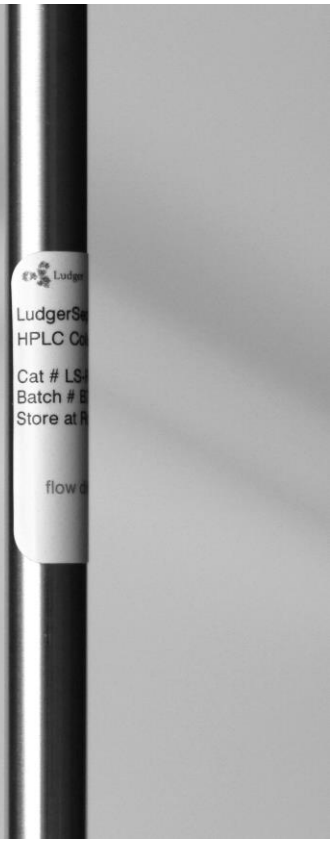
Product Guide for LudgerSep™ R1 HPLC Column for DMB labelled Sialic Acid Analysis



Product # LS-R1-4.6x150

Ludger Document # LS-R1-DMB-Guide-v5.1

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Contents

Specifications for LudgerSep™ R1 Column

Applications	Analysis of sialic acid variants labelled with DMB (1,2-diamino-4,5 methylenedioxybenzene.2HCl)
Description	The LudgerSep™ R1 HPLC column contains particles with an octadecylsilane coating optimized for hydrophobic chromatography.
Particles	3 µm silica derivatized with octadecylsiliane coating. 120 Angstrom pore size.

Column Size	**Cat #**	**Diameter x Length**	**Column Volume**
	LS-R1-4.6x150	4.6 x 150 mm	2.49 ml

Column Tube	Stainless steel
Flow Rates	Typical flow rates = 0.3 to 2.0 mL/min.
Pressure	Pressure should not exceed 2000 psi.
pH Range	2 - 8
Temperature	Typical operating temperature, 30°C, but increasing the temperature may improve resolution for some samples. Temperature range = 15 to 50°C.
Solvents	A typical solvent system for DMB labelled sialic acid analysis uses 7:9:84 methanol:acetonitrile:water as solvent A and acetonitrile as solvent B. Solvents should be degassed. Avoid strong oxidants and anionic detergents.
Column Protection	It is good practice to install a 0.2 µm in-line filter in front of the column.
Suitable Samples	DMB labelled sialic acids
Sample	Filter samples, if required, through a 0.2 µm filter. Avoid exposure of DMB labelled samples to light.
Preparation	Dilute samples in water (see LT-KDMB-A1 guide).
Sample Detection	Fluorescence: Excitation: 373 nm, Emission: 448 nm

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 Safety Data Sheets (SDS'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 solvents 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. We recommend keeping sample injection volumes at or below 25 µL, ideally use full loop injection. Although an example chromatogram is shown in this guide, retention times will vary dependent on the HPLC system used.

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 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 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 DMB labelled sialic acid analysis, flush the column at a flow rate of 0.5 ml/min with 7:9:84 methanol:acetonitrile:water.

Column Cleaning and Storage

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

Peptide or other components in the sample may cause retention times to shift over a period of time. To keep the column in good condition we recommend cleaning with 10% 7:9:84 methanol:acetonitrile:water, 90% acetonitrile at the end of each run (see methods below). A more prolonged wash for 30 min may be required for heavy contamination.

The LudgerSep R1 column should be stored in a low aqueous solvent. We recommend acetonitrile (minimum 50%).

Analysis of DMB-labelled Sialic Acids

For release of sialic acids from glycoproteins and labelling 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).

Prepare the LC system. Ensure that the solvent lines are primed.

Solvent A = Acetonitrile:Methanol:Water 9:7:84

Solvent B= Acetonitrile

Fluorescence: Excitation: 373 nm, Emission: 448 nm

Column temp = 30°C; Sample temp = 10°C.

Time (min)	Flow mL/min	%A	%B
0	0.5	100	0
19	0.5	100	0
19.5	0.5	10	90
23.5	0.5	10	90
24	0.5	100	0
30	0.5	100	0

Table 1. 30 minute running method for HPLC analysis using a LudgerSep-R1 column column (4.6 x 150 mm, 3 µm particles) LS-R1-4.6x150.

Injection volume = 25 µL.

The DMB labelled sialic acid reference panel (a component of the DMB sialic acid labelling kit) is a good system suitability standard to run on the LudgerSep™ R1 column to ensure efficient performance of the column for DMB labelled sialic acid identification. An example DMB sialic acid reference panel chromatogram is shown (Figure 1).

