HMGB1 Detection Kit

Catalog # 6010

For Research Use Only - Not Human or Therapeutic Use

INTRODUCTION

HMGB1 (high mobility group box 1) (1) was recently rediscovered as a late lethal mediator of endotoxin (10) and is currently considered a pro-inflammatory cytokine that plays crucial roles in a variety of acute and chronic inflammatory diseases. HMGB1 contains 216 amino acids (6) and maintains 99% of its sequence identity among mice (2), rats (3), bovines (4), and humans (5). HMGB1 consists of three structural domains (7), termed "A box (9-85)", "B box (88-162)", and a negatively charged carboxyl terminus (186-216). Moreover, it has been previously shown that the B box recapitulates the pro-inflammatory activity whereas the A box acts as an antagonist of HMGB1 (8, 9).

Several lines of evidence highlight the significance of HMGB1 in the immune inflammatory response. For example, it has been shown that HMGB1 is actively released by a variety of cells such as macrophages when stimulated by lipopolysaccharides (LPS), TNF-α, and IL-1 (10), and is passively released by injured or necrotic cells associated with collapsing cell structures. In fact, patients who died from septic shock had higher serum HMGB1 levels than surviving sepsis patients (13). Similarly, high serum HMGB1 levels are observed in sepsis animal models and in collagen-induced arthritis animal models (14). With regard to the function of the protein itself, HMGB1 has also been shown to stimulate the release of TNF-α and IL-1 (11, 12), as well as bind LPS and synergistically increase peripheral blood mononuclear cell IL-6 production (19). Together, these observations demonstrate that HMGB1 plays important roles in the inflammatory cascade.

Chondrex, Inc. provides a capture ELISA kit to determine HMGB1 levels in cell culture media and sera (see "Notes Before Using Assay" on page 3). This kit contains enough reagents to measure 40 samples in duplicate together with standards.

KIT COMPONENTS

Item	Quantity	Amount	Storage
HMGB1 Standard (60101)	2 vials	50 µl/vial	-20°C
Capture Antibody (Anti-HMGB1 Monoclonal Antibody) (60102)	1 vial	100 µl/vial	-20°C
Detection Antibody (Anti-HMGB1 Monoclonal Antibody) (60103)	1 vial	Lyophilized	-20°C
Solution A - Capture Antibody Dilution Buffer (60104)	1 bottle	10 ml	-20°C
Solution B - Sample/Standard Dilution Buffer (601010)	1 bottle	20 ml	-20°C
Solution C - Detection Antibody Dilution Buffer (60106)	1 bottle	10 ml	-20°C
Solution D - Streptavidin Peroxidase Dilution Buffer (9055)	1 bottle	20 ml	-20°C
Streptavidin Peroxidase (9029)	2 vials	50 µl	-20°C
TMB Solution (contains DMSO) (90023)	2 vials	0.2 ml	-20°C
Chromogen Dilution Buffer (90022)	1 bottle	20 ml	-20°C
Stop Solution - 2N Sulfuric Acid (9016)	1 bottle	10 ml	-20°C
Wash Buffer, 20X (9005)	1 bottle	50 ml	-20°C
ELISA Plate (9026)	1 each	96-well (8-well strips x 12)	-20°C

