Product Guide for LudgerSep™ C3 anion exchange HPLC Column for Glycan Analysis

(Ludger Product Code: LS-C3-7.5x75)

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Ludger Ltd
Culham Science Centre
Oxford OX14 3EB
United Kingdom

Tel: +44 1865 408 554
Fax: +44 870 163 4620
Email: info@ludger.com
www.ludger.com

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Specifications for LudgerSep™ C3 Column

Application Charged-based analysis and purification by HPLC of LudgerTag™ fluorophore and UV-chromophore labeled glycans.

Description The C3 HPLC column contains macroporous (1000 Angstrom) anion exchange particles optimized for anion exchange chromatography of complex glycan mixtures.

Particles 10 μm polymer based hydrophilic particles. Functional group $–CH_2CH_2N^+(CH_2H_5)_2$

Column Size	**Cat #**	**Diameter x Length**	**Volume**
	LS-C3-7.5x75	7.5 x 75 mm	3.3 cm^3

Column Tube Stainless steel

Flow Rates Typical flow rates = 0.3 – 1.0 ml/min
Maximum flow rate = 1.2 ml/min

Pressure Pressure should not exceed 150psi

pH Range 2 – 12

Temperature Typical operating temperature = 30 °C,
Maximum temperature range = 10 - 45 °C.

Solvents Shipping Solvent – deionized water.
Typical solvent systems for glycan analysis include gradients of aqueous buffers containing acetonitrile and either ammonium formate, ammonium acetate, or sodium acetate salts (maximum 20% acetonitrile and 0.5 Molar salt).

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 an in-line 0.2 μm filter with minimal dead volume between the injector and the head of the column to prevent damage to the column by particles.

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 0.1 pmol - 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-AB (2-aminobenzamide)

Sample Filter samples to 0.2 μm then dry using a centrifugal evaporator.

Preparation Re-dissolve in 5 - 50 μl of water then inject.

Sample Detection Fluorescence detector.

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 – safety glasses, 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 C3 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. 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 7.5 mm diameter 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).

A fluorescence detector is required with the following detection wavelengths:

Fluorescence Label		λ_{ex} (nm)	λ_{em} (nm)
2-AB	[2-aminobenzamide]	330	420

For optimal detection, use wide slit widths (e.g. 10 – 20 nm). Sub-picomole levels of 2-AB labelled glycans

can be detected with good signal-to-noise (depending on the sensitivity of the detector used).

To improve repeatability and intermediate precisions for glycan analyses use a column temperature controller.

Installation of the Column

During column installation we recommend that :

- You should connect the LudgerSep C3 column to the HPLC using PEEK fittings and tubing (10.32 (1/16") ID). Hand-tightened fittings 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 a 0.2 µm in-line filter with minimal dead volume either immediately before the injector or between the injector and the head of the LudgerSep C3 column to prevent damage to the column by particles.
- Before analysing any samples, the newly installed column should be conditioned using the protocol described below.

Preconditioning of the Newly Installed Column

The following preconditioning steps are recommended prior to use of the LudgerSep C3 column :

Flush the column sequentially at a flow rate of 1.0 ml/min with the following eluants in order :

- Solvent A - 20% acetonitrile for 30 minutes.
- Solvent B - 500 mM ammonium formate pH 9.0 in 20% acetonitrile (50mL of Ludger product #LS-C-BUFFx4 diluted with 200mL water and 50mL acetonitrile) for 30 minutes.
- Equilibrate with Solvent A for 30 minutes.

Prepare your newly installed column for glycan analysis as follows:

- Precondition the column by running two complete solvent gradient cycles (see below) without any sample injected.

Column Cleaning and Storage

After heavy use, your LudgerSep C3 column may become contaminated with strongly adsorbed sample constituents that will lead to a loss in column performance.

- Clean the column following the protocol in the 'Column Preconditioning' section above. Afterwards,

equilibrate in the appropriate starting buffer and run a gradient without injecting a sample to check the baseline.

- Store the column in Solvent A – 20% acetonitrile.

Sample Preparation

Samples intended for charge mode analysis on LudgerSep C3 columns should be free of salt or anionic detergent and free of any particulates. Samples should be made up in water or 20% acetonitrile for injection onto the HPLC.

Sialylated glycans can become desialylated if exposed to acidic conditions with elevated temperatures. Avoid desialylation of such samples by (a) minimising exposure to acidic conditions (if possible, keep the pH above 5, and (b) minimising exposure to temperatures greater than 25^{o}C.

Particulates can be removed from samples using microcentrifuge filters with 0.2 μm pore size membranes.

Charge Mode Analysis of Anionic and Neutral Glycans

Picomole quantities (or less depending on the sensitivity of the detector) of oligosaccharides may be analysed by fluorescent labeling with 2-aminobenzamide (2-AB) using the LudgerTag™ 2-AB Glycan Labeling Kit (Cat. No. LT-KAB-A2) followed by LudgerSep™ C3 HPLC.

