Validated Biosystems

quarterly resource guide
for downstream processing Volume 1, No. 3

http://www.validated.com

Welcome to the third issue of Validated Biosystems! We've made a couple of changes since last time. We've combined the contents of The Jungle and The Wishlist under The Jungle. This puts all the short-subject practical tips under a single heading.

This issue also marks the introduction of a new feature: S.W.A.T. (Special Weapons and Tactics). This is in response to requests for a feature dealing with real process development and manufacturing problems. We'll select one topic each issue and do our best to suggest practical solutions. We're relying on you to submit engaging problems. All sources will be kept strictly confidential and products will remain anonymous. E-mail us as much detail as you can concerning physicochemical features of the product, what's been tried, what's worked, and what' hasn't.

—Pete Gagnon, Editor

Contents...

The Consultant

Every issue we'll address one of a series of practical topics in downstream processing, on which we'll share insights and technical tips we've developed from over 15 years of hands-on idustrial process development. There will be a strong emphasis on the practical steps required to achieve the best results, along with whatever theory is necessary for it all to make sense. We welcome follow-up discussion, which we will post in the next issue. Our topic for this issue is:

Linear and Step Gradient Elution: Data verus Dogma

by Pete Gagnon, VBI

The relative value of linear and step gradients remains a point of controversy in purification process design. Like most such controversies, its persistence reflects incomplete articulation of the merits and limitations of the two formats. In this article we'll discuss some of the major process parameters affecting or affected by gradient format, with the goal of revealing how the 2 formats can be applied most productively.

Product concentration. Step gradients have a reputation for eluting product at higher concentrations than linear gradients. They do so in many contexts, but not all, and there are limits to their concentrating ability in any case. Figures 1 and 2 contrast peak volume as a function of step or linear gradient interval. As shown, gradient format is a minor determinant of peak volume. The key factor is the magnitude of the step or slope. Depending on sample composition and resolution requirements, peak volume from linear gradients can be competitive with peak volume from steps.

As implied by Figures 1 and 2, there is an inverse relationship between peak concentra-

tion and resolution (Figures 3,4). The sacrifice of resolution to achieve a high product concentration is generally more severe with step gradients. Within a linear gradient, the relative relationships among the eluting proteins tend to be well-preserved. In a step gradient, setting a broader interval to achieve higher product concentration automatically compromises purity. Such intervals are feasible with step gradients only when the requirement for resolution is low.

It is important to look beyond the method at hand when evaluating resolution requirements. If the contaminants flanking the product in one method are easily removed by another method in the same process, then resolution requirements for method at hand are low, despite the flanking contaminants (Figure 5). Broad gradient steps can be employed to elute the product at high concentration without imparing overall process performance.

Figure 6 illustrates the opposite situation where flanking contaminants are shaired by a pair of separation methods. Linear gradients would be challenged by such a situation, but step gradients would be wholly unsuitable. This highlights the point that the foundation of a good process is built on complementarity of separation methods. High resolution linear gradients can be used to maximize the degree of inherent complementarity, but they are not a substitute for the lack of it.

When developing gradient specifications for step gradients, set the broadest interval that doesn't compromise overall process performance. With linear gradients, set the steepest slope. These actions will yield the highest eluting product concentration for whichever format you use.

Eluted product concentration is limited by a number of other factors, regardless of gradient setpoints. One of the most important is diffusional limitations. The slow diffusion constants of proteins makes peak volume a function of media particle and pore size distribution. No matter how extreme your elution step, a given gel will always have a fixed minimum peak width, as a function of particle and pore size distribution. Packing quality will have an effect,

