SPIFE® 3000 Alkaline Hemoglobin Procedure For Plastic Blades

Cat. No. 3415

The SPIFE Alkaline Hemoglobin method is intended for the qualitative and semi-quantitative determination of hemoglobins using agarose electrophoresis in alkaline buffer on the SPIFE 3000 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 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.[3] The protocol for hemoglobin electrophoresis involves stepwise use of two systems.[4-9] Initial electrophoresis is performed in alkaline buffer. Cellulose acetate used to be the major support medium used, but agarose also 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 alkaline electrophoretic buffer and the agarose support. The SPIFE Alkaline Hemoglobin procedure is a simple procedure requiring minute quantities of sample lysate to provide a screening method for the presence of abnormal hemoglobins such as HbS, HbC and HbF.

PRINCIPLE

Very small quantities of lysates prepared from washed, packed cells are automatically applied to the SPIFE 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 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 1L 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.

INSTRUMENT

A SPIFE 3000 Analyzer must be used to apply samples, electrophorese, stain, destain and dry the gels. The gels may be scanned on a separate densitometer such as the QuickScan Touch/2000 (Cat. No. 1690/1660). Refer to the appropriate Operator's Manuals 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 washed, packed cells may be stored up to 1 week at 2 to 8°C. Frozen samples may produce an artifact band anodic to HbA.
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
AA_2 (Cat. No. 5328) no dilution is necessary
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 Alkaline Hemoglobin Kit (Cat. No. 3415). Individual items are not available.

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

Materials available but not contained in the kit:

ITEM	CAT. NO.
SPIFE 3000 Analyzer	1088
QuickScan Touch	1690
QuickScan 2000	1660
AFSC Hemo Control	5331
AA_2 Hemo Control	5328
REP Prep	3100
Gel Block Remover	1115
Applicator Blade Weights	3387
Disposable Sample Cups	3369
SPIFE Dispo Cup Tray	3370
Applicator Blade Weights	3387
SPIFE Alkaline Hb Electrodes	3707

Materials needed but not provided:

5% 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. Place the Applicator Blade into the vertical slots numbered 2 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.
3. Place an Applicator Blade Weight on top of the Applicator Blade. When placing the weight on the blade, position the weight with the thick side to the right.
4. Slide the Disposable Sample Cup strip into the top channel of the Cup Tray (numbered 1 to 20).
5. Pipette 17 µL of patient or control lysates into the Disposable Cups. Cover until ready for use.

II. Gel Application

1. Remove the gel from the protective packaging and discard the overlay.
2. Place a SPIFE Blotter C on the gel with the longer edge parallel with gel blocks. Gently blot the entire surface of the gel using slight fingertip pressure on the blotter, and remove the blotter.
3. Dispense approximately 2 mL of REP Prep onto left side of SPIFE chamber.
4. Place the left edge of the gel over REP Prep aligning the round hole on the left pin. Gently lay the gel down on the REP Prep, starting from the left side 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 <u>flat</u> 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 square 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 **ALKALINE HEMOGLOBIN** option appears on the displays.

III. Electrophoresis/Staining Parameters

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

***A dry time of 30 minutes is recommended. Due to variation in environmental conditions, a dry time range of 20-40 minutes is acceptable.**

Electrophoresis Unit

1) No Prompt Load Sample 1	00:30	20°C	SPD 4	
2) No Prompt Apply Sample 1	00:30	20°C	SPD 4	LOC 1
3) No Prompt Electrophoresis 1	20:00	21°C	725V	100mA
4) No prompt END OF TEST				

Stainer Unit

1) No Prompt Stain 1	4:00	REC = OFF	VALVE = 3
2) No Prompt Destain 1	0:30	REC = ON	VALVE = 2
3) No Prompt Dry 1	*30:00	70°C	
4) No Prompt Destain 2	1:30	REC = ON	VALVE = 2
5) No Prompt Destain 3	1:30	REC = ON	VALVE = 2
6) No Prompt Dry 2	7:00	70°C	
7) No Prompt END OF TEST			

IV. Electrophoresis

1. Open the chamber lid. Place the Cup Tray with sample lysates on the instrument. Align the holes in the tray with the pins on the instrument. Close the chamber lid.
2. With **ALKALINE 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. Dispose of blade and cups as biohazardous waste.

