XBridge Glycan BEH Amide, 130Å, 2.5 µm *XP* and 3.5 µm Columns and Standards

CONTENTS

I. INTRODUCTION

Thank you for choosing a Waters® XBridge® Glycan BEH Amide, 130Å Guard or Column containing our 2.5 µm ***XP*** or 3.5 µm particles designed for HILIC separations of 2-aminobenzamide (2-AB), 2-aminobenzoic acid (2-AA), or Waters *Rapi*Fluor-MS™ (p/n: 176003635) labeled glycans. This column chemistry, when used on an appropriately configured LC instrument, is capable of separating both neutral and charged labeled glycan species. Retention of 2-AB, 2-AA, or *Rapi*Fluor-MS labeled oligosaccharides is based on the hydrophobicity of the molecule, a parameter that is broadly related to hydrodynamic volume or molecular size. The resolving power of these columns is due in part to the particle size of the fully porous packing materials. Chemical and mechanical stability of the column are the consequence of Waters ethylene bridged hybrid (BEH Technology™) particle composition.

The column may be calibrated using one of the labeled Waters Dextran Calibration Ladder Standards (p/n: 186006841), such that elution may be expressed in terms of glucose units (GU). Under the suggested chromatographic conditions, the retention of Waters *Rapi*Fluor-MS, 2-AB, or 2-AA labeled oligosaccharides on the XBridge Glycan BEH Amide 2.5 µm ***XP*** or 3.5 µm Column may be predicted based on the hydrophilic contributions of the individual constituent monosaccharides.

II. GETTING STARTED

Each XBridge Glycan BEH Amide Column has a Certificate of Analysis and a Performance Test Chromatogram. The Certificate of Analysis is specific to each batch of packing material and includes the batch number and analyses of the physical and chemical properties of the particle. Particle size and pore structure are analyzed prior to bonding. The carbon and nitrogen content of the bonding are measured to insure consistent coverage. The selectivity of each batch is also assessed with the chromatographic separation of Waters Glycan Performance Standard (p/n: 186006349), a 2-AB labeled N-linked glycan standard mix spiked with Man-5 and Man-6, derived from human IgG. The complex mixture of IgG glycans includes high mannose structures as well as neutral and acidic complex structures. The retention times and retention time differences of selected components are used as the quality control test for each batch of packing material. The Performance Test Chromatogram is specific to each individual column and contains the following information: batch number, column serial number, backpressure, USP plate count, reduced plate height (RPH), USP tailing factor, retention factor (k'), peak width, and chromatographic conditions. These data can be found in the documentation supplied for each column and should be stored for future reference.

a. Column Installation

*Note: The flow rates given in the procedure below are for a typical 2.5 µm **XP** packing in a 2.1 mm I.D. column. It is also highly recommended that a column heater be used with our XBridge Glycan BEH Amide Column to help ensure consistent and reproducible separated component retention times.*

1. Purge the solvent delivery system of any buffer-containing or water-immiscible mobile phases and connect the inlet end of the column to the injector outlet. An arrow on the column identification label indicates the correct direction of solvent flow.
2. Flush the column with 100% organic mobile phase (acetonitrile) by setting the pump flow to 0.1 mL/min and increase the flow to 0.17 mL/min over 3 minutes. Increase the aqueous phase to 90% over 15 minutes. Note the backpressure. Decrease aqueous phase to starting conditions (22% aqueous in the test chromatogram).
3. When the mobile phase is flowing freely from the column outlet, stop the flow and attach the column outlet to the detector. This prevents entry of air into the detection system and gives more rapid baseline equilibration.
4. Gradually increase the flow rate from 0.17 to 0.34 mL/min over 3 minutes.
5. Once a stable backpressure and baseline have been achieved, proceed to the next section

b. Column Equilibration

Glycan Separation Technology Columns are shipped in 100% acetonitrile. It is important to ensure mobile phase compatibility before changing to a different mobile-phase system. Equilibrate the column with a minimum of 10 column volumes of the mobile phase to be used (refer to Table 1 for column volumes).

