Mouse High mobility group protein B1 ELISA Kit

A Complete ELISA kit for the detection of Mouse Hmgb1

(Cat. # IT13761)

INTRODUCTION

The microtiter plate provided in this kit has been pre-coated with an antibody specific to HMGB1. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for HMGB1 and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. Only those wells that contain HMGB1, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of HMGB1 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

ITEMS SUPPLIED

Description	Size
Coated Microtiter Plate	1
Lyophilized Standard	2 vials
Sample Diluent	20ml
Assay Diluent A	10ml
Assay Diluent B	10ml
Detection Reagent A	120µl
Detection Reagent B	120µl
Wash Buffer(25 x concentrate)	30ml
Substrate	10ml
Stop Solution	10ml
Plate sealer for 96 wells	5

STORAGE CONDITIONS

The Assay Plate, Standard, Detection Reagent A and Detection Reagent B should be stored at -20℃ upon being received. After receiving the kit , Substrate should be always stored at 4℃.Other reagents are kept according to the labels on vials. But for long term storage, please keep the whole kit at -20℃. The unused strips should be kept in a sealed bag with the desiccant provided to minimize exposure to damp air. The test kit may be used throughout the expiration date of the kit (six months from the date of manufacture). Opened test kits will remain stable until the expiring date shown, provided it is stored as prescribed above.

SPECIFICATIONS

- **Reactivity:** Mouse
- **Range:** 0.156-10ng/mL
- **Sensitivity:** 0.082ng/mL
- **Specificity:** Natural and recombinant mouse High mobility group protein B1

ADDITIONAL ITEMS NEEDED

- Microplate reader
- Precision single and multi-channel pipette and disposable tips
- Clean tubes and Eppendorf tubes
- Deionized or distilled water

PRECAUTIONS

- The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.
- The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
- 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, further dilute the samples with the Sample Diluent and repeat the assay. Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.

SAMPLE COLLECTION & STORAGE

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000 × g. Remove serum and assay immediately or aliquot and store samples at -20℃ or -80℃.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000 × g at 2℃ - 8℃ within 30 minutes of collection. Store samples at -20℃ or -80℃. Avoid repeated freeze-thaw cycles.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at ≤ -20℃. Avoid repeated freeze-thaw cycles.

Tissue homogenates - The preparation of tissue homogenates will vary depending upon tissue type. For this assay, tissue was rinsed with 1X PBS to remove excess blood, homogenized in 20 mL of 1X PBS and stored overnight at ≤ -20℃ After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g. Remove the supernate and assay immediately or aliquot and store at ≤ -20℃.

Cell culture supernates and Other biological fluids - Remove particulates by centrifugation and assay immediately or aliquot and store samples at -20℃ or -80℃. Avoid repeated freeze-thaw cycles.

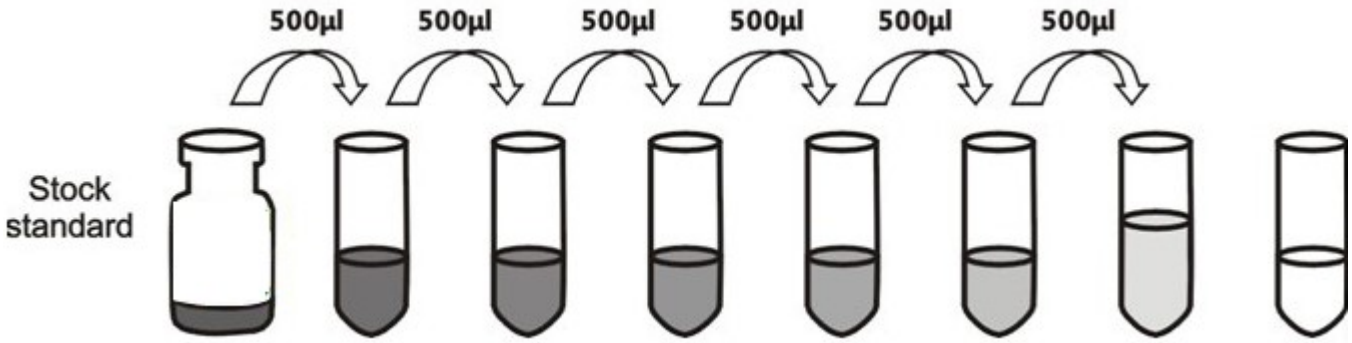
Note:

1. Samples to be used within 5 days may be stored at 2-8℃ , otherwise samples must stored at -20℃ (1 month) or -80℃ (2 months) to avoid loss of bioactivity and contamination.
2. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts of certain chemicals.
3. 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
4. Sample hemolysis will influence the result, so hemolytic specimen can not be detected.
5. When performing the assay slowly bring samples to room temperature.
6. Do not use heat-treated specimens.

PREPARATION BEFORE USE

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 30 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 750 mL of Wash Buffer.

