



Ludger

LudgerSep™ N2 High Resolution Amide HPLC Column for Glycan Analysis

Product Code LS-N2-xx

Product Guide

Version 1.2

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LudgerSep N2 Glycan Analysis HPLC Column - Specifications

Application Analysis and purification by HPLC of LudgerTag™ fluorophore and UV-chromophore labeled glycans.

Description The N2 HPLC column contains particles with a polymeric amide coating optimized for high resolution chromatography of complex glycan mixtures.

Particles 3 µm particle size with 80 angstrom pores and polymeric amide coating.

Column Size	Cat #	Description	Dimensions
	LS-N2-4.6x150	LudgerSep N2 HPLC Column	4.6 x 150 mm
	LS-N2-2.0x150	LudgerSep N2 HPLC Column	2.0 x 150 mm

Flow Rates Typical flow rates = 0.4 - 1.0 ml/min for 4.6mm column
= 0.15 - 0.22ml/min for 2.0mm column

Column Pressure Maximum pressure = 2250 psi (150 kg/cm²) for 4.6mm column
= 2900 psi (20MPa) for 2.0mm column

pH Range 2.0 - 7.5

Temperature Typical operating temperature = 30 °C.
Temperature range = 10 - 40 °C.

Solvents Typical solvent systems for glycan analysis include gradients of acetonitrile(aq) and buffers containing ammonium formate, pH 4.4 .

Shipping Solvent 75% acetonitrile - 25% water

Cleaning Solvents

1. Water [to remove very polar solutes from the bonded phase]
2. 45% acetonitrile (aq) [to desorb hydrophobic compounds]
3. 0.1% triethylamine in 80% acetonitrile [to remove desorbed basic compounds]
4. 50 mM ammonium formate pH 4.4 / acetonitrile (1:1 v/v) [to remove ionic compounds]

Storage Before long-term storage flush the column with at least 5 column volumes of 75% acetonitrile (aq).

- Column Protection** Filter all solvents to 0.2 µm and degas using either helium sparging or vacuum degassing.
Filter all samples using a 0.2 µm filter membrane before loading onto the column.
Install a good quality in-line filter between the sample injector and the column.
Please call us for advice on the most suitable sample and in-line filters to use.
- Amount of Sample** The maximum amount of glycan sample that can be loaded on the column depends on the number and type of glycan components as well as the nature of any non-glycan material. The typical range for successful analytical runs is 10fmol - 1 nmol per sample peak and up to 200 nmol of total glycans.
- Suitable Samples** Suitable samples include glycans labeled with the following LudgerTag labels :
2-AA (2-aminobenzoic acid), 2-AB (2-aminobenzamide), AA-Ac (3-(acetilamino)-6-aminoacridine
- Sample** Filter samples to 0.2 µm then dry using a centrifugal evaporator.
- Preparation** For the 4.6mm column inject the sample in up to 100 µl of the starting buffer (i.e. the solvent mixture used at the very start of the HPLC gradient) or up to 10µl of water.

For the 2mm column inject the sample in up to 10 µl of the starting buffer (i.e. the solvent mixture used at the very start of the HPLC gradient) or up to 1µl of water
- Sample Detection** Either fluorescence, mass spectrometry or UV-absorbance depending on the dye used (see the appropriate LudgerTag instruction guide).
- 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.
- Safety:** Please read the Material Safety Data Sheets (MSDS's) for all chemicals used.
All processes involving labeling 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

For research use only. Not for human or drug use

HPLC System Requirements

LudgerSep N2 columns can be used with an HPLC system capable of delivering accurate gradients at a flow rate of 0.3 to 1.0 ml/min (for 4.6 mm diameter column) and 50 to 300 µl/min (for 2mm diameter column). In general, systems which mix eluants at high pressure (after the pump head) have lower dead volumes and supply more accurate gradients that are appropriate at the flow rate needed for LudgerSep columns.

For the 4.6mm column inject the sample in up to 100 µl of the starting buffer (i.e. the solvent mixture used at the very start of the HPLC gradient) or up to 10µl of water. For the 2mm column inject the sample in up to 10 µl of the starting buffer or up to 1µl of water

A fluorescence detector is required with the following detection wavelengths:

Fluorescence Label		λ_{ex} (nm)	λ_{em} (nm)
2-AB	[2-aminobenzamide]	360	425
2-AA	[2-aminobenzoic acid]	360	425
AA-Ac	[3-(acetylamino)-6-aminoacridine]	382 or 445	525

For optimal detection, use wide slit widths (e.g. 10 – 20 nm). Typically, LudgerSep N2 column containing sub-picomol levels of 2-AB or 2-AA labelled glycans can be detected with good signal-to-noise.

To improve repeatability and intermediate precisions for glycan analyses use a column temperature controller. Good results can be obtained with a column temperature of 35°C. Do not use column temperatures higher than 40°C or lower than 20°C.

Installation of the Column

During column installation we recommend that :

- You should connect the LudgerSep N2 column to your HPLC system using standard 1/16" OD tubing and 10-32 (1/16") fittings in either stainless steel or PEEK (polyetheretherketone). Hand-tight PEEK fittings and tubing (0.17 mm / 0.007" ID) are recommended for ease of connection and to minimise damage to the column threads. Flow direction is indicated by an arrow on the column label.
- Keep the lengths of tubing between the injector to column and column to detector as short as possible to minimise dispersion effects.
- Install an in-line filter with minimal dead volume either immediately between the injector and the head

of the LudgerSep N2 column to prevent damage to the column by particles.

