PROzyme®

SIGNAL™ DMB LABELING KIT

Convenient labeling of sialic acid with 1,2-diamino-4,5-methylenedioxybenzene (DMB) with subsequent identification by comparison with a Sialic Acid Reference Panel

- High sensitivity and specificity
- Good resolution of O-acetylated derivatives.
- Minimal artifacts due to O-acetyl ester migration from the 7- to the 9- position
- Samples may contain up to 2.5 nmols sialic acid
- Up to 20 individual samples (2 batches of 10)

Product Code: GKK-407

NOTICE: ProZyme was purchased by Agilent in July 2018. Documents for products and product lots manufactured before August 2019 will contain references to ProZyme. For more information about these products and support, go to: **www.agilent.com/en/contact-us.**

Agilent

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KIT CONTENTS

Item	Qty
Vial A - Reaction Solution (Acetic acid/β-mercaptoethanol solution)	2
Vial B - Reductant (Sodium dithionite)	2
Vial C - DMB (1,2-Diamino-4,5-methylenedioxybenzene dihydrochloride)	2
Vial D - Release Agent (Acetic acid solution)	2
Sialic Acid Reference Panel	2

ADDITIONAL REQUIRED ACCESSORIES

Heating block or oven (set at 50°C for labeling, 80°C for release reaction)
HPLC system for analysis
GlykoSep R™ HPLC column (Product Code GKI-4727, available from ProZyme)
Water, HPLC grade
Methanol, HPLC grade
Acetonitrile, HPLC grade

SAFETY AND HANDLING

Please read the Safety Data Sheets (SDS) included with the kit.

All procedures involving labeling reagents should be performed using appropriate personal safety protection, eyeglasses, chemically resistant gloves (e.g. nitrile) and, where appropriate, work in a laboratory fume hood.

Storage Conditions

Upon arrival store at -20°C (± 10°C). The dye in vial C is light sensitive and must be stored in the dark.

The fluorescence intensity of DMB-labeled sialic acids will decrease gradually over several days as the conjugate oxidizes. Samples should be analyzed soon after labeling, preferably within a few hours. If necessary, labeled samples may be stored at -20°C in the dark, but should be analyzed within 72 hours.

General Laboratory Procedures

Use powder-free gloves for all sample handling procedures. Make sure that any glassware, plasticware, solvents or reagents used are free of glycosidases and carbohydrate contaminants.

All steps involving labeling reagents must be performed in a dry environment with dry glassware and plasticware.

Once individual vials of reagents are opened, their contents should be used immediately and the excess discarded.

Minimize exposure of sialic acid-containing samples to elevated temperatures or extremes of pH; high temperatures or low pH will cause degradation of the sialic acids.

INTRODUCTION

Sialic acids are a family of 9-carbon carboxylated 2-keto sugars found on many glycans associated with glycoproteins[1,2]. Over 25 sialic acid derivatives have been identified in nature and have been shown to play a major role in a variety of biological situations.

Sialic acids released from glycoconjugates have been identified by a variety of analytical techniques including TLC, GC, GC-MS, FAB-MS and HPLC. Of these techniques, derivatization of sialic acids with DMB with subsequent separation by reverse-phase HPLC offers many advantages[3,4,5]:

- High sensitivity and specificity
- Good resolution of O-acetylated derivatives.
- Minimal artifacts due to O-acetyl ester migration from the 7- to the 9- position

Using the Kit

Once the component reagents have been opened and mixed, the Labeling Reagent must be used within 1 hour. Two sets of reagents have been included to allow more flexibility for the user.

A maximum of 10 samples including the control Sialic Acid Reference Panel may be labeled with one set of reagents.

Sialic Acid Reference Panel

The Sialic Acid Reference Panel contains a dried mixture of six sialic acid derivatives (see table below) in various amounts totaling ~1.25 nmol/vial.

