

# Pharma and Biopharma

## Biotherapeutic Non-Reduced Peptide Mapping

### *Routine non-reduced peptide mapping of biotherapeutics on the X500B QTOF System*

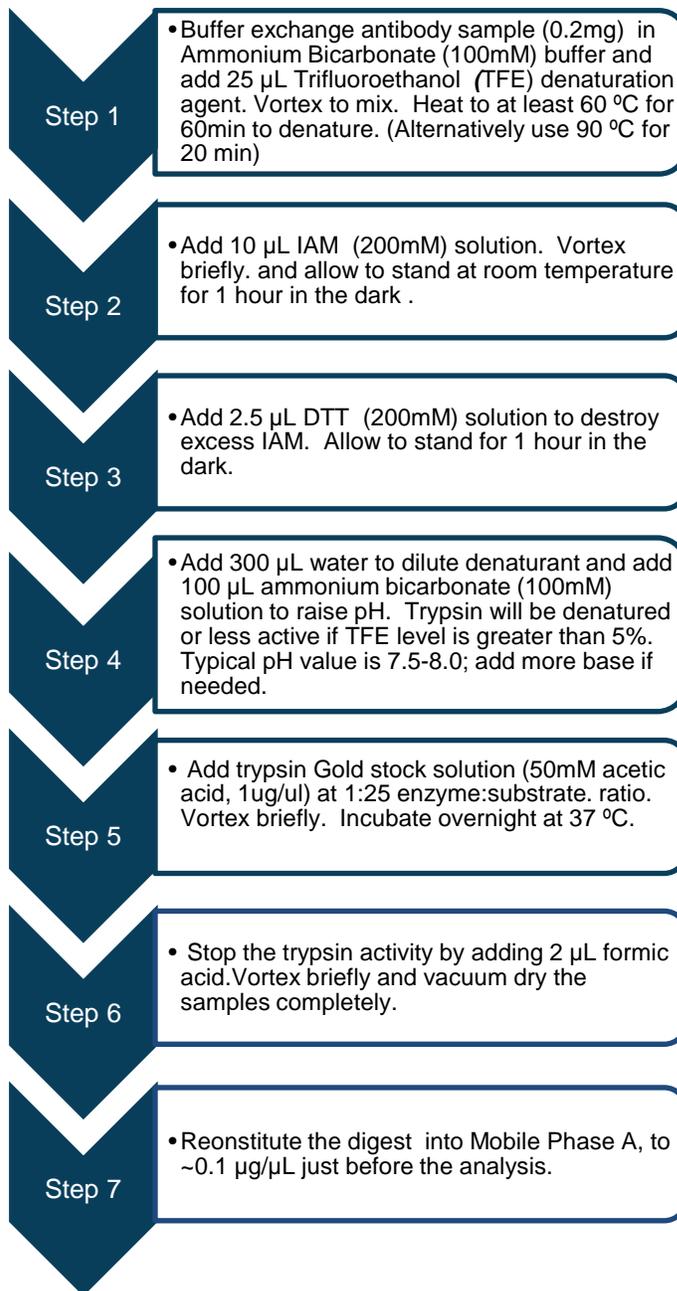
Method details for the routine non-reduced peptide mapping of a biotherapeutic monoclonal antibody (mAb) protein by high-resolution accurate mass analysis on the X500B QTOF System, powered by SCIEX OS Software. An information independent SWATH® Acquisition method was employed to acquire MS and MS/MS level data on the digested biologic protein product for the purpose of localizing disulfide bonds. Either an information dependent acquisition (IDA) method or an information independent SWATH acquisition method can be employed for non-reduced peptide mapping analyses.

SWATH Acquisition utilizes either fixed or variable Q1 mass isolation window, transmitting all precursor ions in the defined Q1 window through to the collision cell. Transmitted ions are fragmented and analyzed at high-resolution. The Q1 isolation window is stepped across the entire mass range, with an LC compatible cycle time, resulting in the comprehensive acquisition of high-resolution MS/MS spectra for every precursor ion in a sample. This unbiased data acquisition approach ensures data completeness is maximized, thus limiting the need to reanalyze a sample to obtain sufficient MS/MS spectra for disulfide bond location confirmation.



