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Establishment of a versatile histidine-rich glycoprotein (HRG) measurement methodology bearing a highly compatible design for patients with sepsis in daily clinical practice

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A B S T R A C T

Sepsis is a life-threatening condition that requires reliable biomarkers for appropriate clinical assessment. Histidine-rich glycoprotein (HRG) has emerged as a promising biomarker candidate associated with sepsis severity. We previously developed an ELISA method using monoclonal antibodies, 69-1 A and 75-2D for sensitive and specific quantification of plasma HRG. However, the assay required extensive sample dilution and further optimization to improve analytical robustness. In the present study, we developed an optimized ELISA by reducing sample dilution ratio and extending the measurable range toward higher HRG concentrations through a competitive reaction system incorporating a small amount of free 75-2D antibody. The optimized assay covered a plasma HRG range of 16–250 μg/mL and showed lower coefficients of variation than the previous method. The optimized method also showed good correlation with the previous ELISA. In addition, we evaluated recombinant single-isoform HRG protein as candidate calibrator material for this assay system. Because both isoforms showed comparable reactivity and dilution linearity, recombinant Form-1 HRG (an isoform bearing Pro at 204-position, 75 kDa) was selected as a practical candidate calibrator based on its simpler glycosylation-related characteristics and quantitative assignment by amino acid analysis. Our new findings support the optimization of ELISA system and a single-isoform recombinant calibrator for more reproducible HRG measurement. It is expected that analytical standardization and clinical validation will subsequently be performed to establish in vitro diagnostic application in actual clinical use.

1. Introduction

Sepsis is defined as a clinical state in which the biological response to infectious illness becomes unregulated, leading to severe organ damage according to The Japanese Clinical Practice Guidelines for Management of Sepsis and Septic Shock 2024 (J-SSCG 2024, Shime et al., 2024). Once septic shock occurs, abnormalities in circulation and intracellular metabolism cause a more severe condition with a significantly high mortality rate (Rhee et al., 2017; Fleischmann et al., 2016; Rudd et al., 2020). Various blood biomarkers and physiological assessments are used for diagnosis and monitoring of disease progression (Wu et al., 2017; Bloos and Reinhart, 2014). Biomarkers such as procalcitonin, presepsin, interleukin-6, and C-reactive protein are typically used as commercially available in vitro diagnostics (IVD) in clinical practice (Liu et al., 2016; Wacker et al., 2013; Mat-Nor et al., 2016; Kobayashi et al., 2021).

Although biomarkers such as procalcitonin, C-reactive protein, presepsin, and interleukin-6 are used in clinical practice, each has limitations in sensitivity, specificity, timing of elevation, or interpretation in complex inflammatory states. Since sepsis is a heterogeneous syndrome involving dysregulated inflammation, coagulation, endothelial dysfunction, and multiorgan injury, no single existing biomarker is sufficient for comprehensive assessment in all clinical settings. Under these circumstances, other biomarkers are also assumed to complement currently available tests and have the potential to improve patient stratification and clinical decision-making for patients. Therefore, additional biomarkers that complement currently available tests may improve patient stratification and clinical decision-making.

However, because sepsis presents with a variety of symptoms and indications of severe damage to multiple organs, a comprehensive evaluation of the patient's condition based on multiple clinical test results is needed (Liu et al., 2015; Yang et al., 2018; Parlato et al., 2018). A broader set of biomarker information associated with sepsis severity may contribute to more appropriate clinical assessment and management. Given this background, we explored the behavior of histidine-rich

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glycoprotein (HRG) in blood to clarify the characteristic features of HRG, which offers potential as a sepsis biomarker (Kuroda et al., 2018; Nishibori et al., 2018; Kuroda et al., 2021). This background makes HRG an attractive complementary biomarker that reflects pathophysiological processes relevant to sepsis including immunothrombosis, endothelial dysfunction and inflammatory dysregulation. A reliable method for measuring plasma HRG that is suitable for routine clinical use may encourage further investigation for its possible roles in early risk assessment, disease stratification, and treatment monitoring.

HRG is a type of protein found abundantly in human plasma that contains a large number of histidine residues, accounting for approximately 12% of its amino acid residues (Poon et al., 2011; Blank and Shoenfeld, 2008). Previous studies have identified HRG as a multifunctional protein that is involved in various physiological processes, including the regulation of blood coagulation, angiogenesis, and immune responses. HRG is assumed to regulate these processes through interactions with various molecules in the blood, similar to substances such as fibrinogen, heparin, and growth factors (Blank and Shoenfeld, 2008; Shannon et al., 2010; Nishimura et al., 2019; Wake et al., 2016; Gao et al., 2019). With the aim of advancing clinical research on HRG, we previously attempted to construct an HRG measurement reagent with high performance for the analysis of human blood samples. Our trials successfully afforded a new enzyme-linked immunosorbent assay (ELISA) prototype system for quantitating HRG in human plasma samples (Uchiumi et al., 2025). ELISA methods involve ligand binding assays with high sensitivity and offer potential robustness and versatility for clinical tests, whether the measurements are manual or automated. The measurement procedure is also very straightforward. Therefore, ELISA methods have become a foundation for the development of automated, high-volume immunoreaction-based reagents.

Because sepsis is usually a severe and potentially fatal condition, high reproducibility is required for biomarker measurement to support patient stratification and appropriate clinical assessment. Therefore, we started to improve the practical performance and reproducibility of our HRG ELISA system. Working in parallel, we tried to evaluate a more consistent calibrator strategy for HRG quantification. In this report, the development of an optimized ELISA system covering a wider and clinically relevant concentration range for plasma HRG is discussed together with an evaluation of a recombinant single-isoform HRG preparation as a candidate calibrator material.

2. Materials and methods

2.1. Purification of HRG protein in human plasma (standard antigen derived from human plasma)

Commercially available human plasma (Japanese Red Cross Society, Tokyo, Japan) underwent nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Qiagen, Hilden, Germany) followed by high-performance liquid chromatography (HPLC) purification with a Mono Q column (GE Healthcare, Chalfont St. Giles, UK) to give purified HRG solution containing two isoforms: wild type (CAS reg. 481,289–16-1) and its mutant (CAS reg. 953,489–04-8). The HRG fraction was further dialyzed against phosphate-buffered saline (PBS) to adjust the HRG concentration to 1000 μg/mL and used as a standard HRG antigen (Wake et al., 2016).

2.2. Preparation of anti-human HRG mouse monoclonal antibodies (mAbs) for sandwich immunoassays

Anti-HRG monoclonal antibodies used in this study were prepared by employing the murine iliac lymph node method (ITM Co., Ltd., Nagano, Japan) which was developed in previous research (Sado et al., 2006). Briefly, BALB/c mice were immunized with standard HRG antigen, and hybridomas were established and screened by ELISA for reactivity to HRG. The monoclonal antibodies used in the present study (Uchiumi et al., 2025) were obtained from respective hybridoma clones, which were selected by their reactivity in HRG ELISA screening, followed by purification by Protein A chromatography, dialyzing against Dulbecco's PBS, and sterilizing by membrane filtration. The mAb 75-2D and mAb 69-1 A were adopted as the most suitable antibody pair for sandwich ELISA. The mAb concentration was determined by absorbance at 280 nm using an extinction coefficient ($E^{1\%}_{280}$) of 13.8, resulting in concentrations of 0.984 mg/mL (75-2D) and 0.843 mg/mL (69-1 A).

