A rapid one-step kinetics-based immunoassay procedure for the highly-sensitive detection of C-reactive protein

Sandeep Kumar Vashist (sandeep.vashist@yahoo.com)
HSG-IMIT - Institut für Mikro- und Informationstechnik; Laboratory for MEMS Applications, Department of Microsystems Engineering -IMTEK, University of Freiburg, Georges-Koehler-Allee 103, 79110 Freiburg, Germany

Gregor Czilwik
HSG-IMIT - Institut für Mikro- und Informationstechnik, Georges-Koehler-Allee 103, 79110 Freiburg, Germany

Thomas van Oordt
HSG-IMIT - Institut für Mikro- und Informationstechnik, Georges-Koehler-Allee 103, 79110 Freiburg, Germany

Felix von Stetten
HSG-IMIT - Institut für Mikro- und Informationstechnik; Laboratory for MEMS Applications, Department of Microsystems Engineering -IMTEK, University of Freiburg, Georges-Koehler-Allee 103, 79110 Freiburg, Germany

Roland Zengerle
HSG-IMIT - Institut für Mikro- und Informationstechnik; Laboratory for MEMS Applications, Department of Microsystems Engineering -IMTEK, University of Freiburg, Georges-Koehler-Allee 103, 79110 Freiburg, Germany

E. Marion Schneider
Sektion Experimentelle Anaesthesiologie, University Hospital Ulm, Albert Einstein Allee 23, 89081 Ulm, Germany

John H.T. Luong
Innovative Chromatography Group, Irish Separation Science Cluster (ISSC), Department of Chemistry and Analytical, Biological Chemistry Research Facility (ABCRF), University College Cork, Cork, Ireland

---

Method Article

Keywords: One-step kinetics, immunoassay, C-reactive protein, Dynabeads, in vitro diagnostics

Posted Date: May 27th, 2014

DOI: https://doi.org/10.1038/protex.2014.017

Abstract

A rapid one-step kinetics-based sandwich enzyme-linked immunosorbent \(ELISA) procedure has been developed for highly-sensitive detection of C-reactive protein \(CRP) in less than 30 min. With minimal process steps, the procedure is highly simplified and cost-effective. The analysis only involves sequentially the formation of a sandwich immune complex on capture anti-CRP antibody \(Ab)-bound Dynabeads, followed by two magnet-assisted washings and an enzymatic reaction. The developed immunoassay \(IA) detected CRP in the dynamic range of 0.3-81 ng mL^{-1} with a limit of detection and analytical sensitivity of 0.4 ng mL^{-1} and 0.7 ng mL^{-1}. Its analytical precision for analysis of CRP spiked in diluted human serum, whole blood, and ethylenediaminetetraacetic acid \(EDTA) plasma samples of patients was validated by conventional ELISA, unravelling its immense potential for _in vitro_ diagnostics \(IVD).

Introduction

As the gold standard for the detection of CRP in clinical diagnostics, ELISA exhibits high-throughput, excellent reproducibility, high precision and remarkable sensitivity. Last five decades have accumulated over 300,000 peer-reviewed articles related to ELISA. In particular, the last decade has also witnessed considerable advances in diversified CRP assay formats[1-22], lateral flow[20], immunoturbidimetry[1,11], surface plasmon resonance[14], piezoresistive cantilever-based IA[10], chemiluminescent IA[3], impedimetry[23], electrochemistry[17], reflectometric interference spectroscopy[19], microfluidics[7] and homogenous bead-based IA[24]. In general, most of these assay formats are based on a complex procedure comprising of many process steps with a lengthy analysis time[25-28]. Therefore, there is uttermost importance for the development of rapid and cost-effective IA procedures with high precision and minimal process steps to guide healthcare professionals to decide on the desired intervention at an early stage. CRP, a pentameric protein with a molecular weight of 118 kDa, is a member of a class of acute-phase reactants indicating activation of innate and adaptive immunity[13,29,30]. It plays an important role in host defense by binding to phosphocholine and related microbial molecules. As an early indicator of infectious or inflammatory conditions[31,32], CRP is usually elevated in patients with neonatal sepsis[33-35], meningitis, pancreatitis, pneumonia and pelvic inflammatory disease and occult bacteremia. The significantly elevated serum CRP levels are associated with malignant diseases, bacterial infections and correlate with increased 30-day mortality rates in hospitalized patients[36]. The monomeric CRP binds to the surface of damaged cells and platelets, thereby activating the complement cascade that plays an important role in inflammation. The American Heart Association/Center for Disease Control has considered CRP as the best inflammatory marker for clinical diagnosis[37]. The precise and rapid determination of human C-reactive protein \(CRP) is essential for diagnosis and management of neonatal sepsis[33-35,38,39], cardiovascular diseases[40-44], infectious/inflammatory conditions[31] and diabetes[45-47]. The ability for repeated CRP measurements with high precision in an acute setting provides clinicians with the valuable information to assess disease diagnosis and circumvent any unnecessary administration of antibiotics. The normal

