Intuitive software workflow for intact and subunit analysis of monoclonal antibodies (mAbs)

Featuring the ZenoTOF 7600 system and Biologics Explorer software

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This technical note describes an integrated and intuitive workflow for intact mass analysis of therapeutic proteins and their subunits using the new Biologics Explorer software from SCIEX. In addition to providing highly confident deconvolution results, Biologics Explorer software offers powerful visualization tools for quick assessment of impurity level in protein therapeutics and in-depth comparisons between different batches or processes.

The characterization of therapeutic proteins, such as mAbs and antibody-drug conjugates (ADCs) or next-generation biologics, poses analytical challenges due to sample heterogeneity and complexities that arise from post-translational modifications (PTMs) or truncation products, for instance. Intact protein and subunit analyses, routinely performed at different stages of the development process, are essential for confirmation of drug identity, monitoring of product quality and comparative studies of innovator versus biosimilar products. One of the challenges with intact protein analysis is achieving accurate, consistent and automated deconvolution and correct annotation of intact LC-MS data.

In this technical note, an intact protein analysis workflow template in Biologics Explorer software is employed to analyze intact and subunit LC-MS data of an mAb acquired using the ZenoTOF 7600 system from SCIEX. The ease of use and flexibility aspects of Biologics Explorer software as well as its powerful visualization tools are highlighted.

Key features of Biologics Explorer software

- Purity/impurity assessment and comparative data analysis can be done quickly by leveraging powerful visualization tools (Figure 1)

- Ease of use and a reliable output of results is provided by an intuitive workflow template with predefined parameters, which allows for flexibility in processing method optimization

- Confidence in results is achieved by state-of-the-art algorithms for baseline subtraction, noise reduction and mass deconvolution

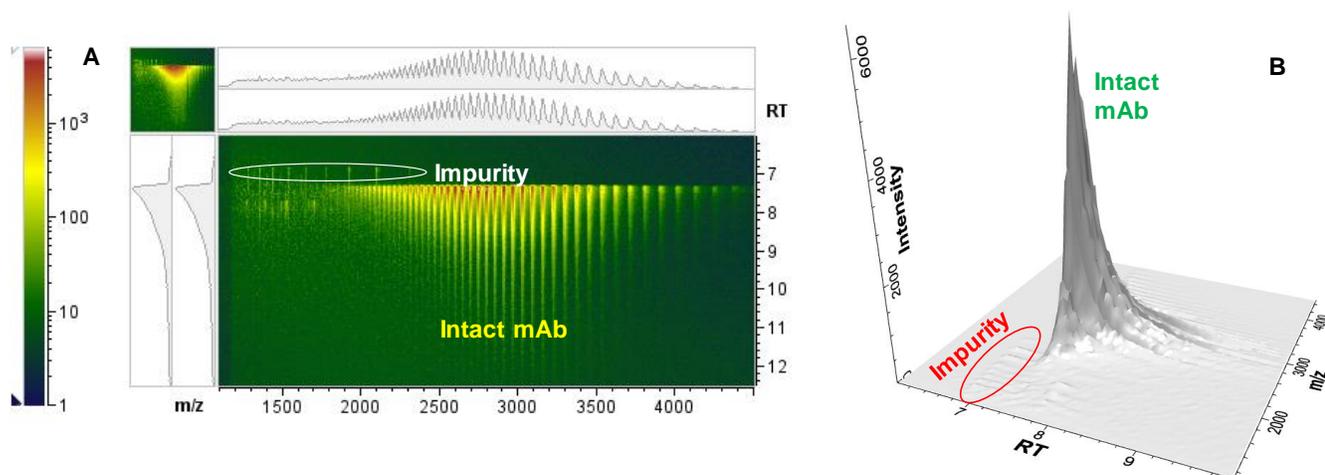- Quick data re-processing is enabled using snapshots saved at different stages of the workflow

Figure 1. Biologics Explorer software with powerful visualization tools. The heat map in the topographic color scheme (A) and the 3D display (B) provide a multi-dimensional view of data files to facilitate quick assessment of data quality and impurity level for intact protein therapeutics. This example shows an intact NISTmAb with lower molecular weight impurities.

