

Agilent Enzymatic Deglycosylation Kit for N-Linked and Simple O-Linked Glycans

This kit contains all enzymes and reagents needed to completely remove all N-linked and simple O-linked carbohydrates from glycoproteins:

- Deglycosylates up to 2 mg of glycoprotein
- Native and denaturing protocols
- No degradation of protein
- Removes O-linked sugars containing polysialic acid
- Control glycoprotein provided

Product Code GK80110

The Agilent Extender Kit for Complex O-Linked Glycans removes complex Core 2 O-linked carbohydrates including those containing poly lactosamine (optional).

Product Code GK80115 (formerly ProZyme)

Storage conditions

Kits are shipped with a cold pack for next day delivery, and should be stored at 2 to 8 °C. DO NOT FREEZE. Sterile pipette tips should be used since preservatives have not been added.

Kit contents

Item	Qty
N-Glycanase PNGase F (≥5 U/mL)	20 µL
Sialidase A (≥5 U/mL)	20 µL
O-Glycanase (≥1.25 U/mL)	20 µL
Denaturation Solution (2% sodium dodecylsulfate [SDS] and 1 M β-mercaptoethanol [βME])	0.5 mL
Detergent Solution (15% NP-40)	0.2 mL
Bovine Fetuin Control (heat-treated, lyophilized)	0.5 mg
5x Incubation Buffer (0.25 M sodium phosphate, pH 7.0)	1 mL
Agilent Extender Kit for Complex O-Linked Glycans (GK80115, Optional)	
β(1-4) Galactosidase (≥2 U/mL)	20 µL
β-N-Acetylglucosaminidase (≥40 U/mL)	20 µL

Introduction

Carbohydrates in the form of asparagine-linked (N-linked) or serine/threonine-linked (O-linked) oligosaccharides are major structural components of many eukaryotic proteins. They perform critical biological functions in protein sorting, immune recognition, receptor binding, inflammation, pathogenicity, and many other processes. The diversity of oligosaccharide structures often results in heterogeneity in the mass and charge of glycoproteins. N-linked oligosaccharides may contribute 3.5 kd or more per structure to the mass of a glycoprotein (see Figure 1). Variations in the structures and different degrees of saturation of available glycosylation sites in a glycoprotein all contribute to mass heterogeneity. The presence of sialic acid (N-acetylneuraminic acid) affects both the mass and charge of a glycoprotein.

Other modifications to the carbohydrate such as phosphorylation or sulfation also affect charge.

O-linked sugars, although usually less massive than N-linked structures, may be more numerous, and are also heterogenous in structure (see Figures 2 and 3).

To study the structure and function of a glycoprotein, it is desirable to remove either all or only a select class of oligosaccharides. This approach allows the assignment of specific biological functions to particular components of the glycoprotein. For example, the loss of ligand binding to a glycoprotein after removal of sialic acid may implicate this sugar in the binding process.

Researchers may wish to remove sugars from glycoproteins for a number of reasons:

- For simplifying amino acid sequence determination of glycoproteins
- To remove heterogeneity in glycoproteins for X-ray crystallographic analysis
- To remove carbohydrate epitopes from antigens
- To enhance or reduce blood clearance rates of glycoprotein therapeutics¹⁵
- To investigate the role of carbohydrates in enzyme activity and solubility
- To investigate ligand binding
- For quality control of glycoprotein pharmaceuticals
- To study the peptide portion of the glycoprotein by SDS PAGE

Deglycosylation methods

Both chemical and enzymatic methods exist for removing oligosaccharides from glycoproteins. Hydrazinolysis of glycoproteins,⁹ although capable of removing both N- and O-linked sugars, results in the complete destruction

of the protein component, and is not suitable if recovery of the protein is desirable. Milder chemical methods such as trifluoromethanesulphonic acid (TFMS),¹² even when optimized, result in incomplete sugar removal and partial protein destruction. The amino acid-linked sugar residue of both N- and O-linked oligosaccharides is retained. Only the enzymatic method provides complete sugar removal with no protein degradation.

Enzymatic removal of N-linked oligosaccharides

Use of the enzyme N-Glycanase (PNGase F) is the most effective method of removing virtually all N-linked oligosaccharides from glycoproteins¹⁷.

