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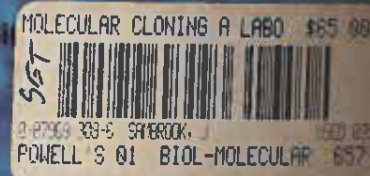
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A LABORATORY MANUAL

SECOND EDITION

J. Sambrook

UNIVERSITY OF TEXAS SOUTHWESTERN MEDICAL CENTER

E.F. Fritsch

GENETICS INSTITUTE

T. Maniatis

HARVARD UNIVERSITY

Associate Author

Nina Irwin

Managing Editor

Nancy Ford

Editor

Chris Nolan

Associate Editor

Michele Ferguson

Illustrator

Michael Ockler



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Lysis of Cells

This is perhaps the most crucial step in immunoprecipitation. The aim is to find a method that will solubilize all of the target antigen in a form that is immunoreactive, undegraded, and, for some purposes, biologically active. In view of the wide range of physical and biological properties of mammalian proteins, it is not surprising that no single method of lysis is sufficient for every purpose. Among the variables that have been found to influence the efficiency of solubilization and subsequent immunoprecipitation of proteins are the ionic strength and pH of the lysis buffer; the concentration and type of detergent used; and the presence of divalent cations, cofactors, and stabilizing ligands.

Although there are exceptions, many soluble nuclear and cytoplasmic proteins can be solubilized by lysis buffers that contain the nonionic detergent Nonidet P-40 (NP-40) and either no salt at all or relatively high concentrations of salt (e.g., 0.5 M NaCl). However, the efficiency of extraction is often greatly affected by the pH of the buffer and the presence or absence of chelating agents such as EDTA and EGTA. Extraction of membrane-bound and hydrophobic proteins is less affected by the ionic strength of the lysis buffer but often requires a mixture of ionic and nonionic detergents.

When attempting to solubilize a protein for the first time, there are two different strategies that can be employed. At one extreme, harsh conditions can be used in an effort to ensure that the protein is released quantitatively from the cells; however, this may result in loss of immunoreactivity. At the other extreme, gentle conditions can be used to help preserve the protein in a native state; however, this may result in inefficient extraction of the protein from the cells. Which of these approaches should be used is determined in large part by the properties of the antiserum available for immunoprecipitation. For example, as discussed earlier in this chapter, monospecific antisera raised against synthetic peptides may react only with denatured forms of the target protein, whereas monoclonal antibodies directed against native epitopes may be specific for the correctly folded form of the protein. To minimize problems, try to use polyclonal antisera or mixtures of monoclonal antibodies that react with all forms of the protein. It is usually then possible to tailor the extraction conditions to fit the characteristics of the target protein rather than the properties of the available antisera.

Many methods of solubilization, particularly those that involve mechanical disruption of cells, release intracellular proteases that can digest the target protein. The susceptibility of different proteins to attack by proteases varies widely, with cell-surface and secreted proteins generally being more resistant than intracellular proteins. Denatured proteins are much more likely to be degraded than native proteins. It is therefore advisable to take steps to minimize proteolytic activity in cell extracts, especially when harsh conditions of extraction are used. It is important to keep the extracts cold (i.e., at 0°C or below, depending on the sensitivity of the target protein to freezing and thawing). In addition, inhibitors of proteases are commonly included in lysis buffers (Table 18.1). For additional information about inhibitors of proteases, see Barrett and Salvesen (1986).

TABLE 18.1 Properties of Commonly Used Protease Inhibitors

Inhibitor	Active against	Inactive against	Effective concentration	Stock solution
Aprotinin ^a (Trasylol)	kallikrein trypsin chymotrypsin plasmin	papain	1–2 µg/ml	10 mg/ml in 0.01 M HEPES (pH 8.0)
Leupeptins (supplied as a 3:1 mixture of propionyl and acetyl derivatives)	plasmin trypsin papain cathepsin B	chymotrypsin pepsin cathepsins A and D	1–2 µg/ml	10 mg/ml in water
Pepstatin A	pepsin cathepsin D	trypsin plasmin chymotrypsin elastase thermolysin	1 µg/ml	1 mg/ml in ethanol
Antipain	cathepsins A and B papain trypsin	plasmin chymotrypsin pepsin	1–2 µg/ml	1 mg/ml in water
PMSF ^b (phenylmethylsulfonyl fluoride)	chymotrypsin trypsin		100 µg/ml	1.74 mg/ml (10 mM) in isopropanol
TLCK (tosyllysine chloromethyl ketone)	trypsin	chymotrypsin	50 µg/ml	1 mg/ml in 0.05 M sodium acetate (pH 5.0)
TPCK (tosylphenylalanine chloromethyl ketone)	chymotrypsin	trypsin	100 µg/ml	3 mg/ml in ethanol
EDTA	metalloproteases		1 mM	0.5 M in water

