

Amintra

Affinty Resins

Protein A

Technical Data and Instruction Manual



expedeon
PROTEIN SOLUTIONS

Innovators of Protein Technologies

info@expedeon.com

Expedeon Protein Solutions: Innovators of Protein Technologies

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Introduction

Affinity purification of monoclonal antibodies has been largely confined to the use of Protein A and Protein G chromatography. The Amintra Protein A and Protein G resins are designed for simple, one-step and rapid antibody purification from serum, ascites and tissue culture supernatant such as those derived from static cultures and bioreactors. Antibody samples purified using this affinity resin may be used in a wide range of laboratory procedures such as 1D or 2D polyacrylamide gel electrophoresis, Western blotting, ELISA etc. The antibodies are sufficiently pure for radiolabelling, conjugations (for example fluorescein) or preparation of immuno-affinity columns.

Storage

The resin is formulated in 0.01 % Thimerosal. The Protein A resin as supplied is stable for up to 2 year at 2-8 °C from the date of manufacture. Please see packaging for expiry date. Do not freeze the resin or store it at room temperature. Freezing the suspension will damage the agarose beads. The resin is pre-swollen and de-fined.

Specifications

| | |
|---------------------------|---|
| Protein A | Recombinant Protein A expressed in <i>E. coli</i> deficient in albumin binding domain |
| Supporting matrix: | 6% crosslinked agarose resin |
| Ligand Density: | 3.5 mg Protein A/ml resin |
| Bead size range: | 60-165 µm |
| Recommended working pH: | pH 2.5-10.0 |
| Typical binding capacity: | Upto 30 mg Human IgG /ml resin |
| Maximum Flow rate | Upto 300 cm/h |
| Maximum pressure | 0.1MPa (1 bar) |
| Chemical stability: | High |
| Solubility in water: | Insoluble |
| Toxin Levels | Free of Staphylococcus enterotoxins and hemolysins |

Chemical compatibility

All resins are susceptible to oxidative agents. Avoid high temperatures. Amintra Protein A resin is resistant to short term exposure to organic solvents (e.g. 70 % ethanol, 5.8M acetic acid) and are stable in all aqueous buffers commonly used for Protein A chromatography. Protein A is resistant to 6 M guanidine-HCl, 8 M urea and 2 M sodium isothiocyanate.

Disclaimer

This product is for research use only and is not intended for use in clinical diagnosis. No claims beyond replacement of unacceptable material or refund of purchase price shall be allowed.

Protein A chromatography

Protein A is a cell wall protein from *Staphylococcus aureus* with a molecular weight between 35-50 kDa. The quality of the Protein A agarose (or equivalent) is important to avoid leakage of protein A during the elution procedure. Immobilized Protein A resins linked via an amide bond between the amino groups of protein A and either oxirane or N-hydroxysuccinimide ester groups form the most stable cross-links. Immobilized Protein A binds specifically to the Fc region of immunoglobulin molecules of many mammalian species.

Protein A affinity chromatography is a rapid one-step purification, which removes most non-IgG contaminants and can achieve purities close to homogeneity. It is particularly useful for purifications of tissue culture supernatant, where 10-100 fold concentrations can be achieved.

Improving binding conditions

Any sample, such as a crude biological extract, a cell culture supernatant, serum, ascites or an artificial standard can be used with the Protein A resin. Aggregation/precipitation of proteins is common during storage and repeated freeze/thaw cycles of sera, ascites and tissue culture supernatants. It is important that the sample is first filtered through a 0.45-1.2 µm filter to remove particulates that could clog the resin flow channels. All samples should be filtered just prior to loading even if they have been filtered several days before the chromatographic run. When protein aggregation or protein solubility problems are observed, Expedeon's Stabil-PAC product can be used to enhance protein stability and solubility.

Lipids, which can be found at high levels in serum or ascites should also be removed as much as is possible prior to loading, please refer to our recommended Delipidation Protocol on Page 6. Of equal importance is the ability to process the samples rapidly and, if the need arises, to be able to purify the target protein at 4 °C.

Optimal conditions for binding the target molecule to a resin are critical for successful separation of the protein. If the binding conditions are not optimal with respect to pH, salt concentration, presence or absence of metal ions etc, the interaction could be weak or non-existent. In many instances, the sample needs to be dialysed or diafiltered by ultrafiltration before it is applied to an affinity or ion exchange chromatographic support. In Protein A or G separations, the sample should simply be diluted 1:1 (v/v) in 1 x binding buffer.