CRP levels in human serum are usually below 10 µg mL^{-1} [30] but up to 350-400 µg mL^{-1} in several disease states. The CRP levels in the ranges of 10-40 µg mL^{-1}, 40-200 µg mL^{-1}, and >200 µg mL^{-1} are the indicators of mild or chronic inflammation and viral infections; acute inflammation and bacterial infections; and severe bacterial infections and burns, respectively[30]. The CRP levels beyond the cut-off point of 5 µg mL^{-1} are indicative of neonatal sepsis, which is diagnosed based on the determination of two CRP concentration ranges normal \(0.2-480 µg mL^{-1}) and high sensitivity \(0.08-80 µg mL^{-1})[30]. The high sensitivity CRP assay is performed first, but the normal CRP assay is also performed if the CRP levels are >80 µg mL^{-1}. The existing analytical techniques for the determination of CRP have limitations in terms of prolonged IA duration and lower analytical sensitivity, as reviewed recently by Algarra _et al._[22]. The clinical laboratory-based CRP assays, based on latex agglutination or nephelometry, and phosphocholine and O-phosphorylethanolamine based immunoassays can detect CRP only in the detection range of µg mL^{-1}. On the other hand, the recently developed immunoassay formats, based on electrochemical techniques, nanoparticles, nanocomposites, chemiluminescence, total internal reflection and micromosiac immunoassays, have higher sensitivities in the range of ng mL^{-1} to µg mL^{-1}. Similarly, the surface plasmon resonance based real-time and label-free immunoassay formats have sensitivity between ng mL^{-1} to g mL^{-1}. Therefore, there is a critical need for a highly simplified, cost-effective, precise and highly sensitive IA format for the rapid detection of CRP. We have developed a rapid one-step kinetics-based sandwich ELISA procedure[48] \(**Figure 1**) to detect CRP in human whole blood and serum in less than 30 min. It has critically reduced the IA duration by more than 12-fold and the analysis cost by 2.5-fold in comparison to the conventional procedure. This novel ELISA format required also significantly reduced number of process steps and only two washing steps in comparison to the conventional counterpart \(**Table S1**). Moreover, the high analytical precision of the developed procedure implies its tremendous potential for rapid analyte detection in clinical and bioanalytical settings.

Reagents

• Human CRP Duoset kit \(R & D Systems, UK, cat. no. DY1707E) **\!CAUTION** Store reconstituted Ab and antigen at 2-8 °C, if they are used within a month. Otherwise, make aliquots and store at -20 °C to -70 °C for up to 6 months. The kit comprises of • Mouse anti-human CRP capture Ab \(360 µg mL^{-1}) • Recombinant human CRP \(90 ng mL^{-1}) • Biotinylated mouse anti-human CRP detection Ab \(22.5 µg mL^{-1}) • Streptavidin-conjugated horseradish peroxidase \(SA-HRP) **\!CAUTION** Do not freeze. Store in the dark as streptavidin is light-sensitive. The Human CRP Duoset kit's components can also be procured separately, i.e. human CRP antibody \(cat. no. MAB17071), human CRP biotinylated antibody \(cat. no. BAM17072) and recombinant human CRP \(cat. no. 1707-CR). • Blocker BSA in PBS \(10X), pH 7.4, 10% \(w/v) \(Thermo Scientific, Ireland, cat. no. 37525) **CRITICAL** Filter with 0.2 µm pore size filter paper prior to use to avoid contamination. • Dynabeads® M-280 Tosylactivated \(Thermo Fisher Scientific, Ireland) • Sulfuric acid \(Aldrich, cat. no. 339741) **\!CAUTION** Avoid skin contact as it is a strongly corrosive agent and an irritant. Use personal protective equipment \(PPE), such as chemical safety glasses, chemical-resistant shoes and lab coats, for handling. Handle only in a fume cabinet. In case of

skin contact, wash immediately with acid neutralizers and seek medical advice as soon as possible. • BupH Phosphate Buffered Saline Packs \(0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.2) \(Thermo Scientific, Ireland, cat. no. 18372) **\!CAUTION** Avoid inhalation. **CRITICAL** Prepare in autoclaved DIW \(18Ω), see **REAGENT SETUP**. • TMB substrate kit \(Thermo Scientific, Ireland, cat. no. 34021) • TMB solution \(0.4 g L^{-1}) **\!CAUTION** Skin, eye and lung irritant. In case of skin contact, wash with plenty of water. **CRITICAL** The TMB to peroxide ratio is critical for color development. Maintain the ratio 1:1. • Hydrogen peroxide solution \(containing 0.02 % v/v H_2O_2 in citric acid buffer) \(Thermo Scientific, Ireland). **\!CAUTION** Strong oxidizing agent. Harmful if swallowed. Severe risk of damage to eyes. Rinse immediately with plenty of water in case of contact and seek medical attention. Wear suitable protection and work in a safety cabinet or fume cupboard. • Human whole blood \(HQ-Chex level 2, cat. no. 232754, Streck) 180 day closed-vial stability and 30 day open-vial stability. • Human serum \(CRP free serum, cat. no. 8CFS, HyTest Ltd., Finland) • Deionized water \(18 Ω, DIW). \(Direct-Q®3 Water Purification System, Millipore, USA) • Nunc microwell 96-well polystyrene plates, flat bottom \(non-treated), sterile \(Sigma Aldrich, cat. no. P7491) • Eppendorf microtubes \(1.5 mL; Sigma Aldrich, cat. no. Z606340) • Sigmaplot software version 11.2 from Systat **REAGENT SETUP** **PBS.** Add a BupH Phosphate Buffered Saline Pack to 100 mL of autoclaved DIW, dissolve well and make the volume up to 500 mL using autoclaved DIW. Each pack makes 500 mL of PBS at pH 7.2, which can be stored at RT for a week and at 4 ºC for up to four weeks. **Binding buffer.** 0.1% BSA in PBS, pH 7.2. **Washing buffer.** 0.05% Tween® 20 in PBS, pH 7.2. **Anti-CRP capture Ab-bound Dynabeads®.** The anti-CRP capture Ab was bound to the tosylated Dynabeads® using the standard immobilization procedure provided by the manufacturer \(Invitrogen). The prepared stock solution of anti-CRP capture Ab-bound Dynabeads® was then stored at 4 °C. **Biotinylated anti-CRP detection Ab conjugated to SA-HRP.** Biotinylated anti-CRP detection Ab conjugated to SA-HRP was prepared by adding 1 µL of biotinylated anti-CRP detection antibody \(0.5 mg mL^{-1}) to 1 µL of SA-HRP to 2998 µL of the binding buffer followed by 20 min of incubation at room temperature \(RT). As a result, the concentration of biotinylated anti-CRP detection Ab used was 0.17 µg mL^{-1}, while SA-HRP dilution employed was 1:3000.

