XBRIDGE *XP* 2.5 µm COLUMNS

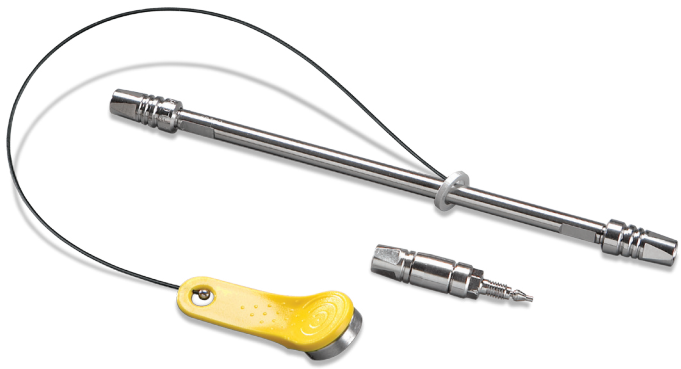
CONTENTS

I. INTRODUCTION

Thank you for choosing a Waters XBridge® Ethylene Bridged Hybrid [BEH] e***X***tended ***P***erformance [***XP***] 2.5 µm Column. The manufacture of XBridge ***XP*** 2.5 µm Columns begins with ultrapure reagents and are manufactured in a cGMP, ISO 9001 certified facility to control the chemical composition and purity of the final product. Well-controlled manufacturing processes result in industry-leading batch-to-batch reproducibility. Every column is individually tested. A Performance Chromatogram and Certificate of Batch Analysis are provided on the eCord™ Intelligent Chip.

XP 2.5 µm Columns are based on the same base particle technology and bonded-phase chemistry as 1.7 µm ACQUITY UPLC® Columns as well as XBridge 3.5, 5 and 10 µm HPLC Columns, thus enabling seamless transferability between HPLC, UHPLC and UPLC® platforms.

XBridge ***XP*** 2.5 µmColumns will exhibit maximum chromatographic performance when used on a member of the ACQUITY UPLC System family.



XBridge™ COLUMNS

II. GETTING STARTED

Each XBridge ***XP*** 2.5 µm Column comes with a Certificate of Analysis and a Performance Test Chromatogram embedded within the eCord intelligent chip. The Certificate of Analysis is specific to each batch of packing material contained in the XBridge ***XP*** 2.5 µm Column and includes the gel batch number, analysis of unbonded particles, analysis of bonded particles and chromatographic results and conditions. The Performance Test Chromatogram is specific to each individual column and contains such information as: gel batch number, column serial number, USP plate count, USP tailing factor, retention factor and chromatographic test conditions. These data should be recorded and stored for future reference or can be accessed via the ACQUITY UPLC console.

a. Column Connection

XP 2.5 µm Columns are designed to operate on any HPLC, UHPLC or UPLC System. Due to the absence of an industry standard, please be aware that the type of fittings and connections on each system will vary by manufacturer and should be mated specifically to a column when it is installed.

The chromatographic performance can be negatively impacted, or leaking can occur, if the style of the column endfitting does not properly match that of the compression screw/ferrule tubing depth setting.

b. Column Installation

Note: The flow rates given in the procedure below are described for a 2.1 mm ID column. Scale the flow rate according to the flow rate and pressure guidelines described in Section VI (Additional Information).

1. Purge the pumping system of any buffer-containing mobile-phases and connect the inlet of the column.
2. Flush the column with 100% organic mobile-phase (methanol or acetonitrile) by setting the pump flow rate to 0.1 mL/min and increase the flow rate to 0.5 mL/min over 5 minutes.
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 air entering the detection system and provides a more rapid baseline equilibration.
4. Gradually increase the flow rate as described in step 2.
5. Monitor until a steady backpressure and baseline have been achieved.

c. Minimizing Band Spread Volume

Band spreading is a measurement of the system dispersion that impacts the chromatographic performance. Internal tubing diameter and fluidic connections can significantly impact system band spreading and chromatographic performance. Larger tubing diameters cause excessive peak broadening and reduced sensitivity (Figure 1).

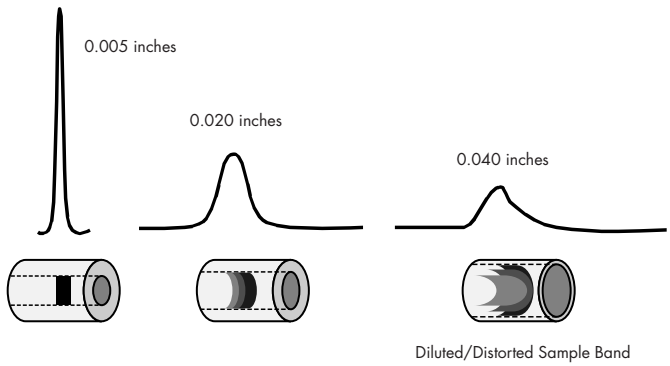


Figure 1: Impact of tubing diameter on band spread.

d. Measuring Band Spread Volume

Note: This test should be performed on an LC system equipped with a UV detector.

