

# A Ceramic Hydroxyapatite–Based Purification Platform

## Simultaneous Removal of Leached Protein A, Aggregates, DNA, and Endotoxins from MAbs

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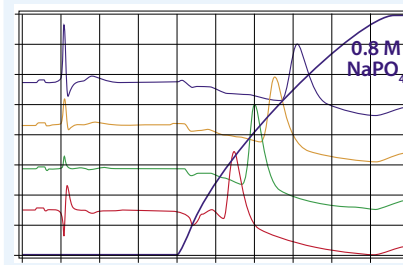
The recent dramatic increase in the number of monoclonal antibodies (MAbs) entering biopharmaceutical product pipelines has created the need for highly efficient purification process development. The concept of platform purification has evolved in response to that need. Platforms are semigeneric multistep procedures that are applicable to most antibodies and give good purification performance with minimal development (1–5). A primary goal of this approach is to accelerate product entry into clinical trials and identify the most qualified candidates as early as possible.

MAbs are sufficiently similar to one another to make this approach practical, but there remains sufficient diversity among clones to challenge the ability of any single platform to accommodate all (5). The challenge is compounded by relatively high therapeutic dosages for many monoclonal products—up to grams

per patient per year. That elevates the possibility that trace contaminants could accumulate and lead to adverse effects. A “toolbox” of plug-and-play platforms can facilitate the ability of process developers to meet these challenges without having to resort to “from-scratch” process development for every new product.

Protein A has become the method of choice for antibody capture and initial purification. Its abilities to remove host-cell proteins, nucleic acids, endotoxins, and viruses are primary contributors to its status as such, but its ability to accommodate a wide range of feedstreams with essentially no requirement for modifying pH, conductivity, or other media characteristics is equally important. Removal of substances that might interfere with downstream purification fortifies its position. Protein A chromatography also creates two of the most serious challenges in MAb purification: Elution conditions produce aggregates, and protein A leaches into the product (1). Even to the extent that aggregates may have been present already in a feedstream, protein A chromatography concentrates them with the product. Aggregates are a concern because of their potential to promote thrombotic events and stimulate patient production of neutralizing antibodies, with many potentially adverse consequences (6–10). Protein A is an immunotoxin with documented clinical ramifications as well as an adjuvant protein capable of promoting formation of neutralizing antibodies (11–15). Removal of such contaminants, as well as DNA and endotoxins, is essential.

**Figure 1:** Enhanced retention of DNA in the presence of NaCl (sheared salmon sperm DNA sample; CHT Type I, 40  $\mu$ m, 2-mL MT-2 column; flow rate 600 cm/hr; equilibration with 10 CV 10 mM sodium phosphate, pH 7.0, inject 100- $\mu$ L sample, wash 5 CV equilibration buffer, elute 20 CV linear gradient to 0.8 M sodium phosphate; hold gradient 10 CV)



Anion-exchange chromatography on quaternary amine ion exchangers is frequently used for polishing protein-A-purified antibodies. It is often used in flow-through mode to reduce DNA, endotoxin, and retrovirus contamination. However, this limits its ability to reduce aggregate and leached protein A levels, leaving the burden of their removal to a subsequent intermediate purification step (4, 10). Cation-exchange chromatography has proven effective in this context (16–18). Here we address the ability of CHT brand (ceramic hydroxyapatite) chromatographic media to fulfill that role.

### MATERIALS AND METHODS

The majority of our work was performed with a monoclonal mouse–human IgG chimeric antibody expressed by NS0 cells grown in serum-supplemented media. The cell line was selected expressly for its

**PRODUCT FOCUS:** MONOCLONAL ANTIBODIES

**PROCESS FOCUS:** DOWNSTREAM PROCESSING

**WHO SHOULD READ:** PROCESS DEVELOPERS, QA/QC, AND MANUFACTURING

**KEYWORDS:** HYDROXYAPATITE, PROTEIN A, CHROMATOGRAPHY, PURIFICATION PLATFORMS

**LEVEL:** ADVANCED

tendency to aggregate. Additional experiments were conducted with a protein-A-purified human IgG<sub>4</sub>, which was provided by GTC Biotherapeutics of Framingham, MA ([www.transgenics.com](http://www.transgenics.com)). Polyclonal human IgG, lysozyme, endotoxin, and DNA were obtained from Sigma Chemical Company of St Louis, MO ([www.sial.com](http://www.sial.com)).

