



ThermoFisher
SCIENTIFIC

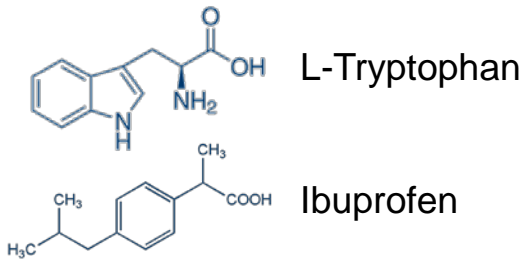
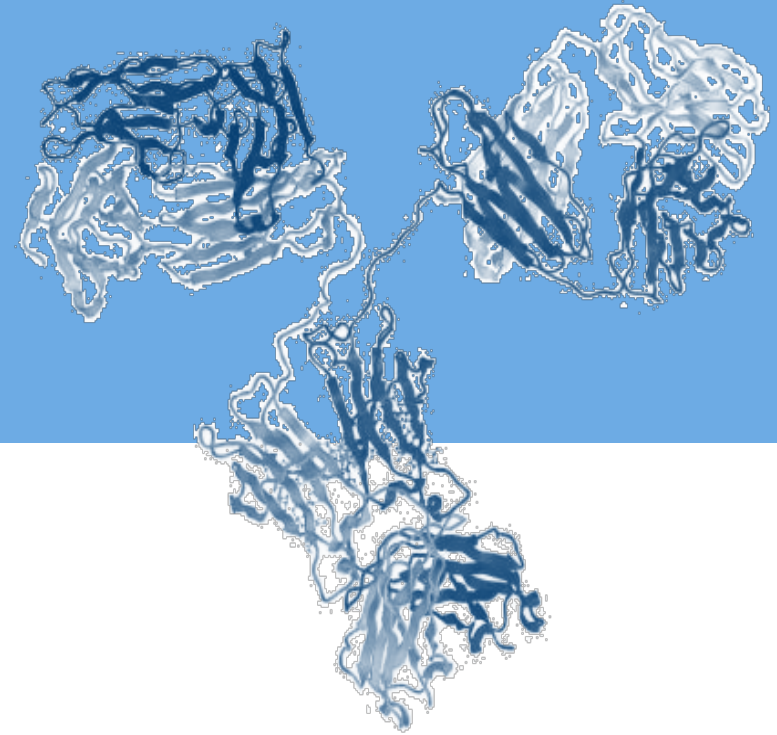
Rapid Automated Peptide Mapping

Kate Erickson
Application Scientist

The world leader in serving science

Why is there a growth in biotherapeutics?

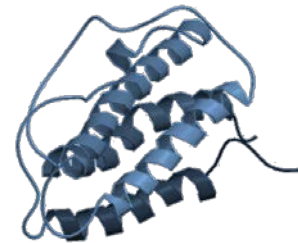
- 8/10 drugs in 2016 Biologics
- Biopharma growing rapidly ~10% over the next 5 years
- \$160 Billion
- Success rate at clinical phase I
 - Classic synthetic drugs ~7%
 - Biologics ~ 12%



**Typical
Pharmaceutical**
MW ~ 200 Da
e.g. Ibuprofen



Insulin
MW ~ 6000 Da
51 amino acids
e.g. Lantus



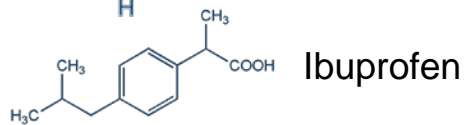
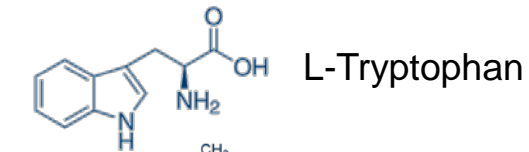
Erythropoietin (EPO)
MW ~ 18000 Da
165 amino acids
e.g. Eprex

mAbs
MW ~ 145000 Da
> 1300 amino acids
e.g. Humira

Complexity and Heterogeneity of Biological Drug Substances

- Substantial increase of structural complexity with size
- Biotechnological production (cell culture, fermentation)
- Often functional requirement for post-translational modifications
- Structural complexity provides a high degree of freedom for modification and variation (micro-heterogeneity)
 - Inert to the production in a biological system
 - Related to processing, storage, sample handling ...

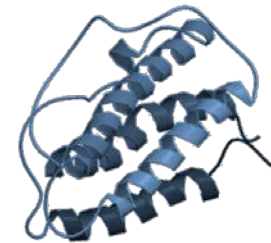
⇒ **Risk to impact safety and efficacy for the patient**



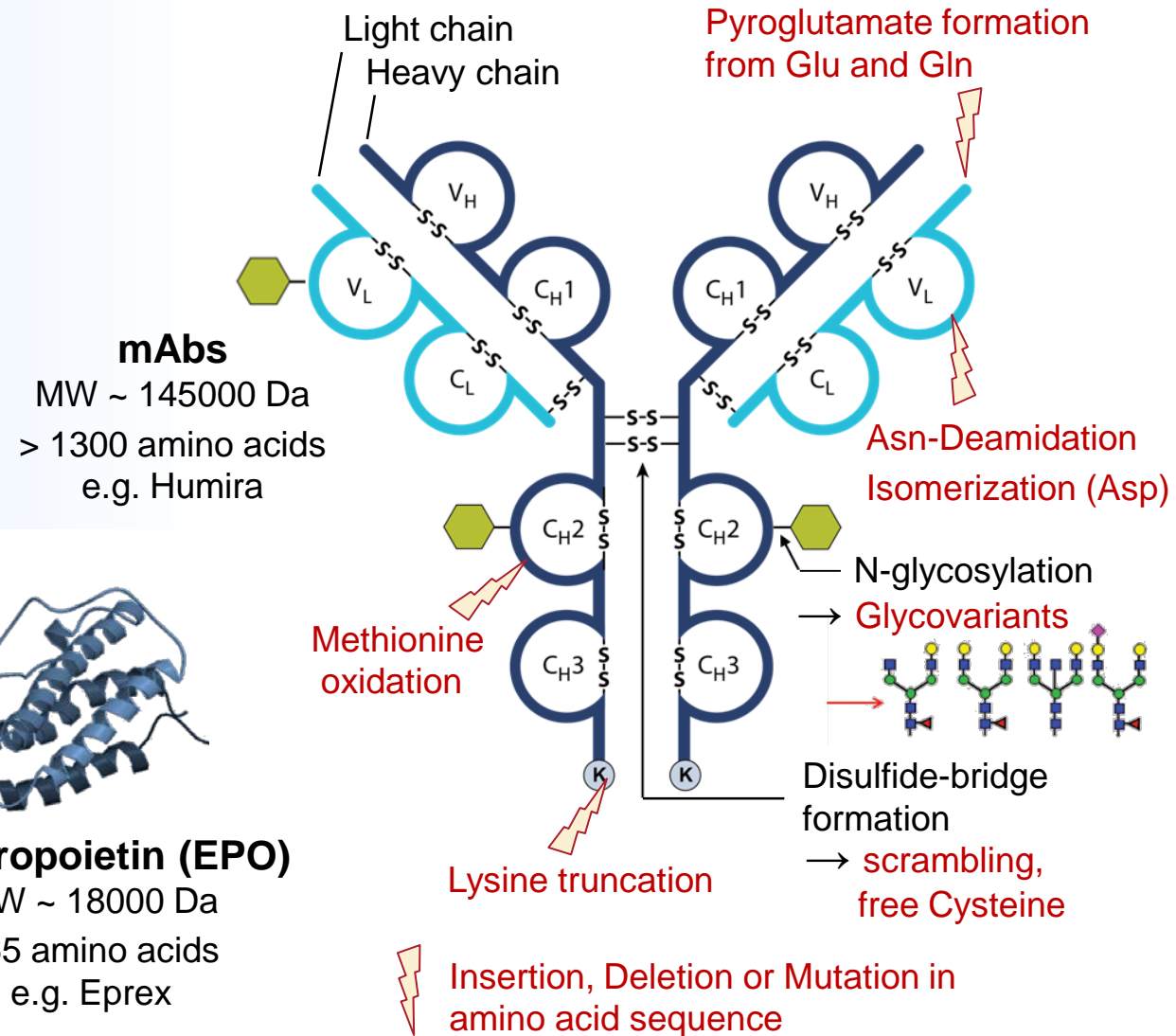
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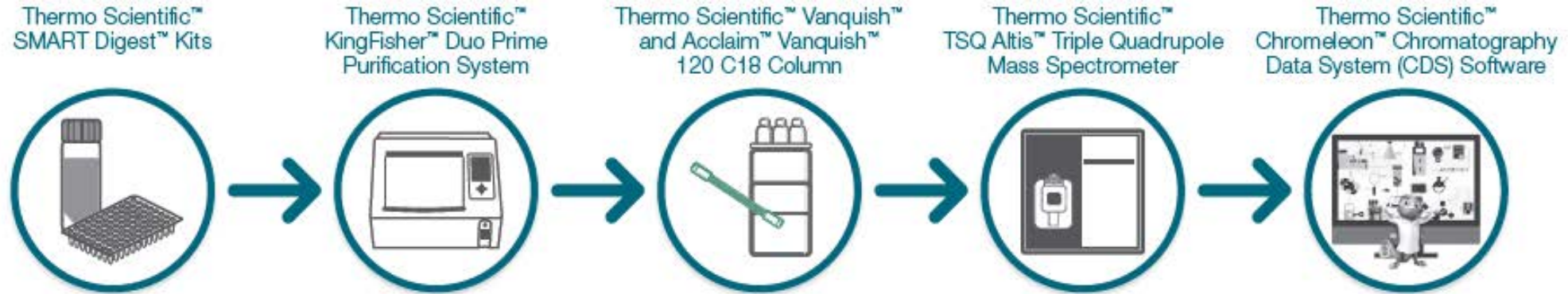


Workflows

- Peptide mapping:



- Peptide Quantitation:



Peptide Mapping Workflow in Biotherapeutic Characterization



Drug Discovery



Drug Development



Pre-Clinical &
Clinical Drug Testing



Chemistry, Manufacturing
& Controls (CMC)



Pharmaceutical QA/QC

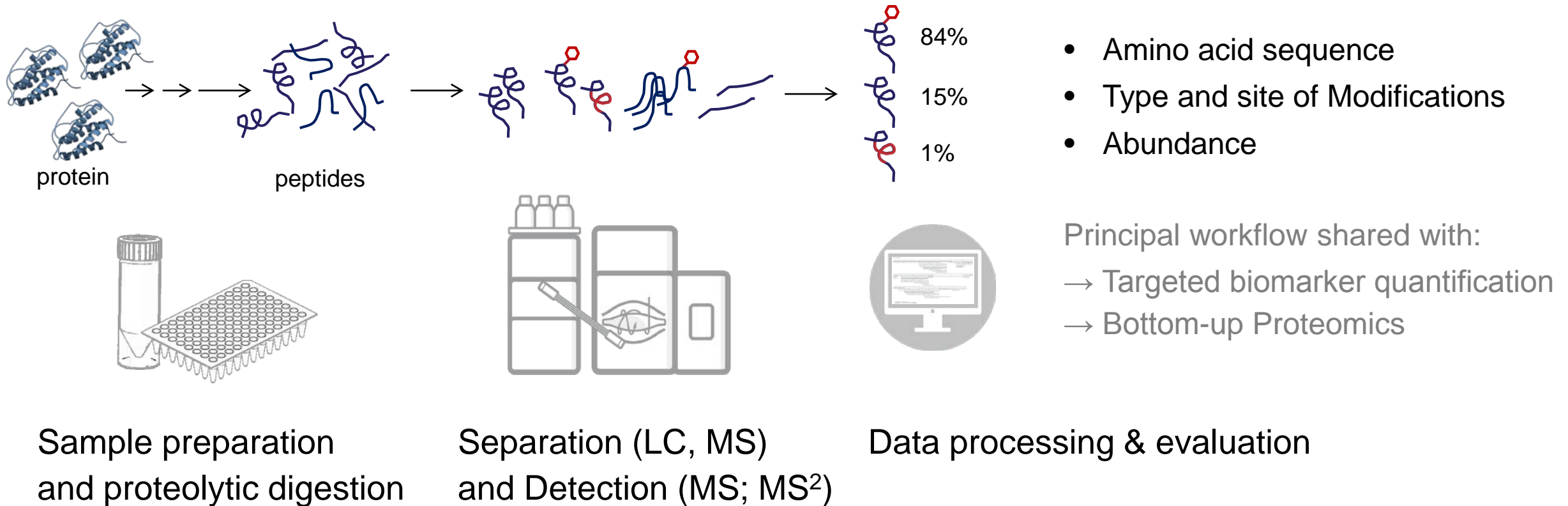
Peptide Mapping

Peptide Mapping Workflow in Biotherapeutic Characterization

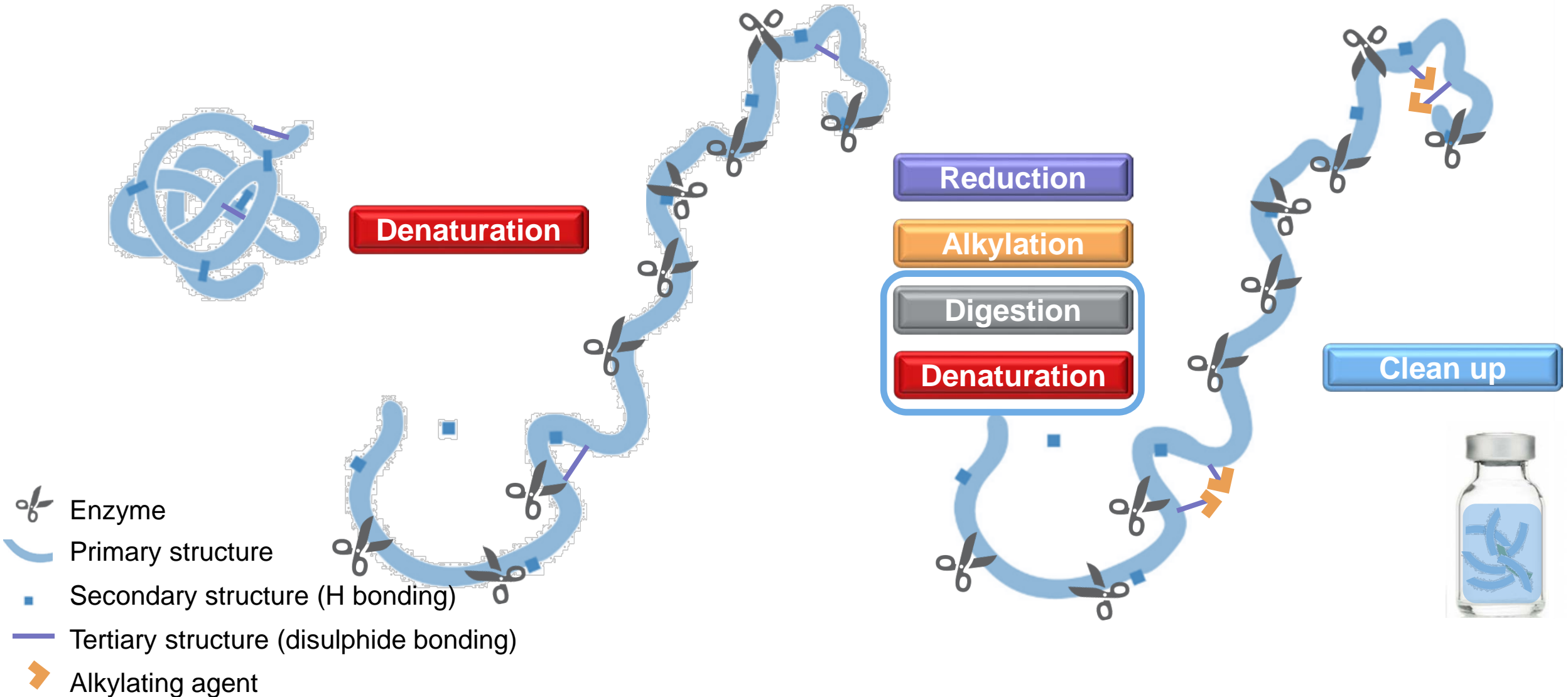
Crucial workflow for biotherapeutic characterization from development to QC

