

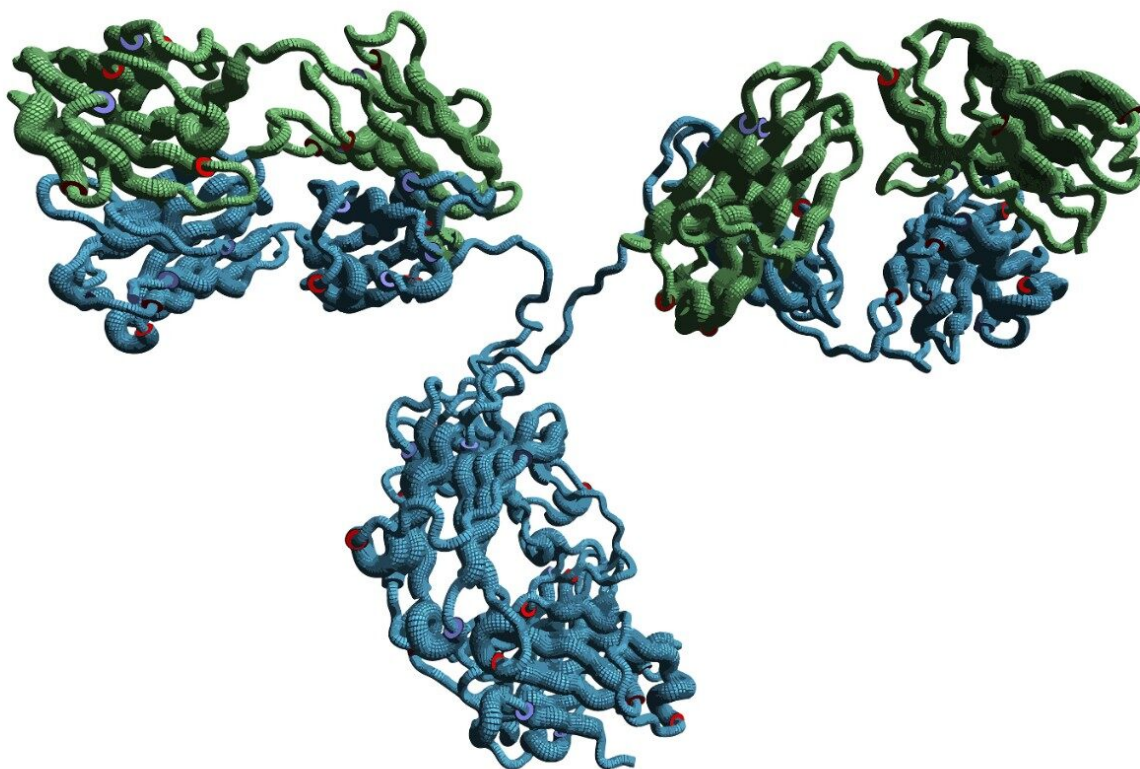
Application Note

## Enabling Routine and Reproducible Intact Mass Analysis When Data Integrity Matters

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Abstract

This application note demonstrates the performance of an integrated intact mass analysis workflow using the BioAccord System for automated data acquisition, processing and reporting, capable of deployment in a regulated lab environment.

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## Introduction

Mass spectrometric (MS) analysis of biotherapeutic proteins at the intact and subunit level is conducted throughout the product development life cycle. These analyses provide information to confirm sequence integrity and product variation. The increased utility and accessibility of this technology for intact mass analysis has led to its widespread use in support of regulatory filings for innovator and biosimilar molecules.<sup>1</sup>

Despite of the wide practice of intact protein mass analysis, experienced MS users are typically still required to manage the instruments operation, data processing, and interpretation. The desire to expand intact mass capability later into development and ultimately into a QC role requires even greater accessibility to non-expert MS users to generate the high quality results generated routinely by experienced analysts.

The benefits of powerful MS capabilities in regulated labs have been realized by a select group of companies.<sup>2-3</sup> Challenges (perceived and real) that have slowed adoption of MS by analysts more familiar with optical detection methods are attributable to the inherent complexities of MS technology and relatively recent availability of compliant-ready informatics platforms capable of converting raw mass spectrometric data into product quality attribute results.

