Technical Tips from the Jungle

Validated Biosystems, Inc.

The first two years of this website carried a regular feature called the jungle, in which readers were invited to submit practical suggestions based on their experiences in the lab. Here they are, as they originally appeared. Please note that some mention web addresses and telephone numbers that may no longer be in service.

A "New" Agent for Viral Inactivation.
"New" is in quotes here because this agent has actually been around since 1972. It is sold under the trademark Citicidal. It is synthesized from a natural extract of grapefruit seeds, secondarily modifed to quarternize some otherwise unstable amino groups. The resulting polycationic diphenol benzene reagent is a remarkable microbicide, effective against bacteria, fungi, amoebae, protozoans, and virus. 10 minutes exposure to Citiricidal at a 1:256 dilution reduced Herpes and Influenza virus to undetectable levels. It has also been tested effective by the USDA against measles virus, foot and mouth disease, African swine fever, swine vesicular virus, and avian influenza virus. Another remarkable property of this agent is that its human toxicity is nil. Yet another remarkable property: after viral inactivation, Citricidal is easily removed by passing your sample through a cation exchanger. The reagent is inexpensive, and a search of "Citricidal" on any of the major Net search engines will turn up several bulk suppliers.

Note that Citricidal can also be used as a column sanitizing agent or column preservative -- except for media with a cation-exchange or hydrophobic functionality.
Pete Gagnon, VBI

Avoiding Accidental Inactivation of Protein A/G affinity chromatography Media During Sanitization.
A problem commonly encountered with current generation Protein A and other protein-based affinity ligands, is the use of inadequate rinse protocols prior to sanitization with a base. The common practice for most manufacturers has been to ship these materials in >20% ethanol. EtOH is, however, a surprisingly difficult material to remove, especially from a polymeric base matrix, due to mildly hydrophobic interactions. The result is that it may require 10 or more column volumes of buffer to completely flush the EtOH from the media. Like the hydroxyl proton of water, the hydroxyl proton of an alcohol is a weak acid -- actually an even weaker acid, having a dissociation constant of 1.3x10-16 to -19. Removal of the proton under alkaline conditions creates a very strong base. Once formed, the ethoxide radical (EtO-) is an extremely potent nucleophile capable of an array of potential reactions in the presence of any protein. The result is a protein (as in immobilized protein A or protein G) that is significantly modified and no longer functional. Functionality lost by this mechanism cannot be restored. On a more positive note, do keep in mind that you can turn this phenomenon to your adavantage for enhancement of column sanitization with nonbiological chromatography media.
Terry Mayes, Amersham Pharmacia Biotech

A New Preservative for chromatography media and in vitro diagnostic reagents.
Finding an ideal preservative for chromatography media frequently turns out to be trickier than it really ought to be. The reason is that most of the common preservatives are

fairly hydrophobic and tend to stick to the media, rather than being out in the interstitial space killing microbes. Ion exchangers can also bind preservatives of complementary charge. This is why azide is ineffective on anion exchangers. Sodium Hydroxymethyl Glycinate (HMG) is broad spectrum antimicrobial that offers a useful alternative. It is effective against gram positive and gram negative bacteria, yeasts and molds. HMG is perfectly suited to cation exchangers, size exclusion, HIC, RPC, hydroxyapatite, IMAC, and bioaffinity media. Start at 0.2%. You probably won't need to go any higher, although you can probably go lower. Unlike most preservatives, which become inactive at high pH, HMG maintains its full microbicidal properties even in concentrated sodium hydroxide -- also in detergents. This can provide you with opportunities for enhanced effectiveness during sanitization. HMG is negatively charged, which could cause it to interact with anion exchangers, but inclusion of physiological sodium chloride is generally adequate to prevent this from being a problem. HMG is manufactured under the trademark Suttocide by Sutton Laboratories. It is marketed by International Specialty Products. For a sample, call 1-800-234-6788. From outside the US, call 1-973-628-3305.
Pete Gagnon, VBI

