



## Ceramic Hydroxyapatite Application Guide for Process Development and Scale Up





<b>Chapter 1 Introduction.....</b>	<b>3</b>	<b>Chapter 5 Column Packing Protocols .....</b>	<b>29</b>
<b>Chapter 2 Product Description .....</b>	<b>7</b>	5.1	General Guidelines .....
2.1	What is CHT Ceramic Hydroxyapatite?.....	8	30
2.2	CHT Ceramic Hydroxyapatite Selection Criteria.....	8	5.2
2.3	What is MPC Ceramic Hydroxyfluoroapatite?.....	10	General Handling and Slurry Preparation .....
2.4	Purity.....	10	31
			5.2.1
			Recommended Column Packing Buffers .....
			31
			5.2.2
			Recommended Slurry Mixing .....
			31
			5.2.3
			Recommended Slurry Transfer.....
			32
<b>Chapter 3 Chromatography.....</b>	<b>13</b>	5.3	Open-Column Packing.....
3.1	CHT Ceramic Hydroxyapatite Protein		32
	Binding Mechanism.....		5.4
			Closed-Column Packing .....
			33
3.2	CHT Ceramic Hydroxyapatite Chemistry and		5.4.1
	Considerations for Process Development .....		Media Transfer Stations.....
			33
			5.4.2
			Suction Transfer Method .....
			34
			5.4.3
			Unpacking Method for Columns with
			Adjustable Inlet Adaptors .....
			34
3.2.1	CHT Chemistry: Role of the Common Ion Effect .....		5.5
			Media Packing Station Methods .....
			35
3.2.2	CHT Surface Chemistry and the Influence of		5.6
	Buffer Cation Concentration Changes.....		Packed Column Qualification .....
			36
3.3	Method Development Guidelines.....		5.7
			Laboratory-Scale Column Packing Protocols .....
			37
3.3.1	Chemical Compatibility/Load Preparation .....		5.7.1
			Reference Methods for Packing Small-Scale CHT
			Columns.....
			37
3.3.2	Elution .....		5.7.2
			Slurry Transfer and Column Packing .....
			37
3.3.3	Use of Co-Buffers During Elution .....		5.7.3
			Packed Column Evaluation/Qualification .....
			38
3.3.4	Use of Surface Neutralization System During Elution .....		5.7.4
			Post-Column Packing Considerations .....
			38
3.3.5	Calcium Addition Increases CHT Column Life .....		5.7.5
			Tips to Ensure Proper Small-Scale CHT Column Packing ..
			38
3.3.6	Phosphate.....		5.7.6
			Summary: CHT Packing – Things to Remember .....
			38
3.3.7	Trace Metal Contamination.....		5.7.7
			Troubleshooting Tips.....
			39
3.3.8	Preparing Phosphate Buffers .....		<b>Appendices .....</b>
			<b>41</b>
3.3.9	Other Suggested Washes .....		A.1
			CHT Ceramic Hydroxyapatite
			Resin and Slurry Calculations .....
			42
3.3.10	Scouting Protocol IA: IgG.....		A.2
			EDTA Complexometric Determination of Calcium
			Concentrations in Column Effluents .....
			42
3.3.11	Scouting Protocol IB: IgG.....		A.3
			References.....
			44
3.3.12	Scouting Protocol II: General Phosphate Elution .....		A.4
			Additional Resources .....
			44
3.3.13	Protocol III: Plasmids .....		A.5
			Ordering Information .....
			45
3.3.14	Protocol IV: Acidic Proteins .....		A.6
			CHT FAQs.....
			46
3.3.15	Protocol V: Viruses .....		
3.3.16	Scouting Tips .....		
3.3.17	Optimization Tips for Protocols I–V .....		
<b>Chapter 4 Regeneration, Sanitization,</b>			
<b>and Storage .....</b>	<b>25</b>		
4.1	Regeneration .....		
4.2	Sanitization .....		
4.3	Storage .....		





**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 25 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. Bio-Rad produces three types of ceramic hydroxyapatite, CHT Types I and II and CHT XT. The temperature of the sintering step in part determines whether the ceramic hydroxyapatite is CHT Type I or Type II or CHT XT. CHT is a mixed-mode resin. Details of the binding mechanism are described in Chapter 3.

CHT is a leading medium for the purification 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).

**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 four main topics:

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

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 Bio-Rad's Technical Support.

### Technical Support

The Bio-Rad Technical Support Department in the U.S. is open Monday through Friday, 5:00 AM to 5:00 PM, Pacific time.

Phone: 1-800-424-6723, option 2

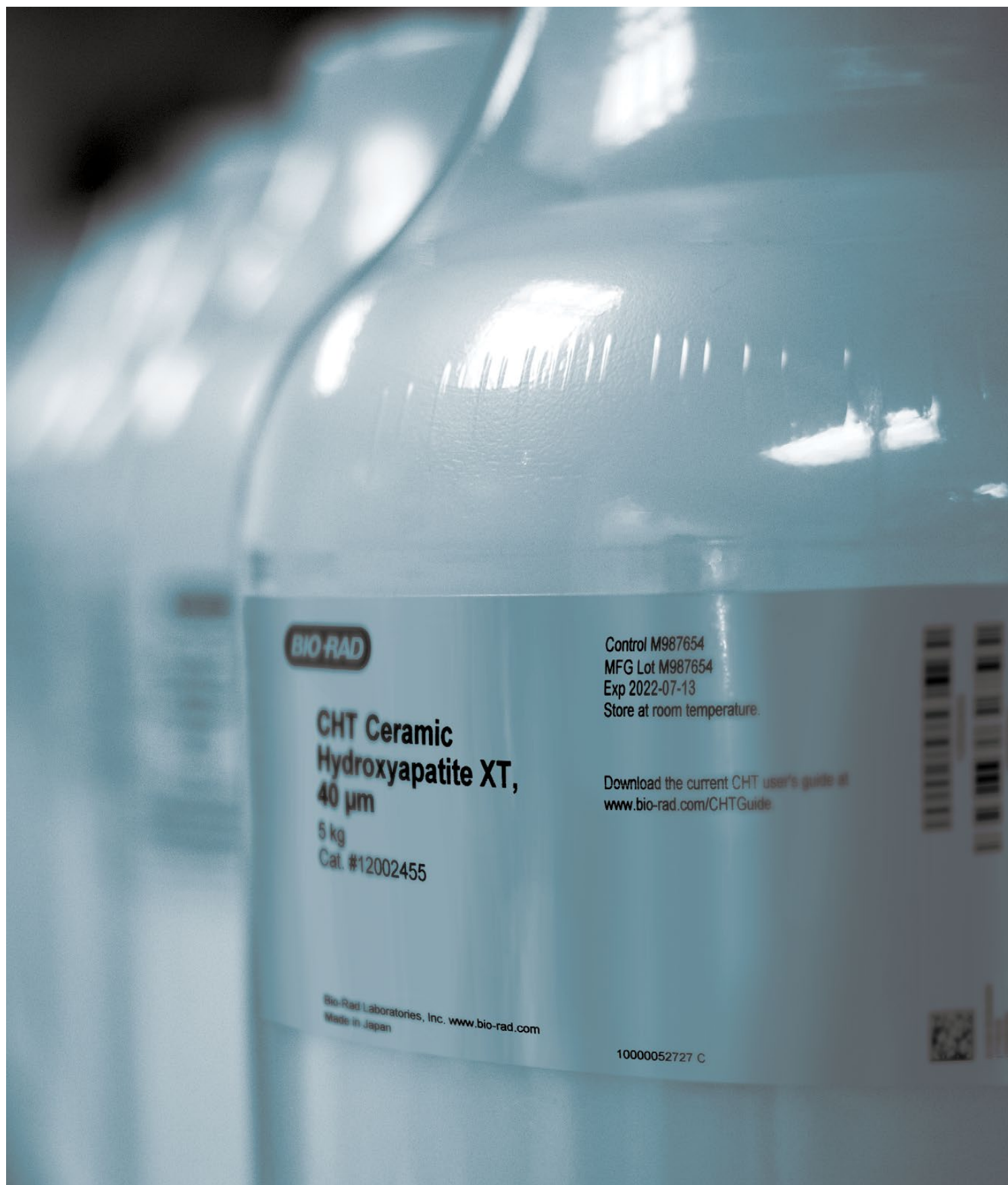
Email: [support@bio-rad.com](mailto:support@bio-rad.com) (U.S./Canada only)

For technical assistance outside the U.S. and Canada, contact your local technical support office or click the Contact us link at [bio-rad.com](http://bio-rad.com).

To contact your process chromatography specialist, email [process@bio-rad.com](mailto:process@bio-rad.com).









**CHAPTER 2**

# Product Description

## 2.1 What is CHT Ceramic Hydroxyapatite?

Hydroxyapatite,  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ , 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 that appear to be homogeneous by electrophoretic and other chromatographic techniques.

CHT Ceramic Hydroxyapatite is made from purely inorganic compounds and is free of any animal/plant components. The manufacturing equipment used is also not exposed to any materials with animal/plant origins. The product is noninfectious and nonhazardous.

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.



