

# Immunoprecipitation

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Selective immunoprecipitation of proteins is a useful tool for characterizing proteins and protein-protein interactions. Clear step-by-step protocols are provided for preparing lysates of cells and yeast under a variety of conditions, for binding the antibody to a solid matrix, and for performing the actual immunoprecipitation. An additional method is provided for increasing the specificity of the technique by reprecipitating the antigen with the same or a different antibody. © 2016 by John Wiley & Sons, Inc.

Keywords: immunoprecipitation • immunoprecipitation-recapture • antigen • antibody • agarose bead • magnetic bead

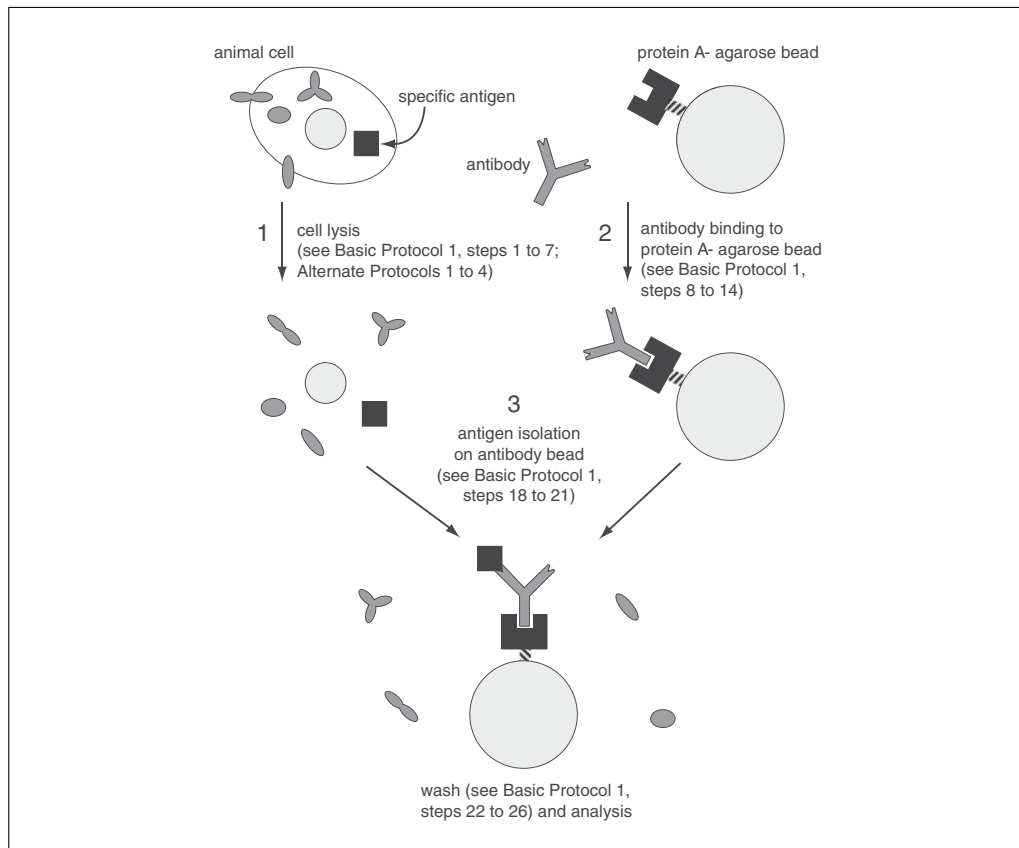
### How to cite this article:

Bonifacino, J.S., Gershlick, D.C., and Dell'Angelica, E.C. 2016. Immunoprecipitation. *Curr. Protoc. Cell Biol.* 71:7.2.1-7.2.24. doi: 10.1002/cpcb.3

Immunoprecipitation is a technique in which an antigen is isolated by binding to a specific antibody attached to a sedimentable matrix. The source of antigen for immunoprecipitation can be unlabeled cells or tissues, metabolically or extrinsically labeled cells (UNIT 7.1; Bonifacino, 1998), subcellular fractions from either unlabeled or labeled cells (see Chapter 3), or in vitro-translated proteins (UNIT 11.2; Jagus et al., 1998). Immunoprecipitation is also used to analyze protein fractions separated by other biochemical techniques such as gel filtration or sedimentation on density gradients (UNIT 5.3; Marks, 1998). Either polyclonal or monoclonal antibodies from various animal species can be used in immunoprecipitation protocols. Antibodies can be bound noncovalently to immunoadsorbents such as protein A- or protein G-agarose, or can be coupled covalently to a solid-phase matrix.

Immunoprecipitation protocols consist of several stages (Fig. 7.2.1; see Basic Protocol 1). In stage 1, the antigen is solubilized by one of several techniques for lysing cells. Soluble and membrane-associated antigens can be released from cells grown either in suspension culture (see Basic Protocol 1) or as a monolayer on tissue culture dishes (see Alternate Protocol 1) with nondenaturing detergents. Cells can also be lysed under denaturing conditions (see Alternate Protocol 2). Soluble antigens can also be extracted by mechanical disruption of cells in the absence of detergents (see Alternate Protocol 3). All of these lysis procedures are suitable for extracting antigens from animal cells. Yeast cells require disruption of their cell wall in order to allow extraction of the antigens (see Alternate Protocol 4). In stage 2, a specific antibody is bound to a solid-phase matrix such as derivatized agarose or magnetic beads to allow isolation by low-speed centrifugation or a magnet, respectively. This unit presents the use of antibodies noncovalently bound to protein A- or protein G-agarose beads (see Basic Protocol 1) or covalently conjugated to magnetic beads (see Alternate Protocol 5). Stage 3 consists of incubating the solubilized antigen from stage 1 with the immobilized antibody from stage 2, followed by extensive washing to remove unbound proteins (see Basic Protocol 1). Immunoprecipitated antigens can be dissociated from antibodies and reprecipitated by a protocol referred to as





**Figure 7.2.1** Schematic representation of the stages of a typical immunoprecipitation protocol. (1) Cell lysis: antigens are solubilized by extraction of the cells in the presence or absence of detergents. To increase specificity, the cell lysate can be precleared with protein A–agarose beads (steps 15 to 17, not shown). (2) Antibody immobilization: a specific antibody is bound to protein A–agarose beads. (3) Antigen capture: the solubilized antigen is isolated on antibody-conjugated beads.

“immunoprecipitation-recapture” (see Basic Protocol 2). This protocol can be used with the same antibody for further purification of the antigen, or with a second antibody to identify components of multisubunit complexes or to study protein-protein interactions (Fig. 7.2.2). Immunoprecipitated antigens can be analyzed by one-dimensional electrophoresis (UNIT 6.1; Gallagher, 2007), two-dimensional electrophoresis, or immunoblotting (UNIT 6.2; Gallagher et al., 2011). In some cases, immunoprecipitates can be used for structural or functional analyses of the isolated antigens. Immunoprecipitates can also be used as sources of immunogens for production of monoclonal or polyclonal antibodies.

**BASIC  
PROTOCOL 1**

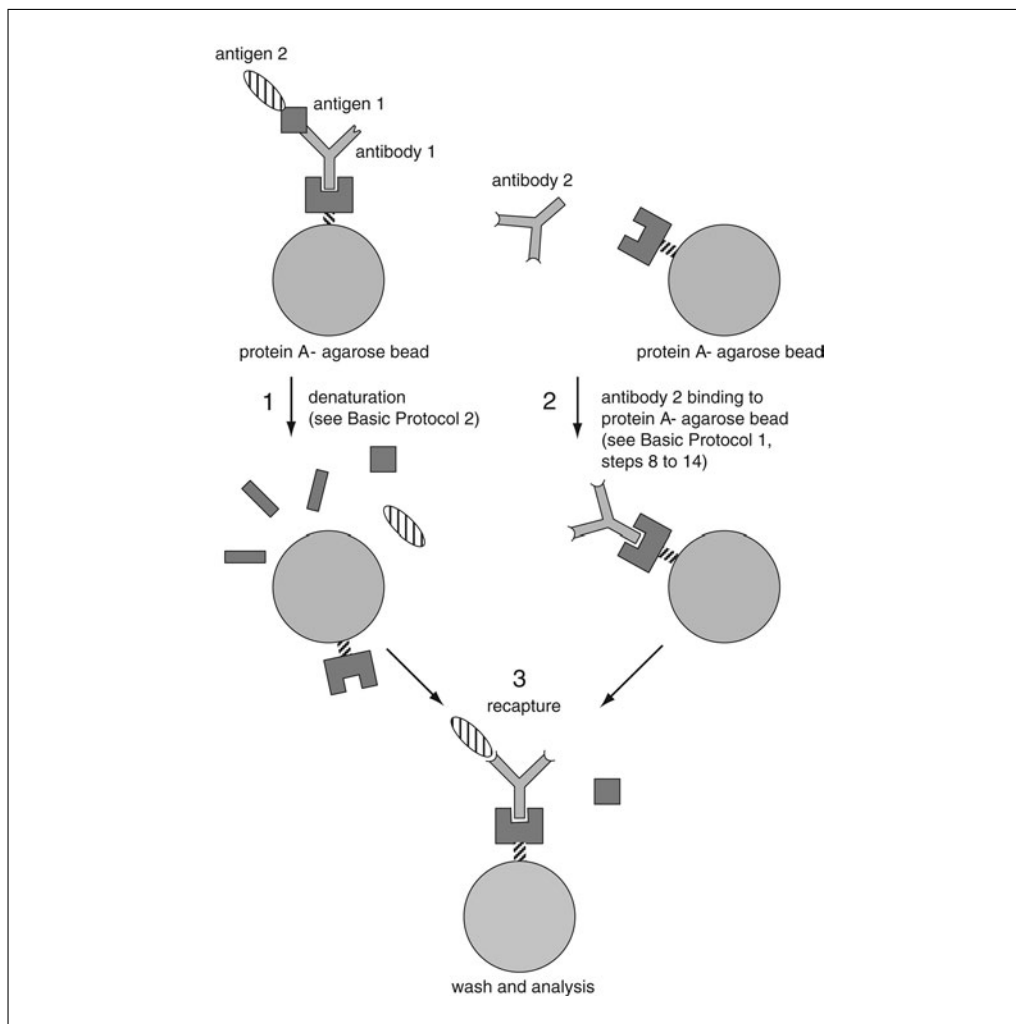
**IMMUNOPRECIPITATION USING CELLS IN SUSPENSION LYSED WITH A  
NONDENATURING DETERGENT SOLUTION**

In this protocol, unlabeled or labeled cells in suspension are extracted by incubation in nondenaturing lysis buffer containing the nonionic detergent Triton X-100 (steps 1 to 7). This procedure results in the release of both soluble and membrane proteins; however, many cytoskeletal and nuclear proteins, as well as a fraction of membrane proteins, are not efficiently extracted under these conditions (see UNIT 5.1; Petty, 1998). The procedure allows immunoprecipitation with antibodies to epitopes that are exposed in native proteins.

