

Agilent AdvanceBio Gly-X N-Glycan Prep with InstantQ Kit, 96-ct  
(formerly ProZyme)

## User Manual



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# Introduction

The Agilent Gly-X N-Glycan Rapid Release and Labeling with InstantQ kit (formerly ProZyme) uses a five minute in-solution enzymatic protein deglycosylation followed by rapid labeling of released N-glycans with InstantQ dye. After a simple clean-up step, the glycan samples are ready for analysis using the Gly-Q instrument. With deglycosylation and labeling carried out in solution, the method is simple, rapid, and suitable for cleanup automation.

The InstantQ dye is specifically designed for glycan separation and detection using the Gly-Q instrument and a Gly-Q InstantQ cartridge. Other benefits include:

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

# Kit Components

The Gly-X with InstantQ (GX96-IQ) consists of five modules. (See [Table 1.](#)) Each module provides enough reagents for up to 96 samples per run.

**Table 1** Kit components

Module	Component	Units	Storage
Gly-X Deglycosylation module for InstantQ Dye GX96-300	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 Blocker, 300 µL	1	4 °C
Gly-X InstantQ Labeling module GX96-301	InstantQ dye (lyophilized)	4	-20 °C
	InstantQ dye solvent, 1 mL	2	-20 °C
	InstantQ Activation reagent 1 mL	2	-20 °C
Gly-X InstantQ Cleanup module GX96-302	InstantQ Cleanup plate A	1	RT
	Gly-X Collection plate, 96 wells	1	RT
	Waste tray for vacuum manifold	1	RT
	Gly-X Used-well sealing caps, black (for cleanup plate)	1	RT
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 module 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

## NOTE

Allow for the Agilent Gly-Q cartridge to equilibrate to room temperature before first use (approximately one hour). The cartridge can be stored at RT after the first use.

## NOTE

Agilent Gly-X with InstantQ 24ct kit (GX24-IQ) (formerly ProZyme) contains 30 µL Gly-X N-Glycanase, one vial Gly-X Digestion Buffer, two vials InstantQ Dye, one vial InstantQ Dye Solvent, one vial InstantQ Activation Reagent and all other kit components listed in the [Table 1.](#)

## NOTE

Agilent Gly-X Vacuum Manifold spacer (GX100) (formerly ProZyme) is required, please contact Agilent for more information.



Figure 1. GX100, Gly-X Vacuum manifold spacer

# Equipment and Reagents Provided By User

A 96-well Thermocycler or two independent heat blocks, set for 90 °C and 50 °C. (for example, Corning THERM-1001, 110 V; THERM-1000, 230 V).

## NOTE

**Two GlykoPrep Heaters, WS0271, can be fitted with VWR 13259-260 Modular Heating Blocks.**

- Vacuum manifold (Millipore MSVMHTS00)
- Vacuum pump (Millipore WP6211560, 110 V; WP6122050, 220 V)

## NOTE

**If you have a vacuum manifold and pump other than the Millipore model suggested, please contact Agilent at [www.agilent.com](http://www.agilent.com) for guidance. A Waters vacuum manifold may be used with a GX200 Spacer in place of GX100. (See “FAQs” on page 27.)**

- Gly-X Vacuum Manifold Spacer (GX100)
- Acetonitrile (ACN)
- Trifluoroacetic acid (TFA)
- DI water

## NOTE

**Agilent Gly-X InstantQ Eluent is ultrapure water (provided by user).**

- Optional - 0.1% Formic acid (for Protein A elution)
- Optional - VWR 10 kDa Centrifugal filters (82031-348)
- Optional - 100% ethanol

# 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 and nucleophiles such as amines. Higher concentration samples should be diluted in water or 50 mM HEPES, pH 7.9.

Other sample considerations include:

- Amine buffer components (for example, Tris, arginine, glycine, histidine) should be avoided as these will react with the InstantQ glycan labeling dye. A 10 kDa molecular weight cut-off spin centrifugal filter is recommended for buffer exchange before the deglycosylation step for these samples. Water or noninterfering buffer can be used for the resuspension of the protein samples.
- When using samples prepared by protein A affinity chromatography, 0.1% formic acid should be used as an eluent rather than a glycine buffer.
- Samples in salt-containing buffers (~150 mM salt) are compatible with the kit, however, higher salt concentrations may decrease the sensitivity of the method. 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), but some glycoproteins can be added in higher concentrations than 2 mg/mL.
- 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 2 on page 10**.
- For citrate-containing buffers, dilute sample with water or 50 mM HEPES, pH 7.9 to reduce citrate below 20 mM or exchange buffer with molecular weight cut-off spin centrifugal filters.
- Use of Gly-X Blocker is recommended for smaller glycoproteins (<50 kDa) or complex sample matrices with ethanol cleanup. (See **"Appendix C"** on page 22 and **"FAQs"** on page 27.)
- Use of a more stringent clean up method is recommended for complex sample matrices, such as cell culture supernatants or biological fluids. (See **"Appendix C"** on page 22.)