**Chromatography:** All experiments were conducted on a BioLogic DuoFlow system from Bio-Rad Laboratories of Hercules, CA ([www.bio-rad.com](http://www.bio-rad.com)). Chromatography media were packed in a Bio-Scale MT2 column (0.7 × 2.6 cm), also from Bio-Rad. CHT columns were dry packed with Type I resin, 20- and 40- $\mu$ m, from Bio-Rad using a density of 0.60 gm/mL, then wetted with 10 column volumes (CV) of 20% ethanol before equilibration. Buffers (vacuum filtered to 0.22  $\mu$ m) and salts came from Sigma, with conditions as specified in the "Protocol" boxes except as indicated in the text and/or figure captions. All phosphate buffers were made with monosodium phosphate titrated to target pH levels with 1 M NaOH, which produces a mix of di- and trisodium phosphates in solution that is difficult to designate/quantify. So herein we refer to "sodium phosphate" or NaPO<sub>4</sub>.

**Analysis:** We determined IgG concentration by absorbance measurement at 280 nm using an extinction coefficient of 1.38. IgG purity was assessed at various stages of purification with reduced sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Criterion precast 4–20% gels and visualized with Flamingo stain, which gives the same sensitivity as silver staining (19). All reagents, protein standards, and apparatus came from Bio-Rad. Aggregate content was measured by high-performance size-exclusion chromatography (HPSEC) on a Bio-Sil SEC 400-5 column (Bio-Rad) equilibrated in 50 mM HEPES at pH 7.2, containing 2.0 M urea and 1.0 M NaCl. Sample volume was 50  $\mu$ L, and flow rate was 0.8 mL/min.

We measured leached protein A by an immunoenzymetric assay from Cygnus Technologies, Inc. of Southport, NC ([www.cygnustechnologies.com](http://www.cygnustechnologies.com)). Absorbance at 405 nm was measured using a  $\mu$ Quant universal

microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT). DNA was measured by picogreen (Invitrogen of Carlsbad, CA, [www.invitrogen.com](http://www.invitrogen.com)). We measured fluorescence by excitation at 480 nm and emission 520 nm on a Cary Eclipse spectrophotometer from Varian, Inc. of Walnut Creek, CA ([www.varianinc.com](http://www.varianinc.com)).

We measured endotoxin content using a kinetic assay by Endoscan-V software and a Tecan Sunrise plate reader from Charles River Laboratories of Wilmington, MA ([www.criver.com](http://www.criver.com)).

## RESULTS AND DISCUSSION

Calcium hydroxyapatite (CHT)—Ca<sub>10</sub>(PO<sub>4</sub>)<sub>6</sub>(OH)<sub>2</sub>—is a mixed-mode chromatography support with a long history of applications in antibody purification (6, 20–27). It can theoretically retain solutes by anion exchange with positively charged calcium, by metal affinity with calcium, by cation exchange with phosphate groups, and by hydrogen bonding with crystal hydroxyl groups (28–30).

The metal affinity of proteins for CHT calcium is generally described as a classical chelating mechanism by which closely neighboring protein carboxyl groups approximate the carboxyl configuration of chelating agents such as ethylene diamine tetraacetic acid (EDTA). Such interactions are much stronger than electrostatic interactions and fail to dissociate even in saturated sodium chloride (28–31). This concomitantly demonstrates that anion exchange between CHT calcium and protein carboxyl groups does not make a significant contribution to protein binding. Elution requires phosphate, which outcompetes protein carboxyl clusters for CHT calcium.

