

# Fractionation of Bispecific Antibody Charge Variants by MauriceFlex™ and their Identification by Mass Spectrometry

Srinivasa Rao<sup>1</sup>, Peter Johnson<sup>1</sup>, Andreas Nägeli<sup>2</sup>, Chris Heger<sup>1</sup>

<sup>1</sup>ProteinSimple, A Bio-Techne Brand, San Jose, CA; <sup>2</sup>Genovis AB, Kävlinge, Sweden

## Abstract

Mosunetuzumab-axgb is a bispecific CD20-directed CD3 T-cell engaging antibody (BsAb). It was approved by the FDA for refractory follicular lymphoma. Mosunetuzumab-axgb binds to the CD3 receptor expressed on the surface of T-cells and CD20 expressed on the surface of lymphoma cells to enable T-cell mediated killing of lymphoma cells.

In this study we characterized charge variant differences between Mosunetuzumab-axgb (Lunsumio) and a research-grade biosimilar by leveraging the MauriceFlex™ instrument which separates charge variants, mobilizes them, and enables fractionation. Individual charge variant peak fractions were digested with IdeS (FabRICATOR®) and analyzed by mass spectrometry at the subunit level to identify the modifications contributing to the charge variant profile.

## Materials and Methods

All reagents used in the study were of analytical grade unless otherwise specified. The MauriceFlex instrument, Maurice cIEF and Flex cartridges are shown in Figure 1. Equipment and reagents used in the study are listed in Table 1.

### Meet MauriceFlex™



Figure 1. MauriceFlex, cIEF, and cIEF fractionation cartridges are shown.

Material	Vendor	Catalog #
Mosunetuzumab-axgb	Genentech	50242-0159-01
Innovator (Lunsumio)		
Mosunetuzumab biosimilar	Ichorbio	ICH5026
MauriceFlex		090-158
Maurice cIEF Cartridge		PS-MC02-C
Maurice cIEF Method Development Kit	ProteinSimple	PS-MDK01-C
MauriceFlex Cartridge		
MauriceFlex cIEF Fractionation Method Development Kit		046-432
FabRICATOR	Genovis	AD-FR1-020
BioAccord LC-MS System		176004402W
BioResolve RP mAb Polyphenyl Column	Waters	186008944
Acetonitrile with 0.1% Formic Acid	Fisher	LS1201
Water with 0.1% Formic Acid	Fisher	LS1181

Table 1. Reagents required for a MauriceFlex run.

