

## Preparation of Bacterial Samples for 2-D PAGE

Brian Berg Vandahl, Gunna Christiansen, and Svend Birkelund

### 1. Introduction

Sample preparation is a very crucial step in two-dimensional (2-D) gel electrophoresis, in which the proteins of the sample must be brought into a state where they can be separated by isoelectric focusing in the first dimension. That is, they must be denatured, reduced, and solubilized, and they must be kept so during electrophoresis without changing their pI. The sample buffer for this purpose is traditionally called the lysis buffer.

In most bacterial studies it is the aim to solubilize as many proteins as possible to obtain the best possible representation of the total protein content or protein expression under the investigated biological circumstances. However, prefractionation or the successive application of different chemical reagents can be used to investigate bacterial proteins with certain characteristics. Be aware that the gels will reflect the proteome of the bacteria at the time the proteins are solubilized, and that preceding centrifugations or other manipulations may stress the bacteria and thus influence the protein profile.

The solubilization procedure is highly dependent on the nature of the sample. Some bacteria are readily lysed by the constituents of the lysis buffer, whereas others must be disrupted mechanically, and for some it may be necessary to remove the cell wall by enzymatic digestion prior to mechanical disruption. In **Subheading 3.1.**, a general protocol for solubilization will be given. It is important to stress that both the lysis buffer and the protocol always must be optimized for the sample in question. All reagents are described in the notes, together with common alternatives.

Some samples may contain nonprotein substances in amounts that are incompatible with first or second dimensional electrophoresis and thus have to be removed from the sample prior to the addition of lysis buffer. Salts and most other compounds that may disturb the first dimension can be removed by protein precipitation, as described in **Subheading 3.2.1.** If nucleic acids are present in high amounts, these may have to be removed by enzymatic digestion, which is described in **Subheading 3.2.2.**

Also, highly abundant proteins may cause problems by preventing optimal focusing in the first dimension or by masking large areas of the gel. In such cases, it may be necessary to carry out prefractionations or to specifically remove the abundant proteins by immunoprecipitation. Prefractionation can be obtained by isolation of specific organelles, by chemical extractions, or by chromatographic or electrophoretic techniques (**I**), but these methods fall beyond the scope of this chapter.

All procedures should be kept as simple as possible to ensure reproducibility and because proteolytic degradation must be considered a risk. When bacteria are disrupted, proteases that are present in the periplasmic space in a high number will be released and start degrading proteins in the sample if not inhibited. As folded proteins are less susceptible to proteolysis than denatured proteins, and as proteases are often more resistant to denaturation than most other proteins, solubilization in urea will often make the problem worse. However, most proteases will be inactivated by disruption in lysis buffer containing thiourea, in 2% sodium dodecyl sulfate (SDS), or in precipitation solution containing 10% trichloroacetic acid (TCA). Still, all handling of the sample after disruption of the bacteria should be carried out as quickly as possible and on ice to minimize proteolytic degradation, and it may be necessary to add protease inhibitors.

Several reagents used for the sample preparation are toxic and/or carcinogenic. For safety reasons, use protective gloves and glasses, and work in a fume hood when mixing the lysis buffer and handling samples in lysis buffer, during presolubilization with reducing agents, during precipitation, and when working with protease inhibitors.

## 2. Materials

1. Lysis buffer (*see Note 1*): 7 M urea (2.10 g) (*see Note 2*), 2 M thiourea (0.76 g) (*see Note 3*), 65 mM dithioerythritol (DTE) (650  $\mu$ L of a 0.5 M stock) (*see Note 4*), 4% (W/V) CHAPS (0.2 g) (*see Note 5*), 2% (v/v) Pharmalyte pH 3.0–10.0 (*see Note 6*), 40 mM Tris base (*see Note 7*), a trace of bromphenol blue (*see Note 8*).
2. Presolubilization solution: 2% SDS, 65 mM DTE.
3. Precipitation solution: 10% TCA in acetone, 20 mM DTE.
4. DNase/RNase solution: 1 mg/mL DNase I, 0.25 mg/mL RNase A, 50 mM MgCl<sub>2</sub>.

