A: Cellulose Acetate Hemoglobin Alkaline Electrophoresis

Summary
This electrophoretic method is commonly utilized for hemoglobin (Hb) separation. Electrophoresis is typically performed at pH 8.6 using cellulose acetate as the support medium. At this pH, the overall hemoglobin molecule is negatively charged and when placed in an electric field, will move towards the positive terminal (anode). This procedure is based on the fact that if an amino acid substitution alters the overall charge of the molecule, then the mobility of the variant hemoglobin will be different from that of Hb A. Although this method is extremely useful, it has limitations in that many hemoglobin variants will migrate in the same position.

For example, Hb’s S, D, G, and Lepore all migrate in the same position (S position). Hb’s C, E, and O-Arab all migrate as seen in figure 1?

Figure 1

Principle
The specimen is applied to a cellulose acetate plate that has been presoaked in a tris-barbital buffer at pH 8.8. The lipoprotein fractions are separated by electrophoresis and then stained with a methanol solution of Fat Red 7B at an alkaline pH. The stained bands may be visually inspected for qualitative results or may be quantitated in a scanning densitometer using a 525 nm filter.

Reagents
Tris-barbital-Sodium Barbital buffer
Lipoprotein Stain: Fat Red 7B in dimethylformamide (25ul 5 mg/liter activated with 5ul NaOH, 0.1 mol/liter and one drop of Triton X-100 after dissolution in methanol)

Sample
Serum should be collected no later than one week prior to testing. The use of plasma should be avoided, as fibrinogen will appear as a distinct narrow band between the beta and gamma fractions. Cerebrospinal fluid may be used if concentrated approximately 100 times; urine may be used if concentrated up to 300 times, depending on original protein concentration.

Note - Hemolysis may cause false elevation in the alpha2 and beta fractions.
Procedure

Membrane Preparation

1. Cut the cellulose acetate membrane to the desired size, and mark the sample location with a straight line with a pencil. This will correspond to the origin for application of the samples.
2. Float the cellulose acetate membrane on the Tris-barbital-Sodium Barbital buffer to allow the membrane to absorb the buffer into the pores by capillary action. Never immerse the membrane as air will be trapped within the pores, disrupting electrical conduction. Soak the membrane for 10 minutes.
3. With gloved hands, remove the membrane from the liquid using tweezers and blot gently with filter a kim wipe.

Electrophoresis Chamber Preparation

1. Place the tank in a suitable position on a level bench surface next to an appropriate power supply. Hoefer recommends the PS300B power supply unit.
2. Move the adjustable bridge pieces to the required positions on either side of the fixed central partition. Serrations beneath the ends of each adjustable bridge piece will locate into those on the tank sides, making further clamping unnecessary. The adjustable bridge pieces should now be parallel to the central partition.
3. Slot the removable partitions into the partition guides positioned parallel to the central partition.
4. Add 250-ml Tris-barbital-Sodium Barbital buffer to each chamber on either side of the central partition. Smaller volumes may be used, provided the electrodes are covered to ensure complete electrical contact.
5. Because the cellulose acetate membrane should not be allowed to come into direct contact with the buffer, cut out two filter paper wicks (e.g. 20 x 10 cm) to maintain electrical contact between the membrane and the tank. Immerse the wicks in buffer solution.
6. Place one wick onto each adjustable bridge piece, having removed the clamping bar. The longer edge of the paper should meet the side of the adjustable bridge piece facing the central partition, while the lower edge dips into the buffer compartment. It is advisable to change the filter wicks with each electrophoresis run.

Sample Application

1. Spot the samples onto the strip with a micropipette or precision bore glass capillary tube.
2. Replace the lid and switch on the power supply. A constant current setting, corresponding to a potential drop of 50 to 80 Volts across the membrane, is advisable to prevent the excessive build-up of heat. Separations normally take 1½ to 3 hours.
3. At the end of the separation, remove the membrane and either dry in an oven at 80 to 100 ºC or fix in a fixing bath accordingly. The membrane should now be ready for immersion in a suitable stain for visualization, or second-dimension electrophoresis, according to the user’s requirements.

