A New Level of Compliant-Ready Intact Biotherapeutic Protein Quantification using Reconstructed Masses

SCIEX OS 1.7 Software with SCIEX TripleTOF® 6600 System or X500B QTOF System

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With biopharmaceuticals in development showing a high level of diversity, there is the need for a high degree of flexibility for analytical methods being used to quantify these molecules. Bioanalysis of larger protein-based therapeutics is widely focusing on the enzymatic digestion of the protein, followed by the quantification of surrogate peptides on MS or MS/MS level (bottom-up approach). Bottom-up approaches show significant strengths in sensitivity, quantification performance and multiplexing capability. However, there are multiple challenges associated: 1) the potential misinterpretation of the actual concentration in a given sample due to unknown biotransformation in matrices; 2) the introduction of artifact during extensive sample preparation and additional variation because of more sample preparation steps.[1] To lower these risks, while increasing the efficiency, scientists started to evaluate the quantification of large proteins such as monoclonal antibodies (mAb) on intact level using accurate mass spectrometry (MS), often referred to as top-down approach.[1]

SCIEX OS 1.7 Software with the TripleTOF® 6600 System and the X500B QTOF System.

Key Features of Intact Protein Reconstructed Mass Quant

- SCIEX OS 1.7 enabling intact protein quantification based on peak height or peak area of reconstructed masses
- 21 CFR Part 11 compliance ready intact quantification workflow
- Ease-of-use of SCIEX OS for getting to answers in a time efficient manner

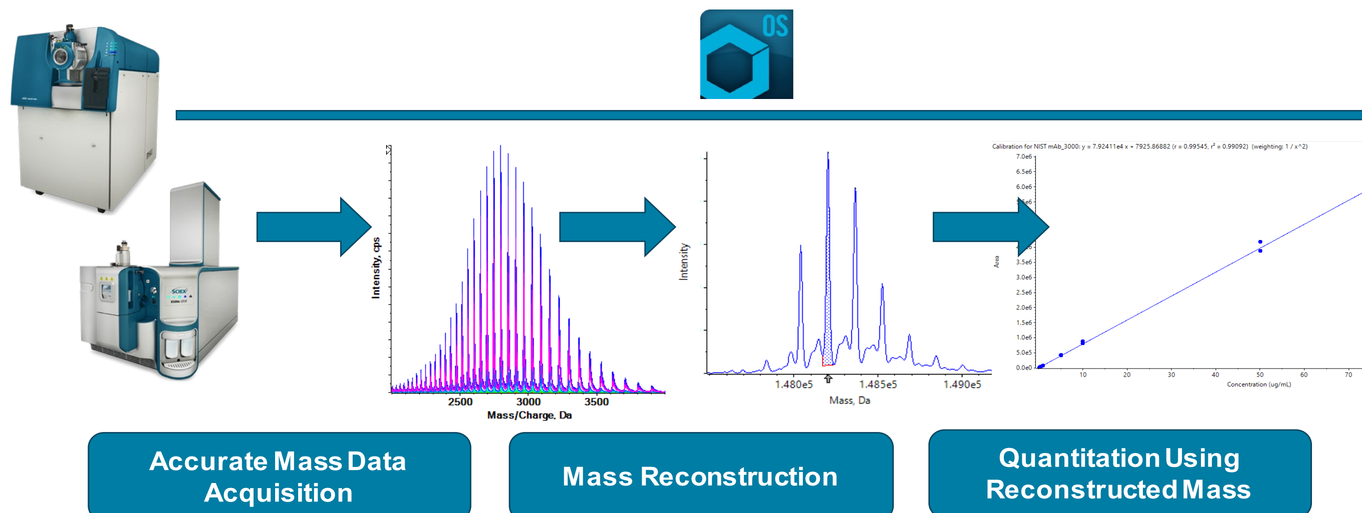


Figure 1. Overview of Intact Protein Quantification Workflow Using Peak Areas of Reconstructed Masses. Upon data acquisition using accurate mass time of flight instrumentation, the raw data was reconstructed, integrated and used for quantification in the SCIEX OS 1.7 Software.