## 3. Methods

### 3.1. General Solubilization Protocol

It is crucial that all bacteria be disrupted, so that the lysis buffer gains access to all proteins. In studies where multiple extractions with different chemical reagents are employed sequentially, it will mask the result if more and more bacteria are disrupted during the procedure. The best method for disruption is dependent on the type of bacteria. In most cases, disruption by sonication will do, but it may be necessary to add lysosyme to break down cell walls. It is advantageous to perform the disruption in SDS or in lysis buffer containing thiourea, in which most proteases are denatured (2). If prolonged manipulation, such as fractionation by different steps of centrifugation, must be performed, protease inhibitors should be added (*see Note 9*). In the procedure described below, the bacteria are sonicated in 2% SDS, 65 mM DTE, and boiled in order to enhance the protein solubilization in general (3). It has been suggested (4) that SDS used for presolubilization does not interfere with first-dimensional electrophoretic separation because it forms micelles with the nonionic detergent of the lysis buffer and migrates out of the strip. Still, the amount of SDS should be kept low compared to the amount of detergent in the lysis buffer (5) (*see Note 10*). **Figure 1** shows a silver-stained gel loaded with 100  $\mu$ g of *Chlamydia pneumoniae* protein that was presolubilized by boiling in 1% SDS, 50 mM Tris-HCl, and diluted in the described lysis buffer to a final concentration of 0.1% SDS (*see Note 11*).

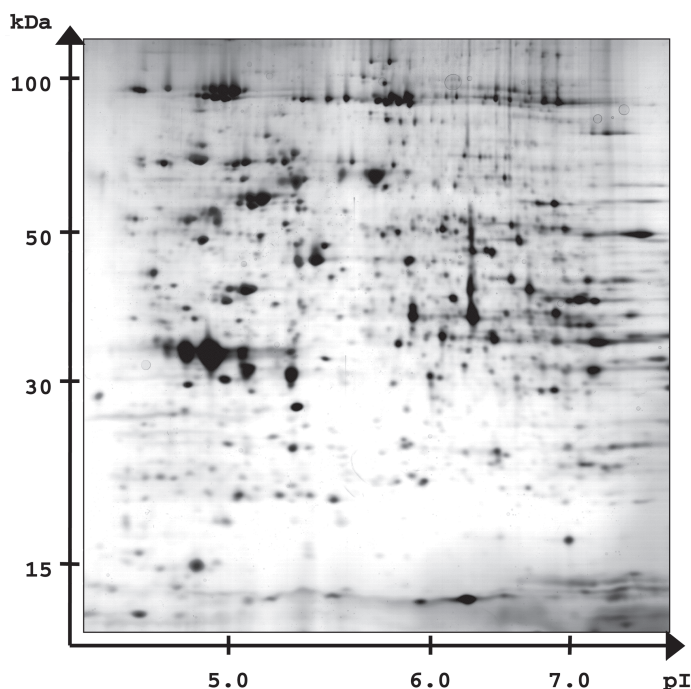


Fig. 1. A pellet of purified *Chlamydia pneumoniae* elementary bodies (**12**) was resuspended in 1% SDS, 50 mM Tris-HCl, pH 7.0, sonicated and boiled for 5 min. Cooled sample was diluted 1:10 in lysis buffer, sonicated briefly, left at room temperature for 1 h and centrifuged at 20,000g for 15 min. 350  $\mu$ L of the supernatant containing 100  $\mu$ g protein was loaded onto a pH 3.0–10.0 nonlinear immobilized pH gradient strip (Amersham Biosciences) and focused for 120,000 Volt hours. Second dimension was 9–16% linear gradient SDS-PAGE. The gel was silver stained.

The protocol below describes the solubilization of proteins from pelleted bacteria. If proteins have been precipitated as a purification step, the lysis buffer should be added directly (**step 8**) in the highest possible amount (*see Note 12*).