Driven by increasing industry demand for a robust system for biotherapeutic late development and quality organizations, the BioAccord System (Figure 1) was purposefully designed to offer operational modes that have been simplified and optimized to deliver automated, accurate, and reproducible mass measurements for proteins, peptides, and glycans. The BioAccord System is a compact high performance LC-MS platform integrating ACQUITY RDa Mass Detector with the ACQUITY UPLC I-Class PLUS System under control by the UNIFI Scientific Informatics System. The integrated nature of this UNIFI-based system streamlines data acquisition, processing, and reporting using method-based, workflow-driven processes. A built-in calibration reference standard for automated instrument calibration and guided instrument and method setup combines to achieve the goals of workflow automation, increased user accessibility, and performance standardization.

# BioAccord System



*Figure 1. The BioAccord System comprised of an ACQUITY UPLC I-Class PLUS configured with an optical detector (TUV/FLR) coupled in-line to the ACQUITY RDa Mass Detector.*

This application note describes the performance characteristics of the BioAccord System for intact mass analysis of an intact monoclonal antibody and IdeS generated subunits. The data shows the high quality, reproducible intact mass data generated by the BioAccord System, with a reduced burden of data processing and management for the user, enabling more labs to generate information-rich results to make better analytical decisions more efficiently.

## Experimental

### Sample Preparation

Humanized mAb Mass Check Standard (Waters P/N=[186009125](https://www.waters.com/nextgen/us/en/shop/standards--reagents/186009125-humanized-mab-mass-check-standard.html) <  
<https://www.waters.com/nextgen/us/en/shop/standards--reagents/186009125-humanized-mab-mass-check-standard.html>> ) and mAb Subunit Standard (Waters P/N=[186008927](https://www.waters.com/nextgen/us/en/shop/standards--reagents/186008927-mab-subunit-standard.html) <  
<https://www.waters.com/nextgen/us/en/shop/standards--reagents/186008927-mab-subunit-standard.html>> ) were used for this study. For intact mass analysis, 400  $\mu\text{L}$  of water was added to the sample vial (contains 80  $\mu\text{g}$  of intact mAb material) to produce a solution of 0.2  $\mu\text{g}/\mu\text{L}$  before injection (2  $\mu\text{L}$ ). For subunit analysis, 250  $\mu\text{L}$  of water was added to the sample vial (contains 25  $\mu\text{g}$  of subunit mAb material) to produce a solution of 0.1  $\mu\text{g}/\mu\text{L}$  before injection (2  $\mu\text{L}$ ).

### BioAccord System:

ACQUITY UPLC I-Class PLUS

ACQUITY RDa Mass Detector

TUV Optical Detector

UNIFI Scientific Information System v1.9.4

### Intact Mass Analysis–LC-MS Method Setup

Column:	ACQUITY UPLC-BEH300 C4, 2.1 x 50 mm (Waters P/N=186004495)
Column temperature:	80 °C
Mobile phase A:	Water with 0.1% formic acid (or 0.1% TFA, data shown in Figure 6)
Mobile phase B:	Acetonitrile with 0.1% formic acid (or 0.1% TFA, data shown in Figure 6)
Optical detection:	UV 280 nm

### LC Gradient Table for Intact mAb Analysis

Time (min)	Flow rate (mL/min)	Composition A (%)	Composition B (%)	Curve
0.00	0.400	95.0	5.0	Initial
1.00	0.400	95.0	5.0	6
3.50	0.400	15.0	85.0	6
3.70	0.400	15.0	85.0	6
4.00	0.400	5.0	95.0	6
4.50	0.400	15.0	85.0	6
5.00	0.400	95.0	5.0	6
5.50	0.400	95.0	5.0	6
7.00	0.400	95.0	5.0	6

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*Total run time: 7.0 min.*

## MS Conditions for Intact Mass Analysis

### Acquisition Settings

Mode: Full scan

Mass range: High (400–7000  $m/z$ )

Polarity: Positive

Scan rate: 2 Hz

Cone voltage:	Custom (70V) (or 150V with 0.1% TFA in the mobile phase)
Capillary voltage:	Custom (1.50 V)
Desolvation temperature:	Custom (550 °C)
Intelligent data capture (IDC):	Off

