The common hemoglobin abnormalities:

Sickle Cell

- This is a heterogeneous state showing HbA and HbS and a small amount of HbF may also be present.

C Disease

- This is a heterogeneous state demonstrating HbS and HbC.

Sickle-Cell-Thalassemia Disease

- This condition shows HbA, HbF, and HbS. In Sickle Cell-Thalassemia HbA is absent.

Thalassemia Disease

- This condition shows HbA, HbF and HbC.

Disease

- This is a heterogeneous state showing almost exclusively HbS.

Thalassemia Major

- This condition shows HbF, HbA, and HbC.

REFERENCES

11. Personal communication from Dr. Virgil Fantasia.

For Sales and Order Information, and Technical Service, call 1-800-233-5672 toll free.

Hemoglobin Electrophoresis Procedure

Hemoglobin electrophoresis is generally considered the best method for separating and identifying hemoglobinopathies. One protocol for hemoglobin electrophoresis involves the use of two systems. Initial electrophoresis is performed in alkaline buffer. Sickle cell anemia is the major support medium used because it provides a rapid separation of HbA, S and C and many other mutants with minimal preparation time. However, because of the electrophoretic similarity of many structurally different hemoglobins, the evaluation must be supplemented by a procedure that measures some other property. A simple method which confirms the identification of both HbS and HbC, as well as HbA, HFP and other mutant hemoglobins, is reported here. The method is based on the interaction of the hemoglobin with the electrophoretic buffer (acid pH) and the agar support. Electrophoresis is a simple procedure requiring only minute quantities of hemolyte to provide highly specific (but not absolute) confirmation of the presence of HbS, HbF and HbC as well as several other abnormal hemoglobins.

**PRINCIPLE**

Very small samples of hemolytes prepared from whole blood are applied to the Titin® Carbohydrate Acetate Plate. The hemoglobin in the sample are separated by electrophoresis and stained using an alkali buffer (pH 8.2-8.6), and are stained with PermaClear. The patterns are scanned on a scanning densitometer and the relative percent of each band determined.

**REAGENTS**

1. **Supra-Hem** Buffer (Cat. No. 5902)

**Ingredients:**

- The buffer contains Tris-EDTA and boric acid.

**WARNING:** FOR IN-VITRO DIAGNOSTIC USE ONLY. NEVER PIPETTE BY MOUTH. Do NOT INGEST. Ingestion of sufficient quantities of boric acid and EDTA can be toxic.

**Preparation for Use:**

- Dissolve one package of buffer in 980 mL deionized water.
- Store the prepared clearing solution at 15 to 30°C and is stable until the expiration date indicated on the package and box.
- The buffer solution is stable two months when stored at 15 to 30°C.

**Signs of Deterioration:**

- Do not use packaged buffer if the material shows discolouration or decomposition. Discard the solution if it shows signs of bacterial contamination.

2. **Hemolyte Reagent (Cat. No. 5129)**

**Ingredients:**

- The reagent contains 0.015 M EDTA in deionized water with 0.07% potassium cyanide added as a preservative.

**WARNING:** FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT PIPETTE BY MOUTH. The reagent contains a small amount of potassium cyanide.

**Preparation for Use:**

- The reagent is ready to use as packaged.

**Storage and Stability:**

- The reagent should be stored at 15 to 30°C and is stable until the expiration date indicated on the bottle.

**Signs of Deterioration:**

- The reagent should be clear and colorless.

3. **Ponceau S Stain (Cat. No. 5526)**

**Ingredients:**

- The reagent contains 0.5% (w/v) Ponceau S in an aqueous solution of 10% (w/v) sulfoisoyl acid.

**WARNING:** FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT INGEST. HARMFUL IF SWALLOWED.

**Preparation for Use:**

- Dissolve one vial of stain in 1 L of deionized water.

**Storage and Stability:**

- The solution should be stored at 15 to 30°C and is stable until the expiration date indicated on the bottle. It may be stored in the bottle or in a tightly closed staining dish and may be reused multiple times if properly protected.

**Signs of Deterioration:**

- Do not use the solution stain if excessive evaporation occurs or if large amounts of precipitate appear.

