Enhancing the sensitivity of peptide quantification for the targeted host cell proteins analysis

Using SCIEX Triple Quad™ 7500 LC-MS/MS System – QTRAP® Ready, powered by SCIEX OS Software

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Host cell proteins (HCPs) are a major class of process-related impurities that accompany a recombinant biotherapeutic product during production. As their levels impact the potential toxicity and efficiency of the therapeutics, there are significant requirements for the quantitative measurement of HCP's across the entire development paradigm, from discovery to quality control.

In downstream manufacturing and QC stages, the HCP targets for quantification are typically predefined, and their levels in the final drug substance or late purification steps can be at the trace level. This results in the increased need for targeted HCP analysis methods with high sensitivity, reduced analysis time, robustness and multiplexing capability (quantify significant numbers of analytes in one injection). Triple quadrupole and QTRAP LC-MS/MS Systems are ideal instrument platforms for such work due to their high quantitative performance.

Herein, a targeted HCP analysis workflow utilizing the SCIEX Triple Quad 7500 LC-MS/MS System – QTRAP Ready has been developed. Multiple hardware improvements on the ion source and the front end of the mass analyzer significantly boost the instrument sensitivity.[1]

The Scheduled MRM™ Algorithm[2] was used to enable the simultaneous quantification of 48 proteins (4 transitions per protein) in an 8 min LC-MS analysis, with LLOQs ranging from 0.02 to 4.54 ppm.

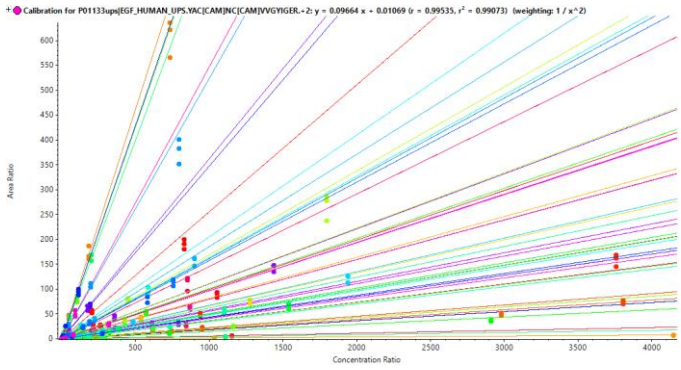

Figure 1. Representative calibration curves for selected peptides from 48 host cell proteins.

Key features of targeted HCP quantification workflow

- Enhanced sensitivity over previous generation systems, with a 4-fold S/N improvement compared to the SCIEX Triple Quad 6500+ LC-MS/MS System[1]
- Hardware advances in ion generation (OptiFlow® Pro Ion Source with E Lens™ Technology) and ion sampling (D Jet™ Ion Guide)
- SCIEX OS Software for data acquisition and processing—single easy to use platform for acquisition, processing and management
- Scheduled MRM Algorithm enabled high multiplexing to measure over 200 MRM transitions in an 8 min LC-MS/MS run by maximizing duty cycle
- The assay shows a high level of reproducibility, accuracy, and linearity, proving the robustness and performance of the developed method

Methods

Sample preparation: NISTmAb monoclonal antibody (NISTmAb) and the Universal Proteomics Standard (UPS) were purchased from Sigma-Aldrich. In this experiment model, NISTmAb was serving as the biotherapeutic molecule, while the 48 human proteins in the UPS mix were used to mimic the targeted HCPs for quantification. Bovine serum albumin (BSA) was used as the internal standard. The UPS proteins and BSA were spiked into NISTmAb solution to prepare high concentration standard sample. It was then serial diluted using the NISTmAb solution with BSA. The levels of NISTmAb and BSA remains consistent among all samples.[3]

Samples were 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 overnight, with an enzyme-protein ratio at 1:25. Formic acid was spiked into the samples to abort digestion. The samples were then centrifuged at the speed of 12000 g and injected into LC-MS analysis.