### Subunit Mass Analysis LC-MS Method Setup

Column:	BioResolve RP mAb Polyphenyl 450, 2.7 $\mu$ m, 2.1 mm x 50 mm (Waters p/n=186008944)
Column temp.:	80 °C
Mobile phase A:	Water with 0.1% formic acid
Mobile phase B:	Acetonitrile with 0.1% formic acid
Optical detection:	UV 280 nm

### LC Gradient Table for Intact mAb Analysis

Time (min)	Flow rate (mL/min)	Composition A (%)	Composition B (%)	Curve
0.00	0.300	80.0	20.0	6
10.00	0.300	60.0	40.0	6
10.30	0.300	20.0	80.0	6

Time (min)	Flow rate (mL/min)	Composition A (%)	Composition B (%)	Curve
11.30	0.300	20.0	80.0	6
11.60	0.300	80.0	20.0	6
15.00	0.300	80.0	20.0	6

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*Total run time: 15.0 min.*

## MS Conditions for Subunit Analysis

### Acquisition Settings

Mode:	Full scan
Mass range:	High (400–7000 $m/z$ )
Polarity:	Positive
Scan rate:	2 Hz
Cone voltage:	Custom (30 V)
Capillary voltage:	Custom (1.00 V)
Desolvation temp.:	Custom (450 °C)
Intelligent data capture (IDC):	Off

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## Results and Discussion

## Intact mass analysis workflow Setup within BioAccord System

Under the control of the UNIFI Software platform, the BioAccord System streamlines the complete intact protein mass analysis process, from acquisition method creation, data processing to final reporting, by automating and standardizing a common analytical workflow (Figure 2). Special attention has been paid to reduce the complexity of ACQUITY RDa instrument operation into four standardized modes.

