thermoscientific

Evaluation of factors affecting detection of host cell proteins in biotherapeutic proteins using an Orbitrap Fusion Lumos Tribrid mass spectrometer.

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INTRODUCTION

The detection of host cell proteins (HCPs) in biotherapeutic proteins is an important analytical requirement because HCPs can present potential safety risks or impact product stability. HCP analysis is a challenging workflow because an extensive dynamic range is required to be able to detect low ppm concentrations of residual HCPs. Also, to be widely adopted by the Biopharmaceutical industry, the analytical solution has to be extremely robust and relatively fast.

MATERIALS AND METHODS

Sample Preparation

Tryptic Smart Digestion: NIST mAb (50 ul at 10 ug/uL) was digested with the Thermo Scientific™ SMART Digest™ trypsin kit. The sample was diluted in 150 uL of the SMART Digest buffer and transferred in the digestion tube containing the SMART Digest trypsin slurry. The sample was incubated at 70C for 90 min and 1400 rpm shaking. After centrifugation, the supernatant was transferred to a new tube and the centrifugation step was repeated two times. Finally, the sample was reduced with DDT at a final concentration of 12 mM for 40 min at 40C.

Liquid Chromatography

LC: Thermo Scientific™ Vanquish™ UHPLC

Columns: Three Thermo Scientific™ Acclaim™ columns (C18, 250mm * 2.1 mm; 2.2 um) were connected together.

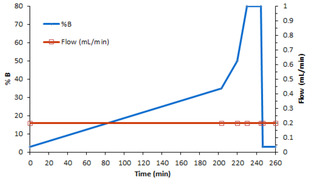
Mobile phase:

Buffer A: 0.1% Formic acid, in H_2O

Buffer B: 0.1% Formic acid, in CH_3CN

LC gradient

time	%B	Flow (mL/min)
0	3	0.2
203	35	0.2
220	50	0.2
230	80	0.2
245	80	0.2
247	3	0.2
260	3	0.2



Mass Spectrometry

Mass spectrometer: Thermo Scientific™ Orbitrap™ Fusion Lumos™ mass spectrometer

Data were acquired in data dependent acquisition (DDA) mode with HCD fragmentation and an isolation window of 1.2 Da,

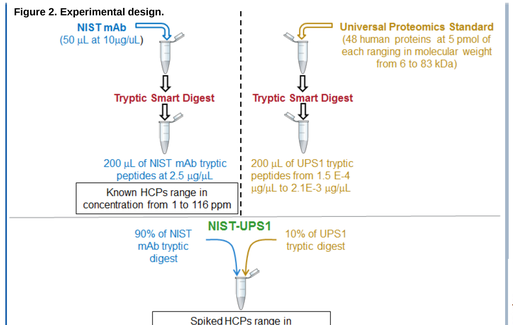
TopS (2s) DDA method	
MS1 OT res: 120K at 200 m/z	MS2 OT res: 30K at 200 m/z
AGC MS1: 4E5	AGC MS2: 1E5
MS1 Max Inject time: 200 ms	MS2 Max Inject time: 250 ms
m/z: 300-1500	Min Intensity threshold 5E4

Data Analysis

Spectral .raw files from data dependent acquisition were analyzed using Thermo Scientific™ Proteome Discoverer™ 2.1 software with SEQUEST® HT search. Data was searched against mouse UniProt combined with NIST mAb and UPS proteins at a 1% spectrum level FDR. MS1 and MS2 mass tolerances were respectively 10 ppm and 0.02 Da. Deamidation of Asparagine (+0.984) and/or oxidation of Methionine (+15.995) were considered as variable modifications.

Figure 1. Data were collected using a Vanquish UHPLC (a) coupled to a Fusion mass spectrometer (b) and processed with Proteome Discoverer software (c). The 3 columns (d) fit in the Vanquish UHPLC column compartment

Figure 2. Experimental design.



For this study three different types of sample were generated. The universal proteomics standard (UPS) was first digested with the SMART Digest trypsin kit. The same digestion protocol was used for the NIST mAb and the UPS sample. The digested UPS was spiked in the digested NIST mAb (NIST-UPS1) to test the effect of sample loading on HCPs identification. Also, a small amount of the digested UPS was analyzed by itself to verify the digestion and define the maximum number of UPS1 proteins that could be identified in the NIST-UPS1 sample. Finally the digested NIST mAb was analyzed to identify HCPs present in the NIST mAb.

