Product Guide for LudgerTag™ 2-AB (2-aminobenzamide) Glycan Labelling Kit

(Ludger Product Code: LT-KAB-A2)

Ludger Document # LT-KAB-A2-Guide-v3.2

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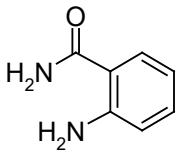
Application For labelling of free glycans with 2-aminobenzamide acid (2-AB).

Description The kit contains reagents for the conjugation of dye to the free reducing end of the glycan by a reductive amination reaction.

Dye Properties Mass = 136.15

Fluorescence, λ_{ex} = 320 nm, λ_{em} = 420 nm.



Number of Samples Typically, up to 15 separate analytical samples per set of labelling reagents.

Amount of Sample From 25 pmol up to 25 nmol glycans per sample.

Suitable Samples Any purified glycans with free reducing termini can be labelled.

Structural Integrity No detectable (< 2 mole per cent) loss of sialic acid, fucose, sulfate, or phosphate.

Labelling efficiency Typically > 85 % (dependent on sample).

Labelling Selectivity Essentially stoichiometric labelling.

Storage: Store at room temperature in the dark. Protect from sources of heat, light, and moisture. The reagents are stable for at least two years as supplied.

Shipping: The product can be shipped at ambient temperature.

Handling: Ensure that any glass, plasticware or solvents used are free of glycosidases and environmental carbohydrates. Use powder-free gloves for all sample handling procedures and avoid contamination with environmental carbohydrate.
All steps involving labelling 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 excess then discarded according to local safety rules.

Safety: **For research use only. Not for human or drug use**
Please read the Material Safety Data Sheets (MSDS's) for all chemicals used. All processes involving labelling reagents should be performed using appropriate personal safety protection - eyeglasses, chemically resistant gloves (e.g. nitrile), etc. - and where appropriate in a laboratory fume cupboard.

Kit Contents

Each labelling reaction set consists of one vial of each of the following:

Cat. #	Item	Quantity
LT-2AB-01	2-AB Dye (2-Aminobenzamide)	5 mg
LT-DMSO-01	DMSO	350 µL
LT-ACETIC-01	Acetic acid	200 µL
LT-CYANOB-01	Sodium cyanoborohydride (Reductant)	6 mg

Additional Reagents and Equipment Required

- Heating block, oven, or similar dry heater (a water bath cannot be used) set at 65°C
- Centrifugal evaporator (e.g. Savant, Heto, or similar)
- Reaction vials (e.g. polypropylene microcentrifuge vials)
- Note: Further reagents are required if doing the optional post-labelling sample cleanup (see Section on Sample Cleanup)

Time Line for Labelling

The LudgerTag™ labelling procedure, including the optional post-labelling sample cleanup, typically takes 4 - 5 hours:

Procedure	Time	Elapsed Time (hours)
Transfer samples to a reaction tube and dry	30 min	0.5
Make up and add labelling reagent	15 min	0.75
Incubate samples with reagent	3 hours	3.75
Post-labelling cleanup	1 hour	4.75

The Reductive Amination Reaction

The labelling reaction involves a two-step process (see Figure 1):

1. **Schiff's base formation.**
   This requires a glycan with a free reducing terminus, 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 reducing terminal 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 labelled glycan.

