

# Residual host cell protein analysis of NISTmAb: From simplified sample preparation to reliable results

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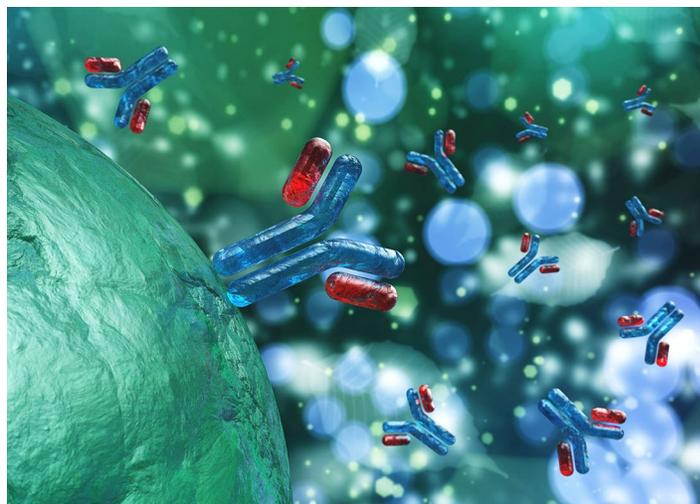
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## Application benefits

- Non-denaturing protein digestion with Thermo Scientific™ SMART Digest™ kit reduces intrasample dynamic range and increases host cell protein (HCP) identifications.
- Efficient and reproducible chromatographic separation with Thermo Scientific™ Acclaim™ VANQUISH™ C18, 2.1 × 250 mm, column.
- High sensitivity detection over large dynamic range, down to sub-1 ppm, using Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer.
- High-resolution accurate-mass (HRAM) MS data combined with Thermo Scientific™ Proteome Discoverer™ 2.4 software provides confident HCP identifications.

## Goal

To demonstrate the benefit of an improved approach for the identification of low ppm HCPs in biotherapeutics using a combination of non-denaturing tryptic digestion, efficient chromatographic separation, sensitive HRAM-MS detection, and advanced data processing to reduce the dynamic range challenge and maximize the identification rate.



## Introduction

Recombinant biotherapeutics are generally produced using non-human host cells, with Chinese hamster ovary (CHO), murine myeloma, and *Escherichia coli* cell-lines most commonly used. During cell growth and harvest, endogenous proteins from these host cells are released that can detrimentally affect final drug product safety and efficacy. Therefore, these host cell proteins must be removed post-harvest through a series of purification steps. The guidelines within the U.S. Pharmacopeia state, “The product purification processes must be optimized to consistently remove as many HCPs as feasible, with the goal of making the product as pure as possible”.<sup>1</sup> Consequently, HCPs levels in monoclonal antibody (mAb) biotherapeutics must be determined prior to drug release.

Current lack of clear regulatory guidance can make defining acceptable concentrations of HCPs in the final drug product challenging. Commonly stated tolerance levels are <100 ppm (100 ng HCP:1 mg mAb) total HCP impurity, with <10 ppm for each individual protein.<sup>2</sup> This approach, however, does not consider the potential for adverse effects of individual HCPs. A particular concentration of one HCP may not elicit any detrimental effect to either patient or drug product, while for another HCP, the same concentration may have disastrous consequences for patients or negatively affect product stability and shelf-life.<sup>3</sup>

The current regulatory gold-standard for host cell protein analysis is “an immunoassay capable of detecting a wide range of protein impurities”, with enzyme-linked immunosorbent assay (ELISA) typically the method of choice due to its high sensitivity and specificity.<sup>1,4</sup> However, developing a new host cell protein ELISA requires suitable anti-HCP reagents to be generated, which can be costly in both time and materials. In addition, each HCP ELISA should be process-specific, in that for each different product manufactured, a new ELISA must be developed, which further impacts costs. Traditional ELISA approaches can also only provide information on the HCPs that are immunologically reactive to the reagent antibodies, with no capacity for individual protein identification or quantification.

Ultra-high-pressure liquid chromatography (UHPLC) combined with high-resolution accurate-mass (HRAM) mass spectrometry (MS), however, can quantify and identify multiple host cell proteins in a single chromatographic separation. This opens the door to the potential for individual HCP monitoring throughout the purification process. The major challenge with liquid chromatography-mass spectrometry (LC-MS) analysis of HCPs is overcoming the huge intrasample dynamic range to detect very low abundant (<10 ppm) residual HCPs within the concentrated biotherapeutic drug product.

