Workflows for the Identification and Relative Quantitation of Glycan Variants

Introduction

Glycosylation is a common post-translational modification (PTM) found on monoclonal antibodies (mAbs). Glycans play an important role in determining the protein structure, solubility, half-life, and antigenicity of the biomolecule. The heterogeneity of glycosylation makes it a challenging modification to characterize given the diversity in their composition, sequence, and site of glycosylation. N-glycans are the most commonly found glycans on mAbs.

We have developed a workflow to both identify released N-glycans based on their accurate mass and confirm the presence of glycan isoforms on intact proteins using LC/MS. Using a predefined (or user defined) list of PTMs, users can now easily update information to add glycans and PTMs to search against their intact or digested LC/Q-TOF data.

Experimental

Sample Preparation

Samples were prepared using an Agilent AssayMAP Bravo liquid handling system (Figure 1). Multiple workflows were used with automated sample preparation including purification of the intact mAb to the release and labelling of the N-glycans off a mAb.[1]

Figure 1 Agilent AssayMAP Bravo

Experimental

Instrumental Analysis

Released glycan samples were analyzed by an Agilent 6545XT AdvanceBio LC/Q-TOF (Figure 2) using an autotune leveraging a particle swarm optimization algorithm to provide best analytical sensitivity and resolution in the mass range of glycans. Intact protein samples were acquired using a variation of this tuning algorithm to optimize on large molecule performance for improved transmission and detection of a mAb.

The Dual Agilent Jet Stream ion source was used for both the intact protein and released glycan experiments.

For intact protein, separation was achieved using an Agilent 1290 Infinity II UHPLC system and an Agilent PLRP-S 1000Å (2.1 × 50 mm, 5 µm) column. Approximately 0.5 µg of mAb sample was injected for each analysis, and separated across a four minute gradient.[2]

For released glycans an Agilent 1290 Infinity UHPLC system and an Agilent AdvanceBio Glycan Mapping (1.8 µm 2.1 x 15 cm) column was used for separation. Each analysis injected the released N-glycans from 0.5 µg of protein, and separated across a 30-minute gradient.

Data analysis was performed using a prerelease version of MassHunter BioConfirm B.09.00.

Figure 2 Agilent 6545XT AdvanceBio LC/Q-TOF.

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Results and Discussion

Identification and Relative Quantitation of Glycoforms

Figure 3 shows the workflow for the data analysis of mAb glycoforms in intact protein data. The user first chooses the sequence of the mAb (in this case, 500 ng of NISTmAb RM 8671[3]) and defines its linkages and PTMs including glycans in the MassHunter BioConfirm software's Sequence Manager (Figure 4).

The BioConfirm software then extracts averaged spectra across chromatographic peaks and applies the Maximum Entropy deconvolution algorithm to construct a zero-charge deconvoluted spectrum. The mass peaks in the spectrum are matched against the protein sequence with linkages and PTMs and labeled (Figure 5).

PTMs for which there is a match are automatically quantitated against the total abundance of all matched PTMs and listed in a table (Figure 6).

The user is able to choose which PTMs to use for relative quantitative analysis by Height or Area, then the BioConfirm software will generate a report.

