Recent advances in the purification of IgM monoclonal antibodies

Pete Gagnon, Validated Biosystems
Frank Hensel, Paul Andrews, Patrys, Ltd.
Richard Richieri, Avid BioServices, Inc.

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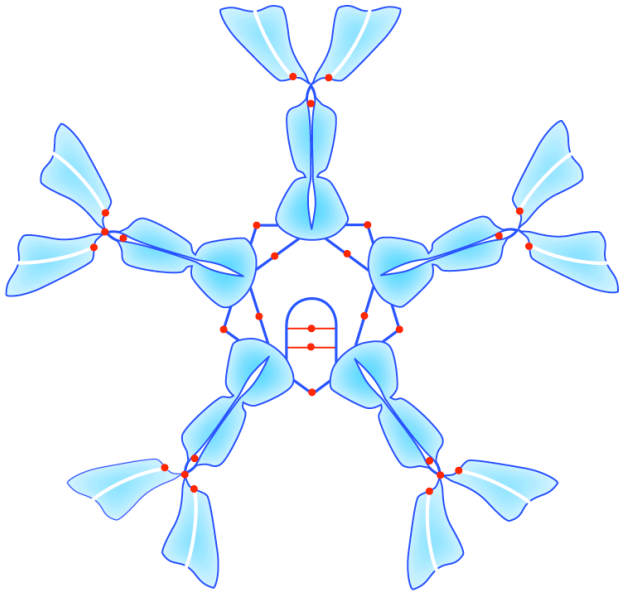
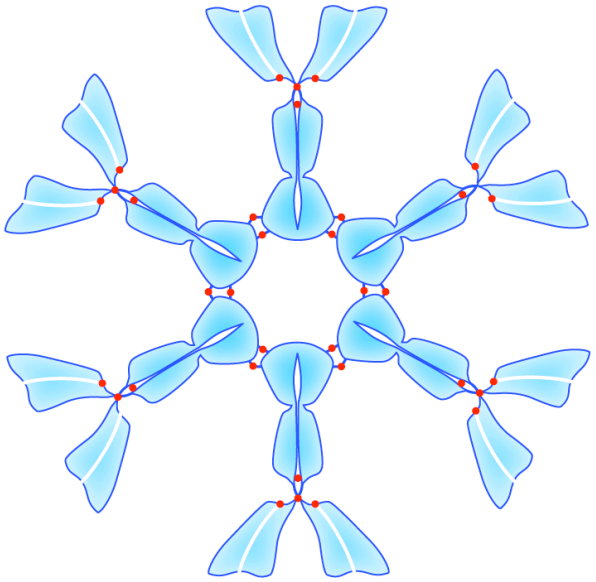
Structure and properties of IgM

Pentameric, 0.96 Md



Hexameric, 1.15 Md



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*

Structure and properties of IgM

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 ion exchangers and hydroxy-apatite to purify most IgMs without exposing them to extreme conditions.*
- *These characteristics also enhance binding capacity and contaminant removal.*

Structure and properties of IgM

Cryoprecipitation

- *Some IgMs form a clear precipitate at low temperatures.*
- *This may be overlooked during cell culture production but it can cause erratic measurements and depress overall productivity estimates. Cold, ambient, and 37°C samples should be assayed early in cell culture development to detect this phenomenon.*
- *If not detected, cryoprecipitation may cause startling losses during filtration or chromatography operations, especially when cell culture supernatant or in-process material is processed directly from storage at 4°C.*

Cryoprecipitation is completely reversible on warming. For more information concerning the mechanism consult: Middaugh et al, 1978, *Proc. Nat. Acad. Sci*., **75** 3440 and *J. Biol. Chem*., **255** 6532

Structure and properties of IgM

- *IgMs commonly precipitate at low conductivity.*[1]
- *Some IgMs may precipitate at pH extremes, even at physiological conductivity.*
- *Light to moderate opacity for brief periods may be reversible. Heavy precipitation is usually permanent.*
- *Operating conditions can usually be developed to avoid precipitation without diminishing the effectiveness of the overall purification process.*

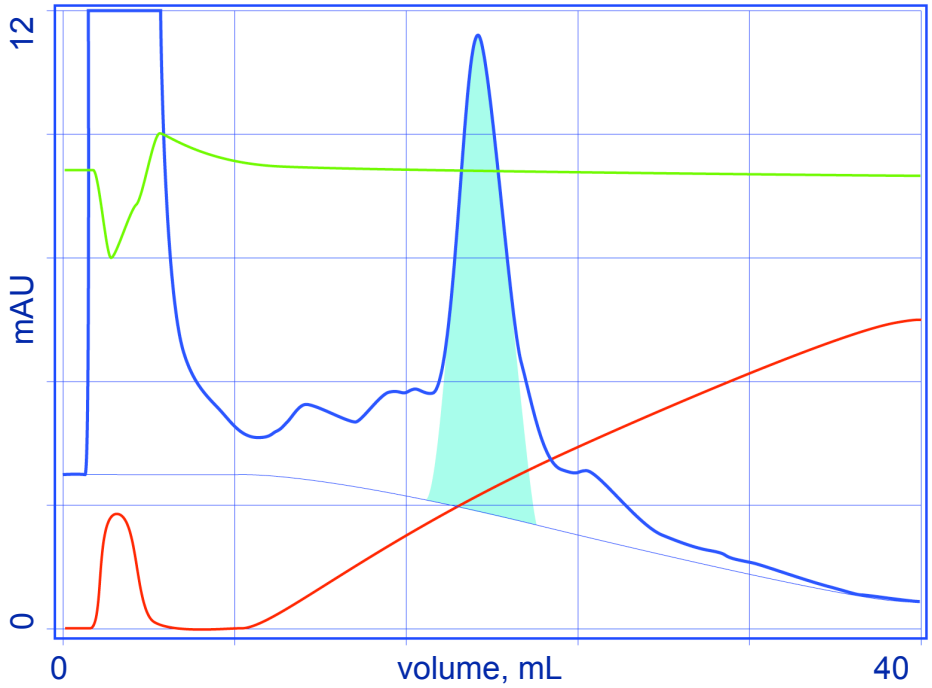
[1] This is the basis of a purification technique called euglobulin precipitation and a chromatography method that has been referred to as euglobulin adsorption, originally described by Bouvet et al, 1984, *J. Immunol. Met*., **60** 299; Clezardin et al, 1986, *J. Chromatogr.,* **354** 425 and **358** 209

