Protocol Article

Blood serum analysis: A modified sandwich enzyme-linked immunosorbent assay protocol

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A B S T R A C T

Blood serum analysis is a versatile tool used in diagnostics, in vivo research, and clinical studies. Enzyme-linked immunosorbent assay (ELISA) is a common method used to analyze blood serum cytokine levels; however, commercial kits are costly and not always available for novel or uncommon targets. Here we present a modified ELISA protocol that, once standardized, can be used to measure blood serum levels of any target and minimize the expense of commercial kits. Additionally, this method can be used for novel or unique targets for which commercial options are unavailable. Ultimately, the modified ELISA method is an efficient, cost-effective method of supplementing clinical and in vivo studies with consistently reliable serum cytokine measurements.

A R T I C L E   I N F O
Method name: Modified sandwich-ELISA  
Keywords: Immunoassay, Plasma, Microplate

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Specifications table

| Subject area: | Biochemistry, Genetics and Molecular Biology |
|---------------|---------------------------------------------|
| More specific subject area: | Immunoassays, ELISAs, plate reader protocols |
| Name of your protocol: | Modified sandwich-ELISA |
| Reagents/tools: | Poly-D-lysine, MES Buffer, EDC, Sulfo-NHS, PBS, PBST, HRP, TMB, Sulfuric Acid, Microplate Reader |
| Experimental design: | Blood serum TNF-a levels in control vs 4-week AngII-induced AAA were analyzed and compared to published data to confirm validity of modified ELISA method. |
| Trial registration: | University of Missouri IACUC, University of Missouri IRB |
| Ethics: | Consistently produces reliable and quality blood serum analysis data |
| Value of the Protocol: | Minimizes cost of commercial ELISA kits |
|                  | Can be used to measure a wide range of blood serum cytokines |
|                  | Protocol can be completed in 1-2 days. |

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Introduction

Serum cytokine levels are powerful markers that can provide insight into the progression of diseases such as cardiovascular disease, cancer, and infection. During in vivo studies, analysis of serum cytokines allows for broader understandings of the systemic trends of disease and facilitates the development of diagnostic tools. For example, Tong et al recently established serum surfactant protein D as a new marker for COVID-19 disease severity [1].

Immunoblotting assays are generally unsuitable for analyzing serum protein levels due to the interference of serum albumin; therefore commercial sandwich ELISA methods, in which unique capture and detection antibodies form a ‘sandwich’ to trap the target factor, are widely cited as reliable and highly sensitive methods of serum cytokine analysis [2,3]. However, in addition to their high cost, commercial kits are not available for all targets and customized ELISA reagents take significant time to optimize. Therefore, we have developed a modified sandwich ELISA protocol for serum analysis that uses covalent antigen attachment to minimize steric hindrance between layers and to allow a single antibody to function as both the capture and detection antibody. Ultimately, our modified ELISA protocol maintains high specificity and is easily customizable with minimal requirement for additional optimization, thereby eliminating the need for highly customized or commercial ELISA kits.

Biochemical modifications

Conventional sandwich ELISAs rely on passive adsorption of a capture antibody to the microplate surface, a physical process which allows variability in antibody orientation as well as non-specific adsorption during additional coating steps. As a result, conventional sandwich ELISAs are subject to capture antibody desorption, steric hindrance between layers, and require two customized primary antibodies to prevent non-specific bioconjugation with exposed capture antibody Fc regions during the detection step [4] (Fig. 1A). To address these drawbacks, we utilized a bioconjugation step in which 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) functions as a crosslinking molecule between a poly-D-lysine pre-coat and the capture antibody (Fig. 1B). This chemical process anchors the capture antibody at the Fc region to prevent non-specific binding of additional layers and increases stability and specificity during antigen detection.

EDC is commonly used in bioconjugation reactions due to its small size and stability after crosslinking is complete. As such, EDC can be utilized as a crosslinking factor without sterically interfering with target capture or detection. For our modified ELISA plate coating method, we also incorporate sulfo-N-hydroxysuccinimide (sulfo-NHS) in the bioconjugation reaction in order to improve EDC crosslinking efficiency, stabilize amine-reactive intermediates, and to prevent additional crosslinking reactions during further coating steps (see full reaction in Fig. 1C). Ultimately, by covalently crosslinking pre-coated poly-D-lysine with the target cytokine’s capture antibody, the EDC/sulfo-NHS bioconjugation step allows us to utilize a single primary antibody for both the capture and detection layers while avoiding steric hindrance and maintaining specificity during the conjugate binding step.

