



Ultra-linked rProtein A resin

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Introduction

Sino Biological Ultra-linked rProtein A resin was produced by immobilizing recombinant protein A to 4% cross-linked agarose matrix. The recombinant protein A contains IgG binding domains, eliminating nonspecific binding sites and reducing steric hindrance.

Specifications

Ligand	Recombinant protein A
Dynamic binding capacity	~30 mg human IgG/ml drained medium
Matrix	4% highly cross-linked agarose
Bead size	45–165 μ m
Mean bead size	90 μ m
Maximum linear flow rate	>150 cm/h
pH stability	Long term: 3–9 Short term: 2–10 Working: 2–9
Sanitization	Sanitize the packed column with 2% Hibitane/20% ethanol or with 70% ethanol
Storage	+4–8 °C in 20% ethanol
Package	1ml, 5ml, 10ml, 25ml, 50ml, Bulk



Application

Protein A affinity chromatography

Pretreatment of the resin

Protein A resin is supplied in a slurry form in an aqueous (20%) ethanol solution. Place the slurry from 4°C to room temperature. Resuspend the resin by shaking the container. Pour the resuspended resin to a sintered glass crucible with fine porosity and apply vacuum. Wash the resin at least three times with distilled (deionized) water to remove the fines and the gas in the beads while stirring the slurry with a glass rod. Transfer the resin to a beaker and exchange the ethanol with neutralization buffer or high-viscosity-imparting packing solution such as 0.8M~1.2M Na₂SO₄ or distilled (deionized) water. The presence of high concentration of Na₂SO₄ increases the viscosity of the packing solution, which can, in turn, decrease back pressure and increase chromatography-column efficiency. The slurry concentration is defined as the volume of the settled gel divided by the total volume of the slurry. The recommended concentration is about 50%. Normally the slurry concentration do not need to be measured as long as the resin can be resuspended in the packing solution and be handled conveniently for packing. Make sure the volume of the slurry is larger than the column volume which can be determined by the dimension of the column.

Packing column

Preparing column

Set up the empty column upright in a clamp on a laboratory stand. Flush the end parts with buffer to avoid air bubbles trapped in the column dead spaces, using a squirt bottle or reverse flow. Close the column outlet with 1~2cm of buffer left in the column.

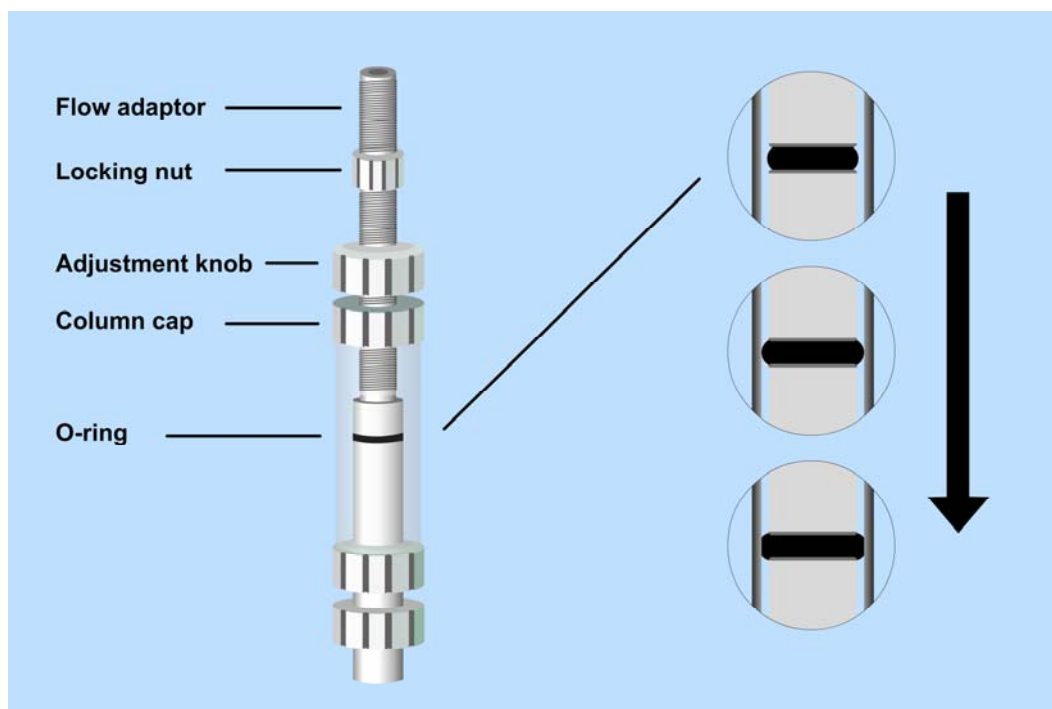
Pouring resin

Put a ruler into the inside of the column and mark the height which is intended to pack. Slowly pour the slurry to the column in one continuous motion down a glass rod held against the wall of the column to prevent introducing air bubbles. When the slurry surface is above the height mark by a few centimeters, stop pouring.