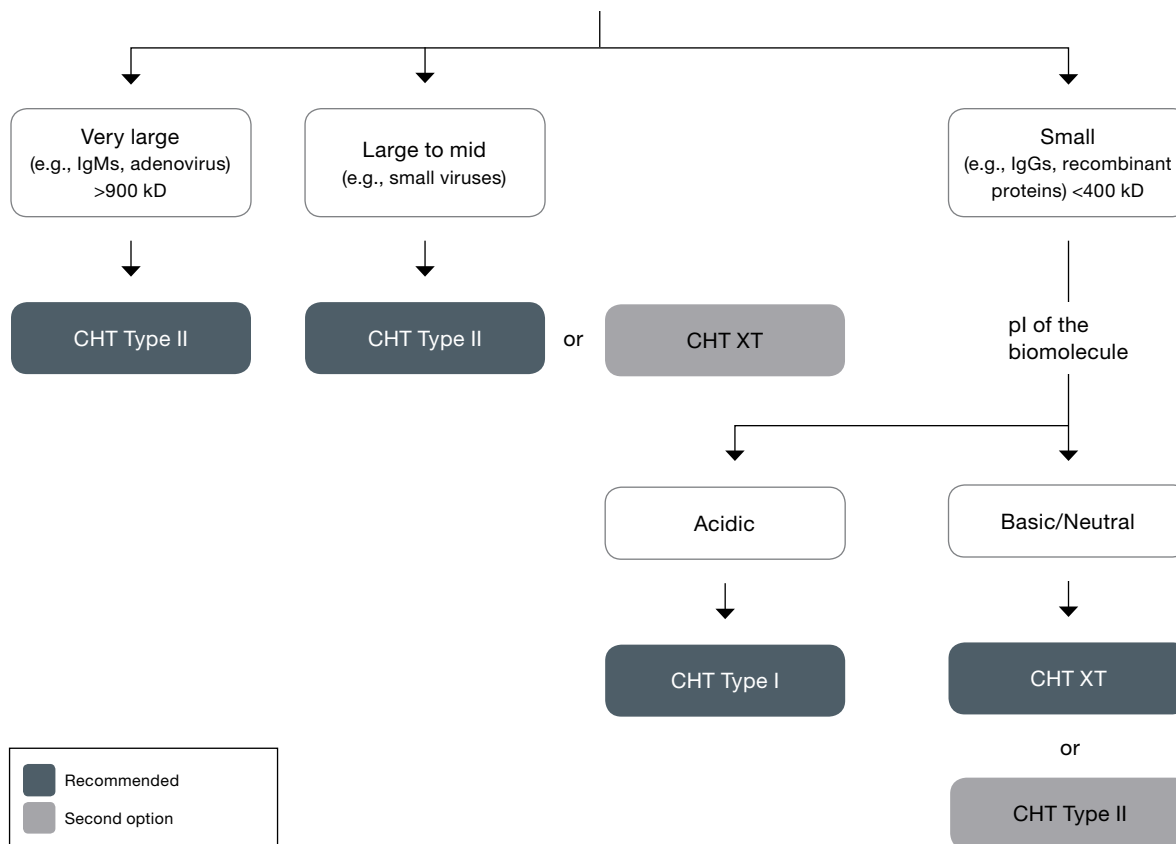
CHT Ceramic Hydroxyapatite Types I and II are available in two particle sizes, 40 and 80  $\mu\text{m}$ .\* CHT Ceramic Hydroxyapatite XT is available in the 40  $\mu\text{m}$  particle size. CHT Type I has the highest overall protein binding capacity among the three varieties. It also has the best capacity for acidic proteins in general. CHT XT is the newest member of the CHT family. It is similar in application usage to CHT Type I and is a more stable matrix, providing longer usable column lifetime at process scale. CHT Type II, with its large pore size, is the most effective for purification of large biomolecules such as IgM and viruses.

\* CHT Ceramic Hydroxyapatite Types I and II are also available in a 20  $\mu\text{m}$  particle size, though we do not recommend using this size in process-scale columns.

## 2.2 CHT Ceramic Hydroxyapatite Selection Criteria

Choosing the appropriate CHT Ceramic Hydroxyapatite from the three available types depends on multiple criteria, as shown in the CHT selection tree. Use this chart to select the CHT Screening Media most pertinent for your application.

## Size of the molecule to be purified



## Notes

Product	Scenario Description
CHT Type I, 40 µm	<ul style="list-style-type: none"> <li>Particle size 40 µm</li> <li>Sinter temperature 400°C</li> <li>Smallest pore size among the CHT types (same pore size as Type I, 80 µm)</li> <li>Highest DBC due to the largest surface area among the CHT types</li> <li>Binds molecules in the broadest range of pI values</li> <li>Strongest binding to acidic molecules among all CHT types</li> </ul>
CHT Type I, 80 µm	<ul style="list-style-type: none"> <li>Particle size 80 µm</li> <li>Sinter temperature 400°C</li> <li>Smallest pore size among the CHT types (same pore size as Type I, 40 µm)</li> <li>Highest DBC due to the largest surface area among the CHT types. May require slower flow to achieve similar DBC to Type I, 40 µm. Pore size is same as Type I, 40 µm, but mass transfer may be less efficient at faster flow due to the larger particle size</li> </ul>
CHT Type II, 40 µm	<ul style="list-style-type: none"> <li>Particle size 40 µm</li> <li>Sinter temperature 700°C</li> <li>Largest pore size among the CHT types (same pore size as Type II, 80 µm)</li> <li>Lowest DBC due to the smallest surface area among the CHT types</li> <li>Binds well to molecules of neutral and basic nature (similar to MPC and CHT XT)</li> </ul>
CHT Type II, 80 µm	<ul style="list-style-type: none"> <li>Particle size 80 µm</li> <li>Sinter temperature 700°C</li> <li>Largest pore size among the CHT types (same pore size as Type II, 40 µm)</li> <li>Lowest DBC due to the smallest surface area among the CHT types</li> <li>Enables faster flow rates due to the larger particle size</li> </ul>
CHT XT, 40 µm	<ul style="list-style-type: none"> <li>Particle size 40 µm</li> <li>Sinter temperature 600°C</li> <li>Robust matrix providing longer usable column life compared to Type I, 40 µm, at process scale</li> <li>Pore size is slightly larger than Type I and smaller than Type II</li> <li>Selectivity is between Type I and Type II</li> <li>Binds well to molecules of neutral and basic nature (similar to MPC and Type II)</li> </ul>

## 2.3 What is MPC Ceramic Hydroxyfluoroapatite?

MPC, like CHT, is macroporous ceramic hydroxyapatite with 25% of the hydroxyl groups substituted with fluoride, lending to its name hydroxyfluoroapatite,  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_{1.5}(\text{F})_{0.5}$ . 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	XT	Type I
Functional groups	$\text{Ca}^{2+}$ , $\text{PO}_4$ , OH	$\text{Ca}^{2+}$ , $\text{PO}_4$ , OH	$\text{Ca}^{2+}$ , $\text{PO}_4$ , OH	$\text{Ca}^{2+}$ , $\text{PO}_4$ , OH, F
Observed dynamic binding capacity lysozyme (Lys)	25–40 mg Lys/g CHT	12.5–20 mg Lys/g CHT	17.4–24.8 mg Lys/g CHT	25–40 mg Lys/g MPC
Nominal mean particle size	20 ± 2, 40 ± 4, and 80 ± 8 µm	20 ± 2, 40 ± 4, and 80 ± 8 µm	40 ± 4 µm	40 ± 4 µm
Tap-settled density* (g/ml tap settled bed)	0.63 g/ml	0.63 g/ml	0.67 g/ml**	0.72 g/ml

	CHT Characteristics			MPC Characteristics
	Type I	Type II	XT	Type I
Observed dynamic binding capacity IgG	25–60 mg polyclonal IgG/ml CHT***	15–25 mg polyclonal IgG/ml CHT***	≥60 mg/ml mAb G <sup>†</sup>	25–50 mg polyclonal IgG/ml MPC***
Typical linear flow rate range	50–400 cm/hr	50–400 cm/hr	50–400 cm/hr	50–400 cm/hr
pH stability	6.5–14	6.5–14	6.5–14	6.5–14
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	At least 1 year in 1 N NaOH
Regeneration	0.4 M sodium phosphate, pH 7–7.5; 1 M trisodium phosphate, pH 11–12. If higher concentrations of sodium phosphate needed, use 0.4–1 M potassium phosphate	0.4 M sodium phosphate, pH 7–7.5; 1 M trisodium phosphate, pH 11–12. If higher concentrations of sodium phosphate needed, use 0.4–1 M potassium phosphate	0.4 M sodium phosphate, pH 7–7.5; 1 M trisodium phosphate, pH 11–12. If higher concentrations of sodium phosphate needed, use 0.4–1 M potassium phosphate	0.4 M sodium phosphate, pH 7–7.5; 1 M trisodium phosphate, pH 11–12. If higher concentrations of sodium phosphate needed, use 0.4–1 M potassium phosphate
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	121°C, 20 min in phosphate buffer, pH 7
Sanitization	1–2 N NaOH	1–2 N NaOH	1–2 N NaOH	1–2 N NaOH
Recommended column storage	0.1 N NaOH	0.1 N NaOH	0.1 N NaOH	0.1 N NaOH
Shelf life (dry, unused material)	5 years stored dry, sealed, and at room temperature	5 years stored dry, sealed, and at room temperature	5 years stored dry, sealed, and at room temperature	5 years stored dry, sealed, and at room temperature

\* Under ideal conditions

\*\* Determined from preliminary data.

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

† At 100 cm/hr with 5 mM sodium phosphate, 25 mM sodium chloride, pH 7 in a 0.5 x 9.5 cm column.

## 2.4 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%
Lead	<0.001%
Cadmium	<0.0001%
Barium	<0.001%
Arsenic	<0.001%







**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 positively charged 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.