→ sequence verification, quantitative and qualitative assessment of modifications

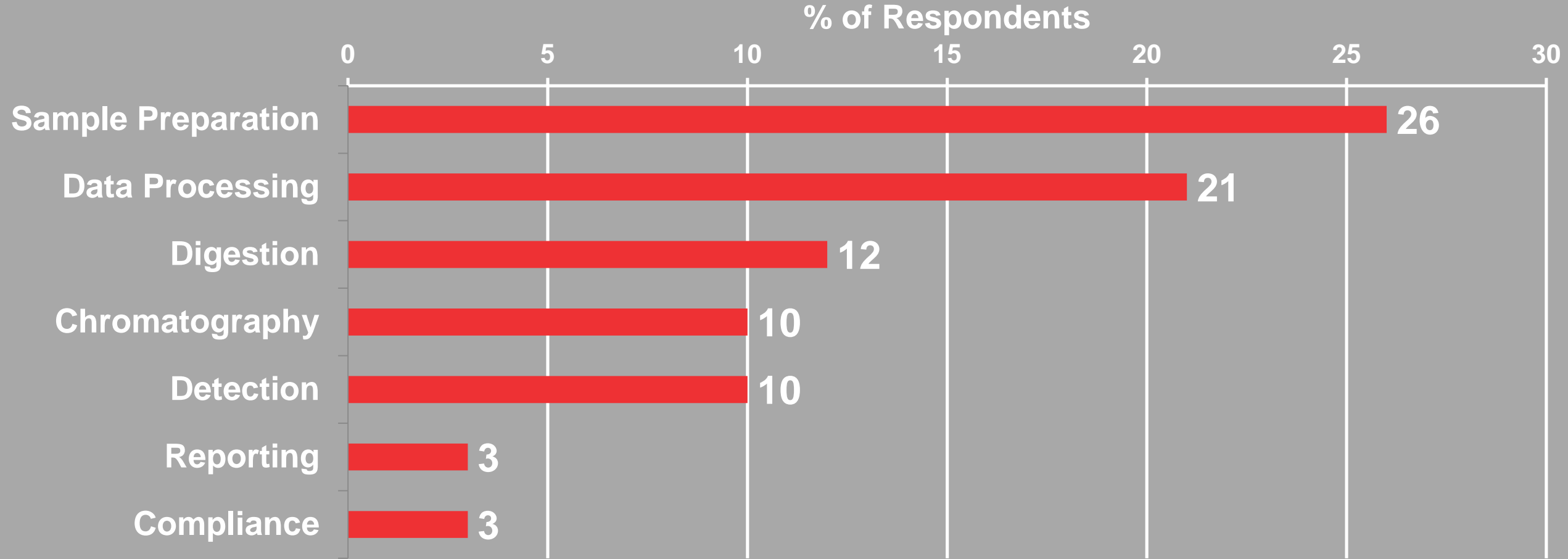
⇒ Identity, Purity and Heterogeneity ⇒ Safety and efficacy for the patient



Traditional Digestion Processes are Inefficient and Introduce Error



What's the pain point?

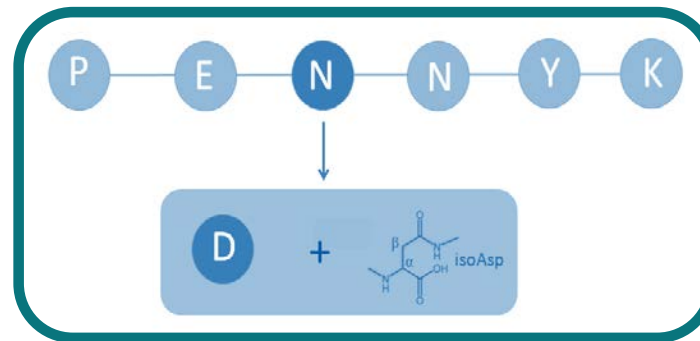
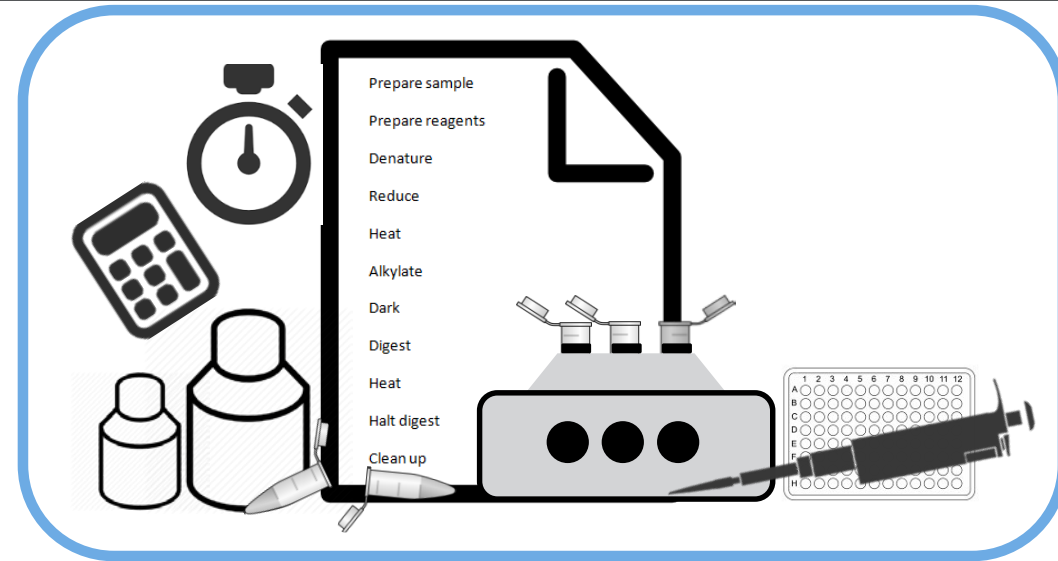


How can this pain be eased?

A workflow that is faster, more reliable, reproducible and easy to use

Peptide Mapping Analysis Has Multiple Bottle Necks

- Lengthy multi-step protocols
- Process-induced PTMs
- Reproducibility
- Throughput/speed
- Method development ease



Sample Preparation Challenges for Bottom-up Analysis of Proteins

Input quality

- **Effort and time consuming**
 - Labour intensive, multi-step sample preparation, with little standardization
 - Handling of toxic and alkylating reagents
 - Overnight digestion causes delay of results
- **Variability in digestion**

Different protocols and operators produce different results
- **Lack of reproducibility** – Leading to a lack of data confidence
- **Not readily amenable to high throughput workflows**
- **Difficult to automate**

Output quality /Quantity

Make up reagents: 8M Urea, DTT, Iodoacetamide, Trypsin

Perform protein quantification

Denaturation in 8M Urea

Reduction of disulfide bridges; 30-60 min

Alkylation of cysteines; 30 min

Quenching of excess Iodoacetamide; 15 min

Dilute to 1 M Urea

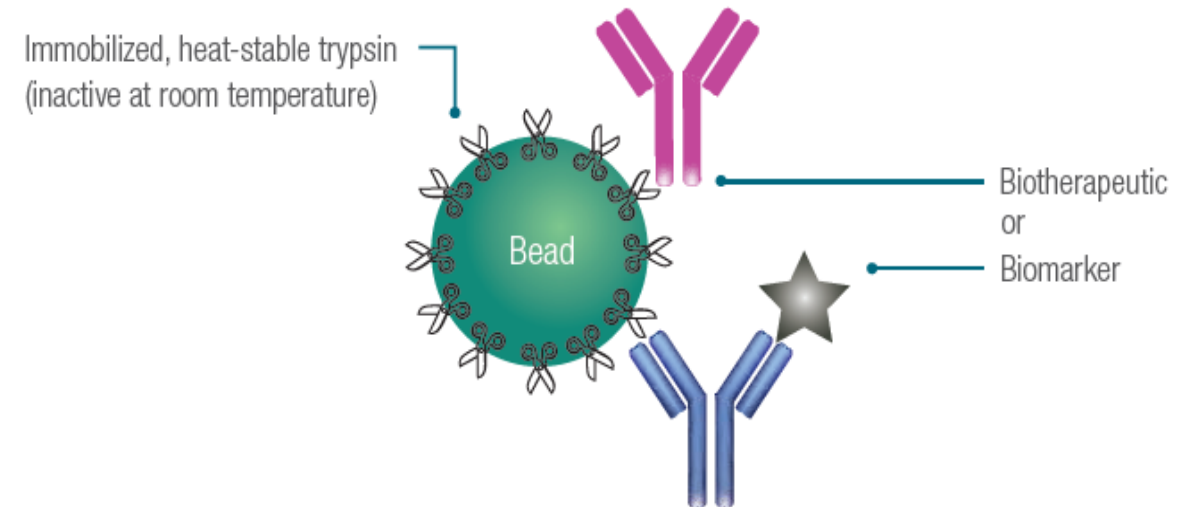
Add Trypsin to vial and digest overnight

Spin to remove particulates

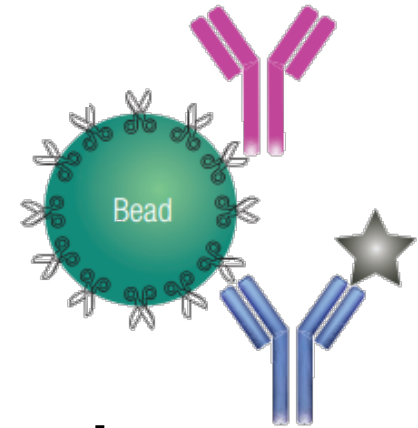
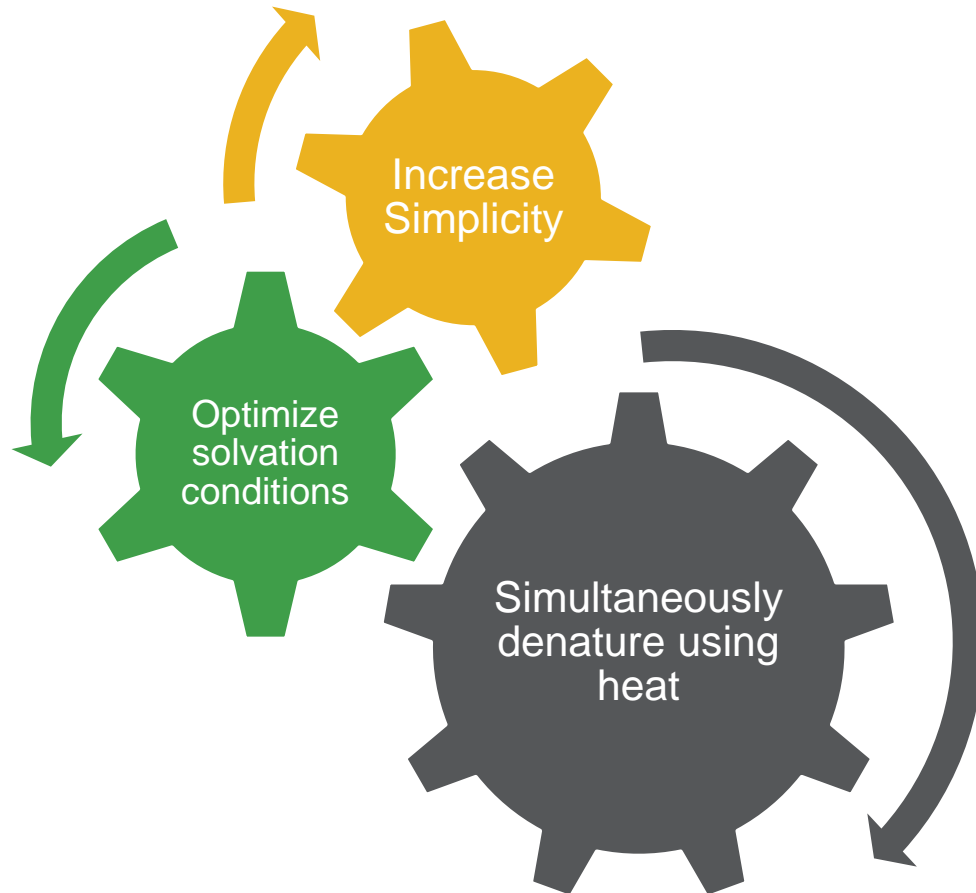
Extract peptides with SPE

Immobilized Heat Stable Enzyme: Reproducible Protein Digestion in Minutes

- **SMART Digest** is a heat-stable immobilized enzyme digestion kit;
 - Proteins are heat-denatured for digestion,
 - Additional denaturing agents or reduction and alkylation is not required
- High-throughput and automation-compatible formats
 - 96 x PCR tubes pre-packed with resin
 - Bulk resin format
 - Magnetic Bulk resin format
- Additional post digestion clean up options:
 - 96 well filter plate
 - SOLA μ SPE plate



Optimized Thermo Scientific SMART Digestion at Key Steps



- **Save time**
 - Reagent prep/denaturation, reduction/alkylation
- **Simplify**
 - Fewer steps
 - Fewer reagents
- **Increase sensitivity**
- **Increase robustness**
 - Stable, reproducible activity