LC-MS conditions: Samples were analyzed in triplicate by a SCIEX Triple Quad 7500 LC-MS/MS System – QTRAP Ready, coupled with an ExionLC™ System. The method details are summarized in Table 1, 2 and Table A1. The same sample set was also analyzed using a SCIEX Triple Quad 6500+ LC-MS/MS System, coupled with the same HPLC system, to compare the performance of the two mass spectrometers. All MRM parameters were optimized on both mass spectrometers for the most accurate performance comparison.

Data processing: *Data are processed using* the Analytics function in SCIEX OS Software 2.0.

Table 1. HPLC conditions 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*

Table 2. Gas/source parameters on the SCIEX 7500 System.

Parameter	Value	Parameter	Value
Curtain gas	*40 psi*	*Source temperature*	550 *°C*
Ion source gas 1	*50 psi*	*Ion source gas 2*	70 *psi*
CAD gas	*11 psi*	*Ion spray voltage*	1500 V

Signature peptide selection and MRM method development

The signature peptide selection and MRM method development process are reported in a previous technical note.[1] An IDA analysis was performed on a TripleTOF® 6600+ LC-MS/MS System to analyze the digested NISTmAb-UPS protein mix. The IDA data was processed by ProteinPilot™ Software 5.0, searched against the protein sequences of NISTmAb, UPS proteins and BSA. The database search result file (serving as the peptide library) and protein sequences were then imported into Skyline software. The list of MRM transitions was generated based on peptide/transitions settings and peptide library matching.

The list of MRM transitions was then imported into SCIEX OS Software and test injections were performed to determined the retention times of each peptide using the fast 8-min gradient (Figure 2). Retention times were then uploaded to the Scheduled MRM Algorithm and the optimized method was computed, maximizing dwell times for every MRM. This time scheduled method was used to acquire all the calibration curve data.

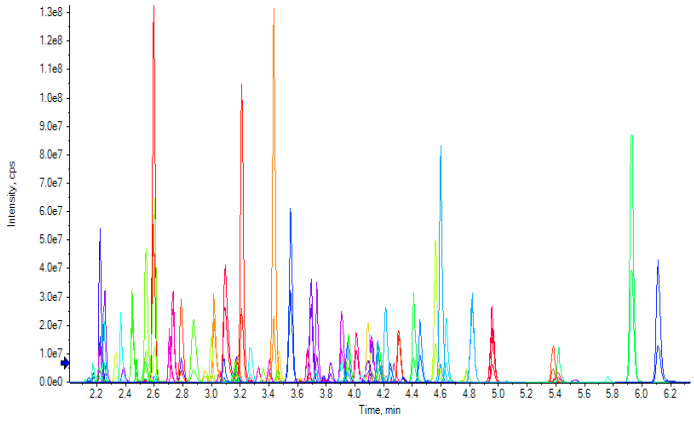

Figure 2. High quality chromatography achieved. The extracted ion chromatograms (XICs) of 48 target proteins (~200 transitions) demonstrate the LC-MRM based targeted HCP quantification workflow.

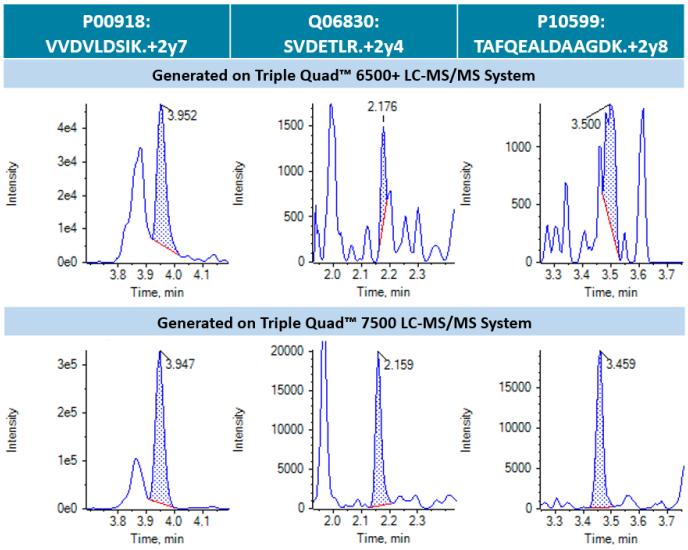

Figure 3. Sensitivity gains observed. MRM XIC comparison between SCIEX 7500 System (bottom) and SCIEX Triple Quad 6500+ LC-MS/MS System (top) on representative peptides.

