GlycoWorks Single Use Sample Preparation Kit

CONTENTS

I. INTRODUCTION

This document provides information regarding the general care and use of the Waters GlycoWorks™ Single Use Sample Preparation Kit for release of N-glycans. This protocol can be used as a guideline and is validated using IgG but can also be used and optimized for other N-linked glycoproteins. This sample preparation offers an inclusive protocol that allows the user to source many of the critical components needed to perform the preparation from one vendor.

The Single Use Cartridge Pack contains 20 1cc cartridges, good for 10 analyses.

For comparison results using this protocol versus the 96 well plate and for recovery information with the control standard.

The guide provides the following:

- An instruction template for new users that highlights the use of a control standard (included in the kit) for first time validation as well as subsequent troubleshooting
- Protocol for LC/FLR and LC/FLR/MS
- Tips and tricks throughout each section to help aid in the success
- An online training video is also available

General Guideline of Sample Preparation from Glycoprotein to enrich FLR Labeled Glycans Using Reductive Amination Reaction.

1. Denaturation and Deglycosylation
   - Rapigest™ (if necessary)
   - DTT Reduction (if necessary),
   - IAM Alkylation (if necessary)
   - Deglycosylation by PNGase F

2. Extraction of Released Glycans
   - GlycoWorks HILIC 1 cc Cartridge

3. FLR Labeling Reaction of Glycans

4. Excess Labeling Reagent Removal
   - GlycoWorks HILIC 1 cc Cartridge

FLR Labeled Glycans

MALDI MS
LC-FLR
LC-FLR-MS

If using MALDI please refer to Product Solution, Literature Code 720004660EN

Figure 1: Sample Preparation from Glycoprotein to Labeled Glycans
Table 1: Kit Components

<table>
<thead>
<tr>
<th>Kit Component</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlycoWorks HILIC 1 cc Cartridge</td>
<td>Extract and clean up of free or FLR labeled glycans</td>
</tr>
<tr>
<td></td>
<td>■ Capture of glycans</td>
</tr>
<tr>
<td></td>
<td>■ Removing contaminants like salt and detergents from hydrophilic analytes, such as carbohydrates, prior to MS</td>
</tr>
<tr>
<td></td>
<td>■ Removing excess labeling reagent</td>
</tr>
<tr>
<td></td>
<td>■ The 10 mg sorbent was tested to have maximum binding capacity for 400 µg glycans</td>
</tr>
<tr>
<td>GlycoWorks Glycoprotein Control Standard</td>
<td>Unlabeled IgG (100 µg/lyophilized) to use as a control for process validation and troubleshooting</td>
</tr>
<tr>
<td>RapiGest SF</td>
<td>An enzyme friendly detergent used to denature the glycoproteins prior to deglycosylation reaction. For stability purposes, there is enough RapiGest included for initial use of the kit with 5 samples, more may need to be ordered for processing all 10 samples.</td>
</tr>
<tr>
<td>Manifold</td>
<td>For collection of non-releasing glycans steps. This is disposable but can be cleaned and used several times before acquiring a new one as long as it is rinsed out.</td>
</tr>
<tr>
<td>GlycoWorks Reagent Kit</td>
<td>Contains ready to use reagents for both denaturation, reduction, and alkylation as well as raw materials for FLR tagging.</td>
</tr>
</tbody>
</table>

II. STORAGE AND DISPOSAL OF CARTRIDGES

Cartridges stored in their original sealed pouch remain stable for long periods. For long term storage of unused cartridges, keep cartridges sealed and in a desiccator.

Note: Dispose of used cartridges safely in accordance with applicable government or local regulations.

III. ABBREVIATIONS

DMSO  Dimethylsulfoxide  IAM  Iodoacetamide  
DTT  Dithiothreitol  2AB  2-aminobenzamide

IV. USING THE GLYCOWORKS SINGLE USE KIT

Below, are general guidelines for utilizing the GlycoWorks Single Use Kit for complete extraction, purification, and concentration of N-linked glycans validated using IgG. Also included is a check list of accessory materials that will be needed to complete the protocol from start to finish as well as tips and tricks throughout the instructions.

