

Tech. Note #13c
COVALENT COUPLING PROTOCOLS

- This is a collection of recipes or methods for covalent coupling of proteins to polymeric microspheres which have not been tested by us. Feel free to substitute other buffers or make changes according to your judgement.

I. General Considerations

A. Microspheres

Particles are usually made and shipped with surfactant present. Although surfactants or detergents may sometimes interfere with, it is often not necessary to clean the microspheres prior to use. The microsphere suspension should ideally contain only the smallest amount of surfactant necessary to maintain its stability. This may mean removing some of the surfactant, e.g., by ion exchange, cross-flow filtration, dialysis, column chromatography, or centrifugation. For extreme dilution of latexes (less than 1% solid content), surfactant at a 0.01-0.1% concentration may be necessary (before protein coating). Note that in adaptation of the following protocols, centrifuge times and speeds must be varied according to the size of the particles. Refer to Tech. Note #37 for tips on washing microspheres.

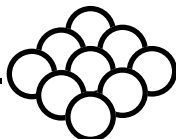
B. Ratio of Protein to Microspheres to be Used

We suggest an excess of protein over that which can be accommodated by the PS surface if you want to ensure maximum coverage.

1. Achieving Surface Saturation

- Polystyrene capacity for BSA $\approx 300 \text{ ng/cm}^2 \approx 0.30 \text{ } \mu\text{g/cm}^2 \approx 3 \text{ mg/m}^2$
(BSA = bovine serum albumin)
- Polystyrene capacity for BlgG $\approx 250 \text{ ng/cm}^2 \approx 0.25 \text{ } \mu\text{g/cm}^2 \approx 2.5 \text{ mg/m}^2$
- Surface Area/Mass for Particles = $6/\rho D$ (m^2/g)
where ρ = density of a particle (g/cm^3) ($\rho = 1.05 \text{ g/cm}^3$ for polystyrene) and
D is in μm .
Then, if $D = 1 \mu\text{m}$, $A/M \approx 6 \text{ m}^2/\text{g}$.
- Therefore, one gram of $1 \mu\text{m}$ diameter PS particles can accommodate
 $\sim 6 \text{ m}^2 \times 3 \text{ mg/m}^2 \approx 18 \text{ mg}$ of BSA or
 $\sim 6 \text{ m}^2 \times 2.5 \text{ mg/m}^2 \approx 15 \text{ mg}$ of BlgG

Note: For non-agglutination tests, if the coating is antigen, to which the antibody will bind, most often the more antigen on the solid phase, the better. If the coating is IgG antibody (Ab), then the antigen to be subsequently bound must have room to access the binding site of the solid-phase IgG, and you may not need complete coverage. Some experienced users report using only enough Ab to cover about



half the particles' surfaces as ideal for latex agglutination tests.

C. Microsphere Resuspension

To remove surfactant or to remove unbound protein after coating we recommend cleaning by dialysis, column methods or ultrafiltration. If you must use centrifugation to wash the particles, then please make certain by microscope or particle size instrument that particles are *completely* resuspended as single particles and redispersed after new buffer is added before proceeding to next steps. This can be done by rolling, sonication, aspiration through a fine pipet tip, vortex mixing, etc.

D. Use of "Surface Diluents"

The use of a surface diluent, blocker or filler is recommended in most covalent coupling protocols. The following are some of the more common blockers:

- BSA/ HSA/ ovalbumin
- BSA/Tween 20 in a 20:1 ratio (1% BSA + 0.05% Tween)
- Surfactants, especially non-ionics (Triton X-100, Tween 20, etc.)
- Polypeptides (e.g. Prionex, safer (?) alternative to BSA)
- "Irrelevant" or neutral IgG
- Gelatin / gelatin hydrolysate (enzymatic) or fish gelatin
- "heterophilic (antibody) blocking reagent" (Scantibodies)¹
- Casein or non-fat dry milk
- KLH (keyhole limpet hemocyanin)
- Blotto (normal goat serum + non-fat dry milk)
- Fish Serum
- Super Block serum-free protein blocker (SLD)²
- Megga-block 3: "components of small molecular size" (Bionostics)³
- α 1-acid glycoprotein
- Polyvinyl alcohol
- Polyethylene glycol