Methods

Sample preparation: For intact analysis, NISTmAb (NIST RM #8671) was diluted to 1 µg/µL with 10% acetonitrile and 0.1% formic acid prior to LC-MS analysis. In subunit analysis, NISTmAb (1 µg/µL) was denatured with 7 M guanidine hydrochloride and reduced by 200 mM dithiothreitol at 37ºC for 1 hour.

Chromatography: Intact and reduced NISTmAb samples were separated using reversed-phase liquid chromatography with an ExionLC system.

Mass spectrometry: The data were acquired in the TOF-MS positive ionization mode using a ZenoTOF 7600 system with intact protein mode enabled.

Data processing: Data was processed with Biologics Explorer software 1.0.1. The Biologics Explorer software can process any SCIEX QTOF data files in WIFF or WIFF2 format. In this technical note, the NISTmAb intact and subunit data files from the ZenoTOF 7600 system were processed in WIFF2 format

using the default IntactProtein_Automated template, which consists of predefined activity nodes connected in a logical manner (Figure 2). The default parameters in each activity node of the template were specifically optimized for SCIEX QTOF-MS data files, providing a quick starting point and an easy-to-use workflow. With this starting point, users can adjust the processing settings to suit their needs.
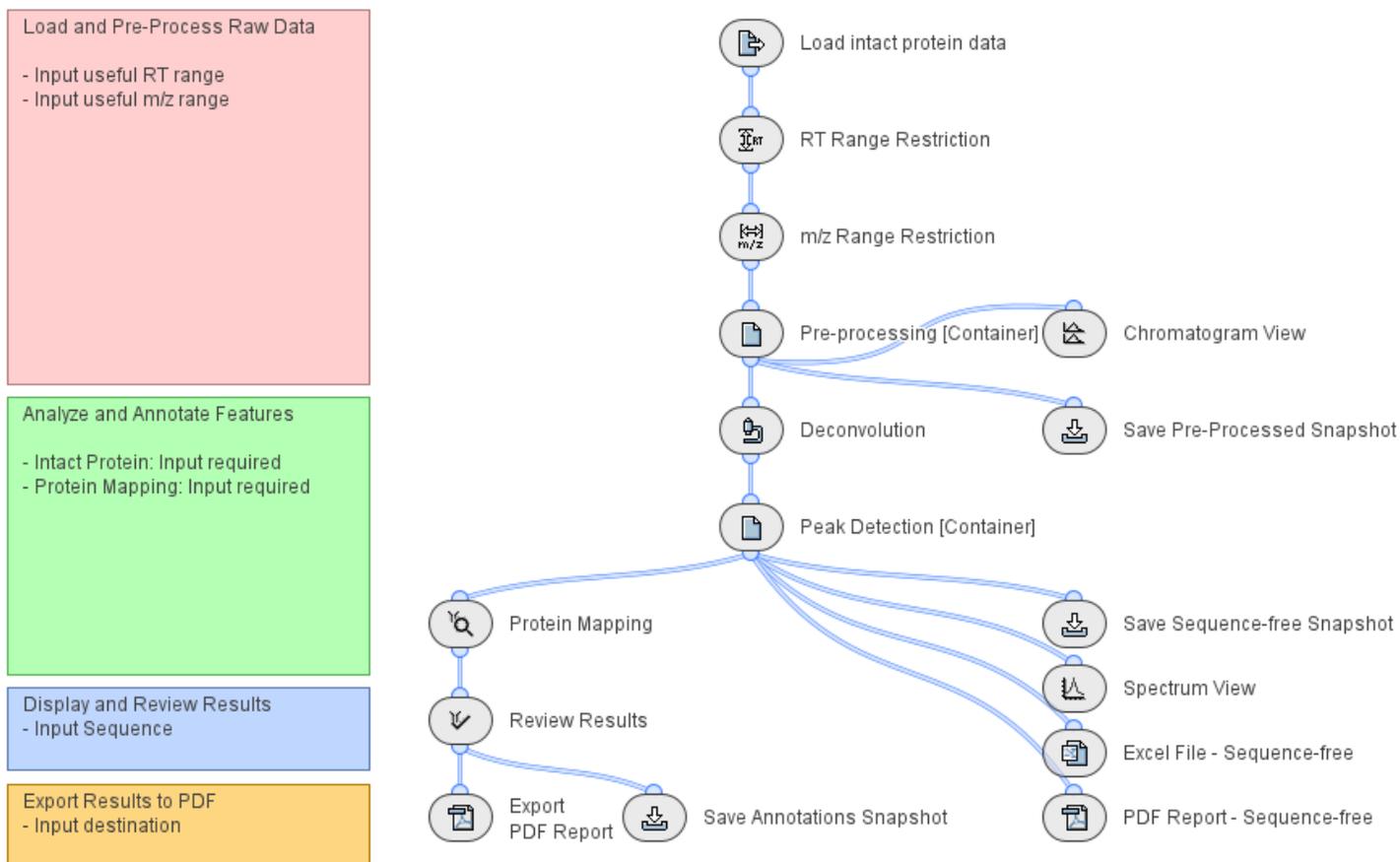

