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N-Glycosidase F Deglycosylation Kit

Kit for the deglycosylation of asparagine-linked glycan chains on glycoproteins.

Cat. No. 1 836 552
Kit for 12 deglycosylation reactions (including controls)

Store at –15 to –25°C

Instruction Manual

Version 2, September 2003

Roche

1. Preface

1.1 Table of Contents

1.2 Kit contents

Bottle	Label	Contents
1	N-Glycosidase F, recombinant	• Lyophilizate • 150 U
2	Denaturation buffer, pH 8.6	• 150 µl • contains sodium phosphate and ionic detergent
3	Control glycoproteins • human transferrin • ribonuclease B, • human α1-acid glycoprotein	• Lyophilized • 150 µg of each protein
4	Reaction buffer, pH 7.2	• 400 µl • Contains sodium phosphate and nonionic detergent (the nonionic detergent is necessary to complex any excess ionic detergent used for denaturing)
5	Premixed protein molecular weight markers	10 µl (10 µg) of each protein • Phosphorylase B, 97.4 kD • Bovine serum albumin, 66.2 kD • Aldolase, 39.2 kD • Triose phosphate isomerase, 26.6 kD • Trypsin inhibitor, 21.5 kD • Lysozyme, 14.4 kD

2. Product overview

Introduction

For structural analysis of N (asparagine)-linked carbohydrate chains of glycoproteins, chemical hydrazinolysis and enzymatic methods are most widely employed to cleave all common classes of oligosaccharides. During hydrazinolysis, however the protein is destroyed and also degradation and modification of the released sugar chains have been observed (1,2).

Enzymatic procedures are therefore the only methods allowing both the structural analysis of the glycan and the protein part. Several endoglycosidases useful for these applications have been described. Endoglycosidase H* and Endoglycosidase F1 for instance act only on high mannose type and hybrid type structures, while Endoglycosidase F2 prefers "biantennary complex" type chains.

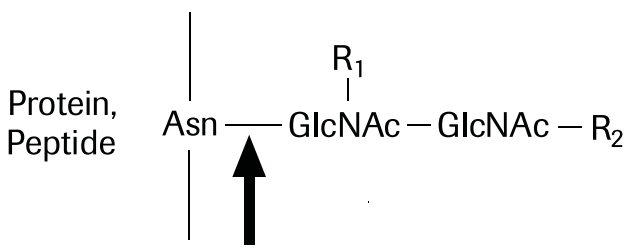
While these enzymes are useful tools for the selective removal and characterization of the individual glycan types (3), only N-glycosidase F is able to release all common classes of N-glycans from the protein backbone (4). N-glycosidase F is not a glycosidase but an amidase converting asparagine to aspartic acid in the following reaction:

Protein-asn-GlcNAc-Glycan $\rightarrow$ Protein-asp + NH_3 + GlcNAc-Glycan

Not all carbohydrate chains on a glycoprotein are equally sensitive to N-glycosidase F. In many cases N-glycosidase F can cleave the intact glycoproteins (*e.g.,* RNAse B, fetuin, human transferrin, ovomucoid and fibrinogen) (4,5). For other glycoproteins like for instance α_1 -acid glycoprotein the reducing of -S-S- bridges and denaturation of the protein using ionic detergents or chaotropic salts prior to the enzymatic treatment was found to be necessary (6,7).

From a variety of denaturing conditions a procedure with optimum results for all glycoproteins has been developed as basis for this kit. It must be stated, however, that some modifications like α1,3-bound core fucose present on some plant and invertebrate glycoproteins allow only deglycosylation with N-glycosidase A* and not with N-glycosidase F (8).

Reaction principle



R_1 = Fucose α-1,3
R_2 = Oligosaccharide

Application

The kit can be used to test for the existence of asparagine-linked glycan chains on glycoproteins. The kit also allows to estimate the number of glycan chains bound to a glycoprotein. After the deglycosylation reaction the protein can be analyzed on SDS-PAGE, where a shift to a lower apparent molecular weight after the reaction indicates the existence of asparagine linked glycan chains. The glycan chains can also be used for further structural and functional analysis.

Purity

N-glycosidase F is produced as a recombinant protein from *E. coli*. It is highly purified and tested to be free of contaminating protease, exo- and endoglycosidase activities.

Control glycoproteins

Transferrin (from human serum): Transferrin has an apparent M_r of 65 kD containing two glycan chains of sialylated bi- and triantennary complex type. The apparent M_r is reduced to 60 kD upon digestion with N-glycosidase F (9).

α_1-acid glycoprotein (from human serum): α_1-acid glycoprotein has an apparent M_r of 45 kD containing five glycan chains of sialylated bi- tri- and tetraantennary complex type. The apparent M_r is reduced to 22 kD upon digestion with N-glycosidase F (10).

Ribonuclease B (from bovine pancreas): Ribonuclease B has an apparent M_r of 17 kD containing one glycan chain of high mannose type. The apparent M_r is reduced to 15 kD upon digestion with N-glycosidase F (11).

