

Chapter 2

Analysis of the Released Nuclear Cytokine HMGB1 in Human Serum

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Abstract

A ubiquitous nuclear protein, the high-mobility group box 1 (HMGB1), is secreted by activated macrophages/monocytes and leaked passively from injured cells. HMGB1 functions as a mediator of infection- and injury-elicited inflammatory diseases. Here, we describe a semiquantitative immuno-blotting method to measure the released HMGB1 in human serum, in comparison with a commercially available HMGB1 ELISA technique.

Key words HMGB1, Western blot, ELISA, Serum, Antibody

1 Introduction

High-mobility group box 1 (HMGB1) is expressed constitutively in most cells, and a large “pool” of preformed HMGB1 is stored in the nucleus due to the presence of two lysine-rich nuclear localization sequences [1, 2]. It contains two internal repeats of positively charged domains (known as “A box” and “B box”) in the N-terminus and a continuous stretch of negatively charged (aspartic and glutamic acid) acidic tail in the C-terminus. These HMG boxes enable HMGB1 to bind chromosomal DNA and fulfill its nuclear functions such as maintaining the nucleosomal structure and stability and regulating gene expression [3]. In response to exogenous bacterial products (such as endotoxin or CpG-DNA) [1, 4], or endogenous inflammatory stimuli (e.g., TNF, IFN- γ , or hydrogen peroxide) [1, 5, 6], innate immune cells actively release HMGB1 in a dose- and time-dependent manner. In addition, HMGB1 can be released passively from damaged cells [7] and similarly trigger an inflammatory response [8]. The accumulated evidence has supported a pathogenic role for extracellular HMGB1 in infection- or injury-elicited inflammatory diseases [9–13]. Thus, it is important to measure plasma or serum HMGB1 levels in patients

with various inflammatory diseases. In this chapter, we describe two immunoassays for measuring HMGB1 in human serum samples: ELISA and Western blotting.

2 Materials

2.1 Sandwich ELISA Kit

Several companies have recently developed sandwich ELISA kits for measuring HMGB1 levels in plasma and serum samples. For instance, the Shino-Test Corporation HMGB1 ELISA kit contains the following components:

1. ELISA plate: 8-well strips coated with the capture polyclonal antibody generated against the peptide KPDAAKKGVVKA EK adjacent to the C-terminal acidic tail of HMGB1 [14, 15] (*see Notes 1 and 2*).
2. Peroxidase (POD)-conjugated detection antibody (lyophilized for making 12 ml solution): Peroxidase-linked anti-HMGB1 monoclonal antibody (generated against human HMGB1 protein).
3. HMGB1 standard: Pig HMGB1 protein.
4. Sample diluent (20 ml).
5. Conjugate solvent (12 ml).
6. Color reagent A (3, 3', 5, 5'-tetramethyl-benzidine).
7. Color reagent B containing 0.005 M hydrogen peroxide.
8. Substrate solution: Equal volumes of color reagents A and B, which have been brought to room temperature, and mixed just before use.
9. Stop solution: 0.35 M Sulfuric acid.
10. 5× Washing buffer (200 ml).
11. ELISA plate seal (two sheets).

2.2 Western Blotting

1. 10× Phosphate-buffered saline (10× PBS): 1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, 18 mM KH₂PO₄, pH 7.4.
2. 10× Tris/glycine/SDS buffer: 0.025 M Tris, 0.192 M glycine, 0.1 % SDS, pH 8.3.
3. 10× Tris/glycine buffer: 0.025 M Tris, 0.192 M glycine, pH 8.3.
4. SDS-PAGE gel: 4–20 % Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad).
5. PageRuler Plus Prestained Protein Ladder 10–250 kDa molecular weight marker (Thermo Scientific).
6. Immuno-blot PVDF membrane.
7. TWEEN® 20.
8. Nonfat dry milk.

