Streamlined therapeutic peptide map analysis with BPV Flex Software

Featuring BPV Flex Software 2.1

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Characterization of biotherapeutics requires the use of many complimentary and orthogonal techniques to identify, characterize and monitor quality attributes. Mass spectrometry plays a pivotal role for characterization due to its ability to identify and quantify a wide range of attributes across product development. In particular, peptide mapping is one of the most common assays due to its ability to characterize, localize, and quantify post translational modifications and quality attributes. While peptide mapping is a common assay, interrogation of mass spectrometry data and comparative studies between samples is challenging due to the complexity of the data and need to correlate results with other assays.

Recently, the use of peptide map based studies has expanded their scope and utility. In particular, the introduction of the multiple attribute method and the increased complexity of biotherapeutics now requires the need to enable access to mass spectrometric capabilities to a greater number of users. As access to a wider number of users increases, there is a need to implement solutions which are intuitive for both data acquisition and analysis to ensure high quality data and results.

Presented in this technical note is the use of the X500B QTOF System and BPV Flex Software for peptide mapping analysis of protein therapeutics. The solution enables access to a wide range of users while providing high quality data for actionable results.

Key feature of BPV Flex Software

- Customizable interface for curation of results and comparison of samples
- Interactive interface for rapid review of MS and MS/MS data
- Powerful sorting, grouping, and filtering capabilities to focus on the scientific question of interest
- Easily compare results processed at different times and with different parameters

Figure 1. Review of MS and MS/MS deamidated NQVVLK peptide of NISTmAb in BPV Flex Software using customizable review interface. MS graph provides information on charge state and overlay with theoretical isotope ratio for increased confidence in the assignment. MS/MS pane shows fragment ions (b and y ions) and precursor (M) in addition to an overview of the MS/MS sequence coverage information.

Methods

Sample preparation: NISTmAb standard (#RM8671) was purchased from NIST. An aliquot of 10 µl was taken and subjected to denaturation and reduction with 10 mM DTT at room temperature for 30 minutes. The sample was then alkylated with iodoacetamide at 20 mM for 20 minutes in the dark at room temperature. After desalting, it was then digested with trypsin (Roche, sequence grade) for 30 min at 37 °C followed by quenching with TFA.

Chromatography: Separation was accomplished using an ExionLC™ System fitted with a 2.1×150 mm Agilent ZORBAX 300 SB-C18 , 1.8 µm column at 50°C with the gradient shown in Table 1. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile.

Mass spectrometry: Experiments were executed on a SCIEX X500B system using information dependent acquisition (IDA). Instrument conditions are listed in Table 2.

Data processing: Data were processed using BPV Flex Software 2.1.

Table 2. MS parameters.

Parameter	Setting
Scan Mode	*Positive*
GS1 and GS2	*50 psi*
Curtain Gas	*45 psi*
Temperature	*400°C*
Ion Spray Voltage	*5200 V*
Time Bins to Sum	*6*
Accumulation Time (TOF-MS)	*0.25 sec*
TOF mass range	*300-1800 m/z*
Accumulation Time (TOF MSMS)	*0.04 sec*
Declustering Potential	*80.0 V*
Max Number of Candidates	*10*
Dynamic CE (Charge States)	*1-5*
Collision Energy Range	*5-80 V*

Table 1. LC gradient.

Time (min)	Flow Rate (ml/min)	%A	%B
Initial	*0.3*	*99*	*1*
5.0	*0.3*	*99*	*1*
6.0	*0.3*	*90*	*10*
50.0	*0.3*	*65*	*35*
55.0	*0.3*	*40*	*60*
56.0	*0.3*	*10*	*90*
60.0	*0.3*	*10*	*90*
62.0	*0.3*	*99*	*1*
64.0	*0.3*	*99*	*1*
66.0	*0.3*	*10*	*90*
70.0	*0.3*	*10*	*90*
72.0	*0.3*	*99*	*1*
74.0	*0.3*	*99*	*1*
76.0	*0.3*	*10*	*90*
80.0	*0.3*	*10*	*90*
82.0	*0.3*	*99*	*1*
95.0	*0.3*	*99*	*1*