1. Disconnect the column from the system and replace with a zero dead volume union.
2. Set the flow rate to 1 mL/min.
3. Use a test mixture (dissolved in the mobile-phase conditions) that delivers a maximum peak height of 0.5 – 1.0 AU (System Start Up Test Mixture can be used, Part No. WAT034544).
4. Inject 2 – 5 µL of this solution.
5. Using the 5-Sigma method, measure the peak width at 4.4% of peak height:

   Band Spreading (µL) = Peak Width (min) x Flow Rate (µL/min)

   (For example, if peak width = 0.1 min and flow rate = 1000 µL/min, band spread = 100 µL)

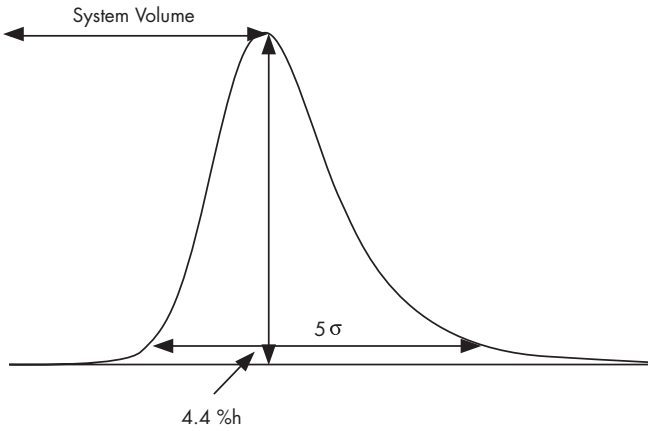


Figure 2: Determination of system band spread volume using 5-Sigma Method.

Table 1: Expected System Band Spread Volumes

System	Band Spread
Alliance 2695 HPLC	29 µL
Vendor A HPLC	41 µL
Vendor B UHPLC (600 bar)	28 µL
Vendor C UHPLC	21 µL
Vendor D UHPLC	17 µL
ACQUITY UPLC	12 µL
ACQUITY UPLC H-Class	9 µL
ACQUITY UPLC I-Class (FTN)	7.5 µL
ACQUITY UPLC I-Class (FL)	5.5 µL

e. Measuring System Dwell Volume

Dwell volume is different than system band spreading. System dwell volume is a measurement of the volume it takes for the initial gradient conditions to reach the head of the column. This calculation is particularly useful when it is necessary to transfer a method between different LC systems.

1. Disconnect the column from the system and replace with a zero dead volume union.
2. Use acetonitrile as mobile-phase A, and acetonitrile with 0.05 mg/mL uracil as mobile-phase B.
3. Monitor UV at 254 nm.
4. Use the flow rate in the original method and the intended flow rate on the target instrument.
5. Collect 100% A baseline for 5 minutes.
6. At 5 minutes, program a step to 100% B, and collect data for an additional 5 minutes.
7. Measure absorbance difference between 100% A and 100% B.
8. Measure time at 5% of that absorbance difference.
9. Calculate time difference between start of step and 50% point.
10. Multiply time difference by flow rate to calculate system volume.

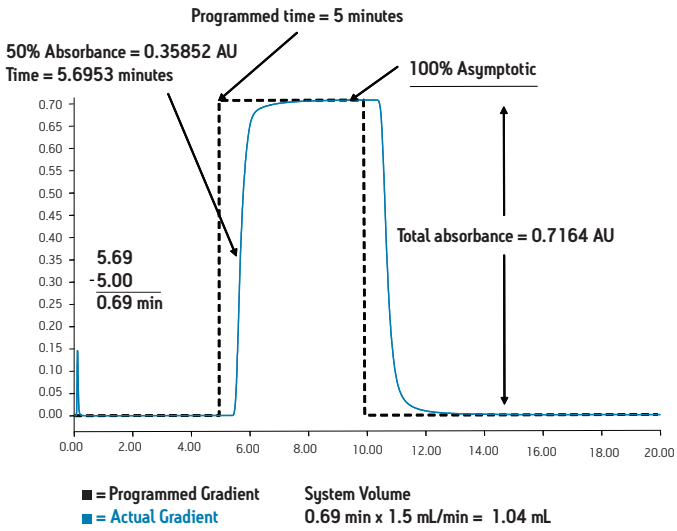


Figure 3: Measuring system band spread volume.

Table 2: Expected System Dwell Volumes

System	Dwell Volume
Alliance 2695 HPLC	900 µL
ACQUITY UPLC	120 µL
ACQUITY UPLC H-Class	350 µL
ACQUITY UPLC I-Class (FTN)	100 µL
ACQUITY UPLC I-Class (FL)	95 µL

f. Column Equilibration

XBridge ***XP*** 2.5 µm 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 3 for a list of column volumes). The column may be considered fully equilibrated once a constant backpressure is achieved.

Table 3: Column Volumes (mL)

Column Length (mm)	Internal Diameter		
	2.1 mm	3.0 mm	4.6 mm
30	0.10	0.21	0.50
50	0.17	0.35	0.83
75	0.26	0.53	1.25
100	0.35	0.71	1.66

To avoid precipitating mobile-phase buffers within the column or system, flush the column with five column volumes of a water/organic solvent mixture using the same, or lower, solvent content as in the desired buffered mobile-phase (i.e., flush the column and system with 60% methanol in water prior to introducing 60% methanol/ 40% buffer mobile-phase).

Note: If mobile-phase additives (i.e., ion-pairing reagents) are present in low concentrations (<0.2% v/v), 100 to -200 column volumes may be required for complete equilibration. In addition, mobile-phases that contain formate (i.e., ammonium formate, formic acid) may require extended equilibration times.

For XBridge HILIC ***XP*** 2.5 µm Columns, flush with 50 column volumes of 50:50 acetonitrile:water with 10 mM final buffer concentration. For XBridge Amide ***XP*** 2.5 µm Columns, flush with 50 column volumes of 60:40 acetonitrile:water with 10 mM final buffer concentration. Prior to the first injection, equilibrate with 20 column volumes of initial mobile-phase conditions (refer to Table 3 for a list of column volumes). See "Getting Started with XBridge HILIC Columns" or "Getting Started with XBridge Amide Columns" sections for additional information.

g. eCord Installation

eCord Technology represents a significant advancement in column usage tracking management which can be realized if the column is installed on an ACQUITY UPLC System. The eCord device can be read by connecting the yellow fob to the reader/writer located on the right-hand side of the ACQUITY UPLC Column heater module. Embedded information such as the column manufacturing QC data and Certificates of Analysis may then be accessed via the ACQUITY UPLC console.

h. Initial Column Efficiency Determination

1. Perform an efficiency test on the column before using it to track column performance over time. This test may consist of:
   a. An analyte test mixture that is commonly used in your laboratory.
   b. An analyte mixture as found on the "Performance Test Chromatogram" which can be accessed via the eCord.

