

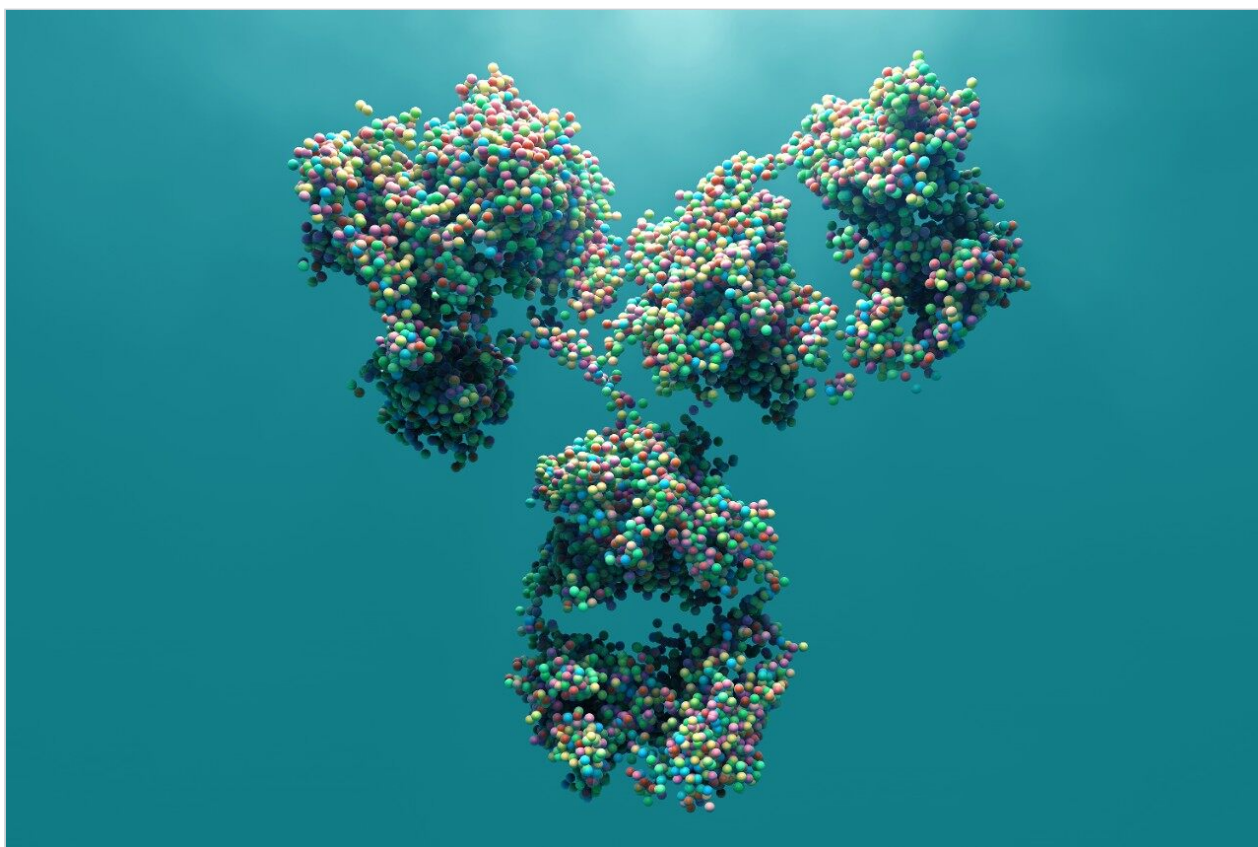
Application Note

## Identification of Host Cell Proteins (HPCs) 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

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.