Equipment

• -70 °C freezer \(operating range -60 to -86 °C) \(New Brunswick) • 2-8 °C refrigerator \(Future, UK) • Direct-Q® 3 water purification system \(Millipore, USA) • Tecan Infinite M200 Pro microplate reader \(Tecan, Austria GmBH) • Mini incubator \(Labnet Inc., UK) • Quadermagnet magnetic holder \(Supermagnete, Germany) • PVC fume cupboard Chemflow range \(CSC Ltd.)

Procedure

Preblocking TIMING ~ 30 min 1. Block the MTP wells by incubating with 300 µL of 5% \(w/v) BSA for 30 min a 37 °C and wash with 300 µL of wash buffer five times. Washing can also be performed with an automatic plate washer. The preblocking is essential to prevent non-specific binding of IA reagents to the MTP wells[49]. **CRITICAL STEP** Use filtered BSA or filter the BSA solution prior to use to remove any

microbial or other contaminants. **?TROUBLESHOOTING** **One-step kinetics-based CRP sandwich ELISA TIMING 30 min** 2. Dispense in the BSA-blocked MTP wells sequentially 2 µL of the diluted stock solution of anti-CRP capture Ab-bound Dynabeads® \(diluted 1:10 in binding buffer), 38 µL of the binding buffer and 40 µL of biotinylated anti-CRP detection Ab \(0.17 µg mL^{-1}) pre-conjugated to SA-HRP \(diluted 1:3000). Finally, dispense 40 µL of CRP \(varying concentrations; 0.3-81 ng mL^{-1}) in the respective MTP wells in triplicate. Place the MTP on the magnetic holder and incubate at 37 °C for 15 min so that the magnets capture the Dynabead®-bound sandwich immune complex. Take out the excess reagents by sucking back the solution using a 300 µL multi-channel pipette. **?TROUBLESHOOTING** 3. Wash the magnetically-captured sandwich immune complex-bound Dynabeads® twice by dispensing and sucking back 300 µL of the washing buffer using a 300 µL multi-channel pipette. Thereafter, suspend the washed magnetically-captured sandwich immune complex-bound Dynabeads® in 50 µL of the binding buffer. 4. Add 100 µL of the TMB-H_2O_2 mixture to each MTP well and incubate at RT for 4 min to allow the enzymatic reaction to develop color. **?TROUBLESHOOTING** 5. Stop the enzymatic reaction by adding 50 µL of 2N H_2SO_4 to each MTP well. 6. Measure the absorbance at a primary wavelength of 450 nm and 540 nm as the reference wavelength in the Tecan Infinite M200 Pro microplate reader. **CRITICAL STEP** Determine the absorbance within 10 min of stopping the enzymatic reaction.

Timing

Steps 1, Preblocking: 30 min Steps 2-6, One-step kinetics-based CRP sandwich ELISA: 30 min

Troubleshooting

Troubleshooting advice is provided in **Table 1**.

