An Integrated Workflow for Sensitive Intact Protein Quantitation of Monoclonal Antibodies from Biological Matrix

Abstract

This Application Note describes a method for the quantitative analysis of intact monoclonal antibodies (mAbs) in a serum matrix. The workflow comprised an Agilent AssayMAP Bravo platform coupled with an Agilent 6545XT AdvanceBio LC/Q-TOF.
**Introduction**

mAbs and their derivative products have quickly become an important class of biopharmaceutical molecules with a wide range of therapeutic applications. The full characterization (detection, identification, monitoring, and quantification) of mAbs requires the development of more advanced sample preparation methods and analytical techniques. As the complexity of mAbs and the demand from *in vivo* pharmacokinetic (PK) studies increase, it is critical to develop a highly sensitive and high-throughput LC/MS method for accurate quantification of these mAbs.

Quantitation of therapeutic antibodies by LC/MS/MS typically uses a bottom-up or so-called surrogate peptide measurements approach. This method relies on the multiple reaction monitoring (MRM) of the targeted peptides from enzymatically digested samples. However, this approach has faced some significant limitations:

- Lower throughput due to time-consuming sample preparation
- The requirement of complete enzymatic digestion with full recovery from the sample cleanup process
- Questions about what is actually being measured in the sample: a homogeneous sample or a mixture of proteoforms resulting from the biotransformation process

Directly quantitating proteins at the intact level addresses these limitations. Analytical throughput is dramatically increased, and various heterogeneous mixtures of mAbs (such as degraded/truncated mAbs, mAbs with PTMs) can be quantitated simultaneously. However, even though this approach has rapidly gained more attention in the biopharmaceutical industry, there are still analytical challenges. The lack of comparable sensitivity to the MRM or ligand-binding assays (LBAs) and the narrow dynamic range in detection may prohibit such an approach from being routinely applied to PK studies.

This Application Note demonstrates a highly sensitive LC/MS workflow for quantifying therapeutic proteins in a serum matrix at the intact protein level. The workflow featured the AssayMAP Bravo automated liquid-handling platform for sample preparation and the 6545XT AdvanceBio LC/Q-TOF system with enhanced MS capabilities for intact protein analysis.

**Experimental**

**Materials and methods**

The formulated Herceptin (trastuzumab) was obtained from Genentech (South San Francisco, California, USA). Bovine serum albumin (BSA) and formic acid (FA) were purchased from Sigma-Aldrich (St. Louis, MO, USA.). The mAbs that were spiked into mouse serum in this study were produced by Amgen Inc. (South San Francisco, CA, USA.).

**Instrumentation**

- Agilent AssayMAP Bravo system (G5542A)
- Agilent 1290 Infinity II LC including:
  - Agilent 1290 Infinity II high-speed pump (G7120A)
  - Agilent 1290 Infinity II multisampler (G7167B)
  - Agilent 1290 Infinity II thermostatted column compartment (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF

**Sample preparation**

**mAb in neat solution**: Herceptin (Trastuzumab) neat solution was prepared by diluting 20 mg/mL formulated stock solution with 0.001% BSA to a concentration range of 0.01 to 50 ng/µL. A 1 µL amount of sample was used for each injection.

**mAbs from biological matrix (mouse serum)**: Different amounts of mAbs were spiked into mouse serum to make a concentration ranging from 35 to 50,000 ng/mL. Immunofinity capture, wash, and enrichment of the mAbs from mouse serum was performed using the AssayMAP Bravo system. Approximately 0.035 to 150 ng of intact mAbs were injected for each LC/MS analysis.

![Figure 1. Agilent intact protein quantitation workflow configuration.](image)
**LC/MS analysis**

LC/MS analyses were conducted on a 1290 Infinity II LC coupled with a 6545XT AdvanceBio LC/Q-TOF system equipped with a dual Agilent Jet Stream source. Agilent MassHunter Acquisition (B.09.00) workstation software with large molecule SWARM autotune feature was used. LC separation was obtained with an Agilent PLRP-S column (2.1 × 50 mm, 1,000 Å, 5 µm). Tables 1 and 2 list the LC/MS parameters used.

**Data processing**

All MS data of the intact mAbs were processed using Agilent MassHunter Quantitative Analysis 10.0 software.

