Released N-Glycan Assay

Table 1. Reagents, Consumables, and Column

Release	Cat. No.
IgG from human serum	I4506
Trizma® HCl	T5941
Urea	U0631
Ammonium Bicarbonate	09830
PNGase F	P7367
30 k centrifugal filter unit (Millipore)	MRCF0R030
Labeling	
Sodium Cyanoborohydride	156159
Procainamide Hydrochloride	P9391
Dimethyl Sulfoxide	D8418
Acetic Acid	320099
Water	39253
Dextran Hydrolysate	31417
Cleanup	
Acetonitrile	34851
Discovery® Glycan SPE	55465-U
UPLC-FLR-MS	
BIOshell™ Glycan, 15 cm × 2.1 mm, 2.7 µm	50994-U
Ammonium Formate	17843
Formic Acid	94318

N-Glycan Release

This follows a Filter Aided Sample Prep (FASP) protocol typically used in proteomics sample prep. Reduction and alkylation of cysteines is not employed here but is warranted if intact protein analysis shows incomplete deglycosylation

1. Sample requirements: 100–500 µg purified glycoprotein at 1–5 mg/mL

a. System suitability: 200 µg IgG from human serum (Sigma I4506)

2. Prepare buffers and enzyme

a. 0.1 M Trizma HCl (Sigma T5941), pH 8.5
- Need 1 mL/sample

b. 8 M Urea (Sigma U0631), prepared in Trizma buffer
- Prepare on day of use: 1.33 mL buffer/g urea
- Need 1.2 mL/sample

c. 50 mM Ammonium Bicarbonate (Fluka 09830)
- Need 2 mL/sample

d. 0.5 UN/µL PNGase F (Sigma 7367)
- Solubilize in water; may be aliquoted and stored for 6 months or longer at -20 °C

3. Denature glycoprotein by buffer exchange

a. Add protein to 30 k centrifugal filter unit (Millipore MRCF0R030)

b. Add urea solution to bring total volume to 400 µL
- Mix by pipette without touching filter

c. Centrifuge 14,000 × g for 15 minutes

d. Add 200 µL urea solution; centrifuge as before

e. Repeat step d.

4. Buffer exchange to ammonium bicarbonate

a. Add 200 µL ammonium bicarbonate buffer

b. Centrifuge 14,000 × g for 15 minutes

c. Repeat steps a. and b. two more times

5. Enzymatic release of glycans

a. Prepare 0.08 UN PNGase F/ µL ammonium bicarbonate buffer

b. Add 75 µL resultant/sample (6 UN enzyme)

c. Transfer filter cups to new collection tubes, cap and vortex 1 min

d. Seal centrifuge device with parafilm

e. Incubate at 37 °C 16–20 h

6. Recover glycans

a. Add 40 µL ammonium bicarbonate buffer

b. Centrifuge 14,000 × g for 10 minutes

c. Add 100 µL ammonium bicarbonate buffer

d. Centrifuge 14,000 × g for 10 minutes

e. Repeat steps c. and d.

f. Transfer glycans in collection tube to 1.5 mL microcentrifuge tubes for labeling

7. Recover deglycosylated proteins (optional)

a. Wet the centrifuge device with 100–300 µL ammonium bicarbonate buffer

b. Vortex 1 min

c. Invert the device into a new collection tube

d. Centrifuge 3,000 × g for 5 minutes

e. Repeat steps a. to d.
- Returning the device to the original collection tube for vortexing
- Pool the recovered protein

f. SpeedVac dry, freeze, or analyze proteins as desired

8. Dry glycans by vacuum centrifugation

Procainamide Labeling

All preparation and labeling must be performed in a fume hood except for weighing reagents

1. Prepare the incubation block

a. Move to the fume hood and set the temperature to 65 °C

2. Prepare the reaction solution

a. Weigh 6–10 mg of sodium cyanoborohydride ($NaBH_3CN$, Aldrich 156159)