### Sample Prep

A generic sample preparation strategy is shown for the digestion of an antibody biotherapeutic prior to a non-reduced peptide mapping LC-MS analysis.



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## LC Method

*Column* Waters Acquity UPLC BEH C18 Column, 130 1.7  $\mu\text{m}$ , 2.1 mm X 100 mm

*Mobile Phase A* Water, 0.1% Formic acid

*Mobile Phase B* Acetonitrile, 0.1% Formic acid

*Flow rate* 200  $\mu\text{L}/\text{min}$

*Column temperature* 40° C

*Injection volume* 10  $\mu\text{L}$ , 1  $\mu\text{g}$  total protein

*Gradient profile*

<b>Time (min)</b>	<b>% B</b>
-------------------	------------

8.0	2
-----	---

40.0	30
------	----

60.0	50
------	----

62.0	90
------	----

66.0	90
------	----

66.5	2
------	---

75.0	2
------	---

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## MS Method

Suggested starting MS and MS/MS method parameters for routine SWATH based peptide mapping analysis as displayed in SCIEX OS user interface. The SWATH acquisition criteria are shown with a 25Da fixed SWATH window from 350-1500 m/z acquiring high-resolution MS/MS in each cycle. For best sequence coverage and sensitivity, the specific SWATH parameters should be optimized for the length of HPLC separation used.

Peptide Map\_SWATH\_75min

Method duration: 75 min | Total scan time: 2.631841 sec | Estimated cycles: 1709 | Add Experiment

Device: X500 QTOF | Ion Source: TurboSpray

SWATH (TOF MSMS Scans: 46) | 0 min - 75 min

**Source and Gas Parameters**

Ion source gas 1: 40 psi | Ion source gas 2: 40 psi | Curtain gas: 35 psi | CAD gas: 7 psi | Temperature: 450 °C

**Experiment** | SWATH

Polarity: Positive

**TOF MS**

TOF start mass: 400 Da | TOF stop mass: 1500 Da | Accumulation time: 0.125 s

**TOF MSMS**

TOF start mass: 50 Da | Accumulation time: 0.05 s

**Autofill SWATH Windows**

Generate an initial set of SWATH windows and then use the windows to autofill the MSMS mass table

Precursor start mass: 350 Da | Window width: 25 Da | Precursor stop mass: 1500 Da | Windows per cycle: 46

