Mouse/Rat HMGB1 ELISA Kit

Enzyme Immunoassay kit for the quantification of Mouse/Rat HMGB1 in Mouse/Rat plasma, cell culture supernatants and cell/tissue lysate samples.

Catalog number: ARG81310

Package: 96 wells

TABLE OF CONTENTS

SECTION	Page
INTRODUCTION	3
PRINCIPLE OF THE ASSAY	3
MATERIALS PROVIDED & STORAGE INFORMATION	4
MATERIALS REQUIRED BUT NOT PROVIDED	5
TECHNICAL HINTS AND PRECAUTIONS	5
SAMPLE COLLECTION & STORAGE INFORMATION	6
REAGENT PREPARATION	9
ASSAY PROCEDURE	11
CALCULATION OF RESULTS	12
EXAMPLE OF TYPICAL STANDARD CURVE	13
QUALITY ASSURANCE	13

MANUFACTURED BY:

Arigo Biolaboratories Corporation

Address: No. 22, Ln. 227, Gongyuan Rd., Hsinchu City 300, Taiwan

Phone: +886 (3) 562 1738

Fax: +886 (3) 561 3008

Email: info@arigobio.com

INTRODUCTION

Multifunctional redox sensitive protein with various roles in different cellular compartments. In the nucleus is one of the major chromatin-associated non-histone proteins and acts as a DNA chaperone involved in replication, transcription, chromatin remodeling, V(D)J recombination, DNA repair and genome stability. Proposed to be a universal biosensor for nucleic acids. Promotes host inflammatory response to sterile and infectious signals and is involved in the coordination and integration of innate and adaptive immune responses. In the cytoplasm functions as sensor and/or chaperone for immunogenic nucleic acids implicating the activation of TLR9-mediated immune responses, and mediates autophagy. Acts as danger associated molecular pattern (DAMP) molecule that amplifies immune responses during tissue injury. Released to the extracellular environment can bind DNA, nucleosomes, IL-1 beta, CXCL12, AGER isoform 2/sRAGE, lipopolysaccharide (LPS) and lipoteichoic acid (LTA), and activates cells through engagement of multiple surface receptors. In the extracellular compartment fully reduced HMGB1 (released by necrosis) acts as a chemokine, disulfide HMGB1 (actively secreted) as a cytokine, and sulfonyl HMGB1 (released from apoptotic cells) promotes immunological tolerance. [Provided by Uniprot]

PRINCIPLE OF THE ASSAY

This assay employs the sandwich enzyme immunoassay technique for the detection of Mouse/Rat HMGB1 in plasma, cell culture supernatants and cell/tissue lysate samples. An antibody specific for HMGB1 has been pre-coated onto a microtiter plate. Standards or samples are pipetted into the wells

and any HMGB1 present is bound on the plate. After washing away any unbound substances, a Horseradish Peroxidase (HRP) conjugated primary antibody binds to HMGB1 is added to each well and incubates. After washing away any unbound antibody-enzyme reagent, a substrate solution (TMB) is added to the wells and color develops in proportion to the amount of total HMGB1 bound in the initial step. The color development is stopped by the addition of acid and the intensity of the color is measured at a wavelength of 450nm ±2nm. The concentration of total HMGB1 in the sample is then determined by comparing the O.D of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Store unopened kit at 2-8 °C. Use the kit before expiration date.

Component	**Quantity**	**Storage information**
antibody-coated microplate	12 X 8 strips	4°C
Standard (100 ng)	1 vial	4°C, store at -20 °C after reconstitution
Standard reconstitution buffer	1.1 ml	4°C
HRP-Conjugated antibody (200X)	115 µl	4°C
Sample & Antibody diluent buffer	50 ml	4°C
10X Wash buffer	20 ml	4°C
TMB substrate	12 ml (ready-to-use)	4°C (Protect from light)
STOP solution	6 ml	4°C
Plate sealer	1 strips	Room temperature

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 450nm
- Pipettes and pipette tips
- Deionized or distilled water
- Automated microplate washer (optional)
- Lysis buffer (optional)

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store the kit at 4°C at all times.
- Briefly spin down the HRP-Conjugated antibody before use.
- If crystals are observed in the 10X Wash buffer, warm to RT (not more than 50°C) until the crystals are completely dissolved. 1X Wash buffer should be prepared and stored at 4°C before use.
- Ensure complete reconstitution and dilution of reagents prior to use.
- It is highly recommended that the standards, samples and controls be assayed in duplicates.
- Change pipette tips between the addition of different reagent or samples.
- Samples contain azide cannot be assayed.
- Cell culture supernatants samples contain animal serum (E.g. fetal bovine serum (FBS) or calf serum (CS)) might contain HMGB1 from the animal serum and it might affect the accuracy of detection. Therefore, it is recommended to use BSA to replace serum in cell culture medium or chemical modified serum free medium (e.g. OPTI-MEM) for culture

cells.

