Technical Manual

# Protein A-Agarose

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## I. Introduction

The Imgen BioSceinces' Protein A-Agarose is 4% agarose beads that have been covalently coupled with recombinant protein A. It is a high quality immunoaffinity matrix for the purification of IgG fractions from biological fluids and from cell culture media. It is also an idea matrix for the isolation of antibody-antigen complexes by immunoprecipitation.

Native protein A, a bacterial cell wall protein from *Staphylococcus aureus*, binds specifically to the Fc region of immunoglobulin molecules of many mammalian species without disturbing their binding of antigen. A native protein A has five IgG binding domains. The recombinant protein A, used to produce Imgen BioSceinces' Protein A-Agarose, has an exact amino acid copy of the native protein A from *S. aureus*, but expressed in and purified from *E. coli*. The purified recombinant protein A functions same as its native form, but with low levels of endotoxin contamination.

## II. Features

1. High IgG binding capacity
2. Stable affinity medium
3. Milder elution condition than protein G resin
4. Resin is reusable up to 10 - 20 times

## III. Characteristics

The matrix is a 4% agarose beads that have been covalently coupled with recombinant protein A by a cyanogen bromide method. The swollen agarose medium has approximately 6 mg of recombinant protein A/ml of drained resin. It's total binding capacity for human IgG is about 25 -30 mg/ml of drained resin. It has high excellent chromatographic and immunoaffinity isolation qualities in high binding capacity, low leakage, and stable affinity medium. It withstands a wide range of pH, and many common denaturing reagents such as 4 M urea, 4 M thiourea and 6 M guanidine hydrochloride. The product characteristics are summarized in Table 1.

**Table 1. Technical Specifications**

| | |
|---|---|
| Ligand | Recombinant protein A (*E. coli*) |
| Ligand coupling | Cyanogen bromide covalently coupling |
| Ligand density | 6 mg recombinant protein A/ml resin |
| Matrix | 4% agarose beads |
| Average beads size | 90 µm (50 – 150 µm) |
| Binding capacity | ≥25 mg human IgG/ml medium |
| pH stability | 2 - 14 (short term); 3-12 (long term) |
| Flow rate | 1 - 4 ml/min |
| Contents | Supplied as 50% aqueous suspension (e.g., 5 ml of settled resin is equivalent to 10 ml of 50% slurry) in buffer containing 20% ethanol, 20 mM sodium phosphate pH 7.4, 0.15 M NaCl, and 0.02% $NaN_3$ (w/v). Product is shipped at ambient temperature. |
| Storage | +4°C to +8°C. Do not freeze. |

## IV. Procedures for Column Purification and Sample

**Procedures**

*Note: The procedures are general guidelines for a column packed with 1.0 ml resin (i.e., 2 ml of 50% slurry). The volume of the resin can be scaled up or down according to the operator's desire and the size of the column.*

In most cases, they are the preferred procedures but may be modified to suit special needs.

1. **Buffer Preparation**:
   (1) Binding buffer/wash buffer: 0.2 M $Na_2HPO_4$, pH 8.0/0.15 M NaCl
   (2) Elution buffer: 50 mM Glycine-HCl pH 2.5 - 3.0
   (3) Neutrolization buffer: 1.0 M Tris-HCl, pH 8.0

2. **Sample Preparation**: To insure proper ionic strength and pH are maintained for optimal binding, it is necessary to dilute serum samples, ascites fluid or tissue culture supernatant at least 1:1 with Binding/wash buffer. Alternatively, the sample may be dialyzed overnight against Binding/wash buffer.

3. **Column and Resin Preparation:** Mix the slurry by gently inverting the bottle several times to suspend the resin completely. Transfer 2.0 ml of Protein A-Agarose slurry to a column, in which 1 ml of Binding/wash

buffer has previously added. Allow the column to flow by gravity to pack the column bed. Equilibrate the column with 10 ml (10 column volumes (CV)) of Binding/wash buffer.

4. **Sample Purification:** Gently apply the sample on the column by layering onto the top of the resin with a flow rate about 0.5 ml/min. Do not let the resin bed to run dry. Wash the column at 1 ml/min with 10 ml (10 CV) of Binding/wash buffer or until the absorbance of elute at 280 nm reaches the background level.

