Antibody Purification Protocol - Protein A Agarose

Protein A is a cell wall protein from Staphylococcus aureus and is commonly used for the affinity purification or pull downs of IgG class antibodies. It binds to immunoglobulins through interaction with the IgG heavy chain within the Fc region. Antibodies from serum (polyclonal antibodies), ascites or cell culture supernatant of hybridoma cell lines (monoclonal antibodies) can thus be easily isolated. Protein A does not bind to all IgG classes from all species equivalently well (for details see table 1).

Species	Subclass	Protein A binding
Human	IgA	variable
	IgD	-
	IgE	-
	IgG_1	++++
	IgG_2	++++
	IgG_3	-
	IgG_4	++++
	IgM	variable
Avian egg yolk	IgY	-
Cow	IgG	++
Dog	IgG	++
Goat	IgG	-
Guinea pig	IgG	++++
Hamster	IgG	+
Horse	IgG	++
Lama	IgG	-
Monkey (rhesus)	IgG	++++
Mouse	IgG_1	+
	IgG_{2a}	++++
	IgG_{2b}	+++
	IgG_3	++
	IgM	-
Pig	IgG	+++
Rabbit	IgG_1	++++
Rat	IgG_1	-
	IgG_{2a}	-
	IgG_{2b}	-
	IgG_3	+
Sheep	IgG	+/-

Table 1

Important Product Information

- 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 cross-linked 6% beaded agarose support can tolerate commonly used water-miscible solvents when they are added in a stepwise gradual manner. The agarose support will compress under pressure causing column flows to slow. Never freeze agarose supports, 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.
- Pass the sample through a 0.45 µm or 0.2 µm filter in order to remove any precipitates. Alternatively, spin down sample right before usage.
- All buffers should be degassed.
- Equilibrate all buffers and Protein A Agarose to room temperature before use.

Gravity-Flow Column Procedure for Antibody Purification using Protein A Agarose

Materials and reagents

- **Storage buffer**: PBS (phosphate buffered saline), 1 mM EDTA, 0.02% NaN_3, pH 7.2-7.4
- **Binding and wash buffer**: PBS (phosphate buffered saline), pH 7.2-7.4
- **Elution buffer**: 0.1 M Glycine/HCl, pH 2.5
- **Neutralization buffer**: 1 M Tris/HCl, pH 8.5
- **Cleaning-in-Place (CIP) solution**: 0.1–0.5 M NaOH

Procedure

1. Carefully pack appropriate column with Protein A Agarose.
2. Equilibrate the column by adding 3x column bed volumes (CVs) of **binding and wash buffer**.
   Note: To avoid air being drawn into the Protein A Agarose resin, remove the top cap before the bottom cap when opening the column.
3. Dilute the sample at least 1:1 with **binding and wash buffer** before application to Protein A column to maintain proper pH and ionic strength for optimal binding. *Note: Plasma may become hazy when diluted with binding & wash buffer due to lipoprotein precipitation. If this occurs or any precipitates are seen in the sample, centrifuge the diluted sample at 10,000x g for 20 min.*
4. Apply the diluted sample slowly to the column and allow it to flow completely into the resin. Do not allow the resin bed to run dry.
   Note: If the 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 flow-through will contain excess antibody. By saving the flow-through, non-bound antibody can be recovered and examined by antibody-specific assays.
5. Wash the Protein A column with 5-10 CVs of **binding and wash buffer**.
   Note: Efficiency of washing can be monitored via OD (280 nm) of the flow-through.
6. Elute the antibody with 5 CVs of **elution buffer** and collect 0.5-1 ml fractions. Immediately adjust eluted fractions to physiological pH by adding 100 µl of **neutralization buffer** per 1 ml of eluate and vortex gently.
7. The antibody concentration of the fractions can be determined by measuring OD at 280 nm. An OD_{280} of 1.38 is equivalent to 1 mg/ml antibody. The desired fractions with the highest absorbance can be pooled, and the final antibody concentration can be determined by OD_{280}.
8. Regenerate the Protein A column by washing with 5 CVs of **elution buffer**. Then, wash with at least 5 CVs of **binding and wash buffer**.

Cleaning-in-place (CIP)

CIP is the removal of tightly bound precipitated or denatured substances from the resin. If such contaminants remain in the system, they may affect the chromatographic properties of the material, reduce the binding capacity and, potentially, come off in subsequent runs.

1. Equilibrate column with 3 CVs of H_2O.
2. Rinse the column with at least 3 CVs of **CIP solution**.
   Note: Contact time should be at least 15 minutes.
3. Equilibrate column with **storage buffer** until pH 7.2-7.4 is reached. Fill the column almost completely with storage buffer and seal the column.
4. Store columns upright at 4°C.

FPLC Procedure for Antibody Purification using Protein A Agarose cartridges

Materials and reagents

- **Storage buffer**: PBS (phosphate buffered saline), 1 mM EDTA, 0.02% NaN_3, pH 7.2-7.4
- **Binding and wash buffer**: PBS (phosphate buffered saline), pH 7.2-7.4
- **Elution buffer**: 0.1 M Glycine/HCl, pH 2.5
- **Neutralization buffer**: 1 M Tris/HCl, pH 8.5
- **Cleaning-in-Place (CIP) solution**: 0.1–0.5 M NaOH

Procedure

1. Set FPLC system flow rate to 0.5-1 ml/min for a 1 ml cartridge and 1-3 ml/min for a 5 ml cartridge.
2. Equilibrate the column by adding 5x column bed volumes (CVs) of **binding and wash buffer**.
3. Dilute the sample at least 1:1 with **binding and wash buffer** before application to Protein A column to maintain proper pH and ionic strength for optimal binding. *Note: Plasma may become hazy when diluted with binding & wash buffer due to lipoprotein precipitation. If this occurs or any precipitates are seen in the sample, centrifuge the diluted sample at 10,000x g for 20 min.*
4. Load sample. *Note: If the sample contains more IgG than can bind to the Protein A cartridge (or is an antibody type that does not bind to Protein A), the flow-through will contain excess antibody. By saving the flow-through, non-bound antibody can be recovered and examined by antibody-specific assays.*
5. Wash the cartridge with 5-10 CVs of **binding and wash buffer**.
6. Elute the antibody with 5 CVs of **elution buffer** and collect fractions. Immediately adjust eluted fractions to physiological pH by adding 100 µl of **neutralization buffer** per 1 ml of eluate and vortex gently.
7. The antibody concentration of the fractions can be determined by measuring OD at 280 nm. An OD_{280} of 1.38 is equivalent to 1 mg/ml antibody. The desired fractions with the highest absorbance can be pooled, and the final antibody concentration can be determined by OD_{280}.
8. Regenerate the Protein A cartridge by washing with 5 CVs of **elution buffer**. Then, wash with at least 5 CVs of **binding and wash buffer**.

Cleaning-in-place (CIP)

CIP is the removal of tightly bound precipitated or denatured substances from the resin. If such contaminants remain in the system, they may affect the chromatographic properties of the material, reduce the binding capacity and, potentially, come off in subsequent runs.

1. Equilibrate cartridge with 3 CVs of H_2O.
2. Rinse the cartridge with at least 3 CVs of **CIP solution**.
   Note: Contact time should be at least 15 minutes.
3. Equilibrate the cartridge with **storage buffer** until pH 7.2-7.4 is reached.
4. Seal the cartridge and store at 4°C.