PROzyme®

SIGNAL™ 2-AB 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 sugar may be labeled

- Labeling efficiency typically >85%
- No detectable loss of sialic acid or fucose
- Samples may contain up to 50 nmols of glycan
- Up to 36 individual samples (2 batches of 18)
- Useful for profiling and quantitation

Product Code GKK-404

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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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
Vial A 2-AB Dye (2-aminobenzamide, 5 mg)	2 ea
Vial B DMSO (350 µl)	2 ea
Vial C Glacial acetic acid (200 µl)	2 ea
Vial D Reductant (sodium cyanoborohydride, 6 mg)	2 ea

Additional Required Reagents/Equipment

GlycoClean™ S Cartridges (Product Code GKI-4726, available from ProZyme; 1 per sample for post-labeling cleanup)
Water, HPLC grade
Acetonitrile, HPLC grade
Acetic acid (glacial), 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

Please read the Material Safety Data Sheets (MSDS) 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, in a laboratory fume hood.

Storage Conditions

Store kit in a dry environment at room temperature. The dye in vial A is light sensitive and must be stored in the dark.

Store glycans labeled with 2-AB at -20°C in the dark. Glycans labeled with 2-AB have the same stability characteristics as the unlabeled glycan; the 2-AB label is retained during subsequent analytical techniques.

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 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.

LICENSE TO USE

By accepting delivery of the 2-AB Kit or labeled standards [Material(s)] and by subsequently using them in glycan analysis, Recipient agrees to be bound by the following terms and restrictions:

1. A Use Sublicense is granted Recipient for in-house use of Material(s) only.

2. The Material(s) will not be made available by Recipient to any outside parties in any form, separately or in combinations, for any monetary or other consideration or at no charge, except that the Materials may be made available to outside parties who agree to be bound by all the terms and restrictions of this Agreement for purposes of evaluation only.

3. Recipient will not make commercial use of the Material(s) unless it first secures a license agreement from ProZyme, Inc. for such commercial use.

4. Recipient is solely responsible for qualification of the products for the Recipient's specific use.

5. The Material(s) will not be used *in vivo* in humans.

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 HPLC and/or MS methods.

The Signal 2-AB 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 causes no detectable loss of sialic acid or fucose. The highly fluorescent 2-AB moiety allows picomolar quantitation of carbohydrates.

The Signal 2-AB Labeling Kit has been optimized by using a large excess of 2-AB, suitable solvents and moderate reaction conditions to ensure high labeling efficiency (>85%) while maintaining the structural integrity of the glycan (see Figure 1). This efficiency is independent of glycan composition or structure, and carbohydrate degradation does not occur due to the short reaction time and mild conditions.

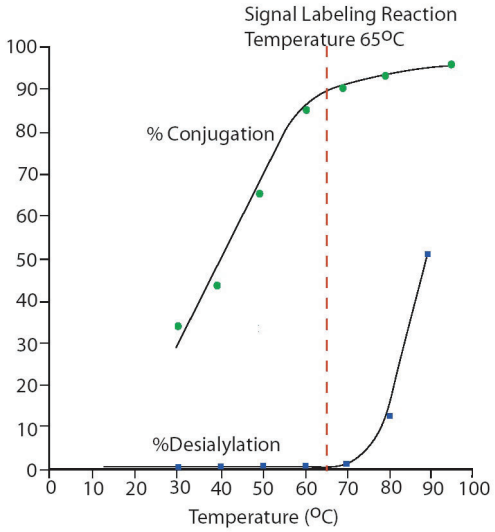

Figure 1 - Optimization of 2-AB Labeling

Glycans labeled with 2-AB may be followed by high-sensitivity fluorescence detection or monitoring of UV-absorbance during various analytical procedures. These include chromatography on GlycoSep™ HPLC columns and sequencing using ProZyme's Glyko exoglycosidases (Bigge *et al.*; Guile *et al.*, 1996 and Townsend *et al.*, 1996).

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 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

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 18 samples may be labeled with one set of reagents, depending on the precision with which 5 µl aliquots are dispensed into the individual reactions.

The size of the labeling reactions has been chosen based on the limitation of the GlycoClean S cartridge used in the post-labeling cleanup. Split larger samples into multiple labeling reactions and pool after the cleanup step.

