# Clone Selection Using the Agilent 1290 Infinity Online 2D-LC/MS Solution

## Application Note

Biopharmaceuticals


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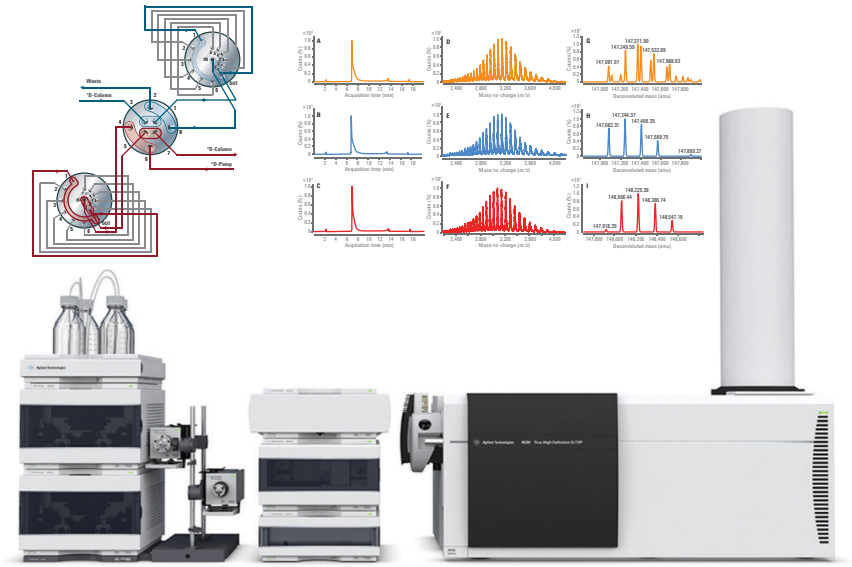
### Abstract

This Application Note describes the Agilent 1290 Infinity online 2D-LC/MS solution and proof of principle for clone selection and quantification of monoclonal antibody (mAb) using an Agilent Bio-Monolith Protein A column in the first dimension followed by a desalting step with an Agilent AdvancedBio RP-mAb diphenyl column in second dimension that is coupled to a mass spectrometer. The first step provides the enrichment of the mAb, and the second step was used for confirmation of right quality of the mAb.

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## Introduction

Monoclonal antibodies (mAbs) are the fastest growing protein therapeutics in recent years[1]. There is also rapid growth of biosimilars on the market[2]. During the development of mAbs, a well-planned strategy for good clone selection, having the correct intended mAb with a good amount of expression, is critical. This is also true for biosimilars development. Currently, clone selection involves enrichment or purification of the mAb, and collection of the enriched fraction, followed by mass spectrometry analysis in a separate experiment. Online 2D-LC/MS analysis provides a solution to perform different analysis methods in one run. This Application Note describes a combination of protein enrichment and purification with affinity chromatography in the first dimension, followed by reversed-phase chromatography coupled to an accurate-mass spectrometer for protein mass confirmation in the second dimension. In this study and as proof of principle, we used a commercial innovator and biosimilar pair of rituximab spiked in Dulbecco's Modified Eagle Medium (DMEM) as a matrix mimic to demonstrate the online 2D-LC/MS methodology. This method can easily be applied during the clone selection process.

## Materials and Methods

Rituximab biosimilar and innovator were purchased from a local pharmacy, and stored according to the manufacturer's instructions. Acetic acid, formic acid, sodium phosphate, monobasic, sodium phosphate dibasic, sodium chloride, and LC/MS grade solvents were purchased from Sigma-Aldrich. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22-µm membrane point-of-use cartridge (Millipak).

### Sample preparation

The antibodies stock solution present at 10 mg/mL was diluted with DMEM medium for construction of a calibration curve. The samples were centrifuged at 5,000 g for 5 minutes prior to the injection.

