PROzyme Advancing Glycosciences

An Integrated Solution for High-throughput, User-friendly Glycoanalysis Using Rapid Separation by Capillary Electrophoresis

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INTRODUCTION

Glycan characterization is becoming necessary in the earliest stages of biotherapeutic cell line development, to the point where cell culture screening often requires glycan profiling. This entails significantly increased throughput for sample preparation, analytical instrumentation, data processing and expertise in glycan characterization. Unfortunately, these factors can cause a bottleneck to results. Here we present a glycan analysis solution that provides rapid sample preparation and analysis combined with a simplified data processing approach, enabling relative N-glycan quantification for 96 cell culture samples within a single workday.

METHODS

Materials: MabThera® lot # B6069B03, Enbrel® lot # 1036826, Glyko® APTS-(Biantennary & High Mannose Partitioned Library) GKSP-520, AssayMAP PA50 (G5524-60010 KIT)

N-Glycan Sample Preparation: Monoclonal antibodies (mAbs) were purified from cell culture media using a Protein A purification module (product code G5524-60001 KIT) by loading samples containing 100 µg of mAb onto Protein A cartridges. Protein A-purified mAbs, as well as Enbrel and MabThera samples, were adjusted to a concentration of 2 mg/mL and were enzymatically deglycosylated using a developmental protocol with a 5-minute in-solution digest. In ProZyme's proprietary method [2], a solution of 20 µL of glycoprotein at 2 mg/mL (40 µg total) was treated with a shelf-stable master mix consisting of buffer, reductant, and denaturant. The denatured protein was incubated with N-Glycanase for 5 minutes at 50 °C. Released N-glycans were labeled with 5 µL of Gly-Q InstantDye®. Clean up of the labeled glycans was performed on a 96-well plate using a vacuum manifold.

Instrument Method: Relative migration times for unknown peaks were determined using bracketing standards consisting of labeled glucose homopolymers with a degree of polymerization (DP) of 2 (maltose, DP2) and 15 (maltopentadecaose, DP15). A mixture of bracketing standards (DP 2, lower; DP 15, upper) was injected at 2 kV for 2 seconds prior to injecting samples. Following a water dip, diluted samples were electrokinetically injected at 2 kV for 2 seconds. Samples were separated at 10 kV for 2 minutes. To flush the cartridge, the capillary was purged with gel buffer at 4 kV for 10 seconds.

Data Processing: After raw data acquisition, electropherograms were automatically exported in AIA (ANDI/netCDF) format (.cdf) for alignment in developmental software. Alignment of glycans migrating between bracketing standards used a linear fit. After alignment, data files were exported to Chromeleon 7.2 Chromatography Data System (Thermo) for integration and peaks were assigned a value (##.##) from 0 to 100 based on the distance between the DP2 and DP15 markers. Using standards, a relative migration time library was constructed to allow streamlined N-glycan assignment in Chromeleon. Relative peak areas were calculated for individual glycans using Chromeleon.

RESULTS

This automation-friendly workflow includes Protein A purification, 5-minute in-solution deglycosylation, Gly-Q InstantDye labeling and sample clean up. The workflow avoids tedious sample preparation steps such as overnight deglycosylation, sample drying, and labeling using reductive amination.

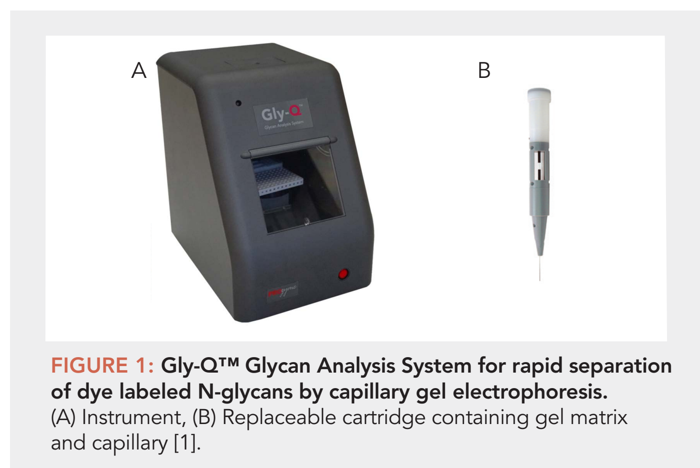

FIGURE 1: Gly-Q™ Glycan Analysis System for rapid separation of dye labeled N-glycans by capillary gel electrophoresis. (A) Instrument, (B) Replaceable cartridge containing gel matrix and capillary [1].