Staining and Evaluation of Lipoprotein Bands

Prepare the staining solution approximately 5 minutes before the end of the electrophoresis time.
1. Add 30 mL of stain to a shallow staining dish. Pipette 10 mL of 1.0 N sodium hydroxide into the stain while swirling the solution.
2. Remove the membrane from the CE-30 chamber at the end of the electrophoresis period, blot gently with a kim wipe and immerse the membrane with the cellulose acetate side up, in the staining solution.
3. Stain the membrane for 15 to 25 minutes.
B: Serum Protein Electrophoresis
For the separation and quantitation of serum proteins using cellulose acetate electrophoresis.

Summary
Serum contains over one hundred individual proteins, each with a specific set of functions and subject to specific variation in concentration under different pathologic conditions. Kaplan and Savay’s use of zone electrophoresis, serum proteins have been fractionated on the basis of their electrical charge into five classical fractions: albumin, alpha1, alpha2, beta, and gamma proteins. Each of these classical electrophoretic zones (with the exception of albumin) normally contains two or more components. Approximately fifteen serum proteins have been studied extensively because they are easily measured.

Principle
Proteins are large molecules composed of covalently linked amino acids. Depending on electron distributions resulting from covalent or ionic bonding of structural subgroups, proteins have different electrical charges at a given pH. In the Serum Protein procedure, the proteins are separated according to their respective electrical charges at pH 8.8 on a cellulose acetate plate using both the electrophoretic and electroendosmotic forces present in the system. After the proteins are separated, the plate is placed in a solution of sulfosalicylic acid and Ponceau S (to stain the protein bands). The staining intensity is related to protein concentration. After dehydration in methanol, the plate background is then rendered transparent by treatment with a de-staining solution.

Reagents
Ponceau S Stain
Tris-barbital-Sodium Barbital buffer
Polyethylene glycol

Additional Instruments Needed
Scanning densitometer with visible transmittance capability may be used.

Procedure
Please follow the same procedures for Section A: Cellulose Acetate Hemoglobin Alkaline Electrophoresis. However, staining protocol is below.

Staining the Protein Bands
1. At the end of the electrophoresis time, remove the membrane(s) from the chamber. Place them in 40-50 mL of Ponceau S stain (sufficient volume to cover the membrane(s)) for 6 minutes. When staining 2 or more plates, carry out the protocol vertically in a rack. The stain may be reused until the plate background contains stain precipitate.
2. Destain in 3 successive 2 minute washes of 5% acetic acid or until the background is white.
3. Migrations patterns should be compared to a known control.

For quantitative determination, use densitometry or elute separated fractions. When using Ponceau S Solution, the densitometry scan should be undertaken at 525 nm.

Reference Values
The reference values for serum protein electrophoresis on cellulose acetate stained with Ponceau S were determined from a study of 51 normal subjects. These values are for illustrative purposes only. Each laboratory should establish its own range.

Protein Fraction Concentration:
- Albumin - 3.63 - 4.91 g/dL
- Alpha1 - 0.11 - 0.35 g/dL
- Alpha2 - 0.65 - 1.17 g/dL
- Beta - 0.74 - 1.26 g/dL
- Gamma - 0.58 - 1.74 g/dL
C: Agarose and polyacrylamide gels

Use the adjustable bridge pieces and removable partitions, set to the required distance to support horizontal polyacrylamide and agarose gels. Filter paper wicks establish contact between the gel and each buffer compartment. Electrophoresis can then be performed according to the user’s desired current, voltage and power settings.