Populate the MSMS table

Append to existing list

Overwrite the existing list

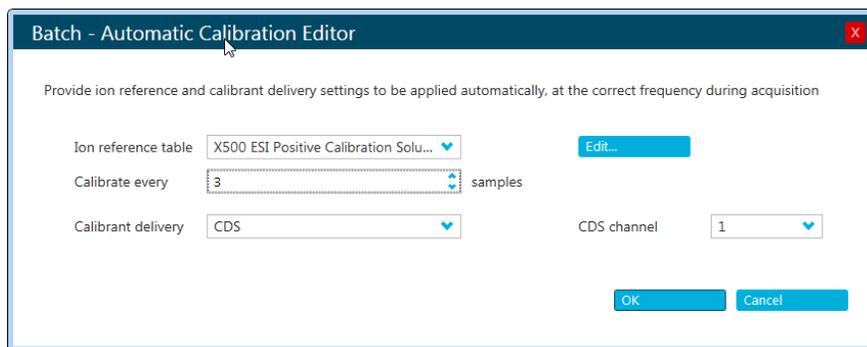
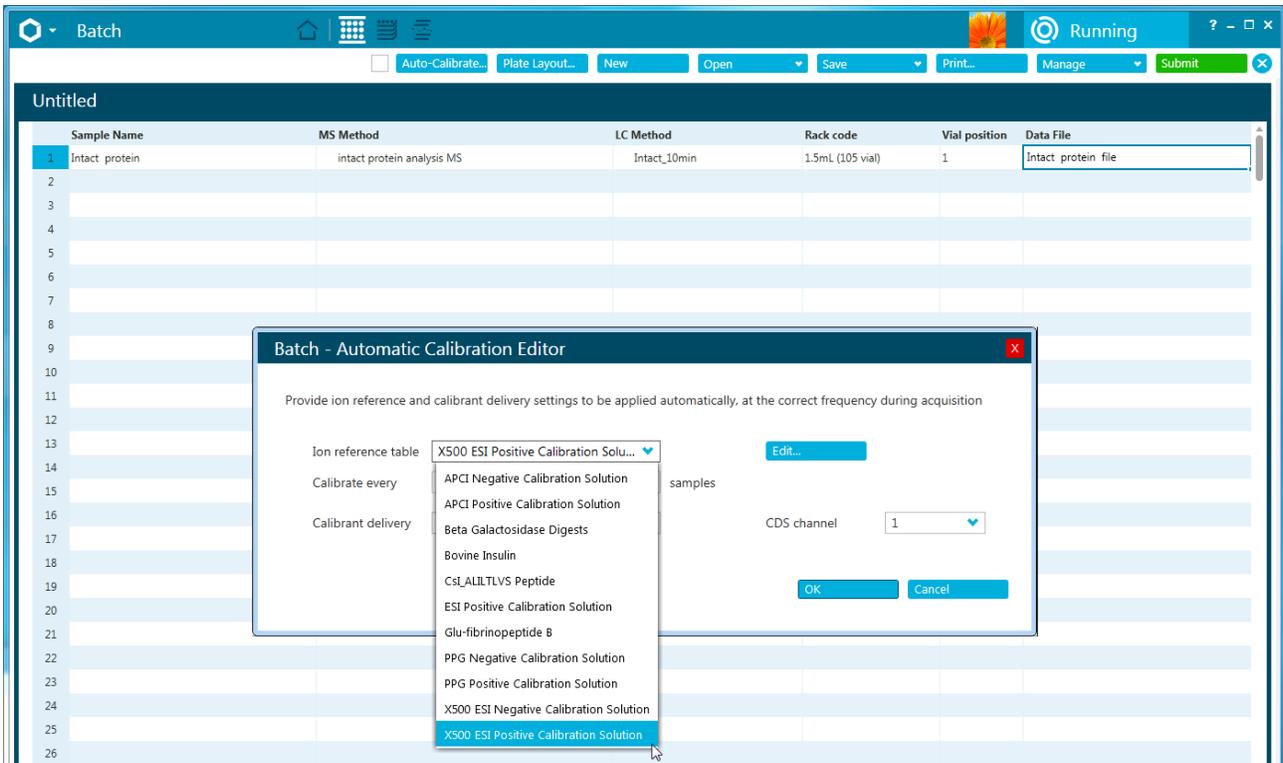
Apply | Cancel

**Mass Table** | [Autofill SWATH windows...](#)

	Precursor ion st...	Precursor ion st...	Declusteri...	DP spread (V)	Collision energy (V)	CE spread (V)
1	350.0000	375.0000	80	0	16	15
2	374.0000	400.0000	80	0	18	15
3	399.0000	425.0000	80	0	19	15
4	424.0000	450.0000	80	0	20	15
5	449.0000	475.0000	80	0	21	15
6	474.0000	500.0000	80	0	22	15
7	499.0000	525.0000	80	0	24	15
8	524.0000	550.0000	80	0	25	15
9	549.0000	575.0000	80	0	26	15

## 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.



## Data Processing

### Process SWATH® biotherapeutic peptide mapping data in BioPharmaView™ Software 2.0.

Input the protein sequence, and assign potential modifications as well as expected localization of disulfide bonds in the 'Assay Information' window.

