XTerra® and XTerra® Prep Column Care and Use Instructions

READ CAREFULLY BEFORE USING THIS COLUMN

Thank you for ordering a Waters XTerra® column. XTerra® packing materials are synthesized using Waters Hybrid Particle Technology. XTerra® particles contain both inorganic (silica) and organic (organosiloxane) components sharing the advantages of both. Hybrid technology allows for the high efficiency of separation and improved pH stability when compared to Silica based reversed phase packing materials. The manufacture of XTerra® columns starts with ultrapure reagents to control the chemical composition and purity of the Hybrid particle. Every manufacturing step is conducted within narrow tolerances and each column is individually tested. Certificates of Batch Analysis and Column Efficiency are provided with each column.

We recommend the use of Waters Sentry™ guard columns to extend the life of your column and protect it from contaminants.

Connecting the column to the HPLC instrument

Handle the column with care. Do not drop or hit column on a hard surface as it may disturb the bed and affect its performance.

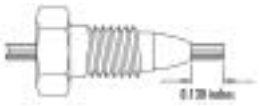
1. Correct connection of 1/16 inch outer diameter stainless steel tubing leading to and from the column is essential for high-quality chromatographic results.
2. When using standard stainless steel compression screw fittings, it is important to ensure proper fit of the 1/16 inch outer diameter stainless steel tubing. When tightening or loosening the compression screw, place the 5/16 inch wrench on the compression screw and the other 3/8 inch wrench on the hex head of the column endfitting.

   Note: If one of the wrenches is placed on the column flat during this process, the endfitting will be loosened and leak.
3. If a leak occurs between the stainless steel compression screw fitting and the column endfitting, a new compression screw fitting, tubing and ferrule must be assembled.
4. An arrow on the column identification label indicates correct direction of solvent flow.

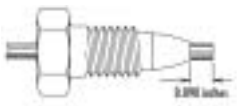
It is important to realize that extra column peak broadening can destroy successful separation. The choice of appropriate column connectors and system tubing is discussed in detail below.

Column connectors and system tubing considerations

Due to the absence of an industry standard, various column manufacturers have employed different styles of chromatographic column connectors. The chromatographic performance of your separation can be negatively affected if the style of your column endfittings do not match the existing instrumentation tubing ferrule setting. This page explains the difference between Waters style and Parker style endfittings, which vary in the required length of the tubing protruding from the ferrule. The XTerra® column is equipped with Waters style endfittings which require a 0.130 inch ferrule depth (see next section for setting ferrule depth). If you are presently using a non-Waters style column, it is critical that you reset the ferrule depth for optimal performance.



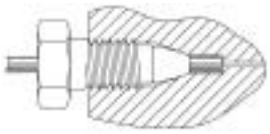
Waters Ferrule Setting



Parker Ferrule Setting

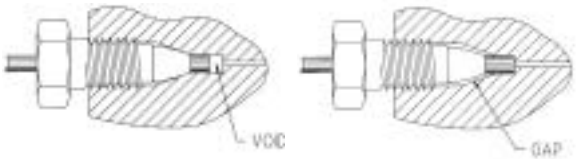
The proper tubing/column connection

Tubing touches the bottom of the column endfitting, with no void between them.



A void appears if a tube with Parker ferrule setting is connected to a Waters style column

The presence of a void in the flow stream downgrades the column performance. There is only one way to fix the problem: Cut the end of the tubing with the ferrule, put a new ferrule on the tubing and make the connection. Before tightening the screw, make sure that the tubing bottoms out in the endfitting of the column.



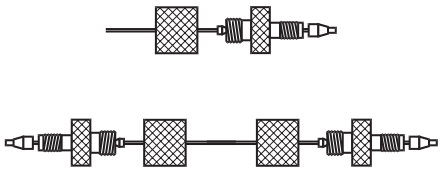
If tubing with a Waters style ferrule setting is connected to a column with Parker style endfitting, the end of the tubing will bottom out before the ferrule reaches its proper sealing position. This will leave a gap creating a leak. There are two ways to fix the problem:

1) Just tighten the screw a little bit more. The ferrule moves forward, and reaches the sealing surface. Do not overtighten because this may end in breaking the screw.
2) Cut the tubing, put a new ferrule on it and make the connection.

