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Deep Identification and Quantitation of Unknown HCPs in NISTmAb Using an Optimized Sample Preparation Combined with NanoLC-MS

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ABSTRACT

Purpose: To develop and demonstrate a sensitive method for unknown HCPs identification and quantitation.

Methods: Optimizing sample preparation and using UPS2 internal standard to evaluate absolute amount of HCPs in NISTmAb on Thermo Scientific™ nanoLC Q Exactive™ Plus system.

Results: Optimized method improves the identification number of HCPs by 60% and saves more time.

INTRODUCTION

Host cell proteins (HCPs) are the predominant class of impurities during manufacturing of therapeutic proteins and can affect the safety and quality of protein drugs[1]. LC-MS/MS methods have successfully shown their ability to identify and quantify such HCPs. However, deep identification of HCPs may be limited by the interference of the antibody drug[2]. Here, a two-hour native digestion method was developed to achieve maximum coverage of HCPs in NIST humanized IgG1κ monoclonal antibody. At the same time, UPS2 was employed as an internal standard for relative quantitation of the HCPs.

MATERIALS AND METHODS

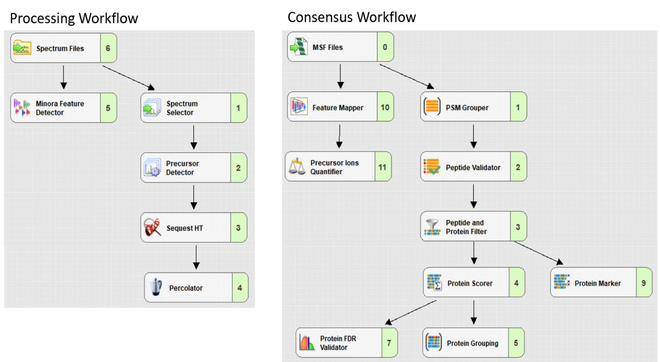
Sample Preparation

NISTmAb (PN#RM 8671) and UPS2 (PN#S5697) were used to evaluate the LOD of the method and quantitate individual HCP. 300 µg of antibody was diluted with purified water to a total volume of 190 µL. Then 10 µL of 1 M Tris/HCL (pH 8.0) and 0.424 µg of UPS2 were added to the sample. 1.5 µg of trypsin was mixed directly with the sample under nondenaturing condition. Preparations were incubated at 37^{0}C for 2h, then reduced with 3 µL of 500 mM DTT and heated to 95^{0}C for 10min. Undigested antibody was precipitated by 5 min centrifugation at 13000g. Supernatant was transferred into a new tube and acidified with 5 µL of 10% formic acid in water. One-third of the sample was injected for nanoLC-MS/MS analysis.

Data Analysis

HCPs were identified using Thermo Scientific™ Proteome Discover™ 2.4 Software. Swiss-Prot Mus musculus, NISTmAb heavy and light chain sequences, UPS2 proteins sequences and contaminant protein sequences were added in the PD2.4. 10 ppm and 0.02 Da were set for MS1 and fragment mass tolerance. Dynamic modifications included methionine oxidation and N-term modification of Met-loss and acetylation. Precursor detector, a new node in PD2.4, was used to search all chimeric MS2 spectrums that precursor masses co-isolated with the targeted precursor and consequently fragmented together. 1% FDR of PSMs and peptides was used to ensure high confidence of the identified HCPs. Unique and razor peptides were used to quantify proteins abundance depending on average abundance of top 3 peptides. Contaminant protein database from MaxQuant software was added in the Protein Marker node. Other parameters were set default.

Figure 1. Detailed nodes in processing and consensus workflow.

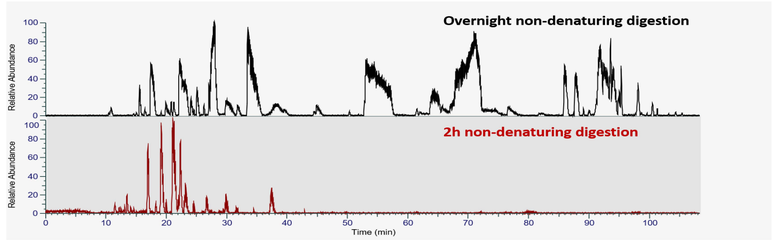


RESULTS

Comparison of sample preparation between two different non-denaturing digestion on Thermo Scientific™ nanoLC-MS system

We first followed Huang, L. et al. [2] non-denaturing method. 117 HCPs were identified based on two or more unique peptides in at least two out of three replicate injections, excluding contaminant proteins, NISTmAb and UPS2 proteins. The sequence coverage of NISTmAb heavy and light chain was up to 78% separately. From the chromatogram, high NISTmAb content and sample loading leaded to poor chromatographic separation. Digestion products of NISTmAb should be reduced to improve the likelihood of more HCPs identification. Digestion time and trypsin dosage were key factors. Finally, the combination of 2 hours digestion time and 1:200 (trypsin: antibody) ratios could achieve the best result. On the Thermo Scientific™ nanoLC Q Exactive Plus system, 189 HCPs were identified using same data processing. The sequence coverage of NISTmAb heavy and light chain was 33% and 46% separately. The interference from antibody was reduced obviously which lead to the number of HCPs identification increased and save sample preparation time (Figure 2).

Figure 2. Effect of different sample preparations on chromatographic separation. Equal amounts of NISTmAb digestion products using overnight and 2h non-denaturing digest conditions was injected on-column.