### MauriceFlex Fractionation Workflow

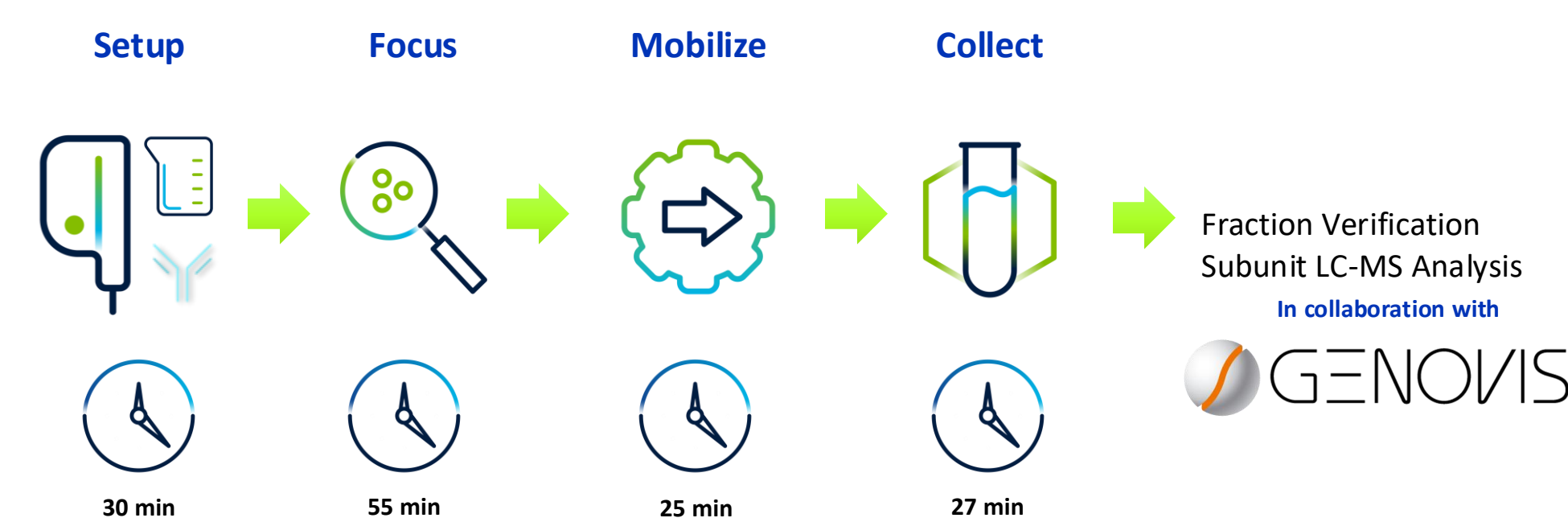


Figure 2. MauriceFlex workflow for Mosunetuzumab fractionation. After preparing sample and instrument (30min) the MauriceFlex will focus the BsAb in the capillary according to their pI (55min). Next, the sample is mobilized (25min) for collection of fractionated capsids (27min). Collected fractions were verified using the analytical cIEF cartridge of Maurice prior to LC-MS analysis by Genovis.

## Methods

### 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 cartridge and focused for 10 min at 250 V, 10 min at 500V, 10 min at 1000V, 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 LC-MS Analysis

Each pooled fraction (80%) collected from the MauriceFlex system was concentrated to 10 µL by SpeedVac. To each fraction, 5 units of FabRICATOR 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 of the subunit peaks (scFc1, scFc2, and Fab'2) were generated and deconvolution was performed using MaxEnt.