### Instrumentation

The Agilent 1290 Infinity 2D-LC solution was composed of the following modules:

- Agilent 1260 Infinity Bio-Inert Quaternary Pump (G5611A)
- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity Autosampler (G4226A) with 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartments (2x G1316C)
- Agilent 1290 Infinity valve drive (G1170A) with 2-position/4-port duo-valve (2D-LC valve head, G4236A)
- Agilent 1290 Infinity valve drives (2x G1170A) with multiple heart-cutting valves (2x G4242-64000) equipped with 40-µL loops
- Agilent 1260 Infinity Diode Array Detectors (2x G1315C) with 1-mm flow cell
- Agilent 6530 Accurate Mass Q-TOF LC/MS (G6530A)

### Columns

- 1D: Agilent Bio-Monolith Column Protein A, 4.6 × 12.5 mm, 5.0 µm (p/n 5069-3639)
- 2D: Agilent AdvanceBio RP-mAb Diphenyl, 2.1 × 50 mm, 3.5 µm (p/n 799775-944)

### Software

- Agilent OpenLab CDS ChemStation Edition software, version C.01.07 [27] with Agilent 1290 Infinity 2D-LC Acquisition software, version A.01.03
- Agilent MassHunter Workstation for instrument control (B.06.01)
- The data obtained from LC/MS were analyzed using Agilent MassHunter Qualitative Analysis software B.07 and Agilent MassHunter BioConfirm software B.07

Table 1. LC/MS instrument parameters.

| Parameter | Value |
|---|---|
| Valve and loop configuration | 2-pos/4-port duo 2 × 6 loops (countercurrent) loop size 40 µL |
| 1D pump | Agilent 1260 Infinity Bio-inert Quaternary Pump |
| 1D mobile phase | A) PBS (Sigma p/n P4417)<br>B) 0.5 M acetic acid in water |
| 1D column temperature | 25 °C |
| 1D gradient | Time (min) %B Flow rate (mL/min)<br>1 0 1<br>1.1 100 1<br>4.0 100 1<br>4.1 0 1<br>10.0 0 1<br>11.0 0 0 |
| Injection volume | 5 µL |
| Thermostat temperature | 4 °C |
| Diode array detector | 280 nm, 4 nm, Ref. 360 nm, 100 nm<br>Peak width 1D > 0.05 minutes<br>Peak width 2D > 0.05 minutes |
| 2D pump | Agilent 1290 Infinity Binary Pump |
| 2D mobile phase | A) 98 % water, 2 % IPA, 0.1 % FA<br>B) 70 % IPA, 20 % acetonitrile, 10 % water, 0.1 % FA |
| 2D column temperature | 80 °C |
| 2D gradient | Time (min) %B Flow rate (mL/min)<br>0 20 0.5<br>4 20 0.5<br>5 40 0.5<br>10 70 0.5<br>11 90 0.5<br>11.1 20 0.5<br>15 20 0.5 |
| Mode | HiRes sampling, 4 cuts, time-based |
| Agilent 6530 Q-TOF LC/MS System | |
| Ion mode | Positive ion mode |
| Source | Agilent Dual Jet Stream |
| Drying gas temperature | 350 °C |
| Drying gas flow | 8 L/min |
| Sheath gas temperature | 400 °C |
| Sheath gas flow | 11 L/min |
| Nebulizer | 35 psi |
| Capillary voltage | 5,000 V |
| Nozzle voltage | 2,000 V |
| MS range (*m/z*) | 2,000–5,000 |
| LC flow diversion | Time segment Start time (min) Diverter valve position<br>1 0 Waste<br>2 5 MS<br>3 10 Waste |

## Results and Discussion

The Protein A purification for the enrichment of mAbs from the matrix medium was developed earlier[3]. In this study, we adapted the method for the first dimension affinity purification of the mAbs (Table 1). Similarly, we adopted the reversed-phase method developed earlier[4] for the second-dimension chromatography involving the online desalting of mAbs, which is coupled to a mass spectrometer. A newer shorter Agilent AdvanceBio Desalting-RP Cartridge for second dimension is also available for faster analysis[5]. Figure 1 shows a plumbing diagram for multiple heart-cutting with the central 2D-LC valve in a countercurrent configuration, and two multiple heart-cutting valves. The multiple heart-cutting setup consists of twelve 40-µL loop in two 6-position/14-port valves, which has excellent peak parking capabilities. The experiments were accomplished using this valve configuration.