Step 1: Obtain purified reference

- *Affinity chromatography is generally unsuitable because harsh elution conditions cause denaturation.*
- *Hydrophobic interaction carries a risk of denaturation and usually requires special sample application conditions.*
- *Size exclusion is gentle and simple but it dilutes, it is slow, and it has low capacity.*
- *Ion exchangers have unpredictable selectivity and usually require special sample application conditions.*
- *Hydroxyapatite is gentle, requires only minor sample modification, concentrates the product, and the IgM peak is almost always recognizable in a consistent region of the elution profile. It is suitable as a protein A analogue for purification during early cell line evaluation.*

Step 1: Obtain purified reference

Elution profile from hydroxyapatite preparation of reference sample



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: human IgM cell supe.

Load: 2 mL

Wash: 5 CV buffer A

Elute: 30 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.

Step 1: Obtain purified reference

Generic hydroxyapatite conditions for preparation of reference IgM

- *Buffer A: 10 mM $NaPO_4$, pH 7.0*
- *Buffer B: 500 mM $NaPO_4$, pH 7.0*
- *Ceramic Hydroxyapatite CHT type II 40µm,*
- *Equilibrate column with buffer A, flow rate: 200 cm/hr*
- *Adjust pH of filtered supernatant to ~pH 7.0. Add phosphate to a final concentration of at least 5 mM (unless already contained).*
- *Apply supernatant up to equivalent of 5 mg IgM per mL of CHT*
- *Wash with buffer A*
- *Elute with a 30 CV linear gradient to 100% buffer B*
- *Expect 70-80% purity from this gradient (typically about 90% purity after gradient optimization).*

Choosing ion exchangers

Diffusion constants for selected proteins

Protein	Mass	K_{diff} cm^2/sec
IgM	960 kD	2.6×10^{-7}
IgA	335 kD	3.7×10^{-7}
IgG	150 kD	4.9×10^{-7}
Albumin	67 kD	6.7×10^{-7}
Light chain	23 kD	9.1×10^{-7}

An IgM molecule would diffuse about 8 cm^2/year at this rate.

Choosing ion exchangers

- *Diffusional limitations — especially with very large molecules such as IgM — reduce capacity and separation performance on chromatography media that rely on diffusion for mass transport.*
- *These losses can be partially compensated by increasing column volume and/or reducing flow rate, but at the expense of raw material costs and process time.*
- *Chromatography media that rely on convection for mass transport are liberated from these restrictions.*
- *Convective alternatives include membranes and monoliths.*

Choosing ion exchangers

- *Membrane based ion exchangers are very effective for removal of contaminants in a flow-through format, but they do not support high resolution separations among eluted components.*
- *This is partly because membranes consist of a single chromatographic plate, and partly because of dispersion from uneven flow distribution and large mixing volumes within the housings.*
- *Monolithic ion exchangers offer separation efficiencies as good or better than the best particle-based media, and they have no void volume.*

A. Strancar, A. Podgornik, M. Barut, and R. Necina, 2002, Short monolithic columns as stationary phases for biochromatography, *Adv. Biochem. Eng. Biotechnol.,* **76** 50

Screening selectivity

Buffers for cation exchange and anion exchange

- *A1: 50 mM MES, pH 5.5*
- *B1: 50 mM MES, 1.0 M NaCl, pH 5.5*

- *A2: 50 mM MES or Hepes, pH 6.5*
- *B2: 50 mM MES or Hepes, 1.0 M NaCl, pH 6.5*

- *A3: 50 mM Hepes, pH 7.5*
- *B3: 50 mM Hepes, 1.0 M NaCl, pH 7.5*

MES and Hepes are both zwitterionic, neither bound nor repelled by either anion or cation exchangers, and thereby provide unimpaired buffer capacity for both. After the basic separation conditions have been established, buffers can be switched for cheaper USP alternatives.

Screening selectivity

- *Anion exchange: CIM® QA monolith, 0.34 mL*
- *Cation exchange: CIM SO_3 monolith, 0.34 mL*
- *Sample prep: Dilute 1 part CHT-purified reference with 2 parts water*
- *Equilibrate: Buffer A, 4 mL/min (~12 CV/min)*
- *Inject: 25-100 µL sample*
- *Wash: Buffer A*
- *Elute: 15-30 CV linear gradient to 50% Buffer B*
- *Clean: 100% Buffer B*

Screening selectivity

What about pH gradients?

- *IgMs will occasionally produce sharp elution peaks within pH gradients at low conductivity, but they will more often elute as long, dilute, poorly defined "zones" — or not elute at all — suggesting insolubility under these conditions.*
- *Similar profiles may be observed with conductivity gradients if the IgM is partly or wholly insoluble at a particular operating pH.*

