Highly sensitive LC-MS/MS workflow for targeted quantification of host cell proteins

***Featuring the* SCIEX QTRAP® 6500+ LC-MS/MS System**

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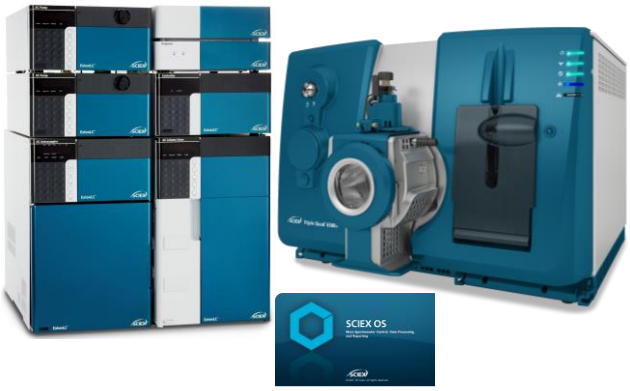

During biotherapeutics manufacturing, process-related impurities and other trace contaminants accompany the recombinant biotherapeutic products. Among them, host cell proteins (HCPs) are a major class of protein impurity derived from the host organism. The detection and quantification of HCPs is an area of particular concern, as these contaminants can elicit an adverse response in patients.

HCP quantification methods have been adopted in manufacturing and quality control processes in biopharmaceutical industry in recent years. The high complexity and the wide dynamic range of protein concentrations in the multiple purification stages of biotherapeutic production pose challenges for the traditional data dependent workflows for HCP quantification. This results in the increasing need for developing targeted HCP analysis methods with high sensitivity, reduced analysis time, robustness and multiplexing capability (quantify significant numbers of analytes in one injection). As the target analytes for quantification have been pre-defined before analysis, triple quadrupole and QTRAP LC-MS/MS Systems are identified as the suitable instrument platforms do to their high quantitative performance. Herein, a targeted HCP analysis workflow utilizing the SCIEX QTRAP 6500+ LC-MS/MS System is presented. A *Scheduled* MRM™ Algorithm is applied to allow the simultaneous quantification of 48 proteins (4 transitions per protein) in an 8 min LC-MS analysis. This method demonstrated LLOQs ranging from 0.09 to 5 ppm.

Key features of the LC-MRM based targeted HCP quantification workflow

- The SCIEX Triple Quad™ and QTRAP 6500+ Systems offer superior quant performance for bioanalysis, reaching low LLOQs (sub ppm level HCP quantification), wide LDR and low CVs
- High analysis throughput to quantify 48 proteins in an 8 min LC-MS/MS run with solid confirmation (4 transitions per protein)
- High-resolution MS/MS peptide library offering reliable signature peptide selection using the SCIEX QTRAP 6500+ LC-MS/MS System
- Seamless integration of Skyline with SCIEX Software allowing fast and robust MRM method optimization
- Comprehensive automated quantification with SCIEX OS-MQ Software combining ease-of-use with confidence in the results