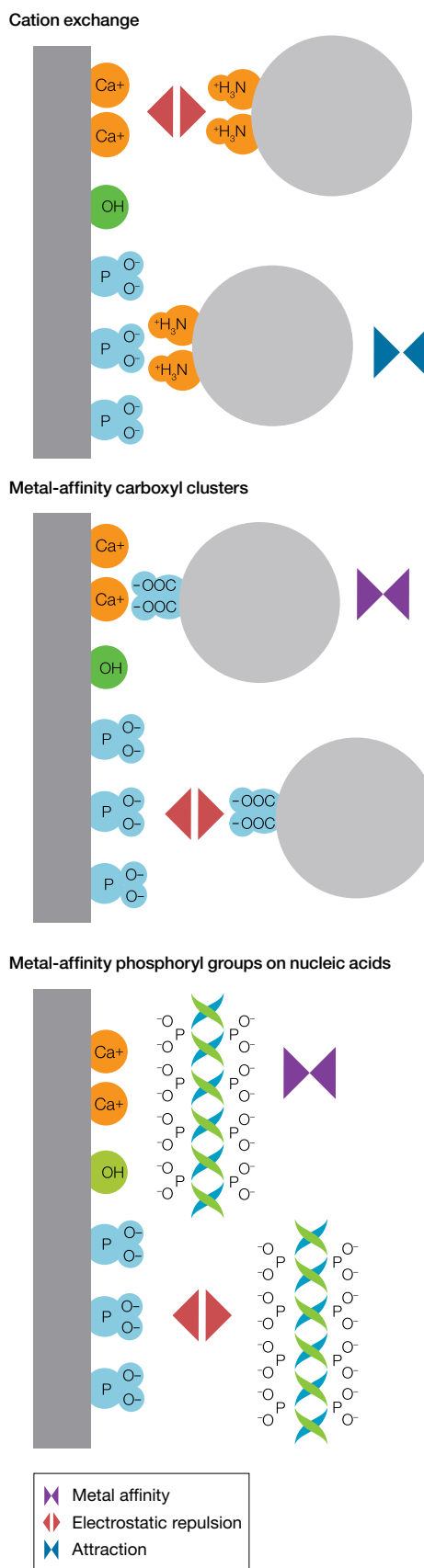


Fig. 3.1. Schematic representation of CHT binding mechanisms.

## 3.2 CHT Ceramic Hydroxyapatite Chemistry and Considerations for Process Development

### 3.2.1 CHT Chemistry: Role of the Common Ion Effect



The commonly accepted value of the solubility product  $K_{sp}$  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.
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

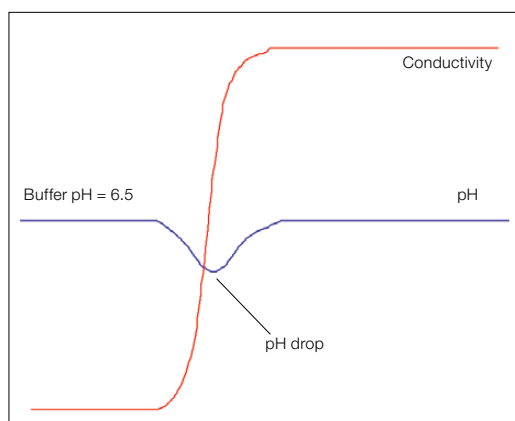


Fig. 3.3. pH drop observed during step increases in solvent cation concentration.

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 exposure to acidic conditions below this point can have a deleterious effect on 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.

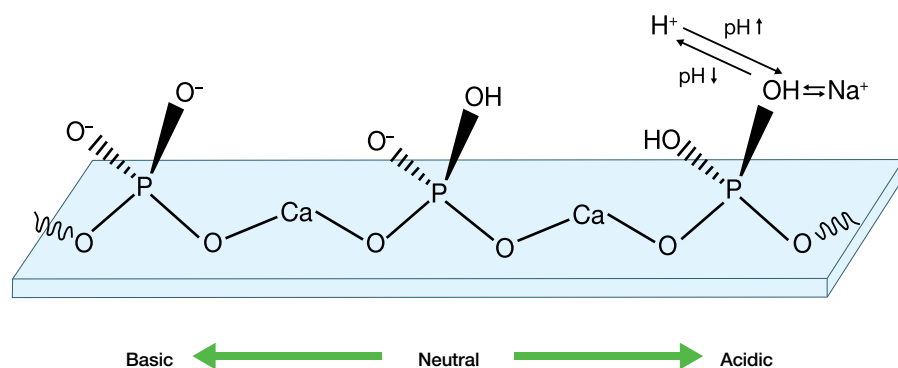


Fig. 3.2. Protonation/deprotonation reactions at the surface of CHT.

### 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 five 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 3.3.10–3.3.15. 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 N NaOH\*
- 6 M guanidine-HCl
- 8 M urea
- 100% acetonitrile
- 100% ethanol
- 1% SDS and other surfactants (not calcium compatible)
- 4 M NaCl
- 1 M potassium phosphate\*\*
- 0.5 M sodium phosphate\*\*

\* No Ca or PO<sub>4</sub> 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) or MOPS (3-(N-morpholino)-propane sulfonic acid) to the elution buffer significantly decreases the exposure of CHT to released protons.

This phenomenon is illustrated in Figure 3.5.

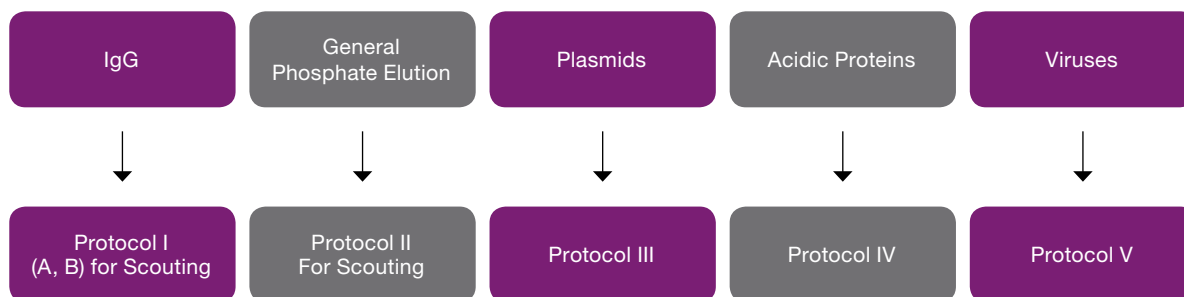
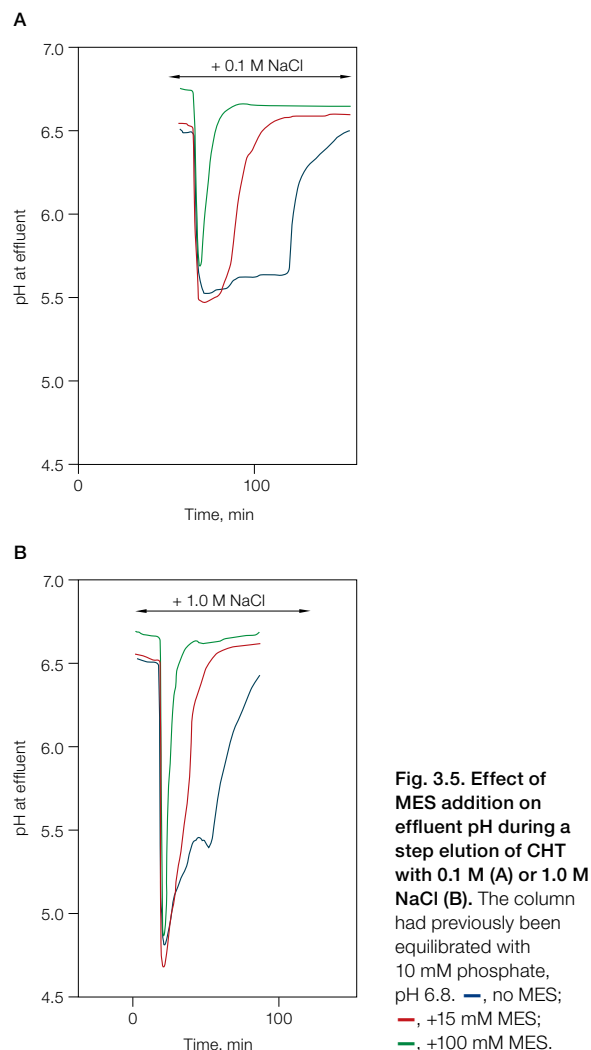


Fig. 3.4. General protocol selection.



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.

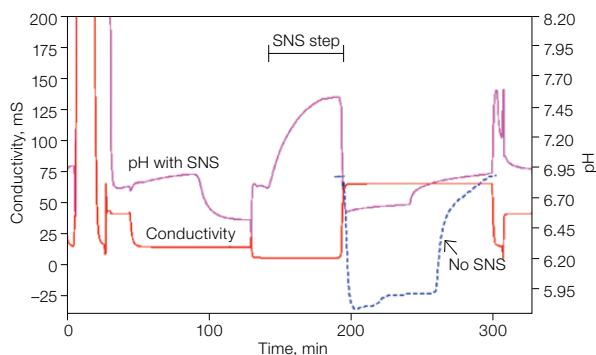
**Note:** With many co-buffers, the acid form is added first and the solution is titrated to the final pH with NaOH. In this case, if the salt concentration for elution has already been predetermined prior to co-buffer studies, the amount of added NaCl to the final eluant buffer composition may need 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 patented\* technology called surface neutralization system, 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.



**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.

The tris acts to keep the overall solution slightly alkaline, providing a 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 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.

\* 8951807, 9212203, 9914749, and others worldwide

### 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, at least 5 mM phosphate should be included in all buffer solutions. 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, contact Bio-Rad for ways to address this.

