An effective platform for purification of IgM monoclonal antibodies using Hydroxyapatite

Frank Hensel, Patrys, GmbH
Pete Gagnon, Validated Biosystems

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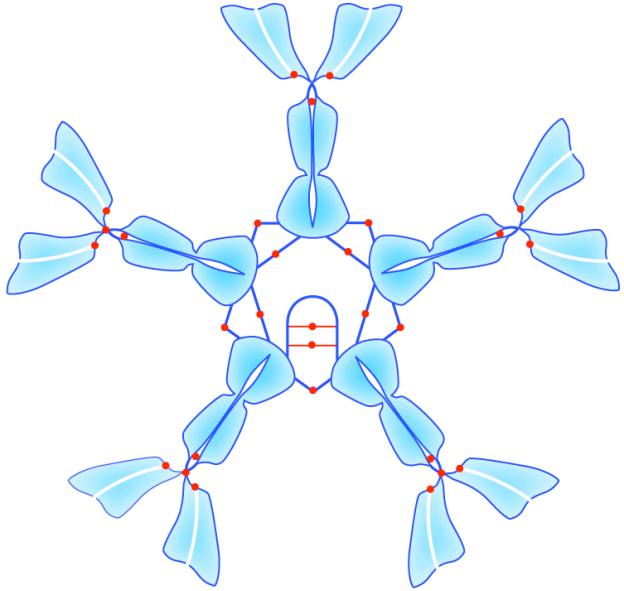
Why work with IgMs?

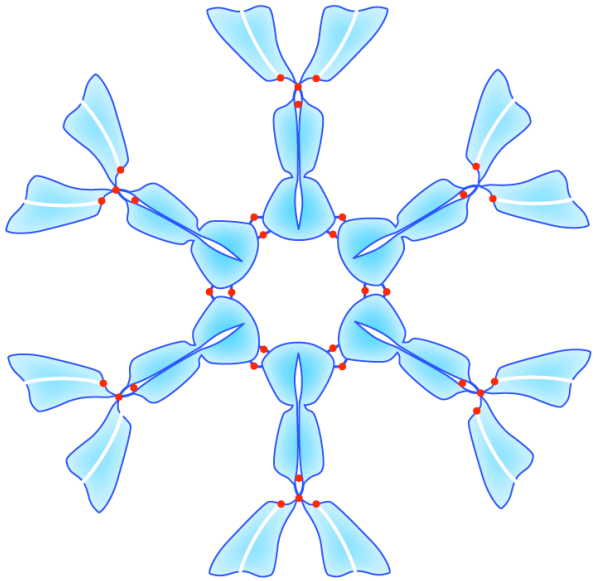
- Natural immunity of humans against cancer cells consists almost exclusively of IgM.
- These antibodies can be isolated from healthy people and cancer patients by fusion technology.
- Natural IgMs are highly tumor-specific.
- Patrys Ltd. has a broad pipeline of tumor-specific and cytotoxic IgMs.
- Lead candidate to be scheduled to enter clinical trials by Q4, 2009.

Structure and properties of IgM

Pentameric, 0.96 MDa



Hexameric, 1.15 MDa



7.5 -12 % carbohydrate, extinction coefficient 1.18

Randall et al, 1990, The biological effects of IgM hexamer formation, Eur. J. Immunol., **20** 1971-1979
Johnstone and Thorpe, 1987, Immunochemistry in Practice, 2nd Ed., Blackwell Scientific Publishers, Oxford

Are IgMs really difficult to purify?

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- IgMs have some characteristics that can limit the application of standard purification tools.
- They are susceptible to denaturation at extremes of pH. This can limit application of affinity chromatography.
- They are poorly soluble at low conductivity. This can limit application of ion exchange chromatography.
- They are susceptible to denaturation from exposure to strongly hydrophobic surfaces. This can limit application of hydrophobic interaction chromatography.
- Size exclusion chromatography is mild and effective but productivity is prohibitively low.

Are IgMs really difficult to purify?

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- The size of IgMs is also a challenge.
- Large size corresponds with slow diffusion constants.
- Porous particle based chromatography media depend on diffusion for mass transport.
- Slow diffusion constants translate into lower capacity and lower resolution, and/or lower flow rates on porous particle based chromatography media.
- Monolithic ion exchangers can overcome the mass transport limitations of diffusion, but solubility issues remain.

Are IgMs really difficult to purify?

