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ab270308 Protein A Resin - Amintra

A product of Expedeon, an Abcam company

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For simple, rapid purification of classes, subclasses and fragments of immunoglobulins from biological fluids and from cell culture media.

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1. Overview

Protein A Resin - Amintra (ab270308) is an affinity chromatography medium designed for easy, one-step purification of classes, subclasses and fragments of immunoglobulins from biological fluids and from cell culture media. The recombinant protein A ligand is coupled to highly cross-linked 4% agarose resin. The coupling is optimized to give high binding capacity for immunoglobulins. The static binding capacity of Protein A Resin - Amintra is greater than 40 mg human IgG per mL settled resin. The dynamic binding capacity varies depending on several factors such as the target antibody, flow rate during binding, etc.

Protein A, a bacterial cell wall protein isolated from Staphylococcus aureus, binds to mammalian IgGs mainly through Fc regions. Recombinant protein A contains five high affinity IgG binding domains with other non-essential domains removed to reduce nonspecific binding. Since only the Fc region is involved in binding, the Fab region is available for binding antigens.

Protein A has a molecular weight between 35-50 kDa. Immobilized Protein A resins linked via an amide bond between the amino groups of protein A and either oxirane or Nhydroxysuccinimide ester groups form the most stable cross-links.

Protein A affinity chromatography is a rapid one-step purification, which removes most nonIgG contaminants and can achieve purities close to homogeneity. It is particularly useful for purifications of tissue culture supernatant.

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2. Materials Supplied and Storage

Store kit at +4°C immediately on receipt**. Do not freeze or store the resin at room temperature. Freezing the suspension will damage the agarose beads.** The resin is pre-swollen and defined. It is formulated as a 50% slurry in 48% water + 2% benzyl alcohol

Item	Quantity			Storage temperature
Protein A Resin - Amintra	5 mL	25 mL	100 mL	+4°C

3. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Binding buffer (see buffer selection Section 4.4)
- Wash buffer (see buffer selection Section 4.4)
- Elution buffer (see buffer selection Section 4.4)
- Neutralization buffer (see buffer selection Section 4.4)

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4. Technical Considerations

4.1 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.

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 in step 5.1. 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 nonexistent. 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 separations, the sample should simply be diluted 1:1 (v/v) in 1 x binding buffer.

The interaction of immobilized Protein A with immunoglobulins (Igs) is pH dependent. The binding capacity for Protein A is optimal at pH 8-9. Salt concentration can significantly affect the binding of mouse Igs to protein A. Mouse IgG_1, rat IgG_1 and rat IgG_{2b} bind well to immobilized protein A when the salt concentration is higher than 1 M, but bind poorly at low salt concentrations.

4.2 Protein Elution:

The most common elution conditions for Protein A 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

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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 immunoreactivity 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. Protein A Resin - Amintra 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.

4.3 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 (IgG_1, IgG_2, IgG_3, and IgG_4) and in mouse (IgG_1, IgG_{2a}, IgG_{2b} and IgG_3).

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 IgG_1, 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

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cases, simply selecting the correct resin and performing a considered purification strategy will provide the best possible separation of your target proteins.

Protein A and Protein G antibody binding affinities:

Species	Subclass	Protein A Binding Affinity	Protein G Binding Affinity
Human	IgA	variable	-
	IgD	-	-
	IgE	-	-
	IgG_1	++++	++++
	IgG_2	++++	++++
	IgG_3	-	++++
	IgG_4	++++	++++
	IgM	variable	-
Avian egg yolk	IgY	-	-
Cow		++	++++
Dog		++	+
Goat		-	++
Guinea pig	IgG_1	++++	++
	IgG_2	++++	++
Hamster		+	++
Horse		++	++++
Koala		-	+
Llama		-	+
Monkey (Rhesus)		++++	++++
Mouse	IgG_1	+	++++
	IgG_{2a}	++++	++++
	IgG_{2b}	+++	+++
	IgG_3	++	+++
	IgM	variable	-
Pig		+++	+++
Rabbit	No distinction	++++	+++
Rat	IgG_1	-	+
	IgG_{2a}	-	++++
	IgG_{2b}	-	++
	IgG_3	+	++
Sheep		+/-	++

++++ Strong binding; ++ Medium binding; - Weak or no binding

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4.4 Recommended buffers:

Water and chemicals used for buffer preparation should be of high purity. We recommend filtering the buffers by passing them through a 0.22 µm or 0.45 µm filter before use.