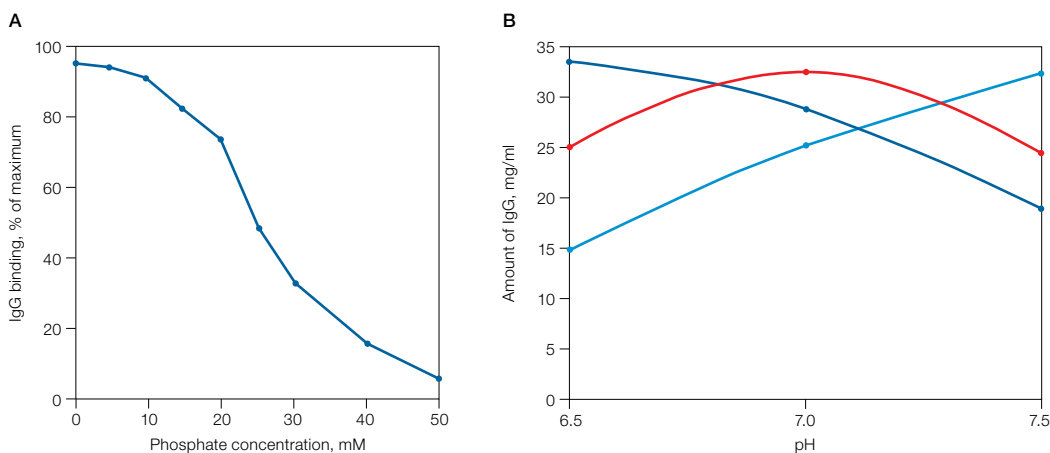
### 3.3.8 Preparing Phosphate Buffers

Hydrated phosphate salts should be used in all buffer preparations. Anhydrous 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 dodecahydrates as these spontaneously decompose to heptahydrates, which results in the same mass of buffer salt having an increasing amount of phosphate over time.

If high concentrations of calcium are included in the phosphate buffer, a precipitate can form over time. When designing a new buffer, it is recommended to let the final preparation sit for 1–2 days to ensure no precipitation forms.

### 3.3.9 Other Suggested Washes

In column use, when concentrated phosphate is immediately followed by concentrated hydroxide, occasional formation of tricalcium phosphate has been observed. This can be mitigated by separating the two solutions with 0.5 CV of a low-phosphate or water wash. Also, if the calcium level is greater than or equal to 10 ppm at the end of elution, wash with 0.5 CV of water or a low-phosphate solution prior to the high-phosphate strip.



**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 150 cm/hr for all steps

Step	Mobile Phase	Column Volumes	Comments
Wash	H <sub>2</sub> O	0.5	Reduce the concentration of 1.0 N NaOH from the sanitization step to prevent formation of trisodium phosphate precipitates
Pre-Equilibrate	400 mM NaPO <sub>4</sub> , pH 6.5	3	Rapidly shifts the pH within the column
Equilibrate	10 mM NaPO <sub>4</sub> , 4–8 ppm Ca <sup>++</sup> , pH 6.5	10	Ca <sup>++</sup> supplementation improves the chemical stability of hydroxyapatite (Section 3.3.5)
Load	Clarified sample 10 mM NaPO <sub>4</sub> , 4–8 ppm Ca <sup>++</sup> , pH 6.5		Maintains chemical stability of the hydroxyapatite
Wash	10 mM NaPO <sub>4</sub> , 4–8 ppm Ca <sup>++</sup> , pH 6.5	5	Maintains chemical stability of the hydroxyapatite
SNS	25 mM Tris 25 mM NaCl, 5 mM NaPO <sub>4</sub> , 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 <sub>4</sub> , linear gradient 0–2 M NaCl, pH 6.5 + 15 ppm Ca <sup>++</sup>	20	If elution does not occur, increase phosphate concentration
Clean	400 mM NaPO <sub>4</sub> , pH 7.0–7.5	5	High phosphate elutes retained contaminants such as aggregates, endotoxin, DNA, and Protein A
Wash	H <sub>2</sub> O	0.5	Reduces the phosphate concentration to prevent formation of trisodium phosphate precipitates
Sanitize	1.0 N NaOH	5	Sanitization

### 3.3.11 Scouting Protocol IB: IgG

Flow rate of 150 cm/hr for all steps

Step	Mobile Phase	Column Volumes	Comments
Wash	H <sub>2</sub> O	0.5	Reduce the concentration of 1.0 N NaOH from the sanitization step to prevent formation of trisodium phosphate precipitates
Pre-Equilibrate	400 mM NaPO <sub>4</sub> , pH 6.5	3	Rapidly shifts the pH within the column
Equilibrate	10 mM NaPO <sub>4</sub> , 8–12 ppm Ca <sup>++</sup> , 100 mM NaCl, pH 6.5	10	Ca <sup>++</sup> 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 <sub>4</sub> , 4–8 ppm Ca <sup>++</sup> , pH 6.5		Maintains chemical stability of the hydroxyapatite
Wash	10 mM NaPO <sub>4</sub> , 4–8 ppm Ca <sup>++</sup> , pH 6.5	5	Maintains chemical stability of the hydroxyapatite
Elute	10 mM NaPO <sub>4</sub> , 15 ppm Ca <sup>++</sup> , linear gradient 0.1–2 M NaCl, pH 6.5 + co-buffer	20	Co-buffer adsorbs the majority of released protons (Section 3.3.3). Ca <sup>++</sup> supplementation improves the chemical stability of hydroxyapatite (Section 3.3.5) by the common ion effect
Clean	400 mM NaPO <sub>4</sub> , pH 7.0–7.5	5	High phosphate elutes retained contaminants such as aggregates, endotoxin, DNA, and Protein A
Wash	H <sub>2</sub> O	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 150 cm/hr for all steps

Step	Mobile Phase	Column Volumes	Comments
Wash	H <sub>2</sub> O	0.5	Reduce the concentration of 1.0 N NaOH from the sanitization step to prevent formation of trisodium phosphate precipitates
Pre-Equilibrate	400 mM NaPO <sub>4</sub> , pH 6.8	3	Rapidly shifts the pH within the column
Equilibrate	5 mM NaPO <sub>4</sub> , 20 ppm Ca <sup>++</sup> , 150 mM NaCl, pH 6.8	10	Ca <sup>++</sup> 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 <sub>4</sub> , 20 ppm Ca <sup>++</sup> , 150 mM NaCl, pH 6.8		Maintains chemical stability of the hydroxyapatite
Wash	5 mM NaPO <sub>4</sub> , 20 ppm Ca <sup>++</sup> , 150 mM NaCl, pH 6.8	5	Maintains chemical stability of the hydroxyapatite
Elute	Linear gradient 5 mM NaPO <sub>4</sub> + 20 ppm Ca <sup>++</sup> – 500 mM NaPO <sub>4</sub> + 12 ppm Ca <sup>++</sup> , 150 mM NaCl, pH 6.8		
Clean	400 mM NaPO <sub>4</sub> , pH 6.8	5	High phosphate elutes retained contaminants such as aggregates, endotoxin, or DNA
Wash	H <sub>2</sub> O	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 150 cm/hr for all steps

Step	Mobile Phase	Column Volumes	Comments
Sanitize	1.0 N NaOH	5	Sanitization
Wash	H <sub>2</sub> O	0.5	Reduce the concentration of 1.0 N NaOH from the sanitization step to prevent formation of trisodium phosphate precipitates
Pre-Equilibrate	400 mM NaPO <sub>4</sub> , pH 7.0	3	Rapidly shifts the pH within the column
Equilibrate	10 mM NaPO <sub>4</sub> , 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 <sub>4</sub> , 1 mM EDTA*, pH 7.0	5	Removes unbound components
Elute	Linear gradient 0–400 mM NaPO <sub>4</sub> , 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 of 150 cm/hr for all steps

Step	Mobile Phase	Column Volumes	Comments
Wash	H <sub>2</sub> O	0.5	Reduce the concentration of 1.0 N NaOH from the sanitization step to prevent formation of trisodium phosphate precipitates
Pre-Equilibrate	400 mM NaPO <sub>4</sub> , pH 6.7	3	Rapidly shifts the pH within the column
Equilibrate	5 mM NaPO <sub>4</sub> , 50–100 mM NaCl, 12–20 ppm Ca <sup>++</sup> , pH 6.7	10	Ca <sup>++</sup> 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 <sub>4</sub> , 50–100 mM NaCl, 12–20 ppm Ca <sup>++</sup> , pH 6.7	2–8	Maintains chemical stability of the hydroxyapatite
Wash	5 mM NaPO <sub>4</sub> , 50–100 mM NaCl, 12–20 ppm Ca <sup>++</sup> , pH 6.7	5	Maintains chemical stability of the hydroxyapatite
Elute	Linear gradient from 5 mM NaPO <sub>4</sub> , 150 mM NaCl, 12–20 ppm Ca <sup>++</sup> , pH 6.7 to 120 mM NaPO <sub>4</sub> , 50–100 mM NaCl, pH 6.7	20	
Clean	400 mM NaPO <sub>4</sub> , pH 6.7	5	High phosphate elutes retained contaminants such as aggregates, endotoxin, or DNA
Wash	H <sub>2</sub> O	0.5	Reduces phosphate levels to prevent formation of trisodium phosphate precipitates
Sanitize	1.0 N NaOH	5	Sanitization

### 3.3.15 Protocol V: Viruses

Flow rate of 150 cm/hr for all steps

Step	Mobile Phase	Column Volumes	Comments
Wash	600 mM NaPO <sub>4</sub> , pH 7.2	5	Cleans the media of any contaminants
Equilibrate	10 mM NaPO <sub>4</sub> , pH 7.2	10	Rapidly shifts the pH within the column
Load	10 mM NaPO <sub>4</sub> , pH 7.2	10	The loading buffer maintains the chemical stability of hydroxyapatite
Wash	10 mM NaPO <sub>4</sub> , pH 7.2	10	Maintains chemical stability of hydroxyapatite
Elute	Gradient elution from 10–600 mM NaPO <sub>4</sub>	15	If precipitation of the phosphate stock is observed, reduce concentration to 0.5 M or use potassium phosphate
Wash	600 mM NaPO <sub>4</sub> , pH 7.2	5	High phosphate elutes retained contaminants such as aggregates, endotoxins, or DNA
Clean	0.8 M potassium phosphate, pH 7–7.5	3	Removes any remnants
Sanitize	1.0 N NaOH	5	Sanitization

#### 3.3.16 Scouting Tips

The slope and amplitude of any gradient can be adjusted based on initial results. In general, retention time of proteins increases with increasing pI.

