PROzyme®

GlykoPrep

Digestion Module

- Rapid, non-selective release and recovery of intact N-Glycans from up to 50 µg of glycoprotein
- Flexible, high-throughput format: process 1 to 192 samples per run (2 kits simultaneously)
- Compatible with microplate liquid handling on a broad range of automation platforms

Product Code: GS96-RX

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.**

Agilent

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page

This product is intended for in vitro research use only.

KIT CONTENTS

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

Item	Qty
WS0253 Digestion (RX) Cartridges	(96 Cartridges)
WS0256 Immobilization Reagent Set	1 ea
WS0226 Denaturation Reagent (30 ml)	
WS0255 Blocking Reagent (6 ml)	
WS0259 Digestion Reagent Set	1 ea
WS0278 N-Glycanase® (300 µl)	
WS0276 25x Digestion Buffer (700 µl)	
WS0229 Finishing Reagent (2 x 1 ml)	
Aluminum Sealing Film (4)	

Storage Requirements

This kit is shipped with cold packs and should be stored at 2–8°C on receipt. For best results, equilibrate to ambient temperature prior to use.

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, available from ProZyme as a complete Starter Set (Product Code AM200) or separately in sets of 10.
Labware: Gilson D-200 tips.
Centrifuge (capable of 50–1000 x g) and microplate rotor with a height capacity of 44 mm
Ultrapure, deionized water (Milli-Q® or equivalent)

Acetonitrile (100%, HPLC-grade)
Pipettors & disposable tips (P5/P10, P200 and P1000)

Optional Reagents and Supplies

Multichannel pipettors & disposable tips (P5/P10 and P200) (Gilson or equivalent, compatible with Gilson Diamond® D200 pipette tips)
Pipette basins
Microplate-compatible, centrifugal evaporator (e.g., SpeedVac® or similar) for optional Finishing of released N-Glycans. A rotor that holds two shallow-well microplates and fits into a small-capacity rotary evaporator may be purchased from Thermo Fisher Scientific (Savant Model RH2MP, Thermo Scientific Catalog number 20-548-127).

SAFETY AND HANDLING

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

http://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.

INTRODUCTION

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

GlykoPrep is modular and can be integrated into any workflow, regardless of throughput or sample type. In order to match any standard sample preparation, Kit components are also available individually as the AssayMAP PA50 (for purification of Fc-containing antibodies only), Digestion Module and dye-specific Labeling & Cleanup Modules.

GlykoPrep is built on AssayMAP technology, microchromatography in a 96-well format, capable of automated high throughput. GlykoPrep 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:

http://www.prozyme.com/documents/TNGP100.pdf

We also provide modified Microfuge Method instructions useful for those interested in using the spin format to run only a handful of samples with a benchtop microfuge and a PCR heater. Microfuge Method Instruction Manuals may be found on our website:

http://www.prozyme.com/tech_notes.html

USING THE KIT

Preparation of Samples

Sample Quantities

The quantitative binding for the RX Cartridge is 50 µg of most standard glycoproteins.

NOTE: The binding capacity for your specific glycoprotein 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; denature replicates together and load them individually.

Less than the maximum quantity may be processed, for example, when the sample is available only in limited amounts. The smallest amount of sample that will give good results depends on the sensitivity requirements of the analytical methods 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 mixture must be 50% Denaturation Reagent or more.

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. Please see the GlykoPrep Guidebook under Digestion Modules or contact us to discuss custom denaturation conditions for your

glycoprotein.

Digest Time & Temperature

Time

The Digest procedure has been optimized to deliver deglycosylation of most N-Glycans 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 (up to 60 minutes). For glycoproteins that are comparatively easy to deglycosylate, such as monoclonal antibodies, a 15-minute incubation is generally sufficient. The selected Incubation Time will be used on page 16.

NOTE: It is critical not to exceed a 60-minute incubation, as the Cartridge resin bed may 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 Digest procedure (deglycosylation and optional Finishing). Please allow a minimum of 1 hour to equilibrate the Blocks

before use. The Incubation Blocks have been designed with a thermometer well in the corner. We have verified that when the thermometer reads 50°C, the temperature in the Cartridge is ~37°C, the optimal temperature for deglycosylation. If using a different heater, confirm the block temperature.