4. **Clear Aid (Cat. No. 5509)**

**Ingredients:**

- The reagent contains polyethylene glycol.

**WARNING:** FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT INGEST.

**Preparation for Use:**

- Clear Aid is used as the clearing solution which is prepared as follows:

  - 30 parts glycerol acetic acid
  - 70 parts absolute methanol
  - 4 parts Clear Aid

**Storage and Stability:**

- Store the prepared clearing solution at 15 to 30°C in a tightly closed container to prevent evaporation of the methanol. When evaporation occurs, the plates may delaminate. Water contamination from over-use of the clearing solution will cause the plate to cloud. The reagent is stable until the expiration date indicated on the bottle.

**Signs of Deterioration:**

- Clear Aid should be a clear, colorless liquid, although it may appear cloudy when cold. Do not use the material upon evidence of gross contamination or discoloration. Discard the prepared Clear Aid if plates appear cloudy after the clearing procedure.

5. **PermaClear Solution (Cat. No. 4950)**

**Ingredients:**

- Polyethylene glycol and PEG.

**WARNING:** FOR IN-VITRO DIAGNOSTIC USE - IRRITANT - DO NOT PIPETTE BY MOUTH. VAPOR HARMFUL.

**Preparation for Use:**

- Add 55 mL PermaClear to 45 mL deionized water.

**Storage and Stability:**

- PermaClear should be stored at 15 to 30°C and is stable until the expiration date indicated on the bottle.

**Signs of Deterioration:**

- Discard the PermaClear Solution if the plates turn white and do not clear as expected.

6. **Titin® Plates (Cat. No. 3012, 3022)**

**Ingredients:**

- Cellulose acetate plates.

**WARNING:** FOR IN-VITRO DIAGNOSTIC USE.

**Preparation for Use:**

- The plates are ready for use as packaged.

**Storage and Stability:**

- The plates should be stored at 15 to 30°C and are stable indefinitely.
The following materials needed for the procedure are:

- Supre-Heme Buffer
- Clear Aid 5005
- III-H Cellulose Acetate
- Hemo AA Control
- Hemo AFSC Control
- Hemo ASA Control
- Hemo AFSC
- Hemo ASA
- Hemo AFSA
- Titan
- Consumables
- Super Z Applicator Kit (8 samples)
- Quickscan 2000
- Helena Glacial acetic acid
- Materials Needed, but not Provided:
  - Cellulose Acetate (76 mm x 60 mm)-8 samples
  - III-H Cellulose Acetate (94 mm x 76 mm)-12 samples
  - Chamber Wicks
  - Prep
  - Prep 5090
  - Preparative Cellulose Acetate (76 mm x 76 mm)-12 samples
  - PermaClear

PROCEDURE

Materials Provided: The following materials needed for the procedure are available from Helena Laboratories.

Hardware

<table>
<thead>
<tr>
<th>Cat. No.</th>
<th>Supr-Z-12 Applicator Kit (12 samples)</th>
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<td></td>
<td>Supr-Z Applicator Kit (8 samples)</td>
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<tr>
<td></td>
<td>Microdispenser and Tubes</td>
<td>6008</td>
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<tr>
<td></td>
<td>1000 Staining Set</td>
<td>5122</td>
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<td>Bufferer</td>
<td>Bufferer</td>
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<tr>
<td></td>
<td>Titan Plus Power Supply</td>
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Summary of Conditions

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<th>Condition</th>
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<td>Titan II-H Cellulose Acetate (84 mm x 76 mm)-12 samples</td>
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</tr>
<tr>
<td>Titan II-H Cellulose Acetate (76 mm x 60 mm)-8 samples</td>
<td>3022</td>
</tr>
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<td>Supre-Heme Buffer</td>
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<td>Hemo AFSC</td>
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<td>Clear Aid</td>
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<td>Identification Labels</td>
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<td>Zone III Chamber Wicks</td>
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<tr>
<td>Glue Stick</td>
<td>5002</td>
</tr>
<tr>
<td>PermaClear</td>
<td>9550</td>
</tr>
</tbody>
</table>

Materials Needed, but not Provided:

- Glacial acetic acid
- Absolute methanol
- 5% acetic acid – Mix 5 parts of glacial acetic acid with 95 parts deionized water

1. Dissolve one package Supre-Heme Buffer in 980 mL deionized water.

2. Properly code the required number of Titan II-H Plates by propery con the glossy hard side with a marker.

3. Soak the required number of plates in Supre-Heme Buffer for 5 minutes.

4. The plates should be soaked in the buffer according to the instructions provided. Alternately, the plates may be wetted by slowly and uniformly lowering a rack of plates into the buffer.

