Electrophoresis and Electroblotting of Proteins

The purpose of the next lab exercises will be to study the relative amounts of β-actin in cells of the B-16 melanoma, liver and muscle of mice. Electrophoresis is used to separate proteins in a mixture and study certain properties, such as molecular weight, subunit structure, and expression level. Electrophoresis is frequently coupled with ‘immunoblotting’, in which antibodies are used to analyze even the rarest (least abundant) proteins in a tissue. Linking electrophoresis and immunoblotting is a methodology called ‘electroblotting’, used to prepare proteins separated by electrophoresis for immunoblotting. This week, you will perform the electrophoresis and electroblotting protocols. The principles of Electroblotting are described later in this lab exercise, and the principles of Immunoblotting are described in the next chapter.

After reading the background information, complete all prelab questions and calculations in your lab notebook.

Objectives
The objectives of this lab exercise are for you to:
- learn the theory, practice, and applications of gel electrophoresis, electroblotting and immunoblotting
- understand how gel electrophoresis can be used to determine the molecular weight of proteins.
- Study the levels of actin in different tissues and compare actin levels in melanoma vs normal tissues.

How does gel electrophoresis work?
"Electrophoresis" refers to the use of an electric field to separate proteins in a mixture. Most proteins have a negative ionic charge due to a preponderance of acidic amino acids on the surface. Thus, when placed in an electric field most proteins move toward the positive electrode through electrical attraction (Figure 1).

In “gel electrophoresis”, electrophoresis is performed through a porous, semirigid matrix (or "gel"). Because different types of protein have unique amino acid composition, they have differences in ionic charge which cause the proteins to move toward the anode at different speeds. After they are separated, the proteins will remain immobilized within the gel in their final positions.

Several different types of materials, including starch, agarose and polyacrylamide can be used to form the gel. Each material has its own unique properties and special applications. The material most widely employed for separation of proteins is polyacrylamide. Polyacrylamide gels are perfectly clear, and this facilitates analysis of the results. After the electrophoresis is complete, the positions of the proteins within the gel can be revealed by staining.
What is a polyacrylamide gel?
Polyacrylamide is formed through the polymerization of acrylamide monomer subunits (Figure 2). Acrylamide monomers are polymerized by adding ammonium persulfate and TEMED (tetramethylenediamine), which produce free-radicals that catalyze the polymerization reaction. Acrylamide monomers polymerize into long linear chains. Also included with the acrylamide is a "crosslinker" molecule called bis-acrylamide, which cross-links the polyacrylamide molecules to form a semirigid, porous matrix though which the proteins migrate. Along with the features of polyacrylamide described above, the concentrations of acrylamide and bis-acrylamide can be varied to yield a matrix with a porosity best suited to a particular application.

One disadvantage of polyacrylamide gels is that the unpolymerized acrylamide monomer is a neurotoxin, and must be carefully handled. In the polymerized form, however, polyacrylamide is not toxic.

When performing gel electrophoresis, a Tracking dye, such as bromphenol blue, is added to the samples. This small, negatively charged molecule is used to monitor progress of the electrophoresis. The electrophoresis is stopped when the tracking dye reaches the bottom edge of the gel.

What factors influence the rate at which proteins move through a polyacrylamide gel?
At the end of the electrophoresis, the final positions of the protein are visualized by staining of the gel. A typical result is shown in Figure 3. Different types of proteins form bands as they move through the gel. Why do proteins move through the gel at different rates?

The relative rate of movement depends upon the charge, molecular weight, and shape of the protein. Proteins with a greater negative charge are attracted more strongly toward the positive electrode, and therefore move faster. The molecular weight and shape of the proteins are factors because of the properties of the gel matrix. The gel matrix imposes a "sieving" effect upon the movement of proteins; the larger the protein the slower its movement. But shape also is important: other factors being equal, small globular proteins move more rapidly than large elongated proteins.
How can protein MW be determined using “SDS - polyacrylamide gel electrophoresis” (SDS-PAGE)?

The type of electrophoresis described above is called native protein electrophoresis because the proteins retain their natural (native) 3-dimensional structure as they move through the gel. This type of electrophoresis is very useful for studying the properties of proteins in their natural form. However, generally, native proteins do not separate very well during gel electrophoresis. Furthermore, native gel electrophoresis cannot be used to determine the molecular weight of proteins, because, as we described above, movement through the gel is also influenced by the shape and charge of the protein. However, the molecular weight of proteins can be determined using a procedure known as sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

For SDS-PAGE, the protein sample is first treated with SDS, an ionic detergent which causes the proteins to unfold, or denature. β-mecaptoethanol (βme) is also included to reduce disulfide bonds and cause separation of protein subunits. When a sample is heated in the presence of SDS, the denatured proteins become long linear molecules completely coated with SDS and assume a uniformly negative ionic charge. Under these conditions, charge and shape are no longer a factor, and the rate of movement through the gel is only influenced by the MW of the protein.

