Ask *"The Particle Doctor®"*

A compilation of the Questions You've Asked over the years.

Bangs Laboratories, Inc.

BEADS ● ABOVE THE REST

Alexa Fluor®

Quantum™ MESF

Quantum™ Simply Cellular®

Volume 24, #1
February 2011

Q: I need to calibrate the fluorescence of cells labeled with Alexa Fluor® 555. How should I go about doing this; is it possible to use one of your Quantum™ MESF kits, perhaps with some sort of conversion factor?

A: In addition to quantitative kits for standard flow cytometry fluorochromes (FITC, PE, PE-Cy™5, etc.), we currently manufacture Quantum MESF products for Alexa Fluor 488 and Alexa Fluor 647. Unfortunately, these wouldn't be suitable for Alexa Fluor 555, as the same fluorochrome must be presented on beads and cells for quantitative determinations

However, if you are working with antibodies, our Quantum™ Simply Cellular® (QSC) kits may offer a solution. QSC bead populations are coated with increasing amounts of capture antibody that the user then labels with the specific fluorochrome-conjugated primary antibody (or indirect staining may be conducted if a labeled primary mAb isn't available). Bead populations are calibrated in terms of their Antibody Binding Capacity (ABC), or the number of primary antibodies that they will bind. When the standard curve is drawn (in QuickCal®, provided with the kits), ABC assignments may be made to labeled cell populations. If monovalent binding is assumed, then the ABC value equals the number of surface receptors. QSC kits are available for mouse, rat, or human monoclonal primary antibodies.

Catalog #	Description
488	**Quantum™ Alexa Fluor® 488 MESF**
647	**Quantum™ Alexa Fluor® 647 MESF**
815	**Quantum™ Simply Cellular® anti-Mouse IgG**
816	**Quantum™ Simply Cellular® anti-Human IgG**
817	**Quantum™ Simply Cellular® anti-Rat IgG**

Sterilizing Microspheres

Volume 24, #1
February 2011

Q: I am hoping to find sterile microsphere preparations for use in an animal study. Do you offer sterile suspensions?

A: While we don't offer sterile microsphere preparations as standard products, microspheres may be sterilized through irradiation or 70% alcohol incubation. It's also possible to conduct stringent washes in sterile medium or pasteurization if the need for a sterile suspension isn't absolute. Our Product Data Sheet 726, *Decontaminating Polystyrene Microspheres*, offers a number of decontamination protocols and may be downloaded from the Technical Literature section of our website (www.bangslabs.com/literature/pds).

BioMag®

COMPEL™

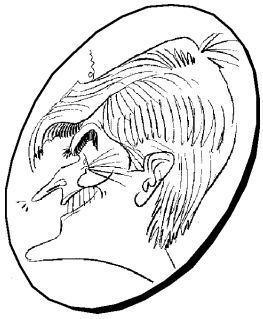
Magnetic Microspheres

ProMag™

Volume 24, #1
February 2011

Q: I'm in need of magnetic microspheres that can be coated with antibody. What options do you have?

A: We offer three different lines of superparamagnetic microspheres – ProMag™, BioMag®, and COMPEL™ – which allow us to uniquely address a wide range of applications, from cell separations and immunoassays to flow cytometric suspension arrays. While each type can be used for varied purposes, we do find that each type offers particular advantages to certain applications. As highly uniform microspheres with high coating capacities and rapid separation rates, ProMag (1µm, 3µm) offer significant benefits for automated immunoassays. BioMag are ~1.5µm high-performance microparticles that are widely used for the efficient separation of cells and purification of biomolecules. The irregular morphology provides tremendous surface area, resulting in high binding capacities and efficient capture of target with conservative use of particles. COMPEL are highly uniform polymer-based microspheres (3µm, 6µm, 8µm) that have size and autofluorescence profiles that are ideal for applications in flow cytometry. The polymer matrix is conducive to dyeing, and standard red and green fluorescent versions are available. Of course, we are always prone to waxing philosophic about our microspheres, and you can find images and more on the characteristics and benefits of each type in TechNotes 102 and 102A on magnetic microparticles. These TechNotes can be found at www.bangslabs.com/literature/technotes.



Antibody Coating

Q: I'm interested in your new pre-activated ProMag™ Bind-IT™ microspheres, however, my process includes an elution step that I'm afraid will be detrimental

ProMag™ Bind-IT™

Volume 23, #2
September 2010

to the antibody coating. Can you address ProMag Bind-IT's resistance to harsh conditions?

A: ProMag Bind-IT microspheres offer a system for highly stable, non-covalent coating of antibody, and we have achieved lengthy stability periods when coated microspheres are stored under normal conditions. Though most applications don't call for re-use of antibody-coated beads after an elution step, we have conducted limited testing, and found that transitory exposure to low pH (2.8) and high pH (10) elution buffers has little or no effect on mAb-coated ProMag Bind-IT beads. We also found that protein-coated Bind-IT beads tolerate boiling in 2% SDS for 10 minutes with only a very small loss of protein detected in silver-stained gels. Ultimately, we believe that they should hold up under normal elution steps. However, we still encourage you to be as kind as you can to them, and of course test the specific conditions both to ensure suitability of the immobilization strategy and to determine effects of the process on the mAb itself – it probably doesn't matter that the protein remains stably bound if it's being denatured.

Binding Capacity

Binding Efficiencies

Biotinylated Proteins

Streptavidin-Coated Microspheres

Volume 23, #2
September 2010

Q: I'm trying to coat a biotinylated protein onto your streptavidin-coated microspheres, however, I'm having trouble getting the expected amount bound. Do you have any ideas of what I could be doing wrong? Are there any tips you could share with me?

A: Firstly, don't despair – a couple of common issues come to mind, and they're generally easy to fix. As a first step (well, second step if we count the not despairing part), you should ensure that the beads are being washed sufficiently prior to coating. The as-supplied storage buffer contains a blocking molecule and other stabilizers that could reduce binding efficiency. As a matter of course, we suggest a few pre-washes (3X - centrifuge, decant, resuspend in buffer) to remove these prior to coating with the biotinylated ligand. You will also want to ensure that your binding buffer is free of (or contains only minimal amounts of) potential interferents such as blocker, surfactant, etc.

I'll also note that we use a biotin-FITC assay to determine binding capacity. As a small conjugate (831 Da), biotin-FITC is able to efficiently access streptavidin binding pockets. The capacity of the beads for biotinylated protein will be somewhat subject to steric effects, i.e. as we expect the far larger protein to mask binding sites that would be accessible to small molecules. Using the "Surface Saturation" equation that is provided in our TechNote 206 will probably give you a better estimate of the amount of protein that can be coated onto the surface, and adding some amount more than this will aid in achieving saturation.

If your protein is of gargantuan proportion (I'm thinking IgM-scale), you might consider biotinylating it with a reagent that incorporates a spacer, such as some of the "long chain" tethers that are available.

Pass / Fail Criteria

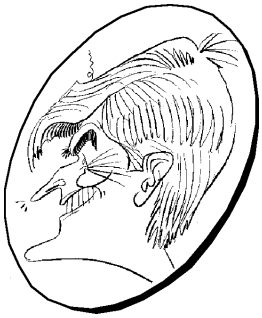
QC Program

ViaCheck™ Viability Standards

Volume 23, #2
September 2010

Q: I just purchased some of your ViaCheck™ Viability Standards and Concentration Controls for use with my Vi-CELL™ and CEDEX analyzers. I see the reported values for viability and concentration on the products' Certificates of Analysis, however, I'm not sure what I should be using as pass/fail criteria when I run them on my instruments.

A: Certificates of Analysis for ViaCheck products provide formal lot-specific values for concentration (and viability). We don't offer firm pass/fail criteria for customer runs as these need to be established by each facility, taking historical instrument performance and study objectives into consideration. Users will often base their pass/fail criteria around the formal result that we report, for example, +/- 10% of our reported viability value. We would encourage you to use several runs over time (and on multiple instruments, if you have them) to establish your specifications. This will allow you to get to know your instruments (their capabilities and any quirks) and to set meaningful specifications.



Carryover

Flash Red

Fluorophores

Q: A number of our fluorescent beads seem to emit throughout the spectrum (including orange and red regions), even though their primary emission is supposed to be in the green. Why is that, and what can we do about it?

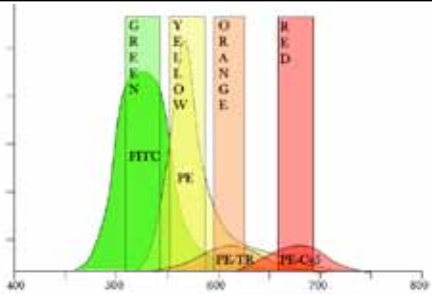
A: This is carryover. It's what's known as "too much of a good thing," and all is lost. (Kidding!)

Glacial Blue

Volume 23, #1
April 2010

To a greater or lesser extent, broad emission, or fluorescence carryover into other (unintended) regions of the spectrum is characteristic of all fluorophores. It is particularly evident with highly sensitive instruments such as confocal microscopes and flow cytometers. It probably wouldn't be apparent with a standard fluorescence microscope, and if you've got one of those black lights, well, you have nothing to fear, as you won't see anything except your white T-shirt and tennies. (Did someone say dance party??)

Remedies might include changing the filter sets (for example to more stringent bandpasses) or the fluorophore itself. Fluorophores in the middle of the spectrum, e.g. with blue or green excitation maxima, tend to have significant carryover into regions that have historically been used in detection of reporters in bioassays, e.g. green, orange, red. It's not that the carryover is more severe with fluorophores in this region, rather it's occurring at an inconvenient place. We have observed that a number of UV/Violet (e.g. Glacial Blue) and Red (e.g. Flash Red) fluorophores tend to have little carryover into the green and orange, and might be better suited to your study if you need low background in these regions of the spectrum. As a final note, another strategy would be to use off-peak excitation, which will reduce fluorescence output, including the carryover signal.



Fluorescence carryover is the region of overlap of emission spectra.

Antibody Binding Capacity

Antibody Weights

Quantum™ Simply Cellular®

Volume 23, #1
April 2010

Q :I just purchased **Quantum™ Simply Cellular®** microspheres for the first time, and want to be sure that I'm using the correct amount of antibody for labeling – what is your recommendation?

A : As **Quantum™ Simply Cellular®** microspheres are intended for quantitative analyses, it is imperative that the beads are stained to saturation. We recommend that an antibody titration be performed for the beads to determine optimal antibody concentration – bear in mind that the amount of antibody needed to saturate the beads may be different than the amount that is appropriate for the cells.

If the antibody concentration is not reported for the conjugate, I would suggest contacting the supplier. They should be able to provide this value, which will aid in establishing the range of concentrations to be used for the titration. Keep in mind, however, that the reported antibody concentration may include the fluorochrome, and a weight-based concentration of PE-conjugated antibody (MW of PE ~280,000 Da) will mean far fewer antibodies on a number basis than the same amount of FITC-conjugated antibody (MW of FITC ~389 Da).

Certified Blank™

Protein A Microspheres

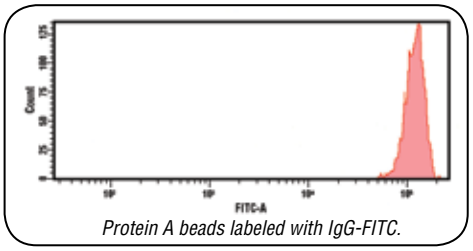
Protein G Microspheres

Simply Cellular® anti-Mouse Compensation Standard

Volume 23, #1
April 2010

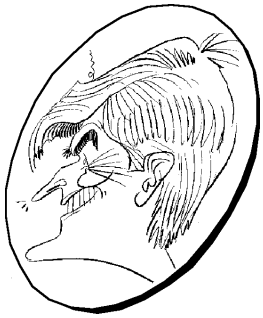
Q : I need compensation beads for flow cytometry, and currently use your Simply Cellular® anti-Mouse Compensation Standard. Do you offer anything for the direct binding of goat, hamster, or rabbit antibodies?

A : Though we don't offer any anti-goat, anti-hamster, or anti-rabbit IgG standards, per se, we now offer single population Protein A and Protein G microspheres that are suitable for binding a range of antibodies, and may be used as reference beads for flow cytometry. Protein G is a strong binder of goat and rabbit antibodies, and Protein A binds hamster antibodies. You may use an unlabeled population or our Certified Blank™ microspheres with the labeled population for compensation purposes.



Protein A beads labeled with IgG-FITC.

Cat. #	Description
553	**Flow Cytometry Protein A Antibody Binding Beads**
554	**Flow Cytometry Protein G Antibody Binding Beads**
890	**Certified Blank™ Reference Standard**



Aggregation

Bangs Bead Buffers

Bangs Bead Solution

Volume 22, #3
November 2009

Q: Quick question. How does Bangs Bead Solution impact subsequent coating – for example, will I need to do a lot of washing to remove it before coating? Will beads aggregate after washing?

A: The **Bangs Bead Solution (SOLN1)** is a good dilution and storage solution for uncoated polymer and magnetic beads. It contains an antimicrobial and stabilizers, and beads should be washed out of it before coating to avoid interference with binding. Washes may be performed in a generic buffer such as PBS or whatever buffer will be used for the next step in the coating process, such as our **Bangs Bead Coupling Buffers (BUFF1-BUFF4)**. As with any wash steps, if these (which effectively reduce the concentration of stabilizers) lead to aggregation, a small amount of stabilizer may be added back in to treat stickiness. The suspension may also be vortexed or carefully sonicated to disrupt aggregates.

For more on washing in general, download TechNote 203, *Washing Microspheres*, from the Technical Literature portion of our website.

Charge Groups

Hydrophobicity

Polystyrene Microspheres

Surfactants

Volume 22, #3
November 2009

Q: As polystyrene is a hydrophobic material, how are polystyrene microspheres stabilized?

A: Polystyrene microspheres in general are stabilized through endogenous charge groups and surfactants:

Charge Groups: PS beads are often synthesized with use of a charged (e.g. sulfate-based) initiator which will impart residual groups (e.g. SO4-) on the surfaces of beads. Beads that have been specifically functionalized (as PS-COOH, which will have a carboxyl monomer co-polymerized with styrene) will also have that charge group on the surface.

Surfactants: PS beads are commonly synthesized in the presence of surfactant, which is essentially a wetting agent. Surfactants are polar molecules with hydrophobic and hydrophilic ends. The hydrophobic tail associates with the hydrophobic bead surface; the hydrophilic head is presented to the aqueous environment. Charged surfactants, like SDS, are often used, which provides charge stabilization in addition to the wetting function. There will be residual surfactant from the synthesis itself, and additional surfactant would be used to treat sticky/aggregated suspensions.

Coupling Strategies

Lipopolysaccharides

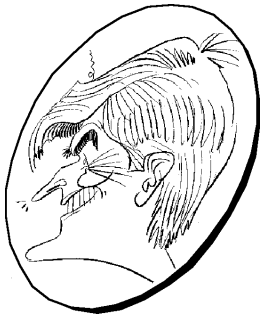
Volume 22, #2
May 2009

Q: I have tried to conjugate lipopolysaccharide (LPS) to carboxylated beads with no success. Any ideas as to what the problem could be? Could you share a proven protocol?

A: Lipopolysaccharides often require specialized coating strategies as they typically lack needed reactive groups for coupling (at least in their native states). Most LPS immobilization schemes feature modification of saccharide moieties for covalent binding. There are also chemistries that are more broadly applicable, such as the use of epoxide-containing reagents, oxidation, etc., though you may want to consider the benefits / drawbacks of each (see *Bioconjugate Techniques*, ISBN: 0-12-342335-X). Polysaccharides may also be immobilized via their affinity binding partners (lectins), however, these are reversible interactions. If the polysaccharides could be biotinylated, they may be (for most application conditions) permanently immobilized to streptavidin-coated beads.

Another option might be to adsorb LPS to microspheres, either through hydrophobic tails of the lipid to non-functionalized polystyrene microspheres, or the hydrophilic region to silica microspheres. However, for adsorbed coatings in general (and particularly where beads will be stored in suspension), shelf life should be considered.

Once you've had a chance to consider the specific structure of the LPS molecule, and factors such as required stability and development time frame, we can provide references associated with a fitting coating strategy.



Flow Cytometry

Q: I'm familiar with the use of polymer beads in flow cytometry, both as instrument QC and set-up standards, and for bead-based assays. However, in looking through

References

Silica Microspheres

Volume 22, #2
May 2009

your catalog, I see that, while you carry silica microspheres, you don't offer silica bead standards for flow. Out of curiosity, are there any known applications for silica beads in flow cytometry?

A: We are delighted that you asked! By happy coincidence, flow cytometry and silica microspheres are two of our favorite things. (Uncanny, isn't it... ?)

Because of silica's unique optical and physical properties (e.g. low autofluorescence and hydrophilic surface), it has been used as an alternative to polystyrene for certain applications in flow cytometry.
For example:

• Silica exhibits less autofluorescence than does polymer when excited with a UV or violet laser. In fact, we featured NH_2-modified silica in our newsletter as a potential substrate for the binding of amine-reactive dyes that are often used in flow, i.e. for user-created reference / compensation standards (see "Amines to an End," July 2008).

• Silica has been used to support lipid bilayers in the creation of "artificial cells" to study things like membrane receptor / ligand dynamics via flow cytometry, and for specialized biosensing applications. For specific examples, see:

Lauer, S., B. Goldstein, R. L. Nolan, J.P. Nolan. 2002. Analysis of cholera toxin-ganglioside interactions by flow cytometry. *Biochemistry*, 41(6):1742-1751.

Zeineldin, R., M.E. Piyasena, T.S. Bergstedt, L.A. Sklar, D. Whitten, G.P. Lopez. 2006. Superquenching as a detector for microsphere-based flow cytometric assays. *Cytometry A*, 69(5):335-41.

• Silica microspheres have proven to be an excellent support for oligonucleotides in hybridization-based assays. The silica surface is negatively charged, which is helpful for deterring the nonspecific binding of DNA. Silica is also highly hydrophilic, and nonspecific binding of proteins (which largely relies on hydrophobic interactions) is less than that seen with many polymer-based beads.

These are just some of the silica microsphere applications that have made an appearance in flow cytometry, and we're sure that we'll continue to see more as investigators explore its unique properties.

Fluorescent Beads

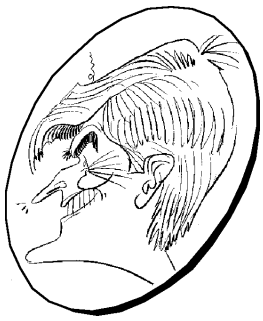
Mounting Mediums

Volume 22, #1
January 2009

Q: I'm having trouble seeing my fluorescent beads after mounting them on a slide. Any ideas as to why this is, or what I can do to prevent it?

A: So, it's lights out, eh? (Sorry, that wasn't punny at all...)
Getting down to business.... Many fixatives, mounting media, and adhesives have components that act as solvents. Organic solvents will swell the polymer matrix, and allow release of fluorophore. A water-soluble mounting medium (e.g. **Mowiol® [Catalog #17951**]. **Aqua Poly/Mount [Catalog #18606] - Polysciences**) should resolve the problem. In fact, aqueous mounting media are used with fluorescent microspheres in the production of **Polysciences' Confocal Multifluorescent Adjustment and Calibration Kits (Catalog #24016**).

Other alternatives include using surface-labeled beads (as in our flow cytometry line, though these won't be as bright as internally dyed beads, i.e. fluorescence intensities are nearer to those for stained biologic samples) or beads synthesized using fluorescent monomer (see our sister company's [**Polysciences'] Fluoresbrite® PolyFluor® Microspheres**). Please note, however, that the polymer base bead will be susceptible to the effects of solvent, so there may be diminished signal.



Antibody Coating

Carboxylated Beads

Coupling Process

Q: I just purchased carboxylated beads, which I have been coating with antibody. Though I have little experience with this, I have had continued problems with reproducibility, sometimes achieving good results, and other times experiencing very low coupling. What can I do to improve my results?

PolyLink Protein Coupling Kit

Volume 22, #1
January 2009

A: Well, first you must stand on one foot... (Sigh. The jokes just don't seem to get any better, do they...?) As a general recommendation, you should rigorously standardize all aspects of the coupling process – both the written protocol and its execution. Though this seems obvious, it's important to take a critical look at your process (including washes, reagent addition, incubation and mixing steps, etc.) and raw materials (reagents, base beads, buffers, etc.). Seemingly small deviations or inconsistencies can show themselves through variability in your results.

If you're confident that reagents and protocols have been consistent, I would suggest examining the EDAC (activator), which should have the appearance of a free-flowing white powder. EDAC is extremely hygroscopic, and will absorb water with disastrous results. Persistent clumps are evidence that the reagent has been contaminated with moisture, and it should be discarded and fresh EDAC obtained. New vials of EDAC should be stored desiccated at -20˚C, and warmed to room temperature in a desiccator before opening to avoid condensation. If possible, the headspace may be flooded with argon before the vial is re-sealed and stored.

If you haven't settled on a specific protocol, you could use our **PolyLink Protein Coupling Kit (Catalog #PL01N)** as a (new) starting point from which to optimize. Our PolyLink coupling kit features standard EDAC-mediated coupling chemistry, and includes a good general protocol (see Product Data Sheet #644).

Affinity Interactions

Crosslinking

Protein A Microspheres

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January 2009

Q: I'll be coating your 5.5µm Protein A-coated beads with IgG, but would prefer to forego the crosslinking step with DMP. Do you think that the IgG coating will be stable without the crosslinking? Can I count on the coating stability to be like that of covalently bound protein?

A: The affinity of protein A for IgG varies by antibody host species and subclass (see a chart in TechNote 101). This means that, without crosslinking, the beads should be used in an environment that is otherwise antibody-free. As an affinity interaction, it may be susceptible to competitive binding (dissociation of the intended antibody through competition with Abs in the sample). You will also want to consider the inherent stability requirements of the application in addition to the desired shelf-life. For example, for quantitative assays, extended stability, or if target is to be eluted, I would suggest crosslinking. If the beads simply need to capture target for a qualitative application, and will be used to fulfill a short-term objective (i.e. a lengthy shelf life isn't required), then crosslinking may not be so important.

Flow Cytometry

Quantum™ Simply Cellular®

Surface Marker Expression

Troubleshooting Tips

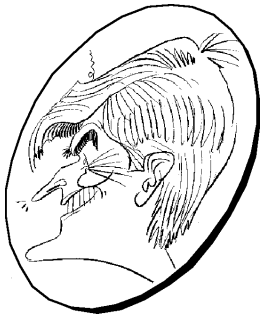
Volume 21, #2
July 2008

Q: I purchased your **Quantum™ Simply Cellular®** beads to evaluate surface marker expression. However, I'm new to this product and flow cytometry in general, and am a bit nervous about performing these analyses. Can you offer any tips beyond the standard protocol? Any common pitfalls I should avoid?

A: First, let us say: Welcome to Flow Cytometry! We like it, and we're sure that (if not immediately, then in time) you will, too.

If your institution has a core flow cytometry facility, you're in luck, as you'll have access to experts in sample handling, instrument operation, etc. Quantum™ Simply Cellular® products are somewhat specialized and do presume a basic proficiency in these techniques, much of which will translate to the use of antibody capture beads.

However, if you're own your own (and even if you're not), we wouldn't think of abandoning you! We are happy to provide you with additional suggestions up front, as well as troubleshooting tips in case things don't go quite as planned.



Getting started:

- Conduct an antibody titration for the beads so that you're confident that saturation is being achieved. (Bear in mind that the antibody concentration used for cells may not be optimal for the beads.)
- Stain and run each antibody-coated population (Beads 1-4) separately for at least the first run to ensure satisfactory labeling and optimal resolution for gating.
- Use the same lot of the same Ab clone for the duration of the study. Where a new lot must be used, run bead samples stained with each lot in parallel to identify

any variation in staining.
- Using a fluorescent bead standard with each run can help in identifying one-off sample preparation problems, etc. For example, use of a suitable Fluorescent Reference Standard would provide a reference point for each run.
- Get to know your instrument. Quantitative fluorescence analyses probably won't be accurate or reproducible if there are problems with instrument linearity, resolution, etc.

And, of course, we've seen our share of troubles over the years. These are often the result of sub-optimal conditions or basic errors, sometimes even of the forehead-smacking variety. (Don't worry, we've done them, too....) And, if error isn't to blame, there are often simple strategies to improve results.

No or poor fluorescence:
- Ensure that the primary mAb species is suitable for the kit. For example, the anti-Mouse kit is intended to bind mouse mAbs, not for the analysis of mouse cells.
- Protect the fluorochrome-conjugated Ab and stained samples from light to prevent photobleaching.
- Ensure that the laser and detector are suitable for the reporter fluorochome.
- In the special case of Fc-tagged proteins, they should be tested to ensure acceptable binding to the Fc specific antibody coated on the beads. We have known some Fc tags to exhibit different binding than their native Ab counterparts, and a lack of binding in rare instances.

Broad fluorescence peaks:
- Use of an indirect staining approach will lead to broader peaks; if this occurs, populations may be stained and run separately for optimal gating.
- Broad peaks may indicate that saturation has not been achieved; an antibody titration will aid in ensuring that bead samples are stained to saturation.
- Ensure that only singlets are gated.
- Do not stain the blank population, which consists of uncoated polymer beads that will be happy to bind antibody nonspecifically.

QuickCal®:
- If the curve doesn't fit in the window, it's likely that the wrong version of the template has been used. To determine "resolution," or the appropriate version of the template, look at the x-axis of the fluorescence histogram. Typically, numbering of 0 - 1000 = 1024 template; 10^0 - 10^4 = BD Relative Linear; 10^{-1} - 10^3 = Coulter Relative Linear.
- An unexpectedly high detection threshold may indicate free dye in the system, or that the blank bead population was stained with the antibody-coated beads.

If you achieve poor results with a particular run, stain and run a new sample. Staining and running peaks separately may provide more specific information for troubleshooting. Labeling beads with a different antibody (clone and fluorophore) will aid in identifying clone- or fluorochrome-specific effects.

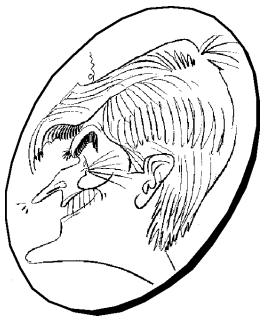
And, of course, we're happy to offer additional comments, support, or a warm shoulder, if needed.

Centrifugation

Volume 21, #1
March 2008

Q: I'm having problems when centrifuging microspheres. My bead counts are so low after staining and washing that I'm afraid they're being broken. Is this possible?

A: Polystyrene-based microspheres can handle the rigors of centrifugation, and we expect this separation technique to work well for spheres ≥ 0.5µm. When you hear us cautioning against overzealous centrifugation, we're most concerned about having too tight of a pellet form, i.e. irreversible aggregation of microspheres.



If spheres are disappearing, it's most likely that they are becoming more hydrophobic with successive washes (as the surfactant concentration is lowered), and are clinging to the sides of tubes. You may centrifuge them with a bit more force to sediment them, or add back a bit of surfactant to aid in wetting.

Some general guidelines for a benchtop microcentrifuge follow (7.3cm rotation radius, 5 minute centrifugation). These may be used as a starting point for further optimization, if needed.

Uncoated Polymer		**Protein Coated Polymer**	
> 0.5µm	6,500 - 14,000 x G	> 0.5µm	8,000 - 11,000 x G
1.0µm	3,000 - 5,500 x G	1.0µm	5,500 - 8,000 x G
< 5.0µm	1,300 - 3,000 x G	< 5.0µm	2,000 - 5,500 x G

Silica	
> 0.5µm	3,000 - 5,500 x G
1.0µm	1,300 - 3,000 x G
< 5.0µm	750 - 1,300 x G

Additionally, to better understand the efficiency of centrifugation steps, you might examine samples of the supernatants under the microscope (40X objective) to determine what isn't being spun down. Also examine the tube, which may have a characteristic smear on the wall if the beads are sticking to it.

If centrifugation isn't an ideal method, or is contraindicated due to small bead size, see the other separation methods that are described in our TechNote 203, *Washing Microspheres*.

Long-Term Storage

Polystyrene Microspheres

Stability

Volume 21, #1
March 2008

Q: I would like to purchase a fairly large batch of polystyrene microspheres, but am concerned about stability. What shelf life can I expect?

A: We don't assign an expiration date to uncoated polymer microspheres. Polystyrene microspheres should be (chemically) stable indefinitely, provided that they are stored under suitable conditions (e.g. in their original buffer at 2-8˚C). Conditions that would be damaging to the product include freezing (irreversible aggregation), high temperatures (> ~95˚C), or exposure to organic solvents (swelling/deformation/sticking of beads).

In general, our primary concerns for long-term storage of uncoated polymer microspheres include:

- ensuring that they do not become contaminated.
- ensuring that they are well dispersed before use.

Though many suspensions contain an antimicrobial, they should be handled with care (aseptic conditions where possible) and stored at 2-8˚C to avoid contamination/proliferation of microbes. If a contamination occurs, it may be possible to decontaminate the suspension; see PDS 726, *Decontaminating Polystyrene Microspheres.*

To ensure that spheres are well-dispersed after prolonged storage, roll the suspension for several hours (perhaps overnight). Monodispersity may be evaluated microscopically, or via an automated particle sizer. If aggregation is observed, it can often be successfully treated with the addition of surfactant; see TechNote 202, *Microsphere Aggregation.*

A periodic re-evaluation of your stored material using the same processes and criteria that you use for qualification of new shipments should provide the needed confidence for long-term storage and use. If you do encounter a problem (e.g. contamination, aggregation, etc), it should be possible to re-work and re-qualify the material.

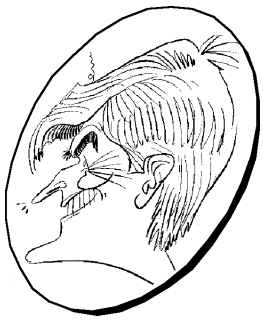
Calibration Standards

NIST-Traceable Particles

Particle Sizing

Volume 20, #3
September 2007

Q: I'm building a particle sizer, and need to obtain calibration standards. What products are available?



