Waters™

Note d'application

INTACT Mass™ - a Versatile waters_connect™ Application for Rapid Mass Confirmation and Purity Assessment of Biotherapeutics

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Abstract

In recent years, liquid chromatography-mass spectrometry (LC-MS) intact mass analysis has become an indispensable tool for attribute characterization and monitoring of biotherapeutics across all stages of their development and commercialization. Functions such as discovery screening, process development, and formulations/stability can generate a large number of samples, and analytical solutions that can achieve high-throughput intact mass analysis and impurity profiling are advantageous to matching these requirements. Here, we report the development of a new versatile software application to streamline LC-MS mass confirmation and quantitative monitoring of biomolecules, suitable for deployment in a compliance-ready environment.

Benefits

- Automatic and efficient workflow for high throughput mass confirmation and purity analysis
- Purity determination using optical chromatogram, total ion chromatogram (TIC) or intra-mass spectra
- Automatically determination of input and output mass ranges and deconvolution parameters, yielding

monoisotopic or average deconvoluted mass results

- Methods can now be applied as platform methods for wide range of biomolecule classes

Introduction

Intact mass analysis can provide a global profile of biotherapeutics, confirming predicted mass, profiling product heterogeneity, and assigning impurities. In recent years, LC-MS intact mass analysis has become an indispensable tool for attribute characterization and attribute monitoring of biotherapeutics across all stages of their development and commercialization. Functions such as discovery screening, process development, and formulations/stability can generate a large number of samples, and analytical solutions that can achieve high-throughput intact mass analysis and impurity profiling are advantageous to matching these requirements.

Here, we report the development of a waters_connect application that streamlines LC-MS data acquisition, data intact mass processing and reporting of results suitable for a compliance-ready environment. The INTACT Mass Application can be applied for simple mass confirmation, high-throughput intact mass screening, and product impurity profiling. In this application note, the key stages of the workflow for high throughput targeted mass confirmation will be demonstrated. Experimental results obtained from six commercial monoclonal antibodies (mAbs), and NISTmAb IdeS digested subunits will be discussed.

Figure 1. The BioAccord™ LC-MS System with ACQUITY™ Premier, comprised of an ACQUITY Premier binary UPLC™ with detection by Tunable UV detector (TUV) coupled in-line with the ACQUITY RDa™ accurate mass detector, operated under the waters_connect informatics platform.

Experimental

Materials and Sample Preparation

LC-MS grade acetonitrile (ACN) was purchased from Honeywell - Burdick & Jackson. LC-MS grade formic acid was purchased from Thermo Fisher Scientific. Commercially available mAbs, Trastuzumab (Genentech), Infliximab (J&J), Bevacizumab (Abbott), Rituximab (Biogen Idec), and Omalizumab (Genentech) were purchased from Besse Medical (www.besse.com <http://www.besse.com/>). All the mAbs were stored at -80 °C before they were thawed and then diluted in Milli-Q water to 0.5 µg/µL for LC-MS analysis. In addition, Waters™ humanized mAb (NISTmAb) Mass Check Standard (Waters p/n: 186009125 <

https://www.waters.com/nextgen/global/shop/standards--reagents/186009125-humanized-mab-mass-check-standard.html> ,) and Waters mAb (NISTmAb) Subunit Standard (Waters p/n: 186008927 < https://www.waters.com/nextgen/global/shop/standards--reagents/186008927-mab-subunit-standard.html>) were used for this study. For the intact NISTmAb sample, 160 µL of Milli-Q water was added to the sample vial (containing 80 µg of protein material) to produce a solution of 0.5 µg/µL before analysis (with 2 µL injection). For subunit analysis, 125 µL of water was added to the sample vial (containing 25 µg of subunit mAb material) to produce a solution of 0.2 µg/µL before analysis (with 2 µL injection).

BioAccord System with ACQUITY Premier

System:	ACQUITY Premier UPLC BSM, ACQUITY Premier UPLC FTN with column heater, ACQUITY RDa Mass Detector, ACQUITY Premier TUV Optical Detector, waters_connect v2.1.2.4

Intact Mass Analysis – LC-MS Method Setup

Column:	ACQUITY Premier Protein BEH™ C_4 Column, 300 Å, 1.7 µm, 2.1 mm x 50 mm (Waters p/n=186010326)
Column temp.:	80 °C
Mobile phase A:	Water with 0.1% formic acid
Mobile phase B:	Acetonitrile with 0.1% formic acid
TUV optical detection:	UV 280 nm

LC Gradient Table for Intact mAb Analysis

	Time (min)	Flow rate (mL/min)	Composition A (%)	Composition B (%)	Curve
1	0.00	0.40	95.0	5.0	Initial
2	1.00	0.40	15.0	85.0	6
3	1.20	0.40	5.0	95.0	6
4	1.50	0.40	95.0	5.0	6
5	2.50	0.40	95.0	5.0	6

Total run time: 2.5 minutes.

MS Conditions for Intact Mass Analysis

Acquisition Settings

Mode:	Full scan
Mass range:	High (400–7000 *m/z*)
Polarity:	ESI Positive
Scan rate:	2 Hz
Cone voltage:	Custom (70 V)
Capillary voltage:	Custom (1.50 kV)
Desolvation temp.:	Custom (550 °C)
Intelligent data capture (IDC):	On

Subunit Mass Analysis LC-MS Method Setup

Column:	ACQUITY Premier Protein BEH C_4 Column, 300 Å, 1.7 µm, 2.1 mm x 50 mm (Waters p/n=186010326)
Column temp.:	80 °C
Mobile phase A:	Water with 0.1% formic acid
Mobile phase B:	Acetonitrile with 0.1% formic acid
TUV optical detection:	UV 280 nm

LC Gradient Table for the Analysis

	Time (min)	Flow rate (mL/min)	Composition A (%)	Composition B (%)	Curve
1	0.00	0.40	80.0	20.0	Initial
2	0.25	0.40	75.0	25.0	6
3	1.75	0.40	60.0	40.0	6
4	2.00	0.40	5.0	95.0	6
5	2.25	0.40	80.0	20.0	6
6	3.00	0.40	80.0	20.0	6

Total run time: 3.0 minutes.

