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# Intact Protein Analysis in Plasma Using Automated 2D-LC/TOF with Multiple Heart-Cutting

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## Introduction

Direct intact and top-down protein analysis enables comprehensive characterization of proteoforms, post-translational modifications, and fragments that are often lost in bottom-up workflows.[1] However, intact mass analysis in complex biological matrices such as whole plasma is challenged by extreme dynamic range and ion suppression, commonly necessitating affinity-based enrichment.

Here, we demonstrate a non-affinity, orthogonal separation strategy using a 2D-LC/TOF workflow with multi-heart-cutting for native intact protein analysis directly in plasma. Therapeutic monoclonal antibodies (mAbs) trastuzumab and antibody-drug conjugate T-DM1, were used as model systems to evaluate performance.

The workflow employs an Agilent 1290 Infinity III 2D-LC system for orthogonal separations and precise multiple heart-cutting, coupled to a high-resolution, high-sensitivity Agilent 6230C Accurate Mass TOF LC/MS System.

## Experimental

### Instrumentation

- Agilent 1290 Infinity III 2D-LC system comprised the following modules:
  - 1290 Infinity III Bio Multisampler with Thermostat (G7137A)
  - 1290 Infinity III Multicolumn Thermostat (G7116B)
  - 1290 Infinity III Diode Array Detector (G7117B) with Max-Light cartridge cell (G7117-60020)
  - 1260 Infinity III Bio Flexible Pump (G7131C)
  - 1290 Infinity III Bio High-speed Pump (G7132A)
  - 1290 Infinity Valve Drive (G1170A) with 2D-LC ASM Valve (G5643B)
  - Two 1290 Infinity III Valve Drives (G1170A) with multiple heart-cutting valves (G5642-64000) equipped with 40 µL Bio loops
  - 1290 Infinity III Valve Drive (G1170A) with a 2-position/6-port valve (G4231A) (used as a diverter valve)
- Agilent 6230C Accurate Mass TOF LC/MS System

### Materials and Sample Preparation

Human plasma were purchased from BioIVT. The formulated trastuzumab and ADC (T-DM1) were from Genentech.

For mAb controls, trastuzumab and T-DM1 were diluted in 200 mM ammonium acetate (AA) buffer to a final concentration of 1 µg/µL.

## Experimental

Formulated trastuzumab and T-DM1 were individually spiked into neat human plasma to a concentration of 1 µg/µL. Plasma blank and mAb-spiked samples were centrifuged at 4 °C and 20,000 × g for 20 min. Supernatants were further cleaned using 30 kDa MWCO ultra centrifugal filters with 200 mM AA buffer, yielding a final volume approximately equivalent to the initial plasma volume.

### LC/MS Analysis

Data acquisition and analysis were carried out using MassHunter acquisition (12.2), BioConfirm (12.1) and MassHunter Qualitative Analysis (13.0) software.

Table 1. LC/MS method.

| LC First Dimension (1D): SEC | | |
|---|---|---|
| Column | AdvanceBio SEC 300Å, 2.7µm, 4.6 mm x 300 mm, (p/n PL1580-5301) | |
| Sampler temp. | 4 °C | |
| Injection volume | 0.5 to 10 µL | |
| Post-injection filter | Bio Inline Filter, titanium frits 0.3 µm (p/n 5720-0020) | |
| Mobile phase | 200 mM ammonium acetate (AA) | |
| Flow rate | 0.35 mL/min | |
| UV Detection | 280 nm | |
| **LC Second Dimension (2D): IEX** | | |
| Column | Agilent Bio WAX, NP5, 2.1 x 250 mm, PEEK (p/n 5190-2491) | |
| Thermostat | 30 °C for both 1st and 2nd D columns | |
| ASM Factor | 5:1 | |
| Mobile phase | A) 10 mM AA<br>B) 500 mM AA | |
| Flow rate | 0.2 mL/min | |
| | trastuzumab | T-DM1 |
| Gradient program | • 0 to 1 min, 0%B;<br>• 1 to 30 min, 0 to 100%B;<br>• 30 to 30.1 min, 100 to 0%B | • 0 to 1 min, 0%B;<br>• 1 to 11 min, 0 to 100%B;<br>• 11 to 11.1 min, 100 to 0%B |
| **Agilent 6230C Accurate Mass TOF LC/MS System** | | |
| Ion source | AJS | |
| Gas temp. | 325 °C | |
| Drying gas | 12 L/min | |
| Nebulizer gas | 35 psi | |
| Sheath gas temp. | 275 °C (trastuzumab)<br>300 °C (T-DM1) | |
| Sheath gas flow | 12 L/min | |
| Capillary | 5500 V | |
| Nozzle | 2000 V | |
| Octopole RF | 750 V | |
| Fragmentor | 300 V | |
| Skimmer | 220 V | |