A: Accurate particle size and distribution analysis is critical to particle-based technologies in industry and research. The particle sizing instruments used to support research, manufacturing and QC efforts in these sectors must be rigorously calibrated and validated to ensure the integrity of results.

We offer a full range of polystyrene-based **NIST-traceable** particle size standards that are suitable for calibrating and validating sizing instruments. With diameters spanning

a range of 40nm to 175µm, we have standards that are suitable for a broad range of sizing methodologies. The standards are supplied as 1% solids aqueous suspensions in dropper bottles.

We also supply a wide range of diameters within our standard catalog if the higher level of traceability isn't required for every run. These products include polymer (25nm-20µm) and silica (150nm-5µm) spheres supplied as 10% solids suspensions. Some dry microsphere products are also available.

For specific sizes and ordering information, see our website or catalog. If you should need further clarification, our friendly Customer Service Representatives are standing by (well, actually, they're probably sitting) and ready to help!

Catalog Code	Description
NT02N-NT40N	**NIST Traceable Size Standards, size ranges from 40nm to 175µm**
PS02N-PS08N	**Polystyrene Plain (Hydrophobic) Microsphere, size ranges from 25nm to 20µm**
SS02N-SS06N	**Silica Plain (Hydrophilic) Microspheres, size ranges from 150nm to 5µm**

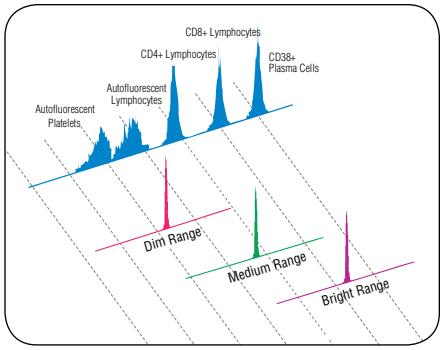
Fluorescence Intensity

Quantum™ FITC MESF

Volume 20, #3
September 2007

Q: We require **Quantum™ FITC** calibration beads for the quantitation of FITC fluorescence intensity in MESF units. It seems 3 types of Quantum FITC beads (low, medium, high) are available. I am confused in choosing the right one for my calibration work. Can you help?

A: The different levels of kits are intended to span the intensity range of common cellular analyses. Low level kits are commonly used for cells with low expression levels, or for small cells that will be dimmer due to their size. Examples include telomere length determination and some cell surface markers (e.g. CD34). Medium level kits are used for many types of analyses, and nicely span the range of typical cell samples. Common analyses include those for many surface markers, including CD4 / CD8. High level kits are often used for cells with very high expression or high autofluorescence, e.g. analysis of tumor cells.



If you're still wondering which is best suited for your assay, you might start with the mid-level kit, which overlaps areas of the low and high kits.

Catalog Code	Description
824	**Quantum™ FITC (low level)**
824p	**Quantum™ FITC (low level) premixed**
825	**Quantum™ FITC (high level)**
825p	**Quantum™ FITC (high level) premixed**
826	**Quantum™ FITC (medium level)**
826p	**Quantum™ FITC (medium level) premixed**

ABC Values

Antibody Quantitation

DNA

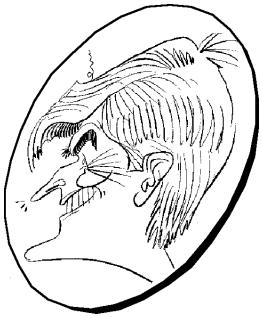
F/P Ratio

MESF Values

Quantum Dots

Quantum™ MESF Kit

Q: I am gathering information on ZAP-70 staining of CCL samples and would like information on which quantification product to select. I have spoken to other labs using ABC binding beads that they are staining with ZAP-70 antibody. Is this preferable over a particle using MESF? What advantages are there, if any, of one over the other?



A: Both **Quantum™ MESF** and **Quantum™ Simply Cellular® (QSC)** kits would be suitable for use, although there are some differences that may lead you to prefer one approach over the other.

Beginning with similarities, both kits include five bead populations: one blank and four populations labeled with increasing amounts of fluorophore (MESF) or antibody (QSC, calibrated in terms of their Antibody Binding Capacity [ABC]). The MESF beads are run as-is; the QSC beads are labeled with the same antibody that is used to stain cells. The

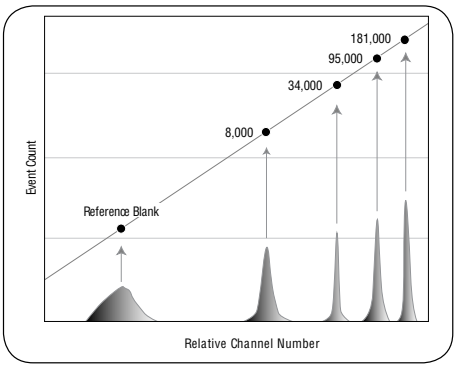
Quantum™ Simply Cellular®

Simply Cellular®

Staining Samples

Volume 20, #2
July 2007

fluorochrome-labeled microspheres are run on the cytometer at the same instrument settings as cells. Their channel values are then used to generate a standard curve relating fluorescence intensity to standardized MESF or ABC values from the beads. The MESF or ABC values of the labeled cell samples may be determined by measuring their fluorescence intensities, and "reading" the corresponding MESF or ABC values from the standard curve using the QuickCal® analysis template that is provided with the kit.



Some differences are as follows:

Quantum™ MESF

- Kits are available in FITC, PE, PE-Cy™5 and APC versions.
- Prelabeled beads are very convenient to use, and are not subject to the same variation that could be introduced through staining (different technicians, antibody lots, etc.).
- There is no mAb consumed to stain the beads.
- Quantum MESF kits are not limited to antibody-based systems. For example, they have been used for DNA-based applications, such as telomere length determination.
- The MESF unit is a standard fluorescence intensity unit. However, if you wish to report the number of antibodies bound, the effective Fluorophore:Protein ratio (effective F/P ratio), or labeling density of the antibody conjugate, would need to be determined. This may be accomplished by staining our single population **Simply Cellular** product (calibrated in terms of ABC) with the antibody conjugate, and determining its MESF value by running it against the appropriate MESF kit. The MESF value is then divided by the ABC value to obtain the effective F/P ratio of the conjugate.

Quantum™ Simply Cellular®

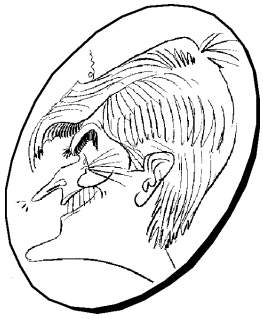
- Kits are available in anti-Mouse, anti-Rat and anti-Human versions, for use with mouse, rat, and human monoclonals, respectively.
- Beads are calibrated in terms of ABC. For cellular analyses, if you presume monovalent binding of antibody to the cell surface receptor, then ABC (# antibodies bound) = marker density. This circumvents the need to determine the effective Fluorophore:Protein ratio of the conjugate.
- Antibody conjugates with any type of fluorescent label may be used, including less-standard fluorochromes and quantum dots.
- Beads are labeled by the user with the same antibodies that are used to stain cells. This presents the benefit of having the identical conjugate on beads and cells. Please note that it is advisable to titrate the antibody conjugate to ensure that the beads are stained at saturation, and it is imperative that standardized staining protocols are used to ensure consistency of results.

Catalog Code	Description
824	**Quantum™ FITC (low level)**
824p	**Quantum™ FITC (low level) premixed**
825	**Quantum™ FITC (high level)**
825p	**Quantum™ FITC (high level) premixed**
826	**Quantum™ FITC (medium level)**
826p	**Quantum™ FITC (medium level) premixed**
827	**Quantum™ R-PE**
828	**Quantum™ PE-Cy™5**
823	**Quantum™ APC**
815	**Quantum™ Simply Cellular® anti-Mouse IgG**
816	**Quantum™ Simply Cellular® anti-Human IgG**
817	**Quantum™ Simply Cellular® anti-Rat IgG**

High Throughput Format

ProMag™

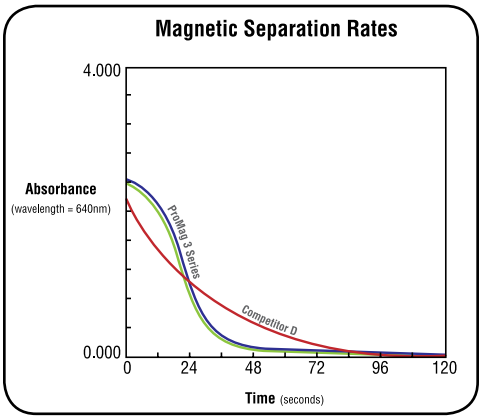
Q: We are developing an automated magnetic chemiluminescent immunoassay, but have had difficulty with uniform separation of the spheres. Do you have any products that are suitable for a higher throughput format?



Uniform Separation

Volume 20, #1
March 2007

A: There are many factors that impact the separation of superparamagnetic microspheres, including bead uniformity, the form and size of iron oxide inclusions, and homogeneity of magnetite distribution. **ProMag™**, our new line of uniform superparamagnetic microspheres, have been carefully designed and rigorously tested to ensure rapid, uniform separations, as reflected in the graph.



Biotinylated Oligo

Quantum™ MESF Kit

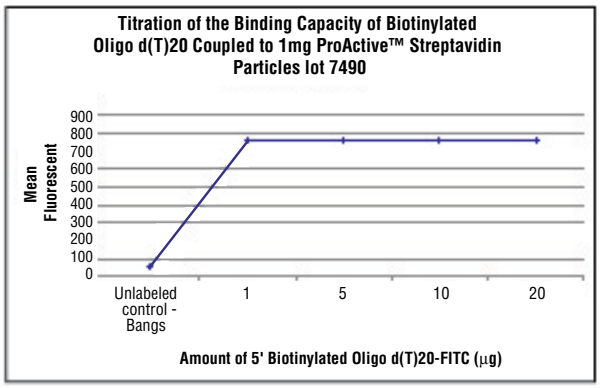
Streptavidin-Coated Microspheres

Volume 20, #1
March 2007

Q: I want to attach a **biotinylated oligo** to streptavidin microspheres, but I don't know how much to include in the reaction. I would also like to determine the amount of biotinylated-oligo that is actually bound. What method can I use?

A: The binding ability of our **streptavidin-coated microspheres** is assessed through the binding of biotin- FITC. The binding capacity is reported on the Certificate of Analysis in terms of µg biotin-FITC/mg microspheres. This value may be used to estimate the capacity for your biotinylated oligo. For a basic attachment protocol with recommendations for oligo concentration, see our Product Data Sheet 714, *Binding Biotinylated DNA to Streptavidin-coated Microspheres*.

After coating, there are a number of methods for determining the amount of immobilized oligo. You may read the OD260/280 of the supernatant following the binding reaction, or use a fluorescent nucleic acid stain for single-stranded DNA. If the oligonucleotide or its target is labeled with a fluorochrome, flow cytometric analysis may be utilized with an appropriate **Quantum™ MESF** kit (e.g. Catalog Code 826, Quantum MESF FITC) to quantitate fluorescence intensity of the bound oligonucleotide on the streptavidin microspheres.



APC-Cy™7

DAPI

Far-Out Red

Fluorescent Reference Standards

Hoechst

Indo-1

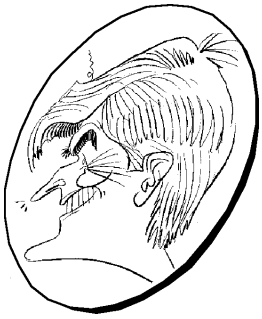
Red Laser/Far-Red Detection

UV & Violet Excitation

Q: We just purchased a customized cytometer, and need additional bead standards for the UV and violet lasers. We would also like something for the red laser with far-red detection. Can you direct us to suitable products?

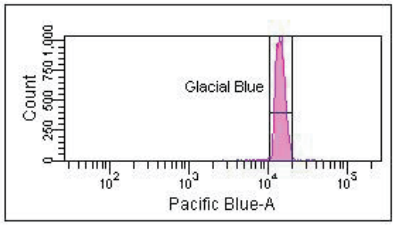
A: Living life on the edge of the visible spectrum, eh? No problem — we're edgy; we can help. Our line of **Fluorescence Reference Standards** includes a number of products for **UV** excitation, such as DAPI, Hoechst, Indo-1, etc. We also recently expanded our offerings to include specific products for violet lasers (**Glacial Blue**), and for excitation with red lasers for far-red emission (**APC-Cy™7 and Far-Out Red**).

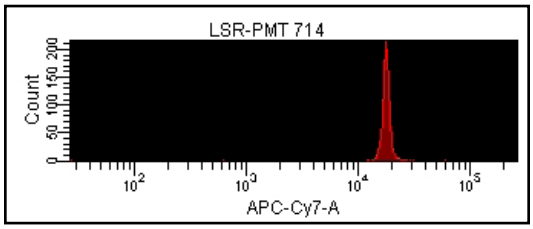
Glacial Blue may be excited using a violet (405nm) laser, with detection in the blue region of the spectrum. Our new surface-labeled **APC-Cy™7** standard is our first dedicated standard for red excitation with far-red emission. (See also Far-Out Red, introduced in July 2008.)



Single-color **Fluorescence Reference Standards** may be used to QC a specific path of the optical system (laser/filter/PMT), to optimize filter and mirror sets for fluorophores, and to establish a test-specific Target Channel Value for instrument set-up.

Volume 19, #2
September 2006





Catalog Code	Description
FC06F/6952	**Glacial Blue Microspheres**
914	**APC-Cy™7 Reference Standard**
913	**Far-Out Red Microspheres**

Flow Cytometry Supplement

Quality Assurance

Standardization

Volume 19, #2
September 2006

Q: I am developing a bead-based assay for use in our laboratory and other clinical research laboratories, and could use some pointers on quality assurance and standardization. Where can I find information?

A: Our new **Flow Cytometry Supplement**, including a technical reference guide, outlines a basic program for quality assurance and standardization in the flow cytometry laboratory, and provides some references that pertain to the clinical research laboratory in particular. (Supplement available for download from our website.) The Clinical and Laboratory Standards Institute (www.nccls.org) and the International Society for Analytical Cytology (ISAC, www.isac-net.org) are excellent sources for information. If you're not already a subscriber, you might also sign up for the Purdue University Cytometry mailing list, which is basically an email forum for all things cytometric. You may submit questions regarding protocols, products, instrumentation, regulatory issues, etc., which will be zealously addressed by "flow-ers" from all over the world (http://www.cyto.purdue.edu/hmarchiv/cytomail.htm). Email archives are also available for searches.

DNA Isolation

DNA Purification

Magnetic Beads

Magnetic Separators

SNARe™

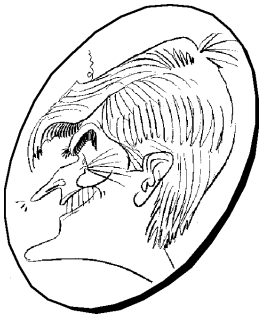
Volume 19, #2
September 2006

Q: We are a contract lab that receives many requests for magnetic particle-based DNA isolation services, particularly for genomic DNA from whole blood. We need a product that will provide exceptional yield, and is amenable for use on our high throughput automated platform. Can you help?

A: Our **SNARe™ Whole Blood Genomic DNA Purification System** (Catalog Code BP691) features our patent-pending DNA Separation Particles for the efficient isolation of dsDNA. We offer protocols for both manual (microcentrifuge tube) and automated (96-well plate) formats so that you may scale up with ease. Both protocols result in exceptional yield, 20µg DNA / 200µL tube whole blood, or 5-20µg DNA per well (100uL lysate from fresh or frozen whole blood, WBCs or MNCs). Protocols are provided in Product Data Sheet 691 (microcentrifuge tubes) and Product Data Sheet 691A (96-well plate).

We also offer two other DNA purification systems (see below), as well as variety of rare earth magnetic separators for use before scale-up to a high throughput system. Our separators are designed to accommodate a complete range of magnetic separation applications, including cell sorting, mRNA and DNA isolation, and purification of biomolecules.

Catalog Code	Description
BP691	**SNARe™ Whole Blood Genomic DNA Purification System**
BP692	**SNARe™ Plasmid DNA Purification System**
BP693	**SNARe™ Plant Genomic DNA Purification System**
LS001	**1.5mL Magnetic Separator**
MS002	**BioMag® Multi-6 Microcentrifuge Tube Separator**
MS003	**BioMag® 96-Well Plate Separator**
MS004	**BioMag® Flask Separator**



Alexa Fluor® 488

Anti-Mouse IgG Beads

Q: I see that you sell standards for some, but not all, of the fluorochromes that I use. For some (Cy-Chrome™, BD Biosciences), I simply need a reference standard, and for others (Alexa Fluor® 488, Molecular Probes, Inc.), I need to be able

Cy-Chrome™

Fluorescence Quantitation

Fluorescent Reference Standards

Protein A Microspheres

Quantum™ Simply Cellular®

Reference Standards

Volume 19, #1
May 2006

to quantitate the fluorescence signal. What can I use?

A: For fluorescence quantitation, the same fluorophore must be on beads and cells. (This ensures that the beads respond to the environment in the same fashion as cells, and quantitative assignments are truly relevant.) Our **Quantum™ Simply Cellular®** kits are comprised of bead populations labeled with anti-mouse, anti-rat, or anti-human antibodies. They may be labeled directly with your fluorochrome-conjugated primary antibody, or indirectly using your unlabeled primary and a labeled secondary. See our online Flow Cytometry catalog for Product Data Sheets.

For a simple reference standard, there are a few options. If, for example, you have a mouse mAb that is labeled with your fluorochrome, you could use it to stain **protein A** or **anti-mouse IgG** microspheres to create your own fluorescent standard. (Be sure to check the Ab subclass if using protein A.) We have a number of options in ~5-10µm polymer beads that would be suitable for flow. See our online Polymer & Silica Beads catalog for details.

We also sell a wide range of fluorescent bead standards that may serve as reasonable surrogates if you simply wish to check the laser or detector for your fluorochrome. Our flow cytometry **Reference Standards** span the spectrum, from UV to Far Red. Additionally, we have several internally-labeled fluorescent microspheres in our standard catalog. Reference spectra for internally-labeled spheres are now available in the Technical Literature section of our website (TechNotes 103 and 103A).

Carboxylated Beads

Directed Immobilization

EDAC Coupling

PolyLink Protein Coupling Kit

Volume 19, #1
May 2006

Q: I'm interested in using PolyLink (EDAC) coupling to attach antibodies to COOH-functionalized beads. However, this method seems to attach to any available amine group of the protein, so I'm not sure if I should use it.

A: Oh, it'll be fine...go ahead and use it. (How we do love to sell beads and reagents!) OK, OK. On a more serious note, using carboxylated beads with **EDAC** will result in some level of nonspecific orientation, although it is generally accepted that the Fc region will preferentially orient toward the bead based on antibody packing, and the slightly greater hydrophobicity of this domain. This immobilization strategy (EDAC activation of bead COOH groups) generally results in sufficient antibody bound, with good activity, and is one of the most common for standard applications, such as immunoassays.

We typically recommend directed binding for special cases, e.g. if the Ab isn't performing when traditional covalent immobilization is used, or for certain ligands such as oligonucleotides, peptides, hormones, etc.

If you decide to explore directed immobilization strategies to orient the antibody, you might consider use of an Fc-binding protein such as protein A or G (although you'll need to confirm the protein's Ab affinity; these proteins do exhibit variable binding, depending on the species in which the Ab was raised as well as Ab subclass). Use of an Fc-specific antibody would also be appropriate. You could also digest the antibody for immobilization of F(ab') fragments, or oxidize the carbohydrate in the Fc region to create aldehydes for direct covalent immobilization or use of a targeted biotinylated linker and a streptavidin support. These methods are certainly valid, but they do involve additional steps. (So … Go **PolyLink**!)

BioMag®

BioMag®Plus

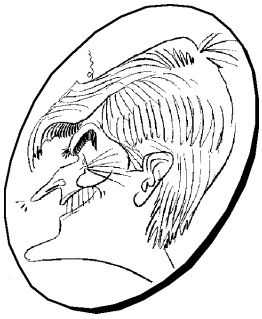
COMPEL™

Flow Cytometric Assays

Magnetization

Purifying Samples

Q: I want to purify my target cells from a fairly nasty sample matrix. Ultra-high purity isn't necessary, but I want to capture as much target as possible. Which type of magnetic bead should I use?



A: Magnetic particle selection is often driven by practical matters, such as availability of an off-the-shelf product for the intended separation. In these instances, further consideration may be given to characteristics of the base particle (such as size, surface area, density, composition) for tailored handling, binding capacity, etc.

Our uniform **COMPEL™** microspheres are well suited for the development of flow cytometric and other bead-based assays. The low density of the polymer matrix permits binding kinetics that approach those of solution- based systems. The polymer matrix

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Fall 2005

is also amenable to dyeing, and the high surface charge allows binding of large amounts of ligand.

BioMag® microparticles are ideal for isolation of cell fractions or purification of target from complex samples. Their tremendous surface area and greater density allow rapid and highly efficient capture of the target species. For this specific application, BioMag is our recommendation.

BioMag® and **BioMag®Plus** are ~1.5µm high-performance superparamagnetic microparticles widely used for the efficient separation of cells and purification of biomolecules. Their irregular shape provides a much greater surface area than similarly-sized spherical particles, resulting in high binding capacities and efficient capture of target with conservative use of particles. The high iron oxide content allows for rapid and efficient magnetic separations, even from difficult, e.g. highly viscous, samples. BioMagPlus particles undergo additional processing for removal of fines.

Composition:	Silanized iron oxide
Morphology:	Irregular/Cluster
Surface groups:	COOH and NH_2 available
Density (g/cm^3):	>2.5
Iron oxide content (%):	>90
Magnetization (emu/g):	25-35
Surface area (m^2/g):	>100
Particles / g:	~$1x10^8$

Once you've determined which type of bead to use, consideration moves to the surface that will be most effective. Often, separation is performed using some sort of affinity system, for example antibody/antigen interaction or charge mediated purification. Protein coated magnetic particles are available off-the-shelf, with streptavidin, protein A, secondary antibody, primary antibody, oligo (dT)20, or anti-CD marker surfaces. Functionalized particles are used in situations requiring the attachment of less common ligands.

Flow-FISH

Fluorescent Beads

Molecular Biology

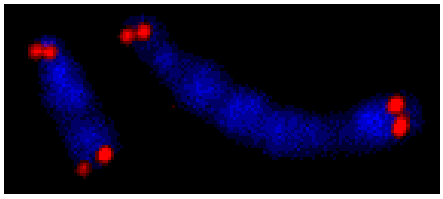
Quantum™ MESF Kit

References

Volume 18, #2
Spring 2005

Q: Do you have any products that support quantitative applications in molecular biology?

A: Our **Quantum™ MESF** kits have been utilized to quantitate fluorescence intensity for Flow-FISH (Fluorescence in situ hybridization)[1] and bead-based hybridization assays[2] via flow cytometry. Provided that you're using a fluorescent reporter for which we offer an MESF kit (FITC, PE, PE-Cy™5, APC) and you're evaluating the fluorescence of a "particle," be it a fluorescently-labeled cell or microsphere, our kits permit quantitation of fluorescence intensity in MESF (Molecules of Equivalent Soluble Fluorochrome) units.

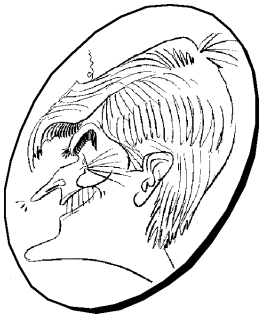


Telomere length measurements via Flow-FISH

To accomplish this, the fluorescent bead set is run on the flow cytometer using the same instrument settings as for the labeled samples. The median channel values (fluorescence intensity) for the beads are entered against their assigned MESF values using the QuickCal® analysis template that we provide; this generates a calibration curve relating fluorescence intensity to MESF value. Channel values for the samples may then be entered into QuickCal to obtain their MESF assignments.

References:

1. Baerlocher G.M., P.M. Lansdorp. 2003. Telomere length measurements in leukocyte subsets by automated multicolor flow-FISH. *Cytometry Part A,* 55A:1-6.
2. Spiro A., M. Lowe. 2002. Quantitation of DNA sequences in environmental PCR products by multiplexed, bead-based method. *Appl Environ Microbiol,* 68(2):1010-1013.



Aggregation

Bead Handling

Dialysis

Filtration

Small Microspheres

Washing Beads

Volume 18, #2
Spring 2005

Q: I am interested in developing an immunoassay using 100nm microspheres. Could you offer me some tips for working with this size of particle?

A: Yes - don't. Just kidding - although it is important to note that small polymer microspheres (≤ 300nm) present unique challenges, and there will be special handling considerations.

Particles in this size range are more prone to **aggregation** than larger spheres due to their very high surface area:volume ratios, and may require more surfactant and sonication than their larger diameter counterparts. In fact, you may find it useful to sonicate the suspension before, during (e.g. ~every 15 minutes), and after coating. An automated particle sizer can aid in determining the level of monodispersity (i.e. fluctuation in mean diameter), as can traditional microscopy. Although you will not be able to visualize individual particles with a standard microscope, aggregates should be visible using 400X magnification.

Regarding washes, centrifugation will not be a suitable means of separation, as pelleting is likely to cause irreversible aggregation. Rather, washes are typically performed using **filtration** or **dialysis**. Filter companies can provide suggestions regarding a suitable filter or dialysis membrane or cartridge, i.e. a pore size or MWCO (Molecular Weight Cut-Off) that will retain particles, while allowing the removal of unbound antibody and blocking molecule.

Quantum™ Simply Cellular®

Volume 18, #1
Winter 2005

Q: I routinely use your Low Level Quantum™ Simply Cellular® Goat anti-Mouse kits (Catalog Code 814) as a quantitative tool in my lab, but I see that 814 is no longer available on your website. HELP!

A: Don't be alarmed! After our new Flow Team put the **Quantum™ Simply Cellular® (QSC)** kits under scrutiny, in support of our ongoing goal to offer the highest quality products, a few changes were made. 1) The Low Level 814 kit and the Medium Level 815 kit were combined to satisfy the requirements of both users, and 815 simply won as the catalog code to maintain. 2) The new QSC Goat anti-Mouse kits (Catalog Code 815), as well as the QSC Goat anti-Human kits (Catalog Code 816), are now being shipped in five separate bottles, a blank and four different Antibody Binding Capacity (ABC) levels. Instead of receiving your normal 1mL, 5mL or 14mL premixed quantities, you will be receiving 1mL, 5mL or 14mL of each bead component. A much better value for you!

Fluorescent Beads

Glacial Blue

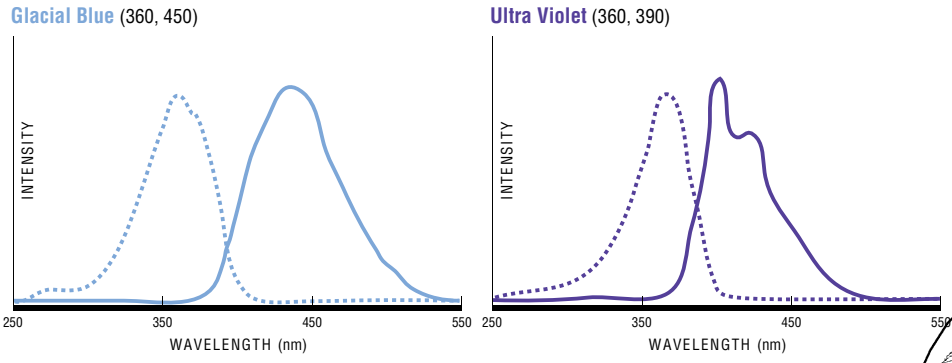
Ultra Violet (Dye)

UV & Violet Excitation

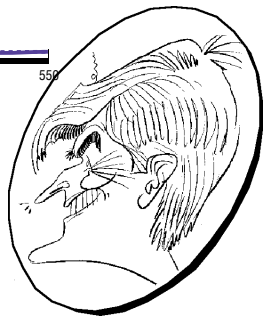
Volume 18, #1
Winter 2005

Q: Do you have any fluorescent beads that are suitable for UV & Violet excitation?

A: In addition to **Plum Purple**, we are pleased to introduce our new violet- and UV-excitable fluorophores, **Glacial Blue** and **Ultra Violet**. We also have a few offerings on our inventory to support applications in flow cytometry.



Our TechNotes 103 and 103A, which may be downloaded from the Technical Literature section of our website, provide spectra for our other fluorophores. If we don't have a product on inventory that is ideal for your application, we would be pleased to provide you with a quotation for a custom project.



Common Window of Analysis

Daily QC

Flow Cytometers

Initial Target Channels

QC3™

QC Windows®

Quality Control

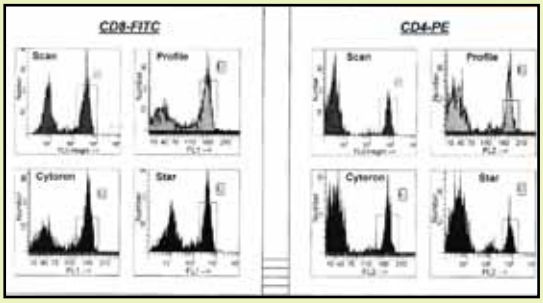
Volume 17, #3
Fall 2004

Q: What type of daily QC do you recommend for flow cytometers?

A: For typical QC needs, we recommend **QC Windows®**, which is the product that we use for daily set up and QC. The QC Windows kit consists of **QC3™** and **Certified Blank™** microbeads. It is supplied with "Initial Target Channels" that, when used in conjunction with labeled control cells, provide a unique approach to unified instrument setup and qualitative evaluation of instrument performance. QC Windows allows multiple users to establish a Common Window of Analysis with respect to the fluorescence intensity. Instruments that have been adjusted to a Common Window of Analysis produce histograms that are nearly identical. Such standardization may be critical when comparing the presence, absence or relative intensity of immunophenotyping cell-clusters in bone marrow or leukemia samples. It also allows data comparison independent of instrument make.

