PROzyme®

Signal™ 2-AB-*plus* Labeling Kit

Convenient fluorescent labeling of glycans with 2-AB (2-aminobenzamide) by reductive amination. Any purified glycan or glycan pool with a free reducing end may be labeled.

- Labeling efficiency typically >85%
- Sufficient Labeling Reagent in each reaction for up to 50 nmols of glycan (up to 48 individual samples).
- Labeling components may be stored and re-used
- Useful for profiling and quantitation

Product Code: GKK-804

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

TABLE OF CONTENTS

This product is intended for in vitro research use only.

KIT CONTENTS

NOTE: We want successful results for our customers, so please read this entire booklet before starting the experiment.

Item		Qty
WS0322	2-AB Solution	1 ea
WS0323	Reductant Solution	1 ea

Additional Required Reagents/Equipment

GlykoPrep Cleanup Module (Product Code GS96-CU) or GlycoClean™ S Cartridges (Product Code GKI-4726), available from ProZyme, 1 cartridge per sample for pre- and post-labeling cleanup

Water, HPLC grade

Acetonitrile, HPLC grade

Heating block, oven or similar dry heater set at 65°C

Centrifugal evaporator (e.g., Savant, Heto or similar)

Reaction vials (e.g., polypropylene microcentrifuge vials)

SAFETY AND HANDLING

Some of the reagents in this Kit are hazardous. Please refer to the Safety Data Sheets (SDS) posted on ProZyme's website under the component name or Product Code.

http://www.prozyme.com

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

Storage Conditions

The 2-AB Solution and Reductant Solution are hygroscopic and the 2-AB Solution is light-sensitive; please store these reagents at -20°C in their original packaging both before and after opening. Allow the 2-AB Solution and Reductant Solution vials to come to room temperature in the sealed desiccant bag before removing them.

Store glycans labeled with 2-AB at -20°C in the dark. Allow labeled glycans to equilibrate to room temperature upon removal from storage.

General Laboratory Procedures

Use powder-free gloves for all sample handling procedures. Ensure that all glass, plasticware or solvents are free of glycosidases and environmental carbohydrates.

All procedures involving Labeling Reagents (2-AB Solution, Reductant Solution and 2-AB Labeling Reagent) should be performed in a dry environment with dry glassware and plasticware, using appropriate personal safety protection, eyeglasses and nitrile gloves, and where appropriate, in a fume hood.

INTRODUCTION

Glycans typically have no or low absorbtivity 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 with fluorescent tags. The resulting labeled glycans may be purified, separated, identified and also quantified using LC and/or MS methods.

The Signal 2-AB-plus Labeling Kit provides a convenient means for derivitizing glycans with 2-AB. The fluorescent label is non-selective, and therefore provides a pool of labeled glycans in truly stoichiometric amounts. The reductive amination procedure is optimized to help preserve labile glycans, such as sialic acid. For highly sialylated samples, shorter incubation times and/or reduced temperatures (e.g., overnight at 37°C) can decrease desialylation.

The Signal 2-AB-plus Labeling Kit has been optimized by using an excess of 2-AB and moderate reaction conditions to ensure high labeling efficiency (>85%) while maintaining the structural integrity of the glycan. This efficiency is independent of glycan composition or structure.

Reductive Amination Reaction

The labeling reaction involves a 2-step process (see Figure 1):

1. Schiff's Base Formation - requires a glycan with a free reducing sugar, which is in equilibrium between the ring closed (cyclic) and ring open (acyclic) forms. The primary amine group of the dye performs a nucleophilic attack on the carbonyl carbon of the acyclic reducing 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 by cyanoborohydride to give a stable, labeled glycan.

Using the Kit

The 2-AB Labeling Reagent should be prepared no more than one hour before use. Prepare only the amount required and store the components for reuse.

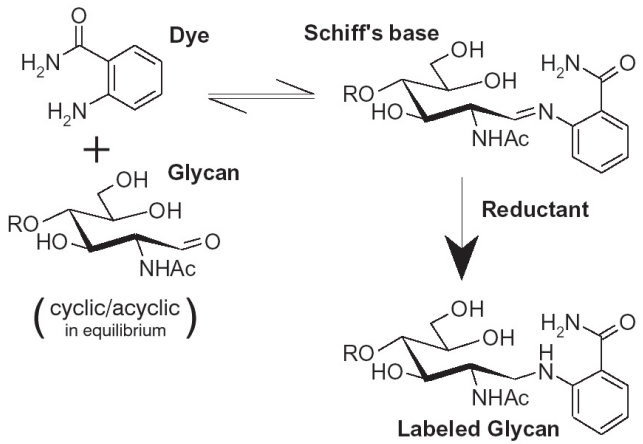

Figure 1 - Labeling a reducing glycan with 2-AB

PROTOCOLS

Overview of the Labeling Procedure

1. Prior to labeling, glycan samples should be purified to remove protein, peptides, salts, detergents and any other contamination that could interfere with the Labeling Procedure. Examples of glycan isolation protocols include:
   - Cold ethanol precipitation
   - Molecular weight cut off filtration
   - Solid phase extraction (e.g., HILIC, normal phase, reversed phase)

2. Each sample is placed in a reaction vial and dried.

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

4. Labeling Reagent is added and the samples incubated at 65°C for 1-4 hours.

5. Excess Labeling Reagent may be removed from the samples using the GlycoClean S Cartridge cleanup

procedure or the GlykoPrep Cleanup Module.