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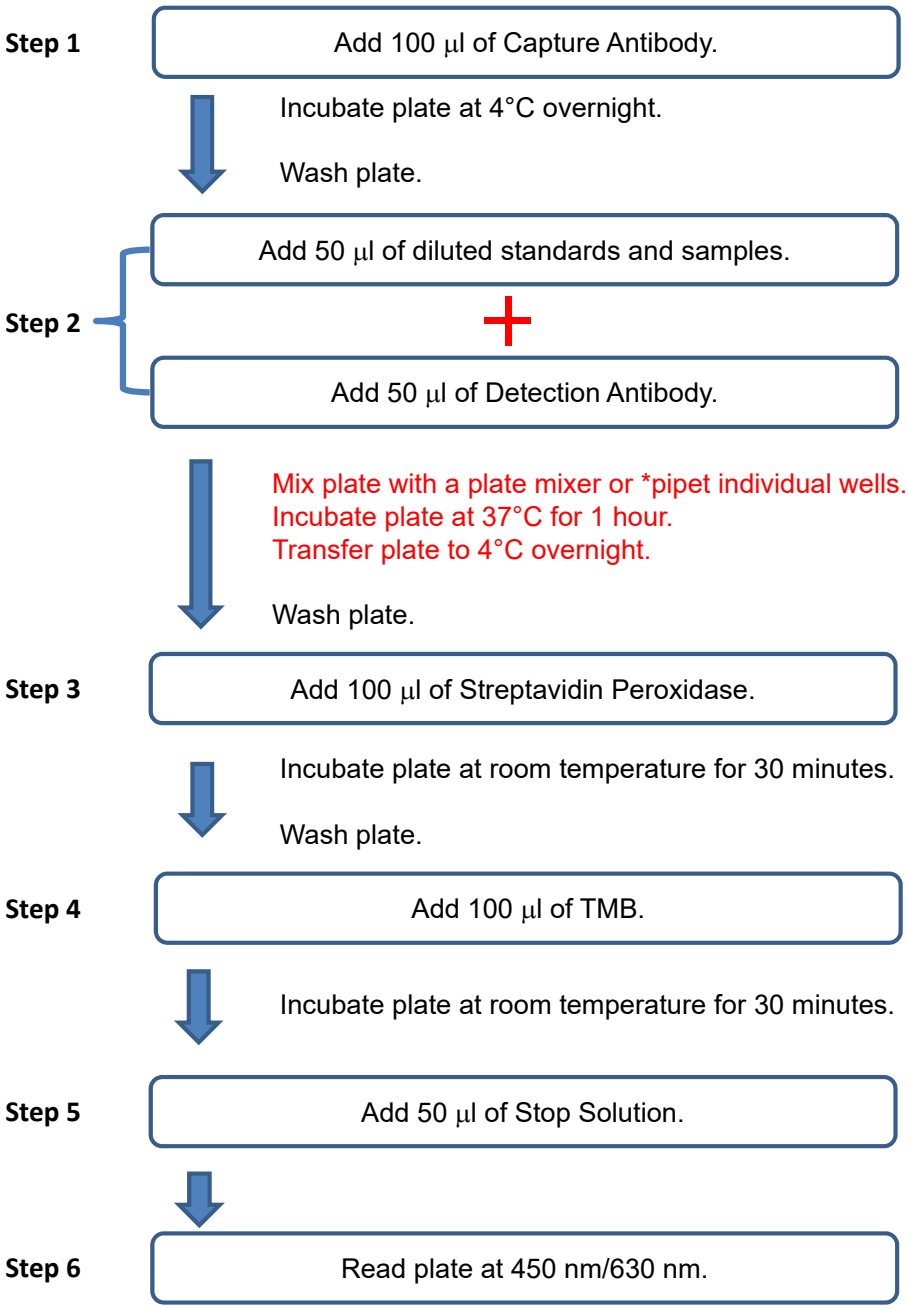
HMGB1 Assay Outline



*Use one tip/sample. Do not cross-contaminate samples by re-using pipet tips.
A multi-channel pipet is recommended.

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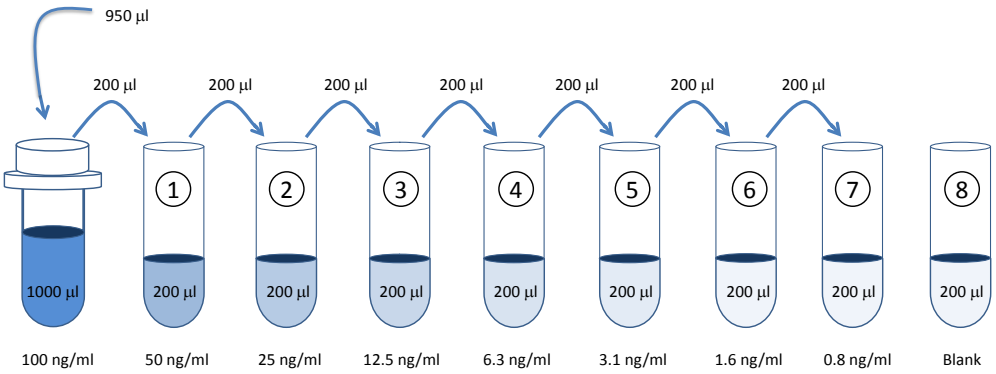
NOTES BEFORE USING ASSAY

