

# Quantitation of Intact Therapeutic Protein in Plasma Matrix by LC/MS

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With unprecedented growth of protein therapeutics in the pharmaceutical industry, therapeutic proteins have been an important component of medical practice and clinical research. This advancement is paralleled with development of analytical technologies and methods to detect, identify, monitor and quantify therapeutic proteins. LC/MS based quantitation of intact therapeutic proteins using high-resolution mass spectrometry (HRMS) is of great interest, as it offers a high throughput platform allowing accurate quantitation and differentiation of major modifications at the intact protein level.

For quantitation in matrices there are several challenges including distribution of signal over many charge states, interference from matrix species and limited guidance on data analysis. A common practice for quantitation is the use of extracted ion chromatograms (XICs) from the non-deconvoluted full-scan mass spectra. This approach may not be feasible in the case of intact therapeutic monoclonal antibodies in biological matrix, due to interference from matrix proteins and low intensity of target ions at low concentrations.<sup>1,2</sup> Another approach is to quantify using the reconstructed spectrum of the target peak in the chromatogram<sup>1</sup>; However, this may lead to loss of original

information during peak selection and impact the robustness of method especially for complex samples.<sup>2</sup>

With the advancement of data analysis software, we developed a novel data processing strategy for quantitation purpose, utilizing the available protein deconvolution algorithm to reconstruct the entire data file by deconvoluting every full-scan mass spectrum recorded in the data file. XICs of major glycoforms of target intact therapeutic monoclonal antibody in the reconstructed data are used for quantitation.

## Key Features of SCIEX Intact Mass Workflow

- Generic workflow to quantify therapeutic proteins in plasma matrix
- Straightforward data processing strategy for enhanced selectivity and sensitivity
- Highly linear quantitative quantification data across a wide dynamic range at intact mass level
- Improved quantitative performance using reconstructed data compared to raw data

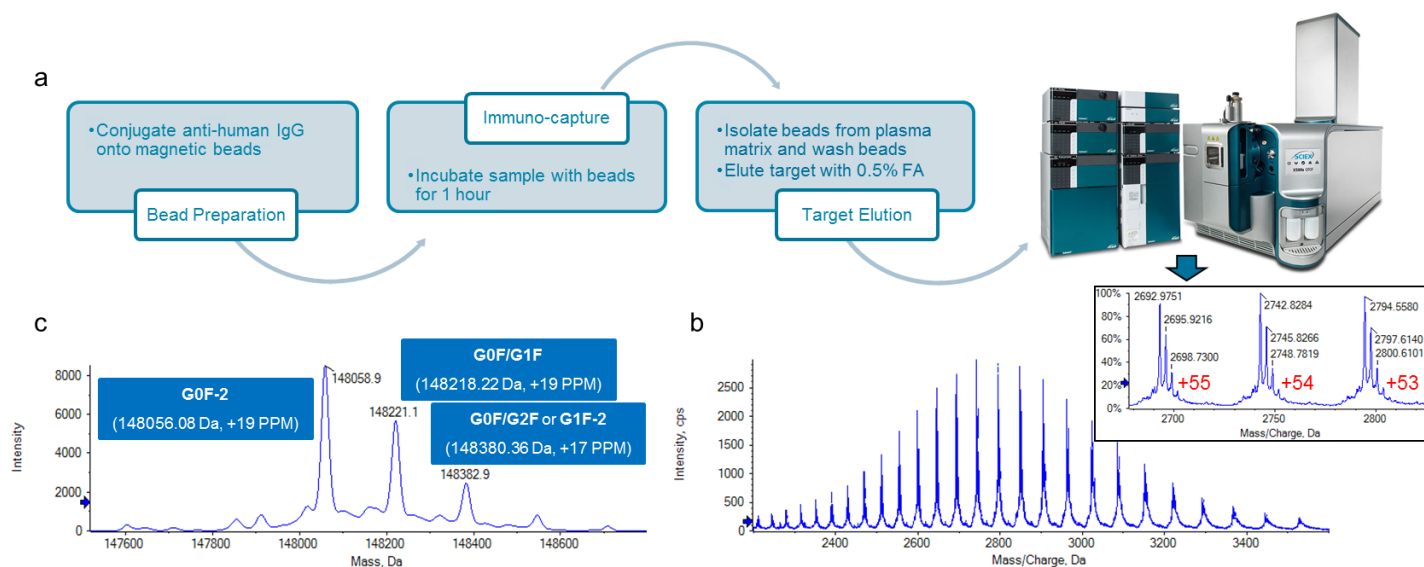


Figure 1. a) Immuno-capture workflow for target enrichment/purification; b) raw spectrum of enriched/purified trastuzumab from rat plasma, generated in SCIEX X500B QTOF system; c) deconvoluted data of raw spectrum in fig.1b (Input m/z range: 2200-3600. Resolution: 5000. Output mass range: 130-170 kDa).