9. Methanol.
10. CL-XPosure™ Film.
11. Amersham™ ECL™ Western Blotting Detection Reagents.
12. Anti-HMGB1 antibody (OncoImmune Inc., MI, USA, Cat# OI0001A-05).
13. ECL Rabbit IgG, HRP-linked whole antibody from donkey.
14. Donkey anti-mouse IgG-HRP antibody.
15. Laemmli 2× buffer/loading buffer: 4 % SDS, 10 % 2-mercaptoethanol, 20 % glycerol, 0.004 % bromophenol blue, 0.125 M Tris-HCl, pH 6.8.
16. Running buffer (Tris-glycine/SDS): 25 mM Tris, 190 mM glycine, 0.1 % SDS, pH 8.3. Add 100 ml of 10× Tris/glycine/SDS buffer to 900 ml of ultrapure water, and mix thoroughly.
17. Transfer buffer: 25 mM Tris, 190 mM glycine, 20 % methanol, pH 8.0. Add 100 ml of 10× Tris/glycine buffer to 200 ml of methanol and 700 ml of ultrapure water, and mix well. Chill buffer at 4 °C.
18. Washing buffer: Add 500 µl of Tween 20–1,000 ml of 1× PBS, and mix thoroughly.
19. Blocking buffer: Add 5 g of nonfat dry milk to 100 ml of washing buffer. Mix thoroughly, filter, and store at 4 °C. Failure to filter can lead to “spotting” tiny dark grains that will contaminate the blot during color development.

3 Methods

3.1 HMGB1 ELISA

Below is a brief protocol adapted from the Shino-Test Corporation HMGB1 ELISA kit for measuring HMGB1 in plasma or serum samples; this method has been used by many investigators to measure HMGB1 levels in serum and plasma [16–26].

1. Prepare serum or plasma samples immediately after blood collection to prevent progressive HMGB1 leakage from blood cells (*see Note 3*).
2. Set each reagent in the ELISA kit at room temperature for at least 30 min before use.
3. Add 100 µl of sample diluent to each well and 10 µl of sample diluent to “zero” well.
4. Add 10 µl of HMGB1 standard and serum or plasma samples to the wells, and shake the plate with a plate mixer (*see Note 4*).
5. Incubate for 20–24 h at 37 °C.
6. Wash the plate five times with wash solution (400 µl/well) using microplate washer.

7. Add 100 μ l of peroxidase-linked anti-HMGB1 MAb to each well, and incubate for 2 h at room temperature (25 °C).
8. Wash the plate five times with wash solution (400 μ l/well) using microplate washer.
9. Add 100 μ l of substrate solution to each well, and incubate for 30 min at room temperature.
10. Add 100 μ l of stop solution to each well in the same sequence and time intervals as the addition of substrate solution.
11. Read the absorbance at 450 nm using a microplate reader within 60 min after adding stop solution.
12. Subtract the absorbance of “zero” well from the absorbance of each well.
13. Read the HMGB1 concentrations of unknown samples from the standard curves prepared from purified human HMGB1 protein (*see* **Notes 5–7**).

3.2 HMGB1 Western Blotting

Western blotting enables indirect detection of protein samples immobilized on a nitrocellulose or a PVDF membrane and serves as a useful tool to quantify HMGB1 in serum or plasma samples. Briefly, plasma or serum protein samples are first resolved by SDS-PAGE and then electrophoretically transferred to the membrane. Following a blocking step, the membrane is probed with a primary antibody raised against HMGB1. After subsequent washings, the membrane is incubated with an enzyme-conjugated secondary antibody that is reactive toward the primary antibody. The activity of the enzyme, such as alkaline phosphatase (AP) and horseradish peroxidase (HRP), is necessary for signal generation. Finally, the membrane is washed again and incubated with an appropriate enzyme substrate (e.g., chemiluminescent substrates for HRP), producing a recordable signal. All procedures are carried out at room temperature unless otherwise specified.

3.2.1 Sample Preparation

1. Use a small volume (50 μ l) of serum or plasma to determine the protein concentration.
2. Mix serum or plasma samples with an equal volume of 2 \times Laemmli sample buffer (*see* **Notes 8 and 9**). We recommend denaturing the sample using the following method unless non-reducing and non-denaturing conditions were intended to investigate HMGB1/protein interactions (*see* **Note 10**).
3. To reduce and denature the proteins, boil the sample mixtures at 100 °C for 5 min and spin briefly in a microcentrifuge to collect the condensed water from the tube cap immediately before loading to SDS-PAGE gel.

3.2.2 Separation of Protein Samples by Gel Electrophoresis

1. Mount the precast SDS-PAGE gels onto the electrophoresis apparatus, and add running buffer to the top and bottom reservoirs. Remove any air bubbles.