Table 1. Empty Column Volumes in mL (multiply by 10 for flush solvent volumes)

Column Length (mm)	Column Internal Diameter (mm)	Empty Column Volume (mL)
50	2.1	0.17
100	2.1	0.35
150	2.1	0.52
30	3.0	0.21
75	3.0	0.53
150	3.0	1.06
50	4.6	0.83
100	4.6	1.66
150	4.6	2.49
250	4.6	4.15

To avoid precipitating mobile-phase buffers on your column or in your system, flush the column with five column volumes of a water/organic solvent mixture using the same or higher acetonitrile content as in the desired buffered mobile phase. For example, flush the column and LC system with 50% acetonitrile in water prior to introducing 50% acetonitrile/50% buffered mobile phase.

The grade and quality of solvents are important for glycan analysis. It is strongly recommended to use only LC-MS grade reagents for glycan analysis mobile phase eluents. Use of Waters ammonium formate solution (p/n: 186007081) is also highly recommended in preparing Eluent A. It is a concentrate made from LC-MS grade reagents and will reduce the generation of undersired labeled glycan adducts.

Column equilibration may be judged initially by stable pressure and by a stable detector baseline. For a specific application, it is, however, necessary to test the required duration of equilibration. The criteria for adequate equilibration include reproducibility of retention time for major and minor peaks, resolution for critical pairs, and consistent baseline characteristics.

Note: Low concentration mobile-phase additives, particularly those with minimal buffering capacity, may require extended equilibration and re-equilibration between gradient analyses.

c. Initial Column Efficiency Determination

1. Perform an efficiency test on the column before using it in the desired application. Waters recommends using the solute mixture and conditions described in the "Performance Test Chromatogram" to test the column upon receipt.
2. Measure the retention of the test compounds and the number of theoretical plates (N).
3. Repeat the test at predetermined intervals to track column performance over time. Slight variations may be obtained on two different LC systems due to the quality of the connections, operating environment, system electronics, reagent quality, condition of column, and operator technique.

d. Conditioning of Previously Unused Columns

It is a good practice to ensure that new (previously unused) Glycan BEH Amide, 130Å columns are well conditioned and providing optimal performance before actual test sample analyses. This can be accomplished via sequential injections of a representative sample until a stable chromatographic profile is achieved.

It should also be noted that even if a Glycan BEH Amide column is to be used with an ammonium formate mobile phase, it can still prove useful to first condition it with a gradient and mobile phases containing 0.1% TFA. TFA can be effective in cleaning a column's stationary phase by both neutralizing and ion pairing contaminants that in their ionic form might otherwise strongly adsorb to a HILIC stationary phase.

e. Useful Functional Tests for Benchmarking a New Column

We suggest use of Waters 2-AB labeled Glycan Performance Standard (p/n: 186006349) to benchmark your new column and monitor its performance during use. *Note: This same 2-AB Glycan Performance Standard is used by Waters manufacturing to quality control test each batch of XBridge Glycan BEH Amide 130Å 2.5 µm **XP** and 3.5 µm material designed for this application (See example in Figure 1).*

To prepare the standard, add 100 µL of 50 mM ammonium formate buffer pH 4.4 and 100 µL of acetonitrile directly to the vial for a total volume of 200 µL.

Gently mix the sample by inversion.

Glycan Performance Test Standard

The separation shown in Figure 1 was generated on an ACQUITY® UPLC® whose total system volume and post column dispersion characteristics make it ideally suited for this application. Note that similar results can be obtained on an appropriately configured HPLC system. It is also important to take note of the injection solvent and injection volume for this application. Larger glycans have limited solubility in solutions that contain more than 50% acetonitrile. Gradual precipitation and loss of these larger glycans will be observed under these condtions. However, also note that large injections of water-containing samples will distort the peak shape in HILIC chromatography. Consequently, the optimum injection volume for these applications is <3 µL.

