REFERENCES
8. Personal communication from Dr. Virgil Fairbanks.

HELENA TITAN® IV CITRATE HEMOGLOBIN ELECTROPHORESIS

INTERPRETATION OF RESULTS
HB Electrophoresis
Citrate agar electrophoresis is a necessary followup test for confirmation of abnormal hemoglobins detected on cellulose acetate. Hemoglobins are genetically controlled, and the presence of abnormal hemoglobins is often associated with functional, physical and morphologic abnormalities in the erythrocyte, as well as pathological manifestations, such as hemolytic anemia.

Sickle Trait
This is a heterozygous state showing HB and Hbs and a normal amount of Hba, on cellulose acetate. Results on citrate agar show hemoglobins in the Hba and Hbs migratory positions (zones).

Sickle Cell Anemia
This is a hemoglobinopathy state showing HbS and HbC.

Sickle Cell - Thalassemia Disease
This condition shows HbS, HbC, and the Thalassemia trait.

Sickle-C Disease
This is a heterozygous state demonstrating Hbs and Hbc.

Sickle Cell - Thalassemia Disease
This condition shows HbA, HbS, HbC, and the Thalassemia trait.

Sickle Cell Major
This condition shows HbS, HbC, and the Major trait.

Hemoglobin Electrophoresis
The following items, needed for the performance of the Titan IV Citrate Hemoglobin Electrophoresis Procedure, must be ordered individually.

Item
Cat. No.
Titan IV Citrate Agar Plates
2400
Citrate Buffer
5121
AFSC Hemo Control
5331
o-Dianisidine
5036
o-Tolidine
5048
Hemolysate Reagent
5215
Blotter Pads (76 x 102 mm)
5034
Hemolysis Marker
5000
Zip Zone® Applicator
4080
Zip Zone® Sample Well Plate (2)
4081
Titan IV Aligning Base
4092
Titan Gel Electrophoresis Chamber
4063
Microdispenser and Tubes (5 ml)
6008
Zip Zone® Sponge Wicks
9014
Titan Plus Supply Wicks
1504

For Sales, Technical and Order Information and Service Assistance, call 800-231-5663 toll free.

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

Procedures for Use: Dissolve one package of buffer in 1000 mL of deionized water. The buffer is ready for use when all material is dissolved and completely mixed.

For Storage and Stability: The packed buffer should be stored at room temperature (15° to 30°C) and is stable until the expiration date indicated on the package. Diluted buffer is stable for one month at 4° to 30°C.

Signs of Deterioration: Do not use packaged buffers if the material shows signs of dampness or discoloration. Discard diluted buffer if it becomes turbid.

3. Hemolytic Reagent (Cat. No. 5125)

Ingredients: Hemolytic Reagent is an aqueous solution of 3%, 40 times, 0.07% potassium cyanide as hemoglobin preservatives.

WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT EAT, CHEW, MOUTH, HARMFUL IF SWALLOWED.

Preparation for Use: The reagent is ready for use as packed.

Storage and Stability: The reagent should be stored at room temperature (15° to 30°C) and is stable until the expiration date indicated on the vial.

Signs of Deterioration: The reagent should be a clear, colorless solution.

4. Stains

a. o-Dianisidine (Cat. No. 5306)

Ingredients: 0.2% (w/v) 3,3 dimethoxybenzidine in methanol.

WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. CARCINOGEN. DO NOT INGEST, AVOID CONTACT WITH SKIN. The reagent is toxic and can cause skin irritation. Should reagent come into contact with skin, wash with copious amounts of water. Harmful if swallowed.

Preparation for Use: Dissolve one vial of stain with 1 L methanol.

Storage: The stain should be stored at room temperature (15° to 30°C) and is stable until the expiration date on the vial.

REFERENCES
10. Personal communication from Dr. Virgil Fairbanks.
STEP-BY-METHOD

A. Preparation of Titan IV Citrate Agar Plate

1. Remove the cover from the Titan IV Citrate Agar Plate from the refrigerator and place the plate to room temperature (15° to 30°C) for 5 to 10 minutes. Plate the patient samples.

2. Remove the plate from the plastic bag and properly identify it by marking with a marker on the plastic backing of the agar. Place the mark in one corner so that it will be aligned with sample No. 1.

