CEB409Bo 96 Tests
Enzyme-linked Immunosorbent Assay Kit
For Hemoglobin (HB)
Organism Species: *Bos taurus; Bovine* (Cattle)
Instruction manual

FOR RESEARCH USE ONLY
NOT FOR USE IN CLINICAL DIAGNOSTIC PROCEDURES

Version 13.0

[INTENDED USE]

The kit is a competitive inhibition enzyme immunoassay technique for the in vitro quantitative measurement of hemoglobin in bovine serum, plasma, tissue homogenates, erythrocyte lysates, cell culture supernates and other biological fluids.

[REAGENTS AND MATERIALS PROVIDED]

Reagents	Quantity	Reagents	Quantity
Pre-coated, ready to use 96-well strip plate	1	Plate sealer for 96 wells	4
Standard	2	Standard Diluent	1×20mL
Detection Reagent A	1×120μL	Assay Diluent A	1×12mL
Detection Reagent B	1×120μL	Assay Diluent B	1×12mL
TMB Substrate	1×9mL	Stop Solution	1×6mL
Wash Buffer (30 × concentrate)	1×20mL	Instruction manual	1

[MATERIALS REQUIRED BUT NOT SUPPLIED]

1. Microplate reader with 450 ± 10nm filter.
2. Single or multi-channel pipettes with high precision and disposable tips.
3. Microcentrifuge Tubes.
4. Deionized or distilled water.
5. Absorbent paper for blotting the microplate.
6. Container for Wash Solution.
7. 0.01mol/L (or 1X) Phosphate Buffered Saline, pH7.0-7.2.

[STORAGE OF THE KITS]

1. **For unopened kit:** The whole kit could be stored at -20°C in shelf life, while up to one month at 4°C. For experiment convenience, reagents could also be stored seperately, **Standard**, **Detection Reagent A**, **Detection Reagent B** and **96-well strip plate** should be stored at -20°C while the others could be at 4 °C.
2. **For used kit:** When the kit is used, the remaining reagents need to be stored according to the above storage condition. Besides, please return the unused wells to the foil pouch containing the desiccant pack, and zip-seal the foil pouch.

Note:

It is highly recommended to use the remaining reagents within 1 month provided this is prior to the expiration date of the kit. For the expiration date of the kit, please refer to the label on the kit box. All components are stable up to the expiration date.

[SAMPLE COLLECTION AND STORAGE]

Serum - Allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 20 minutes at approximately 1,000×g. Assay immediately or store samples in aliquot at -20°C or -80°C. Avoid repeated freeze/thaw cycles.

Plasma - Collect plasma using EDTA as an anticoagulant. Centrifuge samples for 15 minutes at 1,000×g at 2 - 8°C within 30 minutes of collection. Remove plasma and assay immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Tissue homogenates - The preparation of tissue homogenates will vary depending upon tissue type.

1. Tissues were rinsed in ice-cold PBS to remove excess blood thoroughly and weighed before homogenization.
2. Minced the tissues to small pieces and homogenized them in fresh lysis buffer (catalog: IS007, different lysis buffer needs to be chosen based on subcellular location of the target protein) (w:v = 1:20-1:50, e.g. 1mL lysis buffer is added in 20-50mg tissue sample) with a glass homogenizer on ice (Micro Tissue Grinders woks, too).
3. The resulting suspension was sonicated with an ultrasonic cell disrupter till the solution is clarified.
4. Then, the homogenates were centrifuged for 5 minutes at 10,000×g. Collect the supernates and assay immediately or aliquot and store at ≤-20°C.

Erythrocyte lysates:

1. Centrifuge the whole blood for 20 minutes at approximately 1,000×g, remove the supernatant and collect the cells.
2. Wash cells three times in cold PBS(0.01mol/L, pH 7.0-7.2).
3. Resuspend cells in ice-cold PBS and the cells was subject to freeze cells at ≤-20°C. Thaw cells with gentle mixing. Repeat the freeze/thaw cycle for 3 times.
4. Centrifuge at 5,000×g for 10 minutes at 2 - 8°C to remove cellular debris.
5. Assay the supernatant immediately or store samples in aliquot at -20°C or -80°C. Avoid repeated freeze/thaw cycles.