An alternative is to replace the conventional compression screw fitting with an all-in-one PEEK fitting (Waters part number PSL613315) that allows you to reset the ferrule depth. Another approach is to use a Keystone, Inc. Slipfree® fitting to always ensure the correct fit. The finger-tight Slipfree® connectors automatically adjust to fit all compression screw type fittings without the use of tools.

SLIPFREE® Connectors

Guarantees a void-free connection because it pushes the tubing into the endfitting; This design comes installed on the tubing. Fingertight to 10,000 psi – Never needs wrenches. Readjusts to all column endfittings. Compatible with all commercially available endfittings. Unique design separates tube-holding function from sealing function.



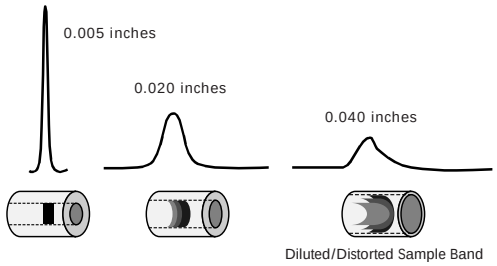
Waters Part Numbers for SLIPFREE® Pre-assembled Tubing

Tubing Length	0.005" i.d.	0.010" i.d.	0.020" i.d.
Single SLIPFREE® 6 cm	PSL 618000	PSL 618006	PSL 618012
Single SLIPFREE® 10 cm	PSL 618002	PSL 618008	PSL 618014
Single SLIPFREE® 20 cm	PSL 618004	PSL 618010	PSL 618016
Double SLIPFREE® 6 cm	PSL 618001	PSL 618007	PSL 618013
Double SLIPFREE® 10 cm	PSL 618003	PSL 618009	PSL 618015
Double SLIPFREE® 20 cm	PSL 618005	PSL 618011	PSL 618017

Minimization of band spreading

The following figure shows the influence of tubing internal diameter on system band spreading and peak shape. As can be seen, the larger tubing diameter causes excessive peak broadening and lower sensitivity.

Effect of Connecting Tubing on System



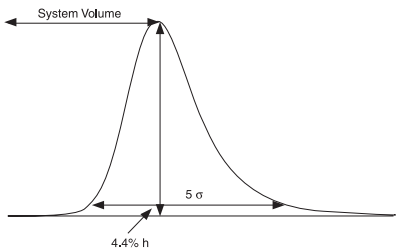
Measuring System Bandspread Volume

1) Disconnect column from system and replace with a zero dead volume union
2) Flow rate 1 mL/min. This should be performed on a single wavelength detector (not a PDA/DAD)
3) Dilute a test mix in mobile phase to give a detector sensitivity 0.5-1.0 AUFS (can use the system start up test mix which contains uracil, ethyl and propyl parabens; Waters part number WAT034544)
4) Inject 2 to 5 µL of this solution
5) Using 5 sigma method measure the peak width at 4.4% of peak height: Bandspread (µL) = PW (seconds)/60 (see Figure 1)

Figure 1: Determination of System Bandspread Volume using the 5-Sigma Method

Typical LC system should be 100 µL ± 30 µL

Microbore (2.1mm i.d. and smaller) system should be no greater than 20-40 µL

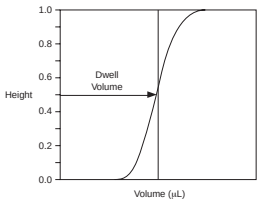


Measuring Gradient Delay Volume

1. Replace the column with a zero dead volume union.
2. Determine the gradient-delay or dwell volume for your system by performing the following test. Prepare eluent A (pure solvent, such as methanol) and eluent B (solvent plus sample, such as 5.6 mg/mL propylparaben in methanol).
3. Equilibrate the system with eluent A until a stable baseline is achieved. Switch to 100% eluent B and record the half height of the step. Refer to Figure 2 for an illustration.

The dwell volume should be less than 1 mL. If this is not the case, see section on System Modifications to reduce your system volume.

