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Roche

DIG Glycan Differentiation Kit

Cat. No. 11 210 238 001

Version 17
Content version: August 2018

Store at +2 to +8°C

1. What this Product Does

For the detection on 5 × 5 blots for 5 lectins, 10 × 10 cm each.

Kit Contents

Vial	Label	Contents / Function
1	GNA, Digoxigenin-labeled	• 50 µl solution (1mg/ml), in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% sodium azide (w/v), pH 7.0. • **GNA** (Galanthus nivalis agglutinin): recognizes terminal mannose, (1–3), (1–6) or (1–2) linked to mannose; thus it is suitable for identifying "high mannose" N-glycan chains or O-glycosidically linked mannoses in yeast glycoproteins.
2	SNA, Digoxigenin-labeled	• 50 µl solution (1 mg/ml), in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% sodium azide (w/v), pH 7.0 • **SNA** (Sambucus nigra agglutinin): recognizes sialic acid linked (2–6) to galactose; thus it is suitable for identifying complex, sialylated N-glycan chains in combination with the lectin MAA; correspondingly linked sialic acids in O-glycan structures are also recognized.
3	MAA, Digoxigenin-labeled	• 250 µl solution (1 mg/ml), in 50 mM Tris-HCl, 0.5 M NaCl, 0.05% sodium azide (w/v), pH 7.0 • **MAA** (Maackia amurensis agglutinin): recognizes sialic acid linked (2–3) to galactose; in combination with SNA (see above) it is suitable for identifying a) complex, sialylated carbohydrate chains and b) type of sialic acid linkage. Lectin MAA identifies (2–3)-linked sialic acids in O-glycans too.
4	PNA, Digoxigenin-labeled	• 500 µl solution (1 mg/ml), in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% sodium azide (w/v), pH 7. • **PNA** (Peanut agglutinin): recognizes the core disaccharide galactose (1–3) N-acetylgalactosamine and is thus suitable for identifying O-glycosidically linked carbohydrate chains (with exception of yeast glycoproteins). Should the disaccharide be substituted, it is necessary to split off the substitute group forst e.g. sialic acid with the aid of neuraminidase [neuraminidase from *Arthrobacter** or from *Vibrio cholerae**].
5	DSA, Digoxigenin-labeled	• 50 µl solution (1 mg/ml), in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% sodium azide (w/v), pH 7. • **DSA** (Datura stramonium agglutinin): recognizes Gal-(1–4)GlcNAc in complex and hybrid N-glycans, in O-glycans and GlcNAc in O-glycans.
6	Anti-DIG AP	0.3 ml polyclonal sheep anti-Digoxigenin Fab fragments, conjugated with alkaline phosphatase (750 U/ml).
7	NBT/BCIP	5 x 1.1 ml NBT/BCIP solution, each. NBT = 4-Nitro blue tetrazolium chloride; BCIP = 5-Bromo-4-chloro-3-indolyl-phosphate.
8	Glucose-Oxidase (control glycoprotein) (for GNA)	Dissolving the contents in 1 ml double dist. water results in a concentration of 1 mg glycoprotein/ml and 0.05% sodium azide (w/v).
9	Transferrin (control glycoprotein) (for SNA)	Dissolving the contents in 1 ml double dist. water results in a concentration of 1 mg glycoprotein/ml and 0.05% sodium azide (w/v).
10	Fetuin (control glycoprotein) (for SNA, MAA and DSA)	Dissolving the contents in 1 ml double dist. water results in a concentration of 1 mg glycoprotein/ml and 0.05% sodium azide (w/v).
11	Asialofetuin (control glycoprotein) (desialylated fetuin; for PNA and DSA)	Dissolving the contents in 1 ml double dist. water results in a concentration of 1 mg glycoprotein/ml and 0.05% sodium azide (w/v).
12	Blocking Reagent	100 ml 10x conc. Blocking Reagent. Blocking solution: Mix well the content of the bottle by gentle shaking, take out 10 ml blocking reagent by sterile handling and mix with 90 ml TBS, pH 7.5.
13	Ponceau S solution	0.2% Ponceau S solution (w/v) in 3% acetic acid (w/v) for general protein staining on nitrocellulose.

Storage and Stability

Store the kit at +2 to +8°C until the expiration date printed on the label.

