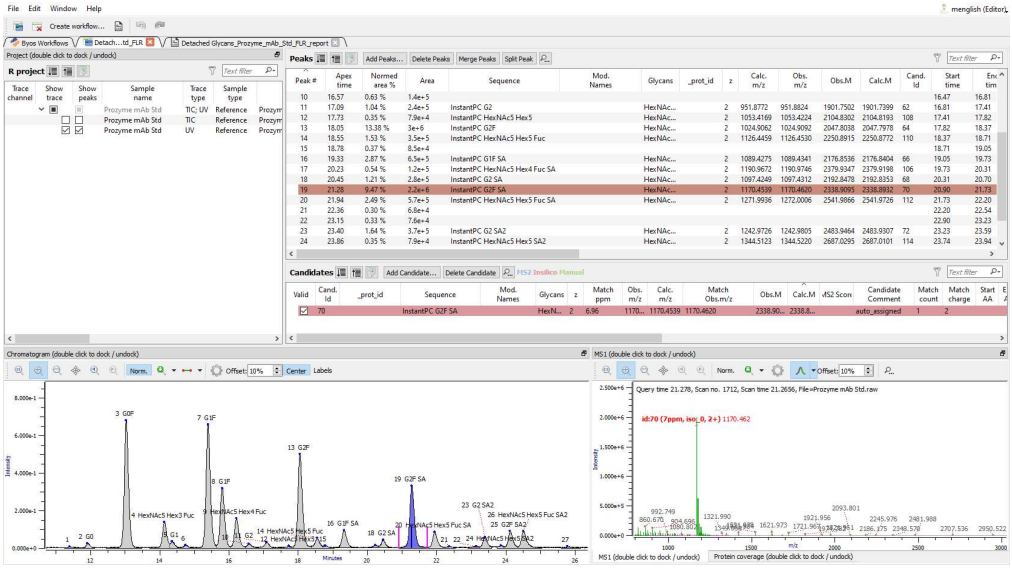
Detached Glycan Analysis in Byos®

Protein Metrics – Customer Success Team, August 2019

Introduction

The analysis of glycosylated proteins used for biotherapeutics involves multiple techniques to complete the characterization. At the intact, subunit, and peptide levels the overall profile, site occupancy, and some degree of structural information is obtainable. However, detailed analysis of the glycans can add significant information and is used as a technique for data in regulatory filings, development, and quality control. The detached glycan assay can provide critical information about linkages, antennary structure, and isomers to complement other experiments.

In the example below, we show detached glycans from a glycan standard on a HILIC-FL-MS system with a column, an LC, and a mass spectrometer, all from different vendors. Mixed systems such as this are often the norm in modern laboratories. The practicalities of performing this analysis might therefore mean that the mass spectrometry data is acquired with one manufacturer's software, and the fluorescence trace with another. In Byos Software, they can all be brought together in one platform allowing faster review and analysis.

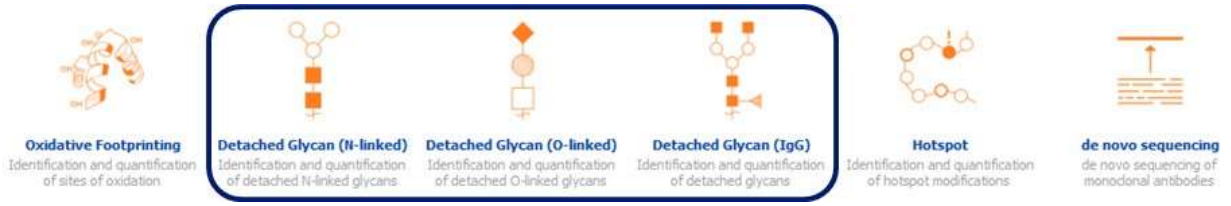


In this application, note we describe detached glycan analysis using Byos, a vendor-neutral, technique-neutral, automated processing and reporting software. With optional tools for GMP environments, the Byos platform can be used from research to QC.

