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SMART Digest and SMART Digest ImmunoAffinity (IA) Kit Technical Guide

Smarter protein preparation

ThermoFisher SCIENTIFIC

SMART Digest and SMART Digest ImmunoAffinity (IA) Kits

Smarter protein preparation

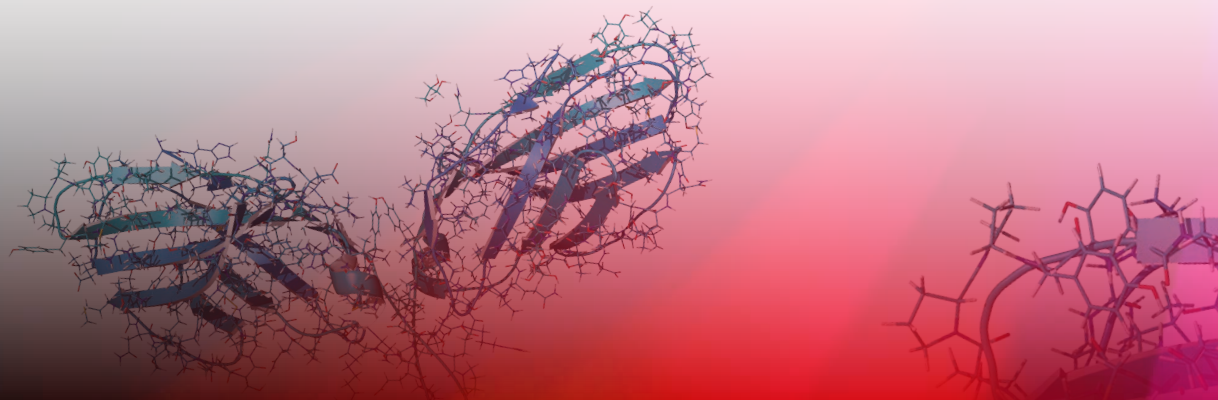
The Thermo Scientific™ SMART Digest™ and the SMART Digest ImmunoAffinity (IA) Kits are designed for biomarker and bio-therapeutic characterization and quantitation. The kits deliver sample preparation of proteins that is:

- **Fast**
- **Simple**
- **Highly reproducible**
- **Sensitive**
- **Compatible with automation**

Challenges for biopharmaceutical laboratories

Modern biopharmaceutical laboratories are tasked with providing high quality quantitative and qualitative analytical results, often in high-throughput, regulated environments. This is exacerbated by the increase in efficacy of biotherapeutics and the often low levels of biomarkers in complex biological matrices.

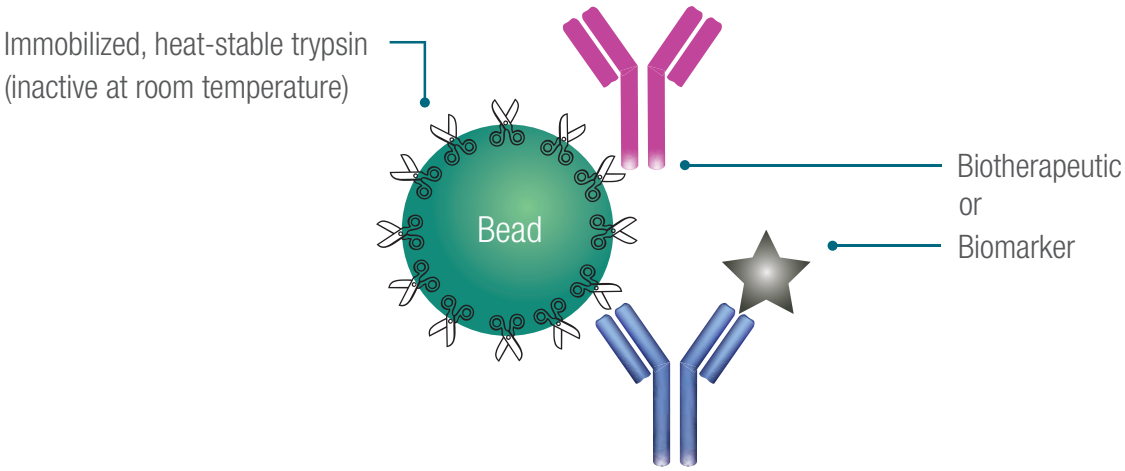
One of the key areas which affects these requirements is sample preparation. Current technologies employed are subject to high levels of irreproducibility, poor sensitivity, and protracted methodologies that are not amenable to automation and often require 24 hours to deliver results.