2.3. Preparation of enzyme-labeled antibody

The suspending buffer of mAb 69-1 A (1.0 mg) was exchanged with buffer A (0.1 M phosphate buffer [pH 6.0] containing 5 mM EDTA 2Na dihydrate; Nacalai Tesque, Inc., Kyoto, Japan) using a gel filtration column (PD-10; GE Healthcare) followed by concentration by centrifugal ultrafiltration (Amicon Ultra-4; Millipore, Billerica, MA, USA) to afford the purified mAb 69-1 A solution. Next, one-twentieth volume of 1 M 2-mercaptoethylamine in buffer A was added to this solution and allowed to stand overnight at 37 °C. The reaction mixture was purified by gel filtration HPLC column(TSK gel G3000SWXL, i.d. 7.8 mm × 300 mm [TOSOH, Tokyo, Japan]; mobile phase, buffer A; flow rate, 0.5 mL/min; detection, UV 280 nm) followed by concentration by centrifugal ultrafiltration (Amicon Ultra-4; Millipore) to afford reduced concentration immunoglobulin G (IgG) of mAb 69-1 A (0.18 mL, 4.4 nmol). Horseradish peroxidase (HRP; 1.6 mg, Roche, Basel, Switzerland) in 0.2 mL of 0.1 M phosphate buffer (PB; pH 7.0) was mixed with 35 μg of N-(8-maleimidocapryloxy) sulfosuccinimide (Sulfo-HMCS; Dojindo, Kumamoto, Japan) in 0.2 mL of 0.1 M PB (pH 7.0) for 1 h at room temperature. HMCS-activated HRP was purified using a gel filtration column (PD-10) and concentrated using a centrifugal concentrator (Amicon Ultra-4; Millipore). The HMCS-activated HRP solution (1.2 mg in 0.23 mL) was added to 49 μL (6.5 nmol) of the reduced IgG of 69-1 A solution in 49 μL (4.4 nmol) of 0.1 M PB (pH 7.0) and mixed overnight at 4 °C. The reaction mixture was purified by gel filtration HPLC column(TSK gel G3000SWXL, i.d. 7.8 mm × 300 mm [TOSOH, Tokyo, Japan]; mobile phase, 0.1 M phosphate buffer [pH 6.5], flow rate, 0.5 mL/min; detection, UV 280 nm) to afford HRP-labeled IgG of 69-1 A (84.4 μg/mL, 1 mL).

2.4. Preparation of clinical specimens

Eighteen EDTA-2 K plasma samples were prepared from ten patients with sepsis and eight healthy adult volunteers. The Sepsis-3 diagnostic criteria were used to select the population with sepsis. All samples were obtained by blood collection followed by prompt separation of plasma, which was frozen at −80 °C until HRG concentrations were measured. All patients and volunteers provided informed consent for inclusion in this study. The plasma samples were diluted 2500-fold with PBS containing 0.1% bovine serum albumin (BSA; Proliant Biologicals, Boone, IA, USA) or 101-fold with PBS containing 0.1% BSA and 0.01% mouse gamma globulin (Rockland, Philadelphia, PA, USA) to obtain test subject solution for the ELISA measurement systems. Regarding the previous method, 2500-fold dilution was adopted, whereas 101-fold dilution was applied to the new ELISA method.

2.5. The previous method

A sandwich ELISA kit was prepared using of two original purified anti-HRG antibodies (a combination of a capture antibody [mAb 75-2D] and a detection antibody [mAb 69-1 A]). In this study, a 96-well MaxiSorp plate (NUNC, Rochester, NY, USA) was used as the routine measurement instead of the 384-well plate used in our previous study. Capture antibody (mAb 75-2D) (5 μg/mL) was added into the 96-well MaxiSorp plate (NUNC) at 150 μL/well and allowed to react at room temperature for 2 h. After the reaction was complete, each well was washed twice with 300 μL of wash solution (PBS containing 0.05%

Tween 20) followed by the addition of 300 μL/well of blocking solution (PBS containing 1% BSA, pH 7.4) into each well. After incubation at room temperature for 1 h, the blocking solution was removed. Subsequently, 50 μL of PBS containing 0.1% BSA (pH 7.4) was added to all wells. Then, 50 μL/well of six calibration standards (five serial dilutions of HRG: 0.05 μg/mL, 0.025 μg/mL, 0.0125 μg/mL, 0.0063 μg/mL and 0.0031 μg/mL) and a blank (0 μg/mL HRG) or a plasma sample diluted 2500-fold with PBS (pH 7.4) containing 0.1% BSA were added. After incubating the plate for 2 h at room temperature, each well was washed three times with 300 μL of wash solution followed by the addition of 100 μL/well of detection antibody solution (HRP-labeled anti-HRG antibody [mAb 69-1 A; 100 ng/mL]) to incubate at room temperature for 1 h. After washing each well three times with 300 μL of wash solution, chromogenic substrate solution (3,3′,5,5′-tetramethylbenzidine [TMB]; TMB-Plus Substrate-Chromogen; Dako, Glostrup, Denmark) was added at 100 μL/well and allowed to react at room temperature for 15 min. Subsequently, 0.5 M sulfuric acid (FUJIFILM Wako Pure Chemical, Osaka, Japan) was added into each well at 50 μL/well, and then absorbance was measured at 450 nm using an ELISA plate reader (Nivo5S; PerkinElmer, Waltham, MA, USA).

2.6. Construction of a new ELISA method

2.6.1. Exploration of immobilization conditions for a new ELISA method

Aiming to identify the most suitable conditions for the preparation of an mAb immobilized plate, we examined anti-HRG antibody concentrations and specifications for an immobilization buffer. The incubation temperature and reaction time were fixed at room temperature and 3 h, respectively. These evaluations were performed using the same procedure as that in our previous study (Uchiumi et al., 2025). Five concentrations of solid-phase anti-HRG antibody were used: 3, 5, 7, 10, and 15 μg/mL. In addition, two types of buffers for the solid phase were used: carbonate-bicarbonate buffer (50 mM, pH 9.6) and PBS (pH 7.4). This optimization was performed through the measurement of the following dilution series for HRG antigens prepared in Section 2.1: 0.05, 0.025, 0.0125, 0.0063, and 0.0031 μg/mL.

2.6.2. Design of a composition of buffer solutions for diluting clinical samples

To improve the method described in our previous report, which involved a procedure for highly diluting human samples, we attempted to improve the sample solution derived from human specimens for the ELISA plate. The processing method for controlling the reactivity of human specimens was examined as follows to add free 75-2D antibody to the sample solution. Using the above previous ELISA method, the sample solution was added to each well preliminarily dispensed with the free 75-2D antibody, which was the same as the immobilized one, followed by ELISA to evaluate the difference in reactivity depending on the amount of free 75-2D antibody added. The new sample solution was prepared by adding free 75-2D antibody (free 75-2D antibody containing buffer) diluted to concentrations of 0, 1.5, 3, 4, 5, 7, 10, 15, and 50 μg/mL with 50 mM PB buffer containing 0.3 M NaCl, 0.1% BSA, 0.01% mouse gamma globulin, 0.05% Tween 20, and 0.1% Proclin 150 (Sigma Aldrich, St. Louis, MO, USA). Next, 100 μL of free 75-2D antibody containing buffer was dispensed per well, and then 25 μL of serially diluted human purified HRG (five-step dilution from 0.13 to 50 μg/mL) was added. The measurement of samples was successively performed according to the previous ELISA method. A calibration curve was created by plotting each absorbance against the HRG concentration of the calibration standard.

2.6.3. Typical experimental protocols for a new ELISA method: competitive reaction method

Capture antibody mAb 75-2D (5 μg/mL) at 150 μL/well was added to a 96-well MaxiSorp plate (NUNC). This was allowed to react at room temperature for 2 h. After the reaction was complete, each well was washed twice with 300 μL of wash solution (PBS containing 0.05% Tween 20) followed by the addition of 300 μL/well of blocking solution (PBS containing 1% BSA) into each well. After overnight incubation at 4 °C, the blocking solution was removed. Subsequently, 100 μL of 7 μg/mL of free 75-2D antibody containing buffer was added to all wells. Then, 25 μL/well of five calibration standards of the HRG solution: five serially diluted solutions (2.5, 1.25, 0.625, 0.313, and 0.156 μg/mL of HRG) and a blank (0 μg/mL of HRG) or plasma sample diluted 101-fold with PBS containing 0.1% BSA and 0.01% mouse gamma globulin were added. After incubating the plate at room temperature for 2 h, each well was washed three times with 300 μL of wash solution, followed by the addition of 100 μL/well of detection antibody solution for incubation at room temperature for 1 h. After washing each well three times with 300 μL of wash solution, chromogenic substrate solution (TMB) was added at 100 μL/well and allowed to react for 15 min at room temperature. Subsequently, 0.5 M sulfuric acid was added into each well at 50 μL/well, followed by the measurement of absorbance at 450 nm using an ELISA plate reader (Nivo5S).

2.6.4. Validation of the new ELISA system

Two types of commercially available human plasma (EDTA-2 K; BioIVT, Woodbury, NY, USA; KOHJIN BIO, Saitama, Japan) were used for the validation of the ELISA system.

Standard curve

For HRG concentrations diluted serially from 2.5 to 0.156 μg/mL, a standard curve was constructed by plotting the mean absorbances against the log values of each HRG concentration, followed by transformation into a Cubic Spline equation using Workout Version 8.0 (PerkinElmer).

