

HMGB1 ELISA Kit II

For research use only

Read this package insert before use.

1. INTRODUCTION¹⁻⁸⁾

High mobility group box 1 (HMGB1) is a protein of approximately 30 kDa. HMGB1 is the major component of the non-histone nuclear protein group and is known as a transcriptional regulator. The major receptor of HMGB1 includes the receptor for advanced glycation end products (RAGE). HMGB1 is recently focused as a late inflammatory mediator in septic shock. The concentration of HMGB1 in blood is increased in many diseases including rheumatoid arthritis (RA), acute lung injury (ALI), cancer, disseminated intravascular coagulation (DIC) and surgery.

2. INTENDED USE

This kit is intended for the quantitative determination of HMGB1 without interference from the analogous protein HMGB2⁹⁾ in human, bovine, pig, rabbit, rat and mouse serum/plasma, cell culture supernatant, cerebrospinal fluid (CSF) and bronchoalveolar lavage fluid (BALF).

This kit has been configured for research use only, and not for diagnostic or therapeutic use.

3. KIT COMPONENTS

Package	96 tests X 1	
Product code	326054329	
Antibody-coated Plate anti-HMGB1 polyclonal antibody	8 wells	X 12 strips
POD-conjugate (lyophilized) peroxidase-linked anti-HMGB1,2 monoclonal antibody	for 12 mL	X 1 vial
Standard (lyophilized) pig HMGB1		X 1 vial
Positive Control (lyophilized) pig HMGB1		X 1 vial
Sample Diluent buffer containing additives and preservative	20 mL	X 1 vial
Conjugate Solvent buffer containing additives and preservative	12 mL	X 1 vial
Color Reagent A 3, 3', 5, 5'-tetramethyl-benzidine	6 mL	X 1 vial
Color Reagent B buffer containing 0.005 mol/L hydrogen peroxide	6 mL	X 1 vial
Stop Solution 0.35 mol/L sulfuric acid	12 mL	X 1 vial
5x Wash Concentrate 5-fold concentrated buffer containing Tween 20	100 mL	X 2 vials
Plate Seal		X 2 sheets

4. PRINCIPLE OF THE ASSAY

This assay employs a 2-step sandwich enzyme-linked immunosorbent assay (ELISA). A polyclonal antibody specific for HMGB1 has been pre-coated onto the wells of the microtiter strips. Samples are added to the wells. HMGB1 in samples binds specifically to the immobilized antibody. After addition of a POD-conjugate anti-HMGB1,2 monoclonal antibody to form an antigen-antibody complex, substrate solution is added to the wells and color develops in proportion to the amount of HMGB1 in the sample. The color development is stopped and the color intensity is measured at 450 nm.

5. PROCEDURE

5-1. MATERIALS REQUIRED

- Micropipettes and tips
- Graduated cylinder
- Vortex mixer and plate mixer
- Microplate washer
- Plastic tubes for diluting standards and samples
- Incubator at 37°C (also possible to use CO₂ incubator at 37°C)
- Incubator at 25°C (also possible at room temperature around 25°C)
- 96-wells microplate reader capable of measurement at 450 nm

5-2. REAGENT PREPARATION

Antibody-coated Plate It is ready to use. Repack unused strips in the aluminum package with desiccant agent and store at 2-8°C.

Sample Diluent It is ready to use.

Standards Reconstitute *Standard* to 320 ng/mL with *Sample Diluent*. Refer to the vial label for instruction. Mix gently and allow to stand for

10 minutes to ensure complete reconstitution. Prepare a standard dilution series using *Sample Diluent* and plastic tubes (see Table 1). Reconstituted and undiluted standard may be stored in plastic tubes as aliquots and remain stable for 1 month below -30°C. Avoid repeated freeze-thaw cycles. Make a standard dilution series with each assay.

