Analysis of Highly Sialylated and low input glycoprotein samples on the GlycanAssure™ System

Shaheer Khan, Raymond Lee, Natalee Gautam, Jenkuei Liu, Bharti Solanki-Nand, Baburaj Kunnummal, and Peter A. Bell
Pharma Analytics, BioProduction Division, Thermo Fisher Scientific
180 Oyster Point Blvd., South San Francisco, California, 94080

ABSTRACT

In this study, we evaluated APTS-labeling conditions on highly sialylated glycans, specifically Fetuin. Furthermore, we also tested 3500xl CE detection capability for analysis of glycans at low sample inputs and labeling with APTS dye. Results reinforce the robustness of the GlycanAssure workflow, even with shorter labeling time and low protein inputs. The 3500xl CE platform achieves quantitative, high resolution glycan analysis, with high-sensitivity and high-reproducibility.

INTRODUCTION

Glycosylation plays many important roles in biological processes involving function, pharmakinetics stability, and immunogenicity, which is why it is important to monitor recombinant protein heterogeneity to ensure safety, effectiveness, as well as consistency in manufacturing production for biopharmaceuticals. The most widely used method in laboratories today for glycan analysis has a huge disadvantage in that sample prep is very time consuming with overnight N-Glycan release steps, additional purification steps to remove excess salts, excess labeling reagents, large protein input, and difficulty in producing data on highly sialylated glycans.

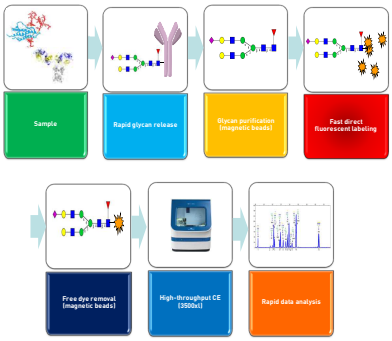
Because sialylated glycans elute at the back end of the LC profile, analysis and accurate quantitation of highly sialylated glycans consistently is a challenge in LC methods. CE offers an advantage in analysis of sialylated glycans as sialylated glycans migrate at the front end of the elctropherogram, making it an ideal platform to analyze and accurately quantitate highly sialylated glycans. CE also offers an opportunity to analyze samples with low glycoprotein input as sample requirement is very low, in comparison to typical LC methods.

The complete GlycanAssure™ Workflow is shown in Figure 1, from the processing of samples to data analysis after the CE run. Glycans are cleaved from proteins using the enzyme PNGase F for 1 hour at 50° C. The digestion mixture is then purified using our magnetic bead based protocol.

Post-purification, glycans are labeled with APTS; 2 hours at 50° C. This workflow was used to detect varying levels of IgG inputs for labeling with APTS. However, to evaluate the labeling of highly sialylated proteins such as Fetuin, three APTS labeling conditions were also evaluated (Figure 4).

The GlycanAssure Data Collection Software is a user friendly, intuitive, easy to use data collection software that allows the user to specify sample types, including references, positive and negative controls. The software, combined with the Protein Analysis enabled CE (3500xl) can process two 96-well plates in about 6 hours. Samples can then be analyzed in GlycanAssure Analysis Software, which allows analysis of small to large sample sets, retaining the sample designations from the collection software. The software allows the user to visualize glycan profiles as well as quantitate relative area with the click of few buttons. Lastly, the user can identify patterns in their trending with the built in trending tool in the software.

Figure 1. GlycanAssure Workflow: 7-9 hours to process and finish CE analysis of 96 samples; hands on time < 3 hours; data analysis ≤ 1 hour.



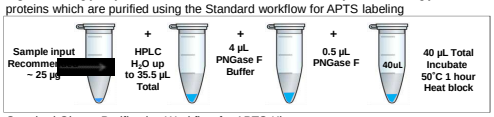
MATERIALS, METHODS AND WORKFLOW

Purified human serum IgG was obtained from Invitrogen (P/N 27102). Bovine Fetuin was obtained from Sigma (P/N F2379). Sample preparation, labeling reaction, dye removal, and CE runs were performed as described in the user guide (GlycanAssure user guide, Thermo Fisher Scientific, Publication Number MAN0014008). Figures 4B and C represent the samples which deviated from the GlycanAssure User guide workflows.