Note: If [b] is performed, the isocratic efficiencies measured in your laboratory may be less than those given on the Waters Performance Test Chromatogram. This is normal and expected. The Waters isocratic column testing systems have been modified in order to achieve extremely low system dispersion. This presents a more challenging test of how well the column was packed. This also guarantees the highest quality packed column. These special testing systems have been modified to such an extent that they are not commercially viable and have limited method flexibility other than isocratic column testing.

2. Determine the number of theoretical plates (N) and use this value for periodic comparisons.
3. Repeat the test periodically to track column performance over time. Slight variations may be obtained on different LC systems due to the quality of the connections, operating environment, system electronics, reagent quality, column condition and operator technique.

i. VanGuard Pre-Columns

VanGuard™ Pre-Columns are 2.1 mm ID x 5 mm length guard column devices designed specifically to protect an analytical column while minimizing the negative dispersion impact of utilizing such a device. VanGuard Pre-Columns are packed with the same stationary phases as the ***XP*** 2.5 µm column offering. VanGuard Pre-Columns are designed to be directly attached to the inlet of a e***X***tended ***P***erformance 2.5 µm Column.

*Note: VanGuard Pre-Columns are shipped with a collet and ferrule that are NOT pre-swaged. This enables the end user to mate the VanGuard Pre-Column to a specific **XP** 2.5 µm Column and ensures void-free and leak-free connections. Care must be taken when removing the O-ring that holds these two pieces on the pre-column tubing.*

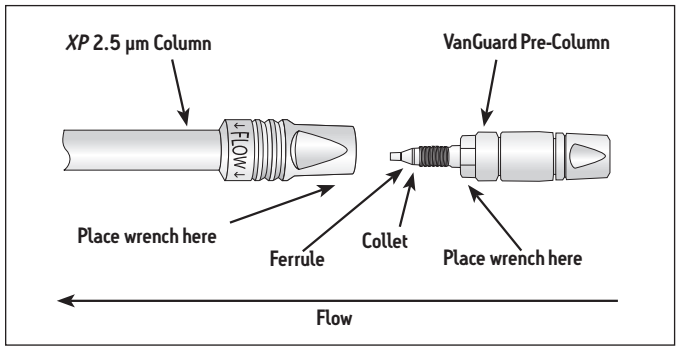


Figure 4: Installing a VanGuard Pre-Column.

VanGuard Pre-Column Installation Instructions

1. Remove the VanGuard Pre-Column from its box and shipping tube and remove the plastic plug.
2. Orient the pre-column so that the male end is facing up and carefully remove the black O-ring that holds the collet and ferrule in place during shipment (collet and ferrule are not permanently attached).
3. Orient the ***XP*** 2.5 µm Column perpendicular to the work surface so that the column inlet is on the bottom.
4. From below, insert the VanGuard Pre-Column into the column inlet; turn the assembled column and pre-column 180° so that the pre-column is now on top.
5. Tighten with two 5/16" wrenches placed onto the ***XP*** 2.5 µm Column flats and VanGuard Pre-Column hex nut (male end) as shown in Figure 4.
6. While keeping pressure on the VanGuard Pre-Column against the ***XP*** 2.5 µm Column, tighten turn to set the collet and ferrule.
7. Check that the ferrule is set by loosening the connection and inspecting the ferrule depth.
8. Reattach the pre-column to the ***XP*** 2.5 µm Column, apply flow and inspect for leaks.

III. COLUMN USE

To ensure the continued high performance of XBridge ***XP*** 2.5 µm Columns, follow these guidelines:

a. Sample Preparation

1. Sample impurities and/or particulates often contribute to column contamination. One option to avoid column contamination is to use Waters Oasis® or Sep-Pak Solid-Phase Extraction (SPE) devices. To select the appropriate sorbent for a specific sample type, visit www.waters.com/sampleprep
2. It is preferable to prepare the sample in the initial mobile-phase conditions or a weaker solvent for the best peak shape and sensitivity.
3. If the sample is not prepared in the mobile-phase, ensure that the sample, solvent and mobile-phases are miscible in order to avoid sample and/or buffer precipitation.
4. Filter sample with a 0.2 µm membrane to remove particulates. If the sample is dissolved in a solvent that contains an organic modifier (e.g., acetonitrile, methanol, etc.) ensure that the membrane/filter material is compatible with the solvents in use. Alternatively, centrifuge the sample for 20 minutes at 8000 rpm, followed by the transfer of the supernatant to an appropriate vial could be considered.

b. pH Range

Table 4: Recommended pH Range

Chemistry	pH Range
XBridge BEH C_{18}	1 - 12
XBridge BEH C_8	1 - 12
XBridge BEH Phenyl	1 - 12
XBridge BEH Shield RP18	2 - 11
XBridge BEH HILIC	1 - 9
XBridge BEH Amide	2 - 11

Column lifetime will vary depending on the combination of temperature, mobile-phase pH and type of buffer/additive used. Table 5 lists the recommended buffers and additives for XBridge ***XP*** 2.5 µm Columns.

Note: Working in combinations of extreme pH, temperature and pressure may result in reduced column lifetime.

