INTRODUCTION

It has been widely recognized that glycosylations of proteins are responsible for their functions in many aspects. Therefore, the glycosylation of glycoconjugates are of interest for understanding their structure-function relationships. Analysis of N-linked glycans has been facilitated by means of N-glycosidases such as PNGase F, while that of O-linked glycans is left behind due to lack of practical releasing method. Each technique to prepare free O-glycans from glycoproteins appears to possess pros and cons in terms of safety, yield, processing time, higher rate of undesired side reaction (peeling), and so on.

Here we introduce a newly developed research tool for O-glycan analysis of glycoproteins; **EZGlyco® O-Glycan Prep Kit**. The kit operation will be accomplished within 5 to 6 hours prior to data acquisition, allowing one-day O-glycan analysis. The kit utilizes totally a new combination of chemical reagents and O-glycan enrichment bead, enabling a rapid and reliable recovery of O-linked sugars from glycoproteins. The method utilized in the kit generates a minimum amount of peeling products and efficiently liberates O-linked sugars. Sugars are recovered as a reducing form so that the kit granted 2-aminobezamide labeling of the recovered carbohydrates for efficient detection with fluorescent detector equipped with LC system such as HPLC, UHPLC, and LC-MS.

We have incorporated features such as simple operation, safety of reagents, rapid process of releasing O-linked glycans, a negligible amount of peeling, effective enrichment of released oligosaccharides, and easy fluorescent labeling of purified O-linked glycans. While conventional processes take 2-3 days to complete a series of treatments, the whole procedure of the kit could be carried out only in about 5 hours.

Avoiding complicated manipulations and disadvantages of currently available methods, the well-designed kit would finally convince researchers for the choice of O-glycan preparation kit.

In this note, we present a detailed investigation of the new kit including its robustness and flawless integration in the O-glycan analysis. We believe that the **EZGlyco® O-Glycan Prep Kit** is far more practical choice than any other conventional methods in all aspect.

OVERVIEW OF THE KIT

- **Easy handling**
  - Streamlined process

- **Minimal decomposition**
  - Suppression of "peeling" during O-glycan release

- **High Recovery**
  - Comparable results to hydrazinolysis and reductive β-elimination

- **Improved safety**
  - No hazardous reagent handing

Other features included:

- The **EZGlyco® O-Glycan Prep Kit** utilizes novel chemical reagents for stable liberation of O-linked oligosaccharides in the form of hemiacetal reducing sugars.
- Released, small-sized O-glycans are efficiently enriched with a propriety developed Glycan Capturing Bead.

Comparison of operation time

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Glycan Release</th>
<th>Purification &amp; Labeling</th>
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</thead>
<tbody>
<tr>
<td>EZGlyco® O-Glycan Prep Kit</td>
<td>Hydrazinolysis</td>
<td></td>
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</table>

Time [hours]
Workflow of EZGlyco® O-Glycan Prep Kit for HPLC and LC-MS-ready sample preparation in around 5 hours

1. Release of O-glycans
2. Enrich on Glycan Capturing Bead
3. Elution with 2AB-Labeling solution
4. Labeling at 50°C for 2.5 hr
5. Cleanup
6. Recovery
7. Analysis

**Required Equipment, Labware, and Reagents**
- Acetic acid (AcOH), reagent grade
- Acetonitrile (ACN), reagent grade
- Methyl alcohol (MeOH), reagent grade
- 1.5-mL microcentrifuge tubes
- Pipette and tips for 1000, 200, 20, 10, and 2 µL
- Heating block for use at 37°C and 50°C
- Vortex mixer
- Microcentrifuge (used at 500 and 3,000 x g)

**EXPERIMENTAL CONDITIONS**
- All samples were subjected to O-glycan sample preparation using EZGlyco® O-Glycan Prep Kit or other commercially available kits.
- Recovered glycans were analyzed with a HILIC mode UHPLC as follows;
  - LC system: Waters ACQUITY UPLC system equipped with UV and Fluorescence detectors
  - Column: ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 mm X 150 mm, Waters
  - Column Temp: 40°C
  - Flow rate: 0.2 mL/min
  - Fluorescence detection: Ex 330 nm/Em 420 nm
  - Injection volume: 1 µL out of 50 µL recovery
  - Gradient:
    - Mobile Phase A: aq. 40% acetonitrile containing 0.1% formic acid
    - Mobile Phase B: aq. 90% acetonitrile containing 0.1% formic acid

<table>
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<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
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<tbody>
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<td>100</td>
</tr>
<tr>
<td>50.0</td>
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Table 1. Reproducibility data of major O-glycans in bovine fetuin prepared by EZGlyco® O-Glycan Prep Kit.

