Simultaneous removal of aggregates, leached protein A, endotoxin, and DNA from protein A purified IgG with CHT™ ceramic hydroxyapatite and CFT™ ceramic fluorapatite

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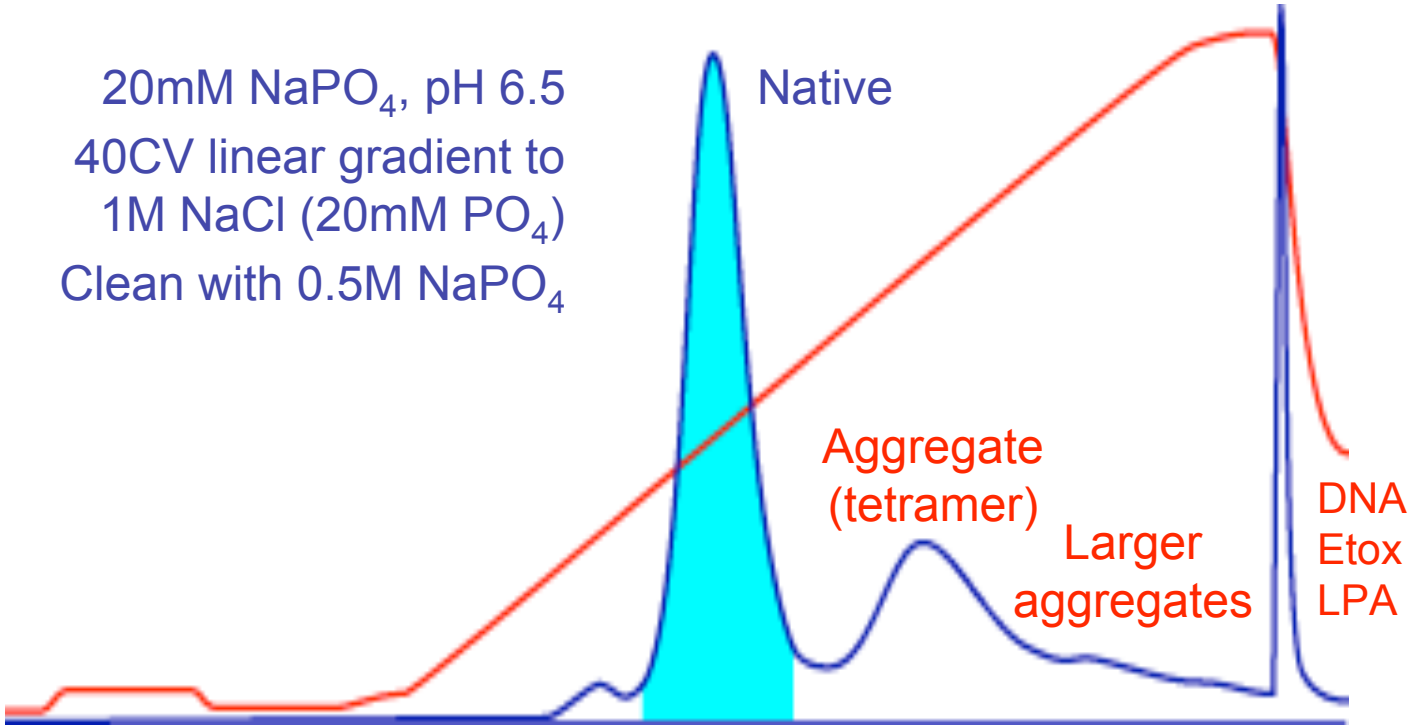
Chemical structure, hydroxyapatite

- **Calcium hydroxyapatite**
- $Ca_{10}(PO_4)_6(OH)_2$
- Calcium participates in metal affinity interactions
- Phosphate participates in cation exchange/exclusion interactions
- Stable down to pH 6.5 in the presence of 5mM phosphate

CHT fractionation of contaminants

20mM $NaPO_4$, pH 6.5
40CV linear gradient to
1M NaCl (20mM PO_4)
Clean with 0.5M $NaPO_4$



