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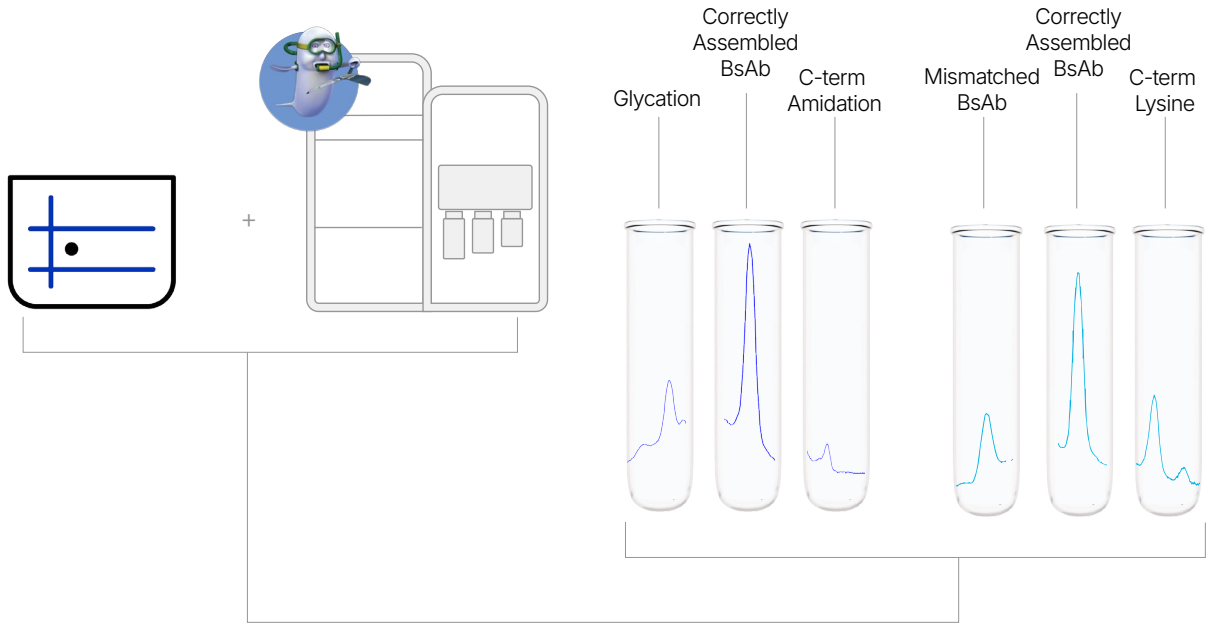
in collaboration with GENOVIS

## Comparing Charge Variants:

# Innovator vs Biosimilar Using the MauriceFlex System & Mass Spectrometry


## Abstract

This application note demonstrates an efficient workflow for the characterization of charge variants of a therapeutic bispecific antibody (BsAb) and a research-grade biosimilar. Imaged capillary isoelectric focusing (icIEF) on the MauriceFlex™ system was used to separate and collect various charge isoforms, which were treated with FabRICATOR® for further digestion prior to LC-MS analysis. A comparison of the results highlighted critical differences between the innovator and biosimilar, including the presence of incorrectly paired species.

# Introduction

Imaged capillary isoelectric focusing (icIEF), the industry gold-standard for protein charge heterogeneity analysis, used to be limited in its ability to isolate charge variants for downstream analysis. The introduction of capillary electrophoresis-mass spectrometry (CE-MS) systems sought to address that limitation but presents the disadvantage of being tied to a specific MS system.

In contrast, an offline fractionation system leverages the power of CE while affording the flexibility to utilize any preferred MS system, and potentially other downstream assays. The MauriceFlex™ system enables charge-based protein fractionation in addition to routine icIEF and CE-SDS assays, and lets you use any MS system of your choice.

This application note showcases fractionation of the bispecific antibody (BsAb) Mosunetuzumab-axgb (brand name Lunsumio™) and a research-grade biosimilar on the MauriceFlex system, followed by subunit analysis of fractions on an LC-MS system.

