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Faster and More Sensitive Protein Characterization and Quantitation

Sample preparation challenges in the modern biopharmaceutical laboratory

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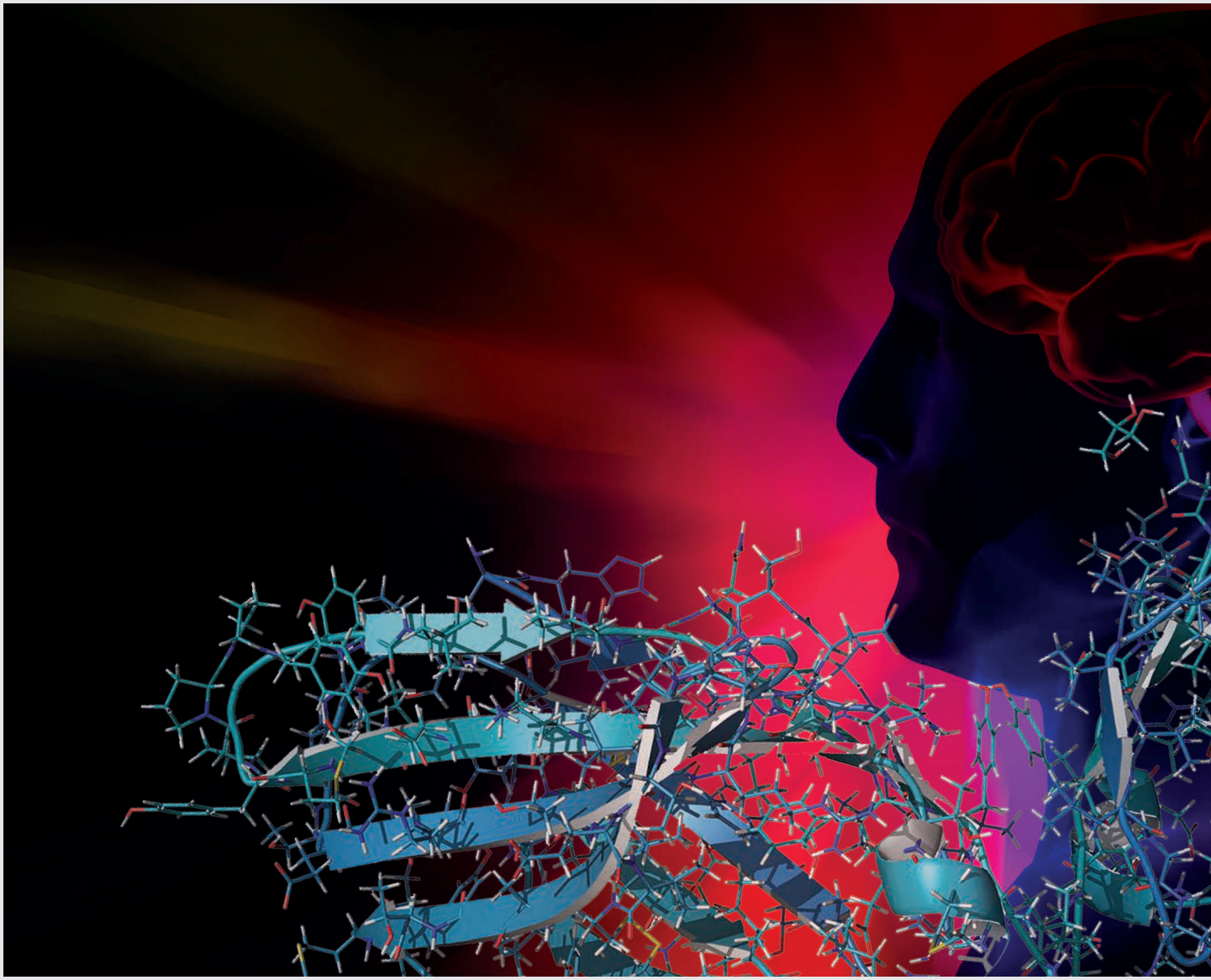
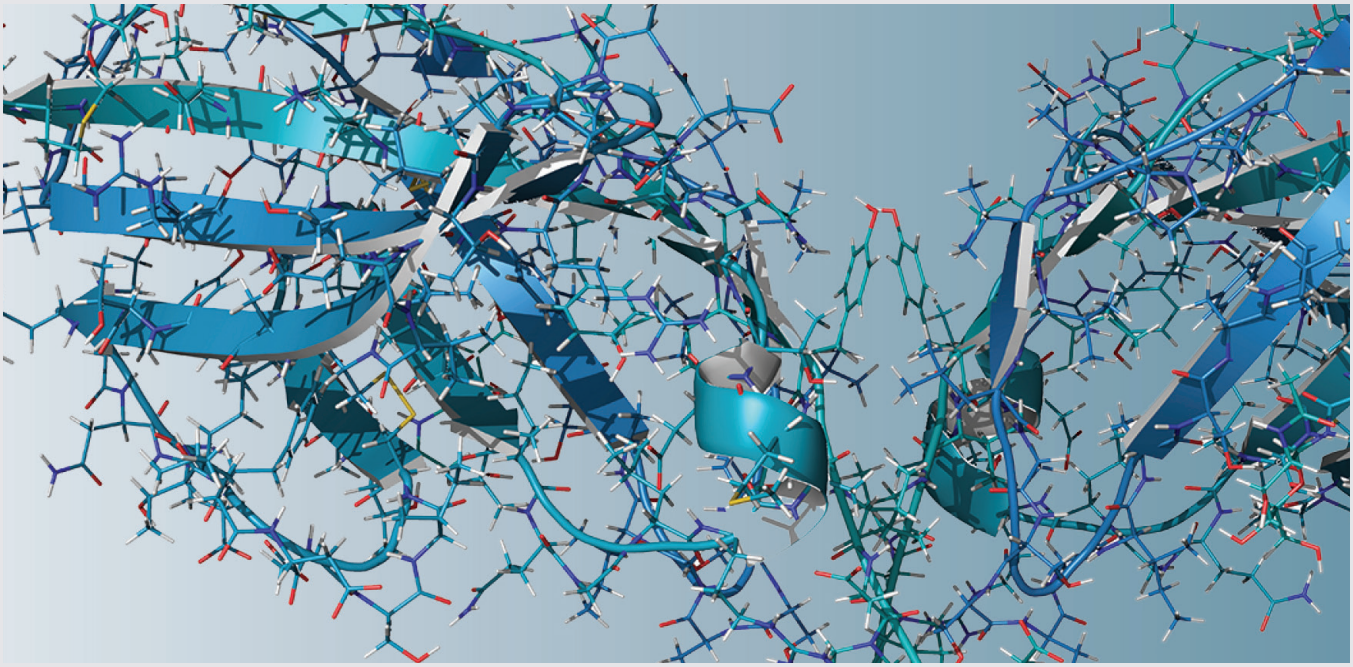


