



GLYCOSEP™ N HPLC COLUMN

Profiling of Signal™ 2-aminobenzamide (2-AB)- and 2-aminobenzoic acid (2-AA)-labeled oligosaccharides.

Product Code: GKI-4728

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INTRODUCTION

NOTE: Please read this entire booklet before beginning.

GKI-4728 GlycoSep N HPLC Column (GlycoSep N) is designed for the profile analysis of Signal 2-aminobenzamide (2-AB)-labeled and 2-aminobenzoic acid (2-AA) oligosaccharides. Both neutral and charged species are resolved using a binary gradient. Retention of Signal-labeled oligosaccharide is based on the hydrophilicity of the molecule, a parameter that is broadly related to hydrodynamic volume or molecular size. The column may be calibrated using a Signal-labeled dextran hydrolysate ladder, such that elution may be expressed in terms of glucose units (GU). Under the chromatographic conditions detailed in this booklet, the retention of Signal-labeled oligosaccharides on GlycoSep N may be predicted based on the hydrophilic contributions of the individual constituent monosaccharides. Retention predictability aids structural identification of unknown peaks.

Specifications for GlycoSep N are listed in Appendix A.

SYSTEM REQUIREMENTS

GlycoSep N may be used with any HPLC system capable of delivering an accurate, reproducible binary gradient at the flow rate specified by the various procedures (0.4 ml/min or 0.4 - 1.0 ml/min). The column is compatible with higher flow rates, which may be used during washing or equilibration. 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 N.

Low dead volume injectors are recommended with sample loops of up to 100 μ l.

Control of column temperature is important for reproducibility of the chromatography on GlycoSep N. A column oven set to 30°C is recommended for optimal resolution; a higher or lower temperature will start to compromise resolution. In any case, control your selected temperature within 1°C to assure run-to-run reproducibility.

Detection of labeled oligosaccharides is by fluorescence (see settings for common fluorophores shown below), although UV absorbance (254 nm) may also be used with a reduced sensitivity (10- to 100-fold). Suitable fluorescence detectors are 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 instruments¹.

Fluorescence Settings

| Fluorophore | Excitation λ_{\max} | Emission λ_{\max} |
|-------------|-----------------------------|---------------------------|
| 2-AB | 330 nm | 420 nm |
| 2-AA | 315 nm | 400 nm |
| DMB | 373 nm | 448 nm |

Column Protection

Use a guard column 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, when peaks become excessively wide or when peaks show splitting. ProZyme offers GlycoSep N Guard (product code GKI-4728G), specifically for use with GlycoSep N.

Connecting to the HPLC System

Connect GlycoSep N Guard and GlycoSep N 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.

NOTE: It is important to keep the tubing length between the injector to column and column to detector as short as possible to minimize dispersion effects.

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

Although no specific preconditioning is required, GlycoSep N should be flushed sequentially with 10 column volumes each of solvents A and B (see "Typical Running Conditions") prior to equilibration and injection of the first sample.

Cleaning, Regeneration and Storage

If cleaning is required, 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 use 0.1% triethylamine in water or 0.1% trifluoroacetic acid. Always flush through with solvents A and B and then thoroughly re-equilibrate the column after cleaning.

Column shipping solvent is 85% acetonitrile/15% water (v/v). Store the column in the shipping solvent when it will not be used the next day. For overnight storage, flush with 10 column volumes of mobile phase A at 50% of the recommended flow rate (~0.2 ml/min).

Store at room temperature; do not refrigerate.

SAMPLE PREPARATION

Salt-free, dry glycan samples (25 pmol – 50 nmol) should be labeled using Signal Labeling Kits^{2,3}. Labeled samples should be dried and redissolved in 5 - 50 μ l of 70% acetonitrile:30% water (v/v) before injection onto the column.

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. Samples up to 0.4 μ g and containing predominantly one species have been successfully chromatographed on GlycoSep N. The solubility of large sample amounts in the starting solvent of the gradient should be checked before injection.

Sample Collection and Finishing

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. Lyophilize or evaporate samples to remove solvents and ammonium formate.

Standards

We recommend ProZyme's Glucose Homopolymer labeled with 2-AA (product code GKSA-503) or 2-AB (product code GKSB-503) as an external standard to calibrate GlycoSep N. Alternatively, it may be prepared by labeling ProZyme's Glucose Homopolymer (product code GKI-4503) with the appropriate Signal Labeling Kit.

