Operating Instructions for the Bio-Gel® HPHT Column Set

For Technical Service
Call Your Local Bio-Rad Office or
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(1-800-424-6723)

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LIT07 Rev B

The Bio-Gel HPHT column set consists of a high-performance hydroxylapatite column and its required guard column. The HPHT column is packed with a specially prepared high-resolution derivative of Bio-Rad's hydroxylapatite, and is suitable for high-speed HPLC separations of biological macromolecules on both an analytical and a preparative scale. The guard column is packed with sperical polymer beads and functions as an inert matrix to trap any particulate material that may enter the system. Its use to protect the HPHT column is required for reproducible separations and longest column life.

Table 1 details the specifications for the HPHT and guard columns, and Table 2 gives important installation recommendations. Please review this data carefully. Since the HPHT packing material is relatively fragile compared to silica and polymer-based HPLC packings, adherence to the recommendations in these instructions is strongly suggested for best performance.

The liquid chromatography specialists at Bio-Rad are ready to help you obtain optimal performance from this column set. If you have any questions about column performance or applications, please contact your local Bio-Rad Technical Representative or Chromatography Technical Services at Bio-Rad Laboratories in Hercules, CA. Call 1-800-4BIORAD(1-800-424-6723).

Unpacking

While unpacking the column set, check it carefully for evidence of shipping damage, rough handling, or leaking buffer. Save the shipping container to store the columns. If there is evidence of damage, immediately noti-

fy the carrier and your local Bio-Rad Technical Representative.

Table 1. Column Specifications

HPHT Column

Packing material	High-resolution derivative of Bio-Rad's hydroxylapatite
Column dimensions	100 x 7.8 mm, 4.8 ml bed volume
Protein loading capacity	100 mg Best resolution with loads of 20 mg or less
Nucleic loading capacity	5 mg Best resolution with loads of 1 mg or less
pH range	5.5 - 10.5 For longest column life maintain above pH 6.5
Temperature range	0-85 °C Autoclavable in storage buffer if tightly sealed with screws and washers originally supplied
Flow rate	1.2 ml/min maximum Best resolution obtained from 0.5 - 1.0 ml/min 1.0 ml/min recommended for standard viscosity phosphate buffers and analytical loads. Reduce flow rate with viscous buffers (*e.g.* 8 M urea; 1 M phosphate buffer) to 0.5 ml/min maximum Reduce flow rate with preparative (greater than 5 mg protein or 0.25 mg nucleic acid) or viscous (*e.g.* ascites fluid) sample loads to keep within pressure limits (0.5 ml/min recommended)
Pressure limits	300 psi maximum For longest column life maintain pressure below 200 psi (see Section 4) 100-200 psi nominal backpressure at 1.0 ml/min in low ionic strength phosphate buffer (note that addition of the guard column results in a total system pressure of 700-1,100 psi) Pressure will increase slightly during normal gradient elution due to increasing buffer viscosity
Compatible buffers	All aqueous buffers and inorganic salts Common ionic and non-ionic detergents (*e.g.* SDS and Triton X-100) Reducing agents (*e.g.* DTT and 2-ME) Chaotropes (*e.g.* urea and formamide)
Conditions to avoid	Acid pH less than 5.5 – irreversibly damages column packing Extremely high viscosity buffers (*e.g.* containing high-percentage glycerol) Extremely high viscosity samples (*e.g.* highly concentrated high molecular weight DNA) Potassium phosphate buffers, or $CaCl_2$ at >0.01 mM, in the presence of SDS – form insoluble precipitates Presence of particulates or precipitates in samples and buffers

Guard Column

Packing material	Inert, hydrophilic, spherical polymer matrix
	10 µm mean particle size
Column dimensions	50 x 4.0 mm 0.6 ml bed volume
Protein loading capacity	Non-binding
Nucleic acid loading capacity	Non-binding
pH range	2-13
Temperature range	Same as HPHT column
Flow rate	Same as HPHT column
Pressure limits	2,500 psi maximum For longest column life maintain pressure below 1,500 psi (see Section 4)
	600-900 psi nominal back-pressure at 1.0 ml/min in low ionic strength phosphate buffer
	Pressure may increase by several hundred psi during normal gradient elution
Compatible buffers	Same as HPHT column – with the addition of strong acids (*e.g.* 0.1 N H_2SO_4 or HCl) and strong bases (*e.g.* 0.1 N HaOH or KOH)
Conditions to avoid	Organic solvents – irreversibly damage the column matrix
	Particulates or precipitates in samples or buffers will clog the guard column rapidly, requiring column cleaning (see Section 5)

