

## Methodological and Preanalytical Evaluation of an HMGB1 Immunoassay

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**Abstract.** *Background:* Soluble high-mobility group box 1 (sHMGB1) is a promising biomarker for the prognosis and the monitoring of cancer and of acute diseases such as trauma and sepsis. *Materials and Methods:* We investigated the methodological characteristics of an ELISA for sHMGB1 (Shino-Test, Tokyo, Japan and IBL, Hamburg, Germany) including intra- and inter-assay imprecision, dilution linearity and differences in serum and plasma materials. Furthermore, the influence of various preanalytical factors such as time and storage temperature before and after centrifugation prior to definite deep freezing, as well as multiple freeze-thaw cycles were tested. By the use of sera from 28 healthy individuals, a reference range and the dependency on patient characteristics were established. *Results:* Intra-assay imprecision (coefficients of variation (CV)=1.2-4.8%) and inter-assay imprecision (10.3-14.0%) were in an acceptable range of manual assays. HMGB1 levels were found to be considerably lower in EDTA plasma as compared to serum samples. Linearity testing yielded satisfying results with dilution recoveries of 100-121% (mean=112.3%). sHMGB1 results were the same when samples were kept at 4°C and 25°C after centrifugation, for up to 7 days (recoveries 87-128%). Delay before centrifugation led to a considerable increase in some samples. The median values for healthy individuals was 1.3 ng/ml, and the 95th percentile was 4.1 ng/ml. HMGB1 levels correlated inversely with age ( $R=0.33$ ). *Conclusion:* The sHMGB1 ELISA is a robust and safe assay producing reliable quantitative results in sera.

High mobility group box 1 (HMGB1, also known as HMG1 or amphoterin) was identified as a chromatin-associated protein acting as a chromatin-binding factor (1, 2). In this function, it causes DNA bending and initiates the binding of regulatory protein complexes to DNA (2, 3). HMGB1 causes interaction between DNA and several different molecules, such as p53, nuclear factor- $\kappa$ B and the estrogen receptor (1, 4). HMGB1 overexpression is observed in many tumor cells (1).

Besides its nuclear function, soluble HMGB1 (sHMGB1) can also be found in the extracellular milieu. The release of HMGB1 takes part during necrosis as well as during apoptosis, which has only recently been discovered (5, 6). HMGB1 can also be secreted actively, for example after stimulation of macrophages by interferon- $\gamma$  (5). A close association of the protein to circulating nucleosomes has also been found, particularly if the latter it is released during apoptotic cell death (7). When HMGB1 is found in the extracellular milieu, it functions as a cytokine and can stimulate monocytes to produce tumor necrosis factor- $\alpha$  (8) as well as several interleukins (IL), including IL-1 $\alpha$  and IL-1 $\beta$  (2). It also binds to different receptors, such as toll-like receptors (TLR) 2, 4 and 9, as well as the receptor for advanced glycation end products (RAGE), thus promoting inflammation (1, 2). Consequently, HMGB1 has been found to play an important role in sepsis (1, 9), trauma (5), chronic inflammatory diseases, such as rheumatoid arthritis and systemic lupus erythematosus (SLE) (1, 5, 7), as well as in cancer (1, 10). High extracellular levels of sHMGB1 have already been found for several types of cancer including lung cancer (11). Therefore, HMGB1 is a promising biomarker for the prognosis and the monitoring of cancer and for acute diseases such as trauma and sepsis. Serum HMGB1 levels have also been found to be elevated in autoimmune diseases and may be informative for the estimation of the disease activity and monitoring of SLE and rheumatoid arthritis (12, 13).

For study purposes, the levels of HMGB1 are often measured retrospectively after several months or years of conservation. Furthermore, the preanalytical conditions often vary when the blood samples are not centrifuged and stored at

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once. In addition, samples are often reused after thawing if enough sample volume is left. In order to analyze the methodological characteristics of an HMGB1 immunoassay that is commercially available and to test potential influencing factors during the preanalytical treatment of blood samples affecting the HMGB1 levels, the present study was performed.