Data analysis strategies for intact protein quantification imply additional challenges as the signal is distributed over a wider range of charges with increasing molecular weight: Usually the bigger the protein, the more charge states and therefore the less absolute intensity for a certain charge state. Consequently, the quantification on a given extracted ion chromatogram (XIC) of a given charge state of the MS spectra is not the method of choice as it is limiting the sensitivity of the data analysis strategy. In addition, interferences from matrix proteins can be a burden resulting in missing desired limits of quantification (LOQ). To overcome these problems, SCIEX introduces a new workflow of quantification of proteins based on reconstructed masses within the SCIEX OS 1.7 software. With Good Laboratory Practice (GLP) and 21 CFR Part 11 compliance being needed quite often for the quantitative analyses in bioanalytical studies, the workflow presented can be set up in order to fully meet the required criteria.[2]

Methods

Sample Preparation:

Rat plasma was processed using biotinylated anti-human-Fc-antibodies (Southern Biotech, #2049-08) and streptavidin-coated paramagnetic beads (bioZen MagBeads, Phenomenex, #KS0-9531) as a matrix. Humanized IgG mAb was obtained from the National Institute of Standards (NIST) (#RM8671) and spiked into the processed rat plasma in different concentrations as an intact antibody analyte model.

Chromatography:

Generic conditions for intact protein analysis using denaturating conditions with reversed phase chromatography were used as listed in Tab. 1 and Tab. 2.

Table 1. Chromatographic Conditions.

Parameter	Value
Column	*Agilent PLRP-S 2.1 mm × 50 mm, 5 µm, 300 Å*
Mobile Phase A	*Water + 0.1 % formic acid*
Mobile Phase B	*Acetonitrile + 0.1 % formic acid*
Flow Rate	*500 µL/min*
Column Temperature	*80 °C*
Injection Volume	*5 µL*

Table 2. Gradient for Intact Protein Analysis.

Time [min]	Mobile Phase A [%]	Mobile Phase B [%]
Initial	*75*	*25*
3.0	*75*	*25*
4.5	*55*	*45*
4.7	*10*	*90*
7.9	*10*	*90*
8.0	*75*	*25*
10.0	*75*	*25*

Mass Spectrometry:

An ExionLC™ system coupled to a SCIEX X500B System was used. Data was acquired using the intact protein mode turned on with the large protein option and the detector decrease option being ticked. Detailed MS settings are listed in Tab. 3.

Table 3. MS Parameters.

Parameter	Setting
Scan Mode	*TOF-MS positive*
Gas 1	*60 psi*
Gas 2	*60 psi*
Curtain Gas	*40 psi*
Source Temperature	*500 °C*
Ion Spray Voltage	*5500 V*
Time Bins to Sum	*80*
Accumulation Time	*0.5 sec*
Mass Range	*2,000-4,000 m/z*
Declustering Potential	*250 V*
Collision Energy	*10 eV*

Data Processing:

Data were processed using SCIEX OS 1.7 Software with mass reconstruction feature. Peak integration was performed using the MQ4 algorithm. The data processing details are described in the later session.

Table 4. Processing Parameters for Reconstructed Mass.

Parameter	Setting
Resolution	*3,000*
Reconstruction Start Mass	*147,000 Da*
Reconstruction Stop Mass	*150,000 Da*
Number of Iterations	*20*
Step Mass	*1.00 Da*
Input m/z Range Start	*2,000*
Input m/z Range Stop	*4,000*

Intact Protein Quantification Workflow

The charge state envelope for an intact monoclonal antibody analyzed under denaturing conditions via reversed phase chromatography usually ranges from about *m*/*z* 2,000-4,000 (Fig. 2). Here, an intensity maximum at a charge state of *z* = 53 was observed (Fig. 2). Since the model antibody sample was not deglycosylated, several glycoforms were detected for each charge state (Fig. 2 inset spectrum).

