Agilent

Agilent Gly-X N-Glycan Rapid Release and Labeling with APTS Express Kit (formerly ProZyme)

User Manual

Notices

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GX96-APTS and GX96-APTS-GQ
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Contents

Introduction

The Agilent Gly-X N-Glycan Rapid Release and Labeling with APTS Express kit (formerly ProZyme) utilizes a novel in-solution enzymatic protein deglycosylation with N-Glycanase (PNGase F) followed by fast labeling of released N-glycans with APTS (8-aminopyrene-1,3,6-trisulfonate) dye (Figure 1). The APTS labeling reaction is performed on the cleanup plate, eliminating the need to dry the sample before labeling. After a simple free dye cleanup step, the glycan samples are ready for analysis by capillary electrophoresis methods. The protocol is simple, rapid (~2.5 hours) and suitable for automation. The APTS dye delivers proven fluorescence performance within well-established glycan analysis workflows.

The kit is designed in two versions:

- **Agilent GX96-APTS-GQ (formerly ProZyme)**: Includes Gly-Q cartridge and labeled migration standards for APTS-labeled glycan separation and detection on the Agilent Gly-Q CE instrument (formerly ProZyme).
- **Agilent GX96-APTS (formerly ProZyme)**: Sample preparation only, for separation of APTS-labeled glycans on other CE and LC platforms.

Other benefits include:

- Flexible, high-throughput format: process 1 to 96 samples
- High sensitivity detection
- Two-minute CE separations using the Gly-Q system
- Rapid N-glycan analysis using Gly-Q Manager software

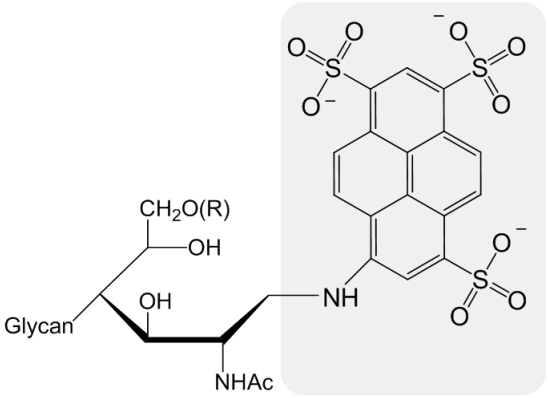


Figure 1. N-Glycan labeled with APTS dye

Kit Components

The Agilent Gly-X with APTS Express kit (formerly ProZyme) (GX96-APTS) consists of 3 modules. Each module provides enough reagents for up to 96 samples per run.

Table 1 Kit components

Module	Component	Units	Storage
Gly-X Deglycosylation Module GX96-100	Gly-X Deglycosylation Plate, 96 wells	1	RT
	Gly-X N-Glycanase, 1 mg/mL, 120 µL	1	4 °C
	Gly-X Digestion Buffer, 240 µL	2	4 °C
	Gly-X Denaturant, 240 µL	1	4 °C
Gly-X APTS Labeling Module GX96-601	Gly-X APTS Dye Solution, 336 µL	1	-20 °C
	Gly-X APTS Reductant, 136 µL	1	-20 °C
	Gly-X APTS Catalyst, 370 µL	1	-20 °C
	Gly-X APTS Finishing Reagent, 230 µL	1	-20 °C
Gly-X APTS Cleanup Module GX96-602	Gly-X Cleanup Plate B	1	RT
	Gly-X APTS Cleanup Solution Concentrate (100 mM ammonium formate), 1.3 mL	1	RT
	Gly-X Collection Plate B, 96 wells	1	RT
	Waste Tray for vacuum manifold	1	RT
	Gly-X Used-Well Sealing Caps, Black (for Cleanup Plate)	1	RT

Two additional modules are included with GX96-APTS-GQ kit:

Module	Component	Units	Storage
Gly-Q Cartridge Module GQ103	Gly-Q Separation Buffer	1	RT
	Gly-Q Mineral Oil	1	RT
	Gly-Q Reagent Tray	1	RT
	200 µL tubes (clear) for optional standards	2	RT
	Gly-Q Cartridge	1	RT
Gly-Q Alignment Standards Set			
GKSQ-505	Gly-Q GU Ladder (GKSQ-503), 100 µL	1	-20 °C
	200 µL tubes (blue) for Migration Standards and (yellow) for GU Ladder	4 each	RT
	Gly-Q Migration Standards GKSQ-500, 100 µL	1	-20 °C
Gly-Q Injection Plate	Gly-X APTS Transfer Plate (96 well PCR plate)	1	RT

NOTE **Gly-X APTS Express Heat Block (GX500) is required for APTS Express labeling on the Cleanup plate. Please contact Agilent for more information.**

NOTE **Gly-X Heater Lid (GX600) is required. Please contact Agilent for more information.**

NOTE **Gly-X with APTS Express 24-ct kit (GX24-APTS) contains 30 µL Gly-X N-Glycanase, one vial Gly-X Digestion Buffer, one vial APTS Dye Solution (24-ct, 84 µL), one vial APTS Reductant (24-ct, 50 µL), one vial APTS Catalyst (24-ct, 94 µL), and all other components listed in the table.**

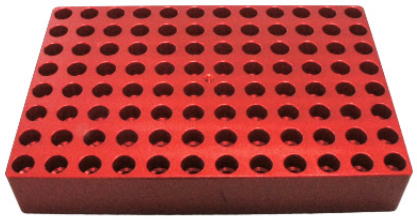


Figure 2. Gly-X APTS Express Heat Block (GX500)

Figure 3. Gly-X Heater Lid (GX600)

Equipment and Reagents Provided By User

- Two dry block heaters with 96 well PCR heat blocks. One of the heaters can be substituted with a 96-well thermocycler. Denaturation and deglycosylation can be performed using a thermocycler or dry block heater for example, Thermo Scientific 88871001 Compact Dry Block Heater; a custom heat block (GX500) is required for the labeling step. If necessary, it is possible to perform all incubations using the GX500 custom red heat block, but this option is to be avoided as it requires additional time for block equilibration.
- Gly-X Heater Lid (GX600)
- Vacuum manifold (Millipore MSVMHTS00)
- Vacuum pump (Millipore WP6211560, 110 V; WP6122050, 220V; Welch WOB-L Pump 2522)

NOTE **If you have a vacuum manifold and pump other than the Millipore model suggested, please contact Agilent at https://www.agilent.com/en/contact-us/page for guidance.**

- DI water, ~100 µL per sample
- Acetonitrile (ACN), MS-grade (Fisher A955-4 recommended), ~4 mL per sample
- Trifluoroacetic acid (TFA), 15 µL per sample
- Optional - VWR 10 kDa Centrifugal Filters (USA 82031-348; EU 516-0229)

Sample Prep Considerations

In general, glycoprotein samples should be prepared to a maximum of 2 mg/mL in a low salt neutral buffer, free of detergents. Higher concentration samples should be diluted in water or 50 mM HEPES, pH 7.9.

