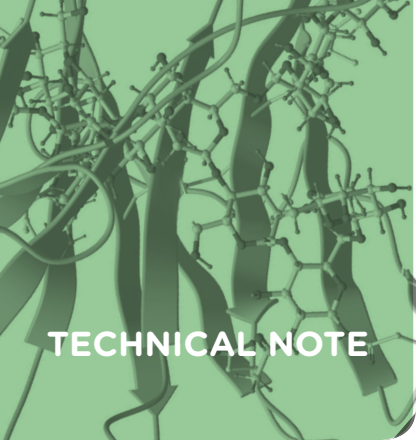

PROzyme

Gly-Q
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GlykoPrep
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InstantAB
InstantQ
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APTS
PhycoLink
PhycoPro
RPE & APC Conjugates
Streptavidins

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

Michael Kimzey, Andres Guerrero, Shirley Ng, Alexander Gyenes, Aled Jones, John Yan, Justin Hyche, Emily Dale, Ted Haxo, Sergey Vlasenko

SUMMARY

Here we present a glycan analysis solution that provides rapid sample preparation and analysis combined with a simplified data processing approach. The sample preparation includes a 5-minute deglycosylation step to release N-glycans, followed by glycan labeling and cleanup, and may completed in under 1 hour. Labeled N-glycans are separated using a small and user-friendly capillary electrophoresis (CE) instrument, with a run time of 2 minutes per sample. This process enables relative N-glycan quantification for up to 96 cell culture samples within a single workday.

Keywords

Biotherapeutic
Capillary Electrophoresis
Enbrel
Fluorescent dye
Glycan Standards
Glycoprotein
InstantQ
MabThera
N-Glycan Labeling
PNGase F
Rapid Deglycosylation

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.

The automation-friendly workflow presented here includes Protein A purification, 5-minute in-solution deglycosylation, InstantQ dye labeling and sample clean up. The workflow avoids tedious sample preparation steps such as overnight deglycosylation, sample drying, and labeling using reductive amination. Following sample prep the samples can be directly loaded into the Gly-Q Instrument for rapid CE separation and analysis with Gly-Q Manager software.

RESULTS

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 minute) separation. Analysis of 96 samples took 4 hours of instrument time.

Data Acquisition and Analysis

A mixture of upper and lower migration standards was electrokinetically injected prior to the injection of samples, followed by a 2-minute separation. Gly-Q Instrument operation and data acquisition was performed by ProZyme Gly-Q Manager™ software, which was also used to align electropherograms. Alignment of glycans migrating between standards consisting of labeled glucose homopolymers with a degree of polymerization (DP) of 2 or 3 (maltose, DP2 or maltotriose, DP3) for the lower migration standard, and 15 (maltopentadecaose, DP15) for the upper migration standard. DP2/3 and DP15 migration standards used a linear fit, enabling labeled N-glycans to be assigned glucose unit (GU) values between 3 and 15. Alignment and integration was performed in Gly-Q Manager or data files were exported to Chromeleon (Thermo). Using standards, a GU library was constructed to allow streamlined N-glycan assignment. Relative peak areas were calculated for individual glycans using Chromeleon for data in figures 7–9.

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).

METHODS AND MATERIALS

Materials

MabThera® lot # 938802, Enbrel® lot # 1058467, AssayMAP Protein A Purification Module PA50 (G5524-60010 KIT)

N-Glycan Sample Preparation

Monoclonal antibodies (mAbs) were purified from cell culture media 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. A total of 40 µg of each glycoprotein were enzymatically deglycosylated using a 5-minute in-solution digest [1]. Released N-glycans were labeled with InstantQ™ dye. Cleanup of the labeled glycans was performed on a 96-well plate using a vacuum manifold.

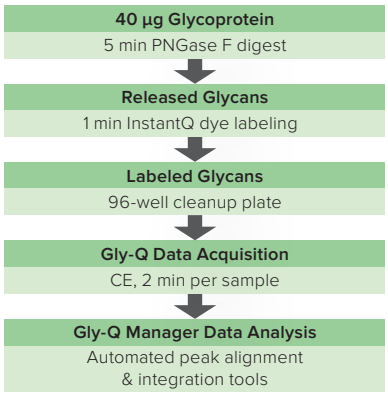

Figure 1: Gly-Q workflow. Gly-Q: a simplified glycan analysis workflow.

