CHAPTER 3

Two-Dimensional Electrophoresis with Carrier Ampholytes

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1 Introduction

Two-dimensional (2-D) gel electrophoresis, which was originally described by O'Farrell (1975), separates proteins in the first dimension according to their isoelectric point, and in the second dimension according to their molecular weight. It offers the opportunity of separating several hundred proteins from a total cellular extract. In combination with the recent development of methods of protein identification based on microsequencing, amino acid composition and mass spectrometry, the technique of 2-D gel electrophoresis now provides an invaluable tool for proteomic studies.

There are presently two different ways of separating proteins in the first dimension on the basis of their isoelectric point. According to the first one, proteins are separated in a pH gradient generated by applying an electric field to a gel containing a mixture of free carrier ampholytes (An der Lan and Chrambach, 1985). Carrier ampholytes are low molecular mass components with both amino and carboxyl groups. According to the second way, the pH gradient is generated by a different type of chemicals, the immobilines (Bjellqvist et al. 1982). The immobilines are acrylamide derivatives carrying amino or carboxyl groups. These immobilines are copolymerized with the acrylamide gel matrix such that an immobilized pH gradient is generated.

The relative advantages and drawbacks of 2-D gel electrophoresis using carrier ampholytes or immobilines have been discussed in several reports (Corbett et al. 1994; Blomberg et al. 1995; Klose and Kobalz 1995; Lopez and Patton 1997). If it is clear that each method has its own advantages and drawbacks, it is also clear that both of them can yield reproducible gels for intra- and interlaboratory studies (Blomberg et al. 1995; Lopez and Patton 1997). This chapter is devoted to 2-D gel electrophoresis based on the use of carrier ampholytes, while Chapter 4 is devoted to 2-D electrophoresis with immobilized pH gradients. We will first report general aspects of 2-D gel electrophoresis with carrier ampholytes. Then we will describe step by step the different procedures that lead from protein sample preparation to visualization of proteins on a 2-D pattern.

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1.1 Principles of Two-Dimensional Gel Electrophoresis

Two-Dimensional gel electrophoresis of proteins is carried out under denaturing conditions. In order to separate proteins in the first dimension, proteins are solubilized in the presence of urea which essentially works by disrupting hydrogen bonds. This denaturant has the advantage that it does not affect the intrinsic charge of proteins so that it allows us to separate proteins only on the basis of their charge. When loaded on a pH gradient of adequate porosity, proteins will migrate until they have no net charge, ie when they reach the pH of the gradient corresponding to their isoelectric point (pI).

After separation of proteins according to their charge, proteins are separated in a second dimension in the presence of sodium dodecylsulfate (SDS). SDS is an anionic detergent that binds to proteins according to a constant weight ratio (1.4 g SDS per gram of protein) independent of the nature of the protein (Reynolds and Tanford 1970). The intrinsic charges of polypeptides are negligible compared to the negative charges provided by SDS, so that SDS-polypeptide complexes have essentially identical charge densities. Under this condition, proteins migrate in polyacrylamide gels strictly according to their size (Weber and Osborn 1969).

1.2 General Aspects of Two-Dimensional Gel Electrophoresis with Carrier Ampholytes

1.2.1 Sample Preparation

The method of sample preparation varies greatly depending on the cell type or the tissue from which proteins are extracted. It must, however, satisfy four main rules:
(1) efficient protein solubilization,
(2) avoidance of proteolysis and of other protein modification that would result in artefactual spots,
(3) avoidance of interfering substances such as nucleic acids, lipids, particulate material or salts that would alter protein migration and
(4) compatibility with the first dimension electrophoresis.

Usually protein solubilization is achieved by using a sample buffer containing high (8–9.5 M) urea concentration. To further improve the solubilizing effect of urea, this denaturant is used in combination with a nonionic detergent such as NP-40 or Triton X-100 or a zwitterionic detergent like CHAPS. Ionic detergents such as SDS can be used in low amounts, provided nonionic or zwitterionic detergent are present in a large excess to ensure their complete removal from proteins prior to electrophoresis. Solubilization of proteins also requires disruption of disulfide bonds. This is obtained either by use of β-mercaptoethanol or by use of dithiothreitol.

It must be emphasized that any step carried out to inactivate proteases or to remove interfering substances brings some risk of altering the 2-D protein pat-

tern. For example, use of protease inhibitors may induce some modification of protein charge (Dunn 1993). TCA precipitation in order to remove interfering material can be followed by a poor resolubilization of some proteins. Finally, it is strongly advised to extract proteins as quickly as possible, in order to avoid the action of enzymes susceptible to altering protein migration, and to avoid any step which is not absolutely necessary.

This section was intentionally limited to general considerations. For more details, the reader should consult Chapter 2. The reader may also consult Dunbar (1987), Rickwood et al. (1990) and Rabilloud (1996).

1.2.2 The Different Two-Dimensional Gel Electrophoresis Techniques

There are three major 2-D electrophoresis techniques based on the use of carrier ampholytes. They essentially differ by the separation of proteins in the first dimension.

Standard Two-Dimensional Gel Electrophoresis: IEF/SDS Gel Electrophoresis. This 2-D method is based on the use of isoelectric focusing (IEF) for separating proteins in the first dimension. It corresponds to the technique originally devised by O'Farrell in 1975. It is generally used to resolve proteins with a pH ranging from 4 to 7. It can also be used for narrow pH intervals. According to this technique, proteins are loaded on the basic side of the gel and migration is towards the anode. Polypeptides migrate through the pH gradient determined by ampholytes until they have no net charge. This takes several hours under the electrophoretic conditions generally used. Although size may affect the rate at which polypeptides migrate through the gel, their final position is determined only by their isoelectric point. When combined with SDS polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension, IEF allows us to visualize 1000 to 2000 proteins from the total cellular protein extract.

It must be emphasized that under the IEF/SDS-PAGE conditions, proteins are separated according to two parameters, isoelectric point and molecular weight, that can be deduced from their amino acid composition. Migration of a known protein can thus be predicted from its sequence. Generally, predictability of protein migration is ± 0.15 pH units for migration on the IEF gel and ± 5000 daltons for migration on the SDS gel (Boucherie et al. 1995; Garrels et al. 1997; Link et al. 1997). The difference between the expected migration of an identified protein and its observed 2-D gel location may be used for investigating post-translational modifications. Conversely, migration of an unknown protein provides valuable information regarding its identity.

NEPHGE/SDS Gel Electrophoresis. Nonequilibrium pH gradient electrophoresis (NEPHGE) separates basic proteins (O'Farrell et al. 1977). The proteins are applied to the acidic end of the first-dimensional gel and separated in a basic pH gradient. Electrophoresis is towards the cathode. Under these conditions, if the gel is allowed to run to equilibrium, there is a collapse of the basic end of the pH gradient. Most of the basic proteins would then migrate out of the gel. To avoid the run off of basic proteins the gel is not run to equilibrium. Accordingly, opti-

mum resolution of proteins is obtained after relatively short periods of electrophoresis. The pH range of the ampholytes varies depending upon the range of proteins to be resolved. Large ampholyte ranges (pH 3–10) are generally used. In this case, following migration, acidic proteins are compressed at the anodic end of the gel and basic proteins are well resolved. Narrow basic pH ranges (pH 7–10) can also be used for detailed studies of basic proteins.