Reduced Solvent Effects with Thermo Scientific SMART Digestion

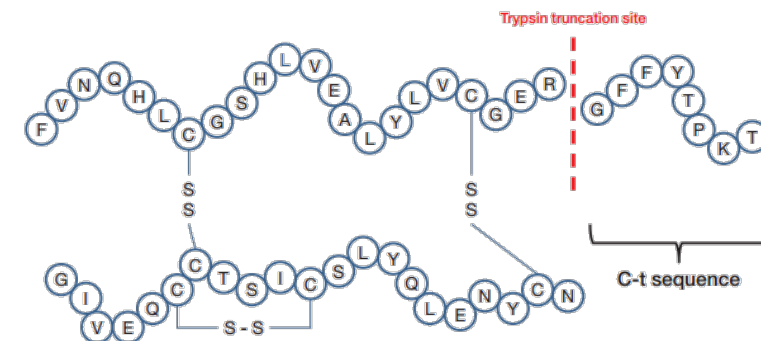


Enzyme

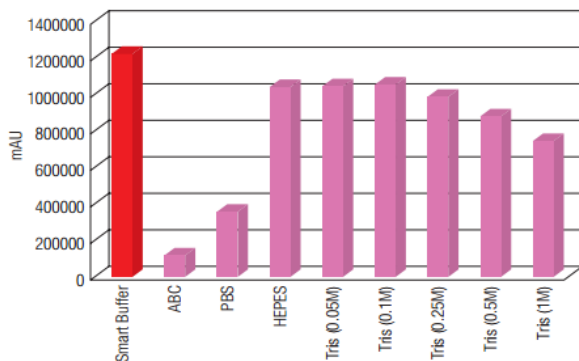
Solubility

Denaturation

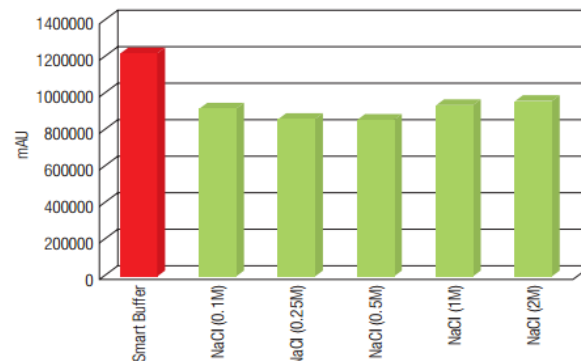
Diffusion



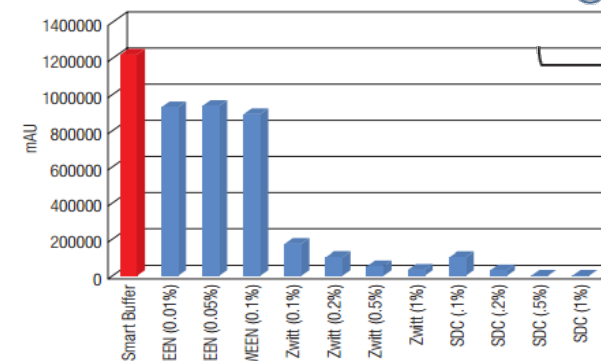
A Effect of buffering ion



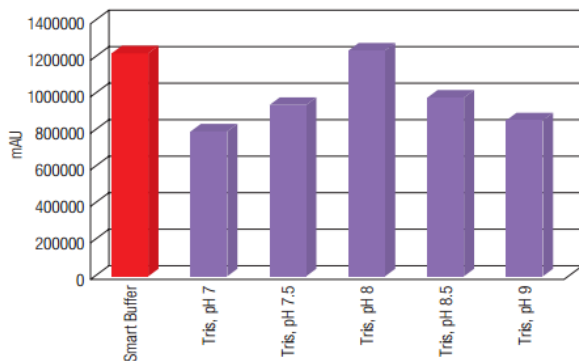
B Effect of salt concentration



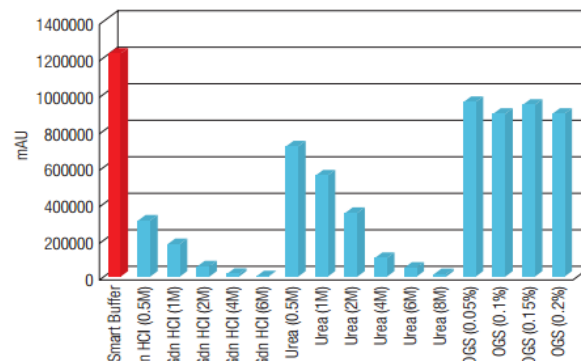
E Effect of detergents



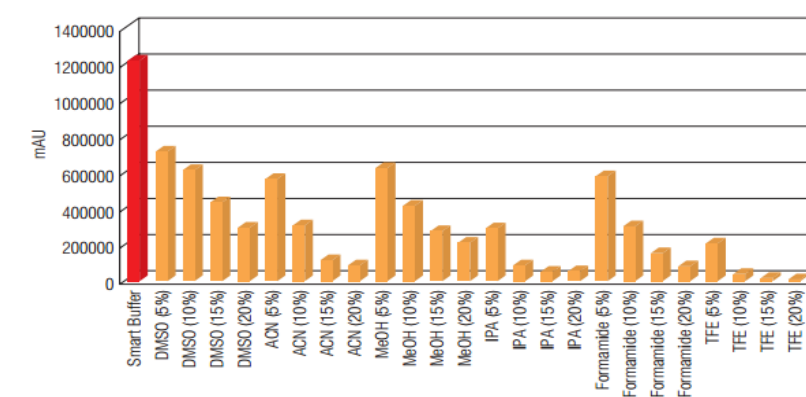
C Effect of pH



D Effect of chaotropes



F Effect of co-solvents



Insulin amino acid sequence, C-t peptide was monitored

Selecting Buffers to Remove Uncertainty in Tryptic Digestion

Valeria Barattini, Philip Humphries, Thermo Fisher Scientific, Runcom, UK

Application Note 21179

Relieve the Effects of Detergents and Chaotropes - Denaturation



Enzyme

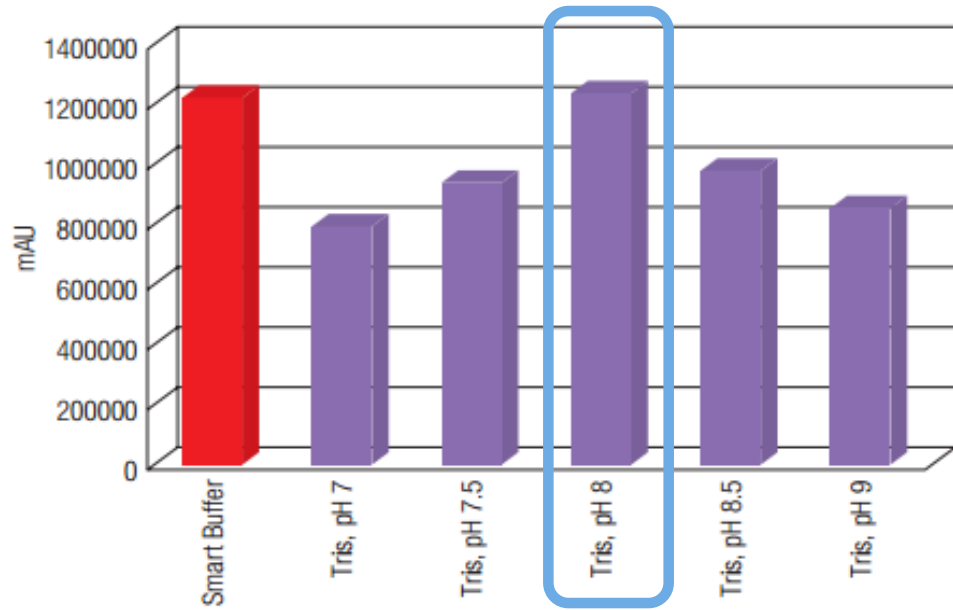
Solubility

Denaturation

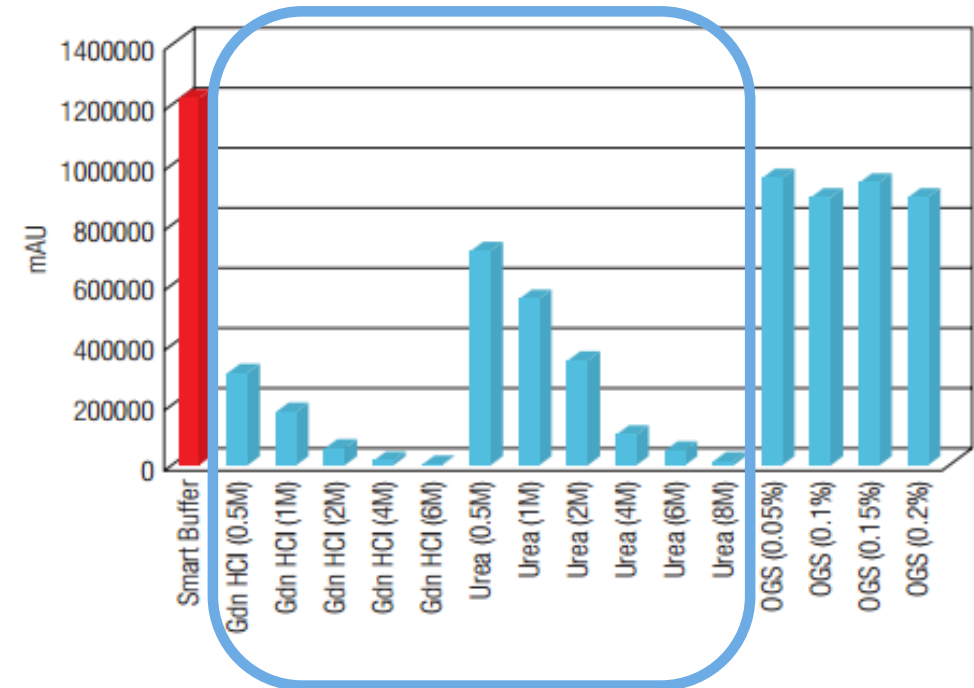
Diffusion

Chemical Name	CAS No.	EINECS No.	Kit Component	Weight %
Water	7732-18-5	231-791-2	2	50-95%
Glycerol	56-81-5	200-289-5	2	< 20%
Tris Base	77-86-1	201-064-4	2	< 10%
Tris-HCl	1185-53-1	214-684-5	2	< 10%
Calcium Chloride	10043-52-4	233-140-8	2	< 10%
Sodium Azide	26628-22-8	247-852-1	2	< 0.1%

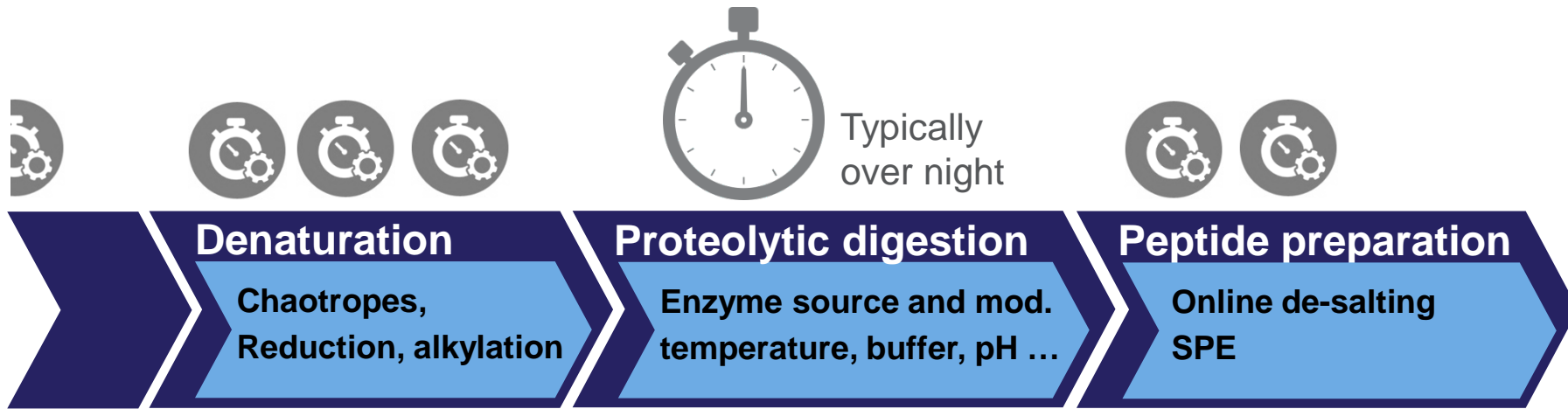
C Effect of pH



D Effect of chaotropes



Accelerate Digestion for Peptide Mapping and Targeted MS Analysis



Options for acceleration

Reagent-free denaturation ←

- No additional steps
- No chaotropes
- No reduction and alkylation
- No handling of alkylating substances

- Enzyme immobilization
- Heat
- Microwave
- Ultrasound
- Infrared (IR)
- Solvents and surfactants

- Independent of E:S ratio
- Reduced enzyme autolysis
- Easy enzyme removal after digestion
- Allows to use enzyme in excess to substrate

~~Make up reagents: 8M Urea,
DTT, Iodoacetamide, Trypsin~~

~~Perform protein quantification~~

~~Denaturation in 8M Urea~~

~~Reduction of disulfide bridges;
30-60 min~~

~~Alkylation of Cysteines; 30 min~~

~~Quenching of excess
Iodoacetamide; 15 min~~

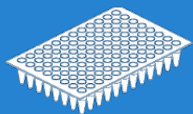
~~Dilute to 1 M Urea~~

Add Trypsin to vial and
digest overnight

Spin to remove particulates

Extract peptides with SPE

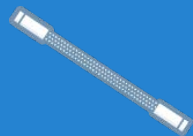
Easy to Use Sample Preparation



Thermo Scientific™
SMART Digest™



Thermo Scientific™
Vanquish™ Flex
UHPLC



Thermo Scientific™
Acclaim™ 120 C18
column



Thermo Scientific™
Q Exactive™ Plus
HRAM-MS



Thermo Scientific™
BioPharma Finder™
informatics platform

Sample preparation: Easy to use

1

Add 150 μ L of buffer



Add 150 μ L of **SMART Digest buffer** to the SMART Digest tube.

2

Add 50 μ L of sample



Add 50 μ L of **sample** to the SMART Digest tube (final volume of 200 μ L per sample).

3

Digestion



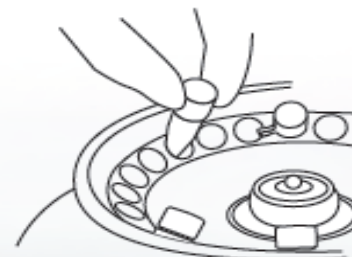
Set your heater/shaker to 70 °C/1400 RPM, allow to equilibrate for 5 minutes. Then add your samples for the required time for digestion.



Note: Refer to the Digestion Optimization Section.

4

Cleanup



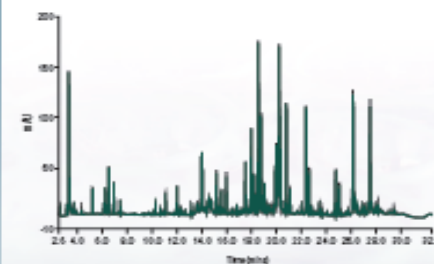
Remove the SMART Digest tube from the heater/shaker and perform one of the sample cleanup methods based on the complexity of your starting matrix.



Note: Refer to the Post Digestion Process.

5

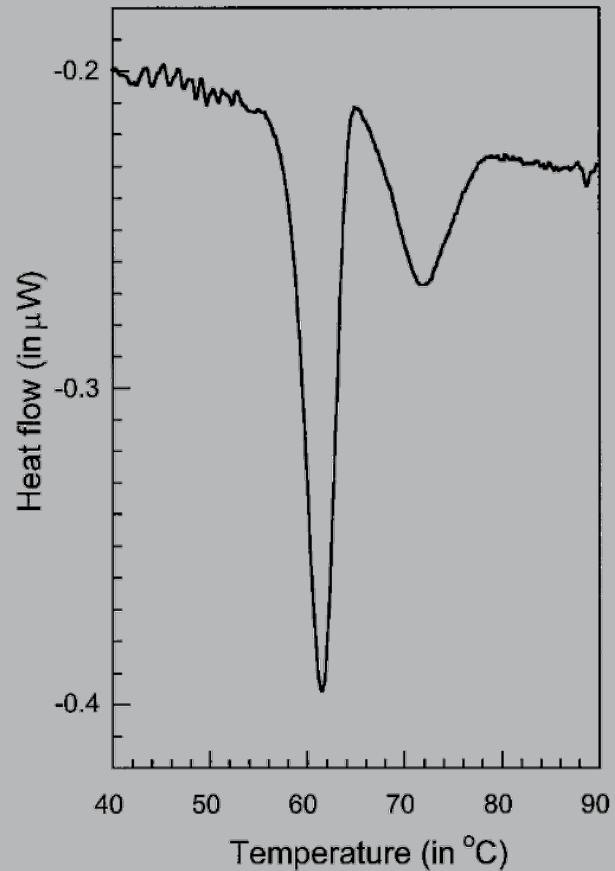
Analyze



Analyze sample.