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 (50 V)
Capillary voltage:	Custom (1.00 kV)
Desolvation temp.:	Custom (450 °C)
Intelligent data capture (IDC):	On

Results and Discussion

The INTACT Mass Application, residing within the waters_connect informatics platform HUB (Figure 2) can be used to confirm the mass and assess the purity for a wide range of biomolecule classes. Untargeted mass analyses can be performed as well to support discovery studies. Additionally, purity assessments can be produced using either optical chromatogram, TIC or mass spectra.

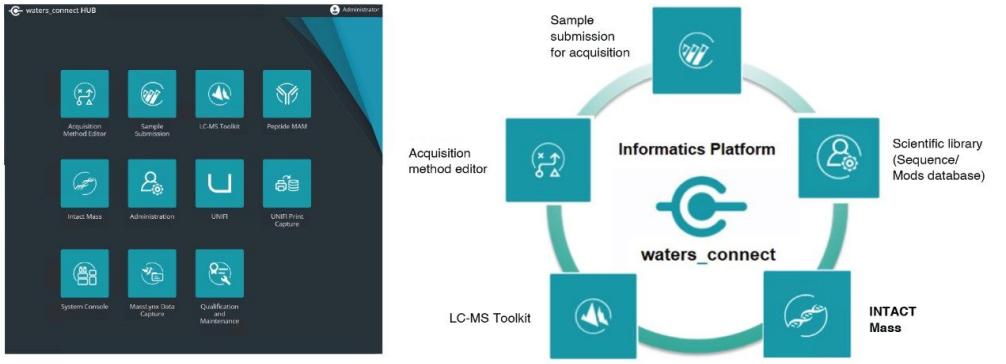


Figure 2. waters_connect HUB contains the application icons that are associated with intact mass analysis workflow, including the Acqusition Method Editor, Sample Submission, Scientific Library, INTACT Mass, and LC-MS Toolkit.

The waters_connect informatics platform HUB contains the applications utilized for the intact mass analysis workflow. The Acquisition Method Editor App is used to define the data acquiring conditions, such as LC gradients and MS settings. The INTACT Mass Application operates the integrated workflow for data acquisition, processing, review and reporting. The Sample Submission App can be launched independently, but when the users selects the option to acquire and process, it is executed within the INTACT Mass application. The Scientific Library contains an extensive list of variable modifications for various biomolecule types that can be selected to search for product variants, impurities, and MS adducts. The LC-MS Toolkit App can be used to manually examine the data when needed for result confirmation or troubleshooting.

Creating the Acqusition Method

To facillitate acquisition of intact mass LC-MS data, a method is defined using the Acqusition Method Editor App in the waters_connect HUB. This Acquisition method includes settings for the solvent manager, sample manager, column compartment, tunable UV (TUV), and ACQUITY RDa MS detectors. To facillitate a higher throughput analysis, a 2.5 minutes total runtime fast LC desalting method was defined, featuring the gradient table and chart (Figure 3A), and MS settings (Figure 3B) indicated. The “scheduled lockmass” was selected in the MS method for this experiment to shorten the total acquisition time, by reducing inter-injecton system check activities from a per injection basis. With the “scheduled lockmass” selected in the INTACT Mass Application, it takes 136 minutes to finish the LC-MS data acquisition and data processing for 48 sample runs using a 2.5 minutes fast LC method.

