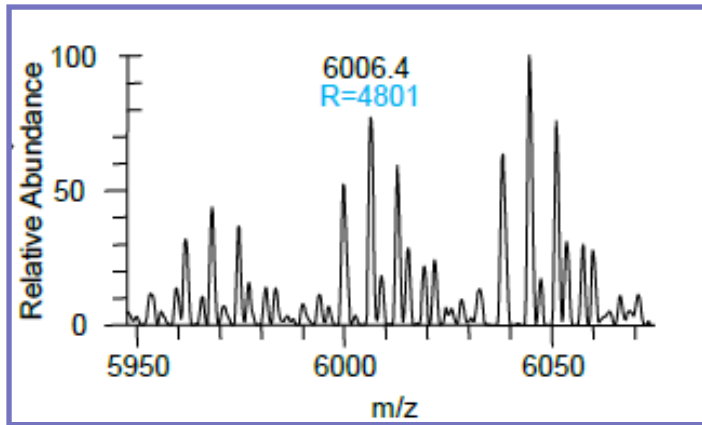
# Trastuzumab-Emtansine ADC

## Native- SEC

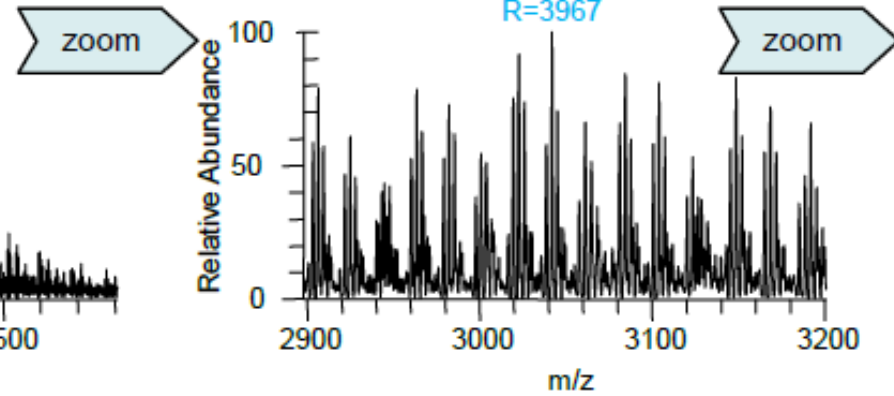
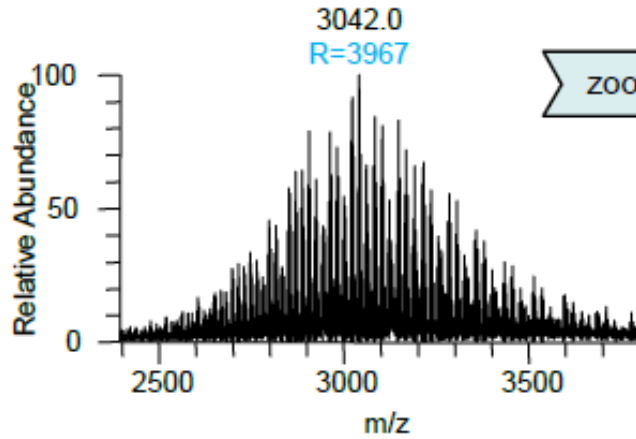
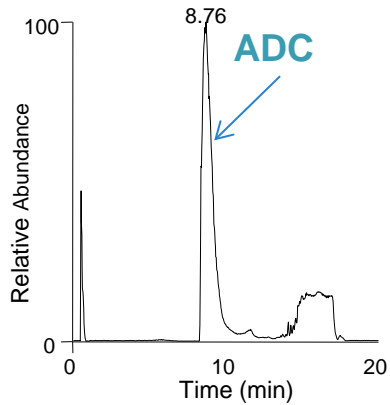