## Methods

### Sample Preparation:

Biotinylated anti-human IgG antibody was conjugated onto streptavidin coated magnetic beads. The conjugated beads were incubated with plasma samples, to capture trastuzumab by immuno-affinity. The beads were then isolated from plasma matrix by magnetic attraction. After plasma was removed, the beads were washed twice to clean up residual plasma proteins. 50  $\mu$ L of 0.5% formic acid was added to elute trastuzumab from beads.

### Chromatography:

SCIEX ExionLC™ AD system coupled to SCIEX X500B QTOF system was used with an Agilent PLRP-S reversed-phase, 50 x 2.1 mm, 300 Å, 5  $\mu$ m column. The mobile phase used for the analysis was composed of buffer A (99.9% water, 0.1% formic acid) and buffer B (99.9% acetonitrile, 0.1% formic acid) at a flow rate of 500  $\mu$ L/min. The injection volume was set to 30  $\mu$ L.

**Table 1. Chromatographic Gradient.**

Time	Flow Rate (ml/min)	%A	%B
0.0	0.5	75	25
3.0	0.5	75	25
4.5	0.5	55	45
4.7	0.5	5	95
7.5	0.5	5	95
7.6	0.5	75	25
10.0	0.5	75	25

### Mass Spectrometry:

Mass spectrometric analysis was accomplished using a SCIEX X500B QTOF operated using SCIEX OS Acquisition software. Instrument parameters used are outlined in Table 1.

**Table 1. Mass Spectrometry Conditions.**

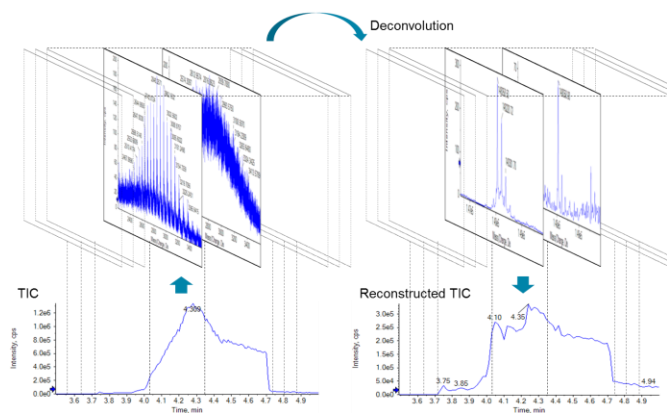
Parameter	Setting
Ion Source Gas 1	60
Ion Source Gas 2	60
Curtain Gas	40
Temperature (°C)	500
Scan Type	TOF MS
Polarity	Positive
Ion Spray Voltage (V)	5500
Time Bins to Sum	80
TOF start mass (m/z)	2200
TOF stop mass (m/z)	3600
Accumulation time (sec)	0.25
Declustering Potential (V)	250
Collision Energy (V)	10

### Data Processing:

Data was processed using a research version of PeakView® Software 1.2.2.0 with Bio Tool Kit 1.0.0.0.

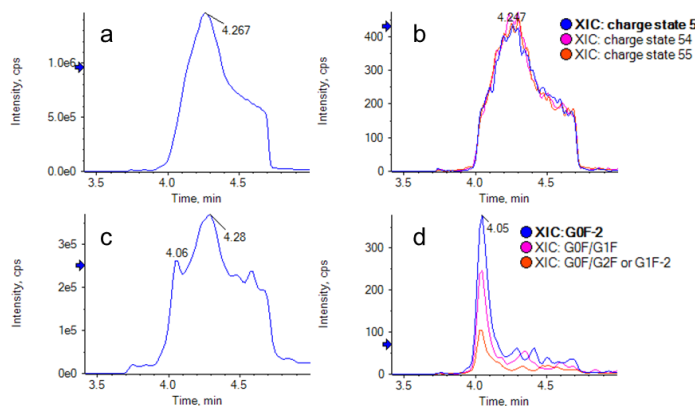
## Results and Discussion

One of the challenges quantifying intact therapeutic proteins in biological matrix is interference from sample matrix. Though the majority of sample matrix is removed during sample preparation using immuno-capture workflow, interference from residual matrix proteins is frequently observed in the final data sets. The ions from residual matrix proteins (endogenous IgG, etc.) might overlap with targeted ions for quantification. As a result of this, generating XICs from non-deconvoluted spectra is challenging. In this study this approach could not isolate trastuzumab signals from interference (see Fig. 3b). As a result, the accuracy of quantitation suffered at low concentrations. Compared to the approach using XICs from non-deconvoluted mass spectra, data processing strategy utilizing deconvoluted data provided clear advantages in case of complex samples. For optimal results adjustment of both input  $m/z$  range and output protein mass range were defined in protein deconvolution algorithm, so that matrix proteins not in the output mass range were filtered out.