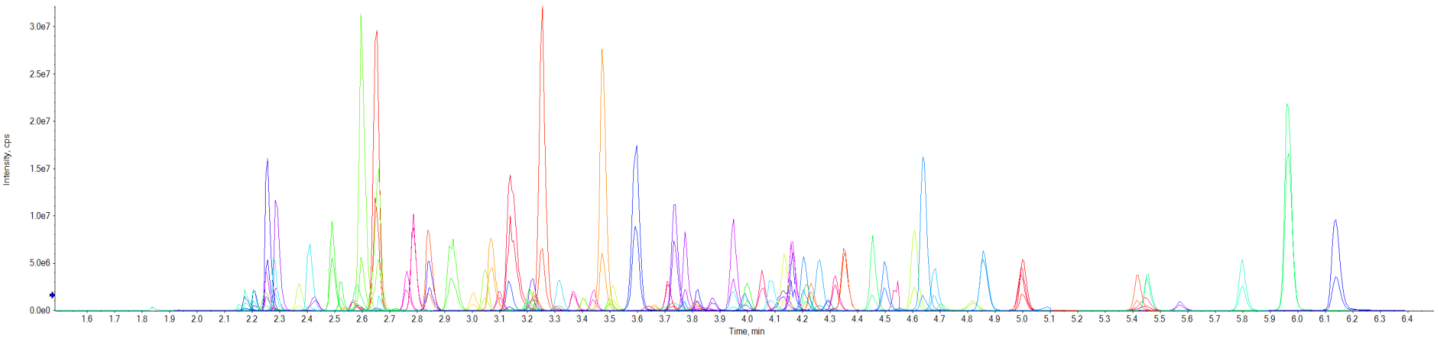

Figure 1. The extracted ion chromatograms (XICs) of 96 peptides (two MRM transitions per protein) from 48 target proteins demonstrate the LC-MRM based targeted HCP quantification workflow. The total LC run time is 8 min, with a 5 min linear gradient for separation.

Methods

Sample preparation: NISTmAb monoclonal antibody (NISTmAb) and the Universal Proteomics Standard (UPS) are purchased from Sigma-Aldrich. In this experiment model, NISTmAb is serving as the biotherapeutic molecule, while the 48 human proteins in the UPS mix are mimicking the targeted HCPs for quantification. Bovine serum albumin (BSA) is used as the internal standard. The UPS proteins and BSA are spiked into NISTmAb solution and serial diluted. In the serial dilution samples, the level of BSA remains consistent as 100 ppm, and the level of UPS proteins ranges from 1.22 to 5000 fmol per 100 µg NISTmAb. The ppm level range for each individual protein varies based on the protein molecular weight (MW). For the smallest protein, epidermal growth factor (P01133) with MW 6353 Da, its concentrations are 0.08 – 317.65 ppm among serial dilution. For the largest protein, gelsolin (P06396) with MW 82959 Da, its concentrations are 1.01 – 4147.95 ppm.

Samples are denatured by incubating with N-octyl-glucoside (OGS), reduced by dithiothreitol (DTT) and alkylated by iodoacetamide (IAM). A trypsin/Lys-C digestion was performed at 37 °C for overnight, with an enzyme-protein ratio at 1:25. Formic acid was spiked into the samples to abort digestion. The samples are centrifuged at the speed of 12000 g and injected into LC-MS analysis.

LC-MS conditions: Two types of LC-MS analysis are performed either on a TripleTOF® 6600+ LC-MS/MS System to build peptide library, or on a SCIEX QTRAP 6500+ LC-MS/MS System for protein quantification.

Peptide library creation: An information dependent acquisition (IDA) analysis is performed to analyze the digested NISTmAb-UPS protein mix. The LC-MS details are summarized in Table 1 and 2.

Table 1. HPLC condition for IDA analysis.

Parameter	Value
Stationary phase	*C18 column, 2.1 X 150 mm, 1.7 µm*
Mobile phase A	*0.1% formic acid in water*
Mobile phase B	*0.1% formic acid in acetonitrile*
Gradient	*B% ramping from 5% to 40% in 42 min*
Total run time	*60 min including equilibration*
Flow rate	*0.2 mL/min*
Column temperature	*40 °C*
Injection volume	*20 µL*

Table 2. Mass spectrometric parameters for IDA analysis.

Parameter	Value	Parameter	Value
MS range	*350-1500*	*MS/MS range*	150-1500
MS accumulation time	*150 ms*	*MS/MS accumulation time*	*30* ms
Curtain gas:	*30 psi*	*Source temperature:*	450 *°C*
Ion source gas 1:	*50 psi*	*Ion source gas 2:*	50 *psi*
Number of MS/MS triggered per circle	*25*	*Ion spray voltage:*	5500 V

Table 3. HPLC condition for MRM analysis.

