NOTICE: ProZyme was purchased by Agilent in July 2018. Documents for products and product lots manufactured before August 2019 will contain references to ProZyme. For more information about these products and support, go to: **www.agilent.com/en/contact-us.**

Rapid N-Glycan Preparation with InstantPC™

Enzymatic deglycosylation, fluorescent labeling with InstantPC and cleanup of excess dye for analysis by LC, LC-MS, or other methods.

- Optional protein A purification for monoclonal antibodies and Fc proteins
- Non-selective, rapid release and recovery of intact N-Glycans from up to 96 glycoprotein samples at a time using a microplate centrifuge
- Non-selective chemistry for stoichiometric labeling of glycans, independent of structure
- Flexible, high-throughput format: process 1 to 192 samples per run (2 kits simultaneously)
- InstantDye™ labeling of N-glycans with InstantPC provides high fluorescence and MS signal
- Labeled glycans are eluted in low volume and ready for analysis without concentrating or drying

Product Code: GP96NG-PC

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This product is intended for in vitro research use only.

NOTE: The following suggestions and data are based on information we believe to be reliable. They are offered in good faith, but without guarantee, as conditions and methods of use of our products are beyond our control. We recommend that the prospective user determine the suitability of our materials and suggestions before adopting them on a commercial scale. Suggestions for use of our products or the inclusion of descriptive material from patents and the citation of specific patents in this publication should not be understood as recommending the use of our products in violation of any patent or as permission to license to use any patents of ProZyme, Inc. See prozyme.com/patents.

KIT CONTENTS

NOTE: We want successful results for our customers, so please read this entire booklet before starting the procedure.

Item	Qty
G5524-60010 KIT AssayMAP PA50 (purchased separately)	Optional
G5524-60010 AssayMAP PA50 (96 Cartridges)	
WS0296 Protein A Solution Set	
WS0294 10x Wash Buffer (15 ml)	
WS0251 Eluent (30 ml)	
GP96NG-PC GlykoPrep® Rapid N-Glycan Preparation with InstantPC	
GS96-RX GlykoPrep Digestion Module (2–8°C)	1 ea
WS0253 Digestion (RX) Cartridges (96 Cartridges)	
WS0256 Immobilization Reagent Set	
WS0226 Denaturation Reagent (30 ml)	
WS0255 Blocking Reagent (6 ml)	
WS0259 Digestion Reagent Set	
WS0278 N-Glycanase® (300 µl)	
WS0276 25x Digestion Buffer (700 µl)	
WS0229 Finishing Reagent (optional, not applicable to this kit)	
Aluminum Sealing Film (4)	
GS96-PC InstantPC Labeling Module (-20°C to Room Temp.)	1 ea
WS0338 InstantPC Dye (30 mg, 4 ea)	
WS0339 Dye Solvent (600 µl)	
GS96-CU GlykoPrep Cleanup Module (2–30°C)	1 ea
WS0263 Cleanup (CU) Cartridges (96 Cartridges)	
Aluminum Sealing Film (2)	

Storage Requirements

This kit is a mixed temperature shipment (-20 to 30°C). Store components as indicated. For best results, equilibrate materials to ambient temperature prior to use. The GS24-PC InstantPC Labeling components are hygroscopic and light-sensitive, please store in a dry environment protected from light. Note: the two GS24-PC components may be stored at different temperatures: InstantPC Dye (WS0338) at -20°C; Dye Solvent (WS0339) at -20°C to room temperature.

Additional Required Reagents/Equipment

Heater and Incubation Blocks, capable of 50–100°C, available from ProZyme as product code GS150
AssayMAP Labware: Racks, Receiver Plates and Lids
Other Labware: Waste Plates/Cleanup Collection Plates, 450 µl well volume (Thermo Fisher Scientific part number 07-202-502/Corning part number 3343 or equivalent)

NOTE: Labware needed to use this kit is available from ProZyme as a complete Starter Set (Product Code AM200), or purchased separately in sets of 10.

Centrifuge (capable of 50 - 1000 x g) and deep microplate rotor with a height clearance of ≥44 mm
Ultrapure, deionized water (Milli-Q® or equivalent)
Acetonitrile (ACN, 100%, HPLC-grade)
Pipettors & disposable tips (P5/P10, P200 and P1000)
Formic acid (HPLC-grade)

Optional Reagents and Supplies

Microplate reader (capable of reading A_{280}) for measurement of antibody concentration after Purification
Pipette basins

SAFETY AND HANDLING

Please refer to the Safety Data Sheets (SDS) included with the kit or posted on ProZyme's website under the component name or Product Code:

www.prozyme.com

General Laboratory Procedures

Use powder-free gloves for all sample handling procedures. Ensure that all glass, plasticware and solvents are free of glycosidases and environmental carbohydrates.

