GlykoPrep® Microfuge Method - Rapid N-Glycan Preparation with 2-AB

GlykoPrep Rapid N-Glycan Preparation with 2-AB (product codes GP24NG-AB and GP96NG-AB) using a microfuge and PCR heat block

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REQUIRED REAGENTS/EQUIPMENT

Centrifuge (capable of 50–1000 x g) and rotor for 1.5/2.0-ml microcentrifuge tubes

Heater and heat block capable of 50-100°C that accommodates 0.2-ml PCR tubes

Centrifugal evaporator (e.g., SpeedVac®) for drying released N-glycans prior to labeling

Vortexer

Fume hood

Glass, graduated cylinder, 25-ml

Vials, 1-ml, polypropylene for use with organic solvents

Ultrapure, deionized water (Milli-Q® or equivalent)

Acetonitrile

Pipettors & disposable tips (P5/P10, P200 and P1000)

Nitrile gloves

Required Labware (per sample)

1 x Cartridge Adaptor A from ProZyme, Product Code AM400 (3 x 8 ea)

1 x Cartridge Adaptor B from ProZyme, Product Code AM400 (3 x 8 ea)

NOTE: Adaptors are reusable. Do not discard.

1 x 0.5-ml Microtube, screw cap, Sarstedt part number 72.730.711 or equivalent

GlykoPrep Cartridges (supplied with each Kit)

1 x 0.2-ml PCR tube, domed cap, Axygen® part number PCR-02D-C or equilvalent

2 x 1.75-ml Microcentrifuge tube, flip top, E&K Scientific part number 290175 or equivalent

NOTE: Some brands of PCR tubes form a tight seal between the tube and Cartridge. Do not use these brands.

SAFETY & HANDLING

Some of the reagents in the GlykoPrep Kits are hazardous. 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, nitrile gloves for all sample handling procedures. Ensure that all glass, plasticware and solvents are free of glycosidases and environmental carbohydrates.

All procedures involving 2-AB Labeling Reagent or its components should be performed in a dry environment with dry glassware and plasticware, using appropriate personal safety protection, eyeglasses and nitrile gloves, and where appropriate, in a fume hood.

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. Components are available individually as a Purification Module (optional), Digestion Module and dye-specific Labeling & Cleanup Modules.

GlykoPrep is built on AssayMAP technology, performed using centrifugation to move liquid through the Cartridges (spin format). The Microfuge Method is useful for those interested in using the spin format to run only a handful of samples. Using the spin format with a 96-well microplate and microplate centrifuge, up to 192 samples can be processed simultaneously with 2 Kits. GlykoPrep-plus employs the Syringe Head on the Agilent AssayMAP Bravo Liquid Handling Workstation to move liquid through the Cartridges, for automated high-throughput.

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:

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

USING THE KIT

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

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.

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).

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.

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.

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), as in Example 1 below. More Denaturation Reagent may be used for problematic glycoproteins, as shown in Example 2 (sample to Denaturation Reagent ratio of 9:1).

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

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.

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

Digestion Time, Temperature and Finishing

Time

The Digest procedure has been optimized to deliver deglycosylation of 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 in the Digestion Module.

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

Temperature

The PCR heat block is set to 45°C for the Digest procedure (deglycosylation and optional Finishing step).

NOTE: Optimal temperatures for the Microfuge Method differ from those used with a microplate centrifuge.

Finishing

Finishing Reagent converts the glycosylamine produced by N-Glycanase digestion to a free reducing end, required for labeling with 2-AB or APTS via Rapid-Reductive-Amination™. The incubation time for Finishing is fixed at ten minutes.