Protein A-purified human IgG1
CHT type I, 20 micron, 300 cm/hr

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Analysis of CHT fractions

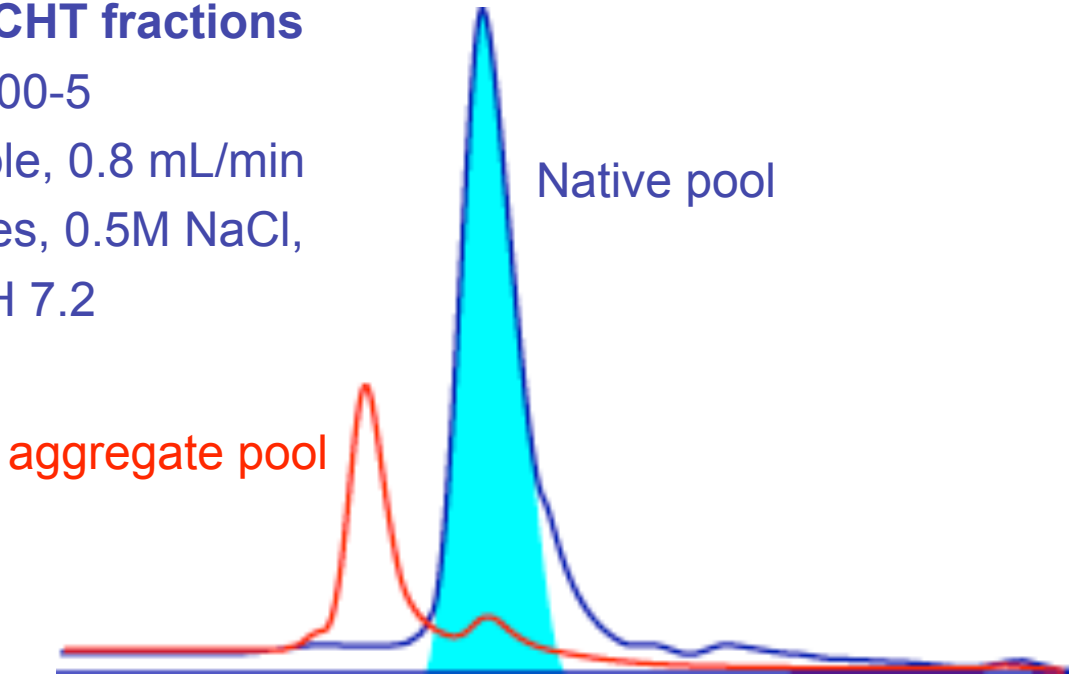
HPSEC of CHT fractions

Bio-Silect 400-5

50 µL sample, 0.8 mL/min

50mM Hepes, 0.5M NaCl,

2M urea, pH 7.2



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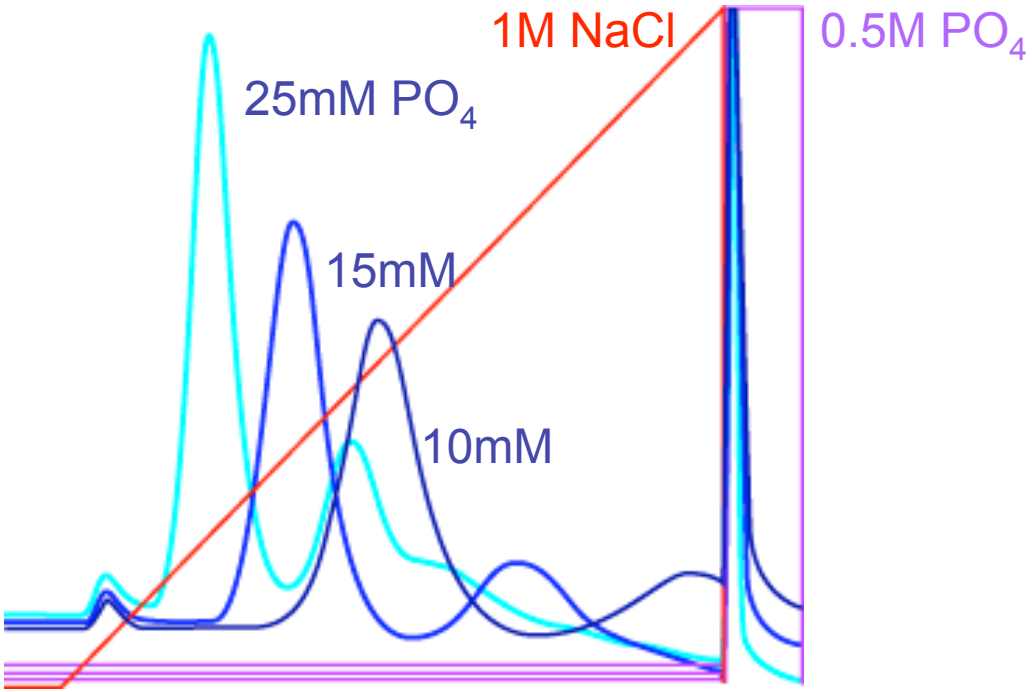
The effect of PO_4 on CHT