**TABLE // 01**

| Material | Vendor | Catalog Number |
|---|---|---|
| Mosunetuzumab innovator | Genentech | |
| Mosunetuzumab biosimilar | Ichorbio | ICH5026 |
| MauriceFlex system | ProteinSimple, a Bio-Techne brand | **090-158** |
| Maurice cIEF Cartridge | | **PS-MC02-C** |
| Maurice cIEF Method Development Kit | | **PS-MDK01-C** |
| MauriceFlex cIEF Fractionation Cartridge | | **PS-MC02-F** |
| MauriceFlex cIEF Fractionation Method Development Kit | | **PS-MDK01-F** |
| FabRICATOR Lyophilized 2000 Units | Genovis | **A0-FR1-020** |
| BioAccord LC-MS System | Waters | 176004402W |
| BioResolve RP mAb Polyphenyl Column | | 186008944 |
| Acetonitrile (ACN) with 0.1% Formic Acid | Fisher | LS1201 |
| Water with 0.1% Formic Acid | Fisher | LS1181 |

Table 1. The kits and reagents used in this study.

# icIEF Method

The innovator and biosimilar samples were prepared at a final concentration of 0.1 mg/mL in an ampholyte solution containing Pharmalytes (4%) 8-10.5 and 3-10 (3:1), 5 mM arginine, pI markers 7.05 and 9.50. The samples were loaded onto the MauriceFlex™ instrument along with the Maurice cIEF cartridge and focused for 1 minute at 1500 V, then 12 minutes at 3000 V.

# Fractionation Method

Samples were prepared at a final concentration of 2 mg/mL in an ampholyte solution containing Pharmalytes (4%) 8-10.5 and 3-10 (3:1), 30 mM arginine, 33% SimpleSol, pI markers 7.05 and 9.50. The samples were loaded onto the MauriceFlex instrument along with the MauriceFlex cIEF Fractionation Cartridge and focused for 10 min at 250 V, 10 min at 500 V, 10 min at 1000 V, and 25 min at 1500 V. The detected peaks mobilized for 25 min at 1000 V, followed by fraction collection for 45 sec at 1000 V. All data were analyzed using Compass for iCE software.

# Subunit Analysis

Each pooled fraction collected from the MauriceFlex system was concentrated to 10 µL by SpeedVac. To each fraction, 5 units of FabRICATOR Lyophilized (Genovis) were added and the reaction was incubated for 90 min at 37˚C. The resulting subunits were analyzed on a Waters BioAccord System equipped with a Waters BioResolve RP mAb column (2.1x50 mm). Mobile phases were water and ACN with 0.1% formic acid and separation was achieved using a gradient of 22-38% ACN over 10 minutes. Column temperature was set to 60˚C. MS data was acquired with a capillary voltage of 1.2 kV, a desolvation temperature of 450˚C, and a cone voltage of 40 V. Average mass spectra of each subunit peak (scFc 1, scFc 2, and Fab'2) were generated and deconvolution was performed using MaxEnt.

# Results

### Charge Heterogeneity Analysis and Fractionation

The Mosunetuzumab innovator and biosimilar samples have been analyzed with imaged cIEF and CE-SDS assays on the Maurice™ system, with data published in another study[1]. To collect charge variant fractions, it is essential to first establish cIEF data comparability between the Maurice cIEF and MauriceFlex cIEF Fractionation cartridges. Figures 1A and 1B show cIEF data comparability between both cartridges for the innovator molecule, while Figures 1C and 1D show data comparability between both cartridges for the biosimilar.

