

# 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.<sup>1</sup>

The Scheduled MRM™ Algorithm<sup>2</sup> 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.



## 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<sup>1</sup>
- 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

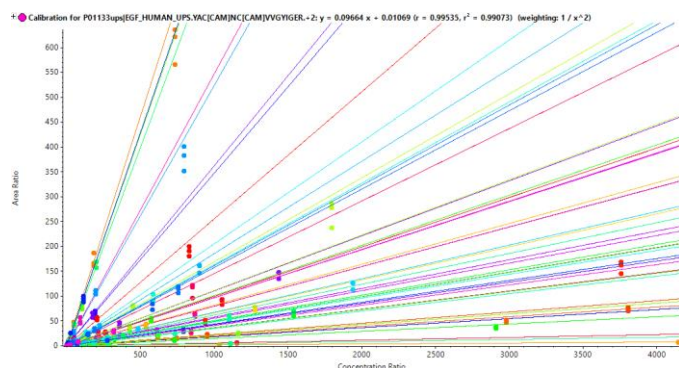


Figure 1. Representative calibration curves for selected peptides from 48 host cell proteins.

## 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.<sup>3</sup>

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

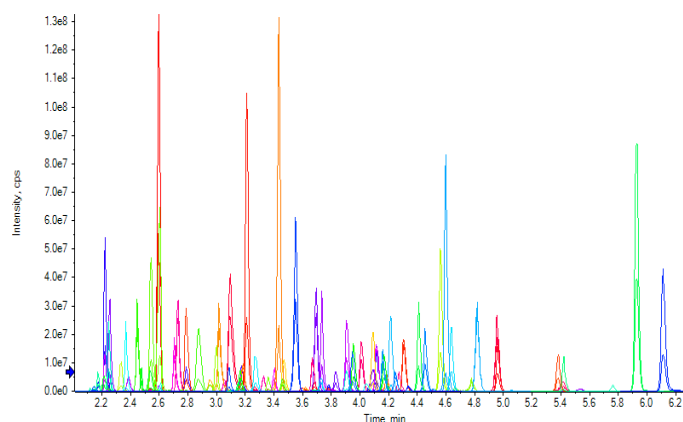
**Table 2. Gas/source parameters on the SCIEX 7500 System.**

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

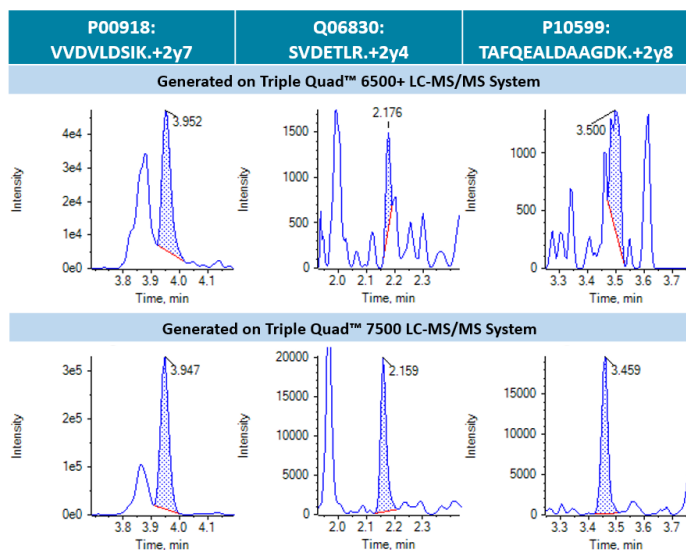
## Signature peptide selection and MRM method development

The signature peptide selection and MRM method development process are reported in a previous technical note.<sup>1</sup> 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.

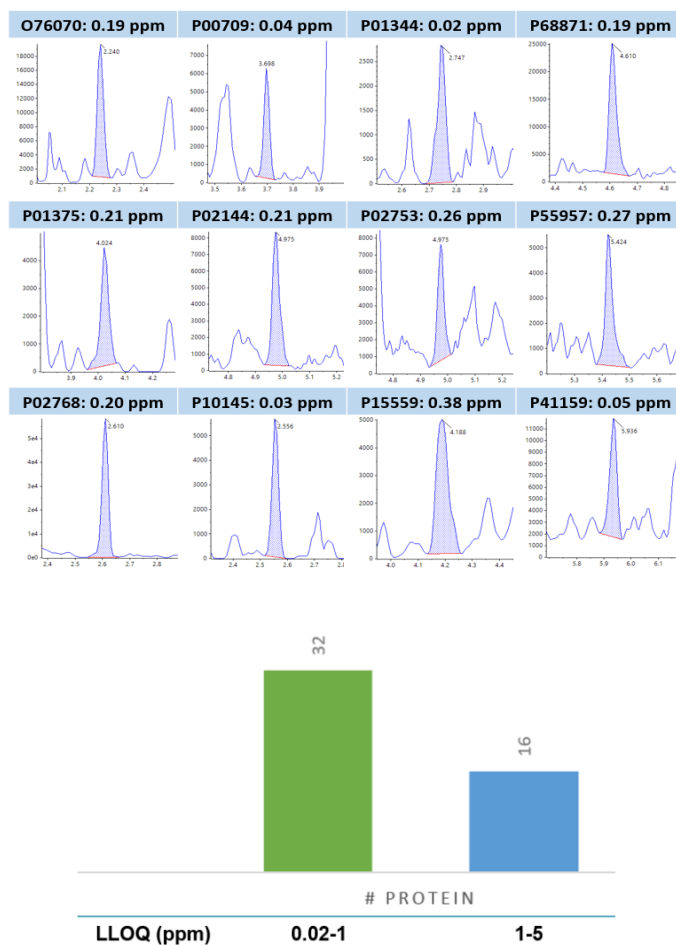
## HCP quantification using the SCIEX Triple Quad 7500 LC-MS/MS System – QTRAP Ready

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<sup>1</sup>
- 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

- Enabling new levels of quantification. [SCIEX technical note RUO-MKT-02-11886-A](#).
- [The Scheduled MRM™ Algorithm Pro. SCIEX Technical note RUO-MKT-02-8539-A](#).
- 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	Ribosyl-dihydronicotinamide dehydrogenase [quinone] [Quinone oxidoreductase 2] [NQO2]		
P63279	Ubiquitin-conjugating enzyme E2 I [UbcH9]		
P01008	tithrombin-III		
P61769	Beta-2-microglobulin		

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