

ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Columns and Glycoprotein Performance Test Standard

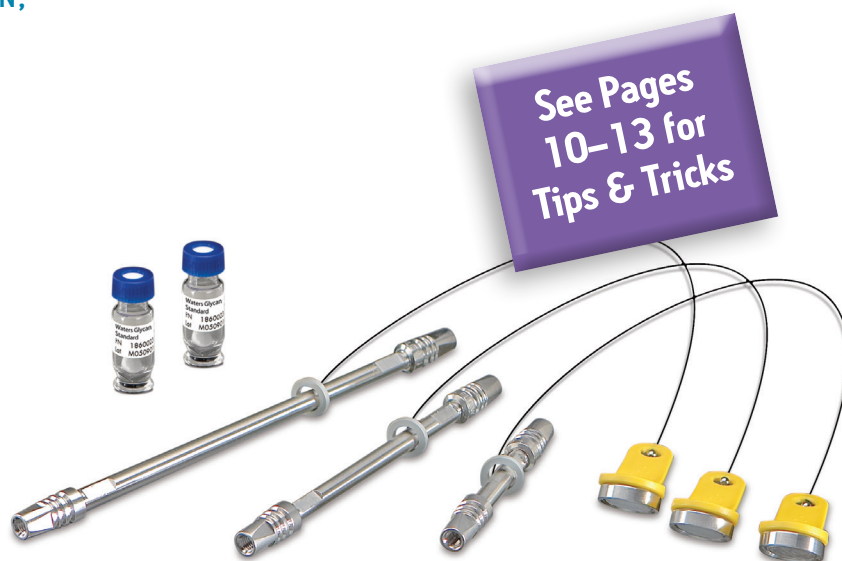
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I. INTRODUCTION

Thank you for choosing a Waters ACQUITY UPLC® Glycoprotein BEH Amide, 300Å, 1.7 µm Column that has been purposefully designed for intact glycoprotein profiling, subunit fragment analysis, and glycopeptide mapping using hydrophilic interaction chromatography (HILIC). These orthogonal analytical techniques provide additional means of characterizing protein glycosylation by assisting in glycan identification or in elucidating sites of glycan occupancy as documented in Waters Application Notes Entitled: “Developing High Resolution HILIC Separations of Intact Glycosylated Proteins Using a Wide-Pore Amide-Bonded Stationary Phase” (720005380EN), “Mapping IgG Subunit Glycoforms Using HILIC and a Wide-Pore Amide Stationary Phase” (720005385EN), and “HILIC Glycopeptide Mapping with a Wide-Pore Amide Stationary Phase” (720005409EN).

The high resolving power of this amide-based, UPLC® Column is due in part to the small particle size (1.7 µm) of the fully-porous packing material when used on an appropriately configured UPLC System. Outstanding chemical and mechanical stability of the column, that translates into long column life, are the result of using Waters ethylene bridged hybrid (BEH) particle coupled to innovative Amide bonding technology.



To help ensure column-to-column consistency, each batch of Waters ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm material is quality control tested with Waters Glycoprotein Performance Test Standard (p/n 186008010). This same glycoprotein standard (i.e, ribonuclease glycoforms) is included with each shipped column to assist users in obtaining optimal performance with a new column, and monitoring column and system performance over time.

The ACQUITY UPLC Glycoprotein BEH Amide, 300Å Column is based on the 1.7 µm particles that are characteristic of UltraPerformance LC®. The small particle size packing reduces dispersion and band broadening so that improved resolution, sensitivity, and speed are obtained in glycan separations. It is commonly expected that very high system backpressures will be observed with such small particles. The high pressure capability of the ACQUITY UPLC, ACQUITY UPLC H-Class, and ACQUITY UPLC H-Class Bio Systems is required for UPLC glycan analysis. With HILIC, back pressure increases with the increasing water content during the gradient. If an aqueous wash is chosen before re-equilibration, it might be necessary to use a lowered flow rate to prevent excessive back pressure.

Note: ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Columns are optimally designed for use with the ACQUITY UPLC, ACQUITY UPLC H-Class, and ACQUITY UPLC H-Class Bio Systems. The expected separations will not be obtained on a conventional HPLC system because of excessive dispersion and pressure limitations.

II. GETTING STARTED

Each ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm 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 Glycoprotein Performance Test Standard that consists of a mixture of Ribonuclease A and Ribonuclease B glycoforms (p/n 186008010). The retention times and resolution 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 on the eCord supplied with each column and should be stored for future reference.

a. eCord Installation

The eCord button should be attached to the side of the column heater module. The eCord button is magnetized and does not require specific orientation.

b. Column Connectors

The ACQUITY UPLC, ACQUITY UPLC H-Class, and ACQUITY UPLC H-Class Bio Systems utilize tubing and gold plated compression screws which have been designed to meet stringent tolerance levels and to minimize system dispersion.