All steps involving labeling reagents (InstantPC Dye and Dye Solvent) should be performed in a dry environment with dry glassware and plasticware. Procedures involving these reagents should be performed using appropriate personal protective equipment, eyeglasses, chemically resistant gloves (e.g., nitrile) and, where appropriate, in a laboratory fume hood.

INTRODUCTION

The GlykoPrep Sample Preparation Platform (GlykoPrep) dramatically streamlines glycoanalysis by facilitating optional protein purification, quantitative deglycosylation and separation of glycans, complete fluorescent labeling and efficient cleanup to reduce excess reagent peaks.

GlykoPrep is modular and can be integrated into most workflows. Components are available individually as AssayMAP PA50 (for purification of Fc-containing glycoproteins), Digestion Module and dye-specific Labeling & Cleanup Modules. Product Codes are indicated in the Kit Contents section.

GlykoPrep is built on AssayMAP technology, microchromatography in a 96-well format, and may be performed using centrifugation to move liquid through the Cartridges (spin format), or with the Syringe Head on the Agilent AssayMAP Bravo Liquid Handling Workstation (GlykoPrep-plus). Using the spin format with a microplate centrifuge, up to 192 samples can be processed simultaneously with 2 Kits. Important general information for achieving success with the spin format, as well as special tips particular to individual Modules, may be found in the GlykoPrep Guidebook under Using Specific Kits and Modules:

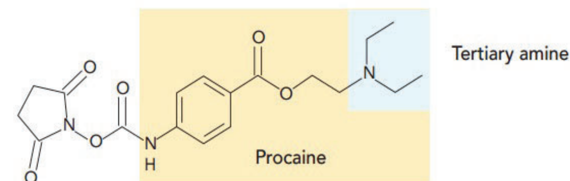
www.prozyme.com/documents/TNGP100.pdf

We also provide a modified Microfuge Method for those interested in using the spin format to run only a handful of samples with a benchtop microfuge and a PCR heater:

www.prozyme.com/InstantPC_Microfuge_Method

InstantPC Label

InstantPC (Instant Procaine) forms a stable urea linkage with glycosylamines released by N-Glycanase (PNGase F) digestion., and is designed for optimal performance in fluorescence detection (FLD) and mass sepctrometry (MS) applications [1]. InstantPC contains a tertiary amine which generates high MS signal in positive mode. For further details, see the "Analysis of Labeled Glycans" section.



InstantPC Dye

USING THE KIT

GlykoPrep Rapid N-Glycan Preparation with InstantPC combines the Digestion Module, the Rapid N-Glycan Preparation with InstantPC Labeling Module and the Cleanup Module, which may be purchased individually. Optional purification modules may be employed just prior to Digestion to allow glycoanalysis directly from cell culture as a single workflow (directions included for your convenience). For information on purification modules under development, please contact us.

Preparation of Samples

Sample Quantities

The quantitative binding for each Cartridge is:

AssayMAP PA50 cartridge	125 µg of MAb or Fc-fusion protein
RX Cartridge	50 µg of most standard proteins
CU Cartridge	30 µg of N-glycans

NOTE: The binding capacity for specific glycoproteins may need to be determined.

Cartridges are capable of binding more target, but will do so with increasing breakthrough, making the process non-quantitative.

For quantitative loading, prepare an excess of 10% or more sample, and prepare replicates together. For example, for Digestion, samples should be denatured together and loaded individually.

Less than the maximum quantity may still be processed with this Kit, for example, when the sample is available only in limited amounts. The smallest amount of sample that will still give good results will depend on the sensitivity requirements of the analytical methods used and the specific application (e.g. screening vs. QC release).

Sample Denaturation

Prior to deglycosylation, the samples are denatured by pre-mixing with Denaturation Reagent. The suggested sample concentration prior to deglycosylation is 1–5 mg/ml, and sufficient reagents have been provided for the standard sample concentration range.

NOTE: If quantitation is desired, pipetting less than 10 µl is not recommended; pipetting smaller volumes introduces variability, especially when samples are highly concentrated. If necessary, dilute the sample to within the 1-5 mg/ml range with Digestion Buffer before starting.

The Kit is useful for very dilute samples without requiring further concentration, by expanding this load step to multiple spins. See the GlykoPrep Guidebook section “Loading.”

When performed in a single spin, the amount loaded to each RX Cartridge should be 10–100 µl. The recommended starting ratio of Denaturation Reagent to sample is 1:1 (v/v).

NOTE: The final denatured Sample must be at least 50% Denaturation Reagent.

Example 1:
Sample concentration 1 mg/ml
Sample amount needed: 50 µg

50 µl (50 µg) Sample + 50 µl Denaturation Reagent
= 100 µl denatured sample

Example 2:
Sample concentration 5 mg/ml
Sample amount needed: 50 µg

10 µl (50 µg) Sample + 90 µl Denaturation Reagent
= 100 µl denatured sample

The current protocol employs a 5-minute, relatively gentle denaturation, but any custom denaturation may be performed and the subsequent protocol followed as described, as long as no SDS or other detergents are used. See the GlykoPrep Guidebook under Digestion Modules or contact us to discuss custom denaturation conditions for your glycoprotein.