Other sample considerations include:

- Samples in salt-containing buffers (~150 mM salt) including PBS are compatible with the kit. The preferred diluents are water or a matching buffer of 50 mM HEPES, pH 7.9.
- Samples below 2 mg/mL can be used depending on the protein sample and desired number/volumes used for injections.
- The maximum amount of protein suggested for each reaction is 40 µg (20 µL of a 2 mg/mL solution), though it may be possible to use more than 40 µg depending on the glycoprotein (see **"FAQs"** on page 28).
- Protein samples should not be below pH 5.5. Adjust the pH before starting the protocol or add 3 µL of the Gly-X Digestion Buffer per 20 µL sample in **step 1** on **page 12**.

If a precipitate is observed upon incubation at 90 °C (step 2.4), review the sample prep and sample buffers for salts, low pH and/or possible interfering detergents. If you have questions on the compatibility of your sample buffer with the Gly-X protocol, please contact Agilent at **https://www.agilent.com/en/contact-us/page**.

Optional sample buffer exchange with 10 kDa MWCO spin columns (for example, VWR cat# 82031-348):

- 500 µL DI water added to spin column
- Add glycoprotein (40 µg, 20 µL of 2 mg/mL)
- Centrifuge at 12,000 x g, 10 minutes
- Add additional 500 µL DI water, centrifuge for additional 10 minutes at 12,000 X g
- Bring sample up to initial starting volume with DI water (20 µL)

Protocol

Getting started

1 Prepare samples (see **"Sample Prep Considerations"** on page 9).

2 Set up heaters and heat blocks using one of these options:

Available equipment	Instructions
Two heaters with regular 96 well PCR plate heat block and GX500 custom red heat block	Set two independent heat blocks to 90 °C and 50 °C. Use the GX500 custom red heat block for the 50 °C set point, as this will be reset to 55 °C for APTS labeling.
Thermocycler, heater with GX500 custom red heat block	Program thermocycler to 90 °C and set the GX500 custom red heat block to 55 °C.
Single heater with GX500 custom red heat block	Set a heater containing the GX500 custom red heat block to 90 °C. It is possible to perform all incubations using the GX500 heat block, but this is to be avoided as it requires additional time for block equilibration.

NOTE

One heater must be fitted with custom heat block (GX500) to accommodate the Cleanup plate for the APTS labeling step (55 °C). The 55 °C step is recommended to be carried out in a laboratory fume hood.

3 Prepare the working solutions as shown in **Table 2**, **Table 2A**, and **Table 2B**.

Table 2 Working solution instructions. For solutions containing ACN, use tubes, basins or plates that are compatible with organic solvents (polystyrene is not compatible)

Working Solution	Instructions	Notes
N-Glycanase Working Solution	Mix N-Glycanase and Gly-X Digestion Buffer 1:1 (v/v). Per sample, prepare 2.4 µL of working solution; for eight wells mix 9.6 µL N-Glycanase and 9.6 µL Digestion Buffer.	2 µL required per sample, mix 20% overage of working solution.
APTS Working Solution	See **Table Table 2A** on page 11 for Working Solution instructions for various sample numbers. ACN, APTS Reductant, APTS Catalyst and APTS Solution are mixed in 6.25:1:2.5:2.75 ratio. Always add APTS Solution last. Mix with pipette or vortex.	15 µL required per sample. Make 25% overage. APTS Working Solution is stable at -20 °C, one month and 10 freeze thaw cycles. **Note: Use tubes or plates that are compatible with organic solvents (polystyrene is not compatible)**
Loading Solution 99% ACN/1% TFA	Prepare 99% ACN:1% TFA solution.	1.4 mL required per sample. Always prepare fresh with at least 20% overage (1.68 mL per sample)
Cleanup Solution A 90% ACN/10% water	Prepare 90% ACN:10% DI water solution.	1.2 mL required per sample. Always prepare fresh with at least 20% overage (1.44 mL per sample).

Table 2 Working solution instructions. For solutions containing ACN, use tubes, basins or plates that are compatible with organic solvents (polystyrene is not compatible) (continued)

Working Solution	Instructions	Notes
Cleanup Solution B 85% ACN/15% 25 mM ammonium formate	See **Table Table 2B** for volumes needed for various sample numbers. 1. Dilute APTS Cleanup Solution Concentrate in DI water in 1:3 ratio (4X dilution). 2. Mix with ACN for 85% ACN:15% diluted Cleanup Solution.	0.3 mL required per sample. Always prepare fresh with at least 20% overage (0.36 mL per sample).

Table 2A APTS Working Solution instructions, includes ~20% overage. Always add APTS Solution last. Mix with pipette, or vortex.

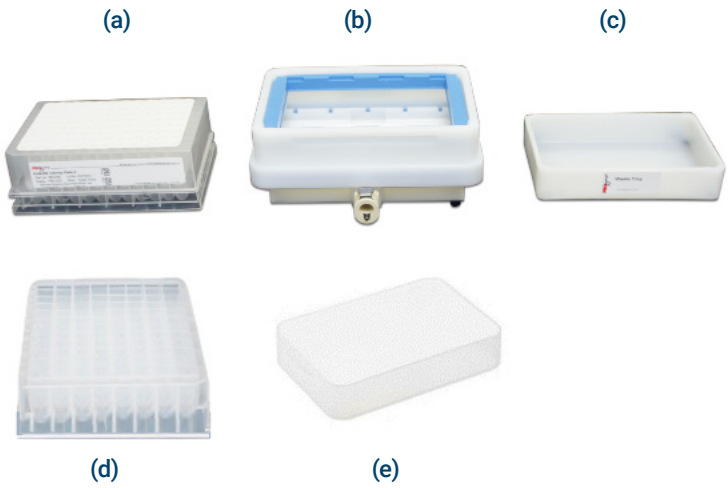
Number of Samples	ACN (µL)	APTS Reductant (µL)	Catalyst (µL)	APTS Solution (µL)	Total (µL)	Vol/Sample (µL)
n	9.375/sample	1.5/sample	3.75/sample	4.125/sample	18.75	15
4	37.5	6	15	16.5	75	15
8	75	12	30	33	150	15
24	225	36	90	99	450	15

Table 2B Cleanup Solution B instructions, includes 20% overage.

