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ProductInformation

GlycoProfile® IV, Chemical Deglycosylation Kit

Product Code **PP0510**
Storage Temperature 2–8 °C

TECHNICAL BULLETIN

Product Description

Glycosylation is one of the most common post-translational modifications of proteins that can add complexity to the proteome of many cell types. Glycoproteins are involved in a wide range of biological functions such as receptor binding, cell signaling, immune recognition, inflammation, and pathogenicity. Mammalian glycoproteins contain one or more of the three major classes of oligosaccharides (glycans); N-linked, O-linked, and glycosaminoglycans (GAGs). N-Linked glycans are linked to the protein backbone via an amide bond to asparagine (Asn) residues in an Asn-Xaa-Ser/Thr motif, where Xaa can be any amino acid, except proline. O-Linked glycans are attached to the hydroxyl group of serine or threonine. GAGs are linked to serine in the protein backbone of proteoglycans. However, variations in the glycan structures and different degrees of occupancy of available glycosylation sites often result in heterogeneity in the mass and charge of glycoproteins.

The complexity of a protein population as a result of glycosylation can be reduced by enzymatic or chemical deglycosylation. Enzymatic methods are relatively mild and allow removal of a selected class of glycans without protein or glycan degradation, although complete removal of glycans often requires denaturation of the protein.

Overall, chemical deglycosylation methods are generally less selective. Depending upon the method used, either the protein or the glycans can be recovered intact without significant degradation. A number of chemical methods have been developed with different efficiencies. Hydrazine is used to release intact and unreduced forms of N- and O-linked glycans. The glycans can be released quantitatively and selectively by using the appropriate hydrolysis temperature and time. This method, however, results in complete destruction of the protein component.

β-Elimination under strongly alkaline conditions is another method for removal of N- and O-linked glycans and, like hydrazinolysis, results in total destruction of the protein backbone.

Unlike hydrazinolysis and β-elimination, treatment with anhydrous trifluoromethanesulfonic acid (TFMS) is very effective in preserving the core protein. TFMS-mediated deglycosylation efficiently removes a variety of oligosaccharide motifs including N-linked (except the innermost Asn-linked GlcNAc or GalNAc), O-linked glycans, GAGs linked to the proteoglycan core, and collagen saccharides (Hyp linkage). TFMS deglycosylation has been found to be effective on plant, bacterial, and fungal glycoproteins that are often complex and difficult to digest enzymatically. The TFMS hydrolysis reaction results in minimal protein degradation; however, the released glycans are destroyed. It is reported that the biological, immunological, and receptor-binding activities of certain glycoproteins are retained upon deglycosylation by this method although this may not be true for all glycoproteins.

The GlycoProfile™ IV, Chemical Deglycosylation Kit has been optimized to accommodate a broad range of glycoprotein samples. Simple or low molecular weight glycoproteins can be deglycosylated in as little as 30 minutes without the use of a scavenger. For high molecular weight or complex, non-mammalian glycoproteins, an optional scavenger species (anhydrous anisole) is provided to ensure the highest protein yield possible. The use of anisole as a scavenger along with an extended incubation period may result in higher protein recovery and more protection for amide bonds and/or side chains that may be present. Either protocol provides a convenient and reproducible method to remove glycans from glycoproteins by reaction with TFMS. The deglycosylated protein can then be recovered using suitable downstream processing, such as dialysis or gel filtration.

Components
The kit contains sufficient reagents and a glycoprotein standard for a minimum of 10 reactions when the sample size is between one to two mg of a typical glycoprotein.

Trifluoromethanesulfonic acid, anhydrous (Product Code 34,781-7)	5 X 1.0 g
Ribonuclease B Glycoprotein Standard (Product Code R 1153)	3 X 1.0 mg
Pyridine Solution, 60% (Product Code P 5496)	1 X 10 ml
Bromophenol Blue Solution, 0.2% (Product Code B 1560)	1 X 0.5 ml
Anisole, anhydrous (Product Code 29,629-5) (for optional procedure)	5 X 1 ml
Reaction Vials with Caps (Product Code 27265/27273)	10 each

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

WARNING!! Portions of the procedure involve **highly exothermic reactions**. Instructions must be carefully followed and the procedure carried out in a chemical fume hood. It is strongly recommended to read the entire Technical Bulletin prior to starting the procedure.

Storage/Stability
The kit is stable for at least one year if stored unopened at 2–8 °C. TFMS is highly corrosive and very hygroscopic. Therefore, a fresh vial must be used for each group of deglycosylation reactions and any unused reagent should be disposed of properly (see Material Safety Data Sheet).

Procedure
This procedure has been developed to achieve optimal deglycosylation using RNase B as a model glycoprotein. The procedure can be used as a general guideline for the treatment of other glycoproteins. However, the reaction conditions should be optimized for each individual glycoprotein to achieve complete deglycosylation.