Here, detection of low abundance HCPs is facilitated by non-denaturing protein digestion. Exploiting the resistance of native mAb protein structure to tryptic proteolysis enables efficient removal of undigested material post-digestion. This reduces the dynamic range of the sample, subsequently increasing the likelihood of HCP detection.<sup>5</sup>

The Orbitrap Exploris 480 mass spectrometer provides significantly improved sensitivity and faster acquisition rates supporting HCP identification by data-dependent acquisition (DDA) even at very low ppm levels. Furthermore, increasing the MS<sup>2</sup> isolation window to promote co-isolation of peptide ions and consequent acquisition of high-resolution chimeric spectra (multiple peptides per spectrum) can increase protein identifications in complex samples with a large dynamic range.<sup>6</sup> A new node in Protein Discoverer 2.4 software, Precursor Detection, increases identification of proteins from chimeric spectra.

## Experimental

### Automated protein digestion

- NIST<sup>®</sup> monoclonal antibody (NISTmAb) reference material (RM 8671) was concentrated to approximately 60 µg/µL using 0.5 mL 3 kDa molecular weight cutoff spin filters.
- 1.2 mg NISTmAb (at a final concentration of 6 µg/µL) was digested for 3 hr at 37 °C with 10 µL SMART Digest magnetic bulk resin (mAb:trypsin ~130:1).
- The digestion was automated using a Thermo Scientific<sup>™</sup> KingFisher<sup>™</sup> 96 Deepwell plate (Table 1) with the Thermo Scientific<sup>™</sup> KingFisher<sup>™</sup> Duo Prime purification system.
- The mixing parameters within the Thermo Scientific<sup>™</sup> KingFisher<sup>™</sup> BindIt<sup>™</sup> 4.0 software were set to medium for 5 min, slow for 20 min, with a loop count of 7.
- Undigested protein was removed via precipitation at 95 °C for 10 min, followed by centrifugation at 14,000 × g for 5 min.
- Recovered supernatant was reduced with 1 µL Thermo Scientific<sup>™</sup> Bond-Breaker<sup>™</sup> TCEP Solution.

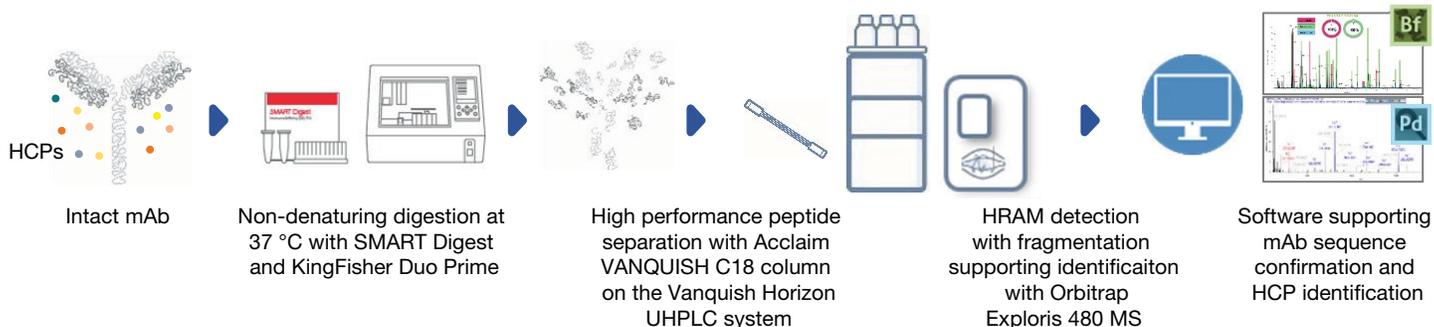


Figure 1. Overview of experimental set-up

Table 1. Layout of KingFisher Deepwell, 96 well plate

Lane	Description	Contains	Volume per well
A	Sample	Sample	50 µL*
		SMART Digest buffer	150 µL
B	Tips	12-tip comb	
C	Empty	-	-
D	SMART Digest Trypsin	Magnetic Bead Bulk Resin	10 µL
		SMART Digest buffer	100 µL
E	Bead wash buffer	Water (at least 18.2 MΩ·cm)	160 µL
		SMART Digest buffer	40 µL
F	Waste	Water (at least 18.2 MΩ·cm)	250 µL

\*Use 1.2 mg mAb, diluted with water (at least 18.2 MΩ·cm) if required, to maintain 50 µL sample volume.

## LC-MS

- Peptides in a 20 µL injection (equivalent to 160 µg mAb, if digestion were complete) were separated using an Acclaim VANQUISH C18 UHPLC 250 × 2.1 mm (2.2 µm) column over a 90 min linear gradient:

- A: 0.1% formic acid in water
- B: 0.1% formic acid in acetonitrile
- 1 min hold at 3% B with eluent sent to waste via a 2-position, 6-port switching valve
- 3–35% B over 89 min, increase B to 85% to wash column, and then re-equilibrate at 3% B for 19.9 min (Table 2)

Table 2. Chromatographic separation - LC gradient conditions.

