SPIFE® 3000 Acid Hemoglobin Procedure for Plastic Blades

Cat. No. 3418

The SPIFE Acid Hemoglobin method is intended for the qualitative determination of hemoglobins using agar in acidic buffer on the SPIFE 3000 system.

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. The non-alpha chains of fetal hemoglobin are called gamma. A minor (3%) hemoglobin fraction, called HbA_2, contains alpha and delta chains. Two other chains are formed in the embryo.

The major hemoglobin in the erythrocytes of a normal adult is HbA, but 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)

(3) Developmental 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 stepwise use of two systems.[3-8]

Initial electrophoresis is performed in alkaline buffers. Cellulose acetate was the major support medium used because it yields rapid separation of HbA, F, 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 citrate agar electrophoresis which measures a property other than electrical charge.

This method is based on the complex interactions of the hemoglobin with an acid electrophoretic buffer and the agar support. The SPIFE Acid Hemoglobin method is a simple procedure requiring minute quantities of hemolysate to provide a screening method for the presence of abnormal hemoglobins such as HbS, HbC and HbF.

PRINCIPLE

Very small samples of lysates prepared from washed, packed cells are automatically applied to the SPIFE Acid Hb gel. The hemoglobins in the sample are separated by electrophoresis using a citrate buffer and are stained with Acid Blue Stain.

REAGENTS

1. SPIFE Acid Hb Gel

Ingredients: Each gel contains agar in citrate buffer with 0.25% EDTA and thimerosal as a preservative.

Preparation for Use: The gels are ready for use as packaged.

Storage and Stability: The gels should be stored horizontally at room temperature (15 to 30°C) and are stable until the expiration date indicated on the package. The gels must be stored in the protective packaging in which they are shipped. **DO NOT REFRIGERATE OR FREEZE THE GELS**.

Signs of Deterioration: Any of the following conditions may indicate deterioration of the gel: (1) crystalline appearance indicating the agarose has been frozen, (2) cracking and peeling indicating drying of the agarose, (3) bacterial growth indicating contamination, (4) thinning of the gel blocks.

2. Acid Blue Stain

Ingredients: When dissolved as directed, the stain contains 0.5% (w/v) acid blue stain.

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

Preparation for Use: Dissolve the dry stain (entire contents of vial) in 1 L of 5% glacial acetic acid. Mix thoroughly for 30 minutes.

Storage and Stability: The dry stain should be stored at 15 to 30°C and is stable until the expiration date indicated on the package. The diluted stain is stable six months when stored at 15 to 30°C.

Signs of Deterioration: The diluted stain should be a homogeneous mixture free of precipitate. Discard if precipitate forms. The stain must be replaced after processing ten gels to avoid contamination.

3. Hemolysate Reagent

Ingredients: The reagent contains deionized water with 0.005 M EDTA, 0.175% saponin and 0.07% potassium cyanide.

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

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: Discard if solution has precipitate or flocculent.

4. Citric Acid Destain

Ingredients: After dissolution, the destain contains 0.3% (w/v) citric acid.

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

Preparation for Use: Pour 11 L of deionized water into the Destain vat. Add the entire package of Destain. Mix well until completely dissolved.

Storage and Stability: Store the Destain at 15 to 30°C. It is stable until the expiration date on the package.

Signs of Deterioration: Discard if solution becomes cloudy.

INSTRUMENT

A SPIFE 3000 instrument must be used to apply samples, electrophorese, stain, destain and dry the gels. Refer to the appropriate Operator's Manual for detailed instructions.

SPECIMEN COLLECTION AND HANDLING

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

Specimen Storage: If storage is necessary, whole blood and packed cells may be stored up to 1 week at 2 to 8°C. Frozen samples may produce an artifact band between HbF and HbA, and band intensity may diminish, especially with hemoglobin C.

Specimen Preparation: Washed, packed cell lysates must be prepared for each patient specimen.

A) Whole Blood sample
1. Centrifuge anticoagulated blood for 10 minutes to separate cells from plasma.
2. Remove plasma.
3. Wash packed cells 3 times by resuspending in 5 to 10 volumes of normal saline solution (0.85% NaCl), centrifuging and aspirating supernatant.
4. After washing the samples, prepare the lysates by mixing 10 µL sample to 100 µL Hemolysate Reagent. Vortex or shake vigorously for 15 seconds.

B) Control
AFSC (Cat. No. 5331) 1:2 (1 part control + 1 part Hemolysate Reagent)

PROCEDURE

Materials provided: The following materials needed for the procedure are contained in the SPIFE Acid Hemoglobin Kit (Cat. No. 3418). Individual items are not available.