Indicated phosphate concentration maintained across the sodium chloride gradient



protein A purified IgG on CHT type I 20 µm

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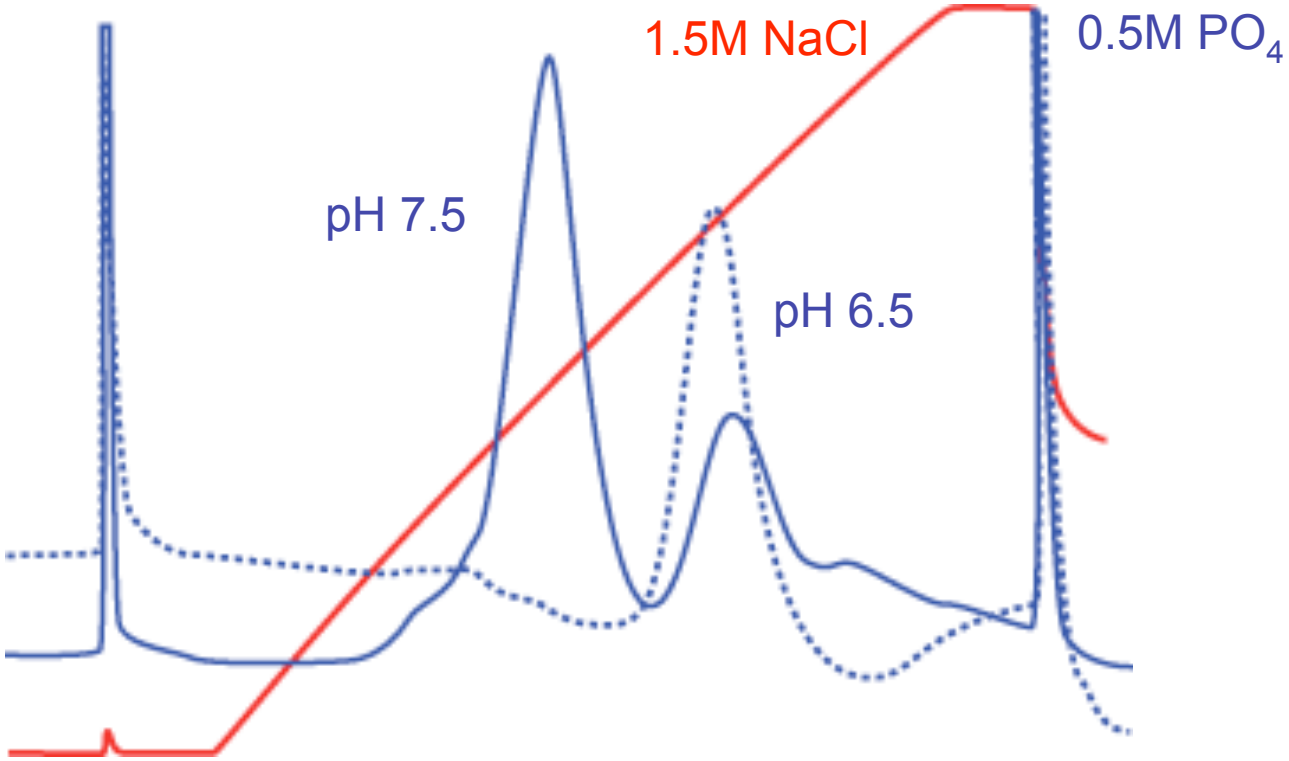
The effect of pH on CHT