### Imaged cIEF of BsAbs used in the Study

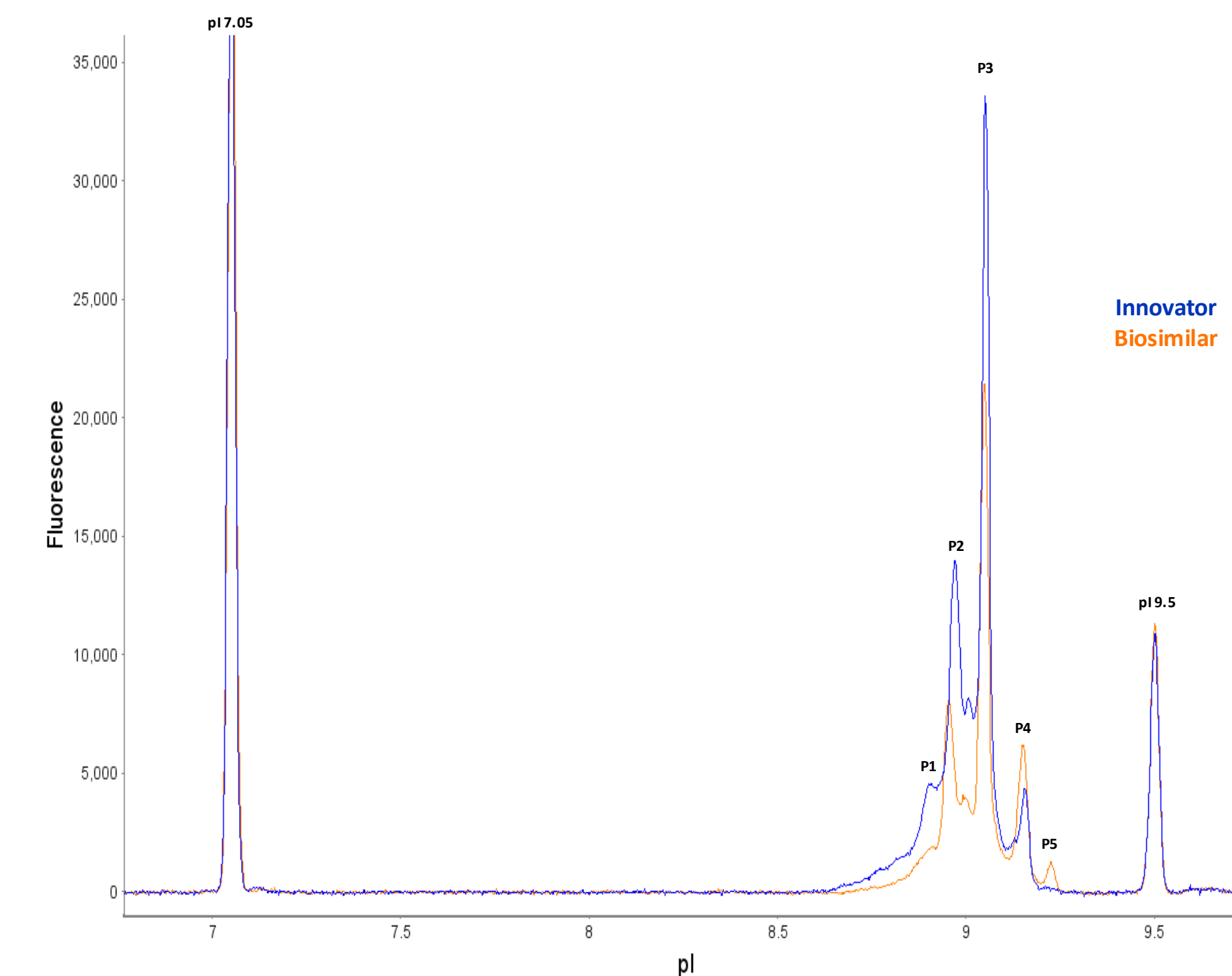