Introduction



In 1996 the first antibody based drug was introduced to the market and since then the growth of the biopharmaceutical market has continued to grow at a rapid pace. Today approximately 20% of the overall pharmaceutical market is based on biological drugs with the market continuing to grow at more than 8% per year, whereas the market based on classic synthetic drugs exhibits little if any growth. The growth of biopharmaceuticals comes from their greater success rate and speed of development. This is in part due to their evolution where engineered fully human monoclonal antibodies (mAbs) blend perfectly with the body's own antibody repertoire to avoid an unwanted immune response, over antibody-drug-conjugates that allow the directed delivery of a small molecule drug to a limited population of carefully defined target cells, e. g. cancer cells, or the combination of different target structures in antibody-fusion proteins or bi-specific antibodies to harness a combinatorial effect, resulting in higher selectivity and efficacy.

As with synthetic drugs, bio-therapeutics require rigorous analytical characterization and quantitation to ensure efficacy and safety for the patient. The analytical challenges especially in terms of characterization of bio-therapeutics (peptide mapping) have been exacerbated due to their tremendous structural complexity. Adding to this are posttranslational modifications (PTMs) of the protein

"As with synthetic drugs, bio-therapeutics require rigorous analytical characterization and quantitation to ensure efficacy and safety for the patient."