For immunoprecipitation, a specific antibody is immobilized on a sedimentable, solid-phase matrix (steps 8 to 14). Although there are many ways to attach antibodies to matrices (see Commentary), the most commonly used methods rely on the property of

**Immuno-  
precipitation**

**7.2.2**



**Figure 7.2.2** Scheme showing the stages of immunoprecipitation-recapture. (1) Dissociation and denaturation of the antigen: an antigen immunoprecipitated with antibody 1 bound to protein A-agarose beads is dissociated and denatured by heating in the presence of SDS and DTT. (2) Immobilization of the second antibody: antibody 2 is bound to protein A-agarose beads. (3) Recapture: the denatured antigen (striped oval) is recaptured on antibody 2 bound to protein A-agarose beads. Alternatively, antibody 1 can be used again for further purification of the original antigen (square).

immunoglobulins to bind *Staphylococcus aureus* protein A, or protein G from group G *Streptococcus* (Table 7.2.1). The best results are obtained by binding antibodies to protein A or protein G that is covalently coupled to agarose beads. In this protocol, Sepharose beads are used (Sepharose is a more stable, cross-linked form of agarose). Immunoprecipitation is most often carried out using rabbit polyclonal or mouse monoclonal antibodies, which, with some exceptions (e.g., mouse IgG1), bind well to protein A (Table 7.2.1). Antibodies that do not bind to protein A-agarose can be adsorbed to protein G-agarose (Table 7.2.1) using exactly the same protocol. For optimal time management, incubation of antibodies with protein A-agarose can be carried out either before or during lysis of the cells.

The final stage in immunoprecipitation is combining the cell lysate with the antibody-conjugated beads and isolating the antigen (steps 18 to 26). This can be preceded by an optional preclearing step in which the lysate is absorbed with either “empty” protein A-agarose beads or with an irrelevant antibody bound to protein A-agarose (steps 15 to 17). The need for preclearing depends on the specific experimental system being studied and the quality of the antibody reagents. The protocol described below incorporates a

**Table 7.2.1** Binding of Antibodies to Protein a and Protein G<sup>a,b,c</sup>

Antibody	Protein A binding	Protein G binding <sup>d</sup>
<i>Monoclonal antibodies<sup>e</sup></i>		
Human IgG1	++	++
Human IgG2	++	++
Human IgG3	–	++
Human IgG4	++	++
Mouse IgG1	+	++
Mouse IgG2a	++	++
Mouse IgG2b	++	++
Mouse IgG3	++	++
Rat IgG1	+	+
Rat IgG2a	–	++
Rat IgG2b	–	++
Rat IgG2c	++	++
<i>Polyclonal antibodies</i>		
Chicken	–	–
Donkey	–	++
Goat	+	++
Guinea pig	++	+
Hamster	+	++
Human	++	++
Monkey	++	++
Mouse	++	++
Rabbit	++	++
Rat	+	+
Sheep	+	++

<sup>a</sup> ++, moderate to strong binding; +, weak binding; –, no binding.

<sup>b</sup> A hybrid protein A/G molecule that combines the features of protein A and protein G, coupled to a solid-phase matrix, is available from Pierce.

<sup>c</sup> Information from Harlow and Lane (1988), and from Pharmacia Biotech, Pierce, and Jackson Immunoresearch.

<sup>d</sup> Native protein G binds albumin from several animal species. Recombinant variants of protein G have been engineered for better binding to rat, mouse, and guinea pig IgG, as well as for avoiding binding to serum albumin.

<sup>e</sup> Protein A binds some IgM, IgA, and IgE antibodies in addition to IgG, whereas protein G binds only IgG.

preclearing step using protein A–agarose. Protein fractions separated by techniques such as gel filtration or sedimentation on sucrose gradients (*UNIT 5.3*; Marks, 1998) can be used in place of the cell lysate at this stage. After binding the antigen to the antibody-conjugated beads, the unbound proteins are removed by successive washing and sedimentation steps.

### Materials

Unlabeled or labeled cells in suspension (*UNIT 7.1*; Bonifacino, 1998)

PBS (*APPENDIX 2A*), ice cold

Nondenaturing lysis buffer (see recipe), ice cold

50% (v/v) protein A–Sepharose bead (Sigma, Pharmacia Biotech) slurry in PBS containing 0.1% (w/v) BSA and 0.01% (w/v) sodium azide (NaN<sub>3</sub>)

Specific polyclonal antibody (antiserum or affinity-purified immunoglobulin) or monoclonal antibody (ascites, culture supernatant, or purified immunoglobulin)

Control antibody of same type as specific antibody (e.g., preimmune serum or purified irrelevant immunoglobulin for specific polyclonal antibody; irrelevant ascites, culture supernatant, or purified immunoglobulin for specific monoclonal antibody; see Critical Parameters)

10% (w/v) BSA (APPENDIX 2A)

Wash buffer (see recipe), ice cold

Microcentrifuge with fixed-angle rotor (Eppendorf 5415C or equivalent)

Tube rotator (capable of end-over-end inversion)

**CAUTION:** When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by the local radiation safety officer (also see APPENDIX 1D; Meisenhelder and Semba, 1998).

**NOTE:** All solutions should be ice cold and procedures should be carried out at 4°C or on ice.

### ***Prepare cell lysate***

1. Collect cells in suspension by centrifuging 5 min at  $400 \times g$ , 4°C, in a 15- or 50-ml capped conical tube. Place tube on ice.

*Approximately  $0.5\text{--}2 \times 10^7$  cells are required to yield 1 ml lysate, which is 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. Aspirate supernatant with a Pasteur pipet attached to a vacuum trap.

**CAUTION:** *Dispose of radioactive materials following applicable safety regulations (APPENDIX 1D; Meisenhelder and Semba, 1998).*

3. Resuspend cells gently by tapping the bottom of the tube. Rinse cells twice with ice-cold PBS as in steps 1 and 2, using the same volume of PBS as in the initial culture.

4. Add 1 ml ice-cold nondenaturing lysis buffer per  $\sim 0.5\text{--}2 \times 10^7$  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.*

5. Keep suspension on ice 15 to 30 min and transfer to a 1.5-ml conical microcentrifuge tube.

*Tubes can have flip-top or screw caps. Screw-capped tubes are preferred because they are less likely to open accidentally during subsequent procedures. They are also recommended for work with radioactivity.*

6. Clear the lysate by microcentrifuging 15 min 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 temperature 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). If it is necessary to reduce background, the lysate can be spun for 1 hr at  $100,000 \times g$  in an ultracentrifuge.*

7. Transfer the supernatant to a fresh microcentrifuge tube using an adjustable pipet fitted with a disposable tip. Do not disturb the pellet, and leave the last 20 to 40  $\mu$ l

of supernatant in the centrifuge tube. Keep the cleared lysate on ice until preclearing (step 15) or addition of antibody beads (step 18).

**IMPORTANT NOTE:** *Resuspension of even a small amount of sedimented material will result in high nonspecific background due to carryover into the immunoprecipitation steps. A cloudy layer of lipids floating on top of the supernatant will not adversely affect the results of the immunoprecipitation.*

*When the lysate is highly radioactive—as is the case for metabolically labeled cells—the use of tips with aerosol barriers is recommended to reduce the risk of contaminating internal components of the pipet.*

*Cell extracts can be frozen at  $-70^{\circ}\text{C}$  until used for immunoprecipitation. However, it is preferable to lyse the cells immediately before immunoprecipitation in order to avoid protein degradation or dissociation of protein complexes. If possible, freeze the cell pellet from step 3 rather than the supernatant from step 7.*

### **Prepare antibody-conjugated beads**

8. In a 1.5-ml conical microcentrifuge tube, combine 30  $\mu\text{l}$  of 50% protein A–Sepharose bead slurry, 0.5 ml ice-cold PBS, and the following quantity of specific antibody (select one):

1 to 5  $\mu\text{l}$  polyclonal antiserum

1  $\mu\text{g}$  affinity-purified polyclonal antibody

0.2 to 1  $\mu\text{l}$  ascitic fluid containing monoclonal antibody

1  $\mu\text{g}$  purified monoclonal antibody

20 to 100  $\mu\text{l}$  hybridoma culture supernatant containing monoclonal antibody.