Figure 2. Intact protein analysis template in Biologics Explorer software. This template can be used for intact and subunit data derived from SCIEX QTOF instrumentation. It offers a prebuilt workflow with optimized settings for all activity nodes.

Intact protein workflow template

The default intact protein workflow template (Figure 2) provides a useful starting point for analyzing SCIEX intact and subunit data. Minimal user input is required to run this workflow template, saving time during method development for data processing. The workflow can be executed either in a stepwise manner or automatically. Running each activity node separately allows for quick review of the immediate outcome, facilitating optimization of processing parameters, such as those for baseline subtraction, smoothing and peak detection. Once an optimal set of parameters is chosen, the template can be saved for automated routine analysis of similar data files. The Biologics Explorer software also provides the option of saving pre-processed information and fully annotated results as snapshots that can be archived and reused at a later point in time. This can be particularly helpful for post-analysis review of many different end results at the same time.
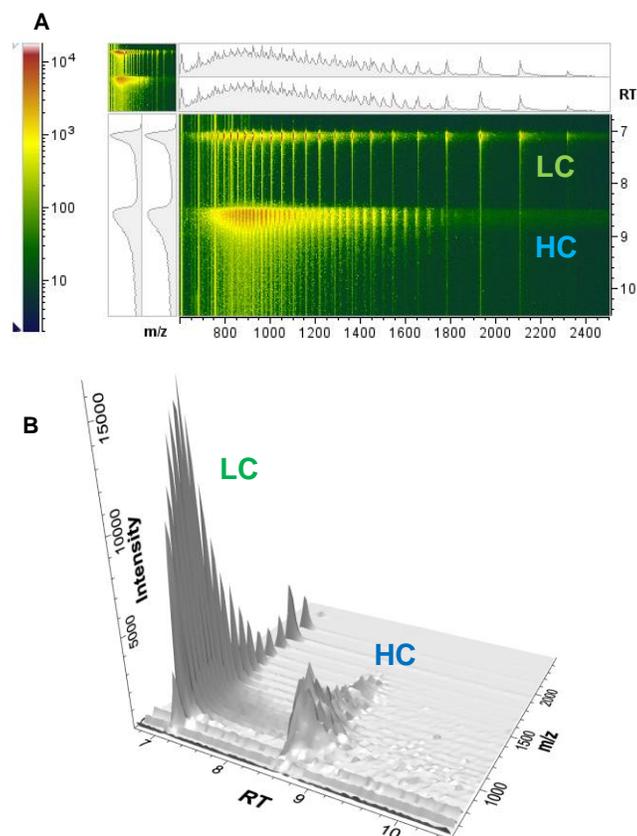

Figure 3. Visualization of subunits of NISTmAb. A heat map (A) and a 3D display (B) of NISTmAb subunits are shown. The 2 distinct peak series at ~7 min and ~8.5 min correspond to the LC and HC, respectively.