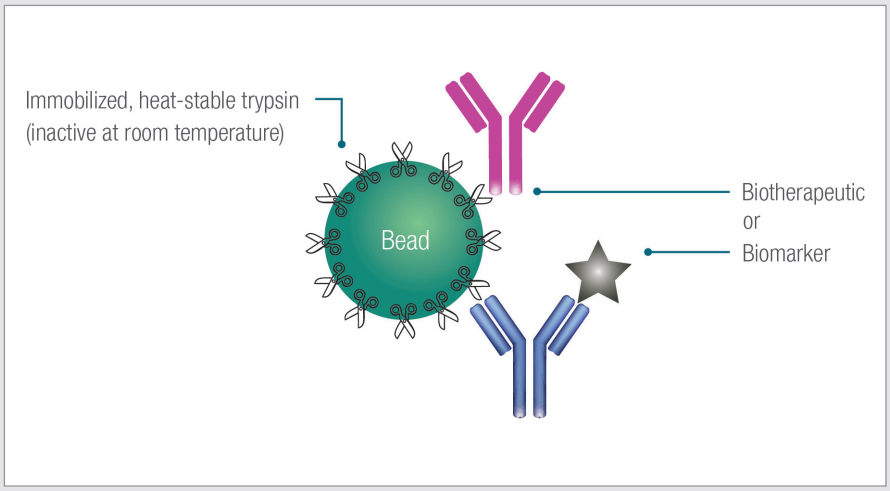


Figure 1. Heat-stable, immobilized enzyme design

such as glycosylation or the specific formation of disulfide-bridges that are crucial requirements for the functionality of the protein. This complexity however can give rise to a virtually unlimited number of potential different combinations of modifications. Within bioanalyitcal workflows (where the drug is evaluated in terms of its adsorption, distribution, metabolism and excretion (ADME) in the target organism) the challenges are from complex matrices which cause suppression of signal in mass spectrometers and need to be effectively removed, the drive towards higher efficacy drugs which require greater sensitivity in analytical workflows and the nature of protein based bio-therapeutics which are prone to adsorption and non-specific binding.

These challenges are compounded by sample preparation approaches required in these workflows where the protein is typically digested into its constituent peptides normally via an in-solution based enzyme reaction. These approaches are often subject to a number of issues that are not aligned to the needs of the modern biopharmaceutical laboratories, which are tasked with providing high quality analytical results, often in high-throughput, regulated environments. These issues include:

1. Complex procedures–prior to digestion the protein must be denatured. This is done in order to unravel and open the protein structure to allow for optimal enzyme interaction. This requires a multiple steps including disulfide bond reduction, and free sulphydryl alkylation.
2. Reproducibility of results–due to the complexity of the process and the chemicals involved (potential for introduction of chemically induced post translational modifications [PTMs]). there is the potential for poor reproducibility which has a significant effect on the confidence in the analytical results produced
3. Speed of analysis–depending on the size and complexity of the protein being analyzed, the digestion process can take up to 24 hours to complete. This combined with the complexity of the process does not lend itself to automation and high throughput processing.

Bioanalytical workflows are further complicated by the use of a complicated immunoaffinity step to remove the protein of interest from complex matrices e.g. plasma prior to digestion.

In order to meet these challenges a number of sample preparation options have shown promise, in particular the Thermo Scientific™ SMART Digest™ Kit, which is a heat stable, immobilized resin based enzyme design (Figure 1).

The SMART Digest kit offers significant advantages over traditional in-solution based protocols in terms of:

- Speed – up to 24 time faster than in-solution digest
- Simplicity – simple procedure
- Reproducibility of results.

More Information

PDF: SMART Digest Kit Technical Guide

Video: See how SMART Digest works

Faster digestion – SMART Digest Kit approach minutes compared to overnight with in-solution digestion

In-solution digest procedures typically require an overnight digestion. This does not fit with the high speed and throughput of analysis required within biopharmaceutical environments. Due to the excess of resin-based, heat-stable enzyme in the SMART Digest kit the speed of digestion can be greatly accelerated, providing procedures which can be performed in just a few minutes or hours compared to overnight.

In order to show the speed of the workflow offered by the SMART Digest kit compared to an in-solution based protocol, a commercially available monoclonal antibody rituximab drug product (Hoffmann La Roche, Basel, Switzerland) was digested via in-solution based protocols using urea and heat respectively for denaturation with the SMART Digest kit procedure and the data compared.

Both in-solution digest protocols required reduction and alkylation steps prior to overnight digestion with trypsin at 37 °C prior to addition of trifluroacetic acid (TFA) to stop the reaction.