Upon acquisition of the TOF-MS raw data, the SCIEX OS 1.7 Software offers two approaches for quantification: The first approach utilizes the XIC of a certain charge state of a protein or the sum of several XICs linked to either several charge states or to several protein forms (e.g. different glycoforms such as G0F/G1F and G1F/G1F) or both (Fig. 3). The second quantification approach is based on the reconstruction of masses, which is uniquely offered in SCIEX OS 1.7. The raw data of many charge states can be calculated to a so-called zero charge state with the help of an algorithm, referred to as mass reconstruction (Fig. 1), the peak area integration is performed on the MS peaks from the reconstructed MS spectrum. To evaluate the applicability of the reconstruction based quantification within the SCIEX OS 1.7 Software, the whole mass range and the most abundant glycoforms G0F/G1F of the analyte model were used with generic reconstruction parameters (Tab. 4). For this model in plasma matrix a linear dynamic range of 3 orders of magnitude (Tab. 5 and Fig. 4). In this dataset, matrix proteins was partially

Table 5. Quantification Results for G0F/G1F Using Reconstructed Masses.

Actual Conc. [ug/ml]	Reconstructed Peak Area G0F/G1F	Calculated Conc. [ug/ml]	Accuracy [%]
100	*7.00E+06*	*88.2*	*88.2*
50.0	*4.03E+06*	*50.8*	*101.6*
10.0	*8.53E+05*	*10.7*	*106.6*
5.00	*4.23E+05*	*5.23*	*104.6*
1.00	*8.79E+04*	*1.01*	*100.9*
0.500	*4.57E+04*	*0.477*	*95.4*
0.200	*2.44E+04*	*0.208*	*104.1*
0.100	*1.57E+04*	*0.099*	*98.6*

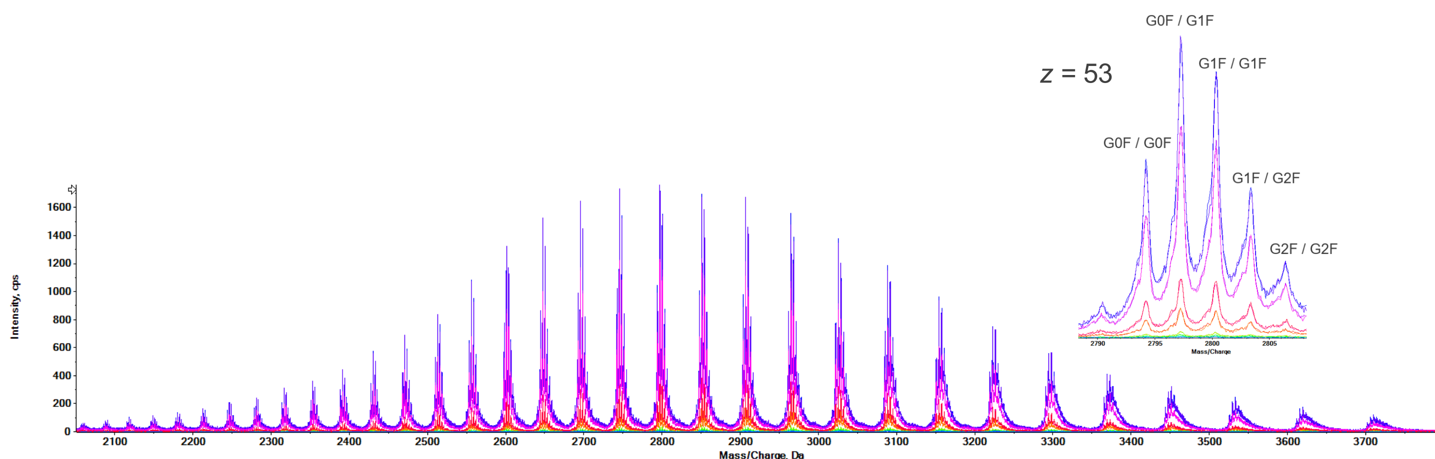
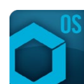


Figure 2: TOF-MS Raw data for Replicate Injections of Different Concentrations of NIST mAb Analyte in Processed Plasma Matrix. Upper right hand side: Zoom into highest abundant charge state *z* = 53 with indication of the different glycoforms of the antibody sample.