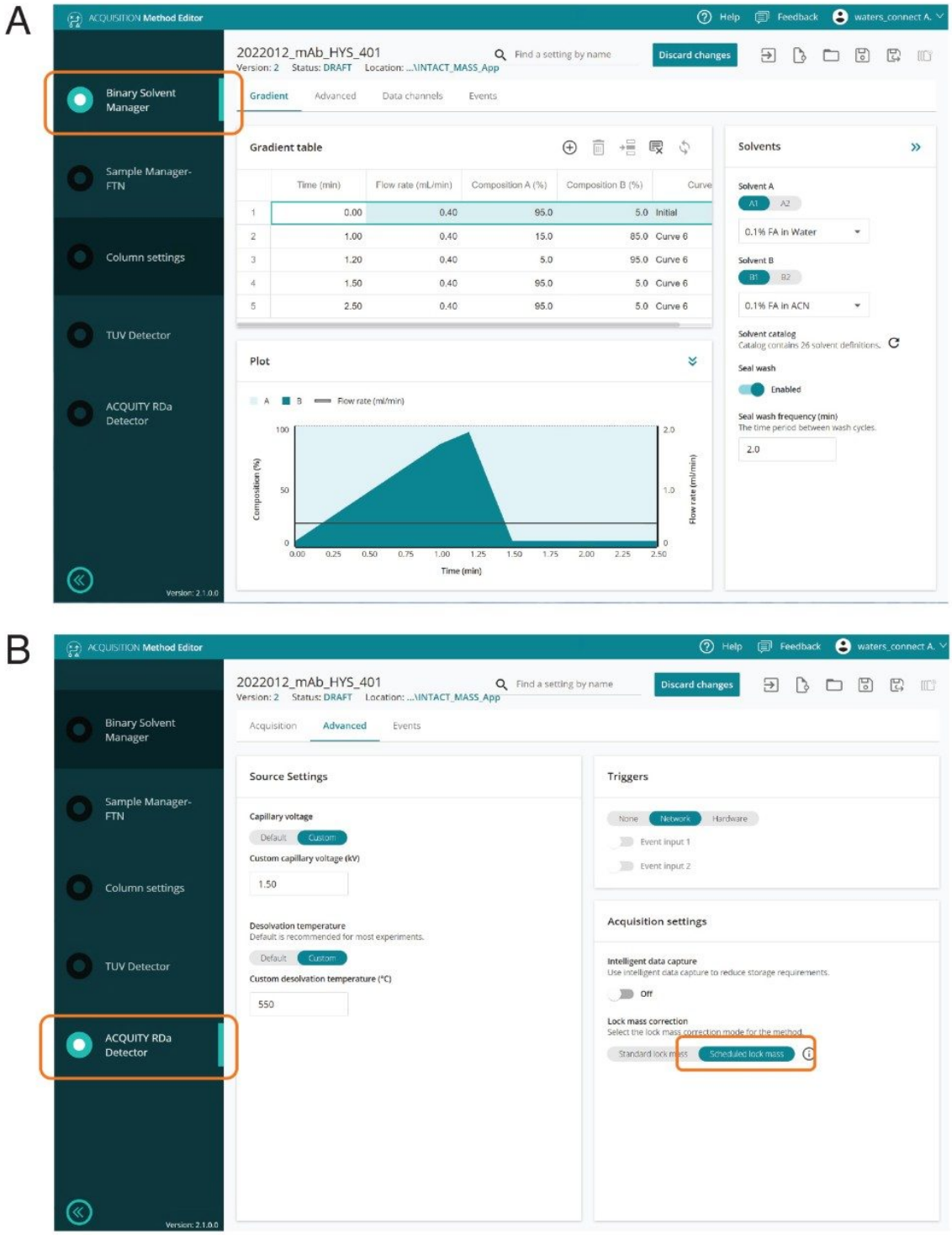


Figure 3. The Acquisition Method Editor was used to generate a higher throughput intact mAb LC-

MS acquisition method. It shows a 2.5 minutes total runtime, as indicated on the gradient table and chart (A), and selecting the scheduled lockmass under the advanced MS settings (B).

Creating the Processing Method

The Data Processing method is created within the INTACT Mass Application. It can be cloned from an existing method, or newly generated. The welcome page of the INTACT Mass Application (Figure 4) allows the user to generate a process only analysis for an existing data set or combine acquiring and processing for a new data set.

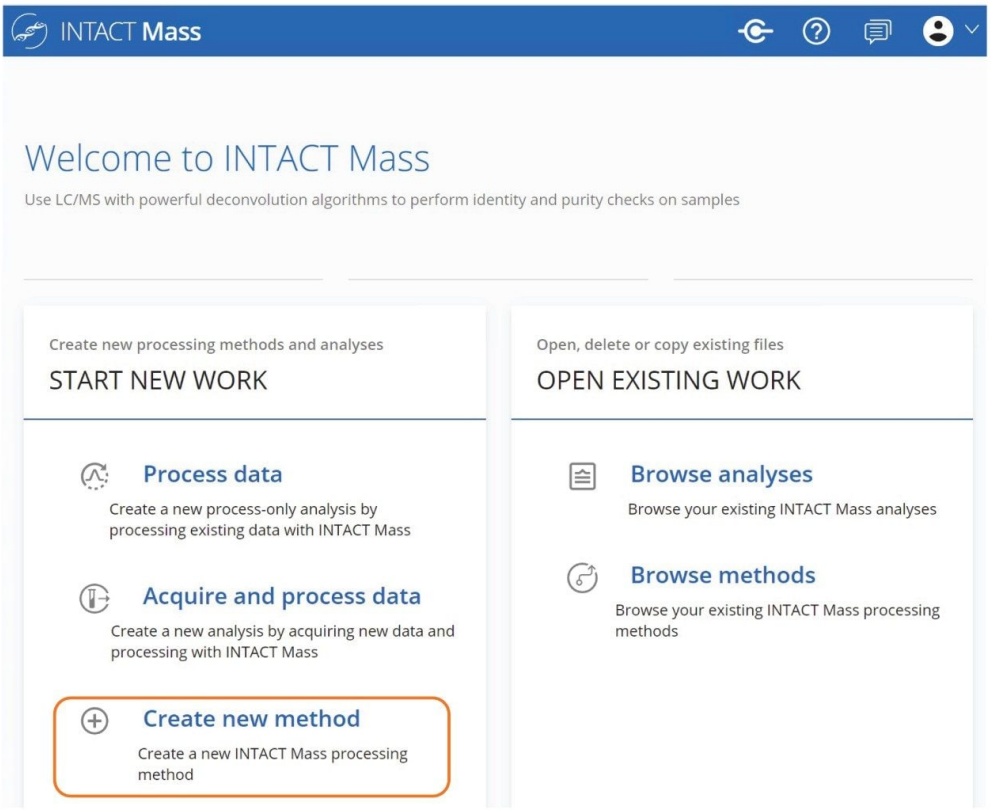


Figure 4. The main page of the INTACT Mass Application. Processing methods can be executed on exisiting data, executed as part of a combined acquire and process selection, or created a new data processing.