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

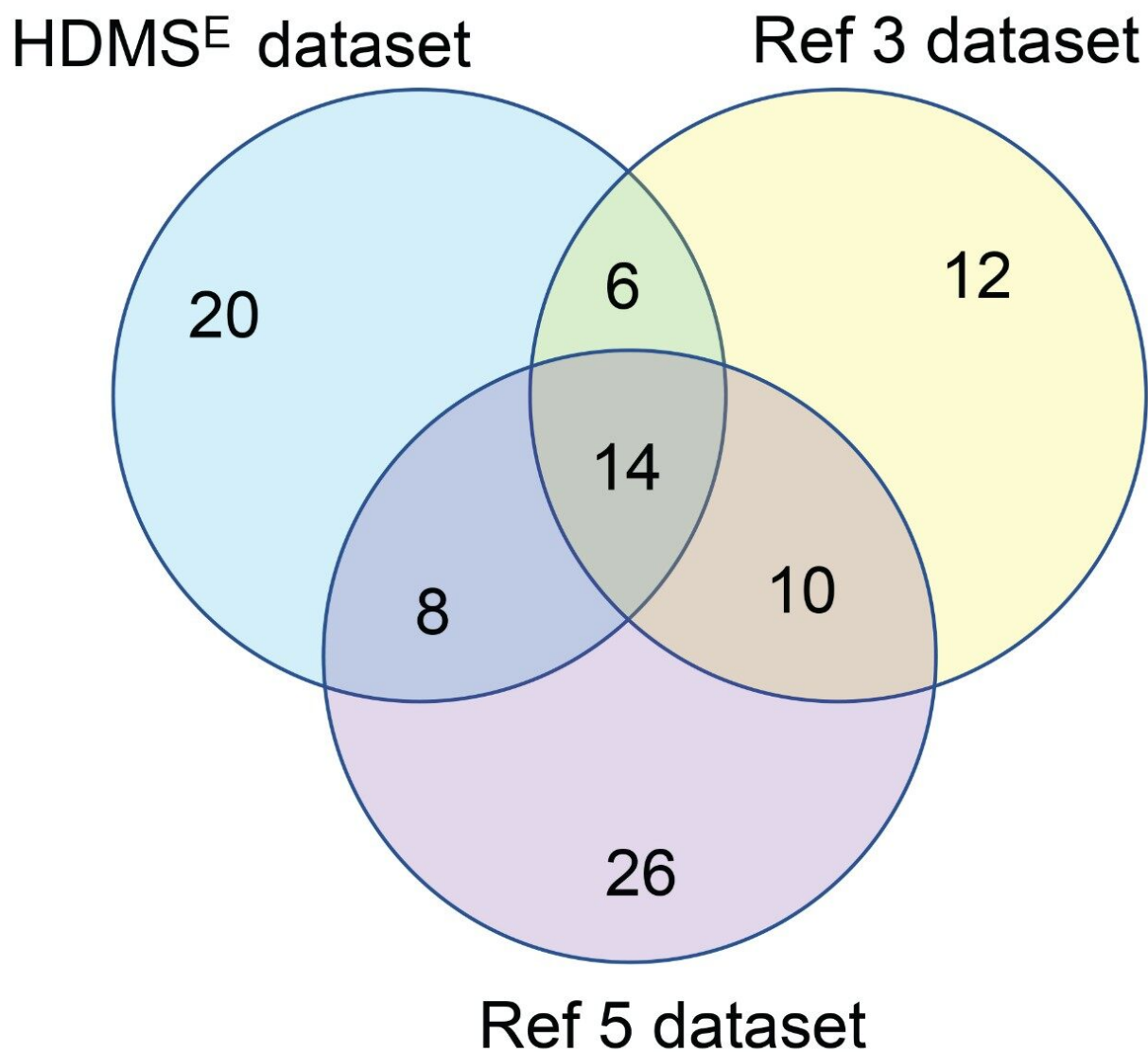
with the IMS drift time of peptide precursors.