Screening selectivity

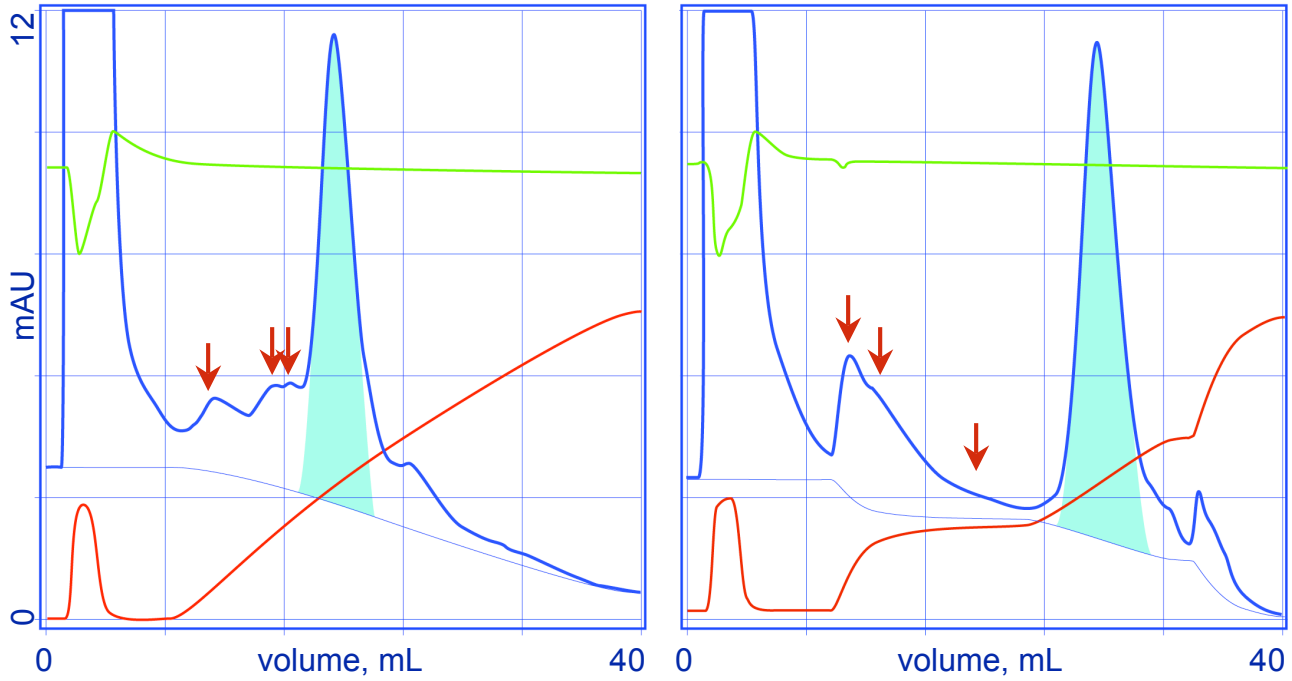
Cation exchange: Look for the highest pH at which the IgM remains bound. This has two important process ramifications:

- *The highest pH will be the pH at which the antibody elutes at the lowest conductivity. Whether the next step in the process is hydroxyapatite or anion exchange, low conductivity will require less dilution to prepare the cation exchange pool for loading.*
- *The highest pH will also be the pH at which the fewest contaminants bind to a cation exchanger.*

Anion exchange: Look for the lowest pH at which the IgM remains bound.

Optimizing selectivity

Adjusting gradient set points



Hydroxyapatite. Left: screening gradient. Right: optimized gradient. Highlighted peaks indicate IgM. Once the set points are established, the linear gradient segment can be converted to a step.

Optimizing selectivity

Additional options for optimizing hydroxyapatite

- *Elution pH may be increased as high as the particular IgM will tolerate. The usual response is weaker binding, which may improve contaminant elimination.*
- *Eluting with NaCl in conjunction with phosphate has been shown to improve removal of aggregates, host cell proteins, endotoxin, DNA, and virus from IgG preparations.[1]*
- *Eluting with polyethylene glycol in conjunction with either NaCl or phosphate gradients has been shown to have similar effects, although by a different mechanism.[2]*

[1]P. Gagnon et al, 2006, Practical issues in the industrial use of hydroxyapatite for purification of monoclonal antibodies, 232nd meeting of the American Chemical Society, San Francisco

[2]P. Gagnon et al, 2007, Nonionic polymer enhancement of aggregate removal by ion exchange and hydroxyapatite chromatography, 12th Waterside Conference, San Juan, Puerto Rico

Preparative loading conditions

Hydroxyapatite

- *Titrate 1 part unpurified sample with 0.1 parts 1.0 M Tris, pH 7.0.*
- *For bulk sample loading, dilute 1:1 with 10 mM $NaPO_4$ pH 7.0. Higher bulk dilution factors risk precipitation*
- *For higher dilution factors, load titrated sample by in-line dilution: 1 part titrated sample with 1-4 parts equilibration buffer.*
- *Use these conditions initially to load enough material to support meaningful analysis with PAGE and other methods. Optimize titration and dilution factors later.*

Preparative loading conditions

Anion exchange

- *Titrate 1 part CHT-purified reference with 0.2 parts 1.0 M Tris, pH 8.0. Titrate unpurified sample with 0.1 parts 1.0M Tris.*
- *Test at small scale. If you observe precipitation, lower the pH of the Tris!*
- *Load titrated sample by in-line dilution. 1 part sample diluted with 2-4 parts equilibration buffer. Bulk dilution risks precipitation!*
- *Use these conditions initially to load enough material to support meaningful analysis with PAGE and other methods. Optimize titration and dilution factors later.*

Preparative loading conditions

Cation exchange

- *Titrate 1 part CHT-purified reference with 1 part 0.75 M MES, pH 5.5. Titrate 1 part cell supernatant with 0.1 parts MES.*
- *Test at small scale. If you observe precipitation, raise the pH of the MES!*
- *Load titrated sample by in-line dilution. 1 part sample diluted with 2-4 parts equilibration buffer. Bulk dilution risks precipitation!*
- *Use these conditions initially to load enough material to support meaningful analysis with PAGE and other methods. Optimize titration and dilution factors later.*