In general, the procedural variables are incubation time, reaction temperature, and the optional use of a scavenger for each individual glycoprotein, to achieve efficient deglycosylation. Longer incubation times may be required for complete release of certain O-linked glycans. This procedure has been used successfully with RNase B and other glycoproteins, namely bovine fetuin, human IgG, ovalbumin, and horseradish peroxidase. The latter glycoprotein is a plant glycoprotein, normally resistant to the action of PNGaseF, as it contains a fucose residue bound through an $\alpha(1\rightarrow3)$ linkage to the core glycan structure.

It is recommended to carry out the entire procedure in a chemical fume hood wearing appropriate personal protection equipment (see MSDS).

A. Rapid Deglycosylation Procedure

1. Use one vial of the salt-free, lyophilized RNase B Glycoprotein Standard (1 mg in a 2 ml screw-top glass vial, Product Code R 1153) as a control. For each sample glycoprotein, lyophilize 1 mg of the sample in an empty Reaction Vial (Product Code 27265). Ensure that the sample and the vial are **absolutely dry**. Cool the sample reaction vial to 2–8 °C. Note: One ampule of TFMS contains sufficient volume to analyze four 1 mg samples of glycoprotein. Due to the hygroscopic nature of the reagent, it is recommended that any unused portion of TFMS should be disposed of properly.
2. Cool the Trifluoromethanesulfonic acid (TFMS, (Product Code 34,781-7) to 2–8 °C. Add 150 µl of the pre-cooled TFMS to each pre-cooled sample reaction vial and rapidly seal the vial with the supplied cap (Product Code 27273).
   Note: TFMS may form a white vapor in the vial. The vapor will dissipate in several minutes.
3. Gently shake the mixture for 2–5 minutes until the glycoprotein is completely dissolved.
   Note: Do not vortex the mixture as the solution may foam, which could lead to protein degradation.
4. Incubate the sample reaction vial on ice or at 2–8 °C for an additional 25 minutes with occasional shaking.
5. Cool the 60% Pyridine Solution (Product Code P 5496) and the sample reaction vial to approximately –15 °C in a methanol-dry ice bath. Take appropriate precautions during the cooling of materials in the methanol-dry ice bath as the bath temperature is below –70 °C. Occasional inversion or gentle shaking may be required to avoid solidification of the pyridine solution. Should the pyridine solution solidify, remove the vial from methanol-dry ice bath and warm gently until it is completely melted.

6. Add 4 µl of the 0.2% Bromophenol Blue Solution (Product Code B 1560) as an indicator dye along the inside wall of the pre-cooled sample reaction vial and gently shake to mix. The color of the solution should now turn red.
7. Immediately after adding the indicator solution, **dropwise**, add the pre-cooled 60% Pyridine Solution to the sample reaction vial, taking care to mix and cool the sample reaction vial between drops.
   Note: The addition of the first drop of the 60% Pyridine Solution is **highly exothermic** and needs rapid cooling in the methanol-dry ice bath (–20 to –15 °C). With subsequent drops there is less need to cool to these temperatures. It is recommended to use as low a temperature as possible without freezing the reaction mixture. It is essential that the reaction mixture be maintained in a liquid state at all times.
8. The color of the solution gradually changes from red to yellow as the pH is raised with the addition of the 60% Pyridine Solution.
9. After having added approximately an equal volume of the 60% Pyridine Solution (150 µl), a fine precipitate may develop in the reaction mixture. If a precipitate does form, add 20 µl of water and shake. The precipitate will soon dissolve.
10. Continue the addition of the 60% Pyridine Solution until the color of the solution changes to light purple or blue. The pH is now approximately 6. Routinely, a total of approximately 300 µl of the 60% Pyridine Solution is added to the 150 µl of TFMS, resulting in a final volume of less than 500 µl.
    Note: The entire process of neutralization should be carried out quickly, keeping the reaction mixture cold at all stages to minimize protein degradation.
11. The deglycosylated protein can now be purified for MS and/or SDS-PAGE analysis using a suitable desalting method.

B. Optional Procedure with Scavenger

It has been reported that a free-radical scavenger species (anisole or toluene) may neutralize reactive groups formed during the reaction. The presence of a scavenger may not be essential to achieve deglycosylation of a glycoprotein. This kit includes anisole as an optional component should a reaction in the presence of a scavenger species be desired. The use of anisole as a scavenger has been shown to provide a higher yield of protein when horseradish peroxidase, a complex, non-mammalian glycoprotein, was deglycosylated both in the presence and absence of the anisole scavenger.