Number of Samples	APTS Cleanup Solution Concentrate (4X) (µL)	Water (µL)	ACN (µL)	Total (µL)	Vol/Sample (µL)
n	13.5/sample	40.5/sample	306/sample	360	300
4	54	162	1224	1440	300
8	108	324	2448	2880	300
24	324	972	7296	8640	300

4 Have on hand items for the cleanup station:

- **a** Gly-X Cleanup Plate B
- **b** Vacuum manifold connected to vacuum pump
- **c** Gly-X Waste tray
- **d** Gly-X Deep Well Collection Plate B
- **e** Heater Lid [Agilent GX600 (formerly ProZyme)]



Gly-X Deglycosylation

Deglycosylation

1 Add 2 µL of Gly-X Denaturant (orange cap vial) to the bottom of the Gly-X Deglycosylation Plate.

NOTE **For samples with a pH of 5.5 or lower, also add 3 µL Gly-X Digestion Buffer (white cap vial), mix thoroughly with pipette.**

2 Add 20 µL of each glycoprotein sample (~2 mg/mL) to the bottom of the Gly-X Deglycosylation Plate. After each addition, mix thoroughly with a pipette.

3 Tap plate on benchtop to collect samples on bottom of wells (or spin).

4 Incubate uncovered at 90 °C for 3 minutes.

NOTE **If a precipitate forms at this point, review the sample buffer composition (See page 9).**

5 Remove plate, place at room temperature for two minutes before adding N-Glycanase.

NOTE **If using a thermocycler or single GX500 custom red heat block, reset temperature to 50 °C for digestion.**

6 Add 2 µL of N-Glycanase Working Solution to each sample. Mix well using a pipette.

7 Tap on benchtop to collect samples on bottom of wells (or spin).

8 Incubate uncapped at 50 °C for 5 minutes.

Finishing

1 Add 2 µL of Finishing Reagent, mix well.

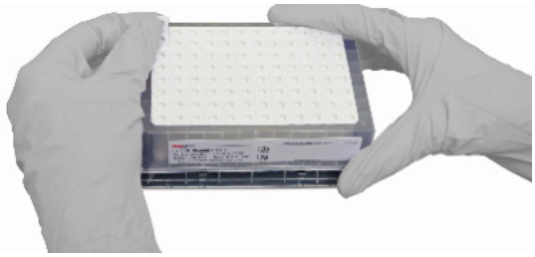
2 Incubate uncapped at 50 °C for 10 minutes.

NOTE **A precipitate is normal. Finishing Reagent converts the N-glycans into the -OH form suitable for reductive amination labeling by APTS.**

3 Remove plate from heat block and proceed to Glycan Loading. Reset the GX500 custom red heat block temperature to 55 °C for APTS labeling.

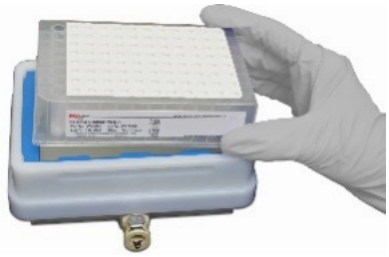
Glycan loading onto matrix

1 Prepare Gly-X Cleanup Plate B for the required number of wells by carefully removing white caps.



2 Place Waste Tray in the Vacuum Manifold and assemble the complete manifold.

3 Remove Cleanup Plate B from the storage plate (round bottom) and install on the Vacuum Manifold over waste tray.



NOTE **Cleanup Plate B ships with a storage plate (round bottom) to protect the tips. Retain the storage plate for later use.**

4 With a multichannel pipette, add 700 µL of the Loading Solution (ACN/1% TFA) to the required number of wells in APTS Cleanup Plate B. Do not apply vacuum.

NOTE **Use basins or plates that are compatible with organic solvents (polystyrene is not compatible).**

5 Transfer the entire sample (~25 µL) from Deglycosylation Plate into the corresponding wells of Cleanup Plate B. Mix by pipette (discard tips after mixing).

6 Repeat steps 4 and 5 until all rows have been transferred to Cleanup Plate B.

7 Apply <5 inHg vacuum. Let the solution pass through, this may take three to five minutes. Increase vacuum to 10 inHg after five minutes at <5 inHg, if needed.

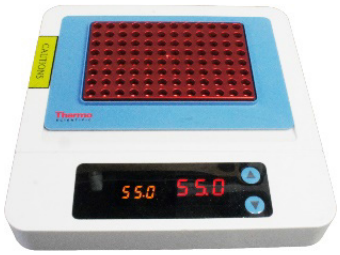
NOTE **Vacuum guidelines should be strictly followed. Performing vacuum steps at a higher than suggested level can impact results.**

8 Add 700 µL of Loading Solution (ACN/1% TFA) to each well. Apply <5 inHg vacuum. Let the solution pass through.

9 The glycans are now loaded on the Cleanup Plate B matrix, proceed to APTS Express labeling.

On-Matrix APTS express labeling

1 Add 15 µL of APTS Working Solution to each well of Cleanup Plate B. The solution should be added to the membrane, not on the plate walls.



2 Place Cleanup Plate B directly onto the surface of the 55 °C custom red heat block.

NOTE **A heater must be fitted with Agilent modified red 96 well PCR heat block (GX500) to accommodate Cleanup Plate B.**

3 Cover the Cleanup Plate with Heat Block Lid and incubate at 55 °C for one hour.

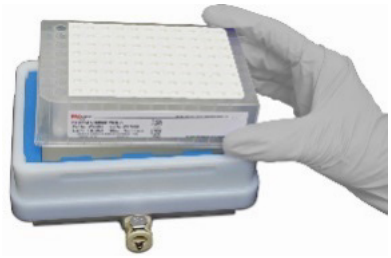
NOTE **A laboratory fume hood is recommended for APTS labeling, even though the heater is covered.**

APTS cleanup

Wash

1 Remove the Waste Tray from manifold, empty. Reinstall the Waste Tray on to the manifold.

2 Install Gly-X Cleanup Plate B on top of the vacuum manifold.



3 Add 500 µL of Cleanup Solution A (ACN/10% water) to each well in the Cleanup Plate. Add the solution to the walls to wash down excess labeling reagent.

4 Turn on the pump, apply <5 inHg vacuum and let the solution pass through. This should take ~three minutes.

NOTE

Follow the vacuum strength (5 inHg) and time guidelines - passing the solutions through the plate too quickly will impact results. If the wells are not empty, increase vacuum to 10 inHg and wait a further three minutes. Remember to reduce vacuum to <5 inHg before the next step. The same applies to subsequent Cleanup steps. Collect the eluent into the Waste Tray.

5 Add 300 µL of Cleanup Solution B (ACN/15% 25 mM ammonium formate) to each well in the Cleanup Plate, washing any leftover Cleanup Solution A from the walls.

NOTE

Avoid leaving drops on the walls - tapping the plate will help (always with the vacuum off)

6 Apply <5 inHg vacuum and let the solution pass through. This should take ~three minutes.

7 Add 300 µL of Cleanup Solution A (ACN/10% water) to each well in the Cleanup Plate, washing any leftover Cleanup Solution B from the walls.