For most glycoproteins, standard denaturation is sufficient to allow N-Glycanase access to glycans; for others, additional denaturation by reduction may improve results in some cases. For your convenience, we provide two alternative denaturation protocols. The first procedure is Reduction-Denaturation with TCEP, the second is Reduction-Denaturation-Alkylation. For more information please visit:

www.prozyme.com/GlykoPrep-reduction-denaturation

Enzyme Incubation

Time

The Digest procedure has been optimized to deliver deglycosylation of most glycoproteins in 15-60 minutes. The optimal incubation time will vary depending on the specific glycoprotein; those which have proven to be resistant to deglycosylation via conventional enzymatic methods may require longer incubation times (e.g. 60 minutes).

For glycoproteins that are comparatively easy to deglycosylate, such as monoclonal antibodies, a 15-minute incubation is sufficient. The selected Incubation Time will be used in the Digestion Module.

NOTE: It is critical not to exceed a 60-minute incubation, as the Cartridge resin bed will dry out, yielding uncertain results.

Temperature

The GS150 Heater and Incubation Blocks are specially designed to provide rapid heat transfer through the Receiver Plate and into the packed bed of each Cartridge. The Incubation Blocks are sold separately (ProZyme Product Code WS0272) and can be used in any standard dry-block heater of the proper size, or pre-heated and used in an oven. Custom Incubation Blocks compatible with robotic systems are also available from ProZyme.

NOTE: If using the Microfuge Method format, PCR heat block substitutes for the GS150 Heater (with Incubation Blocks)

The GS150 Heater (with Incubation Blocks) is set to 50°C for the Digestion procedure. Please allow a minimum of 1 hour to equilibrate before beginning the procedure. The Incubation Blocks have been designed with a thermometer well in the corner. We have verified that when the thermometer there reads 50°C, the temperature is 37°C, the optimal temperature for deglycosylation. If using a different heater, confirm the block temperature.

PROTOCOLS

Overview of the Procedure

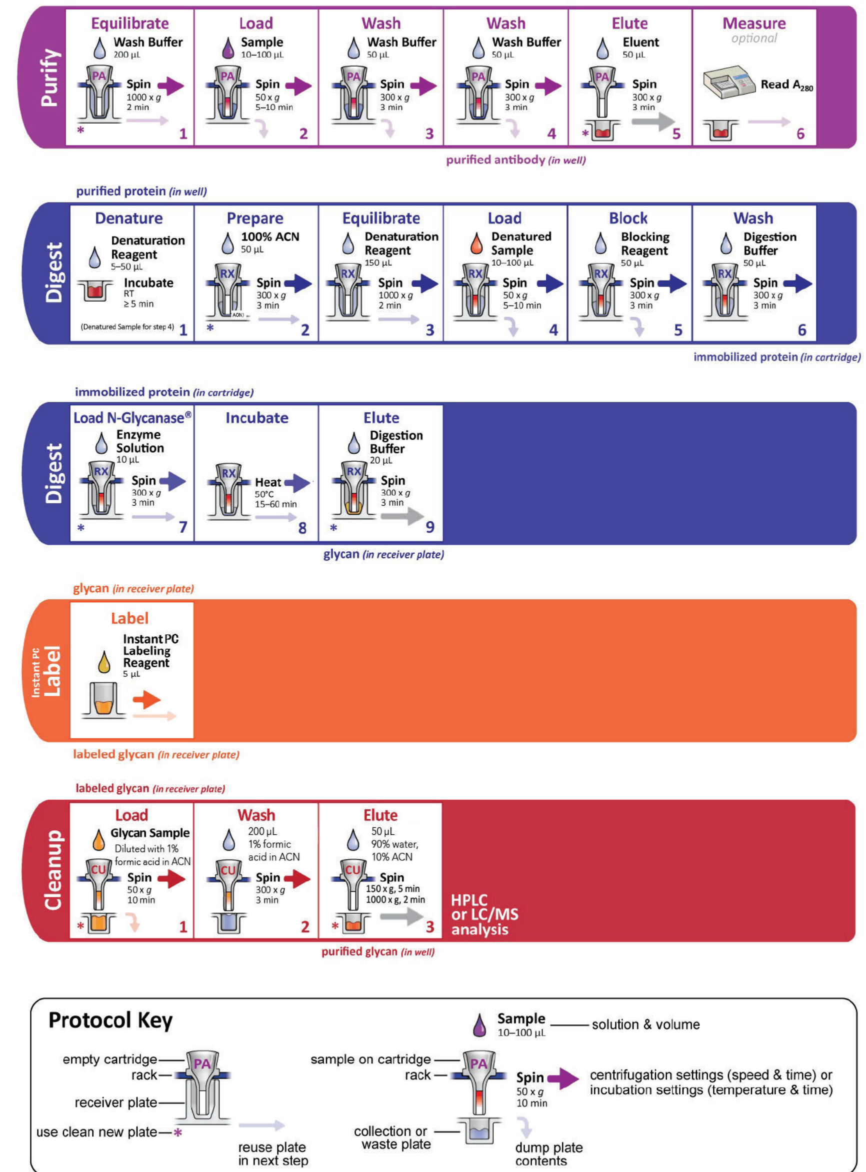
Purify (optional, purchased separately)
Antibodies or Fc-fusion proteins may be purified from crude samples using Protein A.

