A rapid, simple high throughput sample prep method for N-glycan analysis by capillary electrophoresis on Applied Biosystems™ DNA sequencers

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ABSTRACT

Glycosylation is one of the key critical quality attributes of mAb based biotherapeutics. Glycosylation changes can impact biotherapeutics safety, efficacy, clearance and immunogenicity, making it necessary to accurately detect and quantitate the changes. Glycan profiling begins at cell line development and continues through process development.

Current glycan sample prep methods are laborious and tedious multi-day processes containing long enzymatic deglycosylation, purification of released glycans, fluorescent labeling and excess dye removal steps. The labeling reaction typically requires vacuum drying of purified glycans and the use of toxic reducing chemicals such as sodium cyanoborohydride. Growth in bio- pharmaceuticals and adjacent markets necessitated the need for a rapid, simple high throughput N-glycan sample prep method that can also preserve the integrity of glycan structure.

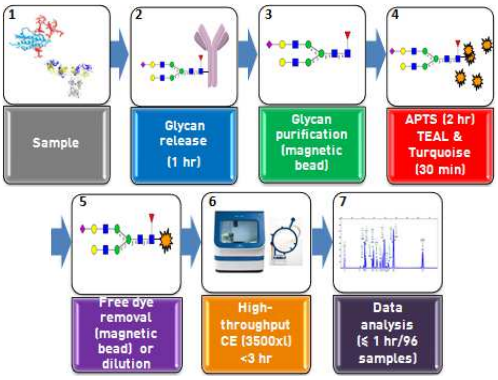
Here we report development of a N-glycan sample prep method consisting of rapid deglycosylation followed by magnetic bead based glycan labeling and excess dye removal steps. We eliminated time consuming vacuum centrifugation from the workflow and the use of toxic sodium cyanoborohydride, resulting in a streamlined automatable method that can process 96 samples in about 8 hours. Our workflow is analysis platform independent and the labeled N-glycans can be separated and quantitated over a CE or LC platform.

INTRODUCTION

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 PNGase F (Figure 2). This workflow is used for all three dye labeling kits. For APTS, the standard workflow is sufficient in purifying glycans post digestion. For the more sensitive, proprietary dyes, TEAL™ and TURQUOISE™ (Figure 2), the Supplement Wash workflow cleanly separates cleaved glycans in most cases.

Most common conditions will require the Standard or Supplement Wash protocol. However, in some cases, with difficult samples, it may be necessary to dilute samples or perform buffer exchange using Amicon Microcon™ filters before digestion.

Figure 1. GlycanAssure Workflow 7-9 hours to process and finish CE analysis of 96 samples with no vacuum centrifugation; hands on time < 3 hour; data analysis ≤ 1 hr.



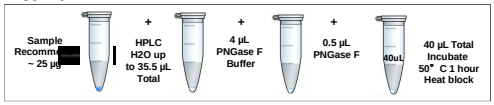
MATERIALS, METHODS AND WORKFLOW

The purified human serum IgG was obtained from Invitrogen (P/N 27102). Ribonuclease B was obtained from Sigma (P/N R7884). M3 (high salt) and M4 (drug substance) test matrices were used to test for interference. 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). 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:

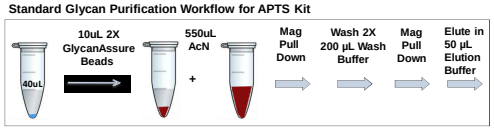
- Polymer used in CE capillary: POP7 (P/N 4393708)
- Anode Buffer (P/N 4393927); Cathode buffer (P/N 4408256)
- 3500xL Genetic Analyzer Capillary Array, 50 cm (P/N 4404689)
- LIZ Size Standard v2.0 (P/N 4408339) used in every injection
- Injection conditions: 1.6 kV
- Voltages: Pre-run at 19.5 kV and Run at 19.5 kV
- Run time: 1330 sec
- Capillary oven temperature: 60ºC
- APTS EX 475nm EM 501nm; TEAL™ Dye EX 466nm EM 505nm; TURQUOISE™ Dye EX 493nm EM 520nm

Figure 2. Deglycosylation and Purification PNGase F enzyme cleaves glycans from proteins and glycans are purified using the Standard workflow for APTS labeling or Supplement Wash workflow for TEAL or TURQUOISE Labeling.