The oligosaccharide is left intact and suitable for further analysis (the asparagine residue from which the sugar was removed is deaminated to aspartic acid, the only modification to the protein). A tripeptide with the oligosaccharide-linked asparagine as the central residue is the minimal substrate for N-Glycanase. However, oligosaccharides containing a fucose linked α(1-3) to the asparagine-linked N-acetylglucosamine (commonly found in glycoproteins from plants or parasitic worms) are resistant to N-Glycanase;¹ endoglycosidase A, isolated from almond meal, must be used in this situation.¹⁶ However, this enzyme is ineffective when sialic acid is present on the N-linked oligosaccharide.

Steric hindrance slows or inhibits the action of N-Glycanase in some cases. Denaturation of the glycoprotein by heating with SDS and βME greatly increases the rate of deglycosylation. For some glycoproteins, no cleavage occurs unless the protein is denatured. For others, some or all of the oligosaccharides can be removed from the native protein after extensive incubations of three days or longer. N-Glycanase will remain active under

reaction conditions for at least three days allowing extended incubations of native glycoproteins. In general, it appears that particular glycans can be resistant to N-Glycanase due to their location in the native protein structure; such glycans cannot be removed unless the protein is denatured (see Figure 4, lane 6).

Note: Prior to the addition of N-Glycanase, a nonionic detergent such as NP-40 must be added in excess to the SDS-denatured glycoprotein to complex any free SDS. Otherwise, reduction in the rate of N-Glycanase cleavage will result.

Other commonly used endoglycosidases such as endoglycosidase H⁸ and the endoglycosidase F¹⁸ series are not suitable for general deglycosylation of N-linked sugars because of their limited specificities, and because they leave one N-acetylglucosamine residue attached to the asparagine.

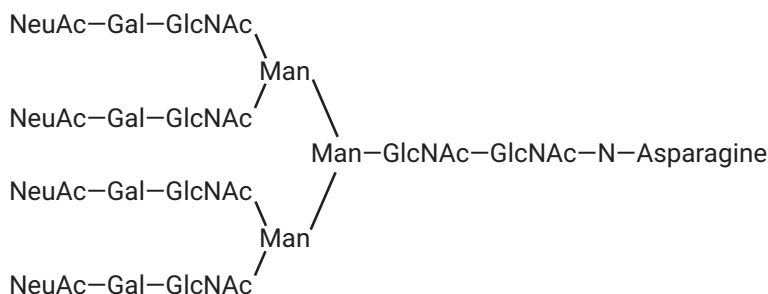


Figure 1. Tetraantennary N-linked Sugar.

Gal - galactose; Man - mannose; GalNAc - N-acetylgalactosamine; GlcNAc - N-acetylglucosamine; NeuAc - N-acetylneuraminic acid (sialic acid)

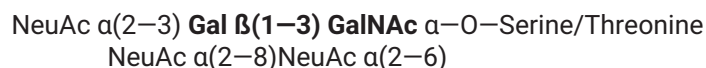


Figure 2. Di- and Tri-sialylated O-linked Core (core shown in bold).

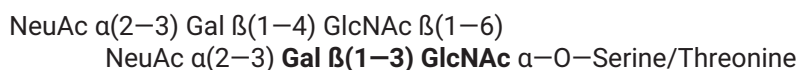


Figure 3. O-linked Core-2 Hexasaccharide.

O-linked oligosaccharides

There is no enzyme comparable to N-Glycanase for removing intact O-linked sugars. Monosaccharides must be removed by a series of exoglycosidases until only the Gal $\beta(1-3)$ GalNAc core remains attached to the serine or threonine. O-Glycanase (endo- α -N-acetylgalactosaminidase)⁶ can then remove the core structure intact with no modification of the serine or threonine residues. Denaturation of the glycoprotein does not appear to significantly enhance de-O-glycosylation.

Any modification of the core structure will block the action of O-Glycanase. By far the most common modification of the core Gal $\beta(1-3)$ GalNAc is mono-, di- or tri-sialylation.^{3,10,11,13}

These residues are easily removed by a suitable sialidase. The trisialyl structure can be removed by the *Arthrobacter ureafaciens* sialidase (Sialidase A)¹⁹ since this enzyme is capable of efficient

cleavage of the NeuAc $\alpha(2-8)$ NeuAc bond.

A less common but widely distributed O-linked hexasaccharide structure contains $\beta(1-4)$ -linked galactose and $\beta(1-6)$ -linked N-acetylglucosamine as well as sialic acid.^{5,20}

Complete removal of this O-linked structure or its derivatives would require, in addition to Sialidase A, a $\beta(1-4)$ -specific galactosidase and a β -N-acetylglucosaminidase. The galactosidase must be $\beta(1-4)$ -specific since a nonspecific galactosidase would remove the $\beta(1-3)$ galactose from the core Gal $\beta(1-3)$ GalNAc leaving O-linked GalNAc, which cannot be removed by O-Glycanase. The Agilent Extender Kit for Complex O-Linked Glycans (GK80115, optional) provides the appropriate additional enzymes and reagents for degrading these and any other O-linked structures containing $\beta(1-4)$ -linked galactose or β -linked N-acetylglucosamine such as polylactosamine.