Migration of the proteins in NEPHGE gels is dependent upon both their electrophoretic mobility and their isoelectric point. Using NEPHGE/SDS PAGE gels, several hundred proteins not resolved by IEF can be visualized.

Giant Gels. As shown above, the complete 2-D separation of a complex protein sample using carrier ampholytes requires two separate 2-D runs, one based on an IEF separation and the other on an NEPHGE separation, and results in two partially overlapping protein patterns. Since 1975, Klose et al. (Klose 1975; Klose and Feller 1981; Klose and Kobalz 1995) combining several refinements of the 2-D technique, have devised a 2-D method that offers a good separation of both acidic and basic proteins. As for NEPHGE separation, proteins are loaded on the anodic side of the gel. A wide pH range is used in combination with the use of very long first-dimensional gels (46 cm). Focusing is stopped before the basic proteins reach the cathodic end of the gel. This technique provides the most powerful resolving 2-D method so far reported in the literature. More than 10 000 polypeptide spots can be resolved on these giant gels (46×30 cm). However, this technique, given its sophistication, is not accessible to the beginner and remains limited to a few experienced laboratories. It has been described in detail by Klose and Kobaltz (1995), as well as the special equipment required.

1.2.3 Two-Dimensional Gel Electrophoresis: The Choices

The beginner will have to face several important technical choices at each step of 2-D gel electrophoresis before deciding on a definitive strategy for running 2-D gels. Each of these choices will have an impact on the final quality of 2-D separation. The following section deals with the main choices that will be encountered.

Equipment. The first dimension based on the use of carrier ampholytes is generally carried out in vertical cylindrical gels and the second dimension is most often run on vertical slab gels. Many different commercialized apparatus for both the first and the second dimension of these types are available, e. g. the Multi Cell (Bio-Rad), Iso-Dalt 2-D gel electrophoresis system (Amersham Pharmacia-Biotech) and Esa 2-D system (Esa Inc.). However, first dimension and second dimension apparatus are very conventional and can be easily constructed in the laboratory.

Alternatively, the first dimension can be run on horizontal slab gels; the strips corresponding to each sample run are sliced, and applied to the surface of a horizontal slab gel for running the second dimension (Multiphor II horizontal electrophoresis apparatus; Amersham Pharmacia-Biotech).

First Dimension. The length of the first-dimensional gel routinely used can vary from 13 to 25 cm. It should be kept in mind that the number of spots to be detected is highly dependent on the size of the gel. The diameter of the cylindrical gels is also important: depending on its size (more or less than 1 mm), equilibration prior to the second dimension may or may not be required (see below). Finally, it must be taken into account that the acrylamide concentration of first dimensional gels must be low enough in order to render protein migration independent of the size of the protein. However, it should be sufficient to allow easy manipulation of the gels. This is generally obtained by acrylamide concentration around 3.5 to 4.5 %.

Equilibration Versus Nonequilibration. To facilitate transfer of proteins from the first-dimensional gel to the second dimension, it is generally recommended to equilibrate gels in the presence of SDS. The main objective of equilibration is to coat proteins with SDS before starting migration in the second dimension. Then, all molecules of the same protein will run out of the first-dimensional gel at the same time and proteins will be separated as round spots in the second dimension. In the absence of equilibration, the SDS is provided by the electrophoresis buffer of the upper chamber. At the onset of the second dimension, SDS enters the first-dimensional gel, binds to proteins, and subsequently SDS-coated proteins enter the second-dimensional gel. A progressive binding of SDS to proteins at this stage may occur, resulting in streaking.

However, it should be kept in mind that prolonged equilibration results in protein loss, particularly of low molecular weight species. The loss of proteins during equilibration can be as high as 15 to 25 % (Garrels 1989; Rickwood et al. 1990). It is thus better to avoid this step when not necessary. This is the case when first-dimensional gels are 1 mm in diameter or less.

Second Dimension. In the second dimension, a uniform separating gel (generally 10 or 11 % acrylamide) or a gradient separating gel (10–15 %) can be used. However, it is advisable to use gradient gels only when necessary. It increases the difficulty of obtaining reproducible gels as it is difficult to ensure that gradients are exactly the same over a long period of months. For proteomic studies, the aim of which is to obtain an overview of whole cell proteins, it should be kept in mind that the average size of proteins is 50 000. Gradient gels often allow a good resolution of proteins between 7000 and 30 000 whereas the vast majority of proteins (30 000 to 100 000) are compressed in the upper part of two-dimensional gels.

To avoid protein streaking in the second dimension, a stacking gel is often recommended. The addition of this stacking gel may provide a further source of nonreproducibility. When the diameter of first-dimensional gels is small enough, no stacking gel is required.

Precast Gels. Precast gels for the first dimension with carrier ampholytes and for the second dimension can be purchased from Esa Inc. (Chelmsford, Massachusetts) and from Amersham Pharmacia-Biotech (Uppsala, Sweden).

1.2.4
Post-Two-Dimensional Gel Electrophoresis Procedures

There are many different ways to detect proteins after two-dimensional gel separation, e. g. staining, autoradiography, fluorography, fluorescence. Special mention should be given to the use of the phosphor screen technology. This technique presently offers the best way to quantify spots, provided radioactive labelling of protein is possible. It is the most sensitive technique presently known for the detection of radioactive proteins. Also, phosphor imaging plates have a linear dynamic range for radioactivity detection which covers five orders of magnitude (Johnston et al, 1990). This allows us to easily quantify all polypeptide spots detectable on a gel in only one gel exposure. In comparison, X-ray films for autoradiography are 10 to 250 times less sensitive. In addition, they require several time exposures as the linear dynamic range of their response to radioactivity covers only two orders of magnitude. For similar reasons, quantification of proteins after silver staining requires the running of several gels with different loading of protein sample. Phosphor screens allowed us to detect ^{14}C, ^{35}S, ^{32}P and ^{3}H radioisotopes.

Identification of separated polypeptides can be carried out by microsequencing, mass spectrometry (Schevchenko et al. 1996) or amino acid composition (Garrels et al. 1994; Maillet et al. 1996; see also part II of the present book). When the amount of protein contained in the polypeptide spot to be identified is too low, it is necessary to concentrate the protein by using several spots excised from different gels. Two different elution-concentration gel systems can be used. One is based on the use of a vertical slab gel containing a large polyacrylamide stacking gel and a small resolving gel (Rasmussen et al. 1991). The other is based on the use of a Pasteur pipette containing only a small polyacrylamide stacking gel (Gevaert et al. 1996; Kristensen et al. 1997).

2
Sample Preparation

The sample preparation described here was originally devised for extracting proteins from yeast cells (Boucherie et al. 1995, 1996). Because yeast cells are surrounded by a cell wall, protein extraction cannot be simply carried out by an osmotic shock as it is, for example, with mammalian cells. Extensive breakage of yeast cells is required. This is obtained by vigorously shaking cells in the presence of glass beads. To avoid proteolysis, this step is performed on lyophilized cells, in the absence of buffer. Apart from preventing proteolysis, a further interest of this procedure is the possibility of sending cells for protein extraction by ordinary mail without the requirement of frozen ice. Another particularity of this sample preparation is the heating of the sample in the presence of SDS at the first step of protein solubilization. The rationale for this heating is not only to increase solubilization, but also to inactivate proteases or other enzymes that may alter protein size or charge.