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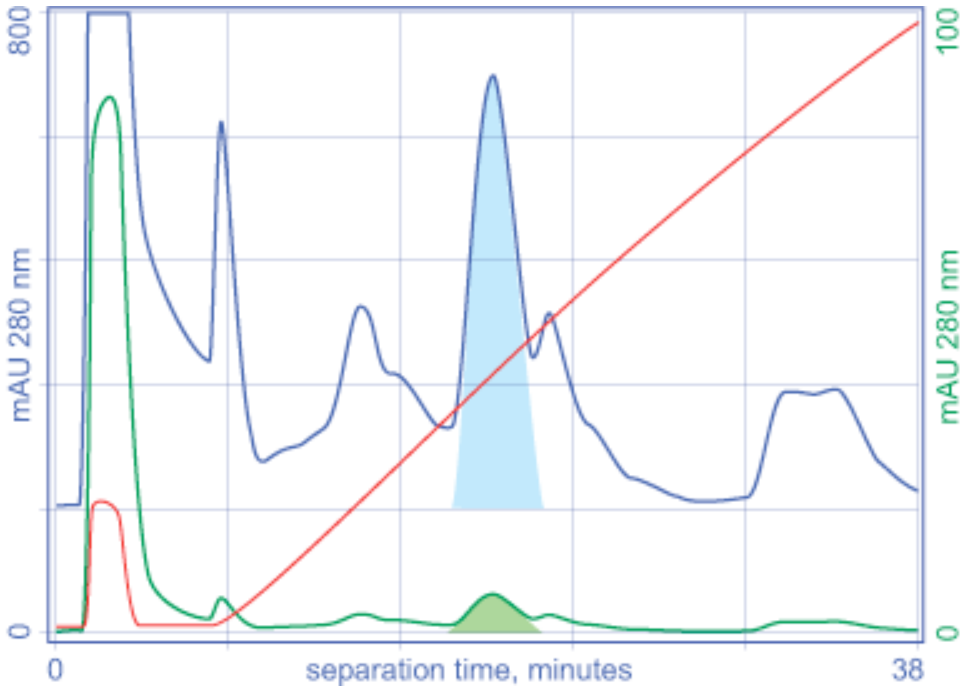
On the positive side, IgMs are typically more charged than IgGs.

- They bind much more strongly than IgG to anion exchangers, or cation exchangers, or both.
- They also bind more strongly than IgG to hydroxyapatite, and much more strongly than most contaminants.
- These characteristics allow hydroxyapatite and ion exchangers to purify most IgMs without exposing them to extreme conditions.

Method scouting

Initial screening, hydroxyapatite

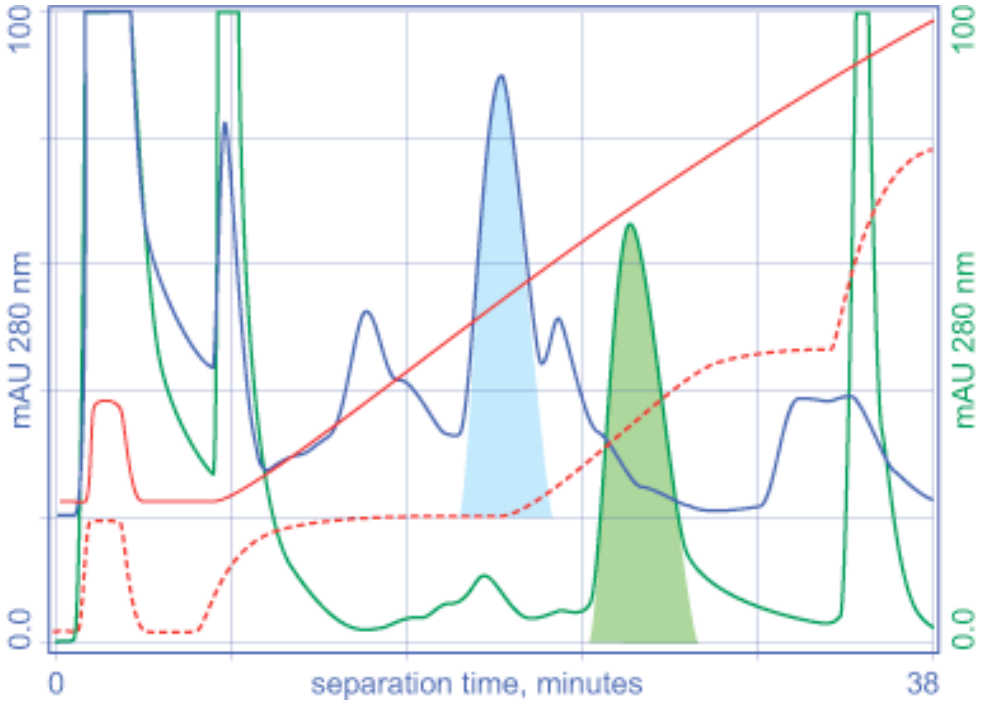


Media: 1 mL CHT™ type II 40 µm
Column: MediaScout® 5 x 50 mm
Flow rate: 0.67 mL/min
Buffer A: 10 mM NaPO4, pH 7.0
Buffer B: 500 mM NaPO4, pH 7.0
Equilibrate: buffer A
Sample: IgM CCS
Load: 2 mL
Wash: 5 CV buffer A
Elute: 20 CV LG to buffer B

Highlighted peak is IgM. This profile is typical of IgMs, most of which elute from hydroxyapatite in a well defined peak between 200 and 300 mM phosphate. The green trace illustrates the same profile at lower sensitivity.

