Agilent

Agilent AdvanceBio Gly-X N-Glycan Prep with 2-AB Express Kit, 96-ct (formerly ProZyme)

User Manual

Notices

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Contents

Introduction

The Agilent Gly-X N-Glycan Rapid Release and Labeling with 2-AB Express kit uses a novel in-solution enzymatic protein deglycosylation followed by fast labeling of released N-glycans with 2-AB dye. The 2-AB 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 LC-FLR, mass spectrometry or other methods. The protocol is simple, rapid (~2.5 hours), and suitable for automation. The 2-AB dye delivers proven fluorescence performance within well-established glycan analysis workflows. Other benefits include:

- Small molecule size for optimal chromatographic peak resolution
- Flexible, high-throughput format: process 1 to 96 samples
- Optimized cleanup removes excess free dye, protein, and other interfering compounds

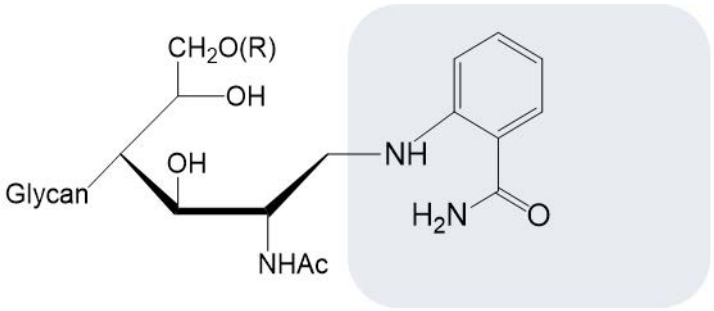

Figure 1. N-Glycan labeled with 2-AB dye

Kit Components

The Gly-X with 2-AB Express Kit (GX96-2AB) consists of three modules. (See **Table 1**.) Each module provides enough reagents for up to 96 samples.

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 2-AB Express Labeling Module GX96-401	Gly-X 2-AB Dye solution, 50 µL	4	-20 °C
	Gly-X 2-AB Reductant, 50 µL	4	-20 °C
	Gly-X 2-AB Catalyst, 100 µL	4	-20 °C
	Gly-X 2-AB Finishing reagent, 230 µL	1	-20 °C
Gly-X 2-AB Express Cleanup Module GX96-402	Gly-X Cleanup plate A	1	RT
	Gly-X Load plate, deep well plate, 96 wells	1	RT
	Gly-X Used-well sealing cap mat, black (for cleanup plate)	1	RT
	Gly-X Collection plate, 96 wells plate, clear	1	RT
	Sealing film (preslit)	1	RT
	Storage sealing foil	1	RT
	Waste tray for vacuum manifold	1	RT
	Cover film for heat block	1	RT

NOTE

Gly-X 2-AB Express Starter Kit (GX400) is required. (See Figure 2.) Includes Vacuum Manifold Spacer (GX100) and Heat Block Lids (two sizes). Please contact Agilent for more information.

NOTE

Gly-X with 2-AB Express 24-ct kit (GX24-2AB) contains 30 µL Gly-X N-Glycanase, one vial Gly-X Digestion Buffer, one vial 2-AB Dye Solution, one vial 2-AB reductant, one vial 2-AB Catalyst, and all other components listed in Table 1.

Heater covers **Vacuum manifold spacer**

Figure 2. GX400, Gly-X 2-AB Express Starter Kit, required

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, a heat block is required for the labeling step.

Recommended:

- Two Thermo Scientific 88871001 Compact Dry Block Heaters with 96 well PCR heat blocks
- Agilent Gly-X Vacuum Manifold Spacer and heater block lid (formerly ProZyme) (included in GX400 Gly-X 2-AB Express Starter Kit; or use GX100 Spacer and alternate heater lid)
- 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. A Waters vacuum manifold may be used with a GX200 spacer in place of the GX100, see "FAQs" on page 22.

- DI water, ~100 µL per sample
- Acetonitrile (ACN), MS-grade (Fisher A955-4 recommended), ~4 mL per sample
- Optional - sample buffer exchange with AdvanceBio Spin columns (p/n **1980-1103**) or 96-sample plates (p/n **1980-1104**). For AdvanceBio Spin columns, collection tubes are included. For AdvanceBio Spin 96-sample plates, the recommended accessory plates are p/n 5043-9308 for wash steps, p/n **5043-9312** for collection, with p/n **5042-1389** sealing mat for storage. See user guide **5994-7244EN** for detailed AdvanceBio Spin instructions.

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 22).
- 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 10**.

