

AREA of DETAIL

Adduct formation

Site of Aggregation

N-terminal
pyroE
formation

Conjugation Site (ADC)

Conjugation Site (ADC)

ThermoFisher
SCIENTIFIC

SMART Digest Kits: Simple, Fast and Reproducible Protein Digestion and Immuno Affinity Capture Extensions

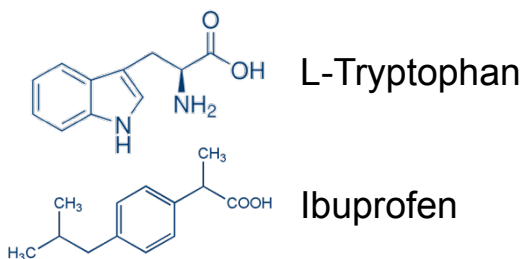
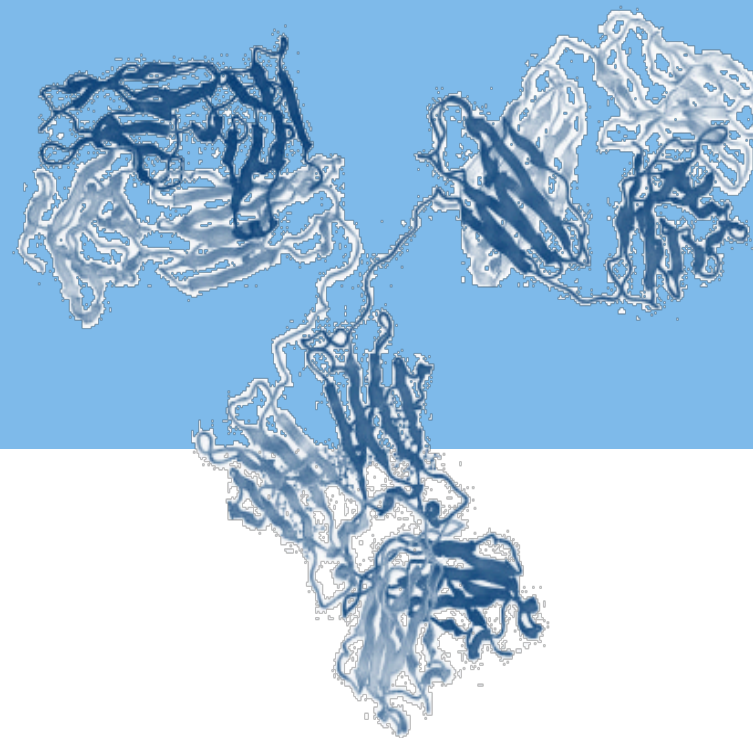
reaction as the antibody for a specific antigen | There are five main types:
IgA, IgD, IgE, IgG, and IgM

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

STRUCTURAL INSIGHTS

Why is There a Growth in Biotherapeutics?

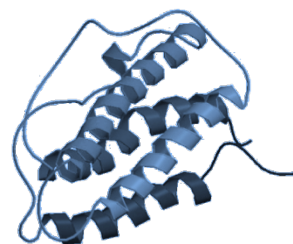
- 8/10 drugs in 2016 biologics
- Biopharma growing ~10% over the next five years
- \$160 billion
- Success rate at clinical phase I
 - Classic synthetic drugs ~7%
 - Biologics ~ 12%



Typical pharmaceutical
MW ~ 200 Da
e.g. Ibuprofen



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



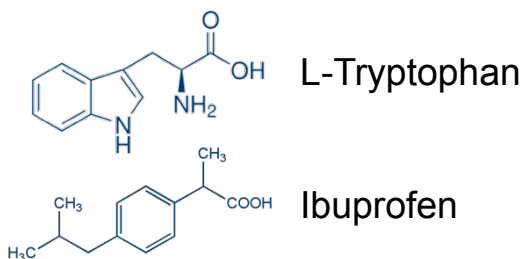
Erythropoietin (EPO)
MW ~ 18000 Da
165 amino acids
e.g. Eprex

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**



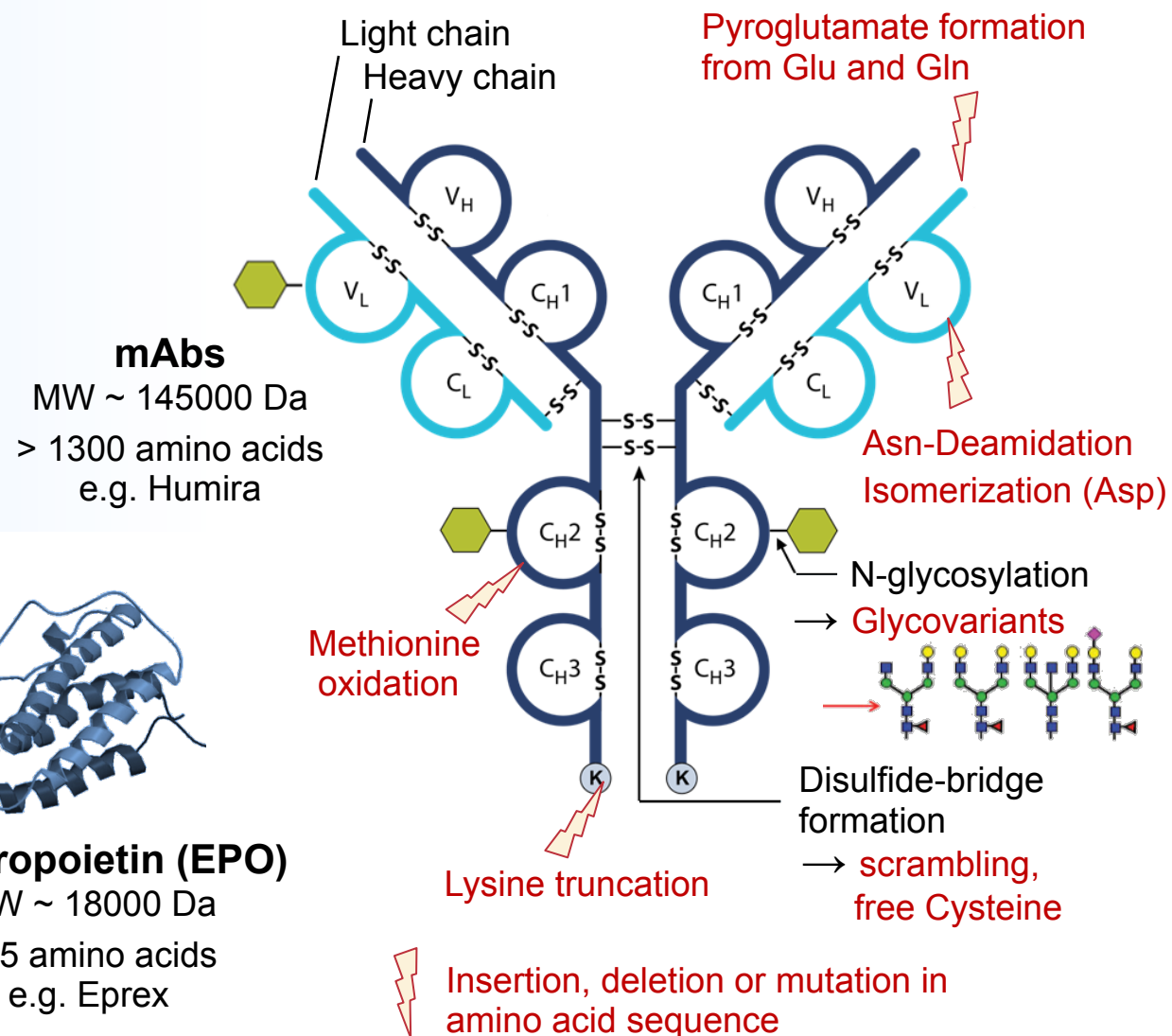
Typical pharmaceutical
 MW ~ 200 Da
 e.g. Ibuprofen



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



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



Peptide Mapping Workflow in Biopharmaceutical Characterization



Drug Discovery



Drug Development



Pre-Clinical &
Clinical Drug Testing



Chemistry, Manufacturing
& Controls (CMC)



Pharmaceutical QA/QC

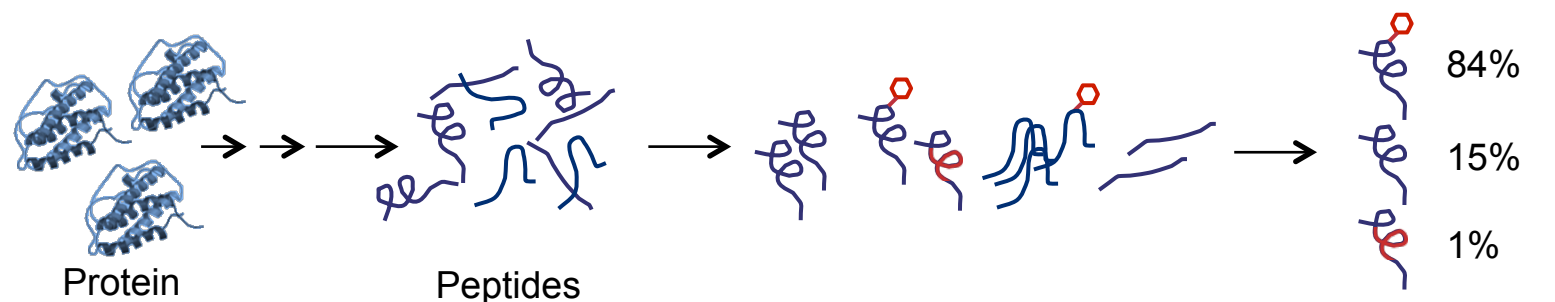
Peptide mapping

Peptide Mapping Workflow in Biotherapeutic Characterization

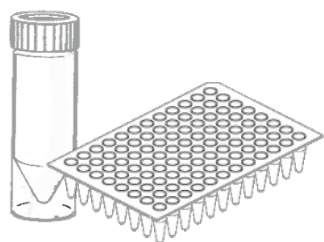
Crucial workflow for biotherapeutic characterization from development to QC

→ Sequence verification, quantitative and qualitative assessment of modifications

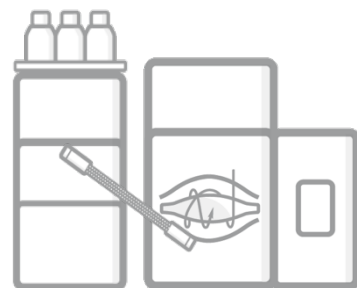
⇒ Identity, purity and heterogeneity ⇒ Safety and efficacy for the patient



- Amino acid sequence
- Type and site of modifications
- Abundance



Sample preparation
and proteolytic digestion



Separation (LC, MS)
and detection (MS; MS²)



Data processing & evaluation

Principal workflow shared with:

- Targeted biomarker quantification
- Bottom-up Proteomics

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

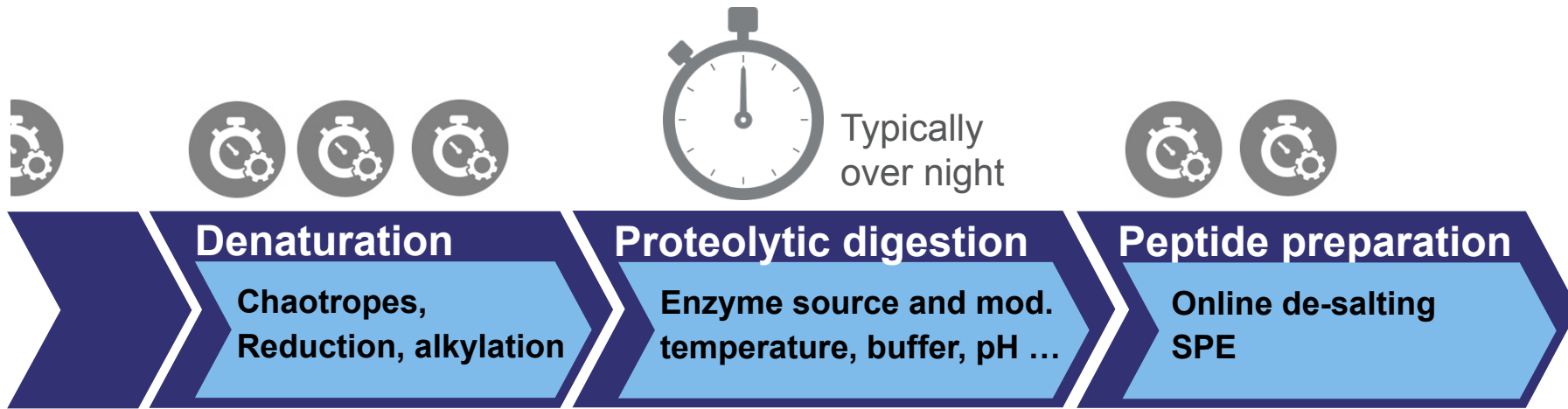
Dilute to 1 M Urea

Add Trypsin to vial and digest overnight

Spin to remove particulates

Extract peptides with SPE

Accelerating 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~~

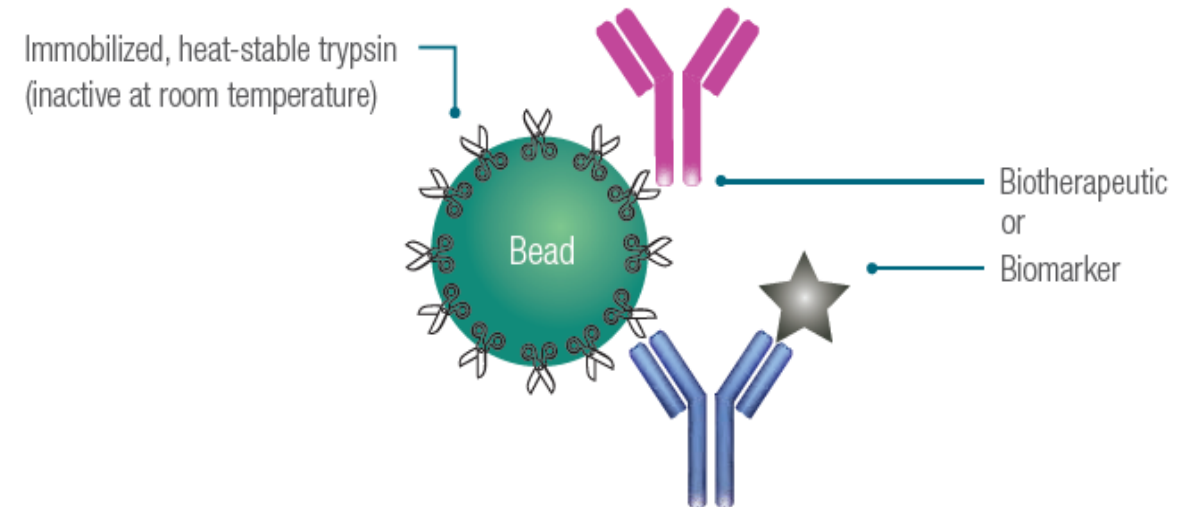
Add Trypsin to vial and
digest overnight

Spin to remove particulates

Extract peptides with SPE

SMART Digest Kits: Easy, Reproducible Protein Digestion Within Minutes

- Thermo Scientific™ SMART Digest™ kit are heat-stable immobilized enzyme digestion kits:
 - 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
 - Thermo Scientific™ SOLA μ ™ SPE plate



SMART Digest Kits: Easy, Reproducible Protein Digestion Within Minutes

- SMART Digest kits are a heat-stable immobilized enzyme digestion kit:
 - Proteins are heat-denatured for digestion
 - Additional denaturing agents are not required

Enzyme options:

- Trypsin
- Soluble Trypsin – complex samples
- Chymotrypsin
- Proteinase K

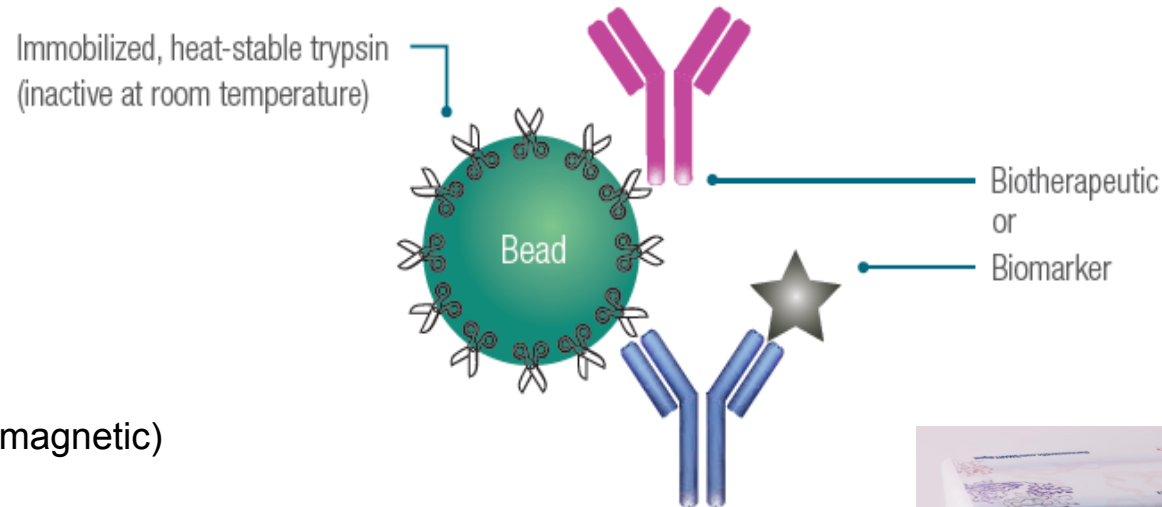
Resin options:

- Pre-packed into SMART Digest tubes (non magnetic)
- Magnetic bulk
- Non-magnetic bulk

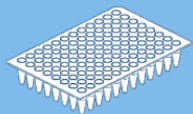
Post digestion clean up options:

- Thermo Scientific™ SOLA μ with collection plate
- Filtration

All kits are supplied with digestion buffer



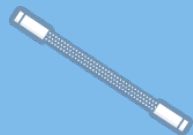
SMART Digest: Easy to Use



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



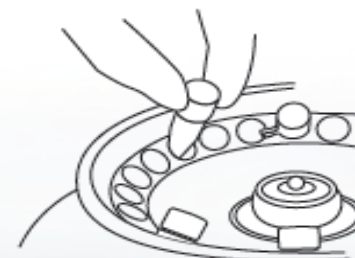
Set your heater/shaker to 70 °C/1400 RPM, allow to equilibrate for 5 minutes. Then add your samples for the required time for digestion.



Note: Refer to the Digestion Optimization Section.

4

Cleanup



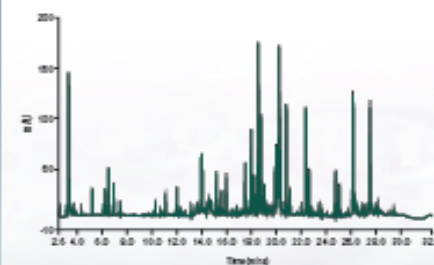
Remove the SMART Digest tube from the heater/shaker and perform one of the sample cleanup methods based on the complexity of your starting matrix.



Note: Refer to the Post Digestion Process.

5

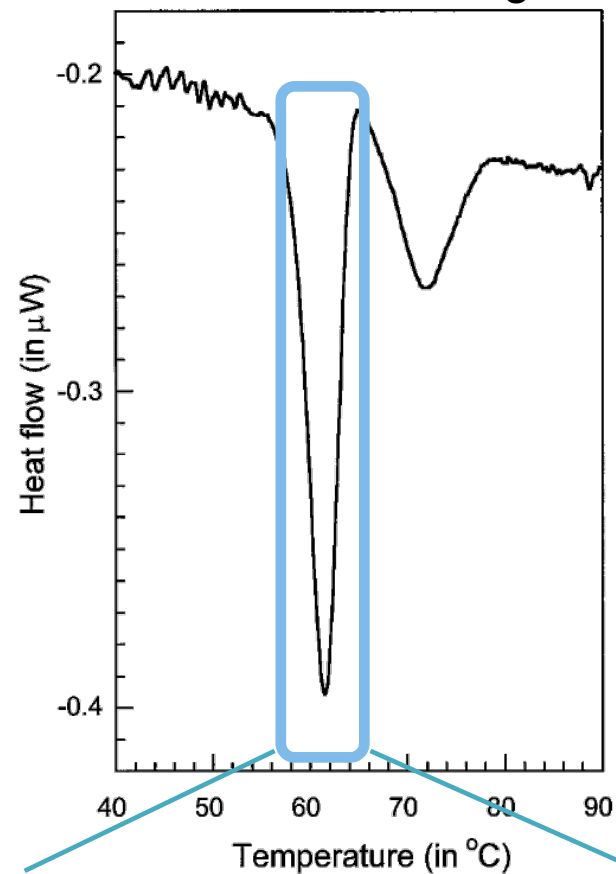
Analyze



Analyze sample.