Digest
Samples (antibodies or other proteins) are denatured and immobilized.

N-Glycans are released by N-Glycanase digestion and eluted.

Label
Released glycans are labeled with InstantPC.

Cleanup
Buffer salts and excess labeling reagents are removed; labeled glycans are eluted with a 10% Acetonitrile solution in water.



Getting Started

Heater Setting

Turn on the GS150 Heater (with 2 Incubation Blocks). Set to 50°C and allow to equilibrate for a minimum of 1 hour.

Centrifuge Settings

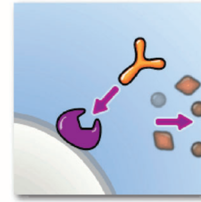
If the centrifuge does not have x g settings, determine the setting for the centrifuge and the specific microplate rotor by consulting the operation manual or the manufacturer's website.

_______________ rpm = 50 x g

_______________ rpm = 150 x g

_______________ rpm = 300 x g

_______________ rpm = 1000 x g



Purify (optional)

Protein A purifies antibodies or Fc-fusion proteins from cell-culture supernatants. All other samples must be purified by other methods; proceed to Digestion Module.

Overview

1 Equilibrate

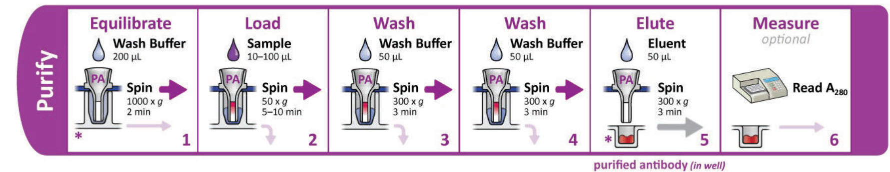
2 Load

3 Wash

4 Wash (second time)

5 Elute

6 Measure (optional)



Reagents and other Supplies

AssayMAP PA50 (supplied in the G5524-60010 Kit, 1 per sample)
    Prepare two balanced AssayMAP PA50 assemblies (Cartridges on Racks on Receiver Plates with Lids)
Wash Buffer (prepared below)
Eluent (supplied with the kit)

NOTE: The prepared eluent must NOT contain glycine because it leaves a signature peak on the LC chromatogram.

Purification Collection Plate (supplied in the AM200 Starter Labware Set, or equivalent)
UV-compatible, flat-bottom, half-area plate for direct protein assay (optional)
PCR plate (optional)
Crude antibody or Fc-fusion protein samples (samples may contain NO MORE than 125 µg total protein, the quantitative binding capacity of the AssayMAP PA50; the amount loaded onto the downstream RX Cartridge may contain NO MORE than 50 µg total protein, the quantitative binding capacity of the downstream RX Cartridge). Samples should be between pH 6.5 and 8.5 and clear of particulates.

Preparation of Reagents

Wash Buffer

NOTE: May be prepared up to one week before use. Store at 2–8°C.

10x Wash Buffer (supplied with the Kit)
Ultrapure water

Dilute one volume of 10x Wash Buffer stock with nine volumes of ultrapure water to obtain Wash Buffer. Specifically, add 4 ml of 10x Wash Buffer stock to 36 ml of ultrapure water to make 40 ml of Wash Buffer.

For fewer samples, prepare 400 µl of Wash Buffer for each sample to be processed.

Procedure

Equilibrate

1.a Pipet 200 µl of Wash Buffer into the Sample Cup of each AssayMAP PA50.

1.b Spin at 1000x g for 2 minutes; do not empty the Receiver Plates.

Load

2.a Load 10–100 µl of sample into each AssayMAP PA50 (see Sample Loading Technique in the GlykoPrep Guidebook).

2.b Remove the Racks from the Receiver Plates. Empty the Receiver Plate and blot with a paper towel to avoid cross-contamination. Replace Racks.

2.c Spin at 50x g until all Sample Cups are empty. The estimated spin time is 5 minutes for volumes between 10 and 50 µl or 10 minutes for volumes up to 100 µl.

Wash

3.a Pipet 50 µl of Wash Buffer into the Sample Cup of each AssayMAP PA50.

3.b Empty the Receiver Plate and blot with a paper towel.

3.c Spin at 300x g for 3 minutes.

Wash (second time)

4.a Pipet 50 µl of Wash Buffer into the Sample Cup of each AssayMAP PA50.