Data visualization

The Biologics Explorer software offers powerful visualization tools for reviewing and comparing data. The heat map and the 3D display (Figure 1 and Figure 3) provide excellent visualization of the data in both *m/z* and retention time (RT) spaces, allowing quick assessment of data quality and detection of impurities, which might remain undetected otherwise. Using these visualization tools, a low-abundant impurity (~2% in relative abundance compared to the main intact mAb species), eluting earlier than the intact species, was quickly detected. This prompted further identification of this impurity, as will be described later. In another example, multidimensional visualization of NISTmAb subunit data (Figure 3) shows 2 distinct peak series for the light chain (LC) and heavy chain (HC), respectively, confirming great sample quality based on high-quality raw data. When necessary, the mass spectra and extracted ion chromatograms (not shown here), easily accessible via heat map, can be further used for in-depth inspection of the raw data at each RT.

Deconvolution of QTOF-MS raw data

The Biologics Explorer software performs spectrum deconvolution to a zero-charge state using a proven maximum entropy deconvolution method. This method uses a proprietary algorithm to determine the best deconvolution using a probabilistic approach, and it is performed for each RT range containing automatically identified signals. The raw data is pre-processed, prior to deconvolution. A baseline subtraction and smoothing transformation are applied, followed by peak detection based on the deconvoluted data as the last step. All of the steps are transparent to the user and have suggested default settings that can be adjusted. A number of peak detection strategies are available for both RT selection and for the deconvoluted data. While default settings, such as , are suggested in the software (ascent-based for peak detection of deconvoluted data, Figure 4), the selection of the peak detection
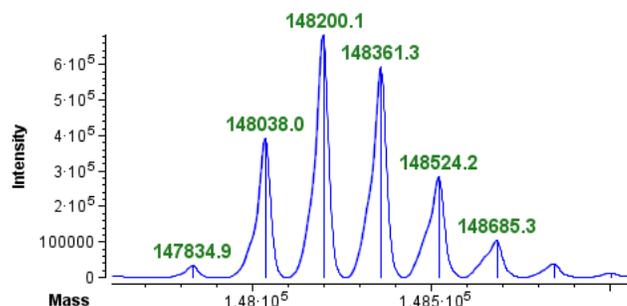

Figure 4. Ascent-based peak detection strategy for deconvoluted data. The centroided data in sticks represent the peaks detected by the 2 strategies.

strategy can be adjusted, depending on the chromatographic resolution and spectrum complexity.

Protein mapping and peak annotation

The detected peaks in the deconvoluted data are annotated by matching the sequences and modifications defined in the protein mapping activity node of the workflow. To facilitate the annotation, a few glycosylation libraries (GlycomeDB, CHO N-linked glycans and O-linked glycans) are available for use. Furthermore, the state of disulfide bonds needs to be specified. The Biologics Explorer software offers the options of fully connected, partially reduced and fully reduced to expedite data analysis by eliminating the need to manually enter detailed disulfide bonding information. The results of NISTmAb intact and subunit analysis are displayed in Figure 5. Excellent mass accuracies (<10 ppm) were obtained for all of the major species detected in both experiments. The low-abundant impurity (~2% in relative abundance compared to the main intact mAb species) observed in the heat map and 3D display of the intact NISTmAb sample (Figure 1) was identified as the LC by intact mass analysis (data not shown), demonstrating again the power of using visualization tools for quick impurity assessment.