Other rare O-linked structures have been found, removal of which would require the use of additional enzymes not included in the kit. These structures include oligosaccharides containing α -linked galactose or fucose,^{4,20} and directly O-linked N-acetylglucosamine (found on nuclear proteins)⁷ or N-acetylgalactosamine (found in mucins). Fucose¹⁴ and mannose² directly O-linked to proteins cannot be removed enzymatically.

Monitoring deglycosylation

The simplest method of assessing the extent of deglycosylation is by mobility shifts on SDS polyacrylamide gels (PAGE). The amount of enzyme added to a reaction in this kit is <500 ng for each enzyme; bands corresponding to the enzymes in the gels should be barely visible compared with the glycoprotein.

For bovine fetuin, Figure 4 shows that the sequential addition of each of the three enzymes N-Glycanase, Sialidase A, and O-Glycanase results in a noticeable increase in mobility. The greatest shift (lane 3) is a result of removal of N-linked sugars by N-Glycanase. Removal of sialic acid from O-linked sugars by Sialidase A results in the shift in lane 4. Finally, removal of the O-linked core Gal β (1-3) GalNAc by O-Glycanase is responsible for the small shift in lane 5. The ability to detect obvious mobility shifts when the disaccharide core structure is removed depends on the size of the protein and the relative mass contribution of the disaccharides removed. Thus, it may be difficult to establish the presence of O-linked sugars based solely on mobility shifts following O-Glycanase treatment of very large proteins with small numbers of O-linked sugars.

Other methods can be used in conjunction with SDS PAGE gels to monitor deglycosylation. These methods, used in gel or blot formats, directly detect the carbohydrate portion of the glycoprotein. In each method,

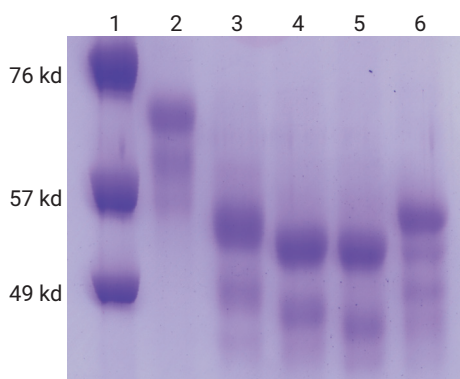


Figure 4. Enzymatic Deglycosylation of Bovine Fetuin.

Lane 1 - Molecular weight markers
 Lane 2 - Denatured bovine fetuin (DBF)
 Lane 3 - DBF + PNGase F (three hour incubation)
 Lane 4 - DBF + PNGase F + Sialidase A (3 hour incubation)
 Lane 5 - DBF + PNGase F + Sialidase A + O-Glycanase (three hour incubation)
 Lane 6 - Native Fetuin + PNGase F + Sialidase A + O-Glycanase, (three day incubation)

the carbohydrate is oxidized with periodate. The oxidized carbohydrate is either directly stained (Alcian Blue or silver stain), or is reacted with biotin hydrazide, which biotinylates the sugar. An enzyme-linked streptavidin conjugate and the appropriate indicator substrate is used to detect the carbohydrate in a blot. Completely deglycosylated protein should produce no signal with these methods.

Protocols

The two conventional enzymatic methods of deglycosylation, denaturing versus nondenaturing, differ in the final conformation of the protein. While the denaturing protocol ensures the removal of all N-linked sugars, the native conformation of the protein is disrupted. If native conformation must be preserved to maintain enzymatic activity or antigenicity, the nondenaturing protocol is recommended. A sample of the protein should always be deglycosylated using the denaturing protocol to use as a standard of the fully deglycosylated protein for comparison with the nondenatured sample in gel-shift experiments.

Denaturing protocol reagents

- Glycoprotein
- 5x Incubation buffer (supplied in kit)
- Denaturation solution (supplied in kit)
- N-Glycanase (supplied in kit)
- Sialidase A (supplied in kit)
- O-Glycanase (supplied in kit)
- Detergent solution (supplied in kit)
- β (1-4) Galactosidase (Extender Kit for Complex O-Linked Glycans (GK80115, optional))
- β -N-Acetylglucosaminidase (Extender Kit for Complex O-Linked Glycans (GK80115, optional))

Procedure

1. Dissolve 100 μ g or less of the glycoprotein in 30 μ L deionized water in an Eppendorf tube.
2. Add 10 μ L 5x Incubation Buffer and 2.5 μ L denaturation solution. Mix gently.
3. Heat at 100 $^{\circ}$ C for five minutes.