Standard - Reconstitute the Standard with 1.0 mL of Sample Diluent. This reconstitution produces a stock solution of 1000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making serial dilutions (Making serial dilution in the wells directly is not permitted). The undiluted standard serves as the high standard (1000 pg/mL). The Sample Diluent serves as the zero standard (0 pg/mL).



pg/mL	1000	500.	250.	125.	62.5	31.2	15.6	0

Detection Reagent A and B - Dilute to the working concentration using Assay Diluent A and B (1:100), respectively.

PROTOCOL

Allow all reagents to reach room temperature (Please do not dissolve the reagents at 37℃ directly.). All the reagents should be mixed thoroughly by gently swirling before pipetting. Avoid foaming. Keep appropriate numbers of strips for 1 experiment and remove extra strips from microtiter plate. Removed strips should be resealed and stored at 4℃ until the kits expiry date. Prepare all reagents, working standards and samples as directed in the previous sections. 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

1. Add 100 µl of Standard, Blank, or Sample per well. Cover with the Plate sealer. Incubate for 2 hours at 37℃.
2. Remove the liquid of each well, don't wash. Add 100 µl of Detection Reagent A working solution to each well. Cover with the Plate sealer. Incubate for 1 hour at 37℃. Detection Reagent A working solution may appear cloudy. Warm to room temperature and mix gently until solution appears uniform.
3. Aspirate each well and wash, repeating the process three times for a total of three washes. Wash by filling each well with Wash Buffer (approximately 400 µl) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. 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.
4. Add 100 µl of Detection Reagent B working solution to each well. Cover with a new Plate sealer. Incubate for 1 hour at 37℃.
5. Repeat the aspiration/wash as in step 4.
6. Add 90 µl of Substrate Solution to each well. Cover with a new Plate sealer. Incubate within 15-30 minutes at 37℃. Protect from light.
7. Add 50 µl of Stop Solution to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
8. Determine the optical density of each well at once, using a microplate reader set to 450 nm.

Note:

1. Absorbance is a function of the incubation time. Therefore, prior to starting the assay it is recommended that all reagents should be freshly prepared prior to use and all required strip-wells are secured in the microtiter frame. This will ensure equal elapsed time for each pipetting step, without interruption.
2. Please carefully reconstitute Standards or working Detection Reagent A and B according to the instruction, and avoid foaming and mix gently until the crystals have completely dissolved. The reconstituted Standards Detection Reagent A and B can be used only once. This assay requires pipetting of small volumes. To minimize imprecision caused by pipetting, ensure that pipettors are calibrated. It is recommended to suck more than 10µl for once pipetting.
3. 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.
4. For each step in the procedure, total dispensing time for addition of reagents to the assay plate should not exceed 10 minutes.
5. 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.
6. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
7. Duplication of all standards and specimens, although not required, is recommended.
8. Substrate Solution is easily contaminated. Please protect it from light.

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

CALCULATIONS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. 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 HMGB1 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. It is recommended to use some related software to do this calculation, such as curve expert 1.3. 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.

Important note ：

- Limited by the current condition 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
- The final experimental results will be closely related to validity of the products, operation skills of the end users and the experimental environments. Please make sure that sufficient samples are available.
- 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 information.
- 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.
- A microtiter plate reader with a bandwidth of 10nm or less and an optical density range of 0-3 OD or greater at 450nm wavelength is acceptable for use in absorbance measurement.

- Use fresh disposable pipette tips for each transfer to avoid contamination.
- Do not substitute reagents from one kit lot to another. Use only the reagents supplied by manufacturer.
- Even the same operator might get different results in two separate experiments. In order to get better reproducible results, the operation of every step in the assay should be controlled. Furthermore, a preliminary experiment before assay for each batch is recommended.
- 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.

ELISA TROUBLESHOOTING GUIDE

Problem	Possible Source	Solution
Poor Standard Curve	Improper standard solution	Confirm dilutions are made correctly
	Standard improperly reconstituted	Briefly spin vial before opening, inspect for undissolved material after reconstituting
	Standard degraded	Store and handle standard as recommended
	Curve does not fit scale	Try plotting using different scales e.g. log-log, 5 parameter logistic curve fit
	Pipetting error	Use calibrated pipettes and proper pipetting technique
	Standard was incompletely reconstituted or was inappropriately stored	Reconstitute standard according to protocol. Store reconstituted standard in appropriate vials. Store reconstituted standard at -70 °C
	Reagents added to wells with incorrect concentrations	Check for pipetting errors and correct reagent volume.
	Incubations done at inappropriate temperature, timing or agitation	Assay conditions need to be checked
No signal	Incubation time too short	Incubate samples overnight at 4°C or follow the manufacturer guidelines.
	Target present below detection limit of assay	Decrease dilution factor or concentrate samples.
	Incompatible sample type	Detection may be reduced or absent in untested sample types. Include a sample that the assay is known to detect a positive control.
	Recognition of epitope impeded by absorption to plate	To enhance detection of a peptide by direct or indirect ELISA, conjugate peptide to a large carrier protein before coating onto the microtiter plate.
	Assay buffer compatibility	Ensure assay buffer is compatible with target of interest (e.g. enzymatic activity retained, protein interactions retained)
	Incorrect or no Detection Antibody was added	Add appropriate Detection Antibody and continue.
	Avidin-HRP was not added	Add Avidin-HRP according to protocol and continue.
	Substrate solution was not added	Add substrate solution and continue.
	Wash buffer contains sodium azide	Avoid sodium azide in the wash buffer.
	Multichannel pipette errors	Calibrate the pipettes.
	Plate washing was not adequate or uniform	Make sure pipette tips are tightly secured. Confirm all reagents are removed completely in all wash steps.
	Non-homogenous samples	Thoroughly mix samples before pipetting
	Samples may have high particular matter	Remove the particular matter by centrifugation.