Fixing adaptor

Before inserting the flow adaptor into the column, be sure to read the instructions appropriate for the adjustable adaptor you are using.

Insert the flow adaptor carefully into the column above the slurry. Make sure that the tubing in the adaptor is open and the sealing O-ring is loosened for the adaptor to move freely in the column. Gently lower the adaptor till it contacts with the slurry. Eliminate the air bubbles between the adaptor and the slurry by slowly moving the adaptor up and down. Tighten the adjusting knob to compress the O-ring until it just seal against the column wall without impeding its sliding. Lower the adaptor slowly to the top of the resin bed while the buffer should come out through the adaptor tubing. If buffer does not come out through the tubing, but flows above the adaptor, the O-ring is not compressed enough to seal the resin. Back out the adaptor, adjust the adaptor tighter and repeat. Adjust the adaptor to compress the O-ring to position the adaptor firmly. Tighten the column cap to lock the adaptor.



Flow packing the column

- 1) Connect the column to a pump, preferably with a pressure detector. Be careful not to introduce air bubbles to the tubings or system.
- 2) Place a waste beaker under the column outlet. Then open the outlet.
- 3) Pump the packing solution to the column at the maximum flow rate close to the pressure limits of the



column or at least 133% of the flow rate recommended during normal operation.

- 4) Maintain the packing flow rate for 2~5 bed volumes after a constant bed height is reached and a head space at the top of the column is formed.
- 5) Turn the adjusting knob to loosen the O-ring to the extent that it seals the adaptor to allow buffer flowing through the tubing in it instead of flowing around the adaptor. Readjust the adaptor to touch the resin bed and lower an additional 0.5 to 1 cm to ensure a complete contact with the top of the bed.
- 6) Lock the adaptor by tightening the O-ring and the column cap. The column is now ready for use or storage after equilibration with binding buffer or pumping 5 volumes with storage buffer.

Binding

Protein A resin can bind IgG from most species and molecules tagged with an antibody Fc-region at neutral or alkaline pH.

Table 1. IgG binding of protein A

Species	Subclasses	Protein A
Human	IgG1	++
	IgG2	++
	IgG3	—
	IgG4	++
	IgA	Variable
	IgD	—
	IgM	Variable
Rabbit	No distinction	++
Guinea pig	IgG1	++
	IgG2	++
Bovine		+
Mouse	IgG1	+
	IgG2a	++
	IgG2b	+
	IgG3	+
	IgM	Variable
Chicken	IgY	—

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The binding buffer commonly used in purification is:

20 mM sodium phosphate, pH 7.0;

50 mM Tris buffer, pH 7.0

PBS (7 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic), pH7.4

Dynamic binding capacity of Protein A resin is affected by various factors, such as IgG type, binding buffer, flow rate, sample concentration, ligand density, etc. Therefore, the sample volume applied to the column should refer to previous relevant data.

Washing

Apply the sample to an appropriate volume and pump the neutralization buffer to wash the column till the absorbance level decrease to the baseline. Larger washing volume may wash off some impurities binding to the column by non-specific interaction. To remove contaminants from the target protein, a mediate washing step may be efficient by adding salts or detergents.

Elution

The general elution buffer for Protein A resin is 0.1 M glycine buffer pH 3.0 or 0.1 M citric acid pH 3.0 as elution buffer. It is also recommended to use histidine or arginine buffer at pH 3.0 as elution buffer. Practical experience and researches showed improved stability and reduced aggregation from elution by these buffers.

Because most antibodies and proteins are stable at neutral pH, neutralizing the elution peak with 1M Tris buffer at pH8.0 or 9.0 is necessary after elution as soon as possible.

Regeneration

Regeneration step should be operated after elution to remove the residue proteins and impurities on the resin for repeated use. Wash the resin with 2–3 bed volumes of regeneration buffer followed by re-equilibration with 2–3 bed volumes of binding buffer. Sometimes the regeneration buffer is the same with the elution buffer. When the elution buffer has a milder condition to avoid aggregation or degradation, the regeneration buffer is different, with a lower pH or containing different salts. But this procedure does not guarantee removing all kinds of impurities like denatured proteins or lipids. In this case, cleaning in place procedure is indispensable.



