2 Preparation of Cellular and Subcellular Extracts

Richard J. Simpson

Joint ProteomicS Laboratory (JPSL) of the Ludwig Institute for Cancer Research and the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

TISSUES AND CELLS CAN BE DISRUPTED BY MECHANICAL OR CHEMICAL MEANS, 41

RECOMBINANT PROTEIN CAN BE RECOVERED FROM INCLUSION BODIES BY DENATURATION AND RENATURATION, 44

PREPARATION OF SUBCELLULAR EXTRACTS ENRICHES FOR TARGET PROTEINS, 45

PROTOCOLS

1. Homogenization of Mammalian Tissue, 47
   - Additional Protocol: Removal of Mucin from Tissue Homogenates, 50
2. Lysis of Cultured Cells for Immunoprecipitation, 51
3. Lysis of Cultured Animal Cells, Yeast, and Bacteria for Immunoblotting, 53
4. Disruption of Cultured Cells by Nitrogen Cavitation, 55
5. Small-scale Extraction of Recombinant Proteins from Bacteria, 59
6. Large-scale Extraction of Recombinant Proteins from Bacteria, 60
7. Preparation of Clarified *E. coli* Extract Containing Histidine-tagged Proteins, 62
8. Solubilization of *E. coli* Recombinant Proteins from Inclusion Bodies, 64
9. Preparation of Extracts from Yeast, 66
10. Differential Detergent Fractionation of Eukaryotic Cells, 68
    - Additional Protocol: Precipitation of Tubulins and MAPs Using Magnesium, 75

Extraction of protein from tissues and cells is perhaps the most critical step in any protein purification or proteomics strategy because this step influences protein yield, biological activity, and the structural integrity of the specific target protein. Thus, care must be taken in selecting the specific extraction conditions employed. The principal aim must be to reproducibly achieve the highest degree of cell breakage using minimal disruptive forces while maintaining protein integrity. A summary of commonly used methods for homogenizing cells and tissues is given in Table 2.1.

It is important to avoid altering the native structure of the target protein and, therefore, possibly its biological activity. Perturbation of native protein structure during preparation of cell extracts can occur by exposure to extremes of pH, temperature, mechanical stress (shearing forces), pressure, and proteolytic degradation. Although proteolytic degradation may not alter the biological activity per se, it may influence the association of the target protein with other cellular regulatory components. Such nonspecific alterations can result in irreproducibility of behavior of the target protein from one preparation to the next, which makes interpretation of biological studies extremely difficult. For

TABLE 2.1. Methods for homogenizing cells and tissues

Method	Underlying basis of cell disruption	Type of tissue
Gentle		
Osmotic shock	Osmotic disruption of cell membranes	bacteria, erythrocytes
Detergent lysis	Detergent disruption of cell membranes	tissue culture cells
Enzymatic digestion	Digestion of cell wall; contents released by osmotic disruption	bacteria, yeast
Dounce homogenizer and/or Potter-Elvehjem homogenizer[a]	Cells forced through a narrow gap with a clearance of 0.05–0.08 mm (tight fitting) to 0.1–0.3 mm (loose fitting); cell membrane disrupted by liquid shear forces The smaller the clearance, the greater the shearing force; the clearance of a Teflon pestle (Potter-Elvehjem homogenizer) is normally 0.05–0.06 mm[b]	soft animal tissues and cells
Moderately harsh		
Homogenization (Waring Blendor)	Cells broken by rotating blades	most animal, plant tissues
Grinding (with sand, alumina, or glass beads)	Cell walls broken by abrasive action of particles	cell suspensions
Vigorous		
French pressure cell	Cells forced through small orifice at high hydraulic pressure (100–150 MPa or 15,000–20,000 psi) and disrupted by shear forces	bacteria, yeast, plant cells
Explosive decompression (nitrogen cavitation)	Cells equilibrated with inert gas (e.g., N_2) at high pressure (typically, 5500 kPa or 800 psi); on exposure of cells to 1 atm, disruption occurs	bacteria, yeast, plant cells
Bead mill	Rapid vibration with glass beads disrupts cell wall	cell suspensions
Ultrasonication	High-pressure sound waves cause cell rupture by cavitation and shear forces	cell suspensions

[a]Sometimes called Teflon-and-glass homogenizer; it is power-driven, the pestle typically rotates at 500–1000 rpm.

[b]For soft tissue such as liver, a Potter-Elvehjem homogenizer with a clearance of ~0.09 mm is recommended. Smaller clearances can lead to damage to released organelles (especially nuclei) as well as cause difficulty in moving the glass vessel relative to the pestle in the early stages of homogenization (Graham 1997).

discussions on avoiding proteolytic degradation of proteins in extracts, see Beynon and Oliver (1996) and Beynon and Bond (1989).

It is not always necessary to break open cells to extract recombinant proteins. For example, various high-expression mammalian cell lines (in particular, Chinese hamster ovary [CHO] cells) and strains of yeast (e.g., *Pichia pastoris*) have been engineered to secrete recombinant proteins that can be purified directly from cell-conditioned media and culture filtrates. When using these approaches, it is important to concentrate the large volumes of cell-conditioned media into a "protease-free" environment as quickly as possible.

Key variables that determine the successful preparation of crude extracts include

- the method of cell lysis,
- the control of pH,
- temperature, and
- avoidance of proteolytic degradation.

Note: A trial-and-error approach in pilot experiments is often required to optimize cell lysis conditions.

A different problem can occur with *Escherichia coli* expression systems, where the expressed recombinant protein often appears in the crude extract (lysate) as insoluble aggregates, referred to as "inclusion bodies." In this case, the purification of a target protein often involves initial solubilization of the inclusion bodies in a strong denaturant (e.g., guanidine hydrochloride or urea) and subsequent refolding. These procedures are described in Protocols 6 and 8.

When choosing a cell disruption strategy, it is important to consider the intended use of the cell lysate. For example, lysis conditions (choice of buffer, detergents, and so on) will vary markedly, depending on whether the lysate is to be used for

- immunoprecipitation studies,
- western blotting,
- two-dimensional gel electrophoresis,
- native target protein isolation using conventional chromatographic purification procedures, or
- recombinant protein purification procedures that rely on the target protein expressed as a fusion protein including a purification "handle" or "tag."

The first part of this introduction describes commonly used methods for cell lysis, including procedures for preparing crude cell extracts for immunoprecipitation and immunoblotting studies. The range of methods discussed is by no means exhaustive and does not include plant tissues and fungi. For more detailed reviews describing the preparation of crude extracts from eukaroytes, prokaryotes, and plants, see Deutscher (1990), Doonan (1996), Graham (1997), and Spector et al. (1998). The second section deals with the problem of solubilizing *E. coli*-derived recombinant proteins from inclusion bodies. The third section discusses the advantages of isolating subcellular fractions and describes the differential detergent fractionation (DDF) of eukaryotic cells for the isolation and analysis of proteins. The protocols described in this chapter are sufficiently general and thus can be applied to a variety of different tissues and cell types with only minor modifications.

TISSUES AND CELLS CAN BE DISRUPTED BY MECHANICAL OR CHEMICAL MEANS

Preparation of Protein Extracts from Mammalian Tissues

The preparation of protein extracts from most animal tissues is relatively simple because the cell membranes are weak and easily disrupted by a combination of osmotic and mechanical forces. The first steps of a typical protein isolation procedure usually consist of washing the tissue, disrupting the tissue in a suitable buffer using a homogenizer, and clarifying the homogenate by centrifugation. The centrifugation step separates the soluble proteins from the membrane fraction and insoluble cell debris. A generalized procedure that may be applied to a number of different tissues and cell types with only minor modifications is given in Protocol 1.

The choice of tissue for extraction of soluble proteins depends on several criteria. Clearly, the tissue distribution of a particular target protein varies markedly. Hence, it is always desirable to perform small-scale pilot experiments to measure the relative content of the target protein in various tissues. However, the final choice of starting tissue will, invariably, be determined by the balance between target protein abundance, ready availability and cost of the tissue, and various technical issues such as minimization of proteolytic activity. It should be borne in mind that certain animal tissues (e.g., liver, spleen, kidney, and macrophages) are rich in lysosomal proteases, particularly cathepsins, and should be avoided unless the goal of the study is the proteolytic enzyme(s) itself. Fresh tissue is preferable, but in some cases, frozen tissue may be acceptable, provided it is frozen rapidly in small pieces and not stored for too long. It is recommended that frozen tissue be stored below –50°C, since some proteolytic degradation of proteins can be expected due to release of proteases from lysosomes as a result of ice-crystal formation (Dignam 1990).

Preparation of Protein Extracts from Mammalian Cultured Cells

Mammalian cultured cells can be lysed by several different methods, the method of choice depending on the final use of the target protein (e.g., immunoprecipitation, immunoblotting, two-dimensional gel electrophoresis or conventional purification). Mammalian cells lack a cell wall and thus are easily lysed by treatment with mild detergents. If the final preparation of the target protein need

not retain its structural integrity (three-dimensional structure) or biological activity, the cells can be lysed under harsh denaturing conditions (e.g., RIPA lysis buffer). If gentler conditions are required, the Nonidet P-40 (NP-40) lysis buffer (or variations thereof) should be used.

Many extraction conditions release proteolytic enzymes in the lysis buffer. If proteolytic degradation of the target protein becomes a problem, two approaches can be employed to lessen its effect:

- Keep the sample cold (temperature has a profound effect on the catalytic activity of most proteases).
- Add protease inhibitors to the lysis buffer (for a list of some of the commonly used protease inhibitors, see Table 2.2 on p. 48).

For immunoprecipitation studies, the conditions used for lysis should be as gentle as possible to maintain the structural integrity of the target protein and to minimize the solubilization of irrelevant proteins. This is best accomplished by using nonionic detergents instead of ionic ones, lower concentrations than higher, and single detergents rather than mixtures. The two most widely used lysis buffers for the extraction of proteins from mammalian culture cells are NP-40 lysis buffer and RIPA lysis buffer. The former buffer releases cytoplasmic and nuclear proteins without releasing chromosomal DNA, which, because of its viscous nature, can cause numerous problems during protein purification and analysis.

The lysis conditions can be easily tailored to suit the target protein. Variables that can affect the release of a target protein from a cell include salt concentration, type of detergent, presence of divalent cations, and pH. To determine the optimum conditions for extracting a target protein, the variables listed below should be monitored in pilot experiments (Harlow and Lane 1999):

- Salt concentration should be varied from 0 to 1 M.
- Nonionic detergent concentrations between 0.1% and 2%.
- Ionic detergent concentrations between 0.01% and 0.5%.
- Divalent cation concentrations between 0 and 10 mM.
- EDTA concentrations between 0 and 5 mM.
- pH values between 6 and 9.

A generalized procedure for lysing tissue culture cells for the purpose of performing immunoprecipitation is given in Protocol 2.

For immunoblotting studies, the conditions used for lysis can be harsher than those used for immunoprecipitation studies. The most widely used buffer system is the Laemmli sample buffer (Laemmli 1970) containing 2% SDS. A procedure for lysing tissue culture cells for the purpose of performing immunoblotting is given in Protocol 3. An alternative procedure for lysing any tissue culture cells (and microorganisms such as bacteria) that relies on gaseous shear to disrupt cells is nitrogen cavitation (see Protocol 4).

Disruption of Bacterial Cells

Due to the advent of recombinant DNA technology, bacteria are now a particularly convenient vehicle for generating large quantities of recombinant protein. Enormous numbers of bacteria can be grown under defined conditions and are relatively easy to break open for extraction purposes. A number of methods, based on mechanical and enzymatic means, are available for lysing bacteria, and to a large extent, the choice will depend on the scale of the process (for a review, see Cull and McHenry 1990).

The mechanical procedures for lysing bacteria (e.g., French pressure cell, nitrogen cavitation, ultrasonication, grinding with abrasive agents, and vortexing with glass beads) rely on shearing forces to disrupt the tough outer cell wall (for a review of these procedures, see Graham 1997). However, mechanical methods are more likely to damage the cellular contents (compared with enzymatic methods) and are not easily scaled-up.

The *French Press* lyses cells by applying hydraulic pressure to the cell suspension (typically, 8000–20,000 psi, or 550–1400 kg/cm^2), followed by a sudden release to atmospheric pressure. In this device, the cell suspension is propelled by a piston through a narrow orifice, often the annulus around a ball bearing. The rapid change in pressure causes a liquid shear and consequent bursting of cells. In some cases, two to three passes of the cells through the French Press are required to obtain adequate lysis (Cull and McHenry 1990). The French Press method works well for cell suspension volumes of 10–30 ml (ratio of cell wet weight to lysis buffer volume can range from 1:1 to 1:4 g/ml), but it is considered too time-consuming for larger volumes and can be technically difficult for smaller volumes.

In *nitrogen cavitation* devices, the pressure cell consists of a robust stainless steel cylinder with an inlet port for delivery of nitrogen gas from a cylinder and an outlet tube with a needle valve. During pressurization (i.e., 5500 kPa [800 psi] for 10–30 minutes), nitrogen dissolves in the cell suspension buffer as well as in the cytosol of the cells. When the needle valve is opened, the suspension is forced through the outlet tube, and at 1 atm, it experiences a rapid decompression that causes cell disruption due to the sudden formation of bubbles of nitrogen gas. This method can be used with bacteria or any tissue culture cell in volumes ranging from 1 to 1000 ml. Nitrogen cavitation (see Protocol 4) eliminates the heat buildup associated with mechanical and ultrasonic disruption because the cells are actually cooled by the expanding gas.

Sonication disrupts cells by creating vibrations that cause mechanical shearing of the cell wall. This method is suitable for small-scale purifications (up to 1 g of cells or tissue can be lysed at a time). Generation of heat during sonication can be a problem and may result in protein denaturation (as evidenced by foaming); this problem can be overcome by sonicating the cell suspension in short bursts and allowing the sample to cool on ice between treatments.

