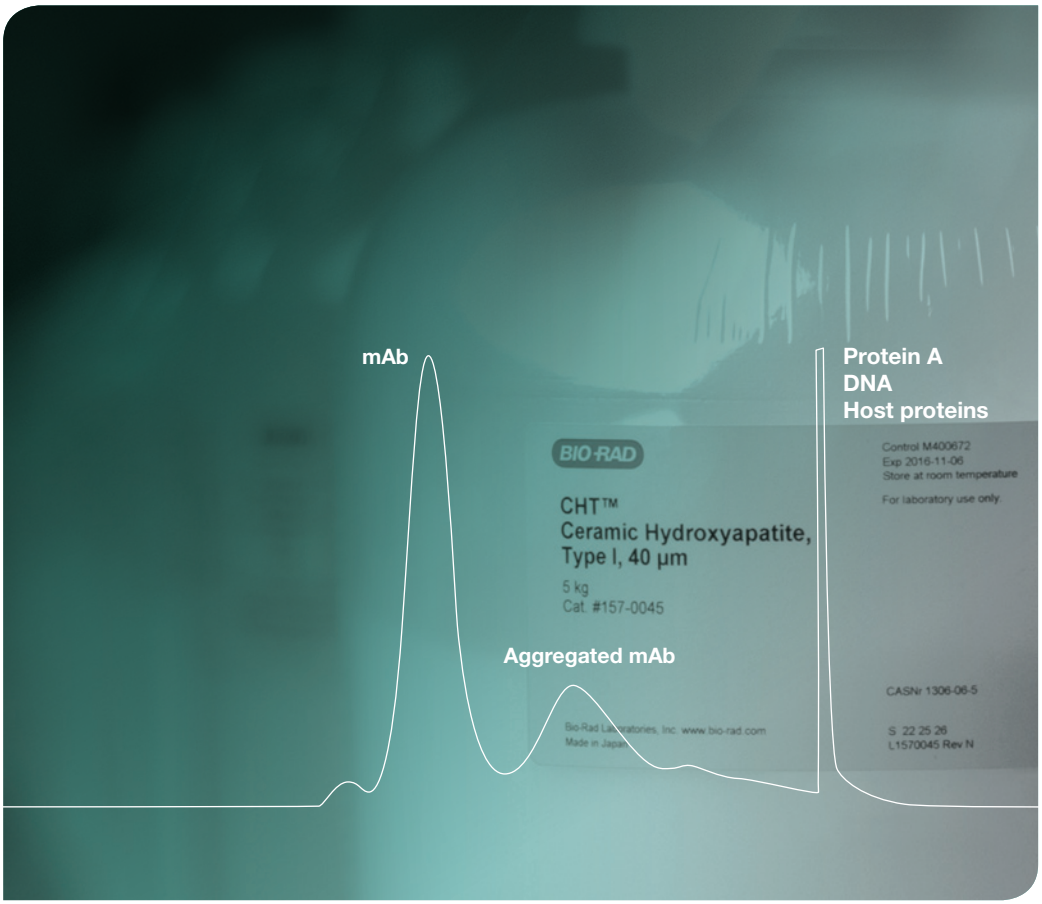

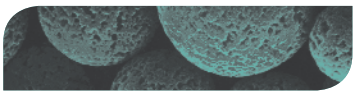

Ceramic Hydroxyapatite Application Guide for Process Development and Scale-Up

BIO-RAD


BIO-RAD

CHAPTER 1
Introduction

Bio-Rad was founded in 1952 and has been in the chromatography industry for over 50 years. Many of our products are used in established research techniques, biopharmaceutical production processes, and food testing regimens. In particular, CHT™ ceramic hydroxyapatite has been well accepted for over 10 years in biopharmaceutical manufacturing processes.

Hydroxyapatite is formed from the chemical combination of calcium and phosphate salts. The crystalline material so derived is widely used in analytical and preparative biomolecule research, as well as in industrial production of biologically active substances. However, the fragility of the crystals limits flow rates and column longevity. In the 1980s, scientists discovered how to agglomerate and sinter the crystals at high temperature to form robust spherical, macroporous beads that could withstand very high flow rates and pressures without breakage. This is the material that is manufactured today and used for the fractionation and purification of a wide variety of biological molecules as described in this guide. Product descriptions and specifications are provided in Chapter 2. Two types of chemically pure ceramic hydroxyapatite, CHT™ ceramic hydroxyapatite Types I and II, are produced. The duration and temperature of the sintering step determine whether the ceramic hydroxyapatite is CHT Type I or Type II. CHT is a mixed-mode resin. Details of the binding mechanism are described in Chapter 3.

CHT is a leading purification medium of biomolecules in today's demanding downstream process industry. Its mixed-mode support offers unique selectivities and often separates biomolecules that appear homogeneous with other chromatographic methods. The diverse binding capabilities of CHT for host cell proteins, leached protein A, antibody dimers and aggregates, nucleic acids, and viruses allow its use at any stage from initial capture to final polishing.

Table 1.1. Contaminant removal.

Contaminants	Reduction, logs
Aggregates	1–2
Protein A	1–2
Host cell proteins	2
DNA	>3
Endotoxin	>4
X-MuLV (log_{10})	4
MVM (log_{10})	2

The properties of CHT ceramic hydroxyapatite improve efficiency, yield, and financial value through:

- Excellent capture at elevated flow rates enabling processing at all scales
- Large capacity for higher-titer upstream feedstocks
- Exceptional selectivity allowing for a two-step chromatographic process

This is a guide for the use of CHT as a media support in your purification process. The guide is organized into five main topics:

- Product Description
- Chromatography
- Regeneration, Sanitization, and Storage
- Column Packing Protocols
- Case Studies

Throughout this guide, we have incorporated recommendations ranging from method scouting and optimization to column packing techniques that represent feedback from process chromatographers worldwide. Should you have further questions, contact either your local Bio-Rad process chromatography sales representative or the Bio-Rad chromatography technical support department at 1-510-741-6563 or 1-800-876-3425 extension 6563. You can also send an email to lsg_techserv_us@bio-rad.com.


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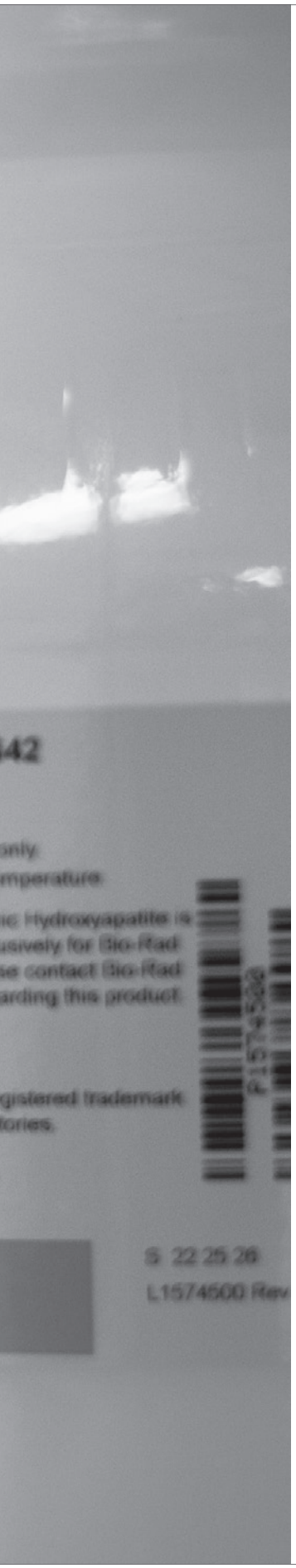
Ceramic
Hydroxyapatite
Type II
40 µm
5 kg
Catalog #157-4500
Bio-Rad Laboratories, Inc.
www.bio-rad.com
Lot # S7006
Made in Japan



CHAPTER 2
Product Description

2.1 What is CHT™ Ceramic Hydroxyapatite?

Hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, is a form of calcium phosphate used in the chromatographic separation of biomolecules. Sets of five calcium doublets (C-sites) and pairs of –OH containing phosphate triplets (P-sites) are arranged in a repeating geometric pattern. Space-filling models and repeat structure from Raman spectroscopy have also been constructed. Hydroxyapatite has unique separation properties and unparalleled selectivity and resolution. It often separates proteins shown to be homogeneous by electrophoretic and other chromatographic techniques.

Applications of hydroxyapatite chromatography include the purification of:

- Different subclasses of monoclonal and polyclonal antibodies
- Antibodies that differ in light chain composition
- Antibody fragments
- Recombinant proteins
- Viral particles
- Vaccines
- Isozymes
- Supercoiled DNA from linear duplexes
- Single-stranded from double-stranded DNA

CHT ceramic hydroxyapatite is a spherical, macroporous form of hydroxyapatite. It has been sintered at high temperatures to modify it from a nanocrystalline to a ceramic form. The ceramic material retains the unique separation properties of crystalline hydroxyapatite, and lot-to-lot control assures reproducibility in large-scale production columns. Unlike most other chromatography adsorbents, CHT is both the ligand and the support matrix. Separation protocols originally developed on crystalline hydroxyapatite can often be transferred directly to the ceramic material with only minor modifications.

Two types of CHT ceramic hydroxyapatite, Type I and Type II, are available in three particle sizes, 20, 40, and 80 µm. Although both types have elution characteristics similar to crystalline hydroxyapatite, they also have some important differences. CHT Type I has a higher protein binding capacity and better capacity for acidic proteins. CHT Type II has a lower protein binding capacity but has better resolution of nucleic acids and certain proteins. The Type II material also has a very low affinity for albumin and is especially suitable for the purification of many species and classes of immunoglobulins.

2.2 What is MPC™ Ceramic Hydroxyfluoroapatite?

MPC is a second-generation CHT mixed-mode chromatography media. MPC, like CHT, is macroporous ceramic hydroxyapatite with 25% of the hydroxyl groups substituted with fluoride, lending to its name hydroxyfluoroapatite, $Ca_{10}(PO_4)_6(OH)_{1.5}(F)_{0.5}$. The incorporation of fluoride creates a more chemically stable form of the matrix and provides greater useable column lifetime at process scale. The utility of MPC is similar to that of CHT, including binding capacity, protein separations, clearance, and packing.

Methods described throughout the guide for the optimal usage of CHT also apply to MPC. Note that the tap-settled density for MPC is greater than that for CHT.

Table 2.1. Chromatography media.

	CHT Specifications		MPC Specifications
	Type I	**Type II**	**Type I**
Functional groups	Ca^{2+}, PO_4, OH	Ca^{2+}, PO_4, OH	Ca^{2+}, PO_4, OH, F
Observed dynamic binding capacity lysozyme (Lys)	≥25 mg Lys/g CHT	≥12.5 mg Lys/g CHT	≥25 mg Lys/g MPC
Nominal pore diameter	600–800 Å	800–1,000 Å	600–800 Å
Maximum backpressure	100 bar (1,500 psi)	100 bar (1,500 psi)	100 bar (1,500 psi)
Nominal mean particle size	20 ± 2, 40 ± 4, and 80 ± 8 µm	20 ± 2, 40 ± 4, and 80 ± 8 µm	40 ± 4 µm
Tap-settled density* (g/ml tap settled bed)	0.63 g/ml	0.63 g/ml	0.72 g/ml
	CHT Characteristics		**MPC Characteristics**
	Type I	**Type II**	**Type I**
Observed dynamic binding capacity IgG	25–60 mg IgG/ml CHT**	15–25 mg IgG/ml CHT**	25–50 mg IgG/ml MPC**
Typical linear flow rate range	50–1,000 cm/hr	50–1,000 cm/hr	50–1,000 cm/hr
pH stability	6.5–14 pH	6.5–14 pH	6.5–14 pH
Base stability	at least 1 year in 1 N NaOH	at least 1 year in 1 N NaOH	At least 1 year in 1 N NaOH
Regeneration	500 mM sodium phosphate, pH 7; 1 M trisodium phosphate, pH 11–12	500 mM sodium phosphate, pH 7; 1 M trisodium phosphate, pH 11–12	500 mM sodium phosphate, pH 7; 1 M trisodium phosphate, pH 11–12
Autoclavability (bulk)	121°C, 20 min in phosphate buffer, pH 7	121°C, 20 min in phosphate buffer, pH 7	121°C, 20 min in phosphate buffer, pH 7
Sanitization	1–2 N NaOH	1–2 N NaOH	1–2 N NaOH
Recommended column storage	0.1 M NaOH + 10 mM sodium phosphate	0.1 M NaOH + 10 mM sodium phosphate	0.1 M NaOH + 10 mM sodium phosphate
Shelf life (dry, unused material)	60 months stored dry, sealed, and at room temperature	60 months stored dry, sealed, and at room temperature	5 years

* Under ideal conditions
** 40 µm particles, 300 cm/hr, 5 mM sodium phosphate, 25 mM NaCl, pH 6.5.

2.3 Purity

In the manufacture of ceramic hydroxyapatite and ceramic hydroxyfluoroapatite, the use of high-purity raw materials results in low levels of contaminants as determined by inductive coupled plasma (ICP) mass spectrometry for metal analysis and ion chromatography for anions.

Table 2.2. Purity.

Impurity	Levels
Chloride	<0.005%
Sulfate	<0.01%
Carbonate	<0.01%
Lead	<0.001%
Cadmium	<0.0001%
Barium	<0.001%
Arsenic	<0.001%


POWER ON
MONO 110VAC
Column
TOP OF COLUMN
RESET
Back
XV26
XV27
XV24
XV25
XV23
XV33
XV34
XV37
XV47
PT29
0.62 Bars
AS45
Duration: 00:00:00:00
INLETS1 : XV61 OPENED

CHAPTER 3
Chromatography

3.1 CHT™ Ceramic Hydroxyapatite Protein Binding Mechanism

Hydroxyapatite contains two types of binding sites, positively charged calcium and negatively charged phosphate groups. These sites are distributed regularly throughout the crystal structure of the matrix. Solute species dominantly interact through cation exchange via the phosphate groups and/or metal affinity via the calcium atoms.

Cation exchange occurs when protein amino groups interact ionically with the negatively charged phosphates. The amino groups are similarly repelled by the calcium sites. Binding depends upon the combined effects of these interactions. These ion exchange interactions can be disrupted by adding neutral salts such as sodium chloride or buffering species such as phosphate to the mobile phase. Cation exchange interactions also weaken with increasing pH. Hence, the addition of salt or phosphate, or an increase in pH, can be used to weaken the interaction. Studies with model proteins have demonstrated that anion exchange, which might be expected from interactions of negatively charged surface residues with calcium, does not make a significant contribution.

Calcium affinity occurs via interactions with carboxyl clusters and/or phosphoryl groups on proteins or other molecules (for example, nucleic acids); these groups are simultaneously repelled by the negative charge of the CHT phosphate groups. The affinity interaction is between 15 and 60 times stronger than ionic interactions alone and, like classical metal-affinity interactions, is not affected by increasing ionic strength using typical elution ions (for example, chloride). Species binding through calcium affinity may adsorb more strongly as the ionic strength increases due to ionic shielding of the charge repulsion from the CHT phosphate sites. Metal affinity interactions can be dissociated by phosphate in the mobile phase.

Figure 3.1 illustrates cation exchange metal affinity to carboxyl and to phosphoryl groups.

Dominantly acidic proteins, such as albumin, bind chiefly by metal-affinity interactions. Sodium chloride at 1.0 M reduces retention time by approximately 10% in the presence of phosphate gradients, indicating a minor contribution by cation exchange. To elute acidic proteins, phosphate buffers are required.

Dominantly basic proteins such as IgG bind chiefly by cation exchange interactions. Sodium chloride reduces retention time in the presence of phosphate gradients, indicating a minor contribution by metal affinity. Basic proteins may be selectively eluted with either phosphate or salts.

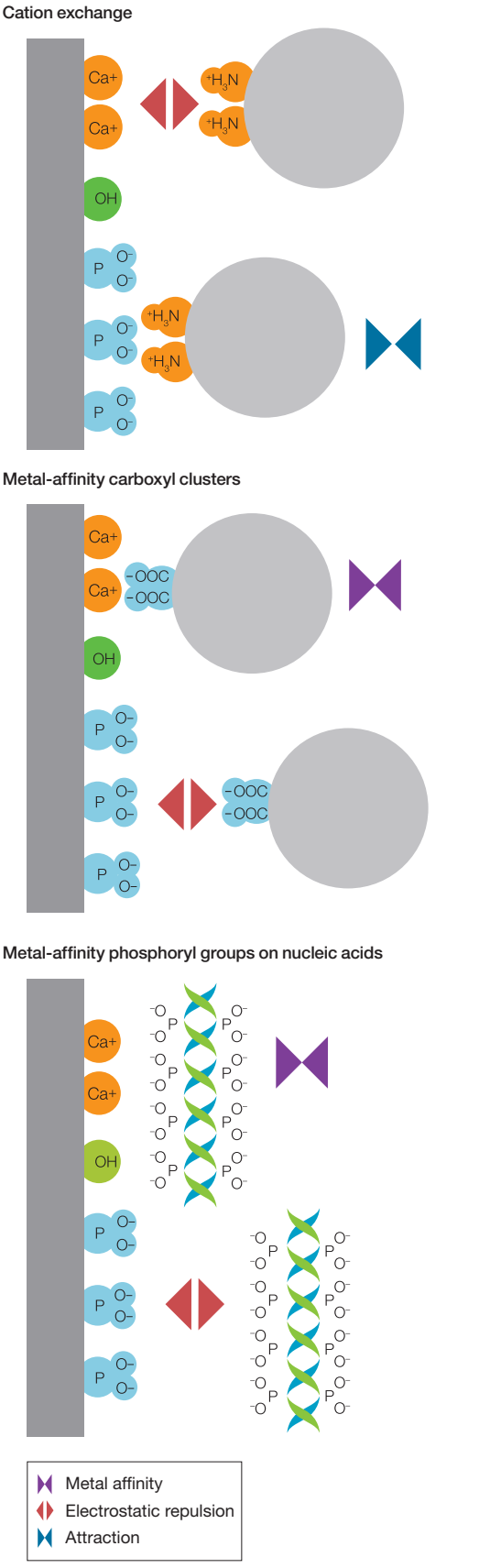


Fig. 3.1. Schematic representation of CHT binding mechanisms.