Method scouting

Gradient optimization, hydroxyapatite

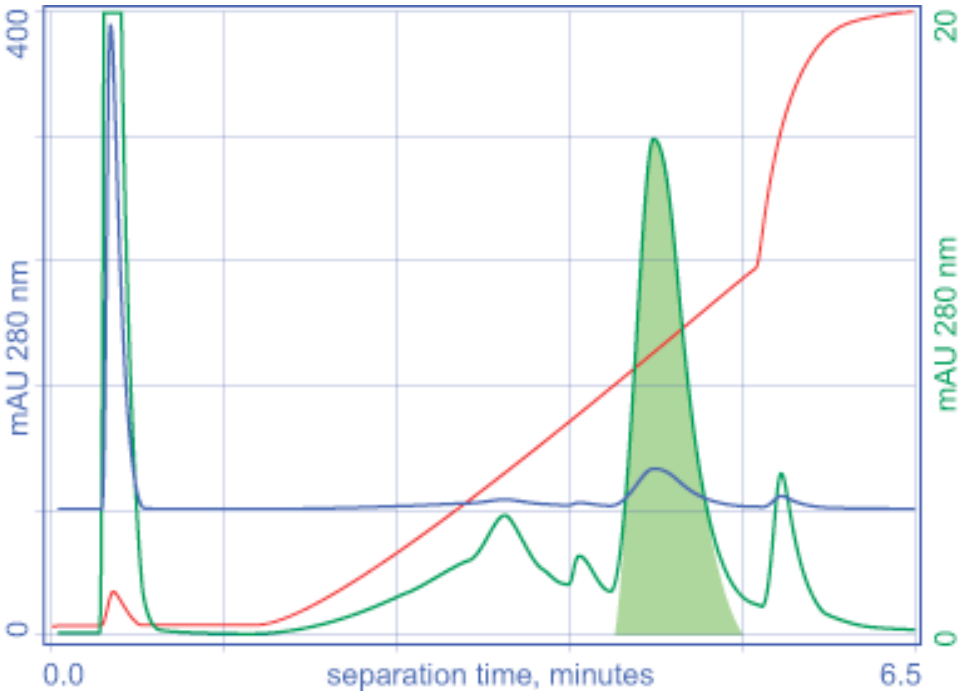


Media: 1 mL CHT™ type II 40 µm
Column: MediaScout® 5 x 50 mm
Flow rate: 0.67 mL/min
Buffer A: 10 mM NaPO4, pH 7.0
Buffer B: 500 mM NaPO4, pH 7.0
Equilibrate: buffer A
Sample: IgM CCS
Load: 2 mL
Wash: 5 CV buffer A
Elution gradients indicated in red

Highlighted peak is IgM. Blue trace reproduces screening results from previous slide. Lower trace illustrates improved purity from optimized gradient set points.

