AdvanceBio N-Glycan Sample Preparation Kit User Guide (96 samples)

p/n 5190-8005

Agilent Technologies

Introduction

The AdvanceBio N-Glycan Sample Preparation Kit is a solution for the analysis of protein glycosylation by liquid chromatography of released, labeled N-glycans. The kit is comprised of four components that perform the steps of N-glycan release, N-glycan purification, labeling with 2-aminobenzamide, and labeled glycan cleanup. The workflow yields samples that are suitable for analysis by liquid chromatography, typically by hydrophilic interaction chromatography (HILIC) using the Agilent AdvanceBio Glycan Mapping Column. The goal of this analysis is typically to achieve relative quantitation of the various N-glycan structures in the sample, rather than absolute quantitation.

AdvanceBio N-glycan deglycosylation kit (p/n 5190-8006) includes an enzyme, PNGase F, that cleaves the amide bond between N-glycans and asparagine residues. This gives released N-glycans in the glycosylamine form, as well as deglycosylated proteins in which glycosylated asparagines are converted to aspartates. The kit includes a choice of sodium phosphate or *TRIS*-HCl reaction buffers depending on user preference, *TRIS*-HCl is preferred for MS detection. Released N-glycans immediately begin to convert from the glycosylamine form into free reducing end glycans, and this process can be accelerated by brief incubation in acidic aqueous conditions.

The PNGase F concentration is 100 U/mL. One unit is defined as the amount of enzyme that catalyzes the release of N-linked oligosaccharides from 1 µmol of denatured ribonuclease B (RNase B) per minute at pH 7.5 and 37 °C.

	Contents	Storage
Enzymatic deglycosylation (release of N-glycans) p/n 5190-8006 AdvanceBio N-glycan deglycosylation kit	40 µL/100 U Peptide-N-glycosidase F (PNGase F) EDTA-free	2–8 °C
	1 mL 5x Na phosphate reaction buffer (100 mM, pH 7.5, 0.1% sodium azide)	
	1 mL 5x *TRIS* reaction buffer (50 mM *TRIS*-HCl, pH 8.0)	
	1 mL detergent solution (15% NP-40)	
	1 mL denaturation solution (2% SDS, 1 M, β-ME)	
↓		
Purify released N-glycans by SPE p/n 5190-8007 AdvanceBio N-glycan deglycosylation cleanup cartridges	96 x HILIC SPE cartidges designed to extract free glycans	Ambient
↓		
Labeled N-glycans with fluorescent tag p/n 5190-8008 AdvanceBio 2-AB glycan labeling kit	300 µL 2-AB solution (2-aminobenzeamide, acetic acid, DMSO)	–25 °C to –10 °C
	300 µL reductant solution (Sodium cyanoborohydride, DMSO)	
↓		
Clean up labeled N-glycans by SPE p/n 5190-8009 AdvanceBio 2-AB glycan labeling cleanup cartridges	96 x HILIC SPE cartidges designed to clean up 2-AB labeled glycans	Ambient
↓		
Analysis by LC-FLD with optional MS	**All above sub-kits can be purchased individually*	

The deglycosylation kit also includes two optional reagent vials that allow the analyst to denature their sample protein prior to deglycosylation, if required. The denaturing solution consists of a surfactant and a reducing agent that breaks disulfide bonds. If this solution is used, the analyst must subsequently add the detergent solution, which contains a nonionic surfactant that prevents the components of the denaturing solution from interfering with deglycosylation by PNGase F. The kit contains sufficient reagent for 24 samples.

AdvanceBio N-glycan deglycosylation cleanup cartridges (p/n 5190-8007) are single-use solid phase extraction devices that are used to extract released N-glycans from the deglycosylation reaction solution. N-glycans bind to the highly polar HILIC sorbent in the presence of acetonitrile. Subsequent washes with acetonitrile-water mixture remove unwanted components left over from the deglycosylation reaction, reducing interferences downstream. Finally, the adsorbed N-glycans are eluted from the SPE device with water or aqueous solutions. The cartridges are suitable for use with the Agilent Vacuum Manifold (p/n 5133000) and 96-well plate (p/n 5190-8010). The kit includes 24 cartridges.

AdvanceBio 2-AB glycan labeling kit (p/n 5190-8008) includes reagents to label released glycans with a tag that allows fluorescence detection and promotes efficient chromatography. The kit consists of solutions of the fluorescent tag (2-aminobenzamide) and a reductant (sodium cyanoborohydride) that should be mixed in a 1:1 ratio immediately prior to use. This allows a highly convenient, low volume one-pot reductive amination reaction without the need for weighing out hazardous reagents in an analytical balance. The kit contains sufficient reagent for at least 24 samples.