Verification and adjustment of peptide matching criteria

Following processing of peptide mapping data, it is important to verify the matching criteria for both MS and MS/MS data to ensure accurate assignment for MS level data and auto-validation of MS/MS spectra. BPV Flex Software has an interactive peptide analytics tool that allow users to refine matching criteria based on the minimum score required for identification of peptides as well as the matching tolerance in parts per million (ppm). As shown in Figure 2, each criteria may be adjusted independently. To enable visualization of the adjustment in acceptance criteria, there are two plots which adjust in an interactive manner without the need to reprocess data. The left hand plot displays two sets of circles which represent identified components (Figure 2). The circles in green fall within the mass tolerance criteria while those which are blue fall outside; the filled circles represent identified peptides with MS/MS evidence (Figure 2). As the values for acceptance are adjusted the number of matched components changes and is reflected by a change in the plot. Similarly, the right hand plot shows the false discovery rate vs the MS/MS score (Figure 2).

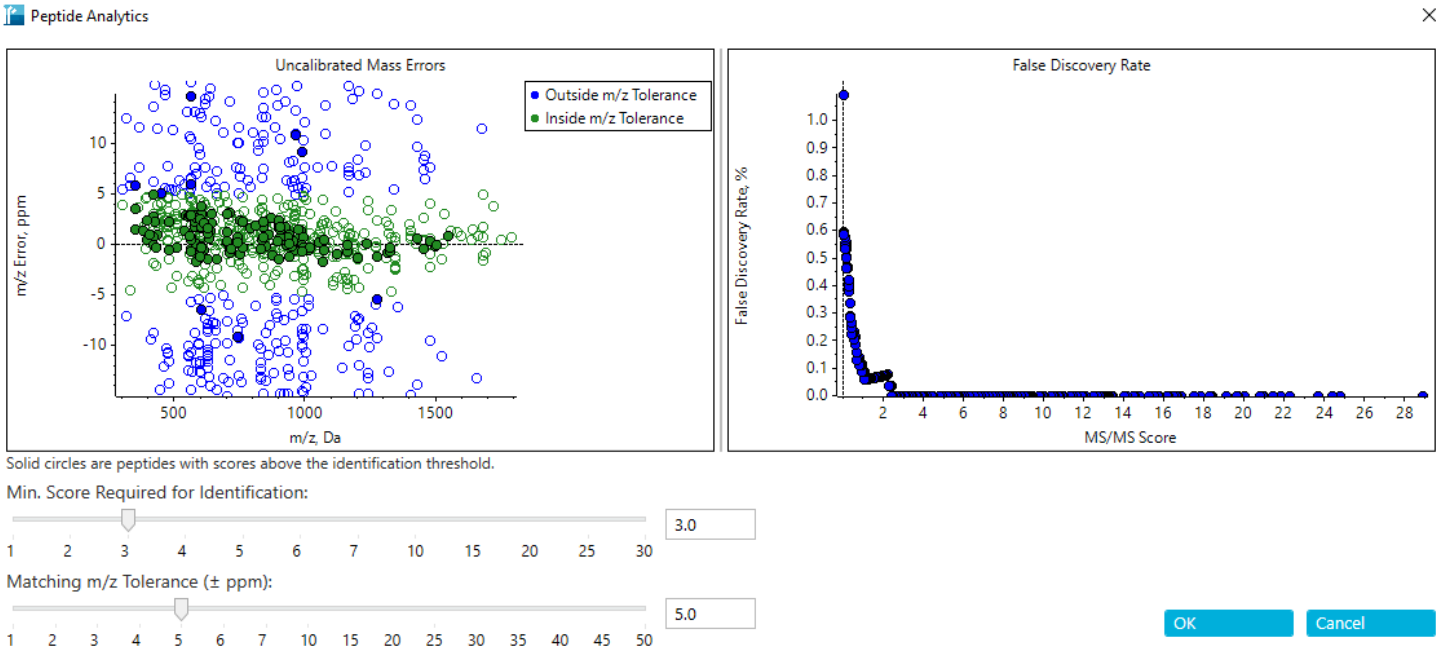


Figure 2. Interactive peptide analytics to refine matching criteria for peptide mapping experiments. MS/MS scores required for automatic validation can be adjusted as well as matching tolerance in ppm. Both graphs adjust in an interactive manner.

