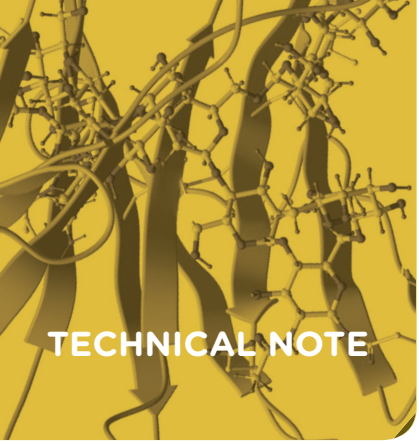

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Automation of Gly-*X* N-Glycan Sample Prep with InstantPC and InstantQ dyes

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SUMMARY

The Gly-*X*™ N-glycan sample preparation protocol, including 5-minute rapid deglycosylation, instant labeling and cleanup is carried out in 96-well format plates compatible with most liquid handling workstations. Here we present methods for automation of Gly-*X* N-glycan sample prep with InstantPC™ (LC/MS) and Gly-*X* with InstantQ™ (CE) on the Hamilton Microlab NIMBUS liquid handler.

The method is designed to process 8–24 samples, utilizing up to 3 full columns of 8 samples. Samples were prepared in a 96-well PCR plate and reagents were prepared per kit instructions. Samples, reagents and any appropriate dilutions of reagents were placed on the platform deck in either vials or into troughs. All subsequent steps were fully automated on the NIMBUS system without further user intervention. Samples were ready for analysis by LC/MS (InstantPC dye) or CE (InstantQ dye) on the Gly-Q™ Glycan Analyzer in about 1.5 hours total time.

Keywords

Automation
Biotherapeutic
Capillary Electrophoresis
Enbrel
Fluorescent Dye
Glycoprotein
HILIC
InstantPC
InstantQ
MabThera
N-Glycans

INTRODUCTION

Biopharmaceutical research and development of monoclonal antibodies has grown exponentially to become the fastest growing class of therapeutic agents. Recombinant monoclonal antibodies contain a conserved N-glycosylation site at Asn297 in the Fc region, with differences in glycosylation patterns observed depending on the host cell line and culture conditions used during protein production.[1] Glycan heterogeneity has been shown to impact the physiochemical and pharmacokinetic properties of therapeutic antibodies, with particular glycoforms having very specific effects on antibody function[1,2] For example, minimizing N-glycans that contain core fucosylation has been shown to enhance Fc receptor binding and antibody dependent cell-mediated cytotoxicity (ADCC) activity.[2] These findings have given rise to engineering efforts to control glycoform profiles for optimum bioefficacy and reliable batch-to-batch consistency.[2,3] Various approaches have been tested to alter glycoprofiles including directed mutations to the protein Fc region, and modifications to the host cell line, either by molecular or chemical interference that redirect cellular glycosylation pathways.[3,4] In light of these efforts, glycan analysis has become an important tool for quality control and analytical characterization of this critical quality attribute (CQA) during therapeutic antibody production and development.[5,6] Here we test two purified recombinant monoclonal antibodies with the goal of developing a streamlined, walkaway N-glycan sample preparation workflow that can be implemented as a part of cell line screening and process development using a standard liquid handler and off-the-shelf sample preparation kits.

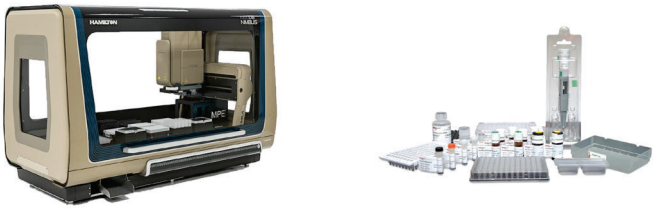


Figure 1: HAMILTON NIMBUS workstation and Gly-*X* N-Glycan sample prep kit.