No protease inhibitors are added to the sample according to our procedure (some inhibitors are known to induce charge alteration; Dunn 1993). In contrast,

exogenous DNase and RNase are added in order to eliminate nucleic acids. Care should be taken to use protease-free DNase and RNase. The entire procedure, as applied to the extraction of proteins from yeast cells, is described in Protocol 1 (Sect. 2.4). This extraction procedure has been revealed to be also well adapted to protein extraction of bacterial cells. With modifications (see Sect. 2.4) it has been successfully used for extracting proteins from mammalian cells.

The final concentration of the protein sample is 0.03 M Tris-HCl pH 8.0, 9.5 M urea, 0.1 % SDS, 0.7 % CHAPS, 1.75 % β-mercaptoethanol, 0.2 % ampholytes. We observed that increasing the SDS concentration to 1 % or (and) the CHAPS concentration to 4 % does not improve protein solubilization.

2.1 Chemicals

CHAPS, 4.9 M, $MgCl_2$ solution, RNase A type XII-A from bovine pancreas, Trizma Base, Trizma hydrochloride (Sigma); dodecylsulfate sodium salt LAB (SDS), urea beads ultra pure, β-mercaptoethanol for molecular biology (Merck); Pharmalyte 3–10 (Amersham Pharmacia-Biotech); DNase I, RNase free, from bovine pancreas, 10 000 units/ml (Boehringer Mannheim).

2.2 Buffers

- *Extraction Buffer A* (0.1 M Tris-HCl pH 8.0, 0.3 % SDS). To prepare 100 ml of lysis buffer, dissolve 888 mg of Trizma hydrochloride, 530 mg of Trizma Base and 300 mg of SDS in deionized water and make up to 100 ml. Filter through a 0.45-μm pore size filter and store as 500-μl aliquots at -20 °C.
- *RNase A Solution* (0.05 M $MgCl_2$, 200 Kunitz units/ml RNAse A, 0.5 M Tris-HCl pH 7.0). To prepare 2.5 ml of RNase solution, dissolve 5 mg RNase A in 1.7 ml of 0.75 M Tris-HCl pH 7.0. Add 25.5 μl of 4.9 M $MgCl_2$ and 0.775 ml deionized water. Store as small aliquots (25 μl) at -20 °C.
- *Extraction Buffer B* (4.75 M urea, 4 % CHAPS, 1 % Pharmalyte 3–10, 5 % β-mercaptoethanol). To prepare 10 ml sample buffer, dissolve 2.85 g urea, 0.4 g CHAPS, 0.5 ml β-mercaptoethanol and 0.25 ml Pharmalyte 3–10 in 7 ml deionized water. Store as aliquots (150 μl) at -20 °C.

2.3 Equipment

MiniBeadBeater (Biospec Products); acid-washed glass beads (0.45 mm, glass beads; B. Braun).

2.4 Procedure

Protocol 1. Sample Preparation

1. The amount of cells to be used for protein extraction is calculated such that the final protein concentration of the sample ranges between 3 and 10 mg/ml. Washed cells are transferred into a "screw cap" microcentrifuge tube, resuspended in a small amount of ice-cold deionized water (50 to 100 µl) and frozen at –80 °C prior to being lyophilized. It is of importance not to lyophilize cells as a pellet. This would greatly impair the efficiency of cell breakage.
2. Lyophilize cells, taking care not to exceed too much the minimum time required for complete lyophilization. Overpassing this time results in a decrease in cell disruption efficiency.
3. Add acid-washed glass beads (0.45 mm in diameter) to the lyophilized cells. The volume of glass beads must be equivalent to the volume of resuspended cells prior to lyophilization.
4. Disrupt cells by shaking lyophilized cells in the presence of glass beads on a MiniBeadBeater. The tubes are agitated five times for 20 s, at 20-s intervals, leaving on ice between bursts of shaking.
5. Solubilize proteins by adding extraction buffer A previously kept at 100 °C. Briefly vortex the sample.
6. Maintain the sample for 10 s in a water bath at 100 °C.
7. Leave on ice for 1 min.
8. Add per microliter of extraction buffer A, 0.1 µl RNase A solution and 0.02 µl DNase I (10 000 units/ml) to yield a final concentration per milliliter of 20 000 units RNase A and 200 units of DNase I. Add β-mercaptoethanol to bring the final concentration to 2.5 % (v/v).
9. Incubate for 1 min at 4 °C.
10. Add per microliter of sample: 1.4 mg of urea and 0.5 µl of extraction buffer B. Mix by gently moving the tube upside–down several times.
11. Leave 5 min at room temperature.
12. Gently vortex the sample and centrifuge for 3 min at 13 000 rpm .

Note: the final volume of the sample will be 3.12 × the initial volume of extraction buffer A added at step 5. Steps 3 and 4 can be omitted when using mammalian cells and more generally cells which can be easily lysed by an osmotic shock. The samples can be used immediately or stored at -80 °C for several months. Samples should not be thawed and frozen again. Accordingly, prepare aliquots that will be thawed only once.

Protocol 2. Rapid Sample Preparation

When there is no risk of proteolysis, heating of extraction buffer A is not necessary, and steps 5 and 6 can be omitted. In practice, after step 4, proteins are solublized in a mixture of extraction buffer A, RNase and DNase solutions and β-mercaptoethanol, prepared according to the ratios reported in step 8. Continue then as described in step 9.

2.5
Measurement of Sample Protein Concentration

The amount of protein loaded on the first dimension varies depending on the technique to be used for protein visualization. It is better to load as small an amount of protein as possible in order to prevent alteration of the pH gradient of the first dimension. When yeast proteins are radioactively labelled (^{14}C or ^{35}S), the equivalent of 5×10^6 cpm is loaded. This generally corresponds to a 10-μl sample containing 30 μg of proteins. Using phophor screen technologies, proteins can be visualized after a one-night exposure. When yeast proteins have to be revealed by silver staining 300 μg is loaded. Usually, 30 μl of a sample with a protein concentration of 10 mg/ml is loaded.

2.5.1
Assay for Protein Radioactivity in Samples

Pipette 2 μl of protein sample on a glass microfibre filter (GF/C, Whatman). Let filters dry at room temperature for 15 min. Soak filters twice for 10 min in 5 % TCA containing 1 g/l of the same amino acid as the one used for labelling proteins. Then place dried filters in a counting vial. Counting is done in 5 ml of liquid scintillaton (Ready value; Beckman).

2.5.2
Measurement of Protein Concentration

Protein samples prepared according to our procedure can be assessed for protein concentration using the modified Bradford assay of Ramagli and Rodriguez (1985). The modification consists of an acidification of the samples prior to determining protein concentration.