B. Preparation of Patient Sample and the Control

1. To prepare a hemolysate of the patient sample, add one (1) part whole blood to 19 parts Hemolysate Reagent. Alternatively, if examination of denatured hemoglobin from the sample is deemed necessary, washed cells should be used.

   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 (v/v) three times. After each wash, centrifuge the cells for 10 minutes at 3500 RPM.

   c. Add 1 volume deionized water and 14 volume toluidine (or carbon tetrachloride) to the washed red cells. Vortex at high speed for one minute. Centrifuge the samples at 3500 RPM for 10 minutes.

2. If a sample is used, the top layer in the tube will contain cell stroma and should be removed with a 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.

3. Fill the clear red solution through two layers of Whamart filter paper.

4. Prepare the AFSC Hemolysate Control by adding one (1) part Hemolysate Reagent to one (1) part Hemolysate Reagent.

5. Mix all hemolysate preparations well. Cover the tubes and place the control to one (1) part Hemolysate Reagent.

C. Preparation of Titan Gel Chamber

1. Pour approximately 100 mL of Citrate Buffer into each outer section of the Titan Gel Chamber.

2. Wet two sponge wicks in the buffer, and place the sponges in each outer compartment so that the top surface protrudes approximately 2 mm above the inner chamber ridges. (Care should be taken to rinse all buffer from the sponges before each use.) Gently press the sponges to assure complete saturation with buffer.

D. Sample Application

1. Mix the hemolysates solutions once more to ensure complete lysis.

2. Place 5 μL of each prepared hemolysate (patient and control) in separate wells of the Titan IV Sample Well Plate using the Microdispenser. Cover the Sample Well Plate with a glass slide if the samples are not used within 2 minutes.

3. To prime the Zip Zone® Application Point, quickly place 0.5 D.L. tips into the sample wells 3 or 4 times and apply to a blotter. Place the applicator mouth next to the second loading more uniform. Do not load the applicator again at this point, but proceed quickly to the next step.

4. Remove the cover from the Titan IV Citrate Agar Plate. Position the plate in the Titan IV Aligning Base. The identification mark should be aligned with sample No. 1. If desired, the spring can be removed from the applicator, allowing the applicator tip into the agar without cutting it.

5. To apply the sample to the plate, place the applicator tips into the sample wells 3 or 4 times and promptly transfer the applicator to the second set of stanchions on the Titan IV Aligning Base. Gently press the applicator tips down onto the gel surface. Allow the samples to soak into the agar for about one minute. To run 16 samples on one plate, use a second Zip Zone® Sample Well Plate and fill the wells with a second hemolysate (patient and control).

6. Using a clean Zip Zone® Applicator, place the applicator in the second set of stanchions on the Titan IV Aligning Base and apply the samples to the plate in the same manner as before.

E. Electrophoresis of the Sample Plate

1. Pour out the agar, gel side down, in the Titan Gel Chamber so that the agar layer makes good contact with the top surface of the sponges. The first application point should be nearest the anode (+).

2. Place the lid on the chamber and ensure that it is completely sealed.

3. Electrophoresis for 45 minutes at 50 volts. Electrophoresis time may be increased to 60 minutes, if additional separation of HbS from the HbA is desired.

F. Visualization of the Hemoglobin Bands

1. Prepare the staining solution while electrophoresis is in progress. The reagents in this staining solution should be kept in separate bottles, mixed just prior to use, and discarded after each use. Prepare the staining solution as follows:

   5 mL 0.2% o-Tolidine (o-Tolidine may be substituted) 10 mL 5% acetic acid 1 mL 3% hydrogen peroxide 1 mL 1% sodium nitro ferricyanide

2. Upon completion of electrophoresis, remove the plate from the chamber and place on the counter top, gel side up.

3. Puddle the stain over the entire surface of the plate and stain for 5 to 10 minutes. Plates may also be immersed in the stain, but a greater volume of stain is required.

4. The hemoglobin gels in the present patient samples should be identified by comparison to the migration pattern of the AFSC Hemo Control. For immediate visualization, pour off the stain.

5. If permanent storage is desired:

   a. Wash in 5% acetic acid for 30 minutes.

   b. Rinse in deionized water for 10 minutes.

   c. Hold the plate under gently running water.

   d. Cut off the agar in half, then slide a 3 x 5 card under the stained half of the agar and remove it from the holder.

   e. Flood the agar surface with 2% glyceral for 35 minutes.

   f. Tilt and drain the plate onto a blotter for 2 minutes.