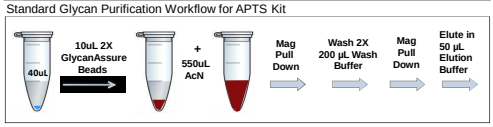
Capillary electrophoresis was performed using Applied Biosystems™ 3500xL, a system configured with a 505 nm solid state laser and laser induced fluorescence detection. Experimental details for this work were as follows:

- Polymer used in CE capillary: POP7 (P/N 4393708)
- Anode Buffer (P/N 4393927); Cathode buffer (P/N 4408256)
- 3500xL Protein Quality Analyzer Capillary Array, 50 cm (P/N 4404689)
- GlycanAssure APTS Kit (P/N 28676)
- LIZ Size Standard used in every injection
- Injection conditions: 1.6 kV
- Voltages: Pre-run at 19.5 kV and Run at 19.5 kV
- Run time: 1330 sec
- Capillary oven temperature: 60ºC
- APTS EX 450nm EM 515nm

Figure 2. Deglycosylation and Purification - PNGase F enzyme cleaves glycans from proteins which are purified using the Standard workflow for APTS labeling



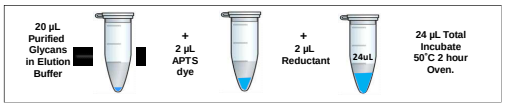
Standard Glycan Purification Workflow for APTS Kit



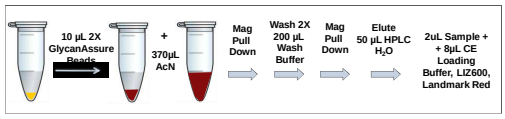
Purified glycans can be labeled with APTS for 1 hour, which is a rapid post digestion APTS dye labeling method. This rapid method was used only on highly sialylated glycans from Fetuin (Figure 4B). APTS labeled glycans require excess dye removal step post labeling, similar to GlycanAssure standard workflow. To avoid off scale peaks, labeled glycan samples were diluted before CE run. Recommended dilutions are 1:2 to 1:5 for APTS samples.

Figure 3. APTS Dye Labeling and Excess Dye Removal Protocol.

(A) APTS GlycanAssure Dye Labeling Workflow



(B) Excess APTS Dye Removal Workflow



RESULTS

Figures 2 and 3 show the workflow for glycan cleavage, purification and APTS dye labeling for Fetuin, a highly sialylated glycoprotein. Results shown in figure 4 indicate that even though intensity is lower from shorter labeling time, the GlycanAssure workflow still delivers adequate labeling efficiency. Figure 4 also shows the results from the standard 2 hour and shorter 1 hour APTS labeling and compared to the traditional overnight labeling at 37° C. These results show the robustness of the GlycanAssure kit with high intensity peaks and adequate labeling efficiency, even after reducing labeling time.

Figure 4. GlycanAssure APTS workflow (50°C, 2 hour) vs. GlycanAssure APTS 50°C, 1 hour and 37° Overnight. 25µg Bovine Fetuin was used in all cases.

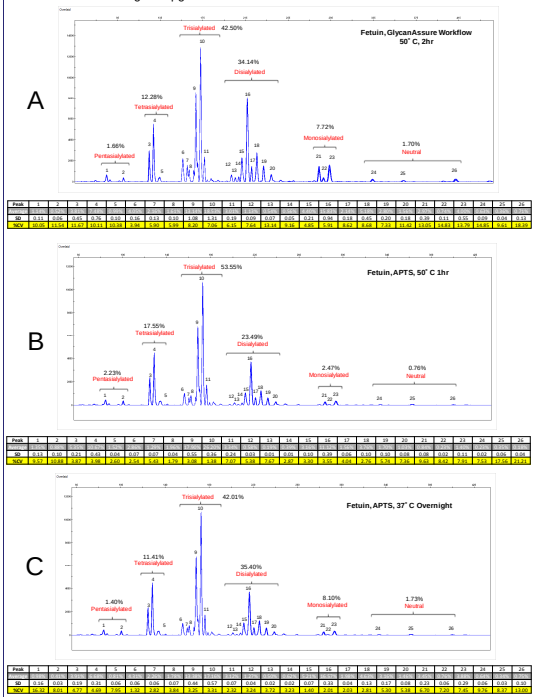
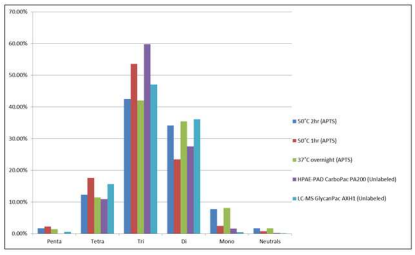


Figure 5: CE analysis of APTS-labeled bovine fetuin N-linked glycans from 3 different labeling conditions.



In Figure 5 Glycans have been groups by their degree of sialylation, and displayed percentage values are percentage of the total peak area. APTS labeling at 50° C for 1 hour minimizes desialylation of glycans with higher level of sialylation. Reported percentage areas of unlabeled glycans by HPAE-PAD and LC-MS are included for reference purpose only,
Ref: *Anal. Biochem.*, 458 (2014) 27-36

To test the robustness of the GlycanAssure system, we tested low to high protein inputs. Figure 6 shows data from APTS labeled samples with no protein input (top) as a negative control to indicate which peaks fall out of the region of interest and electropherogram (bottom) shows the separation of all major IgG glycans (25µg input). Table 1 shows the variation of Human IgG inputs from as low as 1µg to 100µg. CV% for APTS labeled samples were less than 15% in all cases for peaks above 1%. While relative peak intensities were low for the lower inputs, there were no significant differences in the relative peak ratios for APTS labeled IgG glycans (Table 1).

