Principles and Reactions of Protein Extraction, Purification, and Characterization

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Electrophoretic Analyses of Protein

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3 Electrophoretic Analyses of Protein

In electrophoresis, proteins are separated in an electric field by virtue of their charge and size. Electrophoresis of macromolecules is normally carried out by applying a thin layer of a sample to a solution stabilized by a porous matrix. The matrix can be composed of a number of different materials including paper, cellulose, acetate, or gels made of starch, agarose, or polyacrylamide. Agarose and polyacrylamide can act as a size-selective sieve in the separation. However, polyacrylamide is the most common matrix for separating proteins, probably due to its versatile applications.

3.1 DRIVING FORCE OF ELECTROPHORESIS

The movement of molecules in electrophoresis is dependent on the applied voltage (V), which equals the product of current (I) and resistance (R).

\[ V = IR \] (Ohm’s Law)

The following power equations are also used in electrophoresis:

\[ P = VI \text{ or } P = I^2R \text{ or } P = \frac{V^2}{R} \]

where \( P \) = power, which provides amount of heat produced in the circuit.

\[ H = I^2RT, \text{ } H = \text{ heat produced over time (T).} \]

In electrophoresis, voltage and current are supplied by a DC (direct current) power supply, and the electrodes, buffer, and gel are considered to be resistors. Power supply is used to hold one electrical parameter (current, voltage, or power) constant. Most power supplies have more than one pair of outlets. When two gels are connected in parallel to one outlet of a power supply, gel currents are additive. When two gels are connected in series to one outlet of a power supply, gel voltages are additive. Gel currents are additive when two gels are connected in parallel to adjacent outlets of a power supply.

Choice of Driving Force: Constant Current or Constant Voltage?

The resistance of the circuit does not remain constant during electrophoresis. For example, in a discontinuous system (separating and stacking gels) of SDS-PAGE
running at constant current, resistance increases. Therefore, voltage will increase over time, leading to increased heat generation and may require active heat removal. When running SDS-PAGE at constant voltage, current drops as the resistance increases. This will not result in a high increase of heat, since the main determinant factor (square root of current) is decreased, although resistance is increased. In contrast, in a continuous system (only separating gel) of SDS-PAGE, resistance decreases, resulting in a heat gain when running at constant voltage.

### 3.2 POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide (Figure 3.1) gels are formed by copolymerization of acrylamide monomer, \( CH_2 = CH-CONH_2 \), and a cross-linking comonomer, \( N,N' \)-methylenebisacrylamide, \( CH_2 = CH-CO-NH-CH_2-NH-CO-CH = CH_2 \) (bisacrylamide).

**Mechanism of Gel Formation**

The mechanism of gel formation is vinyl addition polymerization and is catalyzed by a free radical-generating system composed of ammonium persulfate (the initiator) and an accelerator, \( N,N,N',N' \)-tetramethylethylenediamine (TEMED). TEMED catalyzes the decomposition of the ammonium persulfate to yield a free radical (unpaired electron), which activates the acrylamide monomer. The activated
monomer then reacts with an unactivated monomer to begin the polymer chain elongation as shown below:

\[(\text{NH}_4)_2\text{S}_2\text{O}_8 + e^- \rightarrow (\text{NH}_4)_2\text{SO}_4 + \text{SO}_4^{2-}\].

If S represents \(\text{SO}_4^{2-}\), its reaction with acrylamide monomer (A) can be written as follows:

\[\text{S} \cdot + \text{A} \rightarrow \text{SA} \cdot\]
\[\text{SA} \cdot + \text{A} \rightarrow \text{SAA} \cdot\]
\[\text{SAA} \cdot + \text{A} \rightarrow \text{SAAA}\]

and so on.

During the polymer chain elongation, bisacrylamide is randomly cross-linked, resulting in closed loops and a complex “web” polymer (see Figure 3.1) with a characteristic porosity, which depends on the polymerization conditions and monomer concentrations.

In some applications (e.g., acid urea PAGE), riboflavin (or riboflavin-5′-phosphate) is used as an initiator of polymerization of acrylamide, as ammonium persulfate interferes with the stacking of the protein. In the presence of light and oxygen, riboflavin is converted to its leuco form, which is active in initiating polymerization.

Oxygen, a radical scavenger, interferes with polymerization, so that proper degassing to remove dissolved oxygen from acrylamide solutions is crucial for reproducible gel formation.

The effective pore size depends on the acrylamide concentration of a gel. The pore size decreases as the acrylamide concentration increases. Usually, gels are characterized by the two parameters, \(\%T\) and \(\%C\), where \(\%T\) refers to the total monomer (acrylamide + cross-linker), and \(\%C\) is the ratio of cross-linker (i.e., bisacrylamide) to acrylamide monomer (w/w). The following formulas are used to calculate:

\[\%T = \frac{\text{Acrylamide\,(g)} + \text{Bisacrylamide\,(g)}}{\text{Volume\,(ml)}} \times 100\]
\[\%C = \frac{\text{Bisacrylamide\,(g)}}{\text{Acrylamide\,(g)} + \text{Bisacrylamide\,(g)}} \times 100\]

The effective pore size is established by the three-dimensional network of fibers and pores that are formed by cross-linking acrylamide with bifunctional bisacrylamide.

When the gel is poured into a tube or slab mold, the top of the solution forms a meniscus. If the meniscus is ignored, the gel will polymerize with a curved top,
which will cause the separated sample bands to have a similar curved pattern. To 
eliminate the meniscus, a thin layer of water, water-saturated n-butanol, or isopro-
panol is carefully floated on the surface of the gel mixture before it polymerizes. 
The layer of water or water-saturated butanol should be deaerated; otherwise, it will 
inhibit polymerization on the gel surface.

Various polyacrylamide gel electrophoresis (PAGE) systems are known, and the 
choice of PAGE depends on the nature of the protein sample and the applications 
after electrophoresis (see Table 3.1).

### 3.2.1 PAGE UNDER DENATURING CONDITIONS (SDS-PAGE)

Denaturing PAGE in the presence of sodium dodecyl sulfate (better known as SDS-
PAGE) is a low-cost, reproducible, and rapid method for analyzing protein purity 
and for estimating protein molecular weight. SDS-PAGE is also employed for the 
following: (a) monitoring protein purification; (b) verification of protein concentra-
tion; (c) detection of proteolysis; (d) detection of protein modification; and (e) 
identification of immunoprecipitated proteins. SDS-PAGE can also be performed in 
a preparative mode to obtain sufficient protein for further studies. After electro-
phoresis the protein of interest is recovered from polyacrylamide by electroelution

<table>
<thead>
<tr>
<th>PAGE System</th>
<th>Application</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS-PAGE (Laemmli)</td>
<td>Determination of subunit molecular weight</td>
<td>Native protein activity is lost</td>
</tr>
<tr>
<td></td>
<td>Homogeneity test of a purified protein</td>
<td>Not suitable for low molecular weight proteins/peptides (&lt;10 kDa)</td>
</tr>
<tr>
<td>SDS-urea PAGE</td>
<td>Separation of membrane proteins</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Suitable for low molecular weight proteins</td>
<td></td>
</tr>
<tr>
<td>Non-denaturing PAGE</td>
<td>Homogeneity test of a purified protein</td>
<td>Native protein activity usually retained</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Not reliable for molecular weight estimation</td>
</tr>
<tr>
<td>Tricine PAGE</td>
<td>Separation of low molecular weight proteins/peptides (1 – 40 kDa range)</td>
<td>Protein band in the gel can be excised for amino acid sequencing without significant interference.</td>
</tr>
<tr>
<td>Non-urea SDS-PAGE (modified Laemmli)</td>
<td>Separation of low molecular weight proteins/peptides (as low as 5 kDa)</td>
<td>—</td>
</tr>
</tbody>
</table>
| Acid-urea PAGE      | Separation of basic proteins such as histones | Long run
Proteins move toward cathode
Electrode connection is opposite to the SDS-PAGE configuration |
| CTAB-PAGE           | Determination of native molecular weight Native activity assay | Proteins move toward cathode
Electrode connection is opposite to the SDS-PAGE configuration |

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(see Section 3.2.1.7). The protein that is obtained by this process is generally used for raising antibodies or sequencing.

**Mechanism**

In SDS-PAGE, the sample applied to the electrophoresis has been treated with sodium dodecyl sulfate, an anionic detergent. This detergent denatures the proteins in the sample and binds strongly to the uncoiled molecule. Approximately one SDS molecule binds per two amino acids. The SDS molecules mask the surface charge of the native proteins and create a net negative charge resulting from the sulfate groups on the SDS molecule (Figure 3.2). Therefore, charge/size ratio is equal for all proteins, and separation can be achieved only on the basis of size. Low molecular weight proteins travel faster in the gel, and proteins of high molecular weight move slower in the gel. Because proteins are separated on the basis of size, their molecular weights can be estimated by running appropriate standard proteins of known molecular weights on the same gel.

The quality of the SDS is very important, because differential protein-binding properties of impurities such as C10, C14, and C16 alkyl sulfates can cause single proteins to form multiple bands in gels.

Electrophoresis can be performed in two buffer systems: continuous and discontinuous. A continuous system has only a single separating gel and uses the same buffer in the tanks and the gel. The widely used SDS-PAGE, a modification by...
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Laemmli\(^1\) from those described in Ornstein\(^2\) and Davis,\(^3\) is a discontinuous system consisting of two contiguous but distinct gels: a resolving or separating (lower) gel and a stacking (upper) gel (Figure 3.3). The two gels are cast with different porosities, pH, and ionic strength. In addition, different mobile ions are used in the gel and electrode buffers.

**How Are Proteins Concentrated in the Stacking Gel?**

The buffer discontinuity acts to concentrate large volume samples in the stacking gel, resulting in better resolution than is possible using the same sample volumes in gels without a stacking gel. Proteins, once concentrated in the stacking gel, are separated in the resolving gel. In SDS-PAGE, samples prepared in a low-conductivity buffer (0.06 M Tris-HCl, pH 6.8) are loaded between the higher conductivity electrode buffer (0.025 M Tris, 0.192 M glycine, pH 8.3) and the stacking gel buffer (0.125 M Tris-HCl, pH 6.8). When power is applied, a voltage drop develops across the sample solution, which drives the proteins into the stacking gel. During electrophoresis, glycinate ions from the electrode buffer follow the proteins into the stacking gel. Between the highly mobile chloride ions in the front and the relatively slow glycinate ions in the rear, a high-voltage gradient forms. This causes SDS-protein complexes to form into a thin zone (stack) and migrate between the chloride and the glycinate ions. Most proteins usually move in the stacking gel (3 to 4%) due to large pore size, but at the interface of the stacking and resolving gels, the proteins experience a sharp increase in retardation due to the restrictive pore size of the resolving gel. In the resolving gel, the glycinate ions overtake the proteins, which continue to be slowed by the sieving of the matrix. Molecular sieving causes the SDS-polypeptide complexes to separate on the basis of their molecular weight. The molecular weight ranges of proteins that are separated depend on the percentage of the acrylamide gel (Table 3.2).

SDS-PAGE yields the molecular weight of the subunit that is non-covalently linked. To obtain the molecular weight of the subunit that is linked by disulfide bond, the presence of a reducing agent, such as 2-mercaptoethanol or dithiothreitol...
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(DTT), is necessary in the sample buffer. The reducing agent breaks the disulfide bonds in the protein as follows:

\[ \text{P}_1\text{S-S-P}_2 + \text{RSH} \rightarrow \text{P}_1\text{SH} + \text{P}_2\text{SH} \]

Of two gel formats (tube gel and slab gel), slab gels (formed between two sheets of supporting glass) are most widely used, since many samples can be run on the same gel, thereby providing uniformity during polymerization, staining, and destaining. Reagents for SDS-PAGE and electrophoresis cells of various designs are available from several vendors. For most analytical applications, the mini slab gel (8 \times 10 cm) is generally used, due to the increased resolution and reduced amounts of time and materials needed for electrophoresis. The experimental procedures and reagents described below have been calculated for a mini gel system; however, working procedures for other systems are adapted easily.

3.2.1 Preparation of Gels

Polyacrylamide gels with various pore sizes are made by varying the concentration of acrylamide. The choice of acrylamide concentration is determined by the molecular weight range of proteins to be separated (see Table 3.2). Recipes for making gels of varying concentration are shown in Table 3.3. Gels of fixed acrylamide concentrations are typically used on a daily basis because they are simple to prepare. When proteins of broad molecular weight range or higher resolution are desired, gradient polyacrylamide gels can be made (see Section 3.2.3). In practice, acrylamide solution (from the recipe in Table 3.3) is poured into a cassette made by joining two gel plates (usually made of glass) to form a separating gel (Figure 3.4 A). Spacers are placed between plates to make the cassette. Once the separating gel is polymerized, stacking gel is then made on top of the separating gel (Figure 3.4 B). Gels can be made in various thicknesses according to the thickness of the spacers (0.75 mm and 1.5 mm are common). Single or multiple gels can be made at a time. Gel casters of various sizes are commercially available for this purpose.