LC CONDITIONS

LC system:	ACQUITY UPLC H-Class Bio
Detection:	ACQUITY UPLC FLR Detector/ 2475 FLR Detector
Excitation:	330 nm
Emission:	420 nm
Scan rate:	10 Hz
Time const.:	0.2 sec
Gain:	1.00
Column:	XBridge Glycan BEH Amide, 130Å, 2.5 µm ***XP***, 2.1 x 150 mm (p/n: 186007265)
Column temp.:	60 °C
Sample temp.:	15 °C
Mobile phase A:	100 mM ammonium formate, pH 4.5
Mobile phase B:	Acetonitrile (ACN)
Vials:	LCGC Certified Clear Glass 12 x 32 mm Screw Neck Qsert Vial (p/n: 186001126C)

Gradient:

Time (min)	Flow Rate (mL/min)	Profile %A	%B
0.00	0.34	22.00	78.00
56.62	0.34	44.10	55.90
58.09	0.1	80.00	20.007
65.44	0.17	80.00	20.00
68.38	0.34	22.00	78.00
75.53	0.34	22.00	78.00

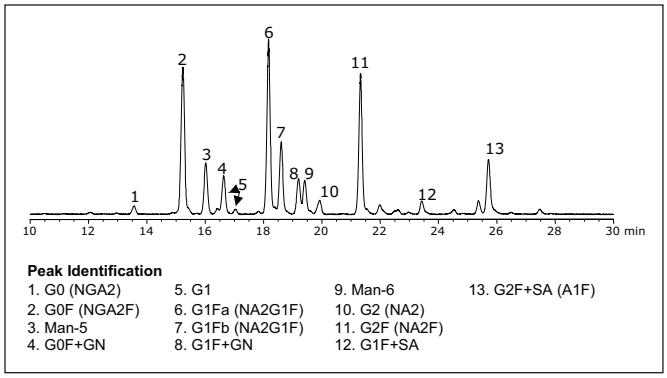

Figure 1. Typical chromatogram of 2-AB labeled human IgG N-linked glycans using the Glycan Performance Test Standard (p/n: 186006349).

*Note: Use of the scaled gradient shown below on an XBridge Glycan BEH Amide, 3.5 µm 130Å, 2.1 x 150 mm Column (p/n: 186007504) should yield results similar to Figure 1 that was generated on a XBridge Glycan BEH Amide, 130Å, 2.5 µm, **XP**, 2.1 x 150 mm Column (p/n: 186007265).*

Column: XBridge Glycan BEH Amide, 130Å, 3.5 µm, 2.1 x 150 mm (p/n: 186007504)

Gradient:

Time (min)	Flow Rate (mL/min)	Profile %A	%B
0.00	0.24	22.00	78.00
79.26	0.20	44.10	55.904
81.32	0.12	80.00	20.00
91.62	0.12	80.00	20.00
95.74	0.24	22.00	78.00
102.94	0.24	22.00	78.00

f. Scalable UPLC and HPLC BEH Glycan Column Offerings

Waters Glycan BEH Amide chemistry offerings are available in three highly scalable particle sizes that address UPLC (i.e. 1.7 µm) and HPLC-based (2.5 µm ***XP*** and 3.5 µm) application needs. (See Waters ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm Columns Care and Use Manual, literature code: 720003042EN.) Figure 2 clearly demonstrates the comparatively shorter analyses time with improved component resolution as the particle size of the XBridge Glycan BEH Amide Column chemistry is decreased. For this single variable study, the same low system dispersion/volume ACQUITY UPLC System was used with all three columns since use of different LC systems with different system volumes would affect the final recorded results.

III. COLUMN USE

To ensure the continued high performance of XBridge Glycan BEH Amide Columns, observe the following guidelines:

a. Sample Preparation

Sample impurities often contribute to column contamination. Samples should be free of particles before injection into the system.