Evaluation of system sensitivity on the Thermo Scientific™ nanoLC-Q Exactive Plus platform

Proteomics dynamic range standard (UPS2) was used to evaluate the sensitivity of system and the absolute amount of individual HCP depending on the protein abundance. The absolute amount of proteins in added UPS2 ranged from 6.7 amol to 0.67 pmol in every injection. In our results, 23 proteins of UPS2 were identified ranging from 6.7 fmol to 0.67 pmol (as low as 0.98 ppm, 0.098 ng in 100 µg antibody) (Table 1). So the detection range of this system is at least from 6.7 fmol to 687 pmol (100 µg antibody in every injection), 6 orders of magnitude. The sensitivity of this system can satisfy the need of biopharmaceutical companies.

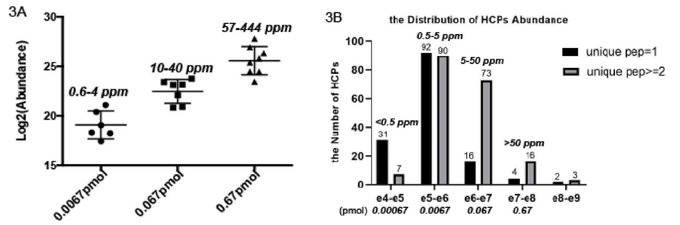
Table 1. The identification and absolute amount of UPS2 proteins. *represent that the protein was also endogenous expressed.

Accession	Absolute amount in 100 µg antibody (pmol)	MW (Da)	Absolute amount (ng)	Measured ppm	Protein average abundance
P00918ups*	0.67	29115	19.50	195.0	2.32E+08
P62988ups	0.67	10597	7.10	71.0	1.27E+08
P41159ups	0.67	16158	10.82	108.2	8.45E+07
P69905ups	0.67	15126	10.13	101.3	5.85E+07
P01031ups	0.67	8563	5.74	57.4	4.36E+07
P00915ups	0.67	28739	19.26	192.6	2.29E+07
P68871ups	0.67	15867	10.63	106.3	2.33E+07
P02768ups	0.67	66357	44.46	444.6	1.14E+07
Q06830ups	0.067	21979	1.47	14.7	1.41E+07
P15559ups*	0.067	30736	2.06	20.6	1.10E+07
P63165ups	0.067	38815	2.60	26.0	9.08E+06
P04040ups	0.067	59625	3.99	39.9	9.23E+06
P00167ups	0.067	16022	1.07	10.7	4.45E+06
P62937ups	0.067	20176	1.35	13.5	1.96E+06
P02144ups	0.067	17053	1.14	11.4	1.87E+06
P12081ups	0.0067	58233	0.39	3.9	2.24E+06
P16083ups	0.0067	25821	0.17	1.7	1.37E+06
P02753ups	0.0067	21071	0.14	1.4	3.04E+05
P06732ups	0.0067	43101	0.29	2.9	3.23E+05
Q15843ups	0.0067	9072	0.06	0.6	5.40E+05
P61626ups	0.0067	14701	0.098	0.98	1.76E+05
P01008ups	0.00067	49039	0.03	0.3	1.63E+04
P02787ups	0.000067	75181	0.005	0.05	2.51E+04

Individual HCP quantitation

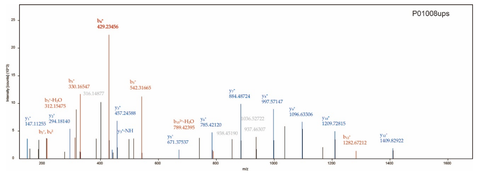
The relationship between absolute molar content and protein abundance of UPS2 had certain linear (Figure 3A). This can be used to evaluate the approximately absolute molar content or content for individual HCP. Figure 3B shows the evaluated quantitation results of HCPs in NISTmAb. For example, the abundance of 92 HCPs with only one unique peptide and 90 HCPs with more than one unique peptide were in the range of between e+05 and e+06, their absolute molars were about 0.0067 pmol and occupies about 0.5 to 5 ppm according to the UPS2 internal standard.

Figure 3. Individual HCP quantitation with USP2 as internal standard. (A) Display the relationship between protein abundance and absolute molar content of UPS2. (B) The distribution of HCPs abundance and approximate absolute content.



Another thing should be taken into consideration, there are many HCPs containing only one unique peptide and the abundance below e+05, however they still have high confident MS2 spectrum such as internal standard of P01008ups (Figure 4), so the HCP with one unique peptide should be also monitored in the commercial antibody product. To ensure high confidence of low abundant HCPs identification, it is recommended to manually check matched spectrum of each peptide.

Figure 4. Representative MS2 spectrum of very low abundant UPS2 containing one unique peptide.



CONCLUSIONS

- The optimized sample preparation method reduces the interference of the antibody and increases the identified number of HCPs.
- The system of Thermo Scientific™ nanoLC-Q Exactive Plus has enough sensitivity and dynamic range to identify HCPs as low as sub-ppm. This can enough satisfy the need of biopharmaceutical companies.

REFERENCES

1. Wang, X et al, Biotechnology and bioengineering (2009). Host cell proteins in biologics development: Identification, quantitation and risk assessment. Biotechnology and bioengineering.
2. Huang, L et al, Analytical chemistry (2017). A Novel Sample Preparation for Shotgun Proteomics Characterization of HCPs in Antibodies.

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TRADEMARKS/LICENSING

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