Positive control Reconstitute *Positive Control* with *Sample Diluent*. Refer to the vial label for instruction. Mix gently and allow to stand for 10 minutes to ensure complete reconstitution. The concentration range of the positive control is indicated on the vial label. Reconstituted positive control may be stored in plastic tubes as aliquots and remain stable for 1 month below -30°C. Avoid repeated freeze-thaw cycles.

POD-conjugate solution Reconstitute *POD-conjugate* with entire volume of *Conjugate Solvent*. Mix gently and allow to stand for 10 minutes to ensure complete reconstitution. Reconstituted POD-conjugate solution may be stored in plastic tubes covered with aluminum foil as aliquots and remain stable for 1 month below -30°C. Avoid repeated freeze-thaw cycles.

Substrate solution Immediately before use, mix in a clean tube equal volumes of *Color Reagent A* and *B*, which have been brought to room temperature prior to use.

Stop Solution It is ready to use.

Wash solution Dilute *5x Wash Concentrate* with four volumes of distilled water.

Table 1: Example of standard dilution method

Dilution method	Concentration (ng/mL)
S1: Add 100 µL of 320 ng/mL standard solution to 300 µL of <i>Sample Diluent</i> and mix.	80
S2: Add 100 µL of S1 to 100 µL of <i>Sample Diluent</i> and mix.	40
S3: Add 100 µL of S2 to 100 µL of <i>Sample Diluent</i> and mix.	20
S4: Add 100 µL of S3 to 100 µL of <i>Sample Diluent</i> and mix.	10
S5: Add 100 µL of S4 to 100 µL of <i>Sample Diluent</i> and mix.	5
S6: Add 100 µL of S5 to 100 µL of <i>Sample Diluent</i> and mix.	2.5

5-3. SAMPLE PREPARATION

Collect serum/plasma immediately from blood collection tube after centrifugation for 15 minutes at over 1,000xg. HMGB1 level may increase due to cytolysis, when centrifugation is not sufficient or serum/plasma is in contact with blood cell for considerable period of time. Aliquots of samples into plastic tubes can be stored at -80°C. Avoid repeated freeze-thaw cycles.

HMGB1 is measured using samples from cell culture supernatant, cerebrospinal fluid (CSF) and bronchoalveolar lavage fluid (BALF). In the case of cell culture supernatant with fetal bovine serum (FBS), bovine HMGB1 in FBS is measured. Therefore negative control should be taken.

If samples are containing HMGB1 exceeding the highest standard, measure after dilution with *Sample Diluent* using plastic tubes.

5-4. PROCEDURAL NOTES

1. Each reagent should be brought to room temperature at least 30 minutes before use.
2. Crystals may be observed in some wells of the microtiter strips but it shall not affect the assay performance. There is no need to wash the wells prior to use.
3. A standard curve must be run with each assay.
4. Use plastic tubes for diluting standards and samples.
5. See the section **6 'TEST PERFORMANCE'**, when higher sensitivities are required.

5-5. ASSAY PROCEDURE

1. Add 100 µL of *Sample Diluent* to each well.
2. Add 10 µL of *Sample Diluent* to zero well.
3. Add 10 µL of standards and samples to the wells. When including positive control, add 10 µL to the well.
4. After shaking the plate with a plate mixer, cover all wells tightly using *Plate Seal* and incubate for 20-24 hours at 37°C. When shaking the plate, be careful not to spill liquids from the wells.

5. Wash the wells 5 times with wash solution (400 μ L/well) using an automated or manual microplate washer. After the final wash, turn over the plate and gently tap 4 or 5 times on a lint-free paper towel to remove any remaining wash buffer.
6. Add 100 μ L of POD-conjugate solution to each well.
7. Cover all wells tightly with *Plate Seal* and incubate for 2 hours at 25°C.
8. Wash the wells 5 times with wash solution (400 μ L/well) using an automated or manual microplate washer. After the final wash, turn over the plate and gently tap 4 or 5 times on a lint-free paper towel to remove any remaining wash buffer.
9. Clean the back of the wells. Be careful not to scratch the wells as this may interfere with measurements.
10. Add 100 μ L of substrate solution to each well at a regular time interval. Incubate for 30 minutes at room temperature. During the reaction, cover the plate with a plate cover or plastic wrap to avoid contamination during the reaction.
11. Add 100 μ L of *Stop Solution* to each well in the same sequence and at the same time intervals to the additions of substrate solution.
12. Read the absorbance of each well at 450 nm using a microplate reader within 60 minutes after adding *Stop Solution*.
13. Subtract the absorbance of zero well from the absorbance of each well. Read the HMGB1 concentrations of unknown samples from the standard curve (see Fig. 1). If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