^aAprotinin is a basic polypeptide of 58 amino acids that aggregates if repeatedly frozen and thawed (for review, see Trautschold et al. 1967). The stock solution should be stored in small aliquots at –20°C. Each aliquot should be discarded after use.

^bCaution: PMSF is extremely destructive to the mucous membranes of the respiratory tract, eyes, and skin. PMSF may be fatal if inhaled, swallowed, or absorbed through the skin. In case of contact, immediately flush eyes or skin with copious amounts of water. Discard contaminated clothing.

PMSF is inactivated in aqueous solutions. The rate of inactivation increases with pH and is faster at 25°C than at 4°C. The half-life of a 20 µM aqueous solution of PMSF is about 35 minutes at pH 8.0 (James 1978). This means that solutions of PMSF can be safely discarded after they have been rendered alkaline (pH > 8.6) and stored for several hours at room temperature.

The lysis buffers that are commonly used to prepare extracts of mammalian cells for immunoprecipitation are shown below. In the absence of any information about the target antigen, we recommend trying the triple- and single-detergent lysis buffers before turning to more specialized methods of extraction.

Triple-detergent lysis buffer

50 mM Tris · Cl (pH 8.0)
150 mM NaCl
0.02% sodium azide
0.1% SDS
100 µg/ml phenylmethanesulfonyl fluoride (PMSF)
1 µg/ml aprotinin
1% Nonidet P-40 (NP-40)
0.5% sodium deoxycholate

Single-detergent lysis buffer

50 mM Tris · Cl (pH 8.0)
150 mM NaCl
0.02% sodium azide
100 µg/ml PMSF
1 µg/ml aprotinin
1% Triton X-100 or 1% NP-40

High-salt lysis buffer

50 mM HEPES (pH 7.0)
500 mM NaCl
1% NP-40
1 µg/ml aprotinin
100 µg/ml PMSF

No-salt lysis buffer

50 mM HEPES (pH 7.0)
1% NP-40
1 µg/ml aprotinin
100 µg/ml PMSF

Cautions: PMSF is extremely destructive to the mucous membranes of the respiratory tract, the eyes, and skin. PMSF may be fatal if inhaled, swallowed, or absorbed through the skin. In case of contact, immediately flush eyes or skin with copious amounts of water. Discard contaminated clothing.

Sodium azide is poisonous. It should be handled with great care wearing gloves, and solutions containing it should be clearly marked.

PMSF, which is labile in aqueous solution, should be added from a stock solution just before the lysis buffer is used. The rate of inactivation in aqueous solution increases with pH and is faster at 25°C than at 4°C. The half-life of a 20 µM aqueous solution of PMSF is about 35 minutes at pH 8.0

(James 1978). This means that aqueous solutions of PMSF can be safely discarded after they have been rendered alkaline (pH > 8.6) and stored for several hours at room temperature. PMSF is usually stored as a 10 mM or 100 mM stock solution (1.74 or 17.4 mg/ml in isopropanol) at -20°C.

LYSIS OF CULTURED MAMMALIAN CELLS

1. Add the lysis buffer of choice (cooled to 0°C) to chilled, washed cell monolayers (step 5, page 18.28). Incubate for 20 minutes on a flat aluminum tray on a bed of crushed ice.

Volume of lysis buffer (ml)	Size of petri dish or well (mm)
1.0	90
0.5	60
0.25	35
0.25	30 (well)

2. Scrape the cells to one side of the dish with a policeman. Using an automatic pipetting device, transfer the cell debris and lysis buffer to a chilled microfuge tube.

The best policemen are made as follows:

- a. Using a scalpel, slice silicon rubber stoppers (2 cm in diameter) into quarters.
- b. Place each piece of stopper on the bench and impale it on a pasteur pipette.
- c. Use the straight edge of the sliced stopper for scraping cells.
- d. After use, store the policemen in detergent solution before washing. The policemen can be sterilized by autoclaving for 20 minutes at 15 lb/sq. in.

