AN EFFICIENT LC-MS WORKFLOW FOR IDENTIFICATION AND MONITORING OF HOST CELL PROTEINS FOR ASSISTING MONOCLONAL ANTIBODY PURIFICATION

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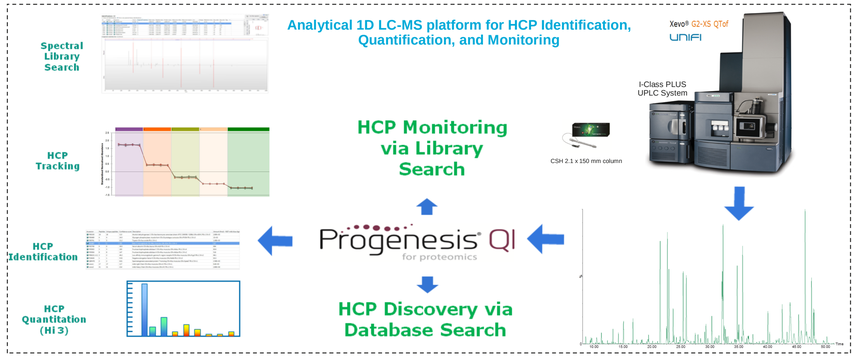
OVERVIEW

An HCP Discovery assay using data-independent (MS^E) acquisition in UNIFI was used for identification of unknown HCPs in the NIST mAb and in 5 other mAb preparations. The assay was then used to build an MS/MS spectral library to facilitate other high-throughput HCP quantification / monitoring assays employing MS^E acquisition.

INTRODUCTION

- In recent years, the LC-MS assays have been adopted as orthogonal techniques to ELISA for HCP analysis due to their flexibility and potential for full proteome-wide applications. Here we describe an efficient analytical scale LC/MS workflow that allows the identification and quantification of HCPs during mAb purification using a CHO cell line.
- Here we explored the capabilities of a 1D LC-MS assay for *individual* HCP identification, quantification and monitoring.
- The first step of the HCP identification and quantification workflow is the HCP Discovery Assay, employing data-independent MS^E acquisition using 90 min peptide separations.
- In the second step, samples are analyzed by higher–throughput HCP Monitoring Assays using MS^E acquisitions with 30 min peptide separations.
- A real-life example is presented in which the 1D LC-MS platform is used to compare four different SCX protocols in their ability to remove two HCPs present in the Protein A eluate of a mAb expressed in CHO (Chinese hamster ovary) cells.
- The HCP workflow described here was able to identify which SCX purification protocols produced the mAb target with the lowest concentration of low-level HCP impurities.
- The data for both assays was acquired in UNIFI 1.9.4, exported in the RAW file format and processed with Progenesis QI for proteomics 4.2 for HCP identification and monitoring.



METHODS

Sample Preparation

A well characterized mAb (NIST mAb) in terms of the HCP content [1,2], was digested using a previously reported protocol designed to deplete a significant amount of the mAb-derived peptides [2]. The NIST mAb was diluted into 50 mM ammonium bicarbonate to a protein concentration of 25 mg/mL and digested overnight with a mixture of Lys-C and porcine trypsin (Promega). Next day, the undigested mAb was denatured with RapiGest surfactant (60°C, 15 min), reduced with DTT (60°C, 1h) and alkylated with IAM (RT, 30 min) to achieve the precipitation of the undigested mAb. Three MassPREP protein digest standards (ADH—yeast alcohol dehydrogenase, PHO - rabbit phosphorylase b, and BSA - bovine serum albumin) were spiked at different concentration levels (See Table I) post-digestion.
In a different experiment, a different mAb was purified by Protein A-chromatography and then by SCX chromatography using four different elution protocols. All five mAb preparations were digested with the same digestion protocol and spiked with the same MassPREP protein digests.

LC Conditions

Discovery HCP Assays were performed on an ACQUITY™ UPLC® I-class Plus system equipped with a CSH (charged-surface hybrid) C18 column (2.1 x 150 mm, 1.7 µm particles, P/N 186005298). Peptide separations were performed at a flow rate of 0.2 mL/min with a gradient from 0% to 45% Solvent B in 90 min, at a column temperature of 60°. The mobile phases were: 0.1% FA (formic acid) in DI water (Solvent A) and 0.1% FA in acetonitrile (Solvent B). Monitoring HCP Assays were performed on the same LC-MS system using shorter, 30 min gradient separations.

Mass Spectrometry

Data-independent acquisitions were performed in MS^E mode on a Xevo® G2-XS QTof mass spectrometer operated by UNIFI 1.9.4. data acquisition software. For the Discovery HCP Assays, the data was acquired with 0.5 s scans over a mass range of 100-2000 Da. For the HCP Monitoring Assays, the data was acquired using 0.3 s scans over the same mass range. For both acquisition modes, low-energy scans were acquired with a CE of 4 eV, while the high-energy fragmentation scans used CE ramping from 15 to 45 V.