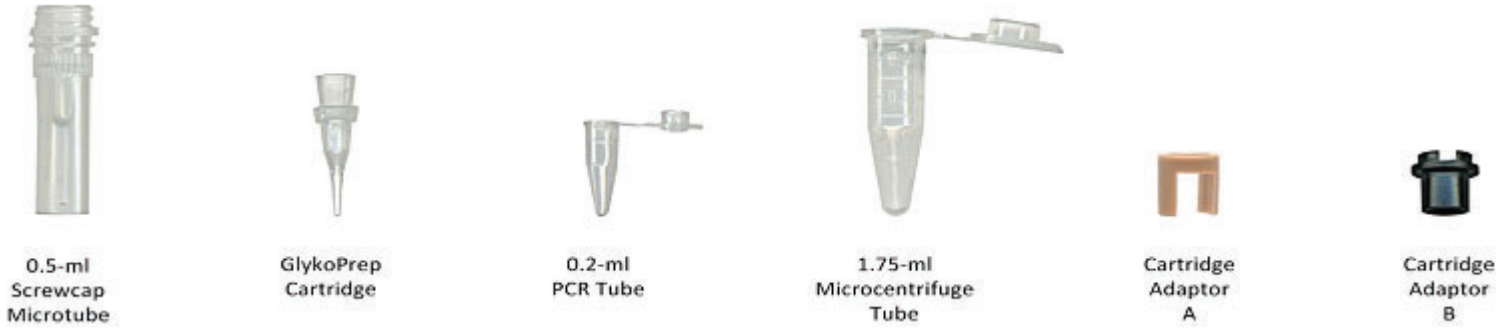
Labeling Times & Temperatures

The Rapid-Reductive-Amination with 2-AB Labeling incubation time is 60 minutes.

The Labeling procedure requires the PCR heat block to be equilibrated at 65°C.

NOTE: Optimal temperatures for the Microfuge Method differ from those used with a microplate centrifuge.

USE OF ADAPTORS AND CONSUMABLES



Most Standard Operations

Basic Assembly: Nest the RX Cartridge into Cartridge Adaptor A, then into a 0.5-ml screw cap Microtube.

RX Cartridge (only)

Cartridge Adaptor A

0.5-ml screw-cap Microtube

Equilibrate, Prepare, Block, Wash, Load Sample (except Cleanup).

In this configuration, flow-through remains in contact with the tip of the RX Cartridge in the Microtube ("Tips Wet").

Operations with Special Requirements

"Tips Wet" Adaptor Assembly: Insert an RX Cartridge into a 0.2-ml PCR tube. Insert into an AssayMAP Cartridge Adaptor and nest into a 1.75-ml, flip-top Microcentrifuge Tube.

RX Cartridge (only)

0.2-ml PCR tube

Cartridge Adaptor B

Flip-top, 1.75-ml Microcentrifuge Tube

Incubation Assembly: Perform all incubations (including N-Glycanase, Finishing and Labeling incubations) in a 0.2-ml capped PCR tube. Note that the RX Cartridge will be nested in the uncapped PCR tube for the N-Glycanase Digestion step.

RX Cartridge (only)

0.2-ml PCR tube

OR

0.2-ml capped PCR tube

Load N-Glycanase and Elute (Digestion steps 7 & 9)

In this configuration, flow-through remains in contact with the tip of the RX Cartridge inside the 0.2-ml PCR tube ("Tips Wet").

- For N-Glycanase Digestion: when ready to Incubate, remove the RX Cartridge with the 0.2-ml PCR tube from the "Tips Wet" Assembly and place them on the PCR heat block. When the incubation is complete, return the RX Cartridge/PCR tube to the "Tips Wet" Assembly for elution of N-glycans.
- For Finishing (Rapid-Reductive-Amination with 2-AB or APTS protocols): after elution with Finishing Reagent, remove the 0.2-ml PCR tube that holds the eluted N-glycan Sample Replicates from the "Tips Wet" Adaptor Assembly, cap it and place it on the PCR heat block to incubate. The N-glycans are then dried in the same PCR tube.
- For Labeling Incubation: add Labeling Reagent directly to the 0.2-ml PCR tube with the N-glycans and perform incubation as instructed.

"Tips Free" Assembly:

CU Cartridge (only, no PCR tube)

Cartridge Adaptor B

Flip-top, 1.75-ml Microcentrifuge tube

For superior removal of free dye, the Cleanup procedure requires that the tip of the Cartridge not be in direct contact with the flow-through at the bottom of the microtube. This is accomplished by placing an adaptor on a 1.75-ml Microcentrifuge tube and then positioning the CU Cartridge on top.

Add the N-glycan Sample to the Sample Cup of the CU Cartridge in the "Tips Free" Assembly. Load the Assembly into the Microcentrifuge, balance and spin as instructed.

Keep the Cartridge in the "Tips Free" Assembly to Wash and Elute, but prior to eluting the labeled N-glycans from the CU Cartridge, transfer the Adaptor and CU Cartridge to a fresh 1.75-ml Microcentrifuge tube.

PROTOCOLS

Getting Started

Heater Setting

Turn on the heat block. Set to 45°C and allow to equilibrate for a minimum of 1 hour.

Centrifuge Settings

Convert the required RCF values (i.e., 50 x g, 300 x g and 1000 x g) to the microcentrifuge's RPM settings based on the radius of the rotor to be used.