Limit of blank (LOB) and limit of detection (LOD)

The analytical detection capability of an ELISA system was assessed by determining its LOB and LOD. The LOB was calculated from replicate measurements of the blank sample as the mean value plus 1.645 times as much as the standard deviation (SD) of the blank. The LOD was determined from the LOB together with replicate measurements of a low-concentration HRG sample (Armbruster et al., 2008).

Intra- and inter-day assay coefficient of variation (CV)

The precision of the method was evaluated using two pooled EDTA-2 K human plasma samples in this study. Intra-assay precision was assessed by measuring each sample five times on the same plate, and inter-assay precision was assessed by measuring the same samples once daily over three days. Inter-measurer reproducibility was evaluated from duplicate measurements performed by three different measurers.

The results of each assay were expressed as the CV%, which was calculated using the following equation: CV% = SD / Mean x 100%.

2.6.5. Verification of the effects by interfering substances in plasma

To confirm the effects of interfering substances on HRG assay systems for free bilirubin, conjugated bilirubin, hemolyzed hemoglobin, and chyle were evaluated using pooled EDTA-2 K plasma from healthy adults and Interference Check A Plus (Sysmex, Hyogo, Japan). Free bilirubin and free bilirubin (blank) with Interference Check A Plus were dissolved by adding 2 mL of distilled water. The dissolved free bilirubin and free bilirubin (blank) were then added to the pooled EDTA-2 K plasma at a ratio of 10 vol%.

The plasma samples to which free bilirubin and free bilirubin (blank) were added were designated as Samples A and B, respectively. A six-step dilution series was prepared with mixing ratios of the Sample A to Sample B set at 0:10, 2:8, 4:6, 6:4, 8:2, and 10:0. The concentrations of free bilirubin were 0, 4.1, 8.2, 12.3, 16.4, and 20.5 mg/dL. Conjugated bilirubin (0, 3.9, 7.8, 11.8, 15.7, and 19.6 mg/dL), hemolytic hemoglobin (0, 104, 208, 312, 416, and 520 mg/dL), and chyle (0, 340, 680, 1020, 1360, and 1700 FTU) were prepared in the same manner. The HRG values of the prepared plasma were measured using the new ELISA method.

HRG values were expressed as relative values, with the blank

condition defined as 100%, which did not contain any interfering substances. Considering the analytical variability of the assay, a relative value within the 100 ± 20% range of the corresponding blank condition was defined in advance as the allowable criterion for interference evaluation.

2.7. Construction of a candidate calibrator material

2.7.1. Preparation of recombinant HRG proteins

Two types (wild type; 75 kDa [Form-1], and mutant type; 77 kDa [Form-2]) of recombinant HRG proteins were prepared using standard recombinant protein expression procedures (FUJIFILM Wako Pure Chemical). The expression vector was constructed by inserting the synthesized sequences based on gene sequence information into expression vectors (pCAG_Hyg). Successively, each expression vector was also prepared in large quantities. Freestyle 293F cells (Invitrogen, Carlsbad, CA, USA) were cryopreserved and subcultured using Freestyle 293 Expression Medium. After appropriate subculture, the cells were growing as expected, so each expression vector was transfected into cells in accordance with the manufacturer's recommended protocol (293fectin Transfection Reagent, Thermo Fisher Scientific, Waltham, MA, USA). The culture medium was collected 6 days after completion of transfection, and then centrifugation was performed to separate the culture supernatant and cell fraction. The HRG protein was purified using Ni affinity chromatography, where the cell culture supernatant was first loaded onto a HisTrap HP column (GE Healthcare) equilibrated with Binding Buffer (20 mM Imidazole-HCl, 20 mM HEPES-NaOH, 500 mM NaCl, 5% (*v*/v) Glycerol, pH 7.4), followed by washing with wash buffer (20 mM Imidazole-HCl, 20 and 50 mM HEPES-NaOH, 500 mM NaCl, 5% (v/v) Glycerol, pH 7.4) to remove nonspecifically bound proteins, and the target protein was eluted by applying a linear imidazole gradient ranging from 50 to 500 mM, with fractions collected throughout the gradient. The target protein was purified from each expression product using Ni affinity followed by dialysis to give it as PBS (−) replaced solution.

2.7.2. Western blot analysis for recombinant human HRG proteins

The recombinant HRG proteins were treated with sodium dodecyl sulfate (SDS) sample buffer (NuPAGE LDS Sample Buffer (4×), Invitrogen) at 70 °C for 10 min followed by separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; NuPAGE Bis-Tris Gel, Invitrogen) and subsequent transfer onto polyvinylidene difluoride membranes (iBlot Transfer Stack; Invitrogen). The membranes were blocked with Tris-buffered saline containing 1% BSA, followed by incubation with a commercially available anti-human HRG mouse mAb (Clone No. 227901; R&D Systems, Minneapolis, MN, USA) and treatment with ECL anti-mouse IgG and HRP-linked whole antibody. Finally, the HRG signals were visualized using ECL reagent (GE Healthcare).

2.7.3. Amino acid analysis for new recombinant HRG proteins

Amino acid quantification within the protein was performed using amino acid analysis in accordance with standard procedures conducted at Medicinal Chemistry Pharmaceutical, Co., Ltd., Japan, the samples were acid-hydrolyzed followed by labeling with derivatization reagents for separation by ultra-performance liquid chromatography (UPLC). Calibration curves for 17 amino acid standards were generated to analyze quantitatively the amino acid content present in the subject samples. For the preparation of samples, 100 μL of recombinant HRG (Form-1) was mixed with 50 μL of MilliQ water and 150 μL of hydrochloric acid (FUJIFILM Wako Pure Chemical), and then heated at 110 °C in an oven for 40 h. The resulting reaction mixture was dried by a centrifugal concentrator to obtain a powdery product. This dried powder was reconstituted in 500 μL of MilliQ water to generate the sample solution. Five serial dilutions (0.25, 0.125, 0.050, 0.025, and 0.010 μmol/mL) of the 17 amino acid standard substances (Amino Acid Standard H, Waters, Milford, MA, USA) were prepared to construct the standard curves. For derivatization, the AccQ·Tag Ultra Derivatization Kit was used to label both the standard substances and sample solutions. Each standard substance and sample solution (20 μL) was added to a UPLC analysis tube. A mixture of 60 μL borate buffer and 20 μL AccQ·Tag reagent from the AccQ·Tag Ultra Derivatization Kit was incubated at 55 °C for 10 min. Both the derivatized samples and standard substances were injected into the UPLC system under the following conditions: reverse-phase separation was performed on a Waters AccQ·Tag Ultra C18, 1.7 μm, 2.1 × 100 mm column. The derivatives were eluted with specified dilutions of Waters AccQ·Tag Ultra Eluent A and Waters AccQ·Tag Ultra Eluent B. The derivatives were quantitatively analyzed using UV detection.

2.8. Statistical analysis

Calibration curves were constructed by mean absorbance values using Cubic Spline analysis with Workout Version 8.0 (PerkinElmer). Coefficients of variation (CVs) were calculated by SD/mean × 100%. Correlation analyses between methods or calibrators were evaluated by linear regression, and the coefficient of determination (R^2) was used to assess the strength of their linear relationship.

3. Results

3.1. Optimization of the solid-phase antibody concentration

mAb 75-2D solutions at various concentrations (3, 5, 7, 10, and 15 μg/mL) were added to each well of the plate. Using the respective plates, the standard HRG protein was measured at five points with a twofold serial dilution (0.05, 0.025, 0.0125, 0.0063, and 0.0031 μg/mL) to give the results constructed in Fig. 1a and b. At solution concentrations of 3, 5, and 7 μg/mL, the absorbance values for a standard HRG concentration of 0.05 μg/mL were greater than 2.5, indicating the suitability of adopting this concentration range for the immobilizing methodology. On the other hand, a decrease in absorbance was observe using mAb 75-2D solutions at concentrations of 10 μg/mL or more in comparison with mAb 75-2D solutions at concentrations less than 7 μg/mL. These trials suggested a threshold around 7 μg/mL based on the evaluation of the absorbance of the measurement sample at a wavelength of 450 nm. In contrast, antibody concentrations between 3 and 7 μg/mL showed comparable reactivity within a sufficient absorbance range for the ELISA system. We also investigated the effect of HRP-labeled anti-HRG antibody (mAb 69-1 A) in concentrations from 50 to 150 ng/mL, and the results indicated that the linearity of the standard curve decreased above 100 ng/mL. This was assumed to be caused by the hook effect, which inhibits the formation of antigen-antibody complexes because of the presence of an excessive amount of antibody. Accordingly, an HRP-labeled anti-HRG antibody (mAb 69-1 A) concentration of 75 ng/mL was selected (data not shown).

