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Protocol

Lysis of Cultured Cells for Immunoprecipitation

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INTRODUCTION

Cell lysis with mild detergent is commonly used with cultured animal cells. If low detergent concentrations are sufficient to cause cell lysis (e.g., 1% Nonidet P-40 [NP-40] or 1% Triton X-100), this method can be gentler to the protein of interest than mechanical homogenization methods. The choice of detergent must be tailored to the nature of the epitope recognized by the immunoprecipitating antibody. If the antibody recognizes a linear peptide epitope (e.g., a synthetic peptide), then use a harsh denaturing lysis buffer (e.g., RIPA buffer). On the other hand, if the antibody is directed toward a conformational epitope, use NP-40 lysis buffer (or 1% Triton X-100). This protocol presents separate methods for lysing cells grown as monolayer cultures and for cells grown in suspension.

RELATED INFORMATION

For details on protease inhibitor selection, see Table 1. Table 2 provides information on preparation of a general protease inhibitor cocktail. **Homogenization of Mammalian Tissue** (Simpson 2010) gives more information on protease inhibitors and also describes mechanical homogenization methods for animal tissue.

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <I>, and recipes for reagents marked with <R>.

Reagents

Cell culture, suspension or monolayer

Lysis buffer

See Table 3 for more information on the choices. As described there, good first choices are NP-40 lysis buffer (Triton X-100 may be substituted for NP-40) or RIPA lysis buffer. Chill the lysis buffer to 4°C prior to use.

<R>Phosphate-buffered saline (PBS) (ice-cold)

Equipment

Centrifuge

<I>Dry ice/ethanol (optional; see Steps 6 and 12)

Ice

Pipettes

Tubes, centrifuge

Vortex mixer

Adapted from *Proteins and Proteomics*, by Richard J. Simpson. CSHL Press, Cold Spring Harbor, NY, USA, 2003.
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Table 1. Inhibitor cocktails used to control proteolysis during protein isolation

Tissue type	Protease inhibitors (working concentration)	Target protease type ^a	Stock solution ^b
Animal tissues	AEBSF (0.2 mM)	Serine	AEBSF: 20 mM in methanol
	or DCI (0.1 mM)		DCI: 10 mM in DMSO
	or PMSF (0.2 mM)		PMSF: 200 mM in ethanol or isopropanol
	Benzamidine (1 mM)	Serine	100 mM
	Leupeptin (10 µg/mL)	Serine/cysteine	1 mg/mL
	Pepstatin (10 µg/mL)	Aspartic	5 mg/mL in methanol
	Aprotinin (1 µg/mL)	Serine	0.1 mg/mL
	EDTA or EGTA ^c (1 mM)	Metallo	100 mM
Plant tissues	AEBSF (0.2 mM)	Serine	AEBSF: 20 mM in methanol
	or DCI (0.1 mM)		DCI: 10 mM in DMSO
	or PMSF (0.2 mM)		PMSF: 200 mM in ethanol or isopropanol
	Chymostatin (20 µg/mL)	Serine/cysteine	1 mg/mL in DMSO
	EDTA or EGTA ^c (1 mM)	Metallo	100 mM
Yeasts and fungi	AEBSF (0.2 mM)	Serine	AEBSF: 20 mM in methanol
	or DCI (0.1 mM)		DCI: 10 mM in DMSO
	or PMSF (0.2 mM)		PMSF: 200 mM in ethanol or isopropanol
	Pepstatin (15 µg/mL)	Aspartic	5 mg/mL in methanol
	1,10-Phenanthroline (5 mM)	Metallo	1 M in ethanol
Bacteria	AEBSF (0.2 mM)	Serine	AEBSF: 20 mM in methanol
	or DCI (0.1 mM)		DCI: 10 mM in DMSO
	or PMSF (0.2 mM)		PMSF: 200 mM in ethanol or isopropanol
	EDTA or EGTA ^c (1 mM)	Metallo	100 mM

Adapted from North (1989).

Abbreviations: AEBSF: 4-(2-aminoethyl)-benzenesulfonyl fluoride; DCI: 3,4-dichloroisocoumarin; DMSO: dimethyl sulfoxide; EDTA: ethylenediamine tetraacetic acid; EGTA: ethylene glycol bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; PMSF: phenylmethylsulfonyl fluoride.

M_r values of inhibitors: 1,10-phenanthroline: 198; AEBSF: 240; aprotinin: 6500; benzamidine hydrochloride: 157; chymostatin: 605; DCI: 215; EDTA (disodium salt, dihydrate): 372; leupeptin: 427; pepstatin: 686; PMSF: 174.

^aFor a review of proteolytic enzymes, see Neurath (1989) and Perlmann and Lorand (1970).

^bAqueous solution unless otherwise indicated.

^cAn efficient chelator of divalent metal cations other than Mg²⁺ (for which it has a 103-fold lower affinity) (Gegenheimer 1990).

