DATA INDEPENDENT LC-MS ASSAYS FOR IDENTIFICATION, QUANTIFICATION AND MONITORING OF HOST CELL PROTEINS IN MONOCLONAL ANTIBODIES

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OVERVIEW

Two analytical scale LC-MS workflows were developed for identification (HCP Discovery workflow) and compliant, fast monitoring (HCP Monitoring workflow), for tracking host-cell-protein (HCP) contaminants in mAb preparations down to 5 ppm

INTRODUCTION

- The HCP Discovery Assay employs data-independent MS^E acquisition on a high-resolution QTof instrument (Xevo™ G3, 30,000 MS resolution) along with extensive chromatographic separations (90 min gradient) for analysis of mAb digests
- In the second workflow, samples are analyzed by higher throughput HCP Monitoring Assays on a BioAccord™ Tof instrument, using MS^E acquisitions following 30 min gradient separations of spiked mAb digests
- A sample preparation protocol involving mAb precipitation [2] and protein digestion with a newly introduced enzyme—RapiZyme™ trypsin [6] is introduced for the HCP Discovery assay
- The HCP Monitoring Assay can achieve a similar sensitivity with the HCP Discovery Assay (5ppm), as demonstrated by the spiking of MIX-5 protein digest standards in the NIST mAb digest

METHODS

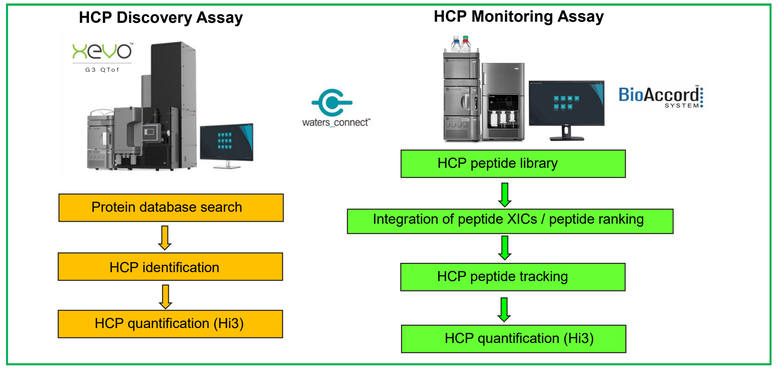
Sample Preparation

A mAb well characterized in terms of the HCP content [1-5], (NIST mAb Reference Material 8671), was digested using a modified version of a previously reported protocol designed to deplete a significant amount of the mAb-derived peptides [2]. In a 500 µL Protein LoBind Eppendorf tube (catalogue no 0224431064), 200 µL of NIST mAb were mixed with 20 µL of 1M Tris HCl buffer (containing 1 M $CaCl_2$) and 10 µL of 4 µM RapiZyme trypsin [6] (Waters P/N 186010107) and digested overnight (~ 16 hours) at 37 °C. Following enzymatic digestion, the undigested mAb was denatured by heating and reduction with 4 mM DTT (90 °C, 15 min). The sample was then spun in a centrifuge for 5 minutes at 12,000 g and the supernatant was recovered and acidified with 1 µL of formic acid (Millipore Sigma). For the HCP Discovery Assay, performed in MS^E mode on the Xevo G3 QTof, four protein digest standards (MIX-4: ADH—yeast alcohol dehydrogenase, BSA - bovine serum albumin, ENL – yeast enolase and PHO - rabbit phosphorylase b), were spiked post-digestion in the NIST mAb digest at concentrations listed in Table I.

For the HCP Monitoring Assay, performed in MS^E mode on the BioAccord LC-MS system, five protein digests (MIX-5: ADH, BSA, ENL, PHO and CLP-B—chaperone Ecoli digest) were spiked post digestion in the NIST mAb at various concentration levels as illustrated in Figure 4 panels A-D.

LC Conditions

The HCP Discovery Assay were performed on an ACQUITY™ UPLC® Premier BSM equipped with a ACQUITY Premier CSH (charged-surface hybrid) C18 column (2.1 x 150 mm, 1.7 µm particles, P/N 186009462). 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 °C. The mobile phases were: 0.1% FA (formic acid) in DI water (Solvent A) and 0.1% FA in acetonitrile (Solvent B). HCP Monitoring Assays were performed on the same LC system using shorter (30 min) gradient separations from 0% to 45% Solvent B.