If a precipitate is observed upon incubation at 90 °C (**step 5 on page 10**), review the sample prep and sample buffers for salts, low pH 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 (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)

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>.



# Protocol

## Getting started

- 1 Prepare samples (see “**Sample Prep Considerations**” on page 8).
- 2 Set the thermocycler to 90 °C, or set two independent heat blocks to 90 and 50 °C.
- 3 Prepare the working solutions in **Table 2**.

**Table 2 Working solution instructions**

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.
InstantQ Working dye solution	<p><b>a</b> Warm to room temperature the InstantQ Dye vial, Dye solvent, and Activation reagent. Remove from desiccant pouch.</p> <p><b>b</b> Add 400 µL of Dye solvent to the InstantQ Dye vial, vortex until dissolved. This can be stored at -20 °C.</p> <p><b>c</b> Mix solubilized InstantQ Dye and Activation reagent 1:1. Prepare a total volume of 20 µL per sample with ~20% overage. For eight wells, mix 96 µL InstantQ Dye and 96 µL Activation reagent, mix well.</p> <p><b>Note:</b> Use tubes or plates that are compatible with organic solvents (polystyrene is not compatible).</p>	<p>20 µL of InstantQ Working solution is required per sample, mix ~20% overage.</p> <p>Solubilized dye (without addition of Activation reagent) is stable for one month at -20 °C. Minimize exposure of InstantQ Dye to air.</p> <p>Final InstantQ Dye working solution is stable for three hours.</p>

## Prepare cleanup station

Prepare cleanup station with the following items shown in **Figure 2**:

- a InstantQ Cleanup plate A
- b Vacuum manifold connected to vacuum pump
- c Waste tray
- d InstantQ Collection plate
- e Gly-X Manifold spacer (GX100)
- f ACN/TFA Solution; mix acetonitrile (ACN), trifluoroacetic acid (TFA) and water in a ratio 87:2:11 (v/v). Make up 1440 µL of each solution per sample (includes 20% overage). (Not shown in **Figure 2**.)
- g 90% ACN Solution (ACN:water, 90:10 v/v). Make up 1440 µL of each solution per sample (includes 20% overage). (Not shown in **Figure 2**.)

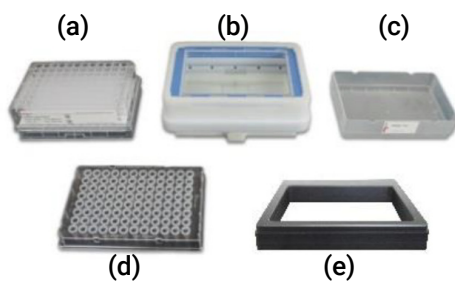


Figure 2. Cleanup station items

## Gly-X Deglycosylation

- 1 Add 2  $\mu$ L of Gly-X Denaturant (orange cap vial) to the bottom of the Gly-X Deglycosylation plate. (See [Figure 3](#).)



Figure 3. Gly-X Deglycosylation plate

- 2 Optional: Add 2  $\mu$ L of Gly-X Blocker to each well.

### NOTE

**Gly-X Blocker is not required for most biotherapeutics; for example, mAbs and Fc-fusions. See “FAQs” on page 27 for further details.**

### NOTE

**For samples with a pH of 5.5 or lower, add 3  $\mu$ L Gly-X Digestion Buffer (white cap vial), mix thoroughly with pipette.**

- 3 Add 20  $\mu$ L of each glycoprotein sample (~2 mg/mL) to each well.
- 4 Mix well using a pipette, and tap on benchtop to collect samples at the bottom of wells.
- 5 Incubate at 90 °C for three minutes.

### NOTE

**If a precipitate forms, review the sample buffer composition, avoid histidine and glycine in samples.**

- 6 Remove the plate, place at room temp for two minutes.
- 7 Add 2  $\mu$ L of N-Glycanase working solution to each sample. Mix well using a pipette.
- 8 Tap the plate on benchtop to collect samples on the bottom of wells (or spin).
- 9 Incubate uncapped at 50 °C for five minutes. Do not exceed five minutes, longer incubation with enzyme can lead to reduced signal.
- 10 Remove the plate from heat, and proceed directly to **“InstantQ labeling”** on page 11.

