

Intact biotherapeutic mass analysis

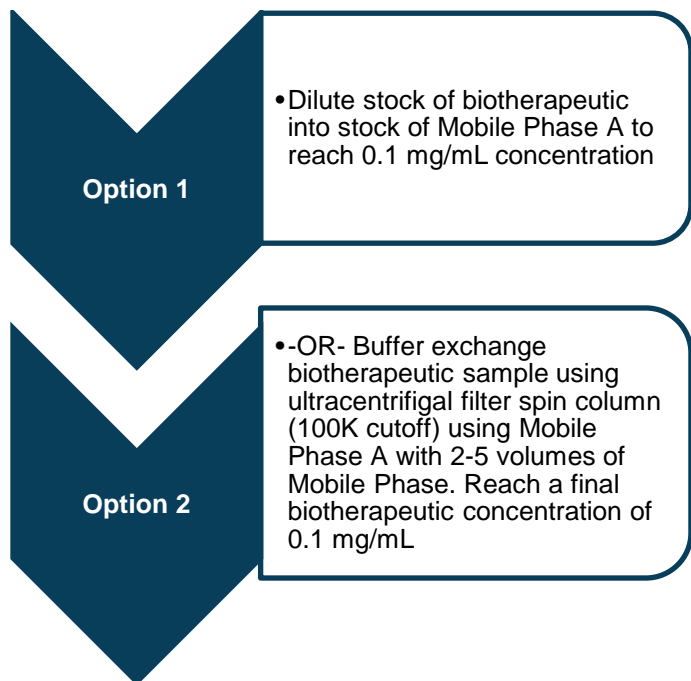
Routine high-resolution accurate mass analysis of intact biologics on the X500B QTOF System

Method details for the routine characterization of rituximab biotherapeutic protein by high-resolution intact mass analysis using HPLC coupled with the X500B QTOF System, powered by SCIEX OS Software.



Sample Prep

A generic sample preparation strategy is shown for general clean-up of an intact biotherapeutic if necessary prior to LC-MS analysis.



LC Method

<i>Column</i>	Waters Acquity UPLC BEH C4, 2.1mm x 50mm, 300A, 1.7 um	
<i>Mobile Phase A</i>	Water, 0.1% Formic acid	
<i>Mobile Phase B</i>	Acetonitrile, 0.1% Formic acid	
<i>Flow rate</i>	200 µL/min	
<i>Column temperature</i>	80° C	
<i>Injection volume</i>	10 µL, 1 µg total protein	
<i>Gradient profile</i>	Time (min)	% B
	2.0	5
	6.0	90
	7.0	90
	8.1	5
	10	5

MS Method

Suggested starting MS method parameters for routine intact mAb analysis as displayed in SCIEX OS. For best sensitivity and resolution, the declustering potential (DP) and collision energy (CE) parameters should be optimized for each individual biotherapeutic.

The screenshot shows the 'MS Method' configuration window for 'intact protein analysis MS'. The interface includes a top navigation bar with 'MS Method' and 'Running' status, and a toolbar with 'New', 'Open...', 'Save', 'Print...', and 'Advanced' options. The main configuration area is divided into several sections:

- Method Overview:** Device: X500 QTOF, Ion Source: TurboSpray.
- TOF MS:** 0 min - 10 min.
- General Parameters:** Method duration: 10 min, Total scan time: 0.526388 sec, Estimated cycles: 1139, Intact protein mode: ON. Checkboxes for 'Large proteins (>70 kDa)' and 'Decrease detector voltage' are present.
- Source and Gas Parameters:** Ion source gas 1 and 2: 45 psi; Curtain gas: 30; CAD gas: 7; Temperature: 450 °C.
- Experiment (TOF MS):** Polarity: Positive; TOF start mass: 900 Da; TOF stop mass: 4000 Da; Accumulation time: 0.5 s; Spray voltage: 5000 V; Declustering potential: 275 V; DP spread: 0 V; Collision energy: 20 V; CE spread: 0 V.
- Advanced Experiment Settings:** Time bins to sum: 120; Channel 1, 3, and 4 are checked; Channel 2 is unchecked.

The bottom status bar shows 'Data Acquisition' with 'MS' selected, and 'Start', 'Stop', and 'Save...' buttons.

Pharma and Biopharma

Batch

In the Batch setup, open the 'Automated Calibration Editor' window in order to select the use of the autocalibration function. Designate use of the 'X500 ESI Positive Calibration Solution', and then determine how often you would like the system to perform a fast, automated calibration. These short calibrations will be added automatically to your queue once you have submitted a sample batch.