3
First Dimension: Standard Isoelectric Focusing

The pH range of protein separation according to this standard isoelectric focusing is from 4.5 to 7. Isoelectric focusing is carried out on gel rods which are 24 cm long and 1 mm in diameter. Given the small diameter of these gels, equilibration is not required prior to submitting proteins to the second dimension. An example of 2-D protein separation obtained by using this type of isoelectric focusing in combination with standard 2-D gel electrophoresis (described in Sect. 4) is shown in Fig. 3.1.

Acrylamide concentration: 3.6 % T, 4.7 % C
Gel composition: 3.4 % acrylamide, 0.17 % bisacrylamide, 9.5 M urea, 3.6 % CHAPS, 4 % ampholytes
Gel size: 24 cm long, 1 mm in diameter
pH gradient: 4.5–7

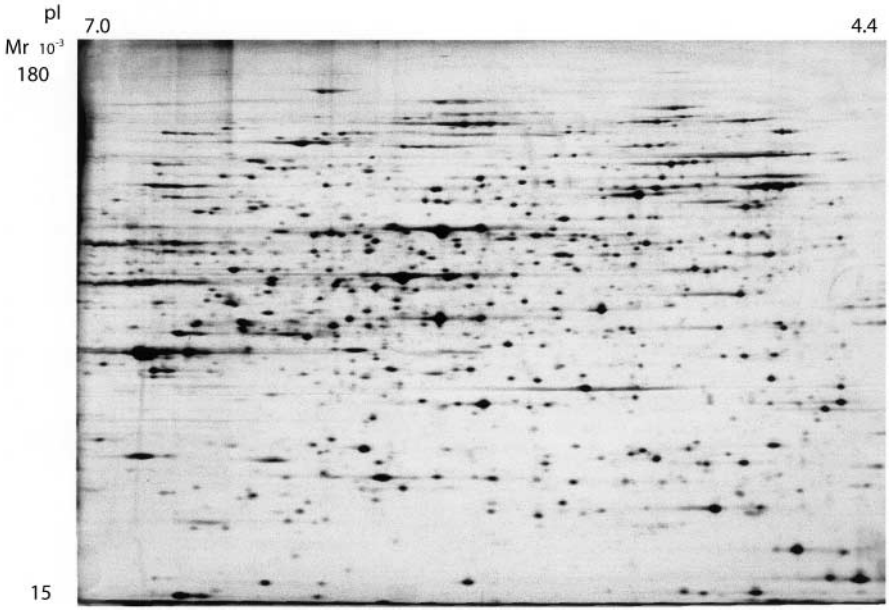

Fig. 3.1. Two-dimensional electrophoresis using standard isoelectric focusing. First dimension IEF. Second dimension SDS-PAGE (11 % acrylamide). Silver stain. Sample: *Saccharomyces cerevisiae*

3.1 Chemicals

N, N'-methylenebisacrylamide ultrapure, electrophoretic grade, ammonium persulfate (Boehringer Mannheim); CHAPS (Sigma); urea beads ultra pure (Merck); acrylamide IEF PlusOne, Pharmalyte 3–10, Pharmalyte 5–6, Pharmalyte 5–8 (Amersham Pharmacia-Biotech).

3.2 Reagent Solutions

- *IEF Acrylamide Solution* (29.8 % T, 4.7 % C). 28.4 % acrylamide (w/v) and 1.4 % (w/v) N, N'-methylenebisacrylamide. To prepare 25 ml of the solution, dissolve 7.1 g acrylamide and 0.35 g bisacrylamide in 20 ml deionized water. Make up to 25 ml, filter through a 0.45-μm pore size filter and store at 4 °C for no more than 2 weeks. Keep in a brown bottle to protect from light.
- *CHAPS Solution*. 10 % (w/v) in deionized water. Dissolve 2.5 g of CHAPS in 20 ml deionized water and make up to 25 ml. Filter through a 0.45-μm pore size filter and keep at 4 °C for no more than 1 month.
- *Ammonium Persulfate Solution* (10 % APS). To prepare 2 ml of solution, dissolve 200 mg ammonium persulfate in 2 ml deionized water. This solution should be prepared just before use.

- *Cathodic Solution* (0.1M NaOH). To prepare 1 l of cathodic solution, dissolve 4 g NaOH in 1 l deionized water. Deaerate under vacuum for 30 min while continuously stirring. The cathodic solution is prepared just before use.
- *Anodic Solution* (0.08 M H_3PO_4). To prepare 1 liter of anodic solution dissolve 5.5 ml of 85 % phosphoric acid in 1 l of deionized water. Prepare just before use.
- *Overlay Solution* (2.37 M urea, 2 % CHAPS, 0.5 % Pharmalyte 3–10). To prepare 10 ml of overlay solution, dissolve 1.4 g urea and 0.2 g of CHAPS in deionized water, add 125 µl Pharmalyte 3–10 and make up to 10 ml. Store as aliquots (200 µl) at -20 °C.
- *Sample Buffer* (0.03 M Tris-HCl pH 8.0, 9.5 M urea, 0.1 % SDS, 0.7 % CHAPS, 1.75 % β-mercaptoethanol, 0.2 % ampholytes). To prepare sample buffer, mix the various components used for sample preparation in the same proportion as described in Section 2.4, only omitting RNase and DNase solutions.

3.3 Equipment

We use glass tubes specially designed for isoelectric focusing (see Fig. 3.2; available on special request from Atelier Jean Premont, 36 av de Labarde, 33300 Bordeaux, France). The part of the tube in which the gel is polymerized is 24 cm long and has an inner diameter of 1 mm. It is surmounted by a 2.2-cm glass tube which has the same external diameter (8 mm) and an inner diameter of 2.5 mm. This part of the tube is used as a funnel in which the sample is loaded. A 10-ml pipette-pump is required to fill the tubes with the gel solution (Poly Labo).

In our laboratory the isoelectric focusing gel tubes are mounted into a homemade vertical electrophoresis apparatus with an upper and a lower electrode chamber. This apparatus can hold 12 gel rods. Silicone rubber grommets convenient for holding the tubes can be purchased from BioRad (Cat no. 165–1985). However, any commercialized apparatus for isoelectric focusing capable of accomodating glass tubes which are 26 cm long can be used.

The isoelectric focusing gels are polymerized and run in an incubator maintained at 26 °C.

3.4 Preparation of Isoelectric Focusing Gels

Recipe for 12 tubes. The first-dimensional gels are prepared the day before isoelectric focusing. We observe a better reproducibility under this condition.

