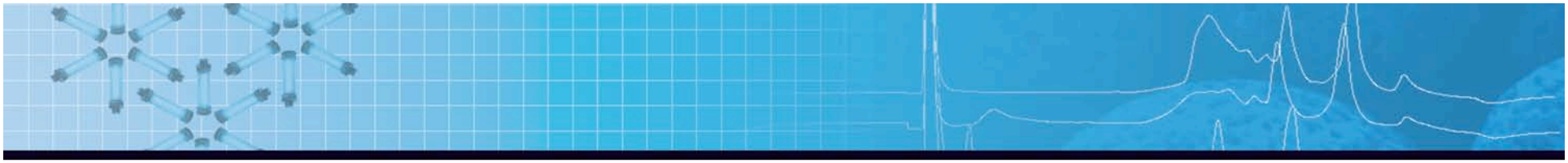

A Tale of Two Platforms:

2-step purification of monoclonal IgG with protein A and CHT™ ceramic hydroxyapatite

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IBC Production and Purification of Monoclonal Antibodies
Carslbad CA, March 1-3, 2006

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The challenge

- Protein A affinity chromatography is an effective capture method but its use is associated with two downstream purification challenges:
  - formation of aggregates
  - leaching of protein A
- Aggregates promote formation of neutralizing antibodies and can promote thrombotic events.
- Protein A is immunotoxic and is an adjuvant protein that can promote formation of neutralizing antibodies.

How can CHT help?

- Hydroxyapatite has been known for more than a decade to remove leached protein A and aggregates from monoclonal antibodies.
- It also reduces endotoxin, DNA, and virus levels.
- Dynamic binding capacity for IgG monoclonals ranges from 25 to more than 60 mg IgG/mL (CHT type I, 40 micron).

How does CHT work?

- **Cation Exchange**
  - Protein amino groups form ionic bonds with negatively charged CHT phosphates.
  - These interactions can be controlled with neutral salts like sodium chloride, or with buffering salts like sodium phosphate.
- **Metal Affinity**
  - Proteins and other biomolecules participate in metal affinity interactions with CHT calcium.
  - These interactions are impervious to neutral salts. They can be eluted only with phosphate.

How does CHT work?

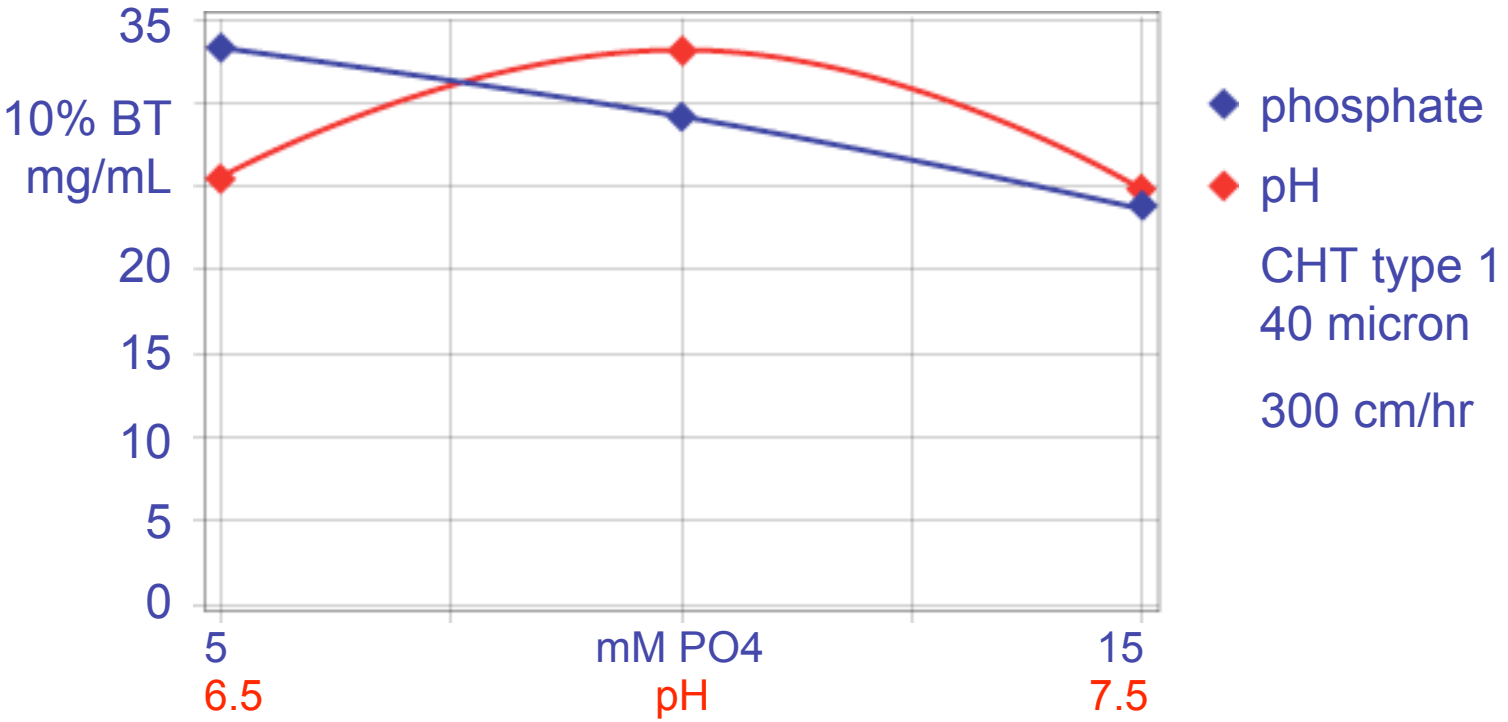
- Most large proteins bind by a combination of mechanisms:
  - Dominantly acidic proteins bind chiefly by metal affinity. Albumin is an example. NaCl weakly reduces capacity and retention time (in phosphate gradients) indicating a minor contribution by cation exchange. Phosphate is required for elution.
  - Dominantly basic proteins bind chiefly by phosphoryl cation exchange. IgG is an example. NaCl substantially reduces capacity and retention time (in phosphate gradients). Very little phosphate is required for elution at high concentrations of NaCl, indicating a small contribution by metal affinity.