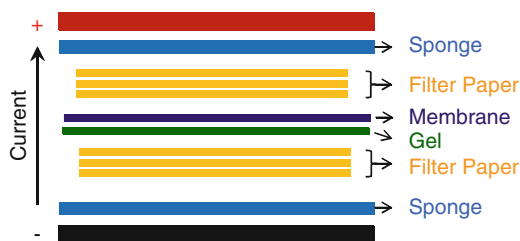


Fig. 1 Assembling the sandwich transfer stack

2. Load equal amounts of serum or plasma proteins (20–30 μg) into the wells of the SDS-PAGE gel, along with molecular weight markers (*see Note 11*), as well as purified HMGB1 protein at several concentrations (e.g., 1, 5, 20 ng/well).
3. Attach the electrophoresis apparatus to an electric power supply. The positive electrode should be connected to the bottom buffer reservoir.
4. Run the gel for 1–2 h at 110 V. The time and voltage may require some optimization. We recommend following the manufacturer's instructions. A reducing gel should be used unless non-reducing conditions were intended to investigate HMGB1/protein interactions (*see Note 10*).
5. Remove the plates from the electrophoresis apparatus, and place them on a paper towel. Using a spatula, pry the plates apart.
6. Remove the gel from the electrophoresis apparatus, and incubate it in transfer buffer for approximately 10 min to equilibrate the gel (*see Note 12*).

3.2.3 Transferring Proteins from the Gel to the Membrane

1. Cut the PVDF membrane to the dimensions of the gel, immerse it in 100 % methanol for 5 min, and rinse with transfer buffer before preparing the transfer stack (*see Note 13*). Mark and/or clip one corner for orientation. Handle only with flat forceps.
2. Soak the member, filter paper, and fiber pads in transfer buffer for 10 min.
3. Prepare the transfer stack as shown below (Fig. 1):
 - (a) Place the cassette, with the gray side down, on a lean surface.
 - (b) Place one pre-wetted sponge/fiber pad on the gray side of cassette.
 - (c) Place a sheet of filter paper on the sponge pad.
 - (d) Place the equilibrated gel on the filter paper.
 - (e) Place the pre-wetted PVDF membrane on the gel.

- (f) Complete the sandwich by placing another piece of filter paper on the membrane.
 - (g) Using a glass tube gently roll all air bubbles out.
 - (h) Add the last sponge pad.
4. Close the cassette firmly and carefully so as not to move the gel and filter paper sandwich. Lock the cassette closed with the white latch.
 5. Place the cassette in the electrophoresis module.
 6. Add the frozen Bio-Ice cooling unit. Place in tank, and completely fill the tank with buffer. Add standard stir bar to help maintain even buffer temperature and ion distribution in the tank.
 7. Put on the lid, and plug the cable into the power supply.
 8. Transfer the protein at 200 mA for 60 min.
 9. The time and voltage may require some optimization. We recommend following the manufacturer's instructions. Transfer to the membrane can be checked using Ponceau Red staining before the blocking step.
 10. After the transfer, unclamp the blot sandwich and remove the sheets of blotting paper, exposing the blot membrane.

3.2.4 Antibody Staining

1. Block the membrane for 2 h at room temperature or overnight at 4 °C using 5 % nonfat milk blocking solution (*see* **Notes 14** and **15**).
2. Incubate membrane with appropriate dilutions of primary anti-HMGB1 antibody in 5 % or 2 % blocking solution overnight at 4 °C or for 2 h at room temperature (*see* **Notes 16** and **17**).
3. Wash the membrane three times for 10 min each in washing buffer to remove unbound antibody.
4. Incubate the membrane with the recommended dilution of labeled secondary antibody in 5 % blocking buffer at room temperature for 1 h.
5. Wash the membrane three times for 10 min each in washing buffer containing 0.05 % Tween-20. Rinse the membrane with washing buffer without Tween-20 (*see* **Note 15**).
6. To prepare the substrate, proceed according to the kit manufacturer's recommendations. For instance, mix the black and white ECL solutions (1:1 ratio) of the Amersham ECL kit.
7. Aliquot sufficient volume of substrate solution to cover and wet the membrane, and incubate the blot with the substrate for 1 min (0.1 ml/cm²) when using Amersham ECL or 5 min when using SuperSignal Substrates.
8. Remove excess reagent, and cover the membrane in transparent plastic wrap. A plastic sheet protector works well, although

plastic wrap also may be used. Remove all air bubbles between the blot and the surface of the membrane protector.

