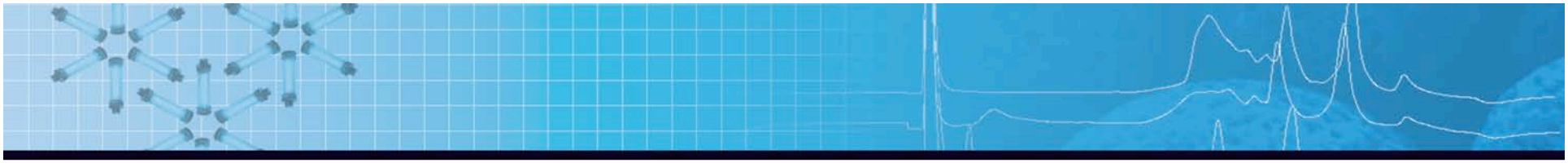

The practical task of monoclonal IgG purification with CHT™ ceramic hydroxyapatite

Pete Gagnon, Jie He, Paul Ng, Julia Zhen,
Cheryl Aberin, Heather Mekosh

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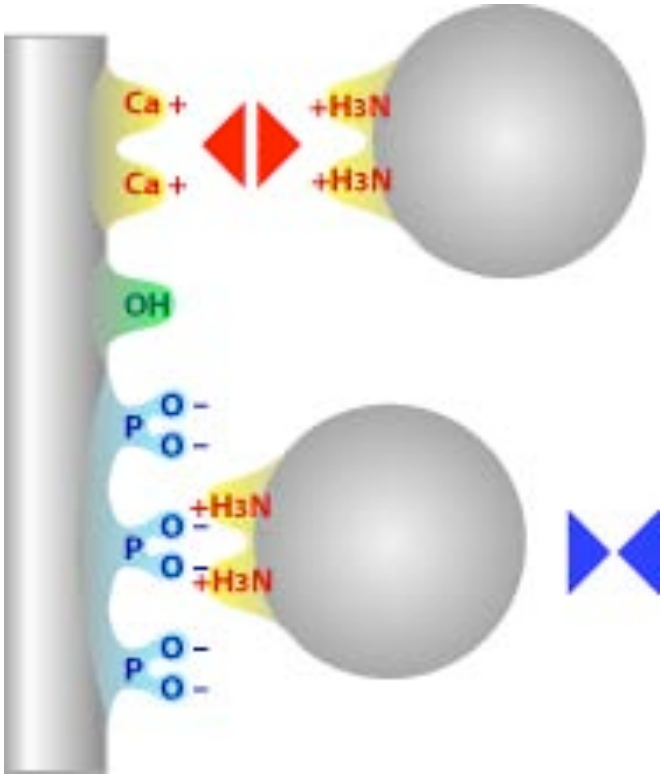
Antibody purification with hydroxyapatite

- 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.
- The introduction of ceramic hydroxyapatite CHT has made it possible to exploit these capabilities in a high throughput chromatographic format.

How CHT works

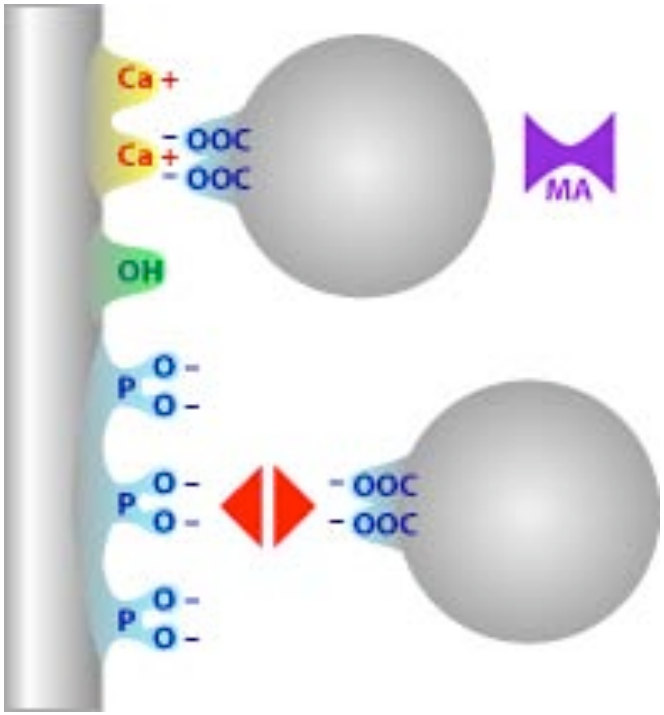
- Hydroxyapatite is a crystalline mineral of calcium and phosphate with the structural formula $Ca_{10}(PO_4)_6(OH)_2$
- It is a mixed mode adsorbent, interacting with biomolecules principally through two mechanisms: cation exchange and calcium metal affinity.

How CHT works



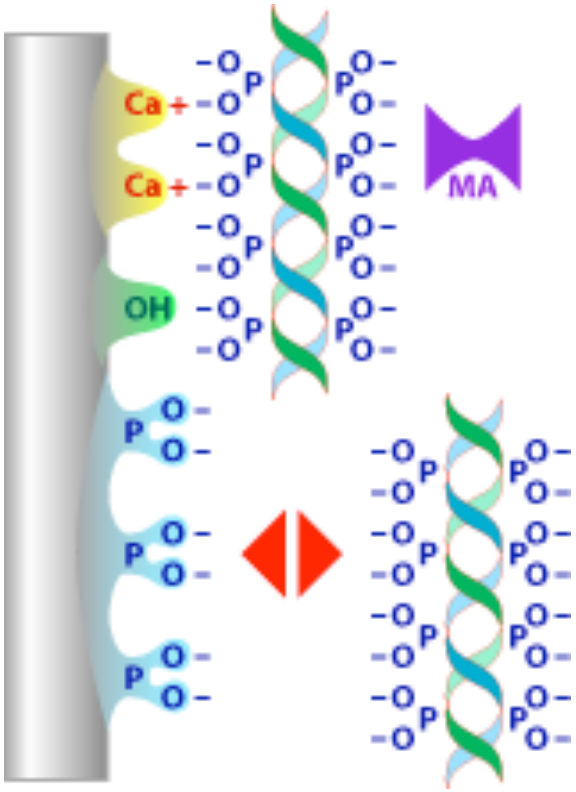
- **Amino residues**
- Classical cation exchange
- Dissociate with neutral salts like sodium chloride or with buffering salts like phosphate.
- Weaken or dissociate with increasing pH