Figure 1. Effect of step amplitude on peak width

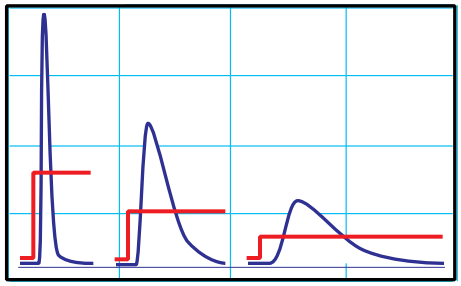


Figure 2. Effect of linear gradient slope on peak width

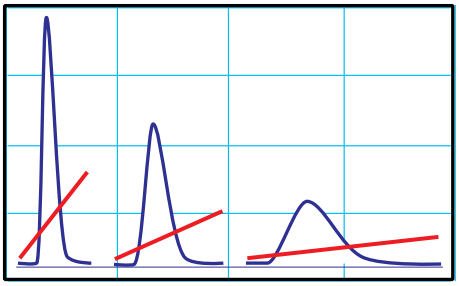


Figure 3. Effect of step amplitude on resolution vs peak concentration

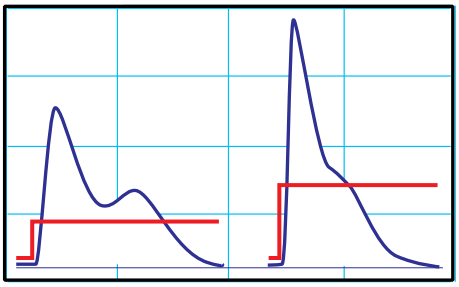
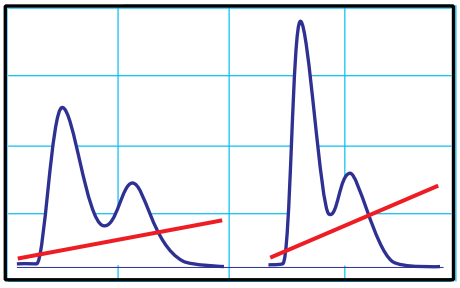


Figure 4. Effect of gradient slope on resolution vs peak concentration



and dependency on diffusion makes peak width strongly dependent on flow rate as well. Figure 7 illustrates increasing peak width in a linear gradient as a function of flow rate for BSA on a HIC matrix and an anion exchanger. The differences in the relative response, despite column dimensions, sample load, particle and pore size distribution all being identical, demonstrate the influence of other factors. In this case, the higher viscosity of the high-salt HIC buffer was judged to be the dominant cause. However, differential kinetics of the respective elution mechanisms cannot be discounted.

Figure 5. Contaminant mapping

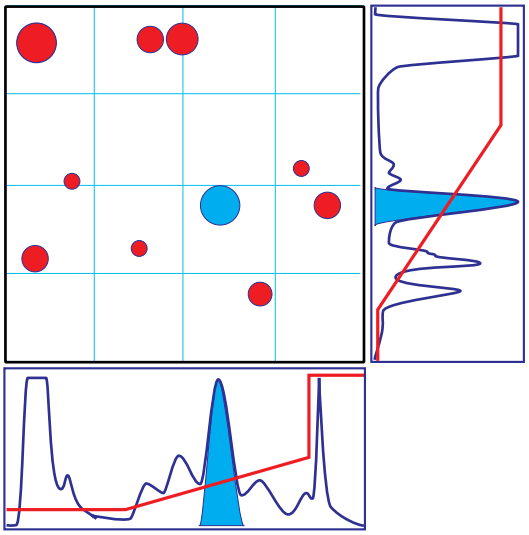
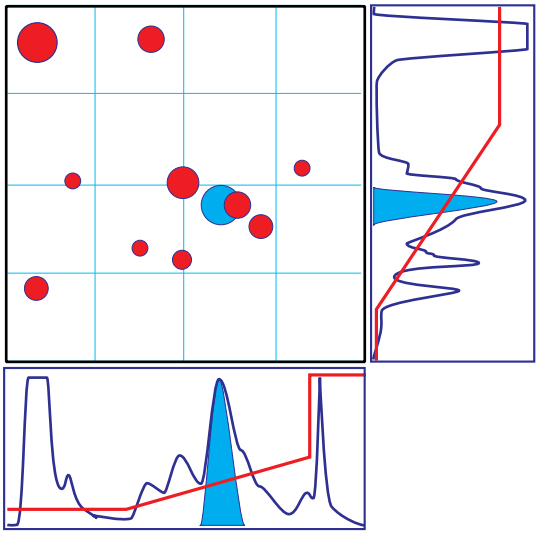


Figure 6. Contaminant mapping



Product purity and recovery. Product purity within a given method is seldom as good with step gradients as with linear gradients, and when it is, it's usually is achieved at the expense of recovery. Narrowing the gradient intervals to partition out flanking contaminants almost always requires sacrificing the leading or trailing fractions of the product peak (Figure 8). This re-emphasizes the importance of maximizing complementarity among process methods as the foundation to process development.