For the ACQUITY UPLC System, columns should be attached to the injector with a column stabilizer, of which there are 4 types:

- 205000291 50 or 100 mm column
- 205000365 150 mm column
- 205000489 HTCH 50 or 100 mm
- 205000494 HTCH 150 mm

The first two parts are for the original heater and differ in the tubing arrangement to allow 150 mm columns to be used with a VanGuard™ pre-column or in-line filter while stabilizing the solvent temperature. The second two parts are for the newer high temperature column heater (HTCH). Optimized column inlet tubing is supplied with the ACQUITY UPLC System. The inject valve end of the tubing is clearly marked with a blue shrink tube marker. Insert the opposite end of the tubing into the ACQUITY UPLC Column and tighten the compression fitting using two 5/16-inch wrenches (or finger tighten the knurled nut).

If this column will be used on an ACQUITY UPLC H-Class or ACQUITY UPLC H-Class Bio System, simply connect the column to the active preheater supplied on the system using the gold fingertight fittings. There is only one configuration of column stabilizer on the ACQUITY UPLC H-Class and ACQUITY UPLC H-Class Bio Systems.

For more information on fittings and connective tubing, please refer to the relevant sections of the operator's guides for the ACQUITY UPLC, ACQUITY UPLC H-Class, and ACQUITY UPLC H-Class Bio Systems.

c. Column Installation

Note: The flow rates given in the procedure below are for a typical 1.7 μm packing in a 2.1 mm I.D. column.

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 0.25 mL/min over 3 minutes. Increase the aqueous phase to 90% over 10 minutes. Note the backpressure. Decrease aqueous phase to starting conditions (15% 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 0.25 to 0.5 mL/min over 3 minutes.
5. Once a stable backpressure and baseline have been achieved, proceed to the next section.

d. Column Equilibration

Glycoprotein 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	Volume in mL (2.1 mm I.D.)
50	0.17
100	0.35
150	0.52

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 UPLC system with 50% acetonitrile in water prior to introducing 50% acetonitrile/50% buffered mobile phase.

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.

e. 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. These conditions can be found on the eCord attached to the column.
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 UPLC Systems due to the quality of the connections, operating environment, system electronics, reagent quality, condition of column, and operator technique.

f. Conditioning of Previously Unused Columns and LC System Considerations

New (previously unused) ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Columns should be conditioned, before actual test sample analyses. This can be accomplished via sequential injections of a representative sample until a stable chromatographic profile is achieved. Alternatively two 40 µg injections of Glycoprotein Performance Test Standard (10 µL at 4 mg/mL in 0.1% TFA, 80% ACN) could be used.

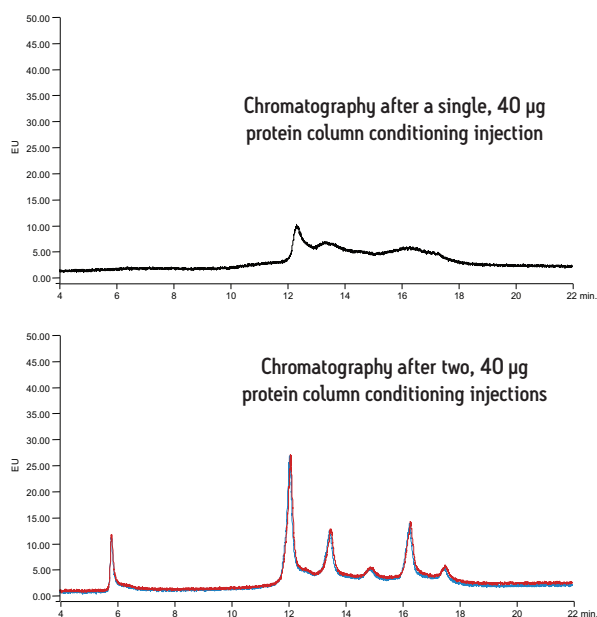


Figure 1. Conditioning of a previously unused ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Column. Top: Separation of Glycoprotein Performance Test Standard after a single column conditioning with 40 µg of same Glycoprotein Performance Test Standard. Bottom: Overlay of two sequential chromatograms of the Glycoprotein Performance Test Standard after two, 40 µg injections (i.e., 80 µg total protein) of same Glycoprotein Performance Test Standard.

It should also be noted that even if the Glycoprotein BEH Amide column is to be used with an alternative mobile phase system, such as ammonium formate, 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.

In addition, LC systems might require passivation in order to achieve optimal HILIC separations. It is advised to use metallic flow paths where ever possible, including post column tubing that extends to optical detectors and/or the divert valve of a mass spectrometer. Stainless steel and MP35N alloy materials

have been found to show better compatibility with large molecule HILIC than PEEK.

g. Useful Functional Tests for Benchmarking a New Column: Glycoprotein Performance Test Standard

We suggest use of Waters Glycoprotein Performance Test Standard (p/n 186008010) to benchmark your new column and monitor its performance during use.

Stability: Please store the standard in the original packaging at -20 °C upon arrival for long-term storage before solubilization. Once solubilized, the standard can be aliquoted and stored frozen for up to 3 months or stored at 4-10 °C for no more than a week, but avoid any freeze/thaw that could lead to degradation.