Like the French Press method, grinding cells with *abrasive materials* such as alumina or sand is an efficient method of lysing unicellular organisms, as well as plant cells. Unlike the French Press, grinding requires inexpensive materials (e.g., mortar, pestle, and either sand or alumina) and is effective for moderate quantities of cells (up to 30 g wet weight of cells) (Fahnestock 1979; Sebald et al. 1979). Abrasives can also be added to sonication mixtures.

An extension of the grinding method is the *glass bead vortexing procedure*, which has been described for lysing unicellular organisms, particularly yeast (Schatz 1979). Glass bead vortexing is suitable for small samples (~3 g wet weight of cells) that can withstand being repeatedly vortexed with glass beads. A number of instruments are now commercially available for lysing larger quantities of cells (e.g., Manton-Gaulin homogenizer, Braun MSK Glass Bead Mill).

Methods based on *enzymatic breakdown* of the cell wall use the activity of lysozyme, which cleaves the glucosidic linkages in the bacterial cell-wall polysaccharide, to cause disruption (the inner cytoplasmic membrane can be readily disrupted by detergents, osmotic pressure, or mechanical methods). Enzymatic methods are much gentler than mechanical methods and are more easily applied to large-scale processing. Two generic methods for preparing bacterial extracts are given in Protocols 5 and 6. A method for preparing bacterial extracts containing histidine-tagged proteins is given in Protocol 7.

Disruption of Yeast Cells

A number of procedures exist for preparing yeast extracts, including autolysis (e.g., addition of toluene to yeast suspension), French pressure cell, abrasives (e.g., glass beads), and enzymatic lysis (for an overview, see Jazwinski 1990; Bridge 1996). Of these, the most widely used procedure is the use of abrasives. The abrasive action of well-agitated glass beads (typically, 0.5-mm diameter) on yeast cells yields up to 95% cell breakage (Jazwinski 1990). Although this method is useful for preparing enzymes and some cell organelles, it is considered too harsh to preserve the integrity of nuclei as well as protein complexes (enzymatic methods are recommended for this purpose). Major differences between abrasive procedures reside in the method employed to agitate the glass

beads. One of the simplest methods for agitating glass beads involves the use of a vortex mixer (Baker et al. 1988). A generic method for lysing small quantities of yeast cells employing the glass bead vortexing approach is given in Protocol 9. For a discussion on commercially available equipment for lysing yeast, see Jazwinski (1990).

RECOMBINANT PROTEIN CAN BE RECOVERED FROM INCLUSION BODIES BY DENATURATION AND RENATURATION

A significant impediment to overexpressing recombinant proteins in *E. coli* is the tendency for the targeted protein to form inclusion bodies, which are in vivo agglomerates of proteins that appear in the cytoplasm as large, dense bodies in scanning electron micrographs (Williams et al. 1982). Inclusion bodies are relatively rare in nature (most proteins are expressed in soluble form), with sickle cell anemia and other related blood diseases being the notable exception (Carrell et al. 1966). Overexpression of recombinant proteins from strong promoters on multiple-copy plasmids—with expression levels up to 40% of total cell protein—is thought to be the underlying reason for inclusion body formation in *E. coli.*

It is thought that inclusion body protein is partially or incorrectly folded, especially a protein containing disulfide bonds. An important difference between the expression of eukaryotic proteins in *E. coli* and their native environment is the inability of *E. coli* to form disulfide linkages, due to the reducing environment of its cytoplasm. Inclusion body formation of overexpressed recombinant protein in *E. coli* is found not only for foreign eukaryotic proteins, but also for overexpressed bacterial proteins that are normally soluble (Gribskov and Burgess 1983). Temperature-sensitive denaturation can be overcome (by reducing the frequency of inclusion body formation, and therefore increasing the soluble fraction of the target protein) by lowering the expression temperature from 37°C to 30°C (Schein and Noteborn 1988).

In addition to growth temperature, a number of other growth parameters have been manipulated to prevent inclusion body formation and increase the soluble fraction of the target protein. Most notable of these are the following:

- ***Varying the media composition and using different host strains*** (Schein and Noteborn 1988).
- ***Coexpression of molecular chaperones.*** For example, coexpression of DNAK increased the percentage of soluble human growth hormone in *E. coli* by ~87% (Blum et al. 1992), and coexpression of GroES and GroEL facilitated the purification of milligram quantities of recombinant p50^{csk} (Amrein et al. 1995).
- ***Fusion of the target protein with a highly soluble protein*** such as glutathione-*S*-transferase (Smith and Johnson 1988) or thioredoxin (La Vallie et al. 1993).
- ***Growing cells in the presence of sorbitol and glycyl betaine.*** Sorbitol facilitates the cellular uptake of the "protein stabilizer" glycyl betaine (Blackwell and Horgan 1991).

It should be emphasized that none of the above-mentioned approaches for increasing the soluble fraction of the target protein are general for all applications (Hockney 1995), and pilot experiments are recommended to ascertain the best approach for a particular protein. The following are two major advantages of inclusion bodies.

- By sequestering recombinant protein, these bodies permit the cell to express the protein at high levels.
- The inclusion bodies can be readily purified away from bacterial cytoplasmic proteins by centrifugation, yielding an effective purification step.

The major disadvantage of inclusion bodies is that extraction of the target protein requires the use of detergents. This problem is exacerbated where natively folded protein is required, particularly if the target protein contains more than three disulfide bonds. The protein contained within

inclusion bodies is generally insoluble in nonionic detergents and salts. The most widely used solubilizing agents are the water-soluble chaotropic agents, such as urea (8 M) and guanidine hydrochloride (6 M), that cause complete denaturation and are more compatible with protein refolding. Thus, the target protein can be renatured by simply removing the denaturant under conditions that favor complete refolding of the target protein over the formation of aggregates due to intermolecular protein–protein interactions. Such refolding conditions include low temperature and very low protein concentration (~1 μg protein/ml).

For cysteine-containing target proteins that contain disulfide bonds in their native structure, solubilization of inclusion bodies is usually performed in the presence of a reducing agent, such as dithiothreitol or β-mercaptoethanol, in order to fully reduce the target protein and to prevent the formation of disulfide-bonded aggregates (i.e., to favor intramolecular versus intermolecular disulfide bonds) during the refolding process. A generic procedure for the isolation and solubilization of inclusion bodies and renaturation of target protein is given in Protocol 8.

PREPARATION OF SUBCELLULAR EXTRACTS ENRICHES FOR TARGET PROTEINS

Two Main Steps in the Subcellular Fractionation Process

Although the aim of subcellular fractionation is to separate cellular compartments with minimal damage to them, it is evident that it is never possible with the use of current fractionation techniques to recover cellular organelles in a completely undamaged state. Indeed, it may never be possible to separate cell organelles in a natural state. There are two main steps in the subcellular fractionation process: homogenization of tissues and cells followed by separation of cellular organelles. The principal aims of any homogenization method are to

- achieve maximal cell breakage in a reproducible manner,
- use disruptive forces that minimize damage to the organelles of interest (e.g., protein denaturation and proteolytic degradation), and
- retain the original structure and functional integrity of the organelles of interest.

The principal methods for disrupting cells (osmotic shock, ultrasonic vibration, mechanical grinding or shearing, and nitrogen cavitation) are detailed in earlier parts of this chapter and in the protocols that follow. The components of the homogenate are then separated by procedures based on the variations in physical properties of the subcellular components, for example,

- centrifugal methods that separate organelles by size and/or buoyant density in gradient media,
- immunoisolation methods that use antibodies, which bind to specific surface proteins, and
- electrophoresis methods that separate proteins on the basis of surface charge distribution.

Detergents Can Be Used in Combination to Fractionate Eukaryotic Cells

Differential detergent extraction is an established method for cell fractionation, which partitions subcellular constituents into functionally and structurally distinct compartments (Lenk et al. 1977; Lenstra and Bloemendal 1983; Fey et al. 1984; Ramsby et al. 1994; Ramsby and Makowski 1999). The use of differential detergent fractionation (DDF) to obtain cell fractions enriched in cytosolic, membrane, nuclear, and cytoskeletal proteins for direct analysis by 2D electrophoresis has been described recently (Ramsby et al. 1994; Ramsby and Makowski 1999). DDF is applicable for the fractionation of cells grown in suspension or monolayer culture, as well as for fractionation of whole tissue, and can be modified to achieve specific fractionation goals, including further subfractionation.

DDF preserves the structural and functional integrity of cellular proteins, including the cytoskeleton, and permits direct biochemical analysis of detergent extracts by a variety of meth-

ods, including enzymatic assays, autoradiography, immunoblotting, immunoprecipitation, 2D electrophoresis, and mass spectrometry (Lenk et al. 1977; Lenstra and Bloemendal 1983; Fey et al. 1984; Ramsby and Kreutzer 1993; Ramsby et al. 1994; Patton 1999; Ramsby and Makowski 1999). Thus, DDF is useful in a variety of proteome research applications, and has been used to

- determine the subcellular localization of biologics,
- semipurify compartment-specific macromolecules,
- enrich for low-abundance proteins,
- investigate dynamic interactions between cytosolic and structural entities (i.e., membranes and the cytoskeleton), and
- monitor treatment-induced compartmental redistributions of macromolecules.

Protocol 10 describes a procedure for the fractionation of proteins from eukaryotic cells, and the preparation of those proteins for analysis by 2D electrophoresis.

2D or Not 2D?

It is apparent that two-dimensional (2D) gel electrophoresis (see Chapter 4), the classical method for studying the global expression of cellular proteins, has major limitations (Gygi et al. 2000):

- 2D electrophoresis gels are unable to resolve all proteins (although improvements in the technology, such as isoelectric focusing [IEF] gels covering only a single pH unit, have increased the resolution of the method) (Corthals et al. 2000; Gorg et al. 2000; Hanash 2000; Hoving et al. 2000; Wildgruber et al. 2000).
- Some protein classes (e.g., the membrane proteins) are underrepresented on 2D electrophoresis because of their poor solubility. Efforts to overcome this problem have been made by using novel detergents (see Santoni et al. 2000) and differential solubilization methods (see Molloy et al. 1998).
- The limited sample capacity and limited detection sensitivity of 2D electrophoresis reduce the ability to detect low-abundance proteins. For example, if a total yeast cell lysate is fractionated by 2D electrophoresis and silver-stained, proteins present at <1000 copies per cell (a large portion of the yeast proteome) are not detected (Gygi et al. 2000).

2D electrophoresis, like most other protein separation methods, is capable of revealing only a "narrow slice" of a cell's total proteome. To address this limitation, some measure of subcellular fractionation prior to 2D electrophoresis is required to bring low-abundance proteins into view. Other non-2D electrophoresis proteomic approaches for identifying low-abundance proteins include a combination of ion-exchange chromatography and reversed-phase high-performance liquid chromatography (RP-HPLC) (see Chapter 7).

Protocol 1

Homogenization of Mammalian Tissue

To purify or characterize an intracellular protein, it is important to choose an efficient method for disrupting the cell or tissue that rapidly releases the protein from its intracellular compartment into a buffer that is not harmful to the biological activity of the protein of interest. One of the most widely used methods for disrupting soft tissues is homogenization. In this protocol, three processes for tissue homogenization using mechanical shear are discussed: chopping the tissue in a Potter-Elvehjem glass-Teflon homogenizer, a Dounce hand homogenizer, or a hand-held Waring Blendor. These methods are rapid and pose little risk to proteins other than the release of proteases from other cellular compartments. Proteolytic degradation can be minimized by the inclusion of protease inhibitors in the homogenization buffers.

MATERIALS

CAUTION: See Appendix 5 for appropriate handling of materials marked with <!>.

Reagents

Appropriate animal tissue

Working with human tissue <!> presents a unique set of hazards.

Dithiothreitol (DTT) (0.5 M) <!>

Prepare a 0.5 M stock solution in cold H_2O and store frozen. Add the reagent to cold buffers at the indicated concentration just prior to use.

Homogenization buffer A
- 50 mM Tris-Cl (pH 7.5)
- 2 mM EDTA
- 150 mM NaCl
- 0.5 mM DTT <!>

Homogenization buffer B
- 50 mM Tris-Cl (pH 7.5)
- 10% (v/v) glycerol (or 0.25 M sucrose)
- 5 mM magnesium acetate
- 0.2 mM EDTA
- 0.5 mM DTT <!>
- 1.0 mM phenylmethylsulfonyl fluoride (PMSF) <!>

The choice of homogenization buffer will depend on the nature of the extract required. Generally, use a buffer of moderate ionic strength at neutral pH (e.g., 0.05–1.0 M phosphate or Tris, pH 7.0–7.5). The appropriate buffer ionic strength should be chosen by trial and error to optimize the yield of the target protein. For example, the addition of 0.1 M NaCl or KCl <!> will increase the yield of those proteins that have a tendency to attach electrostatically to cell debris/membrane fragments. On the other hand, the association-dissociation behavior of some proteins is influenced markedly by ionic strength. If the purpose of the extraction is to isolate organelles, it is important to use low-ionic-strength buffers (e.g., 5–20 mM Tris, HEPES, or TES at pH 7.4) containing iso-osmotic sucrose or mannitol (0.25 M). Avoidance of proteolytic degradation of the target protein in a crude extract is a primary concern. In many cases, it may not be

essential to add protease inhibitors to the homogenization buffer (due to the protective effect of bulk protein on a target protein), but some proteins are more susceptible to proteolysis than others, and some tissues (e.g., liver and pancreas) have much higher levels of proteases than others (e.g., heart). The use of protease inhibitor cocktails can be expensive if the extract volumes are large. Hence, carry out pilot experiments over a period of a few hours to ascertain whether there are measurable losses of the target protein activity. If proteolytic degradation is a problem, then include protease inhibitors in the homogenization buffers (for a list of protease inhibitors, see Table 2.2 and for the preparation of protease inhibitor cocktails, see Table 2.3). If the target protein is susceptible to oxidation or its activity is inhibited by heavy metals, then add DTT (1 mM) <!> (or 0.1 M β-mercaptoethanol <!>) and EDTA (0.1 M), respectively, to the extraction buffer.

