

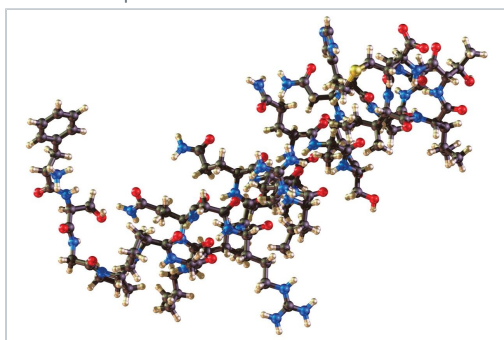
Application Note

## Application of a Mass Confirmation Workflow for Biotherapeutics Screening

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## Abstract

In the study, we report the development of a higher throughput intact mass confirmation workflow using the BioAccord LC-MS System<sup>1-10</sup> operated under the control of waters\_connect informatics. We have implemented a faster gradient LC-MS analysis in combination with an enhanced informatics capability for matching target mass of the proteins. In addition, a color-coded sample plate can be displayed to show the mass confirmation status of each sample using a simple custom calculation. The mass confirmation workflow was applied to reversed phase and native SEC LC-MS intact mass analysis, with a total LC runtime of 2.5 minutes for each analysis. This workflow should improve analytical efficiency of LC-MS intact mass analysis for the clone screening, process development, and other functions where sample throughput is a concern for biotherapeutics analysis.

## Benefits

- Demonstrated application to both reversed phase and native SEC LC-MS intact mass analysis
- A color-coded sample plate for rapid visualization of the mass confirmation status for each sample
- High throughput LC-MS workflow on the compliance ready waters\_connect platform can be deployed across a biopharmaceutical organization
- Targeted Molecular Weight based intact mass confirmation

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## Introduction

Across biopharmaceutical development, analytical support for the characterization of biotherapeutics, such as monoclonal antibodies (mAbs) is essential. Intact mass analysis by mass spectrometry provides important information, such as molecular weight and sample heterogeneity. Because of the large amount of biotherapeutics sample/candidates generated, analytical methods that achieve high-throughput analysis can be used to screen product attributes in early development (e.g. clone screening) as well as support intensive QbD initiatives across process development. In this study, we report the development of a high throughput intact mass confirmation workflow using the BioAccord LC-MS System<sup>1-10</sup> operated under the control of waters\_connect informatics. We have implemented a faster gradient LC-MS analysis method in combination with enhanced informatics capability for matching target mass for high throughput intact mass confirmation analysis. A color-coded sample plate display rapidly presents the mass confirmation status of each sample using a simple custom calculation. The mass confirmation workflow was applied to reversed phase and native SEC LC-MS intact mass analysis, with a total LC runtime of 2.5 minutes for each analysis. Six commercially purchased monoclonal antibodies and a cysteine-conjugated antibody drug conjugate sample were examined in this study. The developed workflow should have a positive impact in improving analytical efficiency of LC-MS intact mass analysis areas of biotherapeutics development where sample throughput is a critical consideration.



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*Figure 1. Waters BioAccord LC-MS System and the waters\_connect informatics platform.*

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## Experimental

Both reversed-phase LC-MS and native SEC-MS methods were developed for higher throughput intact mass confirmation screening. Six monoclonal antibodies and one cysteine-conjugated ADC samples were examined in this study to demonstrate the mass confirmation workflow. Replicate samples were placed across a 48-vial sample plate for the analysis.

### Sample Description

NIST Reference mAb (National institute of Standards and Technology), Trastuzumab (Genentech, South San Francisco, CA), Infliximab (Janssen), Retuximab (Biogen-Idec), Bevacizumab (Genentech), and Adalimumab (Abbvie) were all obtained commercially in the original formulations. The proprietary ADC sample was obtained through a research collaboration. Samples were diluted to 0.2 mg/mL in 50 mM ammonium acetate (Sigma 431311, pH) before reversed-phase LC-MS analysis and to 2.5 mg/mL in 50 mM ammonium acetate (pH ~7.0) for native SEC LC-MS analysis. LC-MS grade water and acetonitrile (Fisher Scientific Optima) were used to make the mobile phases and system solutions. Formic acid (p/n= 85178) was purchased from Fisher Scientific.