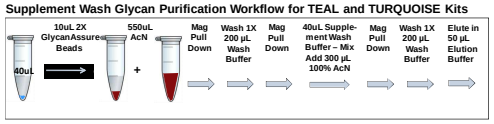
Deglycosylation



Standard Glycan Purification Workflow for APTS Kit



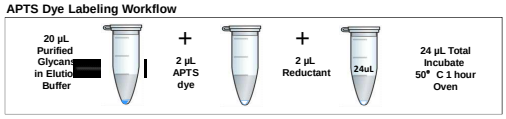
Supplement Wash Glycan Purification Workflow for TEAL and TURQUOISE Kits



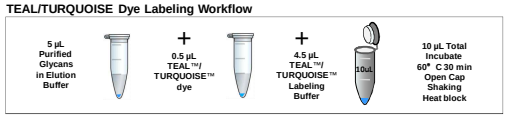
Post-digestion, Standard purified glycans can be labeled with APTS for 1 hour and Supplemental Wash purified glycans can be labeled with either TEAL or TURQUOISE dyes (Figure 3) on a shaking heat block with open caps. Post dye labeling, APTS and TURQUOISE labeled glycans require excess dye to be removed. The TEAL labeled glycans can be re-suspended in 50 µL of HPLC water and run on CE immediately. To avoid off scale peaks, dilute labeled samples before CE run. Recommended dilutions are 1:2 to 1:5 for APTS and 1:2 to 1:20 for TEAL and TURQUOISE samples.

Figure 3. Dye Labeling and Excess Dye Removal APTS, TEAL and TURQUOISE labeling protocol. Excess dye removal for APTS and TURQUOISE dyes.

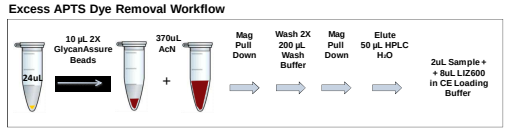
APTS Dye Labeling Workflow



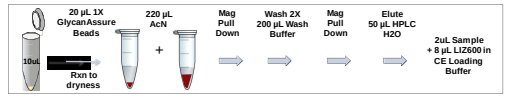
TEAL/TURQUOISE Dye Labeling Workflow



Excess APTS Dye Removal Workflow



Excess TURQUOISE Dye Removal Workflow



The GlycanAssure workflow can also be performed in 96-well plates to process large numbers of samples. Figure 4 shows the workflow for glycan cleavage and purification for all three dyes in a 96-deep well plate. APTS , TEAL and TURQUOISE labeling are done in a 96-well plate and APTS and TURQUOISE dye removal uses the same 96-well plate. As in the tube protocol, TEAL dye samples are re-suspended in HPLC water and run on the CE. Similar to the tube protocol, off scale peaks can be avoided by diluting labeled samples before CE run. Recommended dilutions are 1:2 to 1:5 for APTS and 1:2 to 1:20 for TEAL and TURQUOISE samples.

Figure 4. Plate Protocol Workflows Glycan cleavage and purification in a 96-deep well plate. APTS labeling and excess dye removal in a 96-well plate. TEAL and TURQUOISE dye labeling in a 96-well plate and TURQUOISE dye removal in a 96-well plate.

