Human Hemoglobin ELISA Kit

Cat. No. E88-134
Lot No. 260520

Components Supplied

- **Human Hemoglobin Pre-Coated 96-well Strip Plate,** 1 each
- **Human Hemoglobin Standard**, 200 ng/vial, 2 each
- **Human Hemoglobin Detection Antibody,** 12 ml
- **10X Dilution Buffer B**, 25 ml
- **HRP Solution A,** 12 ml
- **TMB Substrate,** 12 ml
- **Stop Solution,** 12 ml
- **20X Wash Buffer,** 50 ml
- **Sealing Tape,** 6 sheets

Shelf Life: 6 months from date of receipt.

For In vitro laboratory use only. Not for any clinical, therapeutic, or diagnostic use in humans or animals. Not for animal or human consumption.

Introduction

This enzyme linked immunosorbent assay (ELISA) is for the detection of human hemoglobin in whole blood, serum, plasma, CSF or cell culture media. This kit contains sufficient components to quantitate Human Hemoglobin in up to 40 samples, tested in duplicate.

Background

Hemoglobin (Hb) is an iron-containing protein that makes up about 97% of the red blood cells. The Hb molecule is composed two 141-residue α-chains and two 146-residue β-chains, forming an α2β2 complex. A heme moiety containing a ferrous iron (Fe^{2+}) binds oxygen in the lungs and transports it to the rest of the body, where it is released for cell use.

The most abundant form of hemoglobin in human blood is hemoglobin A (HbA or HbA1), but there are many variants. For example, in the fetus hemoglobin F (HbF) is the predominant form, which is gradually replaced by HbA during the first few months after birth. Other hemoglobin variants are either present normally but in low levels (such as HbA2), or from genetic abnormalities that result in specific diseased states (such as HbS, HbC, and HbH).

Principle of the Assay

This kit is based on a sandwich ELISA. Human hemoglobin present in the test sample is captured by anti-human hemoglobin antibody that has been pre-adsorbed on the surface of microtiter wells. After sample binding, unbound proteins and molecules are washed off, and a biotinylated detection antibody is added to the wells to bind to the captured hemoglobin. A strepavidin-conjugated horseradish peroxidase (SA-HRP) is then added to catalyze a colorimetric reaction with the chromogenic substrate TMB (3,3',5,5'-tetramethylbenzidine). The colorimetric reaction produces a blue product, which turns yellow when the reaction is terminated by addition of dilute sulfuric acid. The absorbance of the yellow product at 450 nm is proportional to the amount of hemoglobin analyte present in the sample and a four-parameter standard curve can be generated. The hemoglobin concentrations in the test samples can then be quantified by interpolating their absorbance from the standard curve generated in parallel with the samples. After factoring sample dilutions, the Hemoglobin concentrations in the original sample can finally be calculated.

Procedure Overview

1. Add 100 µl of standard or sample to designated wells.
   Note: Run each standard or sample in duplicate.



2. Cover the plate and incubate at room temperature (20-25°C) for 1 hour.



3. Wash the plate FOUR times.



4. Add 100 µl of anti-Hemoglobin Detection Antibody to each well.



5. Cover the plate and incubate at room temperature for 1 hour.



6. Wash the plate FOUR times.



7. Add 100 µl of HRP Solution A to each well.



8. Cover the plate and incubate at room temperature for 30 minutes.



9. Wash the plate FOUR times.



10. Add 100 µl of TMB Substrate Solution to each well.



11. Incubate the plate in the dark at room temperature for 30 minutes.



12. Stop the reaction by adding 100 µl of Stop Solution to each well.



13. Measure absorbance on a plate reader at 450 nm.

Additional Materials Required

- Ultrapure water
- Precision pipettors, with disposable plastic tips
- Polypropylene or polyethylene tubes to prepare standard and samples
  Do not use polystyrene, polycarbonate or glass tubes.
- A container to prepare 1X Dilution Buffer B
- A container to prepare 1X Wash Buffer
- A 96-well plate washer or a wash bottle
  An autoclavable plate washer is recommended, if available.
- Disposable reagent reservoirs
- A standard microtiter plate reader for measuring absorbance at 450 nm for end-point ELISA.