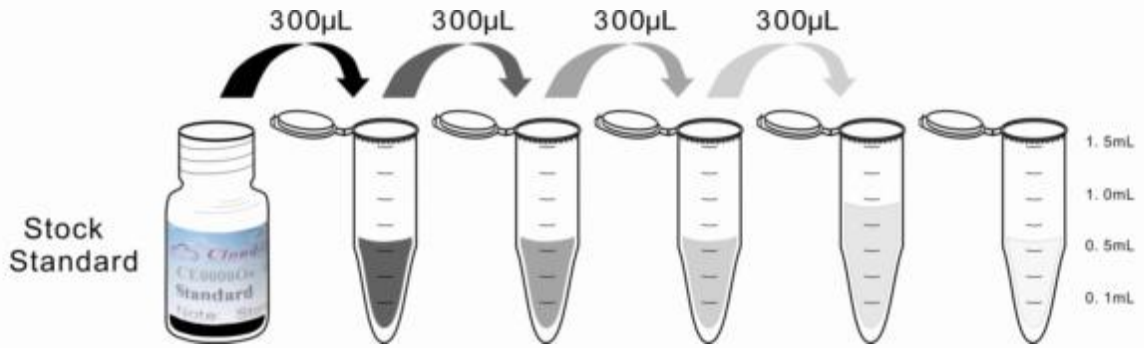
Cell culture supernates and other biological fluids - Centrifuge samples for 20 minutes at 1000×g. Collect the supernates and assay immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Note:

1. Samples to be used within 5 days may be stored at 4°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination.
2. When performing the assay, bring samples to room temperature.
3. It is highly recommended to use serum instead of plasma for the detection based on quantity of our in-house data.
4. Erythrocyte lysates must be diluted with Standard Dilute before running.

[REAGENT PREPARATION]

1. Bring all kit components and samples to room temperature (18-25°C) before use. If the kit will not be used up in one time, please only take out strips and reagents for present experiment, and leave the remaining strips and reagents in required condition.
2. **Standard -** Reconstitute the **Standard** with 0.5mL of **Standard Diluent,** kept for 10 minutes at room temperature, shake gently(not to foam). The concentration of the standard in the stock solution is 1,000μg/mL. Please prepare 5 tubes containing 0.6mL Standard Diluent and produce a triple dilution series according to the picture shown below. Mix each tube thoroughly before the next transfer. Set up 5 points of diluted standard such as 1,000μg/mL, 333.33μg/mL, 111.11μg/mL, 37.04μg/mL, 12.35μg/mL, and the last EP tubes with **Standard Diluent** is the blank as 0μg/mL.



Tube	1	2	3	4	5	6
μg/mL	1,000	333.33	111.11	37.04	12.35	0

3. **Detection Reagent A and Detection Reagent B -** Briefly spin or centrifuge the stock Detection A and Detection B before use. Dilute them to the working concentration 100-fold with **Assay Diluent A** and **B**, respectively.
4. **Wash Solution** - Dilute 20mL of Wash Solution concentrate (30×) with 580mL of deionized or distilled water to prepare 600mL of Wash Solution (1×).
5. **TMB substrate** - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Note:

1. Making serial dilution in the wells directly is not permitted.
2. Prepare standard within 15 minutes before assay. Please do not dissolve the reagents at 37°C directly.
3. Detection Reagent A and B are sticky solutions, therefore, slowly pipette them to reduce the volume errors.
4. Please carefully reconstitute Standards or working Detection Reagent A and B according to the instruction, and avoid foaming and mix gently until the crystals are 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 one pipetting.
5. The reconstituted Standards, Detection Reagent A and Detection Reagent B can be **used only once**.

6. If crystals have formed in the Wash Solution concentrate (30×), warm to room temperature and mix gently until the crystals are completely dissolved.
7. Contaminated water or container for reagent preparation will influence the detection result.

