



**Product Guide for LudgerSepTM R1
HPLC Column
for Glycan Analysis**

(Ludger Product Code: LS-R1-4.6x150)

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Specifications for LudgerSep™ R1 Column

Applications	<p>Analysis of sialic acid variants labeled with DMB (1,2-diamino-4,5 methylenedioxybenzene.2HCl)</p> <p>Analysis of monosaccharides labeled with 2-AA (2-aminobenzoic acid)</p> <p>Analysis of glycans labeled with 2-AB (2-aminobenzamide). Two columns joined with a zero dead volume connector are required for this application.</p>		
Description	<p>The LudgerSep™ R1 HPLC column contains particles with an octadecylsilane coating optimized for hydrophobic chromatography.</p>		
Particles	<p>3 µm silica derivatized with octadecylsilane coating. 120 Angstrom pore size.</p>		
Column Size	Cat #	Diameter x Length	Column Volume
	LS-R1-4.6x150	4.6 x 150 mm	2.49 ml
Column Tube	<p>Stainless steel</p>		
Flow Rates	<p>Typical flow rates = 0.3 – 2.0 ml/min.</p>		
Pressure	<p>Pressure should not exceed 2000psi.</p>		
pH Range	<p>2 - 8</p>		
Temperature	<p>Typical operating temperature = 30 °C, but increasing the temperature may improve resolution for some samples.</p> <p>Maximum temperature range = 15 - 50 °C.</p>		
Solvents	<p>A typical solvent systems for sialic acid analysis is an isocratic gradient of 7:9:84 methanol:acetonitrile:water</p> <p>Avoid strong oxidants and anionic detergents.</p>		
Column Protection	<p>Filter all solvents to 0.2 µm and degas using either helium sparging or vacuum degassing.</p> <p>Filter all samples using a 0.2 µm filter membrane before loading onto the column.</p> <p>Install a good quality in-line filter between the sample injector and the column.</p> <p>Please call us for advice on the most suitable sample and in-line filters to use.</p>		
Suitable Samples	<p>DMB labelled sialic acids and monosaccharides labeled with 2-AA.</p>		

Sample	Filter samples to 0.2 μm and avoid exposure to light. Ensure DMB labelled samples are kept frozen and out of direct light if they are not to be used immediately.
Preparation	Dissolve samples in 7:9:84 methanol:acetonitrile:water.
Sample Detection	Fluorescence
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 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

For research use only. Not for human or drug use

HPLC System Requirements

LudgerSep R1 columns can be used with any HPLC pumping system capable of delivering accurate gradients at a flow rate of 0.3 to 2.0 ml/min. In general, systems that 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. Low dead volume injectors should be used (Rheodyne 7125 / 9125 or similar are recommended). The loop size to be used depends on the separation mode and amount of sample. For analytical runs it is desirable to minimise the sample volume and, typically, a 10 μl loop is used with sample injection volumes of 1 to 5 μl (partial fill) or > 10 μl (complete fill). For charge mode separations, generally, anionic glycans that are retained by the column (and are therefore effectively concentrated on the column) are reasonably tolerant of larger injection volumes whereas non-anionic glycans are not retained by the column matrix and will elute in a volume proportional to the injection volume.

Installation of the Column

During column installation we recommend that :

- You should connect the LudgerSep™ R1 column to your HPLC system using standard 1/16" OD tubing and Valco compatible 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 before the injector or between the injector and the head of the LudgerSep R1 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 step is recommended prior to use of the column :

For sialic acid analysis flush the column at a flow rate of 1.0 ml/min with 7:9:84 methanol:acetonitrile:water.

For 2-AA monosaccharide analysis flush the column at a flow rate of 0.8 mL/min with BPT solvent (see page 10 for recipe).

Column Cleaning and Storage

After heavy use, your LudgerSep™ R1 column may become contaminated with strongly adsorbed sample constituents that will lead to a loss in column performance.

A high acetonitrile solvent will aid removal of hydrophobic compounds. Long (overnight), low flow rate washes at 0.2 ml/min are better than fast (1-2 hour) high flow rate (1ml/min) washes, for efficient contaminant removal.