## Materials and Methods

**Methodological characteristics of the HMGB1 immunoassay.** In order to assess the methodological performance of the HMGB1 immunoassay (sHMGB1, Shino-Test, Tokyo, Japan and IBL, Hamburg, Germany), the intra- and inter-assay imprecisions were tested in three sera with different HMGB1 concentrations, in five and eight repetitions, respectively. Dilution linearity was assessed in four serial two-step dilutions (1:1, 1:2, 1:4, 1:8) using two sera with high HMGB1 concentrations.

Regarding different materials, serum and EDTA plasma from four volunteers were tested.

**Evaluation of preanalytical potentially influencing factors.** Sera of five volunteers were exposed to several potentially influencing factors, such as prolonged time of transportation, storage at different temperatures after centrifugation and several freeze-thaw cycles.

In the first experiment, blood samples that were obtained in serum separator tubes (Sarstedt GmbH, Nümbrecht, Germany), were stored at 4°C (refrigerator temperature) and 25°C (room temperature) for 0 h, 6 h, and 24 h prior to centrifugation at 3000 ×g for 15 min. Subsequently, serum was aliquoted and deep-frozen at -80°C immediately.

In a second experiment, blood samples were centrifuged 15-30 min after venous puncture under the same conditions (3000 ×g for 15 min). Then they were aliquoted and were kept for 0 h, 6 h, 24 h, 48 h and 7 days at different temperatures (4°C and 25°C) before freezing at -80°C.

Finally, samples were thawed and refrozen at -80°C once, twice and three times in order to assess the influence of multiple thawings.

Samples were analyzed in batches, examining all samples from a single patient in one immunoassay run in order to minimize interassay analytical errors.

**HMGB1 assay.** A commercially available sandwich-enzyme immunoassay (ELISA; Shino-Test, Tokyo, Japan and IBL, Hamburg, Germany) was used for the quantitative measurement of HMGB1 in serum and in plasma. The analysis was performed according to the instructions of the manufacturer. Microplates were coated with a purified anti-HMGB1 antibody, which specifically binds for HMGB1. Diluent buffer (50 µl) was pipetted in the wells of the microtiter plate, then 50 µl of standard, positive control and serum or plasma samples were added to each well. The plate was covered with adhesive foil and was incubated at 37°C for 24 h in order to allow for the binding of HMGB1 to the antibodies on the plate. Subsequently, the plate was washed five times and 100 µl of enzyme conjugate was added. Once again, the plate was sealed and left to incubate for 2 h at room temperature. After a washing procedure more, 100 µl of color solution were added and left for another 30 min of incubation time at room temperature. Finally, 100 µl of stop solution were added and the optical density was measured photometrically at 450 nm. The final concentration values were calculated by the use of an optimized

standard curve (eight-step dilution of 1:2 with a starting concentration of 20 ng/ml) included in the assay kit.

**Statistics.** For all comparisons, means (or medians) and ranges were calculated. Coefficients of variation were assessed for imprecision experiments. Recoveries were calculated for the dilution, the material and the stability experiments. For healthy individuals, median and 95th percentiles of HMGB1 values were established.

## Results

The intra-assay imprecision tested in five repeats of three serum samples was 1.2%, 2.6% and 4.8% for HMGB1 concentrations of 6.7, 10.9 and 11.8 ng/ml, respectively. The interassay imprecision was assessed in eight repeats of three serum samples (4.4, 4.8 and 18.9 ng/ml) with a coefficient of variation of 10.3%, 14.0% and 11.2%, respectively.

Dilution linearity of the two sera with high HMGB1 concentrations (undiluted values of 10.4 and 10.9 ng/ml) yielded satisfying results with a mean dilution recovery of 112.3% (range=103-121%).

Measurement of HMGB1 in EDTA plasma yielded considerably lower results than what was measured in sera with a mean recovery of only 27% (range=14-32%).