What's new?

- Sodium chloride gradients conducted at a constant low concentration of sodium phosphate provide higher capacity, better IgG purification, and more consistent performance among different monoclonals than traditional phosphate gradients.

Capacity versus phosphate and pH

Dynamic binding capacity, polyclonal human IgG



phosphate experiments conducted at pH 7.0
pH experiments conducted in 5mM phosphate

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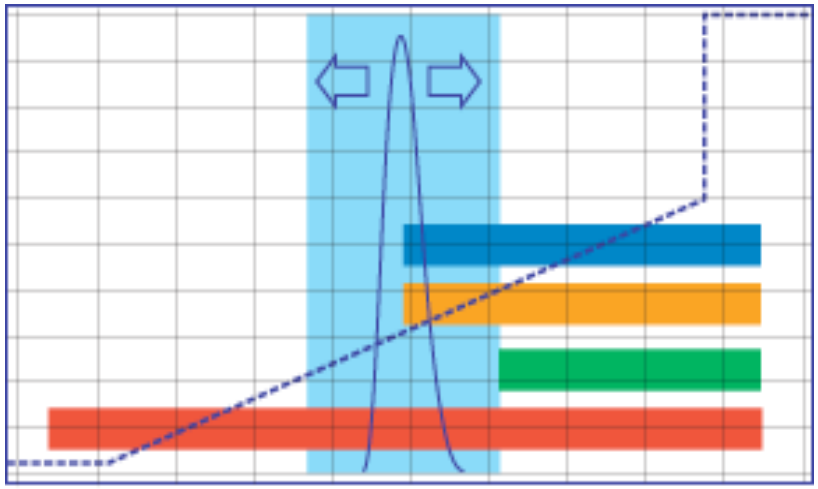
Phosphate versus chloride gradients

Contaminant removal from protein A purified IgG

CHT-I, 40µm	Phosphate	Chloride
Aggregates	50-90%	95-99%
Protein A	50-95%	95-99.9%
CHOP	50%	98%
DNA	<1 log	>3 logs
Endotoxin	<1 log	>4 logs

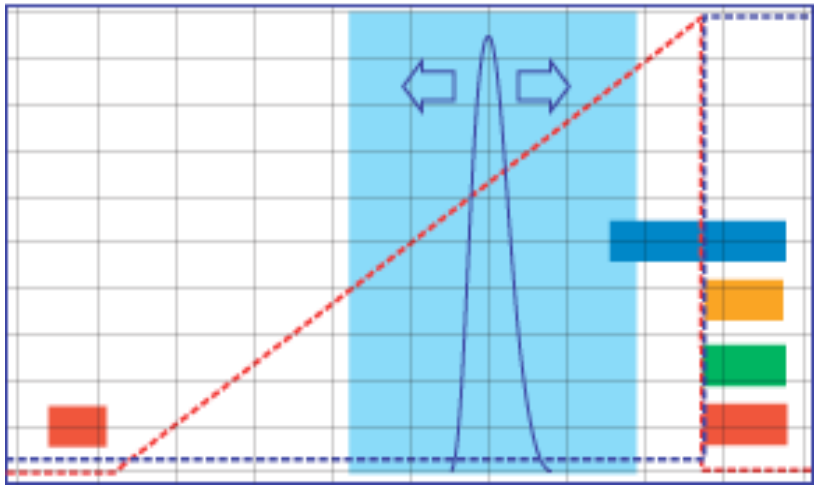
Phosphate versus chloride gradients

Phosphate Gradient
Equilibrate 5 mM NaPO4
Gradient to 0.3M NaPO4
Clean with 0.5M NaPO4