*Table 5. Buffer Recommendations for XBridge **XP** 2.5 µm Columns.*

Additive/Buffer	pKa	Buffer Range	Volatility (±1 pH unit)	Used for Mass Spec	Comments
TFA	0.3	-	Volatile	Yes	Ion pair additive, can suppress MS signal, used in the 0.02-0.1% range.
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.
Acetate (CH_3COO-)	4.76	3.76 – 5.76	Volatile	Yes	Used in the 1-10 mM range. Note that sodium or potassium salts are not volatile.
Formate (HCOO-)	3.75	2.75 – 4.75	Volatile	Yes	Used in the 1-10 mM range. Note that sodium or potassium salts are not volatile.
Phosphate 1	2.15	1.15 – 3.15	Non-volatile	No	Traditional low-pH buffer, good UV transparency.
Phosphate 2	7.2	6.20 – 8.20	Non-volatile	No	Above pH 7, reduce temperature/concentration and use a guard column to maximize lifetime.
Phosphate 3	12.3	11.3 - 13.3	Non-volatile	No	Above pH 7, reduce temperature/concentration and use a guard column to maximize lifetime.
4-Methylmorpholine	~8.4	7.4 – 9.4	Volatile	Yes	Generally used at 10 mM or less.
Ammonia (NH_4^+)	9.2	8.2 – 10.2	Volatile	Yes	Keep concentration below 10 mM and temperatures below 30 °C.
Ammonium Bicarbonate	10.3 (HCO_3^-) 9.2 (NH_4^+)	8.2 – 11.3	Volatile	Yes	Used in the 5-10 mM range (for MS work keep source >150 °C). Adjust pH with ammonium hydroxide or acetic acid. Good buffering capacity at pH 10. *Note: use ammonium bicarbonate (NH_4HCO_3), not ammonium carbonate ($(NH_4)_2CO_3$).*
Ammonium (Acetate)	9.2	8.2 – 10.2	Volatile	Yes	Used in the 1-10 mM range.
Ammonium (Formate)	9.2	8.2 – 10.2	Volatile	Yes	Used in the 1-10 mM range.
Borate	9.2	8.2 – 10.2	Non-volatile	No	Reduce temperature/concentration and use a guard column to maximize lifetime.
CAPSO	9.7	8.7 – 10.7	Non-volatile	No	Zwitterionic buffer, compatible with acetonitrile, used in the 1-10 mM range. Low odor.
Glycine	2.4, 9.8	8.8 – 10.8	Nton-volatile	No	Zwitterionic buffer, can give longer lifetimes than borate buffer.
1-Methylpiperidine	10.2	9.3 – 11.3	Volatile	Yes	Used in the 1-10 mM range.
CAPS	10.4	9.5 – 11.5	Non-volatile	No	Zwitterionic buffer, compatible with acetonitrile, used in the 1-10 mM range. Low odor.
Triethylamine (as acetate salt)	10.7	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.
Pyrrolidine	11.3	10.3 – 12.3	Volatile	Yes	Mild buffer, gives long lifetime.

c. Solvents

To maintain maximum column performance, use high quality HPLC or MS grade solvents. Filter all aqueous buffers prior to use through a 0.2 µm filter. Solvents containing suspended particulate materials will generally clog the outside surface of the inlet of the column. This may result in higher backpressure or distorted peak shape.

d. Pressure

XBridge ***XP*** 2.5 µm Columns are compatible with HPLC, UHPLC and UPLC pressures. Table 6 depicts the maximum operation pressure.

Table 6: Maximum Operation Pressure

Column ID	Pressure Range
2.1 mm	18,000 psi [1034 bar]
3.0 mm	18,000 psi [1034 bar]
4.6 mm	9000 psi [620 bar]

e. Temperature

XBridge ***XP*** 2.5 µm Columns can be used up at intermediate temperatures to enhance selectivity, reduce solvent viscosity and increase mass transfer rates.

Chemistry	Temperature Limit Low pH	Temperature Limit High pH
XBridge BEH C_{18}	80 °C	60 °C
XBridge BEH C_8	60 °C	60 °C
XBridge BEH Phenyl	80 °C	60 °C
XBridge BEH Shield RP18	50 °C	45 °C
XBridge BEH HILIC	45 °C	45 °C
XBridge BEH Amide	90 °C	90 °C

Note: Working in combinations of extreme pH, temperature and pressure may result in reduced column lifetime.

IV. COLUMN CLEANING, REGENERATION AND STORAGE

a. Cleaning and Regeneration

Changes in peak shape, peak splitting, shouldering peaks, shifts in retention, change in resolution or increasing backpressure may indicate contamination of the column. Flush with a neat organic solvent to remove the non-polar contaminant(s), taking care not to precipitate any buffered mobile-phase components. If this flushing procedure does not solve the problem, purge the column with the following cleaning and regeneration procedures.

Use a cleaning routine that matches the properties of the samples, stationary-phase type (reversed-phase, normal-phase or HILIC) and will solubilize the suspected contaminate. Flush with 20 column volumes of solvent at an intermediate temperature of 45°C. Return to the initial mobile-phase conditions by reversing the sequence.

If using a reversed-phase column, purge the column with a sequence of progressively more non-polar solvents (i.e., water–tomethanol–to–tetrahydrofuran–to–methylene chloride).

If using a HILIC column, purge the column with a sequence of progressively more polar-organic solvents (i.e., acetonitrile-to-acetonitrile/methanol-to-acetonitrile/water-to-water).

If column performance has not improved after regeneration/cleaning procedures, contact your local Waters representative for additional support.

b. Storage after Reversed-Phase Use

For periods longer than four days, store the ***XP*** 2.5 µm Column in 100% acetonitrile. For separations utilizing elevated temperature, store immediately after use in 100% acetonitrile. Do not store columns in buffered eluents. If the mobile-phase contained a buffer salt, flush the column with 10 column volumes of HPLC grade water (see Table 3 for column volume information) followed by 10 column volumes of acetonitrile. Failure to perform this intermediate step could result in precipitation of the buffer salt in the column when 100% acetonitrile is introduced. Completely seal the column to avoid solvent evaporation and drying out of the chromatographic bed.