In building the processing method for intact mAb screening (Figure 5A), user has the flexibility to choose either the latest BayesSpray[1] or the traditional MaxEnt1[2] algorithm for data deconvolution. BayesSpray can be applied to generate either average or monoisotopic spectra, while MaxEnt1 delivers average mass.[3] When selecting the automated peak deconvolution settings, the mass range of the raw spectra and deconvolved spectra and other deconvolution parameters will be self-optimized, with the results of this optimization available for locking down future methods, if desired. In this study, the MaxEnt1 deconvolving agerithm was selected for processing. The user can choose the molecular type (Protein was selected for this study) from the pull down list (Figure 5B), allowing the deconvolution algorithm to model a proper elemental composition to obtain optimal results, or select a custom elemental composition to support atypical classes of molecule. This feature gives optimum charge deconvolution results with little user intervention, and, enables the creation of platform methods that can be applied to sample sets containing a diverse range of molecules. As an example, an oligo-focused analysis using the INTACT Mass Application titled "LC-MS Analysis of Single Guide RNA Impurities Using the BioAccord System with ACQUITY Premier and Automated waters_connect INTACT Mass Application" is listed in the references.[4]

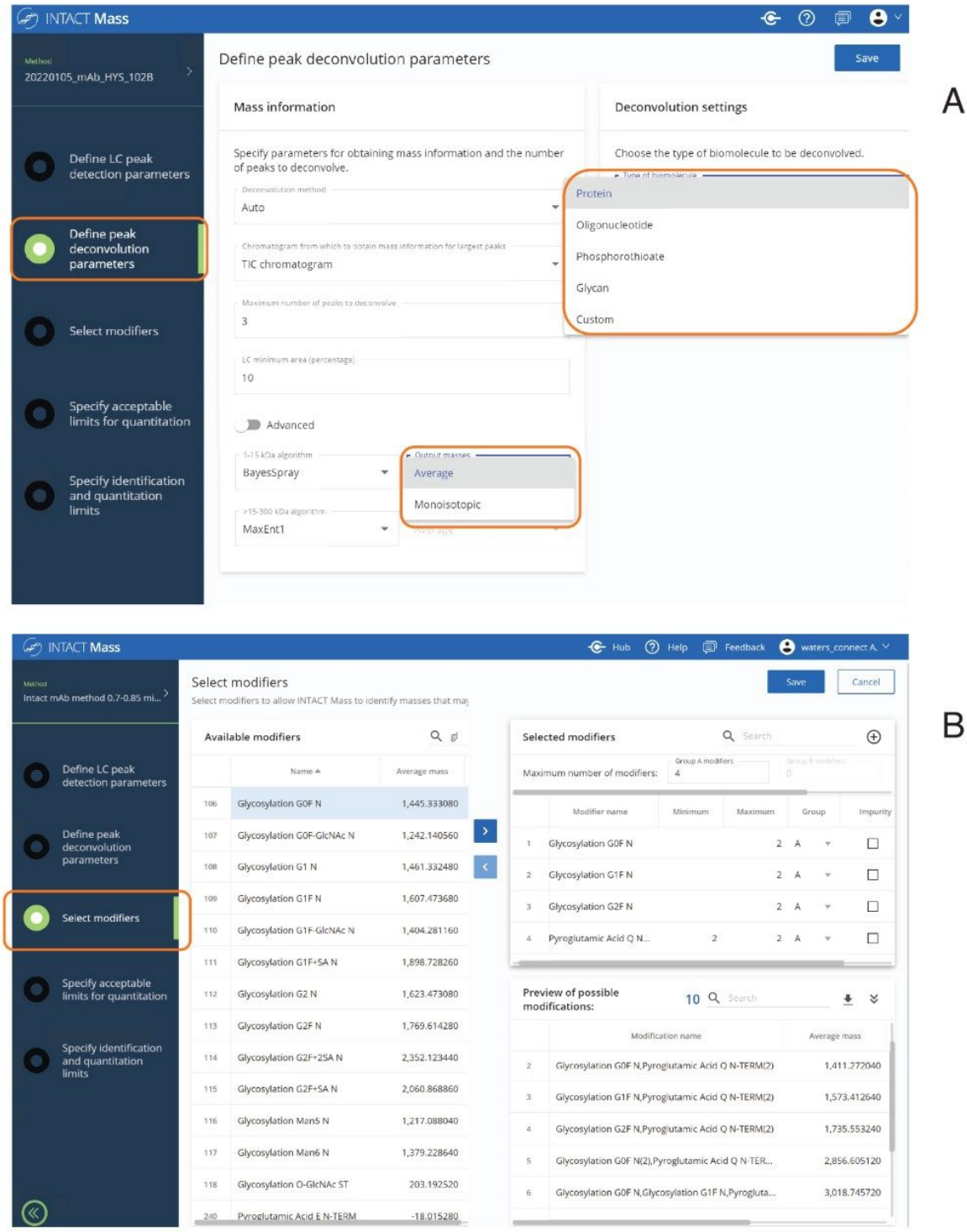


Figure 5. Selecting Processing Parameters. The App supports both the BayesSpray or traditional MaxEnt1

algorithms for data deconvolution: the BayesSpray output can be average or monoisotopic mass while MaxEnt 1 provides average mass. Using the default automated setting enables the mass range of the raw and deconvolved spectra and other deconvolutuion settings to be intelligently self-optimized during data processing (Figure 5A). Furthermore, various sample types can be selected, optimizing the elemental composition model for more optimum charge deconvolution (Figure 5B).

Executing the Analysis

Selecting the methods following selection of the Acquire and Process option, opens a Sample List (Figure 6) where sample information, such as targeted masses, sample, and molecule types, any pre-sample treatment (***e.g.,*** IdeS digestion) can be specified.