How CHT works



- **Carboxyl clusters**
- Calcium chelation modulated by ion exclusion
- 15–60x stronger than ionic interactions alone
- Will not dissociate at any concentration of sodium chloride
- Dissociation requires phosphate

How CHT works



- **Phosphoryl residues**
- Calcium coordination modulated by ion exclusion
- 15–60x stronger than ionic interactions alone
- NaCl causes *stronger* DNA binding by suppressing charge repulsion between phosphates
- Dissociate with phosphate

How CHT works with IgG

- Most published applications report the use of phosphate gradients for IgG purification.
- Phosphate gradients simultaneously dissociate calcium affinity and cation exchange.
- Recent experience indicates that more effective contaminant clearance can be achieved with sodium chloride gradients at constant low phosphate concentrations.

How CHT works with IgG

- Most IgG monoclonals have weak affinity for CHT calcium but fairly strong charge interactions with CHT phosphates.
- Setting a constant low level of phosphate suspends weak calcium affinity interactions but leaves strong ionic interactions intact.
- A sodium chloride gradient can then dissociate ionic bonds. This elutes monomeric IgG. Aggregates elute at higher sodium chloride concentrations.
- Contaminants with a strong calcium affinity remain bound to the column until it is cleaned with concentrated phosphate.

Contaminant clearance

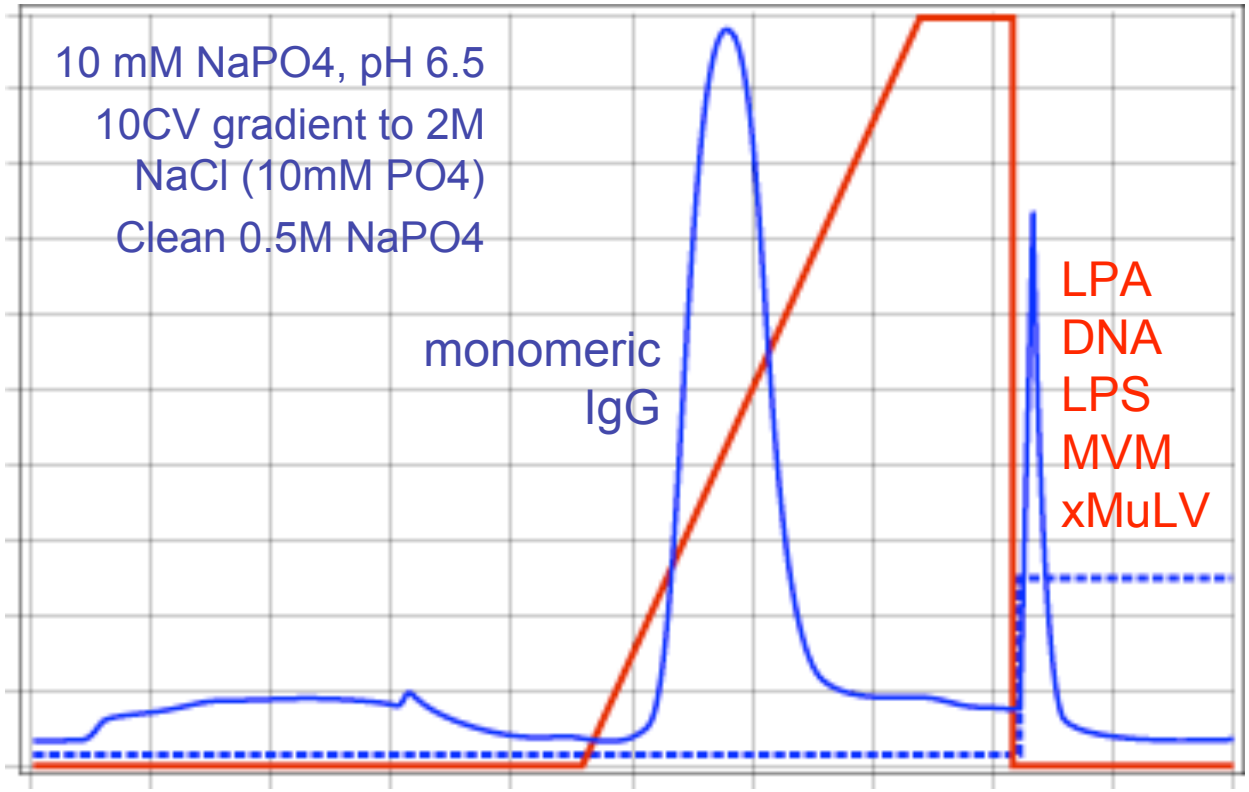
Contaminant clearance in NaCl gradients

Contaminant	Method	Clearance
Aggregates	HPSEC	1-2 logs
Protein A	Cygnus	1-2 logs
CHOP	ELISA	2 logs
DNA	Picogreen	>3 logs
Endotoxin	LAL (chromo)	>4 logs
xMuLV MVM	Infectivity* Infectivity*	>3 logs 2 logs