Molecular weight of an unknown protein is determined using standard curve of proteins of known molecular weight. The “MW standard proteins” are electrophoresed and their relative mobility (Rf) is calculated. RF is the distance traveled by each protein divided by the distance traveled by the tracking dye. A linear relationship is observed when RF is plotted versus the log of the protein MW (Figure 4). The equation of the trendline can then be used to calculate the MW of another protein.

Discontinuous gel electrophoresis

Discontinuous gel electrophoresis is modification that increasing the sharpness and resolution of the protein bands. The significant feature of this system is the presence of a stacking gel, above the regular resolving gel. The stacking gel has a lower concentration of acrylamide and lower pH than that of the resolving gel (Figure 5). As the proteins move into the stacking gel, the proteins in the sample accumulate, or "stack," into a tight band. When the stacked sample reaches the resolving gel, the proteins begin to migrate a rate proportional to their molecular weight. The result is better separation of the bands than if
Summary of Exercise

WEEK 1
1. Each group will prepare and load samples into a polyacrylamide gel.
2. The electrophoresis will be carried out for approximately 2.5 hours.
3. The class will reconvene later to electroblot the proteins to nitrocellulose.

WEEK 2
1. The membrane segment stored in blocking buffer will be probed with antibody, and the protein band corresponding to the antigenic proteins will be identified.

Running the gel
Record all calculations and sample preparation information in your lab notebook

Supplies (composition of buffers is given below)
5 Eppendorf centrifuge tubes
Sample Denaturation Buffer (SDB)
β-actin standard (APHL95, from Cytoskeleton, Inc)
MW Standards (MWS; SigmaMarkerM-3913 from Sigma-Aldrich)
SDS-polyacrylamide gel (4% acrylamide stacking gel, pH 6.8; 12% acrylamide resolving gel, pH 8.8)

A. Sample Preparation.
All samples to be analyzed will be loaded in a 50μL volume containing SDB. All tissue samples will contain 50μg of protein; the B16 and muscle samples will be provided by the instructor, and you will prepare two samples for your liver homogenate. The instructor will also provide the β-actin standard sample (containing 0.2 μg protein) and the MW standards (these do not have a specified protein content).
1. Label the lids of 5 Eppendorf centrifuge tubes (or 6 if your group is preparing the MW standard sample) with the lane numbers for each sample (See Figure 6).
2. Obtain from the front bench 50μL samples and place them directly into the appropriately numbered tubes:
3. For each of your liver samples, combine the following in the Eppendorf centrifuge tubes:
   A. 15 μL Sample Denaturation Buffer
   B. __ μL of liver homogenate containing 50 μg protein (from Protein Measurement lab).
   C. __ μL of Volume Adjustment Buffer (= the volume needed to have a final 50μL volume) for the sample; i.e., 50 – (A + B)
   D. Mix the samples by gently passing it into and out of the pipet tip.
4. Visually check all tubes to see that they contain the same volume.
5. Heat the samples. All samples except the MWS will be heated
   A. Make sure that the lid is securely snapped on the tubes,
   B. Heat all but the MWS at 95°C for 3 minutes.
   C. Allow the tube to cool in your test tube rack; do not store on ice.
B. Prepare the sample wells of the gel.
1. Slowly and carefully remove the teflon sample well comb from the top of the gel.
2. Rinse the inside of the sample wells by squirting reservoir buffer into each with a 5cc syringe, and sucking the wells dry again. Be careful not to damage the bottoms or the side partitions of the wells.
3. Fill each well that will receive a protein sample approximately ½ full with reservoir buffer; fill the other sample wells fully with reservoir buffer.

C. Load the samples onto the gel. See Figure 6.
1. Load each sample to the appropriate sample well using a P-200 Pipetman. Discharge the samples slowly near the bottom of the well; the high density of the sample will cause it to settle at the bottom of the well.
2. After all of the samples have been added, carefully add enough reservoir buffer to the top of the gel to completely fill all of the sample wells. Be sure not to disturb the samples in the wells when doing this.
3. Assemble the gel apparatus, add 500 ml Reservoir Buffer to the upper and lower electrode reservoirs, and add the safety cover to the apparatus. These steps will be demonstrated during the lab period.

D. Running the gel
1. Attach the electrode wires to the electrophoresis power supply: RED = ANODE, BLACK = CATHODE.
2. We will run the gels at 30 mA per gel until the tracking dye has just reached the bottom of the gel (about 3.5 hours).

**Caution**
THE CURRENT RUNNING AN ACRYLAMIDE GEL IS ENOUGH TO KILL!! NEVER HANDLE A GEL APPARATUS WHILE THE CURRENT IS TURNED ON!!
How are proteins in a gel electroblotted to nitrocellulose?

In next week's lab exercise we are going to perform the Immunoblotting (also called “Western blotting”) procedure to identify which band in our homogenates is the protein B-actin. To do this, we must "probe" the electrophoretically resolved sample with antibodies that bind specifically and exclusively with the β-actin protein. It is not possible to probe polyacrylamide gels directly with antibodies because the gel matrix blocks access to the proteins embedded within it. Instead, the proteins in the gel are transferred to a thin sheet (called a membrane) of nitrocellulose, in such a way as to preserve the original banding pattern. The transfer process is called electroblotting, and is performed by again subjecting the polyacrylamide gel to an electric field. This time the gel is applied flat upon a piece of the nitrocellulose membrane, and an electric field is applied that drives the proteins from gel onto the membrane. Nitrocellulose has the property of tenaciously binding proteins. When the proteins move from the gel, they bind to the nitrocellulose membrane, preserving the original banding pattern. The arrangement of the electroblotting apparatus is shown in Figure 7.