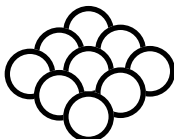
II. Covalent Coupling of Protein to Carboxylate-Modified Particles Via -COOH + -NH₂ Binding

A. One-Step Coupling (Average Conditions)

1. Reagent Preparation

- a. Microspheres: Carboxylic acid-modified, 10% solids content.
*Note that in this protocol, microspheres are used without prior cleaning.
- b. MES/Protein Solution: Dilute purified IgG (1 mg/ml) in MES buffer [Morpholino ethane sulfonic acid (Merck, Sigma)], 0.05M, pH 5.5.
- c. WSC Solution: 1- (3-dimethylaminopropyl) -3-ethylcarbodiimide (Aldrich-Sigma) solution freshly prepared at 10 mg/ml in deionized water.
- d. TPG Wash/Storage Buffer:

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7.8g NaH₂PO₄ · 2H₂O (0.05M)

1.0 g NaCl (0.1%)

Add approximately 800 ml of deionized water, adjust pH to 6.6 with NaOH. Add 2 g Gelatin (dissolved in hot H₂O, while stirring). Dilute with deionized water to 1 liter.

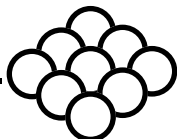
2. Coupling
 - a. Place 0.1 ml of carboxylate-modified microspheres in a glass tube.
 - b. Add 1 ml MES/Protein Solution - vortex.
 - c. Add 0.1 ml WSC solution - vortex.
 - d. Gently shake for 2 hours at room temperature or incubate 10 min. at 50°C.
3. Wash/Storage
 - a. Centrifuge at 4°C, discard supernatant, resuspend pellet in 1ml TPG Wash/Storage Buffer. Repeat twice.
 - b. Store at 4°C.

**Other variations on the preceding one-step coupling procedure are offered by Nathan⁴, Hager⁵ or Quash⁶.

B. Two-Step Coupling Procedure⁷

*The first step is run at an acid pH to ensure that carboxylic acid groups are in COOH form. The second step is run at basic pH to ensure that amine groups are in NH₂ form.

1. Reagent Preparation
 - a. Microspheres: Carboxylate-modified polystyrene 10% solids.
 - b. Water-Soluble Carbodiimide (WSC) Solution: 2 % (w/v) 1- ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma Chem. Co.) solution freshly prepared in deionized water.
 - c. Pre-activation Buffer: 0.05M KH₂PO₄, pH 4.5
 - d. Coupling Buffer: 0.2M Borate Buffer, pH 8.5
 - e. Protein Solution: 1.2mg egg white avidin or streptavidin / 5ml Coupling Buffer.
 - f. Quenching Solution: 5mM ethanolamine
 - g. Wash/Storage Buffer: 0.1M glycine-saline, pH 8.2 with 0.2% NaN₃, 0.2% BSA and 0.05% Tween-20
2. Washing/Pre-Activation
 - a. To 1ml microsphere suspension, add 3ml deionized water.
 - b. Wash three times with deionized water.
 - c. After third wash, resuspend particles in 4ml deionized water. Add 1ml of Pre-Activation Buffer.
 - d. Place suspension on a magnet stirrer and maintain at 22°C. Add 5ml of WSC Solution.
 - e. Allow to react for 3.5 hours.
3. Washing/Protein Coupling
 - a. Wash particle suspension in saline, and resuspend in 5ml saline.
 - b. Add 5ml of microsphere suspension to 5ml Protein Solution.