Plate Protocol: Glycan Release and Purification

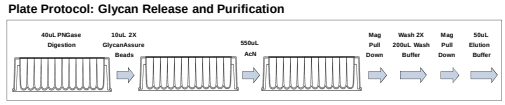


Plate Protocol: APTS Labeling and Dye Removal

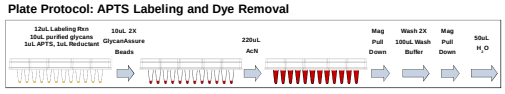
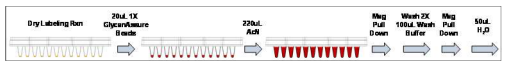


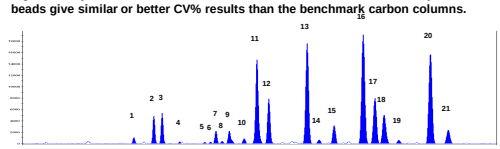
Plate Protocol: TEAL/TURQUOISE Labeling and TURQUOISE Dye Removal



9 Types of beads were tested during the development process and each type of bead was compared to the benchmark carbon column used currently in this field. Out of the 9 types of beads tested, the GlycanAssure Beads gave the best results (Figure 5). CV% for the GlycanAssure Beads were similar or better than samples processed using the carbon column. Other considerations taken into account when selecting beads were handling of the beads and automation.

RESULTS

Figure 5. GlycanAssure Beads vs. the Benchmark Carbon Column GlycanAssure beads give similar or better CV% results than the benchmark carbon columns.

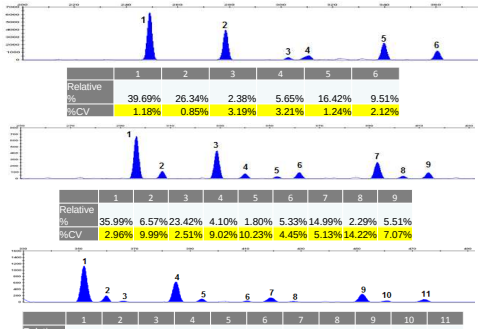


	Peak	1	2	3	4	5	6	7	8	9	10
Carbon column	Rel % Area	0.69%	3.11%	3.54%	0.20%	0.16%	0.16%	1.55%	0.29%	1.96%	0.68%
	CV%	5.12%	4.88%	5.75%	4.31%	5.38%	3.29%	3.80%	0.39%	2.52%	1.76%
Glycan-Assure Beads	Rel % Area	0.68%	2.82%	3.09%	0.23%	0.16%	0.18%	1.66%	0.26%	1.94%	0.68%
	CV%	1.91%	3.63%	4.13%	1.69%	1.17%	1.52%	0.50%	2.28%	1.37%	1.12%

	Peak	11	12	13	14	15	16	17	18	19	20	21
Carbon column	Rel % Area	12.28%	6.49%	15.64%	0.51%	2.80%	18.15%	7.58%	5.25%	0.56%	15.95%	2.43%
	CV%	2.64%	1.76%	0.95%	18.63%	1.26%	1.19%	2.15%	1.41%	1.57%	1.68%	2.62%
Glycan-Assure Beads	Rel % Area	12.77%	6.44%	15.76%	0.53%	2.66%	18.38%	7.67%	5.16%	0.54%	16.00%	2.39%
	CV%	0.88%	1.46%	1.07%	1.53%	0.85%	0.59%	1.00%	0.46%	2.69%	0.60%	0.64%

Inter-assay variation was also tested with 8 independent samples preps and those samples were run on the same capillary. As shown in Figure 6, 8 Ribonuclease B samples run on a single capillary give CV% of less than 15%. 50 µg of Ribonuclease B was used for each sample prep. Instrument to instrument variation also showed CVs less than 15% when Human IgG was run on 3 instruments (Table 1).

Figure 6. APTS, TEAL and TURQUOISE Labeled Ribonuclease B Glycan profiles of Ribonuclease B labeled with APTS, TEAL and TURQUOISE dyes. 8 independent sample preps on the same capillary show CV% under 15% for all three dyes.



