MBP008 **Alkaline Hemoglobin Electrophoresis Kit**

(No Destaining Required)

Kit Contents

Product Code	Reagents provided	MBP008
		224 Runs
DS0205	Pre-weighed Agarose, special, low EEO	0.16 g X 8 vials
DS0059	10x Wash Solution (SCW)	100 ml
DS0060	Gel Loading Buffer (SGL)	15 ml
DS0061	10x Gel Running Buffer (SGR)	500 ml
DS0062	Hemolysing Solution (SHL1)	100 ml
DS0063	Hemolysing Solution (SHL2)	80 ml
ML091	X-Press Blue™	500 ml
ML024	Molecular Biology Grade Water	9 X 500 ml
PW1139	Collection Tube, 2.0ml	200nos.

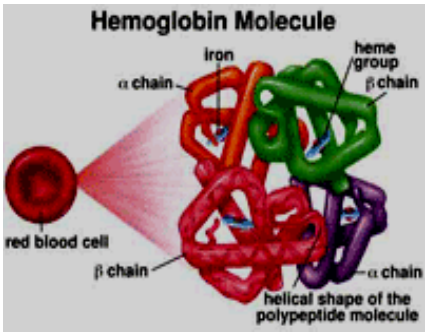
NOTE: Use of Electrophoresis unit (LA666) is recommended as it has provision for placing two combs of 14-teeth and 10-teeth in each gel. Hence, 24 samples can be run at a time during a single electrophoretic run making the sample analysis fast and cost-effective with multiple sample processivity.

Intended Use

Recommended for isolation of hemoglobin from human blood samples.

Introduction

Red blood cells (RBC's) deliver oxygen to cells throughout the body. Hemoglobin is an oxygen-carrying protein within the red blood cells. Sickle cell disease is a group of genetic disorders having effect on hemoglobin. In sickle cell disease the RBC's become crescent or sickle shaped. These cells have a short life span as compared to normal, round red blood cells, which lead to anemia.Anemia can cause shortness of breath, fatigue, and delayed growth and development in children. The rapid breakdown of red blood cells may also cause yellowing of the eyes and skin, which are signs of jaundice. Painful episodes can occur when sickled red blood cells, which are stiff and inflexible, get stuck in small blood vessels. These episodes deprive tissues and organs of oxygen-rich blood and can lead to organ damage, especially in the lungs, kidneys, spleen, heart and brain.



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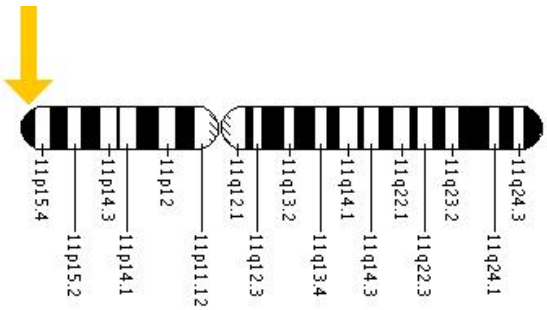
Sickle cell disease

The functional hemoglobin molecule is made up of four protein subunits. Two subunits called alpha hemoglobin and two subunits called beta hemoglobin. Each protein subunit of hemoglobin carries an iron-containing molecule called heme. Heme molecules are necessary for red blood cells to pick up oxygen in the lungs and deliver it throughout the body. A complete hemoglobin protein is capable of carrying four oxygen molecules at a time. Oxygen binding to hemoglobin gives blood bright red colour. The gene encoding beta hemoglobin is HBB. Mutations in the HBB gene result in formation of different beta hemoglobin. The mutant forms of hemoglobin are hemoglobin S (HbS), hemoglobin C (HbC) and hemoglobin E (HbE). In people with sickle cell disease, at least one of the beta hemoglobin subunits in hemoglobin is replaced with hemoglobin S. In sickle cell anemia, which is a common form of sickle cell disease, hemoglobin S replaces both beta hemoglobin subunits in hemoglobin. In other types of sickle cell disease, just one beta hemoglobin subunit in hemoglobin is replaced with hemoglobin S. The other beta hemoglobin subunit is replaced with a different abnormal variant, such as hemoglobin C.

