

# PURIFICATION OF PROTEINS BY PRECIPITATION

## SECTION VI

Protein precipitation refers to the formation of protein aggregates that can be isolated by centrifugation. Globular proteins can be precipitated from aqueous solutions by the addition of salt (e.g., ammonium sulfate), organic solvent (e.g., acetone), organic polymers (e.g., polyethylene glycol) or trichloroacetic acid. However, a problem that arises when using these agents with complex protein mixtures is that more than one protein will often selectively precipitate a given protein from a complex protein mixture, provided that specific antibodies directed against the protein are available.

## Immunoprecipitation

### UNIT 10.16

Immunoprecipitation consists of multiple ordered steps (Fig. 10.16.1): lysing the cell with detergent if the antigen (usually a protein) to be precipitated is membrane-bound; binding of a specific antigen to an antibody; precipitating the antibody-antigen complex; washing the precipitate; and dissociating the antigen from the immune complex. The dissociated antigen is then analyzed by electrophoretic methods (UNITS 10.2-10.4). The protocols for immunoprecipitation differ in the method of precipitation used and whether or not the antigen being precipitated is radiolabeled (UNIT 10.18).

The basic protocol details the immunoprecipitation of a radiolabeled antigen with a specific antibody (polyclonal or monoclonal) covalently linked to Sepharose. Preparation of Ab-Sepharose is described in the support protocol. The first two alternate protocols present methods for precipitating or isolating the soluble immune complexes formed between a specific antibody and a radiolabeled antigen. Immunoprecipitation is achieved with polyclonal anti-immunoglobulin (Ig) serum, anti-Ig-Sepharose, *Staphylococcus* protein A or *Streptococcus* protein G bound to Sepharose, or *Staphylococcus aureus* bacteria which contain protein A on the cell surface. The third alternate protocol should be used for immunoprecipitation of antigens that are nonspecifically associated with other proteins. The fourth alternate protocol describes immunoprecipitation of unlabeled protein antigens with Ab-Sepharose.

### IMMUNOPRECIPITATION OF RADIOLABELED ANTIGEN WITH ANTIBODY-SEPHAROSE

#### BASIC PROTOCOL

This protocol follows the steps presented in Figure 10.16.1. It relies on the formation of an insoluble immune complex between a protein antigen and an antigen-specific monoclonal (or polyclonal) antibody bound to Sepharose.

#### Materials

Surface-labeled cells (with  $^{125}\text{I}$ ) or biosynthetically  $^{35}\text{S}$ -,  $^3\text{H}$ -, or  $^{14}\text{C}$ -labeled cells (UNIT 10.18)

Lysis buffer

Dilution buffer

Antibody (Ab)-Sepharose (see support protocol)

Activated, quenched (control) Sepharose, prepared as for Ab-Sepharose (support protocol) but eliminating Ab or substituting irrelevant Ab during coupling

Tris/saline/azide (TSA) solution

0.05 M Tris-Cl, pH 6.8

2× SDS/sample buffer (UNIT 10.2)