Anticipated Results

The developed CRP sandwich ELISA critically reduced the IA assay duration by 12-fold, from 6 h \(commercial CRP sandwich ELISA) to just 30 min, based on the use of Ab-bound Dynabeads®/MTPs. The developed ELISA is cost-effective and highly-simplified as attested by the minimal process steps and 2.5-fold reduced IA components in comparison to the conventional ELISA procedure \(**Tables S1, S2**). It detects 0.3-81.0 ng mL^{-1} of CRP with linearity in the range of 1-81 ng mL^{-1} \(**Figure 2A**). LOD, analytical sensitivity and correlation coefficient \(R^2) are determined to be 0.4 ng mL^{-1}, 0.7 ng mL^{-1}, and 0.998, respectively \(**Table S2**). The intraday and interday variability determined from five assay repeats \(in triplicate) in a single day and five consecutive days, respectively, are in the ranges of 0.7-10.8 and 1.6-11.2, respectively. The developed ELISA can detect the entire pathophysiological range of hsCRP from 3-80 µg mL^{-1} in human whole blood and serum after appropriate dilution, as demonstrated by the detection of CRP spiked in diluted whole blood and plasma \(**Figure 2A**). It has high specificity for CRP, as demonstrated by the use of various experimental process controls \(**Figure 2B**) that shows no detectable interference with the immunological reagents. The optimum duration for the formation of the

sandwich immune complex by the one-step kinetics-based procedure is 15 min, while the optimum number of magnet-assisted washings thereafter is only two \(**Figure S1**). The developed and conventional sandwich ELISAs have the same analytical precision for the detection of CRP in diluted whole blood and serum as the results are in agreement with each other \(**Table 2**). The percentage recovery for the CRP-spiked diluted human whole blood is in the range of 93.3-107.0 and 94.0-103.3 for the developed and conventional sandwich ELISAs, respectively. Similarly, the percentage recovery for CRP-spiked diluted human serum ranges from 103.3 -108.0, compared to 93.3-113.0 for the developed and conventional sandwich ELISAs, respectively. The results obtained for the detection of CRP in the EDTA plasma samples of patients by the developed ELISA are also in good agreement with those obtained by the conventional ELISA \(**Table 3**). The anti-CRP capture Ab-bound Dynabeads® can be stored for more than 4 months at 4 ºC without compromising the CRP detection response \(**Figure 2C**). The production variability for the preparation of anti-CRP capture Ab-bound Dynabeads®, using the same lots of Dynabeads® and anti-CRP capture Ab, is less than 3 percent \(**Figure 2D**). Moreover, the developed ELISA using SA-HRP/biotinylated anti-CRP Ab conjugate is similar to the variant of the developed ELISA procedure that employs the two-step binding of biotinylated anti-CRP Ab and SA-HRP \(**Figure S2A**). The one-step kinetics-based sandwich ELISA solution \(comprising anti-CRP capture Ab-bound Dynabeads® and biotinylated anti-CRP detection Ab preconjugated to SA-HRP, stored at 4 ºC in BSA-preblocked MTPs) exhibited no noticeable decrease in its functional activity for up to 4 weeks \(**Figure S2B**). Therefore, taking into account the attributes of the developed generic sandwich ELISA procedure, it can be reliably employed in clinical and bioanalytical settings. Moreover, it has immense potential for the development of novel and fully automated rapid IVD kits in combination with lab-on-a-chip technologies, microfluidics, nanotechnology and smart system integration.

References

1. Deegan, O., Walshe, K., Kavanagh, K. & Doyle, S. Quantitative detection of C-reactive protein using phosphocholine-labelled enzyme or microspheres. _Anal. Biochem._ **312**, 175-181 \(2003). 2. Kumar, D. & Prasad, B. B. Multiwalled carbon nanotubes embedded molecularly imprinted polymer-modified screen printed carbon electrode for the quantitative analysis of C-reactive protein. _Sensor Actuat. B-Chem._ **171**, 1141-1150 \(2012). 3. Islam, M. S. & Kang, S. H. Chemiluminescence detection of label-free C-reactive protein based on catalytic activity of gold nanoparticles. _Talanta_ **84**, 752-758 \(2011). 4. Islam, M. S., Lee, H. G., Choo, J., Song, J. M. & Kang, S. H. High sensitive detection of C-reactive protein by total internal reflection fluorescence microscopy on rapidly making nanoarray protein chip. _Talanta_ **81**, 1402-1408 \(2010). 5. Islam, M. S., Yu, H., Lee, H. G. & Kang, S. H. Molecular switching fluorescence based high sensitive detection of label-free C-reactive protein on biochip. _Biosens. Bioelectron._ **26**, 1028-1035 \(2010). 6. Shiesh, S. C., Chou, T. C., Lin, X. Z. & Kao, P. C. Determination of C-reactive protein with an ultra-sensitivity immunochemiluminometric assay. _J. Immunol. Methods_ **311**, 87-95 \(2006). 7. Lee, W. B., Chen, Y. H., Lin, H. I., Shiesh, S. C. & Lee, G. B. An integrated microfluidic system for fast, automatic detection of C-reactive protein. _Sensor Actuat. B-Chem._ **157**, 710-721 \(2011). 8. Baldini, F., Carloni, A., Giannetti, A., Porro, G. & Trono, C. An optical PMMA biochip