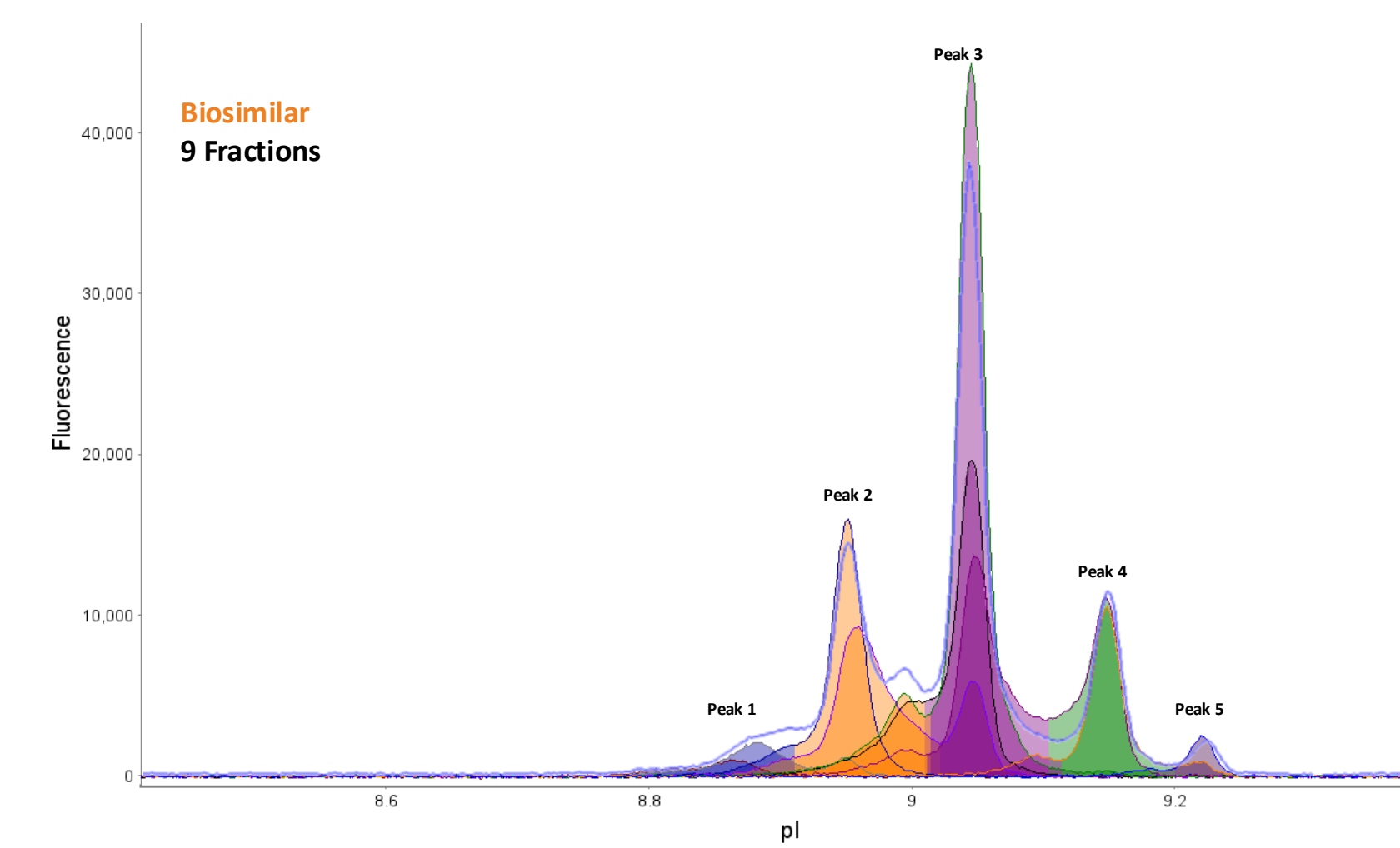
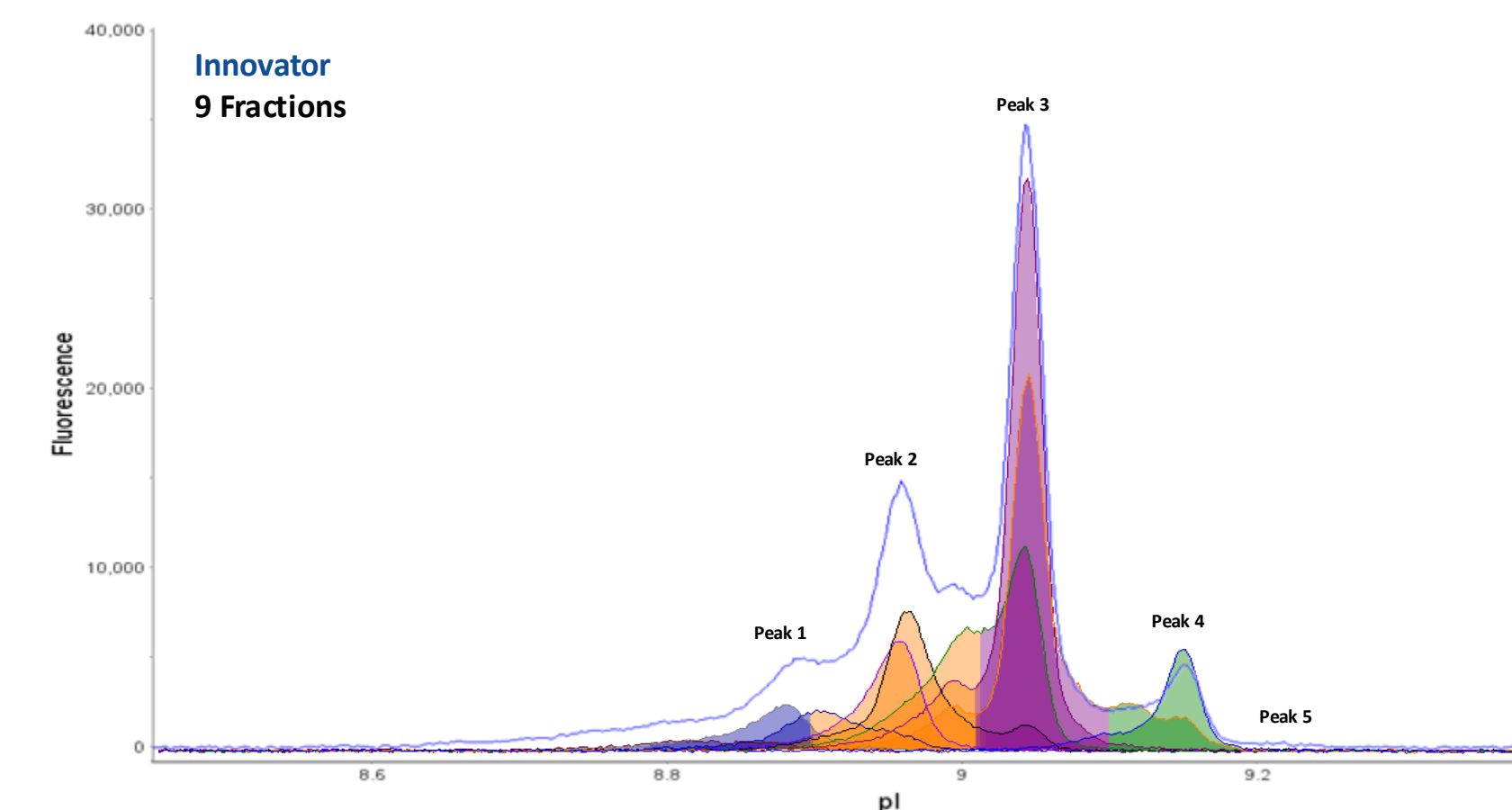