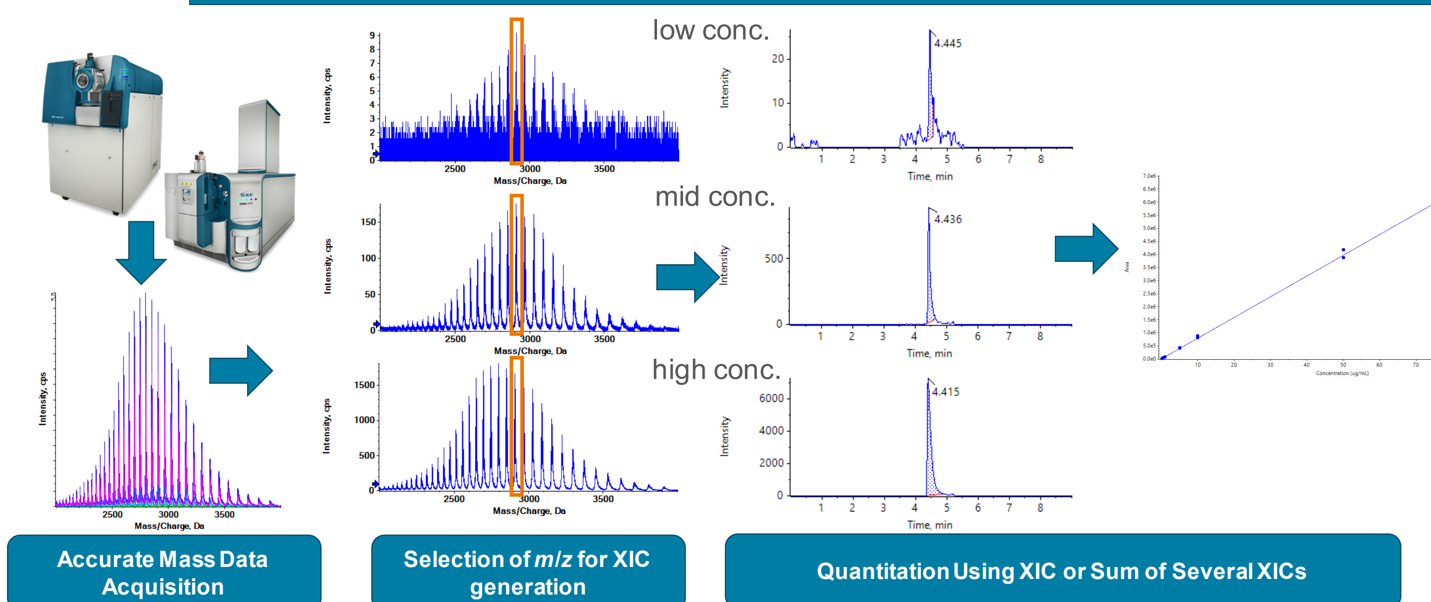


Figure 3. Overview of the intact quantification workflow based on XICs. Upon data acquisition using accurate mass time of flight instrumentation, the XIC or sum of XIC of one or multiple charge states or protein forms was used for integration and quantification in the SCIEX OS Software.

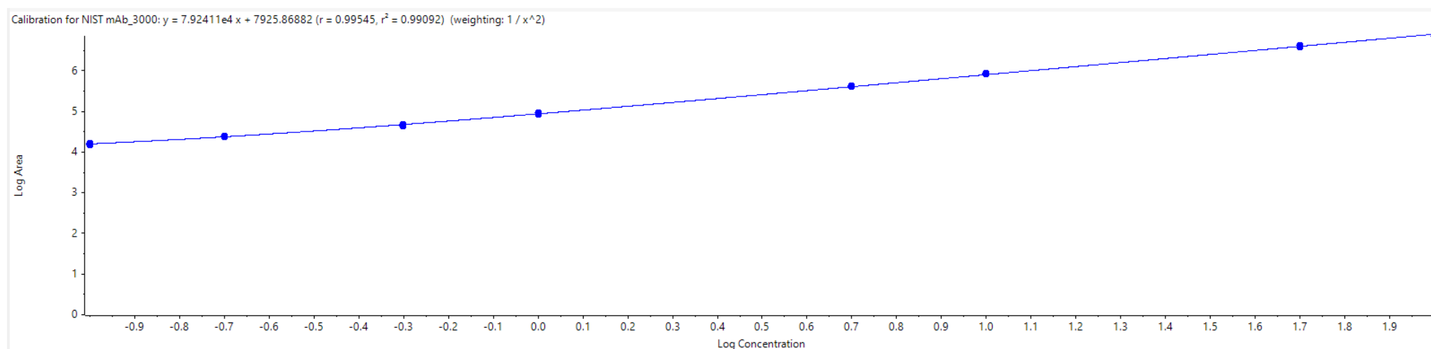


Figure 4. Calibration Curve of Intact NIST mAb in Processed Plasma Matrix. Log-log plot of areas of reconstructed masses of glycoform G0F/G1F versus the concentration in ng/mL. A linear fit was applied using a $1/x^2$ weighting. Regression details and R^2 value are indicated in the graph.