3.2 CHT Ceramic Hydroxyapatite Chemistry and Considerations for Process Development

3.2.1 CHT Chemistry: Role of the Common Ion Effect

$Ca_{10}(PO_4)_6(OH)_2 \rightarrow 10\ Ca^{2+} + 6\ PO_4^{3-} + 2\ OH^-$

The commonly accepted value of K_{sp} for hydroxyapatite is 10^{-58}. Although the equilibrium constant is very low, it cannot be ignored that if hydroxyapatite is in an aqueous environment, it will form a pH-dependent equilibrium with calcium and phosphate in solution.

This equation implies the following for CHT use:

1. Keep the pH above 6.5 — that is, keep the hydroxyl content as high as possible — to stabilize CHT by keeping it in its insoluble, native state.
2. Adding calcium and phosphate to the various process solutions has a significant positive effect on CHT stability.

Strategies for proper implementation of these points are discussed in more detail below.

3.2.2 CHT Surface Chemistry and the Influence of Buffer Cation Concentration Changes

The surface active structure of CHT is shown in Figure 3.2.

The phosphate groups on the solvent-exposed faces are protonated to varying degrees depending upon the solvent pH. Typically a significant part of the phosphoryl oxygen atoms are protonated. If the concentration of cations in solution (here shown as sodium) increases, the sodium displaces some of the protons into solution. This can cause a pH drop, as shown in Figure 3.3. This phenomenon, as indicated in Figure 3.2, is freely reversible: a decrease in solvent cation concentration causes CHT to readsorb protons, leading to an increase in solvent pH. The phenomenon of proton adsorption/desorption has been thoroughly studied and modeled (Bankston et al. 2010, Pabst et al. 2008). During process development, therefore, it is critical to monitor the pH of the column effluent and address any issues that lead to a pH value of less than 6.5, since prolonged exposure to acidic conditions below this point can have a deleterious effect on the usability of CHT.

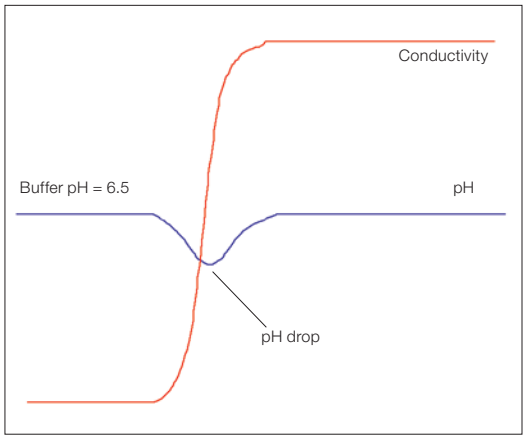


Fig. 3.3. pH drop observed during step increases in solvent cation concentration.

This pH drop, in turn, can decrease the number of cycles that one can obtain from CHT. In the following sections, several methods that significantly alleviate or eliminate this pH drop will be presented.

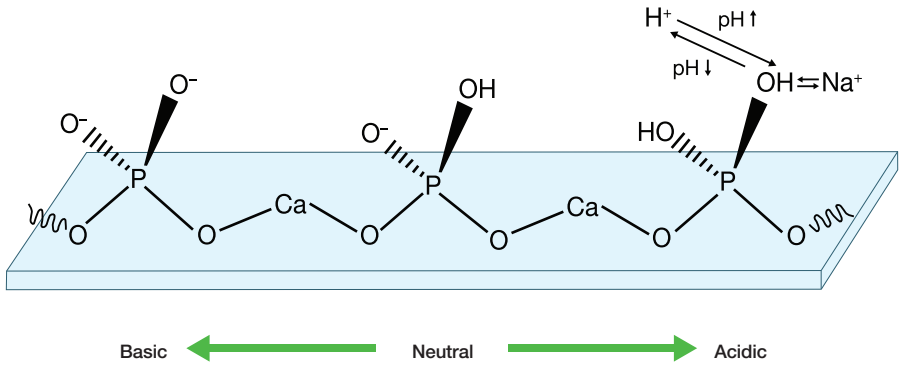


Fig. 3.2. Protonation/deprotonation reactions at the surface of CHT.

3.3 Method Development Guidelines

Process optimization requires the management of multiple variables including matrix interaction, elution characteristics, scale-up from bench, regulation and drug safety requirements, and process robustness and economics. The following four protocols have been developed as general starting guidelines for the purification of most proteins and nucleic acids (Figure 3.4) and may help to reduce time spent in methodology development.* Step-by-step descriptions for each protocol are offered as guidelines in Sections 3.3.10–3.3.14. Detailed explanations of the key points are described in this chapter.

* Optimal experimental conditions vary. These protocols serve only as a screening tool for biomolecule purification.

3.3.1 Chemical Compatibility/Load Preparation

Loads should be free of agents such as citrate or EDTA that could degrade CHT ceramic hydroxyapatite via chelation. CHT is chemically compatible with the following solutions at pH 6.5–14 in the presence of calcium and phosphate.

- 2 M NaOH*
- 6 M guanidine-HCl
- 8 M urea
- 100% acetonitrile
- 100% ethanol/methanol
- 1% SDS and other surfactants (not calcium compatible)
- 4 M NaCl
- 1 M potassium phosphate**
- 0.5 M sodium phosphate**

* No Ca or PO_4 required.
** No Ca required.

3.3.2 Elution

During the course of operation, any change (particularly a step), in the buffer conditions (increase or decrease in salt, buffering species, or other components) can lead to a transient change in the pH of the mobile phase. This phenomenon can be attributed to the interaction of the mobile phase ions with the phosphate surface groups of CHT. The extent of this pH shift depends on the degree to which components are increased or decreased.

3.3.3 Use of Co-Buffers During Elution

The addition of a co-buffer such as MES (2-(N-morpholino)-ethane sulfonic acid), MOPS (3-(N-morpholino)-propane sulfonic acid), or histidine, to the elution buffer significantly decreases the exposure of CHT to released protons. This phenomenon is illustrated in Figure 3.5.

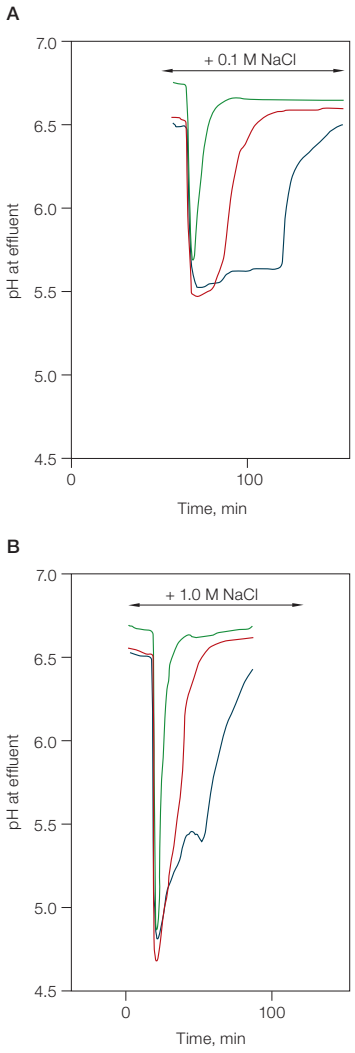


Fig. 3.5. Effect of MES addition on effluent pH during a step elution of CHT with 0.1 M (A) or 1.0 M NaCl (B). The column had previously been equilibrated with 10 mM phosphate, pH 6.8. —, no MES; —, +15 mM MES; —, +100 mM MES.

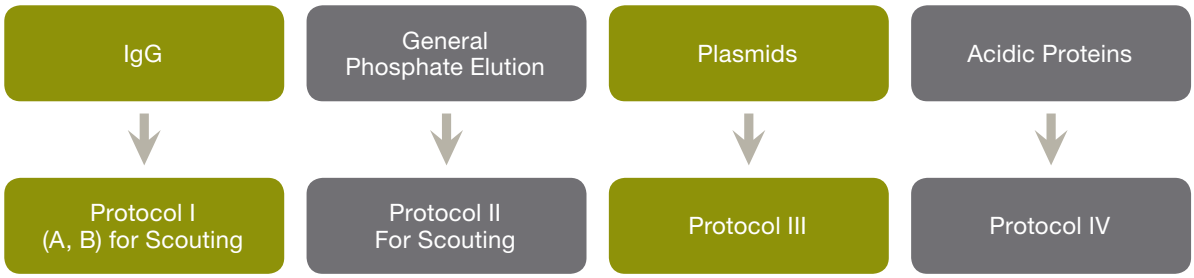


Fig. 3.4. General protocol selection.

Although the magnitude of the pH drop is not significantly lessened, the total time of exposure to low pH is dramatically decreased. This has been shown to significantly enhance the number of CHT cycles. In at least one case, the co-buffer was present during only the first column volume (CV) of elution, when most of the protons are released. The amount of co-buffer needed will vary depending upon a variety of circumstances; Table 3.1 gives some general guidelines for starting amounts to be tested.

Note: With many co-buffers, the acid form is added first and the solution is titrated to the final pH with NaOH. In this case, if the salt concentration for elution has already been predetermined prior to co-buffer studies, the amount of added NaCl to the final eluant buffer composition may need to be reduced because the co-buffer neutralization process itself adds sodium.

Table 3.1. Suggested starting levels of co-buffer as a function of step changes in cation concentration.

Increase in cation concentration, mM	Suggested amount of co-buffer to add, mM MES
Up to 300	30
500	70
1,000	100

3.3.4 Use of Surface Neutralization System During Elution

Ideally, if all of the protons on the surface of CHT could be substituted with a neutral cation immediately prior to elution, without affecting column performance, the increase in cation concentration during elution would have no effect on pH. Bio-Rad has taken this idea and developed a patent-pending technology called surface neutralization solution, or SNS. In brief, this involves inserting a slightly alkaline wash step immediately prior to elution. This phenomenon is illustrated in Figure 3.6. A typical protocol would involve 6–8 CV of 25 mM Tris, 25 mM NaCl, 5 mM phosphate, pH 7.75.

The Tris acts to keep the overall solution slightly alkaline, providing an essentially infinite reservoir of hydroxyl groups, which quickly form water with the released protons, and has been shown to dramatically improve lifetime at the 20 × 20 cm scale. A variety of other buffering species (arginine, lysine, histidine, PIPES, HEPES, ACES MOPS, MOPSO) have been shown to be extremely effective at the 3.2 cm diameter column scale. Studies on monoclonal antibodies have indicated that use of SNS compared to traditional methods produces an eluant with the same quality outputs (yield, peak elution point in a gradient, aggregate level, DNA, host cell protein). In addition, use of SNS for >40 cycles does not alter the binding capacity of CHT for individually tested antibodies. In some cases, acidic proteins may elute during the SNS step. In this case the eluant should still be checked as the quality attributes may warrant the use of SNS directly as an elution buffer.

3.3.5 Calcium Addition to Increase CHT Column Life

As implied in Section 3.2.1, the addition of calcium also enhances CHT column life. The amount of calcium added is determined by measuring the calcium in the effluent pool from a particular step (equilibration, load, elution). This can be done in a single mock (protein-free) run in a small-scale column, or run several times to obtain a statistically valid number. The test itself takes approximately 10 min per sample and is directly derived from a standard colorimetric titration water hardness analysis method (see the Appendices section A.2 for the exact protocol). A 96-well test format is also available; contact Bio-Rad for details.

Because calcium and phosphate will precipitate in solution, ensure that the amounts of each are below the solubility product for the given buffer. It should be noted that calcium ion has significant stabilizing abilities and should be measured and added to all buffers exhibiting calcium leaching as needed. Note that typical regeneration and sanitization solutions do not require added calcium.

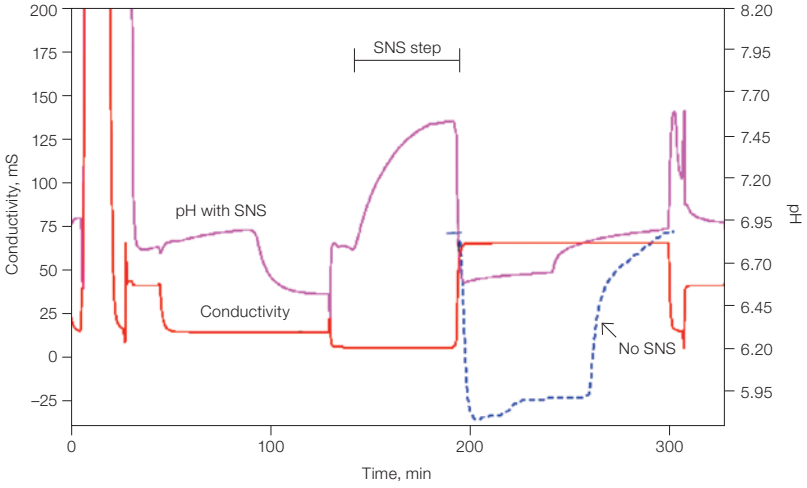


Fig. 3.6. Effect of SNS on effluent pH during step elution with 1.0 M NaCl. The column had previously been equilibrated with 10 mM phosphate, pH 6.8. —, conductivity; - - -, mS; —, pH.

3.3.6 Phosphate

As implied in Section 3.2.1, the addition of phosphate improves CHT robustness by favorably affecting the solubility equation. Generally, 5 mM phosphate should be included in all buffer solutions. When operating at pH >7.0, lower amounts of phosphate, down to 2 mM, may suffice. Phosphate concentrations above 5 mM in these buffers may decrease protein binding. As illustrated in Figure 3.7, CHT binding capacity decreases in 50 mM MES with increasing phosphate concentration.

3.3.7 Trace Metal Contamination

CHT will also bind to trace metals, such as iron, that may be present in buffer solutions. The metal contaminants may originate from production media, buffers and salts, process water, and/or corroded stainless steel. The degree of trace-metal deposition will manifest itself as a visible discoloration at the top of the column over time. If this becomes an issue, either pretreat buffers by incubating with a small amount of CHT prior to filtration, or install a CHT guard column for use during buffer and/or load application. An additional method involves adding low levels of calcium-saturated EDTA. Contact your Bio-Rad representative for more details.

3.3.8 Preparing Phosphate Buffers

Hydrated phosphate salts should be used in all buffer preparations. Nonhydrated phosphates should not be used because the manufacturing process for these salts leads to pyrophosphate formation. Pyrophosphates inhibit the binding of some macromolecules and reduce CHT selectivity. Avoid back-titrating buffer pH as this increases conductivity and may reduce target protein binding on CHT. Avoid dodecahydrates as these spontaneously decompose to heptahydrates.

If calcium is used in excess, it can bind ionically to the surface of phosphate sites and later form precipitates with phosphate regeneration buffer.

3.3.9 Other Suggested Washes

If the calcium level is greater than or equal to 10 ppm at the end of elution, wash with three CV of a low phosphate buffer at pH 7.5–8.0 prior to the high phosphate strip. It is also recommended to include a 0.5 CV wash between sequential steps involving high phosphate and high NaOH solutions.

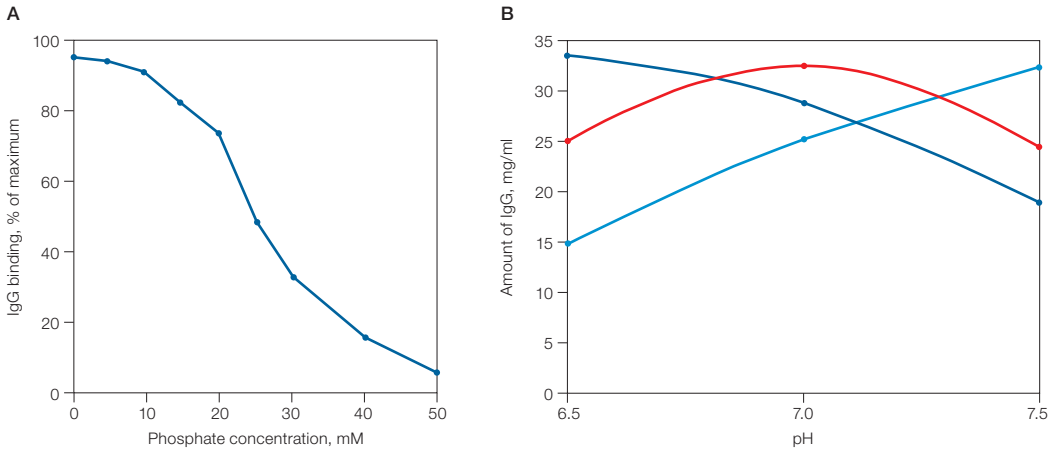


Fig. 3.7. A, IgG binding capacity of CHT vs. phosphate concentration and pH. **B**, IgG binding capacity of CHT vs. pH. —, Human IgG; —, Chimeric IgG; —, Murine IgG.