Cleaning in place (CIP)

The most common CIP procedure for Protein A resin is washing the resin with 2 column volumes of 6 M guanidine hydrochloride to remove precipitated or denatured substances, followed by re-equilibrating with at least 5 column volumes of binding buffer.

For strongly bound hydrophobic proteins, lipoproteins and lipids, wash the resin with a non-ionic detergent, for instance, 0.1% Triton X-100, at 37 °C for one minute. Immediately re-equilibrate with at least 5 column volumes of binding buffer.

If the above methods are still not effective to remove the impurities, wash the medium with 70% ethanol and let it stand for 12 hours. Re-equilibrate with at least 5 column volumes of binding buffer.

Unpacking

Under some circumstances, the column has to be unpacked. Position the column in a clamp of a stand. Connect the top of the column with a pump and disassembling the column bottom end fitting. Place a container large enough to contain the unpacked resin below the column. And start the pump to flow through the column and press the resin to come out of the column. Collect the resin slurry in the container.

Sanitization

Use a buffer containing 2% hibitane digluconate and 20% ethanol for sanitization. Stand for 6 hours after neutralization with the sanitization buffer. Alternatively, equilibrate the resin with 70% ethanol and keep it for 12 hours. Re-equilibrate the medium with at least 5 bed volumes of sterile binding buffer. The cleaning in place procedures or sanitization procedures described above normally do not significantly change column performance.

Storage

For long-term storage, store Protein A resin at 4–8 °C in 20% ethanol. Do not freeze the resin.



Trouble shooting



High back pressure



There is a clog somewhere in the system.
Column has clogged.



Disassemble the column from the system and check the backpressure.
Cell debris in the sample may clog the column. If the column is clogged, clean the column and ensure that samples have been filtered or centrifuged. Use low flow rate to clean the column with CIP buffer. However, if the back pressure is still high, unpack the column and wash the resin with cleaning buffer.



Low binding to the purification column



The binding condition is not optimal, including flow rate, pH, buffer composition, temperature.
Proteases are degrading Fc tag.



Do trial experiments to optimize loading parameters.
Decrease the flow rate to improve binding.
Load at neutral pH.
Adjust salt and ion concentration in Binding Buffer.
Prevent degradation by low-temperature operation within a short period.



Poor elution from the column



The elution condition is not suitable.



Decrease the flow rate to improve elution.
Check pH and composition of elution buffer.
Change to a different eluent.
Use reverse (upward) flow to elute the column.



Low purity



The target protein is unstable. Degradation or aggregation occur sometimes. Impurities bind to the resin by non-specific binding or interaction with the binding target protein.



To avoid degradation, operate at low temperature or minimize the purification time. To avoid aggregation, optimize the elution buffer. Try other milder eluants like histidine, arginine instead of glycine or citric acid. Increase the pH of elution buffer. Neutralize the elute immediately after elution. To minimize non-specific binding, prepare a binding buffer containing enough salt. Wash columns with a more stringent second Wash Buffer (for example, with higher salt) after sample application. Collect the elution peak by fraction and pool the fractions of high purity. Load the collected peaks on the column for another run to increase the purity.



Introduce bubbles in column



Column poured and stored at one temperature, but used at another temperature
The pump is not stopped immediately when all the sample has already loaded on the column.
Buffers are not degassed.



Degas under vacuum and remix resin to remove bubbles before using.
Pour column at the same temperature that it will be used.
Back wash the column if the bubbles are just trapped in a small upper part of the column. If the bubbles have spread to the total column, disassemble the column and repack it.



Immunoprecipitation

Immunoprecipitation is a purification method for detection or analysis of a particular antigen by precipitation of a sedimentable matrix (e.g. Protein A resin) with the complex of the antigen and its antibody. The protein can then be examined for quantity or physical characteristics (molecular weight, isoelectric point, etc.) by SDS-PAGE and immunoblotting.

Protein A resin plays a critical role in immunoprecipitation for removal of immune complexes formed between an antigen and its specific antibody.

Immunoprecipitation procedure comprises several steps which should be optimized for specific application according to actual conditions. The protocol should be developed empirically to obtain optimal results. The following protocol is just a generic reference for immunoprecipitation using Protein A resin in single protein precipitation. Co-immunoprecipitation or chromatin immunoprecipitation is not included.