3. Centrifuge the lysate at 12,000g for 2 minutes at 4°C in a microfuge.
4. Transfer the supernatant to a fresh microfuge tube, and store it on ice or at -70°C, depending on the sensitivity of the target antigen to freezing and thawing.

Notes

- i. When experimenting with conditions for solubilization of the protein of interest, it is sometimes useful to extract the cell debris (step 4) with a stronger lysis buffer.
- ii. Cells growing in suspension should be concentrated by centrifugation and then resuspended in the appropriate lysis buffer (chilled to 0°C) at a concentration of 10⁷ cells/ml. After 30 minutes at 0°C, transfer the cell suspension to a chilled microfuge tube and proceed as described in step 3.
- iii. After thawing, samples that have been stored at -20°C should be centrifuged at 12,000g for 5 minutes at 0°C in a microfuge. This removes aggregates of cytoskeletal elements.

MECHANICAL LYSIS OF YEAST

This method can be used only when the antibody recognizes denatured forms of the target protein.

1. Collect the cells from a 1-ml culture by centrifugation at 12,000g for 1 minute at 0°C in a microfuge or at 3000 rpm for 5 minutes at 0°C in a Sorvall RT6000 (or equivalent). Discard the supernatant (unless the protein of interest is a secretory protein).
2. Resuspend the cells by vortexing in 1 ml of ice-cold 50 mM Tris · Cl (pH 8.0). Recover the cells by centrifugation as described in step 1. Pour off the supernatant, and remove as much of the residual fluid as possible with a disposable pipette tip attached to a vacuum line.
3. Resuspend the pellet in 5 volumes (~ 250 μl) of yeast lysis buffer.

Yeast lysis buffer

50 mM Tris · Cl (pH 8.0)
0.1% Triton X-100
0.5% SDS

4. Add acid-washed glass beads (0.45–0.50 mm in diameter) to the level of the meniscus. The total volume of the mixture of glass beads and yeast lysis buffer should be approximately 400 μl.

At one time, glass beads of the correct size could be purchased only from one supplier, located in Germany (B. Braun, Melsungen). Recently, however, several companies in the United States have begun to sell beads of the correct size of acceptable quality.

5. Vortex the suspension on a heavy-duty vortexing machine for five periods of 20 seconds each. Cool the suspension on ice for 1 minute between each cycle of vortexing. Monitor cell lysis by phase-contrast microscopy.
6. Recover the cell extract by punching a small hole through the bottom of the microfuge tube. This is best done with a red-hot hypodermic needle (23 gauge). Place the microfuge tube containing the glass beads inside an empty microfuge tube. Centrifuge the piggybacked pair of tubes in a 15-ml Corex tube at 2000g for 2 minutes at 4°C.
7. Recover the bottom microfuge tube, which should contain the cell extract. Clarify the extract by centrifuging the tube at 12,000g for 5 minutes at 4°C in a microfuge. Transfer the supernatant to a fresh microfuge tube.
Extracts of yeast cells are usually stable when stored at -20°C. However, if the protein of interest is sensitive to proteolysis, add protease inhibitors to the extracts (see Table 18.1) and store them at 0°C. Try to minimize the time between cell lysis and immunoprecipitation.
8. Immunoprecipitate the protein of interest from the cell extract as described on pages 18.44–18.46.

ENZYMATIC LYSIS OF YEAST

This method is used when the available antibody recognizes the native form of the target protein.

1. Collect the cells from a 1-ml culture by centrifugation at 12,000g for 1 minute at 0°C in a microfuge or at 3000 rpm for 5 minutes at 0°C in a Sorvall RT6000 (or equivalent). Discard the supernatant (unless the protein of interest is a secretory protein).
2. Resuspend the cells by vortexing in ice-cold phosphate-buffered saline (PBS; see Appendix B). Recover the cells by centrifugation as described in step 1. Discard the supernatant.
3. Resuspend the pellet in a volume of stabilizing buffer A (at room temperature) equivalent to the volume of the original culture (usually 1 ml as in step 1). Incubate the suspension for 10 minutes at 30°C.

Stabilizing buffer A

1 M sorbitol
10 mM MgCl₂
2 mM dithiothreitol
50 mM potassium phosphate (pH 7.8)
100 µg/ml phenylmethylsulfonyl fluoride (PMSF)

Caution: PMSF is extremely destructive to the mucous membranes of the respiratory tract, the eyes, and skin. PMSF may be fatal if inhaled, swallowed, or absorbed through the skin. In case of contact, immediately flush eyes or skin with copious amounts of water. Discard contaminated clothing.