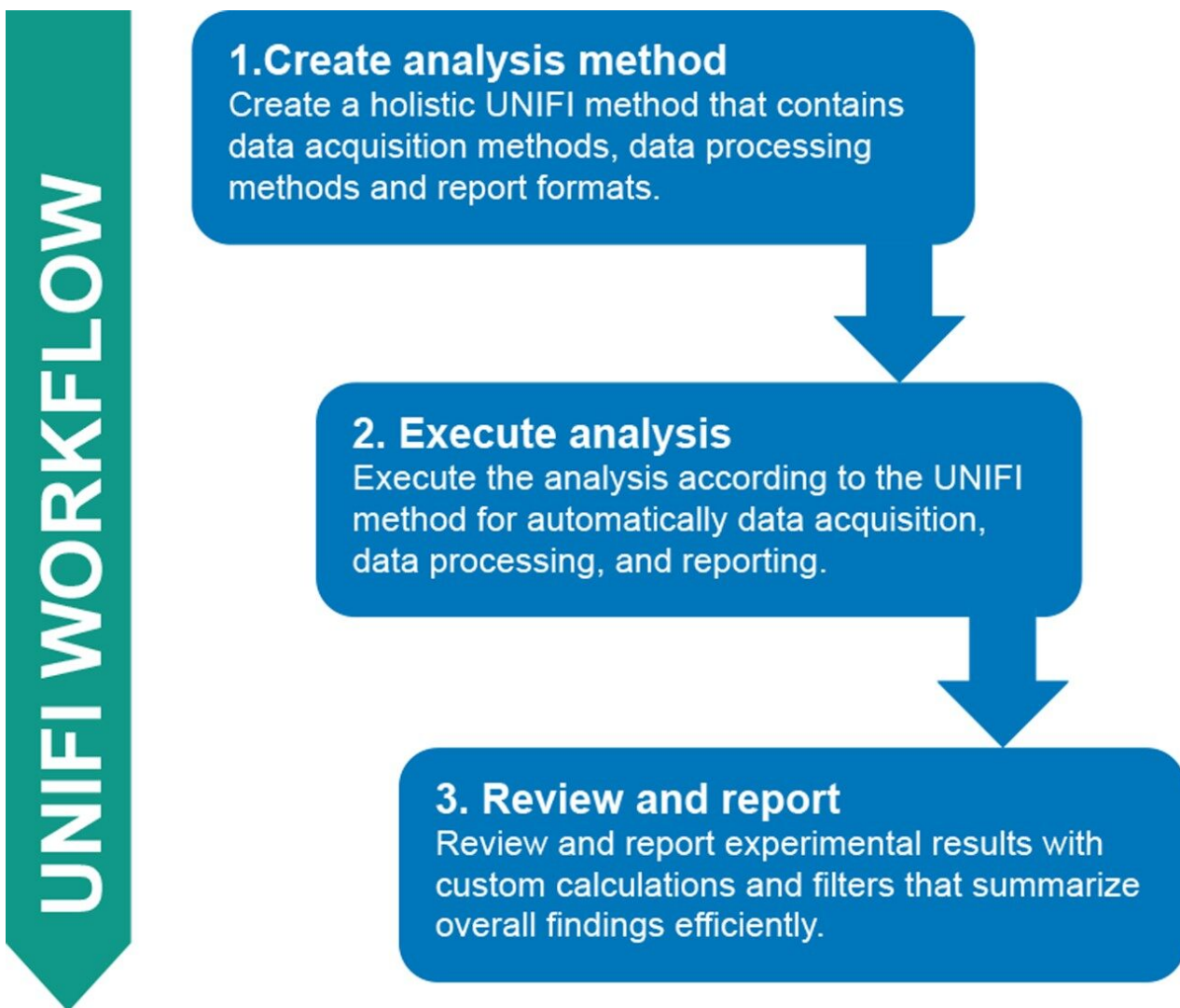


Figure 2. Automated and standardized intact mass workflow.

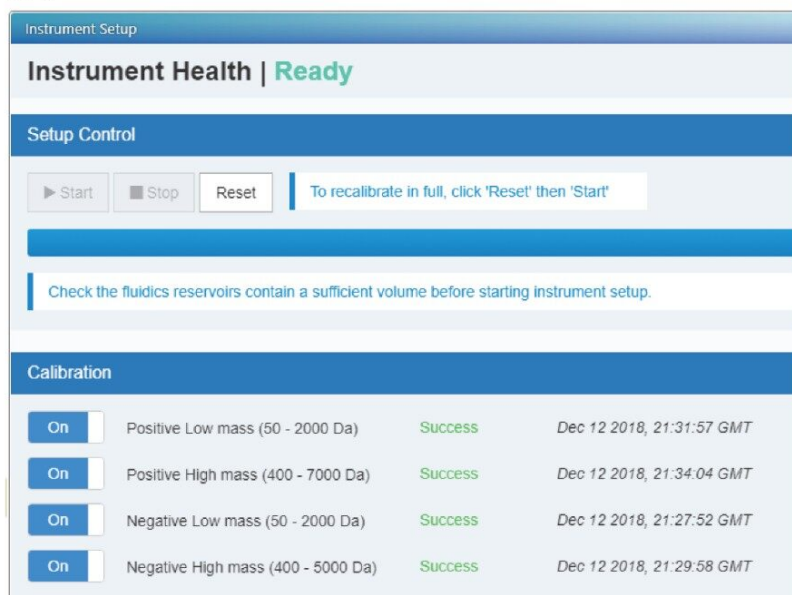
- (1). Positive lower mass analysis (up to  $m/z = 2000$  data acquisition, suitable for peptide mapping);
- (2). Positive high mass analysis (up to  $m/z = 7000$ , suitable for intact mass and subunit analysis);
- (3). negative lower mass analysis (up to  $m/z = 2000$ ); and



(4). negative higher mass analysis (up to  $m/z = 5000$ ).

This standardization of operation modes together with simple and intuitive user interface, automated optimization of advanced tuning parameters, and the pre-programmed calibration checks facilitate the integration of the system into standard operating procedures and ensure consistency between different operators and different laboratories. Figure 3 shows the screen capture of the ACQUITY RDa instrument control window, where the typical settings for the tuning parameters are displayed.

3A.



3B.