**FIGURE // 01**

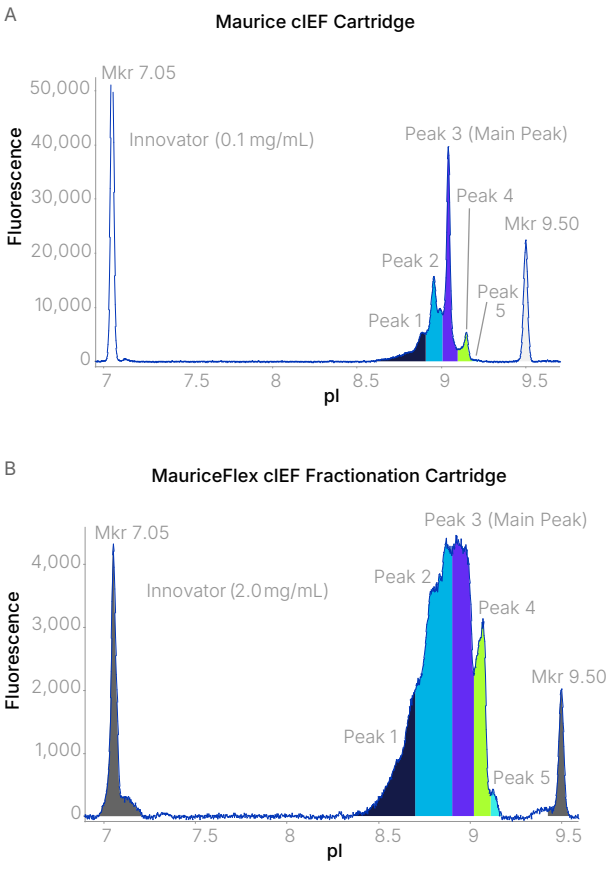


**FIGURE // 01 CONTINUED**

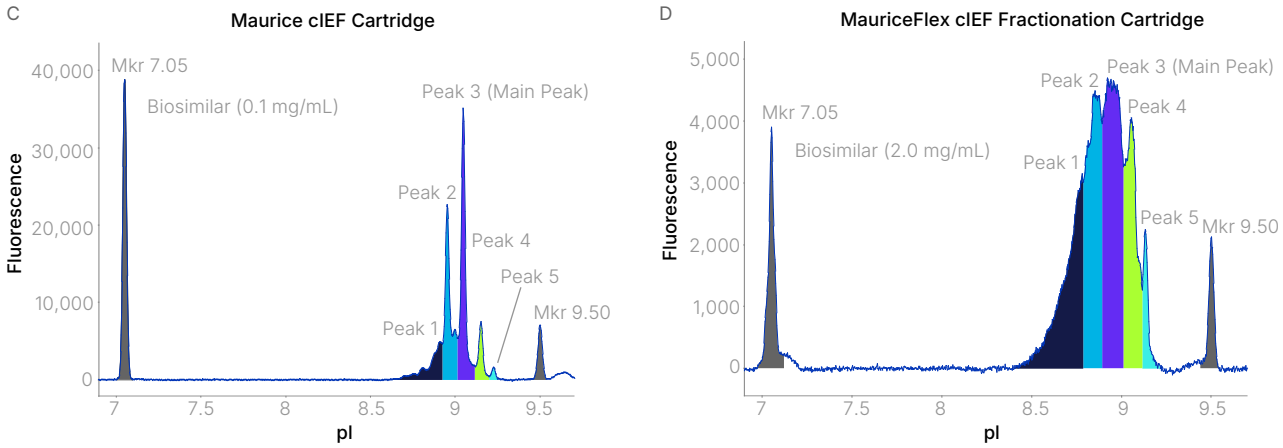



Figure 1. Maurice cIEF and MauriceFlex cartridges generate comparable results. Charge profiles of the innovator molecule using the cIEF cartridge (A) and the MauriceFlex cartridge (B); similarly, profiles for the biosimilar molecule using the cIEF cartridge (C) and the MauriceFlex cartridge (D) are shown. All four profiles display five peaks of interest. Broader peaks in the MauriceFlex cartridge results are attributed to the higher sample load required for analysis.

Five independent fractionation runs were conducted for both innovator and biosimilar samples, from which fractions were pooled. It should be noted that pooling is optional and largely depends on the sensitivity of the MS instrument being used. Specific fractions were then selected for subsequent icIEF peak verification and purity estimation, as illustrated in Figure 2. These fractions were overlaid with unfractionated Mosunetuzumab that served as a reference for peak verification. Both innovator and biosimilar samples revealed 14 out of 18 fractions that were >75% pure, which are listed in Table 2.