Buffers and solutions

Before starting immunoprecipitation, the target protein (antigen) must be extracted from the cells by proper lysis buffer, which should be prepared in advance. The lysis buffer can be mild or harsh depending on the nature of the protein.

Soluble proteins within cells (e.g., cytosolic or luminal organellar proteins) may not require the use of detergents to be released from cells. Instead, cells can be mechanically disrupted by repeated passage through a needle, and soluble proteins can be separated from insoluble material by centrifugation. A PBS-based detergent-free lysis buffer is proper for these proteins.

Soluble and membrane proteins can be released by nondenaturing lysis buffer containing the nonionic detergent such as Triton X-100, Nonidet P-40, CHAPS, digitonin, or octyl glucoside. But many cytoskeletal and nuclear proteins, as well as a fraction of membrane proteins are not efficiently extracted under these conditions. Extraction with nondenaturing lysis buffer allows immunoprecipitation with antibodies to epitopes that are exposed on native proteins.

If epitopes of native proteins are not accessible to antibodies, or if the antigen cannot be extracted from the cell with nonionic detergents, cells should be solubilized under denaturing conditions. Denaturation is achieved by heating the cells in a denaturing lysis buffer that contains an ionic detergent such as SDS or



Sarkosyl (N-lauroylsarcosine). The denaturing lysis buffer also contains DNase I to digest DNA released from the nucleus. Supernuclease (alternative to Benzonase) is recommended to replace Dnase I.

Table 2. Summary of lysis buffer for different proteins



Mild

Medium

Harsh



Component	Detergent-free soluble	soluble (low salt)	Detergent-soluble native	soluble cytoplasmic or nuclear (RIPA)	Detergent-free denaturing
Tris		50mM	20 mM	50mM	
Salt: 0-1M	PBS		137 mM NaCl	150 mM NaCl	
EDTA: 0-5mM	5mM		2 mM		5 mM
Non-ionic detergent : 0.1 – 2%		1% NP-40	1% NP-40		
Ionic detergent 0.01%-0.5%				0.1% SDS 0.5% sodium deoxycholate	1%SDS
Denaturant					10mM dithiothreitol or beta-mercaptoethanol
Protease inhibitor	√	√	√	√	√
Additive	0.02 % Sodium Azid		10% glycerol	Supernuclease(optional)	15 U/ml DNase1 or Supernuclease

Reagents in lysis buffer

Protease inhibitors

In addition to place the lysis procedure at 4°C or on ice, protease inhibitor should be added fresh to the lysis buffer to slow down proteolysis, dephosphorylation and denaturation along with lysis. Some researcher strongly recommended lysing the cells in a cold room. The following protease inhibitors are commonly used. Select whatever is effective and convenient for you to obtain.

Cocktail is commercially available. You can also prepare it yourself following the recipe.

Protease Inhibitor Cocktail (100X):

PMSF, 5mg (50µg/ml)

Aprotinin, 100ug (1µg/ml)

Leupeptin, 100ug (1µg/ml)

Pepstatin, 100ug (1µg/ml)

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100% Ethanol bring up to 1ml, aliquot and keep at -20°C.

Sodium vanadate inhibits all tyrosine protein phosphatases. 200 micromolar is recommended. Add it fresh for each experiment from a stock made in water and store in a plastic tube at room temperature. Storage in glass leads to the appearance of precipitates.

50mM sodium fluoride is recommended as an inhibitor of Serine/Threonine protein phosphatases.

Other reagents or solutions

EDTA prevents phosphorylation in the lysate. 100 mM EDTA stock solution is made with 1.86 g into 40 ml H₂O. Add NaOH to dissolve and adjust pH to 7.4. Finally, adjust the total volume to 50 ml.

Sterile PBS pH 7.4

Sterile PBS-BSA 1% (filtered)

Loading/sample buffer for Western blotting

Phosphate is a good buffer at pH 7.2 and also functions as an inhibitor of phosphatases. Tris is a poor buffer at pH 7.2 and does not inhibit phosphatases. Tris is helpful if you need to add calcium or manganese, both of which will precipitate phosphate. Otherwise, phosphate is preferable.