Figure 3. Imaged cIEF analysis of Mosunetuzumab samples used in this study. Using the method described above, the innovator (blue) and biosimilar (orange) samples were analyzed. Five unique peaks were identified, P1 (Acidic 2), P2 (Acidic 1), P3 (Main), P4 (Basic 1), and P5 (Basic 2).

## Results

### Bispecific Ab Fractionation with MauriceFlex



Innovator						Biosimilar					
Sample	Peak1	Peak2	Peak3	Peak4	Peak5	Sample	Peak1	Peak2	Peak3	Peak4	Peak5
Ref	14.3	35.3	42.5	7.5	0.3	Ref	7.6	28.8	46.4	15	2.3
F1			12.8	<b>87.1</b>	0.1	F1			15.8	<b>84.2</b>	
F2		11.6	73.7	14.7		F2		7	14.3	<b>79.3</b>	6.4
F3		16.1	<b>83.9</b>			F3		7	54.5	38.5	
F4		44.7	55.3			F4		16.6	<b>82.2</b>		
F5		<b>88</b>	12			F5		16.4	<b>83.3</b>		
F6	7.3	<b>92.7</b>				F6		<b>75.1</b>	24.4		
F7	36.7	63.3				F7	11.5	<b>88.5</b>			
F8	<b>89</b>	11				F8	<b>80.2</b>	19.8			
F9	<b>99.7</b>	0.3				F9	<b>99</b>				

Figure 4. MauriceFlex fractionation of BsAbs. Each BsAb was fractionated using the same method, producing 9 fractions for both the innovator (top) and biosimilar (bottom) respectively. Unfractionated molecule (overlaid blue line) is shown to illustrate coverage obtained with fractionation. Assessment of fraction purity is summarized in tables below where purity levels >75% are shown in bold.