1. Keep the glass tubes for at least 1 hour at 26 °C.
2. Prepare the 10 % ammonium persulfate solution.

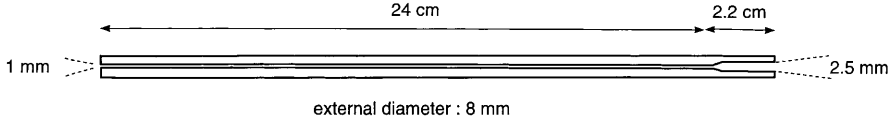

Fig. 3.2. Schematic representation of glass tube for IEF gels

3. Prepare the first-dimensional acrylamide gel solution in a corex tube by adding the components in the following order:
   – 4 g of urea
   – 0.85 ml of IEF acrylamide solution
   – 2.6 ml of 10 % CHAPS
   – 166 µl of Pharmalyte 3–10
   – 166 µl of Pharmalyte 5–6
   – 332 µl of Pharmalyte 5–8.
4. Dissolve urea by gentle mixing. Warm the urea solution by keeping the corex tube in the palm of the hand. Do not heat the urea solution!
5. Deaerate the solution under vacuum for 3 min.
6. Initiate polymerization by adding 20 µl of 10 % APS freshly prepared.
7. Swirl gently the corex tube in the hand. Take care not to re-introduce oxygen into the solution.
8. Glass tubes can be filled simultaneously with the first-dimensional gel acrylamide solution, using a gel-casting apparatus such as the one described by Garrels (1983) or Dunbar (1987). According to this procedure, the gel solution is displaced upwards into the glass tubes by overlaying the gel solution with water. Alternatively, glass tubes can be filled individually by using a pipette-pump. For this purpose, insert the upper end of the glass tube ("loading funnel") into the pipette-pump. Pump the gel solution until it enters a few millimeters into the "loading funnel". Maintain the tube horizontally and remove the pipette-pump. Immediately press the thumb on the upper end of the tube in order to prevent leakage of the gel solution. Eliminate excess acrylamide solution by carefully releasing the pressure of the thumb (acrylamide solution must be only 1 mm inside the loading funnel) and lay the tube horizontally for polymerization. This procedure is routinely used in our laboratory.
9. Leave the gels to polymerize overnight at 26 °C.

Note: TEMED is not required for polymerization.

3.5 Running Standard Isoelectric Focusing Gels

According to this procedure the samples are loaded at the basic end of the gels. A prefocusing step is performed prior to loading the samples.

1. Place the isoelectric focusing tubes into the electrophoresis stand. Fill the lower chamber with 0.08 M H_3PO_4. If necessary remove air trapped at the bottom end of the tubes.
2. Overlay the gels with 15 µl of sample buffer. Fill up the tubes with the 0.1-M NaOH solution previously deaerated.
3. Fill the upper chamber with the 0.1-M NaOH solution, taking care not to disturb the sample buffer layer on the top of the gels.
4. Prefocus the gels as follows:
   – 500 V for 30 min
   – 1000 V for 45 min
   – 1500 V for 15 min.

5. Remove the upper electrode buffer and the sample buffer.
6. Load the protein sample. Cover with 15 µl of overlay solution. Fill up the tubes and the upper chamber with fresh upper electrode buffer.
7. Focusing is carried out at:
   - 500 V for 15 min
   - 1000 V for 30 min
   - 1600 V for 21 h.
8. After focusing, the gels are immediately extruded from the glass tubes. To extrude the gels, use a 2.5-ml syringe filled with water and fitted with a yellow pipette tip. Insert the tip into the top end of the glass tube and push out the gel by pressure on the syringe. The gels are directly extruded onto a piece of Parafilm and are kept at –80 °C. A corner of the Parafilm is cut, indicating the basic side of the gel.

The prefocusing and the focusing are carried out in an incubator maintained at 26 °C.

4 Second Dimension: Standard Slab Gel Electrophoresis

The second dimension is run on a vertical slab gel. This slab gel (90 cm large) allows us to run three second-dimensional gels in parallel, which maximizes comparability of two-dimensional gels. There is no stacking gel and the first-dimensional gels are layed on the top of the slab gel without any sealing with agarose. Another particularity of this second dimension procedure is that the gel composition does not contain SDS as usual. The only SDS present in the gel during the second dimension electrophoresis comes from the electrophoresis buffer of the upper chamber. It enters the gel when the current is applied. We found that the absence of SDS in the slab gel has a "stacking" effect on proteins when they leave the first-dimensional gel and enter into the slab gel. This second-dimensional gel resolves proteins with molecular weight that range between 180 000 and 17 000.

Acrylamide concentration: 11 % T, 3.3 % C
Gel composition: 0.36 M Tris-HCl pH 8.5, 10.6 % acrylamide, 0.35 % bisacrylamide
Gel size: 85 cm long, 20 cm high, 1 mm thick
Molecular weight resolution: 17 000 to 180 000

4.1 Chemicals

Acrylamide, N, N'-methylenebisacrylamide, ammonium persulfate (APS), tetramethylethylenediamine (TEMED) (Boehringer Mannheim); Trizma base, Trizma hydrochloride (Sigma); glycine for electrophoresis, dodecylsulfate sodium salt LAB (SDS) (Merck).

4.2 Solutions for Second-Dimensional Gels

- *Slab Gel Acrylamide Solution* (30,1 % T, 3.3 % C). 29.1 % acrylamide (w/v) and 0.99 % N, N'-methylenebisacrylamide. To prepare 1 l of acrylamide solution, dissolve 291 g acrylamide and 9.9 g bisacrylamide in 800 ml deionized water. Fill up to 1000 ml. Store at 4 °C, for no more than 2 weeks. Keep in a brown bottle to protect from light.
- *Slab Gel Buffer* (1.5 M Tris-HCl pH 8.5). Dissolve 130 g Trizma base and 66.3 g Trizma hydrochloride in 800 ml deionized water. Fill up to 1 l with deionized water. Store at 4 °C.
- *Ammonium Persulfate Solution.* 10 % in deionized water. To prepare 2 ml of solution, dissolve 200 mg ammonium persulfate in 2 ml deionized water. This solution should be prepared just before use.
- *Electrode Buffer* (192 mM glycine, 25 mM Trizma base, 0.2 % SDS). To make 4000 ml electrode buffer add 12.12 g Trizma base, 57.6 g glycine and 8 g SDS. Dissolve in deionized water and fill up to 4000 ml. Prepare before use.

4.3 Equipment

The polymerization cassette consists of two glass plates, a back plate and a front plate (Fig. 3.3a, b) each 925 mm long, 215 mm high and 4 mm thick. The cassette allows us to cast a wide slab gel corresponding to three second-dimensional gels to be run in parallel. The front plate has a notch 15 mm deep and 845 mm long. A PVC U-frame 1 mm thick (Fig. 3.3c) is used to delimit the gel. PVC strips 1 mm thick are used as spacers (Fig. 3.3d). Plexiglas spacers (1 mm thick) are used to fix the distance between the glass plates.

The apparatus for running slab gels is a modification of the one described by Garrels (1983; Fig. 3.4; available on special request from Deco Volume, 30 rue Denfert Rochereau, 33400 Talence, France). It is a vertical system that allows us to run only one wide slab gel at the same time. Usually, two apparatuses are run in parallel.

4.4 Preparation of the Casting Cassette

1. Before use, wash the glass plates with deionized water and carefully air-dry.
2. Place the U-frame and the PVC spacers on the back plate (Fig. 3.3c, d). Make sure that the U-frame and the spacers do not overlap.
3. Place the front plate on the U-frame (Fig. 3.3e)
4. Hold the plates together with clamps (Fig. 3.3f)
5. Slightly insert Plexiglas spacers between the plates in order to maintain the distance between the plates (Fig. 3.3g, h). Press the two glass plates against these spacers by means of plastic U-clamps.