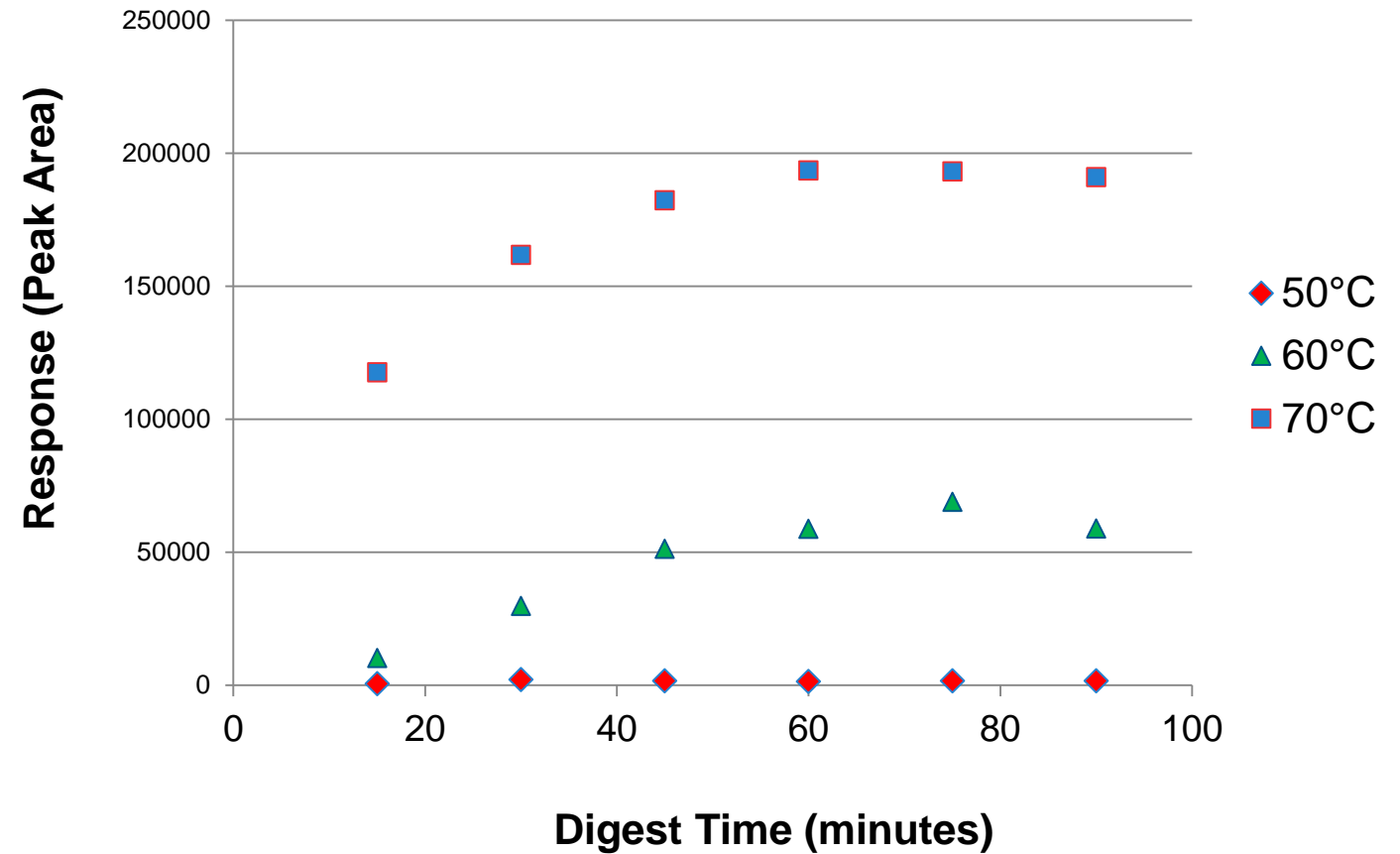
Accelerated Protein Digestion

Thermal denaturation of IgG



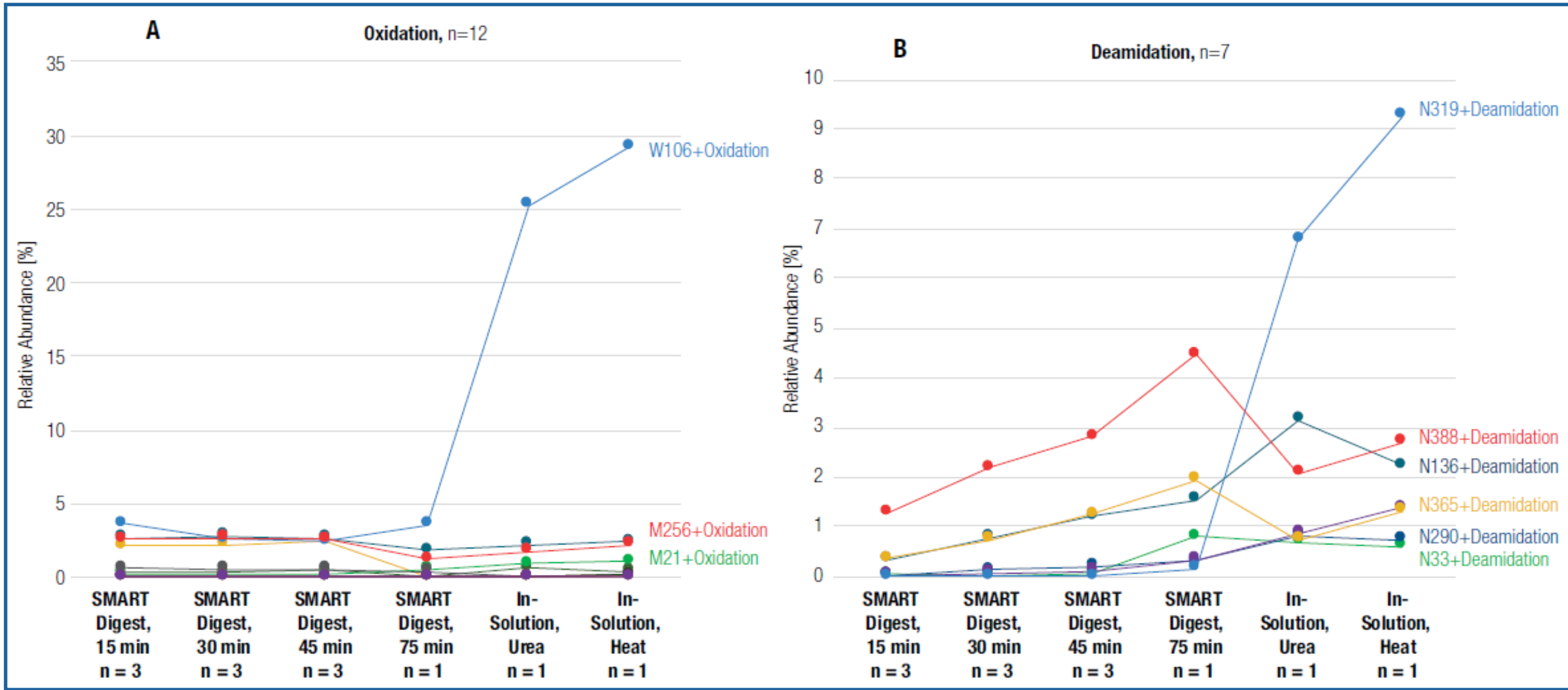
DSC thermogram of IgG (6 mg/ml; mouse IgG2b)
in a 10 mM phosphate buffer pH 8.1; 0.5 $^{\circ}\text{C}/\text{min}$ [*]

Native IgG Digest Profile monitoring VSVLTVLHGDWLNGK

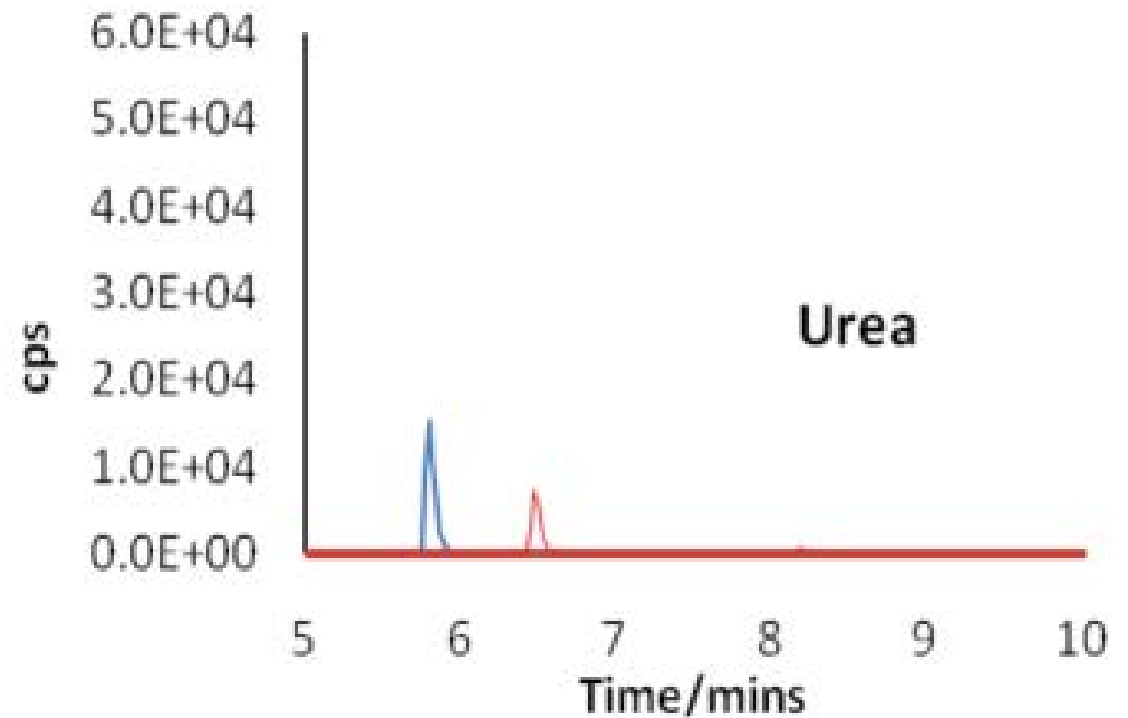
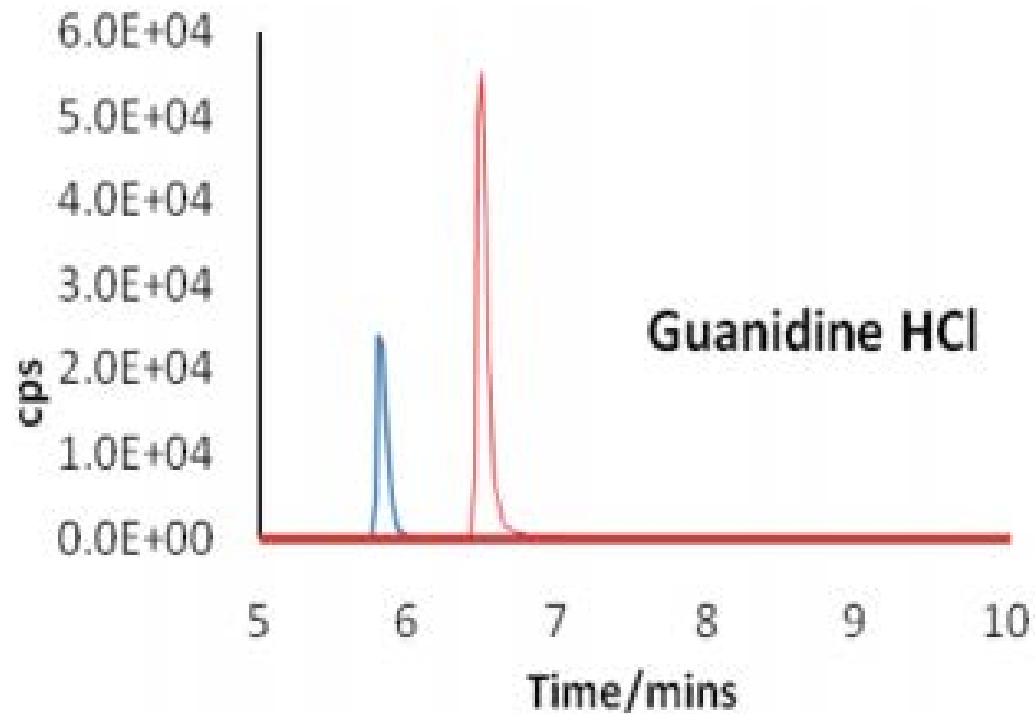


* Vermeer & Norde (2000), Biophysical Journal 78: 394 – 404

Identification of Oxidation and Deamidation

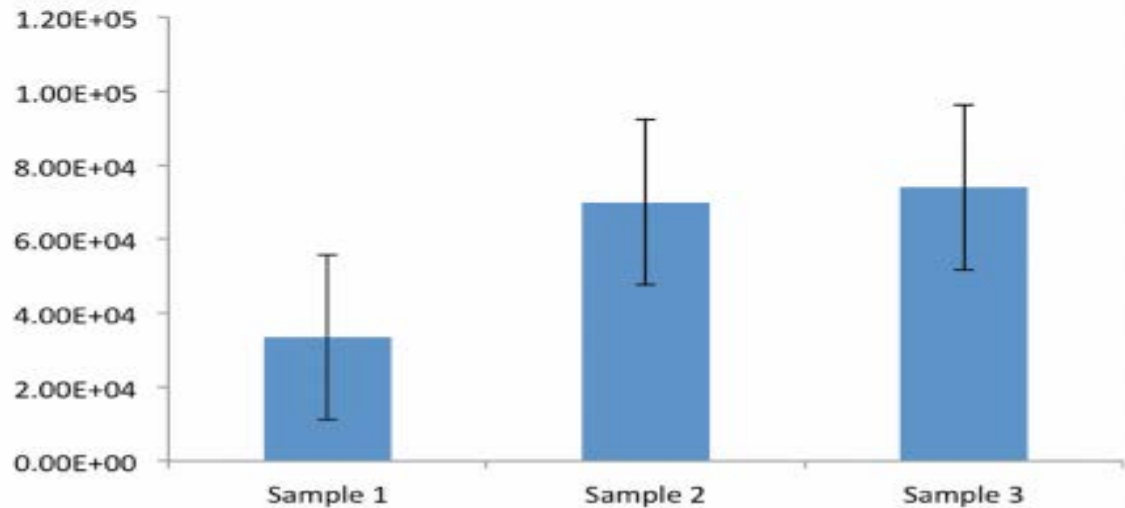
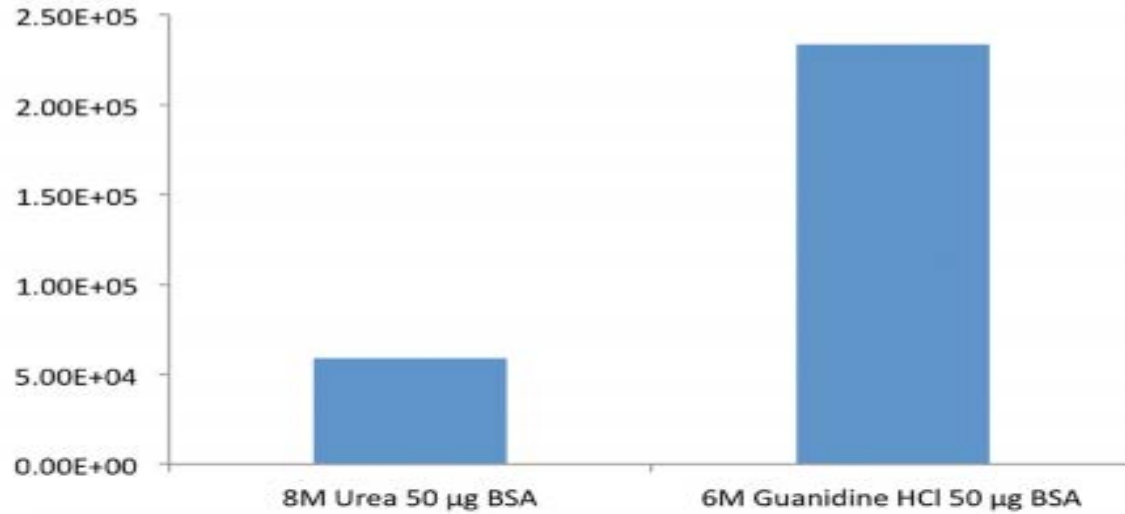


Chaotropes Reduce Accuracy in Quantitation



Denaturing agents alter the way digestion products behave during detection

Denaturing Agents Have a Negative Effect on Digestion Reproducibility



Experimental Conditions

- 100 µl solution of 500 µg/ml of BSA
- 100 mM ammonium bicarbonate
- 22°C room temperature over 30 minutes.

