

## Identification of Host Cell Proteins (HCPs) in Monoclonal Antibodies At Sub-ppm Levels Using the SYNAPT XS Mass Spectrometer

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### Abstract

In this application note we describe a highly sensitive discovery HCP assay that can identify and quantify sub-ppm levels of HCPs from a highly purified monoclonal antibody.

### Benefits

- A highly sensitive HCP discovery assay using the HDMS<sup>E</sup> data independent acquisition (DIA) mode identifies very low levels of HCPs (down to 100 ppb) from a highly purified monoclonal antibody
- When coupled with a literature reported sample preparation method, forty eight (48) HCPs were identified from the NIST mAb, demonstrating a considerable improvement in our ability to identify low abundance HCPs

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### Introduction

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Residual host cell proteins (HCPs) are low-level (typically <100 ppm) process-related impurities, that might be present in protein biopharmaceuticals even after extensive purification. HCPs could produce unwanted immunogenic response in patients, reduce the efficacy or the stability of the drug or be responsible for drug degradation.<sup>1</sup> For these reasons, the regulatory agencies require that HCPs are identified and quantified prior to drug approval. The biopharmaceutical industry relies on ELISA assays for measuring the total HCP concentration expressed in ppm (or ng HCPs/mg biopharmaceutical). Mass spectrometry-based HCP analysis has emerged in recent years as a powerful alternative to ELISA<sup>2-6</sup> because it provides more extensive (proteome-wide) HCP coverage and is able to identify and measure individual HCP levels.



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*Figure 1. SYNAPT XS Mass Spectrometer.*

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## Experimental

### Sample Preparation

A highly purified mAb (NIST mAb candidate reference LRM 8670) produced in a murine cell culture was acquired from the National Institute of Standards and Technology (NIST) at a concentration of 100 mg/mL. The NIST mAb was digested using a modified protocol firstly introduced by Huang *et. al* from Eli Lilly & Company.<sup>3</sup> mAb digestion was performed in a glass vial containing 100 µg of the Lys-C/Trypsin enzyme mixture (Promega, Madison, WI). A volume of 125 µL NIST mAb was mixed with 50 µL of 100 mM CaCl<sub>2</sub> and 625 µL of 100 mM ammonium bicarbonate. The digestion was allowed to proceed overnight (~ 16 hours) at 37 °C. The mAb sample was then denatured in the presence of 0.05% *Rapi*Gest (1 h at 60 °C), reduced in the presence of 25 mM dithiothreitol - DTT (1 h at 60 °C) and alkylated with 12 mM iodoacetamide - IAM (45 min in the dark at room temperature). Precipitation of the mAb started to occur during the alkylation step. After adding 5 µL of formic acid (FA), the digest was incubated for 30 min at 37 °C and centrifugated (10 min, 13,000 x g) to separate the precipitated mAb and the *Rapi*Gest surfactant. All digestion reagents (CaCl<sub>2</sub>, NH<sub>4</sub>HCO<sub>3</sub>, DTT, IAM, and FA) were obtained from Sigma-Aldrich (St. Louis, MO). The supernatant was transferred to an autosampler vial (Waters P/N 186000327C <<https://www.waters.com/nextgen/us/en/shop/vials-containers--collection-plates/186000327c-lcgc-certified-clear-glass-12-x-32-mm-screw-neck-vial-max-recover.html>> ) and five MassPREP protein digest standards were spiked at the following concentrations: 100 nM ENL (yeast enolase P/N 186002325 <<https://www.waters.com/nextgen/us/en/shop/standards--reagents/186002325-massprep-enolase-digestion-standard.html>> ), 40 nM ClpB (Hi3 *E. coli* P/N 186006012 <<https://www.waters.com/nextgen/us/en/shop/standards--reagents/186006012-hi3-e-coli-standard.html>> ), 20 nM ADH (yeast alcohol dehydrogenase P/N 186002328), 20 nM PHO (rabbit phosphorylase b P/N 186002326), and 5 nM BSA (bovine serum albumin P/N 186002329 <<https://www.waters.com/nextgen/us/en/shop/standards--reagents/186002329-massprep-bsa-digestion-standard.html>> ). The amounts of each of the spiked proteins loaded on-column in 50 µL injected sample were: 5000 femtomoles ENL, 2000 fmoles ClpB, 1000 fmoles ADH, 1000 fmoles PHO and 250 fmoles BSA. LC-MS-grade solvents (acetonitrile-ACN and water) were purchased from Thermo Fisher Scientific (Waltham, MA).