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**Table 1. Liquid chromatography parameters.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agilent 1290 Infinity II LC</strong></td>
<td></td>
</tr>
<tr>
<td>Column</td>
<td>Agilent PLRP-S, 2.1 × 50 mm, 1,000 Å, 5 µm (p/n PL 1912-1502)</td>
</tr>
<tr>
<td>Autosampler Thermostat</td>
<td>4 °C</td>
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<tr>
<td>Solvent A</td>
<td>0.1% formic acid in DI water</td>
</tr>
<tr>
<td>Solvent B</td>
<td>0.1% formic acid in 100% acetonitrile</td>
</tr>
<tr>
<td>Gradient</td>
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<tr>
<td>Multicolumn Thermostat Temperature</td>
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<tr>
<td>Flow rate</td>
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<tr>
<td>Injection Volume</td>
<td>1 to 3 µL</td>
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**Table 2. MS acquisition parameters.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
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<tr>
<td><strong>Agilent 6545XT AdvanceBio LC/Q-TOF</strong></td>
<td></td>
</tr>
<tr>
<td>Source</td>
<td>Dual Agilent Jet Stream</td>
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<td>350 °C</td>
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<tr>
<td>Gas Flow</td>
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<td>Nebulizer</td>
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<td>Sheath Gas Temperature</td>
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<tr>
<td>Sheath Gas Flow</td>
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<td>VCap</td>
<td>5,500 V</td>
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<tr>
<td>Nozzle Voltage</td>
<td>2,000 V</td>
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<tr>
<td>Fragmentor</td>
<td>380 V</td>
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<tr>
<td>Skimmer</td>
<td>140 V</td>
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<tr>
<td>Quad AMU</td>
<td>m/z 500</td>
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<tr>
<td>Mass Range</td>
<td>m/z 500 to 8,000</td>
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<tr>
<td>Acquisition Rate</td>
<td>1.0 spectra/s</td>
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<tr>
<td>Reference Mass</td>
<td>922.0098</td>
</tr>
<tr>
<td>Acquisition Mode</td>
<td>Positive, extended (m/z 10,000) mass range</td>
</tr>
</tbody>
</table>
Results and discussion

Method optimization for intact mAb quantitative analysis

To improve the detection sensitivity and reproducibility for intact mAb quantitative analysis, the following method developments have been made:

- The use of the AssayMAP Bravo platform along with the streptavidin affinity cartridge (SA-W) for the biological sample enabled a larger sample loading capacity, which led to more concentrated sample eluent. The AssayMAP Bravo system has proven to be more reproducible than manual sample preparation.

- Intact Herceptin sample was diluted with DI water containing 0.001% BSA (w/v) to prevent intact mAbs from self-aggregation and column binding. Figure 2 shows that the intact Herceptin and BSA were well separated in the short HPLC gradient (five minutes), and no protein coeluting was detected.

- The AssayMAP Bravo system has proven to be more reproducible than manual sample preparation.

- HPLC method (PLRP-S column and higher column temperature) was optimized using the conventional flow rate (0.5 mL/min) to achieve better reproducibility for high-throughput intact mAb analysis compared to the nanoflow protocol. The higher column temperature did not cause sample degradation or precipitation; instead, it improved the MS sensitivity significantly under such high flow conditions.

- The 6545XT AdvanceBio LC/Q-TOF system is not only equipped with new design attributes in terms of ion optics, heater system, and vacuum but also with the large molecule SWARM autotune feature, which can improve the MS sensitivity on intact mAb by more than a factor of four compared to the Agilent 6550 iFunnel Q-TOF LC/MS.

Intact Herceptin quantitative analysis

The formulated stock solution of Herceptin (20 mg/mL) was diluted with 0.001% BSA into a series of concentrations ranging from 0.01 to 50 ng/µL (12 levels).

Figure 2. (A) Representative total ion chromatograms (TIC) of various amounts of intact Herceptin analyzed in the neat solution. (B) The corresponding charge state distributions of the targeted mAb.
Triplicate sample injections were made at each concentration, and all quantitative data analyses were processed using MassHunter Quantitative Analysis 10.0 software.

To achieve the highest analytical sensitivity in the intact mAb quantitation, several data processing methods have been evaluated. They were:

- Peak integration on the intact deconvoluted MS spectrum.
- Various peak summations of the protein charge envelope (summing top number of glycoforms in several charge states).

Based on these methods, the best result on intact mAb quantitation was generated from summing the top two glycoforms in five charge states, as shown in Figure 3. Specifically, data processing parameters included:

- Mass tolerance window ±25 ppm
- Retention time window ±0.5 minutes
- Calibration curve fitting: quadratic regression with 1/x weighting

Figure 3 demonstrates the excellent quantitation curve (sum of 10 top G0F/G1F peaks) of intact Herceptin in neat solution with 3.3 orders of dynamic range (0.025 to 50 ng) with \( R^2 = 0.9955 \). The limit of detection (LOD) of 0.025 ng and limit of quantitation (LOQ) of 0.05 ng were achieved using the above optimized data processing parameters.