Data Processing

Progenesis QI for proteomics 4.2 software was used for data processing. The MS^E datasets produced by the Discovery Assays were searched against a mouse protein database containing 16,644 entries (for the NIST mAb sample) or against the CHO proteome (25,485 proteins) for the other five mAb preparations. The HCPs identified were compiled into a spectral library for easier, faster identification in the subsequent HCP Monitoring Assays.

RESULTS

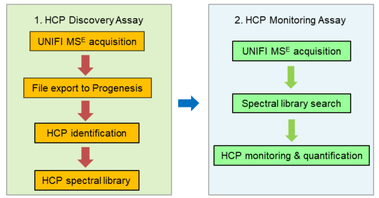


Figure 1. Flow-chart summarizing the two-step HCP identification and monitoring/quantification workflow.

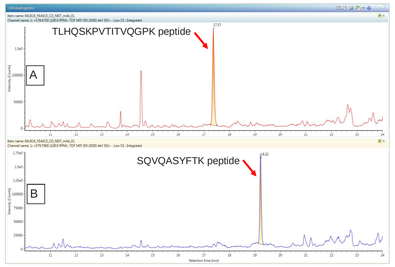
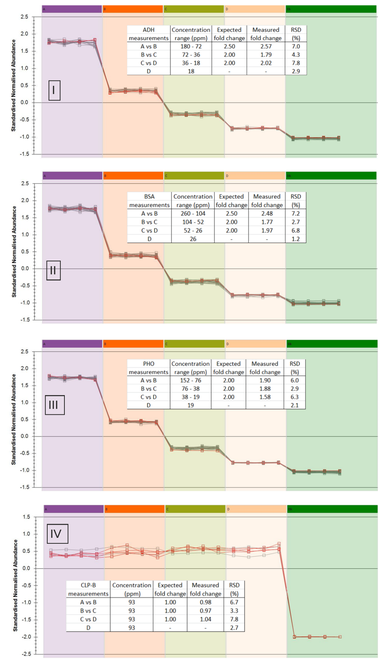


Figure 2. Extracted mass chromatograms of two low-abundance HCP peptides identified in the NIST mAb digest using the Discovery Assay performed with UNIFI acquisition: (A) peptide TLHQSKPVTITVQGPK (precursor 578.67, +3); (B) peptide SQVQASYTFK peptide (precursor 579.79, +2). Both peptides belong to an HCP identified as *low affinity IgG gamma Fc region receptor* (UniProt accession no P08101) present at 16 ppm in the NIST mAb.



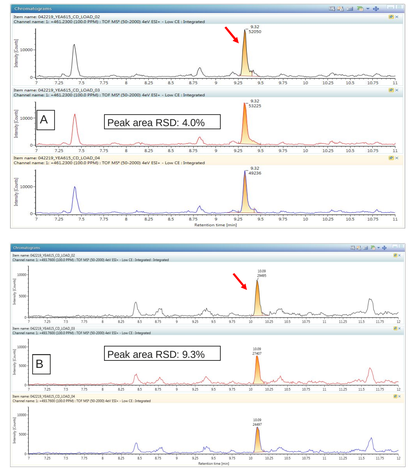
ADH measurements	Concentration range (ppm)	Expected fold change	Measured fold change	RSD (%)
A vs B	180 - 72	2.50	2.57	7.0
B vs C	72 - 36	2.00	1.79	4.3
C vs D	36 - 18	2.00	2.02	7.8
D	18	-	-	2.9

BSA measurements	Concentration (ppm)	Expected fold change	Measured fold change	RSD (%)
A vs B	260 - 104	2.50	2.48	7.2
B vs C	104 - 52	2.00	1.77	2.7
C vs D	52 - 26	2.00	1.97	6.8
D	26	-	-	1.2

PHO measurements	Concentration range (ppm)	Expected fold change	Measured fold change	RSD (%)
A vs B	152 - 76	2.00	1.90	6.0
B vs C	76 - 38	2.00	1.88	2.9
C vs D	38 - 19	2.00	1.58	6.3
D	19	-	-	2.1

CLP-B measurements	Concentration (ppm)	Expected fold change	Measured fold change	RSD (%)
A vs B	93	1.00	0.98	6.7
B vs C	93	1.00	0.97	3.3
C vs D	93	1.00	1.04	7.8
D	93	-	-	2.7

Figure 3. Peptide level results of the HCP monitoring assay. Three protein standards (ADH, BSA and PHO) were spiked at four different concentration levels in four NIST mAb digests, while one protein digest (CLP-B) was spiked at the same concentration in all four samples labeled A-D (see the inset tables for the spiked protein concentrations). Panels I-IV display the Progenesis QI Proteomics plots observed for each spiked protein across four replicate injections of each sample (A-D). The Blk sample is the non-spiked NIST mAb digest. Protein measurements were obtained from multiple peptides (11 ADH peptides, 15 BSA peptides, 14 PHO peptides and 5 CLP-B peptides) and excellent correlation was obtained between the spiked and measured fold changes with RSDs under 10% for all measurements.