Preparative loading conditions

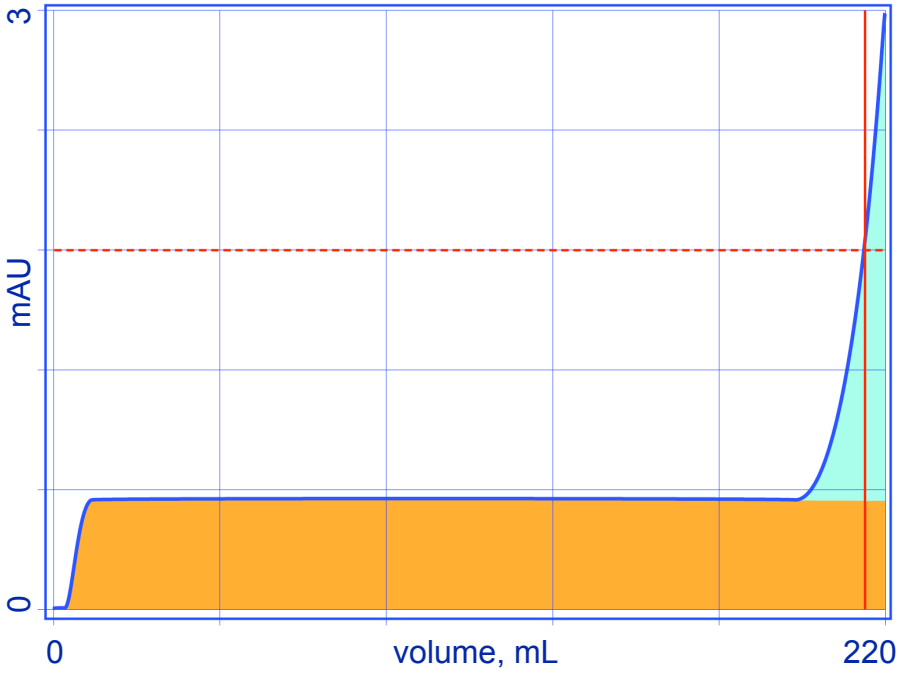
To maximize capacity in the final process

- *Keep in mind that it is not necessary to load ion exchangers at the intended elution pH.*
- *On cation exchangers, consider loading at lower pH than you intend to elute at; on anion exchangers, consider loading at a higher pH.*

The dilution factor is a compromise between process time and column volume. The higher the dilution factor, the higher the column capacity, the smaller the column you can use, and the more concentrated the eluted IgM. However, it will require more buffer and take longer to load.

Preparative loading conditions

Measure dynamic binding capacity at each step



Media (shown): CIM QA 0.34 mL

Flow rate: 5 mL/min

Sample: CHT-purified human IgM

Sample prep: titrate pH, dil. in-line

Load: 220 mL at ~ 0.15mg/mL

IgM DBC at 1% bt: >31 mg/mL

Media: CIM SO_3 0.34 mL

Same sample, flow rate, loading.

IgM DBC at 1% bt: >38 mg/mL

Blue highlighted area indicates IgM breakthrough. Orange highlighted area indicates contaminants flowing through. Red lines indicates 1% IgM breakthrough.

Process sequencing

Hydroxyapatite is generally well suited for IgM capture.

- *mild operating conditions.*
- *minimal sample conditioning.*
- *most contaminants flow through.*
- *recommended flow rate for IgM: 100-200 cm/hr*
- *IgM dynamic capture capacities may range from 5 to more than 20 mg/mL[1] depending on product binding characteristics, product and contaminant concentration, buffer composition of the feedstream, and flow rate.*

[1]Capacities are higher with purified IgM.

Process sequencing

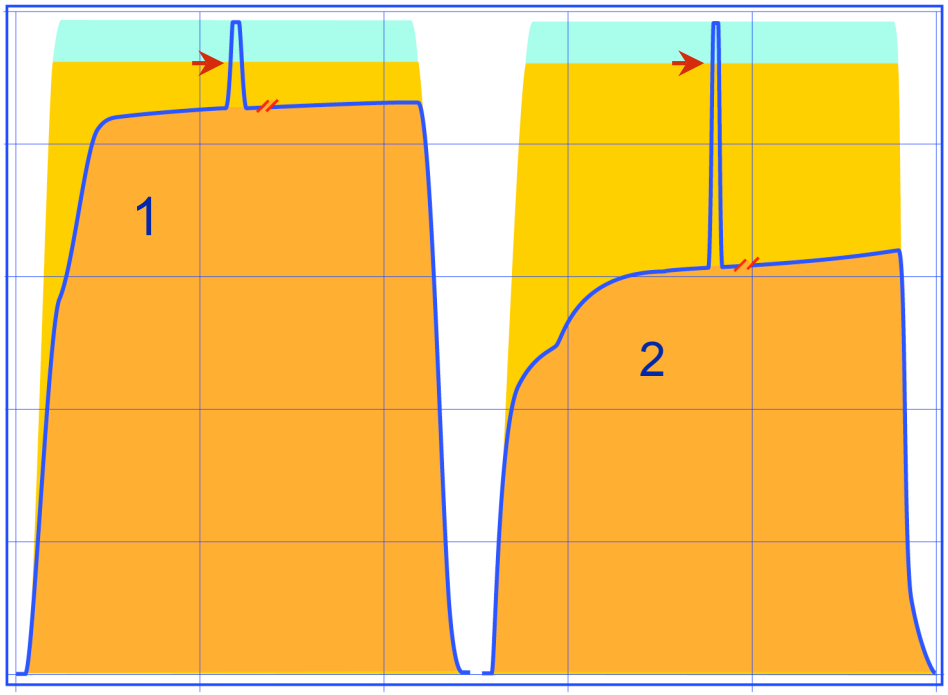
- *Cation exchangers may bind some IgMs strongly enough to be practical for capture. Dilution will still be required to reduce conductivity, but the flow capacity of monoliths may make the compromise worthwhile.*
- *Quaternary amine anion exchangers are poorly suited for capture because phosphorylated contaminants in cell supernatants bind very strongly. These include DNA and phospholipids. This burdens development and validation of column cleaning. If you want to use anion exchange for capture, use DEAE. The same contaminants will compete for capacity but it can be cleaned effectively with NaOH.*

Process sequencing

- *In general, the best candidate for capture will be the one from which the antibody elutes latest and which binds the lowest proportion of contaminants.*
- *This combination of conditions generally supports the highest capacity and purity.*
- *One way to evaluate capture potential is to take the column out of line briefly during sample application and compare the UV absorbance versus absorbance with the column in-line. The difference indicates the proportion of applied protein being bound.*

Process sequencing

Process sequencing: Comparison of capture potential.



