

SPIFE® 4000 Acid Hemoglobin Procedure

Cat. No. 2322

The SPIFE 4000 Acid Hemoglobin method is intended for the qualitative determination of hemoglobins using agar in acidic buffer on the SPIFE 4000 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 HbA₂ contains alpha and delta chains. Two other chains are formed in the embryo. The major hemoglobin in the erythrocytes of the normal adult is HbA, but there are small amounts of HbA₂ 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. Wintrobe¹ 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 4000 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 hemolysates prepared from washed, packed cells are automatically applied to the SPIFE 4000 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 4000 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 one year 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 precipitates 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. Pour the entire contents of the Destain Additive bottle into the prepared Destain and mix.

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.

5. Destain Additive

Ingredients: The product is a wetting agent.

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

Preparation for Use: Pour the entire contents of the Destain Additive bottle into 11 L of prepared Citric Acid Destain.

Storage and Stability: The additive should be stored at 15 to 30°C and is stable until the expiration date indicated on the package.

Signs of Deterioration: The additive should be free of precipitate.

INSTRUMENT

A SPIFE 4000 must be used to apply samples, electrophorese, stain, destain and dry the gels. Refer to the 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 hemolysates 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 samples 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 4000 Acid Hemoglobin Kit (Cat. No. 2322). Individual items are not available.

SPIFE 4000 Acid Hemoglobin Gels (10)

Acid Blue Stain (1 vial)

Hemolysate Reagent (25 mL)

Citric Acid Destain (1 pkg)

SPIFE 4000 Blotter C (10)

SPIFE 4000 Applicator Blades (10)

Destain Additive (28 mL)

Materials available but not contained in the kit:

ITEM	CAT. NO.
SPIFE 4000 Analyzer	1620, 1621
Gel Block Remover	1115
AFSC Hemo Control	5331
SPIFE 4000 White Sample Trays	2315
SPIFE 4000 Cassettes	1630
SPIFE 4000 Maintenance Blotters	2307
SPIFE 4000 Gel Staging Lid	2308
SPIFE 4000 Replacement Electrodes	1625

Materials needed but not provided:

5% glacial acetic acid

0.85% saline

STEP BY STEP METHOD

I. SPIFE 4000 Preparation

1. Place SPIFE 4000 Applicator Blades (one per gel) beginning in the first position at the top of the Applicator Tray.
2. Fill the designated bottles with deionized water and destain.
3. Add prepared acid blue stain to the appropriate stain bottle. The stain must be replaced after processing ten gels to avoid contamination.
4. Fill the DI Water Surfactant jar with deionized water and replace the lid and tubing. Ensure that the ends of the tubing are below the water level.
5. Remove the antisera/water reservoir from the antisera station. Lift the cover, fill the "H₂O" well and replace the cover.
6. Turn on the SPIFE 4000. Wait about 3 minutes after turning on the lower unit. Click the SPIFE 4000 icon on the screen for the instrument to initialize.

7. Using the prompts, prime the surfactant delivery system according to the instructions in the Operator's Manual.

II. Sample and Gel Preparation

1. Prepare hemolysates of patient specimens and controls as instructed in the "Specimen Preparation" section.
2. With the notch in the SPIFE 4000 White sample tray on the left, hand pipette 40 µL of patient or control hemolysate into each sample cup in the top row. Load samples in cups 1-7, skip the smaller cup in the center of the row and then load samples in cups 8-14.
3. Stack the appropriate number of disposable sample trays, with samples loaded, into the sample tray holder (one tray per gel), placing the first tray on the bottom. Place an additional empty sample tray on top of the stack to prevent evaporation of sample.
4. Carefully open one end of the pouch, remove the gel from the protective packaging and discard the overlay.
5. Using a SPIFE 4000 Blotter C, gently blot the entire gel. Discard the blotter.
6. Hold the gel so that the barcode is at the top. Place the gel into the cassette by holding the gel backing in one hand and gently bending the gel. Slide each end of the gel backing into the slots of the cassette to hold it in place. Align the cutout in the gel backing with the alignment pin in the cassette.
7. Ensure the gel blocks make good contact with the electrodes to prevent skewed patterns.
8. Place the cassette with the gel into the humidior and cover the topmost cassette with the Gel Staging Lid. Close the humidior lid to minimize gel dehydration.
9. Repeat Steps 4-8 for each gel needed.

NOTE: A maximum of 3 gels can be run at a time.

III. Electrophoresis Parameters

Using the instructions provided in the Operator's Manual, select

-- **Test Type: Acid Hb**

Test Name: Acid Hb

Check the programmed parameters for each of the following processes.