Negatively charged CHT phosphate groups participate in cation exchange interactions with protein amino groups. Proteins bound exclusively by this mechanism (e.g., lysozyme) can be eluted with either neutral salts (e.g., sodium chloride, NaCl) or buffering salts such as phosphate (data not shown). Acidic proteins are also retained more weakly with increasing pH, an indication that the contribution of anion exchange with crystal calcium is a weak interaction (32).

CHT binds to most proteins by a combination of calcium metal affinity and phosphoryl cation exchange, but their

relative contributions are distinctive for every protein. For example, bovine serum albumin (BSA) is carboxyl rich and has a strong affinity for CHT calcium. It elutes at about 110 mM sodium phosphate in a linear phosphate gradient at pH 6.5 (31). When the same gradient is run in the presence of 1.0 M NaCl, the phosphate concentration required for elution drops to about 100 mM (33). The conspicuously small change caused by such a high concentration of salt indicates that ion exchange is a minor contributor to the binding energy here and that calcium affinity dominates retention.

The majority of IgG monoclonal antibodies (MAbs) elute in the range of 100–200 mM sodium phosphate (6, 20–28). Unlike BSA, IgG retention time and dynamic capacity are reduced sharply by even modest levels of NaCl (31). These phenomena demonstrate that phosphoryl cation exchange is a major contributor to IgG binding. Calcium affinity is also a factor but less so than with albumin; as little as 5 mM phosphate weakens binding to the point where NaCl can elute IgG. However, even this weak calcium affinity must be overcome to achieve elution. Unless a threshold concentration of phosphate is present, most IgGs remain bound to CHT even in saturated NaCl.

The eluting ability of phosphate buffer correctly implies that phosphorylated solutes bind strongly to CHT. Phosphoproteins bind more strongly than their unphosphorylated counterparts (24, 34). DNA binds very strongly, with an apparent correlation between strand size and retention time. Small fragments begin to elute at about 0.1 M phosphate, and chromosomal DNA begins at 0.2–0.3 M phosphate (35–38). Phosphate at 0.5 M is recommended for quantitative elution of all size classes (39). The presence of NaCl causes DNA to elute at higher phosphate concentrations (Figure 1). That has been attributed to NaCl suppressing charge repulsion between phosphate groups on DNA and CHT. It may also reflect DNA existing in a less rigid conformation at higher conductivity, thus better able to conform to the geometry of available CHT calcium groups (38).

Endotoxins are also phosphorylated and may require up to 1.0 M phosphate for complete removal; subpopulations can elute over the entire range of 0.0–1.0 M

potassium phosphate (43). See Figure 2.

**Optimizing Selectivity:** Traditional applications of hydroxyapatite have almost exclusively used phosphate gradients. Phosphate fulfills the dual role of competing for calcium affinity and cation-exchange interactions. Several studies have demonstrated the ability of such gradients to reduce both aggregate and leached protein A levels in purified IgG (40–42). Although phosphate gradients are convenient, their use sacrifices independent control over the two dominant retention mechanisms. That eliminates the ability to accommodate differences in retention properties among various MAbs as well as their inevitably different relationships with major contaminants.

Fully independent control of both mechanisms is a practical impossibility. NaCl should affect only ion-exchange interactions, but as noted above most proteins bind CHT by ion exchange *and* calcium affinity. The phosphate required to control calcium interaction also affects electrostatic interactions. But it is possible to alter the proportional contribution of each mechanism and optimize selectivity for a particular application. One way is to elute with a sodium chloride gradient

## ANION-EXCHANGE PROTOCOL

### UNOsphere Q Packing (Bio-Rad) in Flow-Through Mode

Flow Rate: 300 cm/hr

Equilibrate/Wash (A): 0.05 M Tris, 0.05 M NaCl, pH 7.0

Clean (B): A + 1.0 M NaCl, pH 7.0

Equilibrate: 5 CV buffer A

Apply equilibrated sample

Wash: 5 CV buffer A

Clean: 10 CV buffer B

Sanitize: 1.0 M NaOH

Store: 0.1 M NaOH

while a calcium affinity-weakening concentration of phosphate is held constant. Phosphate concentration can be adjusted incrementally (5 mM is a practical place to start) over a series of runs to optimize separation of IgG from its major contaminants.