Table 2. Installation Recommendations

Upon receipt	Run a blank gradient prior to first sample load. This cleans and regenerates the column set and equilibrates into starting buffer. Buffer strength, gradient shape, elution time and flow rate may subsequently be adjusted to achieve optimal separation.
Start-up	Always pressure-up slowly to normal flow rates (ca. 2-5 min). This method is gentle on the column set and provides maximum longevity and retention of resolution and efficiency. Starting the column set at full flow rate may compress the HPHT packing and create an inlet void with subsequent reduced performance.
pH	Always maintain pH greater than 5.5. If possible, use the column system at pH greater than 6.5. Remember that laboratory grade water may have a pH less than 5.5 if not freshly prepared.
Sample and buffer preparation	Filter all buffers and samples through 0.2 µm membrane filters.
	Degas all buffers at least 10 min. under vacuum, and carefully purge solvent delivery lines of all bubbles.
	Include 0.01 mM $CaCl_2$ in all buffers, if possible, to help maintain HPHT crystal integrity. Use of chelating agents, such as EDTA, is therefore not suggested.

Table 2. Installation Recommendations (*continued)*

High temperature	After high temperature separation, continue to pump buffer until the column system returns to room temperature. If pumps are stopped immediately, air may be sucked into the column by contraction of solvent.
Column cleaning	Disconnect the HPHT coumn from flow when back-flushing or cleaning the guard column. This prevents contaminating materials from washing onto the HPHT matrix. **Do not** reverse flow direction of the HPHT column since this will irreversibly damage the column packing.
Storage	Store the column set with 0.02% sodium azide in low ionic strength phosphate buffer containing calcium. **Do not** store in high ionic strength buffers.

Section 1 Sample Preparation

Samples should be equilibrated in starting buffer prior to column loading. Dialysis or buffer exchange (*e.g.* chromatography on Bio-Gel® P-6DG; request Bulletin 2068) provides the most thorough equilibration. This is especially important if the solubility of any component of the sample in the starting buffer is unknown. Alternatively, resuspension of sample material lyophilized from volatile buffers, simple dilution of sample into starting buffer (*e.g.* 1:10), or slight dilution of sample with a concentrated stock solution (*e.g.* 10 x) may be utilized.

Following buffer equilibration, and prior to injection, all samples must be particulate free. Filter samples through a 0.2 µm membrane filter. If filtration is not feasible, hard centrifugation o the sample (20,000 x G for 10-15 min) may be substituted. Inspect centrifuged samples carefully for any evidence of suspended material before injecting them onto the column.

Section 2 Buffers

The HPHT system is compatible with all aqueous buffers and salts, chaotropes, reducing agents and common ionic and non-ionic detergents, provided the pH is buffered at greater than 5.5 and that minimum phosphate (1 mM - see Section 3) and calcium (0.01 mM - see Table 2) concentrations are used. Although the hydroxylapatite material is also stable to many organic solvents, the guard column material is not, and the use of organic modifiers for separations is therefore not suggested.

In general, the HPHT column is operated under the same extremely mild conditions as conventional hydroxylapatite. Samples are usually applied to the HPHT column in a phosphate buffer of low ionic strength, and are selectively eluted by increasing the phosphate concentration in a gradient or stepwise manner. Other elution schemes have used combinations of NaCl gradient elution, chaotropes and/or detergents (*e.g.* urea and/or SDS), as well as temperature and pH shifts to achieve separations (see References 1-5 for applications).

When using other than standard elution buffers, be aware of the effects of increased vis-

cosity on the system back pressure. Generally, **do not** pump viscous buffers (*e.g.* greater than 0.5 M phosphate or NaCl, urea buffers, etc.) at flow rates above 0.5 ml/min. In all cases, **do not** exceed the flow rate and back pressure limits, since even short exposure to a higher flow rate or back pressure can crush the HPHT packing.

Standard Elution Buffers: Listed below are useful starting concentrations for most protein and nucleic acid loads. Binding is accomplished in low ionic strength buffer and elution is normally achieved by a linear or step gradient from low to high ionic strength.

Low	10 mM sodium phosphate, pH 6.8 0.01 mM $CaCl_2$
High	350 mM sodium phosphate, pH 6.8 0.01 mM $CaCl_2$
MUP buffers	For plasmid purification (see Reference 4)
Equilibration and load	8 M urea (deionized with mixed bed ion exchange resin, *e.g.* AG 501-X8, Catalog Number 142-6424) 0.24 M sodium phosphate, pH 6.8 0.01 mM $CaCl_2$
Wash	10 mM sodium phosphate, pH 6.8 0.01 mM $CaCl_2$
Elution	Linear or step-gradient to 350 mM sodium phosphate, pH 6.8 0.01 mM $CaCl_2$

Phosphate Buffer Preparation: Phosphate buffers are conveniently and reproducibly prepared from concentrated stock solutions stored at room temperature. Only high purity reagent grade chemicals should be used. Recommended stock concentrations are: 1.0 M sodium phosphate at the desired pH; 10 mM $CaCl_2$ (1,000 x); and 2% sodium azide (100 x). Stock solutions should be prepared in distilled and/or deionized water, filtered through 0.2 µm membrane filters and stored at room temperature in tightly closed glass bottles.