The outline of the procedure is as follows:

- The oligosaccharides are labeled by reductive amination with 2-AB.
- Excess labeling reagents are then removed using LudgerClean S cartridges.
- The labeled oligosaccharides are analysed by anion exchange HPLC on a LudgerSep™ C3 column with fluorescence detection.

The following protocols are intended as a guide to the conditions for using LudgerSep™ C3 columns. Always use HPLC grade buffer salts and solvents together with pure water. We recommend using LS-C buffer (500 mM ammonium formate buffer pH 9, 20 % acetonitrile v/v) rather than lower pH solvents because this higher pH ensures that **all anionic glycans** (sialic acids, sulphated and phosphorylated sugars) are in a negatively charged state. A lower pH solvent (e.g. pH 4.4) can be used for separation of sialylated glycans, but this is below the pKa value for phosphorylated glycans.

Below are the details for three different of solvents. Ammonium formate is a particularly useful solvent because it is volatile and can be easily removed following collection of samples after elution from the column before they are run on the LudgerSepN (or other HILIC) column for orthogonal separation.

LS-C 500 mM Ammonium Formate pH 9 Solvent.

The LS-C solvent is recommended for separation of all negatively charged glycans including phosphorylated and sulphated glycans.

(50mL of Ludger product #LS-C-BUFFx4 diluted with 200mL water and 50mL acetonitrile)

Column: LudgerSep C3 – 7.5 x 75 mm

Solvents: Solvent A = 20% acetonitrile.

Solvent B = LS-C (500 mM ammonium formate pH 9 in 20% acetonitrile).

Flow rate: 0.4-0.8 mL/min.

Gradient :

time / min	% A	% B	Flow Rate mL/min
0	100	0	0.4
5	100	0	0.4
21	96	4	0.4
61	74	26	0.4
72	59	41	0.4
77	51.5	48.5	0.4
80	51.5	48.5	0.4
81	100	0	0.8
89	100	40	0.8
90	100	0	0.4

Note: Higher percentage solvent B may be required for glycans with five or more sialic acids.

Detection by fluorescence :

λ_{ex} = 330 nm, λ_{em} =420 nm

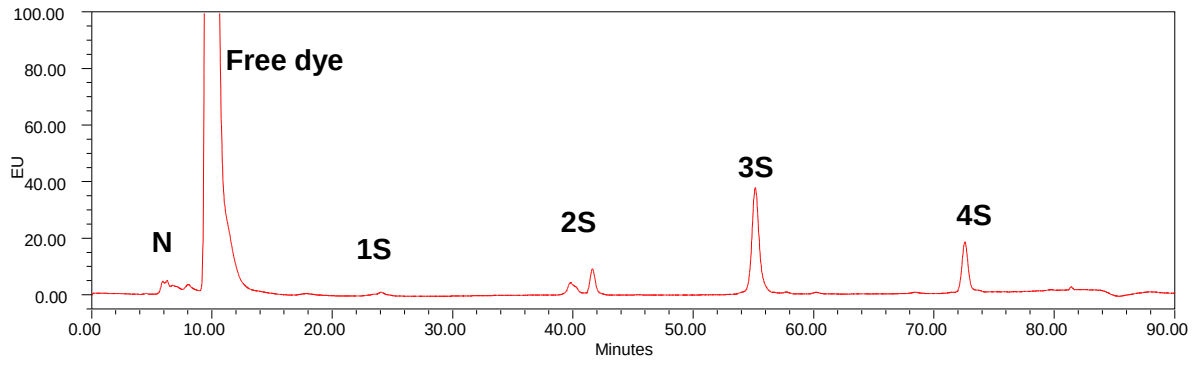

Figure 1: Separation of 2AB labeled N-glycans from bovine fetuin on LudgerSepC3 7.5 x 75mm using LS-C pH 9 solvent.

1S, 2S, 3S & 4S indicate the mono-, di-, tri- and tetra-sialylated glycan peaks. N = neutral glycans.

500mM Ammonium Formate pH 4.4 Solvent

This solvent is only recommended for separation of sialylated glycans

(50mL of Ludger product #LS-N-BUFFx40 diluted with 200mL water and 50mL acetonitrile)

Column: LudgerSep C3 – 7.5 x 75 mm

Solvents: Solvent A = 20% acetonitrile.

Solvent B = 500 mM ammonium formate pH 4.4 in 20% acetonitrile.

Flow rate: 0.4-0.8 ml/min.

Gradient :

time / min	% A	% B	Flow Rate ml/min
0	100	0	0.4
5	100	0	0.4
21	96	4	0.4
61	75	25	0.4
72	60	40	0.4
75	60	40	0.4
76	100	0	0.8
90	100	0	0.8

Note: Higher percentage solvent B may be required for glycans with five or more sialic acids.