If a precipitate is observed upon incubation at 90 °C (**step 4** on **page 11**), 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 **www.agilent.com**.

See User Guide **5994-7244EN** for detailed instructions, and supporting data in Application Note **5994-7239EN** for optional sample buffer exchange with AdvanceBio Spin columns or 96-sample plates.

Protocol

Getting started

1 Prepare samples (see **"Sample Prep Considerations"** on page 8).

2 Program the thermocycler to 90 °C and set a heat block set to 65 °C, or set two independent heat blocks to 90 °C and 50 °C.

NOTE

A heat block that is set to 90 °C will need to be reset to 65 °C for the labeling step. (See step 4 on page 13.) The 65 °C step is recommended to be carried out in a laboratory fume hood.

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 8 wells mix 9.6 µL N-Glycanase and 9.6 µL Digestion Buffer.	2 µL required per sample, mix 20% overage of working solution.
2-AB Working solution	Mix 2-AB Solution, 2-AB Reductant, 2-AB Catalyst and ACN in 1:1:2:44 ratio per sample with overage. For example, for 8 wells mix 16 µL 2-AB Solution, 16 µL 2-AB Reductant, 32 µL 2-AB Catalyst, 704 µL ACN. Mix with pipette or vortex. See **Table 3** for volumes needed for various sample numbers.	80 µL required per sample. Make 20% overage. A 2-AB 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).

Table 3 2-AB Working solution volumes. Includes 20% overage.

Number of Samples	2-AB Solution (µL)	2-AB Reductant (µL)	Catalyst (µL)	ACN (µL)	Total (µL)	Vol/Sample (µL)
n	2/sample	2/sample	4/sample	88/sample	96	80
4	8	8	16	352	384	80
8	16	16	32	704	768	80
24	48	48	96	2112	2304	80

4 Prepare cleanup station with the following items shown in **Figure 3**:

- **a** Gly-X Cleanup plate A
- **b** Vacuum manifold connected to vacuum pump
- **c** Gly-X Waste tray
- **d** Gly-X Collection plate (PCR plate)
- **e** Cly-X Load plate (deep well plate)
- **f** Gly-X Manifold spacer (GX100)

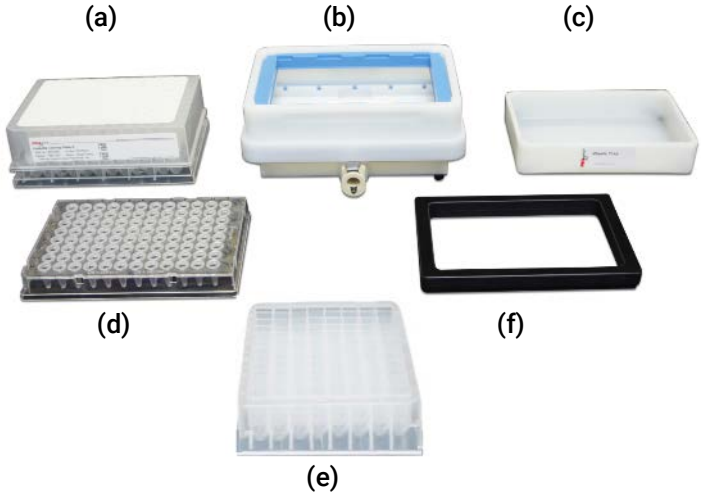

Figure 3. Cleanup station items

Gly-X Deglycosylation

Deglycosylation

1 Add 2 µL of Gly-X Denaturant (orange cap vial) to the bottom of the Gly-X Deglycosylation plate. (See **Figure 4**.)

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.**

Figure 4. Gly-X Deglycosylation plate

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

4 Incubate at 90 °C for three minutes.

NOTE **If a precipitate forms at this point, review the sample buffer composition.**

5 Remove plate, place at room temp for two minutes before adding N-Glycanase. Reset heater to 65 °C in preparation for the labeling step.

