SPIFE® Nexus Acid Hemoglobin Procedure

Cat. No. 2418

INTENDED USE

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

For In Vitro Diagnostic Use.

Rx Only

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 the 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]

Gel electrophoresis is routinely used for identifying hemoglobinopathies. The protocol for hemoglobin electrophoresis involves stepwise use of two systems.[3-8]

Initial electrophoresis testing is performed in an alkaline buffer system, followed by further typing using acid buffers, which measures a property other than electrical charge. Historically, cellulose acetate with alkaline buffers was used to rapidly separate HbA, F, S and C and other variants. Further testing using citrate agar with acid buffers was used to differentiate between variants with similar electrophoresis properties. This testing can now be performed using acid and alkaline buffers on agarose gel with greater automation.

This method is based on the complex interactions of hemoglobin with an acid electrophoretic buffer and agar support media. The SPIFE Nexus Acid Hemoglobin method is a simple procedure requiring minute quantities of hemolysate to provide specific (but not absolute) confirmation of 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 Nexus 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. **SPIFE Nexus Blue**

   Ingredients: The stain contains 0.5% (w/v) acid blue stain, 5% acetic acid and surfactant.

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

   Preparation for Use: The stain is ready for use as packaged.

   Storage and Stability: Stable for one year stored at 15 to 30°C in a closed container.

   Signs of Deterioration: The prepared stain should be a homogeneous mixture free of precipitate. Discard if precipitate forms.

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 precipates 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 Nexus analyzer must be used to apply samples, electrophorese, stain, destain, dry and then scan the gels. The gels may also be scanned on a separate densitometer such as the QuickScan Touch Plus (Cat. No. 1640). 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 at application, between HbS and HbC, 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 hemolysates 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)

Alternate control preparation 2:1 (2 parts control + 1 part Hemolysate Reagent)

PROCEDURE

Materials provided: The following materials needed for the procedure are contained in the SPIFE Nexus Acid Hemoglobin Kit (Cat. No. 2418). Individual items are not available.

SPIFE Acid Hemoglobin Gels (10)
SPIFE Nexus Blue (1 vial)
Hemolysate Reagent (2 bottles)
Citric Acid Destain (2 pkgs)
REP Blotter C (10)
Serrated Blade Applicator Kit, 20 Sample (10)

Materials available but not contained in the kit:

ITEM	CAT. NO.
SPIFE Nexus Analyzer	1650
QuickScan Touch Plus	1640
Gel Block Remover	1115
AFSC Hemo Control	5331
SPIFE Dispo Sample Cups	3369
SPIFE 20,40,60 Dispo Cup Tray	3370
SPIFE Nexus Cassette	2580
SPIFE Nexus Applicator Templates	2570
SPIFE Nexus Applicator Blade Weights	2572
SPIFE Nexus Dispo Stain Cups	2575
Pos ID Barcode Labels for Touch and SPIFE Nexus Systems	1696
REP Prep	3100
SPIFE Nexus Reagent Roller	2583
SPIFE Nexus Ready Run Kit	2582
SPIFE Nexus Carbon Electrode Insert	2576

Materials needed but not provided:

0.85% saline

STEP BY STEP METHOD

I. Sample Preparation

A. Prepare lysates of patient specimens and controls as instructed in the "Specimen Preparation" section.

B. Slide the Disposable Sample Cup strip into the center channel of the Cup Tray (numbered 21 to 40). Ensure the Sample Cup strip is fully inserted to allow correct placement of tray onto platform.

C. Pipette 17 µL of patient or control lysate into each of the Disposable Cups. Cover until ready for use.

II. SPIFE Nexus Preparation

A. Fill designated bottles with 0.85% saline, deionized water, and destain.

B. Turn on the SPIFE Nexus. Click on the SPIFE Nexus icon to initialize.

C. If this is the first test of the day, prime the instrument according to the instructions in the SPIFE Nexus Operator's Manual.

D. Open the main door of the instrument and prepare the items onboard the instrument.

1. Ensure that the following items are in their respective onboard storage locations: **Platen Cover** with the Carbon Electrode Insert and **Dryer Cover** with the red sticker toward the back of the instrument.
2. Place the prepared **SAMPLE CUP TRAY** onto the sample tray platform.

3. Stain/Reagent Dispenser

a. Fill three Stain Cups each with 700 µL of SPIFE Nexus Blue stain and place a Stain Cup in each slot of the Stain/Reagent Dispenser.

b. Place a clean Reagent Roller bar between the hooks on the Stain/Reagent Dispenser.

