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## BEADS ABOVE THE REST™

### Description

Protein A is a 42 kD polypeptide that is a normal constituent of the cell wall of *S. Aureus*. It was discovered in early work with this bacteria when researchers noticed that one fraction, isolated during purification of the individual components of the cell wall, contained a protein that would bind to rabbit and human antibodies. Surprisingly, this protein bound to antibodies isolated not only from animals that had been immunized with protein A, but also from animals that had never been exposed to this antigen.

Although Protein A has four antibody binding sites, only two of these can be used at one time. It is known that there are at least two protein A binding sites on any antibody, and that these are located in the Fc region of the antibody. Because Fc-directed binding is desired in order to maximize the antibody's biological activity, protein A pre-conjugated to a solid support, such as our microspheres, has become an important reagent in many immunochemical applications.

Protein A coated microspheres have a couple of advantages when compared to conventional surface-functionalized microspheres. Antibodies are bound to protein A beads in a simple one-step process, and the antibody can be eluted if necessary. Traditionally, coupling antibodies to polystyrene microspheres has been done by passive adsorption or covalent coupling. Passive adsorption is the easier of the two, but the possibility of reversibility and undesirable antibody orientation exists. Covalent coupling is irreversible and, with the proper chemistry, allows for controlled orientation of antibody attachment. The disadvantages of any covalent coupling protocol are the time and costly reagents involved in optimization.

Protein A conjugated microspheres are, for many applications, the answer to these problems. Antibody coupling is Fc-directed (to maximize biological activity), done in one step (simply by mixing the reagents in their proper concentrations), and the bond formed is very strong ( $K_a=10^9$ )<sup>1</sup>, yet reversible at low pH.

Protein A is a very sturdy molecule. Research has been done in which protein A is subjected to very harsh conditions (6M Guanidine HCl, 4 M Urea, 4 M thiocyanate, or pH 2.5), and, in each case, the protein A was renatured to full binding capacity when returned to normal physiological conditions.

While these features make protein A useful in a wide variety of applications, it does have its limitations. Protein A's affinity for various polyclonal antibodies is species-specific (Table 1). Also, when using monoclonal antibodies, its affinity for IgGs will vary with the different sub-classes within a species (Table 2).<sup>2</sup>

**Table 1: Protein A Affinities for Polyclonal Antibodies from Various Species**

Species	Affinity for Protein A
Rabbit, Guinea Pig	++++
Human, Pig	+++
Horse, Cow, Mouse	++
Sheep, Rat	+/-
Hamster	+
Goat, Chicken	-

**Table 2: Protein A Affinities for Various Monoclonal Antibodies**

Antibody	Affinity for Protein A
Human IgG <sub>1</sub> , IgG <sub>2</sub> , IgG <sub>4</sub>	++++
Human IgG <sub>3</sub>	-
Rat IgG <sub>1</sub> , IgG <sub>2a</sub> , IgG <sub>2b</sub>	-
Rat IgG <sub>2c</sub>	+
Mouse IgG <sub>1</sub>	+
Mouse IgG <sub>2a</sub>	++++
Mouse IgG <sub>2b</sub>	+++

In practice, sera from humans, donkeys, rabbits, dogs, pigs, and guinea pigs can be used without worry for all tests that rely on protein A. Most immunochemical assays will not be affected by using polyclonal antibodies from mice, cows, or horses. However, depending on the type of assay, antibodies from sheep, goats, rats, or chickens will often need a secondary antibody.

As Table 2 shows, subclasses that are more difficult to use include human IgG<sub>3</sub>, mouse IgG<sub>1</sub>, and all rat subclasses, except possibly IgG<sub>2c</sub>. In all these cases, protein A may need to be supplemented with a second bridging antibody layer, or be replaced by a secondary antibody layer to ensure quantitative binding.

## Physical Parameters

### Microsphere Types

Polystyrene:	0.02µm to 1mm, plain or dyed in a variety of colors, including fluorescent
Superparamagnetic Polystyrene:	Polydisperse polystyrene/magnetite with nominal mean diameter of ~1µm
Two types:	<i>Classical</i> , with magnetite exposed at surface, and <i>Encapsulated</i> , with outer polymer shell.
	Magnetite percentage ranges from 12-66% by weight (density ranges from 1.16-2.24 g/cm <sup>3</sup> ).
Silica:	0.15-5.0µm (density=1.96 g/cm <sup>3</sup> )
Concentration:	10mg microspheres/mL (1% solids w/v)
Storage Buffer:	100 mM Borate, pH 8.5 + 0.01% BSA + 0.05% Tween 20 + 10 mM EDTA + 0.1% NaN <sub>3</sub> (unless otherwise specified)
Binding Capacity:	Supplied on the Certificate of Analysis for each lot.

## Procedure

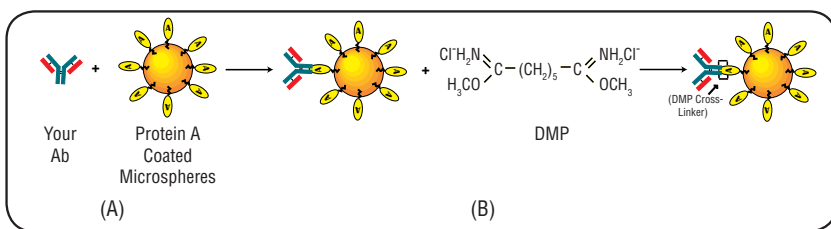
Researchers are advised to optimize the use of particles in any application.