*Testing performed by Charles River Laboratories

Contaminant distribution

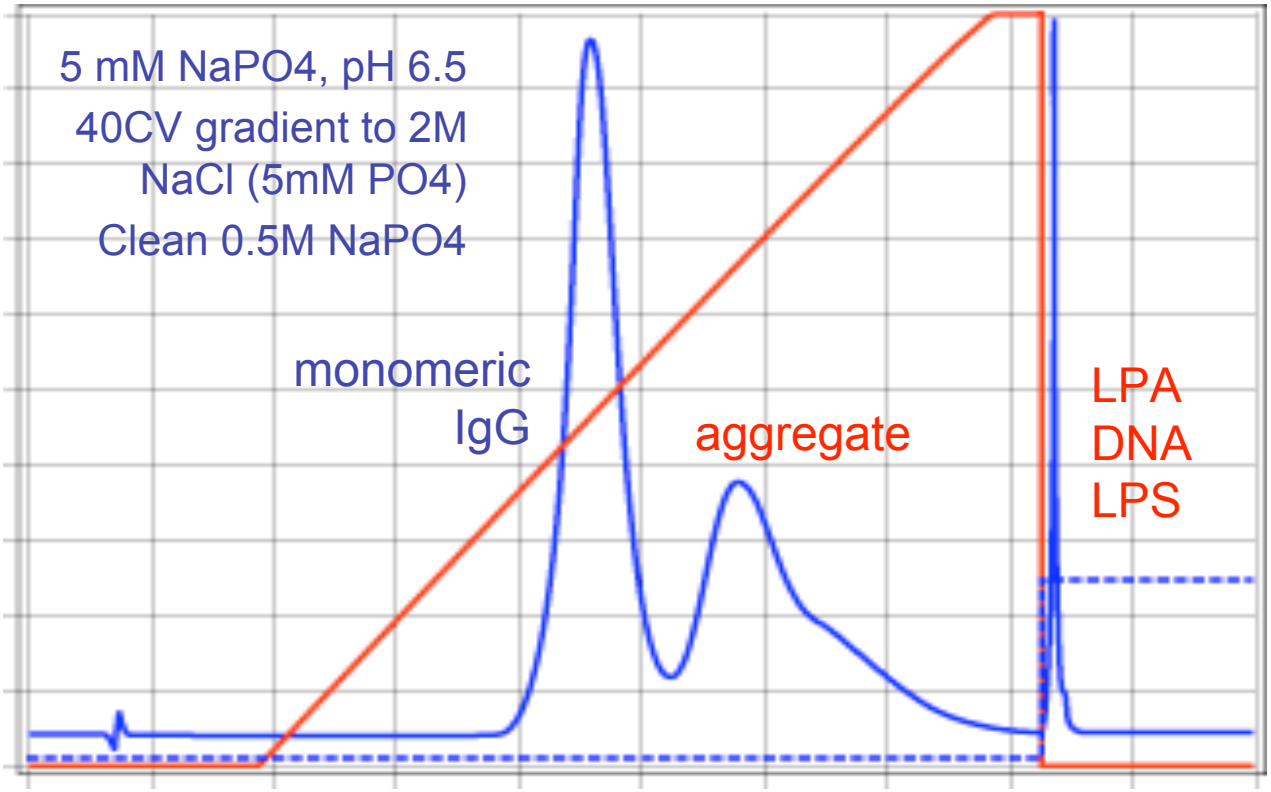
protein A purified human IgG1



CHT type I, 40 micron, 300 cm/hr

Contaminant distribution

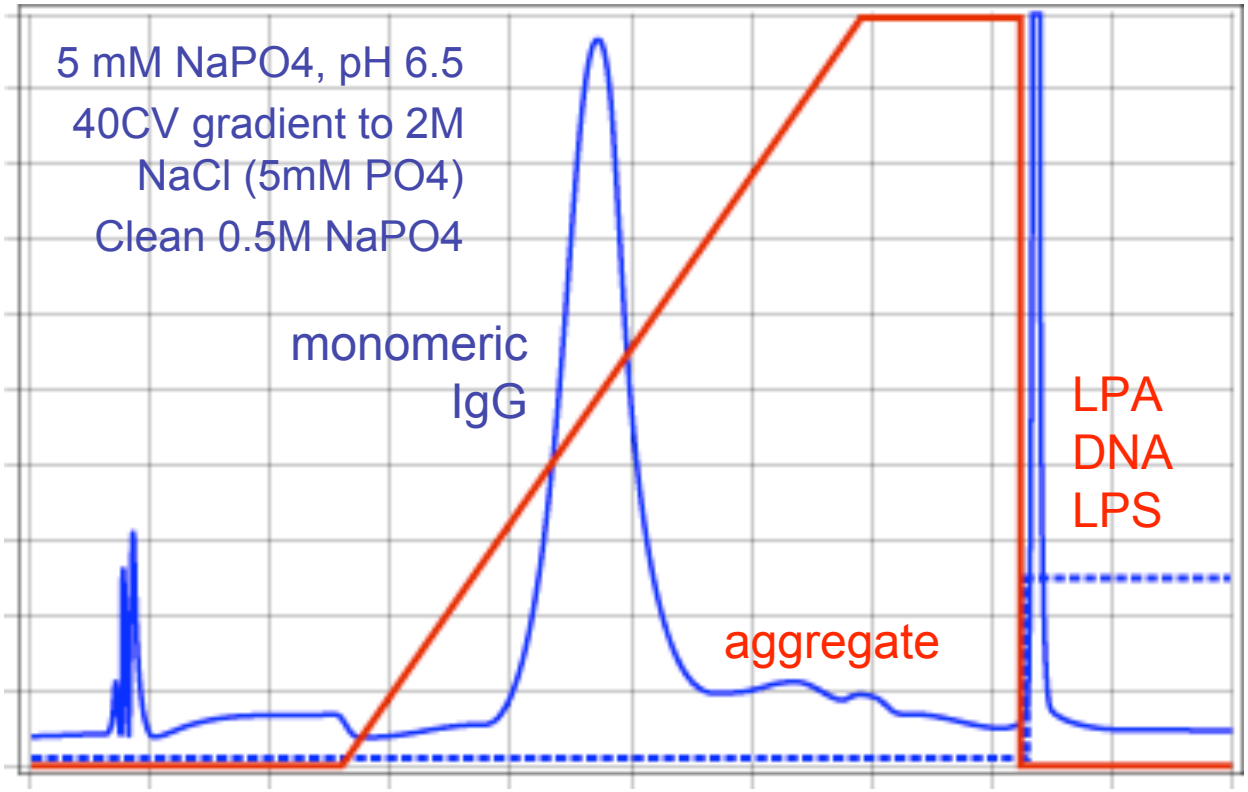
protein A purified IgG1 chimera



CHT type I, 20 micron, 300 cm/hr

Contaminant distribution

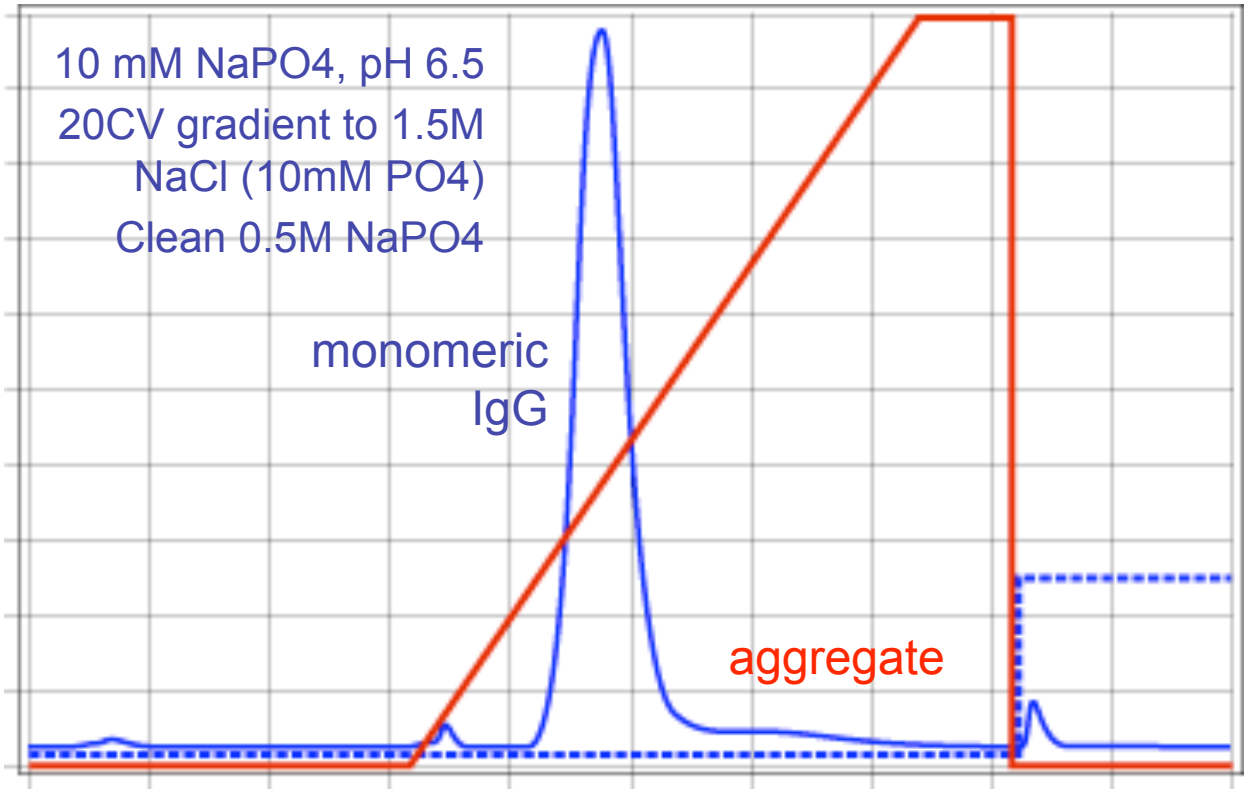
protein A purified human IgG1



CHT type I, 20 micron, 300 cm/hr