**NOTE:** Carry out all procedures in a 4°C cold room or on ice.

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Analysis of  
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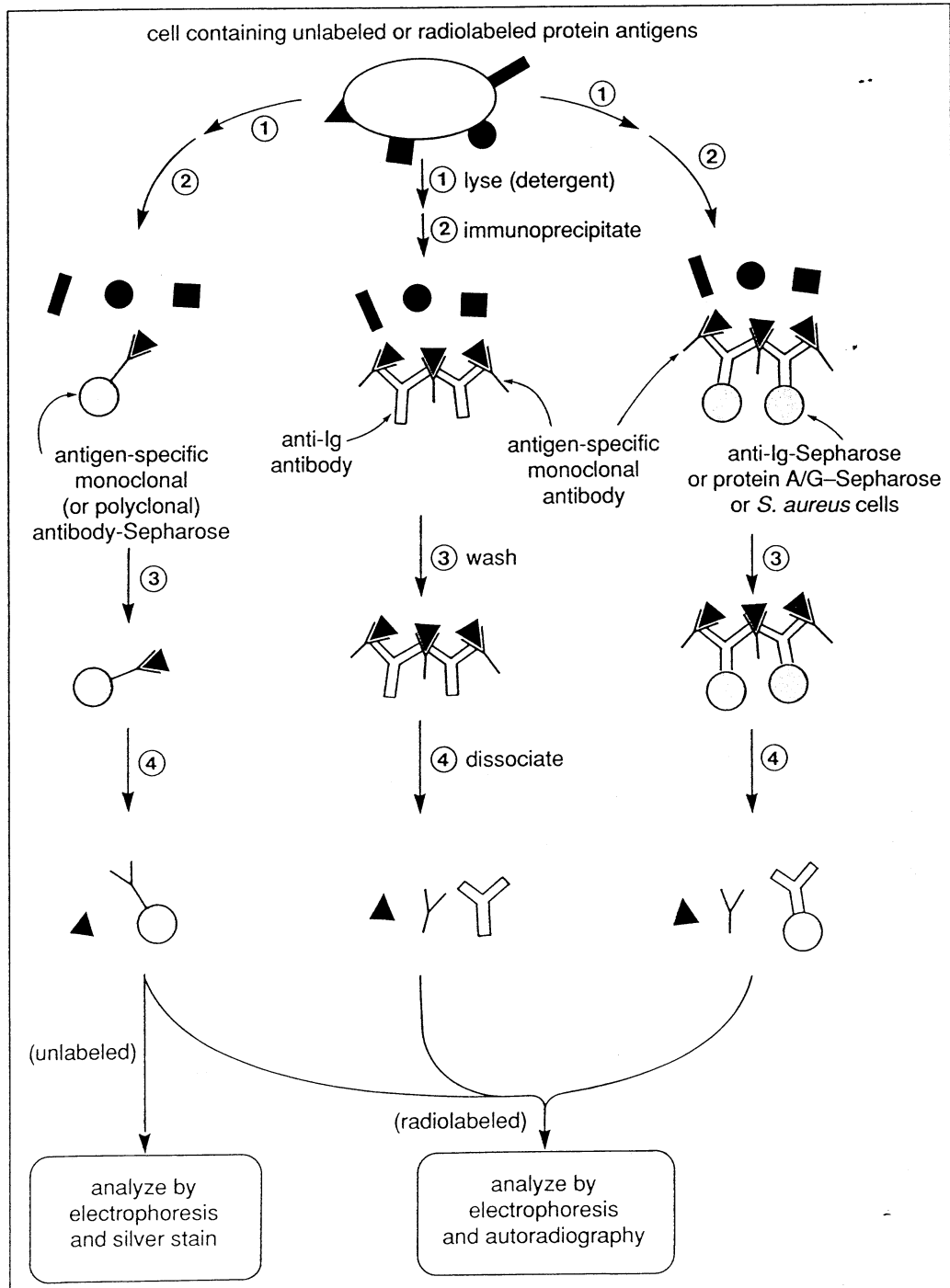
**10.16.1**

Supplement 13

**Lyse labeled cells and preclear the lysate**

1. Incubate surface- or biosynthetically-labeled cells in lysis buffer ( $5 \times 10^7$  cells/ml) for 1 hr at  $4^\circ\text{C}$ .
2. Centrifuge the lysate 10 min at  $3000 \times g$  to remove nuclei and save the supernatant.
3. Centrifuge the supernatant 1 hr at  $100,000 \times g$  and save the supernatant.

*Supernatants may also be prepared by microcentrifugation ( $10,000 \times g$ ) for 30 min. The supernatant must be used within several days or stored at  $-70^\circ\text{C}$ . The length of storage is limited by autoradiolysis and the half-life of the isotope.  $^3\text{H}$ - and  $^{14}\text{C}$ -labeled samples can often be stored frozen for years. Storage of  $^{125}\text{I}$ -labeled samples*



**Figure 10.16.1** Immunoprecipitation for the isolation of protein antigens.

is usually limited to 1 to 2 months because of autoradiolysis. The usefulness of  $^{35}\text{S}$ -labeled samples is usually limited to 6 months because of half-life. Repeated freezing and thawing may disrupt antigenic determinants and dissociate some protein complexes, especially those that are noncovalently associated.