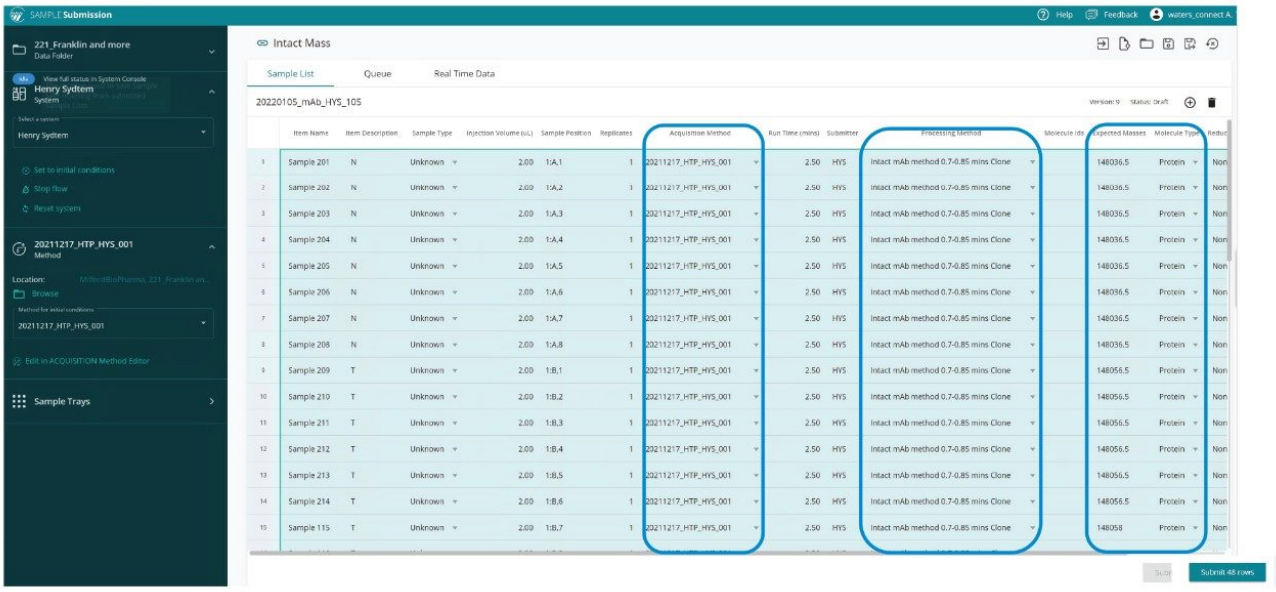


Figure 6. Acquire and Process Sample List. Instrument system, acqusition method, processing method can be selected accordingly. In additional, sample specific information can be added as needed to enable intelligent acquisition and processing on a sample-by-sample basis.

When the analysis is submitted, the automated data acquistion and processing will start. Data processing will

take place in parallel with subsequent data acquisition, with deconvolution of up to five peaks from one or more samples occuring concurrently. Users can examine these results in real-time to make decisions on continuing acquisition, or determine any needed method enhancements.

Review and Reporting of Results

The dashboard view of the results (Figure 7) from an intact mAb screening experiment summarizes the results for a plate of 48 injections. This represents six antibodies, prepared as described in the Experimental, and analyzed with eight replicate wells per antibody. In the processing method, each antibody is searched against its appropriate target average mass.

The color flags displayed on the sample plate reflect the status of the samples analyzed, and reflect whether targted masses were confirmed, and if any product purity specifications were exceeded. In these experiments no purity criteria were specified.

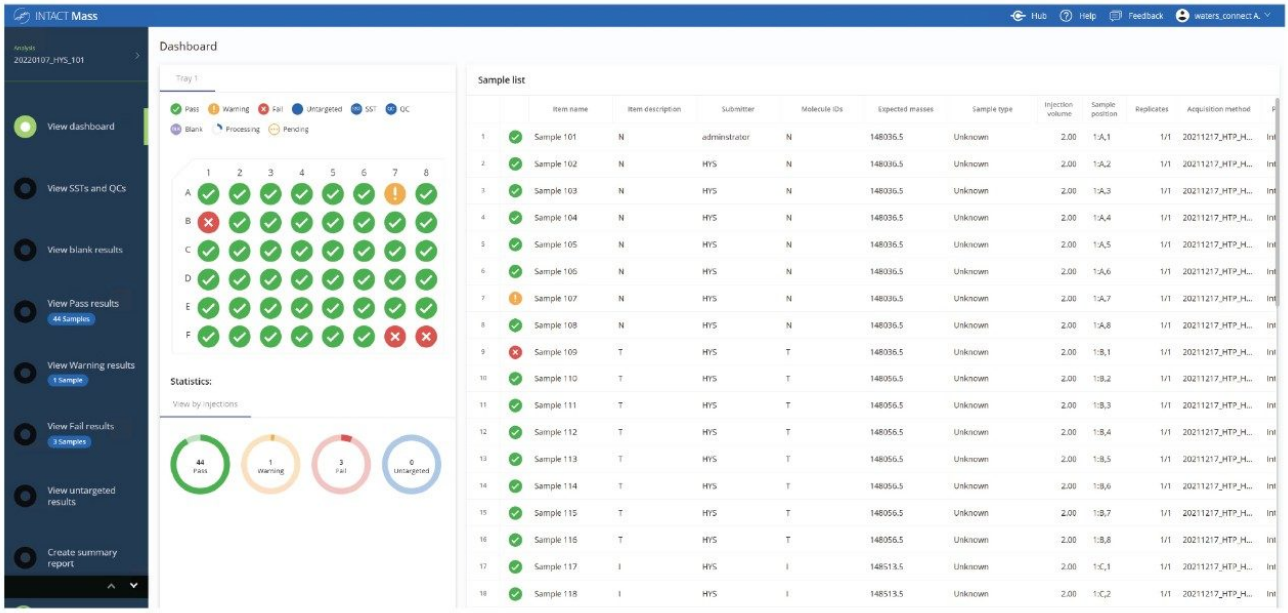


Figure 7. The dashboard results view. Results are from an intact mAb screening experiment with 48 injections representing eight replicates of six different antibodies. The color codes on the sample plate reflect the result status of the samples analyzed. The detailed individual sample results can be reviewed by clicking within the sample list.