SMART Digest: Accelerated Protein Digestion

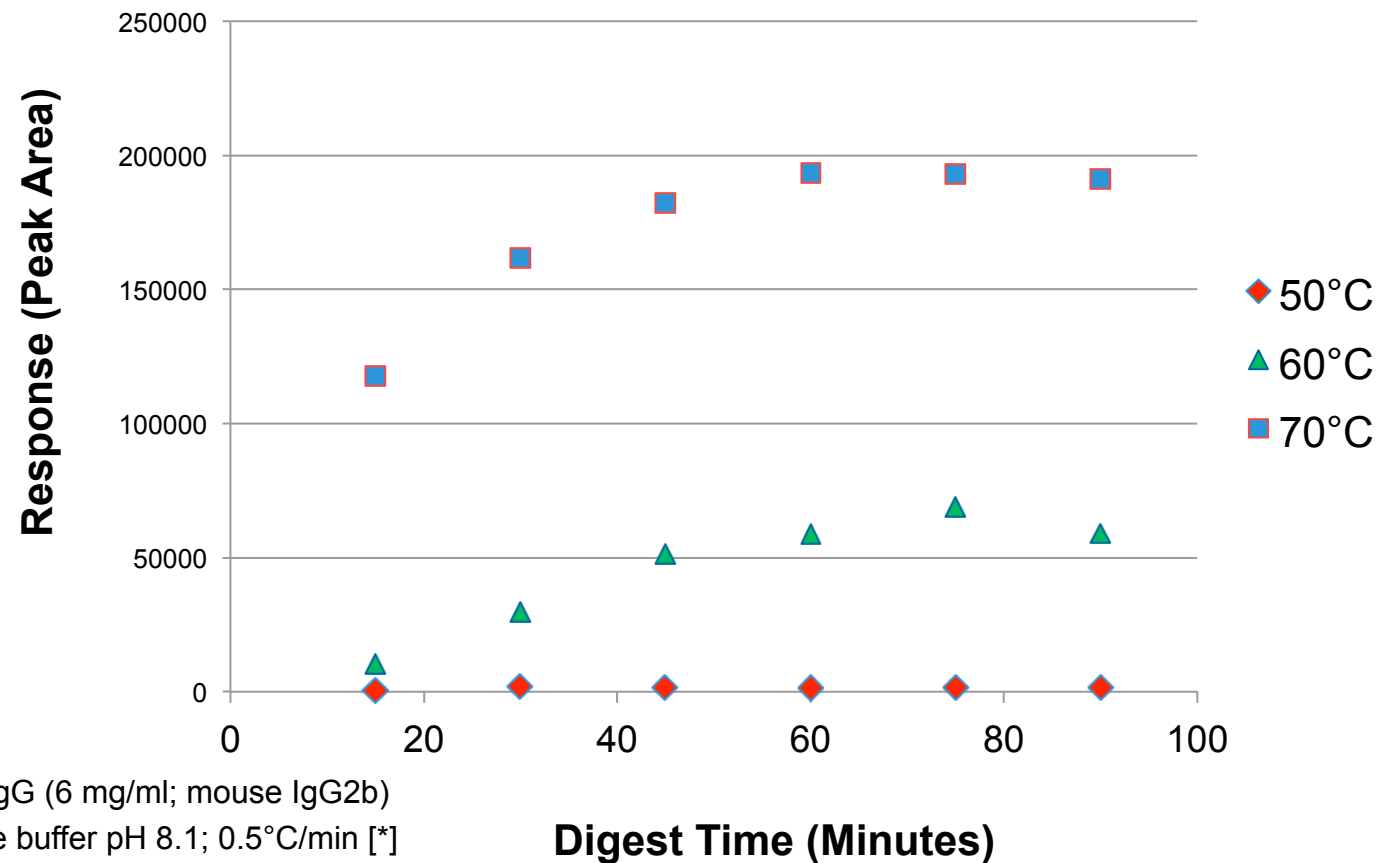
Thermal denaturation of IgG



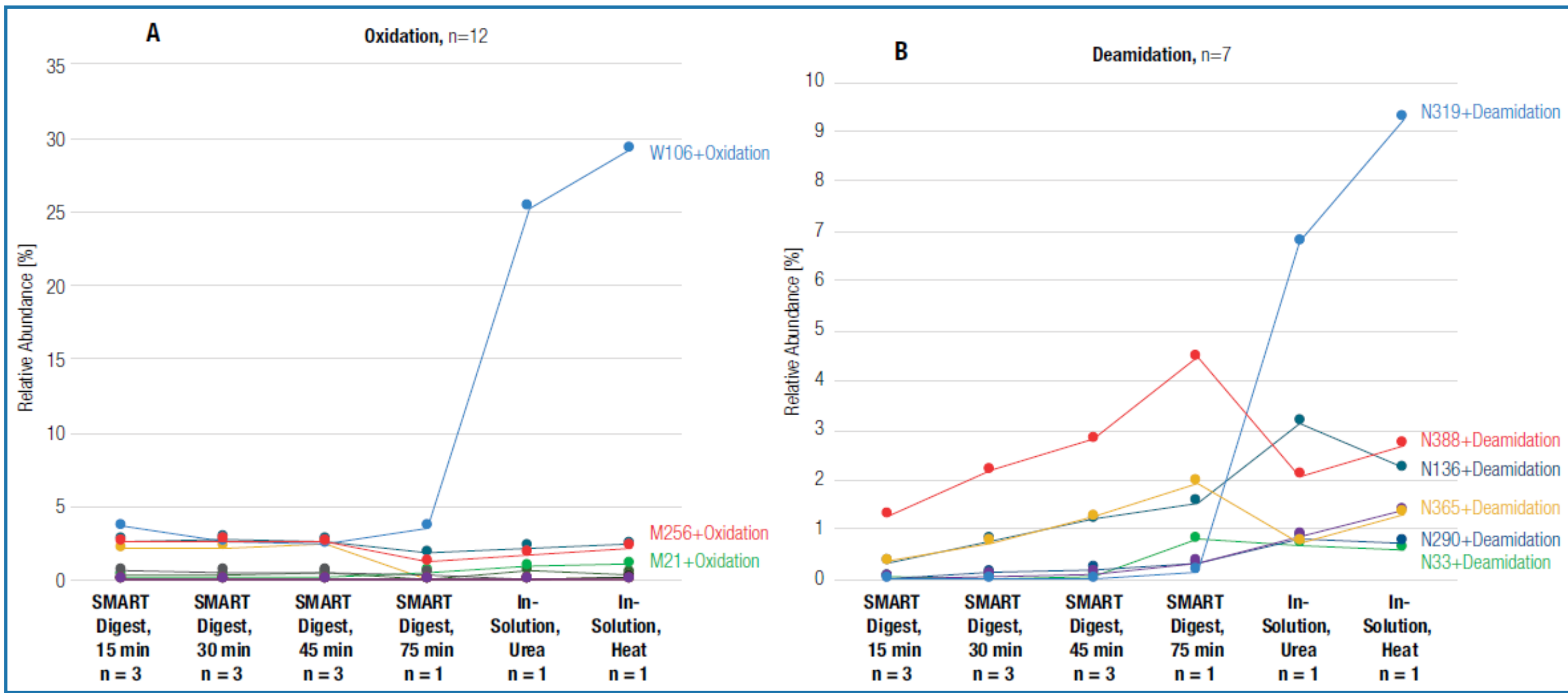
DSC thermogram of IgG (6 mg/ml; mouse IgG2b)
in a 10 mM phosphate buffer pH 8.1; $0.5^{\circ}\text{C}/\text{min}$ [*]

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

Native IgG digest profile monitoring VSVLTVLHQDWLNGK



SMART Digest: Identification of Oxidation and Deamidation



SMART Digest: Accelerated Protein Digestion

Trypsin digests within minutes

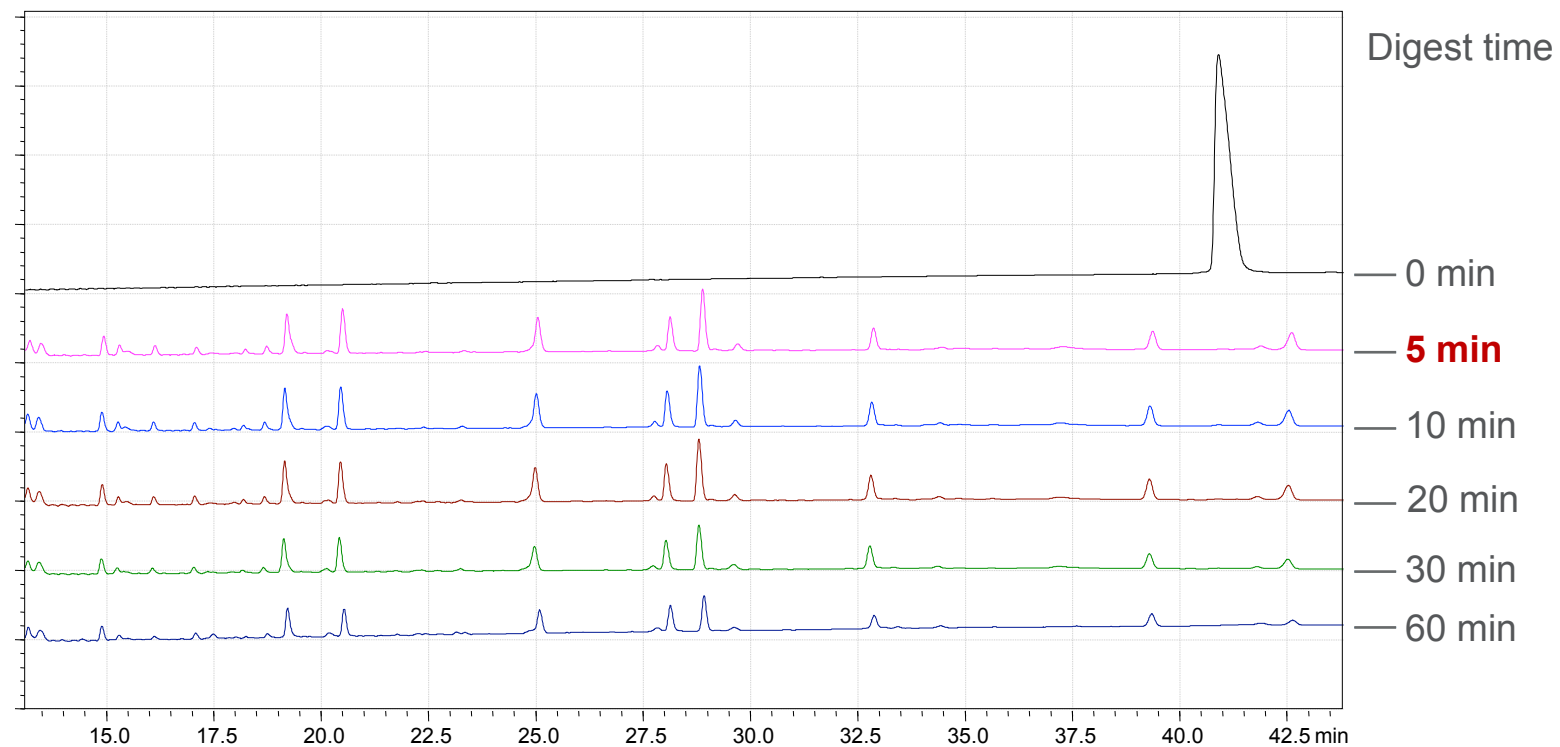
Recommended digestion starting conditions for known proteins*	
Protein	Digest Time (min)
Insulin	4
BSA	< 5
Carbonic anhydrase	< 5
Lysozyme	< 5
Apo-B	30
IgG	45
IgG in 50 μ L plasma	75
Ribonuclease A	150
Thyroglobulin	240
C-reactive protein	240

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

IgG in plasma: 17.5 μ g/mL

Temperature: 70°C

Carbonic anhydrase, 29 kDa



SMART Digest: Accelerated Protein Digestion

Trypsin digests within minutes

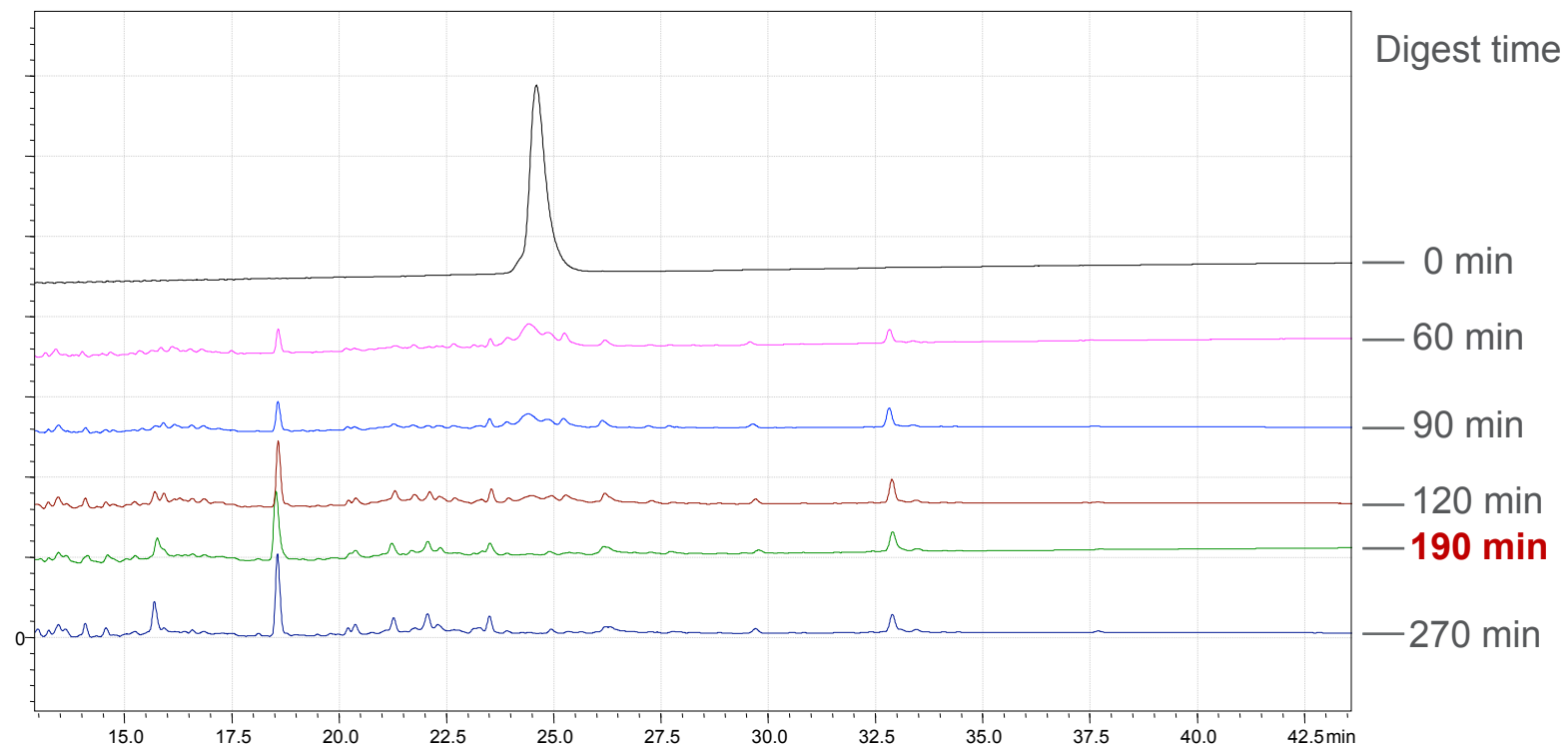
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Insulin	4
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Ribonuclease A	150
Thyroglobulin	240
C-reactive protein	240