*The quantities of antibody suggested are rough estimates based on the expected amount of specific antibodies in each preparation. Quantities can be increased or decreased, depending on the quality of the antibody preparation (see Commentary).*

*Substitute protein G for protein A if antibodies are of a species or subclass that does not bind to protein A (see Table 7.2.1).*

*If the same antibody will be used to immunoprecipitate multiple samples (e.g., samples from a pulse-chase experiment; UNIT 7.1; Bonifacino, 1998), the quantities indicated above can be increased proportionally to the number of samples and incubated in a 15-ml capped conical tube. In this case, the beads should be divided into aliquots just prior to the addition of the cleared cell lysate (step 18).*

*Antibody-conjugated beads can be prepared prior to preparation of the cell lysate (steps 1 to 7), in order to minimize the time that the cell extract is kept on ice.*

9. Set up a nonspecific immunoprecipitation control in a 1.5-ml conical microcentrifuge tube by incubating 30  $\mu\text{l}$  of 50% protein A–Sepharose bead slurry, 0.5 ml ice-cold PBS, and the appropriate control antibody (select one):

1 to 5  $\mu\text{l}$  preimmune serum as a control for a polyclonal antiserum

1  $\mu\text{g}$  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  $\mu\text{l}$  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  $\mu$ g purified irrelevant monoclonal antibody as a control for a purified monoclonal antibody

20 to 100  $\mu$ l hybridoma culture supernatant containing irrelevant monoclonal antibody as a control for a hybridoma culture supernatant containing specific monoclonal antibody

*The amount of irrelevant antibody should match that of the specific antibody and the antibody should be from the same species as the specific antibody.*

10. Mix suspensions thoroughly. Tumble incubation mixtures end over end  $\geq 1$  hr at 4°C in a tube rotator.

*Addition of 0.01% (w/v) Triton X-100 may facilitate mixing of the suspension during tumbling. Incubations can be carried out for as long as 24 hr. This allows preparation of the antibody-conjugated beads prior to immunoprecipitation.*

11. Microcentrifuge 2 sec at 16,000  $\times$  g (maximum speed), 4°C.
12. Aspirate the supernatant (containing unbound antibodies) using a fine-tipped Pasteur pipet connected to a vacuum aspirator.
13. Add 1 ml nondenaturing lysis buffer and resuspend the beads by inverting the tube 3 or 4 times.

*For lysates prepared with detergents (this protocol and see Alternate Protocols 1 and 2), use 1 ml nondenaturing lysis buffer; for lysates prepared by mechanical disruption (see Alternate Protocol 3), use detergent-free lysis buffer (see recipe).*

*Use of a repeat pipettor is recommended when processing multiple samples.*

14. Wash by repeating steps 11 to 13, and then steps 11 and 12 once more.

*At this point the beads have been washed twice with lysis buffer and are ready to be used for immunoprecipitation. Antibody-bound beads can be stored up to 6 hr at 4°C until used.*

#### ***Preclear lysate (optional)***

15. In a microcentrifuge tube, combine 1 ml cell lysate (from step 7) and 30  $\mu$ l of 50% protein A–Sepharose bead slurry.

*The purpose of this step is to remove from the lysate proteins that bind to protein A–Sepharose, as well as pieces of insoluble material that may have been carried over from previous steps. If the lysate was prepared from cells expressing immunoglobulins—such as spleen cells or cultured B cells—the preclearing step should be repeated at least 3 times to ensure complete removal of endogenous immunoglobulins.*

*If cell lysates were frozen and thawed, they should be microcentrifuged 15 min at 16,000  $\times$  g (maximum speed), 4°C, before the preclearing step.*

16. Tumble end over end 30 min at 4°C in a tube rotator.
17. Microcentrifuge 5 min at 16,000  $\times$  g (maximum speed), 4°C.

#### ***Immunoprecipitate***

18. Add 10  $\mu$ l of 10% BSA to the tube containing specific antibody bound to protein A–Sepharose beads (step 14), and transfer to this tube the entire volume of cleared lysate (from step 7 or 17). 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  $\mu$ l of supernatant on top of the pellets in the preclearing tubes. Discard beads and remaining*

supernatant. The BSA quenches nonspecific binding to the antibody-conjugated beads during incubation with the cell lysate.

19. Incubate 1 to 2 hr at 4°C while mixing end over end in a tube rotator.

*Samples can be incubated overnight, although there is an increased risk of protein degradation, dissociation of multiprotein complexes, or formation of protein aggregates.*

20. Microcentrifuge 5 sec at 16,000 × g (maximum speed), 4°C.

21. Aspirate the supernatant (containing unbound proteins) using a fine-tipped Pasteur pipet connected to a vacuum aspirator.

*The supernatant can be kept up to 8 hr at 4°C or up to 1 month at –70°C for sequential immunoprecipitation of other antigens or for analysis of total proteins. To reuse lysate, remove the supernatant carefully with an adjustable pipet fitted with a disposable tip. Before reprecipitation, preabsorb the lysate with protein A–Sepharose (as in steps 15 to 17) to remove antibodies that may have dissociated during the first immunoprecipitation.*

CAUTION: Dispose of radioactive materials following applicable safety regulations.

22. Add 1 ml ice-cold wash buffer, cap the tubes, and resuspend the beads by inverting the tube 3 or 4 times.

*Use of a repeat pipettor is recommended when processing multiple samples.*

23. Microcentrifuge 2 sec at 16,000 × g (maximum speed), 4°C.

24. Aspirate the supernatant, leaving ~20 µl supernatant on top of the beads.

25. Wash beads three more times (steps 22 to 24).

*Total wash time (steps 22 to 26) should be ~30 min, keeping the samples on ice for 3 to 5 min between washes if necessary (see Critical Parameters).*

26. Wash beads once more using 1 ml ice-cold PBS and aspirate supernatant completely with a drawn-out Pasteur pipet.

*The final product should be 15 µl of settled beads containing bound antigen.*

*Immunoprecipitates can either be processed immediately or frozen at –20°C for later analysis. For subsequent analysis of the isolated proteins prior to electrophoresis (e.g., comparison of the electrophoretic mobility of the antigen with or without treatment with glycosidases), samples can be divided into two or more aliquots after addition of PBS. Transfer aliquots of the bead suspension to fresh tubes, centrifuge and aspirate as in the previous steps. Immunoprecipitates can be analyzed by one-dimensional electrophoresis (UNIT 6.1; Gallagher, 2007), two-dimensional electrophoresis, or immunoblotting (UNIT 6.2; Gallagher et al., 2011).*

## ALTERNATE PROTOCOL 1

## IMMUNOPRECIPITATION USING ADHERENT CELLS LYSED WITH A NONDENATURING DETERGENT SOLUTION

Immunoprecipitation using adherent cells can be performed in the same manner as with nonadherent cells (see Basic Protocol 1). This protocol is essentially similar to steps 1 to 5 of Basic Protocol 1, but describes modifications necessary for using the same nondenaturing detergent solution to lyse cells attached to tissue culture plates. It is preferable to use cells grown on plates rather than in flasks, because the cell monolayer is more easily accessible.

*Additional Materials (also see Basic Protocol 1)*

Unlabeled or labeled cells grown as a monolayer on a tissue culture plate (UNIT 7.1; Bonifacino, 1998)



*NOTE:* All solutions should be ice cold and procedures should be carried out at 4°C or on ice.

1. Rinse cells attached to a tissue culture plate twice with ice-cold PBS. Remove the PBS by aspiration with a Pasteur pipet attached to a vacuum trap.

*CAUTION: Dispose of radioactive materials following applicable safety regulations.*

2. Place the tissue culture plate on ice.
3. Add ice-cold nondenaturing lysis buffer to the tissue culture plate.

*Use 1 ml lysis buffer for an 80% to 90% confluent 100-mm-diameter tissue culture plate. Depending on the cell type, a confluent 100-mm dish will contain  $0.5\text{--}2 \times 10^7$  cells. For other plate sizes, adjust volume of lysis buffer according to the surface area of the plate.*

4. Scrape the cells off the plate with a rubber policeman, and transfer the suspension to a 1.5-ml conical microcentrifuge tube using an adjustable pipettor fitted with a disposable tip. Vortex gently for 3 sec and keep tubes on ice for 15 to 30 min.

*Tubes can have flip-top or screw caps. Screw-capped tubes are preferred because they are less likely to open accidentally during subsequent procedures. They are also recommended for work with radioactivity.*

5. Clear the lysate and perform immunoprecipitation (see Basic Protocol 1, steps 6 to 26).

#### **IMMUNOPRECIPITATION USING CELLS LYSED WITH DETERGENT UNDER DENATURING CONDITIONS**

#### **ALTERNATE PROTOCOL 2**

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. This protocol is based on that for nondenaturing conditions (see Basic Protocol 1, steps 1 to 7), with the following modifications. 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. Prior to immunoprecipitation, the denatured protein extract is diluted 10-fold with nondenaturing lysis buffer, which contains Triton X-100; this step protects the antigen-antibody interaction from interference by the ionic detergent. Immunoprecipitation is performed as described (see Basic Protocol 1).

The following protocol is described for cells in suspension culture, although it can be adapted for adherent cells (see Alternate Protocol 1). Only antibodies that react with denatured proteins can be used to immunoprecipitate proteins solubilized by this protocol.

##### ***Additional Materials*** (also see Basic Protocol 1)

Denaturing lysis buffer (see recipe)  
Heating block set at 95°C (Eppendorf Thermomixer 5436 or equivalent)  
25-G needle attached to 1-ml syringe

1. Collect cells in suspension culture (see Basic Protocol 1, steps 1 to 3). Place tubes on ice.
2. Add 100  $\mu$ l 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.*

#### **Protein Labeling and Immuno- precipitation**

#### **7.2.9**

*Tubes can have flip-top or screw caps. Screw-capped tubes are preferred because they are less likely to open accidentally during subsequent procedures. They are also recommended for work with radioactivity.*

4. Heat samples 5 min at 95°C in a heating block.
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 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 (see Basic Protocol 1, steps 6 to 26).