Column 1: hydroxyapatite, human IgM cell culture supernatant

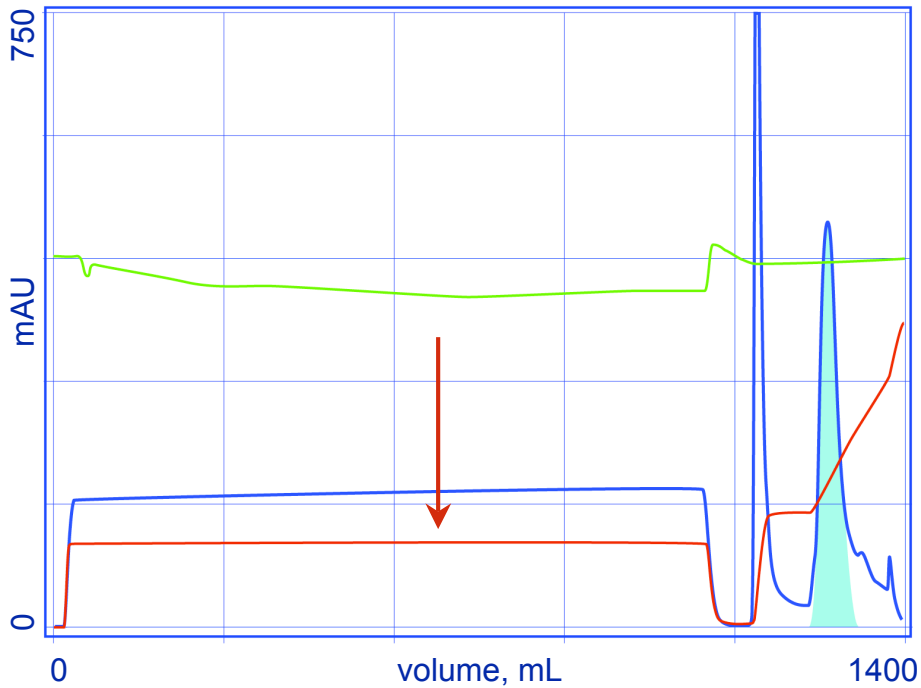
Column 2: cation exchange, murine IgM cell culture supernatant

The spike on each profile occurred when the column was taken off line to determine UV absorbance of the sample load. Blue indicates the proportion of IgM in the sample load. Yellow indicates bound contaminants. Orange indicates flow-through contaminants.

Note that the hydroxyapatite column binds a smaller proportion of contaminants, leaving more capacity for IgM.

Purification Process 1, IgM 1

Step 1. Hydroxyapatite chromatography



Media: 10 mL CHT type II 40µm

Column: MediaScout 11.8 x 100

Flow rate: 3.4 mL/min

Equilibrate: 10 mM $NaPO_4$ pH 7.0

Titrate, dilute, load: 1000 mL

Wash: 75 mM $NaPO_4$ pH 7.0

Elute: LG to 225 mM $NaPO_4$

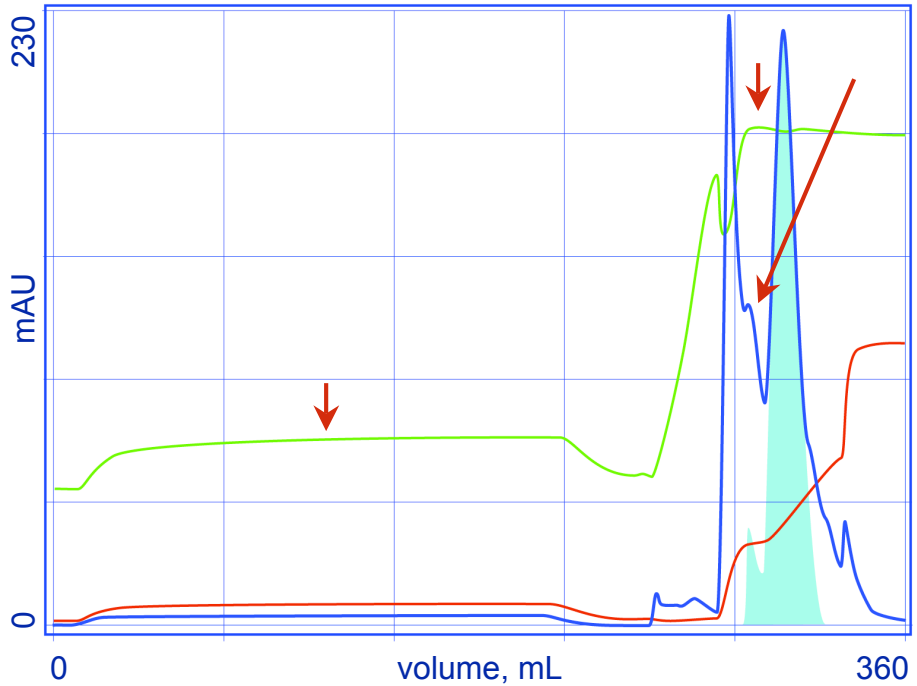
Clean: 500 mM $NaPO_4$ pH 7.0

Sanitize/store: 1.0/0.1 M NaOH

The high conductivity of the sample load is mostly from NaCl, which has less effect on binding than PO_4 but still reduces capacity. Consider membrane concentration before loading but keep conductivity about physiological, then dilute during sample loading.