Contaminant distribution

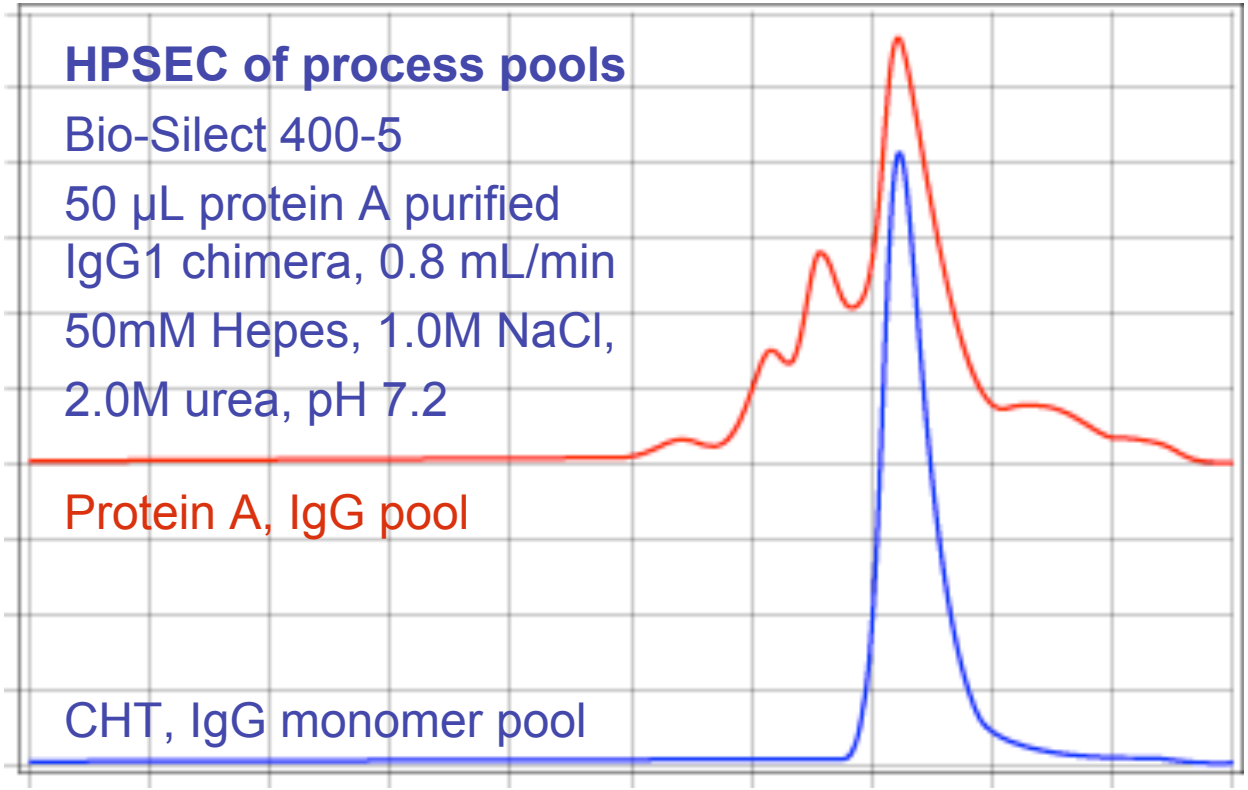
protein A purified mouse IgG1



CHT type I, 20 micron, 300 cm/hr

Contaminant distribution

Aggregate clearance with NaCl gradients



Ramifications

Equivalent clearance in bind/elute or flow-through mode

CHT-I 40µm	**OM**	**Protein A** IgG pool	**CHT pool** bind/elute	**CHT pool** flow-through
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