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

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

8 Incubate uncapped at 50 °C for five minutes.

Finishing

1 Add 2 µL of finishing reagent (found in the labeling module), 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 animation.**

3 Remove plate from heat block and proceed to Glycan loading.

Glycan loading onto matrix

1 Prepare Gly-X Cleanup plate (**Figure 5**) for the required number of wells by carefully removing white caps.

Figure 5. Gly-X Cleanup plate

2 Place Waste tray in the Vacuum manifold, and assemble the complete manifold.

Figure 6. Vacuum manifold with waste tray

3 Install Cleanup plate on the Vacuum manifold over the waste tray.

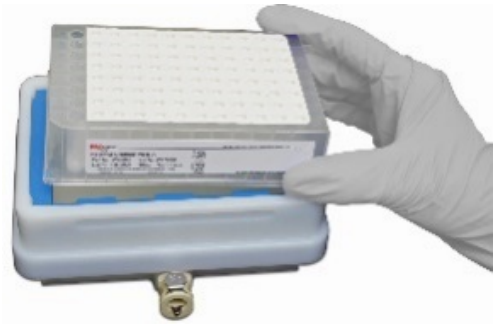

Figure 7. Vacuum manifold with waste tray and cleanup plate

4 With a multichannel pipette, add 450 µL of ACN to one column or row of wells in the Cleanup Plate. Do not apply vacuum.

NOTE

There will be some loss of ACN through the Cleanup Plate. Proceed to step 5 immediately.

5 With a multichannel pipette, add 150 µL of ACN to each sample in the deglycosylation plate, mix well, and then transfer the entire sample (~172 µL) from deglycosylation plate into the corresponding wells of the Cleanup plate. Mix.

6 Repeat steps **4** and **5** until all rows have been transferred to the Cleanup plate.

NOTE

When using a single channel pipette, perform steps 4 and 5 one sample at a time, and repeat steps for all samples.

7 Apply <5 in Hg vacuum. Let the solution pass through.

8 Add 600 µL of acetonitrile to each well. Apply <5 in Hg vacuum.

9 The glycans are now loaded on the Cleanup plate matrix, proceed to **"On-Matrix 2-AB express labeling"** on page 13.

On-Matrix 2-AB express labeling

1 Add 80 µL of 2-AB Working solution to each well of the Cleanup plate.

2 Apply <5 in Hg vacuum. Let the solution pass through.

Figure 8. Working solution added to the Cleanup plate

3 Place the Cleanup plate directly onto the surface of the 65 °C heater block.

Figure 9. Cleanup plate on the heater block

4 Cover the Cleanup plate with Heat Block lid, and incubate at 65 °C for one hour.

Figure 10. Cleanup plate with heat block lid

NOTE **A laboratory fume hood is recommended for 2-AB labeling, even though the heater is covered.**

2-AB Cleanup

Rinse

1 Remove the Waste tray from the manifold, empty. (See **Figure 11**.)

Figure 11. Waste tray

2 Install the Load plate (deep well plate) into the manifold. (See **Figure 12**.)

Figure 12. Load plate (deep well plate)

3 Install Gly-X Cleanup plate on top of the manifold. (See **Figure 13**.)

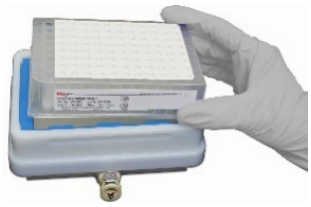

Figure 13. Gly-X Cleanup plate on top of manifold

4 Add 600 µL of ACN to each well in the Cleanup plate.

5 Apply <5 in Hg vacuum, and collect the eluent into the Load plate (deep well).

6 Take the Cleanup plate along with manifold top off the manifold base. Set aside on the storage plate to avoid damaging or contaminating the tips. (See **Figure 14**.)

Figure 14. Cleanup plate with manifold and storage plate

7 Take the Load plate (deep well) off the manifold. (See **Figure 15**.)

Figure 15. Load plate (deep well)

8 Install the Waste tray. (See **Figure 16**.)

Figure 16. Waste tray

9 Install the Cleanup plate on the vacuum manifold over the Waste tray. (See **Figure 17**.)

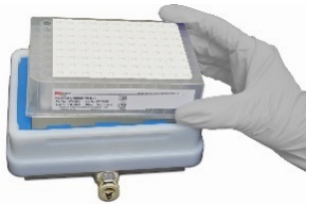

Figure 17. Gly-X Cleanup plate over the Waste tray and vacuum manifold

Reload

1 Transfer the entire ~600 µL of the collected eluent from the Load plate (deep well) into the corresponding wells of the Cleanup plate.

2 Apply <5 in Hg vacuum.

NOTE **The Reload step is to maximize glycan recovery.**

Wash

1 Wash three times with 600 µL of acetonitrile with vacuum to <5 in Hg.

2 After final wash, increase vacuum to ~ 20 in Hg for 30 seconds to fully dry wells.

NOTE **If processing more than 48 samples, empty the Waste tray after the second wash (Waste tray holds approximately 120 mL). Dispose of organic waste in the appropriate stream.**

NOTE **If shutting off the vacuum by turning off the pump, set the valve to 5 in Hg in preparation for elution.**

3 Remove the Cleanup plate from the Vacuum manifold and blot the bottom with a lab wipe to remove any remaining residual solvent. Set aside.