1.0 M sodium phosphate buffer at pH 6.8 is easily prepared by equimolar mixture of monobasic (*e.g.* $NaH_2PO_4 \cdot H_2O$) and dibasic (*e.g.* $Na_2HPO_4 \cdot 7H_2O$) phosphate. For example, to prepare 2 liters of 1.0 M sodium phosphate, pH 6.8:

Step 1. Dissolve 1 mole of monobasic (monohydrate; FW = 137.99) in approximately 1 liter of distilled and/or deionized water.

Step 2. When fully dissolved, increase the volume to approximately 1.5 liters and add 1 mole of dibasic (heptahydrate: FW = 268.07) slowly, with stirring. Mild heat will increase the rate of dissolution.

Step 3. When fully dissolved, make up to 2.0 liters and filter through a 0.2 µm membrane filter. Upon dilution, the pH should be 6.8±0.05.

Section 3 Buffer Preparation Notes

A. Binding Buffers: Most protein and nucleic acid samples will bind to the HPHT column in 10 mM sodium phosphate buffer. However, some lightly binding samples may require loading in 1 mM buffer, the lowest recommended buffer concentration. Alternatively, if the sample component of interest is determined to be more tightly bound to the HPHT column than other contaminants, then loading at an ionic strength slightly below the elution concentration will effectively increase the column capacity for the sample, since lightly binding contaminants will simply wash through the column.

B. Elution and Regeneration Buffers: 350 mM sodium phosphate buffer is normally sufficient to desorb most samples and regenerate the HPHT column for subsequent use. If necessary, buffer concentrations up to 1.0 M, or the use of NaCl to 1.0 M, can be used to remove tightly bound samples or stubborn contaminants. Remember that the back-pressure limit of the HPHT column must be observed when using high viscosity buffers, *i.e.* reduce flow rate to prevent potential overpressure condition.

C. Phosphate/SDS Buffers: Potassium phosphate buffers may be substituted for sodium phosphate in most procedures. Potassium phosphate is recommended for separations which require cold room operation (*e.g.* 4 °C) because of its greater solubility. However, if SDS is used in a procedure, it must be used with sodium phosphate since SDS is incompatible with potassium containing buffers. Also, SDS-sodium phosphate separations should be performed at elevated temperatures (*e.g.* 37 °C), especially with high phosphate concentrations, to avoid SDS precipitation from solution.

D. Buffer Exchange: When changing column buffers, exchange at slow flow rates (0.5 ml/min or less) preferably by use of a gradient. Always degas buffers, especially when changing solvents, to reduce the possibility of bubble generation.

E. Buffer Preservation: If preferred, 0.02% to 0.05% sodium azide may be included in elution buffers. Buffers preserved with sodium azide and 0.2 µm filtered may be used for up to one week, or longer if refrigerated. Buffers not containing azide should be made fresh daily, or refiltered to prevent bacterial contamination.

F. Storage Buffer: Store in 10 mM phosphate buffer, pH 6.8, containing 0.01 mM $CaCl_2$ and 0.02% sodium azide to prevent microbial growth.

Section 4 Pressure Checks

When first connecting the column to an HPLC system, check the pressure while operating at 1.0 ml/min (after slow start-up - see

Table 2) in 10 mM phosphate buffer. The total back pressure of the system will be approximately 700-1,100 psi (Note: some HPLC pumps do not give accurate pressure readings at low pressures; use of a 0-1,000 gauge placed upstream of the injector may be necessary). To determine the back pressure of the HPHT column, read the total system operating pressure, disconnect the tubing between the guard column and the HPHT column, and note the pressure drop. The decrease should be approximately 100-200 psi (Note: remember to turn off the pumps before re-connecting the HPHT column, then slowly return over 2-5 minutes to the full flow rate).

If the total back pressure of the system increases during use (other than the normal increase caused by the higher concentration buffers used during elution), repeat the above procedure to determine the cause. If the system increase is small, and is due to the guard column, the system can be operated normally. If the increase in guard column pressure is greater than 150% of the pressure when new, the guard column should be cleaned (see Section 5) or replaced.