8 Apply <5 inHg vacuum and let the solution pass through. This should take ~three minutes.

9 Add 300 µL of Cleanup Solution A (ACN/10% water) to each well in the Cleanup Plate.

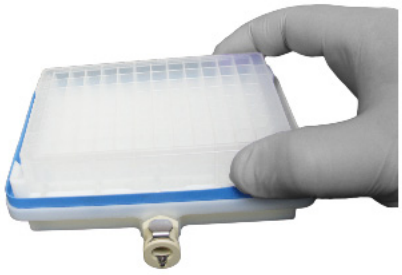
10 Apply <5 inHg vacuum and let the solution pass through. This should take ~three minutes.

11 Take the Cleanup Plate with manifold top off the manifold base. Set aside.

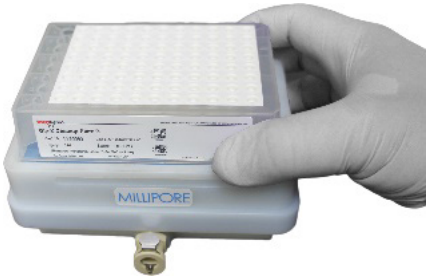
12 Take the Waste Tray off the manifold.

Elute

1 Install the Gly-X Deep Well Collection Plate B.



2 Assemble the manifold and install the Cleanup Plate B on the vacuum manifold over Deep Well Collection Plate.



3 Add 150 µL of DI Water to each well of the Cleanup Plate.

4 Apply <5 inHg vacuum and collect cleaned labeled glycan samples into the Collection Plate B.

NOTE **Wells should empty in ≤ five minutes. If the wells are not empty, increase the vacuum to 7.5 inHg and wait a further five minutes.**

5 Increase the vacuum to 10 inHg to collect the remaining eluent.

6 Turn off pump and tap the manifold on the bench to release drops from bottom of clean up plate.

7 Release the vacuum, remove the cleanup plate to fully release vacuum, then disassemble manifold and remove the Collection Plate.

8 Mix each sample with a pipette.

NOTE **This final, post-elution mixing step is critical for consistent results.**

9 Seal the Deep Well Collection Plate B with Sealing Film.

10 Add Black Used Well Sealing Caps (g) to used Cleanup Plate B wells, place the plate on the round bottom storage plate (h), return to bag and store at RT.

11 APTS-labeled glycan samples are ready for analysis. Samples may be stored at -20 °C for at least six months, or 4 °C for up to five days.

NOTE **Black cap strips (g) should be placed on wells used in previous cleanup procedures to prevent reuse of wells.**

NOTE **Cleanup Plate storage (round bottom) plate (h) should be used to store Cleanup Plate. Store in bag provided at room temperature.**

Analysis Of Labeled Glycans

Excitation/Emission wavelengths for APTS Glycans

- Excitation: 473 nm
- Emission: 520 nm

APTS-labeled N-glycans are typically separated by capillary electrophoresis (CE), although hydrophilic interaction liquid chromatography (HILIC) may also be used (see See **"FAQs"** on page 28.). Instructions are given below for setting up a sequence and running APTS-labeled samples on Agilent Gly-Q Glycan Analysis CE system with detection by LED-induced fluorescence (LEDIF).

For separation of APTS glycans on other CE instruments, please follow the manufacturer's instructions. Examples of APTS glycan separations using the Sciex PA800 plus may be found in our poster "Orthogonal Methods for Glycoanalysis: CE and UPLC" and associated method details. Methods for separation of APTS glycans using a DNA sequencer is described by Reusch *et al.*[1].

Analysis of APTS Glycans with the Agilent Gly-Q system

GLY-Q and GLY-Q manager startup

1. Open Gly-Q Manager software.
2. Turn on the Gly-Q instrument.
3. Turn on the Gly-Q vacuum pump.
4. Prepare Gly-Q Cartridge and insert into instrument (see See **"Appendix A"** on page 23.)
   - Each cartridge can be used for up to 120 injections, see the Gly-Q Manager info bar display for the number of Cartridge Runs.
5. Prepare a Gly-Q Reagent Tray by adding 4 mL each of Park, Wash, Separation and Clean solutions to their respective reservoirs.
   a. Park: 4 mL DI water, layer on mineral oil to cover if cartridge is stored on system >five hours.
   b. Wash and Clean: 4 mL DI water
   c. Separation: 4 mL Gly-Q separation buffer (provided with kit)

Preparing sample plate

1. Transfer 40 µL APTS-labeled N-glycan samples from the Deep Well Collection Plate into wells of the Gly-X APTS Transfer Plate (96 well PCR plate).
2. Store the remaining APTS-labeled N-glycan samples in the Deep Well Collection Plate after sealing. Samples may be stored at -20 °C for at least six months, or 4 °C for up to five days.

Setting up a sample sequence

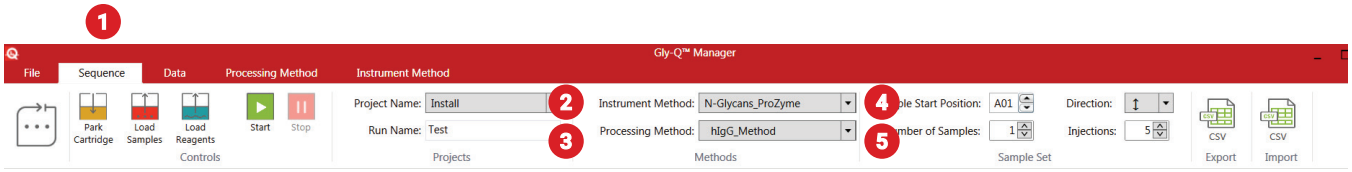
1. In Gly-Q Manager, navigate to the **Sequence Tab** (default start up tab).
2. Select or enter a Project Name.
3. Name your Run.
4. Select **Instrument Method**.
5. Select **Processing Method**.

NOTE **Pre-installed Methods are recommended.**

NOTE **The Instrument Method defines the location of the Migration Standards (upper and lower) (MS) and the GU ladder (GU). To change these positions, or to use additional standards, navigate to the Instrument Methods Tab, create a new method, save, and then select the new method in the Sequence Tab.**

NOTE **An Instrument Method can only be modified prior to data acquisition.**

NOTE **A Processing Method can be changed prior to and after data acquisition.**



6 Select starting position of the 96-well plate (first sample to be analyzed).

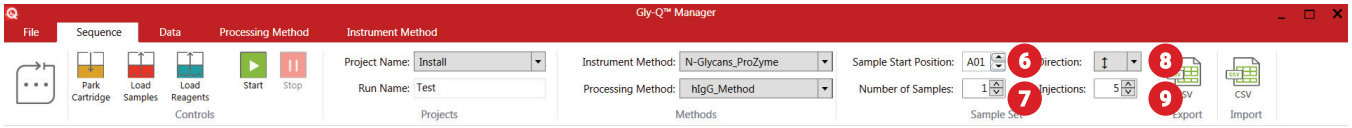
7 Enter number of samples.