5. Remove the wetted Titan III Plate from the buffer with the fingerpicks and blot ones firmly between two blotters. Place the plate in the aliquoting base, cellulose acetate side up, aligning the top edge of the plate with the black slot in the frame.

6. Apply the sample to the plate with depressurizing the applicator tips into the sample well 4 times and promptly transferring the applicator to the aligning base. Press the button down and hold it 5 seconds.

Alternate Sample Preparation Procedure:

if removal of denaturated hemoglobins from the sample is deemed necessary, perform the following steps:

a. Centrifuge the blood sample at 3500 RPM for 5 minutes.

b. Remove the plasma from the sample and wash the red blood cells in 0.85% saline (+/−) three times. After each wash, centrifuge the cells for 10 minutes at 3500 RPM.

c. Add 1 volume deionized water and 1/4 volume toulene (or carbon tetrachloride) to the washed red cells. Vertax high for one minute. Centrifuge the sample at 3500 RPM for 10 minutes.

d. If toulene is used, the top layer in the tube will contain cell stroma and should be removed with a capillary pipette before proceeding to the next step. The clear middle layer contains the desired sample. If carbon tetrachloride is used, all red cell waste material will be contained in the bottom of the tube after centrifugation.

7. Staining the Hemoglobin Bands

a. Place the plate, side by side, on a water bath at 3500 RPM for 5 minutes.

b. Destain in 3 successive washes of 5% acetic acid. Allow the plates to stay in each wash 2 minutes or until the background is white.

c. The plates may be dried and stored for a permanent record at this point. If a transparent background is desired for densitometry, proceed to the next step.

If using Clear Aid Solution:

4. Drain off excess solution. Then place the plate, sample end is toward the cathodic(-) side of the chamber. Place a weight (glass slide, etc.) on the plate to inscruect the wicks.

5. Place the plate(s) into the chamber, and electrophoresis the plate for 25 minutes at 350 V.

Staining the Hemoglobin Bands

1. Remove the plates from the electrophoresis chamber and stain in Poncaue 5 for 5 minutes.

2. Destain in 3 successive washes of 5% acetic acid. Allow the plates to stay in each wash 2 minutes or until the background is white.

3. The plates may be dried and stored for a permanent record at this point. If a transparent background is desired for densitometry, proceed to the next step.

If using Clear Aid Solution:

4. Drain off excess solution. Then place the plate, sample end is toward the cathodic(-) side of the chamber. Place a weight (glass slide, etc.) on the plate to inscruect the wicks.

5. Place the plate(s) into the diluted PermaClear clearing solution for 2 minutes.

6. Drain off excess solution by holding plate(s) vertically for 1 minute. Then place the plate, acetic side up, onto a blotter, and into an I. O. D., or other µL of the Hemo Controls into the wells of the Sample Well Plates using the Microdispenser. Do not prepare a hemolyzed of the Hemo Controls.

3. To prevent evaporation, cover the Sample Well Plate Blotter Pads, if the samples are not used within 2 minutes.

4. Prime the applicator by depressing the tips into the sample well(s) 4 times. Apply this loading to a piece of blotter paper. Priming the applicator makes the second loading much more uniform. Do not load applicator again at this point, but proceed quickly to the next step.

5. Remove the wetted Titan III Plate from the buffer with the fingerpicks and blot ones firmly between two blotters. Place the plate in the aliquoting base, cellulose acetate side up, aligning the top edge of the plate with the black slot in the frame.