- Serum/plasma from various autoimmune diseases animal model might contain HMGB1 autoantibodies which might result in interference of this assay.

SAMPLE COLLECTION & STORAGE INFORMATION

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Plasma: Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. It is important to ensure a platelet free preparation since platelets can release HMGB1. Assay immediately or aliquot and store at ≤ -20°C. Avoid repeated freeze-thaw cycles. Avoid using haemolytic, icteric or lipaemic samples and samples contain azide cannot be assayed.

Cell Culture Supernatants: It is recommended to use serum-free medium or use BSA to replace serum in cell culture medium or use chemical modified serum free medium for cell culture. Users should choose suitable culture medium based on the cell types. Remove particulates by centrifugation and aliquot & store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: Due to the expression/releasing levels of different cell lines or conditions are variant, samples from Cell Culture Supernatant might need to be concentrated by speedvac or protein concentrator spin columns before assay. (It is recommended to do pre-test to determine the suitable concentration factor. For our experience, we concentrated 5X for the Cell Culture Supernatant from LPS treated Huh7 cells before assay).

Lysis buffer

1. **Mild cell/tissue lysis buffer: RIPA buffer**

   10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl

2. **Harsh cell/tissue lysis buffer: 0.2% SDS lysis buffer**

   0.2% SDS in 1XPBS

3. **RBC lysis buffer**

   8.3 g/L ammonium chloride in 0.01 M Tris-HCl buffer.

Note: Since HMGB1 locates at cytoplasm, membrane and nucleus, we would recommend use 0.2% SDS lysis buffer to lyse the samples to completely lyse the samples.

For cell lysate

For suspension cell line:

1. Wash the cells three times with ice-cold PBS by centrifugation at 200 x g for 5 minutes at 4°C. Remove the supernatant and collect the cell pellet.
2. Add 50 $\mu l/10^6$ cells ice cold protease inhibitor containing lysis buffer and keep it on ice for 15-30 mins.
3. Optional, but recommended: Sonicate the cell lysate 5 sec with 30% amplitude.
4. Centrifuging at 10,000 x g for 15 min, and collect the supernatant.
5. Aliquot & store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

For adherent cell line

1. Wash the cells three times with ice-cold PBS.
2. Add 50 $\mu l/10^6$ cells ice cold protease inhibitor containing lysis buffer and

keep on ice for 10-30 mins. Collect the lysate in a tube.

3. Optional, but recommended: Sonicate the cell lysate 5 sec with 30% amplitude.
4. Centrifuging at 10,000 x g for 15 min, and collect the supernatant.
5. Aliquot & store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

For tissue lysate

Notice: Red blood cells (RBCs) contain high level of HMGB1, so samples contain RBCs will result in a high HMGB1 background. Therefore, it is important to remove RBCs from tissue samples before lysis.

1. Tissues should be performed single cell suspension in PBS or serum free culture medium by ground glass slides or other methods.
2. Wash the cells will by PBS once.
3. Centrifuge at 300 x g for 10 minutes at room temperature to collect the cells. Remove the supernatant.
4. Add RBC lysis buffer (10^8 cells/ml) for 5 min at room temperature.
5. Wash three times by PBS. Remove the PBS.
6. Add 50 μl/10^6 cells ice cold protease inhibitor containing lysis buffer and keep on ice for 10-30 mins. Collect the lysate in a tube.
7. Optional, but recommended: Sonicate the cell lysate 5 sec with 30% amplitude.
8. Centrifuging at 10,000 x g for 15 min, and collect the supernatant.
9. Aliquot & store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

- **Standard**: Reconstitute the standard with 1.0 ml Standard reconstitution buffer to yield a stock concentration of 100 ng/ml. Mix the standard by inversion or brief vortex for at least 5-10 seconds (do not vortex at high-speed) and stand for 10 minutes at RT to completely dissolve contents. Aliquot and store at ≤ -20°C for up to 2 weeks (store at -80°C is recommended, freeze-thaw the reconstituted standard for only once).
  Make sure the standard is dissolved completely before making serial dilutions. The Sample & Antibody diluent buffer serves as zero standard (0 ng/ml), and the rest of the Sample & Antibody diluent buffer can be diluted as according to the suggested concentration below: 20 ng/ml, 10 ng/ml, 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml and 0.3125 ng/ml.

