DATA SHEET

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 currently considered as a pro-inflammatory cytokine that plays crucial roles in a variety of acute and chronic inflammatory diseases.

HMGB1 contains 216 amino acids (6) that share more than 99% sequence identity in mice (2), rats (3), bovines (4), and humans (5). HMGB1 consists of three structural domains (7), termed “A box (9-85)” and “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 indicate the significance of HMGB1 in the immune inflammatory response. For example, it has been shown that HMGB1 is actively released from a variety of cells such as macrophages stimulated by lipopolysaccharides (LPS), TNF and IL-1 (10), and is passively released by injured or necrotic cells associated with collapsing cell structures. In fact, deceased patients with septic shock have higher serum HMGB1 levels than surviving patients from sepsis (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 and IL-1 (11, 12), as well as the ability to bind LPS and synergistically increase peripheral blood mononuclear cell IL-6 production (19). Taken together, these observations demonstrate that HMGB1 plays important roles in the inflammatory cascade.

Chondrex provides a capture ELISA kit to determine HMGB1 levels in cell culture medium and mouse sera. This kit contains enough reagents to measure 40 samples in duplicate together with standards.

KIT COMPONENTS

Item	Quantity	Amount	Storage
HMGB1 Standard	2 vials	50 µl/vial	-20°C
Capture Antibody (Anti-HMGB1 Monoclonal Antibody)	1 vial	100 µl/vial	-20°C
Detection Antibody (Anti-HMGB1 Monoclonal Antibody)	1 vial	Lyophilized	-20°C
Solution A - Capture Antibody Dilution Buffer	1 bottle	10 ml	-20°C
Solution B - Sample/Standard Dilution Buffer	1 bottle	20 ml	-20°C
Solution C - Detection Antibody Dilution Buffer	1 bottle	10 ml	-20°C
Solution D - Streptavidin Peroxidase Dilution Buffer	1 bottle	20 ml	-20°C
Streptavidin Peroxidase	2 vials	50 µl	-20°C
TMB Solution (contains DMSO)	2 vials	0.2 ml	-20°C
Chromagen Dilution Buffer	1 bottle	20 ml	-20°C
Stop Solution - 2N Sulfuric Acid	1 bottle	10 ml	-20°C
Wash Buffer, 20X	1 bottle	50 ml	-20°C
ELISA Plate	1 each	96-well (8-well strips x 12)	-20°C

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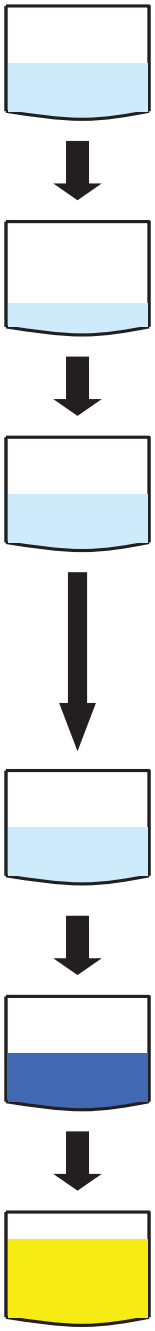
HMGB1 Assay Summary



Add 100 µl Capture Antibody
Incubate at 4°C overnight
Aspirate and wash 3X

Add 50 µl Detection Antibody

Add 50 µl Standards/Samples
Mix the plate.
Incubate at 37°C for 1 hour, then settle at 4°C for 16-24 hours
Aspirate and wash 3X

Add 100 µl Streptavidin Peroxidase
Incubate at room temperature for 30 minutes
Aspirate and wash 3X