Figure 2: Determination of Dwell Volume



Use of Narrow-Bore Columns – (≤3.0 mm i.d.)

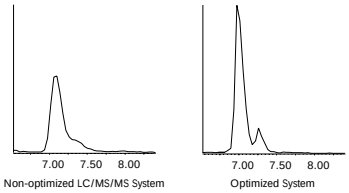
This section describes how to minimize extra column effects and gives some guidelines on how to maximize the advantages of your narrow-bore column. The 3.0 mm i.d. narrow-bore column usually requires no system modifications. With the 2.1 mm i.d. column, however, modifications to your HPLC system may be required in order to eliminate excessive system bandspread volume. Without proper system modifications, excessive system bandspread volume causes peak broadening and has a large impact on peak width as peak volume decreases.

Impact of bandspreading on column performance (2.1 mm i.d. column)

System with 70 µL bandspread >> 10,000 plates

System with 130 µL bandspread >> ~8,000 plates (same column)

Note: Flow splitters after the column will introduce additional bandspreading.



Optimizing a system, especially one using flow splitters can have a dramatic effect on sensitivity and resolution. Use of correct ferrule depth connectors and minimizing tubing diameter and lengths showed a doubling of sensitivity and enabled resolution of the metabolite on this LC / MS / MS system.

System Modification Guidelines

1) Use a microbore detector flow cell with the 2.1 mm columns. Recall that due to the shorter pathlength, detector sensitivity is reduced to achieve lower band spread volume.
2) Injector sample loop should be reduced to minimum.
3) Use 0.009 inch (0.25 mm) tubing between pump and injector.
4) Use 0.009 inch (0.25 mm) tubing for rest of connections in standard systems and 0.005 inch (0.12 mm) tubing for narrowbore (≤2.1 mm i.d.) systems.
5) Use perfect (pre-cut) connections (with a variable depth inlet if using columns from different suppliers)
6) Time constants should be shortened <0.2

Waters Small Particle Size (2.5 µm and 3.5 µm) Columns – Fast Chromatography

The Waters columns with 2.5 µm and 3.5 µm packings provide faster and more efficient separations without sacrificing column lifetime. This section describes five parameters to consider when performing separations on the 2.5 µm and 3.5 µm columns.

Note: All 3.5 µm and 2.5 mm materials have smaller outlet frits to retain packing material. These columns should not be backflushed.

1) Flow Rate — Compared with the 5 µm columns, the 2.5 µm and 3.5 µm columns have a higher optimum flow rate. These columns are used for high efficiency and short analysis times. The higher flow rates, however, lead to increased backpressure. Use a flow rate that is practical for your system.
2) Backpressure — The backpressures on the 2.5 µm and 3.5 µm columns are higher than for the 5 µm columns of the same dimesion.

Use a shorter column to compensate for increased backpressure and obtain a shorter analysis time.

3) Temperature — Use a higher temperature to reduce backpressure caused by smaller particle sizes.

4) Sampling Rate — Use a sampling rate of about 10 points per second.

5) Detector Time Constant – Use a time constant of 0.1 seconds for fast analysis.

Column Performance Validation

Each pre-packed column has an individual quality control report that provides significant information about the column. This report is available as a ready reference and should be kept in your files. It indicates the column specifics: gel lot, column dimensions, bonding chemistry type, particle shape, particle size, porosity, and chromatographic test conditions.

1. Perform an efficiency test on your column before you use it. Waters recommends using a suitable solute mixture, such as found in the "Column Test Report", to immediately analyze the column once you receive it. Determine the number of theoretical plates (N) and use for periodic comparison. Repeat the test periodically to track column performance over time. Slight variations may be obtained on two different HPLC systems due to the quality of the connections, operating environment, system electronics, reagent quality, column condi tion and operator technique. Please report any column problems observed upon receipt of the column.

Sample Preparation

1) It is preferable to prepare sample in the mobile phase or a weaker solvent than the mobile phase.

2) If the sample is not dissolved in the mobile phase, ensure sample, solvent and mobile phases are miscible to avoid sample or buffer precipitation.

3) Filter sample with 0.2 µm membrane to remove particulates.