Where high resolution is required, linear gradients are the best option. Figure 9 illustrates a frequent pattern in linear gradient development. As gradient slope is reduced, initially, resolution increases more than peak volume. This reflects the rate of change in mobile

Figure 7. Peak width as a function of flow rate and elution mechanism

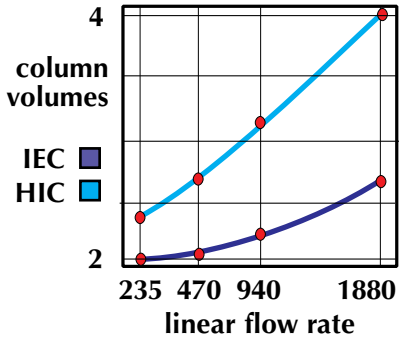
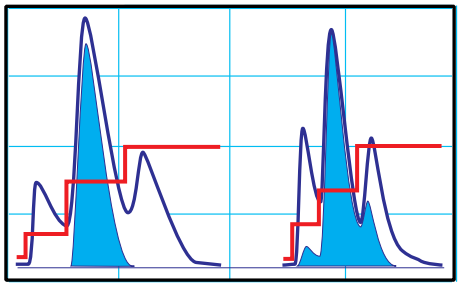


Figure 8. Effect of step amplitude on peak purity vs recovery



phase composition coming into phase with the kinetic limitations of the ligand:protein interaction. With further slope reduction, peak volume increases more than resolution. This doesn't mean that resolution won't continue to improve, just that you will pay an increasingly high price for it. The transition point in resolving efficiency versus peak volume can be estimated by comparing height ratios of the product peak with the adjacent valleys.

Process reproducibility. Linear gradients have the ability to buffer minor process variations. So long as the product elutes near gradient center and the gradient amplitude exceeds the range of process variation, external variations cause little more than a modest deviation of gradient slope. The relative relationships among the eluting proteins remain relatively unchanged. If uncontrolled external process variation is high, maintaining the slope while extending the gradient start and endpoints increases its insulating capability. Even substantial variances are absorbed with little consequence. This is important because the sources of variation are diverse and many of them are substantial.

One such source is variation in the fluidics architecture of process chromatographs, especially as they compare with process development systems. Systems vary with respect to accuracy of both flow and solvent proportioning. Equally important, they vary with respect to the amount of internal solvent mixing that occurs between the proportioning valve and the column. Each system has a characteristic "dispersion volume" -- the volume of solvent required for complete transition from one gradient setpoint to another. The larger the dispersion volume, the larger the volume of solvent required to achieve a programmed setpoint. The effects on step gradients can be devastating.

Figure 10 illustrates process variation resulting from differences in dispersion volume relative to column volume. The process was developed with a small column on a chromatograph

Figure 9. Effect of gradient slope on peak purity vs recovery

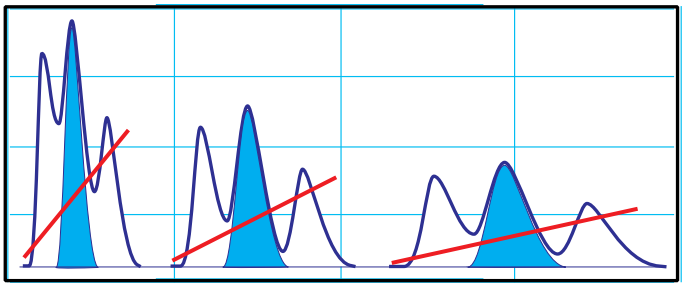
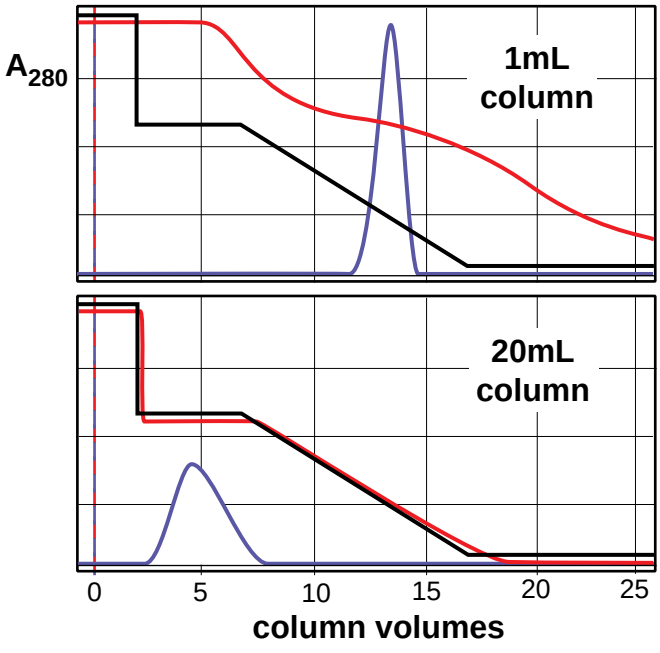


Figure 10. Process variation due to diffrent ratios between column volumn and chromatograph dispersion volume



with a high dispersion volume. During development, the wash step never reached target concentration within the programmed volume. When the process was scaled to a larger column on the same system, the dispersion to column volume ratio diminished, the wash step did reach its programmed value, and the product eluted prematurely.