Table 2: Checklist of Additional Materials Needed Before You Begin

<table>
<thead>
<tr>
<th>Material Description</th>
<th>Recommended Suppliers</th>
<th>Check List: Circle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium bicarbonate</td>
<td>Sigma Aldrich Cat No. 09830</td>
<td>Yes No</td>
</tr>
<tr>
<td>Ammonium acetate</td>
<td>Sigma Aldrich Cat No. 73594 FLUKA</td>
<td>Yes No</td>
</tr>
<tr>
<td>Formic acid (to make 1% [v/v] formic acid, prepared in 50% acetonitrile)</td>
<td>Sigma Aldrich Cat No. F0507</td>
<td>Yes No</td>
</tr>
<tr>
<td>15,000 units (500,000 units/mL) PNGase F*</td>
<td>New England Biolabs Cat. No. P0704S</td>
<td>Yes No</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>Sigma Aldrich Cat No. 360457</td>
<td>Yes No</td>
</tr>
<tr>
<td>25 mM ammonium bicarbonate in water, pH 8 - 9</td>
<td>See Above</td>
<td>Yes No</td>
</tr>
<tr>
<td>100 mM ammonium acetate in 5% [v/v] acetonitrile, pH 7</td>
<td>See Above</td>
<td>Yes No</td>
</tr>
<tr>
<td>85% [v/v] acetonitrile</td>
<td>See Above</td>
<td>Yes No</td>
</tr>
<tr>
<td>2-AB**</td>
<td>Sigma P/N A89804</td>
<td>Yes No</td>
</tr>
<tr>
<td>SPE vacuum pump</td>
<td>115, 60 Hz: Waters P/N 176002986</td>
<td>Yes No</td>
</tr>
<tr>
<td>Centrifugal vacuum evaporator</td>
<td>N/A</td>
<td>Yes No</td>
</tr>
<tr>
<td>Heat block</td>
<td>N/A</td>
<td>Yes No</td>
</tr>
<tr>
<td>Fisherbrand Premium 1.5 mL MCT graduated, RNase/DNase free</td>
<td>Fisher Scientific Cat No. 05-408-129</td>
<td>Yes No</td>
</tr>
</tbody>
</table>

*PNGase F comparison table: https://www.neb.com/tools-and-resources/usage-guidelines/glycobiology-unit-conversion-chart

**Inclusive Kits are also offered by ProZyme, Ludger, and Sigma covered under US Patent No. 5,747,347 and its foreign equivalents.
Step 1. Denaturation and Enzymatic Deglycosylation

RapiGest, DTT, IAM are included in the GlycoWorks Reagent Kit box labeled “Enzymatic Deglycosylation Reagents” for your convenience in following this protocol:

1) Prepare RapiGest solution by adding 500 μL of 25 mM ammonium bicarbonate to 1 mg RapiGest vial.
2) For the GlycoWorks Control Standard, add 100 μL of 25 mM ammonium bicarbonate to vial.
3) Add 90 μL of the 0.2% RapiGest solution to the glycoprotein solution in a sterile 1.5 mL centrifuge tube.

Note: For use with other samples, it is recommended that the protein concentration be 1–5 mg/mL.

3) Prepare 0.5 M DTT by adding 500 µL of 25 mM ammonium bicarbonate to the tube. Vortex to mix into solution.

■ Add 2 μL of 0.5 M DTT and incubate sample(s) at 37 °C for 30 minutes. Cool to room temperature.

4) Prepare 0.5 M IAM by adding 500 µL of 25 mM ammonium bicarbonate to the tube. Vortex to mix into solution.

■ Iodoacetamide is light sensitive. Cover the tube in foil and place it in a dark drawer when not in use. This solution should be made fresh daily.

■ Add 4 μL of 0.5 M IAM and incubate at room temperature in the dark for 30 minutes.

5) Add 2.5 mU (1 μL of 2.5 mU μL-1 stock) of PNGase F to each sample and incubate at 37 °C overnight. If a more concentrated enzyme stock is purchased, dilute with included buffer first to get to a 2.5 mU/μL concentration.

Note: If digesting glycoprotein samples greater than 1 mg/mL, it is recommended to use more PNGase F.

Step 2a. Extracting the Released Glycans

1) Condition each cartridge by adding 1,000 μL of Milli-Q® water to each cartridge and aspirate using the vacuum manifold.
2) Add 1,000 μL of 85% acetonitrile to each cartridge and aspirate using the vacuum manifold.
3) Take 100 μL of the PNGase F-digested protein mix and add this to 700 μL of acetonitrile.

Note: If precipitation occurs, do not centrifuge! Centrifugation causes reduced glycan recovery.

4) Load the sample onto the resin bed using a very low vacuum setting (1–2 in Hg).
5) Wash each cartridge 3 times with 400 μL of 85% acetonitrile, collecting the filtrate after each wash into the waste tray.
6) Remove the waste tray and replace with sterile 1.5 mL Eppendorf tubes.
7) Elute the glycans with 100 μL of 100 mM ammonium acetate in 5% acetonitrile.

Note: The concentration of acetonitrile and ammonium acetate in the elution buffer may require optimization for some glycan samples.

8) Repeat the elution two more times.
9) Place the collection plate and dry released glycans by vacuum evaporation to a concentrate of ≤ 2 μL in volume.