APPLICATION

1. Place a line of droplet solution in the center of the chamber.

2. Place the plate(s) into the diluted PermaClear clearing solution for 2 minutes.

3. Drain off excess solution by holding plate(s) vertically for 1 minute. Then place the plate, acetic side up, onto a blotter, and into an I. O. D., or other

4. Place the plate(s) into the distilled PermaClear clearing solution for 2 minutes.

5. Drain off excess solution by holding plate(s) vertically for 1 minute. Then place the plate, acetic side up, onto a blotter, and into an I. O. D., or other

6. Place the sample to the plate by depressing the applicator tips into the sample well 4 times and promptly transferring the applicator to the aligning base. Press the button down and hold it 5 seconds.

Stability of End Product: The dried plates are stable for an indefinite period of time, and may be stored in Titan Plastic Envelopes.

Calculation: A calibration curve is not necessary because relative concentration of the bands is the only parameter determined.

Quality Control: Four different types of globin electromorphs are available from Helena Laboratories: AA, Hemo Control (Cat. No. 5328), AS, Hemo Control (Cat. No. 5329), AS, Hemo Control (Cat. No. 5330), and AS, Hemo Control (Cat. No. 5331). The controls should be used as markers for the identification of the hemoglobin bands, and they may be used for verification of the accuracy of the procedure. Refer to the package insert provided with the controls for assay values and migration patterns. Use at least one of these controls on each plate run.

RESULTS

Figure 1 illustrates how the combination of cellulose acetate and citrate agar electrophoresis can be used in tandem for the identification of hemoglobin types.

Figure 2 lists the relative mobilities of various hemoglobin mutants on cellulose acetate and citrate agar plates.

Calculation of Unknown: The Helena Quickscan 2000 will automatically print the relative percent and absolute values for each band. Alternatively, the relative percent of each band can be calculated manually by referring to the Operator’s Manual provided with the densitometer. The relative percent of each band is calculated by the following formula:

\[
\text{Relative Percent} = \frac{\text{Density of the Band}}{\text{Density of the Reference Band}} \times 100
\]

LIMITATIONS

Some abnormal hemoglobins have similar electrophoretic mobilities and must be differentiated by other methodologies. Further testing required:

1. Citrate agar electrophoresis may be a necessary follow-up test for confirmation of abnormal hemoglobin detected on cellulose acetate.

2. Isoelectric focusing, high performance liquid chromatography, globin chain analysis (both acid and alkaline) and structural studies may be necessary in order to positively identify some of the more rare hemoglobin.

Some low levels of HbF (1-10%) may be accurately quantitated using any commercially available HbF method.

Figure 1. Electrophoretic Mobilities of Hemoglobins on Titan II-H Cellulose Acetate and on Titan IV Citrate Agar
The following materials needed for the procedure are such that the QuickScan 2000 will automatically print the.

**Materials Needed, but not Provided:**
- Glue Stick 5002
- Helena Marker 5000
- Titan Plastic Envelopes 5052
- Hemolysate Reagent 5125
- Hemo AFSC Control 5331
- Hemo AA Control 5329
- Hemo AFSC Control 5331
- Hemo ASA Control 5329
- Hemolysate Reagent 5125
- Ponceau S 5256
- Clear Aid 5005
- Titan Blister Pads 5034
- Zip Zone® Prep 5090
- Titan Plastic Envelopes 5052
- Helena Marker 5000
- Identification Labels 5006
- Zip Zone® Chamber Wicks 5081
- Glue Stick 5002
- PermaClear 4950

**PROCEDURE**

**Materials Provided:** The following materials needed for the procedure are available from Helena Laboratories.

**Hardware**
- Super Z-1 Blotter Kit (2 samples) 4093
- Super Z-2 Applicator Kit (8 samples) 4088
- Microdispenser and Tubes 6008
- 1009 Staining Set 5122
- Buffer 5093
- Titan Plus Power Supply 1504

**Consumables**
- Titan® III-H Cellulose Acetate (96 µm x 76 µm)-12 samples 3021
- Titan® Cellulose Acetate (76 µm x 60 µm)-8 samples 3022
- Supre-Heme Buffer 5330
- Hemo ASA Control 5329
- Hemo AFSC Control 5331
- Hemo ASA Control 5329
- Hemolysate Reagent 5125
- Ponceau S 5256
- Clear Aid 5005
- Titan Blister Pads 5034
- Zip Zone® Prep 5090
- Ponceau S 5052
- Hemolysate Reagent 5125
- Identification Labels 5006
- Zip Zone® Chamber Wicks 5081
- Glue Stick 5002
- PermaClear 4950

**Materials Needed, but not Provided:**
- Glacial acetic acid (absolute methanol)
- 5% acetic acid – Mix 5 parts of glacial acetic acid with 95 parts deionized water.