Aggregates
Protein A-IgG
DNA
Endotoxin

Chloride Gradient
Equilibrate 5 mM NaPO4
Gradient to 2.0M NaCl
Clean with 0.5M NaPO4



Aggregates
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Phosphate versus chloride gradients

Phosphate Gradient
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CHT, type I, 40 µm

Chloride Gradient
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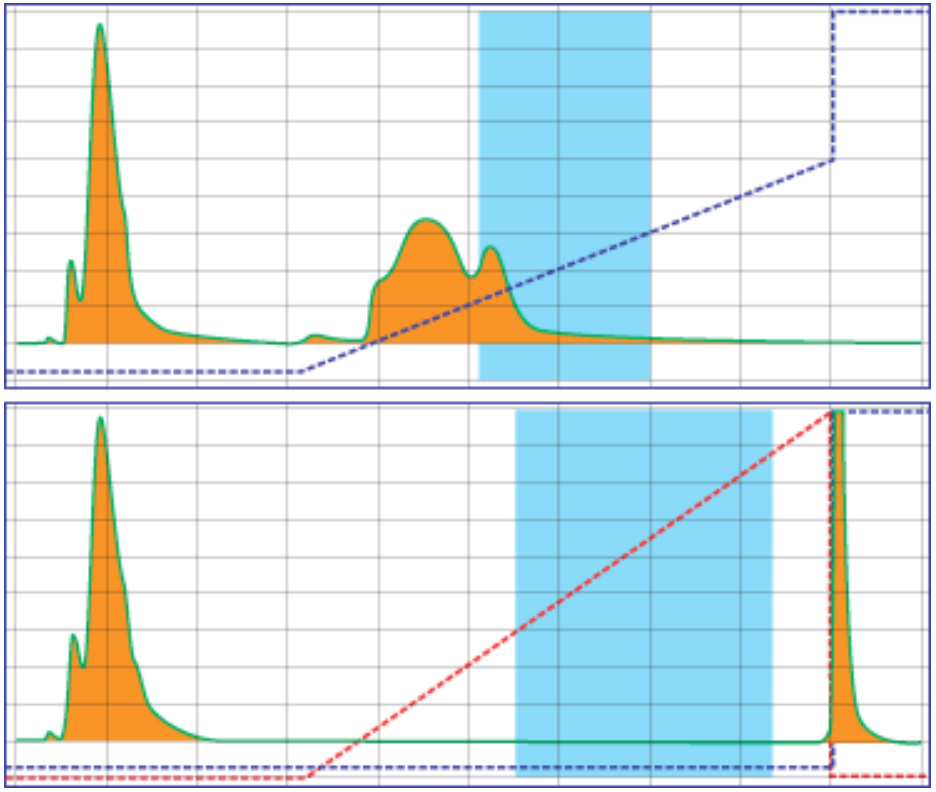

Sample: an equal mixture of purified endotoxin from E.coli, P. aeruginosa, and S. minnesota

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Why are chloride gradients better?

- The phosphate in traditional gradients acts simultaneously as a salt, dissociating cation exchange interactions, and an affinity competitor for CHT calcium, but provides no latitude for independent control of the two mechanisms.
- Setting a constant low level of phosphate weakens metal affinity, but leaves ionic bonds mostly intact. The sodium chloride gradient then dissociates ionic bonds, eluting IgG but leaving contaminants that require high phosphate for elution still bound to the column.

The two platforms…

- **Platform I.**
- Microfiltration
- **Protein A affinity**
- Virus inactivation
- **CHT, type I, 40µm**
  bind/elute mode
- Virus filtration
- Conc/diafiltration
- Microfiltration

- **Platform II.**
- Microfiltration
- **Protein A affinity**
- Virus inactivation
- **CHT, type I, 40µm**
  flow-through mode
- Virus filtration
- Conc/diafiltration
- Microfiltration