### LC Conditions

LC system: ACQUITY UPLC I-Class PLUS

Column: ACQUITY Premier CSH C<sub>18</sub> Column 1.7 μm, 2.1 x 150 mm (P/N: 186009462)

Column temperature: 60 °C

Flow rate: 50 μL/min

Mobile phases: Solvent A: 0.1% FA in DI water  
Solvent B: 0.1% FA in acetonitrile

Injection volumes: 50 μL

Wash solvents: Purge solvent: 50% MeOH  
Sample Manager wash solvent: 50% MeOH

## Gradient

Time (min)	Flow rate (mL/min)	Solvent A composition (%)	Solvent B composition (%)	Curve profile
0.00	0.2	100	0	Initial
2.00	0.2	100	0	6
2.10	0.05	100	0	6
25.00	0.05	90	10	6
82.00	0.05	80	20	6
112.00	0.05	70	30	6
113.00	0.4	70	30	6
120.00	0.4	50	50	6
122.00	0.4	50	50	6
123.00	0.4	100	0	6
124.00	0.4	100	0	6
134.00	0.4	100	0	6

## MS Conditions

MS System:	SYNAPT XS QTof Mass Spectrometer
Ionization mode:	ESI+
Capillary voltage:	2.5 kV
Cone voltage:	40 V
Source offset:	4 V
Source temperature:	120 °C
Desolvation temperature:	300 °C
Cone gas flow:	35 L/h
Desolvation gas flow:	600 L/hr
Nebuliser gas Pressure:	7 bar
Data acquisition:	HDMS <sup>E</sup> mode
Mass range ( <i>m/z</i> ):	50–2000
Scan rate:	1.0 sec
Low energy CE:	6 V
High energy CE ramp:	Transfer Look-up Table (LUT) with CE in the range of 0–85 V

Data acquisition software: MassLynx 4.2 SCN 1009

Data processing software: Progenesis QI for Proteomics v4.2

Note: the CE values are correlated with the IMS drift times of peptide precursors (UDMS<sup>E</sup> acquisition)

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## Results and Discussion

The HCP impurities contained in the NIST mAb (100 mg/mL) have been previously characterized.<sup>2-6</sup> Three laboratories using the same experimental setup - two dimensional microflow chromatography (high pH RP/low pH RP), coupled to a SYNAPT G2-*Si* Mass Spectrometer, collectively identified 35 HCPs, among which 14 HCPs were in common. This approach reached a detection limit of 1 ppm.<sup>2</sup>

In recent years, several alternative sample preparation protocols have been introduced in order to increase the sensitivity of the LC-MS assay for identifying even lower levels of HCPs in mAb samples. These protocols either rely on the removal of the major drug species through mAb precipitation,<sup>4</sup> or use low molecular-weight filters to remove the mAb and concentrate the HCPs<sup>5</sup> or employ HILIC fractionation at the protein level to remove the major sample component and enrich the HCPs.<sup>6</sup> Here we adapted a protocol introduced by Eli Lilly and Company<sup>4</sup> to precipitate the NIST mAb, thus greatly minimizing the ion suppression and interference phenomena imposed by the more abundant NIST mAb peptides during the LC-MS analysis. Unlike the traditional digestion protocols using protein denaturation, reduction and alkylation before tryptic digestion, this approach relies on mAb resistance to trypsin digestion while in its native conformation. For this reason, HCPs non-covalently attached to the mAb are more easily digested by trypsin compared to the mAb, which stays mostly intact even during prolonged (overnight) digestion. According to this protocol, the sample is denatured, reduced, and alkylated after tryptic digestion, in order to precipitate and remove through centrifugation the major sample component. Our digestion protocol (detailed in the Experimental section) can be applied to other mAb products.