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

c. Storage after HILIC Use

For periods longer than four days, store the ***XP*** 2.5 µm Column in 95/5 acetonitrile/water. Do not store columns in buffered eluents. If the mobile-phase contained a buffer salt, flush the column with 10 column volumes of HPLC grade water (see Table 3 for column volume information) followed by 10 column volumes of 95/5

acetonitrile/water. Failure to perform this intermediate step could result in precipitation of the buffer salt in the column when 95% acetonitrile is introduced. Completely seal the column to avoid solvent evaporation and drying out of the chromatographic bed.

V. eCORD INTELLIGENT CHIP TECHNOLOGY

a. Introduction

The eCord Intelligent Chip Technology represents a significant advancement in column usage tracking management which can be realized if the column is installed on an ACQUITY UPLC System.

The eCord Intelligent Chip provides a paperless tracking history of the column's performance and usage throughout its lifetime. The eCord is permanently attached to the column body via a tether that cannot be removed. This ensures that the history of the column is always accessible to the user of that column.

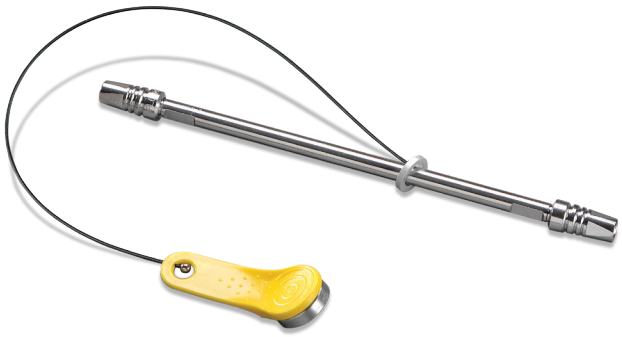


Figure 5: eCord Intelligent Chip.

At the time of manufacture, information such as the performance test chromatogram, column manufacturing QC data and Certificates of Analysis is downloaded onto the eCord. This information may then be accessed via the ACQUITY UPLC console once the column is installed.

b. Installation

The eCord device can be read by connecting the yellow fob to the reader/writer located on the right-hand side of the ACQUITY UPLC Column heater module. Once the eCord is connected to the magnetic catch on the column heater, column identification and overall column usage information can be accessed.

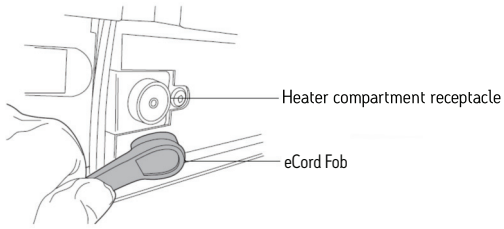
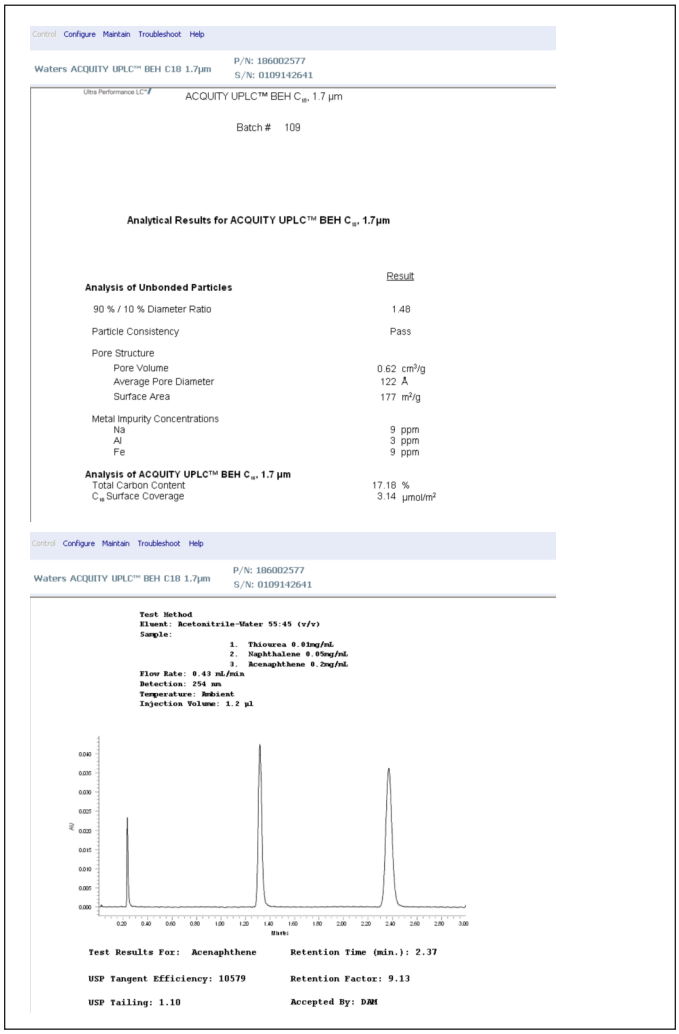


Figure 6: Installing the eCord Intelligent Chip.

c. Manufacturing

The eCord Chip provides the user with the Batch Certificate of Analysis and Performance Test Chromatogram.



Control Configure Maintain Troubleshoot Help

Waters ACQUITY UPLC™ BEH C18 1.7µm
P/N: 186002577
S/N: 0109142641

ACQUITY UPLC™ BEH C_{18}, 1.7 µm

Batch # 109

Analytical Results for ACQUITY UPLC™ BEH C_{18}, 1.7µm

	Result
Analysis of Unbonded Particles	
90 % / 10 % Diameter Ratio	1.48
Particle Consistency	Pass
Pore Structure	
Pore Volume	0.62 cm^3/g
Average Pore Diameter	122 Å
Surface Area	177 m^2/g
Metal Impurity Concentrations	
Na	9 ppm
Al	3 ppm
Fe	9 ppm
Analysis of ACQUITY UPLC™ BEH C_{18}, 1.7 µm	
Total Carbon Content	17.18 %
C_{18} Surface Coverage	3.14 µmol/m^2