## InstantQ labeling

- 1 Add 20  $\mu\text{L}$  of InstantQ Dye working solution, prepared above, per sample. After each addition, mix thoroughly with a pipette. Repeat until InstantQ Dye solution has been added to each sample.
- 2 Incubate at 50  $^{\circ}\text{C}$  for one minute.

### NOTE

the InstantQ Dye working solution must be used within three hours.

## InstantQ cleanup

The following cleanup method including TFA is suggested as of Rev AH of this protocol. It was developed for complex sample matrices, such as biological fluids. However, the method enables better residual salt and protein removal that results in higher signal and improved baseline for most sample types. Total fluorescence signal may be more variable. For the previous cleanup method using 100% ethanol see “Appendix C” on page 22.

### Prepare load and wash solutions

### NOTE

A total of 1200  $\mu\text{L}$  TFA/ACN solutions and 90% ACN solution is needed per sample, make up 1440  $\mu\text{L}$  of each solution per sample (including 20% overage).

- 1 Prepare ACN/TFA solution, mix acetonitrile (ACN), trifluoroacetic acid (TFA), and water in a ratio 81:2:11 (v/v).
- 2 Prepare 90% ACN solution (ACN:water, 90:10 v/v).

### Load

- 1 Mark the Gly-Q Cleanup plate A (Figure 4) for the required number of wells.

### NOTE

Black cap strips (Figure 4) should be placed on wells used in previous cleanup procedures to prevent reuse of wells.

### NOTE

Cleanup plate storage plate (Figure 4) should be set aside and used to store Cleanup plate after use. Store in foil bag provided.



Figure 4. Gly-Q Cleanup plate A

- 2 Place the Waste tray in the Vacuum or Pressure manifold. (See **Figure 5**.)



Figure 5. Vacuum manifold with Waste tray

- 3 Assemble the complete manifold.
- 4 Install InstantQ Cleanup plate A on top of the manifold. (See **Figure 6**.)

#### NOTE

**No priming is required.**



Figure 6. InstantQ Cleanup plate A with complete manifold

- 5 With a multichannel pipette, add 150  $\mu\text{L}$  of ACN/TFA solution to each sample in the deglycosylation plate, mix with pipette.
- 6 With a multichannel pipette, add 450  $\mu\text{L}$  ACN/TFA solution to one column of wells in the Gly-X Cleanup plate. Do not apply vacuum.

#### NOTE

**There will be some loss of ACN/TFA solution due to pass through of the cleanup plate. Proceed to step 7 immediately or increase the ACN/TFA solution volume to 500  $\mu\text{L}$  to allow for more time ( $\leq 2$  minutes).**

- 7 Transfer the entire sample ( $\sim 172 \mu\text{L}$ ) to corresponding wells in the Gly-X Cleanup plate.
- 8 Repeat until all samples have been transferred to the Cleanup plate.
- 9 Apply vacuum to  $< 5$  in Hg. Let the solution pass through until the wells are empty.

#### NOTE

**This step loads the sample onto the Gly-X Cleanup plate.**

#### Wash

- 1 Wash with 600  $\mu\text{L}$  of ACN/TFA solution, and apply vacuum to  $< 5$  in Hg, collecting wash in the Waste tray.
- 2 Wash twice with 600  $\mu\text{L}$  of 90% ACN solution applying vacuum to  $< 5$  in Hg and collecting wash in the Waste tray.

**NOTE**

All wells should be completely empty, if not, after two minutes, increase the vacuum to 10 in Hg.

**NOTE**

If processing more than 48 samples, empty the Waste tray after the second wash (Waste tray holds approximately 120 mL).

- 3 Release the vacuum, remove the Cleanup plate and blot on paper. Empty the Waste tray.

**Elute**

- 1 Remove the Waste tray from the manifold. (See **Figure 7.**)



Figure 7. Waste tray

- 2 Place the Gly-X Manifold spacer (GX100) into the manifold, with the cut-away edge of the spacer facing up. (See **Figure 8.**)

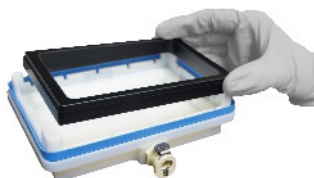


Figure 8. Gly-X Manifold spacer (GX100) with manifold

- 3 Place the Collection plate on top of the Spacer plate, and reassemble the vacuum manifold. (See **Figure 9.**)

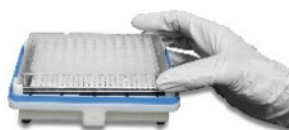


Figure 9. Collection plate

- 4 Install the Gly-X InstantQ Cleanup plate A on top of the vacuum manifold. (See **Figure 10.**)



Figure 10. Gly-X InstantQ Cleanup plate A with vacuum manifold

- 5 Add 100  $\mu$ L of DI water to each well. Using vacuum, ( $\leq 5$  in Hg) elute samples into the Collection plate.
- 6 Maintain the vacuum for at least one minute to collect the eluent.