<table>
<thead>
<tr>
<th>Percentage (%) of Acrylamide Gel</th>
<th>Separating Resolution (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>15–45</td>
</tr>
<tr>
<td>12.5</td>
<td>15–60</td>
</tr>
<tr>
<td>10</td>
<td>18–75</td>
</tr>
<tr>
<td>7.5</td>
<td>30–120</td>
</tr>
<tr>
<td>5</td>
<td>60–212</td>
</tr>
</tbody>
</table>

*Adapted from Reference 4.
### TABLE 3.3

Recipes for Polyacrylamide Separating Gel

<table>
<thead>
<tr>
<th>Stock Solutions</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>12</th>
<th>13</th>
<th>15</th>
<th>17</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide/0.8% bisacrylamide</td>
<td>2.50</td>
<td>3.00</td>
<td>3.50</td>
<td>4.00</td>
<td>4.50</td>
<td>5.00</td>
<td>6.00</td>
<td>6.50</td>
<td>7.00</td>
<td>7.50</td>
<td>8.5</td>
</tr>
<tr>
<td>4× separating gel buffer</td>
<td>3.75</td>
<td>3.75</td>
<td>3.75</td>
<td>3.75</td>
<td>3.75</td>
<td>3.75</td>
<td>3.75</td>
<td>3.75</td>
<td>3.75</td>
<td>3.75</td>
<td>3.75</td>
</tr>
<tr>
<td>Water</td>
<td>8.75</td>
<td>8.25</td>
<td>7.75</td>
<td>7.25</td>
<td>6.75</td>
<td>6.25</td>
<td>5.75</td>
<td>5.25</td>
<td>4.75</td>
<td>4.25</td>
<td>3.75</td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>
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Working Procedure

Equipment

1. Glass plates
2. Spacers
3. Comb
4. Gel caster (Hoefer, Bio-Rad)

Reagents

1. Acrylamide, electrophoresis grade
2. Bisacrylamide (N,N'-methylene bisacrylamide)
3. Tris (2-hydroxyethyl-2-methyl 1,3 propanediol)
4. SDS (Sodium dodecyl sulfate or sodium lauryl sulfate)
5. TEMED (N,N,N’,N’-tetramethylethylenediamine)
6. Ammonium persulfate
7. 2-Mercaptoethanol
8. Glycerol
9. Bromophenol blue

FIGURE 3.4 Preparation of gel and loading samples on the gel. A, Gel cassette; B, Pouring stacking gel on top of separating gel; C, Inserting comb into the stacking gel; and D, Loading samples into wells.
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10. Glycine
11. Hydrochloric acid (HCl)
12. Dithiothreitol

Stock Solutions

1. 2 M Tris-HCl (pH 8.8), 1 liter: weigh out 242 gram Tris base and add 900 ml distilled water. Adjust pH to 8.8 by adding concentrated HCl slowly and finally add distilled water to a total volume of 1 liter.
2. 1 M Tris-HCl (pH 6.8), 100 ml: to 12.1 gram of Tris base add 80 ml distilled water and adjust pH to 6.8 with concentrated HCl. Add distilled water to a total volume of 100 ml.
3. 10% SDS (w/v), 100 ml: weigh out 10 gram SDS and add distilled water to a total volume of 100 ml. Store solution at room temperature.
4. 50% glycerol (v/v), 100 ml: add 50 ml distilled water to 50 ml 100% glycerol.
5. 1% bromophenol blue (w/v), 10 ml: weigh out 100 mg bromophenol blue and add 10 ml distilled water. Stir until dissolved and filter to remove particulates.

Working Solutions

1. 30% acrylamide stock solution: dissolve 29.2 gram acrylamide and 0.8 gram bisacrylamide with distilled water to make 100 ml. The solution is stable at 4°C for months.
   (Note: unpolymerized acrylamide is a skin irritant and a neurotoxin and thus should be handled with gloves. Use of a mask is also recommended to avoid inhalation. Unused acrylamide solution should be polymerized and disposed of with solid waste.)
2. 4× separating gel buffer, 100 ml:
   a. 75 ml 2M Tris-HCl (pH 8.8); final conc. 1.5 M
   b. 4 ml 10% SDS; final conc. 0.4%
   c. 21 ml water
   (The solution is stable for months at 4°C.)
3. 4× stacking gel buffer, 100 ml:
   a. 50 ml 1 M Tris-HCl (pH 6.8); final conc. 0.5 M
   b. 4 ml 10% SDS; final conc. 0.4%
   c. 46 ml water
   (The solution is stable for months at 4°C.)
4. 10% ammonium persulfate, 5 ml: dissolve 0.5 gram ammonium persulfate in 5 ml distilled water. Aliquot 100 μl in 0.5 ml microfuge tubes and store at −20°C (stable for months).
5. Electrophoresis buffer, 1 liter:
   a. 3 gram Tris base; final conc. 25 mM
   b. 14.4 gram glycine; final conc. 192 mM
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- 1 gram SDS; final conc. 0.1%
- Water to make 1 liter
  (pH should be approximately 8.3; 10× stock solution can also be made and stored at 4°C; stable for months.)

6. 5× sample buffer, 10 ml:
   - 0.6 ml 1M Tris-HCl (pH 6.8); final conc. 60 mM
   - 5 ml 50% glycerol; final conc. 25%
   - 2 ml 10% SDS; final conc. 2%
   - 0.5 ml 2-mercaptoethanol; final conc. 14.4 mM
   - 1 ml 1% bromophenol blue; final conc. 0.1%
   - 0.9 ml water
     (Aliquot in small volume in microfuge tubes and store at –20°C. Stable for months.)

Procedure

Making the Gel Sandwich

1. Clean the gel plates with detergent and dry.
2. Make a sandwich with two gel plates by placing spacers (0.75 or 1.5 mm) between them.
3. Assemble single or multiple sandwiches into the appropriate gel caster following the manufacturer’s instructions (see Figure 3.4 A).
   (Note: preparation of multiple gels has advantages. Since all gels are identical, the effect of gel-to-gel variation in protein separation is minimum. Additionally, the time to make multiple gels compared to single gel is only slightly more.)

Pouring the Separating Gel

4. From the recipe in Table 3.3, combine the appropriate volume of gel components without ammonium persulfate and TEMED in a 250 or 500 ml Erlenmeyer flask with a side tube. The volume of the gel components depends on number and percentage of gels.
5. Cover the flask with a rubber cork and degas the solution under vacuum for 5 to 10 min by connecting the side tube to a laboratory vacuum line.
6. Add appropriate volume of ammonium persulfate and TEMED (see Table 3.3) and mix by swirling. Work without delay at this point, as polymerization begins upon addition of ammonium persulfate and TEMED.
7. Carefully pour the gel solution into gel sandwich using a pipette by touching with the surface of the glass plate (see Figure 3.4 B). Pouring the gel this way minimizes the formation of air bubbles. Do not fill the whole cassette. Leave empty about 1.5 cm from top of the sandwich for making stacking gel (on top of the separating gel).
8. Without any delay, gently layer 100 to 200 µl of isopropanol (for mini gel, 0.75 mm thickness) on top of the separating gel solution. This allows the gel surface to remain flat.

9. Allow gel to polymerize at room temperature for 30 to 60 min. During polymerization, the gel sandwich should not be disturbed. Leave the unused gel solution in the flask to allow polymerization and discard. This serves two purposes. Unlike acrylamide monomer, polyacrylamide is not toxic and thus can be considered as non-hazardous waste. Second, it serves as a check for polymerization. At this point, the gel sandwich can be checked for polymerization. Once the acrylamide solution has been polymerized, a distinct interface should appear between the separating gel and the isopropanol. At this point, isopropanol can be flicked off, and after washing the gel surface with 1× separating gel buffer, stacking gel can be added. Separating gels can be stored for several weeks at 4°C. If separating gels are to be stored, replace isopropanol with 1× separating gel buffer and cover with parafilm or plastic wrap.

### Pouring the Stacking Gel

10. Combine the following reagents in a 50 ml sidearm flask:
   a. 30% acrylamide stock solution: 0.65 ml
   b. 4× stacking gel buffer, pH 6.8: 1.25 ml
   c. Water: 3.05 ml
   (The solution is good for making two stacking gels for mini gels of 0.75 mm thickness).

11. Degas under vacuum for 10 to 15 min at room temperature.

12. Add 25 µl of 10% ammonium persulfate and 5 µl of TEMED, mix by swirling.

13. Rapidly add stacking gel solution on top of separating gel. Fill up the empty space completely.

14. Carefully insert comb into gel sandwich (Figure 3.4 C). Make sure not to trap air bubbles in between teeth. The distance between the separating gel and the bottom of the comb teeth should be about 0.5 cm.

15. Allow stacking gel to polymerize at room temperature for about 30 min.

### 3.2.1.2 Running Gels

Once a gel has been made, the comb is removed from the gel. After washing the wells with the electrophoresis buffer, the gel sandwich is placed in the electrophoresis tank. The tank is filled with the electrophoresis buffer following the manufacturer’s instructions. Electrophoresis tanks from various commercial sources vary in size and shape. But, in all cases, the gel sandwich is submerged in the electrophoresis buffer and protein samples are loaded into wells through the buffer. Once samples are loaded, the electrophoresis lid is carefully closed; the chamber electrodes are attached to the power supply.
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Working Procedure

Equipment

1. Mini gel apparatus (Hoefer, Bio-Rad, Life Technologies)
2. Power supply (capacity 200 V)
3. Heat block (100°C) or boiling water bath
4. Disposable gel loading tips for micropipette or Hamilton syringe
5. Eppendorf tubes
6. Plastic or glass container with lid
7. Rocking shaker
8. Gel dryer

Reagents

1. Electrophoresis buffer (see Working Solutions, Section 3.2.1.1)
2. Sample buffer (see Working Solutions, Section 3.2.1.1)
3. Protein markers (Bio-Rad, Sigma, Invitrogen)

Procedure

1. With marker place a line indicating the bottom of each well on the front plate. This marking provides a guide for loading the samples onto wells. Alternatively, bromophenol blue to a final concentration of 1 mg/ml in the stacking gel can be added to visualize the gel wells.
2. Remove the comb from the stacking gel and rinse the wells with 1x running electrophoresis buffer.
3. Place the gel sandwich in the electrophoresis tank following manufacturer’s protocol.
4. Fill up the electrophoresis chamber with 1x electrophoresis buffer according to the manufacturer’s instructions.
5. Prepare the protein samples as follows:
   a. Mix protein sample or standard protein and 5x sample buffer (4:1 ratio) in Eppendorf tube. (For 10-well comb and 0.75 mm gel thickness, 20 µl of protein sample and 5 µl sample buffer can be mixed and after heating 5 to 20 µl can be loaded on each well. Amount of protein to be loaded depends on the gel thickness, size of the comb, and the staining method. For 0.75-mm-thick gel with 10-well comb, 5 to 25 µg of protein is recommended per well for complex protein when staining with Coomassie blue. If silver staining is used, 10- to 100-fold less protein can be applied.)
   b. Heat the mixture at 100°C for 2 to 5 min.
   c. Spin down protein solution for a second in a microfuge. The sample is now ready to load on gel.
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6. Load protein sample (as prepared in the previous step) onto well using a disposable gel loading tip or a Hamilton syringe as shown in Figure 3.4 D. Carefully layer protein sample (without introducing any bubbles) onto bottom of the well.

7. Load also a mixture of protein standards into a separate well. Mixture of protein standards in various molecular weight ranges is commercially available, such as Sigma, Bio-Rad, and Novex.

8. Connect the power supply to the electrophoresis chamber. Ensure the electrodes connection is in correct polarity (red to red, black to black). In SDS-PAGE, all proteins move to the anode (+).

9. Turn on power supply to 100 volt when GIBCO BRL apparatus is used. Once the sample passes stacking gel, voltage can be increased to 150 volt. When running gel at constant current at 20 to 30 mA (e.g., Hoefer mini gel system), the temperature of the running buffer should be controlled (10 to 20°C) with a circulating cold-water bath (usually set at 10°C) to prevent “smiling” (curvature of the migratory band).

10. Disconnect the power supply when bromophenol blue tracking dye reaches the bottom of the separating gel.

11. Remove the gel sandwich and orient the gel so that the order of the sample wells is known.

12. Carefully slide one of the spacers halfway along the edge of the sandwich and, using the exposed spacer as a lever, pry open the glass plate to obtain gel.

13. Mark one corner by cutting gel (a small triangle) to avoid any loss of sample well orientation during staining.

14. Carefully remove the gel from the plate and proceed with the staining.

3.2.1.3 Detection of Proteins in Gel

After electrophoresis, proteins are detected on the gel by using various stains (Coomassie blue, silver, Amido Black, etc.). Staining with Coomassie blue is rapid and the most common protein stain for routine work (Table 3.4). Compared to Coomassie stain, silver staining is a time-consuming, but more sensitive, method for staining proteins in gels. Silver staining should be used to assess the purity of a protein preparation, such as antigen preparation for development of polyclonal antibodies. Reversible stains such as Ponceau and India ink are generally used to visualize protein bands in gels prior to Western transfer or on membranes prior to protein elution (see Section 3.5). Protein staining with Procion blue can be used for quantification of protein in gels. Many stains are commercially available (Table 3.5).

3.2.1.3.1 Coomassie Brilliant Blue Stain

Coomassie Brilliant Blue dye (see Figure 2.6 A) is widely used to visualize proteins in polyacrylamide gels. The staining is simple and can detect as little as 0.1 µg of protein in a single band. The dye binds primarily to positively charged amino acids, such as lysine and arginine. Thus, basic proteins tend to stain more strongly than acidic proteins. The advantage of the Coomassie stain is that it is rapid and
<table>
<thead>
<tr>
<th>Stain</th>
<th>Sensitivity</th>
<th>Time Required</th>
<th>Advantage</th>
<th>Disadvantage</th>
<th>Recommended Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie</td>
<td>100 ng</td>
<td>5–10 min staining, 1–3 h destaining</td>
<td>Rapid, Low cost</td>
<td>Accumulates large volume of methanol present in staining and destaining solution</td>
<td>Routine work</td>
</tr>
<tr>
<td>EZBlue™ (Sigma)</td>
<td>2 ng</td>
<td>5–10 min</td>
<td>No fixation step</td>
<td>Expensive</td>
<td>Routine work</td>
</tr>
<tr>
<td>Colloidal Coomassie</td>
<td>10 ng</td>
<td>5–10 min</td>
<td>No destaining step</td>
<td>Fixation required</td>
<td>Routine work</td>
</tr>
<tr>
<td>CBB in acid</td>
<td>20 ng</td>
<td>30–60 min</td>
<td>No fixation step, No destaining step</td>
<td>Longer staining time</td>
<td>Quantitation of protein in gel</td>
</tr>
<tr>
<td>Silver (alkaline method)</td>
<td>0.1 ng</td>
<td>2 h</td>
<td>Most sensitive, when sensitized with glutaraldehyde prior to staining</td>
<td>Complex reagent preparation, unstable reagent</td>
<td>To assess purity of protein preparation</td>
</tr>
<tr>
<td>Silver (acid method)</td>
<td>0.6–1.2 ng</td>
<td>90 min</td>
<td>Few steps, Simple reagent preparation</td>
<td>Less sensitive than alkaline silver stain method</td>
<td>To assess purity of protein preparation</td>
</tr>
<tr>
<td>Zinc</td>
<td>5 ng</td>
<td>25–40 min</td>
<td>No fixation step</td>
<td>Multiple steps when toning reaction intended</td>
<td>Peptide sequencing, antibody development</td>
</tr>
<tr>
<td>Nile Red</td>
<td>100 ng</td>
<td>As little as 6 min</td>
<td>Rapid</td>
<td>UV light box and camera required for documentation</td>
<td>Routine work</td>
</tr>
<tr>
<td>Calconcarboxylic acid</td>
<td>10 ng</td>
<td>30–70 min</td>
<td>Migration of stained proteins during electrophoresis</td>
<td>Simultaneous staining is less sensitive than post staining</td>
<td>Routine work</td>
</tr>
<tr>
<td>25 ng (post electrophoresis)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosin Y</td>
<td>10 ng</td>
<td>30 min</td>
<td>Antigenicity of the stained protein retained</td>
<td>Transilluminator required</td>
<td>Antibody development</td>
</tr>
<tr>
<td>Procion blue</td>
<td>100 ng</td>
<td>1.5 h staining, 48 h destaining</td>
<td>Proteins in gel can be quantitated</td>
<td>Time consuming</td>
<td>Quantitation of proteins</td>
</tr>
<tr>
<td>Amido Black</td>
<td>&gt;100 ng</td>
<td>2–4 h</td>
<td>—</td>
<td>Less sensitive</td>
<td>—</td>
</tr>
<tr>
<td>Fast Green FCF</td>
<td>200 ng</td>
<td>2–4 h</td>
<td>—</td>
<td>Less sensitive</td>
<td>—</td>
</tr>
</tbody>
</table>
The gel can be stained in 5 to 10 min, followed by destaining that requires about 1 to 2 h. Coomassie blue turns the entire gel blue, and after destaining, the blue protein bands appear against a clear background. If staining appears to be incomplete after destaining, gel can be restained. If staining is not sensitive enough to detect all proteins, the gel can be rinsed and then subjected to the more sensitive silver staining procedure (see Section 3.2.1.3.2).