The digested sample was analyzed in triplicate using both MS<sup>E</sup> and HDMS<sup>E</sup> acquisition modes and the datasets

were processed in Progenesis QI for Proteomics v4.2 for HCP identification and quantification. Only the HCPs identified in all three LC-MS replicates with 3 or more peptides per protein were reported. The MS<sup>E</sup> acquisition was able to identify only 5 of the most abundant HCPs, reaching a detection limit of about 10 ppm (data not presented). In contrast with MS<sup>E</sup>, the HDMS<sup>E</sup> acquisition was able to identify significantly more HCPs - forty eight (48), improving the detection limit by 100 fold and reaching a detection level of 100 ppb. A detailed list of all the HCPs identified in all three replicates across 3 orders of magnitude is presented in Table 1. Compared to MS<sup>E</sup>, the sensitivity of HDMS<sup>E</sup> is greatly enhanced because in this acquisition mode the coeluting peptide precursors are separated by very fast (15-20 millisecond) ion mobility separations before fragmentation. As a result, the SYNAPT XS Mass Spectrometer provides cleaner MS/MS fragmentation spectra enabling many more HCP identifications. In addition, the collision energy (CE) used for peptide fragmentation is further optimized in the HDMS<sup>E</sup> mode, by synchronizing the applied CE on the transfer cell with the IMS drift time of peptide precursors.



