GLYCOSEP™ C HPLC COLUMN

Charge profiling of Signal™ 2-aminobenzamide (2-AB)-labeled oligosaccharides.

Product Code GKI-4721

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INTRODUCTION

NOTE: We want successful results for our customers, so please read this entire booklet before beginning.

GKI-4721 GlycoSep C HPLC Column (GlycoSep C) is an anion-exchange high performance liquid chromatography (HPLC) column that has been selected for optimal separation of negatively charged fluorescent Signal 2-AB-labeled glycans. Retention and separation of glycans on the column is predominantly governed by their overall anionic charge (the number of negatively charged groups). Glycan size also has a small effect on separation, which may result in a separation of glycans within a given charge group. The number of negatively charged groups most commonly relates to the degree of sialylation of N- and O-linked glycans, while sulfate groups, phosphate groups and glutamic acid represent less common modifications of glycans.

SYSTEM REQUIREMENTS

GlycoSep C may be used with any HPLC system capable of delivering an accurate, reproducible binary gradient at a flow rate between 0.5 ml/min and 1.0 ml/min. In general, systems that mix eluants at high pressure (after the pump head) have lower dead volumes and supply more accurate gradients at the flow rate needed for GlycoSep C. Low dead volume injectors are recommended.

2-AB labeled oligosaccharides are preferably detected by fluorescence (see settings shown below), although UV absorbance (254 nm) may also be used with reduced sensitivity (10- to 100-fold). However, the absorption of the buffers/solvents becomes a problem when using UV detection at short wavelengths.

Fluorescence Settings

Fluorophore	Excitation λ_{max}	Emission λ_{max}
2-AB	330 nm	420 nm

Fluorescence may be detected by either filter or monochromator instruments. The detection limit for 2-AB-labeled glycans on a commercially available filter instrument has been measured to be about 200 fmol, while 10-fmol detection is possible on more sensitive monochromator instruments[1]. Typically, 2 - 5 pmols of 2-AB-labeled glycans can be detected with a good signal-to-noise ratio.

Column Protection

Use of a guard column is highly recommended to prolong the life of the analytical column. Guard-column life depends greatly on sample cleanliness. As a general rule, guard columns should be replaced after every 30 - 40 sample injections or when peaks become excessively wide or show splitting. ProZyme offers GlycoSep C Guard Column,

GKI-4721G, specifically for use with GlycoSep C.

Connecting to the HPLC System

Connect GlycoSep C Guard and GlycoSep C to HPLC systems using standard 1/16" OD tubing and 10/32 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. An arrow on the column tube indicates flow direction.

Purge air from each section of the tubing prior to connecting each column. Make sure each column flow indicator is aligned in the proper direction.

Column Preconditioning

Preconditioning of GlycoSep C is required prior to use. Sequentially, flush the column with at least 25 ml of these eluants: water, 0.5 M ammonium formate and water. Then, take the column through two complete separation cycles using Gradient 1 (see page 8) without sample injection.

Cleaning, Regeneration and Storage

Follow the protocol in the Column Preconditioning section. Afterwards equilibrate in the appropriate starting buffer and run a gradient without injecting a sample to check the baseline.

If a more rigorous cleaning is required, use a 1 M solution of ammonium formate. Alternatively, if resolution is still poor, a 0.1 - 0.2 M NaOH solution and/or a 20 - 40% acetic acid solution may be injected in 250 µl aliquots, not to exceed one column volume (3 - 3.5 ml). Always flush with solvents A and B and then thoroughly equilibrate the column after cleaning.

NOTE: Do not run base or acid wash from buffer reservoir as this may cause permanent damage to the matrix.

For overnight storage, flush GlycoSep C with ~25 ml of mobile phase A: 20% acetonitrile/80% water (v/v).

For long-term storage, flush GlycoSep C with ~25 ml of 20% methanol/80% water (v/v).

Store at room temperature; do not refrigerate.