HCP quantification using the SCIEX Triple Quad 7500 LC-MS/MS System – QTRAP Ready

Improvements to the ion generation and sampling have led to increased sensitivity on the SCIEX 7500 System. To characterize the impact on host cell protein quantification workflows, the UPS protein serial dilution samples were analyzed on both the SCIEX 7500 System and the SCIEX Triple Quad 6500+ LC-MS/MS System. On average, a 4-fold difference in S/N was observed (Figure 3).

Holding the amount of NISTmAb constant per injection at 20 µg, the LLOQs for the UPS proteins (the HCPs) were reported in the format of ppm. All 48 proteins were quantified using 2 peptides per protein for most proteins and 2 transitions per peptide (Figure 3) for confidence in detection and quantification. The LLOQs determined ranged from 0.02 ppm to 4.54 ppm, with 2/3 of them between 0.02 and 1 ppm. The rest were between 1 and 4.54 ppm (Figure 4). Accuracies of all peptides are 85-115% and the %CV are within 15%. Representative XICs of UPS proteins at their LLOQ levels, calibration curves, and quantification results are shown in Figures 1, 4 and 5.

Conclusions

- A targeted HCP quantification workflow using the SCIEX 7500 System has been developed that demonstrates the high sensitivity, high analysis throughput, robustness and multiplexing capability of the platform

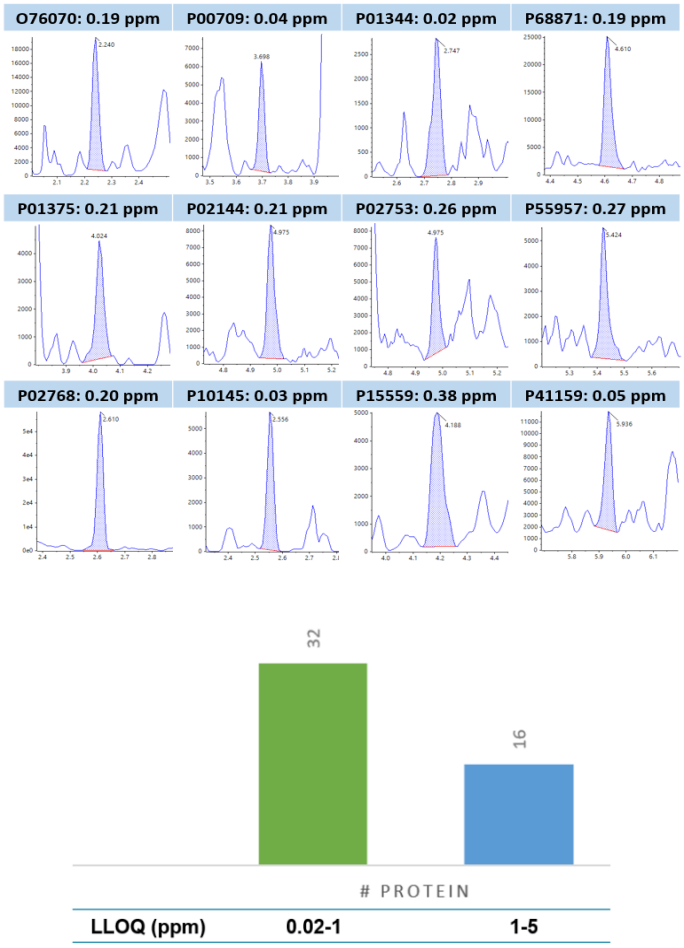

Figure 4. The summary of LLOQs of UPS proteins. (Top) MRM XICs of peptides from HCPs at their LLOQs, UniProt accession numbers and LLOQ concentrations are listed. (Bottom) 32 out of 48 proteins (67%) have LLOQs between 0.02 and 1 ppm.16 out of 48 proteins (33%) have LLOQs between 1 and 5 ppm.