The labeled glycans are now ready for analysis.

Sample Preparation

Clean glycan samples - the amount of sample should be in the range of 100 picomoles to 50 nanomoles for a glycan pool obtained from a typical glycoprotein. With a single pure glycan, as little as 5 picomoles may be labeled.

Dry the aqueous samples in a centrifugal evaporator. NOTE: Lyophilization may be used with caution. Specifically, ensure that the sample dries to a small, compact mass at the very bottom of the tube.

2-AB Labeling Reaction

Reagents

2-AB Solution (supplied with the Kit)
Reductant Solution (supplied with the Kit)

Preparation of 2-AB Labeling Reagent

NOTE: 2-AB Labeling Reagent should be prepared no more than one hour before use.

NOTE: 2-AB Labeling Reagent components are hazardous. Please refer to the Safety Data Sheets included in the Kit or on our website.

NOTE: Both the 2-AB Solution and Reductant Solution are hygroscopic; minimize exposure to air and protect from exposure to light. The individual reagents may be resealed, repackaged with the desiccant in the resealable bag, and frozen (-20°C) for storage up to 6 months; return to RT before opening for use to minimize condensation.

Allow the 2-AB Solution and Reductant Solution vials to come

to room temperature in the sealed desiccant bag before removing them. Before opening each vial, flick it or gently tap it on a flat surface to dislodge any liquid adhering to the underside of the cap and ensure that the contents collect at the bottom.

In a separate vial, prepare a Labeling Reagent master mixture: Determine the number of samples to be labeled. For each sample to be processed, add 3 µl of 2-AB Solution and 3 µl of Reductant Solution.

Cap tightly and vortex on high for 10 seconds to mix; briefly spin down in a centrifuge.

Tightly cap the 2-AB Solution and Reductant Solution vials, return to the desiccant-containing bag and store at -20°C.

Labeling Procedure

Add 5 µl of Labeling Reagent to each dried glycan sample, cap the microtube, mix thoroughly, and gently tap or centrifuge at low speed to ensure the contents are at the bottom of the vial.

Place the reaction vials in a heating block, sand tray or dry oven set at 65°C. Incubate for 3 hours.

NOTE: The incubation should be performed in a dry environment (see Appendix A: Tips & Hints, page 20).

NOTE: In most cases, the incubation time may be shortened to 1 hour or extended to 4 hours without significantly changing the outcome.

After incubation, centrifuge each reaction tube briefly to incorporate any liquid that may have condensed on the top and sides.

Allow to cool completely to room temperature.

NOTE: Proceed to post-labeling cleanup immediately after the incubation.

Post-labeling Cleanup

Sample cleanup to remove excess dye and other labeling reagents is necessary for certain applications, e.g., subsequent analysis by liquid chromatography. Cleanup can be achieved using ProZyme's GlykoPrep Cleanup Module or GlycoClean S Cartridges, or equivalent technologies.

Instructions for use of the GlykoPrep Cleanup Module (product code GS96-CU) may be found on ProZyme's webpage:

prozyme.com/documents/GS96-CU_Instruction_Manual.pdf

Instructions for use of GlycoClean S Cartridges (product code GKI-4726) may be found on ProZyme's webpage:

prozyme.com/pdfs/gki-4726.pdf

Analysis of Glycans Labeled with 2-AB

Use standard techniques, such as Liquid Chromatography (LC), Mass Spectrometry (MS), or a combination of the two, to analyze the aqueous eluate containing eluted, labeled N-glycans.

UPLC® Analysis

Suggestions for rapid UPLC analytical techniques may be found in TechNote TNJP101, UPLC Rapid Methods, on ProZyme's webpage:

http://www.prozyme.com/tech_notes.html

Enzymatic Analysis

ProZyme's Glyko® line of high purity, sequencing-grade enzymes is suitable for structural analysis of both N- and

O-linked glycans labeled with 2-AB.

Mass Spectrometry

Mass spectrometry and various types of spectroscopic methods may also be used to analyze glycans labeled with 2-AB. The label is stable under acidic and alkaline conditions and does not interfere with the action of exoglycosidases (Bigge et al.; Guile et al., 1996; Townsend et al., 1996 and Hardy, 1997). Note, however, that glycan structures may not be stable under extremes of pH. For this reason, users are advised not to subject 2-AB-labeled glycans to strongly acidic or alkaline conditions.