### Working Procedure

#### Equipment

1. Plastic or glass container with lid
2. Rocking shaker

#### Reagents

1. Coomassie gel stain stock solution, 1 liter:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie Brilliant Blue R-250</td>
<td>1 g</td>
</tr>
<tr>
<td>Methanol</td>
<td>450 ml</td>
</tr>
<tr>
<td>Water</td>
<td>450 ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

**TABLE 3.5**

**Commercial Stains of Protein on Gels**

<table>
<thead>
<tr>
<th>Based on</th>
<th>Stain</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie Blue</td>
<td>GelCode Blue Stain (Colloidal properties of Coomassie G-250)</td>
<td>Pierce</td>
</tr>
<tr>
<td></td>
<td>InstaStain Blue Gel Stain Paper (Coomassie dye in a solid phase)</td>
<td>Pierce</td>
</tr>
<tr>
<td></td>
<td>Coomassie</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td></td>
<td>Bio-Safe Coomassie</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td></td>
<td>EZ Blue Gel Stain</td>
<td>Sigma</td>
</tr>
<tr>
<td>Silver</td>
<td>GelCode Silver Stain</td>
<td>Pierce</td>
</tr>
<tr>
<td></td>
<td>GelCode Silver SNAP Stain</td>
<td>Pierce</td>
</tr>
<tr>
<td></td>
<td>Silver Stain (Meril)</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td></td>
<td>Silver Stain Plus (Gottlieb and Chavco)</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>Zinc Reverse</td>
<td>GelCode E-Zinc Reversible Stain</td>
<td>Pierce</td>
</tr>
<tr>
<td></td>
<td>Zinc Stain</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>Fluorescent</td>
<td>SYBR Red (Based on Nile Red)</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td></td>
<td>SYPRO Tangerine</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td></td>
<td>SYPRO Orange</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td></td>
<td>SYPRO Red</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Luminescent</td>
<td>SYPRO Ruby Stain</td>
<td>Molecular Probes</td>
</tr>
</tbody>
</table>
Electrophoretic Analyses of Protein

2. Coomassie gel destain stock solution, 1 liter:
   Methanol 100 ml
   Water 800 ml
   Glacial acetic acid 100 ml

Procedure

1. After electrophoresis, transfer gel to a small container and add Coomassie stain enough to submerge.
2. Incubate the gel in staining solution for 5 to 10 min (for 0.75 mm). Thicker gels require longer incubation times (10 to 20 min for 1.5 mm gel). Gels can also be kept in staining solution overnight.
3. Discard the stain and incubate the gel in destaining solution (40 to 50 ml) with agitation. Strong bands become visible within a few minutes. To destain completely, agitate gel in several changes of fresh destaining solution. Complete destaining of the gel usually requires several hours. A piece of Styrofoam or a Kimwipe paper may be added in the container during destaining to facilitate the destaining. Styrofoam or Kimwipe paper absorbs Coomassie stain, which diffuses from the gel. Replace with a new foam when original becomes dark blue.

Other Variations of Coomassie Stain

Coomassie Brilliant Blue G (0.04%, w/v) in 3.5% (w/v) perchloric acid can be used to stain proteins on SDS and non-denaturing polyacrylamide gels as well as agarose gels. Proteins are stained in 30 to 60 min. No destaining step is required. Fixation step is also not required as perchloric acid can fix proteins during staining. However, SDS-PAGE gels are pre-fixed to remove SDS prior staining.

EZBlue™ Gel staining reagent (Sigma-Aldrich) is a one-step ultrasensitive stain based on Coomassie Brilliant Blue G-250. EZBlue stain can detect as little as 2 ng protein. It fixes proteins during staining, and thus a separate fixing step is not required. Destaining is also not required with this stain, although a water rinse after staining enhances sensitivity.

A colloidal concentrate of Coomassie Brilliant Blue G is available from Sigma-Aldrich. After dilution, the suspension contains 0.1% Brilliant Blue G, 0.29 M phosphoric acid, and 16% saturated ammonium sulfate. The stain is about tenfold more sensitive than the regular Coomassie stain. A fixation step is required prior to staining.

An advantage of these variations of the Coomassie stain is the absence of methanol, which is a regulated chemical waste.

3.2.1.3.2 Silver Stain

Switzer et al. introduced a silver stain, which is at least 100 times more sensitive than Coomassie stain. Several variations and modifications have been developed. However, silver staining is primarily achieved in two ways: an alkaline method based
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on the use of ammoniacal silver or silver diamine, and the use of silver nitrate in weakly acidic solution. Both procedures are based on the reduction of cationic silver to metallic silver. Amino groups, especially the epsilon-amino group of lysine and sulfur residues of cysteine and methionine, are believed to react with silver cations.

**Reaction**

In the first method, ammoniacal silver or silver diamine is prepared by mixing silver nitrate with sodium hydroxide resulting in a precipitate of silver hydroxide, which is brought back into solution with the slow addition of ammonia as follows:

\[
\text{AgNO}_3 + \text{NaOH} \rightarrow \text{AgOH} \\
\text{AgOH} + \text{NH}_3 \rightarrow \text{Ag(NH}_3)_2\text{OH}
\]

The gel is impregnated with silver diamine to allow the formation of a complex with the proteins. After removing the excess silver diamine from the gel, the complexed silver cations are reduced to metallic silver with formaldehyde in the presence of acid, usually citric acid.

The second method requires an initial gel soak in a weakly acidic silver nitrate solution and development with formaldehyde in the presence of alkali, usually sodium carbonate or sodium hydroxide. Sodium carbonate or other bases buffer the formic acid produced by the oxidation of formaldehyde, so that the silver ion reduction can continue until the protein bands appear in the gel.

Of the two methods, the alkaline silver nitrate method is more sensitive than the acidic silver nitrate method, but the former is more time consuming than the latter.

In some cases, prior to the silver nitrate step, gels are primed with a reducing agent like dithiothreitol or an oxidizing reagent like permanganate or dichromate. With Bio-Rad’s Silver Stain, the formation of a positive image is enhanced by dichromate oxidation, which may convert protein hydroxyl and sulfhydryl groups to aldehydes and thiosulfates, thereby altering the redox potential of the protein. Complexes formed between the proteins and dichromate may further form nucleation centers for silver reduction.

Among the various modifications, a method combining the use of glutaraldehyde treatment and the use of silver diamine to soak the gel was found to be most sensitive. The increased sensitivity is probably due to increased reduction rate of silver on the proteins.

Before a protein gel can be stained, the proteins must be fixed, in order to minimize the diffusion of molecules in the gel. Fixation also elutes substances from the gel that may interfere with the establishment of the oxidation/reduction potential differences and with silver reduction. Ampholytes, detergents, reducing agents, initiators or catalysts, and buffer ion (glycine, chloride, etc.) must be removed. Water used in all silver stain reactions must be of 1 μmho conductance or less and free of organic contaminants.
Although silver staining normally produces a dark brown image, other colors may be produced when dense protein zones become saturated after prolonged development. Color production largely depends on the size and the distribution of the silver particles within the gel and the refractive index of the gel.\(^{16}\)

**Working Procedure**

**Silver Diamine Method**

**Reagents**

1. Gel fixation solution: 20% (w/v) trichloroacetic acid solution.
2. Sensitization solution: 10% (w/v) glutaraldehyde solution.
3. Silver diamine staining solution: add 4 ml of 20% (w/v) silver nitrate dropwise with stirring to a mixture of 21 ml of 0.36% (w/v) NaOH and 1.4 ml of 35% (w/v) ammonia. Mixture will form a brown precipitate. Add a minimum amount of ammonia to dissolve the precipitate. Dilute the solution to 100 ml with water. (Note: the resulting silver diamine solution is unstable and should be used within 5 min.)
4. Developing solution: combine 2.5 ml of 1% citric acid and 0.26 ml of 36% (w/v) formaldehyde. Dilute the solution to 500 ml with water.
5. Stopping solution: 40% (v/v) ethanol/10% (v/v) acetic acid in water.
6. Destaining solution: 0.3% (w/v) potassium ferricyanide/0.6% (w/v) sodium thiosulfate/0.1% (w/v) sodium carbonate.

**Procedure**

All steps are performed at room temperature.

1. After electrophoresis, incubate the gel in 200 ml of fixing solution for at least 1 h. (Note: gels with a high percentage of acrylamide may require longer period of fixation. Alternatively, the gel can be soaked in fixing solution overnight.)
2. Wash the gel twice for 30 min each (2 × 30 min) with 200 ml of 40% ethanol/10% acetic acid in water.
3. Rehydrate the gel in excess water for 2 × 20 min.
4. Incubate the gel in sensitizing solution for 30 min.
5. Wash the gel in water for 3 × 20 min.
6. Incubate the gel in staining solution for 30 min. (Note: dispose silver diamine reagent after treating with 1 N HCl.)
7. Wash the gel in water for 3 × 5 min.
8. Develop the gel in developing solution until the appearance of proteins as dark brown zone (color development should be within 10 min).
9. Stop the staining with the stopping solution.
10. For destaining, wash the gel in water for 5 min and incubate the gel in destaining solution until the background clears.
11. Terminate destaining by incubating the gel in stopping solution.

**Acidic Silver Nitrate Method**

**Reagents**

1. Gel fixation solution:
   - 250 ml ethanol
   - 60 ml acetic acid
   - 0.25 ml formaldehyde
   Add distilled water to 500 ml
2. Staining solution:
   - 100 mg silver nitrate
   - 70 µl formaldehyde
   Add distilled water to 100 ml
3. Developing solution:
   - 3 g sodium carbonate (anhydrous)
   - 50 µl formaldehyde
   - 1 mg sodium thiosulfate (Na₂S₂O₃·5H₂O)
   Add distilled water to 100 ml
4. Stop solution: 0.01 M EDTA

*(Note: all solutions should be made fresh except EDTA.)*

**Procedure**

1. After electrophoresis, incubate the gel in fixative solution for 30 to 60 min.
2. Wash the gel three times for 20 min each with 50% ethanol.
3. Wash the gel in 0.01% (w/v) sodium thiosulfate (Na₂S₂O₃·5H₂O) solution for 1 min.
4. Wash the gel three times for 20 sec each with distilled water.
5. Incubate the gel in staining solution for 20 min.
6. Wash the gel three times for 20 sec each with distilled water.
7. Incubate the gel in developing solution until color develops.
8. Rinse the gel with distilled water for 20 sec and stop the reaction with the stopping solution.
3.2.1.3.3 **Procion Blue Stain**

Procion blue MX-2G-125 dye can be used to quantitate proteins on gels (6). The lower detection limit is about 1 µg per band.

**Reaction**

Procion dye contains a dichlorotriazine group which reacts with hydroxyl and amino groups of proteins.

**Working Procedure**

**Reagents**

1. **Procion blue stain (0.2%, w/v):** dissolve 0.4 gram dye in 100 ml methanol. Add 20 ml glacial acetic acid and 80 ml distilled water.
2. **Destaining solution:** to 800 ml distilled water, add 100 ml methanol and 100 ml glacial acetic acid.

**Procedure**

1. At the end of electrophoresis, stain gels in 0.2% Procion blue for 2 to 6 h at room temperature.
2. Clear the background with several changes of destain solution.
3. Measure the band intensity by scanning densitometry.
4. Determine the protein content from a standard curve of known amount of proteins.

3.2.1.3.4 **Nile Red Stain**

Staining of proteins with Nile Red (9-diethylamine 5 H-benzo [α] phenoxazine-5-one) (Figure 3.5) is rapid. It detects as low as 0.1 µg protein/band.

**Reaction**

Nile Red is a fluorescent hydrophobic dye. It binds protein-SDS complexes. Since it also interacts with SDS micelles, SDS-PAGE is usually performed at a lower SDS

![Diagram of Nile Red](image)

**FIGURE 3.5** Diagram of Nile Red.
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concentration (0.05% instead of typical 0.1%) in order to reduce background. This concentration is lower than the critical micelle concentration.