Addressing sample preparation challenges for biopharma

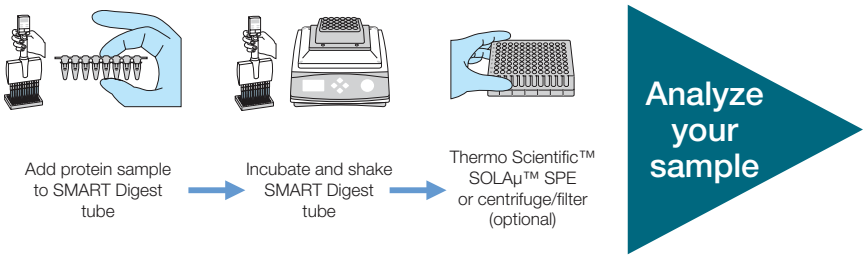
The SMART Digest Kit

The kits deliver fast, simple and highly reproducible digestion of proteins for characterization and quantitation applications, achieved due to the **heat-stable immobilized trypsin design**.

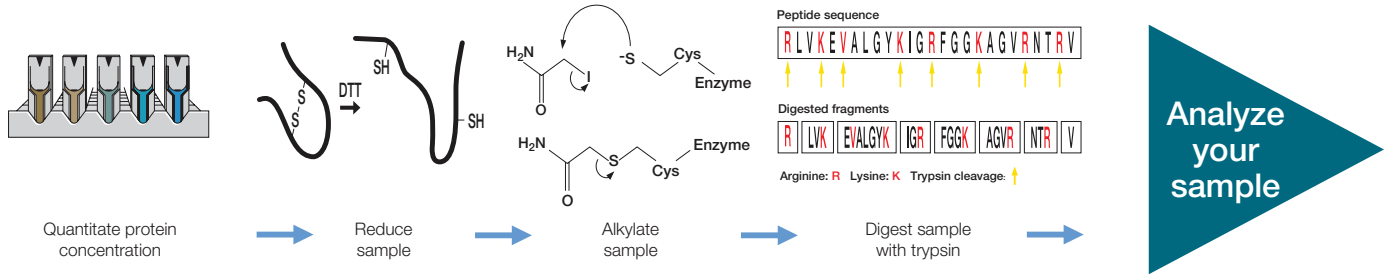


This design allows the digestion process to be significantly simplified compared to traditional in-solution digest protocols.

SMART Digest Kit
Simple and easy to use workflow



Standard In-Solution Digest
Complex and laborious workflow



Questions Answers

Q: Why is the temperature set to 70 °C?

This has been shown to be the optimum temperature for enzyme activity and the unfolding of proteins. Higher or lower temperatures may reduce the efficiency of the digestion.

Q: What concentration of protein can I add to each SMART Digest tube?

You can add 200 pg to 3.5 mg of total protein to each SMART Digest tube (protein/sample dependent).

Q: Do I have to reduce and alkylate my protein?

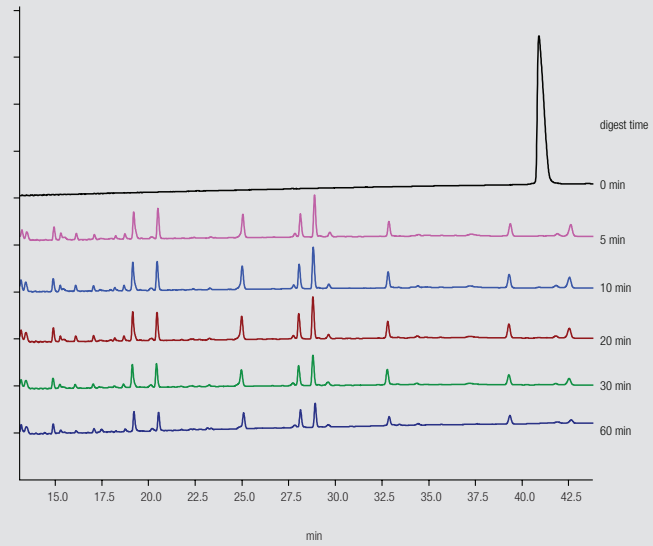
The SMART Digest kits are thermally stable. When operated at high temperatures denaturation and digestion occur simultaneously. Therefore, for many quantitative workflows, there is no need to perform the additional steps of denaturation, reduction and alkylation. However, during this process many disulfide bonds will remain intact. As a result, for characterization workflows where maximum sequence coverage is required, it is recommended that you perform reduction and alkylation after digestion.

SMART Digest Kit
Fast digestion of proteins

Using the SMART Digest kit simplifies the process and reduces the time needed for sample preparation. This not only speeds up your workflows, but also significantly reduces method development time. In the example below it can be seen that for carbonic anhydrase full digestion is achieved in 5 minutes.

Carbonic Anhydrase, 29 KDa

Time course experiment for digestion optimization



Typical Digestion Times

Protein	Digest Time (min)
Insulin	4
BSA	< 5
Carbonic anhydrase	< 5
Lysozyme	< 5
Apo-B	30
IgG	45
IgG in 50 µL plasma*	75
Ribonuclease A	150
Thyroglobulin	240
C-reactive protein	240

200 µL protein solution (100 µg/mL) at 70 °C
*IgG in plasma (17.5 mg/mL total protein) at 70 °C

Q: My protein is very difficult to unfold so I need to use urea. What concentration of urea can I use?