AdvanceBio 2-AB glycan labeling cleanup cartridges (p/n 5190-8009) are single-use solid phase extraction devices that are used to extract labeled N-glycans from the labeling reaction solution. Labeled N-glycans bind to the highly polar HILIC sorbent in the presence of acetonitrile. Subsequent washes with acetonitrile-water mixture remove unwanted components left over from the labeling reaction, particularly the large excess of 2-aminobenzamide, which can otherwise interfere with fluorescence detection. The adsorbed labeled N-glycans are eluted from the cartridge with water or aqueous solution. Each kit contains 24 cartridges, which are suitable for use with the Agilent Vacuum Manifold (p/n 5133000) and 96-well plate (p/n 5190-8010).

TIPS

During shipping and handling, some of the vial contents may adhere to different parts of the vial, including the cap. We recommend a brief centrifuge to ensure that all the contents are at the base of the vial.

Other key equipment (purchased separately)

Equipment	Ordering information
Vacuum centrifuge	N/A, general lab equipment
Vortex mixer	N/A, general lab equipment
Incubation oven or heating block (30 °C–100 °C range)	N/A, general lab equipment
Vacuum pump	N/A, general lab equipment
Pipettors and disposable tips (2 µL–1 mL)	N/A, general lab equipment
Agilent 96-well manifold, 1/pk	p/n 5133000
Agilent 96-well plate adapter for deglycosylation and labeling, 1/pk	p/n 5190-8010
Agilent Versaplate sealing strips, 240/pk	p/n 12236108
Agilent 96 deep well plates, 1 mL, 50/pk	p/n 5042-6454
Agilent VersaPlate Shimset, 1/pk	p/n 12236104
Disposable Reservoir Tray	p/n 5185-5782

Protocols

For easy reference, the protocol for the AdvanceBio N-Glycan Sample Preparation Kit is divided into sections that correspond to the four steps of the workflow

Deglycosylation using AdvanceBio N-glycan deglycosylation kit (p/n 5190-8006)

1. In a 1.5 mL sample tube, mix sample containing 50–500 µg glycoprotein with 7.5 µL chosen Reaction Buffer and water to make a total volume of 35 µL.

2. Add 2 µL of denaturation solution.

3. Denature the glycoprotein mixture by heating at 95 °C for 10 minutes. Allow to cool to room temperature.

4. To the cooled mixture, add 2 µL detergent solution.

5. Add 0.4 µL PNGase F solution (~4 milliunits) and incubate for 3 hours at 37 °C.

TIPS for deglycosylation

- *Some native proteins contain glycosylation sites that cannot be accessed by PNGase F. Steps 2–4 of the protocol denature the protein by heating it in the presence of a surfactant and a reagent that reduces disulfide bonds. However, some N-glycan sites are easily cleaved when the protein is in its native form, for example, the N-glycan in the Fc region of IgG antibodies. If you are certain that your sample only contains N-glycans that can be cleaved from the native form of your protein, you can skip steps 2–4.*

- *50 µg of glycoprotein are normally sufficient for the analysis of released, labeled glycans by LC with fluorescence detection. Larger amounts of glycoprotein may be required for some mass spectrometry methods or for sophisticated workflows that require additional sample handling and multiple analyses. If your sample is very dilute, it may be necessary to concentrate it by diafiltration prior to combining it with the deglycosylation reagents. If the sample has poor solubility, it may be necessary to scale up the reaction volumes to process sufficient protein.*

- *For ease of handling, PNGase F can first be diluted by 4X with DI water then add 1.6 µL of the diluted PNGase F to each sample.*

- *Deglycosylation efficiency is highly dependent on the sample protein. When using the recommended 0.4 µL of PNGase F (~4 milliunit) for 250 µg of IgG, deglycosylation of the Fc region will approach completion after a 3 hour digestion, regardless of whether the protein is denatured (Steps 2–4). Yield may be reduced if the incubation time, enzyme quantity, or enzyme-to-protein ratio are reduced. Conversely, increasing the reaction time to an overnight incubation may allow complete deglycosylation with a lower quantity of enzyme, or with a lower enzyme-to-protein ratio.*

Deglycosylation cleanup using AdvanceBio N-glycan deglycosylation cleanup cartridges (p/n 5190-8007)

1. Assemble the vacuum manifold apparatus with the disposable reservoir tray underneath the 96-well plate adaptor. Install one cartridge for each sample to be processed, and cover the remaining positions with the Agilent Versaplate sealing strips so that vacuum can be maintained within the manifold.