The screenshot displays the BioPharmaView software interface for processing Rituximab data. The 'Assay Information' window is active, showing the 'Sequence Features' tab. The protein sequence is entered as 'Antibody' with an unmodified protein molecular weight of 144286.27. The sequence is divided into four chains: Chain 1 (Light Chain 1), Chain 2 (Heavy Chain 1), Chain 3 (Heavy Chain 2), and Chain 4 (Light chain 2). Each chain has its amino acid sequence and AA indexes displayed. Below the sequence, the 'Modifications' table is shown, listing various modifications such as N-terminal Gln->pyro-Glu, Deamidated, Oxidation, G1F, G2F, G0, G0F-GlcNAc, and G0F-HexNAc. The 'Disulfide Bonds' table shows 16 disulfide bonds between chains 1-4, with columns for From Chain, To Chain, From Cysteine, and To Cysteine.

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

From Chain	To Chain	From Cysteine	To Cysteine
1	1	1	23
1	1	1	87
2	1	1	133
2	1	1	193
3	1	2	213
3	1	2	224
4	2	2	22
4	2	2	96
5	2	2	148
5	2	2	204
6	2	2	265
6	2	2	325
7	2	2	371
7	2	2	429
8	4	4	23
8	4	4	87
9	4	4	133
9	4	4	193
10	4	3	213
10	4	3	224
11	3	3	22
11	3	3	96
12	3	3	148
12	3	3	204
13	3	3	265
13	3	3	325
14	3	3	371
14	3	3	429
15	2	3	230
15	2	3	233
16	2	3	233
16	2	3	233

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Navigate to the 'Peptide Mapping' tab complete processing parameters and to generate all peptide forms for matching.

## Rituximab

Create Open Save Save As Close

Project

Assay Information

Intact Protein

Characterize Standard

Create Batch

Review Results

Peptide Mapping

Characterize Standard

Create Batch

Review Results

System

View Queue

Create Report

Assay Information
Sequence Features
Intact Protein
Peptide Mapping

**Processing Parameters**

m/z Tolerance, ppm: ±5.0 ppm      RT Range Processing: Time Selection

Minimum Score for Auto-Validation: 3.0      Start RT: 0.00 min

MS/MS Matching Tolerance: 0.03 Da      Stop RT: 58.36 min

**Batch Processing Parameters**

Retention Time Tolerance: ± 0.50 min

**Batch Processing Pass / Fail Criteria**

XIC Area Limits: ± 10.0 %

Minimum Sequence Coverage: ≥ 85.0 %

Required Form Minimum: ≥ 80 %

Restricted Form Maximum: ≤ 120 %

**Annotated Protein Sequence**

Chain 1 - Light Chain1

QIVLSQSPAILSASPGKVTMTCRASSVSYIHWFPQKPGSSPKPFIYATSNLASGVPVRFSGSGSGTSYSLTISRVEAEDAATYYCQQWTSNPPTFGGGTKLEIKRRTVAAPSVFIFPPSDEQLKSGTASVIVCLLNNFYPREAKVQWIKVDNALQSGNSQESVTEQDSKDSYLSSTLTLSKADYEKHKVYACEVTHOGLSSPVTKSFNRGEC

Chain 2 - Heavy Chain 1

QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWYFNWVAGTTVTVAASSTKGPSVFLPASPSSKSTSGGTAALGCLVKDYFPEPTVSWNSGALTSVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHPKSTKVDKKAEPKSCDKHTHTCPCPAPELGGPSVFLFPPKPKDTLMISSRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTSKARGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFPYSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSSVMHEALHNHYTQKSLSLSPG

Chain 3 - Heavy Chain 2

QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWYFNWVAGTTVTVAASSTKGPSVFLPASPSSKSTSGGTAALGCLVKDYFPEPTVSWNSGALTSVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHPKSTKVDKKAEPKSCDKHTHTCPCPAPELGGPSVFLFPPKPKDTLMISSRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTSKARGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFPYSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSSVMHEALHNHYTQKSLSLSPG

