INSTRUCTIONS

Protein A IgG Purification Kit

PIERCE
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44667

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Number	Description
44667	**Protein A IgG Purification Kit,** sufficient materials to perform 45 purifications

Kit Contents:

AffinityPak™ Protein A Columns, 5 × 1 ml resin-packed columns

Support: Crosslinked, 6% beaded agarose resin

Supplied Format: Packed resin in 0.02% sodium azide storage solution

Binding Capacity: 6-8 mg mouse IgG per ml of settled resin

Protein A IgG Binding Buffer, 1 L, pH 8.0, contains EDTA as a preservative

IgG Elution Buffer, 500 ml, pH 2.8, contains amine

D-Salt™ Excellulose™ Desalting Columns, 5 × 5 ml

Storage: Upon receipt store at 4°C. Kit is shipped at ambient temperature.

Introduction

The Protein A IgG Purification Kit is useful for routine purification of antibodies. Samples containing IgG are incubated with immobilized Protein A in a buffer that facilitates binding. After non-IgG components are washed from the column, the bound IgG is recovered by elution. The supplied buffers provide for maximum immunoglobulin binding and elution efficiency with the Protein A column. The pre-packed Protein A columns and desalting columns supplied in the kit make IgG purification easy and efficient.

Protein A is a cell wall component produced by several strains of *Staphylococcus aureus* and consists of a single 42 kDa polypeptide chain and contains little or no carbohydrate.[1,2] The unique functional property of Protein A is its ability to bind specifically to the Fc region of immunoglobulins, especially IgG. The Protein A molecule contains four high affinity ($K_a = 10^8\ M^{-1}$) binding sites capable of interacting with the Fc region of several species.[3] The molecule is heat-stable and retains its native conformation when exposed to denaturing agents such as 4 M urea, 4 M thiocyanate and 6 M guanidine hydrochloride.[4]

Protein A covalently coupled to agarose has been used extensively as an affinity support for the isolation IgG from several mammal species.[5,6,7] However, the interaction between Protein A and IgG is not equivalent for all species.[8,9] Even within a species, Protein A interacts with some subclasses of IgG and not others. For example, human IgG_1, IgG_2, and IgG_4 bind strongly but IgG_3 does not[3] and mouse IgG_1 binds poorly to Protein A.[10] Despite its variable binding characteristics, Protein A possesses IgG-binding properties that make it ideal for affinity purification of IgG from the serum. When using samples from species not well referenced in the literature, conduct preliminary experiments to determine if a particular immunoglobulin binds.

The Immobilized Protein A resin supplied in this kit is superior to other commercially available Protein A agarose resins in that the protein is immobilized to the beaded agarose resin by more stable secondary amine bonds. The result is an affinity resin with excellent stability and binding characteristics, enabling the column to be re-used multiple times without significant loss in binding capacity.

Important Product Information

- The Protein A Binding Buffer supplied provides the highest efficiency of IgG binding and elution for most species. Using other buffer formulations may significantly alter the binding capacity and the wash volumes required for efficient purification. Therefore, optimization may be necessary when using other buffers.
- For optimal recovery, use a sample size such that the expected IgG load on the column is less than 80% of the maximum binding capacity. The total IgG content of serum is approximately 10-15 mg/ml. The concentration of antibody in tissue culture supernatant varies considerably among hybridoma clones. Be aware that antibodies from fetal bovine serum (FBS) culture media supplement will be purified along with the antibody of interest.
- The crosslinked 6% beaded agarose resin can tolerate commonly used water-miscible solvents when they are added in a stepwise gradual manner. The agarose resin will compress under pressure causing column flows to slow. Never freeze agarose resin, as this will cause irreversible damage to the bead structure.
- Serum samples, ascites fluid, plasma or tissue culture supernatant may be used with this product.

Reagent Preparation

Kit components	Equilibrate Immobilized Protein A and all buffers to room temperature.
Neutralization Buffer	Prepare 1 ml of high-ionic strength alkaline buffer such as 1 M phosphate or 1 M Tris; pH 7.5-9
Antibody Sample	To ensure proper ionic strength and pH for optimal binding, dilute serum, ascites fluid or tissue culture supernatant samples with an equal volume of Binding Buffer. Centrifuge cloudy samples and use only the clear supernatant. To obtain good IgG recovery from lipemic plasma or serum, centrifuge diluted samples at 10,000 × *g* for several minutes.

Procedure for Purification of IgG

A. Equilibrate Protein A Column

1. Sequentially remove top and bottom cap from Protein A column.

   Note: Always remove top cap first and bottom cap second to prevent air bubbles from being drawn into the gel.

2. Pour off and discard the storage solution, which contains 0.02% sodium azide.
3. Equilibrate Protein A column by applying 3-5 ml of Binding Buffer and allowing it to drain through the column.

   Note: In all steps column flow will stop when solution drains down to the disc at the top of the gel bed. This prevents the gel bed from drying out. However, do not leave drained column uncapped for more than a few minutes.