## Method Conditions

LC Conditions (reversed-phase LC-MS analysis)

LC system:	ACQUITY UPLC I-Class PLUS
Detection:	ACQUITY RDa Mass Detector
Vials:	Waters total recovery vials (p/n= 186000385C)
Column(s):	BioResolve RP mAb Polyphenyl Column, 450 Å, 2.7 µm, 2.1 mm x 50 mm (p/n= 186008944)
Column temp.:	80 °C
Sample temp.:	6 °C
Injection volume:	4 µL
Flow rate:	0.4 mL/min
Mobile phase A:	0.1 % Formic acid
Mobile phase B:	0.1% Formic acid in acetonitrile

## Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
0.00	0.40	95.0	5.0	6
1.00	0.40	15.0	85.0	6
1.20	0.40	5.0	95.0	6
1.50	0.40	95.0	5.0	6
2.50	0.40	95.0	5.0	6

## MS Conditions

MS system: ACQUITY RDa Mass  
Detector, BioAccord  
System

Ionization mode: Positive mode

Acquisition range:  $m/z = 400$  to  $7000$

Scan rate: 2 Hz

Capillary voltage: 1.5 KV

Desolvation temp.: 550 °C

Cone voltage: 70 V

Intelligent data capture: Off

## LC Conditions (native SEC LC-MS analysis)

LC system:	ACQUITY UPLC I-Class PLUS
Detection:	ACQUITY Rda Mass Detector
Vials:	Waters Total Recovery (p/n= 186000385C)
Column(s):	ACQUITY UPLC Protein BEH SEC Column, 200 Å, 1.7 µm, 2.1 mm x 150 mm (p/n=186008471)
Column temp.:	30 °C
Sample temp.:	6 °C
Injection volume:	10 µL
Flow rate:	0.2 mL/min
Mobile phase A:	50 mM Ammonium acetate

## Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
0.00	0.20	100.0	0.0	6
2.50	0.20	100.0	0.0	6

## Data Management

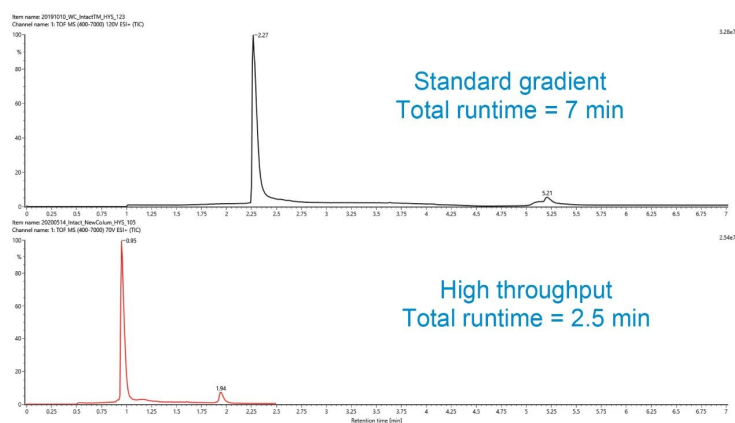
Informatics: waters\_connect

System control and data  
processing: UNIFI (version 1.9.4)



## Results and Discussion

To develop the higher throughput mass confirmation workflow rapid LC gradients were produced to shorten experimental runtime. For the reversed-phase LC-MS analysis, the 2.5 min gradient (5 %B to 85 %B in a standard method – 7 min total runtime – were reduced to a 1.0 min 5 %B to 85 %B gradient in the higher throughput method – 2.5 min total runtime). The flow rate was kept at 0.4 mL/min. The TIC (Total Ion Chromatogram) comparison of the two gradient methods is shown in Figure 2.



*Figure 2. TIC comparison of a standard reversed-phase LC-MS method (top) and the higher throughput reversed-phase LC-MS method (bottom). The total runtime for the higher throughput method is reduced to 2.5 min.*

For the native SEC LC-MS analysis, the flow rate of the LC was increased from 0.075 mL/min to 0.2 mL/min, reducing the total runtime from 7.0 min to 2.5 min for the native SEC LC-MS analysis.

Figure 3 shows the TUV comparison of the standard SEC LC-MS analysis vs the higher throughput method.

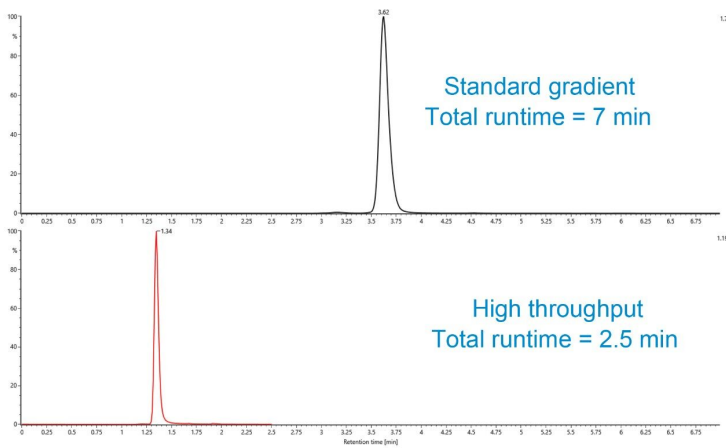


Figure 3. TUV comparison of the standard SEC LC-MS method (top) and the higher throughput SEC LC-MS (bottom). The total runtime for the throughput method is shorted to 2.5 min.