No crt	Accession number	Protein description	Sequence coverage (%)	Average MW (kDa)	Amount on column		Concentration		RSD (%)
					fmoles	ng	ng/mL	ppm	
1	P00924	Enolase 1 yeast (ENL) - 5000 fmoles	51.4	46.6	3747	175	27938	279	9.6
2	P63284	Chaperone protein ClpB (CLP) - 2000 fmoles	11.2	95.6	1617	155	24734	247	5.3
3	P05064	Fructose-biphosphate aldolase A	55.5	39.3	2675	105	16820	168	4.2
4	P00489	Glycogen phosphorylase rabbit (PHO) - 1000 fmoles	43.2	97.1	1000	97	15536	155	0.0
5	P05063	Fructose-biphosphate aldolase C	42.4	39.4	1416	56	8926	89	7.3
6	P00330	Alcohol dehydrogenase yeast (ADH) - 1000 fmoles	39.2	36.7	1028	38	6036	60	3.3
7	Q922R8	Protein disulfide-isomerase A6	8.8	48.1	413	20	3178	32	16.5
8	P02769	Bovine serum albumin (BSA) - 250 fmoles	31.3	66.3	232	15	2461	25	0.4
9	P17225	Polypyrimidine tract-binding protein 1	11.4	56.5	208	12	1880	19	1.0
10	P06745	Glucose-6-phosphate isomerase	22.4	62.7	164	10	1645	16	2.2
11	P40142	Transketolase	7.2	67.6	126	9	1363	14	2.1
12	Q61937	Nucleophosmin	18.3	32.6	247	8	1288	13	2.1
13	Q60864	Stress-induced-phosphoprotein 1	11.7	62.5	83	5	830	8	4.8
14	Q9Z0X1	Apoptosis-inducing factor 1	5.1	66.8	75	5	802	8	4.6
15	Q9CZY3	Ubiquitin-conjugating enzyme E2 variant 1	15.5	16.3	296	5	772	8	0.8
16	P08101	Low affinity immunoglobulin gamma Fc region receptor	19.7	36.7	121	4	711	7	14.7
17	Q8K4F5	Alpha/beta hydrolase domain-containing protein 11	9.7	33.4	129	4	689	7	6.2
18	Q91YR9	Prostaglandin reductase 1	8.1	35.5	109	4	619	6	7.1
19	Q9CZ44	NSFL1 cofactor p47	8.3	40.7	76	3	495	5	16.6
20	Q8C7U7	Polypeptide N-acetylgalactosaminyltransferase 6	6.6	71.5	40	3	458	5	10.2
21	Q6PB93	Polypeptide N-acetylgalactosaminyltransferase 2	3.9	64.5	44	3	454	5	2.3
22	Q3UEB3	Poly(U)-binding-splicing factor PUF60	6.2	60.2	44	3	424	4	8.5
23	P17892	Pancreatic lipase-related protein 2	4.9	52.6	46	2	387	4	1.2
24	Q9WVA4	Transgelin-2	6.7	22.4	104	2	373	4	3.8
25	Q923D2	Flavin reductase (NADPH)	11.5	22.2	104	2	369	4	5.1
26	P60335	Poly(rC)-binding protein 1	6.3	37.5	60	2	360	4	9.8
27	O88569	Heterogeneous nuclear ribonucleoproteins A2/B1	5.6	37.4	56	2	335	3	20.1
28	P01887	Beta-2-microglobulin	18.3	13.8	145	2	320	3	15.7
29	P49312	Heterogeneous nuclear ribonucleoprotein A1	9.5	34.2	58	2	317	3	3.0
30	P26928	Hepatocyte growth factor-like protein	2.3	80.6	21	2	271	3	22.3
31	Q9CQF3	Cleavage and polyadenylation specificity factor subunit 5	4.4	26.2	64	2	268	3	5.5
32	Q9CQE1	Protein NipSnap homolog 3B	5.2	28.3	49	1	222	2	3.1
33	Q8BL97	Serine/arginine-rich splicing factor 7	5.2	30.8	44	1	217	2	20.2
34	Q9WTP6	Adenylate kinase 2 mitochondrial	7.8	26.5	41	1	174	2	2.8
35	P33174	Chromosome-associated kinesin KIF4	0.6	139.5	7	1	156	2	14.3
36	Q9ER00	Syntaxin-12	3.4	31.2	27	1	135	1	19.2
37	Q9DBP5	UMP-CMP kinase	2.9	22.2	34	1	121	1	7.8
38	P35700	Peroxiredoxin-1	4.5	22.2	33	1	117	1	19.3
39	P45878	Peptidyl-prolyl cis-trans isomerase FKBP2	4.6	15.3	42	1	103	1	2.4
40	P10126	Elongation factor 1-alpha 1	3.4	50.1	11	1	88	0.9	5.4
41	Q01768	Nucleoside diphosphate kinase B	3.6	17.4	31	1	86	0.9	6.8
42	P57722	Poly(rC)-binding protein 3	3.2	39.3	12	0.5	75	0.8	8.3
43	P02798	Metallothionein-2	1.5	6.2	51	0.3	51	0.5	3.0
44	P99029	Peroxiredoxin-5	6.7	21.8	14	0.3	49	0.5	7.1
45	P56812	Programmed cell death protein 5	2.7	14.3	18	0.3	41	0.4	5.6
46	Q9D7P6	Iron-sulfur cluster assembly enzyme ISCU	2.5	18.1	14	0.3	41	0.4	7.1
47	Q35166	Golgi SNAP receptor complex member 2	4.1	24.7	10	0.2	40	0.4	10.0
48	P07724	Serum albumin mouse	5.3	68.7	3	0.2	33	0.3	21.7
49	P15532	Nucleoside diphosphate kinase A	3.3	17.2	9	0.2	25	0.2	11.1
50	Q9JIX0	Transcription and mRNA export factor ENY2	2.2	11.5	11	0.1	20	0.2	10.8
51	Q9CR16	Peptidyl-prolyl cis-trans isomerase D	1.5	40.7	3	0.1	20	0.2	17.3
52	Q9WV55	Vesicle-associated membrane protein-associated protein A	3.3	27.9	4	0.1	18	0.2	13.3
53	P62843	40S ribosomal protein S15	1.8	17.0	3	0.1	8	0.1	21.7