Degree of column loading also has disproportionate importance for step gradients. Figure 11 illustrates variation in peak width and elution position as a function of column load. Not only does the peak become wider with increasing load, it elutes earlier. Step specifications set at a given column load are valid only at that load. This is a particular problem in situations where the product concentration and its proportion to contaminants in the feedstream vary from lot to lot.

This is also an impediment to process development. Development columns must be loaded to their intended process capacity throughout process development. This is a circular trap since capacity varies according to the run conditions. Setpoints for linear gradients, on the other hand, can be set preliminarily with low subcapacity column loads, then adjusted to compensate for the load-shift after other process specifications have been set. This is much simpler and it conserves sample.

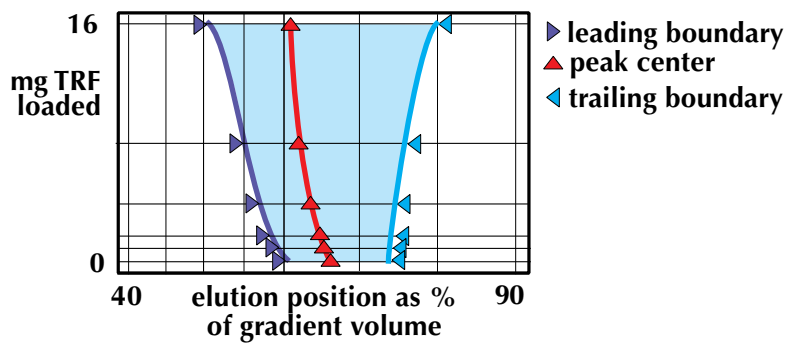
Other external variations also have a significant impact on the efficacy of step gradient setpoints. Hydrophobic interaction and protein A separations are very sensitive to temperature. Variations of a few degrees can render steps invalid, sacrificing purity, recovery, or both. Ion exchange is sensitive to minor variations in conductivity. As with column load, these effects have process development as well as reproducibility ramifications. The process must be modeled, and setpoints validated across the range of process variation that may affect the process. If resolution requirements are very permissive then broad steps pose no serious reproducibility concern. Otherwise, the "buffering capacity" of linear gradients makes their use essential.

Process sequencing. Step gradients offer process sequencing opportunities that linear gradients rarely match. For example, you can often elute product from a HIC column with a low salt buffer, and proceed directly to an ion exchanger with little intermediate sample re-equilibration. Products eluting within a linear gradient are likely to have a higher salt content, requiring either a higher degree of dilution or complete buffer exchange. The same principle applies to other process sequences.

Process control. Step gradients on ion exchangers can cause gross pH aberrations within the column. With anion exchangers, a large step in chloride concentration can liberate a sufficient concentration of hydroxide to raise the local pH to 12 and potentially denature the product. Acidification by hydronium ion displacement can occur on cation exchangers. This puts more constraints on buffer formulation to ensure adequate pH control. The gradual increase of salt in linear gradients avoids this problem.

Process monitoring. Steps provide no information as to the composition of a peak. Three gradient steps taken on a column loaded with a complex mixture will produce 3 peaks, regardless of the gradient intervals. This makes extensive secondary testing essential during process development, and also means that large-scale process failures can be masked. Even linear gradients can't indicate the com-

Figure 11. Peak position as a function of column load



plete composition of a peak, but the relationship among eluting peaks does provide an index that allows immediate visual assessement as to whether or not the process is within specified control limits. Linear gradient profiles also make it possible to abbreviate the requirement for secondary testing during method devlopment.

Process simplicity. The purported simplicity of step gradient applies to mechanical simplicity only. When large-scale chromatography systems were limited to simple switch valves this was an overiding factor, but no longer. Virtually all of the current generation large scale systems have linear gadient capability equivalent to the most sophisticated HPLCs.

With the wide availability of large-scale linear gradient chromatography systems, step gradients have become more -- not less -- complicated than linear gradients. The complications begin in development, as noted above, where setting reproducible specifications requires comprehensive full-load scale modeling. Accommodating all of the factors requires tedious balancing and rebalancing of the step intervals to support the best combination of purity, recovery, product concentration, and reproducibility. With linear gradients, once the slope is defined, accommodating external process variation is a simple matter of extending the start and endpoints sufficiently to insulate the "core" segment.