The SMART Digest kit uses heat to unfold the protein. If urea is required then it is recommended to dilute the sample to 0.5 M or less of urea prior to transferring to the SMART Digest tube.

Q: What if I have less than 50 µL of sample?

If your sample is less than 50 µL adjust to 50 µL with ultrapure water.

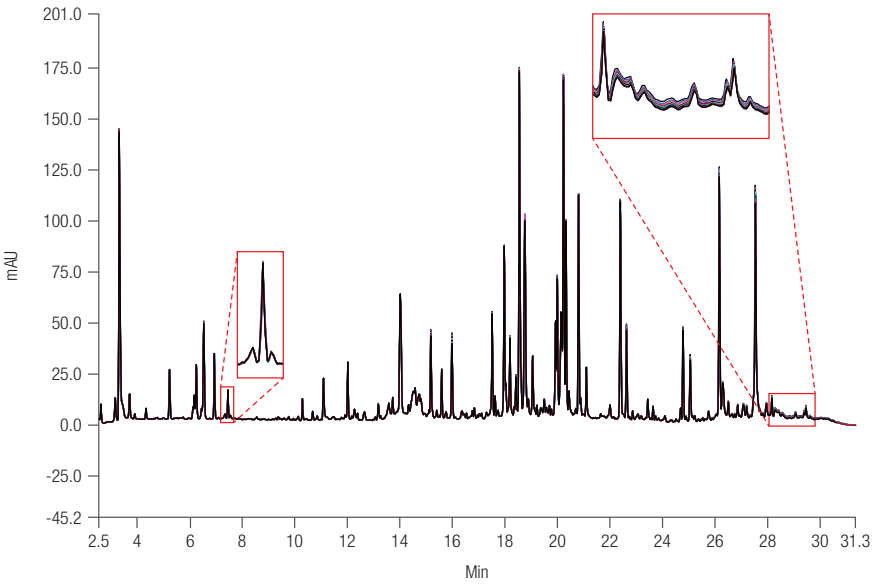
Q: Does digestion using the SMART Digest kit at high temperatures result in an increase in post-translational modifications?

In comparison to in-solution digests a comparable number of PTMs have been observed when screening for deamidation, amidation, methylation and oxidation. No modifications to existing PTMs, such as phosphorylated sites, have been observed.

SMART Digest Kit
High quality analytical results

The SMART Digest kit provides significant improvements in reproducibility over existing protocols, which results in fewer sample failures, higher throughput and the ability to more easily interrogate data.

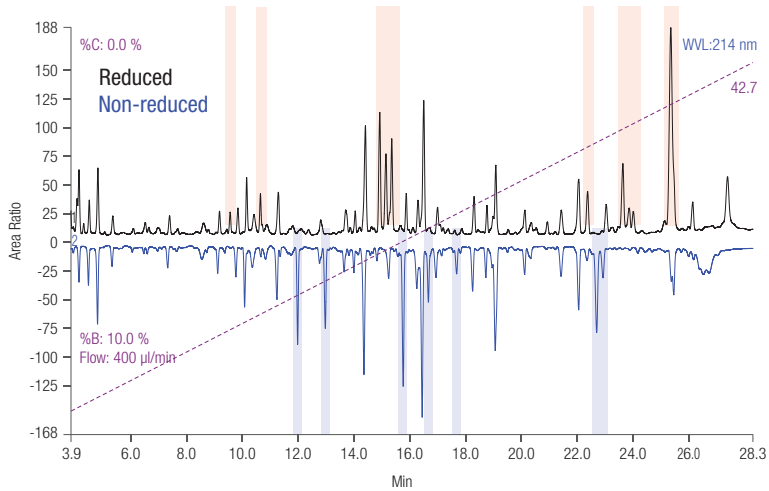
The following chromatogram shows 13 overlaid traces of mouse mAb peptides, digested with the SMART Digest kit. Excellent reproducibility of <0.082 %RSD is achieved when using the Thermo Scientific™ Vanquish™ UHPLC system, as indicated in the table below.



Overlay of 13 consecutive chromatographic runs of a peptide sample separated on an analytical Thermo Scientific™ Acclaim™ VANQUISH C18 column (2.2 µm, 2.1 × 250 mm) and prepared from a mAb, digested with the 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



Monitoring of conditional differences

Reproducible peptide maps and excellent retention time repeatability enable confidence in direct comparisons.