Sodium chloride gradient at constant 5mM $NaPO_4$



protein A purified IgG on CHT type I 20 µm

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Summary of CHT performance

- **Aggregate removal**
  - > 99% by HPSEC
  - from > 40% to < 1%
- **Leached protein A removal**
  - 90% by Cygnus*
  - from 55 to 5 ng/mL
- **DNA removal**
  - > 3 logs by PCR
  - down to < 1ng/mL by picogreen
- **Endotoxin removal**
  - 7 x 10^4 by LAL
  - down to 1EU/mL

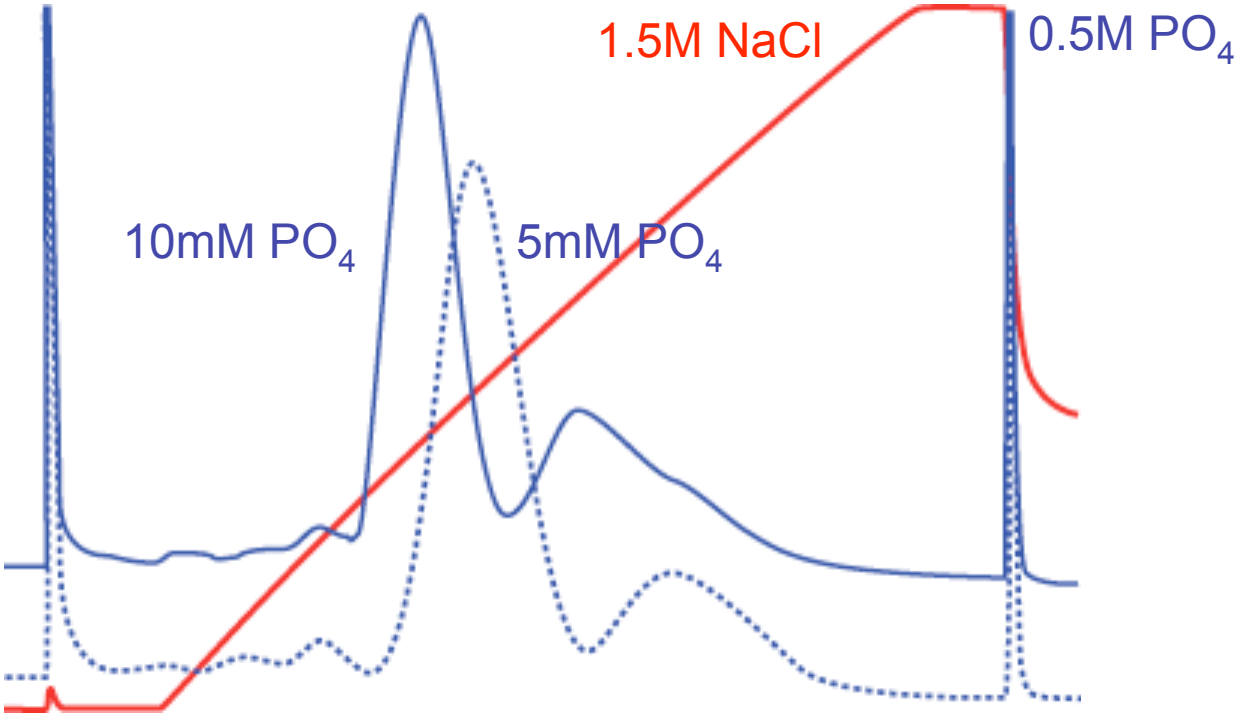
* at 20 mM $NaPO_4$, >99% LPA removal at 5mM

Chemical structure, fluorapatite

- **Calcium fluorapatite**
- $Ca_{10}(PO_4)_6F_2$
- Calcium participates in metal affinity interactions
- Phosphate participates in cation exchange/exclusion interactions
- Stable to pH 5.5
- 4-5 times more mechanically stable than CHT