### **ALTERNATE PROTOCOL 3**

#### **IMMUNOPRECIPITATION USING CELLS LYSED WITHOUT DETERGENT**

Immunoprecipitation of proteins that are already soluble within cells (e.g., cytosolic or luminal organellar proteins) may not require the use of detergents. Instead, cells can be mechanically disrupted by repeated passage through a needle, and soluble proteins can be separated from insoluble material by centrifugation. The following protocol describes lysis of cells in a PBS-based detergent-free lysis buffer. Other buffer formulations may be used for specific proteins.

#### *Additional Materials (also see Basic Protocol 1)*

Detergent-free lysis buffer (see recipe)  
25-G needle attached to 3-ml syringe

**NOTE:** All solutions should be ice-cold and procedures should be carried out at 4°C or on ice.

1. Collect and wash cells in suspension (see Basic Protocol 1, steps 1 to 3).
2. Add 1 ml of ice-cold detergent-free lysis buffer per  $\sim 0.5\text{--}2 \times 10^7$  cells in a pellet.
3. Resuspend the cells by gentle agitation for 3 sec with a vortex mixer set at medium speed.
4. Break cells by passing the suspension 15 to 20 times through a 25-G needle attached to a 3-ml syringe.

*Extrusion of the cell suspension from the syringe should be rapid, although care should be exercised to prevent splashing and excessive foaming. Cell breakage can be checked under a bright-field or phase-contrast microscope. Repeat procedure until >90% cells are broken.*

*It is helpful to check ahead of time whether the cells can be broken in this way. If the cells are particularly resistant to mechanical breakage, they can be swollen for 10 min at 4°C with a hypotonic solution containing 10 mM Tris·Cl, pH 7.4 (APPENDIX 2A) before mechanical disruption.*

5. Clear the lysate and perform immunoprecipitation (Basic Protocol 1, steps 6 to 26).

## IMMUNOPRECIPITATION USING YEAST CELLS DISRUPTED WITH GLASS BEADS

## ALTERNATE PROTOCOL 4

Unlike animal cells, yeast cells have an extremely resistant, detergent-insoluble cell wall. To allow extraction of cellular antigens, the cell wall needs to be broken by mechanical, enzymatic, or chemical means. The most commonly used procedure consists of vigorous vortexing of the yeast suspension with glass beads. The breakage can be done in the presence or absence of detergent, as previously described for animal cells (see Basic Protocol 1, Alternate Protocol 2, and Alternate Protocol 3). The protocol described below is suitable for mechanical disruption of most yeast species, including *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. A protocol for metabolic labeling for yeast has been described by Franzusoff et al. (1991).

### *Additional Materials (also see Basic Protocol 1)*

Unlabeled or radiolabeled yeast cells

Lysis buffer, ice cold: nondenaturing, denaturing, or detergent-free lysis buffer (see recipes)

Glass beads (acid-washed, 425- to 600- $\mu\text{m}$  diameter; Sigma)

**NOTE:** All solutions should be ice-cold and procedures should be carried out at 4°C or on ice.

1. Collect 10 ml of yeast culture at 1 OD<sub>600</sub> per immunoprecipitation sample, and centrifuge 5 min at 4000  $\times$  g, 4°C. Place tube on ice.
2. Remove supernatant by aspiration with a Pasteur pipet attached to a vacuum trap.

*CAUTION: Dispose of radioactive materials following applicable safety regulations.*

3. Loosen pellet by vortexing vigorously for 10 sec. Rinse cells twice with ice-cold distilled water as in steps 1 and 2.

*Radiolabeled yeast cells are likely to have been pelleted earlier as part of the labeling procedure. If the pellets are frozen, they should be thawed on ice prior to cell disruption.*

4. Add 3 vol ice-cold lysis buffer and 3 vol at 1 OD<sub>600</sub> glass beads per volume of pelleted yeast cells.

*Use nondenaturing lysis buffer or detergent-free lysis buffer as required for the antigen under study. If the experiment requires denaturation of the antigen, the procedure can be adapted (see Alternate Protocol 2 for higher eukaryotic cells); however, the yeast cells must be broken with glass beads before heating the sample at 95°C.*

5. Shake cells by vortexing vigorously at maximum speed for four 30-sec periods, keeping the cells on ice for 30 sec between the periods.

*Check cell breakage under a bright-field or phase-contrast microscope. It is helpful to check ahead of time if the cells can be broken in this way.*

6. Remove the yeast cell lysate from the beads using a pipettor with a disposable tip. Transfer to a fresh tube.
7. Add 4 vol (see step 4) lysis buffer to the glass beads, vortex for 2 sec, and combine this supernatant with the lysate from step 6.
8. Clear the lysate and perform immunoprecipitation (see Basic Protocol 1, steps 6 to 26).

## **IMMUNOPRECIPITATION OF A TAGGED PROTEIN USING ANTIBODY-CONJUGATED MAGNETIC BEADS**

In recent years, there have been significant advances in the quality of commercially available reagents for immunoprecipitation. These include a variety of primary antibodies covalently conjugated to agarose or magnetic beads. These reagents are particularly useful for immunoprecipitation of transgenically expressed proteins appended with epitope or protein tags (e.g., FLAG, HA or Myc epitopes, GFP), and for subsequent identification of co-precipitated proteins by mass spectrometry. For this latter purpose, cell lysis is performed under nondenaturing detergent conditions as in Basic Protocol 1. As controls, it is best to use cells transfected with a plasmid encoding an unrelated tagged protein, although cells transfected with a plasmid encoding only the tag or untransfected cells can also be used. Additionally, some companies sell unconjugated beads that can be used for control experiments and for pre-clearing the cell lysate if desired. The following protocol describes the use of an antibody to GFP covalently coupled to magnetic beads to immunoprecipitate a GFP-tagged protein expressed by transfection.

### **Materials**

Cells expressing the tagged protein of interest, as well control cells, plated in 100-mm-diameter cell culture dish  
PBS (*APPENDIX 2A*), ice cold  
Co-immunoprecipitation lysis buffer (see recipe), ice cold  
Dilution buffer: wash buffer (see recipe) supplemented with protease inhibitors (e.g., Roche Complete Mini, EDTA-free, 1 tablet per 10 ml buffer), ice cold  
Magnetic beads conjugated to antibody against GFP or epitope tag of interest (e.g., GFP-Trap-M; Cromotek; usually provided as a slurry with concentrations ranging from ~3 to 10 mg/ml, depending on the manufacturer)  
Wash buffer (see recipe), ice cold  
1× SDS-PAGE sample buffer (*UNIT 6.1*; Gallagher, 2007)

Rubber policeman  
Round-bottomed 2-ml microcentrifuge tubes and 1.5-ml conical-bottom microcentrifuge tubes (Eppendorf)  
Microcentrifuge with fixed-angle rotor (Eppendorf 5415 C or equivalent)  
Magnetic separation rack  
Tube rotator (capable of end-over-end inversion)  
95°C heat block

**NOTE:** Before starting the protocol, carefully read the product sheet for the magnetic beads to check for any specific adjustments recommended by the manufacturer.

1. Remove culture medium from 100-mm-diameter dish containing cells expressing protein of interest, as well as any relevant control dishes. Place dishes on ice.

*Cells can be either stably or transiently transfected. The size of the culture dish can be varied, but 100-mm diameter is a good starting point.*

2. Wash cells by adding 5 ml ice-cold PBS to the dish using a repeat pipettor. After gentle swirling, remove wash by aspiration with a Pasteur pipet attached to a vacuum trap. Repeat wash twice.

*Unless otherwise stated, all solutions from this point on should be ice-cold.*

3. Add 500  $\mu$ l co-immunoprecipitation lysis buffer and scrape cells from plate using a rubber policeman. Transfer the suspension to a 1.5-ml conical microcentrifuge tube using a repeat pipettor fitted with a disposable tip.
4. Vortex suspension gently for 3 sec and incubate for 30 min on ice.

5. Microcentrifuge for 15 min at maximum speed, 4°C, to pellet cell debris.
6. Transfer supernatant to a fresh, 2-ml round-bottomed microcentrifuge tube on ice, carefully avoiding the cell pellet.

*It is preferable, but not essential, to use round-bottomed tubes, as it is easier to avoid disturbing the magnetic beads when removing supernatant in later steps.*

7. Add 500  $\mu$ l ice-cold dilution buffer to each tube from step 6 and mix by gentle, but thorough, up-and-down pipetting.
8. Transfer 100  $\mu$ l of cell lysate from step 7 into a 1.5-ml conical microcentrifuge tube.
9. Mix 1.5 ml of co-immunoprecipitation lysis buffer with 1.5 ml dilution buffer per sample in a 15-ml conical centrifuge tube. For each sample, aliquot 1 ml into a 2-ml round-bottomed microcentrifuge tube for equilibration of the beads.

*This is considered the input and should be loaded next to the immunoprecipitate when performing an immunoblot assay.*

10. Add 50  $\mu$ l of the magnetic bead suspension to the equilibration buffer from step 9 and immediately place into magnetic separation rack. Leave for 1 min, or until the supernatant is clear, before removing the supernatant. Add 1 ml of wash buffer and repeat the magnetic separation to wash the beads. Repeat the wash procedure for a second wash.

*The amount of beads added here should be determined as per the supplier's instructions, commonly 20 to 50  $\mu$ l of the supplied suspension (depending on the manufacturer). If information is not available,  $\sim$ 0.2 mg of the bead suspension is a good starting point.*

*It is important not to let the beads dry on the side of the tube proximal to the magnetic separation rack, to avoid aggregation and clumping.*

11. After the final wash of the beads, add the cell lysate left over from step 8 and resuspend the beads with gentle pipetting.
12. Incubate for 1 to 2 hr at 4°C on a end-over-end tube rotator.
13. Separate beads and sample on a magnetic separation rack for 1 min or until the supernatant is clear. Remove and keep the supernatant.