IgG aggregates generally elute later from CHT than native IgG in both phosphate and NaCl gradients (20), which suggests that the retention characteristics of individual components are additive in the complex. As shown in Figures 3 and 4,

## PROTEIN A AFFINITY PROTOCOL

### ProSep-A Packing (Millipore, Bedford, MA)

Flow Rate: 300 cm/hr

Buffer A: 0.05 M, 0.1 M NaCl, NaPO<sub>4</sub>, pH 7.2

Buffer B: 0.05 M NaPO<sub>4</sub>, 1.0 M NaCl, 2.0 M urea, 10 mM EDTA, pH 7.2

Buffer C: 0.10 M glycine, 0.05 M NaCl, pH 3.8

Equilibrate: 10 CV buffer A

Apply sample

Wash: 5 CV buffer A

Optional Wash: 5 CV buffer B, followed by 2 CV buffer A

Elute: 10 CV buffer C

Clean: 6 M guanidine-HCl, pH 3.5

Store: 0.2% sodium azide

a sodium chloride gradient with a fixed phosphate concentration gives much better resolution than a phosphate gradient. HPSEC profiles of the IgG pool from both gradient modes emphasize the superiority of the NaCl gradient (Figure 5). From analytical and preparative perspectives, aggregate separation on CHT with the NaCl gradient system is arguably superior even to SEC: Resolution between native and aggregated antibody is higher, as are capacity and the effective range of flow rates (43).

Free protein A, when eluted by either phosphate or NaCl gradients, overlaps to varying degrees with the elution zone for most IgGs under the same conditions. This could imply limited potential for leachate reduction, but it is important to remember that that leached protein A is affinity complexed to IgG. As with aggregates, the retention properties of the components appear to be additive in a complex, with the result that IgG-complexed protein A elutes after native IgG. Leachate levels can be reduced to undetectable levels in NaCl gradients at 5 mM phosphate (Table 1). IgG-complexed protein A remains bound to the column until the 0.5 M phosphate cleaning step.

As noted above, eluting even small fragments of DNA from CHT requires at least 0.1 M phosphate. The consequence is that DNA fails to elute in NaCl gradients

## HYDROXYAPATITE SCREENING PROTOCOLS

### NaPO<sub>4</sub> Elution System

Flow Rate: 300 cm/hr

CHT, type I, 20 or 40 μm, as indicated (Bio-Rad)

Buffer A: 5 mM sodium phosphate, pH 6.5

Buffer B: 0.50 M sodium phosphate, pH 6.5

Equilibrate: 10 CV buffer A

Apply sample, 5% CV if unequilibrated, up to 20 mg/mL of media if equilibrated

Wash: 5 CV buffer A

Elute: 30 CV linear gradient to 60% buffer B

Clean: 10 CV buffer B

Sanitize: 1.0 M NaOH

Store: 0.1 M NaOH

### NaCl Elution System

Flow Rate: 300 cm/hr

CHT, type I, 20 or 40 μm, as indicated (Bio-Rad)

