SPIFE® 4000 Hemoglobin IEF Procedure

Cat. No. 2325

The SPIFE 4000 Hemoglobin IEF system is intended for the qualitative identification of hemoglobin variants using isoelectric focusing on the SPIFE 4000. 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 4000 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 4000 must be used to load the sample tray (if appropriate), apply samples, 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. For automated sample tray loading, a minimum of 500 µL of lysate is needed. Prepare patient sample lysates using a 1:4 dilution with Hemolysate Reagent. Mix one part (125 µL) whole blood with three parts (375 µL) Hemolysate Reagent.
   b. For manual sample tray loading, prepare patient sample lysates using a 1:4 dilution with Hemolysate Reagent. Mix one part (25 µL) whole blood with three parts (75 µL) Hemolysate Reagent.
   c. 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.
   d. For automated sample tray loading, a minimum of 500 µL of lysate is needed. Prepare patient sample lysates using a 1:8 dilution with Hemolysate Reagent. Mix one part (75 µL) washed, packed cells with seven parts (525 µL) Hemolysate Reagent.
   e. For manual sample tray loading, prepare patient sample lysates using a 1:8 dilution with Hemolysate Reagent. Mix one part (10 µL) washed, packed cells to seven parts (70 µL) Hemolysate Reagent.
   f. 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. 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 are provided in the SPIFE 4000 Hemoglobin IEF Kit (Cat. No. 2325). Individual items are not available.

SPIFE 4000 Hemoglobin IEF Gels (10)
Hemolysate Reagent (1 x 25 mL)
SPIFE 4000 Applicator Blades (10)

Materials needed but not contained in the kit:

Item	Cat. No.
SPIFE 4000 Hb IEF Cassettes and Electrodes	2327
SPIFE 4000 Analyzer	1620, 1621
REP Prepper	1359
AFSC Control	5331
SPIFE 4000 Blue Sample Trays	2316
SPIFE 4000 Gel Staging Lid	2308

Materials needed but not provided:

0.85% NaCl

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 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.
3. Remove the antisera/water reservoir from the antisera station. Lift the cover, fill the "H_2O" well and replace the cover.
4. 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.
5. 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.

1. Lysate volumes measuring 500 µL or more can be automatically dispensed into the sample tray by the SPIFE 4000. Lysate volumes measuring less than 500 µL must be hand-pipetted into the sample tray.
2. For automated sample tray loading, place test tubes containing 500 µL or more of each sample and control lysate in the sample carousel. Load test tubes into the carousel counterclockwise starting at the space numbered 1. Multiple carousels are loaded counterclockwise. Place a SPIFE 4000 Blue Sample Tray in the sample tray holder. Proceed to Section III.
3. For manual sample tray loading, with the notch in the SPIFE 4000 Blue Sample Tray on the left, hand pipette 75 µL of patient or control lysate into each sample cup in the top row. Load samples 1-7, skip the smaller cup in the center of the row and then load samples 8-14.
4. Place the sample tray, with samples loaded, into the sample tray holder.
   NOTE: Do not place a sample tray on top of the loaded sample tray.

III. Gel Preparation

1. Carefully open one end of the gel pouch, remove the gel from the protective packaging and discard the overlay.
2. Use a Rep Prepper to remove excess moisture from the gel.
3. Hold the gel so that the barcode is at the top. Place the gel into the SPIFE 4000 Hb IEF 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 makes 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 manually loaded sample trays, proceed to Step 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: Isoelectric Focusing (IEF)**
Test Name: Hemoglobin IEF

Check the programmed parameters for each of the following processes.

Sample Preparation
Sample Application
Electrophoresis
Predry

Sample Preparation	Volume (µL)	75
	Primary Wash Time (mm:ss)	00:02
	Primary Wash Cycles	1
	Samples per gel	14
	Application per sample	1
	Dilutions 1-14	Neat
Sample Application	Applicator Load Time (mm:ss)	00:30
	Applicator Load Speed	175
	Application Rows	1
	Row 1 Location (mm from gel edge):	80.0
	Apply Time (mm:ss)	02:00
	Apply Cycles	5
	Absorption Time (mm:ss)	00:00
	Inter-Gel Start Delay (mm:ss)	00:00
Electrophoresis	Voltage	600
	Minimum Current (mA)	0
	Maximum Current (mA)	100
	Temperature (°C)	14
	Time (hh:mm:ss)	00:45:00
Pre Dry	Temperature (°C)	60
	Time (hh:mm:ss)	00:15:00

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

2. Volumes less than 500 µL

a. The SPIFE 4000 User Setup menu should be used to create "Add a Test" parameters for small volumes of lysates which needs 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 and dry 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, 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 carbon electrodes should be washed with deionized water and dried after each use. Refer to the Operator's Manual for instructions.

VI. Evaluation of the Hemoglobin Bands

The hemoglobin gels may be visually inspected for the presence of hemoglobin bands. The Helena AFSC Control provides a marker for band identification.

Stability of End Product

Gels should be examined the day of electrophoresis.

Quality Control

Use of the AFSC Control (Cat. No. 5331) 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 4000 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.

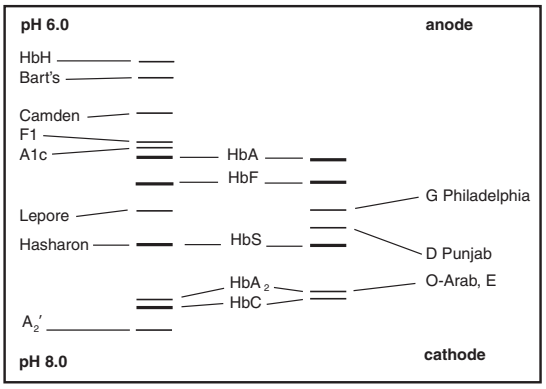

Figure 1

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 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.

INTERPRETATION OF RESULTS

Clinically important hemoglobinopathies include variants whose presence cause sickling disorders (as HbS-S, HbS-D Los Angeles and HbS-O-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.

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

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® 4000 Hemoglobin IEF System

Cat. No. 2325

SPIFE 4000 Hemoglobin IEF Gels (10)
Hemolysate Reagent (1 x 25 mL)
SPIFE 4000 Applicator Blades (10)

Other Supplies and Equipment

The following items, needed for performance of the SPIFE 4000 Hemoglobin IEF Procedure, must be ordered individually.

Item	Cat. No.
SPIFE Hb IEF Cassetts and Electrodes	2327
SPIFE 4000 Analyzer	1620, 1621
REP Prepper	1359
AFSC Control	5331
SPIFE 4000 Blue Sample Trays	2316
SPIFE 4000 Gel Staging Lid	2308

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

Pro 213
12/14(2)

HELENA LABORATORIES

Beaumont, Texas USA 77704