HMGB1 Detection ELISA kit

Catalog # 6010

For Research Use Only - Not Human or Therapeutic Use

PRODUCT SPECIFICATIONS

DESCRIPTION:	ELISA kit to quantify HMGB1
FORMAT:	96-well ELISA Plate with removeable strips
ASSAY TYPE:	Sandwich ELISA
ASSAY TIME:	2 hours*
STANDARD RANGE:	50 ng/ml to 0.8 ng/ml
NUMBER OF SAMPLES:	Up to 40 (duplicate) samples/plate
SAMPLE TYPES:	Cell culture, Serum, and Plasma
RECOMMENDED SAMPLE DILUTIONS:	1:1 (at least)
CHROMOGEN:	TMB (read at 450 nm)
STORAGE:	-20°C
VALIDATION DATA:	Human Serum: Intra-Assay (2.7-3.6%)/Inter-Assay (1.3-5.1%)/Recovery (101+/-25.1%)
	Mouse Serum: Intra-Assay (1.4-8.1%)/Inter-Assay (0.9-4.5%)/Recovery (104+/8.3%)
NOTES:	*This kit has an overnight incubation step

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INTRODUCTION

HMGB1 (high mobility group box 1) (1) was recently rediscovered as a late lethal mediator of endotoxin (2) 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 (3) and maintains 99% of its sequence identity among mice (4), rats (5), bovines (6), and humans (7). HMGB1 consists of three structural domains (8), 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 (9,10).

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 (2), 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 (11). Similarly, high serum HMGB1 levels are observed in sepsis animal models and in collagen-induced arthritis animal models (12). With regard to the function of the protein itself, HMGB1 has also been shown to stimulate the release of TNF-α and IL-1 (13,14), as well as bind LPS and synergistically increase peripheral blood mononuclear cell IL-6 production (15). Together, these observations demonstrate that HMGB1 plays important roles in the inflammatory cascade.

Chondrex, Inc. provides an HMGB1 Detection ELISA kit (Cat # 6010) to determine HMGB1 levels in cell culture media and sera. 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 - Coating Buffer (9052)	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	1 each	96-well (8-well strips x 12)	-20°C

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ASSAY OUTLINE

Add 100 µl of diluted Capture Antibody Solution to wells.

Incubate plate at 4°C overnight.

Wash plates.

Add 50 µl of diluted standards and samples to wells.

+

Add 50 µl of diluted Detection Antibody Solution to wells.

Mix plate with a plate mixer or *pipet individual wells.
Incubate at 37°C for 1 hour.
Transfer plate to 4°C overnight.

Wash plates.

Add 100 µl of diluted Streptavidin Peroxidase Solution to wells.

Incubate plate at room temperature for 30 minutes.

Wash plates.

Add 100 µl of diluted TMB solution to wells.

Incubate plate at room temperature for 30 minutes.

Add 50 µl of Stop Solution to wells.

Read plate at 450 nm/630 nm.

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

PLATE LAYOUT

	1	2	3	4	5	6	7	8	9	10	11	12
A	50	50	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
B	25	25	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
C	12.5	12.5	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
D	6.3	6.3	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
E	3.1	3.1	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
F	1.6	1.6	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
G	0.8	0.8	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
H	B	B	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

Standards | Samples

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NOTES BEFORE USING ASSAY

NOTE 1: It is recommended that the standard and samples be run in duplicate.

NOTE 2: Warm up all buffers to room temperature before use.

NOTE 3: Crystals may form in Wash Buffer, 20X when stored at cold temperatures. If crystals have formed, warm the wash buffer by placing the bottle in warm water until crystals are completely dissolved.

NOTE 4: Measure exact volume of buffers using a serological pipet, as extra buffer is provided.

NOTE 5: Cover the plate with plastic wrap or a plate sealer after each step to prevent evaporation from the outside wells of the plate.

NOTE 6: For partial reagent use, please see the assay protocol's corresponding step for the appropriate dilution ratio. For example, if the protocol dilutes 50 µl of a stock solution in 10 ml of buffer for 12 strips, then for 6 strips, dilute 25 µl of the stock solution in 5 ml of buffer. Partially used stock reagents may be kept in their original vials and stored at -20ºC for use in a future assay.

NOTE 7: This kit contains animal components from non-infectious animals and should be treated as potential biohazards in use and for disposal.

NOTE 8: 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 (16-19). 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.

ASSAY PROCEDURE

1. **Add Capture Antibody**: Dilute 1 vial (100 µl) of Capture Antibody with 10 ml of Coating Buffer (Solution A). Add 100 µl of capture antibody solution to each well and incubate at 4°C overnight. If planning to use less, the remaining stock solution can be stored in its original vial at -20°C.