4.b Empty the Receiver Plate and blot with a paper towel.

4.c Spin at 300 x g for 3 minutes.

Elute

5.a Remove the Racks from the Receiver Plates and place on top of a collection plate.

NOTE: For a colorimetric measurement (A_{590}/A_{450}) of protein concentration, use the Purification Collection Plate. For direct measurement (A_{280}) of concentration, use a UV-compatible, flat-bottom, half-area plate. If no protein determination will be made, a PCR plate may be used.

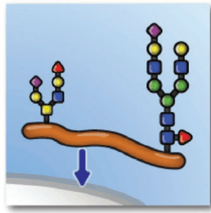
5.b Pipet 50 µl of Eluent into the Sample Cup of each AssayMAP PA50.

5.c Spin at 300 x g for 3 minutes.

5.d Remove the Racks and dispose of the Cartridges.

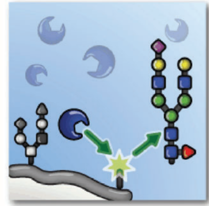
Measure (optional)

6. Measure the absorbance on a plate reader at 280 nm.



Digest

Samples (antibodies or other glycoproteins) are denatured and immobilized.



N-glycans are released by N-Glycanase and eluted.

Overview

Proceed through the Digestion, Labeling and Cleanup modules without delay.

1 Denature

2 Prepare

3 Equilibrate

4 Load

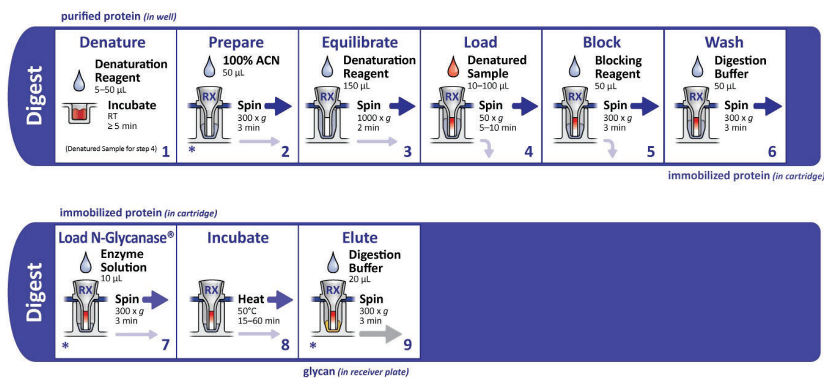
5 Block

6 Wash

7 Load N-Glycanase

8 Incubate

9 Elute



Reagents and other Supplies

Glycoprotein Samples

NOTE: The quantity of purified sample loaded to the RX Cartridge may contain NO MORE than 50 µg total protein, the quantitative binding capacity of the RX Cartridge.

RX Cartridges (supplied with the Kit, 1 per sample)
Prepare two balanced RX Cartridge assemblies (Cartridges on Racks on Receiver Plates with Lids)
Denaturation Reagent (supplied with the Kit)
Acetonitrile (100%, HPLC-grade), 50 µl/sample
Blocking Reagent (supplied with the Kit)
Digestion Buffer (prepared below)
Enzyme Solution (prepared below)

Preparation of Reagents

Digestion Buffer

NOTE: May be prepared up to one week before use. Store at 2–8°C.

25x Digestion Buffer (supplied with the kit)
Ultrapure water

Dilute one volume of 25x Digestion Buffer with twenty-four volumes of ultrapure water to obtain Digestion Buffer. Specifically, add 0.4 ml of 25x Digestion Buffer to 9.6 ml of ultrapure water to make 10 ml of Digestion Buffer.

For fewer samples, prepare 100 µl of Digestion Buffer for each sample to be processed.

Enzyme Solution

NOTE: Should be prepared on the day of use. Store at RT.

N-Glycanase (supplied with the kit)
Digestion Buffer (prepared above)

Spin the N-Glycanase briefly prior to use to collect the contents in the base of the vial, pipet up and down several times to mix prior to use.

In a separate vial, prepare a mixture of 3 µl of N-Glycanase and 9 µl of Digestion Buffer for each sample to be processed. Pipet up and down several times to mix.

To prepare 96 samples, add 288 µl of N-Glycanase to 864 µl of 1x Digestion Buffer in a pipette basin. Pipet up and down several times to mix.

NOTE: The pipette basin requires a minimum of ~100 µl volume, so for fewer than 8 samples, do not use a basin.

Procedure

NOTE: An incubation at elevated temperature is required for full deglycosylation. Before beginning, be sure each Incubation Block has equilibrated to 50°C: a thermometer may be placed in the corner well of Incubation Block to monitor the temperature.

Denature

1.a Add Denaturation Reagent to each sample as described in Sample Denaturation (page 7).

1.b Pipet up and down to mix.

1.c Incubate at room temperature for at least 5 minutes.

NOTE: Proceed through the Prepare, Equilibrate and Load steps without interruption, as evaporation can lead to airlock.