**Figure 2. Data Reconstruction.** Every full-scan mass spectrum was deconvoluted individually; then the original spectrum was replaced by the corresponding deconvoluted data, to generate a reconstructed data file.

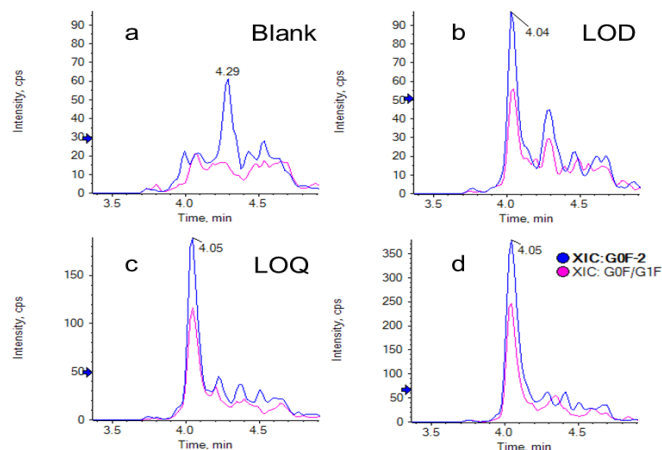
With conventional data analysis software, deconvolution is only executed on selected raw mass spectra from selected chromatographic peaks in individual data files. To overcome the concern of loss of original information and robustness of analysis, an automated data reconstruction workflow in Research PeakView Software 1.2.2.0 with Bio Tool Kit (protein/peptide reconstruction) 1.0.0.0 was developed: In this software every raw mass spectrum in the chromatogram is deconvoluted, resulting in a reconstructed ion chromatogram which corresponds to the reconstructed data. (Fig.2) Automated batch analysis of multiple data files is also enabled using this approach.



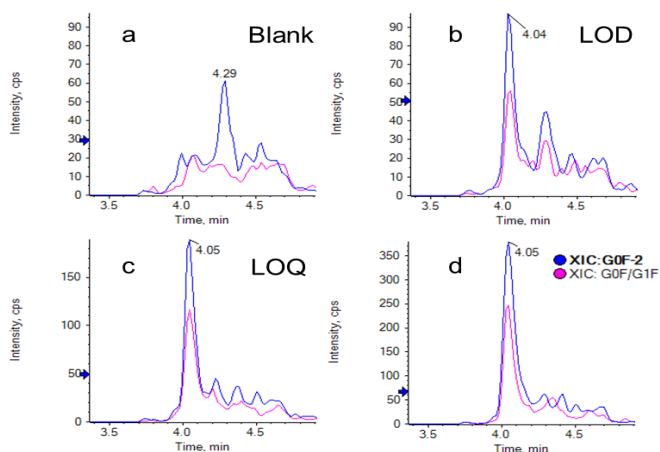
**Figure 3. Chromatograms of 200 ng/mL Trastuzumab in Rat Plasma.** a) TIC, non-deconvoluted; b) XICs of the most abundant charge states (see Fig.1b) from non-deconvoluted data, isolation window  $\pm 0.1$  Th; c) TIC after data reconstruction; d) XICs of major glycoforms (see Fig.1c) in deconvoluted data after data reconstruction, isolation window  $\pm 2$  Da.

After data reconstruction, extracted ion chromatograms of major glycoforms of trastuzumab were generated based on theoretical average masses. As shown in Figure 3d, interference from matrix proteins was efficiently filtered out using this reconstruction approach and clean trastuzumab peaks formed in LC chromatograms enabling facile quantification of intact protein species.

Peak selection and integration in resulting XICs were automatically completed in Research PeakView software, with information of peak area and retention time provided. Quantitation was carried out using peak area of major glycoforms as shown in Figure 4.