Chain 4 - Light chain 2

QIVLSQSPAILSASPGKVTMTCRASSVSYIHWFPQKPGSSPKPFIYATSNLASGVPVRFSGSGSGTSYSLTISRVEAEDAATYYCQQWTSNPPTFGGGTKLEIKRRTVAAPSVFIFPPSDEQLKSGTASVIVCLLNNFYPREAKVQWIKVDNALQSGNSQESVTEQDSKDSYLSSTLTLSKADYEKHKVYACEVTHOGLSSPVTKSFNRGEC

**Peptide Mapping**

Cysteine Alkylation: None      Maximum Number of Combined Modifications per Peptide: 4

Digest Agent: Trypsin      Maximum Missed Cleavages: 4

Reduced Protein Form      Sequence coverage of 0 Matched peptides = 0.0 %

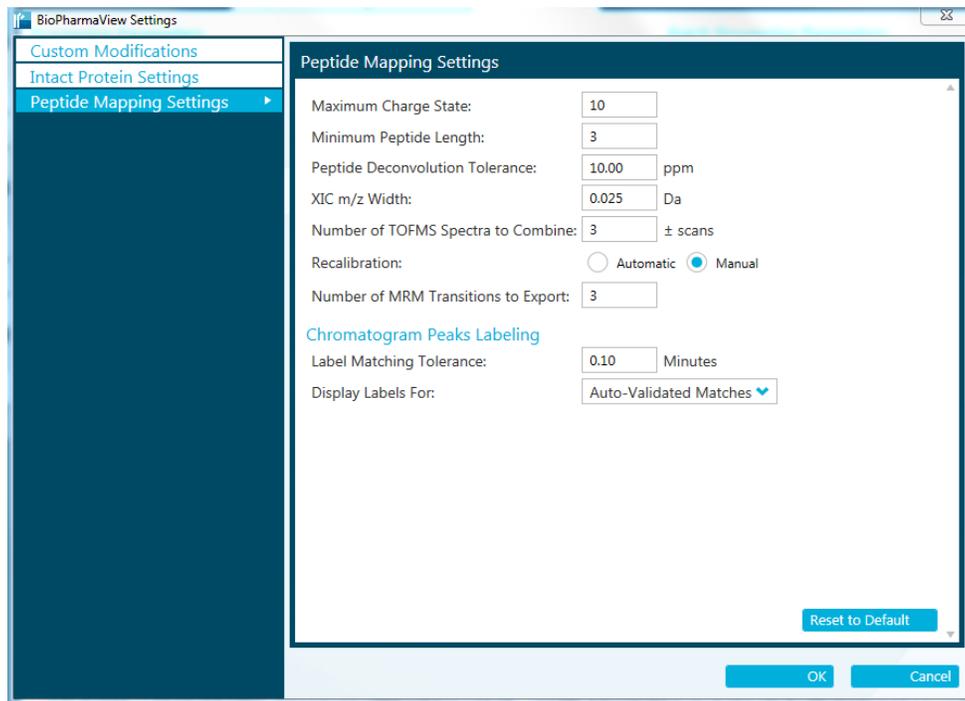
Filter Digest

#	Chains	Peptide	AA Index	Sequence	Modifications	Disulfide Bonds	Mono. Mass	Mono. m/z	Charge	XIC Area	Retention Time
1	1,4	T1	1-18	QIVLSQSPAILSASPGK			1823.9993	-	-	-	-
2	1,4	T14-15	183-189	ADYEKHK	Oxidation@*		905.4243	-	-	-	-
3	1,4	T14-15	183-189	ADYEKHK	Oxidation@*, Oxidator		921.4192	-	-	-	-
4	1,4	T15	188-189	HK			283.1644	-	-	-	-
5	1,4	T15	188-189	HK	Oxidation@*		299.1594	-	-	-	-
6	1,4	T15	188-189	HK	Oxidation@1(188), Oxidator		315.1543	-	-	-	-
7	1,4	T8	108-125	TVAAPSVFIFPPSDEQLK	Deamidated@16(123), Oxidator		1977.9935	-	-	-	-
8	1,4	T8	108-125	TVAAPSVFIFPPSDEQLK	Oxidation@*, Oxidator		1977.0095	-	-	-	-
9	1,4	T8	108-125	TVAAPSVFIFPPSDEQLK	Deamidated@16(123), Oxidator		1961.9986	-	-	-	-
10	1,4	T8	108-125	TVAAPSVFIFPPSDEQLK	Oxidation@*		1961.0146	-	-	-	-
11	1,4	T14-15	183-189	ADYEKHK			889.4294	-	-	-	-
12	1,4	T8	108-125	TVAAPSVFIFPPSDEQLK	Deamidated@16(123), Oxidator		1946.0037	-	-	-	-
13	1,4	T7-8	107-125	RTVAAPSVFIFPPSDEQLK	Deamidated@1(107), D		2135.0787	-	-	-	-
14	1,4	T7-8	107-125	RTVAAPSVFIFPPSDEQLK	Deamidated@*, Oxidator		2134.0946	-	-	-	-
15	1,4	T7-8	107-125	RTVAAPSVFIFPPSDEQLK	Deamidated@1(107), D		2119.0837	-	-	-	-
16	1,4	T7-8	107-125	RTVAAPSVFIFPPSDEQLK	Oxidation@*, Oxidator		2133.1106	-	-	-	-
17	1,4	T7-8	107-125	RTVAAPSVFIFPPSDEQLK	Deamidated@*, Oxidator		2118.0997	-	-	-	-