Users may adjust the values as desired to refine the matched results to their requirements

Peptide map sequence viewer

When reviewing peptide map experiments, it is important to verify overall peptide mapping sequence coverage. BPV Flex Software offers a highly interactive coverage map which provides a rapid view of overall sequence coverage as well as the peptides which result in that coverage. Each identified peptide is displayed under the defined protein sequence. If peptides are identified in multiple charge states or in modified forms, each are captured individually in a graphical manner (Figure 3 top, left). Users may select to view all matched peptides, selected peptides, auto-validated peptides, or those that have been designated for use in the downstream calculations. Shown in Figure 3 (top, left) is the sequence viewer with all auto-validated peptides displayed. These peptides meet the criteria defined for mass tolerance and minimum MS/MS score. For each matched peptide, if a modification is present and has been assigned to a specific residue, the portion of the sequence corresponding to this modification is highlighted in magenta. Hovering over any peptide within the sequence view provides rapid access to the MS/MS b and y-ion coverage and the MS/MS coverage in percent for the individual peptide (Figure 3, top left).

Peptide results curation

In addition to peptide results being presented in a sequence view, results are also tabulated within the peptide results table (Figure 3, bottom left). The table contains the preliminary assignments of peptides identified following processing. The data in the table is easily sorted by any header in ascending or descending order simply by clicking on the heading. Data may also be readily filtered by typing filter criteria directly into the top row of the results table. As shown in Figure 3, the results from this study have been filtered to display peptides that contain the sequence DTLMISR and have been automatically validated due to fulfilling MS and MS/MS acceptance criteria. As shown, missed cleavage products, oxidized peptides and the fully tryptic unmodified peptide are presented based on the filter criteria being used. In addition, the peptide sequence coverage based on identified fragments in percent is accessible in the table enabling quick evaluation of the MS/MS spectra quality without the need to go through each entry individually. Selection of any of the peptides from the table returns the chromatographic and underlying MS and MS/MS data associated with peptide selected in the table (Figure 3, right). The chromatographic data displayed may be the total ion chromatogram (TIC), base peak chromatogram (BPC), the extracted ion chromatogram (XIC) of the precursor or UV trace, or any combination of these. As shown, the MS data for the selected peptide is shown. The user has the option to overlay the theoretical isotope pattern for the same peptide with that observed in the raw data for greater confidence in the peptide identification. In addition, the MS/MS