3.2. Setting the conditions for adding free 75-2D antibody to the test sample reaction solution: development of new ELISA method (competitive reaction method)

Aiming to shift the measurable range toward higher HRG concentrations, we evaluated the effect of adding free 75-2D antibody to the reaction system. Increasing the concentration of free 75-2D progressively shifted the calibration behavior toward a range more suitable for less diluted plasma samples. Under the condition of 101-fold sample dilution, 7 μg/mL free 75-2D provided the most appropriate calibration profile for quantifying HRG concentrations corresponding to 0.16–2.5 μg/mL in the assay solution (Fig. 2).

Aiming to optimize the assay conditions, we systematically evaluated each experimental factor, and the selected conditions are summarized in Table 1. Some procedures and conditions constituting key ELISA steps have been renewed compared with the previous ELISA. Candidate

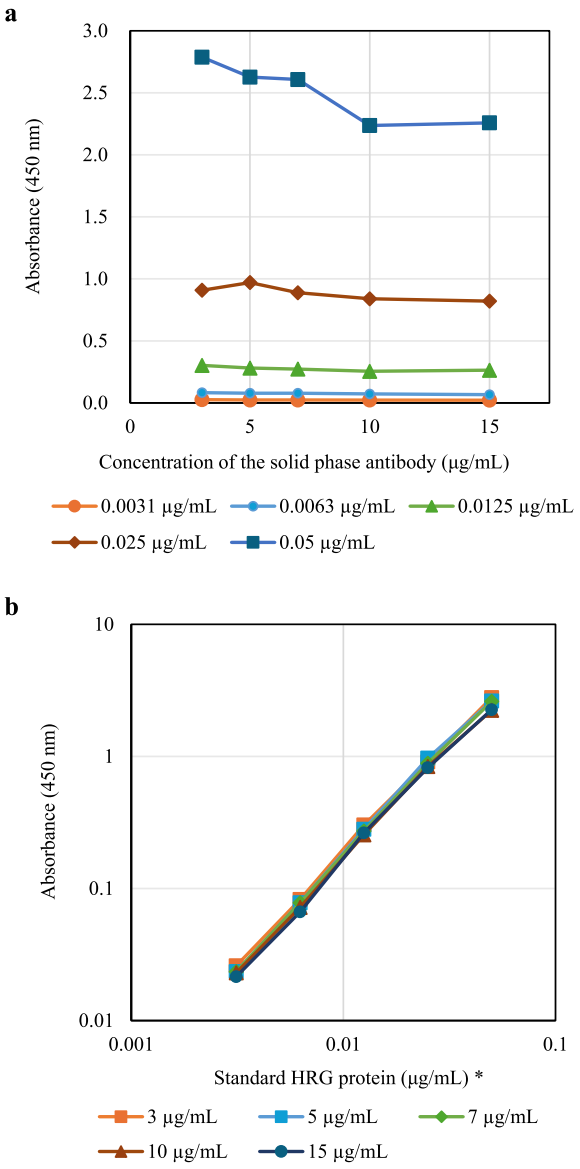


Fig. 1. Optimization of solid-phase antibody concentration. a) Absorbance of standard HRG protein diluted twofold (0.05, 0.025, 0.0125, 0.0063, and 0.0031 μg/mL) using plates immobilized with mAb 75-2D at concentrations of 3, 5, 7, 10, and 15 μg/mL. b) Calibration curve of the HRG protein using plates immobilized with mAb 75-2D at concentrations of 3, 5, 7, 10, and 15 μg/mL.
* Standard HRG protein was purified from human plasma, with the concentration of the protein measured by the Bradford method.

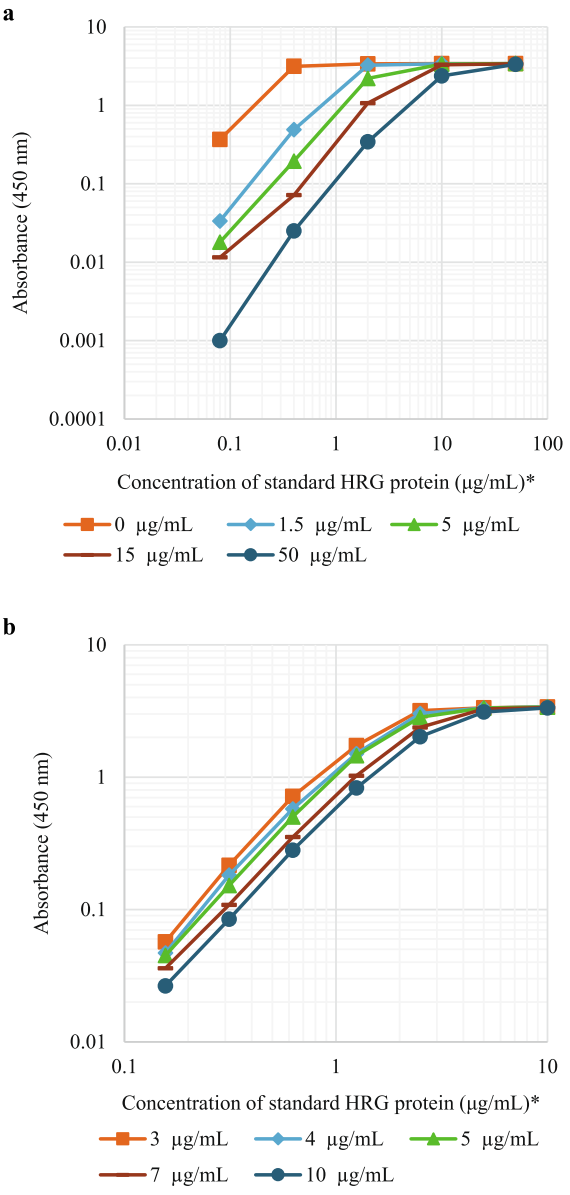


Fig. 2. Setting the conditions for adding a capture antibody to the test sample reaction solution.
a) Calibration curve for a five-step dilution series of HRG standard protein from 0.13 to 50 μg/mL. The capture antibody was added to the antibody-containing buffer at concentrations of 0, 1.5, 5, 15, and 50 μg/mL. b) Calibration curve with capture antibody concentrations of 3, 4, 5, 7, and 10 μg/mL in antibody-containing buffer.
* Standard HRG protein was purified from human plasma, with the concentration of the protein measured by the Bradford method.

conditions were selected based on signal intensity, calibration curve profile, and suitability for measuring clinically relevant HRG concentrations.

3.3. Validation trials for the sandwich ELISA method: a new ELISA method (competitive reaction method)

3.3.1. Limit of blank (LOB), limit of detection (LOD), and precision

The analytical performance of the optimized sandwich ELISA was evaluated by determining the limit of blank (LOB), limit of detection (LOD), and assay precision (Armbruster et al., 2008). The LOB and LOD were 0.04 μg/mL and 0.16 μg/mL, respectively, under the test conditions. Given the 101-fold dilution applied to plasma samples, the practical lower measurable concentration in plasma was calculated to be approximately 16 μg/mL. Precision studies using two pooled EDTA-2 K human plasma samples resulted in 3.6% and 1.8% CVs for intra-assay and 7.3% and 9.3% CVs for inter-assay, respectively. Inter-measurer reproducibility testing yielded CVs of 8.4% and 6.6% (Table 2). These results indicate that the optimized ELISA comprising the antigen-antibody reaction under the presence of 7 μg/mL of free 75-2D antibody provided good analytical sensitivity and reproducibility. (See Table 3.)

3.3.2. Evaluation of effects of plasma interfering substances

Measurements were conducted for samples prepared from pooled EDTA-2 K plasma from healthy adults by adding different concentrations of free bilirubin (4.1, 8.2, 12.3, 16.4, and 20.5 mg/dL) to give values in the range of 92%–101% compared with the case of free bilirubin (Fig. 3a). Similarly, trials in the presence of various levels of

Table 1
Typical procedure constituting the new ELISA system for human HRG measurement.