NOTE: ProZyme's Glucose Homopolymer is a mixture of dextran hydrolysates with $\alpha(1-6)$ linkages. Other commonly available standards may be maltooligosaccharides, which are $\alpha(1-4)$ linkages. At the same molecular weight, these standards will run similarly on GlycoSep N, but will not coelute. All data presented here was generated with Glucose Homopolymer.

The carbohydrate standards listed in this booklet are available from ProZyme. A set of these qualitative standards may be used for identification of samples instead of or in addition to the Glucose Homopolymer and calculated GU values (see "Applications"). However, the Glucose Homopolymer is useful to examine GlycoSep N performance from day to day.

TYPICAL RUNNING CONDITIONS

Two solvent systems are described here, using two different concentrations of ammonium formate buffer for mobile phase B. These have been designated **High Salt Solvent System** (HSSS) and **Low Salt Solvent System** (LSSS)¹:

Solvent A: acetonitrile

Solvent B: 50 mM ammonium formate pH 4.4 (**LSSS**)
or 250 mM ammonium formate pH 4.4 (**HSSS**)

NOTES:

Always use HPLC-grade solvents, buffers and water.

Ammonium formate may 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.

Thoroughly degas buffers and solvents prior to use, or use on-line degassing (e.g. by sparging with helium or with a vacuum degassing module).

Three typical gradients are shown in Table 1. Gradients 1 and 2 are suitable for all neutral and sialylated glycans (up to tetraantennary, tetrasialylated). Gradient 3 gives improved

resolution of O-linked oligosaccharides and for larger sugars containing polylactosamine repeats, but with somewhat extended run time.

Table 1 - Typical Gradients for GlycoSep N HPLC

| Gradient 1 | | | |
|-------------------|----|-----|--------------------|
| Time (min) | %A | %B | Flow rate (ml/min) |
| 0 | 65 | 35 | 0.4 |
| 72 | 47 | 53 | 0.4 |
| 75 | 0 | 100 | 0.4 |
| 77 | 0 | 100 | 1.0 |
| 92 | 0 | 100 | 1.0 |
| 95 | 65 | 35 | 1.0 |
| 100 | 65 | 35 | 0.4 |

| Gradient 2 | | | |
|-------------------|----|-----|--------------------|
| Time (min) | %A | %B | Flow rate (ml/min) |
| 0 | 65 | 35 | 0.4 |
| 70 | 50 | 50 | 0.4 |
| 71 | 0 | 100 | 0.4 |
| 80 | 0 | 100 | 0.4 |
| 81 | 65 | 35 | 0.4 |
| 110 | 65 | 35 | 0.4 |

Table 1 - Typical Gradients for GlycoSep N HPLC

| Gradient 3 | | | |
|------------|----|-----|--------------------|
| Time (min) | %A | %B | Flow rate (ml/min) |
| 0 | 80 | 20 | 0.4 |
| 132 | 47 | 53 | 0.4 |
| 135 | 0 | 100 | 0.4 |
| 137 | 0 | 100 | 1.0 |
| 142 | 0 | 100 | 1.0 |
| 145 | 80 | 20 | 1.0 |
| 180 | 80 | 20 | 0.4 |

APPLICATIONS

GlycoSep N may be used to separate both charged and neutral Signal-labeled glycans using a single binary gradient.

Retention is based on hydrophilicity of the glycan, which increases with molecular size in a predictable manner under the chromatographic conditions described herein. Generally, small glycans and monosaccharides are eluted first, followed by larger neutrals and, finally, sialylated structures. Small sialylated structures may elute before large neutrals since retention depends on size (irrespective of charge) and linkage.

The most common use of GlycoSep N is the analysis of samples against purified labeled standards. However, glycan

composition may be predicted from HPLC results. Elution of Signal-labeled glycans may be calibrated, and glucose unit (GU) values assigned, by reference to the peaks in the Signal-labeled Glucose Homopolymer [chromatographed under the same conditions (Figure 1)].

Table 2 shows the retention (expressed as 2-AB glucose units) of some 2-AB-labeled oligosaccharide standards on GlycoSep N. It is clear that using HSSS results in increased retention of sialylated oligosaccharides and slightly improved reproducibility compared to LSSS. Differences between GU values of neutral oligosaccharides obtained using the two solvent systems were small, although enhanced selectivity of some species has been reported using LSSS, particularly between arm-specific isomers. GU values were found to vary significantly with temperature, but only slightly from column to column.

