SPIFE® Touch Hemoglobin IEF Procedure

Cat. No. 3428

The SPIFE Touch Hemoglobin IEF procedure is intended for the qualitative identification of hemoglobin variants using isoelectric focusing on the SPIFE Touch system. The system is used to aid in the diagnosis of hemoglobinopathies.

SUMMARY

Hemoglobins (Hb) 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), and (3) development 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 screening and diagnosis of hemoglobinopathies. Routine testing of adults and newborns consists of alkaline electrophoresis followed by citrate agar electrophoresis, in order to confirm the presence of HbS or HbS-like variants (as HbG-Philadelphia and D-Los Angeles) or the presence of HbO-Arab and HbE, which are indistinguishable from HbA on alkaline electrophoresis. The resolving power of these systems is rather poor for many abnormal hemoglobins, however, and positive identification must be determined by other methods, such as globin chain electrophoresis described by Schneider.[3]

Pioneering work by Vesterberg and Svensson[4] in the manufacture and use of ampholyte buffers has enabled isoelectric focusing to become a powerful diagnostic tool in the identification of hemoglobinopathies. Other methods of technology have been described by Drysdale, Righetti, and Bunn;[5] Basset, Beuzard, Garel, and Rosa;[6] Monte, Beuzard, and Rosa;[7] and Galacteros, Kleman, Caburi-Martin, Rosa, and Lubin.[8]

Because the isoelectric point of any protein is an absolute value, isoelectric focusing may reveal many more abnormal hemoglobins than conventional electrophoretic or column methods which, when combined with interpretation of clinical data, minimizes the chance of missing a “silent” variant.

PRINCIPLE

By using ampholyte buffers appropriate for isoelectric focusing of hemoglobin (pH 6-8), the separation and identification of many abnormal hemoglobins, indistinguishable from other more common forms on alkaline and acidic electrophoresis, are possible since the migration is only affected by the isoelectric point of the protein. Hemoglobins with a pH difference of less than 0.05 pH units could be resolved.

REAGENTS

1. **SPIFE Hemoglobin IEF Gel**

   Ingredients: Each gel contains 1% w/v agarose, 5.3% v/v carrier ampholytes and 0.01% thymol as a preservative.

   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY.

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

   Storage and Stability: The gels should be stored horizontally at 15 to 30°C, in the protective packaging, and are stable until the expiration date indicated on the package. **DO NOT FREEZE THE GELS OR EXPOSE THEM TO EXCESSIVE HEAT.**

   Signs of Deterioration: Discard the gel if any of the following conditions occur, indicating deterioration of the gel: (1) crystalline appearance indicating the agar has been frozen, (2) cracking and peeling indicating drying of the agar, (3) bacterial growth indicating contamination.

2. **Hemolysate Reagent**

   Ingredients: The reagent is an aqueous solution containing 0.07% potassium cyanide, 0.005 M (0.18%) ethylenediaminotetraacetate (EDTA), and 0.175% saponin.

   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. NEVER 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 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.

INSTRUMENT

A SPIFE Touch unit (Cat. No. 1068) is used to electrophorese and dry the gels. Refer to the Operator’s Manual for detailed instructions.

SPECIMEN COLLECTION AND HANDLING

Specimen: The specimen of choice is whole blood collected in EDTA tubes. However, washed, packed cells may also be used.

Specimen Storage: Whole blood specimens and packed cells may be stored at 2 to 8°C for one week.

Specimen Preparation: Specimen hemolysates must be prepared for each patient sample.

1. Whole Blood Samples
   a. Prepare the patient sample lysate using a 1:4 dilution with Hemolysate Reagent. Mix one part (25 µL) of whole blood with three parts (75 µL) Hemolysate Reagent.
   b. Vortex briefly and allow the sample to stand at least 5 minutes prior to use. It is important that the cells be completely lysed (forming a clear red hemolysate) before applying the sample to the gel.
2. Washed, Packed Cell Samples

   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 as before.

d. Make a 1:8 dilution by mixing one part (10 µL) washed, packed cells to seven parts (70 µL) Hemolysate Reagent.

e. Vortex briefly, and allow the sample to stand at least 5 minutes prior to use. It is important that the cells be completely lysed (forming a clear red hemolysate) before applying the sample to the gel.