Column Equilibration

Waters delivers the column in 100% acetonitrile. It is important to ensure solvent compatibility before changing to a new solvent. Equilibrate your column with a minimum of 10 times its internal volume with the mobile phase to be used (refer to Table 1 for some standard column volumes).

1. Purge your pumping system and then connect the inlet end of the column to the injector outlet. Turn on the pump flow at 0.1 mL/min. and increase to 1 mL/min over 5 minutes.
2. When the solvent is flowing freely from the column outlet, attach the column to the detector. This procedure prevents entry of air into the detection system and gives more rapid equilibration.
3. When the mobile phase is changed, gradually increase the flow rate of the new mobile phase from zero mL/min to 1.0 mL/min in 0.1 mL/min increments.
4. Once a steady backpressure and baseline have been achieved, the column is ready to be used.

Note: If mobile phase additives are present in low concentrations (such as ion-pairing reagents, at 5 to 10 mmol/L) 100 to 200 column volumes may be required for complete equilibration.

Table 1. Volume of standard columns (mL), multiply by 10 for flush solvent volume

Column Length	Column internal diameter (mm)									
	1.0	2.1	3.0	3.9	4.6	7.8	10	19	30	50
30 mm		0.1	0.2		0.5		2.4	8		
50 mm	0.1	0.2	0.3		0.8	2.4	4	14	35	98
100 mm	0.1	0.4	0.7	1.2	1.7	5	8	28	70	
150 mm	0.1	0.5	1.0	1.8	2.5	7	12	42	106	294
250 mm		0.9	1.8		4		20	70	176	490
300 mm						14	24	85	212	589

Column Usage

To ensure the continued high performance of your columns and cartridges, follow these guidelines:

1. Guard columns

Samples: Sample impurities very often contribute to column contamination. Two ways to avoid this are:

a. Use of Waters Oasis® solid-phase extraction sample clean-up cartridges or columns or Sep-Pak® cartridges of the appropriate chemistry to clean up your sample before analysis.

b. Use of a Waters guard cartridge of matching chemistry and particle size between the injector and main column. It is important to use a high-performance matching guard column to protect the main column while not compromising analytical resolution.

2. pH Range

XTerra® columns have a widened useable pH range over silica based columns. The pH range is pH 1-12 for the MS columns and 2-12 for the RP columns. Lifetime although greater is still finite and will vary depending upon what buffers are used, the concentration of those buffers and the temperature at which they are used. Here is a table of recommended and non-recommended buffers to be used as a guideline when developing methods. Note that high pH use of Phosphate is not recommended even though it will generally give longer lifetimes than silica based columns. For a table on appropriate buffers to use, please see Tables 2, 3 and 4.

Table 2: XTerra® buffers for use from pH 1-7

Additive or Buffer	pKa	Buffer Range (±1 pH unit)	Volatile or Non-Volatile	Recommended Use with XTerra® Packings
TFA	<1.0		Volatile	Yes (0.02-0.1%)
Acetic Acid	4.76		Volatile	Yes (0.1-1.0%)
Formic Acid	3.75		Volatile	Yes (0.1-1.0%)
Acetate (Ammonium)	4.76	3.76 – 5.76	Volatile	Yes (1-10mM) note Na+, K+ salts are not volatile
Formate (Ammonium)	3.75	2.75 – 4.75	Volatile	Yes (1-10mM) note Na+, K+ salts are not volatile
Phosphate 1	2.15	1.15 – 3.15	Non-volatile	Yes
Phosphate 2	7.2	6.20 – 8.20	Non-volatile	pH's >7.0 lifetime decreases significantly with this buffer. See also note on Phosphate 3 below. The lower the temperature and buffer molarity, the longer the column lifetime achievable.