Phenylmethylsulfonyl fluoride (PMSF) (0.2 M) <!>

Prepare a 0.2 M stock solution in 2-propanol, and add the reagent to cold buffers with adequate stirring just prior to use; the reagent will crystallize from 2-propanol when stored at –20°C. Aminoethylbenzenesulfonyl fluoride (AEBSF <!>) is a water-soluble alternative to PMSF that can be used at the same molar concentration (0.1–1.0 mM) for most applications.

Equipment

Centrifuge

Cheesecloth and filter funnel

Knife or meat grinder

Homogenizer (see note to Step 2)

- Power-driven Potter-Elvehjem glass-Teflon homogenizer
  - Clearance range: 0.05–0.6 mm.
- Dounce hand homogenizer
  - Loose fitting: 0.1–0.3-mm clearance. Tight fitting: 0.05–0.08-mm clearance.

TABLE 2.2. 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) <!> (*or* DCI [0.1 mM]or PMSF [0.2 mM]) <!>	serine	20 mM in methanol (DCI: 10 mM in DMSO; 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 (trasylol) (1 µg/ml) <!>	serine	0.1 mg/ml
	EDTA or EGTA[c] (1 mM)	metallo	100 mM
Plant tissues	AEBSF (0.2 mM) <!> (*or* DCI [0.1 mM] or PMSF [0.2 mM]) <!>	serine	20 mM in methanol (DCI: 10 mM in DMSO; 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) <!> (*or* DCI [0.1 mM]or PMSF [0.2 mM]) <!>	serine	20 mM in methanol (DCI: 10 mM in DMSO; 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) <!> (*or* DCI [0.1 mM] or PMSF [0.2 mM]) <!>	serine	20 mM in methanol (DCI: 10 mM in DMSO; 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)-benzenesulfonylfluoride; (DCI) 3,4-dichloroisocoumarin; (DMSO) dimethylsulfoxide; (EDTA) ethylenediamine tetraacetic acid; (EGTA) ethylene glycol bis (β-aminoethyl ether) *N,N,N,′N′*-tetraacetic acid; (PMSF) phenylmethylsulfonyl fluoride.

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

[a]For a review of proteolytic enzymes, see Neurath (1989) and Perlmann and Lorand (1970).

[b]Aqueous solution unless otherwise indicated.

[c]An efficient chelator of divalent metal cations other than Mg^{2+} (for which it has a 10^3-fold lower affinity) (Gegenheimer 1990).

TABLE 2.3. Preparation of a general protease inhibitor cocktail

Inhibitor	Trial working concentration	Stock (100x) concentration	Recipe	Use[a]	Target protease type
AEBSF <!>	<1 mM	20 mM	239.5 mg/10 ml H_2O (100 mM)	4 ml	serine proteases
EDTA	1–10 mM	100 mM	19 mg/100 ml H_2O (0.5 M)	4 ml	metallo proteases
Leupeptin <!>	10–100 μM	2 mM	18.9 mg/2 ml H_2O (20 mM)	2 ml	cysteine/serine proteases
Pepstatin	1 μM	100 μM	6.8 mg/10 ml methanol (1 mM)	2 ml	aspartic proteases

Adapted from Calbiochem Technical Bulletin CB0578-0998.

[a]Mix the inhibitor solutions and bring to a final volume of 20 ml with H_2O or appropriate aqueous buffer. The resulting solution, 20 ml of a stock 100x protease inhibitor cocktail, can be aliquoted into microfuge tubes and stored at –20°C until required.

Waring Blendor

A mechanical shear homogenizer that uses rotating metal blades or teeth to disrupt the material. There are many variations of the traditional domestic food liquidizer in which the material is placed in a glass reservoir with the blades driven by a motor beneath it. Other models resemble the modern hand-held blenders in which the motor is overhead. The Waring Blendor is typically used to macerate large amounts (100–1000 ml) of hard animal tissue and plant tissue. For smaller volumes (~1–5 ml), the Ultra-Turrax (IKA Works) and its successor, the Polytron homogenizer, are widely used.

METHOD

Carry out all procedures at 0–4°C.

1. After the tissue is excised from the animal, trim and discard fat and connective tissue from the tissue. Place the tissue in cold Homogenization buffer A.
2. Dice the washed tissue into small pieces (i.e., 1-cm cubes) with a knife or, alternatively, pass the tissue through a meat grinder twice.

   Tissues such as liver, brain, kidney, and heart are readily homogenized in a Waring Blendor, but tissues such as skeletal muscle and lung are tougher, and it is advisable to grind them in a domestic meat grinder prior to homogenization. Very fibrous tissues such as mammary glands must be frozen prior to homogenization to facilitate disruption (the Ultra-Turrax homogenizer is widely used for this purpose). Cultured mammalian cells and small amounts (1–5 g) of soft tissue such as brain can be homogenized conveniently using a Dounce hand homogenizer (Dignam 1990). In all cases, prechill the homogenizers and glassware to 4°C, and work in a cold room while using the blender.
3. Add 3–4 volumes of Homogenization buffer B per volume of tissue, and transfer the mixture to the homogenizer.
4. Prepare the homogenate using one of the following methods:

   Potter-Elvehjem homogenizer: Homogenize the tissue with the apparatus set at 500–1500 rpm, allowing 5–10 seconds per stroke.

   Dounce hand homogenizer: Homogenize the tissue with 10–20 strokes of the pestle.

   Waring Blendor: Homogenize the tissue three to four times for 20–30 seconds each (no longer), pausing for 10–15 seconds between each homogenization.
5. Pour the homogenate into a glass beaker, place the beaker on ice, and stir the homogenate gently for 30–60 minutes at 4°C to allow further extraction of proteins.

   Do not allow the homogenate to foam.
6. Remove cell debris and other particulate matter from the homogenate by centrifugation at 10,000*g* for 10–20 minutes at 4°C.

7. Filter the supernatant through two layers of cheesecloth (or a plug of glass wool) in a filter funnel to remove any fatty material that has floated to the surface. Carefully squeeze the cloth to obtain the maximum amount of filtrate (referred to as the "crude extract").
8. Proceed with the appropriate fractionation or analysis strategy as quickly as possible (see Chapter 1).

ADDITIONAL PROTOCOL: REMOVAL OF MUCIN FROM TISSUE HOMOGENATES

The presence of mucin in bulk biological extracts (e.g., colonic mucosa) complicates subsequent purification because the mucin binds to most chromatographic supports causing blockage of the column. Mucin can be selectively removed from tissue homogenates using this protocol, which was developed for the isolation and characterization of novel growth factors in colonic mucosa (Nice et al. 1991).

Additional Materials

CAUTION: See Appendix 5 for appropriate handling of materials marked with <!>.

Ammonium carbonate buffer (7.85 g/liter, pH 9.2, ice-cold) containing the proteolytic inhibitors pepstatin (3 mg/liter), HCl (1 M), <!> and PMSF (22 mg/liter) <!>
HCl (1 M) <!>

Method

1. Homogenize mucosa scraped from the descending colon, using an Ultra-Turrax homogenizer, in 5 volumes of ice-cold carbonate buffer (pH 9.2) containing pepstatin and PMSF.
2. Remove the tissue debris by centrifugation at 10,000*g* for 20 minutes.
3. Slowly adjust the pH of the resulting supernatant to 4.5 (>2 hours) by the dropwise addition of 1 M HCl (~100 ml required) with continuous stirring at 4°C.

   This results in a flocculent precipitate of mucin (Glass 1964).
4. Remove the precipitate by centrifugation at 10,000*g* for 20 minutes at 4°C.

   The supernatant is the crude extract, which can be used as starting material in the desired purification strategy.

Protocol 2

Lysis of Cultured Cells for Immunoprecipitation

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% NP-40 or 1% Triton X-100), this method may be more gentle to the protein of interest than the homogenization methods discussed in Protocol 1. 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) (for lysis buffer details, see Table 2.4). This protocol was contributed by Hong Ji (Joint ProteomicS Laboratory of the Ludwig Institute for Cancer Research, and Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia).

MATERIALS

CAUTION: See Appendix 5 for appropriate handling of materials marked with <!>.

Reagents

Cell culture, suspension, or monolayer

Lysis buffer

Good first choices are NP-40 lysis buffer (Triton X-100 <!> may be substituted for NP-40) and RIPA lysis buffer. For additional information on choosing a lysis buffer, see Table 2.4. Chill the lysis buffer to 4°C prior to use.

TABLE 2.4. Commonly used lysis buffers for lysing cultured cells

Buffer	Comments
NP-40 lysis 150 mM NaCl 1.0% NP-40 50 mM Tris-Cl (pH 7.4)	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% NP-40 0.5% sodium deoxycholate <!> 0.1% SDS <!> 50 mM Tris-Cl (pH 7.4)	A much harsher denaturing lysis buffer than NP-40, due to the inclusion of two ionic detergents (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 NaF, 40 mM β-glycerol phosphate, 100 μM Na_3VO_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 PMSF (50 μg/ml). Alternatively, commercially available protease cocktail tablets (e.g., from Boehringer) can be included. Also see Tables 2.2 and 2.3.

Phosphate-buffered saline (PBS)
- 8.0 g of NaCl (final concentration 137 mM)
- 0.2 g of KCl (final concentration 2.7 mM) <!>
- 1.44 g of Na_2HPO_4 (final concentration 10.1 mM)
- 0.24 g of KH_2PO_4 (final concentration 1.8 mM)
- in 800 ml of H_2O

Adjust pH to 7.4, and then adjust volume to 1 liter with H_2O.

METHODS

Method 1: Lysing Cells Grown as Monolayer Cultures

1. Discard the culture medium, and wash the cells twice with ice-cold PBS.
2. Place the culture dishes on ice.
3. Add 1.0 ml of lysis buffer (chilled to 4°C) per 100-mm dish. For culture dishes of other sizes, adjust the volume of lysis buffer accordingly.
4. Incubate the cells for 10–30 minutes (dependent on cell lines being studied) on ice with occasional rocking of the dishes.
5. Tilt a dish on the bed of ice and allow the buffer to drain to one side; remove the lysate with a pipette and transfer it to a microfuge tube or other suitable centrifuge tube. Repeat with all of the remaining dishes.

   Although some researchers prefer to scrape the cells from the tissue-culture dish, this does cause some stress to the cells and is only required in unusual cases.
6. Centrifuge the lysate at 20,000*g* for 10 minutes at 4°C.
7. 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.

Method 2: Lysing Cells Grown in Suspension

1. Harvest the cells by centrifugation at 480*g* for 10 minutes. Pour off the supernatant and discard.
2. Carefully wash the cell pellet twice with ice-cold PBS, and then place the washed cell pellet on ice.
3. Resuspend the pellet in 1.0 ml of lysis buffer (chilled to 4°C) per 1×10^7 to 5×10^7 cells.
4. Incubate the cells for 15 minutes on ice with occasional vortexing of the tube.
5. Centrifuge the lysate at 20,000*g* for 10 minutes 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.

Protocol 3

Lysis of Cultured Animal Cells, Yeast, and Bacteria for Immunoblotting

Cell lysis with detergents is commonly used with cultured animal cells. Typically, the ionic detergent SDS (e.g., 2% SDS) is sufficient for lysing cells for the purpose of immunoblotting studies. Both cultured animal cells and bacteria such as *E. coli* may be lysed in this manner. If the antigenic determinant recognized by the antibody being studied is dependent on the native spatial conformation and sensitive to reducing conditions, then dithiothreitol should be omitted from the lysis buffer and nonreducing/non-urea gels may need to be employed (Ji et al. 1997; Ji and Simpson 1999). This protocol was contributed by Hong Ji (Joint ProteomicS Laboratory of the Ludwig Institute for Cancer Research, and Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia).

MATERIALS

CAUTION: See Appendix 5 for appropriate handling of materials marked with <!>.

Reagents

Cell culture, suspension or monolayer, from bacteria or yeast cells

10^9 tissue culture cells is ~1 ml or ~1 g.

Dithiothreitol (DTT) (1 M) (10x stock solution) <!>

Store DTT in aliquots at –20°C.

1x Laemmli sample buffer

- 2% SDS <!>
- 10% glycerol
- 60 mM Tris-Cl (pH 6.8)
- 0.01% bromophenol blue <!>

It is often convenient to prepare Laemmli sample buffer as a 2x or 5x stock. Just prior to use, add DTT <!> to a final concentration of 100 mM.

Equipment

Centrifuge

Water bath or heating block preset to 95°C

METHOD

1. Add 1 ml of Laemmli sample buffer containing 100 mM DTT to 1×10^7 to 5×10^7 cells.
2. The cell lysate becomes highly viscous in Laemmli buffer due to the presence of released DNA. Two options are available for surmounting this viscosity problem.
   - Centrifuge the lysate at 100,000*g* for 20 minutes to remove DNA.
   - Lyse the cells by sonicating the mixture using four bursts of 15–30 seconds each. Transfer the samples to ice for 15 seconds between each sonication step.

3. Heat the collected supernatant or sonicated sample for 5 minutes at 95°C.
4. Centrifuge at 20,000*g* for 5 minutes.
5. Transfer the supernatant to a fresh tube.
6. Prepare the samples (supernatant) for electrophoresis (see Chapter 3, Protocol 1) and immunoblotting (see Appendix 4). When preparing cell culture extracts for immunoblotting, the protein sample must be
   - in a solution that is compatible with the gel electrophoresis system (e.g., the pH of the solution should be ~7.0 and the salt concentration ~200 mM) and
   - at a protein concentration that does not exceed the loading capacity for a particular gel system (for a discussion of gel electrophoresis variables, see Chapter 3). As a rule of thumb, for a conventional gel, do not load >150 µg of total protein per lane for a minigel.