2. To condition each cartridge, add 1 mL water, and then open the vacuum until the water drops to the level of the bed. Then add 1 mL of 96% ACN/water and open the vacuum until the liquid drops to the level of the bed.

3. Add 35 µL of deglycosylated mixture to 315 µL of acetonitrile to make a 90% ACN solution. Load the sample onto the conditioned cartridge.

4. Open the vacuum valve partially to pull the sample through (~1 drop/2–3 seconds) until the liquid drops to the level of the bed. Turn off the vacuum and wait 2 minutes.

5. Wash with 3 × 750 µL of 96% ACN/water to elute matrix reagents. Stop the vacuum, and add each additional wash when the liquid level falls to the level of the bed.

6. Remove the disposable reservoir tray and replace with the 1 mL × 96-well plate. It may be necessary to add some shims (p/n 12236104) to elevate the well plate by ~ 5 mm so that the tips of the cartridges are located within the wells in the plate.

7. Elute the N-glycans with 2 × 500 µL 1% formic acid in water. Control the flow by partially opening the vacuum to pull each aliqout of 500 µL through the cartridge over the course of 1 minute. For the second aliquot, maintain the vacuum until all of the liquid is pulled through the cartridge.

8. Dry the glycans in a vacuum centrifuge for 2 hours to overnight, as necessary.

Once the sample is vacuum dried, it is ready for the 2-AB labeling protocol. If it is not to be labeled immediately, store at 4 °C for up to 1 week. For longer term storage, keep at –20 °C.

TIPS for deglycosylation cleanup

- *The deglycosylation mixture will sometimes form a precipitate, or a precipitate may form after acetonitrile is added. Some of the released glycans may be bound to this precipitate, so it should not be removed through centrifugation. Rather, vortex or aspirate the solution so that the precipitate is in suspension, then transfer the entire contents of the sample tube to the SPE cartridge.*

- *Different cartridges will flow at different rates. Vacuum should be maintained until the liquid in all cartridges is drawn down to the level of the bed. It is acceptable to pull air through the beds of the faster flowing cartridges while parallel processing a set of samples, as the glycans will not prematurely elute from the cartridges.*

- *Depending on the number of samples, drying can either be done in the 96-well plate or the samples may first be transferred to individual sample tubes. Drying rates may vary widely depending on the efficiency of the vacuum centrifuge.*

- *It is important to keep the temperature of the vacuum centrifuge below 33 °C while drying down the glycans that are eluted from the cartridge in 1% formic acid. This is because glycans that contain sialic acids can be hydrolyzed by exposure to heat under acidic conditions. If you suspect that you are losing sialic acids due to the formic acid, you can substitute the formic acid solution for pure water and compare the results.*

Labeling using an AdvanceBio 2-AB glycan labeling kit (p/n 5190-8008)

1. Mix equal parts 2-AB tag (vial 1) and reductant (vial 2) solutions. The volume of the mix required will be 5 µL for every sample (that is, 2.5 µL of both vial 1 and vial 2 for each sample), plus a little extra to cover inevitable losses by evaporation during handling.

2. Resolubilize dried released glycans in 5 µL of dye/reductant mix per sample.

3. Heat at 65 °C for 3 hours.

4. After heating, spin the tube briefly to collect any condensate, and allow the labeled glycan solution to cool to room temperature.

TIPS for labeling

- *The labeling reaction uses a very small volume, which may not entirely immerse the film of glycans that are dried onto the lower interior surfaces of the sample tube. Improve the labeling yield by ensuring that all of the dried glycans dissolve in the labeling solution. Suggestion: after dispensing the labeling solution, use the autopipette tip to drag the solution across any part of the sample tube that is likely to have a film of dried glycans. Vortex the tube, and spin briefly to collect the solution at the bottom of the vial.*

Labeling cleanup with AdvanceBio 2-AB glycan labeling cleanup cartridges (p/n 5190-8009)

1. Add 8 µL DI H_2O to the labeled sample solution with gentle aspirating. Then add 193 µL ACN.

2. Set up the vacuum manifold with the disposable reservoir tray. Install one cartridge for each sample.

3. To each cartridge, add 1 mL of water, then open the vacuum until the liquid falls to the level of the sorbent bed. Add 1 mL of 96% ACN/water, and repeat.