**FIGURE // 02**

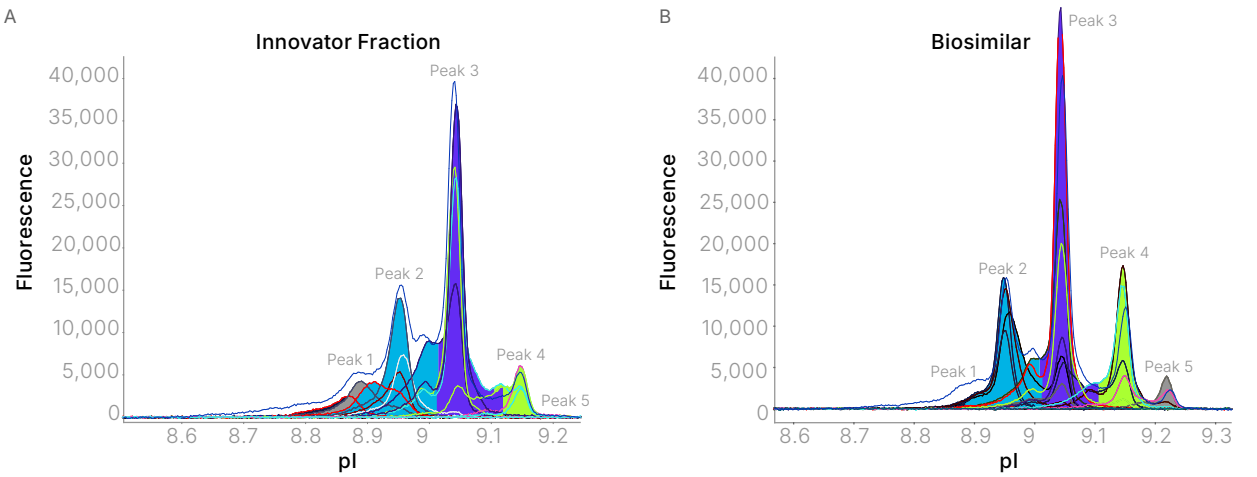



Figure 2. The MauriceFlex system fractionates both BsAb samples, providing relevant charge variant fractions for further analysis. Each BsAb was fractionated using the same method, producing 9 fractions each for the innovator (A) and biosimilar (B). The unfractionated molecule (overlaid blue line) is shown to illustrate coverage obtained with fractionation. The collected fractions were quantitated and used for subsequent downstream analysis.

TABLE // 02

| Fraction Peak Purity (% of Total) | | | | | |
|---|---|---|---|---|---|
| **Innovator** | | | | | |
| **Sample** | **Peak 1** | **Peak 2** | **Peak 3** | **Peak 4** | **Peak 5** |
| Unfractionated Sample | 14.3 | 35.3 | 42.5 | 7.5 | 0.3 |
| Fraction 1 | | | 12.8 | **87.1** | 0.1 |
| Fraction 2 | | 11.6 | 73.7 | 14.7 | |
| Fraction 3 | | 16.1 | **83.9** | | |
| Fraction 4 | | 44.7 | 55.3 | | |
| Fraction 5 | | **88** | 12 | | |
| Fraction 6 | 7.3 | **92.7** | | | |
| Fraction 7 | 36.7 | 63.3 | | | |
| Fraction 8 | **89** | 11 | | | |
| Fraction 9 | **99.7** | 0.3 | | | |
| **Biosimilar** | | | | | |
| **Sample** | **Peak 1** | **Peak 2** | **Peak 3** | **Peak 4** | **Peak 5** |
| Unfractionated Sample | 7.6 | 28.8 | 46.4 | 15 | 2.3 |
| Fraction 1 | | | | 15.8 | **84.2** |
| Fraction 2 | | | 14.3 | **79.3** | 13.2 |
| Fraction 3 | | 7 | 54.5 | 38.5 | 1 |
| Fraction 4 | | 16.6 | **82.2** | | |
| Fraction 5 | | 16.4 | **83.3** | | |
| Fraction 6 | | **75.1** | 24.4 | | |
| Fraction 7 | 11.5 | **88.5** | | | |
| Fraction 8 | **80.2** | 19.8 | | | |
| Fraction 9 | **99** | | | | |

Table 2. The MauriceFlex system generates high-purity fractions of both the innovator and biosimilar. Peaks displaying >75% purity are emboldened.

## Mass Spectrometry Analysis of Fractions

Prior to analysis with mass spectrometry, the selected fractions were treated with FabRICATOR Lyophilized to cleave the bispecific antibody at a single amino acid site below the hinge region. The BsAb samples were successfully digested into Fab'2 and two scFc fragments, which were separated by reversed-phase HPLC for subunit analysis. This workflow is summarized in Figure 3.