Accelerated Protein Digestion

Trypsin digests within minutes

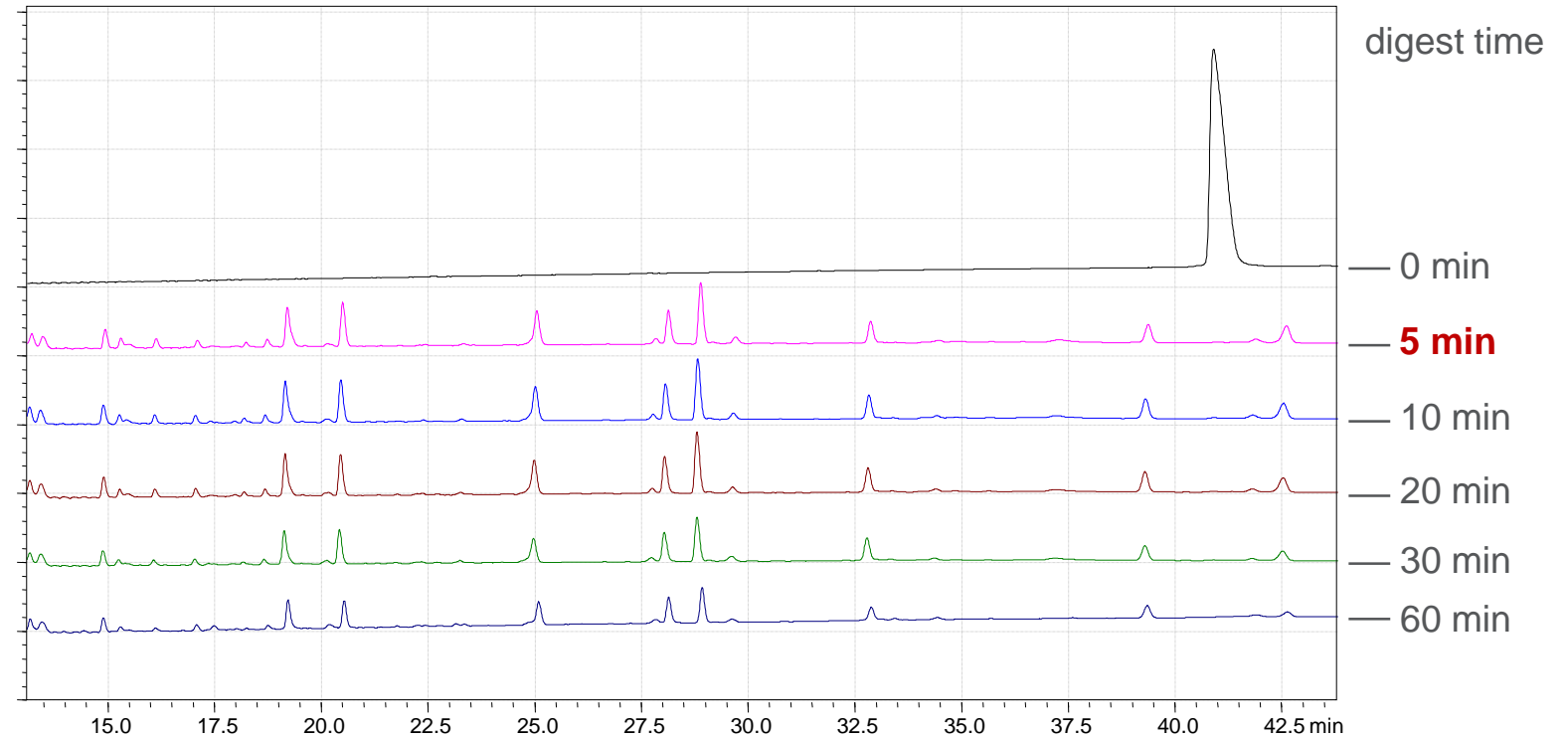
Recommended digestion starting conditions for known proteins*	
Protein	Digest Time (min)
Insulin	4
BSA	< 5
Carbonic anhydrase	< 5
Lysozyme	< 5
Apo-B	30
IgG	45
IgG in 50 μ L plasma	75
Ribonuclease A	150
Thyroglobulin	240
C-reactive protein	240

* 200 μ L protein solution (100 μ g/mL);

IgG in plasma: 17.5 μ g/mL

Temperature: 70°C

Carbonic Anhydrase, 29 kDa



Accelerated Protein Digestion

Trypsin digests within minutes

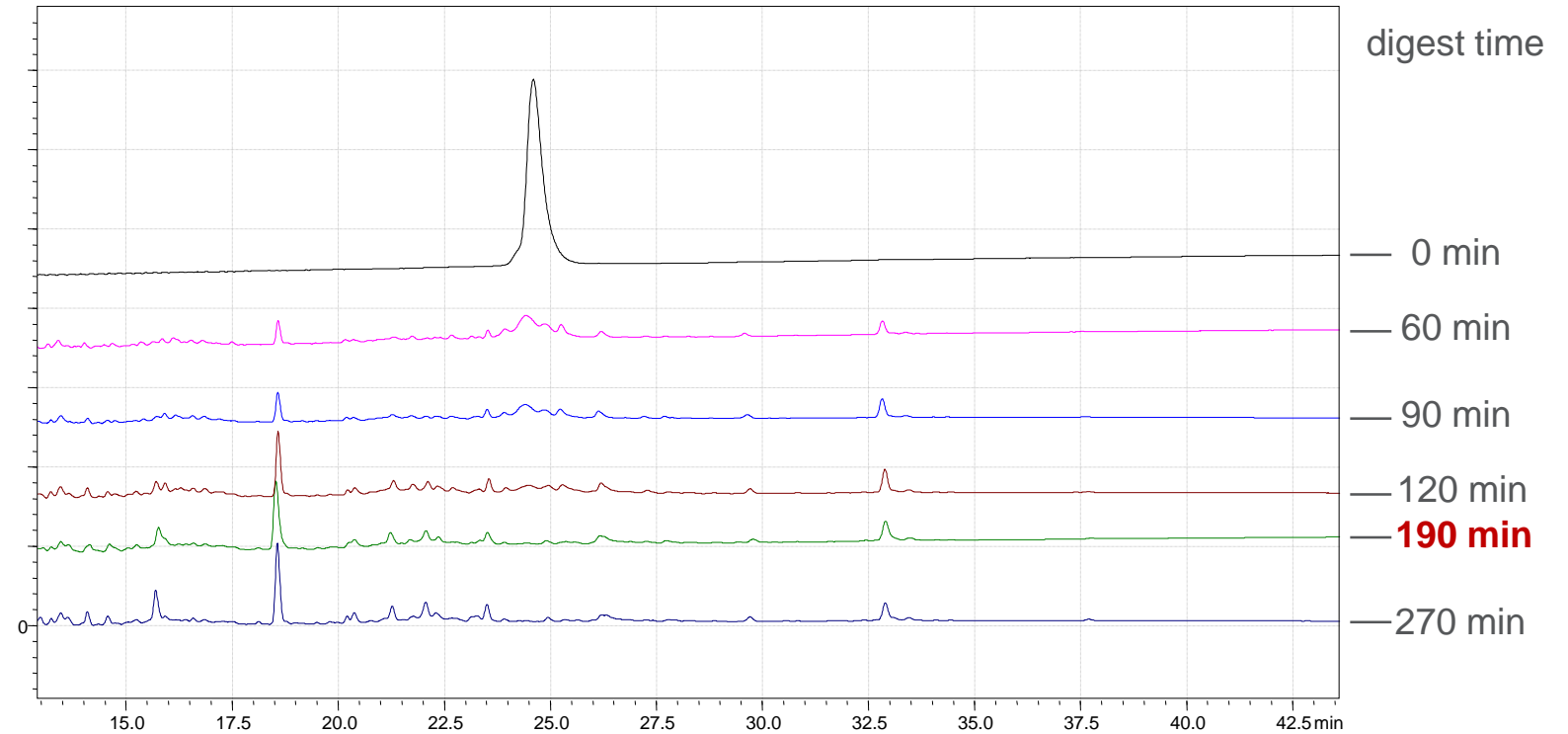
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* 200 μ L protein solution (100 μ g/mL);

IgG in plasma: 17.5 μ g/mL

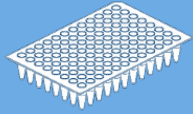
Temperature: 70°C

Ribonuclease A, 13.7 KDa



- “*Highly stable toward unfolding*” Protein Eng. (2001) 14 (10): 791-796.
- “*Amazingly stable*” David Goodsell Protein Data Bank

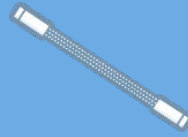
Reproducible Digestion User to User



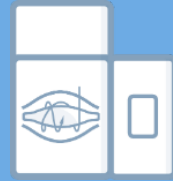
Thermo Scientific™
SMART Digest™



Thermo Scientific™
Vanquish™ Flex
UHPLC



Thermo Scientific™
Acclaim™ 120 C18
column

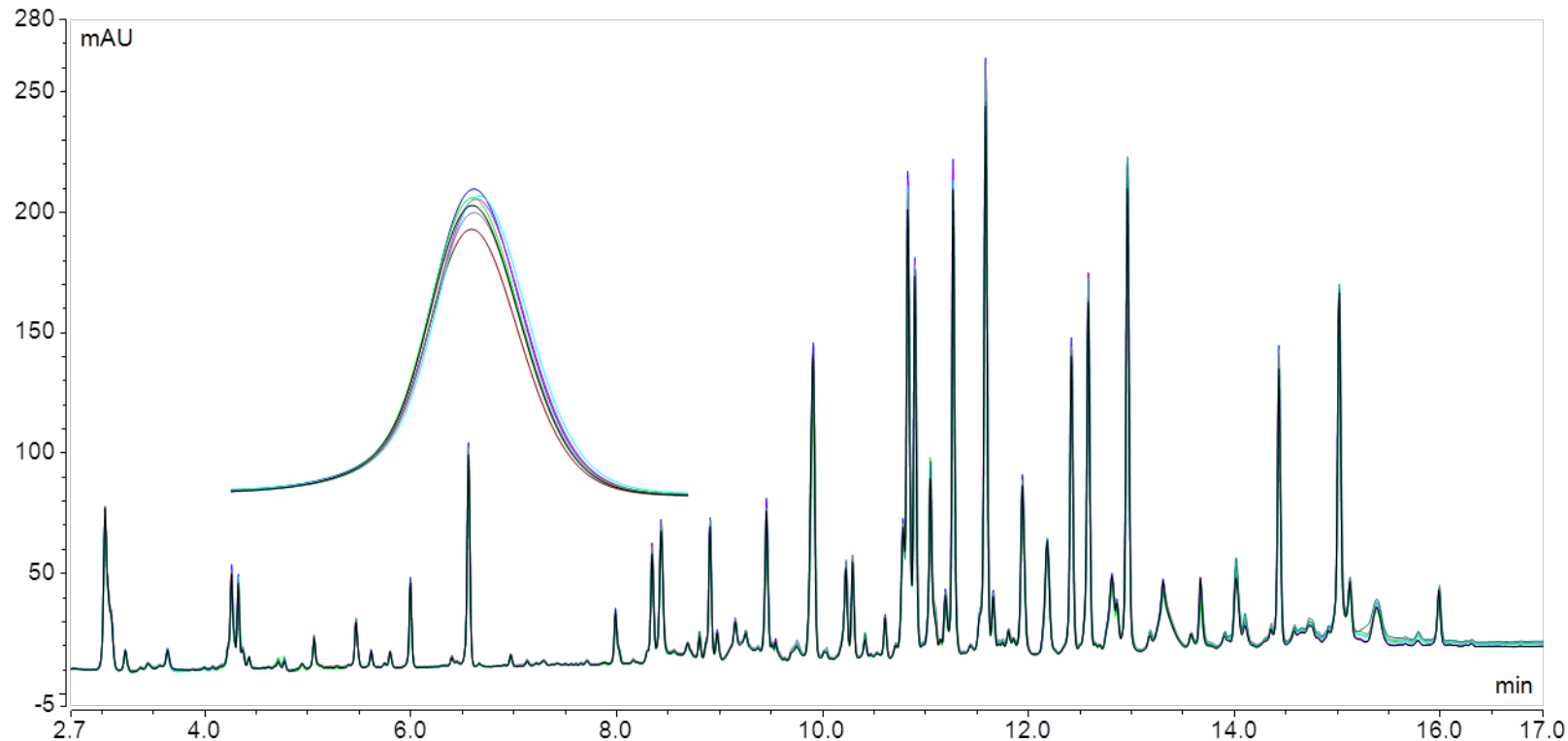


Thermo Scientific™
Q Exactive™ Plus
HRAM-MS



Thermo Scientific™
BioPharma Finder™
informatics platform

**Sample preparation:
High reproducibility digestion
user to user**



**7 independent digests of
Rituximab, conducted by
individual operators**

Digest time: 45 min (70°C)

LC-MS run time: 40 min

Sequence coverage: 100%

average %RSD (A_{rel}) < 3%

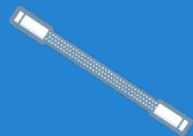
Reproducible Chromatography and Digestion Results