SPIFE Acid Hemoglobin Gels (10)
Acid Blue Stain (1 vial)
Hemolysate Reagent (50 mL)
Citric Acid Destain (1 pkg)
REP Blotter C (10)
Blade Applicator Kit - 20 Sample (10)

Materials available but not contained in the kit:

ITEM	CAT. NO.
SPIFE 3000 Analyzer	1088
AFSC Hemo Control	5331
REP Prep	3100
Gel Block Remover	1115
Applicator Blade Weights	3387
Disposable Sample Cups	3369
SPIFE Disposable Cup Tray	3370

Materials needed but not provided:

5% glacial acetic acid
0.85% saline

STEP BY STEP METHOD

I. Sample Preparation

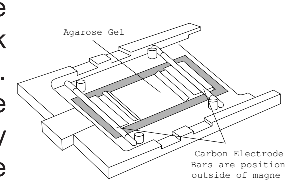
1. Prepare lysates of patient specimens and controls as instructed in the "Specimen Preparation" section.
2. Remove one Applicator Blade from the packaging.
3. Place the Applicator Blade into the vertical slot numbered "8" in the Applicator Assembly.

NOTE: The Applicator Blade will only fit into the slots in the Applicator Assembly one way; do not try to force the Applicator Blade into the slots.

4. Place an Applicator Blade Weight on top of each Applicator Blade. When placing the weight on the blade, position the weight with the thick side to the right.
5. Slide the Disposable Sample Cups strip into the center channel of the Cup Tray (numbered 21 to 40).
6. Pipette 17 µL of patient or control lysate into each of the Disposable Cups. Cover until ready for use.

II. Gel Preparation

1. Remove the gel from the protective packaging and discard the overlay.
2. Place a REP Blotter C on gel with the longer end parallel with the gel blocks. Gently blot the entire surface of the gel using light fingertip pressure on the blotter, and remove the blotter.
3. Dispense approximately 2 mL of REP Prep onto the left side of SPIFE chamber.
4. Place the left edge of the gel over the REP Prep aligning the round hole on the left pin. Gently lay the gel down on the REP Prep, starting from the left and ending on the right side, fitting the obround hole over the right pin. Use lint-free tissue to wipe around the edges of the gel backing, especially next to electrode posts to remove excess REP Prep. Make sure that the gel lays flat in the chamber and that no bubbles remain under the gel.
5. Clean the electrodes with deionized water and wipe with lint-free tissue before and after use.
6. Place a carbon electrode on the outside ledge of each gel block outside the magnetic posts. Improper contact between the electrode and the gel block may cause skewed patterns. Close the chamber lid.



7. Press the **TEST SELECT/CONTINUE** buttons located on the Electrophoresis and Stainer sides of the instrument until the **ACID HEMOGLOBIN** option appears on the displays.

III. Electrophoresis Parameters

Using the instructions provided in the appropriate Operator's Manual, set up parameters as follows for the SPIFE 3000:

Electrophoresis Unit

1)	No Prompt				
	Load Sample 1	00:30	20°C	SPD4	
2)	No Prompt				
	Apply Sample 1	00:30	20°C	SPD4	LOC 1
3)	No Prompt				
	Electrophoresis 1	25:00	17°C	250V	85mA
4)	Remove gel blocks, (continue)				
	Dry 1	7:00	62°C		
5)	No prompt				
	END OF TEST				

Stainer Unit

1)	No Prompt			
	Stain 1	4:00	REC = OFF	VALVE = 3
2)	No Prompt			
	Destain 1	1:00	REC = REV	VALVE = 2

3) No Prompt
Dry 1 7:30 54°C
4) No Prompt
Destain 2 2:00 REC = REV VALVE = 2
5) No Prompt
Destain 3 2:00 REC = REV VALVE = 2
6) No Prompt
Dry 2 20:00 54°C
7) No Prompt
END OF TEST

IV. Electrophoresis

1. Open the chamber lid. Place the Cup Tray with prepared lysates on the SPIFE 3000. Align the holes in the tray with the pins on the instrument. Close the chamber lid.
2. With **ACID HEMOGLOBIN** on the display, press the **START/STOP** button. An option to either begin the test or skip the operation will be presented. Press **START/STOP** to begin. The SPIFE 3000 will apply the samples, electrophorese and beep when completed.

V. Visualization

1. After electrophoresis is complete, open the chamber lid and use the Gel Block Remover to remove the gel blocks. Place one electrode across each end of the gel to prevent curling during drying. Dispose of blades and cups as biohazardous waste.
2. Close the chamber lid and press the **TEST SELECT/CONTINUE** button to dry the gel.
3. After the gel has been dried, carefully remove the gel from the electrophoresis chamber.
4. Remove the Gel Holder from the stainer chamber. Attach the gel to the holder by placing the round hole over the left pin and the obround hole over the right pin on the holder.
5. Place the Gel Holder with the attached gel facing backwards into the stainer chamber.
6. With **ACID HEMOGLOBIN** on the display, press the **START/STOP** button. An option to either begin the test or skip the operation will be presented. Press **START/STOP** to begin. The instrument will stain, destain and dry the gel.
7. When the gel has completed the process, the instrument will beep. Remove the Gel Holder from the stainer. Take the gel off of the holder and replace the holder.