The screenshot shows the 'Batch' software interface. At the top, there is a toolbar with buttons for 'Auto-Calibrate...', 'Plate Layout...', 'New', 'Open', 'Save', 'Print...', 'Manage', and 'Submit'. Below the toolbar is a table with the following columns: Sample Name, MS Method, LC Method, Rack code, Vial position, and Data File. The table contains one row of data: 'Intact protein', 'intact protein analysis MS', 'Intact_10min', '1.5mL (105 vial)', '1', and 'Intact protein file'. Overlaid on the table is the 'Batch - Automatic Calibration Editor' dialog box. The dialog has a title bar with a close button (X). The main text reads: 'Provide ion reference and calibrant delivery settings to be applied automatically, at the correct frequency during acquisition'. There are three main sections: 'Ion reference table' with a dropdown menu showing 'X500 ESI Positive Calibration Solu...' and an 'Edit...' button; 'Calibrate every' with a dropdown menu showing '3' and the unit 'samples'; and 'Calibrant delivery' with a dropdown menu showing 'CDS' and a 'CDS channel' dropdown showing '1'. There are 'OK' and 'Cancel' buttons at the bottom right of the dialog.

This is a close-up view of the 'Batch - Automatic Calibration Editor' dialog box. The title bar shows the window name and a close button (X). The main text is: 'Provide ion reference and calibrant delivery settings to be applied automatically, at the correct frequency during acquisition'. The 'Ion reference table' dropdown is set to 'X500 ESI Positive Calibration Solu...' with an 'Edit...' button to its right. The 'Calibrate every' dropdown is set to '3' with the unit 'samples' to its right. The 'Calibrant delivery' dropdown is set to 'CDS'. The 'CDS channel' dropdown is set to '1'. There are 'OK' and 'Cancel' buttons at the bottom right.

Pharma and Biopharma



Data Processing

Process intact biotherapeutic data in BioPharmaView™ Software 2.0.

Input the protein sequence, and assign potential modifications in the 'Assay Information' window.

BioPharmaView Rituximab

Assay Information | **Sequence Features** | Intact Protein | Peptide Mapping

Summary

Protein Name: Rituximab
 Description: Rituximab

Protein Sequence

Protein Type: Antibody Add Chain Unmodified Protein MWs:
 Monoisotopic: 144195.3139 Average: 144286.27

Chain 1 Light Chain 1 Delete Chain
 AA Indexes:
 1-110 QIVLSQSPAILASDPGERVTMTCRASSVSYSIHWFQOKPGSSPKPWIYATSNLASGVVPRFSGSGSSTYSYSLTISRVEAEDAATYYCQOWTSPNPPTFGGGTKLEIKRTVA
 111-213 APSVFI PPPSDEQLKSGTASVVCLLNNFYPREAKVQKVDNALQSGNSQESVTEQDSKSTYSLSSTLTLSKADYKHKHYACEVTHQGLSSPVTKSFRNGEC

Chain 2 Heavy Chain 1 Delete Chain
 AA Indexes:
 1-110 QVQLQOPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPNGDTSYNQKFKGKATLTADKSSSTAYMQLSSTLSEDSAVYYCARSTYYGGDYFNV
 111-220 WGAGTIVTVSAASTKGPSVFLPAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKRAE
 221-330 PKSCDKHTCTPCPAPELLGGPSVFLFPPKPKDTLMI SRTPETCVVVDVSHEDPEVRFNWIYDGVVHNARTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
 331-440 ALPAPIERTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSEFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNNY
 441-450 TQKSLSLSPG

Chain 3 Heavy Chain 2 Delete Chain
 AA Indexes:
 1-110 QVQLQOPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPNGDTSYNQKFKGKATLTADKSSSTAYMQLSSTLSEDSAVYYCARSTYYGGDYFNV
 111-220 WGAGTIVTVSAASTKGPSVFLPAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKRAE
 221-330 PKSCDKHTCTPCPAPELLGGPSVFLFPPKPKDTLMI SRTPETCVVVDVSHEDPEVRFNWIYDGVVHNARTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
 331-440 ALPAPIERTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSEFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNNY
 441-450 TQKSLSLSPG

Chain 4 Light chain 2 Delete Chain
 AA Indexes:
 1-110 QIVLSQSPAILASDPGERVTMTCRASSVSYSIHWFQOKPGSSPKPWIYATSNLASGVVPRFSGSGSSTYSYSLTISRVEAEDAATYYCQOWTSPNPPTFGGGTKLEIKRTVA
 111-213 APSVFI PPPSDEQLKSGTASVVCLLNNFYPREAKVQKVDNALQSGNSQESVTEQDSKSTYSLSSTLTLSKADYKHKHYACEVTHQGLSSPVTKSFRNGEC

Modifications Cysteine Modifications Can Replace Disulfide Bonds **Disulfide Bonds - (16)** Import Export...