The LudgerSep R1 column should be stored in a low aqueous solvent (recommend 80% acetonitrile).

Sample Preparation

Samples intended for charge mode analysis on LudgerSep™ R1 columns must be free of salt or anionic detergent and free of any particulates.

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

Analysis of DMB-labeled Sialic Acids

For release of sialic acids from glycoproteins and labeling with 1,2-diamino-4,5-methylenedioxybenzene.2HCl (DMB), please follow the guide provided with the LudgerTag™ DMB Sialic Acid Labelling Kit, (Cat. No. LT-KDMB-A1)

The DMB labeled sialic acid reference panel (a component of the DMB sialic acid labelling kit) is an excellent sample to run on the LudgerSep™ R1 column to ensure efficient performance of the column for sialic acid identification. An example DMB sialic acid reference panel chromatogram is shown below in Figure 1.

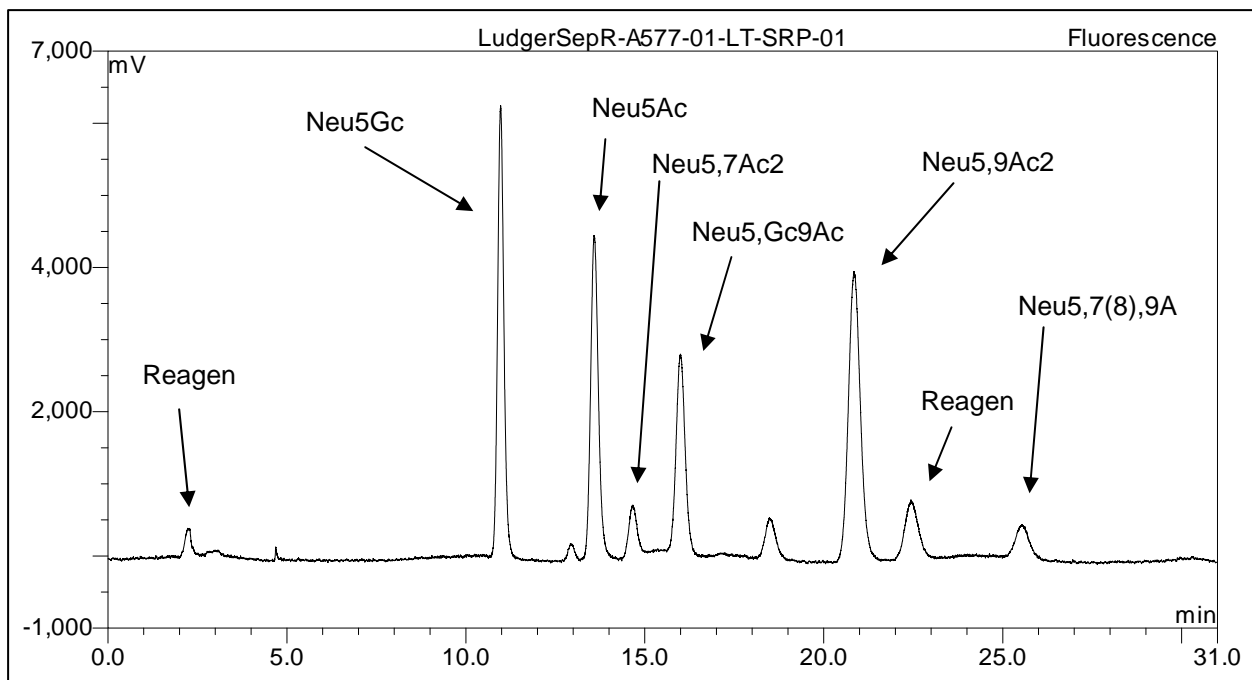


Figure 1: DMB Labeled Sialic Acid Reference Panel Run on the LudgerSep™ R1 HPLC column.

Prepare test samples at a range of dilutions e.g. 1 in 10, 1 in 20 and 1 in 30 (to determine the dilution at which optimal peak resolution is achieved) See LudgerTag™ DMB Sialic Acid Labelling Kit Product Guide for instructions. Figures 2 and 3 show the NeuAc and NeuGc standards run at 1 in 10 dilution.