4 Remove Waste tray from vacuum manifold.

Elute

1 Install the Gly-X Vacuum manifold spacer with mitered edge facing up in the manifold. (See **Figure 18**.)

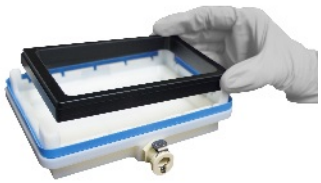

Figure 18. Gly-X Vacuum manifold spacer

2 Install the Collection plate (PCR plate). (See **Figure 19**.)

Figure 19. Collection plate (PCR plate)

3 Assemble the manifold, and install the Cleanup plate on the vacuum manifold over the Collection plate. (See **Figure 20**.)

Figure 20. Cleanup plate over Collection plate

4 Add 100 µL of DI Water to each well of the Cleanup plate.

5 Apply <5 in Hg vacuum, and collect cleaned labeled glycan 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 one to two minutes. Do not increase vacuum during the elution step.

7 Tap the manifold on the bench to release drops from bottom of Cleanup plate.

8 Release the vacuum, remove the Cleanup plate to fully release vacuum, then disassemble manifold and remove the Collection plate.

9 Mix each sample with a pipette.

NOTE

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

10 Seal the Collection plate with Sealing film.

11 Add Black Used Well Sealing Caps to used Cleanup plate wells, return to bag and store at RT.

12 2-AB-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 (Figure 21) should be placed on wells used in previous cleanup procedures to prevent reuse of wells.

NOTE

Cleanup plate storage (round bottom) plate (Figure 21) should be used to store Cleanup plate after use. Store in bag provided at room temperature.

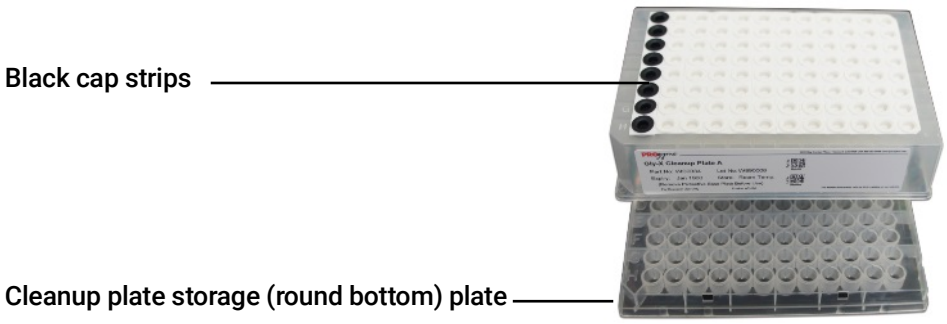

Figure 21. Cleanup plate with black cap strips

Analysis of Labeled Glycans

Excitation/Emission wavelengths for 2-AB Glycans

Optimal excitation/emission wavelengths for 2-AB conjugated to an N-glycan may vary depending upon the optical configuration of the instrument used. Excitation/emission pairs that have been used together include:

- 360/428 nm (used by Agilent with the Waters Acquity UHPLC)
- 260/430 nm (used by Agilent with the Agilent 1290 Infinity)
- 250/428 nm (Melmer *et al.*, 2010)
- 330/420 nm (Bigge *et al.*,1995)

LC Injection volumes for 2-AB Glycans

Injection of 1 µL 2-AB-glycans in water is recommended for UHPLC.

For larger injection volumes (>1 µL) of 2-AB-glycans, dilute with acetonitrile up to 75% ACN.

If a more concentrated injection is needed, the glycans in 100 µL may be dried down using SpeedVac (no heat) and reconstituted in a smaller volume.

Recommended HILIC methods for 2-AB-labeled Glycans

Agilent AdvanceBio Amide HILIC Column

Agilent AdvanceBio Amide HILIC columns are designed for HILIC separations of N-glycans. The totally porous, 1.8 µm silica particles and unique bonding chemistry provide for exceptional long-term stability and reproducibility, while providing optimal separation of complex N-glycan mixtures.

Recommended starting conditions

Please consult the AdvanceBio Amide HILIC column User Manual (**5994-6915EN**) for instructions including installation, column conditioning and operating parameters (pH, temperature, and pressure).