In biotherapeutic discovery through clone screening, very often sequence information is not needed for the question of mass confirmation. This is also commonly observed when samples are outsources to a contract organization for analysis, and companies wish to keep the sequence proprietary. The waters\_connect platform supports this function so that targeted masses can be directly entered into the analysis method for automated mass conformation during data processing. Figure 4 shows the six antibody masses that were entered in the processing method for reversed-phase LC-MS analysis experiment. Post translational modifications (PTMs) existing or added to the software library can be used in combination with the target masses for component identification in the method.

The screenshot shows the 'Manage Components' window in a software application. It features a table with the following data:

Component n...	Expected RT (min)	Time window (min)	Expected mass (Da)	Description
1 mAb1	0.96	0.3	148037.0	mAb 1
2 mAb2	0.96	0.3	148058.0	mAb 2
3 mAb3	0.96	0.3	149199.0	mAb 3
4 mAb4	0.96	0.3	147078.0	mAb 4
5 mAb5	0.96	0.3	148078.0	mAb 5
6 mAb6	0.96	0.3	148512.0	mAb 6

Figure 4. Target masses are entered in the Expect mass (Da) field of the analysis method for automated mass confirmation during data processing.

Two custom fields were generated to display the mass confirmation status for each sample on the sample plate. One custom field (named ID\_comp) is used to communicate the status for the identified (matched) components (masses) and the other (named ID\_count) is used to link the

identification status to the signal response of the identified components (masses). These custom fields are included in the analysis method as shown in Figure 5A. The response and the custom fields of ID\_count are set in the analysis method "Limit Checks" (as shown in Figure 5B) to color code the status of the mass confirmation on the sample plate. A detailed description of the intact mass analysis data processing using custom fields in UNIFI/waters\_connect can be found in this reference.<sup>11</sup>

Include in results	Name	Type	Data sub-type	Description	Formula
<input type="checkbox"/>	ID Comp	Calculated	Component		1 #Identification status: "Identified"; 1.0
<input checked="" type="checkbox"/>	ID_Count	Calculated	Component		11 COUNT(MS Response Identification status: "Identified" & MS Response: "...")

A - 2 custom fields

Node	Field name	Component	Sample type	Level	Error minimum	Warning minimum
1	Component Response	All	All	All	10000	40000
2	Component ID_Count	All	All	All	0.5	

B - Limit check for color coding

Figure 5A. Two custom fields are generated and selected in the analysis method, and in combination with the limit checks (Figure 5B) set up to display and color code the mass confirmation status (Confirmed: green, Questionable: yellow, and Not Confirmed: red) in the analysis method.

With the method established, the streamlined experiment can then be run with integrated and automated data acquisition and processing. Figure 6 displays the example of mass confirmation status for each of the sample replicates on a 48-vial plate before and after the automated acquisition of a reversed-phase LC-MS experiment on the BioAccord LC-MS System.