Platform I & II: screening conditions

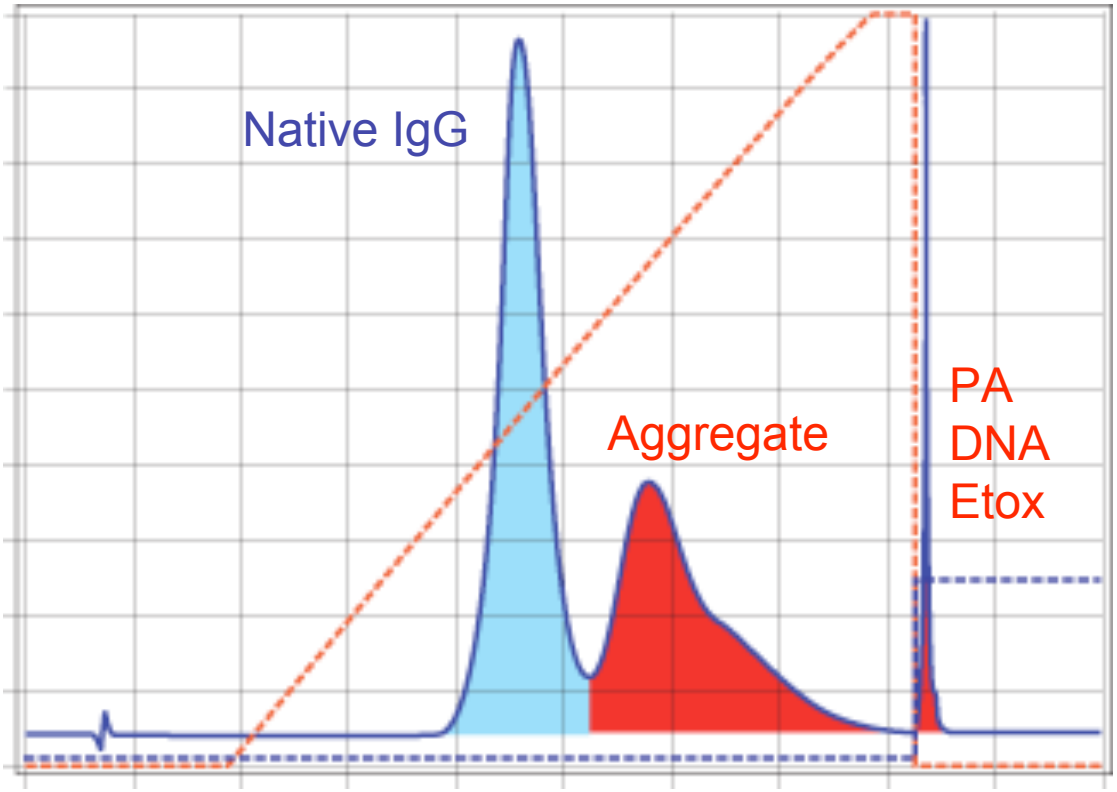
- Elute protein A with 0.1M glycine, 0.05M NaCl, pH 3.8 (no citrate or EDTA).
- Hold for virus inactivation (see resources)
- Raise pH to 6.5 by addition of 0.5M NaPO4, pH 10.4, 1% v:v.
- Equilibrate CHT with 5mM NaPO4, pH 6.5
- Load, wash, 40 CV linear gradient to 2.0M NaCl
- Clean with 0.5M NaPO4

Platform I & II. initial screen

15 mM NaPO4, pH 6.5

40CV gradient to 2M NaCl (15mM PO4)

Clean 0.5M NaPO4



Protein A purified IgG1

CHT type I, 20 micron, 300 cm/hr

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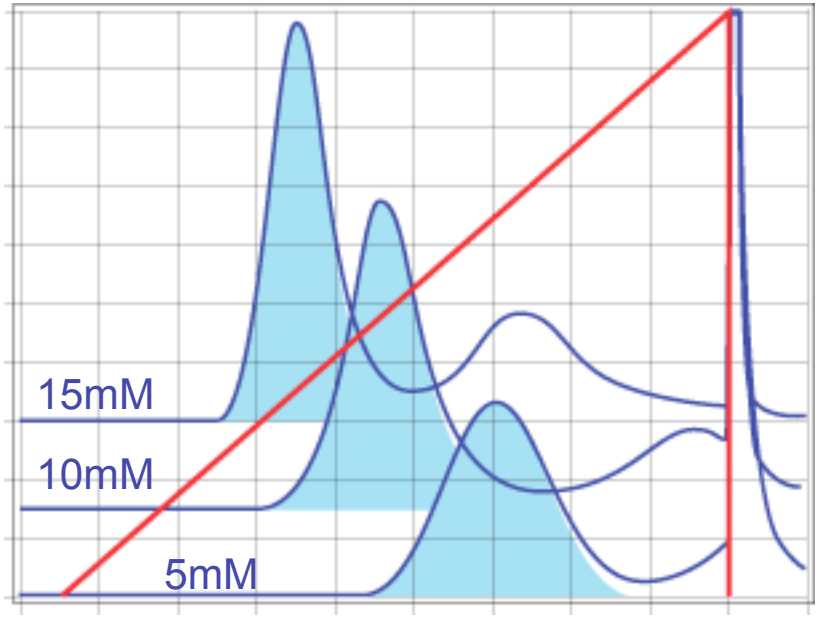
Platform I & II: optimization

- Determine the critical phosphate threshold:
  - Conduct a chloride gradient at a constant 5mM phosphate*.
  - If native IgG fails to elute within the chloride gradient at 5 mM phosphate, repeat the experiment at 10 mM.
  - If it fails to elute at 10 mM, try 15 mM, etc.