In most separations it is preferable to prepare the sample in the gradient initial composition. However, the labeled glycans are often insoluble in the high acetonitrile concentrations which typify HILIC initial conditions. Since small volume injections are being made, the sample diluents may contain higher aqueous content (e.g. 50%) than the initial composition.

Note: A mixture of acetonitrile and dimethylformamide is recommended when analyzing Waters RapiFluor-MS Labeled glycans (see: GlycoWorks RapiFluor-MS N-Glycan Kit Care and Use Manual p/n: 715004793EN).

If the sample is not dissolved in the mobile-phase or solvent combinations specified in this manual, ensure that the sample, solvent, and mobile phases are miscible in order to avoid sample and/or buffer precipitation. Preparation of labeled glycans could involve one or two steps of solid-phase extraction. As a result, protein precipitate has typically been removed. If not, remove protein particles by centrifugation at >10,000 rpm for more than 2 minutes.

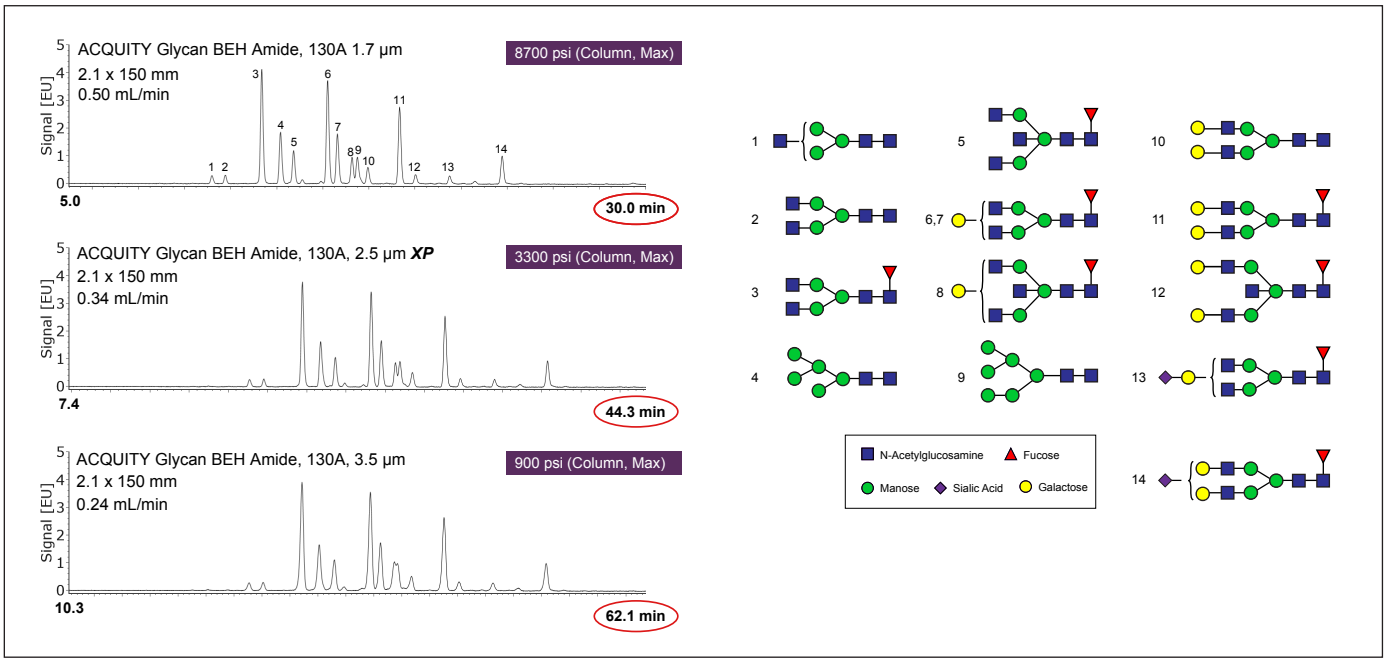

*Figure 2. Comparative scaled separations (based on column particle size differences) using Waters Glycan Performance Test Standard (p/n: 186006349) on ACQUITY UPLC H-Class System with ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm; XBridge Glycan BEH Amide, 130Å, 2.5 µm **XP**; and XBridge Glycan BEH Amide, 130Å, 3.5 µm Columns.*