- c. Incubate at 22°C for 20 hours.
4. Quenching/Blocking/Washing
 - a. To neutralize surface carboxyl groups that are not bound to avidin, add Quenching Solution, then BSA (blocker) to a concentration of 2% w/v
 - b. Wash microsphere suspension once in Wash/Storage Buffer.
5. Storage
 - a. Resuspend coated particles in Wash/Storage Buffer.
 - b. Store at 4°C.

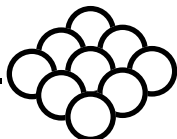
III. Covalent Coupling to Carboxylate-Modified Superparamagnetic Beads⁸

- A. Reagent Preparation
 1. Microspheres: Superparamagnetic Carboxylate-Modified at 10% solids.
 2. Wash Buffer: 0.1M Diethanolamine pH 10.5
 3. WSC Solution: 45 mg/ml water soluble carbodiimide (eg. 1-cyclohexyl-3(2-morpholinoethyl) carbodiimide) in Wash Buffer.
 4. Coupling Buffer: ~3 mg/ml of protein in Wash Buffer.
- B. Wash
 1. Separate 50µl of microsphere suspension (5mg beads) with a magnet (or centrifuge). Decant and discard supernatant.
 2. Resuspend microspheres in 5ml of Wash Buffer (<0.1% Tween 20, optional).
 3. Separate solids/liquid with magnet (or centrifuge). Decant and discard supernatant.
- C. Microsphere Activation
 1. Resuspend microspheres in 1ml of WSC Solution. (High pH ensures that COO⁻ groups will be present to provide colloidal stability.) *Non-ionic surfactant such as Tween 20 or Triton X-100 may be included.
 2. Incubate for 30 minutes at 25°C.
- D. Activated Microsphere Washing
 1. Separate solids/liquid with magnet (or centrifuge). Discard supernatant.
 2. Resuspend particles in 1ml of Wash Buffer.
 3. Separate, decant, resuspend in 1ml of Wash Buffer.
 4. Separate, decant and discard supernatant.
- E. Protein Coupling
 1. Resuspend microspheres in 1ml of Coupling Buffer.
 2. Incubate of 60 minutes at 25°C.
- F. Washing/Storage
 1. Add 5ml of Wash Buffer to the microsphere/protein solution above.
 2. Separate solids/liquid with magnet (or centrifuge). Decant and discard supernatant.
 3. Resuspend coated microspheres in 1ml of a BSA-containing buffer.
 4. Store at 4°C.

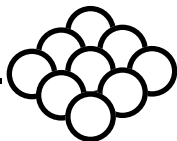
IV. Covalent Coupling of Protein to Amino-Modified Particles⁹

