

ACQUITY UPLC BEH GLYCAN, 1.7 μm Columns

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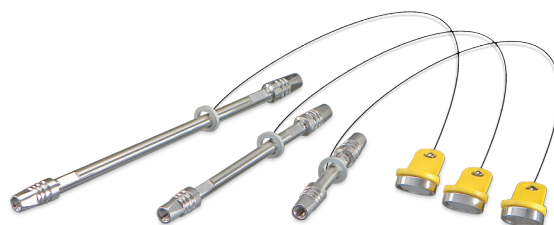
VII. ORDERING INFORMATION

I. INTRODUCTION

Thank you for choosing Waters ACQUITY UPLC® BEH Glycan column designed for HILIC-mode separations of 2-aminobenzamide (2-AB) labeled glycans.* This column chemistry and the UPLC® instrument conditions recommended in this manual are capable of separating both neutral and charged species. Retention of 2-AB-labeled oligosaccharides is based on the hydrophilicity of the molecule, a parameter that is broadly related to hydrodynamic volume or molecular size. The high resolving power of this column is due in part to the small particle size (1.7 μm) of the fully porous packing material. Chemical and mechanical stability of the column are the consequence of Waters ethylene bridged hybrid (BEH) particle composition.

The column may be calibrated using a labeled dextran hydrolysate ladder, such that elution may be expressed in terms of glucose units (GU). Under the suggested chromatographic conditions, the retention of 2-AB labeled oligosaccharides on the ACQUITY UPLC BEH Glycan column may be predicted based on the hydrophilic contributions of the individual constituent monosaccharides.

The ACQUITY UPLC BEH Glycan 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 mode, back pressure increases with the increasing water content during the gradient. If an aqueous wash is chosen before re-equilibration, the flow rate must be lowered to prevent excessive back pressure.



Note: ACQUITY UPLC BEH Glycan, 1.7 μ m 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 BEH Glycan 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 2-AB labeled *N*-linked glycans 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 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 μ m 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 (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 0.25 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

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	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. Useful Functional Tests for Benchmarking a New Column

The performance test uses a Glycan Performance Test Standard (Part No. 186006349). This test sample consists of 2-AB Human IgG and is QC verified to contain the components needed to benchmark and monitor a new column, as shown in Figure 1.

To prepare the standard, add 100 µL of 100mM ammonium formate buffer pH 4.5 and 100 µL of acetonitrile directly to the vial for a total volume of 200 µL.

Gently mix the sample by inversion.

Human IgG Glycans Performance Test

The conditions shown below are for use on an ACQUITY UPLC system. If you are using an ACQUITY UPLC H-Class or ACQUITY UPLC H-Class Bio system, use 50/50 acetonitrile/water for the purge and wash solvents. All of the other conditions can remain the same. 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 50/50 acetonitrile/water.

It is also important to note 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 conditions. However, also note that large injections of water-containing samples will distort the peak shape in HILIC chromatography. The optimum injection volume for these applications is < 3 µL.

Injection Volume:	1.5 µL
Injection Mode:	Partial loop (20 µL loop)
Column:	ACQUITY UPLC BEH Glycan, 2.1 x 150 mm , 1.7 µm
Eluent A:	100 mM ammonium formate, pH 4.5
Eluent B:	Acetonitrile
Weak Needle Wash:	Acetonitrile/HPLC grade water, (90/10 v/v)
Strong Needle Wash:	Acetonitrile/HPLC grade water, (10/90 v/v)
Seal Wash:	Acetonitrile/water (50/50 v/v)
Temperature:	60 °C
Detection:	Fluorescence: $\lambda_{ex} = 330$ nm, $\lambda_{em} = 420$ nm
Gradient:	Time Flow Rate
	(min) (mL/min) %A %B
	Init. 0.50 22.0 78.0
	38.5 0.50 44.1 55.9
	39.5 0.25* 100.0 0
	44.5 0.25* 100.0 0
	46.5 0.50 22.0 78.0

Note: The peptide needle and additional mixers are not required to perform the separations in this care and use manual. However, if these components are already installed on the system from other applications, it is not necessary to remove them. No changes in the separation quality of N-linked glycans should be observed if these components are installed.

