An improved EasyPep sample preparation method for enrichment and quantitation of host cell proteins

Jae Choi[1], Philip Remes[2] ,Oleg Silivra[2], Cristina C. Jacob[2], Terry Hicks[3], Matthew Daniels[3], Nikki Jarrett[1], Kay Opperman[1], Bhavin Patel[1]
[1]Thermo Fisher Scientific, Rockford, IL, [2]Thermo Fisher Scientific, San Jose, CA, [3]Thermo Fisher Scientific, St. Louis, MO

Abstract

Purpose: To explore the effectiveness of protein A depletion as a viable solution to address the wide dynamic range issue encountered in Liquid chromatography (LC) to mass spectrometry (MS) analysis of host cell proteins (HCPs).

Methods: We used magnetic protein A beads for antibody removal followed by semi-tryptic digestion to recover the HCPs bound to the beads or antibody. This modified Thermo Scientific™ EasyPep™ sample preparation method was compared to a native digest with and without protein A depletion.

Results: The protein A-depleted EasyPep sample preparation method exhibited superior performance compared to both the native digest method and the other two sample preparation methods. Specifically, the modified method showed higher numbers of HCP protein identification and quantification.

Introduction

HCPs, a diverse mixture of impurities in biotherapeutics, pose a challenge for biologic drug product safety. Monitoring and controlling these critical contaminant proteins during biologics drug production is vital. LC-MS/MS analysis, alongside ELISA, is increasingly used to detect and quantify HCP. However, the large dynamic range of the biologic drugs compared to HCPs protein levels presents an analytical challenge for HCP detection and quantitation.

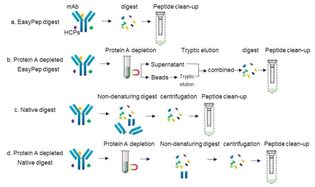
We have developed a modified EasyPep sample preparation method that uses protein A-based depletion and heavy-labeled peptide standards for the detection and quantitation of HCPs. One of the challenge with this approach is potential loss of HCPs during depletion via non-specific interactions with the biologic drug or depletion resin. To address this concern, we developed a “trypsin elution” protocol that uses trypsin to partially digest and recover HCPs from the depleted sample. In addition, we have developed a targeted MS assay to monitor 28 critical HCPs from CHO cell lines, helping maximize the acquisition efficiency of the MS platform. This novel approach can be used to monitor HCPs at each step of the downstream processes.

Materials and methods

Sample Preparation

We used the EasyPep™ MS sample prep kit (PN: A45734) to digest 1mg of downstream process intermediate (1st affinity-purified trastuzumab) or ultrafiltration and diafiltration (UF/DF) which is equivalent to drug substance, respectively. In addition, we used Pierce™ high-capacity protein A MagBeads alkali stable (PN: A53035) for antibody depletion before the EasyPep digest. We have also compared with native digestion with and without the protein A depletion.

Figure 1. Sample preparation workflows



LC-MS/MS analysis

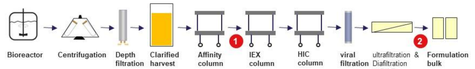
A CHO critical HCP AQUA peptide panel was developed for the quantification of 28 high-risk HCPs[1]. Two hundred fmol of internal standard (IS) peptide mixture was spiked into 0.5µg of digested sample. Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano System coupled to the Thermo Scientific™ Q Exactive plus™ Mass Spectrometer was used to acquire data in DDA or PRM mode. Raw files were analyzed using Thermo Scientific™ Proteome Discoverer™ (PD) 3.0 or Skyline software.

Results

Evaluation of different sample prep methods for HCP analysis

We utilized four distinct sample preparation workflows, as illustrated in Figure 1, to prepare four digest samples starting with 1mg of trastuzumab that had undergone affinity column purification, as shown below (❶) in Figure 2.

Figure 2. The manufacturing process of a monoclonal antibody drug



The data analysis revealed that the protein A-depleted EasyPep digest sample (Figure 3) exhibited the highest number of identified HCPs. This sample showed twice as many HCPs compared to the native digest sample or the protein A-depleted native digest sample. Our results suggest that both protein A depletion (proA) and semi-tryptic elution from the beads are effective in reducing dynamic range differences and efficient recovery of HCPs from the beads, thereby improving the identification of HCPs. Additionally, the protein A-depleted EasyPep digest sample produced the highest number of unique HCPs compared to the other three sample preparation methods (Figure 4a-d).