1. Start out with an appropriate amount of bacteria as pellet (*see Note 13*).
2. Add four times the pellet volume of 2% SDS, 65 mM DTE (*see Note 14*).
3. Sonicate three times for 2–20 s depending on sample size (*see Note 15*).
4. Spin briefly to collect the sample (*see Note 16*).
5. Resuspend any pellet that may have formed.
6. Boil for 5 min (*see Note 17*).
7. Allow the sample to cool.
8. Add 8 vols of lysis buffer to one vol of extract (*see Note 18*).
9. Sonicate three times for 5 s, cool between sonications (*see Note 19*).
10. Leave the sample on a rocking table for 30 min.
11. Spin at 20,000g for 15 min and collect the supernatant (*see Note 20*).
12. Assess the protein concentration (*see Note 21*).
13. Run first dimension immediately or store the sample at  $-70^{\circ}\text{C}$  for several months (*see Note 22*).

### 3.2. Sample Purification

Common contaminants in 2-D PAGE studies are salts, small ionic compounds, polysaccharides, nucleic acids, and lipids. Salt is the most likely reason if bad first-dimensional focusing is observed. Enhanced conductivity and water migration in the strip due to high concentrations of salts will cause horizontal streaks. The concentration of salt should be below 10 mM when samples are loaded by strip rehydration. Small charged substances may likewise disturb the isoelectric focusing. Polysaccharides may clog the gel of the strip and may complex proteins by electrostatic interactions. Lipids may also clog the gel but are mainly a problem due to complexing of hydrophobic proteins and binding of detergent. Nucleic acids may clog the gel, bind proteins through electrostatic interactions, and cause streaking, especially in silver staining.

Dialysis and precipitation (**Subheading 3.2.1.**) are straightforward and effective ways to reduce the concentration of salt and small ionic compounds to an acceptable level. Dialysis causes a minimal loss of sample, but requires relative large volumes of solute and is rather time consuming. Spin dialysis using, for instance, Amicon Ultra from Millipore is faster and requires no extra volume of solute, but protein may be lost by adsorption onto the dialysis membrane. Precipitation may also be used to remove polysaccharides and to some extent lipids. Large polysaccharides can be removed by ultracentrifugation. If lipids are causing major problems, the amount and nature of detergent must be optimized for the particular sample. High amounts of nucleic acids may require treatment with DNase/RNase (**Subheading 3.2.2.**).

The presence of proteases is likely to be a problem during sample purification, and in that case protease inhibitors must be added (*see Note 9*). It must be stressed that sample purification preceding addition of lysis buffer should be carried out only if necessary and not as a standard part of the sample preparation.

#### 3.2.1. Precipitation

Precipitation is very efficient for removal of most contaminants, including salts, but no precipitant will precipitate all proteins, and some proteins will be difficult to resuspend following precipitation. This is especially a problem when a picture of the total protein content is desired.

A combination of TCA and acetone is the most common precipitant in 2-D PAGE studies, as it is more effective than either of these reagents alone. Besides, very few proteases are active in 10% TCA. Resolubilization is easier after precipitation with acetone alone (75% final concentration), but this gives a less complete precipitation. The TCA/acetone precipitation described here is essentially as in **ref. 6**.

1. Add 10% TCA in ice-cold acetone with 20 mM DTE to the sample (*see Note 23*).
2. Leave at  $-20^{\circ}\text{C}$  for 2 h (*see Note 24*)
3. Centrifuge at 10,000g for 10 min.
4. Wash with cold acetone containing 20 mM DTE.
5. Repeat wash.
6. Let the pellet dry to remove residual acetone.
7. Resuspend pellet in lysis buffer (*see Subheading 3.1.*).

### 3.2.2. DNase/RNase Treatment

If nucleic acids are present in high amounts, the sample will appear viscous and a smear will be seen after silver staining. If ultracentrifugation does not solve the problem, enzymatic digestion will.

1. Add 1/10 of the sample volume of a solution containing 1 mg/mL DNase I, 0.25 mg/mL RNase A, and 50 mM MgCl<sub>2</sub> (see **Note 25**).
2. Incubate on ice for 20 min.