Recommended Reconstitution: Reconstitute the contents of each vial with 25 µL of 0.1% TFA in 80% ACN. For conditioning a previously unused 2.1 mm ID ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Column, perform two sequential gradient separations of the standard injected at a volume of 10 µL. For benchmarking and monitoring the performance of a 2.1 mm ID ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Column, perform gradient separations of the standard injected at a volume of 0.5 µL.

Generic Chromatographic Method

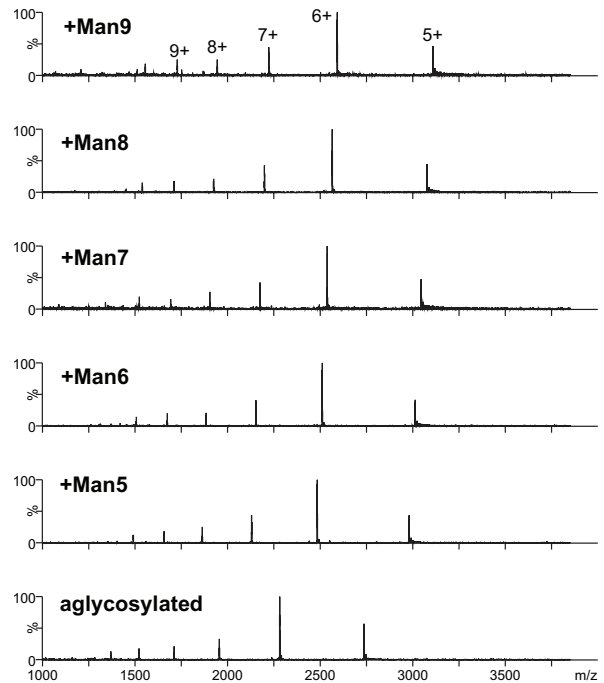
The conditions shown below are for use on an ACQUITY UPLC H-Class or ACQUITY UPLC H-Class Bio System, wherein 70/30 acetonitrile/water is used for the purge and wash solvents. Note that the ACQUITY UPLC H-Class and ACQUITY UPLC H-Class Bio Systems do not have a strong and weak needle wash. Instead, they have one purge and one wash solvent, both of which should be in 70/30 acetonitrile/water.

It might be necessary to avoid high organic diluents for some samples due to the propensity for proteins to precipitate under ambient conditions. A 2.1 mm ID column can typically accommodate up to a 1.2 µL aqueous injection before chromatographic performance is negatively affected.

High ACN diluents can sometimes be used in intact protein HILIC, but care must be taken to enhance the solubility of the protein sample through either the use of TFA ion pairing at concentrations between 0.2 to 1%, the combined application of TFA and hexafluoroisopropanol (HFIP), or by use of co-solvents, such as dimethylsulfoxide (DMSO).

Injection volume: 1.0 µL
 Column: ACQUITY UPLC Glycoprotein
 BEH Amide, 300Å, 1.7 µm
 2.1 x 150 mm (p/n 186007963)
 Eluent A: 0.1% (v/v) TFA, H₂O
 Eluent B: 0.1% (v/v) TFA, ACN
 Wash and purge solvents: Acetonitrile/HPLC grade water,
 (70/30 v/v)
 Temperature: 45 °C
 Native Fluorescence
 Detection: Fluorescence: λ_{ex} = 280 nm,
 λ_{em} = 320 nm
 UV Detection: 214 / 280 nm, 2 Hz
 Flow: 0.2 mL/min

Gradient:	Time	%A	%B	Curve
	0.0	15.0	85.0	6
	0.5	15.0	85.0	6
	1.0	33.0	67.0	6
	21.0	40.0	60.0	6
	22.0	100.0	0.0	6
	24.0	100.0	0.0	6
	25.0	15.0	85.0	6
	35.0	15.0	85.0	6



KETAA AKFER QHMDSSTSAA CNQMMKSRNL
 TKDRC KPVNT AVCSQ KNVAC KNGQT
 Disulfides
 NCYQSYSTMSITD CR ETGSS KYPNC AYKTT QANKH
 IIVA C EGNPYVPVHF DASV

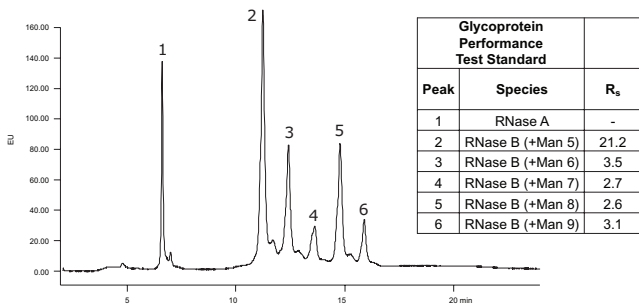


Figure 2. Separation of the Glycoprotein Performance Test Standard (RNase A + RNase B glycoforms) using an ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column. Fluorescence detection at Ex 280 nm and Em 320 nm and a column temperature of 45 °C were employed in this example.

