Gly-*X*™ N-Glycan Rapid Release and Labeling with InstantPC™ Kit

User Manual

Product Code GX96-IPC, GX24-IPC
Version AA

PROzyme
Advancing Glycosciences

This product is intended for *in vitro* experimental research use only

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INTRODUCTION

The Gly-*X* N-Glycan Rapid Release and Labeling with InstantPC kit utilizes a novel in-solution enzymatic protein deglycosylation followed by rapid labeling of released N-glycans with InstantPC dye. After a simple clean-up step, the glycan samples are ready for analysis by UHPLC, LC-MS, MS/MS and other methods. With deglycosylation and labeling carried out in solution, the method is simple, rapid and suitable for automation. The InstantPC dye delivers unmatched fluorescent brightness and MS signal, which enables a single labeling method to be deployed across different glycan analysis workflows. Other benefits include:

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

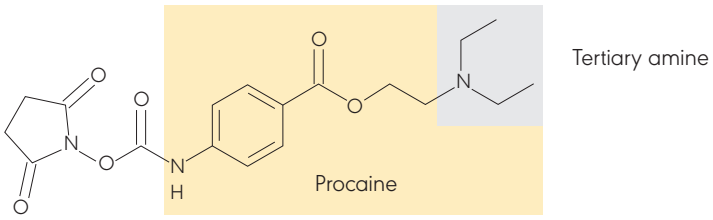


Figure 1: InstantPC dye (IPC)

KIT COMPONENTS

The Gly-*X* with Instant PC (GX96-IPC) consists of three 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 or 4 °C
	Gly-*X* N-Glycanase, 120 µl	1	4 °C
	Gly-*X* Digestion Buffer, 240 µl	2	RT or 4 °C
	Gly-*X* Denaturant, 240 µl	1	RT or 4 °C
Gly-*X* InstantPC Labeling Module GX96-101	InstantPC Dye (lyophilized)	4	-20°C
	Gly-*X* InstantPC Dye Solvent, 600 µl	1	-20°C
Gly-*X* InstantPC Clean-up Module GX96-102	Gly-*X* Cleanup Plate A	1	RT or 4 °C
	Gly-*X* InstantPC Eluent	1	RT or 4 °C
	Gly-*X* Used-Well Sealing Caps, Black (for Cleanup Plate)	96	RT or 4 °C
	Gly-X Collection Plate, 96 vials	1	RT or 4 °C
	Gly-*X* Collection Plate Sealing Caps, Red	96	RT or 4C
	Waste Tray for vacuum manifold	1	RT or 4 °C

Note: Gly-X InstantPC Eluent is 160 mM Ammonium Formate w/10% (v/v) Acetonitrile
Note: Gly-X with InstantPC 24ct kit (GX24-IPC) contains 30ul Gly-X N-Glycanase, 1 vial Gly-X Digestion Buffer, 1 vial InstantPC Dye and all other kit components listed in the table.

EQUIPMENT & REAGENTS PROVIDED BY USER

- 96-well Thermocycler or two independent heat blocks, set for 90 °C and 50 °C.
  - Thermocycler (Corning THERM-1001, 110V; THERM-1000, 230V)
  - *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, 220V)
- Formic acid, MS-grade (Fisher A117-50 recommended)
- Acetonitrile (ACN), MS-grade (Fisher A955-4 recommended)

SAMPLE PREP CONSIDERATIONS

In general, glycoprotein protein 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. Samples in salt-containing buffers (~150mM salt) are compatible with the kit, however, the use of PBS is not recommended (see FAQs). The preferred dilient is water. 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).

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 2.1. For citrate-containing buffers, dilute sample with water to reduce citrate below 20 mM.