# Trastuzumab-Emtansine ADC

## Native- SEC

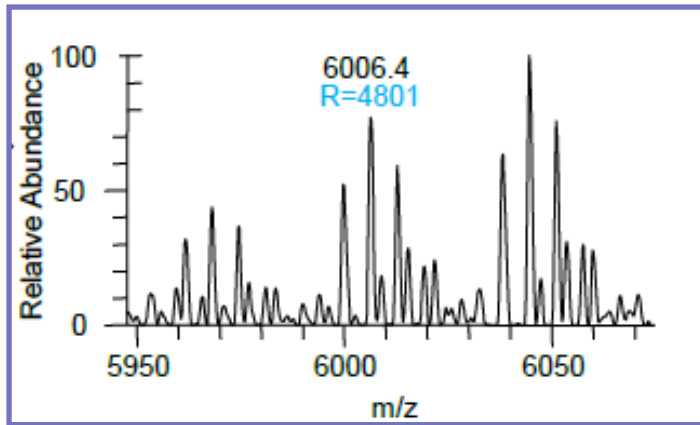


## Denaturing - RP



# Trastuzumab-Emtansine ADC

## Native- SEC

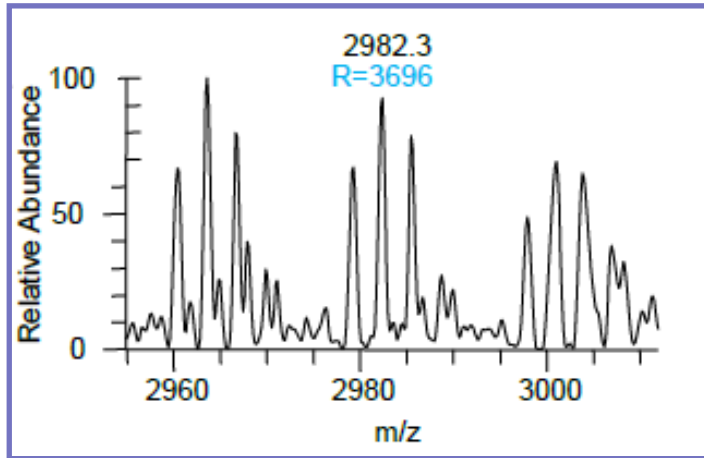


Under native conditions a **higher spatial resolution** is obtained

Detection at higher *m/z* gives improved, baseline resolved peaks

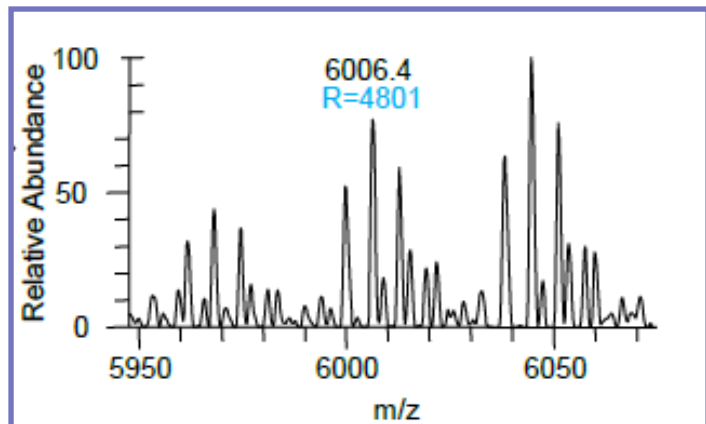
**Even for overlapping peak patterns.**

## Denaturing - RP



# Trastuzumab-Emtansine ADC

## Native- SEC

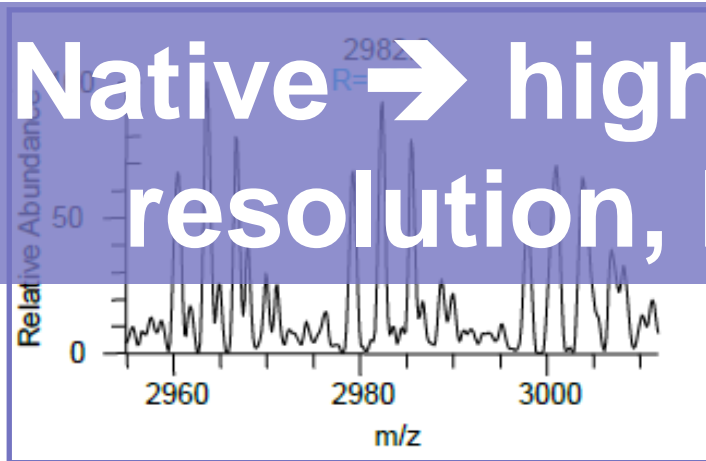


Under native conditions a **higher spatial resolution** is obtained

Detection at higher *m/z* gives improved, baseline resolved peaks

**Even for overlapping peak patterns.**

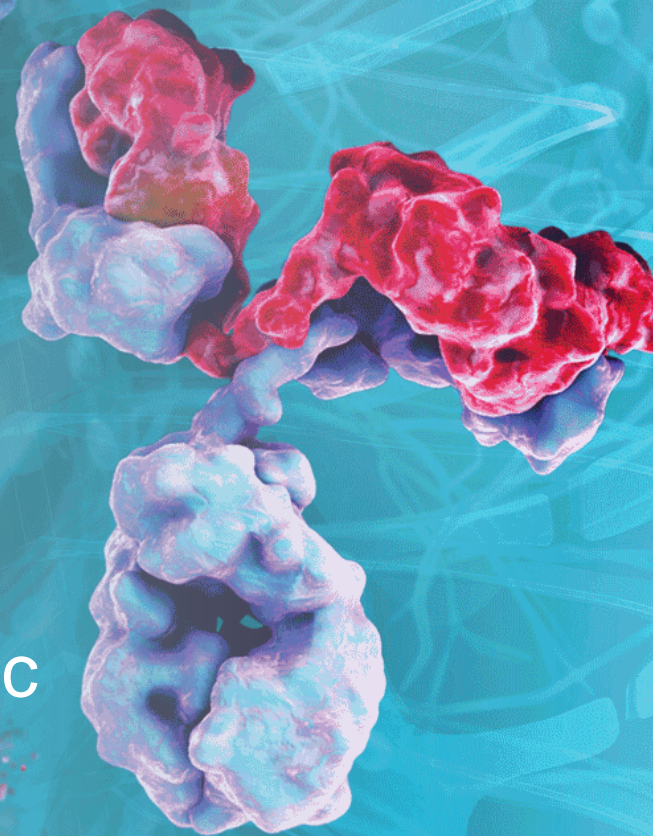
## Denaturing - RP



**Native → higher *m/z*, higher spatial resolution, higher S:N, simpler**

The use of appropriate **UHPLC-HRAM MS** workflows and tools is the key to unlocking the secrets of biomolecules

Our **Built for Biopharma** solutions are designed so that you can stay ahead in the evolving biotherapeutic landscape





# Acknowledgements

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