A - Plate review before and after

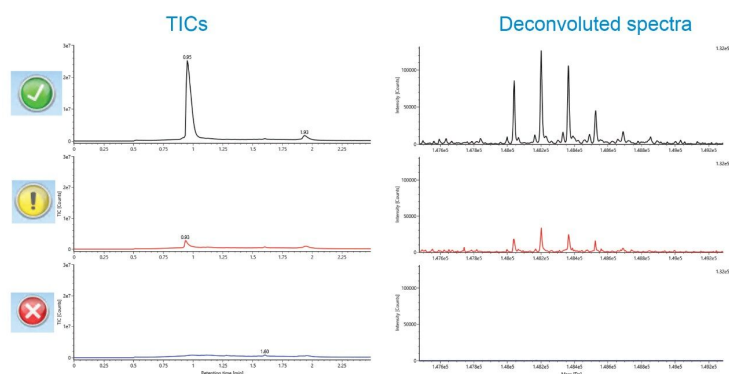


B - 48 (or 96) samples queue

Item name	Sample type	Sample position	Run time (min)	Injection volume (µL)	Replicates
1 WC_20200417HWFS_202	Blank	1.A.1	2.00	10.00	1
2 WC_20200417HWFS_203	Unknown	1.A.2	2.00	4.00	1
3 WC_20200417HWFS_204	Unknown	1.A.3	2.00	4.00	1
4 WC_20200417HWFS_205	Unknown	1.A.4	2.00	4.00	1
5 WC_20200417HWFS_206	Unknown	1.A.5	2.00	4.00	1
6 WC_20200417HWFS_207	Unknown	1.A.6	2.00	4.00	1
7 WC_20200417HWFS_208	Unknown	1.A.7	2.00	4.00	1
8 WC_20200417HWFS_209	Reference	1.A.8	2.00	4.00	1
9 WC_20200417HWFS_210	Unknown	1.B.1	2.00	4.00	1
10 WC_20200417HWFS_211	Unknown	1.B.2	2.00	4.00	1
11 WC_20200417HWFS_212	Unknown	1.B.3	2.00	4.00	1
12 WC_20200417HWFS_213	Unknown	1.B.4	2.00	4.00	1
13 WC_20200417HWFS_214	Unknown	1.B.5	2.00	4.00	1
14 WC_20200417HWFS_215	Unknown	1.B.6	2.00	4.00	1
15 WC_20200417HWFS_216	Unknown	1.B.7	2.00	4.00	1
16 WC_20200417HWFS_217	Unknown	1.B.8	2.00	4.00	1
17 WC_20200417HWFS_218	Unknown	1.C.1	2.00	3.00	1
18 WC_20200417HWFS_219	Unknown	1.C.2	2.00	3.00	1
19 WC_20200417HWFS_220	Unknown	1.C.3	2.00	3.00	1
20 WC_20200417HWFS_221	Unknown	1.C.4	2.00	3.00	1
21 WC_20200417HWFS_222	Unknown	1.C.5	2.00	3.00	1
22 WC_20200417HWFS_223	Unknown	1.C.6	2.00	3.00	1
23 WC_20200417HWFS_224	Unknown	1.C.7	2.00	3.00	1
24 WC_20200417HWFS_225	Unknown	1.C.8	2.00	3.00	1
25 WC_20200417HWFS_226	Unknown	1.O.1	2.00	4.00	1
26 WC_20200417HWFS_227	Unknown	1.O.2	2.00	4.00	1

Figure 6. Mass confirmation status for each sample on a 48 vials plate before and after the automated data acquisition and processing in waters\_connect.

Example TICs and deconvoluted mass spectra of several mass confirmation samples are shown in Figure 7. The green color code on the sample plate indicates that the sample position is mass confirmed against one of the targeted masses included in the method. The yellow color code indicates that one of the targeted masses in the method is matched, but with signal intensity that fell between warning minimum but above the failure minimum level in the limit check settings. When this happens, manual review of the data is suggested. A red color code indicates that none of the target masses were matched. In the case of Figure 7, no real signal was obtained for this sample, therefore, there is no potential match.



*Figure 7. Examples of TICs and deconvoluted spectra with different mass confirmation status in the reversed phase LC-MS experiment; (top) Green: mass confirmed, (middle) Yellow: questionable-manual review suggested, and (bottom) Red: Mass not confirmed.*

TICs and the deconvoluted spectra from the reversed-phase LC-MS analysis of the six antibody samples are shown in Figure 8. High quality data are shown mass accuracies for the six antibodies typically in the 5–15 ppm range.

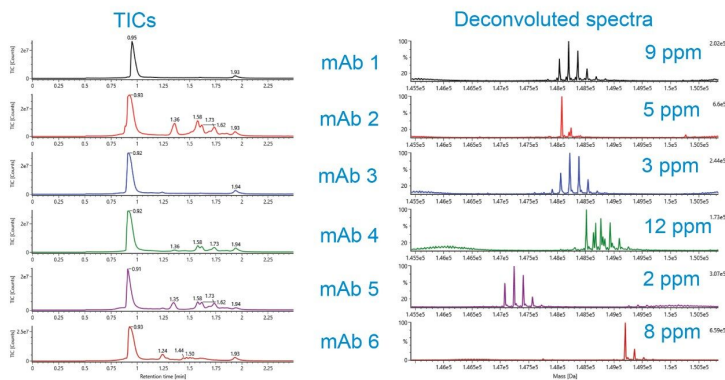


Figure 8. High quality TICs and deconvoluted spectra for the six antibody samples run by the reversed-phase higher throughput LC-MS method.