* At a minimum of 5mM phosphate, pH 6.5, CHT exhibits no loss of mass, flow properties, or separation performance after 50 cycles under experimental conditions. Operating pH should not be reduced below 6.5 nor phosphate below 5mM.

Platform I & II: optimization

40 CV linear gradient to 1.0M NaCl at constant phosphate concentrations as indicated



protein A purified IgG on CHT type I 20 µm

Blue areas indicate native IgG

Red line indicates NaCl gradient trace

NaCl gradient followed by cleaning with 0.5M phosphate

All experiments at pH 6.5
300 cm/hr

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Platform I & II. optimization

Phosphate mM	5	10	15
Protein A ng/mL	<0.2	<0.2	<0.2
DNA ng/mL	<1	<1	3.9
Endotoxin EU/mL	<0.05	1.0	1.6

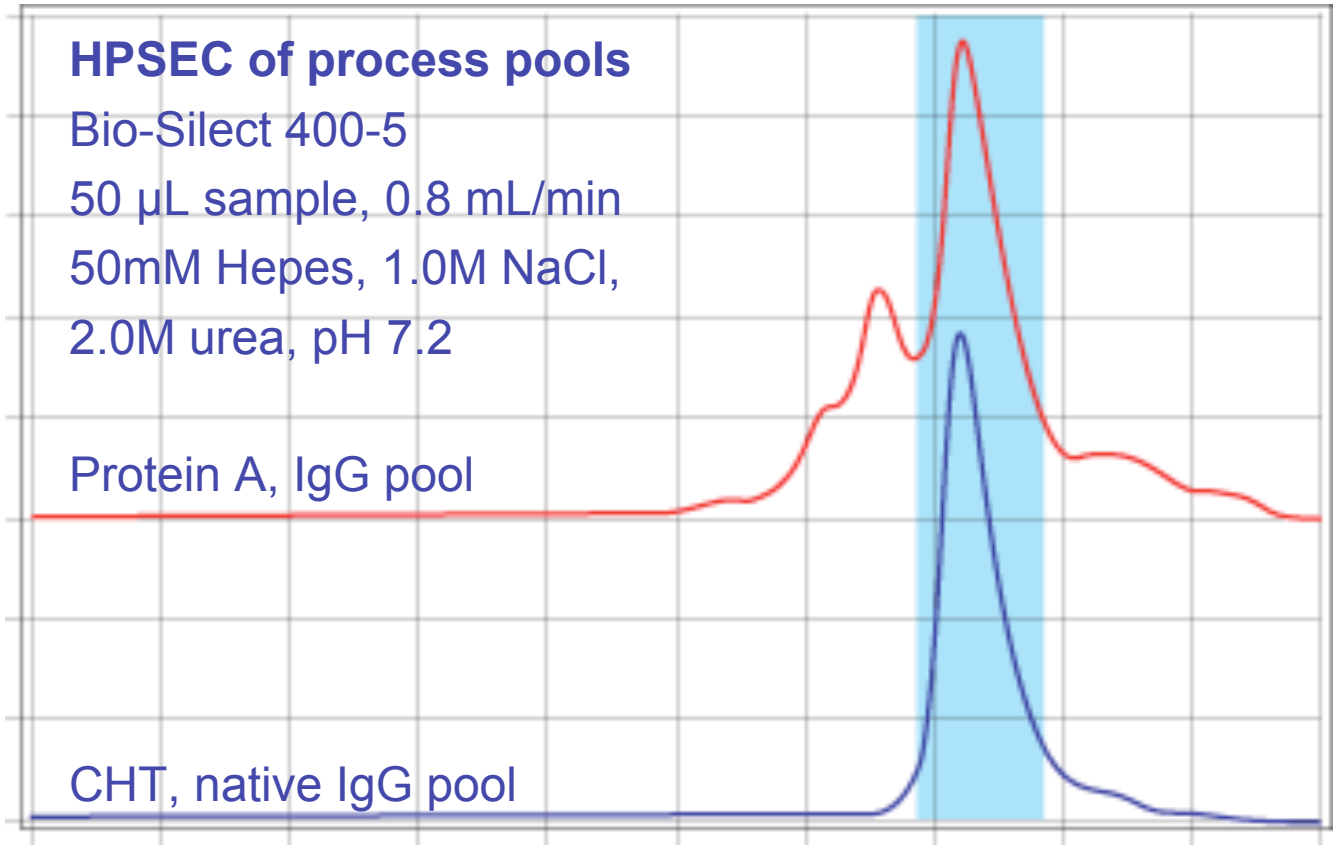
Sample: protein A purified IgG. 22 ng/mL leached protein A, 2.3×10^3 ng/mL DNA, 1.9×10^4 EU/mL endotoxin

