



## **Rat Hemoglobin (Hb) ELISA Kit**

**Catalog Number. CSB-E13036r**

**For the quantitative determination of rat hemoglobin (Hb) concentrations in serum, plasma, lysate for RBC.**

This package insert must be read in its entirety before using this product.

### **If You Have Problems**

#### **Technical Service Contact information**

Phone: 86-27-87582341

Fax: 86-27-87196150

Email: [tech@cusabio.com](mailto:tech@cusabio.com)

Web: [www.cusabio.com](http://www.cusabio.com)

In order to obtain higher efficiency service, please ready to supply the lot number of the kit to us (found on the outside of the box).

## **PRINCIPLE OF THE ASSAY**

This assay employs the competitive inhibition enzyme immunoassay technique. The microtiter plate provided in this kit has been pre-coated with an antibody specific to Hb. Standards or samples are added to the appropriate microtiter plate wells with Horseradish Peroxidase (HRP) conjugated Hb. The competitive inhibition reaction is launched between with HRP-conjugated Hb and Hb in samples. A substrate solution is added to the wells and the color develops in opposite to the amount of Hb in the sample. The color development is stopped and the intensity of the color is measured.

## **DETECTION RANGE**

0.78 µg/ml-50 µg/ml.

## **SENSITIVITY**

The minimum detectable dose of rat Hb is typically less than 0.39 µg/ml.

The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest rat Hb concentration that could be differentiated from zero.

## **SPECIFICITY**

This assay has high sensitivity and excellent specificity for detection of rat Hb. No significant cross-reactivity or interference between rat Hb and analogues was observed.

**Note:** Limited by current skills and knowledge, it is impossible for us to complete the cross-reactivity detection between rat Hb and all the analogues, therefore, cross reaction may still exist.

## **PRECISION**

### **Intra-assay Precision (Precision within an assay): CV%<8%**

Three samples of known concentration were tested twenty times on one plate to assess.

### **Inter-assay Precision (Precision between assays): CV%<10%**

Three samples of known concentration were tested in twenty assays to assess.

## **LIMITATIONS OF THE PROCEDURE**

- **FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with Sample Diluent and repeat the assay.
- Any variation in Sample Diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Immunoassay, the possibility of interference cannot be excluded.

## **MATERIALS PROVIDED**

| Reagents                          | Quantity    |
|-----------------------------------|-------------|
| Assay plate                       | 1(96 wells) |
| Standard (Freeze dried)           | 2           |
| HRP-conjugate (100 x concentrate) | 1 x 60 µl   |
| HRP-conjugate Diluent             | 1 x 10 ml   |
| Sample Diluent                    | 2 x 20 ml   |
| Wash Buffer (25 x concentrate)    | 1 x 20 ml   |
| TMB Substrate                     | 1 x 10 ml   |
| Stop Solution                     | 1 x 10 ml   |
| Adhesive Strip (For 96 wells)     | 4           |
| Instruction manual                | 1           |

## **STORAGE**

|               |  |   |
|---------------|--|---|
| Unopened kit  | Store at 2 - 8°C. Do not use the kit beyond the expiration date. |   |
| Opened kit    | Coated assay plate   | May be stored for up to 1 month at 2 - 8°C. Try to keep it in a sealed aluminum foil bag, and avoid the damp. |
|               | Standard   | May be stored for up to 1 month at 2 - 8°C. If don't make recent use, better keep it store at -20°C.          |
|               | HRP-conjugate  |   |
|               | HRP-conjugate Diluent  | May be stored for up to 1 month at 2 - 8°C.   |
|               | Sample Diluent   |   |
|               | Wash Buffer  |   |
|               | TMB Substrate  |   |
| Stop Solution |  |   |

**\*Provided this is within the expiration date of the kit.**

### **OTHER SUPPLIES REQUIRED**

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- An incubator which can provide stable incubation conditions up to 37°C±0.5°C.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- Absorbent paper for blotting the microtiter plate.
- 100ml and 500ml graduated cylinders.
- Deionized or distilled water.
- Pipettes and pipette tips.
- Test tubes for dilution.

