GLYCOWORKS *Rapi*Fluor-MS QUICK START PROTOCOL

Waters™

Streamlined Protocol for Disulfide Rich Glycoproteins – 96 sample (4 x 24 Format)

- Set heat blocks to at least 90 °C and 50 °C.
- Protocol is based on 1.5 mg/mL glycoprotein starting concentration.
- DTT is not provided.

STEP 1: Rapid Deglycosylation

1. Reconstitute 1 vial of the Intact mAb Mass Check Standard (1 mg/vial) in 670 µL 18.2 MΩ water to create a 1.5 mg/mL solution.
   Note: For glycoproteins with a formulation buffer containing nucleophiles or anionic reagents (e.g., His, Gly, Tris, PO_4^{3-}), if a Glycan C_{18} AX Column is applied for LC separation, it is highly recommended to desalt the sample with water prior to Step 1.
2. Prepare *Rapi*Gest™/DTT denaturing buffer *Rapi*Gest 3% (w/v); DTT 2 µg/µL (approx. 15 mM):
   a) Dissolve 10 mg *Rapi*Gest SF Surfactant in 200 µL of rapid buffer, vortex.
   b) Dissolve DTT in water to 5 µg/µL. Add 135 µL DTT solution to *Rapi*Gest SF solution, vortex.
   Note: Use of TCEP, as opposed to DTT, has been shown to interfere with the reversed-phase anion-exchange mixed mode (RP/AX) separation of RFMS-labeled glycans.
3. Dilute PNGase F enzyme (35 µL) with 255 µL water for a total of 290 µL.
4. Add 10 µL of 1.5 mg/mL glycoprotein into the reaction tube or well.
5. Add 10 µL of 3% (w/v) *Rapi*Gest SF/2 µg/µL DTT denaturing buffer to above tube, aspirate to mix.
6. Heat at least to 90 °C for 3 minutes.
7. Cool at room temperature for 3 minutes.
8. Add 10 µL Rapid PNGase F and aspirate to mix.
9. Incubate at 50 °C for 5 minutes.
10. Cool at room temperature for 3 minutes.

STEP 2: Rapid Labeling of Glycosylamines

1. Aliquot 15 µL of deglycosylated glycoprotein reaction mixture to a new tube and dilute with 15 µL of 18.2 MΩ water.
   Note: For maximum consistency in yield, 2x concentration of RapiFluor-MS reagent is recommended, as compared to the original GlycoWorks protocol (720005343EN). This protocol describes halving the volume of the deglycosylated mixture to achieve the 2x increase in RapiFluor-MS label concentration.
2. Add 280 µL of anhydrous DMF or DMSO directly to one vial of 23 mg of *Rapi*Fluor-MS™ Reagent. Mix to solubilize.
3. Add 10 µL of the *Rapi*Fluor-MS solution to the deglycosylation mixture and aspirate to mix.
4. Allow the labeling to proceed at room temperature for 5 minutes.
   Note: For a Glycan C_{18} AX separation sample, add 5 µL ammonium acetate solution (1 M, neutral pH) to sample mixture, and incubate at room temperature for 5 minutes.
5. Dilute the reaction with 360 µL of acetonitrile (ACN) and aspirate to mix.

STEP 3: HILIC Cleanup of Labeled Glycosylamines

1. Set up a GlycoWorks™ HILIC µElution Plate and add in shims or spacer and waste tray.
2. Condition wells by adding 200 µL of water per well.
3. Equilibrate wells by adding 200 µL 85% ACN.
4. Load ACN-diluted samples (~400 µL).
5. Wash wells with two (2) 600 µL volumes of 1% formic acid, 90% ACN.
6. Replace waste tray with sample collection tray loaded with 600 µL tubes.
7. Elute glycans with three (3) 30 µL volumes of SPE elution buffer into 600 µL tapered bottom inserts.
8. Dilute SPE eluate with 310 µL of the GlycoWorks SPE Diluent (DMF/ACN). Aspirate to mix.
   Note: For a Glycan C_{18} AX separation sample, either skip dilution Step 8 or dilute with 310 µL of water.
9. Cap the tubes with pre-slit cap mats.

► For the complete Care and Use Manual, visit waters.com and search 715004793EN.

► For more details on this method, download Application Notes 720005506 and 720007038.