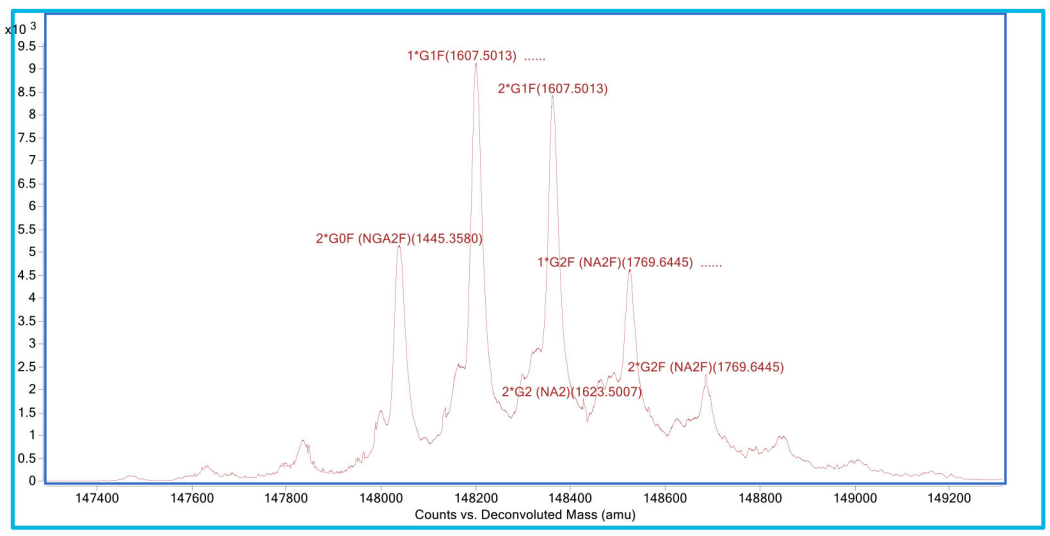


Figure 5 Deconvoluted spectrum of NISTmAb

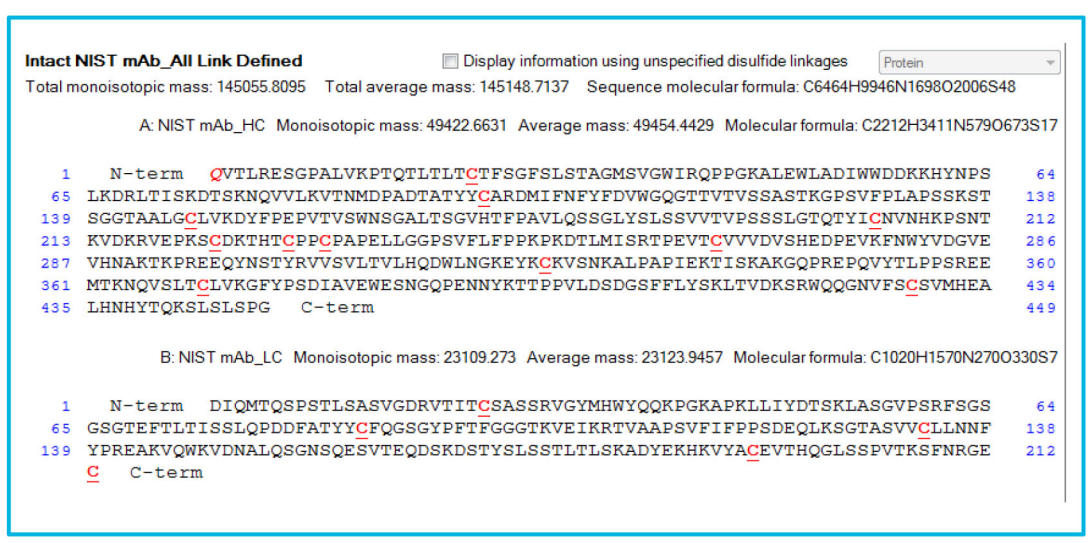
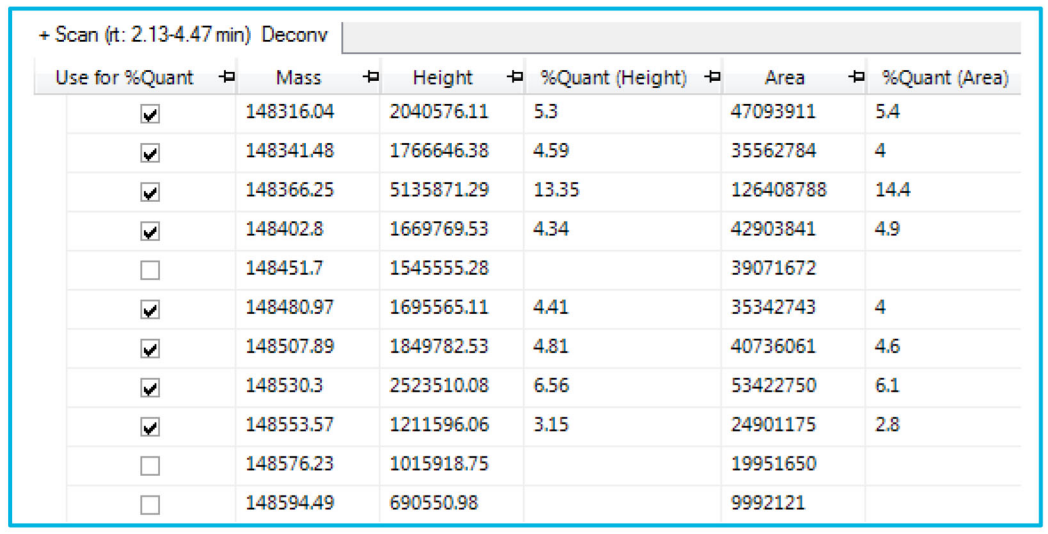