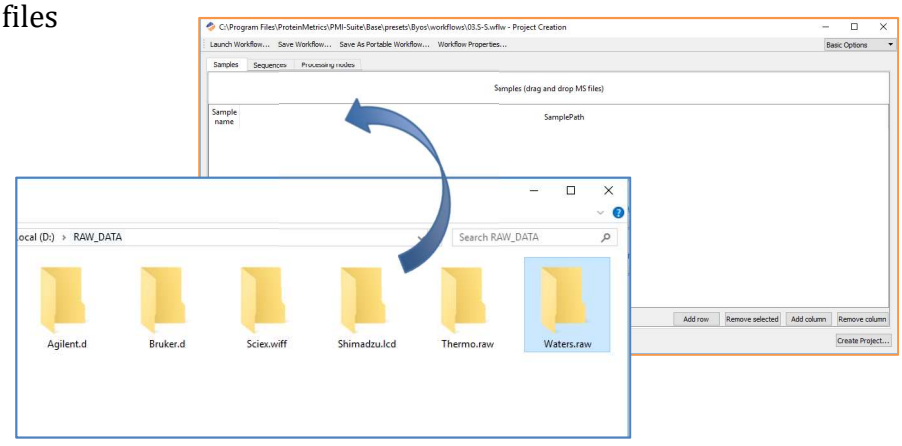
Summary

I. Executing the Byos N-Glycan Workflow

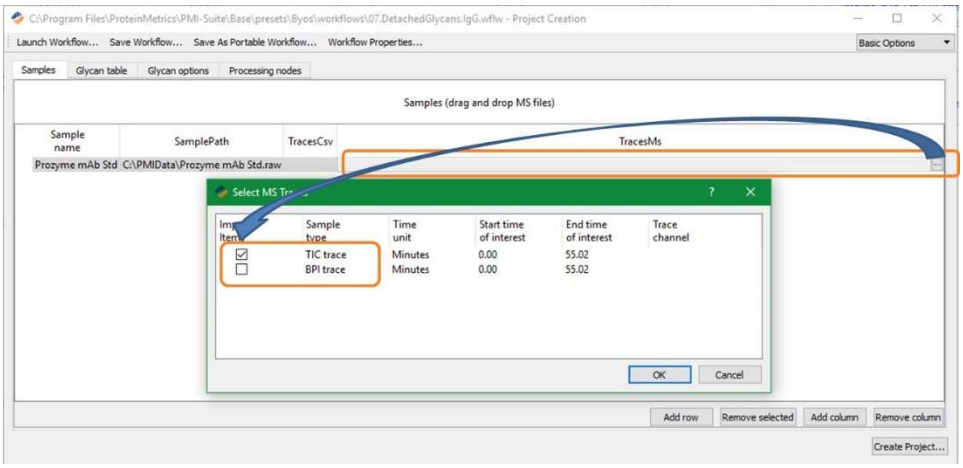
- Click one of the Detached Glycan icons to launch a workflow



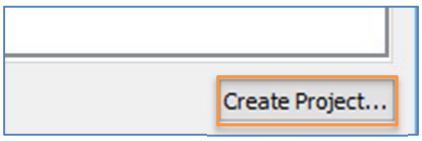
- Drag & drop raw data files



- Select MS Trace



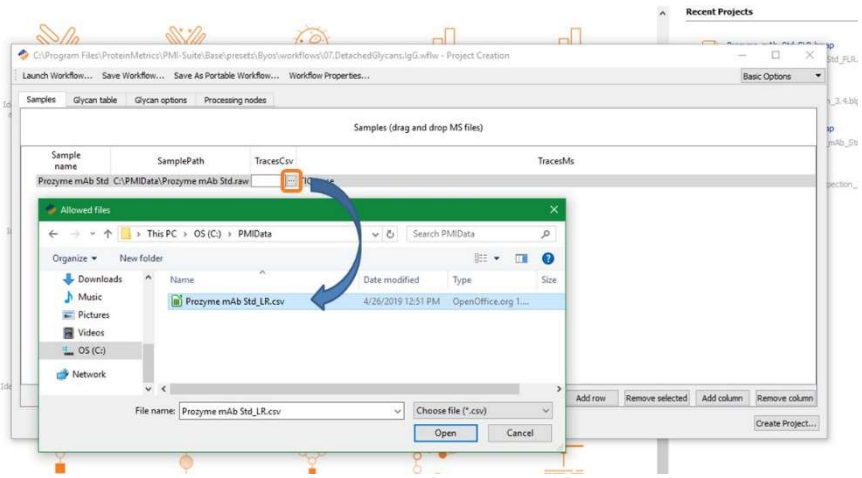
- Create the project



II. Configuring the Workflow

Optionally Adding a CSV Optical Trace

Even if the fluorescence or UV trace is not captured by the MS control software, it is still possible annotate this data in the Byos project. To do so, export the fluorescence traces to X, Y (intensity, time) data in a CSV format. Add this CSV file to the appropriate line on the Samples tab as shown below.



Modifying the Glycan Table

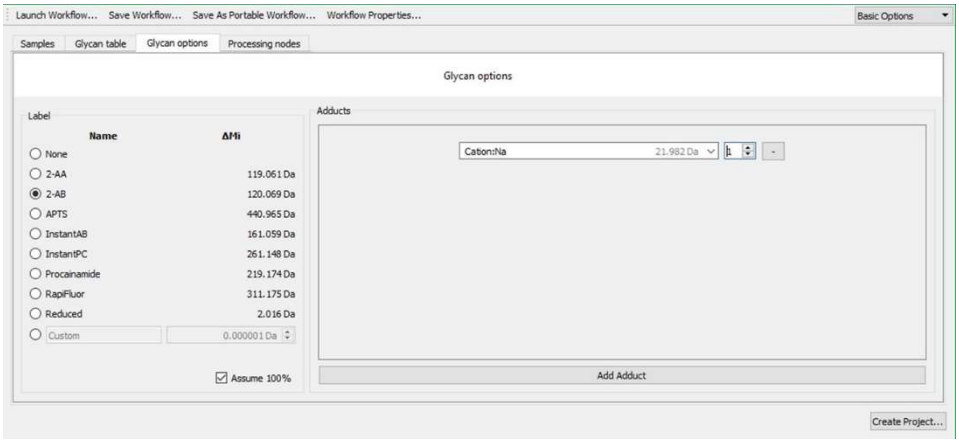
The glycans included in the default workflows, while representative of many common therapeutic molecules, are not exhaustive. For example, the default file has only G1F (and by default will only automatically assign this annotation to one peak), but you may know the relative retention times your organization observes for G1Fa & G1Fb and would like to include this information to refine the resultant annotations. This is easily done by importing a CSV file with user-specific annotation information. The default glycan files are located in the following directory (if default file paths are selected during installation of the software): C:\Program Files\ProteinMetrics\PMI-Suite\Base\presets\Byomap\glycan_tables. These files can be copied and edited with additional glycans, short name, and retention time boundaries.

