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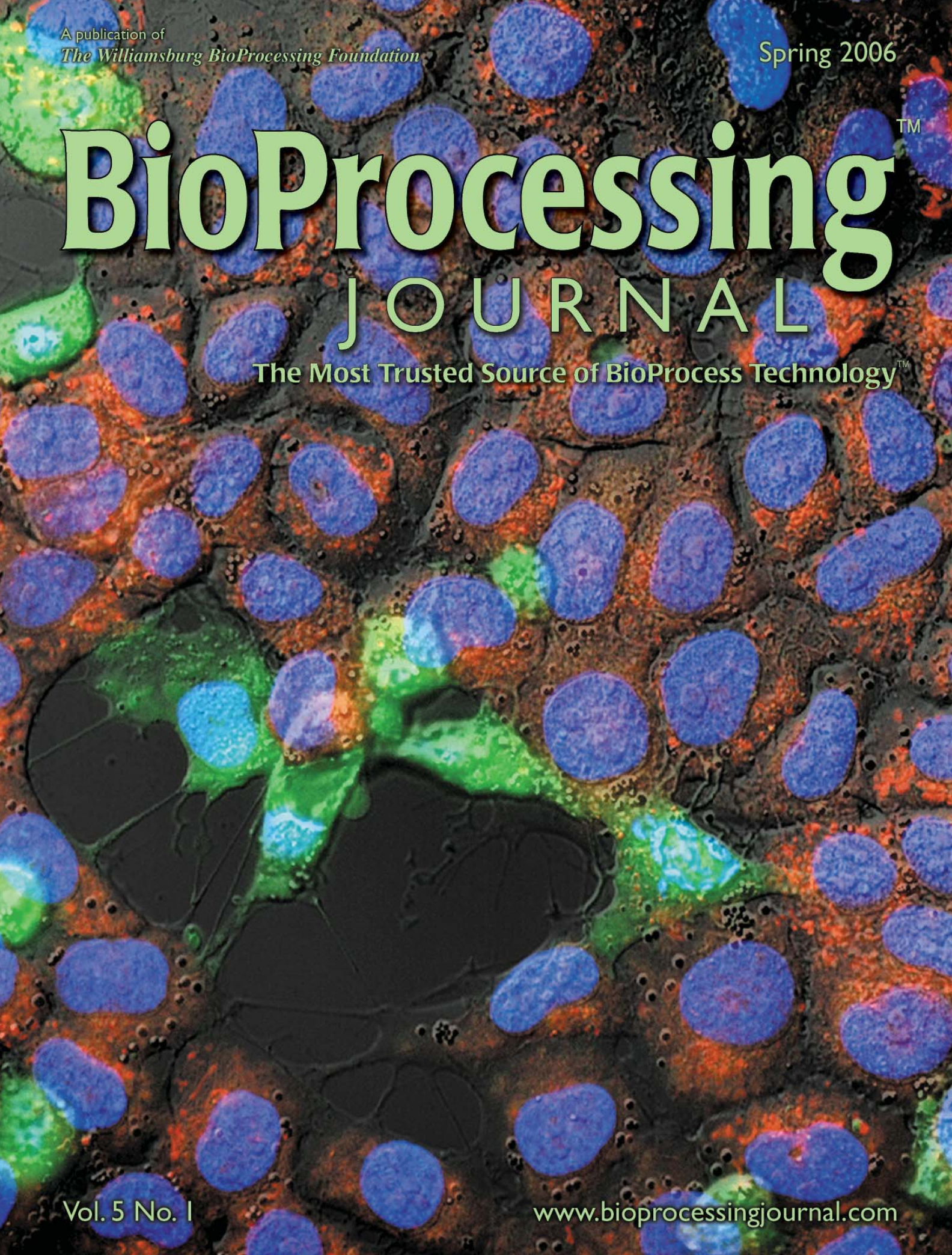

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The Quest for a Generic IgG Purification Process

By PETE GAGNON

The outstanding success and safety record of first generation monoclonal products has created an immense increase in the number of product candidates that need to be evaluated clinically. The concept of platform purification has emerged in response to this need. A platform is a semigeneric, multistep purification procedure that can be applied to a wide range of monoclonal antibodies without extensive method-scouting and optimization. This approach can substantially accelerate process development and hasten inception of clinical trials.