## 4. Notes

1. Absolute amounts are to make 5 mL. All reagents must be analytical grade. Use doubly distilled water. The solution is best mixed in a 10-mL tube on a rotating device. The solution should be made fresh before use or alternatively frozen in aliquots at  $-70^{\circ}\text{C}$  and only thawed once. The solution must not be heated above  $37^{\circ}\text{C}$ .  
The composition of the lysis buffer is essential for the final result of 2-D PAGE, and different lysis buffers will be optimal for different samples, and for different proteins in one sample. The function of the lysis buffer described here is to bring as many proteins in the sample as possible into solution and keep them in solution during electrophoresis. As isoelectric focusing is best carried out under denaturing and reducing conditions, the lysis buffer should solubilize, denature, and reduce the proteins of the sample. At the same time, the lysis buffer must not change the pI of the proteins, and it must not be highly conductive; hence, uncharged components are preferred. Most lysis buffers are still based on that introduced by O'Farrell in 1975 (4), containing urea as denaturing agent, a detergent, a reducing agent, and carrier ampholytes. The standard lysis buffer described here is based on (3), and the characteristics of each reagent are described in the following notes.
2. Urea— $(\text{NH}_2)_2\text{CO}$ —is a noncharged chaotrope that disrupts noncovalent bonds and thereby denatures proteins. It is the main denaturant in all lysis buffers used in 2-D PAGE, and it can be brought into solution in concentrations up to 9.8 M if no thiourea is added. Urea in solution is in equilibrium with ammonium cyanate, which in the form of isocyanic acid will react with amino groups of lysine and arginine residues and the amino terminus of proteins causing carbamylation. The carbamylation of an amine group removes a positive charge from the protein, causing a shift towards the acidic side in the gel. Furthermore, it prevents N-terminal sequencing and some enzymatic digests. To avoid carbamylation, use only freshly prepared urea solutions. A urea solution should not be left at room temperature for long periods and should never be heated above  $37^{\circ}\text{C}$  (7).
3. Thiourea— $(\text{NH}_2)_2\text{CS}$ —improves the solubilization of especially hydrophobic proteins during first dimension (8), and in combination with urea it can be used in concentrations up to 2.5 M. The addition of thiourea reduces the solubility of urea, and combinations of 7 M urea and 2 M thiourea or 8 M urea and 0.5 M thiourea are most common. The addition of thiourea to the lysis buffer has a pronounced inhibitory effect on proteases, which may still be active in high concentrations of urea alone (2). As thiourea can hinder the binding of SDS to proteins, it should not be included in the buffers used to equilibrate strips prior to second dimension (8).
4. DTE—MW: 154.3. Make a 0.5 M stock solution and store at  $-20^{\circ}\text{C}$ . DTE has the same strong reducing power as dithiothreitol (DTT), and both can be used in concentrations from 10–100 mM. At alkaline pH, both DTE and DTT are charged and migrate towards the anode during first dimension, which may leave the basic end of the strip without reducing agent and hence cause streaking due to reoxidation and precipitation. Also, 2-mercaptoethanol can be a problem to use in first dimension due to ionization at alkaline pH. Besides, 2-mercaptoethanol does not have the same reducing power as DTE and DTT.

An alternative and very strong reducing agent is tributyl phosphine (TBP). It can be used in concentrations as low as 2 mM and is noncharged, meaning that it keeps all proteins reduced throughout the first dimension, thereby enhancing the resolution (9). TBP is stable, but spontaneously inflammable in air. Make a 200-mM stock in anhydrous isopropanol and store under nitrogen at 4°C (9).