Precautions

- Store all reagents at 2-8ºC. *Do not freeze reagents*.
- All reagents must be at room temperature (20-25°C) before use.
- Vigorous plate washing is essential.
- Use new disposable pipette tips for each transfer to avoid cross-contamination.
- Use a new adhesive plate cover for each incubation step.
- Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.
- Avoid microbial contamination of reagents and equipment. Automated plate washers can easily become contaminated and result in erroneous data.
- Do not contaminate the TMB Solution. *Do not expose TMB Substrate solution to glass, foil, or metal*. If the solution is blue before use, DO NOT USE IT.
- Individual components may contain preservatives. Wear gloves while performing the assay and follow proper disposal procedures.

Handling and Preparation of Reagents, Standards, and Samples

Preparation of 1X Dilution Buffer B

- Prepare 1X Dilution Buffer B by diluting 25 ml of 10X Dilution Buffer B into 225 ml of ultra pure water. Mix well. Store reconstituted 1X Dilution Buffer B at 2-8ºC for up to six (6) months. Do not use 1X Dilution Buffer B if it becomes visibly contaminated during storage.

Preparation of Standard Solutions

- Standard solutions should be treated as a biological material and universal precautions should be followed.

1. Reconstitute the 200-ng Hemoglobin standard vial with 1.0 ml of 1X Dilution Buffer B to achieve a final concentration of 200 ng/ml. Mix well.
2. Label seven (7) tubes, one for each standard curve point: 66.6, 22.2, 7.4, 2.5, 0.82, 0.27, and 0 ng/ml. The original vial of re-suspended standard solution represents the top standard at 200 ng/ml.
3. Add 300 µl of 1X Dilution Buffer B into each of the seven tubes.
4. Serially dilute 1:3 by adding 150 µl of the 200 ng/ml standard into the first tube containing 300 µl of 1X Dilution Buffer B. Mix well. Continue the dilution by adding 150 µl of the previous standard into 300 µl of 1X Dilution Buffer B in the next tube until the sixth tube (0.27 ng/ml).
5. The seventh tube containing 300 µl of 1X Dilution Buffer B serves as the zero standard value or blank.

- Using this dilution scheme, only 350 µl of the re-suspended standard solution should be used. The remaining standard solution can be used for another assay within the same day. Otherwise, use the second vial of lyophilized standard.

Sample Handling

- This ELISA assay can be used for whole blood, serum, plasma, CSF or cell culture media.
- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions when handling and disposing of infectious agents.

- 100 µl of sample or standard is required per well.
- Samples must be assayed in duplicate each time the assay is performed.
- Store samples to be assayed within 24 hours at 2-8ºC. For long-term storage, aliquot and freeze samples at -70°C. Avoid repeated freeze-thaw cycles when storing samples.
- If particulates are present in samples, centrifuge prior to analysis.
- If blood samples are clotted, grossly hemolyzed, lipemic, or the integrity of the sample is of concern, make a note on the Plate Template and interpret results with caution

Sample Preparation

- The dilution schemes indicated below are only suggestions. Dilutions should be based on the expected concentration of the unknown sample such that the diluted sample falls within the dynamic range of the standard curve. Prepare one or more dilutions of the sample in 1X Dilution Buffer B using the same serial dilution technique described below until the desired concentration is obtained. Not all listed sample types have been specifically tested by our laboratory.
- Whole Blood – Recommended starting dilution is 1:50,000. With this large dilution one must strive for accurate pipetting. A typical dilution scheme starts by adding 10 µl of plasma into 990 µl of 1X Dilution Buffer B to give a 1:100 dilution. This is repeated by adding 10 µl of the 1:100 diluted sample to 990 µl of 1X Dilution Buffer B to give 1:10,000 dilution. Then add 200 µl of the 1:10,000 dilution to 800 µl of 1X Dilution Buffer B for a final 1:50,000. It is important to mix thoroughly at each dilution step.
- CSF - Recommended starting dilution is 1:100.
- Serum, plasma, or cell culture supernatant - Recommended starting dilution is 1:10 – 1:100.