Mobile phase A: water with 0.1% formic acid. Mobile phase B: acetonitrile with 0.1% formic acid. Column temperature: 60 °C

Time (min)	Flow rate (mL/min)	Mobile phase (%)	
		A	B
0	0.30	97	3
1	0.30	97	3
90	0.30	65	35
95	0.30	15	85
100	0.30	15	85
105	0.30	97	3
110	0.30	15	85
115	0.30	85	85
115.1	0.30	97	3
135	0.30	97	3

- Data were acquired by data-dependent acquisition (dd-MS<sup>2</sup>, Top15) with an Orbitrap Exploris 480 mass spectrometer, using a resolution setting of 120,000 for MS<sup>1</sup> and 30,000 for MS<sup>2</sup>, with a MS<sup>2</sup> isolation window of 4 *m/z* (Table 3).

Table 3 (part 1). Orbitrap Exploris MS parameters

MS parameters	
Vaporizer temperature	350 °C
Ion transfer tube temperature	325 °C
Application mode	Peptide
Pressure mode	Standard
Advanced peak determination	True

**Table 3 (part 2). Orbitrap Exploris scan properties**

Scan properties	
Full Scan properties	
Orbitrap resolution	120,000
Scan range	350–1200
RF lens (%)	40
AGC target	Custom – 300%
Injection time	Custom – 25 ms
Microscans	1
Data type	Profile
Polarity	Positive
ddMS2 scan properties	
Isolation window ( <i>m/z</i> )	4
Collision energy	Fixed, Normalized
HCD collision energy (%)	30
Orbitrap resolution	30,000
Scan range mode	Define first mass (120 <i>m/z</i> )
AGC target	Custom – 100%
Injection time	Custom – 70 ms
Microscans	1
Data type	Centroid

**Instrumentation and software**

- Thermo Scientific™ Vanquish™ Horizon UHPLC system consisting of:
  - System Base Vanquish Horizon/Flex (P/N VF-S01-A-02)
  - Vanquish Binary Pump H (P/N VH-P10-A)
  - Vanquish Split Sampler HT (P/N VH-A10-A)
  - Vanquish Column Compartment H (P/N VH-C10-A-02)
  - Switching valve (2 position – 6 port) (P/N 6036.1560)
- Thermo Scientific Orbitrap Exploris 480 mass spectrometer with BioPharma option (extended mass range to *m/z* 8000) (P/N BRE725539)
- Thermo Scientific Proteome Discoverer 2.4, options available

**Recommended consumables**

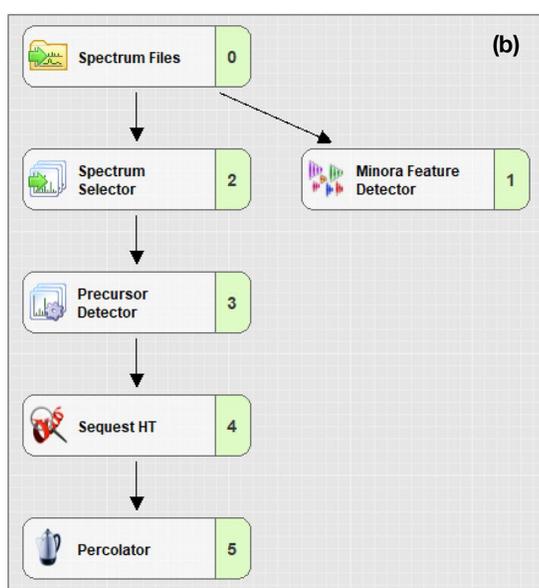
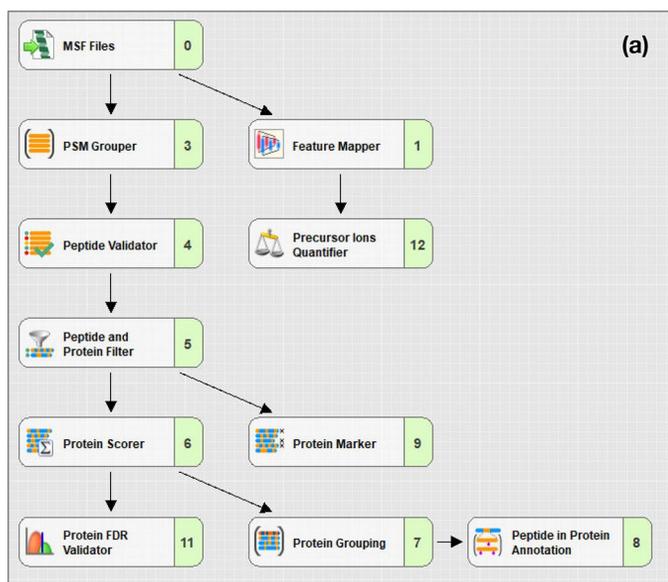
- Thermo Scientific SMART Digest Trypsin Kit, Magnetic Bulk Resin option (P/N 60109-101-MB)
- Thermo Scientific KingFisher Deepwell, 96 well plate (P/N 95040450)