Figure 4 Protein sequence in Sequence Manager



Use for %Quant	Mass	Height	%Quant (Height)	Area	%Quant (Area)
☑	148316.04	2040576.11	5.3	47093911	5.4
☑	148341.48	1766646.38	4.59	35562784	4
☑	148366.25	5135871.29	13.35	126408788	14.4
☑	148402.8	1669769.53	4.34	42903841	4.9
☐	148451.7	1545555.28		39071672	
☑	148480.97	1695565.11	4.41	35342743	4
☑	148507.89	1849782.53	4.81	40736061	4.6
☑	148530.3	2523510.08	6.56	53422750	6.1
☑	148553.57	1211596.06	3.15	24901175	2.8
☐	148576.23	1015918.75		19951650	
☐	148594.49	690550.98		9992121	

Figure 6 Columns to choose PTMs for relative quant.

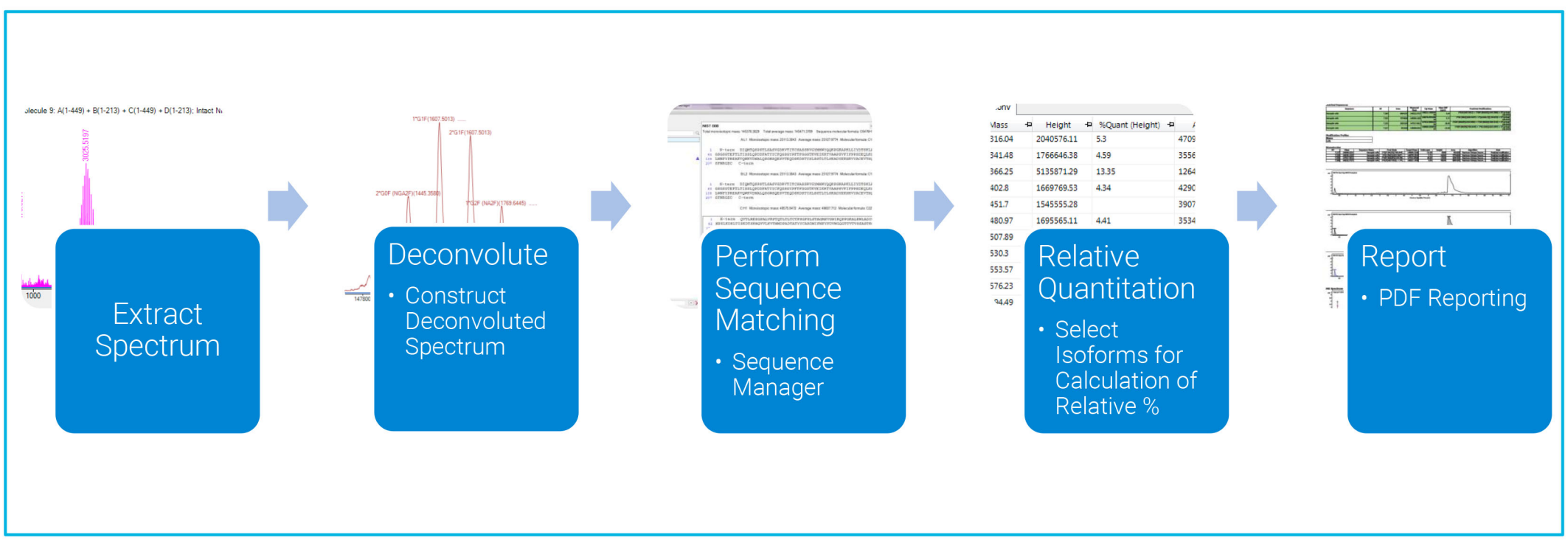


Figure 3 Workflow for the data analysis of glycoforms in intact protein data.