#### 3.3.17 Optimization Tips for Protocols I-V

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 400 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.

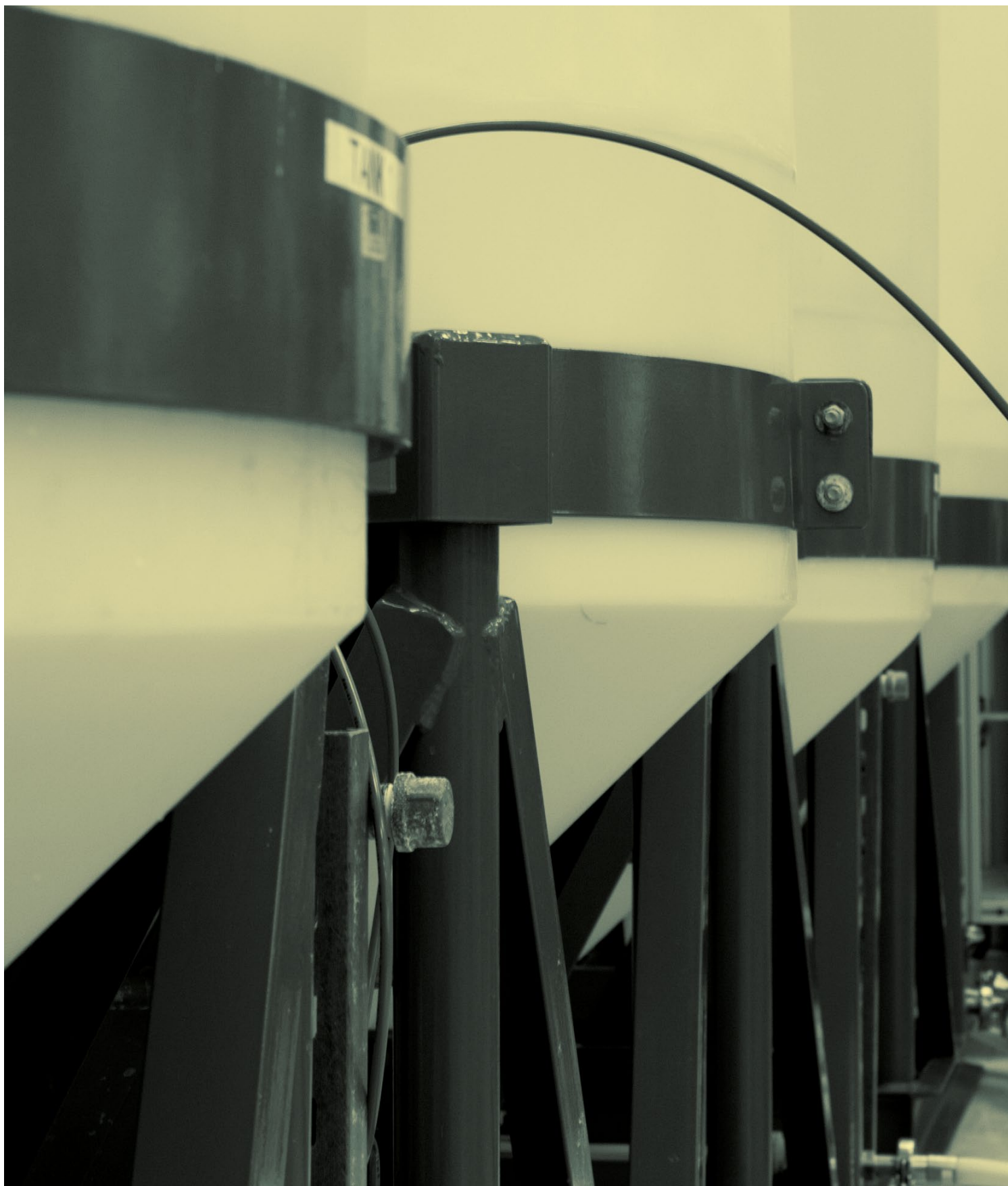


6. Where appropriate, convert linear gradient elution to step elution. Use the information from the gradient to devise an intermediate wash step if desired for increased purity.
7. Determine the pH that gives the highest binding capacity at a phosphate concentration of 5 mM.
8. With further method development, conversion of bind/elute to flowthrough conditions is also possible.

**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	Protocol V: Viruses
Flow rate	150 cm/hr for all steps	150 cm/hr for all steps	150 cm/hr for all steps	150 cm/hr for all steps	150 cm/hr for all steps	150 cm/hr for all steps
Sanitize				1.0 N NaOH		
Wash	H <sub>2</sub> O	H <sub>2</sub> O	H <sub>2</sub> O	H <sub>2</sub> O	H <sub>2</sub> O	600 mM NaPO <sub>4</sub> , pH 7.2
Pre-Equilibrate	400 mM NaPO <sub>4</sub> , pH 6.5	400 mM NaPO <sub>4</sub> , pH 6.5	400 mM NaPO <sub>4</sub> , pH 6.8	400 mM NaPO <sub>4</sub> , pH 7.0	400 mM NaPO <sub>4</sub> , pH 6.7	
Equilibrate	10 mM NaPO <sub>4</sub> , 4–8 ppm Ca <sup>++</sup> , pH 6.5	10 mM NaPO <sub>4</sub> , 8–12 ppm Ca <sup>++</sup> , 100 mM NaCl, pH 6.5	5 mM NaPO <sub>4</sub> , 20 ppm Ca <sup>++</sup> , 150 mM NaCl, pH 6.8	10 mM NaPO <sub>4</sub> , 1 mM EDTA, pH 7.0	5 mM NaPO <sub>4</sub> , 50–100 mM NaCl, 12–20 ppm Ca <sup>++</sup> , pH 6.7	10 mM NaPO <sub>4</sub> , pH 7.2
Load	Clarified sample 10 mM NaPO <sub>4</sub> , 4–8 ppm Ca <sup>++</sup> , pH 6.5	Clarified sample 10 mM NaPO <sub>4</sub> , 4–8 ppm Ca <sup>++</sup> , pH 6.5	Clarified sample 5 mM NaPO <sub>4</sub> , 20 ppm Ca <sup>++</sup> , 150 mM NaCl, pH 6.8	Clarified sample in load buffer	Clarified sample 5 mM NaPO <sub>4</sub> , 50–100 mM NaCl, 12–20 ppm Ca <sup>++</sup> , pH 6.7	10 mM NaPO <sub>4</sub> , pH 7.2
Wash	10 mM NaPO <sub>4</sub> , 4–8 ppm Ca <sup>++</sup> , pH 6.5	10 mM NaPO <sub>4</sub> , 4–8 ppm Ca <sup>++</sup> , pH 6.5	5 mM NaPO <sub>4</sub> , 20 ppm Ca <sup>++</sup> , 150 mM NaCl, pH 6.8	10 mM NaPO <sub>4</sub> , 1 mM EDTA, pH 7.0	5 mM NaPO <sub>4</sub> , 50–100 mM NaCl, 12–20 ppm Ca <sup>++</sup> , pH 6.7	10 mM NaPO <sub>4</sub> , pH 7.2
SNS	25 mM Tris, 25 mM NaCl, 5 mM NaPO <sub>4</sub> , pH 7.75					
Elute	10 mM NaPO <sub>4</sub> , linear gradient 0–2 M NaCl, pH 6.5 + 15 ppm Ca <sup>++</sup>	10 mM NaPO <sub>4</sub> , 15 ppm Ca <sup>++</sup> , linear gradient 0.1–2 M NaCl, pH 6.5 + co-buffer	Linear gradient 5 mM NaPO <sub>4</sub> + 20 ppm Ca <sup>++</sup> – 500 mM NaPO <sub>4</sub> + 12 ppm Ca <sup>++</sup> , 150 mM NaCl, pH 6.8	Linear gradient 0–400 mM NaPO <sub>4</sub> , 1 mM EDTA, pH 7.0	Linear gradient from 5 mM NaPO <sub>4</sub> , 150 mM NaCl, 12–20 ppm Ca <sup>++</sup> , pH 6.7 to 120 mM NaPO <sub>4</sub> , 50–100 mM NaCl, pH 6.7	Gradient elution from 10–600 mM NaPO <sub>4</sub>
Wash						600 mM NaPO <sub>4</sub> , pH 7.2
Clean	400 mM NaPO <sub>4</sub> , pH 7.0–7.5	400 mM NaPO <sub>4</sub> , pH 7.0–7.5	400 mM NaPO <sub>4</sub> , pH 6.8		400 mM NaPO <sub>4</sub> , pH 6.7	0.8 M potassium phosphate, pH 7–7.5
Wash	H <sub>2</sub> O	H <sub>2</sub> O	H <sub>2</sub> O		H <sub>2</sub> O	
Sanitize	1.0 N NaOH	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 400 mM sodium phosphate, pH 7.0–7.5, or 400 mM trisodium phosphate, pH 11–12. If higher concentrations of phosphate are needed to remove tightly bound species, use potassium phosphate. 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. Note that sodium phosphate at pH 6.7–6.8 was used in the scouting protocols for convenience.