based on fluorescence anisotropy: Application to C-reactive protein assay. _Sensor Actuat. B-Chem._ **139**, 64-68 \(2009). 9. Ahn, J. S. _et al._ Development of a point-of-care assay system for high-sensitivity C-reactive protein in whole blood. _Clin. Chim. Acta_ **332**, 51-59 \(2003). 10. Lee, J. H. _et al._ Label free novel electrical detection using micromachined PZT monolithic thin film cantilever for the detection of C-reactive protein. _Biosens. Bioelectron._ **20**, 269-275 \(2004). 11. Kjelgaard-Hansen, M., Martinez-Subiela, S., Petersen, H. H., Jensen, A. L. & Ceron, J. J. Evaluation and comparison of two immunoturbidimetric assays for the heterologous determination of porcine serum C-reactive protein. _Veterinary J._ **173**, 571-577 \(2007). 12. Aguiar, M., Masse, R. & Gibbs, B. F. Mass spectrometric quantitation of C-reactive protein using labeled tryptic peptides. _Anal. Biochem._ **354**, 175-181 \(2006). 13. Kushner, I. & Somerville, J. A. Estimation of the molecular size of C-reactive protein and CX-reactive protein in serum. _Biochim. Biophys. Acta_ **207**, 105-114 \(1970). 14. Kim, H. C. _et al._ Detection of C-reactive protein on a functional poly\(thiophene) self-assembled monolayer using surface plasmon resonance. _Ultramicroscopy_ **108**, 1379-1383 \(2008). 15. Kim, N., Kim, D. K. & Cho, Y. J. Development of indirect-competitive quartz crystal microbalance immunosensor for C-reactive protein. _Sensor Actuat. B-Chem._ **143**, 444-448 \(2009). 16. Algarra, M. _et al._ Thiolated DAB dendrimer/ZnSe nanoparticles for C-reactive protein recognition in human serum. _Talanta_ **99**, 574-579 \(2012). 17. Bryan, T., Luo, X., Bueno, P. R. & Davis, J. J. An optimised electrochemical biosensor for the label-free detection of C-reactive protein in blood. _Biosens. Bioelectron._ **39**, 94-98 \(2013). 18. Koskinen, J. O. _et al._ Fluorescent nanoparticles as labels for immunometric assay of C-reactive protein using two-photon excitation assay technology. _Anal. Biochem._ **328**, 210-218 \(2004). 19. Choi, H. W., Sakata, Y., Kurihara, Y., Ooya, T. & Takeuchi, T. Label-free detection of C-reactive protein using reflectometric interference spectroscopy-based sensing system. _Anal. Chim. Acta_ **728**, 64-68 \(2012). 20. Leung, W. _et al._ InfectCheck CRP barcode-style lateral flow assay for semi-quantitative detection of C-reactive protein in distinguishing between bacterial and viral infections. _J. Immunol. Methods_ **336**, 30-36 \(2008). 21. Ibupoto, Z. H., Jamal, N., Khun, K. & Willander, M. Development of a disposable potentiometric antibody immobilized ZnO nanotubes based sensor for the detection of C-reactive protein. _Sensor Actuat. B-Chem._ **166**, 809-814 \(2012). 22. Algarra, M., Gomes, D. & Esteves da Silva, J. C. Current analytical strategies for C-reactive protein quantification in blood. _Clin. Chim. Acta_ **415**, 1-9 \(2013). 23. Vermeeren, V. _et al._ Impedimetric, diamond-based immmunosensor for the detection of C-reactive protein. _Sensor Actuat. B-Chem._ **157**, 130-138 \(2011). 24. Punyadeera, C., Dimeski, G., Kostner, K., Beyerlein, P. & Cooper-White, J. One-step homogeneous C-reactive protein assay for saliva. _J. Immunol. Methods_ **373**, 19-25 \(2011). 25. Dixit, C. K., Vashist, S. K., MacCraith, B. D. & O'Kennedy, R. Multisubstrate-compatible ELISA procedures for rapid and high-sensitivity immunoassays. _Nat. Protoc._ **6**, 439-445 \(2011). 26. Dixit, C. K. _et al._ Development of a high sensitivity rapid sandwich ELISA procedure and its comparison with the conventional approach. _Anal. Chem._ **82**, 7049-7052 \(2010). 27. Vashist, S. K. A sub-picogram sensitive rapid chemiluminescent immunoassay for the detection of human fetuin A. _Biosens. Bioelectron._ **40**, 297-302 \(2013). 28. Vashist, S. K. Graphene-based immunoassay for human lipocalin-2. _Anal. Biochem._ **446**, 96-101 \(2014). 29. Clyne, B. & Olshaker, J. S. The C-reactive protein. _J. Emerg. Med._ **17**, 1019-1025 \(1999). 30. Vashist, S. K. C-Reactive Protein: An Overview. _J. Basic Appl. Sci._ **9**, 496-499 \(2013). 31.

