SPIFE® 4000 Alkaline Hemoglobin Procedure

Cat. No. 2320

The SPIFE 4000 Alkaline Hemoglobin Electrophoresis Procedure is intended for the qualitative and semi-quantitative determination of hemoglobins using agarose electrophoresis in alkaline buffer on the SPIFE 4000 system. The system is a screening method for in-vitro diagnostic use.

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. Two other chains are formed in the embryo.

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)
(3) Developmental anomalies (e.g. hereditary persistence of fetal hemo-globin (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.[3] The protocol for hemoglobin electrophoresis involves stepwise use of two systems.[4-9] Initial electrophoresis is performed in alkaline buffers. Cellulose acetate used to be the major support medium used; however, agarose also yields rapid separation of HbA, F, S and C and many other mutants with minimal preparation time. 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 alkaline electrophoretic buffer and the agarose support. The SPIFE 4000 Alkaline Hemoglobin procedure 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 packed cells are automatically applied to the SPIFE 4000 Alkaline Hemoglobin gel. The hemoglobins in the sample are separated by electrophoresis using an alkaline buffer and are stained with Acid Blue Stain. The patterns are scanned on a densitometer, and the relative percent of each band is determined.

REAGENTS

1. **SPIFE 4000 Alkaline Hemoglobin Gel**
   Ingredients: Each gel contains agarose in tris, glycine buffer with 0.05% EDTA and sodium azide as a preservative.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. To prevent the formation of toxic vapors, sodium azide should not be mixed with acidic solutions. When discarding reagents containing sodium azide, always flush sink with copious quantities of water. This will prevent the formation of metallic azides which, when highly concentrated in metal, are potentially explosive. In addition to purging with water, plumbing should occasionally be decontaminated with 10% NaOH.
   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% 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 is an aqueous solution of 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: The reagent should be a clear, pale yellow solution.
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.
5. **Destain Additive**
   Ingredients: The product is a wetting agent.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. IRRITANT–DO NOT INGEST.
   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 instrument must be used to load the sample tray (if appropriate), apply samples, electrophorese, stain, destain, dry and scan 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.

1. Whole Blood sample
   Prepare washed, packed cells from whole blood.
   a. Centrifuge anticoagulated blood for 10 minutes to separate cells from plasma.
   b. Remove plasma.
   c. Wash packed cells 3 times by resuspending in 5 to 10 volumes of normal saline solution (0.85% NaCl), centrifuging and aspirating supernatant.
   d. For automated sample tray loading, a minimum of 500 µL of lysate is needed. Prepare patient sample lysates by mixing one part (50 µL) washed, packed cells with ten parts (500 µL) Hemolysate Reagent.
   e. For manual sample tray loading, prepare patient sample lysates by mixing one part (10 µL) washed, packed cells with ten parts (100 µL) Hemolysate Reagent.
   f. Vortex or shake vigorously for 15 seconds. It is important that the cells be completely lysed (forming a clear red hemolysate) before applying the sample to the gel.
2. Control
   a. For automated sample tray loading, a minimum of 500 µL of lysate is needed. Prepare AFSC (Cat. No. 5331) control lysate using a 1:2 dilution with Hemolysate Reagent. Mix one part AFSC control with one part Hemolysate Reagent. AFSC control lysate can be stored at 2 to 8ºC for four months.
   b. For manual sample tray loading, prepare AFSC control lysate using a 1:2 dilution with Hemolysate Reagent. Mix one part (50 µL) AFSC control with one part (50 µL) Hemolysate Reagent.

PROCEDURE

Materials provided: The following materials needed for the procedure are contained in the SPIFE 4000 Alkaline Hemoglobin Kit (Cat. No. 2320). Individual items are not available.

SPIFE 4000 Alkaline 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 Alkaline Hb Cassettes and Electrodes	2324
SPIFE 4000 Maintenance Blotters	2307
SPIFE 4000 Gel Staging Lid	2308

Materials needed but not provided:

5% acetic acid
0.85% saline

STEP BY STEP METHOD

I. SPIFE 4000 Preparation

1. Place a SPIFE 4000 Applicator Blade 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 "H_2O" 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 Preparation

Prepare lysates of patient specimens and AFSC control as instructed in the "Specimen Preparation" section.

NOTE: Only one gel can be run at a time.