Step 1 Tips and Tricks

■ The minimum amount of glycoprotein advised is 10 μg (100 μL of 0.1 mg/mL).
■ Avoid temperature extremes with glycan samples.
■ Exposure to high pH may result in epimerization of reducing sugars.
Step 2b. Formic Acid Treatment of Released Glycans

1) Prepare 1% formic acid by adding 10 μL formic acid to 990 μL of 50% acetonitrile.
2) Add 100 μL of 1% formic acid solution to each glycan sample.
3) Incubate for 40 minutes at room temperature.
4) Dry glycans using vacuum evaporation bringing them to complete dryness.

Step 3. Labeling of Glycans

Reference: Legal Information


Below is only an example of a sample procedure that one can follow for labeling. Acetic Acid, DMSO, and Sodium cyanoborohydride are included in the GlycoWorks Reagent Kit box labeled “Additional Reagents” for your convenience in following this protocol with the control standard.

Note: Prepare labeling solution immediately prior to labeling.

1) Add 300 μL of acetic acid vial to 700 μL of DMSO vial in the kit.
2) Weigh out 10 mg of 2AB.
3) Add 800 μL of the acetic acid/DMSO mixture to the entire vial of chosen label.
4) Mix until dissolved (may require vortexing).
5) Add the entire contents of the labeled mixture to the vial of sodium cyanoborohydride in the kit and mix until completely dissolved.
6) Add 20 μL of the labeling solution to each dried glycan sample. Ensure glycans are fully reconstituted in the 2AB label.
7) Incubate glycan samples for a minimum of 3 h at 65 °C in a heating block (if samples are in eppendorfs), dry oven, or sand tray.
8) Following incubation, briefly spin the vials to recollect each sample at the bottom of the vial if samples are in Eppendorfs.
9) Allow samples to cool to room temperature.

Step 2 Tips and Tricks

- The concentration of ammonium acetate and acetonitrile in the elution buffers of Steps 2a and 4 may require optimization for some glycans.
- The reason for using low concentrations of formic acid is to convert all glycans to free reducing glycans, hence improving the overall yield of the FLR-labeling via reductive amination.
- Drying down the sample does cause some sample loss but it essential to have the sample completely dry before proceeding to Step 3.

Step 3 Tips and Tricks

- Protect the final preparation of labeling reagent from light.
- The sodium cyanoborohydride once it is solubilized, should be used within an hour.
- Left over reagent which contains sodium cyanoborohydride has to be disposed separately in a clearly marked bottle due to its toxicity (see MSDS).
- To ensure high yield of labeled glycans, it may be necessary, for some samples, to increase the concentrations of the reducing and labeling reagents.
Step 4. Removal of Excess Label

1) Condition a NEW well of the GlycoWorks cartridge by adding 1,000 μL of Milli-Q water to each cartridge and aspirate using the vacuum manifold.

2) Add 1,000 μL of 85% acetonitrile to each well and aspirate using the vacuum manifold or positive pressure manifold.

3) Add 200 μL of acetonitrile to the 20 μL of 2AB labeled glycan sample and load into the cartridge using low vacuum setting (1-2 in Hg).

4) Wash each well with 400 μL of 85% acetonitrile. Remove filtrate to waste.

5) Replace the waste tray with sterile 1.5 mL Eppendorf tubes.

6) Elute glycans with 100 μL of 100 mM ammonium acetate in 5% acetonitrile.

Note: The concentration of ammonium acetate and acetonitrile in the elution buffer may require optimization for some glycan samples.

7) Repeat elution two more times.

8) Place 96-well collection plate in a centrifugal vacuum evaporator and dry the glycans to a concentrate of ≤ 2 μL in volume.

Note: If a rotor is not available for plates, transfer each eluent to a sterile 1.5 (or 0.5) mL Eppendorf and place in the vacuum evaporator. This may contribute to a small sample loss due to extra transfer.

9) Glycans can be stored in Milli-Q water at -20 °C until required.

V. PREPARING LABELED GLYCANS FOR HILIC-FLR WITH AN ACQUITY UPLC BEH GLYCAN COLUMN

The following steps are suggested for preparing the labeled glycans for HILIC-FLR with an ACQUITY UPLC BEH Glycan column when using this kit with the Waters Total Glycan Application Solution.

1. Reconstitute the labeled glycans in 100 μL of Milli-Q water by gently aspirating, avoiding vortexing and sonication.

2. Add 150 μL of ACN to the reconstituted glycans immediately prior to and in preparation for analysis by HILIC-FLR.

3. Inject 4 μL of the labeled glycan containing 60% acetonitrile solution onto the column.

Note: Preparations of non-IgG glycoproteins or glycoprotein samples with concentrations other than 1 mg/mL may need to be reconstituted with different volumes to achieve desired results. Similarly, more concentrated samples can be obtained by reconstituting with up to 10 times lower volumes. The optimum injection volume for this application is ≤4 μL.