Evaluation of the Hemoglobin Bands

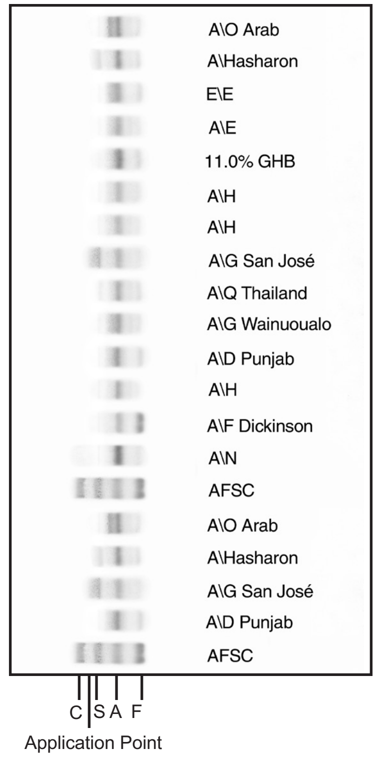
The hemoglobin gels should be inspected visually for the presence of abnormal hemoglobin bands. Glycated hemoglobin migrates with HbF. The Helena AFSC Hemo Control provides a marker for band identification.

Stability of End Product: The dried gels are stable for an indefinite period of time.

Quality Control: The Helena AFSC Hemo Control (Cat. No. 5331) should be run on each SPIFE Acid Hemoglobin Gel. The control verifies all phases of the procedure and acts as a marker to aid in the identification of the hemoglobins in the unknown samples.

RESULTS

Figure 1 illustrates the electrophoretic mobility of bands on the SPIFE Acid Hemoglobin Gel.



LIMITATIONS

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

1. Globin chain analysis (both acid and alkaline) and structural studies may be necessary in order to positively identify some of the more rare hemoglobins.
2. When a particular hemoglobin concentration varies significantly from the control, the migration will be affected.

REFERENCE VALUES

At birth, the majority of hemoglobin in the erythrocytes of the normal individual is fetal hemoglobin, HbF. Some of the major adult hemoglobin, HbA, and a small amount of HbA_2 are also present. At the end of the first year of life and through adulthood, the major hemoglobin present is HbA with up to 3.7% HbA_2 and less than 2% HbF.[9]

INTERPRETATION OF RESULTS

Most hemoglobin variants cause no discernible clinical symptoms, so are of interest primarily to research scientists. Variants are clinically important when their presence leads to sickling disorders, thalassemia syndromes, life long cyanosis, hemolytic anemias, erythrocytosis or if the heterozygote is of sufficient prevalence to warrant genetic counseling. The combinations of HbSS, HbSD-Los Angeles and HbSO-Arab lead to serious sickling disorders.[2] Several variants including HbH, E-Fort Worth and Lepore cause a thalassemic blood picture.[2] The two variant hemoglobins of greatest importance in the U.S., in terms of frequency and pathology, are HbS and HbC.[2] Sickle cell anemia (HbSS) is a lethal disease that first manifests itself at about 5-6 months of age. 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 individual with homozygous HbCC suffers mild hemolytic anemia which is attributed to the precipitation or crystallization of HbC within the erythrocytes. Cases of HbSC disease are characterized by hemolytic anemia that is milder than sickle-cell anemia.

The thalassemias are a group of hemoglobin disorders characterized by hypochromia and microcytosis due to the diminished synthesis of one globin chain (the α or β) while synthesis of the other chain proceeds normally.[10,11] This unbalanced synthesis results in unstable globin chains. These precipitate within the red cell, forming inclusion bodies that shorten the life span of the cell. In α-thalassemias the α-chains are diminished or absent, and in the β-thalassemia the β-chains are affected. Another quantitative disorder of hemoglobin synthesis, hereditary persistent fetal hemoglobin (HPFH), represents a genetic failure of the mechanisms that turn off gamma chain synthesis at about four months after birth which results in a continued high percentage of HbF. It is a more benign condition than the true thalassemias, and persons homozygous for HPFH have normal development, are asymptomatic and have no anemia.[11]

The most common hemoglobin abnormalities:

Sickle Cell Trait

This is a heterozygous state showing HbA and HbS and a normal amount of HbA_2 on cellulose acetate. Results on citrate agar show hemoglobins in the HbA and HbS migratory positions (zones).

Sickle Cell Anemia

This is a homozygous state showing almost exclusively HbS, although a small amount of HbF may also be present.

Sickle-C Disease

This is a heterozygous state demonstrating HbS and HbC.

Sickle Cell-Thalassemia Disease

This condition shows HbA, HbF, HbS and HbA_2.

In Sickle Cell $\beta°$-Thalassemia HbA is absent.

In Sickle Cell β^+-Thalassemia HbA is present in reduced quantities.

Thalassemia-C Disease

This condition shows HbA, HbF and HbC.

C Disease

This is a homozygous state showing almost exclusively HbC.

Thalassemia Major

This condition shows HbF, HbA and HbA_2.

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