18	1,4	T7-8	107-125	RTVAAPSVFIFPPSDEQLK	Deamidated@1(107), D		2103.0888	-	-	-	-
19	1,4	T7-8	107-125	RTVAAPSVFIFPPSDEQLK	Oxidation@*		2117.1157	-	-	-	-
20	1,4	T7-8	107-125	RTVAAPSVFIFPPSDEQLK	Deamidated@*		2102.1048	-	-	-	-
21	1,4	T7-8	107-125	RTVAAPSVFIFPPSDEQLK			2101.1208	-	-	-	-
22	1,4	T8	108-125	TVAAPSVFIFPPSDEQLK			1945.0197	-	-	-	-
23	1,4	T14	183-187	ADYEK	Oxidation@*, Oxidator		656.2653	-	-	-	-
24	1,4	T14	183-187	ADYEK	Oxidation@*		640.2704	-	-	-	-
25	1,4	T14	183-187	ADYEK			624.2755	-	-	-	-

Settings
?
!

# Pharma and Biopharma

Navigate to the 'Settings' icon and review your global 'Peptide Mapping Settings'



Data extraction, including peptide matching can be performed in minutes, on either a single datafile, or on multiple samples using the batch processing function. Review your peptide mapping results in the BioPharmaView Software window. Full sequence coverage of matched peptides can be viewed by clicking 'View Sequence'. Peptide matches can be reviewed in the 'Peptide Results' window. For each selected peptide, corresponding TOF-MS raw spectrum (lower left) and high-resolution, annotated MS/MS spectrum (lower right) are shown for easy confirmation. Disulfide bond locations are automatically identified and high-resolution MS/MS spectra for disulfide bond containing peptides are annotated to allow for fast review and bond confirmation.

**Project:** Rituximab

**Assay Information:** Open File... 20160713-Ritu\_NR\_SWATH02.wiff2

**Processing Parameters:** m/z Tolerance, ppm: 5.0; Minimum Score for Auto-Validation: 3.0; MS/MS Matching Tolerance: 0.03 Da

**RT Range Processing:** Automatic (selected)

**BPC/TIC/XIC Graph:** BPC from 20160713-Ritu\_NR\_SWATH02.wiff2 (sample 1) - 20160713-Ritu\_NR\_SWATH, Experiment 1, +SWATH TOF MS (400 - 1500)