ELISA steps	Differences between previous and new ELISA method	
	Previous ELISA	New ELISA
Plate coating	Coat MaxiSorp plate with 100 μL/well of 5 μg/mL of monoclonal antibody (mAb) 75-2D diluted in 10 mmol/L of pH 7.4 phosphate-buffered saline (PBS) at room temperature for 2 h	Coat MaxiSorp plate with 200 μL/well of 5 μg/mL of mAb 75-2D diluted in 10 mmol/L of pH 7.4 PBS at room temperature for 2 h
Washing	Wash twice with 300 μL/well of 10 mmol/L PBS containing 0.05% Tween 20 (pH 7.4)	Wash three times with 300 μL/well of 10 mmol/L PBS containing 0.05% Tween 20 (pH 7.4)
HRG antigen binding	Add 50 μL/well of PBS containing 0.1% (*w/v*) BSA (pH 7.4) Subsequently, add 50 μL/well of sample or HRG standard solution and then incubate at room temperature for 2 h	Add 100 μL/well of 7 μg/mL of mAb 75-2D diluted in the mixture of 50 mmol/L PB containing 0.1% (w/v) BSA, 0.3 mol/L NaCl, 0.05% Tween 20, and 0.02% NaN_3 Subsequently, add 25 μL/well of sample or HRG standard solution and then incubate at room temperature for 2 h
Binding HRP conjugate antibody, 69-1 A	Add 100 μL/well of HRP enzyme conjugate antibody 69-1 A (100 ng/mL) in PBS containing 0.1% (w/v) BSA (pH 7.4) and then incubate at room temperature for 1 h	Add 100 μL/well of HRP enzyme conjugate antibody 69-1 A (75 ng/mL) in 10 mmol/L PBS containing 0.4% (w/v) Block ACE and 0.02% NaN_3, and then incubate at room temperature for 1 h

Table 2
The limit of blank and the limit of detection.

	The limit of blank (LOB)		The limit of detection (LOD)	
HRG (μg/mL)	0.04	μg/mL	0.16	μg/mL

Table 3
Verification of optimized ELISA.

HRG (μg/mL)	Intra-assay		Inter-assay	
	Mean ± SD (μg/mL) (n = 5)	CV (%)	Mean ± SD (μg/mL) (*n* = 3)	CV (%)
Pool EDTA-2 K human plasma No. 1	76.9 ± 2.7	3.6	81.1 ± 6.0	7.3
No. 2	102.9 ± 1.9	1.8	115.5 ± 10.8	9.3

HRG (μg/mL)	Inter-measurer reproducibility	
	Mean ± SD (μg/mL) (n = 3)	CV (%)
Pool EDTA-2 K human plasm No. 1	82.7 ± 7.0	8.4
No. 2	124.3 ± 8.2	6.6

conjugated bilirubin (3.9, 7.8, 11.8, 15.7, and 19.6 mg/dL) gave HRG concentrations within 105%–116% compared with the value at 0 mg/dL of conjugated bilirubin (Fig. 3b). The cases containing hemolyzed hemoglobin (104, 208, 312, 416, and 520 mg/dL) showed HRG concentrations in the range of 101%–110% for 0 mg/dL of hemolyzed hemoglobin (Fig. 3c). Lastly, HRG concentrations in the range of 94%–100% compared with 0 FTU were seen in the cases of samples having various chyle concentrations (340, 680, 1020, 1360, and 1700 FTU) (Fig. 3d). The results of these trials are depicted in Table 4. Therefore, interfering substances, which can often contaminate human plasma specimens, affected the measured values of HRG in the range of 92%–116% compared to samples without any interfering substances. The new ELISA method showed no significant concentration-dependent interference over the test range of interfering substances, and all measured values remained within the predefined acceptance criterion of 100 ± 20% for interference evaluation.

3.3.3. Comparison between the new ELISA method (competitive reaction method) and the previous ELISA method

The correlation between the newly optimized ELISA method and the previous ELISA method was evaluated using 10 EDTA-2 K plasma samples, resulting in the following correlation formula: $y = 1.1703x - 7.7106$, $R^2 = 0.9542$, $n = 10$ (Fig. 4).

Since the new ELISA showed good correlation with the previous method, the optimized assay was considered suitable for measuring HRG over a higher practical concentration range with reduced sample dilution and lower assay variability. These features indicate the improvement of operability and reproducibility compared to the previous format.

3.4. Construction of a candidate calibrator material for the new ELISA system

3.4.1. Validation of standard proteins

Next, we investigated the feasibility of substituting the previous HRG protein prepared from human plasma with recombinant proteins which are the candidate calibrator materials for our new ELISA method. This trial was intended to enhance the reproducibility and reliability of the standard curves. As such, we attempted to synthesize two types of recombinant HRG proteins (Form-1 and Form-2). As a result, Form-1 and Form-2 had molecular weights of 75 kDa and 77 kDa, respectively, in the purified fractions by SDS-PAGE. The identity of the prepared purified HRG products was confirmed by western blotting. Purified HRG and a commercial HRG mAb (Clone No. 227901; R&D Systems) showed reactivity to the 75 kDa and 77 kDa bands (Fig. 5). It was confirmed that the expected difference in molecular weight between the two proteins was caused by differences in glycosylation. Accordingly, this result indicated that the protein expression and purification of HRG proteins in this study were successful.

3.4.2. Linearity verification using two kinds of HRG isoforms for a new ELISA method (competitive reaction method)

Next, HRG ELISA standard curves were constructed using the two recombinant proteins as reference standards. Using three HRG proteins—a plasma-derived purified protein and two recombinant proteins respective calibration curves ranging from 0.16 to 2.5 μg/mL were drawn, followed by a comparison of each absorbance value. The recombinant human HRG proteins were diluted to prepare the fourfold dilution series, followed by measurement with the new ELISA. The mean value of the measurement outcomes at each level was plotted via comparisons with the theoretical concentrations, as shown in Fig. 6a. To confirm the dilution linearity of the two types of recombinant HRG proteins, these proteins were serially diluted, and the HRG concentration was determined using the new ELISA method. The results showed good linearity for both proteins ranging from 0.37 to 5 μg/mL (Fig. 6b).

These findings indicated that recombinant Form-1 and Form-2 HRG showed comparable reactivity and dilution linearity in the optimized ELISA system. On this basis, recombinant Form-1 was selected as a candidate calibrator material for this assay because of its analytically favorable single-isoform composition and simpler glycosylation-related characteristics.

3.4.3. Amino acid analysis

The recombinant proteins were subjected to amino acid analysis to quantify the amino acid composition. The calibration curve given by the standards for 17 amino acids enabled quantitative analysis of each