# Results and Discussion

## Intact Native Trastuzumab in Plasma

- Agilent 2D-LC solutions enable rapid and intuitive method development, leveraging 1D UV-guided heart-cutting to streamline method setup and minimize development time for complex biological samples.
- High-resolution, precise heart-cutting provides flexible and selective isolation of intact proteins from plasma, enabling efficient transfer to the second dimension for orthogonal separation while preserving native structure.
- Native MS detects intact trastuzumab (**Figure 1**) directly from neat plasma at 1 µg/µL with excellent mass accuracy, and preserved glycoform integrity, achieved without any affinity-based protein enrichment or immunocapture.

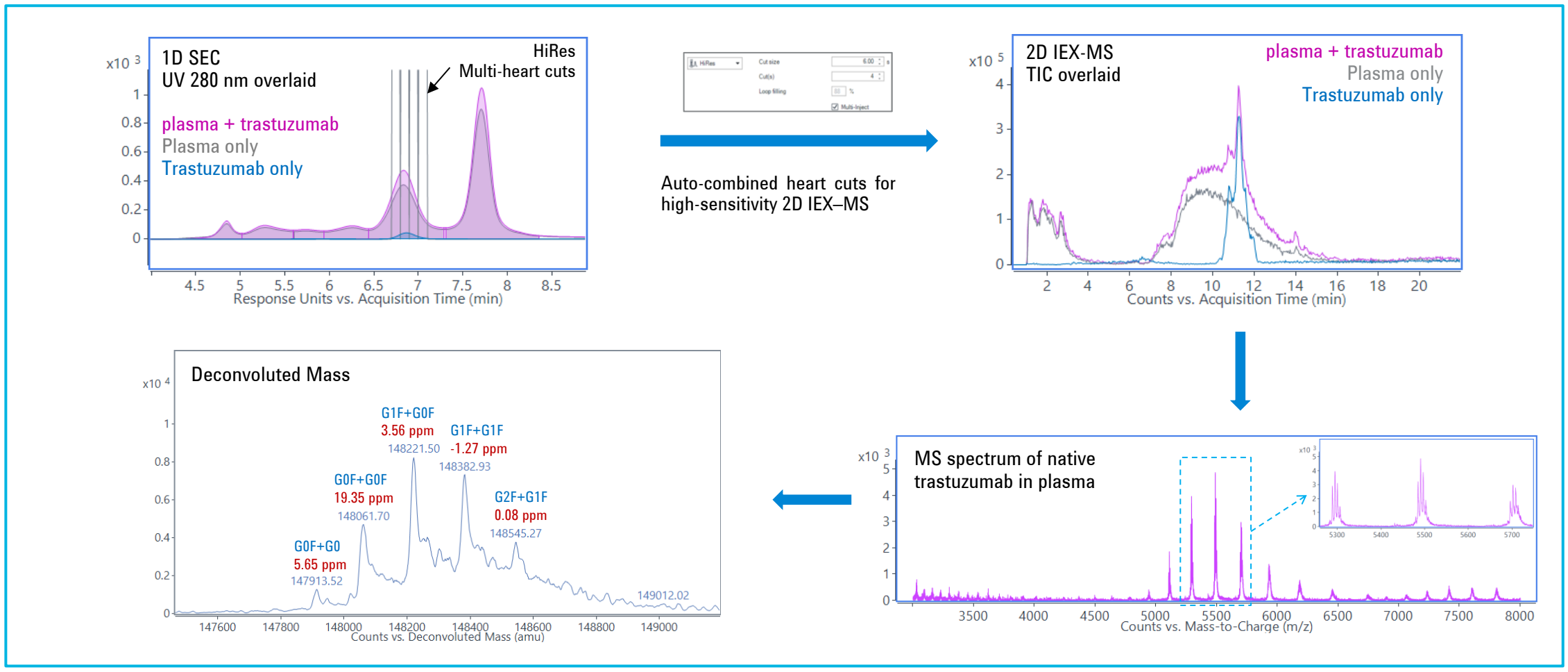


Figure 1. 2D-LC/TOF analysis of intact native trastuzumab in neat plasma.

To further evaluate the performance of this 2D-LC/TOF system for low level intact protein characterization and quantification in complex biological matrices, trastuzumab was spiked into cleaned human plasma at 1 µg/µL and serially diluted with plasma to final concentrations ranging from 62.5 ng/µL to 1 µg/µL.

All the samples were analyzed using this 2D-LC/TOF system.

**Figure 2** presents the native MS spectra of trastuzumab observed at the 27+ charge state in human plasma across the tested concentrations, demonstrating excellent analytical sensitivity and glycoproteoform characterization in a highly complex biological matrix.