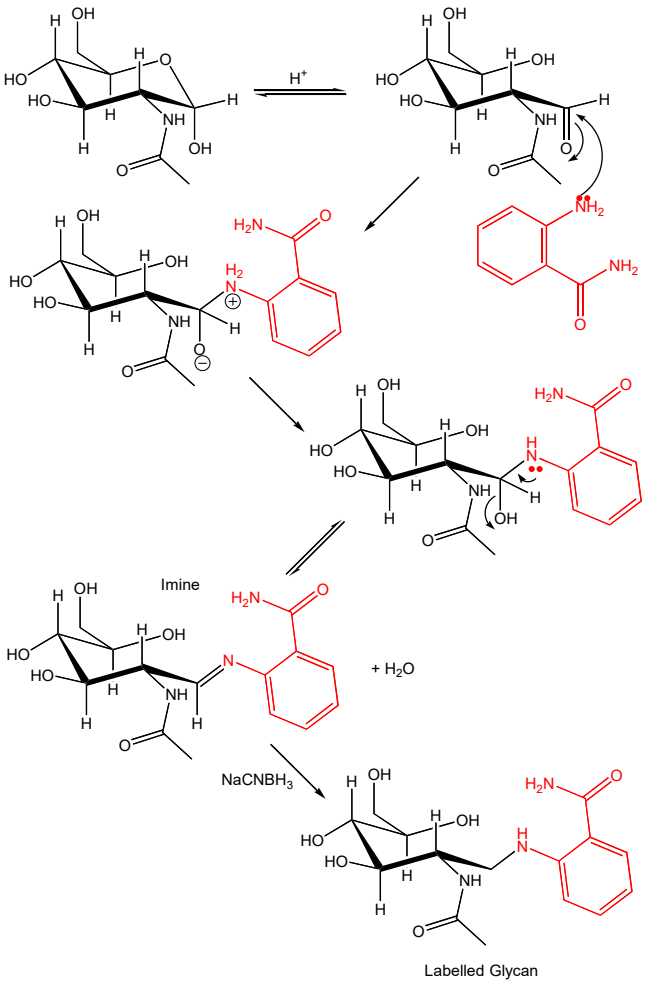


Figure 1: Labelling of a glycan with 2-aminobenzamide acid (2-AB) by reductive amination.

Outline of Labelling Protocol

LudgerTag™ glycan labelling kits are designed for the fluorophore or chromophore labelling of glycans with a free reducing terminus. Labelled glycans may be followed by either high-sensitivity fluorescence detection or monitoring of UV-absorbance during various chromatographic and structure sequence analyses. These include chromatography on LudgerSep™ HPLC columns and sequencing using exoglycosidases (See refs 1-5, 7).

The outline of the labelling procedure is as follows:

1 Prepare the glycans.

Prepare the glycan samples by removing contaminants such as salts and detergents that could interfere with the labelling procedure.

2 Dry the glycans

Place the samples in reaction vials and dry down.

3 Prepare labelling reagent

Prepare a fresh dye labelling solution by mixing the reagents in the kit.

4 Add labelling reagent to glycans

Add a small amount of labelling solution to each sample.

5 Incubate

Incubate the samples to allow the labelling reaction to progress.

6 Post-labelling cleanup

After incubation, if required (depending on the subsequent analysis procedures), remove the excess labelling reagents using a straightforward cleanup procedure.

7 Store or analyse the labelled glycans

The labelled glycans are now ready for analysis.

Sample Preparation

The glycan sample to be labelled, whether a purified glycan or a glycan mixture, must contain a free reducing terminus, be particle and salt-free, and be presented in a volatile solvent system (preferably pure water).

The following may interfere with the labelling reaction and must be removed from the glycan samples before LudgerTag™ labelling:

- Non-volatile solvents
- Non-volatile salts, in particular, transition metal ions
- Detergents
- Dyes and stains such as Coomassie Blue

A range of LudgerClean™ kits for cleaning glycan samples before LudgerTag™ labelling is available from Ludger. These are detailed in the LudgerClean Glycan Cleanup Guide [ref 6].

The standard sample preparation protocol is as follows:

1 Purify the glycans

If necessary, remove non-carbohydrate contaminants from the samples using one of the strategies outlined in the Glycan Cleanup Guide [ref 6].

2 Transfer the sample to a reaction vial

The amount of sample should be in the range 100 picomoles - 50 nanomoles for a glycan pool obtained from a typical glycoprotein. With a single pure glycan, as little as 5 picomoles can be labelled and detected in subsequent HPLC analysis. Suitable reaction vials include small polypropylene microcentrifuge tubes and tubes for PCR work.

3 Dry the samples

Ideally, samples should be dried using a centrifugal evaporator. If this is not possible, then freeze drying (lyophilisation) can be used with caution (in particular, ensure that the sample dries to a small, compact mass at the very bottom of the vial).

Do not subject samples to high temperatures (> 28°C) or extremes of pH, as these conditions will result in acid-catalysed loss of sialic acids (high temperatures, low pH) or epimerization of the glycan reducing terminus (at high pH).

Preparation of Labelling Reagent

Prepare fresh labelling reagent as follows:

4 Prepare a DMSO-acetic acid mixture

Add 150 µL glacial **acetic acid** to the vial of **DMSO** and mix by pipette action.

The Catalogue #s for the acetic acid and DMSO are LT-ACETIC-01 and LT-DMSO-01, respectively.
Open the ampoules by carefully tapping or flicking to dislodge any contents in the upper half, then carefully break open the ampoule.
If the DMSO is frozen, then gently warm up the vial (before opening) in an oven or heating block to between 30 °C and 65 °C.

5 Add the dye

Add 100 µL of the DMSO-acetic acid mixture to a vial of LudgerTag™ **2-AB (2-Aminobenzamide Acid) Dye** and mix until the dye is dissolved.