3. Controls
   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 are provided in the SPIFE Hemoglobin IEF Kit (Cat. No. 3428). Individual items are not available.

SPIFE Hemoglobin IEF Gels (10)
Hemolysate Reagent (1 x 25 mL)

Materials needed but not contained in the kit:

Item	Cat. No.
SPIFE Touch Analyzer	1068
SPIFE IEF Electrodes and Adapter	3704
REP Prepper	1359
AFSC Control	5331
AA_2 Control	5328
SPIFE IgG IEF Square Electrode	3703

Materials needed but not provided:
0.85% NaCl

STEP-BY-STEP METHOD

NOTE: If the staining chamber was last used to stain a gel, the SPIFE Touch software has an automatic wash cycle prompted by the initiation of the SPIFE Touch Hemoglobin IEF test. To verify the status of the stainer chamber, use the arrows under the **STAINER UNIT** to select the appropriate test, place the empty Gel Holder into the stainer chamber and press **START**. If washing of the stainer chamber is necessary, the prompt "Vat must be washed. Remove gel and install gel holder." will appear. Press **RETRY** to begin the stainer wash. The cleaning process will complete automatically in about 7 minutes. To avoid delays, this wash cycle should be initiated at least 7 minutes prior to the end of electrophoresis.

I. Gel Preparation

1. Carefully open one end of the pouch, remove the gel from the protective packaging, and discard the overlay.
2. Use the REP Prepper to remove excess moisture from the sample wells.
3. Do **NOT** use REP Prep with this procedure.
4. Place left edge of gel over the chamber, aligning the round hole on the left pin. Gently lay the gel down on the chamber, starting from the left side and ending on the right side, fitting the obround hole over the right pin.
5. Use a lint-free tissue to wipe around the edges of the gel.
6. Insert the SPIFE IEF Electrode Adapter marked FRONT between the two magnetic posts located at the front of the chamber floor. Insert the IEF Electrode Adapter marked REAR between the two magnetic posts located at the back of the chamber floor.
7. Clean and wipe the three IEF electrodes with a lint-free tissue.
8. Place a round electrode into the slots created by the adapter – one on <u>each</u> outside edge. Place the square electrode in the middle. Be sure all three electrodes are seated firmly against the gel and electrode posts. Close the chamber lid.
9. Use the arrows under **SEPARATOR UNIT** to select the appropriate test. To check parameters, select Test and press **SETUP**.

II. Parameters

1. Using the instructions provided in the SPIFE Touch Operator's Manual, set up the parameters as follows:
   ***Due to Variation in environmental conditions, a dry time of 25 minutes is recommended, but a range of 25-30 minutes is acceptable.**

	<u>Separator Unit</u>
Pause	Prompt: None Time: 0:30 Temperature: 14°C
Electrophoresis	Prompt: To Continue Time: 40:00 Temperature: 14°C Voltage: 650 V mA: 50 mA
End	
	<u>Stainer Unit</u>
Dry	Prompt: None Time: 25:00* Temperature: 70°C
End	

2. Use the arrows under **SEPARATOR UNIT** to select the appropriate test. Press **START** and choose the option **PAUSE**.
3. Open the chamber lid and pipette 2 µL of sample lysate into each well. Close the chamber lid and press **START** to proceed. Sample absorption will be timed and electrophoresis will begin.
4. Open the chamber lid, and remove the electrodes and adapters.
5. Immediately remove the gel from the electrophoresis chamber.
6. Remove 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.
7. Place the Gel Holder with the attached gel facing backwards into the stainer chamber.
8. Use the arrows under **STAINER UNIT** to select the appropriate test. Press **START** and choose **DRY** to proceed. The instrument will dry the gel.
9. When the process is completed, the instrument will beep. Remove the Gel Holder from the stainer and the gel from the holder.