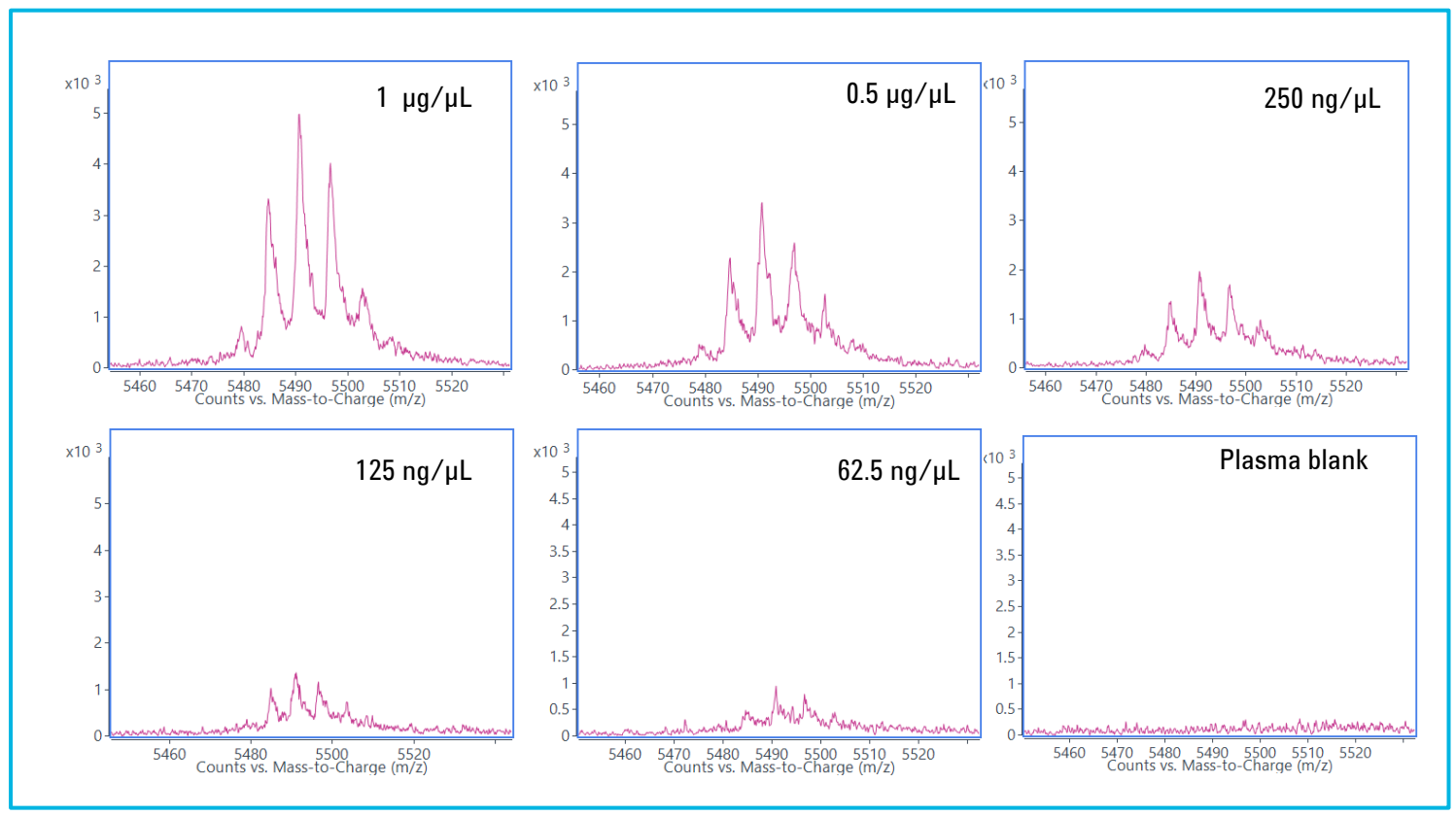


Figure 2. MS spectra of intact native trastuzumab acquired in neat plasma across a range of concentrations, demonstrating excellent analytical sensitivity and glycoproteoform characterization using the Agilent 2D-LC/TOF system.

## Results and Discussion

### Intact Native T-DM1 in Plasma

T-DM1 is an antibody–drug conjugate (ADC) designed to treat HER2-positive breast cancer.[2] It combines the targeted trastuzumab with the chemotherapy agent DM1. Detection of intact T-DM1 by LC/MS is more challenging than trastuzumab due to ADC-associated mass heterogeneity (DAR distribution) and increased structural complexity.

Native MS analysis revealed substantial interference from co-eluted endogenous plasma proteins with T-DM1, which could not be resolved by extending the IEX gradient. By leveraging multiple heart-cutting, the 2D-LC system enables each fraction to be analyzed separately with high-resolution IEX-MS. Using this approach, T-DM1 species with different DARs were successfully detected directly from plasma (**Figure 3**).