Many microcentrifuges allow direct setting of RCF values.

_____ rpm = 50 x g

_____ rpm = 300 x g

_____ rpm = 1000 x g

Preparation of Reagents

Glycoprotein Samples

This protocol begins with purified Glycoprotein Samples (see page 5 for details). Glycoprotein Samples must not contain any particulates, as they will plug the top frit, or sit on the top of the resin bed and impede the flow. Spin samples to remove particulates before processing.

Digestion Buffer

25x Digestion Buffer (supplied with the Kit)

Ultrapure water

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

Dilute one volume of 25x Digestion Buffer with twenty-four volumes of ultrapure water to obtain Digestion Buffer.

Prepare 100 µl of Digestion Buffer for each sample to be processed. For example, for 10 samples add 40 µl of 25x Digestion Buffer to 960 µl of ultrapure water to make 1 ml of Digestion Buffer.

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

Enzyme Solution

N-Glycanase (supplied with the Digestion Module)

Digestion Buffer (prepared previously)

0.6-ml microcentrifuge vial

Prepare only on the day of use. Store at RT.

Spin the N-Glycanase briefly to collect the contents in the base of the vial. Vortex the solution prior to dispensing.

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 75 +15 = 90 µl of Digestion Buffer.

96% Acetonitrile Solution

Ultrapure water

Acetonitrile (100%, HPLC-grade)

Glass, graduated cylinder, 25-ml

May be prepared up to one week before use. Store sealed at room temperature.

To make 10 ml of 96% Acetonitrile Solution (v/v), add 0.4 ml of ultrapure water to a glass, graduated cylinder. Bring the volume up to 10 ml with 100% acetonitrile. Transfer to a glass storage vessel, cap tightly and swirl gently to mix.

For fewer samples, prepare 250 µl of 96% Acetonitrile Solution for each sample to be processed: pipette 10 µl of ultrapure water into a polypropylene microtube, add 240 µl of 100% acetonitrile, cap the tube tightly and vortex on high for 10 seconds to mix.

Labeling Reagent

For instructions to prepare 2-AB Labeling Reagent, please see Labeling, page 14.

Digest

GlykoPrep Digestion Module (product codes GS24-RX and GS96-RX)

Reagents and other Supplies

Glycoprotein Samples

RX Cartridges (supplied with the Digestion Module, 1 per sample)

0.5-ml Microtube, screw cap, 0.2-ml PCR tubes, Cartridge Adaptors A and B and flip-top, 1.75-ml Microcentrifuge tubes

Denaturation Reagent (supplied with the Digestion Module)

Acetonitrile (100%, HPLC-grade), ~2 ml

Blocking Reagent (supplied with the Digestion Module)

Digestion Buffer (prepared previously)

Enzyme Solution (prepared previously)

Finishing Reagent (optional, supplied with the GlykoPrep 2-AB and APTS Kits)

Denature

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

The Denaturation Reagent is viscous and needs to be mixed well.

1.b Pipet up and down to mix well.

1.c Allow to incubate at room temperature for at least 5 minutes.

Prepare

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

2.a Prepare a Basic Assembly for each Sample by nesting the numbered RX Cartridges into Cartridge Adaptor A, then into 0.5-ml screw cap Microtubes.

RX Cartridge (only)

Cartridge Adaptor A

0.5-ml screw-cap Microtube

2.b Pipet 50 µl of 100% Acetonitrile into the Sample Cup of each RX Cartridge in the Basic Assemblies.

2.c Place the Basic Assemblies in the centrifuge and spin at 300 x g for 3 minutes.

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.

Load

4.a Load 100 µl of the denatured Samples into the Sample Cup of each RX Cartridge (see Sample Denaturation, page 5).

4.b Empty the flow-through by lifting each RX Cartridge and pouring out the liquid collected in the Microtube below. Dispose of the liquid as organic waste and return each RX Cartridge to its Microtube.

4.c Spin at 50 x g until all Sample Cups are empty (~15 minutes).

Block

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

5.b Empty the flow-through (as described in 4.b).

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

Wash

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

6.b Empty the flow-through (as described in 4.b).

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

Load N-Glycanase

7.a Prepare a "Tips Wet" Adaptor Assembly for each sample by nesting PCR tubes into Cartridge Adaptor B and 1.75-ml Microcentrifuge tubes.

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

7.c Transfer the RX Cartridges into the corresponding "Tips Wet" Adaptor Assemblies. Dispose of Microtubes and flow-through from 6.