Method scouting

Initial screening, cation exchange, pH 7.0



Media: CIM SO3, 0.34 mL

Flow rate: 4 mL/min

Buffer A: 10 mM NaPO4, pH 7.0

Buffer B: 500 mM NaPO4, pH 7.0

Equilibrate: buffer A

Load: 100 µL IgM CCS

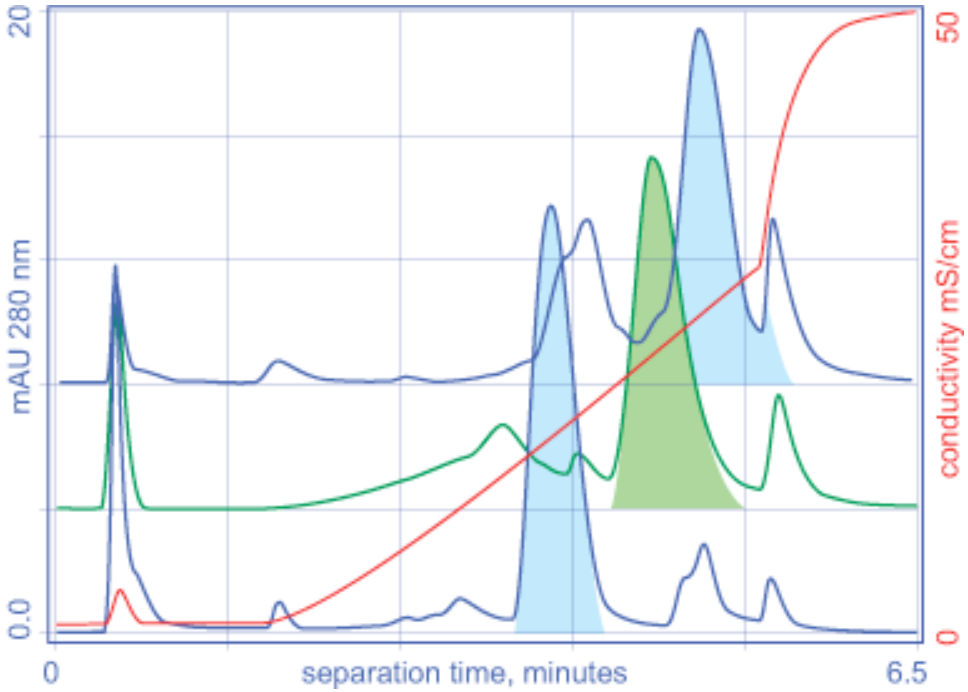
Wash: buffer A

Elute: 34 CV LG to 50% buffer B

Clean with 100% buffer B

Method scouting

Initial screening, cation exchange, pH 6.0, 7.0, 8.0



Media: CIM SO3, 0.34 mL

Flow rate: 4 mL/min

Buffer system A: pH 6.0

Buffer system B: pH 7.0

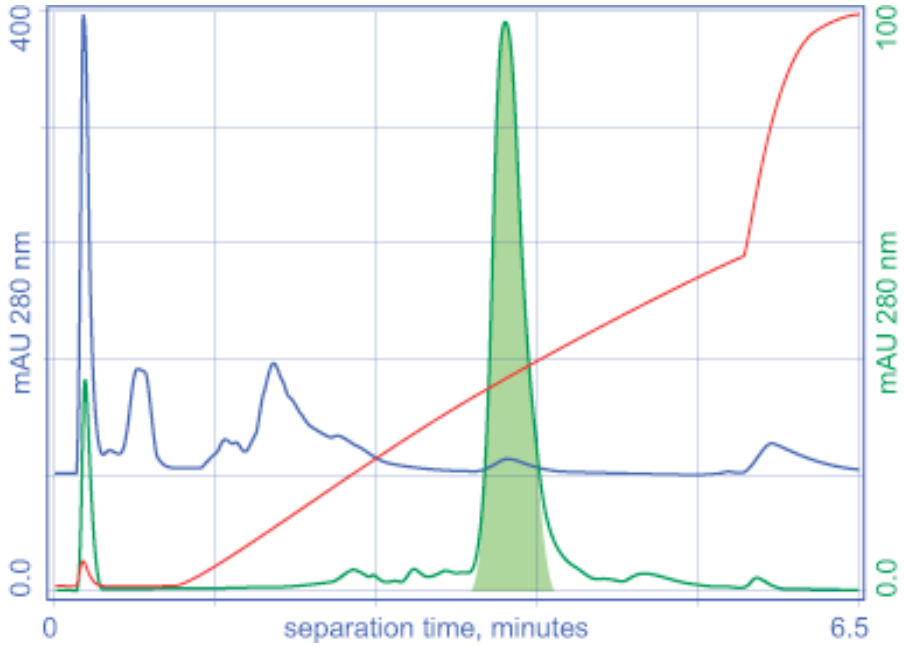
Buffer system C: pH 8.0

Same sample and run configuration as previous slide

Highlighted peak is IgM. Uppermost trace shows results at pH 6.0. Center trace, pH 7.0. Lowermost trace, pH 8. Note stronger binding at pH 6, better purification at 8.0. Further improvement is achievable with gradient optimization.

Method scouting

Initial screening, anion exchange, pH 7.0



Media: CIM QA, 0.34 mL

Flow rate: 4 mL/min

Buffer A: 10 mM NaPO4, pH 7.0

Buffer B: 500 mM NaPO4, pH 7.0

Equilibrate: buffer A

Load: 100 µL

Wash: buffer A

Elute: 34 CV LG to 50% buffer B

Clean with 100% buffer B

Highlighted peak is IgM. Blue trace, IgM CCS. Green trace, CHT-purified IgM. Strong anion exchange retention is typical of IgMs.

Sample loading and capacity

Hydroxyapatite

- Directly compatible with cell culture supernatant
- Filter to 0.2 µm
- Titrate pH to 7.0
- Add phosphate to 5 mM
- Capacity up to 25 mg/mL

Sample loading and capacity

Ion exchange

- In order to avoid product precipitation at low conductivity, load sample by in-line dilution.

  Titrate sample pH to target, load through inlet A

  Dilute with buffer, through inlet B

  Dilution factor depends on the charge characteristics of the antibody, the starting conductivity, and the desired capacity. Typical dilution factors range from 1:2 to 1:9

  Capacity: 20-40 mg/mL at 12 CV/min on monoliths, depending on sample conductivity.

Sample loading and capacity

- Lower in-line dilution factors reduce sample loading time.
- Higher in-line dilution factors increase capacity, which reduces column size and buffer volumes. This also increases eluted product concentration, which reduces sample volume going into the next step.
- In-line dilution suspends the need to employ diafiltration for intermediate sample preparation. This eliminates costly equipment, materials, preparation/operating/maintenance time, validation, and the inevitable losses that occur at each processing step.

Process sequencing

Hydroxyapatite is generally well suited for IgM capture.

- Mild operating conditions.
- Minimal sample conditioning (no significant dilution).
- Most contaminants flow through, leaving capacity available for product.
- Most IgMs elute at 200-300 mM phosphate
- HA may discolor from iron in CCS. Chelating agents may reduce column life.

Process sequencing

Substantial dilution will probably be required for capture on ion exchangers. This is undesirable, especially at the first step, where product concentration will be the lowest of the entire process.

In this case, the IgM bound to the anion exchanger at higher conductivity than to the cation exchanger. Since the HA eluate had the highest conductivity in the process, it was followed with anion exchange. Cation exchange was positioned as a final polishing step.

Process modeling

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- After establishing a preliminary process order, loading conditions, capacity, and separation conditions, it is useful to run the integrated process to provide a benchmark of overall process performance and economics.
- A good working process model highlights its own deficiencies, provides an order of priority for addressing them, and allows each one to be evaluated in a meaningful context.
- Defer optimization of individual steps until after the benchmark has been run.

Process modeling

Our modeling run revealed that DNA co-eluted with the IgM.

This was a serious concern because DNA and IgM form charge complexes that can be fully dissociated only at high salt concentrations* – usually higher salt concentrations than are employed to elute IgM from ion exchangers.

If no steps are taken to control this phenomenon, DNA tends to be transported through the entire process, and fails to be reduced to adequate levels in the final product.