All results for a sodium chloride gradient to 1.5 M at pH 6.5 with phosphate concentration held at the indicated level

Platform I: optimization

- Once the critical phosphate threshold is determined:
- Refine the chloride gradient set points
- Convert to a step gradient (optional timing).
- Consider loading at pH 7.0 to optimize capacity.

Platform I. aggregate removal



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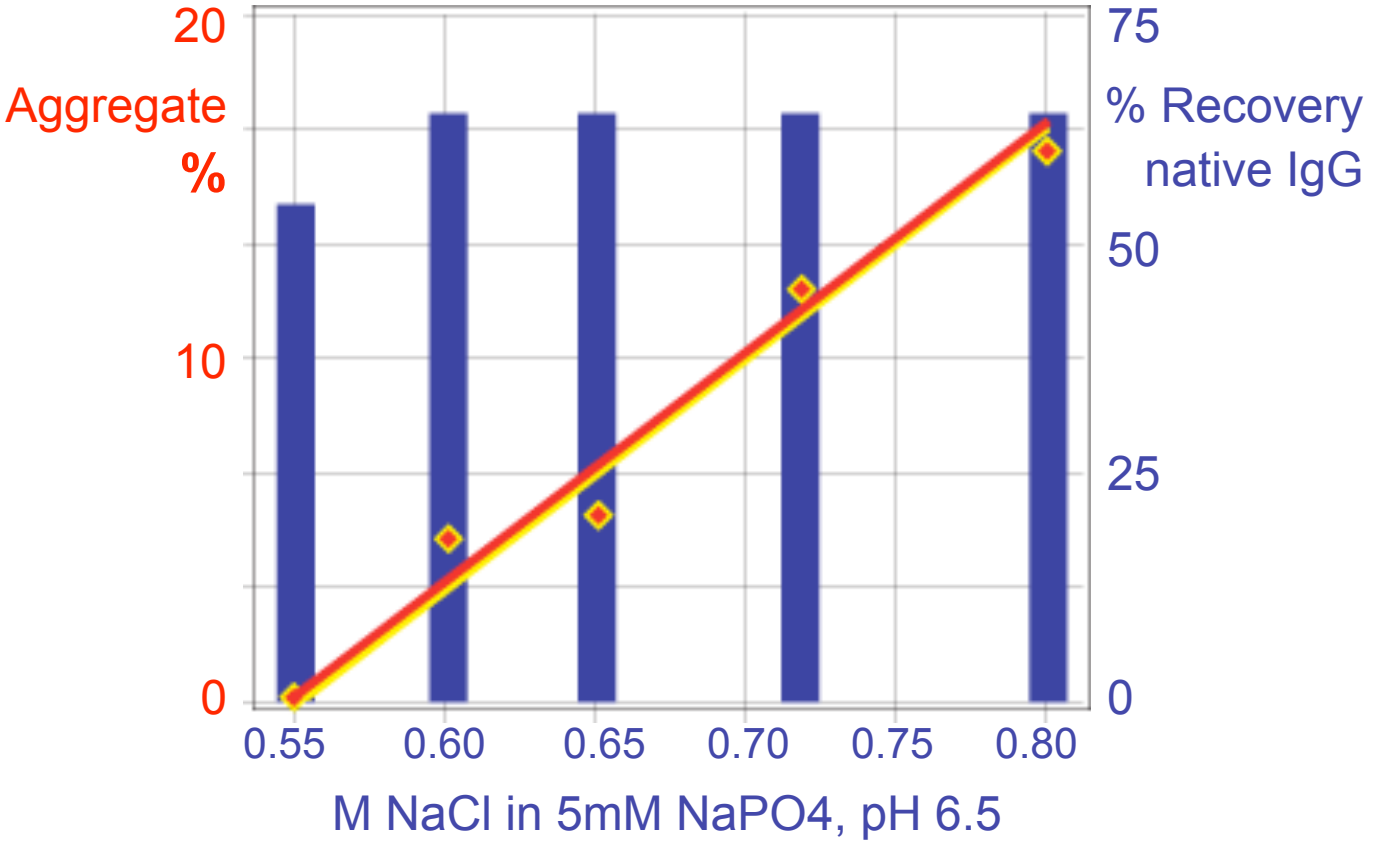
Platform II: optimization

- Once the critical phosphate threshold is determined:
- Define the sodium chloride concentration for the flow-through phase.
- Consider loading at pH 7.0 to optimize capacity.