*The supernatant can be considered the unbound fraction and is useful for tracking the fate of the protein of interest when troubleshooting. This can be stored at  $-80^{\circ}\text{C}$ .*

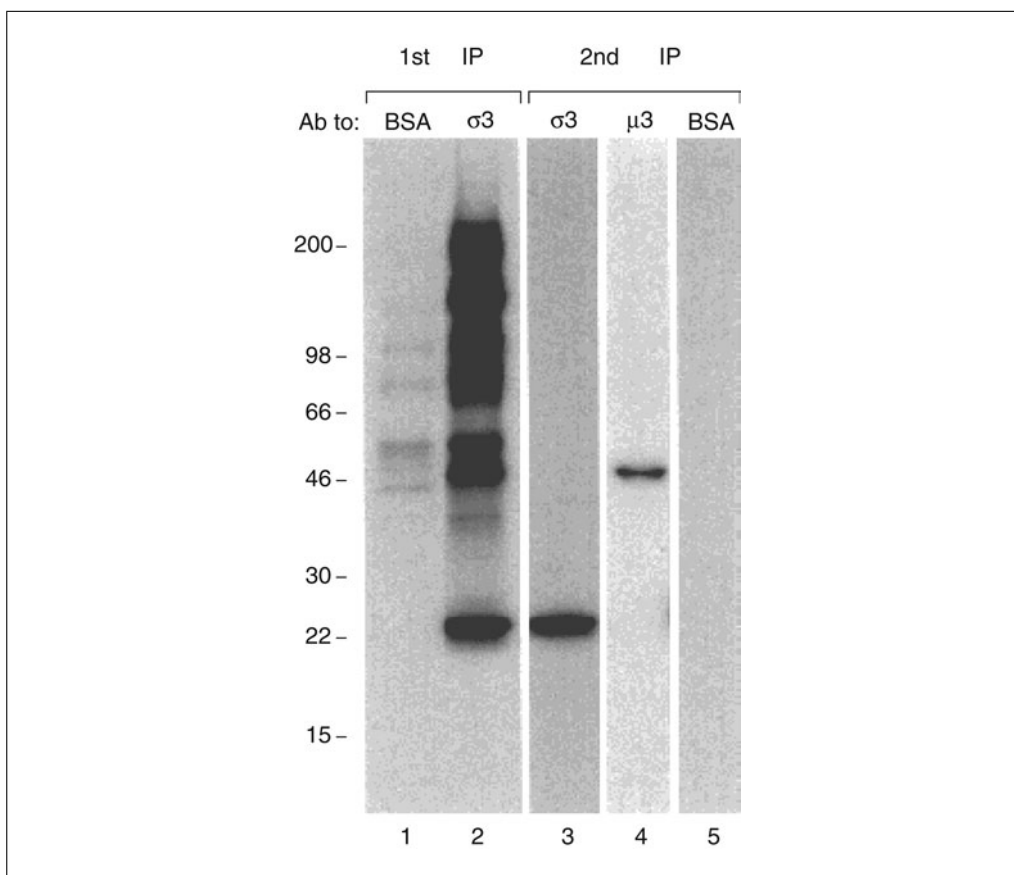
14. Resuspend beads in 1 ml wash buffer and separate on magnetic rack for 1 min or until the supernatant is clear. Repeat wash twice with wash buffer and one final time with PBS.
15. After final separation, add 100  $\mu$ l of room temperature 1  $\times$  SDS-PAGE sample buffer to the beads, and vortex for 3 sec.
16. Heat sample to 95°C for 5 min, and centrifuge 1 min at 5,000  $\times$  g, room temperature, to pellet beads and condensate. Resuspend the beads by pipetting up and down, and finally separate on the magnetic rack for 1 min. Transfer the supernatant to a fresh into 1.5-ml conical microcentrifuge tube.

*Immunoprecipitates can either be processed immediately or frozen at  $-20^{\circ}\text{C}$  for later analysis. Immunoprecipitates can be analyzed by one-dimensional electrophoresis (UNIT 6.1; Gallagher, 2007), two-dimensional electrophoresis (UNIT 6.4; Harper et al., 1999), or, most commonly, immunoblotting (UNIT 6.2; Gallagher et al., 2011).*

**IMMUNOPRECIPITATION-RECAPTURE**

Once an antigen has been isolated by immunoprecipitation, it can be dissociated from the beads and reimmunoprecipitated (“recaptured”) with either the same antibody used in the first immunoprecipitation or with a different antibody (Fig. 7.2.2). Immunoprecipitation-recapture with the same antibody allows identification of a specific antigen in cases where the first immunoprecipitation contains too many bands to allow unambiguous identification. By using a different antibody in the second immunoprecipitation, immunoprecipitation-recapture can be used to analyze the subunit composition of multi-protein complexes (Fig. 7.2.3). The feasibility of this approach depends on the ability of the second antibody to recognize denatured antigens.

Dissociation of the antigen from the beads is achieved by denaturation of antigen-antibody-bead complexes at high temperature in the presence of SDS and DTT. Prior to recapture, the SDS is diluted in a solution containing Triton X-100, and the DTT is neutralized with excess iodoacetamide. Recapture is then performed as in the first immunoprecipitation (see Basic Protocol 1, step 26).



**Figure 7.2.3** Example of an immunoprecipitation-recapture experiment. Human M1 fibroblasts were labeled overnight with [<sup>35</sup>S]methionine (UNIT 7.1; Bonifacino, 1998) and extracted with non-denaturing lysis buffer (see Basic Protocol 1). The cell extract was then subjected to immunoprecipitation with antibodies to BSA (irrelevant antibody control; lane 1) and to the AP-3 adaptor (σ3; lane 2), a protein complex involved in protein sorting. Notice the presence of several specific bands in lane 2. The AP-3 immunoprecipitate was denatured as described in Basic Protocol 2 and individual components of the AP-3 complex were recaptured with antibodies to two of its subunits: σ3 ( $M_r \sim 22,000$ ; lane 3) and μ3 ( $M_r \sim 47,000$ ; lane 4). An immunoprecipitation with an antibody to BSA was also performed as a nonspecific control (lane 5). The amount of immunoprecipitate loaded on lanes 1 and 2 is  $\sim 1/10$  the amount loaded on lanes 3 to 5. Notice the presence of single bands in lanes 3 and 4. The positions of  $M_r$  standards (expressed as  $10^{-3} \times M_r$ ) are shown at left. IP, immunoprecipitation.

## Materials

Elution buffer (see recipe)

Beads containing bound antigen (see Basic Protocol 1, step 26)

10% (w/v) BSA (APPENDIX 2A)

Nondenaturing lysis buffer (see recipe)

Heating block set at 95°C (Eppendorf Thermomixer 5436 or equivalent)

1. Add 50 µl elution buffer to 15 µl beads containing bound antigen. Mix by vortexing.

*The DTT in the elution buffer reduces disulfide bonds in the antigen and the antibody, and the SDS contributes to the unfolding of polypeptide chains.*

2. Incubate 5 min at room temperature and 5 min at 95°C in a heating block. Cool tubes to room temperature.

3. Add 10 µl of 10% BSA. Mix by gentle vortexing.

*BSA is added to prevent adsorption of antigen to the tube, and to quench nonspecific binding to antibody-conjugated beads.*

4. Add 1 ml nondenaturing lysis buffer.

*The iodoacetamide in the nondenaturing lysis buffer reacts with the DTT and prevents it from reducing the antibody used in the recapture steps. The presence of PMSF and leupeptin in the buffer is not necessary at this step.*

5. Incubate 10 min at room temperature.

6. Clear the lysate and perform second immunoprecipitation (see Basic Protocol 1, steps 6 to 26).

## REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A.

### Co-immunoprecipitation lysis buffer

0.5% (w/v) Triton X-100

50 mM Tris·Cl, pH 7.4 (APPENDIX 2A)

300 mM NaCl

5 mM EDTA (APPENDIX 2A)

0.02% (w/v) sodium azide

Store up to 6 months at 4°C

Immediately before use, add protease inhibitor tablets (e.g., Roche Complete, Mini, EDTA-free: 1 tablet per 10 ml buffer)

### Denaturing lysis buffer

1% (w/v) SDS

50 mM Tris·Cl, pH 7.4 (APPENDIX 2A)

5 mM EDTA (APPENDIX 2A)

Store up to 1 week at room temperature (SDS precipitates at 4°C)

Add the following fresh before use:

10 mM dithiothreitol (DTT, from powder)

1 mM PMSF (APPENDIX 2A)

2 µg/ml leupeptin (store 10 mg/ml stock in H<sub>2</sub>O up to 6 months at –20°C)

15 U/ml DNase I (store 15,000 U/ml stock solution up to 2 years at –20°C)

*1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), added fresh from a 0.1 M stock solution in H<sub>2</sub>O, can be used in place of PMSF. AEBSF stock can be stored up to 1 year at –20°C.*

### ***Detergent-free lysis buffer***

*PBS* (APPENDIX 2A) containing:

5 mM EDTA (APPENDIX 2A)

0.02% (w/v) sodium azide

Store up to 6 months at 4°C

Immediately before use add:

10 mM iodoacetamide (from powder)

1 mM PMSF (APPENDIX 2A)

2 µg/ml leupeptin (store 10 mg/ml stock in H<sub>2</sub>O up to 6 months at –20°C)

*1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), added fresh from a 0.1 M stock solution in H<sub>2</sub>O, can be used in place of PMSF. AEBSF stock can be stored up to 1 year at –20°C.*

### ***Elution buffer***

1% (w/v) SDS

100 mM Tris·Cl, pH 7.4 (APPENDIX 2A)

Store up to 1 week at room temperature

10 mM dithiothreitol (DTT, add fresh from powder before use)

### ***Nondenaturing lysis buffer***

1% (w/v) Triton X-100

50 mM Tris·Cl, pH 7.4 (APPENDIX 2A)