Procedure

1. Take a few minutes to become familiar with the system components. Notice the buffer chamber is already filled with barbitone buffer (pH 8.6).
2. Soak the acetate membrane in buffer for 10 min by floating it on the buffer. Handle the membrane with forceps or gloved hands ONLY! Be careful not to bend it, as it will break.
3. Using forceps remove the membrane from the buffer and blot off excess fluid by placing between 2 paper towels.
4. Mount the strip on the bridge, making sure the spring-loaded clip is locked in the up position and the strip is centered. Lock the strip retainers and carefully release tension (be careful not to rip the strip). The strip should be flat (no ripples).
5. Place the loaded bridge assembly in the chamber and push both ends of the strip down into the buffer. The strip must bend into the buffer on both ends in order to complete the circuit. Place the cover on the chamber.
6. Fill sample wells with serum and plasma as instructed (plasma in odd wells [1,3,5,7], serum in even wells [2,4,6,8]). Be sure to fill the wells completely otherwise it will evaporate before use.
7. Place the sample applicator over the sample wells (it should settle into 3 depressions), depress the applicator into the wells, and HOLD for 15 sec.
8. Transfer the applicator to the chamber lid (it should settle into 3 depressions) and depress the applicator for 15 sec. If properly aligned, you should see 8 yellow rectangles on the strip.
9. Remove the applicator and connect the power supply to the chamber. Adjust the power to 225 volts and set the timer for 20 min.
10. Wash the applicator as instructed by the Professor. Do not attempt to clean it until after the demonstration.
11. When complete, turn off the power, disconnect the unit, remove the chamber lid, and lift out the bridge assembly. Lock the spring-loaded retaining clip in the up position.
12. Release the retaining clips, use forceps to remove the strip from the bridge, and float the strip on top of the stain for 10 min. The stain contains 7.5% trichloroacetic acid (TCA), which denatures the proteins, binding them to the strip. The red stain is Ponceau S, which reacts with peptide bonds. The color intensity is directly proportional to the mass of the protein in each band.
13. Using forceps, remove the strip from the stain and gently shake off excess stain over paper towels.
14. To remove the unbound stain float the strip on dilute (5%) acetic acid in wash tray #1, gently agitating the tray for 5 min. Remove, blot, and transfer to wash tray #2. Repeat with wash tray #3. At this point, no dye should elute from the membrane (5 min).
D: Two-dimensional (2-D) Electrophoresis of Urinary Glycosaminoglycans - a Diagnostic Indicator of Mucopolysaccharide Disorders

**Buffer 1:** Pyridine: Acetic Acid: Distilled Water (10:1:89 v/v)
**Buffer 2:** 0.1M Barium Acetate, pH 6.0
**Stain:** Alcian Blue
**Destain:** 5% Acetic Acid (v/v)

1. Spot 2x 1µl aliquots of extracted urine sample, stained with Alcian Blue, onto a 78 x 75mm cellulose acetate membrane, previously soaked and electrophoresed in Buffer 1 for 10 minutes at 55V (7.5V/cm).
2. Run the membrane at 55V for a further 75 minutes in Buffer 1.
3. Remove the cellulose acetate membrane from the CE-30 Cellulose Acetate System allowing it to dry in a fume hood for 1 hour.
4. Float the cellulose acetate membrane face down for 5 seconds in Buffer 2, before rotating it at 90º to its original orientation and then spotting a 0.5µl aliquot of MPS Type III urine as a positive control.
5. Dry and then run the cellulose acetate membrane at 55V for 3 1/2 hours.
6. Stain the cellulose acetate membrane face down for 15 minutes, before rinsing it in water and standing it overnight in de-stain.
7. After de-staining, rinse the cellulose acetate membrane with water and dry it flat between two sheets of filter paper.

**Diagnostic Analysis of Mucopolysaccharide Disorders with the CE-30 unit.**

Extracted urine sample was applied to a 78 x 75mm cellulose acetate membrane, electrophoresed at 55V for 75' in the first dimension, and then electrophoresed in the second dimension for a further 3½ hours at same voltage as described in the outlined protocol. *(Courtesy of the NHS)*