3.3.10 Scouting Protocol IA: IgG

Flow rate of 300 cm/hr for all steps

Step	Mobile Phase	Column Volumes	Comments
Wash	H_2O	0.5	Reduce the concentration of 1.0 N NaOH from the sanitization step to prevent formation of trisodium phosphate precipitates
Pre-equilibrate	500 mM $NaPO_4$, pH 6.5	3	Rapidly shifts the pH within the column
Equilibrate	10 mM $NaPO_4$, 4–8 ppm Ca^{++}, pH 6.5	10	Ca^{++} supplementation improves the chemical stability of hydroxyapatite (Section 3.3.5)
Load	Clarified sample 10 mM $NaPO_4$, 4–8 ppm Ca^{++}, pH 6.5		Maintains chemical stability of the hydroxyapatite
Wash	10 mM $NaPO_4$, 4–8 ppm Ca^{++}, pH 6.5	5	Maintains chemical stability of the hydroxyapatite
SNS	25 mM Tris 25 mM NaCl, 5 mM $NaPO_4$, pH 7.75	6	Controls localized pH while absorbing protons released from hydroxyapatite surface protonation sites by an ion exchange neutralization reaction (Section 3.3.4)
Elute	10 mM $NaPO_4$, linear gradient 0–2 M NaCl, pH 6.5 + 15 ppm Ca^{++}	20	If elution does not occur, increase phosphate concentration
Wash	10 mM $NaPO_4$, 100 mM NaCl, pH 7.5–8.0	1	Increases pH prior to high cation step to mitigate pH drop.
Clean	500 mM $NaPO_4$, pH 7.0	5	High phosphate elutes retained contaminants such as aggregates, endotoxin, DNA, and protein A
Wash	H_2O	0.5	Reduces the phosphate concenration to prevent formation of trisodium phosphate precipitates
Sanitize	1.0 N NaOH	5	Sanitization

3.3.11 Scouting Protocol IB: IgG

Flow rate of 300 cm/hr for all steps

Step	Mobile Phase	Column Volumes	Comments
Wash	H_2O	0.5	Reduces the concentration of 1.0 N NaOH from the sanitization step to prevent formation of trisodium phosphate precipitates
Pre-equilibrate	500 mM $NaPO_4$, pH 6.5	3	Rapidly shifts the pH within the column
Equilibrate	10 mM $NaPO_4$, 8–12 ppm Ca^{++}, 100 mM NaCl, pH 6.5	10	Ca^{++} supplementation improves the chemical stability of hydroxyapatite (Section 3.3.5). NaCl competes with hydrogen for hydroxyapatite surface protonation sites
Load	Clarified sample 10 mM $NaPO_4$, 8–12 ppm Ca^{++}, 100 mM NaCl, pH 6.5		Maintains chemical stability of the hydroxyapatite
Wash	10 mM $NaPO_4$, 8–12 ppm Ca^{++}, 100 mM NaCl, pH 6.5	5	Maintains chemical stability of the hydroxyapatite
Elute	10 mM $NaPO_4$, 50–120 ppm Ca^{++}, linear gradient 0.1–2 M NaCl, pH 6.5 + co-buffer	20	Co-buffer adsorbs the majority of released protons. Ca^{++} supplementation improves the chemical stability of hydroxyapatite (Section 3.3.5) by the common ion effect
Wash	10 mM $NaPO_4$, 100 mM NaCl, pH 7.5–8.0	1	Increases pH prior to high cation step to mitigate pH drop.
Clean	500 mM $NaPO_4$, pH 7.0	5	High phosphate elutes retained contaminants such as aggregates, endotoxin, DNA, and protein A
Wash	H_2O	0.5	Reduces the phosphate concentration to prevent formation of trisodium phosphate precipitates
Sanitize	1.0 N NaOH	5	Sanitization

3.3.12 Scouting Protocol II: General Phosphate Elution

Flow rate of 300 cm/hr for all steps

Step	Mobile Phase	Column Volumes	Comments
Wash	H_2O	0.5	Reduces the concentration of 1.0 N NaOH from the sanitization step to prevent formation of trisodium phosphate precipitates
Pre-equilibrate	500 mM $NaPO_4$, pH 6.8	3	Rapidly shifts the pH within the column
Equilibrate	5 mM $NaPO_4$, 20 ppm Ca^{++}, 150 mM NaCl, pH 6.8	10	Ca^{++} supplementation improves the chemical stability of hydroxyapatite (Section 3.3.5). NaCl competes with hydrogen for hydroxyapatite surface protonation sites
Load	Clarified sample 5 mM $NaPO_4$, 20 ppm Ca^{++}, 150 mM NaCl, pH 6.8		Maintains chemical stability of the hydroxyapatite
Wash	5 mM $NaPO_4$, 20 ppm Ca^{++}, 150 mM NaCl, pH 6.8	5	Maintains chemical stability of the hydroxyapatite
Elute	Linear gradient 5 mM $NaPO_4$ + 20 ppm Ca^{++} - 500 mM $NaPO_4$ +12 ppm Ca^{++}, 150 mM NaCl, pH 6.8		
Clean	500 mM $NaPO_4$, pH 6.8	5	High phosphate elutes retained contaminants such as aggregates, endotoxin, or DNA
Wash	H_2O	0.5	Reduces phosphate levels to prevent formation of trisodium phosphate precipitates
Sanitize	1.0 N NaOH	5	Sanitization

3.3.13 Protocol III: Plasmids*

Flow rate of 300 cm/hr for all steps

Step	Mobile Phase	Column Volumes	Comments
Sanitize	1.0 N NaOH	5	Sanitization
Wash	H_2O	0.5	Reduces the concentration of 1.0 N NaOH from the sanitization step to prevent formation of trisodium phosphate precipitates
Pre-equilibrate	500 mM $NaPO_4$, pH 7.0	3	Rapidly shifts the pH within the column
Equilibrate	10 mM $NaPO_4$, 1 mM EDTA, pH 7.0	5	Conditions column for binding plasmid
Load	Clarified sample in load buffer		Load buffer consists of nonacetate alkaline lysate constituents
Wash	10 mM $NaPO_4$, 1 mM EDTA, pH 7.0	5	Removes unbound components
Elute	Linear gradient 0–400 mM $NaPO_4$, 1 mM EDTA, pH 7.0	10	Elutes purified plasmid
End	In general, plasmid purification is a single use process; multiple cycles require methods development		

* Presence of EDTA will shorten lifetime. See Section 3.3.1 and Section 3.3.9 for additional information.

3.3.14 Protocol IV: Acidic Proteins

Flow rate 300 cm/hr for all steps

Step	Mobile Phase	Column Volumes	Comments
Wash	H_2O	0.5	Reduces the concentration of 1.0 N NaOH from the sanitization step to prevent formation of trisodium phosphate precipitates
Pre-equilibrate	500 mM $NaPO_4$, pH 6.7	3	Rapidly shifts the pH within the column
Equilibrate	5 mM $NaPO_4$, 50–100 mM NaCl, 12–20 ppm Ca^{++}, pH 6.7	10	Ca^{++} supplementation improves the chemical stability of hydroxyapatite (Section 3.3.5). NaCl competes with hydrogen for hydroxyapatite surface protonation sites
Load	Clarified sample 5 mM $NaPO_4$, 50–100 mM NaCl, 12–20 ppm Ca^{++}, pH 6.7	2–8	Maintains chemical stability of the hydroxyapatite
Wash	5 mM $NaPO_4$, 50–100 mM NaCl, 12–20 ppm Ca^{++}, pH 6.7	5	Maintains chemical stability of the hydroxyapatite
Elute	Linear gradient from 5 mM $NaPO_4$, 50–100 mM NaCl, 12–20 ppm Ca^{++}, pH 6.7 to 120 mM $NaPO_4$, 50–100 mM NaCl, pH 6.7	20	
Clean	500 mM $NaPO_4$, pH 6.7	5	High phosphate elutes retained contaminants such as aggregates, endotoxin, or DNA
Wash	H_2O	0.5	Reduces phosphate levels to prevent formation of trisodium phosphate precipitates
Sanitize	1.0 N NaOH	5	Sanitization

3.3.15 Scouting Tips

The target protein will usually elute within the phosphate gradient. The slope and amplitude can be adjusted based on initial results. The flow rate may also be converted to a step format or run in flow-through mode. In general, retention time of proteins increases with increasing pI.

3.3.16 Optimization Tips for Protocols I-IV

1. Select the optimum buffering agent making sure to add phosphate and calcium to stabilize the CHT matrix.
2. The ionic strength in samples containing a high concentration of salt should be reduced to be equivalent to the starting buffer. Dilution, diafiltration, or buffer exchange using Bio-Gel® P-6DG gel may also be used.
3. As with any chromatographic step, buffer solutions and samples should be filtered through a 0.20–0.45 µm filter before use.
4. If 500 mM sodium phosphate is not sufficient for protein elution (this is rare), try higher concentrations of potassium phosphate.
5. If the elution peak is not sharp enough, try 10 CV linear gradient elution.
6. Where appropriate, convert linear gradient elution to step elution. Use the information from the gradient to devise an intermediate wash step if desired for increased purity.
7. Determine the pH that gives the highest binding capacity at a phosphate concentration of 5 mM.

Table 3.2. Protocols in table format for convenient review.

	Scouting Protocol IA IgG	Scouting Protocol IB IgG	Scouting Protocol II General Phosphate Elution	Protocol III Plasmids	Protocol IV Acidic Proteins
Flow rate	300 cm/hr for all steps	300 cm/hr for all steps	300 cm/hr for all steps	300 cm/hr for all steps	300 cm/hr for all steps
Sanitize				1.0 N NaOH	
Wash	H_2O	H_2O	H_2O	H_2O	H_2O
Pre-equilibrate	500 mM $NaPO_4$, pH 6.5	500 mM $NaPO_4$, pH 6.5	500 mM $NaPO_4$, pH 6.8	500 mM $NaPO_4$, pH 7.0	500 mM $NaPO_4$, pH 6.7
Equilibrate	10 mM $NaPO_4$, 4–8 ppm Ca^{++}, pH 6.5	10 mM $NaPO_4$, 8–12 ppm Ca^{++}, 100 mM NaCl, pH 6.5	5 mM $NaPO_4$, 20 ppm Ca^{++}, 150 mM NaCl, pH 6.8	10 mM $NaPO_4$, 1 mM EDTA, pH 7.0	5 mM $NaPO_4$, 12–20 ppm Ca^{++}, 50–100 mM NaCl, pH 6.7
Load	Clarified sample 10 mM $NaPO_4$, 4–8 ppm Ca^{++}, pH 6.5	Clarified sample 10 mM $NaPO_4$, 8–12 ppm Ca^{++}, 100 mM NaCl, pH 6.5	Clarified sample 5 mM $NaPO_4$, 20 ppm Ca^{++}, 150 mM NaCl, pH 6.8	Clarified sample in load buffer	Clarified sample 5 mM $NaPO_4$, 12–20 ppm Ca^{++}, 50–100 mM NaCl, pH 6.7
Wash	10 mM $NaPO_4$, 4–8 ppm Ca^{++}, pH 6.5	10 mM $NaPO_4$, 8–12 ppm Ca^{++}, 100 mM NaCl, pH 6.5	5 mM $NaPO_4$, 20 ppm Ca^{++}, 150 mM NaCl, pH 6.8	10 mM $NaPO_4$, 1 mM EDTA, pH 7.0	5 mM $NaPO_4$, 50–100 mM NaCl, 12–20 ppm Ca^{++}, pH 6.7
SNS	25 mM Tris, 25 mM NaCl, 5 mM $NaPO_4$, pH 7.75				
Elute	10 mM $NaPO_4$, linear gradient 0–2 M NaCl, pH 6.5 + 15 ppm Ca^{++}	10 mM $NaPO_4$, 50–120 ppm Ca^{++}, linear gradient 0.1–2 M NaCl, pH 6.5 + co-buffer	Linear gradient 5 mM $NaPO_4$ + 20 ppm Ca^{++} - 500 mM +12 ppm Ca^{++}, 150 mM NaCl, pH 6.8	Linear gradient 0–400 mM $NaPO_4$, 1 mM EDTA, pH 7.0	Linear gradient from 5 mM $NaPO_4$, 50–100 mM NaCl, 12–20 ppm Ca^{++} to 120 mM $NaPO_4$, 50–100 mM NaCl, pH 6.7
Wash	10 mM $NaPO_4$, 100 mM NaCl, pH 7.5–8.0	10 mM $NaPO_4$, 100 mM NaCl, pH 7.5–8.0			
Clean	500 mM $NaPO_4$, pH 7.0	500 mM $NaPO_4$, pH 6.8	500 mM $NaPO_4$, pH 7.0		500 mM $NaPO_4$, pH 6.7
Wash	H_2O	H_2O	H_2O		H_2O
Sanitize	1.0 N NaOH	1.0 N NaOH	1.0 N NaOH		1.0 N NaOH

CHAPTER 4
Regeneration, Sanitization, and Storage

4.1 Regeneration

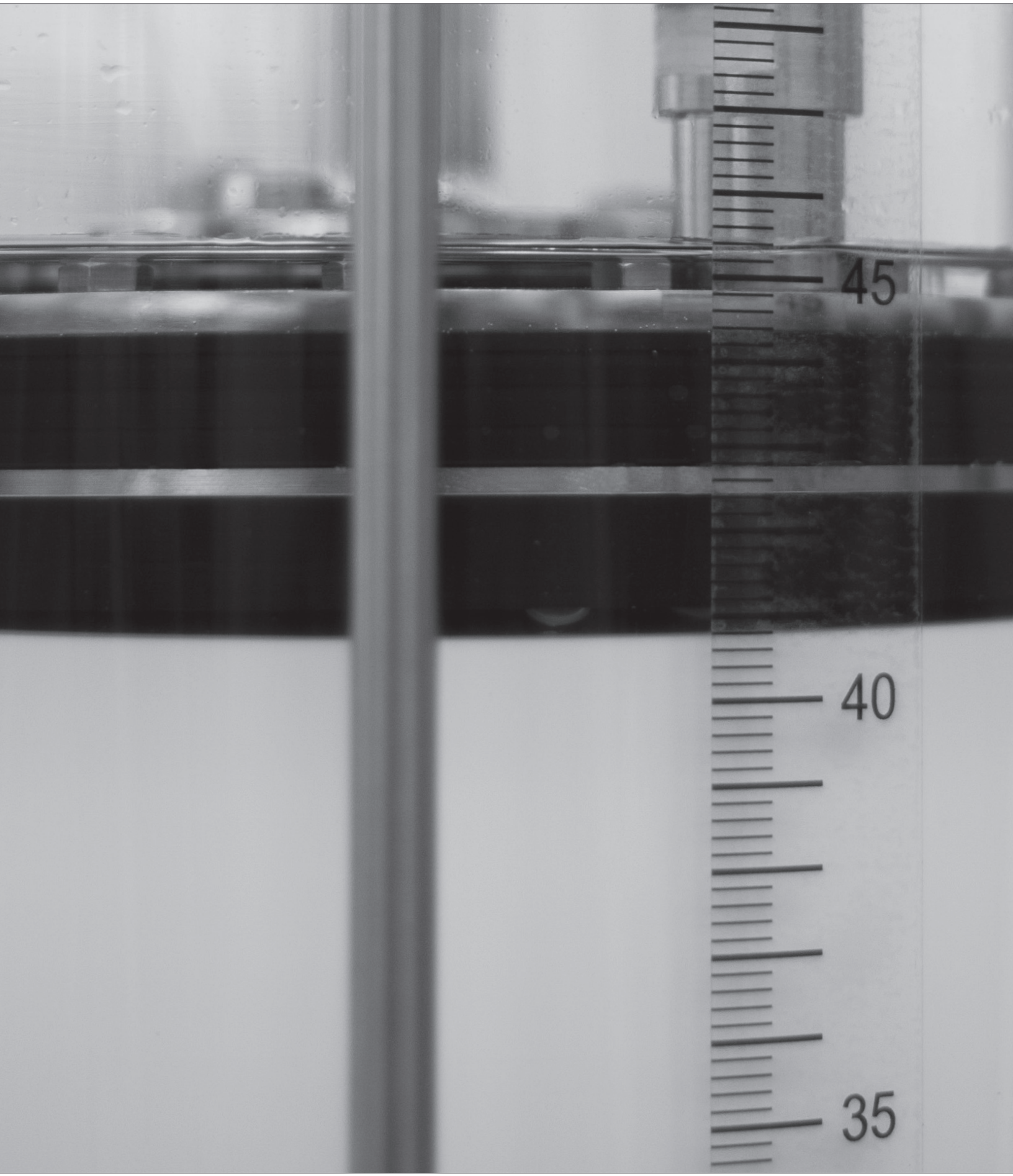
CHT™ ceramic hydroxyapatite columns should be regenerated at the completion of each run with 3–5 CV of 500 mM potassium or sodium phosphate buffer at neutral pH, or 400 mM trisodium phosphate, pH 11–12. The column can also be stripped with other cleaning solutions (1–2 M KCl or NaCl, 8 M urea, or 6 M guanidine-HCl) containing 5 mM phosphate at neutral pH.

4.2 Sanitization

The column can be sanitized in up to 2 N NaOH and stored in 0.1–1.0 N NaOH if desired.

4.3 Storage

Unused CHT ceramic hydroxyapatite should be stored in the original container at room temperature. Once wetted, CHT may be stored at room temperature in 0.1 N NaOH. If concentrations of NaOH lower than 100 mM are used, include 10 mM phosphate in the solution. Higher concentrations of NaOH may be used if desired. Used CHT, after being regenerated and sanitized, can be stored in solution up to 1.0 N NaOH at room temperature and away from direct light.


45
40
35

CHAPTER 5
Column Packing Protocols

This section offers guidelines for packing process scale columns. Please carefully read over and follow the protocols for packing your specific column. Should you have additional questions, contact your local process chromatography sales representative or the chromatography technical support department for further assistance (1-510-741-6563).

Well-packed columns, in which the beds are homogeneous and continuous from top to bottom, exhibit the best chromatographic separations. It is therefore very important to pack your columns according to the suggested guidelines. The following sections discuss recommended packing solutions, packed column qualification, and column conditioning for process scale applications.