Maintaining consistent effectivity of heter - obifunctional reagents
is an important part of achieving good lot-to-lot reproducibility when making conjugates. One of the obvious things -- that most heterobifunctional manufacturers specify -- is to store them frozen in vacuum dessicators. Even a tiny amount of water can effectively cleave off the functional succinimidyl groups. And, it's important to appreciate that once a given water molecule cleaves off one group, it's free to move on to the next, and the next, and the next... A little water can go ALL the way. Even the hydration water associated with a heterobifuntional can cleave it.. A precaution most manufacturers don't mention is the importance of bringing the container to room temperature before opening it. If you open it cold, condensation will form inside, and the water will wreak havoc on the reagent. Generally it is best to let it come to temperature gradually, sitting at room temperature. If you are in a hurry, warm it up it in your pocket. You won't see this on an SOP, but it's better than opening it cold. This assumes, of course, that the closure is secure. When you do open it, make the opening as bief as possible and return the reagent to the dessicator as quickly as you can. Even with precautions, many users prudently specify that a given container must be discarded after five openings, no matter how much reagent remains. Some users specify three. Obviously, it's usually advantageous to purchase the reagent in the smallest containers possible.

Another frequent cause of heterobifunctional-related inconsistency comes from the way you solubilize it in your working solution. Many people solubilize the reagent first in DMSO and then add that solution to their aqueous protein solution. The problem here is that DMSO has a fairly short shelf life. Sniff a freshly opened bottle and then compare that to one that's been sitting around for a week or so at room temperature. Bad DMSO breaks down heterobifunctionals with incredible speed. You may, for example, see your coupling efficiency drop 50% or more. Buy your DMSO in small containers and set very short expiration dates for opened containers -- then throw them away. The expense of the discarded DMSO is trivial compared to the expense of a blown batch of conjugate. DMF has better handling characteristics but many users don't like the relatively higher toxicity. Most people avoid alcohol because of the potential detriment to protein.
Pete Gagnon, VBI

Simplifying and improving sensitivity when scanning slab gels

Even the most experienced operators of slab-gel dryers for polyacrylamide PAGE or IEF gels occasionally have gels that crack and fragment during drying. For the rest of us, this -- along with curling to various degrees -- is an all too frequent annoyance, often requiring gels to be rerun. An easy way to avoid this is to digitize the gels on a flatbed scanner. Lay the wet gel carefully on a sheet of transparency film (the kind you use to make overhead projections) then starting at one corner of the gel, lay another transparency sheet on top of it, squeezing out the bubbles as you go. Set this "protected" gel on your scanner, then set a sheet of yellow paper on top of it. The yellow is complementary to the blue (assuming you've used coomassie stain) and improves sensitivity in weakly stained areas (this assumes you're using the grayscale option for the scan). You can experiment with resolution, brightness and contrast settings to get a "true" reproduction (as judged by the screen image), then simply store the gel to disk in whatever format will make it most available to people that need to reference it. Do note that even small gels may require a hefty amount of disk space, so if you decide to implement this approach on a broad basis you may want to invest in some type of high-capacity storage device. Whether or not you obtain a high capacity storage system, you can reduce the file size substantially with a data compression package. As hinted above, also stick with grayscale. Color scans produce much larger data files. One final note, a nice thing about these digital gel-files is that you can make back-up copies for regulatory archival purposes. They'll last a lot longer than any dried gel. And -- if you really want to dry the gel, you can still do so after it's been digitized.
Pete Gagnon, VBI