Buffer A: 5 mM sodium phosphate, pH 6.5

Buffer B: 5 mM sodium phosphate, 1.0 or 1.5 M NaCl, pH 6.5

Buffer C: 0.50 M sodium phosphate, pH 6.5

Equilibrate: 10 CV buffer A

Apply sample, 5% CV if unequilibrated, up to 20mg/mL of media if equilibrated

Wash: 5 CV buffer A

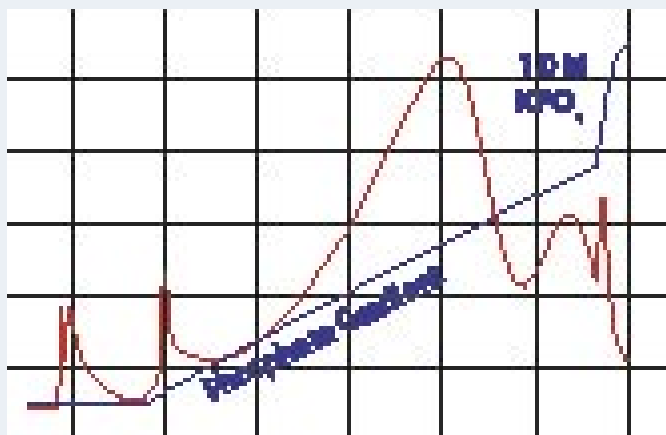
Elute: 30 CV linear gradient to buffer B

Clean: 10 CV buffer C

Sanitize: 1.0 M NaOH

Store: 0.1M NaOH

**Figure 2:** Endotoxin elution profile in a phosphate gradient (purified endotoxin from *Pseudomonas aeruginosa* sample; CHT Type I, 20- $\mu$ m, 2-mL MT-2 column; flow rate 600 cm/hr; equilibration with 10 CV 10 mM potassium phosphate, pH 7.0, inject 100- $\mu$ L sample, wash 5 CV equilibration buffer, elute 20 CV linear gradient to 0.5 M potassium phosphate, clean with 1.0 M potassium phosphate)



at 5–15 mM phosphate concentrations, reducing levels up to 3 logs or more in the native antibody pool (Table 1). DNA is eliminated in the 0.5 M phosphate cleaning step. Despite more complex elution behavior in phosphate gradients, endotoxins too remain bound until the cleaning step, leading to reductions of >4.5 logs in a native IgG pool (44).

Such results recommend a very simple approach to method optimization: Begin with a sodium chloride gradient over a baseline phosphate concentration of 5 mM. The native form of most antibodies will elute within a gradient to 1.5 M NaCl. For those that fail to elute, increasing the phosphate concentration to 10 mM will usually result in elution at a lower NaCl concentration (Figure 6). It is rarely necessary to increase phosphate concentration further. Doing so may be counterproductive. Our preliminary results

indicate a trend of decreasing efficiency in removal of aggregates, leached protein A, DNA, and endotoxins with increasing phosphate concentration (Table 2).

#### Integration of CHT into a Purification Platform Architecture:

CHT integrates easily with protein A affinity in a two-step platform. The only concession required at the protein A step is avoidance of citrate and chelating agents such as EDTA. Elution buffers such as 0.1 M glycine or arginine, 0.05 M sodium chloride, at pH 3.8 work effectively in most cases. Following a hold for virus inactivation, pH can be adjusted to 6.5 and phosphate concentration raised to 5 mM by adding 1% (vol:vol) of 0.5 M sodium phosphate at pH 10.5 to the sample. Glycine and arginine are both zwitterionic at pH 6.5 and thus do not contribute significantly to conductivity. IgG capacity on CHT

varies from one MAB to another, with lows of about 25 mg/mL to more than 60 mg/mL (6, 31, 40). Initial platform evaluation can be conducted with  $\leq 20$  mg IgG/mL CHT until capacity is determined experimentally.

A three-step platform including anion exchange can be conducted by placing a Q column immediately following protein A, with CHT last in sequence. This allows the process to flow directly from step to step without requiring additional buffer exchange to accommodate high NaCl concentration in the IgG pool from CHT. Buffers and conditions for running this platform are provided in Table 3.

When we ran two- and three-step platforms in parallel, leached protein A was undetectable in both IgG pools, DNA was less than 1 ng/mL, and endotoxin was less than 0.05 EU/mL. Figure 7 illustrates HPSEC aggregate profiles for our protein

**Table 1:** Comparison of contaminant clearance between phosphate gradients and sodium chloride gradients

	Endotoxin EU/mL	DNA ng/mL	ppm	Protein A ng/mL	ppm
Starting material	>500	1194	117	19.96	2.00
Phosphate pool	16.6	3.80	6	1.27	1.8
NaCl pool	<0.05	0.67	2	0.00	0.00