*linear detection limit of the assay

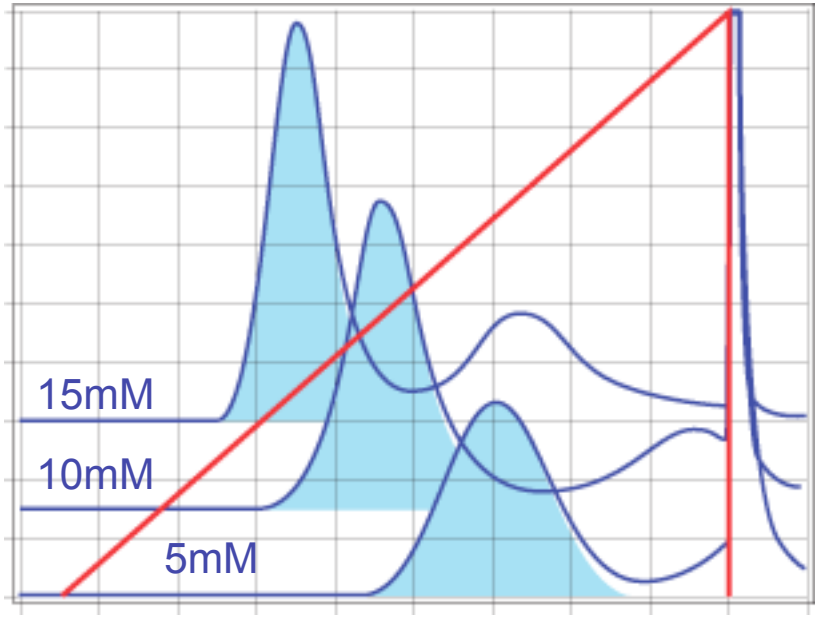
Assessing the potential

- **Initial screening conditions**
- CHT type I, 20µm
- Equilibrate: 5mM NaPO4, pH 6.5
- Load, 5%CV, protein A purified MAb
- Wash: 5CV EQ buffer
- Elute: 40 CV linear gradient to 2.0M NaCl (5mM NaPO4)
- Clean: 10 CV 0.5M NaPO4

- If the MAb fails to elute, repeat at 10mM NaPO4
- If it still fails to elute, repeat at 15mM

Select the phosphate concentration

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



Blue areas indicate monomeric IgG, trailing peak is aggregate

Red line indicates NaCl gradient trace

NaCl gradient followed by cleaning with 0.5M phosphate

All experiments at pH 6.5
300 cm/hr

Protein A purified IgG1 chimera on CHT type I 20 µm

Select the phosphate concentration

Phosphate mM	5	10	15
Protein A ng/mL	0.03	0.03	0.01
DNA ng/mL	<1.0	<1.0	3.9
Endotoxin EU/mL	<0.05	1.0	1.6

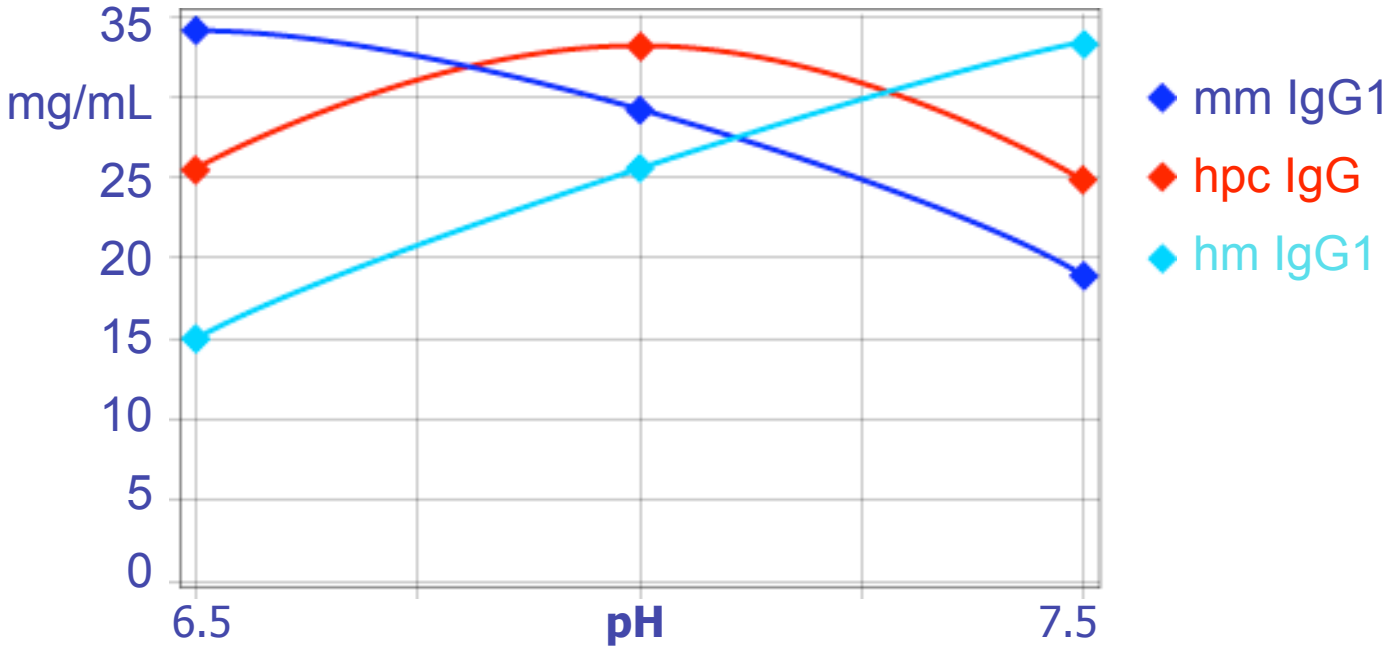
Sample: protein A purified chimeric monoclonal IgG1. 22 ng/mL leached protein A, 2.3×10^3 ng/mL DNA, 1.9×10^4 EU/mL endotoxin

Linear detection limit of protein A assay: 0.2ng/mL

All results for the monomeric IgG pool from a sodium chloride gradient to 1.5 M at pH 6.5 with phosphate concentration held at the indicated level. CHT Type I, 40 µm, 300cm/hr.