Thermo Scientific™
SMART Digest™



Thermo Scientific™
Vanquish™ Flex
UHPLC



Thermo Scientific™
Acclaim™ 120 C18
column



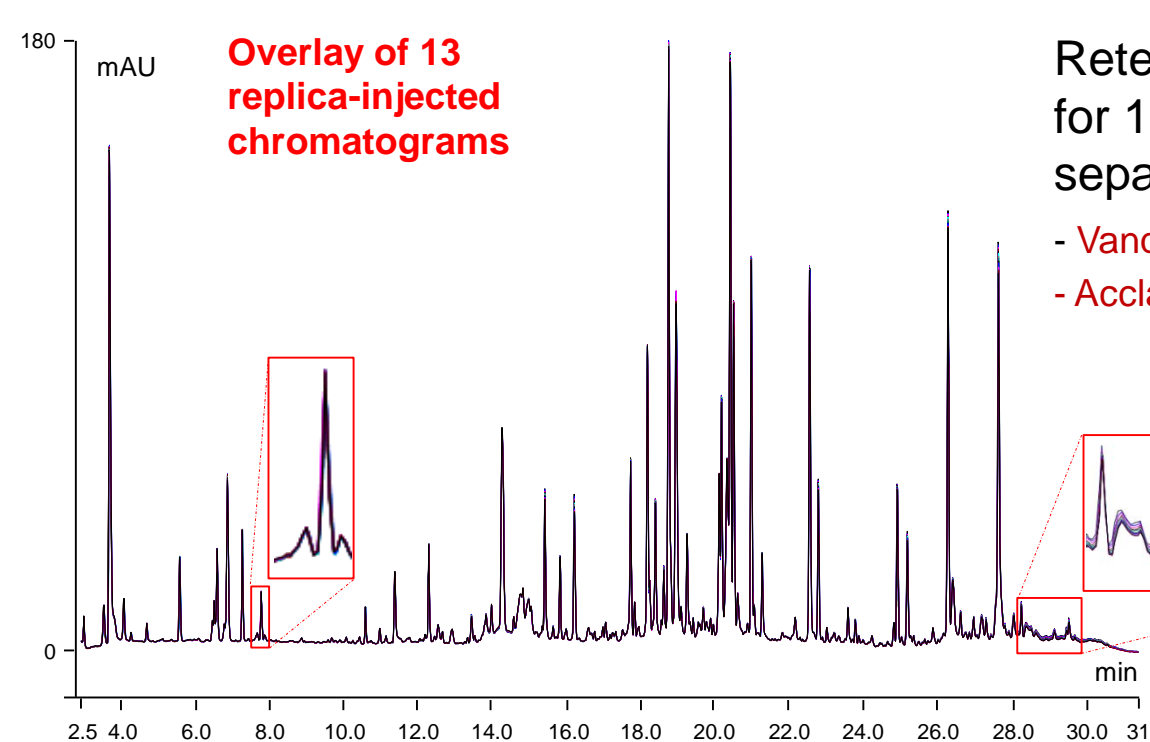
Thermo Scientific™
Q Exactive™ Plus
HRAM-MS



Thermo Scientific™
BioPharma Finder™
informatics platform

UHPLC:

High chromatographic reproducibility



Retention time repeatability for 13 consecutive peptide separations

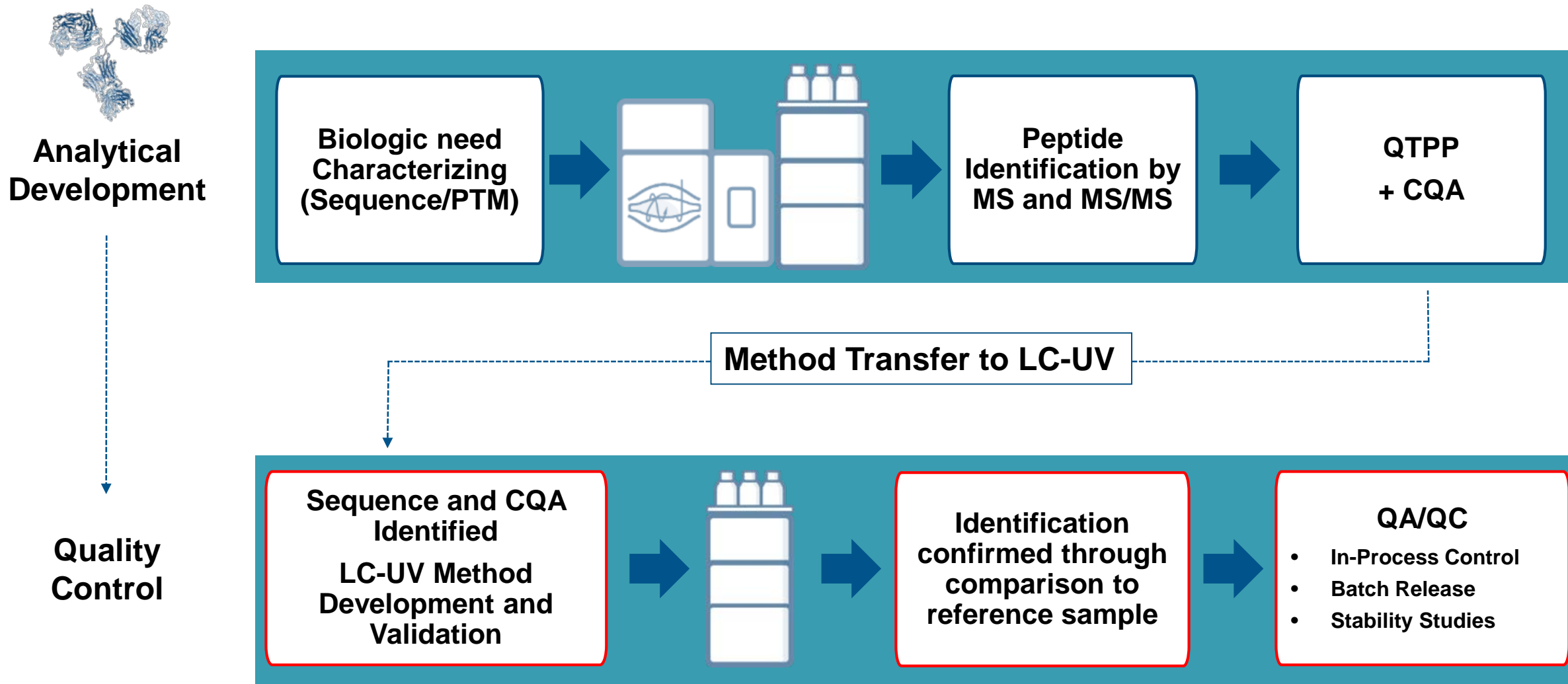
- Vanquish Horizon UHPLC
- Acclaim 120 C18 (2.2 μ m, 2.1x250 mm)

peak	t_R [min]	%RSD (t_R)
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



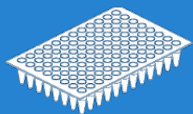
Vanquish Horizon
Vanquish Flex

Customer Need: Method Transfer from LC-MS to LC-UV for a Biotherapeutic



Confidence in reproducibility of results to achieve Transfer to LC-UV only platform

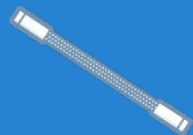
Confidence in Digestion Results with HRAM



Thermo Scientific™
SMART Digest™



Thermo Scientific™
Vanquish™ Flex
UHPLC



Thermo Scientific™
Acclaim™ 120 C18
column



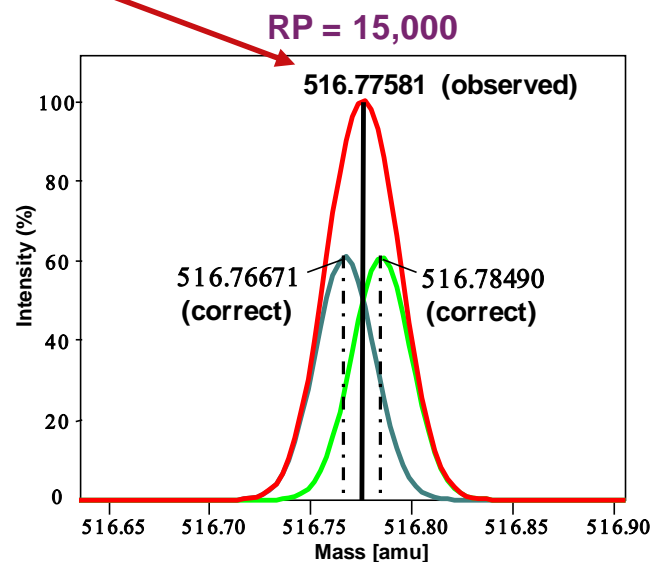
Thermo Scientific™
Q Exactive™ Plus
HRAM-MS



Thermo Scientific™
BioPharma Finder™
informatics platform

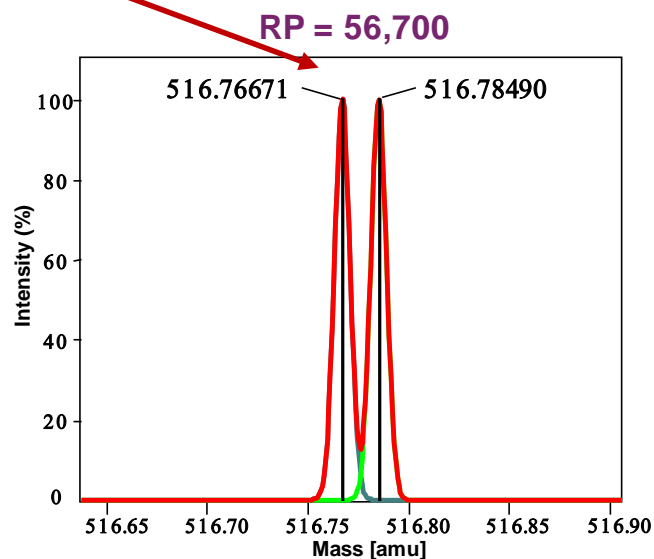
High Resolution Accurate Mass -
Providing confidence in results

Wrong Answer for Both Peptides



Peptide mixture:
Sequence: [Val⁹]-Angiotensin II
DRVYVHPF
Formula: C₄₉H₆₉N₁₃O₁₂
Exact mass: [M+2H]²⁺ = 516.76671
Dm (mmu): 18.2 mmu

Right Answer for Both Peptides



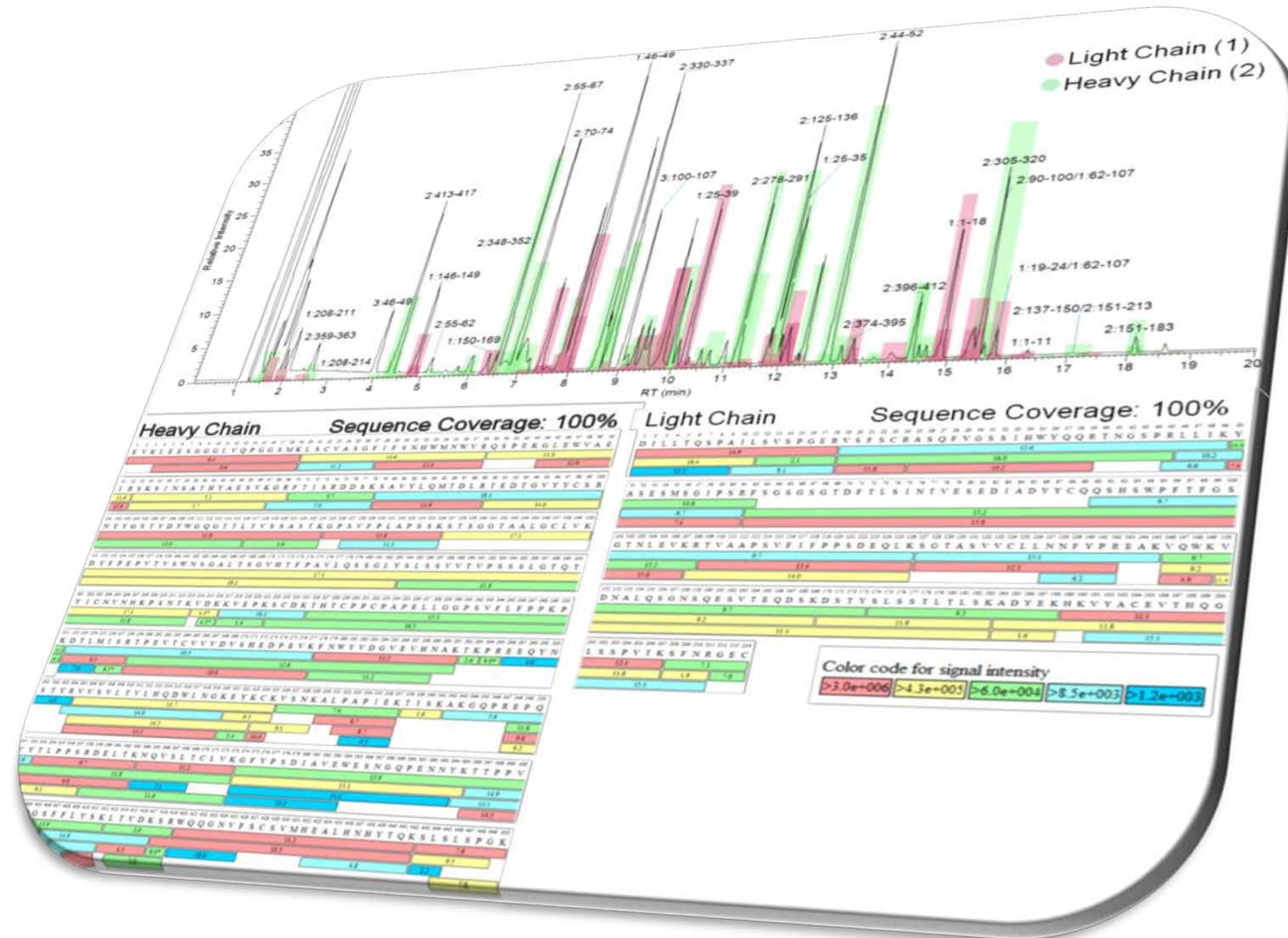
Lys-des-Arg⁹-Bradykinin
KRPPGFSPF
Formula: C₅₀H₇₃N₁₃O₁₁
Exact mass: [M+2H]²⁺ = 516.78490



Joshua J. Coon, et al. ASMS 2012 oral, MOB pm

100% Sequence Coverage of a Monoclonal Antibody Light Chain and Heavy Chain

- Inflixumab



Site of Aggregation

Adduct formation

N-terminal
pyroE
formation

Conjugation Site (ADC)

Conjugation Site (ADC)

AREA of DETAIL

ThermoFisher
S C I E N T I F I C

Thermo Scientific SMART Digest – Automation

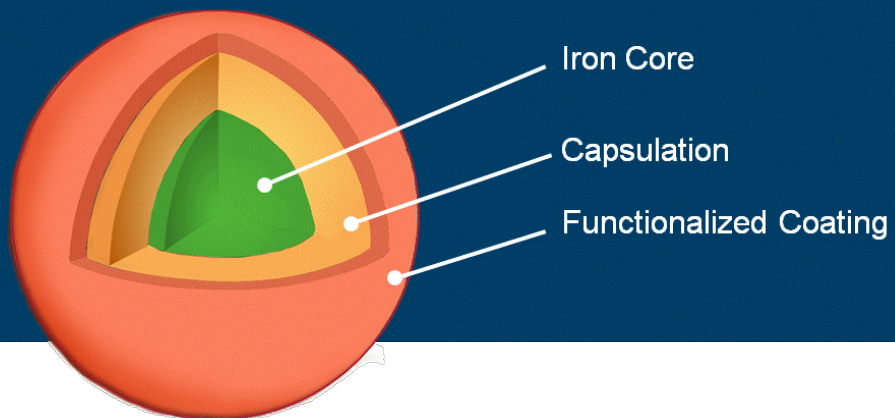
Immunoglobulin protein | ca. 150,000 Daltons | participates in the immune reaction as the antibody for a specific antigen | There are five main types: IgA, IgD, IgE, IgG, and IgM

Humanized IgG antibody fragment (Fab) | 50,000 Daltons | VH, CH1 and VL, CL regions, linked by an intramolecular disulfide bond.