8 Select direction (rows versus columns) and number of injections per well.

9 Select number of injections per sample.

NOTE **Sample names can be edited on the Sequence Preview table.**

NOTE **Sample lists can be imported and exported using the Import/Export Sequence functions. Supported file types are.xls and .csv.**

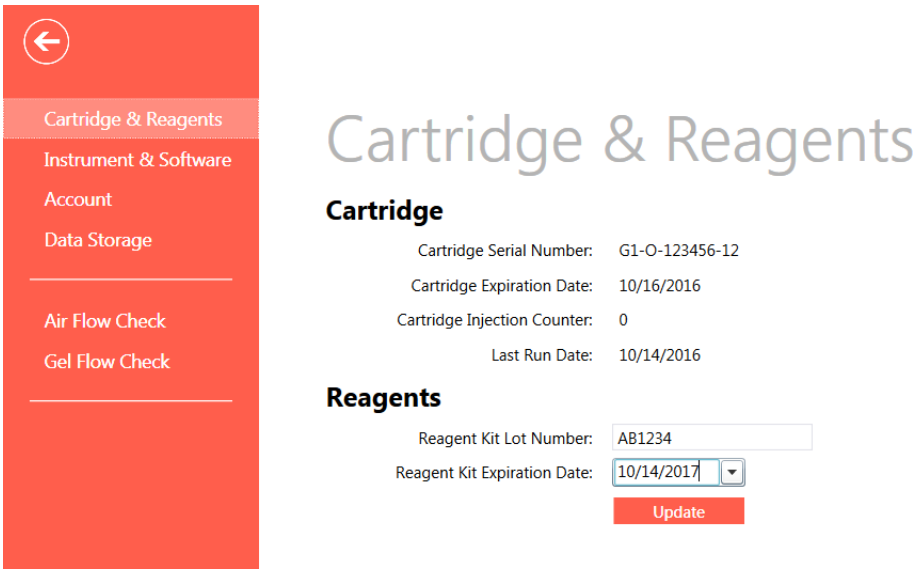


Checking/Editing reagent lot information

1 In Gly-Q Manager, navigate to the **File** tab.

2 Under Cartridge and Reagents verify Reagent Kit Lot Number and Reagent Kit Expiration Date. Edit if needed.

NOTE **Cartridge Serial Number, Expiration Date, Injection Counter and Last Run Date are updated automatically.**



Starting a run

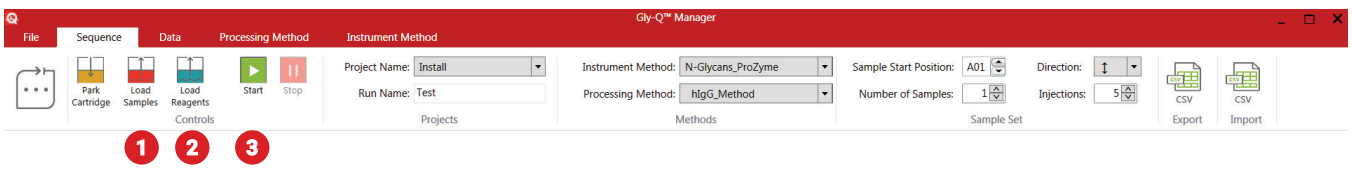
1 Select the **Load Samples** button at the top left of the Sequence Tab. This will prompt the instrument to rotate the sample plate holder to the front of the instrument window. Insert Sample Plate.

2 Select the **Load Reagents** button, at the top left of the Sequence Tab. This will rotate the reagent wells to the front of the instrument window. Transfer 50 µL each of Migration Standards (MS, blue dot on cap) and GU Ladder (GU, yellow dot on cap), into the color-coded PCR tubes provided. Pipette 30 µL of mineral oil on top of both the Migration Standard and the GU Ladder. Place into the appropriate locations as shown in the Sequence Tab Reagents and Standards diagram. Install the Reagents Tray with Park, Wash, Separation and Clean buffer solutions.

NOTE **Remember to close the Gly-Q instrument door after loading samples and reagents.**

3 Select **Start** from Sequence Tab screen to begin sample sequence.

NOTE **Instrument first runs a long high voltage prime before injection of the GU ladder.**



Completing a run

1. After the samples have been processed, the system Cartridge will return to Park.
2. Use the “Load Sample” and “Load Reagents” buttons to remove sample plate and reagent tray.
   a. Migration Standard and GU Ladder tubes can be capped and stored at -20 °C directly in PCR tubes. For reuse, thaw and centrifuge briefly to bring mineral oil to the surface.
   b. APTS-labeled N-glycan samples may be stored at -20 °C for at least six months, or 4 °C for up to five days.
3. Store the cartridge.
   a. To store on the instrument: Press the **Park** button before shutting down the system. The cartridge can remain on the system for up to three days. Mineral Oil must be layered onto the Park solution.
   b. For longer term storage (>three days), remove the cartridge from the instrument and place tape over the cartridge cap (to cover the pin hole). Return it to the original clamshell packaging. The cartridge tip must be in contact with the gel block inside the package. Place inside the foil pouch, and store vertically at room temperature.

Appendix A

Gly-Q cartridge preparation

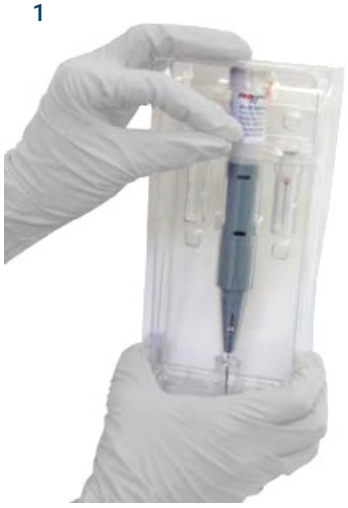
1 Open clamshell container (keep this for storage between uses).