Selection of antibodies

Polyclonal antibodies

In terms of antigen, polyclonal antibodies can be categorized into antibodies to whole proteins (native or recombinant) and antibodies to peptides. Antibodies to whole proteins have the advantage that they frequently recognize multiple epitopes on the target antigen, enhance the avidity of the interactions and increase the efficiency of immunoprecipitation. However, the features lead to a disadvantage of higher backgrounds and possible misidentification of antigens due to cross-reaction with epitopes on other proteins. Anti-peptide polyclonal antibodies have lower chance to cross-react with other proteins. But their immunogenicity is limited to the specific epitopes. Moreover, the purity of polyclonal antibodies also affects immunoprecipitation. Unfractionated antisera contain serum proteins and antibodies to other antigens, which respectively will cause non-specific binding to resin and cross-reaction with cellular proteins. Affinity-purified antibodies are a better alternative when antisera do not yield clean immunoprecipitation.

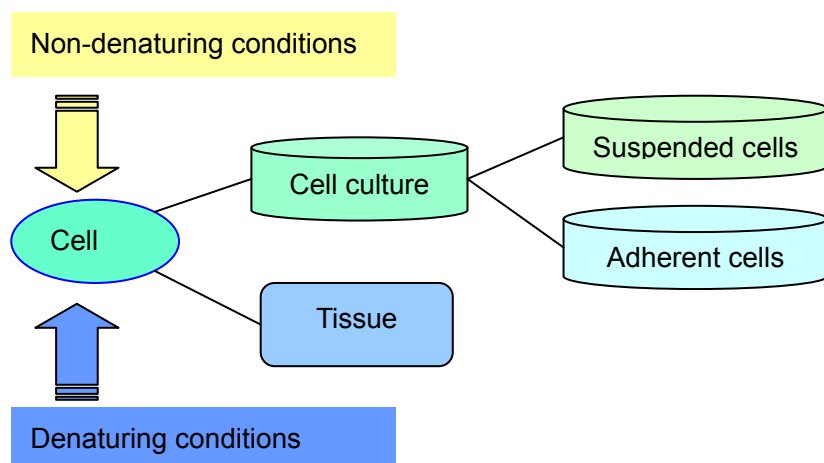
Monoclonal antibodies

Monoclonal antibodies are more specific, which reduces background. But their less affinity may lead to failure of immunoprecipitation. Ascites may also contain endogenous antibodies to other antigens and proteins such as transferrin that can bind to other proteins in the lysate.

In conclusion, selection of antibodies for immunoprecipitation should be determined empirically or by specific tests.

Immunoprecipitation protocol

1. Preparation of lysates



■ Non-denaturing condition

Cell culture-Suspension cell

- 1) Collect cells in suspension by low-speed centrifugation (e.g. 200g or 400g) for 5 min at 4°C, in a 15- or 50-ml capped conical tube. Place tube on ice. Carefully pipet supernatant.

Approximately $0.5-2 \times 10^7$ cells are required to yield 1 ml lysate, which is the amount generally used for each immunoprecipitation. Labeled cells are likely to have been pelleted earlier as part of the labeling procedure. If the cells are frozen, they should be thawed on ice before solubilization.

- 2) Wash cells twice with ice-cold PBS by resuspension and centrifugation, using the same volume of PBS as in the initial culture.
- 3) Add 1 ml ice-cold lysis buffer per $\sim 0.5-2 \times 10^7$ cells and resuspend pellet by gentle agitation for 3 sec with a vortex mixer set at medium speed.



Do not shake vigorously, as this could result in loss of material or protein denaturation due to foaming.

- 4) Keep suspension on ice 15 to 30 min (maintain constant agitation or keep cells still according to the cell type and lysis buffer) and transfer to a 1.5-ml conical microcentrifuge tube.
See steps 5)~6) described in the part of adherent cell.

Cell culture-Adherent cell

- 1) Wash cells attached to a tissue culture plate twice with ice-cold PBS. Remove the PBS with a pipet.
CAUTION: Dispose of radioactive materials following applicable safety regulations.
- 2) Place the tissue culture plate on ice or operate in a cold room.
- 3) Add ice-cold lysis buffer to the tissue culture plate to a concentration of 10^6 – 10^7 cells/ml.
- 4) Scrape the cells off the plate with a cell scraper, and transfer the suspension to a 1.5-ml conical microcentrifuge tube with a pipet. Vortex gently for 3 sec and keep tubes on ice for 15 to 30 min.
Optional: Maintain constant agitation for 30 minutes at 4°C. Sonicate each sample on a 70% duty cycle or less by placing only the very tip of the pin into the vial, then slowly lowering it into the lysate until it foams completely and then stop. Alternatively, pass the lysate through a 21 gauge needle to shear the DNA & incubate 30–60 minutes on ice.
- 5) Clear the lysate by microcentrifuging 15 min or longer at $16,000 \times g$ (maximum speed), 4°C.
Centrifugation can be carried out in a microcentrifuge placed in a cold room or in a refrigerated microcentrifuge. Take precautions to ensure that the 4°C is maintained during the spin (e.g., use a fixed-angle rotor with a lid, as the aerodynamics of this type of rotor reduces generation of heat by friction). The microfuges in the cold room are not satisfactory because they heat up to room temperature in 15 minutes. If it is necessary to reduce background, the lysate can be spun for 1 hr at $100,000 \times g$ in an ultracentrifuge.
- 6) Transfer the supernatant to a fresh microcentrifuge tube with a pipet. Do not disturb the pellet, and leave the last 20 to 40 μ l of supernatant in the centrifuge tube. Keep the cleared lysate on ice until preclearing or addition of antibody beads.