Table 1. Complete list of HCPs identified and quantified in the NIST mAb using the HDMS<sup>E</sup> assay developed on a

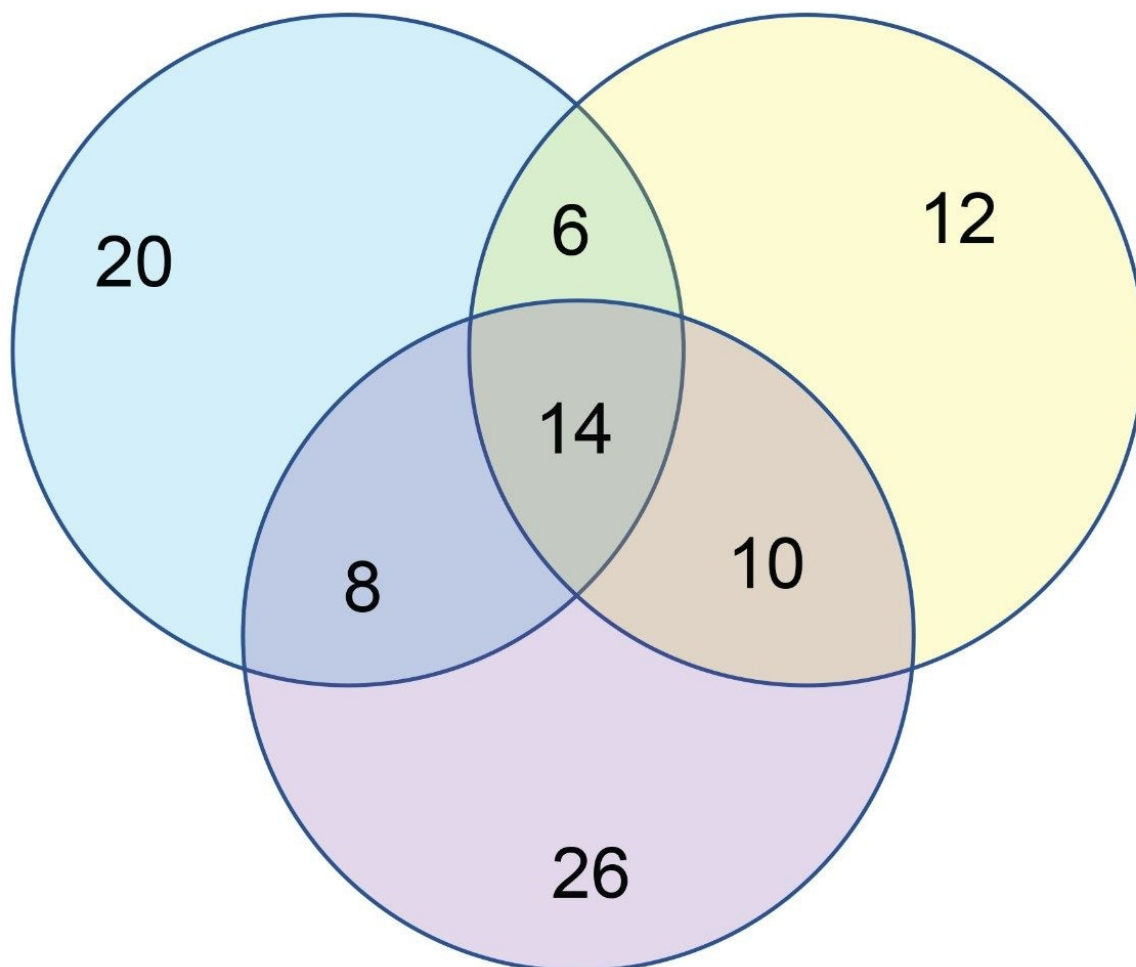
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*SYNAPT XS Mass Spectrometer. Five spiked proteins (ENL, ClpB, ADH, PHO, and BSA) along with 48 HCPs were identified in all three replicate injections. Fourteen HCPs were identified at concentrations below 1 ppm, with the detection limit of the assay being at 100 ppb.*