RESULTS

A) UPS1 sample.

table 1. UPS1 proteins identified after tryptic digestion and loading 0.25 pmol on-column.

#	Accession	Description	# PSMs	# Unique	MW [kDa]
1	O00762ups	Ubiquitinconjugating enzyme E2 C (Chain 1179, Nterminal His tag) (Human)	3	2	20.5
2	O76070ups	Gammasynuclein (Chain 1127) (Human)	25	10	15.5
3	P00441ups	Superoxide dismutase [CuZn] (Chain 2154) (Human)	9	4	15.8
4	P00709ups	Alphalactalbumin (Chain 20142) (Human)	1	1	14.1
5	P00915ups	Carbonic anhydrase 1 (Chain 2261) (Human)	7	5	28.7
6	P00918ups	Carbonic anhydrase 2 (Chain 2260) (Human)	2	2	29.1
7	P01008ups	AntithrombinIII (Chain 33464) (Human)	5	4	49
8	P01031ups	Complement C5 (C5a anaphylatoxin) (Chain 678751) (Human)	9	5	8.3
9	P01112ups	GTPase HRas (Chain 1189) (Human)	1	1	21.3
10	P01127ups	Plateletderived growth factor B chain (Chain 82190) (Human)	22	8	12.3
11	P01133ups	ProEpidermal growth factor (EGF) (Chain 9711023) (Human)	1	1	6.3
12	P01344ups	Insulinlike growth factor II (Chain 2591) (Human)	9	3	7.5
13	P01375ups	Tumor necrosis factor, soluble form (Chain 77233) (Human)	1	1	17.3
14	P01579ups	Interferon Gamma (Chain 23166) (Human)	9	4	16.8
15	P02144ups	Myoglobin (Chain 2154) (Human)	7	3	17
16	P02753ups	Retinolbinding protein 4 (Chain 19201) (Human)	4	3	20.6
17	P02768ups	Serum albumin (Chain 26609) (Human)	11	7	66.3
18	P02787ups	Serotransferrin (Chain 20698) (Human)	19	15	75.1
19	P02788ups	Lactotransferrin (Chain 20710) (Human)	24	15	76.1
20	P04040ups	Catalase (Chain 2527) (Human)	7	6	59.6
21	P05413ups	Fatty acidbinding protein, heart (Chain 2133) (Human)	20	8	14.7
22	P06396ups	Gelsolin (Chain 28782) (Human)	5	5	82.9
23	P06732ups	Creatine kinase Mtype (Chain 1381) (Human)	7	6	43.1
24	P08263ups	Glutathione Stransferase A1 (Chain 2222) (Human)	7	4	25.5
25	P08758ups	Annexin A5 (Chain 2320) (Human)	1	1	35.8
26	P10145ups	Interleukin8, IL8 (Chain 2899) (Human)	11	4	8.4
27	P10599ups	Thioredoxin (Chain 2105, Nterminal His tag) (Human)	1	1	12.4
28	P10636-8ups	Microtubuleassociated protein tau (Isoform TauF (Tau4)) (Chain 2441) (Human)	40	14	45.7
29	P41159ups	Leptin (Chain 22167) (Human)	5	2	16.1
30	P61626ups	Lysozyme C (Chain 19148) (Human)	7	2	14.7
31	P61769ups	Beta2microglobulin (Chain 21119) (Human)	6	2	11.7
32	P62937ups	Peptidylprolyl cistrans isomerase A (Chain 1165, N terminal His tag) (Human)	6	3	20.2
33	P62988ups	Ubiquitin (Chain 176, Nterminal His tag) (Human)	15	4	10.7
34	P63165ups	Small ubiquitinrelated modifier 1 (Chain 197, Nterminal GST tag) (Human)	1	1	38.8
35	P63279ups	SUMOconjugating enzyme UBC9 (Chain 1158) (Human)	1	1	18
36	P68871ups	Hemoglobin subunit beta (Chain 2147) (Human)	4	2	15.9
37	P69905ups	Hemoglobin subunit alpha (Chain 2142) (Human)	1	1	15.1
38	P99999ups	Cytochrome c (Chain 2105) (Human)	9	5	11.6
39	Q06830ups	Peroxiredoxin 1 (Chain 2199) (Human)	6	4	22
		total	329	170	