Mass Spectrometry

For the HCP Discovery Assays, data-independent acquisitions were performed in MS^E mode on a Xevo® G3-QTof mass spectrometer operated by waters_connect software. Data was acquired with 0.5 s scans over a mass range of 400-2000 Da. Low-energy MS^E scans were acquired with a CE (collision energy) of 6 V, while the high-energy fragmentation scans used CE ramping from 15 to 45 V.

For the HCP Monitoring Assays, data was acquired in MS^E mode on a BioAccord LC-MS system using 0.5 s scans over the mass range of 50-2000 Da. Low-energy MS^E scans were acquired with a CV (cone voltage) of 40 V, while the high-energy fragmentation scans used CV ramping from 60 to 80 V.

Data Processing

Two proteomics software packages were used for processing of the HCP Discovery datasets: 1) Progenesis QI for proteomics 4.2 and 2) Protein Metrics Byonic (version 5.2.31). The Xevo G3 MS^E datasets for the NIST mAb digest were searched against a mouse protein database containing 16,644 entries and the processing results obtained from search engines are displayed in Figure 3 screenshots, while the combined results are presented in Table I.

The HCP Monitoring dataset of MIX-5 spiked peptides is similar to a dataset containing various levels of HCP derived peptides. This dataset (25 runs, including 5 replicates for each sample) were processed using the Accurate Mass Screening Workflow available in waters_connect.

A list of 43 best responding peptides in ESI-MS, comprising 10 peptides from each proteins (ADH, BSA, ENL and PHO), along with 3 peptides from the CLP-B protein digest standard, was automatically imported in the Component Table, a section of the data processing method. The list contained the only the most abundant precursor for each peptide.

Extracted mass chromatograms were generated for all 43 spiked peptides and the corresponding chromatographic peaks were integrated and ranked according to their peak area. The most abundant peptides from each protein (based on their peak area counts) were selected for Hi3 protein quantification [7]. Concentration of four spiked proteins (ADH, BSA, ENL and PHO) was measured against the CLP-B protein digest which was spiked at the same concentration level (120 ppm) in all samples. Monitoring of representative peptides from MIX-4 proteins is illustrated in Figure 4, panels A-D. A graphical representation of the complete HPC workflow can be found in the section above.

RESULTS

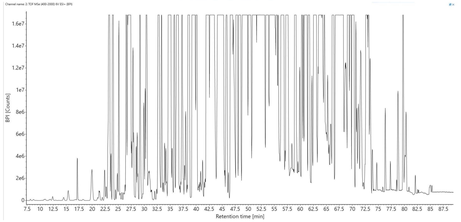


Figure 1. Base-peak chromatogram showing the separation of the NIST mAb digest peptides with a 90-min gradient using the Discovery HCP Assay performed on a Xevo G3 QTof instrument. The CSH Premier column is maintaining a good chromatographic performance under sample overloading conditions.

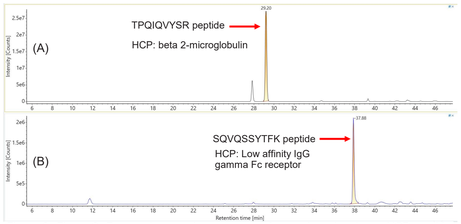
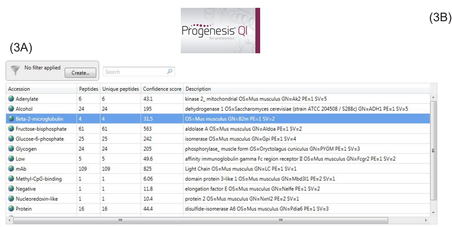


Figure 2. Extracted mass chromatograms for two low-abundance HCP peptides identified in the NIST mAb digest using the Discovery HCP Assay: (A) peptide SQVQASYTFK (precursor 579.79, +2) from *Low affinity IgG gamma Fc receptor protein*; (B) peptide TPQIQVYSR (precursor 546.30, +2) from *beta-2-microglobulin*. These two HCPs were identified in the NIST mAb at a concentration of 19 ppm (*Low affinity IgG*) and 13 ppm (*beta-2-microglobulin*) according to the data presented in Table I.

(3A)



(3B)

(D)

Figure 3. Screenshots showing the HCPs identified in the NIST mAb by the Discovery Assay performed on the Xevo G3 instrument:
(A) Progenesis QIP results; (B) PMI (Protein Metrics) Byonic results.