There are many options for platforms, nearly all of which employ protein A affinity chromatography for antibody capture and initial purification. Affinity is typically followed by an intermediate step to remove residual host cell proteins (HCP), product aggregates (Agg), leached protein A (LPA) and virus. Most platforms conclude with a polishing step of anion exchange chromatography to remove DNA, endotoxins (Etox), and retrovirus (Table 1). The protein A and anion exchange steps can be applied effectively under truly generic conditions. This makes it possible for process developers to focus scouting and optimization on the intermediate step. It also invites speculation that a single platform might work effectively for all antibodies. A platform based on protein A, followed by cation exchange and a polishing step

Table 1. A Basic Platform for Purification of Monoclonal IgG.

STEP	PRINCIPAL PURPOSE
Concentration (optional)/microfiltration	Remove particulates
Capture on immobilized protein A	**Remove host cell proteins, virus**
Low pH incubation	Inactivate virus*
Intermediate purification	**Remove protein A, aggregates, virus**
Virus filtration	Remove virus**
Polishing on anion exchange	**Remove DNA, endotoxin, retrovirus**
Ultrafiltration/dialfiltration	Concentration, formulation

*Cation exchange, hydrophobic interaction, or ceramic hydroxyapatite. **Anion exchange.

Table 2. Candidates for Intermediate Purification.

METHOD	STRENGTHS	WEAKNESSES
CEX	High capacity	MAbs insoluble under binding conditions, usually require in-line dilution
	High flow rates	Mabs form complexes with DNA and endotoxins at low pH, low conductivity, poor clearance
	Base stable	Unpredictable clearance aggregates
	Good clearance LPA	Complex method development
	Good clearance HCP	
HIC	Good capacity	Weak ligands require excessive salt to achieve acceptable capacity
	Good flow rates	MAbs insoluble under binding conditions, can require in-line dilution
	Base stable	Salts stress equipment, costly disposal
	Good clearance Agg	Strong ligands can denature product, give poor recovery, can create aggregates
	Good clearance HCP	Complex method development
	High clearance DNA	Poor clearance LPA
	Good clearance Etox	
HAC	Good capacity	Unstable below pH 6.5 or in EDTA
	Good flow rates	Requires phosphate in buffers
	Base stable	Complex binding mechanism
	Good clearance HCP	High density requires special packing
	High clearance LPA	Media damaged by rough handling
	High clearance Agg	
	High clearance DNA	
	High clearance Etox	

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on anion exchange, has been suggested for this application. But even though there are sufficient chemical similarities among antibodies to make a platform approach feasible, the diversity among clones exceeds the ability of any single platform to accommodate them all. The stakes are increased by therapeutic dosages up to grams per patient per year, elevating the risk of cumulative adverse effects by trace contaminants. Given that a single platform is too restrictive to meet these needs, the next best solution is a toolbox of platform templates that collectively cover a broader range of product behaviors.

Options for Platform Development

The major candidates for intermediate purification of monoclonal antibodies are: cation exchange (CEX), hydrophobic interaction (HIC), and hydroxyapatite chromatography (HAC), as shown in Table 2. The dominant tasks

Table 3. A platform template based on an intermediate step of cation exchange chromatography

PROTEIN A ELUTION BUFFER: Any, but with low conductivity (see comments)

CATION EXCHANGE. High capacity industrial cation exchanger of choice
Sample preparation: Titrate sample to the pH of the equilibration buffer
Buffers:
A: 0.05M acetate, pH 4.5 or 0.05M MES, pH 5.5
B: A + 1.0 M NaCl, pH the same as buffer A
Fractionation:
Flow rate: per gel manufacturer's recommendation
Equilibrate: buffer A until pH of column effluent matches buffer A
Load sample: volume equivalent to 20 mg IgG per mL of gel, load by on-line dilution, 1 part sample to 9 parts buffer A. See comments concerning lower dilution factors.
Wash: 5CV buffer A
Elute: 15 CV linear gradient to 30% buffer B
Strip: 5CV buffer B
Comments: The biggest challenge with this prep is having enough salt in the protein A elution buffer to maintain antibody solubility without having an excess that will increase the dilution factor required to permit the antibody to bind the cation exchanger with high capacity. Increase the in-line dilution factor as necessary to achieve good capacity, but first consider using a high capacity exchanger which will be more tolerant of higher pH and conductivity, and generally permit lower in-line dilution factors, possibly even batch sample equilibration. Consider pH gradient elution to reduce conductivity of the sample going into the anion exchange step.