**Working Procedure**

**Equipment**

1. Transilluminator with midrange ultraviolet (UV) bulbs (approximately 300 nm).
2. Camera and photo films: Polaroid instant films such as 667 (black/white positive film), 665 (black/white positive/negative film), 669 (color positive film).
3. Optical filter: Wratten (Eastman Kodak) filters No. 9 (yellow) and 16 (orange) to eliminate the UV and visible light from the transilluminator. Filters are placed together, 16 facing camera lens and 9 facing gel.
4. Negative cleaning solution: 18% sodium sulfate. This is required when 665 films are used.

**Reagent**

Nile Red: 50× concentrated stock (0.4 mg/ml) in dimethyl sulfoxide. Stable at room temperature for at least 3 months. Nile Red is commercially available from vendors such as Sigma (St. Louis, MO) and Eastman Kodak (Rochester, NY).

Ready-to-use Nile Red solution is available from Molecular Probes (Eugene, OR) under the trade name SYBR Red Protein Gel Stain.

**Procedure**

1. At the end of electrophoresis, transfer gel to a plastic box.
2. Dilute Nile Red just before staining. Take 5 ml of concentrated Nile Red in 500 ml Erlenmeyer flask and quickly add 245 ml distilled water.
3. Soak the gel in diluted Nile Red and agitate vigorously using an orbital shaker (speed 300 rpm) for about 5 min.
   (Note: Nile Red is stable in DMSO, but it precipitates rapidly in aqueous solution. So as soon as the dye is diluted to 1× with water, this should be used immediately for staining protein bands with vigorous agitation. This ensures the staining of protein bands (reaction with SDS-protein) before it precipitates in the gel.)
4. Discard the starting solution and rinse the gel with distilled water (four times, 10 sec each) to remove the excess Nile Red precipitates.
5. Place the gel on a UV transilluminator and document the protein bands by photography.
3.2.1.3.5 Zinc Reverse Stain

Unlike traditional staining methods such as Coomassie and silver stains, reverse staining methods stain the whole gel except the area of the protein bands. The sensitivity of zinc stain is comparable to Coomassie stain. Zinc reverse staining is achieved in three steps: (a) incubate the gel in sodium carbonate, (b) incubation in imidazole, and (c) finally incubation with zinc sulfate. No fixative solution is used in this method. Reverse stain is particularly useful when elution of unstained protein is intended for further analyses. Usually, the gel is kept in water after staining. However, a toning reaction with a mixture of potassium ferricyanide, o-tolidine, and sulfuric acid is necessary if gels should be dried.

Reaction

At alkaline pH, Zn$^{2+}$ forms a white insoluble precipitate with imidazole. The white precipitate turns into a deep blue with toning reaction.

Working Procedure

Reagents

1. Staining solution I: 1% (w/v) sodium carbonate
2. Staining solution II: 0.2 M imidazole containing 0.1% SDS (w/v)
3. Staining solution III: 0.2 M zinc sulfate
4. (Optional) Toning reaction I: 0.2% (w/v) potassium ferricyanide
5. (Optional) Toning reaction II: 0.2% (w/v) o-tolidine
6. (Optional) Toning reaction III: 0.36 N sulfuric acid

Procedure

1. At the end of electrophoresis, soak the gel in staining solution I for 5 min.
2. Soak the gel in staining solution II for 15 min.
3. Wash the gel briefly (about 10 sec) with water.
4. Soak the gel in staining solution III for about 40 sec. White background should develop in a few seconds, while protein bands remain transparent. *(Note: incubation time should be limited to 40 sec, because the white precipitate in the gel surface can be redissolved with excess of Zn$^{2+}$.)*
5. Wash the gel with abundant water to remove excess Zn$^{2+}$ solution (twice 10 sec each and twice 5 min each).
7. For toning reaction, mix equal volumes of toning reaction I and II and soak the gel for 4 min. In this step, the white background of the gel turns brown-yellowish, while the protein bands turn colorless to yellow.
8. Wash the gel briefly with water (10 sec). This will eliminate any trace amount of precipitate.
9. Soak the gel in a mixture of equal volume of toning reaction II and III for 5 min. Within 2 min, the brown background of the gel turns blue, while the yellow protein bands become colorless again.
10. Wash the gel several times with water to remove excess reagents. Gels can now be dried.

3.2.1.3.6 Calconcarboxylic Acid Stain

Calconcarboxylic acid [1-(2-hydroxy-4-sulfo-1-naphthylazo)-2-hydroxy-3-naphthoic acid, CNN] (Figure 3.6) can be used for simultaneous as well as post-electrophoretic protein staining. For simultaneous staining of proteins during electrophoresis, CNN is simply added in the upper reservoir. The sensitivity of this stain is about 10 ng and 25 ng by post-staining and simultaneous staining, respectively.

**Reaction**

Staining of proteins with CNN is pH dependent (intense staining at pH 1.6 to 4.4 and weak at alkaline pH). At acidic pH, various functional groups of CNN (carboxyl, sulfonic acid, hydroxyl) probably form electrostatic bonds with protonated amino groups in proteins. The lower staining intensity in simultaneous staining is probably due to alkaline pH of the electrophoresis buffer.

**Working Procedure**

**Reagents**

1. Destaining solution: 530 ml distilled water/400 ml methanol/70 ml glacial acetic acid.
2. Simultaneous staining solution: 1% (w/v) CNN. Dissolve 1 gram CNN in 100 ml reservoir buffer with stirring at 50 to 60°C.
3. Post-staining solution: 0.05% (w/v) CNN. Dissolve 0.05 gram CNN in 100 ml destaining solution.

**Procedure for Simultaneous Staining**

1. Load the sample electrophorese for about 10 min to allow protein migration into the stacking gel.
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2. Turn off power supply and add 1% CNN in the upper reservoir to give a final concentration of 0.01 to 0.015%.
3. At the end of the electrophoresis, remove stained gel and destain for 30 min.

Procedure for Post-electrophoretic Staining

1. Soak the gel in post-staining solution with agitation for 30 min.
2. Remove staining solution and destain with destaining solution until the background clears.

3.2.1.3.7 Eosin Y Stain

The Eosin Y staining method detects proteins on gels as well as on membranes more rapidly than most Coomassie and silver staining methods. The stain can detect as little as 10 ng of protein. An advantage of this stain is that the antigenicity of the stained protein is retained.

Reaction

Protein staining may occur by means of hydrophobic interaction between aromatic rings of eosin Y and the protein and by hydrogen bonding between hydroxyl groups of eosin Y and the protein.

Working Procedure

Equipment

1. Black plastic board
2. Transilluminated fluorescent white light box

Reagents

1. Fixation solution: 10% glacial acetic acid/40% methanol
2. Staining solution: 1% (w/v) Eosin Y in 40% methanol and 0.5% glacial acetic acid
3. Destaining solution: 10% glacial acetic acid/40% methanol

Procedure

1. At the end of electrophoresis, fix the gel in fixation solution for 10 min.
2. Rinse the gel with distilled water twice.
3. Soak the gel in staining solution for 15 min with gentle agitation.
4. Remove the staining solution and soak the gel in destaining solution for about 15 sec.
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5. Rinse with water.
6. View the stained protein bands by using transilluminated fluorescent light. Stained protein bands can also be viewed by placing the gel on a black plastic board with illuminated light on top of the gel. Stained gel can be kept in water for a month without losing sensitivity.

3.2.1.3.8 Amido Black Stain
Amido Black (also known as Naphthol Blue Black, Acid Black 1, or Buffalo Black NBR) can be used to stain proteins on gels. The detection sensitivity is lower than that of Coomassie Blue. Fixation is recommended for this stain.

Working Procedure

Reagents

1. Fixation solution: 10% glacial acetic acid/40% methanol
2. Staining solution: 0.1% (w/v) Amido Black in 7% (v/v) acetic acid
3. Destaining solution: 7% acetic acid

Procedure

1. At the end of electrophoresis, fix the gel in fixation solution for 10 min.
2. Rinse the gel with distilled water twice.
3. Soak the gel in staining solution for 2 h with gentle agitation.
4. Remove the staining solution and soak the gel in destaining solution.

3.2.1.3.9 Fast Green FCF
Fast Green FCF dye is used for protein staining in SDS-PAGE, native PAGE, and isoelectric focusing gels. After electrophoresis, fixing is required for maximum sensitivity. Sensitivity is about two times less than Coomassie staining.

Working Procedure

Reagents

1. Staining solution: 0.1% (w/v) Fast Green FCF in 30% (v/v) ethanol and 10% (v/v) acetic acid
2. Destaining solution: 30% (v/v) ethanol and 10% (v/v) acetic acid

Procedure

1. At the end of electrophoresis, soak the gel in staining solution for about 2 h.
2. Destain the gel with several changes of destaining solution.
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3.2.1.3.10 Other Stains

Vendors of several commercial protein stains (Molecular Probes, Bio-Rad, Pierce, Sigma) offer ready-to-use convenient packs (Table 3.5). The sensitivity of some of these is comparable to silver stain. For example, Molecular Probes’ SYPRO® Tangerine, SYPRO® Orange, and SYPRO® Red are fluorescent-based stains (Ex/Em wave lengths are 490/640, 470/570, and 550/630, respectively) and can detect as little as 4 ng protein per band. SYPRO® Ruby stain (Molecular Probes) is an ultrasensitive luminescent stain for the detection of proteins on polyacrylamide gels (lower detection limit 75 fmol).

3.2.1.4 Determination of Molecular Weight

Subunit molecular weight of a protein is usually determined on SDS-PAGE, since the migration of protein is proportional to the mass. A standard curve is generated from proteins of known molecular weight (known as standard proteins), and the molecular weight of unknown protein is determined from the curve. The standard curve is obtained by plotting the relative mobility (R_f) value (in x-axis) and log_{10} of the molecular weight (in y-axis). R_f value is determined as follows:

\[ R_f = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}} \]

Following electrophoresis and staining, the migration of proteins and tracking dye (bromophenol blue) can be measured.

3.2.1.5 Quantitation of Proteins in Gels by Densitometric Scan

Following staining of proteins in gels, individual protein bands can be quantitated by densitometric scan over a limited range of protein concentration (1 to 10 µg/band). This technique clearly provides an advantage over the estimation of crude proteins (mixture of proteins) in solution where quantitation of individual proteins cannot be obtained. For densitometric quantitations, the most suitable protein stains are Procion blue stain,6 zinc stain,19 and colloidal Coomassie stain.20 Staining of proteins with these procedures is discussed in Section 3.2.1.3. A standard curve is drawn from known amounts of proteins, and the amount of the unknown protein is then determined from the plot.

Alternatively, protein quantitation is achieved by eluting dye from the stained protein bands.21

3.2.1.6 Drying Gel

For long-term preservation, stained gels can be dried on thick paper backing under vacuum22 or between sheets of cellophane at atmospheric pressure.23 Gels dried between transparent sheets are useful for densitometry.
Principles and Reactions of Protein Extraction, Purification, and Characterization

Working Procedure for Vacuum Drying

Materials

1. Acetate sheets or plastic kitchen wrap
2. Whatman 3 MM paper

Equipment

1. Gel dryer (Bio-Rad)
2. Vacuum pump

Procedure

1. Soak stained and destained gels in 5% glycerol overnight. This reduces the risk of cracking gel during drying.
2. Place the gel on Whatman 3 MM paper.
3. Cover the gel with an acetate sheet or plastic wrap, taking care not to trap air bubbles. A test tube can be used to roll out air bubbles.
4. Place the gel on the gel dryer. Acetate sheet or plastic wrap should face up.
5. Operate the gel dryer according to the manufacturer’s instructions. Usually, gel is covered with sealing gasket and gel drying is performed at 60°C under vacuum.

Working Procedure for Air-Drying

Materials

Cellophane

Equipment

Gel drying frame (Figure 3.7)

Procedure

1. Soak stained and destained gels in 20% ethanol/10% glycerol for 30 min. This reduces the risk of cracking gel during drying.
2. Soak two cellophane sheets in water for about 2 min. This will soften the sheets.
3. Place one sheet of wet cellophane on the solid back plate, beveled edge down, taking care not to trap air bubbles between the plate and the cellophane.
4. Place gel on the center of the cellophane. Avoid trapping air bubbles. Layer a few milliliters of ethanol/glycerol solution on top of the gel.
5. Place a second sheet of wet cellophane on top of the gel. Match the edges of the cellophane with the edges of the backing plate. Avoid trapping air bubbles between the gel and the top sheet of cellophane.

6. Place the open frame over the stack, beveled edge up. Be sure the open frame matches the edges of the back plate and covers all edges of the cellophane.

7. Tie the back plate and open frame together by attaching plastic chips to all four sides of the frame.

8. Leave the frame assembly at room temperature overnight to allow the gel to air dry.

### 3.2.1.7 Extraction of Protein from Gel

Proteins from acrylamide gels can be extracted by electroelution\(^{24}\) or protein diffusion.\(^{25}\) For this purpose, stained gels (usually Coomassie or zinc reverse stained) containing protein bands are cut out with a razor blade, minced, and subjected to elution of proteins employing either method. Various electroelution devices are commercially available (from vendors such as Bio-Rad and Millipore) and should be operated following the manufacturer’s instructions.

For extraction of proteins by diffusion, an appropriate buffer is added to the minced gel slice, incubated for 15 min to several hours, and centrifuged, and the supernatant is collected. Ball\(^{21}\) described an efficient and simple procedure to isolate Coomassie stained protein from gel slices. In this procedure, the gel slice is incubated with 1 ml of 3% SDS in 50% isopropanol at 37°C for 24 h, and after centrifugation supernatant is collected.
3.2.2 SDS-Urea PAGE

SDS-urea PAGE is often used for proteins of low molecular weight and membrane proteins. In SDS-PAGE, the migration of low molecular weight proteins may not be proportional to their molecular weight, as the protein charge properties become significant relative to the mass. SDS-urea PAGE is suitable for membrane proteins, as they may not be soluble at conditions used in SDS-PAGE.

**Working Procedure**

All procedures for SDS-urea PAGE are essentially similar to those described for SDS-PAGE except the composition of gels (both separating and stacking) and sample loading buffer. These should contain 8 M urea. The recipe for 10% separating gel is shown below as an example.