Parameter	Value
Stationary phase	*Phenomenex Kinetex C18 column, 3 X 50 mm, 2.6 µm*
Mobile phase A	*0.1% formic acid in water*
Mobile phase B	*0.1% formic acid in acetonitrile*
Gradient	*B% ramping from 12% to 32% in 5 min*
Total run time	*8 min including equilibration*
Flow rate	*0.5 mL/min*
Column temperature	*40 °C*
Injection volume	*20 µL*
Divert valve set-up	*1-6.2 min to MS*

Protein quantification: A *Scheduled* MRM Algorithm analysis is performed on the serial dilution samples for protein quantification. The LC-MS details are summarized in Table 3, Table 4 and Table A1.

Table 4. Gas/source parameters for MRM analysis.

Parameter	Value	Parameter	Value
Curtain gas:	*30 psi*	*Source temperature:*	550 *°C*
Ion source gas 1:	*65 psi*	*Ion source gas 2:*	65 *psi*
CAD gas:	*12 psi*	*Ion spray voltage:*	5500 V

Data processing: Figure 2 describes the workflow for data generation and processing.

Peptide library creation: The database search is performed on IDA data by using ProteinPilot™ Software 5.0. A .fasta file including sequences of NISTmAb, BSA and USP proteins is

Figure 2. The data generation and processing workflow of targeted HCP quantification. Four major steps are involved: peptide library build-up, MRM method development, data acquisition and quantitative data processing. Details and zoomed-in figure are provided in later sessions. Among them, peptide library creation step can be simplified as in-silico digestion, if a high-resolution MS (HRMS) is not available to generate IDA data.

created for database search. The search result file is generated and imported into Skyline software for peptide library creation.

MRM method development: Skyline software is used for MRM method optimization. The UPS protein sequences are imported, and the MRM transitions are predicted based on peptide and transition settings. The most abundant fragment ions observed in the MS/MS spectra of the peptide library are selected for further MRM optimization, including retention time verification, and the optimization of CE, DP, gas/source parameters.

Protein quantification: The optimized MRM method is used for creation of the calibration curve. The MRM data are processed by using the Analytics function in SCIEX OS Software 1.7.