Table 3: XTerra® buffers for use from pH 7-12

Additive or Buffer	pKa	Buffer Range (±1 pH unit)	Volatile or Non-Volatile	Recommended Use with XTerra® Packings
4-Methyl-Morpholine	~8.4	7.4 – 9.4	Volatile	Yes (10mM)
Ammonia	9.2	8.2 – 10.2	Volatile	<10 mM and <30° C
Ammonium Bicarbonate	10.3 (HCO_3^-) 9.2 (NH_4^+) 7.8 (H_2CO_3)	9.3 – 11.3 8.2 – 10.2 6.8 – 8.8	Volatile	Yes 5-10mM (keep source >150°C) **(do not use carbonate)** (total pH range 6.8- 11.3, natural pH=8.4) adjust pH with either ammonium hydroxide or acetic acid
Ammonium (Acetate) or (Formate)	9.2	8.2 – 10.2	Volatile	Yes (1-10mM)
Borate	9.2	8.2 – 10.2	Non-Volatile	
1-Methyl- Piperidine (Acetate or Formate)	10.3	9.3 – 11.3	Volatile	Yes
Triethylamine (Acetate or Formate)	10.7	9.7 – 11.7	Volatile	Yes (0.1-1%)
Pyrrolidine	11.3	10.3 – 12.3	Volatile	Yes
Phosphate 3	12.3	11.3 – 13.3	Non-Volatile	

Table 4: XTerra® buffers for use from pH 9-12 with alternative buffers

Additive or Buffer	pKa	Buffer Range (±1 pH unit)	Volatile or Non-Volatile	Recommended Use with XTerra® Packings
Glycine	9.8	8.8 – 10.8		Yes
CAPSO	9.7	8.7 – 10.7		Yes (1-10mM)
CAPS	10.5	9.5 – 11.5		Yes (1-10mM)

3. Solvents

To maintain maximum column performance, use high quality chromatography grade solvents. Filter all buffers before use. Pall Gelman Laboratory Acrodisc® filters are recommended. Solvents containing suspended particulate materials will generally clog the outside sur face of the inlet distribution frit of the column. This will result in higher operating pressure and poorer performance.

Degas all solvents thoroughly before use to prevent bubble formation in the pump and detector.

4. Pressure

All XTerra® columns, regardless of dimension, can be operated at pressures up to 6000 psi, 400 bar or 40 Mpa.

5. Temperature

Temperatures between 20 – 60˚C are recommended for operating Waters XTerra® columns to enhance selectivity, lower solvent viscosity and increase mass transfer rates. However, any temperature rise above ambient will have a negative effect on lifetime which will vary depending on the pH and buffer conditions used.

Scaling Up/Down

The following formulas will allow scale up or scale down, while maintaining the same linear velocity (retention time), and provide new sample loading values:

If only column i.d. is changed: $X = (r_2 / r_1)^2$

If both column i.d. and length are altered: $F_2 = F_1(r^2 / r_1)^2$

$Load_2 = Load_1(r_2 / r_1)^2(L_2 / L_1)$

Where: X = Factor by which original flow must be modified (also adjusts sample load)
L = Length of column, in mm
r = Radius of the column, in mm
F = Flow rate, in mL/min.
1 designates the original, or reference column
2 designates the new dimension column.

Column Cleaning, Regenerating and Storage

1. Cleaning and Regeneration

A shift in retention or resolution may indicate contamination of the column. Flushing with a neat organic solvent is usually sufficient to remove the contaminant. If the flushing procedure does not solve the problem, purge the column with a sequence of progressively more nonpolar or hydrophobic solvents. For example, switch from water to tetrahydrofuran (THF) to methylene chloride. Return to the standard mobile phase conditions by reversing the sequence.

Guard columns need to be replaced at regular intervals as determined by sample contamination. When system backpressure steadily increases above a set pressure limit, it is usually an indication that the guard column should be replaced.

2. Storage

For periods longer than four days store the column in 100% acetonitrile. Do not store columns in buffered, acidic or basic eluents. If the mobile phase contained a buffer salt flush the column with 10 column volumes of HPLC grade water (see Table 1) and replace with 100% acetonitrile. Completely seal column to avoid evaporation and drying out of the bed.

Troubleshooting

Changes in retention time, resolution, or backpressure are often due to column contamination. See the Column Cleaning, Regeneration and Storage section of this instruction sheet. Information on column troubleshooting problems may be found in HPLC Columns Theory, Technology and Practice, U.D. Neue, (Wiley-VCH, 1997) or the Waters HPLC Troubleshooting Guide (Literature code # 720000181EN).