Prepare

2.a Pipet 50 µl of 100% Acetonitrile into the Sample Cup of each RX Cartridge.

2.b Spin at 300 x g for 3 minutes; do not empty the Receiver Plates.

Equilibrate

3.a Pipet 150 µl of Denaturation Reagent into the Sample Cup of each RX Cartridge.

3.b Spin at 1000 x g for 2 minutes into the same Receiver Plate used for Step 2.b.

NOTE: Do not empty Receiver Plate prior to loading the denatured Sample.

Load

4.a Load each Denatured Sample into an RX Cartridge (see Sample Loading Technique in the GlykoPrep Guidebook).

4.b Empty the Receiver Plate and blot with a paper towel.

NOTE: Discard waste Acetonitrile Solution according to waste disposal procedures.

4.c Spin at 50 x g until all Sample Cups are empty. The estimated spin time is 5 minutes for volumes between 10 and 50 µl or 10 minutes for volumes up to 100 µl.

Block

5.a Pipet 50 µl of Blocking Reagent into the Sample Cup of each RX Cartridge.

5.b Empty the Receiver Plate and blot with a paper towel.

5.c Spin at 300 x g for 3 minutes; do not remove the Receiver Plate.

Wash

6.a Pipet 50 µl of Digestion Buffer into the Sample Cup of each RX Cartridge.

6.b Spin at 300 x g for 3 minutes.

Load N-Glycanase

7.a Transfer RX Cartridges to fresh Receiver Plates.

7.b Pipet 10 µl of Enzyme Solution into the Sample Cup of each RX Cartridge.

7.c Spin at 300 x g for 3 minutes;

DO NOT DISCARD FLOW-THROUGH.

Incubate

8. Incubate RX Cartridge assemblies on the equilibrated Incubation Blocks (Heater setting 50°C) for the chosen Incubation Time (not to exceed 60 minutes; see Enzyme Incubation, page 8).

Elute

9.a Remove the RX Cartridge assemblies from the Incubation Blocks.

NOTE: If condensation is apparent, spin at 300 x g for 3 minutes and tap dish gently on the bench top to release Cartridges that may be stuck to the Lid.

9.b Pipet 20 µl of Digestion Buffer into the Sample Cup of each RX Cartridge; do not remove Rack from Receiver Plate.

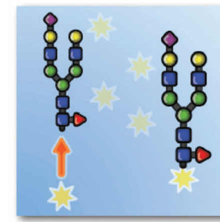
9.c Spin at 300 x g for 3 minutes.

9.d Remove Cartridges and Rack from the Receiver Plate. The eluted glycans are in the Receiver Plate; DO NOT DISCARD.

NOTE: Retain the RX Cartridges to recover the deglycosylated protein for further analysis (see Tips & Hints).

Proceed immediately to Labeling.

NOTE: Labeling with InstantDye requires the availability of reactive glycosylamine ends, such as those resulting from rapid digestion with N-Glycanase. Glycosylamine ends spontaneously hydrolyze over time to reducing ends which are incompatible with InstantDye chemistry. To maximize labeling efficiency, Labeling should be performed immediately following collection of the glycans from the GlykoPrep Digestion Module.



Label

Reagents and other Supplies

InstantPC Labeling Reagent (prepared below)
N-Glycan Samples (eluted N-glycans with reactive glycosylamine ends, in a Receiver Plate)

Preparation of Reagents

InstantPC Labeling Reagent

NOTE: The InstantDye™ is hygroscopic; minimize exposure to air and protect from exposure to light. Reconstituted dye may be resealed, repackaged with desiccant in a resealable bag, and frozen (-20°C) for storage up to 3 months and 10 freeze thaw cycles; return to RT before opening for use.

InstantPC Dye (4 x 30 mg, stored at -20°C)
Dye Solvent (600 µl, stored at -20°C or room temperature)

Bring the Dye Solvent to room temperature before opening.

Add 150 µl of Dye Solvent into a 30 mg InstantPC Dye vial.

Sufficient for 24 samples; prepare all 4 sets to process a full kit.

Replace the cap and vortex the vial to ensure the dye is completely dissolved.

Tightly recap unused Dye Solvent, return to the foil pouch with dessicant, and store at -20°C or room temperature.

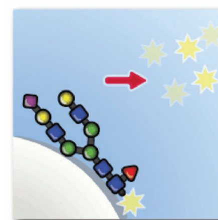
Procedure

Label

1. Pipet 5 µl of InstantPC Labeling Reagent into each well of N-glycan eluate in the Receiver Plate and pipet up and down several times to mix. Allow the samples to incubate at room temperature for 5 minutes.

Glycans are now labeled and ready for Cleanup.

NOTE: A precipitate may form in the labeling reaction over time. This precipitate is removed by Cleanup, but may require longer spin times. To avoid this, proceed directly to Cleanup.