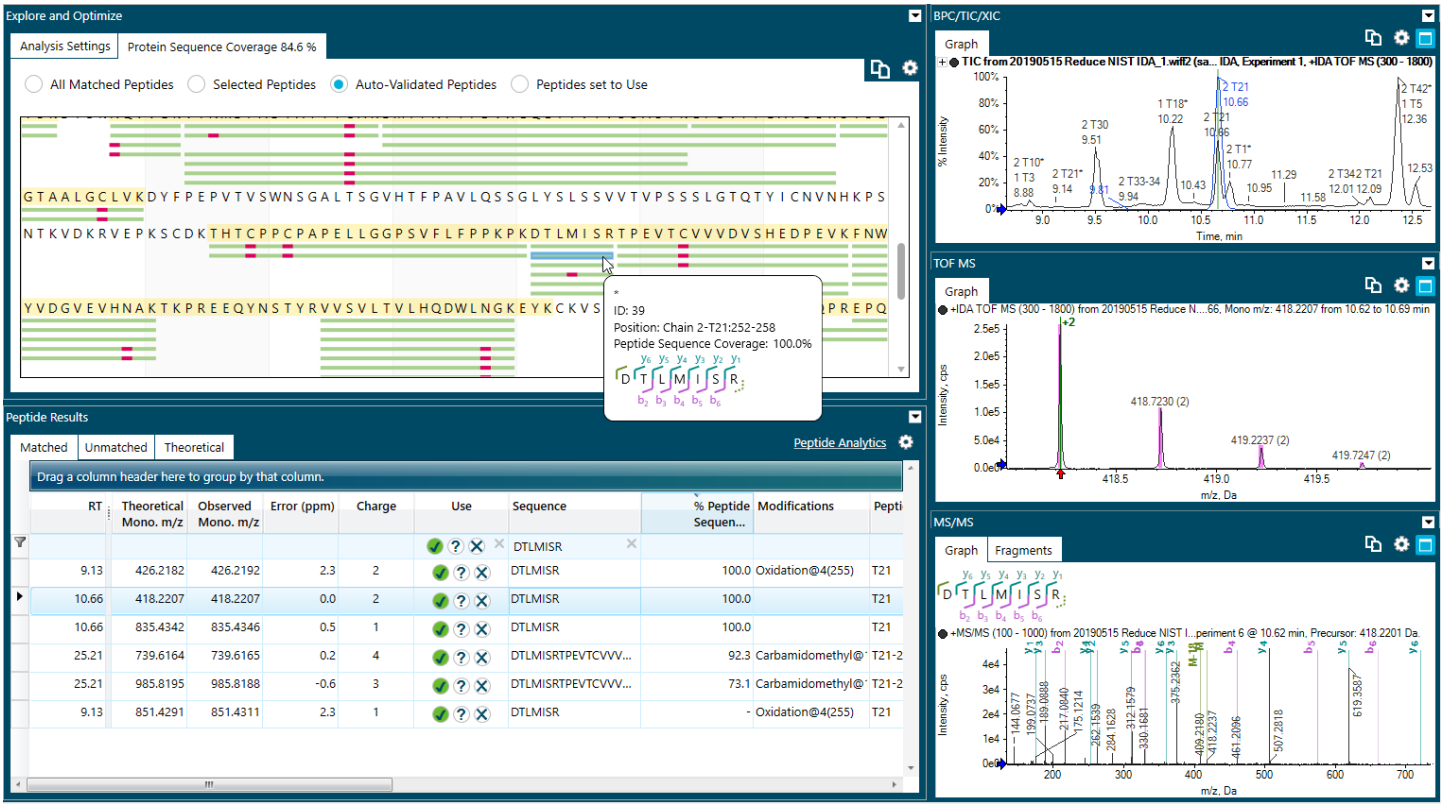


Figure 3. Overview of results in BPV Flex Software. Top left: Interactive Sequence Viewer. Observed peptides (green bars) and modifications (magenta bars) are shown**.** Hovering over any identified peptide provides rapid access to additional information on the position of the peptide and identified fragment ions including the MS/MS sequence coverage in percent. Bottom left: results are tabulated and can be filtered easily. All entries are directly linked to chromatographic, MS and MS/MS information (right hand side).

data is annotated with the identified fragment ions highlighted and an overview of the observed fragments is shown within the MS/MS pane.

There are many options available for each of the review windows which are accessible by clicking the gear icon for that window. Shown in Figure 4 are the options for the MS/MS review window. As shown, there is significant flexibility for what content the user would like to see so that there is flexibility to focus on the aspects of characterization which are important for the current study. If during curation of results any of the identification are found to be incorrect their status is easily changed to uncertain or omit to flag them for review later if required. There is also a column for user comments if there is relevant information about any components that needs to be captured.