Method details

Materials

Reagents
- Poly-D-Lysine
- Phosphate-buffered saline (PBS) and PBST (0.1% Tween20 in PBS)
- Coupling Buffer (0.05M 2-ethanesulfonic acid [MES] in diH2O, pH 5.0)
- Storage Buffer (0.01% Tween20, 0.05% sodium azide (NaN3), 0.1% bovine serum albumin [BSA] in PBS)
Fig. 1. Conventional vs modified ELISA methods. 1A illustrates the layers of a conventional sandwich ELISA in which the capture antibody is passively adsorbed at variable orientations onto the plate surface and a custom detection antibody is needed to maintain antigen-specific bioconjugation. 1B illustrates the layers of our modified sandwich ELISA protocol, where an antigen–capture layer is formed by establishing a covalent crosslink between the primary antibody Fc region and the electrostatically adsorbed poly-D-lysine pre-coat. The same primary antibody can then be used to form a detection layer to which an HRP-conjugate will bind without jeopardizing ELISA specificity. Finally, 1C illustrates the biochemical reaction that allows EDC, in conjunction with sulfo-NHS, to act as a covalent crosslink between poly-D-lysine and the primary antibody. First, EDC reacts with a carboxylic acid group on poly-D-lysine, forming an amine-reactive O-acylisourea intermediate. This intermediate is unstable in aqueous solutions and failure to immediately react with an amine will result in hydrolysis of the intermediate. Therefore, the addition of sulfo-NHS stabilizes the amine-reactive intermediate by coupling to EDC carboxyls producing an amine-reactive sulfo-NHS ester. When a primary antibody is added, the amine-reactive sulfo-NHS ester spontaneously reacts with the primary amines in the Fc region of the antibody to form a stable amide bond and an isourea by-product.
• EDC and sulfo-NHS (50 mg/mL in coupling buffer)
• Blocking Buffer (1% BSA in PBS)
• 3,3',5,5'-Tetramethylbenzidine (TMB)
• 0.18M sulfuric acid (H2SO4)
• Target-specific monoclonal or polyclonal antibody (primary antibody); 1:10 dilution in coupling buffer; 1:100 dilution in PBS
• Horseradish peroxidase (HRP) conjugate (secondary antibody); 1:5000 dilution in PBS

Materials and Equipment
• Polystyrene microplate
• Microplate fluorescent reader
• Aluminum foil to protect microplate from light

Procedure

1) Platepre-coating (Overnight)
   a) Add 20-50 uL of poly-D-lysine, or any cationic extracellular matrix, to desired wells in a 96-well culture plate. Ensure that the entirety of each well surface is coated.
   b) Allow plate to sit under a fan or vacuum for 12-24 hours at room temperature, or until coating is completely evaporated. After 12 hours, excess coating can be aspirated.
   c) Gently wash wells with 100 uL sterile PBS. Do not directly flush the well surface. Allow wells to sit in PBS for 5 minutes at room temperature. Aspirate PBS without touching well surface before the next step.
   d) Repeat PBS wash in step 1(c) twice more. At this stage, plate can be stored at 4 degrees Celsius in PBS and covered to prevent evaporation.

2) Crosslinking reaction (40 - 60 minutes)
   a) Gently wash wells with 100 uL coupling buffer four times, allowing wells to incubate in solution for 5 minutes at room temperature between each wash.
   b) Proceed with EDC coupling reaction: (CRITICAL STEP)
      i) Add 80 uL coupling buffer to each well.
      ii) Add 10 uL EDC (50 ug/uL) and 10 uL sulfo-NHS (50 ug/uL) to each well.
      iii) Keep plate covered and protected from light at room temperature for 20 minutes on a gentle rocker. Proceed immediately to the next step.

3) Antibody coating and blocking (3 - 4 hours)
   a) Add 20 uL coupling buffer and 2 uL undiluted target primary monoclonal antibody to each well. Incubate plate covered and protected from light on a rocker for 2 hours room temperature, or at 4 degrees Celsius overnight.
   b) Repeat coupling buffer wash in step 2(a) four times.
      i) Plate can be stored at 4 degrees Celsius in storage buffer at this step.
   c) Add 100 uL blocking buffer to each well and incubate plate (covered and protected from light) at room temperature on a gentle rocker for 1 hour.
   d) Repeat PBS wash in step 1(c) three times. Proceed to next step immediately following the final wash.