REFERENCES

Bigge, J. C., Patel, T. P., Bruce, J. A., Goulding, P. N., Charles, S.M. and R. B. Parekh. Non-selective and efficient fluorescent labeling of glycans using 2-aminobenzamide and anthranilic acid. Anal Biochem 230: 229-238 (1995).

Guile, G. R., Rudd, P. M., Wing, D. R., Prime, S. B. and R. A. Dwek. A rapid and high-resolution high-performance liquid chromatographic method for separating glycan mixtures and analyzing oligosaccharide profiles. Anal Biochem 240: 210-226 (1996).

Hardy, M. R. Glycan labeling with the fluorophores 2-aminobenzamide and anthranilic acid in Techniques in Glycobiology (Townsend, R. R and Hotchkiss, A. T. Marcel, eds) Dekker Inc, New York (1997).

Townsend, R. R., Lipniunas, P. H., Bigge, C., Ventom, A. and R. Parekh. Multimode high-performance liquid chromatography of fluorescently labeled oligosaccharides from glycoproteins. Anal Biochem 239: 200-207 (1996).

Technical Notes

TechNotes referred to in the text may be found on ProZyme's website at:

http://www.prozyme.com/tech_notes.html

TECHNICAL ASSISTANCE

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

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FAX **(510) 638-6919**
E-MAIL **info@prozyme.com**
WEB **www.prozyme.com**

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For a complete list of ProZyme Patents, please visit our website at

http://www.prozyme.com/patent.html

OTHER PROZYME PRODUCTS & KITS

ProZyme offers a number of glycans labeled with 2-AB to use as qualitative standards. Find them on our webpage.

A wide variety of other glycobiology products are available from ProZyme. A complete listing is accessible on our website:

http://www.prozyme.com

PRODUCT USE AND WARRANTY

Terms and conditions of sale may be found at:

http://www.prozyme.com/terms.html

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For North American destinations: telephone orders may be placed between 8:00 am and 5:00 pm Pacific Time. Telefax or e-mail orders may be sent or messages recorded anytime.

TOLL FREE (800) 457-9444 (US & CANADA)
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E-MAIL info@prozyme.com
WEB www.prozyme.com

Outside North America:

A list of ProZyme's distributors, with contact information, may be found at:

http://www.prozyme.com/distributors.html

If there is no distributor in your area, place an international order directly at:

http://www.prozyme.com/ordering.html

APPENDIX A: TIPS & HINTS

Calculating the Mass of Glycans Labeled with 2-AB

The reductive amination reaction results in the loss of an oxygen atom. The mass of the 2-AB-labeled N-glycan is obtained using the following formula:

$$Mass_{Glycan} + Mass_{2\text{-}AB} = Mass_{2\text{-}AB\text{-}Labeled\ Glycan} - Mass_{Oxygen}$$

Mass Added to Glycan	
Monoisotopic	120.06875
Average	120.2

Estimating the Amount of Glycoprotein Needed for Labeling
Since the degree of glycosylation for a given protein may vary widely, this example gives an approximation of the amount of protein required to generate 50 nmol of glycan for labeling.

Assume glycosylation is 2-5% of the protein by weight; 1 mg of protein contains ~50 µg of glycan. Assuming an average MWof a typical glycan structure is ~1,000 g/mol, 50 µg of glycan is ~50 nmol of sample for labeling.

A water bath may be used if vials are kept tightly sealed during the incubation at 65°C. The presence of water promotes desialylation. Use parafilm to seal Eppendorf tubes so they do not pop open while heating.

Troubleshooting the Labeling Reaction

The GKK-804 2-AB labeling protocol is an efficient, robust method. If problems do arise they can normally be corrected without difficulty. These are the most likely problems, possible causes and solutions:

Poor Incorporation of 2-AB/Low Labeling Yield

The labeling temperature may be incorrect. Ensure that the oven or heating block is equilibrated to 65°C and that the reaction tube is subjected to this temperature for the entire incubation period.

The sample may be incompletely solubilized. The glycans must be completely dissolved in the labeling mixture for maximum labeling efficiency. Ensure that the sample is thoroughly mixed with the labeling reagent prior to the 65°C incubation and, as a precaution, vortex the samples 30 minutes after the start of the incubation as described in the protocol.

The sample may have contained contaminants that interfered with the labeling. Ensure that the glycans are adequately purified before labeling.

The labeling solution may have been inactive. Make up the labeling solution immediately before use; the reagents lose optimal activity within an hour of mixing.