#### 4.2 Sanitization

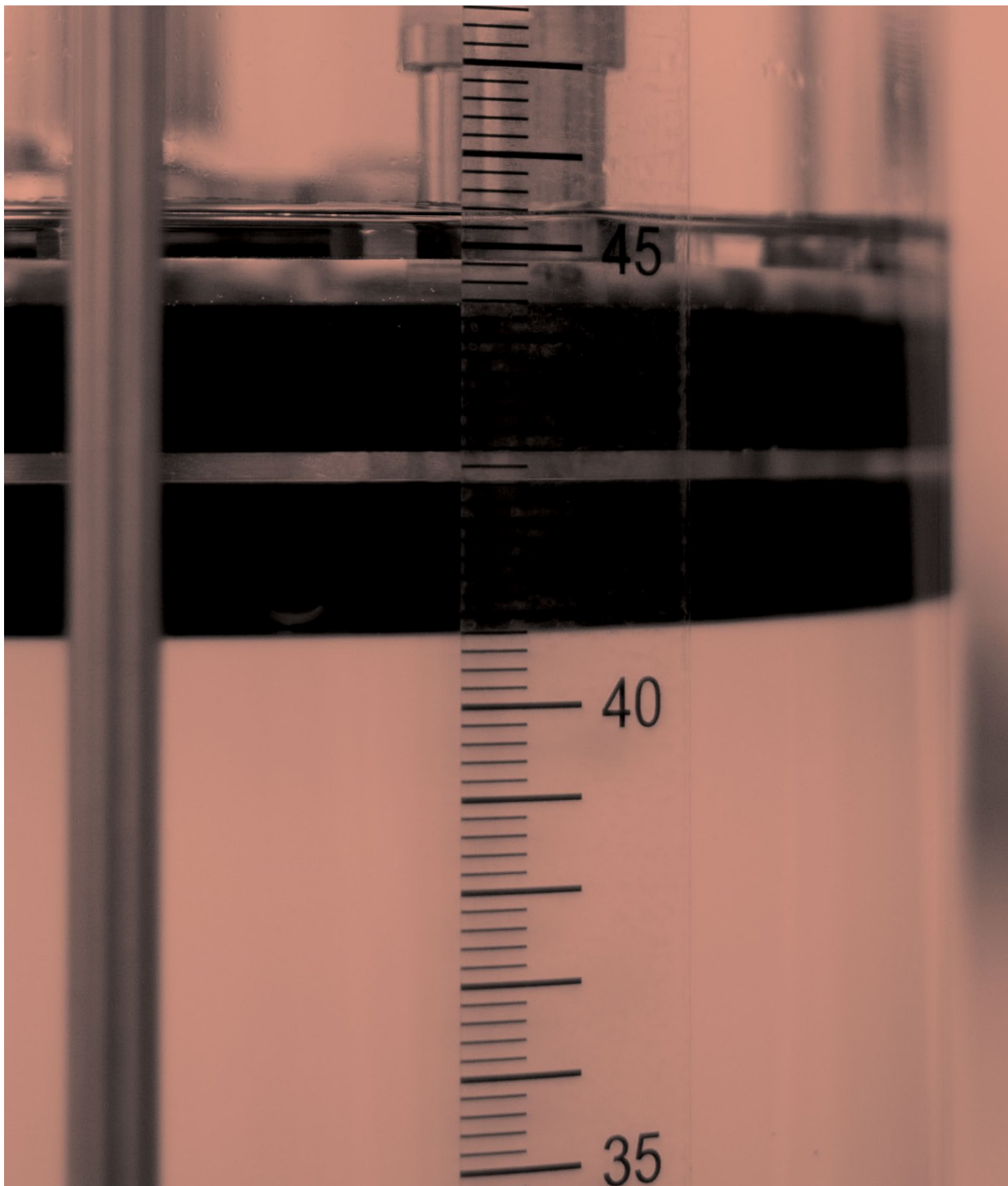
The column can be sanitized in up to 2 N NaOH. Owing to the higher viscosity of concentrated NaOH solutions, the flow rate may need to be lowered to avoid overpressure issues. See Figures 5.1 and 5.2.

#### 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.







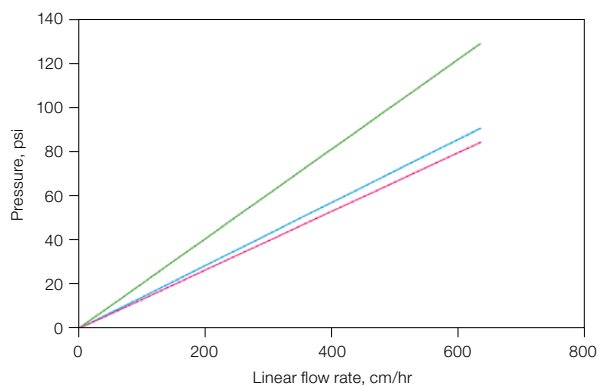
**CHAPTER 5**

# Column Packing Protocols

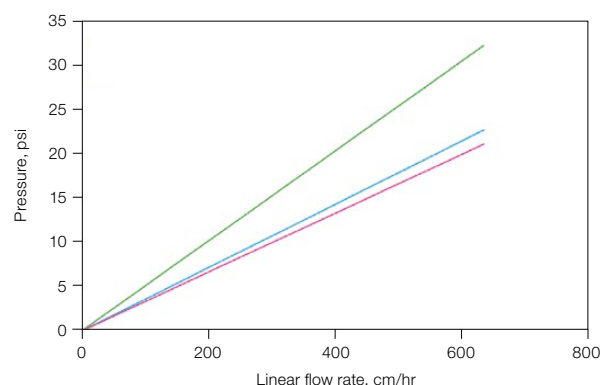
This section offers guidelines for packing process scale columns. Please carefully read over and follow the protocols for packing your specific column. Should you have additional questions, contact your local process chromatography specialist or the chromatography technical support department for further assistance (1-800-4-BIORAD).

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  $\mu\text{m}$  CHT and Figure 5.2 for 80  $\mu\text{m}$  CHT.



**Fig. 5.1. Estimated pressure for 40  $\mu\text{m}$  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  $\mu\text{m}$  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, slightly irregular spheroidal 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 may continue to settle. Additionally, the material in the column may continue to settle slightly over a number of cycles. The results of this phenomenon are twofold:

- An apparent loss in bed height (appearance of headspace) due to further bed compaction
- Small increases 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 1–5 mm.** The gap ensures that the surface of the packed bed is undisturbed by contact with the inlet adaptor. In acrylic and glass columns, the gap may be visually observed. In stainless steel columns with axial compression, it is advisable to stop the piston when it is 10% above the target bed height and check asymmetry and plate count. If satisfactory values are not obtained, the piston can be lowered in increments and the column retested until acceptance criteria are met. 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 upwards. Then continue with downflow at 100 cm/hr for 3 CV and perform bed testing.

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)

- For 40  $\mu\text{m}$  CHT, a frit porosity of 10  $\mu\text{m}$  or less is recommended. For 80  $\mu\text{m}$  material, a frit porosity of 20  $\mu\text{m}$  or less is recommended.

**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. Appropriate measures should be taken to minimize dust formation when dry powder is added to the buffer. 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. Invert the container several times to loosen the CHT into a 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  $\mu\text{m}$  and 80  $\mu\text{m}$  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. Always add powder to buffer, as this will ensure rapid and homogeneous slurry preparation.

**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. 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-mechanical 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.

Damage to the chromatography medium due to excessive physical force is possible. Peristaltic and rotary lobe pumps fracture the particles and must not be used. Pressurized slurry vessels or diaphragm pumps can be used for media transfer. Do not recirculate the slurry through a diaphragm pump for mixing; a single-pass operation of well-mixed slurry is recommended when a pump is used for transfer.

### 5.2.3 Recommended Slurry Transfer

#### Suction or Vacuum Slurry Transfer

Some types of process columns with motorized pistons (for example, the VERDOT Ips<sup>2</sup> InPlace Column) can introduce the slurry without a pump, using the top adaptor in a syringe-style motion to draw the slurry into the column via suction. The transfer should be performed at a minimum speed of 150 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 30% v/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. Use only a diaphragm pump, as rotary lobe and peristaltic systems will damage the particles. Thinner slurries minimize the shear forces and particle-to-particle collisions in piping, bends in piping, other connections, and the packing nozzle. A single-pass operation of the slurry through these pumps, piping, valves, and nozzle minimizes damage.

### 5.3 Open-Column Packing

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

- VERDOT Ips<sup>2</sup> Columns
- BPG Columns (GE Healthcare)
- QuikScale Columns (Millipore)

In these cases it is possible to make the slurry in the column itself rather than in an external tank. To determine the dry weight of CHT and the volume of packing buffer necessary to make up an appropriate slurry, see the formula in Appendix A1. Remember that the height of the column tube should be sufficient to contain the entire slurry volume.

#### 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.
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 150–300 cm/hr for 40 µm material and 300–400 cm/hr for 80 µm material. Do not exceed the maximum recommended pressure for the column. If desired, a downward piston movement can be combined with buffer flow rate to provide the total required velocity.
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 bottom of the top flow adaptor is about 1–5 mm **above** the packed bed. The gap ensures that the top of the packed bed is not crushed by the flow adaptor.  
In acrylic and glass columns, the gap may be visually observed. In stainless steel columns, it is advisable to stop the piston when it is 10% above the target bed height and check asymmetry and plate count. If satisfactory values are not obtained, the piston can be lowered in increments and the column retested until acceptance criteria are met. 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 upwards.
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:

- VERDOT Ips<sup>2</sup> 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 formula in Appendix A1). 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 Column.
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 at 150–300 cm/hr for 40 µm material and 300–400 cm/hr for 80 µm material. Do not exceed the maximum recommended pressure for the column. If desired, a downward piston movement can be combined with buffer flow rate to provide the total required velocity.
11. Continue the descent until the adaptor is about 1–5 mm **above** the packed bed. This ensures that the top of the bed is not crushed by the adaptor. In acrylic and glass columns, the gap may be visually observed. In stainless steel columns, it is advisable to stop the piston when it is 10% above the target bed height and check asymmetry and plate count. If satisfactory values are not obtained, the piston can be lowered in increments and the column retested until acceptance criteria are met. 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 upwards.
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:

- VERDOT Ips<sup>2</sup> 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 150 cm/hr for 40  $\mu$ m and 300 cm/hr for 80  $\mu$ m 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 150–300 cm/hr for 40  $\mu$ m and 300–400 cm/hr for 80  $\mu$ m material 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 about 1–5 mm **above** the packed bed. This ensures that the top of the bed is not crushed by the adaptor. In acrylic and glass columns, the gap may be visually observed. In stainless steel columns, it is advisable to stop the piston when it is 10% above the target bed height and check asymmetry and plate count. If satisfactory values are not obtained, the piston can be lowered in increments and the column retested until acceptance criteria are met. 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 upwards.

