REP® Hemoglobin-30 IEF Procedure

Cat. No. 3250

Helena Laboratories

The REP Hemoglobin IEF system is intended for the qualitative and semi-quantitative identification of hemoglobin variants using isoelectric focusing. 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 on 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 onto three groups; (1) production of 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 indentification 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] 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 pI difference of less than 0.05 pH units could be resolved.

REAGENTS

1. **REP 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 and 0.005 M (0.18%) ethylenediaminotetraacetate (EDTA).

   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. NEVER PIPETTE BY MOUTH.

   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, colorless solution. If microbial contamination should occur, discard the reagent to avoid inaccurate results.

INSTRUMENTS

A Rapid ElectroPhoresis (REP®) unit (Cat. No. 1351) is used to electrophorese, dry and scan the gels. Refer to the Operator’s Manual for detailed instructions. The REP IEF Electrodes and Adapter (Cat. No. 1349) must be used to perform this procedure.

SPECIMEN COLLECTION AND HANDLING

Specimen: The specimen of choice is whole blood col-

lected in EDTA or heparinized tubes. However, cord blood, packed cells or paper discs from heel sticks may also be used. Cord blood has the disadvantage that it may be contaminated with maternal blood.

Specimen Preparation: Specimen hemolysates must be made according to the instructions outlined in the STEP BY STEP METHOD. The paper discs should be eluted with hemolysing reagent.

Specimen Storage: Whole blood specimens may be stored at 2 to 8°C for one week. Filter disc samples are satisfactory if used within one week without degradation.[9]

Interfering Substances: The use of fresh whole blood is recommended to avoid artifactual bands, caused by oxidation of denaturation of the hemoglobin. Especially troublesome are ferric-ferrous hybrids which appear as twin bands cathodal to the parent molecule. The position of these hybrids from HbA are noted on Figure 1. 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.

PROCEDURE

Materials Provided: The following materials are provided in the REP Hemoglobin IEF Kit (Cat. No. 3250). Individual items are not available.

REP Hemoglobin-30 IEF Gels (10)
REP IEF Hemolysate Reagent (1 x 25 mL)
REP Blotter A (10)
REP Sample Cups (300 cups)

Materials Provided by Helena but not contained in the kit:

Item	Cat. No.
IEF Electrodes and Adapter for REP	1349
SUREprep	1574
REP Prepper	1359
AFSC Control	5331
AA_2 Control	5328

SUMMARY OF CONDITIONS

Support Media	REP Hemoglobin IEF Gel
Lysate Sample Volume	75.0 µL
Sample Application Volume	2 µL
Electrophoresis Temperature	16°C
Electrophoresis Time	50:00 mm:ss
Electrophoresis Voltage	600 V
Incubation Time	0 mm:ss
Incubation Temperature	45°C
Drying Time	20:00 mm:ss
Drying Temperature	54°C
Scanning Wavelength	415 nm

STEP-BY-STEP METHOD

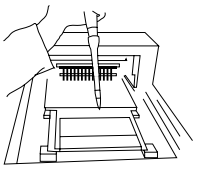
A. Sample Lysate Preparation

1. Whole Blood: To prepare the patient hemolysates, make a 1:4 dilution with IEF Hemolysate Reagnet. Mix 1 part (25 µL) of whole blood with three (3) parts (75 µL) Hemolysate Reagent.
2. Packed Cells: Make a 1:8 dilution by mixing one part (10 µL) packed cells to 7 parts (70 µL) Hemolysate Reagent.
3. Paper Discs: Place three 1/8" diameter discs into a test tube. Reconstitute with 25 µL Hemolysate Reagent. 2 µL must be applied **by hand** to the gel.

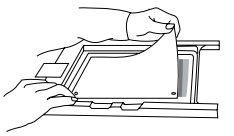
Vortex briefly and allow the sample to stand at least 5 minutes prior to use. It is important that the cells be completely lysed (forming a clear red hemolysate) before applying the sample to the gel.

B. Sample Application

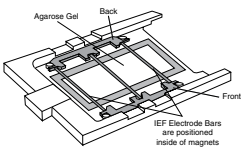
1. Place 30 sample cups into the sample tray. Place 75 µL of sample or control lysates into appropriate sample cups.
   When working with paper discs, the lysate volume is insufficient to put into a cup and must be applied by hand.



2. Place REP Blotter A on sample tray in area adjacent to sample cups. Place approximately 4 mL of SUREprep into outside washwell of sample tray. Place approximately 4 mL of water into inside washwell of sample tray. Do **NOT** use REP Prep with this procedure.
3. Carefully open one end of the pouch and remove the protective packaging. Remove both gels from the protective packaging. Carefully separate the two gels. If each gel has an overlay, either gel may be used for testing. If both overlays remain on one gel, use the gel with no overlay immediately. Replace the unused gel in the protective packaging and return it to the pouch. Reseal the pouch with tape to prevent drying of the gel. The gel must be used within one month after resealing the pouch.
4. Remove the overlay(s) if necessary. Use the REP Prepper to remove excess moisture from the sample wells and trough.