- Thermo Scientific KingFisher Duo 12-tip comb (P/N 97003500)
- Thermo Scientific KingFisher Duo Prime purification system (P/N 5400110)
- Thermo Scientific Bond-Breaker TCEP Solution (P/N 77720)
- Thermo Scientific Acclaim VANQUISH C18 column, 2.2  $\mu\text{m}$ , 2.1  $\times$  250 mm (P/N 074812-V)
- Fisher Scientific™ Water, Optima™ LCMS grade (P/N 10505904)
- Fisher Scientific Water with 0.1% formic acid (v/v), Optima LCMS grade (P/N 10188164)
- Fisher Scientific Acetonitrile with 0.1% formic acid (v/v), Optima LCMS grade (P/N 10118464)

**Data analysis**

Proteins were identified using Proteome Discoverer 2.4 software (Figure 2). Nodes of particular interest are detailed below:

- Feature Mapper: Retention time alignment, maximum shift of 2 min
- Precursor Ions Quantifier: Normalized to total peptide amount
- Peptides filtered to not include any low confidence peptide spectral matches (PSMs) and to have a minimum of two peptides identified per protein
- Minora Feature Detector: Enables creation of ratios and scaled abundance values for label-free quantification
- Precursor Detector: 1.5 S/N Threshold. Precursor Detector node detects all precursor masses that are co-isolated with the targeted precursor and consequently fragmented together to produce the chimeric MS<sup>2</sup> spectrum
- Sequest HT: UniProt *Mus musculus* database used to which NISTmAb heavy and light chain sequences were added, 5 ppm precursor mass tolerance, 0.02 Da fragment mass tolerance. Dynamic modifications – Methionine oxidation, asparagine deamidation, plus N-term modification of Met-loss and/or acetylation
- Percolator – 0.05 relaxed FDR and 0.01 strict FDR



**Figure 2. Proteome Discoverer 2.4 workflow nodes for protein identification.** (a) Consensus Step Workflow, (b) Processing Step Workflow with Precursor Detector node

## Results and discussion

### Non-denaturing digest optimization

The standard protocol for SMART Digest takes advantage of the heat-stable trypsin immobilized on the magnetic beads, enabling high temperature to be used to denature proteins during the digestion process. Here, where the aim is to minimize mAb digestion to reduce the intrasample dynamic range and consequently increase the likelihood of

HCP identification, the digestion temperature was reduced to create non-denaturing digest conditions (Figure 3).

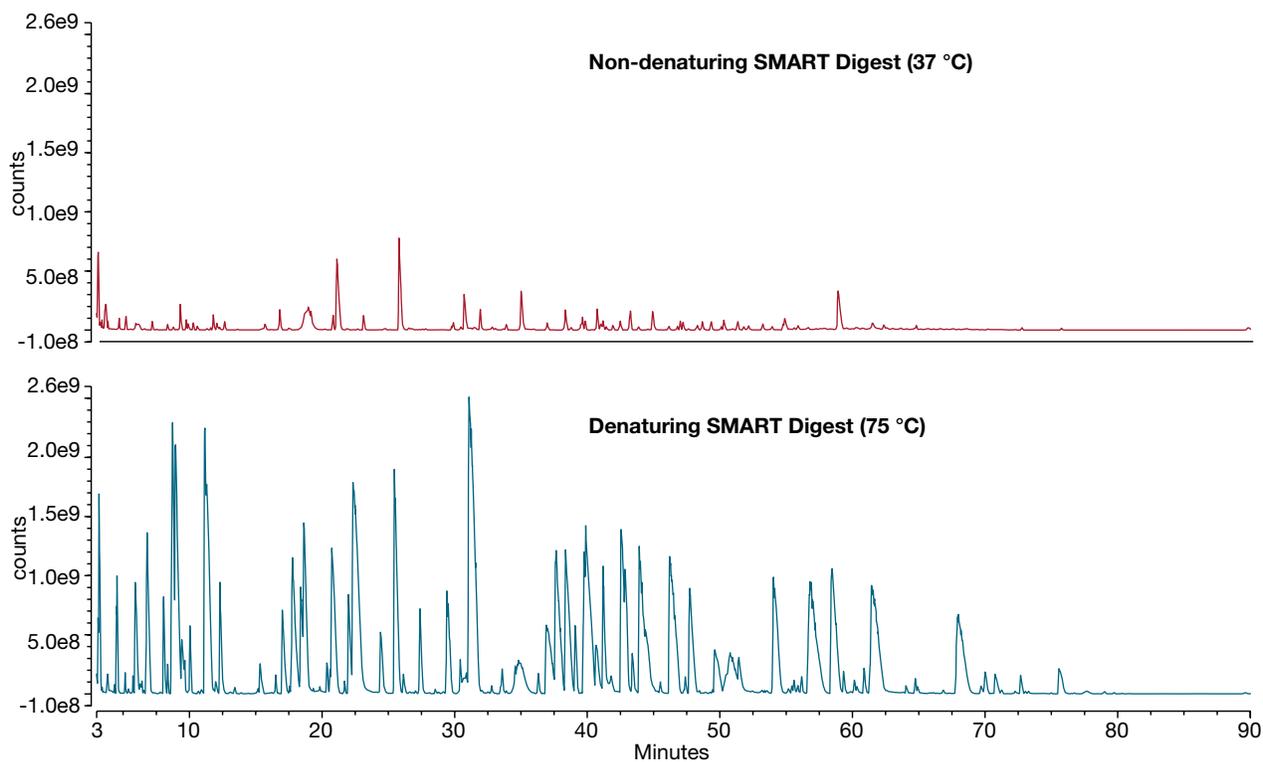
Another factor to consider during non-denaturing digest optimization was the high trypsin concentration immobilized on the SMART Digest magnetic beads. Initial investigations identified slightly fewer HCPs using 15  $\mu$ L magnetic beads compared to digests using 10  $\mu$ L or 5  $\mu$ L. Subsequent analysis comparing 5  $\mu$ L and 10  $\mu$ L SMART Digest beads, analyzed in triplicate, identified 20 additional unique HCPs with the 10  $\mu$ L SMART Digest beads, and so, this volume was used for this and all future experiments.