Control Configure Maintain Troubleshoot Help

Waters ACQUITY UPLC™ BEH C18 1.7µm
P/N: 186002577
S/N: 0109142641

Test Method
Eluent: Acetonitrile-Water 55:45 (v/v)
Sample:
1. Thiourea 0.01mg/mL
2. Naphthalene 0.05mg/mL
3. Acenaphthene 0.2mg/mL
Flow Rate: 0.43 mL/min
Detection: 254 nm
Temperature: Ambient
Injection Volume: 1.2 µl

Test Results For: Acenaphthene Retention Time (min.): 2.37

USP Tangent Efficiency: 10579 Retention Factor: 9.13

USP Tailing: 1.10 Accepted By: DAM

Figure 7: Manufacturing results stored on an eCord.

d. Column Use

The eCord Intelligent Chip provides the user with specific column information as well as column use data including: chemistry type, column dimension, serial number and part number. The overall column use information includes: total number of samples injected, total number of injections as well as the maximum pressure and temperature that the column has been exposed to. Additionally, detailed column history includes the sample set start date, user name and system name.

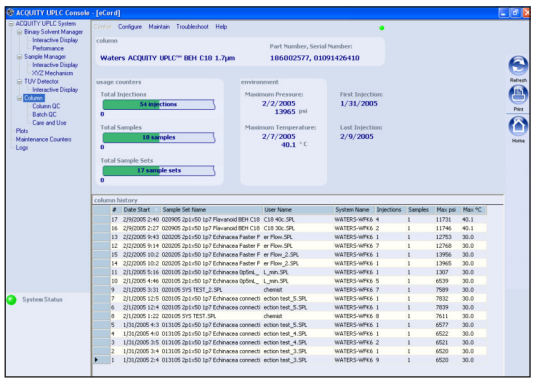


Figure 8: Column use information.

VI. ADDITIONAL INFORMATION

a. Tips for Maximizing XBridge *XP* 2.5 µm Column Lifetime

1. To maximize ***XP*** 2.5 µm Column lifetime, pay close attention to:
   - Water quality (including water purification systems)
   - Solvent quality
   - Mobile-phase preparation, storage and age
   - Sample, buffer and mobile-phase solubilities
   - Sample quality and preparation.
2. When problems arise, systematically troubleshoot potential causes one variable at a time in a systematic fashion.
3. Always remember to:
   - Use an in-line filter unit (Part No. 205000343) or a VanGuard Pre-Column.
   - Discourage bacterial growth by minimizing the use of 100% aqueous mobile-phases where possible.
   - Discard and re-prepare aqueous mobile-phase every 24-48 hours (if 100% aqueous mobile-phase is required).
   - Add 5 – 10% organic modifier to aqueous buffer to minimize bacterial growth (adjust gradient profile as necessary).
   - Filter aqueous portions of mobile-phase through a 0.2 µm filter.
   - Routinely maintain your water purification system to ensure it is functioning properly.
   - Only use ultra-pure water (18 MegaOhm-cm) and highest quality solvent possible.
   - Consider sample preparation (e.g., solid-phase extraction, filtration, centrifugation, etc.) when possible.
4. Avoid when possible:
   - 100% aqueous mobile-phases
   - HPLC-grade bottled water
   - 'Topping off' your mobile-phases
   - Using phosphate salt buffer in combination with high acetonitrile concentrations (e.g., >70%) due to precipitation.
5. Don't assume the column is to blame:
   - Investigate cause of column failure
   - Monitor backpressure
   - Mobile-phase age, bacterial contamination, mobile-phase precipitation...etc.
   - Peak splitting
   - Sample quality
   - Injection solvent strength.
6. Do not prepare excessive amounts of mobile-phase:
   - To reduce the chances of mobile-phase contamination or degradation, prepare enough mobile-phase to last 3 – 4 days. Alternatively, store excess bulk quantities in a refrigerated environment.

b. Troubleshooting Questions

1. Are you using 100% aqueous mobile-phases?
2. What is the age of the mobile-phase?
3. Is the mobile-phase filtered through a 0.2 µm membrane?
4. Was the mobile-phase prepared fresh or topped off?
5. Is the water source of adequate quality?
6. When was the last time the water system was serviced or was the bottle of water unopened?
7. Is bacterial growth a possibility (pH 7 phosphate buffer is susceptible to bacterial growth within 24 hours)?
8. If a neat standard is prepared in the initial mobile-phase conditions and injected, are the problems still observed?
9. If the sample is filtered/purified (i.e., SPE, filtration...etc.) is the problem still observed?
10. Has the quality of the samples changed over time?

c. Recommended Flow Rates and Anticipated Backpressures for Reversed-Phase XBridge *XP* 2.5 µm Columns

XP 2.5 µm, 2.1 mm ID Columns (40 °C)								
Linear Velocity	3 mm/sec		4 mm/sec		5 mm/sec		6 mm/sec	
Column Dimension	Flow Rate [mL/min]	Backpressure [psi]	Flow Rate [mL/min]	Backpressure [psi]	Flow Rate [mL/min]	Backpressure [psi]	Flow Rate [mL/min]	Backpressure [psi]
2.1 x 30 mm	0.45	1760	0.6	2350	0.75	2940	0.9	3520
2.1 x 50 mm	0.45	2640	0.6	3520	0.75	4400	0.9	5280
2.1 x 75 mm	0.45	3740	0.6	4980	0.75	6230	0.9	7470
2.1 x 100 mm	0.45	4830	0.6	6440	0.75	8055	0.9	9670