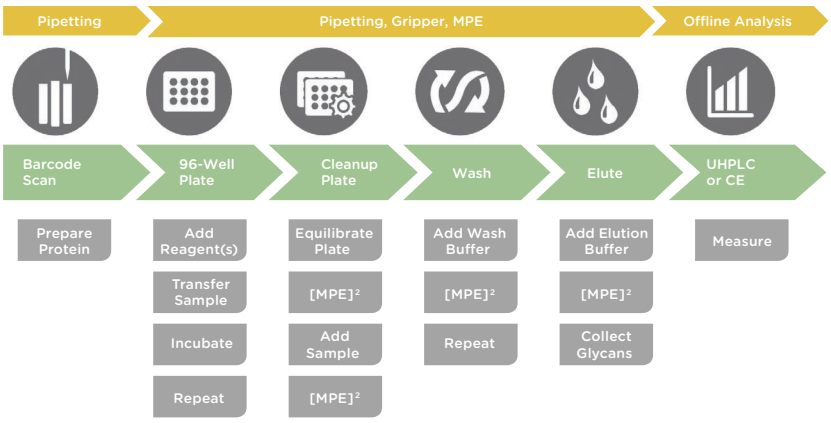


Figure 2: Automation overview: Workflow on Hamilton NIMBUS for Gly-*X* Rapid Release and Labeling with InstantPC™ and with InstantQ™ kits.

METHODS AND MATERIALS

Sample Preparation Kits

Gly-*X* Rapid Release and Labeling with InstantPC Dye Kit (GX96-IPC)

Gly-*X* Rapid Release and Labeling with InstantQ Dye Kit (GX96-IQ)

MabThera® lot # H0102B03,
Enbrel® lot # 1075801

Automation Station

Hamilton Microlab NIMBUS liquid handler with:

- 96-well head
- 2 heater blocks (set at 90°C and 50°C)
- MPE positive pressure automation module

Deck Configuration

- Load tips – 50 µL and 1000 µL, see Figure 4
- Load Reagent Source Plate (empty PCR plate, semi-skirted)
- Load Cleanup Plate stack on deck, not MPE (Gly-*X* Cleanup Plate and waste tray, waste tray remains on deck and is not transferred to MPE)
- Load collection plate
- Preheat heaters to 50°C and 90°C

Reagent Preparation

- Gly-*X* Denaturant – Dilute 1:1 with DI water. Prepare 120 µL for 24 wells. Place in a 0.5 mL Sarstedt screw top vial and place in the reagent block on deck

- N-Glycanase Working Solution – Prepare N-Glycanase working solution according to kit instructions, then dilute 1:1 with DI water. Prepare 120 µL of 1:1 dilution for 24 wells. Place in a 0.5 mL Sarstedt screw top vial and place in reagent block on deck
- InstantDye Working Solutions
  - For InstantPC, prepare 1 vial of InstantPC in 150 µL of InstantPC Dye Solvent per the kit instructions and place on the deck
  - For InstantQ, prepare 1 vial of InstantQ in 400 µL InstantQ Dye Solvent, and mix 1:1 with Activation Reagent, according to the kit instruction. Place in a 0.5 mL Sarstedt screw top vial and place in reagent block on deck
- Cleanup reagents
  - For InstantPC, prepare 100 mL of Load/Wash buffer according to the kit instructions and place into two troughs on deck
  - For InstantQ, place 100 mL ethanol into two troughs on deck
- Elution of labeled N-Glycans
  - For InstantPC, add InstantPC Eluent to trough on deck (100 µL per sample required)
  - For InstantQ, place water in trough on deck. (100 µL per sample required)
- Load Samples
  - Place samples (20 µL each) into columns of a semi-skirted PCR plate and place on deck.

Automation Protocol Design

The automation protocol was developed to provide the most efficient use of the previously installed NIMBUS system, processing 8–24 samples at a time. Glycoprotein samples (20 µL, 2 mg/mL) were placed in the a 300 µL semi-skirted 96-well PCR plate on the NIMBUS deck. Reagents for denaturation, deglycosylation and labeling were placed in vials on the platform deck prior to starting the automation workflow (Figure 3). The automation design begins with transferring required volumes of these stock reagents into a 96-well Reagent Source Plate (using single tips, see Figure 5). The number of columns for each transferred reagent matches the number of columns of glycoprotein samples to be processed (up to 3 columns). The automation design then moves the glycoprotein samples (using 8 channels, see Figure 5) into the wells of the source plate (containing reagents), mixing and then returning to the mixture to the Sample Plate followed by incubation at the specified temperature. In this manner, all incubations are in the Sample Plate, and 8 channels can be used to transfer the samples back and forth from Sample Plate to Reagent Source Plate and back to the Sample Plate. Dye removal and cleanup is performed with the Gly-*X* Cleanup Plate and the NIMBUS MPE positive pressure manifold.