- Before analysing any samples, condition your newly installed column as follows

Preconditioning of the Newly Installed Column

Prepare your newly installed column for glycan analysis as follows:

- Precondition the column by running two complete LudgerSep N2-01 gradient cycles (see below) without any sample injected.
- Check the column performance by running a fluorescently labeled glucose homopolymer (GHP) as a system suitability standard using a LudgerSep N2-01 gradient (see section on suitability suitability below).

Column Cleaning and Storage

After heavy use, your LudgerSep N2 column may become contaminated with strongly adsorbed sample constituents that will lead to a loss in column performance.

- If the contamination is light, clean the column following the protocol in the 'Column Preconditioning' section above. Afterwards, equilibrate in the starting buffer for the N2-01 gradient and do a blank run without injecting a sample to check the baseline.
- Use the following regime if more rigorous cleaning is required:
 - a. Run through one N2-01 gradient.
 - b. Wash with 50% (v/v) 50 mM ammonium formate pH 4.4, 50% (v/v) acetonitrile at 0.1 ml/min overnight
 - c. Run through one N2-01 gradient
 - d. Run a system suitability check
- For long-term storage, the column should be washed with a gradient going from the operational buffer to 100% water then to the storage solvent of 75% acetonitrile, 25% water (v/v).

Sample Preparation

The N2 column should be used for analysis of purified glycans (fluorescently labeled or unlabeled) only. Samples intended for analysis on LudgerSep N2 columns must be free of salt and free of any particulates.

Desalting Glycan Samples

Use EB10 cartridges (Ludger product # LC-EB10-A6) to desalt unlabeled glycans prior to:

- a. fluorescent labeling then N2 HPLC analysis or
- b. injection on the N2 column

Fluorescent Labeling

Fluorescent glycans can be prepared by derivatizing pure glycans using reductive amination with a LudgerTag label and purification on a LudgerClean cartridge. The following are commonly used labeling and purification systems for biopharmaceutical glycosylation analysis:

Labelling System

- 2-AB (LudgerTag kit # LT-KAB-A2)
- 2-AA (LudgerTag kit # LT-KAA-A2)
- AA-Ac (LudgerTag kit # LT-KAAAC-A2)

Post-Labeling Purification System

- S-Cartridge (LudgerClean kit # LC-S-A6)
- S-Cartridge (LudgerClean kit # LC-S-A6)
- D1-Cartridge (LudgerClean kit # LC-D1-A6)

Filtering Samples

Remove particulates from samples by one of the following methods:

- a. Filtering through a spin filter or syringe filter with 0.2 µm pore size membrane.
- b. Spin in a benchtop centrifuge (15 min at 10,000 rpm) the inject from the supernatant.

Sialylated Glycans

Sialylated glycans can become desialylated if exposed to acidic conditions and elevated temperatures. Avoid desialylation with such samples by

- a. minimizing exposure to acid (if possible, keep the pH between 5 - 8), and
- b. minimizing exposure to temperatures greater than 25°C.

Operating Schedule and System Suitability Test

We recommend the following operating schedule for GLP and GMP work:

- Immediately prior to any analyses, condition the system by running a blank profile (no sample) using the N2-01 gradient (see below)
- Run one of the following system suitability standards using the N2-01 gradient:

Product #	Description
CAB-GHP-30	2-AB GHP (2-AB labelled glucose homopolymer ladder)
CAA-GHP-30	2-AA GHP (2-AA labelled glucose homopolymer ladder)

- If the system suitability profile is out specification then troubleshoot and start the operating schedule again
- If the system suitability profile is in specification then run your samples.
- Continue running fluorescent labelled GHP suitability standards every 10 sample runs

Glycan Analysis with the N2 Amide HPLC Column

Solvents

The glycan analysis gradients in this guide are based on the following solvents:

- Solvent A :** 50 mM ammonium formate pH 4.4
Solvent B : Acetonitrile

Gradient # N2-01 : N-Glycan Analysis Gradient

Use as a gradient for analysis of N-glycans labelled with LudgerTag 2-AB and 2-AA labeled.

Time (min)	% A	%B	4.6mm column 2mm column	
			Flow Rate (ml/min)	Flow Rate (ml/min)
0	35	65	0.8	0.15
48	47	53	0.8	0.15
49	100	0	0.8	0.15
53	100	0	0.8	0.15
54	35	65	0.8	0.15
60	35	65	0.8	0.15

Solvent Recipes

40x Stock Solution for Solvent A (2.2 M Ammonium Formate, pH 4.4)

- Weigh 184.12 g of formic acid into a 2-litre glass beaker.
- Place the beaker in an ice bath to which salt has been added to take the temperature down to -10°C
- Add 1 litre of water, and stir with a glass rod.
- Adjust the pH by adding 4 x 50 ml 25% ammonia solution. This causes a rapid rise in temperature, so the ammonia must be added in small amounts. Ensure that the temperature drops between each
- Continue adding 5-ml aliquots of 25% ammonia until pH 4.4 is reached at room temperature.
- Transfer the solution to a 2-litre volumetric flask and make up to 2 litre with water.
- Store this stock solution in a brown Winchester bottle at room temperature.

Solvent A (50mM Ammonium Formate, pH 4.4)

- Dilute 50 ml of 40x Stock Solution for Solvent A to 2 litres with water

Solvent B (Acetonitrile)

Use acetonitrile for HPLC, far UV grade (e.g. Chromasolv acetonitrile, Riedel-de Haën, Sigma, Cat. No. 34888).

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 warranties, 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.

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