Prolonged storage of blood samples before centrifugation at room temperature (25°C) did often lead to higher HMGB1 values in sera, with mean recoveries of 148% after 6 h (range=98-242%) and of 112% after 24 h (range=32-158%). However, the broad range of recovery data indicates a considerable individual variability.

In contrast, storage of serum samples after centrifugation at 4°C and 25°C for a time period of up to 7 days did not affect HMGB1 levels. Mean recovery after 6 h was 98.6% (range=94.3-110%) at 4°C and 97.2% (90.0-103%) at 25°C; after 24 h, it was 120% (range=106-132%) at 4°C and 109% (104-113%) at 25°C; after 48 h it was 114% (range=102-128%) at 4°C and 104% (90.7-113%) at 25°C, and after 7 days it was 107% (range=86.8-126%) at 4°C and 95.3% (79.1-113%) at 25°C, respectively (Figure 1).

Finally, repeated freeze-thaw cycles did not lead to different HMGB1 results, yielding a mean recovery of 97.2% (range=81.4-107%) after two freeze-thaw cycles and of 103% (range=95.3-108%) after three freeze-thaw cycles. The measurement of HMGB1 in sera of 28 healthy individuals showed low levels in most cases. The median of healthy individuals was calculated to be 1.3 ng/ml and the 95th percentile was 4.1 ng/ml. There was a slight inverse correlation between HMGB1 levels and age (R=0.33).

## Discussion

Soluble HMGB1 in serum is a promising biomarker for the prognosis and therapy monitoring of sepsis, trauma, cancer and chronic inflammatory diseases and is currently under