- A. Reagent Preparation

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1. Microspheres: Amino-Modified Polystyrene-based microspheres, 10% solids.
 2. Wash Buffer: 0.1M Phosphate Buffered Saline (PBS) pH 7.4.
Make phosphate buffer by adding 0.1M NaH_2PO_4 to 0.1M Na_2HPO_4 until the pH becomes 7.4. To 200ml of the phosphate buffer above, add 8.77g of NaCl and make up the volume to one liter with deionized water. Adjust pH, if necessary, back to 7.4 with dilute HCl or NaOH.
 3. Glutaraldehyde Solution: 8% Glutaraldehyde (E.M. grade) in 0.1M PBS pH 7.4.
**See note following this protocol regarding monomeric glutaraldehyde.
 4. Protein Solution: 400-500 μg protein in 1ml of Wash Buffer. (~20-30mg protein/gram microspheres)
 5. Quenching Solution: 0.15ml of ethanolamine (2-aminoethanol) in 4.8ml Wash Buffer. [This will quench any unreacted amino groups on the particle surface.]
 6. Blocking Solution: 10 mg/ml BSA in Wash Buffer.
 7. Storage Buffer: Wash Buffer containing 10 mg/ml BSA and 5% glycerol. (0.1% sodium azide (NaN_3) may be added as a preservative if desired.)
- B. Microsphere Preparation
1. Add 1ml Wash Buffer to 250 μl microsphere suspension (25 mg particles).
 2. Centrifuge, discard supernatant, resuspend pellet in 1.5 ml Wash Buffer.
 3. Repeat Step 2 twice.
 4. Centrifuge, decant and discard supernatant.
- C. Glutaraldehyde Activation/Washing
1. Resuspend pellet in 1ml of Glutaraldehyde Solution.
 2. Incubate overnight at room temperature with gentle end-to-end mixing.
 3. Centrifuge, decant and discard supernatant, resuspend in Wash Buffer.
 4. Repeat Step 3.
 5. Centrifuge, decant and discard supernatant.
- D. Protein Coupling/Washing
1. Resuspend pellet in 1ml of Protein Solution.
 2. Incubate 4-5 hours at room temperature with gentle end-to-end mixing.
 3. Centrifuge, decant supernatant. Save the supernatant for protein determination.
- E. Quenching
1. Resuspend the pellet in 1ml of Quenching Solution.
 2. Incubate for 30 minutes at room temperature with gentle end-to-end mixing.
 3. Centrifuge, decant and discard supernatant.
- F. Blocking
1. Resuspend the pellet in 1ml of Blocking Solution.
 2. Incubate for 30 minutes at room temperature with gentle end-to-end mixing.
 3. Centrifuge, decant and discard supernatant. Resuspend the pellet in 1ml of Blocking Solution.
 4. Centrifuge, decant and discard supernatant.
- G. Storage
1. Resuspend the pellet in 1ml of Storage Buffer.
 2. Store at 4°C.



**Purifying Glutaraldehyde (GA):

We recommend using pure monomeric glutaraldehyde for coupling proteins to amino-modified microspheres. Monomeric GA (absorbance peak 280nm) can be easily purified from contaminating polymeric GA (absorbance peak 235nm) with activated charcoal 5% (w/v) and subsequent filtration (repeat 3-4 times). To see the purification, make a UV scan (230-300nm). Subsequent storage of the purified glutaraldehyde at -20°C is recommended.¹⁰

V. Covalent Coupling of Protein to Chloromethyl-Modified Particles¹¹

A. Reagent Preparation

1. Microspheres: 40nm polyvinyl-naphthalene core/chloromethylstyrene shell. Supplied at 10% solids.
*Although this protocol was used with polyvinyl-naphthalene/chloromethylstyrene particles, it is also applicable to particles made of styrene/vinylbenzylchloride.
2. First Wash Buffer: 15 mmol/L phosphate buffer, pH 7.5 containing 0.5 g/L Rhodafac RE610 (an anionic detergent/Rhône-Poulenc, Inc. SDS can be substituted) and 0.1 g/L sodium azide (NaN₃).
3. Protein Coupling Buffer: First Wash Buffer containing protein concentrations in the range of 1.0-3.4 g/L.
4. Quench/Wash Buffer: 50 mM Glycine solution, pH 7.5 containing 0.1 g/L NaN₃.
5. Storage Buffer: 200 mM Glycine solution, pH 7.5 containing 0.1 g/L NaN₃.

B. Microsphere Preparation

1. Dialyze microspheres in First Wash Buffer for 24 hours at room temperature.
2. Dilute dialyzed particles 10-fold with First Wash Buffer to give a 10 g/L suspension.

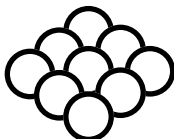
C. Protein Preparation

*The antibody used was the immunological fraction of a polyclonal rabbit antiserum to human sex-hormone-binding globulin.

1. Dialyze antibody in First Wash Buffer for 24 hours at room temperature.
2. Centrifuge the antibody to remove any particulate material.
3. Calculate the protein concentration of the supernate from absorbance measurements at 280nm.
4. Dilute protein to appropriate concentrations in First Wash Buffer to make Protein Coupling Buffer.