QC Windows is an essential part of a uniform set up protocol to establish a **Common Window of Analysis**. The QC3 standard has the same spectral properties and fluorescence intensity as the samples being analyzed. QC Windows establishes the position of the fluorescence intensity Window of Analysis before any samples are run. The Window positioning is accomplished by adjusting the PMT voltage (with the compensation turned off) such that the QC3 standards fall in predetermined Target Channels. After setting the Target Channels, the compensation may then be accurately adjusted using labeled control cells, as they represent the most accurate spectra of the sample cells. Re-running the QC3 standards at these settings (post-compensation) provides the Instrument-Specific Target Channels, which should be achieved daily if the instrument settings are not changed. The instrument noise may then be qualitatively assessed with the Certified Blank microbead standard, by comparing its position relative to the autofluorescence of non-labeled cells to ensure that noise does not interfere with the assay.

The same sample of labeled blood was run after a Common Window of Analysis was established using QC Windows® on each instrument.



Catalog Code	Description
845	**QC Windows® (FITC/PE)**
846	**QC Windows® (FITC/PE/PE-TR)**
847	**QC Windows® (FITC/PE/PE-Cy™5)**
848	**QC Windows® (FITC/PE, Cy™5/APC)**

Anti-CD Markers

BioMag®

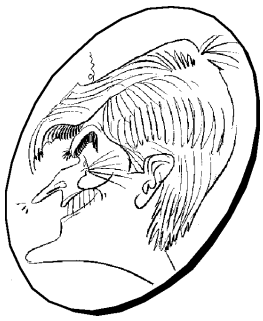
Cell Separations

Volume 17, #3
Fall 2004

Q: I conduct many different types of cell separations. Do you offer any products that support this application?

A: Funny you should ask...! We were just talking about the variety of BioMag® kits and particles for human and mouse cell separations that we offer. (Well, as far as the boss knows, that's what we were talking about....) A list of our anti-leukocyte BioMag particles is provided below. We also offer BioMag T cell enrichment systems and a range of secondary antibody and other affinity coatings to capture cells that have been antibody-labeled.

Catalog Code	Description
BM595	**BioMag® anti-Human CD2**
BM580	**BioMag® anti-Human CD3**
BM581	**BioMag® anti-Human CD4**
BM583	**BioMag® anti-Human CD8**
BM596	**BioMag® anti-Human CD11b**
BM584	**BioMag® anti-Human CD14**
BM585	**BioMag® anti-Human CD16**
BM586	**BioMag® anti-Human CD19**
BM587	**BioMag® anti-Human CD34**
BM588	**BioMag® anti-Human CD45**



BM589	**BioMag® anti-Human CD56**
BM590	**BioMag® anti-Human CD71**
BM592	**BioMag® anti-Mouse CD4**
BM593	**BioMag® anti-Mouse CD8a**
BM594	**BioMag® anti-Mouse CD45R**
BM597	**BioMag® Human CD3+ T cell Enrichment System**
BM598	**BioMag® Human CD4+ T cell Enrichment System**
BM599	**BioMag® Human CD8+ T cell Enrichment System**

BioMag®

COMPEL™

Phage/Yeast Library

ProMag™

Recombinant Antibodies

References

Q: Do you have any products that are suitable for selecting recombinant antibodies from a large antibody phage library?

A: **Magnetic microparticles** have been utilized with success for efficient selection of surface-displayed molecules from phage and yeast libraries. Sequential magnetic bead and flow cytometric sorting has also been reported. A few pertinent references follow:

Feldhaus M.J,. et. al. 2003. Flow-cytometric isolation of human antibodies from a nonimmune Saccharomyces cerevisiae surface display library. *Nature Biotechnology,* 21:163-170.

McConnell S.J., et. al. 1999. Biopanning phage display libraries using magnetic beads vs. polystyrene plates. *Biotechniques,* 26(2):208-210, 214.

Yeung Y.A., K.D. Wittrup. 2002. Quantitative screening of yeast surface-displayed polypeptide libraries by magnetic bead capture. *Biotechnol Prog,* 18(2):212-220.

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We offer **BioMag®**, **COMPEL™,** and **ProMag™** microparticles with a number of coatings for affinity binding (e.g. secondary antibody, streptavidin, protein A, etc.), in addition to **BioMag®** and **COMPEL™** functionalized microparticles for covalent attachment of capture molecules. See our *Magnetic Particles* brochure and TechNote 102 for information on particle characteristics.

BioMag®Plus Amine

BioMag®Plus Carboxyl

Coupling Antibodies

Custom Services

PolyLink Protein Coupling Kit

Q: I would like to couple antibody to microspheres, but I haven't worked with beads before. What do you suggest?

A: Using our custom coating service, naturally. Seriously, although we do offer a custom coating service, we do have products that are intended to introduce users to the wonderful world of microsphere coating in a relatively painless manner.

Our new **PolyLink Protein Coupling Kit** includes EDAC (for activation of –COOH surface groups on microspheres), coupling buffer, and wash/storage buffer. The accompanying protocol (Product Data Sheet [PDS] 644) has been optimized for use with polymer microspheres 1µm and larger.

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We also offer coupling kits featuring **BioMag® Plus Carboxyl** (see PDS 618) or **Amine** (see PDS 617) particles, and a **BioMag® Magnetic Immobilization Starter Kit** that includes a magnetic separator, reaction flask, particles, and buffers (see PDS 546). Our TechNotes 201–206 provide additional support on a range of topics related to microparticle handling.

Simply select the microspheres and/or kit that best meet your needs, review the coupling protocol and pertinent TechNotes, and you'll be off and running. Or, heck, join us at **The Latex Course™**.

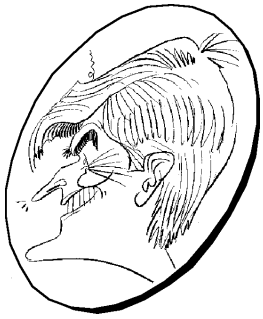
Full Spectrum™

Optical Alignment

Quality Control

Reference Beads

Q: In our flow lab, we run calibration beads daily to verify instrument sensitivity and adjust detector settings. I'm wondering if we should also periodically run reference beads as a check on optical alignment and which ones would be best?



A: Our **Full Spectrum™** microspheres are internally labeled with multiple fluorophores to excite from the **UV** to the **near IR**, and emit over the full range. This product is suitable for instruments with single or multiple lasers, and may be

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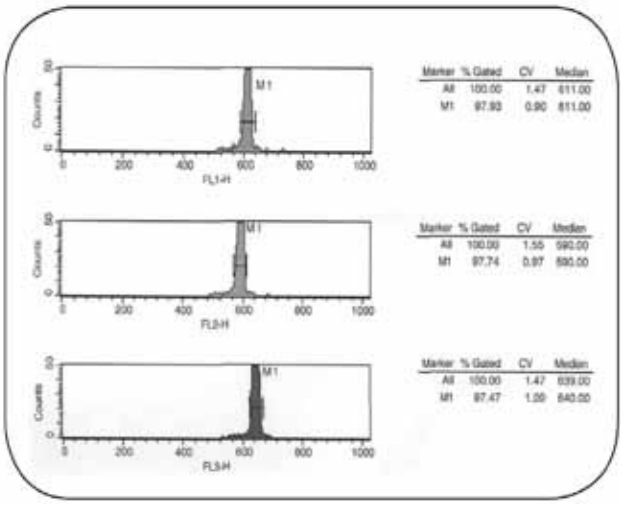
used to check the alignment of all of the lasers and PMTs.



Histogram showing fluorescence and narrow CV's of the Full Spectrum™ (Catalog Code 885) standard.

Cell Quantification

Flow Cytometry Absolute Count Standard™

Volume 17, #1
March 2004

Q: I need to quantify the number of white cells in a sample for a study I am doing. My study is currently qualitative only, but I need to ensure that the changes I am seeing are not just relative, but quantitatively different.

I thought that using beads and adding a known concentration to my sample would allow me to determine the absolute number of white cells that I have. Basically, I want to take a blood sample, add the beads, lyse the red blood cells and then use the flow cytometer to quantify the WBC concentration.

Could you recommend beads that I could use for this process? The less expensive the better.

A: We have just the product for you. It is called **Flow Cytometry Absolute Count Standard™**. The beads are roughly 7-8 micron in size, and exhibit broad-spectrum fluorescence. Just as you mentioned, beads are added to your cells prior to acquiring them on the cytometer, and, using a simple equation provided in the Product Data Sheet, allow you to calculate the concentration and absolute number of cells in the sample.

Catalog Code	Description
580	**Flow Cytometry Absolute Count Standard™**

Immunophenotyping

Quality Control

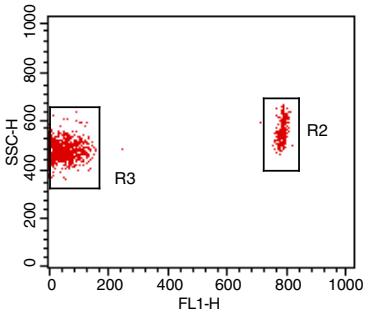
Quantum™ MESF Kit

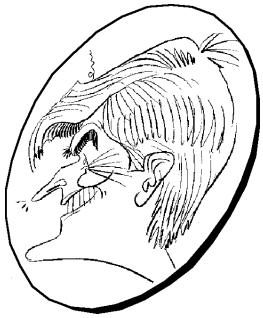
Simply Cellular®

Volume 17, #1
March 2004

Q: I perform standard WBC immunophenotyping, and use your fluorescent beads as controls. I'm worried that differences in my conjugated antibodies either lot-to-lot or over time may be contributing variability to my results. Is there any way to QC my antibodies using the bead controls?

A: **QC** of fluorescently-conjugated antibodies may be performed with our **Simply Cellular®** microspheres. The single population of beads has an **anti-Mouse** (Catalog Code 810) or **anti-Human** (Catalog Code 812) IgG surface. When stained, the beads will bind a known number of your fluorescently-conjugated mouse or human IgG antibodies. QC of the antibody is as simple as monitoring the fluorescence intensity of the stained beads. When used in conjunction with one of our **Quantum™ MESF** kits, the Simply Cellular beads will allow you to determine the effective fluorochrome/protein (F/P) ratio of your antibody.





BioMag®

Biotin

Volume 17, #1
March 2004

Q: I would be interested in using biotin-coated beads. Do you have them available?

A: We certainly do! We offer **BioMag®** biotin-coated superparamagnetic particles as a standard product. We also have custom coating services, and would be pleased to coat a base bead selected from our many polymer, magnetic, fluorescent or dyed microsphere product lines.

Crosslinking

Functionalized Microspheres

Peptide Binding

Spacers

Streptavidin-Coated Microspheres

Volume 17, #1
March 2004

Q: I would like to bind a peptide to microspheres. What type of microsphere do you recommend?

A: For **peptides** and other small molecules, you may wish to employ the use of a **spacing molecule** to ameliorate steric effects, or a crosslinker to target a specific residue and optimally orient the molecule. **Crosslinking agents** are available with a variety of reactive groups for use with functionalized microspheres (covalent coupling), or with a biotin molecule for affinity binding to **streptavidin-coated microspheres**. Depending on reactive groups that are present on the peptide, you may wish to first modify the microspheres (to avoid peptide crosslinking). If a homobifunctional linker is used (like glutaraldehyde), you will want to use it in excess to prevent crosslinking or "hairpin" binding.

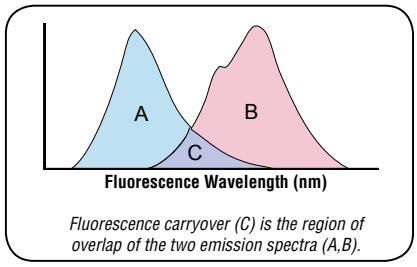
Compensation

Flow Cytometry

Volume 16, #4
December 2003

Q: What is compensation and why is correct compensation important?

A: Flow cytometers are designed to have a primary detector for each fluorochrome label, e.g. FL1 - FITC, FL2 - PE, FL3 - Cy5, etc. Fluorescent signals emitted by fluorochromes can bleed or overlap into the secondary fluorescence detectors. In order to remove this overlap, the proper amount of signal must be subtracted from the secondary detector as a percentage of fluorescence intensity measured in the primary detector. This subtraction is performed by the electrical circuits prior to collecting sample data or by software when analyzing the list mode files. When the mean fluorescence of two populations of labeled standards are adjusted such that they have equal intensities in the secondary fluorescence detectors, then the data from the samples will be accurately compensated.



Fluorescence carryover (C) is the region of overlap of the two emission spectra (A,B).

Compensation

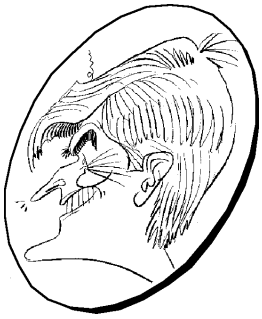
FITC/PE Compensation Standard

Simply Cellular® Compensation Standard

Volume 16, #4
December 2003

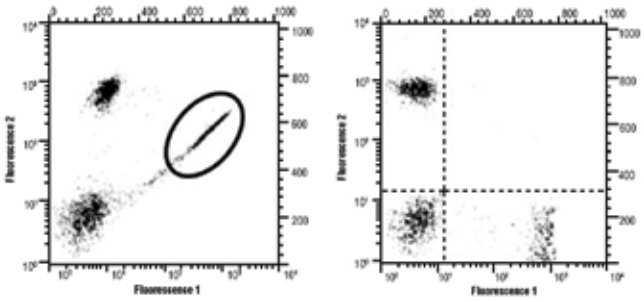
Q: What's the difference between the two compensation kits that you offer? Is there a way I can check to see if my instrument's compensation is correct?

A: *(Trying to sneak in a second question, huh?)* We offer two products for color compensation of your cytometer. The **FITC/PE Compensation Standard** (Catalog Code 820) consists of four different bead populations: one labeled with FITC, one labeled with PE, one labeled with both FITC and PE, and an Autofluor™ population exhibiting a level of fluorescence similar to that of unstained cells. The beads come pre-labeled and ready to use. The product provides a simple means of setting two-color (FITC/PE) compensation.



The **Simply Cellular® Compensation Standard** (Catalog Code 550) consists of two populations of microspheres with goat anti-Mouse (GAM) IgG surfaces. The two populations have the ability to bind different amounts of mouse monoclonal IgG antibody. The user stains these beads with their fluorescently-conjugated mouse monoclonal antibody. The resulting stained beads exhibit "dim" and "bright" levels of fluorescence. One drop of beads is stained for each fluorescent antibody, which may then be analyzed together on the cytometer.

To address your second question, take a look at the graphics. These are dot plots of the FITC/PE Compensation Standard. The one on the left was acquired on a BD FACScan with the compensation circuits turned off. (Note the circled population. See how the fluorescence carry-over from the FITC makes the FITC bead pull away from the axis and appear to have some PE fluorescence?) The dot plot on the right was acquired on the same instrument after the compensation was set.



BioMag®

BioMag® Immobilization Kit

Immunoassays

Volume 16, #4
December 2003

Q: I'm planning the development of an immunoassay using BioMag, but I've never worked with the particles before. Where do I begin?

A: You might consider working with one of our **BioMag® immobilization kits**. These include most of the things that you'll need to get started, such as **BioMag®** particles, chemical crosslinkers, buffers and, in some instances, a reaction vessel and magnetic separator. You will also be supplied with a detailed protocol providing step-by-step instructions for coupling the biomolecule of choice to the particles, and for determining coupling efficiency.

Once you have exhausted the supply of reagents provided with the kit, you may buy the components on an individual basis.

QuickCal®

Volume 16, #3
September 2003

Q: Why do I have to log on to your website to access QuickCal® , and what is the QuickCal® Access Number for? Will my access number stay the same, or will it change with every order?

A: By logging in to our website and using the **QuickCal®** Access Number printed on your **Quantum™ MESF** or **Quantum™ Simply Cellular®** kit, our computer will be able to search your purchases and offer you the files appropriate for the bead kits you have purchased. This way, we can be sure that you always get the correct files, without your having to select the right one from a long list of files.

Adsorption

BioMag®Plus Wheat Germ Agglutinin

Carbodiimide

Crosslinker

Lectin Coating

References

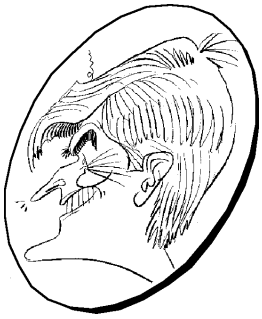
Wheat Germ Agglutinin

Volume 16, #3
September 2003

Q: How do I coat your beads with lectins, such as wheat germ agglutinin (WGA)?

A: For the immobilization of **WGA**, a number of strategies may be utilized. For one common method see: Ertl B., F. Heigl, M. Wirth, F. Gabor. 2000. Lectin-mediated bioadhesion: preparation, stability and caco-2 binding of wheat germ agglutinin-functionalized Poly(D,L-lactic-co-glycolic acid)-microspheres. *J Drug Target,* 8(3): 173-184. They used the "carbodiimide/N-hydroxysuccinimide method," which is a fairly standard method for coupling ligands to COOH-functionalized microspheres (see also our TechNote 205). In any case, lectins will have COOH and NH_2 termini that could be utilized for immobilization.

WGA also possesses numerous cysteine residues, which could be utilized for the formation of disulfide bonds, i.e. a bead could be modified using a heterobifunctional crosslinker that will react with amines (bead) and sulfhydryls (WGA). This is a strategy similar to others presented in TechNote 205. EDAC is a zero-length crosslinker for joining COOH and NH_2 groups; glutaraldehyde is a homobifunctional crosslinker utilized for binding NH_2 groups. (Visit our sister company, Polysciences, at www.polysciences.com, for glutaraldehyde and other reagents such as formamide, nylon wool or ethanol.)



Adsorption of WGA to non-functionalized microspheres might also be considered. A sample adsorption protocol is provided in TechNote 204.

Yet another strategy would be to immobilize the **lectin** through affinity binding. For example, biotinylated WGA (which is commercially available) may be bound to streptavidin-coated spheres.

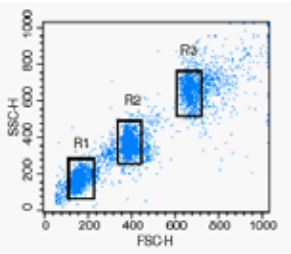
While you may insist on coating the beads yourself, you could also try our newest BioMag®Plus product, Wheat Germ Agglutinin (Catalog Code BP530). Ready to go, we've done the work for you - at least this step.

COMPEL™

Flow Cytometry

Volume 16, #3
September 2003

Q: I was thinking about trying your uniform COMPEL™ magnetic particles for flow cytometry. What do you think?



A: We certainly recommend **COMPEL™** for flow cytometry. The side scatter (SSC) vs. forward scatter (FSC) plot demonstrates the three discrete populations that COMPEL™ 3µm, 6µm, and 8µm magnetic beads yield upon flow cytometric analysis. COMPEL also exhibit low autofluorescence, which is important for bead-based flow assays.

Antibody Source

F/P Ratio

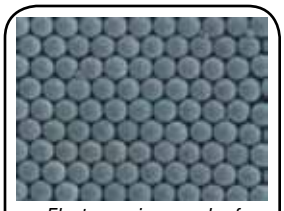
Fluorescence Intensity

Quantum™ Simply Cellular®

Volume 16, #2
June 2003

Q: I use your Quantum™ Simply Cellular® kits to determine CD61 expression of platelets and their precursors. The manufacturer of the anti-CD61-PE I had been using is no longer offering that antibody. If I switch to another antibody source, will my results still compare to the work I've already done? I'm worried about the difference in F/P ratios between the two antibodies.

A: While the **F/P ratio** (fluorochrome/protein ratio - the number of fluorochromes per antibody) will differ between the two antibodies, that difference will not be reflected in the Antibody Binding Capacity (ABC) results obtained from the QSC kit. This is also due to the fact that each of the beads in the **Quantum™ Simply Cellular®** kit is calibrated to bind a specific number of monoclonal antibodies, regardless of the F/P ratio of the antibody. You may notice a difference in the fluorescence intensity of both your samples and the QSC beads with the new antibody, but as long as your samples and the QSC beads are stained with the same antibody, the results should correlate very well with your previous work.



Electron micrograph of 0.97µm uniform silica beads.

Adsorption

Hydrophilic

Nonspecific Binding

References

Silica Applications

Silica Microspheres

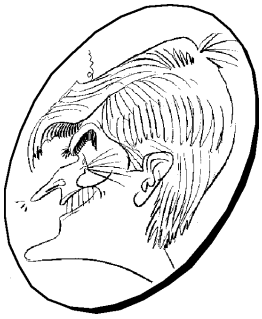
Volume 16, #2
June 2003

Q: I am familiar with the use of plain polystyrene for adsorption of protein (specifically, antibody). I have always heard that silica does not bind protein. If this is the case, what is its use?

A: Silica has become the substrate of choice for many applications, due to its low nonspecific protein binding properties. It is hydrophilic and is less likely to adsorb high amounts of protein nonspecifically than hydrophobic polymer, such as polystyrene. Additionally, its negative charge will cause it to resist nonspecific binding of negatively-charged molecules, such as nucleic acids. However, nothing is absolute- adsorption is dependent upon a number of factors, including characteristics of both the biomolecule (concentration, solubility, charge, pI, etc.) and the solution (pH, salt content, presence of competing molecules, etc.). In fact, our silica microspheres have been utilized for the study of protein adsorption, as reported in:

Docoslis, A., L.A. Ruskinski, R.F. Giese, C.J van Oss. 2001. Kinetics and interaction constants of protein adsorption onto mineral microparticles - measurement of the constants at the onset of hysteresis. *Colloids and Surfaces B: Biointerfaces,* 22: 267-283.

van Oss, C.J., A. Docoslis, R.F. Giese. 2001. Free energies of protein adsorption onto mineral particles - from the initial encounter to the onset of hysteresis. *Colloids and Surfaces B: Biointerfaces,* 22: 285-300.



Our silica microspheres have been proven to be useful for many applications, including the following:

- In solid-phase diagnostics (after functionalization of the surface for coupling of biomolecules).
- For nonspecific immobilization or purification of nucleic acids (in the presence of divalent cations or in the presence of a chaotropic agent and high salt.

- As substrates for the self-assembly of lipid bilayers.
- As spacers for flat panel displays.
- Select lots of highly uniform silica have also been used for microstructure assembly.

Flow Cytometer

Quantum™ MESF

Standardization

Volume 16, #1
March 2003

Q: We have flow cytometers in labs at several different facilities. Is there a way to correlate the values of samples between the different instruments?

A: Yes! You can use **Quantum™ MESF** standards to establish standard curves relating channel values to fluorescence intensity on each instrument. By determining the intensity of your unknowns in **MESF (Molecules of Equivalent Soluble Fluorochrome)** units, you will be able to compare the results of samples run on different machines.

Antibody Quantitation

F/P Ratio

MESF Values

Volume 16, #1
March 2003

Q: How do MESF units relate to the number of antibodies binding to a cell?

A: There is a direct relationship between the MESF value of a cell and the number of antibodies bound to it. The MESF value, however, does not equal the number of bound antibodies. An antibody may have any number of fluorescent molecules (fluorochromes) conjugated to it. The number of fluorochromes per antibody is known as the MESF value of the antibody, or more commonly, the "effective fluorochrome to protein ratio" or simply the "F/P ratio." The F/P ratio of an antibody can usually be obtained from the antibody manufacturer. Alternatively, the F/P ratio may be determined by using the antibody to label a cell or laboratory standard known to bind a specific number of antibodies, and then comparing the MESF value of the labeled sample to its binding capacity. Once the F/P ratio is obtained, it is easy to determine the number of antibodies bound to the cell: simply divide the MESF number by the F/P ratio.

Biotin Binding

PCR Thermocycling

References

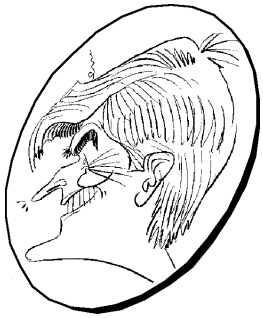
Streptavidin

Volume 16, #1
March 2003

Q: Will streptavidin/biotin binding be stable enough to survive PCR thermocycling?

A: *Short answer:* Several customers have reported that our SA beads work well for PCR work. *Very long answer:* Here are some references from my exhausting (but not exhaustive) literature search regarding stability of [strept]avidin and the [strept]avidin/biotin complex:

1. Bayer, E.A., et al. 1996. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic method for assessing the quaternary state and comparative thermostability of avidin and streptavidin. *Electrophoresis,* 17(8):1319-1324. PubMed: 8874057. On heating "...in the absence of biotin, the quaternary structure of streptavidin is more stable than that of avidin."
2. Wang, C., et al. 1996. Influence of the carbohydrate moiety on the stability of glycoproteins. *Biochemistry,* 35(23):7299-7307. PubMed: 8652506. Apparently, deglycosylated avidin (SuperAvidin™, NeutrAvidin) is less stable than avidin, the carbohydrate moities conferring greater stability to the protein. If unbound streptavidin is more heat stable than avidin, then bound streptavidin should be more stable than bound deglycosylated avidins, although the next reference describes somewhat different findings.
3. Gonzalez, M., et al. 1997. Interaction of biotin with streptavidin. Thermostability and conformational changes upon binding. *J Biol Chem,* 272(17):11288-11294.PubMed: 9111033. Also, Gonzalez, M., et al. 1999. Extremely high thermal stability of streptavidin and avidin upon biotin binding. *Biomol Eng,* 16(1-4):67-72. PubMed:10796986. Biotin binding increases the midpoint temperature of thermal denaturation of streptavidin from 75˚C (unbound) to 112˚C at full saturation (4 biotin: 1 streptavidin); and for avidin, from 83˚C (unbound) to 117˚C at full saturation, i.e., that in both scenarios (unbound and saturated), avidin possesses greater thermal stability. Again, however, deglycosylated avidin is expected to be less thermally stable than native avidin.
4. We expect that 1-2 binding sites are available for binding on each molecule of (strept)avidin (assuming that at least two sites are inaccessible due to immobilization of the molecule on the bead). So, biotin-bound immobilized (strept) avidin (1-2X biotin) should have stability intermediate between that of unbound



(0X) and saturated (4X) (strept)avidin. One might speculate as to whether immobilization will confer further stability to the (strept)avidin molecule (we know that many enzymes are more stable once immobilized).

5. As mentioned before, we have had clients successfully use our streptavidin-coated microspheres during thermocycling. We are not aware of any studies that cite use of deglycosylated avidin-coated beads during PCR thermocycling. Nor do we know of any studies that evaluated the activity of streptavidin, avidin or modified avidin molecules after denaturation and subsequent "renaturation," i.e., studies that investigated "folding errors" during renaturation, or the specific effects of different denaturation procedures.
6. Reznik, et al. 1996. Streptavidins with intersubunit crosslinks have enhanced stability. *Nat Biotechnol,* 14(8):1007-1011. PubMed: 9631041.
7. "Avidin-Biotin" in Pierce Catalog and Handbook, Pierce Chemical Company.

Please note: We do recommend that beads be washed prior to use, as some preservatives and stabilizers can inhibit PCR.

COMPEL™

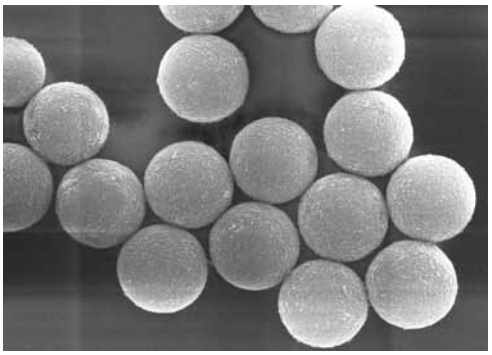
Uniform Magnetic Microspheres

Volume 15, #4
December 2002

Q: I need magnetic beads for my application. Why should I choose yours? I mean, what's so great about these new COMPEL™ beads (other than the very "compelling" name)?

A: Are you kidding? Have you seen the micrograph? Those babies are beautiful! OK, maybe it takes a real bead aficionado to truly appreciate the attractiveness of a round, mostly-plastic ball. But seriously, the **COMPEL™** beads have several features (some of which can be seen in the image), which make them ideal for a whole range of applications.

The first observation to make is that the beads are spherical. There are no nooks, crannies or crevices in which to waste costly antibody, oligonucleotide or other ligand, where it might be inaccessible and unable to do its job.



Next, you will notice that COMPEL beads are not perfectly smooth. The slight surface roughness effectively increases the surface area, allowing you to bind more ligand to the surface than you could to a smooth sphere. Because of this property, the COOH group surface titer and protein binding capacity are much higher than expected for a smooth bead. In our hands, 3µm diameter COMPEL beads bind slightly more streptavidin than the same weight of 1µm diameter magnetic beads.

Finally, you can note that the beads are all the same size. All sizes of COMPEL have extremely uniform size distributions. This property makes separation predictable and easy. You won't have to wait for the small beads from a heterogeneous population to straggle over to the magnet. Additionally, COMPEL beads reflect a nice, tight population on our flow cytometer.

What you can't see in the image is that COMPEL beads respond very quickly to a magnet and redisperse readily when the magnet is removed. And, although they move to the magnet rapidly, their physical density is low enough that they remain suspended in solution long enough for your binding reactions to take place.

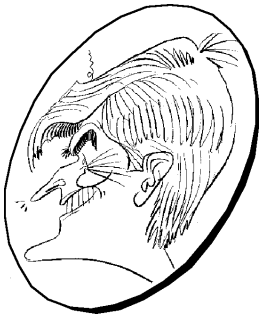
What a great package of features for little, mostly-plastic balls! We encourage you to put them to work in your application... we think you'll like them.

Anti-Human IgG

Antibody Binding Capacity

Quantum™ Simply Cellular®

Q: I saw your recent ad announcing your new anti-human beads. Sounds like a top-secret government conspiracy to me. What's the deal?!