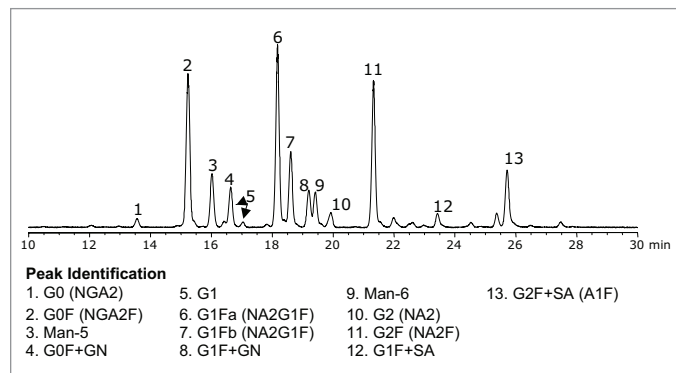


Figure 1: Typical chromatogram of 2-AB labeled human IgG N-linked glycans using the Glycan Performance Test Standard (Part No. 186003649).

This chromatogram is typical of the results obtained in Waters laboratories with the method described above, using an ACQUITY UPLC BEH Glycan, 2.1 x 150 mm , 1.7 µm column. The retention times will be decreased by 33% on a 100 mm length column and 67% on a 50 mm length column, and the resolution of component peaks will also be reduced proportionately, as shown in Figure 2. Since these columns are all designed to be used with an ACQUITY UPLC instrument which has low dead volume, high pressure and high sensitivity, chromatographic retention times will be quite similar. Variances will be due to system volume (e.g. the mixer volume) and dispersion, column oven, and detector cell. This test is exceptionally valuable for monitoring the life of the column and for troubleshooting separation difficulties that may arise.

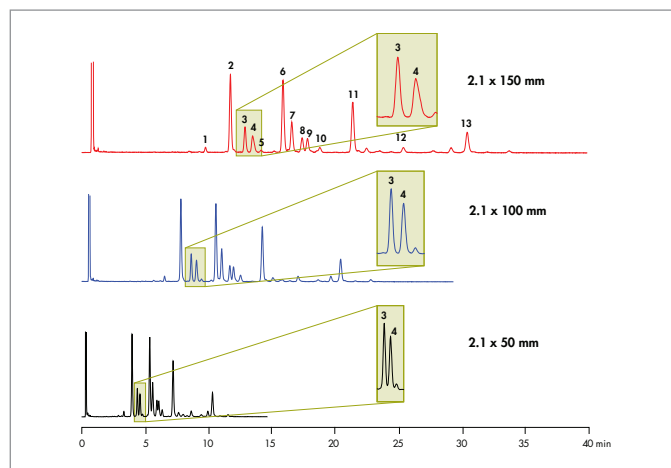
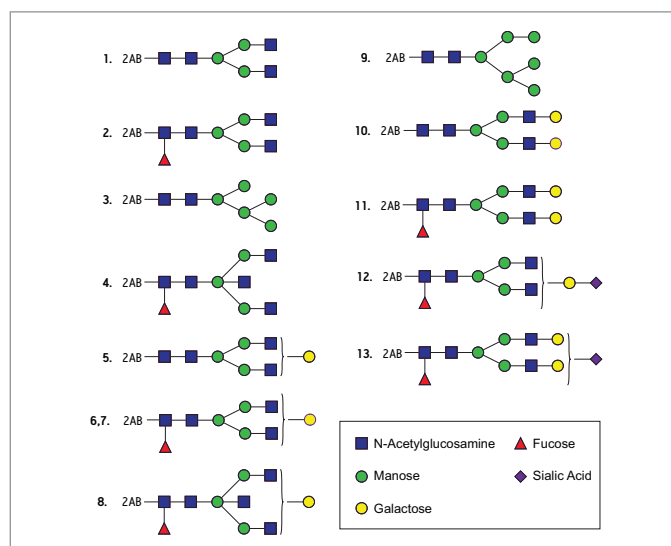


Figure 2: Typical chromatogram of 2-AB labeled human IgG on 2.1 x 150 mm, 2.1 x 100 mm, and 2.1 x 50 mm columns.



III. COLUMN USE

To ensure the continued high performance of ACQUITY UPLC BEH Glycan 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. In most separations it is preferable to prepare the sample in the gradient initial composition. However, the 2-AB 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.
3. 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 2-AB labeled glycans involves 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.

b. Operating pH Limits

The recommended operating pH range for the ACQUITY UPLC BEH Glycan 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.

Table 2: Buffer Recommendations for Using ACQUITY UPLC BEH Glycan 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)	10.70	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.