Increasing column life of prepacked columns

No matter how careful you are with maintenance of your prepacked HPLC columns, they eventually foul and become unusable. For columns with adjustable adapters you can often open the column, scrape off the top 2-5mm of gel, reset the adaptor, and go on about your business. However, adapters have limits, so that in most cases you can only get away with this a time or two before you've lost so much media that you have to retire the column. One way to extend column life after you've come to the limit of the adapter is to replace the removed gel with an inert media like Sephadex G-25 or equivalent. This bypasses the adapter limitation indefinitely. This approach also works with continuous bed columns, like the UNO product line from Bio-Rad. Clearly, there are some limits to this approach, but if you find yourself halfway through a critical series of runs, your column fouls, there's no back-up in the lab, replacement columns just happen to be out of stock for two weeks, and your deadline is in 48 hours (or less), this may just save the day. It also protects the remaining gel -- at least to some degree -- from additional fouling.
Pete Gagnon, VBI

Enhancement of DNA detection by Molecular Probes PicoGreen Reagent

The previous issue of this newsletter carried a review of Molecular Probes new PicoGreen reagent for sensitive detection of DNA. We have found that both sensitivity and accuracy are improved by adding 0.1% SDS to the samples. Using black -bottom fluorescence microtiter plates also improves sensitivity. (Visit Molecular Probes website at <http://www.probes.com>)
Amy Dingle, BioGen

Reagents for virus, DNA, and lipid precipita - tion
LigoChem has recently made available a range of soluble or supended polymer reagents for selective precipitation of various solutes. Viraffinity is used for either removal or purification of enveloped and non-enveloped viruses. Product literature notes that even protein-complexed viral particles are adsorbed. Viraffinity can also be used for Lambda DNA isolation. Cleanascite is a general delipidating agent, effective with biological samples of all types, including serum, ascites, and egg yolk. Loss of IgG upon treatment with Cleanascite is reported to be less than 5%. Other products are available for precipitation of hemoglobin and for general protein precipitaiton.
E-mail: <email@ligochem.com> or call 1-800-935-0628.
Pete Gagnon, VBI

Reagents for monitoring biotinylation
One of the perennial shortcomings of biotinylation is that biotin itself is optically invisible in the range of wavelengths that support easy characterization of biotin:protein (B/P) ratios. This problem has been overcome with the development of reactive biotin reagents that incorporate a patented dinitrophenol chromophore (Molecular Probes, Eugene, OR). However, although the chromophore supports easy characterization of B/P ratios, it adds so much hydrophobicity to the conjugates that it reduces conjugate recoveries and tends to elevate nonspecific interference in the assay. These limitations can be ameliorated, but it requires development work.

Dyes that interact with the biotin-binding site of avidin provide a better alternative. The fluorescent dye 2,6-anilinonapthalene sulfonate (Molecular Probes) and the UV-absorbent dye 4'-hydroxyazobenzene-2-carboxylic acid (HABA, Pierce Chemical, Rockford, IL) both bind the biotin site on avidin. With HABA, this increases absorbance at 500nm. Biotin, whether free or resident on a conjugate, displaces HABA from the avidin. The consequent reduction in absorbance at 500nm is proportional to the number of such displacements. The fluorescent dye operates by the same principle.

These dyes overcome another important limitation with characterization of biotin conjugates. The biotin-binding pocket on avidin is deep within the molecule, and the position of biotin on irregular protein surfaces may preclude unrestricted interaction between the reactants. B/P ratios are therfore likely to overestimate biotin complementarity to avidin, sometimes grossly. Since HABA and the fluorescent dye operate by biotin-mediated dye displacement, they allow detection only of avidin-accessible biotin moieties, thereby providing an accurate model of how the conjugate should behave in the intended assay system. If you want to determine absolute B/P, you'll have to digest the conjugate with pronase, DNAase, or other enzyme as appropriate, then assay for the no-longer encumbered biotin.
Pete Gagnon, Validated Biosystems

Valuable New References for Biomolecule Conjugation and Labeling.
I've recently reviewed 2 new references and found them to be outstanding resouces for anyone doing labeling and conjugation. Bioconjugate Techniques by Greg Hermanson is a remarkably thorough and systematic treatment of the field. It is heavily diagrammed for the benfit of non-hardcore chemists who nevertheless want to understand the mechanisms. It provides detailed protocols and sources of reagents to get you started in the area of your choice. Extensive referencing will help you dig deeper into subjects of interest. Bioconjugate Techniques (ISBN 0-12-342336-8) is available from Pierce Chemical Company, Rockford, IL for $50.00. Don't leave home without it.