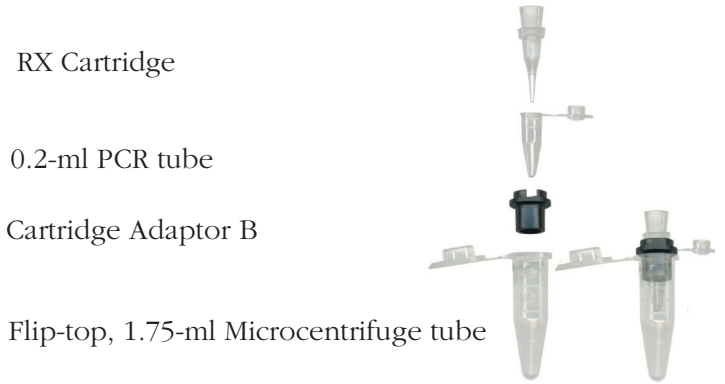
7.d Spin at 300 x g for 3 minutes; do not discard flow-through.

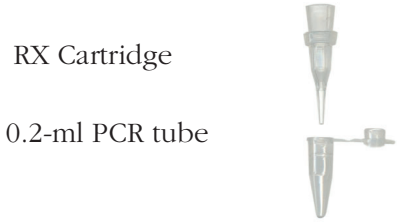
Incubate

8. Transfer the RX Cartridges/PCR tubes (Incubation Assembly) to the equilibrated 45°C PCR heat block and incubate for the chosen incubation time (see page 6).

Use the special sample loading technique to load samples in all protocols to prevent the introduction and entrapment of air bubbles in the neck of the Sample Cup. Use a pipet to remove trapped air bubbles.

Check that Sample Cups are empty before proceeding or yield will be reduced.





Elute (and Finish)

9.a Remove the Incubation Assemblies from the heat block and reinsert into the Microcentrifuge tubes to form "Tips Wet" Adaptor Assemblies again.

9.b Pipet 15 µl of Finishing Reagent into the Sample Cup of each RX Cartridge.

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

9.d Remove RX Cartridges from the PCR tubes. The eluted N-Glycans are in the PCR tubes; DO NOT DISCARD.

10. Close the cap on each PCR tube and incubate on the equilibrated 45°C heat block for 10 minutes.

11. Open the PCR tubes, return them to the "Tips Wet" Adaptor Assemblies (now minus the RX Cartridges). Dry in a centrifugal evaporator (SpeedVac, heat setting turned to the off position) until fully dry (~30 minutes).

Label

Reagents and other Supplies

N-Glycan Samples (dried N-glycans in 0.2-ml PCR tubes)

2-AB Labeling Reagent (prepared no more than one hour before use)

Preparation of 2-AB Labeling Reagent

2-AB Solution (supplied with the Kit)

Reductant Solution (supplied with the Kit)

0.6-ml microcentrifuge vial

Allow the 2-AB Solution and Reductant Solution to come to room temperature in the sealed desiccant bag before removing them. Then, invert gently to mix.

Determine the number of samples to be labeled. In a separate vial, prepare a mixture of 2.5 µl of 2-AB Solution and 2.5 µl of Reductant Solution for each sample to be processed, plus 20% for overage.

Used RX Cartridges may be discarded.

0.2-ml capped PCR tube 

After removing the PCR tubes from the heat block, adjust the temperature setting to 65°C.

The N-glycans are condensed/spun into a pellet small enough to be dissolved by 5 µl of 2-AB Labeling Reagent or 4.5 µl of APTS Labeling Reagent in the next step.

GlykoPrep Rapid-Reductive-Amination 2-AB Labeling Module (product codes GS24-AB and GS96-AB)

2-AB Labeling Reagent should be prepared no more than one hour before use.

NOTE: 2-AB Labeling Reagent components are hazardous. Please refer to the Safety Data Sheets on our website. Perform this procedure using appropriate personal safety protection, eyeglasses and nitrile gloves.

Both the 2-AB Solution and Reductant Solution are hygroscopic; minimize exposure to air and protect from exposure to light.

For example, 10 samples would require 25 + 5 = 30 µl each of 2-AB Solution and Reductant Solution.

Cap tightly and vortex on high for 10 seconds to mix; briefly spin down in a centrifuge.

The individual reagents may be resealed, repackaged with the desiccant in the resealable bag, and frozen (-20°C) for storage up to 6 months; return to RT before opening for use to minimize condensation.