- Note: When adding from CSV, the system will append the contents of the file, so if you have redundant entries (i.e., a complete file) delete the existing entries before adding the CSV.

Configuring Labels and Labeling Options

Glycans are often analyzed after sample processing such as labelling with a fluorophore that enables accurate quantification. The Byos user interface provides a list of the most frequently used labels. Adducts are also a frequent source of complexity; the user also has tools to select those which are likely in the sample. These settings are found on the Glycan options tab and permit control of the related species that are labeled in the final chromatogram. A default list of many common fluorophores is included with the ability select the appropriate reagent simply by clicking the associated radio button. For organizations using novel reagents, a custom option is available.

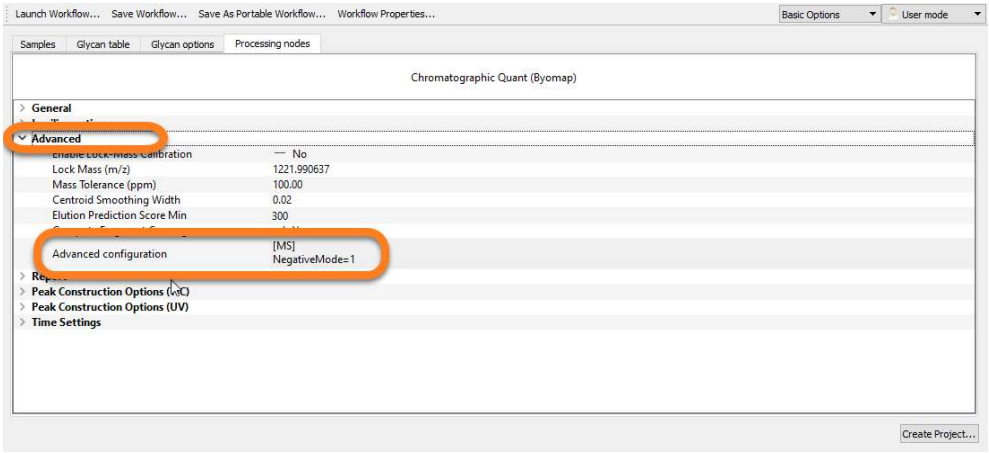
- Note: The mass added along with the selection of the custom option should be the net delta mass of the label, not the reagent mass.



Other options available on this tab include a control to tell the software to look for unlabeled glycans along with the labeled counterparts. This function is helpful when trying to identify unknown species in early analyses. Similarly, the "Add Adduct" button at the bottom of the screen will facilitate the addition of both preconfigured and custom adducts.

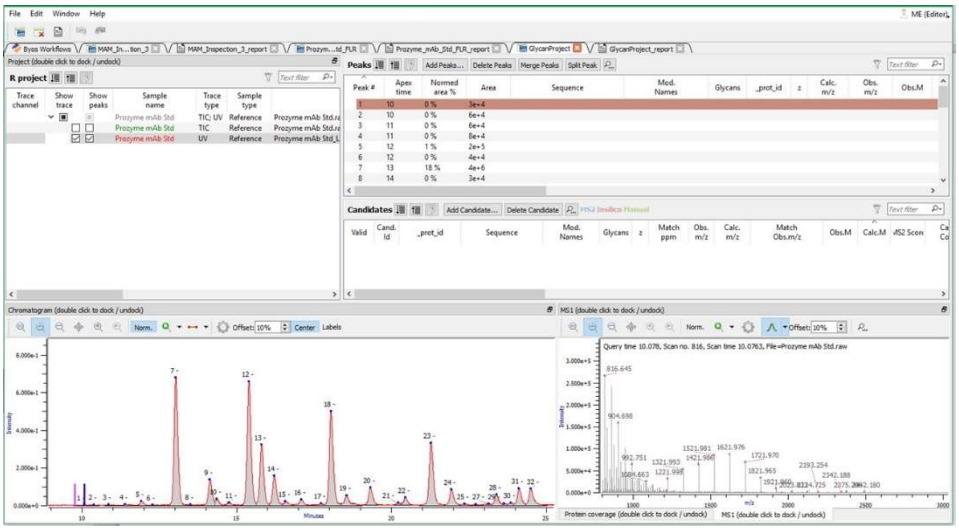
Configuring the MS Polarity

The detached glycan workflows expect negative mode data by default, though this is easily changed using settings available on the Processing Nodes tab. To switch to positive mode, you simply change the default text in the "Advance Configuration" section to "NegativeMode = 0" rather than "NegativeMode = 1".

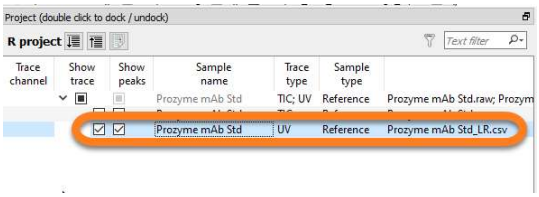


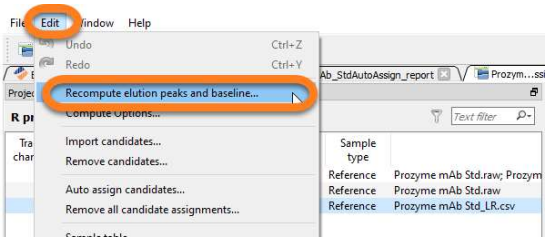
III. Reviewing the Project

Upon completion of project execution, Byos will open a Byomap tab with the project and a report based on the project. At first glance, the report will be blank and no assignments will have been made in the project. This is not an error, it is actually by design so that you can review and adjust the peaks in your chromatogram before you assign identifications to the peaks.