### Subunit Analysis Workflow using FabRICATOR®

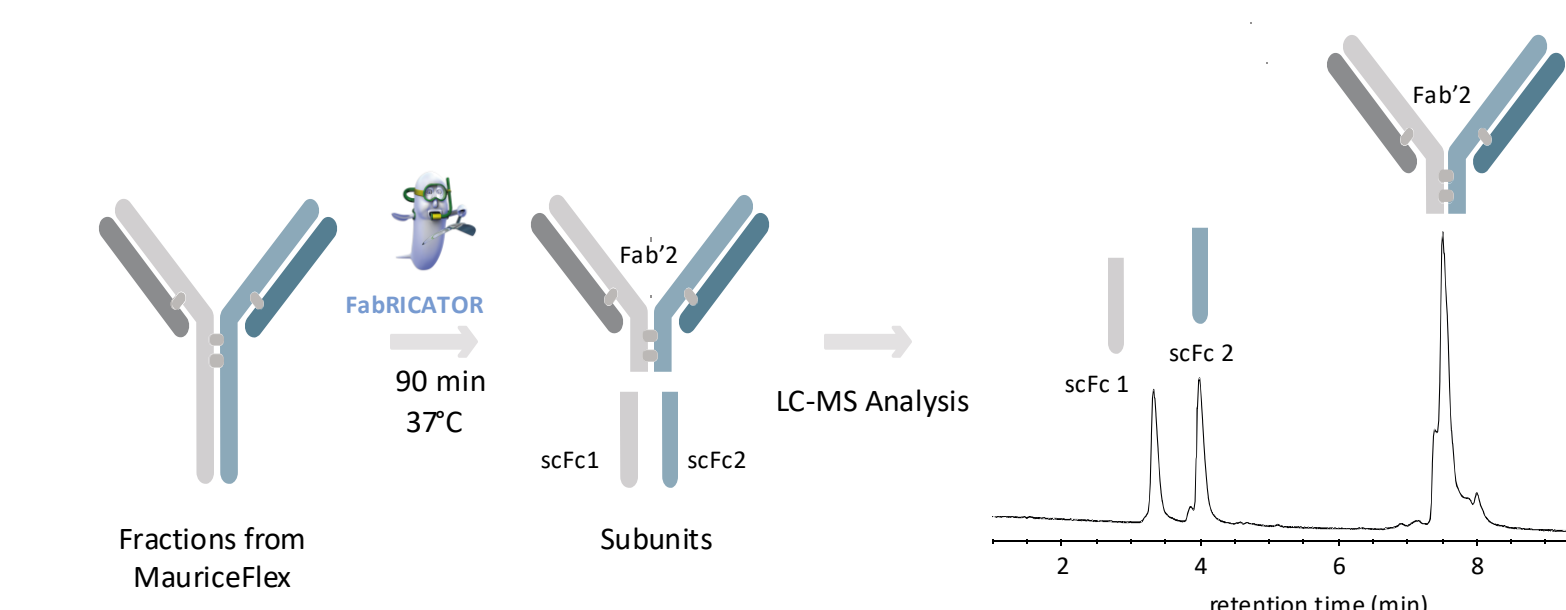


Figure 5. MauriceFlex fractions are digested with FabRICATOR under non-reducing conditions and analyzed by LC-MS.