4. Preclear supernatant to be used in one batch by adding 10  $\mu\text{l}$  activated, quenched (control) Sepharose per 200  $\mu\text{l}$  supernatant. Shake on an orbital shaker 2 hr at room temperature or overnight at 4°C. Centrifuge 1 min at 200  $\times$  g and save supernatant.

*Preclearing removes nonspecifically absorbing material. Control Sepharose can be 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.*

#### **Immunoprecipitate the antigen**

5. Precoat 1.5-ml microcentrifuge tubes by filling with lysis buffer 10 min at room temperature. Remove the solution by aspiration.

*Precoating minimizes antigen absorption to the tube.*

6. Add  $10^5$  to  $10^6$  cpm of radiolabeled ( $^{125}\text{I}$  or  $^{35}\text{S}$ ) supernatant containing antigen (from step 5) to a precoated microcentrifuge tube and bring the volume to 200  $\mu\text{l}$  with dilution buffer.

*The recommended amount of radioactivity is appropriate for eukaryotic cells with >1000 molecules of antigen/cell. It is assumed that detection on slab gels of  $^{125}\text{I}$ -labeled proteins will be carried out with enhancing screens and  $^{35}\text{S}$ -labeled proteins with fluorography.*

7. Add ~10  $\mu\text{l}$  of a 1:1 slurry of Ab-Sepharose/dilution buffer and shake 1.5 hr at 4°C on an orbital shaker.

*The antibody coupled to Sepharose is antigen-specific. As described in the following support protocol, 5 mg/ml antibody per milliliter Sepharose is coupled, and the amount actually coupled can be estimated as described in step 10 of the support protocol. Shaking must be vigorous enough to suspend the Sepharose. Shaking may be extended to 3 hr; longer periods may increase background.*

#### **Wash, dissociate, and analyze the immunoprecipitate**

8. Wash the Ab-Sepharose with 1 ml of the buffers listed below. After each wash, centrifuge 1 min at 200  $\times$  g or microcentrifuge 5 sec. Then, carefully aspirate the supernatant with a fine-tipped Pasteur pipet and leave 10  $\mu\text{l}$  of fluid above the pellet. After the fourth wash, centrifuge again to bring down any residual drops on the side of the tube, aspirate, and leave 10  $\mu\text{l}$  over the pellet.

First wash: dilution buffer

Second wash: dilution buffer

Third wash: TSA solution

Fourth wash: 0.05 M Tris-Cl, pH 6.8.

*Prepare a fine-tipped Pasteur pipet by pulling the pipet in a flame, scoring with a diamond pen, and breaking at the score.*

9. Add 20 to 50  $\mu\text{l}$  of SDS/sample buffer. Because the sample buffer has a higher density than the wash solution, it will sink into the Sepharose. Do not vortex because Sepharose may stick to side of tube above buffer level. Cap the tube securely and incubate 5 min at 100°C. Vortex and centrifuge 1 min at 200  $\times$  g or microcentrifuge 5 sec. Load the supernatant, carefully avoiding the Sepharose, into a gel lane and analyze by SDS-PAGE (UNIT 10.2). Detect labeled proteins by autoradiography (APPENDIX 3) with an enhancing screen ( $^{125}\text{I}$ ) or by fluorography ( $^{35}\text{S}$ ,  $^{14}\text{C}$ , and  $^3\text{H}$ ).

## PREPARATION OF ANTIBODY-SEPHAROSE

This protocol details the procedure for covalently linking an antibody to Sepharose (an insoluble, large-pore-size chromatographic matrix) using the cyanogen bromide activation method. It is necessary to first prepare the antibody and Sepharose separately. Next, the Sepharose is activated with cyanogen bromide (alternatively, CNBr-activated Sepharose can be purchased from Pharmacia LKB and used according to the manufacturer's instructions). Finally, the CNBr-activated Sepharose is coupled to the antibody.