**Supplies**

- 2 sheets of blotting paper (12 x 11 cm)
- 1 sheet of nitrocellulose (12 x 11 cm)
- Electroblotting apparatus *(Hoeffer, TE50x)*
- Amido black stain and destain
- 2 plastic trays
- Razor blade
- Transfer buffer *(you prepared)*
- Blocking buffer

**Electroblotting procedure**

Details of assembling the electroblotting system will be demonstrated in lab. Some key steps are described below.

1. **Before beginning measure the distance traveled (in mm) by the tracking dye from the top of the resolving gel and record this value in your lab notebook.**
2. After disassembling the gel, sliced the gel through lanes 6 and 10, yielding three sections. Discard the stacking gel.
3. Notch the upper left-hand corner of each of the gel sections to mark the gel orientation.
4. The method for assembling the electroblotting apparatus will be demonstrated at this time.
5. Electroblotting will be performed for about 40 minutes using the electroblotting apparatus.
6. After the electroblotting is completed, carefully cut the membrane so that it corresponds in size exactly with the gel sections, then carefully remove the gel and dispose of it.
7. Notch the upper left hand corner of each section of membrane.
8. Stain with amido black or place in blocking buffer the appropriate sections.
**Staining membranes for total protein**

1. Label a plastic tray with your names and "amido black stained".
2. Place the membrane containing lanes 3 - 6 into a plastic tray.
3. Cover with amido black stain, and swirl for about 30 seconds.
4. Pour off the stain solution, and wash the membrane repeatedly with destain solution until the background is reasonably clear.
5. An image of the membrane will be posted for you to use to determine the MW standard curve.

**“Blocking” membranes to be immunoblotted**

This is the first step in the immunoblotting procedure; and the need for this step is described in the Immunoblotting lab exercise.

1. Label a plastic tray with your names and "to be probed".
2. Place the appropriate section of the membrane, in a plastic container, cover with 25 ml of blocking buffer, and seal the lid.
3. Place the container in the refrigerator.

**Preparing the MW standard curve.**

Table 1 contains information about the molecular weight standard proteins. Remember, the largest proteins are near the top of the gel.

1. Transfer Table 1 to your lab notebook, and record there the data and calculations.
2. Using a ruler, measure the distance (mm) each protein moved from the top of the membrane.
3. Calculate the Rf (relative mobility) of the molecular weight standards by dividing the distance each traveled by the distance traveled by the tracking dye. Record the values in the tables.

\[
\text{Rf} = \frac{\text{mm traveled by protein}}{\text{mm traveled by tracking dye}}
\]

4. Plot Rf vs log molecular weight using Excel. Rf should extend from 0 to 1.0 on the y-axis, and molecular weight should be plotted on the x-axis. Fit a trendline to the data, and record the equation. A properly formatted Figure of the standard curve will be included in the lab report.
Table 1. Molecular weight standards resolved by SDS-PAGE. Write this table and complete in your lab notebook.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight</th>
<th>Log (MW)</th>
<th>Distance migrated (mm)</th>
<th>Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>tracking dye</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-lactalbumin</td>
<td>14,200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>trypsin inhibitor</td>
<td>20,100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>trypsinogen</td>
<td>24,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>carbonic anhydrase</td>
<td>29,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glyceraldehyde-3-P-dehydrogenase</td>
<td>36,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ovalbumin</td>
<td>45,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bovine serum albumin</td>
<td>66,000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Place complete table in lab notebook

Equation of the trendline: ______________________________

Solution Composition

**Reservoir Buffer**
- Trizma-base: 0.025 M
- Glycine: 0.192 M
- SDS: 0.1% (w/v)

**Volume Adjustment Buffer**
- Trizma-Base: 0.125 M
- SDS: 0.1% w/v
- pH 6.8

**Sample Denaturation Buffer (4X)**
- Trizma-HCl, pH 6.8: 200 mM
- SDS: 2% (w/v)
- Glycerol: 10% (v/v)
- Brome phenol blue: 0.02%
- β-mercaptoethanol: 4 mM

**Transfer Buffer**
- Trizma-base: 0.025 M
- Glycine: 0.192 M
- Methanol: 20% (v/v)

**Nitrocellulose Blocking Buffer**
- Tris-HCl, pH 7.5: 10 mM
- NaCl: 150 mM
- Tween-20: 0.05% (v/v)
- NFDM*: 5% (w/v)
- (*non-fat dried milk)

**Amido Black stain**
- Amido black: 0.1% (w/v)
- Methanol: 45% (v/v)
- Acetic acid: 10% (v/v)
- **Destain** – same as above, less amido black