Protocol 4

Disruption of Cultured Cells by Nitrogen Cavitation

Cell disruption by nitrogen decompression from a pressurized vessel is a rapid and effective way to homogenize cells and tissues, to release intact organelles, and to prepare cell membranes (Hunter and Commerford 1961). The principle of the method is simple: Cells are placed in a pressure vessel, and large quantities of oxygen-free nitrogen are first dissolved in the cells under high pressure (~5500 kPa, which is equivalent to 800 psi). When the gas pressure is suddenly released, the nitrogen comes out of solution as bubbles that expand and stretch the cell membrane, rupturing it, and releasing the contents of the cell. Nitrogen cavitation is well suited for mammalian and plant cells and fragile bacteria (i.e., bacteria treated to weaken the cell wall), but it is less effective at lysing yeast, other fungi, spores, or other cell types with tough cell walls. Features of the nitrogen cavitation method include the following:

- It is a gentle method for homogenizing or fractionating cells because the chemical and physical stresses that it imposes upon enzymes and subcellular compartments are minimized compared to other ultrasonic and mechanical homogenizing methods. For example, functional intact nuclei and mitochondria can be released from most cell types.
- Unlike many cell lysis methods relying on shear stresses and friction, no heat is generated with the nitrogen cavitation because this method is accompanied by an adiabatic expansion that cools the sample instead of heating it. Hence, there is no heat damage to proteins and organelles.
- Any labile cell components are protected from oxidation by the use of an inert gas, nitrogen. Furthermore, nitrogen does not alter the pH of the suspending medium.
- The process is fast and uniform because the same disruptive forces are applied within each cell and throughout the sample, ensuring reproducible cell-free homogenates.
- Variable sample sizes (e.g., from ~1 ml to 1 liter or more) can be accommodated with most commercial systems.

This protocol, adapted from the Kontes Glass Company "instructions for users," is designed for small volumes (1–15 ml) of tissue culture cells using the Kontes Mini-Bomb cell disruption chamber (see Fig. 2.1).

MATERIALS

CAUTION: See Appendix 5 for appropriate handling of materials marked with <!>.

Reagents

Cultured animal cells or tissues

Homogenization medium

  0.25 M sucrose

  20 mM Tris-Cl (pH 7.4)

For alternative homogenization buffers, see Protocol 1. For nitrogen cavitation, choose any buffer compatible with subsequent purification steps. Isotonic solutions are suitable for most applications. Hypertonic solutions tend to stabilize organelles. Low concentrations of $MgCl_2$ <!>, magnesium acetate, or $CaCl_2$ have been reported to stabilize nuclei.

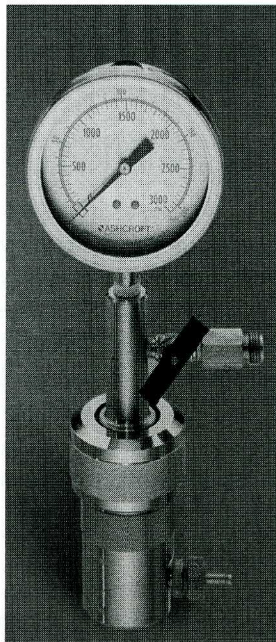

FIGURE 2.1. Nitrogen cavitation vessel. The image shown is the Parr Instrument Company Model 4639 Cell Disruption Vessel, designed specifically for small samples from 30 ml down to less than one milliter. (Parr Instrument Company 4636 and 4639 Cell Disruption Vessels.)

Phosphate-buffered saline

- 8.0 g of NaCl (final concentration 137 mM)
- 0.2 g of KCl (final concentration 2.7 mM) <!>
- 1.44 g of Na_2HPO_4 (final concentration 10.1 mM)
- 0.24 g of KH_2PO_4 (final concentration 1.8 mM)
- in 800 ml of H_2O

Adjust pH to 7.4, and then adjust volume to 1 liter with H_2O.

Equipment

Cell disruption chamber

Commercially available cell disruption chambers (also called nitrogen pressure vessels) include:

- Mini-Bomb cell disruption chamber (Kontes Glass Company, Vineland, New Jersey; www.kimble-kontes.com).
- Parr Cell Disruption Bomb (Parr Instrument Company, Moline, Illinois; www.parrinst.com).
- Baskervilles (Manchester, U.K.) or Artisan Industries (Waltham, Massachusetts).

Magnetic stirrer

Nitrogen (oxygen-free) <!>

Rubber policeman

METHOD

CAUTION: Because high pressures are generated, carry out this procedure behind a shield.

1. Ensure that the outlet port is closed (do not over-tighten), and cool the cell disruption chamber (if necessary) by immersing the unit in an ice bath.
2. Prepare the cells or tissue for disruption.

   For adherent cultured cells: Wash the cells gently two or three times with phosphate-buffered saline (PBS). Use a rubber cell scraper to scrape the cells from the dish into PBS.

For nonadherent cells: Collect the cells by centrifugation at 400*g* for 10 minutes. Wash the pellet of cells two or three times with PBS.

For animal tissue: Mince the tissue using a mechanical homogenizer or by passing through a screen or sieve.

3. Collect the cells by centrifugation at 480*g* for 5 minutes at 4°C.
4. Discard the supernatant and resuspend the cells in 10 volumes (~15 ml) of homogenization medium. Transfer the suspension to a plastic beaker (containing a magnetic stir bar) and gently mix to obtain a uniform suspension.
5. Remove the filter holder from the chilled cell disruption chamber by using a turning and lifting motion.
6. Add up to 15 ml of cell suspension to the cell disruption chamber.

   For larger volumes, an extension is available for the Mini-Bomb. Alternatively, other nitrogen pressure vessels can be used.
7. Replace the filter holder.
8. Attach the unit to the source of oxygen-free nitrogen by screwing on the cap of the pressure vessel, which should already be connected to the source of nitrogen. *HAND TIGHTENING* is all that is required.
9. Replace the pressure vessel in the cooling bath if desired.
10. Pressurize the bomb to the desired pressure by allowing nitrogen to flow from the cylinder into the disruption chamber.

    There must be a gauge on the filling connection that shows the pressure inside the chamber. The amount of pressure required depends on the type of cells being disrupted. Typical pressures used include:

    - 500 psi for KB cells
    - 500 psi for rat liver
    - 1000 psi for chicken red blood cells

    Step 10 must be optimized for each application. In general, lower pressures lead to a greater recovery of intact organelles, whereas higher pressures can result in total homogenization. Cells that are difficult to lyse can be treated more than once for more complete homogenization. For additional information on effective gas pressures to use to disrupt various cells, see Table 2.5.

    IMPORTANT: Inexperienced workers handling high-pressure gases must always seek expert help before using a gas cylinder.
11. Allow the pressurized apparatus to equilibrate for at least 30 minutes to allow the nitrogen to dissolve and come to equilibrium within the cells. Gentle agitation once or twice during this time period with a magnetic stirrer or periodic gentle shaking keeps the cells in a uniform suspension.
12. Place a collection container (e.g., beaker) in an ice bath by the outlet port.
13. With the pressurization unit still activated, *slowly* open the outlet port until the cell suspension begins to flow into the chilled collection container. Make sure that the pressure is maintained until all of the suspension is out.

    A reasonable flow rate is 3–10 drops per second. An indication that the unit is almost empty is the appearance of a "foamier" flow of fluid. The end of the flow is accompanied by a gentle hissing.

    Cell lysis occurs upon release of pressure, but passage through the narrow orifice of the outlet port often aids cell disruption.

    IMPORTANT: The homogenate is expelled with great force, so this operation must be carried out very carefully, particularly if the sample contains hazardous materials. Because of the potential for creating an aerosol, this procedure must always be performed in a safety hood.

TABLE 2.5. Suggested working parameters for disruption of cells using nitrogen cavitation

Cell type	Cell suspension	psi	No. cells used/ml	No. times through	No. cells remaining/ml	No. nuclei remaining/ml	% cells totally lysed
Cultured cells	KB	500	3.4×10^6	1	2.8×10^5	n.d.	93
	KB	500		2	2.0×10^3	n.d.	99.95
	KB	250	3.3×10^6	1	5.5×10^5	4.62×10^5	69.4
	KB	250		2	5.4×10^4	2.2×10^4	97.7
	KB	250		3	0	0	100
	KB	0	1.8×10^6	0	1.08×10^6	7.6×10^5	–
	KB	250		1	2.4×10^5	6.4×10^5	52.2
	KB	250		2	3.4×10^4	5.0×10^4	95.4
	KB	250		3	1.6×10^4	4.6×10^4	96.6
Tissue	rat liver	500	9.2×10^5	1	8.5×10^5	n.d.	7.8
		500		2	0		100
Blood	chicken red blood cells	1000	5.6×10^9	1	9.6×10^8	n.d.	83
Bacteria	*E. coli*	1500	1.3×10^{10}	1	1.2×10^{10}	n.d.	8
		1500		2	6.6×10^9	n.d.	50
	E. coli	1500	6.5×10^8	1	3.6×10^8	n.d.	45
		1500		2	3.3×10^8	n.d.	50

These values were determined with the Mini-Bomb cell disruption chamber. (KB cells) Human oral epidermoid carcinoma. n.d. indicates not determined. (Modified from Kimble/Kontes [http://208.72.236.210/html/pg-881455.html].)

14. Gently stir the "foam" to allow it to subside before any centrifugation or subsequent work up of the homogenate is carried out.
15. Close the outlet port. Do not overtighten it.
16. Turn off the flow of nitrogen at its source.
17. Discharge the gas in the unit by opening the outlet port and venting the unit. Once the pressure inside the bomb has returned to atmospheric pressure, the chamber can be opened for recovery of any remaining sample and thorough cleaning.

    IMPORTANT: If the lysed cell material is potentially hazardous, vent the gas through a suitable trap.

Protocol 5

Small-scale Extraction of Recombinant Proteins from Bacteria

Bacteria are particularly convenient for producing recombinant proteins for purification purposes. To monitor induction as well as the levels of recombinant protein expression, it is important to have a rapid, simple method for estimating bacterial protein expression. This protocol describes the preparation of small-scale bacterial extracts using cell lysis with 0.5% Triton X-100.

MATERIALS

CAUTION: See Appendix 5 for appropriate handling of materials marked with <!>.

Reagents

Bacterial cells expressing target recombinant protein
Dithiothreitol (DTT) (1 M) (10x stock solution) <!>

Store DTT in aliquots at –20°C.

1x Laemmli sample buffer
- 2% SDS <!>
- 10% glycerol
- 60 mM Tris-Cl (pH 6.8)
- 0.01% bromophenol blue <!>

It is often convenient to prepare Laemmli sample buffer as a 2x or 5x stock. Just prior to use, add DTT <!> to a final concentration of 100 mM.

Triton X-100 (0.5% v/v) <!>

Cool to 4°C before use.

Equipment

Boiling-water bath
Electrophoresis equipment (Chapter 3, Protocol 1)
Sonicator <!>

METHOD

1. Harvest the bacterial cells from 1 ml of culture by centrifugation in a microfuge for 1 minute at room temperature.
2. Pour off the supernatant and resuspend the bacterial cell pellet in 1 ml of chilled aqueous 0.5% (v/v) Triton X-100.
3. Sonicate the suspension for three cycles of 20 seconds each, cooling the cells on ice between treatments.
4. Centrifuge the suspension in a microfuge for 1 minute.
5. Add Laemmli buffer containing 100 mM DTT to the supernatant, boil the mixture for 2 minutes, and analyze for the target protein using analytical SDS-PAGE (see Chapter 3, Protocol 1).

Protocol 6

Large-scale Extraction of Recombinant Proteins from Bacteria

Bacteria are particularly convenient for producing recombinant proteins for purification purposes. Suitable extraction methods for bacterial cells include sonication, glass bead milling, grinding with alumina or sand, high-pressure shearing using the French pressure cell (French Press), and lysozyme treatment. These procedures are applicable for preparing extracts from a variety of Gram-negative bacteria such as *E. coli* and *Klebsiella pneumoniae* Gram-positive bacteria such as *Bacillus subtilis*. Disruption of bacterial cells by enzymatic means is commonly used because a relatively uniform treatment is obtained when cells are in suspension. A protocol for enzymatic disruption of *E. coli* follows.

MATERIALS

CAUTION: See Appendix 5 for appropriate handling of materials marked with <!>.

Reagents

Bacterial cells expressing target recombinant protein
Dithiothreitol (DTT) (1 M) (10x stock solution) <!>

Store DTT in aliquots at –20°C.

DNase I
1x Laemmli sample buffer
- 2% SDS <!>
- 10% glycerol
- 60 mM Tris-Cl (pH 6.8)
- 0.01% bromophenol blue <!>

It is often convenient to prepare Laemmli sample buffer as a 2x or 5x stock. Just prior to use, add DTT <!> to a final concentration of 100 mM.

Lysis buffer
- 50 mM Tris-Cl (pH 7.4)
- 25% (w/v) sucrose

EDTA can be included (to a final concentration of 10 mM) to minimize proteolytic degradation due to metalloproteases. Although the inclusion of EDTA and protease inhibitors such as 1 mM PMSF <!> (or AEBSF <!>), 1 µg/ml aprotinin <!>, or 10 µg/ml leupeptin <!> in the lysis buffer may not be necessary when the target protein is packaged in inclusion bodies, they may be important in the solubilization/ refolding steps (unfolded proteins are more susceptible to proteolytic degradation) or when extracting soluble proteins.

Lysozyme (hen egg)
$MgCl_2$ (1 M) <!>
NP-40 (10% v/v)

Equipment

Centrifuge
Electrophoresis equipment (see Chapter 3, Protocol 1)
Waring Blendor (see Step 4)

METHOD

1. Harvest the bacterial cells by centrifugation at 3000*g* for 15 minutes at 4°C.
2. Wash the cells with lysis buffer to remove the residual culture medium and harvest the washed cells by centrifugation as in Step 1.
3. Pour off the supernatant and weigh the wet pellet.
4. Resuspend the washed *E. coli* cells in ~3 ml of lysis buffer per gram of cell pellet and stir the suspension for 30 minutes at 4°C. If the pellet is not fully resuspended after 30 minutes, mix the suspension in a Waring Blendor at low speed for ~1 minute.
5. Add lysozyme to a concentration of 0.1% (w/v) and incubate for 35 minutes at 4°C, shaking gently.