Marnell, L., Mold, C. & Du Clos, T. W. C-reactive protein: ligands, receptors and role in inflammation. _Clin. Immunol._ **117**, 104-111 \(2005). 32. Ridker, P. M. High-sensitivity C-reactive protein, inflammation, and cardiovascular risk: from concept to clinical practice to clinical benefit. _Am. Heart J._ **148**, S19-26 \(2004). 33. Dollner, H., Vatten, L. & Austgulen, R. Early diagnostic markers for neonatal sepsis: comparing C-reactive protein, interleukin-6, soluble tumour necrosis factor receptors and soluble adhesion molecules. _J. Clin. Epidemiol._ **54**, 1251-1257 \(2001). 34. Nguyen-Vermillion, A., Juul, S. E., McPherson, R. J. & Ledbetter, D. J. Time course of C-reactive protein and inflammatory mediators after neonatal surgery. _J. Pediatr._ **159**, 121-126 \(2011). 35. Chiesa, C. _et al._ C reactive protein and procalcitonin: reference intervals for preterm and term newborns during the early neonatal period. _Clin. Chim. Acta_ **412**, 1053-1059 \(2011). 36. Chundadze, T. _et al._ Significantly elevated C-reactive protein serum levels are associated with very high 30-day mortality rates in hospitalized medical patients. _Clin. Biochem._ **43**, 1060-1063 \(2010). 37. Myers, G. L. _et al._ CDC/AHA Workshop on Markers of Inflammation and Cardiovascular Disease: Application to Clinical and Public Health Practice: report from the laboratory science discussion group. _Circulation_ **110**, e545-549 \(2004). 38. Chiesa, C., Panero, A., Osborn, J. F., Simonetti, A. F. & Pacifico, L. Diagnosis of neonatal sepsis: a clinical and laboratory challenge. _Clin. Chem._ **50**, 279-287 \(2004). 39. Tappero, E. & Johnson, P. Laboratory evaluation of neonatal sepsis. _Newborn Infant Nurs. Rev._ **10**, 209-217 \(2010). 40. Kuo, H. K., Yen, C. J., Chen, J. H., Yu, Y. H. & Bean, J. F. Association of cardiorespiratory fitness and levels of C-reactive protein: data from the National Health and Nutrition Examination Survey 1999-2002. _Int. J. Cardiol._ **114**, 28-33 \(2007). 41. Park, H. E. _et al._ Can C-reactive protein predict cardiovascular events in asymptomatic patients? Analysis based on plaque characterization. _Atherosclerosis_ **224**, 201-207 \(2012). 42. Ridker, P. M. High-sensitivity C-reactive protein and cardiovascular risk: rationale for screening and primary prevention. _Am. J. Cardiol._ **92**, 17K-22K \(2003). 43. Sicras-Mainar, A., Rejas-Gutierrez, J., Navarro-Artieda, R. & Blanca-Tamayo, M. C-reactive protein as a marker of cardiovascular disease in patients with a schizophrenia spectrum disorder treated in routine medical practice. _Eur. Psychiatry_ **28**, 161-167 \(2013). 44. Wilson, A. M., Ryan, M. C. & Boyle, A. J. The novel role of C-reactive protein in cardiovascular disease: risk marker or pathogen. _Int. J. Cardiol._ **106**, 291-297 \(2006). 45. Lin, M. S. _et al._ Serum C-reactive protein levels correlates better to metabolic syndrome defined by International Diabetes Federation than by NCEP ATP III in men. _Diabetes Res. Clin. Pract._ **77**, 286-292 \(2007). 46. Morimoto, H. _et al._ Effect of high-sensitivity C-reactive protein on the development of diabetes as demonstrated by pooled logistic-regression analysis of annual health-screening information from male Japanese workers. _Diabetes Metab._ **39**, 27-33 \(2013). 47.Testa, R. _et al._ C-reactive protein is directly related to plasminogen activator inhibitor type 1 \(PAI-1) levels in diabetic subjects with the 4G allele at position -675 of the PAI-1 gene. _Nutr. Metab. Cardiovasc. Dis._ **18**, 220-226 \(2008). 48. Vashist, S. K. _et al._ One-step kinetics-based immunoassay for the highly-sensitive detection of C-reactive protein in less than 30 minutes. _Anal. Biochem._ **456**, 32-37 \(2014). 49. Dixit, C. K., Vashist, S. K., MacCraith, B. D. & O'Kennedy, R. Evaluation of apparent non-specific protein loss due to adsorption on sample tube surfaces and/or altered immunogenicity. _Analyst_ **136**, 1406-1411 \(2011).

Acknowledgements

We thank Dr. Eberhard Barth for providing and anonymizing the leftover EDTA plasma samples of patients treated by intensive care at University Hospital Ulm, Germany for the validation of the developed sandwich ELISA.