5. CHAPS—3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate—is a zwitterionic detergent that is used in many 2-D PAGE studies. Zwitterionic or nonionic detergents are preferred to anionic detergents like SDS, which interfere with the isoelectric focusing in first dimension (*see Note 1*). The efficiency of many zwitterionic detergents has been investigated, and sulfobetaines with a hydrophobic tail of 12–16 alkyl carbons and an empirically determined linker in between have been found to be good alternatives to CHAPS and superior for some samples (10). Which detergent is best for a given sample can still not be predicted. The zwitterionic agent amido sulfobetaine (ASB)-14 ( $C_{22}H_{46}N_2O_4S$ , Calbiochem), with a 14-carbon alkyl tail; the nonionic Triton X-100 ( $C_{14}H_{22}O(C_2H_4O)_n$ , with an average number (n) of ethylene oxide of 9 to 10); or the maltoside *n*-dodecyl  $\beta$ -D-maltoside ( $C_{24}H_{46}O_{11}$ , Sigma-Aldrich) would be good first-choice alternatives if CHAPS does not give satisfactory results (11).
6. Pharmalyte 3–10 can be used for most immobilized pH gradient strips, but if narrow strips or very basic strips are used, carrier ampholytes that match the pH range of the strip should be chosen. Ask the strip supplier if in doubt. Pharmalyte 3–10 is a mixture of carrier ampholytes with pI between 3 and 10. These are small amphoteric compounds with a molecular weight below 1 kDa that have a high buffering capacity at their pI but do not bind proteins due to their high hydrophilicity. When using 2% v/v of Pharmalyte 3–10 (Amersham Pharmacia), it gives a final concentration of carrier ampholytes of 0.72% in the lysis buffer, since Pharmalyte 3–10 is 36% (w/v). Carrier ampholytes help keep proteins in solution during first dimension and especially prevent hydrophobic interactions between proteins and the immobilized pH gradient in the basic end of the strip. Furthermore, the precipitation of nucleic acids is improved by carrier ampholytes.
7. Tris base is added to raise pH of the lysis buffer to 8.5. Without the addition of base, pH of the lysis buffer would be about 5.5. At alkaline pH, more proteins will be anionic and thus not bind to DNA. However, the pH for optimal solubilization will vary between samples, and Tris base is left out in many studies.
8. Bromphenol blue should be added in a small amount to color the solution lightly blue. The color will move towards the anode during first dimension, which can be used to check that the isoelectric focusing is ongoing. However, the color will disappear before the first dimension is finished and cannot be used as an indicator of when to stop.
9. Protease-inhibitor cocktails are available from most commercial laboratory reagent suppliers, but most proteases will be inhibited by adding 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1  $\mu$ M Pepstatin A, and 13  $\mu$ M Bestatin. EDTA chelates free metal ions, thereby inhibiting metalloproteases; make a 0.5-M stock solution in water, pH 8.0. PMSF inhibits serine proteases and some cysteine proteases; make a 100-mM stock solution in methanol. Pepstatin A inhibits aspartic proteases; make a 1-mM stock solution in methanol. Bestatin inhibits aminopeptidases; make a 13-mM stock solution.
10. When NP-40 is used as detergent in the lysis buffer, it has been reported (5) that the ratio of NP-40 to SDS should be at least 8 to avoid streaking. NP-40 (Nonidet P-40) is a nonionic detergent that is very similar to Triton X-100, and the properties are often reported as being identical. NP-40 (Roche) is  $C_{15}H_{24}O(C_2H_4O)_n$ , where *n* = 9–10 on average.
11. No reducing agent was added during presolubilization.



12. If the sample is applied by strip rehydration of 18-cm strips, the maximum amount is 350  $\mu$ L per strip.
13. For most bacteria, 25–100  $\mu$ g of protein is appropriate for silver staining, 100–150  $\mu$ g for immunoblotting, and 0.5–2 mg for preparative gels when 18-cm immobilized pH gradient strips in the pH range of 3.0–10.0 or similar broad range intervals are used.
14. Be sure not to add more SDS than can be diluted to 0.25% in lysis buffer. If all the sample is to be used for one gel using 350  $\mu$ L lysis buffer to rehydrate an immobilized pH gradient strip, this means that no more than 40  $\mu$ L of 2% SDS should be added. However, if streaking is observed, try lowering the amount of SDS used for presolubilization. For some samples, pH must be buffered to optimize solubilization. Use for instance 50–100 mM Tris-HCl, pH 7.0. The optimal pH may vary from sample to sample. Be aware that the final concentration of salt should not exceed 10 mM when samples are loaded by strip rehydration (*see Subheading 3.2.*).
15. Adjust the amplitude of the sonicator so that microbubbles are formed, and keep the tip of the probe deep in the sample to avoid too much foam formation. The sample should be cooled between sonications.
16. It cannot be avoided that some foam is formed during sonication, and this should be spun down before the sample is boiled, in order to avoid protein coagulation in drying bubbles.
17. DTE develops toxic gas upon heating. Boil in a fume hood.
18. Dilute the sample as much as possible in lysis buffer (*see Notes 13 and 14*).
19. When the sample is in lysis buffer containing urea, it is important to keep the temperature below 37°C in order to avoid carbamylation of proteins. If the SDS/boiling step is left out, the sonication may have to be extended.
20. The centrifugation step is important to remove cell debris and precipitated DNA, and it should not be left out.
21. Several constituents of the lysis buffer may cause problems for assessment of protein concentration. Carrier ampholytes, CHAPS, and other detergents will bind most dyes, and reduction of cupric ion cannot be employed in the presence of thiourea and DTE. Hence, the protein must be selectively precipitated and then measured. This may be done with the 2-D Quant Kit from Amersham Pharmacia. Alternatively, the protein may be estimated in a parallel sample that is not solubilized in lysis buffer. This may not give the actual protein concentration in the lysis buffer but will in most cases provide adequate information to determine the load.
22. Repeated freeze-thaw cycles should be avoided due to the risk of carbamylation and because the solubility of some proteins may be changed by the process.
23. The bacteria must be disrupted beforehand by an appropriate method (*see Subheading 3.1.*). If the bacteria are disrupted directly in the precipitation buffer, most proteases will be inactivated by the TCA.
24. The precipitation time should not be longer than the minimal time required for satisfactory precipitation. For some samples, 15 min will do, while others must be incubated overnight. Be aware that prolonged exposure to the very acidic solution may cause protein degradation.
25. The bacteria must be disrupted beforehand by an appropriate method (*see Subheading 3.1.*). If active proteases are present, it may be necessary to add protease inhibitors (*see Note 9*) even if the DNase/RNase treatment is carried out on ice.