300 mM NaCl

5 mM EDTA (APPENDIX 2A)

0.02% (w/v) sodium azide

Store up to 6 months at 4°C

Immediately before use add:

10 mM iodoacetamide (from powder)

1 mM PMSF (APPENDIX 2A)

2 µg/ml leupeptin (store 10 mg/ml stock in H<sub>2</sub>O up to 6 months at –20°C)

*1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), added fresh from a 0.1 M stock solution in H<sub>2</sub>O, can be used in place of PMSF. AEBSF stock can be stored up to 1 year at –20°C.*

### ***Wash buffer***

0.1% (w/v) Triton X-100

50 mM Tris·Cl, pH 7.4 (APPENDIX 2A)

300 mM NaCl

5 mM EDTA (APPENDIX 2A)

0.02% (w/v) sodium azide

Store up to 6 months at 4°C

## **COMMENTARY**

### **Background Information**

The use of antibodies for immunoprecipitation has its origin in the *precipitin* reaction (Nisonoff, 1984). The term precipitin refers to the spontaneous precipitation of antigen-antibody complexes formed by interaction of certain polyclonal antibodies with their antigens. The precipitation arises from formation of large networks of antigen-antibody complexes, due to the bivalent or polyvalent nature of immunoglobulins and to the presence of two or more epitopes in some antigens. This

phenomenon was quickly exploited to isolate antigens from protein mixtures; however, its use remained limited to antibodies and antigens that were capable of multivalent interaction. In addition, the efficiency of precipitate formation was highly dependent on the concentrations of antibody and antigen. Thus, the precipitin reaction was not generally applicable as a method for immunoprecipitation.

A significant improvement was the use of secondary anti-immunoglobulin reagents (generally anti-immunoglobulin serum) to



cross-link the primary antibodies, thus promoting the formation of a precipitating network. Protocols based on the use of cross-linking secondary antibodies are still used in immunoprecipitation and are reputed to give very low backgrounds (Springer, 1996).

In the 1970s, immunoprecipitation became widely applicable to the study of cellular antigens as a result of several technological advances. A critical development was the introduction of methods for the production of monoclonal antibodies (Köhler and Milstein, 1975). The ability to produce unlimited amounts of antibodies with specificity against virtually any cellular antigen had a profound impact in many areas of biology and medicine. The fact that preparation of monoclonal antibodies did not require prior purification of the antigens accelerated the characterization of cellular proteins and organelles, a process in which immunoprecipitation protocols played a major role. To this day, monoclonal antibodies produced in mice or rats continue to be among the most useful tools in cell biology.

Another important development was the discovery of bacterial Fc receptors, proteins found on the surface of bacteria that have the property of binding a wide range of immunoglobulins. Two of the most widely used bacterial Fc receptors are protein A from *Staphylococcus aureus* and protein G from group G streptococci. Protein A and protein G bind both polyclonal and monoclonal antibodies belonging to different subclasses and from different animal species (Table 7.2.1). Protein A was initially used to adsorb immunoglobulins as part of fixed, killed *Staphylococcus aureus* particles. Both protein A and protein G are now produced in large quantities by recombinant DNA procedures and are available coupled to solid-phase matrices such as agarose. In most cases, the binding of polyclonal or monoclonal antibodies to immobilized protein A (or G) avoids the need to use a secondary antibody to precipitate antigen-antibody complexes. Because of their broad specificity and ease of use, protein A-agarose and protein G-agarose (and related products) are the state-of-the-art reagents for the isolation of soluble antigen-antibody complexes in immunoprecipitation protocols.

In the 30 years between 1970 and 2001, there were about 90,000 mentions of “immunoprecipitation” in literature databases. In the 15 years since 2001 (when this protocol was originally published), there have been over 90,000 further mentions. Immunoprecipitation has become a standard technique to

discover and validate protein-protein interactions. Although the fundamental principle has stayed the same, there are now many more high-quality commercially available reagents. For performing immunoprecipitations using proteins modified with common tags (e.g., FLAG, Myc, HA, GFP), many commercial suppliers now produce antibodies that are pre-conjugated to agarose or other matrices. Use of these pre-conjugated antibody beads can make the experiment faster and more reliable. Care needs to be taken in designing appropriate negative controls, as overexpressed tagged proteins can sometimes cause nonspecific interactions. For this reason, it is important to have control samples expressing an unrelated tagged protein or the tag alone (e.g., GFP).

Another addition to the portfolio of reagents available commercially are superparamagnetic beads as an alternative solid-phase matrix. These magnetic nanoparticles are used, in general principle, in the same way as agarose. For bead-supernatant separation, however, a magnetic rack is used instead of centrifugation. The advantage of magnetic particles over traditional matrices is two-fold. First, the method of separation allows total removal of buffer from the tube, preventing either buffer carry-through or accidental loss of beads. Second, magnetic particles are significantly less porous than traditional substrates, allowing faster washes. Although agarose beads have increased binding due to their porous nature, this is offset by the comparatively small size of the magnetic beads (0.5 to 10  $\mu\text{m}$ ; note that the beads range in size from 0.5 to 10  $\mu\text{m}$ ) relative to agarose beads (50 to 100  $\mu\text{m}$ ). In addition, magnetic beads are more suited to large-throughput automated approaches.

Antibody engineering with recombinant production (Rapley, 1995; Irving et al., 1996; Hollinger and Hudson, 1998) and non-antibody (or antibody-derived) binding reagents (Gebauer et al., 2009) have been employed in order to try and avoid the expense and inherent variability of using animals to produce antibodies (Baker et al., 2015). Antibody fragments with high affinity for specific antigens can now be selected from phage display antibody libraries. Selected recombinant antibodies can then be produced in large quantities in *Escherichia coli*. Techniques have been developed for producing antibodies in soluble, secreted form. Affinity tags are added to the recombinant antibody molecules to facilitate purification, detection, and use in procedures such as immunoprecipitation. While

attractive in principle, the production of recombinant antibodies has been plagued by technical difficulties that so far have limited their widespread use in cell biology. However, as technical problems are overcome, recombinant techniques will progressively replace immunization of animals as a way of producing antibodies for immunoprecipitation and for other applications.

In 1993, a novel type of antibody, consisting of only a heavy chain (termed HCAb), was discovered to occur in camelids (e.g., camels and llamas) (Hamers-Casterman et al, 1993; reviewed in Muyldermans et al., 2013). The single-chain HCAb comprises two structural domains (CH2, CH3) and one epitope-recognition domain (VHH). Interaction screens of VHH gene segments expressed as phage-display libraries from either immunized or naïve animal libraries can be used to isolate antibody probes to any given epitope, allowing subsequent cloning and recombinant expression. These recombinant VHH domains are called nanobodies; they are much smaller than conventional antibodies (~15kDa) and exhibit increased stability in solution.

Nanobodies covalently immobilized on both agarose and magnetic beads have recently become commercially available. Nanobodies have several advantages over traditional antibodies for immunoprecipitation. Their small size allows coating of solid-phase matrices at a higher density. Furthermore, direct conjugation to a matrix makes them less prone to dissociation from the beads during the elution of the immunoprecipitate. Finally, as the nanobody is derived from a camelid, antibodies from mouse, rabbit, or sheep can be used to probe the blots without interference from the immunoprecipitating antibody.

## Critical Parameters

### *Extraction of antigens*

Isolation of cellular antigens by immunoprecipitation requires extraction of the cells so that the antigens are available for binding to specific antibodies, and are in a physical form that allows separation from other cellular components. Extraction with nondenaturing detergents such as Triton X-100 (see Basic Protocol 1 and Alternate Protocol 1) or in the absence of detergent (see Alternate Protocol 3) allows immunoprecipitation with antibodies to epitopes that are exposed on native proteins. Other nondenaturing detergents such as Nonidet P-40, CHAPS, digitonin, or octyl glucoside are also appropriate for extrac-

tion of native proteins (*UNIT 5.1*; Petty, 1998). Some of these detergents (e.g., digitonin) preserve weak protein-protein interactions better than Triton X-100. If the antigen is part of a complex that is insoluble in nondenaturing detergents (e.g., cytoskeletal structures, chromatin, membrane “rafts”) or if the epitope is hidden within the folded structure of the protein, extraction under denaturing conditions is indicated (see Alternate Protocol 2).

The number of cells necessary to detect an immunoprecipitated antigen depends on the cellular abundance of the antigen and on the efficiency of radiolabeling. The protocols for radiolabeling (*UNIT 7.1*; Bonifacino, 1998) and immunoprecipitation described in this book are appropriate for detection of antigens that are present at low to moderate levels (10,000 to 100,000 copies per cell), as is the case for most endogenous integral membrane proteins, signal transduction proteins, and transcription factors. For more abundant antigens, such as cytoskeletal and secretory proteins or proteins that are expressed by viral infection or transfection, the quantity of radiolabeled cells used in the immunoprecipitation can be reduced accordingly.

### *Production of antibodies*

Immunoprecipitation can be carried out using either polyclonal or monoclonal antibodies (see discussion of selection below). Polyclonal antibodies are most often prepared by immunizing rabbits, although polyclonal antibodies produced in mice, guinea pigs, goats, sheep, and other animals, are also suitable for immunoprecipitation. Antigens used for polyclonal antibody production can be whole proteins purified from cells or tissues, or can be whole or partial proteins produced in bacteria or insect cells by recombinant DNA procedures. Another useful procedure is to immunize animals with peptides conjugated to a carrier protein. Production of polyclonal antibodies to recombinant proteins and peptides has become the most commonly used approach to obtain specific probes for immunoprecipitation and other immunochemical techniques, because it does not require purification of protein antigens from their native sources. The only requirement for making these antibodies is knowledge of the sequence of a protein, information which is now relatively easy to obtain as a result of cDNA and genomic DNA sequencing projects. Polyclonal antibodies can be used for immunoprecipitation as whole serum, ammonium sulfate-precipitated immunoglobulin fractions, or affinity-purified

immunoglobulins. Although all of these forms are suitable for immunoprecipitation, affinity-purified antibodies often give lower backgrounds and are more specific.