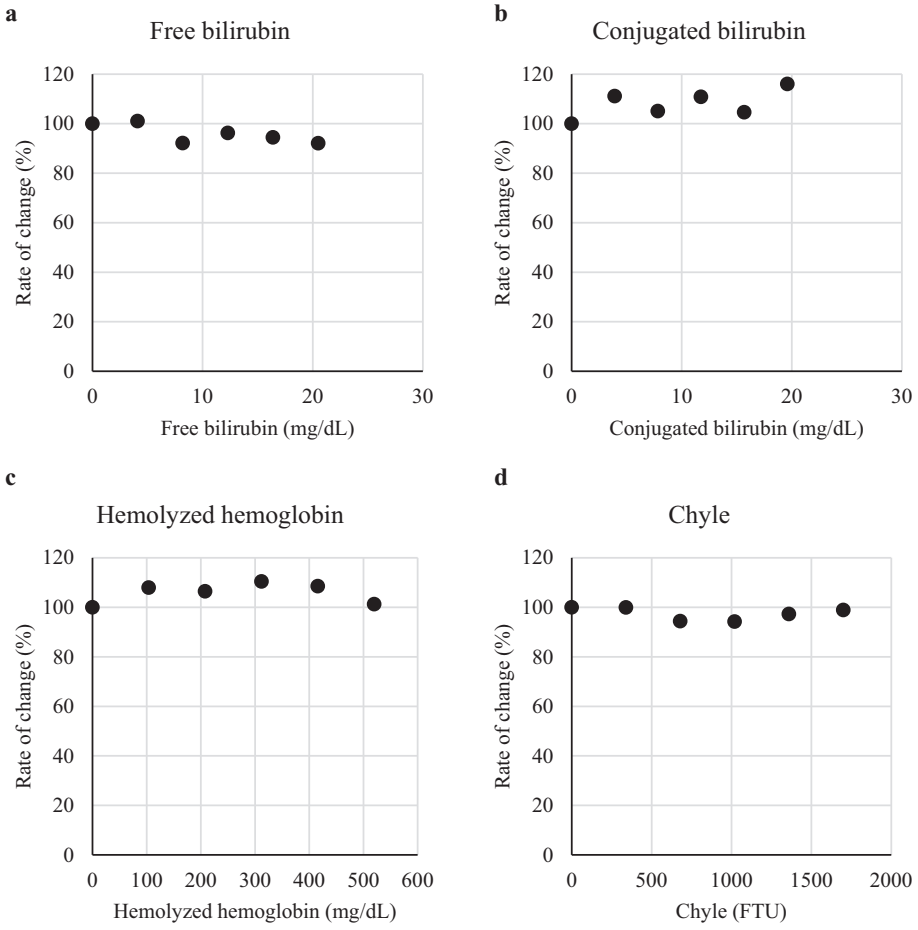


Fig. 3. Verification of the effects of plasma interfering substances. The variation ratio of the HRG concentration at each added concentration of free bilirubin (4.1–20.5 mg/dL), conjugated bilirubin (3.9–19.6 mg/dL), hemolyzed hemoglobin (104–520 mg/dL), and chyle (340–1700 FTU) is expressed as a percentage relative to the reference value of 100% at an added concentration of 0.

Table 4
Verification of the effects of plasma interfering substances.

Free bilirubin	Free bilirubin (mg/dL)					
	0	4.1	8.2	12.3	16.4	20.5
HRG (μg/mL)	121	122	112	116	114	111
% of 0 (mg/dL)	100%	101%	92%	96%	94%	92%
Conjugated bilirubin	**Conjugated bilirubin (mg/dL)**					
	0	3.9	7.8	11.8	15.7	19.6
HRG (μg/mL)	114	127	120	126	119	132
% of 0 (mg/dL)	100%	111%	105%	111%	105%	116%
Hemolyzed hemoglobin	**Hemolyzed hemoglobin (mg/dL)**					
	0	104	208	312	416	520
HRG (μg/mL)	113	122	120	125	123	114
% of 0 (mg/dL)	100%	108%	106%	110%	109%	101%
Chyle	**Chyle (FTU)**					
	0	340	680	1020	1360	1700
HRG (μg/mL)	111	111	104	104	108	109
% of 0 (FTU)	100%	100%	94%	94%	97%	99%

amino acid contained in the sample. Amino acid analysis was performed to measure the precise amount of HRG protein contained in the prepared recombinant HRG protein (Form-1) solution. The amount of protein for each amino acid was determined by dividing the measured amount of each amino acid by the theoretical amount. The protein amount calculated from all amino acids is shown in Table 5. According to the 18th

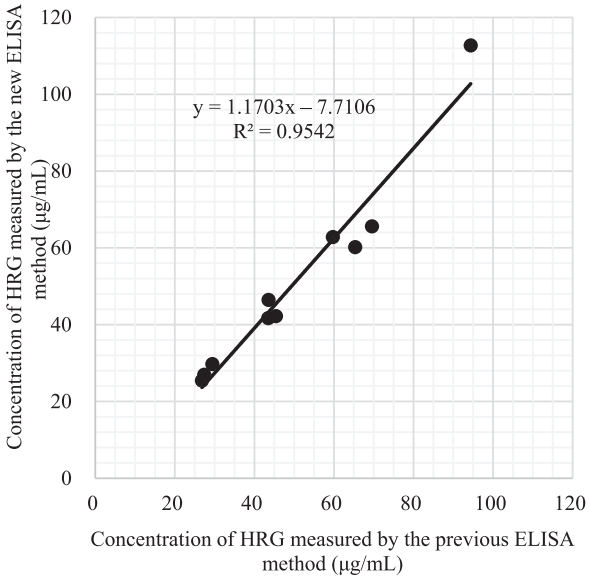


Fig. 4. Correlation between the new and previous ELISA method. HRG concentrations measured using each method in ten EDTA-2 K plasma samples from patients with sepsis.

revised version of the Japanese Pharmacopoeia (Reference Information,

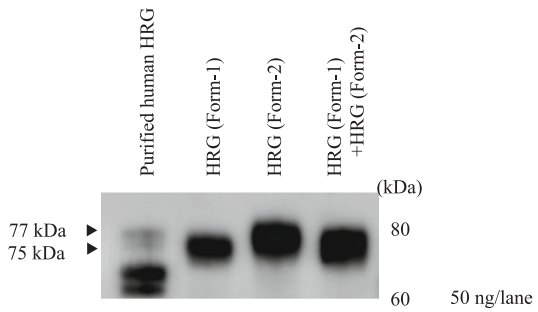


Fig. 5. Western blotting.
The 75-kDa band corresponds to recombinant Form-1 HRG, and the 77-kDa band corresponds to recombinant Form-2 HRG. The native plasma-derived HRG contains the corresponding HRG-immunoreactive bands. These results confirmed the identity of the recombinant proteins and their expected electrophoretic difference.

Amino Acid Analysis <G3–2-171>), amino acids with good recovery rates generally include aspartic acid/asparagine, glutamic acid/glutamine, alanine, leucine, phenylalanine, lysine, and arginine. The protein amount calculated for each amino acid was 1.51, 1.55, 1.25, 1.33, 1.40, 1.49, and 1.41 mg/mL, respectively. Next, the average protein amount was calculated using only the amino acids with good recovery rates. Because the protein amount calculated for each amino acid must be evenly distributed around the average, values that deviated significantly from the average were excluded. Typically, values that deviate from the average value by more than 5% are considered to be subject to exclusion. The average value is recalculated from the remaining values to obtain the protein amount of the sample. The protein concentration calculated from arginine, lysine, and phenylalanine using this method was 1.43 mg/mL. A standard protein was then prepared according to the precise HRG protein amount determined by this result.

3.4.4. Comparison of plasma HRG measurement values between the different calibrations based on human purified and two recombinant HRG proteins

Using two kinds of calibrator materials, namely recombinant HRG (Form-1) and human purified HRG protein, eight EDTA-2 K plasma samples obtained from healthy adult volunteers underwent HRG measurement by the same procedure, followed by quantification based on two standard curves given by the respective calibrator materials. A strong significant correlation was observed between the HRG values measured using the two different calibrator materials, resulting in the following equation: $y = 0.7932\times - 19.319$ ($R^2 = 0.9991$, $n = 8$) (Fig. 7).

4. Discussion

4.1. Analytical issues to be addressed for broader application of the HRG ELISA system

An important aim of this study was to improve the analytical performance and practical usability of our previously developed HRG ELISA. Rather than establishing immediate readiness for routine clinical use or collection of data for in vitro diagnostic application, the present work was intended to provide a more reproducible assay format and a more consistent calibrator strategy that could serve as a foundation for future analytical and clinical validation.

Additionally, we needed to solve the problem of determining standard analyte substances, which are often encountered in immunoassays in clinical examinations. The measurable range of our previous ELISA method was 0.0031–0.05 μg/mL, with intra- and inter-assay CVs of 10% and 15%, respectively. While the previous ELISA method has the advantage of being highly sensitive for HRG measurement, the large variability in the measured values remains a challenge and needs to be solved to realize the reliable detection of HRG in human samples from various patients with different clinical conditions. Therefore, aiming to identify the optimal conditions and suppress variation in our previous ELISA method, an evaluation of multiple factors, including the type of plate, reaction time and temperature, volume of buffer solution, and antibody concentration, was conducted. Regarding the concentration of antibodies mAb 75-2D for immobilization and HRP-labeled mAb 69-1 A, the optimal conditions were 5 μg/mL and 75 ng/mL as in the previous ELISA method, respectively. Despite our attempts to improve the measurement accuracy by reevaluating each step of the ELISA system to determine the optimal conditions, the major factor affecting the variation could not be identified, and since the measurement system itself was not considered the cause, the decision was made to reduce the sample dilution rate.