   Before beginning the elution step, set up 5 tubes (e.g., 1.5 ml eppendrof tubes) to collect entire elution volume of 5 ml (5 CV) as 1 ml fraction per tube. To each collection tube, add 0. 1 ml of Neutralization buffer. To elute the antibody, gently add 1 ml of Elution buffer to the top of the resin and collect the eluate at 1 ml/min with the prepared tubes. Repeat until the entire volume has been collected up to 5 ml. Alternately, the eluate can be collected in a single bulk tube containing 0.5 ml of Neutralization buffer. Elution of bound antibody can be monitored by absorbance at 280 nm, if desired.

5. **Column Regeneration:** wash the column with 5 ml (5 CV) of Elution buffer followed with 5 ml (5 CV) of Binding/wash buffer.

6. **Resin Storage:** Wash the column with 5 ml (5 CV) of Binding/wash containing 20% ethanol and 0.02% $NaN_3$ and store at +4 $^{o}$C to +8 $^{o}$C. Do not freeze.

**Samples**

Fig. 1. (right) Comparison of SDS-PAGE of purified antibodies from normal human serum by chromatography between with Imgen BioSciences' Protein A-Agarose and with a leading competitor's.

Lane 1: Molecular weight marker

Lane 2: Blank

Lane 3: Loaded sample

Lane 4: Flow through

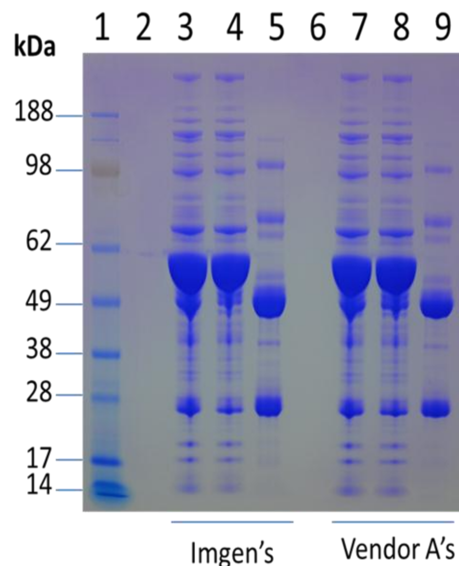
Lane 5: Elute

Lane 6: Blank

Lane 7: Loaded sample

Lane 8: Flow through

Lane 9: Elute




## V. Procedures for Immunoprecipitation (IP)

Immunoprecipitation (IP) is one of the most widely used immunochemical techniques to isolate specific proteins from complex samples such as cell lysates or extracts. Protein A coupled to an insoluble resin, such as agarose beads, is commonly used to capture a protein (antigen): antibody complex in solution. The complex is then "precipitated" together with the resin by centrifugation.

**1. Recommended buffers:**

(1) PBS: 20 mM $Na_2HPO_4$, pH 7.4, 0.15 M NaCl

(2) Modified RIPA buffer: PBS (pH 7.4), 0.25% Na deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM NaF, 1 mM $Na_3VO_4$, 1 mM PMSF, 1 μg/ml Aprotinin, 1 μg/ml Leupeptin, 1 μg/ml Pepstatin A

**2. Recommended Protocol**

*Note: If using a pre-existing cell lysate, proceed directly to step (5).*

**Preparation of Cell Lysate**

(1) Wash adherent cells twice in the dish or flask with ice-cold PBS and drain off PBS. Wash non-adherent cells in PBS and centrifuge at 800 to 1000 rpm in a table-top centrifuge for 5 minutes to pellet the cells.

(2) Add ice-cold modified RIPA buffer to cells (1 ml per 107 cells/100 mm dish/150 $cm^2$ flask; 0.5 ml per 5 x $10^6$ cells/60 mm dish/ 75 $cm^2$ flask).

(3) Scrape adherent cells off the dish or flask witt a rubber policeman or a plastic cell scraper that has been cooled in ice-cold distilled water. Transfer the cell suspension into a centrifuge tube. Gently rock the suspension on either a rocker or an orbital shaker at 4°C for 15 minutes to lyse cells.