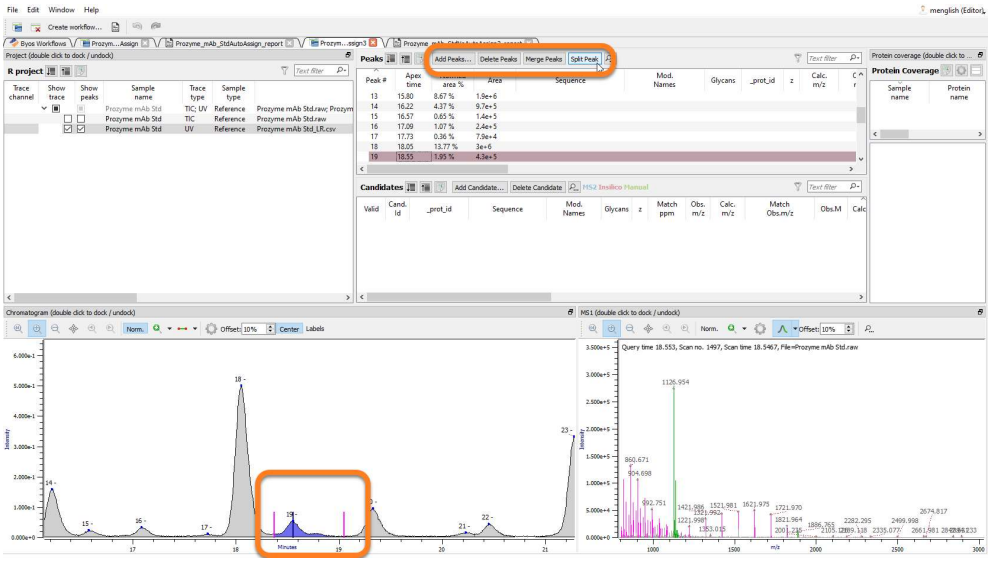
If peaks are not automatically picked in the desired trace, select the trace you desire to annotate then click "Edit"→ "Recompute elution peaks and baseline" then click "OK" on the box that pops up and the software will pick peaks in the selected trace. This feature can also be used to recompute peaks if you want to try different peak picking or time settings.



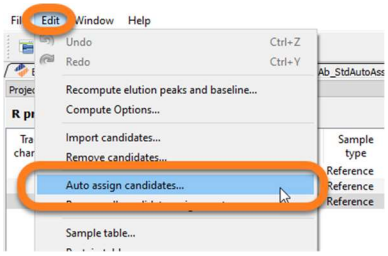


Inspecting the Chromatogram and Auto-Assigning Candidates

While the peak-picking algorithms are quite good, you may still want to adjust some peaks. For example, in the following chromatogram, peak 19 has a tailing bump that might be better annotated as an independent peak. This is easy to do by selecting the peak and pressing the "Split Peaks" button; this will automatically split the peak in two. You may also add, merge, and delete peaks in the same fashion. It is also possible to manually adjust peak areas by moving the pink bars and set the MS1 that is displayed by moving the central blue bar.

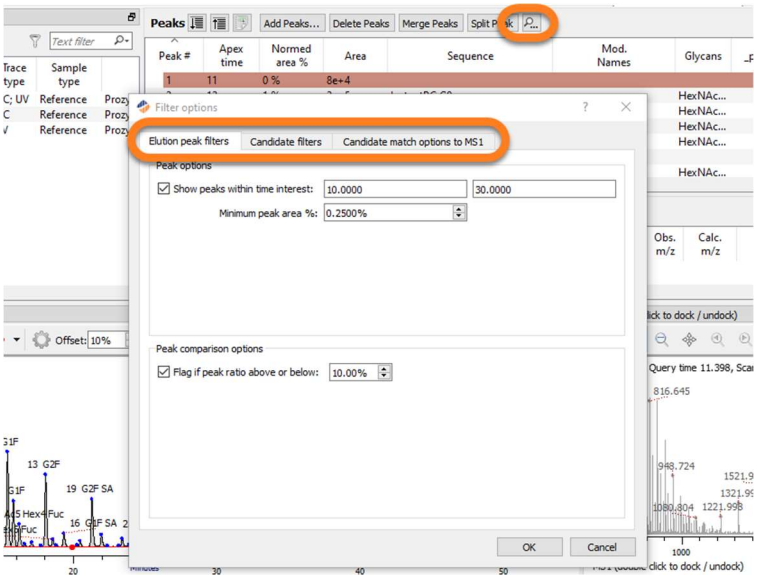


Once the chromatogram is satisfactory, return to the "Edit" menu and select "Auto assign candidates". Within a short time of starting the auto assignment, you should now see that most peaks are identified.