**For 10 ml 10% Separating Gel**

- Acrylamide stock solution (see Section 3.2.1.1) 3.3 ml
- 4× separating gel buffer (see Section 3.2.1.1) 2.5 ml
- Urea 4.8 g (equivalent to 3.6 ml)
- Water 0.6 ml
- 10% ammonium persulfate 50 µl
- TEMED 5 µl

**For 4 ml 5% Stacking Gel**

- Acrylamide stock solution (see Section 3.2.1.1) 0.67 ml
- 4× stacking gel buffer (see Section 3.2.1.1) 1.0 ml
- Urea 1.9 g
- Water 0.93 ml
- 10% ammonium persulfate 30 µl
- TEMED 5 µl

3.2.3 Gradient Gels

Although polyacrylamide gels of fixed concentrations are widely used for routine analyses, the use of gradient polyacrylamide gels (increasing acrylamide concentration and hence decreasing pore size) has at least two advantages over fixed-concentration acrylamide gels. First, a gradient gel allows the separation of proteins of a larger range of molecular weights compared to a fixed-percentage gel. The second advantage of the gradient gels over the fixed-percentage gels is that the proteins of very close molecular weights can be resolved as sharp bands. However, the gradient gel requires additional equipment (such as gradient maker, pump, and tubing) and special attention when pouring the gel mixture into the gel sandwich. Air bubbles lodged in the tubing or in the gradient maker can cause the gradient to form unevenly. Fortunately, precast gradient gels are commercially available from Pharmacia, Bio-Rad, Jule Inc., and other manufacturers. The two common ranges of gradient
Electrophoretic Analyses of Protein

gels are 3 to 30% and 5 to 20%, which resolve 13 to 950 kDa and 15 to 200 kDa, respectively.

**Mechanism**

In gradient gels, proteins of a wide molecular weight range enter the gel. Proteins of high molecular weight start to resolve immediately according to the pore size of the gel. Proteins of low molecular weight migrate freely in the beginning of the gel and start to resolve when they reach the appropriate percentage of gel with the smaller pore size. Proteins travel until they reach critical pore size (pore limit), which impedes further progress. At this point, the pattern of protein bands does not change significantly with time, although migration does not stop completely.28

Regarding the separation of two proteins of very close molecular weights, each protein travels through the gel until it reaches its pore size limit. At this point, the protein stacks up, as the gel pore is too small to allow further migration of protein. A similar protein but with slightly lower molecular weight is able to travel further before it reaches its pore size limit and stacks as a sharp band.

The following procedure shows the preparation of two 0.75-mm-thick gradient gels. The amounts of each component can be scaled up when multiple gels are to be prepared. However, it is important to assemble all gel sandwiches in a single gel caster.

**Working Procedure**

**Equipment**

1. Gradient maker (Bio-Rad, Hoefer, Pharmacia)
2. Peristaltic pump (Millipore, Fisher, Pharmacia)
3. Tygon tubing

**Reagents**

1. 30% acrylamide stock solution (see Section 3.2.1.1)
2. 4× separating gel buffer (see Section 3.2.1.1)
3. 4× stacking gel buffer (see Section 3.2.1.1)
4. 10% ammonium persulfate
5. TEMED
6. Sucrose

**Procedure**

1. Prepare gel sandwich as shown in Figure 3.4 A.
2. Set up gradient maker as shown in Figure 3.8. Place a magnetic bar in the chamber attached to the outlet. This chamber receives heavy solution (higher percentage of acrylamide solution). Place a magnetic stirrer under this chamber separated by a sheet of Styrofoam.
3. Prepare the heavy and light gel solutions without TEMED as follows. Example shows the amounts of solution for a 5 to 20% separating gel.

5% (light solution) 20% (heavy solution)

<table>
<thead>
<tr>
<th>Component</th>
<th>Solution 1</th>
<th>Solution 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide stock solution</td>
<td>1.67</td>
<td>6.67</td>
</tr>
<tr>
<td>4x stacking gel buffer</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Water</td>
<td>5.8</td>
<td>0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0</td>
<td>1.5 g</td>
</tr>
<tr>
<td>(equivalent to 0.8 ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>TEMED (To be added later)</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

4. Add 5 µl of TEMED to each separating gel solution, mix gently, and immediately transfer to the appropriate chamber as shown in Figure 3.8. (Note: work rapidly after the addition of TEMED as the polymerization is under way. To allow more time for pouring gradient gels [i.e., to delay polymerization] gel solution may be chilled. Alternatively, riboflavin [to a final concentration of 0.0005%] may be used to replace ammonium persulfate to allow more time for pouring. If riboflavin used, after pouring polymerization should be initiated by exposure of daylight or white or blue fluorescent lamp.)

5. Turn on magnetic stirrer, open connection between the chambers, and turn on the peristaltic pump to allow gradient gels to fill gel cassettes at a rate of about 3 to 5 ml/min.

6. When the level of the gel reaches about 3 cm from top of the gel cassettes, gently layer about 2 to 3 mm of water on top of gels.

7. Rinse out the gradient maker before the remaining gel solution polymerizes.

8. Allow gel to polymerize for about 30 min at room temperature.

9. Polymerized gels may be stored at 4°C for future use or set up for electrophoresis. For running gel, a stacking gel is made as described before (see Section 3.2.1.1).
3.2.4 **NON-DENATURED PAGE**

Non-denaturing PAGE, also called native PAGE, refers to the electrophoretic separation of the native protein. This can be performed following the standard Laemmli SDS-PAGE protocol described above, except the solutions contain no SDS or reducing agent. In non-denaturing PAGE, separation of proteins depends on many factors such as size, shape, and native charge. Native PAGE is mostly used to determine the homogeneity of the purified protein. Native PAGE is very useful to visualize enzyme or lectin activity after electrophoretic separation. Unlike SDS-PAGE, in which the denatured proteins are uniformly negatively charged and their mobilities are dependent on their molecular weights, determination of the native molecular weight using native PAGE is not reliable, as the mobility of the native proteins depends on both molecular weight and charge.

This difficulty is partly overcome by operating native PAGE at a high pH buffer (pH 8.8). At this pH, most proteins are negatively charged and thus move toward the anode. In order to determine molecular weight using non-denaturing gel electrophoresis, the protein should be run under a variety of acrylamide concentration (usually 4 to 12%). The results from these conditions are used to adjust the effect due to protein charge. In native PAGE, acrylamide concentration may vary from 5 to 15% and acrylamide:bisacrylamide ratio may vary from 20:1 to 50:1 to achieve different sieving effects. The ionic strength is an important factor in the native PAGE, especially when the protein’s activity is to be investigated after electrophoresis. High ionic strength generates heat during electrophoresis, resulting in a loss of protein activity. However, if the ionic strength is too low, proteins may aggregate nonspecifically. Typically, ionic strength is kept in the range of 10 to 100 mM. All steps are usually performed at 0 to 4°C to minimize the loss of protein activity by denaturation and to reduce proteolysis.

Native PAGE is performed in two ways: (a) discontinuous: both stacking and separating gels like SDS-PAGE, and (b) continuous: no stacking gel. Continuous gel electrophoresis is simpler than discontinuous, as no stacking gel is involved. However, the lack of stacking gel often results in diffused or poorly resolved bands. In continuous native PAGE, ionic strength of the protein buffer is kept five- to tenfold lower than the gel buffer in order to obtain the sharpest bands. The volume of the protein sample is kept as small as possible. Thus, the protein concentration should be high (2 to 10 mg/ml). Buffers for continuous native PAGE may be the same as described below except that those pertaining to the stacking gel are omitted. Additional buffers are described elsewhere. The procedure for discontinuous non-denaturing gel electrophoresis is described below.

**Working Procedure**

**Equipment**

1. Mini gel apparatus (Hoefer, Bio-Rad, Life Technologies)
2. Power supply (capacity 200 V)
3. Heat block (100°C) or boiling water bath
4. Disposable gel loading tips for micropipette or Hamilton syringe
5. Eppendorf tubes
6. Plastic or glass container with lid
7. Rocking shaker
8. Gel dryer

**Reagents**

1. Acrylamide, electrophoresis grade
2. Bisacrylamide (N,N’-methylenebisacrylamide)
3. Tris
4. Hydrochloric acid (HCl)
5. TEMED (N,N,N’,N’-tetramethylethylenediamine)
6. Ammonium persulfate
7. Glycine
8. Glycerol
9. Bromphenol blue

**Stock Solutions**

1. 2 M Tris-HCl (pH 8.8), 1 liter: weigh out 242 gram Tris base and add 500 ml distilled water. Adjust pH to 8.8 by adding concentrated HCl slowly and finally add distilled water to a total volume of 1 liter.
2. M Tris-HCl (pH 6.8), 100 ml: to 12.1 gram of Tris base add 50 ml distilled water and adjust pH to 6.8 with concentrated HCl. Add distilled water to a total volume of 100 ml.
3. 1% bromophenol blue (w/v), 10 ml: weigh out 100 mg bromophenol blue and add 10 ml distilled water. Stir until dissolved and filter to remove particulates.

**Working Solutions**

1. 30% acrylamide stock solution: Dissolve 29.2 gram acrylamide and 0.8 gram bisacrylamide with distilled water to make 100 ml. The solution is stable at 4°C for months.
   (Note: unpolymerized acrylamide is a skin irritant and a neurotoxin and thus should be handled with gloves. Unused acrylamide solution should be polymerized and disposed of with solid waste.)
2. 4x separating gel buffer, 100 ml:
   a. 75 ml 2M Tris-HCl (pH 8.8); final conc. 1.5 M
   b. 25 ml water
   (The solution is stable for months in the refrigerator.)
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3. 4× stacking gel buffer, 100 ml:
   a. 50 ml 1 M Tris-HCl (pH 6.8); final conc. 0.5 M
   b. 50 ml water
   (The solution is stable for months in the refrigerator.)
4. 10% ammonium persulfate, 5 ml: dissolve 0.5 gram ammonium persulfate in 5 ml distilled water. Aliquot 100 µl in 0.5 ml microfuge tubes and store at –20°C (stable for months).
5. Electrophoresis buffer, 1 liter:
   a. 3 gram Tris base; final conc. 25 mM
   b. 14.4 gram glycine; final conc. 192 mM
   c. Water to make 1 liter
   (pH should be approximately 8.8; 10× stock solution can also be made and stored at 4°C; stable for months.)
6. 5× sample buffer, 10 ml:
   a. 3.1 ml 1M Tris-HCl (pH 6.8); final conc. 312.5 mM
   b. 5 ml glycerol; final conc. 50%
   c. 0.5 ml 1% bromophenol blue; final conc. 0.05%
   d. 1.4 ml water
   (Aliquot in small volume in microfuge tubes and store at –20°C. Stable for months.)

Preparation of Gel

The recipe for making gels of varying strengths is essentially identical to the amounts shown in Table 3.3. Preparation of gel cassettes, pouring separating and stacking gel into the gel cassettes, and running gels are similar to denaturing PAGE.

3.2.5 Tricine PAGE

Tricine PAGE is mainly used for the separation of low molecular weight peptides (range 40 to 1 kDa), which cannot be resolved in Laemmli SDS-PAGE. In the Laemmli system, SDS and smaller proteins comigrate and thus obscure the resolution. In Tricine (N-Tris [hydroxymethyl] methylglycine) gel electrophoresis, Tricine separates SDS and peptides, thus improving resolution. The Tricine PAGE system has an additional advantage. Since glycine (which interferes with the amino acid sequence analyses) is replaced by Tricine in the electrophoresis buffer, protein bands in the gel can be excised for amino acid sequencing.

Working Procedure

Reagents

1. 30% acrylamide stock solution (see Section 3.2.1.1)
2. Separating/stacking gel buffer: 3 M Tris-HCl/0.3% SDS, pH 8.45
3. Glycerol
4. 10% ammonium persulfate
5. TEMED
6. Cathode buffer: 0.1 M Tris/0.1 M Tricine/0.1% SDS
7. Anode buffer: 0.2 M Tris

Procedure

1. Prepare gel sandwich as shown in Figure 3.4 A.
2. In a 100 ml side-arm flask, combine the following reagents for separating gel without ammonium persulfate and TEMED.

<table>
<thead>
<tr>
<th></th>
<th>Separating gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide stock solution</td>
<td>15.0 ml</td>
<td>1.62 ml</td>
</tr>
<tr>
<td>Separating/stacking gel buffer</td>
<td>10.0 ml</td>
<td>3.10 ml</td>
</tr>
<tr>
<td>Water</td>
<td>1.83 ml</td>
<td>7.78 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>3.17 ml</td>
<td></td>
</tr>
<tr>
<td>10% ammonium persulfate (to be added later)</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>TEMED (to be added later)</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

3. Degas under vacuum for 10 min. Add 50 µl ammonium persulfate and 5 µl TEMED to the gel solution, mix gently, and immediately pour gel solution into the gel cassette, leaving empty about 1.5 cm from top of the cassette as described in Section 3.2.1.1.
4. Once polymerized, combine the above recipe to prepare stacking gel, following protocols as described in Section 3.2.1.1.
5. For running gel, heat sample to 100°C for 3 to 5 min or 40°C for 30 to 60 min.

3.2.6 NON-UREA SDS-PAGE FOR SEPARATION OF PEPTIDES

Okajima et al.31 described a modification of Laemmli SDS-PAGE for separation of peptides as low as 5 kDa. In this modification, the concentration of buffers is increased to provide better separation between the stacked peptides and the SDS micelles.

Working Procedure

Reagents

1. 30% acrylamide stock solution (see Section 3.2.1.1)
2. Separating gel buffer: 3 M Tris-HCl, pH 8.8 (compared to Laemmli SDS-PAGE, this concentration is equivalent to 8×)
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3. Stacking gel buffer: 0.5 M Tris-HCl, pH 6.8  
4. 10% SDS  
5. 10% ammonium persulfate  
6. TEMED  
7. SDS electrophoresis buffer: 0.05 M Tris/0.384 M glycine/0.2% SDS, pH 8.3 (compared to Laemmli SDS-PAGE, this recipe is 2×)

Procedure

1. Prepare gel sandwich as shown in Figure 3.4 A.  
2. In a 50 ml side-arm flask, combine the following reagents for separating gel without ammonium persulfate and TEMED:

<table>
<thead>
<tr>
<th></th>
<th>Separating gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide stock solution</td>
<td>10.0 ml</td>
<td>0.65 ml</td>
</tr>
<tr>
<td>Separating gel buffer</td>
<td>3.75 ml</td>
<td>—</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td></td>
<td>1.25 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.15 ml</td>
<td>50 µl</td>
</tr>
<tr>
<td>Water</td>
<td>1.0 ml</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>10% ammonium persulfate (to be added later)</td>
<td>50 µl</td>
<td>25 µl</td>
</tr>
<tr>
<td>TEMED (to be added later)</td>
<td>10 µl</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

3. Degas under vacuum for 10 min. Add 50 µl ammonium persulfate and 10 µl TEMED to the gel solution, mix gently, and immediately pour gel solution into the gel cassette, leaving empty about 1.5 cm from top of the cassette as described in Section 3.2.1.1.  
4. Once polymerized, prepare stacking gel, following protocols as described in Section 3.2.1.1.  
5. Run gel in 2× SDS electrophoresis buffer.