III. Evaluation of the Hemoglobin Bands

The hemoglobin gels may be visually inspected for the presence of hemoglobin bands. The Helena Hemo Controls provide a marker for band identification.

Stability of End Product

Gels should be examined the same day of electrophoresis.

Quality Control

Use of the AFSC Control (Cat. No. 5331) and AA_2 Control (Cat. No. 5328) is recommended with each run. Dilute the AFSC Control 1:2 with Hemolysate Reagent before use. **Caution:** Bands from fresh whole blood may electrophorese at a slightly slower rate than the control. Older samples may electrophorese at a slightly faster rate than the control. If controls do not perform as expected, test results should be considered suspect or invalid.

RESULTS

Figure 1 shows the relative positions of commonly seen abnormal hemoglobins on the SPIFE Hemoglobin IEF Gel. HbE and HbO-Arab migrate slightly anodal to HbA_2, which itself is slightly anodal to HbC. HbG-Philadelphia and HbD-Punjab are clearly separated from HbS, as is Hb Lepore. HbA, HbF, and HbF_1 (acetylated HbF) are clearly separated from each other, allowing identification between heterozygous AS trait, homozygous SS anemia and HbS/β^+ thalassemia.[5]

Anodal, or fast, hemoglobins such as Bart's, HbH, HbJ and HbN-Baltimore may not be separated by conventional electrophoresis but are clearly separated by isoelectric focusing.

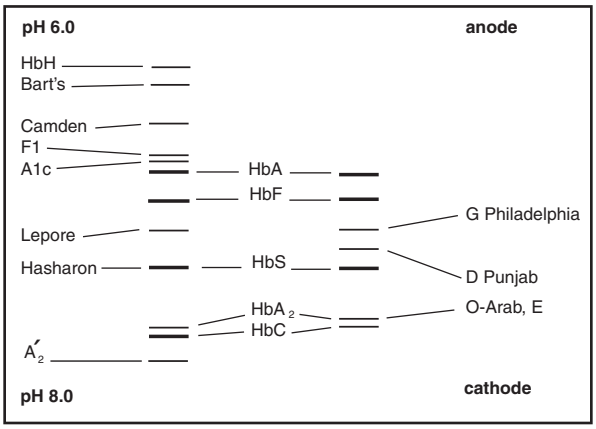

Figure 1

LIMITATIONS

Some abnormal hemoglobins have similar isoelectric points and cannot be distinguished using isoelectric focusing. Examples indistinguishable from HbS are HbG-Galveston and HbG Norfolk. The variants Hb Hammersmith, Brigham, and Bethesda are indistinguishable from HbA. HbE, HbC-Harlem, HbO-Arab and Hb-Koln cannot be separated, as cannot HbN-Baltimore and HbI-Texas. Confirmation by citrate agar or alkaline acetate electrophoresis is recommended. Globin chain analysis and structural studies are recommended as a last resort. It is impossible to distinguish HbSS, HbS/β° thalassemia, and HbS/HPFH in neonatals.[9] The presence of HbS+HBF requires family studies.

HbCC cannot be distinguished from HbC/β° thalassemia, and HbEE cannot be distinguished from HbE/β° thalassemia. Both require family studies.

Beta-Thalassemia major cannot be distinguished from homozygous HPFH or normals with low concentrations of HbA by electrophoresis and require clinical and family studies.[9]

Gels which do not lay flat in the chamber or those with surface artifacts should not be used.

Interfering Substances:

The use of fresh whole blood is recommended to avoid artifactual bands caused by oxidation or denaturation of the hemoglobin. Especially troublesome are ferric-ferrous hybrids, which appear as twin bands cathodal to the parent molecule. In order to use badly hemolyzed or aged specimens, they may be centrifuged, the plasma removed and discarded, and the red cells washed with physiological saline twice to minimize artifactual bands. Washed specimens may be used as packed cells or diluted with physiological saline and treated as whole blood.

