

DESCRIPTION

Protein A is a 42kD polypeptide that is a normal constituent of the cell wall of *S. Aureus*. It was discovered in early work with the 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 (e.g. 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 may be eluted, if necessary. Traditionally, antibody immobilization 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 allows for controlled orientation of antibody attachment with the proper chemistry. The disadvantages of any covalent coupling protocol are the time and costly reagents involved in optimization.

For many applications, Protein A coated microspheres are 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 ($K_a=10^9$)¹, 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). 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 IgG's will vary with the different subclasses within a species (Table 2).²

In practice, sera from humans, donkeys, rabbits, dogs, pigs, and guinea pigs can be used without worry for most 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 IgG₃, Mouse IgG₁, and all rat subclasses, except possibly IgG_{2c}. 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.

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, IgG ₂ , IgG ₄	++++
Human IgG ₃	-
Rat IgG ₁ , IgG _{2a} , IgG _{2b}	-
Rat IgG _{2c}	+
Mouse IgG ₁	+
Mouse IgG _{2a}	++++
Mouse IgG _{2b}	+++

PHYSICAL PARAMETERS

Microsphere Types

Polystyrene:	~20nm - 90µm, plain or dyed in a variety of colors, including fluorescent
Silica:	~150nm - 5.0µm (density = 2.00 g/cm ³)
Superparamagnetic:	~0.35µm - 8.0µm, variety of magnetic particle lines
Concentration:	~10mg beads/mL (1% solids w/v)
Storage Buffer:	PBS, pH 7.4 + 0.1% BSA + ≤ 0.1% NaN ₃ (unless otherwise specified)
Binding Capacity:	Supplied on the Certificate of Analysis for each lot

PROCEDURE

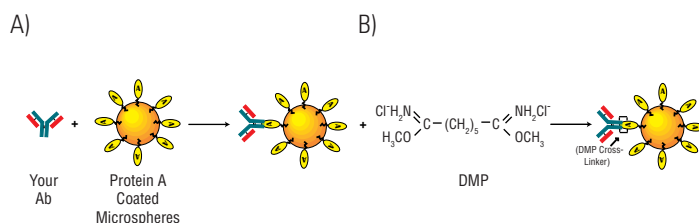
Researchers are advised to optimize reagent concentrations during antibody immobilization and the use of particles in any application.

Preparation of Protein A Coated Microspheres

A preliminary wash is recommended 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*. A good pH to perform preparatory washes at is 4.0 as most impurities coupled to Protein A can be eluted at this pH.

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, though it may be undesirable when forming microsphere/antibody reagents used in immunoassays that require permanently immobilized conjugates. A sample procedure follows that first details a protocol for attaching IgG to Protein A coated microspheres, and then uses DMP (dimethyl pimelimidate) to covalently cross-link the IgG to the immobilized Protein A. DMP is used as an example in this procedure; other homobifunctional cross-linkers (e.g. glutaraldehyde) may be substituted.



Reagents

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 in 10mL of antibody binding buffer. Repeat the wash and remove supernatant after the second wash.
2. Dissolve IgG in 10mL of antibody binding buffer. Up to a 10X IgG excess of the reported binding capacity listed on the accompanying Certificate of Analysis may be used.
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 (i.e., in affinity separation applications), separate microspheres from solution, resuspend in 10mL of elution buffer for 15 minutes, wash, separate supernatant, and dialyze the supernatant into desired storage buffer (normally 0.1 M PBS, pH 7.4).
5. If permanent IgG immobilization is desired, separate microspheres from solution, 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 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), de-ionized water.
10. Wash and resuspend in storage buffer (e.g. PBS, pH 7.4 + 0.1% BSA + 0.1% NaN₃) to desired concentration (often 10 mg/mL).
11. Store at 4°C until used.

NOTES

With regards to wash/separation methods, centrifugation or dialysis is suitable for polymeric and silica microspheres, and magnetic separation is recommended for superparamagnetic beads. Since Protein A's affinity for various antibodies varies (see Tables 1 & 2), some optimization of ligand binding may be necessary. Some of the parameters that play a role in optimal binding are as follows.

pH: Typically, IgG's bind optimally near pH 8.

Salt Concentrations: Typically that of normal saline is sufficient. Salt concentrations >1 M may be beneficial when working with some monoclonal antibodies.³

Buffers: Typically, low ionic strength buffers of borate, HEPES, PBS, TRIS, TBS, or sodium carbonate work well.

Cations: The presence or absence of divalent cations (e.g. Mg²⁺ or Ca²⁺) will often be a factor in binding. Some antibodies will only bind to Protein A in the absence of divalent cations, while for others this hinders binding; thus, the presence of chelating agents (e.g. EDTA) may affect binding.

Temperature: Normally, binding reactions can be carried out at room temperature, but some monoclonal antibodies show enhanced binding at 4°C.⁴

Concentrations: Bead and antibody concentrations usually range from 0.5 g/mL - 10 mg/mL and good binding has been demonstrated using stock solution antibody concentrations of ~10µg Ab/mL - 150µg Ab/mL (at the latter bead concentration). Additionally, a 3-10X excess of the calculated surface antibody monolayer amount may be a good starting point for optimization of the binding protocol (see *TechNote 204, Adsorption to Microspheres*, for surface monolayer calculation).

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 that the Protein A coated microspheres are supplied in 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 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

Cat. Number	Description	Sizes
CP02000	1.00µm Protein A Polymer Microspheres	1mL, 2mL, 5mL, or 10mL
CP02001	5.00µm Protein A Polymer Microspheres	1mL, 2mL, 5mL, or 10mL
CP02002	10.0µm Protein A Polymer Microspheres	1mL, 2mL, 5mL, or 10mL

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