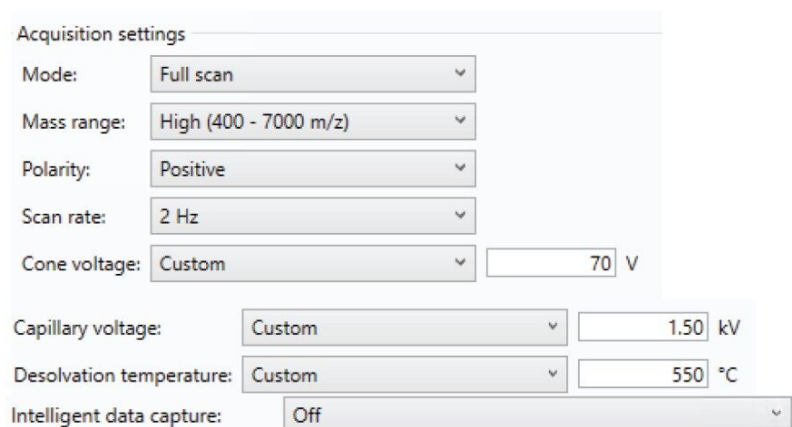


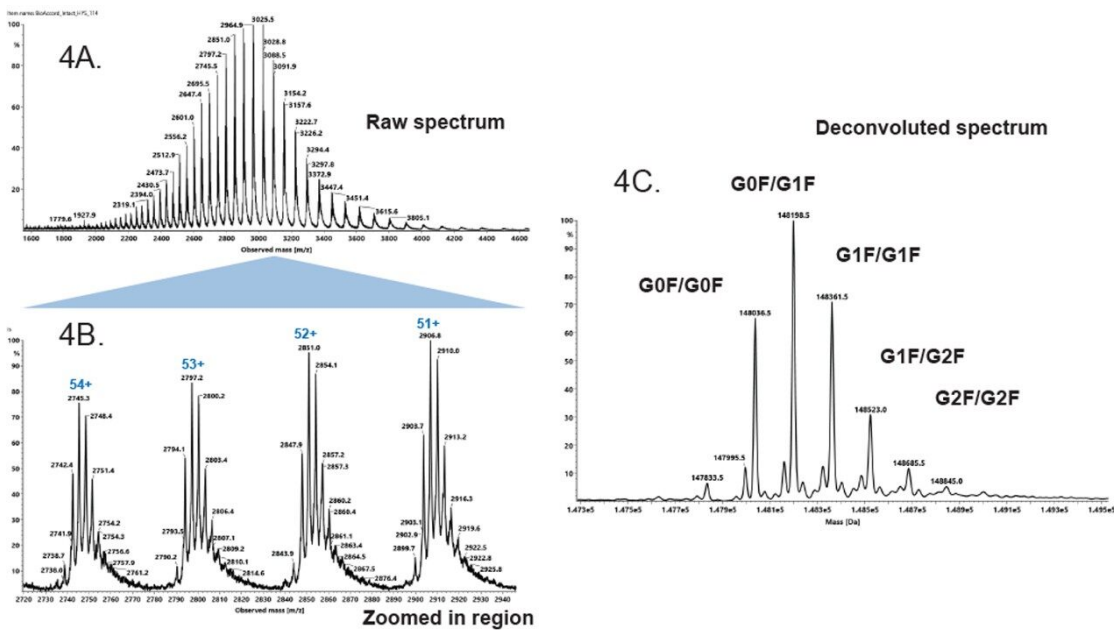
Figure 3. (A) ACQUITY RDa MS source controlling parameters and method setup for intact mass analysis; (B). ACQUITY RDa MS data acquisition parameters for intact mass analysis with mobile phases containing 0.1% formic acid.