A: *Are you related to that guy who always writes in every time we mention "goat anti-mouse" and asks about antimurine bias?* Well, don't worry; there's no evil plot here! The beads you're referring to are the new **Quantum™ Simply Cellular®** Human Antibody Binding Standards. The **"anti-human"** nickname comes from the Goat anti-Human antibody found on the beads' surfaces. The kits allows you to measure

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the **Antibody Binding Capacity (ABC)** of cells when you're using a fluorescently-labeled human antibody as your reporter. Here's how it works:

The kit consists of five bead populations - one blank population and four populations labeled with varying amounts of Goat anti-Human IgG. Each of the four labeled populations is calibrated to bind a specific number of Human IgG (class I or II) monoclonal antibodies (calibrated to ABC values). These beads are stained with your human monoclonal antibody just like you stain your cells. When you run them on the cytometer, you'll get five separate peaks of fluorescence. These are used to set up a standard curve relating fluorescence intensity to Antibody Binding Capacity. Once you've generated that curve, you can read directly from it the ABC values of all the samples that you run.

Just like the Quantum Simply Cellular anti-Mouse Antibody Binding Standards, the new product is offered in 20, 100 and 280 test sizes. Don't let the "anti-human" name fool you - the beads are actually quite friendly!

Freezing Prevention

Shipping Methods

Volume 15, #4
December 2002

Q: How do you avoid freezing - especially during the winter months?

A: I dress warmly myself! And we "dress" our microspheres in packaging and proper labeling to protect them from **freezing**, which is effective except where they are inadvertently left out on a loading dock over the weekend or advertently (intentionally) put in the freezer by some helpful receiving department person, "to keep them safe overnight." We also carefully choose the shipping method and timing, shipping by overnight carriers who will handle our products carefully and get them to you before they can freeze. In "Freezin' Season" we do not ship on Fridays, so your package will not sit on a loading dock over the weekend anywhere. (You *will* have to worry about that guy in receiving, though!)

Blocker Concentration

Buffers

Conformational Changes

IgG Coating Levels

Reagent Stability

References

Volume 15, #3
September 2002

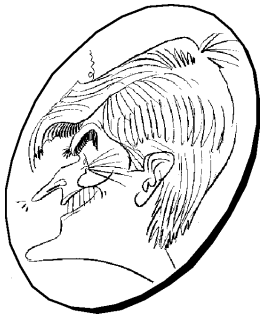
Q: Help! My microsphere reagent has lost activity only one month after adsorbing IgG and blocking BSA. What happened? What can I do?

A: Your problem with reagent stability could be due to one or more of the following:

1. **Concentration of BSA in storage buffer** (too much BSA might cause competition between BSA and IgG molecules);
2. **Level of IgG coating** (perhaps using a higher concentration of protein will allow you to load more on the surface, making it less accessible to BSA molecules in the buffer);
3. **Use of BSA as a blocker** (other blockers might not compete as efficiently for the surface);
4. **Use of PBS buffer** (other buffers might provide improved storage stability).

Another factor to consider is that proteins tend to become more tightly adsorbed to surfaces over time. The loss of activity that you have observed may be caused by this phenomenon - as molecules become more tightly adsorbed, they undergo conformational changes that can reduce activity. If this is the case, using a higher IgG concentration might improve loading, and force molecules into a crowded, upright position.

To investigate, you might utilize a total protein assay to determine if the protein level is remaining constant (on the beads or in the supernatant). If it remains constant, this might indicate competitive desorption (replacement of IgG molecules with BSA molecules), or loss of activity due to conformational changes of protein. See our TechNote 205, *Covalent Coupling*, for references and suggestions on assaying beads for protein load and activity. Here are some other references:



Puela, J.M., et al. 1995. Coadsorption of IgG and BSA onto sulfonated polystyrene latex: I. Sequential and competitive isotherms. *J Biomater Sci Polym Ed,* 7(3):231-240. PubMed ID: 7577826.

Puela, J.M., et al. 1995. Coadsorption of IgG and BSA onto sulfonated polystyrene latex: II. Colloidal stability and immunoreactivity. *J Biomater Sci Polym Ed,* 7(3):241-251. PubMed ID: 7577827.

Zalazar, F.E., et al. 1992. Parameters affecting the adsorption of ligands to polyvinyl

chloride plates in enzyme immunoassays. *J Immunol Methods,* 152(1):1-7. PubMed ID: 1640104.

Adsorption

Equations

Ligand/Bead Calculation

Parking Area

Surface Area

Surface Monolayer

Volume 15, #3
September 2002

Q: How many protein molecules can I adsorb onto 1µm microspheres?

A: If we assume that you are packing them tightly like a monolayer, then you should be able to calculate the number of protein molecules per bead as follows:

1) From the Stokes diameter of the protein molecule, you can calculate that a spherical protein molecule would occupy an area (cast a shadow) of $\pi d^2/4$. If the diameter of IgG is 10nm, then its parking spot on a microsphere would be 78.5 sq. nm.
2) The surface area of a microsphere is πD^2. Then a 1µm (1000nm) microsphere has 3.14×10^6 sq. nm. (or 314×10^4 sq. nm.) of surface area.
3) You can therefore expect to be able to pack a maximum of $\pi D^2 / \pi d^2/4 = 4 (D/d)^2$ molecules per sphere. In this case, it would be $314 \times 10^4 / 78.5 = 4 \times 10^4$ IgG molecules on each microsphere.

Remember, different proteins will have different affinities for a bead surface. Also, more isn't always better, but depends on conformational changes and steric effects. *You* must test to determine how much adsorption is needed for best performance.

NHS Esters

Particle Determination

Technical Literature

Volume 15, #3
September 2002

Q: What size amine microspheres should I try for coupling reactions with NHS esters?

A: *Of course, the devil in me suggests that you try all the sizes!* But seriously, folks, for a proper response, we must ask you for more information: What do you want to do with the microspheres? What sort of assay or application do you have in mind? The size of bead is typically dictated by the application or assay format, etc. Size will impact bead handling, surface area (area for immobilization of biomolecule), settling times, etc. For example, flow cytometric tests and assays typically make use of beads that are ~2-8µm, strip tests typically require beads that are 0.1-0.4µm. Our 300 series of TechNotes describes a number of applications with usual bead sizes noted. Also see TechNote 402 (published article with a link to the publisher's website), which contains recommendations on bead sizes for a number of formats. (Of course, all our TechNotes may be downloaded from our website, www.bangslabs.com.)

Calibration

GFP/EGFP

Volume 15, #2
June 2002

Q: I'm looking for some beads to calibrate my cytometer in terms of GFP molecules. I heard that you made such a kit, but I can't find it on your website.

A: Although we previously produced a quantitative EGFP microsphere standard for BD Clontech, this was discontinued after 2004 due to licensing. The development of GFP microsphere calibration standards is covered by US Patent #6,326,157, Recombinant fluorescent protein microsphere calibration standard.

Coupling Efficiency

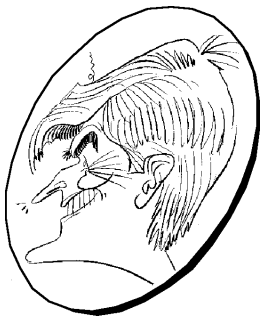
Oligo Binding

References

Volume 15, #2
June 2002

Q: After oligo coupling, how can I determine the efficiency of the oligo immobilization?

A: There are a number of methods for assessing binding efficiency. 1) Binding capacity of oligo dT beads may be determined by binding mRNA and measuring the amount of eluted RNA by wavelength scanning (see our TechNote 302). 2) The amount of bound oligonucleotide may also be estimated by hybridizing it to its biotinylated complement, with detection via a streptavidin-labeled fluorophore. [A similar approach is detailed in: Kumar, A., et al. 2000. *Nucleic Acids Res,* 28(14):e71. Available for free download from the journal website.] 3) Use radiolabeled probes. [Lund, V., et al. 1988. *Nucleic Acids Res,* 16(22):10861-10880; Day, P.J.R., et al. 1991. *Biochem J.* 278:735-740.] 4) Nucleic acid dyes/stains may also be used to determine the amount of DNA attached to beads (or unbound in solution). Walsh, M.K., et al. 2001.



J Biochem Biophys Methods, 47: 221-231 describes use of Molecular Probe's OliGreen for DNA quantitation (suitable for ssDNA and oligonucleotides).

Carbodiimide Reaction

CML Binding

EDAC

Ethanolamine

Quenching

Surfactant

Two-Step Coupling

Volume 15, #2
June 2002

Q: 1) When coupling to a -COOH surface in a two step carbodiimide (EDAC) reaction (where step 1 = EDAC activation of beads in MES pH 4.5 and step 2 = Protein coupling in Carb/bicarb pH 9.6, with excess protein), is there really a need to block the unreacted sites with ethanolamine? 2) Can a similar two step EDAC reaction scheme be used with the encapsulated magnetic particles? I have a protocol that shows diethanolamine at pH 10.5. Is diethanolamine pH 10.5 preferred? 3) What is the surfactant used with the -COOH beads? Will this interfere with the coupling reaction? Is pre-washing recommended? I know, there are really seven questions, not a couple, as I bunched a few together. But, I figured if I worded it 1-7, then by the time you got to #7, you might have inadvertently hit the "delete" button. Thanks for your help.

A: 1) Some protocols call for blocking of unreacted sites; others omit it since the reactive intermediary will hydrolyze back to COOH anyway. You may wish to determine empirically the need for quenching (e.g., the impact on nonspecific binding). 2) A two-step protocol may be used for magnetic particles. The reactive group, particle size and MW of biomolecule will be more important than the composition of the base particle when deciding between one- and two-step protocols. For example, particle size will in part dictate the wash method that is utilized, and whether washes can be accomplished relatively quickly, before the reactive intermediary hydrolyzes. 3) Our TechNote 205 has a more typical (but generic) COOH bead/EDAC/MES buffer procedure. You might have better luck this way (with some optimization), as the optimal pH for an EDAC reaction is considerably lower, i.e. 4.5-7.5. Also, an amine buffer (ethanolamine) might interfere with the reaction between bead/COOH and ligand/NH_2. 4) The surfactant utilized for the synthesis of COOH-functionalized microspheres may vary by lot. As this is something that is generally held as a trade secret by manufacturers, we can't advise with certainty on the specific surfactant utilized, or its concentration. 5) We highly recommend the 'pre-washing' of microspheres. If use of surfactant is indicated (i.e., if beads are aggregating or clinging to pipette tips and tubes), we generally recommend using the lowest possible concentration of a non-ionic surfactant such as Tween® 20 (0.01%).

Biotin Elution

Competitive Detachment

References

Streptavidin-Coated Microspheres

Volume 15, #2
June 2002

Q: OK, I bound SA to your beads and it really grabs the biotinylated thingamabob that I wanted to bind, but enough already! I want to recover my Biotin-Thing conjugate. Make the SA let go! It's hanging on like a 3-year old kid!

A: I am glad to learn that you got good binding. Also, I located a reference that cited use of biotin to competitively detach biotinylated molecules - from modified avidin. [Morag, E., E.A. Bayer, M. Wilchek. 1996. Reversibility of biotin-binding by selective modification of tyrosine in avidin. *Biochem J,* 316 (Pt 1): 193-199. PubMed ID: 8645205] Also, the prebinding of biotin before binding of biotinylated ligand has been cited much more extensively, i.e., it is the basis of several competitive assays. This may help control the level of loading when using streptavidin-coated beads.

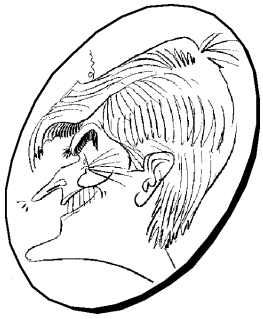
Photobleaching

Quenching

Volume 15, #1
March 2002

Q: What is quenching? Is it the same as photobleaching?

A: Quenching and photobleaching are two different phenomena. Quenching is the loss of fluorescence intensity due to interference between fluorochromes. It usually occurs when fluorochromes are closer together than 50 angstroms – too many dye molecules per bead. Quenching is a reversible proximity issue. Photobleaching, on the other hand, is an actual alteration or destruction of the fluorochrome by the excitation energy or ambient light – like your favorite shirt that just fades in sunlight. That said, if you can find a way of quenching photobleaching, please let us know!



pH

Q: What do you know about the stability of the streptavidin/biotin complex vs. pH? After binding of SA/B, I want to do a reaction at acidic pH ~3.5.

References

Streptavidin/Biotin

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A: You should be OK. The book **Avidin-Biotin Chemistry: A Handbook** notes: "This complex is not significantly affected by pH values between 2 and 13 nor by concentrations of guanidine HCl up to 8M at neutral pH's." This information was originally reported in "Avidin", Green, N.M., 1975. *Advances in Protein Chemistry,* 29: 85-133. 1975. New York: Academic Press (Eds. C.B. Anfinsen, J. T. Edsall, F.M. Richards).

IgG Binding

Latex Agglutination Tests

Volume 15, #1
March 2002

Q: How do I make a latex agglutination test for an internal QC check?

A: Here is some general information regarding latex agglutination tests: 1) LATs make use of microspheres in the range of 0.2-1.0µm. 2) You can calculate material needs (latex, antibody and antigen) for each test from the following data: a) it takes ~100 latex clumps to judge agglutination, b) each clump must be ~50µm in size to be seen by the eye, c) ~10 bonds are required per microsphere to agglutinate them, and d) sample size can be as small as 10µL. See TechNote 301, *Immunological Applications*, for more details.

When you want to bind IgG antibody to microspheres, there are a variety of options. Included are: direct adsorption or covalent coupling of the antibody to the microsphere. Or, you might want to use beads that are precoated with a generic binding protein - perhaps streptavidin plus a biotinylated IgG of your choosing. A discussion of binding strategies is provided in our TechNote 201, *Working with Microspheres*. TechNotes 101, *ProActive® Microspheres*, 204, *Adsorption to Microspheres*, and 205, *Covalent Coupling*, may also help as you consider the development of a coating protocol. Each contains sample protocols. A complete list of TechNotes can be found and TechNotes can be downloaded from our website, www.bangslabs.com.

Brightness

MESF Values

Volume 15, #1
March 2002

Q: What is the value in knowing the brightness of a sample in **Molecules of Equivalent Soluble Fluorochrome (MESF)**?

A: Equal numbers of fluorochrome molecules do not necessarily have the same brightness. Brightness needs to be corrected for changes in extinction coefficient, quenching and small spectral shifts. MESF units account for most of these environmental corrections. For example, a cell with a very high expression of a given marker may be labeled with 2,000,000 FITC molecules, but due to quenching, may exhibit the fluorescence intensity of only 1,500,000 FITC molecules in solution.

Adsorption

Affinity Binding

Coating Beads

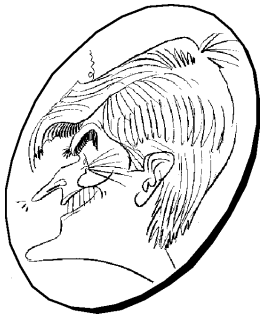
Covalent Coupling

Volume 14, #4
December 2001

Q: Do you have a suggestion as to what is simple and easy as far as coating antibodies to beads? Do you need to know what type of antibody I plan to use?

A: The basic bead (surface) choices for conjugation include non-functionalized (for adsorption protocols), functionalized (for covalent coupling) and protein-coated (for affinity binding). Each has its benefits, which you will need to weigh in the context of your work/timetable/etc. Our TechNote 201, *Working with Microspheres*, provides a discussion of the benefits/drawbacks of the different binding strategies. You may also wish to view our TechNotes 101, *ProActive® Microspheres*, 204, *Adsorption to Microspheres*, and 205, *Covalent Coupling*, for further discussion/sample protocols. The TechNotes may be downloaded from our website, www.bangslabs.com.

Briefly, covalent coupling of the Ab to COOH functionalized beads should result in a very stable reagent; however, covalent coupling protocols generally do require some optimization (protein and activator concentrations, incubation times/temperatures, buffer pH, blocking). Conjugation of ligand to protein-coated beads generally requires little optimization, if any; however, the initial cost of microspheres will be slightly higher, and the reagent may not be as stable (although this is highly dependent upon the protein coating and the bead environment). The streptavidin/biotin system is very stable; however, biotinylation of the antibody (with potentially some optimization of this reaction) or purchase of biotinylated Ab would be required.



If your Ab is IgG, a covalent coupling protocol should be relatively straightforward

(and published protocols are readily available). If you are binding a different Ig (e.g. IgM), the chemistry will be a bit more involved.

Binding Capacity

Streptavidin-Coated Microspheres

Volume 14, #4 December 2001

Q: Is it possible to bind just one biotinylated fluorophore molecule to a streptavidin-coated microsphere?

A: Binding capacities of small microspheres will, in general, be greater than those of larger microspheres due to increased surface area per unit weight. Researchers performing single molecule imaging work (DNA) have utilized our low-binding SA-coated microspheres with success (better results for their applications than some of the higher binding beads). Please note that, regardless of binding capacity of the microspheres utilized, optimal binding conditions (to achieve minimal binding, or binding of a single molecule) will likely need to be determined empirically.

Some references regarding use of streptavidin-coated microspheres in single molecule imaging (AFM) studies are available from our literature library at your request. Orders for beads may be placed through our Customer Service Department (phone, fax, email: info@bangslabs.com), or at our website, http//:www.bangslabs.com.

Particle Sizing

Volume 14, #3 September 2001

Q: Can you please advise how to use our new HIAC / Royco instrument to measure your microspheres?

A: *What's the matter? Don't you trust our numbers?* Previously, we used an Accusizer 770 (Particle Sizing Sytems), which depends upon the same methodology, i.e. photozone method, as the HIAC/Royco. It was at its best with very dilute samples. For the Accusizer 770, we used ~100-500 microspheres/mL. *(Note: We used much more dilute suspensions than the manufacturer recommends.)* The use of dilute suspensions lowers coincidence (multiple particles in the chamber) for instruments that are intended to measure single particles, thereby ensuring more accurate results.

Once you have had an opportunity to review the user manual for your instrument, and perhaps speak with Particle Technology Laboratories in Chicago (our expert guide in this area), please feel free to contact us for the selection of standards that are suitable for your instrument.

Freezing Prevention

Microbial Growth

Shipping Methods

Sodium Azide

Storage

Volume 14, #3 September 2001

Q: I want to send some microspheres to a colleague in Spain for a limnology project in Antarctica. Can they withstand 3-4 days without refrigeration?

A: The short answer is "Yes, but..." The longer answer is, "It depends." And the more correct and complete answer is that to minimize any microbial growth, we always recommend that beads be stored refrigerated (at 2-8˚C), in conjunction with the use of preservatives, such as sodium azide or merthiolate. Room temperature storage is acceptable, too. If uncoated beads are stored properly and handled by aseptic techniques, they should have a shelf-life of more than 5 years. Anyway, it should be possible to ship from the US to Spain without refrigeration, but you could add some ice to the package to ensure cooler travel.

In Antarctica's harsh climate, the bigger hazard will be freeze damage to the beads. Plain, singly-dispersed beads can be turned into badly clumped beads resembling cottage cheese if they are frozen. *So, don't sweat the bugs down there, but do guard against freezing!*

Bicarbonate Buffer

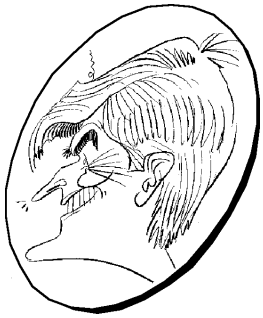
Biotinylated Oligonucleotides

Nonspecific Binding

References

Q: What's the best way to optimize binding of biotinylated oligos to your streptavidin-coated beads, while minimizing NSB?

A: A customer recently advised us about his use of a bicarbonate buffer for the binding of biotinylated oligonucleotides to our SA-coated microspheres, which (in his hands) effectively eliminated NSB (nonspecific binding). For buffer and binding conditions, see Bianco, P.R., L.R. Brewer, et al. 2001. Processive translocation and DNA unwinding by individual RecBCD enzyme molecules. *Nature,* 409: 374-378.



Streptavidin-Coated Microspheres

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September 2001

Other conditions that have been said to reduce nonspecific binding include increased salt concentration, increased pH, decreased probe concentration (or decreased streptavidin binding on the bead). Please note that we do not routinely work with (biotinylated) oligonucleotides in house, and thus have not optimized binding conditions for such.

Blockers

PEG

Protein Adsorption

References

Volume 14, #3
September 2001

Q: How do I attach PEG to microspheres to deter/prevent adsorption of cytoplasmic extracts or other protein adsorption?

A: There appear to be several articles in the literature about attaching PEG via covalent coupling or adsorption to inhibit protein adsorption. Here are two articles that might be of particular interest:

Satulovsky, J., M.A. Cargnano, I. Szleifer. 2000. Kinetic and thermodynamic control of protein adsorption. *PNAS,* 97(16): 9037-9041.

Szleifer, I., J. Satulovsky. 1999. Kinetic and thermodynamic control of protein adsorption by grafted polymer layers. *Polymer Preprints-America,* 40(2):89-90. (Some of their work pertains directly to the attachment of PEG to surfaces to prevent protein adsorption; if these articles are not of direct relevance to your work, their 'references' sections may prove to be helpful.)

Loss on Drying

Solids Concentration

Turbidity

Washing Beads

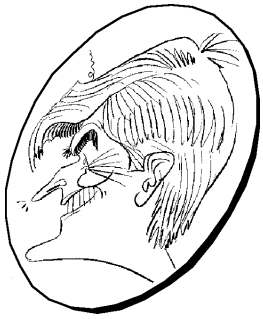
Volume 14, #2
June 2001

Q: In the process of cleaning microspheres, it seems likely that you will change the solids concentration and lose a fair fraction of particles - especially with small amounts of latex. How do we determine the solids concentration after washing?

A: First, we agree that it *is* important to know the amount of particles you have at any stage of your process- to know the weight of particles per mL, so they can be handled conveniently, and to control your process for addition of the proper amount of wash solution and coating materials. Second, you *will* lose some particles in washing - more or less, depending on which kind of particles you have. "Less" if you are working with monodispersed size microspheres which should all behave the same. While there will always be some losses in transferring things, there won't be too many losses unless the particles are caught in filters. More will be lost if you are working with smaller samples and smaller particles (harder to handle). Losses will also depend on your cleaning process. (*Of course, we won't mind if you want to buy more microspheres to ensure that you will have enough.*)

The only reliable way that we know of to measure solids content is by **loss on drying**. We have found good reproducibility and agreement with others' measurements by using as little as 100µL of a well-dispersed (completely resuspended as single particles) suspension at ~10% solids (100 mL at 10% solids = 10 mg which requires a pretty good balance). If solids content is ~1%, then it will certainly take more sample to get good measurable solids. This method may be poorer for low % solids, but it works and it's our method of choice. Automated particle counters are also useful for determining concentrations.

We shouldn't mention any *bad* ideas, but occasionally somebody tries to use a spectrophotometer to measure solids content by measuring the turbidity of particle dispersions. This is a *bad* idea because turbidity depends on particle size and degree of dispersion, *as well as on particle concentration*. Thus, the absorbance of a 1% solids dispersion of *single* microspheres will be significantly different than 1% solids suspension of *doublets*. And, 1% of 0.2µm microspheres will be very different from 1% of 0.8µm microspheres. Also, everything else in the aqueous phase and on the beads will influence the absorbance. This is a really dangerous method - like skiing out of bounds - you are really in avalanche territory!



It **might** be possible to devise a method whereby microsphere solids and water are dissolved in some solvent (maybe DMF?) and measured in a spectrophotometer at a wavelength sensitive to polystyrene. (I do not know of such a method, I'm only *"composing at the keyboard."*)

Dilution

Suspending Dry Microspheres

Volume 14, #2
June 2001

Q: I have purchased BLI microspheres both dry and in suspension. Please advise how to dilute a suspension of microspheres and how to suspend the powdered microspheres; especially, what solutions are needed?

A: Microspheres may be diluted using the buffer they come in (often DI water), or the buffer of choice, following centrifugation or other separation method (see our TechNote 203, *Washing Microspheres*, for more information regarding separation methods). Additional buffer may be added directly to the suspension, or may be added to the pellet following centrifugation.
To suspend dry microspheres, add the buffer of choice and mix (e.g., using an end-over-end mixer, roller, vortexter or sonicator [very carefully]). Surfactant may be added if aggregation is observed - see our TechNote 202, *Microsphere Aggregation*, for details. The duration of the mixing process will depend upon the size and amount of microspheres, i.e. anywhere from a few minutes to a few hours of mixing may be required. You may wish to periodically check the progress of the suspension, i.e., for the presence of aggregates, through microscopy.

DNA Adsorption

References

Silica Adsorption

Silica Microspheres

Volume 14, #2
June 2001

Q: Your TechNote 104, *Silica Microspheres*, says it's possible to reverse the charge of silica (from - to +) to adsorb negatively charged DNA. Do you have any references?

A: Well, it turns out that Leigh Bangs (who suggested rinsing clean silica in a 0.1-1 M $CaCl_2$ solution) hasn't been lying to us all these years. Here is an actual reference: Romanowski, G., et al. 1991. Adsorption of plasmid DNA to mineral surfaces and protection against DNase. *Appl Environ Microbiol,* 57(4):1057-1061. Mg^{++} or Ca^{++} were 100X better than Na^{+}, K^{+}, or NH_4^{+} in the adsorption of plasmid DNA onto sand, indicating a charge-dependent process.

Carboxylated Beads

Parking Area

Surface Titration Value

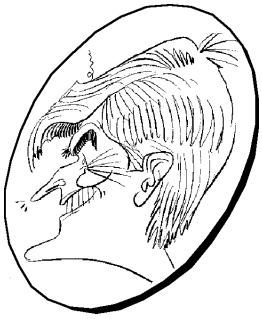
Volume 14, #1
March 2001

Q: We're using some of your COOH-modified microspheres and I just encountered the term "parking area". What's that? Are you guys running a parking lot?

A: *Yes, a parking lot for molecules!* Actually, the "parking area" permits one to compare particles with different titration values (meq/g or µeq/g) and different diameters for their surface charge density, which relates to their relative stabilities and binding capacities for proteins. Calculated parking areas ($Å^2$/ charge group) are the reciprocal of the surface charge density (groups/$Å^2$ or groups/nm^2), and are calculated from the diameter and titration of surface charge of clean microspheres.

If the parking area for any lot of microspheres is ~20 $Å^2$/COOH group, then the microspheres are assumed to be covered with a monolayer of COOH groups. This number comes from the packing density for a close-packed monolayer of fatty acids at an air-water interface. The number for sulfate groups would be about the same; primary amino groups might be smaller.

One envisions a model where all the microspheres are the same diameter and the charged groups are neatly arranged closely-packed on the surface of the microspheres. This is strictly true only for sulfate-modified microspheres and for certain COOH-modified microspheres which have charged groups only at the ends of the polymer chains. Most of our COOH microspheres are made by copolymerizing a small portion of acrylic acid with styrene (S/AA). In this case, the COOH groups are on random polymer chains which will tend to arrange themselves with the hydrophilic COOH groups in the aqueous phase and at the microsphere surfaces. The COOH groups are certainly not arranged as a neat monolayer here, but probably exist as hydrophilic chains attached at one or both ends and extending out into the aqueous phase, rather like tennis ball fuzz. The ~20$Å^2$/COOH group "rule" can very easily be violated here, of course.



If particles with non-uniform size (like our magnetic particles) are considered, then the model, based upon a calculation on spheres of one diameter will fail again, due to errors in estimating surface area per gram.

With S/AA microspheres, often only about half of the acid which is added actually appears on the particles; the rest ends up as water-soluble polymer (WSP, or polymer with so much acid that the chains are fully water-soluble and they completely escape

the particle surface). WSP is removed by ion exchange cleaning of the particles before they are titrated. Of course, some acid may also be buried in the interior of the particles and not titrated.

We report titration values (µeq of COOH/g) and then calculate **apparent** parking area and report it for most lots of these beads. (For more on this topic, see TechNotes 201 and 206).

Coating Beads

Cross-Flow Filtration

Isopropanol

Volume 14, #1
March 2001

Q: We could deposit our special hydrophobic coating on your beads from isopropanol. Would that be okay for polystyrene? If so, are there other ways to get the microspheres out of aqueous solution, besides centrifuging? And finally, since I can work with large sized spheres, what would be an ideal size to remove water and then deposit our coating?

A: 1) Isopropanol would be fine for the beads. And, you could add it to the beads directly because it mixes with water.
2) Cross-flow filtration is a good way to remove some (but not all) solvent - water or isopropanol.
3) If you used >1µm beads, they would be very easy to spin down and to resuspend. Larger beads will also settle easily and rather quickly without centrifuging (the larger the bead, the faster they will settle, of course.) They will also settle faster in isopropanol than in water. You can do "dead-end" or bed filtration of large beads more easily, too. (Flow through a filter cake is much easier with 1µm beads than with 0.1µm beads.) But, since they have a lower specific surface area, you might need to use more beads. I would suggest choosing the size by what surface area you need and by how easy it will be to clean them before and after coating. (For more information, see our TechNotes - especially 201, 203, and 206.)

Binding Protein

Covalent Coupling

Magnetic Beads

ProActive®

Thiol Preservation

Volume 13, #4
December 2000

Q: How do I bind a protein, via the protein's carboxyl or amino terminus, to 2-9µm magnetic beads and then use these beads for pulling out antibodies that bind to protein? (I don't want to use a chemistry that might modify the cysteine residues on my protein). Also, would it be easier to use preactivated beads that I can just react with my protein so I don't have to do the chemical reactions myself?

A: *You folks keep sneaking in two questions. OK this time.* 1) To avoid the possibility of binding to the cysteine residues on the protein, try our COOH-functionalized mag beads. Activate the beads with EDAC and NHS; wash the microspheres; and add protein. Unlike the o-acylisourea intermediate formed by the EDAC reaction, the succinimidyl ester formed by the NHS will not react with thiols. (See our TechNotes 205, *Covalent Coupling*, and 102, *Magnetic Microspheres.*) 2) Our largest magnetic microspheres are ~8µm. Orders may be placed through our website or by contacting our Customer Service Department. 3) If you can biotinylate your protein, then you could choose our streptavidin-coated ProActive® magnetic beads. (Ask if you need help.)