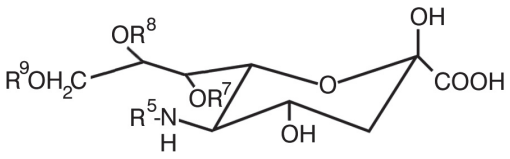

Figure 1 - Sialic Acid Reference Panel

	Position:			
R=	5	7	8	9
Neu5Ac	Ac	H	H	H
Neu5Gc	Gc	H	H	H
Neu5,7Ac2	Ac	Ac	H	H
Neu5Gc9Ac	Gc	H	H	Ac
Neu5,9Ac2	Ac	H	H	Ac
Neu5,7(8),9Ac3	Ac	(Ac) or	(Ac)	Ac

where Ac = Acetyl, Gc = Glycolyl

Handling of the Sialic Acid Reference Panel

The Sialic Acid Reference Panel is shipped as a dried solid. Allow the unopened vial to reach ambient temperature and tap unopened on a solid surface to ensure that most of the material is at the bottom of the vial. Gently remove the cap, add the desired volume of water or buffer, re-cap and mix thoroughly to redissolve all the oligosaccharide. For maximal recovery, ensure that the cap lining is also rinsed and centrifuge the reconstituted vial briefly before use.

DMB-Labeling of Sialic Acids

Glycans typically have no or low absorptivity in both UV and visible light, so detection systems associated with most analytical techniques require the glycan to be labeled with a suitable marker molecule that allows sensitive and quantitative detection. Using reductive amination chemistry, the free reducing end of released glycans may be labeled using fluorescent tags. The resulting tagged glycans may be separated, identified and also quantified using HPLC and/or MS methods.

The Signal DMB Labeling Kit provides a convenient means for derivitizing glycans with DMB. The DMB fluorescent label is non-selective, and therefore provides a pool of labeled sialic acid derivatives in truly stoichiometric amounts. The highly fluorescent DMB moiety allows picomolar quantitation of the carbohydrates.

The Signal DMB Labeling Kit has been optimized by using a large excess of DMB, suitable solvents and moderate reaction conditions to ensure high labeling efficiency while maintaining the structural integrity of the glycan. This efficiency is independent of glycan composition or structure, and degradation of the carbohydrate does not occur due to the short reaction time and mild conditions.

Glycans labeled with DMB may be analyzed by high-sensitivity fluorescence detection using a GlycoSep R HPLC column.

Reductive Amination Reaction

The labeling reaction involves a 2-step process (see Figure 2):

1. Schiff's Base Formation - requires a glycan with a free aldehyde or keto-group, which is in equilibrium between the ring closed (cyclic) and ring open (acyclic) forms. The primary amino group of the dye performs a nucleophilic attack on the carbonyl carbon of the acyclic sugar residue to form a partially stable Schiff's base.

2. Reduction of the Schiff's Base - the Schiff's base imine group is chemically reduced to give a stable labeled glycan.

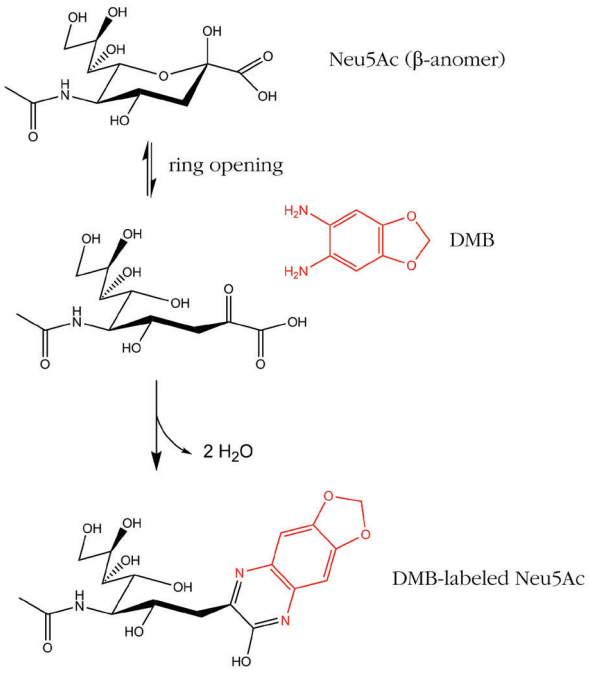

Figure 2 - DMB Labeling Reaction

PROTOCOL

NOTE: Please read this entire booklet before beginning your experiment to obtain successful results.