Gly-Q Glycan Analysis System: The analytical system introduced herein is a small, simple, user friendly, low-maintenance Capillary Electrophoresis (CE) instrument with an easily-replaceable gel cartridge (Figure 1). The capillary mounted into the cartridge contains gel matrix that is replenished from the top reservoir of the cartridge between sample runs. The system provides high throughput results with a rapid (2 min) separation. Analysis of 96 samples took 4 hours of instrument time.

Relative migration time reference library: Using standards, the relative migration time (RMT) values for 66 pharmaceutically relevant N-glycans were determined (Figure 2). N-glycans with α(2,3)-linked terminal sialic acid were found to migrate more slowly than those with α(2,6)-linkages. This is consistent with migration order in conventional CE and differs from the elution order observed with UHPLC-HILIC, where α(2,3)-linkages appear first. Also noteworthy is the separation between fucosylated and afucosylated glycan pairs (G0/G0F, G1/G1F and G2/G2F) which can also be observed in MabThera (Figure 3) and Enbrel (Figure 4).

0 10 20 30 40 50 60 70 80 90 100
Relative Migration Time

NNF 14.16	MNN 19.74	MNNF 24.47	A4 (α-2,6) 27.94	A3 (α-2,6) 30.95	A3 (α-2,6/2,6/2,3) 32.20	A2 (α-2,6) 32.46	A4 (α-2,3) 33.33	A3 (α-2,3/2,3/2,6) 33.35	A2B (α-2,6) 33.59	A2F (α-2,6) 34.09
A3 (α-2,3) 34.52	A2FB (α-2,6) 35.16	A4G4S3 (α-2,6) 35.39	A2 (α-2,3) 35.89	A2B (α-2,3) 36.23	Man3 36.90	A2F (α-2,3) 37.46	A2FB (α-2,3) 37.55	G1[6]S1 (α-2,6) 40.29	Man3F 40.87	G1[3]S1 (α-2,6) 41.11
A3G3S2 (α-2,6) 41.25	A4G4S3 (α-2,3) 41.57	G1[6]S1 (α-2,3) 42.64	G1[6]FS1 (α-2,6) 42.75	G1[3]S1 (α-2,3) 43.45	G1[3]FS1 (α,2,6) 43.46	G1FS1 (α-2,3) 45.17	A1 (α-2,6) 45.67	A3G3S2 (α-2,3) 45.81	G0[6]-N 46.26	A4G4S2 (α-2,6) 47.01
G0[3]-N 47.09	A1F (α-2,6) 48.02	A1 (α-2,3) 47.22 & 49.78	G0[6]F-N 49.89	A1B (α-2,3) 50.76	G0[3]F-N 51.08	Man5 51.12	A1F (α-2,3) 49.62 & 51.90	A1FB (α-2,3) 52.81	G1[6]-N 54.12	G0 55.45
G1[3]-N 56.70	G1[6]F-N 57.58	G0B 57.83	Man6 58.14	G0F 58.97	G1[3]F-N 60.58	G0FB 61.30	G1[6] 63.05	NGA3 63.86	G1[3] 64.47	Man7 64.87
A4G4S1 (α-2,6) 66.11	G1[6]F 66.47	G1[3]F 68.04	NGA4 70.75	Man8 71.33 & 72.93	G2 71.89	G2B 72.88	G2F 75.24	Man9 75.41	G2FB 75.96	NA3 88.34

FIGURE 2: Relative Migration Time (RMT) Library or 66 N-glycans separated using the Gly-Q system. The RMT scale is set to 100, where RMT=0 is the lower bracketing standard and RMT=100 is the upper bracketing standard. The time for the separation between the lower and upper bracketing standards is approximately 1 minute.

