*Title:*
**Serum, plasma, and dried blood spot C-reactive protein enzyme immunoassay for population research.**

*Running Title:*
CRP ELISA for population research

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**ABSTRACT**

*Background:* C-reactive protein (CRP) is increasingly used as a biomarker of morbidity and mortality risk in studies of population health, creating a need for efficient, inexpensive assays for large-scale studies. Antibodies for a previously described assay suitable for population research are no longer available, making a replacement assay necessary, preferably one yielding comparable results from any type of blood specimen.

*Methods:* We developed an enzyme immunoassay using readily available monoclonal antibodies to measure CRP in serum, plasma, or dried blood spots made from venous or capillary blood collection. CRP ELISA performance was evaluated by standard methods, and by comparing CRP concentrations measured in the new assay with those from a previously described assay no longer available. Effects of sample type were tested by measuring CRP in serum, plasma, and dried blood spot specimens collected from ten individuals.

*Results:* Experiments to assess assay limits of detection, parallelism, recovery, imprecision, and concordance with an established method (Pearson correlation = 0.988, n = 20) demonstrated the validity of the new assay. We found excellent agreement among CRP measurements in serum, plasma, and dried blood spot specimens (Pearson correlations ranged from 0.949 to 0.994, n = 10).

*Conclusions:* This CRP assay is a reliable, efficient, and inexpensive new tool appropriate for use in large-scale population health research.

C-reactive protein (CRP) is a widely-used marker of both chronic and acute inflammation (1, 2), and high-sensitivity assays have revealed its importance as an indicator of morbidity and mortality risk (3, 4), particularly for cardiovascular disease (i.e. (5-7) and others). Several methods for measuring CRP are available (8-10), including those designed for clinical diagnostic use and commercial assay kits designed for non-clinical research. However, these can be prohibitively expensive for large-scale research, may be limited in the sample types demonstrated to yield valid results, and may not offer the flexibility needed to optimize assay efficiency for high-throughput. The functional range of the available CRP assays is also a concern (11), as accurate quantification of both acute-phase inflammatory levels of CRP and subtle low-level variability in CRP may be needed for the same research study (for example, (5)). The value of CRP as a biomarker of health has created a demand for less expensive and more efficient methods particularly for large-scale, non-clinical population research with limited budgets and facilities. An assay described by Wu and colleagues (12) for use in serum, and later described by McDade et al. (13) for use in dried blood spots (DBS), filled the demand for appropriate methods very effectively, but the polyclonal antibodies needed have recently become unavailable.

Comparisons between studies are hindered by the differences in assay methods from study to study necessitated by the ongoing problem of consistency in antibody supplies for longitudinal and/or large-scale biomarker research, or by the need for specialized equipment for each assay format. Specimen collection protocol differences can compound this problem, as assay methods may be validated for only one sample type. Our goal was to simplify and streamline population research by creating a simple enzyme immunoassay method requiring basic equipment, and based on commercially available monoclonal antibodies (MAb) from an

established supplier. Further, we sought an assay that would be useful for measuring the entire physiological range of CRP in serum, plasma, and dried blood spots made using either capillary or venous blood collection methods. We report here on a new, high-sensitivity CRP ELISA that meets those requirements, yet is designed to be efficient and inexpensive for use in large-scale population health research.

Capture and detection antibodies recommended for use as a matched pair were purchased from BIODESIGN International / OEM Concepts. The detection antibody (clone C6 anti-CRP MAb, cat. no. M86284M) was conjugated to biotin in our lab following the method of Hermanson (14). 1 mole of antibody to 12 moles of NHS-LC–biotin ester (Sigma-Aldrich) dissolved in dimethylformamide were combined in pH 7.2 phosphate buffered saline, and allowed to react at room temperature with stirring for 1 hour. The conjugated antibody was separated from free excess biotin by dialysis against phosphate buffered saline, pH 7.2, for 48 h at 4°C. Biotinylated C6 anti-CRP monoclonal antibody was aliquoted and stored at −80°C.

Microtiter plates (Nunc, cat. no. 442404) were coated with 100µl of 1µg/ml anti-CRP MAb (clone C5, cat. no. M86005M, BIODESIGN International / OEM Concepts) diluted in coating buffer (0.20 M $NaHCO_3$, pH 9.6), and incubated overnight or up to five days at 4°C. Plates were then washed (0.15 M NaCl; 0.05% Tween 20), then blocked for 30 minutes with 200µl per well of assay buffer (0.01M phosphate buffer, 0.5M NaCl, 0.1% v/v Tween 20, pH 7.2). Plates were washed again, and 100µl of samples, controls, or calibrators diluted in assay buffer were added to the wells. DBS specimens were eluted from filter paper (Whatman 903, cat. no. 10534612) by immersing one 1/8" punch made avoiding the center of a blood spot (equivalent to 1.525µl serum, (15)), in a volume of assay buffer (ranging from 0.25 to 5.00 ml) needed to achieve the desired dilution. An 8-point calibration curve (Fitzgerald Industries

International, Inc., cat. no. 30-AC10) was run on every plate (0, 0.0003, 0.0006, 0.0013, 0.0025, 0.0050, 0.0101, and 0.0202 mg/l). For serum and plasma specimens, liquid calibrators were used; for DBS specimens, calibrator was combined with washed erythrocytes and preserved on filter paper (Whatman 903, cat. no. 10534612) following the method described in (13) at concentrations that would yield the same calibration curve range when 1/8" punches from the spot were eluted in 2ml assay buffer.