4) Target capture (2 - 3 hours)
   a) Add 20-40 uL of serum sample (1:10 dilution in PBS) to each well. Incubate wells (covered and protected from light) on a gentle rocker for 1 hour at room temperature or at 4 degrees Celsius overnight.
   b) Repeat PBS wash in step 1(c) 2 times.
   c) Add 50-100 uL primary antibody (1:100 dilution in PBS) to each well. Incubate plate (covered and protected from light) on a gentle rocker for 1 hour at room temperature.
   d) Repeat PBS wash in step 1(c) 3 times and then proceed immediately to next step.

5) Conjugationand measurement (1.5 - 2 hours)
   a) Add 30-50 uL of freshly prepared secondary antibody (1:5000 dilution in PBS) to each well. Incubate plate (covered and protected from light) with gentle rocking for 1 hour at room temperature (CRITICAL STEP).
b) Repeat PBS wash in step 1(c) 4 times (CRITICAL STEP).

c) Add 20-50 μL TMB to each well. Incubate plate covered and in a dark room for 30 minutes at room temperature.

d) To stop the TMB reaction, add 20-50 μL of 0.18 M H₂SO₄ to each well. The amount of H₂SO₄ added at this step should be equal to the amount of TMB added in step 5(c). Proceed immediately to next step.

e) Measure absorbance at 450nm using a plate reader. Cytokine levels can be reported as fold-change values relative to a control group or as exact concentration values based on a recombinant protein standard curve.

Troubleshooting

Crosslinking reaction: Always utilize freshly prepared EDC and sulfo-NHS solutions. Ensure that these solutions are kept in the dark prior to use, and that the 96-well microplate is protected from light for the duration of the reaction. If the quality of the crosslinking reagents or the stability of the crosslinking reaction is in question, the researcher may use the bicinchoninic acid (BCA) protein assay to verify the crosslink reaction after step 3. If the reaction mixture absorbs strongly at 562 nm after the assay is complete, then the crosslink reaction was successful [5,6]. After completing the BCA assay, the researcher may gently wash the wells with PBS 3x and proceed to step 4 normally.

Target capture: We recommend optimizing serum sample dilutions. If the target is typically observed at high concentrations in serum, consider increasing the sample dilution during the target capture step. If the specificity of the primary antibody is in question, we recommend validating the antibody using western blot assay. If non-specific binding is observed, consider increasing the duration of the blocking step to minimize blood albumin interference.

Conjugation: We recommend optimizing the secondary antibody dilution before utilizing this protocol. Additionally, always make sure that the secondary antibody dilution is freshly prepared no more than one hour prior to use. If non-specific binding is observed or the signal is saturated, try (a) rewashing each well with PBST three times for five minutes and repeating the TMB reaction or (b) stripping the HRP conjugate and repeating step 5 using a different secondary antibody dilution.

Method validation

To establish the validity of this immunoassay we analyzed TNF-α levels in human and mouse blood serum samples from subjects with and without abdominal aortic aneurysm (AAA). The AAA model, blood serum extraction protocol, and clinical protocols are detailed in a previous publication [7]. Two TNF-α monoclonal antibodies, Mouse anti-TNF-α (Cat No.: 602291-1-Ig, ProteinTech, Rosemont, IL) and rabbit anti-TNF-α XP (Cat No: D2D4, Cell Signaling Technology, Danvers, MA), were used as primary antibodies for the mouse and human serum samples, respectively. TNF-α serum concentrations were determined using a standard curve generated for both antibodies using recombinant human TNF-α (Cat No: 210-TA, R&D Systems, Minneapolis, MN) (Fig. 2C). HRP-conjugates for mouse and rabbit IgG were acquired from ProteinTech. Statistical analyses were conducted using Microsoft Excel’s t-test: paired two sample for means.