Outline of the labeling procedure:

1. Sialic acid is released from the glycoconjugate by acid hydrolysis or enzymatic cleavage.

2. A sample aliquot is placed in a reaction vial.

3. Labeling Reagent is prepared fresh by mixing components supplied in the kit.

4. Labeling Reagent is added and the samples incubated at 50°C for 3 hours.

5. The DMB-labeled glycans are analyzed by HPLC within 72 hours.

Sialic Acid Release (Sample Preparation)

Sialic acid must be released from the glycoconjugate prior to labeling with DMB. This kit contains acetic acid for release of sialic acids, irrespective of linkage type, by mild acid hydrolysis, which may be labeled without further purification. However, linkage-specific enzymes may also be used to remove sialic acid for subsequent labeling with DMB. Appendix A lists various ProZyme sialidases and their linkage specificities.

Reagents

Samples of glycoprotein (typically 1-10 µg) or oligosaccharide (0.1-1 µg) containing up to 2.5 nmols of sialic acid. Samples may be dried or dissolved in 5 µl of pure water or buffer.

Vial D - Release Agent (supplied with kit)

Procedure

Transfer samples to 0.5 ml Eppendorf (or similar) tubes.

Add 100 µl of Release Agent (Vial D) to each sample, cap the vial and mix well.

Incubate samples at 80°C for 2 hours.

Cool to room temperature, mix again.

Remove a 5 µl aliquot for labeling.

NOTE: For very small amounts of sialic acid, dry the entire sample using a centrifugal evaporator (Savant, Heto or similar), make up to 5 µl with water, and use the entire sample in the Labeling Protocol.

Labeling Protocol

NOTE: Prepare the labeling reagent immediately prior to use.

Reagents & Supplies

Samples containing 20-500 µM free sialic acid

Sialic Acid Reference Panel (supplied with kit; dissolve in water before labeling as described below)

Vial A - Reaction Solution (supplied with kit)

Vial B - Reductant (supplied with kit)

Vial C - DMB (supplied with kit)

Procedure

Pipette 5 µl sialic acid sample (0.1 - 2.5 nmol) into an Eppendorf tube.

Tap Reductant tube (Vial B) to ensure all solid material is at the base of the vial.

Transfer 220 µl of Reaction Solution (Vial A) to Vial B. Mix by pipette action until the solid in Vial B is fully dissolved.

Transfer the contents of Vial B to Vial C and mix by pipette action. This is the Labeling Reagent.

Add 20 µl of freshly prepared Labeling Reagent to each of the sialic acid samples and to the Sialic Acid Reference Panel.

Cap the reaction vials, mix by vortexing and incubate for 3 hours at 50°C in the dark.

Terminate the labeling reaction by adding 0.5 ml water to each reaction vial and mix thoroughly.

Store at 4°C in the dark. Samples should be analyzed soon after labeling, preferably within a few hours.

Discard remaining reagents from all open vials.

NOTE: The fluorescence intensity of DMB-labeled sialic acids will decrease gradually over several days as the conjugate oxidizes. Labeled samples may be stored at -20°C in the dark to slow oxidation, but should be analyzed within 72 hours.

Analysis of DMB-Sialic Acids

The preferred method of analysis for DMB-sialic acids is reverse-phase HPLC on a GlycoSep R column. A typical chromatogram of DMB-labeled sialic acid reference panel is shown in Figure 3.

Suggested analytical system:

Column: GlycoSep R HPLC column

Solvent: Acetonitrile:methanol:water (9:7:84). In some cases it may be convenient either to change the proportion of acetonitrile in the solvent or to run a gradient of acetonitrile (from 5 to 10% v/v).

Flow rate: 0.5-0.9 ml/min.

Detection: Fluorescence (λ_{ex} = 373 nm, λ_{em} = 448 nm)

While differences in retention times may occur with different columns, flow rate, solvent or laboratory conditions, the elution order of DMB-sialic acids is consistent. The profile obtained should be compared with Figure 3 to identify the components.