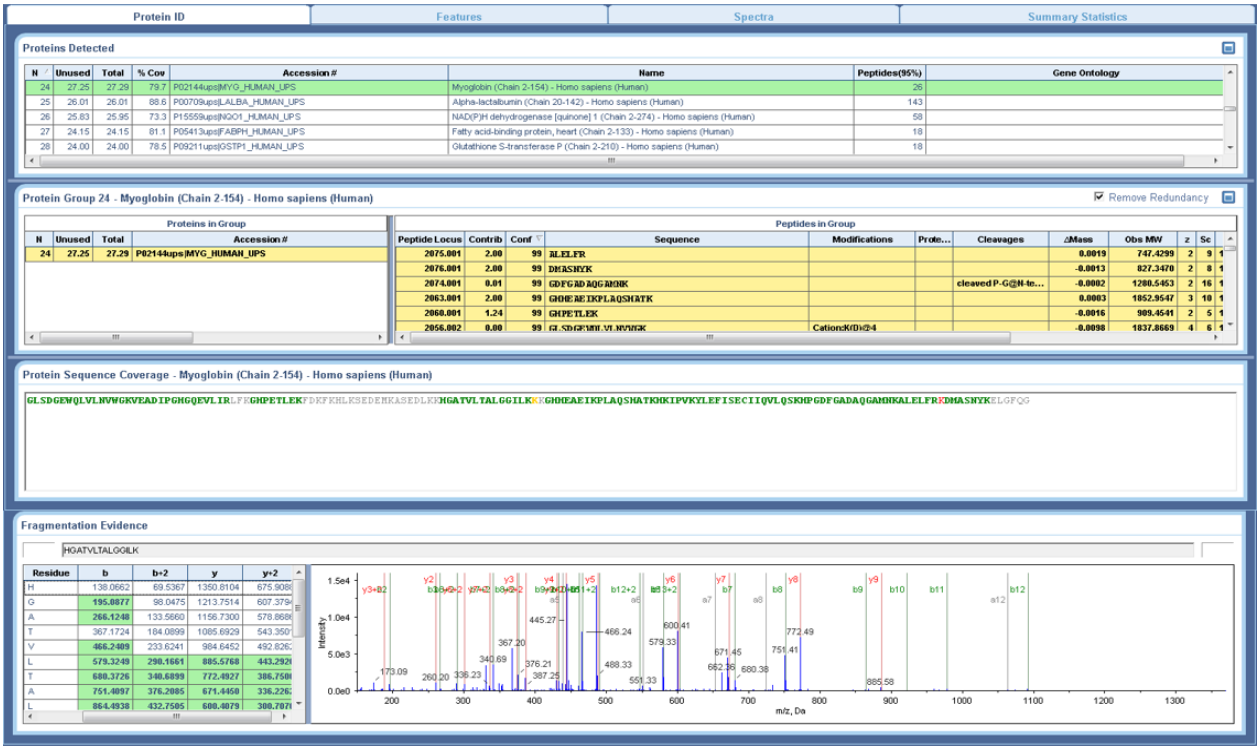

Figure 3. Example database search result shown in ProteinPilot Software 5.0. The result file provides information including (from top to bottom panel): the list of identified protein, the summary of peptides identified per protein, the sequence coverage map, the MS/MS fragment ion assignment for each peptide.

Peptide library creation

For any signature peptide-based protein quantification workflow, selecting the appropriate peptide targets is a critical requirement to ensure the assay's success. Several criteria for signature peptide selection are commonly applied: 1) the uniqueness of peptide sequences: selected peptides should be unique from any matrix protein to minimize endogenous interference; 2) the abundance of LC-MS/MS signal: selected peptides should have good ionization and fragmentation efficiency for the optimal assay sensitivity; 3) no missed cleavages or variable post-translation modification (PTM) site: the peptides should include neither missed cleavages, nor amino acids (e.g. methionine) that can be easily modified during the biological process or sample preparation.

There are two methods commonly used for signature peptide selection: in-silico digestion of the target protein and IDA analysis for peptide mapping on the authentic protein digest sample. When an HRMS instrument is available for IDA analysis, the later method is usually adopted for the most appropriate and reliable peptide selection based on real data. Herein, an IDA peptide mapping analysis is performed on a TripleTOF 6600+ LC-MS/MS System to analyze the digested NISTmAb-UPS protein mix. The IDA data is processed by ProteinPilot Software 5.0 (Figure 3), searched against the protein sequences of NISTmAb, UPS proteins and BSA. All 48 UPS proteins, together with BSA and NISTmAb, are identified during database search. The information of peptide ID, sequence coverage and MS/MS fragment ion assignment can be found in the search result file. This result file is imported into Skyline software, served as the peptide library.

MRM method development

The combination of Skyline software (Version 20.1.0.31) (Figure 4) together with Analyst® Software 1.7 is adopted for MRM development. After protein sequences are imported into Skyline, the list of peptides is generated based on peptide settings and peptide library matching. The MRM transitions are created by selecting the fragment ions with the strongest MS/MS signal per peptide in the peptide library. The MRM acquisition method can be generated in Skyline and imported into Analyst Software 1.7. The method format can be standard MRM, *Scheduled* MRM Algorithm Pro with expected retention time for each peptide defined, or MRM for compound parameter optimization. The data generated by using these methods can also be imported back to Skyline for MRM method optimization, by identifying the optimal CE and DP values, or adjusting the expected analyte retention time.

