HbS-D Los Angeles, and HbS-O-Arab), thalassemia derived anemias (Beta and Alpha-thalassemias), life long cyanosis, hemolytic anemias, polycythemias, and erythrocytosis. 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 HbA2. Beta-thalassemia minor is characterized by having elevated HbA2 and reduced levels of HbA.

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

**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 HbA2 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 HbA2 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 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).


**REAGENT**

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.

**REAGENTS**

**REP® Hemoglobin-30 IEF System**

Cat. No. 3250

REP® Hemoglobin-30 IEF Gels (10) 1349
REP® IEF Hemolyte Reagent (1 x 25 mL) 1351
REP® Blotter A (10) 1574
REP® Sample Cups (300 cups) 1359

**Other Supplies and Equipment**

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

- IEF Electrodres and Adapter for REP Cat. No. 1349
- REP® (Rapid Electrophoresis) Analyzer 1351
- SUREprep 1574
- REP Preparer 1359
- AFSC Control 5331
- AA Control 5328

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

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**REFERENCES**

7. Lubin8.

The specimen of choice is whole blood collected in Pro. 125

**Ingredients:**

Each gel contains 1% w/v agarose, 5.3% v/v carrier ampholytes and 0.01% thymus 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.

**Hemolysate Reagent**

**Ingredients:**

The reagent is an aqueous solution containing 0.07% potassium cyanide and 0.005 M (0.18%) ethylenediaminetetraacetate (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 occurs, 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® Electrodres and Adapter (Cat. No. 1349) must be used to perform this procedure.

**SPECIMEN COLLECTION AND HANDLING**

Specimen: The specimen of choice is whole blood collected.
STEP-BY-STEP METHOD

A. Sample Lysate Preparation

1. Whole Blood: To prepare the patient hemolysates, make a 1:4 dilution with IEF Hemolysate Reagent. 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 1/16 diameter discs into a test tube using a micropipette with 25 µL Hemolysate Reagent. 2 µL must be applied by hand to the gel.

B. Sample Application

1. Place 30 sample cups into the sample tray. Place 75 µL of sample or controls lysates into appropriate sample cups.

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 to achieve a water:water interface. Use the REP Prepper 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.

4. The gel must be used within one month after resealing the pouch.

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 into 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 the parameters as follows:

   - Sample Location [Row] ........................................ AB
   - Sample Application Time: 2 sec
   - Application Volume .................................................. 2
   - Sample Absorption Time ............................................... 0:01 mm:ss
   - Needle Wash Cycles .......................................................... 2
   - Needle Blot Time ............................................................... 1 sec
   - Electrophoresis Voltage .................................................. 500 mm:ss
   - Electrolysis Temperature .................................................. 16°C
   - Air Dry Time ................................................................. 0:20 mm:ss
   - Reagent Pour Time ........................................................... 0 sec
   - Reagent Spread Cycles ...................................................... 0
   - Incubation Time ................................................................. 0:00 mm:ss
   - Incubation Temperature .................................................... 45°C
   - Dry Time .................................................................. 0:20 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, III, 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 Control (Cat. No. 5328) is recommended with each run. Dilute the AFSC Control 1:2 with Hemolysate Reagent before use. Care should be taken to ensure that 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 invalid or invalid.

RESULTS

Figure 1 shows the relative positions of commonly seen abnormal hemoglobin on the Helena REP IEF Gel. HbE and HbO-Arab migrate slightly anodal to HbA, 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, (acetylated Hbf) are clearly separated from each other, allowing identification between heterozygous AS trait, homozygous SS anemia, and Hbs/B thalassemia.

Anodal, or fast hemoglobins such as Bart’s, HbH, HbJ and HbO-Arab migrate slightly anodal to HbA2, which itself is slightly anodal to HbS.

LIMITATIONS

Some abnormal hemoglobins have similar isoelectric points and cannot be distinguished using isoelectric focusing. Examples indistinguishable from Hbs are HbG-Galveston and HbN-Harlem. The variants HbHammersmith and HbD-Los Angeles are indistinguishable from Hba. HbE, HbC-Harlem, HbO-Arab and HbKöln cannot be separated, as cannot HbN-Baltimore and HbTexas.

Confirmation by nitro blue tetrazolium or alkaline acetate electrophoresis is recommended. Golin chain analysis and structural studies are recommended as a last resort. It is impossible to distinguish HBSS from HbE/B thalassemia and HbE/β thalassemia.