STRUCTURAL INSIGHTS

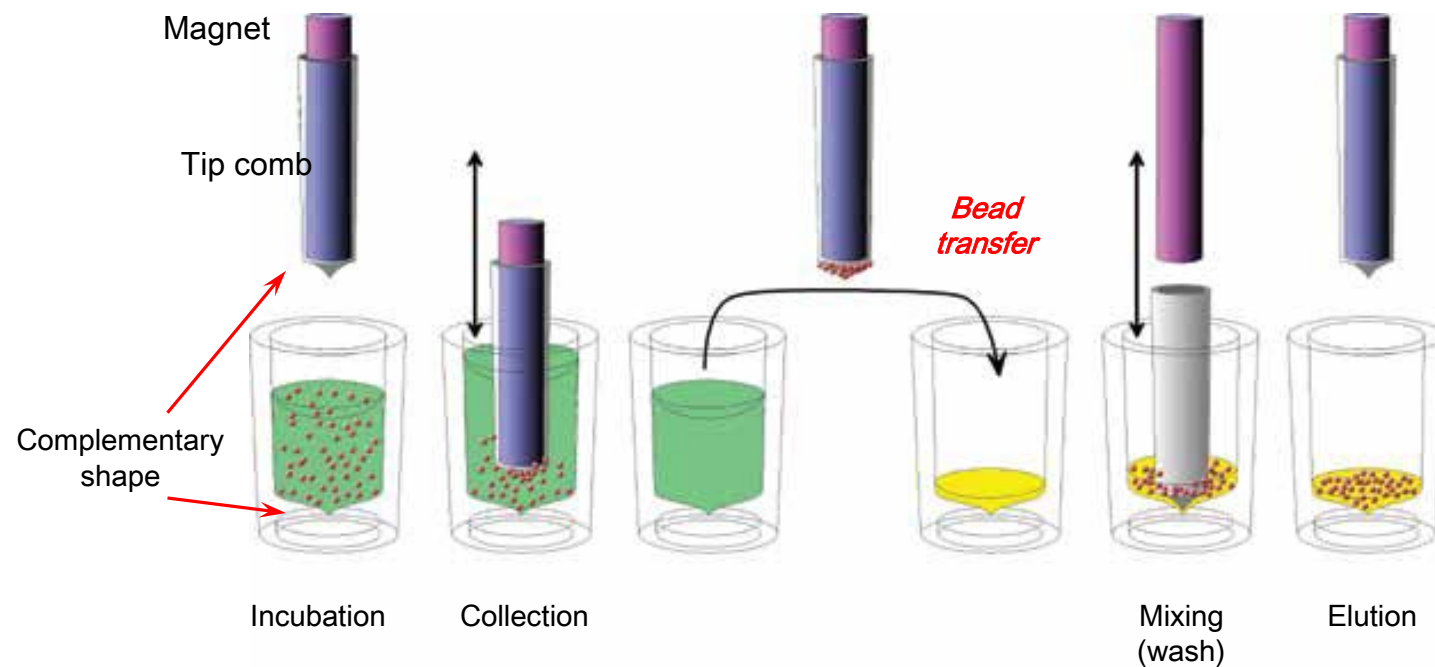
Easy Automation of Digestion

Magnetic Particle



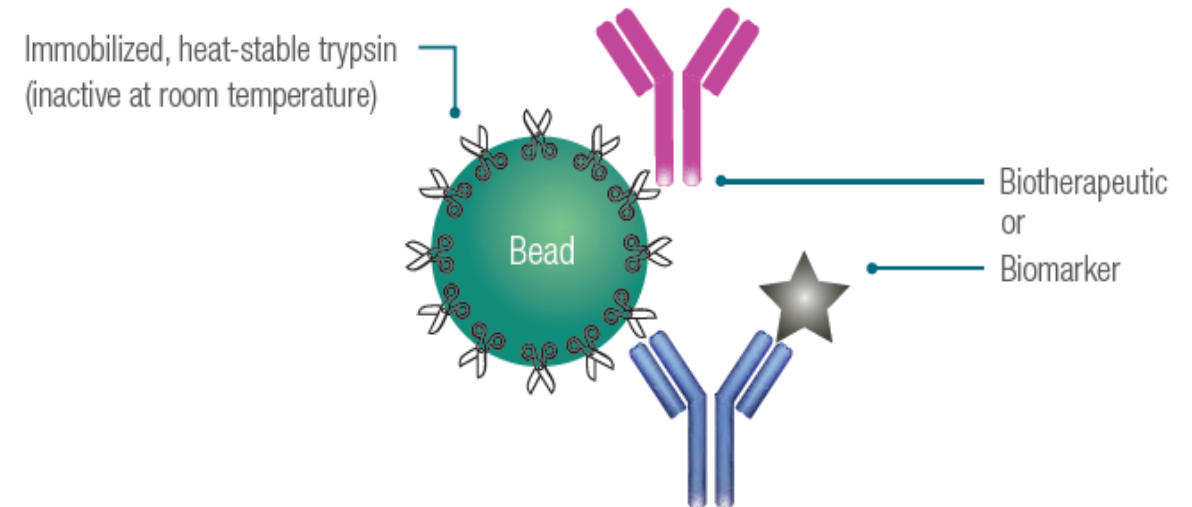
Thermo Scientific™ KingFisher™ purification systems:

- Only the magnetic beads are transferred
- High-speed purification
- Contaminants are left behind
- High-quality, concentrated samples
- Operator-independent
- Utmost reproducibility



Immobilized Heat Stable Enzyme: Reproducible Protein Digestion Within Minutes

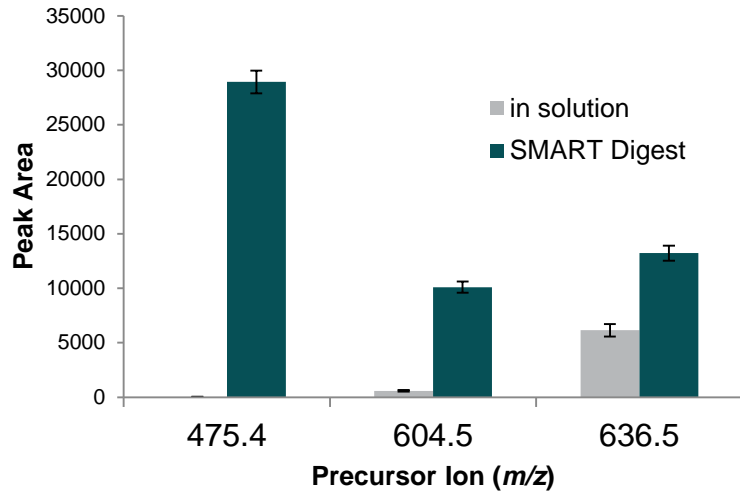
- **SMART Digest** is a heat-stable immobilized enzyme digestion kit;
 - Proteins are heat-denatured for digestion,
 - Additional denaturing agents or reduction and alkylation is not required
- High-throughput and automation-compatible formats
 - 96 x PCR tubes pre-packed with resin
 - Bulk resin format
 - Magnetic Bulk resin format
- Additional post digestion clean up options:
 - 96 well filter plate
 - SOLA μ SPE plate



Increased Sensitivity for Targeted MS Analysis

Confident detection of biomarkers with high sensitivity within a wide dynamic range

Case study: Thyroglobulin in plasma

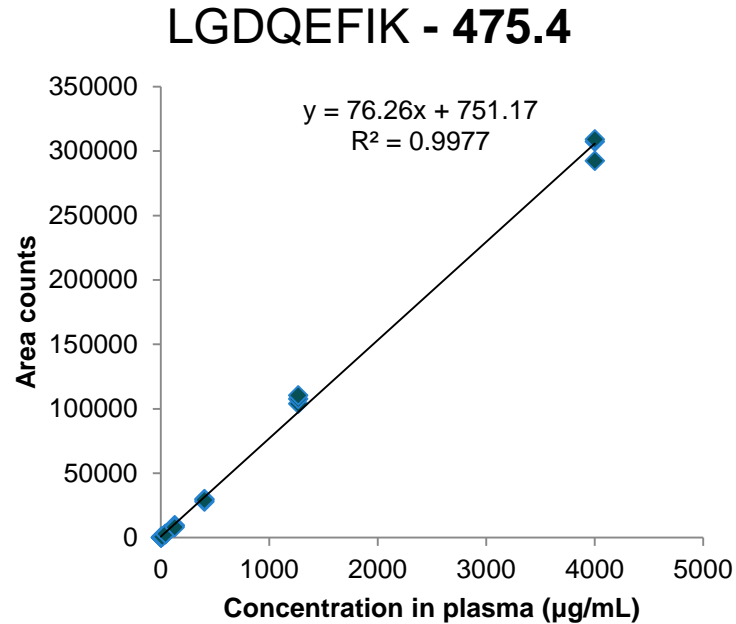


Measurement of serum Thyroglobulin after tryptic digestion of serum samples

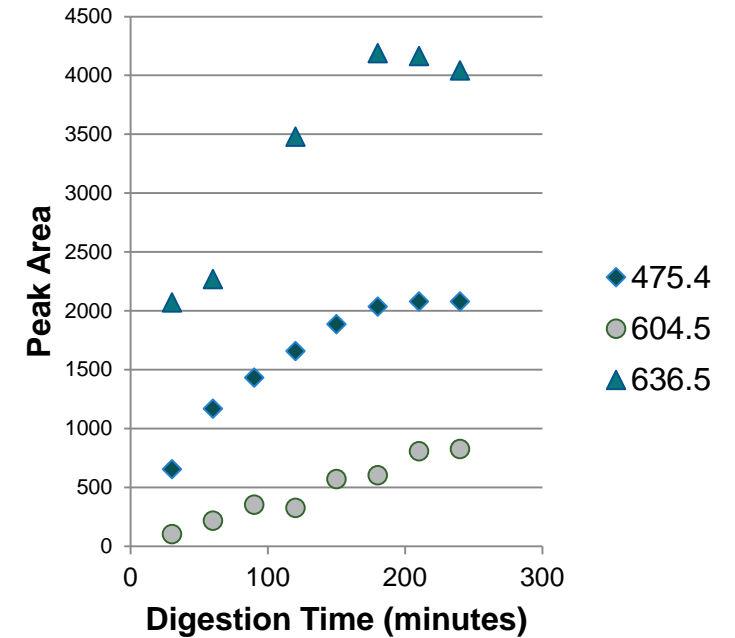
SMART Digest: 25% plasma, 3.5 h digestion

in-solution digest: 20% plasma, R/A, 4 + 16 h digestion

Clarke et al. (2012), J. Investigative Medicine, 60(8)



Calibration curve for thyroglobulin signature peptide in murine plasma (4 – 4000 µg/mL)



Digestion time curve (70°C)

Immuno Affinity Capture and Digestion in One

Combination of heat-stabile, immobilized trypsin with affinity capture: SMART-Digest
Streptavidin
Protein A
Protein G

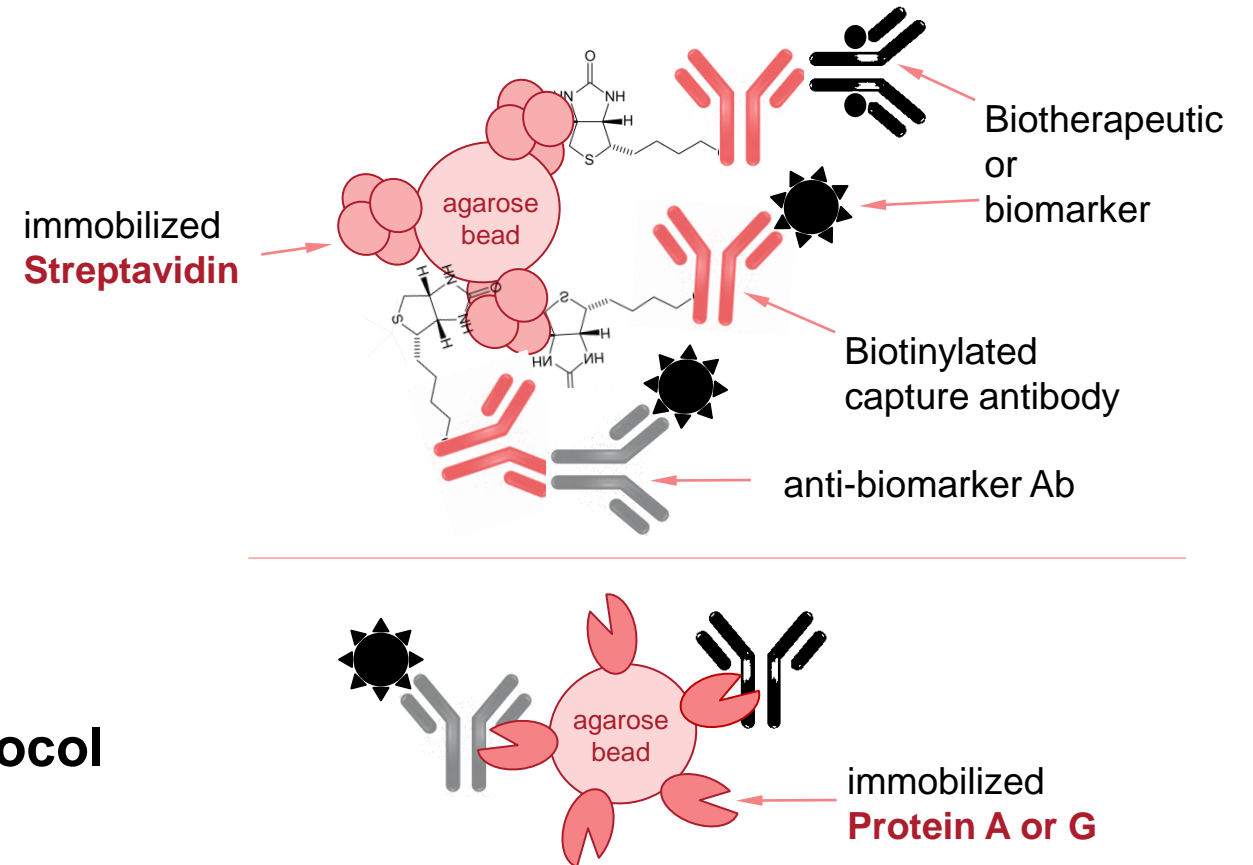
Biomarker quantitation is a challenging task:

- Many biomarker proteins are present at low levels
- Samples are typical body fluid matrices and matrix interferences are common.

Immunoaffinity capture is an established and effective protein concentration technique ...