The interaction of immobilized Protein A or Protein G with immunoglobulins (Igs) is pH-dependent. The binding capacity for Protein A is optimal at pH 8-9, whereas the binding capacity of Protein G is high over a broader pH range. Salt concentration can significantly affect the binding of mouse Igs to protein A. Mouse IgG1, rat IgG1 and rat IgG2b bind well to immobilized protein A when the salt concentration is higher than 1 M, but bind poorly at low salt concentrations.

Table 1. Affinity of Protein A for IgG subclasses.

| Species | Subclass | Binding pH | Elution pH |
|------------|----------|------------|------------|
| Mouse | IgG1 | 8.5-9.0 | 6.0-7.0 |
| Mouse | IgG2a | 8.0-9.0 | 4.5-5.5 |
| Mouse | IgG2b | 8.0-9.0 | 3.5-4.5 |
| Mouse | IgG3 | 8.0-9.0 | 4.0-7.5 |
| Rat | IgG1 | 8.0-9.0 | 6.0-8.0 |
| Rat | IgG2a | 9.0 | 7.5-9.0 |
| Rat | IgG2b | 8.0-9.0 | 7.0-8.0 |
| Rat | IgG2c | 8.0-9.0 | 3.0-7.0 |
| Human | IgG1 | 7.0-7.5* | 2.5-4.5 |
| Human | IgG2 | 7.0-7.5* | 2.5-4.5 |
| Human | IgG3 | 7.0-7.5* | 3.0-7.0 |
| Human | IgG4 | 7.0-7.5* | 2.5-4.5 |
| Rabbit | IgG | 7.5 | 3.0-7.0 |
| Guinea pig | IgG1 | 7.5-9.0 | 4.0-5.0 |
| Guinea pig | IgG2 | 7.5-8.0 | 3.0-4.5 |

*Experiments in our laboratory show that human IgG binds well to Proteus Protein A spin columns at pH 9.0 (the pH of the binding Proteus Protein A kit). However, Proteus Protein G spin columns may be more suited to the purification of IgG from human serum.

Fig. 1. Typical pH dependence of binding of mouse IgG1 to Protein A

Protein Elution

The most common elution conditions for Protein A or G affinity and immuno-affinity separations involve a reduction in pH to between pH 2.5 and 5.0. It is important to appreciate that a few proteins (e.g. some monoclonal antibodies) are acid-labile and they can lose their activity at very low pH values. Above all, the elution conditions must preserve the integrity and activity of the target protein. Most observed denaturation is caused by harsh elution conditions. Acidic pH is known to reduce the antibody titre, decrease immuno-reactivity and distort the antibody structure. It is therefore critical that the pH is restored to neutrality after elution.

Flow rate is an important consideration for achieving optimal separation. Flow rate through the column support is related to the efficiency of the separation; too fast and the mobile phase will move past the beads quicker than the diffusion time necessary for the antibodies to reach the internal bead volume. This will also result in lower apparent binding capacity for the resin. Amintra Protein A resin chemistry provides very rapid association kinetics between the protein molecule and the immobilized ligand, giving optimal diffusional flow through the internal bead structure and allowing the use of high flow rates.

Protein A or Protein G

Immunoglobulin G from most species consists of several subclasses with different biological properties. Four subclasses of IgG have been identified in human (IgG1, IgG2, IgG3, and IgG4) and in mouse (IgG1, IgG2a, IgG2b and IgG3). For immunological studies, it is often necessary to isolate one particular subclass of IgG from the other subclasses.

Protein G binds to all major Ig classes except IgM and therefore has a wider reactivity profile than Protein A. However, the binding of Igs to Protein G is often stronger, requiring more stringent elution conditions for complete recovery of the immunoglobulin compared to Protein A.

Protein A can withstand more harsh conditions which can be beneficial for deep cleaning and regeneration. Different mouse IgG subclasses will exhibit varying strength of association to Protein A. Customization of the purification strategy may be required for the affinity separation, e.g. mouse IgG1, the most common subclass used, does not bind well to Protein A at low ionic strength. However, the use of high salt concentrations (2-3 M NaCl) and high pH (pH 8-9), these antibodies will bind to Protein A and provide good separation.

The needs of the researcher dictate that the speed of sample processing, the cost and the reproducibility are key criteria for selecting purification tools. Amintra purification resins have been designed to offer the optimal solution to each criterion. In the vast majority of cases, simply selecting the correct resin and performing a considered purification strategy will provide the best possible separation of your target proteins.