- AdvanceBio Amide HILIC column, 2.1 × 150 mm, 1.8 µm (p/n 859750-913)
- Mobile phase A: 50 mM Ammonium formate, adjusted to pH 4.4 with formic acid, or prepared from Agilent AdvanceBio Ammonium Formate HILIC Mobile Phase concentrate (p/n G3912-00000)
- Mobile phase B: Acetonitrile
- Column temperature: 60 °C
- Flow rate: 0.5 mL/min

The following methods for 2-AB glycans are used in Application Note **5994-7477EN**, Glycan Peak Assignment Tool (GPAT):

Table 4 LC gradient for 2-AB glycan samples containing neutral, S1, and S2 glycans

Time (min)	%B
0	74
50	54
51	40
52	40
54	74
64	74

Table 5 LC gradient for 2-AB glycan samples containing neutral through S4 glycans

Time (min)	%B
0	74
75	44
76	40
77	40
79	74
90	74

Glycan Peak Assignment Tool (GPAT)

HILIC retention time (RT) alone is often insufficient to confidently match the numerous possible glycan structures to the resulting chromatographic peaks. It is possible to predict peak identities based on a library of glucose unit (GU)-normalized glycan retention times. The Glycan Peak Assignment Tool (GPAT) contains libraries of GU values for over 100 InstantPC- and 2-AB-labeled N-glycans separated using the AdvanceBio Amide HILIC column and mobile phase. The tool is web-based, free of charge, and getting started requires only the input of retention times (RTs) acquired with a 2-AB-labeled ladder standard (p/n GKSB-503). Using the RTs from the ladder standard, the tool generates a list of predicted N-glycan RTs using the libraries, avoiding the need to input potentially sensitive sample information.

In Application Note **5994-7477EN**, we used the tool to annotate glycans present in cetuximab and have characterized the prediction accuracy of GPAT on a variety of glycan structures.

The tool is designed for use with data collected on the 2.1 × 150 mm version of the Agilent AdvanceBio Amide HILIC column (p/n 859750-913, and is freely available through the Agilent website at the following link: **www.agilent.com/biopharma/gpat**.

15-Minute UHPLC methods for Waters BEH Glycan separation technology column

2.1 x 100 mm, 1.7 µm column temperature 60 °C, excitation 360 nm, emission 428 nm

Table 6 15-minute method, Waters column

Time (min)	Flow Rate (mL/min)	% Acetonitrile	% 100 mM Ammonium Formate, pH 4.4
0.0	1.0	80.0	20.0
2.0	1.0	80.0	20.0

Table 6 15-minute method, Waters column (continued)

Time (min)	Flow Rate (mL/min)	% Acetonitrile	% 100 mM Ammonium Formate, pH 4.4
12.0	1.0	52.5	47.5
12.1	0.5	40.0	60.0
12.5	0.5	40.0	60.0
12.6	0.5	75.0	25.0
12.7	1.0	80.0	20.0
15.0	1.0	80.0	20.0

60-Minute UHPLC methods for Waters BEH Glycan separation technology column

2.1 x 150 mm, 1.7 µm column temperature 60 °C, excitation 360 nm, emission 428 nm

NOTE

This method was used in application note 5994-0942EN, Comparison of Common Fluorescent Labels for LC/MS Analysis of Released N-Linked Glycans.

Table 7 60-minute method, Waters column

Time (min)	Flow Rate (mL/min)	% ACN	% 50 mM Ammonium Formate, pH 4.4
0.0	0.4	80.0	20.0
2.0	0.4	80.0	20.0
2.5	0.4	75.0	25.0
50.0	0.4	62.0	38.0
52.0	0.4	40.0	60.0
53.5	0.4	40.0	60.0
55.0	0.4	80.0	20.0
60.0	0.4	80.0	20.0

Suggested MS conditions for 2-AB-labeled Glycans

MS conditions

Agilent Jet Stream ESI source, any MS, positive mode, sheath gas 300 °C at 10.0 L/min, dry gas 150 °C at 9.0 L/min, nebulizer pressure 35 psig, VCap 2,500 V, Nozzle 500 V, Fragmentor 100 V, (if applicable).

Waters Xevo G2-S QTof, positive or negative mode, capillary voltage 2.8 kV, cone voltage 30 V, source temperature 120 °C, desolvation temperature 350 °C, scan time 0.8 second, *m/z* range 300 to 2,000 Da.