The effect of PO_4 on CFT

Sodium chloride gradient pH 6.5



protein A purified IgG on CFT type II 40 µm

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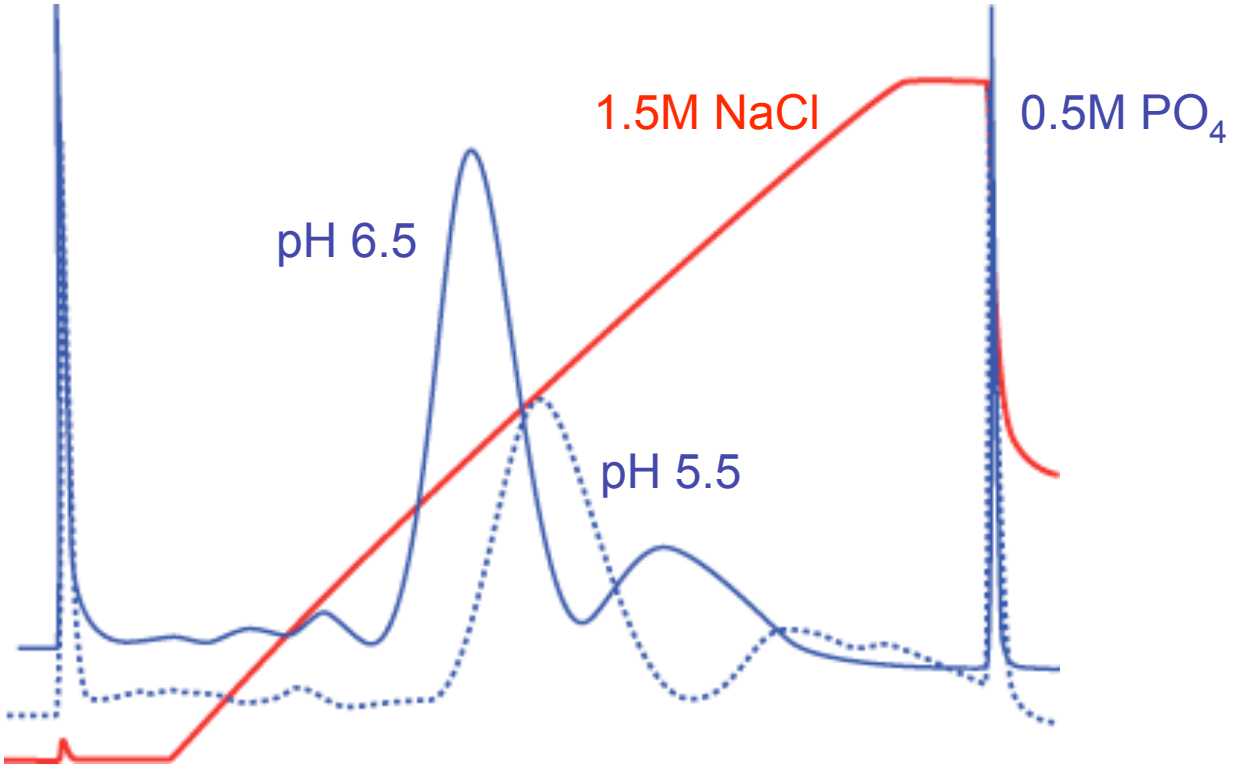
The effect of pH on CFT