**FIGURE // 03**

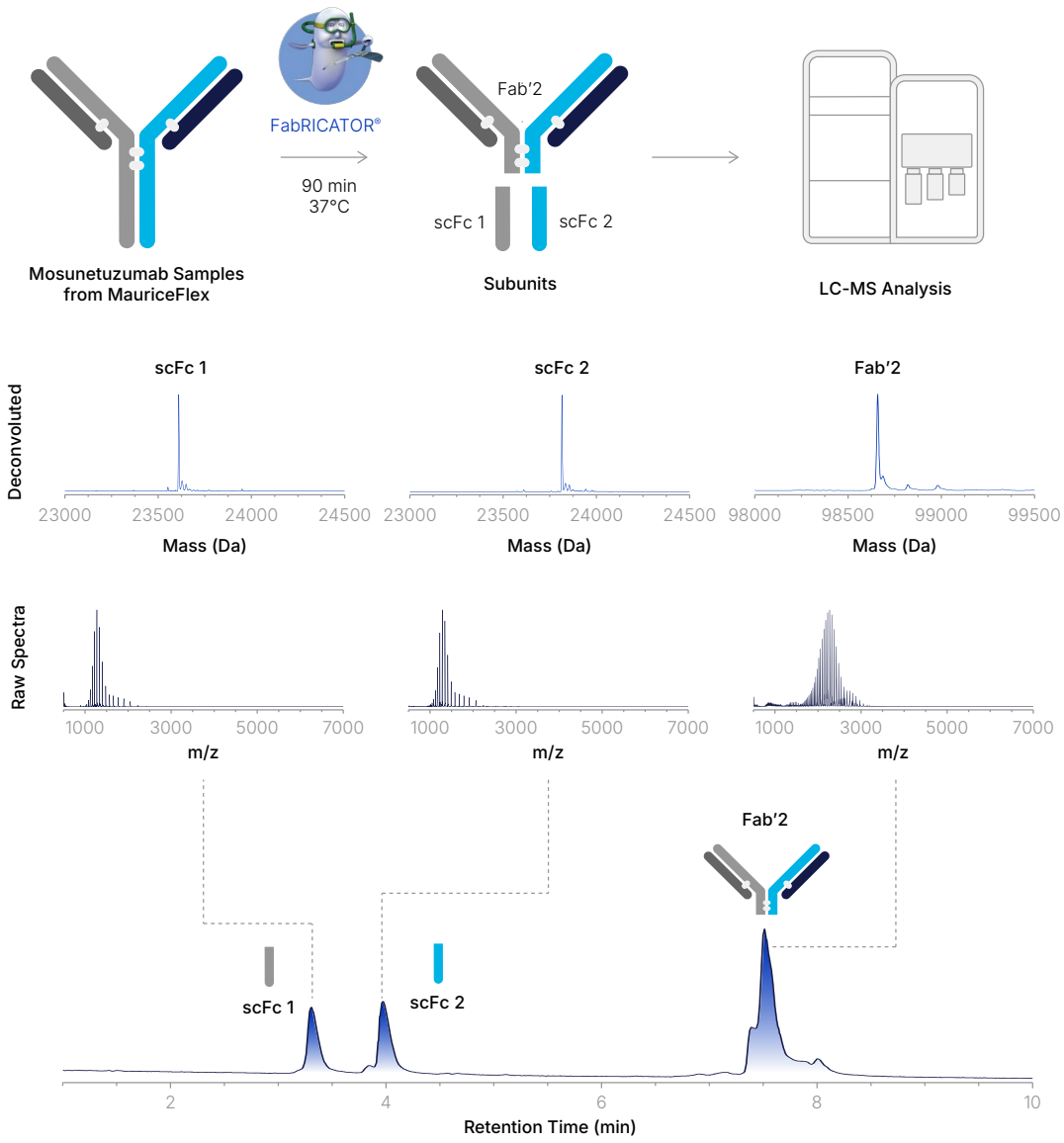



Figure 3. Workflow of subunit analysis for collected charge variant fractions. The antibody was digested using FabRICATOR to generate subunits, followed by LC-MS analysis. This analysis resulted in distinct chromatogram peaks for the subunits Fab'2, scFc1, and scFc2. Both raw and deconvoluted spectra are also displayed.