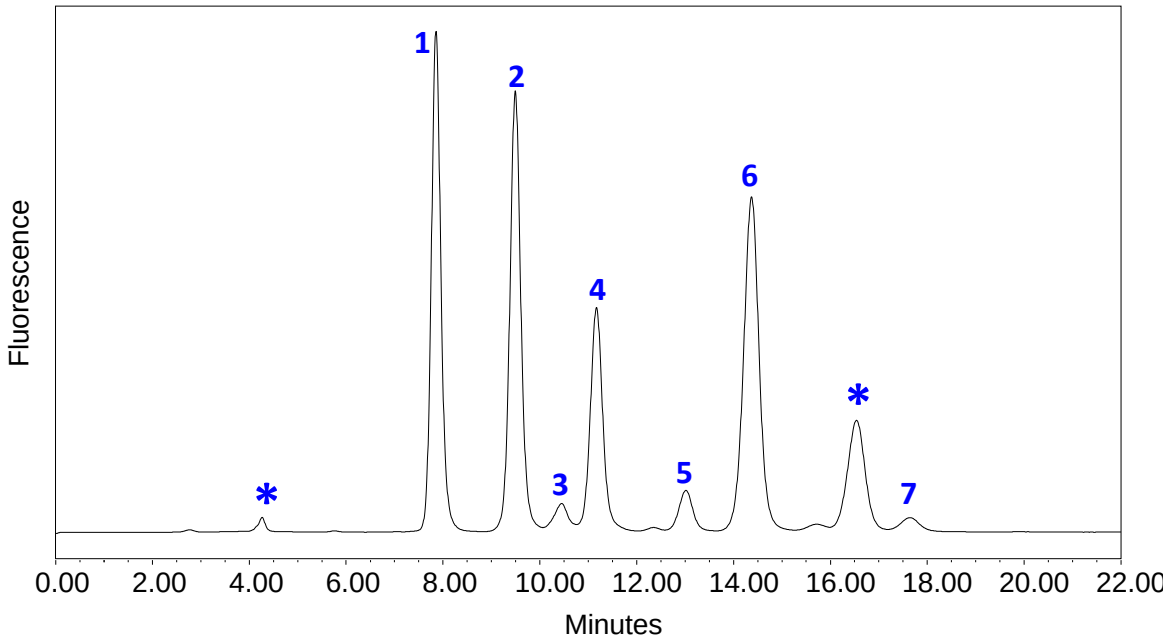

Figure 1: Chromatogram of DMB Labelled Sialic Acid Reference Panel (CM-SRP-01), run on the LudgerSep™ R1 HPLC column.

*Peaks: 1 = Neu5Gc; 2 = Neu5Ac; 3 = Neu5,7Ac$_2$; 4 = Neu5Gc,9Ac; 5 = Neu5,8Ac$_2$; 6 = Neu5,9Ac$_2$; 7 = Neu5,x,xAc$_3$ (where x is an unknown acetyl position); * = Reagent*

Note: This chromatogram is provided as an example only. Peak width, resolution and retention are dependent on the HPLC system setup in your laboratory.

References

1. Ludger Document: DMB-kit-Validation-Report-GP-0057-v1.0. Validation of the DMB kit with Ludger Standards.
2. Ludger Document: S-GP-0048-WG-50381-Report-v1.0. Determination of the effect of freezing of DMB Labelled Sialic Acids.

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.

Troubleshooting Guide

1. **Low signals on HPLC.**
   - Incomplete acid hydrolysis: We recommend using an oven rather than a heating block for the acid hydrolysis step. Some heating blocks cause evaporation and condensation of the acid in the sample vial lid causing incomplete acid hydrolysis. We also recommend the use of small sample vials, no greater than 0.5 mL in volume, for the acid hydrolysis.
   - Salts in the sample interfering with labelling: Salts and buffers can interfere with the sialic acid labelling method. If you suspect salt interference with your sample, dialyse the sample into a salt free solvent before analysis.
2. **High levels of free dye peaks in chromatograms.**
   - This can be caused by too much light exposure. Ensure that incubation steps are performed in the dark. Once the samples are labelled it is ideal to run them immediately on the LC to avoid degradation as prolonged exposure of samples to light and heat causes an increase in non-sialic acid specific chromatogram peaks. The issue may also be caused by contamination of the LC column over time, see below.
   - The amounts of Neu5Ac and Neu5Gc have been shown to be stable when the DMB labelled samples are stored at in the dark at 10°C for up to 72 hours, provided that the calibration standards have been stored in the same conditions and are analysed at the same time [Ref 1]. If this is not possible then the DMB labelled samples can be frozen for up to 2 days [Ref 2].
3. **Variation in LC chromatogram peaks retention times; unstable baseline.**
   - Incorrect or old LC solvent. Always prepare the solvents in the same way (making a solvent up to one litre in a measuring cylinder, for example, by mixing two solvents together, is not the same as measuring out the two solvents separately and mixing in a bottle). Isocratic gradients are particularly sensitive to variations in solvent preparation. Solvent composition can change over time due to evaporation.
   - Contamination of the column with excess free dye/ peptides etc can lead to retention time shifts and extra peaks on the chromatogram. This can be more of a problem for sample with low levels of sialylation where larger amounts of protein are injected onto the columns. Wash the column at the normal flow rate with a 10:90 mixture of normal running solvent and acetonitrile.
4. **Problem: There is precipitate in the labelling solution**
   - Although rare, it is possible that a slight precipitate may form during the preparation of the DMB labelling solution (mixture of sodium dithionite, mercaptoethanol and DMB dye). We have also observed this occurrence, have tested this mixture for its labelling efficiency and can confirm that the precipitate does not impact the labelling reaction.

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