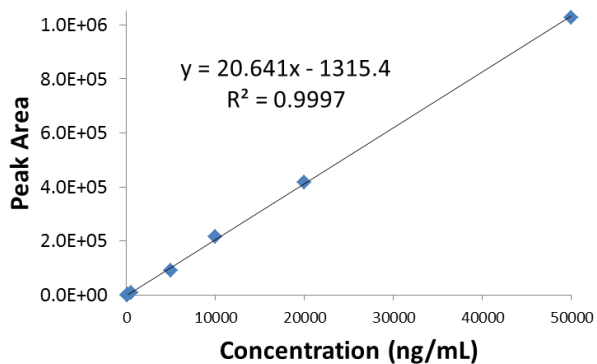


**Figure 4. XICs of Glycoforms G0F-2 and G0F/G1F.** a) blank; b) 50 ng/mL, LOD; c) 100 ng/mL, LOQ; d) 200 ng/mL.

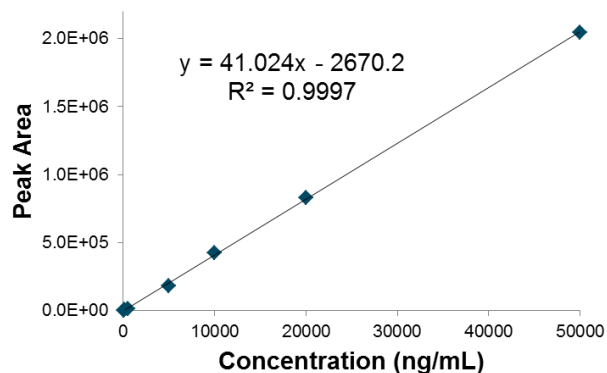


**Figure 5. Deconvoluted Data of Trastuzumab at 4.05 min.** a) blank; b) 50 ng/mL, LOD; c) 100 ng/mL, LOQ; d) 200 ng/mL.

Glycoforms of G0F-2 and G0F/G1F were detected in 50 ng/mL of trastuzumab in rat plasma (Fig.5b), with S/N ratios of 4 and 2.25. Quantitation was achieved with a linear dynamic range of 100 to 50 000 ng/mL, using either peak area of glycoform G0F-2 or summed peak area of glycoforms G0F-2 and G0F/G1F (Fig. 6 and 7).



**Figure 6. Calibration Curve using Peak Area of Glycoform G0F-2.**



**Figure 7. Calibration Curve using Peak Area of Glycoform G0F-2.**

By comparing data in Table 1 and 2, the observed consistency between calculated concentrations and accuracy confirmed that the intact reconstruction data processing strategy for each spectrum provides consistent results evidenced by data deconvolution/reconstruction and XIC peak selection / integration. Based on this observation, the ratio of glycoform G0F-2 peak area and glycoform G0F/G1F peak area was used to estimate the concentration ratio of these two major glycoforms in the samples.

**Table 1. Data of Calibration Standards using Peak Area of Glycoform G0F-2.**

Actual Conc. (ng/mL)	RT (Min)	Peak Area	Calculated Conc. (ng/mL)	Accuracy %
100	4.05	1030.46	113.65	113.65
200	4.05	2366.06	178.36	89.18
500	4.05	7629.86	433.37	86.67
5000	4.05	92003.36	4521.04	90.42
10000	4.04	214493.22	10455.34	104.55
20000	4.05	418274.19	20327.97	101.64
50000	4.04	1027517.28	49844.13	99.69

**Table 2. Data of Calibration Standards using Summed Peak Area of Glycoforms G0F-2 and G0F/G1F.**

Actual Conc. (ng/mL)	RT (Min)	Peak Area	Calculated Conc. (ng/mL)	Accuracy %
100	4.05	2087.54	115.97	115.97
200	4.05	4668.67	178.89	89.45
500	4.05	15363.03	439.58	87.92
5000	4.05	180089.79	4454.95	89.10
10000	4.04	425333.87	10433.02	104.33
20000	4.05	832089.10	20348.07	101.74
50000	4.04	2041509.55	49828.87	99.66

## Conclusions

Here we report a complete workflow for quantitative analysis of therapeutic monoclonal antibody trastuzumab in rat plasma, achieving 2.7 orders of magnitude in linear dynamic range at the intact level. This method minimizes sample manipulation, and instead focuses on a data processing strategy to improve selectivity and sensitivity. Interference from matrix is filtered out thoroughly using XIC approach after targeted data reconstruction performed in batch processing mode. Validated in terms of selectivity, sensitivity, accuracy and consistency, this workflow may serve as a generic quantitation method for intact therapeutic proteins in complex biological matrix.

## References

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