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

IgG in plasma: 17.5 μ g/mL

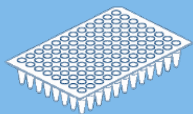
Temperature: 70°C

Ribonuclease A, 137 KDa



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

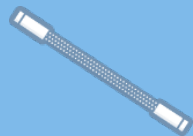
SMART Digest: Reproducible Digestion User to User



Thermo Scientific™
SMART Digest™



Thermo Scientific™
Vanquish™ Flex UHPLC



Thermo Scientific™
Acclaim™ 120 C18
column

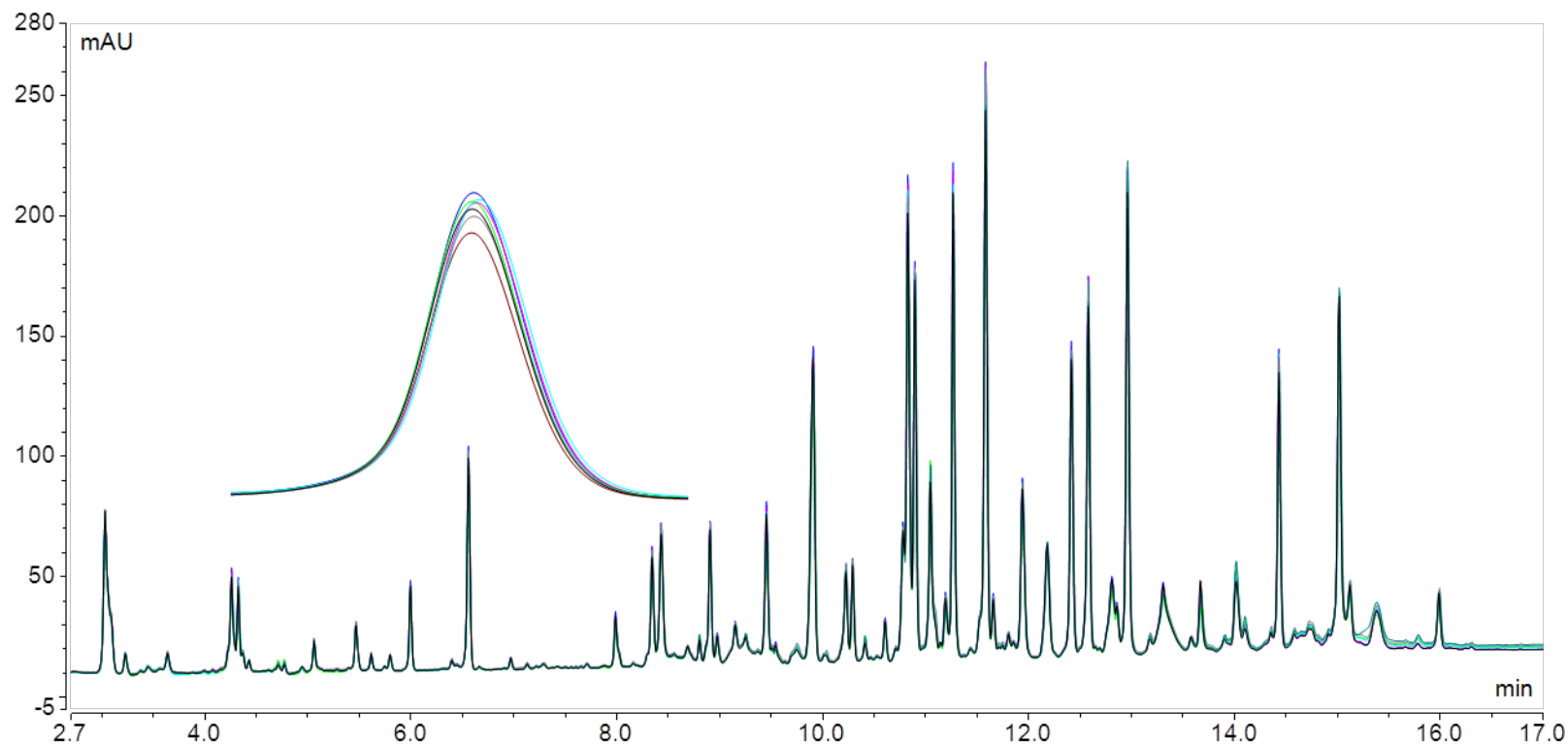


Thermo Scientific™
Q Exactive™ Plus
HRAM-MS



Thermo Scientific™
BioPharma Finder™
informatics platform

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



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

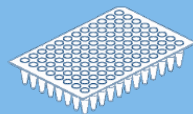
Digest time: 45 min (70°C)

LC-MS run time: 40 min

Sequence coverage: 100%

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

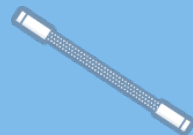
SMART Digest: Reproducible chromatography and digestion results



Thermo Scientific™ SMART Digest™



Thermo Scientific™ Vanquish™ Flex UHPLC



Thermo Scientific™ Acclaim™ 120 C18 column

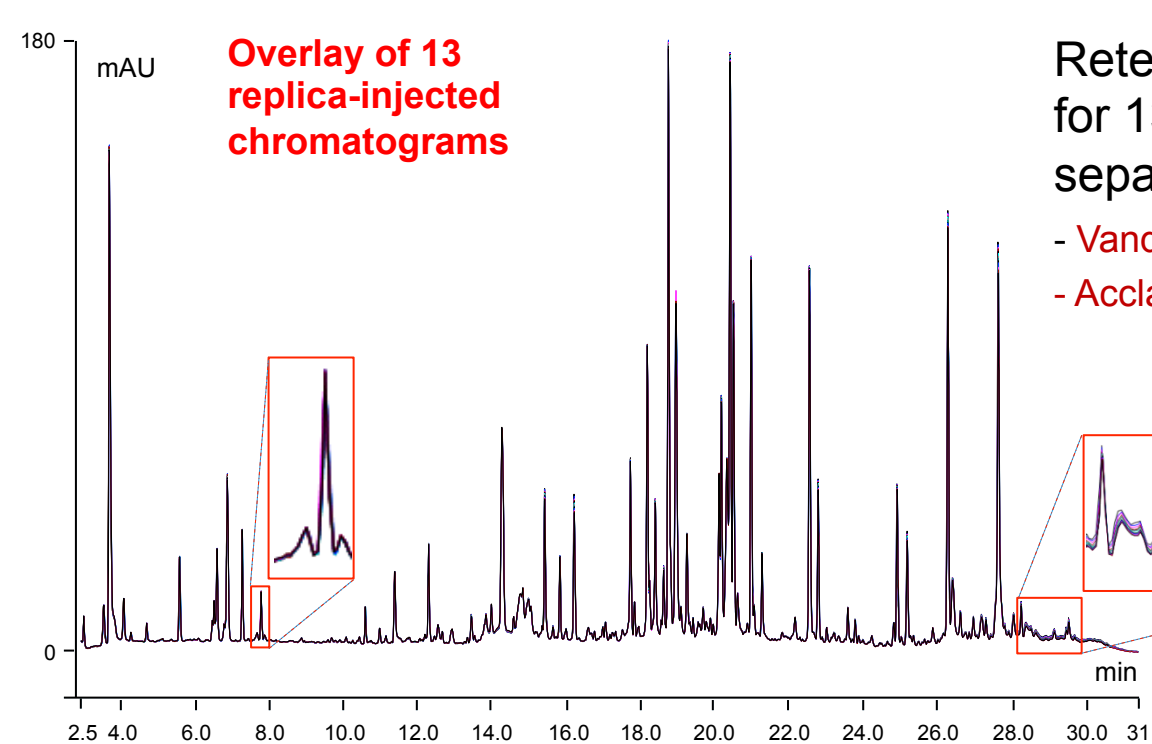


Thermo Scientific™ Q Exactive™ Plus HRAM-MS



Thermo Scientific™ BioPharma Finder™ informatics platform

UHPLC:
High chromatographic reproducibility



Retention time repeatability for 13 consecutive peptide separations

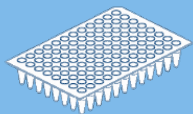
- Vanquish Horizon UHPLC
- Acclaim 120 C18 (2.2 μ m, 2.1x250 mm)

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

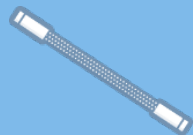
SMART Digest: Confidence in Digestion Results with HRAM



Thermo Scientific™ SMART Digest™



Thermo Scientific™ Vanquish™ Flex UHPLC



Thermo Scientific™ Acclaim™ 120 C18 column



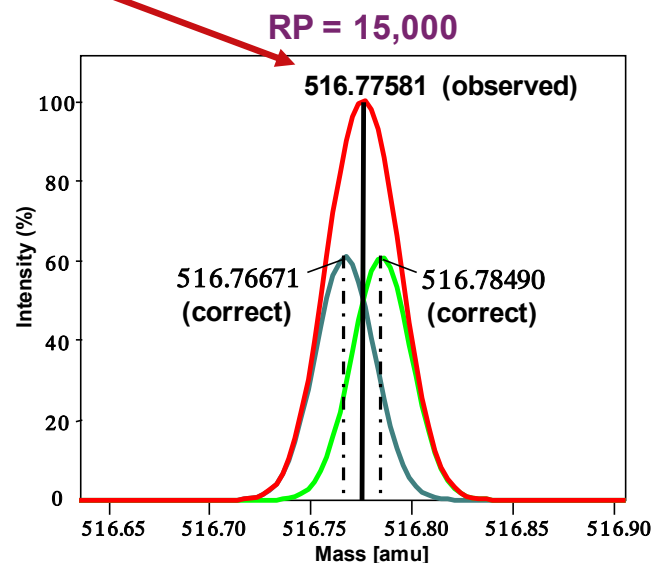
Thermo Scientific™ Q Exactive™ Plus HRAM-MS