* IgM purification on immobilized DNA: M. Abdullah et al, 1985, J. Chromatogr., 347 129-136

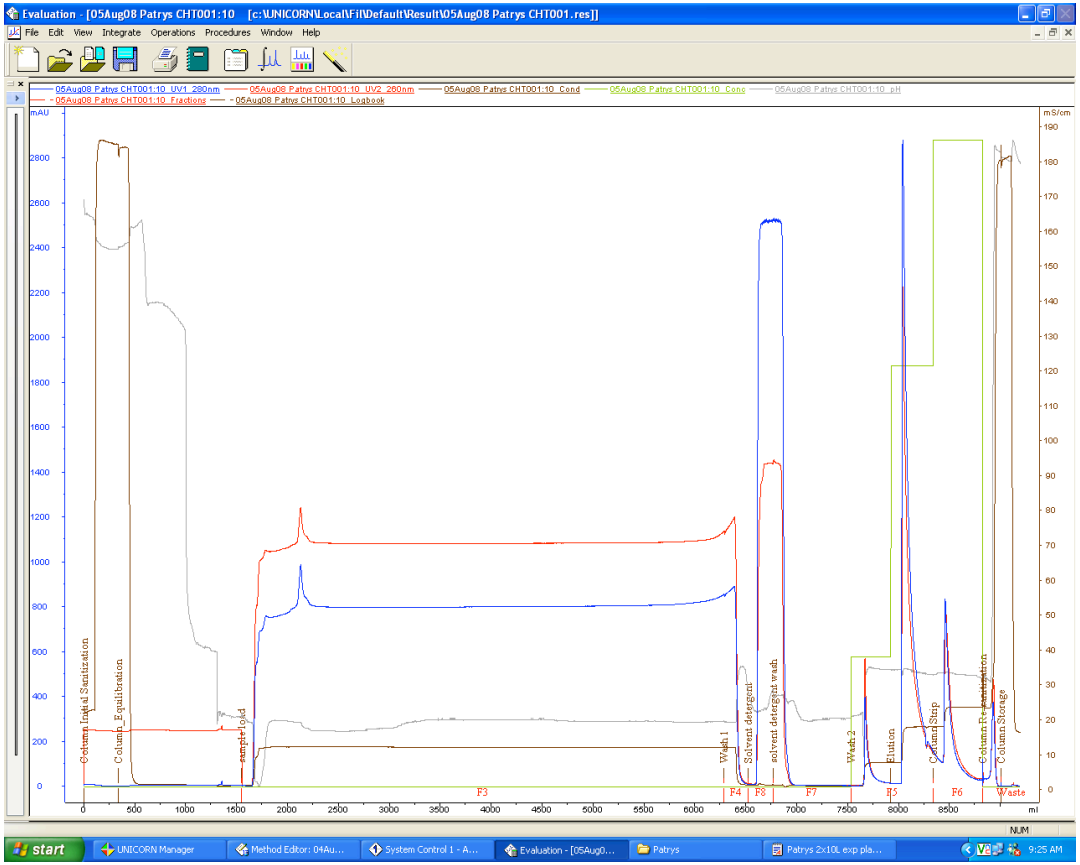
Process modeling

The point of highest conductivity in the process was the elution step from CHT. We evaluated insertion of a membrane anion exchanger at this point to reduce DNA levels going into the remainder of the process. DNA binds anion exchangers at NaCl concentrations up to 0.6 M, so no sample dilution was required. The IgM was not retained under these conditions.

We were able to plumb the membrane in tandem with the CHT column but ultimately chose to run it as a separate unit operation to simplify validation and documentation.