D. Protein Coupling

1. Mix dialyzed particle suspension and Protein Coupling Buffer in equal volumes (usually 1 or 2 ml).
2. Incubate overnight (15-18 hours) at 37°C with continuous shaking.
*We recommend quenching any unreacted chloromethyl groups with glycine or ethanolamine, as in this protocol and the next. (section VI).



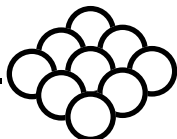
- E. Quenching/Washing
1. Centrifuge protein/particle mixture at 40,000 x g for 60 minutes at 20°C. Decant and discard the supernatant. Resuspend the pellet in 4 ml of Quench/Wash Buffer.
 2. Repeat Step 1 three times.
 3. Centrifuge, decant and discard supernatant.
- F. Storage
1. Resuspend the pellet in Storage Buffer at half the volume of the original mixture of antibody and microspheres.
 2. To ensure total resuspension of the particles, sonicate the mixture twice for 30 seconds each.
 3. Store at 4°C.

VI. Covalent Coupling of Protein to Aldehyde-Modified Particles¹²

- A. Reagent Preparation
1. Microspheres: 0.3µm diameter acrylic microspheres with aldehyde surface functional groups. (Also applicable to polyacrolein microspheres.)
*Microspheres are used without prior cleaning of surfactant .
 2. Borate Buffer: 0.1M borate buffer, pH 8.2 containing 0.3M NaCl.
 3. Protein Coupling Solution: 3.3 g/L human thyroglobulin (hTg) in Borate Buffer.
 4. Quenching Solution: 2.4M ethanolamine (2-aminoethanol), pH 8.2.
 5. Storage Buffer: Borate Buffer containing sodium azide (NaN₃) 2 g/L.
- B. Protein Coupling
1. Mix 10g of microspheres in 1L of Protein Coupling Solution.
 2. Incubate for 2 hours at room temperature.
 3. Incubate for 18 hours at 4°C.
- C. Quenching
1. To mixture from B3 above, add 50ml of Quenching Solution.
 2. Incubate for 2 hours at room temperature to quench the unreacted aldehyde groups of the microspheres.
 3. Separate the conjugate from the binding mixture by centrifuging at 8000 x g for 1 hour at 4°C on a discontinuous sucrose gradient (200/800 g/L in Borate Buffer).
- D. Storage
1. Collect the conjugate at the interface of sucrose solutions.
 2. Suspend conjugate in Storage Buffer. Store at 4°C.

VII. Covalent Coupling of Protein to Epoxy-Modified Particles¹³

- A. Reagent Preparation
1. Microspheres: Styrene/Glycidyl Methacrylate Particles ~0.2µm diameter, with epoxy surface-functional groups.
 2. Protein Coupling Solution: 5000IU of human chorionic gonadotropin (HCG) in 7ml phosphate buffer pH 8.0 with 0.1M NaCl and 1:10,000 thimerosal.
 3. Wash Buffer: Phosphate buffer, pH 7.0, with 0.1M NaCl.



4. Quenching Buffer: 0.1M Glycine Buffer, pH 8.2.
- B. Protein Coupling
1. Mix 7ml Protein Coupling Solution with 1.61g microspheres.
 2. Incubate for 4 days at 5°C with stirring.
- C. Washing/Quenching
1. Wash by membrane filtration ~3 hours in a Diaflo® filter cell with 145ml Wash Buffer.
 2. Dialyze the washed latex in cellophane against Quenching Buffer for 2 days.
- *In general, the coupling reaction is carried out at a pH of from about 7.5-8.5 with a pH of about 8.0 preferred. Phosphate buffer is generally used to couple HCG to these microspheres. Borate buffers are generally unsatisfactory with proteins such as HCG which have a high carbohydrate content. The pH and ionic strength of the coupling buffer can be varied over a wide range (pH 0-12, concentration 0.01-1.0M) according to the optimal conditions for binding of a particular protein.
- *Coupling reaction time can be reduced to about one day or less by conducting the reaction at room temperature instead of 5°C.
- *If the final microsphere-HCG conjugate is buffered with pH 8.2 glycine buffer, it is unnecessary to discharge or quench the residual epoxy groups.
- *If a neutral buffer system is desired, an extra step to discharge the residual epoxy groups is desirable. This can be done with amino-compounds of low molecular weight (amino acids such as glycine) or non-interfering proteins:

Add to protein coated particles, 1M glycine in 0.5M potassium phosphate buffer solution pH 8.0 or 10% ethanolamine hydrochloride in water (pH adjusted to 8.0 with NaOH), or 5% 2-mercaptoethanol in water (pH adjusted to 8.0 with NaOH). Let stand at room temperature for 16 hours.

VIII. Covalent Coupling of Protein to Hydrazide-Modified Particles¹⁴

A. Reagent Preparation

1. Microspheres: Hydrazide-modified styrene/acrylamide.
2. Glutaraldehyde Solution: 3mg glutaraldehyde per 1ml deionized water.
3. PBS: 3.68 ml of 0.5M KH₂PO₄
32.2 ml of 0.5M Na₂HPO₄
5.84 g of NaCl
10 ml of 1% Merthiolate

Bring to 1L with deionized water. This gives pH of 8.0 at 5°C.

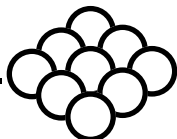
4. Protein Solution: The HCG preparation was lyophilized and contained in septum bottles having 5,000 I.U. of HCG, 50 mg of mannitol, and PBS such that solution in 10ml of deionized water gave a pH of 7.2.

B. Activation

1. Mix 5 ml microsphere suspension (0.68g solids) with 3 ml of deionized water and 1.7 ml Glutaraldehyde Solution.
2. Incubate with stirring at room temperature for 4 hours.

C. Protein Coupling

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1. To the particle/glutaraldehyde mixture above, add 10,000 I.U. of HCG in PBS (Protein Solution).

2. Incubate with stirring at 5°C for 5 days.

D. Washing/Storage

1. Dilute microsphere/protein solution 1-fold with PBS.

2. Centrifuge for 30 minutes at 20,000 rpm (32,000 x g) at 5°C.

3. Carefully remove supernatant and discard.

4. Resuspend pellet in PBS and centrifuge again.

5. Resuspend pellet in PBS. Filter mixture by gravity through a thin mat of glass wool supported in a small polyethylene funnel.

6. Rinse funnel with a small amount of PBS.

7. Store at 4°C.

**Although the preceding protocol used glutaraldehyde as the difunctional linker, other compounds may be used, such as 1,5-difluoro-2,4-dinitrobenzene, 4,4'-di-fluoro-3,3'-dinitrodiphenyl sulfone or 2,4-dichloro-6-carboxymethylamino-s-triazine.

IX. Miscellaneous Coupling Ideas

A. Try the homobifunctional linkers: parabenzoquinone (P-BZQ) and bis-epoxirane (butanediol diglycidyl ether) for reacting with -NH₂ (α -NH₂ or ϵ -NH₂ of lysine), -OH (tyrosine or sugars), and -SH (cysteine) groups.

1. P-BZQ: Run the first reaction at pH 5.0 for 1 hr. at room temperature, in the dark. Second reaction: pH 8-9, 48 hr, at 4°C.

2. Bis-epoxirane: Must use at high pH (8.5-12). Best for sugars or nucleic acids.

B. Try the NHS-ester maleimide heterobifunctional crosslinkers (available from Pierce). The NHS-ester reacts with primary amine groups, and the maleimide reacts with sulfhydryl groups.

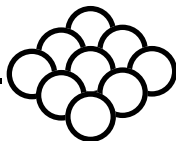
1. The NHS-ester reaction is performed first to minimize hydrolysis of the NHS ester in aqueous systems.