⚠ Keep the NBT/BCIP solution (vial 7) away from light!

- Once the kit is opened, store the kit components as described in the following table:

Vial	Function/Description	Storage
1	GNA, Digoxigenin-labeled	Store at +2 to +8°C
2	SNA, Digoxigenin-labeled	
3	MAA, Digoxigenin-labeled	
4	PNA, Digoxigenin-labeled	
5	DSA, Digoxigenin-labeled	Store at –15 to –25°C
6	Anti-Digoxigenin-AP*	Store at +2 to +8°C
7	NBT/BCIP	• Store at –15 to –25°C. • **Keep away from light!**
8	Control glycoprotein glucose-oxidase, GOD rec. (for GNA)	Store at –15 to –25°C
9	Control glycoprotein transferrin* (for SNA)	
10	Control glycoprotein fetuin (for SNA, MAA and DSA)	
11	Control glycoprotein asialofetuin* (desialylated fetuin; for PNA and DSA)	
12	Blocking Reagent*	• Aliquot and store at +2 to +8°C. • The diluted blocking solution is stable at +2 to +8°C for one month.
13	Ponceau S solution	• Store at +2 to +8°C • **Do not freeze!**

Additional Equipment and Reagents Required

- TBS (Tris buffered saline): 0.05 M Tris-HCl*, 0.15 M NaCl, pH 7.5
- Buffer 1: TBS; 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.5.
- Buffer 2: 0.1 M Tris-HCl, 0.05 M $MgCl_2$, 0.1 M NaCl, pH 9.5.

Application

With this method carbohydrate moieties of glycoproteins, bound to nitrocellulose or other mem-branes can be characterized. It is also generally suitable for the analysis of other, correspondingly fixed, glycoconjugates. The suitability for the application on tissue sections has been described. The following method has been developed for the structural characterization of carbohydrate chains of glycoproteins bound to nitrocellulose which have been separated on an SDS-polyacrylamide gel and transferred. “Dot-blot” samples can be analyzed using the same procedure.

2. How to Use this Product

2.1 Before You Begin

Preparation of Sample Material

The glycoproteins are transferred either onto nitrocellulose or other membranes after gel electrophoretic separation or directly blotted onto the membrane (dot-blot). For the SDS-polyacrylamide gel electrophoresis the standard system described by Laemmli (Laemmli, UK, 1970) can be used. Transfer can be done according to Burnette (Burnette, WN, 1981).

⚠ About 0.01 µg–1 µg glycoprotein should be bound to the filter.

Protein Staining (optional)

After immobilization on nitrocellulose the proteins can be stained with the Ponceau S solution (bottle 13). Incubate the filter in the Ponceau S solution for 5 min (use just enough solution to cover it; the solution can be used at least 10 times) and then rinse with double dist. water until the bands are visible. The filter can then be photographed or the standard proteins can be marked with a pencil for documentation. The Ponceau S staining disappears during the following incubation in the Blocking solution.

⚠ Staining with Ponceau S cannot be used with nylon membranes, because the stain binds irreversibly to these membranes).

Negative Control

Recombinant proteins from *E. coli*, or N-Glycosidase F treated with Glucose-Oxidase or transferrin, respectively, can be used as a negative control.

⚠ Bovine serum albumin (BSA) is not suited as negative control, because it can be chemically glycosylated.

2.2 Procedure for Glycoprotein Detection

⚠ Stabilizers that are added to enzyme preparations (like sucrose or BSA) are detected. The volumes stated refer to a 50 – 100 cm^2 filter. Incubate all filters by gentle agitation at +15 to +25°C, except for color development which should be done without shaking.

❶ Incubate filter for at least 30 min in approx. 20 ml Blocking solution.

⊘ If necessary, the glycoprotein detection can be interrupted at this stage and the filter kept in the blocking solution at +2 to +8°C, for *e.g.,* overnight.

❷ Wash twice for 10 min each in approx. 50 ml TBS and once with Buffer 1.

❸ Lectin incubation: Add the required amount of lectin solution (for GNA, SNA and DSA 10 µl each; for MAA 50 µl and for PNA 100 µl) to 10 ml buffer 1 and incubate the filter for 1 h in this solution.

❹ Wash the blot 3 times for 10 min each with approx. 50 ml TBS.

