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Protein-Pak Hi Res HIC Column and HIC Protein Standard

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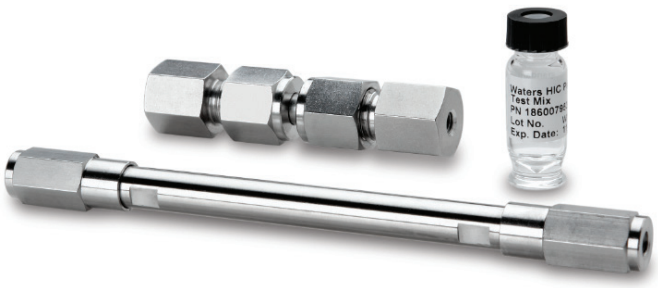

I. INTRODUCTION

This offering contains non-porous, polymethacrylate-based particles (2.5 µm) functionalized with a butyl ligand coating and is well suited for the characterization of proteins and biotherapeutics including monoclonal antibodies (mAb) and antibody drug conjugates (ADC).

While reversed-phase chromatography is a frequently used bioanalytical technique, HIC offers attractive orthogonal separation advantages. In reversed-phase LC, proteins are retained by hydrophobic interaction with alkyl groups (e.g., C18) on the packing material. However, the butyl ligand density on Waters Protein-Pak Hi Res HIC Column is comparatively less resulting in fewer protein-ligand hyrdophobic interactions. Consequently, HIC-based elution is possible using gradients of decreasing salt concentration at physiological pH values. Use of denaturing organic solvent eluents (e.g., acetonitrile in 0.1% TFA) thus allowing biotherapeutics (e.g., acid labile, cysteine-linked ADCs) to be analyzed in non-denaturing conditions.

In addition, Waters has developed HIC Protein Standard Test Mix (that we include with each shipped column) designed for user verification of HPLC/UPLC instrument and Protein-Pak Hi Res HIC column performance prior to sample analyses. This HIC-based intact protein validation mix, when used on a regular basis, helps monitor system and column performance and is also highly valuable in method development and /or troubleshooting. As indicated in Figure 1, the HIC protein standard mix contains a carefully chosen set of six proteins that provide good chromatographic representation using a gradient of decreasing salt concentration.

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a. Mobile Phase

Prevent air bubbles from entering the column during its installation, use, and storage since this may cause degradation of column performance through the formation of channels in the packed bed. Mobile phases must be thoroughly degassed before use. This can be accomplished by vacuum filtration, helium sparging, or in-line vacuum degassing. In addition to degassing the solvent, vacuum filtration of the solvent will also prevent small particles from plugging the column frit. You can use 0.20 or 0.45 µm membrane to filter aqueous and aqueous/organic mobile phases.

Note: *Consult with filtration membrane manufacturer for details on solvent compatibility.*

Note: *Use high quality reagents, HPLC-grade water, and HPLC-grade solvents for preparing buffers to maximize column efficiency especially when using Waters Protein-Pak Hi Res HIC, non-porous columns.*

The useful column lifetime is a function of numerous factors including: the cleanliness and composition of the mobile phase and the sample; the flow rate and pressure used; and the temperature. Refer to the section below about "Cleaning" for information on extending column life.

Note: *Cleaning is not effective when the column is damaged by irreversible sample adsorption, channeling, or packing material exposure to excessive heat or shock.*

b. Flow Direction

The recommended flow direction through the column is indicated by the arrow on the tag. Operating the column with the flow in the reverse direction is only recommended as part of a cleaning procedure when removing particulates from a clogged frit.

II. CONNECTING COLUMN TO LC SYSTEM

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 column endfitting does not match the existing instrumentation tubing ferrule setting. Waters Protein-Pak Hi Res HIC Columns require endfittings that have a 0.175" depth between the ferrule and the end of the extending stainless steel tubing (Figure A) that must perfectly seat to the end of the Protein-Pak Hi Res HIC Column. Connecting a non-Protein-Pak Hi Res HIC style connector to a Protein-Pak Hi Res HIC Column will leave a gap between the end of the stainless steel tubing and the column that will result in undesired peak broadening (Figure B). To correct this problem, cut the tubing, place a new ferrule on it and re-make the connection (i.e., 0.175" depth) to the Waters Protein-Pak Hi Res HIC Column.

Figure A.