(4) Centrifuge the lysate at 14,000 x g in a precooled centrifuge for 15 minutes. Immediately transfer the supernatant to a fresh centrifuge tube and discard the pellet.

**Immunoprecipitation**

*Note: perform the following experiment in a microcentrifuge tube.*

(5) To prepare Protein A-Agarose resin, wash the beads twice with PBS and restore to a 50% slurry with PBS. It is recommended to cut the tip off of the pipette when manipulating agarose beads to avoid disruption of the beads.

(6) Pre-clear the cell lysate by adding 100 μl of Protein A-Agarose bead slurry (50%) per 1 ml of cell lysate and incubating at 4°C for 10 minutes on a rocker or orbital shaker. Pre-clearing the lysate will reduce non-specific binding of proteins to the agarose when it is used later on in the assay.

(7) Remove the Protein A-Agarsoe beads by centrifugation at 14,000 x g at 4°C for 10 minutes. Transfer the supernatant to a fresh centrifuge tube.

(8) Determine the protein concentration of the cell lysate (e.g., if performing a Bradford assay one must dilute the cell lysate at least 1:10 before determining the protein concentration because of the interference of the detergents in the lysis buffer with the Coomassie-based reagent.).

(9) Dilute the cell lysate to approximately 1 μg/μl total cell protein with PBS to reduce the concentration of the detergents in the buffer. To precipitate a protein with low expression levels in a cell model, a more concentrated cell lysate (e.g., 10 μg/μl) may be necessary.

(10) Add the recommended volume of immunoprecipitating antibody (refer to antibody datasheet for detailed information) to 500 μl (500 μg) of cell lysate. The optimal amount of antibody for the quantitative immunoprecipitation of the protein of interest should be empirically determined for individual samples.

(11) Gently rock the cell lysate/antibody mixture for either 2 hours at room temperature or overnight at 4°C on a rocker or an orbital shaker. A 2 hour incubation time is recommended for the immunopecipitation of active enzymes for kinase or phosphatase assays.

(12) Capture the immunocomplex by adding 100 µl Protein A-Agarose slurry (i.e., 50 µl drained beads) and gently rocking on either a rocker or orbital shaker for either 1 hour at room temperature or overnight at 4°C.

(13) Pull down the beads by centrifugation at 12,000 rpm for 5 seconds. Discard the supernatant and wash the beads 3 times with 800 µl ice-cold PBS or RIPA buffer.

(14) Resuspend the beads in 60 µl 2X SDS-PAGE sample buffer and mix gently. This will allow for sufficient volume to run three lanes.

(15) Boil the beads for 5 minutes to dissociate the immunocomplexes from the beads. The beads are collected by centrifugation and SDS-PAGE is performed with the supernatant. Alternatively, the supernatant can be transferred to a fresh microcentrifuge tube and stored frozen at –20°C for later use. Frozen supernatant should be reboiled for 5 minutes immediately prior to loading on a gel.

## VI. Trouble Shooting

| Problem | Possible Cause | Solution |
|---|---|---|
| Flow rate of column is extremely low (e.g., <0.5 ml/min) | Tiny air bubbles derived from buffer or sample are blocking the gel pores | Degas buffers and sample |
| Considerable antibody is purified, but no or only trace amount of antibody of interest detected | Concentration of antibody of interest is very low | 1. Use serum free medium for cell culture supernatant samples<br>2. Affinity purify the antibody using resins coupled with specific antigen |
| Purified antibody is degraded | Antibody is sensitive to low-pH Elution buffer | 1. Neutralize the eluted fractions immediately<br>2. Try Elution buffers with high pH (e.g., pH 3.0) |
| No antibody is detected in any elution fraction | Sample does not contain antibody species or isotype that binds to protein A | Affinity purify the antibody using resins coupled with specific antigen |

## VII. Order Information

| Product Number | Description |
|---|---|
| B-2001-5 | Protein A-Agarose, 5 ml settled resin (10 ml of 50% slurry) |
| B-2001-25 | Protein A-Agarose, 25 ml settled resin (50 ml of 50% slurry) |

*Note: For research use only, not for use in diagnosis or therapeutics.*