**NOTE**

**All wells should be completely empty, if not, keep the vacuum at  $<5$  in Hg for another 1 to 2 minutes. Do not increase vacuum during the elution step.**

- 7 Release the vacuum, remove the Cleanup plate and rest on the Storage plate.
- 8 Remove the collection plate. Optional: seal the Collection plate with preslit sealing film included in the kit for use on UHPLC. For long term storage at  $-20$   $^{\circ}$ C, use the foil sealing film included in the kit, recommended for overnight use of Gly-Q to prevent evaporation of samples.
- 9 Vortex to mix, and spin/tap to collect samples at the bottom of the wells, or mix with a pipette.

**NOTE**

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

- 10 Add Black Used Well Sealing Caps to used Cleanup plate A wells, return Cleanup plate to bag and store at RT.
- 11 InstantQ labeled glycan samples are ready for analysis. Samples may be stored at  $-20$   $^{\circ}$ C for at least one month or  $4$   $^{\circ}$ C for up to five days.

**NOTE**

**Black cap strips (Figure 11) should be placed on wells used in previous cleanup procedures to prevent reuse of wells.**

**NOTE**

**Cleanup plate storage (round bottom) plate (Figure 11) should be used to store Cleanup plate after use. Store in foil bag provided.**

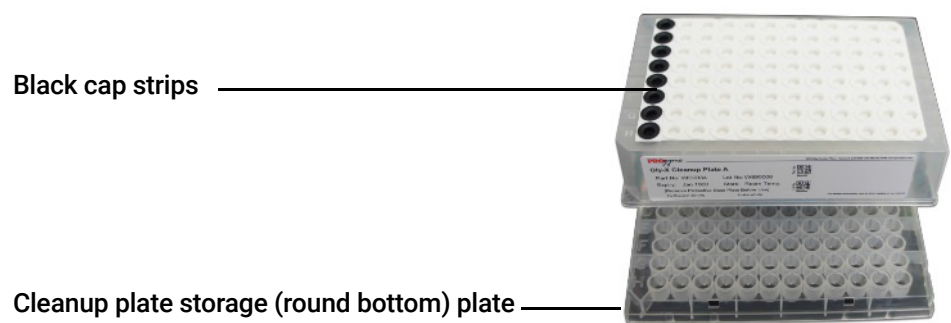


Figure 11. Cleanup plate with black cap strips

# Analysis of Labeled Glycans

## 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 **“Appendix A”** on page 19.)
  - 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.
  - **Park:** 4 mL DI water, layer on mineral oil to cover if cartridge is stored on system >five hours
  - **Wash and Clean:** 4 mL DI water
  - **Separation:** 4 mL Gly-Q separation buffer (provided with kit)

## 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

Preinstalled 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.

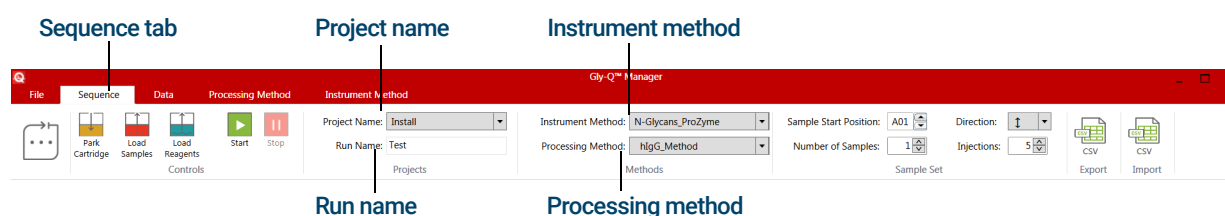


Figure 12. Setting up a sample sequence steps 1 through 5



- 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.