Sodium chloride gradient at 5mM $NaPO_4$



protein A purified IgG on CFT type II 40 µm

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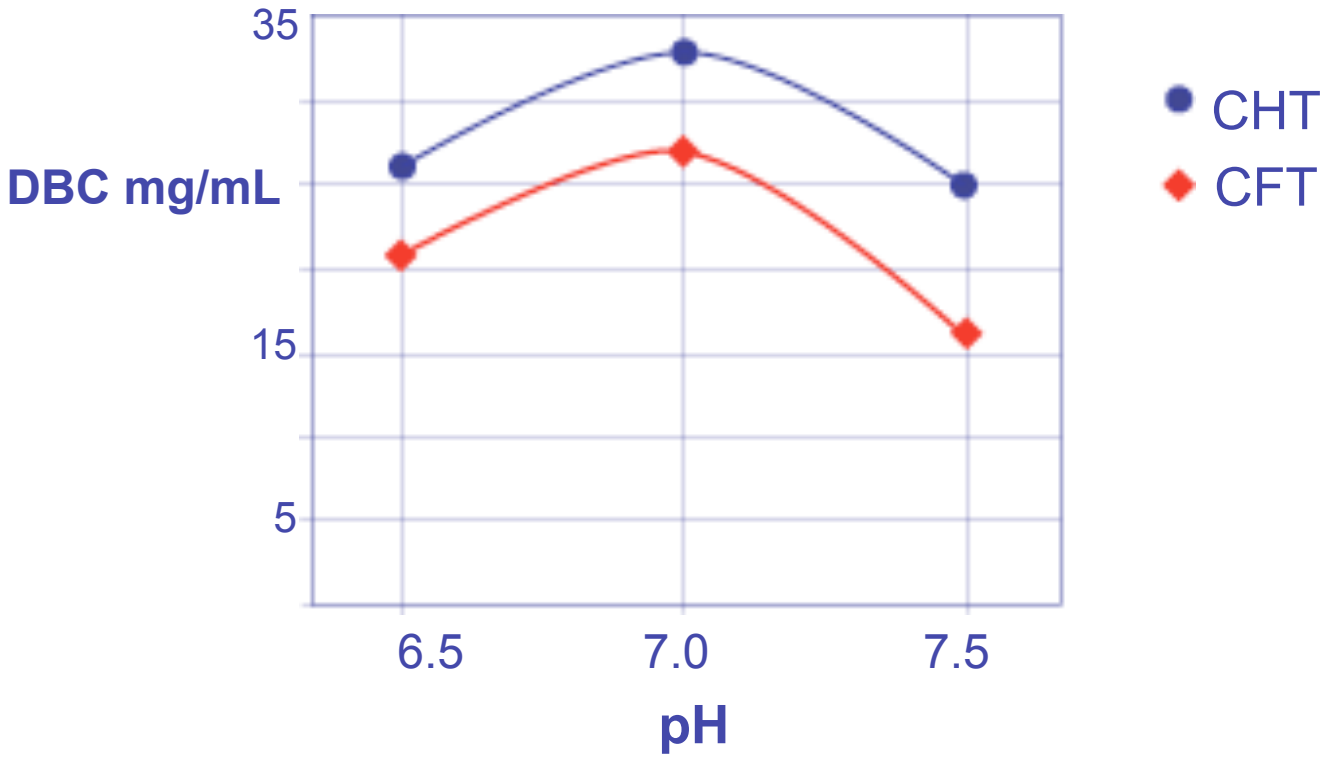
Capacity CHT vs CFT

Dynamic binding capacity of polyclonal human IgG
on CHT type I 40 µm and CFT type II 40 µm
10% breakthrough, 300 cm/hr



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Contaminant removal CHT vs CFT

	CHT, type I, 40 µm			CFT, type II, 40 µm		
$[PO_4]$ mM	5	10	15	5	10	15
PA ng/mL	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
DNA ng/mL	<1.0	<1.0	3.9	1.7	<1.0	<1.0
Etox, EU/mL	<0.05	1.0	1.6	3.9	6.2	9.5

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, followed by a cleaning step at 0.5 M $NaPO_4$, pH 6.5

Initial screening conditions CFT/CHT

- Equilibrate column with 5 mM $NaPO_4$, pH 6.5
- Inject 5% CV protein A purified IgG
- Wash 5 CV equilibration buffer
- Elute 30 CV linear gradient to 1.5 M NaCl (5mM $NaPO_4$)
- Clean with 0.5M $NaPO_4$.

- If native MAb peak fails to elute within the NaCl gradient, raise the phosphate concentration enough to bring it in (increments of 5mM or less).
- Optimize by adjusting slope and amplitude of NaCl gradient. Convert to step or flow-through for scale-up.