### LC-MS Analysis of BsAb Fractions Uncover a Mismatch

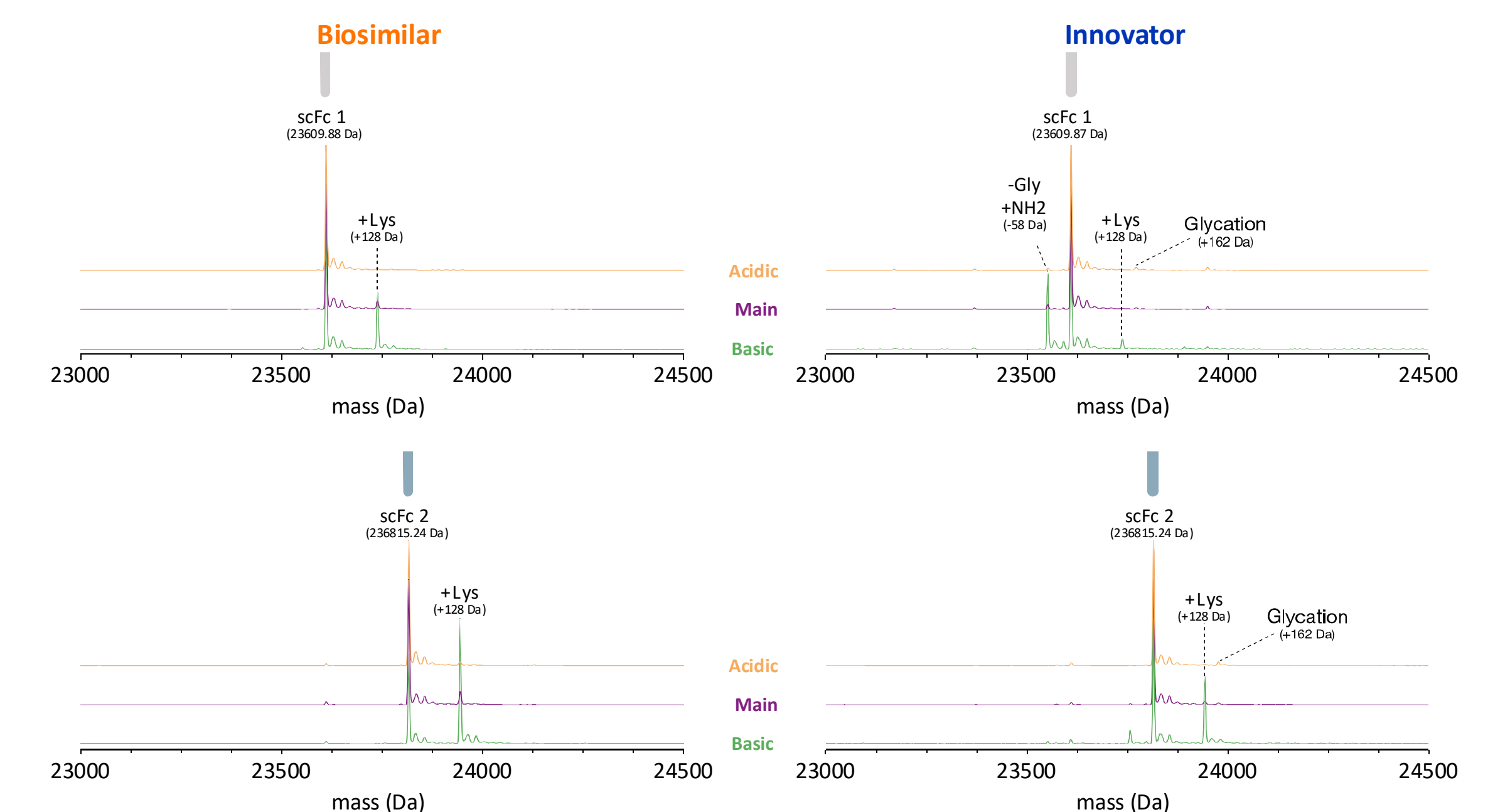


Figure 6. Analysis of BsAb scFc1 and scFc2 samples from MauriceFlex after digestion with FabRICATOR. Deconvoluted MS spectra of peak fractions P2-P4 from Innovator and Biosimilar are shown. Sample prep details are given in methods section. C-terminal lysine variants are observed in the basic fraction.