**SUMMARY OF CONDITIONS**

<table>
<thead>
<tr>
<th>Plate</th>
<th>Titan® III-H Buffer</th>
<th>Supre-Heme® dissolved in 980 mL deionized water</th>
<th>Soaking Temperature for Plates</th>
<th>5 minutes</th>
<th>Sample Size (hemolysate)</th>
<th>5 µL</th>
<th>Number of Applications</th>
<th>One (1)</th>
<th>Voltage</th>
<th>25 minutes</th>
<th>Station Time (total)</th>
<th>20 minutes</th>
<th>Drying Time</th>
<th>10 minutes at 50°C</th>
<th>Scanning Wavelength</th>
<th>525 nm</th>
</tr>
</thead>
</table>

**STEP BY STEP METHOD**

1. **Preparation of the Titan® III-Plate**
   - Dissolve one package Supre-Heme® Buffer in 980 mL deionized water.
   - Pour approximately 100 mL of Supre-Heme® Buffer into each of the smaller sections of the Zip Zone® Chamber. Do not load the applicator again at this point, but instead do a gentle loading of the applicator to keep thestyling.
   - Place the plate in the plate holder, cellulose acetate side up, aligning the top edge of the plate with the black stripe on the micro-dialysis cathode.
2. **Application of the Sample**
   - Load the sample into the applicator, and then press the applicator tip into the sample well 3 or 4 times and promptly transferring the applicator to the aligning base. Do not load the applicator again at this point, but instead do a gentle loading of the applicator to keep thestyling.
   - Do the same thing for all the samples.
   - Place the plate into the diluting chamber using a pipette to load the plate.
   - Place the plate, aqueous side up, onto a blotter and into an I. O. D., Micro-Hood, or other drying oven at 50-60°C for 15 minutes or until dry.

**RESULTS**

**Quality Control:** Four internal standards of electrophoresis are available from Helena Laboratories: AA, Hemo Control (Cat. No. 5328), ASA, Hemo Control (Cat. No. 5329), AFSA, Hemo Control (Cat. No. 5330) and AFSIC Hemo Control (Cat. No. 5331). The controls should be used as markers for the identification of the hemoglobin bands, and they may be used for verification of the accuracy of the procedure. Refer to the package insert provided with the controls for assay values and migration patterns. Use at least one of these controls on each plate run.

**LIMITATIONS**

Some abnormal hemoglobin bands have similar electrophoretic mobilities and may be differentiated by other methodologies.

**Further testing required:**
- C.ite agar electrophoresis may be a necessary follow-up test for confirmation of abnormal hemoglobin electrophoresis.
- Acidic and alkaline performance chromatography, globin chain analysis (both acid and alkaline) and structural studies may be necessary in order to positively identify some of the more rare hemoglobin bands.

**Stability of End Product:** The dried plates are stable for an indefinite period of time, and may be stored in Titan Plastic Envelopes.

**Calibration:** A calibration curve is not necessary because relative concentration of the bands is the only parameter determined.

**Quality Control:** Four internal standards of electrophoresis are available from Helena Laboratories: AA, Hemo Control (Cat. No. 5328), ASA, Hemo Control (Cat. No. 5329), AFSA, Hemo Control (Cat. No. 5330), and AFSIC Hemo Control (Cat. No. 5331).

**Sample Preparation:**
- Whole blood collected in tubes containing EDTA or heparin is the preferred sample. Alternately, the plates may be wetted by slowly and uniformly soaking two wicks in the buffer and draping one over each support bridge being sure it makes contact with the buffer and that there are no air bubbles under the wicks.
- Cover the chamber to prevent buffer evaporation. Discard the buffer and wicks after use.

**Sample Preparation and Application**

1. Prepare a hemolysate of the patient samples as follows:
   - Using whole blood: Add 1 part whole blood to 3 parts hemolysate Reagent. Mix well and allow to stand 5 minutes.
   - Using packed cells: Mix 1 part packed red blood cells to 6 parts hemolysate Reagent. Mix well and allow to stand 5 minutes.