9. Acquire image using darkroom development techniques for chemiluminescence or normal image scanning methods for colorimetric detection.
10. The relative band intensity is quantified by using the NIH image 1.59 or other software to determine HMGB1 levels with reference to standard curves generated with purified HMGB1 at various dilutions (*see* **Notes 18–22**).

4 Notes

1. According to the manufacturer, the Shino-Test Corporation capture anti-HMGB1 antibodies are highly specific to HMGB1, but not to HMGB2. However, their cross-reactivities with other plasma or serum protein are not yet known (Table 1) and should be a subject of future investigation.
2. As a highly charged molecule, HMGB1 can interact with various plasma or serum proteins such as immunoglobulins (IgGs) and thrombomodulin [14, 15]. It is not yet known how these and as-yet-unidentified HMGB1-binding molecules affect the detection of HMGB1 by using the Shino-Test or other HMGB1 ELISA kits [14].
3. Prolonged storage of blood samples before centrifugation at room temperature often leads to higher HMGB1 levels in sera [17], possibly due to leakage of HMGB1 from stressed/damaged blood cells. However, storage of serum samples after centrifugation for up to 7 days does not affect HMGB1 levels [17].
4. The minimal measurable HMGB1 concentrations are calculated by adding two standard deviations to the mean optical density value of several zero standard replicates. According to the Shino-Test Corporation, the limit of detection of HMGB1 ELISA is approximately 0.3–1 ng/ml, making it suitable for measuring plasma or serum HMGB1 in patients with sepsis or other inflammatory diseases. If serum HMGB1 levels are relatively low, consider using the recommended sensitive HMGB1 ELISA method by loading more serum samples and less HMGB1 standard on the ELISA plates (Fig. 2a).
5. In agreement with previous report [16], the measurement of HMGB1 in healthy individuals showed low levels by using the Shino-Test Corporation HMGB1 ELISA kit (Fig. 2a).
6. Research in Dr. Stoetzer's laboratory has shown that the measurement of HMGB1 in EDTA plasma samples yields considerable lower values than in sera with a mean recovery <30 % [17].

Table 1
Commercial available anti-HMGB1 antibodies for Western blotting analysis

Company	Sigma-Aldrich		Abcam		Cell signaling	Oncolmmune Inc
Antibody type (Clone #)	M-MAb (Clone 2F6)	R-PAb	M-MAb	R-PAb	R-PAb	M-MAb (Clone 3B1 or 3E8)
Catalog no.	WH0003146M8	SAB2101049	ab77302	ab18256	3935	OI0001A or OI0001B
Species cross-reactivity	Human	Human Mouse Rat Bovine Canine Chicken Pig	Human	Human Mouse Rat	Human Mouse Rat Monkey	Human Mouse Rat Monkey Canine
Immunogen	Human HMGB1 residues 1–91	Human HMGB1, residues 1–91	Recombinant HMGB1, residues 1–216	Human HMGB1 residues 150–216	Synthetic peptide	Recombinant HMGB1 residues 1–216
Specificity to whole-cell lysate ^a	Hela	Hela	HMGB1-transfected 293T cell	NIH/3T3 MEF1 e PC12	NIH/3T3 DLD-1 C6 COS	Mouse spleen cells
Cross-reactivity to serum proteins	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown

Note: *M-MAb* murine monoclonal antibodies, *R-PAb* rabbit polyclonal antibodies

^aOne specific band was detected in lysates of indicated cells

- The ELISA method has been used by many investigators to measure HMGB1 levels in serum or plasma samples [17–26].
- Almost all serum or plasma proteins can be readily solubilized by sodium dodecyl sulfate (SDS), making SDS-PAGE the most widely used method for determining the molecular mass of serum or plasma proteins.
- As an ionic detergent, SDS denatures proteins by wrapping around the polypeptide backbone at a fixed ratio (1.0 g of SDS:0.7 g of polypeptide). Thus, more SDS can be added in the sampling buffer if excessive amount of plasma or serum proteins (up to 50–100 µg) are loaded onto SDS-PAGE gels in order to detect relative lower HMGB1 levels.
- To investigate the possibility whether HMGB1 interacts with itself (aggregation) or other proteins in plasma and serum