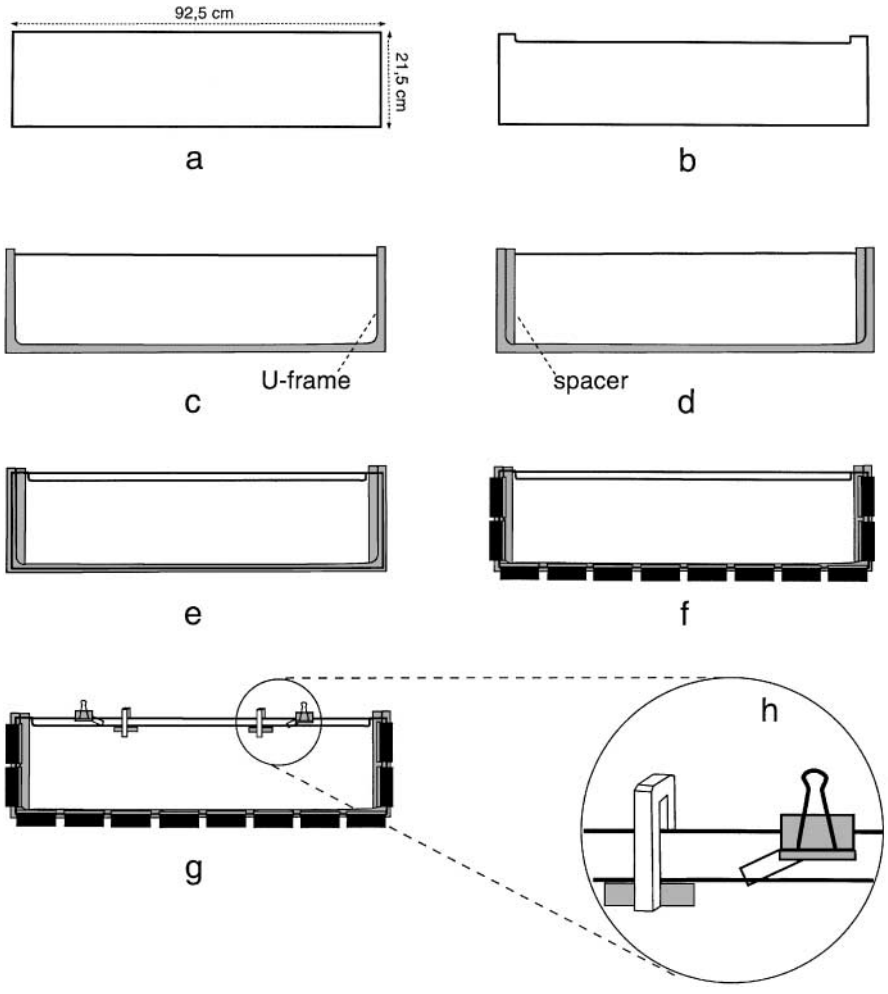

Fig. 3.3. Preparation of the casting cassette for second-dimensional gels. *a* back plate; *b* front plate; *c-h* preparation of the casting cassette (see text)

4.5 Preparation of Slab Gels

1. In a 500-ml vacuum flask add successively:
   - 51 ml of slab gel buffer
   - 76.5 ml of slab gel acrylamide solution
   - 81 ml of deionized water
   - Deaerate under vacuum for 3 min
   - Add 1 ml of 10 % APS freshly prepared
   - Mix gently
   - Add 136 μl of TEMED

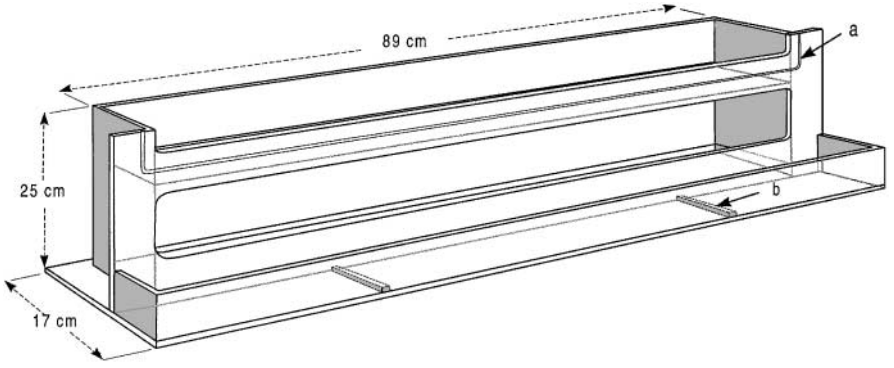

Fig. 3.4. Schematic representation of slab gel electrophoresis apparatus. The gel cassette is clamped against the front of the upper electrode chamber. A silicon sponge cord is placed in a notch (*a*) to seal the upper chamber against the front glass plate. Spacers (*b*) maintain the glass plates off the bottom of the lower electrode chamber

– Transfer to a 500-ml beaker. Take care not to re-introduce oxygen into the solution.

2. Pump the gel solution with a 50-ml syringe and pour it down inside the cassette, by making the solution run down alternately on the left and right side of the plates (never in the middle of the cassette). Add the gel solution up to a level 2 or 3 mm below the bottom of the notch.
3. Gently overlay the gel solution with deionized water.
4. Allow the slab gel to polymerize for at least 1 h at room temperature.

Note: there is no SDS in the gel composition.

4.6 Running Two-Dimensional Gels

Three two-dimensional gels are run simultaneously on each of the slab gels.

1. Prepare the electrophoresis buffer (192 mM glycine, 25 mM Trizma base, 0.2 % SDS).
2. Remove the U-frame of the casting cassette. Remove any residual liquid on the top of the gel by blotting or aspiration. Rinse the gel surface with deionized water and dry before loading isoelectric focusing gels.
3. Lay the SDS gel cassette at 45° with the front plate upward in order to facilitate the application of the first-dimensional gels.
4. Transfer the first dimensional gel from the Parafilm to the back glass (internal side) of the casting cassette, taking care that the first-dimensional gel is parallel to the top of the slab gel.
5. With a blunt-ended spatula push the first-dimensional gel between the glass plates, seating it carefully on the top of the slab gel. A few drops of running buffer will help to slide the gel between the glass plates. Be sure that no air bubbles are trapped between the IEF gel and the surface of the acrylamide slab gel.

6. Do the same with the two other first-dimensional gels.
7. Insert the cassette into the electrophoresis apparatus.
8. Carefully fill the space of the cassette above the rod gels with electrophoresis buffer using a pipette. Take care not to displace the first-dimensional gels.
9. Fill the upper and higher chamber with electrophoresis buffer.
10. Take care that no air bubbles are trapped between the lower surface of the slab gel and the two glass plates of the cassette. If so, remove the bubbles by a stream of buffer from a bent needle connected to a 50-ml syringe.
11. Run the slab gel at room temperature as follows:
    - 5 W for 15 min
    - 25 W until the Bromophenol blue tracking dye reaches the bottom of the gel. The running time is about 6 h.
12. After running, open the cassette with a spatula. Cut the slab gel into three individual gels, each corresponding to one of the first-dimensional gel loaded. Cut the lower corner of the gel corresponding to the basic side of the first dimensional gel to indicate its orientation. Then process the gels to detect polypeptides.