Characterization

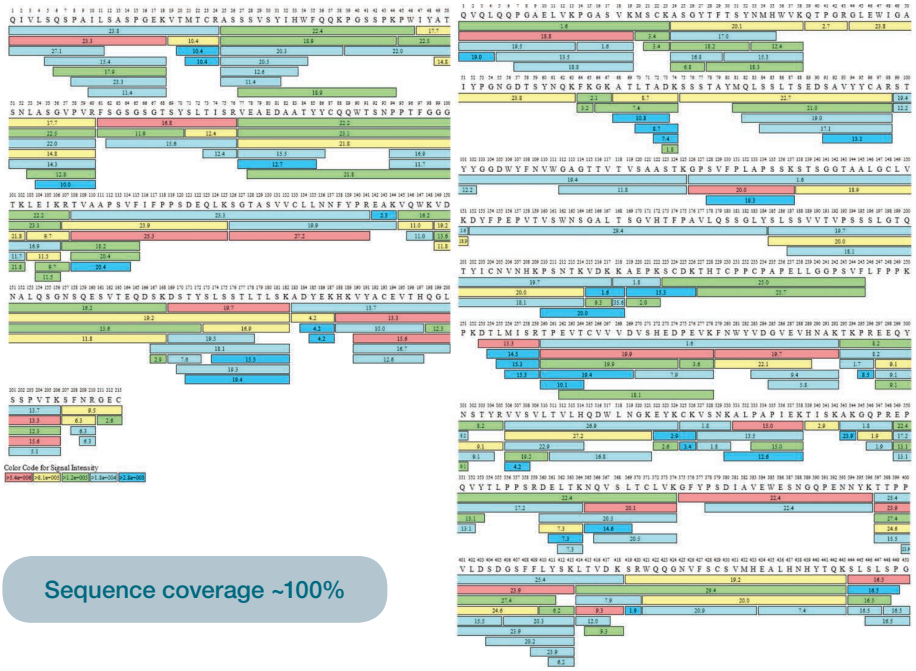
The SMART Digest kit, in combination with the High Resolution Accurate Mass (HRAM) capabilities of the Thermo Scientific™ Q Exactive™ mass spectrometers, and high resolution chromatographic separation with the Vanquish UHPLC family provides us with high quality data sets that can be more effectively interrogated with Thermo Scientific™ BioPharma Finder™ Software.

The BioPharma Finder software allows us to map our protein, in the case for Rituximab ~100% sequence coverage is achieved, as well as quantitation of modifications on both the light and heavy chains.

This is of particular importance in understanding how the molecule will interact within a biological system.

Rituximab light chain

Rituximab heavy chain

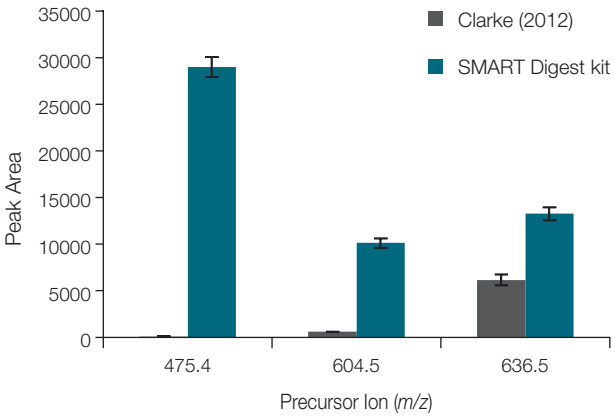


Sequence coverage ~100%

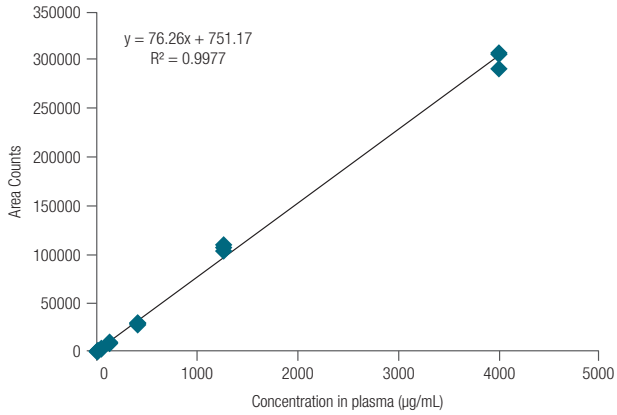
Protein	Modification	Recovery	Abundance
Rituximab_LC	Q1+NH_3 loss	Good	87.81%
Rituximab_LC	W90+Oxidation	Good	2.06%
Rituximab_HC	~Q1+NH_3 loss	Good	100.00%
Rituximab_HC	W281+Oxidation	Good	4.98%
Rituximab_HC	N301+A1G0F	Fair	2.87%
Rituximab_HC	N301+A1G1F	Fair	1.22%
Rituximab_HC	N301+A2G0	Fair	1.30%
Rituximab_HC	N301+A2G0F	Fair	37.69%
Rituximab_HC	N301+A2G1F	Fair	44.86%
Rituximab_HC	N301+A2G2F	Fair	10.77%
Rituximab_HC	N301+M5	Fair	1.07%
Rituximab_HC	N365+Deamidation	Good	2.72%
Rituximab_HC	W385+Oxidation	Good	5.37%
Rituximab_HC	G450+Lys	Good	3.2683%