Species	M _{Average} (Da)
Aglycosylated (4 disulfides)	13682.3
RNase B (4 disulfides), Man5	14899.4
RNase B (4 disulfides), Man6	15061.6
RNase B (4 disulfides), Man7	15223.7
RNase B (4 disulfides), Man8	15385.8
RNase B (4 disulfides), Man9	15548.0

Figure 3. Sequence Information and MS Data for the Glycoprotein Performance Test Standard.

Table 2. Buffer Recommendations for Using ACQUITY UPLC Glycan BEH Amide, 300Å, 1.7 µm Columns

Additive/Buffer	pKa	Buffer range (±1 pH unit)	Volatility	Used for Mass Spec	Comments
Trifluoroacetic acid	0.23	-	Volatile	Yes	TFA ion pairing is often essential in large molecule HILIC separations as it improves the solubility of proteins in high ACN mobile phase and shields the proteinaceous residues from interacting with the amide stationary phase. TFA ion pairing therefore improves peak shape, selectivity and resolution of the hydrophilic modifications on proteins and peptides (Waters Application Note 720005380EN)
Formic acid	3.75	-	Volatile	Yes	Maximum buffering obtained when used with ammonium formate salt. Used in 0.1-1.0% range.

III. COLUMN USE

To ensure the continued high performance of the ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Columns, observe the following guidelines:

a. Sample Preparation

1. Sample impurities often contribute to column contamination. Samples should be free of particles before injection into the system.
2. 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. It is good practice to remove any precipitate or particulates by centrifugation at >10,000 rpm for more than 2 minutes.

b. Operating pH Limits

The recommended operating pH range for the ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Column is 2 to 10. 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 ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Columns will have greatly increased backpressure when operated in 90-100% aqueous mobile phases. When employing long column lengths or investigating high flow rates, it might be necessary to be mindful of this increase in pressure and to potentially reduce flow rates during the aqueous regeneration step of the HILIC separation. ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Columns can tolerate pressures of up to 15,000 psi (1034 bar or 103 Mpa).

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 ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm 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 ribonuclease A and B glycoforms in the Glycoprotein Performance Test Standard may reveal more subtle changes in surface chemistry that affect the application.

There are several common symptoms of change in the column.

1. 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.

2. 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 a separation of the Glycoprotein Performance Test Standard. If both the plate count and glycoprotein 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.
3. Change in peak shape, resolution, or relative retention of peaks. Follow the same steps as for loss of retention (Section II).
4. 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 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 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. 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), 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 μ L 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. 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.

VI. INTRODUCING eCORD INTELLIGENT CHIP TECHNOLOGY

a. Introduction

The eCord intelligent chip provides the history of a column's performance throughout its lifetime. The eCord is permanently attached to the column to assure that the column's performance history is maintained in the event that the column is moved from one instrument to another.

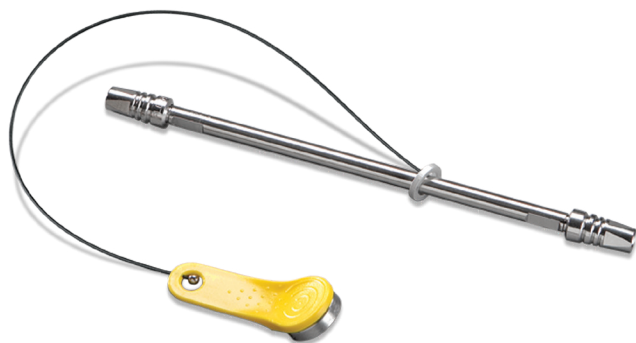


Figure 4. eCord Intelligent Chip.

At the time of manufacture, tracking and quality control information will be downloaded to the eCord. This includes the conditions and results for the Performance Test Chromatogram. Storing this information on the chip will eliminate the need for a paper Certificate of Analysis. Once the user installs the column, the software will automatically download key parameters into a column history file stored on the chip. In this manual, we explain how the eCord will provide a solution for easily tracking the history of the column, reduce the frustration of paperwork trails, and give customers the reassurance that a well performing column is installed onto their instruments.

b. Installation

Install the column into the column heater. Plug the eCord into the side of the column heater. Once the eCord is inserted into the column heater, the identification and overall column usage information will be available in the ACQUITY UPLC Console, allowing the user to access column information on their desktop.