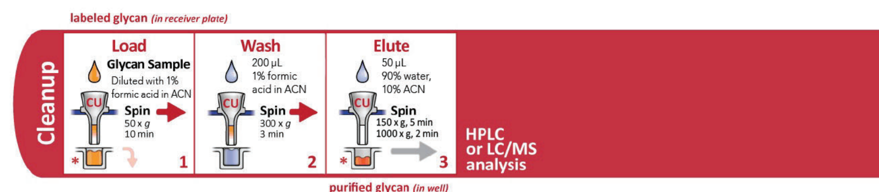


Cleanup

CU Cartridges allow most hydrophobic, non-glycan contaminants to be washed through; glycans are then eluted with a 10:90 Acetonitrile:water solution.

Overview

1	Load
2	Wash
3	Elute



Reagents and other Supplies

N-Glycan Samples labeled with InstantPC
CU Cartridges (supplied with the kit, 1 per sample)
Prepare two balanced CU Cartridge assemblies (Cartridges on Racks on Waste Plates with Lids)

Load Solution: 1% Formic acid in Acetonitrile
Wash Solution: 1% Formic acid in Acetonitrile
Elute Solution: 10% Acetonitrile in water

Cleanup Collection Plate (supplied in the AM200 Starter Labware Set, or equivalent)

Preparation of Reagents

NOTE: Store all solutions sealed at room temperature.

Load and Wash Solution: 1% Formic acid in Acetonitrile

To make 45 ml (enough for a full 96-well Kit) of 1% Formic acid in Acetonitrile (v/v), add 450 µl of Formic acid to a glass, graduated cylinder. Bring the volume up to 45 ml with 100% Acetonitrile. Transfer to a glass storage vessel, cap tightly and swirl gently to mix. Scale as necessary (prepare 450 µl per sample).

Elute Solution: 10% Acetonitrile in water

To make 30 ml (enough for a full 96-well Kit) of 10% Acetonitrile in water (v/v), add 3.0 ml of Acetonitrile to a glass, graduated cylinder. Bring the volume up to 30 ml with ultrapure water. Transfer to a glass storage vessel, cap tightly and swirl gently to mix. Scale as necessary (prepare 250 µl per sample).

Procedure

NOTE: DO NOT use Receiver Plates in this procedure; build the stack with Waste Plates (~450 µl well volume) instead. This entire section is performed with the Cartridges "tips free."

Load

1.a Pipet 180 µl of Load Solution into each well of glycan eluate in the Receiver Plate. Pipet up and down to mix.

1.b Transfer each N-Glycan Sample into the Sample Cup of a CU Cartridge. This must be done quickly because Acetonitrile has very low viscosity and may drip from the pipette tip; each sample may be pipetted in multiple rounds in order to achieve a quantitative transfer.

NOTE: Air bubbles are not a concern with this concentration of Acetonitrile.

1.c Spin at 50 x g for 10 minutes or until CU Cartridges are empty.

1.d Empty the Waste Plate.

NOTE: Discard waste containing acetonitrile according to waste disposal procedures.

Wash

2.a Pipet 200 µl of Wash Solution into the Sample Cup of each CU Cartridge.

2.b Spin at 300 x g for 3 minutes.

2.c Empty the Waste Plate.

Elute

3.a Place each racked set of CU Cartridges over a clean Cleanup Collection Plate.

NOTE: Because the eluate contains organic solvent, polystyrene plates should NOT be used. Any polypropylene ANSI/SBS 96-well microplate may be used as a collection plate. To facilitate complete product recovery, we recommend plates with conical bottoms, such as PCR plates or the Cleanup Collection Plates provided in the AM200 Starter Labware Set.

3.b Pipet 50 µl of Elute Solution (10% Acetonitrile) into the Sample Cup of each CU Cartridge.

NOTE: Up to 200 µl of Elute Solution may be used if more dilute glycans are desired.

3.c Spin at 150 x g for 5 minutes.

3.d Spin at 1000x g until all Sample Cups are empty. The estimated spin time is 2 minutes.

The Cleanup Collection Plate now contains the purified glycans; DO NOT DISCARD.

Mix the eluate by pipette action or vortexing prior to analysis to ensure homogeneity. Glycan Samples are now ready to be analyzed. If not analyzed immediately, store sealed at -20°C in the dark.

ANALYSIS OF LABELED GLYCANS

Standard techniques such as Hydrophilic Interaction Liquid Chromatography (HILIC) may be used to analyze labeled glycans.

Optimizing Excitation/Emission Wavelengths

The Optimal excitation/emission wavelengths for InstantPC Dye conjugated to an N-glycan are:

Excitation: 285 nm
Emission: 345 nm

LC Injections

Injection of 1 µl InstantPC-glycans in InstantPC Eluent (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. Use 1 part sample in eluent to 3 parts 50:50 ACN:DMF, for a final concentration of 22.5% aqueous buffer, 37.5% DMF, 40.0% ACN.