- Tare a microcentrifuge tube
- Transfer $NaBH_3CN$ to the tube in the fume hood; a pencil eraser-head volume should be sufficient
- Cap the tube and blow off any dust with N_2 gas in the fume hood
- Weigh the tube

b. Weigh procainamide hydrochloride (Sigma P9391)

- Must have at least 1.833 times more procainamide, by mass, than $NaBH_3CN$

c. Prepare 70% dimethyl sulfoxide (DMSO, Sigma D8418) 30% acetic acid (AcOH, Aldrich 320099) solution

- 350 µL DMSO + 150 µL AcOH in a microcentrifuge tube

d. Solubilize the procainamide with the 70% DMSO 30% AcOH solution

- 100 µL/11 mg procainamide
  - Divide procainamide mass by 11 and multiply by 100 for the required volume in µL
- Ensure solution is homogenous by pipette mixing and/or vortexing

e. Add solubilized procainamide to $NaBH_3CN$

- 111 µL/6 mg $NaBH_3CN$
  - $NaBH_3CN$ will not be fully solubilized
  - As exposure to strong acid releases cyanide gas, this step especially warrants working in the fume hood

f. Complete solubilization of $NaBH_3CN$ by adding water (DI water or Fluka 39253)

- 30 µL/6 mg $NaBH_3CN$
  - Cap and mix by vortex in the fume hood to fully solubilize $NaBH_3CN$

3. Label glycans

a. Add reaction solution

- 40 µL/0.5-10 µg dry glycans
- Control for labeling: 2 µg dextran hydrolysate (Fluka 31417)
- Fully solubilize invisible residue by repeatedly aspirating and dispensing solution along bottom ¼ sides of the tube

b. Place capped tube in incubator block and incubate at 65 °C for 3 hr

- Cover with foil to limit condensation on the lid and keep dark

SPE Cleanup

1. Prepare glycans for loading

a. Add 30 µL water to glycans in their 40 µL labeling solution; mix by pipette

b. Add 70 µL acetonitrile (Sigma-Aldrich 34851); mix by pipette

2. Prepare Discovery Glycan 50 mg cartridges (Supelco 55465-U)

a. Place Falcon tube under cartridge for waste collection

b. 1 mL water, with minimum pressure gradient by vacuum manifold

c. 1 mL 99% acetonitrile, with minimum pressure gradient by vacuum manifold

- Stop flow when meniscus completely enters top frit

3. Load samples

a. Place microcentrifuge tube under cartridge for breakthrough collection

b. Add full sample volume to bed

c. Pass sample through bed by gravity

d. When meniscus completely enters top frit, add 500 µL 99% acetonitrile

e. Pass volume through by gravity, collecting in same tube

- Stop flow when meniscus completely enters top frit

f. Place Falcon tube under cartridge for waste collection

g. Add breakthrough + 99% acetonitrile to bed

h. Pass volume through bed by gravity

- Stop flow when meniscus completely enters top frit

4. Wash

a. 1 mL 99% acetonitrile, with minimum pressure gradient by vacuum manifold

b. Repeat four more times

5. Elute

a. Place microcentrifuge tube under cartridge for purified glycan collection

b. Add 100 µL 20% acetonitrile to bed

c. Pass volume through bed by gravity

d. When meniscus completely enters top frit, add 100 µL 20% acetonitrile to bed

e. Repeat c.-d. two more times

f. After the 4th elution volume has completely entered the bed and the collection drip has stopped, apply medium vacuum manifold pressure to evacuate all liquid from SPE to the collection tube