Table 2. Buffer Recommendations for Using ACQUITY Glycan BEH Amide, 130Å Columns from pH 3 to 8

Additive/Buffer	pKa	Buffer Range (±1 pH unit)	Volatility	Used for Mass Spec	Comments
Acetic acid	4.76	–	Volatile	Yes	Maximum buffering obtained when used with ammonium acetate salt. Used in 0.1–1.0% range.
Formic acid	3.75	–	Volatile	Yes	Maximum buffering obtained when used with ammonium formate salt. Used in 0.1–1.0% range.
Ammonium (acetate)	9.20	8.2–10.2	Volatile	Yes	Up to 100 mM.
Ammonium (formate)	9.20	8.2–10.2	Volatile	Yes	Up to 250 mM.
Triethylamine (as acetate salt)		9.7–11.7	Volatile	Yes	Used in the 0.1–1.0% range. Volatile only when titrated with acetic acid (not hydrochloric or phosphoric). Used as ion pair for DNA analysis at pH 7–9.

b. Operating pH Limits

The recommended operating pH range for the XBridge Glycan BEH Amide, 130Å Column is 3 to 8. A listing of commonly used buffers and additives is given in Table 2. Additionally, the column lifetime will vary depending on the operating temperature as well as the type and concentration of buffer used.

c. Solvents

To maintain maximum column performance, use high quality chromatography grade solvents. If filtering, Acrodisc® filters are recommended. Solvents containing suspended particulate materials can damage the fluidic components of the UPLC System and will generally clog the inlet distribution frit of the column. This will result in higher operating pressure and poor performance.

d. Pressure

The XBridge Glycan BEH Amide, 130Å, 3.5 µm and 2.5 µm ***XP*** Columns will exhibit increased backpressure when operated in 80–100% aqueous mobile phases. As shown in the gradient table for Figure 1, the flow rate needs to be lowered when washing a glycan column. XBridge Glycan BEH Amide, 130Å, 3.5 µm Column can tolerate pressures up to 5000 psi (345 bar or 34 Mpa) while the maximum operating pressure for the 2.5 µm ***XP*** column is approximately 18,000 psi.

Note: Working at the extremes of pressure, pH and/or temperature will result in shorter column lifetimes.

e. Temperature

Temperatures between 20 °C–90 °C are recommended for operating XBridge Glycan BEH Amide, 130Å Columns in order to enhance selectivity, lower solvent viscosity, and increase mass transfer rates. However, higher temperature will have a negative effect on lifetime that will vary depending on the pH and buffer conditions used.

IV. TROUBLESHOOTING

The first step in systematic troubleshooting is comparing the column, in its current state, to the column when it was functioning properly. The method suggested in Section II for measuring plate count is an essential first step. This technique detects physical changes to the packed bed and chemical changes in the bonded-phase surface. The functional test with the 2-AB labeled Dextran Calibration Ladder (p/n: 186006841) or the Glycan Performance Test Standard (p/n: 186006349) may reveal more subtle changes in surface chemistry that affect the application.

There are several common symptoms of change in the column.

An increase in pressure is often associated with lost performance in the application. The first step in diagnosis is to ensure that the elevated pressure resides in the column rather than somewhere else in the system. This is determined by measuring pressure with and without the column attached to the instrument. If the system is occluded, the blockage should be identified and removed. If the pressure increase originates from the column, it is helpful to know whether the problem was associated with a single injection or whether it occurred over a series of injections. If the pressure gradually built up, it is likely that the column can be cleaned as described below (Section V). For future stability, it may be useful to incorporate a stronger regeneration step in the method. If a single sample caused the pressure increase, it likely reflects particulates or insoluble components. Cleaning is still an option, but using the more aggressive methods. The sudden pressure increase suggests that the user should consider some sample preparation, such as high speed centrifugation.