Binding/Wash buffer: 20 mM NaH_2PO_4,150 mM NaCl; pH 7.0
Elution buffer: 100 mM glycine; pH 3.0
Neutralization buffer: 1 M Tris-HCl; pH 8.5

4.5 Characterization of the Resin:

Supporting matrix	Highly cross-linked 4% agarose supplied as 50% slurry
Ligand	Recombinant Protein A
Bead size range	45-165 µm
Recommended working pH	pH 2.5-9.0
Typical binding capacity	>40 mg Human IgG/mL resin
Maximum Flow rate	Up To 300 cm/hr
Maximum pressure	0.3 MPa (3 bar)
Chemical stability	High - Stable in all aqueous buffers commonly used in Protein A chromatography: • 10 mM HCl (pH 2) • 1 mM NaOH (pH 11) • 0.1 M sodium citrate/HCl (pH 3) • 6 M guanidine-HCl • 20% ethanol
Storage buffer	48% water + 2% benzyl

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5. Assay Procedure

5.1 Delipidation procedure:

All Protein A 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.

5.1.1 Add 0.04 mL 10% dextran sulphate solution and 1 mL 1 M calcium chloride per mL sample.

5.1.2 Mix for 15 minutes.

5.1.3 Centrifuge at 10,000 x *g* for 10 minutes.

5.1.4 Discard the precipitate.

5.1.5 Exchange the sample into Tris-buffered saline (TBS) using dialysis, ultrafiltration or a desalting column.

⚠ Note: <u>Do NOT buffer exchange into a phosphate-containing buffer such as PBS.</u>

5.2 Standard purification protocol:

5.2.1 <u>Pre-equilibration:</u>
Equilibrate the resin with three to five column volumes of binding buffer.

5.2.2 <u>Sample loading:</u>
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 >40 mg IgG/mL sedimented resin. Collect the sample flow-through for further analysis.

5.2.3 <u>Washing:</u>
Wash the column 5 times, each time with three column volumes of wash buffer. Collect the washes for further analysis to ensure that all unbound protein is removed.

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5.2.4 Elution:
Apply ten to fifteen column volumes elution buffer to the resin and collect appropriate fractions sizes (e.g 1 CV) for further analysis.

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

The eluate must be neutralized rapidly. The purified IgG can either be eluted directly into a high pH neutralization buffer e.g. 1M Tris-HCl, pH 8.5 or dialysed.

5.2.5 Always check the protein content of each fraction before pooling to avoid unnecessary dilution of the purified target protein.

5.2.6 Regeneration:
Wash the column with 10 CVs of elution buffer followed by 5 CVs of binding buffer. Proceed to the preequilibration step of another bind-wash-elute cycle if the column is to be re-used immediately. Columns can be regenerated up to 10 times without significant loss of binding capacity

5.3 Packing the column for use with an AKTA system:

Column packing:

5.3.1 Remove air from the column dead spaces by flushing the end-piece and adapter with packing buffer. Make sure no air has been trapped under the column net.

5.3.2 Close the column outlet leaving the net covered with packing buffer.

5.3.3 Resuspend the resin medium in its container by shaking (avoid stirring the sedimented medium). Pouring the slurry down a glass rod held against the column wall will minimize the introduction of air bubbles.

If using a packing reservoir, immediately fill the remainder of the column and reservoir with packing buffer. Mount the adapter or lid of the packing reservoir and connect the column to a pump. Avoid trapping air bubbles under the adapter or in the inlet tubing.