N-Glycan Separations: Glycan characterization in biopharma requires quantification of biologically important glycans early in product development. Therefore, identification of high mannose (especially Man5), sialylated, and afucosylated glycans may be desired. As shown by Figure 3, Man5 is partially resolved from A1F and well separated from G0 for MabThera N-glycans. In addition, the system demonstrates resolving power towards linkage and positional isomers. An example of this is the separation of A1F (aka G2FS1 or FA2G2S1) isomers shown in the Enbrel N-glycan profile (Figure 4. The Gly-Q System has comigrations for certain glycans. Examples of pharmaceutically important comigrating glycan pairs include G0F and Man6 (from RNase B).

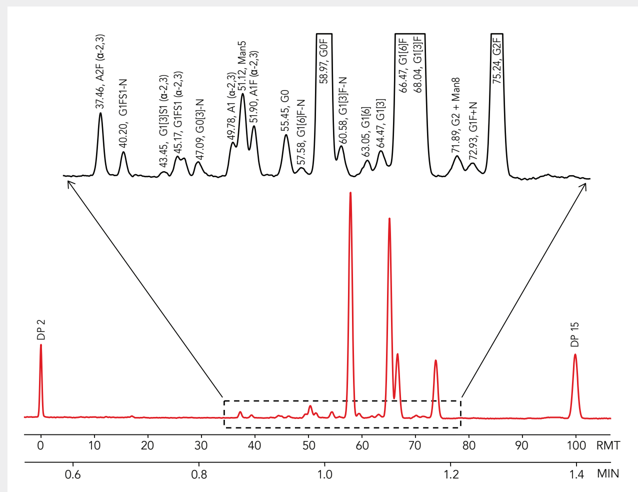

FIGURE 3: **MabThera N-glycans on the Gly-Q System.** Top x-axis is relative migration time between the bracketing standards set on a scale of 100. Bottom x-axis is actual time for the separation (minutes). Expanded inset shows minor peaks and glycan assignments made using the Gly-Q RMT library. Assignments were confirmed using orthogonal methods. G1FS1-N was not found in the RMT reference library and was assigned through additional exoglycosidase treatments not described here.

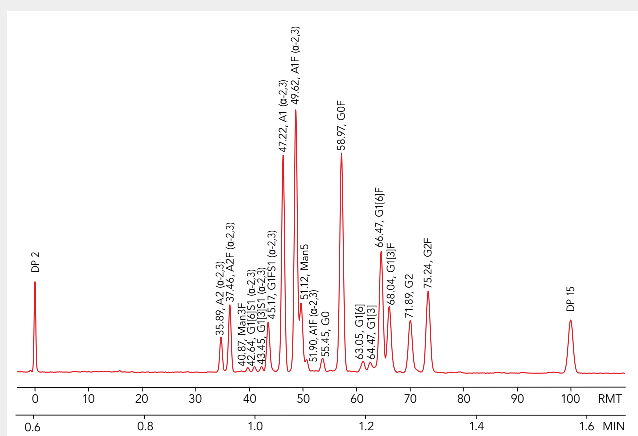

FIGURE 4: **Enbrel N-glycans on the Gly-Q System.** Top x-axis is relative migration time between the bracketing standards set on a scale of 100. Bottom x-axis is actual time for the separation (minutes). Glycan assignments shown were made using the Gly-Q RMT library.

Cell Culture Samples: The glycosylation of biotherapeutics produced in mammalian cell lines can be affected by cell culture conditions [3]. Screening of N-glycans during bioprocess development assures that biotherapeutics with an optimal N-glycan profile are produced. Figure 5 shows aligned electropherograms of 72 cell culture samples processed by the Gly-Q System (mAb purification, N-glycan release, glycan labeling, cleanup, separation). Figure 6 shows the relative abundance of Man5 in a shake flask experiment where growth media was supplemented with different additives to target a particular mAb N-glycan profile. Most of the additives minimally impacted the relative abundance of Man5 relative to Controls 1 & 2, however, with Treatment 5 the abundance of Man5 approaches the target range (blue bar).