6. Lay the plate on a fresh blotter, then dry at 50°C for 1 hour and 20 minutes or dry at 37°C for 3 to 4 hours.

7. (Optional) Staining of End Product: The unprepared plates are stable for three months if kept tightly closed. Dried plates are stable indefinitely.

8. Quality Control: The Helena AFSC Hemo Control (Cat. No. 5331) should be run on each Titan IV Citrate Agar Plate. The control verifies the absence of procedures and acts as a marker in the identification of the hemoglobin in the unknown samples.

**RESULTS**

Figure 1 illustrates a comparison of Citrate Agar and Cellulose Acetate plates for electromobility (both media are prepared with 8% acetic acid). The electrophoresis was performed on a Titan IV Aligning Base, 45 minutes at 50 volts. Figure 2 lists the relative mobilities of various hemoglobin mutants on cellulose acetate and citrate agar plates.
**Materials required:**
- Liver oil
- Glacial acetic acid (Dilute 5 parts with 95 parts deionized water, to yield 5% solution.)
- Hydrogen peroxide (3%)
- Whatman #1 filter paper.
- Sponge Wicks
- Steel applicator, 1/4 in. diameter, 11.8 in. long.
- Plastic plate with a glass slide if the samples are not used within 2 hours.
- 3 x 5 cards
- Polyvinyl chloride film
- Toluene.
- Carbon tetrachloride.

**Preparation for Use:**
- Centrifuge the blood sample at 3500 RPM for 5 minutes.
- Add 1 volume deionized water and 1/4 volume toluene (or benzyl alcohol). Centrifuge the cells for 5000 RPM for 3 to 5 minutes.
- Stain at room temperature for 5 to 10 minutes.
- Discard the stain, then wash the cells with buffer, and place the sponges in deionized water for 10 minutes. Rinse in the buffer, and place the sponges in deionized water for 10 minutes.
- Allow the samples to stand in the buffer until required, then drain and place in the buffer.
- Stain the plates for 50 minutes before drying. Allow to dry at 37°C for 3 to 4 hours.

**Storage and Stability:**
- Glue the applicator tips down onto the gel surface. Allow the samples to soak into the agar for about one minute. If the 5% blood sample isuke desired; washed cells should be used. The stain should be stored at room temperature.
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**Materials needed but not supplied:**
- Hydrogen peroxide (3%)
- Glacial acetic acid (Dilute 5 parts with 95 parts deionized water, to yield 5% solution.)

**Application Point**
- Centrifuge the blood sample at 3500 RPM for 5 minutes.
- Add 1 volume deionized water and 1/4 volume toluene (or benzyl alcohol). Centrifuge the cells for 5000 RPM for 3 to 5 minutes.
- Stain at room temperature for 5 to 10 minutes.
- Discard the stain, then wash the cells with buffer, and place the sponges in deionized water for 10 minutes. Rinse in the buffer, and place the sponges in deionized water for 10 minutes.
- Allow the samples to stand in the buffer until required, then drain and place in the buffer.
- Stain the plates for 50 minutes before drying. Allow to dry at 37°C for 3 to 4 hours.

**Procedure:**
- Pour approximately 100 mL of Citrate Buffer into each outer section of the Titan-IV Gel Chamber.
- Wet two sponge wicks in the buffer, and place the sponges in each outer compartment so that the top surface of the sponges are approximately 2 mm above the inner chamber ridges. (Care should be taken to rinse all buffer from the sponges before each use to prevent the applicator from clogging.)
- Place the Osaka and Osaka plates on one plate, use a second Zip Zone applicator, quickly press the applicator, and allow the samples to soak into the agar for about one minute. If the 5% blood sample isuke desired; washed cells should be used. The stain should be stored at room temperature.
- Glue the applicator tips down onto the gel surface. Allow the samples to soak into the agar for about one minute. If the 5% blood sample isuke desired; washed cells should be used. The stain should be stored at room temperature.
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HELENA TITAN® IV CITRATE HEMOGLOBIN ELECTROPHORESIS

REFERENCES
9. Personal communication from Dr. Virgil Fairbanks.

HELENA TITAN® IV CITRATE HEMOGLOBIN ELECTROPHORESIS

The following items, needed for the performance of the Titan® IV Citrate Hemoglobin Electrophoresis Procedure, must be ordered individually.