Procedure

Label

1.a Add 5 µl of 2-AB Labeling Reagent to each N-Glycan Sample.

1.b Return the PCR tubes to the "Tips Wet" Adaptor Assemblies (minus the RX Cartridges) and spin at 300 x g for 1 minute to ensure the liquid is collected at the bottom of the wells.

Incubate

Before use, be sure each heat block has equilibrated to 65°C; a thermometer may be placed in the corner thermometer well of the heat block to test the temperature.

2.a Close the cap on each PCR tube and transfer it to the heat block.

2.b Incubate at 65°C on the equilibrated heat block for 1 hour.

2.c Remove the PCR tubes from the heat block and allow to cool to room temperature (~5 minutes).

Prepare the 2-AB Labeling Reagent during sample drying.

2.d In a fume hood, open each PCR tube.

0.2-ml capped PCR tube

It is normal for condensate to collect on the underside of the lid. DO NOT centrifuge the tube to collect the condensate. Proceed immediately to Cleanup.

Cleanup

GlykoPrep Cleanup Module (product codes GS24-CU and GS96-CU)

Reagents and other Supplies

This entire section is performed with the CU Cartridges "Tips Free."

N-Glycan Samples from 2-AB Labeling (in PCR tubes)

CU Cartridges (supplied with the Kit, 1 per N-glycan sample)

Cartridge Adaptor B and flip-top, 1.75-ml Microcentrifuge tubes, 2 per sample

Acetonitrile

96% Acetonitrile Solution (prepared previously)

Ultrapure water

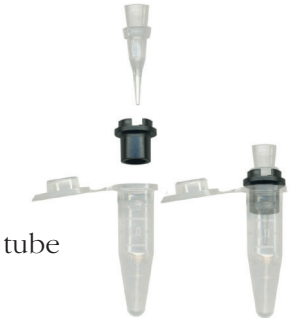
Procedure

Prepare one “Tips Free” Assembly per N-Glycan Sample by nesting a CU Cartridge into a Cartridge Adaptor B and a flip-top, 1.75-ml Microcentrifuge tube.

CU Cartridge

Cartridge Adaptor B

Flip-top, 1.75-ml Microcentrifuge tube



Load

1.a Add 20 µl of water to each N-Glycan Sample in the PCR tubes.

1.b Add 180 µl of 100% Acetonitrile to each sample. Pipet up and down to mix thoroughly

Transfer each sample into the Sample Cup of a CU Cartridge in a “Tips Free” Assembly.

Transfer the mixture as quickly as possible; Acetonitrile solution 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.

1.c Spin at 50 x g until the Sample Cup of each CU Cartridge is empty (~10 minutes).

Wash

2.a Pipet 200 µl of 96% Acetonitrile Solution into the Sample Cup of each CU Cartridge in the “Tips Free” Assembly.

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

Elute

3.a Prepare a clean, 1.75-ml Microcentrifuge tube for each sample.

3.b Transfer each CU Cartridge with its Adaptor to the new Microcentrifuge tube.

3.c Pipet 25 µl of water into the Sample Cup of each CU Cartridge.

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

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

The 1.75-ml Microcentrifuge tubes now contain the 2-AB-labeled N-Glycans with free dye and buffer salts removed; DO NOT DISCARD.

Used CU Cartridges may be discarded. Adaptors are reusable, DO NOT DISCARD.

N-Glycan Samples are now ready to be analyzed. If not analyzed immediately, store sealed at -20°C in the dark.

ANALYSIS OF LABELED N-GLYCANS

Eluted N-glycans labeled with 2-AB may be analyzed using standard techniques such as High Performance Liquid Chromatography (HPLC), Mass Spectrometry (MS), or a combination of the two (see Tips and Hints, below).

TIPS & HINTS

Optimizing Excitation/Emission Wavelengths

Optimal excitation/emission wavelengths for 2-AB Dye 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:

250/428 nm (Melmer et al., 2010), 330/420 nm (Bigge et al., 1995)

360/428 nm (used by ProZyme with the Waters® Acquity® UPLC®; Haxo et al., 2012).

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.

Calculating the MW of Labeled N-glycans

The MW of the 2-AB-labeled N-glycan is:

MW_{Glycan} + 120.0687

REFERENCES

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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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http://www.prozyme.com

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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

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FAX (510) 638-6919
E-MAIL info@prozyme.com
WEB www.prozyme.com

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PROzyme®

Agilent AssayMAP

3832 Bay Center Place
Hayward, CA 94545-3619
USA

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