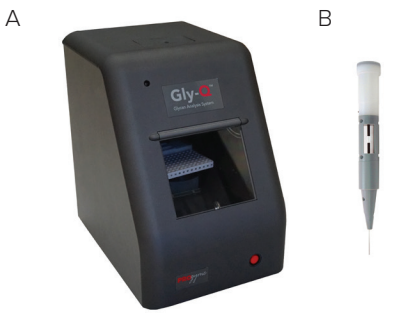

Figure 2: Gly-Q™ Glycan Analysis System for rapid separation of dye labeled N-glycans by capillary gel electrophoresis. (A) Instrument, (B) Replaceable cartridge containing gel matrix and capillary [2].

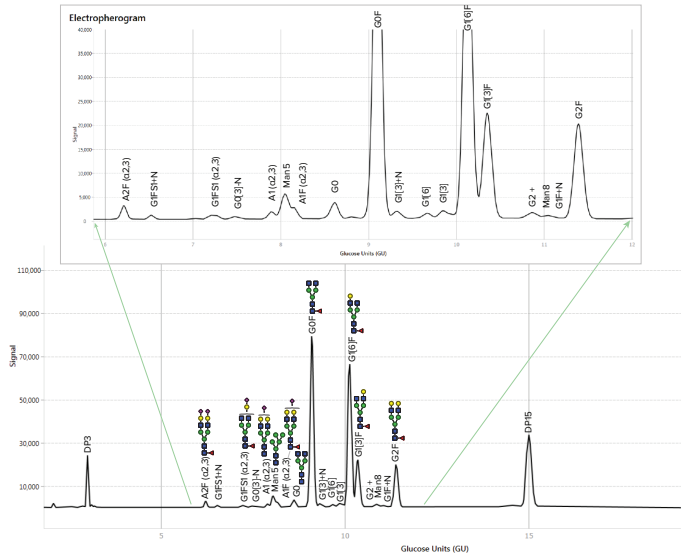

Figure 3: MabThera N-glycans on the Gly-Q System. X-axis represents Glucose Unit (GU) values as calculated based on the relative migration time between the bracketing standards. Bottom x-axis is actual time for the separation (minutes). Expanded inset shows minor peaks. Assignments were confirmed using orthogonal methods. G1FS1-N was not found in the GU library and was assigned through additional exoglycosidase treatments not described here.

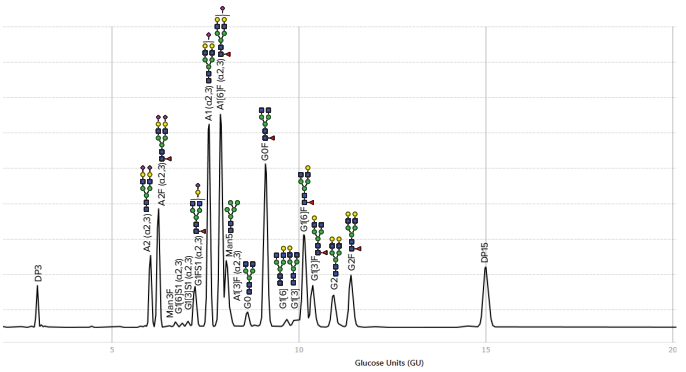

Figure 4: Enbrel N-glycans on the Gly-Q System. X-axis represents Glucose Unit (GU) values as calculated based on the relative migration time between the bracketing standards set on a scale of 100. Glycan assignments shown were made using the Gly-Q GU library. The time for the separation between the lower and upper migration standards is approximately 1 minute.

Relative migration time reference library

Using standards, the GU values for 46 pharmaceutically relevant N-glycans were determined (Figure 6). 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 4) and Enbrel (Figure 5).

Glycan	GU	Structure
MNN	4.050	
MNNF	4.630	
A3 (2,6)	5.390	
A3 (2,6/2,6/2,3)	5.530	
A2 (2,6)	5.570	
A3 (2,6/2,3/2,3)	5.690	
A2 (2,3/2,6)	5.770	
A2F (2,6)	5.800	
A3 (2,3)	5.830	
A2FB (2,6)	5.920	
A2 (2,3)	6.010	
Man3	6.190	
A2F (2,3)	6.230	
Man3F	6.690	
G1[6]S1 (2,3)	6.860	
G1FS1 (2,6)	6.920	
G1[3]S1 (2,3)	7.030	
G1FS1 (2,3)	7.210	
A1 (2,6)	7.310	
G0-N[6]	7.420	
G0-N[3]	7.540	
A1 (2,3)	7.580	
A1F (2,6)	7.590	

Glycan	GU	Structure
A1FB (2,6)	7.710	
G0F-N[6]	7.900	
A1[6]F (2,3)	7.900	
G0F-N[3]	8.060	
Man5	8.060	
G0	8.640	
Man6	9.010	
G0F	9.120	
G0FB	9.450	
G1[6]	9.680	
NGA3	9.820	
G1[3]	9.870	
Man7	9.950	
G1F[6]	10.140	
G1F[3]	10.350	
G1FB[6]	10.350	
NGA4	10.760	
Man8	10.850	
G2	10.920	
G2F	11.390	
Man9	11.450	
G2FB	11.530	
G3	13.300	

Figure 5: Glucose Unit (GU) Library for 46 N-glycans separated using the Gly-Q system. Numbers in parentheses indicate sialic acid linkage position.