Location of the Sickle Cell Disease Gene

Cytogenetic Location: 11p15.5

Molecular Location on chromosome 11: base pairs 5,203,271 to 5,204,876



The HBB gene is located on the short (p) arm of chromosome11 at position 15.5.More precisely, the HBB gene is located from base pair 5,203,271 to base pair 5,204,876 on chromosome11.

Types of Hemoglobin

Depending upon the type of mutations different types of hemoglobin (Hb) exist. The most common ones are HbA, HbA2, HbF, HbS, HbC, Hgb H, and Hgb M. Healthy adults only have significant levels of HbA and HbA2. Some people may also have small amounts of HbF (which is the main type of hemoglobin in an unborn baby's body). Certain diseases are associated with high HbF levels (when HbF is more than 2% of the total hemoglobin). HbS is an abnormal form of hemoglobin associated with sickle cell anemia. HbC is an abnormal form of hemoglobin associated with hemolytic anemia. In adults, these hemoglobin molecules make up the following percentages of total hemoglobin:

- Hgb A1: 95% to 98%
- Hgb A2: 2% to 3%
- Hgb F: 0.8% to 2%
- Hgb S: 0%
- Hgb C: 0%

In infants and children, these hemoglobin molecules make up the following percentages of total hemoglobin:

- Hgb F (newborn): 50% to 80%
- Hgb F (6 months): 8%
- Hgb F (over 6 months): 1% to 2%

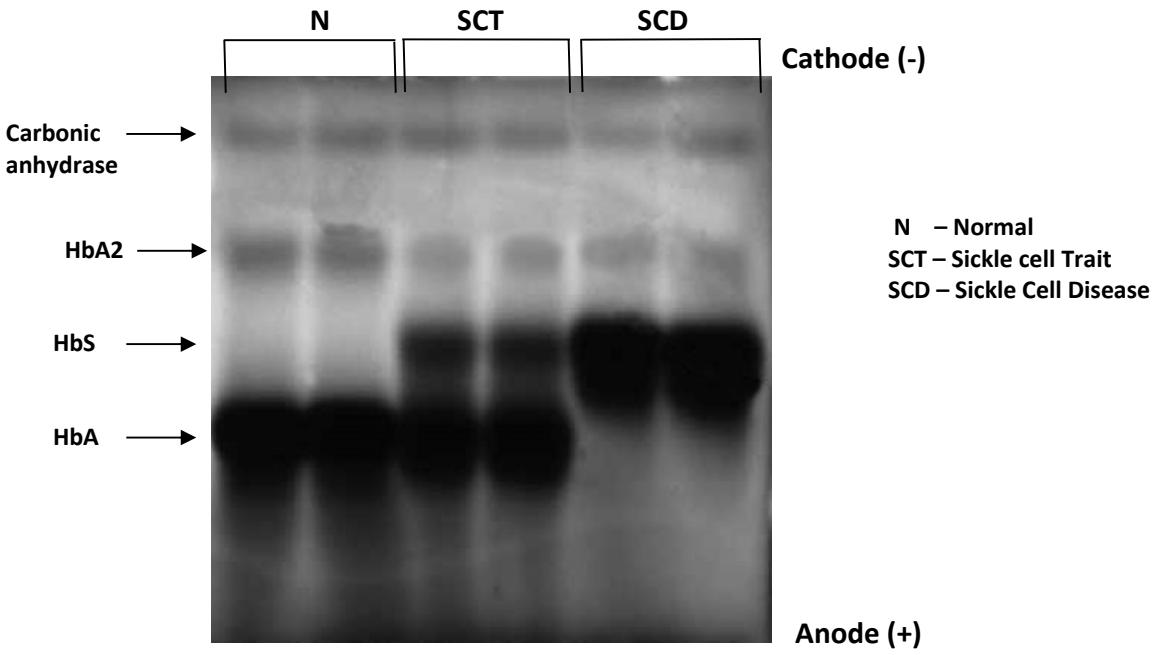
Deviation from any of the above is an indicative of diseased condition.

- **Hemoglobin S**. This type of hemoglobin is present in sickle cell anemia.
- **Hemoglobin C**. This is another type of hemoglobin found in sickle cell anemia.
- **Hemoglobin E**. This type of hemoglobin is found in people of Southeast Asian descent.
- **Hemoglobin D**. This type of hemoglobin may be present with sickle cell anemia or thalassemia.
- **Hemoglobin H (heavy hemoglobin)**. This type of hemoglobin may be present in certain types of thalassemia.