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Results and Discussion

Identification of Released Glycans

Figure 7 shows the workflow for the data analysis of released glycans from mAb data.. The user chooses a tag in the BioConfirm software based on the sample preparation technique. A feature finding algorithm is run which will find all glycans which have formulas in a provided database with optional filtering by retention times. The scoring of matches is based on the monoisotopic mass, the isotope distribution, and the isotope spacing (Figure 8). A list of glycans that have been found is displayed with a score that indicates the confidence in the match (Figure 9). The BioConfirm software will generate a report with the identifications.

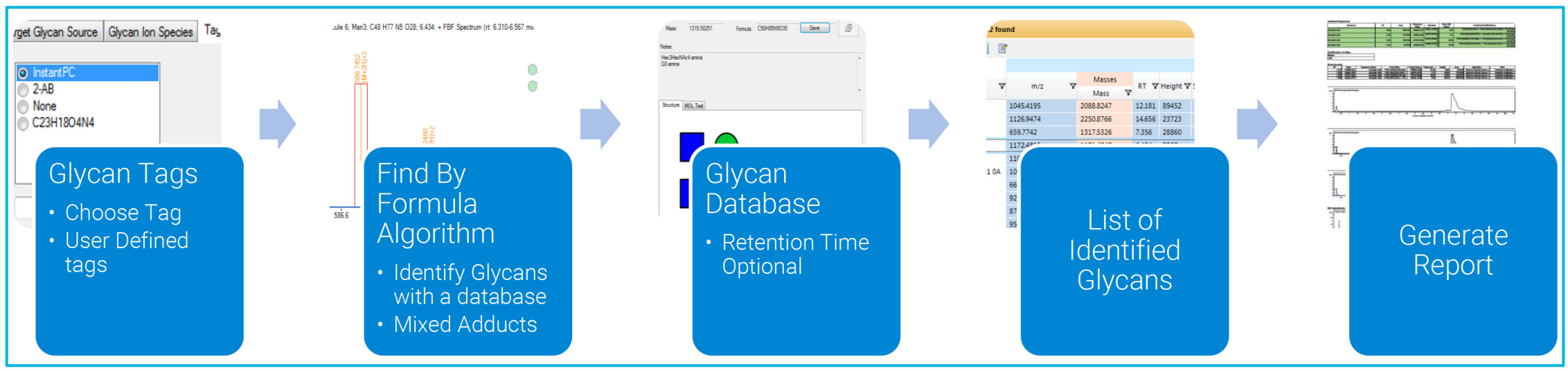


Figure 7 Workflow for data analysis of released glycans

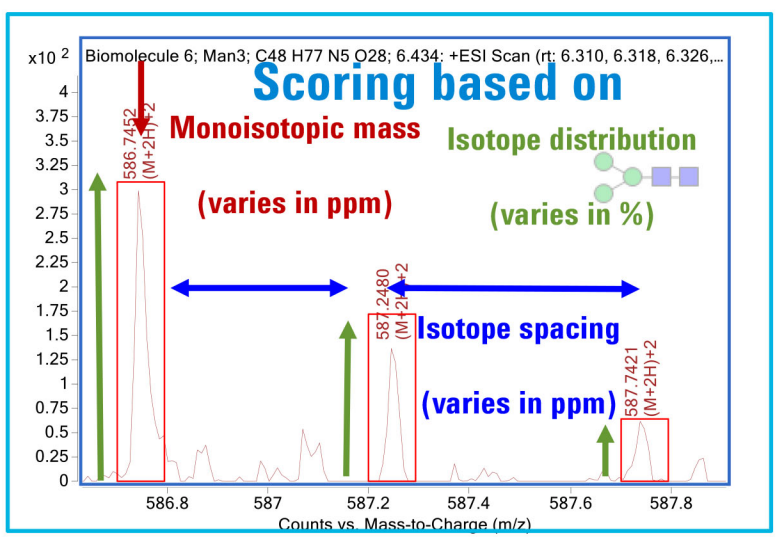
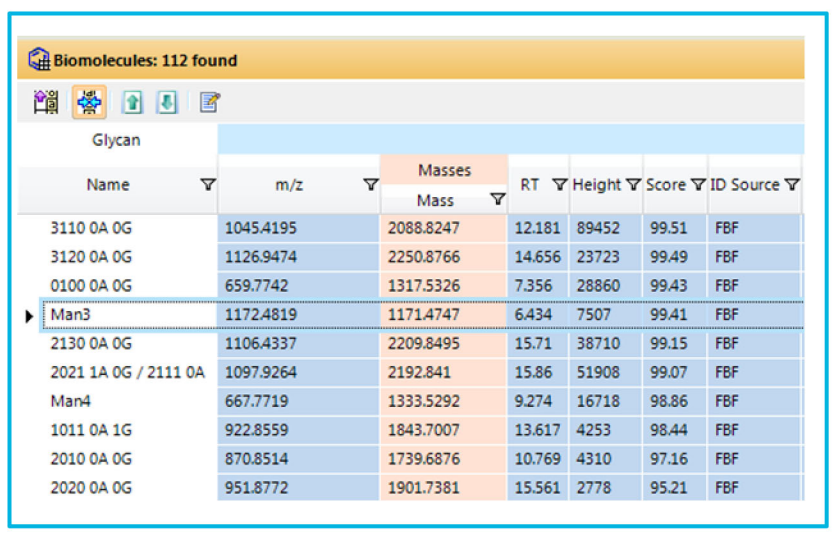


Figure 8 Scoring by feature finding algorithm



Biomolecules: 112 found

Glycan Name	m/z	Masses Mass	RT	Height	Score	ID Source
3110 0A 0G	1045.4195	2088.8247	12.181	89452	99.51	FBF
3120 0A 0G	1126.9474	2250.8766	14.656	23723	99.49	FBF
0100 0A 0G	659.7742	1317.5326	7.356	28860	99.43	FBF
Man3	1172.4819	1171.4747	6.434	7507	99.41	FBF
2130 0A 0G	1106.4337	2209.8495	15.71	38710	99.15	FBF
2021 1A 0G / 2111 0A	1097.9264	2192.841	15.86	51908	99.07	FBF