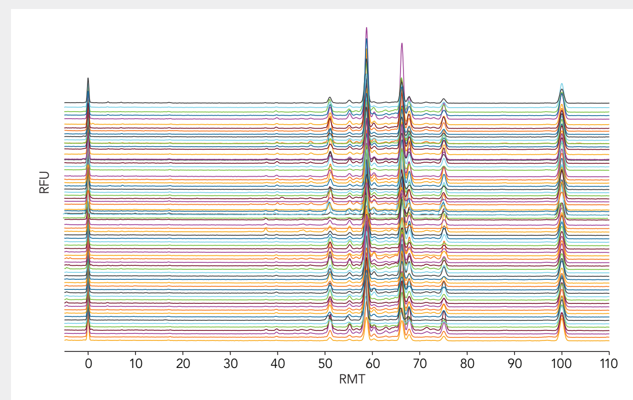

FIGURE 5: **Aligned N-glycan profiles for cell culture screen.** Raw electropherograms for 72 samples in AIA format (.cdf) were auto exported to developmental alignment software. The aligned outputs are processed using Chromeleon 7.2 which integrates the peaks and uses the RMT library to assign glycan ID.

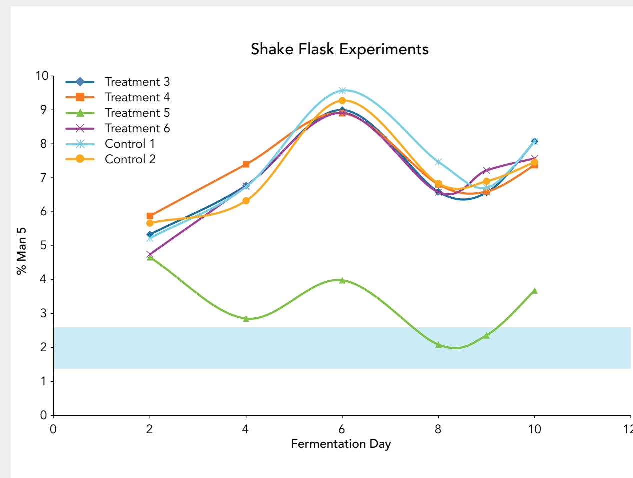

FIGURE 6: **Use of Gly-Q System to screen for the effect of culture conditions on the relative abundance of Man5 on mAb N-glycans produced by a cell line.** Shake flask experiment with different media additives. Blue bar represents target Man5 abundance.

Reproducibility: In order to test the reproducibility of the system, N-glycan relative % peak area was calculated for 24 replicates of a MabThera sample prepared and separated with the Gly-Q System. Average % CV calculated for relative peak area (peaks greater than 1%) was 3.7% (Figure 7A). Results are comparable to data from the same MabThera lot obtained using GlykoPrep/InstantAB labeling and UHPLC profiling (Figure 7B).

APTS Labeled Glycans: The Gly-Q System is also capable of analyzing glycans labeled with APTS if the samples are cleaned up of excess salt (e.g., using GlykoPrep APTS Cleanup Module GS24-C2 or GS96-C2). Figure 8 shows three APTS-labeled N-glycan libraries separated by Gly-Q. The lower (DP2) and upper (DP15) Gly-Q InstantDye bracketing standards may also be used for APTS samples.