Process modeling

Capture on hydroxyapatite

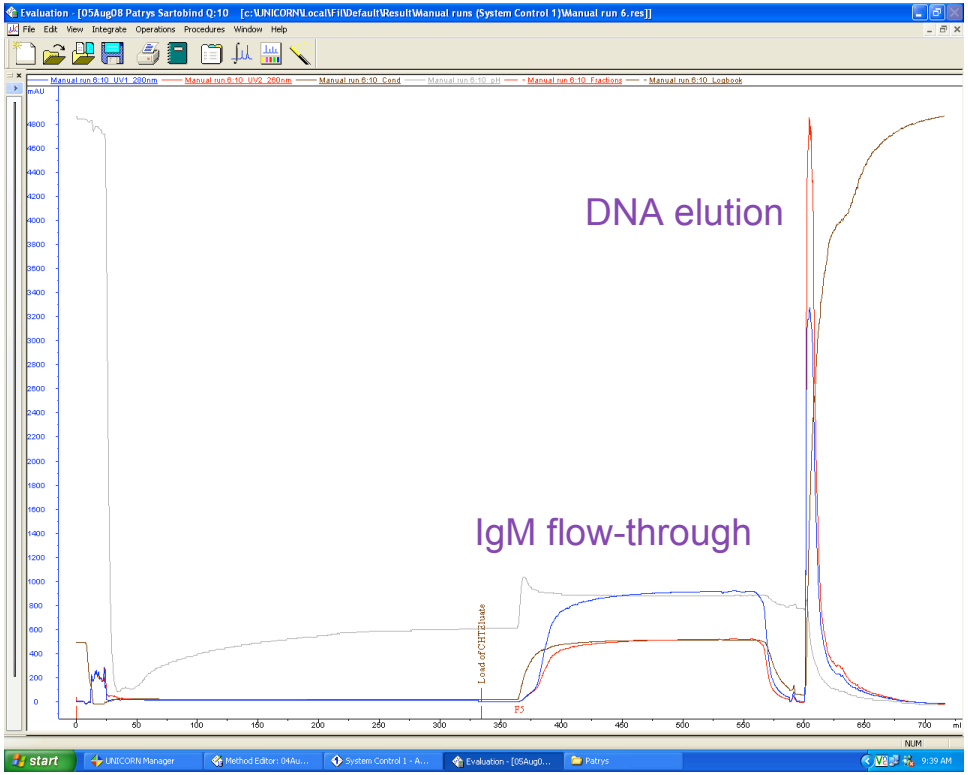


Load: 4.65 L of clarified harvest
Column: 3.2 cm x 15 cm (120.6 mL)
CHT Type II, 40 micron
Run velocity: 150 cm/hr
Load adjusted to 1% (v/v) 0.5 M sodium phosphate, pH 7.0 to achieve a final [phosphate] of 5 mM.
Buffer A: 10 mM sodium phosphate, 10% PEG-600, pH 7.0
Buffer B: 500 mM sodium phosphate, 10% PEG-600, pH 7.0
Buffer C: 1% Triton-X 100, in 10 mM sodium phosphate, pH 7.0
Buffer D: 1 N NaOH
Buffer E: 0.1 N NaOH

See Gagnon, 2009, J. Immunol. Met., 336 222-228 for explanation of aggregate removal in the presence of PEG.

Process modeling

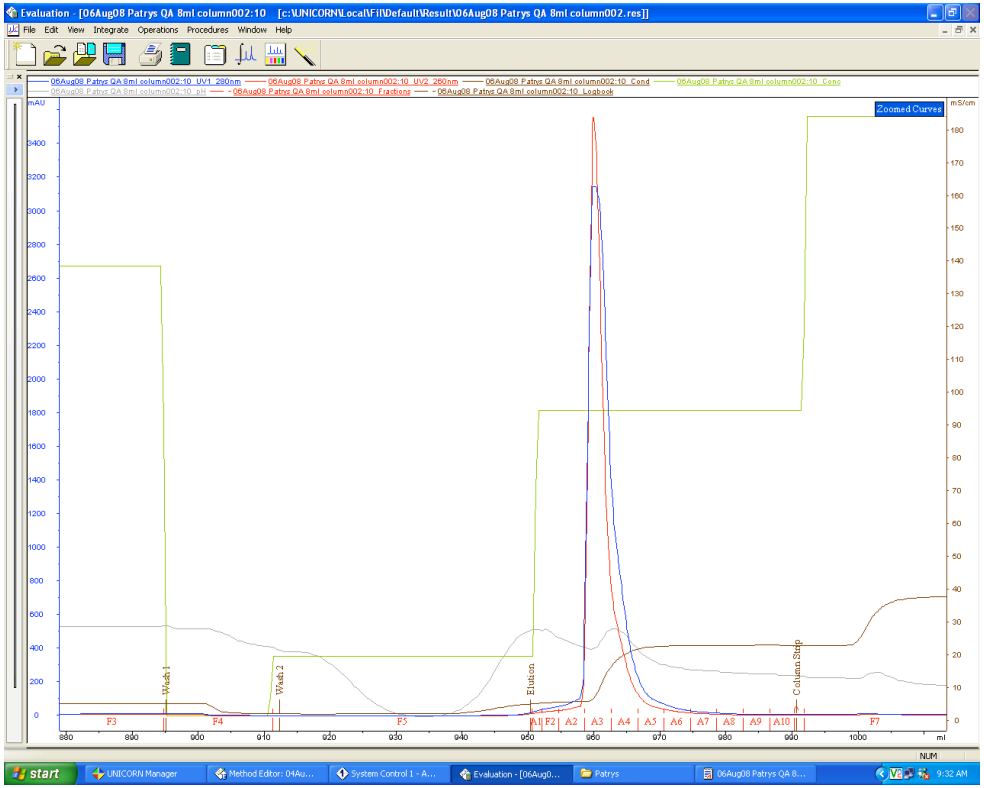
DNA removal by membrane anion exchange



Load: 210 mL pooled CHT eluate
Membrane: Sartobind Q Mini
Membrane Area: 0.025 m^2
Equilibrate filter with 10 mM sodium phosphate, 10% PEG-600, pH 7.0
Collect flow-through
Strip filter with 2 M NaCl
Note the difference in UV absorbance at 254 nm (red) and 280 nm (blue).