Platform II: optimization



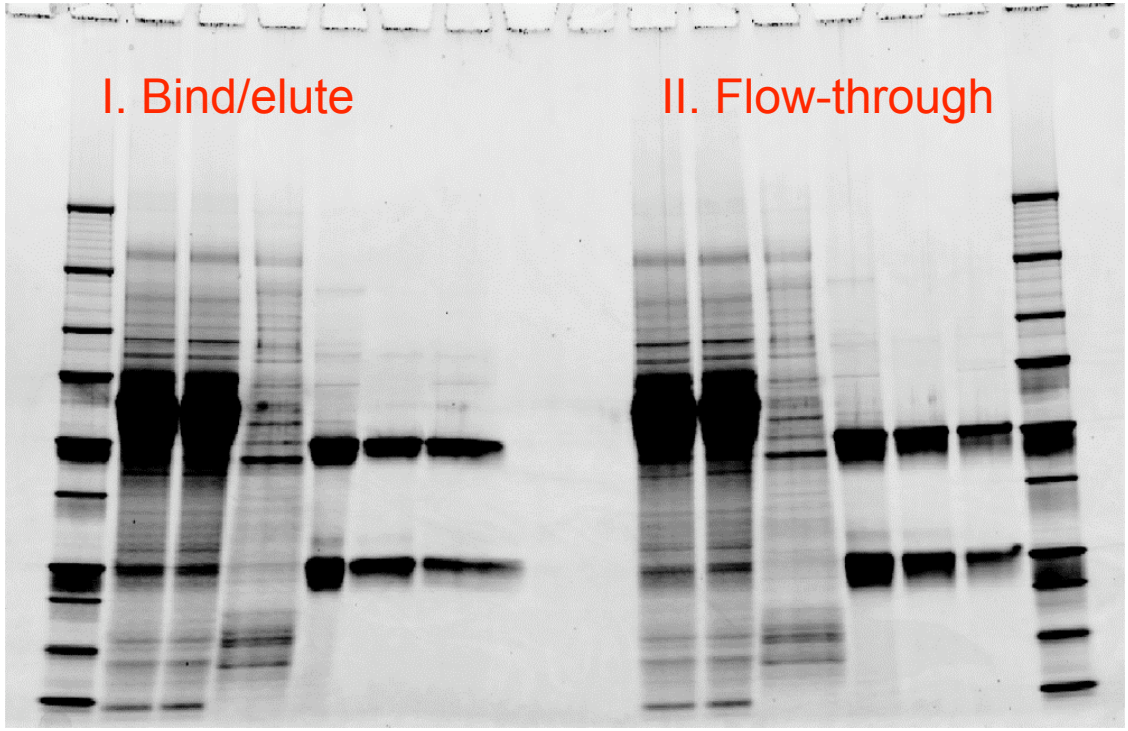
Platform comparison

	OM	Protein A IgG pool	Platform I native pool	Platform II native pool
Aggregate %, HPSEC	n.d.	>40%	<1%	<1%
Protein A ng, Cygnus	0	162	<0.2*	<0.2
DNA, ng picogreen	9.9×10^5	3.8×10^4	<1	<1
Endotoxin EU, LAL	2.8×10^3	5.0×10^2	<0.05	<0.05
% Recovery	100	25**	75***	54***

*detection limit of the assay, **low recovery due to aggregation, ***native IgG

Platform comparison

Reduced SDS PAGE (Flamingo stain)



1. OM
2. PA flow-through
3. PA wash (KS)
4. PA pool
5. CHT native pool
6. CHT clean step

KS: 1M NaCl, 2M urea, 10mM EDTA 0.05M PO_4 pH 7.0

Sensitivity equals silver stain

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Platform comparison

- The bind/elute platform achieves good recovery plus outstanding removal of leached protein A, aggregates, endotoxin, and DNA.
- The flow-through platform supports equivalent removal of leached protein A, endotoxin, and DNA, but imposes a compromise between aggregate removal and product recovery.
- Given that aggregate content could prove to be variable from lot to lot of purified IgG, even if aggregate content is low (thereby permitting optimization of recovery) the bind/elute platform will generally be the more prudent choice.

Is a 2-step platform feasible?