1. It is recommended that the standard and samples be run in duplicate.
2. Warm up all buffers to room temperature before use.
3. For partial reagent use, use the appropriate dilution ratio for each reagent as stated in the protocol. For example, if the protocol dilutes 50 µl of stock solution in 10 ml of buffer, then for 6 strips, dilute 25 µl of stock solution in 5 ml of buffer. Partially used capture antibody stock, detection antibody stock, streptavidin peroxidase, and TMB reagents may be kept at in their original vials at –20°C.
4. Crystals may form in the 20X wash buffer when stored at cold temperatures. If crystals have formed, it is necessary to warm the wash buffer by placing the bottle in warm water until crystals have dissolved completely.
5. Measure exact volumes of buffers using a serological pipette, as extra buffer is provided.
6. Cover the plate with plastic wrap or a plate sealer after each step to prevent evaporation from the outside wells of the plate.
7. This kit can be used to determine HMGB1 levels in sera and cell culture media samples. However, special concern should be considered for assaying HMGB1 in human serum, because autoantibodies to HMGB1 are determined in 9-89% of sera from patients with autoimmune and inflammatory diseases (15-18). These reports indicate that human serum polyclonal antibodies to HMGB1 might mask the epitopes recognized by the capture and detection antibodies used in this kit, resulting in interference against the assay. Therefore, it is important to use this kit with background knowledge of patients.
8. This kit contains components of animal origin from non-infectious animals, but should be treated as potential biohazards in use and for disposal.
9. Human samples should be handled as if they are capable of transmitting infectious diseases.

ASSAY PROCEDURE

All reagents must be at room temperature before use.

1. **Add Capture Antibody**: Dilute 1 vial (100 µl) of Capture Antibody with 10 ml of Capture Antibody Dilution Buffer (Solution A). Add 100 µl of capture antibody solution to each well and incubate at 4°C overnight. If you plan to use less, the remaining stock solution can be stored in its original vial at -20°C.
2. **Prepare Standard Dilutions**: The recommended standard range is 0.8-50 ng/ml. Dilute one vial of HMGB1 Standard with 950 µl of Sample/Standard Dilution Buffer (Solution B) - 100 ng/ml. Prepare serial dilutions of the standard by mixing 200 µl of the 100 ng/ml standard with 200 µl of Solution B - 50 ng/ml. Then repeat this procedure to make six more serial dilutions of standard - 25, 12.5, 6.25, 3.1, 1.6, and 0.8 ng/ml solutions. Partially used 100 ng/ml standard stock cannot be saved for future assays. Discard unused, diluted standard solution. We recommend making fresh standard and serial dilutions for each assay.



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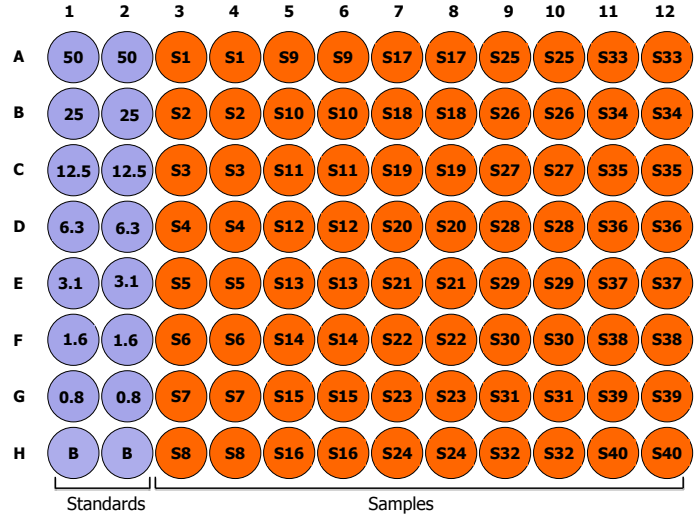
3. **Prepare Sample Dilutions**: Centrifuge samples at 10,000 rpm at 4°C for 3 minutes to remove insoluble materials and lipids, and use the supernatant as samples. Dilute sample, at minimum, with an equal volume of Solution B. For example, take 100 µl of a serum, and mix with 100 µl of Solution B.

4. **Prepare Detection Antibody**: Reconstitute one vial of Detection Antibody with 50 µl of distilled water to make a stock solution. Dilute the 50 µl of detection antibody stock solution in 5 ml of Detection Antibody Dilution Buffer (Solution C). If you plan to use less, the remaining stock solution can be stored in its original vial at -20°C.