### *Additional Materials*

- 1 to 30 mg/ml antigen-specific monoclonal or polyclonal antibody
- 0.1 M NaHCO<sub>3</sub>/0.5 M NaCl
- Sepharose CL-4B (or Sepharose CL-2B for high-molecular-weight antigens; Pharmacia)
- 0.2 M Na<sub>2</sub>CO<sub>3</sub>
- Cyanogen bromide (CNBr)/acetonitrile
- 1 mM and 0.1 mM HCl, ice-cold
- 0.05 M glycine (or ethanolamine), pH 8.0
- Dialysis tubing (molecular weight cutoff >10,000)
- Whatman No. 1 filter paper
- Buchner funnel
- Erlenmeyer filtration flask
- Water aspirator

### *Prepare the antibody*

1. Dialyze 1 to 30 mg/ml antibody against 0.1 M NaHCO<sub>3</sub>/0.5 M NaCl at 4°C with three buffer changes during 24 hr. Use a volume of dialysis solution that is 500 times the volume of antibody solution.

*Dialysis is performed to remove all small molecules containing free amino or sulfhydryl groups (see UNIT 10.5 for hints on dialysis membrane selection).*

2. Centrifuge 1 hr at 100,000 × g, 4°C, to remove aggregates. Save the supernatant.

*Removal of aggregates is important. Because only some of the antibody molecules in an aggregate will be directly coupled to the Sepharose, the noncoupled antibody molecules may leach out during elution.*

3. Measure the A<sub>280</sub> of an aliquot of the solution and determine the concentration of the antibody (mg/ml IgG = A<sub>280</sub>/1.44). Dilute with 0.1 M NaHCO<sub>3</sub>/0.5 M NaCl to 5 mg/ml (or to the same concentration as desired for Ab-Sepharose) and keep at 4°C. Measure the A<sub>280</sub> of this solution for later use in step 10.

### *Prepare the Sepharose*

4. Allow the Sepharose slurry to settle in a beaker and decant and discard the supernatant. Weigh out the desired quantity of Sepharose (assume density = 1.0).
5. Set up a filter apparatus using Whatman No. 1 filter paper in a Buchner funnel and an Erlenmeyer filtration flask attached to a water aspirator. Wash the Sepharose on the filter apparatus with 10 vol water.

*Sintered-glass funnels are traditionally recommended but rapidly become clogged unless coarse porosity funnels are used.*

### *Activate Sepharose with cyanogen bromide*

6. Transfer Sepharose to 50-ml beaker and add an equal volume of 0.2 M Na<sub>2</sub>CO<sub>3</sub>.

7. Activate Sepharose at room temperature using 3.2 ml CNBr/acetonitrile per 100 ml Sepharose. Add CNBr/acetonitrile dropwise with a Pasteur pipet over 1 min, while slowly stirring the slurry with a magnetic stirrer. Continue stirring slowly for 5 min.

*Excessive and vigorous stirring may fracture the Sepharose beads; this may cause flow problems during column chromatography. The protocol uses 2 g CNBr/100 ml Sepharose. Two to four grams of CNBr/100 ml Sepharose can be used to couple 1 to 20 mg of antibody/ml Sepharose.*

CAUTION: Activation should be carried out in a fume hood.

8. Rapidly filter the CNBr-activated Sepharose as in step 5. Aspirate to semidryness (i.e., until the Sepharose cake cracks and loses its sheen). Wash with 10 vol ice-cold 1 mM HCl. Wash with 2 vol of ice-cold 0.1 mM HCl. Hydrate the cake with enough ice-cold 0.1 mM HCl so the cake regains its sheen, but so there is no excess liquid above the cake.

*Washing is most efficient if the wash solution is added evenly over the surface of the cake at about the same rate as the solution is removed by filtration. CNBr-activated Sepharose is very unstable at the alkaline pH necessary for activation; it is much more stable in dilute HCl. CNBr-activated Sepharose can be purchased from Pharmacia, but the coupling capacity will be lower.*

#### **Couple antibody to CNBr-activated Sepharose**

9. Immediately transfer a weighed amount of Sepharose (assume density = 1.0) to a beaker. Add an equal volume of a solution of antibody dissolved in 0.1 M NaHCO<sub>3</sub>/0.5 M NaCl (from step 2). Stir gently with a magnetic stirrer or rotate end-over-end 2 hr at room temperature or overnight at 4°C.
10. Add 0.05 M glycine (or ethanolamine), pH 8.0, to saturate the remaining reactive groups on the Sepharose and allow the slurry to settle. Remove an aliquot of the supernatant, centrifuge to remove any residual Sepharose, and measure  $A_{280}$ . Compare absorbance to that of the  $A_{280}$  of the antibody solution from step 2 to determine the percentage coupling.
11. Store the Ab-Sepharose in TSA solution.