PMSF, which is labile in aqueous solution, should be added from a stock solution just before the stabilizing buffer is used. The rate of inactivation in aqueous solution increases with pH and is faster at 25°C than at 4°C. The half-life of a 20 µM aqueous solution of PMSF is about 35 minutes at pH 8.0 (James 1978). This means that aqueous solutions of PMSF can be safely discarded after they have been rendered alkaline (pH > 8.6) and stored for several hours at room temperature. PMSF is usually stored as a 10 mM or 100 mM stock solution (1.74 or 17.4 mg/ml in isopropanol) at -20°C.

4. Recover the cells by centrifugation as described in step 1, and resuspend the cell pellet in a volume of stabilizing buffer B equal to the volume of the original culture. Incubate the suspension for 2 minutes in a water bath set at 30°C.

Stabilizing buffer B

1 M sorbitol
10 mM MgCl₂
2 mM dithiothreitol
25 mM potassium phosphate (pH 7.8)
25 mM sodium succinate (pH 5.5)
100 µg/ml PMSF

5. Add 0.25 volume of a stock solution of zymolase 100T (10 mg/ml).

Zymolase is an enzyme, isolated from cultures of *Arthrobacter luteus*, that catalyzes lysis of cell walls by hydrolyzing glucose polymers with 1 → 3 linkages. As supplied by commercial manufacturers, zymolase 100T is contaminated with other enzymes, including proteases. It should therefore be used in conjunction with protease inhibitors.

6. Incubate the suspension at 30°C. After 15 minutes, take out two small aliquots of the suspension. To one sample, add Nonidet P-40 (NP-40) to a final concentration of 1%. Examine the two samples by phase-contrast microscopy. Protoplasts formed by the action of zymolase will be visible in the untreated sample but should be lysed in the sample containing detergent. Continue incubation until the majority of the cells have been converted to protoplasts. This usually takes 20–30 minutes.

7. Collect the protoplasts by centrifugation at 500g for 10 minutes at 4°C. Discard the supernatant.

8. Resuspend the pellet (which consists of protoplasts and intact cells) in a volume of lysis buffer equivalent to 0.01 volume of the original culture. Store the suspension at 0°C for 30 minutes.

Either NP-40 high- or no-salt lysis buffer (see page 18.32) or RIPA buffer (see page 18.38) may be used, depending on the properties of the available antibody. It is essential to include protease inhibitors in the lysis buffer (see Table 18.1).

9. Centrifuge the suspension at 12,000g for 10 minutes at 4°C in a microfuge. Transfer the supernatant to a fresh microfuge tube.

Extracts of yeast cells are usually stable when stored at -20°C. However, if the protein of interest is sensitive to proteolysis, add protease inhibitors to the extracts (see Table 18.1) and store them at 0°C. Try to minimize the time between cell lysis and immunoprecipitation.

10. Immunoprecipitate the protein of interest from the cell extract as described on pages 18.44–18.46.

Note

When experimenting with conditions for solubilization of the protein of interest, it is sometimes useful to extract the cell debris (step 9) with a stronger lysis buffer.

RAPID LYSIS OF YEAST CELLS

This method can be used only when the antibody recognizes denatured forms of the target protein.

1. Collect the cells from a 1-ml culture by centrifugation at 12,000g for 1 minute at 0°C in a microfuge or at 3000 rpm for 5 minutes at 0°C in a Sorvall RT6000 (or equivalent). Discard the supernatant (unless the protein of interest is a secretory protein).
2. Resuspend the cells in 0.5 ml of 25% trichloroacetic acid (TCA).
3. Recover the cells by centrifugation as described in step 1, and resuspend them in 1 ml of 90% acetone.
4. Recover the cells by centrifugation as described in step 1, and estimate the volume of the pellet. Resuspend the pellet in an equal volume of 1% SDS.

5. Add acid-washed glass beads (0.45–0.50 mm in diameter) to the level of the meniscus.

At one time, glass beads of the correct size could be purchased only from one supplier, located in Germany (B. Braun, Melsungen). Recently, however, several companies in the United States have begun to sell beads of the correct size of acceptable quality.