Process economy. The higher resolution supported by linear gradients frequently allows purifications to be conducted with fewer methods. A pair of linear methods will often support purification performance equivalent or better than a triplet of step methods, and triplets of linear gradient methods consistently outperform quads of steps. This is an important distinction for process economics. It reduces media requirements. It reduces column hardware requirements. It reduces labor. It reduces storage space requirements. It means that expensive manufacturing space is tied up for shorter periods per product --thereby increasing capcity, and it reduces validation requirements. The economic advantage is amplified by simpler development and better reproduciblity.

Conclusions. Gradient elution is the means by which the inherent complementarity among separation methods is exploited to its greatest advantage. Step gradients may be preferable where the relative selectivities among separation methods make high resolution fractionation unnecessary. The more permissive the fractionation requirements for a given method, the steeper the steps, the higher the eluted product concentration, and the less the results will be affected by external process variation. This tends to favor steps in processes with more methods, and where external sources of process variability are tightly controlled, as with manufacture of injectable products.

Linear gradients are generally a stronger option where resolution requirements are high, where external process variables are poorly controlled, where time pressure requires accelerating the development cycle, and where there is economic pressure to minimize the number of fractionation methods. This combination of requirements is more characteristic of in vitro reagent manufacturing environments and preparation of investigational materials.

In practice, every purification represents a unique challenge, as well as a unique set of opportunities. No preconceived philosophy about gradient modes is going to give you the flexibility you need to achieve the best process performance. Evaluate both formats, and apply them as they serve you best.

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S.W.A.T.

The Strange Case of the Golden Gamma Globulin

By Pete Gagnon, VBI

This case concerns a mouse IgG1 monoclonal used in a diagnostic application. When the antibody was produced in ascites and purified by ammonium sulfate precipitation followed by anion exchange chromatography, the antibody produced a water-clear solution. When production was converted to in vitro cell culture, antibody purified by the same process was bright yellow. There was minor reduction of color intensity after the anion exchange step.

This is the 4th monoclonal antibody we've heard of exhibiting this behavior: one other diagnostic and 2 intended for injectable application. This isn't an epidemic problem by any means, but it crops up enough that we thought it would be a worthwhile discussion topic.

First of all, this isn't a case of the antibody actually becoming colored. The monoclonal is complexing with a component from the culture media that causes it to appear yellow. The fact that the complex survives ammonium sulfate precipitation suggests that there is a strong hydrophobic component to the interaction. If it was solely ionic, the complexant would have been mostly removed in the supernatant. On the other hand there evidently is a significant ionic component, since the complex also survives in low ionic strength (ion exchange) environments.

The first step is to dissociate the complex. Begin by dialyzing a sample of the antibody versus 1.0 M sodium chloride, and a sub-CMC concentration of your favorite nonionic detergent, in a neutral pH buffer. The 1.0M sodium chloride is to dissociate ionic interactions. Since this salt is an extremely weak promotor of hydrophobic interactions, you don't have to worry about compensatory hydrophobic stabilization of the complex. Buffer exchange chromatography may work, but if the asociation is very strong, dissociation kinetics may be too retarded to allow complete dissociation.

If the problem persists, which is unlikely, there are a couple of semi-exotic things you can try. Add 1.0 M urea to the disociating buffer. Urea is strong hydrogen donor/acceptor and will dissociate hydrogen bonds. At 1.0M it can be used without risk to protein conformation. Another possibility is 1.0M Tris or triethylamine. Both are strong electron donor/acceptors, and will weaken or disrupt pi-pi interactions.

If the complexant doesn't dialyze or buffer exchange out, it suggests that it may simply be too large. Try putting the high-salt/detergent treated antibody on a higher exclusion limit size exclusion gel. Sephacryl 200, ToyoPearl HW55, and Superdex 200 are all candidates.

Assuming that the complex is dissociated by some combination of the above treatments, the next step is to develop a treatment you can integrate with your purification scheme. Size exclusion chromatography is a good option because the fractionation mechanism is independent of the chemical enviroment in which the sample resides. So long as you treat the sample in advance, you needn't include any additives in the running buffer. However, size exclusion is unattractive because of its slowness and low capacity. Retry the ion exchange step, including the nonionic detergent in the buffer. Urea is nonionic and will also be tolerated. With a little luck, the complexant will either be washed through or bind to the column. This brings up the point that if anion exchange doesn't work, try cation exchange. Besides offering the complexant an alternative binding partner, the effects of the pH differential on the titration states of the protein and the complexant may enhance dissociation.