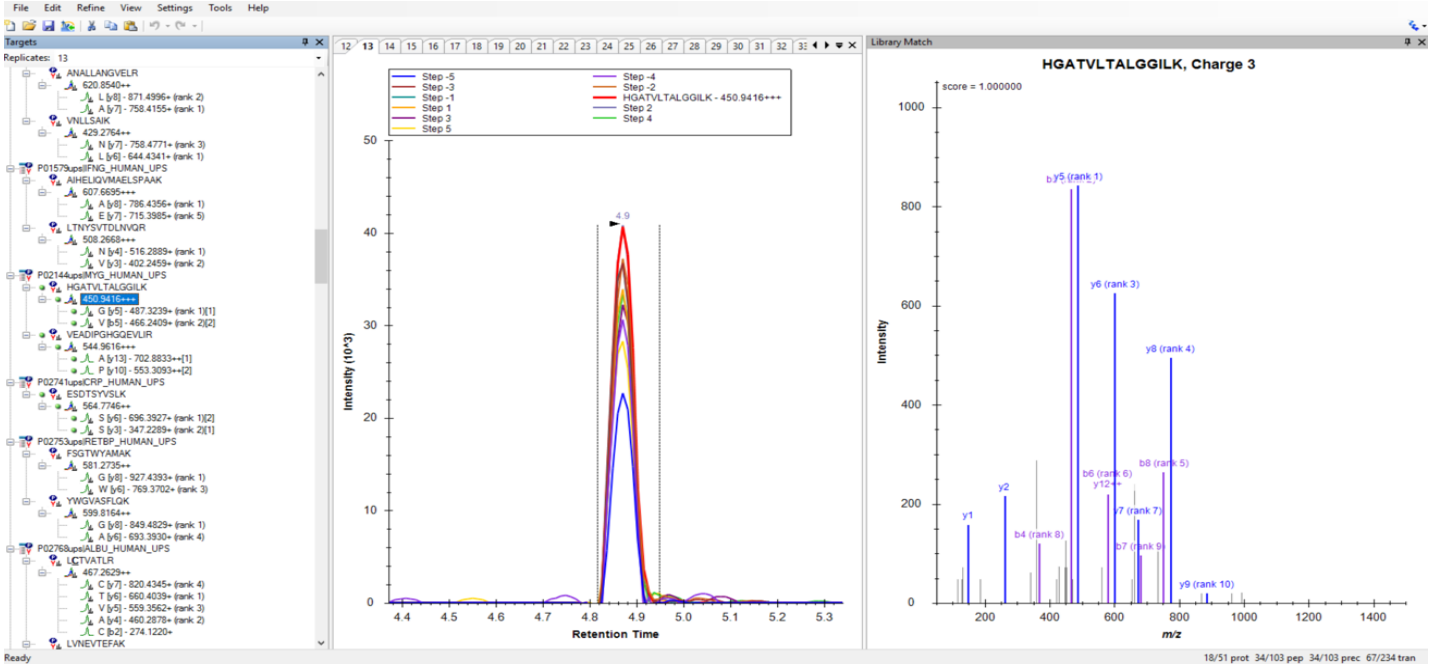

Figure 4. A screenshot of Skyline software for MRM optimization. Left: the list of selected peptides and fragment ions to create MRM method. Middle: the XICs of one MRM transition with different CE values for MRM optimization. The CE associated with the largest peak area is the optimal CE value. Right: the representative MS/MS spectra from the imported peptide library. The most abundant fragment ions (ranked and color labeled) are listed as the fragment ions in the left panel for MRM method generation.