Alkaline Hemoglobin Electrophoresis Kit

Alkaline Hemoglobin Electrophoresis Kit is intended for discrimination of hemoglobin variants on agarose gel using alkaline buffer. Each hemoglobin molecule, normal as well as abnormal variant has a particular distinct charge on basis of which it can be separated on agarose gel. Use of Agarose, special, low EEO (DS0205) is recommended for separation of hemoglobin molecule components. Red Blood Cells are pelleted down by centrifuging blood sample and these RBC's are further lysed to separate out hemoglobin from them. The isolated hemoglobin is then loaded into Agarose, special, low EEO (DS0205) and electrophoresed at high voltage and high current.

The abnormal hemoglobins have a sufficiently altered charge distribution and can therefore be easily identified by electrophoresis. The presence of HbS variant of hemoglobin can be clearly visualized and identified on a agarose gel stained with X-Press Blue™ (ML091).



Hemoglobin variants separated in Agarose, special, Low EE0 using Alkaline Hemoglobin Electrophoresis Kit

Alkaline Hemoglobin Electrophoresis Kit can be used to run 24 samples at a time in a single electrophoretic run using HiMedia Electrophoresis Unit (LA666) with one comb of 14-teeth and other comb of 10-teeth each per gel, making the sample analysis fast and cost-effective with multiple sample processivity. The kit provides a convenient and efficient method for preliminary screening of the population for hemoglobin related disorders by alkaline agarose gel electrophoresis. The analysis and detection can be later confirmed by HPLC.

Materials needed but not provided

- Electrophoresis unit (Product Code: LA666)
- Electrophoresis power supply (Product Code: LA690)
- Spatula (for detaching the gel from the casting tray for staining purpose)
- Box tray for staining
- Centrifuge machine (with 2ml rotor and upto 10,000rpm speed)
- Pipettes [1000µl (Product Code: LA614), 200µl (Product Code: LA613), 50µl (Product Code: LA612)and 10µl (Product Code: LA611) capacity]

Storage

1. Alkaline Hemoglobin Electrophoresis Kit can be stored at room temperature (15-30°C) for up to 15 months without showing any reduction in performance.
2. The diluted Gel Running Buffer (SGR) (DS0061) should be freshly prepared prior to use as indicated in general preparation instructions.

General Preparation Instructions

1. **Dilute 10X Gel Running Buffer (SGR) (DS0061) as follows:**

Number of Gels	10X Gel Running Buffer (SGR)	Molecular Biology Grade Water (ML024)
1	50 ml	450 ml

Important Note: Diluted Gel Running Buffer (SGR) should be freshly prepared before each use.

2. **Dilute 10X Wash Solution (SCW) (DS0059) as follows:**

Number of Gels	10X Wash Solution (SCW)	Molecular Biology Grade Water (ML024)
1	10 ml	90 ml

Centrifugation

All centrifugation steps can be carried out in conventional laboratory centrifuge e.g. Beckman 6KR, Heraeus Varifuge 3.0R, or Sigma 6k10 with fixed angle rotor. Perform the centrifugation at room temperature at specified g-force. The corresponding rpm can be calculated using the formula:

$$RPM = \sqrt{RCF/1.118 \times 10^{-5}\, r}$$

where RCF = required gravitational acceleration (relative centrifugal force in units of g); r = radius of the rotor in cm; and RPM = the number of revolutions per minute required to achieve the necessary g-force

Specimen Handling and Collection

Collect whole blood in an anticoagulant tube (an EDTA tube is preferred) under sterile conditions (if to be used for future). Ensure that the blood sample is at room temperature before beginning the protocol.

Procedure

I. Sample preparation

NOTE: Blood sample used should be freshly collected.

Red blood cells are to be separated from the fresh blood sample to isolate hemoglobin from them. Centrifuge 250 µl of fresh whole blood sample at 8,000x g (≈10,000 rpm) for 10 minutes at room temperature. Discard the supernatant plasma carefully such that the red blood cell pellet obtained, is not disturbed. Wash the red blood cell pellet with 1 ml of diluted Wash Solution (SCW) (DS0059) **(Refer General Preparation Instructions)** by resuspending the pellet with a pipette. Centrifuge the resuspended pellet at 8,000x g (≈10,000 rpm) for 10 minutes. Again discard the supernatant carefully and wash the pellet with another 1 ml of diluted Wash Solution (SCW). Perform this wash step one more time with diluted Wash Solution (SCW) to obtain a red blood cell pellet. Discard the supernatant carefully at the end of the washing steps such that the red blood cell pellet remains undisturbed.