Filter Options

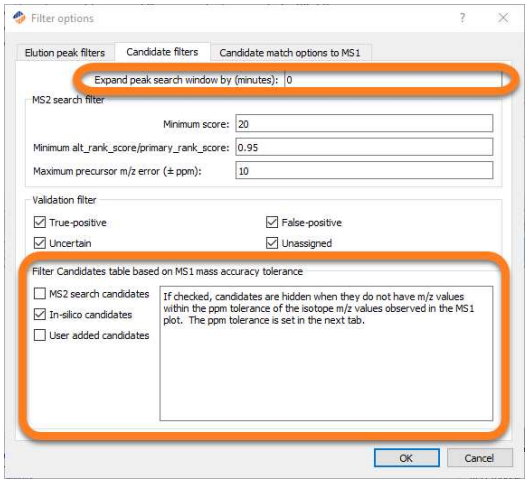
Filters that control the peaks picked and automatic candidate assignments can be adjusted using the "Filter options" dialog box. The box is accessed by clicking on the magnifying glass icon. The resultant pop-up box contains three tabs; one for filtering elution peaks, one for filtering candidates, and another that controls candidate match options.



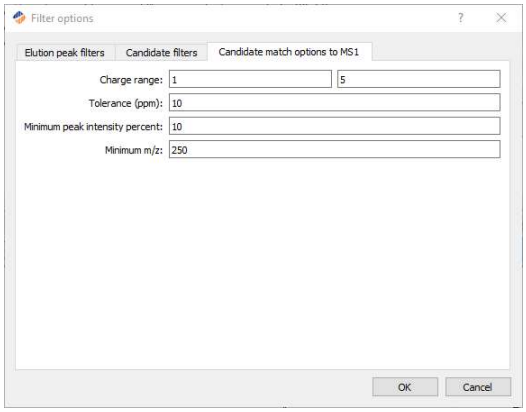
Key options on the "Elution peak filters" tab include a setting to limit the retention times between which peaks will be picked. A second control establishes the minimum peak area of a feature to be integrated and assigned. The retention time limits are particularly useful for omitting contaminating species that are either too hydrophilic or too hydrophobic. The "Minimum peak area %" can be used to set the sensitivity level at which peaks will be annotated.

The "Candidate filters" tab is largely aimed at defining which candidates are available for matching to your MS1 spectrum. Since glycan assignments are currently based solely on MS1, only a few of the options on this tab are relevant to detached glycan processing. The "Expand peak search area by" option allows an automatic increase the time range within which a peak may be identified. If options are checked in the "Filter Candidates table based on MS1 mass accuracy tolerance" section, those candidate types will be removed from

consideration if they do not meet the mass accuracy specified on the "Candidate match options to MS1 tab" (as described in the following section).



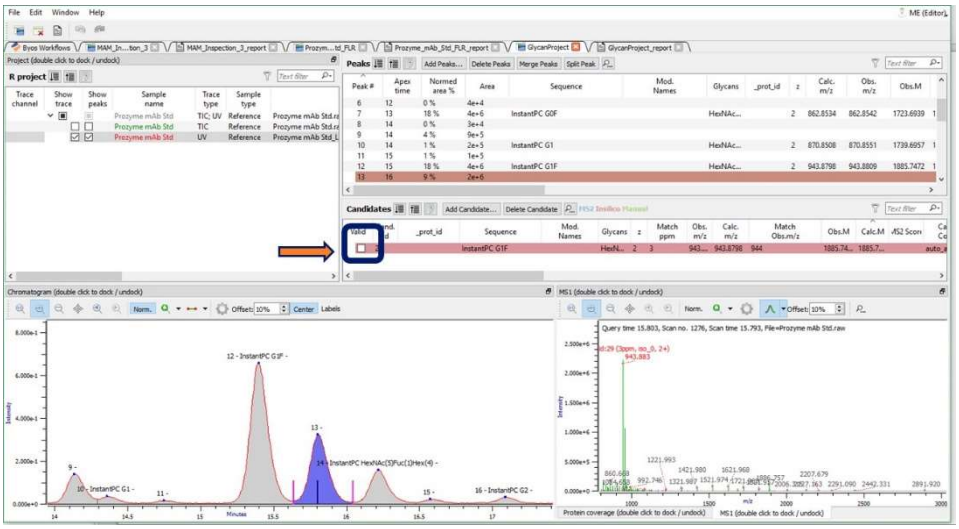
The final tab, "Candidate match options to MS1", is perhaps one of the most important, as the choices here define how candidates will be matched to MS1 spectra. "Charge range" limits the acceptable range of z values used in making assignments. "Tolerance (ppm)" sets the maximum mass deviation allowed for assignment, while "Minimum peak intensity percent" establishes the minimum relative abundance of a peak that might be annotated in a given spectrum. This option is used to assure that annotated species are sufficiently abundant before annotation. Finally, ions below the "Minimum m/z" limit are ignored.