Number of reactions

The contents of the kit are sufficient for 10 deglycosylation reactions and two additional deglycosylations with the control glycoproteins.

Storage and stability

The kit is stable at -15 to -25°C until the expiration date printed on the label. The stability of the reconstituted solutions is indicated in chapter 3.

Advantages

Benefit	Feature
fast	The deglycosylation can be completed within 1 – 2 hours
easy	Only 3 easy working steps are necessary
versatile	Unlike to chemical procedures, both, the glycan and the protein part can be analyzed after the reaction.
complete	All required buffers and reagents (except ß-mercaptoethanol) are supplied with the kit.

3. Procedures and required materials

3.1 Before you begin

Additional equipment and reagents required

- Incubator for 37°C and 95°C
- equipment for SDS-PAGE and staining of gels
- lab centrifuge
- SDS-sample buffer: 125 mM Tris-HCl, pH 6.8, 10% glycerol (v/v), 2% SDS (w/v), 5% ß-mercaptoethanol, (v/v), 0.01% bromophenol blue (w/v).
- SDS-PAGE staining reagents

Preparation of kit working solutions

Please refer to the following table

Solution	Composition/Preparation	Storage/ Stability
N-Glycosidase F	Dissolve the lyophilizate in 125 µl of double dist. water.	several months at 2-8°C
Reduced denaturation buffer	• Thaw the frozen solution and warm to 37°C to dissolve all contents. • Take the needed amount of denaturation buffer and add 1% ß-mercaptoethanol (v/v) before use.	up to 2 months at –15 to –25°C
Control glycoproteins	Dissolve the content in 50 µl of double dist. water.	-15 to -25°C
Reaction buffer	Thaw the frozen solution and warm up to 37°C to dissolve all contents.	-15 to -25°C
Premixed protein molecular weight markers	Dilute the molecular weight markers with 190 µl of SDS-sample buffer and heat for 3 min at 95°C immediately prior to use. ***Note:*** 5 µl of this solution is necessary for a 6.5 × 9 cm minigel and staining with Coomassie.	

3.2 Protocol for complete deglycosylation

Before you begin

For each sample to be analyzed label two eppendorf vials with *e.g.,* "+" and "-". In the same manner label two vials for the Control glycoproteins with "C+" and "C-".

Handling instructions

- Usually the glycoprotein samples can be used without further treatment prior to digestion. If, however, the sample material contains ionic detergents, potassium ions or acidic buffers in high concentration a dialysis against double distilled water or neutral buffers is recommended.
- The denaturation buffer makes proteins very sensitive against proteolytic attack. Therefore, if a protease contamination of the sample can not be excluded, it is recommended to use Complete[1)] Mini* (protease inhibitor cocktail tablets) for protease inhibition.
- The maximum amount of glycoprotein for 1 reaction is 100 µg. If higher amounts of glycoproteins are to be digested longer incubation times or multiple reactions are needed.

Procedure

Please refer to the following table.

Step	Action
1	• Add 5 µl of your glycoprotein sample to the vials labeled with "+" and "-". • Add 5 µl of Control glycoprotein to vials the labeled with "C+" and "C-".
2	• Add 5 µl of Reduced denaturation buffer to each vial. • Incubate for 3 min at 95°C. • Centrifuge down the contents after the heating step.
3	Add 10 µl of Reaction buffer to each vial and mix.
4	• Add 10 µl of reconstituted N-glycosidase F to all vials labeled with "+" (including "C+"). • Add 10 µl of Reaction buffer to all vials labeled with "-" (including "C-"). • Incubate for 1 hour at 37°C. ***Note:*** Both the Denaturation buffer and the Reaction buffer contain detergents which may interfere with further analysis. To remove these detergents add 1 ml of cold acetone to each vial, mix and centrifuge for 5 min at 10 000 × g. Then aspirate carefully 950 µl of the supernatant and reextract the pellet once again with acetone. Then dry the pellet using a vacuum concentrator. The dried pellet containing detergent free carbohydrates (together with proteins and buffer salts) can now be dissolved in water and further analyzed.
5	Mix an appropriate aliquot of the samples with an equal amount of SDS-sample buffer and heat for 3 min to 95°C. ***Note:*** 10 µl of the sample solution is sufficient for a 6.5 × 9 cm minigel and staining with Coomassie.

Example of the digested control glycoproteins

An example of the digested control glycoproteins is shown in fig 2.

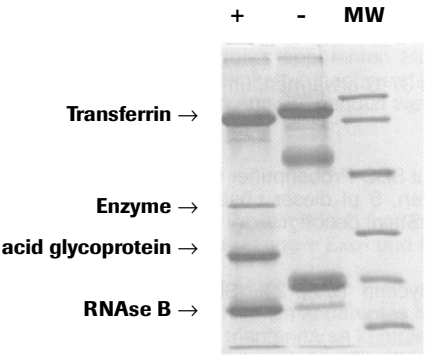


Fig. 2:
Lane 1, digested glycoproteins
Lane 2, undigested glycoproteins
Lane 3, protein molecular weight markers

3.3 Working procedure for estimating the number of glycan chains bound to a glycoprotein

Handling instructions

- Protocol 3.3 should only be performed, if protocol 3.2 worked properly and indicated the existence of N-glycosidic chains.
- Usually the glycoprotein samples to be digested can be used without further pre-treatment. If, however, the sample material contains ionic detergents, potassium ions or acidic buffers in high concentration a dialysis against double distilled water or neutral buffers is recommended.