### Confident low abundant protein identifications

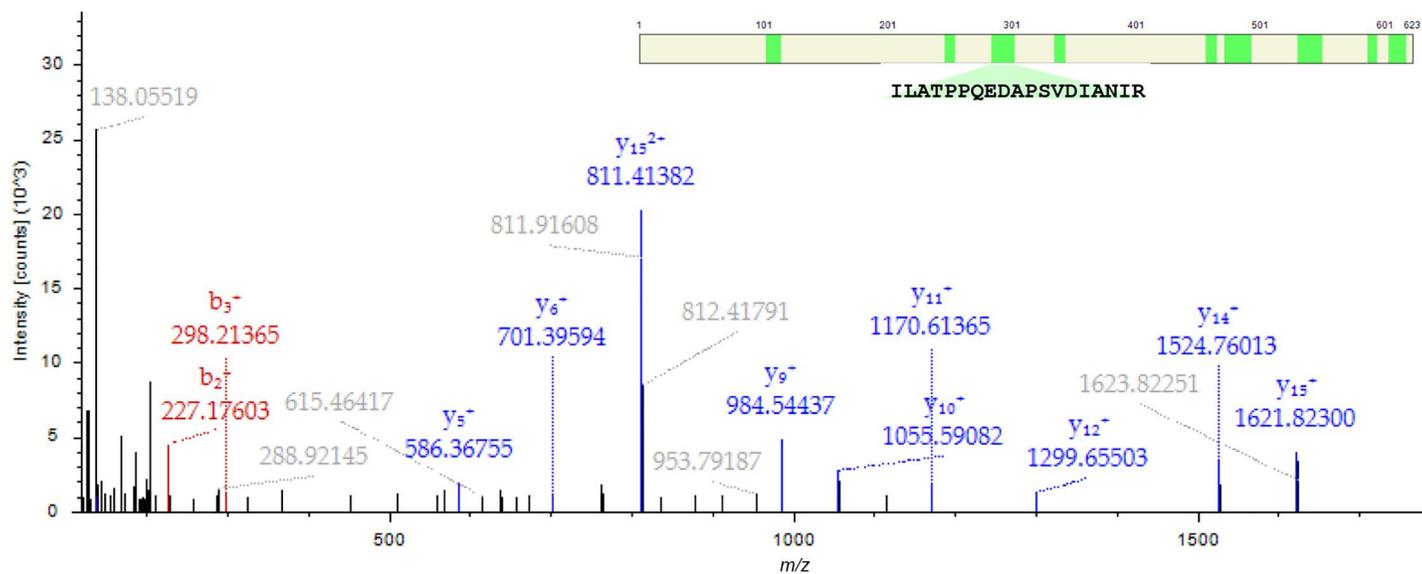
A previously published analysis of NISTmAb using a similar digestion strategy identified 60 HCPs with two or more peptides (42 HCPs with three or more peptides) using a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer.<sup>5</sup> The measured ppm levels for these HCPs correlate well with the 14 HCPs quantified using a 2D-LC approach analyzed with an ion-mobility time of flight MS,<sup>7</sup> so they were used as a reference for levels of specific HCPs in this identification study.