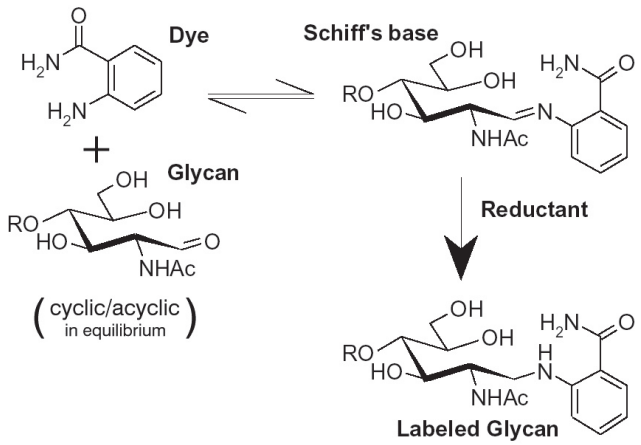

Figure 2 - Labeling a reducing glycan with 2-AB

Opening the Component Ampules

Gently tap the ampule to settle the contents on the bottom. Take proper safety measures for handling the contents as described in the enclosed MSDS's. To open, hold both the body and the top of the ampule, then gently but firmly snap open at the colored break-ring. Snap away from your body.

Fluids may be pipetted into or out of the ampules with standard pipettors or syringes with slim tips or needles. Be careful of the sharp edges of the opening *(be sure to wear gloves and safety glasses during these operations).*

PROTOCOLS

Glycan samples to be labeled, whether purified glycans or a glycan mixtures, must contain a free reducing sugar, be relatively particle and salt-free, and be presented in a volatile solvent system (preferably in water). Suitable glycan samples may be prepared from glycoproteins using one of the range of endoglycosidases (including N-Glycanase®) available from ProZyme.

Outline 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.

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 3 hours.

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

The labeled glycans are now ready for analysis.

Sample Preparation

Reagents

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 and detected in subsequent GlycoSep HPLC analysis.

Procedure

Dry the aqueous samples in a centrifugal evaporator.

NOTE: Do not subject samples to high temperatures (>28° C) or extremes of pH as these conditions will result in acid catalyzed loss of sialic acids (high temperatures, low pH) or epimerization of the reducing sugars (high pH).

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 Dye (Vial A, supplied with the kit)

DMSO (Vial B, supplied with the kit)

Acetic acid (Vial C, supplied with the kit)

Reductant (Vial D, supplied with the kit)

Procedure

Add 150 µl of acetic acid (from Vial C) to a vial of DMSO (Vial B) and mix by pipette action.

NOTE: Tap or flick the vials to dislodge any contents in the upper half before opening.

NOTE: *If the DMSO is frozen, gently warm the vial (before opening) in an oven or heating block set between 30° C and 65° C.*

Add 100 µl of acetic acid/DMSO mixture to a vial of 2-AB Dye (Vial A) and mix until the dye is dissolved.

Add all of the acetic acid/DMSO/2-AB dye mixture to a vial of Reductant (Vial D) and mix by pipette action until the reductant is completely dissolved. This is the Labeling Reagent; protect from exposure to moisture and use within 1 hour.

NOTE: If the reductant is difficult to dissolve, gently warm the vial for up to three minutes in the 65° C incubation oven or stand on a heating block at 65°, then mix by pipette action. If undissolved reductant is still visible add 10 µl water (HPLC grade) to the vial and mix.

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 22).

NOTE: The samples must be completely dissolved in the Labeling Reagent for efficient labeling. To encourage complete dissolution, the samples may be vortexed briefly 30 minutes after the start of the 65° C incubation and the incubation continued.

NOTE: In most cases, the incubation time may be shortened to 2 hours 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. The cleanup procedure and sample finishing should be conducted reasonably quickly to avoid acid-catalyzed desialylation.

Post-labeling Cleanup

Sample cleanup to remove excess dye and other labeling reagents is necessary for certain applications, *e.g.* subsequent analysis by HPLC. Cleanup can be achieved using GlycoClean S cartridges.

GlycoClean S Cartridge Cleanup

GlycoClean S cartridges contain a membrane that retains a wide range of glycans in >90% acetonitrile solutions; monosaccharides and disaccharides generally interact too weakly for efficient retention. Most hydrophobic non-glycan contaminants either pass through the membrane or are retained weakly and may be washed off. The glycans are then eluted from the membrane with water.

The cartridge is first primed with acetonitrile and then a sample loaded. The glycans adsorb while excess dye is removed by washing with acetonitrile. The glycans are then desorbed by washing with water.

S cartridges should be used only once.

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

http://www.prozyme.com/pdfs/gki-4726.pdf

Sample Finishing

Filter the sample (if appropriate) and evaporate to dryness using a centrifugal evaporator.

Redissolve in a desired volume of water or other suitable solvent for further analysis.

Store at -20°C in the dark.

Analysis of Glycans Labeled with 2-AB

Glycans labeled with 2-AB may be studied by a number of analytical methods including HPLC and mass spectrometry.