   A faster rate of lysis may be obtained by increasing the lysozyme concentration to 1.0% (w/v) (10 mg/ml). Under these conditions, satisfactory lysis can be accomplished in as little as 5 minutes at temperatures as low as 4°C (Bollag et al. 1996).

6. Add in the following order:

   NP-40 to a final concentration of 0.5% (v/v)
   $MgCl_2$ to a final concentration of 5 mM
   DNase I to a final concentration of 40 μg/ml

   Stir the suspension for 30 minutes at 4°C to remove the viscous nucleic acid.

   Bacterial extracts are 40–70% protein, 10–30% nucleic acid, 2–10% polysaccharide, and 10–15% lipid (Worrall 1996). The release of DNA upon cell lysis often results in a highly viscous extract that can cause serious problems in subsequent chromatographic purification steps. In addition to DNase I treatment, DNA can be removed from the cell extract (along with other nucleic acids, and in some cases, highly acidic proteins) by the addition of a neutralized solution of positively charged compounds such as protamine sulfate (up to 5 mg/g wet weight of cell pellet) (Scopes 1994) or polyethyleneimine (Burgess and Jendrisak 1975). Methods for DNA removal involving positively charged compounds should not be used with inclusion body preparations, since the precipitated DNA will cocentrifuge with the inclusion bodies.

7. Centrifuge the suspension at 23,000*g* for 30 minutes at 4°C.
8. Resuspend a small portion of the pellet in Laemmli buffer containing DTT.
9. Analyze aliquots of both the soluble protein fraction (supernatant) and pellet fraction for the presence of target protein using analytical SDS-PAGE (see Chapter 3, Protocol 1).

   If the bulk of the target protein is found in the insoluble pellet fraction, then inclusion bodies have likely formed, and the target protein will need to be solubilized and purified according to Protocol 8. If the target protein is found in the supernatant, this material should be stored at 4°C in readiness for the next purification protocol(s).

Protocol 7

Preparation of Clarified *E. coli* Extract Containing Histidine-tagged Proteins

Recombinant protein fused to a histidine peptide tag can be readily expressed in *E. coli* and subsequenctly purified on an immobilized metal ion affinity (IMAC) column (see Chapter 9). The goals of the extract preparation are to release the target protein from the cells and remove insoluble material that may foul the IMAC column filters and bed. The viscosity of the extract can be reduced by adding DNase (e.g., Benzonase) together with $MgCl_2$ to fragment the bacterial DNA. To avoid proteolysis of the target protein, PMSF or other protease inhibitors (but preferably not EDTA) can be included. Lysozyme, DNase, and $MgCl_2$ can often be omitted when using a high-pressure homogenizer. This protocol describes a typical lysis method. Other methods and conditions may be required for some proteins depending on the protein stability, solubility, and tendency to adsorb to cell debris.

MATERIALS

CAUTION: See Appendix 5 for appropriate handling of materials marked with <!>.

Reagents

E. coli cells expressing histidine-tagged target recombinant protein

Imidazole-HCl (2 M, pH 7.4) <!>

Imidazole purchased from Merck (Darmstadt, Germany) is essentially colorless. Other imidazole products on the market have been successfully used in IMAC, although many of them are discolored by impurities that affect the baseline of the UV trace in chromatograms.

NaCl (4 M)

Supplemented Buffer P

- 50 mM sodium phosphate (pH 7.4) <!>
- 0.5 M NaCl
- 0.5 mg/ml lysozyme <!>
- 1 mM PMSF <!>
- 1.7 units/ml Benzonase (Merck, Darmstadt, Germany)
- 1 mM $MgCl_2$ <!>

Add the lysozyme <!>, PMSF <!>, and Benzonase just prior to use in Step 3.

Equipment

Filter apparatus (0.45 µm)

Manton-Gaulin homogenizer (APV Gaulin model 30 CD)

Optional, see Step 4.

Sonicator, probe-type <!>

METHOD

1. Harvest *E. coli* cells from a 1-liter suspension culture (10–100 g of sedimented cells) by centrifugation at 7000–8000*g* for 10 minutes at 4°C or 1000–1500*g* for 30 minutes at 4°C.
2. Discard the supernatant and place the centrifuge bottles on ice.

   If the cell paste will not be used immediately, store it at –70°C. Freeze the cell paste in flat containers or flattened plastic bags to make thawing easy or to allow frozen pieces to be used.
3. Suspend 1–2 g of cell paste in 10 ml of supplemented Buffer P, and incubate the suspension for 30–60 minutes on ice. After incubation, check that the pH is 7–8, and if necessary, adjust the pH with dropwise addition of 0.5 M NaOH with vigorous mixing.

   Lysozyme concentrations as high as 10 mg/ml may be used to reduce incubation time to 5 minutes.
4. Use a probe sonicator to sonicate the cell suspension four times for 20 seconds each on ice. Wait 2 minutes between each cycle to cool the sample. Alternatively, if the protein is stable enough, freeze and thaw the suspension at least twice to disrupt the cells. This may be more convenient but less efficient. Check that the pH is still 7–8. If not, adjust it as in Step 3.

   When processing a large volume of cells, instead of performing Steps 1–4, homogenize the cells from a 10-liter fermentor (50–500 g of *E. coli* cells) as follows:

   a Centrifuge the cells at 7000*g* for 10 minutes at 4°C.

   b. Resuspend them in ice-cold 20 mM Na-phosphate buffer (pH 7.4) and 0.5 M NaCl supplemented with 1 mM PMSF, to a final volume of 1 liter.

   c. Pass the suspension three times through a high-pressure homogenizer at 800 bar.
5. Centrifuge the homogenate at 12,000*g* for 10 minutes at 4°C to remove cell debris.

   Depending on the lysis method, higher *g* values and longer run times (e.g., 50,000*g* for 30 minutes) may be required to clarify the sample. Cell debris that remains in the supernatant may lead to increased back pressure and reduce the life span of the IMAC column.
6. Decant and, if required, filter the supernatant through a 0.45-μm filter.

   Extract may be stored in aliquots at –70°C or used in Step 7. When thawed, frozen extract should be filtered through a 0.45-μm filter or centrifuged before use.
7. Adjust the extract to 20 mM imidazole and 500 mM NaCl by adding 116 μl of 2 M imidazole and 1.45 ml of 4 M NaCl per 10 ml of sample.

   The previous concentration of NaCl is not taken into account because a higher ionic strength will not be a problem. Addition of imidazole and NaCl may also be done before clarification (Steps 5 and 6).
8. Analyze the concentration of target protein by ELISA (enzyme-linked immunosorbent assay) using anti-His_6 antibodies or target-protein-specific antibodies. Alternatively, estimate the concentration by applying a small volume of the sample onto a small IMAC column (see Chapter 9).

   SDS-PAGE can also be used to determine a rough estimate of the target protein concentration. Knowing the protein concentration aids in selecting the proper column size for the IMAC purification and in estimating recovery.
9. Purify the histidine-tagged protein using an IMAC column (see Chapter 9, Protocols 2 and 3).

Protocol 8

Solubilization of *E. coli* Recombinant Proteins from Inclusion Bodies

Because molecular cloning techniques allow high levels of expression in bacteria, this is a particularly convenient system for producing recombinant proteins. Regrettably, these proteins are often difficult to purify due to their tendency to aggregate and precipitate within the bacteria to form insoluble inclusion bodies. The formation of inclusion bodies is especially common for nonbacterial proteins. Although no single method can be applied to every protein, a number of strategies are available to solubilize inclusion body proteins. One of these strategies is described in this protocol. A number of steps must be considered in solubilizing inclusion body proteins:

- cell lysis
- isolation of inclusion bodies
- washing of inclusion bodies
- solubilization of inclusion bodies
- renaturation (if required) of recombinant protein

First, the washing of inclusion bodies prior to solubilization is an important step for removing nontarget proteins, as well as nonproteinaceous material. For example, washing inclusion bodies with 1 M guanidine hydrochloride was used to remove contaminating proteins from recombinant IL-2 (Weir et al. 1987), and 4.0 M urea was used in the case of IL-6 inclusion bodies (Zhang et al. 1992). It is important to remove nonproteinaceous (lipid-like) material in order to prevent the clogging of RP-HPLC columns and therefore prolong column lifetimes. This can be accomplished by extensive washing of inclusion bodies with 4.0 M urea (Zhang et al. 1992) or extraction of the inclusion bodies with butanol-1-ol/10 mM EDTA (Weir et al. 1987). Whereas some target proteins will refold due to air oxidation (e.g., IL-6 [Zhang et al. 1992]), the most common methods to induce proper refolding of proteins use extensive dilution and dialysis in the presence of thiol reagents (e.g., 1 mM DTT) to gradually reduce the concentration of denaturant and promote correct disulfide bond formation.

MATERIALS

CAUTION: See Appendix 5 for appropriate handling of materials marked with <!>.

Reagents

Bacterial cells expressing the target recombinant protein

Renaturation buffers

See Step 9 for examples.

Solubilization buffer (8 M guanidine hydrochloride <!> in 50 mM Tris-Cl [pH 7.5])

The ideal solubilization buffer for a particular protein may have to be determined empirically. See Steps 8 and 9.

Washing buffer

100 mM Tris-Cl (pH 8.0)

2–4.0 M urea <!>

1% (v/v) Triton X-100 <!>

Equipment

Centrifuge
Electrophoresis equipment (Chapter 3, Protocol 1)

METHOD

1. Lyse the cells as described in Protocol 6.
2. Centrifuge the cell lysate at 23,000*g* for 30 minutes at 4°C.
3. Pour off the supernatant and measure the mass of the pellet (wet mass).
4. Resuspend the pellet in ~10 volumes of washing buffer. Stir the suspension for 1 hour at room temperature.
5. Centrifuge the mixture at 23,000*g* for 30 minutes at 4°C.
6. Pour off the supernatant and recover the pellet.
7. Repeat Steps 4–6 three more times (or until the recombinant protein is >80% pure, as judged by analytical SDS-PAGE).
8. Dissolve the pellet from Step 7 in ~8 ml of solubilization buffer per gram wet mass pellet (determined in Step 3), and gently stir the mixture for 16–20 hours at 4°C.

   Do not exceed a protein concentration of 2–3 mg/ml.

   Alternative solubilization buffers:

   - 8 M guanidine hydrochloride, 10 mM DTT, 50 mM Tris-Cl (pH 8.5).

     Use 15 ml per 12–15 g wet weight of harvested cells. Incubate the mixture for 1 hour at 37°C (Weir et al. 1987). Renature the protein as described in Step 9a.
   - 8 M urea, 2 mM reduced glutathione/0.2 mM oxidized glutathione.

     Use 9 volumes of buffer per gram wet weight of inclusion body pellet. Incubate the mixture for 1 hour at room temperature. Do not exceed a protein concentration of 2.5 mg/ml (Bollag et al. 1996). Renature the protein as described in Step 9b.
9. Refolding conditions for a specific target protein must be optimized in pilot-scale experiments. Examples of renaturation conditions include the following:

   a. Dilute the solubilized pellet 25-fold with 50 mM Tris-Cl (pH 8.5) containing 1.5 μM copper sulfate to give final concentrations of 0.04 M DTT, 0.24 M guanidine hydrochloride, and ~1.4 μg/ml target protein (Weir et al. 1987). Incubate the mixture for 2 hours at 20°C.

   b. Slowly add 9 volumes of 50 mM phosphate, 50 mM NaCl, and 1 mM EDTA containing 2 mM reduced glutathione/0.2 mM oxidized glutathione to the solubilized pellet. Incubate the mixture for 2–4 hours at 25°C.
10. Recover the folded protein using a concentration step that is appropriate for the target protein (e.g., RP-HPLC, lyophilization, or ultrafiltration).

Protocol 9

Preparation of Extracts from Yeast

Because yeast is exceptionally well suited to genetic analysis, both classical and molecular, it is an attractive system for expressing recombinant animal proteins for purification purposes. For a discussion on cell growth and harvesting yeast, especially the genus of the budding yeast *Saccharomyces*, see Jazwinski (1990). A number of methods available for lysing yeast cells include autolysis, pressure cells (e.g., French pressure cell), abrasives (glass bead vortexing), and enzymatic lysis (e.g., zymolase). One of the simplest methods, discussed in this protocol, involves the abrasive action of well-agitated glass beads. This is a very effective method for both low volumes (e.g., <1 ml using a microfuge tube) and many liters using a specialized DynoMill apparatus. Cell breakage is typically >95%, as assessed by phase-contrast microscopy.

MATERIALS

CAUTION: See Appendix 5 for appropriate handling of materials marked with <!>.

Reagents

Lysis buffer (RIPA buffer)
- 150 mM NaCl
- 1% NP-40
- 0.5% sodium deoxycholate <!>
- 0.1% SDS <!>
- 50 mM Tris-Cl (pH 7.2)

Just prior to use, add protease inhibitors to the following final concentrations: 1 μg/ml aprotinin <!>, 1 μg/ml leupeptin <!>, 1 μg/ml pepstatin, and 50 μg/ml PMSF <!>.

Phosphate-buffered saline (PBS)
- 8.0 g of NaCl (final concentration 137 mM)
- 0.2 g of KCl (final concentration 2.7 mM) <!>
- 1.44 g of Na_2HPO_4 (final concentration 10.1 mM)
- 0.24 g of KH_2PO_4 (final concentration 1.8 mM)
- in 800 ml of H_2O

Adjust pH to 7.4, and then adjust volume to 1 liter with H_2O.