Lipemic samples may give erroneous results. Toluene or chloroform extraction of lipids is not recommended. If using lipemic samples, removal of the plasma by saline washing is sufficient for satisfactory performance. Elevated levels of plasma proteins may cause water droplets to appear over the sample application area, causing diffusion of nearby hemoglobin bands. In severe cases, removal of the plasma and washing the red cells with saline will eliminate the problem.

Interfering Factors:

1. Under very low ambient humidity, the gel may exhibit drying, resulting in skewed bands and uneven band migration. Electrophoresis at a slightly lower temperature will eliminate this problem.
2. A band will migrate in an arched manner if the concentration of hemoglobin is too high for that band. Resolution should be adequate, but the phenomena can be prevented by application of less sample. A sample application of 1 µL should rectify the problem.

INTERPRETATION OF RESULTS

Clinically important hemoglobinopathies include variants whose presence cause sickling disorders (as HbSS, HbSD-Los Angeles, and HbSO-Arab), thalassemia derived anemias (Beta and Alpha-thalassemias), life long cyanosis, hemolytic anemias, polycythemias and erythrocytosis.[6-8]

The two major variant hemoglobins in the U.S. in terms of frequency and pathology are HbS and HbC. The early diagnosis of sickle cell disease (HbSS, HbSC, HbSD, HbSO and HbS/β-thal) is crucial to treat against severe infections. Homozygous HbCC and the heterozygous traits HbAS, HbAC, HbAE and HbAO cause mild hemolytic anemia. The diagnosis of their presence is also important for genetic counseling. The thalassemias are quantitative disorders in which the globin chain producing cells are diminished or absent. Alpha-thalassemia trait is characterized by the presence of Hb Barts (gamma 4) in newborns and HbH (Beta 4) in adults.

Beta-thalassemia major is characterized by the lack of HbA and the presence of HbF and HbA_2. Beta-Thalassemia minor is characterized by having elevated HbA_2 and reduced levels of HbA.

Another quantitative disorder is hereditary persistent fetal hemoglobin (HPFH), in which HbF remains present in adults.

BIBLIOGRAPHY

1. Wintrobe. M.M., Clinical Hematology, 6th Edition, Lea and Febiger, Philadelphia, 1967.
2. Fairbanks, V.F., Diag Med, Nov/Dec: 53-58, 1980.
3. Schneider, R.G., Clin Chem 20:1111-1115, 1974.
4. Vesterberg, O. and Svensson, H., Acta Chem. Scand. 20:820, 1966.
5. Drysdale, J.W., Righetti, P., Bunn, H.F., Biochem Biophys Acta, 229:42-50, 1971.
6. Basset, P., Beuzard, M., Garel, M.C., and Rosa, J., Blood, 51, #5 (May), 1978.
7. Monte, M., Beuzard, Y., and Rosa, J., Am J Clin Pathol, 66:753-759, 1976.
8. Galacteros, F., Kleman, K., Caburi-Martin, J., Rosa, J., and Lubin, J., Blood, 56:1068-1071, 1980.
9. International Committee for Standardization in Haematology, Clin Lab Haemat 10:335-345, 1988.

SPIFE® Touch Hemoglobin IEF System

Cat. No. 3428

SPIFE Hemoglobin IEF Gels (10)
Hemolysate Reagent (1 x 25 mL)

Other Supplies and Equipment

The following items, needed for performance of the SPIFE Hemoglobin IEF Procedure, must be ordered individually.

	Cat. No.
SPIFE Touch Analyzer	1068
SPIFE IEF Electrodes and Adapters	3704
REP Prepper	1359
AFSC Control	5331
AA_2 Control	5328
SPIFE IgG IEF Square Electrode	3703

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