[SAMPLE PREPARATION]

1. We are 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. 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. Sample should be diluted by PBS.
3. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
4. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts from certain chemicals.
5. Due to the possibility of mismatching between antigen from other origin 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.
6. Influenced by the factors including cell viability, cell number or sampling time, samples from cell culture supernates may not be detected by the kit.
7. Fresh samples without long time storage is recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.

[ASSAY PROCEDURE]

1. Determine wells for diluted standard, blank and sample. Prepare 5 wells for standard points, 1 well for blank. Add 50μL each of dilutions of standard (read Reagent Preparation), blank and samples into the appropriate wells, respectively. And then add 50μL of Detection Reagent A to each well immediately. Shake the plate gently (using a microplate shaker is recommended). Cover with a Plate sealer. Incubate for 1 hour at 37°C. Detection Reagent A may appear cloudy. Warm to room temperature and mix gently until solution appears uniform.
2. Aspirate the solution and wash with 350μL of 1X Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let it sit for 1-2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Repeat 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.
3. Add 100μL of Detection Reagent B working solution to each well. Incubate for 30 minutes at 37°C after covering it with the Plate sealer.
4. Repeat the aspiration/wash process for total 5 times as conducted in step 2.
5. Add 90μL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for 10 - 20 minutes at 37°C (Don't exceed 30 minutes). Protect from light. The liquid will turn blue by the addition of Substrate Solution.

6. Add 50μL of Stop Solution to each well. The liquid will turn yellow by the addition of Stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
7. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450nm immediately.

Note:

1. **Assay preparation:** Keep appropriate numbers of wells for each experiment and remove extra wells from microplate. Rest wells should be resealed and stored at -20°C.
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. 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 standards, samples, and reagents. Also, use separated 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 are added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be controlled.
4. **Washing:** The wash procedure is critical. Complete removal of liquid at each step is essential for 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 false elevated absorbance reading.
5. **Controlling of reaction time:** Observe the change of color after adding **TMB Substrate** (e.g. observation once every 10 minutes)**,** 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. Please protect it from light.
7. The environment humidity which is less than 60% might have some effects on the final performance, therefore, a humidifier is recommended to be used at that condition.

[TEST PRINCIPLE]

This assay employs the competitive inhibition enzyme immunoassay technique. An antibody specific to hemoglobin has been pre-coated onto a microplate. A competitive inhibition reaction is launched between biotin labeled hemoglobin and unlabeled hemoglobin (Standards or samples) with the pre-coated antibody specific to hemoglobin. After incubation the unbound conjugate is washed off. Next, avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. The amount of bound HRP conjugate is reverse proportional to the concentration of hemoglobin in the sample. After addition of the substrate solution, the intensity of color developed is reverse proportional to the concentration of hemoglobin in the sample.

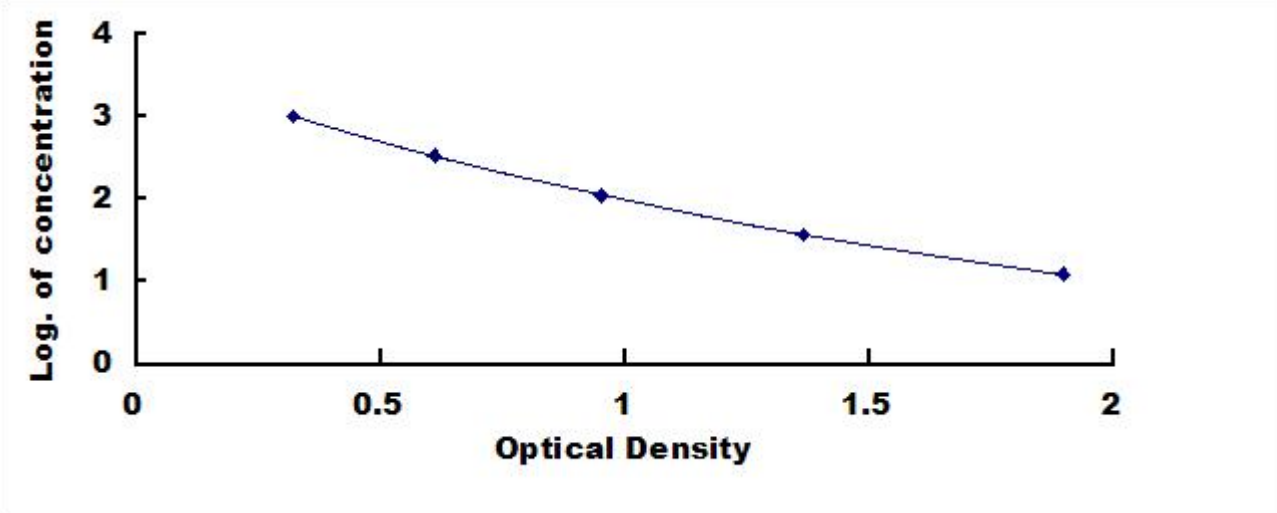
[CALCULATION OF RESULTS]