ANION EXCHANGE. High capacity quaternary amine based exchanger
Sample preparation: Dilute IgG pool with 1.0M Tris pH 8.5 until pH is 7.0 – 7.5. This will be approximately 5% volume to volume (v:v). If necessary add NaCl until conductivity is 10-12 mS/cm.
Buffers:
A: 0.05M Tris, pH 7.0–7.5
B: A + 1M NaCl, pH same as buffer A
Fractionation:
Flow rate: per gel manufacturer's recommendation
Equilibrate: buffer A until pH of column effluent equals buffer A
Load sample: volume equivalent to 10-20 mg IgG per mL of gel
Wash: 5CV buffer A
Elute/Clean: 10 CV 100% buffer B
Comments: In almost all cases, the antibody will flow through the column during sample application. Host cell proteins, DNA, endotoxin, and retrovirus will elute in buffer B. If the antibody elutes at a high salt concentration and requires substantial dilution, it may be run in advance of the CEC step.

for the intermediate purification step are removal of leached protein A and aggregates. Protein A removal is important because it is a documented immunotoxin with clinical ramifications, and because it is an adjuvant protein with the potential to promote formation of neutralizing antibodies in the host that may block therapy.[1-2] Removal of aggregates is important because they increase the frequency of embolisms in conjunction with therapy, and also promote formation of neutralizing antibodies.[3-8]

The ability of the intermediate step to address leached protein A and aggregates simultaneously is the primary determinant of which method will be best suited to a particular antibody. Qualified candidates can only be identified by experimentation. One particular method may support adequate removal of leached protein A but demonstrate poor removal of aggregates — or the reverse — or it may remove both well,

Table 4. A platform template based on an intermediate step of hydrophobic interaction chromatography

PROTEIN A ELUTION BUFFER: Any

HIC: Industrial phenyl media of choice
Sample preparation: Titrate pH to 7.0. Immediately prior to sample application, gradually dissolve dry NaCl in sample to a final concentration of 4M (see comments)
Buffers:
A: 0.05M Na phosphate, 4.0M NaCl, 7.0
B: 0.05M Na phosphate, 2.0M urea, pH 7.0
Fractionation:
Flow rate: per gel manufacturer's recommendation
Equilibrate: 5CV buffer A
Load sample: volume equivalent to 10–20 mg IgG per mL of gel
Wash: 5CV buffer A
Elute: 15 CV linear gradient to buffer B
Strip: 5CV buffer B
Comments: Precipitation of IgG may be apparent at the solid:liquid interface of dissolving NaCl crystals. This will mostly disappear after the salt is completely in solution. Resist the temptation to filter out the haze since the salt will cause massive losses through antibody adsorption to the filter membrane. NaCl was selected for this application because it minimizes the possibility of the product precipitating before it is loaded onto the column. If initial results indicate that HIC is a good candidate for intermediate purification, the binding salt can be converted to ammonium sulfate. The urea in the elution buffer will improve resolution and recovery.

ANION EXCHANGE: High capacity quaternary amine based exchanger
Sample preparation: Dilute IgG pool with 1.0M Tris pH 8.5 until pH is 7.0 – 7/5. This will be approximately 5% volume to volume (v:v). Dilute with water until conductivity is 10-12 mS/cm.
Buffers:
A: 0.05M Tris, pH 7.0–7.5
B: A + 1M NaCl, pH same as buffer A
Fractionation:
Flow rate: per gel manufacturer's recommendation
Equilibrate: buffer A until pH of column effluent equals buffer A
Load sample: volume equivalent to 10-20 mg IgG per mL of gel
Wash: 5CV buffer A
Elute/Clean: 10 CV 100% buffer B
Comments: In almost all cases, the antibody will flow through the column during sample application. Host cell proteins, DNA, endotoxin, and retrovirus will elute in buffer B. If the antibody elutes at a high salt concentration and requires substantial dilution, it may be run in advance of the HIC step.

but each under different conditions. That platform may still be feasible if the polishing step is able to compensate for the deficiency, but the anion exchange polishing step is most often conducted in flow-through mode which severely limits its effectiveness for both aggregate and leached protein A removal.