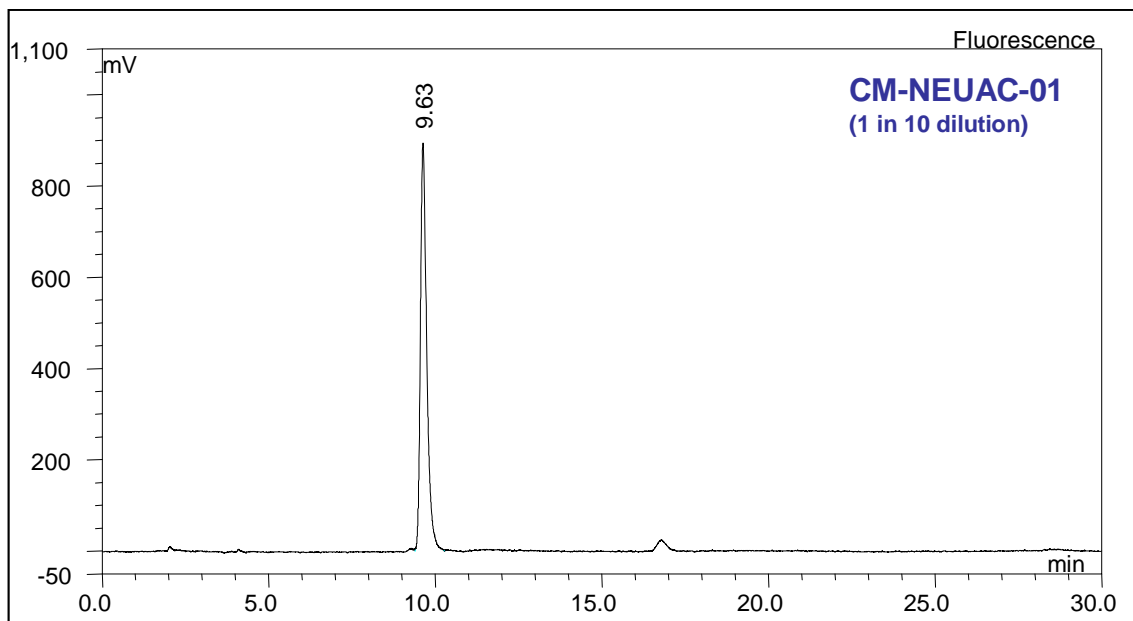


Figure 2: Chromatogram of DMB Labeled Neu5Ac (CM-NEUAC-01) (1 in 10 dilution) run on the LudgerSep™ R1 HPLC column.