When NISTmAb was digested under non-denaturing conditions (37 °C, 10  $\mu$ L SMART Digest magnetic beads) and analyzed by UHPLC combined with HRAM-MS using an Orbitrap Exploris 480 mass spectrometer, more than 80 HCPs were identified based on three or more unique peptides (more than 120 HCPs with two or more unique peptides), in at least two out of three replicate injections (as a fixed acceptance criteria). HCPs spanning the previously referenced published abundance range were identified from 222 ppm (fructose-bisphosphate aldolase A) to <0.5 ppm (malate dehydrogenase, serine/arginine-rich splicing factor 1, cystatin-C). Many additional HCPs not detected in the previous study were also identified (Table 4).

The accurate mass of precursor ions (predominantly less than 2 ppm) plus excellent MS<sup>2</sup> spectral quality provided by the Orbitrap Exploris 480 mass spectrometer further increases the confidence in HCP identifications, even for proteins present at very low abundance (Figure 4). The excellent sensitivity and dynamic range capabilities also facilitate the identification of multiple peptides per protein.



**Figure 3. Effect of SMART Digest temperature on mAb digestion and intrasample dynamic range.** Equal amounts of commercially available nivolumab (160  $\mu$ g) digested using denaturing (75  $^{\circ}$ C) and non-denaturing (37  $^{\circ}$ C) SMART Digest conditions. Equal volume (20  $\mu$ L) injected on-column post digestion.



**Figure 4. Representative MS<sup>2</sup> spectrum of the spectral quality for very low abundant HCPs.** This HCP, transketotase, was previously quantified in NISTmAb at 1 ppm by Huang *et al.*<sup>5</sup> Even at such low abundance, 11 unique peptides were identified, providing additional confidence.

## Reproducibility

To assess the overall consistency in digestion, MS acquisition, and data processing, replicate digestions using the non-denaturing SMART Digest approach were analyzed in triplicate by LC-MS. The number of unique

peptides identified per HCP was highly reproducible (Table 4), especially considering that with proteomic-type analysis there is generally some variance in the peptides identified between replicates.

**Table 4. NISTmAb HCP identifications.** List of 30 HCPs identified in this study, ranked by overall sequence coverage, illustrating the number of unique peptides identified across two replicate digestions. Measured ppm value (as previously determined by Huang *et al.*<sup>9</sup>) also provided as reference.

HCP name	Unique peptides (rep 1)	Unique peptides (rep 2)	Measured ppm
1 Fructose-bisphosphate aldolase A	31	32	222
2 Fructose-bisphosphate aldolase C	23	22	55
3 Glucose-6-phosphate isomerase	28	27	20
4 Programmed cell death protein 5	5	5	
5 Beta-2-microglobulin	5	4	12
6 Peptidyl-prolyl cis-trans isomerase FKBP2	5	6	2
7 Protein Abhd11	12	12	10
8 Adenylate kinase 2	9	9	2
9 Ubiquitin-conjugating enzyme E2 variant 2	3	8	4
10 Syntaxin-12	9	9	4
11 Nucleoside diphosphate kinase b	3	3	
12 Heterogeneous nuclear ribonucleoproteins A2/B1	10	10	6
13 Heterogeneous nuclear ribonucleoprotein A1	9	8	4
14 Cystatin-C	3	2	<0.5
15 Poly(RC)-binding protein 1	6	6	
16 Nucleoside diphosphate kinase A	3	3	
18 Prostaglandin red uctase 1	10	9	5
19 Flavin reductase (NADPH)	6	6	3
20 Polypeptide N-acetylgalactosaminyltransferase 2	12	10	3
21 Peroxiredoxin 1	6	5	1
22 UMP-CMP kinase	5	5	1
23 Transgelin-2	2	3	
24 NSFL1 cofactor p47	11	13	3
25 Protein NipSnap homolog 3B	7	6	
26 Iron-sulfur cluster assembly enzyme ISCU	4	3	
28 Polypeptide N-acetylgalactosaminyltransferase 6	11	11	6
29 Polyubiquitin-C	2	2	
30 Peroxiredoxin-5	3	4	1
29 Transketolase	8	9	1
30 Serine/arginine-rich splicing factor 1	4	4	<0.5

The consistency of the non-denaturing digestion approach can be further demonstrated by considering the mAb peptides present in each digest. Using Thermo Scientific™ BioPharma Finder™ 3.2 software, comparing sequence coverage and number of MS peaks detected across the technical replicates for NISTmAb heavy and light chain confirmed the excellent reproducibility of this non-denaturing digestion strategy. Further analysis of these data, when compared to a denaturing peptide mapping

approach, verifies the efficacy of the non-denaturing approach in reducing the sample dynamic range and complexity for improved HCP analysis: The non-denaturing digestion reduced the sequence coverage and the number of detected MS peaks per protein for both the heavy and light chains (Figure 5). This reaffirms that digestion under non-denaturing conditions reduces proteolysis of the mAb, thus increasing the potential for low abundant HCP detection.