Chai...	Type	Name	Position	Maximum Mods per Chain	Modified AA	Applies To	Workflow Usage	Mass Shift
1	1-4 Internal	Deamidated	-	5	n/a	NQ	Peptide Mapping	0.9840
2	1-4 Internal	Oxidation	-	5	n/a	M	Peptide Mapping	15.9949
3	1-4 N-terminal	Gln->pyro-Glu	-	-	Q	Q	Both	-17.0265
4	2-3 Internal	G1F	301	-	N	N	Both	1606.5867
5	2-3 Internal	G2F	301	-	N	N	Both	1768.6395
6	2-3 Internal	G0	301	-	N	N	Both	1298.4760
7	2-3 Internal	G0F-GlcNAc	301	-	N	N	Both	1241.4545
8	2-3 Internal	G0-HexNAc	301	-	N	N	Both	1095.3966
9	2-3 Internal	G0F	301	-	N	N	Both	1444.5339

From Chain	To Chain	From Cysteine	To Cysteine
1	1	1	23
2	1	1	133
3	1	2	213
4	2	2	22
5	2	2	148
6	2	2	265
7	2	2	371
8	4	4	23
9	4	4	133
10	4	3	213
11	3	3	22
12	3	3	148
13	3	3	265
14	3	3	371
15	2	3	230
16	2	3	233

Add modifications... Delete selected modifications Edit bond... Add bonds... Delete selected bonds

Pharma and Biopharma



Navigate to the 'Intact Protein' tab complete processing parameters and to generate the protein forms for matching.

Project: Rituximab

Assay Information: Sequence Features | **Intact Protein** | Peptide Mapping

Processing Parameters:
 Matching Tolerance: ± 5.00 Da
 Start m/z: 1600.00
 Stop m/z: 4000.00
 Start Mass: 139185.89 Da
 Stop Mass: 152793.28 Da
 RT Range Processing: Time Selection
 Perform LC Peak Detection
 Start RT: 4.17 min
 Stop RT: 6.69 min

Batch Processing Parameters:
 Retention Time Tolerance: ± 1.00 min

Batch Processing Pass / Fail Criteria:
 Reconstruction Area Limits: ± 40.0 %
 Required Form Minimum: ≥ 80 %
 Restricted Form Maximum: ≤ 120 %

Maximum Number of Combined Modifications per Protein: 20

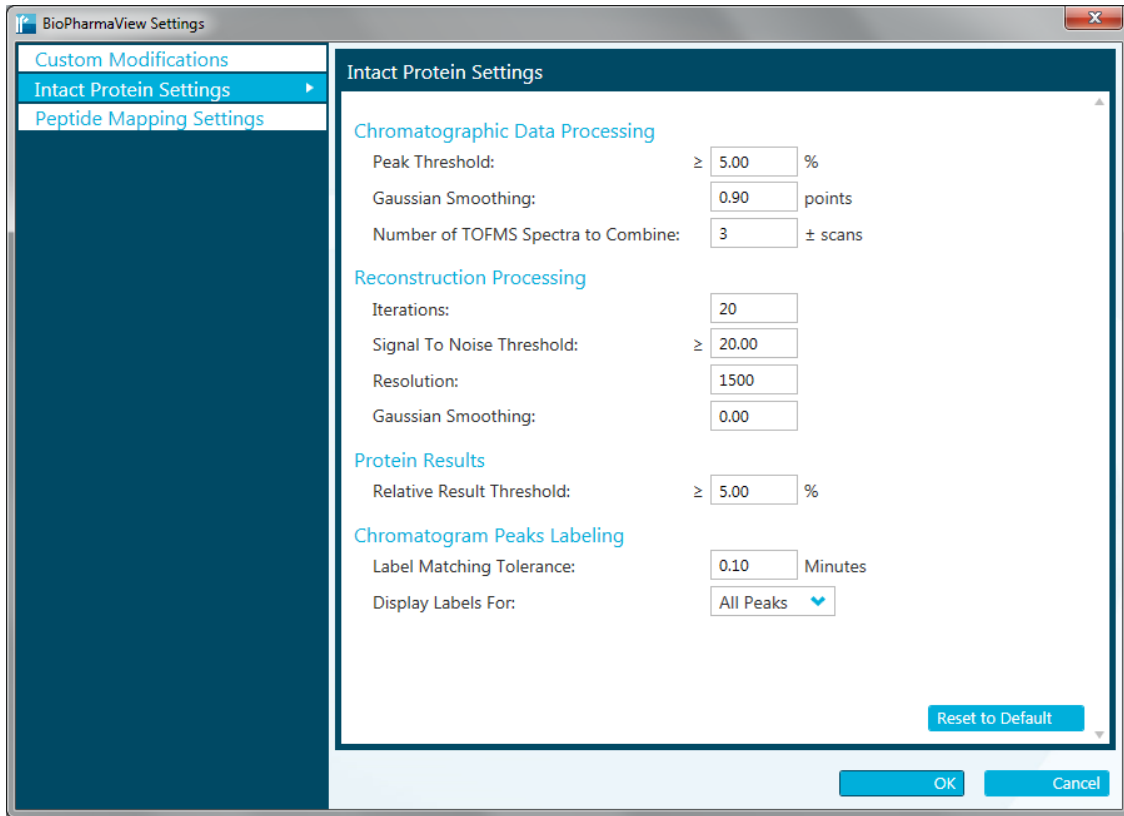
Characterized Proteins Reduced Protein Form Filter Generate Protein Forms