A green color indicates a pass for the sample when measured against the threshold settings for the mass accuracy matched to the targeted masses, and for the expected purity of the sample. An orange color indicates a warning is issued, either the mass error is above the threshold, or the purity of the sample is lower than expected. A red color indicates that either mass accuracy measured against the targeted masses is too large or unmatched, and/or the purity is below thresholds.

Table 1 summarizes the mass error measured against the expected masses of a 48 injections representing eight replicates of six different antibodies (NISTmAb, Trastuzumab, Infliximab, Bevacizumab, Rituximab, and Omalizumab). The mass accuracy for all the injections were found to be around 10 ppm, and the standard deviation of the replicate injections for each antibody are less than 5 ppm. The data in this table indicate that the INTACT Mass Application can produce consistent and useful results for fast intact mass screening projects.

mAbs name	Expected mass (Da)	Sample mass error (ppm)								Average mass error (ppm)	Mass error st. dev. (ppm)
		1	2	3	4	5	6	7	8		
NISTmAb	148036.5	8.8	9.8	11	7.3	11.2	10.7	9.2	13.2	10.15	1.79
Trastuzumab	148056.5	9.7	12.1	14.5	11.9	14.8	15.7	16.3	5.0	12.5	3.75
Infliximab	148513.5	6.7	0.5	6.5	-3	3.2	4.3	10.7	7.7	4.58	4.33
Bevacizumab	149197.5	7.8	8.8	7.6	8.2	9.4	10.8	7.8	9.1	8.69	1.08
Rituximab	147075.5	9.2	7.6	7.7	8.8	8.8	6.1	6.1	6.3	7.58	1.29
Omalizumab	149171.5	1.5	-0.6	-3.8	-2.5	-1.8	-1.5	-0.3	-1.9	-1.36	1.59

Table 1. Mass accuracy summary for 48 injections that representing six antibodies with eight replicate injections each.

Using a NISTmAb sample (Sample 5 in the overview) as an example, we can access summarized individual sample information and its automatically generated report (Figure 8).

Dashboard > Sample 105

< Sample 105 Sample report

INTACT **Mass**

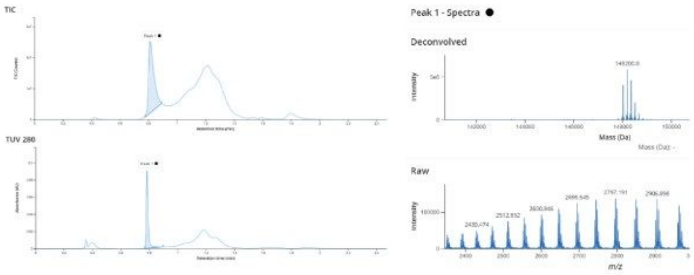
Version: 1.0.0.0

Analyst name: administrator
Analysis name: 20220208-Intact-HYS-102
Sample set name: 20220105_mAb_HYS_105_1 - v2

Sample 105
Pass

Created by: administrator Time acquired: Jan 30, 2022 02:23:12 (+00:00) Time processed: Feb 08, 2022 17:28:04 (-05:00) Time report generated: Feb 15, 2022 09:47:45 (-05:00)

Item description:	N	Molecule IDs:	-
Sample position:	1:A,5	Expected masses:	148036.5
Sample type:	Unknown	Submitter:	HYS
Injection volume:	2	Molecule type:	Protein
Replicates:	1	Reduction state:	None
Acquisition method:	20211217_HTP_HYS_001	Enzymatic treatment:	None
Processing method:	Intact mAb method 0.7-0.85 mins Clone - v5	Acquisition status:	Complete



Results

Identity: Pass Purity: Pass

#	Type	Molecule ID	Component	Observed mass (Da)	Expected mass (Da)	Mass error (ppm)	Identity result	LC amount (%)	MS amount (%)	Purity result
1	Summary	-	-	-	-	-	Pass	-	100	Pass
2	Product	148036.5	148036.5 All Forms	-	-	-	Pass	100.00	100	-
3	Product	148036.5	148036.5	148,037.8	148,036.5	9	Pass	100.00	59.31	-
4	Product	148036.5	148036.5 Glycosylation	148,200.3	148,198.6	11.1	Pass	100.00	100	-
5	Product	148036.5	148036.5 Glycosylation(2)	148,362.3	148,360.8	10.4	Pass	100.00	81.3	-
6	Product	148036.5	148036.5 Glycosylation(3)	148,524.5	148,522.9	10.7	Pass	100.00	36.66	-
7	Product	148036.5	148036.5 Glycosylation(4)	148,685.8	148,685.1	4.8	Pass	100.00	12.98	-

TIC

#	Peak	Spectrum	RT (mins)	Start (mins)	End (mins)	Area	% Area	Height	% Height
1	Peak 1	●	0.81	0.78	0.89	1017457	100.00	24148927	100.00