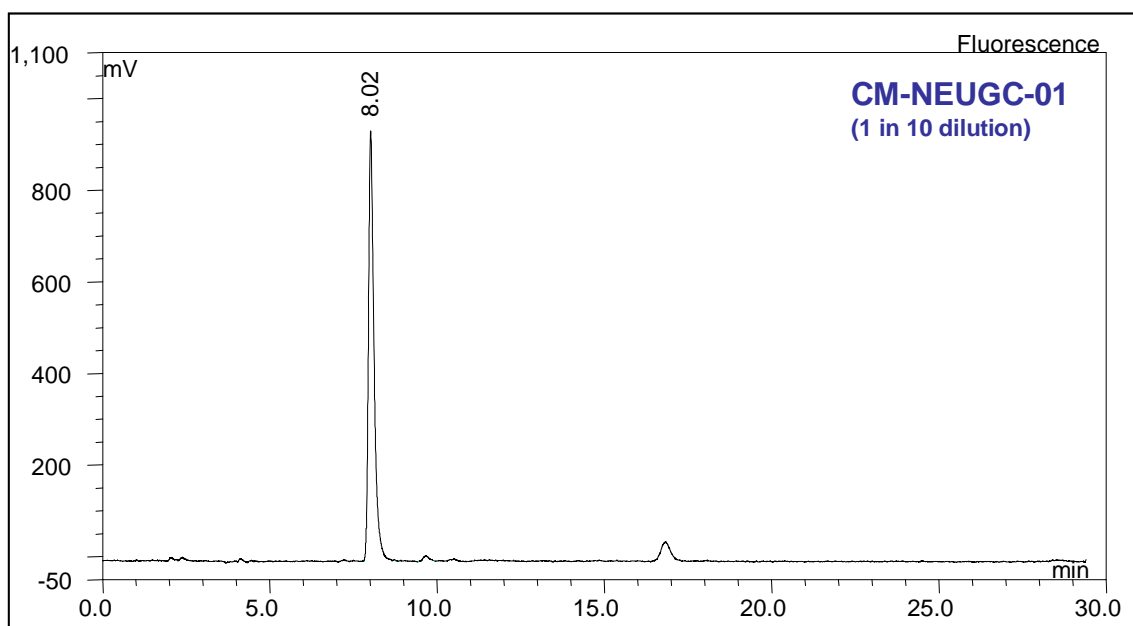


Figure 3: Chromatogram of DMB Labeled Neu5Gc (CM-NEUGC-01) (1 in 10 dilution) run on the LudgerSep™ R1 HPLC column.

Separation Conditions:

An isocratic gradient at room temperature over a 30 min period is recommended.

Flow rate: 0.5 ml/min

Solvent: Methanol:Acetonitrile:Water (7:9:84)

Note: Store samples at -18°C in the dark after use (DMB is light sensitive)

Analysis of 2-AA labeled Monosaccharides

For release of monosaccharides from glycoproteins and labeling with 2-aminobenzoic acid (2-AA), please follow the guide provided with the LudgerTag™ Monosaccharide release and Labeling Kit, (Cat. No. LT-MONO-96).

The LudgerSep™R1 column gives very good separation of the seven main monosaccharides found in most N-link and O-link glycans. Samples are diluted into the butylamine/ortho-phosphoric acid/tetrahydrofuran (BPT) HPLC running solvent. For the monosaccharide standards we recommend diluting the samples 100 fold, but exact dilutions are dependent on the sensitivity of the fluorescence detector and the glycan content of the glycoprotein. As a guide, antibody samples should be diluted 20 to 50 fold as there is usually only one glycosylation site per heavy chain. Bovine fetuin standard (Cat. No. GCP-Fet-50) is usually run at 100 fold dilution, where fetuin is approximately 20% w/w glycan. After samples dilution we recommend sonicating the samples for 30 minutes to remove any gas produced at the dilution stage. Vials can remain capped during sonication. If a sonicator is not available samples can be left for approximately 12 hours after dilution and then lightly mix to remove bubbles. No further gas release will occur.

Solvents

The glycan analysis gradients are based on the following solvents:

Solvent A : purified water based solvent of 0.2 % butylamine (2 mL per litre), 0.5 % phosphoric acid 5 mL per litre), 1 % tetrahydrofuran (10 mL per litre) (henceforth called BPT solvent).

Solvent B : 50 % acetonitrile : 50 % solvent A

We have also tested acetonitrile free solvent systems. This requires a slightly different gradient. Please enquire for further details. Column can be stored in 7% solvent B long term.

Gradient

Column temperature: 30°C

Fluorescence detector settings : Excitation wavelength: 360 nm, Emission wavelength: 425 nm

Time (min)	%B	Flow Rate (ml/min)
0.00	7	0.8
7.00	7	0.8
25	17	0.8
26	100	0.8
36	100	0.8
37	7	0.8
45	7	0.8

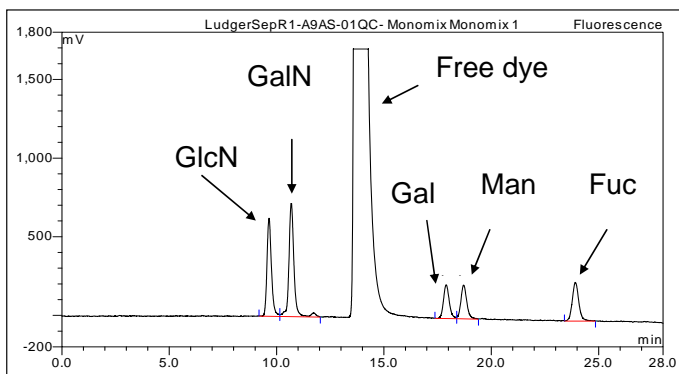


Figure 4: 2-AA fluorescence chromatogram of monosaccharides glucosamine (GlcN), galactosamine (GalN), galactose (Gal), mannose (Man) and fucose (Fuc). Glucose elutes immediately after the Man peak. Xylose elutes before the Fuc peak.