Fig. 2 illustrates the average TNF-α concentration (ng/μL) detected by our modified plate reader method in AAA compared to control human and mouse blood serum samples, where two samples from each group were analyzed in triplicates and average cytokine concentration value was calculated for each sample. In mouse serum, TNF-α levels in mice with late-stage AAA were significantly lower than those in control mice, with a relative fold change of 0.925 (Fig. 2A). In human serum samples, TNF-α levels in symptomatic AAA patients appear lower than those in control mice, with a relative fold change of 0.997 (Fig. 2B). These data are consistent with previous findings that TNF-α levels decrease in human serum during late-stage and symptomatic AAA compared to controls [7–9]. Thus, we conclude that our modified ELISA protocol is a reliable method of analyzing blood serum cytokine levels in clinical and in vivo studies.
Fig. 2. Method validation. Reliability of our modified ELISA protocol was tested by analyzing murine blood serum TNF-α levels from control versus four-week AngⅡ-induced abdominal aortic aneurysm (AAA) subjects. Standard curve was measured using recombinant TNF-α at 0, 2.5, 5, and 10 ng/ul and calculated using Microsoft Excel (2C). Statistical analysis of blood serum data was conducted using Excel’s t-test: paired two sample for means. N=2 and all samples were run in duplicate and averaged before analyzed for significance. 2A shows the TNF-α levels in control versus AAA mouse serum. AAA mice exhibited significantly lower TNF-α levels than control mice, with a fold-change of 0.925 and p = 0.045. 2B illustrates the TNF-α levels in control versus AAA human serum. Serum TNF-α levels in patients with AAA appear lower than serum TNF-α levels in patients without AAA, with a relative fold change of 0.997 and p = 0.44.
Conclusion

While blood serum analysis has gained widespread relevance in clinical diagnostics and translational research, cytokine detection is limited by the need for expensive, highly customized analysis methods such as conventional ELISAs, mass spectrometry, and flow cytometry. Our modified ELISA protocol is an affordable method of serum cytokine analysis which can be used for any target for which a primary antibody is available with minimal need for re-optimizations. Furthermore, although our protocol does take more time to complete than commercial ELISA kits, the accessibility and versatility of this modified ELISA method is ideal for analyzing uncommon or novel targets for which commercial kits are unavailable or too costly. Ultimately, our modified ELISA protocol represents an accessible method of serum cytokine analysis that allows a diverse range of targets to be measured with high specificity and minimal variability.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

References

[1] M. Tong, Y. Xiong, C. Zhu, Q. Zheng, Y. Jiang, L. Zou, X. Xiao, F. Chen, X. Yan, C. Hu, Y. Zhu, Serum surfactant protein D in COVID-19 is elevated and correlated with disease severity, BMC Infect. Dis. 2021 (21) (2021) 737.
[2] R. Mende, F.B. Vincent, R. Kandane-Rathnayake, R. Koelmeyer, E. Lin, J. Chang, A.Y. Hoi, E.F. Morand, J. Harris, T. Lang, Analysis of serum interleukin (IL)-1B and IL-18 in systemic lupus erythematosus, Front. Immunol. 9 (2018) (2018) 1250.
[3] J.W. Song, K.H. Do, S.J. Jang, T.B. Colby, S. Han, D.S. Kim, Blood biomarkers MMP-7 and SP-A, Chest 143 (5) (2012) 1422–1429.
[4] Crowther, J. R. (2009). Methods in Molecular Biology: The ELISA Guidebook (J. M. Walker, Ed.). Humana Press. doi:10.1007/978-1-60327-254-4.
[5] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, et al., Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[6] K. Wiechelman, R. Braun, J. Fitzpatrick, Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation, Anal. Biochem. 175 (1988) 231–237.
[7] D. Cai, C. Sun, G. Zhang, X. Que, K. Fujise, N.L. Weintraub, S. Chen, A novel mechanism underlying inflammatory smooth muscle phenotype in abdominal aortic aneurysm, Circ. Res. 129 (10) (2021) e202–e214.
[8] V Treska, O Topolcan, J Kocová, J Moláček, K Houdek, Z Tonar, J Vrzalová, I Tresková, V Krížková, L. Boudová, Plasmatic levels of proinflammatory cytokines in abdominal aortic aneurysms, Rozhl. Chir. 90 (1) (2011) 37–41.
[9] V. Treska, O. Topolcan, L. Pecen, Cytokines as plasma markers of abdominal aortic aneurysm, Clin. Chem. Lab. Med. 38 (11) (2000) 1161–1164.