If a precipitate is observed upon incubation at 90 °C, 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 ProZyme at info@prozyme.com

PROTOCOL

1) Getting Started

1. Prepare samples (see considerations above)
2. Set the thermocycler to 90 °C, or set two independent heat blocks to 90 °C and 50 °C.
3. Prepare the working solutions in the table below

Table 2: Working solution preparation

Working Solution	Instructions	Notes
N-Glycanase Working Solution	Mix N-Glycanase & Gly-*X* Digestion Buffer 1:1 (v/v). For each well mix 2.2 µl of working solution; for 8 wells mix 8.8 µl N-Glycanase and 8.8 µl Digestion Buffer.	2 µl required per sample, mix 20% overage of working solution.
InstantPC Dye Solution	Remove InstantPC Dye & Gly-*X* InstantPC Dye Solvent from -20 °C. Warm to room temperature, remove from desiccant pouch. Add 150 µl of Gly-*X* InstantPC Dye Solvent to the InstantPC Dye vial, vortex until dissolved.	5 µl required per sample. InstantPC Dye Solution is stable at -20 °C, 1 month & 10 freeze thaw cycles in desiccant pouch.
Load/Wash Solution 2.5% Formic acid/97.5% Acetonitrile	Stock solution for 96 samples: Add 3 ml of Formic acid to a glass graduated cylinder. Bring the volume up to 120 ml with 100% Acetonitrile. Transfer to a glass storage vessel, cap tightly, swirl to mix.	Approximately 1.2 ml required per sample Tightly cap, store up to 6 months at RT

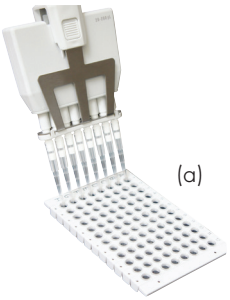
4. Prepare cleanup station.
   - Have at hand:
     - Vacuum manifold
     - Gly-*X* Cleanup Plate A
     - Black Caps
     - Gly-*X* Collection Plate
   - Connect the vacuum manifold to vacuum pump
   - Place waste tray in manifold

2) Deglycosylation

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

   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.
3. Mix well using a pipette, then tap on benchtop to collect samples on bottom of wells (or spin).
4. Incubate at 90 °C for 3 min.



(a)

   Note: If a precipitate forms at this point, review the sample buffer composition.

5. Remove plate, place at room temp for 2 minutes before adding N-Glycanase.
6. Add 2 µl of N-Glycanase Working Solution to each sample.
7. Mix well using a pipette, then tap on benchtop to collect samples on bottom of wells (or spin).
8. Incubate uncapped at 50 °C for 5 min.
9. Remove plate from heat and proceed directly to InstantPC labeling.

3) InstantPC Labeling

1. Add 5µL of InstantPC Dye Solution, prepared above, per sample. After each addition, mix thoroughly with a pipette. Repeat until InstantPC Dye Solution has been added to each sample.

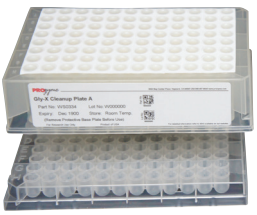
   Note: A precipitate is normal.

2. Incubate at 50 °C for 1 min.
3. Tightly cap the InstantPC Dye Solution, place in desiccant pouch and return to -20 °C.

4) InstantPC Cleanup

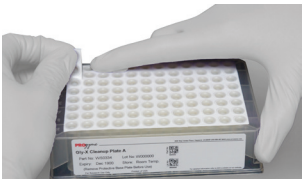
LOAD

1. Prepare Gly-*X* Cleanup Plate A for the required number of wells.



2. Use only the required wells by carefully removing white caps (b), or cutting away white caps for fewer samples.

   Note: Used-Well Caps should be placed on wells that were used in previous cleanup procedures to improve the vacuum.



3. Place Waste Tray in vacuum or pressure manifold.

4. Install Gly-*X* Cleanup Plate A.

5. Add 180 µl of Load/Wash Solution to the first sample in the deglycosylation plate, mix with pipette.
6. Transfer the entire sample to the corresponding well in the Gly-*X* Cleanup Plate A.
7. Repeat step 5 & 6 for all samples, transferring each to the Gly-*X* Cleanup Plate A.
8. Add 200 µl Load/Wash Solution to each sample.
9. Apply the vacuum to 5 inHg for for 2 min, then increase to 24–25 inHg until all wells are empty. This step loads the sample to the Gly-*X* Cleanup A matrix.