Calculating the mass of Glycans labeled with 2-AB

Mass added to glycan with a free reducing end:

- Mass of Glycan (free reducing end) + $C_7N_2OH_8$-O= Mass of 2-AB-Labeled Glycan
- Mass added by $C_7N_2OH_8$-O
  - Monoisotopic: 120.06875 Da
  - Average: 120.2 Da

Appendix A

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 6** on page 19). The ratio of reagents in the automated protocol are equivalent to the standard protocol, but minimum pipetting volume changes from 2 to 5 µL to improve reliability of automated pipetting.

Table 8 Working Solution instructions for Sample, Denaturant and N-Glycanase, to accommodate a minimum pipetting volume of 5 µL. Prepare other working solutions as directed in Table 2 and Table 3 on page 9.

Sample and Reagents	Standard Protocol	Automation Protocol
Sample	20 µL at up to 2 mg/mL (40 µg max)*	15 µL at up to 2.67 mg/mL (40 µg max)*
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 Denaturation 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 eight 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).

* It may be possible to use up to 100 µg glycoprotein, depending on the sample type (see **"FAQs"** on page 22).

FAQs

Q: What are the most common adducts seen in MS analysis of 2-AB-labeled glycans?

A: Please refer to the list of most common 2-AB-labeled glycan adducts and their masses in both positive and negative MS mode at **www.agilent.com**.

Q: Which is better for MS analysis of 2-AB-labeled glycans, positive or negative mode?

A: There are pros and cons with each. Positive mode: less noisy background, stronger signal. Negative mode: less adducts and hence MS looks less convoluted.

Q: Can I place the collection plate directly into an UHPLC autosampler? What are the plate dimensions?

A: Yes.

For an Agilent LC, operated with OpenLab 2, in the Control Panel close the connection to the instrument and select "Configure Instrument." Right-click to open the autosampler module, at the bottom of that window click "Define Sample Containers" to see the list of options. Add a new option with the dimensions shown in **Figure 22**.

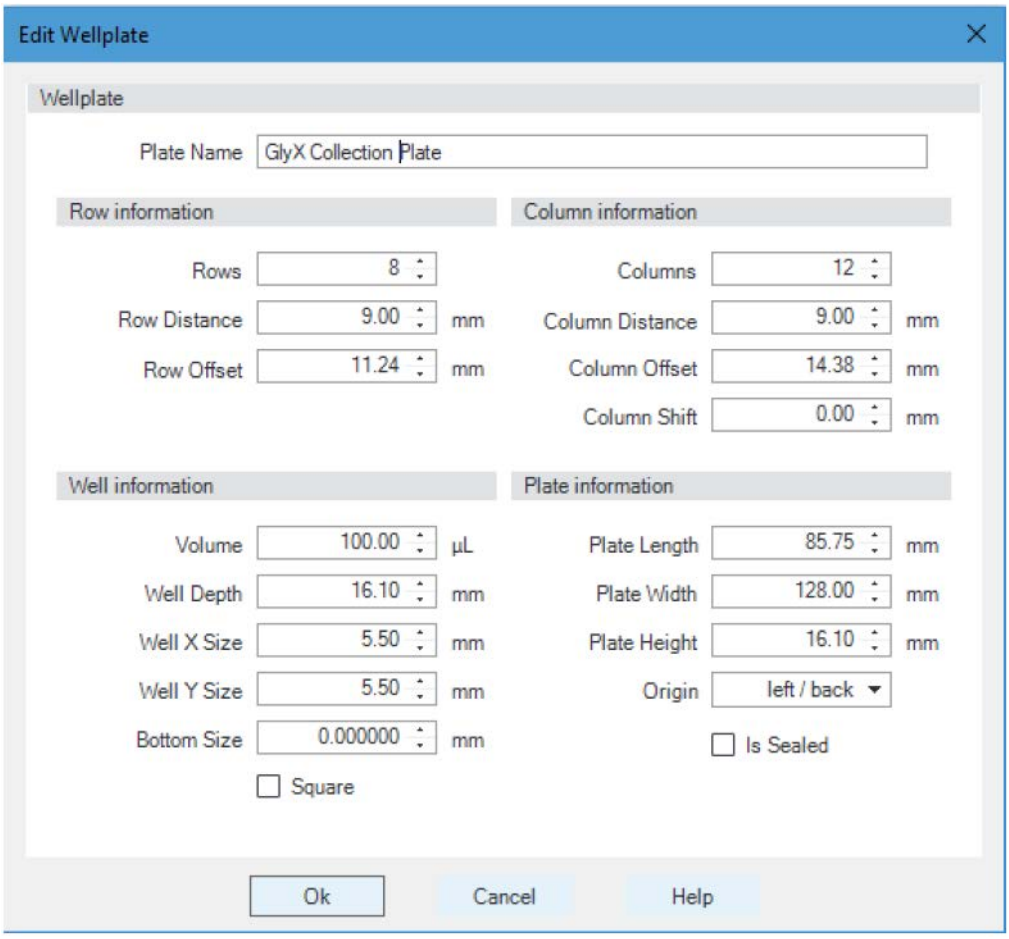

Figure 22. Edit well plate settings

For Agilent LCs operated using MassHunter, the collection plate can be configured in a similar way using the Instrument Configuration tool.