- An average 4-fold S/N improvement was observed over the SCIEX Triple Quad 6500+ System[1]
- Superior sensitivity was achieved, with 2/3 of target proteins having LLOQs in the 0.02-1 ppm range, and the rest of the proteins in the range of 1-4.54 ppm.
- A total of 48 proteins were quantified in an 8 min LC-MS/MS run with high confidence (4 transitions per protein)

References

1. Enabling new levels of quantification. SCIEX technical note RUO-MKT-02-11886-A.
2. The Scheduled MRM™ Algorithm Pro. SCIEX Technical note RUO-MKT-02-8539-A.
3. Highly sensitive LC-MS/MS workflow for targeted quantification of host cell proteins. SCIEX technical note RUO-MKT-02-11418-A.

Component Name	Actual Conc...	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
P01344ups|IGF2_HUMAN_UPS.GIVEEC[CAM]C[CAM]FR.+2y6.	0.02	3 of 3	2.191e-2	2.346e-3	10.71	95.28
P01344ups|IGF2_HUMAN_UPS.GIVEEC[CAM]C[CAM]FR.+2y6.	0.09	3 of 3	1.087e-1	8.379e-3	7.71	119.40
P01344ups|IGF2_HUMAN_UPS.GIVEEC[CAM]C[CAM]FR.+2y6.	0.37	3 of 3	3.563e-1	2.084e-2	5.85	97.63
P01344ups|IGF2_HUMAN_UPS.GIVEEC[CAM]C[CAM]FR.+2y6.	1.46	3 of 3	1.471e0	8.870e-2	6.03	100.73
P01344ups|IGF2_HUMAN_UPS.GIVEEC[CAM]C[CAM]FR.+2y6.	5.84	3 of 3	5.192e0	6.381e-2	1.23	88.90
P01344ups|IGF2_HUMAN_UPS.GIVEEC[CAM]C[CAM]FR.+2y6.	23.36	3 of 3	2.345e1	1.417e0	6.04	100.38
P01344ups|IGF2_HUMAN_UPS.GIVEEC[CAM]C[CAM]FR.+2y6.	93.44	3 of 3	9.126e1	1.041e1	11.41	97.67
Component Name	**Actual Conc...**	**Num. Values**	**Mean**	**Standard Deviation**	**Percent CV**	**Accuracy**
P01375ups|TNFA_HUMAN_UPS.VNLLSAIK.+2y7.light	0.21	3 of 3	2.131e-1	3.175e-2	14.89	100.54
P01375ups|TNFA_HUMAN_UPS.VNLLSAIK.+2y7.light	0.85	3 of 3	8.228e-1	2.495e-2	3.03	97.15
P01375ups|TNFA_HUMAN_UPS.VNLLSAIK.+2y7.light	3.39	3 of 3	3.493e0	4.035e-1	11.55	103.07
P01375ups|TNFA_HUMAN_UPS.VNLLSAIK.+2y7.light	13.56	3 of 3	1.318e1	1.560e0	11.84	97.21
P01375ups|TNFA_HUMAN_UPS.VNLLSAIK.+2y7.light	54.23	3 of 3	5.784e1	4.111e0	7.11	106.66
P01375ups|TNFA_HUMAN_UPS.VNLLSAIK.+2y7.light	216.91	3 of 3	2.262e2	1.523e1	6.74	104.26
P01375ups|TNFA_HUMAN_UPS.VNLLSAIK.+2y7.light	867.65	3 of 3	7.906e2	4.000e1	5.06	91.12
Component Name	**Actual Conc...**	**Num. Values**	**Mean**	**Standard Deviation**	**Percent CV**	**Accuracy**
P02144ups|MYG_HUMAN_UPS.HGATVLTALGGILK.+3b5.light	0.21	3 of 3	2.083e-1	2.865e-2	13.76	100.15