1. Cool the TFMS (Product Code 34,781-7) and the Anisole (Product Code 29,629-5) to 2–8 °C. Prepare a volume of a 10% anisole in TFMS solution sufficient for the number of reactions (150 µl per sample). For each sample add 15 µl of the pre-cooled anisole to 140 µl of the pre-cooled TFMS in an empty Reaction Vial. Invert or gently shake to mix after securely closing the vial. Prepare in a chemical fume hood. Avoid exposure to atmospheric moisture. Cool the 10% anisole in TFMS solution to 2–8 °C. Note: One ampule of TFMS contains sufficient volume to analyze four 1 mg samples of glycoprotein. Due to the hygroscopic nature of the reagents, it is recommended that any unused portion of either the anisole or TFMS should be disposed of properly.
2. Use one vial of the salt-free, lyophilized RNase B Glycoprotein Standard (1 mg in a 2 ml screw-top glass vial, Product Code R 1153) as a control. For each sample glycoprotein, lyophilize 1 mg of the sample in an empty Reaction Vial (Product Code 27265). Ensure that the sample and the vial are **absolutely dry**. Cool the sample reaction vial to 2–8 °C.
3. Add 150 µl of the pre-cooled 10% anisole in TFMS solution to the sample reaction vial and rapidly seal the vial tightly with the supplied cap.
   Note: A white vapor due to the TFMS forms in the vial. The vapor will dissipate in several minutes.
4. Gently shake the mixture for 2–5 minutes until completely dissolved.
   Note: Do not vortex the mixture as the solution may foam, which could lead to protein degradation.
5. Incubate the sample reaction vial on ice or at 2–8 °C for a further 3 hours with occasional shaking.
6. Proceed from step 5 in the Rapid Deglycosylation Procedure (section A).

C. Purification of Deglycosylated Protein

The use of gel filtration or dialysis is recommended for the purification of the deglycosylated protein. Gel filtration is a rapid method for separation of the deglycosylated protein from the pyridinium salt of TFMS. A 1 cm diameter column of Sephadex® G-25 (fine), with a bed height of 5 cm or longer, equilibrated in water, can be used for this purpose. Commercially available pre-packed gel filtration columns are not suitable for the present application as the resolution between the protein and the salt is poor, leading to a low yield of desalted protein. Determine the exclusion volume of the column using Blue Dextran (Product Code D 5751). Then apply the neutralized TFMS reaction mixture onto the column and elute with water. The deglycosylated protein elutes with the void volume.

Collect the eluate in small fractions over the entire separation range of the column. Dry the fractions by vacuum centrifugation. The fractions containing salt can be identified by the presence of a pellet at the bottom of the collection tube.

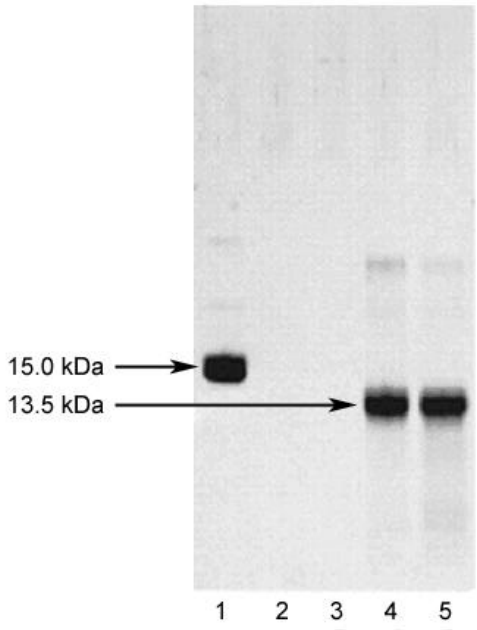
Dialysis is also suitable for purification of the deglycosylated protein, although incubation periods are extended. Typically, dialysis membranes, sacks, tubing, or cassettes with a molecular weight cut-off of 2,000–3,500 Da are suitable for removing salts and cleaved glycans. Samples may be dialyzed overnight against a suitable buffer (10 mM phosphate buffer). Small samples dialyzed against a large volume of buffer with frequent buffer changes may require less time.

Sample precipitation that may occur during gel filtration or dialysis purification procedure is normal and does not adversely affect the quality of subsequent analytical results. Resolubilization schemes appropriate for subsequent analyses may be employed.

Results

One of the simplest methods to assess the extent of deglycosylation is by mobility shift on SDS-PAGE gels. Figure 1 illustrates the deglycosylation of RNase B. Lanes 4 and 5 show a band at 13.5 kDa, representing deglycosylated RNase B, compared to the band at 15 kDa for the RNase B control (lane 1). This ability to detect mobility shifts upon deglycosylation will depend on the molecular weight of the native protein and the relative mass contribution of the glycans.

Figure 1.
Analysis of the chemical deglycosylation of RNase B on 12% homogeneous SDS-PAGE gel.



Lane 1 is the RNase B control (Product Code R 1153), while lanes 2 to 5 represent fractions collected from the gel filtration column. Lanes 2 and 3 are pre-void volume fractions and lanes 4 and 5 show bands at 13.5 kDa, corresponding to deglycosylated RNAse B.

Related Products

Product Name	Product Code
GlycoProfile I, Enzymatic In-Gel N-Deglycosylation Kit	PP0200
GlycoProfile II, Enzymatic In-Solution N-Deglycosylation Kit	PP0201
Enzymatic Protein Deglycosylation Kit	E-DEGLY
PNGase F, Proteomics Grade	P 7367
Trypsin, Proteomics Grade	T 6567

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