Quantitation

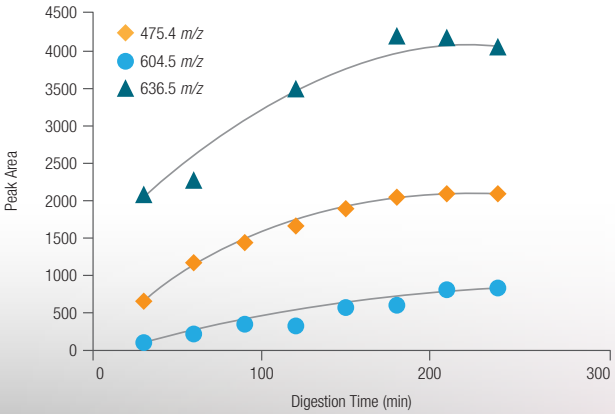
The SMART Digest kit allows confident detection of biomarkers with high sensitivity within a wide dynamic range, as can be seen below with the example of Thyroglobulin. Greater sensitivity is achieved in 3.5 hours compared to an in-solution digest protocol taking 20 hours to complete.



Measurement of serum Thyroglobulin after tryptic digestion of serum samples.
■ SMART Digest kit: 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).



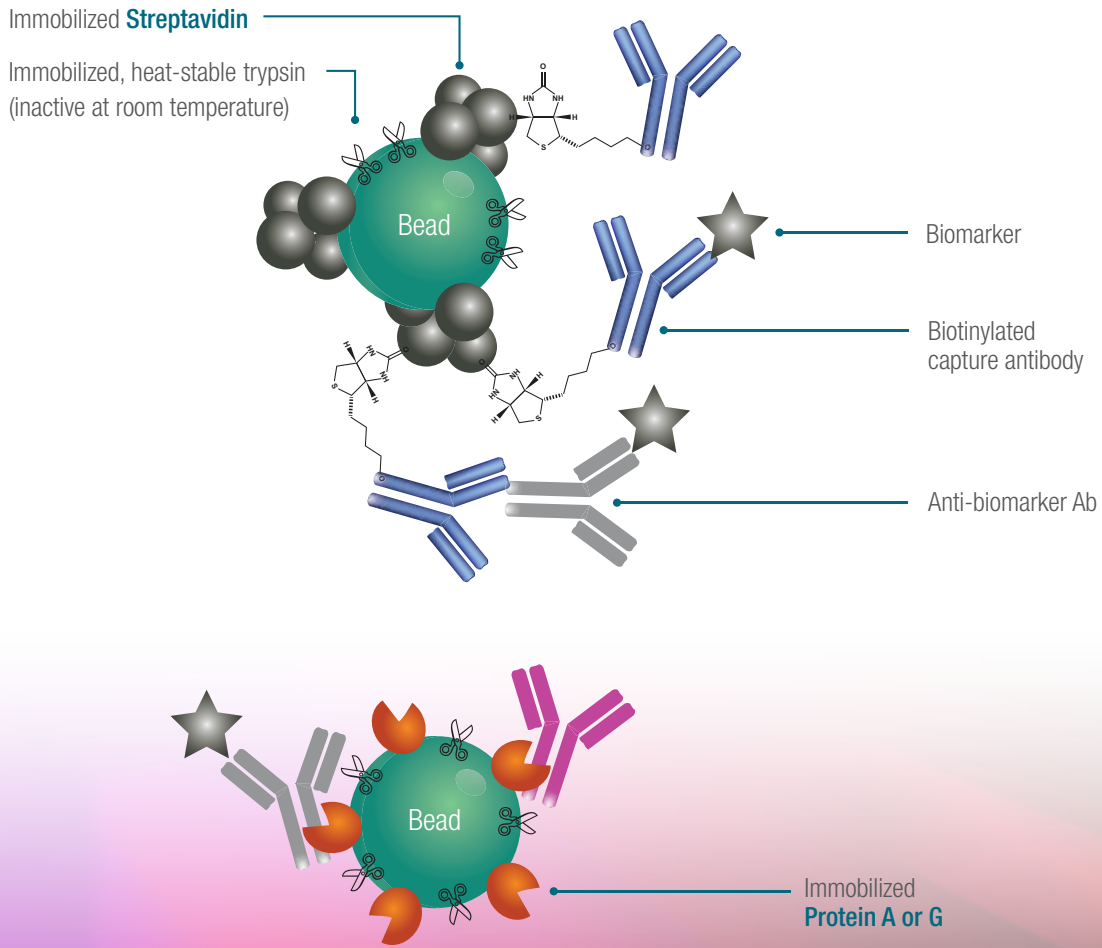
Digestion time curve 70 °C. Optimum digestion time of 3.5 hours for Thyroglobulin was determined by monitoring the peak area response for the three signature peptides.

The SMART Digest IA Kit

The SMART Digest IA kits have all the advantages previously outlined for fast, easy and reproducible protein digestion for quantitation and characterization applications with the added advantage of combining an immunocapture and the digestion process into a single well.

This has significant benefits for quantitation studies where immunoaffinity capture is typically employed to increase sensitivity by purifying low level proteins from complex biological matrices. This step is then followed by protein digestion.