   Note: Difficult samples may take up to 3 min or more to load; matrix appears white when wells are empty.

WASH

1. Wash with 400 µl of Load/Wash Solution (vacuum at 5 inHg for 2 min and then 24–25 inHg to clear any slow flowing wells), collecting wash in the Waste Tray.
2. Release the vacuum and empty the Waste Tray into organic waste.
3. Repeat Wash with 400 µl of Load/Wash Solution, collecting wash in the Waste Tray.
4. Release vacuum. Remove the Waste Tray (fig 2.1) from manifold. Remove storage lid from collection plate (fig 2.2) and install collection plate in vacuum manifold (fig 2.3).

Figure 2.1

Figure 2.2

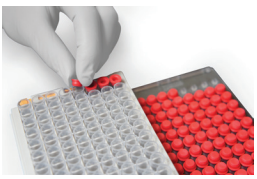
Figure 2.3

5. Install the Gly-*X* Cleanup Plate A on top of the vacuum manifold, apply the vacuum (5 inHg), press to seal.

ELUTE

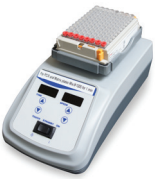
1. Add 100 µl of Gly-*X* InstantPC Eluent to each well. Using vacuum, (5 inHg for 2 min, then 24–25 inHg until all wells are dry) elute samples into the Collection Plate.
2. Release the vacuum, remove the collection plate. Seal the Collection Plate with Red Sealing Caps.

   Note: Solid red caps can be removed for sample access, or pierced a single time.

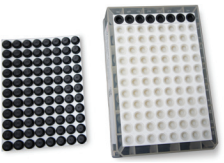


3. Vortex to mix, and spin/tap to collect samples at the bottom of the wells, or mix with a pipette.

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



4. Add Black Used Well Sealing Caps to used Cleanup Plate A wells, return to in clear bag and store at RT or 4 °C.
5. InstantPC-labeled glycan samples are ready for analysis. Samples may be stored at -20 °C for up to a month in Gly-*X* InstantPC eluent, or 4 °C for up to 5 days in either Gly-*X* InstantPC Eluent or after dilution with ACN/DMF (see next section).



ANALYSIS OF LABELED GLYCANS

The optimal excitation and emission wavelengths for InstantPC Dye conjugated to an N-glycan are:

- Excitation: 285 nm
- Emission: 345 nm

Injection of 1 µl InstantPC-glycans in Gly-*X* InstantPC Eluent (160 mM Ammonium Formate, 10% (v/v) Acetonitrile) is recommended for UHPLC.

- For larger injection volumes (> 1 µl) of InstantPC-glycans, do not use ACN alone to dilute the sample as this may cause sialylated glycans to precipitate. Use 1 part sample in eluent to 3 parts 1:1 [v/v] ACN:DMF, for a final concentration of 22.5% aqueous buffer, 37.5% DMF, 40.0% ACN to more closely match the high organic % at the start of a HILIC method.

Dilution examples for Gly-*X* InstantPC-labeled glycan samples for HILIC injections of > 1 µl:

Table 3: **Recommended dilutions for InstantPC-labeled glycan samples and standards**

Total volume needed (µl)	IPC-labeled glycan samples in 10% organic Gly-X Eluent (µl)	IPC-labeled glycan standards (µl)*	1:1 [v/v] ACN:DMF (µl)	% Organic	% Aqueous
10	2.5		7.5	77.5	22.5
20	5		15	77.5	22.5
40	10		30	77.5	22.5
10		2.5	7.5	75	25
20		5	15	75	25
40		10	30	75	25

** InstantPC-labeled standards previously reconstituted in water according to the instructions provided with the standards.*

Recommended HILIC Methods for InstantPC-labeled Glycans

5-Minute screening UHPLC method for Agilent AdvanceBio Glycan Mapping column:

2.1 x 100 mm, 2.7 µm. Column temperature 35 °C, excitation 285 nm, emission 345 nm.