SAMPLE PREPARATION

2-AB-labeled samples (see Signal 2-AB Labeling Kit, ProZyme product code GKK-404) intended for charge analysis on GlycoSep C must be relatively free of salt or anionic detergents, and free of particulates.

Anionic glycans can be desalted using a GlycoClean™ H Cartridge (product code GKI-4025, available from ProZyme); trifluoroacetic acid (used as a additive for elution of glycans from the cartridge) should be removed by repetitive evaporation in vacuum centrifugal evaporator. Volatile salts (ammonium carbonate, formate, acetate, etc.) can be removed by repetitive evaporation in a vacuum centrifugal evaporator, followed by dissolution in water until there is no visible salt residue.

Purified, 2-AB-labeled samples should be redissolved in 5 - 50 µl of water before injection onto GlycoSep C. Anionic glycans are retained by the matrix and are therefore effectively concentrated on the column, while uncharged glycans will elute in a volume proportional to the injection volume. The maximum amount of sample to be loaded is dependent on the number and type of glycans in the sample and the degree of resolution required.

Sample Collection and Finishing

If desired, collect the required peaks or fractions either manually or with a fraction collector; a suitable fraction size range is 0.2 - 0.5 ml. When connecting a fraction collector, ensure that the tubing length and internal bore are minimized to reduce sample dispersion after the detector. Repeatedly lyophilize or evaporate samples to remove solvents and ammonium formate.

Standards

We recommend using any one of ProZyme's 2-AB labeled charged glycans as an external standard to calibrate GlycoSep C. Suggested glycans include GKSB-311 2-AB-A1 (mono-sialylated, galactosylated biantennary glycan), GKSB-312 2-AB-A2 (di-sialylated, galactosylated biantennary glycan) and GKSB-314 2-AB-A3 (tri-sialylated, galactosylated triantennary glycan).

Alternatively, these may be prepared by labeling the corresponding ProZyme glycan standards (product codes GKC-124300, GKC-224300 and GKC-335300, respectively) with the GKK-404 Signal™ 2-AB Labeling Kit.

Also available are the GKLB-002 Bovine Fetuin N-linked Glycan Library (GKSB-002 for 2-AB-labeled library) and the GKLB-005 Human IgG N-linked Glycan Library (GKSB-005 for 2-AB-labeled library), used as examples in this booklet.

TYPICAL RUNNING CONDITIONS

Solvent A: 20% acetonitrile/80% water (v/v)

Solvent B: 20% acetonitrile/30% water/50% 500 mM ammonium formate, pH 4.5

NOTES:

Always use HPLC-grade solvents, buffers and water.

Ammonium formate should be prepared by titrating formic acid with ammonium hydroxide; these are generally available in a higher purity than the salt.

Buffer concentrations are expressed in terms of the anion.

Mix separately measured volumes of acetonitrile with water or aqueous buffer.

Before use, thoroughly degas buffers by sonication, sparging with helium, or vacuum degassing.

Three typical gradients are shown in Table 1: Gradient 1, the standard gradient, gives the highest resolution; Gradients 2 and 3 have shorter run times, but show partial decreases in resolution.

Table 1 - Typical Gradients for GlycoSep C HPLC

Gradient 1

Time (min)	% A	% B	Flow rate (ml/min)
0	100	0	0.5
5	100	0	0.5
35	0	100	0.5
40	0	100	0.5
41	100	0	0.5
60	100	0	0.5

Gradient 2

Time (min)	% A	% B	Flow rate (ml/min)
0	100	0	0.75
5	100	0	0.75
25	0	100	0.75
30	0	100	0.75
31	100	0	0.75
45	100	0	0.75

Table 1 - Typical Gradients for GlycoSep C HPLC

Gradient 3			
Time (min)	% A	% B	Fl ow rate (ml/min)
0	100	0	0.75
5	100	0	0.75
20	0	100	0.75
22.5	0	100	0.75
23	100	0	0.75
30	100	0	0.75

APPLICATIONS

GlycoSep C is most commonly used for analysis of the charge profile of 2-AB-labeled glycans. It determines the molar distribution of uncharged and negatively charged glycans, for example, the distribution of sialylated glycans.