The down-side of binding the contaminant to your ion exhanger is that it may foul the gel irreversibly. In this case you may want to pretreat the antibody with the dissociating formulation in the presence of low-exclusion limit ion-exchange beads. For example, add Dowex AG1X2 (400 mesh) or DEAE-A25 (~1% w:v) to the sample mixture before loading onto an anion exchanger. The exclusion limits are too low to permit inclusion of the antibody, so they selectively bind small contaminants. Simply filter the beads out before sample application. In the case of phenol red, you can actually watch the beads turn color as they scavenge the complexant. Consult your Supelco catalogue for alternatives, including low-exclusion cation exchange beads. Note that if this pre-scavenging approach works, you can omit the dissociating additives from your ion exchange buffers.

Supposing that some variation of the detergent treatment works but you prefer not to expose your product to it, there are a couple of alternatives. Ethylene glycol is a nonionic polarity/surface tension reductant, but unlike detergents, it is excluded from protein surfaces. This means that it is protein-stabilizing and that it can be subsequently removed by either dialysis or buffer exchange chromatography. The stabilizing/exclusion effect is concentration

dependent. At concentrations greater than ~50% (v:v) it begins to bind to protein surfaces and becomes destabilizing. Nevertheless, lower concentrations can be very effective for dissociation of hydrophobic interactions. If you are working with very small proteins or peptides, you can try more agressive organic solvents, but with large proteins, risk of denaturation is a concern. Combination of organic solvents with sodium chloride up to 1.0M suspends ionic interactions without strengthening hydrophobic interactions.

Another option is to add beta-cyclodextrin (BCD) to ~1% (w:v). BCD forms stable complexes with hydrophobic moieties, effectively blocking their hydrophobic contribution to complexation. The reaction may take some time to achieve completion, but the treatment can be very effective. As with organic solvents, combining BCD treatment with up to 1.0M sodium chloride will ensure that the ionic component of the interaction is suspended along with the hydrophobic component.

A point to be aware of is that BCD forms insoluble complexes with a wide variety of lipids. Gross precipitates form upon addition to raw ascites or cell culture supernatant. This necessitates a filtration step immediately before chromatography. Since uncomplexed BCD remains in solution, precipitation continues to occur after filtration. If you are using disposable buffer exchange media, you can simply apply the treated sample -- precipitate and all -- to the buffer exchange column. This removes the free BCD along with the precipitate.

BCD's mechanism of action imposes a potential restriction for injectable products. It also forms stable complexes with hydrophobic side groups on proteins. The net effect is to improve protein solubility, but it also raises validation issues. There is circumstantial evidence that the BCD preferentially transfers to hydrophobic ligands during hydrophobic interaction chromatography, but nothing has been published that could be used directly to support validation requirements.

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Solid gold

Every issue we showcase a product or service that we consider to be an exceptional resource to downstream processing professionals. Our primary selection criterion is the ability to enable significant enhancements in downstream processing efficiency. Along with our reasons for naming a particular product, we'll include hot-links to the supplier's webpage (if they have one) so you can get more information. We welcome suggestions for future columns.

Molecular Devices' Threshold System: An Overlooked Asset in Purification Process Development

Pete Gagnon, VBI

Molecular Devices' Threshold system has been on the market for several years, during which it has developed a solid reputation as an essential validation tool. Some users have recognized its potential as a supporting technology in purification process development as well, and are achieving DNA and other contaminant clearance effectivities far above industry averages. In this review, we suggest that these improvements serve as a model for a broader paradigm shift in development of purification processes for injectable products.

The Threshold system employs a 3-stage ligand assay, in either a sandwich or competitive format, depending on the size and characteristics of the analyte to be detected. In the reaction stage, single-strand DNA is combined with a combination of biotinylated single-strand binding protein and urease-labelled anti-single strand antibody. Streptavidin is added, binding to the biotin. Second, the complex is captured and concentrated by interaction of the streptavidin with a biotinylated membrane. Third, the captured complex is provided with urea, enzymatic conversion of which is measured continuously for 90 seconds by a potentiometric silicon sensor. Software compares the kinetic response with a standard curve and converts the data to metric values. Linear accuracy is maintained down to the low picogram range of total DNA.

Variations on this format allow the Threshold to provide uniquely sensitive and precise measurements for a variety of other anlytes including leached protein A and G, both down to the picogram level; host cell proteins, down to the nanogram level; and media components (such as bovine BSA and transferrin), also down to the low nanogram per mL range. In addition, kits are available with generic reagents and protocols, with which it is possible to develop similarly sensitive assays to any analyte for which you have specific antibodies. As with the DNA assay, accuracy and precision are typically far better than achievable with traditional ELISA formats, usually as good or better than the best isotopic assays.