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 BEH Glycan columns will have greatly increased backpressure when operated in 90-100% aqueous mobile phases. As shown in the gradient table for Figure 1, the flow rate needs to be lowered to 0.25 mL/min when washing a 2.1 x 150 mm Glycan column in 100% A. ACQUITY UPLC BEH Glycan columns can tolerate pressures of up to 15,000 psi (1034 bar or 103 Mpa), although pressures greater than 13,000 psi should be avoided in order to maximize column and system lifetimes.

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 BEH Glycan 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 ladder or IgG glycans 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 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.
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 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), 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. 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.
5. 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. 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.

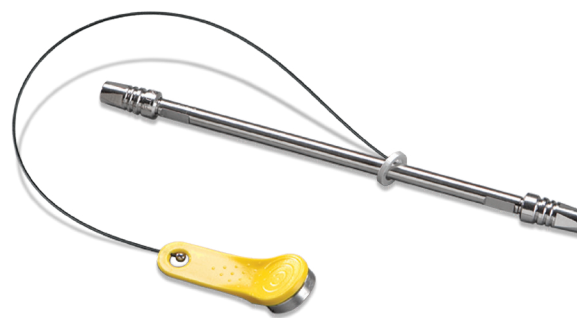


Figure 3: eCord Intelligent Chip.

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.

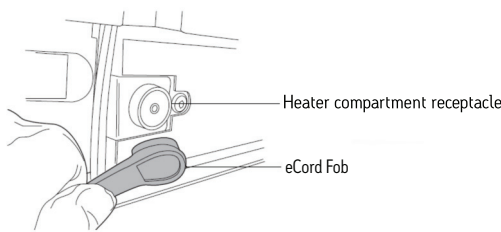
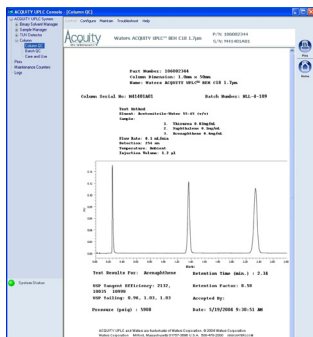
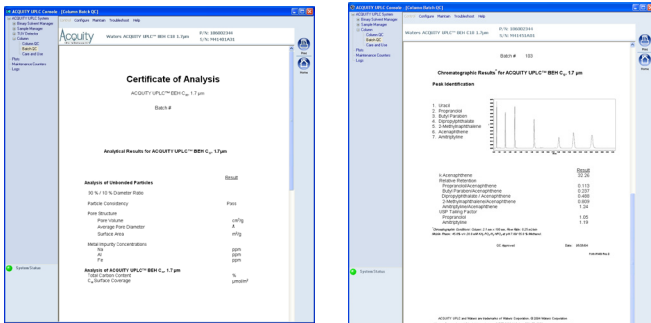


Figure 4: Installing the eCord Intelligent Chip.

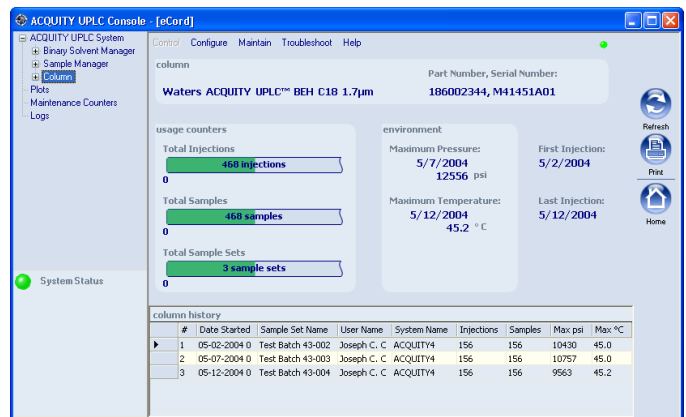
c. Manufacturing Information

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. ORDERING INFORMATION

Description	Part Number
ACQUITY UPLC BEH Glycan Column, 2.1 x 50 mm, 1.7 μm	186004740
ACQUITY UPLC BEH GlycanColumn, 2.1 x 100 mm, 1.7 μm	186004741
ACQUITY UPLC BEH GlycanColumn, 2.1 x 150 mm, 1.7 μm	186004742
ACQUITY UPLC BEH Glycan Vanguard Pre-Column, 2.1 x 5 mm, 1.7 μm, 3-pack	186004739
Glycan Performance Test Standard Mix	186006349

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The Netherlands 31 76 508 7200

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