separated on a chromatographic level (Fig. 5, middle, XIC pane), therefore reducing the interference on spectral level.

However, depending on the matrix and the sample preparation being used upfront, matrix interferences can be much more abundant and not necessarily be separated via liquid chromatography. Especially in such cases, the quantification using reconstructed mass information offers an additional advantage for quantification, since matrix proteins might interfere on spectral level, but not necessarily on reconstructed data level if differing in their molecular weight from the analyte of interest.

This strategy being shown for intact antibodies is not limited to such, but can potentially be extended to subunit protein analysis or deglycosylated samples in order to enhance sensitivity as well as other proteins.

Compliance Readiness

Bioanalysis studies supporting new drug applications or new formulations of already approved pharmaceuticals are required to comply with authorities' regulations such as the 21 CFR Part 11 from the US Food and Drug Administration (FDA).

SCIEX OS meets these criteria when being set up as a closed system including the requirement for records and signatures on an electronical basis (compliant-ready). Data can be stored directly to a network location. For performing quantification, SCIEX OS software has the ability to open raw data files from any visible storage location, which offers the flexibility to work within a closed network with processing work stations. As a combination of SCIEX offering technical controls and the user's responsibility for appropriate configuration of SCIEX OS software, supporting network and operating system security as well as providing policies, procedures and user training, the presented workflow is fully compliant (Fig. 6).

Conclusions

- SCIEX OS 1.7 software provides a comprehensive intact protein quantification workflow by offering the unique peak integration on reconstructed mass spectra, and reduces the complexity for LC-MS method development and cumbersome sample preparation
- The ease-of-use of the SCIEX OS software gets new users up to speed quickly and the compelling data review options within the software speed up the time to get to correct answers
- Regulated environments can use the intact quantification workflow meeting GLP and 21 CFR part 11 criteria having the option to submit studies electronically to the FDA.