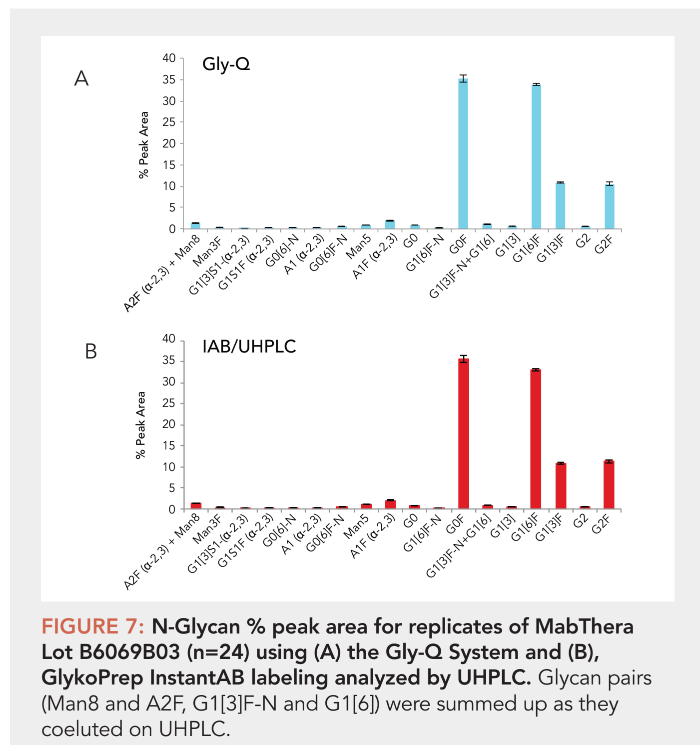

FIGURE 7: **N-Glycan % peak area for replicates of MabThera Lot B6069B03 (n=24) using (A) the Gly-Q System and (B), GlykoPrep InstantAB labeling analyzed by UHPLC.** Glycan pairs (Man8 and A2F, G1[3]F-N and G1[6]) were summed up as they coeluted on UHPLC.

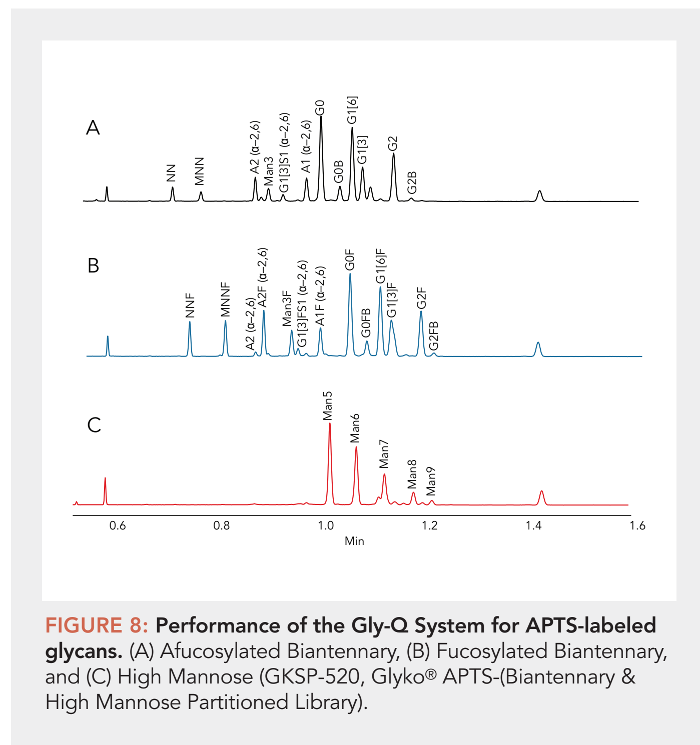

FIGURE 8: **Performance of the Gly-Q System for APTS-labeled glycans.** (A) Afucosylated Biantennary, (B) Fucosylated Biantennary, and (C) High Mannose (GKSP-520, Glyko® APTS-(Biantennary & High Mannose Partitioned Library).

CONCLUSIONS

We present a novel system for rapid N-glycan analysis.

- At the front end, simple and automatable sample preparation uses InstantDye chemistry to label released N-glycans from Protein A purified cell culture samples.
- For N-glycan separation and detection, we introduce a simplified CE system that has taken a cue from the speed, ruggedness and reproducibility of DNA analysis with a total run time of 2 minutes per sample.
- At the back end, peaks are integrated and glycan assignments are made using an automated data analysis approach.
- The demonstrated sample prep, separation and analysis was completed in less than 8 hours. The high-throughput capability of the Gly-Q System and rapid turnaround of results is suited to applications requiring the N-glycan profiling of large number of samples, including biotherapeutic cell line development.

REFERENCES

1. US Patent 8,784,626
   US Patent 8,778,155
   US Patent D698,458
   Patents Pending
2. Patent Pending
3. Hossler et al. Glycobiology. 2009 Sep;19(9):936-49

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