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ProductInformation

GlycoProfile™ I
Enzymatic In-Gel N-Deglycosylation Kit

Product Code **PP0200**
Storage Temperature 2–8 °C

TECHNICAL BULLETIN

Product Description

Recent developments in analytical methods for protein characterization have led to the emergence and development of the field of proteomics. Two-dimensional (2D) gel electrophoresis is a common technique used in proteomics for the separation of proteins prior to obtaining structural information by mass spectrometry (MS). One of the distinguishing features of the proteome in eurkaryotic cells is that most proteins are subject to post-translational modification, of which glycosylation is the most common form. It is estimated that more than half of all proteins are glycoproteins.

Removal of the carbohydrate groups on a glycoprotein is recommended prior to protein identification. In general, glycoproteins and glycopeptides do not ionize completely during MS analysis, leading to inadequate spectral data. Glycopeptides have lower detection sensitivity due to microheterogeneity of the attached glycans, resulting in signal suppression in MS analysis. Thus, removing the glycans increases protein sequence coverage for protein identification. Furthermore, proteolytic cleavage is a prerequisite for the elution of peptide fragments out of gels and identification by MS. However, proteolytic digestion of the native glycoprotein is often incomplete due to steric hindrance from the bulky oligosaccharides present on the molecule. Information gained from MS analysis of deglycosylated tryptic peptides can be used to locate glycosylation sites on the glycoprotein.

The GlycoProfile™ I Kit is optimized to provide a convenient and reproducible method to N-deglycosylate and digest protein samples from 1D or 2D polyacrylamide gel pieces for subsequent MS or HPLC analysis. The procedure is suitable for Coomassie® Brilliant Blue and colloidal Coomassie stained gels. Silver stained gels may also be used if properly destained.

The kit includes PNGase F and trypsin enzymes necessary for N-linked deglycosylation and tryptic digestion, respectively. The samples can then be desalted and concentrated for analysis by MALDI-TOF MS or Electrospray MS with subsequent database searching.

Proteomics Grade PNGase F is extensively purified and lyophilized from potassium phosphate buffer to produce a stable product. The material is free from glycerol and other stabilizers, and contains very low levels of buffer salts. This enzyme gives excellent performance when used for in-gel N-linked deglycosylation of glycoproteins and glycopeptides. PNGase F releases asparagine-linked oligosaccharides from glycoproteins and glycopeptides by hydrolyzing the amide of the asparagine (Asn) side chain. A tripeptide with the oligosaccharide-linked asparagine as the central residue is the minimum substrate for PNGase F. The oligosaccharides can be high mannose, hybrid, or complex type. However, N-glycans with fucose linked $\alpha(1\rightarrow3)$ to the asparagine-bound N-acetylglucosamine are resistant to the action of PNGase F.[1-8] After removal of glycans, the protein can be digested with trypsin.

Proteomics Grade Trypsin is chemically modified through reductive methylation of the ε-amino groups of lysine to reduce autolysis and minimize autolytic fragments. In addition, it has been TPCK treated to remove residual chymotrypsin activity and then further purified by affinity chomatography, yielding a highly purified trypsin suitable for proteomics work. Trypsin is a pancreatic serine protease, which hydrolyzes peptide bonds specifically at the carboxyl side of arginine and lysine residues. The rate of hydrolysis is slower if an acidic residue is on either side of the cleavage site and cleavage may not occur if a proline residue is on the carboxyl side. [9-13] Tryptic digestion of the protein of interest results in a highly specific cleavage and a limited number of peptide fragments.

The GlycoProfile™ I Kit provides adequate reagents to deglycosylate and digest a minimum of 10 samples according to the procedure described here using yeast invertase as a model glycoprotein.

Reagents
The GlycoProfile™ I Kit is composed of 8 reagents:

- Destaining Solution – Solution to remove dye bound to the protein of interest. One bottle of powder that reconstitutes to a final volume of 10 ml (Product Code D 0316).

- Proteomics Grade PNGase F – The enzyme is supplied in a vial containing 50 units (50 IUB milliunits) of PNGase F (Product Code P 7367).