2 Remove cartridge and pin (red) from package.

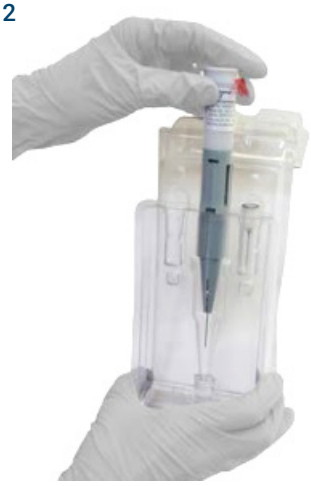
NOTE **The pin is used to puncture the top of the cartridge prior to first use; it is stored in the clamshell container.**

3 Remove tape from cartridge cap (retain top tape for storage of cartridge between uses).

1



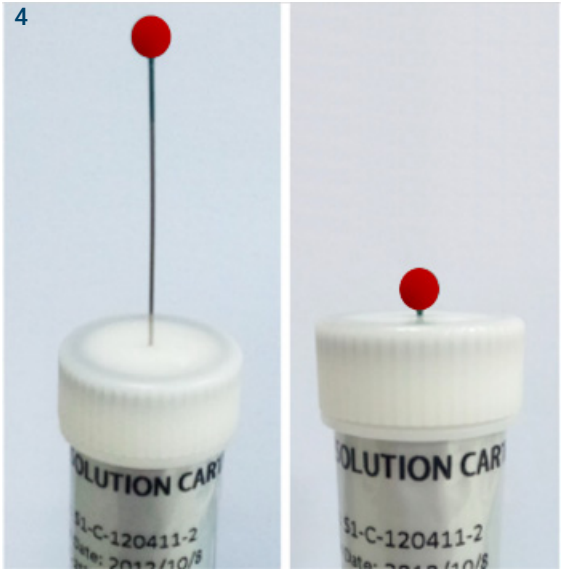
2



3

4 Use pin to puncture the top of the cartridge prior to initial use. Push pin all the way in, a slight resistance should be encountered when performing this step.

4



5 Prime the Cartridge for use, and insert into the Gly-Q Instrument as directed in the Gly-Q System User Manual (GQ2100).

NOTE **Handle the cartridge tip with care. The glass capillary extends beyond the metal tip. Do not allow the tip to contact hard surfaces.**

Appendix B

Peak assignment with Sialidase A

Glyko Sialidase A is available to aid in Gly-Q peak assignment. Sialidase A (GK80040) releases α(2,3)-, α(2,6)-, α(2,8)-, and α(2,9)-linked sialic acids from APTS-labeled glycans causing the peaks on Gly-Q electropherogram to move from sialylated to neutral GU windows. Other exoglycosidases are available and digestion conditions may vary depending on the enzyme, please contact Agilent for details. For example, Sialidase S (GK80021) releases α(2,3)-linked sialic acid from glycans.

Desialylation with Sialidase A

Sialidase A (GK80040) releases α(2,3)-, α(2,6)-, α(2,8)-, and α(2,9)-linked sialic acid from APTS-labeled glycans.

1 Add 1 µL of Sialidase A (product code GK80040) to PCR plate wells.

2 Transfer 75 µL of APTS-labeled glycans to each well.

3 Incubate at 50 °C for 10 minutes.

4 Load the plate onto Gly-Q (**Figure 4**), and run with the same Instrument and Processing methods as untreated samples. Separation by HILIC is also an option (**Figure 5** on page 26).

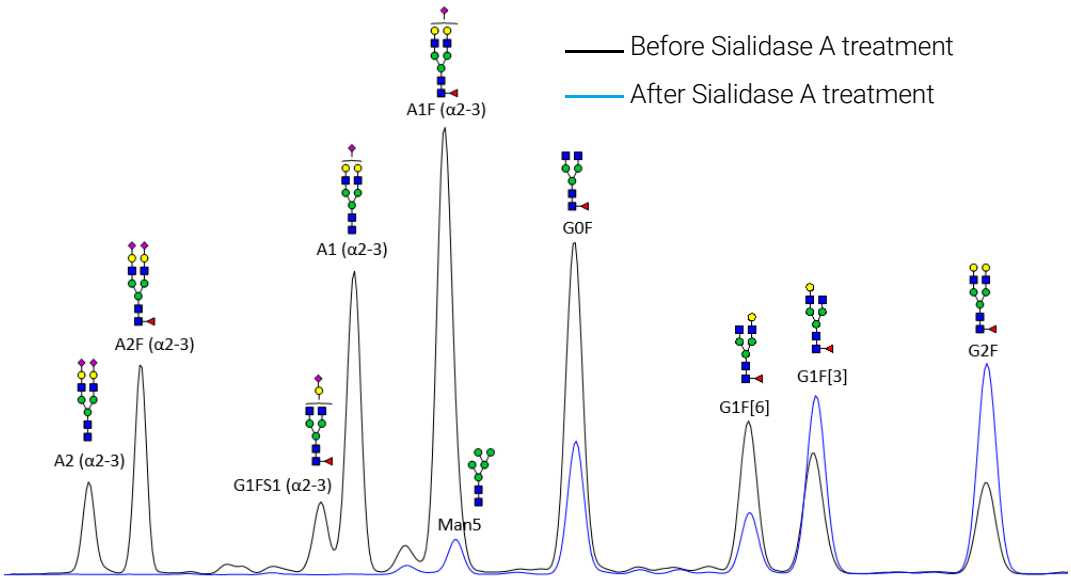


Figure 4. Gly-Q separation of APTS-labeled N-glycans from Enbrel (etanercept) before and after sialidase A digestion.

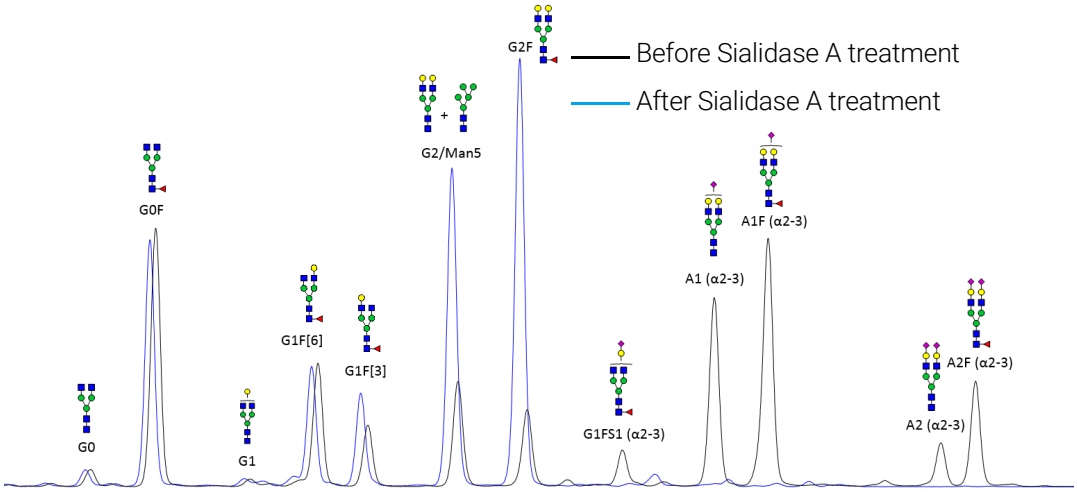


Figure 5. HILIC separation of APTS-labeled N-glycans from Enbrel (etanercept) before and after Sialidase A digestion. For HILIC methods, see **"FAQs"** on page 28.