Recommended Buffers

Binding buffers:

- ❖ 1 -1.5 M Glycine/NaOH, 2-3 M NaCl pH 9.0
- ❖ 1 M Sodium borate, 2 M NaCl pH 9.0
- ❖ 0.1 M Sodium phosphate, 0.1 M NaCl pH 7.4 (PBS)

Elution buffer:

- ❖ 0.1 M Sodium citrate pH 3.0-6.0
- ❖ 0.1-0.2 M Glycine/HCl pH 2.5-3.0
- ❖ 0.1 M Sodium phosphate pH 3.0-6.0

Neutralization buffer:

- ❖ 1M Tris/HCl pH 9.0

Protein Purification Protocol

Delipidation Procedure:

All protein A & G affinity columns are affected by the presence of lipids and lipoproteins, which are especially common in antibody samples derived from ascites fluid. For end users who have antibody solutions which they need to delipidate, the following protocol is a gentle and easy method for removing lipids and lipoproteins.

- 1) Add 0.04 ml 10% dextran sulphate solution and 1 ml 1 M calcium chloride per ml sample.
- 2) Mix for 15 min.
- 3) Centrifuge at 10,000 g for 10 min.
- 4) Discard the precipitate.
- 5) Exchange the sample into TBS (Tris Buffered Saline) using dialysis, ultrafiltration or a desalting column. **Do not buffer exchange into a phosphate-containing buffer such as PBS.**

Pre-equilibration:

Equilibrate the resin with 3-5 column volumes of binding buffer.

Sample loading:

Load an appropriate amount of 0.45 µm filtered cleared lysate on to column or mix the resin with the cleared lysate and let the resin settle for 30 minutes at room temperature or at 2-8 °C. The binding capacity of the resin is approximately 20-30 mg IgG/ml sedimented resin. Collect the sample flowthrough for further analysis.

Washing:

Wash the column 5 times, each time with 3 column volumes of wash buffer. Collect the washes for further analysis to ensure that all unbound protein is removed.

Elution:

Apply 10-15 column volumes elution buffer to the resin and collect appropriate fractions sizes (e.g 1CV) for further analysis.

Alternatively you can apply 2 column volumes of elution buffer to the resin, mix and allow the resin to. Following this, collect the supernatant. Repeat this process at least 5 times.

The eluate must be neutralized rapidly. You can either elute the purified IgG directly into a high pH neutralization buffer e.g. 1M Tris/HCl pH 9.0 or dialyse. Always check the protein content of each fraction before pooling to avoid unnecessary dilution of the purified target protein.

Regeneration:

Wash the column with 10 CVs of elution buffer followed by 10 CVs of binding buffer. Proceed to the pre-equilibration step of another bind-wash-elute cycle if the column is to be re-used immediately. After regeneration, the resin can also be stored in a screw capped bottle in 0.1 % sodium azide (made up in distilled water) at 2-8 °C until further use.

Determination of Antibody Concentration

By Bradford Assay

Expedeon's BradfordUltra assay provides a robust assay for the determination of protein concentrations from 1 µg/ml to 1.5 mg/ml. For determination of antibody concentrations using BradfordUltra, it is recommended that a standard curve be prepared using known samples of pure antibody.

By UV Absorbance

For pure solution rearranging the Beer-Lambert law, $A = \epsilon \cdot c \cdot l$, can be used to determine the protein concentration of IgG (mg/ml). A = Absorbance at 280 nm, ϵ = Extinction Coefficient, c = Concentration of Sample (mg/ml) and l = path length (cm)

| | Extinction Coefficient (ml.mg ⁻¹ .cm ⁻¹) |
|-----|---|
| IgG | 0.72 |
| IgM | 0.84 |
| IgA | 0.94 |

ELISA and SDS-PAGE

Sandwich ELISA assay can also be used to accurately measure antibody concentrations within a range of 1 mg/ml to 20 mg/ml sample. Antibody samples can also be monitored for purity by SDS-PAGE under reducing or non-reducing conditions. Note that IgG appears in a reducing SDS-PAGE as 25 kDa and 50-55 kDa bands and IgM appears as 25 kDa and 70-80 kDa bands. Recovery of immunoglobulins can be quantified by a standard protein assay, scanning densitometry of reducing or non-reducing SDS-PAGE gels or ELISA.

Ordering Information

| Cat # | Description |
|---------|---------------------------|
| APA0005 | Amintra Protein A – 5ml |
| APA0025 | Amintra Protein A – 25ml |
| APA0100 | Amintra Protein A – 100ml |

Contact Information

Expedeon Protein Solutions

Unit 1A, Button End,
Harston,
Cambridgeshire.
CB22 7GX
United Kingdom

Phone: +44 (0)1223 873364

Fax: +44 (0)1223 873371

E-mail: info@expedeon.com

Web : www.expedeon.com

Expedeon Protein Solutions

Unit 1A, Button End,
Harston,
Cambridgeshire.
CB22 7GX
United Kingdom

Phone: +44 (0)1223 873364

Fax: +44 (0)1223 873371

E-mail: info@expedeon.com

Web: www.expedeon.com

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