BioMag®Plus Concanavalin A

Coating Beads

Concanavalin A

Volume 13, #4
December 2000

Q: As a complete novice to beads, I would like some advice on how to coat them with Concanavalin A. Passive adsorption? On what kind of beads? Or is covalent coupling a better option?

A: *Wow, three questions this time!* 1) For general advice on handling beads and especially covalent coupling vs. adsorption, see TechNote 201, *Working with Microspheres*. Passive adsorption on PS beads works best with larger proteins, like IgG, which stay adsorbed; otherwise, try COOH-modified beads and EDAC coupling. See TechNotes 204, *Adsorption to Microspheres,* and 205, *Covalent Coupling*. (BioMag®Plus Concanavalin A are also now available.)

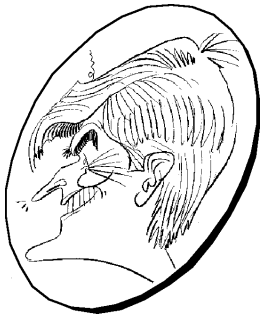
Aggregation

Covalent Coupling

Non-Ionic Surfactant

Small COOH-Modified Beads

Q: Why do the < 50nm COOH-modified fluorescent-dyed particles clump when I try to couple protein to them? Is it the dye?



A: The inability to successfully couple to the small COOH-modified particles is somewhat puzzling. Surely it is more difficult working with these tiny particles, but you should be able to keep them dispersed in surfactant. You raise several interesting points:

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1) The particles are stabilized with negative surface charge-sulfate-groups from the emulsion polymerization initiator, sulfonate or sulfate groups on the surfactant used to emulsify the monomers, and the COOH groups from the vinyl carboxylic acid comonomer added with the styrene. Even if all the surfactant is removed as by washing, the particles should be stable in deionized water from the $SO4^-$ and COO^- surface groups. If necessary, you can add a very small amount (0.001-0.01%) of nonionic surfactant (like Tween® 20 or Triton X-100) to further assist in stabilizing particles. Keep surfactant concentration as low as possible, so as not to interfere with protein binding. Keep the ionic strength of all solutions in contact with the particles as low as possible to avoid aggregation. Keep the particles as dilute as possible to minimize chance of aggregation.
2) Dyed particles may have less surfactant after the dyeing process and the attendant clean-up to remove dye from the aqueous phase.
3) You did not mention how you were coupling your protein, so we will assume water-soluble carbodiimide (WSC) coupling. Adding protein and WSC should not destabilize the particles. If you add protein first, it should adsorb on and assist in stabilizing the particles. Also, you may have better luck if you add the particles to the protein solution, rather than protein to particles (order of addition is often important in colloid chemistry).

Binding IgG

COOH-Modified Beads

Covalent Coupling

Equilibrium Adsorption

Maximum Adsorption Rate (IgG)

Monolayer

Protein Loading

Q: How can I covalently bind more IgG on your carboxylate-modified beads? I used 3-5 g IgG/liter and didn't see much difference in protein concentration before and after coupling.

A: Here are a few ideas for how to load on more protein:

1) The particles should have plenty of carboxylic acid groups on their surfaces - usually about a monomolecular layer of acid groups covers the particles.
2) Our particles also have sodium dodecyl sulfate added to ensure uniform dispersion. This surfactant will be adsorbed onto the particles. We recommend that you clean the particles and remove surfactant to make more room on the surface for the protein.
3) For particles with mean diameters of ~1µm, the specific surface area (m^2/g) is ~6/d, where d is diameter in µm. Thus, 1µm particles have ~6 m^2/g. The maximum adsorption rate of IgG onto polystyrene is ~2-3 mg/m^2 (this is a monolayer of complete coverage of protein). Therefore, one can put on ~6 x 3 = 18mg of IgG/g of ~1µm particles. (If you used ~1mg IgG/10mg of particles you should have ~5-fold excess over monolayer amount (~0.18mg protein/10mg of particles) or plenty to ensure complete coverage.

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4) Equilibrium adsorption of BSA onto polystyrene occurs at ~0.1g BSA/liter. One must add enough protein to achieve monolayer coverage and with as little as 0.1 protein/liter one can maintain equilibrium and keep the protein on the particles. Your 3-5mg of IgG/mL (3-5 g/L) solution is much more concentrated than necessary. So, you may see no detectable difference in absorbance of a solution before and after coupling.
5) The recipes in our *Covalent Coupling* TechNote (TechNote 205) should be used with your own good judgment. Feel free to change buffers, pH's, and concentrations of ingredients to optimize conditions for your specific situation. You might try to simply *adsorb* the IgG onto the particles - just to see if you get any more protein onto the particles by adsorption. This would tell you if the coupling chemistry is at fault somehow.

Custom Coupling

DNA Attachment

ProActive®

Silica Microspheres

Q: 1) Do you have any polystyrene beads with DNA covalently attached to the surface? I notice that you sell PS beads with certain functional groups, proteins, and fluorescent labels. 2) DNA probes/PCR was listed as one of the applications of the beads. Does it mean that a DNA primer is attached to the surface of the bead or just via electrostatic interaction via a functionalized bead. Please, let me know so I can decide what kind of beads to buy and if any chemical modification has to be performed on the beads.

A: We don't offer microspheres with DNA directly attached to the surface, but do have various types of microspheres to which DNA can be easily attached. 1) Our silica beads are useful for nonspecific attachment of DNA (Reference 1, Volume 13, #2, June 2000). 2) You can electrostatically or covalently attach DNA to our carboxyl-modified polystyrene microspheres (Reference 2, Volume 13, #2, June 2000 and TechNote 205). 3) You can biotinylate the DNA and attach these to our ProActive® streptavidin-coated microspheres. (See TechNote 302 and Kathy Turner's 2000 AACC OEM Lecture; available on our website.) We can also talk about custom-coupling your favorite material to our beads

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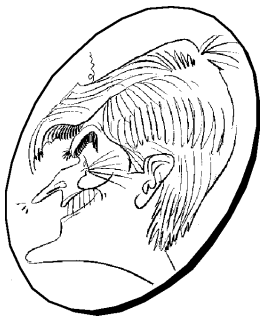


Absorbance

Agglutination

Light-Scattering Assays

Volume 13, #1
March 2000

Q: Upon agglutination of 0.8µm beads, the absorbance went down and I expected it to go up.

A: *Kinda like the stock market, eh; you didn't expect a downturn?* Let me reassure you that what you saw was probably normal. You didn't say what wavelength of light you were using, but, there are two possibilities. 1) If the bead diameter is much lower than the wavelength of light used, then the beads may not scatter light very well (e.g., 100nm particles and 300nm light). On agglutination, however, they grow to a size where they do scatter light and the absorbance will increase. 2) Beads which are larger than the wavelength of light will scatter that light well (e.g., 800nm particles and 400nm light). On agglutination, there are fewer "scatterers" around to scatter the light, and the absorbance drops. Thus, it is normal for absorbance to drop on agglutination unless you were using far-out infrared light. *Feel better now?*

Particle Determination

PETIA

ProActive®

Turbidimetric Assays

Volume 13, #1
March 2000

Q: We are interested in developing a **particle-enhanced turbidimetric immunoassay (PETIA**). The monoclonal antibodies (mAb's) we have are only suitable if they are coated to latex beads. What type of latex beads would you suggest?

A: First, try some ~100nm PS beads with direct adsorption of mAb plus adsorption of blocker to fill in empty spaces between mAb. (See TechNote 204, *Adsorption to Microspheres*.) Some mAb's do not adsorb well directly, and they must be covalently bound. For that, try COOH-modified beads of the same size. (See TechNote 205, *Covalent Coupling*.)

To get a product more quickly, try some of our goat anti-mouse (GAM)-coated ProActive® beads. Our GAM was selected and tested for binding to almost all common mAb's.

Settled Microspheres

Suspension Dispersity

Volume 13, #1
March 2000

Q: I had to use twice the amount of antibody (Ab) to sensitize the microspheres in the last 10% of the bottle. What went wrong?

A: It could be several things:

1) Incomplete dispersion of any settled microsphere solids could yield a more concentrated latex by the end of the bottle and more solids would require more Ab. If solids have settled to the bottom of the bottle, then, before every aliquot is removed, we recommend several hours of rolling the bottle to thoroughly resuspend the beads and to ensure that any clumps are broken up, so you will have singly dispersed beads. (Rolling action avoids foaming which can result from shaking.)
2) Beads can become concentrated by evaporation after repeated bottle openings and a possibly loose cap sometime. Higher solids requires more Ab/mL to coat. And, if evaporation occurred, then the increased concentration of surfactant could also interfere with the adsorption of protein and yield a less stable product.
3) Although not related to your problem, microbial contamination can result from continual reopening of a bottle of beads. Azide or merthiolate will prevent this. We believe that the particles are stable for years or even decades, unless contaminated. Surfactants are chemically stable, too.

Thus, we encourage customers to add antimicrobial to the beads and to repackage them into appropriately sized aliquots as soon as they receive them. We can also prepare aliquots of any size and add your favorite antimicrobial when we prepare your order, if you wish. (*Hint:* One of our customers has their beads packaged in aliquots which exactly fit their production recipe. So, no need to measure beads during production. They just pour it in!)

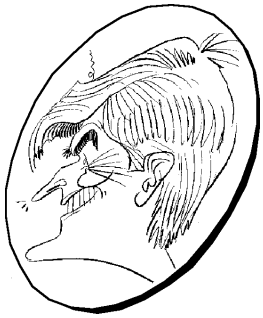
Desorption

Protein Quantification

Volume 13, #1
March 2000

Q: I have coated protein on some 0.2µm dyed beads by simple absorption. I would like to know if there is any way to desorb the protein from the beads, so that a quantification of these proteins is possible.

A: Removing protein from PS beads is about as much fun as getting chewing gum out of your sister's hair! There are recipes using high surfactant, temperature, and pH, but you are likely to get incomplete removal and what you remove may very well be destroyed in the process.



It may be easier to measure protein in solution (with BCA or other protein reagent - from Pierce or others) before and after adsorption on particles. Then, by difference, you will know how much adsorbed. You may also use a total protein assay directly with the coated beads.

Agglutination

DNA Attachment

DNA Hybridization

Latex Agglutination Tests

Volume 12, #4 December 1999

Q: How can I make agglutination tests for DNA with ~1µm microspheres? How fast can one perform such a test?

A: *Still trying to sneak in two questions at a time, aren't you?*

1) DNA hybridization based tests and assays can be made which are analogous to antigen-antibody tests and assays. If the microspheres were coated with a complementary strand of DNA or RNA, they could react with the target DNA or RNA in a sample. We know of some DNA agglutination work and DNA strip tests. BUT (that was a *big* but), the method of binding the DNA onto the beads will be critical to your success.

   If you bind by adsorption, then the DNA will probably be bound by several or many points of contact to the beads (analogous to the adsorption of proteins onto polystyrene). Will the target DNA bind to complementary DNA which is bound by several points of attachment? Alternatively, will the DNA stay bound if you try to hybridize while it is on the beads?

   It may be necessary to bind to the beads by covalent attachment by one end of the complementary DNA molecule, so it is free to hybridize properly (See TechNotes 205 and 302 for more information.)

2) The old "latex" agglutination tests (LATs) - using 0.3-0.8µm particles or microspheres at ~1% solids on glass or paper slides - take about 2-3 minutes with active rocking or rotating. With 1µm particles, it should be about the same time. For reverse passive agglutination (RPLA) tests done in 96-well plates (like hemagglutination tests), it may take 1 hour or longer, depending on particle and liquid density, since the particles must settle to the bottom of the wells and form a lacy pattern or button. Of course, those estimates were for protein-based immunoassays. For DNA/RNA-based assays, more time may be required for the strand hybridization reaction to occur. (See our TechNotes 201 and 301 for more answers about LATs.)

Ab/Ag Binding Reactions

Binding Quantities

IgG Spacing

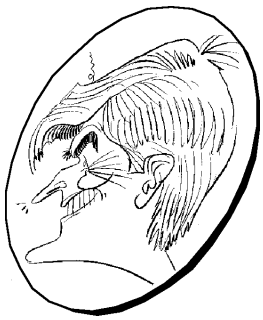
Latex Agglutination

Volume 12, #4 December 1999

Q: I have successfully coated some plain PS beads with polyclonal rabbit IgG using your advice. I coated 250µL of 10% ~250nm beads with 0.8mg of IgG (I added 2mg, but only 0.8 bound = 1/2 the capacity of the beads by my calculations). My assay is in glycine-buffered saline (pH 8.2) - the same buffer used to coat the latex. The total assay volume is 900µL, including the 300µL of antigen at concentrations 2-4000 µg/L. I tried 0.1-0.5% latex solids in the assay. I can detect no antigen-dependent change in 700nm absorbance with these coated beads, yet I saw agglutination under a microscope when I put 5µL of 2.5% beads on a slide and added 5µL of 800µg antigen/ mL. Lower concentrations were ineffective.

A: The specific surface area of your beads is 6 / (0.25 x 1.05) = 23 m^2/g of beads. So, you could adsorb 23 x 3 = 69mg IgG/g beads (maximum adsorption rate of IgG onto polystyrene is ~2-3 mg/m^2). If you used 250µL of 10% solids (~0.025g of beads), it would take 69 x 0.025g or ~1.7mg of IgG to coat the beads with a monolayer of protein. You added 2mg IgG. So there would be little extra protein to achieve equilibrium concentration in solution.

If you used 250µL of 10% solids latex coated at 1% solids, then you had 2500µL of coating solution. You added 2mg IgG. In coating the beads, you bound 0.8mg IgG, so there must have been 1.2mg IgG in 2500µL of equilibrium aqueous solution or 1.2mg IgG/2.5mL = 0.48mg IgG/mL. Compared to data from The Latex Course, this is normal performance. You put 0.8mg IgG onto 0.025g x 23 m^2/g or 0.575 m^2 of particles; this is 0.8mg/0.575 m^2 = 1.4 mg/m^2. Published data would predict a monolayer at 2-7 mg/m^2 (depending on pH) at an equilibrium concentration of 0.5mg IgG/mL. You should get best orientation (Fc portion down) and maximum packing around pH 7.8 for rabbit IgG. If the coating pH is very different from the assay pH, you may lose protein as it changes conformation on the surface. Also, you may not want a monolayer of IgG for



your agglutination test anyway. Since we know nothing about your antigen, we cannot help with ratios of Ag to Ab except to give a general answer. It looks as if you have only a fraction of a monolayer (maybe 1.4mg IgG/m^2 / 7mg IgG/m^2 = 20% of monolayer), but still it is possible that you have too much IgG on the surface. To get bridging with antigen (Ag), you must have an Ag with at least two epitopes per molecule. If both epitopes on Ag can react with adjacent IgGs on the same microsphere, you will get no bridging. You must space IgG molecules on the bead surface, so Ag reacting with one IgG cannot react with another IgG on the same microsphere. You can try using less IgG to coat latex. It is recommended that you do a box titration, varying both. If too much Ag is used, then no bridging will occur because each Ag can find only one IgG. (Prozone or "hook" effect.) You could also try higher solids content - even 0.5% may be too low for your assay since your microscope test was done at higher % solids. (See TechNotes 201, 204, 206, 301, and 304 for more information.)

DNA Adsorption

DNA Purification

Superparamagnetic Beads

Volume 12, #3 September 1999

Q: I read in the protocols of Whitehead Institute that for purifying single- or double-stranded DNA, you use "carboxyl particles." How can you convince DNA to bind to negative charges? Or do metal ions create suitable bridges?

A: *Short answer:* Yes! *(But you know we won't leave it at that.) Long answer:* The folks at Whitehead in their work on the Human Genome Project use 10 mM $MgCl_2$ salt and 13% polyethylene glycol 8000 (PEG) to cause the DNA to bind to the surface of the COOH-modified magnetic beads. We believe that it is the divalent Mg^{++} which is causing the DNA to adsorb onto the magnetic microspheres, which they then use to separate the DNA from cell debris.

Gravimetric Analysis

Percent Solids Determination

Spectrophotometer

Volume 12, #3 September 1999

Q: How do you determine the solids content of your microspheres? On a spectrophotometer?

A: No, a spectrophotometric method to determine the solids content of microspheres is valid only when a standard curve is generated using the same batch of microspheres, and when they are always handled in the same manner (aggregated microspheres scatter differently than single microspheres, and particles coated with protein scatter differently than non-coated beads). It is because of these drawbacks that we do not use spectrophotometric concentration measurement. Our method is gravimetric and is, we feel, much more reliable. Although it may require quite a bit more product, we determine the dry weight of a specific weight of suspension.

Lectin Coupling

Ligand Orientation

Streptavidin/Biotin

Volume 12, #3 September 1999

Q: I want to couple lectin to your magnetic beads. Will I get more lectin bound and better oriented if I covalently bind the lectin directly to the beads or if I buy your streptavidin-coated beads and hook on some biotinylated lectin?

A: Proper orientation when coupling ligands to carboxylate-modified microspheres can normally be controlled by optimizing the reaction conditions, but I believe that the simpler approach is to biotinylate the lectin and attach these to streptavidin-coated magnetic microspheres. Most commercially available biotinylation kits allow the attachment of biotin by a linker, which allows better orientation and less steric hindrance of the ligand at the microsphere surface. We absolutely don't *know* which coupling method would give higher activity, but we would bet on the streptavidin (SA) beads, since we load the surface so heavily with SA, and it is possible to get more than one biotin-lectin bound per molecule of SA.

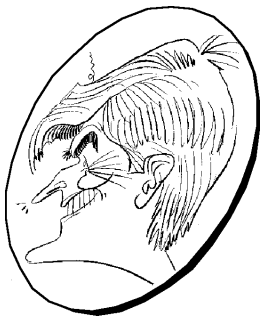
Antibody Attachment

Cleavable Linkers

Denaturation Elution

NHS-Iminobiotin

Q: I am looking for some type of beads that would covalently bind an antibody developed in a chicken (IgY). I would like to bind the antibody to the beads, add proteins and have the antibody "pull out" the proteins it recognizes, and use the beads to isolate this complex (through centrifugation). I would then like to break the covalent bond (by boiling) between the antibody and the bead, leaving the antibody-protein immunoprecipitate. Do you carry any type of bead that would allow this covalent bond with an IgY?



Streptavidin/Biotin

Volume 12, #2
June 1999

A: My guess is that the boiling step would denature the protein-antibody immunoprecipitate. In any case, it would likely soften the polymeric microspheres used to separate the complex. (Beware of irreversible clumping of soft microspheres.) I think that you would have better luck attaching biotin to the chicken antibody and attaching these to our streptavidin-coated microspheres. While the streptavidin/biotin bond is strong, it can be broken more easily than a covalent attachment. See *Biotechniques*, 26:249-254 (1999), for a discussion on a procedure for using 2-mercaptoethanol to disrupt this bond. Another option might be to attach NHS-Iminobiotin (from Pierce), rather than biotin, to the chicken antibody. Iminobiotin has a lower affinity for streptavidin, and therefore can be displaced simply by controlling the pH. (The folks at Pierce can probably supply an elution protocol.)

Our TechNote 101 (download directly from our website, www.bangslabs.com) lists a protocol for attaching biotinylated ligands to our streptavidin-coated microspheres. We have other ideas, such as binding the biotin to the Ab's using cleavable linkers: then binding Ab's to SA beads. *Please ask for more information if you are interested.*

Divinylbenzene

Extreme Temperatures

Glass Transition Temperature

PCR Thermocycling

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June 1999

Q: I have gotten PCR to work with one of the primers conjugated to your magnetic particles via biotin-streptavidin. I really want to do this with non-magnetic beads, since they are easier to keep in suspension during the reaction. Will non-magnetic beads handle the extreme temperature cycling?

A: If PCR works with our mag beads, then you should have *no* problem with our other microspheres. The magnetic microspheres are made of polystyrene (PS) or styrene/divinylbenzene (S/DVB) copolymers with 6-90+% DVB. The glass transition temperature (GTT) of pure PS is ~105˚C, (well above the normal PCR maximum temperature of 95˚C). So *any* of our magnetic or non-magnetic PS beads *should* survive PCR.

Since GTT increases with DVB content (ask for a chart of DVB content vs. GTT), if you have any doubts about or problems with the PS beads, then you can also choose plain (non-magnetic) microspheres with various DVB contents.

Centrifugation

G Forces

Settling Velocity

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March 1999

Q: How many "G's" should I use to centrifuge 350nm microspheres? (Someone recommended 3200rpm for ~20 minutes. That's about 2000-2400G.)

A: In order to really specify how to centrifuge microspheres, one must describe the centrifuge geometry and speed (n. rpm). If someone says only 'Centrifuge at 3200 rpm,' you really don't know what to do, since you don't know the diameter of the rotor or the distance the beads must settle. Likewise, if they say 'Centrifuge at 2400 G's,' you still don't know how far the beads are to settle. When you know the geometry, then you can decide how long you should spin the beads.

In his book, *Uniform Latex Particles* (p. 26, 27), Leigh Bangs used the diameter of the centrifuge at the top of the spinning tubes or bottles (D) and the height (h) of the liquid level in the bottles. Note also that G forces are at a minimum at the top of the liquid, but become higher as beads settle to the bottom of the tube, since the effective diameter of rotation is larger at the bottom. Thus G-force increases linearly from top to bottom of the tube, so the average G force is at the midpoint of the tube. If you pick the top of the liquid for calculating, you are safest since the beads will settle faster than that, not slower. See TechNote 206 for settling velocity equation incorporating $G = 5.59 \times 10^{-6} \bullet n^2 \bullet D$, where G = multiples of earth gravitation constant (G forces), n = rotation, revolutions per minute (rpm), and D = rotor diameter to top of liquid (cm).

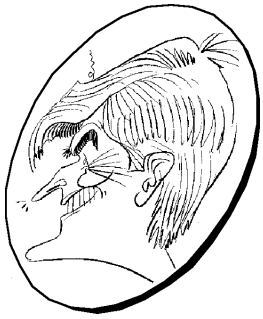
DNA Sequencing

Dye Terminator

Reaction Purification

Streptavidin-Coated Microspheres

Q: Dear Particle Dude, we have a sequencing lab and are looking for an easy way to clean up excess dye terminators from our sequencing reactions. Do you have any ideas?



A: Give our streptavidin-coated beads a shot. You will need to use biotin-labeled primers (easily made or obtained from a vendor) for the sequencing reaction. When the primers have extended to the terminator, they can be removed from the reaction vessel by binding the strands to streptavidin-coated beads and subsequently

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separated magnetically or by centrifugation. By this positive selection, excess dye terminator is left in solution and the sequences of interest are captured on the beads. The captured strands can then be eluted from the bead using 95% formamide at 90˚C. The isolated sequences can now be run on the gel. (See TechNote 302.) *Hint:* Be sure to denature the strands, and put the vessel immediately on ice, prior to bead capture of the strands. Capture of double-stranded DNA may be 50% less efficient.

Custom Coupling

IgG-Coated Beads

ProActive®

Streptavidin-Coated Microspheres

Q: To recover/concentrate bacteria, we wish to coat magnetic particles with our rabbit anti-[bacteria] antibodies (in serum form). We are thinking of your **ProActive®** magnetic beads coated with goat anti-rabbit antibody. Could you please suggest the right beads, plus instructions for using them?

A: We have goat anti-mouse (GAM) IgG-coated magnetic microspheres, but no goat anti-rabbit (GAR) beads yet. To use GAM beads, you would need to bind mouse anti-rabbit (MAR) antibodies to the GAM, then bind your rabbit anti-bacteria (RAB) antibodies. (Are you confused yet?)

We also offer **streptavidin-coated microspheres**. If you biotinylate your antibodies, you could attach these in a one-step coupling reaction with a bond strength nearing that of a covalent bond. (For this approach, you would probably need to purify the serum to increase the efficiency of the biotinylation step.)

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Another approach would be to use our uncoated, COOH-functionalized microspheres. By first purifying the serum, you could attach the antibodies of interest to the microspheres via covalent coupling reaction (see TechNote 205). For protocols to work with streptavidin-coated microspheres, see our TechNote 101. (Download from our website.)

We can also talk about custom preparation of GAR-coated beads.

COOH-Modified Beads

Surfactant-Free

Washing Beads

Water-Soluble Polymer (WSP)

Q: We have some of your surfactant-free carboxyl-modified particles [P(S/V-COOH)]. Are the COOH groups strongly bound to the particles or may some of them be released?

A: The COOH groups are a result of a copolymerization of styrene and a vinyl carboxylic acid (such as acrylic or methacrylic acid). In most cases, the microspheres are sold diluted with deionized water to 10% solids. Thus, surfactant (if any), buffer salts (for pH control), counterions and especially water-soluble polymer (WSP) remains "in the soup." No surfactant was used in your beads, but there are other solutes. WSP is probably composed of styrene/acrylic acid (AA) copolymer molecules which have enough AA to make the polymer chain soluble enough to leave the particle. (If methacrylic acid is used, the WSP will be significantly reduced, due to the lower solubility of MAA and its copolymers with styrene.) About 15-20 years ago, some Dow guys found that about half of the AA added to make COOH beads goes into WSP (they could only account for 50% of acid dose in or on the beads). That is why we always recommend that you clean your microspheres before use, so you don't bind to WSP and lose your precious ligand when you wash after binding. If you don't want to wash before binding (to save time), then you will pay for that time-saving with extra protein coupled to WSP and discarded. Note that after removal of WSP, your ligand will be firmly attached to the particle-bound COOH groups.

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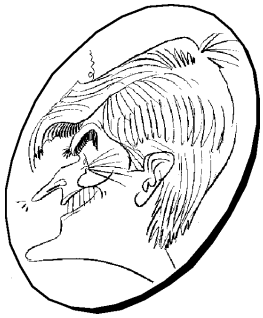
COOH-Modified Beads

Titration Data

Q: Are all the COOH groups on the outer surface of the microsphere or also within the microspheres?

A: Surely some of the carboxyl groups are buried beneath the surface. However, the microspheres are non-porous, and therefore the titration data is representative of the carboxyl groups which are present at the outer surface and available for covalent coupling.



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Agglutination

Chemiluminescent-Based ELISA

Q: Do you know of a good system to automate small bead assays that doesn't cost a zillion dollars? Any ideas?

A: You bet! Try quantitative turbidimetric or nephelometric assays to measure agglutination of ~100nm Ab-coated beads by small samples of all your antigens.

ProActive®

(See new TechNote 304.)

Turbidimetric/ Nephelometric Assays

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If you have no luck with (or enthusiasm for) that approach, then perhaps you can try chemiluminescent-based ELISA, using magnetic microspheres. We can offer magnetic beads with whatever ProActive® coating you want. If you want to "do it yourself," then we can always supply bare mag beads - and we'll help you all the way to a successful coating of these.

DNA Adsorption

References

Silica Microspheres

Volume 11, #4
December 1998

Q: How can I bind DNA to your silica beads non-covalently?

A: W.R. Boom seems to use salt and chaotropic agents to make the liquid phase so nasty ("Let's get out of this place,") that the nucleic acids are more comfortable on the particle surface. We don't think that there is any specific interaction between the silica and nucleic acid. See our TechNote 104 and our TechNote 302 for ideas on the reversal of silica charge (- to +) for DNA pick-up. See also Melzak, K.A., C.S. Sherwood, R.F.B. Turner, C.A. Haynes. 1996. *J Col Interface Sci,* 181: 635-644.

COOH-Modified Beads

Ligand Orientation

Volume 11, #3
September 1998

Q: I tried three different lots of your CML (COOH-modified latex) microspheres. All three lots (A, B &C) were about the same size and had the same amount of acid on the surface. Without cleaning them, I adsorbed protein, washed away excess protein with buffer and added EDAC to covalently link the protein onto the surface. All three beads adsorbed the same amount of protein and had the same amount bound after covalent coupling, *but "C" had zero reaction with the antigen.* So, what happened?

A: While they *were* all the same diameter and acid concentration, the three beads you ordered were made by two different processes. A & B were made with surfactant and C was made with near-zero surfactant.

Our hypothesis is that with A & B the protein adsorbed into the proper orientation (Fab parts up and Fc part down), but in the third case the protein adsorbed "face-down" or "Fab-buried."

If we are right, then adding ~0.1% anionic (SDS) or nonionic (Tween® 20) surfactant to C might fix the problem. (Conversely, if you cleaned up A & B, removing the excess surfactant, then they might bind the IgG face-down also.

Bead Characteristics

Particle Determination

Replicate Lots

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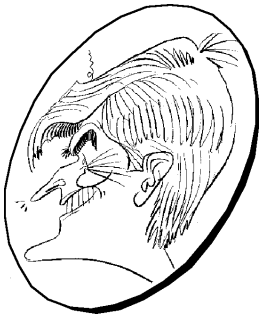
September 1998

Q: What else could I do to fix this problem?

A: You might reverse the order of addition in the binding steps. Most folks doing covalent binding to CML will activate with EDAC, clean to remove excess EDAC, add protein to activated beads, and, finally, wash to remove excess protein. Your method was obviously successful for latexes A & B, but with C it seems *not* to be working. Please let us know how these ideas work out and we can share the results here next time.

Important take - home messages for all you bead binders:

1) Not all beads are created equal. They might have the same specifications of diameter, standard deviation, and acid content, but they could still be *made* in many different ways. We'll be glad to explain these differences to you.
2) You need to know what kinds of beads you are using, and whether different bead lots are replicates or made by different producers or different processes, using acrylic or methacrylic acid, with or without surfactant, etc.
3) During early experimentation (feasibility) you might try beads made by different processes/manufacturers until you find the combination of bead and process which yields the desired result.
4) After you optimize the binding of your protein to one microsphere lot, then when you try to make a replicate binding lot, make sure you are really using a replicate bead lot.



5) When testing your process for robustness (Will your process work if you change bead source or manufacturing process?), you again might want to try beads made by different processes.
6) Talk to us and tell us what you are trying to do at *all* stages of your work. If we know you are in early feasibility testing, we can point you at beads from different processes. When we know that you are trying for replication, we'll be sure you get replicate bead lots, even if we must custom produce another lot made for you.