### Intact mAb Analysis by BioAccord System

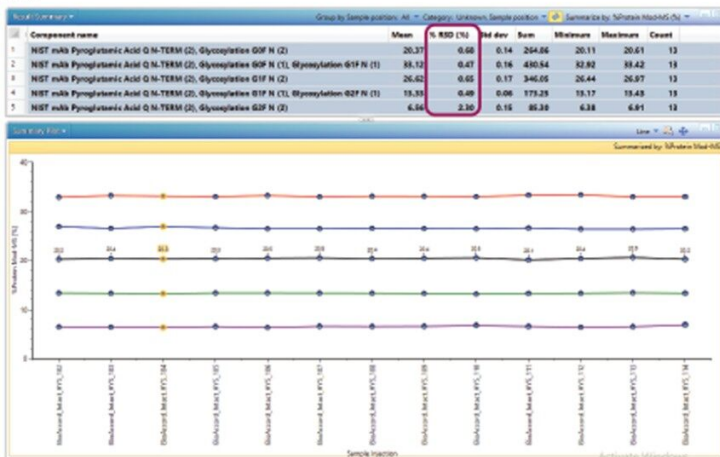
The full MS spectrum obtained from 400 ng Waters Humanized mAb Mass Check Standard (P/N=186009125 <<https://www.waters.com/nextgen/us/en/shop/standards--reagents/186009125-humanized-mab-mass-check-standard.html>> ) applied to a 2.1 x 50 mm ACQUITY BEH Column is displayed in Figure 3. The mass spectrum, acquired over  $m/z$  400–7000 shows the typical charge distribution observed for a large protein. The

spectrum from four high abundant charge states (from 51<sup>+</sup> to 54<sup>+</sup>) spanning *m/z* range from 2730 to 2950, represented in the zoomed figure (Fig 4B), nicely shows the five most abundant glycoforms of the intact antibody and the consistency in the relative abundance of glycoforms across the charge states.

The intact masses of these five most abundant glycoforms and a series of less abundant glycoforms were obtained after the deconvolution of the full MS spectrum (Fig 4C). The assignment of the peaks was based on the *in-silico* calculation from protein sequence, including bi-antennary glycan structures common to a murine mAb. The deconvoluted spectrum (Fig 4C) shows a very similar pattern for the relative abundance of the major glycoforms compared to the raw spectrum.



5A.



G0F/G1F  
G1F/G1F  
G0F/G0F  
G1F/G2F  
G2F/G2F

5B.