Most monoclonal antibodies are produced in mice or rats. The sources of antigen for monoclonal antibody production are the same as those for production of polyclonal antibodies, namely, proteins isolated from cells or tissues, recombinant proteins or protein fragments, and peptides. A significant advantage of monoclonal antibodies is that antigens do not need to be purified to serve as immunogens, as long as the screening method is specific for the antigen. Another advantage is the unlimited supply of monoclonal antibodies afforded by the ability to grow hybridomas in culture or in ascitic fluid. Many monoclonal antibodies can now be produced from hybridomas deposited in cell banks or are directly available from companies. Ascitic fluid, cell culture supernatant, and purified antibodies are all suitable sources of monoclonal antibodies for immunoprecipitation. Ascitic fluid and purified antibodies should be used when a high antibody titer is important. Cell culture supernatants have lower antibody titers, but tend to give cleaner immunoprecipitations than ascitic fluids due to the lack of contaminating antibodies.

#### ***Selection of antibodies: Polyclonal versus monoclonal***

What type of antibody is best for immunoprecipitation? There is no simple answer to this question, as the outcome of both polyclonal and monoclonal antibody production protocols is still difficult to predict. Polyclonal antibodies to whole proteins (native or recombinant) have the advantage that they frequently recognize multiple epitopes on the target antigen, enabling them to generate large, multivalent immune complexes. Formation of these antigen-antibody networks enhances the avidity of the interactions and increases the efficiency of immunoprecipitation. Because these antibodies recognize several epitopes, there is a better chance that at least one epitope will be exposed on the surface of a solubilized protein and thus be available for interaction with antibodies. Thus, the likelihood of success is higher. These properties can be a disadvantage, though, as some polyvalent antibodies can cross-react with epitopes on other proteins, resulting in higher backgrounds and possible misidentification of antigens. By being directed to a short peptide sequence, anti-peptide polyclonal antibodies are less likely to cross-react with other proteins. However, their

usefulness is dependent on whether the chosen sequence turns out to be a good immunogen in practice, as well as on whether this particular epitope is available for interaction with the antibody under the conditions used for immunoprecipitation.

Unfractionated antisera are often suitable for immunoprecipitation. However, there is a risk that serum proteins other than the antibody will bind nonspecifically to the immunoadsorbent, and in turn bind proteins in the lysate that are unrelated to the antigen. For instance, transferrin can bind nonspecifically to immunoadsorbents, potentially leading to the isolation of the transferrin receptor as a contaminant (Harford, 1984). Polyclonal antisera can also contain antibodies to other antigens (e.g., viruses, bacteria) to which the animal may have been exposed, and these antibodies can also cross-react with cellular proteins during immunoprecipitation. Affinity-purified antibodies are a better alternative when antisera do not yield clean immunoprecipitations. Affinity-purification can lead to loss of high-affinity or low-affinity antibodies; however, the higher specificity of affinity-purified antibodies generally makes them “cleaner” reagents for immunoprecipitation.

The specificity, high titer, and limitless supply of the best immunoprecipitating monoclonal antibodies are unmatched by those of polyclonal antibodies. However, not all monoclonal antibodies are useful for immunoprecipitation. Low-affinity monoclonal antibodies can perform acceptably in immunofluorescence microscopy protocols (*UNIT 4.3*; Donaldson, 2015) but may not be capable of holding on to the antigen during the repeated washes required in immunoprecipitation protocols. The use of ascitic fluid has the same potential pitfalls as the use of polyclonal antisera, as 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, an informed empirical approach is recommended in order to select the best antibody for immunoprecipitation. In general, it is advisable to generate and/or test several antibodies to a particular antigen in order to find at least one that will perform well in immunoprecipitation protocols.

#### ***Antibody titer***

The importance of using the right amount of antibody for immunoprecipitation cannot be overemphasized. This is especially the case for quantitative immunoprecipitation studies,

in which the antibody should be in excess of the specific antigen. For instance, in pulse-chase analyses of protein degradation or secretion (*UNIT 7.1*; Bonifacino, 1998), it is critical to use sufficient antibody to deplete the antigen from the cell lysate. This is particularly important for antigens that are expressed at high levels, a common occurrence with the growing use of high-yield protein expression systems such as vaccinia virus or replicating plasmids in COS cells. Consider for example a protein that is expressed at high levels inside the cell, and of which only a small fraction is secreted into the medium. If limiting amounts of antibody are used in a pulse-chase analysis of this protein, the proportion of protein secreted into the medium will be grossly overestimated, because the limiting antibody will bind only a small proportion of the cell-associated protein and a much higher proportion of the secreted protein. The same considerations apply to degradation studies. Thus, it is extremely important in quantitative studies to ensure that the antibody is in excess of the antigen in the cell samples. This can be ascertained by performing sequential immunoprecipitations of the samples (see Basic Protocol 1, annotation to step 21). If the second immunoprecipitation yields only a small amount of the antigen relative to that isolated in the first immunoprecipitation (<10%), then the antibody titer is appropriate. If, on the other hand, the amount of antigen isolated in the second immunoprecipitation is >10%, either more antibody or less antigen should be used.

Too much antibody can also be a problem, as nonspecific immunoprecipitation tends to increase with increasing amounts of immunoglobulins bound to the beads. Thus, titration of the antibody used for immunoprecipitation is strongly advised.

#### **Immuno-adsorbent**

If cost is not an overriding issue, the use of protein A- or protein G-agarose is recommended for routine immunoprecipitation. Protein A- or protein G-agarose beads (or equivalent products) have a very high capacity for antibody binding (up to 10 to 20 mg of antibody per milliliter of gel). Both protein A and protein G bind a wide range of immunoglobulins (Table 7.2.1). Backgrounds from nonspecifically bound proteins are generally low. Protein A- and protein G-agarose beads are also stable and easy to sediment by low-speed centrifugation. A potential disadvantage, in addition to their cost, is that some polyclonal or monoclonal antibodies

bind weakly or not at all to protein A or protein G (Table 7.2.1). This problem can be solved by using an intermediate rabbit antibody to the immunoglobulin of interest. For example, a goat polyclonal antibody can be indirectly bound to protein A-agarose by first incubating the protein A-agarose beads with a rabbit anti-goat immunoglobulin, and then incubating the beads with the goat polyclonal antibody. Anti-immunoglobulin antibodies (e.g., rabbit anti-goat immunoglobulins) coupled covalently to agarose can also be used for indirect immunoprecipitation in place of protein A- or protein G-agarose.

Fixed *Staphylococcus aureus* particles (Pansorbin) can be used as a less expensive alternative to protein A-agarose. They have a lower capacity, can give higher backgrounds, and take longer to sediment. However, they work quite well in many cases. In order to establish if they are appropriate for a particular experimental setup, conduct a preliminary comparison of the efficiency of protein A-agarose with *Staphylococcus aureus* particles as immuno-adsorbent.

Specific antibodies coupled covalently to various affinity matrices can also be used for direct immunoprecipitation of antigens. After binding to protein A-agarose, antibodies can be cross-linked with dimethylpimelimidate (Gersten and Marchalonis, 1978). Purified antibodies can also be coupled directly to derivatized matrices such as CNBr-activated Sepharose (Springer, 1996). This latter approach avoids having to bind the antibody to protein A-agarose. Covalently bound antibodies should be used when elution of immunoglobulins from the beads complicates further analyses of the complexes. This is the case when proteins in immunoprecipitates are analyzed by one- or two-dimensional gel electrophoresis (*UNIT 6.1*; Gallagher, 2007) followed by Coomassie blue or silver staining, or are used for microsequencing. Also, the released immunoglobulins could interfere with detection of some antigens by immunoblotting (*UNIT 6.2*; Gallagher et al., 2011) following immunoprecipitation.

#### **Nonspecific controls**

For correct interpretation of immunoprecipitation results, it is critical to include appropriate nonspecific controls along with the specific samples. One type of control consists of setting up an incubation with an irrelevant antibody in the same biochemical form as the experimental antibody (e.g., serum, ascites, affinity-purified immunoglobulin,

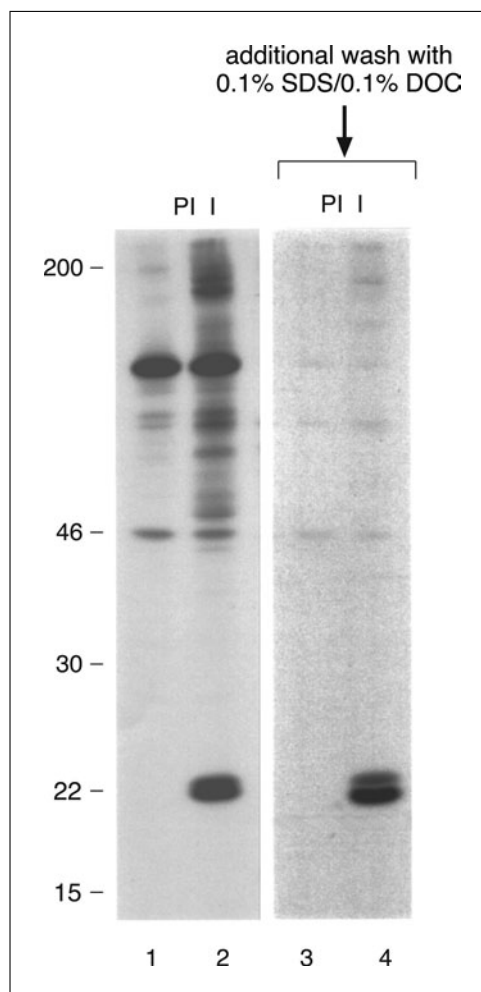
antibody bound to protein A–agarose or directly conjugated to agarose), and belonging to the same species and immunoglobulin subclass as the experimental antibody (e.g., rabbit antiserum, mouse IgG2a). For an antiserum, the best control is preimmune serum (serum from the same animal obtained before immunization). Nonimmune serum from the same species is an acceptable substitute for preimmune serum in some cases. “No-antibody” controls are not appropriate because they do not account for nonspecific binding of proteins to immunoglobulins. In immunoprecipitation–recapture experiments, control immunoprecipitations with irrelevant antibodies should be performed for both the first and second immunoprecipitation steps (Fig. 7.2.3). Another type of control is to perform an immunoprecipitation from cells that do not express a specific antigen in parallel with immunoprecipitation of the antigen-expressing cells. For instance, untransfected cells are a perfect control for transfected cells. In yeast cells, null mutants that do not express a specific antigen are an ideal control for wild-type cells.