After overnight incubation at 4°C, the plates were again washed, and 100µl/well of biotinylated detection MAb, diluted to 250ng/ml in assay buffer, was added to the plate, and incubated for 2 hours at room temperature. Plates were washed and 100µl/well of horseradish peroxidase streptavidin (Invitrogen Corporation, cat. no. 438323) diluted 1:3000 in assay buffer was added. After incubating one hour at room temperature, plates were washed again, and developed in citrate buffer (50 mmol citrate, pH 4.0) combined with 0.4 mmol 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich) and 1.6 mmol of hydrogen peroxide (100µl/well). Color was allowed to develop on an orbital plate shaker for 45 minutes - 1 hour before plates were read (test 405nm, ref 570nm) with a microtiter plate reader (Synergy HT, BioTek Instruments, Inc.), and concentrations were estimated with a four-parameter logistic calibration curve (Gen5, BioTek Instruments Inc.).

Controls were run on every plate. For serum and plasma specimens, controls were made from plasma collected from a healthy US male and diluted to three concentrations. For DBS specimens, controls were eluted from DBS made from three US adults' venous blood, collected with anticoagulant and dropped onto filter paper (Whatman 903, cat. no. 10534612), 50µl per spot, using a wide-bore pipette tip. Assay imprecision was estimated using a variance components model (16). For plasma controls, within assay CVs were 2.5%, 2.0%, and 11.6% for

low, medium, and high controls, respectively, and between assay CVs were 5.8%, 8.8%, and 12.4% (n=10 batches). For DBS, low, medium, and high controls, eluted independently for 10 assay batches gave CVs of 2.9%, 2.1%, and 4.5% within assay runs, and 15.2%, 9.2%, and 17.1% between assays. The higher between-assay imprecision for DBS controls likely reflects the variability introduced by the DBS elution process.

Analytical sensitivity, defined as the concentration 3 SD above the zero dose calibrator (17, 18), was 0.00007mg/l (n=20 plates). Functional sensitivity, estimated as the concentration at which the within-assay CV is consistently less than 10% (19), was 0.00015mg/l (n=230 specimens, run in duplicate, across 8 plates). Ten anti-coagulated venous DBS specimens serially diluted after elution demonstrated parallelism with a calibration curve eluted from DBS, and 10 serially diluted plasma specimens demonstrated parallelism with a liquid calibration curve. Assay recovery of CRP from a plasma matrix was estimated by spiking low, medium, and high endogenous CRP plasma specimens diluted 1:400 in assay buffer, with low, medium, and high doses of calibrator diluted in assay buffer. Varying doses were added as 10% of the total sample volume to maintain consistency in sample dilution from dose to dose, and spiked specimens were run in triplicate in each of 10 independent assay batches. Recovery calculated as [observed CRP value / expected CRP given the concentration native to the sample plus the known added dose of calibrator], averaged 97%, 88%, and 85% for low, medium, and high doses respectively.

To compare CRP measurements in common specimen types, plasma, serum, DBS made from anti-coagulated venous blood, and DBS made from capillary blood collected by a finger prick were all collected at the same time from nine healthy US men and women, ages 25-45, with one additional subject contributing only plasma and anti-coagulated venous DBS.

Participants provided written informed consent, and procedures were approved by the Institutional Review Board of the University of Washington. Whatman 903 filter paper cards were used for all DBS specimens. CRP measurements from serum, plasma, and both types of DBS specimens were highly correlated, with Pearson correlations ranging from 0.949 to 0.994 (Table 1), and absolute values were also in excellent agreement (Figure 1).

CRP measurements in the assay described here using antibodies from BIODESIGN were compared with the method described by McDade et al. (13) using antibodies supplied by DAKO (cat. no. A0073 coating antibody and P0227 detection antibody, recently discontinued). CRP was measured in 4 DBS specimens (both venous and capillary) run at 5 dilutions each to test the full range of the calibration curve (n = 20 specimen/dilution combinations). Results of the two assays were highly correlated: Pearson correlation was 0.989 (n=20), and absolute values did not differ significantly (p=0.843), with DAKO assay results averaging 95% ± 9% of the BIODESIGN assay results (n=20).

The protocol used here differs from that described in (13), in that assay steps are carried out over three days instead of two. This format is intended to be more efficient for large-scale work, as it makes assay of larger batches of specimens practical. However, modifications to the protocol to shorten the total time needed to complete the assay may be possible.

Practical considerations, including cost (about $0.25 per specimen for assay supplies versus $5.00 - $15.00 for commercial kits) and efficiency, and the performance characteristics described here support this CRP assay as a useful tool for population research.

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**Table 1.** Pearson correlations for CRP in plasma, serum, and DBS (from venous and capillary blood) collected at the same time from 9 individuals.

| | Serum | Plasma | Capillary DBS |
|---|---|---|---|
| Venous DBS | 0.974 | 0.973[1] | 0.949 |
| Capillary DBS | 0.976 | 0.958 | |
| Plasma | 0.994 | | |

CRP, c-reactive protein; DBS, dried blood spot

[1]n=10 individuals for plasma and venous DBS

**FIGURE LEGEND**

**Figure 1.** CRP in serum, plasma, capillary DBS, and venous DBS specimens collected at the same time from 10 individuals (plasma and venous DBS only for subject 1). Mean results of four replicate wells, error bars are 2 SE.