	1	2	3	4	5	6
Relative %	39.69%	26.34%	2.38%	5.65%	16.42%	9.51%
%CV	1.18%	0.85%	3.19%	3.21%	1.24%	2.12%

	1	2	3	4	5	6	7	8	9
Relative %	35.99%	6.57%	23.42%	4.10%	1.80%	5.33%	14.99%	2.29%	5.51%
%CV	2.96%	9.99%	2.51%	9.02%	10.23%	4.45%	5.13%	14.22%	7.07%

	1	2	3	4	5	6	7	8	9	10	11
Relative %	41.82%	5.73%	1.00%	24.31%	2.92%	1.55%	5.81%	0.91%	10.44%	1.46%	4.05%
%CV	4.20%	8.19%	14.38%	3.17%	14.25%	11.83%	8.60%	14.87%	8.96%	9.40%	13.50%

Table 1. Instrument to Instrument Variation CV% of Human IgG samples run on three 3500xl CE instruments shows CVs of less than 15%.

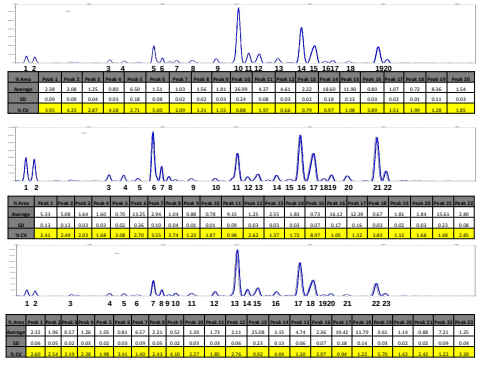
APTS	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8	Peak 9	Peak 10	Peak 11	Peak 12
Average	1.86	1.50	1.86	12.24	2.68	14.05	2.69	21.86	9.42	5.91	23.86	2.09
SD	0.12	0.10	0.07	0.32	0.09	0.23	0.03	0.27	0.13	0.10	0.43	0.07
% CV	6.55	6.95	3.88	2.58	3.55	1.61	1.05	1.23	1.34	1.61	1.80	3.56

TEAL	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8	Peak 9	Peak 10	Peak 11	Peak 12
Average	1.27	1.07	1.71	6.12	1.06	30.60	1.75	6.51	1.35	24.90	13.89	9.76
SD	0.12	0.11	0.11	0.29	0.08	0.26	0.17	0.07	0.14	0.31	0.18	0.16
% CV	9.58	10.37	6.48	4.70	7.49	0.85	9.76	1.01	10.36	1.25	1.33	1.69

TURQUOISE	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6
Average	6.85	31.86	5.71	29.14	13.73	12.71
SD	0.46	0.43	0.18	0.50	0.43	0.23
% CV	6.65	1.34	3.15	1.73	3.16	1.79

To test for interference, matrices M3 (high salt) and M4 (drug substance) were created and used to mimic customers samples. Samples were digested in H_2O and in the presence of M3 and M4 matrices. Supplement Wash protocol was used to purify glycans and samples were labeled with APTS, TEAL and TURQUOISE dyes. Figure 7 shows data from TEAL labeled samples. CV% for all samples from TEAL labeled samples were less than 15%. APTS and TURQUOISE samples also showed similar results.

Figure 7. Matrix Interference No matrix interference after dilution in TEAL labeled samples. Control (H_2O), M3 and M4 matrices respectively.



SUMMARY

The GlycanAssure Kits have been designed and developed for:

- **Easy and rapid high throughput workflow** Analysis of 96 samples can be finished in 7-9 hours (Figures 1-4)
- **High precision in glycan analysis and reproducible alignment of glycan peaks** Very low variation in relative quantities of glycan among 24 capillaries (Figure 5) and nice overlay of glycan peaks from multiple injections (Figure 6, 7 and Table 1).
- **Higher resolution by new fluorescent dyes** Availability of three dyes allows selection of a dye for the best resolution of glycans from any particular sample (Figure 7).

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