CHT™ ceramic hydroxyapatite is rigid and typically exhibits low backpressure at high flow rates relative to its average particle size; refer to Figure 5.1 for 40 µm CHT and Figure 5.2 for 80 µm CHT.

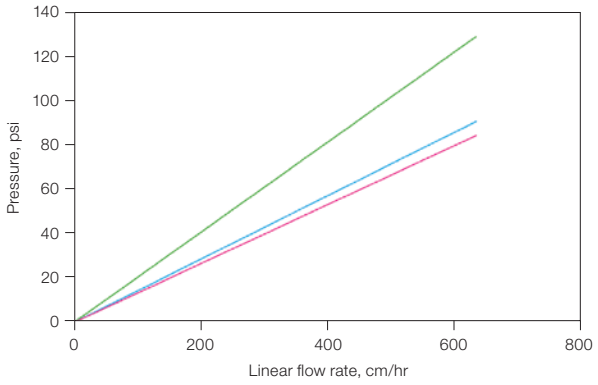


Fig. 5.1. Estimated pressure for 40 µm CHT packed to 20.0 cm bed height vs. 1.0 M NaOH (1.80 cps), 0.5 M sodium phosphate buffer, pH 6.8 (1.27 cps), and phosphate buffered saline (1.18 cps). —, 1.18 cps; —, 1.27 cps; —, 1.80 cps.

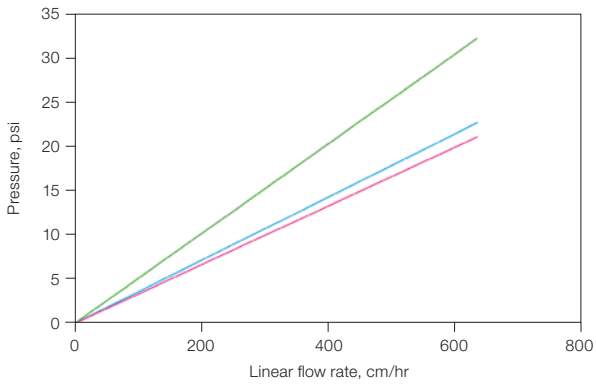


Fig. 5.2. Estimated pressure for 80 µm CHT packed to 20.0 cm bed height vs. 1.0 M NaOH (1.80 cps), 0.5 M sodium phosphate buffer, pH 6.8 (1.27 cps), and phosphate buffered saline (1.18 cps). —, 1.18 cps; —, 1.27 cps; —, 1.80 cps.

5.1 General Guidelines

1. CHT ceramic hydroxyapatite is composed of incompressible, nonspherical particles. As such it is almost impossible to achieve perfect cubic packing during initial bed preparation. If the column is exposed to external vibration (for example, if the column is rolled across the floor from the packing location to the processing location), the material in the column will continue to settle. Additionally, the material in the column may continue to settle slightly over many cycles. The results of this phenomenon are twofold:

- An apparent loss in bed height (appearance of headspace) due to further bed compaction
- Increase in operating pressure as the more well-packed bed becomes increasingly resistant to flow

2. Do not compress the packed bed by lowering the top adaptor into the bed; always leave a small gap of 0.1–1.0 cm. The gap ensures that the surface of the packed bed is undisturbed by contact with the uneven geometry characteristic of most inlet adaptors. In acrylic and glass columns, the gap may be visually observed. In stainless steel columns with axial compression, it is advisable to reduce the speed of the piston when it is 10% higher than the target bed height. If sudden resistance is noted as the adaptor approaches the target height, stop the descent immediately. Close the outlet valve. Open the inlet valve, then pump packing buffer into the inlet valve at 100 cm/hr while raising the adaptor at 50 cm/hr. Stop when the adaptor has traveled 0.5 cm.

3. Flow and perform all chromatography steps only in downflow mode, not in upflow mode.

The following packing methods can be used for packing CHT ceramic hydroxyapatite:

- Axial packing of open columns with motorized adjustable inlet adaptors
- Flow packing of open columns with adjustable adaptors
- Axial packing of closed columns with motorized adjustable inlet adaptors
- Pressure packing of closed columns (using media packing stations)

Best Practices

CHT can be packed into all types of axial flow process-scale columns. Always read the relevant column and associated media transfer skid or media packing skid instruction manuals carefully. Where appropriate, make the recommended changes according to these guidelines.

5.2 General Handling and Slurry Preparation

CHT ceramic hydroxyapatite is supplied as a dry powder. Using personal protective equipment including a dust mask, gloves, safety glasses, and a laboratory coat is advisable while transferring the powder. The 5 kg containers of CHT have a plastic seal covering the container and screw closure. The seal ensures that the container has not been opened after it was filled. The screw closure is a secondary closure that secures a powder seal onto the container's opening.

Clean the container surface if it has accumulated dust. Wipe it with a clean damp cloth and dry it with a clean dry cloth. Invert the container several times to loosen the CHT into a dry free-flowing powder. Perform this step just prior to dispensing the powder.

No defining or decanting steps are required or recommended for new CHT media.

5.2.1 Recommended Column Packing Buffers

Use a packing solution that is at least 150 mM in ionic strength and pH 6.8 or greater. The following packing solutions have been used to successfully pack efficient columns of 40 µm and 80 µm CHT.

- 20 mM phosphate buffer, 150 mM sodium chloride, pH 7.2–8.0 (phosphate-buffered saline)
- Sodium or potassium phosphate buffer, 200–400 mM, pH 6.8–10
- 0.15–1 N NaOH

Best Practices

Avoid using packing solutions that are less than 150 mM in ionic strength for the first contact of the powder with liquid. Lower concentrations may result in turbid supernatants in freshly prepared slurries of CHT. The turbidity results from the first contact of the powder with the solution but is minimized or eliminated when using solutions with an ionic strength greater than 150 mM and a pH of 6.8 or above.

5.2.2 Recommended Slurry Mixing

Manual Mixing

When mixing manually either in a container or in an open column, it is recommended to use a one-piece polypropylene paddle in a "J-stroke" pattern or a back-and-forth motion. Mixing in a standard circular motion may result in uneven settling.

Gas-Assisted Mixing

In columns with supported stainless steel frits, gas-assisted mixing may be used. Gas-assisted methods use clean compressed air, argon, helium, or nitrogen gas at a low pressure to uniformly agitate the mixture of CHT and packing buffer within the column. The gas is applied through the bottom frit by connecting the air supply to the process outlet. This method can be used with slurries up to 50% (v/v). Do not use gas-assisted mixing in columns with porous polyethylene or polypropylene bed supports or unsupported nylon or stainless-steel screens. If you are unsure of your column type, contact the column manufacturer.

Slurry Tanks

For mixing in a slurry tank, two methods are recommended. The first is using a low-shear hydrofoil impeller set at a speed just fast enough to keep the particles in suspension without causing a vortex. The second method is gas-assisted slurry mixing, where the air is connected to the slurry tank outlet. Do not use a sparging ring that is suspended above the bottom of the tank; the sparging will not be sufficient to keep all of the particles in suspension.

Always begin by adding the appropriate volume of buffer to the slurry tank, followed by the powdered CHT.

Note: Excessive mixing or use of impellers other than low-shear hydrofoil impellers may fracture the particles. The fine particles resulting from excessive mixing or use of improper impellers may contribute to increased column backpressure.

Do not recirculate the slurry through a diaphragm or peristaltic pump for mixing. Damage to the chromatography medium due to excessive physical force is possible. Peristaltic and diaphragm pumps fracture the particles and are generally the major cause of particle damage. A single-pass operation of the slurry with these pumps minimizes damage. Never recycle CHT slurries with these pumps or any other pumps as the shear forces and particle-to-particle collisions in piping, bends in piping, and other connections accelerate damage. Whenever possible, avoid pumps for media transfer. Pressurized slurry vessels are preferred.

5.2.3 Recommended Slurry Transfer

Suction or Vacuum Slurry Transfer

Some types of process columns with motorized pistons (for example, the Bio-Rad InPlace™ column) can introduce the slurry without a pump, using the top adaptor in a syringe-style to draw the slurry into the column via suction. The transfer must be performed at a minimum speed of 200 cm/hr for 40 µm CHT and minimum of 300 cm/hr for 80 µm CHT due to the rapid settling rate.

Media Transfer Stations

Media transfer stations can be used to transfer slurries from a mixing tank to an empty (open-style) column. When transferring the CHT slurry to the column using a diaphragm pump, the concentration should be less than 50% v/v. Lower concentrations ensure more efficient packing of columns; however, a further increase in efficiency is negligible with slurries below 15% v/v (9.5% w/v).

Media Packing Stations

Media packing stations can be used to pack thin slurries from a mixing tank into a closed column through a packing nozzle containing multiple small-diameter orifices. When packing CHT using this method, the slurry concentration should be less than 25% v/v (15.8% w/v). Lower concentrations ensure more efficient packing; however, a further increase in efficiency is negligible below 15% v/v (9.5% w/v). Damage to the chromatography medium due to excessive physical force is possible. Peristaltic and diaphragm pumps fracture the particles and are generally the major cause of particle damage. Thin slurries minimize the shear forces and particle-to-particle collisions in piping, bends in piping, other connections, and the packing nozzle. Thin slurries are defined as less than 26% v/v or 16.0% w/v. A single-pass operation of the slurry through these pumps, piping, valves, and nozzle minimizes damage. Never recycle CHT with these pumps or any other pumps as the shear forces and particle-to-particle collisions accelerate damage. Whenever possible, avoid pumps for media transfer. Pressurized slurry vessels are preferred.

5.3 Open-Column Packing

Open columns such as the following are commonly used at the pilot and small manufacturing scale:

- Bio-Rad EasyPack™ columns
- BPG columns (GE Healthcare)
- Quikscale columns (Millipore)

To determine the dry weight of CHT ceramic hydroxyapatite and the volume of packing solutions, see the Appendices, Tables A.1–A.5, which cover tube lengths of 500, 600, 700, and 900 mm where the maximum fill heights are 400, 500, 600, and 800 mm, respectively. The 100 mm difference between tube height and fill height is due to the length required by the inlet adaptor. For example, to pack a 45 cm ID column with a final bed height of 15 cm, 15.03 kg of CHT and 58.6 L of packing solution are required to make a 37.5% v/v slurry.

Packing

1. Level the column; otherwise, the distance between packed bed surface and inlet adaptor surface will not be uniform across the column's diameter.
2. Wet the bottom frit and ensure that no air is trapped in the bottom adaptor. Close the bottom process valve.
3. Measure the amount of packing solution and CHT. Invert the containers of CHT repeatedly to loosen the powder so that it will pour easily into the column.
   a. Dispense the packing solution into the column.
   b. If using gas-assisted slurry mixing, turn the gas line to the recommended pressure so that the packing solution is slightly turbulent.
   c. If using manual mixing, insert the paddle and stir the solution.
   d. Dispense the CHT into the column while continuing to mix.
4. When all of the CHT has been added, rinse the paddle and the walls of the column with a squirt bottle.
5. Insert the flow adaptor into the column with the inlet valve open, lower it to 2 cm above the liquid level, and secure its position.
6. Seal the adaptor's sealing device marginally (O-ring, inflatable bladder, or compression seal). Marginal sealing is the minimum sealing force recommended for the column where liquid will not bypass the seal while the adaptor is lowered.
7. Allow the CHT to settle for 1 min. Lower the inlet adaptor to release the air trapped between the bed support and the packing solution.
8. Fully seal the adaptor's sealing device.
9. Open the outlet valve, then flow-pack the column at 300 cm/hr until the bed is consolidated, or until approximately equivalent to 3 times the slurry volume has passed through the column.
10. Stop the flow. Close the outlet valve, leaving the inlet open.
11. Lower the inlet adaptor, allowing the packing solution to exit through the inlet port.

12. Continue the descent until the adaptor is 10% above the target height. A gap of about 0.1–1.0 cm is acceptable between the packed bed and the inlet adaptor bed support. The gap ensures that the surface of the packed bed is undisturbed by contact with the uneven geometry characteristic of most inlet adaptors.

    In acrylic and glass columns, the gap may be visually observed. In stainless steel columns, it is advisable to reduce the speed of the piston when it is 10% higher than the target bed height. If sudden resistance is noted as the adaptor approaches the target height, stop the descent immediately. Close the outlet valve. Open the inlet valve, then pump packing buffer into the inlet valve at 100 cm/hr while raising the adaptor at 50 cm/hr. Stop when the adaptor has traveled 0.5 cm.

13. Condition with 3–5 CV of equilibration buffer at the selected operating flow rate.

14. Continue with packed column qualification.

5.4 Closed-Column Packing

Larger process-scale chromatography columns are typically closed systems where the slurry must be introduced through slurry valves. The slurry may be transferred into the column in a number of ways, including the following:

- Media transfer (via a pump or pressurized slurry vessel)
- Suction or vacuum transfer
- Media packing station for stall packing (via a pump or pressurized slurry vessel)

5.4.1 Media Transfer Stations

The media transfer method is used for systems designed to inject particle slurries into specially designed columns, such as the following:

- Bio-Rad InPlace columns
- BioProcess LPLC columns (GE Healthcare)

For these columns, it is necessary to calculate the exact amount of CHT ceramic hydroxyapatite needed for your desired bed height; then transfer the entire volume of slurry into the column.

1. Level the column; otherwise, the distance between the packed bed surface and inlet adaptor surface will not be uniform across the column's diameter.
2. Determine the dry weight of CHT and the volume of packing solutions for your column (See the Appendices, Table A.5). Be sure to allow for extra buffer volume to rinse the transfer lines and pumps, if appropriate.
3. Prepare the slurry in a slurry tank as described previously.
4. Leave the inlet adaptor at the uppermost height on the InPlace column or just below the upper slurry transfer port on the BioProcess LPLC.
5. Transfer the CHT slurry to the column through the bottom port(s) at a fast rate to facilitate adequate mixing as it enters the column.
6. Close the filling port or slurry valve.
7. Allow the CHT to settle for 1 min or until a supernatant layer has developed. Lower the inlet adaptor into the supernatant layer and activate the seal.
8. With the outlet closed and the inlet open, lower the top adaptor by 2–4 cm to release air trapped under the top frit.
9. Close the inlet process valve and open the outlet valve.
10. For columns with motorized inlet adaptors, lower the piston to 300 cm/hr on the Bio-Rad InPlace column or down to 700 cm/hr (80 µm CHT) and 500 cm/hr (40 µm CHT) on the LPLC. Do not exceed the maximum recommended pressure for the column.
11. Continue the descent until the adaptor is 10% above the target height of the packed bed. A gap of about 0.1–1.0 cm is acceptable between the packed bed and the inlet adaptor bed support. In acrylic and glass columns, the gap may be visually observed. In stainless steel columns, it is advisable to reduce the speed of the piston when it is 10% higher than the target bed height. If sudden resistance is noted as the adaptor approaches the target height, stop the descent immediately. Close the outlet valve. Open the inlet valve, then pump packing buffer into the inlet valve at 100 cm/hr while raising the adaptor at 50 cm/hr. Stop when the adaptor has traveled 0.5 cm.
12. Condition with 3–5 CV of equilibration buffer at the selected operating flow rate.
13. Continue with packed column qualification.

5.4.2 Suction Transfer Method

The suction transfer method is used for systems designed with motorized inlet adaptors, such as the following:

- Bio-Rad InPlace columns
- AxiChrom columns (GE Healthcare)

For these columns, it is necessary to calculate the exact slurry percentage. Use this value to determine the total height of slurry to introduce into the column. For this type of slurry transfer, preparing an excess amount of slurry of approximately 5–10% is recommended.

1. Level the column; otherwise, the distance between the packed bed surface and inlet adaptor surface will not be uniform across the column's diameter.
2. Prepare the slurry in a slurry tank as described previously.
3. Ensure that the inlet adaptor is positioned at the lowest height, the column and associated transfer lines are primed with packing buffer, and the column is sealed.
4. Transfer the CHT slurry to the column through the bottom port(s) by raising the piston at a minimum speed of 200 cm/hr for 40 µm and 300 cm/hr for 80 µm CHT.
5. Stop the upward piston movement when the calculated height has been reached. Close the filling port(s).
6. Immediately begin consolidating the bed by lowering the motorized piston at 300 cm/hr with the top inlet closed and the bottom inlet open. Do not exceed the maximum recommended pressure for the column.
7. Continue the descent until the adaptor is 10% above the target height of the packed bed. A gap of about 0.1–1.0 cm is acceptable between the packed bed and the inlet adaptor bed support. In acrylic and glass columns, the gap may be visually observed. In stainless steel columns, it is advisable to reduce the speed of the piston when it is 10% above the target bed height. If sudden resistance is noted as the adaptor approaches the target height, stop the descent immediately. Close the outlet valve. Open the inlet valve, then pump packing buffer into the inlet valve at 100 cm/hr while raising the adaptor at 50 cm/hr. Stop when the adaptor has traveled 0.5 cm.
8. Condition with 3–5 CV of equilibration buffer at the selected operating flow rate.
9. Continue with packed column qualification.

5.4.3 Unpacking Method for Columns with Adjustable Inlet Adaptors

The unpacking procedure is similar for all columns with adjustable inlet adaptors, whether open columns or contained systems.