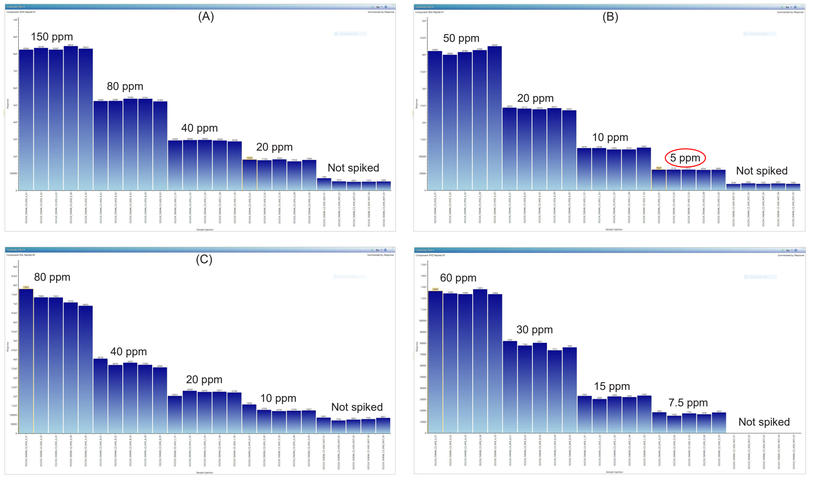


Figure 4. Peptide level results of the HCP Monitoring Assay. Four protein standards (ADH, BSA, ENL and PHO) were spiked at four different concentration levels in four NIST mAb digests, while one protein digest (CLP-B) was spiked at the same concentration (120 ppm) in all four samples. Panels A-D display the summary plots generated for a peptide from each of the four proteins standards spiked at different concentrations: (A) VVGLSTLPEIYEK (724.41, +2) from ADH; (B) LVNELTEFAK (582.32, +2) from BSA; (C) TAGIQIVADDLTVTNPK (585.99, +3) from ENL; and (D) VAAAFPGDVDR (559.29, +2) from PHO. The HCP Monitoring Assay is clearly able to find all the spiked proteins in the NIST mAb digest and it can achieve a similar detection limit with the HCP Discovery Assay (5ppm) in the case of BSA (see panel 4B).

No crt	Accession Number	Protein Description	Sequence Coverage (%)	Average MW (kDa)	Amount on column fmoles	ng	Concentration ng/mL	ppm	RSD (%)
1	P05064	Fructose-bisphosphate aldolase A	55.5	39.3	3032	119	2562	256	5.7
2	P00330	Alcohol dehydrogenase yeast (ADH) - 1000 fmoles	39.2	36.7	1323	49	1064	104	15.2
3	P00489	Glycogen phosphorylase rabbit (PHO) - 200 fmoles	43.2	97.1	200	19	418	42	0.0
4	P06745	Glucose-6-phosphate isomerase	22.4	62.7	242	15	326	33	8.3
5	P08103	Low affinity immunoglobulin gamma Fc region receptor	19.7	36.7	237	9	187	19	14.8
6	P01887	Beta-2-microglobulin	18.3	13.8	444	6	132	13	7.5
7	O93298	Protein disulfide-isomerase A6	8.8	48.1	171	6	125	13	12.4
8	Q99R90	Syntaxin-12	3.4	33.2	120	4	80	7	4.7
9	Q9WTP6	Adenylate kinase 2 mitochondrial	7.8	26.5	65	2	37	(2)	14.3
10	P02769	Bovine serum albumin (BSA) - 20 fmoles	-	66.3	30	1	29	-	-
11	P00924	Enolase 1 yeast (ENL) - 10 fmoles	-	46.6	10	0	75	1	-

Table I. HCPs identified and quantified in the NIST mAb using the Discovery HCP Assay performed on the Xevo G3 instrument. Seven HCPs and two spiked proteins (ADH and PHO) were identified in two out of three replicate injections by both search engines. The detection limit of the assay was **5 ppm**.

CONCLUSIONS

- Two spiked reference proteins, as well as seven HCPs from NIST mAb, were identified and quantified ssing data-independent MS^E acquisition on the Xevo G3 QTof instrument, with an LLOQ of the HCP Discovery Assay of **5 ppm**
- For the HCP Monitoring Assay, four spiked proteins (MIX-4) were monitored and quantified in a biopharmaceutical sample (NIST mAb digest) using the BioAccord LC-MS System and the Accurate Mass Screening workflow from waters_connect
- The HCP Discovery and Monitoring Assays were able to achieve the same LLOQ for HCP quantification: 5 ppm
- The HCP Monitoring Assay is performed in a data compliant environment, indicating the possibility to establish GMP QC assays for HCPs

References

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