User Guide

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Agilent Biomolecule Reversed-Phase LC Columns User Guide

This user guide provides general information on Agilent Altura HPLC columns with Ultra Inert technology, Agilent AdvanceBio, and Agilent BioHPLC reversed-phase columns. For more detailed information about your specific phase or family, see: www.agilent.com/chem/biopharma-analysis.

This guide features the following columns:

- Altura HPH-C18 1.9 µm
- Altura Oligo HPH-C18 2.7 µm
- Altura Peptide Plus
- Altura ZORBAX Eclipse Plus C18
- AdvanceBio Peptide Mapping
- AdvanceBio Peptide Plus
- AdvanceBio Oligonucleotide
- AdvanceBio Amino Acid Analysis
- AdvanceBio RP-mAb
- PLRP-S
- ZORBAX 300
- Poroshell 300

Getting started

A quality control (QC) column performance report, including a test chromatogram, is enclosed with every Agilent column. To minimize system dead volume, the QC test system has been modified from the standard configuration. As a result, it may differ from the system used in your lab. This modification allows for better evaluation of column efficiency and helps ensure a more consistent product. An optimized LC system will generate similar results to the chromatogram on your QC performance report.

Individual column performance reports may be found by searching by part and serial number at www.agilent.com/chem/cop.

Using your column

Installation

- The direction of flow is marked on the column.
- 1.8 µm columns (ZORBAX RRHT, ZORBAX RRHD) can only be operated in the direction flow marked on the column.

For removable, zero-dead-volume column connections, Agilent recommends the use of the InfinityLab Quick Connect and Quick Turn family. Choices are shown in Table 1.

Table 1. Agilent InfinityLab Quick Turn and Quick Connect fittings.

Maximum System Pressure	Recommended Fitting	Part Numbers
Up to 400 bar	InfinityLab Quick Turn fitting (finger-tight)	Fitting: 5067-5966
Up to 800 bar	InfinityLab Quick Turn fitting (with Mounting tool)	Fitting: 5067-5966 Mounting Tool: 5043-0915
Up to 1300 bar	InfinityLab Quick Connect fitting	Fitting: 5067-5965

For more information and part numbers, please see the Agilent InfinityLab Fitting Brochure (5991-5164EN).

InfinityLab Quick Connect assembly part number 5067-5961

InfinityLab Quick Turn fitting part number 5067-5966

Learn more at: www.agilent.com/chem/infinitylabfittings

Column conditioning

Every column is tested before shipment. Before first use, the shipping solvent must be replaced with eluent, taking care that all components are miscible and soluble. If mobile phase additives are used (such as buffers or ion-pair reagents), it is advisable to do an intermediate flush with a mobile phase of the correct composition, but without these additions. Flushing with 10 to 20 column volumes should help in transitioning to your mobile phase. Check that the column has been properly equilibrated before use. This will ensure reproducibility and help prevent retention time drifting. When using formic acid as a mobile phase additive, particularly with the AdvanceBio Peptide Plus column, condition the column as recommended in Table 2.

Table 2. Column conditioning method conditions for formic acid.

Column id (mm)	Mobile Phase	Flow Rate (mL/min)	Column Temp (°C)	No. Column Volumes	After Use
2.1	95:5 H_2O + 0.1% formic acid/CH_3CN + 0.1% formic acid	0.1	60	50	Flush and store in 100% CH_3CN
3.0	95:5 H_2O + 0.1% formic acid/CH_3CN + 0.1% formic acid	0.2	60	50	Flush and store in 100% CH_3CN
4.6	95:5 H_2O + 0.1% formic acid/CH_3CN + 0.1% formic acid	0.4	60	50	Flush and store in 100% CH_3CN

Note: Conditioning overnight, or up to 24 hours, may be beneficial, especially for longer columns or acidic analytes. This conditioning step may not be needed for the Altura columns.

Important safety considerations

- All connection points in liquid chromatographic systems are potential sources of leaks. Users should be aware of the toxicity or flammability of their mobile phases.
- Because of the small particle size, dry column packings are respirable. Agilent does not recommend removing the column end fittings and exposing the media. Columns should only be opened by trained personnel in a well-ventilated area.
- Please adhere to operating pressure limits noted for each column (see Table 3). Exceeding these limits will compromise chromatographic performance and column lifetime, and could be unsafe.

Table 3. Maximum operating parameters: columns up to 4.6 mm.