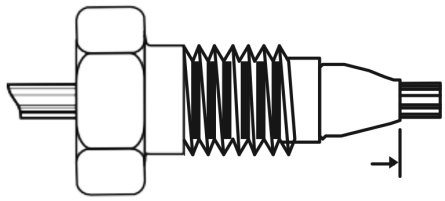
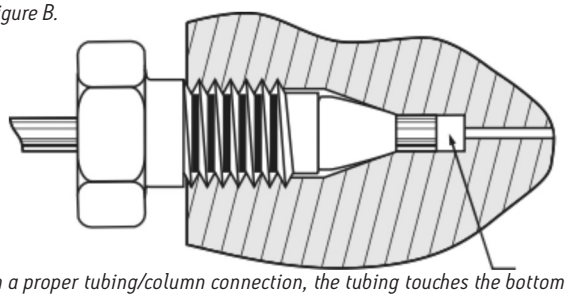
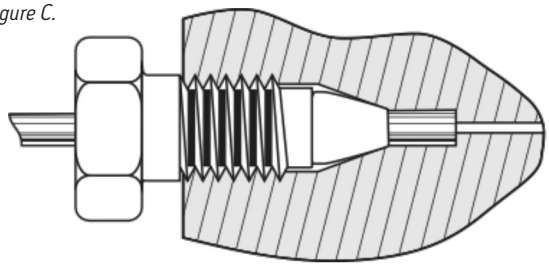


Figure B.



In a proper tubing/column connection, the tubing touches the bottom of the column end fitting with no void between them. (Figure C).

Figure C.



After manufacturing and quality control, the column has been flushed with storage solvent and closed with caps to prevent solvent evaporation. When installing the column to the liquid chromatograph, it is important to prevent air from entering the column. The following steps can be used to minimize this potential problem:

- Remove the cap from the column's inlet side. Solvent should be visible at the inlet fitting (if not, see below).
- Start solvent flow through the liquid chromatograph before connecting the Protein-Pak Hi Res HIC Column to the system. Turn off the pump after observing steady flow of solvent from the system. You can now connect the Protein-Pak Hi Res HIC Column to the liquid chromatograph and slowly begin delivering solvent to the column

Note: *If the Waters Protein-Pak Hi Res HIC Column inlet fitting appears dry, we recommend that you first disconnect the bottom cap and hook up the flow in this reversed flow direction until a few drops of mobile phase exit from the column. Turn off the flow, let the pressure go to zero, and disconnect the column from the system. Turn the column around and hook it up so that the flow is now in the direction of the arrow. Start the flow at a low setting and stop it as soon as the mobile phase exits from the bottom fitting. Now you can hook up the column to the detector inlet and increase the flow to the desired setting.*

- Be sure to set the flow within the recommended range shown in the following tables.

III. COLUMN SPECIFICATIONS AND USE

Shipping solvent:	Distilled Water
Max. flow rate:	1.2 mL/min: Protein-Pak Hi Res HIC , 4.5 x 35 mm 1.5 mL/min: Protein-Pak Hi Res HIC , 4.5 x 100 mm When a buffer with high viscosity is used, the maximum flow rate may have to be reduced so as not to exceed the maximum pressure drop.
Max. pressure:	3000 psi
pH range:	2–12 (pH above 12 or below 2 can only be used for a short time)
Salt conc.:	< 4 Molar
Organic conc.:	< 50% (salt precipitation should be avoided when adding salts to the mobile phase containing organic solvents)
Temperature:	10–60 °C. Reduce flow rate when operating at low temperatures (e.g. 10 °C) to avoid excessive column pressure.
Column washing and regeneration:	Repeated 100–250 µL injections of 0.1–0.2M NaOH are recommended for cleaning or regenerating the column. When this procedure is not effective, we recommend repeated injections of 100–250 µL of 20% aq. acetic acid.

Note: *The above cleaning step using 0.1–0.2M NaOH is best performed after each day of column use.*

Following use of cleaning solvent, rinse the column with 5–10 column volumes of HPLC-grade water before storage.