Suggested HILIC Conditions

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

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

Time (min)	Flow rate (ml/min)	% ACN	% 100 mM Ammonium Formate, pH 4.4
0.00	1.4	77	23
4.00	1.4	60	40
4.15	0.75	40	60
4.30	0.75	40	60
4.40	1.4	77	23
5.00	1.4	77	23

60-Minute high resolution UHPLC method, Agilent AdvanceBio Glycan Mapping column:

2.1 x 150 mm, 2.7 µm. Column temperature 45 °C, excitation 285 nm, emission 345 nm.

Time (min)	Flow rate (ml/min)	% ACN	% 50 mM Ammonium Formate, pH 4.4
0.0	0.4	80	20
43.5	0.4	54	46
45.0	0.4	0	100
50.0	0.4	0	100
52.0	0.4	80	20
60.0	0.4	80	20

For examples of HILIC separations of Instant-PC-labeled N-glycans using Agilent AdvanceBio Glycan Mapping columns, please see reference [1].

15-Minute UPLC method, Waters BEH GST column:

2.1 x 100 mm, 1.7 µm. Column temperature 60 °C, excitation 285 nm, emission 345 nm.

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 high resolution UPLC method, Waters BEH GST column:

2.1 x 150 mm, 1.7 µm. Column temperature 45 °C, max pressure 15,000 psi, excitation 285 nm, emission 345 nm.

Time (min)	Flow rate (ml/min)	% ACN	% 50 mM Ammonium Formate, pH 4.4
0.0	0.4	80	20
43.5	0.4	54	46
45.0	0.4	0	100
50.0	0.4	0	100
52.0	0.4	80	20
60.0	0.4	80	20

MS Analysis of InstantPC-labeled N-glycans

Suggested 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, 0.8 second scan time, m/z range 300–2000 Da.

Suggested 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. InstantPC is suitable for Collision Induced Dissociation (CID) MS/MS. As with other positively charged tags such as Procainamide, the CID profile contains mostly glycosidic cleavages with some cross-ring fragmentation. For an example, please see reference [1].

Adducts in MS analysis of InstantPC-labeled glycans:

In positive mode MS, most biantennary InstantPC- N-glycans will give $[M+2H]^{+2}$, larger sialylated will be majority $[M+3H]^{+3}$. InstantPC-glycan masses for some major N-glycan species present on biotherapeutics:

Glycan ID	IPC-Glycan Monoisotopic 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

Calculating the Mass of Glycans Labeled with InstantPC

Mass added to glycan with a free reducing end:

$\text{Mass}_{\text{Glycan (free reducing end)}} + C_{14}N_3O_2H_{19} = \text{Mass}_{\text{InstantPC-Labeled Glycan}}$

Mass added by $C_{14}N_3O_2H_{19}$ (Da):

Monoisotopic: 261.14773
Average: 261.3

Mass added to glycosylamine:

$\text{Mass}_{\text{Glycan (glycosylamine)}} + C_{14}N_2O_3H_{18} = \text{Mass}_{\text{InstantPC-Labeled Glycan}}$

Mass added by $C_{14}N_2O_3H_{18}$ (Da):

Monoisotopic: 262.13174
Average: 262.3

FAQs

Q. Why do you suggest 100 mM ammonium formate (pH 4.4) for 5- and 15-minute HILIC separations, 50 mM ammonium formate for the 60-minute separations?

A. For faster HILIC separations we use 100 mM ammonium formate. For MS in conjunction with longer separations we use 50 mM ammonium formate.

Q. Is there a method to recover the deglycosylated protein from the Digestion (RX) cartridge for analysis?

A. Please contact us for guidelines for eluting your glycoprotein from the RX Cartridge.

Q. Can inject glycans onto LC directly from the Cleanup Collection Plate?

A. If N-Glycan Samples will be analyzed by LC directly following elution from the CU Cartridges, the 96-well, polypropylene plate with a pierceable lid available from MicroLiter Analytical Supplies (cat# 07-1211N) may serve as a Collection Plate.

Alternatively, cleanup plates (PCR plates or Cleanup Collection Plates) may be heat sealed with pierceable foil (e.g., Thermo Easy Pierce 20 µm Foil, #AB-1720) using a microplate heat sealer (e.g., Thermo ALPS 50 V Semi automated Microplate Heat Sealer, #AB-1443). The dimensions of the cleanup plates can be used to program positioning of the LC sample probe. We advise against direct LC injection after sealing plates with the aluminium sealing film included with this kit. Using sealing film with adhesive may cause system problems.

REFERENCES

1. Kimzey et al. Development of an Instant Glycan Labeling Dye for High Throughput Analysis by Mass Spectrometry. Poster presented at ASMS 2015, St. Louis MO.

prozyme.com/posters/instantpc

Visit ProZyme's website for additional information, downloadable posters and instructional videos:

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TechNote TNGP100 GlykoPrep Guidebook - General tips, tricks and troubleshooting suggestions when using GlykoPrep kits or modules:

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