❺ Incubation with anti-Digoxigenin-AP: Add 10 µl conjugate (vial 6) to 10 ml TBS and incubate the filter for 1 h in this solution.

❻ Wash 3 times for 10 min each in approx. 50 ml TBS.

❼ Staining reaction:
Staining solution (prepare just before use): 10 ml Buffer 2, 200 µl NBT/BCIP solution (vial 7).

⚠ A sometimes appearing precipitation in the NBT/BCIP solution must be removed by heating to 37°C before dilution.

❽ • Immerse the filter without shaking in the staining solution and observe development of the grey to almost black color, which is normally complete within a few minutes.
• Rinse the filter several times with double dist. water to stop the reaction, then dry the blot on paper towels.

⊘ The filter can now be photographed or photocopied and kept for documentation.

⚠ The Ponceau S staining and the glycoprotein detection using anti-Digoxigenin-AP have to be regarded as different procedures. As the protein concentration required for the protein specific staining is higher than for the visualization of the glycoprotein, the detection of the glycoprotein can carried out in spite of a negative result of a previous protein staining.

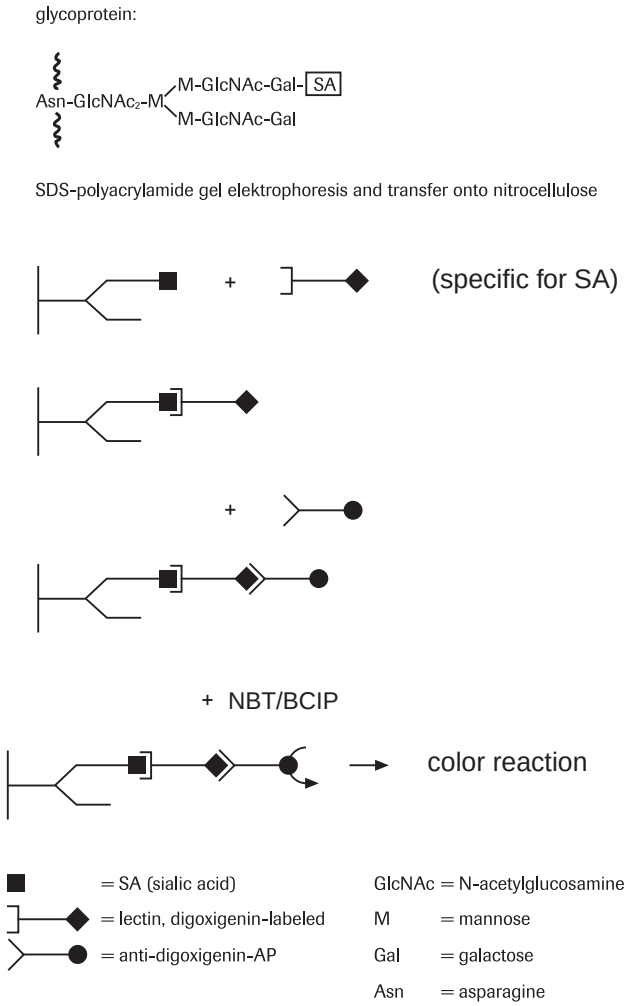
3. Additional Information on this Product

Quality Control

With the lectins GNA, SNA, MAA, DSA and PNA 0.1 µg of the control glycoproteins are detectable after a one-minute incubation in the staining solution.

How this Product Works

The specific binding of lectins to carbohydrate moieties is used to identify these structures. The lectins applied are conjugated with the steroid hapten digoxigenin which enables immunological detection of the bound lectins. When differentiating between carbohydrate structures, lectins which selectively recognice the terminal sugars are used, thus allowing the carbohydrate chain to be identified. In addition to the individual lectins, the kit contains control glycoproteins for demonstrating the specificity of the lectins and for verifying the function of the kit.



Sensitivity

The sensitivity depends greatly on the respective glycoprotein, and also varies between the individual lectins. 0.1 µg of the control glycoproteins transferrin, fetuin and glucose-oxidase is required to produce a positive signal with the lectins SNA, MAA, GNA and DSA after incubation for one minute in the staining solution. 1 µg asialofetuin shows a positive results with the lectin PNA under similar conditions.