Process modeling

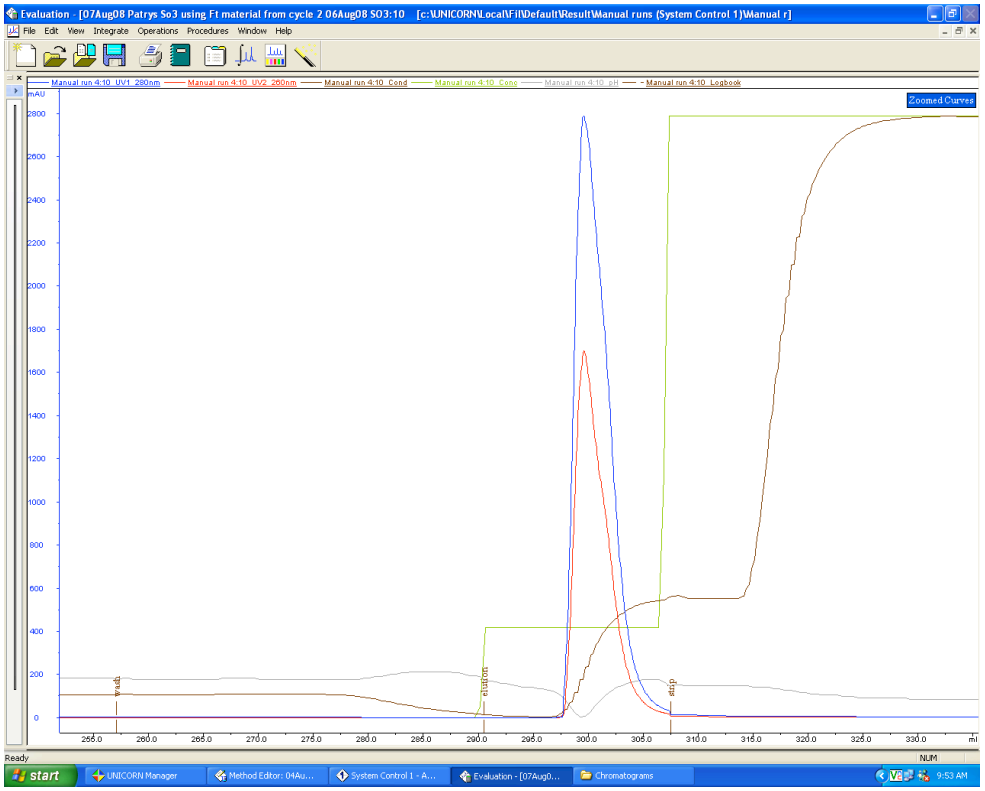
Intermediate purification by anion exchange chromatography



Load: 53 mL Sartobind Q filtrate diluted 5X with Buffer A
Column: 8 mL QA Monolith
Flow rate: 30 mL/min; 3.75 CV/min
Buffer A: 50 mM Tris, 2 M Urea, 2 mM EDTA, pH 8.0
Buffer B: 10 mM sodium phosphate, 2 M Urea, pH 7.0
Buffer C: 500 mM sodium phosphate, pH 7.0
Buffer D: 1 N NaOH
Buffer E: 0.1 N NaOH

Process modeling

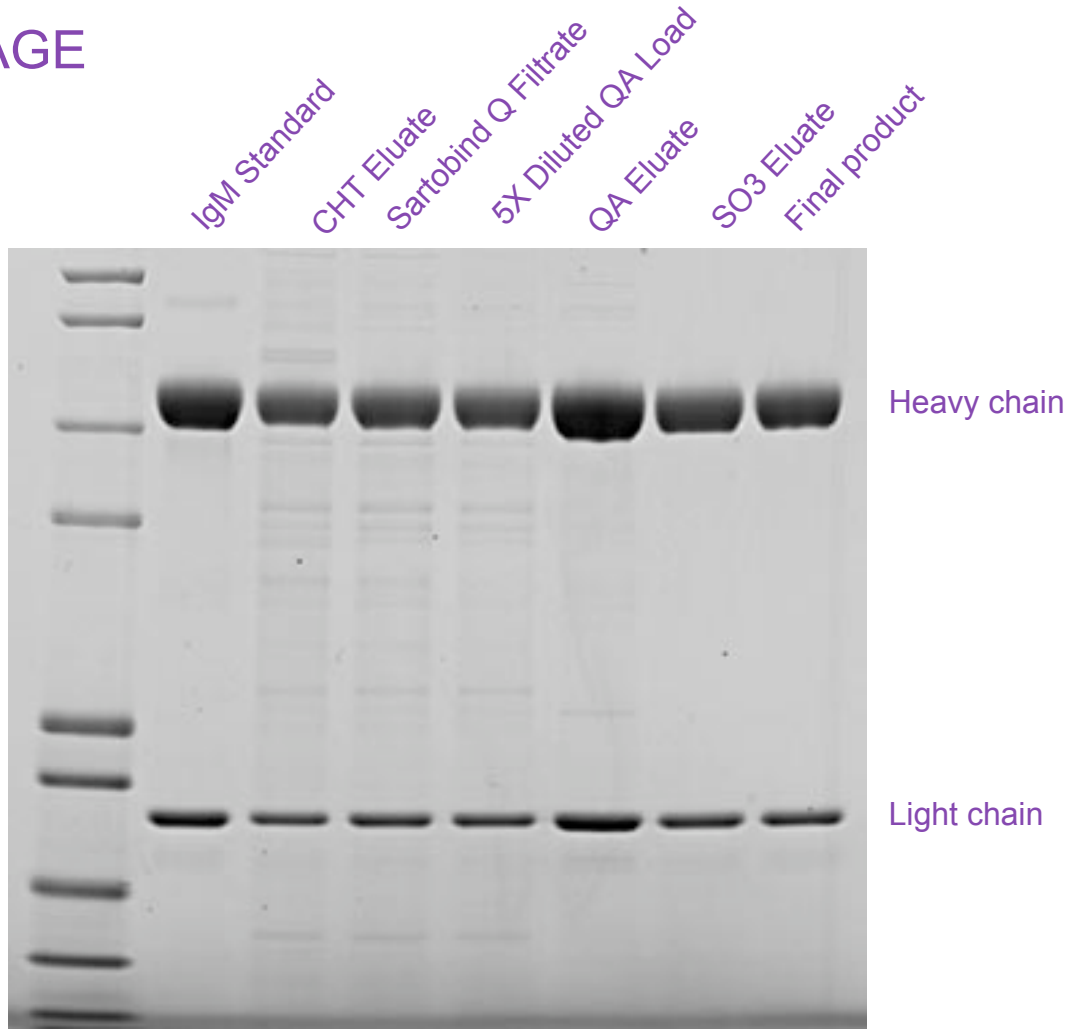
Final purification by cation exchange chromatography