Finishing Options

The Elution step will differ depending on the dye used to label the N-Glycans. If using InstantDyes™, which require the glycosylamine produced by N-Glycanase digestion, no Finishing is necessary. Elute in Digestion Buffer following Finishing Option A (No Finishing, steps 9a–9d).

If proceeding to a Labeling Module using reductive amination (e.g., the GlykoPrep Rapid-Reductive-Amination™ 2-AB Labeling Module) or another label requiring a free reducing end, or for label-free analysis, elute in Finishing Reagent following Finishing Option B (steps 9–11). Finishing Reagent converts the glycosylamine produced by N-Glycanase digestion to a free reducing end.

PROTOCOL

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 and the specific microplate rotor combination by consulting the operation manual or the manufacturer's website:

_____ rpm = 1000 x g

_____ rpm = 300 x g

_____ rpm = 50 x g

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.

Cap tightly and vortex on high for 10 seconds to mix.

Enzyme Solution

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

N-Glycanase (supplied with the Kit)
Digestion Buffer (prepared previously)

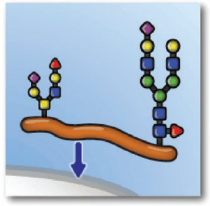
Spin the N-Glycanase briefly to collect the contents in the base of the vial, and pipette up and down several times to mix prior to use.

In a separate vial, prepare a mixture of 2.5 µl of N-Glycanase and 7.5 µl of Digestion Buffer for each sample to be processed, plus 20% for overage.

For example, 10 samples would require 25 + 5 = 30 µl of N-Glycanase and 90 µl of Digestion Buffer.

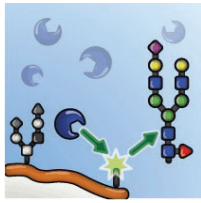
To prepare 96 samples, add 288 µl of N-Glycanase to 864 µl of 1x Digestion Buffer in a pipette basin. Mix the solution several times by pipette action.

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



Digest

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



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

Overview

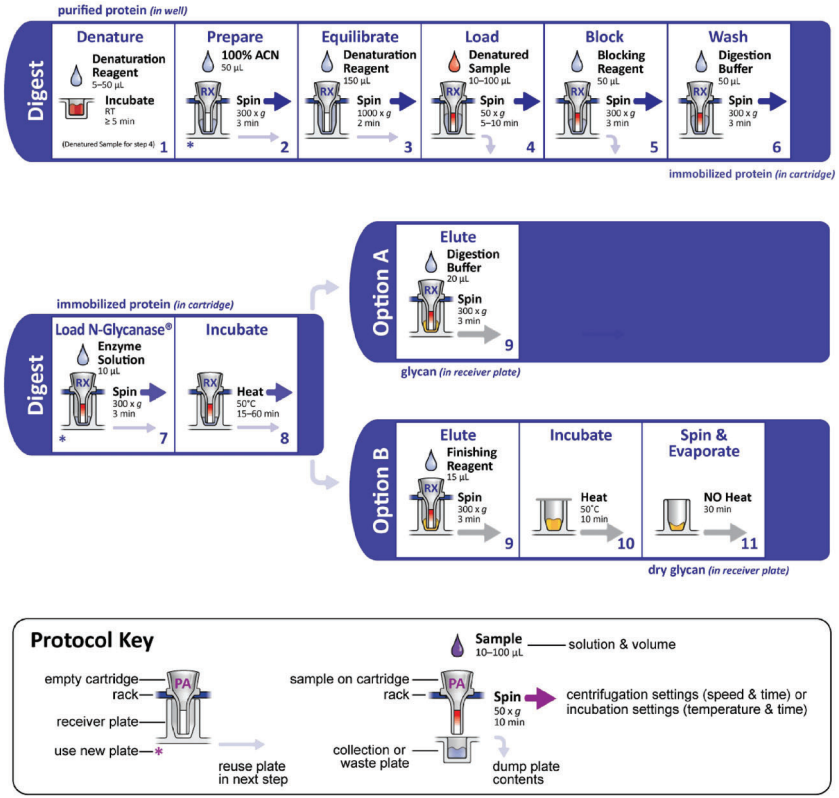
1 Denature
2 Prepare
3 Equilibrate
4 Load
5 Block
6 Wash
7 Load N-Glycanase
8 Incubate

Finishing Option A (No Finishing)

9 Elute

Finishing Option B

9 Elute (and Finish)
10 Incubate
11 Spin & Evaporate



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 previously)
Enzyme Solution (prepared previously)
Finishing Reagent (supplied with the Kit)
Aluminum Sealing Film (supplied with the Kit; film may be cut with scissors if using less than the full Kit)

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 the Incubation Block to monitor the temperature.