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 the same height as the packed bed itself.
3. Decrease the flow rate to 100 cm/hr and change the flow direction to upflow. When the lifted bed reaches the top flow adaptor, turn off the flow and allow the bed to collapse.
4. If the bed has not collapsed, but instead remains up against the top flow adaptor, use downflow to push the bed back to the bottom of the column. Repeat steps 3–4 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. At this point, the slurry percentage is approximately 50%. If desired, 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 containers 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

Refer to the formula in Appendix A1 for required slurry buffer volumes.

#### 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 until the slurry is homogeneous.
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

For other than paddle mixing, always use a low-shear hydrofoil impeller. It is advisable to use multiple impellers on the shaft spaced approximately 1 m apart for deep or large volume 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 may 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.
    - iv) Remove 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 any particulates developed during the unpacking process.
4. Clean the column according to the manufacturer's guidelines.

### 5.6 Packed Column Qualification

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 acetone or NaCl, to the column. The preferred running 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, equilibrate the column with 3–5 CV of running buffer using a flow rate of 75–150 cm/hr.

#### Test conditions

- Sample volume: 1–5% of the bed volume
- Sample concentration: 2.0% (v/v) acetone in running buffer or running buffer with added NaCl up to 1 M

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

Flow rate: 75–150 cm/hr

UV: 280 nm, 1 cm path length, 0.1 AU full scale (acetone)

Conductivity: 100 mS/cm full scale (NaCl)

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

Typical HETP values for 40  $\mu\text{m}$  CHT Ceramic Hydroxyapatite range from 0.016–0.021 cm, and the range for 80  $\mu\text{m}$  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 small trailing shoulder peak during asymmetry testing is normal when NaCl is used as a test probe. Consult Bio-Rad for further explanation.

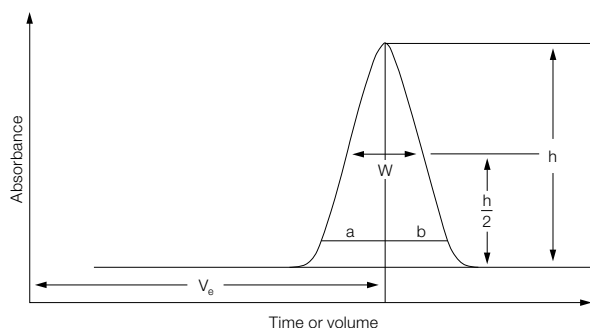


Fig. 5.3. UV or conductance trace for test.

## 5.7 Laboratory-Scale Column Packing Protocols

This section describes the packing of CHT into laboratory-scale columns.

### 5.7.1 Reference Methods for Packing Small-Scale CHT Columns

#### Column Selection/Preparation

- Frit/filter pore size:
  - $\leq 10 \mu\text{m}$  for  $40 \mu\text{m}$  CHT
  - $20 \mu\text{m}$  for  $80 \mu\text{m}$  CHT

#### Slurry Preparation

CHT is provided as a dry powder. Wear proper protective equipment (PPE) when transferring the powder. The first step is to hydrate the resin; recommended buffers are shown in Table 5.1. Calculate the amount of dry powder and buffer needed to make the required volume of slurry. For columns  $\leq 20 \text{ cm}$ , use the density values in Section A.1 to determine the amount of material necessary to pack the column. CHT absorbs  $\sim 90\%$  of its volume in buffer during the initial hydration. A sample calculation is shown below:

- To make 10 ml of 50% slurry (5 ml packed bed)
  - Amount of powdered CHT required:  $3.15 \text{ g}$  ( $5 \text{ ml} \times 0.63 \text{ g/ml}$ )
  - Amount of buffer required:  $5 \text{ ml} + 4.5 \text{ ml}$  (90% of 5 ml).

Table 5.1. Recommended buffers for CHT Columns.

	Hydrating	Equilibrating	Testing
20 mM NaPi, 150 mM NaCl pH 7.2–7.4 (PBS)	•	•	
200–400 mM NaPi, pH >7.2	•		
0.15–1 N NaOH	•		
20 mM NaPi, 0.6–1.2 M NaCl, pH 7.2–7.4			•
20 mM NaPi, 1–2% acetone, pH 7.2–7.4			•

#### Tips for Proper CHT Slurry Preparation

- Do not use a magnetic stir bar for mixing
- Use an orbital shaker or manual inversion in a capped tube or other vessel for proper mixing if required

### 5.7.2 Slurry Transfer and Column Packing

#### Column Packing of Millipore Vantage L Columns

For Millipore Vantage L Biochromatography Laboratory Columns of diameters up to 4.4 cm, follow these steps:

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 the appropriate amount of water to wet the bed support and purge the outlet channel. Drain completely and block the outlet.
3. Attach the column to a stand or other support and ensure that it is level.
4. Add water or buffer to cover the frit.
5. Dispense the prepared CHT slurry into the column and allow settling until  $\sim 1 \text{ cm}$  of clear buffer layer is visible. It may be necessary to use a column extension, as we do not recommend slurry concentrations of  $>50\%$ .
6. Insert the inlet seal and the assembly on top of the buffer layer and connect the column to inlet and outlet lines as desired.
7. Pack at a linear flow rate of 150–200 cm/hr for  $40 \mu\text{m}$  and at least 300 cm/hr for  $80 \mu\text{m}$  material until the bed is fully consolidated.
8. Lower the inlet so that it is 1–5 mm above the packed bed.
9. Continue buffer application at 150 cm/hr for 15 min.

**Note:** Do not allow the adaptor to touch the bed surface. This can cause particle damage.

#### Column Packing of Econo-Column Columns

The following applies to packing CHT into Bio-Rad's Econo-Column Columns of the following sizes:

1.0 × 30 cm (catalog #7371032) and 1.5 × 30 cm (#7371532). Additional required items are flow adapters, 1.0 × 14 cm (#7381015) and 1.5 × 14 cm (#7381016), an Econo-Column Funnel (#7310003), and Luer fittings to connect to liquid chromatography systems. We do not recommend using larger diameter Econo-Column Columns with CHT.

**Important Considerations before Using Econo-Column Columns:**

The upper limit of pressure for the Econo-Column Columns is approximately 14 psi (1 bar). The flow rates we provide in the procedure are suggested 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. Pressure contributed by chromatography system components downstream of the column outlet must be minimized to less than 1 psi at the selected flow rate.

The column is packed similarly to the directions provided for Millipore Vantage L Columns. A funnel can be used to add additional slurry volume into the column. Once the CHT Media has completely settled out from the funnel and into the column tube, remove the funnel and proceed per step 6 in the directions for the Vantage L Columns with the suggested flow rates in this section. The flow adapter is attached and adjusted using the directions in Section 3.1 of the Econo-Column Flow Adaptor Instruction Manual (bulletin M7380014).

**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.

**5.7.3 Packed Column Evaluation/Qualification**

See Section 5.6 for instructions on column qualification and evaluation.

**5.7.4 Post-Column Packing Considerations**

- CHT beds can continue to consolidate after initial packing, as is also seen with silica and controlled-pore glass
- Bed compaction and an apparent loss in bed height (appearance of headspace) can occur over many cycles or if the column is moved from one room to another
- If necessary, lower the flow adapter further to minimize the additional headspace

- CHT binds metals that are in process solutions, which may cause discoloration at the top of the column. Consult your process specialist for mitigating strategies
- The lifetime of CHT can be enhanced through a variety of methods. Consult your process specialist for further details

**5.7.5 Tips to Ensure Proper Small-Scale CHT Column Packing**

- Do not compress CHT
- Defining or decanting steps are not required
- Ensure that the column is as level as possible
- Never perform buffer upflow with packed CHT except during unpacking
- At small scale it can be difficult to achieve a good plate count and/or asymmetry. If this is the case, while the column is flowing at 100–200 cm/hr, place a source of vibration (such as a vortexer or electric toothbrush) up to the column barrel itself. The column bed may settle by as much as an additional 2 cm, at which point the top adapter can be further lowered and the packing test repeated

**5.7.6 Summary: CHT Packing – Things to Remember**

Slurry concentration: 30–50% w/v

Headspace: 1–5 mm

CHT settling time: <10 min

CHT settling rate: 40 µm: 35–125 cm/hr;