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 7 shows aligned electropherograms of 72 cell culture samples processed by the Gly-Q System (mAb purification, N-glycan release, glycan labeling, cleanup, separation). Figure 8 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).

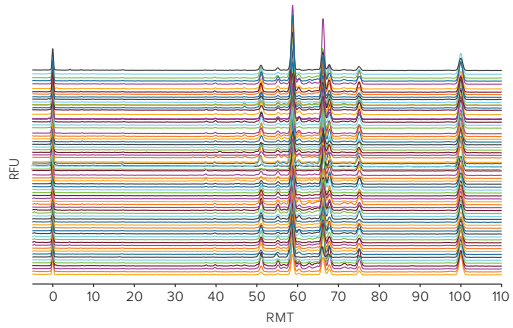

Figure 6: Aligned N-glycan profiles for cell culture screen. Raw electropherograms for 72 samples in AIA format (.cdf) were auto exported to developmental alignment software. The aligned outputs are processed using Chromeleon 7.2 which integrates the peaks and uses a relative migration time library to assign glycan ID.

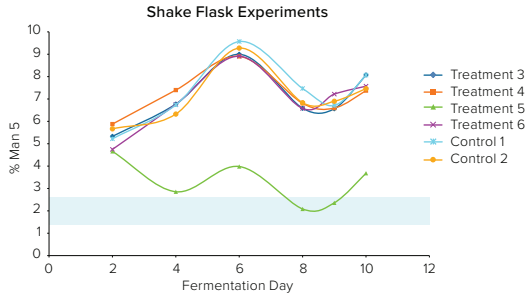

Figure 7: Use of Gly-Q System to screen for the effect of culture conditions on the relative abundance of Man5 on mAb N-glycans produced by a cell line. Shake flask experiment with different media additives. Blue bar represents target Man5 abundance.

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 9A). Results are comparable to data from the same MabThera lot obtained using GlykoPrep/InstantAB labeling and UHPLC profiling (Figure 9B).

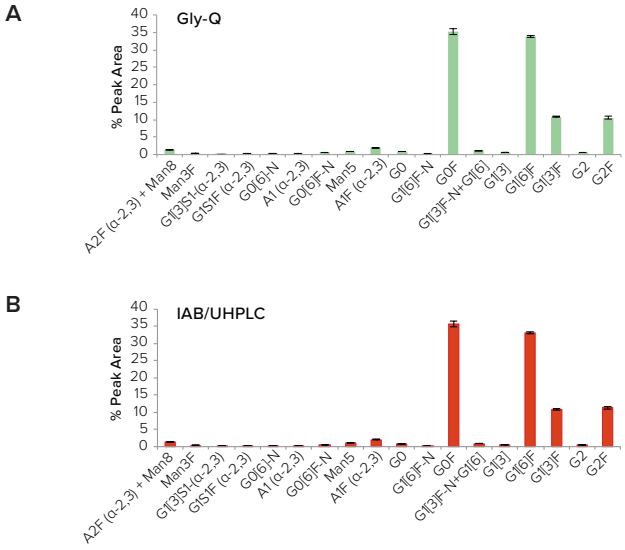

Figure 8: N-Glycan % peak area for replicates of MabThera Lot B6069B03 (n=24) using (A) the Gly-Q System and (B), GlykoPrep InstantAB labeling analyzed by UHPLC. Glycan pairs (Man8 and A2F, G1[3]F-N and G1[6]) were summed up as they coeluted on UHPLC.

CONCLUSIONS

1. We present a novel system for rapid N-glycan analysis.
2. At the front end, simple and automatable sample preparation uses InstantQ dye chemistry to label released N-glycans from Protein A purified cell culture samples.
3. For N-glycan separation and detection, we introduce a simplified CE system with a total run time of 2 minutes per sample.
4. At the back end, peaks are integrated and glycan assignments are made using an automated data analysis approach.
5. The demonstrated sample prep, separation and analysis of 96 samples can be completed in less than 6 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. trices, lacus velit mattis mauris, nec tristique purus elit ac quam

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3. Hossler et al. Glycobiology. 2009 Sep;19(9):936-49

Acknowledgements: BiOptic, Inc.

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