The procedure using the SMART Digest kit involved adding the sample and pre-made SMART Digest buffer to a reaction tube containing 15 µL of the SMART Digest resin slurry, corresponding to 14 of heat-stabile, immobilized trypsin. A time course experiment was performed and tryptic digestion was allowed to proceed at 70 °C for 15, 30, 45, and 75 min at 1400 rpm; a digestion time of

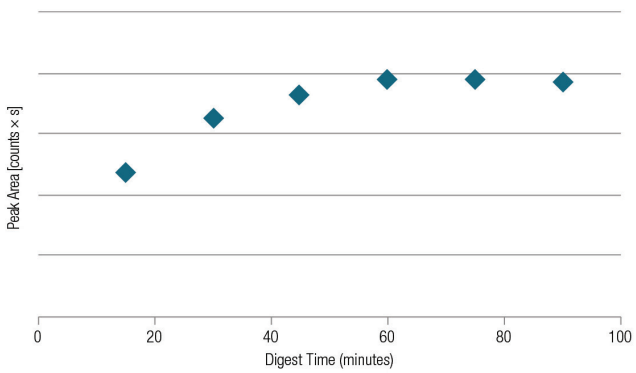


Figure 2. IgG digest profile, monitoring the mAb peptide VVSVLTVLHQDWLNGK for digestion times from 15–90 min using the SMART Digest kit.[2]

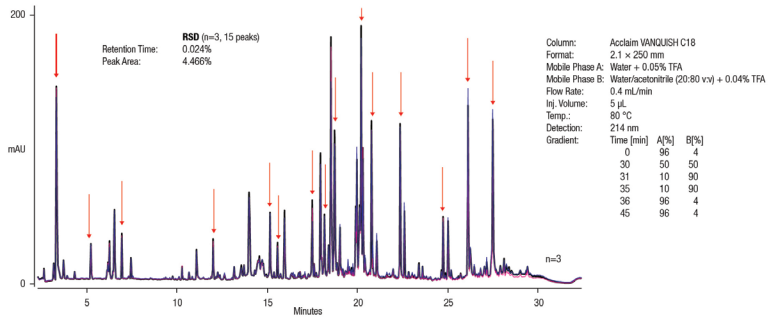


Figure 3. UV chromatogram overlay from three separate SMART digestions from the same mAb, conducted by three individual operators. The 15 marked peptides in each sample were used for inter-user/inter-day RSD value calculations.

"These results highlight the reproducibility that can be achieved when using this novel digestion technique."

Proteins	Number of MS Peaks	MS Peak Area	Sequence Coverage	Relative Abundance	Sample
1: Rituximab Light Chain	521	26%	100%	40%	SMART Digest, 15 min
	532	24%	100%	38%	SMART Digest, 30 min
	526	22%	100%	38%	SMART Digest, 45 min
	516	19%	100%	36%	SMART Digest, 75 min
	404	28%	100%	37%	In-Solution, Urea
	407	31%	100%	38%	In-Solution, Heat
2: Rituximab Heavy Chain	827	43%	100%	54%	SMART Digest, 15 min
	833	47%	100%	56%	SMART Digest, 30 min
	827	45%	100%	55%	SMART Digest, 45 min
	855	37%	100%	59%	SMART Digest, 75 min
	638	54%	100%	62%	In-Solution, Urea
	619	52%	100%	61%	In-Solution, Heat

Table 1. Sequence coverage with different digestion methods.

45–60 min was found to be sufficient to achieve digestion completeness for mAb samples (Figure 2). Disulfide bonds were reduced by incubation for 30 minutes at 37 °C with 5 mM DTT. (Sample names: SMART Digest, 15, 30, 45, 75 min)

The SMART Digest kit provides fast and simple protein digestion with outstanding reproducibility, and digestion completeness for mAb samples is typically achieved within 45–60 min (Figure 2). Here, the relative standard deviation (RSD) was used to evaluate reproducibility, as demonstrated in Figure 3. Three separate digestions of the same mAb sample were conducted by three different analysts on different days. The peptide maps generated perfectly overlap with an average RSD for the peak area of less than 5%. These results highlight the reproducibility that can be achieved when using this novel digestion technique in combination with the Thermo Scientific™ Vanquish™ Flex UHPLC system featuring Thermo Scientific™ SmartInject (intelligent sample pre-compression technology) for high levels of retention time reproducibility.

# Identified Components	Total MS area [counts × s]	Sample
1702	3.48 × 109	SMART Digest, 15 min
1678	4.12 × 109	SMART Digest, 30 min
1688	3.96 × 109	SMART Digest, 45 min
1551	3.13 × 109	SMART Digest, 75 min
1171	3.65 × 109	In-Solution, Urea
1145	4.04 × 109	In-Solution, Heat

Table 2. Number of identified components and TIC area for the different runs.

In peptide mapping analysis of mAbs, 100% sequence coverage for the heavy and light chains must be achieved. The sequence coverages for the different digest conditions are shown in Table 1. For all six methods, including the fast digestion methods of 15 and 30 min, 100% coverage was achieved for light as well as heavy chains. Strikingly, an incubation time of only 15 min is sufficient to achieve 100% sequence coverage for both the heavy and light chains of the antibody when the SMART Digest kit is used. The number of detected MS peaks in the samples digested with the SMART Digest kit was generally higher than in the in-solution digested samples. The same trend was observed when the number of identified components, including all peptides and charge states, and the total MS ion count were compared (Table 2).