B. Apply Sample to Column

1. Apply up to 5 ml of Antibody Sample to the equilibrated Protein A column.

   Note: With very dilute antibody samples (for which 10-50 ml of sample is needed to approach the binding capacity of the column), better yield can be obtained more quickly by incubating the sample and immobilized Protein A in batch format. Remove the top disc from the column and use a portion of the sample to wash the resin out of the column into a tube that is large enough to hold the entire volume of sample and resin. Incubate the resin-sample slurry for 1-2 hours at room temperature or overnight at 4°C with gentle mixing. Pour the slurry back into the column to re-pack the resin bed before proceeding to wash and elution steps.

2. Allow Antibody Sample to flow through the column.

   Note: If desired, collect the solution in a clean collection tube and reserve it for later analysis of binding efficiency. If Antibody Sample contains more IgG than can bind to the Protein A column (or is an antibody type that does not bind to Protein A), the collected flow-through will contain the excess antibody. By saving the flow-through, non-bound antibody can be recovered and examined by antibody-specific assays.

3. Wash column by adding 5-15 ml of Binding Buffer and allowing it to drain through the column.

   Note: If desired, verify that all unbound proteins are thoroughly washed away by collecting separate 2 ml fractions of the solution as it drains and measuring their absorbance at 280 nm. Complete washing is indicated by fractions having absorbances similar to Binding Buffer alone.

C. Elute Antibody

1. Elute the bound IgG with 5-10 ml of the IgG Elution Buffer, collecting separate 1 ml fractions of the eluate that drains from the column.

   Note: If desired, each 1 ml fraction can be neutralized by adding 50 µl of 1 M Tris, pH 9.5 or 100 µl of Binding Buffer. Neutralization helps to stabilizes antibodies that may otherwise be inactivated by long-term storage in acidic buffer.

2. Determine which fractions contain antibody by measuring the absorbance of each one at 280 nm. Pool fractions having the highest absorbance values (typically fractions 2, 3 and 4) compared to neutralized Elution Buffer alone.
3. The purified antibody in neutralized Elution Buffer suitable for storage, BCA Protein Assay (Product No. 23225), SDS-PAGE or dilution for immunoassays. Alternatively the buffer may be exchanged to a system compatible with the specific downstream application (see optional procedure that follows).
4. Regenerate the Immobilized Protein A column by washing with 5-10 ml of Elution Buffer. Columns may be regenerated a minimum of 10 times without significant loss of binding capacity.
5. For storage, wash column with 5 ml of water containing 0.02% sodium azide. When approximately 2 ml of solution remains above the top disc, replace the bottom cap on the column followed by the top cap. Store column upright at 4°C.

Exchange Eluted Antibody into Different Storage Buffer (Optional)

A. Alternative Methods for Buffer Exchange

In the purification procedure, IgG is eluted from the Immobilized Protein G using low-pH, amine-containing buffer and then then neutralized by addition of Tris or phosphate buffer. Often, this neutralized buffer is suitable for long-term storage of the antibody. However, certain applications will require a buffer exchange. For example, most biotinylation or enzyme-labeling procedures for the antibody will necessitate removal of amines from the buffer.

Two options exist for buffer exchange. One is to dialyze the purified antibody against the buffer of choice. For this purpose, choose a Slide-A-Lyzer® Dialysis Cassette (see Related Pierce Products) appropriate for the volume of purified antibody solution. Use Slide-A-Lyzer Concentrating Solution to concentrate the antibody solution after dialysis.

The second option for buffer exchange is size-exclusion chromatography or gel filtration. For this purpose, the kit includes 5 × 5 ml D-Salt Excellulose Desalting Columns. The porous resin beads have an average molecular weight exclusion limit of 5,000. Therefore, molecules with molecular mass greater than 5 kDa will pass by the resin beads and emerge in the column void volume, while smaller molecules will pass through the column more slowly as they migrate into and through the resin beads. In this case, the purified IgG (approximately 150 kDa) will emerge from the column first and be separated from the buffer salts that will emerge later. To achieve adequate separation, apply less than 1.25 ml of sample to the 5 ml desalting column. As such, two or three separate columns may have to be used to process the entire volume of purified IgG sample.

The choice between dialysis and desalting is a matter of speed vs. effort. Dialysis requires several hours to overnight to perform, but the entire sample can be processed at once with minimum hands-on time. Desalting can be performed in much less time but requires careful sample collection and identification of fractions containing the molecule or interest.

B. Procedure for Buffer Exchange Using 5 ml D-Salt Excellulose Desalting Columns

1. Desalting Buffer: Prepare 100 ml of the buffer into which the purified antibody will be exchanged.
2. Equilibrate Desalting Columns to room temperature. Use a separate column for each 1.25 ml of antibody sample.
3. Remove top cap from column and pour off the storage solution containing 0.02% sodium azide.
4. Remove bottom cap and wash column by adding 10 ml of Desalting Buffer and allowing it to drain through.