Tissue

- 1) Dissect the desired tissue with clean tools on ice or in a cold room if possible, and as quickly as possible to prevent degradation by proteases.
- 2) Place the tissue slice in microfuge tubes and freeze it by immersing in liquid nitrogen. Preserve samples at -80°C for future use or keep at 4°C (on ice or in a cold room) for immediate extraction.
- 3) Add proper volume of lysis buffer rapidly to achieve a concentration of 5-20mg tissue/ml. Homogenize with a dounce homogenizer or a sonicator, maintaining temperature at 4°C throughout



the treatment.

- 4) Centrifuge the lysis mixture for 10~20 min at 12,000 rpm at 4°C in a microcentrifuge. Pipet the supernatant and transfer to a new tube, discard the pellet. A longer centrifugation may be necessary to obtain a clear lysate. Make sure that the procedure is performed at 4°C on ice or in a cold room and the pellet is not resuspended.

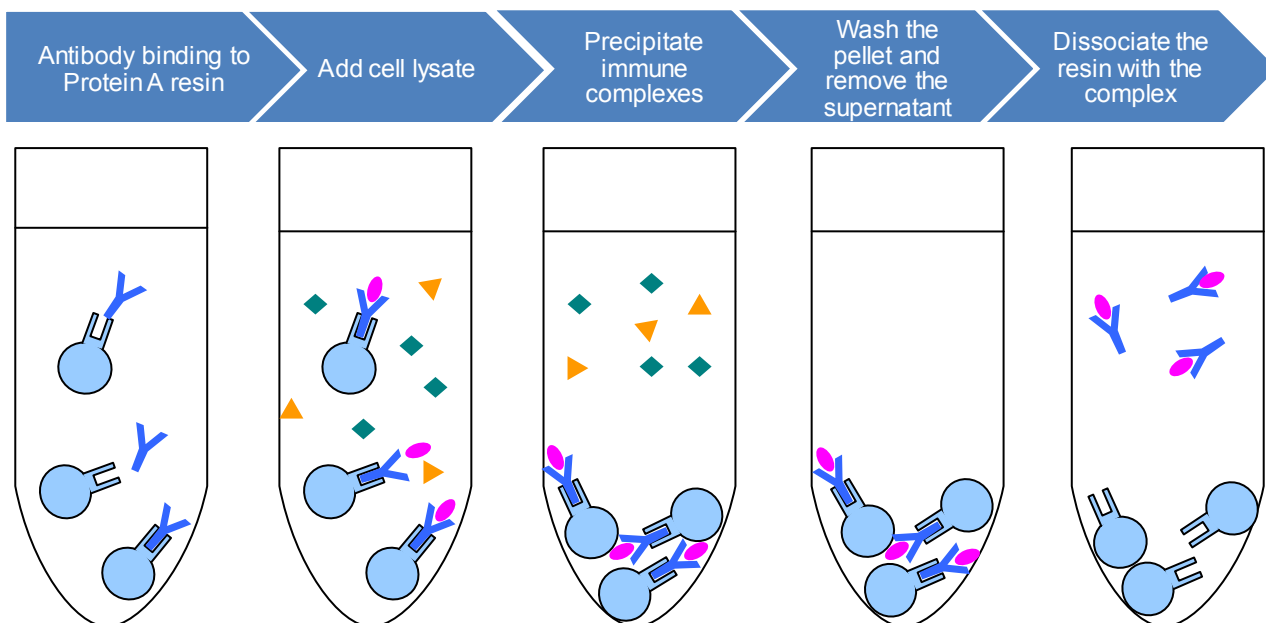
■ Denaturing condition

Cell culture

- 1) Collect cells in suspension culture or adherent culture (see the steps above). Place tubes on ice.
- 2) Add 100 µl denaturing lysis buffer per $\sim 0.5\text{--}2 \times 10^7$ cells in the pellet.
- 3) Resuspend the cells by vortexing vigorously 2 to 3 sec at maximum speed. Transfer suspension to a 1.5-ml conical microcentrifuge tube.
The suspension may be very viscous due to release of nuclear DNA. Addition of Supernuclease is recommended to effectively reduce viscosity.
- 4) Heat samples for 5 min at 95°C.
- 5) Dilute the suspension with 0.9 ml nondenaturing lysis buffer. Mix gently.
The excess 1% Triton X-100 in the nondenaturing lysis buffer sequesters SDS into Triton X-100 micelles.
- 6) Shear DNA by passing the suspension five to ten times through a 25-G needle attached to a 1-ml syringe.
If the DNA is not digested by DNase I or Supernuclease in the denaturing lysis buffer or thoroughly sheared mechanically, it will interfere with the separation of pellet and supernatant after centrifugation. Repeat mechanical disruption until the viscosity is reduced to manageable levels.
- 7) Incubate 5 min on ice.
- 8) Clear the lysate and perform immunoprecipitation

2. Preparation of Protein A Resin

- 1) Centrifuge the resin at 12 000 x g for 20 seconds and discard the supernatant. Add lysis buffer or PBS to the resin to exchange 20% ethanol. Repeat 3 times for buffer exchange.
- 2) Prepare a 50% slurry by mixing equal volumes of resin and lysis buffer or PBS.
- 3) Store at 4 °C and mix well before use.



3. Antibody binding to Protein A resin