2-Step platform, protein A/CHT

- Elute protein A with 0.1M glycine* or arginine* 0.05M NaCl, pH 3.8 (no citrate or EDTA).
- Hold for viral inactivation.
- Raise pH to 6.5 by addition of 0.5M $NaPO_4$ pH 10.5, 1% v:v.
- Equilibrate CHT to 5mM $NaPO_4$, pH 6.5
- Run optimized CHT fractionation conditions

* Glycine and arginine concentration can be raised to 1-2M to reduce aggregation. Both are dielectric constant enhancers preferentially excluded from protein surfaces. They improve solubility at the same time that they stabilize proteins. Since both are zwitterionic above pH 5 they contribute nothing to conductivity when neutralized.

2-Step platform, protein A/CHT

	OM	PPA	PCHT
Aggregate %IgG	-----	>40	<1
Protein A ng	-----	162	6
DNA ng	9.9×10^5	3.8×10^4	12
Endotoxin EU	2.6×10^3	5.0×10^2	<0.05
IgG %	100	25*	45*

OM: original material

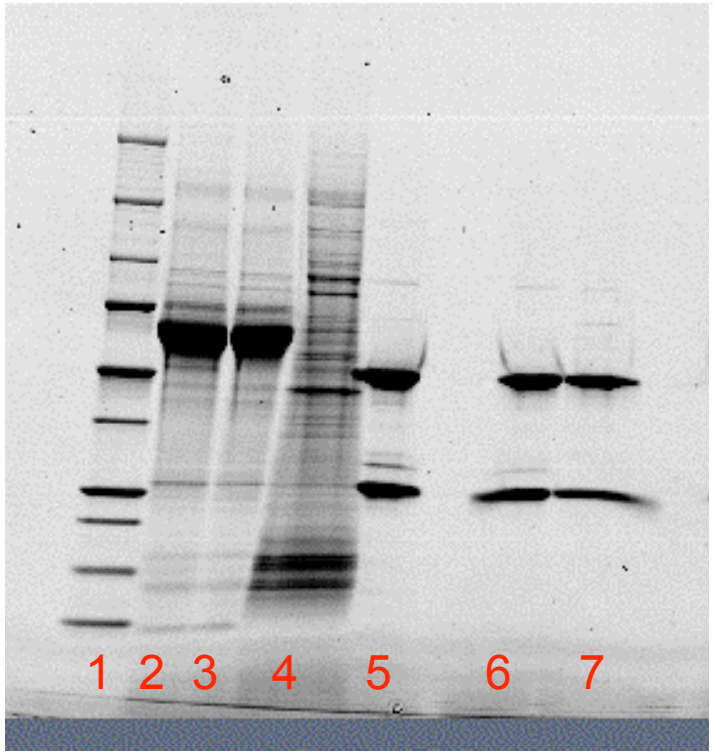
PPA: IgG pool from protein A

PCHT: native IgG pool from CHT

*low recovery PPA due to aggregation; PCHT due to aggregate removal

2-Step platform, protein A/CHT

Reduced SDS PAGE (Flamingo stain*)



1. MW stds
2. OM
3. PA flow-through
4. PA wash (KS)
5. PA pool
6. CHT native pool
7. CHT aggregate pool

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

*sensitivity equivalent to silver

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Conclusions

- CHT and CFT, when eluted with a sodium chloride gradient at a low concentration of phosphate, have a unique ability to simultaneously achieve major reductions in the levels of aggregates, leached protein A, DNA, and endotoxin.
- Scouting/feasibility can often be covered by a single experiment with a sodium chloride gradient at 5mM $NaPO_4$ pH 6.5; sometimes another with 10mM. Increments of pH can also be investigated.
- The method is easily integrated into a 2-step platform with protein A, or with a variety of 3-step platforms that exploit additional fractionation mechanisms.

Conclusions

- The selectivities of CHT and CFT, although similar and based on the same mechanisms, are distinct.
- CHT supports better resolution from endotoxin but performance is roughly equivalent for removal of aggregates, protein A, and DNA.
- CHT supports about 20% higher capacity
- CFT is more stable chemically and mechanically.

Acknowledgments

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