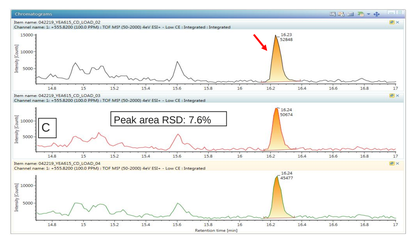


Figure 4. Excellent retention time and peak area reproducibility was obtained for three low-abundance HCP peptides identified in the Protein A preparation of a mAb. The extracted mass chromatograms shown in panels A-C correspond to the following HCP peptide precursors: (A) m/z= 461.23 (+2); (B) m/z = 493.76 (+2) and (C) m/z = 555.82 (+2).

No crt	UniProt Number	Protein Description	Sequence Coverage (%)	Average MW (kDa)	Amount on column fmoles	Amount on column ng	Concentration ng/mL	Concentration ppm	RSD (%)
1	P00330	Alcohol dehydrogenase yeast (ADH) - 5000 fmoles	57.4	36.7	4533	166	26618	266	18.4
2	P05064	Fructose biphosphate aldolase A isoform	62.3	39.3	2614	103	16437	164	14.3
3	P00489	Glycogen phosphorylase rabbit (PHO) - 1000 fmoles	35.3	97.1	1000	97	15536	155	0.0
4	P05063	Fructose biphosphate aldolase C isoform	29.4	39.4	1529	60	9639	96	8.7
5	P02769	Bovine serum albumin (BSA) - 250 fmoles	30.7	66.3	232	15	2461	25	11.3
6	P08101	Low affinity immunoglobulin gamma Fc region receptor	15.3	36.7	265	10	1556	16	16.8
7	P06745	Glucose 6-phosphate isomerase	8.3	62.7	118	7	1184	12	19.4
8	P01887	Beta-2-microglobulin	16.0	13.8	451	6	996	10	18.4
9	Q8BL97	Serine/arginine-rich splicing factor 7	6.4	30.8	111	3	547	5	15.7

Table I. HCPs identified and quantified in the NIST mAb using the Discovery Assay performed with UNIFI acquisition. Three spiked proteins (ADH, PHO and BSA) along with 6 HCPs (highlighted in red) were identified in 3 replicate injections. The detection limit of the assay was **5 ppm**.

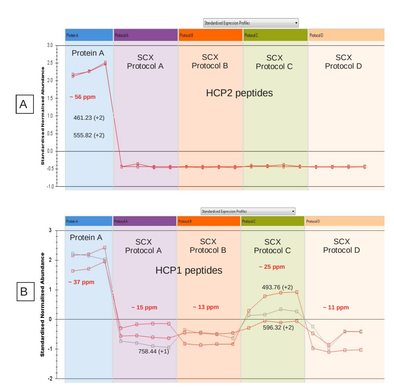


Table II

Protein ID	Peptide precursor m/z	Charge state	RT (min) 30 min gradient	Average peak area	Peak area RSD (%)
HCP1	546.79	2	8.9	31524	4.5
HCP1	596.32	2	9.1	43121	8.8
HCP1	537.78	2	9.4	27387	9.4
HCP1	758.44	1	7.1	35668	4.9
HCP1	**493.76**	**2**	**10.1**	**27266**	**9.3**
HCP1	658.82	2	15.1	29256	7.6
HCP2	**461.23**	**2**	**9.3**	**51504**	**4.0**
HCP2	644.84	2	10.8	37556	6.7
HCP2	**555.82**	**2**	**16.2**	**49666**	**7.6**

Figure 5. Peptide level monitoring of two HCPs (identified as HCP1 and HCP2 in Table II across five mAb preparations (one Protein A eluate and 4 SCX (strong cation exchange) chromatographic purifications using four different protocols (A-D). Panels A and B display the trend plots and ppm concentrations for three HCP1 peptides and two HCP2 peptides. The HCP monitoring data was acquired in MS^E mode and searched against a spectral library. Table II displays the monoisotopic peaks, charge states, retention times, peak areas and peak area RSDs for 9 HCP peptides identified by the Discovery assay in the Protein A eluate. As illustrated here, Protocol D provided the best results. Extracted mass chromatograms for the three peptides highlighted in bold are shown in Figure 4.

CONCLUSIONS

- Three spiked proteins and 6 HCPs were identified by the Discovery Assay in the NIST mAb, with a detection limit of 5 ppm.
- Using the recently developed library search functionality in Progenesis QI for proteomics, the LC-MS platform can be used in timely fashion to monitor/track HCP clearance across samples from bioprocess development, offering a sensitive and high throughput assay for *individual* HCPs.

References:

1. Doneanu et al. Anal Chem, 2015, 87, 10283.
2. Huang et al. Anal Chem, 2017, 89, 5436.

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