Add 100 µl TMB
Incubate at room temperature for 30 minutes

Add 50 µl Stop Solution
Read at 450/630nm

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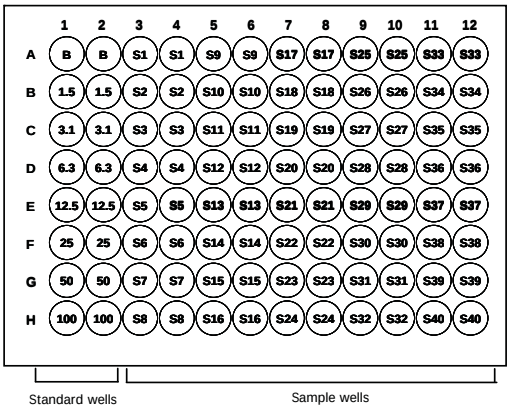
NOTES BEFORE USING ASSAY

1. It is recommended that the standard and samples be run in duplicate.
2. Partially used diluted capture, detection and streptavidin peroxidase reagents may be kept at –20°C.
3. 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.
4. Measure exact volume of buffers using a serological pipette prior to diluting. Extra buffer is provided.
5. This kit can be used to determine HMGB1 in mouse serum and cell culture medium. However, special concern should be considered for assaying HMGB1 in human serum, because it has been reported that autoantibody to HMGB1 is determined in 9 to 89% of sera from patients with autoimmune and inflammatory diseases (15-18). If it is true, polyclonal antibodies in human sera might mask the epitopes recognized by the capture and detection antibodies used in this kit, and interfere with the assay. Therefore, it is important to use this kit with background knowledge of patients.

ASSAY PROCEDURE

All reagents must be at room temperature before use.

1. **Add Capture Antibody**: Dilute one vial 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.
2. **Prepare Standard Dilutions**: The recommended standard range is 1.6-100 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 250 µl of the 100 ng/ml standard with 250 µl of Solution B - 50 ng/ml. Then repeat this procedure to make five more serial dilutions of standard - 25, 12.5, 6.25, 3.1 and 1.6 ng/ml solutions. The 100 ng/ml standard stock can not be stored for future assay. Discard unused extra standard solution. We recommend making fresh standard and serial dilutions for each assay.
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. If the HMGB1 level is more than 100 ng/mL, re-assay the sample at a higher dilution.
4. **Prepare Detection Antibody**: Dissolve one vial of Detection Antibody in 5 ml of Detection Antibody Dilution Buffer (Solution 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 detection antibody solution and sample/standard solutions in all wells by pipetting or using a plate shaker. Cover the plate with a plate sealer and incubate at 37°C for 1 hour, then settle at 4°C overnight.



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7. **Prepare Streptavidin Peroxidase**: Dilute one vial of Streptavidin Peroxidase in 10 ml of Streptavidin Peroxidase Dilution Buffer (Solution D).

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. *Do not incubate the plate more than 60 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**: Dilute one vial of TMB in 10 ml Chromagen 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.

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

13. **Read Plate**: Read the OD values within 30 minutes by dual wavelength at 450 nm (sample) and 630 nm (reference).

CALCULATION OF HMGB1 CONCENTRATION

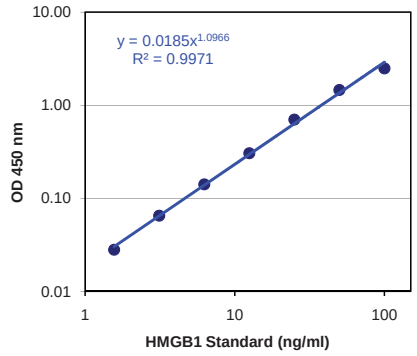
1. Average the duplicate OD values for the blank, standards and samples.

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 1.6 to 100 ng/ml.

4. The amounts of HMGB1 (ng/ml) in samples can be calculated using regression analysis.

Figure 2 - A typical standard curve



ADDITIONAL INFORMATION

Reproducibility:
Intra-assay coefficient of variation is less than 4.2%. Inter-assay coefficient of variation is less than 7.6%.

Recovery:
Recovery of HMGB1 added to mouse serum is 87-137%. Recovery of HMGB1 added to culture medium is 113-118%.

Specificity:
Cross reaction with bovine HMGB2 is the average 12.4%.

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