- Several major companies believe that a 2-step platform can meet all the requirements for an injectable protein, and are pursuing this goal.
- For the capture step, success will require development of wash buffers to remove contaminants that are nonspecifically bound to protein A media.
- For the polishing step, success will require focusing on the challenges associated with protein A: leachate and aggregates; while achieving further reduction of DNA, virus, and endotoxin.
- CHT is a strong candidate for this polishing application.

Column hygiene

- **Clean:** 0.5M phosphate*
- **Sanitize:** 1.0M NaOH
- **Store:** 0.1M NaOH

* In the absence of other salts.

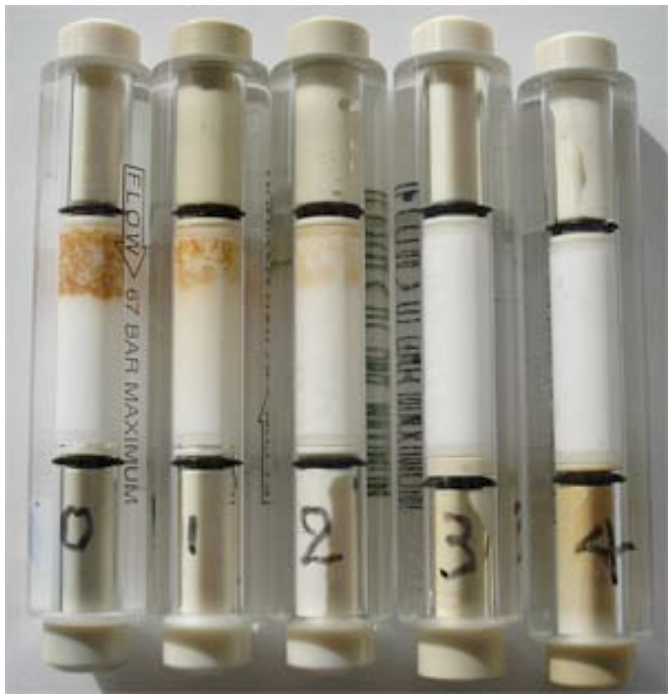
Column hygiene

- CHT binds metals from process solutions, causing discoloration at the top of the column.
- These metals may come from production media, buffers and salts, process water, or corroded stainless steel process equipment.
- To prevent discoloration, add 100 mg CHT (type I, 40µm)* per liter of buffer during formulation.
- Incubate 1 hour.*
- Microfilter buffers as usual.

* Suggested starting points. Experiment with quantity and time to accommodate specific needs.

Column hygiene

Prevention of discoloration by prophylactic buffer treatment



0 15 30 60 120

Minutes buffer exposure to adsorbent before filtration

CHT type I, 40 micron, 1 mL columns
Original buffer: 50µM $FeCl_3$
Each buffer adsorbed with 10 mg CHT type I 40 micron for the indicated time, then filtered to 0.22µm.
800 mL of treated buffer flowed over a new column at 300 cm/hr.

Visible ferric contamination was also removed by 100 mg CHT after 15 minute incubation, or with 1 mg after 16 hours.

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Resources

Viral Inactivation

K. Brorson et al, 2003, Bracketed generic inactivation of rodent retroviruses by low pH treatment for monoclonal antibodies and recombinant proteins, Biotechnol. Bioeng., 83(3) 321-329

CHT, Protein A Removal

S. Franklin, Protein A removal from IgG on CHT Ceramic Hydroxyapatite, Bio-Rad Tech Note 2849, http://www.bio-rad.com/lifescience/pdf/bulletin_2849.pdf

J. Chen et al, 2005, Physical-chemical antibody properties determine optimal separation modes for protein A removal, Poster, Waterside Conference, Miami, jchen@dyax.com

CHT, Aggregate Removal

S. Frankin, Removal of aggregate from an IgG_4 product using CHT ceramic hydroxyapatite, Bio-Rad Tech Note 2940 http://www.bio-rad.com/lifescience/pdf/bulletin_2940.pdf

CHT, Column Packing

CHT Ceramic hydroxyapatite, 40 µm - Optimized packing in GelTec™ Columns, Tech Note 3199, http://www.bio-rad.com/cmc_upload/Literature/113783/Bulletin_3199.pdf

Flamingo Protein Stain

T. Berkelman, 2005, Detection and quantitation of proteins using a novel fluorescent dye. http://www.expressionproteomics.com/LifeScience/pdf/Bulletin_5352.pdf

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- For a copy of this presentation, or other Bio-Rad resources concerning CHT, please contact <Andrew_Cohen@bio-rad.com>
- For technical questions concerning application development, you are welcome to contact <Peter_Gagnon@bio-rad.com>