Loss of retention can reflect a change in the column surface chemistry. Before proceeding with diagnostic or corrective measures, check that the mobile phases have been correctly prepared and the correct method has been selected. Then repeat the plate count test and the glycan test standard.

If both the plate count and glycan test show loss of retention, it is likely that a significant fraction of the bonded phase has been lost, and the column will require replacement. If the changes are small and reflected only for some glycans, one of the cleaning procedures may be effective.

Change in peak shape, resolution, or relative retention of peaks. Follow the same steps as for loss of retention (Section II).

Carryover and memory effects are defined as the appearance of the constituents of one sample in the next gradient analysis. First determine whether the column or the system is the source of carryover. Define a gradient method that includes an "internal gradient". That is, the analytical gradient is repeated within a single method. If the glycan peaks appear in both gradients, at the same time after start, the carryover came from the column in what is often described as a "memory effect". If the glycan peaks only appear when an injection is made, they likely originated from adsorption to some system component. In that case follow the instrument manufacturer's recommendations. Memory effects as a source of carryover may be reduced or eliminated in several ways. First, raising the temperature of the separation reduces the possibility of non-specific adsorption. Second, memory effects may be more pronounced with steep gradients. Keep the gradient slope at 1% per column volume or less. Third, memory effects may be exacerbated by high flow rates. Reduce the flow rate by one half while doubling the gradient time to maintain a constant slope. Finally, apparent memory effects may actually reflect the solubility of the sample in the mobile phase. Reducing the amount injected may eliminate the effect.

Note: Useful general information on column troubleshooting problems may be found in HPLC Columns Theory, Technology and Practice, U.D. Neue, Wiley-VCH, 1997 (p/n: WAT038216), the Waters HPLC Troubleshooting Guide (Literature code # 720000181EN), or visit www.waters.com.

V. COLUMN CLEANING, REGENERATION, AND STORAGE

a. Cleaning and Regeneration

Changes in peak shape, peak splitting, shoulders on the peak, shifts in retention, change in resolution, carryover, ghost peaks, or increasing backpressure may indicate contamination of the column. Choose a cleaning option that may be expected to dissolve the suspected contaminant.

1. All cleaning procedures will be more effective at higher temperatures. It is reasonable to conduct cleaning at 70 °C.
2. It may be useful to conduct cleaning procedures at one half the flow rate typically used with that column. In this way, the possibility of high pressure events is reduced.
3. The first and simplest cleaning procedure is to run a series of gradients from 0–100% water. Be sure to reduce the flow rate for gradients with higher than 75% aqueous content. Columns of 150 mm length should be operated at 250 µL per minute or less during washes. The gradients can be as short as 5 column volumes and 3–5 repetitions may be effective.
4. Regeneration steps and flushing procedures using 100% aqueous mobile phase can help to maintain the optimal peak shape and selectivity of a HILIC separation. Additionally, an analyst can perform gradients with mobile phases containing 0.1% TFA as a means to maintain or recover the performance of a Glycoprotein BEH Amide column. TFA can be effective in cleaning a column's stationary phase by both neutralizing and ion pairing contaminants that in their ionic form might otherwise strongly adsorb to a HILIC stationary phase.
5. Several different cleaning solutions may be injected to strip strongly adsorbed material or particulates from the column. Make the largest injection possible with the system configuration. With such strong cleaning solutions, it is best to disconnect the detector from the column and to direct the flow to waste.
6. Flow reversal or backflushing is often suggested as part of a cleaning procedure. This should be reserved as a last resort. It may further damage the column or provide a short-lived improvement in performance.

b. Storage

For periods longer than four days at room temperature, store the column in 100% acetonitrile. Immediately after use with elevated temperatures and/or at pH extremes, store in 100% acetonitrile for the best column lifetime. Do not store columns in highly aqueous (<50% organic) mobile phases, as this may promote bacterial growth. If the mobile phase contained a buffer salt, flush the column with 10 column volumes of HPLC grade water (see Table 1 for common column volumes) and replace with 100% acetonitrile for storage. Failure to perform this intermediate step could result in precipitation of the buffer salt in the column or system when 100% acetonitrile is introduced. Completely seal the column to avoid evaporation and drying out the bed.