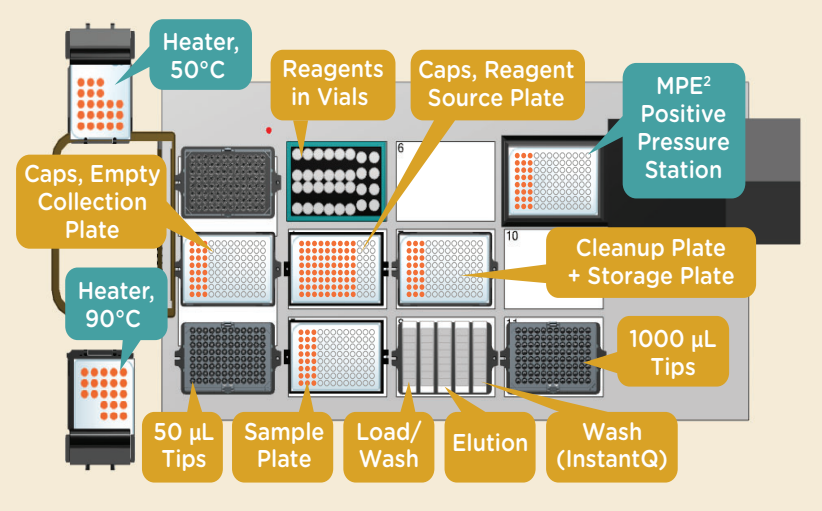


Figure 3: Hamilton NIMBUS deck layout. Location of plates, vials, troughs, heaters and MPE positive pressure manifold.

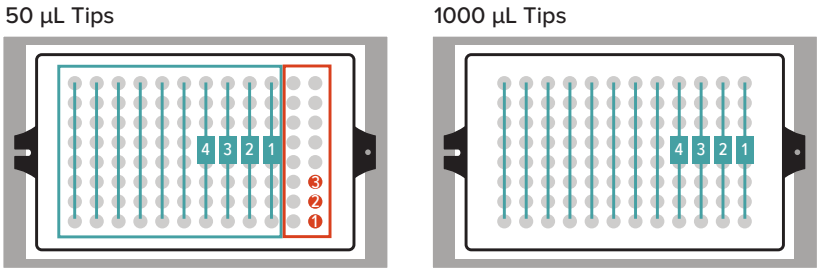


Figure 4: Tip layout. A) 50 µL tips. The first two columns (red) are designated for single tips use tips for dispensing reagents from vials into the Reagent Source Plate. The remaining columns are for multichannel dispensing and transfers. B) 1000 µL tips. All columns are for multichannel dispensing and transfers.

Automation Details

DISPENSE REAGENTS INTO REAGENT SOURCE PLATE

Using single tip mode of the 96-channel head, transfer each of the 3 reagents from tubes into sections of a semi-skirted PCR plate. Dispense such that the number of columns in the Reagent Source Plate for each reagent matches the number of columns of the glycoprotein samples. For 24 samples, dispense 4 µL Gly-*X* Denaturant into columns 1–3; dispense 4 µL of Working N-Glycanase solution into columns 4–6; for InstantDye, dispense into columns 7–9; 5 µL for InstantPC, 20 µL for InstantQ (for 8 samples use columns 1, 4, and 7). For each transfer, pick up a larger volume and dispense in multiple wells to shorten the overall operation.

DENATURE SAMPLES

Using 8 channels on the 96-channel head, pick up the samples and transfer to the corresponding Gly-*X* Denaturant location on the Reagent Source Plate, mix and return to the Sample Plate. Pick up plate and move to 90°C heater. Wait 3 minutes. Move back to original Sample Plate location. Wait 1 minute.