The SMART Digest IA kits achieve this due to their unique design where the immunoaffinity reagents (either streptavidin, protein A or protein G) and heat activated thermally stable trypsin are co-immobilized onto a single bead. Following the binding of a capture reagent to the bead, and enrichment of the target, the enzyme is activated at elevated temperatures for accelerated digestion under protein denaturing conditions. The resulting workflow is as easy as enrich, wash and digest. Magnetic and non-magnetic versions of the beads are available.

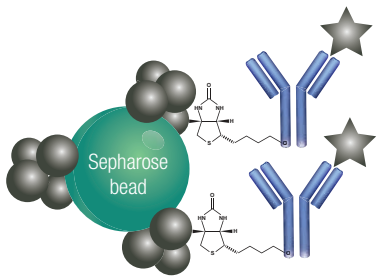


How does the SMART Digest IA Kit work?

Conventional protein enrichment and digestion

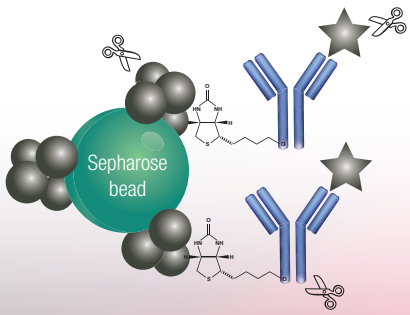
Expose biotinylated capture antibody to biological sample

Incubate with affinity capture resin



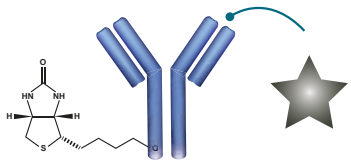
Conventional tryptic digest

Reduction, TCEP: 60 °C, 30–60 min
Alkylation, IAA: RT, 30 min
Quenching, ACC: RT, 15 min
Digestion: 37 °C, 12–16 h

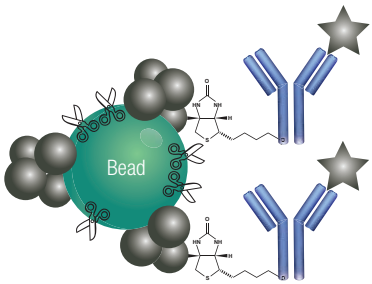


Total time
15–24 hours

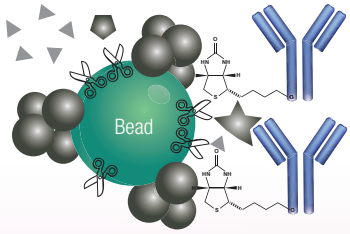
Protein enrichment and digestion using the SMART Digest IA kit



Use SMART Digest IA resin for affinity capture.
Incubate at room temperature.



Digestion with SMART Digest IA resin
70 °C, 1 h



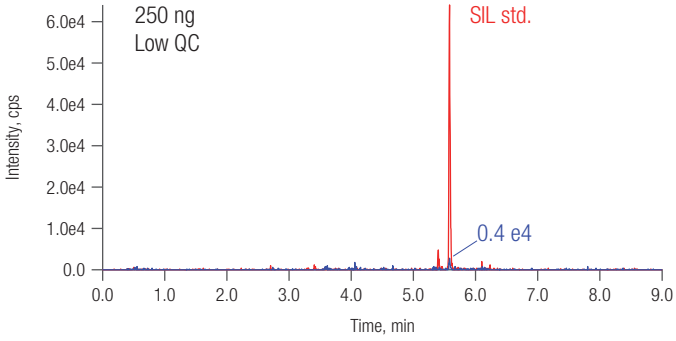
Total time
3–4 hours

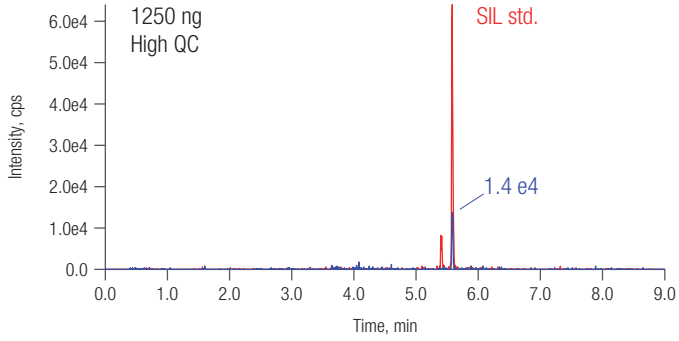
Quantitation with the SMART Digest IA Kit compared to a conventional approach