II. Lyse

1. Add 250 µl of Hemolysing Solution (SHL1) (DS0062) to the red blood cell pellet obtained after performing sample preparation step. Resuspend the red blood cell pellet completely in the Hemolysing Solution (SHL1) by pipetting gently.

2. Add 125 µl of Hemolysing Solution (SHL2) (DS0063) to the hemolysate obtained in Step 1 and vortex thoroughly for 2 minutes.

3. Centrifuge the hemolysate obtained in Step 2 at 8,000x g (≈10,000 rpm) for 10 minutes.

4. Collect the uppermost layer of supernatant containing the isolated hemoglobin protein, carefully without disturbing the intermediate pellet layer and transfer it to a separate tube.

   NOTE: Three layers will be obtained after centrifuging the hemolysate in step 3. A lowermost clear phase, an intermediate floating precipitate containing the red blood cell debris and an uppermost layer of supernatant containing the hemoglobin is observed. The uppermost layer is to be collected carefully without disturbing the debris.

   NOTE: The hemolysate obtained in Step 3 should be freshly prepared before every electrophoretic run using a fresh blood sample.

III. Agarose gel preparation

1. Prepare agarose gel by adding pre-weighed 0.16 g of Agarose (1 vial), special, low EE0 (DS0205) and dissolve in 30 ml of diluted Gel Running Buffer (SGR) (DS0061) by boiling.

   NOTE: Prepare fresh diluted Gel Running Buffer (SGR) as indicated in general preparation instructions

   NOTE: The agarose powder should be dissolved in diluted Gel Running Buffer (SGR) by boiling and swirling intermittently such that the agarose dissolves completely. Do not over boil the agarose so as to minimize water loss due to evaporation.

2. Cool the melted agarose for about 10 minutes and then pour the melted agarose while warm in the casting unit of electrophoresis unit (LA666) with the two combs placed in their respective notches. Ensure that the gel poured spreads evenly on the surface of the casting tray to form a thin gel. Allow the gel to set. The gel will solidify completely in 15 minutes.

   NOTE: Do not pour the gel when it is boiling hot as it leads to water loss due to evaporation which will alter the concentration of agarose in the gel. The formation of a thin uniform gel is essential to minimize resistance produced, which leads to generation of heat due to high voltage and high current required for the electrophoretic run.

3. Position the casting tray after the gel has set such that the wells are oriented towards the cathode.

4. Pour 450 ml of diluted Gel Running Buffer (SGR) into the electrophoretic tank **(Prepare fresh diluted Gel Running Buffer as indicated in general preparation instructions)**. Ensure that the agarose gel is submerged completely in the diluted Gel Running Buffer (SGR).

IV. Electrophoretic migration of hemolysate samples

1. Prepare the sample by mixing 10 µl of supernatant hemolysate obtained in Step 4 with 10 µl of Gel Loading Buffer (SGL) (DS0060) by pipetting gently.

   NOTE: The color of Gel Loading Buffer (SGL) (DS0060) may become light blue in color on storage. But this color change will not affect the application of gel loading buffer, which is to load the hemoglobin sample in the agarose gel in any form.

2. Load 10 µl of diluted sample-dye mixture from Step 1 into each well.

3. Connect the electrodes of the Electrophoresis unit to Electrophoresis power supply unit (LA690) and run the gel at 250 V and 90 mA for 45minutes.

   NOTE: To ensure that the run has started the user can observe bubbles in the buffer from the sides of the electrophoresis unit.

V. Staining of gel for visualization of hemoglobin protein bands

1. Slice the gel carefully along the edges of the casting tray using a spatula, such that gel can slide down easily into the staining tray (not provided with the sample kit). Avoid breakage of gel during handling.

2. Pour 50 ml of X-Press Blue™ (ML091) onto the gel such that the gel is completely submerged in the staining liquid. Allow staining by shaking the gel in the staining solution for 10 minutes.

3. Decant the X-Press Blue™ (ML091) and discard.

   NOTE: No De-staining step is required.