These capabilites allow the Threshold to fill an important gap in purification process development. Despite the importance of DNA and other contaminants of regulatory signficance, their levels are seldom evaluated as a matter of routine during process develoment. Processes are developed, and their clearance efficiencies assessed secondarily. Figure 1 illustrates a hazard of this approach. In this case, an IgM antibody was forming ionic complexes with DNA and carrying it through the entire purification. Protein purity at the end ot the prep was consistently excellent, but DNA levels were prohibitive. Although this example is itself extreme, the phenomenon it illustrates is widespread. It is not limited to antibodies. It is not limited to DNA. It is practically inevitable in situations where PAGE gels and product activity assays provide the sole method selection and process sequencing criteria.

Figure 2 illustrates the DNA reduction profile of the IgM purification process revised on the basis of data obtained with the Threshold system. These data immediately revealed the nature of the problem, which in turn suggested the types of purification methods best suited to solve it. Subsequent Threshold analysis was equally valuable in identifying the most productive process sequence. Along with bringing DNA contamination under control, product recovery was improved by >25%. These data also provided the backbone of the validation package and significantly reduced the burden of characterizing the finished process.

The Threshold system has 2 limitations. Neither are inherent to the technology itself, but they are important to be aware of. The first has to do with secondary complexation between the product and the contaminant being measured. The same ionic complexation that caused DNA to be carried through the IgM process, caused protein to be carried into the DNA analysis, confounding the assay. Test values were erratic until this was recognized and a method developed to clear the protein in advance of DNA measurement.

Far from being a liability, assay interference of this nature provides an early warning system for critical validation issues, and helps to ensure that validation -- as well as the purification process -- will be completed on time, the first time. Molecular Devices has technical bulletins describing protocols that address protein:DNA complexation, and can assist with refinements as necessary. Note that phenome-

Figure 1. pg DNA per mg product

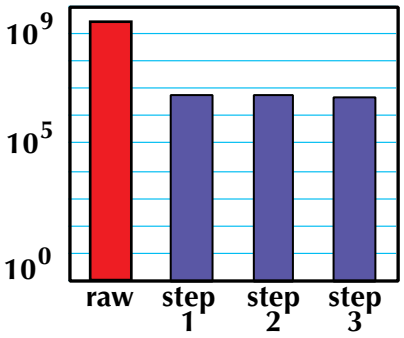
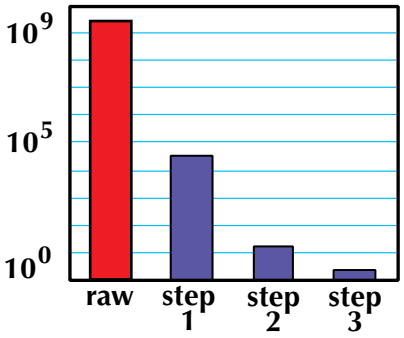


Figure 2. pg DNA per mg product

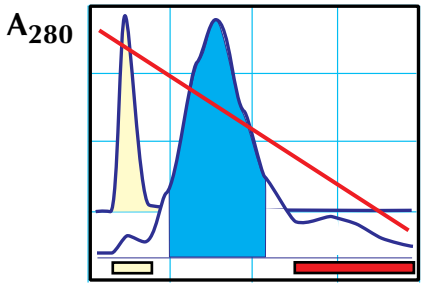


na like this affect most assay formats, not just the Threshold. If you encounter unexpectedly poor sensitivity or reproducibility, regard complexation as a prime suspect and deal with it at the source.

The second limitation has to do with the biotin reagent used for developing custom assays. It includes a patented dinitrophenol (DNP) chromophore that greatly simplifies measurement of the biotin incorporation ratio in the finished conjugate. However, the DNP group also elevates conjugate hydrophobicity, reducing its solubility, and in turn affecting recovery and performance. Strict adherence to Molecular Devices' labelling protocol consistently produces good results with polyclonal antibodies. However, recovery tends to be low and the conjugates must be stored frozen.

Figure 3 illustrates secondary fractionation of a DNP-biotin immunoconjugate by hydrophobic interaction chromatography. This permits removal of uncoupled antibody (upper profile and yellow bar) as well as overcoupled antibody (red bar). The blue zone indicates the fractions with the best balance of activity and solubility. Such fractionation improves signal to noise ratios, sometimes by a factor of 10 or more, and often makes it possible to store the reagent at 4°C. This treatment reduces mass recovery even further, but the mass it removes is mass you're better off without.

Figure 3. HIC fractionation of DNP-biotin-IgG



Another way to avoid DNP's hydrophobicity limitations is to use conventional biotinylation reagents. This sacrifices easy measurement of labelling efficiency, but given equal biotin incorporation, assay performance seems to be equivalent. Even though conventional biotinylation reagents moderate conjugate hydrophobicity, residual uncoupled antibody will still depress assay sensitivity. Secondary fractionation is still required to achieve the best assay performance and stability.