Table 4: **5-Minute method, Agilent column**

Time (min)	Flow rate (ml/min)	% ACN	% 100 mM Ammonium Formate, pH 4.4
0.00	1.4	77.0	23.0
4.00	1.4	60.0	40.0
4.15	0.75	40.0	60.0
4.30	0.75	40.0	60.0
4.40	1.4	77.0	23.0
5.00	1.4	77.0	23.0

60-Minute high-resolution UHPLC method for Agilent AdvanceBio Glycan Mapping column:
2.1 x 150 mm, 2.7 µm. Column temperature 45 °C, excitation 285 nm, emission 345 nm.

Table 5: 60-minute Method, Agilent column

Time (min)	Flow Rate (ml/min)	% ACN	% 50 mM Ammonium Formate, pH 4.4
0.0	0.4	80.0	20.0
43.5	0.4	54.0	46.0
45.0	0.4	00.0	100.0
50.0	0.4	00.0	100.0
52.0	0.4	80.0	20.0
60.0	0.4	80.0	20.0

15-Minute UPLC methods for Waters BEH Glycan Separation Technology column:
2.1 x 100 mm, 1.7 um column temperature 60 °C, excitation 285 nm, emission 345 nm.

Table 6: 15-minute method, Waters column

Time (min)	Flow Rate (ml/min)	% ACN	% 100 mM Ammonium Formate, pH 4.4
0.0	1.0	75	25
12.0	1.0	50	50
12.1	0.5	40	60
12.5	0.5	40	60
12.6	0.5	75	25
13.0	1.0	75	25
15.0	1.0	75	25

60-Minute UPLC methods for Waters BEH Glycan Separation Technology column:
2.1 x 150 mm, 1.7 µm column temperature 45 °C, excitation 285 nm, emission 345 nm.

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
43.5	0.4	54.0	46.0
45.5	0.4	40	60
50.0	0.4	40	60
52.0	0.4	80.0	20.0
60.0	0.4	80.0	20.0

Recommended MS Conditions for InstantPC-labeled Glycans

MS conditions: Waters Xevo G2-S QTof, + 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 300–2000 Da.

MS/MS conditions: Collision energy ramp of 40–60 V for +1; 15–30 V for +2; 15–25 V for +3; 1.0 second scan time, m/z range 50–2000 Da.

Calculating the Mass of Glycans Labeled with InstantPC

Mass added to glycan with a free reducing end:

- Mass of Glycan (free reducing end) + $C_{14}N_3O_2H_{19}$ = Mass of InstantPC-Labeled Glycan
    - Mass added by $C_{14}N_3O_2H_{19}$

      Monoisotopic: 261.14773 Da

      Average: 261.3 Da

Mass added to glycosylamine:

- Mass of Glycan (glycosylamine) + $C_{14}N_2O_3H_{18}$ = Mass of InstantPC-Labeled Glycan
    - Mass added by $C_{14}N_2O_3H_{18}$

      Monoisotopic: 262.13174 Da

      Average: 262.3 Da

FAQS

Q: ***Why is PBS not recommended as a diluent for samples?***

A: PBS is compatible with Gly-X InstantPC kit, however, use of PBS for sample dilution may result in artifact peaks. These peaks elute early in the separation, near the free dye peak and should not interfere with N-glycan analysis. For optimal performance, dilution of samples with water is preferred.

Q. ***You suggest 100 mM ammonium formate for the 5-minute HILIC separation, 50 mM ammonium formate for the 60-minute separations. Is this correct?***

A. For fast separations we stick with 100mM buffer, for MS in conjunction with longer separations we use 50mM ammonium formate.