Appendix C

Recommended instructions for automated protocols

The following sample and reagent volume instructions have been developed to accommodate pipetting requirements for automated Gly-X protocols (**Table 3**). The ratio of reagents in the automated protocol remains unchanged while minimum pipetting volume changes from 2 µL to 5 µL to improve reliability of automated pipetting.

Table 3 Sample and Working Solution instructions for Denaturant and N-Glycanase, to accommodate a minimum pipetting volume of 5 µL. Prepare other working solutions as directed in Table 2A and Table 2B on page 11.

Sample and Reagents	Standard Protocol	Automation Protocol
Sample	20 µL at 2 mg/mL (40 µg total)	15 µL at 2.67 mg/mL (40 µg total)
Denaturant	Use 2 µL of neat Denaturant reagent per sample.	Dilute Denaturant with water 2:3 (v/v). Use 5 µL of diluted Denaturant reagent per sample. Always prepare diluted Denaturant reagent with 20% overage. For example, for eight samples prepare 48 µL (19.2 µL Reagent, 28.8 µL water).
N-Glycanase Working Solution	Prepare N-Glycanase Working Solution by mixing N-Glycanase and Gly-X Digestion Buffer 1:1 (v/v). Use 2 µL of N-Glycanase Working Solution per sample. Always prepare N-Glycanase Working Solution with 20% overage. For example, for 8 samples prepare 20 µL.	Prepare N-Glycanase Working Solution by mixing N-Glycanase, Gly-X Digestion Buffer and water 1:1:3 (v/v/v). Use 5 µL of N-Glycanase Working Solution per sample. Always prepare N-Glycanase Working Solution with 20% overage. For example, for eight samples prepare 48 µL (9.6 µL N-Glycanase, 9.6 µL Gly-X Digestion Buffer, 28.8 µL water).

FAQs

Q. Can I use Agilent AssayMAP Protein A cartridges (fomerly ProZyme) to purify proteins ready for Gly-X with APTS?

A. Yes. AssayMAP PA50 Protein A Cartridges are available from Agilent in 24-count (G5524-60001) and 96-count (G5524-60010) kits, and 0.1% formic acid may be used as an eluent.

Q. What is the lower limit of glycoprotein I can use with the Gly-X/APTS protocol?

A. Each laboratory will need to establish the lower limit for each specific protein. 0.125 mg/mL can be used as a starting point.

Q: Can I use more than the recommended upper limit of 40 µg protein per reaction?

A: It depends on the protein. When using >40 µg protein, the user should check that relative percent area data maintains linearity.

Q: Can I use Eppendorf microtubes for the Denaturation and Digestion Steps, rather than a PCR plate?

A: Yes, leave the tubes open for the heating steps, and ensure that material does not get in the lid during mixing.

Q: What is the Finishing Reagent prior to the APTS labeling step?

A: The Finishing Reagent is a weak acid which drives conversion of the glycosylamine ($-NH_2$) to a glycan with a free reducing end (-OH) prior to APTS labeling by reductive amination.

Q: Can I run glycans labeled with APTS dye on other CE systems?

A: The LIF detector typically used on the Beckman PA800-plus for APTS glycans is compatible with the APTS Dye excitation wavelength. Suitability on other systems may be determined by the user.

Q: How do I calculate the mass of APTS-labeled glycans?

A: Mass added to glycan with a free reducing end:

- Mass of Glycan (free reducing end) + APTS = Mass of APTS-Labeled Glycan
- Mass added by APTS:

  Monoisotopic: 440.96468 Da
  Average: 441.5 Da

Q: What are the suggested conditions for analysis of APTS-labeled glycans by mass spectrometry (MS)?

A: Analytical systems vary, here are our suggested LC/MS conditions using a Waters Xevo G2-S Q-Tof:

Negative mode, capillary voltage 2.8 kV, cone voltage 30 V, source temperature 120 °C, desolvation temperature 350 °C, scan time 1.0 seconds, *m/z* range 500-2000 Da.

LC/MS conditions for APTS glycans using a Thermo Orbitrap Velos are described in (1).

Q: Can I run APTS-labeled glycans on a HILIC column?

A: APTS-labeled glycans can be separated by hydrophilic interaction liquid chromatography on UPLC or HPLC. However, APTS glycan peaks are broader than with other dyes that are more regularly used for LC (for example, InstantPC, 2-AB). A HILIC gradient starting at lower % of organic may be needed as APTS glycans are "stickier" (more retained on HILIC) than 2-AB and so forth, glycans.

Example of HILIC separations of APTS-labeled N-glycans from Rituxan and Enbrel are shown below in **Figure 6**, with method details in **Table 3** on page 27. Peak assignments are based on those of Reusch *et al.*[1].