Load: 43 mL QA eluate pool
Column: 8 mL SO_3 monolith
Flow rate: 30 mL/min; 3.75CV/min
Buffer A: 10 mM sodium phosphate, 2 M Urea, pH 7.0
Buffer B: 500 mM sodium phosphate, pH 7.0
Buffer C: 1 N NaOH
Buffer D: 0.1 N NaOH

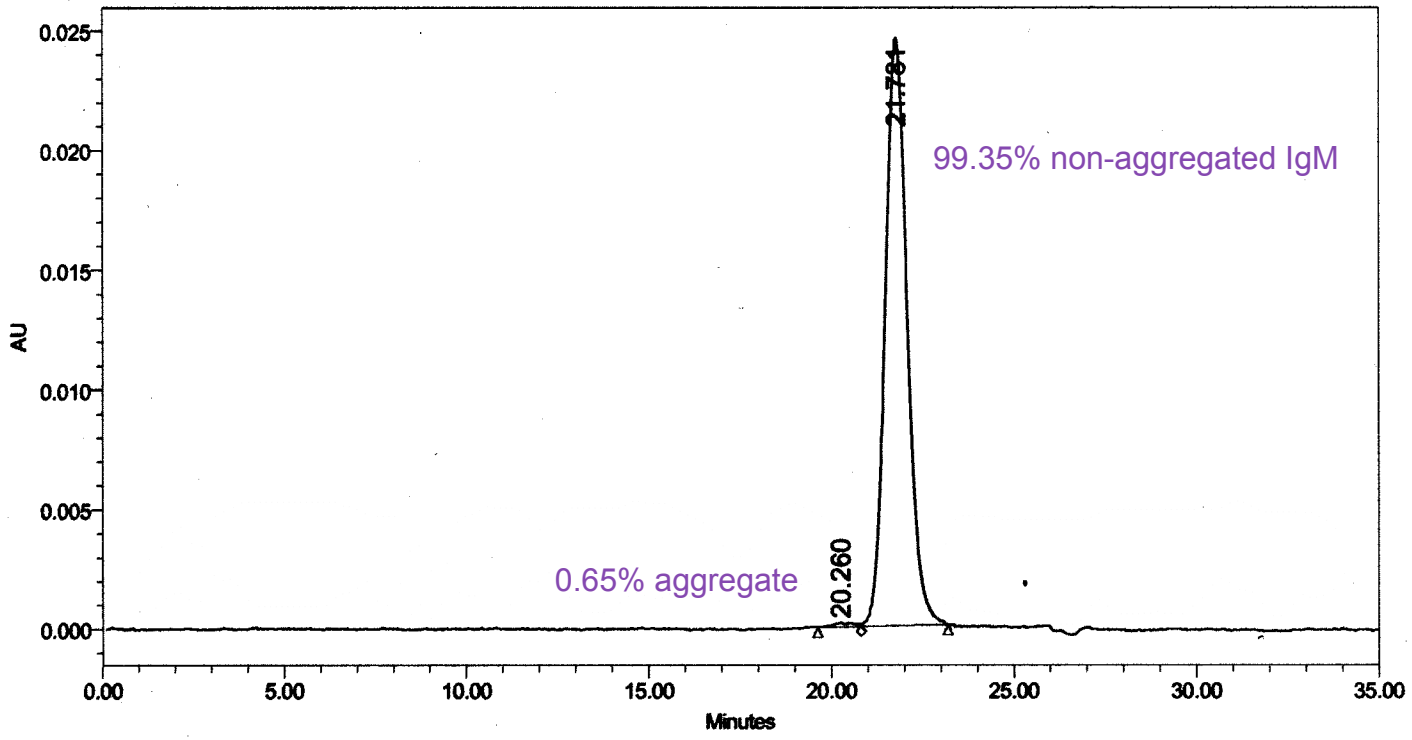
Process modeling

Reduced SDS-PAGE



Process modeling

Analytical SEC



Conclusions

- IgM purification is different than IgG purification, but not inherently more difficult.
- Clinical grade material can be purified with non-affinity methods, with step recoveries comparable to those achieved with IgG.
- Patrys successfully transferred the present process to a CMO for production of antibody for clinical evaluation.
- This platform has also been applied successfully to other IgM monoclonal antibodies.

Conclusions

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- Lower solubility and higher sensitivity to denaturation must be accommodated.
- Binding capacities are generally lower than for IgG but still support economical fractionation.
- Lack of an affinity step makes a positive contribution to process economy.
- Lack of intermediate diafiltration steps, made possible by loading ion exchangers by in-line dilution, also contributes to process economy.

Acknowledgements

Thanks to the Process Applications group at Bio-Rad for performing early characterization of IgM behavior on hydroxyapatite.