Column storage:	Store the column in shipping solvent at the end of each day of use. For overnight storage, continuously flush the column with the mobile phase at 10–20% of the maximum recommended flow rate. Store the column in the HPLC-grade water when it will be used within 24 hrs or in either 20% ethanol or methanol for long term storage. (Caution: Rinse column in with 5–10 column volumes of HPLC-grade water before switching to 20% organic solvent to avoid salt precipitation in column.
Column protection:	No guard column is available for the Protein-Pak Hi Res HIC Column. Be sure to use a filter after the injector with 0.5 µm pores to avoid plugging of the one micron pore size column frit. We also recommend a pre-injector membrane filter to prevent particles from pump seal wear to reach the column. Also, use high quality reagents, water, and solvents for preparing buffers. Fouling of the resin, leading to a loss in retention and/or efficiency, occurs faster due to the small surface area of non-porous resin particles.

IV. USEFUL FUNCTIONAL TEST

It is very valuable to benchmark the column for the first use to obtain a useful measure of the physical state of the packed bed and chemical integrity of the bonded phase. Once this is established, it is recommended to use this same test on a regular basis. The HIC Protein Standard Test Mix (P/N 186007953) was specially designed to provide a relevant standard for this type of testing. This standard comes with each new column purchase and can be reordered to continue to benchmark and monitor the column efficiency and lifetime. It is also a very valuable tool for method development and troubleshooting. The standard contains 6 lyophilized intact proteins that provide good chromatographic representation throughout the example gradient. It is package in a Waters TruView™ Vial for direct solubilization which can be dissolved in distilled water or initial gradient conditions.

Protein	Concentration/Vial
Bovine Ribonuclease A	0.05 mg
Horse Cytochrome c	0.025 mg
Horse Myoglobin	0.05 mg
Chicken Lysozyme	0.03 mg
Yeast Enolase	0.10 mg
Alpha-chymotrypisinogen A	0.05 mg

Table 1: HIC Protein Standard Test Mix Contents

a. Sample Preparation

Prior to injection of your sample onto your Protein-Pak Hi Res HIC Column, it is important to determine the appropriate salt concentration to dissolve the sample without having it precipitate out of solution yet sufficiently high enough to allow the proteins to bind to the hydrophobic butyl groups contained on the non-porous, 2.5 µm particles. While results will vary depending on proteins to be analyzed, we suggest dissolving your sample in 1M $(NH_4)_2SO_4$ containing 50 mM NaH_2PO_4 / Na_2HPO_4, pH 7 since the proteins should remain in solution yet be sufficiently "hyrdophobic" to bind to the column.

***Note** Be sure to adequately flush the LC eluent lines and system with water to remove any organic solvent to prevent HIC solvent precipitation (i.e., salt precipitation if exposed to high organic solvent concentration).*

b. HIC Protein Mixture Performance Test Example

Column: Protein-Pak Hi Res HIC, 2.5 µm
4.6 x 100 mm (P/N 176003576)

Sample: HIC Protein Standard Test Mix (P/N 186007953)
Dissolved in 100 µL 50%A / 50%B

Mobile phase A*: 2 M $(NH_4)_2SO_4$ in 50 mM NaH_2PO_4/Na_2HPO_4, pH 7

Mobile phase B*: 50 mM NaH_2PO_4/Na_2HPO_4, pH 7

***Note** it is recommended to filter all Mobile Phases with 0.2 µm GVWP filters*

Gradient: 0 – 100%B in 15 mins

Flow rate: 0.6 ml/min

Detection: 220 nm

Temperature: 30 °C

Sample: 2 µL injection

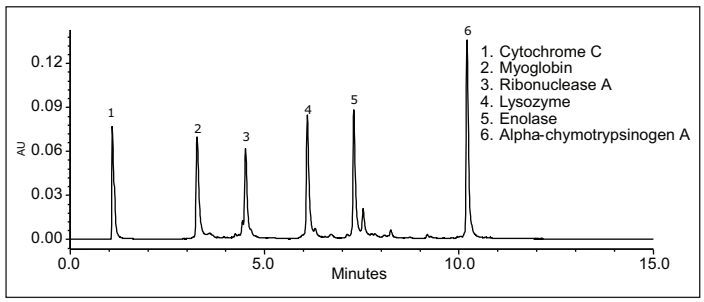


Figure 1: Example Protein Test Mixture Chromatogram.

TIPS AND TRICKS

- Equilibrate at the initial condition for 10 column volumes before running the sample set.
- After running HIC application, be sure to purge the LC eluent lines and system with water to prevent salt precipitation that could occur when system is not being used.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com