Less glycan than was originally estimated

The glycans may not contain a free reducing sugar. 2-AB conjugates to the aldehyde group of the free reducing sugar. Alditols and glycans already conjugated via their reducing terminus (e.g., glycopeptides, glycolipids, and previously labeled glycans) do not contain a free reducing sugar and so cannot conjugate to 2-AB.

Glycans were lost during post-Labeling cleanup. Ensure that the removal of excess labeling reagents in the Post-Labeling Cleanup protocol is performed as specified and that the wash reagents are correctly made. Be especially careful during preparation of Acetonitrile solutions; high percentages of water will cause glycans (especially small molecular mass sugars) to inappropriately elute from the Cleanup (CU) Cartridge or the S Cartridge adsorption disc.

Labeled Samples Contain Fluorescent Non-glycan Material

The glycan samples contained aldehyde-bearing or other electrophilic contaminants. Ensure that the glycans are adequately purified before labeling.

The post-labeling cleanup step did not work correctly. Ensure that the cleanup steps are performed as specified and that the wash reagents are made and stored correctly. For example, 96% Acetonitrile is necessary to wash glycans; use of a lower percent solution or a drop in percent Acetonitrile due to evaporation from inadequately sealed vials can lead to preferential loss of smaller glycans.

Selective Loss of Smaller Glycans

The GlycoClean S cartridge may not have been primed correctly. Ensure the cartridge is prepared as described, and that the adsorption disc is still wet with acetonitrile when the sample is applied to the disc.

The glycans may not have adsorbed onto the GlycoClean S cartridge correctly. If using the GlycoClean S Cartridge, ensure the sample is left to adsorb on the disc for 15 minutes before washing.

Incorrect wash reagents may have been used during post-labeling cleanup. Ensure that the wash reagents are correctly prepared. Be especially careful during preparation of acetonitrile solutions; high percentages of water will cause glycans (especially small molecular-mass sugars) to inappropriately elute from the adsorption disc.

NOTE: CU Cartridges may typically be used for sugars or oligosaccharides as small as trisaccharides (GU > 3).

Selective Loss of Larger Glycans

The sample was incompletely solubilized. Glycans must be completely dissolved in the labeling mixture for maximum labeling efficiency. Larger glycans tend to be less soluble in the labeling mixture than smaller sugars. Ensure that the sample is thoroughly mixed with Labeling Reagent prior to the 65°C incubation and, as a precaution, samples may be vortexed 30 minutes after the start of the incubation.

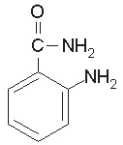
Desialylation of Glycans

The sample may have been subjected to acidic conditions in aqueous solutions at elevated temperatures. Avoid prolonged exposure of samples containing sialylated glycans in aqueous solutions to low pH and elevated temperature. Reductive amination of glycans is carried out under essentially anhydrous conditions; therefore, sialic acid loss should be minimal. To minimize the risk of acid-catalyzed desialylation of glycans, promptly remove samples from incubation after labeling is complete and proceed to post-labeling cleanup. In general, glycans in aqueous solution should be kept between pH 5 - 8.5 at temperatures below 30°C. For long-term storage, keep at -20°C in the dark.

APPENDIX B: PROPERTIES OF 2-AB

Product: 2-aminobenzamide (2-AB)

Structure:



Monoisotopic Mass: 136.0637

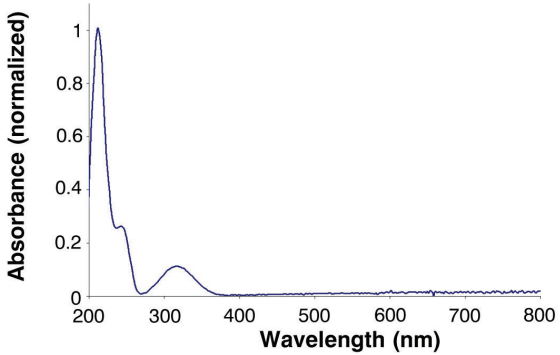


Figure 2- Absorbance Spectrum (peak location at 330 nm)

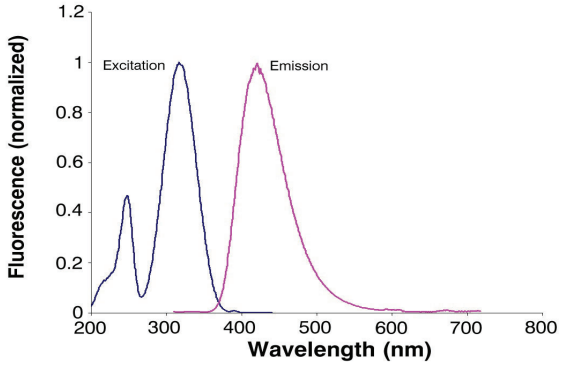


Figure 3 - Fluorescence, peak emission at 420 nm (arbitrary units normalized to absorbance. excitation 200-450 nm, emission 300-750 nm)

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