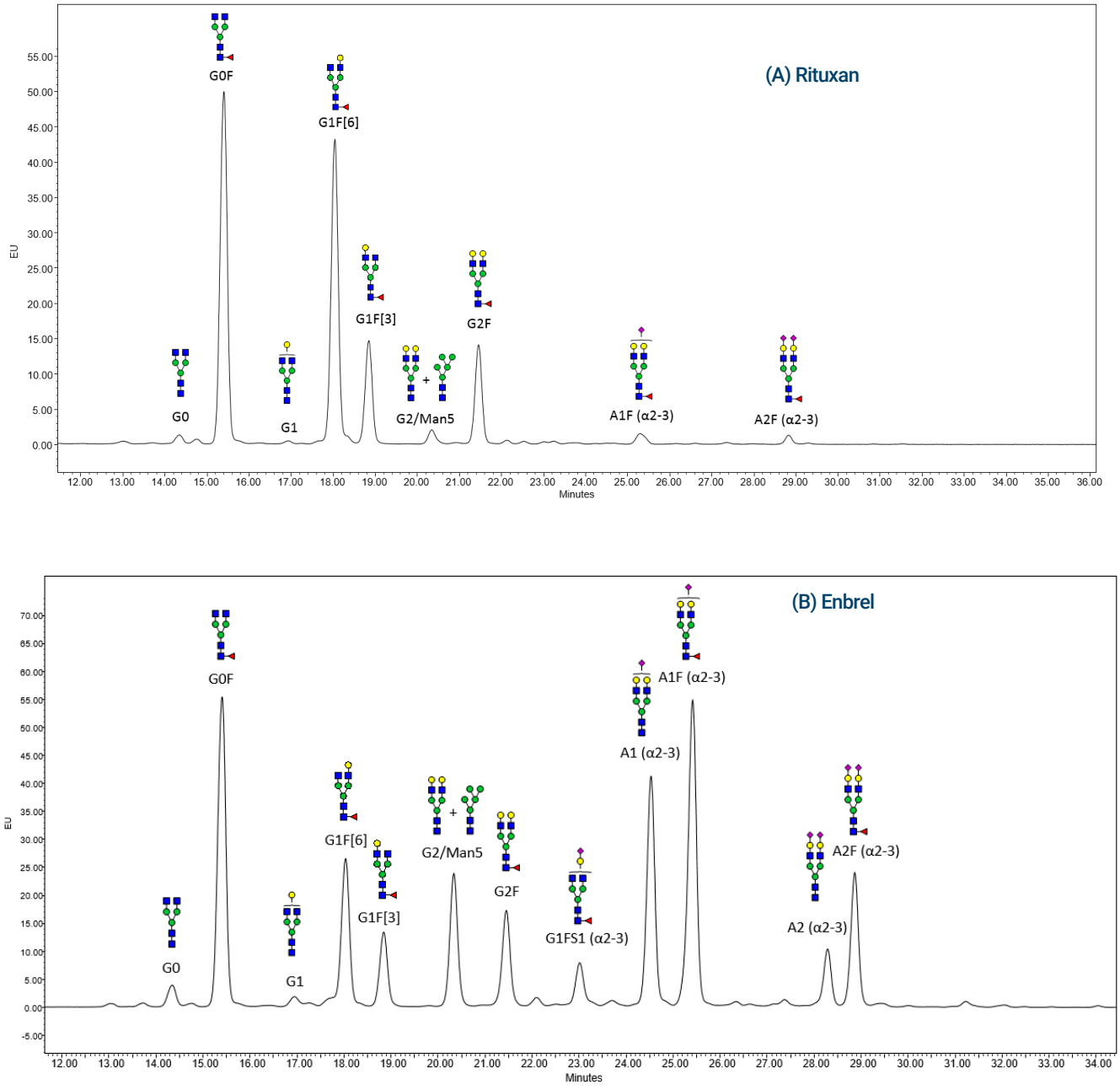


Figure 6. HILIC separation of APTS-labeled glycans from (A) Rituxan and (B) Enbrel using a 60-minute method (see **Table 3** on page 27 for method details).

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Table 4 Example of a HILIC method for ATPS-labeled N-glycans. Waters BEH GST column, 2.1 x 150 mm, 1.7 µm. Column temperature 60 °C, excitation 473 nm, emission 520 nm.

Time (min)	Flow Rate (mL/min)	% ACN	% 50 mM Ammonium Formate, pH 4.4
0	0.4	70	30
2	0.4	70	30
45	0.4	55	45
50	0.4	40	60
52	0.4	40	60
56	0.4	70	30
60	0.4	70	30

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Reference

1 Reusch D *et al.* High-throughput glycosylation analysis of therapeutic immunoglobulin G by capillary gel electrophoresis using a DNA analyzer. MAbs. 2014 Jan 1; 6(1): 185-196.

TECHNICAL ASSISTANCE

Agilent is committed to developing rapid, automatable methods for glycan analysis. Call us to discuss products in development.

If you have any questions or experience difficulties regarding any aspect of our products, please contact us at **https://www.agilent.com/en/contact-us/page**.

Agilent values customer opinions and we encourage you to contact us. We welcome your suggestions about product performance or new applications and tech notes.

Ordering Information

Table 5 Kits and Modules

Product Code	Description
GX96-APTS	Gly-X with APTS Express Kit (96-ct)
GX96-APTS-GQ*	Gly-X with APTS Express Kit for Gly-Q (96-ct)
GX24-APTS**	Gly-X with APTS Express Kit (24-ct)
GX96-601APTS	Gly-X with APTS Express Deglycosylation and Labeling Module Set (96-ct)
GX24-601APTS	Gly-X with APTS Express Deglycosylation and Labeling Module Set (24-ct)
GX96-601	Gly-X APTS Express Labeling Module (96-ct)
GX24-601	Gly-X APTS Express Labeling Module (24-ct)
GX96-602	Gly-X Cleanup Module for APTS Express (96-ct)
GX96-100	Gly-X Deglycosylation Module (96-ct)
GX24-100	Gly-X Deglycosylation Module (24-ct)
GX500	Gly-X APTS Express Heat Block
GX600	Gly-X Heater Lid
G5524-60010 KIT	AssayMAP PA50 Protein A Affinity Purification Kit (96-ct)

*GX96-APTS-GQ kit contains GX96-APTS kit, GQ103 Cartridge Module and GKSQ-505 Gly-Q Alignment Standards Set

** 24-ct kit (GX24-APTS) contains a 96-well Cleanup Plate and 24-ct APTS Labeling Module. Store the cleanup module at room temperature and order 24-ct refills of Gly-X APTS Express Deglycosylation and labeling Module Set (GX24-601APTS).

Table 6 APTS Labeled Glycan Standard Libraries

Product Code	Description
GKSP-005	Human IgG N-Linked Glycan Library
GKSP-520	Biantennary and High Mannose Partitioned Library
GKSP-500	APTS Bracketing Standard (dp2 and dp15)
GKSP-503	APTS Maltodextrin Ladder
GKSP-232	α(2-3) Sialylated Biantennary Library
GKSP-262	α(2-6) Sialylated Biantennary Library
GKSP-233	α(2-3) Sialylated Triantennary Library
GKSP-263	α(2-6) Sialylated Triantennary Library
GKSP-234	α(2-3) Sialylated Tetraantennary Library
GKSP-264	α(2-6) Sialylated Tetraantennary Library

Table 7 APTS Labeled Individual Glycan Standards

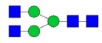
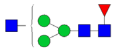
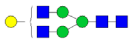
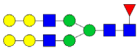
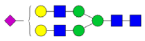
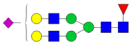
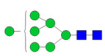
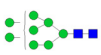
Product Code	Description	
GKSP-401	G0-N	
GKSP-301	G0	
GKSP-402	G0F-N	
GKSP-302	G0F	
GKSP-317	G1	
GKSP-316	G1F	
GKSP-304	G2	
GKSP-305	G2F	
GKSP-318	NA2Ga2F	
GKSP-311	A1 (α2,6)	
GKSP-315	A1F (α2,6)	
GKSP-312	A2 (α2,6)	
GKSP-313	A2F (α2,6)	
GKSP-103	Man5	
GKSP-104	Man6	
GKSP-105	Man7	
GKSP-106	Man8	
GKSP-107	Man9	

Table 8 Gly-Q System

Product Code	Description
GQ2100	Gly-Q Glycan Analysis System*
GQ001	Cartridge storage rack
GQ2050	Gly-Q Computer (Windows OS)

* Gly-Q System includes instrument, pressure pump, priming station, Gly-Q Manager software (GQSW2100), 1-year warranty.

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