Protein quantification

The UPS protein serial dilution samples are analyzed on a SCIEX QTRAP 6500+ LC-MS/MS System with the optimized *Scheduled* MRM Algorithm method. The lower limit of quantification (LLOQ) and linear dynamic range (LDR) was investigated. The LLOQ is reported in the format of ppm, and the amount of NISTmAb per injection is consistent as 20 µg. All 48 proteins are quantified using this 8 min LC-MRM method with solid confirmation (2 peptides per protein for most proteins, 2 transitions per peptide). In summary, 23 out of 48 proteins (48%) can be quantified at 0.09-1 ppm and 24 out of 48 proteins (50%) can be quantified at 1-5 ppm (Figure 5). Accuracies of all peptides are 85-115% and the CV%s are within 15%. Representative XICs of UPS proteins at their LLOQ levels, calibration curves, and quantification results are shown in Figure 6, 7 and 8.

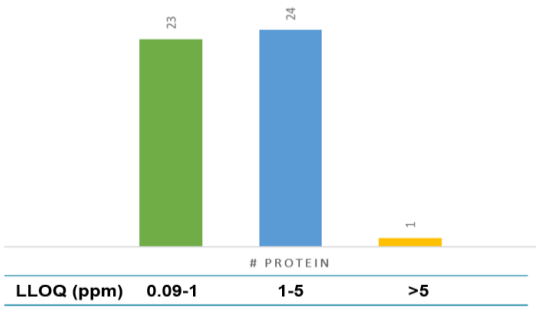

Figure 5. The summary of LLOQ of UPS proteins. 23 out of 48 proteins (48%) have LLOQs between 0.09 and 1 ppm, 24 out of 48 proteins (50%) have LLOQs between 1 and 5 ppm, 1 protein (small protein with limited peptide selection) have LLOQ larger than 5 ppm.

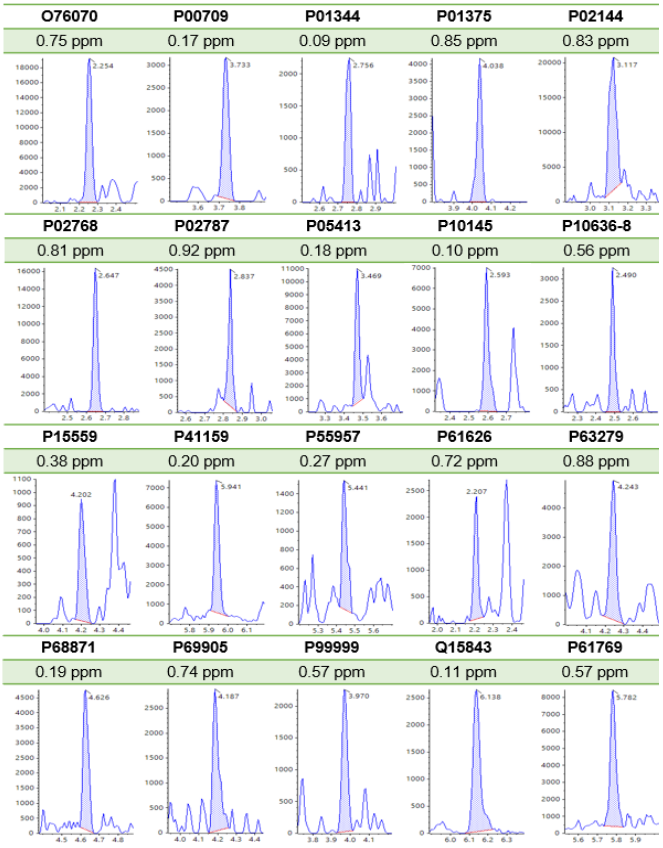

Figure 6. The XICs of the representative HCPs at their LLOQs. UniProt accession numbers and LLOQ concentrations are listed. The data are processed by using the Analytics function in SCIEX OS Software 1.7.