To set up the online 2D-LC/MS method for clone selection, a previously recorded first-dimension chromatogram of affinity purification was loaded using the preview option in the 2D pump setup. The peak from Protein A purification of mAb was heart-cut for the second-dimension analysis, as shown in Figure 2. Figure 2 shows the 2D pump setup with the Protein A purification of mAb at the bottom. The 2D time segments of the Protein A purified peak, which were sampled using high-resolution heart-cutting, is shown as the light orange bar on the chromatogram.

Figure 3 shows the 2D Heart-cut viewer, displaying the third cut position of the first-dimension chromatogram (A). It also shows the 2D UV trace as recorded by the diode array detector (DAD) for the cut portion in the bottom panel (D). The viewer also shows the sampling table with the marked third heart-cut (B) and a peak table (C).

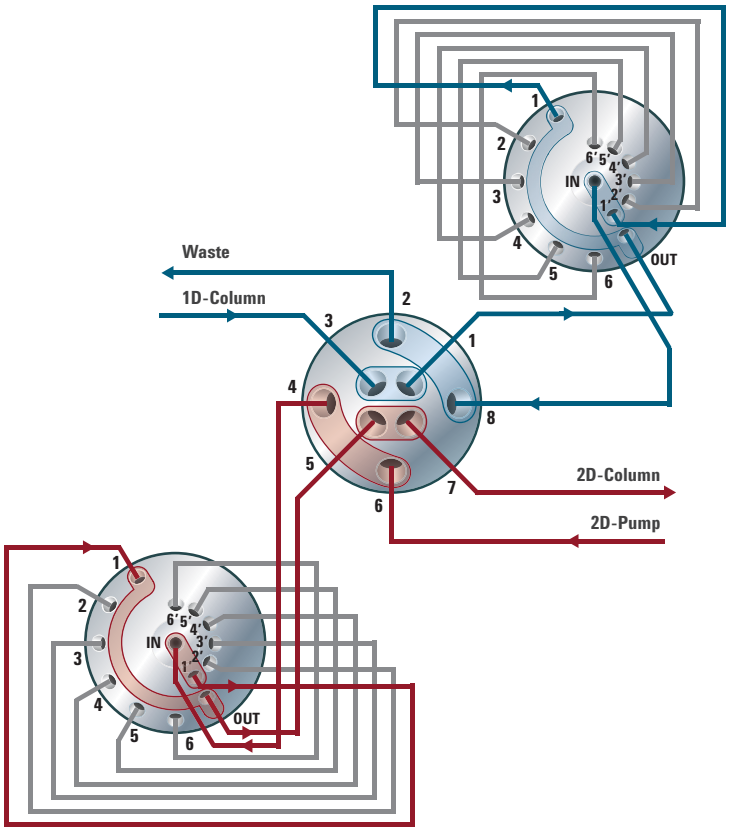


Figure 1. Plumbing diagram for multiple heart-cutting with central 2D-LC valve in countercurrent configuration and two multiple heart-cutting valves.