Blog: BioPharMoore Episode 2: SMARTening up on Protein Digestion

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Speed of method development

The SMART Digest kit not only provides significantly quicker digestion, but also quicker method development. In order to determine the optimum digestion time for a given protein a time course experiment can be used to monitor disappearance of the intact protein via LC-UV or LC-MS.

The example in Figure 4 shows chromatogram overlays of the digestion points of the protein ribonuclease A, (a small, heat stable protein with a molecular weight of 13.7 kDa. Ribonuclease A is a particularly resistant to tryptic digestion due to its heat stability and ability to quickly refold) beginning at T=0 min and ending at T=210 minutes of digestion with the SMART Digest kit.

By monitoring the disappearance of the intact peak seen in isolation on the T=0 chromatogram it can be seen that complete digestion is achieved in 2 hours.

Another example of a time course experiment is seen with IgG in Figure 5. The digestion of mouse IgG proceeds quickly with most of the sample being digested in the first 15 minutes; after 60 minutes the peptide fingerprint is stable.

This demonstrates that method development is extremely quick, with optimization being achieved within a few chromatographic runs. In the examples shown for subsequent analyses the digestion time for ribonuclease A and IgG is 120 and 60 minutes respectively. Table 3 shows the rapid digestion times for a number of proteins that have been prepared with the SMART Digest kit protocol.

Protein	SMART Digestion Time
Insulin	4
BSA	<5
Carbonic Anhydrase	<5
Lysozyme	<5
Apo-B	30
IgG in 50 µL plasma	75
Thyroglbulin	240
C-reactive protein	240

Table 2. Typical SMART digestion times of a series of proteins.

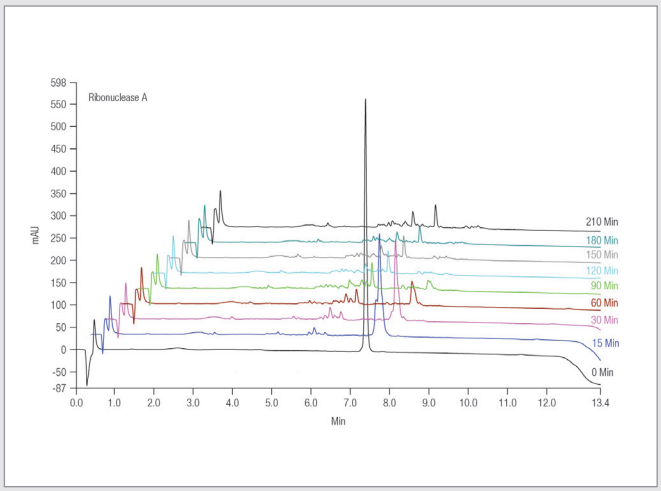


Figure 4. Chromatogram overlay of the digestion time points for ribonuclease A. Complete digestion is achieved in 120 minutes, when the peptide fingerprint is stable and the intact peak is no longer visible.

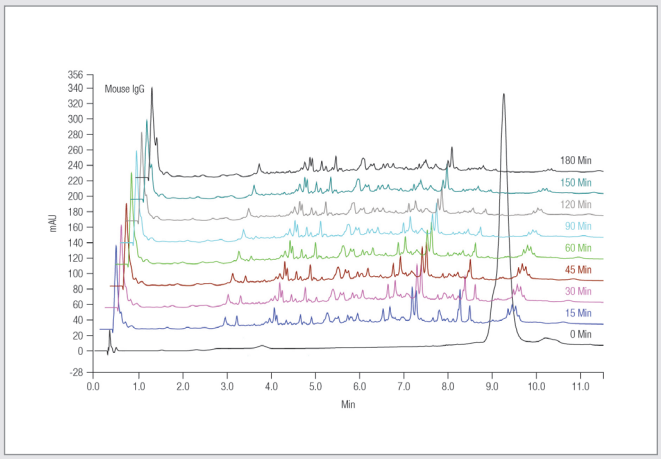
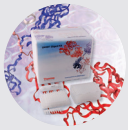


Figure 5. Chromatogram overlay of the digestion time points for mouse IgG. Initial digestion proceeds very fast and most of the sample is digested in the first 15 minutes. After 60 minutes the peptide fingerprint is stable and the intact peak is visible only in trace amounts.