**Table 2:** Contaminant removal efficiency in sodium chloride gradients as a function of phosphate concentration<sup>1,2</sup>

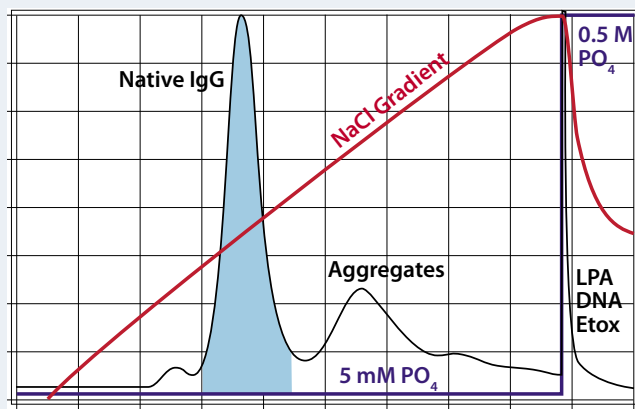
Contaminants	Concentration		
	5	10	15
Phosphate in buffers (mM)	5	10	15
Protein A, IgG pool (ng/mL)	0.03 <sup>3</sup>	0.03	0.01
DNA, IgG pool (ng/mL)	<1.0	<1.0	3.9
Endotoxin, IgG pool (EU/mL)	<0.05	1.0	1.6

<sup>1</sup> Sample: protein-A-purified IgG; 22 ng/mL leached protein A,  $2.3 \times 10^3$  ng/mL DNA,  $1.9 \times 10^4$  EU/mL endotoxin

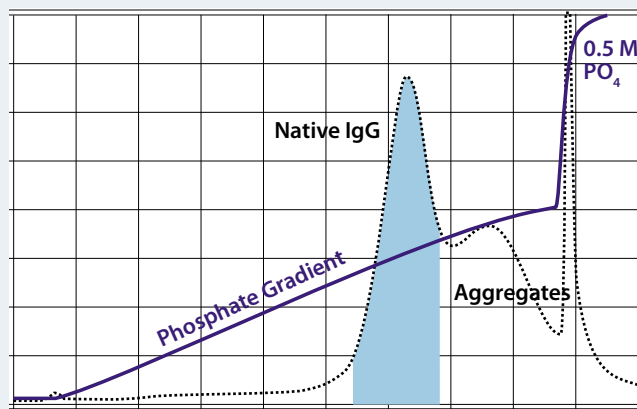
<sup>2</sup> All results for a sodium chloride gradient to 1.5 M at pH 6.5 with phosphate

<sup>3</sup> 0.2 ng/mL is the lower linear sensitivity limit of the assay

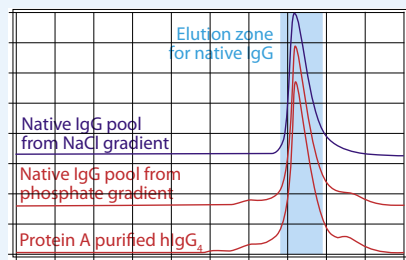
**Figure 3:** Distribution of native IgG and major contaminant classes in a sodium chloride gradient at 5 mM phosphate (protein A purified mouse human IgG chimera, CHT type I, 20 mM, 2 mL MT-2 column). The blue peak contains native IgG as indicated by analytical SEC (Figure 5). Refer to methods section for additional details.



**Figure 4:** Distribution of native IgG and aggregates classes in a phosphate gradient at from 5 to 300 mM (protein A purified mouse human IgG chimera, CHT type I, 20 mM, 2 mL MT-2 column). The blue peak contains native IgG as indicated by analytical SEC (Figure 5). Refer to methods for additional details.



**Figure 5:** Relative aggregate removal efficiency on CHT type I 40  $\mu\text{m}$  in phosphate or sodium chloride gradients (measured by HPSEC); Bio-Sil 400-5 column; run-time 20 minutes; 0.0 to 0.4 AU scale, 280 nm UV



A pool, native IgG pool from CHT, and CHT cleaning step from the two-step platform. The IgG pool from CHT is aggregate free, as it was for the three-step platform (not shown). In Figure 8, reduced SDS PAGE compares both purifications.