Note: as already mentioned in the previous isoelectric focusing section, equilibration of first-dimensional gels prior to loading on top of the second-dimensional gels is omitted.

5
Modifications of the Standard Two-Dimensional Gel Method

5.1
Extension of the pH Gradient and of the Molecular Weight Scale

The extension of the pH gradient and of the molecular weight scale described here allows us to visualize, in addition to the proteins separated by the standard 2-D gel system, some acidic and basic proteins as well as low molecular weight proteins. For this purpose, a modification in the ampholyte composition of the first-dimensional gel is used to improve the resolution of basic and acidic proteins. An increase in the acrylamide concentration of the slab gels allows us to visualize low molecular weight proteins. In return, proteins separated by the standard 2-D gel method are restricted to an area of gel smaller than on standard gels. Protein separation ranges from pH 3.4 to 7.2 in the first dimension and from molecular weight 180 000 to 8000 in the second dimension. An example of gels obtained according to this procedure is shown in Fig. 3.5.

Preparation of a protein sample for loading on the first-dimensional gels is the same as for standard IEF gels.

First dimension:
Acrylamide concentration: 3.6 % T, 4.7 % C
Gel composition: 3.4 % acrylamide, 0.17 % bisacrylamide, 9.5 M urea, 3.6 % CHAPS, 4 % ampholytes
Gel size: 24 cm long, 1 mm in diameter
pH gradient: 3.4–7.2

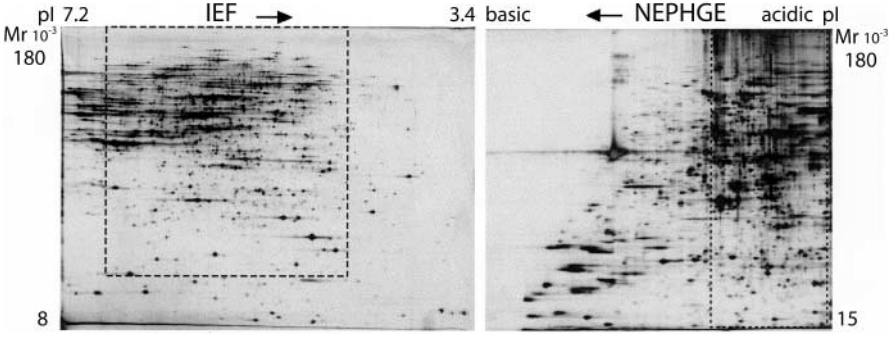

Fig. 3.5. Two-dimensional electrophoresis using modifications of standard 2-D gel electrophoresis. *Left* Extension of the pH gradient and of the molecular weight scale. First dimension modified IEF procedure (see Sect. 5.1). Second dimension SDS-PAGE (13 % acrylamide). *Right* Separation of basic proteins. First dimension NEPHGE (see Sect. 5.2). Second dimension SDS-PAGE (11 % acrylamide). Silver stain. Sample: *Saccharomyces cerevisiae*. *Delimited area* indicates proteins which are resolved by standard IEF/SDS PAGE electrophoresis

Second dimension:
Acrylamide concentration: 13 % T, 3.3 % C
Gel composition: 12.53 % acrylamide, 0.45 % bisacrylamide
Running buffer: 0.192 M glycine, 25 mM Trizma base, 0.2 % SDS
Gel size: 85 cm large, 20 cm high, 1 mm thick
Molecular weight separation: 8000 to 180 000

5.1.1 Chemical

Pharmalyte 2–4, Pharmalyte 7–9 (Amersham Pharmacia-Biotech). Other chemicals are as for IEF gels (see Sect. 3.1).

5.1.2 Procedure

First Dimension. Reagent solutions for preparing first-dimensional gels are as for IEF gels (see Sect. 3.2). To prepare the first-dimensional acrylamide gel solution for 12 gels, add the components in a corex tube in the following order:
- 4 g of urea
- 0.85 ml of IEF acrylamide solution
- 2.6 ml of 10 % CHAPS
- 249 µl of Pharmalyte 3–10
- 166 µl of Pharmalyte 2–4
- 124 µl of Pharmalyte 5–8
- 124 µl of Pharmalyte 7–9.

All other steps, for preparation of the first-dimensional gel and running the first dimension, are as for IEF gels (see Sects. 3.4 and 3.5).

Second Dimension. Chemicals and reagent solutions for preparing the second dimensional gels are as for standard slab gels (see Sects. 4.1 and 4.2). To prepare the slab gel solution, add successively in a 500-ml vacuum flask:
- 51 ml of slab gel buffer
- 90.4 ml of slab gel acrylamide solution
- 67 ml of deionized water
- Deaerate under vacuum for 3 min
- Add 1 ml of 10 % APS freshly prepared
- Mix gently
- Add 136 µl of TEMED
- Transfer to a 500-ml beaker.

All other steps, for preparation of the second dimensional gel and running the second dimension, are as for standard slab gels (see Sects. 4.5 and 4.6).

5.2 Separation of Basic Proteins by NEPHGE

Prepartion of a protein sample for loading NEPHGE gels is the same as for standard IEF gels. In contrast to IEF gels, the sample is loaded at the acidic end of the gel, and the gel is run without prefocalization. The second dimension is the same as for standard two-dimensional gel electrophoresis.

Acrylamide concentration: 3.6 % T, 5.4 % C
Gel composition: 3.4 % acrylamide, 0.19 % bisacrylamide, 9.35 M urea, 5.6 % CHAPS, 2 % ampholytes
Gel size: 24 cm long, 1 mm in diameter
pH gradient: 4–10

5.2.1 Chemicals

Acrylamide IEF PlusOne, Pharmalyte 3–10, Ampholine 3.5–10 (Amersham Pharmacia-Biotech); N, N'-methylenebisacrylamide ultrapure, electrophoretic grade, ammonium persulfate, tetramethylethylenediamine (TEMED) (Boehringer Mannheim); CHAPS (Sigma); urea beads ultra pure (Merck).

5.2.2 Solutions

First dimension solutions (ammonium persulfate solution, anodic and cathodic solutions) except acrylamide solution are as described in Section 3.2. Second dimension solutions (slab gel acrylamide solution, gel buffer and APS) are as described in Section 4.2.

NEPHGE Acrylamide Solution (30 % T, 5.4 % C). 28.38 % Acrylamide (w/v) and 1.62 % (w/v) N, N'-methylenebisacrylamide. To prepare 50 ml of the solution, dis-

solve 14.19 g of acrylamide and 0.81 g of bisacrylamide in deionized water. Filter and store at 4 °C for no more than 2 weeks. Keep in a brown bottle to protect from light.

5.2.3 Procedure

NEPHGE Gel Preparation. Recipe for 12 tubes. The gels are prepared the day before use.

1. Prepare the NEPHGE solution in a corex tube by adding in the following order:
   - 5.5 g of urea
   - 1.16 ml of NEPHGE acrylamide solution
   - 0.55 g of CHAPS
   - 250 μl of Ampholines 3.5–10
   - 250 μl of Pharmalytes 3–10
   - 4.17 ml of deionized water.
2. Dissolve urea by gentle mixing.
3. Deaerate for 3 min under vacuum.
4. Initiate polymerization by adding 20 μl of 10 % APS freshly prepared and 12.75 μl of TEMED.
5. Mix by gentle agitation.
6. Fill glass tubes with the first-dimensional gel acrylamide solution by using a pipette-pump.
7. Leave the tubes to polymerize horizontally at 26 °C for one night.