DEGLYCOSYLATE

Using 8 channels of the 96-channel head, pick up the samples and transfer to the corresponding N-Glycanase location on the Reagent Source Plate. Mix and return samples to the Sample Plate. Pick up plate and move to the 50°C heater. Wait 5 minute. Move plate back to the original Sample Plate location and wait 1 minute.

LABEL

Using 8 channels on the 96-channel head, pick up the samples and transfer to the corresponding InstantDye location on the Reagent Source Plate, mix and return to the Sample Plate. Pick up plate and move to 50°C heater. Wait 1 minute. Move back to original Sample Plate location. Wait 1 minute.

CLEANUP AND ELUTE (InstantPC DYE)

Load. Using 8 channels on the 96-channel head, transfer 400 µL of Load/Wash buffer into the first column of the Cleanup Plate. Using the same 8 tips, transfer 150 µL load/Wash buffer into the first column of samples and mix. Using the same tips, transfer the samples to the Cleanup Plate positions with load wash buffer and mix. Repeat for additional columns if appropriate. Once all samples are loaded onto the Cleanup Plate, move the plate to the positive pressure manifold. Apply a step pressure gradient to the plate. (see Figure 5). When complete, move the plate back to original deck location.

Step	Pressure	Duration
1	1 psi	40 seconds
2	3 psi	20 seconds
3	6 psi	20 seconds
4	10 psi	20 seconds
5	55 psi	10 seconds

Figure 5: Pressure Gradient for MPE positive pressure manifold.

Wash. Using 8 new tips per column, dispense 600 µL of Load/Wash buffer to each column of the Cleanup Plate with samples. Move Cleanup Plate to manifold and apply pressure gradient (Figure 5). When complete, move the Cleanup Plate back to original deck location. Using the same tips, repeat wash and pressure steps 2 more times. Move the Cleanup Plate over the top of the Collection Plate.

Elute. Using 8 new tips, transfer 100 µL of InstantPC Eluent to each of the columns containing samples. Move the stack containing Elution Plate and Cleanup Plate onto the manifold and apply pressure gradient (Figure 5). When complete, move the stack back to original location. Move the Cleanup Plate off the Collection Plate back to the waste plate location. Using 8 new tips per column, mix eluted samples. Procedure is complete.

CLEANUP AND ELUTION (InstantQ DYE)

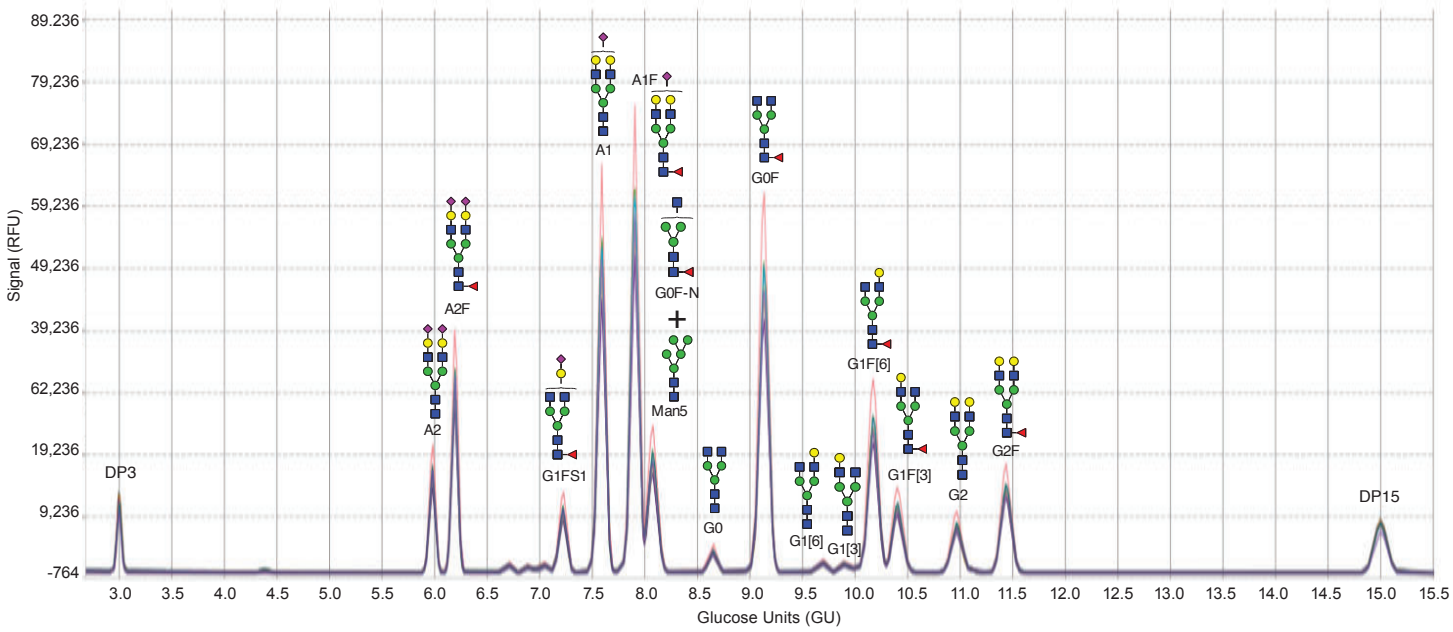
Prime. Using 8 channels on the 96-channel head, transfer 400 µL of water to each well on the Cleanup Plate. Once all columns to be used are loaded with water, move the Cleanup Plate to the positive pressure manifold. Apply a step pressure gradient to the plate (Figure 5). When complete, move the Cleanup Plate back to original deck location. Using 8 channels on the 96-channel head, transfer 600 µL of ethanol to each well on the Cleanup Plate. Once all columns to be used are loaded with ethanol, move the Cleanup Plate to the positive pressure manifold. Apply a step pressure gradient to the plate (Figure 5). When complete, move the Cleanup Plate back to original deck location. Repeat ethanol wash a second time.