<table>
<thead>
<tr>
<th>Peak #</th>
<th>entry1</th>
<th>entry2</th>
<th>entry3</th>
<th>CV</th>
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<tr>
<td>1</td>
<td>6.3%</td>
<td>6.3%</td>
<td>6.3%</td>
<td>0.2%</td>
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<tr>
<td>2</td>
<td>73.1%</td>
<td>73.1%</td>
<td>73.0%</td>
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<tr>
<td>3</td>
<td>17.6%</td>
<td>17.6%</td>
<td>17.6%</td>
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<tr>
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<table>
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<tr>
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<tr>
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<tr>
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<td>3.1%</td>
<td>3.1%</td>
<td>3.2%</td>
<td>2.4%</td>
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</table>

- 20 µg of bovine fetuin was subjected to O-glycan preparation using EZGlyco® O-Glycan Prep Kit (N=3 x 3 days).
- Providing data with low CV and high reproducibility
  - glycan profile: less than 5% of CV
  - deviation in total recovery: less than 10%

Figure 1. Linear recovery of 2-AB-labeled O-glycan in bovine fetuin.

- A varying amount of bovine fetuin (0.5 - 200 µg) was subjected to O-glycan preparation using EZGlyco® O-Glycan Prep Kit (N=1).
- 1 µL of the recovered solution containing O-glycans were analyzed using a HILIC mode UHPLC.
- Recovery (intensity: sum of the area of peaks 1-4 in table 1) plotted for each input.
- EZGlyco® O-Glycan Prep Kit gives a good linear recovery over a wide-range of sample input.
As shown in the chromatograms, 1 µL of the recovered solution containing O-glycans were analyzed using a HILIC mode UHPLC.

Product A (Alkaline β-elimination.)

Product B (Alkaline β-elimination.)

Recovered O-glycans were analyzed using a HILIC mode UHPLC.

As shown in the chromatograms, O-glycan total recovery and the suppression of “peeling” appear to be the best with EZGlyco® O-Glycan Prep Kit.

Figure 2. 2-AB-labeled bovine fetuin O-glycan analysis prepared with different sample preparation kits.

- 200 µg of bovine fetuin was subjected to O-glycan preparation using:
  a) EZGlyco® O-Glycan Prep Kit
  b) Product A (Alkaline β-elimination.)
  c) Product B (Alkaline β-elimination.)

Figure 3. LC chromatograms of 2-AB-labeled O-glycans derived from various glycoproteins prepared using EZGlyco® O-Glycan Prep Kit.

- Each sample was dissolved in 10 µL of pure water and subjected to O-glycan preparation using EZGlyco® O-Glycan Prep Kit.
- 1 µL of the recovered solution containing O-glycans were analyzed using a HILIC mode UHPLC.
- As shown in the chromatograms, O-glycans were detected in all samples with a low peeling ratio (less than 10%).
Figure 4. LC chromatograms of 2-AB-labeled O-glycans derived from human serum prepared using EZGlyco® O-Glycan Prep Kit.

• Following 20 µL of human serum was dried using centrifugal evaporator, the dried human serum was dissolved in 10 µL of pure water and subjected to O-glycan preparation using EZGlyco® O-Glycan Prep Kit.
• 1 µL of the recovered solution containing O-glycans were analyzed using a HILIC mode UHPLC.
• Even from a small amount of human serum, O-glycans were recovered with simple operation using EZGlyco® O-Glycan Prep Kit.

CONCLUSIONS

• A streamlined concept of EZGlyco® O-Glycan Prep Kit has been proven with a reproducible releasing and enrichment of O-glycan.
• By using the novel Kit, highly efficient O-glycan liberation has been achieved with a negligible amount of peeling from various samples including human serum.
• We believe that the novel kit has a great potential to be applied in a wide range of O-glycan studies that requires a precise preparation of oligosaccharides from target glycoproteins. Thus, we expect that the kit would be widely employed by researchers in the field of glycobiology, O-glycan-related biomarker discovery, quality control of biopharmaceuticals, and development of new diagnostics in the near future.

ORDERING INFORMATION

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<th>Catalog No.</th>
<th>Product Name</th>
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<tr>
<td>BS-41601Z</td>
<td>EZGlyco® O-Glycan Prep Kit</td>
<td>For 10 tests</td>
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