Figures

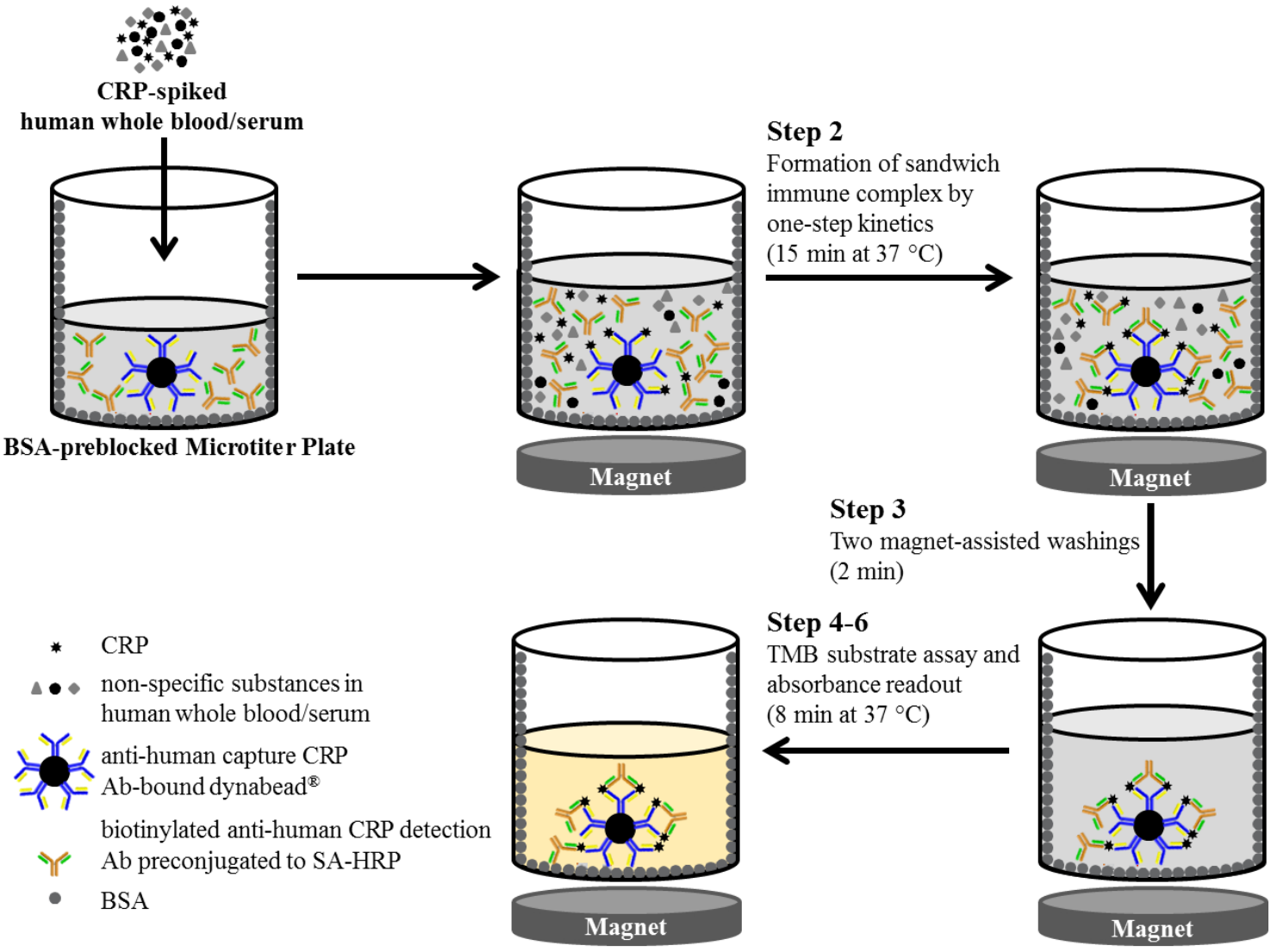

Figure 1

Schematic of the protocol One-step kinetics-based sandwich ELISA procedure for the rapid detection of C-reactive protein (CRP)^48^. Reproduced with permission from Elsevier Inc.