Catalog Code Meanings

OEM Catalog Numbers

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June 1998

Q: Please help me understand your catalog codes and lot numbers. How do I choose the microspheres I need?

A: Our Certificates of Analysis (COAs) have two product identification numbers on them. The first number specified is the **catalog code**, e.g., DS02B. This is a generic code that applies to all plain polystyrene particles between 0.01µm and 0.49µm that are dyed blue. On each of these COAs, the catalog code is DS02B. The second number on the COA is the **Bangs (or BLI) Lot Number**, e.g., 0193, 3283, & 0248. This number is specific for a particular lot of particles. So each of these three particles have the same catalog code, but different Bangs Lot Numbers specific for each lot of particles. When a new product in that range is made for you, it will have the same catalog code (DS02B) but a new BLI Lot Number.

We also create **OEM Catalog Numbers** for regular customers. Once we receive specifications on a product from you, we can apply an OEM Catalog Code that will encompass any lot that meets those specifications. When ordering the product, you will simply have to give the OEM Catalog Number. We will start this process as soon as we get a copy of your specifications for the different products.

Blocker Concentration

BSA

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June 1998

Q: I got very low binding of my biotinylated ligand to your SuperAvidin™-coated beads using a buffer with 1% BSA to prevent unwanted NSB. What's wrong with your microspheres?

A: *Oh, great! You have a problem and it's our fault.* But seriously, you may have used too much BSA. We found that 0.05% BSA (1/20th of what you mentioned) in the storage buffer is enough. For more blocking, try adding 0.01% Tween® 20 - it worked great for blocking NSB of biotinylated acridinium ester in a binding study we published.

Sonication

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June 1998

Q: What can you advise us about using sonication to redisperse microspheres after centrifugation?

A: You can use **gentle** sonication to redisperse microspheres. Use only enough sonication to redisperse beads to a smooth dispersion, but not enough to heat your suspension. An ultrasound bath would be preferred, because there is less chance of using too much energy and no chance of contamination from a probe.

We have not heard of any problems with sonication of protein-coated beads, but we would exercise caution, especially if protein is adsorbed or coupled via long tethers which could break if the protein-bead link is whipped around too much.

By the way, a customer told us that **our** SuperAvidin™-coated beads show **no losses of binding capacity** after sonication.

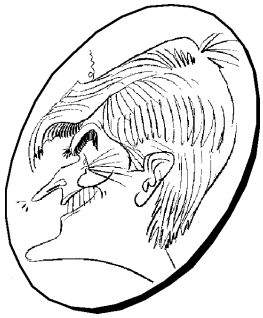
Hydrophilic Beads

Large Microspheres

Nitric Acid

Sulfuric Acid

Q: I have some of your larger beads (100-200µm), but they are hydrophobic. How can I get hydrophilic beads this size? I found some references for creating active surface groups on the beads (one was in your 1984 book *Uniform Latex Particles*), but these references mentioned reacting the beads with "fuming red" nitric acid and sulfuric acid followed by reducing agents to yield amino groups. I'm not a chemist, and all those acids sound scary, and I only want them to be hydrophilic!



A: 1) You are right! Concentrated acids like glacial acetic acid and fuming red nitric acid are *very* nasty to work with. Be *very* careful! You should get some

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help from a chemist at your place. You will need a plastic apron, full face mask, elbow-length rubber or plastic gloves, and boots.

2) If you just want to make hydrophilic beads, then you could simply mix beads with *ordinary* "concentrated nitric acid", which is only saturated (a bit easier to work with) but not as concentrated as "fuming red nitric acid", which is supersaturated in NO_2. The nitric acid will etch the surfaces and create hydrophilic groups (like -OH or -COOH groups), but it probably won't put nitro groups on the surface. If you want to go this way, you can also forget about the glacial acetic acid in that recipe mentioned in my book. For hydrophilic (not nitrated) beads, you can just etch the beads for a few minutes, then drain the beads, by pouring off the nitric acid through a glass frit funnel. Then rinse thoroughly with water (do this *very carefully* since water added to acid will generate lots of heat!) and see if beads are hydrophilic enough. If you place some on the surface of a beaker of water, do they float (hydrophobic) or sink (hydrophilic) and do they cling together in water (hydrophobic) or are they water-dispersible (hydrophilic). If you leave them in the acid too long, they will dissolve, since the acid just keeps on etching.
3) If you do this work with the help of a chemist (this is highly recommended), ask the chemist if he/she has or can make any chromosulfuric acid. This is made by mixing potassium or sodium dichromate into concentrated sulfuric acid. Certain chemists have used this reagent to clean glassware and it chews up anything organic. It will etch and oxidize the bead surfaces, making them hydrophilic. (It will probably make them disappear, if they are left in there long enough.)
4) It is possible to treat the beads with an electrical plasma in the presence of gasses like O_2, NH_2, etc. to create hydrophilic surfaces, but I have no experience with this.
5) Some of our beads are made by suspension polymerization processes which put an inorganic coating on the beads. These will have natural hydrophilicity. Apparently, your beads were not made this way.

Organic Beads

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Q: With all the concern about health now, I just want to be sure: Are your particles grown organically?

A: We assure you that our polystyrene microspheres are 100% organic and our silica beads are 100% inorganic!

Chloromethyl Binding

Stability

Storage

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Q: Regarding chloromethyl latex, do you have any comments about good storage conditions for this bead after protein coupling?

A: As far as we know, chloromethyl latex should be very stable after binding. First, the chloromethyl groups are reacted to form a stable covalent bond with protein. Second, the protein coating should impart colloidal stability.

Active Ester Quenching

Aerosolization

Covalent Coupling

Cross-Flow Filtration

Drying Beads

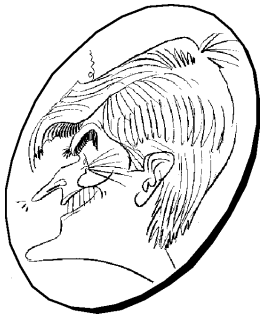
Ethanolamine

Freeze Dry

Tween® 20

Q: I would like to covalently attach a small molecule to your 32nm COOH-modified beads and use this ligand to capture receptor proteins. Here's my plan:

1) Clean the beads by passage through a PD-10 column or use some form of dialysis, then re-concentrate beads to 10% solids. Can I make single, dry beads here?
2) Activate the beads with WSC and NHS or HOBt for 3-4 hours at 22˚C.
3) Dilute the reaction with 0.05% Tween® 20 in 200 mM K_2HPO_4, pH 8.0 which contains the small molecule at 1-5 mM, at 22˚C; then quench residual active esters after 20 hours with 5 mM ethanol-amine.



Main Question: Will Tween® 20 be sufficient to prevent protein adsorption to the derivatized bead, or should I use a neutral protein such as BSA to block as well? I am particularly concerned about the effect that a protein will have on access to the small molecule now linked to the surface of the bead.

Two-Step Coupling

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A: The PD-10 column seems like a good approach. Normal dialysis tubing of the Pierce Slide-A-Lyzer™ should work as well. (Prewash any column or dialysis tubing well, to remove whatever was used to make it.)

Cross-flow filtration (from Microgon, Millipore, Filtron/Pall, Amicon, A/G Technologies, et al.) can be used to concentrate up to 10% solids, or more. The Microgon system, at least, can be used to clean and concentrate the beads.

Some folks have reported cleaning, reacting, removing reactants, and concentrating in one campaign, without changing the filter set-up (only one pot gets dirty).

It may be difficult to make easily redispersible dry beads this small without clumping them. You might try aerosolizing them and drying the spray of droplets, sized so that there is 0-1 bead per drop. Then, when the drops dry, there is only one bead (so two beads cannot be pulled together by surface tension during drying). It may also be possible to freeze dry the beads from dilute suspension so as to produce a free flowing powder of single particles. Either drying process may require addition of surfactants, blockers (like proteins), and sugars. If you can find a way to work with a liquid particle suspension, it will certainly be easier.

Step 2: Some folks use a shorter time.

Step 3: You mention diluting the reaction with a different buffer. If the activation (Step 2) is done at acidic pH, then be sure to add enough of the higher pH buffer (Step 3) to get the pH above neutral, so that the primary amines on your ligand are in the correct form for the covalent reaction. You might try pH adjusting the final solution to achieve this. We like the idea of quenching with ethanolamine, to use up remaining active esters, and to produce a hydrophilic surface, which is less likely to adsorb unwanted protein.

Step 4 sounds good.

As to your **"Main Question"**: We think that surfactant can be used to prevent unwanted binding of proteins after covalent coupling, and then there is no large blocker protein which might cover the small molecule binding site.

Adsorption

Antibody Orientation

Covalent Coupling

ProActive®

Protein A Microspheres

Protein L

References

Secondary Antibody Binding

Q: What is the best way to bind antibodies to microspheres? What about adsorption, covalent coupling, secondary antibody binding, and Protein A?

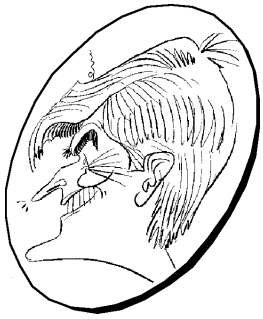
A: 1) Direct adsorption is the oldest and simplest method for putting proteins onto polystyrene particles. It works well, and most proteins will stay on the particles forever- or at least for a long time. (See our TechNote 204 for protocols to start you off.)

2) Many people prefer the more secure covalent coupling of proteins. Water-soluble carbodiimide binding to carboxylate-modified particles is a well-established method for covalent coupling. (See TechNote 205 for protocols and other covalent binding options.)

3) If you adsorb one Ab and use it to bind another Ab (e.g. goat anti-mouse secondary and mouse monoclonal primary), the second Ab will certainly be more accessible, sticking further out into the aqueous phase. It may also be oriented more favorably and therefore significantly more active.

4) You can also use proteins A or G to attach Abs to particles. Some claim superior orientation this way. Since protein A binds specifically to the Fc portion of the IgG, the Fab portions of the Ab are pointed away from the surface. You have a choice of native proteins A or G, recombinant forms of proteins A or G (these have deleted sequences for reduced nonspecific binding potential), or even with recombinant fusion protein A/G. (Try our ProActive® Protein A-coated microspheres and ask about other options.)

5) We have heard recently about "Protein L," which is supposed to be a more universal monoclonal antibody-binding protein. We have not evaluated any yet, but it *might* be easier/better than GAM (worth consideration). For more on Protein L, see Tocaj, A., U. Sjobring, L. Bjorck, O. Holst. 1995. High level expression of protein L, and immunoglobulin-binding protein in *E coli. J Fermentation and Bioengineering,* 80(1):1-5.



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Dye Leaching

Lateral Flow Tests

Volume 10, #3
September 1997

Q: I am experiencing problems in getting your 0.3-0.5µm COOH- and amino-modified latex beads to "chromatograph" on paper. I have conditioned the beads by washing (x10) in aqueous buffer to remove surfactants and other small molecules. At present, I am only able to achieve chromatography by using a mixture of aqueous/organic mobile phase (e.g. 75% aqueous buffer / 25% ethyl acetate). Should I be using another type of bead for this work, or is there another protocol I need to adopt for this application?

A: Particles probably did not move because 1) they were too large to move through the pores of the strip you chose; 2) they were clumped by the buffer, and the clumps couldn't pass through the membrane; or 3) they were stuck to the membrane by hydrophobic bonding.

You probably got chromatographic movement because you dissolved the beads with the ethyl acetate and the **dye** moved up the strip. Try smaller beads, another membrane, or some surfactant to better disperse the beads; or block the membrane with protein and surfactant to make it less sticky for protein-coated beads. (See TechNotes 301 and 303.)

Cleaning Methods

Surfactant Removal

Washing Beads

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Q: How do we know when the particles are clean? That is, how do we know we've removed the surfactant?

A: Microspheres are clean enough when enough surfactant has been removed so that protein coupling proceeds really well. Really, it's not far from the truth. You want to remove surfactant until coupling is uninhibited and reproducible, while the microspheres are singly dispersed. After cleaning, some folks actually add-back surfactant (under their own control) to assist single microsphere coupling.

Most coating protocols call for 3 washes before coating. It also depends on how the microspheres are washed (continuously, by cross-flow filtration or batch process in a centrifuge) and how much supernatant is removed each time (this is related to how much water is left with the microspheres after each wash).

One can measure surfactant or protein coming off the microspheres with instrumental methods, including surface tension or protein analysis, to see when the wash-water is clean. (Remember that you really need *two* good clean-up methods - one for surfactant removal before protein coating and one for excess protein removal after coating.)

Practical Answer: Dr. Seaman says that water is free of surfactant when the foam or bubbles on top of 5mL H_2O shaken in a 10mL test tube collapse in 2-3 seconds. You can use this indicator to test supernatant coming from the microsphere clean-up whether by centrifuge or filter system. We call this the "Seaman Shake Test." (For full details, see TechNote 203, *Washing Microspheres*.)

Cleaning Methods

Contamination

Deionized Water

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Q: How do we know that we have not contaminated the microspheres in the cleaning process?

A: *Possible Answer:* Make sure that water going in is as clean as you can get (no ions, no organics, no microbes). Otherwise, microspheres can pick up stuff from the water. Our deionized (DI) water system has a mixed ion exchange bed with a conductivity meter, organic filter, recirculating filter system with UV light to kill bugs and a 0.2µm final filter. It's the best water we know how to make or get, and we use it directly from the system (no stored water for things to grow in). Monitor things with a microscope to ensure that you always have *singly dispersed* microspheres.

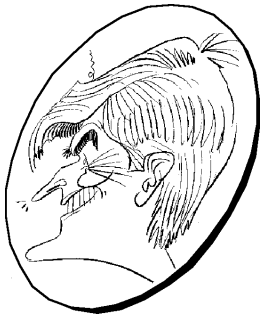
Dissolving Beads

Divinylbenzene (DVB)

Organic Solvents

Toluene

Q: What kind of organic solvents can one use to disperse and dissolve your microspheres, and how do I get them out of water and into the solvent?



A: Most of our microspheres are made of polystyrene (PS) or copolymers of styrene and divinyl benzene (S/DVB). The DVB crosslinks the polymer chains and provides strength and solvent resistance. The microspheres will not swell in several solvents like ethanol, methanol, normal alkanes and others. (We can send a list.)

The PS particles will dissolve to some degree in many other organic solvents, like

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benzene, toluene, some esters, higher ketones, methylene chloride and other chlorinated solvents. If the particles are crosslinked with divinyl benzene (DVB), they will only swell in these same solvents. The degree of swelling depends on the solvent, and is inversely related to the degree of crosslinking, i.e., more DVB = less swelling.

Changing Solvents

Organic Solvents

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Q: How do I change phase to the solvent I want?

A: To change from water to solvent, you can just let the microspheres settle by gravity or centrifuge; decant the supernatant; add a solvent which is miscible with the previous liquid (water), like methanol, ethanol (my favorite), or iso-propanol; and redisperse the beads. Repeat this until you have the beads in the liquid you want.

Changing Solvents

Cross-Flow Filtration

Organic Solvents

Small Microspheres

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Q: Will centrifugation or gravity work for small 0.1µm beads, which don't settle fast?

A: Well, it certainly will be harder (longer) to spin down these small beads and redisperse them to single particle suspensions. By using cross-flow filtration equipment, it is now possible to change phase without causing the beads ever to get clumped or to touch each other.

With the help of Microgon, we recently did a *double* exchange of some 0.1µm beads. Using a 0.05µm polysulfone hollow fiber membrane, we changed our PS and PMMA beads from water to ethanol (it sure smelled good) to a halogenated solvent which was *not* a solvent for PS.

Organic Solvents

PMMA

References

Volume 10, #1
March 1997

Q: What about other polymers like polymethyl methacrylate (PMMA)? What kind of organic solvents will dissolve PMMA?

A: We do not have a list of solvents/non-solvents for PMMA, but we expect that the list will be similar to that for PS. You can get this kind of information from books on solubility parameters, such as "Handbook of Polymer-Liquid Interaction Parameters and Solubility Parameters," by AFM Barton (CRC Press, 1990).

Aerosolization

Changing Phases

Drying Beads

Volume 10, #1
March 1997

Q: How about changing phase from water to air... drying the beads?

A: Drying the larger beads is easy. Let them settle out of suspension, and decant the water or change phase to alcohol (faster settling and faster drying); then let them dry and break up the pellet with a spatula, stir rod or even a mortar and pestle (the latter works well for our silica beads). The smaller beads will settle slower, and will form a harder pellet. In some cases it may be easier to aerosolize the beads. The idea is to disperse them in a liquid (water or alcohol?) in dilute form, then spray the suspension into a bag or other container suitable for collection. If diluted properly, each droplet will have 0 or 1 bead per droplet. Then when the droplet dries, each bead will dry as a single bead, without touching another bead until they are dry. This will lessen the chances of them sticking together in the dry form. Contact TSI for aerosolization equipment and information. (Web: www.tsi.com.)

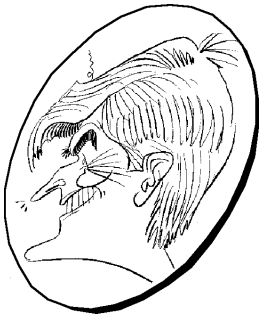
Fluorescent Beads

Regional Blood Flow Tracing

Volume 9, #4
December 1996

Q: What do you know about using dyed microspheres for *regional blood flow tracing?*

A: We have visibly dyed and fluorescent microspheres of all sizes (20nm-20µm), and we are continually making more at customers' requests. Undoubtedly these may be used for many applications. (Look for *Dyed* and *Fluorescent Microspheres* pages in the Products & Ordering section of our website, www.bangslabs.com.)



We don't have experience using our microspheres in regional blood flow experiments. (Please note that our microspheres are not supplied as sterile suspensions.) The real experts in that area are the people at the Fluorescent Microsphere Resource Center at the University of Washington. They have developed a technical manual describing fluorescent microsphere technology for regional blood flow applications. Their Web home page is <http://fmrc.pulmcc.washington.edu> Dr. Robb Glenny will be able to help you. Email: glenny@u.washington.edu.

Adsorption

Binding Issues

Volume 9, #4
December 1996

Q: I have been using 2 and 5 micron beads in an immunoassay where the antibody on the bead needs to bind to a surface bound antigen. I do get adsorption of the beads to the surface, but no significant binding. Is it that the beads are too big and won't attach well to a smooth surface?

A: If your 2 and 5µm beads are coming off the smooth surface, it is possible that they aren't bound tightly (not enough Ab on the surface, Ab binding sites might not be directed outward, or perhaps rinsing or washing following staining is knocking the large beads off the surface). For the first two possibilities, you could try other binding methods. If you suspect the latter, then we offer smaller particles which would sit closer to the smooth surface and be less likely to be removed by any vigorous washing.

Filter Screening

Membrane Challenge

Small Microspheres

Volume 9, #4
December 1996

Q: We are testing a membrane that captures 20nm particles. As part of the membrane challenge, the membrane should demonstrate a "5 log" (10,000x) reduction efficiency. That is, for every 10,000 particles put on the membrane, only 1 gets through. Do you know of any way to accomplish this challenge? How do you detect one or two 20nm particles coming through the membrane? Radioactivity has been ruled out for now.

A: One method might be to heavily load 20nm microspheres with a fluorescent dye (10-20% dye) and challenge the membrane with them. Spheres that escape the membrane could be dissolved in a solvent for polystyrene to release the dye. Then, if enough dye is present, a spectrophotometer or fluorometer could pick up the dye signal from those particles. It *may* be possible to detect the *particles*, if you could find them. In the particles, the dye would be concentrated enough to detect with a fluorescent microscope.

Hemagglutination Tests

Reverse Passive Latex Agglutination (RPLA)

Visibly-Dyed Beads

Volume 9, #3
September 1996

Q: Tell me about the latex beads used in RPLA tests where latex beads are used to simulate red blood cells (RBCs).

A: Microspheres for RPLA should behave like RBCs, since RPLA was developed to mimic hemagglutination tests. So, they should be large/dense enough to settle within about an hour or more (depending on the time allowed for test results). One can use large PS beads with density of 1.05 g/mL, and we can help you calculate the time for settling of various diameter PS beads (TechNote 206). If you want smaller beads with higher surface specific area, try PMMA. At a density of 1.19 g/mL, PMMA beads will settle 4X as fast as same size PS. Silica beads (density 2.00 g/mL) can also be used - these "high speed beads" will settle 19X as fast as the PS.

RPLA beads probably should be dyed to see the pattern in the bottom of the 96 well plate. They don't need to be red, but some dark color is probably best.

Agglutination

Blockers

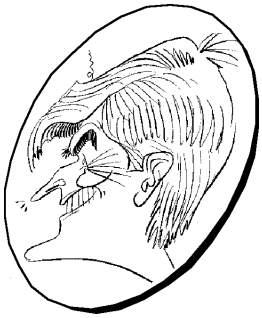
Chloromethyl Beads

COOH-Modified Beads

Nonspecific Binding

Quenching

Q: Protein in urine interferes in one of our urine-based tests using your chloromethyl beads.



A: I can imagine proteins in urine adsorbing on latex (we call it nonspecific binding or NSB) to cause nonspecific agglutination. Alternatively, these proteins might prevent agglutination. NSB can happen with any particles unless they are very hydrophilic and have protein bound covalently, or unless they are well blocked (every hydrophobic site filled with blocker molecules or surfactant). Here are some possible solutions:

1) Try quenching any remaining reactive groups and create hydrophilic surface groups which will not adsorb protein. With your CH_2Cl particles, after you have bound your protein, you might quench unreacted chloromethyl groups with

References

ethanolamine or diethanolamine, converting these hydrophobic chloromethyl groups to hydrophilic, non-adsorptive hydroxyl groups.

Urine-Based Tests

2) After binding IgG (or other desired protein) on the microsphere surface, use plenty of blocker (BSA, casein, Tween® 20, etc.) to fill up uncovered sites which might bind that urine protein.

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3) You can try more hydrophilic COOH-modified microspheres. These can adsorb proteins too, but probably not as much as the more hydrophobic S/VBC particles. Blockers would be appropriate here, too. After protein coupling, you also might want to try quenching any remaining unreacted groups, again with ethanolamine.
4) Avoid a urine centrifuging step to make your test more marketable. You could try filtration of the urine samples to remove the protein.
5) Check out Sokoloff, R.L., J.M. Reno. August 20, 1985. *Method for reducing nonspecific interferences in agglutination immunoassays by adding a halogen-substituted carboxylic acid.* U.S. Patent #4,536,478.

Agglutination

Clumping

Surfactant

Q: To avoid clumping during 2-step covalent coupling of guinea pig polyclonal antibody to carboxylated beads, I added 0.05% Tween® 20 just before EDAC (carbodiimide) addition. The Tween inhibited the clumping induced by EDAC addition, but the agglutination between the bead and antigen showed poor antibody coupling on bead surface. Do you have a solution to my problem?

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A: *Certainly!* If too much Tween is added, it could interfere with coupling by coating antibody or beads to prevent their coming together.

Add only enough Tween to prevent clumping (maybe 0.005% Tween) while permitting good coupling. Test this by adding serial dilutions of Tween to microspheres and mix with EDAC buffer in the absence of antibody. Then when you get Tween dilution which is just enough to yield a stable microsphere dispersion in buffer, test for adequate covalent binding.

Desorption of Protein

Storage

Q: After I bind a protein onto my microspheres, they will be stored in a Tween® 20 containing buffer. Do you expect that the protein will be desorbed under these conditions? Is it possibly better to use covalently binding beads?

Surfactant

A: Yes and Yes! A small amount of surfactant will probably not cause much desorption, but to be safe we would recommend covalent binding.

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Binding Polypeptides

References

Q: What is the best way to couple a decapeptide firmly to a bead without losing activity of the peptide? Others coupled the peptide to IgG via cysteine, using succinimidyl 3-2(pyridyldithio)-propionate and then adsorbed the IgG to the latex bead. [See S. Miyamoto, et. al. 1995. *Science,* 267: 883-885, especially note 4, p. 885]

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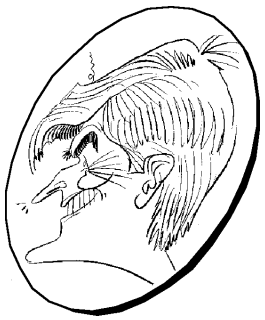
We put a cysteine on the end of our peptide so we can it this way, but could we couple the peptide more directly to the bead?

A: Yes. You can certainly bind your polypeptide to any of several surface functional groups. Popular groups are COOH, NH_2, hydrazide, epoxy, CHO, hydroxyl and chloromethyl.

We expect that you care whether you can put more, active polypeptides on a bead surface by direct tail-down covalent binding OR by binding polypeptides to IgG, followed by adsorption of the IgG onto a polystyrene bead. We suppose that you can put more peptide on the particles with IgG, but we don't know how much will be active. You also have the option of binding your peptides to IgG or to beads using spacers of various lengths to increase activity.



Clumping

Q: I diluted a 10% suspension of your 1.6µm diameter silica microspheres with water, resulting in a solution of 1% solids. However, most of the beads were

Silica Microspheres

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sticking together to form large clusters. What happened?

A: Always make sure that *any* microspheres that you use are singly dispersed before and after dilution. Look at them under a microscope. Even the smallest ones are visible with a light microscope (100X objective, oil immersion lens) if they are clumped. Silica beads are more hydrophilic, but if they do become clumped you must ensure that they are well redispersed. This may require ultrasonication. (See TechNote 202.)

Blockers

BSA

Protein Coating

Volume 9, #2
June 1996

Q: Can I bind two or more different proteins to microspheres at the same time?

A: It should be possible to bind a small amount of one protein to the particles (careful not to cover up all the surface) followed by a second protein. OR Mix and bind the two proteins together. Some folks mix IgG and BSA (or HSA) in a certain ratio and bind them to give proper level of IgG on the microsphere surface, with the albumin acting as blocker. (See TechNote 204.)

Storage

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March 1996

Q: How important is the pH of the storage solution for the shelf life of the carboxylated latex beads?

A: It is best to store latex in water with added surfactant. Keep it fresh in the refrigerator. Increasing ionic strength is bad for particles, in general. Best pH probably is slightly basic to convert COOH groups to COO^- groups.

Shelf-Life

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March 1996

Q: What is the expected life of carboxylated beads?

A: With no bacterial contamination, beads will probably have *decades* of shelf-life (really).

Shelf-Life

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Q: How is shelf life affected by incorrect storage pH and the occurrence of bacterial contamination?

A: If pH is too low, beads will have low surface charge, reduced colloidal stability and, consequently, shorter shelf life. Bugs can eat the surfactant and cause colloidal instability. We, or you, can add antimicrobial (sodium azide?) if you like.

COOH-Modified Beads

Particle Determination

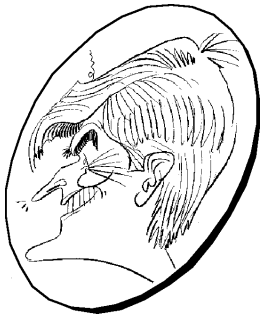
Spacers

Volume 9, #1
March 1996

Q: How do you choose the different carboxylic acid groups for the bead coating (you have three available)?

A: The first COOH modified particles were made by copolymerizing styrene and acrylic aid. We have the widest selection of these. (Methacrylic acid and several other monomeric acids may also be used.)

Styrene and maleic anhydride copolymers are an alternate way to make COOH beads. Maleic anhydride undoubtedly hydrolyzes to dicarboxylic acid groups. These particles have been very popular (our biggest seller in dollars and kilograms) so we know they work well. Perhaps the adjacent COOH groups have special properties?



We have a few particles with spacer arms of 2 or 6 carbons. These were made as examples of what can be done to "improve" binding to particles. We encourage people to try the tether concept especially with very small ligands (like haptens). Pierce and others sell activated spacers for use with COOH particles.

COOH-Modified Beads

Titration Data

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March 1996

Q: Are you able to supply information about the number of carboxylic acid groups on the surface of the beads?

A: We do titrate our carboxylated beads. This information is provided on our website, www.bangslabs.com, and on Certificates of Analysis, where appropriate.

Coulombs per Particle

Derivation

Equations

Parking Area

Surface Charge Density

Titration Data

Volume 9, #1
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Q: The surface charge density in your catalog is mentioned in terms of microequivalents of acid or base per gram of microspheres. What does that mean in terms of number of electronic charges or coulombs per particle?

A: We take an aliquot of ion-exchange or cross-flow filtration cleaned microspheres in suspension, measure % solids (by weight loss on drying) and calculate the weight of microspheres to be titrated. Then, we titrate this volume of microspheres with acid or base of known concentration until the acid-base equivalence point is determined conductimetrically or potentiometrically; from this we calculate microequivalents of COOH groups (for example) per gram of particles.

There are 6.022×10^{23} molecules/mole of 6.022×10^{23} charged groups/microequivalent. [*Coulombs Conversions:* There are 96490 Coulombs/equivalent (of COOH, for example). Therefore, there are $6.022 \times 10^{23} / 96490 = 6.241 \times 10^{18}$ molecules/C (or 1.602×10^{-19} C/molecule).]

For a sphere of diameter, d, the surface area, $A = \pi d^2$; volume, $V = \pi d^3/6 = 0.5236\ d^3$; and mass, $M = V\rho$, where ρ = density (g/mL or g/cm^3).

Then, for 1 micrometer (1 micron) diameter polystyrene spheres: $A = 3.1416 \mu m^2 = 3.1416 \times 10^8$ Å^2/sphere and $V = 0.5236 \mu m^3$/particle.

1cm = 10mm = 10^4 µm = 10^7 nm = 10^8 Å.
1cm^2 = 10^2 mm^2 = 10^8 µm^2 = 10^{14} nm^2 = 10^{16} Å^2.
1cm^3 = 10^3 mm^3 = 10^{12} µm^3 = 10^{21} nm^3 = 10^{24} Å^3.

Density of polystyrene is 1.05 g/mL = 1.05 g/cm^3 x 1 cm^3/10^{12} µm^3 = 1.05×10^{-12} g/µm^3. Then, $M = 0.5236 \times 1.05 \times 10^{-12} = 0.54978 \times 10^{-12}$ g/particle. (Thus $1/M = 1.819 \times 10^{12}$ charges/g.)

