A robust quantitative workflow for the target host cell proteins using denaturing digest, standard-addition and SWATH-based LC-MS/MS platform

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ABSTRACT

Host cell proteins (HCPs) are those produced by the organisms and unrelated to the intended recombinant product. Residual HCPs in the drug product can affect product quality, stability, and safety. LC-MS/MS based methods [1-3] have been widely used for HCP identification and quantitation in biotherapeutics.

A SWATH-based platform was developed to quantify target HCPs to support process development. Two quantitative strategies (compared with reference protein vs standard addition curve) were evaluated for this platform.

- Detection and quantitative limit were established at ~1 ppm and ~2.5 ppm.
- Both native digestion and protein denaturation digestion were assessed for a target protein.
- The relative quantitation results were further confirmed by a protein-specific ELISA.

MATERIALS & METHODS

Sample Preparation:

For the denaturing tryptic digestion condition [1] :

Each sample (500 mg) was denatured with 6 M guanidine HCl in 1 mM sodium acetate (pH 5), then reduced with TCEP for 15 min at 37 C. Following desalting with a NAP-5 column, the pH of the solution was adjusted to 7.2 by addition of MOPS. Trypsin (10 µg) was added to the samples, followed by incubation at 37 °C for 1 hr. The digestion was quenched with TFA and the sample was injected for LC-MS/MS analysis.

For the native tryptic digestion condition [2, 3] :

Each sample (1000 mg) was digested by 2.5 µg trypsin for 2 hr or overnight at 37 C. Samples were reduced by 12 mM TECP and heated at 90°C for 10 min to precipitate the undigested or intact proteins at 13,000 g for 2.5 min. The digestion was quenched with TFA and the sample was injected for each LC-MS/MS analysis.

SWATH LC-MS/MS Analysis:

LC separation was performed on a Waters CSH130 C18 (1.7 mm, 2.1 mm x 150 mm) column with a 40 min LC gradient of 0–40% acetonitrile at 0.3 mL/min. SWATH was performed on Sciex 6600 TTOF using 20 sequential m/z windows of 25 amu (1 amu mass window overlap; 22 ms per window) ranging from m/z 400-1250 interleaved with a single 100 ms full scan TOF MS spectrum per cycle to give a total cycle time of 1 sec.

Data Analysis:

Skyline is used for quantitative data analysis. With the conventional strategy, protein quantitation is based on the average signal responses of the top 3 peptides per mole of protein in comparison with a known spiked reference protein. Using the standard addition quantitative strategy, protein concentration was extrapolated from a standard addition calibration curve generated by spiking various known amounts of the corresponding recombinant protein standard into each sample of interest. Spectronaut was used for peptide identification by SWATH DIA.

RESULTS & DISCUSSION

1. Native digest shows higher detection sensitivity for spiked HCPs by SWATH-LC-MS/MS analysis

	Native digest	Denaturing digest
Spiked HCP	LOD (ppm)	LOD (ppm)
Protein 1	0.625	12.5
Protein 2	1.25	25
Protein 3	1.25	50
Protein 4	0.625	25
Protein 5	1.25	100
Protein 6	2.5	25
Protein 7	2.5	100
Protein 8	2.5	25
Protein 9	5	12.5
Protein 10	2.5	25

LOD: Limit of detection based on >=2 unique peptides per protein (0.01 QValue)
Recombinant proteins were spiked at various levels in a reference mAb to establish the LOD.

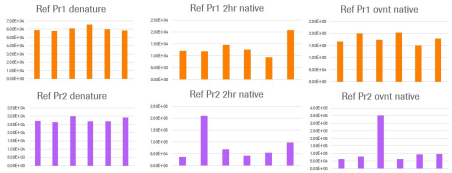
2. SWATH relative quantitation is reference protein dependent; denaturing condition generates more accurate results for a SST protein

SST Protein (theoretical 50ppm, ~70 kD)	Denaturing (ppm)
Based on Ref Protein 1 (~30 kD)	130.0 (+160.1% error)
Based on Ref Protein 2 (~70 kD)	44.1 (-11.8% error)

SST Protein (theoretical 5 ppm)	Native 2hr (measured ppm)	Native ovnt (measured ppm)
Based on Ref Protein 1 (~30kD)	181.2 (3524% error)	185 (3600%)
Based on Ref Protein 2 (~70kD)	1.1 (-78% error)	8.4 (68% error)

More accurate results were observed for the estimation of SST (system suitability protein) using reference protein2 under denaturing condition.
(Note: Error is (measured – theoretical)/ theoretical *100%))

3. Digest consistency is protein-dependent and more consistent digested peptide responses observed under denaturing condition



Two different reference proteins were spiked at the same levels across UFDF pools. Reference protein 2 (purple bars) shows quite different responses under native digest (2 hr or overnight) across replicates.

4. Relative quantitation based on standard addition curve shows good linearity for selected proteins under native and denaturing digest conditions (2.5-200ppm)

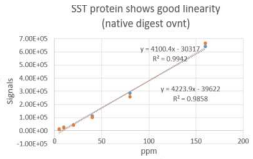


Fig. 4.1

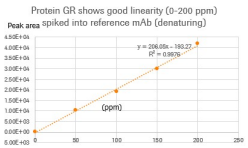


Fig. 4.2

Nine selected proteins were added into a reference mAb at various levels (2.5-160 ppm, or 0-200 ppm). Good linearity and reproducibility was observed for representative proteins under native (Fig. 4.1) or denaturing digest conditions (Fig. 4.2).

5. Quantitation of a target protein GR using different quantitative methods by SWATH LC-MS/MS analysis

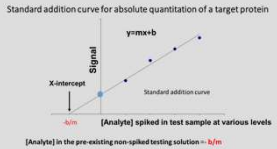


Fig. 5.1 Principle of standard addition to estimate the endogenous HCP levels in a protein drug in-process pools

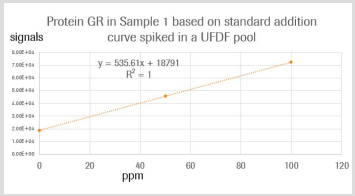


Fig. 5.2 Estimation of endogenous protein GR levels by adding 0ppm, 50ppm, 100ppm of recombinant GR to the UFDF pools and analyzed by SWATH LC-MS/MS method

protein GR in UFDF pools	Based on spiked standard addition (ppm)	Based on comparison with reference protein 2 (ppm)	Commercial ELISA kit (ppm)
Sample 1	35.1	45.1	13.0
Sample 2	47.7	52.2	16.0
Sample 3	21.4	34.6	10.3

Quantitative results are comparable between different quantitative methods using SWATH and correlated with ELISA results

SUMMARY / CONCLUSIONS

- Current native digest is more sensitive than denaturing digest method for protein identification using SWATH method (10-40 folds).
- Digest conditions should be selected based on the target protein and the quantitative method. Relative quantitation based on comparison with a reference protein is highly dependent on the selected protein.
- Denaturing digest may generate more consistent and accurate estimation for relative high levels of protein at >20-50 ppm using reference proteins.
- Quantitation based on standard addition curve is robust under both native and denaturing conditions. Quantitation of a target protein GR shows comparable results analyzed by different quantitative methods using SWATH.

REFERENCES

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