Problem	Possible Source	Solution
	Cross-well contamination	When reusing plate sealers check that no reagent has touched the sealer. Care should be taken when using the same pipette tips used for reagent additions. Ensure that pipette tips do not touch the reagents on the plate.
	Not enough detection reagent	Increase concentration or amount of detection reagent following manufacturer guidelines.
	Sample prepared incorrectly	Ensure proper sample preparation/dilution. Samples may be incompatible with microtiter plate assay format.
	Insufficient antibody	Try different concentrations/dilutions of antibody
	Incubation temperature too low	Ensure the incubations are carried out at the correct temperature. All reagents including plate should be at room temperature or as recommended by the manufacturer before proceeding.
	Incorrect wavelength	Verify the wavelength and read plate again
	Plate washings too vigorous	Check and ensure correct pressure in automatic wash system. Pipette wash buffer gently if washes are done manually.
	Wells dried out	Do not allow wells to become dry once the assay has started. Cover the plate using sealing film or tape for all incubations
	Slow color developments of enzymatic reaction	Prepare substrate solution immediately before use. Ensure the stock solution has not expired and is not contaminated. Allow longer incubation.
High variation in samples and/or standards	Bubbles in wells	Ensure no bubbles are present prior to reading plate
	Wells not washed equally/thoroughly	Check that all ports of the plate washer are not obstructed. Wash wells as recommended.
	Incomplete reagent mixing	Ensure all reagents are mixed thoroughly
	Inconsistent pipetting	Use calibrated pipettes and proper technique to ensure accurate pipetting
	Edge effects	Ensure the plate and all reagents are at room temperature.
	Inconsistent sample preparation	Ensure consistent sample preparation and optimal sample storage conditions (e.g. minimize freeze/thaw cycles).
High background	Wells are insufficiently washed	Wash wells as per protocol recommendations
	Contaminated wash buffer	Prepare fresh water buffer
	Too much detection reagent	Ensure the reagent has been diluted properly or decrease the recommended concentration of detection reagent.
	Blocking buffer ineffective (e.g. detection reagent binds blocker; wells not completely blocked)	Try different blocking reagent and/or blocking reagent to wash buffer.
	Salt concentration of incubation/wash buffers	Increasing salt concentrations may reduce non-specific and/or weak off-target interactions.
	Waiting too long to read plate after adding stop solution.	Read plate immediately after adding stop solution.
	Non-specific binding of antibody	Use suitable blocking buffers e.g. BSA or 5010% normal serum-species same as primary antibody if using a directly conjugated detection antibody or same as secondary if using conjugated.
	Background wells were contaminated	Avoid cross-well contamination by using the sealer appropriately. Use multichannel pipettes without touching the reagents on the plate.
	Matrix used has endogenous analyte or interference	Check the matrix ingredients for cross reacting components (e.g. interleukin modified tissue culture medium).
	TMB Substrate Solution was contaminated	TMB Substrate Solution should be clear and colorless prior to addition to wells. Use a clean container prior to pipetting

Problem	Possible Source	Solution
		substrate solution into wells.
	High antibody concentration	Try different dilutions for optimal results
	Substrate incubation carried out in light	Substrate incubations should be carried out in the dark or as recommended by manufacturer.
	Precipitate formed in wells upon substrate addition.	Increase dilution factor of sample or decrease concentration of substrate
	Dirty plate	Clean the plate bottom.
Low Sensitivity	Improper storage of ELISA kit	Store all reagents as recommended. Please note that all reagents may not have identical storage requirements.
	Not enough target	Concentrate sample or reduce sample dilution
	Inactive detection reagent	Ensure reporter enzyme has the expected activity.
	Plate reader settings incorrect	Ensure plate reader is set to read the correct absorbance wavelength or excitation/emission wavelengths for fluorescent detection.
	Assay format not sensitive enough	Switch to a more sensitive detection system (e.g. colorimetric to chemiluminescence / fluorescence) Switch to a more sensitive assay type (e.g. direct ELISA to sandwich ELISA). Lengthen incubation times or increase temperature.
	Target poorly absorbs to microtiter plate	Covalently link target to microtiter plate.
	Not enough substrate	Add more substrate
	Incompatible sample type (e.g. serum vs. cell extract)	Detection may be reduced or absent in untested sample types. Include a sample that the assay is known to detect as a positive control.
	Interfering buffers or sample ingredients	Check reagents for any interfering chemicals. For example, sodium azide in antibodies inhibit HRP enzyme and EDTA used as anticoagulant for plasma collection inhibits enzymatic reactions.
	Mixing or substituting reagents from different kits	Avoid mixing components from different kits.

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