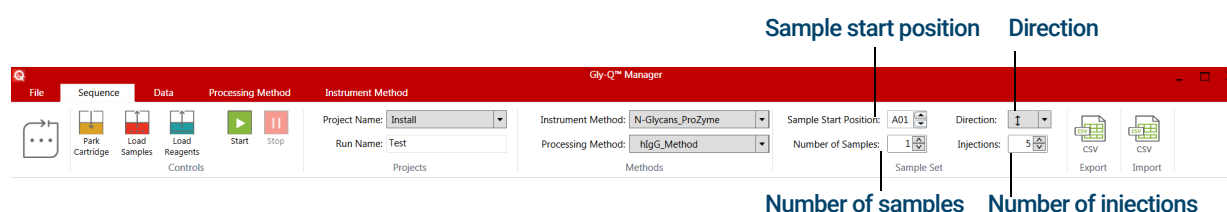


Figure 13. Setting up a sample sequence steps 6 through 9

### 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.

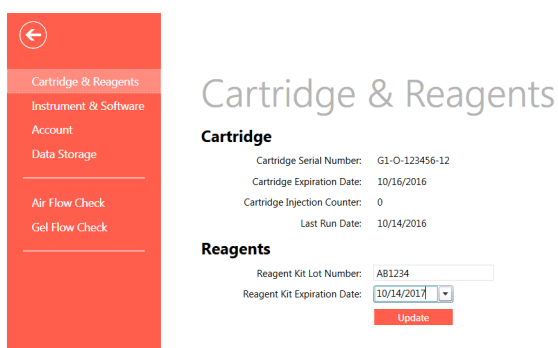


Figure 14. Cartridge and Reagents window

## 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  $\mu\text{L}$  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  $\mu\text{L}$  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 (Figure 15). Install the Reagents Tray with Park, Wash, Separation and Clean buffer solutions (step 5 on page 16).

### 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.

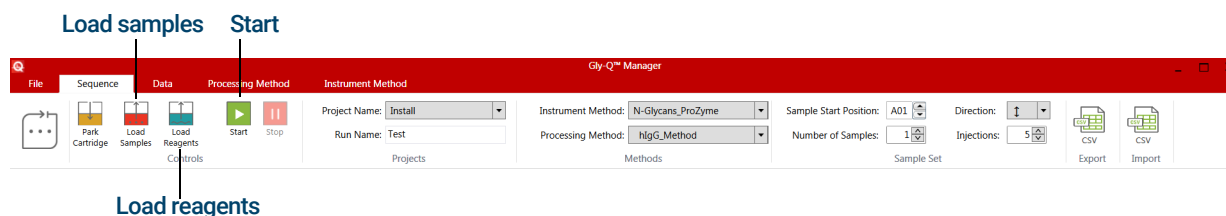


Figure 15. Starting a run

## 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. Migration Standard and GU Ladder tubes can be capped and stored at  $-20\text{ }^{\circ}\text{C}$  directly in PCR tubes. For reuse, thaw and centrifuge briefly to bring mineral oil to the surface.
- 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 RT.

# Appendix A

## Cartridge preparation

- 1 Open clamshell container (keep this for storage between uses). (Figure 16)



Figure 16. Clamshell container

- 2 Remove cartridge and pin (red) from package. (Figure 17)

### NOTE

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



Figure 17. Cartridge

- 3 Remove tape from cartridge cap. (Figure 18)



Figure 18. Red tape on cartridge cap

- 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. (Figure 19)



Figure 19. Red pin installed in cartridge

- 5 Gently wipe the tip of the cartridge. (Figure 20)



Figure 20. Cartridge tip

- 6 Prime the cartridge for use as directed in the *Gly-Q System User Manual*.

**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 C

### Alternative cleanup protocol using 100% ethanol

#### NOTE

The following cleanup protocol using 100% ethanol was suggested up to and including Rev AG of this manual. As of Rev AH this has been replaced with the cleanup method in “InstantQ cleanup” on page 11 of the main protocol, which includes TFA for improved removal of residual salts and protein, with increased signal.

The following are required:

- Wash solution: 100% ethanol
- Priming and Elution solution: DI water

#### Load

- 1 Mark the Gly-Q Cleanup plate A (Figure 22) for the required number of wells.

#### NOTE

Black cap strips (Figure 22) should be placed on wells used in previous cleanup procedures to prevent reuse of wells.

#### NOTE

Cleanup plate storage plate (Figure 22) should be set aside and used to store Cleanup plate after use. Store in foil bag provided.



Figure 22. Gly-Q Cleanup plate A

- 2 Place the Waste tray in the Vacuum or Pressure manifold. (See Figure 23.)



Figure 23. Vacuum manifold with Waste tray

- 3 Assemble the complete manifold.

- 4 Install InstantQ Cleanup plate A on top of the manifold. (See [Figure 24](#).)

**NOTE**

**No priming is required.**



Figure 24. InstantQ Cleanup plate A with complete manifold

- 5 Prime InstantQ Cleanup plate A.
  - a Add 400  $\mu$ L of DI water to each well and apply vacuum at 5 in Hg until the wells are empty.
  - b Add 600  $\mu$ L of 100% ethanol to each well and vacuum at 5 in Hg until the wells are empty.
  - c Repeat Wash with 600  $\mu$ L 100% ethanol.
  - d Empty the Waste tray.