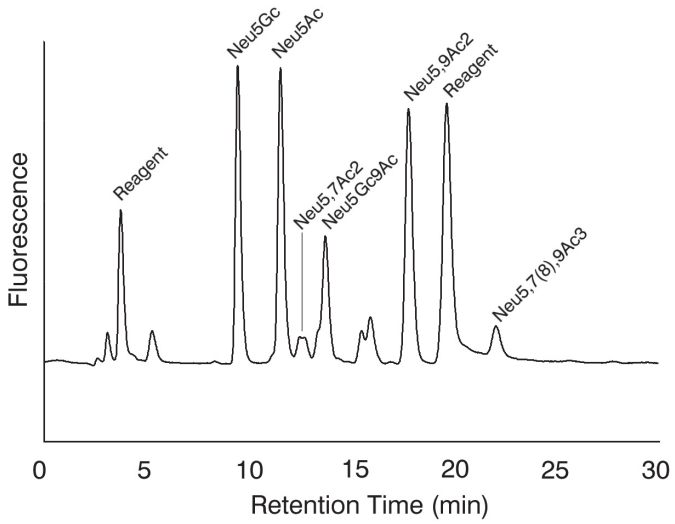

Figure 3 - Profile of DMB-labeled Sialic Acid Reference Panel

Detection: Fluorescence λ_{ex} = 373 nm, λ_{em}= 448 nm
Column: GlycoSep R 150 x 4.6 mm
Flow rate: 0.5 ml/min
Solvent: acetonitrile:methanol:water (9:7:84)
Sample: DMB-labeled Sialic Acid Reference Panel

Confirmation of O-acetylation

O-acetyl groups present on sialic acids are base labile and may be removed by incubating the glycoconjugate in 0.1 M NaOH on ice for 2 hours. Neutralize with an equimolar amount of acetic acid, dry the sample and proceed with the Protocol for release and labeling of sialic acids. Upon analysis, the signals for the O-acetyl groups should disappear and the signals for N-acetyl and N-glycolyl sialic acid should increase correspondingly.

REFERENCES

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3. Hara S, Yamaguchi M, Takemori Y, Furuhata K, Ogura H and Nakamura M. Determination of mono-O-acetylated N-acetylneuraminic acids in human and rat sera by fluorometric high-performance liquid chromatography. Anal Biochem 1989 179:162-166.

4. Manzi AE, Diaz S and Varki A. High-pressure liquid chromatography of sialic acids on a pellicular resin anion-exchange column with pulsed amperometric detection: a comparison with six other systems. Anal Biochem 1990 188:20-32.

5. Butor C, Diaz S and Varki A. High level O-acetylation of sialic acids on N-linked oligosaccharides of rat liver membranes. Differential subcellular distribution of 7- and 9-O-acetyl groups and of enzymes involved in their regulation. J Biol Chem 1993 268:10197-10206.

APPENDIX A: LINKAGE-SPECIFIC SIALIDASES

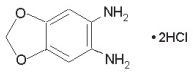
Linkage-specific sialidases may be used to remove sialic acid. No purification is necessary before labeling with DMB. These linkage-specific sialidases are available from ProZyme:

Code	Source	Specificity
GK80040	Arthrobacter ureafaciens	α(2-6,3,8,9)
GKX-5017	Newcastle Disease Virus	α(2-3,8)
GKX-5018	Salmonella typhimurium	α(2-3>6>>8,9)
GKX-5020	Vibrio cholerae	α(2-3,6,8)
GK 80020	Streptococcus pneumoniae	α(2-3)

APPENDIX B: PROPERTIES OF DMB

Product: 1,2-diamino-4,5-methylenedioxybenzene dhydrochloride B)

Structure:



Molecular formula: $C_7H_8N_2O_2 \cdot 2HCl$

Molecular weight: 225.07

Excitation λ_{Max}: 373 nm

Emission λ_{Max}: 448 nm

TECHNICAL ASSISTANCE

If you have any questions or experience difficulties regarding any aspect of our products, please contact us:

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