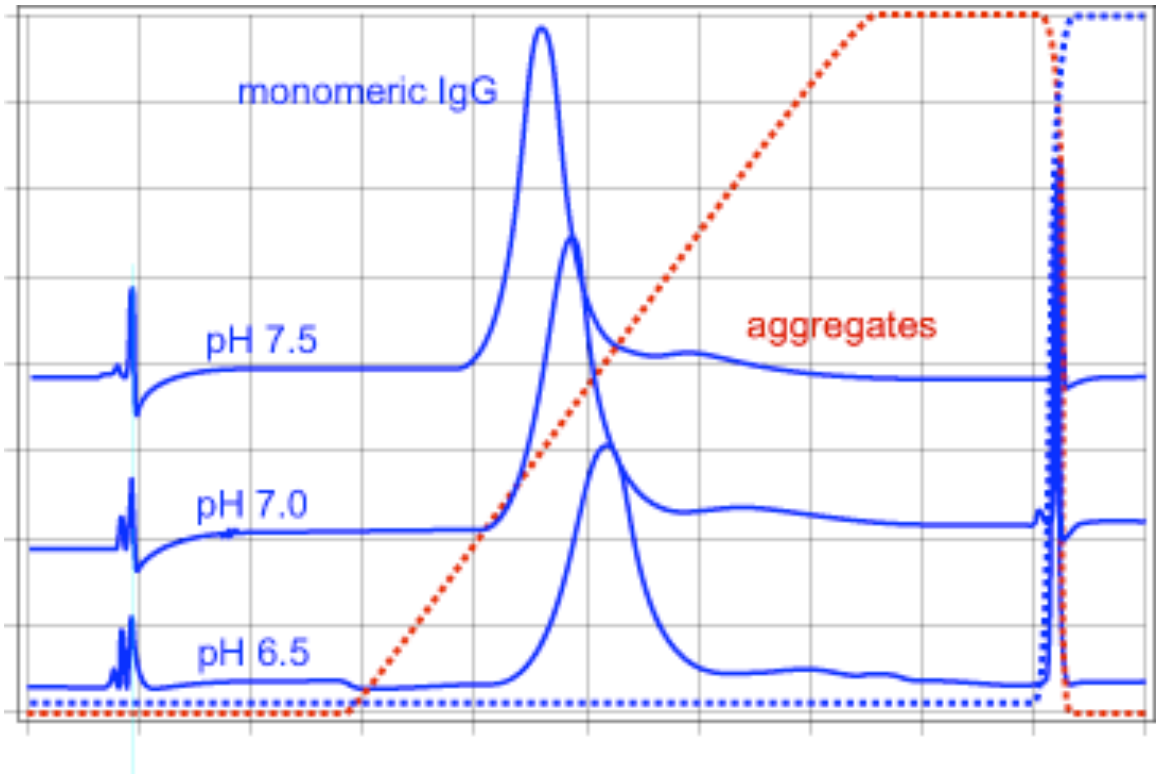
Optimize pH, capacity

Dynamic binding capacity, 10% BT, CHT type I, 40 µm
5mM NaPO4, 300cm/hr



Optimize pH, resolution

Protein A purified monoclonal hIgG1 on CHT type I 20 µm



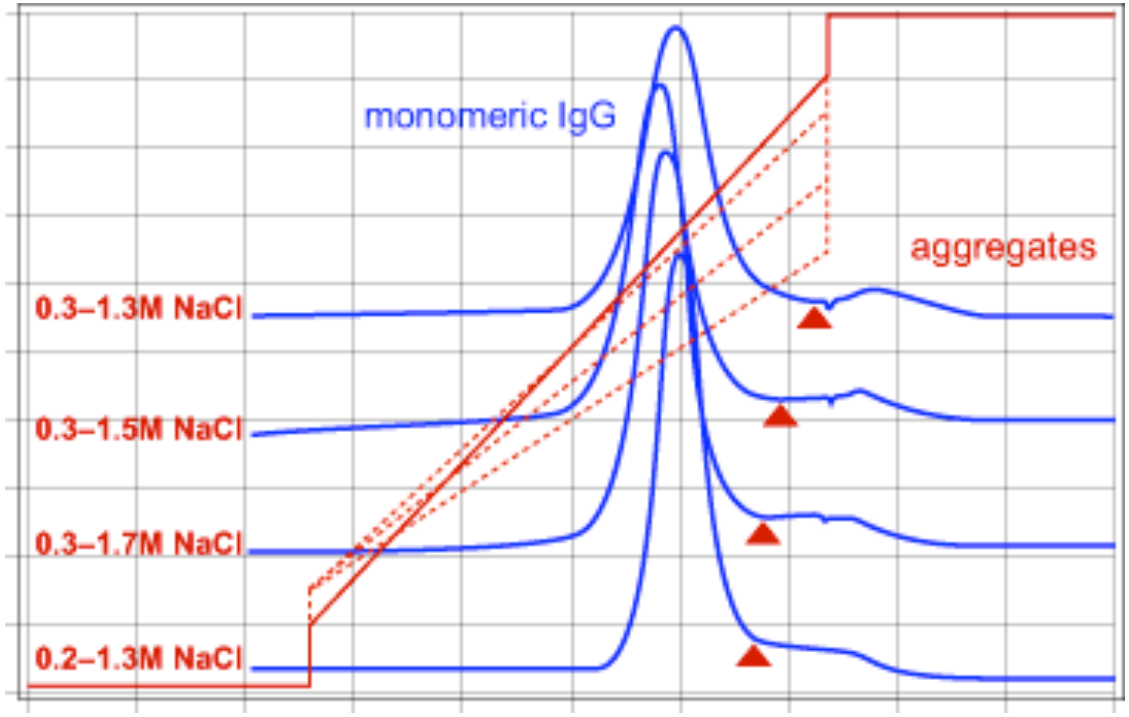
All experiments in 5mM NaPO4 at 300 cm/hr