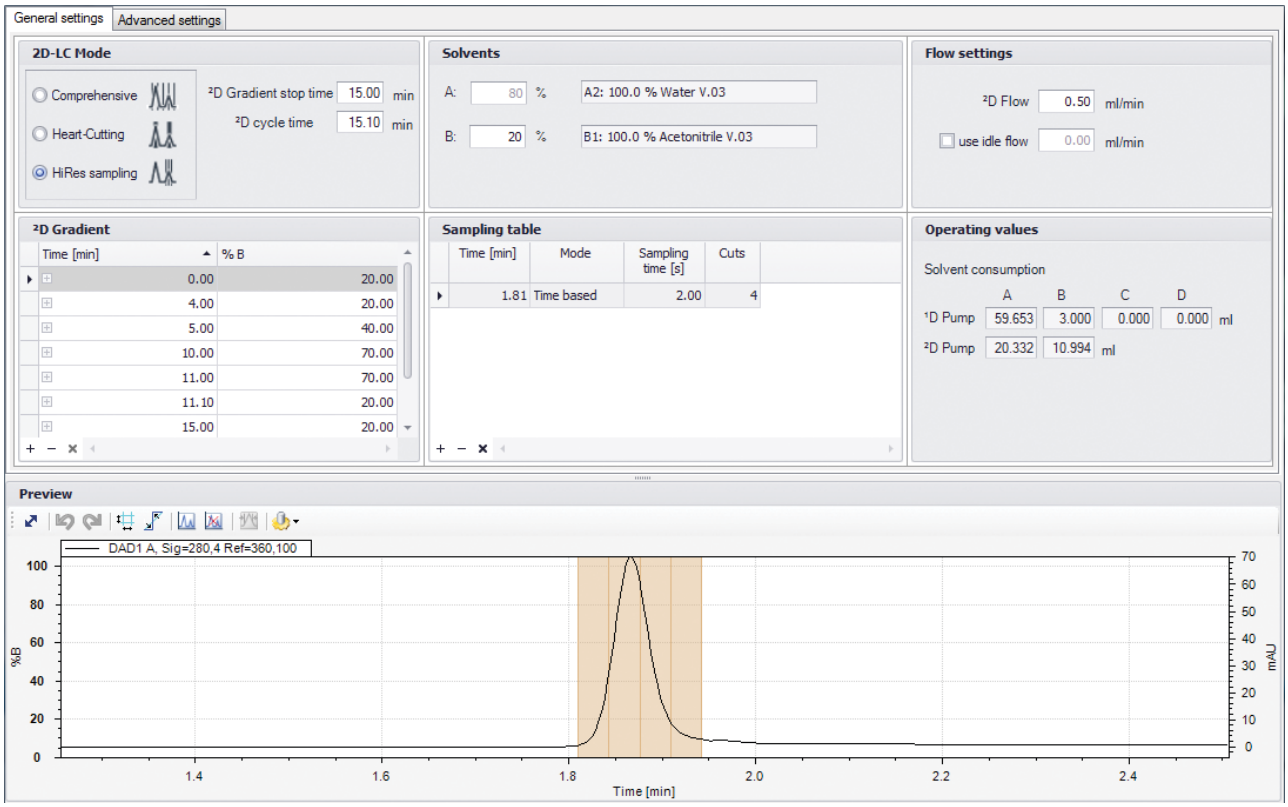


Figure 2. Method setup for high resolution peak sampling. The preview shows the four high-resolution cuts of the Protein A peak stored in different loops.