PDF: Application Note - Fast Digestion Method Optimization



Webinar: Learn how to increase confidence in biopharma peptide mapping.

Blog: Can Protein Digestion Really be Reduced From 2 Days to 60 Mins?

More Information

Improving sensitivity and speed for protein quantitation from complex matrices

e.g. As mentioned protein characterization is just one of the analytical workflows within the biopharmaceutical arena that requires digestion. The other is bioanalysis, where there are additional challenges due to the low abundance of the protein biomarker/bio-therapeutic and the complex biological matrices e.g. plasma in which they reside.

In these workflows immunoaffinity capture is typically employed to increase sensitivity by purifying the low level proteins from the complex biological matrix. This step is then followed by protein digestion. As a consequence these workflows have all the challenges faced when using in-solution digestion protocols, but with the added complexity of additional steps that add labor, the potential for error and that are not amenable to automation.

The SMART Digest ImmunoAffinity (IA) Kits offer a solution to these issues. The SMART Digest IA kits have all the

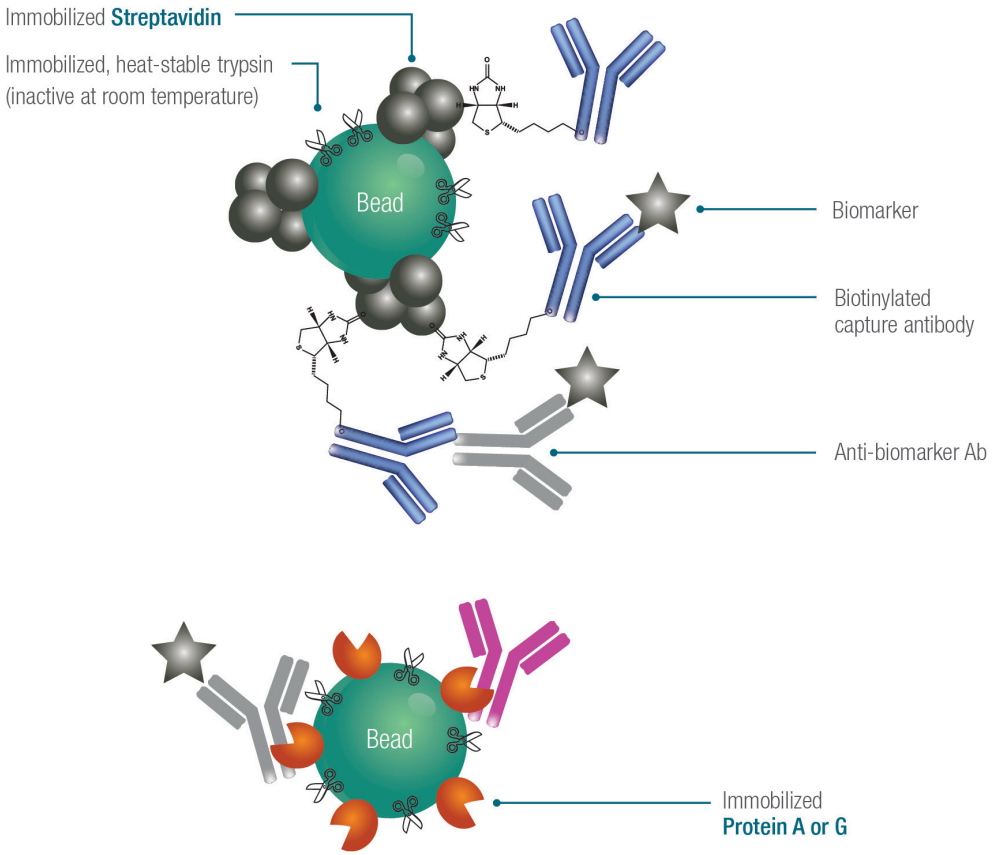


Figure 6. Heat-stable, immobilized enzyme design combines with immunoaffinity capture

