HELENA TITAN® IV CITRATE HEMOGLOBIN ELECTROPHORESIS

The 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 functions are to transport oxygen from the lungs to the tissues and carbon dioxide in the reverse direction. They are composed of polypeptide chains called globin, and iron protoporphyrin heme groups. A specific sequence of amino acids constitutes each of four polypeptide chains. Each normal hemoglobin molecule contains one pair of alpha and one pair of non-alpha chains. In normal adult hemoglobin (HbA), the non-alpha chains are called beta. The non-alpha chains of fetal hemoglobin are called gamma. A minor (3%) hemoglobin fraction called HbA_2 contains alpha and delta chains. In a hereditary inhibition of globin chain synthesis called thalassemia, the non-alpha chains may aggregate to form HbH ($\beta 4$) or Hb Bart's ($\gamma 4$).

The major hemoglobin in the erythrocytes of the normal adult is HbA and there are small amounts of HbA_2 and HbF. In addition, over 400 mutant hemoglobins are now known, some of which may cause serious clinical effects, especially in the homozygous state or in combination with another abnormal hemoglobin. Wintrobe[1] divides the abnormalities of hemoglobin synthesis into three groups;

(1) production of an abnormal protein molecule (e.g. sickle cell anemia),
(2) reduction in the amount of normal protein synthesis (e.g. thalassemia), and
(3) development anomalies (e.g. hereditary persistence of fetal hemoglobin, HPFH).

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.[2] Electrophoresis is generally considered the best method for separating and identifying hemoglobinopathies. The protocol for hemoglobin electrophoresis involves the use of two systems.[3-8] 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 electrical charge. This simple procedure requires only minute quantities of hemolysate to provide highly specific (but not absolute) confirmation of the presence of HbS, HbC and HbF, as well as several other abnormal hemoglobins.

PRINCIPLE

Very small samples of hemolysates 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 an o-Dianisidine or o-Tolidine 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.

REAGENTS

1. **Titan® IV Citrate Agar Plates (Cat. No. 2400)**
   Ingredients: Plates contain 1.5% agar (w/v) in 0.03 M citrate buffer with thimerosal added as a preservative.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY.
   Preparation for Use: The plates are ready for use as packaged.
   Storage and Stability: Plates should be stored flat at 2° to 8°C and are stable until the expiration date indicated on the label. Store in the protective packaging in which the plates are shipped. **DO NOT FREEZE THE PLATES OR EXPOSE THEM TO EXCESSIVE HEAT**.
   Signs of Deterioration: The plates should have a smooth, clear surface. Discard the plates if they appear cloudy, show fungal or bacterial growth, or if they have been exposed to freezing (a cracked or bubbled surface) or excessive heat (a dried, thin surface).
2. **Citrate Buffer (Cat. No. 5121)**
   Ingredients: Each package of Citrate Buffer contains sodium citrate and citric acid.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT INGEST.
   Preparation 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.
   Storage and Stability: The packaged 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 2° to 8°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. **Hemolysate Reagent (Cat. No. 5125)**
   Ingredients: Hemolysate Reagent is an aqueous solution of 0.005 M EDTA and 0.07% potassium cyanide as hemoglobin preservatives.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT PIPETTE BY MOUTH. HARMFUL IF SWALLOWED.
   Preparation for Use: The reagent is ready for use as packaged.
   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. 5036)
   Ingredients: 0.2% (w/v) 3,3 dimethoxybenzidine in methanol after reconstitution.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. CARCINOGEN. DO NOT INGEST. AVOID CONTACT WITH SKIN. The reagent is highly 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.

Signs of Deterioration: The reagent should be light yellow-brown. Discard reagent if it becomes dark brown and/or contains precipitate.

b. o-Tolidine (Cat. No. 5041) (may be substituted for o-Dianisidine)

Ingredients: 0.2% (w/v) o-Tolidine in methanol after reconstitution.

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

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

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

Signs of Deterioration: The reagent should be light yellow-brown. Discard reagent if it becomes dark brown and/or contains precipitate.

SPECIMEN COLLECTION AND PREPARATION

Specimen: Whole blood collected in tubes containing EDTA is the specimen of choice.

Specimen Preparation: Specimen hemolysates are prepared as outlined in the STEP-BY-STEP METHOD.

Specimen Storage: Whole blood samples may be stored up to one week at 2° to 8°C.

PROCEDURE

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

Item	Cat. No.
Zip Zone® Applicator	4080
Zip Zone® Sample Well Plate (2)	4081
Titan IV Aligning Base	4083
Titan Gel Electrophoresis Chamber	4063
Microdispenser and Tubes	6008
Zip Zone® Sponge Wicks	9014
Titan Plus Power Supply	1504
Titan® IV Citrate Agar Plates	2400
Citrate Buffer	5121
AFSC Hemo Control	5331
o-Dianisidine	5036
o-Tolidine	5041
Hemolysate Reagent	5125
Blotters	5034
Helena Marker	5000

Materials needed but not supplied:

Hydrogen peroxide (3%)

Glacial acetic acid (Dilute 5 parts with 95 parts deionized water, to yield 5% solution.)

Absolute Methanol

1% Sodium nitroferricyanide

SUMMARY OF CONDITIONS

Plate	Titan® IV Citrate Agar
Buffer	Citrate Buffer diluted to 1000 mL
Sample volume (Hemolysate)	5µL
Application point	Anode
Number of applications	one (1) or two (2)
Electrophoresis time	45 minutes
Voltage	50 V
Staining time	5 to 10 minutes

STEP-BY-STEP METHOD

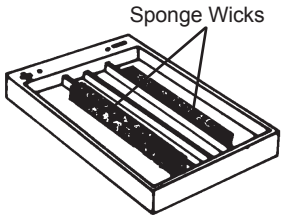
A. Preparation of Titan® IV Citrate Agar Plate

1. Remove the Titan® IV Citrate Agar Plate from the refrigerator and allow the plate to come to room temperature (15 to 30°C) while preparing the patient samples.
2. Remove the plate from the plastic bag and properly identify it by marking with a marker on the plastic support backing of the agar filled half of the plastic plate. Place the mark in one corner so that it will be aligned with sample No. 1.

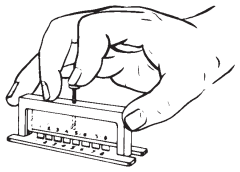
B. Preparation of Patient Sample and the Control

1. To prepare a hemolysate of the patient sample, add one (1) part of whole blood to 19 parts Hemolysate Reagent. Alternatively, if removal of denatured hemoglobins 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 1/4 volume toluene (or carbon tetrachloride) to the washed red cells. Vortex at high speed for one minute. Centrifuge the samples at 3500 RPM for 10 minutes.
   d. If toluene 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.
   e. Filter the clear red solution through two layers of Whatman #1 filter paper.
2. Prepare the AFSC Hemo Control by adding one (1) part of the control to one (1) part Hemolysate Reagent.
3. Mix all hemolysate preparations well. Cover the tubes and allow to stand for five (5) minutes.

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 sponge to assure complete saturation with buffer.



D. Sample Application

1. Mix the hemolysate solutions once more to ensure complete lysis.
2. Place 5 µL of each prepared hemolysate (patient and control) in separate wells of the Zip Zone® 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® 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. Do not load the applicator again at this point, but proceed quickly to the next step.