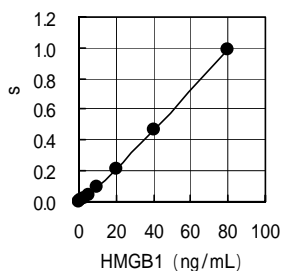


Fig. 1 : Typical standard curve

5-6. ASSAY SUMMARY

Prepare standards (see Table 1)

- ↓
- Add 100 μ L *Sample Diluent* to each well
- ↓
- Add 10 μ L standards and samples
- ↓ Incubate for 20 - 24 hours at 37°C
- Wash wells 5 times
- ↓
- Add 100 μ L POD-conjugate solution
- ↓ Incubate for 2 hours at 25°C
- Wash wells 5 times
- ↓
- Add 100 μ L substrate solution
- ↓ Incubate for 30 minutes at room temperature
- Add 100 μ L *Stop Solution*
- ↓
- Read at 450 nm

6. TEST PERFORMANCE

Dynamic range 2.5 - 80 ng/mL

Sensitivity 1 ng/mL

Specificity Cross-reaction with HMGB2 is < 2 %

Reproducibility

Intra-assay coefficient of variation is < 10 % (n = 8)

Inter-assay coefficient of variation is < 10 % (n = 8)

Recovery 80 - 120 %

Assay sensitivity of 0.2 ng/mL can be achieved by performing the assay in following volumes; (1) Add 50 μ L of *Sample Diluent* to each well. (2) Add 50 μ L of *Sample Diluent* (zero well), standards and samples. (3) Follow the section 5-5. 'ASSAY PROCEDURE' from Item 4 onward. For this level of detection, use standards diluted below 10 ng/mL. The performance of specificity, reproducibility and recovery remains unchanged.

7. STORAGE AND EXPIRATION DATE

Expiration date is printed on the package. Store at 2-8°C and do not freeze.

8. NOTES

1. This kit is for research use only. Do not use for diagnostic or therapeutic use.
2. Read this package insert and the latest MSDS before use.
3. Do not mix components from different kit lot or use reagents beyond the kit expiration date.
4. Each reagent should be brought to room temperature at least 30 minutes before use.
5. Strictly follow the methods for the preparation of the reagent and the sample.
6. Collect serum/plasma immediately from blood collection tube after centrifugation for 15 minutes at over 1,000xg. HMGB1 level may increase due to cytolysis, when centrifugation is not sufficient or serum/plasma is in contact with blood cell for considerable period of time.
7. As described in section 5-2. 'REAGENT PREPARATION', properly store aliquots of the reconstituted standard solution, positive control and POD-conjugate solution. They remain stable for 1 month below -30°C. Avoid repeated freeze-thaw cycles. Repack unused strips in the aluminum package with desiccant agent. Store at 2-8°C together with other reagents.
8. A standard curve must be run with each assay.
9. Handle samples with caution to avoid the possibility of infections.
10. *Sample Diluent* contains a small amount of sodium azide. Sodium azide may produce an explosive azide metal if contaminated with Pb or Cu. Wash with a large volume of water to prevent azide accumulation upon disposal.
11. *Stop Solution* is acidic (below pH 2). Therefore, care should be taken to avoid contact with skin and eyes.
12. All reagents and samples should be considered as potentially hazardous. Take care in handling and disposal. In the case of contact with skin or mucosa, wash immediately with a large volume of water. Consult a physician if any abnormality develops.

9. REFERENCES

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