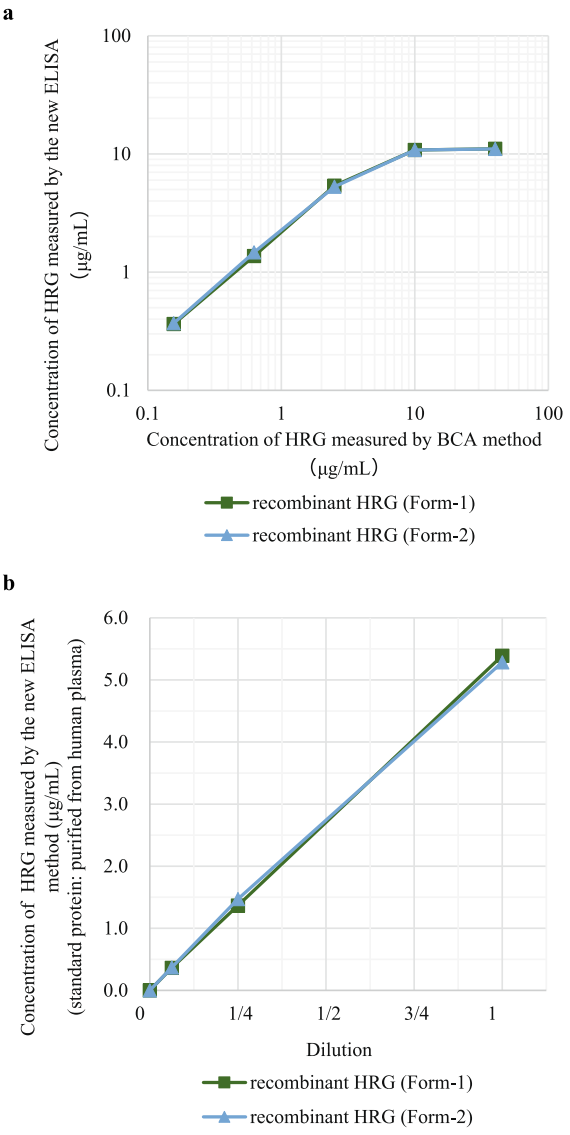
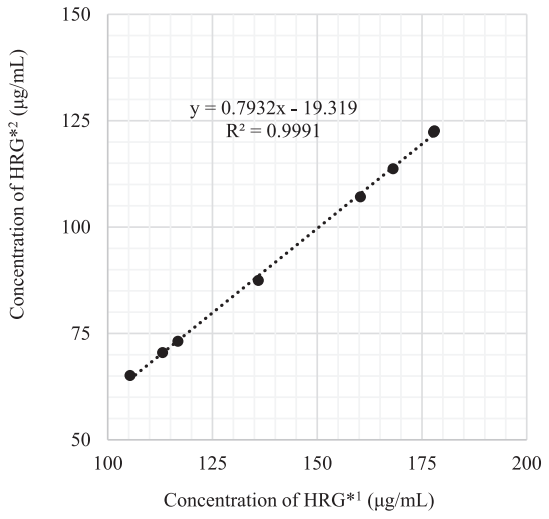


Fig. 6. Linearity verification using two types of HRG isoforms for a new ELISA method.
a) Correlation curve showing the concentration of two types of HRG isoforms measured using the Bradford method and a new ELISA method using a standard curve with purified human plasma HRG. b) Dilution linearity of two types of HRG isoforms. Serial dilutions were measured using the new ELISA method. The dilution curves of recombinant HRG proteins (Form-1 and Form-2) showed linearity with $R^2 = 1.0000$ and 0.9992, respectively.

Table 5
Recombinant protein amino acid analysis.

Amino acid	No. of amino acid residues	Calculated ratio (%)	μmol/100 μL sample *1	mM protein *2	mg/mL *3 (MW: 59578)
Ala	22	4.19	0.046	0.0209	1.25
Cys	17	3.24	0.004	0.0024	0.14
Asp/Asn	51	9.71	0.129	0.0253	1.51
Glu/Gln	46	8.77	0.120	0.0261	1.55
Phe	26	4.95	0.061	0.0235	1.40
Gly	35	6.67	0.084	0.0240	1.43
His	66	12.57	0.158	0.0239	1.43
Ile	13	2.48	0.027	0.0208	1.24
Lys	22	4.19	0.055	0.0250	1.49
Leu	34	6.48	0.076	0.0224	1.33
Met	3	0.57	0.005	0.0167	0.99
Pro	65	12.38	0.150	0.0231	1.37
Arg	33	6.29	0.078	0.0236	1.41
Ser	36	6.86	0.077	0.0214	1.27
Thr	17	3.24	0.040	0.0235	1.40
Val	26	4.95	0.062	0.0238	1.42
Trp	2	0.38	–	–	–
Tyr	11	2.10	0.019	0.0173	1.03

*1: Amount (mol) of each amino acid contained in 100 μL of sample subjected to amino acid analysis.
*2: Amount (mol) of amino acid was divided by the number of amino acid residues for the HRG proteins to calculate the protein concentration from each amino acid.
*3: Protein concentration was determined by multiplying the calculated HRG protein concentration (in mol/L) by the molecular weight of the HRG protein.



*1 Standard protein: purified from Human plasma
*2 Standard protein: Recombinant HRG (Form-1)

Fig. 7. Comparison of plasma HRG measurement values between the different calibrations based on human purified and recombinant HRG proteins (Form-1) HRG concentrations were determined in eight EDTA-2 K plasma samples from healthy adult volunteers using an identical assay procedure with recombinant HRG (Form-1) or human purified HRG protein as the calibrator.
*1 Standard protein: purified from Human plasma.
*2 Standard protein: Recombinant HRG (Form-1).

4.2. Reconstruction of new ELISA measurement system as an IVD suitable for daily clinical care

We focused on the dilution step, which is common to all measurement procedures, as the possible cause of the variability. Thus, we attempted to add the same antibody used for immobilization to the reaction system because it was hypothesized that it could change the measurement range by suppressing the reaction rate between the antigen and the antibody immobilized on the plate via the simultaneous occurrence of a competitive reaction with the capture antibody in the reaction system. Our new methodology was designed to shift the measurement to a higher concentration range in which the same antibody (75-2D) as the immobilized one was added into the reaction buffer in respective wells with the intention of controlling the reaction between HRG (antigen) in the sample and the immobilized antibody. As a result, this method extends the upper measurement limit from 0.05 μg/mL to 2.5 μg/mL, thereby enabling accurate quantification of higher HRG concentrations. It has been reported that the HRG concentration in healthy plasma is approximately 100–150 μg/mL (Poon et al., 2011). Furthermore, our trials indicated that actual clinical samples (from patients with sepsis and healthy individuals) had plasma HRG concentration ranges of 10–200 μg/mL. If plasma HRG measurement were performed using the previous ELISA method, plasma samples have to be diluted several hundred to several thousand times before the measurement process. In this method, a 101-fold dilution procedure is adopted, and the LOD in the assay solution was 0.16 μg/mL under the tested conditions. Since clinical plasma samples are diluted 101-fold prior to measurement, the corresponding practical lower measurable concentration for plasma HRG is approximately 16 μg/mL (0.16 μg/mL x 101), and thus the measurement range of 16–250 μg/mL directly reflects the plasma HRG concentrations in clinical samples. This range is designed to cover the expected plasma HRG concentrations from healthy individuals to patients at various stages of sepsis. Furthermore, compared with the previous ELISA method, because the sample dilution ratio decreased from 2500 to 101, which reduced the dilution ratio to 1/25, it is also expected to contribute significantly to reducing the variability in sample measurement values due to dilution. As expected, this enabled measurement over a higher concentration range without repeated dilution steps, thereby reducing the risk of dilution-related error. In this method, the HRG antigen in the sample reacted competitively with the solid-phase antibody and the free antibody in the antibody-containing buffer. As a result, even when high concentrations of antigen were present in the sample, the solid-phase antibody was not consumed completely; therefore, absorbance values in the high-concentration range were obtained. On the other hand, a decrease in the dilution rate of the plasma sample can actually enhance the inhibitory effect that the plasma matrix (i.e., interfering substances) has on the antigen-antibody reactions in ELISA systems. It is highly preferable for an ELISA HRG system to avoid any effects from such interfering substances because, as commonly known, plasma samples can frequently contain some, especially in severe sepsis cases. Therefore, the HRG measurement was assessed using typical interfering substances, free bilirubin, conjugated bilirubin, hemolyzed hemoglobin, and chyle. These trials were performed using the commercially available Interference Check A Plus kit (Sysmex). It was found that the effects of the interfering substances converged within 10% or less of the theoretical measurement value according to the results of the reactions in the presence of free bilirubin (0–20 mg/dL), conjugated bilirubin (0–16 mg/dL), hemolyzed hemoglobin (0–500 mg/dL), and chyle (0–1700 FTU). Only one sample containing a specifically high concentration of conjugated bilirubin (20 mg/dL) gave 116% of the theoretical measurement value.