4. Consumables Tray

a. Slide the Consumables Tray forward from its home position.

b. Prepare the Applicator Holder

(1) Place an Acid HB Applicator Blade Template (Applicator Blade Template 3) on top of the Applicator Holder.

(2) Place an Applicator Blade in the designated slot corresponding to the sample cups loaded within the sample tray. **NOTE: The Applicator Blades will only fit into the slots in the Applicator Holder one way; do not try to force the Applicator Blades into the slots.**

(3) Place the Applicator Blade Weight on top of the Applicator Blade with the thick side facing the front of the instrument.

c. Slide the Consumables Tray into position in the back of the instrument.

5. Gel Cassette

a. Place the bottom half of the Gel Cassette on the electrophoresis platen with the two pins lined up on the left side.
b. Dispense 2 mL of REP Prep on the platen.
c. Remove the gel from the protective packaging and discard the overlay.
d. Using a REP Blotter C, gently blot the entire gel. Discard the blotter.
e. Place the left edge of the gel into the bottom of the cassette fitting the round hole over the upper pin and the obround hole over the lower pin. Gently lay the gel down over the REP Prep making sure no bubbles remain under the gel.
f. Place the top half of the Gel Cassette over the gel. Make sure the 2D barcode is located in the upper right corner of the cassette.
g. Place a Positive ID Barcode Label on the upper right hand side of the gel backing. Select the barcode that starts with the letter “G”.

E. Close the main door of the instrument.

III. Automated Gel Electrophoresis

A. Click the Start button on the menu bar. Select the **SPIFE Acid Hemoglobin 20 (Acid Blue)** test name from the drop down menu. Ensure the toggles for all Run Processes are set to “Yes”, with the exception of Sample Load which should be set to "No" and click the Start Run button. The analyzer will load samples when appropriate, apply samples, electrophorese, stain, destain, dry and scan the gel. For details of Automated Gel Electrophoresis, contact Technical Services.

B. After scanning, the Gel Cassette with the finished gel will be located in the scanner port on the front side of the instrument. If gel storage is required, remove and discard the two gel blocks.

C. After every test: discard the used blotters, Applicator Blades, Stain Cups and sample cups as biohazardous waste. Clean any residual stain from the electrophoresis platen, Gel Cassette and the Reagent Roller bar. For daily, weekly, and monthly maintenance reference the SPIFE Nexus Operator's Manual.

Evaluation of the Hemoglobin Bands

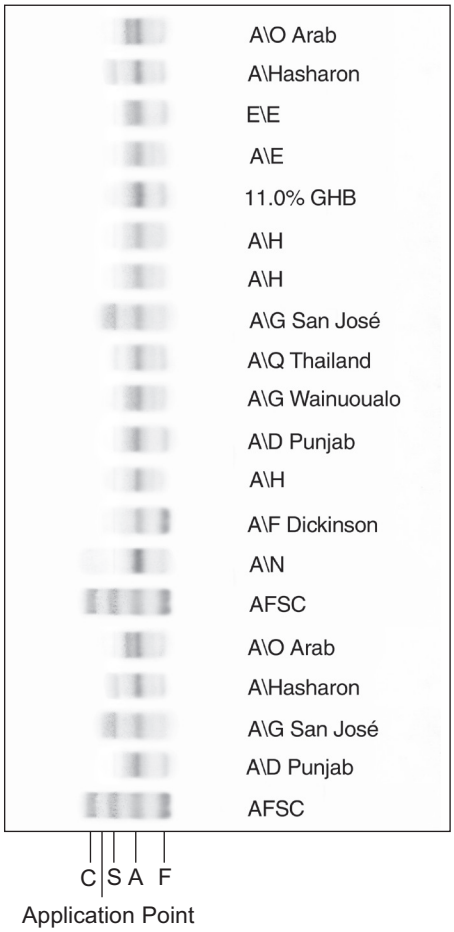
The hemoglobin gel should be inspected visually for the presence of abnormal hemoglobin bands. Glycated hemoglobin migrates with HbF. The Helena AFSC Hemo Control provides a marker for band identification.

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 Nexus 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 Nexus Acid Hemoglobin Gel.



LIMITATIONS

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

1. Due to the high prevalence of HbF the sample may need to be redrawn in one or two weeks.
2. Molecular methods (DNA Analysis) may be required for definitive diagnosis.
3. The relative migration of a hemoglobin variant is concentration dependent, with variants at a lower concentration (g/dL) migrating further from the application point. The migration difference between control vs sample can be mitigated by either preparing the hemoglobin control at a higher concentration (two parts control to one part hemolysate) or diluting the patient sample with hemolysate to normalize the concentration between the two.

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 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.[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 under alkaline condition. Results under acid conditions 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.

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