The other reference is the newly released 6th edition of Richard Haugland's Handbook of Fluorescent Probes and Research Chemicals, available free from Molecular Probes, Eugene, OR. This volume doesn't have the mechanistic information or protocols of Bioconjugate Techniques, but the incredible diversity of thoroughly and clearly explained applications makes it a must-have resource all the same. Topics covered include Fluorophores and their amine-reactive derivatives, Thiol-reactive probes, Reagents for modifying groups other than thiols or amines, Biotins and haptens, Crosslinking and photoreactive reagents, and 22 more categories. This Handbook also doubles as Molecular Probe's Catalog, which far from being a commercial intrusion, simplifies acquisition of all the cool new reagents you'll be dying to try.

You can get a preview of this reference by visiting Molecular Probes website at <http://www.probes.com>. Site contents largely parallel contents of the book.
Pete Gagnon, Validated Biosystems

A New Website for On-line Antibody-Related Resources.
If you have an interest in Antibodies, be sure and visit The Antibody Resource Page: <http://www.antibodyresource.com/> This is unquestionably the best gateway site we've found to all aspects of immunobiology on the Net. It links to some 40 individual sites showcasing a wide range of educational resources, at least 60 sites representing suppliers of immunological reagents, several searchable on-line databases, and a range of other web-based resources.

If you have any gossip-mongering tendencies,
be sure and visit The Biotech Rumor Mill at <http://www.biofind.com/rumor/>.

Improving metal 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 single 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 your gel 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 re-attach 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

Unpacking high density chromatograph y media.
Some older media, like hydroxyapatite, have very high densities. The same is true of many newer media, including Bioprocessing Ltd. controlled pore glass supports and fluidized bed media. High particle density facilitates consistent high quality packing, but it defeats the methods normally used to unpack most polymer beds. A simple effective way to unpack these beds is to fluidize them by pumping water in from the bottom. Once the bed is suspended, you can decant the media from small columns or pump it out of larger ones.
Anne Moschella, Bioprocessing Ltd.

Affinity chromatography media for non-IgG antibodies.
Although there are dozens of products for affinity chromatography of IgGs, options for other classes limited to say the least. This is unfortunate because besides being useful for purification, affinity can be a good tool for monitoring expression levels during bioproduction and for obtaining small amounts of reference material to use as a marker when developing nonaffinity purifications.

Fortunately there are a few products that address this void. Windsor Park Laboratories (Teaneck, NJ, 201-833-4424) makes a mouse IgM purification kit based on an undisclosed biological ligand. Pierce Chemical Company (Rockford, IL, 800-8-PIERCE) markets a kit based on immobilized jacalin for purification of human IgA, and another based on immobilized mannan-binding protein for purification of mouse IgM. Immobilized RCA-1 (Ricinus communis lectin-1) binds human IgM. If you're in the unfortunate situation of having to purify intact mouse IgD, affinity chromatography on immobilized GS-1 (Griffonia simplicifolia lectin) will likely be your best option. Like RCA-1, it can be obtained from both E-Y Laboratories (San Mateo, CA, 415-342-3296 and Vector Laboratories (Burlingame, CA, 415-697-3600). E-Y also sells immobilized jacalin for IgA purification and immobilized GNA (Galanthus nivalis agglutinin) for mouse IgM, but neither their nor Vector's products include kits or application buffers -- which

you will quickly discover can take a significant amount of time to research and perfect. Keep in mind too that protein A will bind up to 30% of IgA and up to 60% of IgM monoclonals.