Note: If a column has been run with a mobile phase that contains formate (e.g., ammonium formate, formic acid, etc.) and is then flushed with 100% acetonitrile, slightly longer equilibration times may be necessary when the column is re-installed and run again with a formate-containing mobile phase.

VI. CAUTIONARY NOTE

Depending on users application, these products may be classified as hazardous following their use and as such are intended to be used by professional laboratory personnel trained in the competent handling of such materials. Responsibility for the safe use and disposal of products rests entirely with the purchaser and user. The Safety Data Sheet (SDS) for this product is available at www.waters.com.

VII. ORDERING INFORMATION (Partial listing. For more information, visit www.waters.com)

Product Description	Pore Size	Particle Size	Dimension	P/N
XBridge Glycan BEH Amide	130Å	2.5 µm	3.0 x 30 mm ***XP***	186008038
XBridge Glycan BEH Amide	130Å	2.5 µm	3.0 x 75 mm ***XP***	186008039
XBridge Glycan BEH Amide	130Å	2.5 µm	3.0 x 150 mm ***XP***	186008040
XBridge Glycan BEH Amide	130Å	2.5 µm	VanGuard Pre-Column	186007262
XBridge Glycan BEH Amide	130Å	2.5 µm	2.1 x 50 mm ***XP***	186007263
XBridge Glycan BEH Amide	130Å	2.5 µm	2.1 x 100 mm ***XP***	186007264
XBridge Glycan BEH Amide	130Å	2.5 µm	2.1 x 150 mm ***XP***	186007265
XBridge Glycan BEH Amide	130Å	2.5 µm	2.1 x 150 ***XP*** MVK	186007266
XBridge Glycan BEH Amide	130Å	2.5 µm	4.6 x 20 mm Guard, 2/pkg	186007267
XBridge Glycan BEH Amide	130Å	2.5 µm	4.6 x 50 mm ***XP***	186007268
XBridge Glycan BEH Amide	130Å	2.5 µm	4.6 x 100 mm ***XP***	186007269
XBridge Glycan BEH Amide	130Å	2.5 µm	4.6 x 150 mm ***XP***	186007270
XBridge Glycan BEH Amide	130Å	2.5 µm	4.6 x 150 ***XP*** MVK	186007271
XBridge Glycan BEH Amide	130Å	3.5 µm	4.6 x 20 mm Guard, 2/pkg	186007272
XBridge Glycan BEH Amide	130Å	3.5 µm	4.6 x 50 mm	186007273
XBridge Glycan BEH Amide	130Å	3.5 µm	4.6 x 100 mm	186007274
XBridge Glycan BEH Amide	130Å	3.5 µm	4.6 x 150 mm	186007275
XBridge Glycan BEH Amide	130Å	3.5 µm	4.6 x 250 mm	186007276
XBridge Glycan BEH Amide	130Å	3.5 µm	4.6 x 150 MVK	186007277
XBridge Glycan BEH Amide	130Å	3.5 µm	2.1 x 10 mm Guard, 2/pkg	186007505
XBridge Glycan BEH Amide	130Å	3.5 µm	2.1 x 50 mm	186007502
XBridge Glycan BEH Amide	130Å	3.5 µm	2.1 x 100 mm	186007503
XBridge Glycan BEH Amide	130Å	3.5 µm	2.1 x 150 mm	186007504
Glycan Performance Test Standard				186006349

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