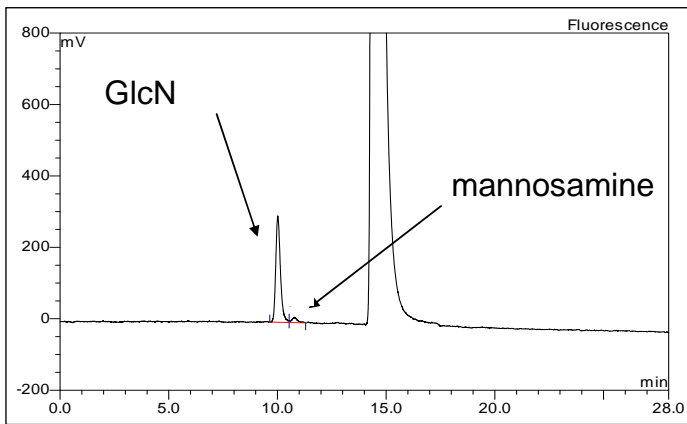


Figure 5: 2-AA fluorescence chromatogram of monosaccharide glucosamine (GlcN). This chromatogram shows the epimer peak from GlcN.

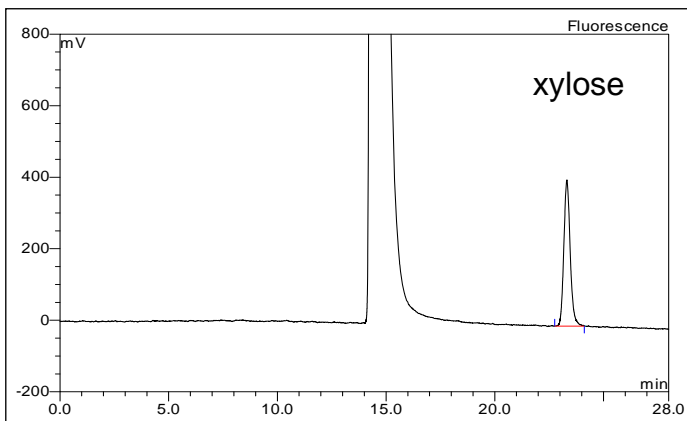


Figure 6: 2-AA fluorescence chromatogram of monosaccharide internal reference xylose (Xyl).

Analysis of 2-AB labeled Glycans

For labeling of glycans with 2-aminobenzamide (2-AB), please follow the guide provided with the LudgerTag™ 2AB Labeling Kit, (Cat. No. LT-KAB-A2). 2AB- labeled glycans can then be analysed using the LudgerSep™ R1 column. This method is particularly well suited for the following applications:

- G0, G1, G2 glycan separation
- fucosylated/non-fucosylated glycan separation,
- bisecting GlcNAc/non-bisecting GlcNAc glycan separations
- separating Man 5 from neutral complex bi-antennary glycans.

In addition this method reduces analysis time by a factor of two (compared to the original method). Two columns joined with a zero dead volume connector are required for this application. This is explained in more detail in the following Product Guide: LS-R1-2AB-Guide-v2.0.doc

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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Appendix 1 : Troubleshooting Guide

Analysis of DMB labelled using the LudgerSep R1 is a reasonably 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 column may not be fully equilibrated.** Ensure that the column is washed thoroughly in 7:9:84 methanol:acetonitrile:water.
- 2. The column is overloaded.** Inject a smaller amount of sample to see if retention is improved.
- 3. The column is contaminated.** Clean the column using the methods described in the guide.

B. Samples are retained and cannot be eluted from the column

- 1. The hplc solvent is not fresh.** Methanol has a low boiling point and will evaporate readily in unsealed bottle. We recommend solvent is prepared on the day of analysis to avoid this problem.