3.2.7 Acid Urea PAGE

Panyim and Chalkley\textsuperscript{32} introduced a continuous acetic acid urea PAGE for the separation of unmodified histone from its monoacetylated or monophosphorylated form. The procedure can separate similar basic proteins based on differences in effective charge as well as differences in size. Proteins of a slightly different charge such as unmodified and acidic acetylated derivative can be separated in acid urea PAGE. Urea is commonly used in this PAGE, mainly to disrupt any aggregation and to increase the density of the loading solution. In this system, riboflavin or riboflavin 5′-phosphate is used as initiator of photo-polymerization of acrylamide, as ammonium persulfate interferes with stacking of the proteins in the gel. Chloride ions also
interfere with the stacking system. Thus, protein samples and glycine used in the electrophoresis buffer should be free of chloride salts.

**Mechanism**

In acid urea PAGE, the samples are electrophoresed in acetic acid buffer (pH around 3). At this pH, basic proteins with a high isoelectric point get positively charged and move toward the cathode under an electric field.33

**Working Procedure**

**Reagents**

1. Acrylamide stock solution: 60% (w/v) acrylamide in water.
2. N,N′-methylenebisacrylamide stock solution: 2.5% (w/v) in water.
3. Urea, ultrapure quality.
4. Glacial acetic acid: 17.5 M.
5. Concentrated ammonium hydroxide: approximately 15 M.
6. Riboflavin 5′-phosphate: 0.006% (w/v) in water. Stable for at least 6 months at dark (4°C).
7. TEMED.
8. Phenolphthalein indicator solution: 1% (w/v) in 95% ethanol.
9. Sample buffer: add 7.7 mg dithiothreitol in 0.9 ml 8 M urea solution and mix. Then add 50 µl 1% phenolphthalein and 50 µl NH₄OH to obtain pink sample buffer.  
   (Note: urea should be free from ionic contaminants such as cyanate to avoid amino acid modification in the protein sample. For example, cysteine residues can be modified by cyanate. To obtain deionized urea, pass urea solution through a column of mixed resin [e.g., AG 501-X8, Bio-Rad]. Freeze dry the deionized solution and store at –20°C. Storage of urea at low temperature minimizes production of ionic contaminants.)
10. Methylene blue solution: 2% (w/v) in sample buffer.
11. Electrophoresis buffer: 1 M acetic acid/0.1 M glycine.

**Procedure**

1. Prepare gel sandwich as shown in Figure 3.4 A.
2. In a 100 ml side-arm flask, combine the following reagents for separating gel without riboflavin 5′-phosphate and TEMED. The recipe is good for making five mini gels.
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3. Degas under vacuum for 10 min. Add 0.35 ml TEMED and 4.67 ml riboflavin 5'-phosphate to the gel solution, mix gently, and immediately pour gel solution into the gel cassette, leaving empty about 1.5 cm from top of the cassette as described in Section 3.2.1.1. Leave the gel cassette under white light or on the regular light box. (Note: since polymerization is initiated by light, high-intensity light boxes close to either side of the gel surface may be required for thick gels of greater than 1.5 mm.)

4. Once polymerized, pour the stacking gel on top of the separating gel as described in Section 3.2.1.1. For the preparation of stacking gel, combine the following gel components.

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide stock solution</td>
<td>17.5 ml</td>
</tr>
<tr>
<td>Bisacrylamide stock solution</td>
<td>2.8 ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>4.2 ml</td>
</tr>
<tr>
<td>Concentrated ammonium hydride</td>
<td>0.23 ml</td>
</tr>
<tr>
<td>Urea</td>
<td>33.6 g</td>
</tr>
<tr>
<td>Water to make 65 ml</td>
<td></td>
</tr>
<tr>
<td>Riboflavin 5'-phosphate (to be added later)</td>
<td>4.67 ml 0.0004%</td>
</tr>
<tr>
<td>TEMED (to be added later)</td>
<td>0.35 ml</td>
</tr>
</tbody>
</table>

3. Degas under vacuum for 10 min. Add 0.35 ml TEMED and 4.67 ml riboflavin 5'-phosphate to the gel solution, mix gently, and immediately pour gel solution into the gel cassette, leaving empty about 1.5 cm from top of the cassette as described in Section 3.2.1.1. Leave the gel cassette under white light or on the regular light box. (Note: since polymerization is initiated by light, high-intensity light boxes close to either side of the gel surface may be required for thick gels of greater than 1.5 mm.)

4. Once polymerized, pour the stacking gel on top of the separating gel as described in Section 3.2.1.1. For the preparation of stacking gel, combine the following gel components.

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide stock solution</td>
<td>1.34 ml</td>
</tr>
<tr>
<td>Bisacrylamide stock solution</td>
<td>1.28 ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>1.14 ml</td>
</tr>
<tr>
<td>Concentrated ammonium hydride</td>
<td>0.07 ml</td>
</tr>
<tr>
<td>Urea</td>
<td>9.6 g</td>
</tr>
<tr>
<td>Water to make 18.6 ml</td>
<td></td>
</tr>
<tr>
<td>Riboflavin 5'-phosphate (to be added later)</td>
<td>1.3 ml 0.0004%</td>
</tr>
<tr>
<td>TEMED (to be added later)</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

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5. Prepare sample by adding 50 µl of sample buffer to the salt-free lyophilized protein (5 to 50 µg). Incubate for 5 min at room temperature for reduction.

(Note: reduction by DTT [in sample buffer] for 5 min at room temperature is optimum for most samples. Longer incubation may modify cysteine residues in the protein by urea contaminant cyanate. To assure complete reduction of proteins by DTT, make sure the pH of the solution is above 8.0. If the pink phenolphthalein color disappears during sample incubation, add a few microliters of concentrated NH$_4$OH to obtain alkaline pH.)

6. Acidify the sample with 2.5 µl glacial acetic acid.

7. Add 2 µl methylene blue to each sample. Sample is now ready for loading onto the gel.

8. Once polymerized, set up the gel in the electrophoresis apparatus, fill the reservoir with the electrophoresis buffer, and load sample as described in Section 3.2.1.2.

9. Connect the electrodes to the power supply.

(Important note: in the acid urea PAGE system, basic proteins are positively charged and thus move toward the cathode. So, upper electrode of the electrophoresis apparatus should be connected to the (+) lead of the power supply and lower electrode to the (-) lead of the power supply. This is opposite to the SDS-PAGE configuration.)

10. Run gel at 200 V in constant voltage mode.

Bonner et al.$^{34}$ described acid urea PAGE system in the presence of non-ionic detergent Triton X-100 for separation of core histones. This is based on the observation that core histones but not linker histones or any other protein bind Triton.$^{35}$

### 3.2.8 CTAB PAGE

Although Laemmli PAGE is widely used for testing the purity of a protein and the determination of its subunit size, this procedure is not suitable to assess the biological activity of proteins treated with SDS. Cetyltrimethylammonium bromide (CTAB) PAGE allows the sample solubilization in CTAB and molecular size-dependent separation of proteins in an arginine/Tricine buffer, with the retention of native activity.$^{36,37}$ The following working procedure is based on Akins et al.,$^{37}$ who described CTAB PAGE in a discontinuous gel format.

**Mechanism**

In the CTAB PAGE system, proteins get positively charged and thus migrate toward the cathode under electric field. The arginine in the electrophoresis buffer also migrates toward the cathode, as arginine is positively charged at the electrophoresis buffer pH 8.2 (pI of arginine is 10.8). However, at the stacking gel (pH 9.96) arginine will have a lower net positive charge and will move slowly. In the interface zone between the upper tank buffer and the stacking gel/sample buffer, sodium ions
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(Tricine-NaOH) move ahead of the slow-moving arginine. The CTAB-coated proteins migrate more quickly in this interface zone than in the sodium-containing zone and “stack” as the interface advances.

**Working Procedure**

**Reagents**

1. 40% acrylamide stock solution: 38.93 gram ultrapure acrylamide and 1.07 gram bisacrylamide in 100 ml of distilled water.
2. Separating gel buffer: 1.5 M Tricine-NaOH, pH 8.0. Dissolve 134.4 gram of Tricine in 400 ml distilled water. Add NaOH until the pH of the solution reaches 8.0 and finally bring the solution to a volume of 500 ml with distilled water.
3. Stacking gel buffer: 0.5 M Tricine-NaOH, pH 10.0. Dissolve 22.4 gram of Tricine in 200 ml distilled water. Add NaOH until the pH of the solution reaches 10.0 and finally bring the solution to a volume of 250 ml with distilled water.
4. Agarose stock solution: prepare a ready-to-use agarose stacking gel solution by combining 25 ml stacking gel buffer, 0.1 gram CTAB, 0.7 gram of electrophoresis grade agarose, and finally bringing the solution volume to 100 ml with distilled water.
5. Electrophoresis buffer: to prepare 1 liter of 5x buffer, dissolve 22.4 gram Tricine and 5 gram CTAB in 900 ml of distilled water. Add 1 M arginine solution (approximately 75 ml) until the pH of the solution reaches 8.2. Finally bring the solution to a volume of 1 liter with distilled water.
6. 10% ammonium persulfate.
7. TEMED.

**Procedure**

1. Prepare gel sandwich as shown in Figure 3.4 A.
2. In a 25 ml side-arm flask, combine the following reagents for separating gel without ammonium persulfate and TEMED. This makes one mini gel of 10%.

   | Reagent                              | Volume  |
---|--------------------------------------|---------|
| 40% acrylamide stock solution        | 2.5 ml  |
| Separating gel buffer                | 2.5 ml  |
| Distilled water                      | 4.89 ml |
| 10% Ammonium persulfate (to be added later) | 0.1 ml  |
| TEMED (to be added later)            | 0.01 ml |
3. Degas under vacuum for about 10 min. Add 100 µl ammonium persulfate and 10 µl TEMED to the gel solution, mix gently, and immediately pour gel solution to the gel cassette, leaving empty about 1.5 cm from top of the cassette as described in Section 3.2.1.1.

4. Once polymerized, pour the stacking gel on top of the separating gel as described in Section 3.2.1.1. For the preparation of stacking gel (4%), combine the following gel components.

40% acrylamide stock solution 1.0 ml
Stacking gel buffer 2.5 ml
Distilled water 6.39 ml
10% ammonium persulfate (to be added later) 0.1 ml
TEMED (to be added later) 0.01 ml

(Note: stacking gels are made from agarose when subsequent protein activity assay is performed, as these are shown to provide better results. For this purpose, melt agarose stock solution in a microwave oven and pour to the gel cassette. Insert the comb immediately. Allow the agarose to cool before removing the comb.)

5. Once polymerized, set up the gel in the electrophoresis apparatus, fill the reservoir with 1× electrophoresis buffer, and load sample as described in Section 3.2.1.2.

6. Connect the electrodes to the power supply.

(Important note: in the CTAB PAGE system, proteins are positively charged with the CTAB and thus move toward the cathode. Therefore, the upper electrode of the electrophoresis apparatus should be connected to the [+] lead of the power supply and lower electrode to the [-] lead of the power supply. This is opposite to the SDS-PAGE configuration.)

7. Run gel at 100 V in constant voltage mode.

3.3 ISOELECTRIC FOCUSING

Isoelectric focusing (IEF) is an electrophoretic method in which amphoteric molecules are separated as they migrate through a pH gradient. Polyacrylamide gels are generally used for focusing proteins. However, for proteins larger than 200,000 dalton (Da), 1% agarose gels can be employed.

**Mechanism**

The net charge on a protein is the algebraic sum of all its positive and negative charges. At physiological pH, lysine, arginine, and histidine residues in a protein are positively charged, while aspartic acid and glutamic acid carry a negative charge. So the net charge of a protein at a specific pH depends on the relative number of positive and negative charges. The pH at which a protein carries no net charge (total
positive charge equal to total negative charge) is called its isoelectric point (pI). Below the pI the protein carries a positive charge, and a negative charge at pHs above pI. When protein is placed in a medium with varying pH and subjected to an electric field, it will initially move toward the electrode with the opposite charge. During migration through the pH gradient, the protein will either pick up or lose protons. As it does, its net charge and mobility will decrease, and at its pI the protein will stop moving. This type of motion is in contrast to conventional electrophoresis, in which proteins continue to move through the medium until the electric field is removed. In proteins migrate to their steady-state positions from anywhere in the system, and thus the location of sample application is arbitrary.

The key to IEF is the establishment of stable pH gradients in electric fields. This is most commonly accomplished by means of commercially available, synthetic carrier ampholytes (amphoteric electrolytes). These compounds are mixtures of relatively small (600 to 900 Da), multicharged, amphoteric molecules with closely spaced pI values and high conductivity. Under the influence of an electric field, carrier ampholytes partition themselves into smooth pH gradients, which increase monotonically from the anode to the cathode. The slope of the pH gradient is determined by the pH interval covered by the carrier ampholyte mixture and the distance between the electrodes. Isoelectric focusing is usually carried out in a denaturing gel system with urea. Charged denaturing agents such as SDS and sodium deoxycholate should not be used, as these interfere with the electrophoresis. Isoelectric focusing can also be carried out in a non-denaturing system, when functions of proteins (e.g., enzyme activity, lectin activity) are studied after focusing. Table 3.6 shows some common problems in isoelectric focusing and their remedies.