Note: in contrast to IEF gels, TEMED is added for polymerization of NEPHGE gels because polymerization is less efficient at alkaline pH. The alkaline pH results from the high amount of basic carrier ampholytes present in the NEPHGE gel composition.

Running NEPHGE Gels

1. The first-dimensional tubes are mounted into a vertical electrophoresis stand with an upper and a lower electrode chamber. The upper chamber is filled with 0.08 M H_3PO_4 (anodic solution) and the lower chamber is filled with 0.1 M NaOH (cathodic solution). The NaOH solution is deaerated for 30 min before use.
2. Samples are loaded at the acidic end of the gels.
3. Electrophoresis is carried out at 500 V for 30 min and then 1400 V for 6 h.
4. After focusing, the gels are processed as standard IEF gels (see Sects. 3.5)

The focusing is carried out in an incubator maintained at 26 °C.

Note: the gel is run from the acidic end to the basic end, i.e. in the opposite direction compared to the typical IEF gel. The H_3PO_4 buffer is placed in the upper chamber and the NaOH solution in the lower. The connections of the power supply are reversed.

6
Visualization of Separated Proteins

Depending on experiments, proteins can be visualized by dye or silver staining, autoradiography and fluorography. All these techniques have been widely described previously, and are described in detail in Chapter 5 of this book. The reader should refer to this chapter for description of detection protocols.

Acknowledgments. We would like to thank those who throughout the years, with their expertise, technical skill or "mistakes", have contributed to the development of 2-D gel electrophoresis in our laboratory. Particular thanks are extended to Marie France Peypouquet, Nelly Bataillé, Didier Thoraval, Manuel Sabiani, Sandrine Piot, Christelle Deletoile, Christophe Lehman and Richard Joubert.

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Appendix: Problems and Troubleshooting

Two-dimensional gel electrophoresis involves many different steps. Each of them is sensitive and may have a dramatic consequence on the final quality of the 2-D protein pattern. This section deals with some of the major problems that may be encountered by the novice starting to practise this methodology. They are categorized according to the step from which they originate. For general electrophoresis troubleshooting, refer to the troubleshooting section of Chapter 4 (Appendices A and B).

Equipment and Chemicals

- Gel plates and glass tubes need to be perfectly clean. After use they must be washed in sulfochromic acid or in strong detergent. Then, they must be extensively rinsed with tapwater and finally with deionized water. Gel plates are dried with filter paper (Kimwipes) and glass tubes are dried with air pressure. Glass tubes used for NEPHGE gels are periodically coated with Repel-Silane (Amersham Pharmacia-Biotech) in order to facilitate the extrusion of first-dimensional gels.
- First dimension and second dimension apparatuses are extensively rinsed with tapwater and, finally, with deionized water, after each run. They are carefully wiped with filter paper (Kimwipe) and periodically cleaned with a detergent.
- Carrier ampholytes of a given pH range are complex mixtures of a large number of different molecules. Their composition may vary from batch to batch. To overcome this problem it is advisable to use a combination of carrier ampholytes corresponding to different ranges of pH (as we do for IEF) or a mixture of carrier ampholytes of the same pH range but different trade marks (as we do for NEPHGE gels).
- The reproducibility and quality of gels are highly dependent upon the quality of reagents and water. Changing the origin of reagents may strongly affect protein separation. Concerning water, our laboratory is equipped with a Milli-Q Water system (Millipore). We found that it is important to avoid the oxidation or hydration of some reagents which may happen when the container has been opened for too long a time. Ammonium persulfate, β-mercaptoethanol and TEMED are particularly sensitive and have to be discarded once the vial has been opened for 2 months.

Sample Preparation

- Abnormal row of spots with the same molecular weight: proteins are carbamylated by isocyanate. Use very pure urea, freshly prepared urea solutions and avoid high temperatures.
- Abnormal proportion of small molecular weight proteins (less than 25 000): protease activity during protein extraction or in the sample. Cells were not completely lyophilized prior to protein extraction. Work rapidly until urea is added to the sample. Some samples may contain robust proteases. If so, use protease inhibitors, but keep in mind that the use of some inhibitors may introduce artefacts such as multiple spots.
- Streaking in the first dimension, mainly at the top of the gel: presence of particulate material in the sample (centrifuge), high concentration of salts (desalt the sample by dialysis) or of nucleic acids (increase the time of incubation in the presence of RNase and DNase). An inadequate solubilization of proteins may be also responsible for streaking: this may result from a too small amount of solubilization buffer added after cell breakage or from an insufficient length of time of solubilization, which in both cases will result in aggregates.

Focusing

- Urea precipitates in the tube after polymerization: precipitate will disappear during focusing.
- Gel breakage during focusing: sample contains high salt concentration or protein concentration is too high.
- All proteins are incorrectly focused. If proteins stay at the top of the first-dimensional gel: air bubble in the sample on top of the gel or air bubble at the bottom end. If proteins are spread all along the first dimension but incompletely focused: lengthen focusing time.
- Partial loss of the basic part of the gradient: (1) protein overloading: apply less protein. If not overloaded: (2) gradient drift. This happens because carrier ampholytes at the basic end of the gel are continuously passing into the reservoir buffer: focus for a shorter time.
- Acidic proteins are incorrectly focused. These proteins have to migrate through the whole length of the gel before they reach their isoelectric point under standard IEF conditions: they have still not reached their isoelectric point. Focus for a longer time.
- High molecular weight proteins or a few proteins are incorrectly focused: (1) the migration of large proteins may be slowed down because of the pore size of the focusing gel; (2) the entry of proteins into the gel may be delayed because of aggregation, interaction with other proteins or with nucleic acids on top of the gel. Lengthen the focusing time.
- Urea precipitates in first-dimensional gels once focusing is arrested, or before extruding the gel from the glass tube: this precipitate increases the risk of breaking the gel during extrusion; warm the gel in order to resolublize urea.
- No proteins in the 2-D gel: the polarity of the electrode did not match the electrode solutions. The acidic solution should be connected to the anode (+ elec-

trode), the basic solution to the cathode (- electrode). This is true for both standard IEF (cathode at the top) and NEPHGE (anode at the top).

Second Dimension

- Individual vertical streaks starting from the top of the second-dimensional gel, or sometimes in the middle of the gel: results from dust particles which have fallen on the gel surface or in the gel solution. Protect the surface of the gel and solutions, and work in a dust-free area. Carefully rinse gloves with water before manipulation.
- The front of migration is crooked: air bubbles between the bottom of the gel and the running buffer.
- Low molecular weight proteins are duplicated: first-dimensional gel was not in contact with the second-dimensional gel when the second dimension started.

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Proteome Research: Two-Dimensional Gel Electrophoresis and Identification Methods
Rabilloud, T. (Ed.)
2000, XVI, 248 p., Softcover
ISBN: 978-3-540-65792-7