Figure 3. Number of HCPs identified from four different sample prep methods

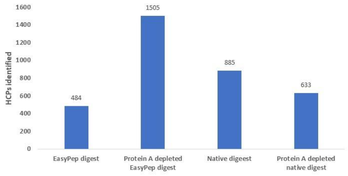
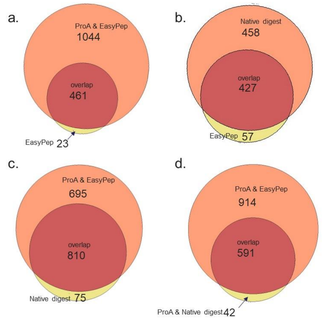


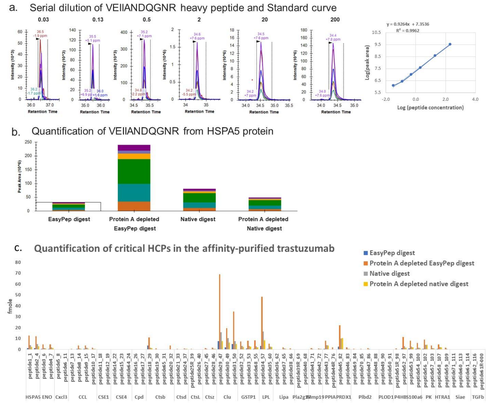
Figure 4. Number of HCPs detected and the pairwise analysis of the four different sample preparation workflows



Targeted MS analysis of CHO HCPs

We utilized PRM method to quantify 28 critical HCPs in four different samples of affinity-purified trastuzumab, each prepared using a distinct method (refer to Figure 1 and 3). To facilitate this analysis, we spiked 200 fmol of a 65 AQUA basic heavy peptide mixture into 0.5 µg of the digest. For quantifying the endogenous light peptide, we used a standard curve generated by serially diluting the heavy peptides (refer to Figure 5a). The data obtained from our analysis revealed that the protein A-depleted EasyPep digest sample had the highest quantification value among the four different sample preparation methods (refer to Figures 5b and 5c). This finding demonstrates that the protein A-depleted EasyPep method offers more accurate quantification of the target HCPs compared to other sample preparation methods.

Figure 5. PRM quantification of four different sample preparation methods using 65 HCPs AQUA heavy peptides



Next, we assessed 1.3mg of post-ultrafiltration and diafiltration (UF/DF) rituximab sample as our starting material, as shown number ❷ in Figure 2, which is equivalent to the final drug substance. We then proceed to prepare digest samples to compare the protein A-depleted EasyPep sample preparation method vs a native digest method. The data obtained from our analysis reveal that the protein A-depleted EasyPep digest method identifies 16 unique HCPs, while the native digest method identifies only 3 unique HCPs. This suggests that the protein A depletion process is effective in reducing dynamic range differences, thereby improving the identification of HCPs (Table 1).

Table 1. Unique and overlap HCPs identified by either native digest or protein A depleted EasyPep digest sample preparation method

Native digest unique protein		Overlap		Protein A depleted EasyPep digest unique protein	
Accession	Description	Accession	Description	Accession	Description
(1)G3XD44: Histone H3, (2)G3B3X4:Fructose-bisphosphate aldolase, (3)G3B8M9: Keratin, type II cytoskeletal 71		(1)Rituximab-mAb-HC, (2)Rituximab-mAb-LC, (3)P13645:Keratin, type I cytoskeletal 10,(4)G3BBF5:Keratin, type II cytoskeletal 2 epidermal		(1) G3HUA1: Anionic trypsin-2-like protein, (2) G3B9K7:Arylacetamide deacetylase, (3) G3B8N5:ATP-dependent RNA helicase DDX18, (4) G3HDV0: Inositol hexakisphosphate and diphosphoinositol-pentakisphosphate kinase 1, (5)G3HWB6:NACHT, LRR and PYD domains-containing protein 9A, (6)G3B2Q8:Serpin A9, (7)G3HIA8:Semaphorin-4D, (8)G3HTC3:Beta-crystallin B1, (9)G3GDS:Annexin, (10)G3HFA8: AP-3 complex subunit delta-1, (11)O76J00:Solute carrier family 35 member C2, (12)G3H272:Vacuolar protein sorting-associated protein 13B, (13)G3B9C7:Arylacetamide deacetylase, (14)G3B8F9:Keratin, type I cytoskeletal 14,(15)G3B9K6:Keratin, type II cytoskeletal 1b, (16)sp|Q011:Hemoglobin light chain sequence	