If the system pressure increase is caused by the HPHT column, the column may be partially clogged and flow rates should be reduced to stay within the 300 psi pressure limit. Column cleaning may then help to reduce the back pressure (see Section 5).

The HPLC pump pressure limit device should be adjusted so that a pressure increase (of 10-20% above the pressure normally experienced when using the high concentration standard buffer) will cause the pumps to shut off. This will protect the system from accidental over-pressure. On some HPLC pumps, the pressure sensor cannot be adjusted to this close a tolerance at the low pressure required. In this event, extra care must be taken to insure that the pressure limit is not exceeded.

Section 5
Column Cleaning

Guard Column: If the guard column back pressure increases, the flow properties can usually be restored by pumping the column in reverse (after first disconnecting the HPHT column) with either high ionic strength buffer (*e.g.* 1.0 M), high salt (*e.g.* 1.0 M NaCl), urea (to 8.0 M), 0.1 N NaOH or 0.1 N HCl. **Do not** use organic solvents. Back pressure may increase to 2,000-2,500 psi during this operation. Continue pumping and/or change washing solvents until the pressure returns to normal values. Re-equilibrate into appropriate buffer and return to forward flow direction before reattaching the HPHT column.

HPHT Column: If the HPHT column back pressure increases, elution with high concentration phosphate buffer (*e.g.* 1.0 M), use of NaCl (to 1.0 M) or the addition of urea (to 8.0 M) may clear contaminants (remember to reduce flow rate with viscous buffers). **Do not** operate the HPHT column in the reverse flow direction at any time.

Trouble-Shooting Guide

Condition	Cause	Characteristics	Resolution
High or increasing back pressure	1. Guard column clogging	Greater than 150% of pressure when new (or >1,500 psi) at 1.0 ml/min with low ionic strength phosphate buffer	See column cleaning
	2. HPHT column contamination	Greater than 300 psi, or gradual pressure run-to-run, at 1.0 ml/min with low ionic strength phosphate buffer	See column cleaning
	3. Obstructed tubing and/or connectors	Low or no flow with high back-pressure	Isolate obstruction and repair or replace tubing
		A sudden catastrophic pressure increase which cannot be traced to equipment failure or clogged connecting tubing may be the result of column bed degradation (*e.g.* by use of inappropriate conditions such as organics with the guard column or acid pH with the HPHT column), column plugging (*e.g.* by particulates or precipitates in samples or buffers, or by bacterial contamination), or column bed colapse. If appropriate column cleaning methods do not reduce the pressure, the column(s) may have to be replaced	

Trouble-Shooting Guide (*continued*)

Condition	Cause	Characteristics	Resolution
Loss of resolution	1. Particulate contamination	Gradual loss in efficiency	See column cleaning
	2. Excessive extra-column dead volume	Inability to match test chromatogram on new column	Check condition of all instrument fittings
		The gradual onset of merging, tailing or skewed peaks may indicate HPHT bed compression, which could lead to bed colapse. If reduction of flow rate (and pression) and the use of appropriate column cleaning methods do not improve the separation or halt the loss of resolution, the column may eventually need to be replaced	
No resolution	1. Tenaciously bound sample	Failure to achieve quantitative recovery	See column cleaning
	2. Incorrectly made buffers	No resolution of standard protein mixture as used for test chromatogram	Check buffers by conductivity or refractive index

References

1. Bio-Rad Catalog: Hydroxylapatite applications and references.
2. Bio-Rad Bulletin 1115: Bio-Gel HPHT for Protein and Nucleic Acid HPLC. New High Performance Hydroxylapatite Column, 4 pp.
3. Compton, S. W. and Engelhorn, S. C., HPHT: High Performance Hydroxylapatite, **LC Magazine, 1,** 294 (1983).
4. Shoyab, M. and Sen, A., The Isolation of Extrachromosomal DNA by Hydroxylapatite Chromatography, in: *Methods in Enzymology*, **68**, p. 199, Academic Press, New York (1979).
5. Juarez-Salinas, H., Engelhorn, S. C., Bigbee, W. L., Lowry, M. A. and Stanker, L. H., Ultrapurification of Monoclonal Antibodies by High Performance Hydroxylapatite (HPHT) Chromatography, **BioTechniques, 2,** 164 (1984).

Product Information

Catalog Number	Product Description
125-0177	**Bio-Gel HPHT Column Set**, consists of Bio-Gel HPHT column, guard column, and connecting tubing
125-0175	**Bio-Gel HPHT Column**, 100 x 7.8 mm
125-0176	**Guard Column**, 50 x 4.0 mm