Thermo Scientific™ BioPharma Finder™ informatics platform

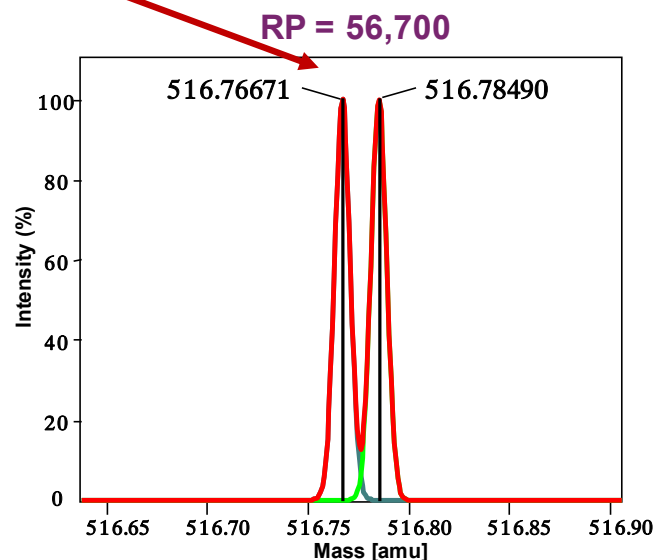
High Resolution Accurate Mass - Providing confidence in results

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



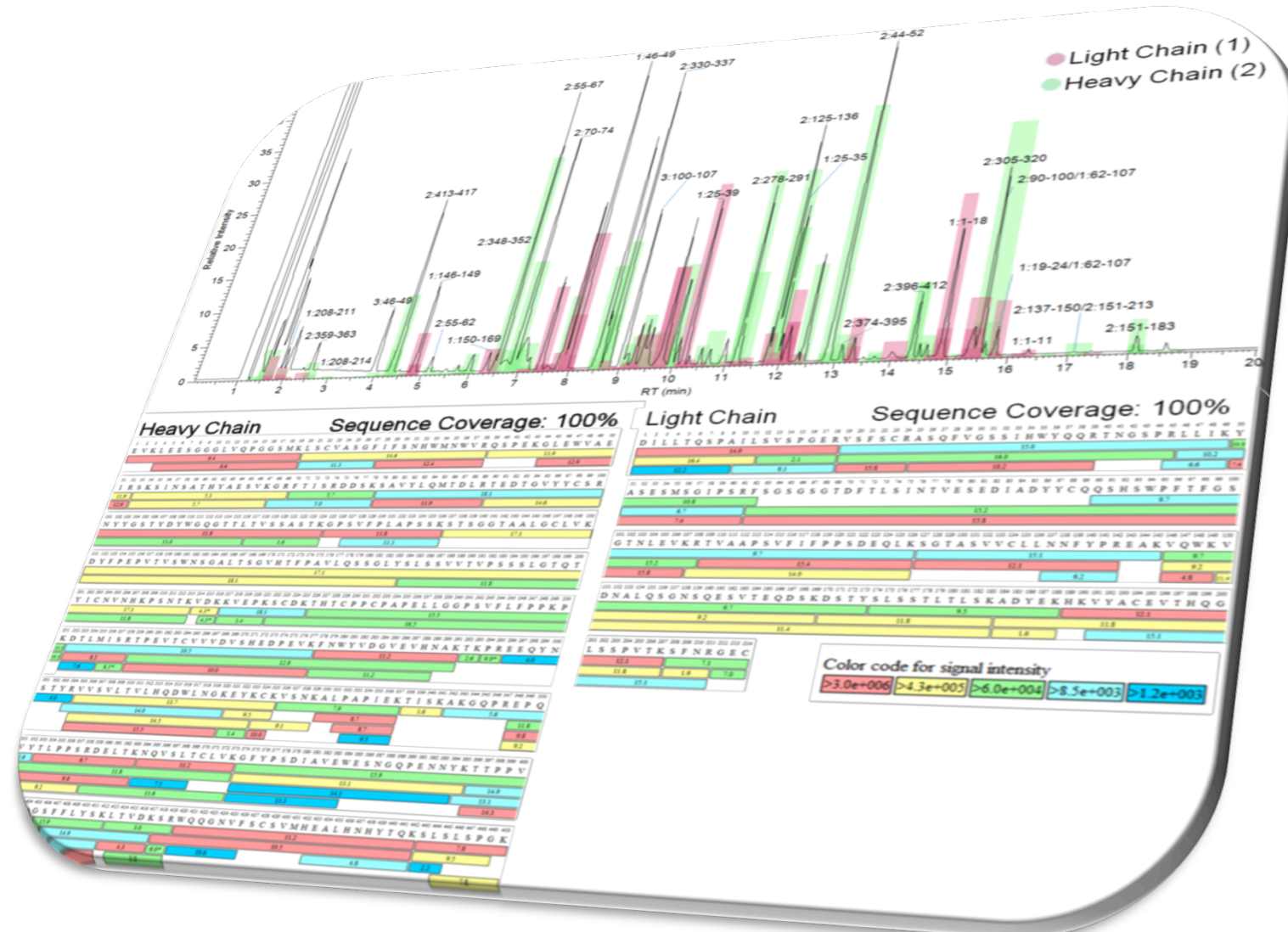
Lys-des-Arg⁹-Bradykinin
 KRPPGFSPF
 Formula: C₅₀H₇₃N₁₃O₁₁
 Exact mass: [M+2H]²⁺ = 516.78490



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

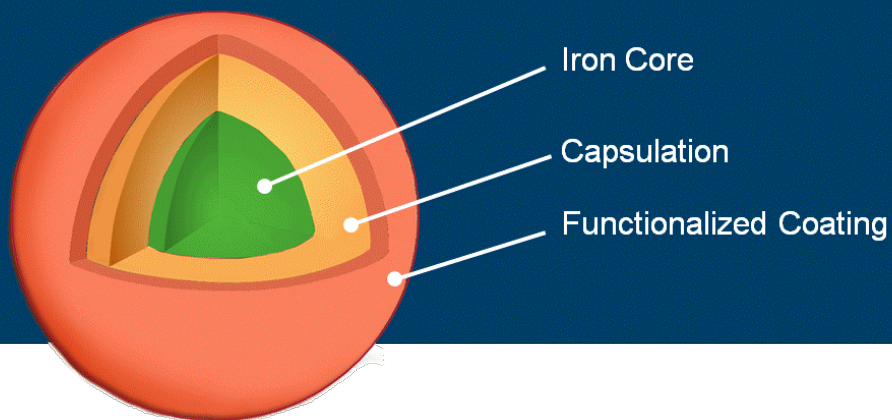
SMART Digest: 100% Sequence Coverage

- Inflixumab



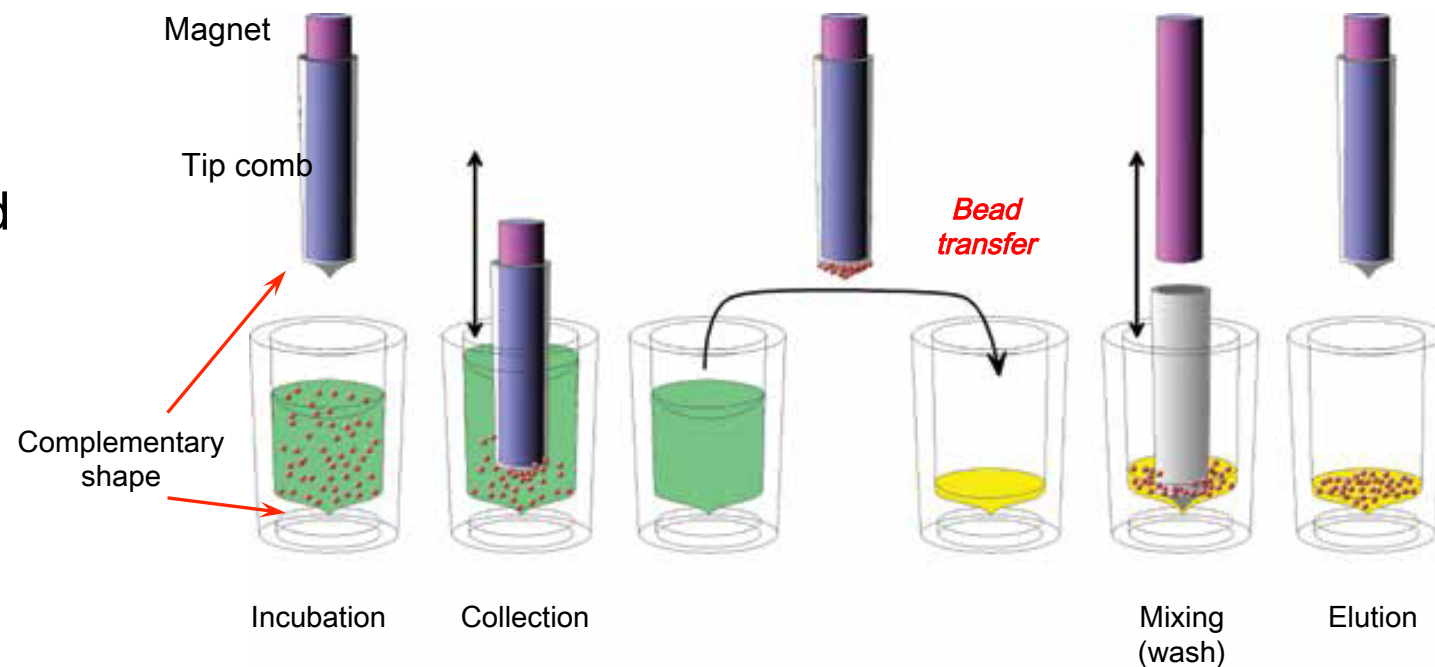
SMART Digest: Easy Automation of Digestion

Magnetic Particle



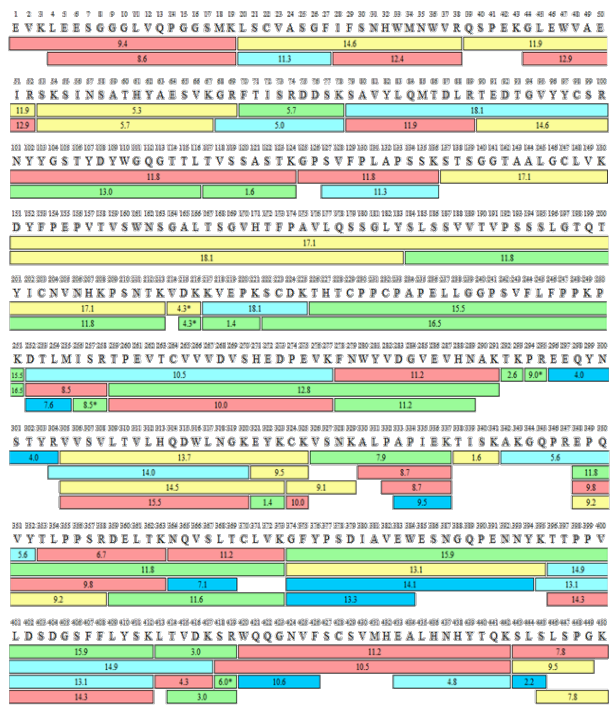
KingFisher purification systems:

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

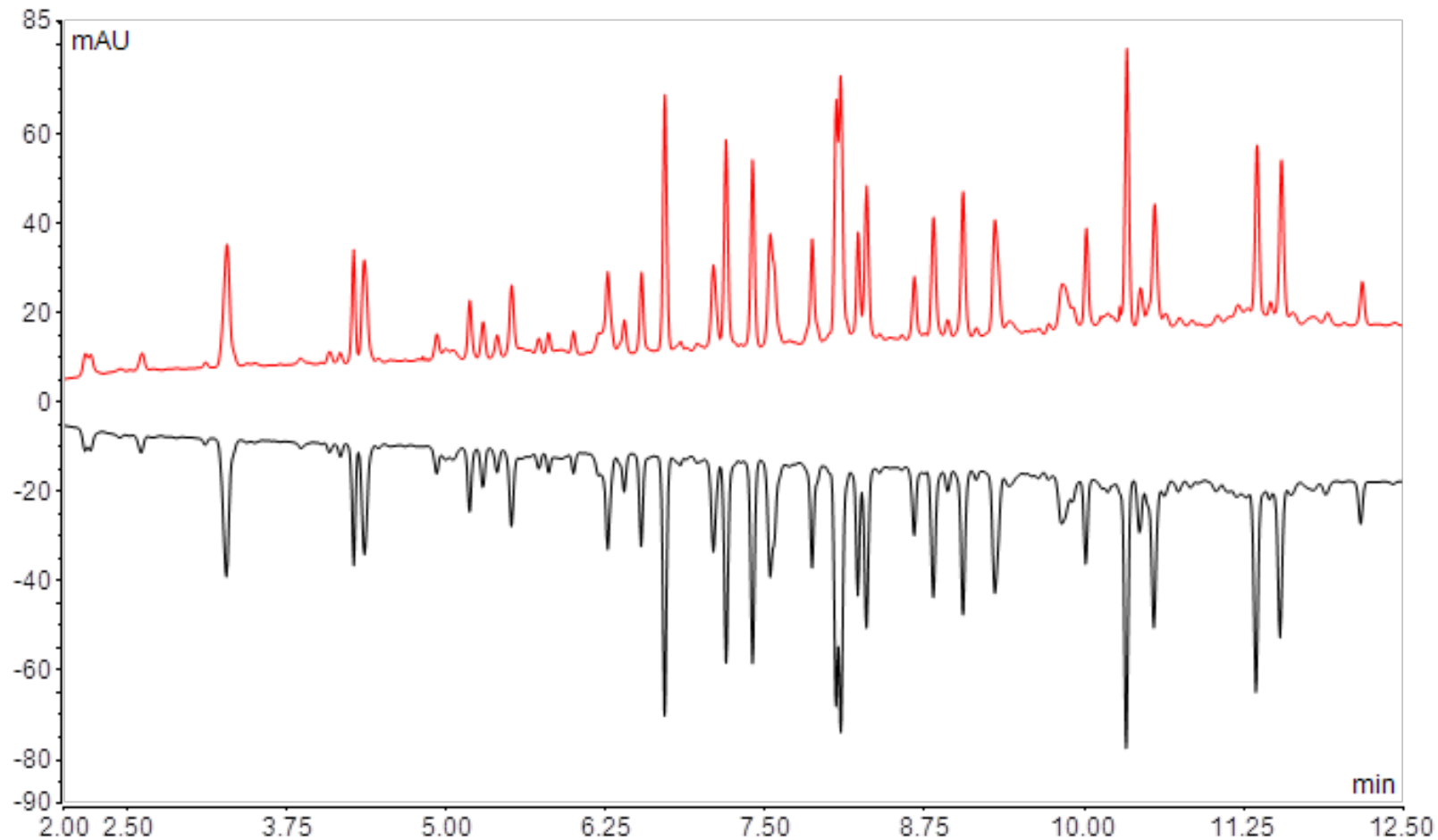
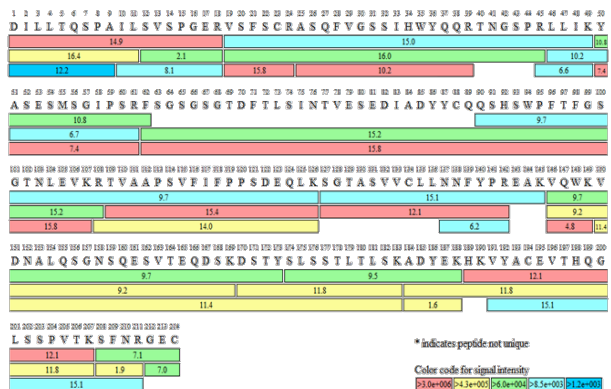


SMART Digest: Automated Digest

Infliximab Heavy Chain Sequence Coverage: 100%

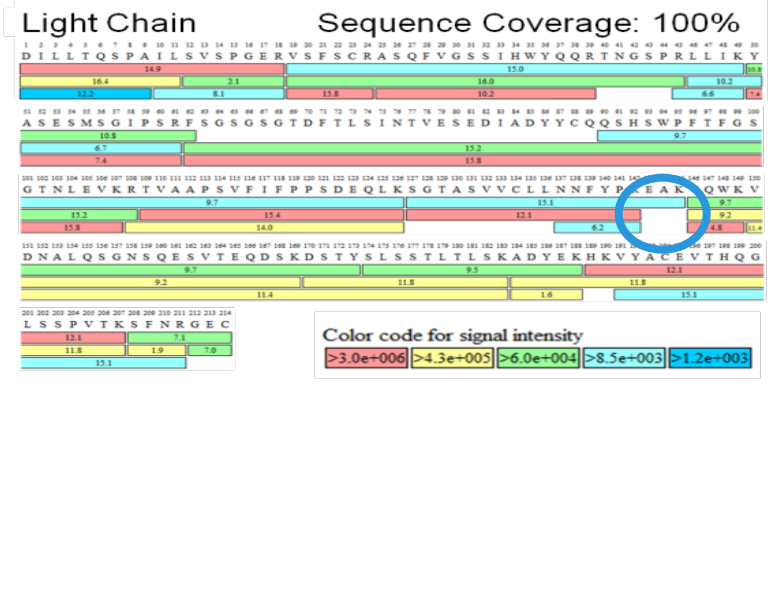
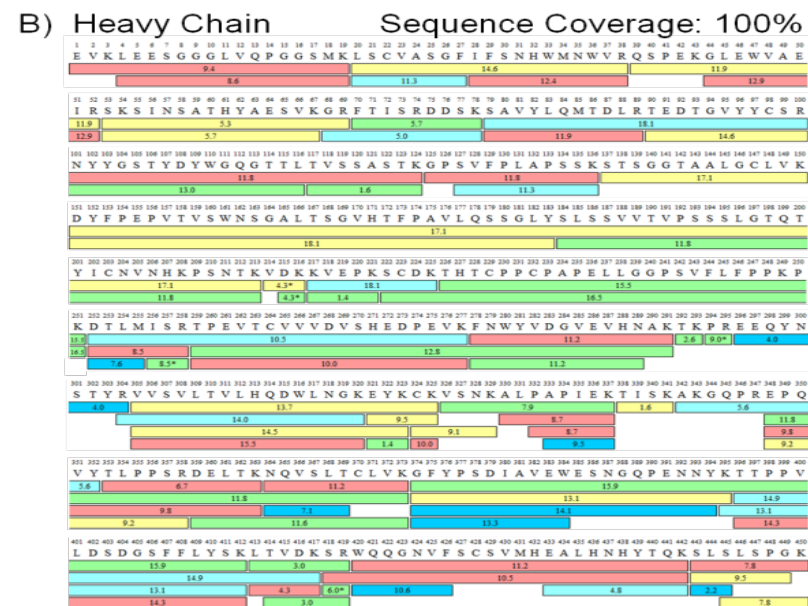
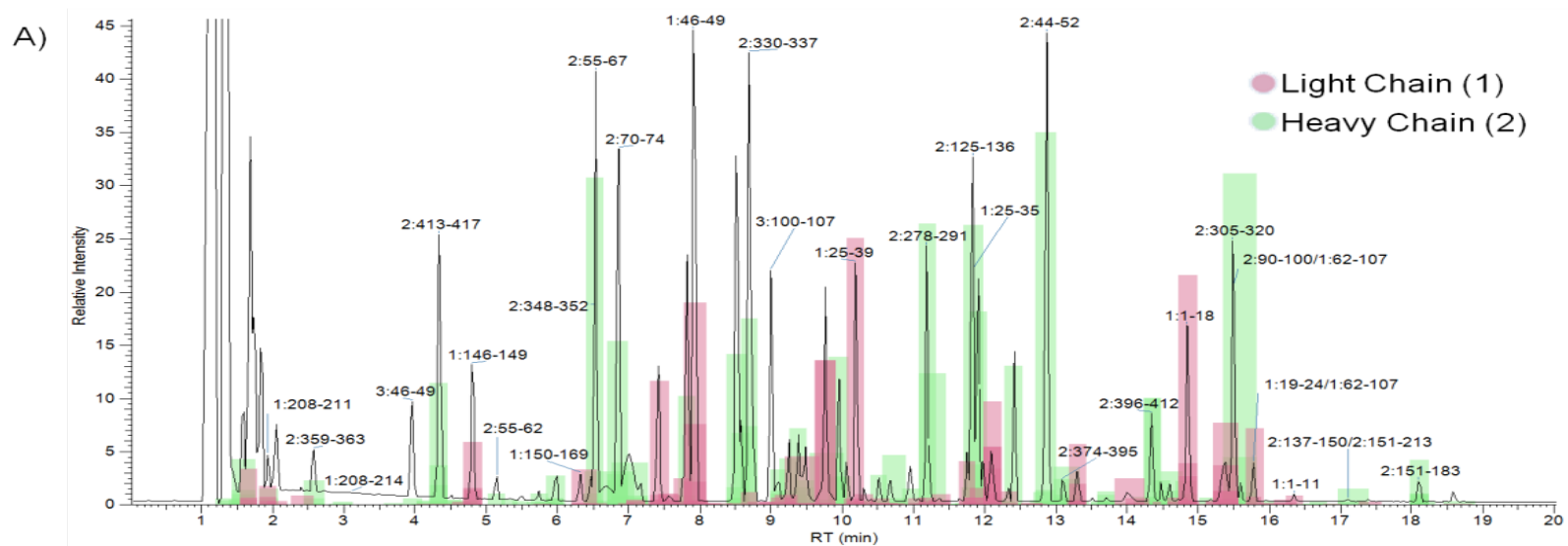


Infliximab Light Chain Sequence Coverage: 100%



Mirror plot for two automated replica digests of Infliximab conducted on the KingFisher Duo system (Digest time: 45 min; Digest temperature: 70°C)

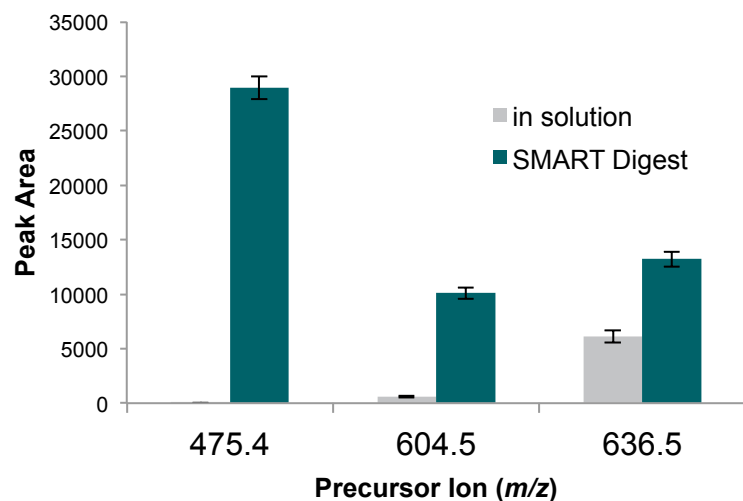
SMART Digest: Sequence Coverage Map from Infliximab



SMART Digest: Increased Sensitivity for Targeted MS Analysis

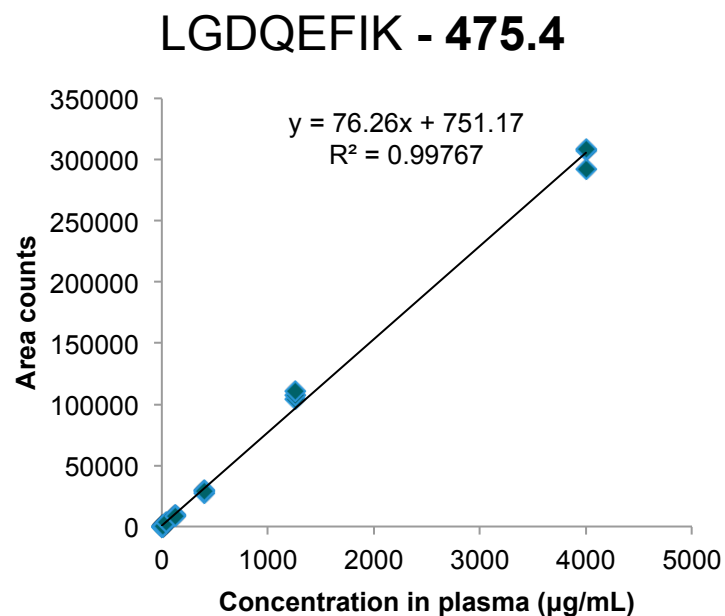
Confident detection of biomarkers with high sensitivity within a wide dynamic range