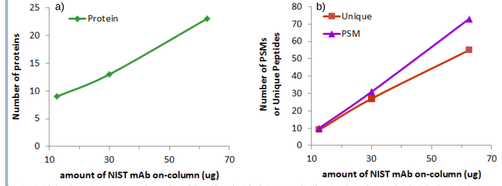
A single raw file of the UPS sample was searched using Proteome Discoverer against the mouse UniProt database combined with NIST mAb and UPS protein sequences. Only 0.25 pmol was loaded on 2.1 mm columns and 329 PSMs and 170 unique peptides were identified corresponding to 39 out of 48 UPS proteins. Nine proteins were identified with only one unique peptide (1% FDR).

B) NIST - UPS1 sample.

Table 2. Number of identified UPS proteins, unique peptides, peptides spectrum matched (PSM) and UPS proteins identified with only one peptide for different on column loading of NIST mAb

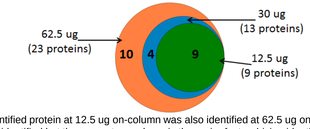
UPS1 proteins spiked in NIST mAb					
Volume Injected (uL)	amount of NIST mAb on-column (ug)	# Protein	# Unique	# PSMs	Only one unique peptide per protein
25	62.5	23	55	73	10
12	30	13	27	31	7
5	12.5	9	9	10	9

Figure 3. Number of proteins (a), unique peptides and peptide spectrum matched (b) versus the amount of NIST mAb on-column



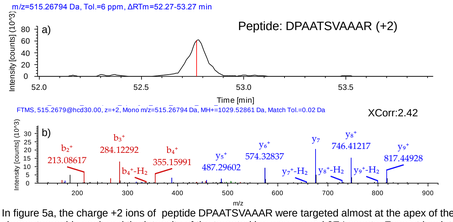
One of the key parameters for HCPs identification is how much digested mAb can be loaded on column without losing chromatographic resolution. In this study, we connected three 25 cm columns together which allowed us to load more than 60 ug of digested mAb per run. The increase of digested mAb amount on column from 12.5 ug to 62.5 ug increased the number of identified UPS proteins spiked in the digested NIST mAb from 9 to 23. Intrinsically, the dynamic range in concentration is identical for the three different NIST mAb loads but higher loadings allowed more spiked proteins to be above the limit of detection which results in the identification of more UPS proteins.

Figure 4. Venn diagram of the identified UPS1 proteins for different on-column loadings.



Every identified protein at 12.5 ug on-column was also identified at 62.5 ug on-column. Proteins are not randomly identified but the amount on-column is the major factor driving identification.

Figure 5. Extracted ion chromatogram (a) and annotated MS2 spectra (b) for the peptide DPAATSVAAAR of Ubiquitinconjugating enzyme E2 (O00762up).



In figure 5a, the charge +2 ions of peptide DPAATSVAAAR were targeted almost at the apex of the chromatographic peak and the intensity of the targeted ion was around 6E4 counts. Even at low intensity, the MS/MS spectrum (5b) recorded in the Orbitrap was identified with a high confidence peptide score.

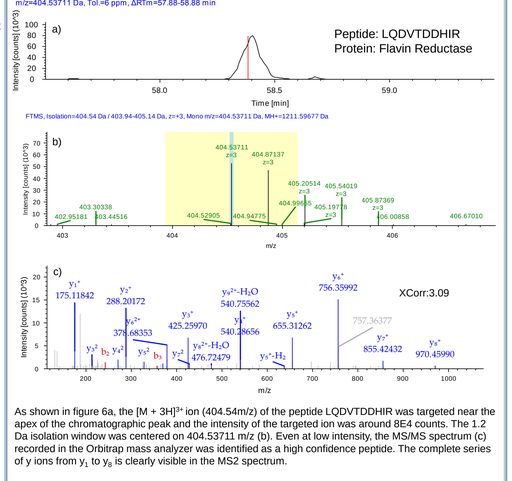
C) NIST sample.