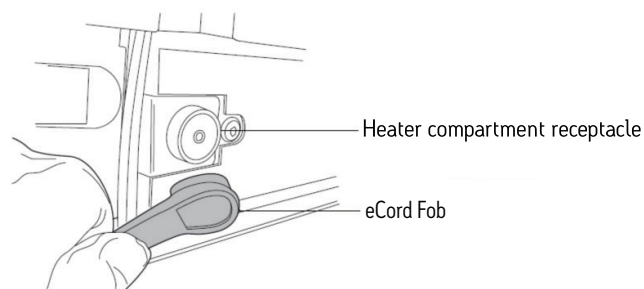
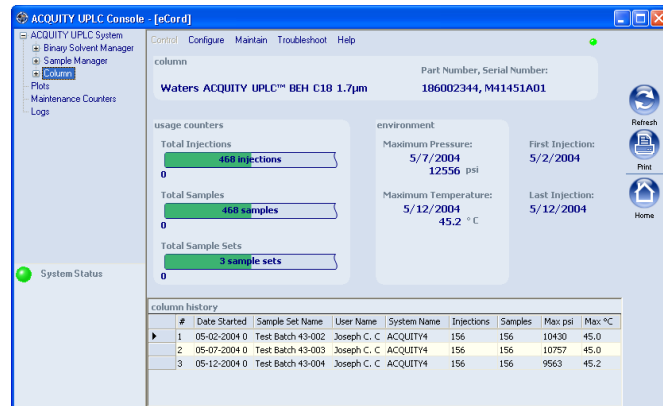
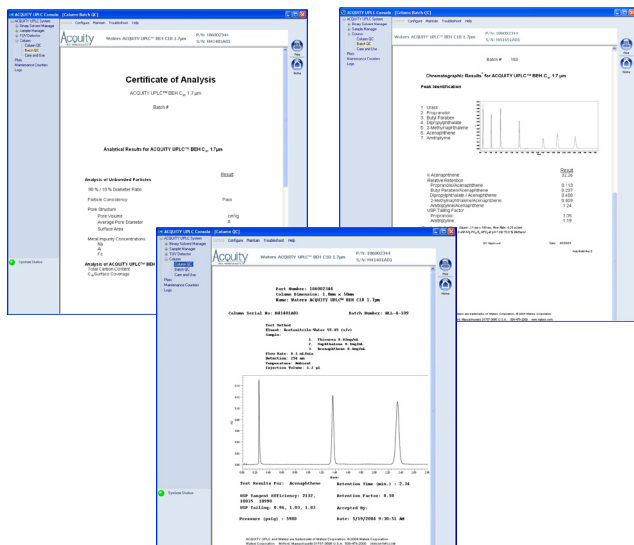


Figure 5. Installing the eCord Intelligent Chip.

c. Manufacturing Information

The eCord chip provides the user with QC test conditions and results on the column run by the manufacturer. The information includes mobile phases, running conditions and analytes used to test the columns. In addition, the QC results and acceptance is placed onto the column.



d. Column Use Information

The eCord chip provides the customer with column use data. The top of the screen identifies the column including chemistry type, column dimensions and serial number. The overall column usage information includes: total number of samples, total number of injections, total sample sets, date of first injection, date of last injection, maximum pressure, and temperature. The information also details the column history by sample set including: date started, sample set name, user name, system name, number of injections in the sample set, number of samples in the sample set, maximum pressure, and temperature in the sample set and if the column met basic system suitability requirements.

VII. 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.

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

Description	Pore size	Particle size	Dimensions	Part no.
ACQUITY UPLC Glycoprotein BEH Amide	300Å	1.7 µm	2.1 x 5 mm Guard 3/pk with Std	176003699
ACQUITY UPLC Glycoprotein BEH Amide	300Å	1.7 µm	2.1 x 50 mm with Std	176003700
ACQUITY UPLC Glycoprotein BEH Amide	300Å	1.7 µm	2.1 x 100 mm with Std	176003701
ACQUITY UPLC Glycoprotein BEH Amide	300Å	1.7 µm	2.1 x 150 mm with Std	176003702
ACQUITY UPLC Glycoprotein BEH Amide	300Å	1.7 µm	2.1 x 100 MVK, 3/pk with Std	176003703
Glycoprotein Performance Test Standard				186008010

HILIC FOR LARGE MOLECULES: PRACTICAL CONSIDERATIONS

Condition Your New Column

- Column conditioning is a common practice used to optimize performance via passivation of secondary interactions.
- HILIC of large molecules is a new frontier! Conditioning of new columns is as important as ever.
- The amide bonding should be primed for its interaction with the analyte, yet once it is conditioned it need not be re-conditioned even after long-term storage.

TIP

- Before using a new column, run 2 injections of your intended sample type at a mass load 10x greater than would be used in a normal analytical run.
 - Per conditioning run, load 20-100 µg protein or 50-250 pmoles of released glycan.
 - If the column is for mAbs, use a mAb; tetrasialylated glycans, use a labeled/unlabeled fetuin N-glycan pool; etc.).
- Run 1 blank and continue with your analyses.