VI. REFERENCES


Step 4 Tips and Tricks

■ Extended periods of time between incubation and analysis may result in desialylation of labeled glycans and consequently should be avoided.
VII. ORDERING INFORMATION

Glycan Sample Preparation Kit and Standards

<table>
<thead>
<tr>
<th>Description</th>
<th>Part No.</th>
</tr>
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<tbody>
<tr>
<td>GlycoWorks Single Use Prep Kit</td>
<td>176003119</td>
</tr>
<tr>
<td>GlycoWorks HILIC 1 cc Cartridge (20/pk)</td>
<td>186007080</td>
</tr>
<tr>
<td>RapiGest SF 1 mg Vial</td>
<td>186001860</td>
</tr>
<tr>
<td>GlycoWorks Control Standard, 100 µg Vial</td>
<td>186007033</td>
</tr>
<tr>
<td>GlycoWorks Reagent Kit</td>
<td>186007034</td>
</tr>
<tr>
<td>GlycoWorks High-throughput Prep Kit</td>
<td>176003090</td>
</tr>
<tr>
<td>GlycoWorks HILIC µElution Plate, 96-well</td>
<td>186002780</td>
</tr>
<tr>
<td>RapiGest SF 1 mg Vial</td>
<td>186001860</td>
</tr>
<tr>
<td>GlycoWorks Control Standard, 100 µg Vial</td>
<td>186007033</td>
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<tr>
<td>GlycoWorks Reagent Kit</td>
<td>186007034</td>
</tr>
<tr>
<td>Manifold Waste Tray</td>
<td>600001282</td>
</tr>
<tr>
<td>Glycan Performance Test Standard</td>
<td>186006349</td>
</tr>
</tbody>
</table>

The Glycan Performance Test Standard is a 2-AB labeled human IgG-like standard that is QC verified to contain the components needed to benchmark and evaluate ACQUITY UPLC BEH Glycan, 1.7 µm Columns.

Dextran Calibration Ladder                        | 186006841 |

The 2-AB labeled, Dextran Calibration Ladder is used to calibrate the HILIC column from retention time to GU values. This calibration ladder provides good peak shape and reliable identification from 2 to 30 Glucose Units.

GlycoWorks HILIC 1cc Flangeless Cartridges Pack   | 186007239 |

This pack of 20 cartridges, good for 10 analyses, are offered for those customers who prefer to use the single use devices with the positive pressure manifold (PPM)

Glycan Analysis System Standards and Mobile Phases

<table>
<thead>
<tr>
<th>Description</th>
<th>Usage</th>
<th>Volume</th>
<th>Part No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alliance with Fluorescence Qualification Standards Test Kit</td>
<td></td>
<td></td>
<td>700002753</td>
</tr>
</tbody>
</table>

Kit contains seven 10 mL vials of varying concentrations of anthracene in 80:20 acetonitrile/water:

- (1) 0.5 pg/µL
- (1) 1.0 pg/µL
- (2) 5.0 pg/µL
- (1) 10.0 pg/µL
- (1) 2.5 ng/mL
- (1) 2.5 µg/mL

One blank 10 mL vial of 80:20 acetonitrile/H2O

Fluorescence Detector Performance Standard Solution | 700003694 |

5.0 pg/µL anthracene in 20:80 water/acetonitrile 1 mL

Fluorescence Detector Performance Standard Solution | WAT047685 |

0.10 mg/L anthracene in 70:30 acetonitrile/water 10 mL

Ammonium Formate Concentrate | 186007081 |

5000 mM Ammonium Formate in a Water w/ 3.8% Formic acid matrix. This allows the end user to dilute the concentrate 10 mL to 1L before using to reach a 50 mM concentration. 10 mL

Additional Consumables

<table>
<thead>
<tr>
<th>Description</th>
<th>Part No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlycoWorks HILIC 1cc Flangeless Cartridges Pack</td>
<td>186007239</td>
</tr>
<tr>
<td>Dextran Calibration Ladder</td>
<td>186006841</td>
</tr>
<tr>
<td>Glycan Performance Test Standard</td>
<td>186006349</td>
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<tr>
<td>RapiGest SF</td>
<td>186001861</td>
</tr>
<tr>
<td>Cap Mats for 1 mL Collection Plate</td>
<td>186002483</td>
</tr>
<tr>
<td>Waters Extraction Manifold, 20 position, without rack</td>
<td>– 186001831</td>
</tr>
<tr>
<td>SPE Vacuum Pump</td>
<td>115, 60 Hz</td>
</tr>
</tbody>
</table>