#### Order of stages

In the immunoprecipitation protocols described in this unit, the antibody is prebound to protein A–agarose before addition to the cell lysate containing the antigen. This differs from other protocols in which the free antibody is first added to the lysate and the antigen–antibody complexes are then collected by addition of the immunoabsorbent. Although both procedures can give good results, the authors prefer the protocols described here because this method allows better control of the amount of antibody bound to the immunoabsorbent. Prebinding antibodies to the immunoabsorbent beads allows removal of unbound antibodies. The presence of unbound antibodies in the incubation mixture could otherwise result in decreased recovery of the antigen on the immunoabsorbent beads. Another advantage of the prebinding procedure is that most proteins other than the immunoglobulin in the antibody sample (e.g., serum proteins) are removed from the beads and do not come in contact with the cell lysate. This eliminates potential adverse effects of these proteins on isolation of the antigen.

#### Washing

The five washes described (see Basic Protocol 1; four with wash buffer and one with PBS) are sufficient for maximal removal of unbound proteins; additional washes are un-



**Figure 7.2.4** Lowering background by washing with SDS and sodium deoxycholate (DOC). In this experiment, BW5147 cells (mouse thymoma) labeled with [<sup>35</sup>S]methionine for 1 hr were extracted with nondenaturing lysis buffer (see Basic Protocol 1). The extracts were subjected to immunoprecipitation with protein A–agarose beads incubated with either preimmune (PI) or immune (I) serum from a rabbit immunized with the ribosomal protein L17 (doublet at  $M_r \sim 22,000$ ). Lanes 1 and 2 correspond to immunoprecipitates obtained using the protocols described in this unit. Notice the presence of nonspecific bands and/or associated proteins in lane 2. Lanes 3 and 4 correspond to beads that were washed an additional time with 0.1% (w/v) SDS and 0.1% (w/v) DOC. Notice the disappearance of most of the nonspecific bands and/or associated proteins. The positions of  $M_r$  standards (expressed as  $10^{-3} \times M_r$ ) are shown at left.

likely to decrease the background any further. The last wash with PBS removes the Triton X-100 that can lead to decreased resolution on SDS-PAGE. It also removes other components of the wash buffer that could interfere with enzymatic treatment of immunoprecipitates.

**Table 7.2.2** Troubleshooting Guide for Immunoprecipitation

Problem	Cause	Solution
<i>No specific radiolabeled antigen band</i>		
Gel is completely blank after prolonged autoradiographic exposure	Poorly labeled cells: too little radiolabeled precursor, too few cells labeled, lysis/loss of cells during labeling, too much cold amino acid in labeling mix, wrong labeling temperature	Check incorporation of label by TCA precipitation ( <i>UNIT 7.1</i> ; Bonifacino, 1998) troubleshoot the labeling procedure
Only nonspecific bands present	Antigen does not contain the amino acid used for labeling	Label cells with another radiolabeled amino acid, or for glycoproteins, with tritiated sugar
	Antigen expressed at very low levels	Substitute cells known to express higher levels of antigens as detected by other methods transfect cells for higher expression
	Protein has high turnover rate and is not well labeled by long-term labeling	Use pulse labeling
	Protein has a low turnover rate and is not well labeled by short-term labeling	Use long-term labeling
	Protein is not extracted by lysis buffer used to solubilize cells	Solublize with a different nondenaturing detergent or under denaturing conditions
	Antigen is not extracted with Triton X-100 at 4°C	Extract with Triton X-100 at 37°C or use another detergent
	Antibody is nonprecipitating	Identify and use antibody that precipitates antigen
	Epitope is not exposed in native antigen	Extract cells under denaturing conditions
	Antibody does not recognize denatured antigen	Extract cells under nondenaturing conditions
	Antibody does not bind to immunoabsorbent	Use a different immunoabsorbent (Table 7.2.1) use intermediate antibody
	Antigen is degraded during immunoprecipitation	Ensure that fresh protease inhibitors are present
<i>High background of nonspecific bands</i>		
Isolated lanes on gel with high background	Random carryover of detergent-insoluble proteins	Remove supernatant immediately after centrifugation, leaving a small amount with pellet if resuspension occurs, recentrifuge
High background in all lanes	Incomplete washing	Cap tubes and invert several times during washes
	Poorly radiolabeled protein	Optimize duration of labeling to maximize signal-to-noise
	Incomplete removal of detergent-insoluble proteins	Centrifuge lysate 1 hr at 100,000 × g
	Insufficient unlabeled protein to quench nonspecific binding	Increase concentration of BSA
	Antibody contains aggregates	Microcentrifuge antibody 15 min at maximum speed before binding to beads
	Antibody solution contains nonspecific antibodies	Use affinity-purified antibodies absorb antibody with acetone extract of cultured cells that do not express antigen for yeast cells, absorb antibody with null mutant cells

*continued*

**Table 7.2.2** Troubleshooting Guide for Immunoprecipitation, *continued*

Problem	Cause	Solution
	Too much antibody	Use less antibody
	Incomplete preclearing	Preclear with irrelevant antibody of same species of origin and immunoglobulin subclass bound to immunoadsorbent
	Nonspecifically immunoprecipitated proteins	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
<i>Immunoprecipitating antibody detected in immunoblots</i>		
Complete immunoglobulin or heavy and/or light chains visible in immunoblot	Protein A conjugate or secondary antibody recognizes immunoprecipitating antibody	Use antibody coupled covalently to solid-phase matrix for immunoprecipitation probe blots with primary antibody from a different species and the appropriate secondary antibody specific for immunoblotting primary antibody

It is not advisable to complete all the washes quickly (e.g., in 5 min), because this may not allow enough time for included proteins to diffuse out of the gel matrix. Instead, beads should be washed over ~30 min, which may require keeping the samples on ice for periods of 3 to 5 min between washes. In order to reduce nonspecific bands, samples can be subjected to an additional wash with wash buffer containing 0.1% (w/v) SDS, or with a mixture of 0.1% (w/v) SDS and 0.1% (w/v) sodium deoxycholate (Fig. 7.2.4). This wash should be done between the fourth wash and the wash with PBS.

### Troubleshooting

Two of the most common problems encountered in immunoprecipitation of metabolically labeled proteins are failure to detect specific antigens in the immunoprecipitates, and high background of nonspecifically bound proteins for antigens that were radiolabeled *in vivo* and analyzed by SDS-PAGE (UNIT 6.1; Gallagher, 2007) followed by autoradiography or fluorography (UNIT 6.3; Voytas and Ke, 1998). When immunoprecipitates are analyzed by immunoblotting (UNIT 6.2; Gallagher et al., 2011), an additional problem may be the detection of immunoprecipitating antibody bands in the blots (Table 7.2.2).

### Anticipated Results

For antigens that are present at >10,000 copies per cell, the radiolabeling and immunoprecipitation protocols described in this

book would be expected to result in the detection of one or more bands corresponding to the specific antigen and associated proteins in the electrophoretograms. Specific bands should not be present in control immunoprecipitations done with irrelevant antibodies. If antigens are labeled with [<sup>35</sup>S]methionine (UNIT 7.1; Bonifacino, 1998), specific bands should be visible within 2 hr to 2 months of exposure. Due to the relatively low yield of the immunoprecipitation-recapture procedure (<10% of that of a single immunoprecipitation step), detection of specific bands is likely to require longer exposure times. This may turn out to be problematic due to the radioactive decay of <sup>35</sup>S (half-life = 88 days). In immunoprecipitation-recapture experiments in which the antibodies used for the first and second immunoprecipitation steps recognize different antigens (e.g., for the study of protein-protein interactions; Fig. 7.2.2), it is advisable to include in the second immunoprecipitation step a positive control with either the antibody used for the first immunoprecipitation (if the antibody recognizes both the native and denatured forms of the antigen) or a different antibody with specificity for the same antigen (Fig. 7.2.3, lane 3).

### Time Considerations

Preparation of cell extracts (Basic Protocol 1 and Alternate Protocols 1 to 4) takes 1 to 3 hr to complete, and isolation of the antigen on antibody-conjugated beads takes 2 to 3 hr. Binding antibodies to

immunoabsorbent beads can be done prior to or simultaneously with preparation of the cell extracts and also takes 1 to 3 hr. Therefore, the whole immunoprecipitation procedure can be completed in 1 day. Immunoprecipitates can be analyzed immediately (e.g., resolved by SDS-PAGE) or frozen and analyzed another day. Immunoprecipitation-recapture experiments (Basic Protocol 2) require an additional 1 to 2 hr to denature and prepare the antigen for immunoprecipitation, and 2 to 3 hr to isolate the antigen. Completion of an entire immunoprecipitation-recapture experiment requires a very long workday. Alternatively, samples can be frozen after the first immunoprecipitation, and the elution and recapture can be carried out another day.

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