The Cat. # for the dye is LT-2AB-01.

6 Add the reductant

Add the dissolved dye to a vial of LudgerTag™ **sodium cyanoborohydride** (reductant) and mix by pipette action until the reductant is completely dissolved to make the final **labelling reagent**.

The Catalogue. # for the sodium cyanoborohydride reductant is LT-CYANOB-01.
If the reductant is difficult to dissolve, then gently warm the vial for up to four minutes in the 65 °C incubation oven or stand on a heating block at this temperature, then mix by pipette action. If undissolved reductant is still visible, add 10 µL pure water to the vial and mix.
Protect the labelling reagent from exposure to moisture and use within 60 minutes.

Labelling Reaction

7 Add labelling reagent to samples

Add 5 µL of labelling reagent to each dried glycan sample, cap the microtube, mix thoroughly, and then gently tap to ensure the labelling solution is at the bottom of the vial.

8 Incubate

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

The incubation must be performed in a dry environment. Use an oven or dry block - please do not use a water bath.

The samples must be completely dissolved in the labelling solution for efficient labelling. To encourage complete dissolution, the samples can be vortexed 30 minutes after the start of the 65°C incubation, then the incubation is continued.

In most cases, the incubation time can be shortened to 2 hours or extended up to 4 hours without significantly changing the outcome of the labelling reaction.

9 Centrifuge and cool

After the incubation period, remove the samples, centrifuge the microtubes briefly, then allow them to cool completely to room temperature.

LudgerClean™ S Post-Labelling Sample Cleanup

Post-labelling sample cleanup (to remove excess dye and other labelling reagents) is necessary for certain applications - e.g. subsequent analysis by HPLC. Such cleanup can be achieved using LudgerClean™ S cartridges (Cat # LC-S-Ax, where x denotes the number of cartridges in the kit) using the standard protocol included with the kit.

Post-labelling sample cleanup is not necessary for applications where the excess labelling reagents do not interfere with subsequent sample analysis. These include carbohydrate electrophoresis, where free dye runs away from the labelled glycans.

Analysis of LudgerTag™ 2AB-Labelled Glycans

LudgerTag™ 2-AB labelled glycans may be studied by a number of different analytical methods, including HPLC, gel electrophoresis, and mass spectrometry. These are covered in detail in reference 8 and overviewed below.

HPLC Analysis

LudgerTag™ 2-AB labelled glycan mixtures may be separated and analysed by a variety of HPLC (high-pressure liquid chromatography) methods, including LudgerSep™ HPLC:

Type of Analysis	Column	Cat. No.
Separation of charged and neutral glycans	LudgerSep™ C	LS-C-01
Profile analysis of neutral and charged glycans	LudgerSep™ N	LS-N-01
Separation of neutral glycans	LudgerSep™ R	LS-R-01

The uses of these columns for glycan analysis are overviewed in References 4 and 8.

The LudgerSep™ N column is an especially powerful tool for the purification and analysis of LudgerTag™ labelled oligosaccharides from complex glycan mixtures. Please contact us for advice regarding your particular application.

Enzymatic Analysis

High purity, sequencing grade enzymes (e.g. exoglycosidases) suitable for structural analysis of both N- and O-linked LudgerTag™ labelled glycans are available from a number of companies.
When selecting glycosidases, be especially careful to choose those with formulations that are compatible with your particular application. For example, some enzymes and enzyme buffers have components that interfere with certain types of analysis. Please call us for guidance in selecting enzymes and reaction conditions for your work.

Mass Spectrometry and Electrophoresis

LudgerTag™ labelled glycans may also be analysed by mass spectrometry, electrophoresis, and various types of spectroscopy. Please call us for advice on the analysis conditions most suitable for your intended analyses.

Warranties and Liabilities

Ludger warrants that the above product conforms to the attached analytical documents. Should the product fail for reasons other than through misuse Ludger will, at its option, replace free of charge or refund the purchase price. This warranty is exclusive and Ludger makes no other warrants, expressed or implied, including any implied conditions or warranties of merchantability or fitness for any particular purpose.
Ludger shall not be liable for any incidental, consequential or contingent damages.

This product is intended for *in vitro* research only.