Yeast cells, from a freshly grown culture

Equipment

Glass beads (500 μm) chilled

Prepare glass beads by washing twice in 1 M HCl <!> and twice in lysis buffer. Store the washed beads at 4°C in a small volume of lysis buffer.

METHOD

1. Harvest the cells by centrifugation in a microfuge for 3 minutes.
2. Discard the supernatant, resuspend the cells in PBS, and centrifuge again for 3 minutes.
3. Discard the supernatant. Estimate the volume of the cell pellet. Add 3 volumes of ice-cold lysis buffer per volume of cell pellet. Keep the suspension on ice.
4. Add a volume of chilled glass beads equal to the total volume of the resuspended yeast cells.
5. Vortex the suspension vigorously for 30 seconds.
6. Repeat Step 5 until the bulk of the yeast cells are lysed, as determined by phase-contrast microscopy.
7. Centrifuge the suspension in a microfuge for 5 minutes at 4°C.
8. Carefully pour off the supernatant and transfer to a fresh tube. Keep it on ice until use.

Protocol 10

Differential Detergent Fractionation of Eukaryotic Cells

Differential detergent fractionation (DDF) involves the sequential extraction of cells with PIPES buffers containing first digitonin, then Triton, and finally Tween/deoxycholate. The procedure yields four biochemically and electrophoretically distinct fractions (see Fig. 2.2 and Table 2.6) composed of the following:

- Cytosolic proteins and extractable cytoskeletal elements: Microtubule components can be semipurified from this fraction by magnesium precipitation (see Additional Protocol: Precipitation of Tubulins and MAPs Using Magnesium, at the end of this protocol).
- Membrane and organelle proteins: Integral membrane proteins can be enriched by using Triton X-114 (Bordier 1981; Pryde and Phillips 1986).
- Nuclear membrane proteins and extractable nuclear proteins.
- Detergent-resistant cytoskeletal filaments and nuclear matrix proteins: Cytoskeleton-associated proteins and interactions can be investigated further by differential urea extraction.

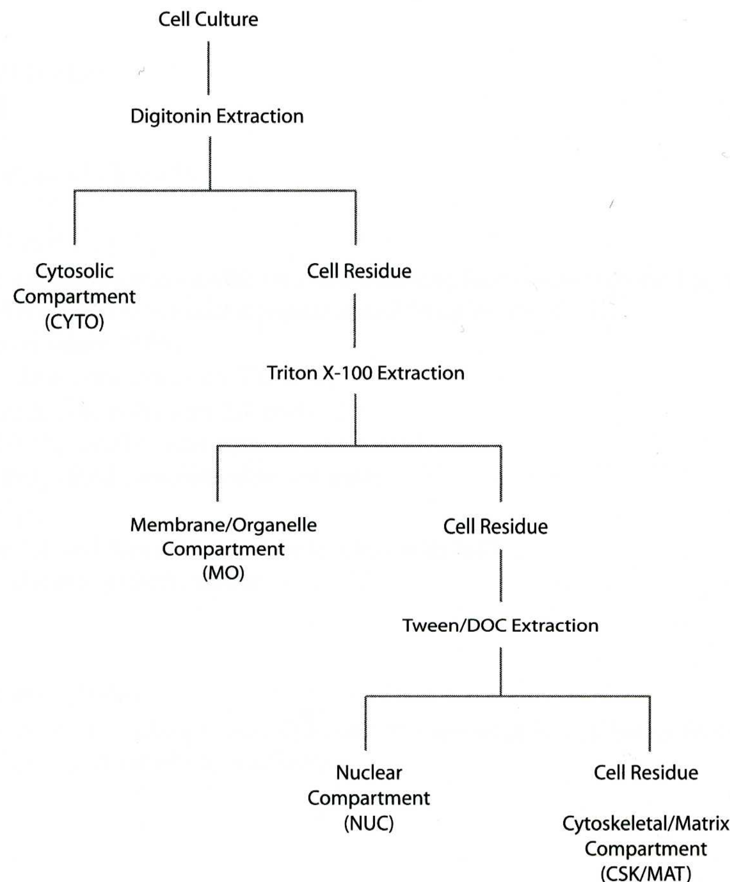

FIGURE 2.2. Schematic for differential detergent fractionation (DDF). (Courtesy of Melinda L. Ramsby and Gregory S. Makowski, University of Connecticut Health Center.)

TABLE 2.6. Protein distribution in detergent fractions of hepatocytes

Cell fraction	Protein distribution in hepatocytes
Digitonin <!>/EDTA extracts (CYTO fraction)	• ~35% total hepatocytic cellular protein • enriched in cytosolic markers (90% LDH activity, 100% carbonic anhydrase <!> immunoreactivity) (Ramsby et al. 1994)
Triton extracts (MO fraction)	• constitutes the bulk of hepatocytic cellular proteins (~50% total protein) • enriched in markers for membrane and organelle proteins (Ramsby et al. 1994)
Tween/DOC (NUC fraction)	• ~5% of total hepatocytic cell protein • contains, exclusively, immunoreactivity for the nuclear protein p38 (Ramsby et al. 1994)
Detergent-resistant fraction (CSK/MAT fraction)	• ~ 7–10% of hepatocytic cellular protein enriched in intermediate filaments, actin, various cytoskeleton-associating proteins, and nuclear matrix • for monolayer cultures, this fraction also contains extracellular matrix (Ramsby et al. 1994)
Magnesium-precipitable fraction (tubulins and microtubule-associated proteins)	• 1–2% of total hepatocytic cellular protein • represents ~4–5% of the protein in digitonin <!>/EDTA extracts • includes tubulins, actin, and proteins presumed to interact with the cytoskeleton

Hepatocytes have a high metabolic rate, which may affect the percent distributions of protein. Distributions will vary depending on cell type and should be determined empirically. Table courtesy of Melinda L. Ramsby and Gregory S. Makowski (University of Connecticut Health Center).

The DDF protocol described here represents a modification of a method used for the fractionation of Madin-Darby canine kidney (MDCK) cells (Fey et al. 1984). Modifications include the addition of a digitonin extraction step, the inclusion of EDTA in digitonin and Triton buffers, and the elimination of a nuclease digestion step (DNA is denatured by shear force in the presence of SDS). The main protocol and additional protocol were generously provided by Melinda Ramsby and Gregory Makowski (University of Connecticut Health Center).

MATERIALS

CAUTION: See Appendix 5 for appropriate handling of materials marked with <!>.

Reagents

Acetone (90%) chilled to –20°C <!>
Cell cultures, suspension or monolayer
Detergent extraction buffers

In general, the detergent extraction buffer formulations are for 100 ml of working solution. Adjust the final volumes for specific use, or freeze aliquots for future use. Solutions should be thawed on ice and not refrozen. The PIPES buffer recipes are listed elsewhere in this Materials list. For additional information on the properties of the detergents, see Table 2.7 and the booklet, A guide to the Preparation and Uses of Detergents in Biology and Biochemistry (available at http://www.calbiochem.com).

- Digitonin extraction buffer (0.015% digitonin, pH 6.8 at 4°C) <!>

Dissolve by heating (boiling) 18.75 mg of digitonin with 10 ml of 4x PIPES buffer in a small flask with a stir bar. Add 1 ml of 100 mM PMSF <!> while the buffer is still warm. Combine with 15

TABLE 2.7. Detergents used in differential detergent fractionation of cells

Detergent	Cell fraction	Volume of detergent used	Comments
Digitonin <!>	CYTO	*suspension cells:* 5 volumes/g (wet weight) of cell pellet *adherent cells:* 1 ml per T-25 flask-equivalent; 3 ml per 100-mm Petri dish	• Digitonin <!> is a steroidal compound that complexes with plasma membrane cholesterol to cause membrane permeabilization and rapid release of soluble cytosolic components (Mackall et al. 1979). • Low concentrations of digitonin <!> (0.015%) preserve ER, mitochondrial, lysosomal, and organelle membrane integrity, hence, ultrastructure, which are damaged at higher concentrations (~0.1%) (Fiskum et al. 1980; Weigel et al. 1983). • EDTA significantly enhances the effectiveness of low concentrations of digitonin <!>, as evidenced by an increased rate of membrane permeabilization (10 minutes in the presence of EDTA vs. 40 minutes in the absence of EDTA) and inhibits calcium-dependent neutral proteases (calpains), typically enriched in cytosolic extracts (Ramsby et al. 1994). • Digitonin <!> releases proteins larger than 200 kD (Weigel et al. 1983), as evidenced by the presence of myosin (>220 kD), desmoplakin II (>250 kD), and the calpain-inhibitor calpastatin (~300 kD) in cytosolic extracts (Ramsby et al. 1994). • The combination of ice-cold temperatures and EDTA results in microtubule depolymerization, which promotes fractionation of tubulins and microtubule-associated proteins in digitonin <!>/EDTA extracts. Tubulins and microtubule-binding proteins can be harvested by repolymerization at 37°C and magnesium precipitation (see Additional Protocol: Precipitation of Tubulins and Maps Using Magnesium).
Triton X-100<!>	MO	*suspension cells:* 5 volumes/g (wet weight) of cell pellet *adherent cells:* 1 ml per T-25 flask-equivalent; 3 ml per 100-mm Petri dish	• Triton <!> is a nonionic detergent, which solubilizes membrane lipids and releases organelle contents. • It has been used in hyper- or hypotonic buffers to obtain cytoskeletal preparations enriched in intermediate filaments (Franke et al. 1978). • Low concentrations of Triton <!> concomitant with iso-osmolar, isotonic buffer composition, as used here, preserve the integrity of the nuclear membrane as well as the microfilament cytoskeleton (Fey et al. 1984). • Triton <!> extraction is possible using either the X-100 or X-114 detergent series. Fractionation with X-114 allows subfractionation of peripheral versus integral membrane proteins (Bordier 1981; Pryde and Phillips 1986) and lends further flexibility to fractionation goals.
Tween/DOC	NUC	*suspension cells:* 2.5 volumes/g (wet weight) of cell pellet *adherent cells:* 0.5–1 ml per T-25 flask-equivalent	• DOC is a weakly ionic detergent that disrupts nuclear membrane and microfilament integrity (Capco et al. 1982; Fey et al. 1984; Reiter et al. 1985). • Tween/DOC extracts contain proteins common to both the membrane/organelle and detergent-resistant cytoskeletal fractions, perhaps representing a more labile, protein compartment.
–	CSK/MAT (detergent-resistant)	–	• DNA present in the detergent-resistant fraction is viscous and difficult to manage. • Denaturation is achieved by mechanical shear, using either Teflon/glass homgenization (for suspension pellets) or tituration with a pipette (for monolayer residues). • The cytoskeletal filaments in this fraction are intact as evidenced by two-dimensional PAGE (Ramsby et al. 1994). Staircase patterns, indicative of cytoskeletal degradation, however, do arise if PMSF <!> or EDTA is absent from extraction buffers. • Solubilization of detergent-resistant samples in SDS phosphate buffer <!>, in the absence of mercaptoethanol <!>, allows direct assay of protein content by the method of Peterson (1983).

Table courtesy of Melinda L. Ramsby and Gregory S. Makowski (University of Connecticut Health Center).

ml of 4x PIPES buffer and 5 ml of 100 mM EDTA. Cool to 4°C and adjust pH to 6.8 with HCl <!>. Add H_2O to a final volume of 100 ml and keep on ice until use. Digitonin buffer without EDTA is stable for 3 hours at 0°C. EDTA prolongs the stability of this buffer.

- Triton X-100 extraction buffer (0.5% Triton X-100, pH 7.4 at 4°C) <!>

  Combine 25 ml of 4x PIPES buffer, 1 ml of 100 mM PMSF <!>, 3 ml of 100 mM EDTA, and 5 ml of freshly prepared 10% (v/v) Triton X-100. Cool to 4°C, adjust pH to 7.4, and add H_2O to 100 ml (final volume). Keep on ice until use.

- Tween-40/deoxycholate extraction buffer (1% Tween-40/0.5% DOC <!>, pH 7.4 at 4°C)

  Dissolve 0.5 g of DOC in 2.5 ml of 10x PIPES buffer, and separately dissolve 1 ml of Tween-40 in 2.5 ml of 10x PIPES buffer (warm if necessary). Combine the Tween and DOC solutions, and then add 5 ml of 10x PIPES buffer and 1 ml of 100 mM PMSF <!>. Cool to 4°C, adjust pH to 7.4, and add H_2O to 100 ml (final volume). Keep on ice until use.

  Triton <!> and Tween are both nonionic detergents, which decompose in aqueous solutions to form peroxides that oxidize protein sulfhydryl groups (Lever 1977; Chang and Bock 1980; Lenstra and Bloemendal 1983). Although EDTA prolongs stability, buffers should be used as freshly prepared or freshly thawed and not refrozen. This avoids artifacts and assures reproducibility between different fractionations.

Cytoskeleton solubilization buffer (5% SDS <!>, 10 mM sodium phosphate <!>, pH 7.4)

For nonreducing buffer, dissolve 0.5 g of SDS in 5 ml of 20 mM sodium phosphate buffer (pH 7.4). Add H_2O to 10 ml (final volume). For denaturing buffer, add 1 ml of β-mercaptoethanol <!>. Adjust H_2O appropriately.

1x O'Farrell lysis buffer

Dissolve 5.7 g of ultrapure electrophoretic-grade urea <!>, 0.2 ml of NP-40, 0.2 ml of ampholines (0.16 ml at pH 5–7 and 0.04 ml at pH 3–10), and 0.5 ml of β-mercaptoethanol <!> in H_2O. Adjust final volume to 10 ml with H_2O. Solution may be warmed to facilitate solubilization. Divide into 1-ml aliquots and store at –70°C.

10x O'Farrell lysis buffer

Combine 0.2 ml of NP-40, 0.2 ml of ampholines (0.16 ml at pH 5–7 and 0.04 ml at pH 3–10), and 0.5 ml of β-mercaptoethanol <!> in H_2O. Adjust final volume to 1 ml with H_2O. Solution may be warmed to facilitate solubilization. Divide into 100-µl aliquots and store at –70°C.