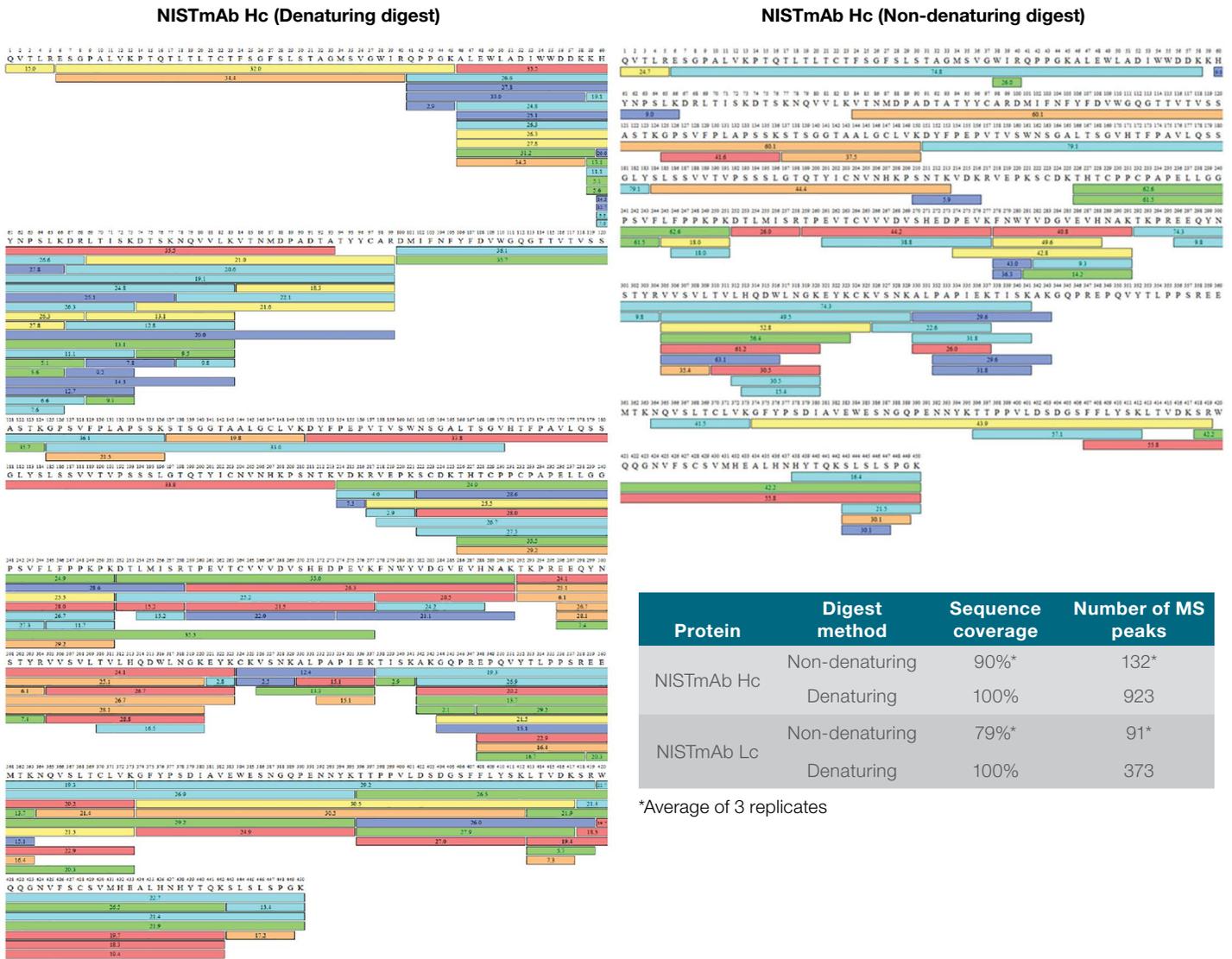


Figure 5. Comparison of NISTmAb sequence coverage from BioPharma Finder software following non-denaturing and denaturing digestion

The Acclaim VANQUISH C18 column used for this study generates reproducibly efficient peptide separations, which is also essential for maximizing detection and subsequent

identification of low abundant HCP peptides when the mAb peptides dominate the separation space (Figure 6).