- Proteomics Grade Trypsin – The enzyme is supplied in a vial containing 20 µg of trypsin (Product Code T 6567).

- Trypsin Solubilization Reagent – Reagent for reconstituting and stabilizing the enzyme. One vial containing 1 ml of reagent (Product Code T 2073).

- Trypsin Reaction Buffer – Buffer to provide optimal pH for the trypsin digestion reaction. One bottle of powder that reconstitutes to a final volume of 11 ml (Product Code R 3527).

- Invertase – A control glycoprotein standard, supplied in a vial containing 0.5 mg protein (Product Code I 0408).

- Peptide Extraction Solution – Solution for extracting peptides from the gel piece. One bottle containing 10 ml of solution (Product Code P 0743).

- Biotech Grade Acetonitrile – Solvent for preparation of other reagents. One 50 ml bottle (Product Code 49,444-5).

Equipment and Reagents Required But Not Provided
- Ultrapure water (18 megaohm or equivalent)
- 50 mM sodium phosphate buffer, pH 7.5
- Flat nosed tweezers
- Siliconized Eppendorf® tubes (Product Code T 4691 or equivalent)
- 37 °C heating block or heating bath
- Scalpel (S 2771 and S 3021) or razor blade
- Bench-top centrifuge (microcentrifuge)
- Centrifugal concentrator (SpeedVac®)
- Sonic bath
- ZipTip® pipette tips

Precautions and Disclaimer
This product is for laboratory use only, not for drug, household, or other uses. Consult the MSDS for information regarding hazards and safe handling practices. It is recommended to read the entire technical bulletin prior to starting the procedure.

Preparation Instructions
It is recommended to use ultrapure water (18 megaohm or equivalent) when reconstituting the following:

- Destaining Solution - Add 6 ml of water and 4 ml of Biotech Grade Acetonitrile to the bottle. After reconstitution, the bottle contains a solution of 200 mM ammonium bicarbonate and 40% acetonitrile.

- PNGase F Solution – Spin vial briefly to collect solid at bottom. Add 100 µl of water, agitate gently, spin, and store on ice. The concentration is 500 units/ml.

- Trypsin Solubilization Reagent – Reagent contains 1 mM HCl and is ready to use.

- Trypsin Reaction Buffer – Add 10 ml of water and 1 ml of Biotech Grade Acetonitrile to the bottle. After reconstitution, the bottle contains a solution of 40 mM ammonium bicarbonate and 9% acetonitrile.

- Trypsin Solution – Add 100 µl of the Trypsin Solubilization Reagent to the vial of trypsin. Mix the vial briefly to ensure the trypsin is dissolved. Add 900 µl of the Trypsin Reaction Buffer to the vial and mix. The final concentration of trypsin is 20 µg/ml. Note: Alternately, the vial of trypsin may be reconstituted with 100 µl of the Trypsin Solubilization Reagent (1 mM HCl) and stored at 2–8 °C for 2 weeks or at –20 °C for up to 4 weeks. When ready to prepare the working Trypsin Solution, an aliquot of the acidic trypsin solution may be combined with the correct amount of Trypsin Reaction Buffer (1 part of acidic Trypsin Solution to 9 parts of Trypsin Reaction Buffer).

- Invertase Control Standard – Spin vial briefly to collect solid at the bottom. Add 100 µl of 50 mM sodium phosphate buffer, pH 7.5, agitate, and spin briefly to obtain a 5 mg/ml solution. Dilute appropriately before use (see Procedure).

- Peptide Extraction Solution – Solution contains 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile and is ready to use.

Storage/Stability

This kit is stable for at least 1 year when stored at 2–8 °C. The Destaining Solution and the Trypsin Reaction Buffer are stable for up to one month after reconstitution when stored at 2–8 °C. The PNGase F Solution should be used within two weeks after reconstitution when stored at 2–8 °C. The ammonium bicarbonate Trypsin Solution may be stored either at 2–8 °C for 2 weeks or as frozen aliquots for up to 4 weeks. The reconstituted PNGase F and Trypsin Solutions are stable for at least 3 freeze-thaw cycles. The reconstituted Invertase Control Standard can be stored frozen for up to one year.