Continuity of process flow is another important consideration. The objective is to avoid concentration or diafiltration between the primary fractionation steps. Extra filtration steps require equipment, development of SOPs, validation, and maintenance; they take time to run, consume space and materials, compound product losses and process costs. If sample preparation going into the intermediate and polishing steps can be limited to modest dilution and/or addition of buffer concentrates, it streamlines both process development and manufacturing with the additional benefit of immense cost savings. Tables 3–5 provide platform templates employing intermediate steps of cation exchange capacity (CEC), HIC, and HAC. Each is set up to avoid extra concentration or diafiltration steps.

Cation exchange is consistently the most challenging because most antibodies are partially insoluble under the binding buffer conditions customarily used for this technique — low pH and low conductivity. One practical solution is to load sample by in-line dilution: sample is loaded though one pump while diluent buffer is loaded simultaneously through another. The two streams meet at the mixer, seconds before reaching the column. Exposure time of the antibody to desolubilizing conditions is too brief for precipitation to become a problem. Another approach is to employ one of the recent generation of high capacity cation exchangers, some of which offer more than three times the capacity of conventional exchangers. High capacity cation exchangers are offered under various trade names by all of the major vendors in the field (UNOsphere™ S, Bio-Rad; Fractogel® EMD SO_3, Merck; Toyopearl™ Super SP, Toso Biosciences; SP Sepharose™ XL, GE Healthcare). Instead of using these exchangers for maximum capacity but still being burdened with the solubility limitations of the antibody, raise the pH or conductivity of the sample to the point where the antibody remains soluble during loading. This will require sacrificing some capacity, but the remaining capacity will probably still be substantially higher than can be achieved with conventional exchangers under desolubilizing conditions.

A third consideration in choosing the intermediate step is the amount of optimization that each requires. In general, the wider the range of variation in retention behavior from one monoclonal to another, the wider the range of conditions that will need to be screened to be sure that the best separation of contaminants is achieved. More variation is observed on cation exchangers than on HIC, and more on HIC than HAC. The screening workload can also be compounded substantially by the availability of multiple chromatography products, each of which gives different capacities and selectivities. The differences among these products can have significant process ramifications. As a result, most developers maintain chromatography media libraries to ensure that they identify the most effective tool for a given task. All other things being equal, the template that meets process requirements with the least development work best serves the spirit of the platform approach.

The platforms offered in templates 3-5 are the most conventional in the sense of being based on the familiar and proven elements of protein A and anion exchange (AEX) chromatography, but there is a wide range of other options. HIC and HAC have the proven ability to remove DNA and endotoxin as effectively as anion exchange, qualifying both as good candidates for polishing. The fact that they do so by different mechanisms makes them potentially even more effective under some circumstances. Both are also tolerant of salt in the feedstream which facilitates process flow following salt gradient elution from an intermediate CEC step. HAC can also remove DNA and endotoxin, as well as LPA and aggregates in flow-through mode. A platform with HIC as the intermediate step, and HAC as the polishing step (or the reverse), also supports good process flow.

As with the primary platforms, the first consideration in evaluating these alternatives is the overall complementary selectivity among the component methods. A two-step platform remains a compelling goal. The potential cost reduction in manufacturing alone is sufficient to make it worthwhile, and both biopharmaceutical companies and chromatography suppliers are actively pursuing it with the expectation that it will prove feasible. Platforms comprising protein A and anion exchange, protein A and ceramic hydroxyapatite (CHT™ ceramic hydroxyapatite, Bio-Rad), and protein A and Capto™ MMC (GE Healthcare), have all been presented as potential candidates for this application. The polishing step for each is run in bind/elute mode to maximize its fractionation potential. Evaluating a two-step platform is a simple matter, but this should not imply that taking it to the point of a commercial manufacturing procedure is as easy. The simpler the eventual manufacturing process, the more work there is in developing it; and the tighter the specifications must be, the more vulnerable it is to external process variation, and the more demanding the validation.

