**O-glycosylation analysis**

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**Why is O-glycosylation analysis important?**

Protein O-glycosylation is one of the most common types of post-translational modifications. It refers to the covalent attachment of a carbohydrate moiety to the polypeptide backbone. In O-link glycosylation the glycan is attached to the side chain of a Ser or Thr residue.

O-glycosylation is known to have a critical impact on protein secretion and protective immunity against cancer, and maintenance of normal development and physiology. Moreover, a substantial portion of the protein-based pharmaceuticals on the market are glycoproteins where O-glycosylation was found to critically modulate the physiochemical properties of proteins, have a functional impact on their therapeutic potentials, and affect the safety profiles of such drugs. O-glycosylation is prevalent in many classes of therapeutic proteins including Erythropoietin (EPO), Follicle stimulating hormone (FSH), Etanercept, Granulocyte-colony stimulating factor (G-CSF) providing evidence of its critical involvement in drug performance and diseases.

In-depth understanding of the O-glycosylation status of these drug substances will elucidate the structure-function relationship of the O-linked sugars, which may lead to the identification of functionally favorable O-glycan structures to improve drug efficacy and safety profile.

**Challenges**

O-linked glycans can vary vastly in size, ranging from a single N-acetylgalactosamine (GalNAc) monosaccharide at the reducing end terminus to large oligosaccharides exhibiting complex glycan motifs resulting from further modifications by the addition of other sugar types including galactose (Gal), N-acetylgalactosamine (GlcNAc), GalNAc, fucose (Fuc), and sialic acids (Neu5Ac and/or Neu5Gc). This substantial heterogeneity can generate eight main core structures, each potentially subject to further elongation and substitution (Figure 1).

![Figure 1. Eight main core O-glycan structures.](image)

The analysis of O-linked glycosylation remains a challenge due to the lack of equivalent enzymes and the inherent structural heterogeneity of O-glycans. The specificities of the reported O-glycanase enzymes are predominantly restricted to T antigen (Galβ1,3-GalNAcα-Ser/Thr). Despite isolated reports describing O-glycanases of broader specificities, a universal O-glycanase that is capable of liberating O-glycans with more sophisticated branching and elongation is still unknown. Therefore, the release of O-linked oligosaccharides is commonly achieved by chemical approaches where a broad range of samples, including but not limited to glycoprotein therapeutics, mammalian cell lines and bacterial cell components, biological fluids and tissues, can be processed.
Ludger offers a range of products for analysis of samples containing O-glycans. This includes:

I. **Enzymes (O-glycosidase)** for release of unsubstituted Galβ1,3-GalNAcα disaccharides attached to the Ser or Thr residues of glycoproteins or glycopeptides; (E-G001)

II. **Enzymes (Enzymatic CarboRelease Kit)** for protein deglycosylation, for the removal of all N-linked oligosaccharides and many O-linked sugars; (KE-DG01)

III. **Enzymes (Enzymatic DeGlycoMx Kit)** containing a premixed cocktail of the enzymes required to remove all N-linked oligosaccharides and most O-linked sugars; (KE-DGMX)

IV. **Kits for complete removal of O-glycans from glycoproteins or glycopeptides:**
   a. Ludger Liberate Hydrazinolysis N- and O-glycan release kit (LL-HYDRAZ-A2)
   b. Ludger Liberate Orela O-glycan release kit (LL-ORELA-A2)

**I. O-Glycosidase**
Cleaves only unsubstituted Gal-β(1-3)GalNAc-α disaccharides attached to the serine or threonine residues of glycoproteins or glycopeptides. Released glycans have free reducing termini to allow for fluorescent tagging by reductive amination and analysis by HILIC-(U)HPLC-ESI-MS.

**II. Enzymatic CarboRelease Kit**
All Ser/Thr-linked (O-linked) Gal-(β1-3)-GalNAc-(α1) and all sialic acid substituted Gal-(β1-3)-GalNAc-(α1) are removed using the combination of Neuraminidase and O-Glycosidase. The addition of β-Galactosidase and β-N-acetylglucosaminidase assists in the deglycosylation of larger O-link structures. Released glycans have free reducing termini to allow for fluorescent tagging by reductive amination and analysis by HILIC-(U)HPLC-ESI-MS.

**III. Enzymatic DeGlycoMx Kit**
All Ser/Thr-linked (O-linked) Gal-(β1-3)-GalNAc-(α1) and all sialic acid substituted Gal-(β1-3)-GalNAc-(α1) are removed using the combination of Neuraminidase and O-Glycosidase. The addition of β-Galactosidase and β-N-acetylglucosaminidase assists in the deglycosylation of larger O-link structures. Released glycans have free reducing termini to allow for fluorescent tagging by reductive amination and analysis by HILIC-(U)HPLC-ESI-MS.

**IV. Kits for complete removal of O-glycans from glycoproteins or glycopeptides:**
   a. **Hydrazinolysis**
The mechanism by which hydrazine removes O-glycans is not fully understood, it is accepted that it proceeds via an initial β-elimination reaction followed by reaction with hydrazine to form hydrazine derivatives. The glycoprotein or glycopeptide samples are prepared by removing salts, detergent and dyes that could interfere with the release by dialysis against water or 0.1 % TFA. Under the release conditions, the N-acetyl groups of the amino sugars are also removed; therefore, it is necessary to perform a re-N-acetylation step with acetic anhydride. Released O-glycans can be labelled by reductive amination using LudgerTag™ labels such as 2-AB (2-aminobenzamide), 2-AA (2-aminobenzoic acid) or procainamide and analyzed by hydrophilic interaction liquid chromatography (HILIC)-(U)HPLC-ESI-MS with fluorescence detection.