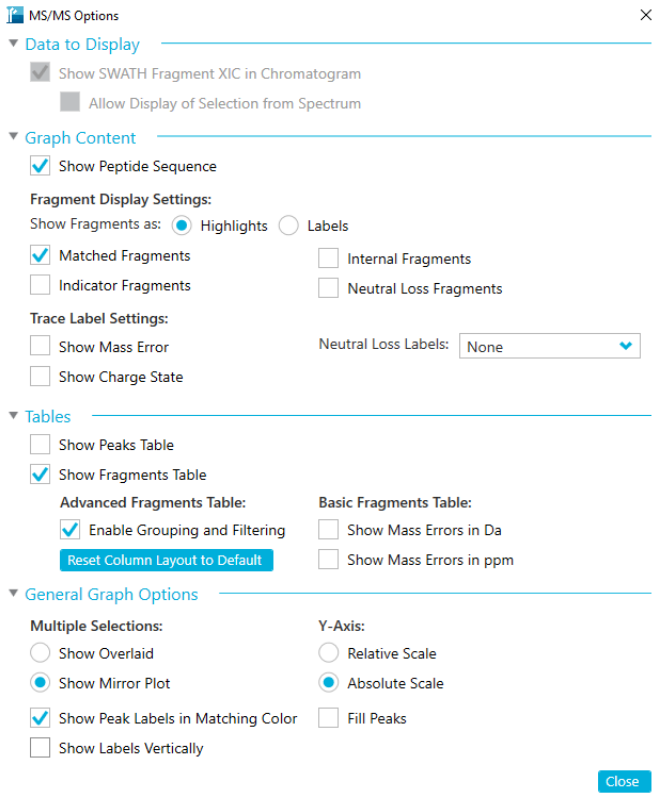


Figure 4. Options menu to allow customization during data review. Shown here is MS/MS options menu.

Focused review of peptide map data

Frequently, there are peptides of specific interest in a study, therefore it is helpful to group data based on specific peptides and/or filter data meeting a set of criteria. As shown in Figure 6, the tabulated data from the results from BPV Flex Software have been grouped based on sequence and modifications and then filtered to show only the peptides set to use. In this study the data were filtered using the peptide associated with glycosylation. This results in aggregation of each glycoforms separately for expedited review of data.

When results from multiple studies need to be compared, BPV Flex Software enables results processed at different times and/or using different processing methods (eg. different enzymes used for increasing the sequence coverage) to be compared simultaneously without the need to reprocess data. This allows for rapid assessment of sample preparation or acquisition parameters or data processing parameters during the development of biomolecules or protocols. As an example, Figure 5 illustrates the function within BPV Flex Software to compare the peptides observed from different results. The peptides from each result are color coded so they are easily distinguished from each other (green and blue bars). When highlighted within the sequence viewer the underlying MS and MS/MS data for the selected peptide are presented for review in the other panels. To expedite review, each panel in the review window may be undocked and reorganized by resizing or