Preparation of 1X Wash Buffer

- Prepare 1X Wash Buffer by diluting 20X Wash Buffer in ultrapure water. For example, if preparing 1 L of 1X Wash Buffer, dilute 50 ml of 20X Wash Buffer into 950 ml of ultrapure water. Mix well. Store reconstituted 1X Wash Buffer at 2-8ºC for up to six (6) months. Do not use 1X Wash Buffer if it becomes visibly contaminated during storage.

Assay Procedure

Sample Incubation

- Determine the number of strips required. Leave these strips in the plate frame. Place unused strips in the foil pouch with desiccant and seal tightly. Store unused strips at 2-8°C. After completing assay, keep the plate frame for additional assays.
- Use a Plate Template to record the locations of the standards and unknown samples within the wells.

1. Add 100 µl of appropriately diluted standards or samples to each well. Run each standard, sample, or blank in duplicate.
2. **Note: Whole blood, serum, plasma, CSF or cell culture media samples must be diluted prior to testing** (see Sample Preparation section above).
3. Carefully cover the wells with a new adhesive plate cover and incubate for one (1) hour at room temperature, 20-25°C.
4. Carefully remove the adhesive plate cover and wash FOUR times with 1X Wash Buffer, as described in the Plate Washing section below.

Plate Washing

- Using a manual or automated plate washer

1. Rinse the tips of the plate washer by dispensing the 1X wash Buffer into the wash trough and aspirating the solution. Repeat this step five times. For automated plate washers, program the rinse step accordingly.

   Note: This initial rinse step is necessary especially if the plate washer has been idle for several days or longer. Automated plate washers are susceptible to microbial growth in the fluid lines and cavities.
2. Aspirate the solutions from the wells. Fill the wells to about 90% full with 1X Wash Buffer and then aspirate the wash solution. Repeat this wash step three more times. For automated plate washers, program 4 washes at 300 µl per wash, according to the manufacturer's instructions.

- Using a squirt bottle

1. Gently squeeze the long sides of plate frame before washing to ensure all strips remain securely in the frame.
2. Empty the plate contents by quickly dumping the contents of the wells into the sink using a quick, flipping motion.

3. Use a squirt wash bottle to fill each well completely with 1X Wash Buffer, and then empty the plate contents. Repeat procedure three more times for a total of FOUR washes. Blot the plate onto paper towels or other absorbent material.

Incubation with Detection Antibody

- Only remove the required amount of Detection Antibody reagent for the number of strips being used.

1. Add 100 µl of Detection Antibody to each well containing standard, sample or blank. Mix well by gently tapping the plate several times.
2. Carefully attach a new adhesive plate cover and incubate the plate for one hour at room temperature, 20-25°C.
3. Carefully remove the adhesive plate cover, discard plate contents and wash FOUR times with 1X Wash Buffer (see Plate Washing section above).

Incubation with HRP Solution A

- Remove only the required amount of HRP Solution A for the number of strips being used.

1. Add 100 µl of HRP Solution A to each well containing standard, sample or blank.
2. Carefully attach a new adhesive plate cover and incubate plate for 30 minutes at room temperature, 20-25°C.
3. Carefully remove the adhesive plate cover, discard plate contents and wash FOUR times with 1X Wash Buffer (see Plate Washing section). Blot off any residual liquid at the bottom of the wells that might interfere later with the absorbance readings.

Incubation with TMB Substrate and Stopping the Reaction

- Remove only the required amount of TMB Substrate Solution and Stop Solution for the number of strips being used.
- Do NOT use glass pipette to measure the TMB Substrate Solution. Do NOT cover the plate with aluminum foil or metalized mylar. Do NOT return leftover TMB Substrate to bottle. Do NOT contaminate the unused TMB Substrate Solution. If the solution is blue before use, DO NOT USE IT!

1. Add 100 µl of TMB Substrate Solution into each well and allow the enzymatic reaction to develop a blue color at room temperature (20-25ºC) in the dark for 30 minutes. Do NOT cover plate with a plate sealer.
2. Stop the reaction by adding 100 µl of Stop Solution to each well. Tap plate gently to mix. The solution in the wells should immediately change in color from blue to yellow.

Absorbance Measurement

Note: Wipe the underside of the wells with a lint-free tissue.

1. For end-point ELISA, measure the absorbance on an ELISA plate reader set at 450 nm within 30 minutes after the addition of the Stop Solution.