Analysis of the Fab'2 subunit revealed a critical difference between the two Mosunetuzumab samples. While the innovator's acidic fractions confirmed the correct assembly of the molecule and detected glycation (Figure 4A), the presence of incorrectly assembled BsAb containing two times the same light chain was observed in the biosimilar's acidic fractions (Figure 4B). Such mispairing of chains is known to occur during the production of BsAbs[2].

**FIGURE // 04**

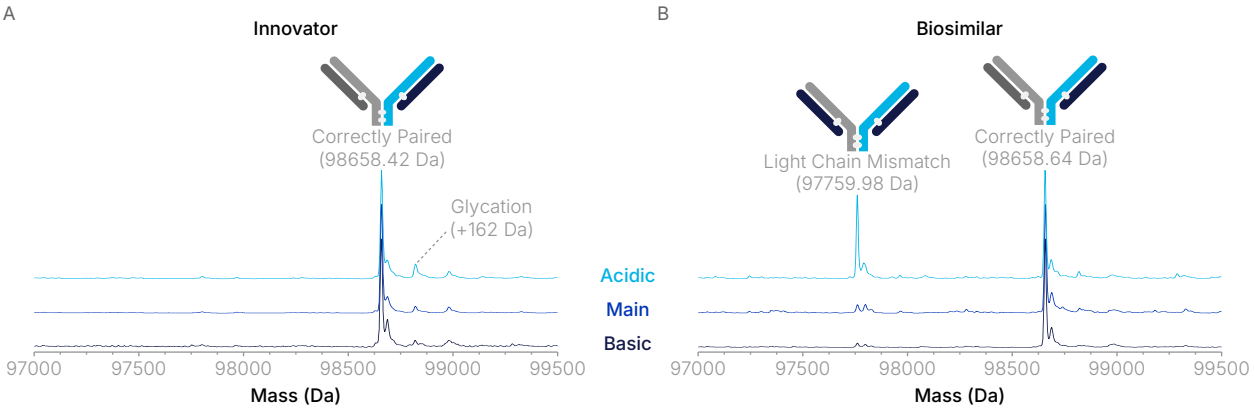



Figure 4. Analysis of the Fab'2 subunit shows critical differences between the innovator and biosimilar. The innovator shows correctly paired BsAbs along with evidence of glycation, especially in its acidic variants (A), while the biosimilar clearly shows the presence of mismatched species in its acidic peaks in addition to the correctly assembled BsAb (B)

The individual scFc subunits highlighted differences in the basic variant profile between the innovator and biosimilar (Figure 5). The basic peaks of the innovator show C-terminal amidation, which is a post-translational modification occurring via glycine removal by PAM monooxygenase[3] (Figure 5A), while the biosimilar's scFc 1 subunit shows retention of C-terminal lysine (Figure 5B). Notably, the basic peaks of the scFc 2 subunit show C-terminal lysine retention for both the innovator and biosimilar albeit to different degrees. (Figures 5C and 5D). This demonstrates the strength of the subunit LC-MS approach in quickly delivering domain specific information on mAb quality. Having uncovered important modifications in acidic and basic peaks of BsAbs, Figures 4 and 5 collectively emphasize the importance of downstream analysis in therapeutic manufacturing and demonstrate how this process can be simplified by taking advantage of the MauriceFlex system.