### **IMMUNOPRECIPITATION OF RADIOLABELED ANTIGEN WITH ANTI-Ig SERUM**

This protocol relies on the formation of soluble immune complexes between a protein and an antigen-specific antibody, followed by immunoprecipitation of the immune complexes by antibodies contained in anti-immunoglobulin (Ig) serum.

#### **Additional Materials**

Normal serum  
Anti-Ig serum (Zymed Laboratories)  
Antigen-specific antiserum *or* antigen-specific purified MAb *or* antigen-specific hybridoma culture supernatant

Follow the procedures in the basic protocol, with the following modifications at the indicated steps:

- 4a. Preclarify by adding normal serum at a concentration of 2 µl/ml radiolabeled antigen. Add the proper amount of anti-Ig serum and let stand 12 to 18 hr at 4°C. Centrifuge 10 min at 1000 × *g* and reserve supernatant.

*Normal serum is the source of carrier Ig. The proper amount of anti-Ig serum must be determined by titration with radiolabeled antigen or Ig. For high-titered anti-Ig*

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serum, this amount would be 20× to 40× the volume of antigen-specific antiserum, 2 to 4 μl/μg purified MAb, or 1/3 the volume of hybridoma culture supernatant.

- 7a. Add 1 μl antigen-specific antiserum, 3 μg antigen-specific purified MAb, or antigen-specific hybridoma culture supernatant (30 μl cloned line or 100 μl uncloned line). Vortex and allow to stand 2 hr at 4°C. Then add the proper amount of anti-Ig serum, vortex, and allow to stand 12 to 18 hr at 4°C.
- 8a. Wash the immunoprecipitate as in the basic protocol, except centrifuge 7 min at 1000 × g.
- 9a. Add 20 to 50 μl of 2× SDS/sample buffer. Do not vortex, as immunoprecipitates may stick to side of tube above buffer level. Cap the tube securely. For immunoprecipitates, first incubate 1 hr at 56°C and then 5 min at 100°C. Load the mixture into a gel lane and analyze as in step 9 of the basic protocol.

*The initial 56°C incubation enhances the dissolution of the immunoprecipitates by reducing irreversible aggregation which occurs when precipitated protein is rapidly heated to 100°C. Proteolytic degradation has never been noted, probably because of the high IgG protein concentration.*

#### ALTERNATE PROTOCOL

#### IMMUNOPRECIPITATION OF RADIOLABELED ANTIGEN WITH ANTI-Ig-SEPHAROSE, PROTEIN A- OR G-SEPHAROSE, OR *S. AUREUS* CELLS

This protocol relies on the formation of soluble immune complexes between a protein and an antigen-specific antibody, followed by immunoprecipitation of the immune complexes by anti-Ig antibodies bound to Sepharose, by *Staphylococcus aureus* protein A or *Streptococcus* protein G bound to Sepharose, or protein A present on the surface of *S. aureus* cells. Monoclonal antibodies to rat and mouse κ chains are useful coupled to Sepharose or as adjuncts with *Staphylococcus* protein A or *Streptococcus* protein G, where antibodies do not couple successfully with protein A or G. In mouse and rat, 90% of immunoglobulins have κ light chains.

##### Additional Materials

1:1 (vol/vol) anti-Ig-Sepharose/dilution buffer (coupled at 10 mg/ml Sepharose as in support protocol), 1:1 (vol/vol) protein A- or G-Sepharose (Pharmacia LKB, Calbiochem, or Sigma)/dilution buffer, or 10% suspension *S. aureus* Cowan strain II bacteria

Follow the procedures in the basic protocol (see Fig. 10.16.1), with the following modifications at the indicated steps:

- 4b. Preclarify as described below in one of the alternatives to step 7b (i, ii, or iii) as appropriate.

*Preclarifying removes nonspecific adsorbing materials.*

- 7b. Add 1 μl antigen-specific antiserum, 3 μg antigen-specific MAb, or antigen-specific hybridoma culture supernatant (30 μl cloned line, 100 μl uncloned line). Then perform (i), (ii), or (iii) below.

(i) Add a 1:1 slurry of anti-Ig-Sepharose/dilution buffer.