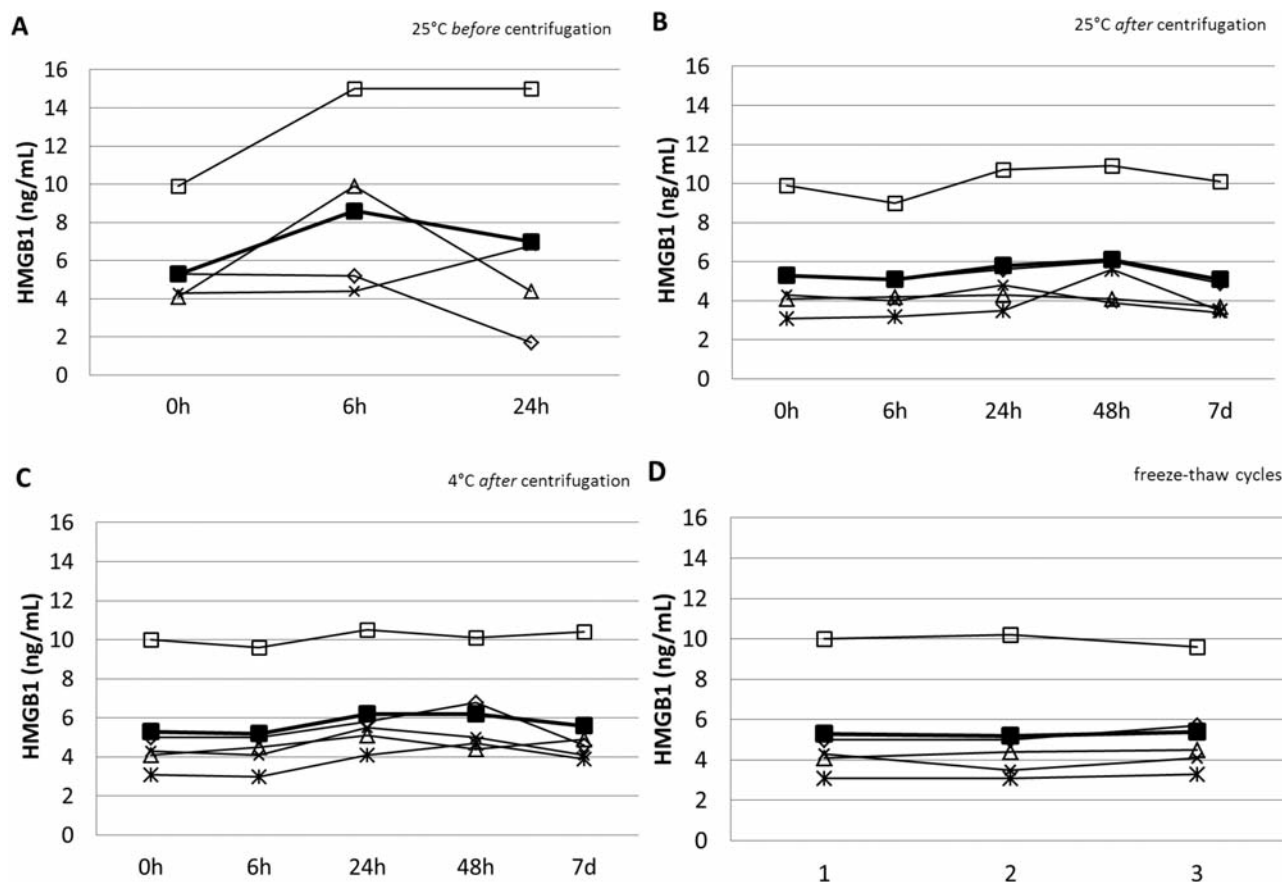


Figure 1. Median and individual courses of high mobility group box-1 (HMGB1) levels exposed to different preanalytical conditions. Storage for different times at 25°C before centrifugation (A), at 25°C after centrifugation (B), at 4°C after centrifugation (C), and after different freeze-thaw-cycles (D).

investigation in several prospective trials. In order to avoid any uncontrolled, non-disease-related bias it is essential to know the methodological characteristics of the assay and the potentially influencing factors in the preanalytical handling of the blood samples.

We therefore tested the methodological characteristics of the HMGB1 immunoassay and found good intra- and inter-assay stability with coefficients of variation being within a satisfying range for manual assays (<15%). Robust and reliable results were found for the dilution linearity. An important finding was the marked decrease in HMGB1 levels in EDTA plasma samples as compared with serum samples of the same individuals. As the results obtained from both materials cannot be compared, further studies should be performed consistently using one type of samples.

Concerning the stability of HMGB1 when blood samples were exposed to adverse preanalytical conditions, our data confirmed the robustness of the analyte and the assay when regular preanalytics were respected. In particular, when serum samples, after centrifugation, were kept for up to

seven days at room temperature or in a refrigerator, HMGB1 results were remarkably stable at both temperatures (4°C and 25°C). This finding is highly relevant as the transport of samples from external sites sometimes takes some days, or may be delayed during the weekend. Furthermore, it shows that HMGB1 results of multicentric studies are comparable even if samples are stored after centrifugation in a different manner.

A further preanalytic feature that is critical for some biomarker levels, such as nucleosomes (14), is delayed centrifugation. Therefore we investigated storage of blood samples at room temperature up to 24 h prior to centrifugation and often found increased HMGB1 levels, however, with an individually considerable scatter. This point is relevant and confirms the importance of adhering to defined preanalytic standards, in particular, early centrifugation within one to two hours after venous puncture in order to minimize preanalytic influences.

An issue of special importance is repeated freezing and thawing, if the serum material is limited in diagnostic

studies, if repetition of the measurement is necessary, or if the cold chain is interrupted for any reason. However, for HMGB1, there was no significant influence of several thaw-freeze cycles on the marker levels obtained.

In line with earlier studies, we found most healthy individuals have low sera HMGB1 levels, with a median of 1.3 ng/ml and a 95th percentile of 4.1 ng/ml. Furthermore, an inverse relationship between HMGB1 and age was found, showing the necessity for using age-matched cases and controls in clinical studies.

## Conclusion

The HMGB1 ELISA was shown to be a robust assay that may be established in research or routine applications for producing reliable, safe results under defined preanalytic conditions.

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