  Dilute HMGB1 standard as according to the table below:

Standard	HMGB1 Conc. (ng/ml)	µl of Sample & Antibody diluent buffer	µl of standard
S7	20	400	100 (100 ng/ml Stock)
S6	10	250	250 (S7)
S5	5	250	250 (S6)
S4	2.5	250	250 (S5)
S3	1.25	250	250 (S4)
S2	0.625	250	250 (S3)
S1	0.3125	250	250 (S2)
S0	0	250	0

- **1X Wash buffer**: Dilute 10X wash buffer with distilled water to yield 1X wash buffer. The diluted Wash buffer should be stored at 4 °C.
- **HRP-Conjugated antibody:** Diluent 200X HRP-Conjugated antibody into Sample & Antibody diluent buffer to yield 1X Detection antibody solution. The 1X HRP-Conjugated antibody solution should be used immediately.
- **Sample:** If the initial assay found samples contain HMGB1 higher than the highest standard, the samples can be diluted with Sample & Antibody diluent buffer and re-assay the samples. For the calculation of the concentrations this dilution factor has to be taken into account.

  Example:

  a) Dilution 1:10: 10 µl sample + 90 µl Sample & Antibody diluent buffer (mix thoroughly).

  b) Dilution 1:100: 10 µl 1:10 diluted a) + 90 µl Sample & Antibody diluent buffer (mix thoroughly).

ASSAY PROCEDURE

All materials except 1X wash buffer should be equilibrated to room temperature (RT) before use. Standards, samples and controls should be assayed in duplicates.

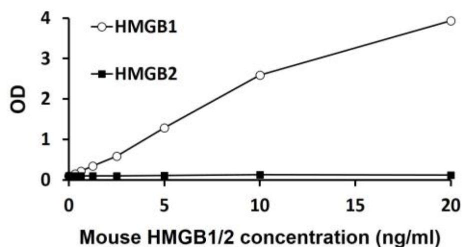
1. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal it.
2. Add **100 µl** of **standards, samples and zero controls** into appropriate wells.
3. Add **200 µl** of **1X HRP-Conjugated antibody** into each well. Mix thoroughly by gently shaking or tapping the plate for few seconds. Cover the plate and **incubate for overnight (~16 hours is recommended) at 4°C** (without shaking).
4. Aspirate each well and wash, repeating the process 4 times for a **total 5 washes**. Wash by filling each well with **1X cold wash buffer** (350 µl) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining buffer by aspirating, decanting or blotting against clean paper towels.
5. Add **100 µl** of **TMB substrate** to each well. Incubate for **10 minutes at RT** in dark. Substrate will change from colorless to different strengths of blue.
6. Add **50 µl** of **Stop solution** to each well. The color of the solution should change from blue to yellow. Mix thoroughly by gently shaking the plate.
7. Read the OD with a microplate reader **at 450 nm** immediately.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards, controls and patient samples.
2. Using linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.
3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.
4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.
5. arigo provides GainData®, an in-house development ELISA data calculator, for ELISA data result analysis. Please refer our GainData® website for details. (https://www.arigobio.com/elisa-analysis)
6. If the samples have been diluted or concentrated, the concentration read from the standard curve must be further converted by the appropriate dilution or concentration factor according to the sample preparation procedure as described above.

EXAMPLE OF TYPICAL STANDARD CURVE

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.



QUALITY ASSURANCE

Sensitivity

Standard range: 0.3125 – 20 ng/ml

Minimum Detectable Concentration: 0.3 ng/ml

Precision:

Intra-assay: 4.3%

Inter-assay: 5.2%

Recovery:

90-99.5%

Specificity

This assay recognizes natural and recombinant total Mouse/Rat HMGB1.

The cross-react to Mouse/Rat HMGB2 is less than 5%, the data tested with 20 ng/ml mouse HMGB2 protein.

The ELISA Kit might react to Human and Rat HMGB1 based on sequence homology.