1. Begin by flowing downflow at 100–200 cm/hr. At the same time, raise the inlet adaptor at a slightly slower linear speed to maintain a net positive downflow.
2. Continue until there is a headspace of approximately 5–10 cm.
3. Decrease the flow rate to 100 cm/hr and change the flow direction to upflow.
4. Continue alternating upflow and downflow until the bed collapses.
5. Unseal the top flow adaptor.
   a. For open columns, remove the inlet adaptor.
   b. For closed systems, raise the inlet to its uppermost position.
6. Continue upflow at 100 cm/hr or less until the desired slurry concentration is achieved.
   a. For open columns, insert a paddle and mix the slurry until it is homogeneous.
   b. For closed columns with stainless steel frits, apply process air to the bottom process outlet at no more than 3 psi to agitate the slurry.
7. Remove the slurry from the column.
   a. For open columns, remove the slurry from the column by using clean pitchers or diaphragm pumps.
   b. For closed systems, use the media transfer station to remove the slurry after opening the slurry transfer valves.
8. For closed systems without stainless steel frits, it may take several cycles of adding buffer and removing slurry through the slurry transfer valve(s).
9. Clean the column according to the manufacturer's recommendations.

5.5 Media Packing Station Methods

The packing station methods are used for contained operating systems with fixed or semimovable pistons, such as the following:

- Chromaflow column (GE Healthcare)
- Resolute column (Pall)
- IsoPak column (Millipore)
- Eastern Rivers column

The Appendices, Table A.5, lists packed bed lengths of 10–20 cm for contained operating system columns that are 40 cm to 240 cm ID. The typical tube length is 50 cm and the inlet adaptor can be fixed so that the packed bed can be between 5 cm and 40 cm in height.

Packing

1. Level the column. Otherwise, the distance between the packed bed surface and inlet adaptor surface will not be uniform across the column's diameter.
2. Set the inlet adaptor to the desired packed bed height.
3. Seal the column's adaptor per the instructions for the specific column.
4. Prime the slurry transfer skid, fill the column completely with packing solution, clear any air from the column, and transfer lines according to the manufacturer's instructions.
5. Determine the amount of packing solution and CHT ceramic hydroxyapatite. Invert the containers of CHT repeatedly to loosen the powder. This will allow the powder to be easily poured when dispensing it into the column.
   a. Dispense the necessary amount of packing solution into the slurry vessel.
   b. Agitate the solution as described previously in the General Handling and Slurry Preparation section (Section 5.2).
   c. Dispense the CHT to the slurry transfer vessel and mix for 5 min following the last addition.
6. Pressure-pack the CHT slurry into the column through the top-filling nozzle at the fastest rate permitted by the pressure limit of the column and media packing station.
   a. Continue until the media packing station stalls. Refer to the manufacturer's instructions.
   b. Close the column outlet valve.
7. Close the packing nozzle, then clear the packing lines immediately. Refer to the manufacturer's instructions.
8. Condition with 5 CV of packing buffer at the selected operating flow rate.
9. Continue with packed column qualification.

Best Practices

Use low-shear hydrofoil impellers for slurry volumes larger than 200 L. It is advisable to use multiple impellers on the shaft spaced approximately 1 m apart for deep slurries. Do not mix or agitate the slurry with the transfer pumps.

Manufacturers of closed columns recommend reverse flow of the column after packing it. This removes entrapped air from the flow distributor of the column inlet. Reverse flow is not recommended for packed beds of CHT.

Unpacking

Do not use the instructions from the manufacturer for unpacking CHT from columns without following the additional instructions in this guideline.
Carefully planned unpacking and repacking strategies are especially necessary for CHT. High solids concentration, prolonged agitation, repeated agitation, and use of the cycling option on slurry packing units will cause irreversible damage to CHT particles.
The media packing skid should be used only to loosen the packed CHT and direct the flow to the slurry or collection tank. Do not cycle the collected slurry back to the column. Never allow any slurry to settle in the packing station, the packing lines, and especially in the nozzles. If this happens, it may be necessary to disassemble any components clogged with settled medium. The unpacking procedure follows.

1. Plan to use about 10 times the packed CV of unpacking solution. For example, you will need a 1,000 L collection capacity for a 100 L packed column.
2. Set both nozzles to "Unpack."

3. Using the packing pump, pump the unpacking solution (test buffer or packing solution) into the column and collect the evacuating slurry in the collection vessel.
   a. It is best to use the slurry tank to collect the evacuated medium; however, the capacity is often too small.
      i) If this is the case, unpack the column in increments equal to the capacity of the slurry tank.
      ii) At each increment, stop the pump, close the nozzles, and clear the evacuation lines of CHT.
      iii) Allow the CHT to settle in the slurry tank. The settling rate is approximately 180 cm/hr for the smallest particles in 40 µm CHT whereas the settling rate for the largest particles in 80 µm CHT is 1,000 cm/hr.
      iv) Aspirate the supernatant from the tank.
   b. Repeat Step 3 until CHT particles are no longer seen in the evacuated unpacking solution. The final aspiration of the collected CHT removes particulate developed during the unpacking process.
4. Clean the column according to the manufacturer's guidelines.

5.6 Packed Column Qualification

Column efficiency should be tested directly after packing, then at regular intervals, and when separation performance is seen to deteriorate. Tests should check the quality of the packing and monitor it during the working life of the column.

The efficiency of a packed column is best determined in terms of the height equivalent to a theoretical plate (HETP) and the asymmetry factor (A_f). These values are easily obtained by applying a test probe, such as 2% acetone/test buffer solution or 1.0 M NaCl in equilibration buffer solution, to the column. The preferred equilibration buffer is 0.15 M NaCl in 20 mM phosphate buffer, pH 7.2–8.0 (PBS).

The calculated plate number (N) will vary depending on the test conditions and should therefore be used as a reference value only. It is also important to maintain constant conditions and use the same equipment when comparing results. Changes in the test probe concentration, test buffer, sample volume, flow rate, liquid pathway, or temperature will affect the results.

For optimal results, the test probe volume should be between 1% and 5% of the CV. The flow rate may range between 75 and 150 cm/hr but should remain consistent from test to test. Flow rates outside of this range are acceptable for specific processes; however, A_f and HETP values will not be optimal.

If an acceptance limit is defined in relation to column performance, the column plate number can be used as part of the acceptance criteria for column use. Avoid sample dilution by applying it as close to the column inlet as possible and without interrupting the flow rate.

To begin the qualification, follow these steps:

Method 1

1. Equilibrate the column with 3–5 CV of test buffer using a flow rate of 75–150 cm/hr.
2. Record the pressure.

Test conditions

- Sample volume: 1–5% of the bed volume
- Sample concentration: 2.0% v/v acetone in test buffer or 1.0 M NaCl in test buffer

Note: When using NaCl, ensure that the injection sample contains the same concentration of phosphate that is in the equilibration buffer.

Flow rate: 75–150 cm/hr

UV: 280 nm, 1 cm, 0.1 AU (acetone)

Conductivity: 100 mS (NaCl)

Method 2*

Pretest conditioning

1. Equilibrate the column with 3 CV of 0.1 N NaOH at 150 cm/hr
2. Equilibrate with 3 CV of 0.10 M NaCl at 150 cm/hr

Test conditions

- Flow rate, 150 cm/hr
- Inject 0.025 CV of 1.0 M NaCl
- Continue with 3 CV of 0.10 M NaCl
- End
- Determine test results

* The NaCl test method following NaOH equilibration was developed by Chugai Pharmaceutical Co., Ltd to avoid conductivity fluctuations observed with other pretest equilibration buffers.

Calculate HETP and the reduced plate height (RPH) from the UV or conductivity curve (Figure 5.3) as follows:

HETP = L/N where

L = bed height (cm)

$N = 5.54(V_e/W_{1/2})^2$

V_e = peak elution distance

$W_{1/2}$ = peak width at half peak height

V_e and $W_{1/2}$ are measured in the same units

RPH = HETP/d where

d = mean particle diameter of the medium in cm

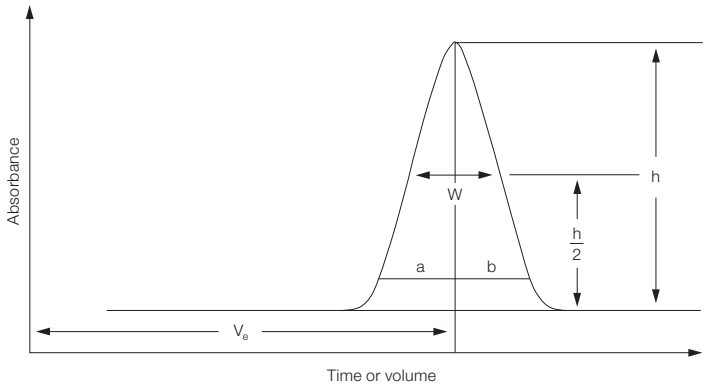


Fig. 5.3. UV or conductance trace for test.

Typical HETP values for 40 µm CHT ceramic hydroxyapatite range from 0.016–0.021 cm, and the range for 80 µm is 0.032–0.041 cm. RPH is often used to compare column performance to that expected for the mean particle size of the chromatography medium. As a guideline for rigid, incompressible chromatography media, an RPH value of less than 5.5 is normally acceptable.

Calculate the peak asymmetry factor, A_f, as follows:

A_f = b/a where

a = 1st half peak width at 10% of peak height
b = 2nd half peak width at 10% of peak height

The asymmetry factor should be as close as possible to 1.0 (values from 0.8–2.3 are usually acceptable). A change in the shape of the peak is usually the first indication of bed deterioration due to use.

5.7 Further Notes on Column Packing

The following notes are provided to further assist your column packing.

5.7.1 Column Qualification for Columns with Adjustable Inlet Adaptors

If the column test failed the initial qualification tests because the A_f is greater than 2.3 but the HETP and RPH are acceptable, the inlet adaptor descended too far and is compressed firmly against the bed support but loosely at the wall of the column. This may occur to packed beds with adjustable inlet adaptors if the advisory statement regarding the inlet adjustment to the target bed height was ignored. The deficiency may be eliminated by following the instructions in the advisory statement.

If the column failed the initial qualification test because the HETP and RPH were too large but the A_f was acceptable, the inlet adaptor did not descend enough. This may occur when the column is not precision-bored. The deficiency may be eliminated by lowering the adaptor by 0.5 cm and then retesting the column. Continue lowering the adaptor in 0.5 cm increments and retesting between each increment until the HETP, RPH, and A_f are acceptable.

5.7.2 Column Qualification for Contained Operating System Pressure-Packed Closed Columns

If the results of the column qualification tests are unsatisfactory, repack the column according to the directions in the appropriate section of this guide. The cause of the deficiency may be channeling, heterogeneous bed formation, or gas entrapped in the packed bed.

5.7.3 Column Qualification for Columns Used in Purification Campaigns

If the column was used for purification cycles and has developed symptoms of deterioration such as higher operating pressure, larger HETP, RPH, and/or poor A_f, we recommend discarding the chromatography medium.

5.7.4 Conditioning the Column for the Purification Application

If the column was tested with the recommended test buffer, proceed directly to the column conditioning protocol established for your purification process.

When using another test buffer at a pH greater than 8, or when packing with buffers above pH 8, adjust the pH of a packed CHT ceramic hydroxyapatite column by equilibrating it with 3 CV of 200 mM sodium phosphate at pH 6.5 to 6.8. Proceed to the column conditioning protocol established for your purification process.

5.8 Laboratory-Scale Column Packing Protocols

This section describes the packing of CHT into laboratory-scale columns to obtain a packed bed height of approximately 20 cm (Sections 5.8.1 and 5.8.2).

5.8.1 Column Packing of Millipore Vantage L Columns

The following applies to packing CHT into Millipore Vantage L Biochromatography laboratory columns of the following sizes: 11 mm × 250 mm, 16 mm × 250 mm, 22 mm × 250 mm, 32 mm × 250 mm, and 44 mm × 250 mm. This procedure applies to 40 µm and 80 µm CHT Types I and II (40 µm and 80 µm).

1. Insert and secure the column outlet seal/bed support to the glass column. Attach the column extension using the appropriate extension kit.
2. Add water to wet the bed support and purge the outlet channel. Use 3 ml for the 11 mm column, 4.5 ml for the 16 mm column, 6 ml for the 22 mm column, 9 ml for 32 mm, and 12 ml for the 44 mm column
3. Drain completely.
4. Insert a ¼–28 plug to block the outlet.
5. Place the column/extension assembly into a three-pronged clamp and secure the column/extension assembly assuring that it is level.
6. Add sufficient water such that a layer of approximately 0.50 cm of water rests above the frit.
7. Prepare a 400 mM solution of sodium phosphate regeneration buffer, pH 7.0–7.5, with a 0.2 µm filtration step.
8. Mix regeneration buffer with water in a beaker to make the packing buffer (see Table 5.1 below).

Table 5.1. Packing buffer preparation for Millipore Vantage L columns.

	Column size				
	11 mm	16 mm	22 mm	32 mm	44 mm
Regeneration buffer	20 ml	40 ml	76 ml	160 ml	305 ml
Water	20 ml	40 ml	76 ml	160 ml	305 ml
Beaker size	50 ml	100 ml	250 ml	500 ml	1,000 ml

9. Weigh the appropriate amount of CHT: use 11.97 g for 11 mm, 25.3 g for 16 mm, 47.9 g for 22 mm, 100 g for 32 mm, 192 g for 44 mm
10. Stir the packing buffer continuously with a plastic spatula while dispensing the CHT to the packing buffer.
11. Dispense the slurry to the column/extension assembly.
12. Allow to settle for 5 min then remove the ¼–28 plug.
13. Let the buffer drain for about 5 min.
14. Insert the ¼–28 plug to stop draining.
15. Rinse any occluded CHT from the column wall into the buffer layer with water.
16. Insert the inlet seal/bed support on top of the column/extension assembly.
17. Insert this assembly/bed support to the top of the buffer layer then secure the inlet.
18. Connect the column inlet to a chromatography system.
19. Remove the ¼–28 plug and connect the outlet to the system's detector line.
20. Pack the CHT at the linear flow rate of 150 cm/hr for 15 min to compact the media: use a flow rate of 2.4 ml/min for the 11 mm column, 5.0 ml/min for the 16 mm column, 9.5 ml/min for the 22 mm column, 20.1 ml/min for the 32 mm column, and 38 ml/min for the 44 mm column.
21. Stop the pump and allow the pressure to decline to ambient.
22. Disconnect the outlet tubing and insert the ¼–28 plug.
23. Remove the inlet tube then disengage the inlet column adjuster lock.

Note: Wear latex gloves while conducting the next steps.

24. Remove the excess buffer from the column extension tube then remove the extension and extension kit.
25. Remove the column from the clamp.
26. Hold the column over a sink while pressing the inlet adapter to advance it into the buffer layer in the column.
27. Trapped air should eject easily during this step.
28. Continue pressing until the seal/bed is 2 mm above the packed bed.
29. Lock the adjustor.
30. Wash the exterior of the column with water to remove buffer.
31. Wipe the exterior of the column dry with paper towels.
32. Remount the column in the clamp but do not connect the column.

Note: The following sequence eliminates the introduction of air to the packed column.

33. In the manual mode, set the flow rate to 1 ml/min.
34. Start the pump to prime the line.
35. Stop the pump then connect the line to the column inlet.

36. Tighten the inlet tubing fitting to assure a firm seal.
37. Remove the ¼–28 plug then connect the outlet to the system's detector line.
38. Set the linear flow rate to 150 cm/hr (note that the maximum flow rate is 300 cm/hr). Use a flow rate of 2.4 ml/min for the 11 mm column, 5.0 ml/min for the 16 mm column, 9.5 ml/min for the 22 mm column, 20.1 ml/min for the 32 mm column, and 38 ml/min for the 44 mm column.
39. Start the pump and continue at the flow rate set in Step 38 for 15 min.
40. Turn off the pump.
41. Turn the inlet adjustor clockwise to advance the inlet seal/bed to 1 mm above the packed bed.

Note: Adjust the adaptor to avoid touching the bed. The axial rotation of the adapter will damage the particles if it contacts the surface of the packed bed.

5.8.2 Column Packing of Econo-Columns

The following applies to packing CHT into Bio-Rad Econo-Column® columns of the following sizes: 1.0 cm × 30 cm (737-1032) and 1.5 cm × 30 cm (737-1532). Additional required items are flow adapters 1.0 × 14 cm (738-1015) and 1.5 × 14 cm (738-1016), an Econo-Column funnel (731-0003), and Luer fittings to connect to liquid chromatography systems. This procedure applies to 40 µm and 80 µm CHT Types I and II (40 µm and 80 µm).

Important Considerations Before Using Econo-Columns:

The upper limit of pressure for the Econo-Columns is approximately 14 psi (1 bar). The flow rates we provide in the procedure are suggestions intended as guidelines. You will need to adjust the flow rate as appropriate for your application.

For a packed bed height of 20 cm, flow rates are limited to approximately 50 cm/hr for 40 µm CHT and approximately 90 cm/hr for 80 µm CHT. As mentioned above, you may need to adjust to reach a slower flow rate.

Pressure contributed by chromatography system components downstream of the column outlet must be minimized to less than 1 psi at the selected flow rate.

We do not recommend using Econo-Columns with diameters of 2.5 cm or 5.0 cm.

1. Add water to wet the bed support and purge the outlet channel: use 3 ml of water for a 1.0 cm bed support and 5 ml of water for a 1.5 cm bed support.
2. Insert a female Luer plug to block the outlet.
3. Place the column into a three-pronged clamp and secure the column assuring that it is level. Attach the funnel to the top of the column.
4. Add sufficient water such that a layer of approximately 0.50 cm of water rests abovethe frit.
5. Mix regeneration buffer with water in a beaker to make the packing buffer (see Table 5.2 below).

Table 5.2. Packing buffer preparation for Econo-Column columns.