Molecular Devices' labelling protocol is a good place to start with monoclonal antibodies, but monoclonals are highly variable with respect to the number of residues available for coupling. Be prepared to invest more time into reagent optimization. Again, these limitations apply to all assay systems, and not just the Threshold. Fortunately, the skills required to optimize these reagents fall squarely within the mainstream of purification techniques. They certainly poses no obstacle to purification process development groups. Besides which, Molecular Devices sells pre-optimized reagents for most of the assays of primary interest.

Overall, we regard the Threshold as the right instrument, in the right place, at the right time. Cycles of blind process development and redevelopment are a luxury that few companies can afford. Even when they do achieve compliant levels for key contaminants, they rarely exhibit the efficiency of processes developed with the benefit of comprehensive contaminant databases. The Threshold system offers virtually all of the assays of interest in a single application format, with high throughput. It focuses process development efforts where they can do the most good, and supports validation at the same time. If you are looking for one step to take that will make a major fundamental improvement in your process development program, take this one: get a Threshold.

Molecular Devices maintains an extensive library of technical bulletins and literature citations describing applications of the Threshold system. For more information, you can contact them by e-mail at <info@moldev.com>. In the United States, call toll-free at 1-800-635-5577. In Germany, call xx-49-898-545050. In the UK, call xx-44-1293-619579.

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Welcome to the jungle. We invite you to share practical tips on any aspect of downstream processing. We also welcome your observations on downstream processing products and services, based on their ability to either enhance or retard fulfilment of your downstream processing goals.

This column relies on your submissions to make it a valuable resource, so we ask that you turn your awareness up a notch when you're in the lab, and when you encounter something useful, please pass it along.

Improving Metal Ion Strip/Recharge Efficiency with Immobilized Metal Affinity Chromatography (IMAC)

Stripping metal from an IMAC column with EDTA is the intuitive choice for most users, and this approach is frequently suggested by chromatography media suppliers. However, its efficiency varies substantially among IMAC media, and may cause serious capacity-reducing losses with some.

Especially with metal ions like nickel, that have 6 coordination sites, it's possible for a singel nickel ion to be bound at 2 ligand sites. This consumes all of its protein-binding valencies. Published data indicate that up to 30% of the nickel bound to a column may be unavailable for protein binding as a result of this phenomenon. Simple proof of its existence is found in the fact that many columns remain colored even after stripping with high concentrations of EDTA. EDTA is effective for stripping single-site bound metal, but does not compete effectively against dual-site binding.

Another problem with EDTA can occur on columns with very high ligand densities. EDTA can serve as a chelating crosslinker, forming ligand-nickel-EDTA-nickel-ligand complexes, each such event making a pair of bound nickel ions unavailable for protein binding. This has led some gel suppliers to recommend that EDTA never be used with their media. It is especially likely to be a problem if you charge your column without having quantitatively removed EDTA from a previous stripping step.

Better stripping effectivity can be obtained by washing the gel at low pH, for example with 0.1-0.5M hydrochloric acid, or equivalent. Combine the acid with 1.0M sodium chloride to suppress any possible residual ion exchange interactions between the positively charged metal ions and the negatively charged chelating ligand.

Pete Gagnon, Validated Biosystems

Removing Air from Column Nets and Frits.

Air in column nets and frits and frits is more than an anoyance, it can prevent you from obtaining the full capability of the media. This may cause you to overlook an effective process tool, or it may cause serious reproducibility problems.

To prepare air-free column nets, remove the net from the adaptor or endpiece and place it in a Petri dish filled with water. Tap it repeatedly to the bottom of the dish, with a glass rod, until it is free of air. Meanwhile, run buffer through the tubing leading up to the adaptor or endpiece. When the air is cleared, then reattach the net.

A more brutal but often effective approach is to put water in a large beaker with a gently domed bottom. Pump buffer into the endpiece or adaptor. To remove trapped air, gently pound the net surface on the bottom of the beaker. One risk with this approach is that you may detach the net at the edges -- maybe not the first time, but if you use this technique as a matter of routine, the risk increases over time. If you do use this approach, and especially if you use large plastic beakers, check the bottom of the beaker for any ragged plastic protuberances. They are sometimes left over from the injection-molding process. Such protuberances can easily puncture the net.

Some columns have "depth-filter" type frits of processed cellulose, sintered glass, or synthetic composites. Trapped air from these assemblies can be removed by sonicating them in 20% ethanol.

Al Williams, Pharmacia

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