The list of HCPs identified in the HDMS<sup>E</sup> experiment was compared against the HCP identification reported in two previous publications<sup>4,6</sup> and the results are summarized in the Venn diagram shown in Figure 2. All three datasets contain a subset of 14 HCPs which was initially reported by our group.<sup>2</sup> In addition, each of the three datasets contains a unique subset of HCPs, identified only by a single laboratory: in our case 20 HCPs were only identified by the HDMS<sup>E</sup> acquisition on SYNAPT XS. Fourteen out of these 20 HCPs (highlighted in yellow in Table 1) were measured to have concentrations in the sub-ppm range (100–1000 ppb) demonstrating the excellent sensitivity of this assay. Clearly, while it is easier to identify the higher abundance HCPs contained in the NIST mAb sample, it appears that each different sample preparation procedure renders some unique very low level HCPs. This observation suggests the need to try different sample preparation methods in order to acquire a comprehensive HCP profile for this challenging mAb sample.

HDMS<sup>E</sup> dataset

Ref 3 dataset



Ref 5 dataset

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Figure 2. Venn diagram showing a comparison between the HCPs identified in the current study against the NIST mAb HCPs previously reported in the literature.

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Conclusion

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- A highly sensitive HCP discovery assay using HDMS<sup>E</sup> data independent acquisition can identify very low levels of HCPs (sub-ppm) from a highly purified monoclonal antibody
- Forty eight (48) HCPs were identified from the NIST mAb, considerably improving our previous results reporting only 14 proteins identifications

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## References

1. Hogwood BD, Smales MC. Measurement and Control of Host Cell Proteins (HCPs) in CHO Cell Bioprocesses, *Curr Opin Biotechnol*, 2014, 30, 153–160.
2. Doneanu CE, Anderson M, Williams BJ, Lauber MA, Chakraborty A, Chen W. Enhanced Detection of Low-Abundance Host-Cell Protein Impurities in High-Purity Monoclonal Antibodies Down to 1 ppm Using Ion Mobility Mass Spectrometry Coupled with Multidimensional Liquid Chromatography, *Anal Chem*, 2015, 87, 10283–10291.
3. Weibin C, Doneanu CE, Lauber MA, Koza S, Prakash K, Stapels M, Fountain KJ. Improved Identification and Quantification of Host Cell Proteins (HCPs) in Biotherapeutics Using Liquid Chromatography-Mass Spectrometry, book chapter in *Technologies for Therapeutic Monoclonal Antibody Characterization, Vol 3, ACS Symposium Series*, 2015, 357–393.
4. Huang L, Wang N, Mitchell CE, Brownlee T, Maple SR, De Felippis MR. A Novel Sample Preparation for Shotgun Proteomics Characterization of HCPs in Antibodies, *Anal Chem*, 2017, 89, 5436–5444.
5. Chen IH, Xiao H, Daly T, Ning L. Improved Host Cell Protein Analysis in Monoclonal Antibody Products through Molecular Weight Cutoff Enrichment, *Anal Chem*, 2020, 92, 3751–3757.
6. Wang Q, Slaney TR, Wu W, Ludwig R, Tao L, Leone A. Enhancing Host-Cell Protein Detection in Protein Therapeutics Using HILIC Enrichment and Proteomic, *Anal Chem*, 2020, 92, 10327–10335.

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