Phosphate-buffered saline (PBS, pH 7.4), ice-cold

8.0 g of NaCl (final concentration 137 mM)
0.2 g of KCl (final concentration 2.7 mM) <!>
1.44 g of Na_2HPO_4 (final concentration 10.1 mM)
0.24 g of KH_2PO_4 (final concentration 1.8 mM)
in 800 ml of H_2O

Adjust pH to 7.4, and then adjust volume to 1 liter with H_2O.

PBS (pH 7.4) containing 1.2 mM PMSF <!>, ice-cold

Add the PMSF just before use.

4x PIPES buffer (piperazine-*N,N'*bis[2-ethanesulfonic acid])

Dissolve 103 g of sucrose and 5.8 g of NaCl in 150 ml of H_2O. Dissolve 3 g of PIPES in a small volume of 1 M NaOH <!>. Mix the PIPES solution with the sucrose/NaCl solution. Add 0.64 g of $MgCl_2 \cdot 6H_2O$ <!> to the mixture. Adjust final volume to 250 ml and filter through a 0.45-µm sterile filter. Store in the dark at 4°C or aliquot and freeze (stable for 2 months at 4°C). Employ aseptic technique when diluting the 4x PIPES buffer for preparation of 1x working solutions or store as single-use aliquots to avoid contamination.

10x PIPES buffer

Dissolve 0.58 g of NaCl in 50 ml of H_2O, and separately dissolve 3 g of PIPES in a small volume of 1 M NaOH <!>. Mix the PIPES solution with the NaCl solution. Add 0.2 g of $MgCl_2 \cdot 6H_2O$ <!> to the solution and adjust final volume to 100 ml with H_2O. Filter and store as described for the 4x PIPES buffer.

Trypan blue

Urea (solid) <!>

Equipment

Centrifuge (refrigerated)
Electrophoresis equipment for 2D gels (Chapter 4)
Homogenizer, Teflon smooth-walled glass
Lyophilizer
Platform mixer

METHOD

Preparation of Cells

1. Cool the cell cultures on ice, and wash them two to three times in ice-cold saline, PBS, or other nondetergent buffer.

   Save an aliquot of the wash buffer (store it at –70°C) to use for future sample normalization or as control material for enzymatic, protein, or RNA analysis.

2. Choose the appropriate method below for working with suspension or monolayer cultures:

   If working with suspension cultures: The volume of DDF solutions for suspension-cultured cells is based on wet weight or cell number.

   i. Transfer an aliquot of the suspension culture to a preweighed plastic tube and centrifuge briefly.

   ii. Decant the culture media and determine the wet weight of the cell pellet.

   iii. Proceed to Step 3.

   If working with monolayer cultures: The volume of DDF buffers for monolayer cell cultures is based on surface area or cell number.

   i. Determine the surface area of the culture flask or dish. Alternatively, count the cells in a representative culture vessel.

   ii. Proceed to Step 19.

Detergent Fractionation of Suspension Cell Cultures

DDF is performed by the sequential addition and removal of DDF buffers. All extractions are performed on ice with gentle agitation. Attention to extraction times is important for reproducibility between different fractionations. Extracts are maintained on ice until used for assay or frozen for storage (–70°C is best). Measure the recovered volume of each detergent extract to calculate net yields or percent distributions. Save an aliquot of each DDF buffer for future sample normalization or control material for assays.

3. Add 5 volumes of ice-cold digitonin extraction buffer per gram wet weight to the washed cell pellets. Gently resuspend the cell pellets by swirling.

4. Incubate the cells on ice with gentle agitation on a platform mixer until 95–100% of the cells are permeabilized (~10 minutes) as assessed by Trypan blue exclusion.

   It is important to maintain consistent extraction times between different fractionations.

5. Centrifuge the extraction mixture at 480*g* for 10 minutes.

6. Transfer the supernatant to a clean tube. Record the supernatant (cytosolic fraction) volume, divide the cytosolic fraction into aliquots, and store them at –70°C. This is the CYTO fraction (see Fig. 2.2 and Table 2.6).

7. Carefully resuspend the digitonin-insoluble pellets in 5 volumes (relative to starting wet weight of the cell pellet [determined above in Step 2]) of ice-cold Triton X-100 extraction buffer to obtain a homogeneous suspension.

   Triton X-114 can be substituted for Triton X-100 in DDF protocols without obvious difference on two-dimensional gels. Relative to Triton X-100, Triton X-114 has a lower cloud point (60°C vs. 20°C) and can partition into lipid-rich and lipid-poor phases at nondenaturing temperatures. This enables subfractionation of integral and peripheral membrane proteins as well as soluble organelle contents (Bordier 1981; Pryde and Phillips 1986).

8. Incubate the cells on ice with gentle agitation on a platform mixer for 30 minutes.
9. Centrifuge the extraction mixture at 5000*g* for 10 minutes.
10. Transfer the supernatant to a clean tube. Record the supernatant (membrane and organelle fraction) volume, divide this fraction into aliquots, and store them at –70°C. This is the MO fraction (see Fig. 2.2 and Table 2.6).
11. To the Triton-insoluble pellets, add Tween-40/deoxycholate extraction buffer at one-half the volume used for Triton extraction (Step 7). Resuspend the pellets with five strokes at medium speed using a Teflon, smooth-walled glass homogenizer.
12. Centrifuge the extraction mixture at 6780*g* for 10 minutes.
13. Transfer the supernatant to a clean tube. Record the supernatant (nuclear fraction) volume, divide the nuclear fraction into aliquots, and store them at –70°C. This is the NUC fraction (see Fig. 2.2 and Table 2.6).
14. Add ice-cold PBS (pH 7.4) containing 1.2 mM PMSF to the detergent-resistant pellets. Resuspend the pellets using three strokes in a Teflon/glass homogenizer. Collect the pellets by centrifugation at 12,000*g* for 10 minutes.
15. Repeat Step 14 two more times to thoroughly wash the pellets.
16. Discard the supernatant, and wash the pellets once with 90% acetone (previously chilled to –20°C).
17. Lyophilize the pellets (cytoskeletal/nuclear matrix fraction) overnight.
18. Determine the pellet weights in tared microfuge tubes. Store the samples at –70°C. This is the CSK/MAT fraction (see Fig. 2.2 and Table 2.6).

    Typically, the yield of the CSK/MAT fraction from cell suspensions is large. Thus, it is usually more convenient to store the fraction as dried pellets, and resuspend measured aliquots in non-denaturing cytoskeleton solubilization buffer as needed.

Detergent Fractionation of Monolayer Cell Cultures

DDF is performed by the sequential addition and removal of DDF buffers. All extractions are performed on ice with gentle agitation. Attention to extraction times is important for reproducibility between different fractionations. Extracts are maintained on ice until used for assay or frozen for storage (–70°C is best). Measure the recovered volume of each detergent extract to calculate net yields or percent distributions. Save an aliquot of each DDF buffer for future sample normalization or control material for assays.

19. Add 1 ml of ice-cold digitonin extraction buffer directly to the cell monolayer in a T-25 flask.

    A typical T-25 culture flask contains ~5 x 10^6 cells. Use 3 ml of digitonin extraction buffer for a T-75 flask or 100-mm diameter Petri dish.

20. Incubate the cells on ice with gentle agitation on a platform mixer until 95–100% of the cells are permeabilized (~10 minutes) as assessed by Trypan blue exclusion.

    It is important to maintain consistent extraction times between different fractionations.

21. Tilt the culture flask and decant the liquid (the cytosolic fraction) into a culture tube. Use a pipette to transfer any residual liquid from the flask into the culture tube. Record the volume of the cytosolic fraction, divide it into aliquots, and store them at –70°C. This is the CYTO fraction (see Fig. 2.2 and Table 2.6).
22. Add 1 ml of Triton extraction buffer per T-25 flask equivalent (~5 x 10^6 cells).

    Triton X-114 can be substituted for Triton X-100 in DDF protocols without obvious difference on two-dimensional gels. Relative to Triton X-100, Triton X-114 has a lower cloud point (60°C

vs. 20°C) and can partition into lipid-rich and lipid-poor phases at nondenaturing temperatures. This enables subfractionation of integral and peripheral membrane proteins as well as soluble organelle contents (Bordier 1981; Pryde and Phillips 1986).

23. Incubate the cells on ice with gentle agitation on a platform mixer for 30 minutes.
24. Tilt the culture flask and decant the liquid (the membrane and organelle fraction) into a culture tube. Use a pipette to transfer any residual liquid from the flask into the culture tube. Record the volume of this fraction, divide it into aliquots, and store them at –70°C. This is the MO fraction (see Fig. 2.2 and Table 2.6).
25. Extract cell monolayers with 0.5–1.0 ml of Tween-40/deoxycholate extraction buffer per T-25 flask equivalent (~5×10^6 cells).
26. Tilt the culture flask and decant the liquid (the nuclear fraction) into a culture tube. Use a pipette to transfer any residual liquid from the flask into the culture tube. Record the volume of the nuclear fraction, divide it into aliquots, and store them at –70°C. This is the NUC fraction (see Fig. 2.2 and Table 2.6).
27. Rinse the monolayers in situ with PBS.
28. Add 1–3 ml of nondenaturing cytoskeleton solubilization buffer without β-mercaptoethanol to the detergent-resistant residue in the culture flasks. Suspend the residue by titurati0n. Record the volume of this material (the cytoskeletal and matrix fraction), divide it into aliquots, and store them at –70°C. This is the CSK/MAT fraction (see Fig. 2.2 and Table 2.6).

    The volume of nondenaturing cytoskeleton solubilization buffer required will vary depending on the cell type and length of time in culture (i.e., the amount of extracellular matrix). In general, 1–3 ml is an appropriate range.

Determination of Protein Concentration

29. Thaw detergent buffers and extracts on ice.
30. Dilute the digitonin/EDTA and Triton/EDTA extracts with H_2O (4 volumes for extracts from suspension cultures, 2–3 volumes for extracts from monolayers). Use the Tween/DOC extracts undiluted.
31. Solubilize the lyophilized CSK/MAT pellets in cytoskeleton solubilization buffer without β-mercaptoethanol. Use 1 ml of buffer per 10 mg (dry weight) of pellet. Dilute CSK/MAT preparations from monolayers as necessary.
32. Assay 20–50 µl of each sample in duplicate, using the bicinchoninic acid (BCA) method.

    An alternative assay is the Folin-phenol method of Peterson (1983), which is not susceptible to interference by detergents. Assay by the standard Lowry method is not recommended because it results in detergent-induced flocculation (M. Ramsby, unpubl.).

Analysis of Protein Fractions Using Two-dimensional Gel Electrophoresis

DDF samples obtained from a variety of cell types can be utilized for two-dimensional PAGE under both IEF and nonequilibrium pH gradient electrophoresis (NEpHGE).

33. Normalize fresh or thawed DDF extracts (kept on ice) to contain protein in equal volumes and then bring them to 9.5 M urea by addition of solid urea.
    - For a 100-µl sample, add 85 mg of urea and 15 µl of 10x O'Farrell lysis buffer and warm to room temperature.
    - For the dried CSK extract, solubilize directly in 1x O'Farrell lysis buffer (O'Farrell 1975; O'Farrell et al. 1977).

- For CSK extracts in nonreducing SDS buffer, adjust to 9.5 M urea by the addition of solid urea and add 10x O'Farrell lysis buffer.

Solid urea and 10x O'Farrell lysis buffer are added to minimize dilutional effects and enable detection of low-abundance proteins. Samples may be warmed slightly to facilitate urea solubilization, but avoid excessive warming (increased temperature and/or prolonged heating), which may result in carbamylation artifacts.

34. Prepare DDF samples for analysis by 2D electrophoresis using established methods (O'Farrell 1975; O'Farrell et al. 1977; also see Chapter 4).

- IEF gels (see Chapter 4) contain a total of 3.5% ampholines (2% pH 5–8, 1% pH 3–10, 0.5% pH 2–5), and samples are electrophoresed for a total of 9800 Volt-hours with hyperfocusing at 800 V for the final hour (Duncan and Hershey 1984; Ramsby et al. 1994).
- NEpHGE gels contain 2% pH 3–10 ampholines, and samples are electrophoresed for 2400 Volt-hours (Ramsby et al. 1994).

In general, samples containing 25–100 µg of protein in 15–60 µl are ample for visualization by Coomassie Blue staining or autoradiography; low-abundance proteins are detected by silver staining. In control experiments, the volume of detergent buffer contained in the samples did not adversely affect the linear range of the pH gradient in IEF gels. However, a slight shift to more acidic values may occur with samples containing Tween/DOC.

ADDITIONAL PROTOCOL: PRECIPITATION OF TUBULINS AND MAPS USING MAGNESIUM

Tubulins and microtubule-associated proteins present in cytosolic extracts can be separated from other cytosolic constituents by incubation at 37°C, followed by magnesium-induced precipitation (Sahyoun et al. 1982).

Additional Materials

CAUTION: See Appendix 5 for appropriate handling of materials marked with <!>.

Imidazole buffer (pH 7.5) <!>
$MgCl_2$ (0.1 M) <!>

Method

1. Adjust an aliquot of the digitonin/EDTA extract (the CYTO fraction from the main protocol) to 7–35 mM magnesium by addition of 0.1 M $MgCl_2$. Incubate the samples for 20–30 minutes at 37°C.

   The total volume of starting digitonin/EDTA extract used will depend on experimental goals. Final magnesium concentration will depend on the origin of the starting material. In general, 35 mM magnesium is excessive, but broadly applicable for complete recovery.

2. Harvest tubulins and associated proteins by centrifugation in a microfuge for 8 minutes.
3. Transfer the supernatant to a clean tube. Wash the pellet in a large volume of imidazole buffer (pH 7.5), or other suitable buffer, and resuspend the pellet in cytoskeleton solubilization buffer for determination of protein concentration and electrophoretic analysis (see Steps 29–34 of the main protocol and Table 2.6).