### **PRECAUTIONS**

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

### **SAMPLE COLLECTION AND STORAGE**

- **Serum** Use a serum separator tube (SST) and allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 15 minutes at 1000 xg. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.
- **Plasma** Collect plasma using EDTA, or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g, 2 - 8°C within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles. Centrifuge the sample again after thawing before the assay.
- **Lysate for RBC** Collect a certain volume of anticoagulant blood sample (e.g. 1ml). Centrifuge at 10000rpm for 10min at 4°C to collect the RBC. Remove supernate and add cold pure water to redissolve RBC to the previous volume (e.g. 1ml). Then store at -20°C for about 30-60 minutes.

After three freeze-thaw cycles to break up the cell membranes, the cell lysates were centrifuged for 10 minutes at 10000rpm, 4°C. Collect the supernatant. RBC lysates should be assayed immediately or aliquotted and stored at -20°C. Centrifuge the sample again after thawing before the assay. Avoid repeated freeze-thaw cycles

### **SAMPLE PREPARATION**

- Serum and plasma samples require a 10-fold dilution into Sample Diluent. The suggested 10-fold dilution can be achieved by adding 25µl sample to 225µl of Sample Diluent. The recommended dilution factor is for reference only. The optimal dilution factor should be determined by users according to their particular experiments.
- Lysate for RBC samples require a 1000-fold dilution into Sample Diluent. The suggested 1000-fold dilution can be achieved by adding 5µl sample to 95µl of Sample Diluent first, then complete the 1000-fold dilution by adding 5µl of this solution to 245µl of Sample Diluent. The recommended dilution factor is for reference only. The optimal dilution factor should be determined by users according to their particular experiments.

**Note:**

1. CUSABIO is only responsible for the kit itself, but not for the samples consumed during the assay. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
2. Samples to be used within 5 days may be stored at 2-8°C, otherwise samples must be stored at -20°C (≤1month) or -80°C (≤2month) to avoid loss of bioactivity and contamination.
3. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
4. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
5. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts of certain chemicals.
6. Owing to the possibility of mismatching between antigen from other resource and antibody used in our kits (e.g., antibody targets conformational epitope rather than linear epitope), some native or recombinant proteins from other manufacturers may not be recognized by our products.
7. Influenced by the factors including cell viability, cell number and also sampling time, samples from cell culture supernatant may not be detected by the kit.
8. Fresh samples without long time storage are recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.

## **REAGENT PREPARATION**

### **Note:**

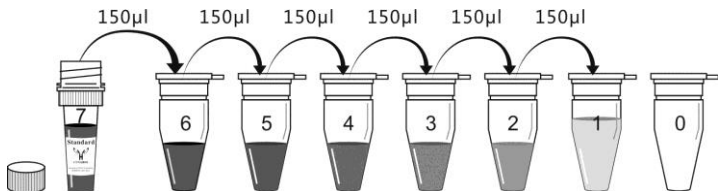
- **Kindly use graduated containers to prepare the reagent. Please don't prepare the reagent directly in the Diluent vials provided in the kit.**
  - Bring all reagents to room temperature (18-25°C) before use for 30min.
  - Prepare fresh standard for each assay. Use within 4 hours and discard after use.
  - Making serial dilution in the wells directly is not permitted.
  - Please carefully reconstitute Standards according to the instruction, and avoid foaming and mix gently until the crystals have completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettors are calibrated. It is recommended to suck more than 10µl for once pipetting.
  - Distilled water is recommended to be used to make the preparation for reagents. Contaminated water or container for reagent preparation will influence the detection result.
1. **HRP-conjugate (1x)** - Centrifuge the vial before opening.  
**HRP-conjugate** requires a 100-fold dilution. A suggested 100-fold dilution is 10 µl of **HRP-conjugate** + 990 µl of **HRP-conjugate Diluent**.
  2. **Wash Buffer(1x)**- If crystals have formed in the concentrate, warm up to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 ml of Wash Buffer Concentrate (25 x) into deionized or distilled water to prepare 500 ml of Wash Buffer (1 x).