Estimating GU Values for Additional Structures or Predicting Glycan Composition From GU Values

Experimental GU values for a series of standards may be used to calculate rules for GU contribution of each constituent monosaccharide using simple empirical calculations (Table 3). These derived rules may be further used to predict the GU values for additional standards or assign possible structures for unknowns. Although GU-value contribution for each monosaccharide varies slightly with the exact structure and/or linkage (particularly fucose and bisecting GlcNAc), the generalized rules shown in Table 3 give good agreement ($\pm 2\%$) with the experimentally determined values when used to calculate GU values for the standards in Table 2.

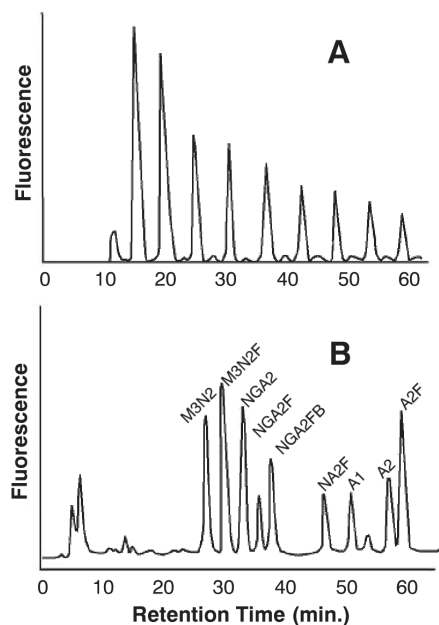


Figure 1 - Separation of 2-AB-labeled Oligosaccharide Standards on GlycoSep N

0.5 μ g of 2-AB-labeled dextran standard (panel A) or approximately 10 pmol each of the 2-AB-labeled oligosaccharide standards (Panel B) were dried, redissolved in 20 μ l 70% acetonitrile/30 % water (v/v) and injected onto GlycoSep N equilibrated with 65% acetonitrile/35% 250 mM ammonium formate, pH 4.4 (HSSS). Standards were eluted using Gradient 2 and detected fluorometrically.

Table 2 - GU Values for Standard Oligosaccharides Chromatographed on GlycoSep N at 30°C Using 2-AB-labeled Glucose Homopolymer

| Oligosaccharide Structure (Code) | 2-AB-GU | |
|----------------------------------|-----------------|-----------------|
| | HSSS | LSSS |
| MNN | 2.52 \pm 0.01 | 2.41 \pm 0.01 |
| MNNF | 3.04 \pm 0.01 | 2.96 \pm 0.01 |
| M3N2 [†] | 4.31 \pm 0.01 | 4.26 \pm 0.01 |
| M3N2F | 4.80 \pm 0.01 | 4.78 \pm 0.02 |
| MAN-5 | 6.14 \pm 0.01 | 6.12 \pm 0.02 |
| MAN-6 | 7.04 \pm 0.01 | 7.01 \pm 0.02 |
| MAN-7 (D1) [†] | 7.92 \pm 0.01 | 7.86 \pm 0.02 |
| HYBRID | 7.02 \pm 0.01 | 7.01 \pm 0.02 |
| NGA2 [†] | 5.32 \pm 0.01 | 5.28 \pm 0.01 |
| MAN-8 (D1,D3) | 8.84 \pm 0.02 | 8.82 \pm 0.03 |
| MAN-9 | 9.52 \pm 0.01 | 9.52 \pm 0.03 |
| NGA2F [†] | 5.76 \pm 0.02 | 5.72 \pm 0.01 |
| NGA2FB [†] | 6.08 \pm 0.02 | 6.07 \pm 0.01 |
| NA2 [†] | 7.05 \pm 0.01 | 7.00 \pm 0.01 |

Table 2 - GU Values for Standard Oligosaccharides Chromatographed on GlycoSep N at 30°C Using 2-AB-labeled Glucose Homopolymer

| Oligosaccharide Structure (Code) | 2-AB-GU | |
|----------------------------------|--------------|-------------|
| | HSSS | LSSS |
| NA2F [†] | 7.48 ± 0.02 | 7.42 ± 0.02 |
| NA2B [†] | 7.18 ± 0.02 | 7.16 ± 0.02 |
| NA2FB | 7.59 ± 0.01 | 7.56 ± 0.02 |
| NGA3 | 5.75 ± 0.01 | 5.69 ± 0.02 |
| NA3 | 8.33 ± 0.01 | 8.25 ± 0.03 |
| NA3F | 8.71 ± 0.01 | 8.62 ± 0.02 |
| NGA4 | 6.41 ± 0.01 | 6.34 ± 0.02 |
| NA4 | 9.74 ± 0.01 | 9.65 ± 0.03 |
| A1 (2-6) | 8.22 ± 0.02 | 7.44 ± 0.02 |
| A2 (2-6, 2-6) | 9.34 ± 0.02 | 7.93 ± 0.02 |
| A3 (2-3, 2-3, 2-6) | 10.56 ± 0.02 | 8.69 ± 0.02 |
| A3 (2-6, 2-6, 2-3) | 10.98 ± 0.02 | 9.04 ± 0.02 |

