I. PRINCIPLE:

Abnormal hemoglobin screen results by HPLC are confirmed by qualitative cellulose acetate and citrate agar electrophoresis. For citrate agar electrophoresis, very small samples of hemolysates are prepared from whole blood are applied to the Titan IV Citrate Agar Plate. The hemoglobins in the samples are separated by electrophoresis using citrate buffer, pH 6.0 to 6.3 and are stained with O-Dianisidine staining solution. Separation of hemoglobins under these conditions depends both on the location of the substituted residue and on its electrophoretic charge. The method is based on the complex interactions of the hemoglobin with the electrophoretic buffer (acid pH) and the agar support.

II. SPECIMEN:

Hemolysate from EDTA specimen used for screen. Procedure for preparation of hemolysate included later in this procedure.

III. REAGENTS:

A. Citrate Buffer - 0.3 Ionic strength - dilute package to 1000 ml deionized water pH 6.0-6.3. **Stable 1 month** in refrigerator. Cat. #5121 Helena, 10 pkg./box.

B. Sponge strips (EC Apparatus) - cut 3 strips to 6-1/2" long; then cut one in half lengthwise.

C. Agar plates - each plate contains 1.5% agar in .05 m citrate buffer - **stable for 1 year** in refrigerator. Cat. #2400 Helena - Box of 20.

D. 0.2% Diansidine in methanol (Cat. #5032 Helena) (0.5gm of Diansidine dilute with 250 ml of methanol) (diansidine in cabinet at room temp.) **Stable for 6 months** stored at room temperature.

E. 1.0% Sodium Nitroferricyanide in water (Cat. #5111 Helena). (1.0gm Sodium Nitroferricyanide in 100 ml deionized water) **Stable for 1 year** stored at room temperature.

F. 5% acetic acid (5 ml glacial acetic acid in 95 ml deionized H_20) **Stable for 1 year** stored at room temperature.

G. Hydrogen peroxide 3%, **stable 3 months from open date** stored at room temperature.

H. Zip Zone applicator (Cat. #4080 Helena).

I. Zip Zone sample well plate (Cat. #4081 Helena).

J. Zip Zone aligning base for agar (Cat. #4083).

K. Labels (Cat # 5006 Helena)

L. 2% Glycerol (2 ml glycerol in 98 ml H_2O) **stable indefinitely** stored at room temperature.

M. Hemolysate reagent (Cat. #5125 Helena 250 ml/bottle)

N. Whatman #3 filter paper for blotting

O. Epson #4 Matte paper (Office Max S041257)

IV. CONTROL:

AFSC Hemoglobin Control (Cat. # 5331 Helena)
Normal Hb AA patient control

V. PROCEDURE:

A. Preparing electrophoresis chamber:

1. Place sponge strip that was cut in half lengthwise on bottom of chamber and another strip on top making it perpendicular to the bottom sponge strip. Do the same on the other side.

2. Place 100 mL of citrate buffer equally in both chambers. Place frozen sponge strips in middle chamber for cooling purposes.

3. Connect the positive and negative leads of the electrophoresis chamber to the plugs in the front panel of the power supply.

B. Preparing Agar Plate:

1. Remove Titan IV citrate agar plate from plastic bag and remove the tape and cover from the agar plate.

2. Let plate sit at room temperature for a few minutes to let the moisture evaporate.

3. Place agar plate into the Titan IV aligning base.

C. Preparation of sample and application:

1. Write a patient grid to refer to when adding patient samples and controls to the wells.

2. Label 12 x 75 tubes for each sample and dispense 0.5 ml of hemolysate reagent to each tube. (**CAP samples: 50µl of sample with 150µl of hemolysate reagent.)**

3. Add 90 µl from the red cell layer to the hemolysate reagent.

4. Cap tube, vortex and allow specimen to sit for 5 min.

5. Place hemolysates of patients and controls in sample wells using the pipette set to 5µl. – Use an AFSC and a normal patient control on every plate.

6. To prime the Zip Zone applicator, quickly press the tips into the sample wells 3 or 4 times and apply to a blotter. Priming the applicator makes the second loading more uniform.

7. Once the applicator is primed, apply the samples to the plate by repressing the applicator into the sample wells 3 or 4 times and promptly transfer the Zip Zone applicator to the stanchions on the Titan IV aligning plate.

8. **Gently press the applicator tips down onto the gel surface.** Allow the samples to soak into the agar for 1 minute.

   a) To run 16 samples on one plate, use a second Zip Zone Sample Well Plate and fill the wells with a second set of hemolysates(patient and controls). Using a clean zip zone applicator, repeat steps 6 and 7 using the other stanchions.

9. Place agar plate on sponge wicks, placing agar side down and making sure the plate is in contact with the sponge wicks. Place the lid on the chamber and ensure it is completely seated.

10. Turn on the power supply, and turn the time dial past 60 minutes

11. Adjust the Voltage to 50 Volts

12. Turn the time dial down to 60 minutes

13. Electrophoresis will continue at the selected voltage and the time programmed. At the end of the run, a beep will sound and power will be turned off.

D. Staining Procedure:

1. In a flask prepare stain by combining:

   10 ml 5% acetic acid
   5.0 ml 0.2% Dianisidine
   1.0 ml 1.0% Sodium nitroferricyanide
   1.0 ml 3% hydrogen peroxide

2. Remove agar plate from cell and place it in a staining dish, agar side up.

3. Puddle the stain over the entire surface of the plate and stain for 10 minutes.

4. For permanent storage:
   a. Wash in 5% acetic acid for 30 minutes in staining dish.
   b. Rinse in deionized water for 10 minutes in staining dish.
   c. Hold the plastic plate under gently running water.
   d. Cut the agar in half, invert agar, then slide a 3 x 5 piece of Epson #4 paper under the stained half of the agar and remove it from the plastic plate.
   e. Flood the agar surface with 2% glycerol for 35 minutes.
   f. Tilt and drain the paper onto a blotter for 2 minutes.
   g. Lay the paper on a fresh blotter, then dry at 37° C overnight.
   h. Secure the ends of the Epson paper with the plastic racks in the incubator. Otherwise, the ends of the Epson paper will curl and the agar will crack.
   i. Label the agar results-date, FASC normal AA patient controls and patients.

VI. INTERPRETATION:

Reported by attending and resident, comparison of various patterns to known mobility patterns and standards help to determine the hemoglobin type.

VII. REFERENCE:

1. Technical Bulletin, "Titan IV Citrate Hemoglobin Procedure", Helena Laboratories, Beaumont, Texas.

VIII. HISTORY:

H-1 This procedure was written by P. Morris on 10-08-97.
H-2 This procedure was revised by S. Richardson on 3/5/2012**.**
H-3 This procedure was revised by A. Link on 10/24/2013