## References

1. Righetti, P. G., Castagna A., Herbert B., Reymond F., and Rossier J. S. (2003) Prefractionation techniques in proteome analysis. *Proteomics* **3**, 1397–1407.

2. Castellanos-Serra, L., and Paz-Lago, D. (2002) Inhibition of unwanted proteolysis during sample preparation: evaluation of its efficiency in challenge experiments. *Electrophoresis* **23**, 1745–1753.
3. Harder, A., Wildgruber, R., Nawrocki, A., Fey, S. J., Larsen, P. M., and Gorg, A. (1999) Comparison of yeast cell protein solubilization procedures for two-dimensional electrophoresis. *Electrophoresis* **20**, 826–829.
4. O'Farrell, P. H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol Chem.* **250**, 4007–4021.
5. Ames, G. F. and Nikaido, K. (1976) Two-dimensional gel electrophoresis of membrane proteins. *Biochemistry* **15**, 616–623.
6. Jacobs, D. I., van Rijssen, M. S., van der Heijden, R., and Verpoorte, R. (2001) Sequential solubilization of proteins precipitated with trichloroacetic acid in acetone from cultured *Catharanthus roseus* cells yields 52% more spots after two-dimensional electrophoresis. *Proteomics* **1**, 1345–1350.
7. McCarthy, J., Hopwood, F., Oxley, D., et al. (2003) Carbamylation of proteins in 2-D electrophoresis—myth or reality? *J. Proteome Res.* **2**, 239–242.
8. Rabilloud, T., Adessi, C., Giraudel, A., and Lunardi, J. (1997) Improvement of the solubilization of proteins in two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* **18**, 307–316.
9. Herbert, B. R., Molloy, M. P., Gooley, A. A., Walsh, B. J., Bryson, W. G., Williams, and K. L. (1998) Improved protein solubility in two-dimensional electrophoresis using tributyl phosphine as reducing agent. *Electrophoresis* **19**, 845–851.
10. Tastet, C., Charmont, S., Chevallet, M., Luche, S., and Rabilloud, T. (2003) Structure-efficiency relationships of zwitterionic detergents as protein solubilizers in two-dimensional electrophoresis. *Proteomics* **3**, 111–121.
11. Luche, S., Santoni, V., and Rabilloud, T. (2003) Evaluation of nonionic and zwitterionic detergents as membrane protein solubilizers in two-dimensional electrophoresis. *Proteomics* **3**, 249–253.
12. Vandahl, B. B., Birkelund, S., and Christiansen, G. (2002) Proteome analysis of *Chlamydia pneumoniae*. *Methods Enzymol.* **358**, 277–288.





<http://www.springer.com/978-1-58829-593-4>

The Proteomics Protocols Handbook

Walker, J.M. (Ed.)

2005, XVIII, 988 p., Softcover

ISBN: 978-1-58829-593-4

A product of Humana Press