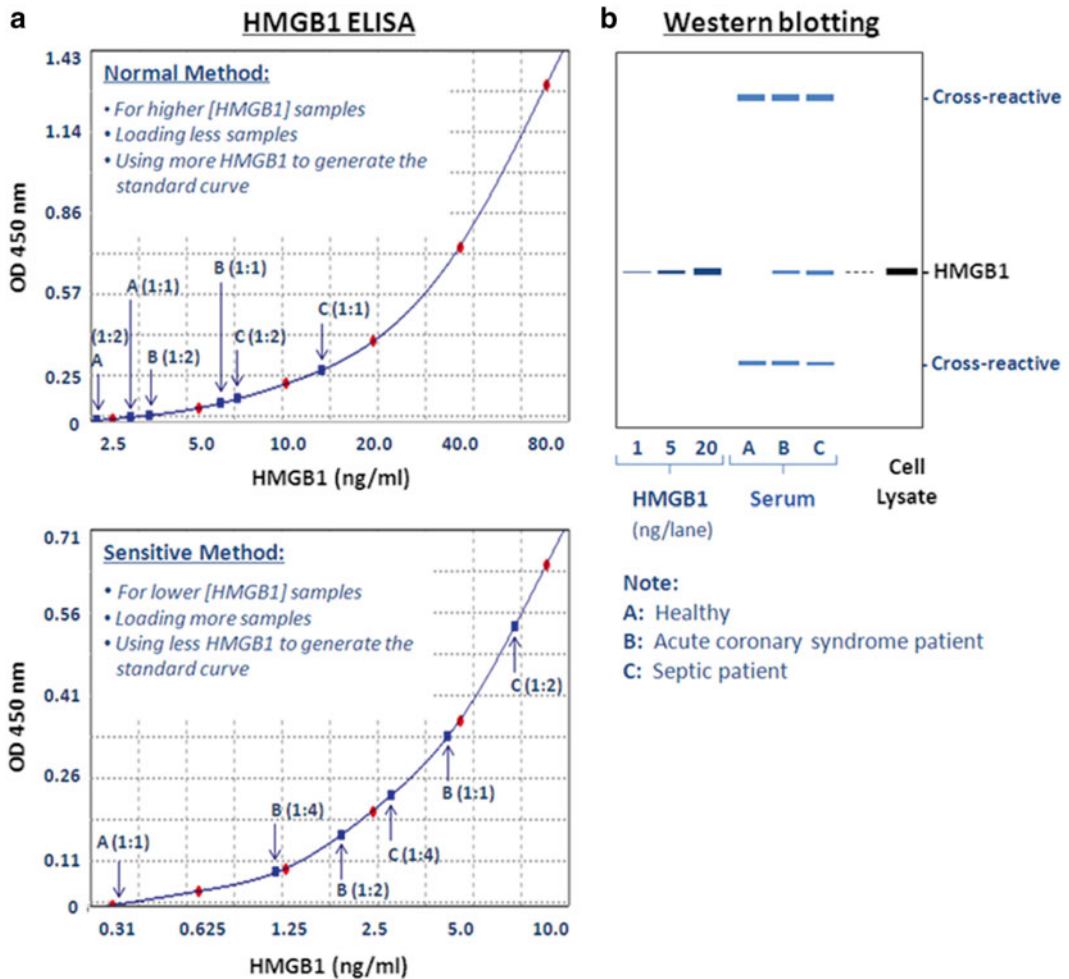


Fig. 2 Representative HMGB1 ELISA and Western blotting results of human serum samples. (a) Measurement of human serum HMGB1 levels using the Shino-Test Corporation ELISA kit. As per the manufacturer's recommendation, two methods with different sensitivities were used to measure HMGB1 in human serum at various dilutions. (b) Measurement of human serum HMGB1 by Western blotting analysis. Polyclonal antibodies were generated against recombinant HMGB1 in the authors' laboratory and used in the Western blotting analysis of human serum HMGB1 levels

samples, non-denaturing native conditions can be employed, where HMGB1 protein is electrophoresed in its native form based on charge-to-mass ratio.

- When using different SDS-PAGE buffer systems (e.g., different pH), the charges and SDS-binding capacities of chemically modified proteins (e.g., pre-stained molecular weight standards) might be slightly affected. Consequently, there might be a slight deviation from the calculated molecular weight (based on the amino acid sequence). To confirm the identity of antibody-reactive band, whole-cell lysate can be used in parallel lanes as a reference size marker.