### 3. **Standard**

Centrifuge the standard vial at 6000-10000rpm for 30s before opening. Reconstitute the **Standard** with 1.0 ml of **Sample Diluent**. Do not substitute other diluents. This reconstitution produces a stock solution of 50  $\mu\text{g/ml}$ . Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 150  $\mu\text{l}$  of **Sample Diluent** into each tube (S0-S6). Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Standard serves as the high standard (50  $\mu\text{g/ml}$ ). **Sample Diluent** serves as the zero standard (0  $\mu\text{g/ml}$ ).



| Tube             | S7 | S6 | S5   | S4   | S3   | S2   | S1   | S0 |
|------------------|----|----|------|------|------|------|------|----|
| $\mu\text{g/ml}$ | 50 | 25 | 12.5 | 6.25 | 3.12 | 1.56 | 0.78 | 0  |

## ASSAY PROCEDURE

**Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. It is recommended that all samples and standards be assayed in duplicate.**

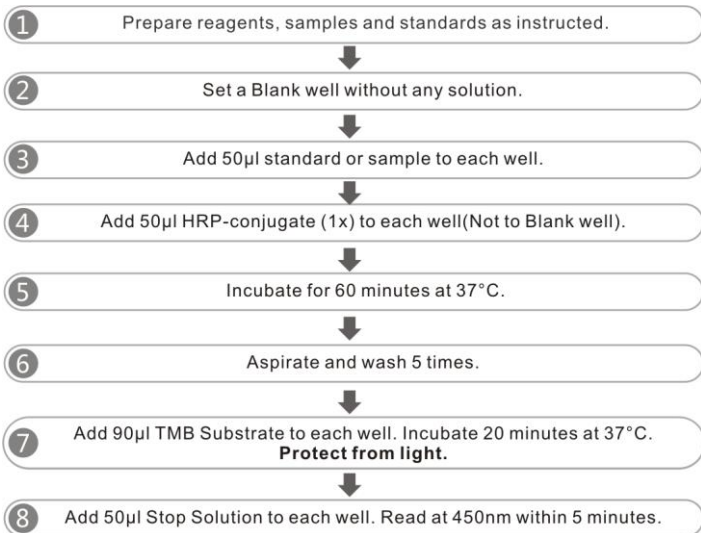
1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells and the desiccant back into the pouch and seal the ziploc, store unused wells at 4°C.
3. Set a **Blank** well without any solution.
4. Add 50µl of **standard** and **sample** per well. Add 50µl **HRP-conjugate (1x)** to each well immediately (not to Blank well). Mix well with the pipette or shake the plate gently for 60 seconds. A plate layout is provided to record standards and samples assayed.
5. Cover with the adhesive strip provided. Incubate for 60 minutes at 37°C.
6. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with **Wash Buffer** (200µl) using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher, and let it stand for 2 minutes, complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
7. Add 90µl of **TMB Substrate** to each well. Incubate for 20 minutes at 37°C. **Protect from light.**
8. Add 50µl of **Stop Solution** to each well, gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well within 5 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. Subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

**\*Samples may require dilution. Please refer to Sample Preparation section.**

Note:

1. The final experimental results will be closely related to validity of the products, operation skills of the end users and the experimental environments.
2. Samples or reagents addition: Please use the freshly prepared Standard. Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall as possible. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all standards and specimens, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
3. Incubation: To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents have been added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be observed.
4. Washing: The wash procedure is critical. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drop of water and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and falsely elevated absorbance reading. When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
5. Controlling of reaction time: Observe the change of color after adding TMB Substrate (e.g. observation once every 10 minutes), TMB Substrate should change from colorless or light blue to gradations of blue. If the color is too deep, add Stop Solution in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.
6. TMB Substrate is easily contaminated. TMB Substrate should remain colorless or light blue until added to the plate. Please protect it from light.
7. Stop Solution should be added to the plate in the same order as the TMB Substrate. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the TMB Substrate.

## ASSAY PROCEDURE SUMMARY



**\*Samples may require dilution. Please refer to Sample Preparation section.**

## **CALCULATION OF RESULTS**

**Using the professional soft "Curve Expert" to make a standard curve is recommended, which can be downloaded from our web.**

Average the duplicate readings for each standard and sample and subtract the average optical density of Blank.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the Hb concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.