TUV 280

#	Peak	Spectrum	RT (mins)	Start (mins)	End (mins)	Area	% Area	Height	% Height
1	Peak 1	●	0.78	0.77	0.90	86530	100.00	90643	100.00

MS

#	Type	Molecule ID	Component	Spectrum	Observed TIC RT (mins)	Observed UV RT (mins)	Observed RT delta (mins)	Response	%	Observed neutral mass (Da)	Observed m/z	Spectrum type	Expected mass (Da)	Mass error	Alternative assignments
1	Product	148036.5	148036.5 Glycosylation	●	0.81	0.78	0.03	22751971	100.00	148,200.3	-	MaxEnt1 Average Mass	✓ 148,198.6	11.1	-
2	Product	148036.5	148036.5 Glycosylation(2)	●	0.81	0.78	0.03	18496448	81.30	148,362.3	-	MaxEnt1 Average Mass	✓ 148,360.8	10.4	-
3	Product	148036.5	148036.5	●	0.81	0.78	0.03	13494191	59.31	148,037.8	-	MaxEnt1 Average Mass	✓ 148,036.5	9	-
4	Product	148036.5	148036.5 Glycosylation(3)	●	0.81	0.78	0.03	8341845	36.66	148,524.5	-	MaxEnt1 Average Mass	✓ 148,522.9	10.7	-
5	-	-	-	●	0.81	0.78	0.03	3883065	17.07	148,328.4	-	MaxEnt1 Average Mass	-	-	-
6	-	-	-	●	0.81	0.78	0.03	3083296	13.55	148,165.2	-	MaxEnt1 Average Mass	-	-	-
7	Product	148036.5	148036.5 Glycosylation(4)	●	0.81	0.78	0.03	2953769	12.98	148,685.8	-	MaxEnt1 Average Mass	✓ 148,685.1	4.8	-

Figure 8. Report for Sample 5 (NISTmAb) from the 48-well sample plate. The report includes the experimental

information, TIC, and TUV chromatograms with the integrated peaks noted, the identified components with their assigned modifications, and the calculated mass accuracy and purity information. The raw and deconvolved spectral data are also visualized in the formatted report.

The experimental results for sample 5 (a NISTmAb injection) from the 48-well plate sample set are presented in Figure 8. The easy to read standardized report template includes information such as TIC and TUV chromatograms with the integrated peaks, the identified targeted components with their assigned modifications, the calculated mass accuracy and sample purity, as well as the raw and deconvolved spectra. The use of the automated MaxEnt1 setting facilitated a single platform acquisition and processing method that could be applied to the different samples. For NISTmAb sample 5, the measured mass accuracy for the top five major glycoforms were about 10 ppm. Similar glycoform mass accuracies were observed for the Trastuzumab, Infliximab, Bevacizumab, Rituximab, and Omalizumab samples (data not shown).

The relative percentage of the indentified components (against the base peak) was also calculated and was found to be consistent across replicated samples (data not shown). Additional quantitative information derived from the TIC, TUV, and MS data is also reviewable in the tables of the sample centric report.

In addition to the intact mAb high throughput analysis experiment, we also conducted an IdeS digested mAb subunit analysis experiment using the Waters mAb (NISTmAb) Subunit Standard as the test sample. This provides the opportunity to display the capabilities of the automated chromatographic peak detection, and spectral deconvolution, in the context of a multi-analyte sample.