- Hb Electrophoresis

1. Titan® IV Citrate Agar Plates
   - Cat. No. 2400

2. Citrate Buffer
   - Cat. No. 5121

3. AFSC Hemo Control
   - Cat. No. 3531

4. O-Dianisidine
   - Cat. No. 5036

5. O-Toluene
   - Cat. No. 5041

6. Hemolysate Reagent
   - Cat. No. 5125

7. Blotted Pads (76 x 102 mm)
   - Cat. No. 5034

8. Helena Marker
   - Cat. No. 5000

9. Zip Zone® Applicator
   - Cat. No. 4080

10. Zip Zone® Sample Well Plate (2)
    - Cat. No. 4081

11. Titan IV Aligning Base
    - Cat. No. 4082

12. Titan Gel Electrophoresis Chamber
    - Cat. No. 4083

13. Microdispenser and Tubes (5 mL)
    - Cat. No. 6008

14. Zip Zone® Sponge Wicks
    - Cat. No. 9014

15. Titan Plus Power Supply
    - Cat. No. 1504

For Sales, Technical and Order Information and Service Assistance, call 800-231-5663 toll free.

HELENAPlex warrants its products to meet our published specifications and to be free from defects in materials and workmanship. Helena’s liability under this contract or otherwise shall be limited to replacement or refund of any amount not to exceed the purchase price attributable to the goods as to which such claim is made. These alternatives shall be buyer’s exclusive remedy.

In no case will Helena Laboratories be liable for consequential damages even if Helena has been notified of the possibility of such damages. The foregoing warranties are in lieu of all warranties expressed or implied, including but not limited to, the implied warranties of merchantability and fitness for a particular purpose.

HELENA TITAN® IV CITRATE HEMOGLOBIN ELECTROPHORESIS Procedure is intended as a qualitative procedure for the identification of human hemoglobins.

SUMMARY
Hemoglobins (Hb) are a group of proteins whose chief functional property other than electrical charge. This simple procedure is based on the complex interactions of the hemoglobin substituted residue and on its electrophoretic charge. The two mutant hemoglobins most commonly seen in the United States are HbS and HbC. Hb Lepore, HbE, HbG-Arab may be seen occasionally. The two mutant hemoglobins most commonly seen in the United States are HbS and HbC. Hb Lepore, HbE, HbG-Philadelphia, HbD-Los Angeles, and HbO-Arab may be seen less frequently. Electrophoresis is generally considered the best method for separating and identifying hemoglobinopathies. The protocol for hemoglobin electrophoresis involves the use of two systems.

Initial electrophoresis is performed in alkaline buffer. Celluloseacetate is the major support medium used because it yields rapid separation of HbA, HbF, HbS and HbC 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 citrate agar electrophoresis which measures a property other than electrophoretic charge. This simple procedure requires only minute quantities of hemosya to provide highly specific (but not absolute) confirmation of the presence of HbS, HbC, HbF, and HbA2, as well as several other abnormal hemoglobins.

PRINCIPLE
Very small samples of hemolysates prepared from whole blood are applied to the Titann® 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 a o-Dianisidine or o-Toluene staining solution. Separation of hemoglobins under these conditions depends both on the location of the substained 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.

REFERENCES
9. Personal communication from Dr. Virgil Fairbanks.

Storage and Stability: The plates should be stored at room temperature (15° to 30° C) and is stable until the expiration date indicated on the package. Diluted buffer is stable for one month at 2° to 8°C.

Signs of Deterioration: Do not use packaged buffers if the material shows signs of dapples or discoloration. Discard diluted buffer if it becomes turbid.

Storage and Stability: The reagent should be stored at room temperature (15° to 30° C) and is stable until the expiration date indicated on the vial.

Signs of Deterioration: The reagent should be a clear, colorless solution.

Stains
- a. o-Dianisidine (Cat. No. 5306)
  - Ingredients: 0.2% (w/v) 3,3-dimethoxybenzidine in methanol.

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

  Preparation for Use: Dissolve 1 vial of stain with 1 mL methanol. Store the stain should be stored at room temperature (15° to 30° C) and is stable until the expiration date on the vial.

- b. o-Toluene (Cat. No. 5307)
  - Ingredients: 0.2% (w/v) 3,3-dimethoxybenzidine in methanol.

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

  Preparation for Use: Dissolve 1 vial of stain with 1 mL methanol.