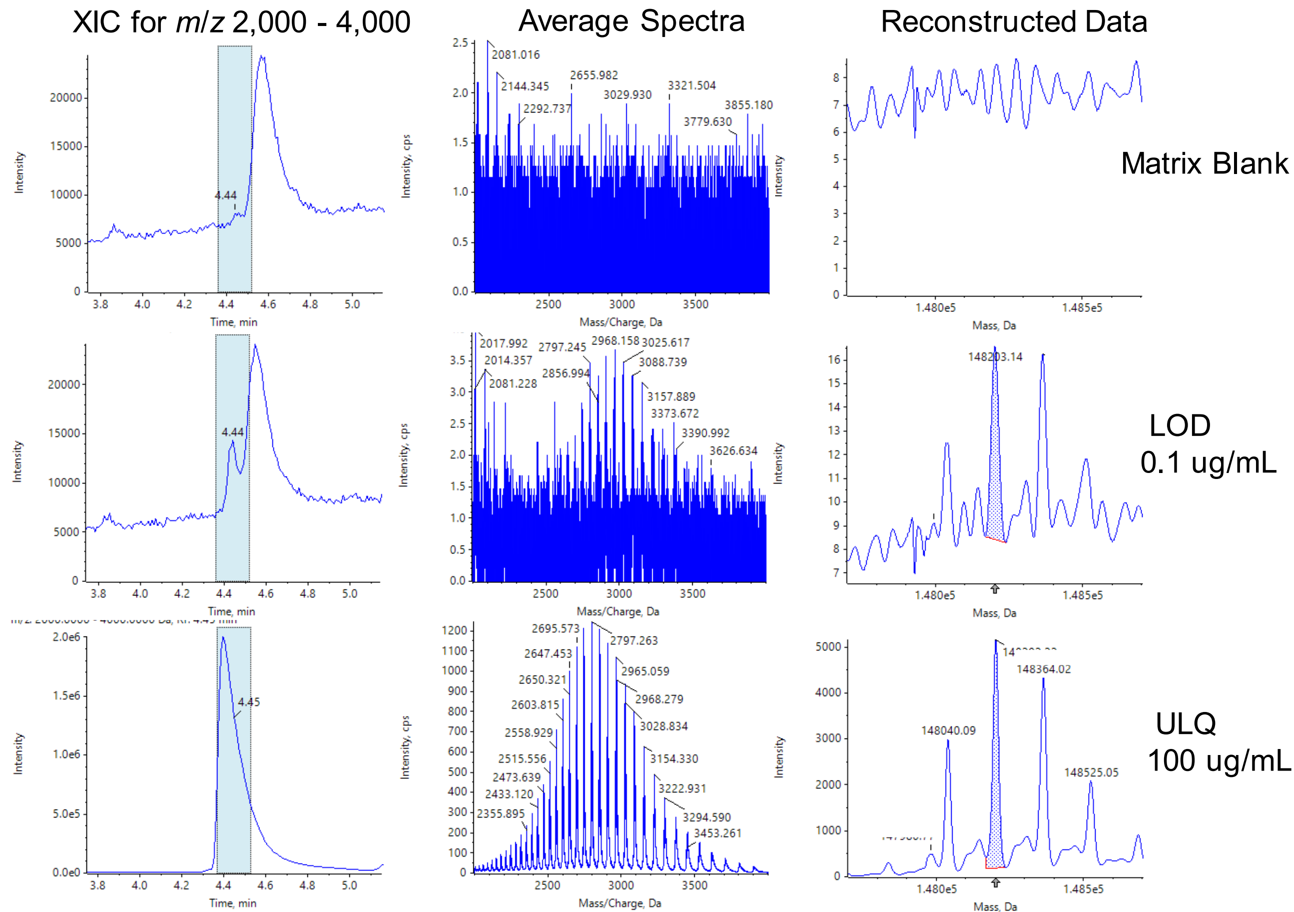


Figure 5: Overview of Quantification Results from SCIEX OS Software. XIC, average spectra and reconstructed data information for the matrix blank, the lowest concentration and the highest concentration used for quantification. The analyte protein is eluting at 4.44 min, the additional peak being visible in the matrix blank and the LOD at 4.55 min is related to the elution of matrix proteins.

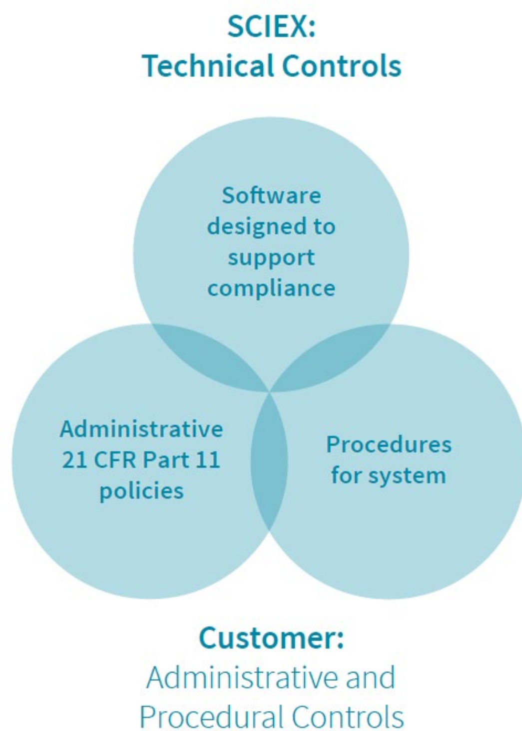


Figure 6. Three Types of Controls Required for Compliance According to 21 CFR Part 11.

References

1. Jian, W., Kang, L., Burton, L., Weng, N.: A workflow for absolute quantitation of large therapeutic proteins in biological samples at intact level using LC-HRMS. Bioanalysis **8** 1679-1691 (2016).
2. White Paper, SCIEX OS LC/MS Software and 21 CFR Part 11 Regulations RUO-MKT-19-10018.

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