	Column size	
	1.0 cm	**1.5 cm**
Regeneration buffer	20 ml	40 ml
Water	20 ml	40 ml
Beaker size	100 ml	150 ml

6. Weigh the appropriate amount of CHT: use 9.90 g for a 1.0 cm column and 22.3 g for a 1.5 cm column.
7. Stir the packing buffer with a plastic spatula continuously while dispensing the CHT to the packing buffer.
8. Dispense the slurry to the column/funnel assembly.
9. Allow to settle for 1 min then remove the Luer plug.
10. Drain the buffer and periodically rinse occluded CHT from the funnel surface with deionized water.
11. Continue draining until the liquid layer is about 3 cm above the packed bed.
12. Insert the Luer plug to stop draining. Remove the funnel.
13. Attach and adjust the flow adapter using the directions in Section 3.1 of the Econo-Column Flow Adaptor Instruction Manual (M7380014).
14. Insert the flow adapter by following steps 4–7 in Section 3.2 of the instruction manual until the edge of the bed support housing is 1 mm above the packed bed.
15. Connect the column inlet to a chromatography system.
16. Remove the Luer plug and connect the outlet to the system's detector line.
17. Condition the CHT at the flow rates listed below and for the number of column volumes suggested by the guideline sections 3.3.10–3.3.14 of this instruction manual.
    a. For 40 µm CHT at 50 cm/hr use 0.65 ml/min for a 1.0 cm column and 1.5 ml/min for a 1.5 cm column.
    b. For 80 µm CHT at 190 cm/hr use 2.50 ml/min for a 1.0 cm column and 5.60 ml/min for a 1.5 cm column.

Note: We do not recommend using GE X/K columns. CHT particles can lodge between the column wall and the plunger/net-ring assembly to produce poor results during HETP testing.

CHAPTER 6
Case Studies

Examples of protocols for packing CHT™ ceramic hydroxyapatite into different types of columns used in the industry have been provided to help guide you. Read each protocol thoroughly.

Variance in specifications and construction of the columns should be taken into consideration when packing your own column.

6.1 Packing Results — Custom GE Healthcare Chromaflow 900/200–400

Objective

To pack approximately 222 L of Type II 40 µm CHT to a height of 35 cm in a 90 cm ID Chromaflow column as a 45% v/v (28% w/v), slurry in 200 mM disodium phosphate buffer (DSPB), pH 9–10 by pressurizing a 700 L tank containing 510 L of the CHT slurry.

Slurry Preparation

Dispense 400 L of DSPB to the slurry tank (316 SS 60° taper, hydrofoil impeller at end of shaft, flat blade impeller at midpoint of shaft). Agitate the buffer while deaerating with nitrogen for 10 min. Prime the line to the column with 10 L of buffer.

Mix 145 kg of CHT into the remaining 390 L of DSPB while mixing (approximately 3% more slurry than is required to fill the column). Mix for an additional 10 min following the addition of the last CHT container while closing and sealing the column hatch.

Column Packing

Pressurize the tank to 45 psi with nitrogen gas and hold for 1 min. Open the tank valve to fill the column while maintaining the tank pressure of 45 psi until the packing nozzle on the column stalls. Immediately close the packing nozzle.

Post-Packing Cleanup

Close the tank valve and release the pressure on the tank. Disconnect the line from the slurry tank at the column filling connection and direct it to a collection container. Open the tank valve, then pressurize the tank if necessary to empty it of excess slurry. Turn off the nitrogen line if used in the prior step. Rinse the interior with WFI with the tank spraying system and collect the contents in the collection container.

Connect the GE Healthcare Chromaflow Pack 50 packing station to the column filling line. Flush and clean the filling line according to the instructions in the Chromaflow column and packing station instruction manuals.

Column Evaluation

Connect the column to the process chromatography workstation. Apply phosphate buffered saline (PBS) test solution to the column at 25 cm/hr in an upward direction for 5 min to purge air from the column's inlet distributor. Apply PBS buffer in a downward direction at 170 cm/hr until the pH and conductivity of the effluent equals that of the PBS (the pressure will be about 38 psi).

Program the workstation for 350 L of test buffer at 100 cm/hr following the sample injection of 5.55 L of 2% acetone in PBS. Obtain and summarize the results.

Results

Slurry preparation	45 min CHT addition time for 29 × 5 kg 13 min additional mixing time including 3 min pressurization
Column packing duration	16 min
Average packing flow rate	30.8 L/min
Flow rate range start to finish	75.0–21.6 L/min
Peak retention	193.53 L

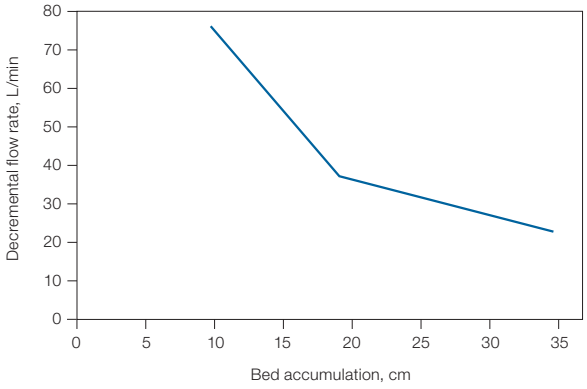


Fig. 6.1. Decremental flow rate chart.

Peak retention %CV	87.17
Plates for column	2,827.5
Plates/meter	8,079
HETP	0.01238 cm
Reduced plate height	3.10
A_f	1.14
Peak width volume	26.75 L
Injection volume	5.55 L
Dilution factor	4.82

6.2 Packing Results — Prototype Millipore IsoPak IPP350/500

Objectives

To demonstrate that the IsoPak packing system is able (1) to pack the two media types CHT Type I 40 µm and 80 µm without damage, (2) to give good chromatographic results, and (3) to determine how many times the media can be repacked before extensive damage occurs.

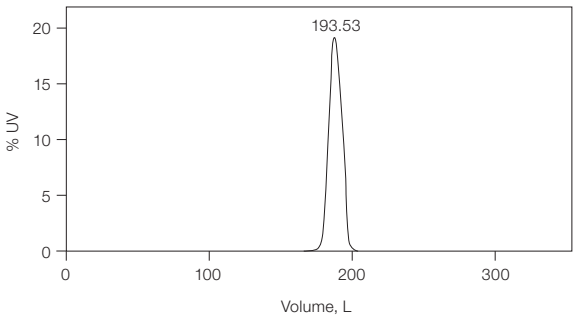


Fig. 6.2. Acetone test chromatogram.

Slurry Preparation

CHT Type I, 40 and 80 µm, was obtained as slurries from single flow-packed experiments conducted to establish baselines for the medium and relevant packed CV. The slurry was transferred from the column to a 45° taper polypropylene slurry tank after agitating by upflow with 200 mM disodium phosphate buffer (DSPB), pH 8–9 to a 25% v/v consistency. It was diluted to 11–14% v/v with DSPB in the tank. The slurry was mixed with a polypropylene paddle to homogeneity just prior to packing the column with the IsoPak packing station.

Column Preparation

The clean, dry IPP350 packing station was adjusted to a volume of about 5% less than the available amount of CHT. The line to the slurry tank was backflushed with air to clear the exit port of any settled CHT. The packing line was connected to the bottom-filling valve on the IPP350 packing station. The inlet adaptor was set to effluent mode and the bottom-filling valve was opened.

Column Packing

The packing station was set to 30 psi and the filling line engaged. The slurry filled the column from the bottom, expelling air and excess DSPB through the inlet frit. The bed accumulated against the frit and increased in length from top to bottom during the filling. The system stalled after completely filling the column. The bottom-filling valve was closed when the system stalled.

Post-Packing Cleanup

The bottom-filling valve and lines were cleaned following the steps in the column and packing station instruction manuals.

Column Evaluation

The column was connected to the process chromatography workstation. We applied 75 mM sodium phosphate/100 mM sodium chloride, pH 7.4 test solution (PBNaCl) to the column at 200 cm/hr for 2.6 CV.

The column was tested at 200 cm/hr with 500 mL of 2% acetone in PBNaCl, loaded in a 10 sec pulse.

Conclusion

CHT Type I (both 40 and 80 µm) was shown to efficiently pack into the Millipore IsoPak IPP350 column using a slurry packing station. IsoPak provided additional convenience, containment, and greatly reduced packing and unpacking time. Moreover, the reproducibility of the IsoPak pump-packed results is excellent. However, after 40 passes through the packing station, the back pressures increased by 15–18%, indicating that CHT is friable when excessively packed and repacked.

Table 6.1. Summary for packing CHT Type I, 40 µm in Isopak IPP350.

Packing number	1	2	3	4	5	6
Slurry concentration, %v/v	13	14	15	8	14	14
Average exhaust flow rate, L/min	10.9	10.9	11.1	11.1	9.75	10.4
Packing time to stall	8.25	7.50	6.30	13.0	8.00	8.00
Test flow rate	200	200	200	200	200	200
Peak retention time	4.02	3.90	3.88	3.90	3.90	3.92
HETP, cm	0.0109	0.0134	0.0157	0.0132	0.0147	0.0151
Asymmetry at 10% peak height	1.064	1.047	0.990	0.909	0.879	0.866
Sample loading time, sec	10	10	10	10	10	10
Peak width at 10% peak height, min	0.526	0.544	0.603	0.544	0.571	0.587
Dilution factor, D1	3.16	3.26	3.62	3.26	3.43	3.52
Column inlet pressure, bar	1.2	1.18	1.21	1.27	1.25	1.3
Bed height, cm	13	13	13	13	13	13

Table 6.2. Summary for CHT Type I, 80 µm in Isopak IPP350.

Packing number	1	2	3	4	5	6	7
Slurry concentration, %v/v	25	25	16	25	11	21	16
Average exhaust flow rate, L/min	27.7	24.6	23.1	23.5	21.8	21.3	20.0
Packing time to stall	1.70	1.95	3.60	2.20	6.25	3.00	4.50
Test flow rate	200	200	200	200	200	200	200
Peak retention time	4.02	4.02	3.98	3.97	3.83	3.93	3.85
HETP, cm	0.026	0.030	0.029	0.030	0.030	0.032	0.039
Asymmetry at 10% peak height	1.265	1.090	1.133	1.137	1.429	1.347	1.545
Sample loading time, sec	10	10	10	10	10	10	10
Peak width at 10% peak height, min	0.805	0.819	0.804	0.812	0.888	0.895	1.008
Dilution factor, D1	4.83	4.91	4.82	4.87	5.33	5.37	6.05
Column inlet pressure, bar	0.66	0.70	0.73	0.69	0.76	0.74	0.75
Bed height, cm	13	13	13	13	13	13	13

Appendices

A.1 CHT™ Ceramic Hydroxyapatite to Buffer Tables

Tables A.1–A.5 cover tube lengths that are 500, 600, 700, and 900 mm where the maximum fill heights are 400, 500, 600, and 800 mm respectively. The 100 mm difference between tube height and fill height is due to the minimum length required by the inlet adaptor. Tables A.1– A.5 also list the appropriate dry weight of CHT and volume of packing solution necessary to prepare the slurry in the column to obtain a packed bed for the specified diameter and height. Table A.5 lists packed bed lengths from 10–20 cm for contained operating system columns that have an inner diameter of 40 cm to 240 cm. The typical tube is 50 cm in length and the inlet adaptor can be fixed so that the packed bed can be between 5 cm and 40 cm in height.

For example, a BPG 450/500 45 cm ID column with a packed bed height of 15 cm requires 15.03 kg of CHT and 48.6 L of packing solution to make a 37.5% volume slurry. The 500 and 600 mm tube length columns can be packed to no more than 20 cm height beds. A 25 cm bed height can be packed into the 700 mm tube length column and the 900 mm tube length column can be packed up to a 30 cm bed height.

Table A.1. CHT to buffer for packing 50 cm high open columns.

50 cm tube open columns and media transfer methods, weight of CHT, and volume of buffer to prepare recommended slurry volume for packing designated column ID to designated height.

Column ID, cm	Bed height, cm	Packed volume, L	Maximum fill height, cm	Maximum slurry volume, L	CHT, kg	Volume buffer, L	Slurry (%v/v)	Slurry (%w/v)
20	10	3.1	40	12.6	1.98	11.9	25.0	15.8
20	15	4.7	40	12.6	2.97	11.6	37.5	23.6
20	20	6.3	40	12.6	3.96	11.3	50.0	31.5
30	10	7.1	40	28.3	4.45	28.2	25.0	15.8
30	15	10.6	40	28.3	6.68	26.1	37.5	23.6
30	20	14.1	40	28.3	8.91	25.3	50.0	31.5
40	10	12.6	40	50.3	7.92	47.6	25.0	15.8
40	15	18.8	40	50.3	11.88	46.3	37.5	23.6
40	20	25.1	40	50.3	15.83	45.0	50.0	31.5
45	10	15.9	40	63.6	10.02	60.3	25.0	15.8
45	15	23.9	40	63.6	15.03	58.6	37.5	23.6
45	20	31.8	40	63.6	20.04	56.9	50.0	31.5
50	10	19.6	40	78.5	12.37	74.4	25.0	15.8
50	15	29.5	40	78.5	18.56	72.4	37.5	23.6
50	20	39.3	40	78.5	24.74	70.3	50.0	31.5
60	10	28.3	40	113.1	17.81	107.2	25.0	15.8
60	15	42.4	40	113.1	26.72	104.2	37.5	23.6
60	20	56.5	40	113.1	35.63	101.2	50.0	31.5
70	10	38.5	40	153.9	24.25	145.9	25.0	15.8
70	15	57.7	40	153.9	36.37	141.8	37.5	23.6
70	20	77.0	40	153.9	48.49	137.8	50.0	31.5
80	10	50.3	40	201.1	31.67	190.5	25.0	15.8
80	15	75.4	40	201.1	47.50	185.2	37.5	23.6
80	20	100.5	40	201.1	63.33	180.0	50.0	31.5
90	10	63.6	40	254.5	40.08	241.1	25.0	15.8
90	15	95.4	40	254.5	60.12	234.4	37.5	23.6
90	20	127.2	40	254.5	80.16	227.8	50.0	31.5
100	10	78.5	40	314.2	49.48	297.7	25.0	15.8
100	15	117.8	40	314.2	74.22	289.4	37.5	23.6
100	20	157.1	40	314.2	98.96	281.2	50.0	31.5
120	10	113.1	40	452.4	71.25	428.6	25.0	15.8
120	15	169.6	40	452.4	106.88	416.8	37.5	23.6
120	20	226.2	40	452.4	142.50	404.9	50.0	31.5
140	10	153.9	40	615.8	96.98	583.4	25.0	15.8
140	15	230.9	40	615.8	145.47	567.3	37.5	23.6
140	20	307.9	40	615.8	196.96	551.1	50.0	31.5
200	10	314.2	40	1,256.6	197.92	1,190.7	25.0	15.8
200	15	471.2	40	1,256.6	296.88	1,157.7	37.5	23.6
200	20	628.3	40	1,256.6	395.84	1,124.7	50.0	31.5
220	10	380.1	40	1,520.5	239.48	1,440.7	25.0	15.8
220	15	570.2	40	1,520.5	359.23	1,400.8	37.5	23.6
220	20	760.3	40	1,520.5	478.97	1,360.9	50.0	31.5
240	10	452.4	40	1,809.6	285.01	1,714.6	25.0	15.8
240	15	678.6	40	1,809.6	427.51	1,667.1	37.5	23.6
240	20	904.8	40	1,809.6	570.01	1,619.6	50.0	31.5

The following formulas are provided for calculating values not covered by the following tables:

$V_L = (V_b/S) - 0.210 \bullet V_b$ where

$V_T = V_L/S$ where

- V_L Volume of buffer to be added
- V_T Total slurry volume
- V_b Packed bed volume
- S Slurry density (%v/v)

Calculating the V_T value is useful to ensure that the total amount of prepared slurry will fit into the column and/or slurry preparation tank. Bio-Rad best practices recommend values for S ≤50%. For packed bed volumes, the tap-settled density of CHT (0.63) can be used (see section 2.1). Experience with larger columns has shown that a value of 0.60 may provide a closer approximation to the final desired packed bed height. Caution: any significant vibration of the column after packing, such as moving the packed column from one area to another, can cause further bed settling.

Note: MPC has a tap-settled density of 0.72 g/ml, therefore calculations need to be adjusted accordingly.

Table A.2. CHT to buffer for packing 60 cm high open columns.

60 cm tube open columns and media transfer methods, weight of CHT and volume of buffer to prepare recommended slurry volume for packing designated column ID to designated height.