1. For lysate volumes measuring 500 µL or more, the lysates can be automatically dispensed into the sample tray. For lysate volumes measuring less than 500 µL, 40 µL of each lysate must be hand-pipetted into the sample tray for application.
   NOTE: Only 10 samples may be run on each gel.
2. For automated sample tray loading, place test tubes containing 500 µL of normal saline in spaces 1 and 2 of the carousel. Load sample test tubes into the carousel counterclockwise beginning at the space numbered 3. NOTE: The control should be placed in space 7. Place a SPIFE 4000 White Sample Tray in the sample tray holder. Proceed to Section III.
3. For manual sample tray loading, with the notch in the SPIFE 4000 White Sample Tray on the left, hand-pipette 40 µL of patient or control lysate into each sample cup in the top row. Skip the first two cups, load samples into cups 3-7, skip the smaller cup in the center of the row and then load samples into cups 8-12. NOTE: The control should be loaded into cup 7.
4. Manually place the sample tray, with samples loaded, into the interior of the sample tray handler.
   NOTE: Do not put a sample tray on top of the loaded sample tray.

III. Gel Preparation

1. Carefully open one end of the pouch, remove the gel from the protective packaging and discard the overlay.
2. Using a SPIFE 4000 Blotter C, gently blot the entire gel. Discard the blotter. Wipe the back of the gel with an alcohol pad and then with a dry wipe before placing in the cassette.
3. Hold the gel so that the barcode is at the top. Place the gel into the SPIFE 4000 Alkaline Hb 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 cut out in the gel backing with the alignment pin in the cassette.
4. Ensure the gel blocks make good contact with the electrodes to prevent skewed patterns.
5. Place the cassette with the gel into the humidor and cover with the Gel Staging Lid. Close the humidor lid to minimize gel dehydration.
   NOTE: Only one gel can be run at a time.
6. For automated sample tray loading, proceed to Step IV.1. For manual sample tray loading, proceed to IV.2.

IV. Electrophoresis Parameters

1. Volume of 500 µL or Greater

a. Using the instructions provided in the Operator's Manual, select:

-- Test Type: Alkaline Hemoglobin
Test Name: Alkaline Hemoglobin

Check the programmed parameters for each of the following processes.

Sample Preparation
Sample Application
Electrophoresis
Predry
Stain
Destain
Dry
Scan

Sample Preparation	Volume (µL)	40
	Primary Wash Time (mm:ss)	00:02
	Primary Wash Cycles	1
	Samples per gel	12
	Application per sample	1
	Dilutions 1-12	Neat
Sample Application	Applicator Load Time (mm:ss)	01:00
	Applicator Load Speed	95
	Application Rows	1
	Row 1 Location (mm from gel edge):	35.0
	Apply Time (mm:ss)	01:00
	Apply Cycles	1
	Absorption Time (mm:ss)	00:00
	Inter-Gel Start Delay (mm:ss)	14:00
Electrophoresis	Voltage	575
	Minimum Current (mA)	10
	Maximum Current (mA)	100
	Temperature (°C)	16
	Time (hh:mm:ss)	00:13:30
Pre-Dry	Temperature (°C)	60
	Time (hh:mm:ss)	00:14:00
Stain	Stain Type	Acid Blue
	Absorption Time (hh:mm:ss)	00:04:00
Destain	Cycles	4
	Time (hh:mm:ss)	00:02:00
	Agitate	Yes
Dry	Temperature (°C)	60
	Time (hh:mm:ss)	00:12:00
Scan	Sequence	7, 3-6, 8-12
	Aperture	4
	Gain Mode	Auto
	Smooth	No
	Auto Interpretation	No
	Image Contrast	0.0
	Fraction Detection	
	Sensitivity	3.0
	Force Fractions	No
	Combine Split Beta	No

b. Proceed with the testing according to steps in Section V.

2. Volume less than 500 µL

a. The SPIFE 4000 User Setup menu should be used to create "Add a Test" parameters for small volumes of lysate that need to be manually pipetted. The test parameters will be the same as those given in Section IV.1. except "Sample Preparation" will not be checked.

b. Proceed with the testing according to steps in Section V.

V. Electrophoresis

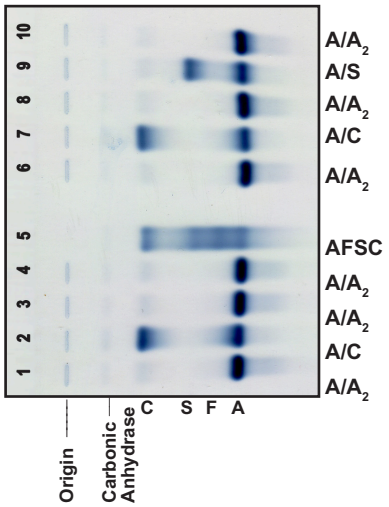
1. Click **START** on the screen and respond to the analyzer prompts. The analyzer will load the sample tray (if appropriate), apply samples, electrophorese, stain, destain, dry and scan the gel.
2. The cassette with the gel will be dropped into the cassette receptacle.
3. Remove the cassette from the 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 gel, applicator blade and sample tray as biohazardous waste.
4. The cassette and electrodes should be washed with deionized water and dried after each use. Refer to the Operator's Manual for instructions.