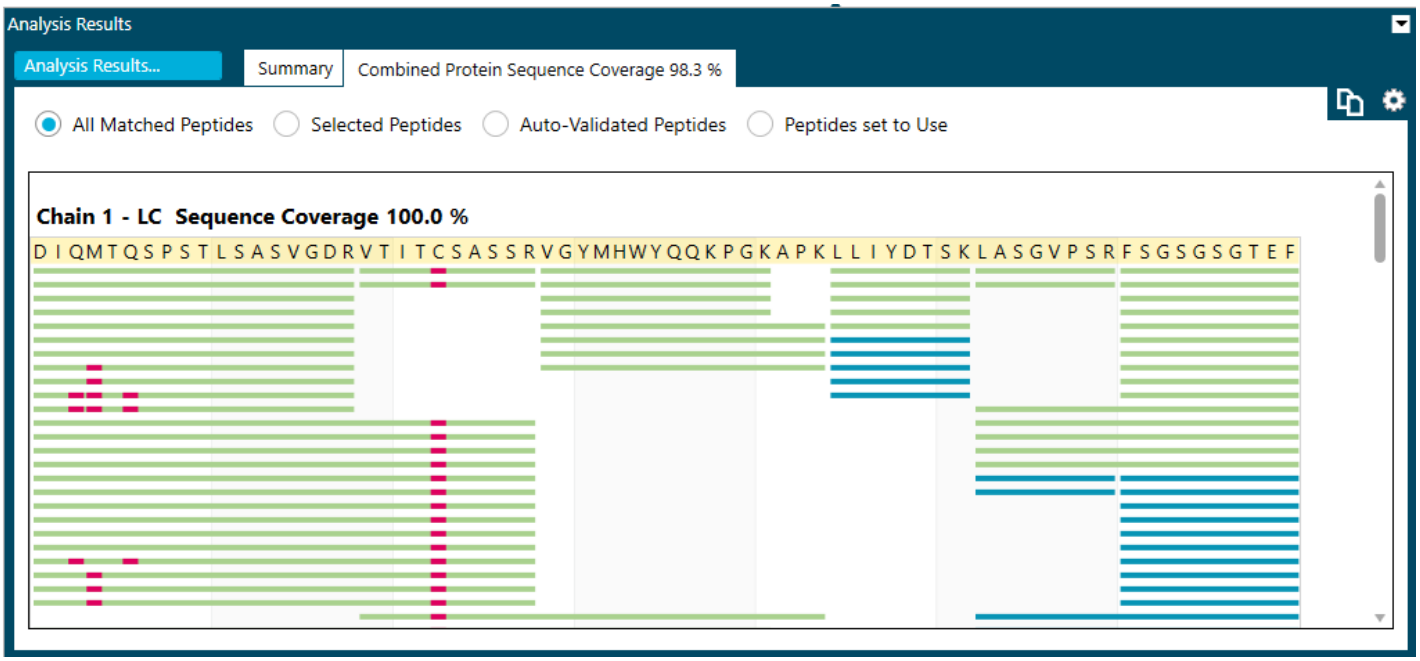
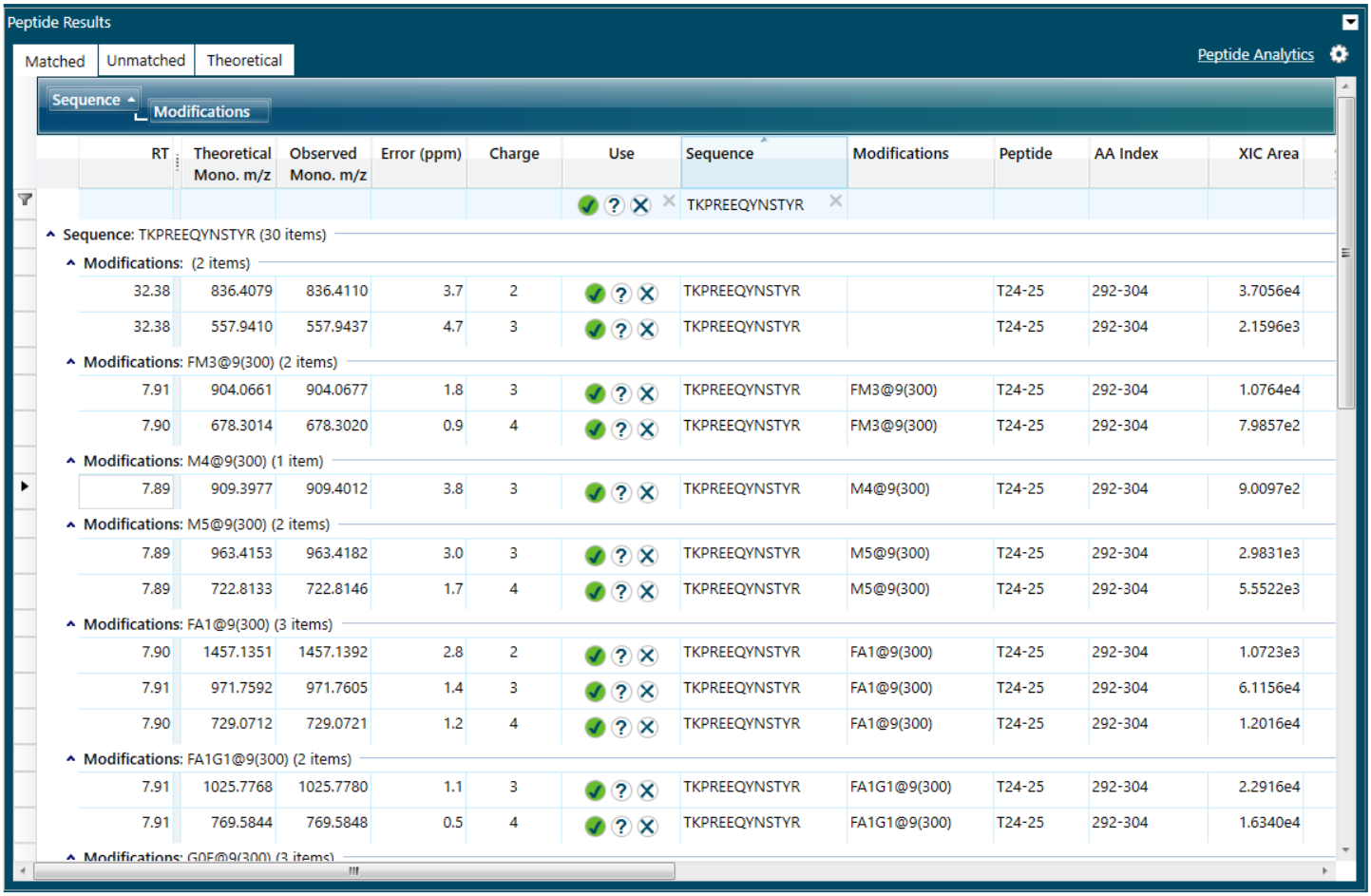