- ⇒ cleaner sample
- ⇒ increased sensitivity

... But it adds a labor intensive step to the protocol

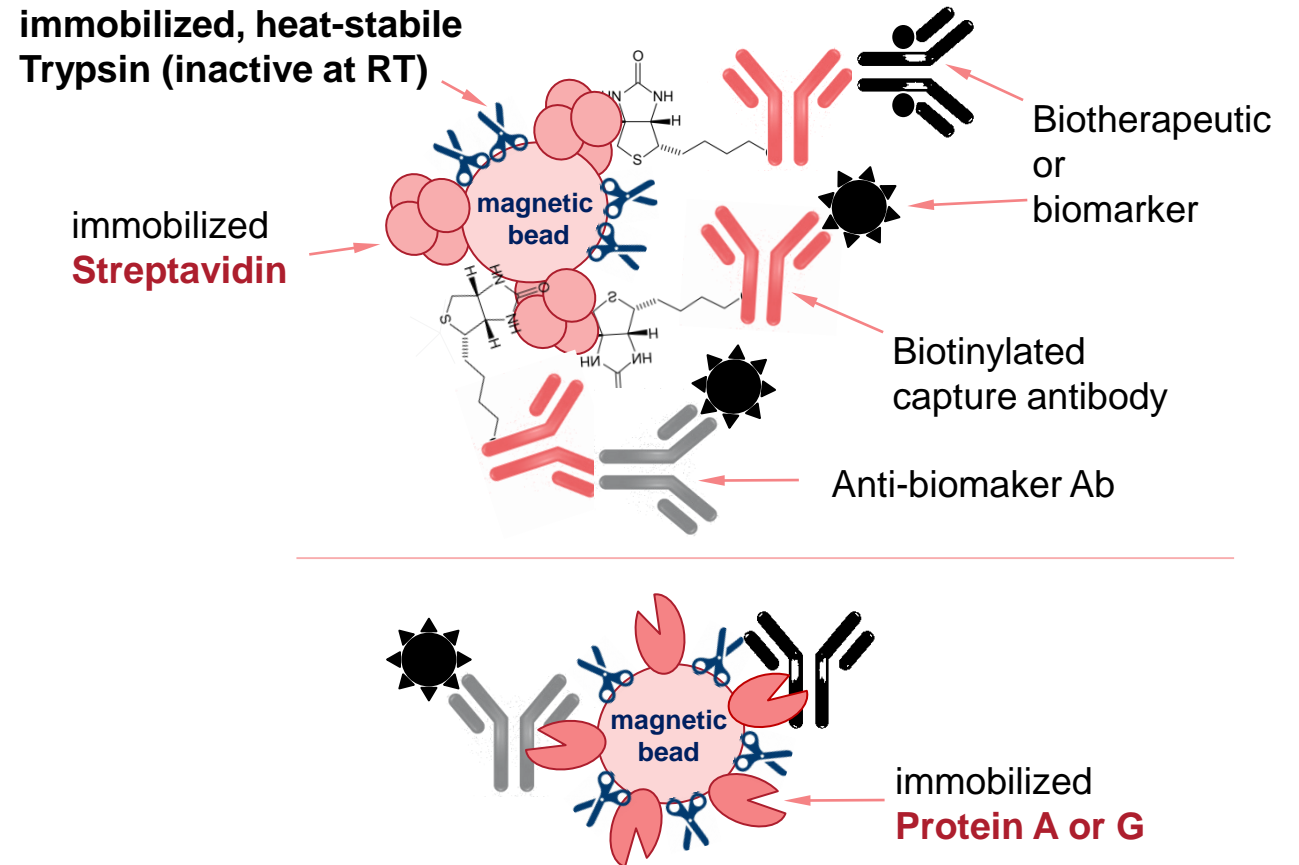


Immuno Affinity Capture and Digestion in One

Combination of heat-stabile, immobilized trypsin with affinity capture: **SMART-Digest Streptavidin**
SMART-Digest Protein A
SMART-Digest Protein G

SMART Digest immunoaffinity kits:

- Combine affinity capture & digestion protocols into a single process
- Enables:
 - Faster sample processing **3-4 hrs**
 - High throughput
 - Greater ROI
- Compatible with automation systems (Kingfisher) due to magnetic bead design
- Greater applicability to high throughput establishments

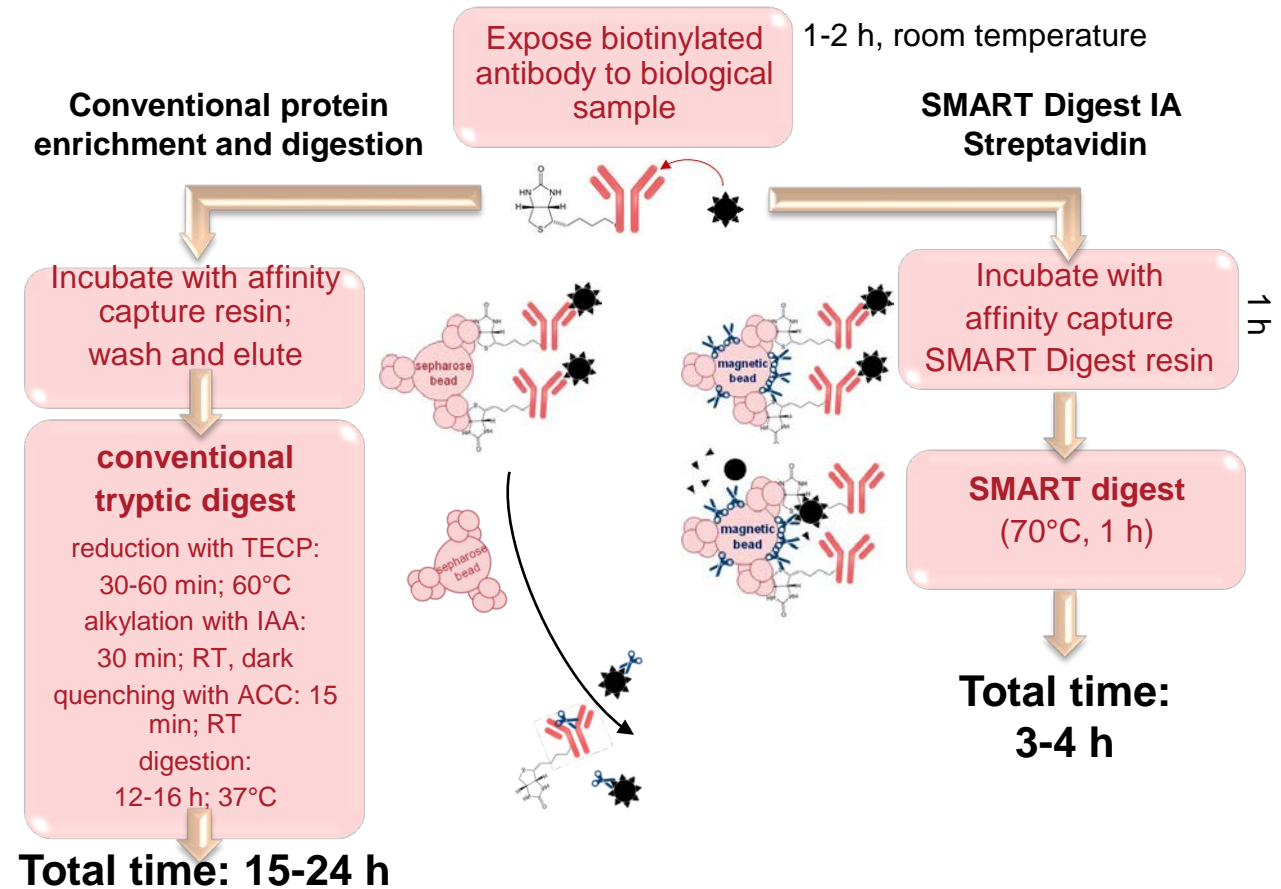


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Immuno Affinity Capture and Digestion in One

Combination of heat-stabile, immobilized trypsin with affinity capture: SMART-Digest Streptavidin
 SMART-Digest Protein A
 SMART-Digest Protein G

Test case:

Soluble plasma protein biomarker

Spike-in SIL peptide

Assay acceptance criteria:

± 20% of nominal for accuracy at all levels

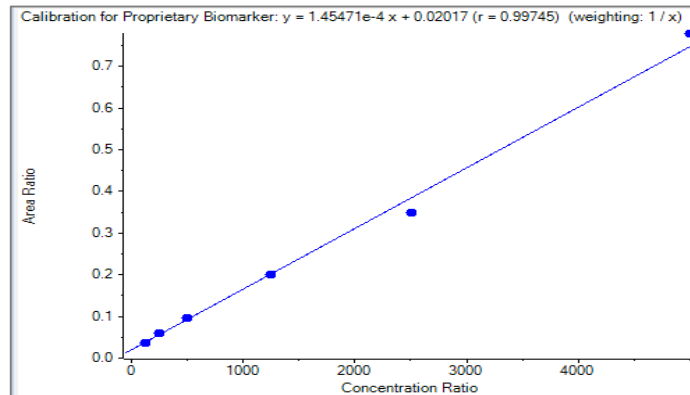
Assay range: 20 - 1000 ng/mL (Peptide A)

100 - 2000 ng/mL (Peptide B)

Method recovery

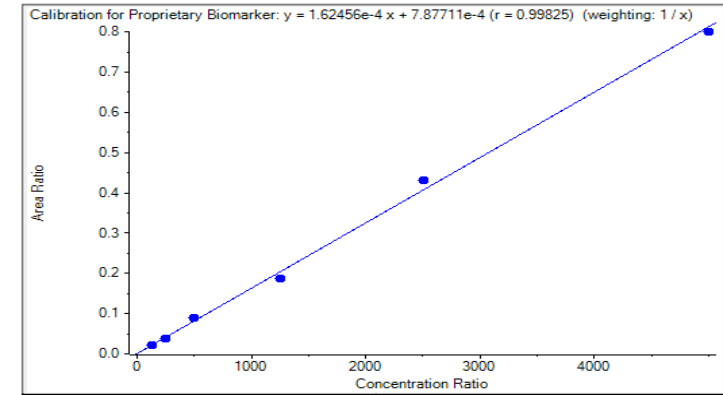
	SMART Digest IA	Streptavidin agarose
500 ng/mL spike:	7330 (cps)	2778 (cps)
% Recovery:	64%	35%

SMART Digest IA - Streptavidin



Standard curve (n = 1)			Quality controls (n = 4)		
Actual Conc (ng/mL)	Accuracy (%)	Calc Value (ng/mL)	Actual Conc (ng/mL)	CV (%)	Accuracy (%)
125	93	116.5	250	11.5	90.2
250	107	266.3			
500	106	531.1			
1250	100	1247			
2500	90	2251			
5000	104	5212	1250	7.4	99.1
15-20 %RSD					

Streptavidin agarose



Standard curve (n = 1)			Quality controls (n = 4)		
Actual Conc (ng/mL)	Accuracy (%)	Calc Value (ng/mL)	Actual Conc (ng/mL)	CV (%)	Accuracy (%)
125	104.7	131	250	14.5	111.2
250	90.0	225			
500	108.8	544			
1250	91.9	1149			
2500	106.2	2654			
5000	98.5	4922	1250	4.1	104.8
20-25 %RSD					

Immuno Affinity Capture and Digestion in One

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 SMART-Digest Protein A
 SMART-Digest Protein G

Test case:

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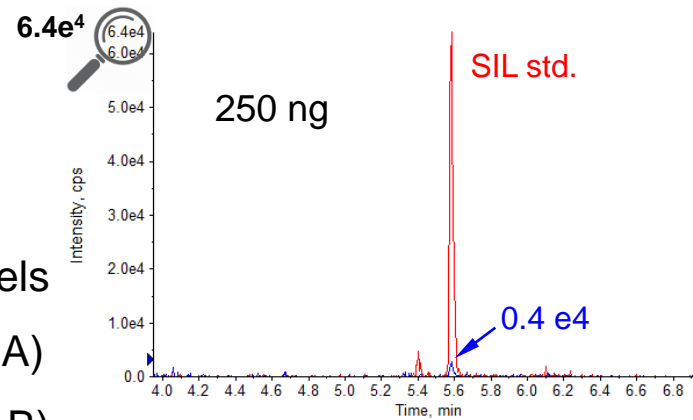
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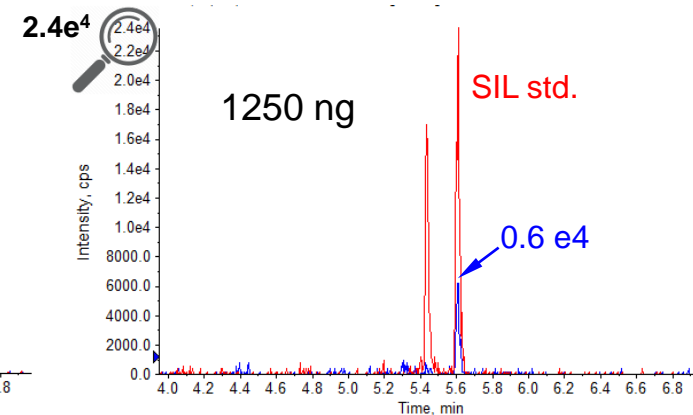
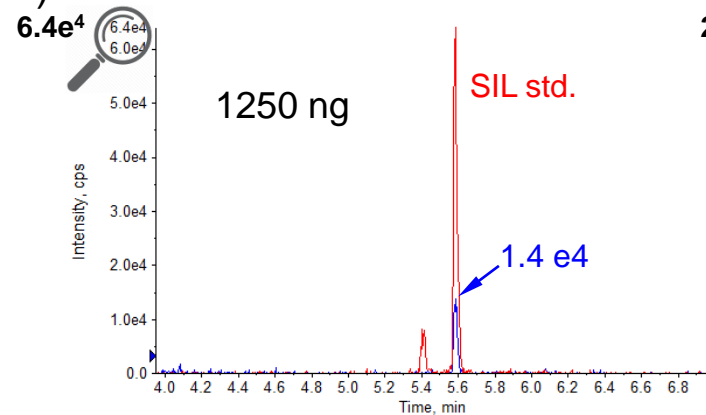
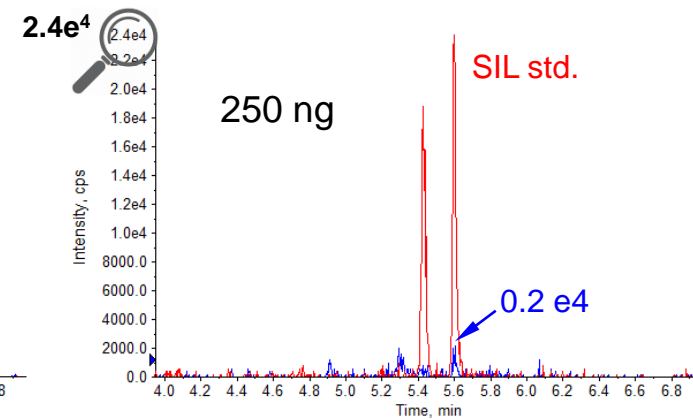
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SMART Digest IA - Streptavidin



Streptavidin agarose



SMART Digest immunoaffinity kits

Improved method recovery

similar quantitative precision and accuracy

up to 7 times faster

Immobilized heat stable enzyme and immuno affinity sample preparation enable workflows which are:

- Easier to use
- Highly reproducible
- Easy to automate
- Sensitive

Thank You

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Visit Booth #2632

Join us Tuesday through
Thursday from 3:30 – 5:00 p.m.
to collaborate with our technical experts

Redeem this ticket
for a complimentary beverage!

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