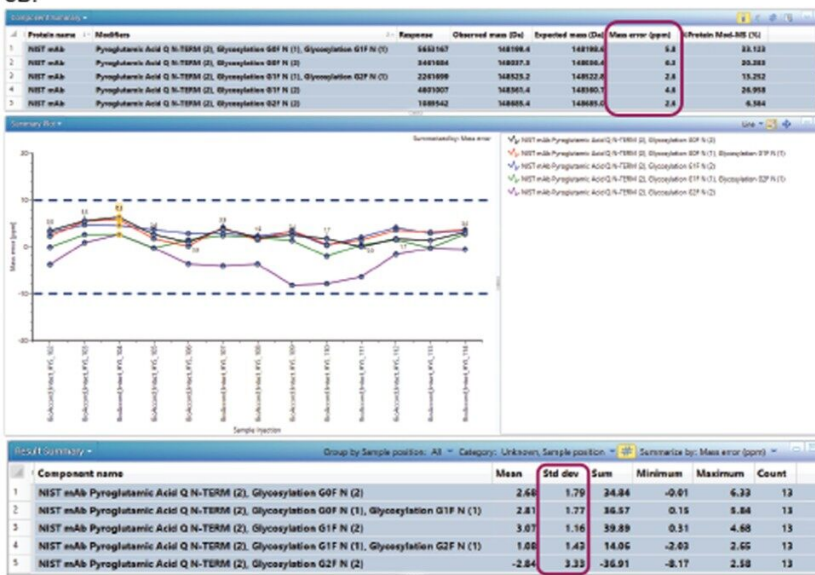


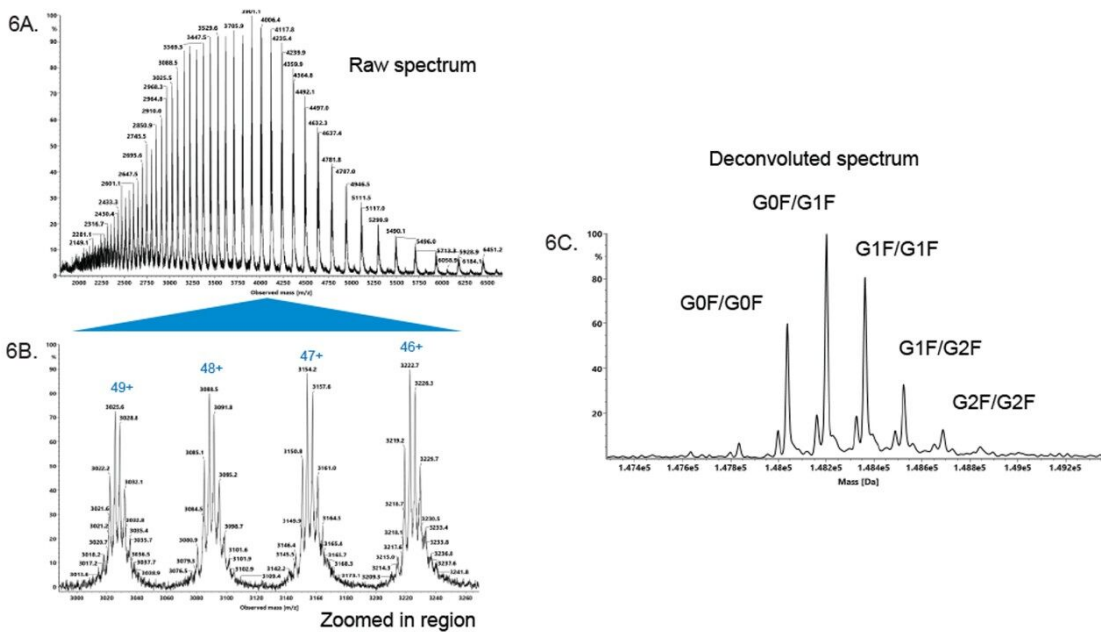
Figure 5A. Spectrum quality and data consistency are the key factors of successful intact mass analysis. Relative quantitation of major glycoforms of the NIST mAb reference standard is displayed here. Across 13 injections, we were able to obtain consistent relative percentage of glycoforms. The RSD% is less than 2.5% as highlighted in red. 5B Automatically calculated NIST mAb intact mass analysis experiment results displayed in the UNIFI review panel. Good mass accuracy is achieved for individual injection and as well as the whole dataset.

Intact mass analysis of the Waters Humanized mAb Mass Check Standard (Fig 5B) includes a component table for the top 5 major glycoforms by MS response, the observed and expected (theoretical) masses, the

calculated ppm mass error, and relative abundance of the respective glycoforms in each injection. The summary plot below shows the mass errors of the 5 major glycoforms identified in 13 injections in one simple display.

Trifluoroacetic acid (TFA) is commonly used as a mobile phase additive for reversed-phase LC (RP-LC) separations of proteins and peptides. TFA works as an ion-pairing agent to improve the chromatographic peak shape of proteins by minimizing the interaction between the protein functional groups of proteins with residual silanol groups on silica LC particles. While TFA can interfere, reduce the MS signal, lowering absolute MS sensitivity of the analysis, TFA has positive effects on quantification by UV detection. As such, TFA has been widely used as an additive in the laboratories traditionally associated with optical-based assays that would likely be the basis for future LC-MS methods.

The LC-MS spectrum of intact Waters Humanized mAb Mass Check Standard using mobile phases with 0.1% TFA shows greater spread of the  $m/z$  charge envelope from  $m/z$  of 2000 to 6500, in comparison with mobile phases containing 0.1% formic acid. Higher sample consumption (~5x) overcomes the TFA ion-suppression effects commonly observed during electrospray process to generate data equivalent to a formic acid analysis. Higher cone voltages (150 volts vs. 60 volts) prove more effective for declustering any TFA adducts, producing high quality spectral data (Fig 6B) from the BioAccord System, comparable to the 0.1% FA data (Fig 5B).



## mAb Subunit Standard (scFc, LC, and Fd) Analysis by BioAccord System

Subunit analysis (Fig 7A-B) was performed using mAb Subunit Standard (Waters P/N=186008927 < <https://www.waters.com/nextgen/us/en/shop/standards--reagents/186008927-mab-subunit-standard.html>> ). The TIC chromatogram (Fig 7A) contained three resolved chromatographic peaks (scFc, LC and Fd) with reported retention times of 4.06, 5.39, and 7.93 min respectively. Combined raw spectra (Fig 7B) corresponding to each peak of Waters Humanized mAb Mass Check Standard (200 ng on column) subunits: scFc, LC, and Fd exhibit high s/n, multiple charged spectral envelopes for the subunits that were automatically processed into deconvoluted results (Fig 7C). The component summary table shows the identified LC, scFc, and Fd subunits with their respective MS response, the mass after deconvolution, the calculated mass error, the retention time and the percentage of modification on the subunit. The review panel also shows the automatically identified and labeled major glycoforms from the scFc after deconvolution in the component plot.