Column ID, cm	Bed height, cm	Packed volume, L	Maximum fill height, cm	Maximum slurry volume, L	CHT, kg	Volume buffer, L	Slurry (%v/v)	Slurry (%w/v)
20	10	3.1	50	15.7	1.98	15.1	20.0	12.6
20	15	4.7	50	15.7	2.97	14.7	30.0	18.9
20	20	6.3	50	15.7	3.96	14.4	40.0	25.2
30	10	7.1	50	35.3	4.45	33.9	20.0	12.6
30	15	10.6	50	35.3	6.68	33.1	30.0	18.9
30	20	14.1	50	35.3	8.91	33.4	40.0	25.2
40	10	12.6	50	62.8	7.92	60.2	20.0	12.6
40	15	18.8	50	62.8	11.88	58.9	30.0	18.9
40	20	25.1	50	62.8	15.83	57.6	40.0	25.2
45	10	15.9	50	79.5	10.02	76.2	20.0	12.6
45	15	23.9	50	79.5	15.03	74.5	30.0	18.9
45	20	31.8	50	79.5	20.04	72.8	40.0	25.2
50	10	19.6	50	98.2	12.37	94.1	20.0	12.6
50	15	29.5	50	98.2	18.56	92.0	30.0	18.9
50	20	39.3	50	98.2	24.74	89.9	40.0	25.2
60	10	28.3	50	141.4	17.81	135.4	20.0	12.6
60	15	42.4	50	141.4	26.72	132.5	30.0	18.9
60	20	56.5	50	141.4	35.63	129.5	40.0	25.2
70	10	38.5	50	192.4	24.25	184.3	20.0	12.6
70	15	57.7	50	192.4	36.37	180.3	30.0	18.9
70	20	77.0	50	192.4	48.49	176.3	40.0	25.2
80	10	50.3	50	251.3	31.67	240.8	20.0	12.6
80	15	75.4	50	251.3	47.50	235.5	30.0	18.9
80	20	100.5	50	251.3	63.33	230.2	40.0	25.2
90	10	63.6	50	318.1	40.08	304.7	20.0	12.6
90	15	95.4	50	318.1	60.12	298.1	30.0	18.9
90	20	127.2	50	318.1	80.16	291.4	40.0	25.2
100	10	78.5	50	392.7	49.48	376.2	20.0	12.6
100	15	117.8	50	392.7	74.22	368.0	30.0	18.9
100	20	157.1	50	392.7	98.96	359.7	40.0	25.2
120	10	113.1	50	565.5	71.25	541.7	20.0	12.6
120	15	169.6	50	565.5	106.88	529.9	30.0	18.9
120	20	226.2	50	565.5	142.50	518.0	40.0	25.2
140	10	153.9	50	769.7	96.98	737.4	20.0	12.6
140	15	230.9	50	769.7	145.47	721.2	30.0	18.9
140	20	307.9	50	769.7	193.96	705.0	40.0	25.2
200	10	314.2	50	1,570.8	197.92	1,504.8	20.0	12.6
200	15	471.2	50	1,570.8	296.88	1,471.9	30.0	18.9
200	20	628.3	50	1,570.8	395.84	1,438.9	40.0	25.2
220	10	380.1	50	1,900.7	239.48	1,820.8	20.0	12.6
220	15	570.2	50	1,900.7	359.23	1,780.9	30.0	18.9
220	20	760.3	50	1,900.7	478.97	1,741.0	40.0	25.2
240	10	452.4	50	2,261.9	285.01	2,167.0	20.0	12.6
240	15	678.6	50	2,261.9	427.51	2,119.5	30.0	18.9
240	20	904.8	50	2,261.9	570.01	2,072.0	40.0	25.2

Table A.3. CHT to buffer for packing 70 cm high open columns.

70 cm tube open columns and media transfer methods, weight of CHT, and volume of buffer to prepare recommended slurry volume for packing designated column ID to designated height.

Column ID, cm	Bed height, cm	Packed volume, L	Maximum fill height, cm	Maximum slurry volume, L	CHT, kg	Volume buffer, L	Slurry (%v/v)	Slurry (%w/v)
20	10	3.1	60	18.8	1.98	18.2	16.7	10.5
20	15	4.7	60	18.8	2.97	17.9	25.0	15.8
20	20	6.3	60	18.8	3.96	17.6	33.3	21.0
20	25	7.9	60	18.8	4.95	17.2	41.7	26.3
30	10	7.1	60	42.4	4.45	40.8	16.7	10.5
30	15	10.6	60	42.4	6.68	40.2	25.0	15.8
30	20	14.1	60	42.4	8.91	39.5	33.3	21.0
30	25	17.7	60	42.4	11.13	38.7	41.7	26.3
40	10	12.6	60	75.4	7.92	72.6	16.7	10.5
40	15	18.8	60	75.4	11.88	71.4	25.0	15.8
40	20	25.1	60	75.4	15.83	70.2	33.3	21.0
40	25	31.4	60	75.4	19.79	68.7	41.7	26.3
45	10	15.9	60	95.4	10.02	91.9	16.7	10.5
45	15	23.9	60	95.4	15.03	90.4	25.0	15.8
45	20	31.8	60	95.4	20.04	88.8	33.3	21.0
45	25	39.8	60	95.4	25.05	87.0	41.7	26.3
50	10	19.6	60	117.8	12.37	113.5	16.7	10.5
50	15	29.5	60	117.8	18.56	111.6	25.0	15.8
50	20	39.3	60	117.8	24.74	109.7	33.3	21.0
50	25	49.1	60	117.8	30.93	107.4	41.7	26.3
60	10	28.3	60	169.6	17.81	163.4	16.7	10.5
60	15	42.4	60	169.6	26.72	160.7	25.0	15.8
60	20	56.5	60	169.6	35.63	157.9	33.3	21.0
60	25	70.7	60	169.6	44.53	154.7	41.7	26.3
70	10	38.5	60	230.9	24.25	222.4	16.7	10.5
70	15	57.7	60	230.9	36.37	218.8	25.0	15.8
70	20	77.0	60	230.9	48.49	215.0	33.3	21.0
70	25	96.2	60	230.9	60.61	210.5	41.7	26.3
80	10	50.3	60	301.6	31.67	290.5	16.7	10.5
80	15	75.4	60	301.6	47.50	285.8	25.0	15.8
80	20	100.5	60	301.6	63.33	280.8	33.3	21.0
80	25	125.7	60	301.6	79.17	275.0	41.7	26.3
90	10	63.6	60	381.7	40.08	367.6	16.7	10.5
90	15	95.4	60	381.7	60.12	361.7	25.0	15.8
90	20	127.2	60	381.7	80.16	355.4	33.3	21.0
90	25	159.0	60	381.7	100.20	348.0	41.7	26.3
100	10	78.5	60	471.2	49.48	453.8	16.7	10.5
100	15	117.8	60	471.2	74.22	446.5	25.0	15.8
100	20	157.1	60	471.2	98.96	438.7	33.3	21.0
100	25	196.3	60	471.2	123.70	429.6	41.7	26.3
120	10	113.1	60	678.6	71.25	653.5	16.7	10.5
120	15	169.6	60	678.6	106.88	643.0	25.0	15.8
120	20	226.2	60	678.6	142.50	631.8	33.3	21.0
120	25	282.7	60	678.6	178.13	618.7	41.7	26.3
140	10	153.9	60	923.6	96.98	889.5	16.7	10.5
140	15	230.9	60	923.6	145.47	875.1	25.0	15.8
140	20	307.9	60	923.6	193.96	859.9	33.3	21.0
140	25	384.8	60	923.6	242.45	842.1	41.7	26.3
200	10	314.2	60	1,885.0	197.92	1,815.2	16.7	10.5
200	15	471.2	60	1,885.0	296.88	1,786.0	25.0	15.8
200	20	628.3	60	1,885.0	395.84	1,754.9	33.3	21.0
200	25	785.4	60	1,885.0	494.80	1,718.5	41.7	26.3
220	10	380.1	60	2,280.8	239.48	2,196.4	16.7	10.5
220	15	570.2	60	2,280.8	359.23	2,161.1	25.0	15.8
220	20	760.3	60	2,280.8	478.97	2,123.4	33.3	21.0
220	25	950.3	60	2,280.8	598.71	2,079.4	41.7	26.3
240	10	452.4	60	2,714.3	285.01	2,613.9	16.7	10.5
240	15	678.6	60	2,714.3	427.51	2,571.9	25.0	15.8
240	20	904.8	60	2,714.3	570.01	2,527.1	33.3	21.0
240	25	1,131.0	60	2,714.3	712.51	2,474.7	41.7	26.3

Table A.4. CHT to buffer for packing 90 cm high open columns.

90 cm tube open columns and media transfer methods, weight of CHT, and volume of buffer to prepare recommended slurry volume for packing designated column ID to designated height.

Column ID, cm	Bed height, cm	Packed volume, L	Maximum fill height, cm	Maximum slurry volume, L	CHT, kg	Volume buffer, L	Slurry (%v/v)	Slurry (%w/v)
20	10	3.1	80	25.1	1.98	24.5	12.5	7.9
20	15	4.7	80	25.1	2.97	24.1	18.8	11.8
20	20	6.3	80	25.1	3.96	23.8	25.0	15.8
20	25	7.9	80	25.1	4.95	23.0	31.9	19.7
20	30	9.4	80	25.1	5.94	23.2	37.5	23.6
30	10	7.1	80	56.5	4.45	55.1	12.5	7.9
30	15	10.6	80	56.5	6.68	54.2	18.8	11.8
30	20	14.1	80	56.5	8.91	53.6	25.0	15.8
30	25	17.7	80	56.5	11.13	51.7	31.9	19.7
30	30	21.2	80	56.5	13.36	52.1	37.5	23.6
40	10	12.6	80	100.5	7.92	97.9	12.5	7.9
40	15	18.8	80	100.5	11.88	96.3	18.8	11.8
40	20	25.1	80	100.5	15.83	95.3	25.0	15.8
40	25	31.4	80	100.5	19.79	91.9	31.9	19.7
40	30	37.7	80	100.5	23.75	92.6	37.5	23.6
45	10	15.9	80	127.2	10.02	123.9	12.5	7.9
45	15	23.9	80	127.2	15.03	121.9	18.8	11.8
45	20	31.8	80	127.2	20.04	120.6	25.0	15.8
45	25	39.8	80	127.2	25.05	116.3	31.9	19.7
45	30	47.7	80	127.2	30.06	117.2	37.5	23.6
50	10	19.6	80	157.1	12.37	153.0	12.5	7.9
50	15	29.5	80	157.1	18.56	150.5	18.8	11.8
50	20	39.3	80	157.1	24.74	148.8	25.0	15.8
50	25	49.1	80	157.1	30.93	143.6	31.9	19.7
50	30	58.9	80	157.1	37.11	144.7	37.5	23.6
60	10	28.3	80	226.2	17.81	220.3	12.5	7.9
60	15	42.4	80	226.2	26.72	216.7	18.8	11.8
60	20	56.5	80	226.2	35.63	214.3	25.0	15.8
60	25	70.7	80	226.2	44.53	206.7	31.9	19.7
60	30	84.8	80	226.2	53.44	208.4	37.5	23.6
70	10	38.5	80	307.9	24.25	299.8	12.5	7.9
70	15	57.7	80	307.9	36.37	294.9	18.8	11.8
70	20	77.0	80	307.9	48.49	291.7	25.0	15.8
70	25	96.2	80	307.9	60.61	281.4	31.9	19.7
70	30	115.5	80	307.9	72.74	283.6	37.5	23.6
80	10	80.3	80	402.1	31.67	391.6	12.5	7.9
80	15	75.4	80	402.1	47.50	385.2	18.8	11.8
80	20	100.5	80	402.1	63.33	381.0	25.0	15.8
80	25	125.7	80	402.1	79.17	367.5	31.9	19.7
80	30	150.8	80	402.1	95.00	370.5	37.5	23.6
90	10	63.6	80	508.9	40.08	495.6	12.5	7.9
90	15	95.4	80	508.9	60.12	487.6	18.8	11.8
90	20	127.2	80	508.9	80.16	482.2	25.0	15.8
90	25	159.0	80	508.9	100.20	465.2	31.9	19.7
90	30	190.9	80	508.9	120.24	468.9	37.5	23.6
100	10	78.5	80	628.3	49.48	611.8	12.5	7.9
100	15	117.8	80	628.3	74.22	601.9	18.8	11.8
100	20	157.1	80	628.3	98.96	595.3	25.0	15.8
100	25	196.3	80	628.3	123.70	574.3	31.9	19.7
100	30	235.6	80	628.3	148.44	578.8	37.5	23.6
120	10	113.1	80	904.8	71.25	881.0	12.5	7.9
120	15	169.6	80	904.8	106.88	866.8	18.8	11.8
120	20	226.2	80	904.8	142.50	857.3	25.0	15.8
120	25	282.7	80	904.8	178.13	827.0	31.9	19.7
120	30	339.3	80	904.8	213.75	833.5	37.5	23.6
140	10	153.9	80	1,231.5	96.98	1,199.2	12.5	7.9
140	15	230.9	80	1,231.5	145.47	1,179.7	18.8	11.8
140	20	307.9	80	1,231.5	193.96	1,166.9	25.0	15.8
140	25	384.8	80	1,231.5	242.45	1,125.6	31.9	19.7
140	30	461.8	80	1,231.5	290.94	1,134.5	37.5	23.6
200	10	314.2	80	2,513.3	197.92	2,447.3	12.5	7.9
200	15	471.2	80	2,513.3	296.88	2,407.6	18.8	11.8
200	20	628.3	80	2,513.3	395.84	2,381.3	25.0	15.8
200	25	785.4	80	2,513.3	494.80	2,297.2	31.9	19.7
200	30	942.5	80	2,513.3	593.76	2,315.4	37.5	23.6
220	10	380.1	80	3,041.1	239.48	2,961.2	12.5	7.9
220	15	570.2	80	3,041.1	359.23	2,913.2	18.8	11.8
220	20	760.3	80	3,041.1	478.97	2,881.4	25.0	15.8
220	25	950.3	80	3,041.1	598.71	2,779.6	31.9	19.7
220	30	1,140.4	80	3,041.1	718.45	2,801.6	37.5	23.6
240	10	452.4	80	3,619.1	285.01	3,524.1	12.5	7.9
240	15	678.6	80	3,619.1	427.51	3,467.0	18.8	11.8

Table A.5. CHT to buffer guide for contained operating system columns.

Contained operating system columns, weight of CHT, and volume of buffer to prepare recommended slurry volume for packing designated column ID.

Column ID, cm	Bed height, cm	Packed volume, L	Recommened slurry volume, L	CHT, kg	Volume buffer, L	Slurry (%v/v)	Slurry (%w/v)
40	10	12.6	50.0	7.92	47.4	25.1	15.8
40	15	18.8	75.0	11.88	71.1	25.1	15.8
40	20	25.1	100.0	15.83	94.9	25.1	15.8
40	25	31.4	125.0	19.79	118.6	25.1	15.8
40	30	37.7	150.0	23.75	142.3	25.1	15.8
45	10	15.9	63.0	10.02	59.8	25.2	15.9
45	15	23.9	95.0	15.03	90.0	25.1	15.8
45	20	31.8	126.0	20.04	119.6	25.2	15.9
45	25	39.8	158.0	25.05	149.4	25.2	15.9
45	30	47.7	189.0	30.06	179.3	25.2	15.9
50	10	19.6	78.0	12.37	73.8	25.2	15.9
50	15	29.5	117.0	18.56	110.7	25.2	15.9
50	20	39.3	156.0	24.74	147.6	25.2	15.9
50	25	49.1	195.0	30.93	184.5	25.2	15.9
50	30	58.9	234.0	37.11	221.4	25.2	15.9
60	10	28.3	112.0	17.81	106.3	25.2	15.9
60	15	42.4	168.0	26.72	159.4	25.2	15.9
60	20	56.5	224.0	35.63	212.5	25.2	15.9
60	25	70.7	280.0	44.53	265.7	25.2	15.9
60	30	84.8	336.0	53.44	318.8	25.2	15.9
70	10	38.5	153.0	24.25	144.6	25.2	15.8
70	15	57.7	230.0	36.37	217.9	25.1	15.8
70	20	77.0	306.0	48.49	289.3	25.2	15.8
70	25	96.2	383.0	60.61	363.1	25.1	15.8
70	30	115.5	459.0	72.74	433.9	25.2	15.8
80	10	50.3	200.0	31.67	189.7	25.1	15.8
80	15	75.4	300.0	47.50	284.6	25.1	15.8
80	20	100.5	400.0	63.33	379.4	25.1	15.8
80	25	125.7	500.0	79.17	474.3	25.1	15.8
80	30	150.8	600.0	95.00	569.1	25.1	15.8
90	10	63.6	254.0	40.08	241.1	25.0	15.8
90	15	95.4	381.0	60.12	361.7	25.0	15.8
90	20	127.2	508.0	80.16	482.2	25.0	15.8
90	25	159.0	635.0	100.20	602.8	25.0	15.8
90	30	190.9	762.0	120.24	723.3	25.0	15.8
100	10	78.5	314.0	49.48	297.7	25.0	15.8
100	15	117.8	471.0	74.22	446.5	25.0	15.8
100	20	157.1	628.0	98.96	595.3	25.0	15.8
100	25	196.3	785.0	12.70	744.2	25.0	15.8
100	30	235.6	942.0	148.44	893.0	25.0	15.8
120	10	113.1	450.0	71.25	426.8	25.1	15.8
120	15	169.6	675.0	106.88	640.3	25.1	15.8
120	20	226.2	900.0	142.50	853.7	25.1	15.8
120	25	282.7	1,125.0	148.13	1067.1	25.1	15.8
120	30	339.3	1,350.0	213.75	1280.5	25.1	15.8
140	10	153.9	615.0	96.98	583.4	25.0	15.8
140	15	230.9	922.0	145.47	875.1	25.0	15.8
140	20	307.9	1,230.0	193.96	1,166.9	25.0	15.8
140	25	384.8	1,537.0	242.45	1,458.6	25.0	15.8
140	30	461.8	1,845.0	290.94	1,750.3	25.0	15.8
200	10	314.2	1,250.0	197.92	1,185.7	25.1	15.8
200	15	471.2	1,875.0	296.88	1,778.5	25.1	15.8
200	20	628.3	3,750.0	395.84	2,371.3	25.1	15.8
200	25	785.4	2,500.0	494.80	2,964.2	25.1	15.8
200	30	942.5	3,125.0	593.76	3,557.0	25.1	15.8
220	10	380.1	3,750.0	239.48	1,440.7	25.0	15.8
220	15	570.2	1,520.0	359.23	2,161.1	25.0	15.8
220	20	760.3	2,280.0	478.97	2,881.4	25.0	15.8
220	25	950.3	3,040.0	598.71	3,601.8	25.0	15.8
220	30	1,140.4	4,560.0	718.45	4,322.1	25.0	15.8
240	10	452.4	1,800.0	285.01	1,707.4	25.1	15.8
240	15	678.6	2,700.0	427.51	2,561.0	25.1	15.8
240	20	904.8	3,600.0	571.01	3,414.7	25.1	15.8
240	25	1,131.0	4,500.0	712.51	4,268.4	25.1	15.8
240	30	1,357.2	5,400.0	855.02	5,122.1	25.1	15.8

A.2 EDTA Complexometric Titration of Hydroxyapatite Column Effluent

Introduction

CHT ceramic hydroxyapatite is a mixed-mode chromatographic media widely used for the purification of proteins and monoclonal antibodies. CHT is a sintered form of hydroxyapatite ($Ca_5(PO_4)_3OH$), which is a form of calcium phosphate. The solubility of CHT increases at an acidic pH (pH <7.0) and it dissolves in its constituent ions (Ca^{2+}, PO_4^{3-}, OH^-). Thus, the solubility of CHT can be estimated by measuring the concentration of the calcium ion, the phosphate ion, or the hydroxyl anion. Of these three ions, calcium can be quantified in a relatively simple and selective manner by complexometric titration (Belcher et al. 1958, Kim and Vipulanandan 2003). The protocol described herein presents the application of EDTA complexometric titration to quantify the total calcium concentration present in the effluent of a given CHT packed bed. The protocol has been modified such that it can be applied to either low buffering or high buffering capacity samples, both typically encountered during purification protocols using CHT.