P02144ups|MYG_HUMAN_UPS.HGATVLTALGGILK.+3b5.light	0.83	3 of 3	8.232e-1	2.467e-2	3.00	98.82
P02144ups|MYG_HUMAN_UPS.HGATVLTALGGILK.+3b5.light	3.33	3 of 3	3.369e0	4.529e-1	13.44	101.15
P02144ups|MYG_HUMAN_UPS.HGATVLTALGGILK.+3b5.light	13.32	3 of 3	1.369e1	1.248e0	9.12	102.74
P02144ups|MYG_HUMAN_UPS.HGATVLTALGGILK.+3b5.light	53.29	3 of 3	5.852e1	6.625e-1	1.13	109.81
P02144ups|MYG_HUMAN_UPS.HGATVLTALGGILK.+3b5.light	213.16	3 of 3	2.070e2	2.198e1	10.62	97.10
P02144ups|MYG_HUMAN_UPS.HGATVLTALGGILK.+3b5.light	852.65	3 of 3	7.694e2	9.460e1	12.30	90.23
Component Name	**Actual Conc...**	**Num. Values**	**Mean**	**Standard Deviation**	**Percent CV**	**Accuracy**
P05413ups|FABPH_HUMAN_UPS.LILTLTHGTAVC[CAM]TR.1	0.18	3 of 3	1.802e-1	1.528e-2	8.48	100.13
P05413ups|FABPH_HUMAN_UPS.LILTLTHGTAVC[CAM]TR.1	0.72	3 of 3	7.108e-1	7.111e-2	10.00	98.86
P05413ups|FABPH_HUMAN_UPS.LILTLTHGTAVC[CAM]TR.1	2.88	3 of 3	2.913e0	3.641e-1	12.50	101.28
P05413ups|FABPH_HUMAN_UPS.LILTLTHGTAVC[CAM]TR.1	11.51	3 of 3	1.186e1	7.617e-1	6.42	103.11
P05413ups|FABPH_HUMAN_UPS.LILTLTHGTAVC[CAM]TR.1	46.02	3 of 3	4.889e1	4.387e0	8.97	106.24
P05413ups|FABPH_HUMAN_UPS.LILTLTHGTAVC[CAM]TR.1	184.09	3 of 3	1.858e2	1.482e1	7.98	100.95
P05413ups|FABPH_HUMAN_UPS.LILTLTHGTAVC[CAM]TR.1	736.35	3 of 3	6.585e2	3.983e1	6.05	89.43
Component Name	**Actual Conc...**	**Num. Values**	**Mean**	**Standard Deviation**	**Percent CV**	**Accuracy**
P41159ups|LEP_HUMAN_UPS.VTGLDFIPGLHPILTLSK.+3y11+2	0.05	3 of 3	4.938e-2	6.649e-3	13.46	100.78
P41159ups|LEP_HUMAN_UPS.VTGLDFIPGLHPILTLSK.+3y11+2	0.20	3 of 3	1.934e-1	2.484e-2	12.84	98.18
P41159ups|LEP_HUMAN_UPS.VTGLDFIPGLHPILTLSK.+3y11+2	0.79	3 of 3	7.431e-1	1.537e-2	2.07	94.18
P41159ups|LEP_HUMAN_UPS.VTGLDFIPGLHPILTLSK.+3y11+2	3.16	3 of 3	3.230e0	1.070e-1	3.31	102.33
P41159ups|LEP_HUMAN_UPS.VTGLDFIPGLHPILTLSK.+3y11+2	12.62	3 of 3	1.224e1	2.971e-1	2.43	96.94
P41159ups|LEP_HUMAN_UPS.VTGLDFIPGLHPILTLSK.+3y11+2	50.49	3 of 3	5.442e1	8.632e-1	1.59	107.78
P41159ups|LEP_HUMAN_UPS.VTGLDFIPGLHPILTLSK.+3y11+2	201.98	3 of 3	2.016e2	7.902e0	3.92	99.80

Figure 5. Representative quantification summary for five proteins. Concentration ranges (unit is ppm), %CV and accuracies are listed.

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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