Load. Using 8 channels on the 96-channel head, transfer 400 µL of ethanol into the first column of the Cleanup Plate. Using the same 8 tips, transfer 150 µL of ethanol into the first set of samples and mix. Using the same tips, transfer the samples to the Cleanup Plate positions with ethanol and mix. Repeat for additional columns if appropriate. Once all samples are loaded on plate, move the plate to the positive pressure manifold. Apply a step pressure gradient to the plate (Figure 5). When complete, move the Cleanup Plate back to original deck location.

Wash. With 8 new tips per column, dispense 600 µL of ethanol to each column of the Cleanup Plate with samples. Move Cleanup Plate to manifold and apply pressure gradient (Figure 5). When complete, move the Cleanup Plate back to original deck location. Using the same tips, repeat wash and pressure steps 2 more times (total of 3 washes).

Elute. Move the Cleanup Plate over the top of the Collection Plate. Using 8 new tips, transfer 150 µL of water to each of the columns containing samples. Move the stack containing Elution Plate and Cleanup Plate onto the manifold and apply pressure gradient (Figure 5). When complete, move the stack back to original location. Move the Cleanup Plate off the Collection Plate back to the waste plate location. Using 8 new tips per column, mix eluted samples. Procedure is complete.

A) Hamilton Method



B) Manual Method

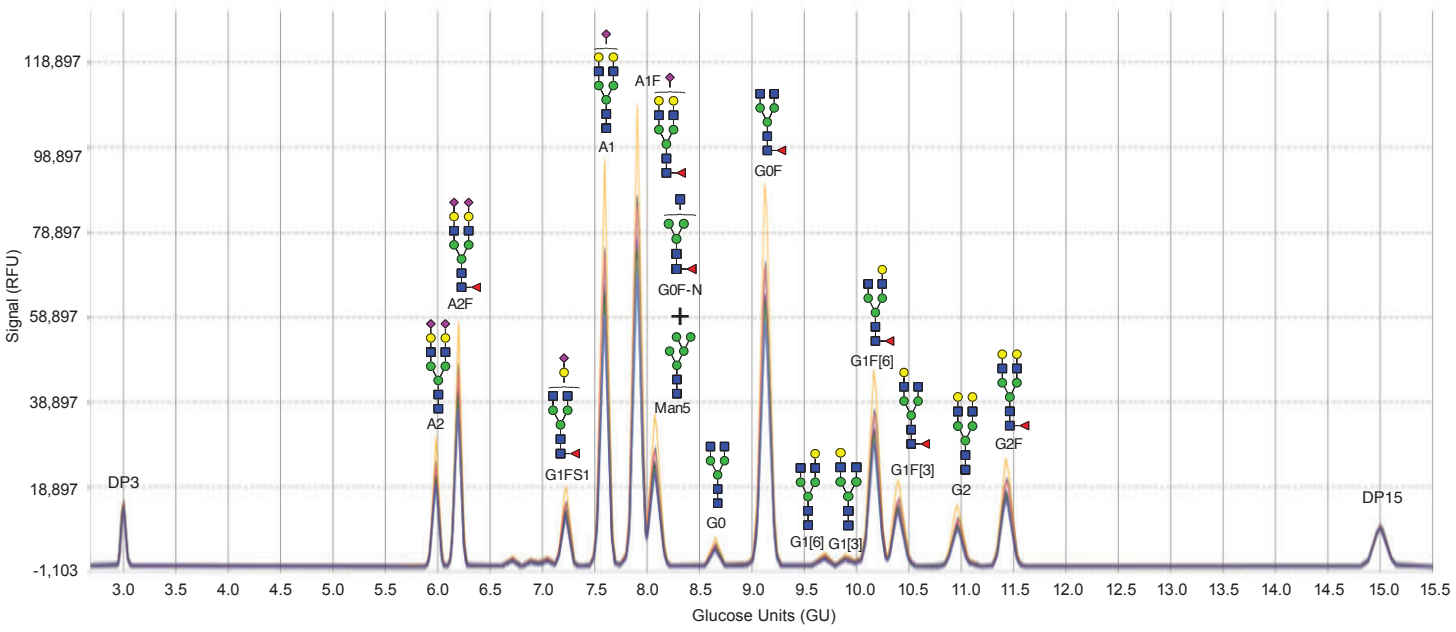