Procedure

The following procedure does not contain reduction or alkylation steps of the protein sample. For most in-gel digestion procedures, reduction and alkylation of the protein sample is suggested prior to running on the gel.[14] This sample treatment results in less streaking and increased resolution of the gel. The ProteoPrep™ Reduction and Alkylation Kit (Product Code PROT-RA) contains reagents and procedures for reduction and alkylation of the protein sample during solubilization, equilibration for 2D electrophoresis, or in-gel tryptic digestion.

The following procedure is performed following Coomassie staining of a 1D or 2D polyacrylamide gel containing a reduced and alkylated protein sample. For silver stained gels, a gel-destaining step different than that used for dye stained gels is required. The ProteoSilver™ Plus Silver Staining Kit (Product Code PROT-SIL2) is recommended for silver destaining prior to tryptic digestion and MS analysis. It contains destaining solutions for silver stained gels and a procedure for preparing gel slices for tryptic digestion. As a positive control, approximately 2 to 5 µg of the reduced and alkylated invertase should be loaded per lane of 1D gel.

1. Carefully cut the band of interest from a 1D gel or the protein spot from a 2D gel, using a clean scalpel or razor blade, taking care to include only stained gel. Remove the gel piece using clean flat nosed tweezers.
   Note: The gel piece may be cut into equal sections of 1 to 1.5 mm size and the sections may be used in place of the intact piece.

2. Place the gel piece(s) in a siliconized Eppendorf tube or equivalent. A siliconized tube reduces binding of the peptides to the tube surface. If unsure of chemicals leaching from the tube, which could interfere or suppress the MALDI-MS signal, prewash the tube with 100 µl of Peptide Extraction Solution and then allow it to dry before use.

3. Cover the gel piece with 200 µl of Destaining Solution and incubate at 37 °C for 30 minutes. Remove and discard the solution from the tube.

4. Repeat step 3 one or two more times to destain the gel.

5. Dry the gel piece in a SpeedVac® for approximately 30 minutes or longer, if required.

6. Add 10 µl (5 units) of the prepared PNGase F Solution to the sample tube and centrifuge briefly.
   Note: The amount of enzyme added can be varied depending on the nature of the glycoprotein and the incubation time.

7. Allow the gel piece to incubate for 30 minutes at 37 °C.

8. Add 20 µl of water to the sample such that the gel piece is covered by the liquid. Continue the incubation for an additional 16 to 18 hours.
   Note: For overnight incubation, ensure that the gel piece remains covered with liquid at all times.

9. Centrifuge briefly and remove the PNGase F Solution from the tube. Retain this solution for glycan analysis, if desired.

10. Add 200 µl of water to the tube, mix at room temperature in a sonic bath for 30 minutes. Centrifuge briefly and remove the supernatant.
    Note: Insufficient washings may lead to PNGase F related peptides being detected in the MS data.

11. Repeat step 10 three more times.
    Note: The supernatant from step 9 and washings from steps 10 and 11 can be combined and retained for glycan analysis, if desired.

12. Dry the gel piece in a SpeedVac® for approximately 30 minutes or longer, if required.

13. Add 20 µl (0.4 µg) of the prepared Trypsin Solution to the gel piece. Allow the gel piece to swell for 30 minutes at 37 °C.

14. Add 50 µl of the Trypsin Reaction Buffer to the gel sample. Centrifuge briefly.

15. Confirm that the gel piece is at the bottom of the tube and covered with liquid.

16. Incubate overnight at 37 °C.
    Note: A shorter digestion time may be sufficient, but may yield slightly lower sequence coverage.

17. After the incubation, remove the liquid from the gel piece and transfer the liquid to a new tube. This solution contains the extracted tryptic peptides. If MALDI analysis is to be performed at this step, acidification with TFA or formic acid prior to matrix addition may be needed.