In other protocols, antibody is first added to the lysate. Binding of antibody to Protein A resin prior to lysate enables better control of the amount of antibody bound to Protein A resin. In addition, it allows for removal of unbound antibodies and other proteins in the antibody sample, which may interfere with the recovery of the antigen.

- 1) In a 1.5-ml conical microcentrifuge tube, combine 30 μ l of 50% protein A resin slurry, 0.5 ml ice-cold PBS, and the following quantity of specific antibody.

1 to 5 μ l polyclonal antiserum

1 μ g affinity-purified polyclonal antibody

0.2 to 1 μ l ascitic fluid containing monoclonal antibody

1 μ g purified monoclonal antibody

20 to 100 μ l culture supernatant containing monoclonal antibody

The quantities of antibody are estimated by expectation of normal antibody preparation. It is advisable to determine the quantities by trial experiment.

Antibody-conjugated beads can be prepared prior to preparation of the cell lysate, to minimize the time the cell extract is kept on ice.

- 2) Set up a nonspecific immunoprecipitation control in a 1.5-ml conical microcentrifuge tube by incubating 30 μ l of 50% Protein A resin slurry, 0.5 ml ice-cold PBS, and the appropriate control



antibody(select one).

1 to 5 μ l preimmune serum as a control for a polyclonal antiserum

1 μ g purified irrelevant polyclonal antibody (an antibody to an epitope that is not present in the cell lysate) as a control for a purified polyclonal antibody

0.2 to 1 μ l ascitic fluid containing irrelevant monoclonal antibody (an antibody to an epitope that is not present in the cell lysate and of the same species and immunoglobulin subclass as the specific antibody) as a control for an ascitic fluid containing specific monoclonal antibody

1 μ g purified irrelevant monoclonal antibody as a control for a purified monoclonal antibody

20 to 100 μ l hybridoma culture supernatant containing irrelevant monoclonal antibody as a control for a hybridoma culture supernatant containing specific monoclonal antibody.

- 3) Mix suspensions thoroughly. Tumble incubation mixtures end over end ≥ 1 hr (up to 24hr) at 4°C in a tube rotator.
- 4) Microcentrifuge 2 sec at 16,000 \times g (maximum speed), 4°C.
- 5) Pipet the supernatant (containing unbound antibodies) and discard it.
- 6) Add 1 ml lysis buffer and resuspend the resins by inverting the tube three or four times.
- 7) Wash twice by resuspension and centrifugation with lysis buffer.

4. Pre-clearing (optional)

Pre-clearing removes nonspecifically adsorbing material by binding to Protein A resin prepared without antibody or coupled with irrelevant (nonspecific) antibody. Irrelevant antibody is an antibody directed against an unrelated protein, and could also be whole IgG; it must not cross-react with the protein being immunoprecipitated. The following steps only use Protein A resin for pre-clearing.