XP 2.5 µm, 3.0 mm ID Columns (40 °C)								
Linear Velocity	3 mm/sec		4 mm/sec		5 mm/sec		6 mm/sec	
Column Dimension	Flow Rate [mL/min]	Backpressure [psi]	Flow Rate [mL/min]	Backpressure [psi]	Flow Rate [mL/min]	Backpressure [psi]	Flow Rate [mL/min]	Backpressure [psi]
3.0 x 30 mm	0.9	2180	1.17	2840	1.53	3710	1.8	4360
3.0 x 50 mm	0.9	3040	1.17	3950	1.53	5170	1.8	6080
3.0 x 75 mm	0.9	4120	1.17	5350	1.53	7000	1.8	8230
3.0 x 100 mm	0.9	5190	1.17	6750	1.53	8825	1.8	10380

XP 2.5 µm, 4.6 mm ID Columns (40 °C)								
Linear Velocity	3 mm/sec		4 mm/sec		5 mm/sec		6 mm/sec	
Column Dimension	Flow Rate [mL/min]	Backpressure [psi]	Flow Rate [mL/min]	Backpressure [psi]	Flow Rate [mL/min]	Backpressure [psi]	Flow Rate [mL/min]	Backpressure [psi]
4.6 x 30 mm	2.1	3360	2.8	4480	3.5	5600	4.2	6720
4.6 x 50 mm	2.1	4210	2.8	5620	3.5	7020	4.2	8430
4.6 x 75 mm	2.1	5280	2.8	7040	3.5	8800	4.2	10560
4.6 x 100 mm	2.1	6350	2.8	8460	3.5	10580	4.2	12700

d. Getting Started with XBridge HILIC *XP* 2.5 µm Columns

Operating Range

1. Because XBridge HILIC Columns do not posses a bonded phase, the pH operating range is 1 to 9, and they can be operated at temperatures up to 45 °C.
2. As with any LC column, operating at the extremes of pH, pressures and temperatures will result in decreased column lifetime.

Column Equilibration

1. When column is first received, flush in 50% acetonitrile/50% water with 10 mM final buffer concentration for 50 column volumes.
2. Equilibrate with 20 column volumes of initial mobile-phase conditions before making first injection.
3. If gradient conditions are used, equilibrate with 8-10 column volumes between injections.
4. Failure to appropriately equilibrate the column could result in drifting retention times.

Mobile-Phase Considerations

1. Always maintain at least 3% polar solvent in the mobile-phase or gradient (e.g., 3% aqueous/3% methanol or 2% aqueous/1% methanol , etc.). This ensures that the XBridge HILIC particle is always hydrated.
2. Maintain at least 40% organic solvent (e.g., acetonitrile) in your mobile-phase or gradient.
3. Avoid phosphate salt buffers to avoid precipitation in HILIC mobile-phases. Phosphoric acid is okay.
4. Buffers such as ammonium formate or ammonium acetate will produce more reproducible results than additives such as formic acid or acetic acid. If an additive (e.g., formic acid) must be used instead of a buffer, use 0.2% (v:v) instead of 0.1%.
5. For best peak shape, maintain a buffer concentration of 10 mM in your mobile-phase/gradient at all times.

Injection Solvents

1. If possible, injection solvents should match the initial mobile-phase conditions.. The polar solvent (i.e., water, methanol, isopropanol) should be minimized to 25% of the total volume.
2. A generic injection solvent is 75/25 acetonitrile/methanol. This is a good compromise between analyte solubility and peak shape.
3. Avoid water and dimethylsulfoxide (DMSO) as injection solvents. These solvents will produce very poor peak shapes.
4. Exchange water or DMSO with acetonitrile by using reversed-phase solid-phase extraction (SPE). If this is not possible, dilute the water or DMSO with organic solvent.

Miscellaneous Tips

1. XBridge HILIC columns are designed to retain very polar bases. Acidic, neutral and/or non-polar compounds will have limited retention.
2. Optimal flow rates for small (<200 daltons) very polar bases are in the 0.4 to 0.8 mL/min range with the XBridge HILIC Columns (2.1 mm ID).
3. As compared to Atlantis® HILIC Silica HPLC Columns, the XBridge HILIC Columns are approximately 20% less retentive for gradient analysis and 35 to 65% less retentive for isocratic analysis. This is due to the lower residual surface silanol concentration of the BEH particle.
4. In HILIC, it is important to remember that water is the strongest solvent. Therefore, it must be eliminated or minimized in the injection solvent.
5. For initial scouting conditions, run a gradient from 95% acetonitrile to 50% acetonitrile. If no retention occurs, run isocratically with 95/3/2 acetonitrile/methanol/aqueous buffer.
6. Alternate polar solvents such as methanol, ethanol or isopropanol can also be used in place of water to increase retention.
7. If using an ACQUITY UPLC System, the weak needle wash should closely match the % organic present in the initial mobile-phase conditions, otherwise, analyte peak shape or retention may suffer.

e. Getting Started with XBridge Amide *XP* 2.5 µm Columns

Operating Ranges

1. XBridge Amide Columns can be used routinely under HILIC conditions between pH 2 to 11, and they can be operated at temperatures up to 90 °C.
2. As with any LC column, operating at the extremes of pH, pressures and temperatures will result in decreased column lifetime.

Column Equilibration

1. When column is first received, flush in 60% acetonitrile/40% aqueous (or initial starting conditons) for 50 column volumes.
2. Equilibrate with 20 column volumes of initial mobile-phase conditions before making first injection.
3. If gradient conditions are used, equilibrate with 8-10 column volumes between injections.
4. Failure to appropriately equilibrate the column could result in drifting retention times.

Mobile-Phase Considerations

1. Always maintain at least 3% polar solvent in the mobile-phase or gradient (e.g., 3% aqueous, 3% methanol or 2% aqueous/1% methanol, etc.).
2. Maintain at least 40% organic solvent (e.g., acetonitrile) in your mobile-phase or gradient.
3. At aqueous concentrations greater than 60%, lower flow rates should be used due to high backpressure. This includes all aqueous wash procedures.
4. Avoid phosphate salt buffers to avoid precipitation in HILIC mobile-phases. Phosphoric acid is suitable.