Figure 6. Separation of labeled N-glycans from human serum IgG.

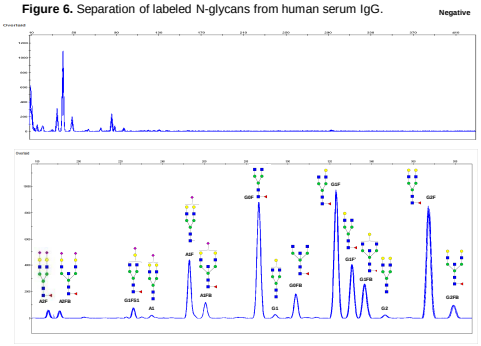
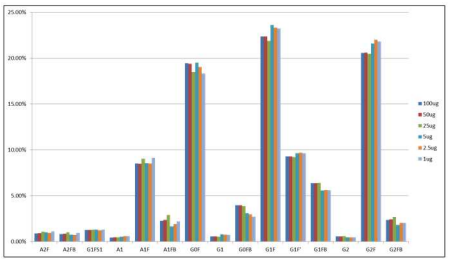


Table 1. Consistent relative quantities of glycans from varying inputs of IgG. Three independent sample preparations were performed for every input amount. Each sample was injected three times in 3500xL CE system

	1µg		2.5µg		5µg		25µg		50µg		100µg	
Peak #	%Area	%CV	%Area	%CV	%Area	%CV	%Area	%CV	%Area	%CV	%Area	%CV
A2F	1.11	12.27	0.96	5.23	1.03	3.60	1.08	4.48	0.93	4.99	0.89	8.25
A2FB	0.96	18.10	0.72	6.87	0.78	11.15	1.01	5.31	0.86	4.28	0.83	8.62
G1FS1	1.32	7.04	1.25	2.83	1.32	4.67	1.32	5.17	1.29	4.49	1.28	3.95
A1	0.61	14.19	0.59	14.17	0.54	13.6	0.47	11.45	0.48	5.16	0.45	3.67
A1F	9.14	4.32	8.53	2.76	8.55	3.97	9.04	1.89	8.51	3.54	8.53	3.64
A1FB	2.20	3.95	1.90	5.78	1.65	3.69	2.92	13.97	2.36	3.60	2.26	7.28
G0F	18.34	6.21	19.03	3.18	19.52	2.83	18.51	0.67	19.39	0.92	19.45	2.00
G1	0.73	12.69	0.77	3.73	0.80	2.86	0.53	2.81	0.56	4.86	0.57	6.40
G0FB	2.73	4.15	2.99	2.25	3.10	1.22	3.86	1.52	3.98	1.39	3.97	1.17
G1F	23.24	1.28	23.35	0.98	23.61	0.40	21.88	1.06	22.39	0.43	22.38	1.07
G1F'	9.66	1.19	9.71	1.82	9.66	1.41	9.21	1.62	9.30	0.83	9.29	1.10
G1FB	5.59	4.89	5.65	2.13	5.56	1.30	6.39	0.58	6.36	0.65	6.36	1.27
G2	0.49	10.45	0.49	5.55	0.46	1.54	0.60	6.16	0.57	3.34	0.57	3.50
G2F	21.83	6.3	22.03	2.02	21.62	1.77	20.49	1.00	20.60	1.19	20.59	1.37
G2FB	2.05	14.73	2.04	4.91	1.82	1.92	2.67	7.78	2.42	2.12	2.37	2.78

Figure 7. Average APTS labeled glycan peak heights from 3 different sample preparations and 3 injections on 3500xL.



SUMMARY

The GlycanAssure system consists of sample prep kit, multi capillary CE instrument and assay specific software. It was evaluated to analyze glycoproteins with highly sialylated glycans to illustrate preservation and accurate quantitation of sialylated glycans. Fetuin analysis data shows the importance of shorter labeling time for sialylated glycans. The significantly higher % peaks areas for higher sialylated glycans (penta-, tetra-, and trisialylated) correlates with lower peak areas for low sialylated species (bi- and monosialylated) suggested the preservation of highly sialylated species at shorter labeling time. GlycanAssure was also tested for it's ability to quantitate glycans from low input glycoproteins. Results from varying glycoprotein inputs illustrate the ability of GlycanAssure system to handle low sample inputs. This is valuable for customers limited with samples, such as customers working with Amber type micro bioreactors. This work suggests that multi capillary CE system can be successfully used to resolve and quantify N-linked glycans associated with IgG and highly sialylated glycoproteins.

ThermoFisher SCIENTIFIC