2. The sulfhydryl (cysteine residues) are reacted with the maleimide group in the second step. This is done in slightly acidic-to-neutral (pH 6.5-7.5) conditions.¹⁵

C. N-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP-also available from Pierce) is a heterobifunctional cleavable crosslinker, which contains one N-hydroxysuccinimide (NHS) residue and one pyridyl disulfide residue. These residues react with primary amines and sulfhydryls, respectively.

1. The NHS-ester reaction is performed first to minimize hydrolysis of the NHS-ester in aqueous systems. NHS-ester crosslinking reactions are pH dependent and the optimum pH for conjugation is between pH 7 and 9. Both the hydrolysis and aminolysis rates increase with increasing pH.

2. The 2-pyridyl disulfide residue reacts with aliphatic thiols to form disulfides. The optimal pH for this reaction is between pH 7 and 8.¹⁶



¹Anon., "Heterophilic Blocking Reagent Minimizes Antibody Interference", *Biomedical Products*, **19/6**, 58 (1994). (Press Release, Scantibodies Laboratory, 9336 Abraham Wan, Santee, CA 92071).

²Press Release, SLD, Rue des Romains, L-8041 Strasse n, Luxembourg.

³Press Release, Bionostics Ltd., The Business Centre, Tythe Farm, Wyboston, Beds MK44 3AT, UK.

⁴ Nathan, Carl F., Zanvil A. Cohn, "Antitumor Effects of Hydrogen Peroxide In Vivo," *J. Exp. Med.*, **154/Nov.**, 1539-1553 (1981).

⁵ Hager, Hans Jacob, "Latex Polymer Reagents for Diagnostic Tests," US Patent # 3,857,931, Dec. 31, 1974.

⁶ Quash, G., A.M. Roch, A. Niveleau, J. Grange, T. Keolouangkhot, J. Huppert, "The Preparation of Latex Particles with Covalently Bound Polyamines, IgG and Measles Agglutinins and Their Use in Visual Agglutination Tests," *J. Immun. Meth.*, **22**, 165-174 (1978).

⁷ Rosenstein, Robert, "Immuno-agglutination Particle Suspensions," US Patent # 4,582,810, Apr. 15, 1986.

⁸ Rhône-Poulenc Technique.

⁹ Deutsch, Alice, Bioscreen, New York, Personal Communication.

¹⁰ Gillett, Roy, Gull, Kieth, "Glutaraldehyde - Its Purity and Stability," *Histochemie*, **30**, 162-167 (1972).

¹¹ Deleo, D.T., I.R. Lee, J.D. Wetherall, D.J. Newman, E.A. Medcalf, C.P. Price, "Particle-Enhanced Turbidimetric Immunoassay of Sex-Hormone-Binding Globulin in Serum", *Clin. Chem.*, **37/4**, 527-531 (1991).

¹² Harchali, A.A., P. Montagne, M.L. Cuilliere, M. Bouanani, B. Pau, J. Duheille, "Detection of Antithyroglobulin Autoantibodies with Defined Epitopic Specificity by a Microparticle-Enhanced Nephelometric Immunoassay", *Clin. Chem.*, **38/9**, 1859-1864 (1992).

¹³ Dorman, L.C., I. Mani, "Method of Coupling a Protein to an Epoxylated Latex", US Patent # 4,210,723, Jul. 1, 1980.

¹⁴ Dorman, L.C., "Method of Coupling a Protein to a Polymer Particle Containing Hydrazide Groups in a Polymer Latex and the Products Formed Therefrom", US Patent # 4,421,896, Dec. 20, 1983.

¹⁵ Pierce Chemical Company, Rockford, IL, "NHS-Ester-Maleimide Heterobifunctional Crosslinkers".

¹⁶ Pierce Chemical Company, Rockford, IL, "SPDP, Sulfo-LC-SPDP, LC-SPDP".

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