Purification Process 1, IgM 1

Step 2. Cation exchange chromatography



Column: CIM SO_3 8 mL radial flow

Flow rate: 20 mL/min

Equilibrate: 50 mM MES, pH 6.0

Load: titrate and dilute: 250 mL

Wash: 50 mM MES, pH 6.0

Wash: 25 mM $NaPO_4$ pH 7.0

Elute: LG to 225 mM $NaPO_4$ pH 7

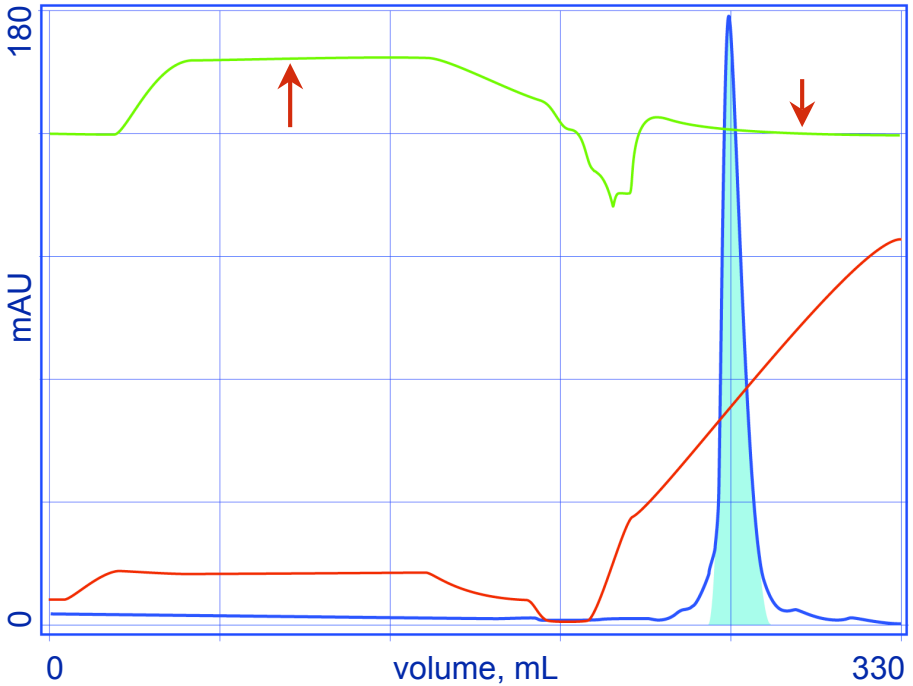
Clean: 500 mM $NaPO_4$ pH 7.0

Sanitize/store: 1.0/0.01 M NaOH

Note that the column is loaded at pH 6 then re-equilibrated to pH 7 to prepare for elution. Also note the second peak during the wash. This is IgM and indicates that the wash-phosphate concentration needs to be reduced.

Purification Process 1, IgM 1

Step 3. Anion exchange chromatography



As with the cation exchange step, the loading pH is different from the elution pH, with the intent to improve both capacity and contaminant removal.

Column: CIM QA 8 mL radial flow

Flow rate: 20 mL/min

Equilibrate: 5 mM $NaPO_4$ pH 7.0

Titrate, dilute, load: 125 mL

Wash: 5 mM $NaPO_4$ pH 7.0

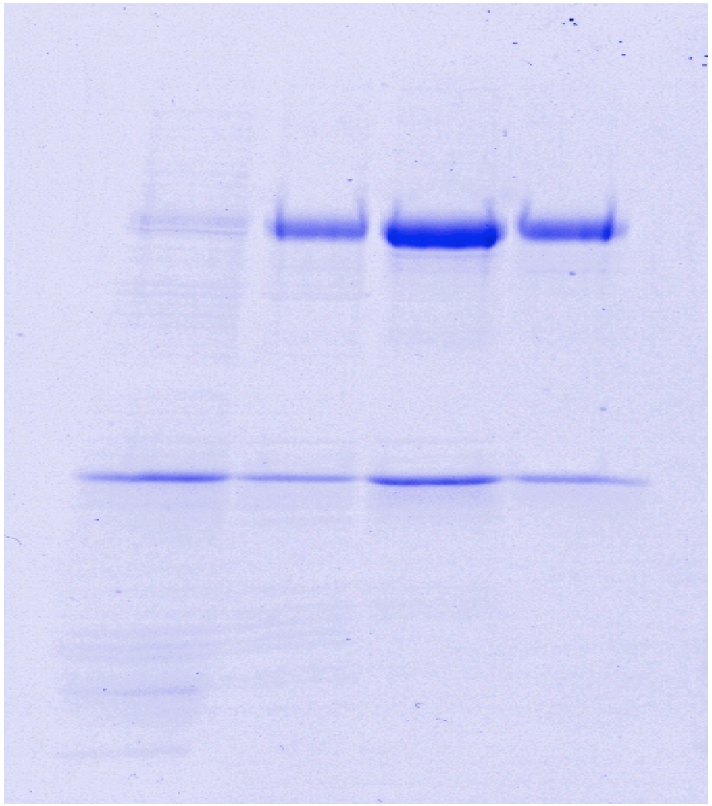
Elute: 15CV LG to 500mM $NaPO_4$

Clean: 500 mM $NaPO_4$ pH 7.0

Sanitize/store: 1.0/0.01 M NaOH

Purification Process 1, IgM 1

Reduced SDS PAGE, 5-15%



1 2 3 4

1. Supernatant
2. Hydroxyapatite pool
3. Cation exchange pool
4. Anion exchange

Purification Process 1, IgM 1

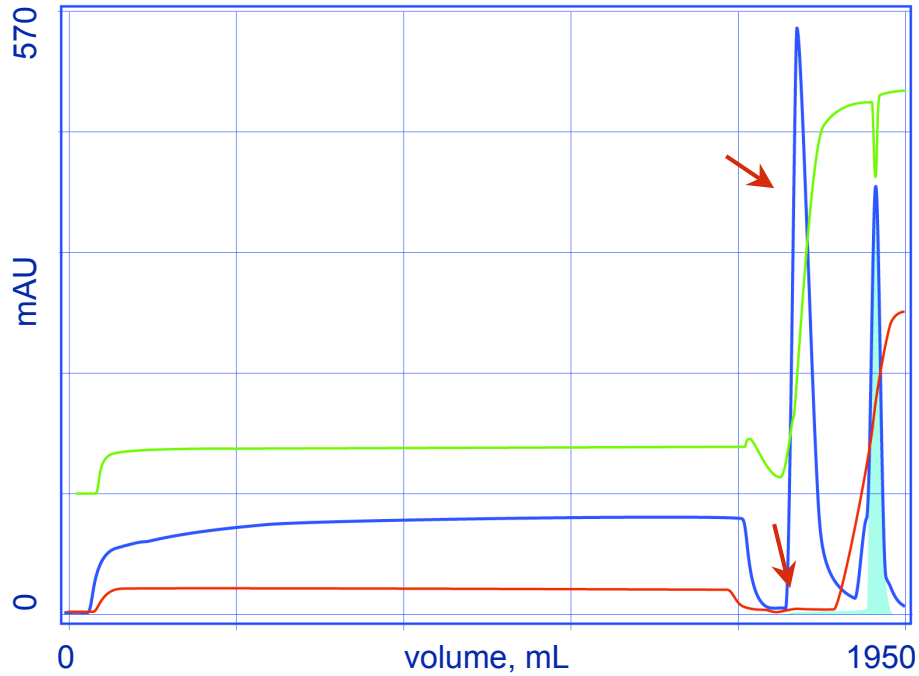
Summary

	Volume	mgs	Recovery	Purity	Time
Supernatant	500 mL	30	100/100%	n/a	n/a
Hydroxyapatite	1000 mL	24	79/79%	90%	390 min
Cation exchange	250 mL	18	73[1]/58%	95%	20 min
Anion exchange	125 mL	16	88/51%	99%	18 min