## AN IDEAL PARTNER

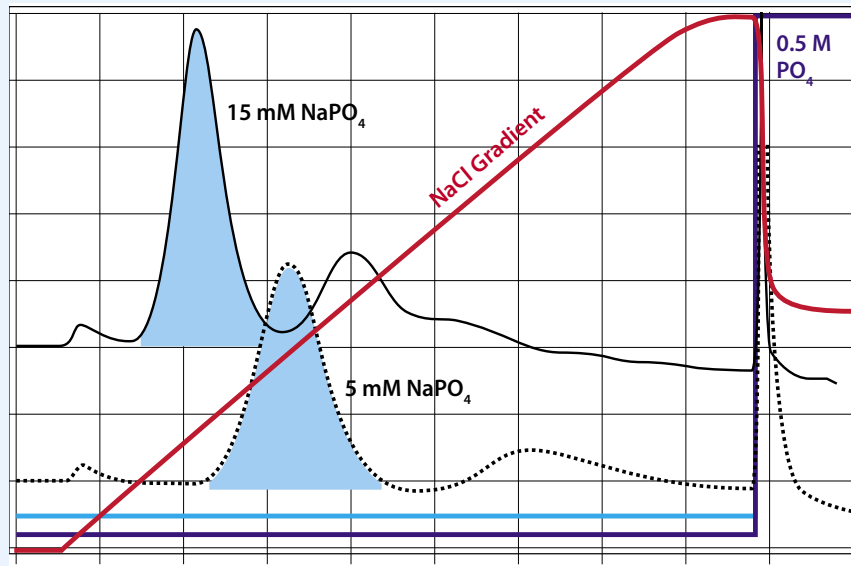
CHT solves the two most serious challenges in using protein A as a capture step—aggregates and leached protein A—thus making it an ideal partner for platform purification of monoclonal IgGs. Its ability to simultaneously remove DNA and endotoxin suggests the prospect of a two-step purification platform with compelling economic incentives: It reduces the number of process steps to be developed, validated, and run in the course of every manufacturing campaign while reducing requirements for hardware, chromatography media, buffer, and water.

For cases in which the contaminant distribution and behavior of a particular antibody might preclude a two-step platform, protein A and CHT can provide a robust and broadly applicable foundation for three-step platforms that include anion-exchange, cation-exchange, or hydrophobic-interaction chromatography. The ability of CHT to remove DNA and endotoxin suggests that it may be substituted for anion exchange in some platforms. In such instances, its relatively higher tolerance for high-conductivity feedstreams make it compatible with a wider range of sample compositions from previous purification steps, thereby requiring less sample treatment and enhancing overall process flow.

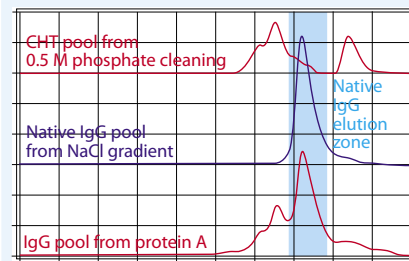
## ACKNOWLEDGMENTS

The authors would like to thank Ursula Snow, Valerie McLaughlin, and Andrew Cohen for their many helpful suggestions in the preparation of this manuscript.