Because the prespecified acceptance criterion was set at 100 ± 20% of the corresponding blank condition, all values observed in this study fell within the acceptable range under the test conditions. These experiments indicated that the newly constructed ELISA system performed acceptably even in the presence of the evaluated interfering substances, supporting its potential as an IVD for measuring plasma HRG in patients typically encountered in daily clinical practice.

These results suggest that the newly constructed ELISA system was reasonably tolerant to the tested interfering substances under the present experimental conditions. However, because these experiments were

conducted using pooled healthy plasma, further evaluation in clinically complex specimens might be required before broader conclusions regarding routine clinical or IVD applicability can be made.

4.3. Selection of a single-isoform recombinant HRG as a candidate calibrator material

Next, we attempted to adopt recombinant HRG protein instead of purified HRG from human plasma, which was used in our previous report, as a candidate calibrator for the measurement. It is widely known that human HRG is a highly polymorphic protein for which many mutations have been reported. In the HRG protein structure, there are five mutation sites with a minor allele frequency greater than 10% (Zou et al., 2023). As major HRG group isoforms, two HRG isoforms (wild type and its mutant) were previously reported. The former (HRG Form-1: 75 kDa) has Pro-residue at position 204 of the amino acid sequence, and the latter (HRG Form-2: 77 kDa) has Ser-residue at the same position. This Pro-Ser exchange at position 204, which can affect the structure and function of HRG, is frequently observed. Furthermore, it has been reported that N-glycosylation proceeds easily at Ser-204. (Herrmann et al., 1998). Although these isoforms can be separated by SDS-PAGE because the difference in glycosylation gives a respective molecular weight, there is no significant difference in physical properties, which makes a crude mixture of Form-1 and Form-2 difficult to separate into each protein with high purity using conventional methods. Numerous studies quantifying plasma HRG have been conducted based on calibrator materials composed of HRG protein purified from human plasma (Kuroda et al., 2018; Kuroda et al., 2021). However, from the viewpoint of analytical chemistry, it is not always preferable to adopt mixtures as a calibrator material. In addition, when using standard substances of human origin, differences in the glycosylation level on the principal chain of the HRG protein among patients should be taken into consideration (Zou et al., 2024). Subsequently, the reactivity of respective isoforms was examined with our new ELISA method. No difference in reactivity was found compared with the results afforded by the dilution linearity test of both isoforms because the same curve was constructed by Form-1 and Form-2. Provided that the recognition site of the antibody overlaps with the mutation site of the antigen, this could lead to discrepancies between the measurement value and actual HRG content when an inhibition due to glycosylation occurs. Our new ELISA method has no vulnerability to genetic HRG polymorphisms and is capable of measuring both isoforms without any difference in sensitivity because no such hindrance by glycosylation was observed. The new ELISA system reacts equally to Form-1 and Form-2, meaning either one of the isoforms can be used as a candidate calibrator material. As described above, the adoption of a single-isoform candidate calibrator material (Form-1 or Form-2) is rational for an IVD because the reproducibility of the measurement value is free of influence by the isoform ratio. Such a protein can be produced recombinantly with stable yield and availability. However, the HRG protein glycosylation level is also an important factor for selecting the most appropriate HRG isoform as a candidate calibrator material. In other words, a substance with fewer glycosylated sites is preferable as a candidate calibrator material because it reduces the probability of reaction inhibition. Accordingly, Form-1 of HRG -which carries a proline residue at amino acid position 204 and therefore lacks an N-glycosylation consensus sequence- was considered suitable as a candidate calibrator material. Form-1 of the HRG protein is the isoform predominantly found in East Asia (Zou et al., 2023). Accordingly, we plan to use recombinant Form-1 HRG as the candidate calibrator material in future studies of plasma HRG in sepsis. As shown in Fig. 7, HRG concentrations were measured in eight EDTA-2 K plasma samples using the same ELISA method, calibrated with either purified human HRG protein or recombinant protein. Although a strong, significant correlation was observed between the purified human HRG protein and recombinant protein, the measured HRG values differed. Three quantitative whole protein analysis methods, the Bradford method, BCA method, and absorptiometry at 280 nm -were examined to assay the HRG protein purified from human plasma at distinctly different values: 2.5, 1.3, and 1.7 μg/mL, respectively. HRG's high histidine content makes these assays susceptible to composition-dependent bias, and the fact that the plasma-derived standard is a mixture of Form-1 and Form-2 with an unknown molar ratio further undermines the accuracy of concentration assessment. In addition, trace nickel potentially eluted during Ni-NTA purification could affect BCA measurements and must be considered (Sprules et al., 1998). It is generally known that the Bradford method is compatible with reducing agents, salt, and so on (Chutipongtanate et al., 2012). Based on these points, we provisionally used the Bradford value in early experiments; however, we judged that assigning molar concentration by amino acid analysis of a single, sequence-known recombinant isoform (Form-1) is preferable from the perspectives of traceability and reproducibility. Even though the amino acid analysis of Form-1 of HRG gave a concentration of 1.43 mg/mL, the BCA method resulted in a concentration of 0.90 mg/mL. Additionally, the analysis of the 280 nm absorbance examination calculated by the absorbance index based on its amino acid sequence gave a different concentration (1.77 mg/mL). As expected, assay method dependent discrepancies were identified. It was necessary to consider carefully any bias in the amino acid content of HRG protein in terms of the protein concentration measurement. Accordingly, in the case that the same sample was measured for HRG protein using the same ELISA method and both purified human HRG and recombinant proteins as calibrators, respectively, the differences in values might be due to the differences in measurement methods for protein concentration, rather than differences due to the reactivity of the HRG protein in the measurement system. The Bradford, BCA, and A280 assays are susceptible to composition-, metal-, and reagent-dependent biases. For traceability, it is therefore preferable to adopt quantitative amino acid analysis as the primary reference method: complete acid hydrolysis followed by quantitative amino acid determination permits direct conversion to the protein's molar amount, enabling traceable assignment in both μmol/mL and mg/mL when the sequence is known. We subsequently compared the measurement values afforded by using two types of calibrator material. The results indicated that the value (x) based on the human purified HRG protein (a mixture of Form-1 and Form-2) could easily be converted to the one (y) based on the recombinant protein, Form-1, through calculation with a simple equation ($y = 0.7932\times - 19.319$), even though there was a small discrepancy between the two measurements. As a result, the new reference standard, recombinant Form-1, which is a versatile and easily accessible substance with stable quality, was successfully established. As such, we decided to start exploring sepsis studies based on plasma HRG using the recombinant protein, Form-1 of HRG, as the reference standard henceforward.

Our data support the use of recombinant Form-1 as a practical candidate calibrator material for the present assay system. This conclusion is based on its comparable assay reactivity, favorable dilution linearity, single-isoform composition, and concentration assignment by amino acid analysis. However, broader qualification will require additional investigation of long-term stability, lot-to-lot consistency, transferability across laboratories, and comparability with native HRG under a wider range of analytical conditions.

There are several limitations in this study. First, the analytical validation was performed on a relatively small scale since the number of clinical samples was limited. Next, precision evaluation was based on only two pooled plasma materials and did not always cover predefined low, medium, and high HRG concentration levels. And on the interference study, it may not fully represent the matrix complexity of clinical sepsis due to our experiments conducted by using pooled healthy plasma. Even our study showed the optimized ELISA assay method and recombinant calibrator strategy promising analytical performance, clinical performance evaluation for development of in vitro diagnostics and broader inter-laboratory standardization of measurement value were beyond the scope in the present study. These points should be

addressed in future studies before routine clinical use.

5. Conclusion

In conclusion, we developed an optimized ELISA method for plasma HRG measurement that reduced the sample dilution requirement and expanded the practical measurement range to 16–250 μg/mL in plasma. Furthermore, it improved reproducibility compared with our previous ELISA. We also showed that recombinant single-isoform HRG, particularly Form-1, is a practical candidate calibrator material for this assay system. These findings provide a methodological basis for more consistent HRG measurement and for future analytical standardization and clinical validation in sepsis-related applications.

CRediT authorship contribution statement

Takaoki Uchiumi: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ken-ichi Shimosako:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Masahiro Nishibori:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Hiroshi Morimatsu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Masahiko Oiwa:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Yoko Inoue:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Hiroshi Nishi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Norio Ota:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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Data availability

The data that has been used is confidential.

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