One thing you need to watch out for with all these products is ligand leaching. Biospecific ligands for antibodies are notorious for their immunomodulatory effects, and potent at incredibly low concentrations. If you're trying to obtain antibody for in vivo evaluation or flow cytometry with mammalian cells, you need to clear any leached ligand first. This is a fairly simple process for most lectins. Bind your harvested antibody to an ion exchanger and wash thoroughly with an excess concentration of the lectin-eluting agent. If the eluting agent happens to be charged, use an exchanger with the same charge or use hydrophobic interaction. Make sure that you obtain some pure lectin and characterize its elution behavior relative to your product to make sure that they don't coelute.
Pete Gagnon, VBI

Addendum:Sterogene Bioseparations (Carlsbad CA, 800-535-2284) markets a line of species-specific anti-light chain affinity media for antibody purification. These media can be used with antibodies from all classes.
Bruce Hoffman, Sterogene

Comparison of In-line Fluorimeters for high sensitivity HPLC

The option of fluorescence monitoring is a useful one in chromatography. I wantto compare the Waters 470 with the PerSeptive Fluor-304 detector for analyzingfluorescent compounds at extremely low concentrations. The added sensitivity,with or without accompanying UV data, can be a powerful aid to analysis.

The Waters features excitation and emission ranges from 220-700 nm, to whichI've added an enhanced far-red PMT. The PerSeptive range is 220-650 nm -- I'veadded the optional PMT for emissions to 800 nm. Both offer scanning andsmoothing capabilities, wavelength accuracy to 2 nm and outputs from 10 mV to 1V full-scale.

The PerSeptive Fluor-304 shows off its more modern computer capability.Programs can be written with more sophistication than the Waters 470, filesnamed, stored and queued up onboard. Adjustment of lamp output has been addedto the gain and attentuation controls for signal processing.

In contrast, Waters' user-friendly design stands out. For a basic workhorsefluorometer, I might forgo the bells and whistles for the more logical 470 layout.Sensitivity is comparable, and you'll spend less time with the manual beforeyou're up and running. It's also quieter, leaving you in peace to ponder your glowing new insights.
Don Ladd, Becton Dickinson Immunocytometry Systems

Centralized database for contract service providers.

I've recently been seeking a contract service provider to help us produce and purify an assortment of monoclonal antibodies for our clinical evaluators. The first problem has been just identifying the candidates. The second problem has been trying to obtain an indication of their range of services and relative competence. For example, what production capabilities are they highly competent with, what purification technologies, is their facility validated, do they have people on staff with experience in the manufacture of products for the end-user market or are they just a Ma & Pa operation looking to cash in on their rodent breeding factilites?

A centralized database would be really helpful. Lacking that, I'd like to see service providers put out some technical literature that accurately reflects their abilities. Say it in

numbers. Forget the fancy 4-color marketing hype. Some of you may be concerned that this will give me a reason to disqualify you without talking to your sales people -- and you're right. I want the best service I can get but I don't have the luxury of a lot of time to investigate every possibility. Besides, if you think being rejected in advance is bad, I assure its nothing compared to the publicity you'll receive if I invest my time and resources only to find you're not up to your sales promises.
Name withheld

Mixed-mode hydrophobic/ion exchange chromatography media.
I would like to see someone introduce a series of hydrophobic ion exchangers. This wish is based upon significant and useful variations in selectivity I've seen among ion exchangers on different materials, or with different spacer arms, that I believe to be conferring different levels of hydrophobicity on the surface of the support. I seem to recall that Rainin at one time introduced such a line on silica. I would much prefer to see these products on a polymer-based media. In my perfect scenario, I'd like to have a series of cation exchangers with perhaps 3 different hydrophobicities: nil, moderate, and strong; and a set of anion exchangers to match. I personally believe that you gel manufacturers are missing an opportunity to help us do our jobs better here. My primary interest is preparative, so if someone out there decides to follow up on this, please plan to introduce a line of products that support both process development and large scale requirements.
Pete Gagnon, VBI

This article was downloaded from Validated Biosystems Quarterly Resource Guide for Downstream Processing
http://www.validated.com

©2002, Validated Biosystems, Inc.