Note: Some proteins may precipitate when heated with SDS. In this event, omit the heat treatment, and increase the incubation time to 24 hours after adding enzymes.

4. Cool to room temperature. Add 2.5 μ L detergent solution. Mix gently.

Note: Failure to add detergent solution may result in the reduction of activity of some enzymes.

5. Add 1 μ L each of N-Glycanase, Sialidase A and O-Glycanase. If using the Extender Kit for Complex O-Linked Glycans (GK80115, optional), add 1 μ L each of β (1-4)-Galactosidase and β -N-Acetylglucosaminidase.
6. Incubate for three hours at 37 $^{\circ}$ C.
7. Analyze by method of choice.

Alternatively, the enzymes may be added individually or sequentially to determine the types of oligosaccharides present on the glycoprotein (Figure 4).

Nondenaturing protocol reagents

- Glycoprotein
- 5x Incubation buffer (supplied in kit)
- N-Glycanase (supplied in kit)
- Sialidase A (supplied in kit)
- O-Glycanase (supplied in kit)
- β (1-4) Galactosidase (Extender Kit for Complex O-Linked Glycans (GK80115, optional))
- β -N-Acetylglucosaminidase (Extender Kit for Complex O-Linked Glycans (GK80115, optional))

Procedure

1. Dissolve up to 100 µg of a glycoprotein in 35 µL deionized water in an Eppendorf tube.
2. Add 10 µL 5x incubation buffer.
3. Add 1 µL each of N-Glycanase, Sialidase A and O-Glycanase. If using the Extender Kit for Complex O-Linked Glycans (GK80115, optional), add 1 µL each of β(1-4)-Galactosidase and β-N-Acetylglucosaminidase.
4. Incubate for 1 to 5 days at 37 °C.

An aliquot should be deglycosylated using the denaturing protocol to provide a gel standard for the fully deglycosylated protein. The position of the native protein can then be compared with this standard to judge the extent of deglycosylation (see Figure 4 lane 6).

Note: Due to steric hindrances, N-Glycanase may fail to remove all N-linked sugars from nondenatured proteins. If after extensive incubation with N-Glycanase your native protein is

still not deglycosylated, contact Agilent to discuss alternative procedures.

Kit capacity

The quantity of enzymes recommended in the protocols is sufficient to deglycosylate approximately 100 µg of an average glycoprotein in the time given. N-Glycanase cleavage is generally the rate-limiting reaction due to the slow removal of some sterically hindered N-linked residues, even when the glycoprotein is denatured. Since all of the enzymes retain activity under reaction conditions for several days, a much larger quantity of glycoprotein may be deglycosylated if incubation time is extended. Conversely, it is not necessary to use the recommended amounts of enzymes if quantities much less than 100 µg of glycoprotein are being cleaved. The enzymes can be diluted into 1x Incubation Buffer. They will remain stable in diluted form at 2 to 8 °C.

Bovine fetuin control protein

Bovine fetuin contains sialylated N- and O-linked oligosaccharides.¹³ Dissolve the 0.5 mg Bovine Fetuin Control in 50 µL of deionized water to yield a 10 mg/mL solution.

Note: Commercial preparations of fetuin contain proteases which will eventually degrade the protein. To inactivate the proteases, the Bovine Fetuin Control has been heat treated at 90 °C for 10 minutes. The reconstituted Bovine

Fetuin Control solution can be stored at 2 to 8 °C.

Technical assistance

Agilent glycohydrolases have been tested for stability, specificity and interfering activities. If you have any questions or experience difficulties regarding any aspect of our products, please contact us:

<https://www.agilent.com/en/contact-us/page>

Agilent customers are an important source of information regarding advanced or specialized uses of our products. We encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

Or, contact your local distributor.

Enzyme specificities

Technical Data Sheets for individual enzymes featuring assay protocols and other information are available upon request, or download them our website at: <http://www.agilent.com>

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Other Agilent products and kits

The individual enzymes in this kit as well as a wide variety of other glycobiology products are available from Agilent. A complete listing is accessible at: <http://www.agilent.com>

Product use and warranty

Terms and conditions of sale may be found at: <http://www.agilent.com>

www.agilent.com/chem

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