Separation of 2-AB labeled N-linked glycans derived from bovine fetuin and from polyclonal human IgG represent such an example of analysis of charge distribution of mono-, di-, tri-, tetra- and/or pentasialylated complex glycans. Profiles for 2-AB-labeled Fetuin N-Linked Library using the three gradients listed in Table 1 are shown in Figures 1, 2 and 3. Figure 4 shows the profile of 2-AB labeled Human IgG N-Linked Library using gradient 1.

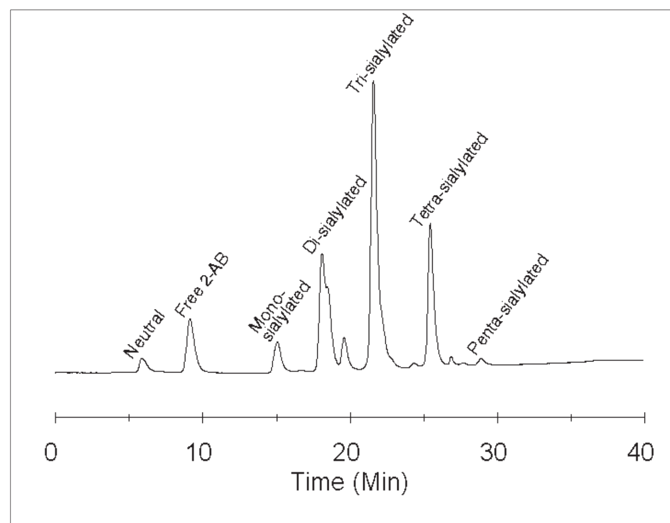

Figure 1 - Separat ion of 2-AB-labeled Fet uin N-linked Glycan Library on GlycoSep C using Gradient 1 (60 min)

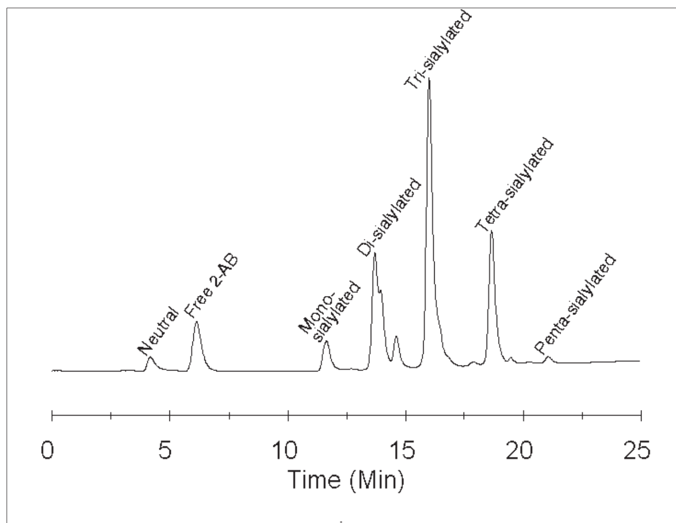

Figure 2 - Separation of 2-AB-labeled Fetuin N-linked Glycan Library on GlycoSep C using Gradient 2 (45 min)