Experimental Procedure

1. Sample pH Adjustment

The pH of the sample should be adjusted to 10 ± 0.1 before the start of the titration. Depending on the buffering capacity of the sample to be analyzed, the pH may be adjusted as follows:

a) Samples with low total buffering capacity (≤10 mM total buffering species):

   The pH of these samples may be adjusted to 10 ± 0.1 with the ammonia buffering solution (solution C) alone. The volume of the ammonia solution may vary per sample; thus, it is recommended to add solution C while monitoring the sample's pH.

b) Samples with relatively high buffering capacity (>10 mM total buffering species):

   To adjust the pH of these samples it is recommended to use a 10 N NaOH solution to neutralize the buffering species, followed by the addition of solution C to adjust the sample pH to 10 ± 0.1.

2. Adding the WEBT Solution

Once the sample pH is within 10 ± 0.1 proceed to add the working indicator solution (WEBT solution). Typically the amount of WEBT needed will be 2–3 µl EBT per ml of sample.

3. Titration

Once the WEBT solution dissolves and a pink color develops titration can begin. Prior to titration record the current EDTA titrant volume (V_1). Begin titrant addition while monitoring the color of the sample. The titration will come to an end once the color of the sample changes from pink to purple to dark blue and finally to sky blue (end point).

End-point reversibility may occur and is typically observed at calcium concentrations ≥5 ppm. Thus, it is recommended to wait at least 2 min after reaching the titration end point before reading the final volume of titrant (V_2).

4. Calculations

Sample volume 100 ml

$$C_{Calcium} = \frac{1 \text{ ppm}}{0.25 \text{ ml titrant}} (V_2 – V_1)$$

Sample volume 40 ml

$$C_{Calcium} = \frac{1 \text{ ppm}}{0.10 \text{ ml titrant}} (V_2 – V_1)$$

where V_1 and V_2 represent the volume of titrant in ml before and after titration, respectively. $C_{Calcium}$ represents the total calcium concentration (ppm).

Materials and Methods

Materials

Eriochrome black T (EBT), triethanolamine (TEA), ammonium chloride (NH_4Cl), magnesium sulfate heptahydrate ($MgSO_4 \cdot H_2O$), ammonium hydroxide solution (NH_4OH), disodium dihydrogen ethylenediaminetetraacetate (Na_2EDTA), 10 N sodium hydroxide (NaOH) solution, 1 N hydrochloric acid (HCl) solution, and deionized (DI) water (18 Ω/cm)

Titration Solutions

Ammonia Buffering Solution

Solution A

Weigh 1.179 g of Na_2EDTA and 750 mg of $MgSO_4 \cdot H_2O$. Dissolve these in deionized water to a final volume of 50 ml. Label this solution as solution A.

Solution B

Weigh 16.9 g of NH_4Cl and dissolve it in 143 ml of ammonium hydroxide solution. Label this solution as solution B.

Solution C

Mix solution A and solution B in a 250 ml glass volumetric flask. Fill the container to 250 ml with deionized water.

0.01 M EDTA (Titrant) Solution

Weigh 3.723 g of Na_2EDTA and dissolve it in deionized water to a final volume of 1,000 ml.

EBT (Indicator) Solution

Stock solution

Weigh 1 g of EBT and 100 g of TEA. Mix them thoroughly and store. Label this solution as EBT stock.

Working Indicator (WEBT) Solution

Dilute the EBT stock solution with deionized water, in a volume ratio of 1 to 4. Label this solution as WEBT.

Equipment and Supplies

Magnetic stir plate, precision pipets, pH meter, 25 ml glass burette, 150 ml glass beaker, 250 ml glass volumetric flask, PTFE-coated stir bar.

Tips

Sample pH Adjustment

- The volume of 10 N NaOH required typically varies between 0.1–1 ml
- It is recommended to use at least 0.1 ml of the ammonia buffer (solution C) after the NaOH step to adjust the final pH to 10 ± 0.1
- If during NaOH addition the pH goes beyond 10 ± 0.1, use 1 N HCl to bring the pH back down such that the volume of ammonia buffer needed to bring the pH finally to 10 ± 0.1 is no less than 0.1 ml

Adding the WEBT Solution

- A pink color should develop upon addition of the EBT solution. The intensity of the color strongly depends on the calcium concentration and volumes of EBT and ammonia buffer added

Titration

- For a sample volume of 100 ml, 0.25 ml of EDTA solution titrates 1 ppm of calcium
- A sample volume of 100 ml is recommended to increase the accuracy of the method, especially at low calcium concentrations (<1 ppm)

A.3 References

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A.4 Additional Resources

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A.5 Ordering Information

Catalog #	Description
CHT Ceramic Hydroxyapatite, Type I	
158-2000	**CHT Ceramic Hydroxyapatite**, 20 µm, Type I, 10 g
157-0020	**CHT Ceramic Hydroxyapatite**, 20 µm, Type I, 100 g
157-0021	**CHT Ceramic Hydroxyapatite**, 20 µm, Type I, 1 kg
157-0025	**CHT Ceramic Hydroxyapatite**, 20 µm, Type I, 5 kg
158-4000	**CHT Ceramic Hydroxyapatite**, 40 µm, Type I, 10 g
157-0040	**CHT Ceramic Hydroxyapatite**, 40 µm, Type I, 100 g
157-0041	**CHT Ceramic Hydroxyapatite**, 40 µm, Type I, 1 kg
157-0045	**CHT Ceramic Hydroxyapatite**, 40 µm, Type I, 5 kg
158-8000	**CHT Ceramic Hydroxyapatite**, 80 µm, Type I, 10 g
157-0080	**CHT Ceramic Hydroxyapatite**, 80 µm, Type I, 100 g
157-0081	**CHT Ceramic Hydroxyapatite**, 80 µm, Type I, 1 kg
157-0085	**CHT Ceramic Hydroxyapatite**, 80 µm, Type I, 5 kg

Larger volumes and special packaging are available upon request.

CHT Ceramic Hydroxyapatite, Type I Cartridges	
732-4322	**Bio-Scale™ Mini CHT-I**, 40 µm, 1 × 5 ml
732-4324	**Bio-Scale Mini CHT-I**, 40 µm, 5 × 5 ml
CHT Ceramic Hydroxyapatite, Type II Cartridges	
732-4332	**Bio-Scale Mini CHT-II**, 40 µm, 1 × 5 ml
732-4334	**Bio-Scale Mini CHT II**, 40 µm, 5 × 5 ml
CHT Ceramic Hydroxyapatite, Type II	
158-2200	**CHT Ceramic Hydroxyapatite**, 20 µm, Type II, 10 g
157-2000	**CHT Ceramic Hydroxyapatite**, 20 µm, Type II, 100 g
157-2100	**CHT Ceramic Hydroxyapatite**, 20 µm, Type II, 1 kg
157-2500	**CHT Ceramic Hydroxyapatite**, 20 µm, Type II, 5 kg
158-4200	**CHT Ceramic Hydroxyapatite**, 40 µm, Type II, 10 g
157-4000	**CHT Ceramic Hydroxyapatite**, 40 µm, Type II, 100 g
157-4100	**CHT Ceramic Hydroxyapatite**, 40 µm, Type II, 1 kg
157-4500	**CHT Ceramic Hydroxyapatite**, 40 µm, Type II, 5 kg
158-8200	**CHT Ceramic Hydroxyapatite**, 80 µm, Type II, 10 g
157-8000	**CHT Ceramic Hydroxyapatite**, 80 µm, Type II, 100 g
157-8100	**CHT Ceramic Hydroxyapatite**, 80 µm, Type II, 1 kg
157-8500	**CHT Ceramic Hydroxyapatite**, 80 µm, Type II, 5 kg

Catalog #	Description
MPC Ceramic Hydroxyfluoroapatite, Type I	
158-0200	**MPC Ceramic Hydroxyfluoroapatite**, 40 µm, Type I, 10 g
157-0200	**MPC Ceramic Hydroxyfluoroapatite**, 40 µm, Type I, 100 g
157-0201	**MPC Ceramic Hydroxyfluoroapatite**, 40 µm, Type I, 1 kg
157-0205	**MPC Ceramic Hydroxyfluoroapatite**, 40 µm, Type I, 5 kg
Foresight Columns	
732-4737	**Foresight™ MPC™ Type I Column**, 40 µm, 1 ml
732-4757	**Foresight MPC Type I Column**, 40 µm, 5 ml
732-4735	**Foresight™ CHT™ Type I Column**, 40 µm, 1 ml
732-4755	**Foresight CHT Type I Column**, 40 µm, 5 ml
732-4736	**Foresight CHT Type II Column**, 40 µm, 1 ml
732-4756	**Foresight CHT Type II Column**, 40 µm, 5 ml
Foresight Plates*	
732-4785	**Foresight MPC Type I Plates**, 40 µm, 20 µl
732-4716	**Foresight CHT Type I Plates**, 40 µm, 20 µl
732-4718	**Foresight CHT Type II Plates**, 40 µm, 20 µl

* Package size: 2 x 96-well plates.

Foresight RoboColumn Units**	
732-4822	**Foresight CHT Type I RoboColumn Units,** 40 µm, 200 µl
732-4823	**Foresight CHT Type I RoboColumn Units**, 40 µm, 600 µl
732-4825	**Foresight CHT Type II RoboColumn Units**, 40 µm, 200 µl
732-4826	**Foresight CHT Type II RoboColumn Units**, 40 µm, 600 µl
732-4828	**Foresight MPC Type I RoboColumn Units**, 40 µm, 200 µl
732-4829	**Foresight MPC Type I RoboColumn Units**, 40 µm, 600 µl

** Package size: one row of eight columns

A.6 CHT FAQs

1. **What are the main advantages of using CHT ceramic hydroxyapatite support?** CHT ceramic hydroxyapatite is a chromatographic, mixed-mode support that offers a unique selectivity for closely related molecules. Its mixed-mode mechanism of separation allows flexibility in optimizing a wide variety of separations. CHT will bind samples in the presence of urea and other chaotropic agents.
2. **What buffers are used with CHT ceramic hydroxyapatite?** A typical binding buffer is 5 mM phosphate buffer at pH 6.8. Basic proteins can be eluted with a step or linear gradient using pH 7.2 phosphate buffer with 1 M NaCl. Acidic proteins and nucleic acids are eluted with a step or linear gradient using 500 mM phosphate. It is critical that the pH of both the buffers and the effluent are at pH 6.5 or higher.
3. **What if the sample does not bind or elute in the recommended buffers?** Rarely, some proteins do not bind to the CHT support in 5 mM phosphate and some will not elute in 500 mM phosphate buffer. If necessary, reduce the pH or lower the phosphate concentration to 1 mM phosphate, but include a buffer that will maintain the pH above 6.5 and add Ca^{++} to the buffer. Note that phosphate concentrations below 5 mM may result in a shorter column lifetime. CFT™ ceramic fluroapatite can be used as an alternative for purifying molecules at a pH as low as 6. Use an alternative method of purifying proteins that will not elute in high phosphate buffers.
4. **What is the difference between CHT Type I and Type II supports?** CHT Type I and Type II supports are chemically identical but are sintered at different temperatures, resulting in a difference in pore size and surface chemistry. CHT Type I has a high capacity for both acidic and basic proteins. However, CHT Type II will typically bind basic proteins well but has a low selectivity for acidic proteins.
5. **Is there a difference in performance with sodium or potassium phosphate buffers?** CHT ceramic hydroxyapatite works well with either sodium or potassium phosphate buffers. However, anhydrous sodium phosphate buffer salts should be avoided for most applications because they may contain pyrophosphates, which can reduce capacity of the support.
6. **What buffer conditions should be avoided when using CHT ceramic hydroxyapatite?** All CHT buffers should contain 5 mM phosphate and the pH should be maintained at pH 6.5 or higher. Buffer components that can damage the support include chelating agents, buffers below 6.5, and metal ions that have a low solubility with phosphate. Washing with pure water extensively will slowly dissolve the support. Using 1 M NaOH immediately after a high phosphate buffer regeneration step is not recommended; use at least ½ CV of water or low phosphate buffer between the high phosphate and the 1 M NaOH regeneration steps. When using buffers containing calcium, use 3 CV of calcium-free buffer before switching to a high phosphate buffer. In addition, washing the column with 3 CV of a low-phosphate buffer after elution and before a high-phosphate strip will prolong CHT lifetime.
7. **How do I ensure longest CHT column life?** Include 5 mM phosphate in all buffers, including the storage buffers, and maintain the pH above 6.5. Always select the highest pH buffer that will work for the application. Addition of calcium at ppm levels will also increase column robustness; consult your Bio-Rad representative for more information. pH excursions will occur when using NaCl as an initial elution buffer due to the elution of hydronium ions, so adding a co-buffer such as MES will dramatically improve robustness. In addition, Bio-Rad has developed a proprietary system for eliminating pH excursions during elution called SNS (surface neutralization system). Consult your Bio-Rad representative for a full discussion of this simple technique. Always regenerate the CHT support with 500 mM phosphate to remove adsorbed proteins and store the column in 0.1 M NaOH. If the NaOH concentration is below 0.1 N, include 5 mM phosphate. Always protect a column stored in NaOH from air to prevent the precipitation of carbonates on the surface of the column. Avoid sudden changes in flow rate to minimize pressure shocks that can damage the CHT bed.

8. **What are the important considerations in packing a CHT column?** A CHT bed is completely stable once it is packed into a column as the resin is incompressible; however the particles are susceptible to damage from shearing during column packing. To avoid this, use a 30% and no greater than 50% v/v slurry when packing a column, use a low-impact impeller to mix slurries, and use diaphragm pumps to transfer a slurry. It is very important to ensure that the column is level before packing the column. When mixing the CHT in a column that does not have gas-assisted mixing, resuspend the support gently by mixing the buffer with minimal contact. Use a 10–13 µm bed support for the 40 µm CHT support, and a 20 µm bed support for the 80 µm CHT support. Use flow packing to pack the column rather than letting the support settle by gravity. Position the inlet adaptor so that it does not touch the top of the CHT bed. Refer to the directions for each column manufacturer for additional information.
9. **What causes discoloration of the CHT support and how can this be avoided?** Metal ions such as Fe and Ni from media and running buffers can integrate irreversibly into the CHT matrix and cause discoloration of the CHT bed. This will not necessarily affect the performance of the column. But this can be avoided by using a CHT guard column to minimize any metal contamination in the sample or buffers or by adding micromolar levels of calcium-saturated EDTA to the suspect solutions. This is the only time that EDTA is recommended for use with CHT.
10. **What is expected HETP and asymmetry of CHT?** When equilibrated with phosphate buffered saline and tested with a 2.5% test of 1 M NaCl in phosphate buffer, the typical HETP values for columns with a diameter greater than 5 cm for 40 µm CHT range from 0.016–0.021 cm, and the range for 80 µm is 0.032–0.041 cm. Asymmetry values from 0.8–2.3 are usually acceptable.
11. **Why are acceptable HETP and asymmetry values difficult to obtain when working with small diameter columns?** Since the CHT support is rigid, it cannot be packed as efficiently in a column with a diameter less than 2.5 cm compared to larger diameter columns.
12. **What is the expected life of a CHT column?** If properly packed and maintained at a pH above 6.5, a CHT column will last at least 40 cycles. If care is taken to avoid damaging the support, it can be successfully packed and repacked 25 times or more.
13. **What is the shelf life of CHT ceramic hydroxyapatite?** CHT support is stable for 5 years from the date of manufacture provided it is stored in the original sealed container between 4 and 30˚C. A packed column should be stored in a sealed column in 0.1 M NaOH containing 5 mM phosphate at room temperature for up to 2 years.
14. **Which type of hydroxyapatite works best for nucleic acid samples?** Both CHT Type I and Type II can be used for nucleic acid removal and plasmid purification. DNA grade Bio-Gel® HTP hydroxyapatite, a type of crystalline hydroxyapatite, is recommended for other applications, such as the separation of single-stranded and double-stranded DNA.
15. **How might co-buffers affect the salt concentrations needed in the elution buffer?** With many co-buffers, the acid form is added first and the solution is titrated to the final pH with NaOH. In this case, if the salt concentration for elution has already been predetermined prior to co-buffer studies, the amount of NaCl added to the final eluant buffer composition may need to be reduced because the co-buffer neutralization process itself adds sodium.
16. **What if 0.5 M sodium phosphate does not adequately clean the resin?** Use a higher concentration of potassium phosphate.

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