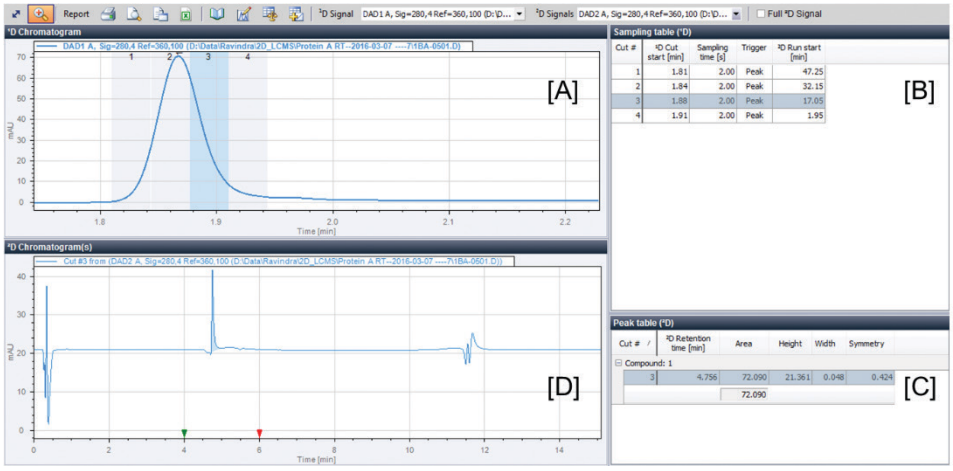


Figure 3. Results of the second-dimension analysis of Protein A. First-dimension chromatogram with marked heart-cut (A), Sampling table (B), Peak table (C), and second-dimension chromatogram (D).

During the biosimilar development programs, low and high-producing clones need to be distinguished. To measure the absolute mAb concentrations, an external calibration can be generated using the innovator molecule. In this study, the calibration curve was constructed using the innovator rituximab. The calibration curve (Figure 4) and corresponding chromatograms (Figure 4B) of innovator rituximab is shown. Good linearity was achieved ranging from 0.2 to 10 µg/µL. Two known spiked sample concentrations were determined using this calibration plot. The inset in Figure 4 summarizes the results. The results suggest good recovery for the spiked sample, and shows the utility of such calibration curves for mAb concentration determination in clones to distinguish between high-producing and low-producing clones.

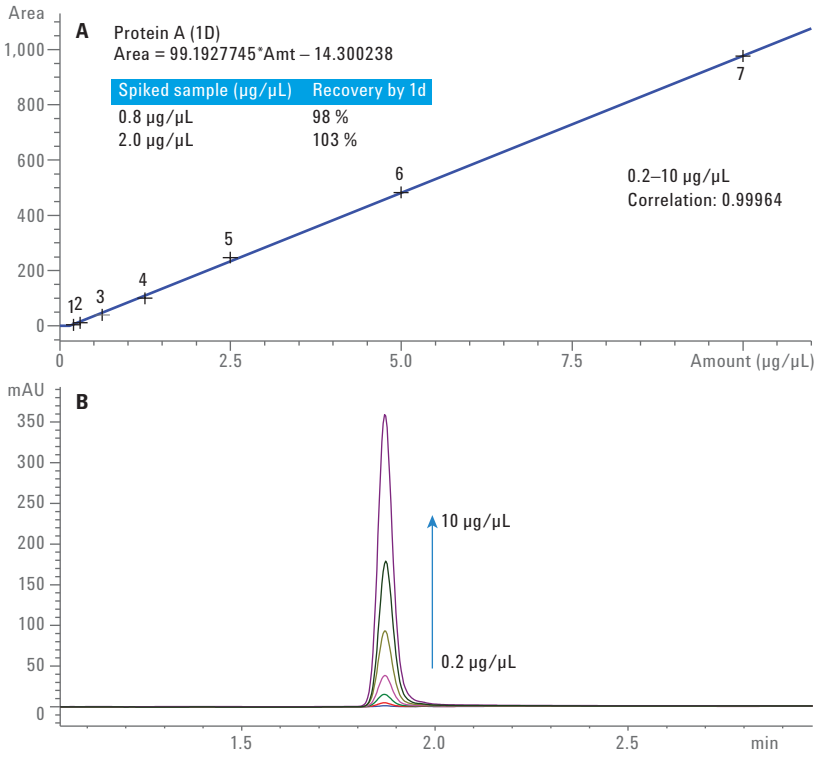


Figure 4. Calibration curve for the rituximab spiked in DMEM using Protein A (A) and overlay of Agilent Bio-Monolith Protein A chromatograms of rituximab calibration points at 280 nm (B).