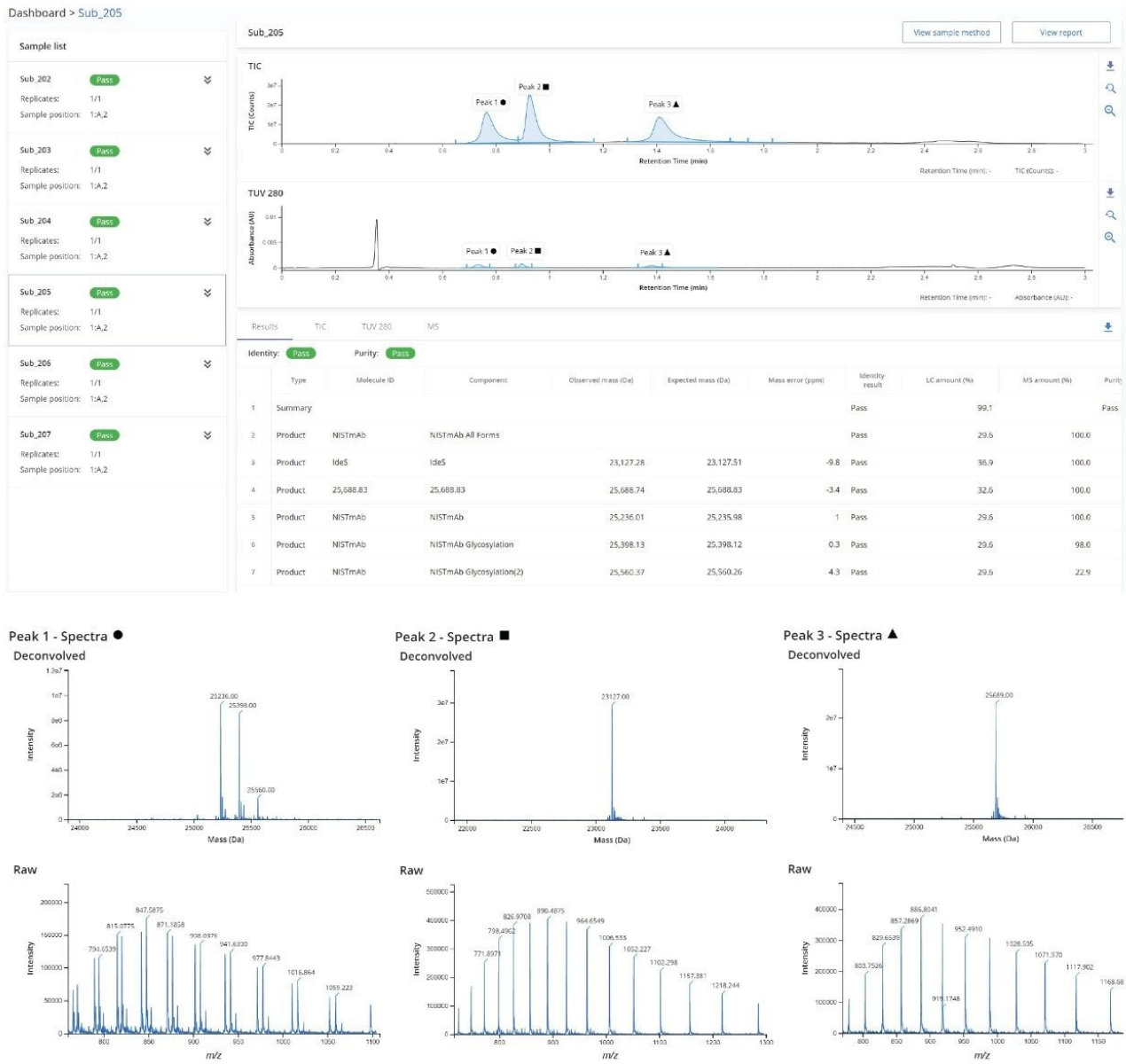


Figure 9. Waters mAb (NISTmAb) Subunit Standard results. The TIC and TUV chromatograms show that the three major peaks of the scFc, LC, and Fd subunits were separated in a 3-minute gradient LC-MS run. The mass accuracies for the assigned 25 kD subunits and their modifications were measured within about 5 ppm). Peak purity was calculated by combining the three subunit TIC peaks and found to be 99.1%. The raw and deconvolved subunit spectra are shown at the bottom panel.

The analysis of the Waters NISTmAb subunit Standard was acquired with a 3-minute LC gradient on a newly launched ACQUITY Premier Protein BEH C4 Column (300 Å, 1.7 µm, 2.1 mm x 50 mm, Waters p/n: 186010326). The three major peaks of the scFc, LC, and Fd subunits were separated using 0.1% formic acid in water and 0.1% formic acid in ACN as the mobile phases, and average mass errors were found to be about 5 ppm. The peak purity was calculated using the combined TIC peak areas of the three subunits and found to be 99.1%.

The ability to automate the processing of intact protein LC-MS data is key to drive the efficiency of laboratories using this methodology to screen samples or monitor attributes, particularly when these studies are conducted with large number of samples. The ability to create platform methods that can be used to analyze diverse sets of samples within one analysis will benefit not only discovery organizations, where this challenge is common, but also those core labs looking to simplify operations by grouping of a multitude of samples for bulk analysis. The combination of efficient separations, robust and simple detectors, and automated acquisition/processing on a compliance-ready platform should enable this approach to see wider deployment, particularly in manufacturing and quality organizations that have been challenged to deploy such methods previously.

Conclusion

- The waters_connect INTACT Mass Application facilitates a streamlined integrated workflow for data acquisition, processing and reporting of deconvoluted mass data for biotherapeutics, deployable in regulated and non-regulated environments.
- The analysis workflows supported by this app include targeted mass confirmation, untargeted profiling, and purity assessments using optical, TIC, or mass spectral approaches.
- Automation of chromatographic peak detection along with the MaxEnt1 and BayesSpray deconvolution algorithms has enabled the development of platform- based methods to look across a diverse set of molecules within a single sample set while preserving the ability to compare against sample-specific specifications.
- In this work, the waters_connect INTACT Mass Application provided low ppm mass confirmation data for assessment of six commercial mAbs analyzed in replicate on a 48-sample plate using a quick desalting LC-MS method, and for a multi-chromatographic peak IdeS digested and reduced NISTmAb subunits sample subjected to a fast gradient separation.

References

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2. Skilling, J., *et al*. Beyond MaxEnt Deconvolution: Increasing the Fidelity and Universal Applicability of Mass Spectral Deconvolution Routines for Biomolecules with the Application of Bayesian Probability Theory Analytical Chromatography, Waters Poster 720003756 < https://www.waters.com/waters/library.htm?lid=10180413> , 2010.

3. Berger. S. Boyce, P., Beyond INTACT Mass Application within waters_connect, *Waters Vblog*, 2022. https://blog.waters.com/automating-intact-mass-deconvolution-its-about-time < https://blog.waters.com/automating-intact-mass-deconvolution-its-about-time>

4. Doneanu, C., *et al*. LC-MS Analysis of Single Guide RNA Impurities Using the BioAccord System with ACQUITY Premier and Automated waters_connect INTACT Mass Application, Waters Application Note, 720007546, 2022.

Featured Products

ACQUITY Premier System <https://www.waters.com/waters/nav.htm?cid=135077739>

ACQUITY RDa Detector <https://www.waters.com/waters/nav.htm?cid=135077027>

ACQUITY UPLC Tunable UV Detector <https://www.waters.com/514228>

BioAccord LC-MS System for Biopharmaceuticals <https://www.waters.com/waters/nav.htm?cid=135005818>

waters_connect <https://www.waters.com/waters/nav.htm?cid=135040165>

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