Figure 4 demonstrates the excellent quantitation curve (sum of 10 top G0F/G1F peaks) of intact Herceptin in neat solution with 3.3 orders of dynamic range (0.025 to 50 ng) with \( R^2 = 0.9955 \). The limit of detection (LOD) of 0.025 ng and limit of quantitation (LOQ) of 0.05 ng were achieved using the above optimized data processing parameters.

**Figure 3.** Zoomed-in charge states of intact Herceptin showing peak selection (summing top two glycoforms in five charge states) for data processing.

**Figure 4.** Calibration curve of intact Herceptin in neat solution (0.001% BSA) in the range of 0.01 to 50 ng/µL. (A) Full range; (B-D) zoomed-in ranges.
Quantitative analysis of intact mAb from mouse serum
To accurately quantitate the intact mAb in biological matrix, it is critical to develop a highly selective and sensitive sample preparation method. Using the AssayMAP platform, we can generate more concentrated elution with higher recovery and increased selectivity and reproducibility compared to the magnetic beads-based methodology.

Figure 5A shows the TIC of an mAb sample from mouse serum prepared by the AssayMAP Bravo system. The presence of few peaks demonstrates good selectivity from the AssayMAP streptavidin cartridge. The extracted ion chromatogram (EIC) of the targeted mAb was generated (Figure 5B) by summing the five most intense peaks over five charge states (this mAb does not have glycoforms), with a mass extraction window of ±25 ppm (Figure 5C).

Figure 5. (A) TIC, (B) EIC, and (C) raw MS spectrum of intact mAb from mouse serum. The top five charge state ions (in the blue box) represent the peak selection and summation for data processing.
Figure 6 shows the quantitation curve (sum of top five charge state peaks) of intact mAb from mouse serum with 3.15 orders of dynamic range (35 to 50,000 ng/mL) with $R^2 = 0.9966$. An LOD of 35 ng/mL and an LOQ of 50 ng/mL were achieved. We have achieved low picogram analytical sensitivity in intact mAb quantitation analysis. Table 3 summarizes the intact mAb quantitation results between the purified mAb in neat solution and in mouse serum using the optimized workflow. We believe the detection sensitivity for mAb from a biological matrix sample was mainly due to the major improvements in both hardware and software for detection of large protein molecules. Moreover, the use of a nonglycosylated mAb in this study could also have contributed to the increase in analytical sensitivity, because the collapsed signal from multiple glycoforms would lead to an increase in signal-to-noise ratio (S/N).

![Figure 6](image-url)

**Table 3.** Summary of quantitative analysis of various intact mAbs.

<table>
<thead>
<tr>
<th></th>
<th>Herceptin (In Neat Solution)</th>
<th>Intact mAb in Mouse Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection Volume</td>
<td>1.0 µL</td>
<td>3.0 µL</td>
</tr>
<tr>
<td>LOD</td>
<td>N/A</td>
<td>35 ng/mL</td>
</tr>
<tr>
<td>LOD: Total On-Column</td>
<td>0.025 ng</td>
<td>0.105 ng</td>
</tr>
<tr>
<td>LOQ</td>
<td>N/A</td>
<td>50 ng/mL</td>
</tr>
<tr>
<td>LOQ: Total On-Column</td>
<td>0.05 ng</td>
<td>0.15 ng</td>
</tr>
<tr>
<td>Standard Curve</td>
<td>0.025 to 50 ng</td>
<td>35 to 50,000 ng/mL</td>
</tr>
<tr>
<td>Dynamic Range</td>
<td>3.3x</td>
<td>3.15x</td>
</tr>
</tbody>
</table>
Conclusion

We reported a highly sensitive LC/MS workflow for the quantitative analysis of intact mAbs from biological matrix. This optimized workflow uses the Agilent AssayMAP Bravo automated liquid-handling platform for sample preparation and the Agilent 6545XT AdvanceBio LC/Q-TOF system with enhanced MS capabilities for intact protein analysis. A detection sensitivity (LOD) of 35 ng/mL (0.105 ng on-column) and an LOQ of 50 ng/mL (0.15 ng on-column) for intact mAb from mouse matrix were achieved using this ultrasensitive, highly reproducible, and extensively automated workflow solution.

Acknowledgement

We would like to thank Dr. Kevin Bateman (Merck & Co.) and his team for providing insightful discussions as well as sharing results and conclusions in this area.

References

1. Precise Characterization of Intact Monoclonal Antibodies by the Agilent 6545XT AdvanceBio LC/Q-TOF, Agilent Technologies Application Note, publication number 5991-7813EN.