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5.3.4 Open the bottom outlet of the column and set the pump to run at the desired flow velocity. Ideally, Amintra Protein A is packed at a constant pressure of approximately 1 bar (0.1MPa). If the packing equipment does not include a pressure gauge, use a packing flow velocity of approximately 400 cm/h (10 cm bed height, 25°C, low viscosity buffer). If the recommended pressure or flow velocity cannot be obtained, use the maximum flow velocity the pump can deliver. This should also give a reasonable well-packed bed. Do not exceed 75% of the packing flow velocity in subsequent chromatographic procedures.

5.3.5 When the bed has stabilized, close the bottom outlet and stop the pump.

If using a packing reservoir, disconnect the reservoir and fit the adapter to the column. If using the column, carefully place the top filter on top of the bed before fitting the adapter.

5.3.6 With the adapter inlet disconnected, push the adapter down, approximately 2 mm into the bed, allowing the packing solution to flush the adapter inlet.

5.3.7 Connect the pump, open the bottom outlet and continue packing. The bed will be further compressed at this point and a space will be formed between the bed surface and the adapter.

5.3.8 Close the bottom outlet. Disconnect the column inlet and lower the adapter approximately 2 mm into the bed. Connect the pump. The column is now ready to use.

<u>Sample Purification protocol:</u>

5.3.9 Fill the syringe or pump tubing with binding buffer. Remove the stopper and connect the column to the syringe (with the provided connector), or pump tubing, "drop to drop" to avoid introducing air into the column. Remove the snap-off end at the column outlet.

5.3.10 Wash the column with ten column volumes of binding buffer.

5.3.11 Apply the sample, using a syringe fitted to the connector or by pumping it onto the column.

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5.3.12 Wash with five to ten column volumes of binding buffer or until no material appears in the effluent.

5.3.13 Elute with five column volumes of elution buffer. Other volumes may be required if the interaction is difficult to break.

Analysis:

5.3.14 Identify fractions using UV absorbance, SDS-PAGE, or the Western blot. We recommend our - RunBlue™ range of precast gels.

Antibody concentration may be determined by UV absorbance or using a protein quantitation reagent (e.g. Bradford reagent).

Bradford assay:

For determination of antibody concentrations using the Bradford assay, it is recommended that a standard curve be prepared using known samples of pure antibody.

UV absorbance:

For pure solution rearranging the Beer-Lambert law, $A = \varepsilon.c.l$, 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).

Immunoglobulin	Extinction Coefficients ($mL.mh^{-1}.cm^{-1}$)
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

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densitometry of reducing or non-reducing SDS-PAGE gels or ELISA.

Regeneration:

5.3.15 Regenerate the column by washing the resin with 10 mL Elution Buffer followed by equilibration with 5 mL Binding/Wash Buffer. Columns can be regenerated up to 10 times without significant loss of binding capacity.

5.4 Resin storage:

Store Protein A Resin - Amintra in Binding/Wash Buffer containing 20% ethanol at 2°C to 8°C. **Do not freeze.**

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6. Troubleshooting

6.1 The flow rate of the column is very low.

6.1.1 Tiny air bubbles from buffer or particles from sample may be blocking the gel pores. De-gas buffers and samples. Do not allow the column to dry.

6.2 A considerable amount of sample has been loaded, but no specific antibody of interest is detected.

6.2.1 The concentration of antibody of interest is probably very low. Purify the antibody using the specific antigen coupled to a support matrix (eg. NHS Activated Resin - Amintra ab270546).

6.3 The antibody is degraded.

6.3.1 The antibody is sensitive to low pH elution buffer. Neutralize the eluted fractions with Neutralization Buffer immediately after elution.

6.4 No antibody is detected in any elution fraction.

6.4.1 The IgG subclass does not bind to protein A. Try other affinity chromatography media to purify the antibody, such as media conjugated with Amintra Protein G.

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