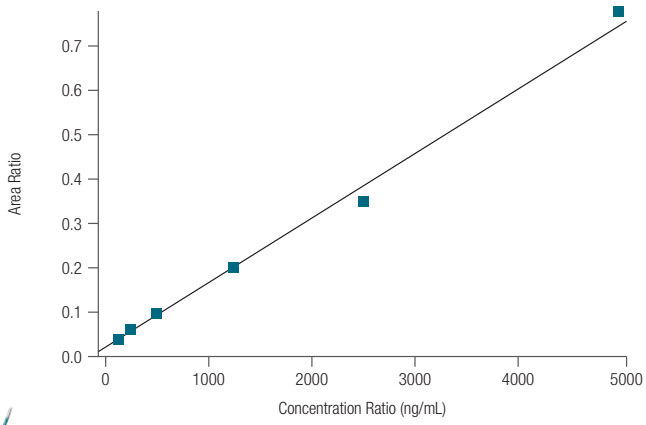
The following is an example of using the SMART Digest IA Streptavidin kit for the quantitation of a biomarker (human interrferon α14) in human plasma, compared to a traditional immunocapture and digestion method.

The SMART Digest IA protocol used involved an immunocapture step, taking 2 hours, followed by a 1 hour, high-temperature digestion with immobilized trypsin. This is compared to immunocapture with a high capacity streptavidin gel followed by overnight tryptic digestion of the biomarker protein. A SIL peptide was spiked into the samples to act as an internal standard.

SMART Digest IA Kit process



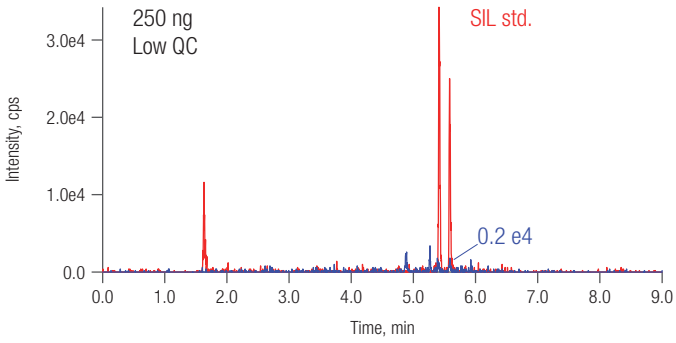


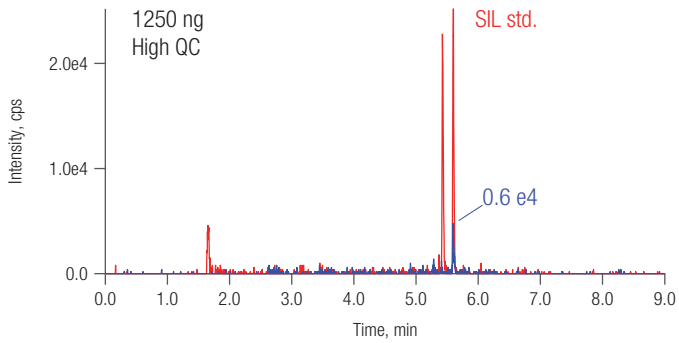


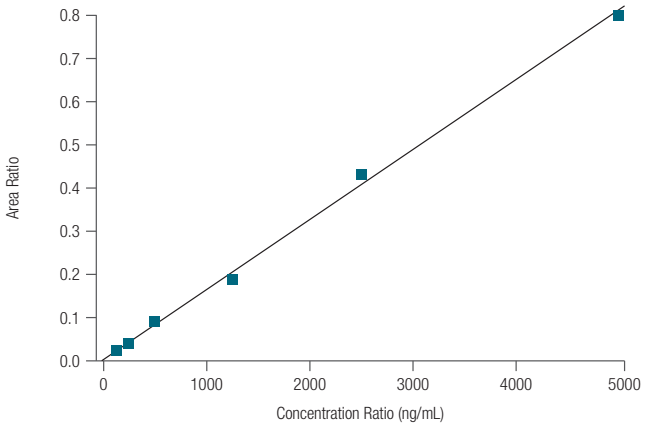
Standard Curve (n = 1)			Quality Controls (n = 4)		
Actual Conc (ng/mL)	Accuracy (%)	Calc Value (ng/mL)	Actual Conc (ng/mL)	CV (%)	Accuracy (%)
125	93	116.5			
250	107	266.3	250	11.5	90.2
500	106	531.1			
1250	100	1247	1250	7.4	99.1
2500	90	2251			
5000	104	2251			

Recovery with SMART Digest IA kit	
500 ng/mL spike	7330 (cps)
Recovery	64%

Conventional streptavidin agarose process







Standard Curve (n = 1)			Quality Controls (n = 4)		
Actual Conc (ng/mL)	Accuracy (%)	Calc Value (ng/mL)	Actual Conc (ng/mL)	CV (%)	Accuracy (%)
125	105	131			
250	90	225	250	14.5	111.2
500	109	544			
1250	92	1149	1250	4.1	104.8
2500	106	2654			
5000	99	4922			

Recovery with conventional approach	
500 ng/mL spike	2778 (cps)
Recovery	35%

Note: Data provided courtesy of PharmaCadence Analytical Services, Hatfield, PA, USA. Carmen Fernández-Metzler, Bonnie Baker, Robyn Buerger.