Strip #	Capture Antibody (µl)	Solution A (ml)
2	17	1.7
4	33	3.3
6	50	5.0
8	66	6.6
10	82	8.2
12	100	10.0

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. Chondrex, Inc. recommends making fresh standard and serial dilutions for each assay.

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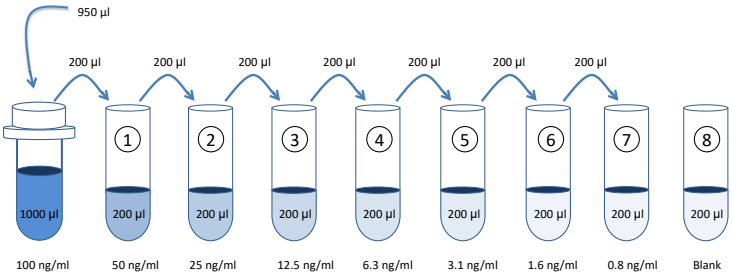

3. **Prepare Sample Dilutions**: Centrifuge samples at 10,000 rpm at 4°C for 3 minutes to remove insoluble materials and lipids and use the supernatant as samples. Dilute samples at least 1:1 with an equal volume of Solution B. For example, take 100 µl of a serum, and mix with 100 µl of Solution B.

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 planning to use less, the remaining stock solution can be stored in its original vial at -20°C.

Strip #	Detection Antibody (µl)	Solution C (ml)
2	8	0.8
4	17	1.7
6	25	2.5
8	33	3.3
10	42	4.2
12	50	5.0

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 blotting on a paper towel to remove excess liquid. *Do not allow the plate to dry out.*

6. **Add Standards, Samples, and Detection Antibody**: Vortex standards, samples, and detection antibody tubes well. Add 50 µl of Solution B (blank), standards, and samples to appropriate wells. 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.

7. **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 blotting on a paper towel to remove excess liquid. *Do not allow the plate to dry out.*

8. **Add Streptavidin Peroxidase**: Dilute one vial of Streptavidin Peroxidase in 10 ml of Streptavidin Peroxidase Dilution Buffer (Solution D). If planning to use less, the remaining stock solution can be stored in its original vial at -20°C. Add 100 µl of streptavidin peroxidase solution to each well and incubate at room temperature for 30 minutes.

Strip #	Streptavidin Peroxidase (µl)	Solution D (ml)
2	9	1.8
4	17	3.4
6	25	5.0
8	33	6.6
10	41	8.2
12	50	10.0

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9. **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 blotting on a paper towel to remove excess liquid. *Do not allow the plate to dry out.*

10. **Add TMB**: Use new tubes when preparing TMB. Dilute one vial of 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 planning to use less, the remaining stock solution can be stored in its original vial at -20°C.

Strip #	TMB (µl)	Chromogen Dilution Buffer (ml)
2	34	1.7
4	66	3.3
6	100	5.0
8	132	6.6
10	164	8.2
12	200	10.0

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

12. **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.

CALCULATING RESULTS

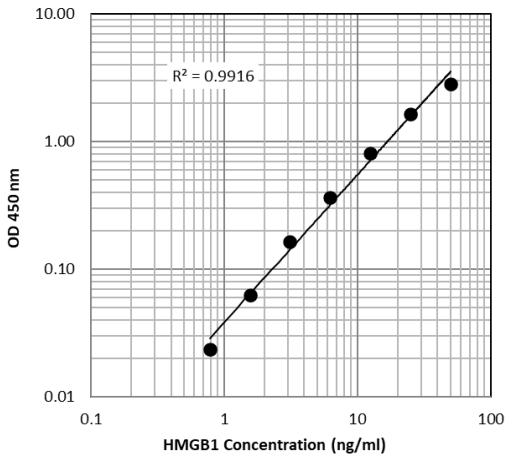
1. Average the duplicate OD values for the blank, standards, and samples.

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 concentration of HMGB1 (ng/ml) using a log scale. Figure 1 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. Multiply the results by the dilution factors (usually 2 without extra dilution). For additional assistance, please download a sample calculation worksheet from www.chondrex.com.

Figure 2 - A Typical Standard Curve for the HMGB1 Detection ELISA Kit



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VALIDATION DATA

Table 1 - Reproducibility Data for the HMGB1 Detection ELISA Kit

Human Serum

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

Mouse Serum

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

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%.

TROUBLESHOOTING

For frequently asked questions about assays and ELISAs, please see Chondrex, Inc.'s ELISA FAQ for more information.

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