Column	Particle Size (µm)	Pressure Limit (bar)
Altura Peptide Plus	2.7	600
AdvanceBio Peptide Plus		
AdvanceBio Peptide Mapping		
AdvanceBio EC-C18		
AdvanceBio Amino Acid Analysis		
Altura Oligo HPH-C18		
AdvanceBio Oligonucleotide	2.7, 4	
AdvanceBio RP-mAb	3.5	
ZORBAX 300SB	3.5, 5, 7	400
Poroshell 300	5	
Altura ZORBAX Eclipse Plus	1.8	1200
ZORBAX RRHD		
Altura HPH-C18	1.9	1200
PLRP-S	3	275
	5, 8, 10	207
	10 to 15, 15 to 20, 30	103

Other operating tips

- Reverse flow will not usually harm the column, but should be avoided except to attempt removal of a clogged frit (see "Column care").
- It is recommended that the flow rate is started at a reduced rate and then gently increased to the desired operating flow rate.
- Always use high-purity reagents and chromatography-grade solvent to prepare your mobile phase. Degas and filter all mobile phases prior to use.
- Disassembling a column will degrade column performance.
- An inline filter or guard column may be used to protect your column and increase its lifetime.
- If the column is used outside of recommended pH ranges for column phase (see Table 4), a reduced lifetime will result.
- Columns should not be maintained at elevated pH or elevated temperature when not in use (see Table 4).
- New columns may contain a mixture of organic solvents and water, which may contain buffer salts. See Table 5 for details of the shipping solvents. Initially, care should be taken not to pass any mobile phase through the column that may cause a precipitate to form or may not be fully miscible.
- Altura, AdvanceBio, and Agilent BioHPLC columns are compatible with all common eluents used for analysis of biomolecules.

Table 4. Column operating parameters: pH and temperature.

Column	Recommended pH Range	Maximum Operating Temperature (°C)
AdvanceBio Peptide Mapping	2.0 to 8.0	60
AdvanceBio EC-C18		
ZORBAX RRHD	1.0 to 8.0	80
ZORBAX 300SB-C8, C3, CN		
ZORBAX 300SB-C18	1.0 to 8.0	90
AdvanceBio RP-mAb		
Poroshell 300		
Altura ZORBAX Eclipse Plus	2.0 to 9.0	60
Altura Peptide Plus	1.0 to 11.0	90
AdvanceBio Peptide Plus		
Poroshell 300 Extend-C18	2.0 to 11.0	60 below pH 8, 40 above pH 8
Altura Oligo HPH-C18	3.0 to 11.0	65
Altura HPH-C18		
AdvanceBio Oligonucleotide		
AdvanceBio Amino Acid Analysis		
PLRP-S	1.0 to 14.0	200

Note: All silica-based packings have some solubility in pH > 6 aqueous mobile phases. When using silica-based columns at pH > 6, best column lifetime is obtained at lower temperatures (40 °C maximum) using low buffer concentrations in the range of 0.01 to 0.02 M.

Table 5. Shipping solvents.

Column	Shipping Solvent	Compatibility
Altura Peptide Plus	100% acetonitrile	Water and all common organic solvents. Avoid tetrahydrofuran (THF).
AdvanceBio Peptide Plus		
AdvanceBio Peptide Mapping	Mixture of acetonitrile and water	
AdvanceBio EC-C18		
Altura ZORBAX Eclipse Plus		
ZORBAX RRHD		
ZORBAX 300SB		
AdvanceBio RP-mAb	Mixture of acetonitrile and water	Water and all organic solvents, including N,N-dimethylformamide and dimethyl sulfoxide.
Poroshell 300 and 300 Extend	Mixture of methanol and water	
Altura Oligo HPH-C18	Mixture of acetonitrile and water	Water and all common organic solvents. Modifiers including HFIP and TEAA.
Altura HPH-C18		
AdvanceBio Oligonucleotide		
AdvanceBio Amino Acid Analysis		
PLRP-S	7:1 acetonitrile:water	Aqueous organic solvents including N,N-dimethylformamide and dimethyl sulfoxide. 100% aqueous is not recommended as it will reduce column performance and lifetime.

Mobile phase selection and operating temperatures

The bonded stationary phase is nonpolar in nature and is best used with polar mobile phases, such as methanol/water or acetonitrile/water mixtures. Increasing the amount of organic component reduces the retention time of the sample.

When the maximum temperature is used for prolonged periods, this will reduce column lifetime.

Using 100% aqueous eluents with PLRP-S columns will significantly reduce the column lifetime and may result in a rapid deterioration in peak width and symmetry.

Recommended starting gradients

Separations of biological molecules typically use gradient conditions—increasing the amount of the organic component to achieve elution from the column. Peptide, polypeptide, and protein separations are most commonly performed using acidic eluents, with trifluoroacetic acid (TFA) or formic acid (FA) added as modifiers to control pH and/or act as ion-pairing additives to achieve the desired retention and selectivity. Organics such as acetonitrile, methanol, and ethanol are used for elution, with acetonitrile being the most commonly used.

Additional information on peptide separations can be found in chapter 11, pages 497–508, Introduction to Modern Liquid Chromotography, Second Edition. L.R. Snyder and J. J. Kirkland, (John Wiley & Sons, 1979).