Injection Solvents

1. If possible, injection solvents should be as close to the mobile-phase composition as possible (if isocratic) or the starting gradient conditions. Acetone should not be used as a sample solvent/diluent unless a Hexane Tetrahydrofuran Compatibility Kit (Part No. 205000464) has been installed.
2. A generic injection solvent is 75/25 acetonitrile/methanol. This is a good compromise between analyte solubility and peak shape. When separating saccharides with limited solubility in in organic solvents, higher concentrations of aqueous solvent in the sample are acceptable. 50/50 acetonitrile/water can provide satisfactory results.
3. The injection solvent's influence on peak shape should be determined experimentally. In some cases, injections of water (or highly aqueous solutions) may not adversely affect peak shape.

Miscellaneous Tips

1. For initial scouting conditions, run a gradient from 95% acetonitrile to 50% acetonitrile. If no retention occurs, run isocratically with 95/3/2 acetonitrile/methanol/aqueous buffer.
2. Alternate polar solvents such as methanol, acetone or isopropanol can also be used in place of water to increase retention.
3. If using an ACQUITY UPLC System with a fixed loop injector, ensure that the weak needle wash solvent/purge solvent is your starting mobile-phase (i.e., high organic), or your peak shapes will suffer. Typical needle wash conditions: 800 µL strong wash in 20/80 ***acetonitrile/water***, 500 µL weak wash in 75/25 ***acetonitrile/water***.
4. Acetone should not be used as a sample solvent/diluent unless a Hexane Tetrahydrofuran Compatibility Kit (Part No. 205000464) has been installed.

Tips for Separating Sugars/Saccharides/Carbohydrates

If separating sugars or sugar-containing compounds that do not include reducing sugars (see below) follow generic 'Getting Started with XBridge Amide Columns' recommendations described above.

If separating reducing sugars, please review the following information.

1. Reducing sugars can undergo mutarotation which produces the undesired separation of the α and β ring forms (anomers).
2. Collapsing anomers into one peak is accomplished through the use of a combination of elevated temperature and high pH:
   a. Use of 35 °C with high pH (0.2% triethylamine (TEA) or 0.1% ammonium hydroxide (NH_4OH)) and/or
   b. Use of >80 °C with 0.05% TEA high temperature (>80 °C)
3. When separating reducing sugars (e.g., fructose, glucose, maltose, lactose, arabinose, glyceraldehyde) please pay attention to the following suggestions. Failure to do so will result in the appearance of split peaks (anomer separation) for these analytes:
   a. Operate at a slow flow rate (e.g., 0.10 - 0.13 mL/min on 2.1 x 50 mm column) to facilitate anomer collapse.
   b. With longer columns, increased flow rates (e.g., up to 0.3 mL/min) can be used. As with all LC separations, optimal flow rates should be determined experimentally.

c. Add triethylamine (TEA) or ammonium hydroxide (NH_4OH) modifiers to both mobile-phase (e.g., A2, B2, etc.) reservoirs.

d. For LC/ELSD separations of mono- and/or disaccharides, typical isocratic conditions include:

i. 75% (acetonitrile) with 0.2% TEA, 35 °C, 0.13 mL/min, 2.1 x 50 mm BEH Amide column;

ii. 77% acetone with 0.05% TEA, 85 °C, 0.15 mL/min, 2.1 x 50 mm BEH Amide column;

iii. 75% acetonitrile with 0.2% TEA, 35 °C, 0.2mL/min, 2.1 x 100 mm BEH Amide column.

e. For LC/ELSD separations of more complex sugar mixtures (e.g., polysaccharides), typical gradient conditions include (add TEA modifier to both mobile-phases A and B):

i. Gradient going from 80% - 50% acetonitrile with 0.2% TEA in 10 minutes, 35 °C, 0.13 mL/min, 2.1 x 100 mm BEH Amide column;

ii. 80% - 55% acetone with 0.05% TEA in 10 minutes, 85 °C, 0.15 mL/min, 2.1 x 100 mm BEH Amide column.

f. For LC/MS separations of mono- and disaccharides, typical isocratic conditions include:

i. 75% acetonitrile with 0.1% ammonium hydroxide, 35 °C, 0.13 mL/min, 2.1 x 50 mm BEH Amide column.

g. For LC/MS separations of more complex sugar mixtures (e.g., polysaccharides), typical gradient conditions include (add NH_4OH modifier to both mobile-phases A and B):

i. Gradient going from 75% - 45% acetonitrile with 0.1% ammonium hydroxide in 10 minutes, 35 °C, 0.2 mL/min, 2.1 x 100 mm BEH Amide column.

5. More complex sample mixtures may require the use of gradient conditions and/or longer column lengths.

6. If acetone is used in one or more mobile-phases, do not use acetone as a sample diluent or needle wash solvent. Refer to injection solvents section for sample diluent recommendations and miscellaneous tip (#3) for needle wash solvent/purge solvent recommendations.

7. Typical sample preparation suggestions for samples that contain sugars /saccharides /carbohydrates:

a. Liquid Samples

i. Dilute with 50/50 acetonitrile/water

ii. Filter using 0.45 µm or 0.22 µm syringe filter (if necessary).

b. Solid Samples

i. Weigh out sample (~3 g) into 50 mL centrifuge tube

ii. Add 25 mL of 50/50 acetonitrile/water and homogenize (mechanically)

iii. Centrifuge at 3200 rpm for 30 minutes

iv. Collect supernatant and filter using 0.45 µm or 0.22 µm syringe filter (if necessary).

c. Depending on sample and/or analyte concentrations, additional sample dilutions may be necessary.

d. More complex samples and/or lower analyte concentrations may require additional sample preparation steps and/or procedures such as solid-phase extraction (SPE).

e. Consider VanGuard BEH Amide Pre-Columns for UPLC Column protection.

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