Red line indicates 40 CV gradient to 2M NaCl

NaCl gradient followed by cleaning with 0.5M phosphate

Optimize gradient slope

Protein A purified human monoclonal IgG1, CHT type I 20 µm



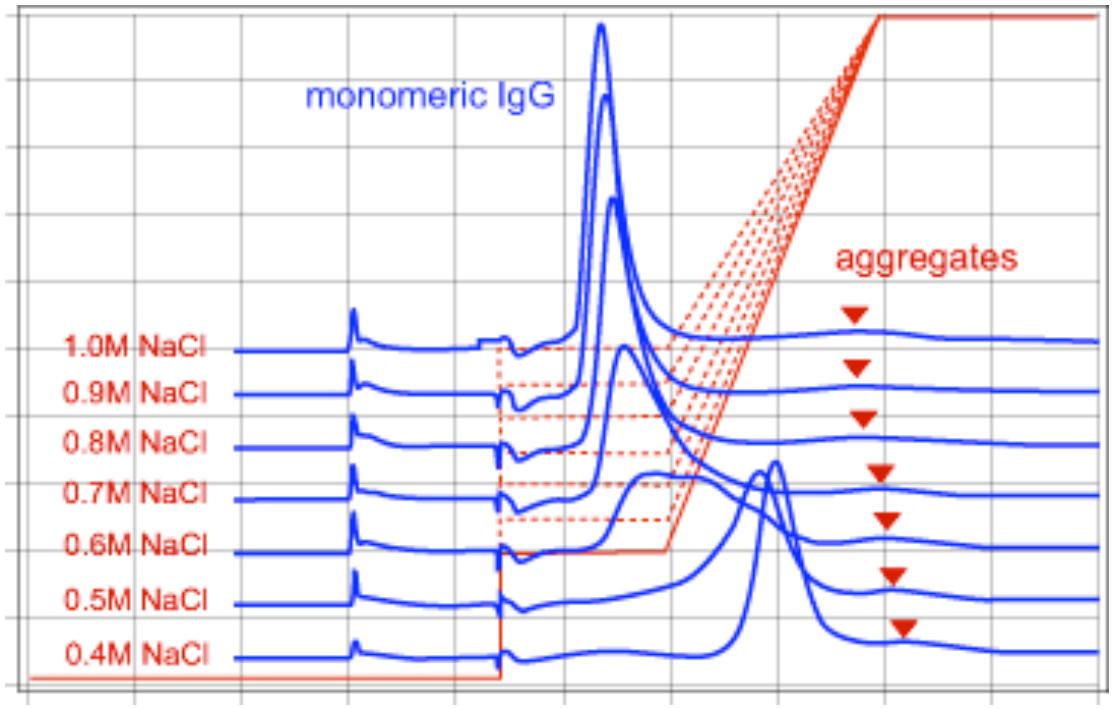
All experiments in 5mM NaPO4 at pH 7.0, 300 cm/hr

Red lines indicate 20CV NaCl gradient traces (max. 2M)

Red notations indicate linear gradient interval

Convert to step gradient

Protein A purified human monoclonal IgG1, CHT type I 20 µm



All experiments in 5mM NaPO4 at pH 7.0, 300 cm/hr

Elution gradients 25CV (step + linear)

Red lines indicate NaCl gradient traces

Red notations indicate step concentration

Scale-up

- Scouting for aggregate resolution can be done effectively with 20 µm media in 1-2mL columns. Visual evaluation from chromatograms saves time and sample by avoiding the need for secondary testing. When the best conditions are identified, *then* confirm aggregate removal with HPSEC.
- Evaluation of IgG binding capacity and clearance of leached protein A, DNA, and endotoxin can also be done in 1-2mL columns but should use 40µm media.
- Scale up with 40µm media in columns with a bed height of 15-30cm.

Platform integration

- **3-Step platform**
- Microfiltration
- **Protein A affinity**
- Virus inactivation
- **Anion exchange** flow-through mode
- **CHT, type I, 40µm** bind/elute mode
- Virus filtration
- Conc/diafiltration
- Microfiltration