FIGURE // 05

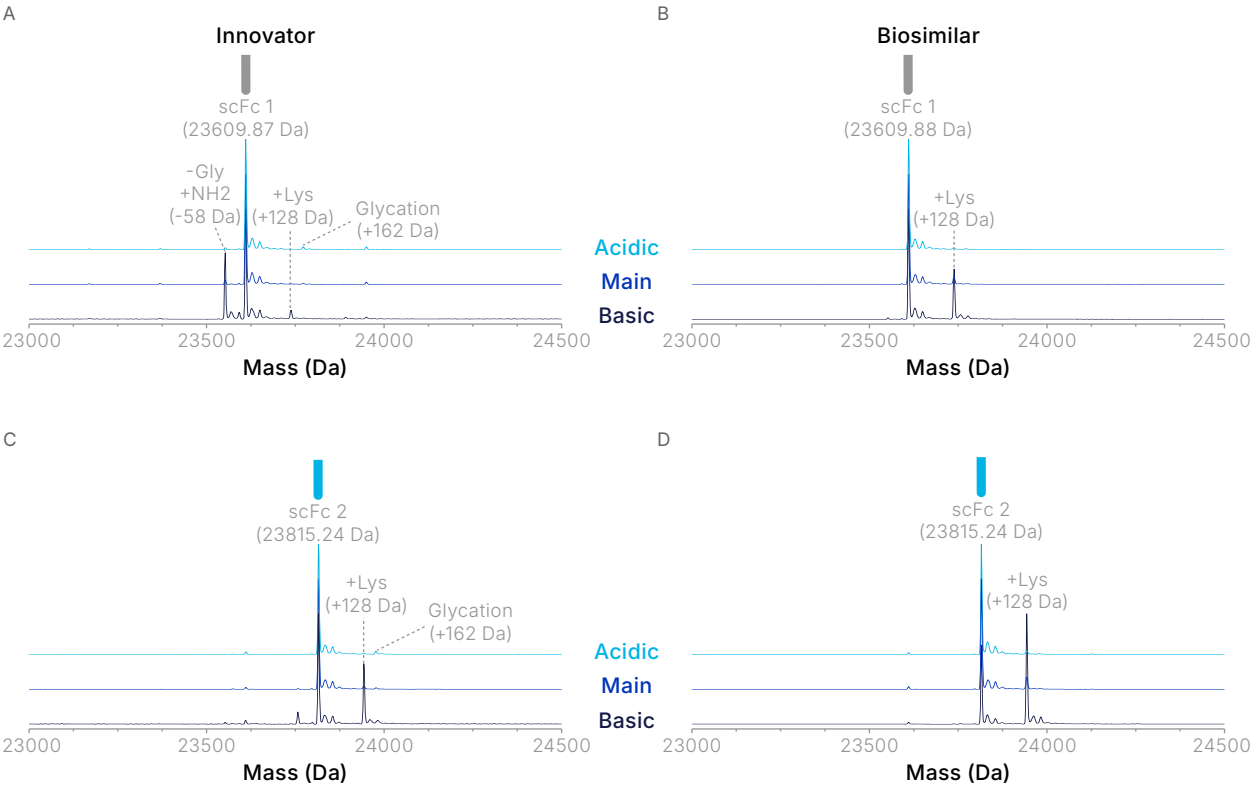



Figure 5. Analysis of the Fc subunits highlight structural differences between the innovator and biosimilar. Innovator scFc 1 subunit analysis indicates C-terminal amidation (-Gly) in its basic peaks (A), while the biosimilar shows the presence of C-terminal lysine (B). For the scFc2 subunit, C-terminal lysine is detected in the basic peaks of both the innovator (C) and the biosimilar (D).

# Conclusion

The MauriceFlex system demonstrates significant advancements in protein characterization by integrating icIEF analysis with icIEF-based charge variant fractionation. This capability eliminates the need for separate method development on traditional IEX platforms, streamlining the workflow significantly. This application note illustrated a comprehensive analysis of charge profiles, fraction collection, and subsequent mass spectrometric analysis of both the innovator and a biosimilar of Mosunetuzumab. The results showed that the MauriceFlex system not only simplifies the analytical process but also provides high-purity fractions, from which detailed insights into the molecular composition of biologics can be gained. LC-MS analysis of the fractions at the subunit level, following FabRICATOR digestion, revealed similarities in main peaks, and some notable differences in acidic and basic variants between the innovator and biosimilar samples. Analysis at the subunit level delivers domain-specific information on mAb quality and facilitates reliable and more confident identification of charge variants. These results underscore the MauriceFlex system's utility in enhancing biopharmaceutical development and quality control by offering reliable, efficient, and integrated characterization capabilities.

# References


1. **Protocol** – Analyzing Mosunetuzumab with Maurice cIEF and CE-SDS

2. Fan, G., Wang, Z., Hao, M., & Li, J. (2015). Bispecific antibodies and their applications. Journal of hematology & oncology, 8, 130. **https://doi.org/10.1186/s13045-015-0227-0**

3. Tak, I.-u.-R., Ali, F., Dar, J. S., Magray, A. R., Ganai, B. A., & Chishti, M. Z. (2019). Posttranslational modifications of proteins and their role in biological processes and associated diseases. In Protein Modificomics: From Modifications to Clinical Perspectives (pp. 1-35). Academic Press. **https://doi.org/10.1016/B978-0-12-811913-6.00001-1**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