Calculation of Results

- Duplicate absorbance values should be within 10% of each other. Care should be taken when interpreting data with differences in absorbance values greater than 10%.

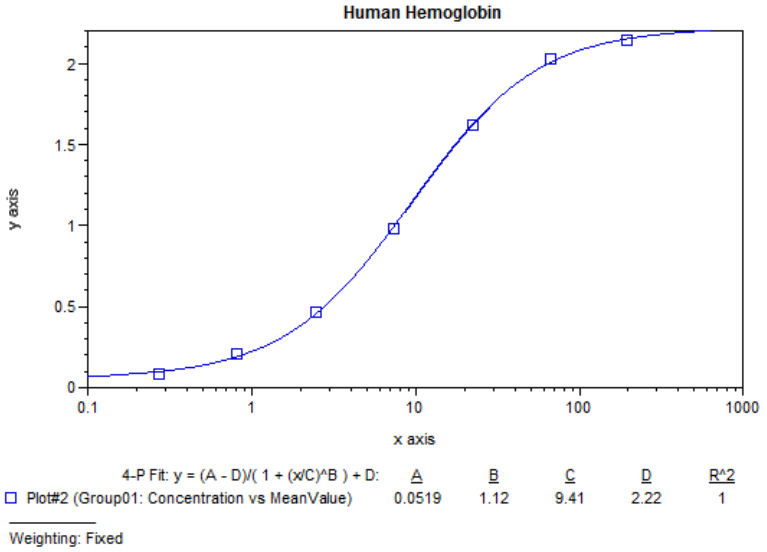
1. Prepare a standard curve to determine the amount of hemoglobin in an unknown sample. Plot the average absorbance obtained for each standard concentration on the vertical (Y) axis versus the corresponding hemoglobin concentration on the horizontal (X) axis using curve-fitting software.
2. Calculate the hemoglobin concentration in unknown samples using the prepared standard curve. Determine the amount of hemoglobin in each unknown sample by noting the hemoglobin concentration (X axis) that correlates with the absorbance value (Y axis) obtained for the unknown sample.
3. If the sample was diluted, multiply the observed Hemoglobin concentration by the dilution factor to determine the concentration of hemoglobin in the original, undiluted sample.

   Note: Most plate readers come with appropriate templates and curve-fitting software. The standard curve of this assay can be fitted into a 4-parameter curve fitting equation that can be programmed to calculate and display a table (or tables) consisting of the raw absorbance readings, net absorbance readings, the analyte concentration in the assay solution, dilution factors, and analyte concentration in the original unknown sample.

Performance Characteristics

Typical Standard Curve

- This typical standard curve was generated using the Human Hemoglobin ELISA Kit Protocol. This standard curve is for demonstration only. A standard curve must be generated for each assay.



Assay Range: 0.27 – 200 ng/ml

- Suggested standard curve points are 200 ng/ml, 66.6 ng/ml, 22.2 ng/ml, 7.4 ng/ml, 2.5 ng/ml, 0.82 ng/ml, 0.27 ng/ml, and 0 ng/ml.

Specificity

- By immunoelectrophoresis and ELISA the antibodies in this kit react specifically with human hemoglobin, not with human immunoglobulins or serum proteins. Cross-reactivity with other species has not been tested.

Warranty

Products are warranted by Bethyl Laboratories, Inc. to meet stated product specifications and to conform to label descriptions when used, handled and stored according to instructions. Unless otherwise stated, this warranty is limited to six months from date of sale. Bethyl Laboratories sole liability for the product is limited to replacement of the product or refund of the purchase price. Bethyl Laboratories products are supplied for research applications. They are not intended for medicinal, diagnostic or therapeutic use. The products may not be resold, modified for resale or used to manufacture commercial products without prior written approval from Bethyl Laboratories, Inc.

Background References

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3. Dickerson RE and Geis I. *Hemoglobin: Structure, Function and Evolution.* Benjamin/Cummings (1983)
4. Huisman THJ et al. *A Syllabus of Human Hemoglobin Variants (1996)*. The Sickle Cell Anemia Foundation, Augusta, GA, USA

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Plate Templates

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
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	1	2	3	4	5	6	7	8	9	10	11	12
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