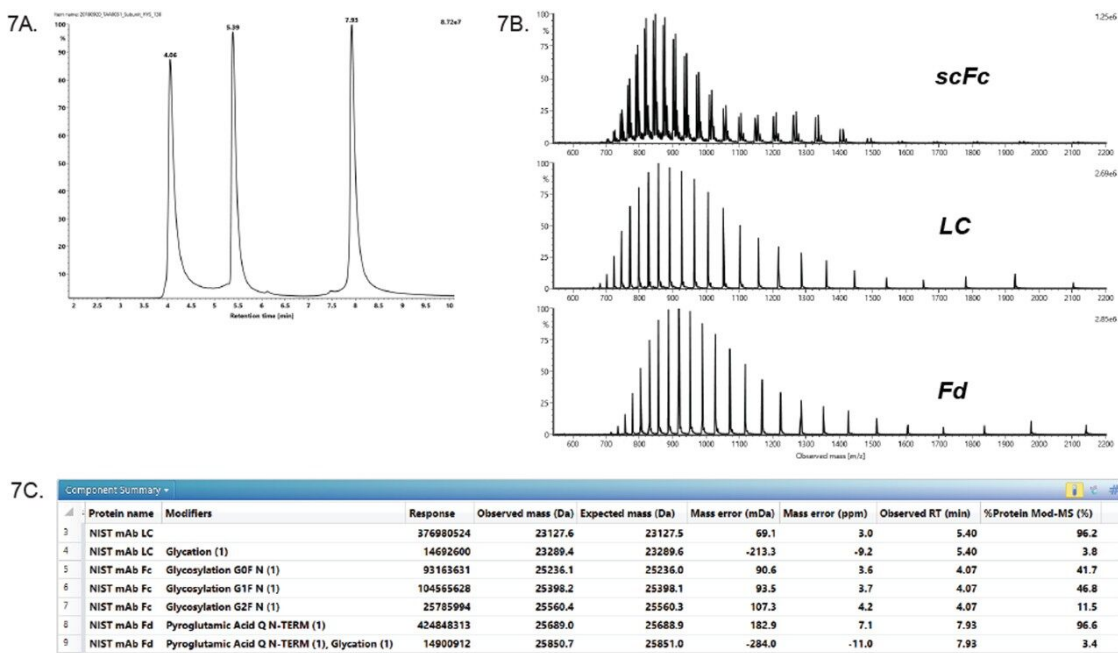


Figure 7. TIC, combined raw spectra and major peaks for the NIST mAb (100 ng on column) IdeS digested subunits scFc, LC, and Fd.

## Conclusion

In this study, the BioAccord System demonstrated highly reproducible chromatography, exceptional performance from a new compact design mass detector, workflow automation and simplified system interaction capability. The UNIFI informatics platform mAb and mAb subunit mass data generated by this system facilitated automated assignment and relative glycoform abundance determination, typical of method validation or routine operation sample sets. The ability to deploy this system in both regulated and non-regulated environments should facilitate rapid method development and simplified downstream transfer of these methods to late development and quality organizations challenged to make more informed analytical decisions faster.

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## References

1. Rogstad, S., Faustino, A., Ruth, A., Keire, D., Boyne, M., and Park, J. *J Am Soc Mass Spectrom.* 2017, Vol 28(5):786–794. doi: 10.1007/s13361-016-1531-9.
2. Sokolowska, I., Mo J., Dong, J., Lewis, M. J. and Hu, P. *MAbs.* 2017, Vol 9, 498–505.
3. Xu, W., Jimenez, B. R., Mowery, R., Luo, H., Cao, M., Agarwal, N., Ramos, I., Wang, X. and Wang, J. *MAbs.* 2017, 9, 1186–1196.

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