Targeted Mass Spectrometry with Stellar MS system

A proof-of-concept study was carried out with the new Thermo Scientific™ Stellar™ MS platform. A PRM assay was developed using a 30-minute active gradient method with a Thermo Scientific™ Vanquish™ Neo. Starting from the list of heavy standard peptide sequences imported into Skyline, an unscheduled, targeted MS2 method was created for the HCP standards plus the Pierce™ Peptide Retention Time Calibration mixture peptides (PN:88321). The Prosit software is tightly integrated into Skyline and was used to predict the peptide spectra and indexed retention times. These *in silico* data closely matched the experimental results and allowed Skyline to pick the LC peaks corresponding to the standards.

Next, a Skyline external tool called PRM Conductor was used to refine the transitions and to create scheduled MS2 and scheduled MS3 instrument methods that included both light and heavy peptides. Dilution curves were created by creating a serial 3x dilution from 20 fmol to 3 atmol, using the Thermo Scientific™ Pierce™ 6 Protein Digest (PN:88342) as diluent. Typically, these data are used to validate the assay and start data collection for samples of interest with data streaming to the Ardia™ server for storage and sharing.

Figure 6. PRM assay-building workflow for absolute quantitation with heavy standards.

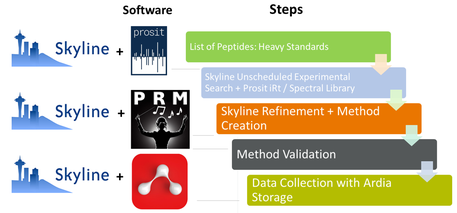
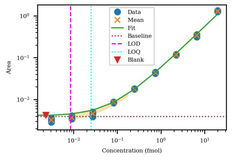
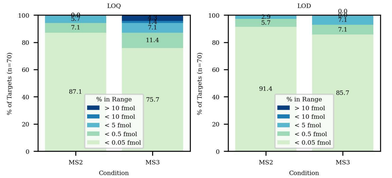


Figure 7. Example targeted MS2 dilution curve.



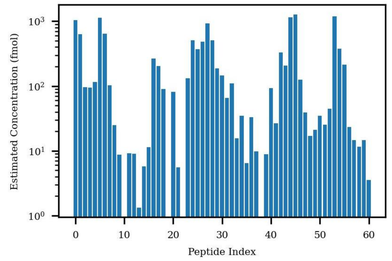
Peak areas were normalized using the PRTC areas. LOD and LOQ were determined with Pinot’s bootstrapping method[2]. For each peptide and acquisition type, a set of ≥ 3 transitions that gave the lowest LOQ was determined[3]. Distributions for dilutions of the heavy and light peptides are shown in Figure 8.

Figure 8. Distributions of LOQ and LOD figures of merit for targeted MS2 and MS3 methods.



The experimentally determined dilution curves were used to determine the concentration of light peptides in a null HCP control sample. This control sample is equivalent to the clarified harvest sample without any antibody expressed and was injected as Unknowns. The data presented in Figure 9 serves as validation for the applicability of the assay in detecting unknown analytes with high concentrations.

Figure 9. The concentrations of unknown peptides in a null HCP control sample



Conclusions

- The protein A-depleted EasyPep method exhibited the highest number of identified HCPs and produced the highest number of unique HCPs compared to other sample preparation methods.
- The protein A-depleted EasyPep method resulted in the highest quantification accuracy among the different methods tested.
- Targeted MSn methods were developed for the HCP heavy/light peptides using the Thermo Fisher Scientific™ Stellar™ MS platform showed excellent ability to detect and quantify HCPs in control samples over a broad dynamic range.

References

1. Jones et al (2021): “High-risk” host cell proteins (HCPs): A multi-company collaborative view. Biotechnol Bioeng. 2021 Aug;118(8):2870-2885.
2. Pino et. al. Matrix-Matched Calibration Curves for Assessing Analytical Figures of Merit in Quantitative Proteomics. 2020, 19, 3, 1147-1153.
3. Heil et. al. Comparison of Unit Resolution Versus High-Resolution Accurate Mass for Parallel Reaction Monitoring. Journal of Proteome Research. 2021, 20, 9, 4435-4442.

Trademarks/licensing

For Research Use Only. Not for use in diagnostic procedures.