Processed UV chromatograms and the combined raw spectra from the native SEC LC-MS analysis of the six antibody samples are shown in Figure 9. The higher  $m/z$  envelope for multiply charged antibody ions were observed (compared to the reversed-phase method) with the combined raw spectra. Mass accuracies of less than 15 ppm were obtained for all six antibodies following deconvolution (not shown).

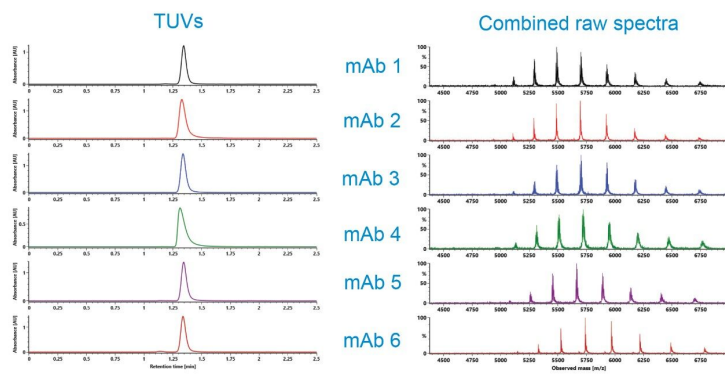


Figure 9. Native SEC LC-MS HTP screening of six mAbs, TUVs and TICs.

Native MS is essential to maintain noncovalent interactions within complex biological samples in order to measure the composite mass of the molecule. In addition to the six antibody samples, we performed a comparison experiment on a cysteine-conjugated ADC sample using both the reversed-phase (data not shown) and the native SEC LC-MS methods (Figure 10). As expected, only the native approach maintained the noncovalent interactions between light chain and the heavy chain, and the two heavy chains that hold the ADC molecule together. This enables us to successfully measure the drug-to-antibody ratio (DAR) at the intact mass level using additional

custom calculations.<sup>6,11</sup>

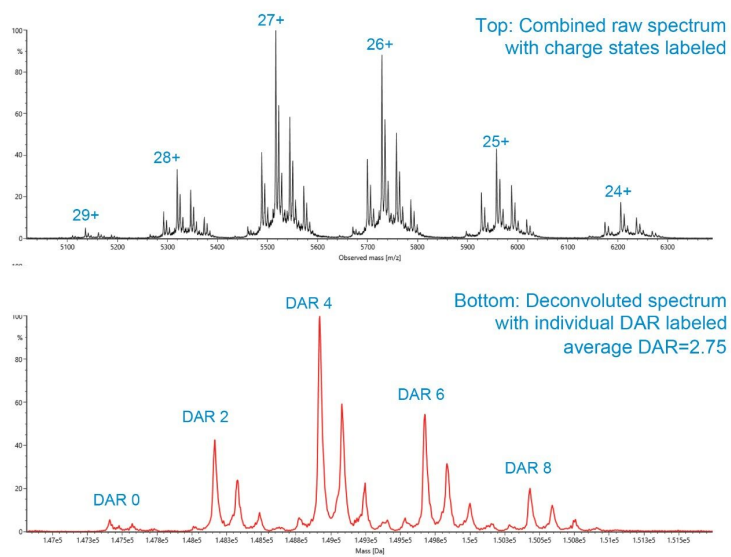


Figure 10. Combined raw spectrum with charge state labeled (top) and MaxEnt1 deconvoluted spectrum (with individual DARs and total average DAR labeled (bottom)). Reversed-phase LC-MS analysis will dissociate the non-covalent bindings between the heavy chains, and the light chain and the heavy chain of the antibody. Only the native approach maintained the noncovalent interactions that hold the ADC molecule together.

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## Conclusion

In the study, we successfully developed a higher throughput intact mass confirmation workflow using the BioAccord LC-MS System operated under the waters\_connect informatics platform. The workflow was demonstrated using reversed phase LC-MS and native SEC LC-MS intact mass confirmation analyses, both with a total LC runtime of only 2.5 minutes per analysis. The color-coded sample well display was shown to provide a rapid visual snapshot of the analysis results. Overall, this faster methodology should improve the laboratory efficiency of analytical organizations tasked with higher throughput intact mass analyses within the biopharmaceutical industry.

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## References

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## Acknowledgements



The authors would like to thank Steve Cubbedge for the creating of the 2 custom fields in this study.

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## Featured Products

[ACQUITY UPLC I-Class PLUS System](#)

[BioAccord LC-MS System for Biopharmaceuticals](#)

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