Q. *What is the most common adduct seen in MS analysis of InstantPC-labeled glycans?*

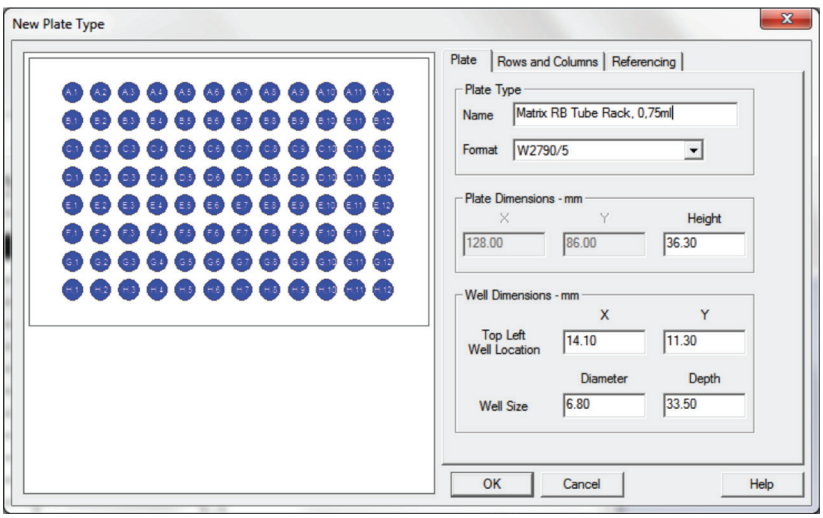
A. In positive mode MS, most biantennary InstantPC- N-glycans will give $[M+2H]^{+2}$, larger sialylated will be majority $[M+3H]^{+3}$.

Table 8: InstantPC-glycan masses for some major N-glycan species

Glycan ID	InstantPC-Glycan Monositopic Mass	$[M+2H]^{+2}$	$[M+3H]^{+3}$
Man5	1495.5811	748.7978	499.5343
G0	1577.6343	789.8244	526.8854
G0F	1723.6922	862.8534	575.5713
G1	1739.6871	870.8508	580.9030
G1F	1885.7450	943.8798	629.5889
G2F	2047.7978	1024.9062	683.6065
A1	2192.8353	1097.4249	731.9524
A1F	2338.8932	1170.4539	780.6383
A2	2483.9307	1242.9726	828.9842
A2F	2629.9886	1316.0016	877.6701

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

A. Yes, here are settings that can be used for example on a Waters Acquity UPLC:
Note: red sealing caps need to be removed.



Q: *Why can I not use ACN to dilute the samples prior to HILIC injection? And can I use something other than DMF for the dilution?*

A: If the InstantPC-labeled glycans in Gly-*X* Eluent are diluted with ACN alone, this may cause the sialylated glycans to precipitate. We have not tested alternatives to DMF for sample dilution.

Q: *My samples are not loading completely onto the* Gly-*X Cleanup Matrix (Load Step 9, page 9). 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: Maybe, although it will depend on the protein. You would have to test to ensure that > 40 µg of protein can be successfully loaded onto the Gly-X Cleanup Matrix without blockage.

RESOURCES AND REFERENCES

Kimzey et al. Development of an Instant Glycan Labeling Dye for High Throughput Analysis by Mass Spectrometry. www.prozyme.com/posters/instantpc

Visit ProZymes's website for additional information, downloadable posters, publications and technotes: http://prozyme.com/pages/resources

ORDERING INFORMATION & ADDITIONAL INFORMATION

Ordering Information

GX96-IPC	Gly-*X* with InstantPC Kit (96 ct)
GX24-IPC	Gly-*X* with InstantPC Kit (24 ct)
GX96-201PC	Gly-*X* with InstantPC Deglycosylation and Labeling Module Set (96 ct)
GX24-201PC	Gly-*X* with InstantPC Deglycosylation and Labeling Module Set (24 ct)
GX96-101	Gly-*X* InstantPC Labeling Module (96 ct)
GX24-101	Gly-*X* InstantPC Labeling Module (24 ct)
GX96-102	Gly-*X* InstantPC Cleanup Module (96 ct)
G5524-60010 KIT	AssayMAP PA50 protein A affinity purification kit (96 ct)
GPKC-005	Human IgG Library with InstantPC
GKPC-503	Dextran Ladder with InstantPC

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GX96-PC Version AA