- 1) Add 50–100 μ l of prepared Protein A resin suspension(50% slurry) to 1 ml cell lysate in an Eppendorf tube.
- 2) Gently mix for 1 hour at 4°C. Prolong the time if necessary.
- 3) Centrifuge at 12 000 \times g for at least 5 min. Save the supernatant.

5. Immunoprecipitation

- 1) Add 10 μ l of 10% BSA and then the clear lysate to the tube containing specific antibody bound to Protein A resin. If a nonspecific immunoprecipitation control is performed, divide lysate in two \sim 0.4-ml aliquots, one for the specific antibody and the other for the nonspecific control.



In order to avoid carryover of beads with precleared material, leave 20 to 40 μ l of supernatant on top of the pellets in the preclearing tubes. Discard beads and remaining supernatant. The BSA blocks nonspecific binding sites on the antibody-conjugated beads during incubation with the cell lysate.

- 2) Incubate 1 to 2 hr at 4°C while mixing end over end in a tube rotator.
- 3) Microcentrifuge 5 sec at 16,000 \times g, 4°C.
- 4) Pipet the supernatant (containing unbound proteins).
- 5) Add 1 ml ice-cold wash buffer, cap the tubes, and resuspend the beads by inverting the tube 3 or 4 times.
- 6) Microcentrifuge 2 sec at 16,000 \times g, 4°C.
- 7) Aspirate the supernatant, leaving 20 μ l supernatant on top of the beads.
- 8) Wash beads three more times as the above method.
- 9) Wash beads once more using 1 ml ice-cold PBS and aspirate supernatant completely with a pipet.
- 10) Analyze immunoprecipitates by one- or two-dimensional electrophoresis, or immunoblotting.

6. Dissociation and analysis

- 1) Suspend the final pellet in 30 μ l SDS-PAGE sample buffer.
- 2) Heat to 95 °C for 3 minutes.
- 3) Centrifuge at 12 000 \times g for 20 seconds to remove the resins. Carefully remove the supernatant.
- 4) Analyse the supernatant by SDS-PAGE, followed by protein staining and/or immunoblotting for detection. Radiolabelled antigens are detected by autoradiography.



Troubleshooting



No or scarce target antigen is detected.



Antigen expressed at very low levels.
Cell lysis is incomplete due to inappropriate conditions.
Epitope is not exposed in native antigen.
Antibody does not bind to denatured antigen.
Antigen is degraded during the process.
Antibody concentration is too low to precipitate antigen.
Antibody does not bind to the resin.
Problems occur in the detection assay.



Transfect cells for higher expression level by replacing the previous cell or optimization.
Adjust the components of the lysis buffer.
Use denaturing lysis buffer instead of non-denaturing buffer and vice versa.
Ensure that fresh protease inhibitor is added and perform the procedure at 4°C on ice or in a cold room.
Increase concentration of precipitating antibody.
Identify and use antibody that precipitates antigen. Otherwise, change the resin.
Check the steps of the detection assay.



High background of nonspecific bands



Incomplete washing or preclearing
Incomplete removal of detergent-insoluble proteins
Antibody contains aggregates.
Antibody solution contains nonspecific antibodies.
Too much antibody
Nonspecifically immunoprecipitated proteins



Prolong the washing time after immunoprecipitation. Increase the stringency of the washes. For example, wash with a buffer containing a higher concentration of detergent or salt. Preclear with Protein A resin or the resin coupled with irrelevant antibody. Centrifuge lysate 30min~1 hr at 100,000 × g to remove aggregated proteins before binding to the resin.



Use affinity-purified antibodies; adsorb antibody with acetone extract of cultured cells that do not express antigen; for yeast cells, adsorb antibody with null mutant cells. Use less antibody. Add saturating amount of competitive protein (i.e. BSA, gelatine, acetone powders). Fractionate cell lysate (e.g. ammonium sulfate precipitation, lectin absorption, or gel filtration) prior to immunoprecipitation; after washes in wash buffer, wash beads once with 0.1% SDS in wash buffer or 0.1% SDS/0.1% sodium deoxycholate.

Ordering Information

Product	Package	Cat. No.
rProtein A	10mg, 1g, 10g	10600-P07E
rProtein G	1mg, 5mg, 10mg	13103-PNAE
rProtein G resin	5ml, 25ml	13103-PNAE-RN
rProtein L	1mg, 5mg, 10mg	11044-H07E
rProtein L resin	5ml, 25ml	11044-H07E-RN

Ordering online:

Please visit our website at www.sinobiological.com and find the product by Cat. No. or searching the product name.

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Reference:

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