Case study: Thyroglobulin in plasma

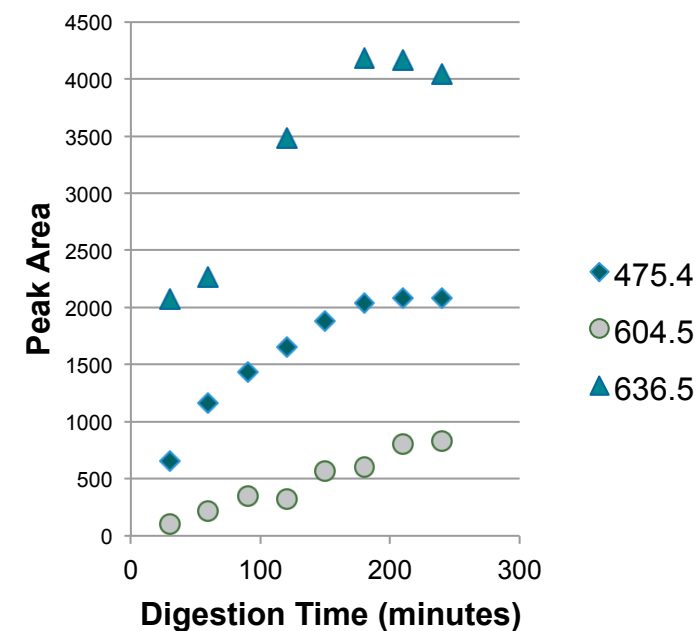


Measurement of serum Thyroglobulin after tryptic digestion of serum samples

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)

SMART Digest: Product Information

Enzyme options

- Trypsin
- Soluble Trypsin – complex samples
- Chymotrypsin
- Proteinase K

Resin options

- Pre-packed into SMART Digest tubes (non magnetic)
- Magnetic bulk
- Non-magnetic bulk

Post digestion clean up option

- Thermo Scientific™ SOLA μ with collection plate
- Filtration

All kits are supplied with digestion buffer



Ordering Information

Part Number	Description
SMART Digest Kits	
60109-101	SMART Digest Kit Trypsin and collection plate
60109-101-B	SMART Digest Kit Trypsin, bulk resin option
60109-101-MB	SMART Digest Kit Trypsin, magnetic bulk resin option
60109-102	SMART Digest Kit Trypsin, filter/collection plate
60109-102-B	SMART Digest Kit Trypsin, bulk resin option with filter/collection plate
60109-102-MB	SMART Digest Kit Trypsin, magnetic bulk resin option with filter/collection plate
60109-103	SMART Digest Kit Trypsin, SOLA μ /collection plate
60109-103-B	SMART Digest Kit Trypsin, bulk resin option with SOLA μ /collection plate
60109-103-MB	SMART Digest Kit Trypsin, magnetic bulk resin option with SOLA μ /collection plate
60113-101	Smart Digest Kit Soluble Trypsin and collection plate
60109-104	SMART Digest Kit Chymotrypsin and collection plate
60109-104-B	SMART Digest Kit Chymotrypsin, bulk resin option
60109-104-MB	SMART Digest Kit Chymotrypsin, magnetic bulk resin option
60109-105	SMART Digest Kit Chymotrypsin, filter/collection plate
60109-105-B	SMART Digest Kit Chymotrypsin, bulk resin option with filter/collection plate
60109-105-MB	SMART Digest Kit Chymotrypsin, magnetic bulk resin option with filter/collection plate
60109-106	SMART Digest Kit Chymotrypsin, SOLA μ /collection plate
60109-106-B	SMART Digest Kit Chymotrypsin, bulk resin option with SOLA μ /collection plate
60109-106-MB	SMART Digest Kit Chymotrypsin, magnetic bulk resin option with SOLA μ /collection plate
60109-107	SMART Digest Kit Proteinase K and collection plate
60109-107-B	SMART Digest Kit Proteinase K, bulk resin option
60109-107-MB	SMART Digest Kit Proteinase K, magnetic bulk resin option
60109-108	SMART Digest Kit Proteinase K, filter/collection plate
60109-108-B	SMART Digest Kit Proteinase K, bulk resin option with filter/collection plate
60109-108-MB	SMART Digest Kit Proteinase K, magnetic bulk resin option with filter/collection plate
60109-109	SMART Digest Kit Proteinase K, SOLA μ /collection plate
60109-109-B	SMART Digest Kit Proteinase K, bulk resin option with SOLA μ /collection plate
60109-109-MB	SMART Digest Kit Proteinase K, magnetic bulk resin option with SOLA μ /collection plate

14

Site of Aggregation

Adduct formation

N-terminal pyroE formation

Conjugation Site (ADC)

Conjugation Site (ADC)

Oxidation

AREA of DETAIL

ThermoFisher
SCIENTIFIC

SMART Digest ImmunoAffinity (IA) Kits

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

SMART Digest IA: Immuno Affinity Capture and Digestion in One

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

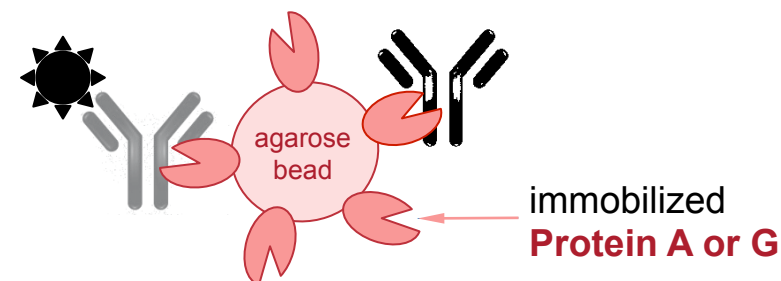
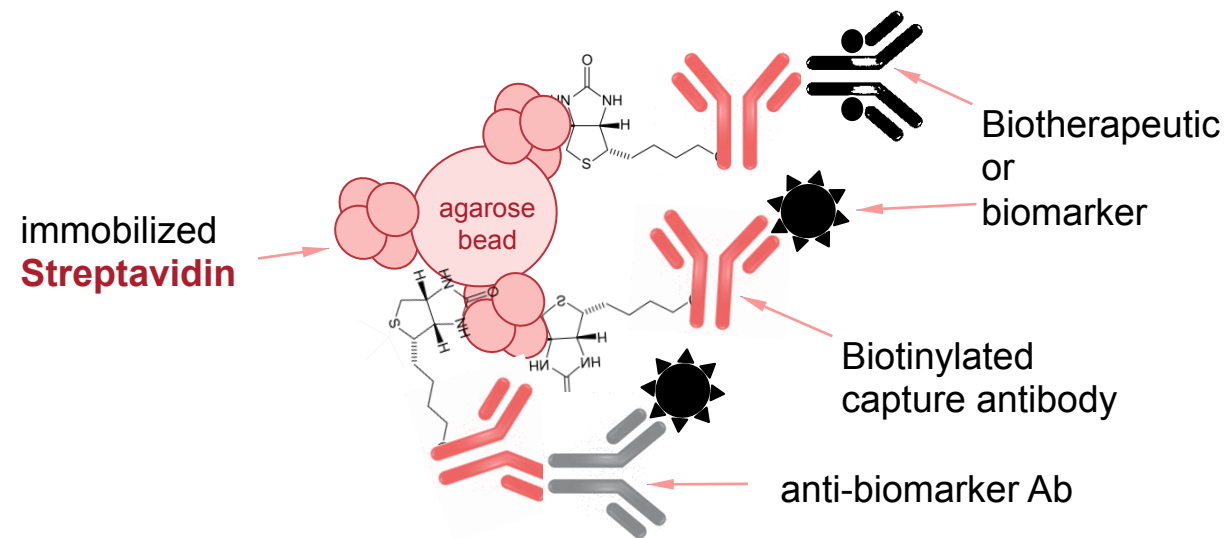
Biomarker quantitation is a challenging task:

- 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

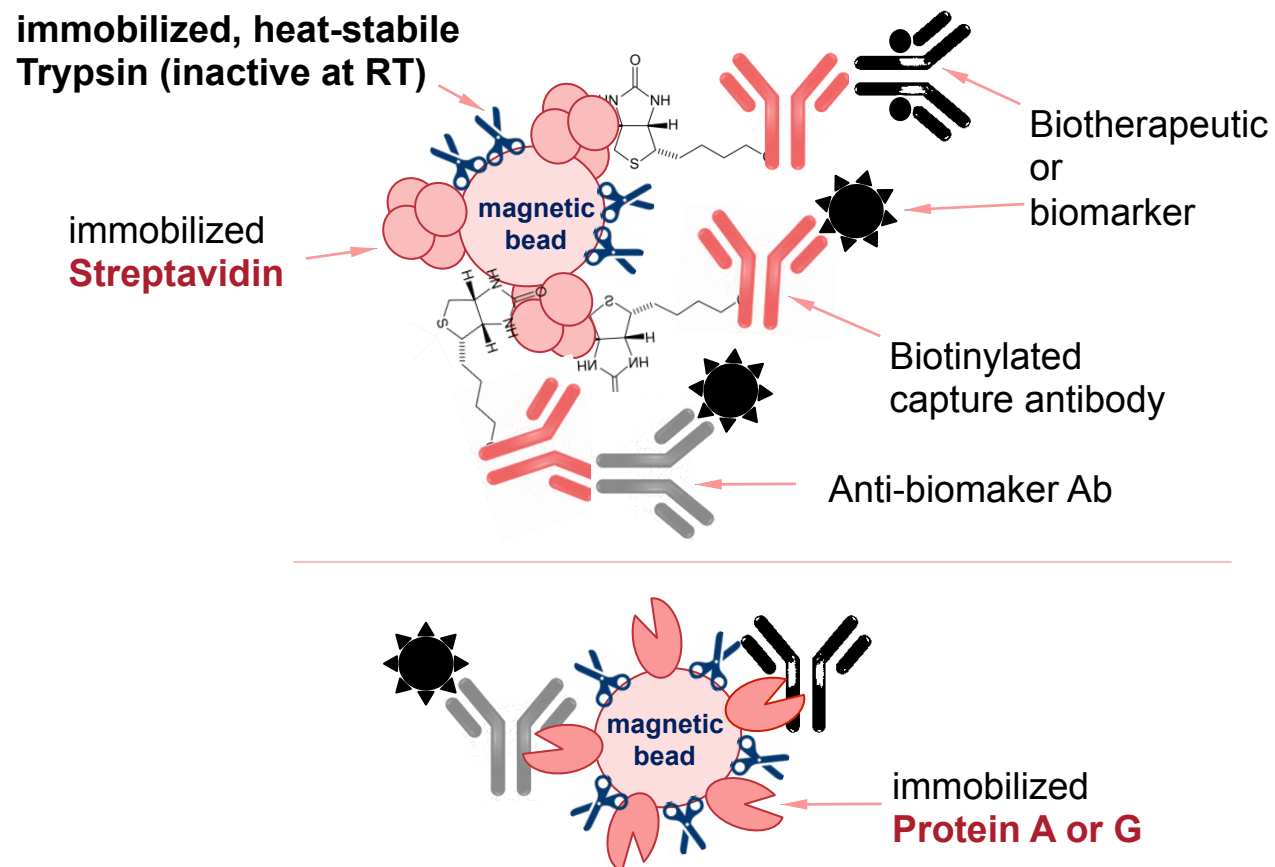


SMART Digest IA: Immuno Affinity Capture and Digestion in One

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 and 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