6. Vortex the suspension on a heavy-duty vortexing machine for five periods of 20 seconds each. Cool the suspension on ice for 1 minute between each cycle of vortexing. Monitor cell lysis by phase-contrast microscopy.
7. Using a red-hot hypodermic needle (23 gauge), make a hole in the lid of the microfuge tube. This prevents the glass beads from exploding out of the tube during heating.
8. Heat the tube in a boiling-water bath for 3 minutes.
9. Transfer the glass beads into a fresh microfuge tube, and wash the original tube with 0.5 ml of RIPA buffer. Add the wash to the fresh microfuge tube containing the glass beads.

RIPA buffer

50 mM Tris · Cl (pH 7.5)
150 mM NaCl
1% Nonidet P-40 (NP-40)
0.5% sodium deoxycholate
0.1% SDS

10. Recover the cell extract by punching a small hole through the bottom of

the microfuge tube. This is best done with a red-hot hypodermic needle (23 gauge). Place the microfuge tube containing the glass beads inside an empty microfuge tube. Centrifuge the piggybacked pair of tubes in a 15-ml Corex tube at 2000g for 2 minutes at 4°C.

11. Recover the bottom microfuge tube, which should contain the cell extract. Clarify the extract by centrifuging the tube at 12,000g for 5 minutes at 4°C in a microfuge. Transfer the supernatant to a fresh microfuge tube. Extracts of yeast cells are usually stable when stored at –20°C. However, if the protein of interest is sensitive to proteolysis, add protease inhibitors to the extracts (see Table 18.1) and store them at 0°C. Try to minimize the time between cell lysis and immunoprecipitation.
12. Immunoprecipitate the protein of interest from the cell extract as described on pages 18.44–18.46.

LYSIS OF BACTERIA

Immunoprecipitation of target proteins from cell lysates is used far more rarely with bacterial expression systems than with mammalian expression systems because foreign proteins are usually expressed in *E. coli* from powerful, inducible promoters. Following induction, therefore, novel gene products frequently accumulate to such a level that they can be detected by the appearance of novel bands after SDS-polyacrylamide gel electrophoresis of total cell proteins. Immunological identification of these novel bands can then be obtained by western blotting using antisera or monoclonal antibodies that react with denatured forms of the target protein. If no novel proteins are detectable by this technique, it makes more sense to test the cloned DNA in a series of different *E. coli* expression systems rather than to attempt immunoprecipitation from cell extracts that contain unusably small quantities of target protein.

Total lysates of small-scale cultures of *E. coli* are easily made as follows:

1. After induction for the appropriate period of time, recover the bacteria from 1 ml of culture by centrifugation at 12,000g for 30 seconds in a microfuge.
2. Remove the medium by aspiration, and then resuspend the pellet by vortexing in 0.5 ml of ice-cold 50 mM Tris · Cl (pH 7.4). Recover the bacteria by centrifugation at 12,000g for 30 seconds at 0°C in a microfuge.
3. Remove the supernatant by aspiration, leaving the bacterial pellet as dry as possible. Take care to remove any beads of fluid that adhere to the sides of the microfuge tube.
4. Resuspend the pellet by vortexing in 25 µl of H₂O. As soon as the pellet is dispersed, add 25 µl of 2 × SDS gel-loading buffer and continue vortexing for 20 seconds.

2 × SDS gel-loading buffer

100 mM Tris · Cl (pH 6.8)
200 mM dithiothreitol
4% SDS (electrophoresis grade)
0.2% bromophenol blue
20% glycerol

2 × SDS gel-loading buffer lacking dithiothreitol can be stored at room temperature. Dithiothreitol should then be added, just before the buffer is used, from a 1 M stock (see Appendix B).

5. Place the sample in a boiling-water bath for 5 minutes.
6. Shear the chromosomal DNA by sonication, using either a sonicator with an immersible tip or a sonicator that can process many samples simulta-

neously in a chilled cup. Depending on the power output of the sonicator and its state of tuning, between 30 seconds and 2 minutes at full power should be sufficient to reduce the viscous lysate to manageable levels.

Larger samples can be sheared by repeated passage through a 23-gauge hypodermic needle.

7. Centrifuge the sample at 12,000g for 10 minutes at room temperature. Transfer the supernatant to a fresh tube and discard the pellet.
8. Analyze 25 µl of the sheared or sonicated lysate by electrophoresis through an SDS-polyacrylamide gel as described on pages 18.47–18.54. Store the unused portion of the sample at –20°C.