METHOD

Lysing Cells Grown as Monolayer Cultures

1. Discard the culture medium. Wash cells twice with ice-cold PBS. Place the culture dishes on ice.
2. Add 1.0 mL of the lysis buffer of choice (prechilled to 4°C) per 100-mm dish.
For culture dishes of other sizes, adjust the volume of lysis buffer accordingly.
3. Incubate the cells for 10-30 min (depending on the cell line being studied) on ice. Rock the dishes occasionally.

Table 2. Preparation of a general protease inhibitor cocktail

Inhibitor	Trial working concentration	Stock (100X) concentration	Recipe	Amount to use ^a	Target protease type
<!--AEBSF	<1 mM	20 mM	239.5 mg/10 mL H ₂ O (100 mM)	4 mL	Serine
EDTA	1-10 mM	100 mM	19 mg/100 mL H ₂ O (0.5 M)	4 mL	Metallo
<!--Leupeptin	10-100 μM	2 mM	18.9 mg/2 mL H ₂ O (20 mM)	2 mL	Cysteine/serine
Pepstatin	1 μM	100 μM	6.8 mg/10 mL methanol (1 mM)	2 mL	Aspartic

Data, in part, from Calbiochem (1998).

^aMix the inhibitor solutions and bring to a final volume of 20 mL with H₂O or an appropriate aqueous buffer. The resulting solution (i.e., 20 mL of the 100X protease inhibitor cocktail stock) can be aliquoted into microcentrifuge tubes and stored at -20°C until required.

4. Tilt a dish on the bed of ice, allowing the buffer to drain to one side. Remove the lysate with a pipette. Transfer to a microcentrifuge tube (or other suitable centrifuge tube). Repeat with each of the remaining dishes.

Some researchers prefer to scrape the cells from the tissue-culture dish. However, this does cause some stress to the cells and is only required in unusual cases.

5. Centrifuge the lysate at 20,000g for 10 min at 4°C.

6. Carefully remove the supernatant to a fresh tube, making sure not to disturb the pellet. Store the lysate on ice until it is needed for the preclearing and immunoprecipitation (see Harlow and Lane 1999).

The cell lysate can be snap-frozen using a dry ice/ethanol mixture and then stored at -70°C for long-term storage. However, for the analysis of protein complexes by immunoprecipitation, the use of a freshly prepared cell lysate is recommended.

Table 3. Commonly used lysis buffers for lysing cultured cells

Buffer	Comments
NP-40 lysis 150 mM NaCl <!--1.0% Nonidet P-40 (NP-40) 50 mM Tris-Cl (pH 7.4)	This is probably the most widely used lysis buffer. It relies on the nonionic detergent NP-40 as the major solubilizing agent, which can be replaced by Triton X-100 with similar results. Variations include lowering the detergent concentration or using alternate detergents such as digitonin, saponin, or CHAPS.
RIPA lysis 150 mM NaCl <!--1.0% Nonidet P-40 (NP-40) <!--0.5% sodium deoxycholate <!--0.1% SDS 50 mM Tris-Cl (pH 7.4)	This is a much harsher denaturing lysis buffer than NP-40, owing to the inclusion of two ionic detergents (sodium dodecyl sulfate [SDS] and sodium deoxycholate). In addition to releasing most proteins from cultured cells, RIPA lysis buffer disrupts most weak noncovalent protein-protein interactions.
When studying the modification of proteins by phosphorylation, phosphatase inhibitors (e.g., 25 mM sodium fluoride [NaF], 40 mM β-glycerol phosphate, 100 μM sodium orthovanadate [Na ₃ VO ₄], or 1 μM microcystin) should be included. If proteolytic degradation of the target protein is a problem, protease inhibitors should be included in the lysis buffer. Some commonly used inhibitors include aprotinin (1 μg/mL), leupeptin (1 μg/mL), pepstatin (1 μg/mL), and phenylmethylsulfonyl fluoride (PMSF; 50 μg/mL). Alternatively, commercially available protease cocktail tablets (e.g., Boehringer) can be included.	

Lysing Cells Grown in Suspension

7. Harvest the cells by centrifugation at 480g for 10 min. Decant and discard the supernatant.
8. Carefully wash the cell pellet twice with ice-cold PBS. Place the washed cell pellet on ice.
9. Resuspend the pellet in 1.0 mL of the lysis buffer of choice (prechilled to 4°C) per 1×10^7 to 5×10^7 cells.
10. Incubate the cells for 15 min on ice, vortexing the tube occasionally.
11. Centrifuge the lysate at 20,000g for 10 min at 4°C.
12. Carefully remove the supernatant to a fresh tube, making sure not to disturb the pellet. Store the lysate on ice until it is needed for the preclearing and immunoprecipitation (see Harlow and Lane 1999).

The cell lysate can be snap-frozen using a dry ice/ethanol mixture and then stored at -70°C for long-term storage. However, for the analysis of protein complexes by immunoprecipitation, the use of a freshly prepared cell lysate is recommended.

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