Man4	667.7719	1333.5292	9.274	16718	98.86	FBF
1011 0A 1G	922.8559	1843.7007	13.617	4253	98.44	FBF
2010 0A 0G	870.8514	1739.6876	10.769	4310	97.16	FBF
2020 0A 0G	951.8772	1901.7381	15.561	2778	95.21	FBF

Figure 9 List of identified glycans

Conclusions

We have developed two workflows to characterize glycan variants that provide:

- High productivity through automation of sample preparation, acquisition and data analysis
- Fast optimization of the instrument using the SWARM autotune
- Superior high resolution data that can be deconvoluted to accurately reveal mAb glycoforms in intact protein data
- Fast identification of released glycans with an option of multiple tags using the same database of native structures

References

[1] ProZyme Technical Note: “Development of an Instant Glycan Labeling Dye for High Throughput Analysis by Mass Spectrometry” (Bulletin 2003 Rev. B)

[2] Agilent Application Note: “Precise Characterization of Intact Monoclonal Antibodies by the Agilent 6545XT AdvanceBio LC/Q-TOF” (P/N 5991-7813EN)

[3] Dong, Q.; Yan, X.; Liang, Y.; and Stein, S.E. In-depth Characterization and Spectral Library Building of Glycopeptides in the Tryptic Digest of a Monoclonal Antibody Using 1D and 2D LC-MS/MS, J Proteome Res. 2016 May 6;15(5):1472-86. (NISTmAb characterization article)

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