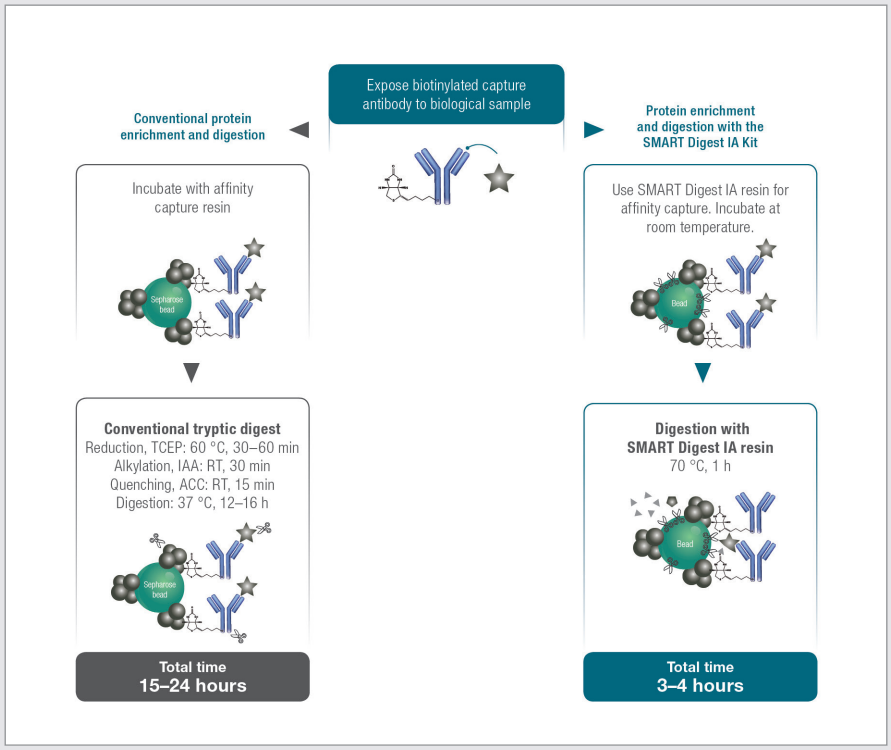
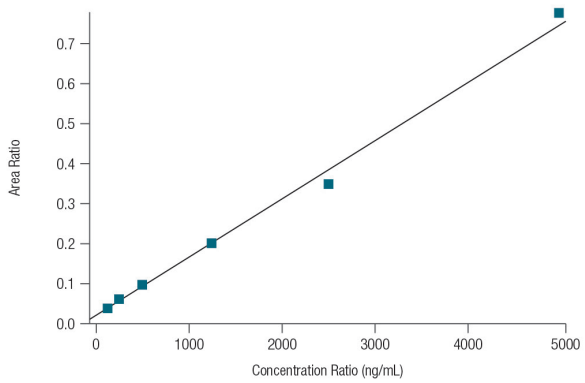


Figure 7. Comparison of SMART Digest IA kit workflow with a conventional workflow.

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 is achieved 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 (Figure 6).

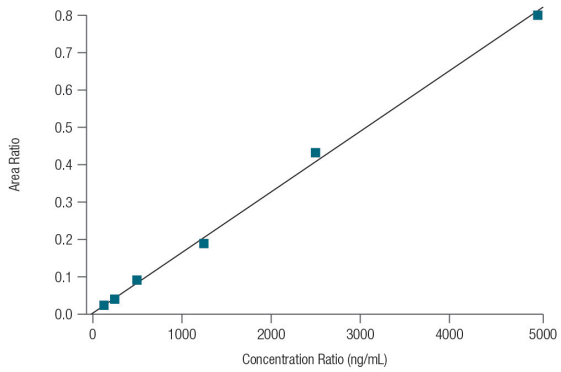
Following the binding of a capture reagent to the bead, and enrichment of the target, the enzyme is activated at elevated temperatures for accelerated digestion under protein denaturing conditions. The resulting workflow is therefore significantly simplified and provides a process where the sample is ready for analysis in just a few hours rather than overnight (Figure 7).

The following example highlights the advantages of using the SMART Digest



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			

Figure 8: Calibration curve for the detection biomarker (human interferon a14), in human plasma using the SMART Digest IA Streptavidin kit.



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			

Figure 9: Calibration curve for the detection biomarker (human interferon a14), in human plasma using a conventional streptavidin agarose process.

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

Table 4: Recovery data for biomarker (human interferon a14), in human plasma using the SMART Digest IA Streptavidin kit.

Recovery with conventional approach	
500 ng/mL spike	2278 (cps)
Recovery	35%

Table 5: Recovery data for biomarker (human interferon a14), in human plasma using a conventional streptavidin agarose process.

IA Streptavidin kit for the quantitation of a biomarker (human interferon a14) 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.

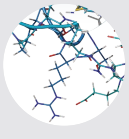
The results show that using the SMART Digest IA kit (Figure 8 and Table 4) good linearity is achieved over a wide dynamic range, with better % CVs and higher recovery than for the traditional approach (Figure 9 and Table 5).