Row	Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
1	P01344ups|IGF2_HUMAN_UPS.GIVEEC[CAM]C[CAM]FR.+2y6.light	0.1	3 of 3	8.916e-2	1.300e-2	14.6	98.0
2	P01344ups|IGF2_HUMAN_UPS.GIVEEC[CAM]C[CAM]FR.+2y6.light	0.4	3 of 3	3.932e-1	3.040e-2	7.7	107.7
3	P01344ups|IGF2_HUMAN_UPS.GIVEEC[CAM]C[CAM]FR.+2y6.light	1.5	3 of 3	1.476e0	1.263e-1	8.6	101.1
4	P01344ups|IGF2_HUMAN_UPS.GIVEEC[CAM]C[CAM]FR.+2y6.light	5.8	3 of 3	5.985e0	6.907e-1	11.5	102.5
5	P01344ups|IGF2_HUMAN_UPS.GIVEEC[CAM]C[CAM]FR.+2y6.light	23.4	3 of 3	2.290e1	1.422e0	6.2	98.1
6	P01344ups|IGF2_HUMAN_UPS.GIVEEC[CAM]C[CAM]FR.+2y6.light	93.4	3 of 3	8.659e1	3.625e0	4.2	92.7
7	P02768ups|ALBU_HUMAN_UPS.LC[CAM]TVATLR.+2y6.light	0.8	3 of 3	7.904e-1	4.325e-2	5.5	97.6
8	P02768ups|ALBU_HUMAN_UPS.LC[CAM]TVATLR.+2y6.light	3.2	3 of 3	3.526e0	1.156e-1	3.3	108.8
9	P02768ups|ALBU_HUMAN_UPS.LC[CAM]TVATLR.+2y6.light	13.0	3 of 3	1.329e1	1.537e0	11.6	102.5
10	P02768ups|ALBU_HUMAN_UPS.LC[CAM]TVATLR.+2y6.light	51.8	3 of 3	5.471e1	2.618e0	4.8	105.5
11	P02768ups|ALBU_HUMAN_UPS.LC[CAM]TVATLR.+2y6.light	207.4	3 of 3	1.955e2	1.086e1	5.6	94.3
12	P02768ups|ALBU_HUMAN_UPS.LC[CAM]TVATLR.+2y6.light	829.5	3 of 3	7.566e2	2.477e1	3.3	91.2
13	P02787ups|TRFE_HUMAN_UPS.YLGEEYVK.+2y6.light	0.9	3 of 3	8.823e-1	4.307e-2	4.9	96.1
14	P02787ups|TRFE_HUMAN_UPS.YLGEEYVK.+2y6.light	3.7	3 of 3	4.224e0	5.885e-1	13.9	115.1
15	P02787ups|TRFE_HUMAN_UPS.YLGEEYVK.+2y6.light	14.7	3 of 3	1.511e1	1.227e0	8.1	102.9
16	P02787ups|TRFE_HUMAN_UPS.YLGEEYVK.+2y6.light	58.7	3 of 3	5.769e1	4.589e0	8.0	98.2
17	P02787ups|TRFE_HUMAN_UPS.YLGEEYVK.+2y6.light	234.9	3 of 3	2.165e2	1.201e1	5.5	92.2
18	P02787ups|TRFE_HUMAN_UPS.YLGEEYVK.+2y6.light	939.8	3 of 3	8.981e2	6.211e0	0.7	95.6

Figure 7. Representative quantification summary for three proteins (insulin-like growth factor II, serum albumin, serotransferrin, [apotransferrin]). Concentration ranges (unit is ppm), CV% and accuracies are listed.

Conclusions

A targeted HCP quantification workflow using the SCIEX QTRAP 6500+ LC-MS/MS System is presented here, demonstrating high sensitivity, analysis throughput, robustness and multiplexing capability.