Refining Assignments

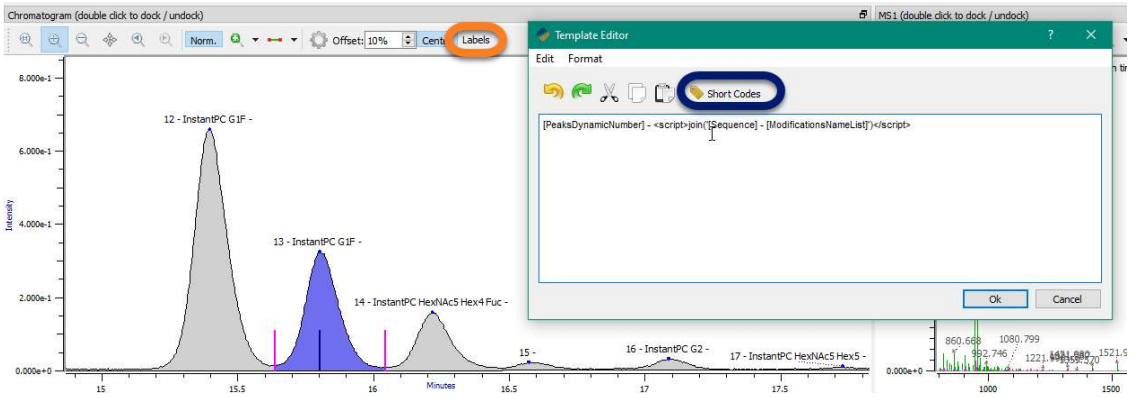
After auto assignments are made, occasionally some peaks may still be unassigned. This often occurs with isobaric species such as G1Fa and G1Fb. The software is currently designed to automatically assign each species to only one peak. For that reason, the largest G1F peak will be annotated with G1F, but the smaller may be unlabeled, as in the following example. It is a simple matter to select the peak, see that the candidate is suggested, and select that

candidate ID for assignment to the peak by checking the "Valid" box. Once validated, the new identification will be shown on the chromatogram. It is also possible to add a candidate that was missing from the candidates list by pressing the "Add Candidate" button.

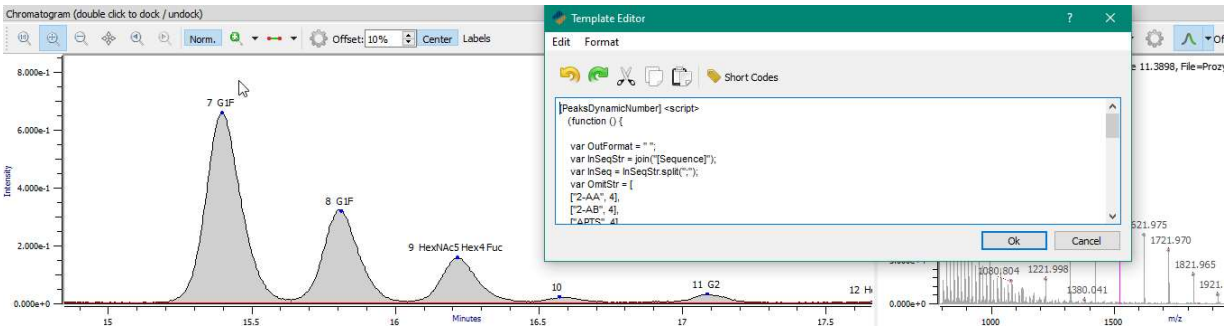


Customizing Chromatogram Labels

The default chromatogram labels will show the glycan, fluorescent tag, and adducts. It is possible, however, to customize these labels by clicking the "Labels" button above the chromatogram. This will bring up a dialog box with the current labeling scheme. On the box, you will find a Short Codes button that will show the fields that can be added to the label (double-clicking a Short Code will add it to the label).

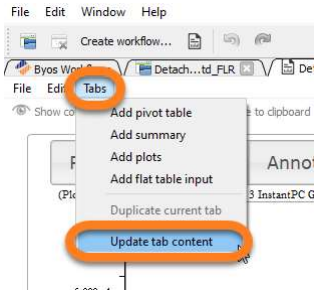


It is also possible to include bits of javascript code to change the data values. For example, one popular script removes the fluorescent tags and adducts from chromatogram labels. This sample script is included in the appendix and could be cut and pasted into the label dialog as shown to obtain more concise labels. For help customizing other labels, contact support@proteinmetrics.com.

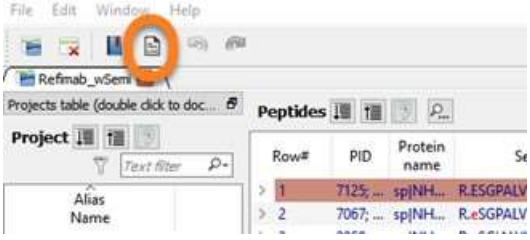


IV. Producing Reports

When the analysis is completed, Byos opens up not only the inspection view, but also the corresponding report as tab within the Byos window. However, because assignments are not made immediately upon project open, the default report will initially be empty. To refresh the report after you have completed assignments in the project, return to the report tab and select "Tabs"→ "Update tab content".