**Figure 6:** Influence of phosphate concentration on IgG elution in a sodium chloride gradient (protein-A-purified mouse IgG chimera sample; CHT Type I, 40  $\mu\text{m}$ , 2 mL MT2 column); solid black line is elution in a sodium chloride gradient to 1.0 M at 15 mM sodium phosphate pH 6.5; dotted black line is elution in sodium chloride gradient to 1.0 M at 25 mM sodium phosphate pH 6.5; later peaks contain aggregates



**Figure 7:** Aggregate content from protein A and CHT purification of monoclonal chimeric IgG (measured by HPSEC); Bio-Sil 400-5 column; run-time 20 minutes; 0.0 to 0.2 AU scale, 280 nm UV; peaks to the left of the colored zone are aggregates



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**Figure 8:** Flamingo-stained, reduced SDS-Page from two- and three-step platforms



- Molecular weight standards
- Protein A feedstream
- Protein A flowthrough
- Protein A, urea, salt, and EDTA wash
- Protein A elution
- blank
- CHT native IgG peak from two-step platform
- CHT aggregate peak from two-step platform
- blank
- UNOsphere Q feedstream from three-step platform
- UNOsphere Q flow-through from three-step platform
- UNOsphere Q, 1.0 M NaCl elution from three-step platform
- blank
- CHT native peak from three-step platform
- CHT aggregate peak from three-step platform

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## THREE-STEP CHT PLATFORM FOR PURIFICATION OF MONOCLONAL IGG

### 1) PROTEIN A: MEDIA OF CHOICE

**Buffers:** A—0.05 M sodium phosphate, pH 7.2; B—0.1 M glycine or arginine, 0.05 M NaCl pH 3.8. No citrate or chelating agents.

**Fractionation:** Flow rate per manufacturer's recommendation; equilibrate with buffer A; load sample (20 mg IgG per mL of gel or manufacturer's recommendation); wash to baseline; elute with buffer B to baseline; clean per manufacturer's recommendation

**Comments:** Elution pH was chosen to conform to published values for virus inactivation (45). Sodium chloride in the elution buffer can improve antibody stability and solubility, which also helps to minimize aggregation (4,6). The choice between glycine and arginine may also affect aggregation and activity recovery but must be determined experimentally (46,47).

### 2) ANION EXCHANGE:

#### FLOW-THROUGH MODE, UNOSPHERE Q

**Sample Preparation:** Dilute IgG pool from protein A step with 1.0 M Tris pH 7.5, 2.8% volume to volume (v:v). This will yield a pH of 7.0 and conductivity of 7.5 mS/cm.

**Buffers:** A—0.05 M Tris, pH 7.0; B—A + 1 M NaCl, pH same as buffer A

**Fractionation:** Flow rate 300 cm/hr; equilibrate with buffer A until pH of column effluent equals buffer A; load sample (volume equivalent to 100 mg IgG per mL gel); wash with 5 CV buffer A; clean with 10 CV 100% buffer B; sanitize with 1 M NaOH

**Comments:** In most cases, antibodies will flow through the column during sample application. If not, conductivity may be raised to 12 mS/cm and conditions will still be within the published range shown to remove retroviruses (16).

### 3) HYDROXYAPATITE: CHT, TYPE I 40 MICRON

**Sample Preparation:** Add 1% v:v 500 mM monosodium phosphate (pH ~4.1) to the IgG pool from the previous step. This will raise the sample phosphate concentration to 5 mM and reduce the pH to about 6.5.

**Buffers:** A—5 mM NaPO<sub>4</sub>, pH 6.5; B—A + 1.5 M NaCl; C—0.5 M NaPO<sub>4</sub>, pH 6.5

**Fractionation:** Flow rate 300 cm/hr; equilibrate with buffer A until column effluent is pH 6.5; load sample (volume equivalent to 20 mg IgG per mL of gel); wash with 5CV buffer A; elute with 40 CV linear gradient to 100% buffer B; clean with 5 CV 100% buffer C

**Comments:** Native antibody will usually elute within the NaCl gradient but if not, then increase the phosphate concentration to 10 mM. The suggested gradient is for screening. Slope and amplitude can be adjusted based on initial results. It may also be converted to a step format or run in flow-through mode. Capacity may be as high as 60 mg/mL for some monoclonals. For the two-step protein A/CHT platform, protein A was run identically. The protein A pool was prepared for the CHT step by a 1% (v:v) addition of 500 mM sodium phosphate at pH 10.5. All other materials and conditions for the CHT step were unchanged.

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