Sample Application

Electrophoresis

Predry

Stain

Destain

Dry

Sample Application	Applicator Load Time (mm:ss)	01:00	
	Applicator Load Speed	85	
	Application Rows	1	
	Row 1 Location (mm from gel edge):	55.0	
	Apply Time (mm:ss)	01:00	
	Apply Cycles	1	
	Absorption Time (mm:ss)	00:00	
	Inter-Gel Start Delay (mm:ss)	22:00	
	Electrophoresis	Voltage	160
		Minimum Current (mA)	10
Maximum Current (mA)		100	
Temperature (°C)		18	
Pre-Dry	Time (hh:mm:ss)	00:20:00	
	Temperature (°C)	62	
Stain	Time (hh:mm:ss)	00:10:00	
	Stain Type	Acid Blue	
Destain	Absorption Time	00:04:00	
	Cycles	4	
Dry	Time (hh:mm:ss)	00:02:00	
	Agitate	Yes	
	Temperature (°C)	60	
	Time (hh:mm:ss)	00:11:00	

IV. Electrophoresis

1. Click **START** on the screen and respond to the analyzer prompts. The analyzer will apply samples, electrophorese, stain, destain and dry the gel(s).
2. The cassette with the gel will be dropped into the cassette receptacle.
3. Remove the cassette(s) from the receptacle. If gel storage is required, remove and discard the two gel blocks. Clean or wipe the non-gel side. If not, discard the used gels, applicator blades and sample trays as biohazardous waste.
4. Cassettes and carbon electrodes should be washed and dried after each use with deionized water. Refer to the Operator's Manual for instructions.

Evaluation of the Hemoglobin Bands

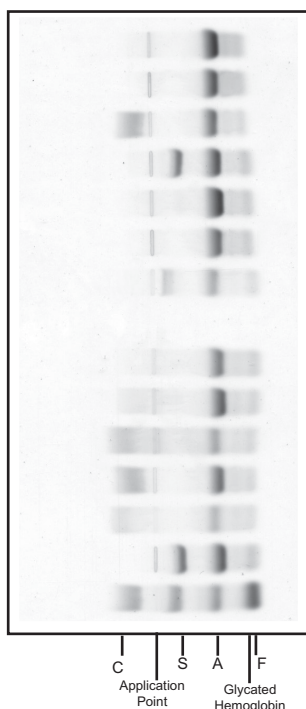
The hemoglobin gel should be inspected visually for the presence of abnormal hemoglobin bands. Glycated hemoglobin migrates with HbF. The Helena AFSC Hemo Control provides a marker for band identification.

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 4000 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 4000 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 HbA₂ are also present. At the end of the first year of life and through adulthood, the major hemoglobin present is HbA with up to 3.5% HbA₂ and less than 2% HbF.

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 or 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 cruel and lethal disease. It first manifests itself at about 5 to 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.^{9,10} 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.¹⁰

The most common hemoglobin abnormalities:

Sickle Cell Trait

This is a heterozygous state showing HbA and HbS and a normal amount of HbA₂ 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 HbA₂.

In Sickle Cell β^0 -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 HbA₂.

BIBLIOGRAPHY

1. Wintrobe, Maxwell M., Clinical Hematology, 6th Edition, LeFebiger, Philadelphia, 1967.
2. Fairbanks, V.F., Diagnostic Medicine, Nov/Dec., 53-58, 1980.
3. Schneider, R.G., et al., Laboratory Identification of the Hemoglobins, Lab Management, August, 29-43, 1981.
4. Center for Disease Control, Laboratory Methods for Detecting Hemoglobinopathies, U.S. Department of Health and Human Services/Public Health Service, 1984.
5. Schneider, R.G., Methods for Detection of Hemoglobin Variants and Hemoglobinopathies in the Routine Clinical Laboratory, CRC Critical Reviews in Clinical Laboratory Sciences, 1978.
6. Schneider, R.G., et al., Abnormal Hemoglobins in a Quarter Million People, Blood, 48(5):629-637, 1976.
7. Huisman, T.H.J. and Schroeder, W.A., New Aspects of the Structure, Function, and Synthesis of Hemoglobins, CRC Press, Cleveland, 1971.
8. Schmidt, R.M., et al, The Detection of Hemoglobinopathies, CRC Press, Cleveland 1974.
9. Weatherall, D.J. and Clegg, J.B., The Thalassemia Syndromes, Blackwell Scientific Publications, Oxford, 1972.
10. Lehman, H. and Huntsman, R.G., Man's Haemoglobins, J.B. Lippincott Co., Philadelphia, 1974.

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