(ii) Add a 1:1 slurry of protein A- or G-Sepharose/dilution buffer.

For (i) and (ii) above, use an amount 20× to 40× the volume of antiserum, 2 to 4 μl/μg MAb, or 1/3 the volume of hybridoma culture supernatants. Shake 1.5 hr at 4°C.

(iii) Wash a 10% suspension *S. aureus* Cowan II bacteria in lysis buffer in a low-speed centrifuge and resuspend at 10% in dilution buffer. Add 50 μl of the 10% suspension. Shake 10 min at 4°C.

- 8b. Wash the immunoprecipitates as in basic protocol, except centrifuge slurries from (i) or (ii) (step 7b above) 1 min at  $200 \times g$  or slurry from (iii) 7 min at  $1000 \times g$ .
- 9b. Add 20 to 50  $\mu\text{l}$  of SDS/sample buffer. Do not vortex since Sepharose and bacteria stick to side of tube above buffer level. Cap the tube securely and heat 5 min at  $100^\circ\text{C}$ . Vortex, microcentrifuge, and load the supernatant into a gel lane and analyze as in step 9 of basic protocol.

### IMMUNOPRECIPITATION USING MORE STRONGLY DISSOCIATING LYSIS AND WASH BUFFERS

ALTERNATE  
PROTOCOL

This protocol should be used when protein antigens are suspected of being nonspecifically associated with other proteins after immunoprecipitation by the basic or alternate protocols.

#### *Additional Materials*

- 10% sodium deoxycholate (Na-DOC)
- 10% SDS
- RIPA buffer

1. To the supernatant obtained after step 3 of the basic protocol, add  $1/10$  vol of 10% Na-DOC and  $1/100$  vol of 10% SDS to the lysate.
2. Repeat steps 3 to 7 of the basic protocol.
3. Repeat step 8 of basic protocol, except use RIPA buffer for first and second washes.
4. Repeat step 9 of the basic protocol.

### IMMUNOPRECIPITATION OF UNLABELED ANTIGEN WITH ANTIBODY-SEPHAROSE

ALTERNATE  
PROTOCOL

Immunoprecipitation of unlabeled antigen followed by visualization with silver staining eliminates radiolabeling, one of the most tedious and expensive steps in immunoprecipitation protocols. Protein antigens present in greater than  $\sim 10^4$  copies per eukaryotic cell may be detected by immunoprecipitation of unlabeled (i.e., not radiolabeled as in the basic protocol) cell lysates with Ab-Sepharose followed by SDS-PAGE (UNIT 10.2) and silver staining (UNIT 10.6). If antigen is eluted from beads in SDS lacking reducing agents, little antibody is coeluted.

#### *Additional Materials*

- Modified lysis buffer
- 0.1% Triton X-100 in TSA solution
- SDS/sample buffer without 2-mercaptoethanol (2-ME; UNIT 10.2)

1. Incubate  $5 \times 10^7$  cells/ml in modified lysis buffer 1 hr at  $4^\circ\text{C}$ .
2. Centrifuge the lysate 15 min at  $3000 \times g$  to remove nuclei, and then centrifuge the supernatant 1 hr at  $100,000 \times g$ . Save the supernatant.  
*Supernatants may also be prepared by microcentrifuging 30 min at  $10,000 \times g$ .*
3. Preclarify the lysate with 50  $\mu\text{l}$  activated, quenched Sepharose (step 4, basic protocol) or irrelevant Ab-Sepharose/ml antigen by gently shaking 1 hr at  $4^\circ\text{C}$ . Centrifuge 5 min at  $200 \times g$  and save the supernatant.

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- Mix 25  $\mu$ l of a 1:1 slurry of Ab-Sepharose/TSA solution per ml lysate and gently shake on an orbital shaker or mix by inversion 1 hr at 4°C.

*To control for antibody eluting from the beads, a control of Ab-Sepharose incubated with mock lysate should be run simultaneously. A control of lysate incubated with irrelevant Ab-Sepharose should also be run.*

- Wash as in step 9 of the basic protocol, except use the following buffers:

First and second washes: 0.1% Triton X-100 in TSA solution

Third wash: TSA solution

Fourth wash: 0.05 M Tris-Cl, pH 6.8.