REFERENCES

Amrein K.E., Takacs B., Steiger M., Molnos J., Flint N.A., and Burn P. 1995. Purification and characterization of human recombinant $p50^{csk}$ protein-tyrosine kinase from an *Escherichia coli* expression system overproducing the bacterial chaperones GroES and GroEL. *Proc. Natl. Acad. Sci.* **92:** 1048–1052.

Baker D., Hicke L., Rexach M., Schleyer M., and Schekman R. 1988. Reconstitution of SEC gene product-dependent intercompartmental protein transport. *Cell* **54:** 335–344.

Beynon R.J. and Bond J.S. 1989. *Proteolytic enzymes: A practical approach.* IRL Press/Oxford University Press, Oxford, United Kingdom.

Beynon R.J. and Oliver S. 1996. Avoidance of proteolysis in extracts. *Methods Mol. Biol.* **59:** 81–93.

Blackwell J.R. and Horgan R. 1991. A novel strategy for production of a highly expressed recombinant protein in an active form. *FEBS Lett.* **295:** 10–12.

Blum P., Velligan M., Lin N., and Matin A. 1992. DnaK-mediated alterations in human growth hormone protein inclusion bodies. *Bio/Technology* **10:** 301–304.

Bollag D.M., Edelstein S.J., and Rozycki M.D., eds. 1996. *Protein methods,* 2nd edition. Wiley-Liss, New York.

Bordier C. 1981. Phase separation of integral membrane proteins in Triton X-114 solutions. *J. Biol. Chem.* **256:** 1604–1607.

Bridge P. 1996. Protein extraction from fungi. *Methods Mol. Biol.* **59:** 39–48.

Burgess R. and Jendrisak J. 1975. A procedure for the rapid large-scale purification of *E. coli* DNA dependent RNA polymerase involving Polymin P precipitation and DNA cellular chromatography. *Biochemistry* **14:** 4634–4645.

Capco D.G., Wan K.M., and Penman S. 1982. The nuclear matrix: Three-dimensional architecture and protein composition. *Cell* **29:** 847–858.

Carrell R.W., Lehmann H., and Hutchinson H.F. 1966. Haemoglobin Koln (beta 98 valine-methionine): An unstable protein causing inclusion body anemia. *Nature* **210:** 915–916.

Celis J.E., ed. 1998. *Cell biology: A laboratory handbook,* 2nd edition, vol. 1. Academic Press, San Diego, California.

Chang H.W. and Bock E. 1980. Pitfalls in the use of commercial nonionic detergents for the solubilization of integral membrane proteins: Sulfhydryl oxidizing contaminants and their elimination. *Anal. Biochem.* **104:** 112–117.

Corthals G.L., Wasinger V.C., Hochstrasser D.F., and Sanchez J.C. 2000. The dynamic range of protein expression: A challenge for proteomic research. *Electrophoresis* **21:** 1104–1115.

Cull M. and McHenry C.S. 1990. Preparation of extracts from prokaryotes. *Methods Enzymol.* **182:** 147–153.

Deutscher M.P., ed. 1990. Guide to protein purification. *Methods Enzymol.,* vol. 182.

Dignam J.D. 1990. Preparation of extracts from higher eukaryotes. *Methods Enzymol.* **182:** 194–203.

Doonan S. 1996. Protein purification protocols. *Methods Mol. Biol.* **59:** 1–16.

Duncan R. and Hershey J.W.B. 1984. Evaluation of isoelectric focusing running conditions during two-dimensional isoelectric focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis: Variation of gel patterns with changing conditions and optimal isoelectric focusing conditions. *Anal. Biochem.* **138:** 144–145.

Evans W.H. 1992. Isolation and characterization of membranes and cell organelles. In *Preparative centrifugation: A practical approach* (ed. D. Rickwood), pp. 233–270. IRL Press/Oxford University Press, Oxford, United Kingdom.

Fahnestock S.R. 1979. Reconstitution of active 50S ribosomal subunits from *Bacillus lichenformis* and *Bacillus subtilis. Methods Enzymol.* **59:** 437–443.

Fey E.G., Wan K.M., and Penman S. 1984. Epithelial cytoskeletal framework and nuclear matrix-intermediate filament scaffold: Three-dimensional organization and protein composition. *J. Cell Biol.* **98:** 1973–1984.

Fiskum G., Craig S.W., Decker G.L., and Lehninger A.L. 1980. The cytoskeleton of digitonin-treated rat hepatocytes. *Proc. Natl. Acad. Sci.* **77:** 3430–3434.

Franke W.W., Schmid E., Osborn M., and Weber K. 1978. The intermediate-sized filaments in rat kangaroo PtK2 cells. II. Structure and composition of isolated filaments. *Cytobiologie* **17:** 392–411.

Gegenheimer P. 1990. Preparation of extracts from plants. *Methods Enzymol.* **182:** 174–194.

Glass G.B.J. 1964. Proteins, mucosubstances, and biologically active components of gastric substances. *Adv. Clin. Chem.* **7:** 235–372.

Gorg A., Obermaier C., Boguth G., Harder A., Scheibe B., Wildgruber R., and Weiss W. 2000. The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* **21:** 1037–1053.

Graham J.M. 1997. Homogenisation of tissue and cells. In *Subcellular fractionation: A practical approach* (ed. J.M. Graham and D. Rickwood), pp. 1–28. IRL Press/Oxford University Press, Oxford, United Kingdom.

Graham J.M. and Rickwood D., eds. 1997. *Subcellular fractionation: A practical approach.* IRL Press/Oxford University Press, Oxford, United Kingdom.

Gribskov M. and Burgess R.R. 1983. Overexpression and purification of the sigma subunit of *Escherichia coli* RNA polymerase. *Gene* **26:** 109–118.

Gygi S.P., Corthals G.L., Zhang Y., Rochon Y., and Aebersold R. 2000. Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proc. Natl. Acad. Sci.* **97:** 9390–9395.

Hanash S. 2000. Biomedical applications of two-dimensional electrophoresis using immobilized pH gradients: Current status. *Electrophoresis* **21:** 1202–1209.

Harlow E. and Lane D. 1999. *Using antibodies: A laboratory manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Hockney R.C. 1995. Recent developments in heterologous protein production in *Escherichia coli. Trends Biotechnol.* **13:** 456–463.

Hoving S., Voshol H., and van Oostrum J. 2000. Towards high performance two-dimensional gel electrophoresis using ultrazoom gels. *Electrophoresis* **21:** 2617–2621.

Hunter M.J. and Commerford S.L. 1961. Pressure homogenization of mammalian tissues. *Biochim. Biophys. Acta* **47:** 580–586.

Hurley J.H. and Meyer T. 2001. Subcellular targeting by membrane lipids. *Curr. Opin. Cell Biol.* **13:** 146–152.

Jazwinski S.M. 1990. Preparation of extracts from yeast. *Methods Enzymol.* **182:** 154–174.

Ji H. and Simpson R.J. 1999. Nonreducing 2-D polyacrylamide gel electrophoresis. *Methods Mol. Biol.* **112:** 255–264.

Ji H., Moriz R.L., Reid G.E., Ritter G., Catimel B., Nice E.C., Heath J.K., White S.J., Welt S., Old L.J., Burgess A.W., and Simpson R.J. 1997. Electrophoretic analysis of the novel antigen for the gastrointestinal-specific monoclonal antibody, A33. *Electrophoresis* **18:** 614–622.

Johnson J.E. and Cornell R.B. 1999. Amphitropic proteins: Regulation by reversible membrane interactions. *Mol. Membr. Biol.* **16:** 217–235.

Laemmli U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

La Vallie E.R., Di Blasio E.A., Kovacic S., Grant K.L., Schendel P.F., and McCoy J.M. 1993. A thioredoxin gene fusion expression

system that circumvents inclusion body formation in the *E. coli* cytoplasm. *Bio/Technology* **11:** 187–193.
Lenk R., Ransom L., Kaufman Y., and Penman S. 1977. A cytoskeletal structure with associated polyribosomes obtained from HeLa cells. *Cell* **10:** 67–78.
Lenstra J.A. and Bloemendal H. 1983. Topography of the total protein population from cultured cells upon fractionation by chemical extractions. *Eur. J. Biochem.* **135:** 413–423.
Lever M. 1977. Peroxides in detergents as interfering factors in biochemical analysis. *Anal. Biochem.* **83:** 274–284.
Mackall J., Meredith M., and Lane L.M. 1979. A mild procedure for the rapid release of cytoplasmic enzymes from cultured animal cells. *Anal. Biochem.* **95:** 270–274.
Molloy M.P., Herbert B.R., Walsh B.J., Tyler M.I., Traini M., Sanchez J.C., Hochstrasser D.F., Williams K.L., and Gooley A.A. 1998. Extraction of membrane proteins by differential solubilisation for separation using two-dimensional gel electrophoresis. *Electrophoresis* **19:** 837–844.
Neurath H. 1989. The diversity of proteolytic enzymes. In *Proteolytic enzymes: A practical approach* (ed. R.J. Beynon and J.S. Bond), pp. 1–13. IRL University Press, New York.
Nice E.C., Fabri L., Whitehead R.H., James R., Simpson R.J., and Burgess A.W. 1991. The major colonic cell mitogenic extractable from colonic mucosa is an N-terminally extended form of basic fibroblast growth factor. *J. Biol. Chem.* **266:** 14425–14430.
North M.J. 1989. Prevention of unwanted proteolysis. In *Proteolytic enzymes: A practical approach* (ed. R.J. Beynon and J.S. Bond), pp. 105–124. IRL Press/Oxford University Press, Oxford, United Kingdom.
O'Farrell P.H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250:** 4007–4021.
O'Farrell P.Z., Goodman H.M., and O'Farrell P.H. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12:** 1133–1142.
Patton W.F. 1999. Proteome analysis. II. Protein subcellular redistribution: Linking physiology to genomics via the proteome and separation technologies involved. *J. Chromatog. B* **722:** 203–223.
Perlmann G.E. and Lorand L., eds. 1970. Proteolytic enzymes. *Methods Enzymol.*, vol. 19.
Peterson G.L. 1983. Determination of total protein. *Methods Enzymol.* **91:** 95–119.
Pryde J.G. and Phillips J.H. 1986. Fractionation of membrane proteins by temperature-induced phase separation in Triton X-114. *Biochem. J.* **233:** 525–533.
Ramsby M.L. and Kreutzer D.L. 1993. Fibrin induction of tissue plasminogen activator in corneal endothelial cells in vitro. *Invest. Ophthalmol. Vis. Sci.* **34:** 3207–3219.
Ramsby M.L. and Makowski G.S. 1999. Differential detergent fractionation of eukaryotic cells. Analysis by two-dimensional electrophoresis. *Methods Mol. Biol.* **112:** 53–66.
Ramsby M.L., Makowski G.S., and Khairallah E.A. 1994. Differential detergent fractionation of isolated hepatocytes: Biochemical, immunochemical and two dimensional gel electrophoretic characterization of cytoskeletal and noncytoskeletal compartments. *Electrophoresis* **15:** 265–277.
Reiter T., Penman S., and Capco D.G. 1985. Shape-dependent regulation of cytoskeletal protein synthesis in anchorage-dependent and anchorage-independent cells. *J. Cell Sci.* **76:** 17–33.
Santoni V., Kieffer S., Desclaux D., Masson F., and Rabilloud T. 2000. Membrane proteomics: Use of additive main effects with multiplicative interaction model to classify plasma membrane proteins according to their solubility and electrophoretic properties. *Electrophoresis* **21:** 3329–3344.
Schatz G. 1979. Biogenesis of yeast mitochondria: Synthesis of cytochrome c oxidase and cytochrome c. *Methods Enzymol.* **56:** 40–50.
Schein C.H. and Noteborn M.H.M. 1988. Formation of soluble recombinant proteins in *Escherichia coli* is favored by lower growth temperature. *Bio/Technology* **6:** 291–294.
Scopes R.K. 1994. *Protein purification: Principles and practice*, 3rd edition. Springer Verlag, New York.
Sebald W., Neupert W., and Weiss H. 1979. Preparation of *Neurospora crassa* mitochondria. *Methods Enzymol.* **59:** 144–148.
Smith D.B. and Johnson K.S. 1988. Single step purification of polypeptides expressed in *E. coli* as fusions with glutathionine-S-transferase. *Gene* **67:** 31–40.
Spector D.L., Goldman R.D., and Leinwand L.A. 1998. *Cells: A laboratory manual*, vols. 1–3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Weigel P.H., Ray D.A., and Oka J.A. 1983. Quantitation of intracellular membrane-bound enzymes and receptors in digitonin-permeabilized cells. *Anal. Biochem.* **133:** 437–449.
Weir M.P., Sparks J., and Chaplin A.M. 1987. Micropreparative purification of recombinant human interleukin-2. *J. Chromatogr.* **396:** 209–215.
Wildgruber R., Harder A., Obermaier C., Boguth G., Weiss W., Fey S.J., Larsen P.M., and Gorg A. 2000. Towards higher resolution: Two-dimensional electrophoresis of *Saccharomyces cerevisiae* proteins using overlapping narrow immobilized pH gradients. *Electrophoresis* **21:** 2610–2616.
Williams D.C., Van Frank R.M., Muth W.L., and Barnett J.P. 1982. Cytoplasmic inclusion bodies in *Escherichia coli* producing biosynthetic human insulin protein. *Science* **215:** 684–687.
Worrall D.M. 1996. Extraction of recombinant protein from bacteria. *Methods Mol. Biol.* **59:** 31–37.
Zhang J.-G., Moritz R.L., Reid G.E., Ward L.D., and Simpson R.J. 1992. Purification and characterization of a recombinant murine interleukin-6: Isolation of N- and C-terminally truncated forms. *Eur. J. Biochem.* **207:** 903–913.