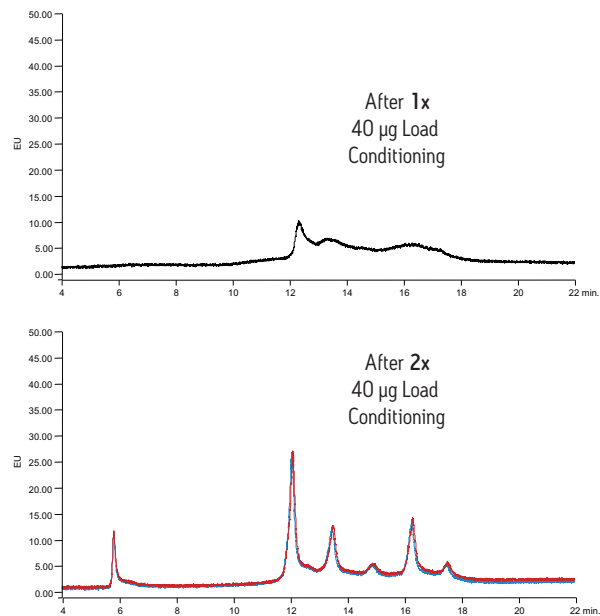


Figure 6. Conditioning of a previously unused ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Column. Shown in the lower panel is an overlay of two analytical runs.

Understand the Role of Ion Pairing

- TFA ion pairing is often essential in large molecule HILIC separations not only because it improves the solubility of proteins in high ACN mobile phase but because it shields proteinaceous residues from interacting with the amide stationary phase.
- TFA ion pairing therefore improves peak shape, selectivity and resolution of the hydrophilic residues and modifications of proteins and peptides.
- The larger the proteinaceous analyte the more critical TFA ion pairing.
- For smaller analytes, like peptides/glycopeptides, some TFA can be replaced with formic acid. However, peaks are likely to be more strongly retained and to exhibit additional broadening due to more complicated, heterogenous analyte to stationary phase interactions. Selectivity between aglycosylated and glycosylated peptides will also be affected.

TIP

- Use mobile phases modified with 0.1% TFA whenever possible.

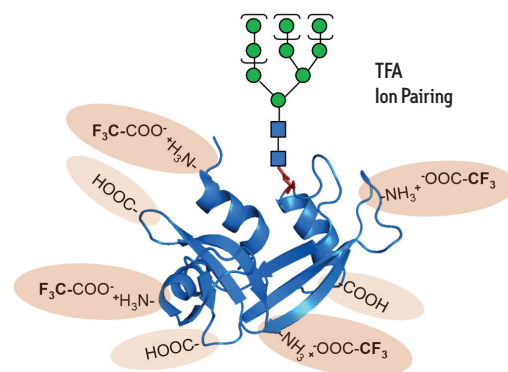
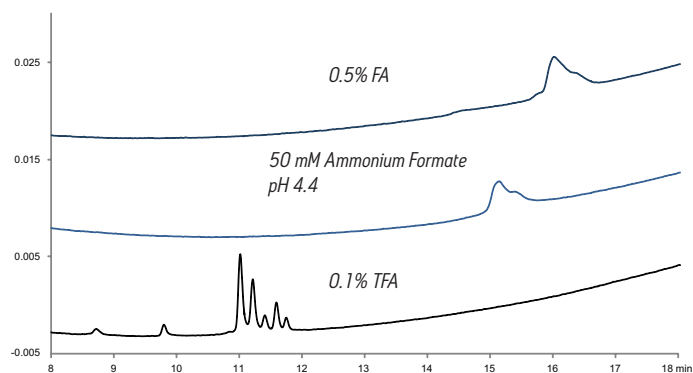


Figure 7. Effect of mobile phase additive on the separation of ribonuclease B.

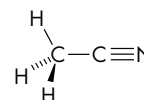
Consider Your Diluents and Sample Solubility

- It is common practice to match the composition of the sample diluent to your initial chromatographic conditions. HILIC of large molecules requires that this be given careful consideration, because some analytes may not be soluble in high organic concentrations. 70-90% ACN could be a condition that initiates the precipitation of your sample.
- Understand that HILIC can in fact be performed on aqueous samples. The only requirement is that very minimal volumes of sample be injected (see next section).
- High ACN diluents can be used, but care must be taken to enhance the solubility of the sample.

TIP

- Add TFA to the sample at concentrations between 0.2 to 1%.
- Use additional additives, like heptafluorobutyric acid (HFBA) or hexafluoroisopropanol (HFIP).
- Use a co-solvent, such as dimethylsulfoxide (DMSO), in concentrations ranging between 1 and 25%.

How do you deal with so much organic solvent?



Can you load from aqueous diluent?



If high ACN..