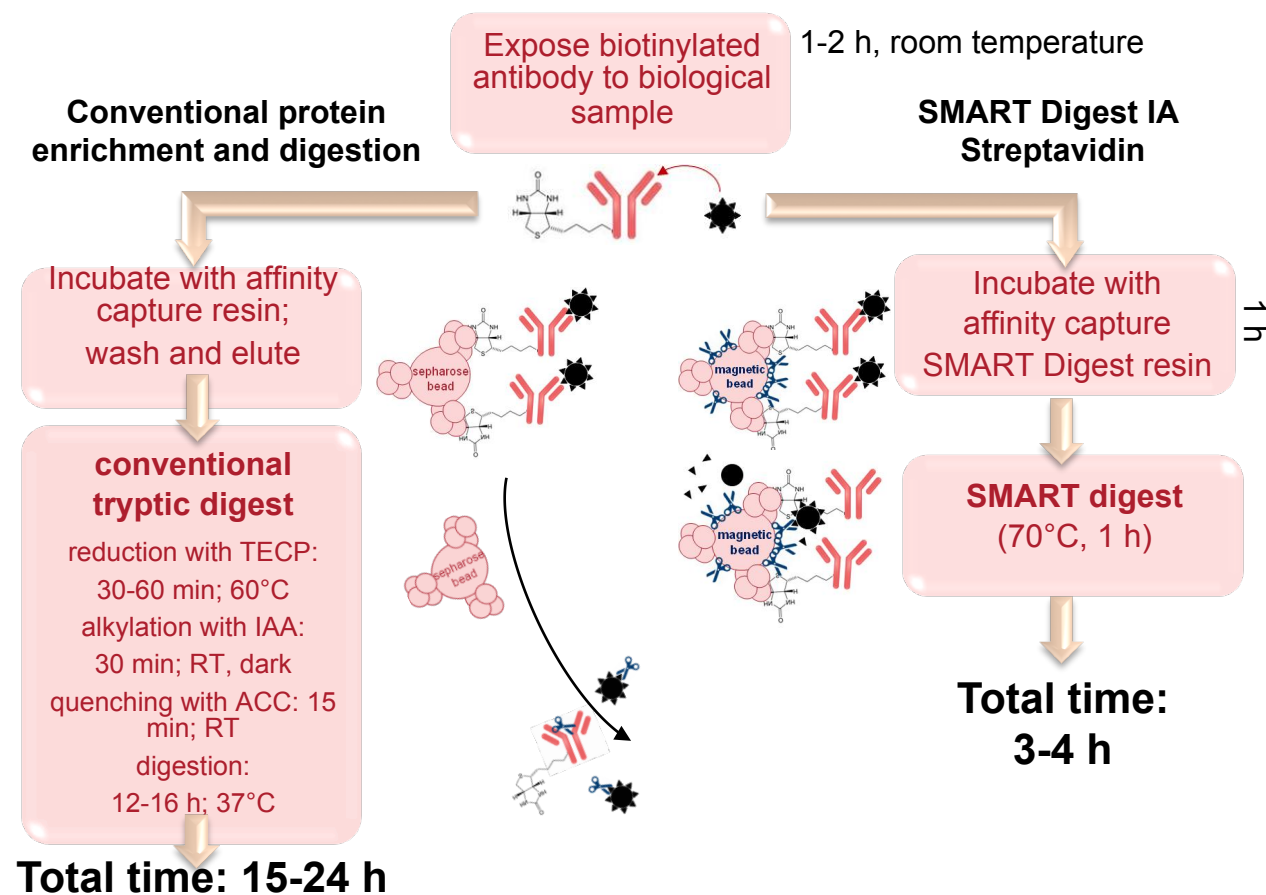


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Test case:

Soluble plasma protein biomarker

Spike-in SIL peptide

Assay acceptance criteria:

± 20% of nominal for accuracy at all levels

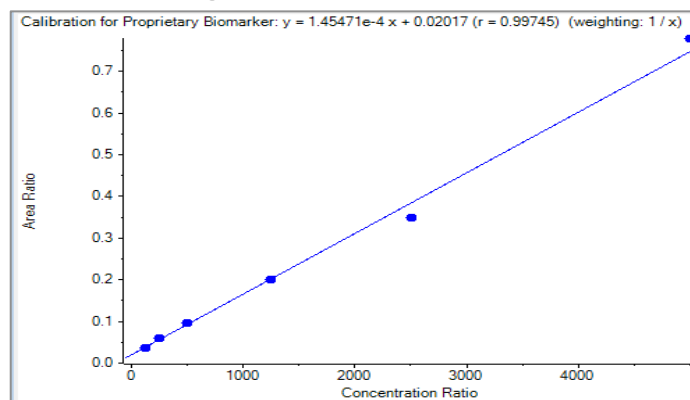
Assay range: 20 - 1000 ng/mL (Peptide A)

100 - 2000 ng/mL (Peptide B)

Method recovery

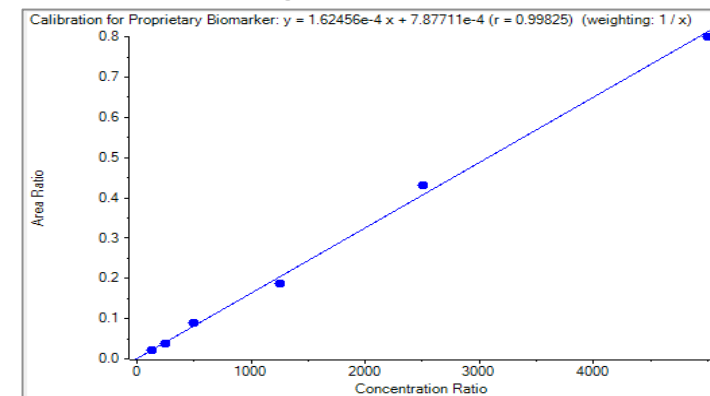
	SMART Digest IA	Streptavidin agarose
500 ng/mL spike:	7330 (cps)	2778 (cps)
% Recovery:	64%	35%

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	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	20-25 %RSD		

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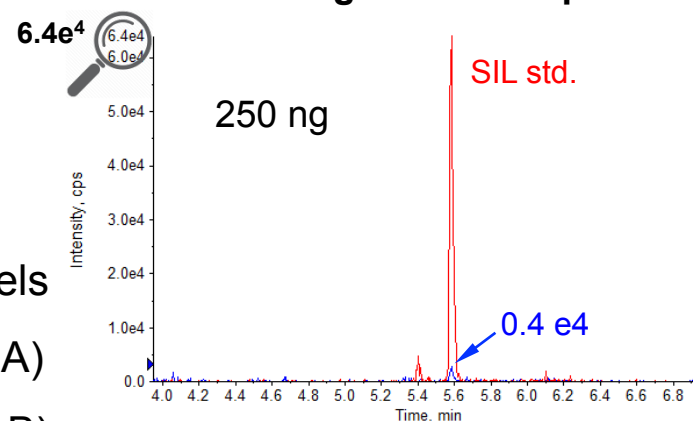
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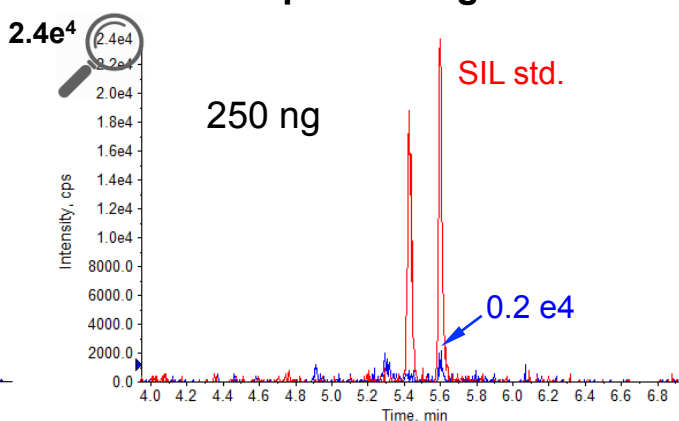
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SMART Digest IA - Streptavidin



Streptavidin agarose

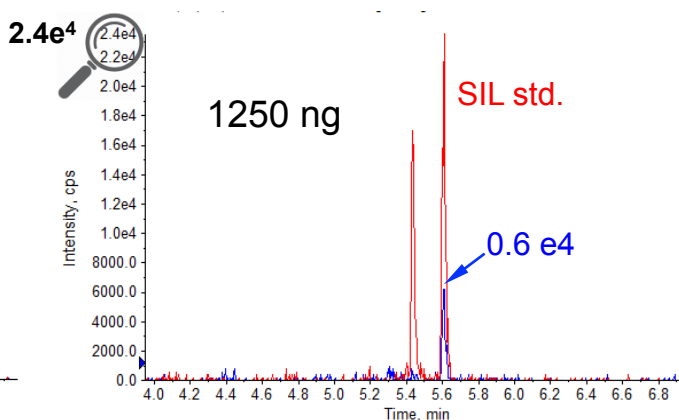
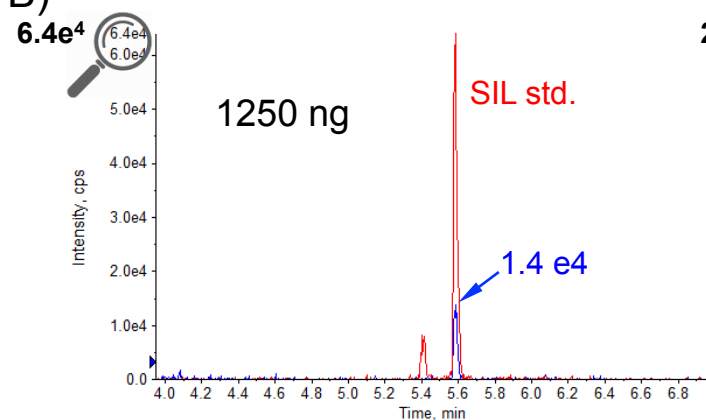


SMART Digest immunoaffinity kits

Improved method recovery

similar quantitative precision and accuracy

up to 7 times faster



SMART Digest ImmunoAffinity (IA) Kits: Product Information

Affinity capture options:

- Streptavidin
- Protein A
- Protein G

Resin options (Trypsin):

- Magnetic
- Non-magnetic

Post digestion clean-up options:

- SOLA μ with collection plate
- Without

All kits are supplied with wash and digestion buffers



Ordering Information

Part Number	Description
Streptavidin	
60110-101	SMART Digest IA Kit, Streptavidin (Av) non-magnetic
60110-102	SMART Digest IA Kit, Av non-magnetic with Thermo Scientific™ SOLA μ ™ SPE and collection plate
60110-103	SMART Digest IA Kit, Av magnetic with SOLA μ SPE and collection plate
60110-104	SMART Digest IA Kit, Av magnetic
Protein A	
60111-101	SMART Digest IA Kit, Protein A non-magnetic
60111-102	SMART Digest IA Kit, Protein A non-magnetic with SOLA μ SPE and collection plate
60111-103	SMART Digest IA Kit, Protein A magnetic with SOLA μ SPE and collection plate
60111-104	SMART Digest IA Kit, Protein A magnetic
Protein G	
60112-101	SMART Digest IA Kit, Protein G non-magnetic
60112-102	SMART Digest IA Kit, Protein G non-magnetic with SOLA μ SPE and collection plate
60112-103	SMART Digest IA Kit, Protein G magnetic with SOLA μ SPE and collection plate
60112-104	SMART Digest IA Kit, Protein G magnetic

SMART Digest and SMART Digest IA enable workflows which are:

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

Learn more about
SMART Digest and SMART Digest IA

www.thermofisher.com/SMARTdigest