Simultaneous Quantitative and Qualitative Analysis of Proteolytic Digests of Therapeutic Monoclonal Antibodies using a TripleTOF® System

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Monoclonal antibodies (mAb) are major target-oriented biotherapeutics that treat an array of human diseases. Current therapeutic monoclonal antibodies are immunoglobulin G (IgG)1 derivatives, typically produced by mammalian cell culture using Chinese hamster ovary (CHO) or other cells1. In recent years, mass spectrometry has emerged as a superior method for the characterization of the heterogeneity of these proteins due to post-translational modifications (PTMs), sequence variations, and purification or formulation alterations/degradation (Figure 1).

There are typically four levels of protein product characterization using mass spectrometric methods. Levels 1 and 2 are the analysis of the intact and reduced molecular weight, respectively. Characterization Level 3, commonly referred to as the “peptide map” involves the analysis of proteolytically digested protein products by LC/MS/MS. Characterization Level 4 focuses on the characterization of protein glycosylation.

Here we describe level 3 characterization: LC/MS/MS analysis of enzymatically digested therapeutic antibodies on the TripleTOF® 5600+ system.

The LC/MS/MS analysis of digested therapeutic antibodies on the TripleTOF system generates simultaneous qualitative and high-fidelity quantitative data. Relative quantitation of every peptide and its modified forms serves as a high-quality snapshot of the current state of a biological product and is invaluable to track lot-to-lot variations including C-terminal lysine clipping, N-terminal blocking, drug conjugates, oxidation, deamidation and other post-translational modifications (Figures 2).

Advantages of the TripleTOF® System for Peptide Mapping of Proteolytic Digests of Therapeutic Antibodies

- TripleTOF system acquisition speed allows simultaneous qualitative and quantitative data acquisition to provide a complete snapshot of the current state of a therapeutic protein product
- High mass accuracy and high resolution TOF MS and MS/MS data provides high confidence in peptide identifications
- Accurate quantitation data for determining relative abundance of each peptide and its modified forms, and lot-to-lot comparisons.
- Sophisticated peptide analysis tools provide accurate identification of peptide sequences, even for highly heterogeneous antibodies with unknown sequences in variable regions.
Experimental Design

Sample Preparation: 1 mg of monoclonal mouse IgG1 isotype (Waters, Milford, MA) was denatured with urea, reduced with DTT and alkylated with iodoacetamide. The resulting denatured protein was then digested with trypsin at 37°C for four hours.

Chromatography: Rapid HPLC analysis of trypsin digested monoclonal antibodies was performed using a Shimadzu HPLC System on a Jupiter C18 column (Phenomenex, 2.1 x 150 mm, 3 μm). The HPLC gradient is shown in Table 1. Solvent A consisted of 2% acetonitrile and 0.1% formic acid, and solvent B consisted of 98% acetonitrile with 0.1% formic acid.

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Table 1. LCMS Gradient Profile.

Mass Spectrometry: High flow LC/MS analysis was performed on the TripleTOF® 5600 System using the Turbo V™ Source. During each LC/MS run, a divert valve was used to divert the initial flow for 2 minutes for desalting. Information dependent acquisition (IDA) was performed as follows: For each cycle of 1.3 sec, a 0.25 sec MS scan was performed followed by 20 50 millisecond MS/MS scans, each of ions with a 2+ or greater charge state. Each selected ion was then put on a dynamic exclusion list for 15 seconds.

Data Processing: IDA data files were searched using ProteinPilot™ Software against a database containing the sequence of the antibody and the sequences of ~1000 other proteins. The MS and MS/MS data was then brought into PeakView® Software and used to generate MS extracted ion chromatograms (XIC) for each identified peptide. The relative quantitation of each peptide and any modified form(s) was then determined. These XICs are true intensity-based quantitative measurements and thus can be imported into the quantitation software, MultiQuant™ Software, for rigorous quantitative analysis, lot-to-lot comparison and automated report generation.

High Quality MS and MS/MS Data Acquisition

TripleTOF® systems generate high resolution MS/MS data (~15 000 – 30 000) with high mass accuracy (~2ppm RMS) and excellent sensitivity at high speed (20-50 Hz) over approximately four orders of linear dynamic range. The TripleTOF system does not sacrifice speed or sensitivity for resolution like some other mass analyzers. Figure 3 (top) depicts the total ion current chromatogram from the MS experiment. In a single 50 millisecond scan, information rich MS/MS spectra are generated with resolution of > 15,000 (bottom) across the entire mass range. The ability to sequence and achieve strong spectra in a short time means a more complete coverage in less run time. Speed also enables the quantitative ability of the TripleTOF® system, as short cycle times yield a greater number of points across each chromatographic peak for high fidelity quantitation.
ProteinPilot™ Software is Uniquely Suited to Take Advantage of High Fidelity MS/MS Data

The unique Paragon™ Database Search Algorithm\(^4\) in ProteinPilot™ Software utilizes high resolution and high mass accuracy MS/MS data to compute sequence tag evidence for each spectrum. For spectra where sequence tag evidence is strong, the Paragon™ Algorithm will expand the search space to include a database of > 300 post translational modifications, amino acid substitutions, and variable digestion rules\(^5\). Because the search space is only expanded for spectra with strong sequence tag evidence, the explosion of false positive identifications that is commonly observed in unscreened error tolerant searches is avoided with ProteinPilot™ software.