### Preparation of ProActive Protein A Coated Microspheres

A preliminary wash is necessary with most applications, to remove various additives including EDTA, antimicrobials, and surfactants. Several washing methods are possible, and a detailed description of these can be found in our TechNote 203, "Washing Microspheres." *Note:* This should be performed at pH 4.0, as this is the pH at which any impurities coupled to protein A will be eluted.

### Fc-directed Attachment (and Elution) of IgG to Protein A Coated Microspheres (A) / Covalent Cross-linking Procedure (B)

Protein A interacts with IgG's in such a way that the bond formed is reversible at low pH (2.5). This can be advantageous in many applications, although it is undesirable when forming microsphere/antibody reagents as are used in many immunoassays (a requirement of these reagent conjugates is that they be permanent). The following procedure first details a protocol for attaching an IgG protein to protein A coated microspheres, and then uses DMP (dimethyl pimelimidate) to covalently cross-link this IgG to the immobilized protein A. DMP is used as an example, although other homobifunctional cross-linkers, such as glutaraldehyde, could be substituted.



### Reagents

- ProActive Protein A coated microspheres (supplied at 1% solids)
- Antibody binding buffer (50 mM sodium borate, pH 8.2)
- Cross-linking buffer (0.2 M triethanolamine, pH 8.2)
- Quenching solution (0.1 M ethanolamine, pH 8.2)
- 1 M NaCl
- 0.1 M glycine, pH 2.8
- Elution buffer (0.1 M glycine-HCl, pH 2.5)

### Procedure

1. Wash 10mL (10 mg/mL) protein A coated microspheres 2 times in 10mL of antibody binding buffer, remove supernatant after the

second wash.

2. Dissolve IgG in up to 10X excess of estimated monolayer amount (based on binding capacity listed on the Certificate of Analysis for Human IgG) in 10mL of antibody binding buffer.
3. Add this antibody suspension to the microsphere pellet, and mix by gently rocking for 45 minutes at room temperature (18-25°C).
4. *If elution is desired*, as in affinity separation applications, separate\* microspheres from solution, resuspend microspheres in 10mL of elution buffer for 15 minutes, wash, separate supernatant, and dialyze this supernatant into desired storage buffer (normally 0.1 M PBS, pH 7.4). (\* Separate polymeric and silica microspheres via centrifugation or dialysis, and with a magnet for superparamagnetic microspheres.)
5. Separate, discard supernatant, and resuspend in 9mL antibody binding buffer:1mL cross-linking buffer. Separate and discard supernatant.
6. Dissolve 66mg DMP into 10mL cross-linking buffer. Immediately add this to the microsphere pellet and mix by gentle rocking.
7. React for 1 hour at room temperature.
8. Separate, discard supernatant, and resuspend in 10mL of quenching solution, and allow to react at room temperature for 10 minutes.
9. Wash sequentially in 10mL volumes of the following: 1 M NaCl, 0.1 M glycine (pH 2.8), water.
10. Wash and resuspend in storage buffer (Page 2, Physical Parameters) to desired concentration (often 10 mg/mL).
11. Store at 4°C until used.

## Notes

Because protein A's affinity for various antibodies varies (see Tables 1-2), some optimization of ligand binding will be required. Some of the parameters that will play a role in optimal binding are as follows.

1. pH: Typically, IgG's bind optimally near pH 8.
2. Salt concentration: Typically that of normal saline is sufficient. However, when working with many monoclonal antibodies, it is often beneficial to work with salt concentrations greater than 1 M.<sup>3</sup>
3. Buffers: Typically, low ionic strength buffers of borate, HEPES, PBS, TRIS, TBS, or sodium carbonate work well.
4. Cations: The presence or absence of divalent cations, such as Mg<sup>2+</sup> or Ca<sup>2+</sup>, will often be a factor in binding. Some proteins will only bind to protein A in the presence of divalent cations, while for others this hinders binding. Therefore, use of a chelating agent, such as EDTA, may affect binding.
5. Temperature: Normally, binding reactions can be carried out at room temperature, but some monoclonal antibodies show enhanced binding at 4°C.<sup>4</sup>
6. Concentrations: A good starting point to achieve maximum binding of human IgG is at a concentration of 130 µg/mL of buffer. Further, microspheres at a concentration of ~0.5 mg/mL often yield optimal binding.

## References

1. **Akerström, B., L. Björck.** 1986. *J Biol Chem*, 261(22): 10240-10247.
2. **Harlow, E., D. Lane.** 1988. *Antibodies: a laboratory manual*. Cold Spring Laboratory, 615-619.
3. **Harlow, E., D. Lane.** 1988. *Antibodies: a laboratory manual*. Cold Spring Laboratory, 524-525.
4. **Wall, J.** 1998. Bangs Laboratories, Indiana. Personal Communication.

## Storage and Stability

Store particles at 2-8°C. Freezing may result in irreversible aggregation and loss of binding activity.

## Safety

The Storage Buffer may contain sodium azide. Sodium azide may react with lead and copper plumbing to form explosive metal azides. Upon disposal of material, flush with a large volume of water to prevent azide accumulation. Please consult the Material Safety Data Sheet for more information.

**This product is for research use only and is not intended for use in humans or for *in vitro* diagnostic use.**



**Ordering Information**

**Catalog Code**

CM02N

CP02N

CP02\*

**Description**

ProActive® Magnetic Protein A Microspheres

ProActive® Polymeric Protein A Microspheres

ProActive® Dyed Polymer Protein A Microspheres

\* See our website for color chart

**Sizes**

1mL, 2mL, 5mL, or 10mL

1mL, 2mL, 5mL, or 10mL

1mL, 2mL, 5mL, or 10mL

Order online anytime at [www.bangslabs.com](http://www.bangslabs.com).