6. Dry glycans by SpeedVac, 2–4 h

a. Labeled glycans can be stored at -20 °C for at least 6 months

UPLC-FLR-MS

1. Solubilize glycans in 100 µL 70% acetonitrile and transfer to autosampler vials

2. Acquisition on Acquity UPLC-Acquity FLR-Thermo LTQ MS

a. HPLC parameters

- 10 µL injection
- BIOshell™ Glycan, 15 cm x 2.1 mm, 2.7 µm (Supelco 50994-U)
- 75 min gradient, 75% B → 59% B, 0.3 mL/min
  - A: 50 mM ammonium formate (Fluka 17843) pH 4.4, pH adjust with formic acid (Fluka 94318)
  - B: Acetonitrile (as above, Sigma-Aldrich 34851)
- 9 min column equilibration

b. Fluorescence detection parameters

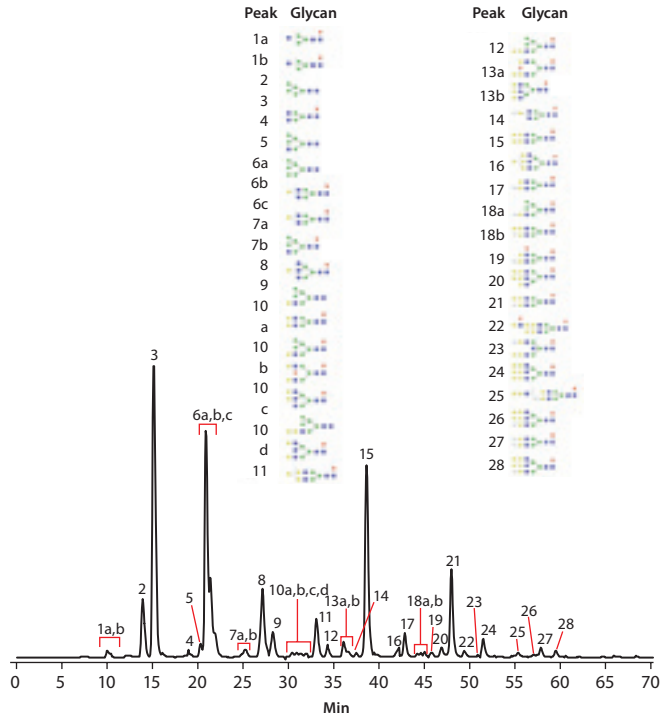
- 308 nm excitation 359 emission

c. MS parameters

- IonMax source, 200 °C, 4 kV
- One MS2 per MS, most abundant ion, any charge state
- 3 s data-dependent exclusion list
- 5 Da isolation width, 30 CID collision energy

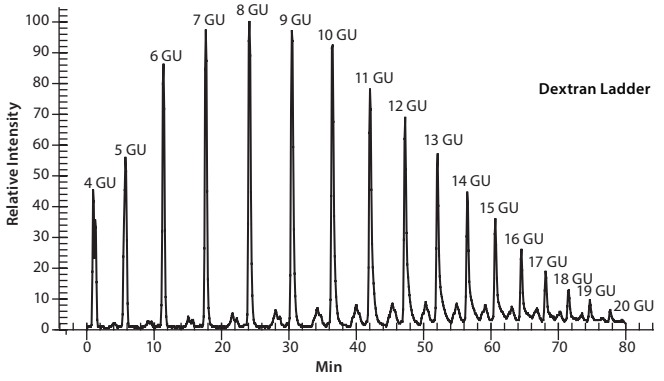
HPLC Analysis of Procainamide-Labeled Cetuximab Glycans on BIOshell™ Glycan Using HILIC-FLR

mobile phase: [A] 50 mM ammonium formate, pH 4.4 (50 mM ammonium hydroxide, adjusted to pH 4.4 with formic acid); [B] acetonitrile
gradient: 75% to 59% B in 75 min
flow rate: 0.3 mL/min
detector: FLR; 308 nm excitation, 359 emission
sample: Cetuximab
injection volume: 10 µL



HPLC Analysis of a Procainamide-Labeled Dextran Ladder on BIOshell™ Glycan Using HILIC-FLR

mobile phase: [A] 50 mM ammonium formate, pH 4.4 (50 mM ammonium hydroxide, adjusted to pH 4.4 with formic acid); [B] acetonitrile
gradient: 75 to 59% B in 75 min
flow rate: 0.3 mL/min
detector: FLR; 308 nm excitation, 359 emission
sample: Dextran Ladder (D3818) after Procainamide Labeling Protocol
injection volume 10 µL



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