This assay employs the competitive inhibition enzyme immunoassay technique, so there is an inverse correlation between hemoglobin concentration in the sample and the assay signal intensity.

Average the duplicate readings for each standard, control, and samples. Create a standard curve with the log of hemoglobin concentration on the y-axis and absorbance on the x-axis. Draw a best fit curve through the points and it can be determined by regression analysis. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

[TYPICAL DATA]

In order to make the calculation easier, we plot the O.D. value of the standard (X-axis) against the log of concentration of the standard (Y-axis), although concentration is the independent variable and O.D. value is the dependent variable. The O.D. values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Typical standard curve below is provided for reference only.



Typical Standard Curve for Hemoglobin, Bovine ELISA.

[DETECTION RANGE]

12.35-1,000μg/mL. The standard curve concentrations used for the ELISA's were 1,000μg/mL, 333.33μg/mL, 111.11μg/mL, 37.04μg/mL, 12.35μg/mL.

[SENSITIVITY]

The minimum detectable dose of hemoglobin is typically less than 4.31μg/mL.

The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined by subtracting two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

[SPECIFICITY]

This assay has high sensitivity and excellent specificity for detection of hemoglobin.

No significant cross-reactivity or interference between hemoglobin and analogues was observed.

Note:

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

[RECOVERY]

Matrices listed below were spiked with certain level of hemoglobin and the recovery rates were calculated by comparing the measured value to the expected amount of hemoglobin in samples.

Matrix	Recovery range (%)	Average(%)
serum(n=5)	88-102	96
EDTA plasma(n=5)	90-99	95

[LINEARITY]

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of hemoglobin and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

Sample	1：2	1：4	1：8	1：16
serum(n=5)	82-92%	80-97%	91-99%	83-98%
EDTA plasma(n=5)	95-105%	92-101%	79-103%	81-95%

[PRECISION]

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level hemoglobin were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level hemoglobin were tested on 3 different plates, 8 replicates in each plate.

CV(%) = SD/meanX100

Intra-Assay: CV<10%

Inter-Assay: CV<12%

[STABILITY]

The stability of ELISA kit is determined by the loss rate of activity. The loss rate of this kit is less than 5% within the expiration date under appropriate storage condition.

To minimize extra influence on the performance, operation procedures and lab conditions, especially room temperature, air humidity, incubator temperature should be strictly controlled. It is also strongly suggested that the whole assay is performed by the same operator from the beginning to the end.

[ASSAY PROCEDURE SUMMARY]

1. Prepare all reagents, samples and standards;
2. Add 50μL standard or sample to each well.
   And then add 50μL prepared Detection Reagent A immediately.
   Shake and mix. Incubate 1 hour at 37°C;
3. Aspirate and wash 3 times;
4. Add 100μL prepared Detection Reagent B. Incubate 30 minutes at 37°C;
5. Aspirate and wash 5 times;
6. Add 90μL Substrate Solution. Incubate 10-20 minutes at 37°C;
7. Add 50μL Stop Solution. Read at 450 nm immediately.