For the demands of the modern biopharmaceutical company where characterization and quantitation workflows prove challenging due to the complexity of the workflow, speed of analysis and quality of results the new innovations offered by SMART Digest and SMART Digest IA kits provide a step change in protein sample preparation by providing workflows which are significantly faster, easier to use highly reproducible and sensitive.

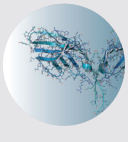
More Information

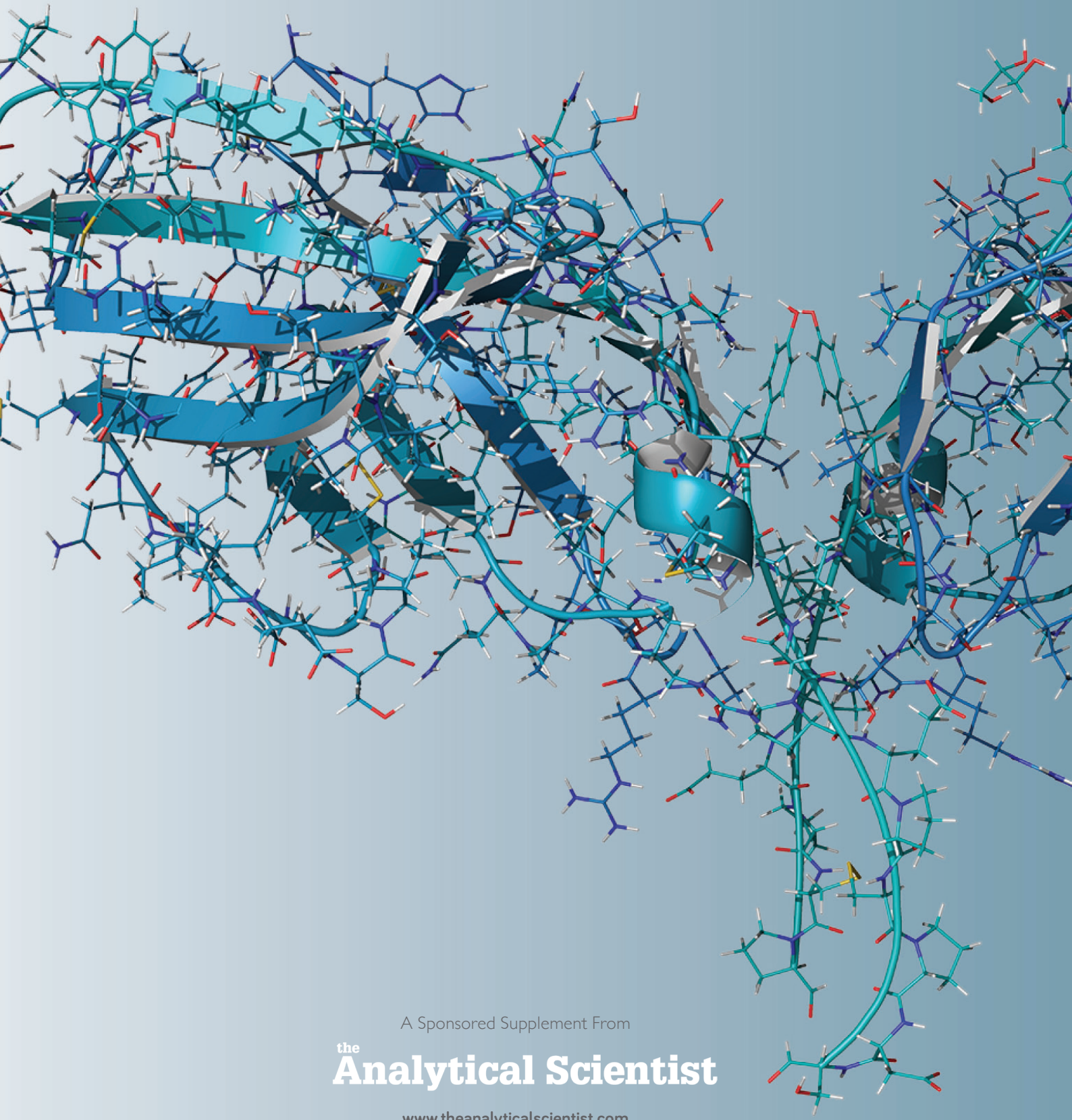
PDF: Application Note - Analysis of Klotho in less than 4 hours.

PDF: Customer Testimonial



PDF: SMART Digest Kit IA – FAQs





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