![Figure 2. Hydrazinolysis Scheme for O-glycan release.](image)

   b. **Orela**
The Orela reagent reacts at the link between the glycan and the peptide backbone to liberate glycans as glycosylamines. The acid-labile glycosylamine derivative is then hydrolysed to produce the free glycan. As in the hydrazinolysis protocol, the glycoprotein or glycopeptide samples are prepared by removing salts, detergent and dyes that could interfere with the release by dialysis against water or 0.1 % TFA. Released glycans have free reducing termini to allow for fluorescent tagging by reductive amination and analysis by HILIC-(U)HPLC-ESI-MS.
Results

Figure 4 shows HILIC-UPLC profiles for hydrazine released O-glycans from fetuin standard. The data is shown in Table 1.

The fetuin sample contains the following O-glycans at the expected GU values: core 1, Galα1-3GalNAc at GU 1.48; mono-sialylated and di-sialylated core 1, Neu5Acα2-3Galβ1-3GalNAc at GU 2.32; Neu5Acα2-6(Galβ1-3)GalNAc at GU 2.97; di-sialylated core 1 O-glycan Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAc at GU 3.55; along with some peeled product*, Neu5Acα2-3Galβ1-3Gal at GU 1.69. Fetuin also contains a small amount of the di-sialylated core 2 structure, Neu5Acα2-3Galβ1-3(Neu5Acα2-3Galβ1-4GlcNAcβ1-6)GalNAc at GU 4.72.

*peeling = site reaction that may be defined as stepwise degradation of the polysaccharide starting at the reducing end and removing one sugar residue at a time.

What information is obtained by using Ludger kits and Standards?

The released O-glycans can be fluorescently labelled and analysed by HILIC-HPLC or LC-ESI-MS/MS.

The HILIC-HPLC data provides:
I. Glucose unit (GU) values for profile comparison between batches where glycan separation is roughly based on glycan size.
II. Relative quantitation of glycan peaks generated (relative % areas) from fluorescence LC data.
III. Preliminary structural assignments by comparison against Ludger standards.

The ESI-MS and MS/MS data and provides:
IV. m/z values
V. mass composition data matched to LC-separated glycan peaks
VI. possible glycan structures from matching m/z values from MS/MS with database of glycans

Figure 3. O-glycosylation analysis workflow.

Figure 4. HILIC-UPLC profiles for hydrazine released fetuin O-glycans.
### Product/Ordering Information

**Glycan Release**

- **E-G001**
  - O-glycosidase for removal of unsubstituted Galβ1,3-GalNAcα disaccharides attached to the Ser or Thr residues

- **LL-HYDRAZ-A2**
  - Ludger Liberate Hydrazinolysis N- and O-glycan release and cleanup kit

- **LL-ORELA-A2**
  - Ludger Liberate Orela O-glycan release and cleanup kit

**Glycan Labelling**

- **LT-KAB-A2**
  - LudgerTag 2-AB (2-aminobenzamide) Glycan Labeling Kit

- **LT-KAB-VP24**
  - LudgerTag 2-AB (2-aminobenzamide) Glycan Labeling Kit containing 2-picoline borane

- **LT-KAB-VP96**
  - LudgerTag 2-AB (2-aminobenzamide) Glycan High Throughput Labeling Kit containing 2-picoline borane

- **LT-KAA-A2**
  - LudgerTag 2-AA (2-aminobenzoic acid) Glycan Labeling Kit

- **LT-KAA-VP24**
  - LudgerTag 2-AA (2-aminobenzoic acid) Glycan Labeling Kit containing 2-picoline borane

- **LT-KPROC-VP24**
  - LudgerTag Procainamide Glycan Labeling Kit with 2-picoline borane

- **LT-KPROC-24**
  - LudgerTag Procainamide Glycan Labeling Kit with sodium cyanoborohydride

- **LT-KPROC-96**
  - LudgerTag Procainamide Glycan Labeling Kit with sodium cyanoborohydride

**Glycan Clean Up**

- **LC-S-A6**
  - LudgerClean S Glycan Cleanup Cartridges

**Process Controls**

- **GCP-FET-250U**
  - Fetuin Glycoprotein Standard

**2-AB Labelled O-glycan Standards**

- **CAB-C1-01**
  - 2AB labelled core 1 O-glycan, 100pmol

- **CAB-C1S(3)1-01**
  - 2AB labelled sialylated core 1 O-glycan, 100pmol

- **CAB-C1S(3)1-02**
  - 2AB labelled sialylated core 1 O-glycan, 50pmol

- **CAB-C1S(3,6)2-01**
  - 2AB labelled di-sialylated core 1 O-glycan, 100pmol

- **CAB-C2S(3,3)2-01**
  - 2AB labelled di-sialylated core 2 O-glycan, 100pmol

**Fetuin O-glycan library**

- **CLIBO-FETUIN-01**
  - A library of O-glycans released from 30μg fetuin glycoprotein

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**Table 1.** HILIC-UPLC data for hydrazine released and procainamide labelled O-glycans from Fetuin replicates (A, B, C).

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LL-ORELA-A2 - Ludger Liberate Orela O-glycan release and cleanup kit