[IMPORTANT NOTE]

1. Limited by the current conditions and scientific technology, we can't completely conduct the comprehensive identification and analysis on the raw material provided by suppliers. So there might be some qualitative and technical risks to use the kit.
2. The final experimental results will be closely related to validity of the products, so the kit should be used prior to the expiration date. And please store the kits exactly according to the instruction.
3. Kits from different batches may be a little different in detection range, sensitivity and color developing time. Please perform the experiment exactly according to the instruction attached in kit while electronic ones from our website is only for reference.
4. Do not mix or substitute reagents from one kit lot to another. Use only the reagents supplied by manufacturer.
5. Protect all reagents from strong light during storage and incubation. All the bottle caps of reagents should be covered tightly to prevent the evaporation and contamination of microorganism. TMB Substrate should remain colorless till it is reacted with the enzyme which binds to the microplate.
6. There may be some foggy substance in the wells when the plate is opened at the first time. It will not have any effect on the final assay results. Do not remove microtiter plate from the storage bag until needed.
7. Wrong operations during the reagents preparation and loading, as well as incorrect parameter setting for the plate reader may lead to incorrect results. A microplate reader with a bandwidth of 10nm or less and an optical density range of 0-3 O.D. at 450 ± 10nm wavelength is acceptable for use in absorbance measurement. Please read the instruction carefully and adjust the instrument prior to the experiment.
8. Variation in sample preparation and each step of experimental opearation may cause different results. In order to get better reproducible results, the operation of each step in the assay should be controlled.
9. Each kit has been strictly passed Q.C test. However, results from end users might be inconsistent with our in-house data due to some unexpected transportation conditions or different lab equipments. Intra-assay variance among kits from different batches might arise from above factors, too.
10. Kits from different manufacturers with the same item might produce different results, since we haven’t compared our products with other manufacturers.
11. The standard of the kit and immunogen used for antibody preparation are commonly recombinant proteins, as different fragments, expression systems, purification methods might be used in recombinant protein preparation, we can not guarantee the kit could detect recombinant protein from other companies. So, it is not recommended to use the kit for the detection of recombinant protein.
12. Please predict the concentration of target molecules in samples, or arrange a preliminary experiment, it is a good way to solve specific problem, eg. the concentration of sampels are beyond the detection range of the kit.
13. The kit might not be suitable for detection of samples from some special experiment, for instance, knock-out experiments, due to their uncertainty of effectiveness.
14. The instruction manual is also for the kit of 48T, but all reagents of 48T kit are reduced by half.
15. The kit is designed for research use only, we will not be responsible for any issue if the kit was used in clinical diagnostic or any other procedures.

[PRECAUTION]

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

[TROUBLE SHOOTING]

Problem	Possible Source	Correction Action
Poor Standard Curve	Improper standard curve preparation	Ensure accurate operation of the dilution
	Incomplete washing and aspiration	Adequate washing and adequate aspiration
	Inaccurate Pipetting	Check and Calibrate pipettes
Poor Precision	Incomplete washing of wells	Ensure sufficient washing
	Inadequate mixing and aspiration reagents	Adequate aspiration and mixing reagents
	Reused pipette tips, containers and sealers	Change and use new pipette tips, containers and sealers
	Inaccurate Pipetting	Check and Calibrate pipettes
Low O.D Values	Inadequate reagent volumes added to wells	Calibrate pipettes and Add adequate reagents
	Incorrect incubation times	Ensure sufficient incubation times
	Incorrect incubation temperature	Reagents balanced to room temperature
	Conjugate or substrate reagent failure	Mix conjugate & substrate, color should develop immediately
	No stop solution added	Follow the assay protocol in the kit manual
	Read beyond suggested reading time	Read within the time recommended in the manual
Sample Values	Improper Sample Storage	Store the sample properly and use the fresh sample
	Improper sample collection and preparation	Take proper sample collection and preparation method
	Low quantity of analyte in samples	Use new sample and repeat assay