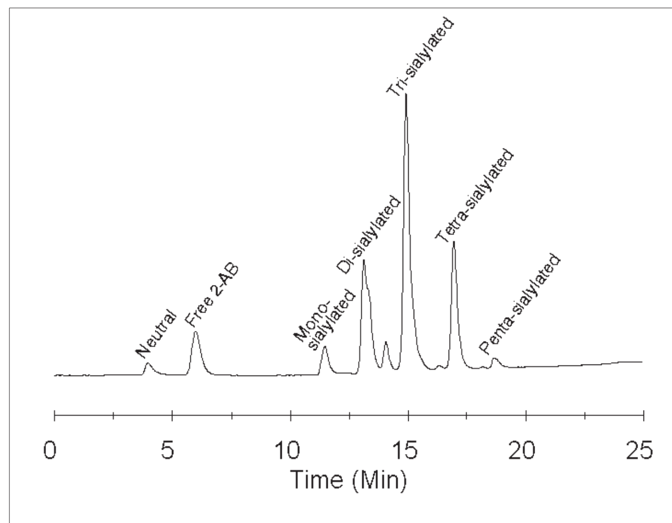

Figure 3 - Separation of 2-AB-labeled Fetuin N-linked Glycan Library on GlycoSep C using Gradient 3 (30 min)

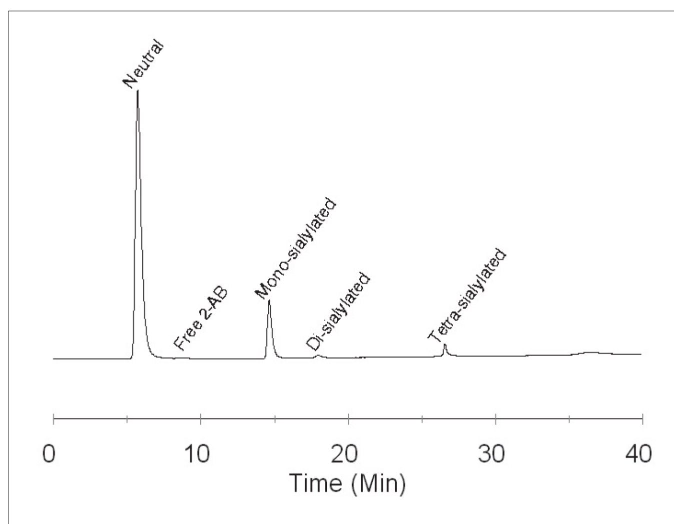

Figure 4 - Separation of 2-AB-labeled Human IgG N-linked Glycan Library on GlycoSep C using Gradient 1 (60 min)

REFERENCES

1. Guile GR, Rudd PM, Wing DR, Prime SB and Dwek RA. A rapid and high-resolution high-performance liquid chromatographic method for separating glycan mixtures and analyzing oligosaccharide profiles. Anal Biochem 1996 Sep 5;240(2):210-226.

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4. Townsend RR, Lipniunas PH, Bigge C, Ventom A and R. Parekh R. Multimode high-performance liquid chromatography of fluorescently labeled oligosaccharides from glycoproteins. Anal Biochem 1996 Aug 1;239(2):200-207.

TECHNICAL ASSISTANCE

If you have any questions or experience difficulties regarding any aspect of our products, please contact us:

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ProZyme values customers opinions and considers customers an important source for information regarding advanced or specialized uses of our products. We encourage you to contact us. We welcome your suggestions about product performance or new applications and techniques.

Also, contact your local distributor:

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APPENDIX A: SPECIFICATIONS

Product name:	GlycoSep C HPLC Column
Product code:	GKI-4721
Base matrix:	10 µm polymer-based resin
Derivatization:	DEAE (diethylaminoethyl)
Column size:	7.5 x 75 mm
Typical flow rate:	0.5 - 1.0 ml/min
Maximal flow rate:	1.2 ml/min
pH compatibility:	pH 2.0 - 12
Temperature range:	10 - 45°C
Maximum pressure:	15 kgf/cm^2 or 225 psi
Solvent compatibility:	organic solvent concentration ≤20%
Typical analysis buffer:	water/acetonitrile with ammonium formate buffer.
Analysis mode:	Anion-exchange
Column tube:	Stainless steel

APPENDIX B: TROUBLESHOOTING

Common problems are presented with a list of possible causes and suggested actions:

Sample not retained	
Possible Cause	Suggested Action
Glycans are non-ionic	The glycans do not bear anionic groups or the anionic groups have been removed (e.g., sample has been desialylated). Minimize desialylation by reducing exposure of the sample to acidic conditions and elevated temperatures.
Sialylated glycans are not ionized	Sialylated glycans will not be ionized in acidic solutions with pH 3, since carboxyl groups will not be deprotonated (therefore will be neutral). Adjust sample pH to the range 7 - 8 with 50 mM ammonia solution (aqueous) or add an equal volume of 10 mM ammonia solution to sample, dry down, then dissolve in water.