Table 3. Identified published NIST HCPs, unique peptides, peptides spectrum matched and NIST HCPs identified with only one peptide for 62.5 ug of NIST mAb on-column.

Accession	Description	# Peptides	# PSMs	# Unique Peptides	MW [kDa]	HCP concentration (ppm) from published study [1]
P05064	Fructose-bisphosphate aldolase A	27	72	21	45.1	116
P05063	Fructose-bisphosphate aldolase C	17	33	11	39.4	97
P08101	Low affinity immunoglobulin gamma Fc region receptor II	2	5	2	36.7	14
Q60864	Stress-induced-phosphoprotein	1	1	1	62.5	13
P06745	Glucose-6-phosphate isomerase	16	27	16	62.7	12
P01887	Beta-2-microglobulin	1	1	1	13.8	7
Q9CZ44	NSFL1 cofactor p47	3	3	3	40.7	7
Q8BL97	Serine/arginine-rich splicing factor	1	1	1	30.8	5
Q9WTP6	Adenylate kinase 2, mitochondrial	1	1	1	26.5	4
Q91YR9	Prostaglandin reductase 1	3	3	3	35.5	3
Q923D2	Flavin reductase (NADPH)	1	1	1	26.6	2

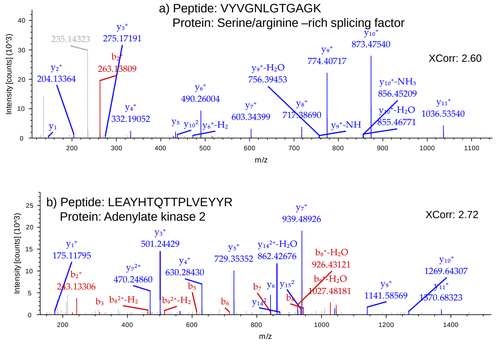
In a published inter-laboratory study on NIST HCPs[1] using a 2 dimensional LC system and loading on-column more than 600 ug of NIST mAb tryptic digest, 14 HCPs were identified by all 3 laboratories. The average concentration of the HCPs between the 3 laboratories ranged from 1 to 166 ppm. In this study, 11 of the 14 known HCPs of the NIST mAb were also identified on a 1D LC system by loading on column 62.5 ug of the NIST mAb. The reported concentration of Flavin reductase was 2 ppm and one peptide from this protein sequence was identified with high confidence (see figure 6). The identification of NIST HCPs at low ppm concentration suggest that a workflow combining the quick and easy SMART Digest protocol with the coupling of 3 x 25 cm 2.1 mm columns on an Orbitrap Fusion Lumos instrument is suitable for HCPs analysis.

Figure 6. Extract ion chromatogram (a), MS1 (b) and annotated MS2 (c) spectra for the identified peptide of Flavin Reductase.



As shown in figure 6a, the [M + 3H]$^{3+}$ ion (404.54m/z) of the peptide LQDVTDDHIR was targeted near the apex of the chromatographic peak and the intensity of the targeted ion was around 8E4 counts. The 1.2 Da isolation window was centered on 404.53711 m/z (b). Even at low intensity, the MS/MS spectrum (c) recorded in the Orbitrap mass analyzer was identified as a high confidence peptide. The complete series of y ions from y_1 to y_8 is clearly visible in the MS2 spectrum.

Figure 7. MS2 spectra of the low concentration NIST HCPs identified with only one peptide.



The MS2 spectra of the lowest concentration NIST mAb HCPs identified with only one unique peptide are displayed in figures 6c, 7a and 7b. Even if the protein is identified with a single unique peptide, the outstanding sensitivity of the Orbitrap Fusion Lumos MS provided high quality MS2 spectra for very confident peptide identifications.

CONCLUSIONS

- The SMART Digest kit provides a fast and easy sample preparation method compatible with HCP analysis.
- Coupling three Acclaim columns (25 cm * 2.1mm ID) is suitable for loading high amounts of digested mAb which is required for extensive detection of low level HCPs .
- The high MS2 spectral quality provided by the Orbitrap Fusion Lumos Tribrid MS allowed the confident identification of HCPs at very low concentration.

REFERENCE

1. Anal. Chem., 2015, 87 (20), pp 10283–10291 (DOI: 10.1021/acs.analchem.5b02103).

TRADEMARKS/LICENSING