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

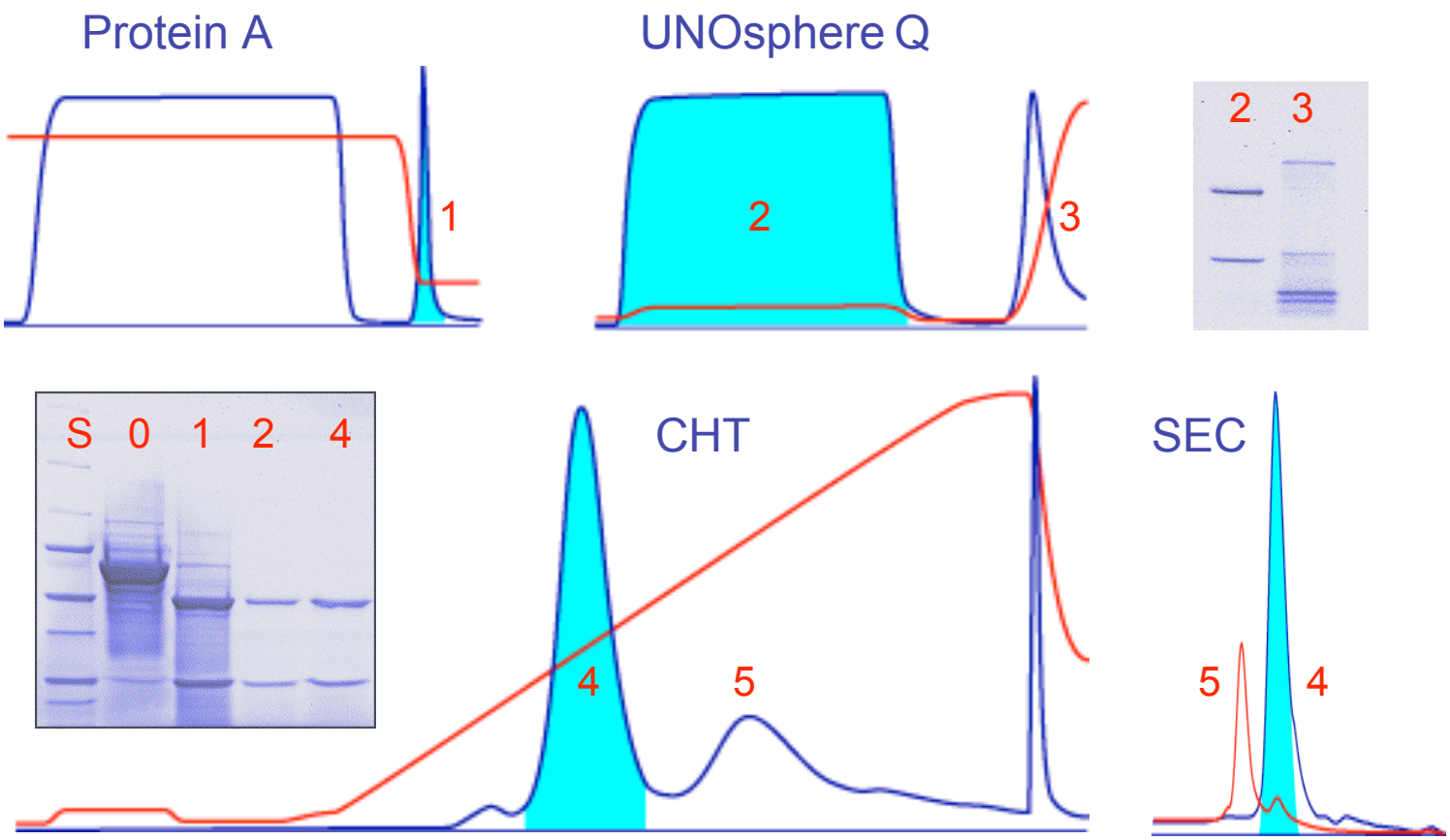
3-Step platform integration

- Elute protein A with 0.1M glycine,* 0.05 M NaCl, pH 3.8**
- Hold for virus inactivation
- Titrate pH to 7.0** with 1M Tris
- EQ UNOsphere Q™ to 0.05M Tris, 0.05M NaCl, pH 7.0** Apply sample. Collect flow-through
- Add 0.5M NaPO4, pH 7.0** to achieve optimal phosphate concentration (1% v:v yields 5mM)
- Conduct virus filtration
- Equilibrate CHT with optimal NaPO4, pH 7.0**
- Load, wash, elute under optimized conditions
- Concentrate/diafilter to final formulation conditions

* Arginine or acetate can also be used for elution. Citrate degrades CHT

** Or other pH according to scouting results

3-Step platform integration



Reduced SDS PAGE fractions: S=standards, 0=original material, 1=protein A elution, 2=UNOsphere Q flow-through, 3=UNOsphere Q elution, 4=CHT monomeric IgG pool

2-step platform integration

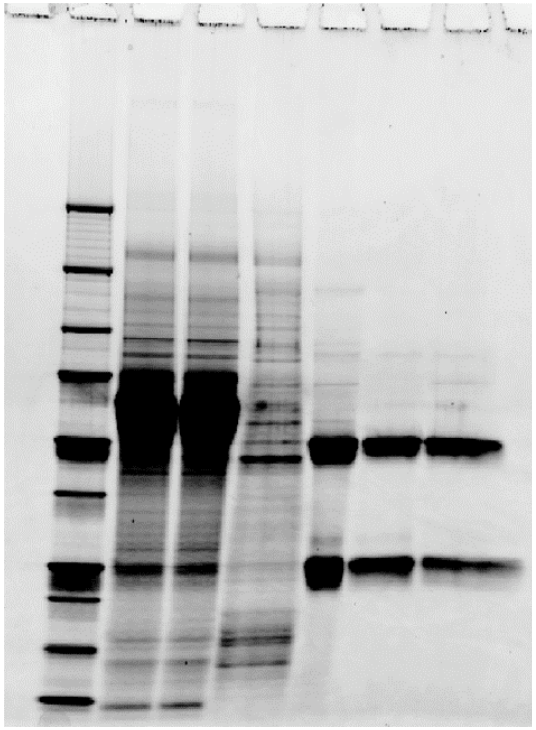
- Elute protein A with 0.1M glycine,* 0.05 M NaCl, pH 3.8**
- Hold for virus inactivation
- Titrate pH to 7.0**
- Add 0.5M NaPO4, pH 7.0** to achieve optimal phosphate concentration (1% v:v = 5mM)
- Conduct virus filtration
- Equilibrate CHT with optimal NaPO4, pH 7.0**
- Load, wash, elute under optimized conditions
- Concentrate/diafilter to final formulation conditions

* Arginine or acetate can also be used for elution. Citrate degrades CHT

** Or other pH according to scouting results

2-Step platform integration

Reduced SDS PAGE (Flamingo stain)



1 2 3 4 5 6

1. OM*
2. PA, flow-through
3. PA, wash (KS)
4. PA, pool
5. CHT, IgG monomer pool
6. CHT, aggregate pool

KS: 1M NaCl, 2M urea, 10mM EDTA, 0.05M PO4, pH 7.0

Sensitivity equivalent to silver stain

* IgG1 chimera, serum supplemented NS0 supernatant

Buffer tips

- Maintain operating pH at 6.5 or above.
- Avoid exposure to chelating agents.
- Include phosphate in all samples and buffers
  - minimum 5mM phosphate at pH 6.5
  - minimum 2mM phosphate at pH 7.5
- If the phosphate level required to achieve the best selectivity is too low to provide adequate buffering capacity, co-formulate with MES, Hepes, Tris, etc., but not citrate.

Column hygiene

- **Clean:** 0.5M phosphate
- **Sanitize:** 1.0M NaOH*
  - 4 hours or more at 23°C
- **Store:** 0.1M NaOH

* >15,000 hours stability at 1.0M NaOH

Acknowledgments

- Special thanks to Avid BioServices of Tustin, CA for generously providing cell culture supernatant to support this work.