This efficient management of search space is especially important to sequence variant analyses in therapeutic protein products. Using traditional PTM-restricted sequence-match search techniques followed by uncurated error tolerant searching generates many false positives and few credible sequence variants. Many groups\(^2,3\) have recently found success with sequence-tag based searching for sequence variant explorations as well as for sequencing proteins with incomplete sequence databases.

The quality of MS and MS/MS data also plays a large role in the identification of peptides, higher mass accuracy data increases the accuracy of sequence tag assignment and confidence in peptide identifications. In Figure 4, the ProteinPilot Software search results are shown for an IgG1 digest analysis performed on the TripleTOF® System. In the upper panel, the sequence coverage results for light and heavy chain are listed as 100% and 98.8% respectively. In the central pane, peptide identifications that have been assigned to the light chain, which is highlighted in the upper pane. The bottom pane shows the identified sequence coverage, which is color coded according to peptide identification confidence. All 100% of the sequence coverage for the light chain is above 95% confidence.

Visualization of Peptide Maps

Once identified, the highly reproducible MS peak areas and fast cycle time of the TripleTOF® system results in very reproducible chromatographic peak areas of each peptide. PeakView\(^8\) Software includes the XIC Manager utility that can associate the peptide assignments from a ProteinPilot Software search result with the MS data file and generate an extracted ion chromatograms (XICs) for each peptide ID. Figure 5 is a plot of the XICs of each identified peptide from the search results in Figure 4, H or L denotes heavy or light chain and T# denotes the tryptic peptide number. The quantitative quality of this strategy is high as these are true intensity based measurements. In addition, the TripleTOF® system enables high speed acquisition
of both MS and MS/MS to maintain a high number of points across each chromatographic peak, even with very narrow UPLC peak widths.

Figure 5 also includes a few other panes that highlight the utility of having a specific XIC for each individual peptide. The center pane features a zoom-in of a specific region of the top pane. Here the difference between a TIC or UV trace and a specific chromatogram for each peptide is tangible. Pink arrows highlight peptide chromatograms that were obfuscated in the TIC but are readily apparent in individual peptide chromatograms.

Comparison between samples is also possible by viewing a mirror plot of any two chromatogram plots, as shown in Figure 1. This can be useful for lot-to-lot comparison or for comparison between innovator and biosimilar molecules. In this case, the comparison is between two preparations of the same antibody, with and without reduction. Loss of some XIC peaks and presence of new XICs in the reduced antibody preparation (bottom) suggests the presence of a disulfide bond.

Quantitative Protein Profiling

MultiQuant™ software is the industry standard for quantitation of AB SCIEX Triple Quad™ and QTRAP® System MRM data. It can also process high resolution full scan data from TripleTOF® System and generate XICs for quantitative from both MS and MS/MS data. After visualization of the peptide maps in PeakView® Software, a quantitation method for MultiQuant Software can be easily generated for final batch processing. Here, there are powerful tools to quickly view multiple XICs simultaneously (Figure 6 top) and process multiple data files in parallel and output custom reports. For example, Figure 6 (bottom) shows the reproducibility of peptide peak area measurements obtained from mouse IgG1 digest that was run in triplicate. Peak area under the XIC for peptides illustrated in Figure 5 (top) were measured for each of the three LCMS runs and their areas are displayed in the graph. The %CV of all measured XIC areas were <10% including the low abundance peptides. The flexible report generation tool within MultiQuant™ Software can be easily configured to automatically generate reports on peak area reproducibility across various lots of product.

Conclusions

- The TripleTOF® 5600+ System provides an excellent solution for the characterization of therapeutic antibodies.
- The high speed of the TripleTOF® 5600 System for peptide mapping experiments provides greater depth of coverage, improved detection of low-level contaminants, as well as enabling more points across each chromatographic peak for high fidelity quantitation.
- The high sensitivity of the TripleTOF® 5600 System reduces sample consumption by providing high quality MS data even for very low amounts of therapeutic antibodies/ low level contaminants.
- The high mass accuracy and resolution of the TripleTOF® 5600 System enables high confidence in both peptide identification as well as enhancing quantitative selectivity by enabling very narrow extracted ion chromatogram widths.
- High linear dynamic range enables reproducible peak intensities, even for low-level analytes.
- Powerful database search algorithms in ProteinPilot™ Software correctly identify more peptides rapidly while keeping the number of false positives to a minimum.

Figure 6. High Quality Quantitation. (Top) MultiQuant™ Software workflow for the quantitative analysis of IgG1 trypic peptides. (Bottom) Report output illustrating three technical replicates of IgG1 digest analysis on a TripleTOF® system, with %CV for all measured peptide XIC areas <10%, including low-abundance peptides.
References


5. ProteinPilot™ Software Overview - High Quality, In-Depth Protein Identification and Protein Expression Analysis. AB SCIEX Technical Note 2780211-01.