   Note: The gravity flow rate of column is approximately 0.1 ml per minute. Column flow will stop when solution drains down to the disc at the top of the gel bed. However, do not leave drained column uncapped for more than a few minutes.

5. Number a set of 6-10 collection tubes.
6. Apply up to 1.25 ml of antibody sample to column and immediately begin to collect a 1.0 ml fraction into the first numbered collection tube.
7. When column flow stops as a result of solution draining down to the disc at top of gel bed, add Desalting Buffer and continue collecting the solution as it drains. After the first 1.0 ml fraction (#1), begin to collect 0.5 ml fractions into successively numbered collection tubes until at least five 0.5 ml fractions (#2-6) have been collected.

8. Measure the absorbance at 280 nm of each 0.5 ml fraction relative to the Desalting Buffer. Fractions #2-4 will likely contain the purified antibody (now exchanged into Desalting Buffer) as indicated by their higher absorbance values. These fractions may be pooled.

   Note: If fraction #6 has absorbance significantly greater than Desalting Buffer alone, go back to step 7 and collect additional fractions until the absorbance approaches that of the Desalting Buffer. Molecules smaller than the exclusion limit of the gel (i.e., the buffer salts in the starting antibody sample) will emerge from the column in subsequent fractions (e.g., fractions #8-10). If collected, these fractions may be discarded after confirming that all fractions containing antibody have been identified and saved.

9. D-Salt Excellulose Desalting Columns can be regenerated for use again by washing with an additional 15 ml of Desalting Buffer after the antibody has been collected. For storage, include 0.02% sodium azide in the buffer used to regenerate the column. Cap column when approximately 2 ml of solution remains above the gel. Securely cap top of column and store at 4°C.

Troubleshooting

Problem	Possible Cause	Solution
Gravity-flow of Protein A column is exceedingly slow (i.e., < 0.1 ml/minute)	Outgassing of buffers or sample on the column, has blocked resin pores with microscopic air bubbles	Degas buffers and remove air bubbles from column (see Tech Tip in Additional Information section)
No protein detected in any elution fractions with absorbance at 280 nm or general protein staining of electrophoresed sample	Accidentally used desalting column instead of Protein A column for purification procedure	Use correct column in kit
	Sample devoid of any antibody species or subclass that binds to Protein A	Ensure by ELISA or isotyping kit, that the sample contains target IgG (see Related Pierce Products)
	Antibody bound to Protein A column (determined by depletion from the starting sample) but did not elute with the low-pH IgG Elution Buffer	Try Gentle Ag/Ab Elution Buffer (see Related Pierce Products)
Considerable antibody purified, but no specific antibody of interest detected	Antibody of interest is at very low concentration or has very low binding affinity for Protein A relative to other immunoglobulins in the sample	Use serum-free medium for cell supernatant samples
		Affinity purify the antibody using the specific antigen coupled to an affinity support (see Related Pierce Products)
Antibody was purified, but it is degraded (determined by lack of function in downstream assay)	Antibody is sensitive to the low-pH IgG Elution Buffer	Try Gentle Ag/Ab Elution Buffer (see Related Pierce Products)
	Downstream application is sensitive to neutralized IgG Elution Buffer	Desalt or dialyze eluted sample into suitable buffer

Additional Information

Please visit the Pierce website for the following related items:

- Tech Tip #27: Optimize elution conditions for immunoaffinity purification
- Tech Tip #7: Remove air bubbles from columns
- Tech Tip #29: Degas solutions for use in affinity columns
- Tech Tip #34: Binding characteristics of Protein A, G, A/G and L for immunoglobulins

Related Pierce Products

21001	**Protein A IgG Binding Buffer**, 1 L
21004	**IgG Elution Buffer**, 1 L
21027	**Gentle Ag/Ab Elution Buffer**, 500 ml
89948-89951	**NAb™ Spin Kits for Antibody Purification (Protein A, G, A/G and L, respectively)**, convenient antibody purification kits using 0.2 ml centrifuge columns
89978-89881	**NAb™ Spin Kits for Antibody Purification (Protein A, G, A/G and L, respectively)**, convenient antibody purification kits using 1 ml centrifuge columns
20333	**Immobilized Protein A**, 5 ml
45212	**Melon™ Gel IgG Purification Kit**, alternative purification method for antibody purification
44894	**AminoLink® Plus Immobilization Kit**, covalent attachment of proteins for affinity purification
37501	**Monoclonal Antibody Isotyping Kit I (HRP/ABTS)**
66382	**Slide-A-Lyzer® Dialysis Cassette Kit**, 10 dialysis cassettes, each appropriate for 0.5-3.0 ml samples
66526	**Slide-A-Lyzer® Concentrating Solution**, 10 x 15 ml

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Current versions of product instructions are available at *www.piercenet.com*. For a faxed copy, call 800-874-3723 or contact your local distributor.