4. Load the sample onto the cartridge. Partially open the vacuum valve to pull the sample solution through until the liquid reaches the level of the bed (~1 drop/2–3 seconds). Wait 2 minutes.

5. Wash with 3 × 750 µL of 96% ACN. Stop the vacuum, and add the next wash each time the liquid level falls to the level of the bed.

6. Replace the waste basin with 96-well plate. It may be necessary to use some shims (p/n 12236104) to elevate the well plate by ~5 mm so that the tips of the cartridges are located within the wells in the plate.

7. Elute the N-glycans with 2 × 500 µL DI water. Control the flow by partially opening the vacuum to pull each aliqout of 500 µL through the cartridge over the course of 1 minute. For the second aliquot, maintain the vacuum until all of the liquid is pulled through the cartridge.

8. Dry the samples in a vacuum centrifuge for 2 hours to overnight, as necessary.

TIPS for labeling cleanup

- *The labeling mixture will sometimes form a precipitate, or a precipitate may form after the acetonitrile is added. Vortex or aspirate the contents of the sample tube so that the precipitate is in suspension, and transfer the entire contents of the sample tube to the SPE cartridge.*

- *Different cartridges will flow at different rates. Vacuum should be maintained until the liquid in all cartridges is drawn down to the level of the bed. It is acceptable to pull air through the beds of the faster flowing cartridges while parallel processing a set of samples. The glycans will not prematurely elute from the cartridges.*

- *Depending on the number of samples, drying can either be done in the 96-well plate, or the samples may first be transferred to individual sample tubes.*

(U)HPLC analysis using FLD or MS detection

1. Reconstitute the labeled glycan in 6 µL of DI water with gentle aspirating. Add 14 µL ACN to make the labeled glycan solution into 70:30 ACN:H_2O.

2. Inject 1 µL into the column.

TIPS for HPLC/FLD analysis

- *For HPLC evaluation of 2-AB labeled N-linked glycan samples, we recommend Agilent AdvanceBio Glycan Mapping Columns. These columns are designed and manufactured to deliver FAST, high resolution, reproducible glycan identification using HILIC chromatography. AdvanceBio Glycan Mapping columns leverage technology that optimizes results for MS and fluorescence detection. Choose from two UHPLC configurations: 2.7 µm superficially porous, for high resolution and lower backpressure, or 1.8 µm for high resolution, fast separations.*

- *Sample solution matrix and injection volume impact separation peak shape and resolution. We recommend dissolving glycan samples in 70% ACN with an injection volume ≤ 2 µL to achieve optimal performance.*

Related products

Agilent offers a high throughput version of the AdvanceBio N-Glycan Sample Preparation Kit for analysts who want to process up to 96 samples in one batch. Agilent also offers a selection of standards for performance testing and retention time based assignment of labeled and unlabeled glycans. These products are listed in the following table.

Description	Part no.
AdvanceBio glycan sample preparation kit (24 samples)	**5190-8000**
AdvanceBio N-glycan deglycosylation kit (24 samples)	5190-8001
AdvanceBio N-glycan deglycosylation cleanup cartridges (24 samples)	5190-8002
AdvanceBio 2-AB glycan labeling kit (24 samples)	5190-8003
AdvanceBio 2-AB glycan labeling cleanup cartridges (24 samples)	5190-8004
Glycan standards	
Dextran Ladder standard, 10 µg, 0.5 mL vial	5190-6997
2-AB labeled dextran ladder standard, 200 pmol	5190-6998
IgG N-linked glycan library, 20 µg, 0.5 mL vial	5190-6995
2-AB labeled IgG N-lined glycan library, 200 pmol	5190-6996

Agilent AdvanceBio Glycan Mapping columns

1.8 µm, stable to 1,200 bar

Description	Part no.
2.1 × 100 mm	858700-913
2.1 × 150 mm	859700-913
Fast Guards, 2.1 mm, 1.8 µm	821725-905

2.7 µm, superficially porous, stable to 600 bar

Description	Part no.
2.1 × 100 mm	685775-913
2.1 × 150 mm	683775-913
2.1 × 250 mm	681750-913
Fast Guards, 2.1 mm, 2.7 µm	821725-906
4.6 × 100 mm	685975-913
4.6 × 150 mm	683975-913
4.6 × 250 mm	680975-913

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