[†] used to calculate GU rules (Table 3)

The incremental values (Table 3, column 3) were calculated from GU values of standards (Table 2) according to the calculations in Table 3, column 2. The total hydrophilicity of a 2-AB-labeled oligosaccharide in terms of GU is approximately equal to the sum of the individual monosaccharide contributions plus a constant term. The GU contribution of a monosaccharide depends on its position, linkage and branching within the molecule. This is particularly noticeable for bisecting GlcNAc where three rules are given:

- Bisect 1 “Short arm bisect” – Both mannose branches are extended by one monosaccharide, whether branched or not.
- Bisect 2 “Long arm bisect” – At least one of the arms is extended by at least two monosaccharide units.
- Bisect 3 “Monoantennary” – At least one of the mannose branches is unsubstituted. The GU contribution for this type of bisect is approximately double that of bisect 1.

To illustrate how glycan structures may be assigned using these rules, glycans were released from two glycoproteins by hydrazinolysis and labeled with 2-AB using the Signal 2-AB Labeling Kit. An aliquot in 70% acetonitrile/30% water (v/v) was loaded onto GlycoSep N and eluted using HSSS and gradient 2. By reference to the 2-AB-(dextran ladder) and the rules for predicting retention time (Table 3), the glycan composition was predicted and compared to the known composition. Results for each glycoprotein are shown in the next section.

Table 3 - Calculated Rules for Predicting Retention Time of 2-AB-labeled Oligosaccharides Expressed in Glucose Units (GU)

| Monosaccharide Rule | Calculation | GU |
|--|---------------------------------|------|
| Man | (M7-M3N2)/4 | 0.90 |
| GlcNAc Bisect 1 | NGA2FB-NGA2F | 0.32 |
| GlcNAc Bisect 2 | NA2B-NA2 | 0.12 |
| GlcNAc Bisect 3 | GlcNAc Bisect 1 x 2 | 0.64 |
| Gal | (NA2-NGA2)/2 | 0.87 |
| GlcNAc | (NGA4-M3N2)/4 | 0.53 |
| Fuc α (1-6) to core GlcNAc | NGA2F-NGA2 | 0.44 |
| Fuc α (1-3) to outer arm GlcNAc | Core Fuc α (1-6) x 2 | 0.88 |
| Fuc α (1-2) to outer arm Gal | Same as core Fuc | 0.44 |
| Neu5Ac α (2-3) to Gal | A2(2-6, 2-3) - A1(2-6) | 0.71 |
| Neu5Ac α (2-6) to Gal | A2(2-6, 2-3) - A1(2-3) | 1.16 |
| Constant term | M3N2 - (3 x Man) - (2 x GlcNAc) | 0.54 |

EXAMPLES: PREDICTING COMPOSITION BASED ON GU VALUES

Tissue Plasminogen Activator

The oligosaccharide profile of recombinant tissue plasminogen activator (r-tPA) obtained using GlycoSep N is shown in Figure 2.

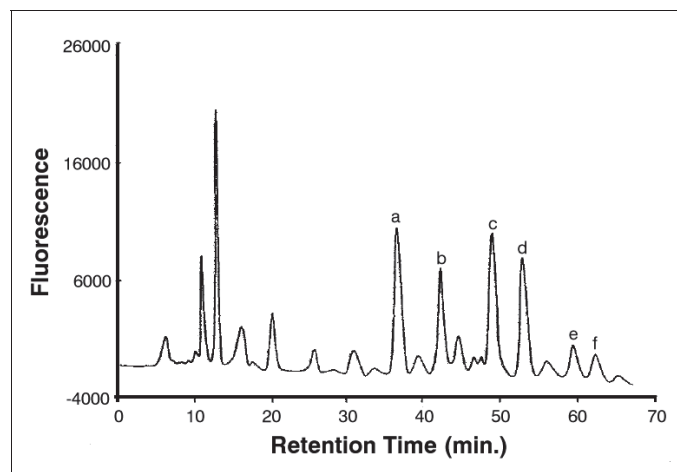


Figure 2 - GlycoSep N profile of 2-AB-labeled r-tPA glycans

A high degree of resolution was obtained and the labeled peaks in Figure 2 (a-f) are consistent with published results:

| Peak | GU | Predicted | Actual Structure |
|------|-------|---------------------|---|
| a | 6.10 | Man-5 | Oligomannose 5 |
| b | 6.99 | Man-6 | Oligomannose 6 |
| c | 8.09 | A1F (2-3) | Monosialylated $\alpha(2-3)$ biantennary core, substituted with fucose |
| d | 8.74 | A2 (2-3, 2-3) | Di-sialylated $\alpha(2-3)$ biantennary |
| e | 9.83 | A2F (2-3, 2-3) | Di-sialylated $\alpha(2-3)$ biantennary core, substituted with fucose |
| f | 10.40 | A3F (2-3, 2-3, 2-3) | Tri-sialylated $\alpha(2-3)$ triantennary core, substituted with fucose |

Mouse IgG

The oligosaccharide profile of mouse IgG obtained using GlycoSep N is shown in Figure 3.

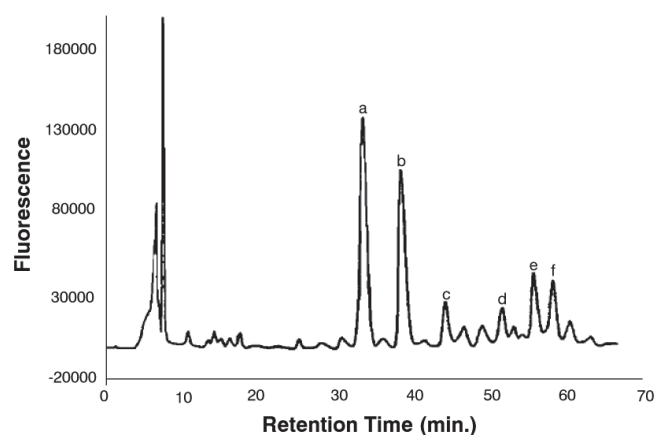


Figure 3 - GlycoSep N profile of 2-AB-labeled mouse IgG glycans

Again, a high degree of resolution was obtained and the labeled peaks in Figure 3 (a-f) are consistent with published results:

| Peak | GU | Predicted | Actual Structure |
|------|------|----------------|---|
| a | 5.70 | NGA2F | Asialo, galactosylated biantennary core, substituted with fucose |
| b | 6.51 | G1, NGA2F | Asialo, monogalactosyl biantennary core, substituted with fucose |
| c | 7.42 | NA2F | Asialo-, galactosylated biantennary core, substituted with fucose |
| d | 8.57 | A1F (2-6) | Monosialylated α (2-6) biantennary core, substituted with fucose |
| e | 9.28 | A2 (2-6, 2-6) | Di-sialylated α (2-6) biantennary |
| f | 9.70 | A2F (2-6, 2-6) | Di-sialylated α (2-6) biantennary core, substituted with fucose |

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APPENDIX A: SPECIFICATIONS

| | |
|--------------------------|--|
| Product name: | GlycoSep N HPLC Column |
| Product code: | GKI-4728 |
| Base matrix: | 5 μ silica |
| Derivatization: | Amide |
| Column size: | 4.6 x 250 mm. |
| Typical flow rate: | 0.3 – 1.2 ml/min |
| pH compatibility: | pH 2.0 - 7.5 |
| Temperature range: | 10 - 80°C |
| Maximum pressure: | 150 kg/cm ² or 2,250 psi |
| Solvent compatibility: | Acetonitrile, methanol, acetone and water |
| Typical analysis buffer: | Descending acetonitrile gradient containing ammonium formate buffer. |
| Analysis mode: | Hydrophilic (normal phase) |
| Column tube: | Stainless steel |

APPENDIX B: TROUBLESHOOTING

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

| 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 |

| 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 | Replace fuse |
| Lamp failed/low energy | 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 | See Appendix B: Fluorescent Settings |
| Sample precipitated | [acetonitrile] or [sample] too high |
| Fluid leak in system | See under "no flow" |
| Sample retained on column | Decrease [acetonitrile] in eluant |

| Sample not retained | |
|---|---|
| Possible Cause | Suggested Action |
| Column overloaded [acetonitrile] too low | Decrease sample size (<250 nmol) Starting gradient conditions should be >65% (v/v) acetonitrile, Sample should be diluted 70% (v/v) acetonitrile |

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

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

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

RELATED PROZYME PRODUCTS

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

<http://www.prozyme.com/glyko>

Find these products referred to in the text:

- Signal Labeling Kits
- Carbohydrate standards
- 2-AB-labeled standards
- GlycoSep Columns

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