Figure 5. Interactive sequence viewer with color-coded peptide coverage for each sample. Each identified peptide is represented by a bar. Green and blue bars represent different samples. Each modification is shown as a magenta highlight. Combined sequence coverage for both samples is shown at the top.



RT	Theoretical Mono. m/z	Observed Mono. m/z	Error (ppm)	Charge	Use	Sequence	Modifications	Peptide	AA Index	XIC Area
						TKPREEQYNSTYR				
Sequence: TKPREEQYNSTYR (30 items)										
Modifications: (2 items)										
32.38	836.4079	836.4110	3.7	2		TKPREEQYNSTYR		T24-25	292-304	3.7056e4
32.38	557.9410	557.9437	4.7	3		TKPREEQYNSTYR		T24-25	292-304	2.1596e3
Modifications: FM3@9(300) (2 items)										
7.91	904.0661	904.0677	1.8	3		TKPREEQYNSTYR	FM3@9(300)	T24-25	292-304	1.0764e4
7.90	678.3014	678.3020	0.9	4		TKPREEQYNSTYR	FM3@9(300)	T24-25	292-304	7.9857e2
Modifications: M4@9(300) (1 item)										
7.89	909.3977	909.4012	3.8	3		TKPREEQYNSTYR	M4@9(300)	T24-25	292-304	9.0097e2
Modifications: M5@9(300) (2 items)										
7.89	963.4153	963.4182	3.0	3		TKPREEQYNSTYR	M5@9(300)	T24-25	292-304	2.9831e3
7.89	722.8133	722.8146	1.7	4		TKPREEQYNSTYR	M5@9(300)	T24-25	292-304	5.5522e3
Modifications: FA1@9(300) (3 items)										
7.90	1457.1351	1457.1392	2.8	2		TKPREEQYNSTYR	FA1@9(300)	T24-25	292-304	1.0723e3
7.91	971.7592	971.7605	1.4	3		TKPREEQYNSTYR	FA1@9(300)	T24-25	292-304	6.1156e4
7.90	729.0712	729.0721	1.2	4		TKPREEQYNSTYR	FA1@9(300)	T24-25	292-304	1.2016e4
Modifications: FA1G1@9(300) (2 items)										
7.91	1025.7768	1025.7780	1.1	3		TKPREEQYNSTYR	FA1G1@9(300)	T24-25	292-304	2.2916e4
7.91	769.5844	769.5848	0.5	4		TKPREEQYNSTYR	FA1G1@9(300)	T24-25	292-304	1.6340e4
Modifications: G0F@9(300) (3 items)										

Figure 6. Filtered and grouped peptide results focusing on targeted glycopeptide TKPREEQYNSTYR and different glycoforms present.

undocked and dragging and dropping into any other panel or additional monitors as desired.

Conclusions

- BPV Flex Software has a customizable interface for curation of results and comparison of samples
- The interactive interface allows rapid review of MS and MS/MS data for accurate results
- Powerful sorting, grouping, and filtering capabilities allow users to focus on their scientific questions of interest
- Results can be compared easily even if processed at different times and with different parameters

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