Document Revision Number

Document # LT-KAB-A2-Guide-v3.1

References

1 Bigge, J.C.; Patel, T.P; Bruce, J.A.; Goulding, P.N.; Charles, S.M; Parekh, R.B. (1995)
'Non-selective and efficient fluorescent labelling of glycans using 2-aminobenzamide and anthranilic acid'. Analytical Biochemistry **230**: 229-238

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

3 Townsend, R.R.; Lipniunas, P.H.; Bigge, C.; Ventom, A.; Parekh, R. (1996)
'Multimode high-performance liquid chromatography of fluorescently labelled oligosaccharides from glycoproteins'.
Analytical Biochemistry **239**: 200-207

4 LudgerSep™ High Resolution HPLC Carbohydrate Profiling Guide (Cat # LS-GUIDE-01)

5 Ludger Enzyme Selection Guide (Cat # EZ-GUIDE-01)

6 LudgerClean™ Glycan Cleanup Guide (Cat # LC-GUIDE-01)

7 Hardy, M.R. (1997)
'Glycan labelling with the fluorophores 2-aminobenzamide and anthranilic acid'
in 'Techniques in Glycobiology', edited by Townsend, R.R and Hotchkiss, A.T.. Marcel Dekker Inc., New York.

8 Ludger Technical Note # TN-AB-01: Analysis of 2-AB (2-aminobenzamide acid) labelled glycans

Appendix 1: Troubleshooting Guide

The LudgerTag™ labelling protocol is an efficient, robust method. If problems do arise, they can normally be corrected without difficulty. The following is a guide to the most likely problems, possible causes, and solutions.

Poor Incorporation of Dye / Low Labelling Yield

The labelling temperature was incorrect.
Please ensure that the oven or heating block is equilibrated to the incubation temperature and that the reaction tube is subjected to this temperature for the entire labelling period.

The sample was incompletely solubilised.
The glycans must be completely dissolved in the labelling mixture for maximum labelling efficiency. Please ensure that the sample is thoroughly mixed with the labelling reagent before the incubation and, as a precaution, carefully mix the samples 15 minutes after the start of the incubation.

The sample contained contaminants that interfered with the labelling.
Please ensure that the glycans are adequately purified before labelling (see protocol step 1 and the LudgerClean™ Glycan Cleanup Guide).

The labelling solution was inactive. Please make up the labelling solution immediately before use - the reagents will lose activity within a few hours of mixing.

There was less starting glycan than was originally estimated.

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

The glycans were lost during the post-labelling cleanup.
Please ensure that the removal of excess labelling reagents is performed as specified in the cleanup protocol and that the wash reagents are correctly made.

The Labelled Samples Contain Fluorescent Non-Carbohydrate Material

The original glycans contained aldehyde-bearing contaminants.
Please ensure that the glycans are adequately purified before labelling (see protocol step 1 and the LudgerClean Glycan Cleanup Guide).

The post-labelling cleanup step did not work correctly.

Please ensure that the removal of excess labelling reagents is performed as specified in the post-labelling cleanup protocol and that the wash reagents are correctly made.

Selective Loss of Smaller Glycans

The cleanup cartridge was not primed correctly.

Please ensure the cartridge is primed correctly and that the cartridge bed is still wet with acetonitrile when the sample is applied to the disc.

Incorrect wash reagents were used during the post-labelling cleanup.

Please ensure that the wash reagents are correctly prepared.

Selective Loss of Larger Glycans

The sample was incompletely solubilised.

The glycans must be completely dissolved in the labelling mixture for maximum labelling efficiency. Larger glycans tend to be less soluble in the labelling mixture than small sugars. Please ensure that the sample is thoroughly mixed with the labelling reagent before the incubation and, as a precaution, carefully mix the samples 15 minutes after the start of the incubation.

Desialylation of the Glycans

The sample was subjected to acidic conditions in aqueous solutions at elevated temperatures

Avoid prolonged periods of exposure of sialylated glycan samples in aqueous solutions to conditions of low pH and elevated temperatures. Note that the reductive amination reaction is carried out in essentially anhydrous conditions under which loss of sialic acids is minimal.

In general, try to keep samples in solutions in the pH range 5 – 8.5 and avoid exposure to temperatures above 30 °C. Samples in pH-buffered aqueous solutions (with a pH between 5 and 8.5) tend to be resistant to acid-catalysed desialylation, even at temperatures higher than 30 °C. However, even then, it is wise to err on the side of caution and keep the samples cool whenever possible.

The samples were not cleaned up correctly after labelling

Ensure that samples undergo post-labelling cleanup immediately after the reductive amination reaction and that the post-labelling drying, and cleanup procedure is conducted promptly.

Labelled samples that have **not** undergone drying and subsequent cleanup will be prone to acid-catalysed desialylation.