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

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

Interpretation of Results: 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 or if the concentration of hemoglobin is too high for that band. Replication should be adequate but the phenomenon 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,
Incubation Time ................................................. 0 mm:ss
Scanning Wavelength ......................................... 415 nm
Electrophoresis Time .................................. 50:00 mm:ss
Lysate Sample Volume ........................................ 75.0 µL

**SUMMARY OF CONDITIONS**

IEF Electrodes and Adapter for REP 1349 are not available.

**Interfering Substances:** The use of fresh whole blood is recommended to avoid artifactual bands, caused by oxida- tion of denaturation of the hemo- globin. Especially trouble- some are ferri-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 cen- trifuged, 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 near hemoglo- bin 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 Hemolyte Reagent (1 x 25 mL)
- REP Bloter A (10)
- REP Sample Cups (300 cups)

**Materials Provided by Helena but not contained in the kit:**

- IEF Electrodes and Adapter for REP
- SUREprep
- REP Preparer
- AFSC Control
- AA Control

**STEP-BY-STEP METHOD**

**A. Sample Lysate Preparation**

1. Whole Blood: Place the patient hemolyses, make a 1:4 dilution with IEF Hemolyte Reagent. Mix 1 part (25 µL) of whole blood with three (3) parts (75 µL) Hemolyte Reagent.
   - Sample Location [Row] ........................................... ABC
   - Sample Application Time ..................................... 2 sec
   - Application Volume ........................................ 10 µL
   - Sample Absorption Time .................................. 0:01 mm:ss
   - Needle Wash Cycles ......................................... 2
   - Needle Blot Time ............................................ 1 sec
   - Electrophoresis Voltage ....................................... 600 V
   - Electrophoresis Temperature ............................... 16°C
   - Air Dry Time ................................................... 50:00 mm:ss
   - Reagent Pour Time ........................................... 0 sec
   - Reagent Spread Cycles ....................................... 0
   - Incubation Temperature ...................................... 45°C
   - Dry Time .......................................................... 20:00 mm:ss
   - Dry Temperature ............................................. 54°C
   - Standby Temperature ........................................ 16°C

2. Packed Cells: Make a 1:8 dilution by mixing one part (25 µL) of packed cells to seven (7) parts (250 µL) Hemolyte Reagent.

   **B. Sample Application**

   1. Place 30 sample cups into the sample tray. Place 75 µL of sam- ple or control lysates into appropriate sample cups.
   2. Place REP Bloter A on sample tray in area adjacent to sample cups. Place approximately 4 mL of SUREprep inside washwell of sample tray. Place approximately 4 mL of water into inside washwell of sample tray. Close the REP Bloter with this procedure.
   3. Carefully open one end of the pouch and remove the protective packaging. Remove both gels from the pro- tective packaging.
   4. Place the sample to the gel.
   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.
   7. Clean and wipe the (3) IEF electrodes with a lint-free tissue.

   **C. Visualization of the Bands**

   Gels may be visually examined or scanned using a 415 nm interference filter. Filter A, Silent 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 Control (Cat. No. 5328) is recommended with each run. Dilute the AFSC Control 1:2 with Hemolyte Reagent before use.

**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, which itself is slightly anodal to HbC.

**LIMITATIONS**

Some abnormal hemoglobins have similar isoelectric points and cannot be distinguished using isoelectric focusing. Results indistinguishable from HbS are HbG-Galveston and HbN-Galveston. The variants HbM-Harris, HbG, and HbE are indistinguishable from HbA. HbE, HbC, and HbN are indistinguishable from HbA. HbE, HbC, and HbN may not be separated by conventional elec- trophoresis. Electrophoresis at a slightly lower tempera- ture will eliminate this problem.

**Interpretation of Results**

Clinically important hemoglobinopathies include variants whose presence causes sickling disorders (as Hbs-S,
Beta-thalassemia major is characterized by the lack of HbA and the presence of HbF and HbAβ. Beta-thalassemia minor is characterized by elevated HbA, reduced levels of HbF, and fetal hemoglobin (HbF), which are normally present only in fetal life. Another quantitative disorder is hereditary persistence of fetal hemoglobin (HPFH), in which HbF remains present in adults.

**BIBLIOGRAPHY**