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

Quality Control: Use of AFSC Hemo Control (Cat. No. 5331) is recommended with each run. The control should be in lane 5 on the gel. The control should be used as a marker for the location of particular hemoglobin bands. It may be quantitated for verification of the accuracy of the procedure. See "LIMITATIONS" section. Refer to the package insert provided with the control for assay values and migration patterns.

RESULTS

Figure 1 illustrates the electrophoretic mobility of bands on the SPIFE 4000 Alkaline Hemoglobin Gel.



SPIFE 4000 ALK HEMOGLOBINS

LIMITATIONS

Some abnormal hemoglobins have similar electrophoretic mobilities and must be differentiated by other methodologies.

Further testing required:

1. Citrate agar electrophoresis may be a necessary follow-up test for confirmation of abnormal hemoglobins detected.
2. Globin chain analysis (both acid and alkaline) and structural studies may be necessary in order to positively identify some of the more rare hemoglobins.
3. Low levels of HbF (1 to 10%) may be accurately quantitated using any commercially available HbF method.

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.[3] A study of forty-seven (47) normal adult specimens was done using the SPIFE system. The data was as follows:

HbA - 97.1% - 99.1%
HbA_2 - 0.9% - 2.9%

These values should only serve as guidelines. Each laboratory should establish its own range.

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 HbS-S, HbS-D-Los Angeles and HbS-O 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 5-6 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 β°-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.

SPECIFIC PERFORMANCE CHARACTERISTICS

PRECISION

Within Run precision was evaluated using an AFSC control in replicate analyses on a single gel. N = 10

Hemoglobin Fraction	Mean %	SD	CV
A	29.2	1.2	4.1%
F	25.7	1.4	5.4%
S	27.8	0.8	2.9%
C	17.4	0.7	4.0%

Between Run precision was evaluated with an AFSC control specimen run in replicate on 6 gels. N = 24

Hemoglobin Fraction	Mean %	SD	CV
A	28.7	1.0	3.5%
F	26.3	1.8	6.9%
S	26.9	1.8	6.8%
C	18.2	1.5	8.1%

CORRELATION

53 patient samples (both normal and abnormal) were run using the SPIFE Alkaline Hemoglobin method on the SPIFE 3000 with the following correlation:

N = 53
Y = 1.0184X - 0.6553
R = 0.9989
X = SPIFE 3000 Alkaline Hemoglobin
Y = SPIFE 4000 Alkaline Hemoglobin

BIBLIOGRAPHY

1. Wintrobe, Maxwell M., Clinical Hematology, 6th Edition, Lea and Febiger, Philadelphia, 1967.
2. Fairbanks, V.F., Diagnostic Medicine, Nov/Dec., 53-58, 1980.
3. Tietz, N.W., Clinical Guide to Laboratory Tests, 3rd Edition, W.B. Saunders Co. Philadelphia, 1995.
4. Schneider, R.G., et al., Laboratory Identification of the Hemoglobins, Lab Management, August, 29-43, 1981.
5. Center for Disease Control, Laboratory Methods for Detecting Hemoglobinopathies, U.S. Department of Health and Human Services/Public Health Service, 1984.
6. Schneider, R.G., Methods for Detection of Hemoglobin Variants and Hemoglobinopathies in the Routine Clinical Laboratory, CRC Critical Reviews in Clinical Laboratory Sciences, 1978.
7. Schneider, R.G., et al., Abnormal Hemoglobins in a Quarter Million People, Blood, 48(5):629-637, 1976.
8. Huisman, T.H.J. and Schroeder, W.A., New Aspects of the Structure, Function, and Synthesis of Hemoglobins. CRC Press, Cleveland, 1971.
9. Schmidt, R.M., et al., The Detection of Hemoglobinopathies. CRC Press, Cleveland, 1974.
10. Weatherall, D.J. and Clegg, J.B., The Thalassemia Syndromes, Blackwell Scientific Publications, Oxford, 1972.
11. Lehman, H. and Huntsman, R.G., Man's Haemoglobins, J.B. Lippincott Co., Philadelphia, 1974.

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

HELENA LABORATORIES
Beaumont, Texas USA 77704

Pro. 217
6/17(4)

Shaded areas indicate that the text has been modified, added or deleted.