⚠ For analyzing low amounts of glycoprotein, a prolonged incubation in the staining solution is required. In this case it is advantageous to keep the membrane longer than 30 min in the blocking solution (*e.g.,* for overnight), which will reduce background staining.

Specificity

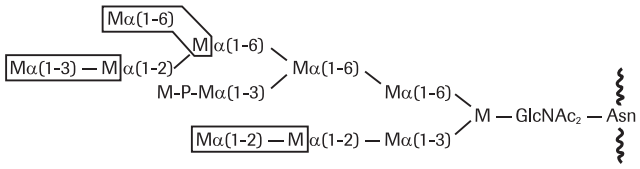
The specificity of lectin affinities enables positive identification of the following sugars:

I. Positive reactions with GNA: indicates mannose, terminally linked.

This reaction confirms the presence of N-glycosidically-linked "high mannose" or "hybrid"-type carbohydrate chains with the exception of yeast glycoproteins, the O-glycosidically-linked mannoses of which also react with GNA. For differentiating between N- and O-linked chains of yeast glycoproteins the mannose residues in the Asn-linked chains may be removed with Endoglycosidase H*, Endoglycosidase F, or N-Glycosidase F* so that only the O-glycosidically-linked mannoses remain.

Example of a high-mannose structure of control glycoprotein

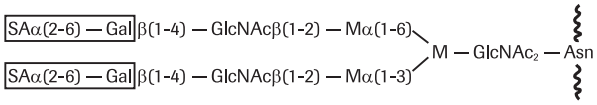
P = phosphate



II. Positive reactions with SNA: indicates sialic acid, terminally linked (2–6) to galactose or N-acetylgalactosamine.

In addition to determining the type of the sialic acid linkage it is possible to ascertain whether the sialic acid is linked to an N-glycan or O-glycan structure, by incubating the respective glycoprotein with N-Glycosidase F*. The absence of a reaction with the lectin SNA after hydrolysis with N-Glycosidase F indicates the presence of sialic acid α(2–6) linked to galactose in a complex N-glycan chain.

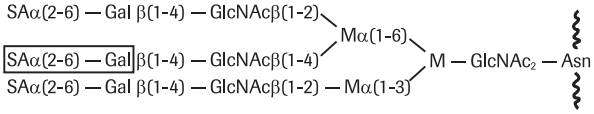
Carbohydrate structure of control glycoprotein transferrin



III. Positive reactions with MAA: indicates sialic acid terminally linked (2–3) to galactose.

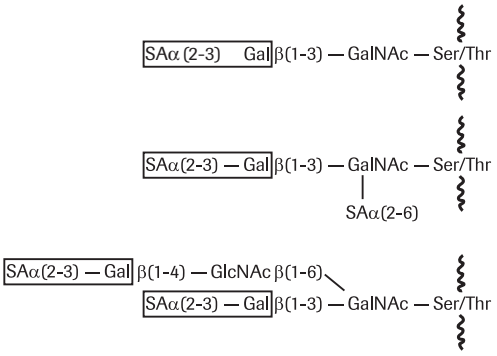
The details given under II. also apply here

Carbohydrate structure of control glycoprotein fetuin which contains three N-glycosidically and three O-glycosidically linked chains.



The structure shows one of numerous variants of the three N-glycan chains.

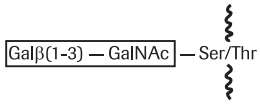
3 O-glycosidically linked chains



IV. Positive reactions with PNA: indicates galactose-β(1–3-N-acetylgalac-tosamine.

This disaccharide usually forms the core unit of O-glycans (exception: yeast glycoproteins). In many cases this disaccharide is substituted by one or two sialic acids which must first be removed with Neuraminidase* in order to obtain a positive result with PNA. In cases of more complex O-glycan structures, such as are found in mucins or membrane glycoproteins, sugars as for example fucose, galactose or N-acetyl-glucosamine must be removed by the respective exoglycosidases in addition to the sialic acid in order to make the core disaccharide accessible to the PNA.