Column care

It is best to filter your samples before injecting them onto any column, as particulates will block the column inlet frit. Where this is not possible, an inline filter or guard column should be used to protect the analytical column and increase the column lifetime. You should also check that all eluents are freshly prepared and filtered before use. Altura columns and columns with a particle size of 1.8 µm have a 0.5 µm inlet frit.

See www.agilent.com/chem/guards for more information about guard columns.

Cleaning your column/extending column life

For columns that can be backflushed (particles > 1.8 µm), clean the column in the reverse direction. Start with a stronger (less polar) solvent.

First, disconnect the column from the detector and run wash solvents into a beaker.

For small molecule contaminants, follow these steps:

1. Start with your mobile phase without buffer salts (water/organic). Run 10 to 20 column volumes through.
2. Next, use 100% organic (methanol or acetonitrile).
3. Check the pressure to see if it has returned to normal. If not, then:
   - Discard the column or consider stronger conditions, such as 75% acetonitrile:25% isopropanol.
   - Increase to 100% isopropanol, 100% methylene chloride, or 100% hexane (if you use methylene chloride or hexane, you will need to flush the column with isopropanol prior to use and before returning to your mobile phase as it is not miscible with aqueous).

Aggressive clean-up cycles using 1 M NaOH or 80:20 1 M HCl:organic can be used with PLRP-S columns.

For columns with 1.8 µm particles, do not backflush the column—you can attempt the cleanup procedure as detailed above but maintain the direction of flow.

To remove proteinaceous contamination from reversed-phase columns, the solvents in Table 6 may be used. It is not recommended to use all of these cleaning solutions sequentially. Choose the most appropriate solution for your probable contaminant. Avoid precipitation of salts, and avoid overpressuring the column due to mobile phase viscosity differences.

Table 6. Solvents used to remove proteinaceous contamination from reversed-phase columns.

Solvent	Composition
Acetic Acid	1% in water
Trifluoroacetic Acid	1% in water
0.1% Trifluoroacetic Acid:Propanol	40:60 (v/v) Viscous – use reduced flow rate
TEA:Propanol	40:60 (v/v) Adjust 0.25 N phosphoric acid to pH 2.5 with triethylamine before mixing
Aqueous Urea or Guanidine	5 to 8 M Adjust to pH 6 to 8
Aqueous Sodium Chloride, Sodium Phosphate, or Sodium Sulphate	0.5 to 1.0 M Sodium phosphate, pH 7.0
DMSO:Water or Dimethylformamide:Water	50:50 (v/v)

Adapted from Cunico, R. L; Gooding, K. M.; Wehr, T. Basic HPLC and CE of Biomolecules. Bay Bioanalytical Laboratory (Richmond, California, 1998), p. 254.

Storage recommendations

In general, columns may be safely stored for short periods in most mobile phases. Long-term storage of silica-based, bonded-phase columns should be in a pure organic solvent. If the column has previously been used with a buffered mobile phase, the buffer should first be removed by purging the column with 20 to 30 column volumes of a 50:50 mixture of methanol or acetonitrile and water, followed by 20 to 30 column volumes of the pure solvent. Before storing, end-fittings should be tightly capped with end-plugs to prevent packing from drying out.

To protect equipment, it is desirable to remove salts from the instrument and column by purging the column with the same mobile phase without the buffer (for example, using 60:40 ACN:H_2O to remove a 60:40 ACN:0.02 M phosphate buffered mobile phase). Re-equilibration is rapid with the original mobile phase when using this approach and any possibility of corrosion from the salts is eliminated.

Tips for the best chromatographic results

- Optimize your instrument by minimizing tubing lengths between components. This will reduce extra column volume and band broadening. Use 0.12 mm id red tubing for Fast LC/high efficiency columns.
- Ensure that the data collection rate is optimized for your column. Use a higher collection rate for Fast LC columns (ZORBAX RRHD).
- Use sample filtration or other sample preparation techniques appropriate for your sample.
- Use certified lamps in your LC instruments for best performance.

Agilent Altura HPLC columns

Enhance your separations with Ultra Inert technology

Altura HPLC columns feature innovative Agilent Ultra Inert technology. This advanced coating blocks active metal sites, ensuring an inert flow path while maintaining the strength, pressure tolerance, and consistency of a traditional stainless-steel HPLC column.

Altura columns are packed with familiar AdvanceBio, Poroshell, or ZORBAX stationary phases, with the added benefit of inert column hardware.

The result? Altura columns unlock the true separation potential of the stationary phase. Experience superior chromatographic performance, faster equilibration, reduced carryover, and enhanced sensitivity for your most challenging metal-sensitive analytes.

All Agilent HPLC columns are rigorously tested to ensure excellent results and are backed by Agilent's 60-day full satisfaction warranty.

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