Data review and reporting

The Biologics Explorer software provides powerful visualization tools for reviewing and comparing results. The deconvoluted data from multiple data files can be compared in a stacked view or via a mirror plot (Figure 6), which offers clear visualization of the differences observed in samples from different batches or processes, for instance. In the result review step, the annotated peaks can be accepted or rejected for automatic calculations of their relative abundances in each sample. Figure 7 shows the relative abundances of all major glycoforms of intact NISTmAb for replicate injections. Consistent results were obtained from these data files, as expected. Finally, the Biologics Explorer software provides the full capability of customized PDF reports to include parameters and results of any of the workflow elements.
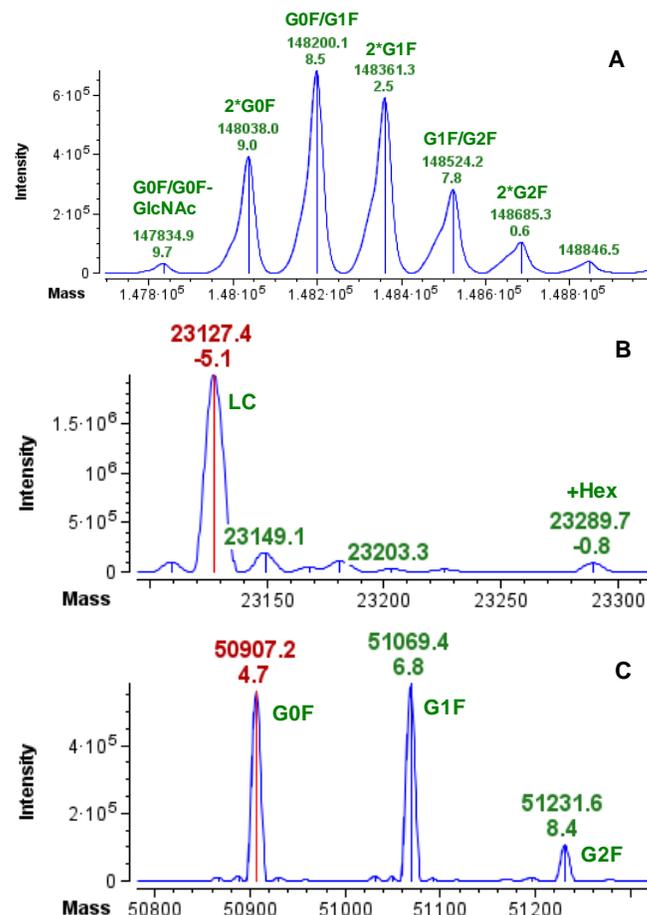
Figure 5. Annotated deconvoluted data of intact and subunits of NISTmAb. (A) Intact NISTmAb and its major glycoforms, including 2x lysine loss and 2x pyro-glutamate formation. (B) LC and LC with 1 glycation (Hex). (C) HC and its main glycoforms (G0F, G1F, G2F), including a lysine loss and pyro-glutamate formation masses, with mass errors indicated for each peak compared to theoretically calculated masses.