12. As an alternative of the classical Western blotting protocol using enzyme-conjugated secondary antibodies, direct detection using a labeled primary antibody can be tried. The direct detection takes less time and has less background signal (from the secondary antibody cross-reactivity) than a classical Western blot. However, it is generally less sensitive than the indirect detection, because a labeled primary antibody cannot provide signal amplification and occasionally loses immunoreactivity to the targeted antigen. One alternative option is biotinylating the primary antibody, which not only amplifies the signal but also eliminates the secondary antibody cross-reactivity.
13. Several types of blotting membranes are commonly used, including nitrocellulose and PVDF membranes. Whereas nitrocellulose binds proteins better and often gives better band signals, PVDF is physically stronger and easier to handle. For best results, determine empirically which membrane type, manufacturer, and lot are optimal for each Western blotting system.
14. Many different blocking reagents are available for Western blotting. Because milk contains variable amounts of endogenous biotin, it may produce higher background when using nonfat milk as a blocking reagent in the avidin/biotin systems.
15. Some systems may benefit from adding a surfactant, such as Tween[®]-20, to the blocking solution. Surfactants can minimize background by preventing the blocking reagent from nonspecifically binding to the target. Adding too much detergent, however, can prevent adequate blocking. Typically, a final concentration of 0.05 % detergent is used; however, for best results, determine if detergents enhance a specific system and at what optimal concentrations. Always use a high-quality detergent that is low in contaminants.
16. HMGB1 is a highly conserved protein, making it difficult to generate highly reactive antibodies in many animal species. Currently, there are several commercial sources for HMGB1-reactive polyclonal or monoclonal antibodies. These antibodies normally recognize one single band on Western blots of lysates of various types of cells (Table 1), but their cross-reactivity with serum components remains largely unknown.
17. Based on our experience, most anti-HMGB1 antibodies tested cross-reacted with several other proteins in serum or plasma samples under denaturing conditions (Fig. 2b).
18. Using highly reactive and specific polyclonal antibodies, we found that Western blotting often gave rise to higher values in serum HMGB1 levels as compared to commercially available HMGB1 ELISA (Table 2). This observation was consistent with previous report by others that sandwich HMGB1 ELISA

Table 2**Comparison of human serum HMGB1 levels measured by ELISA and Western blotting analysis**

Human serum	ELISA				
	Normal method		Sensitive method ^a		Western blotting ^b
	Dilution	[HMGB1] (ng/ml)	Dilution	[HMGB1] (ng/ml)	[HMGB1] (ng/ml)
A	1:1	ND	1:1	ND	ND
	1:2	ND	1:2	ND	
	1:4	ND	1:4	ND	
B	1:1	5.9	1:1	4.5	581
	1:2	5.7	1:2	3.3	
	1:4	8.8	1:4	4.5	
C	1:1	13.2	1:1	20.9	950
	1:2	13.5	1:2	15.3	
	1:4	13.4	1:4	11.5	

Note: ND not detectable

^aThe recommended sensitive method was achieved by increasing sample loading and decreasing HMGB1 standard concentrations for the HMGB1 ELISA assay (*see* Fig. 2a)

^bUsing highly specific and reactive polyclonal antibodies generated in the authors' laboratory

often gave false low or negative results as compared to Western blots [14].

19. Although HMGB1 has been suggested as a feasible therapeutic target for experimental sepsis [3, 11, 27], its levels in unfractionated crude serum of septic patients did not correlate well with their disease severity [28].
20. Following ultrafiltration of serum proteins through membrane with 100 kDa cutoff, a 30 kDa HMGB1 band was detected by Western blotting in both the filtrate (<100 kDa) and retentate (>100 kDa) fractions of some septic patients [1]. Interestingly, HMGB1 levels in the filtrate fraction correlated well with the outcome of sepsis [1]. It supports the possibility that HMGB1 may interact with other serum components to form large (> 100 kDa) complexes.
21. In addition, chemical modification may similarly affect the immuno-detection of HMGB1. For instance, a recent study indicated that reactive oxygen species (ROS) may oxidize HMGB1 to form intramolecular disulfide bond between thiol group of Cys¹⁰⁶ and Cys²³ or Cys⁴⁵ [29]. It will be important to investigate whether oxidization affects the immuno-detection of HMGB1 in future studies.
22. We and others are still routinely using the Western blotting method to measure HMGB1 in serum samples [22, 30–41].

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Cytokine Bioassays

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