[1]Inappropriate wash conditions
Volume refers to sample volume.
mgs refers to mgs recovered in the elution pool at each step.
Recovery values refer to step/cumulative recovery
Time includes column equilibration, loading, washes, elution, and cleaning.
These recoveries are consistent with IgG recoveries obtained at this process scale.
Recovery typically improves at manufacturing bed height and residence time.
Note that process time for hydroxyapatite is about 20 times greater than for monoliths.

Purification Process 2, IgM 2

Step 1. Cation exchange



Column: CIM SO_3 8 mL radial flow

Flow rate: 20 mL/min

Equilibrate: 50 mM MES, pH 6.0

Titrate dilute load: 1500 mL

Wash: 50 mM MES, pH 6.0

Wash: 25 mM $NaPO_4$ pH 7.0

Elute: LG to 225 mM $NaPO_4$

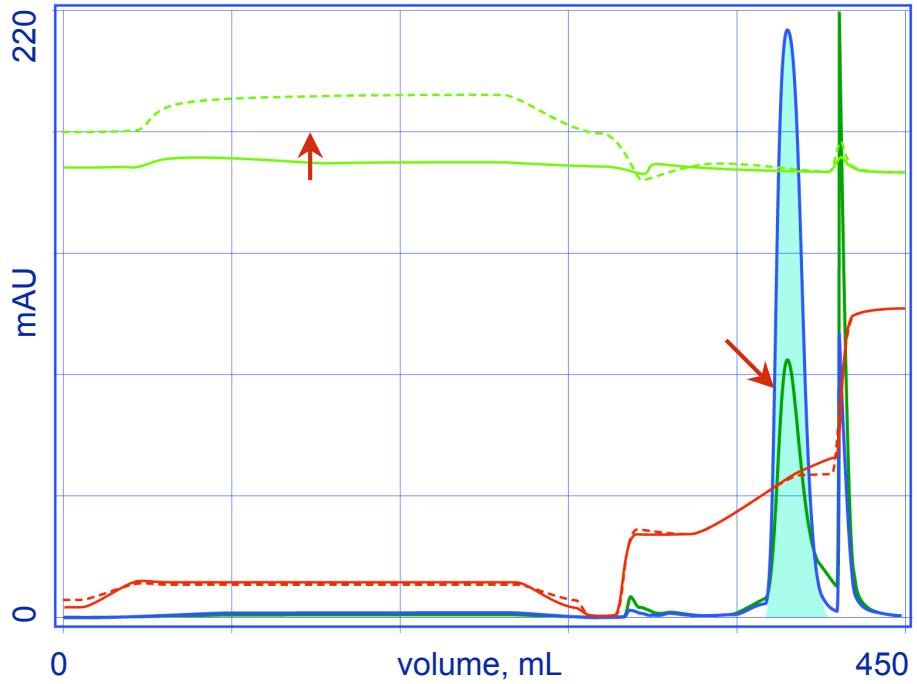
Clean: 500 mM $NaPO_4$ pH 7.0

Sanitize/store: 1.0/0.01 M NaOH

Note the large contaminant peak in the pH 7 wash. These contaminants occupy binding sites during loading, thereby reducing capacity for the IgM. Contaminant binding can be reduced by increasing the loading pH or reducing conductivity, but either will decrease IgM capacity concurrently. Note also the loss of product beginning with the pH 7 wash, suggesting that the wash pH should be reduced.

