Product Guide for LudgerSep™ R1 HPLC Column for 2AB-Labeled Glycan Analysis

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

Ludger Document # LS-R1-2AB-Guide-v2.1

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

Contents

Specifications for LudgerSep™ R1 Column

Applications Analysis by HPLC of 2-aminobenzamide (2-AB) labeled glycans. 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.

Description The LudgerSep™ R1 HPLC column contains particles with an octadecylsilane coating optimized for hydrophobic chromatography of 2-AB labelled glycans.

Particles 3 μm silica derivatized with octadecylsiliane coating. 120 Angstrom pore size.

Column Size

Cat #	Diameter x Length	Column Volume
2 x LS-R1-4.6x150	4.6 x 150 mm	4.98 ml in total

Column Tube Stainless steel

Flow Rates Typical flow rates = 0.3 ml/min.

Pressure Pressure should not exceed 2000psi.

pH Range 2 - 8

Temperature Typical operating temperature = 30 °C.
Maximum temperature range = 15 - 50 °C.

Solvents A typical solvent systems for 2-AB glycan analysis is 0.1% acetic acid for solvent A, and 0.1% acetic acid in an aqueous solution containing 10% acetonitrile.
Avoid strong oxidants and anionic detergents.

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.

Suitable Samples 2-AB labeled glycans.

Preparation Dissolve samples in 0.1% acetic acid or water, and inject in a volume not exceeding 50 µl. Long term storage (e.g. several days) of sialylated glycans at room temperature could result in partial desialylation, but in general it is recommended that samples are prepared immediately prior to use. Five pmols of sample with up to 5 glycans will be readily detectable using most standard fluorometers.

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.

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).

Installation of the Columns

During column installation we recommend that :

- You should connect the two LudgerSep™ R1 columns 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. A zero dead volume connector should be used to connect the two columns.
- 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 Columns

The following preconditioning step is recommended prior to use of the column :

Flush the column at a flow rate of 0.3 ml/min with 100% solvent A increasing to 100% Solvent B over 5 minutes, hold for 5 minutes and then increase to 100% solvent A over another 5 minutes. Repeat.

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

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

Dissolve samples in 0.1% acetic acid or water, and inject in a volume not exceeding 50 μl. Long term storage (e.g. several days) of sialylated glycans at room temperature could result in partial desialylation, but in general it is recommended that samples are prepared immediately prior to use. Five pmols of sample with up to 5 glycans will be readily detectable using most standard fluorometers.

An example chromatogram of 2-AB labelled antibody (IgG) glycans is shown in Figure 1.

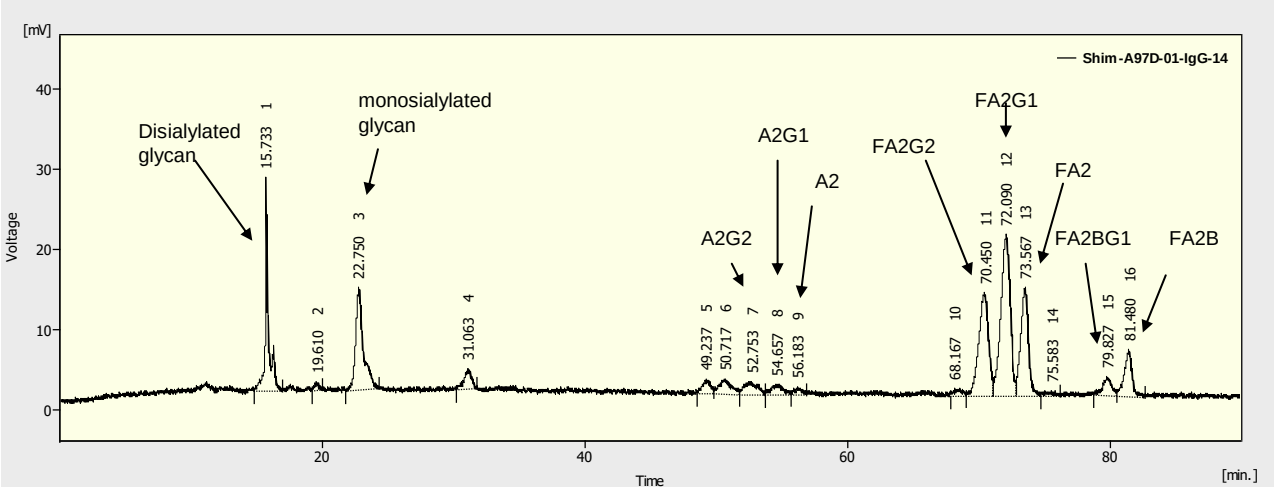

Figure 1: Analysis of 2-AB labeled human IgG immunoglobulin glycans using two LudgerSep™ R1 HPLC columns.

Separation Conditions:

Temperature:	30 °C
Flow rate:	0.3 mL/min increasing up to 0.5 mL/min for wash stage
Solvent A:	0.1% acetic acid in hplc grade water.
Solvent B:	0.1% acetic acid in 10:90 acetonitrile:hplc grade water mix.

Gradient:

Time (min)	%B
0	50 – 0.3 mL/min
5.0	50 – 0.3 mL/min
70.0	85 – 0.3 mL/min
75.0	85 – 0.5 mL/min
80.0	50 – 0.5 mL/min
90.0	50 – 0.5 mL/min

Glycan Retention Times

Glycan - Oxford Nomenclature[2] (alternative names in brackets)	Retention Time (min)
MAN3 (M3N2)	55.2
MAN5	50.2
MAN6	44.7
MAN7	43.1
MAN8	40.3
MAN9	39.9
A2 (NGA2/G0)	57.2
A2G1 (G1)	55.7
A2G2 (NA2/G2)	53.8
A2G2S1 (A1)	16.7
A2G2S2 (A2)	10
FA2 (NGA2F/G0F)	73.6
FA2G1 (G1F)	72.2
FA2G2 (NA2F/G2F)	69.6
FA2G2S1 (A1F)	22.2
FA2G2S2 (A2F)	11.5
A3 (NGA3)	57.9
A3G3 (NA3)	54.7
A3G3S3 (A3)	9.8
A4 (NGA4)	51.5
A4G4 (NA4)	46.1
M5A1B (Hybrid)	51.6

Table 1: Retention times of 2-AB labeled glycans on the 2 x LudgerSep™ R1 HPLC column system. *Values are given as a guide only. Expect different retention times on other HPLC systems, and with*

alternative glycan isomers.

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 # LS-R1-2AB-Guide-v2.1

References

1. Chen, X. and Flynn, G.C. “Analysis of N-glycans from recombinant immunoglobulin G by on-line reversed-phase high-performance liquid chromatography/mass spectrometry” Analytical Biochemistry, 370 (2007), 147-161.

2. Harvey DJ, Merry AH, Royle L, Campbell MP, Dwek RA and Rudd PM. “Proposal for a standard system for drawing structural diagrams of N- and O-linked carbohydrates and related compounds.” Proteomics, 15 (2009), 3796-3801.

Appendix 1 : Troubleshooting Guide

2-AB glycan analysis on LudgerSep™ R1 columns 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 0.1% acetic acid solution.

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 but the retention time varies

1. The solvent preparation may vary from batch to batch. Ensure that the acetic acid and acetonitrile components are accurately and consistently measured.