Figure 23 shows the settings that can be used for example on a Waters Acquity UPLC.

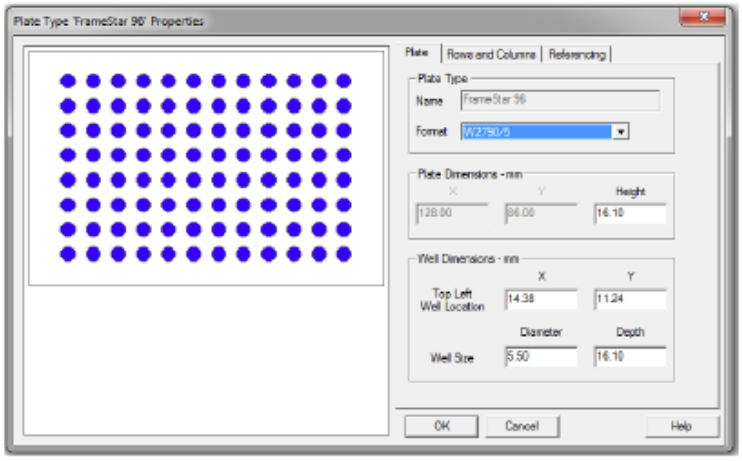

Figure 23. Collection plate settings

NOTE

When injecting from a plate, avoid regular adhesive foils or adhesive sealing film as the adhesive can cause issues with the probe. Adhesive sealing foils and films need to be removed. Use the preslit sealing film included in the kit (no adhesive in the well area), or a heat sealed pierceable foil (do not use the storage sealing foil). Avoid any covers/foils that have adhesive directly above the wells; adhesive can damage the UHPLC probe. The preslit film included in the kit can be covered over with the sealing foil included in the kit for storage. The included sealing foil can be removed prior to analysis leaving the preslit film in place. Additional sealing foil can be ordered from Thermo Scientific (AB-0626).

Collection Plates may be sealed with sealing film (preslit), or heat sealed with pierceable foil (for example, Thermo Easy Pierce 20 µm Foil, #AB-1720) using a microplate heat sealer (for example, Thermo ALPS 50 V Semi-automated Microplate Heat Sealer, #AB-1443).

Collection Plate dimensions are also available on the manufacturers website: Framestar 96-well skirted PCR Plates (#4ti-0960/C, E&K part number 75094). Without needle adjustment, the dead volume using this method is approximately 15 µL. The sample may also be transferred into a vial designed for greater recovery (for example, Waters Total Recovery Vial).

Q: My samples are not loading completely onto the Gly-X Cleanup Matrix ("Glycan loading onto matrix" on page 11). What is happening?

A: This may be caused by the nature of your protein sample, or by matrix effects caused by the composition of your formulation buffer. This can be addressed by using less protein per reaction, or by buffer exchanging your protein prior to starting the Gly-X protocol.

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

A: It depends on the protein. Typically, protein quantities up to 100 ug are compatible with this sample prep, but users should test to ensure that >40 µg protein can be used without a loss of linear response in glycan relative % area values. Please contact Agilent if you need assistance.

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 on the lid during mixing.

Q: Is it possible to use a plate shaker/heater such as the Eppendorf Thermo Mixer C for mixing and heating during the Denaturation and Digestion protocols?

A: Yes, this is possible. Mix rate should be high enough to vortex material in wells.

Q: What is the Finishing Reagent prior to the 2-AB labeling step?

A: The Finishing Reagent is a weak acid, and drives conversion of the glycosylamine (-NH2) to a glycan with a free reducing end (-OH) prior to 2-AB labeling by reductive amination.

Q: For the 2-AB labeling step, the heat block is at 65°C. what is the actual temperature of the reaction?

A: The reaction temperature in the Cleanup Plate Matrix is ~5 degrees lower than the heat block setpoint or ~60°C.

Q: Can I use the GX96-2AB 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 (p12). GX200 provides the correct distance between the Cleanup Place 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.

Q: I'm seeing low fluorescence signal of labeled glycans by LC analysis. How do I address this?