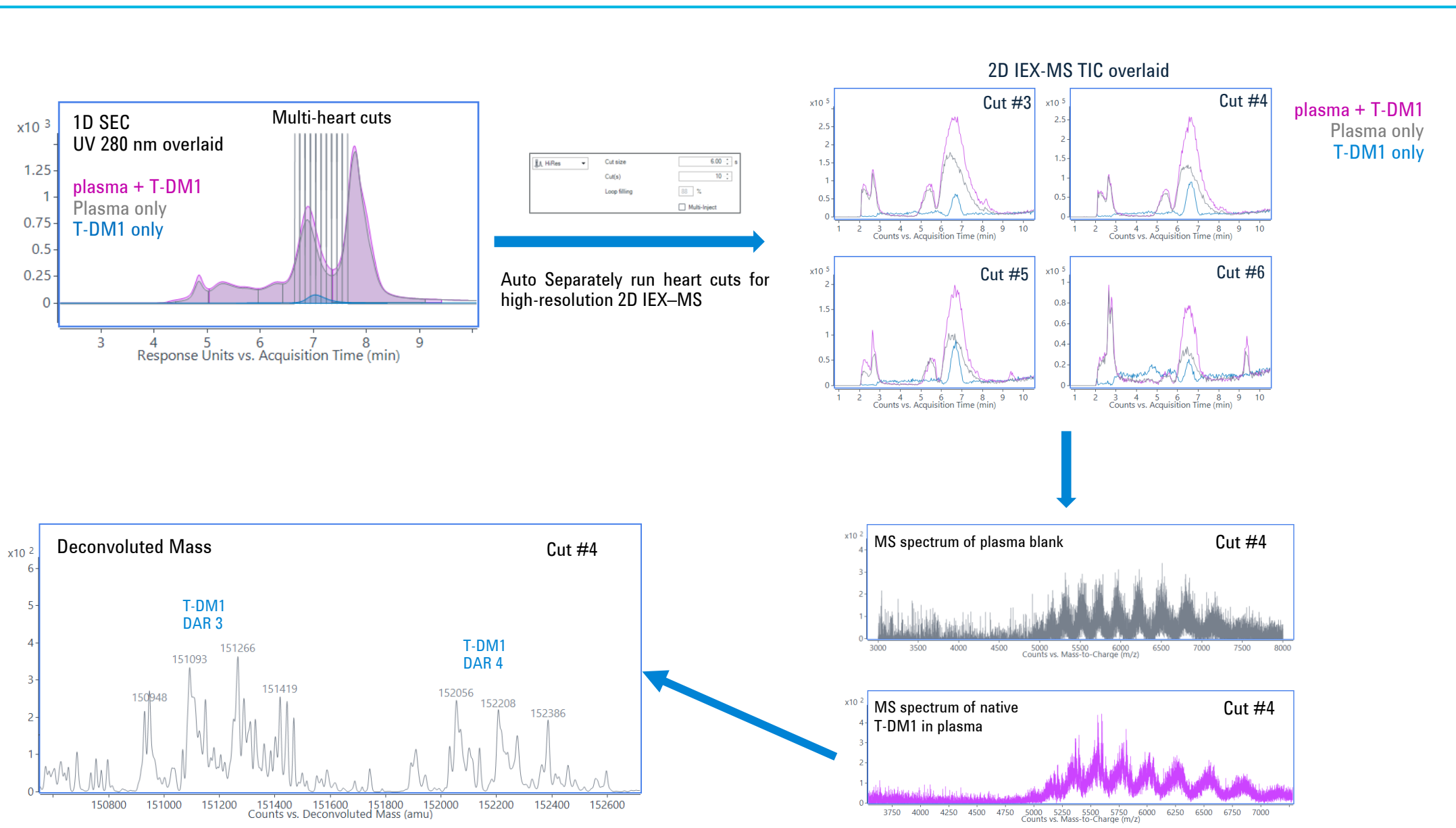


Figure 3. 2D-LC/TOF analysis of intact native T-DM1 in neat plasma using high resolution heart-cutting technology.

## Conclusions

- Agilent 2D-LC enables direct native MS analysis of intact proteins from plasma without affinity-based enrichment.
- UV-guided and multiple heart-cutting simplify 2D-LC method development and provide high-resolution isolation of native proteins from complex biological matrices such as plasma.
- The Agilent 6230C Accurate Mass TOF delivers high sensitivity and excellent mass accuracy, enabling confident intact protein characterization under native conditions.

https://www.agilent.com/en/promotions/asms



## References

[1]Native Top-Down Proteomics of Endogenous Protein Complexes Enabled by Online Two-Dimensional Liquid Chromatography. Fischer MS, et al. *Anal Chem*. 2025

[2]Ado-trastuzumab Emtansine (T-DM1): An Antibody–Drug Conjugate (ADC) for HER2-Positive Breast Cancer. Lambert JM et. al. *J Med Chem*. 2014.

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