Detection by fluorescence :

λ_{ex} = 330 nm, λ_{em} =420 nm

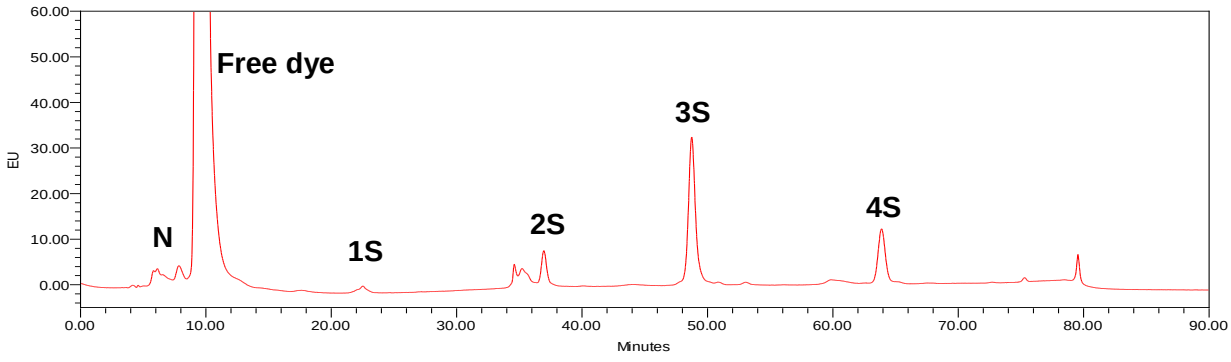

Figure 2: Separation of 2AB labeled N-glycans from bovine fetuin on LudgerSepC3 7.5 x 75mm using LS-C pH 4.4 solvent.

1S, 2S, 3S & 4S indicate the mono-, di-, tri- and tetra-sialylated glycan peaks. N = neutral glycans.

500 mM Ammonium Acetate pH 4.5 Solvent

Column: LudgerSep C3 – 7.5 x 75 mm

Solvents: Solvent A = 20% acetonitrile.

Solvent B = 500 mM ammonium acetate pH 4.5 in 20% acetonitrile.

Flow rate: 0.8 ml/min.

Gradient :

time / min	% A	% B
0	100	0
5	100	0
21	96	4
61	75	25
72	60	40
75	60	40
76	100	0
90	100	0

Note: Higher percentage solvent B may be required for glycans with five or more sialic acids.

Detection by fluorescence :

λ_{ex} = 330 nm, λ_{em} =420 nm

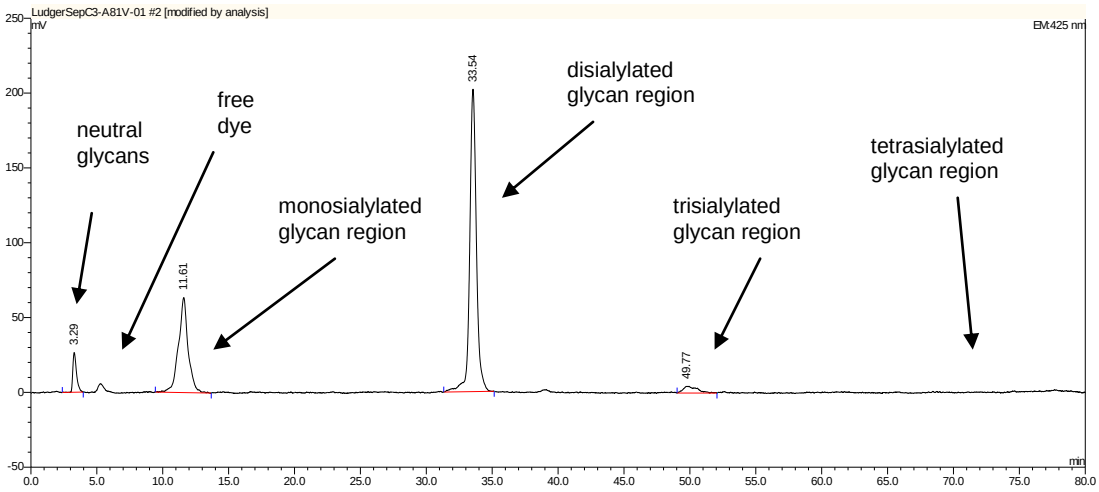

Figure 3: Separation of 2AB labeled glycan standards (picomolar concentration) on LudgerSepC3 7.5 x 75mm using ammonium acetate based solvent.

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

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Appendix 1: Troubleshooting Guide

Charge mode analysis on LudgerSep™ C3 is a 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.

A. Samples are not retained on the column

1. The glycans are non-anionic. The glycans do not bear anionic groups or the anionic groups have been removed (e.g. the sample has been desialylated). Minimize desialylation by reducing exposure of the sample to acid conditions and elevated temperatures.

2. The column may not be fully equilibrated. Ensure that the column is washed thoroughly in solvent A (20% acetonitrile in water) to remove all salts from the column before loading the sample.

3. The sample contains salt that causes self-elution. Ensure that the sample is loaded on in water or solvent A and that all traces of salt have been removed.