5. 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.
6. Insert the IEF electrode adapter marked FRONT between the two magnetic posts located at the front of the chamber floor. Insert the IEF electrode adapter marked BACK between the two magnetic posts located at the back of the chamber floor.



7. Clean and wipe the (3) IEF electrodes with a lint-free tissue.
8. Place an electrode into the slots created by the adapter – one on each outside edge and one in the middle. Be

sure all 3 electrodes are seated firmly against the electrode posts of the adapters.

9. Using the instructions provided in the Operator's Manual, set parameters on the screen as follows:

Sample Location [Row]	AB
Sample Application Time	2 sec
Application Volume	2 µL
Sample Absorption Time	0:01 mm:ss
Needle Wash Cycles	2
Needle Blot Time	1 sec
Electrophoresis Time	50:00 mm:ss
Electrophoresis Voltage	600 V
Electrophoresis Temperature	16°C
Air Dry Time	mm:ss
Reagent Pour Time	0 sec
Reagent Spread Cycles	0
Incubation Time	0 mm:ss
Incubation Temperature	45°
Dry Time	20:00 mm:ss
Dry Temperature	54°C
Standby Temperature	16°C

10. The REP unit will automatically apply samples, electrophorese and dry the gel.
11. Remove the gel from the REP unit for evaluation. If the gel is to be used for future evaluation, it will be necessary to dry the gel by placing the gel into a laboratory oven at 60°C for 10 to 15 minutes.

C. Visualization of the Bands

Gels may be visually examined or scanned using a 415 nm interference filter. Filter A, slit 3 recommended.

Stability of End Product

Gels should be scanned 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 Helena REP IEF Gel. HbE and HbO-Arab migrate slightly anodal to HbA_2, which itself is slightly anodal to HbC. HbG-Philadelphia and HbD-Los Angeles 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/B^+ 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.

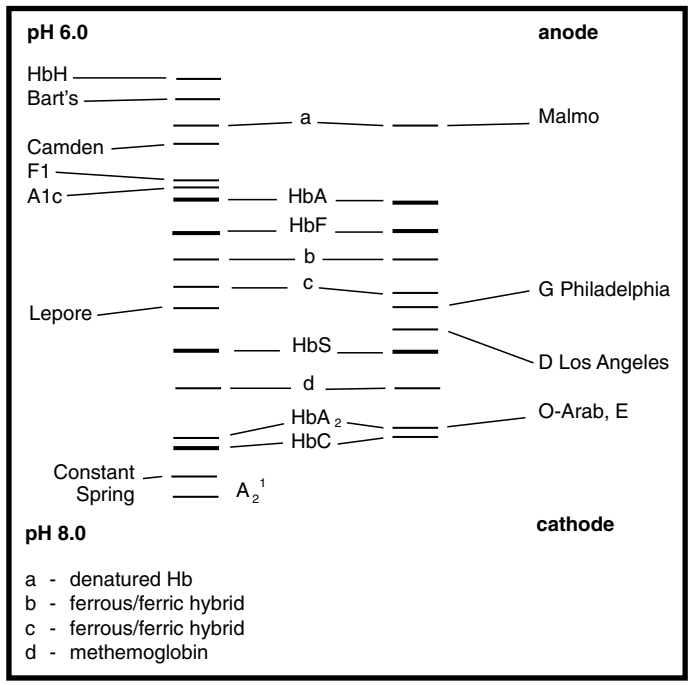


FIGURE 1

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 from HbS/B° thalassemia and HbS/HPFH in neonatals.[9] The presence of HbS+HBF requires family studies.

HbCC cannot be distinguished from HbC/B° thalassemia and HbEE cannot be distinguished from HbE/B° 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 Factors: Refer to SPECIMEN COLLECTION AND HANDLING.

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 causes 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/B-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. Vesterburg, 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 Haemotology, Clin Lab Haemat, 10:335-345, 1988.

REP® Hemoglobin-30 IEF System

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REP Hemoglobin-30 IEF Gels (10)
REP IEF Hemolysate Reagent (1 x 25 mL)
REP Blotter A (10)
REP Sample Cups (300 cups)

Other Supplies and Equipment

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

	Cat. No.
IEF Electrodes and Adapter for REP	1349
REP® (Rapid ElectroPhoresis) Analyzer	1351
SUREprep	1574
REP Prepper	1359
AFSC Control	5331
AA_2 Control	5328

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

Shading indicates that text has been modified, added or deleted.

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