No crt	Accession number	Protein description	Sequence coverage (%)	Average MW (kDa)	Amount on column		Concentration		RSD (%)
					fmoles	ng	ng/mL	ppm	
1	P00924	<i>Enolase 1 yeast (ENL) - 5000 fmoles</i>	51.4	46.6	3747	175	27938	279	9.6
2	P63284	<i>Chaperone protein ClpB (CLP) - 2000 fmoles</i>	11.2	95.6	1617	155	24734	247	5.3
3	P05064	<i>Fructose-biphosphate aldolase A</i>	55.5	39.3	2675	105	16820	168	4.2
4	P00489	<i>Glycogen phosphorylase rabbit (PHO) - 1000 fmoles</i>	43.2	97.1	1000	97	15536	155	0.0
5	P05063	<i>Fructose-biphosphate aldolase C</i>	42.4	39.4	1416	56	8926	89	7.3
6	P00330	<i>Alcohol dehydrogenase yeast (ADH) - 1000 fmoles</i>	39.2	36.7	1028	38	6036	60	3.3
7	Q922R8	<i>Protein disulfide-isomerase A6</i>	8.8	48.1	413	20	3178	32	16.5
8	P02769	<i>Bovine serum albumin (BSA) - 250 fmoles</i>	31.3	66.3	232	15	2461	25	0.4
9	P17225	<i>Polypyrimidine tract-binding protein 1</i>	11.4	56.5	208	12	1880	19	1.0
10	P06745	<i>Glucose-6-phosphate isomerase</i>	22.4	62.7	164	10	1645	16	2.2
11	P40142	<i>Transketolase</i>	7.2	67.6	126	9	1363	14	2.1
12	Q61937	<i>Nucleophosmin</i>	18.3	32.6	247	8	1288	13	2.1
13	Q60864	<i>Stress-induced-phosphoprotein 1</i>	11.7	62.5	83	5	830	8	4.8
14	Q9Z0X1	<i>Apoptosis-inducing factor 1</i>	5.1	66.8	75	5	802	8	4.6
15	Q9CZY3	<i>Ubiquitin-conjugating enzyme E2 variant 1</i>	15.5	16.3	296	5	772	8	0.8
16	P08101	<i>Low affinity immunoglobulin gamma Fc region receptor</i>	19.7	36.7	121	4	711	7	14.7
17	Q8K4F5	<i>Alpha/beta hydrolase domain-containing protein 11</i>	9.7	33.4	129	4	689	7	6.2
18	Q91YR9	<i>Prostaglandin reductase 1</i>	8.1	35.5	109	4	619	6	7.1
19	Q9CZ44	<i>NSFL1 cofactor p47</i>	8.3	40.7	76	3	495	5	16.6
20	Q8C7U7	<i>Polypeptide N-acetylgalactosaminyltransferase 6</i>	6.6	71.5	40	3	458	5	10.2
21	Q6PB93	<i>Polypeptide N-acetylgalactosaminyltransferase 2</i>	3.9	64.5	44	3	454	5	2.3
22	Q3UEB3	<i>Poly(U)-binding-splicing factor PUF60</i>	6.2	60.2	44	3	424	4	8.5
23	P17892	<i>Pancreatic lipase-related protein 2</i>	4.9	52.6	46	2	387	4	1.2
24	Q9WVA4	<i>Transgelin-2</i>	6.7	22.4	104	2	373	4	3.8
25	Q923D2	<i>Flavin reductase (NADPH)</i>	11.5	22.2	104	2	369	4	5.1
26	P60335	<i>Poly(rC)-binding protein 1</i>	6.3	37.5	60	2	360	4	9.8
27	O88569	<i>Heterogeneous nuclear ribonucleoproteins A2/B1</i>	5.6	37.4	56	2	335	3	20.1
28	P01887	<i>Beta-2-microglobulin</i>	18.3	13.8	145	2	320	3	15.7
29	P49312	<i>Heterogeneous nuclear ribonucleoprotein A1</i>	9.5	34.2	58	2	317	3	3.0
30	P26928	<i>Hepatocyte growth factor-like protein</i>	2.3	80.6	21	2	271	3	22.3
31	Q9CQF3	<i>Cleavage and polyadenylation specificity factor subunit 5</i>	4.4	26.2	64	2	268	3	5.5
32	Q9CQE1	<i>Protein NipSnap homolog 3B</i>	5.2	28.3	49	1	222	2	3.1
33	Q8BL97	<i>Serine/arginine-rich splicing factor 7</i>	5.2	30.8	44	1	217	2	20.2
34	Q9WTP6	<i>Adenylate kinase 2 mitochondrial</i>	7.8	26.5	41	1	174	2	2.8
35	P33174	<i>Chromosome-associated kinesin KIF4</i>	0.6	139.5	7	1	156	2	14.3
36	Q9ER00	<i>Syntaxin-12</i>	3.4	31.2	27	1	135	1	19.2
37	Q9DBP5	<i>UMP-CMP kinase</i>	2.9	22.2	34	1	121	1	7.8
38	P35700	<i>Peroxiredoxin-1</i>	4.5	22.2	33	1	117	1	19.3
39	P45878	<i>Peptidyl-prolyl cis-trans isomerase FKBP2</i>	4.6	15.3	42	1	103	1	2.4
40	P10126	<i>Elongation factor 1-alpha 1</i>	3.4	50.1	11	1	88	0.9	5.4
41	Q01768	<i>Nucleoside diphosphate kinase B</i>	3.6	17.4	31	1	86	0.9	6.8
42	P57722	<i>Poly(rC)-binding protein 3</i>	3.2	39.3	12	0.5	75	0.8	8.3
43	P02798	<i>Metallothionein-2</i>	1.5	6.2	51	0.3	51	0.5	3.0
44	P99029	<i>Peroxiredoxin-5</i>	6.7	21.8	14	0.3	49	0.5	7.1
45	P56812	<i>Programmed cell death protein 5</i>	2.7	14.3	18	0.3	41	0.4	5.6
46	Q9D7P6	<i>Iron-sulfur cluster assembly enzyme ISCU</i>	2.5	18.1	14	0.3	41	0.4	7.1
47	O35166	<i>Golgi SNAP receptor complex member 2</i>	4.1	24.7	10	0.2	40	0.4	10.0
48	P07724	<i>Serum albumin mouse</i>	5.3	68.7	3	0.2	33	0.3	21.7
49	P15532	<i>Nucleoside diphosphate kinase A</i>	3.3	17.2	9	0.2	25	0.2	11.1
50	Q9JIX0	<i>Transcription and mRNA export factor ENY2</i>	2.2	11.5	11	0.1	20	0.2	10.8
51	Q9CR16	<i>Peptidyl-prolyl cis-trans isomerase D</i>	1.5	40.7	3	0.1	20	0.2	17.3
52	Q9WV55	<i>Vesicle-associated membrane protein-associated protein A</i>	3.3	27.9	4	0.1	18	0.2	13.3
53	P62843	<i>40S ribosomal protein S15</i>	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 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.



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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

- 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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720007101, January 2021

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