## LC/MS analysis

The second-dimension effluent was also introduced into the Agilent 6530 Accurate Mass Q-TOF LC/MS to determine the masses of mAbs. Figure 5 shows the LC/MS analysis of intact mAbs. Good shapes with narrow peak width is achieved using IPA/ACN/$H_2O$ mobile phase for an Agilent AdvanceBio RP-mAb Diphenyl column (Figures 5A, 5B, and 5C). Figures 5D, 5E, and 5F show the Gaussian distribution of the charge state envelope for the mAbs.

The deconvoluted spectra of the mAbs (rituximab innovator and biosimilar and IgG1) are shown in Figures 5G, 5H, and 5I. The spectra were deconvoluted using the peak modeling deconvolution algorithm in Agilent MassHunter BioConfirm software. Multiple mass peaks are observed, corresponding to different glycoforms attached to the mAbs. The intact mAb analysis reveals that the antibodies have differences in mass. The masses for the biosimilar and innovator pair for some major peaks are similar, but the biosimilar shows additional peaks that may correspond to additional modification in the biosimilar mAb. An Application Note has been published showing charge heterogeneity in biosimilar mAb describing possible charge variants[6,7]. The IgG1 shows different molecular mass when compared with the biosimilar-innovator pair. These results show the utility of accurate mass spectrometric analysis for distinguishing differences between mAbs.