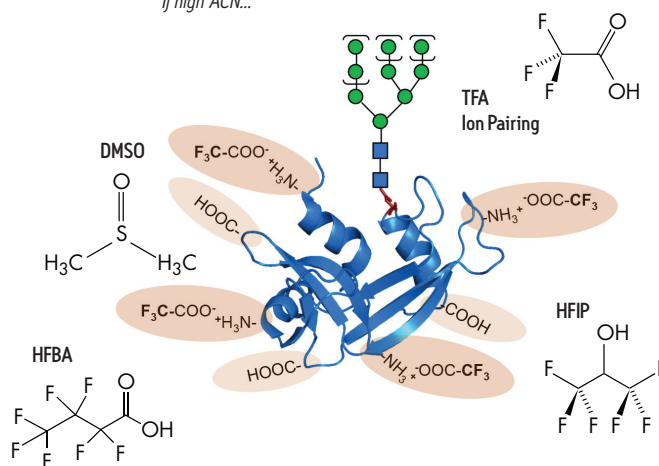


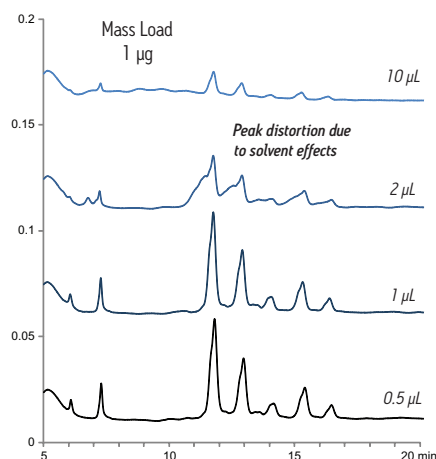
Figure 8. Additives to consider if using ACN/organic solvent diluents.

Minimize Injection Volumes

- In most situations, HILIC injection volumes must be much lower than what can otherwise be used in reversed-phase chromatography.
- 100% AQUEOUS Samples
 - For a 2.1 mm ID column, maximum injection volumes are usually between 0.4 and 1.2 μ L (depending on the dispersion of your LC).
- ACETONITRILE Containing Samples
 - Adding ACN to a sample increases the allowable injection volume but can create solubility issues as noted earlier.
- High ACN initial conditions can facilitate sample loading and the mixing of sample to higher ACN content before it sees the head of the column. Better band formation can thereby be achieved.

TIP

- Empirically determine your maximum injection volume.
 - Inject varying volumes, i.e. 1, 3, and 10 μ L.
 - Select an injection volume that does not show signs of breakthrough or solvent effects.



Time (min)	%A	%B	Time (min)	%A	%B
0	33.0	67.0	0	15.0	85.0
20.0	40.0	60.0	0.5	15.0	85.0
21.0	100.0	0.0	1.0	33.0	67.0
23.0	100.0	0.0	21.0	40.0	60.0
24.0	33.0	67.0	22.0	100.0	0.0
34.0	33.0	67.0	24.0	100.0	0.0
			25.0	15.0	85.0
			35.0	15.0	85.0

Figure 9. Determining the optimal volume load for an aqueous sample of ribonuclease B. An initial gradient condition to improve band formation (shown in red).

Take Advantage of Temperature, Additives, and High Pressures to Avoid On-Column Aggregation

- With HILIC of large molecules, on-column aggregation can sometimes be encountered in the form of distorted, spurious peaks comprised of multiple analyte forms.
- Choose method conditions that enhance the solubility of analytes in ACN.
 - Proteins are most soluble in ACN mixtures when in the presence of hydrophobic, strong acid mobile phase additives.
 - Elevated column temperatures improve solubility.
 - Ultrahigh back pressures can also be used to minimize aggregation.

TIP

- Use hydrophobic mobile phase additives.
 - TFA and/or HFIP.
 - Avoid HFBA if performing LC-MS (adduct formation).
- Explore elevated column temperatures if needed (i.e., 50 to 80 °C).
- Use long column lengths, coupled columns and/or flow restrictors if possible.

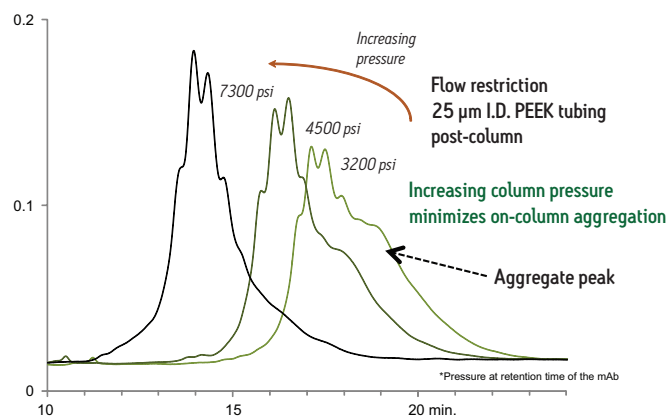


Figure 10. Using flow restriction post-column, pre-detector to eliminate the on-column aggregation of an intact mAb (trastuzumab).

Disrupt Non-Covalent Interactions in Your Sample

- The denaturing conditions of HILIC chromatography cannot always be relied upon to disrupt non-covalent interactions.
- It is best practice to disrupt these interactions in the sample, prior to making an injection.
- Non-covalent complexes can sometimes otherwise be separated to the detriment of accurately profiling a sample.

TIP

- Add GuHCl to your sample (up to 6 M GuHCl into a fully aqueous sample).