Purification Process 2, IgM 2

Step 2. Anion exchange



Column: CIM QA 8 mL radial flow

Flow rate: 20 mL/min

Equilibrate: 10 mM $NaPO_4$ pH 7.0

Load 1: pH 7.0 Load 2: pH 8.0

Wash: 75 mM $NaPO_4$ pH 7.0

Elute: LG to 225 mM $NaPO_4$

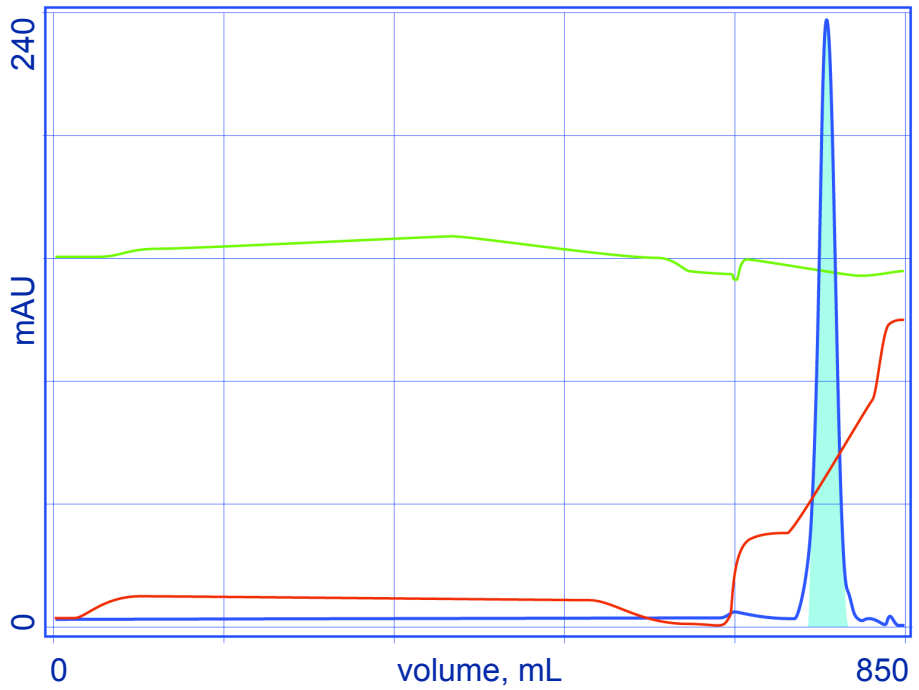
Clean: 500 mM $NaPO_4$ pH 7.0

Sanitize/store: 1.0/0.01 M NaOH

The profile in dark green and the dashed lines correspond to the run loaded at pH 8. Note the smaller IgM elution peak at pH 8. This IgM became opaque when titrated to pH 8, was apparently bound irreversibly, and removed in 1.0 M NaOH (not shown).

Purification Process 2, IgM 2

Step 3. Hydroxyapatite



Media: 10 mL CHT type II 40µm

Column: MediaScout 11.8 x 100

Flow rate: 3.4 mL/min

Equilibrate: 10 mM $NaPO_4$ pH 7

Titrate, dilute, load: 500 mL

Wash: 10 mM $NaPO_4$ pH 7.0

Wash: 75 mM $NaPO_4$ pH 7.0

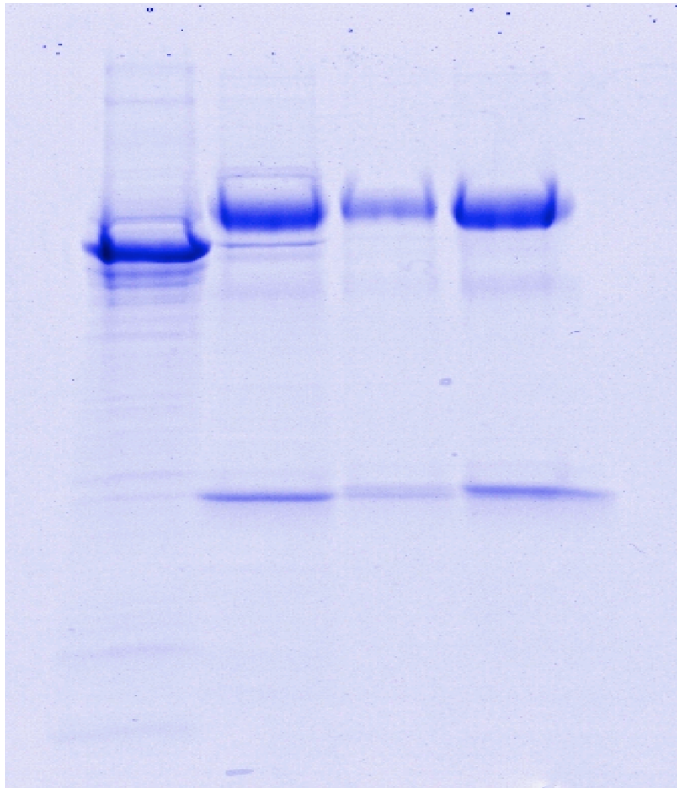
Elute: LG to 225 mM $NaPO_4$ pH 7

Clean: 500 mM $NaPO_4$ pH 7.0

Sanitize/store: 1.0/0.1 M NaOH

Purification Process 2, IgM 2

Reduced SDS PAGE, 5-15%



1 2 3 4

1. Supernatant
2. Cation exchange pool
3. Anion exchange pool
4. Hydroxyapatite pool

Purification Process 2, IgM 2

Summary	Volume	mgs	Recovery	Purity	Time
Supernatant	250 mL	50	100/100%	n/a	n/a
Cation exchange	1250 mL	39	78/78%	90%	70[1] min
Anion exchange	250 mL	33	84[2]/65%	95%	20 min
Anion exchange[3]	250 mL	10	31/26%	95%	20 min
Hydroxyapatite	250 mL	28	88/57%	99%	140[1] min

These recoveries are consistent with IgG recoveries obtained at this process scale. Recovery typically improves at manufacturing bed height and residence time.

[1]This is 5.6 times faster than the loading time for a similarly sized hydroxyapatite column despite the cation exchange load volume being 25% greater, yielding a net productivity improvement of about 700% for the monolith. A similar productivity improvement is realized with the anion exchange monolith.

[2]This value was lower than expected and may indicate a solubility limitation of the IgM.

[3]Sample loading at pH 8.0

Conclusions

IgM purification is not more difficult than purification of IgG, but it is different.

Mass and activity losses from exposure to harsh elution conditions generally prohibit the application of affinity chromatography, however…

The unique charge characteristics of IgM monoclonals typically provide purification opportunities that are rarely or never encountered with IgGs.

Conclusions

Purification of clinical grade IgM can generally be achieved with 3 bind-elute chromatography steps.

Step recoveries are comparable to those achieved with IgG purification.

Lower recoveries may be an indication that a particular IgM is partially insoluble under a specific set of conditions.

In such cases, the conditions can usually be adjusted to avoid the problem and restore recovery to “normal” levels.

Conclusions

- *Monolithic ion exchangers are advantageous because they achieve high dynamic binding capacities and high separation efficiency at high flow rates.*
- *Capacity appears to be 2-3 times higher than conventional ion exchangers.*
- *These features eliminate the productivity bottleneck experienced with conventional ion exchangers, particularly during loading of large volume samples.*
- *Rapid flow rates also permit process development to be conducted more rapidly. This encourages more thorough process development and characterization.*

Acknowledgements

The authors sincerely thank Bio-Rad Laboratories USA for providing hydroxyapatite, ATOLL GmbH Germany for packing hydroxyapatite into their MediaScout minichrom columns to facilitate this work, and BIA Separations, GmbH Austria for providing monolithic ion exchangers. Thanks also to the Process Applications group at Bio-Rad for performing early characterization of IgM behavior on hydroxyapatite, for performing PAGE analysis, and for other lab support.

Copies of this presentation can be downloaded at www.validated.com