80 µm: 125–275 cm/hr

Asymmetry range: 0.8–2.3

HETP: 40 µm: 0.016–0.021 cm; 80 µm: 0.032–0.041 cm



### 5.7.7 Troubleshooting Tips

Observation	Possible Cause	Correction
Turbid supernatant in fresh CHT slurry	<ul style="list-style-type: none"> <li>First contact of the CHT powder is with a low ionic strength solution</li> </ul>	<ul style="list-style-type: none"> <li>Use solutions with an ionic strength &gt;150 mM and a pH of 6.8 or above</li> </ul>
Settling of CHT during slurry preparation	<ul style="list-style-type: none"> <li>Mixing in a standard circular motion</li> </ul>	<ul style="list-style-type: none"> <li>Use a J-stroke for mixing or use an orbital shaker</li> </ul>
High rHETP	<ul style="list-style-type: none"> <li>Clogged column screen/frit</li> <li>Probe volume too big/unoptimized efficiency test condition</li> <li>Unevenly packed column</li> </ul>	<ul style="list-style-type: none"> <li>Clean screen/frit</li> <li>Modify injection loop to reduce probe volume</li> <li>Repack column</li> </ul>
Peak fronting	<ul style="list-style-type: none"> <li>Channel(s) in column</li> <li>Packing pressure/flow rate too high</li> </ul>	<ul style="list-style-type: none"> <li>Repack</li> <li>Use a lower packing pressure/flow rate</li> </ul>
Peak tailing	<ul style="list-style-type: none"> <li>Probe volume too high/unoptimized efficiency test condition</li> <li>Air trapped under column adaptor/piston</li> <li>Space between column adaptor/piston and bed</li> </ul>	<ul style="list-style-type: none"> <li>Modify injection loop to reduce probe volume</li> <li>Eliminate air</li> <li>Adjust adaptor/piston</li> </ul>
High column pressure	<ul style="list-style-type: none"> <li>Clogged column screen/frit</li> <li>Presence of fine particles due to excessive mixing</li> <li>Contaminated resin</li> </ul>	<ul style="list-style-type: none"> <li>Clean column screen/frit</li> <li>Use the recommended mixing method to minimize fines. Do not repack the same slurry</li> <li>Clean or replace resin</li> </ul>
Trailing shoulder peak during asymmetry testing	<ul style="list-style-type: none"> <li>This is normal when NaCl is used as a test probe. Consult Bio-Rad for further explanation</li> <li>Plugged or contaminated resin</li> </ul>	<ul style="list-style-type: none"> <li>Small second peak seen with NaCl tracers</li> <li>Clean or replace resin</li> </ul>
Channeling when packing	<ul style="list-style-type: none"> <li>Hardware configuration</li> </ul>	<ul style="list-style-type: none"> <li>Use slower flow rate to consolidate the bed and then lower the adaptor to the desired bed height followed by conditioning with high flow rate</li> </ul>



# Appendices

## A.1 CHT Ceramic Hydroxyapatite Resin and Slurry Calculations

The following formulas are provided for calculating values:

$$V_L = (V_b/S) - 0.210 \cdot V_b \text{ where}$$

$$V_T = V_L/S \text{ where}$$

$V_L$  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  $S \leq 50\%$ . For determining packed bed volumes, the tap-settled packing density of CHT Type I and II (0.63) and CHT XT (0.67) can be used (see Section 2.1). However, experience with larger columns has shown that a value of 0.60 for CHT Type I and II and 0.64 for CHT XT may provide a closer approximation to the final desired packed bed height.

**Note:** 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.

Make sure that the height of the column tube can accommodate the entire slurry volume.

## A.2 EDTA Complexometric Determination of Calcium Concentrations in Column Effluents

### Introduction

CHT Ceramic Hydroxyapatite is a mixed-mode chromatographic media widely used for the purification of proteins, monoclonal antibodies, and viruses. CHT is a sintered form of hydroxyapatite ( $\text{Ca}_5(\text{PO}_4)_3\text{OH}$ ), which is a form of calcium phosphate. The solubility of CHT increases at an acidic pH ( $\text{pH} < 7.0$ ) and it dissolves in its constituent ions ( $\text{Ca}^{2+}$ ,  $\text{PO}_4^{3-}$ ,  $\text{OH}^-$ ). 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. For details, refer to Bio-Rad bulletins 6067 and 6278.

### Experimental Procedure

#### 1. Sample pH Adjustment

The pH of the sample should be adjusted to  $10 \pm 0.1$  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 ( $\leq 10$  mM total buffering species):

The pH of these samples may be adjusted to  $10 \pm 0.1$  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  $10 \pm 0.1$ .

#### 2. Adding the WEBT Solution

Once the sample pH is within  $10 \pm 0.1$  proceed to add the working indicator solution (WEBT solution). Typically the amount of WEBT needed will be 2–3  $\mu\text{l}$  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  $\geq 5$  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

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

Sample volume 40 ml

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

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

#### Materials and Methods

##### Materials

Eriochrome black T (EBT), triethanolamine (TEA), ammonium chloride ( $\text{NH}_4\text{Cl}$ ), magnesium sulfate heptahydrate ( $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ ), ammonium hydroxide solution ( $\text{NH}_4\text{OH}$ ), disodium dihydrogen ethylenediaminetetraacetate ( $\text{Na}_2\text{EDTA}$ ), 10 N sodium hydroxide (NaOH) solution, 1 N hydrochloric acid (HCl) solution, and deionized (DI) water (18  $\Omega$ /cm).

##### Titration Solutions

##### Ammonia Buffering Solution

##### Solution A

Weigh 1.179 g of  $\text{Na}_2\text{EDTA}$  and 750 mg of  $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ . 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  $\text{NH}_4\text{Cl}$  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  $\text{Na}_2\text{EDTA}$  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  $10 \pm 0.1$
- If during NaOH addition the pH goes beyond  $10 \pm 0.1$ , use 1 N HCl to bring the pH back down such that the volume of ammonia buffer needed to bring the pH finally to  $10 \pm 0.1$  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)

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## A.5 Ordering Information

Catalog # Description

### CHT Ceramic Hydroxyapatite, Type I

1582000 CHT Ceramic Hydroxyapatite, 20 µm, Type I, 10 g  
 1570020 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  
 1584000 CHT Ceramic Hydroxyapatite, 40 µm, Type I, 10 g  
 1570040 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  
 1588000 CHT Ceramic Hydroxyapatite, 80 µm, Type I, 10 g  
 1570080 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

7324322 Bio-Scale Mini CHT-I, 40 µm, 1 × 5 ml  
 7324324 Bio-Scale Mini CHT-I, 40 µm, 5 × 5 ml

### CHT Ceramic Hydroxyapatite, Type II Cartridges

7324332 Bio-Scale Mini CHT-II, 40 µm, 1 × 5 ml  
 7324334 Bio-Scale Mini CHT II, 40 µm, 5 × 5 ml

### CHT Ceramic Hydroxyapatite, Type II

1582200 CHT Ceramic Hydroxyapatite, 20 µm, Type II, 10 g  
 1572000 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  
 1584200 CHT Ceramic Hydroxyapatite, 40 µm, Type II, 10 g  
 1574000 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  
 1588200 CHT Ceramic Hydroxyapatite, 80 µm, Type II, 10 g  
 1578000 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

### CHT Ceramic Hydroxyapatite XT

12002457 CHT Ceramic Hydroxyapatite XT, 40 µm, 10 g  
 12002454 CHT Ceramic Hydroxyapatite XT, 40 µm, 100 g  
 12002456 CHT Ceramic Hydroxyapatite XT, 40 µm, 1 kg  
 12002455 CHT Ceramic Hydroxyapatite XT, 40 µm, 5 kg

Catalog # Description

### MPC Ceramic Hydroxyfluoroapatite, Type I

1580200 MPC Ceramic Hydroxyfluoroapatite, 40 µm, Type I, 10 g  
 1570200 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  
 12003150 Foresight CHT XT Column, 40 µm, 1 ml  
 12003149 Foresight CHT XT 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  
 12003151 Foresight CHT XT 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  
 12003152 Foresight CHT XT RoboColumn Units, 40 µm, 200 µl  
 12003148 Foresight CHT XT RoboColumn Units, 40 µm, 600 µl

\*\* Package size: one row of eight columns

**Note:** 1 and 5 kg CHT containers/lids are made of high density polyethylene (HDPE) while 10 and 100 g CHT containers/lids are made of polypropylene.



## 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?** Most buffers should contain at least 5 mM phosphate. 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 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 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 co-buffer that will maintain the pH above 6.5 and add  $\text{Ca}^{++}$  to the buffer. Note that phosphate concentrations below 5 mM may result in a shorter column lifetime. CFT Ceramic Fluoroapatite can be used as an alternative for purifying molecules at a pH as low as 5.
- 4. What is the difference between CHT Type I, CHT Type II, and CHT XT?** All three CHT supports are chemically identical but are sintered at different temperatures, resulting in a difference in pore size and surface area. CHT Type I has the highest overall protein binding capacity among the three varieties. CHT XT is the newest member of the CHT family. It is similar in application usage to CHT Type I and is a more stable matrix, providing longer usable column lifetime at process scale. CHT Type II, with its large pore size, is the most effective for purification of large biomolecules such as IgM and viruses.
- 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 transition-state metal ions. Washing with pure water extensively will slowly dissolve the support. Using 1 N NaOH immediately after a high phosphate buffer regeneration step is not recommended; use  $\frac{1}{2}$  CV of water or low phosphate buffer between the high phosphate and the 1 N NaOH regeneration steps.
- 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 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 at least 400 mM phosphate to remove adsorbed proteins and store the column in 0.1 N NaOH. If the NaOH concentration is below 0.1 N, include 10 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 stable once it is packed into a column; 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-shear hydrofoil 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  $\mu\text{m}$  bed support for the 40  $\mu\text{m}$  CHT support and a 20  $\mu\text{m}$  bed support for the 80  $\mu\text{m}$  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. Contact your Bio-Rad representative for further details and possible mitigation strategies.
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  $\mu\text{m}$  CHT range from 0.016–0.021 cm, and the range for 80  $\mu\text{m}$  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. Refer to p. 37 for packing small-scale columns.
12. **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 at room temperature. A packed column should be stored in a sealed column in 0.1 N NaOH.
13. **Which type of hydroxyapatite works best for nucleic acid samples?** All three types of CHT, Type I, Type II, and XT, 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.
14. **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.
15. **What if 0.4 M sodium phosphate does not adequately clean the resin?** Use a higher concentration of potassium phosphate. Concentrations of sodium phosphate above 0.4 M may be difficult to prepare..

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