V. Visualization

1. After electrophoresis is complete, open the chamber lid and use the Gel Block Remover to remove both gel blocks from the gel. Lift the Gel Holder from the stainer chamber. Attach the gel to the holder by placing the round hole in the gel mylar over the left pin on the holder and the obround hole over the right pin on the holder.
2. Place the Gel Holder with the attached gel facing backwards into the stainer chamber.
3. With **ALKALINE 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** again to begin. The instrument will stain, destain and dry the gel.
4. When the process is complete, the instrument will beep. Remove the Gel Holder from the stainer. Take the gel off of the holder and replace the holder.

VI. Evaluation of the Hemoglobin Bands

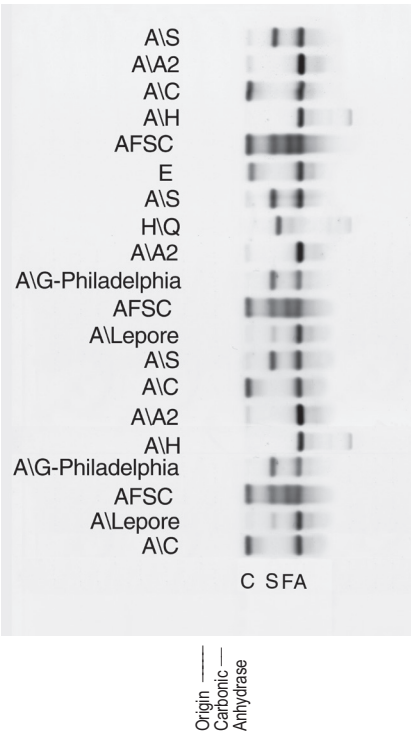
1. **Qualitative evaluation:** The hemoglobin gels may be inspected visually for the presence of hemoglobin bands. The Helena Hemo Controls provide a marker for band identification.
2. **Quantitative evaluation:** Determine the relative percent of each hemoglobin band by scanning the dried gels <u>agarose side up</u> on the QuickScan Touch/2000 using the Acid Blue setting. A slit size of 4 is recommended. Verify that the default setting for Smoothing is "No". "Autoslope" may be used with this test.

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

Quality Control: Two controls for hemoglobin electrophoresis are available from Helena Laboratories: AA_2 Hemo Control (Cat. No. 5328) and AFSC Hemo Control (Cat. No. 5331). The controls should be used as markers for the location of particular hemoglobin bands. They may be quantitated for verification of the accuracy of the procedure (see "LIMITATIONS" section). Refer to the package insert provided with the controls for assay values and migration patterns. Use at least one of these controls on each gel run.

RESULTS

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



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 quantitized 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 47 normal adult specimens was done using the SPIFE system with the following results:

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, 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 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 run twenty times on one gel. n = 20

Hemoglobin Fraction	Mean %	SD	CV
A	28.7	0.8	2.8%
F	28.6	0.8	2.8%
S	23.7	0.6	2.6%
C	19.1	0.2	0.9%

Between Run precision was evaluated with an AFSC control specimen run in replicate on seven gels. n = 140

Hemoglobin Fraction	Mean %	SD	CV
A	28.8	1.2	4.2%
F	28.4	1.0	3.7%
S	24.0	0.8	3.2%
C	18.9	0.7	3.6%

CORRELATION

121 patient samples (both normal and abnormal) were run using the SPIFE Alkaline Hemoglobin method on both the SPIFE 2000 and SPIFE 3000 with the following correlation:

n = 121
Y = 1.002X - 0.103
R = 0.9996
X = SPIFE Alkaline Hemoglobin on SPIFE 2000
Y = SPIFE Alkaline Hemoglobin on SPIFE 3000

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