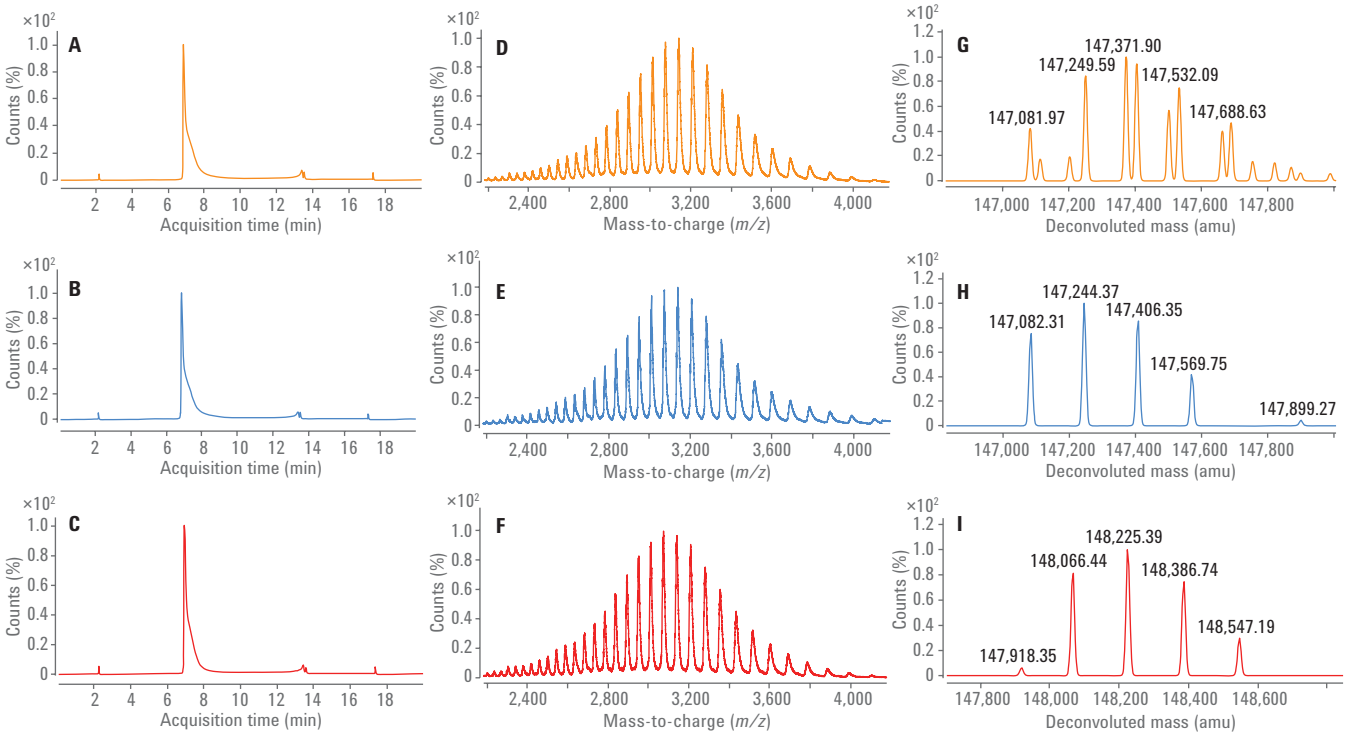


Figure 5. TIC of intact mAbs (A, B, C), charge envelope (D, E, F), and their respective deconvoluted masses (G, H, I). A, D, and G is the biosimilar, B, E, and H is the innovator, and C, F, and I is IgG1.

### Mirror plots

Mirror plots were used to compare the biosimilar-innovator pair. From the mirror plots, it can easily be seen that the biosimilar has more modifications compared to the innovator. These mirror plots help in making quick decisions about the quality of the clone to be selected for further studies.

## Conclusions

- An online 2D-LC/MS analytical method is demonstrated, consisting of multiple heart-cutting with MS detection using affinity chromatography in the first dimension, followed by desalting using reversed-phase chromatography in the second dimension for clone selection.
- The Agilent 1290 Infinity 2D-LC solution with multiple heart-cutting, together with the Agilent 6530 Accurate Mass Q-TOF LC/MS represents a perfect solution for clone selection during mAb development.
- Agilent MassHunter BioConfirm software provided data extraction, deconvolution, and mirror plots for finding the differences between the mAbs.
- This method can be useful during clone selection, and in the area of biosimilar comparison.

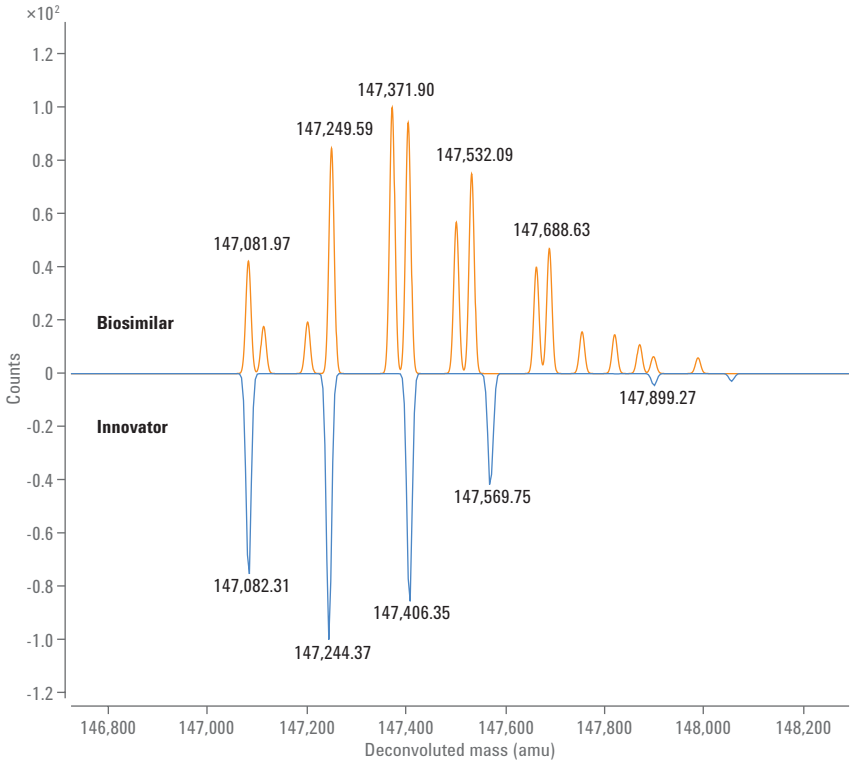


Figure 6. Mirror plots for biosimilar (light brown trace) and innovator (blue trace) comparing intact masses.

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