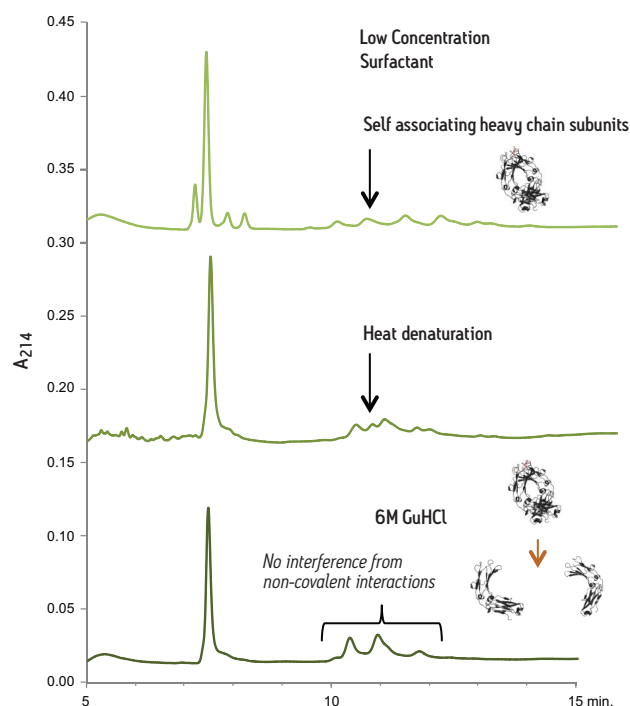
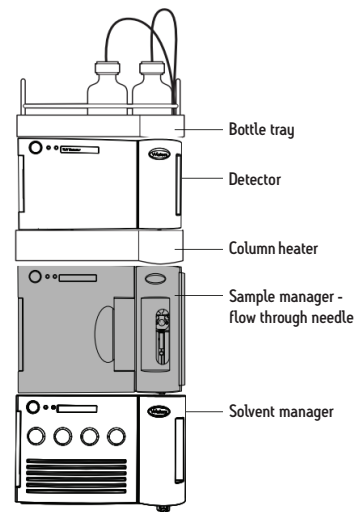


Figure 11. HILIC profiling of reduced mAb samples prepared from various conditions.

Use a Flow Through Needle When Possible

- With the possible solubility issues of proteins under HILIC conditions, it is best to use a flow through needle autosampler so that sample immediately goes to the LC flow path and is quickly pressurized via the pump and the column backpressure.
- If restricted to a fixed loop system, use a full loop injection mechanism to eliminate issues with precipitation, which could occur as analyte is diluted at low pressure with needle wash during a partial loop injection.
 - Weak Needle Wash: 0.1% TFA, 85% ACN.
 - Strong Needle Wash : 0.1% TFA, water.



TIP

- Use flow through needle injection mechanisms whenever possible.

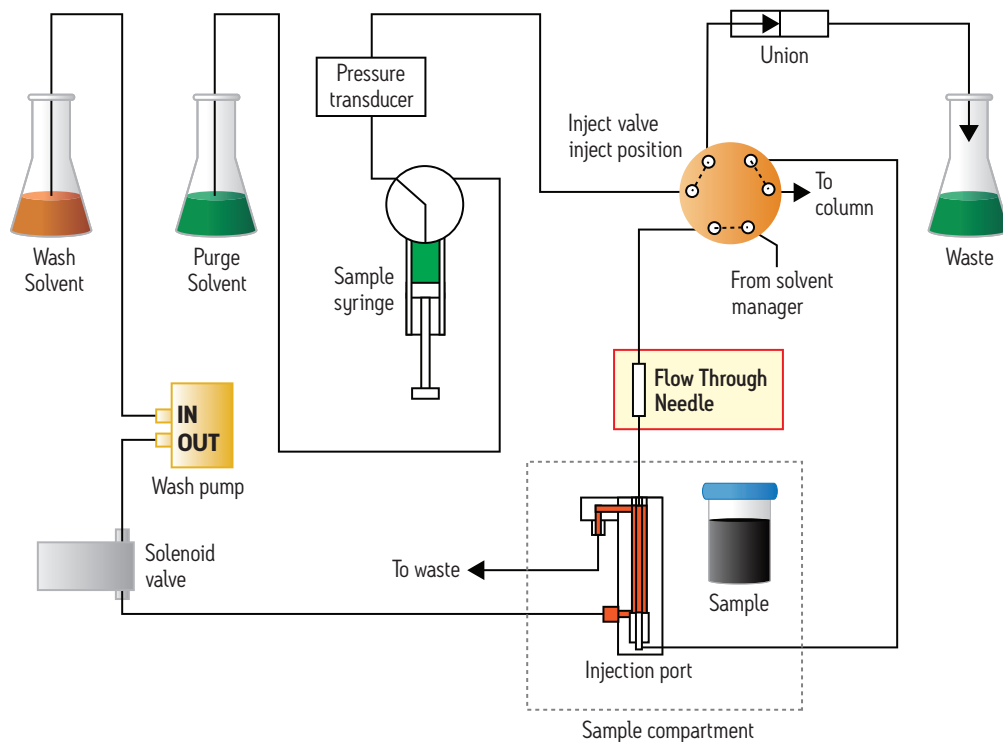


Figure 12. Flow through needle injections.



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