- Add 20 to 50  $\mu$ l of SDS/sample buffer without 2-ME. Do not vortex because Sepharose sticks to side of tube above buffer level. Cap tube securely and heat 5 min at 100°C. Microcentrifuge 5 sec to pellet Sepharose. Save the supernatant and apply to SDS-PAGE directly (nonreducing) or after incubation 1 hr at 37°C with 5% 2-ME (reducing). Load the mixture into a gel lane and analyze by SDS-PAGE (UNIT 10.2). Detect antigen by silver staining (UNIT 10.6).

*Elution of antigen from Sepharose should be done under nonreducing conditions since antibody eluted from the beads under reducing conditions gives background staining. Theoretically, only 1 of the 4 chains in an IgG antibody molecule is covalently linked to the Sepharose, but interchain disulfide bonds keep all the chains linked under nonreducing conditions.*

## REAGENTS AND SOLUTIONS

### *CNBr/acetoneitrile*

To 25 g of cyanogen bromide (*CNBr should be white, not yellow, crystals*), add 50 ml acetoneitrile to make a 62.5% (wt/vol) solution. This may be stored indefinitely at -20°C in a desiccator over silica. Allow to warm before opening.

CAUTION: *CNBr is a highly toxic lachrymator; handle in fume hood.*

### *Dilution buffer*

0.1% Triton X-100 (store at room temperature in dark)

0.1% bovine hemoglobin (store frozen)

Prepare in TSA solution (see below)

### *Lysis buffer*

1% Triton X-100

1% bovine hemoglobin

1 mM iodoacetamide (freshly prepared)

Aprotinin (0.2 trypsin inhibitor U/ml)

1 mM phenylmethylsulfonyl fluoride (PMSF; add fresh from 100 mM stock in absolute ethanol)

Prepare in TSA solution (see below)

### *Modified lysis buffer*

0.5% Triton X-100

1 mM PMSF

5 mM iodoacetamide

Aprotinin (0.2 trypsin inhibitor U/ml)

Prepare in TSA solution (see below)

### *RIPA buffer*

1% sodium deoxycholate

0.1% SDS

Prepare in lysis buffer (see above)

### Tris/saline/azide(TSA) solution

0.01 M Tris·Cl, pH 8.0

0.14 M NaCl

0.025% NaN<sub>3</sub>

CAUTION: Sodium azide (NaN<sub>3</sub>) is poisonous; wear gloves.

## COMMENTARY

### Background Information

Because of their high specificity, antibodies may be used to isolate specific antigens that are minor components of complex mixtures, such as cell lysates. Prior to isolation, membrane proteins must be solubilized into detergent micelles. Nonionic detergents are preferred over bile salts (see APPENDIX 1) as the latter precipitate at acid pH or in the presence of bivalent cations.

Both conventional and monoclonal antibodies may be employed for immunoprecipitation. Procedures used here are similar to those in Kürzinger and Springer (1982), Springer (1981), Ho and Springer (1983), Sanchez-Madrid et al. (1983), and Sastre et al. (1986). Monoclonal antibodies are usually nonprecipitating, as are most polyclonal antibodies at the low antigen concentrations employed. Therefore, a "sandwich" reagent is used to precipitate or isolate the antibody-antigen complex. The sandwich reagent may be anti-Ig serum, affinity-purified or monoclonal anti-Ig, *Staphylococcus* protein A or *Streptococcus* protein G coupled to Sepharose, or *S. aureus* Cowan strain II cells. Protein A methods are not generally applicable to either the rat (only its IgG1 and IgG2c subclasses bind) or the mouse (its IgG1 subclass binds too

poorly for immunoprecipitation). In many cases protein G can be substituted for protein A as a conjugate (Table 10.16.1). However, protein G-Sepharose appears to have a lower binding capacity.

The identification of immunoprecipitated, radiolabeled protein antigen is achieved by SDS-PAGE followed by detection of radiolabeled proteins by autoradiography. Nonlabeled proteins are detected by silver staining (Dustin et al., 1986).