Before you begin

For each sample to be analyzed label five eppendorf vials with "60", "45", "30", "15", and "0" to indicate the time (in minutes) of the N-glycosidase F reaction.

Procedure

Please refer to the following table.

Step	Action
1	Add 5 µl (with 5 –50 µg) of glycoprotein sample to all vials.
2	• Add 5 µl of Reduced denaturation buffer to each vial. • Incubate for 3 min at 95°C. • Centrifuge down the contents after the heating step.
3	Add 10 µl of Reaction buffer to each vial and mix.
4	Add an additional 10 µl of Reaction buffer to the vial labeled with "0" and mix.
5	• Add 10 µl of N-glycosidase F to all vials except of the vial labeled with "0" and mix. • Incubate all vials at 37°C.
6	• At the time indicated on the vials add 30 µl of SDS-sample buffer and immediately heat for 3 min to 95°C. • Then analyze on SDS-PAGE.

4. Interpretation of results

Complete deglycosylation

The following results can be obtained:

Result	Interpretation	Recommendation
The apparent molecular weights for the sample and the control glycoproteins decrease after N-glycosidase F treatment to one single band each upon SDS-PAGE.	The sample is a glycoprotein containing one or more asparagine bound glycosidic chains.	To estimate the number of glycosidic chains present on the sample glycoprotein perform protocol 3.3.
The apparent molecular weights for the control glycoproteins decrease after N-glycosidase F treatment to one band and the sample yields several bands upon SDS-PAGE.	The sample is a glycoprotein containing more than one asparagine bound glycosidic chain but, due to partial resistance to N-glycosidase F, is not cleaved completely.	Add a second aliquot of N-glycosidase F after 1 hour.
	The sample is not denatured properly or it contains inhibiting agents to N-glycosidase F.	The presence of inhibitors can be assessed by co-incubating the control glycoproteins with the sample. Inhibitors may be removed by dialyzing the sample.
	The sample is heterogeneous because of reasons other than glycosylation.	
The apparent molecular weights for the sample and the control glycoproteins decrease after N-glycosidase F treatment to several bands each upon SDS-PAGE.	Incomplete denaturation of proteins or the activity of N-glycosidase has decreased.	Increase the denaturation time to 10 min. If the result is the same use a higher amount or a fresh vial of N-glycosidase.
N-glycosidase F treatment to one band and the sample does not show an increased mobility on SDS-PAGE.	The sample does not contain asparagine bound glycan chains.	The absence of glycosylation may be proved by a glycoprotein specific staining using our DIG Glycan Detection Kit*.
	The sample contains inhibiting agents to N-glycosidase F.	The presence of inhibitors can be assessed by co-incubating the control glycoproteins with the sample. Inhibitors may be removed by dialyzing the sample.

Estimation of the number of glycan chains (3.3)

Protocol 3.3 should only be performed if protocol 3.2 worked properly and indicated the existence of N-glycosidic chains.

The following results can be obtained:

Result	Interpretation	Recommendation
Even at the shortest incubation time only one band at a lower apparent molecular weight appeared.	There is only one glycan chain present.	
	N-glycosidase F removed several chains, which are very sensitive to N-glycosidase.	Repeating the experiment using 6 twofold dilutions of the enzyme starting with 50 U/ml and a constant incubation time of 15 min.
More than one band (= n) appear at a lower molecular weight. The lowest band becomes more intensive with incubation time, while the band of the original undigested sample disappears	The sample contains N-asparagine glycan chains.	

5. Appendix

5.1 References

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5.2 Related products

For further information contact our “Complete site for Protease inhibition at http://www.biochem.roche.com/proteaseinhibitor

A Protease inhibitor guide is available at http://www.biochem.roche.com/proteaseinhibitor/prod07.htm

Product	Pack size	Cat. No.
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Complete EDTA free Inhibitor Cocktail Tablets	20 tablets	1873 580
Complete Mini EDTA free Inhibitor Cocktail Tablets	25 tablets	1 836 170
Protease Inhibitor Set	1 set	1 206 893
Detection of carbohydrates in glycoconjugates		
DIG Glycan Detection Kit	Kit for 100 reactions	1 142 372
Deglycosylation of “high mannose” type glycans		
Endoglycosidase H	1 U (200 µl) 2.5 U (500 µl)	1 088 726 1 643 053
Deglycosylation of O-linked carbohydrates		
O-Glycosidase	25 mU	1 347 101
Deglycosylation of N-linked carbohydrates with α1,3 bound core fucose		
N-Glycosidase A	5 mU	1 642 995

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