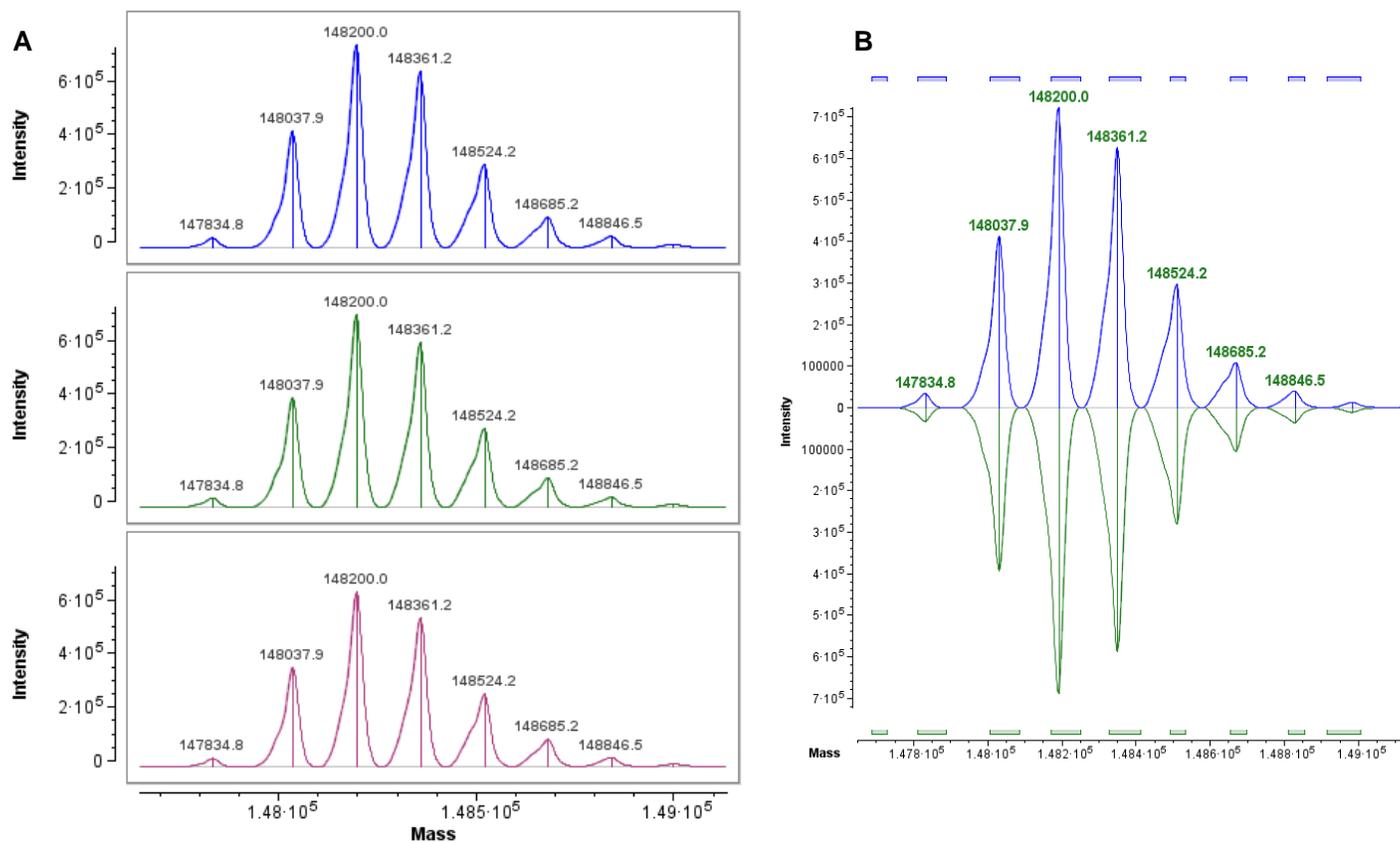
Figure 6. Comparison of deconvolution results from multiple intact data files. A stacked view of 3 replicate injections (A) and a mirror plot showing 2 replicates (B) are shown. Either view can be helpful when comparing multiple samples for consistency, such as different batches or making originator/biosimilar comparisons.

Figure 7. Relative abundances of major NISTmAb glycoforms obtained from intact analysis. Results from replicate injections are shown. Calculated volume was used for obtaining a volume percentage value indicative of the relative abundance of a certain glycoform in the sample.

Conclusions

- An intuitive intact workflow template for analyzing intact and subunit data of protein therapeutics provides ease of use while allowing significant flexibility in optimizing parameters.

- Powerful visualization tools enable quick assessment of impurity level and the quality of samples and data as well as the comparative analysis of multiple samples from different batches or processes.

- The proven algorithms for baseline subtraction, smoothing and noise reduction as well as spectrum deconvolution and peak detection ensure consistent and confident results.