A: There are a number of options for increasing signal, but first check the FLD ex/em wavelengths (**"Excitation/Emission wavelengths for 2-AB Glycans"** on page 18), as these can differ depending on the instrument manufacturer. Options include:

1. Inject more labeled glycans after dilution with ACN (**page 18**).
2. Dry down the glycans using for example, SpeedVac (no heat) and resuspend in a smaller volume.
3. During the protocol, elute labeled glycans with 50 µL of DI Water rather than 100 µL (See **"Elute" step 4** on **page 16**). This should work for most sample types. Please contact Agilent with any questions.

Resources and References

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

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Virtual patent marking

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References

Bigge, J. C., T. P. Patel, J. A. Bruce, P. N. Goulding, S. M. Charles, R. B. Parekh. Nonselective and Efficient Fluorescent Labeling of Glycans Using 2-Amino Benzamide and Anthranilic Acid. Anal Biochem 230: 229-38 (1995).

Melmer, M., T. Stangler, M. Schiefermeier, W. Brunner, H. Toll, A. Rupprechter, W. Lindner and A. Premstaller. HILIC Analysis of Fluorescence-Labeled N-Glycans from Recombinant Biopharmaceuticals. Anal Bioanal Chem 398: 905-914 (2010)

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
GX96-2AB	Gly-X with 2-AB Kit (96-ct)
GX24-2AB*	Gly-X with 2-AB Express Kit (24-ct)
GX24-401AB	Gly-X with 2-AB Express Deglycosylation and Labeling Module Set (24-ct)
GX96-401	Gly-X 2-AB Express Labeling module (96-ct)
GX24-401	Gly-X 2-AB Express Labeling module (24-ct)
GX96-402	Gly-X Cleanup module for 2-AB Express (96-ct)
GX96-100	Gly-X Deglycosylation module (96-ct)
GX24-100	Gly-X Deglycosylation module (24-ct)
GX400	Gly-X 2-AB Express starter pack (contains Gly-X Vacuum manifold spacer and 2 Heater block lids, two sizes)
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)

* 24 ct kit (GX24-2AB) contains a 96-well cleanup plate and 24-ct 2-AB Labeling module. Store the cleanup module at room temperature and order 24 ct refills of Gly-X 2-AB Express Deglycosylation and Labeling module set (GX24-401AB)

AdvanceBio Amide HILIC Columns and Mobile Phase Concentrate

Part Number	Description
859750-913	AdvanceBio Amide HILIC, 2.1 × 150 mm, 1.8 µm
858750-913	AdvanceBio Amide HILIC, 2.1 × 100 mm, 1.8 µm
G3912-00000	AdvanceBio Ammonium Formate Mobile Phase Concentrate (Makes 1 L of 50 mM Ammonium Formate, pH 4.4)

2-AB Labeled Glycan Libraries

Product Code	Description
GKSB-005	Human IgG N-Linked Glycan library
GKSB-520	Biantennary and High Mannose partitioned library
GKSB-001	Human α1-acid glycoprotein N-Linked Glycan library
GKSB-003	Bovine Fetuin N-linked Glycan Library
GKSB-503	Glucose Homopolymer standard
GKSB-232	α(2-3) Sialylated Biantennary Library
GKSB-262	α(2-6) Sialylated Biantennary Library
GKSB-233	α(2-3) Sialylated Triantennary Library
GKSB-263	α(2-6) Sialylated Triantennary Library
GKSB-234	α(2-3) Sialylated Tetraantennary Library
GKSB-264	α(2-6) Sialylated Tetraantennary Library

2-AB Labeled Individual Glycan Standards

Product code	Description	Product code	Description
GKSB-401	G0-N	GKSB-308	G3
GKSB-301	G0	GKSB-314	A3
GKSB-402	G0F-N	GKSB-309	NGA4
GKSB-302	G0F	GKSB-310	G4
GKSB-303	G0FB	GKSB-111	HYBR
GKSB-317	G1	GKSB-100	NN
GKSB-316	G1F	GKSB-101	Man3
GKSB-304	G2	GKSB-102	Man3F
GKSB-305	G2F	GKSB-103	Man5
GKSB-306	G2FB	GKSB-104	Man6
GKSB-318	NA2Ga2F	GKSB-105	Man7
GKSB-311	A1	GKSB-106	Man8
GKSB-315	A1F	GKSB-107	Man9
GKSB-312	A2	GKSB-201	GalGalNAc
GKSB-313	A2F	GKSB-203	3'-SLN
GKSB-307	NGA3	GKSB-204	6'-SLN

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