**NOTE**

**If processing more than 72 samples, empty the Waste Tray after the second wash (Waste tray holds approximately 120 mL).**

- 6 Load samples from Deglycosylation plate into Cleanup plate.
  - a With a multichannel pipette, add 400  $\mu$ L 100% ethanol to each well in the cleanup plate.

**NOTE**

**There may be some loss of 100% ethanol due to pass through of the Cleanup plate. Proceed to [step b](#) immediately, or increase the 100% ethanol volume to 500  $\mu$ L to allow for more time ( $\leq$ two minutes).**

- b With a multichannel pipette, add 150  $\mu$ L 100% ethanol to each sample in the Deglycosylation plate. Mix with a pipette and transfer the entire sample ( $\sim$ 190  $\mu$ L) to the corresponding wells in the InstantQ Cleanup plate A. Mix well with a pipette.

**NOTE**

**When multiple samples are prepared, load the samples row-by-row or column-by-column to minimize ethanol lost from the plate wells.**

- c Repeat until all samples have been transferred to the Cleanup plate.
- d Apply vacuum to 5 in Hg until the wells are empty. Vacuum can be increased to 10 in Hg if any wells are not empty after two minutes.

**NOTE**

**This step loads the sample to the InstantQ Cleanup plate A matrix.**

**Wash**

- 1 Wash with 600  $\mu$ L of Wash solution (100% ethanol), and apply vacuum to  $<$ 5 in Hg, collecting wash in the Waste tray.
- 2 Repeat with a second 600  $\mu$ L ethanol wash.
- 3 Repeat with a third 600  $\mu$ L ethanol wash.

**NOTE**

All wells should be completely empty, if not, after two minutes, increase the vacuum to 10 in Hg.

**NOTE**

If processing more than 48 samples, empty the Waste tray after the second wash (Waste tray holds approximately 120 mL).

- 4 Release the vacuum, remove the Cleanup plate A, and rest on the Storage plate. (See **Figure 29** on page 25.) Empty the Waste tray.

**Elute**

- 1 Remove the Waste tray from the manifold. (See **Figure 25**.)



Figure 25. Waste tray

- 2 Place the Gly-X Manifold spacer (GX100) into the manifold, with the cut-away edge of the spacer facing up. (See **Figure 26**.)

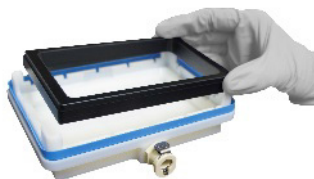


Figure 26. Gly-X Manifold spacer (GX100) with manifold

- 3 Place the Collection plate on top of the Spacer plate and reassemble the vacuum manifold. (See **Figure 27**.)

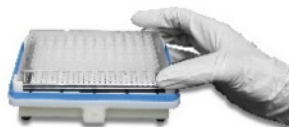


Figure 27. Collection plate



- 4 Install the Gly-X InstantQ Cleanup plate A on top of the vacuum manifold. (See [Figure 28](#).)



Figure 28. Gly-X InstantQ Cleanup plate A with vacuum manifold

- 5 Add 100  $\mu$ L of DI water to each well. Using vacuum, ( $\leq 5$  in Hg) elute samples into the Collection plate.
- 6 Maintain the vacuum for at least one minute to collect the eluent.

**NOTE**

**All wells should be completely empty, if not, keep the vacuum at  $<5$  in Hg for another 1 to 2 minutes. Do not increase vacuum during the elution step.**

- 7 Release the vacuum, remove the Cleanup plate, and rest on the Storage plate.
- 8 Remove the collection plate. Optional: seal the Collection plate with preslit sealing film included in the kit, recommended for overnight use of Gly-Q to prevent evaporation of samples.
- 9 Vortex to mix, and spin/tap to collect samples at the bottom of the wells, or mix with a pipette.

**NOTE**

**This final, postelution mixing step is critical for consistent results.**

- 10 Add Black Used Well Sealing Caps to used Cleanup plate A wells, return Cleanup plate to bag and store at room temperature.
- 11 InstantQ labeled glycan samples are ready for analysis. Samples may be stored at  $-20$   $^{\circ}$ C for at least one month or  $4$   $^{\circ}$ C for up to five days.