HPLC Analysis

Glycan mixtures labeled with 2-AB may be separated and analyzed by HPLC with these GlycoSep HPLC columns (available from ProZyme):

Code	Column	Analyses
GKI-4721	GlycoSep C	Separation of neutral/charged glycans
GKI-4728	GlycoSep N	Profiling of neutral/charged glycans
GKI-4727	GlycoSep R	Separation of neutral glycans

GlycoSep N is the most versatile column of the three GlycoSep columns and is routinely used to purify and/or analyze 2-AB-labeled oligosaccharides from complex glycan mixtures.[2]

Enzymatic Analysis

ProZyme's Glyko range 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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WEB **www.prozyme.com**

ProZyme values customers opinions and considers customers an important source for information regarding advanced or specialized uses of our products. We encourage you to contact us. We welcome your suggestions about product performance or new applications and techniques.

Also, contact your local distributor:

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

APPENDIX A: TIPS & HINTS

Calculating the MW of Glycans Labeled with 2-AB The reductive amination reaction results in the loss of an oxygen atom. So the MW of the labeled glycan is:

$$MW_{Glycan} + MW_{2\text{-}AB} (136.2) - 16$$

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 is ~50 µg of glycan. Assuming an average MW of 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 retards the labeling reaction. Use parafilm to seal Eppendorf tubes so they do not pop open while heating.

Troubleshooting the Labeling Reaction

The GKK-404 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 Dye/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 the 96% Acetonitrile Solution; higher percentages of water will cause glycans (especially small molecular mass sugars) to inappropriately elute from the adsorption disc.

Labeled Samples Contain Fluorescent Non-glycan Material

The glycan samples contained aldehyde-bearing 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 correctly.

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. 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 the 96% acetonitrile solution; higher percentages of water will cause glycans (especially small molecular-mass sugars) to inappropriately elute from the adsorption disc.

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 small sugars. Ensure that the sample is thoroughly mixed with Labeling Reagent prior to the 65°C incubation and, as a precaution, vortex the samples 30 minutes after the start of the incubation.

Desialylation of Glycans

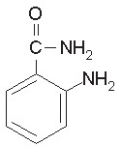
The sample may have been subjected to acidic conditions in aqueous solutions at elevated temperatures. Avoid prolonged exposure of sialylated glycans in aqueous solutions to low pH and elevated temperature. The reductive amination reaction (Signal labeling) is carried out under essentially anhydrous conditions so sialic acid loss should be minimal. In general, glycans in solution should be kept in the pH range 5 - 8.5 at temperatures below 30°C.

The samples may have experienced acid-catalyzed desialylation. Ensure that samples undergo post-labeling cleanup immediately after reductive amination, and that cleanup and finishing are conducted reasonably quickly.

APPENDIX B: PROPERTIES OF 2-AB

Product: 2-aminobenzamide (2-AB)

Structure:



Molecular weight: 136.15

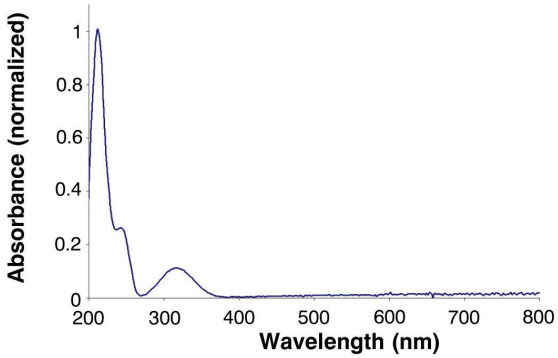


Figure 3 - Absorbance Spectrum (peak location at 330 nm)

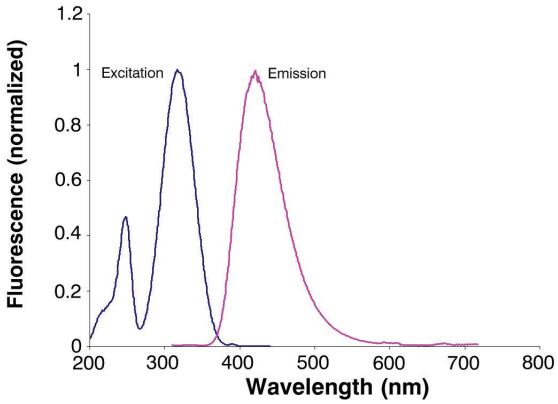


Figure 4 - Fluorescence, peak emission at 420 nm (arbitrary units normalized to absorbance. excitation 200-450 nm, emission 300-750 nm)

Storage: Store the dye at room temperature in the dark. Store glycans labeled with 2-AB at -20°C in the dark.

OTHER PROZYME PRODUCTS & KITS

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

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

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

http://www.prozyme.com/glyko

PRODUCT USE AND WARRANTY

Terms and conditions of sale as well as product warranties may be found at:

http://www.prozyme.com/pdfs/terms-products.pdf

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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.

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WEB **www.prozyme.com**

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

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