4. Rinse the gel with 100 ml distilled water (not provided) once.

5. Sharp hemoglobin bands will be clearly visible on the gel.

   NOTE: The gel can be preserved by keeping it on butter paper for 24 hours without covering it.

Warning

Certified for *in vitro* Diagnostic Use (IVD). Not for Medicinal Use. Read the procedure carefully before beginning the protocol. Wear protective gloves/protective clothing/eye protection/face protection. Follow good clinical laboratory practices while handling clinical samples. Standard precautions should be followed as per established guidelines. Safety guidelines may be referred in safety data sheets of the product.

Precautions

Read the procedure carefully before starting the experiment.

Performance and Evaluation

Each lot of HiMedia's Alkaline Hemoglobin Electrophoresis Kit is tested against predetermined specifications to ensure consistent product quality.

Quality Control

Type of Sample	Bands observed
Normal Human Blood (250µl whole blood)	Yes
Sickle Homozygous Human Blood (250µl whole blood)	Yes
Sickle Heterozygous Human Blood (250µl whole blood)	Yes

References:

1. Frenette P. S. and Atweh G. F. (2007) Sickle cell disease: old discoveries, new concepts, and future promise. J Clin Invest. 117(4): 850–858
2. Ashley-Koch A, Yang Q, Olney RS. (2000) Sickle hemoglobin (HbS) allele and sickle cell disease: a HUGE review. Am J Epidemiol. 151(9):839-45.
3. Lepp C. A. and Bluestein B. I. (1978) Hemoglobin electrophoresis at alkaline pH on agarose gels. Clin.Chem. 24(6):936-937

Troubleshooting Guide

Sr. No	Problem	Possible Cause	Solution
1.	Poor separation of hemoglobin protein bands	Blood sample used is not freshly collected	Freshly collected blood sample should be used. Do not use old and stored blood samples
		Hemolysate obtained in Step 4 is not freshly used	Do not store hemolysate obtained in Step 4. Prepare hemolysate freshly every time before each electrophoretic run
2.	Smearing of hemoglobin protein bands observed	Incomplete removal of Wash Solution (SCW) in Sample preparation	Ensure that Wash Solution (SCW) is completely removed from the red blood cell pellet in the Sample preparation step carefully without disturbing the red blood cell pellet
		Insufficient washing with Wash Solution (SCW)	Wash the red blood cell pellet sufficiently with Wash Solution (SCW) as mentioned in the Sample Preparation step
		Collection of red blood cell debris in step 4	Ensure that while collecting the supernatant hemolysate in step 4, the intermediate layer of red blood debris should not get collected along with upper layer of the supernatant
3.	Melting of Agarose gel	Use of Gel Running Buffer Concentrate (SGR) instead of diluted Gel Running Buffer (SGR)	Use only diluted Gel Running Buffer (SGR) for running the gel as well as for preparing the gel NOTE: Prepare fresh diluted Gel Running Buffer (SGR) as indicated in General Preparation Instructions

4.	Poor resolution of the hemoglobin bands	The band might have not migrated to 4-4.5 cm	Please run the gel for another 15 minutes to obtain proper migration of bands
5.	**Wells not properly formed on the gel**	**The surrounding temperature is too high to solidify the gel**	**Allow the gel to solidify for at least 30 minutes or till the color of the gel becomes white/translucent from colorless.**
			Before pouring the gel on the gel tray, arrange the gel apparatus on the platform with the help of leveler (not provided)

Safety Information

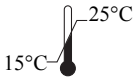
Alkaline Hemoglobin Electrophoresis Kit is for laboratory use only, not for drug, household or other uses. Take appropriate laboratory safety measures and wear gloves when handling. Not compatible with disinfecting agents containing bleach. Please refer the Safety Data Sheet (SDS) for information regarding hazards and safe handling practices.

Disposal

User must ensure safe disposal by autoclaving and/or incineration of used or unusable preparations of this product. Follow established laboratory procedures in disposing of infectious materials and material that comes into contact with clinical sample must be decontaminated and disposed off in accordance with current laboratory techniques.

Technical Assistance

At HiMedia, we pride ourselves on the quality and availability of our technical support. For any kind of technical assistance, mail to mb@himedialabs.com.

IVD	In vitro diagnostic medical device
CE	CE Marking
	Storage temperature
	Do not use if package is damaged
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