**NOTE**

**Black cap strips ([Figure 29](#)) should be placed on wells used in previous cleanup procedures to prevent reuse of wells.**

**NOTE**

**Cleanup plate storage (round bottom) plate ([Figure 29](#)) should be used to store Cleanup plate after use. Store in foil bag provided.**

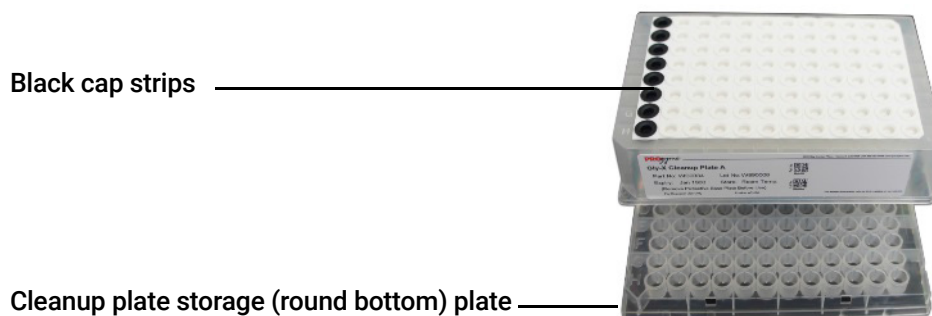


Figure 29. Cleanup plate with black cap strips

## Appendix D

### Recommended instructions for automated protocols

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

**Table 3 Working Solution instructions for Sample, Denaturant and N-Glycanase, to accommodate a minimum pipetting volume of 5  $\mu$ L. Prepare other working solutions as directed in Table 2 on page 9.**

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

\* It may be possible to use up to 100  $\mu$ g glycoprotein, depending on the sample type (see **"FAQs"** on page 27).

# FAQs

**Q: When do I need to use the Gly-X Blocker with my deglycosylation reaction? What does the Blocker do?**

**A:** The Gly-X Blocker is optional when using the ethanol cleanup (“**Appendix C**” on page 22). It is not required for most biotherapeutics for example IgGs, Fc-fusion proteins. However, if you observe an uneven/rounded baseline during analysis we suggest running the protocol with and without the Blocker to test whether it is required. Potential scenarios that may require the Blocker are:

- If your glycoprotein is <50 kDa;
- If the glycoprotein contains a large proportion of basic amino acids
- If your sample is a complex matrix such as a biofluid

The Blocker ensures that labeled protein does not interfere with N- glycan analysis by blocking free amine groups on the glycoprotein before they can be labeled with InstantQ dye.

Using the blocker may cause the reaction to turn yellow, particularly when there is a lot of protein material. This is caused by Maillard reaction products and is not a cause for concern. Please contact Agilent for further details.

**Q: Why should I use 0.1% formic acid to elute samples from Protein A ready for Gly-X with InstantQ, rather than a low pH glycine solution?**

**A:** Glycine has a primary amine which will react with the InstantQ label, so we suggest that 0.1% formic acid is used as a protein eluent. Alternatively, glycine may be used but samples must be desalted before deglycosylation.

**Q: Can I use Agilent AssayMAP Protein A cartridges (formerly ProZyme) to purify proteins ready for Gly-X with InstantQ, and elute with 0.1% formic acid?**

**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/InstantQ 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 of 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: My samples contain polysorbate. Does polysorbate interfere with the InstantQ sample preparation?**

**A:** We have successfully tested samples containing up to 1% Tween 20 on the Gly-Q Analysis System and have not seen any significant difference (>5% CV) between samples with and without Tween 20.

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

**A:** The LIF detector typically used on the Beckman PA800-plus for APTS glycans is not compatible with the InstantQ Dye excitation wavelength. Suitability on other systems may be determined by the user.

**Q: Can I use the GX96-IQ kit with a Waters vacuum manifold?**

**A:** Yes, if GX200 Gly-X Vacuum Manifold Spacer (Waters Manifold) is used in place of GX100 during the **“Elute”** step on **page 13**. GX200 provides the correct distance between the Cleanup plate and Collection plate when using a Waters vacuum manifold (#186001831). This is critical to avoid crosstalk when eluting labeled N-glycans into the Collection plate. Please contact Agilent at <https://www.agilent.com/en/contact-us/page> for further details.

# Resources and References

Visit the Agilent website for additional information, downloadable posters, publications, and technical notes:

[www.agilent.com](http://www.agilent.com)

## **Product use, Warranty and License to use**

Terms and conditions of sale may be found at: [www.agilent.com](http://www.agilent.com)

## **Virtual patent marking**

Visit [www.agilent.com](http://www.agilent.com) for a list of Agilent products and patents.

# 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 encourage you to contact us. We welcome your suggestions about product performance or new applications and techniques.