Figure 10: Overlay of InstantQ labeled N-glycans prepared from Enbrel samples (n=8) separated with the Gly-Q System. A) Sample preparation performed on the NIMBUS automation platform; B) Sample preparation using the manual method.

A

Glycan	Average % Area		Stdev	
	Hamilton	Manual	Hamilton	Manual
G0	1.16	1.17	0.03	0.02
G0F	17.87	18.16	0.19	0.10
G1F[6]	9.95	10.12	0.12	0.04
G1F[3]	4.55	4.55	0.08	0.03
Man5 + G0F-N	6.95	7.05	0.07	0.06
G2	3.57	3.54	0.06	0.04
G2F	6.01	5.98	0.11	0.07
G1FS1 (2,3)	3.20	3.17	0.03	0.02
A1 (2,3)	15.82	15.46	0.12	0.09
A1[6]F (2,3)	18.87	18.64	0.12	0.09
A2 (2,3)	4.15	4.17	0.10	0.02
A2F(2,3)	7.90	8.00	0.16	0.07

B

Glycan	Average % Area		Stdev	
	Hamilton	Manual	Hamilton	Manual
G0F	36.97	37.25	0.14	0.26
G2F	11.68	11.66	0.23	0.13
G1F[6]	35.24	35.46	0.26	0.17
G1F[3]	11.40	11.55	0.12	0.10
Man5 + G0F-N	2.94	2.97	0.12	0.04
A2F(2,3)	1.19	1.26	0.09	0.08

Figure 11: Comparison of Gly-*X* InstantQ sample preparation using NIMBUS automation with the manual method for A) Enbrel InstantQ-labeled N-glycans; B) Rituxan InstantQ-labeled N-glycans.

CONCLUSIONS

1. Adaptation of the Gly-*X* protocol to the Hamilton NIMBUS liquid handler used off-the-shelf InstantPC and InstantQ kits (GX96-IPC, GX96-IQ) and did not require modification of the existing installed NIMBUS system.
2. Automation provides walkaway Gly-*X* N-glycan sample preparation in approximately 1.5 hrs.
3. No crosstalk was detected between N-glycan samples prepared on the NIMBUS system.
4. Results from manual and automated methods are comparable, allowing day to day operational flexibility.

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