Then, 6.02×10^{19} charges/g ÷ 1.819×10^{12} particles/g = 3.31×10^7 charges/particle. That's plenty!

Similarly, you can calculate charges per particle for other sizes, densities and surface charge densities.

The other conversion factors above will let you calculate Coulombs per particle from microeq/g, density and diameter.

We calculate and report the parking area or average area occupied by each charged group on the surface. This parameter gives us a way to compare the amount of acid on the surfaces of different sized particles. (In the example above, one sphere will have a surface area of 3.14×10^8 Å^2 and 3.31×10^7 charges/particle or 31.4/3.31 = 9.5 Å^2/COOH group. COOH groups in a close-packed monolayer of fatty acid each occupy an area of about 25Å^2, so the equivalent of much more than a monolayer of acid groups on the surface.)

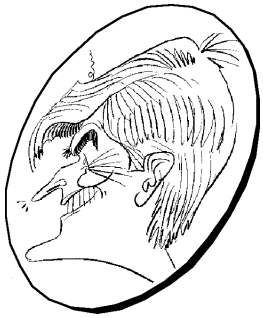
COOH-Modified Beads

Covalent Coupling

One-Step vs. Two-Step Coupling

Q: Do you recommend one step or two step covalent coupling to COOH-modified microspheres?

A: The short answer is "Two step!" The (inevitable) longer answer is that the one step method involves combining microspheres, water-soluble carbodiimide (WSC) and protein in a neutral pH buffer all at once. The advantage is that it is only one step - "only one pot gets dirty."



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One disadvantage to "one step" is that the pH normally used is a compromise between the ideal pH for both halves of the coupling reaction. The BIG disadvantage is that the WSC is indiscriminate; it can crosslink protein as well as bind it to the particles and it is possible to bind everything together - protein, crosslinked protein and microspheres. You can easily form clumps of particles which are covalently bound together by crosslinked protein. The clump sizes are uncontrollable and a wide distribution of clump sizes can be formed. It may be possible to do this coupling reaction successfully in one pot. But, if folds are successful, we don't know about it because they don't call to say "Eureka!" We hear about the problems, if any, so we are constantly teaching ways to avoid the clumping problem. This is easier than breaking clumps once formed because, "Breaking up is hard to do!" (Neil Sedaka)

The two step method involves reaction of microspheres with WSC at a low pH (where the carboxylate groups are in the COOH form) to form an activated intermediate, followed by cleaning to remove unreacted WSC. (Note that the active intermediate is unstable and immediately begins to hydrolyze. Cleaning should be rapid to minimize hydrolysis/maximize # of active sites that will bind protein.) Then protein is added and the pH adjusted to the basic side (pH > 8) so that the amino groups are in the NH_2 form. Again, after coupling protein, the microspheres are cleaned to remove unbound material. While the "two step" process indeed takes more steps, we think you have better control of each step and the whole process, and can easily avoid forming microsphere clumps. Call us for coupling protocols and tips for trouble-free covalent binding. One suggestion is to use crossflow systems to do all coupling and cleaning steps *in the same reactor.*

If you insist on doing a simplified "one pot, one pH" process, then we would suggest the following "1.5 step" method: 1) Carefully calculate how many COOH groups you need to activate (dry weight of particles x millequivalents of COOH/gram). 2) Add only enough WSC to activate *all* surface groups (may require some excess). 3) Let this first part of the reaction proceed for perhaps 1/2 hour at room temperature. 4) Calculate how much protein you intend to bind to the particles. 5) Add only enough protein to saturate the surface. Such a process should allow covalent binding of protein to particles without any extra WSC or protein to permit crosslinking of protein or particles.

Microsphere Dispersion

Q: I have a problem keeping microspheres dispersed, so I keep my 0.8µm latex in a 13% sucrose solution- in order to keep it from settling. Do you have any other suggestions?

Storage Conditions

Sucrose

A: You only need to rotate the bottle occasionally to prevent 0.8µm microspheres from settling; or, if they do settle, you can put the bottle on a set of rollers at >20 rpm for a few hours to thoroughly resuspend the microspheres.

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Also, I'm worried that all that sucrose will only encourage microbial growth (and attract ants!). Polystyrene microspheres smaller than ~0.5µm will *never* settle in H_2O; Brownian motion or diffusivity is greater than settling velocity.

The *best* conditions for storage of microspheres >0.5µm are:
1) Keep with surfactant (to maintain colloidal stability);
2) Add antimicrobials, like sodium azide or thimerosal;
3) Put them on rollers at low speed (5-8 rpm), to keep them suspended;
4) Keep temperature at ~4-8˚C, to suppress growth of microorganisms.

Agglutination Tests

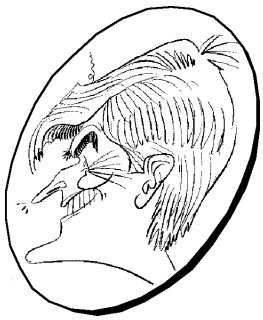
Clumping

Coated Microspheres

Reproducing Clump Sizes

Settling

Q: Why can't I keep my 0.3µm coated microspheres dispersed after I have coated them?



A: Small *singly dispersed* PS microspheres will *never* settle (see question above), even after they are protein coated. However, microspheres can easily become clumped during cleaning (especially in a centrifuge), or during coating with protein. *And* clumps of microspheres, like bunches of grapes, will settle. You must ensure that the microspheres in your product are truly singly dispersed. Check with a microscope or particle measuring instrument for change in mean diameter and distribution during

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your processing. Unfortunately, this clumping can make a *big* difference in the performance of a test or assay. Single microspheres will migrate in a strip test much faster than clumped microspheres, which may not move at all. Agglutination-based tests which have preclumped microspheres will have very different performance (sensitivity) than those using single microspheres.

The worst part of this problem is the *extreme difficulty (impossibility?) of reproducing the needed clump size.* One might need to use elaborate processes like a special stirring protocol to *make* the right clump size. *Or* use elaborate schemes involving mechanical shearing or ultrasonication to try to reproduce the correct clump size by *breaking very large clumps down* to just the right size.

If you think that you may have a problem with a test or assay which uses and *depends on* clumped microspheres, call us. You perhaps inherited a project where the product was *unintentionally* designed around clumped microspheres. If these products were now made with singly-dispersed microspheres, they might not work at all.

We think it's much better to design your product to use **single** microspheres, and we will gladly help you choose the right size and surface chemistry. If you *need* large microspheres for proper sensitivity of an agglutination test or to be caught on a filter of a certain porosity, we may be able to supply these. Then we will help you to be sure that they remain singly-dispersed as you work with them and formulate your final product. Alternatively, if you want a 300nm microsphere product which will never settle, *we'll be able to guarantee it!*

Adsorbing Protein

Adsorption

Coating Beads

Dialysis

Small Beads

Surfactant

Washing Beads

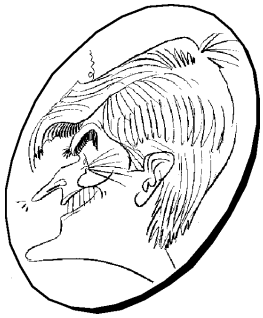
Volume 8, #2
June 1995

Q: I *still* have problems adsorbing protein onto polystyrene microspheres without clumping them. Have you any *new* ideas for coating particles painlessly?

A: This common problem often starts when you try to clean the particles by washing, especially with a buffer. Even if you use cross-flow filtration, you can clump some low surface charge polystyrene microspheres. When you clean the microspheres or "take off their sweaters" (Don't be afraid, it's only a very clever metaphor.), you are removing the surfactant used to make and store them. They may not be stable even in DI water ("In a hostile environment, they may huddle together without their sweaters."), and even less stable in buffers - *any* buffer can be a problem. After you get them coated with protein ("put their overcoats on") they will become very stable ("warm?") again.

You have several options:

1) *Don't* clean the microspheres. Just add your protein. ("Put on the overcoat *while* removing the sweater?"). The high molecular weight protein should have a higher affinity for the PS surface than the relatively low MW surfactant and protein will eventually displace the surfactant. It may work - unless and until you change the conditions. If you establish a protocol with this technique and then switch to another type or lot of microspheres, you might get very different results. This is because the surfactant type and level may be different. If you switch suppliers you could easily get a different surfactant and/or concentration (and they may not tell you what or how much they used). Then both the equilibrium and kinetics of your protein adsorption process may change. This technique can be made to work, by telling us that you need to get the same lot or recipe for your next shipment. Better still would be to test the ruggedness of your procedure by trying several lots of the "same" microspheres while you are developing your protocols.
2) Clean the particles with a dilute surfactant solution of your own choosing; then coat with protein under reproducible conditions. Use only enough surfactant to ensure colloidal stability, but not so much that it interferes with protein adsorption. "Remove sweater in a warm and comfortable environment."
3) Clean microspheres in the presence of protein. (This is a much more active version of option 1, above.) Add protein to raw microsphere suspension and dialyse or ultrafilter the surfactant out. We first heard of using low (maybe 10,000 or 50,000) MW dialysis tubing which would let our surfactant but retain the protein. As the surfactant diffuses out of the solution and off the microspheres, the protein will replace it on the microsphere surfaces. This "dialysis" method is still quite passive; you must wait for the surfactant to diffuse through the membrane.



Now ultrafiltration membranes are available (from Microgon, for example) with 50,000 MW cut-offs. In hollow-fiber form, these membranes can be used to hold protein and

microsphere together, while surfactant is actively (under pressure) washed away, and forced out of the coating zone. After such surfactant cleaning, the microspheres should be quite well coated with protein.

Then transfer the microsphere/protein suspension to a 0.1 or 0.2µm membrane cross-flow filter and remove the unbound protein from the microsphere suspension.

DNA Adsorption

Protein Adsorption

Silica Microspheres

Volume 8, #1
March 1995

Q: How do I adsorb protein onto your silica microspheres?

A: The short answer is, "You don't!" The long answer is that there is good news and bad news.
First the *bad* news. Remember that protein adsorbs onto polystyrene "latex" very well because IgG forms many hydrophobic bonds to the PS surface. These bonds hold the protein onto the surface indefinitely and almost irreversibly (Just try to get it off!).

On the other hand, silica has a very hydrophilic surface with many negative charges (no surfactant to remove). Therefore, we would not expect protein to adsorb as well, unless you can arrange for many positive charges on your protein molecules. Even then, adsorption will probably be reversible by changing the ionic environment (ionic strength and pH).

Good News: 1) You should be able to adsorb protein onto silica when silica is negatively charged (when pH > 3.7) and IgG is positively charged (pH < 8), but bonding is still not as permanent as on PS. 2) DNA/RNA will adsorb onto silica in the presence of chaotropic agents. (See TechNote 302.) 3) We derivatized some of the silica microspheres with silane coupling agents and created amino-modified and COOH-modified surfaces (Ask for *your* favorite flavor). Now you can covalently couple ligands onto the silica microspheres by various chemical schemes. (See TechNote 205, *Covalent Coupling.*)

Lateral Flow Tests

Nitrocellulose Membranes

Strip Tests

Volume 8, #1
March 1995

Q: We are having trouble applying a consistent antibody coating directly onto nitrocellulose membrane. Would it be better to coat microspheres and put them on the membrane?

A: That is a great idea. It may be difficult to get an even coating on your membrane, since the protein solution may want to wet into the membrane (depth) and spread out on the surface (width). You have no control of the adsorption reaction. About 10 years ago, in the early days of particle capture ELISA tests (those blue dot tests, like Hybritech's ICON pregnancy test), researchers had trouble putting reproducible spots of antibody on the membranes. They finally decided to coat particles with protein in a flask (controllable homogeneous chemistry possible) and spot the particles onto the membranes (easier to control where the particles go both in depth and width). We can help with your choice of microspheres to use and how to coat them.

Equations

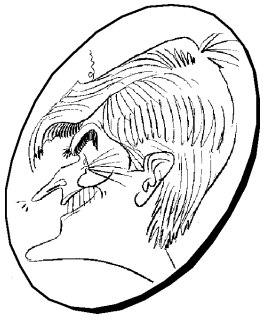
Surface Area

Volume 7, #4
December 1994

Q: What is the surface area of the microsphere, and how many microspheres are there per gram or per mL?

A: See our TechNote 206 for the equations and sample values. For **polystyrene,** ρ_s = 1.05 g/mL. For **silica,** ρ_s = 2.0 g/mL.

We report all calculated N's (particles per g and per mL) and A's on Certificates of Analysis, or you can ask for these values anytime. Of course, if you have any questions, just call, fax or e-mail us.
Snail mail is OK, too.



Equations

Parking Area

Stokes Diameter

Q: In June, Deep Coat reported that you could put 75,000 IgG's on one 0.8µm microsphere. Do you really believe that?

A: That data came from Dr. Singer, so you can ask him about it. When we calculate it using 10nm for the Stokes diameter of IgG, *we* get ~25,600 IgG's/0.8µm.

Surface Area

Volume 7, #4
December 1994

The close packed parking area for a spherical IgG molecule, **a** = $\pi d^2/4$ = 7.85 • 10^{-5} μm^2/IgG, in this case. The surface area of a 0.8µm microsphere, **A** = πD^2 = 2.01μm^2/0.8µm bead. Therefore, you can pack **A/a** = 2.01/7.85 • 10^{-5} ~ 25,600 IgG's/bead. The general solution is simply **A/a** = $\pi D^2/\pi d^2/4 = 4D^2/d^2$. Or, here **A/a** = $4(0.8)^2/(0.01)^2$=25,600. (You can, of course, get different values by considering IgG's elongated or ellipsoid shape and possible orientation standing up or lying down.) Singer used a whip?!

Blockers

Clumping

Coating Bead Concentrations

Coating Beads

Cross-Flow Filtration

Washing Beads

Volume 7, #2
June 1994

Q: After coating my polystyrene particles with IgG, I found that they were clumped. Can you help me?

A: We may not be able to help with the already clumped and coated batch, but we may be able to prevent this from happening again.

1) Examine the particles at every stage of the processing to ensure continual single particle suspension. Look at the particles under a light microscope (1000X magnification, oil immersion). Normal appearance is a continuous sea of single particles "swimming" or vibrating rapidly under Brownian motion, with occasional doublets or very few small clumps of dried particles knocked off the sides of the bottle during mixing. You cannot see single particles < ~0.4µm, but you can detect the larger, slowly moving clumps. Ultrasonics will break up the loosely bonded clumps which result from centrifuging particles, but will not remove tightly bound clumps resulting from freezing, drying or chemical agglomeration. If the particles do become clumped during processing, you may remove very large clumps by settling or with a fine mesh sieve cloth (400 mesh sieve will remove > 37µm particles or clumps).
2) Did you wash the particles before protein coating and, if so, what method did you use? If you centrifuged, did you ensure that the particles were completely resuspended before going on to the next step of your protocol? Small (< 0.5µm) microspheres are more difficult to spin down and to resuspend. Try cross-flow filtration for cleaning particles without clumping them (ask us for details). Many customers use this system and report good results with it.
3) When coating the particles with protein, did you calculate the amount of protein required to coat these small particles? One gram of particles will adsorb ~15/ρ • d mg of IgG, where ρ = density of the particles (1.05 g/cm^3 for polystyrene) and d = diameter of the particles in µm. Therefore, you will need ~57mg of IgG to coat 1 gram of 0.25µm particles, plus some excess to ensure complete coverage. We recommend that you put the clean particles directly into the protein solution (putting them into buffer first may cause clumping due to the ionic strength of the buffer). After cleaning, you want to get them coated with protein quickly. After microspheres become coated with protein, they will be more resistant to ionic strength clumping.
4) Did you use a blocker? You may want to mix blocker and IgG and adsorb them together in one step (avoiding separate adsorption and cleaning steps). Surface area/mass should also be considered here.
5) At what percentage solids did you perform the coating? Working with particles at 0.5-1% solids can help reduce the chance for clumping.
6) If your particles were coated after they became clumped, then you may have great difficulty in trying to break up the clumps. The only possible solution that we've come up with is to add a large amount of your protein to the coated microsphere suspension. After adding the extra protein, sonicate the suspension to try to break up the clumps. If the clumps do break up, the extra protein should coat the single particles before they have a chance to stick back together.

Centrifugation

Continuous Centrifugation

Cross-Flow Filtration

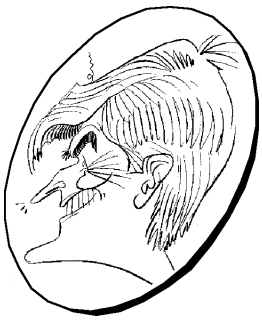
Equations

G Forces

Particle Density

Q: How can I get your particles to "get down" - in our centrifuge?

A: It sounds as if you tried, but failed. Please keep the following points in mind:



1) 99.99% of our products are heavier than and will settle in water (see "density" column in our product list). The maximum settling velocity of a *single sphere* settling in water (cm/sec), $v_{max} = 5.448 \times 10^{-5} (\rho_S - 1) d^2$, where ρ_S = density of the solid sphere (g/cm^3) and d = particle diameter (µm). Where many particles are settling together, they interfere with one another and at 5% solids the hindered settling velocity, $v_h^{5\%} = 2/3\ v_{max}$. Thus, polystyrene particles (ρ_S = 1.05 g/mL) settle in water, but will *not* settle in solutions with densities > 1.05 g/mL. (In a strong

Settling Rates

Washing Beads

Volume 7, #1
March 1994

enough solution of salt or sucrose, they will never settle).

2) Particles with d<0.6µm will *never* settle at normal gravity because Brownian motion keeps them dispersed.
3) Any particles which are more dense than water will settle in water given enough centrifugal force. The usual problem is that people don't spin fast enough or long enough. The minimum G forces generated in your centrifuge can be calculated as follows: $G = 5.59 \times 10^{-6} n^2 D$, where G = "G forces" (multiples of earth's gravitational constant), n = rotation, revolutions/min (rpm), and D = rotor diameter to top of the liquid (cm). [Maximum G forces would be calculated from the rotor diameter to the bottom of the liquid in your tube while it is spinning.] Thus, if your centrifuge has D = 10cm and spins at 10,000 rpm, it will generate $G = 5.59 \times 10^{-6} \times (10{,}000)^2 \times 10 = 5590$ G's and your particles will therefore settle 5590 times as fast as at 1G. Settling time, $t = h/v_h^{5\%}$, where h = distance the particles must settle (top of the liquid to top of the settled layer particle).
4) After they are spun down, the particles may be *very* difficult to redisperse as single particles, and *you may not know this*, unless you are careful to monitor particle size after the redispersion step.
5) Carboxylate-modified particles, other charged or hydrophilic particles, and protein-coated particles will survive centrifugation better than polystyrene particles. PS particles are more likely to become firmly stuck together and therefore hard to redisperse. And, the cleaner they get, the more resistant they are to redispersion.
6) If you are centrifuging particles to wash or clean them, then there are other, better and easier ways to clean them. Ask for our TechNote 203 for an exhaustive (or exhausting) discussion of cleaning methods. One of these "better" ways is **cross-flow filtration** which cleans the particles without clumping them. Gradually the folks at Spectrum Labs are winning over the particle world, converting the biggest particle users to their system. Contact them for more information at www.spectrapor.com.

Are you *still* interested in centrifuging particles? If you are that persistent, then please consider **continuous centrifuging.** In a continuous centrifuge you never need to form a filter cake, which is where the particles become clumped. Rather, you separate the input stream into two fractions and form two output streams - a particle-rich concentrate and a particle-free supernatant, discarding the supernatant. Then you can dilute the concentrated particle suspension with water or buffer and put in through the continuous centrifuge again, until you achieve the appropriate level of cleanliness.

Diagnostic Test Design

Technical Services

Volume 6, #4
December 1993

Q: How can you help us to make a new diagnostic test?

A: Thanks for asking. We can help in several ways, as follows:

1) Give us a chance to supply any particles you need.
2) We offer free technical service and literature to customers (and prospective customers) with questions which we can answer by phone, fax or email. (See our collection of TechNotes, www.bangslabs.com.)
3) We can coat polystyrene spheres with your antibody of choice on a custom basis (contact our Customer Service Department at info@bangslabs.com to request an official quote).
4) We have colleagues who can also help you scale-up for your in-house preparation. Ask us to supply their names and addresses.

Cleavable Crosslinkers

Disulfide Bonds

Protein Binding

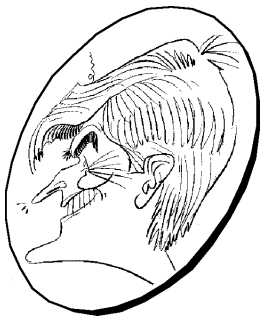
References

Thiol-Activated Particles

Volume 6, #4
December 1993

Q: How can I bind protein to your particles via a disulfide bond?

A: We were not familiar with this chemistry, but found the answer with some help from the book, **Chemistry of Protein Conjugation and Cross-Linking**, by Shan S. Wong, CRC Press (1991), especially pp. 58 & 61 (highly recommended by us as an excellent reference for coupling things to particles via a *wide* variety of chemistries) and from Pierce Chemical Company's excellent booklet on SPDP.



First, react primary amino-modified particles (plain or magnetic), with SPDP [N-succinimidyl 3-(2-pyridyldithio) propionate, a heterobifunctional, cleavable cross-linker] or related analogs. The reaction proceeds via the NHS ester end. Then, react the 2-pyridyl disulfide-activated particles with cysteine groups on protein or peptide. The

SH (thiol) group on the cysteine reacts with the disulfide group on the particles to create a disulfide linkage. By the way, this disulfide bond is later cleavable with dithiothreitol (DTT), to release a protein or peptide, if desired.

You can also create **thiol-activated particles** by cleaving the disulfide bond with DTT after the first, SPDP activation reaction, above.

Hydrophilic Membranes

Lateral Flow Tests

Strip Tests

Volume 6, #4
December 1993

Q: We are trying to make a test where coated dyed particles move along a strip until they are immobilized by an antibody bound on the strip. Any new ideas for drying particles on strips so they will move later?

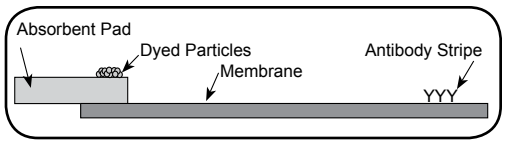
A: The problem has been to pick (or spot treat) a membrane which will adsorb the capture antibody at the site where the colored line is supposed to form *and* which will release the dried particles so they can move down the strip to form a sandwich test. One idea is to dry the particles on a hydrophilic pad which overlaps the strip upstream of the adsorbed protein. When the pad is wet with sample, the particles are transferred from the pad to the strip, and along the strip to the antibody stripe, where they are immobilized, (e.g. Fusion 5 membrane from Whatman).

Antibody Orientation

Spacer Arm

Volume 6, #3
September 1993

Q: When you use antigen-coated particles for immunization, do certain epitopes typically get hidden or exposed with a different Ig response profile than a typical immunization with or without adjuvant (followed by a boost)?



A: Certainly if antigen is adsorbed on a surface, the epitopes on the "down" side will be less likely to stimulate Ig response. You might want to covalently bind molecules so the desired epitope(s) are face up. Alternatively you might want to put the antigen on the end of a tether or spacer arm - like amino caproic acid.

Agglutination Assays

Dyed Microspheres

Sandwich Strip Test

Volume 6, #3
September 1993

Q: Can you make a *sandwich* strip test for small molecules with dyed particles?

A: "Any molecule with MW ≤6000 are too small to allow binding by two antibodies simultaneously, thus all assays on mycotoxins and other small molecules such as steroid hormones use competitive assays." [Special Guest Response by Dr. Roger Collin - Mycotoxin Research Group, AgResearch, New Zealand]

Dry Silica

Redispersing Beads

Silica Microspheres

Volume 6, #3
September 1993

Q: Why do you ship some silica particles dry and how do I redisperse them?

A: We grow silica particles from an alcohol solution. Because it is difficult and expensive to ship them in that flammable solution, we usually evaporate the solvent and ship them dry or resuspend them in deionized water for shipment.

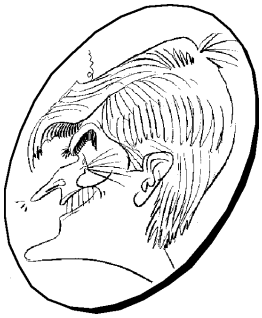
To disperse them in water, we recommend breaking up the dry material by grinding with water in a mortar and pestle plus use of an ultrasonic probe to assist in thorough redispersion. (Don't worry; you can't break the individual particles.)

Lateral Flow Test

Sandwich Strip Test

Volume 6, #2
June 1993

Q: How can I make a *direct* strip test for small molecules?



A: If you can raise Ab's to two different epitopes on the antigen/hapten, then you should be able to put one Ab on the dyed particles and put the second (capture) Ab on the strip at the capture zone. Then if a sample contains antigen (hapten), it should form a sandwich between the dyed particles and the capture Ab.

Centrifugation

Cross-Flow Filtration

Small Microspheres

Volume 6, #2
June 1993

Q: What's wrong with your ~20nm polystyrene particles? I can't spin them down in my centrifuge.

A: Particles are OK. Polystyrene particles of density 1.05 g/mL will settle in pure water (ρ = 1.00 g/mL), if you can apply enough acceleration (G-forces). For 20nm particles, it will require > 3 x 10^6 G's (really!) to achieve 10 cm/hr settling velocity. And, if you do spin them down, they will be very difficult to resuspend.

Try cleaning the particles with cross-flow filtration or a Sephadex G-25 column (See TechNote 203).

Centrifugation

Equations

Settling Rates

Stokes Law

Volume 6, #2
June 1993

Q: How do you calculate the G-forces required to achieve a settling velocity of 10cm/hr?

A: We calculate it from Stokes law formula for settling velocity of spheres: $v_m = 5.448 \times 10^{-5} (\rho_S - 1) d^2$, where v_m (cm/sec) is maximum settling velocity at normal acceleration (1G) for a sphere of diameter, d (µm), and density, ρ_S (g/mL) in water. We divide the desired settling velocity (e.g. 10/3600 cm/sec) by v_m to arrive at the G's necessary to achieve the settling rate.

Loss on Drying

Low Particle Concentration

Scattered Light Measurements

Solids Content

Volume 6, #2
June 1993

Q: How can I measure low particle concentrations?

A: We always measure solids content by loss-on-drying; weigh out 200µL of particle suspension, evaporate water, weigh solids remaining and calculate solids/suspension ratio (% solids). This method would be quite difficult for very low (<<1% solids).

You can try to measure solids content turbidimetrically with a spectrophotometer if you understand that light scattered by the particles is a function of the concentration of particles (the parameter you want to measure). Scattered light is also a strong function of several parameters which you do *not* want to measure, like particle diameter, particle aggregation (singly dispersed particles vs. clumped particles), polymer refractive index, particle coating (may affect refractive index) and wavelength of light used. You can be successful measuring solids if you are *very* careful to observe the following precautions:

1) Carefully prepare serial dilutions of particles with surfactant solution (to ensure complete single particle dispersion). Measure and plot absorbance vs. concentration.
2) Measure your unknown under exactly the same conditions.
3) Coated particles may behave differently from clean, bare particles.
4) Prepare a fresh curve for each different particle type and diameter.

Cross-Flow Filtration

Ion Exchange

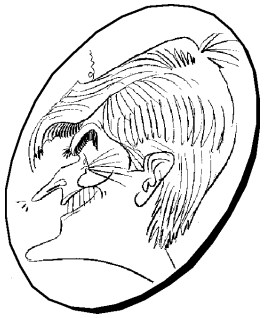
Volume 6, #1
March 1993

Q: What's the difference between ion exchange (IX) and cross-flow (XF) filtration cleaning of particles?

A: IX is a quantitative, fast, one-step process. It removes all ionic species, except those stuck on the particles (like $-SO_4^-$ and COO^-) and converts all the particle groups to the neutral species ($-SO_4H$ and -COOH).

XF filtration removes all water-soluble material. Cleaned particles are usually about half-charged, with half of the sulfate and carboxylate surface groups in the $-SO_4H$ and -COOH forms.

The IX cleaned surfaces thus have a lower charge and the particles are not as stable. XF filtration takes longer, since it depends upon passive desorption of surfactants from the surface. Overall, I would choose XF, first.



Surfactants for Protein

Volume 6, #1
March 1993

Q: Which surfactants are better to use with protein-coated particles?

A: Nonionics, probably. In studies where high levels of surfactant were added to *try* to remove protein, nonionics could only remove 60% of adsorbed protein while anionics removed ~80%.

Adsorption Rates

Equations

Volume 5, #4
December 1992

Q: To detect a certain virus by latex agglutination, we tried to adsorb polyclonal and monoclonal antibodies onto 0.8µm and 2.5µm polystyrene particles. First, we washed 50mg of particles several times with buffer. Then, we resuspended them in 4mL of 0.3mg of Ab/mL buffer solution and incubated (overnight at room temperature). Next, we measured amount of Ab in solution after adsorption and found 0 mg/mL for 0.8µm particles and ~0.3 mg/mL for 2.5µm particles we tried. What happened here?

A: You have *no* problem so far. The surface capacity of polystyrene for adsorption of protein is ~3mg IgG/m^2. The specific surface area of particles, in m^2/g, is ~6/d, where d is in µm. Therefore, 0.8µm particles will have surface area of 6/0.8 or $7.5 m^2/g$. Thus, they will adsorb 7.5 x 3 = 22.5mg IgG/g of particles or 22.5 x 0.05g = 1.1mg IgG/0.05g particles. You added ~1/2mg IgG (4mL x 0.3mg IgG/mL) to 0.05g of particles, or about what we would expect to saturate the surface, if it all were to adsorb onto the particles.

You found 0mg of protein/mL in the supernatant, and this result is consistent with the amount you added and the capacity of the particles. (If the IgG adsorbed to capacity on the polystyrene surface, we would expect to find none left in the solution.)