5. **Dilute Wash Buffer**: Dilute 50 ml of 20X wash buffer in 950 ml of distilled water (1X wash buffer). Wash the plate with 1X wash buffer at least 3 times using a wash bottle with manifold or an automated plate washer. Empty the plate by inverting it and blot on a paper towel to remove excess liquid. *Do not allow the plate to dry out.*

6. **Add Standards, Samples, and Detection Antibody**: Mix standards, samples, and detection antibody tubes well. Add 50 µl of Solution B (blank), standards, and samples to appropriate wells (Figure 1). Add 50 µl of diluted detection antibody solution to all wells. Mix all wells by pipetting or use a plate shaker. Cover the plate with a plate sealer and incubate at 37°C for 1 hour, then transfer plate to 4°C overnight.

Figure 1 - A typical assay layout



7. **Prepare Streptavidin Peroxidase**: Dilute one vial (50 µl) Streptavidin Peroxidase in 10 ml of Streptavidin Peroxidase Dilution Buffer (Solution D). If you plan to use less, the remaining stock solution can be stored in its original vial at -20°C.

8. **Wash**: Wash the plate with 1X wash buffer at least 3 times using a wash bottle with manifold or an automated plate washer. Empty the plate by inverting it and blot on a paper towel to remove excess liquid. *Do not allow the plate to dry out.*

9. **Add Streptavidin Peroxidase**: Add 100 µl of streptavidin peroxidase solution to each well and incubate at room temperature for 30 minutes.

10. **Wash**: Wash the plate with 1X wash buffer at least 3 times using a wash bottle with manifold or an automated plate washer. Empty the plate by inverting it and blot on a paper towel to remove excess liquid. *Do not allow the plate to dry out.*

11. **TMB**: Use new tubes when preparing TMB. Dilute one vial (200 µl) TMB with 10 ml of Chromogen Dilution Buffer just prior to use. Add 100 µl of TMB solution to all wells immediately after washing the plate. Incubate for 30 minutes at room temperature. If you plan to use less, the remaining stock solution can be stored in its original vial at -20°C.

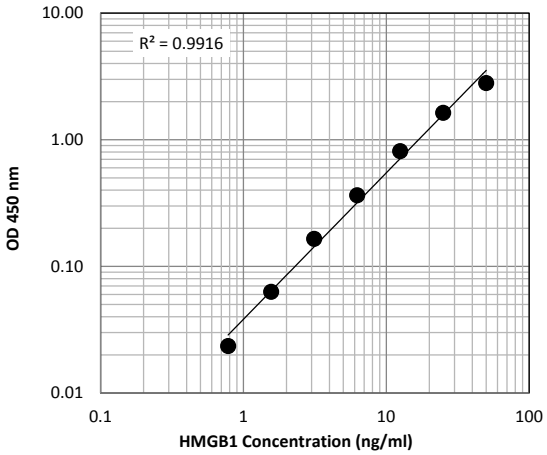
12. **Stop**: Add 50 µl of 2N sulfuric acid (Stop Solution) to each well.

13. **Read Plate**: Read the OD values at 450 nm (a 630 nm filter can be used as a reference) immediately. If the OD values of samples are greater than the OD values of the highest standard, re-assay the samples at a higher dilution.

CALCULATION OF HMGB1 CONCENTRATION

1. Average the duplicate OD values for the blank, standards, and samples.
2. Subtract the averaged blank (B) OD value from the averaged standard and sample OD values.
3. Plot the OD values of standards against the amount of HMGB1 (ng/ml) using a log scale. Figure 2 shows a typical standard curve where the HMGB1 range is from 0.8-50 ng/ml.
4. The concentration of HMGB1 (ng/ml) in samples can be calculated using regression analysis (an Excel calculation sheet can be downloaded from www.chondrex.com). Multiply the results by the dilution factors (usually 2 without extra dilution).

Figure 2 - A typical standard curve



ADDITIONAL INFORMATION

Reproducibility:

Human Sera

Test At	2 ng/ml	7.5 ng/ml	30 ng/ml
Inter-Assay CV (%)	1.3	5.1	3.1
Intra-Assay CV (%)	2.7	3.4	3.6

Mouse Sera

Test At	2 ng/ml	7.5 ng/ml	30 ng/ml
Inter-Assay CV (%)	3.7	4.5	0.9
Intra-Assay CV (%)	1.4	3.2	8.1

Recovery:

Species	Averaged Recovery Results
Human (5 sera)	101 ± 25.1%
Mouse (5 sera)	104 ± 8.3%

Specificity:

Average cross reactivity with bovine HMGB2 is 12.4%.

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