Denature

1.a Add Denaturation Reagent to each sample as described in Sample Denaturation (page 5).

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 the Receiver Plate prior to loading the

denatured sample.

Load

4.a Load each Denatured Sample into the Sample Cup of 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 Time, page 7).

NOTE: If N-Glycans are to be labeled using InstantDyes, now would be a good time to prepare the Labeling Reagent.

Finishing Options

If proceeding to labeling using InstantDyes (e.g., InstantAB™), elute in Digestion Buffer following Option A.

If proceeding to labeling using reductive amination (e.g., Rapid N-Glycan Preparation with 2-AB) or another label that requires a free reducing end, or for label-free analysis, elute in Finishing Reagent following Option B.

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Option A (no Finishing)

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 N-Glycans are in the Receiver Plate; DO NOT DISCARD.

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 N-Glycans from the GlykoPrep Digestion Module.

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

Option B

Elute (and Finish)

9.a Remove the RX Cartridge assemblies from the Incubation Blocks. Keep the heat on for Incubate step.

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

9.b Pipet 15 µl of Finishing Reagent 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 N-Glycans are in the Receiver Plate; DO NOT DISCARD.

Incubate

10. Cover the Receiver Plate with Aluminum Sealing Film and incubate on the equilibrated Incubation Block (with Heater still set at 50°C) for 10 minutes.

Spin & Evaporate

11. Remove the Aluminum Sealing Film and dry the N-Glycans in a centrifugal evaporator (SpeedVac, heat setting turned to the off position) for 30 minutes or until fully dry. This condenses N-Glycans into a pellet small enough to be dissolved by 5 µl of Labeling Reagent if proceeding to labeling using Rapid Reductive Amination (e.g., the GlykoPrep Rapid-Reductive-Amination 2-AB Labeling Module).

NOTE: The Finishing Reagent must be removed promptly by drying. Store N-Glycans at -20 or -80°C in the dark if further processing will not take place immediately.

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

ANALYSIS OF RELEASED N-GLYCANS

Use standard techniques, such as LC, CE and MS, to analyze the released N-Glycans.

TIPS & HINTS

Recovery of the Deglycosylated Protein from the Digestion (RX) Cartridge

Often, the deglycosylated protein is analyzed to evaluate the completeness of deglycosylation using such electrophoretic methods as SDS-PAGE or microfluidic lab-on-a-chip technology. Please contact us for guidelines for eluting your glycoprotein from the RX Cartridge.

REFERENCES

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

http://www.prozyme.com/glykoprep

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

http://www.prozyme.com/documents/TNGP100.pdf

TECHNICAL ASSISTANCE

ProZyme 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:

TOLL FREE **(800) 457-9444** (US & CANADA)
PHONE **(510) 638-6900**
FAX **(510) 638-6919**
E-MAIL **info@prozyme.com**
WEB **www.prozyme.com**

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OTHER PROZYME PRODUCTS & KITS

A wide variety of glycoanalysis products are available from ProZyme. A complete listing is accessible on our website by clicking on Glyko® Tools for Glycobiology:

http://www.prozyme.com

ORDERING INFORMATION

For North American destinations:

Telephone orders may be placed between 8:00 am and 5:00 pm Pacific Time. Telefax or e-mail orders may be sent or messages recorded anytime.

TOLL FREE (800) 457-9444 (US & CANADA)
PHONE (510) 638-6900
FAX (510) 638-6919
E-MAIL info@prozyme.com

Outside North America:

A list of ProZyme's distributors, with contact information, may be found at:

http://www.prozyme.com/distributors.html

If there is no distributor in your area, instructions for placing an international order may be found at:

http://www.prozyme.com/ordering.html

NOTES

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