Sample not retained (Continued)	
Possible Cause	Suggested Action
GlycoSep C not fully equilibrated	Ensure that column is washed thoroughly in Solvent A (20% acetonitrile/80 % water) to remove all salts before injecting the sample.
Sample contains salts that cause self-elution	Ensure that sample is salt-free and is loaded in water or Solvent A. Desalt sample if necessary. Alternatively, dilute sample with water before loading onto column. This reduces ionic strength of the sample and minimizes the possibility of self-elution. However, the peak widths of uncharged glycans (not retained) will increase as the sample injection volume increases.
Column is overloaded	Inject a smaller amount of sample
Column is contaminated	Clean the column using method described in Cleaning, Regeneration and Storage section.

No flow or reduced flow	
Possible Cause	Suggested Action
Pump power disconnected	Reconnect Power
Fuse blown	Replace fuse
Mechanical/electronic failure in pump driver	Call manufacturer service
Check valves not functioning	Remove and clean or replace
Blocked filter or frit	Replace filter or frit
Fluid leak at connectors	Tighten or replace connector and/or tubing
Air bubbles in pump head	Purge pump, degas solvents
Error in pump gradient program	Reprogram gradient

Poor signal to noise ratio	
Possible Cause	Suggested Action
Detector lamp failing	Replace lamp
Dirty flow cell	Clean flow cell
Electrical interference	Check signal cables, re-site HPLC
Bubble in the system	Purge system, degas solvents
Wrong detector settings	Check filters, wavelengths, bandwidth and attenuation.

Baseline drift	
Possible Cause	Suggested Action
Column not equilibrated	Allow at least 10 column volumes for equilibration. Allow extra for mixing chamber/connecting tubing
Ambient temperature change	Site system under stable temperature conditions
Detector lamp failing	Replace lamp
Dirty flow cell	Clean flow cell
Insufficient sample	Inject 5 pmol/peak of labeled glycan

No peaks	
Possible Cause	Suggested Action
Detector power disconnected	Reconnect power
Detector output not connected to chart recorder/data collection	Connect chart recorder/data collection
Detector fuse blown or lamp failed/low energy	Replace fuse or Replace lamp
Detector electronic failure	Call manufacturer service
Not enough sample	Inject 5 pmol/peak of labeled glycan (fluorescence detection)
Inappropriate detection method	Fluorescence detection
Incorrect wavelength	Select correct wavelength
Fluid leak in system	See under “no flow”

Unexpected peaks	
Possible Cause	Suggested Action
Dirty column	See section Cleaning, Regeneration and Storage"
Contaminated solvents	Use HPLC grade solvents
Contaminated buffers	Use analytical/HPLC grade buffers; change buffers regularly to prevent microbial contamination
Contaminated sample	Review sample preparation
Bubble in detector flow cell	Flush and/or clean flow cell

PRODUCT USE AND WARRANTY

Terms and conditions of sale as well as product warranties may be found at:

http://www.prozyme.com/terms.html

RELATED PROZYME PRODUCTS

Find a complete listing of ProZyme's glycobiology products on our website:

http://www.prozyme.com/glyko

Including these products referred to in the text:

Signal Labeling Kits

Carbohydrate standards

2-AB-labeled standards

GlycoSep Columns

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For North American destinations: telephone orders may be placed between 8:00 am and 5:00 pm Pacific Time. Telefax or e-mail orders may be sent or messages recorded anytime.

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