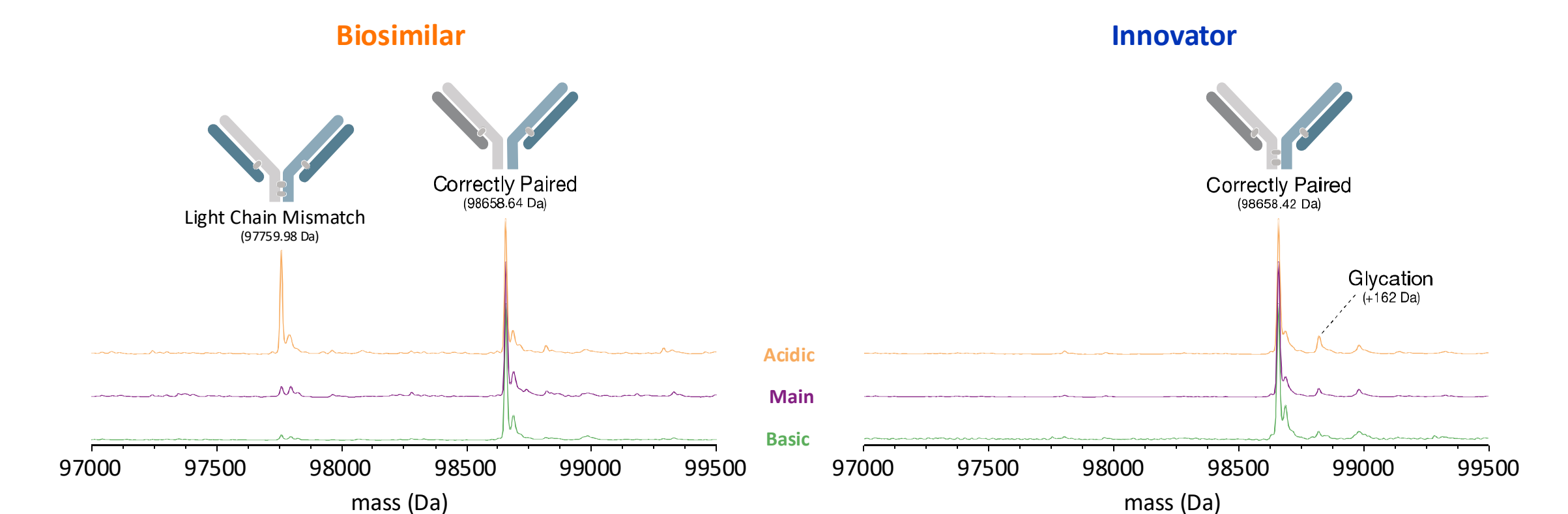


Figure 7. Analysis of BsAb Fab'2 samples from MauriceFlex after digestion with FabRICATOR. Deconvoluted MS spectra of peak fractions P2-P4 from Innovator and Biosimilar are shown. Sample prep details are given in methods section.

## Conclusions

- Biological products, like therapeutic mAbs and BsAbs, are highly complex drugs that require both careful manufacturing and testing to ensure product quality and consistency.
- Imaged cIEF is the gold standard tool for charge heterogeneity analysis, and with MauriceFlex™ you can now characterize your charge variants.
- FabRICATOR® digests IgG at one specific site below the hinge. With minimal sample prep and high specificity, this generates more manageable subunits that are well suited for analysis by LC-MS.
- A workflow using MauriceFlex™ to fractionate charge variants with LC-MS characterization is significantly enhanced by subunit analysis, fueled by the FabRICATOR® enzyme from Genovis.
  - Mismatched LC chain was detected in Mosunetuzumab biosimilar, but not in the innovator
  - Mosunetuzumab innovator contains glycation and amidation

To learn more about MauriceFlex, open the camera on your phone and scan the QR code!