Batch Usage	Protein Name	Modifications	User Defined	Mono. Mass	Avg. Mass	Match...	Reconstruction Area	Retention Time
1 Optional	Rituximab	Gln->pyro-Glu - 4		144094.9573	144185.89		-	-
2 Optional	Rituximab	Gln->pyro-Glu - 3		144111.9839	144202.92		-	-
3 Optional	Rituximab	Gln->pyro-Glu - 2		144129.0104	144219.96		-	-
4 Optional	Rituximab	Gln->pyro-Glu - 1		144146.0370	144236.99		-	-
5 Optional	Rituximab			144163.0635	144254.02		-	-
6 Optional	Rituximab	G0-HexNAc - 1 Gln->pyro-Glu - 4		145190.3539	145281.90		-	-
7 Optional	Rituximab	G0-HexNAc - 1 Gln->pyro-Glu - 3		145207.3805	145298.94		-	-
8 Optional	Rituximab	G0-HexNAc - 1 Gln->pyro-Glu - 2		145224.4070	145315.97		-	-
9 Optional	Rituximab	G0-HexNAc - 1 Gln->pyro-Glu - 1		145241.4336	145333.00		-	-
10 Optional	Rituximab	G0-HexNAc - 1		145258.4601	145350.03		-	-
11 Optional	Rituximab	G0F-GlcNAc - 1 Gln->pyro-Glu - 4		145336.4118	145428.05		-	-
12 Optional	Rituximab	G0F-GlcNAc - 1 Gln->pyro-Glu - 3		145353.4384	145445.08		-	-
13 Optional	Rituximab	G0F-GlcNAc - 1 Gln->pyro-Glu - 2		145370.4649	145462.11		-	-
14 Optional	Rituximab	G0F-GlcNAc - 1 Gln->pyro-Glu - 1		145387.4915	145479.14		-	-
15 Optional	Rituximab	G0 - 1 Gln->pyro-Glu - 4		145393.4333	145485.10		-	-
16 Optional	Rituximab	G0F-GlcNAc - 1		145404.5180	145496.17		-	-
17 Optional	Rituximab	G0 - 1 Gln->pyro-Glu - 3		145410.4598	145502.13		-	-

Settings ? !
 Import Delete Reset Characterized Proteins

Pharma and Biopharma

Navigate to the 'Settings' icon and review your global 'Intact Protein Settings'



Pharma and Biopharma

Intact protein deconvolution can be performed in seconds, on either a single datafile, or on multiple samples using the batch processing function. Review your intact protein deconvolution results in the BioPharmaView Software window. Annotated reconstruction mass graph (bottom right) hyperlinks to the raw spectra (middle right) to confirm peak identity and show fidelity between raw and deconvoluted data. Detailed information on modifications, such as glycosylation, can be found in the 'Modification Summary' window (bottom left).

[Learn more at sciex.com/X500B](http://sciex.com/X500B)

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Document number: RUO-MKT-02-4521-A