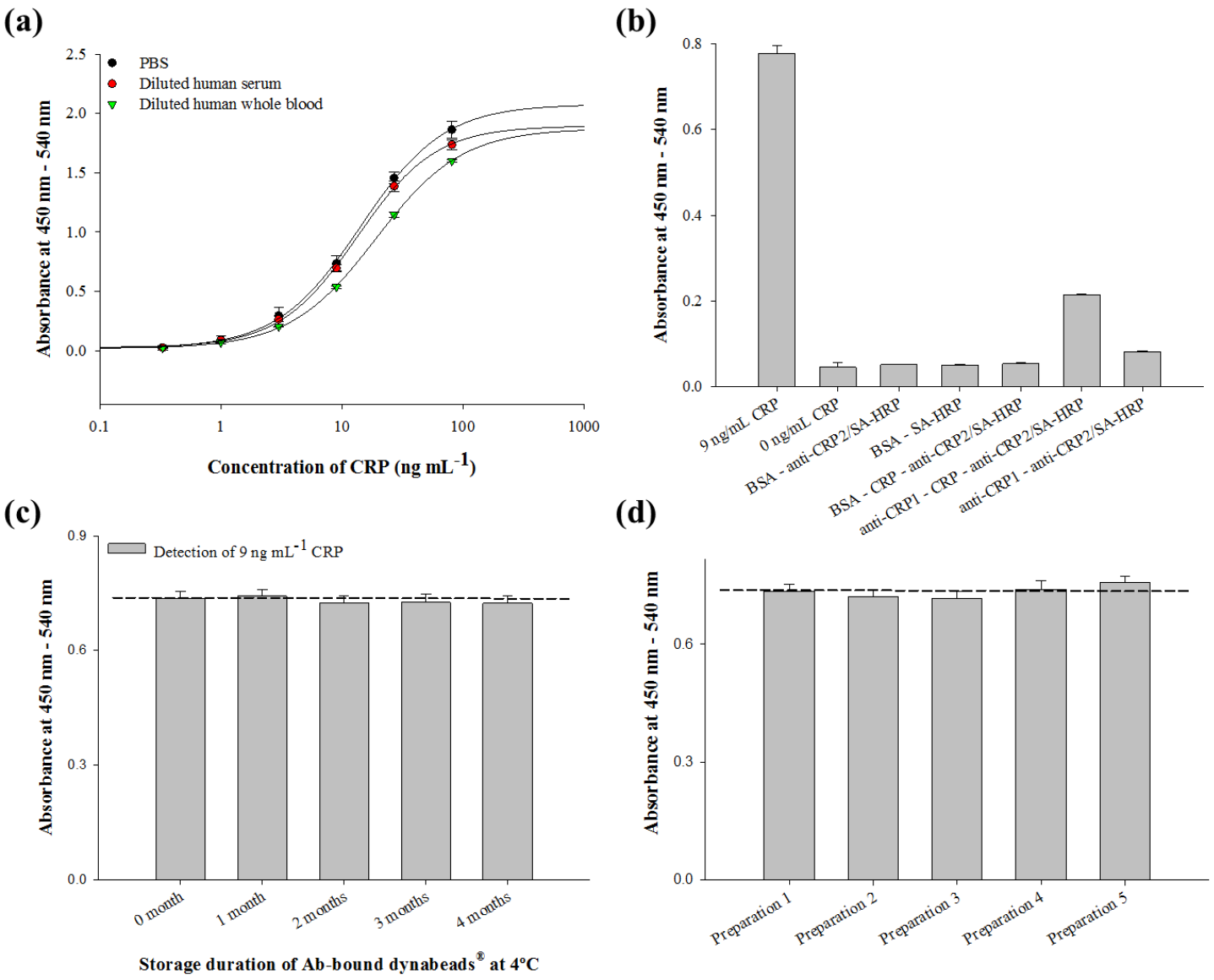

Figure 2

Bioanalytical performance of one-step kinetics-based CRP sandwich ELISA^48^. (A) Detection of CRP in PBS (10 mM, pH 7.4), diluted human serum and diluted human whole blood. (B) Specific CRP detection with respect to various experimental process controls (anti-CRP1 and anti-CRP2 are capture and detection antibodies (Ab), respectively). (C) Stability of anti-CRP capture Ab-bound Dynabeads^®^ stored at 4 ºC. (D) Production variability for the preparation of anti-CRP capture Ab-bound Dynabeads^®^ from the same lots of Dynabeads^®^ and anti-CRP capture Ab. All experiments were performed in triplicate with the error bars representing the standard deviation. Reproduced with permission from Elsevier Inc.

Sample matrix	Added conc. (in ng mL^{-1})	One-step kinetics-based sandwich ELISA		Conventional sandwich ELISA	
		Determined conc. (ng mL^{-1})	*Percentage recovery*	*Determined conc. (ng mL^{-1})*	*Percentage recovery*
Diluted human whole blood	3	2.92±0.19	97.3	3.05±0.18	101.7
	1	1.07 ±0.06	107	0.94±0.05	94
	0.3	0.28±0.01	93.3	0.31±0.01	103.3
Diluted human serum	3	3.16±0.09	105.3	2.89±0.11	96.3
	1	1.08±0.07	108	1.13±0.08	113
	0.3	0.31±0.02	103.3	0.28±0.01	93.3

Figure 3

Table 2 Determination of spiked CRP concentrations in diluted human whole blood and serum by one-step kinetics-based and conventional sandwich ELISAs^48^. The experiments were performed in triplicate with the results presented as mean ± S.D. Reproduced with permission from Elsevier Inc.

Samples	One-step kinetics-based sandwich ELISA (ng mL^{-1})	Conventional sandwich ELISA (ng mL^{-1})
1	4.40±0.19	4.60±0.23
2	10.30±0.36	9.90±0.31
3	13.10±0.42	12.80±0.35
4	14.20±0.31	13.70±0.38
5	35.10±0.45	34.80±0.48
6	36.70±0.42	37.10±0.36
7	45.10±0.51	45.30±0.51
8	63.10±0.63	62.90±0.58
9	81.20±1.23	83.10±1.12
10	129.80±1.61	129.30±2.21
11	226.50±3.84	224.60±4.21
12	248.30±4.13	246.20±3.63
13	264.20±4.52	267.30±4.92
14	274.90±4.92	276.30±5.44
15	298.70±6.11	299.20±5.92

Figure 4

Table 3 Determination of CRP in the EDTA plasma samples of patients using one-step kinetics-based and conventional sandwich ELISAs^48^. The experiments were performed in triplicate with the results presented as mean ± S.D. Reproduced with permission from Elsevier Inc.

Sample matrix	Added conc. (in ng mL^{-1})	One-step kinetics-based sandwich ELISA		Conventional sandwich ELISA	
		Determined conc. (ng mL^{-1})	*Percentage recovery*	*Determined conc. (ng mL^{-1})*	*Percentage recovery*
Diluted human whole blood	3	2.92±0.19	97.3	3.05±0.18	101.7
	1	1.07 ±0.06	107	0.94±0.05	94
	0.3	0.28±0.01	93.3	0.31±0.01	103.3
Diluted human serum	3	3.16±0.09	105.3	2.89±0.11	96.3
	1	1.08±0.07	108	1.13±0.08	113
	0.3	0.31±0.02	103.3	0.28±0.01	93.3

Figure 5

Table 1 Troubleshooting

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement0.pdf