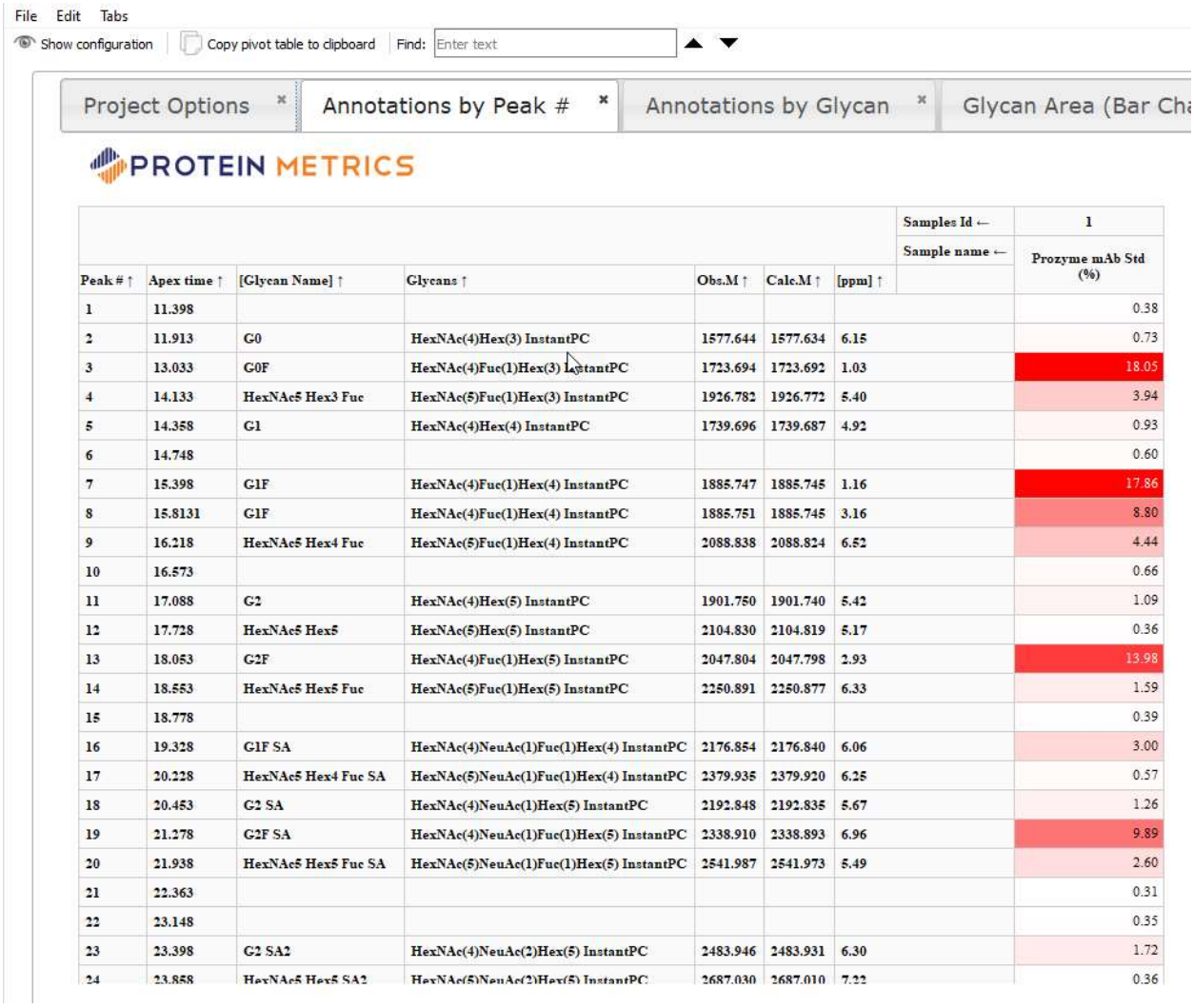


If the report tab is ever closed, it can be reopened by clicking the report tab just below the menu bar or alternatively by selecting "File"→ "Export"→ "Generate configurable pivot summary…".



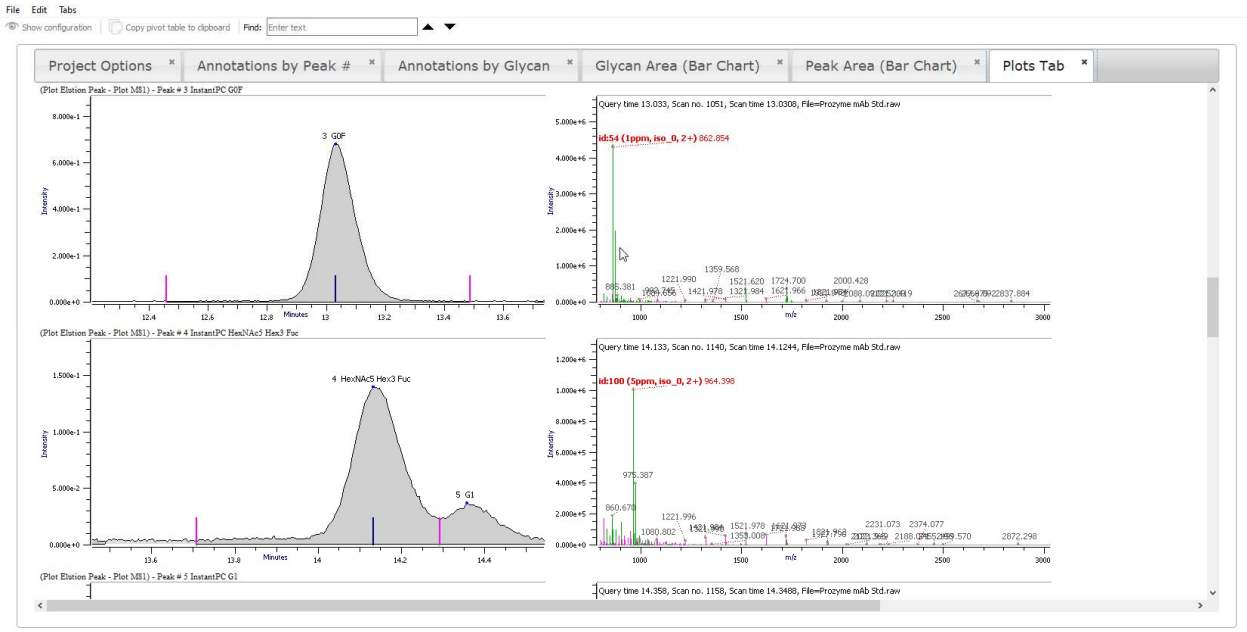
The default glycan report configuration includes project options, annotations by peak number and glycan, and bar charts showing both glycan and peak areas. Although the report

is associated with the project by default, if you ever make changes and want to revert to the original report, you can do so by selecting "File"→ "Presets"→ "Report Presets"→ "Bmap_Detached_Glycan.rptc". An example of the default report follows.