It is important to perform isoelectric focusing on a device where efficient gel cooling is achieved. This is required to maintain high-voltage gradient for better resolution of protein bands. Isoelectric focusing can be performed on either slab gels or tube gels. Several devices are commercially available for running slab gels (vertical and horizontal) and tube gels. A procedure for isoelectric focusing gel electrophoresis on a vertical slab gel format is described here.

**Working Procedure**

**Equipment**

1. Mini Protein II Gel System (Bio-Rad) or other slab gel electrophoresis apparatus
2. Power supply

**Reagents**

1. Acrylamide: 30% acrylamide stock solution (see Section 3.2.1.1)
2. Ampholyte solutions (pH 3.5 to 10; pH 4 to 6; pH 6 to 9; pH 9 to 11)
3. Urea, ultrapure
4. Ammonium persulfate
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5. TEMED
6. Triton X-100
7. 2-mercaptoethanol
8. Phosphoric acid: 10 mM (made fresh)
9. Sodium hydroxide: 20 mM (made fresh)
10. Trichloroacetic acid (TCA): 10% and 1%

### TABLE 3.6
Common Problems in Isoelectric Focusing Gels and Possible Remedy

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Remedy</th>
</tr>
</thead>
<tbody>
<tr>
<td>High background staining</td>
<td>Incomplete removal of ampholytes from the gel</td>
<td>Increase time of fixing with 1% TCA</td>
</tr>
<tr>
<td>Wavy bands</td>
<td>High salt content in the sample Impurities in the ampholyte or electrolyte solutions Dirty electrodes</td>
<td>Dialyze the sample in low salt buffer Use fresh ampholytes and electrolytes Clean electrodes</td>
</tr>
<tr>
<td>Streaking bands</td>
<td>Protein aggregation or precipitation Presence of nucleic acids in the sample Modification of protein may occur such as oxidation of cysteine, deamination of asparagine and glutamine, carbamoylation of protein by isocyanate present in impure urea.</td>
<td>Centrifuge samples before loading Remove nucleic acids Remove isocyanate impurities by prerunning the gel. Handle sample properly to avoid other modification.</td>
</tr>
<tr>
<td>Overlapping bands</td>
<td>Complex protein mixtures</td>
<td>Change the pH range of the gel Verify that the electrodes are clean for good contact</td>
</tr>
<tr>
<td>Skewed bands</td>
<td>Faults in the pH gradient</td>
<td>Increase voltage gradient incrementally towards the end of the run Use more porous agarose gel for large proteins</td>
</tr>
<tr>
<td>Fuzzy bands</td>
<td>Incomplete focusing Large proteins and thus restricted mobility</td>
<td>Increase TCA concentration</td>
</tr>
<tr>
<td>Missing or faint bands</td>
<td>Proteins have not been denatured during fixation</td>
<td>Increase TCA concentration</td>
</tr>
<tr>
<td>Uneven pH gradient</td>
<td>Electrode contact is not parallel to the gel Impurities within the gel Ampholyte concentrations are too low</td>
<td>Make sure electrodes are parallel to the gel Use reagents of highest grade Increase ampholyte concentrations</td>
</tr>
</tbody>
</table>

* Based on Reference 40.
**TABLE 3.7**

<table>
<thead>
<tr>
<th>Gels with pH Range</th>
<th>Ratio of Ampholytes</th>
<th>% in Final Gel Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 3.5–10</td>
<td>pH 3.5–10</td>
<td>2.4</td>
</tr>
<tr>
<td>pH 4–6</td>
<td>pH 3.5–10</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>pH 4–6</td>
<td>2.0</td>
</tr>
<tr>
<td>pH 6–9</td>
<td>pH 3.5–10</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>pH 6–8</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>pH 7–9</td>
<td>1.0</td>
</tr>
<tr>
<td>pH 9–11</td>
<td>pH 3.5–10</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>pH 9–11</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Based on Reference 41.

**Procedure**

**Preparation of Gels**

1. Prepare gel cassettes according to the manufacturer’s instructions.
2. Combine the following gel components in 50 ml Erlenmeyer flask. The following recipe makes two mini gels (0.75 mm) with a gradient from pH 4 to 6. For making gels with other pH ranges, see Table 3.7.

   Acrylamide stock solution (see Section 3.2.1.1) 2.0 ml
   Water 5.4 ml
   Ampholyte solution, pH 3.5-10 48 µl
   Ampholyte solution, pH 4-6 240 µl
   Urea, ultrapure 6 g

3. Swirl gently to dissolve urea.
4. Add 50 µl 10% ammonium persulfate and 20 µl TEMED to the above gel solution. Mix gently and immediately pour into assembled gel cassettes. Be careful to avoid trapped air bubbles. Fill the gel cassette completely with acrylamide solution.
5. Insert comb immediately, not trapping any air bubbles in the teeth of the comb.
6. Allow gels to polymerize at room temperature.
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Set Up Gel

7. Remove comb carefully after gels have polymerized.
8. Attach gel to the electrophoresis tank according to the manufacturer’s instructions.
9. Add catholyte (20 mM sodium hydroxide) to the upper buffer chamber and anolyte (10 mM phosphoric acid) to the lower buffer chamber.

Sample Preparation and Loading

10. Mix protein sample (5 to 10 µg per lane) with an equal volume of 2× loading buffer. To obtain 2× loading buffer, combine the following reagents. This recipe makes 5 ml loading buffer. Aliquot the unused buffer into small volume and store frozen at −20°C until use.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea, ultrapure</td>
<td>6 g</td>
<td>8 M</td>
</tr>
<tr>
<td>Ampholyte solution, pH 3.5 to 10</td>
<td>20 µl</td>
<td></td>
</tr>
<tr>
<td>Ampholyte solution, pH 4 to 6</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>20% Triton X-100</td>
<td>500 µl</td>
<td>2%</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>5 µl</td>
<td>1%</td>
</tr>
<tr>
<td>1% bromophenol blue</td>
<td>200 µl</td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.7 ml</td>
<td></td>
</tr>
</tbody>
</table>

(Note: protein sample should not contain high salt [not more than 50 mM]).

11. Centrifuge the sample, if necessary, for a few minutes in an Eppendorf centrifuge at highest speed to remove any aggregate.

(Note: aggregated protein may cause streaking in the gel).

12. Apply the clear supernatant into the bottom of the well with a disposable gel loading tip or a Hamilton syringe.

Running Gel

13. Connect the electrophoresis chamber with the power supply (lower chamber with the anode, upper chamber with the cathode).
14. Run the gel at 150 V for 30 min and at 200 V for additional 2.5 h.

Cutting Gel Slices for pH Determination

15. After electrophoresis, cut a strip of gel into 0.5 cm slice, keeping track the distance from any electrode (e.g., anode). Place each slice into an Eppendorf tube and label by the distance (in cm) from the anode.
16. Incubate each slice in 1 ml 10 mM KCl for about 30 min.
17. Centrifuge and read pH of the clear supernatant. A standard curve is drawn by plotting pH of the gel slices and the distance from the anode. The pI of the unknown is determined from the plot. (Note: alternatively, pI of the unknown can be determined from a plot of migration of proteins of known pIs [isoelectric focusing markers]. Marker proteins are loaded in the same gel where unknown protein is electrophoresed, and the migration from the electrode is determined after staining the gel.)

Fixing and Staining the Gel

18. Soak gels in 10% TCA for 10 min.
19. Replace with 1% TCA and incubate for 2 h to overnight to remove ampholytes. Removal of ampholytes is important to reduce background staining.
20. Stain gel with Coomassie blue for 10 min (see Section 3.2.1.3 for Coomassie stain composition).
21. Discard staining solution and replace with the destaining solution (see Section 3.2.1.3 for destaining solution).
22. Gel may be dried following protocols as previously described (see Section 3.2.1.6).

Native Isoelectric Focusing Gel

The working procedure for native isoelectric focusing is essentially identical to denaturing isoelectric focusing except some modifications in the recipe of the gel solution and the gel-loading buffer.

Recipe for Native Isoelectric Focusing Gel

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide stock solution (see Section 3.2.1.1)</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Water</td>
<td>9.7 ml</td>
</tr>
<tr>
<td>Ampholyte solution, pH 3.5 to 10</td>
<td>48 µl</td>
</tr>
<tr>
<td>Ampholyte solution, pH 4 to 6</td>
<td>240 µl</td>
</tr>
<tr>
<td>10% ammonium persulfate (to be added later)</td>
<td>50 µl</td>
</tr>
<tr>
<td>TEMED (to be added later)</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

Recipe for Native Gel Sample Buffer (2×)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>Ampholyte solution, pH 3.5 to 10</td>
<td>33.3 µl</td>
</tr>
<tr>
<td>Ampholyte solution, pH 4 to 6</td>
<td>166.7 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.8 ml</td>
</tr>
</tbody>
</table>
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Isoelectric Focusing in Horizontal Slab Gels

Isoelectric focusing in a horizontal slab gel format has several advantages over the vertical format. Cooling of the gel during electrophoresis is very efficient, as the gel remains flat on the cooling plate. Only a few milliliters of electrode solutions (enough to soak electrode strips) are required for electrophoresis. The gel size can be adjusted as needed. Sample can be added at any position in the pH gradient. Larger sample volume (more than 10 to 15 µl) can be added on a slab gel. Precast isoelectric focusing gels (polyacrylamide gel on a plastic support film) with varying pH ranges are commercially available (Amersham Pharmacia). Electrophoresis should be performed according to the manufacturer’s instructions.

3.4 TWO-DIMENSIONAL (2D) GEL ELECTROPHORESIS

In 2D gel electrophoresis, protein separates in two dimensions: the first dimension on the basis of pI and the second dimension based on subunit molecular weight. Usually, isoelectric focusing is performed on a tube gel of very small diameter or on a thin gel strip, and after completion of the run the gel is placed horizontally onto the top of a polymerized slab gel for SDS-PAGE. In this system proteins are separated into many more components than is possible with conventional one-dimensional electrophoresis (Figure 3.9). Due to greater resolution, it is possible to quantitate differentially expressed proteins during a certain biological process. The powerful technique of protein separation and identification under the heading “Proteomics” is based on the principle of 2D electrophoresis. Procedures for 2D gel electrophoresis can be adopted from the isoelectric focusing and SDS-PAGE procedures described above. Additionally, 2D equipment and procedures for 2D electrophoresis are available from various commercial sources such as Hoefer, Bio-Rad, and Millipore.

3.5 WESTERN BLOTTING

Western blotting refers to the electrophoretic transfer of the resolved proteins or glycoproteins from a polyacrylamide gel to a membrane such as nitrocellulose and polyvinylidene difluoride (PVDF). The immobilization of proteins on a membrane is more useful than working on the gel for a number of reasons: (a) proteins are more accessible, (b) membranes are easier to handle than gels, (c) smaller amounts of reagents are needed, and (d) processing time is shorter. Following the transfer of proteins to a membrane, a wide variety of applications can be carried out on the immobilized proteins such as immunodetection (see Chapter 6), carbohydrate detection (in case of glycoprotein see Chapter 7), and amino acid analysis and protein sequencing. Other applications involved in immobilized proteins are (a) epitope mapping, (b) ligand binding, (c) cutting out protein band for antibody production, and (d) structural domain analysis (see Figure 3.10). In most applications, immobilized proteins or glycoproteins can be identified and visualized by using very specific and sensitive detection techniques (immunological or biochemical). For example, as
FIGURE 3.9 2D gel of an aqueous extract of E. coli stained with Coomassie Blue. Isoelectric focusing in the first dimension was performed in Protein cell (Bio-Rad, Hercules, CA) on IPG strips in the pH range 3 to 10. SDS-PAGE in the second dimension was performed on a 4 to 15% gradient gel (Bio-Rad). Protein markers are shown in the left (indicated by arrow).

FIGURE 3.10 Flow diagram showing applications of immobilized proteins.
low as 1 to 10 pg of protein can be detected employing immunological techniques. Because of its wide applications and flexibility in protein detection methods, Western blotting has become a very popular and convenient method for analysis of denatured proteins.

**Mechanism**

Proteins are transferred from the SDS-PAGE gels, in which all proteins are negatively charged due to the SDS treatment. In an electric field, these negatively charge proteins migrate towards the positive and get immobilized on the membrane.

Protein transfer is usually accomplished by one of two electrophoretic methods: semi-dry blotting and wet blotting. In the former method, the gel and immobilizing membrane matrix are sandwiched between buffer-wetted filter papers, and a current is applied for 10 to 30 min. In wet blotting, the gel-membrane matrix sandwich is submerged in a transfer buffer and current is applied for 45 min to overnight. Due to its greater flexibility, wet blotting will be described here.

**Blotting Membrane**

Proteins in acrylamide gels can be transferred to nitrocellulose, PVDF, nylon, or carboxymethyl cellulose. However, for most applications, nitrocellulose and PVDF are preferred for the following reasons (see also Table 3.8). Nitrocellulose is relatively inexpensive, and its non-specific binding to the antibody can largely be blocked. PVDF is more expensive than nitrocellulose, but is ideal for N-terminal amino acid sequencing and amino acid analysis, since the membrane is resistant to acid and organic solvents. In contrast, blocking of the non-specific protein band in nylon is cumbersome because of the high-charge density of the matrices. Protein staining in nylon with common anionic dyes (Coomassie Blue, Amido Black, etc.) is not possible due to the positive charge of the nylon matrix. However, nylon is used when (a) higher protein binding is required, (b) a protein binds weakly to nitrocellulose (especially high molecular weight), and (c) greater resistance to mechanical stress is desired. The binding capacity of nylon is almost eightfold more than that of nitrocellulose (80 µg/cm²).