RT	Sequence	Disulfide Bonds	Theoretical Mono m/z	Observed Mono m/z	Error (PPM)	Score	Charge	XIC Area	Peptide	Chains	User Defn
30.80	VTMTCR VEAEDAATYYCQWTSNPPTFGGGTK	(1,4)T2@5(23)=(1,4) T5@11(87)	1176.8550	1176.8568	1.5	10.977	3	1.1517e5	T2 T5	1,4 1,4	
31	30.80 VTMTCR VEAEDAATYYCQWTSNPPTFGGGTK	(1,4)T2@5(23)=(1,4) T5@11(87)	882.8931	882.8927	-0.4	9.160	4	7.0457e5	T2 T5	1,4 1,4	

**TOF MS Graph:** +SWATH TOF MS (400 - 1500) from 20160713-Ritu\_...\_80. Mono m/z: 882.8927 from 30.65 to 30.90 min

**MS/MS Graph:** +MS/MS (50 - 1500) from 20160713-Ritu\_NR\_SWATH...H. Experiment 21 @ 30.80 min. (874 - 900 Da.)

Sequence Coverage 99.2 %

All Matched Peptides  Auto-Validated  Used for IDs  Selected Peptides

**Chain 1 - Light Chain1 Sequence Coverage 98.6 %**

```
QIVLSQSPAILLSASPGKRVMTTCRASSSVSYIHWFQQRPGSSPKPWIYATSNLASGVPVR
FSGSGSGTSYSLTISRVEAEDAATYYCQWTSNPPTFGGGTKLEIKRRTVAAPSVFIFPPS
DEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKIDSTYLSLSSTLTL
SKADYEHKHKVYACEVTHOGLSSPVTKSFNRGEC
```

**Chain 2 - Heavy Chain 1 Sequence Coverage 98.4 %**

```
QVQLQPGAEELVKGASVSRMSCKASGYTFTSYNHWVKQTPGRGLEWIGAIYPNGDTSY
NQRKFGKATLTADKSSSTAYMQLSLSLTSSEDSAVYYCARSTYGGDWYFNVWAGAGTITVTS
AASRTRGSPVFLPAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSVHTEFPAVLOS
SGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRAEPKSCDKRHTHTCPPAPPELLG
GPEVLEFPPKPKDTLMISRTPEVTCVVDVSHEDDEVEKFNWYDQVEVHNATKRFREEQY
NSTYRVSVLTFLVHODWLNQKEYRCKVSNKALPAPIERTISKAQGRPEPQVYTLPPSRD
ELRKNQVSLTCLVKGFPYSDIAVEWESNGQPENNYKTTTPVPLDSDGSEFFLYSKLTVDKSR
WQOQNVFSCVMHEALHNNHYTKLSLSLSPG
```

**Chain 3 - Heavy Chain 2 Sequence Coverage 100.0 %**

```
QVQLQPGAEELVKGASVSRMSCKASGYTFTSYNHWVKQTPGRGLEWIGAIYPNGDTSY
NQRKFGKATLTADKSSSTAYMQLSLSLTSSEDSAVYYCARSTYGGDWYFNVWAGAGTITVTS
AASRTRGSPVFLPAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSVHTEFPAVLOS
SGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRAEPKSCDKRHTHTCPPAPPELLG
GPEVLEFPPKPKDTLMISRTPEVTCVVDVSHEDDEVEKFNWYDQVEVHNATKRFREEQY
NSTYRVSVLTFLVHODWLNQKEYRCKVSNKALPAPIERTISKAQGRPEPQVYTLPPSRD
ELRKNQVSLTCLVKGFPYSDIAVEWESNGQPENNYKTTTPVPLDSDGSEFFLYSKLTVDKSR
WQOQNVFSCVMHEALHNNHYTKLSLSLSPG
```

**Chain 4 - Light chain 2 Sequence Coverage 100.0 %**

```
QIVLSQSPAILLSASPGKRVMTTCRASSSVSYIHWFQQRPGSSPKPWIYATSNLASGVPVR
FSGSGSGTSYSLTISRVEAEDAATYYCQWTSNPPTFGGGTKLEIKRRTVAAPSVFIFPPS
DEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKIDSTYLSLSSTLTL
SKADYEHKHKVYACEVTHOGLSSPVTKSFNRGEC
```

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