							Samples Id ←	1
							Sample name ←	Prozyme mAb Std (%)
Peak # ↑	Apex time ↑	[Glycan Name] ↑	Glycans ↑	Obs.M ↑	Calc.M ↑	[ppm] ↑		
1	11.398							0.38
2	11.913	G0	HexNAc(4)Hex(3) InstantPC	1577.644	1577.634	6.15		0.73
3	13.033	G0F	HexNAc(4)Fuc(1)Hex(3) InstantPC	1723.694	1723.692	1.03		18.05
4	14.133	HexNAc5 Hex3 Fuc	HexNAc(5)Fuc(1)Hex(3) InstantPC	1926.782	1926.772	5.40		3.94
5	14.358	G1	HexNAc(4)Hex(4) InstantPC	1739.696	1739.687	4.92		0.93
6	14.748							0.60
7	15.398	G1F	HexNAc(4)Fuc(1)Hex(4) InstantPC	1885.747	1885.745	1.16		17.86
8	15.8131	G1F	HexNAc(4)Fuc(1)Hex(4) InstantPC	1885.751	1885.745	3.16		8.80
9	16.218	HexNAc5 Hex4 Fuc	HexNAc(5)Fuc(1)Hex(4) InstantPC	2088.838	2088.824	6.52		4.44
10	16.573							0.66
11	17.088	G2	HexNAc(4)Hex(5) InstantPC	1901.750	1901.740	5.42		1.09
12	17.728	HexNAc5 Hex5	HexNAc(5)Hex(5) InstantPC	2104.830	2104.819	5.17		0.36
13	18.053	G2F	HexNAc(4)Fuc(1)Hex(5) InstantPC	2047.804	2047.798	2.93		13.98
14	18.553	HexNAc5 Hex5 Fuc	HexNAc(5)Fuc(1)Hex(5) InstantPC	2250.891	2250.877	6.33		1.59
15	18.778							0.39
16	19.328	G1F SA	HexNAc(4)NeuAc(1)Fuc(1)Hex(4) InstantPC	2176.854	2176.840	6.06		3.00
17	20.228	HexNAc5 Hex4 Fuc SA	HexNAc(5)NeuAc(1)Fuc(1)Hex(4) InstantPC	2379.935	2379.920	6.25		0.57
18	20.453	G2 SA	HexNAc(4)NeuAc(1)Hex(5) InstantPC	2192.848	2192.835	5.67		1.26
19	21.278	G2F SA	HexNAc(4)NeuAc(1)Fuc(1)Hex(5) InstantPC	2338.910	2338.893	6.96		9.89
20	21.938	HexNAc5 Hex5 Fuc SA	HexNAc(5)NeuAc(1)Fuc(1)Hex(5) InstantPC	2541.987	2541.973	5.49		2.60
21	22.363							0.31
22	23.148							0.35
23	23.398	G2 SA2	HexNAc(4)NeuAc(2)Hex(5) InstantPC	2483.946	2483.931	6.30		1.72
24	23.858	HexNAc5 Hex5 SA2	HexNAc(5)NeuAc(2)Hex(5) InstantPC	2687.030	2687.010	7.22		0.36

To include chromatogram and MS1 images in the report, click "Tabs"→ "Add Plots". This step can take a few minutes depending on the number of glycans included in the analysis.



Hide configuration fields before exporting.

Click "File" →"Export" → "Export to PDF…" to generate a single .pdf file including all of the tabs.

For more information on how to customize reports, please see our related videos available at https://www.proteinmetrics.com/resources/#videos-tutorials or contact the Customer Success Team at support@proteinmetrics.com.

Appendix

Sample Label Script

Copy and paste the following script in its entirety to switch to a more concise label format:

```
[PeaksDynamicNumber] <script>
  (function () {

   var OutFormat = " ";
   var InSeqStr = join("[Sequence]");
   var InSeq = InSeqStr.split(";");
   var OmitStr = [
   ["2-AA", 4],
   ["2-AB", 4],
   ["APTS", 4],
   ["InstantAB", 9],
   ["InstantPC", 9],
   ["Procainamide", 12],
   ["RapiFluor", 9],
   ["Reduced", 7]];

   for (i=0; i < InSeq.length; i++){
      var TruncSeq ="";
      var Omit ="";
      var Len = 0;
      for (j=0; j < OmitStr.length; j++){
         if (InSeq[i].indexOf(OmitStr[j][0])>=0){
            Omit = OmitStr[j][0];
            Len = OmitStr[j][1];
         }
         }
      if  (InSeq[i]) {TruncSeq = InSeq[i].substring(InSeq[i].indexOf(Omit) + Len);
            }
      if (OutFormat == " "){
           OutFormat = TruncSeq;
      } else {
           OutFormat = OutFormat +"; "+" "+TruncSeq;
      }
   }
   return OutFormat;
   })();
</script>
```

Protein Metrics Inc.
Cupertino, California
www.proteinmetrics.com
info@proteinmetrics.com