Conclusions

Ultimately, there are so many potential platforms that surveying all of them can be nearly as time-consuming as doing development from scratch, which defeats the original purpose of the platform approach. Individual companies tend to settle on fairly well defined conventions for creating new clones and for cell culture conditions. This manifests within a company as a substantial degree of similarity in the composition and characteristics of the cell culture media, which may in turn favor a particular purification platform. However, it is important not to restrict the options until a database has been developed indicating the diversity of purification behavior that the various products may exhibit, and it is important to appreciate that the platform that best serves one company may not serve another as well.

A general strategy of evaluating the

three primary platforms offered in this article is a sound approach. The differences in the fractionation mechanisms among the intermediate steps, although not guaranteed to accommodate all antibodies, will certainly accommodate the majority. Individual experience may indicate that a particular platform consistently meets a company's need better than the others, but even if this is the case, there are other benefits to surveying the three primaries as a matter of routine. The surface chemical characteristics of antibodies influence aspects of product behavior beyond purification: product solubility, stability, container compatibility; even pharmacokinetic behavior. Very strong binding to HIC media may reveal a tendency toward nonspecific associations that may affect analytical methods, and warn of aggregation at high product concentrations. Strong binding to CEX media may reveal a tendency to form stable complexes with

Table 5. A platform template based on an intermediate step of hydroxyapatite chromatography

PROTEIN A ELUTION BUFFER: 0.1M glycine or arginine, 0.05M NaCl pH 3.5.
No citrate or chelating agents.

ANION EXCHANGE: High capacity quaternary amine based exchanger
Sample preparation: Dilute IgG pool with 1.0M Tris pH 8.5 until pH is 7.0 – 7/5.
This will be approximately 5% volume to volume (v:v). If necessary add NaCl until conductivity is 10-12 mS/cm.
Buffers:
A: 0.05M Tris, pH 7.0–7.5
B: A + 1M NaCl, pH same as buffer A
Fractionation:
Flow rate: per gel manufacturer's recommendation
Equilibrate: buffer A until pH of column effluent equals buffer A
Load sample: volume equivalent to 10-20 mg IgG per mL of gel
Wash: 5CV buffer A
Elute/Clean: 10 CV 100% buffer B
Comments: In almost all cases, the antibody will flow through the column during sample application. Host cell proteins, DNA, endotoxin, and retrovirus will elute in buffer B. Note that this template has the polishing step earlier, in advance of the intermediate step for continuity of process flow; the high salt concentration of the IgG pool after CHT would require buffer exchange if the polishing step was last.

HYDROXYAPATITE: CHT ceramic hydroxyapatite -Type I 40 micron (Bio-Rad)
Sample preparation: Add 1% v:v 1.0M monosodium phosphate (pH ~4.1) to the IgG pool from the previous step. This will raise the sample phosphate concentration to 5mM and reduce the pH to about 6.5.
Buffers:
A: 5mM $NaPO_4$, pH 6.5
B: A + 1.5 M NaCl
C: 0.5M $NaPO_4$, pH 6.5
Fractionation:
Flow rate 300 cm/hr
Equilibrate: buffer A until column effluent is pH 6.5
Load sample: volume equivalent to 20 mg IgG per mL of gel
Wash: 5CV buffer A
Elute: 40 CV linear gradient to 100% buffer B
Clean: 5CV 100% buffer C
Comments: Unaggregated antibody will usually elute within the NaCl gradient. Aggregates typically elute later. Protein A, endotoxin, and DNA elute in the cleaning step. If the antibody fails to elute within the confines of the NaCl gradient, increase the phosphate concentration to 10mM. The suggested NaCl gradient is a screening gradient. The length and interval can be adjusted based on initial results.

DNA, endotoxin, and other contaminants. It is best not to be surprised by such revelations. The ability of a select toolbox of platform templates to circumvent these surprises makes the basic strategy that much more valuable.

The only certainty about the future of the platform approach is that it will change. The search for alternatives to protein A is a major priority for the industry. New mixed-mode selectivities continue to be introduced, and new application formats, like membranes and ultra-high capacity, porous particulate resins, continue to evolve. It has recently been suggested that phase separation technologies and novel precipitating agents may find a place in the industry as well. To the extent that any of these options has, by their ability to advance the field, they will be well received.

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