A Step Change in Protein Affinity Capture and Digestion

SMART Digest and SMART Digest IA kits provide a significant change in protein sample preparation, by delivering workflows that are:

- **Significantly faster**
- **Easier to use**
- **Highly reproducible**
- **Sensitive**
- **Compatible with automation**

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, SOLAμ/collection plate
60109-103-B	SMART Digest Kit Trypsin, bulk resin option with SOLAμ/collection plate
60109-103-MB	SMART Digest Kit Trypsin, magnetic bulk resin option with SOLAμ/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, SOLAμ/collection plate
60109-106-B	SMART Digest Kit Chymotrypsin, bulk resin option with SOLAμ/collection plate
60109-106-MB	SMART Digest Kit Chymotrypsin, magnetic bulk resin option with SOLAμ/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, SOLAμ/collection plate
60109-109-B	SMART Digest Kit Proteinase K, bulk resin option with SOLAμ/collection plate
60109-109-MB	SMART Digest Kit Proteinase K, magnetic bulk resin option with SOLAμ/collection plate

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

Complementary Products

Part Number	Description
60103-351	Thermo Scientific™ 96 well Vacuum Manifold
60104-243	Vacuum Pump (NA)
60104-241	Vacuum Pump (EU)
60209-001	SOLAμ HRP SPE Plate
60109-102-F	SMART Digest Filter Plate
5400110	KingFisher Duo Prime Purification System
5400630	KingFisher Flex Purification System

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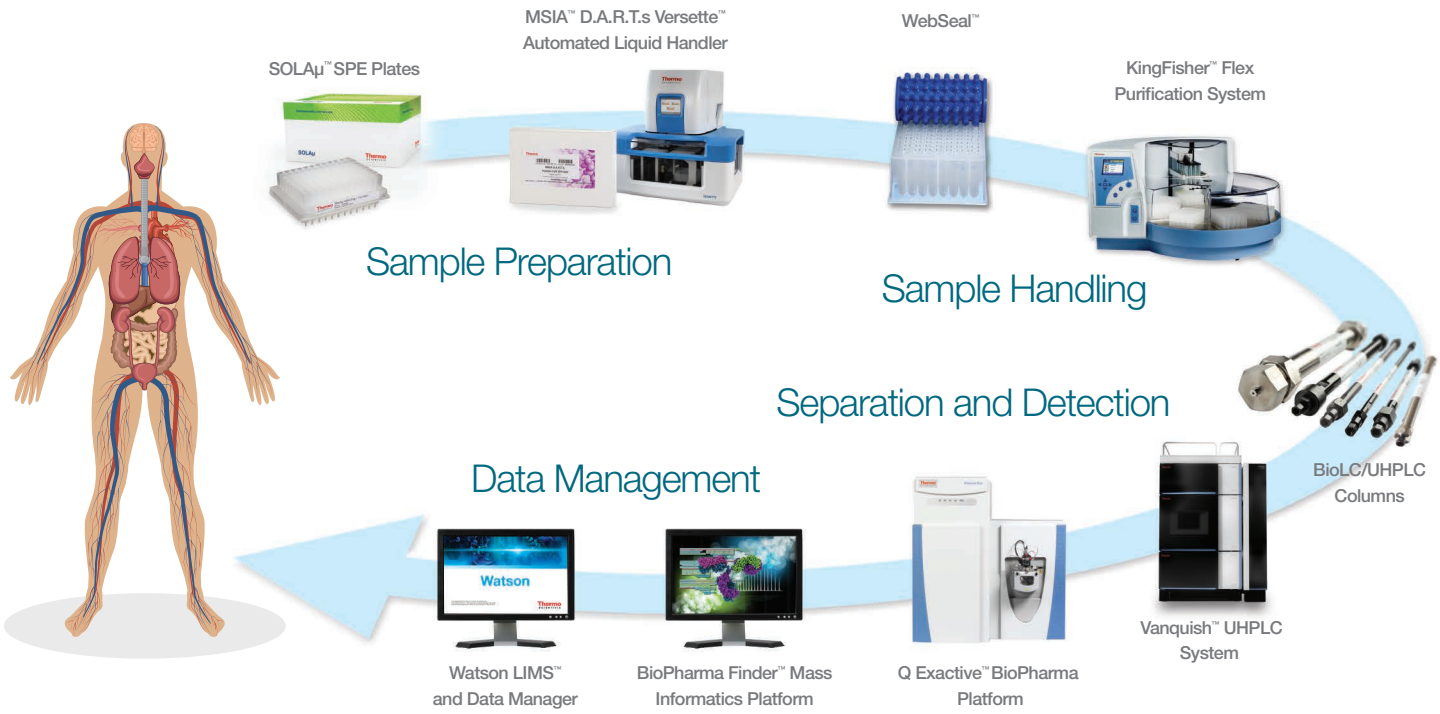
Characterize the full diversity of biomolecules

Thermo Scientific™ solutions for biomolecule separations



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