18. Add 50 µl of the Peptide Extraction Solution to the gel piece and incubate for 30 minutes at 37 °C.
    Note: In some cases, the extraction step may not be required and the sample solution from step 17 can then be analyzed directly.

19. Remove Peptide Extraction Solution and combine with the liquid from step 17. Reduce volume to 10 µl by evaporation in a SpeedVac®.
20. The combined sample solution from step 19 is ready for MALDI-MS analysis.

Note: If digesting low levels of protein, the peptide mixture may need to be concentrated with a ZipTip before spotting on the MALDI target.

Results

Two N-deglycosylated peptide fragments (*m/z* 1318 and 1625) are observed in the MALDI MS of invertase (Figure 1). These fragments are not present in the MS of the native, glycosylated invertase.

Upon deglycosylation the asparagine residue at the site of glycan attachment is converted to an aspartic acid residue and hence the *m/z* of the corresponding peptide fragment is increased by one unit.

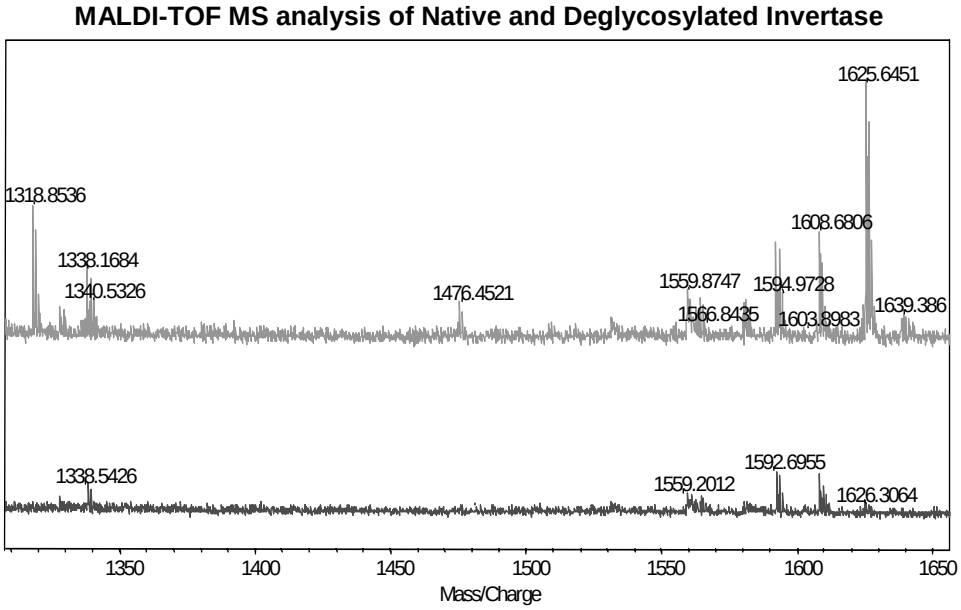

Figure 1: MALDI-TOF mass spectra of the tryptic peptides of native (lower spectrum) and deglycosylated (upper spectrum) Invertase from a 1D gel (Axima CFR spectrometer in a positive reflectron mode using α-cyano-4-hydroxycinnamic acid as matrix). The test gel piece was deglycosylated in-gel with PNGase F and both the control and test gel pieces were further in-gel digested with trypsin, as described in the procedure, before MS analysis.

Related Products	Product Code
ProteoPrep™ Kits	
Total Extraction Sample	PROT-TOT
Membrane Protein Extraction	PROT-MEM
Universal Extraction	PROT-TWO
ProteoPrep™ Reduction and Alkylation Kit	PROT-RA
ProteoSilver™ Plus Silver Staining Kit	PROT-SIL2
ProteoMass™ MALDI-MS Calibration Kits	
Protein and Peptide	MS-CAL1
Peptide	MS-CAL2
Protein	MS-CAL3
Trypsin Profile IGD Kit	PP0100
EZBlue™ Gel Staining Reagent	G 1041
PNGase F, Proteomics Grade	P 7367
Invertase	I 0408
Trypsin, Proteomics Grade	T 6567
α-cyano-4-Hydroxycinnamic acid	C 8982

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