### Critical Parameters

Anti-Ig serum gives very low backgrounds and is preferable to affinity-purified anti-Ig for immunoprecipitation. Anti-Ig precipitation gives lower backgrounds than *S. aureus* cells. Furthermore, there is no advantage to precoating *S. aureus* with antibody, since antibody-antigen complexes will bind preferentially over free antibody anyway (Kessler, 1975). Results comparable in excellence to anti-Ig serum are obtained with anti-Ig or protein A coupled to Sepharose. The use of monoclonal anti-Ig, such as anti-kappa monoclonal antibodies coupled to Sepharose, is also convenient.

The best results in terms of complete precipitation and lower backgrounds are obtained

**Table 10.16.1** Relative Affinities of Proteins A and G for Various IgG Subclasses<sup>a</sup>

Antibody	Affinity for protein A	Affinity for protein G
Human IgG1	++++	++++
Human IgG2	++++	++++
Human IgG3	—	++
Human IgG4	++++	++++
Rat IgG1	+ or ++	+
Rat IgG2a	—	++++
Rat IgG2b	—	++
Rat IgG2c	++ or ++++	++
Mouse IgG1	+	++++
Mouse IgG2a	++++	++++
Mouse IgG2b	+++	+++
Mouse IgG3	++	+++

<sup>a</sup> Adapted from Harlow and Lane (1988).

when an antigen-specific antibody is coupled directly to Sepharose. Sepharose CL-4B appears to give a lower background than CL-2B, perhaps because aggregates are better excluded.

The support protocol for coupling protein antigens to CNBr-activated Sepharose is a modification of the methods of Cuatrecasas (1970) and March et al. (1974). As originally described, the washing was done at alkaline pH. Because activated Sepharose is very unstable at this pH, it was originally recommended that washing, adding the protein ligand, and mixing be done in <90 sec (Cuatrecasas, 1970). However, by using an acid wash buffer (Gelb, 1973) as described in the support protocol, activated groups remain stable for  $\geq 30$  min. The 0.1 M NaHCO<sub>3</sub> buffer in which antibody is dissolved provides an optimal pH of ~8.4, after mixing with activated-Sepharose. The low amount of CNBr recommended is sufficient to obtain a coupling yield of 80% to >99%. Higher amounts of CNBr may result in multipoint attachment of IgG molecules to the matrix, thereby reducing accessibility to antigen. Most investigators purchase CNBr-activated Sepharose, while others, to achieve a higher coupling efficacy or to avoid the expense of the commercial product, prefer to prepare it themselves.

Quantities and ratios recommended in these protocols have been found to work well with several hundred monoclonal antibodies and more than 40 different antigens. However, titration of Ab-Sepharose or sandwich reagents versus the protein antigen may further optimize a given immunoprecipitation.

Reaction between antibody and antigen can be extended overnight, but this may increase the background when using Ab-bound Sepharose. Antigen-antibody complexes should not be left overnight in the midst of washing, as significant dissociation may occur.

In vitro translation products often appear aggregated and require pretreatment. Clear results have been obtained by incubation at 100°C in SDS followed by dilution in nonionic detergent to form mixed micelles prior to precipitation (Anderson and Blobel, 1983).

### Troubleshooting

Distortion of SDS-PAGE separations can result from loading an excessive amount of unlabeled antibody used for immunoprecipitation, since wide zones of migrating heavy and light chains "push" background radioactivity ahead of them in the gel. This can give rise to artifact bands. In the presence of SDS and

2-mercaptoethanol, all IgH and IgL chains, except the 25% actually linked to Sepharose, will be eluted from Ab-Sepharose, which leads to contamination of the sample. Anti-Ig antibodies should always be affinity purified before coupling to Sepharose to reduce the amount of nonspecific antibodies coupled.

### Anticipated Results

Provided that an monoclonal (or polyclonal) antibody directed against the protein of interest is available, it should be possible to immunoprecipitate the protein of interest from a complex mixture of proteins, using the basic or alternate protocols. Coupling yields for Ab-Sepharose are 80% to >99%.

### Time Considerations

The basic, alternate, and support protocols each require ~1 day to complete. Most of the time involves waiting for cell lysis, centrifugation, nonspecific and specific binding reactions, or chemical reactions to be completed.

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*The above references describe various detergents and solubilization conditions.*

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Contributed by Timothy A. Springer  
Center for Blood Research  
Harvard Medical School  
Boston, Massachusetts