- High resolution MS/MS peptide library generated by IDA analysis offers reliable signature peptide selection
- A seamless MRM method development workflow with the combination of Skyline and SCIEX OS Software allows fast and robust MRM method optimization
- A total of 48 proteins are quantified in an 8 min LC-MS/MS run with solid confirmation (4 transitions per protein)
- Superior sensitivity is achieved, with half of target proteins quantified at 0.09-1 ppm level, the other half quantified at 1-5 ppm level

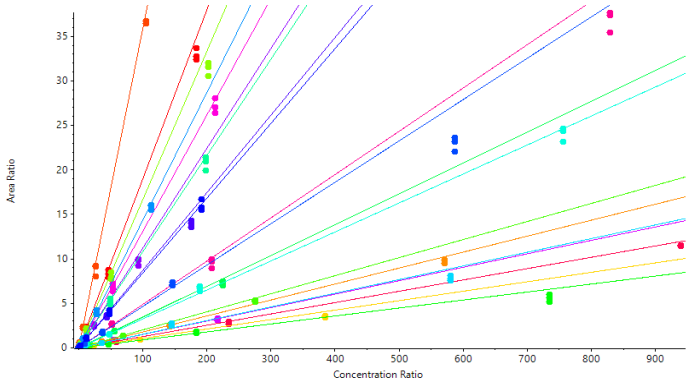

Figure 8. Representative calibration curves for HCPs. Dynamic ranges covers three orders of magnitude or more for most analytes.

Table A1. List of UPS proteins (protein names and uniProt accession numbers are included).

uniProt Accession Number	UniProt Protein Name [Synonym]	uniProt Accession Number	UniProt Protein Name [Synonym]
P00915	*Carbonic anhydrase 1*	*P55957*	*BH3 Interacting domain death agonist [BID]*
P00918	*Carbonic anhydrase 2*	*O76070*	*Gamma-synuclein*
P01031	*Complement C5 [Complement C5a]*	*P08263*	*Glutathione S-transferase A1 [GST A1-1]*
P69905	*Hemoglobin alpha chain*	*P01344*	*Insulin-like growth factor II*
P68871	*Hemoglobin beta chain*	*P01127*	*Platelet-derived growth factor B chain*
P41159	*Leptin*	*P10599*	*Thioredoxin*
P02768	*Serum Albumin*	*P99999*	*Cytochrome c[Apocytochrome c]*
P62988	*Ubiquitin*	*P06396*	*Gelsolin*
P04040	*Catalase*	*P09211*	*Glutathione S-transferase P [GST]*
P00167	*Cytochrome b5*	*P01112*	*GTPase HRas [Ras protein]*
P01133	*Epidermal Growth Factor*	*P01579*	*Interferon gamma (IFN-gamma)*
P02144	*Myoglobin C*	*P02787*	*Serotransferrin [Apotransferrin]*
P15559	*NAD(P)H dehydrogenase [quinone] 1 [DT Diaphorase] C*	*O00762*	*Ubiquitin-conjugating enzyme E2 C [UbcH10]*
P62937	*Peptidyl-prolyl cis-trans isomerase A [Cyclophilin A]*	*P51965*	*Ubiquitin-conjugating enzyme E2 E1 [UbcH6]*
Q06830	*Peroxiredoxin 1*	*P08758*	*Annexin A 5*
P63165	*Small ubiquitin-related modifier 1 [SUMO-1]*	*P02741*	*C-reactive protein*
P00709	*Alpha-lactalbumin*	*P05413*	*Fatty acid-binding protein*
P06732	*Creatine kinase M-type [CK-MM]*	*P10145*	*Interleukin-8*
P12081	*Histidyl-tRNA synthetase [Jo-1]*	*P02788*	*Lactotransferrin*
P61626	*Lysozyme C*	*P10636*	*Microtubule-associated protein tau [Tau protein]*
Q15843	*Neddylin [Nedd8]*	*P00441*	*Superoxide dismutase [Cu-Zn]*
P02753	*Retinol-binding protein*	*P01375*	*Tumor necrosis factor [TNF-alpha]*
P16083	*Ribosyldihydronicotinamide dehydrogenase [quinone] [Quinone oxidoreductase 2] [NQO2]*		
P63279	*Ubiquitin-conjugating enzyme E2 I [UbcH9]*		
P01008	*tithrombin-III*		
P61769	*Beta-2-microglobulin*		

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