The efficiency of Western transfer depends on several factors such as composition of buffer, time, voltage, and size of the protein; percent acrylamide; and the thickness of the gel. An optimization is required for each protein, and the efficiency of transfer can be assessed by staining the blots with any blot stain (see Section 3.5.1). In general, proteins of low molecular weight transfer more easily than those of high molecular weight. Proteins transfer more effectively from low-percent acrylamide gels than from high-percent gels. Methanol improves protein binding to nitrocellulose membrane, but inhibits transfer. SDS is sometimes added to the transfer buffer to improve transfer of large proteins, but unfortunately it inhibits protein binding to the membrane. Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) is commonly used for most proteins. When the transferred protein is used for amino acid analyses, the Towbin buffer is replaced by the CAPS buffer.
### TABLE 3.8
Characteristics of Various Membranes Used in Western Blotting

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Commercial Source</th>
<th>Charge of the Membrane</th>
<th>Nature of Interaction between Membrane and Protein</th>
<th>Remarks</th>
<th>Recommended Applications of the Transferred Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrocellulose</td>
<td>Bio-Rad</td>
<td>Negative</td>
<td>Hydrophobic and electrostatic forces</td>
<td>Low cost</td>
<td>Immuno detection</td>
</tr>
<tr>
<td>PVDF</td>
<td>Millipore</td>
<td>Negative</td>
<td>Hydrophobic forces</td>
<td>Mechanically fragile</td>
<td>N-terminal sequencing, amino acid analysis, immunodetection, ligand binding</td>
</tr>
<tr>
<td>Nylon</td>
<td>Millipore</td>
<td>Positive</td>
<td>Electrostatic forces</td>
<td>Mechanically strong</td>
<td>Multiple reprobing</td>
</tr>
<tr>
<td>Carboxymethyl cellulose</td>
<td>Millipore</td>
<td>Negative</td>
<td>Ionic interactions</td>
<td>Very high capacity for histones Elution step is required before sequencing</td>
<td>Sequencing of basic proteins</td>
</tr>
</tbody>
</table>

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(10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, 10% methanol, pH 11.0) to avoid interference with the analyses due to the presence of glycine. When protein is transferred from acid urea gel or isoelectric focusing gel, acetic acid (0.7%) is used as a transfer buffer. The apparatus for Western transfer is available from several vendors such as Hoefer, Bio-Rad, and Pharmacia and can easily be operated following the manufacturer’s instructions. A procedure for Western transfer using Hoefer apparatus is described below.

**Working Procedure**

**Equipment**

1. Western transfer apparatus
2. Power supply
3. Gel-membrane sandwich cassette
4. Sponges
5. Whatman 3MM paper
6. Blotting membrane (for example, PVDF)
7. Magnetic stirrer
8. Cooling water circulator
9. Plastic or glass tray to assemble gel-membrane sandwich

**Reagents**

1. Towbin transfer buffer: 25 mM Tris, 192 mM glycine, 20% methanol
2. Methanol

**Procedure**

1. Following SDS-PAGE, soak gel in Towbin buffer for 20 min at room temperature.
2. Fill up approximately two thirds of the transfer apparatus with the transfer buffer and cool down to 10°C with cold-water circulator.
3. Cut a piece of PVDF membrane slightly larger than the gel. Soak the membrane in 100% methanol for a few minutes (PVDF will not wet directly in Towbin buffer) and incubate the membrane in transfer buffer for 10 to 15 min.
4. Cut two pieces of Whatman 3MM filter paper slightly larger than the membrane and soak in transfer buffer. Leave gel, membrane, and papers in transfer buffer until a sandwich is made.
5. Unlock the empty cassette and place in a tray. Assemble gel-membrane sandwich as shown in Figure 3.11. In order to prepare sandwich, place a sponge (presoaked in transfer buffer) on top of one backing of the cassette.
Place one filter paper on top of the sponge. Place the gel on top of filter paper. Now layer carefully the membrane on top of the gel, not trapping any air bubbles. A test tube can be rolled over the membrane to remove bubbles. Be careful also not to dry out sponge, gel, or membrane during the assembly. It is helpful to put a few milliliters of transfer buffer in the tray. Place the second filter paper on top of the membrane and finally the second sponge on top of the filter paper. Now lock the cassette and place into the transfer apparatus, facing the membrane side towards the anode and the gel side towards the cathode. Add more transfer buffer if needed.

6. Close the lid of the electrophoresis apparatus and connect the electrodes to the power supply.

7. Run the transfer at 60 to 80 V or at 0.4 amps for 60 to 90 min. Most proteins up to 100 kDa are usually transferred by these conditions.

8. At the end of transfer, turn off power supply and remove the cassette from the transfer apparatus. Separate membrane from the gel and mark the side of the membrane to which the proteins were immobilized. The membrane is now ready for immunodetection or other applications as described. Treat Towbin buffer as MeOH hazardous waste. However, Towbin buffer can be reused three to four times.

3.5.1 Staining Proteins on Blot Transfer Membrane

Membrane-immobilized proteins are often visualized to monitor the efficiency of transfer prior to further processing. Several stains are used for blot membranes, but anionic dyes such as Amido Black or India ink are less satisfactory for nylon membranes.
Staining with Coomassie Brilliant Blue

Immobilized proteins can be visualized in a few minutes with Coomassie staining. As this staining is irreversible, blot membranes that are subject for immunodetection should not be stained with Coomassie. However, one important application of Coomassie blue is to stain a portion of the membrane and match the stained proteins with the immunodetected proteins.

Working Procedure

Reagents

1. Staining solution: 0.1% (w/v) Coomassie Brilliant Blue in 40% methanol/10% acetic acid
2. Destaining solution: 40% methanol/10% acetic acid

Staining Procedure

1. Stain the membrane with the staining solution for 1 to 5 min.
2. Destain with the destain solution until it clears the background.
3. Rinse with water and leave the membrane in water.

Staining with Amido Black

A protein band of lower microgram range can be detected with Amido Black satin.47

Working Procedure

Reagents

1. Amido Black 10B
2. Stain or destain solution: 25% isopropanol/10% acetic acid

Procedure

1. Stain the membrane (nitrocellulose or PVDF) with 0.1% Amido Black in staining solution for a minute.
2. Destain the membrane for 30 min with destaining solution and wash in water before drying.
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Staining with India Ink

With this staining, protein bands appear as black on a gray background. The stained membrane can be stored for at least one month without any loss of sensitivity.

Working Procedure

Reagents

1. Wash buffer: 0.3% Tween 20 in 10 mM sodium phosphate/150 mM NaCl, pH 7.2 (PBS-T)
2. India ink: 1% solution in wash buffer
3. KOH solution: 1%
4. PBS: 10 mM sodium phosphate/150 mM NaCl, pH 7.2

Procedure

1. Incubate the blot membrane in KOH solution for 5 min at 20°C.
2. Rinse the membrane twice with PBS (10 min each).
3. Wash the blot with wash buffer at 37°C (4 times, 5 min each). Rinse with water between wash.
4. Stain the blot with India ink solution with agitation for 15 min to overnight at room temperature.
5. Destain with multiple changes of PBS.

Staining with Ponceau S

Staining of immobilized proteins with Ponceau S is a reversible procedure, since the stain can be washed off completely with water. This stain is not very sensitive. Nonetheless, it is often used to monitor the transfer of protein prior to immunodetection or other applications. The stain is also used to identify bands for micro sequencing.

Working Procedure

1. At the end of transfer, soak the blot in Ponceau S solution (0.1 g Ponceau S/1 ml acetic acid/100 ml water) for 5 min with gentle agitation.
2. Destain with water for 2 min. Blot now can be photographed.
3. For immunodetection or other applications, destain the membrane completely in water for 10 min. Prior to destaining, mark the band and the molecular weight standard with a pencil or pinhole.
Other Stains for Blot Membranes

Several stains for blot membranes are commercially available. They are usually more sensitive than Ponceau or Amido Black. In most cases, the identities of the staining reagents are trade secrets. The staining procedures are available from vendors. MemCode™ Reversible Protein Stain (Pierce, Rockford, IL) is used to stain the protein band on nitrocellulose membranes. The stain on blots can be washed off quickly for immunodetection or other applications. This stain is not suitable for PVDF membrane. SYPRO® Ruby protein blot stain (Molecular Probes, Eugene, OR) is a very sensitive reagent to detect proteins on both nitrocellulose and PVDF membranes.

3.5.2 Recovery of Proteins from Blot Membrane

Recovery of proteins from membranes is often needed for many applications such as amino acid composition analysis, for protein sequencing, and as an immunogen. Several solvents can be used to elute protein from the membrane, and the choice of the solvent system depends on the intended application. For example, acetonitrile or n-propanol usually maintains the protein structure and thus can be used as an immunogen or antigen in radioimmunoassays. Detergent-based systems are used to elute proteins when proteolytic and analytical manipulations are desired. Detergent elution is more effective than elution with organic solvents.

Working Procedure

Elution with an Organic Solvent System

1. Cut out the band of interest from the membrane and place it in a microfuge tube.
2. Incubate with 500 µl elution buffer (40% acetonitrile in 0.1 M ammonium acetate buffer, pH 8.9) at 37°C with shaking for 3 h.
3. Centrifuge the tube for 10 min at highest speed in a microfuge. Collect the supernatant.
4. Incubate the same blot for a second time with 250 µl elution buffer. After centrifugation, pool the second supernatant with the first.
5. Lyophilize to remove the volatile solvent.

Elution with a Detergent-Based Solvent System

1. Cut out the band of interest from the membrane and place it in a microfuge tube.
2. Incubate with 500 µl elution buffer (50 mM Tris-HCl, pH 9.0 containing 2% SDS, 1% Triton X-100, and 0.1% dithiothreitol) at room temperature for 1 to 3 h.
3. Centrifuge the tube for 10 min at highest speed in a microfuge. Collect the supernatant.
4. Incubate the same blot for a second time with 250 µl elution buffer. After centrifugation, pool the second supernatant with the first.

### 3.6 CAPILLARY ELECTROPHORESIS

In this procedure molecules such as proteins, glycoproteins, peptides, and DNA are separated in a capillary tube (usually made of silica, 10 to 100 µm diameter) under a potential difference produced at two ends. The most common type of capillary electrophoresis is capillary zone electrophoresis (CZE), which relies on simple instrumentation consisting of a capillary column, a detector, and a high-voltage power supply (Figure 3.12). The two ends of the capillary tube are immersed in reservoirs containing electrolytes, which serve as electrodes. These electrodes are connected to a high-voltage power supply. A sample is introduced at one end of the capillary (inlet), and upon applying an electric field, sample components are separated as they travel through the capillary toward the other end (outlet). At the far end of the capillary, the separated components are sensed by a detector, and output signal is recorded. Since the walls of the capillary have a standing charge, an electroosmotic flow of water is produced from anode to cathode (Figure 3.13). So migration of a positively charged molecule from anode to cathode depends on the applied voltage gradient and electroosmotic flow. Uncharged molecules are separated.

![Schematic representation of capillary electrophoresis apparatus.](image1)

**FIGURE 3.12** Schematic representation of capillary electrophoresis apparatus.

![Separation of sample in the silica capillary.](image2)

**FIGURE 3.13** Separation of sample in the silica capillary.
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on a silica capillary because of the electroendo-osmotic flow. For charged molecules, the apparent rate of migration is the algebraic sum of electrophoretic mobility and electroosmotic flow. Electrophoretic mobility is dependent on the mass/charge ratio.

The silica capillary columns are usually coated to reduce electroosmotic flow, resulting in improved separation. In the presence of electroosmotic flow, charged molecules migrate in an elliptical shape, but when migration is solely by the applied voltage gradient, the molecule front is plug shaped, resulting in a sharp peak.

The use of a buffer at extreme pH (high about pH 10 and low at about 2) results in a decrease in electrostatic adsorption. The silanol group is negatively charged at high pH and is protonated at acidic pH (Figure 3.14). At pH 10, most proteins (except very basic proteins) are negatively charged, and since the capillary wall is also negatively charged, the electrostatic interaction is minimized. Similarly at very low pH (about 2) both capillary wall and proteins are positively charged, resulting in a reduced electrostatic adsorption of proteins onto capillary wall. However, this practice is not popular because of possible denaturation and the loss of biological activities of proteins at extreme pH.

Detection of Protein

In contrast to standard liquid chromatography, where proteins are usually detected at 280 nm (the path length of the absorbance detector is usually 1 cm), detector signal in a capillary electrophoresis system is not satisfactory due to a very short detection path length (25 to 75 µm). Although the absorbance of protein at 200 nm is about 50- to 100-fold greater than that at 280 nm, detection in the low UV region is also not suitable for many applications in capillary electrophoresis. Alternatively, proteins can be detected by the intrinsic fluorescence of their tryptophan and tyrosine residues. However, the detection of intrinsic protein fluorescence requires very costly laser detectors (49). Thus, for capillary electrophoresis, pre- and postcolumn derivatization techniques have been developed to increase detection sensitivity of proteins.

Detection Using Precolumn Derivatization of Proteins

Precolumn derivatization is widely used for analysis of amino acids using a variety of reagents such as phenylisothiocyanate and o-phthalaldehyde, which react with the amino groups of the proteins. There are some inherent problems in derivatization of proteins prior to electrophoresis. In contrast to amino acids, which have one or two reaction sites, proteins can have multiple reactive sites producing multiple derivatization (heterogeneous) products with varying mobilities. This results in broadening of a protein peak. The production of heterogeneous derivatives can be
minimized, to some extent, by using either mild or drastic derivatization conditions. In the former condition, only the most reactive sites will be derivatized, while in the latter condition all possible reactive sites will be labeled.49

**Capillary Coating**

Capillary walls are coated in several ways in order to reduce the non-specific adsorption of protein onto the capillary wall. The capillary is generally deactivated by silanization, and the negatively charged silanol is then modified by a variety of groups such as methyl cellulose, polyacrylamide, polyethylene glycol, etc.

Capillary walls can be coated temporarily during electrophoresis by several buffer additives. High salts such as sulfates and phosphates of about 0.25 M compete with protein for adsorption, resulting in an improved separation. The only problem associated with high ionic strength buffer is the generation of Joule heat, which needs to be dissipated efficiently. Some zwitterionic salts such as betaine, sacrosine, and triglycine are shown to be advantageous up to 1 to 2 M without contributing significant change of conductivity. No single method is suitable for the separation of all types of proteins. Thus, the type of coating changes according to the nature of protein to be separated. For example, for the separation of hydrophobic proteins, non-ionic surfactants such as Tween 20 or Brij 35 are used to reduce the hydrophobicity of the coated capillary column. Similarly for the improved separation of the cationic proteins, the negative charge of the capillary wall is reversed by cationic surfactants such as CTAB.

**REFERENCES**


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