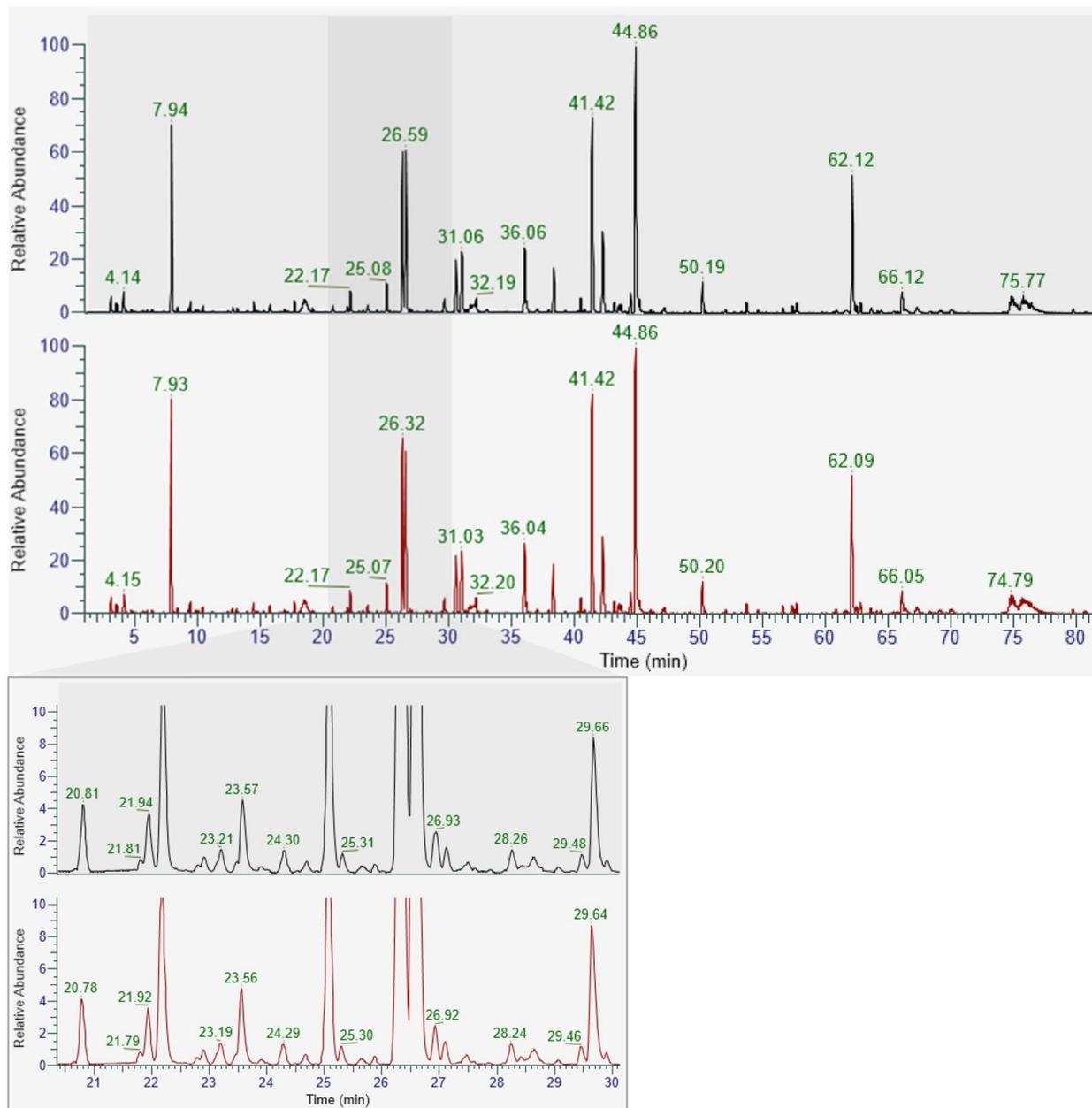


Figure 6. Chromatographic efficiency and reproducibility of replicate non-denaturing digests of NISTmAb

## Conclusion

- Non-denaturing protein digestion facilitates detection of HCPs by exploiting the resistance of native mAb protein structure to proteolysis. Removal of undigested protein with heat-treatment prior to MS analysis reduces the dynamic range of the sample, increasing the likelihood of HCP identification.
- The SMART Digest magnetic bead kit simplifies reproducible protein digestion, especially when automated using a KingFisher Duo Prime purification system.
- The Acclaim VANQUISH C18 column used in this study delivered reproducible high separation efficiency across the entire analysis.
- Using a wide MS<sup>2</sup> isolation window (4 *m/z*) promotes co-isolation of precursor ions with similar *m/z*, resulting in chimeric spectra. The advantage of this for HCP analysis is the increased potential for isolation and subsequent fragmentation of these low abundant peptides. A new workflow node available in Proteome Discoverer 2.4 software called Precursor Detection was used to maximize protein identifications from chimeric spectra.
- The high resolution, accurate mass, and fast scanning speeds, as well as excellent sensitivity of the mass spectrometer used in this study, all aided in the identification of more than 80 HCPs from the NISTmAb reference standard, based on three or more unique peptides over a range of <1 ppm to approximately 200 ppm.

- The showcased analysis approach laid out in this study reduces the dynamic range challenge and maximizes the identification rate, thus increasing overall confidence in identifications both in terms of the number of identified HCPs and unique peptides per protein.

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