# Ordering Information

## Kits and Modules

Product Code	Description
GX96-IQ	Gly-X with InstantQ Kit (96-ct)
GX24-IQ*	Gly-X with InstantQ Kit (24-ct)
GX96-302IQ	Gly-X with InstantQ Deglycosylation and Labeling Module Set (96-ct)
GX24-302IQ	Gly-X with InstantQ Deglycosylation and Labeling Module Set (24-ct)
GX96-301	Gly-X InstantQ Labeling Module (96-ct)
GX24-301	Gly-X InstantQ Labeling Module (24-ct)
GX96-302	Gly-X Cleanup Module (96-ct)
GX100	Gly-X Vacuum Manifold Spacer (2 pack)
GX200	Gly-X Vacuum Manifold Spacer (Waters manifold)
G5524-60010 KIT	AssayMAP PA50 protein A affinity purification kit (96-ct)
G5524-60001 KIT	AssayMAP PA50 protein A affinity purification kit (24-ct)
GQ103	Gly-Q Cartridge module
GK800400	Glyko Sialidase A
GK80021	Glyko Sialidase S

\* 24-ct kit (GX24-IQ) contains a 96-well Cleanup plate. Store the Cleanup plate at room temperature and order 24-ct refills of Gly-X InstantQ Deglycosylation and Labeling modules (GX24-201IQ).

## Gly-Q System

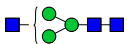
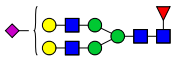

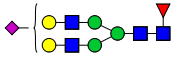
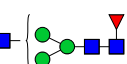
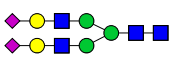
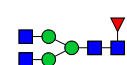
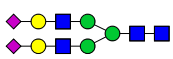

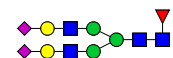
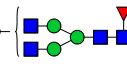
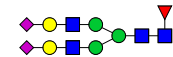
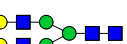
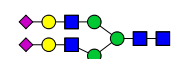
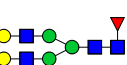


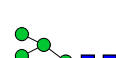
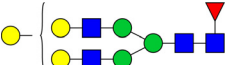

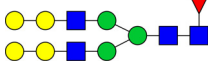
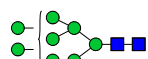
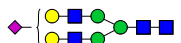

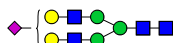
Product Code	Description
GQ2100	Gly-Q Glycan Analysis system*
GQ2050	Gly-Q Computer (Windows OS)

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

## InstantQ Ladder and Migration Standards

Product Code	Description
GKSQ-503	Gly-Q GU Ladder
GKSQ-500	Gly-Q Migration standards (upper and lower)
GKSQ-505	Gly-Q Alignment standards set (GKSQ-500 and GKSQ-503)

## InstantQ Labeled Individual N-Glycan Standards

Product Code	Description		Product Code	Description	
GKSQ-401	G0-N		GKSQ-325	A1F ( $\alpha$ 2, 3)	
GKSQ-301	G0		GKSQ-315	A1F ( $\alpha$ 2, 6)	
GKSQ-402	G0F-N		GKSQ-322	A2 ( $\alpha$ 2, 3)	
GKSQ-302	G0F		GKSQ-312	A2 ( $\alpha$ 2, 6)	
GKSQ-317	G1		GKSQ-323	A2F ( $\alpha$ 2, 3)	
GKSQ-316	G1F		GKSQ-313	A2F ( $\alpha$ 2, 6)	
GKSQ-304	G2		GKSQ-314	A3( $\alpha$ 2, 6)	
GKSQ-305	G2F		GKSQ-103	Man5	
GKSQ-403	G1F + 1aGal		GKSQ-104	Man6	
GKSQ-404	G2F + 1aGal		GKSQ-105	Man7	
GKSQ-318	G2F + 2aGal		GKSQ-106	Man8	
GKSQ-321	A1 ( $\alpha$ 2, 3)		GKSQ-107	Man9	
GKSQ-311	A1 ( $\alpha$ 2, 6)				



## InstantQ N-Glycan Libraries

Product Code	Description
GKSQ-005	Human IgG N-Linked Glycan Library
GKSQ-009	RNase B N-Linked Glycan Library
GKSQ-020	CHO mAb N-Linked Glycan Library
GKSQ-020-P	CHO mAb N-Linked Glycan Library + CHO mAb Glycoprotein
GKSQ-233	$\alpha(2-3)$ Sialylated Triantennary Library
GKSQ-234	$\alpha(2-3)$ Sialylated Tetraantennary Library
GKSQ-263	$\alpha(2-6)$ Sialylated Triantennary Library
GKSQ-264	$\alpha(2-6)$ Sialylated Tetraantennary Library





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