Likewise, the 2.5µm particles will have surface area of $6/2.5 = 2.4 m^2/g$, and will adsorb 2.4 x 3 = 7.2mg IgG/G of particles or 7.2 x 0.05g = 0.36mg IgG/0.05g particles. You added ~1.2mg of IgG/0.05 g of particles - an amount which is about 3.33X what we would expect to saturate the surface. The excess 1.2-0.36 or 0.84mg will be found in the supernatant at a concentration of 0.84/4 = 0.21 mg/mL, which is quite close to the 0.3 mg/mL concentration which you found.

Thus, we would say that your adsorption results are about what we would expect. For more information, check out TechNote 204, *Adsorption to Microspheres*.

Agglutination

Antibody/Antigen Concentration

Volume 5, #4
December 1992

Q: After adsorption of Ab, we washed and resuspended with BSA/buffer. We next tried agglutination of particles using a virus culture, but no agglutination appeared.

A: In this case, agglutination should be caused by a virus attaching itself to two or more antibody-coated particles and binding the particles together. You might need to adjust the amount of virus added to cause agglutination. If your sample contained a very high concentration of virus, they could completely cover the antibody-coated particles and no agglutination could occur, because no bridging between particles could happen. Try diluting your virus concentration by 10X and 100X to see if you can get agglutination this way.

In some cases, you may also need to adjust the amount of antibody on the particle surfaces to less than a monomolecular layer. (See our TechNote 301 regarding coating density of Ab or Ag on the particles and the ratio of agglutinating agent to particles.)

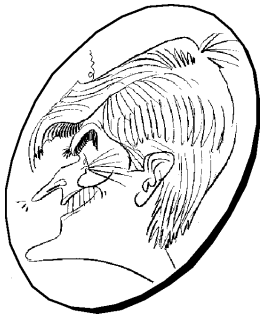
Binding DNA

Binding Oligonucleotides

DNA Adsorption

Streptavidin/ Biotinylated DNA

Q: How do I bind DNA or oligonucleotides on your particles?



A: You can use magnetic particles (amino- or COOH-modified), silica or polystyrene-based particles. There are several binding methods, depending on the molecular weight of the DNA or piece you use, and on the type of particles you choose.

1) DNA will adsorb directly onto our uniform silica particles, if the conditions are right. Silica will be negatively charged at pH >3.7. So, at any pH (>4) where your DNA or fragment is positively charged, DNA should adsorb onto SiO_2. (It is also possible to reverse the silica charge and adsorb negatively charged DNA. (See

Volume 5, #4
December 1992

TechNote 104 for more information.)

2) Polymeric particles (e.g., plain or magnetic polystyrene) will also adsorb (or covalently couple) streptavidin. The streptavidin will bind biotinylated DNA or oligonucleotide very securely. (See Product Data Sheet 714 for the protocol.)
3) Amino-terminated oligos will bind to COOH-modified particles via water-soluble carbodiimide. (See TechNote 304 for the protocol.)

Light-Scattering Assays

Nephelometric Assays

References

Refractive Indices

Volume 5, #3
September 1992

Q: Could I make better nephelometric assays using particles with refractive indices higher than polystyrene's?

A: There is interest in particles with refractive indices *different* from that of polystyrene (both higher and lower refractive indices have been the subject of recent papers). One group showed that higher refractive index polymers can be made using vinylnaphthalene as the core and a shell polymer with active surface groups for covalent coupling. These particles are better light scatterers, especially after agglutination. [Deleo, D.T., I.R. Lee, J.D. Wetherall, D.J. Newman, E.A. Medcalf, C.P. Price. 1991. Particle-enhanced turbidimetric immunoassay of sex-hormone-binding globulin in serum. *Clin Chem,* 37(4): 527-531.]

However, we worked with a customer who says that for these nephelometric assays, the surface groups are more important than the refractive index of the core polymer. Meanwhile, some other work showed that lower refractive index polymers would be useful. [Amiral, J., M. Migaud. 1991. Development and applications of a new photometric method for fast and sensitive immunoassays. *Europ Clin Lab,* 10: 28.] We have many sizes of polymethyl methacrylate (PMMA) particles with lower refractive index for you to try if you like.

Surface Tension

Surfactant Concentration

Volume 5, #3
September 1992

Q: Can I check surfactant concentration on incoming lots of latex by measuring surface tension?

A: You can use surface tension to compare incoming lots of latex if they contain sulfate or sulfonate surfactants. If, however, the surfactant is a fatty acid sale, you should check pH also. Sulfate surfactant molecules will normally all be in the $-SO_4^-$ form, so surface tension will probably be independent of pH at pH>5. Fatty acid surfactants (with pKa's >5.2) are more sensitive to pH and will have -COOH as well as $-COO^-$ species present at higher pH, each contributing differently to surface tension. So, always measure surface tension at the same pH (>11 recommended).

Parking Area

Titration Data

Volume 5, #3
September 1992

Q: What is this "parking area"?

A: *Not* an employee perk, it refers to the area per molecule for titrated acid (or other functional group) on the surface of our particles. We titrate and report the amount of acid as *microequivalents per gram of particles.*

Equations

Parking Area

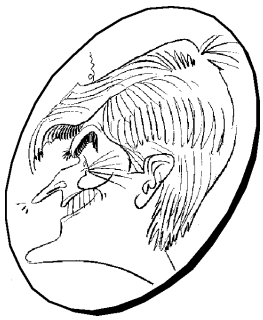
Surface Area

Titration Data

Volume 5, #3
September 1992

Q: But what about the relative number of groups on the *surface*?

A: The specific surface area for a sphere (m^2/g) is ~6/d, where d = diameter in micrometers, so 1µm particles will have surface area = ~6 m^2/g. If you divide surface charge (µeq/g) by the specific surface area for that lot of particles, you will get surface charge per unit area (µeq/m^2). We invert this number and report the **"parking area"** ($Å^2$/COOH group) or area available per molecule of acid on the particle surface. Keep in mind that a close-packed monomolecular layer of acid groups would have a "parking area" of 20-25$Å^2$/COOH group. Then you can see that particles with "parking area" <25 have a complete coating of acid groups, while those with >250 would be only 10% covered with acid groups. The "parking area" is a way to begin to select particles of different sizes with equivalent binding properties.



Lateral Flow Tests

Sensitivity

Strip Tests

Volume 5, #2
June/July 1992

Q: What sensitivity is possible with chromatographic (dyed particle migration) strip tests?

A: I calculated the sensitivity from the following assumptions:

1) Colored line must be ~5mm long x ~0.5mm wide x ~10 particles deep or thick.
2) The particles are ~0.25µm diameter.
3) It will take ~10 molecules to hold each particle at the strip location.
4) Binding will be ~10% efficient (there should be a 10X excess of molecules present to get proper binding).
5) Antigen molecules might be 1000-10,000 MW.

Thus, there will be 20,000 x 2,000 x 10 = 4×10^8 particles and 4×10^9 molecules for binding the beads and 4×10^{10} molecules or **67fM (67-667 picogram) sensitivity** is possible. Please ask for a more complete explanation, if you wish.

Particle Diameter

Reproducibility

Volume 5, #2
June/July 1992

Q: Last year, I bought some 0.300µm particles. How close can you get to 0.300µm again with an entirely new lot?

A: We can usually reproduce lots to ±5% of the original size or within the range of 0.285-0.315µm. Often, we can get closer. We can usually show you two or three replicate lots where we tried to repeat a given recipe several times.

Please try other sizes, since most people find no difference between particles of ±20% from the original size (0.24-0.36µm).

Particle Determination

Sandwich Test

Volume 5, #2
June/July 1992

Q: I want to replace enzymes and some steps by using dyed particles in a sandwich test format on coated tubes. What size particles should I use?

A: Try the smallest particles (<100nm) which can move fast and stick tightly to the tube surface. If larger particles (say 1µm) were used, they might stick out from the surface too far and be more likely to be knocked off by liquid swirling around inside the tube.

Coating Beads

Surface Monolayer

Volume 5, #1
March 1992

Q: What's the matter with your particles? I could only put 200-300ng of protein per cm^2 of particle surface.

A: Congratulations! You achieved maximum coverage of protein on the particles. The best one can do is a monolayer of protein molecules, which is ~3 mg/m^2 (=300 ng/cm^2). See our TechNotes 204 or 205 for details on calculating expected coverage.

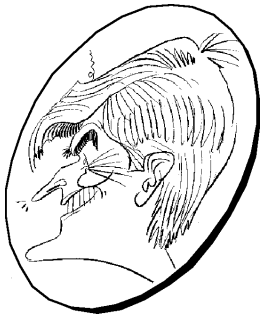
Agglutination Test

Bacteria

Volume 5, #1
March 1992

Q: We can do an agglutination test with bacteria + antibody. Could we mix bacteria + particles + antibody?

A: It sounds like a good idea. Particles would add bulk to the test to enhance visibility and sensitivity. One way would be to put Ab to bacteria cell surface antigens on the particles; then any added bacteria in a sample would cause coagglutination.



Hydrophilic Membranes

Membrane Sticking

Q: My dyed particles don't have any get up and go. How do I put them on a membrane and prevent them from sticking permanently?

A: Covalently bind protein to the particles. Then add lots of surfactant to them and to the membrane to prevent the particles from sticking to nitrocellulose or any

Strip Tests

other hydrophobic membranes. Pretreatment of the membrane with substances that can easily be rehydrated and maintain a small space between the beads and the membrane (e.g. sucrose solution) may also be helpful.

Volume 4, #4
December 1991

Strip Tests

Q: What are these strip tests you refer to?

Volume 4, #4
December 1991

A: Strip tests are like sandwich assays, but the color comes from dyed particles which complete the sandwich, instead of an enzyme and substrate to generate color (see TechNote 303).

Blockers

Nonspecific Binding

Protein Binding

Surfactant

Q: What's the best way to bind protein to particles and avoid NSB?

A: **Answer 1:** Adsorbing protein onto polystyrene and blocking with BSA, gelatin or other protein works well. *But* we cannot guarantee that some other protein won't adsorb also.

Answer 2: Covalent coupling of protein to carboxylate-modified particles permits permanent anchoring of protein to the surface. This may be followed by addition of large amounts (1-5%) of surfactant like Tween® 20, with or without blocker protein. The amount of surfactant or blocker can be increased until complete blocking is achieved without worry that the desired protein will come off.

Volume 4, #4
December 1991

Water-Soluble Carbodiimide

Q: Can I activate COOH-modified particles with WSC (water-soluble carbodiimide) today and couple my protein to the surface tomorrow?

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December 1991

A: We recommend adding protein to the activated particles immediately after activation to prevent hydrolysis of the activated beads.

Dilute Suspensions

Particle Concentration

Solids Content

Turbidity

Q: How do I measure solids content, especially for dilute (<1%) particle suspensions?

A: The best way we know to measure solids content is to weigh suspended beads, evaporate the water and weigh the solids remaining. There is no simple instrumental method to give solids or number concentration of particles with accuracy.

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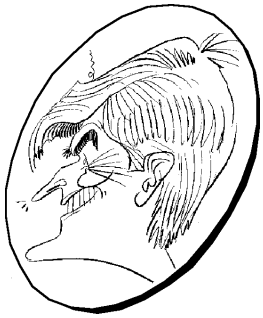
December 1991

Turbidity is sensitive to particle composition (refractive index), diameter, solids content and wavelength of light used. If composition, diameter and wavelength are held constant (i.e., for the same lot of particles measured under the same light conditions), then it is possible to measure and plot turbidity (absorbance in a spectrophotometer) vs. concentration. Such a curve would be most sensitive in very dilute (ppm) concentrations.

The accuracy of the data would be very sensitive to the care involved in doing the work, especially **accuracy of dilution** (taking 10% solids suspension and diluting it carefully to the ppm range), **thorough redispersion** (no particles sticking together) and **absorbance of the diluent** (water + surfactant for dispersing particles + other ingredients). A wavelength of ~700nm is suggested.

Obviously, such a curve would be applicable only for particles of the same lot, measured under identical conditions (spectrophotometer, wavelength, diluent, etc.) and a new curve would be needed for each different lot of particles.

If particles are coated with protein or other chemicals, then a new curve should be generated.



Cell Depletion

Magnetic Particles

Phagocytosis

Volume 4, #3
September 1991

Q: Doing cell purification/depletion studies, I'm trying to tag and remove certain cells with your magnetic particles. How do I stop my cells from engulfing or 'eating' the magnetic particles?

A: Dr. Ramani from Transmed Biotech says there are two ways of stopping cells from eating particles: 1) lower the temperature or 2) add some azide - not enough to kill them, just spoil their "appetites." Also, remember that the more hydrophobic the particle, the more likely it will be phagocytosed.

Filter Method

Fluorescent Beads

Immunoassays

Quenching

References

Volume 4, #3
September 1991

Q: How can I use fluorescent particles in immunoassays?

A: We can think of at least two ways:

Flow method: Imagine that the particles are put through a capillary and individual particle fluorescence is measured. (The fluorescence of the dye is excited at one wavelength and measured at another wavelength.) You should get light flashes of one intensity for single particles. As particles start to agglutinate, you should get flashes of higher intensity as doublets, triplets, etc. pass by.

Quenching method: Now imagine that all the particles are measured at once, dilutely dispersed in a tube. If they are agglutinated or clumped, then some of the fluorescence will be absorbed or scattered and lost and the signal will diminish on agglutination. See also the Gosling paper cited below:

Gosling, James P. 1990. A decade of development in immunological methodology. *Clin Chem*, 36(8): 1408:1427.

Filter Method: If single particles pass through a filter, but agglutinated particles are caught, then fluorescence on the filter will be a measure of antigen content of an agglutinating sample. (See TechNote 103, *Fluorescent Dyed Microspheres*, for a list of different combinations of sizes, surface chemistries and dyes. Dye names and excitation and emission wavelengths are listed.)

Magnet Selections

Rare-Earth Magnets

Volume 4, #2
June 1991

Q: Should we use an electromagnet or a permanent magnet to separate the magnetic particles from suspending liquid?

A: Either should work. The combination of liquid path length (tube diameter) and magnet strength should be arranged to cause separation in a reasonable amount of time. We usually recommend rare earth magnets because they are so strong. An electromagnet should work as well, providing that there is no residual magnetism in the electromagnet when it is turned off. (See TechNote 102, *Magnetic Microspheres*.)

Adsorption

Blockers

Covalent Coupling

Hydrophilic Beads

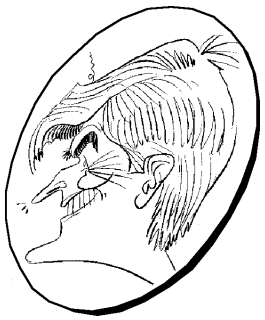
Nonspecific Binding

Volume 4, #2
June 1991

Q: How do I couple protein to particles while avoiding nonspecific adsorption?

A: Two routes have been attempted in the past.

1) Adsorb (or covalently couple) the appropriate protein (usually IgG) onto polystyrene. Then adsorb (or couple) a blocker, like BSA, to fill any empty surface with inert protein, leaving no space for nonspecific adsorption by anything else in the sample. Currently, we recommend this route and can share more details of this approach. Coadsorption or co-coupling of the blocker protein is another option as well.
2) Start with hydrophilic particles with surface functional groups but which have very poor adsorption capacity for proteins. The idea is to covalently link only active protein to these particles. Subsequent nonspecific binding is (theoretically) prevented by this method.



We continue to search for particles which are appropriate for this second approach. The difficulty is to obtain a surface which does not adsorb anything but which has groups suitable for covalent attachment. Possible candidates for this approach are amide-modified particles, with their very hydrophilic surfaces. They can be (partially) derivatized (with hydrazine) to form some hydrazide groups which permit easy covalent coupling.

Particle Determination

Volume 4, #2
June 1991

Q: What size particles should I use for a particle-based test?

A: The diameter you choose is dictated by the type of format you want to use. We suggest the following guidelines:

Test/Assay Type	*Particle Size*
Slide agglutination	0.2 - 0.9µm
Latex immunoassay	0.01-0.3µm
Particle capture ELISA	0.3 - 0.9µm*
Chromatographic strip test:	
(dyed particles moving along strip, like pregnancy tests)	0.1 - 0.4µm**

* depending on particle capture method
** depending on porosity of the strip

Adsorption

Co-Adsorption

Isoelectric Points

Monoclonal Antibodies

Polyclonal Antibodies

References

Q: Why can't we get our monoclonal antibodies to stick onto particles as well as the polyclonal antibodies do?

A: Monoclonal (Mc) and polyclonal (Pc) antibodies may behave very differently during adsorption. One major difference is that they often have very different isoelectric points. Pc IgG adsorbs best at its isoelectric point (pH ~7.8). At this pH one gets maximum adsorption, apparently because the IgG is in its most relaxed state and each molecule takes up lowest area on the surface.

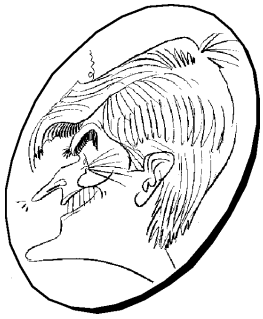
Do you know the isoelectric point of your Mc Ab? Some Mc Ab's have isoelectric points as low as pH 4. Adsorption under these conditions *may* work for you.

Volume 4, #1
March 1991

At these low pH's, some PS (polystyrene) particles *may not* be as colloidally stable (they may tend to spontaneously clump or flocculate), so other tricks may be necessary to get good adsorption. One customer used ascites fluids for coating the particles; he got good coating and activity, but immunological stability was poor, perhaps due to the enzymes which denatured the protein after a few days or weeks (depending upon temperature). This customer is now trying to partially purify his ascites fluid, to remove the problem.

It may be better to start with purified Mc Ab and to add things like coadsorbents or blocking agents. It is well-known that pure surfactants do not adsorb as well as those which have an impurity which is also surface active; the two adsorb better than either alone. So, try mixing Mc Ab with BSA, HSA, non-ionic surfactant or other blocker. It is quite reasonable to expect that some other protein will make a more compatible environment for the Mc Ab. A recent protocol suggests blocking with BSA and non-ionic surfactant in the weight ratio of 20/1 (1% BSA + 0.05% Tween® 20) [See Jenkins, S.H., H.B. Halsall, W.R. Heineman. 1990. The use of ion-pairing reagents to reduce nonspecific adsorption in a solid phase electrochemical enzyme immunoassay. *J Clin Immunoassay,* 13(2): 99-104.]

One can also covalently couple the Mc Ab's to surface-modified particles to ensure their firm binding.



Anti-Microbial

Contamination

Q: What do we do if the particles are contaminated?

pH

Volume 4, #1
March 1991

A: We don't usually have problems with bioburden or wildlife. In many of our particles, the combination of the surfactant used (fatty acid) and pH>8 hinders microbial growth. We also store our particles at 4˚C after they are made. Nevertheless, particles can become contaminated after repeated entry into the bottle. At the request of some customers, we have added an antimicrobial - either sodium azide or thimerosal (sodium ethylmercurithio-salicylate) to some particles. If you have a contamination problem and don't want to add an antimicrobial, try adding base (to pH 11) and store for about two weeks.

COOH-Modified Beads

Covalent Coupling

EDAC Coupling

Two-Step Coupling

Volume 4, #1
March 1991

Q: How should we bind our protein to carboxylic-acid modified particles?

A: Try the two step method. *First,* react the particles with water-soluble carbodiimide (WSC) at acid pH 4-6. Clean to remove unbound WSC. *Second,* react WSC-activated particles with protein at pH 8. Clean to remove unbound protein.

Under these conditions each part of the reaction occurs at its optimum pH. In the first step, the carbodiimide is protonated to make it more susceptible to nucleophilic attack by surface COO^- groups.

In the second part, the amino groups on the protein are in the NH_2 (not NH_{3+}) form.

Adsorption

Antibody Immobilization

COOH-Modified Beads

Covalent Coupling

Volume 3, #2
November 1990

Q: To make a diagnostic test, should I adsorb protein on polystyrene (PS) or couple it to COOH-modified particles?

A: Simple adsorption works fine to put whole polyclonal IgG molecules on particles for agglutination tests. You might need covalent binding if you want to put on IgG pieces (Fab portion, for example), if you are working with monoclonal antibodies (they may not adsorb as well as polyclonals), if you are working with our itty-bitty (<100nm) PS particles which may not be as stable as COOH particles, or if you have a supersensitive assay where a minute amount of ligand coming off the particles will interfere.

You probably *will* need covalent coupling if you are trying to put on small molecules like antigens, peptides, pieces of DNA or RNA, or haptens. They either won't adsorb on the particles or stay very long.

There are about a dozen different coupling chemistries you could use to bind things onto particles. Covalent coupling to COOH-modified particles is easier because there is a better choice of particles – sizes, types, colors and acid levels. Also, the chemistry has been well worked out and recipes published. However, we also have a few particles with other surface groups, like amino and hydrazide, which are also very useful for coupling.

Lateral Flow Test Membranes

Membrane Pre-Coating

Mobility of Particles

Sticking

Strip Tests

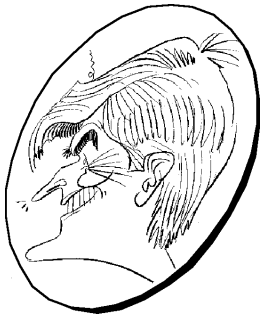
Volume 3, #2
November 1990

Q: After we dry the Ab-coated dyed particles onto our membrane, how do we ensure that they will move when re-wet with sample?

A: Lack of movement could be due to particles too large to move freely in the membrane, particles sticking to the membrane, or a sample flow rate inadequate to dislodge and carry the particles along the strip.

You could investigate the mobility of the particles through the membrane as follows:

1) Spot some particles on the membrane.
2) Before they can dry, add water or buffer to move the particles.
3) If they do *not* move, then there may be a physical barrier to their movement.



Sticking on a bare membrane may be prevented by precoating the membrane with proteins. Alternately or additionally, the membrane surface can be treated with very hydrophilic materials, like sucrose, which should easily rehydrate and release the particles upon re-wetting. Other hydrophilic materials might also work, such as trehalose, other saccharides and oligosaccharides. Gelman suggests using BSA, non-ionic surfactant *and* sucrose. (See TechNote 303, *Lateral Flow Tests*.)

Lateral Flow Tests

Sandwich Strip Test

Strip Tests

Volume 3, #1
April/May 1990

Q: How do we make a test using dyed particles which move along a strip and form a colored line if antigen is present in a sample?

A: Use a pregnancy test as an example. In this test, Ab-coated, blue-dyed particles flow along a nitrocellulose strip to reach an immobilized second Ab; if HCG is present, a sandwich forms and results in a blue line for a positive test. For this type of test, one needs darkly dyed particles which will move easily through the strip. The particles should be large enough to give a strong signal (as large a "light bulb" possible), yet small enough to move freely along the strip. A compromise is made between large, bright particles and small, fast moving particles. (See TechNote 303, *Lateral Flow Tests*.) *Here's the commercial message:* We have a virtual rainbow of colored and fluorescent particles in many sizes and with different surface chemistries.

Aggregation

Electrophoretic Mobility Instruments

Field Flow Fractionation

Latex Immunoassay

Monodispersity

Nephelometers

Particle Counters

Particle Sizers

References

Spectrophotometer

Volume 3, #1
April/May 1990

Q: We are currently assessing the potential of latex particles as a solid phase in immunoassays. During coating or storage the particles may aggregate. We are interested in methods for the determination of the proportion of single particles, doublets, triplets, etc.

A: The detection of particle aggregation is an interesting problem. We have been urging attention to possible aggregation for customers who prefer to clean the particles by centrifugation/decantation/redispersion. Also, a sensitive detection method can be the basis for **Latex ImmunoAssay (LIA).** I can suggest several methods in order of increasing sophistication:

1) If the particles are large enough, >0.5µm, they may be observed in the light microscope and approximate counts of singles, doublets, etc., may be made. This method must be used with caution because the necessity for sample dilution before observation may introduce an artifact (the act of dilution may cause or eliminate the particle aggregation you are trying to observe). Of course, this method will not work for smaller particles.
2) **Spectrophotometers** can be used to monitor particle aggregation. The light scattered by single small particles will change if the same number (or weight) of particles are partially aggregated. The scattered light may be read as "absorbance" on any spectrophotometer. If absorbance changes with time or differs from batch to batch, one can make inferences and conclusions about the aggregation state. However, it may be difficult to quantify the exact numbers of doublets, triplets, etc. Again, dilution may cause changes in the state of aggregation.
3) **Nephelometers** may be used similarly, by reading scattered light directly. Usually, these instruments have a *fixed angle* (often 90°) between the detector and the incident light beam.
4) Particle counters or sizers like those made by Brookhaven Instruments, Coulter and Malvern can be used to monitor the size distribution of a population of single, double, triple, etc., particles.
5) Electrophoretic mobility instruments can be used for particle characterization and may help in aggregation studies.
6) Field-flow fractionation is a technique which can sort out the relative ratios of singles, twins, triplets, etc. FFFractionation, Inc., of Salt Lake City, UT offers these instruments (now available through Postnova Analytics, www.postnova.com). See paper by Giddings, J.C., et al. 1989. Colloid characterization by sedimentation field-flow fractionation. VII. Colloid aggregates. *J Colloid & Interface Sci,* 132(2): 554-565. In this work, they measured singles, twins, triplets, etc., of latex particles which I gave them to test.

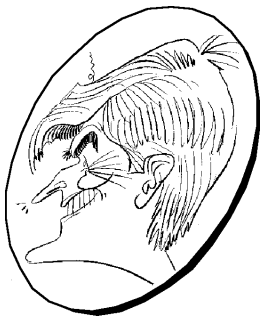
Latex Agglutination Tests

Particle Determination

Volume 3, #1
April/May 1990

Q: What size particles should I use for a (latex) agglutination test kit?

A: The short answer is ~0.8µm. The long answer: People use anything from 0.1-1µm. The most popular sizes are either 0.2-0.3µm or ~0.8µm. In this range, sensitivity should increase with particle diameter, so we'd say try the larger ones first. Because they are popular sizes, there is a good selection of particles in either range. At 0.7-0.9µm, you can choose from several replicate lots of PS particles. COOH-modified particles with different levels of surface COOH groups are also available, for covalent linkage.



Aggregation

Clumping

Electrolytes

Flocculation

Hydrophilic vs. Hydrophobic Surfaces

Surfactants

Volume 2, #4
December 1989

Q: I'm having problems coating particles with protein. The particles are stable when I start, but when I add the protein solution, they form visible clumps.

A: Sometimes there are problems with flocculation (clumping) of the small particles upon addition of protein buffer solution. Often, the buffer (electrolyte) is causing the particles to flocculate before they can become protein coated.

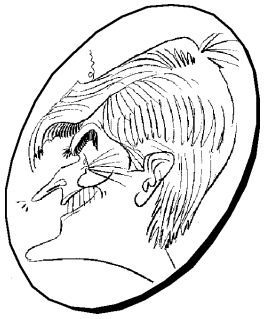
Most of our particles are made with fatty acid surfactants. Added as emulsifiers during polymerization, they act as colloidal stabilizers after polymerization. The hydrophilic COOH groups and, at basic pH's, the charged COO^- groups from the adsorbed fatty acid molecules make the surface of the polystyrene particles more hydrophilic. Permanent charged SO_4^- groups on the ends of the polystyrene polymer chains (from free radical initiator used in polymerization) also add hydrophilic character to the particles. In pure (deionized) water, particles covered with these negatively charged groups are mutually repulsive and therefore completely dispersed (colloidally stable).

Nevertheless, the particles still have exposed hydrophobic polystyrene surface. Once the hydrophobic surfaces of adjacent particles come into contact with each other, they will stick together firmly. Electrolyte added to the water can decrease the surface charge to the point where Brownian motion brings the particles into contact, and they aggregate and stick together in large clumps.

This electrolyte-induced flocculation can be diminished or eliminated by covering the exposed polystyrene surface of the particles with protein, more anionic surfactant, or nonionic surfactant (like Triton™ X-100 or Tween® 20). We suggest the following ways to avoid flocculation during coating:

1) Add a SMALL amount of surfactant to particles before exposure to protein buffer solution. Particles partially precoated with surfactant are more able to survive electrolyte shock during coating in full strength protein/buffer solution. Too much surfactant could coat the particles completely and prevent protein adsorption.
2) Some people rinse the particles with a dilute solution of protein (a few µg/mL in lowest possible ionic strength buffer) to precoat them.
3) Avoid electrolytes (use as little as you can and still prevent protein denaturation).
4) Keep the particles apart (dilute the suspension as much as possible).
5) Use as much protein as possible (stronger concentration than you might normally add).
6) Add particles to protein solution (NOT protein to particles).
7) Use stirring to promote rapid kinetics (get particles diluted and coated quickly).

Explanation: The idea here is to coat each particle with lots of protein before it encounters another uncoated particle in a hostile (high ionic strength) environment. The stoichiometry is adjusted so that the particles become protein-coated before they become flocculated. After coating, they will be more electrolyte resistant.



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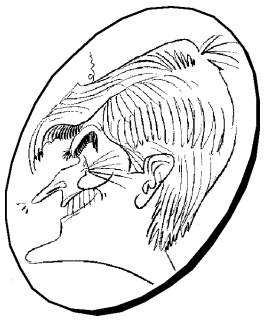

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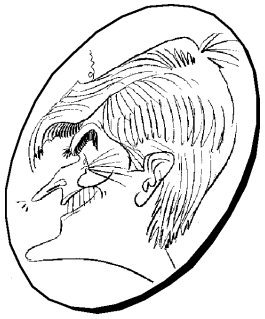

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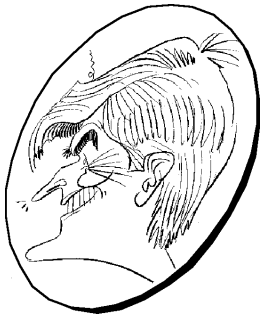

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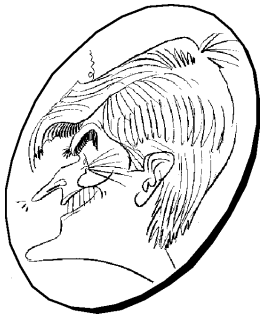

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