O-linked carbohydrate structure of control glycoprotein asialofetuin, which reacts positively with PNA

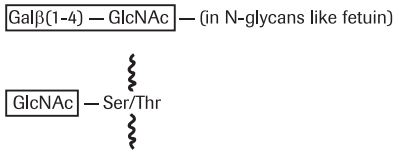


V. Positive reactions with DSA: indicates galactose-ß(1–4)-N-acetylglucosamine.

The disaccharide is present in complex and hybrid N-glycan structures. Thus, complex chains with no terminal sialic acids (which consequently cannot be detected with the SNA or MAA lectins) are identified. DSA does not react with "high-mannose" structures like those found in glucose-oxidase.

DSA also recognizes Gal-b(1–4)GlcNAc structures in O-glycans, such as those found in certain mucins for example. For distinguishing between N- or O-linked glycan chains N-Glycosidase F* may be used as described under II. Furthermore, DSA is suitable for identifying individual N-acetyl-glucosamine residues with O-glycosidic links to serine or threonine in certain proteins from cell nuclei or cytoplasm.

Structures which are recognized:



Summary of lectin affinities for control glycoproteins

Glycoprotein	Lectin				
	GNA	SNA	MAA	DSA	PNA
Glucose-Oxidase	+	-	-	-	-
Transferrin	-	+	-	(+)	-
Fetuin	-	+	+	+	-
Asialofetuin	-	-	-	+	+

+ strong reaction (+) weak reaction - no reaction

Molecular Weight of Control Glycoproteins

In SDS-PAGE [disc-electrophoresis according to Laemmli (7), 10% isocratic gel] the following molecular weights are found for the control glycoproteins:

1. without prior deglycosylation;
2. after (partial) deglycosylation with N-glycosidase F (only the O-linked sugars remain, if present);
3. after complete deglycosylation with N-glycosidase F, O-glycosidase* and neuraminidase.

Molecular weights of main bands are underlined

Control-Glycoprotein	Molecular Weight		
	Without Deglycosylation	N-glycosidase F-treated	Completely deglycosylated
Glucose-Oxidase	80 kDa	68 kDa	68 kDa
Transferrin	80 kDa	75 kDa	75 kDa
Fetuin	68 kDa 65 kDa 61 kDa	53 kDa 45 kDa 38 kDa	51 kDa 43 kDa 38 kDa
Asialofetuin	61 kDa 55 kDa 48 kDa	53 kDa 45 kDa 38 kDa	52 kDa 43 kDa 38 kDa

References

Burnette, W. N. (1981) *Anal. Biochem.* **112,** 195–203.

Laemmli, U. K. (1970) *Nature* **227,** 680–685.

4. Supplementary Information

4.1 Conventions

Text Conventions

To impart information consistent and memorable, the following text conventions are used in this Instruction Manual:

Text Convention	Usage
Numbered Listing using the number symbol type ❶	Numbered working steps which have to be performed according to the succession
Asterisk *	Denotes a product available from Roche Diagnostics.

Symbols

In this Instruction Manual symbols are used as an optical signal to point out important information:

Symbol	Description
	Information Note: Designates a note that provides additional information concerning the current topic or procedure.
⚠	Important Note: This symbol is used to bring your attention to an important annotation.

4.2 Changes to Previous Version

- The control glycoprotein carboxypeptidase Y (vial 8) has been replaced by glucose-oxidase.
- A chapter Quality Control has been added.
- The Typo 'Without glycosilation' within the table 'Molecular Weight' on page 4 has been corrected to 'Without Deglycositation'.
- Editorial changes.

Trademarks

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Disclaimer of License

For patent license limitations for individual products please refer to: List of biochemical reagent products

4.3 Ordering Information

Product	Pack Size	Cat. No.
Endoglycosidases		
N-Glycosidase F, lyophilizate	100 U 250 U	11 365 185 001 11 365 193 001
N-Glycosidase F, solution	100 U (0.1 ml) 250 U (0.25 ml)	11 365 169 001 11 365 177 001
Endoglycosidase H	1 U (200 µl) 2.5 U (500 µl)	11 088 726 001 11 643 053 001
O-Glycosidase	25 U (50 µl)	11 347 101 001
Exoglycosidases		
Neuraminidase, from Arthrobacter ureafaciens	1 U (100 µl)	10 269 611 001
Neuraminidase, from *Clostridium perfringens*, special quality with low protease content	5 U	11 585 886 001
Neuraminidase, from *Vibrio cholerae*	1 U	11 080 725 001

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