2. Wet two chamber wicks in the buffer and place one over each support bridge being sure it makes contact with the buffer and that there are no air bubbles under the wicks.

3. Cover the chamber to prevent buffer evaporation. Discard the buffer and wicks after use.

4. Place the plate into the diluting chamber using a pipette to load the plate.

5. Place the plate into the Clear Aid solution for 5-10 minutes.

6. Apply the sample to the plate by depressing the applicator tips into the sample well 3 or 4 times and promptly transferring the applicator to the aligning base. Press the button down and hold it 5-10 seconds.

Alternative Sample Preparation Procedure:
- If removal of denatured hemoglobins from the sample is deemed necessary, see the Alternate Sample Preparation Procedure.
- Place 5 µL of the patient hemolysates or 5 µL of the Hemo Controls into the wells of the Sample Well Plates using the microdispenser. Do not prepare a hemolysate of the Hemo Controls.

To prevent evaporation, cover the Sample Well Plate Blister Pads with the sample wells not used in 2 minutes.

Prime the applicator by depreciating the tips into the sample wells 3 or 4 times. Apply this loading to a piece of blotter paper. Priming the applicator makes the second loading much more uniform. Do not load applicator again at this point, but instead do a gentle loading of the applicator to keep thestyling.

**CATHODE APPLICATION**

1. Qualitative evaluation: The hemoglobin plates may be inspected visually for the presence of abnormal hemoglobin bands. The Helena Hemo Controls should be used as markers for the identification of the hemoglobin bands, and they may be used for verification of the accuracy of the procedure. Refer to the package insert provided with the controls for assay values and migration patterns. Use at least one of these controls on each plate run.

2. Isoelectric focusing, high performance liquid chromatography, globin chain analysis (both acid and alkaline) and structural studies may be necessary in order to positively identify some of the more rare hemoglobin bands.

3. Low levels of HbF (1-10%) may be accurately quantitated using any commercially available HbF method.

4. Relative Percent of Hemoglobin = Total Hemoglobin x 100

Figure 1. Electrophoretic Mobilities of Hemoglobins on Titan® III Cellulose Acetate and on Titan IV Cite agar.
The most common hemoglobin abnormalities: Sickle Cell

- This is a heterogeneous state showing HbA and HbS, and a normal amount of HbA, on cellulose acetate. Results on citrate agar show hemoglobin in the HbA and HbS migratory positions (zones).

Sickle Cell Anemia

- This is a heterogeneous state showing almost exclusively HbS, although a small amount of HbA may also be present.

Sickle Cell Disease

- This is a heterogeneous state demonstrating HbS and HbC.

Sickle Cell-Thalassemia Disease

- This condition shows HbA2, HbS, HbC, and in Sickle Cell-thalassemia HbA is absent.

Thalassemia Disease

- This condition shows HbA, HbC, and HbE.

C Disease

- This is a heterogeneous state showing almost exclusively HbC.

Thalassemia Major

- This condition shows HbF, HbA2, and HbC.

BIBLIOGRAPHY

11. Personal communication from Dr. Vivien Fananapazir.

Hemoglobin Electrophoresis Procedure

**INTRODUCTION**

Most hemoglobin variants cause no discernible clinical symptoms, so are of interest only to research scientists. Variants are clinically important when their presence leads to sickling disorders, thalassemia syndromes, life long anemia which is attributed to the precipitation or crystallization of HbC within the erythrocyte. The clinical course presents agonizing episodes of pain and temperature elevations with anemia, listlessness, lethargy, and infarct in virtually all organs of the body. The two variant hemoglobins most commonly seen in the United States are HbS and HbC, so are of interest in the diagnosis of sickling disorders.

**INTERPRETATION OF RESULTS**

Hemoglobin electrophoresis involves the use of two systems:

1. Electrophoresis

- A simple procedure which conforms the identification of both HbS and HbC, as well as HbA and HbF.

2. Hemoglobin Precipitation

- The precipitation method is based on the complex interactions of the hemoglobin with the electrophoretic buffer (acid pH) and the agar support.