

## Development of a point-of-care assay system for high-sensitivity C-reactive protein in whole blood

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### Abstract

**Background:** C-reactive protein (CRP) is emerging as a potential risk predictor for future cardiovascular diseases (CVD). High sensitivity assays have been developed and applied for clinical purposes. **Methods:** The fluorescence immunochromatographic assay was employed to detect and quantify CRP in whole blood. It consisted of a fluorescence (FL) antibody detector buffer, a test strip housed in a disposable cartridge, and a laser fluorescence scanner. Whole blood sample was mixed with detector, loaded onto a cartridge, incubated for 10 min, and the concentration of CRP was measured in a laser fluorescence scanner. The linearity, limit of detection (LOD), and performance of new assay system was tested and evaluated. The comparability of assay was examined with an automated reference method. **Results:** With the new assay system, a reliable correlation of coefficient ( $r$ ) was obtained between the ratio value ( $A_T/A_C$ ) and a concentration of CRP in samples. The linearity fell in the range of 0–10 mg/l of CRP, and the analytical detection limit was 0.133 mg/l of CRP. The mean recovery of the control was 105.2% in a working range. The precision of the intra- and inter-assay in a range of 0.5–6 mg/l was CVs <6% and <8%, respectively. The new fluorescence immunochromatographic assay system correlated well with a traditional immunoturbidimetric assay for quantification of CRP concentration ( $r=0.955$ ,  $N=90$ ). **Conclusion:** The fluorescence immunochromatographic assay is fast, reliable, and a reproducible platform for point-of-care testing (POCT) of high-sensitive (hs)-CRP in whole blood.

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**Keywords:** hs-CRP; Fluorescence immunochromatographic assay; Anti-CRP-mAb; Cardiovascular diseases; Laser fluorescence scanner

**Abbreviations:** hs-CRP, high-sensitive C-reactive protein; CVD, cardiovascular disease; mAbs, monoclonal antibodies; NC, nitrocellulose membrane; mAb-FL, monoclonal antibody-fluorescence conjugate; Anti-RIgG-FL, anti-rabbit IgG-fluorescence; ELISA, enzyme-linked immunosorbent assay; CV, coefficient of variation; POCT, point-of-care testing.

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## 1. Introduction

The C-reactive protein (CRP) is synthesized by the liver in response to interleukin-6 and well known as one of the classical acute-phase reactants and as a marker of inflammation. The serum CRP level may rise from a normal level of <5 to 500 mg/l during the body's general, non-specific response to infectious and other acute inflammatory events. For some time, the measurement of CRP concentration has been used as a clinical tool for monitoring autoimmune diseases and infectious processes, such as rheumatoid arthritis. It has recently been suggested that a marker of inflammation, along with serum cholesterol, may be critical component in the development and progression of atherosclerosis [1,2]. Since the early 1990s, a growing body of evidence has supported the idea that cardiovascular diseases (CVD), including coronary heart disease, ischemic stroke, and acute myocardial infarction, as well as peripheral vascular diseases (PVD), develop, at least in part, because of a chronic, low-level CRP of the vascular endothelium [3–8]. However, the prospect of using CRP as a predictor of future vascular risks faced a big obstacle because existing assay methods, such as latex agglutination and capillary immunoprecipitation with 3–8 mg/l of a limit of detection (LOD), were not sensitive enough to detect very low-level of CRP in serum.

Apparently, more sensitive assay systems now exist, which are able to quantify low-level CRP in serum. With the capability of measuring CRP at concentrations from 0.1 to 4.0 mg/l, the manual enzyme-linked immunosorbent assay (ELISA) test was first developed for laboratory research and used for several population studies [3,9]. A latex, particle-enhanced immunonephelometric high-sensitive (hs)-CRP method has been followed and its clinical applications have been evaluated [10,11]. Recently, a new generation of automated immunoturbidimetric and immunoluminometric assays has been developed and gained popularity. These new assays have an improved sensitivity and precision at a low-level of CRP [12,13], and they are able to assess the role of hs-CRP as the strongest and most independent predictive risk factor for atherosclerosis and CVD [14].

In this report, we introduce a new hs-CRP assay system using a principle of traditional immunochromatographic assay with a sandwich type [15].

Fluorochrome dye was used to detect a signal in an immunochromatographic assay system. Consisting of an immunochromatographic strip in a disposable cartridge, a fluorescence (FL) antibody detector buffer, and a laser FL scanner, the developed hs-CRP assay takes advantage of the inherent simplicity of lateral flow immunochromatography, while providing quantification of low-level CRP in whole blood. We evaluated the FL immunochromatographic assay system for quantification of hs-CRP for the followings: limit of detection, linearity, recovery, and precision. We also examined the comparability between the developed assay system and an automated immunoturbidimetric assay with serum samples from healthy individuals.

## 2. Materials and methods

Rabbit IgG, anti-rabbit IgG (anti-RIgG), polyethyleneglycol, sodium azide, and sodium bicarbonate were purchased from Sigma (St Louis, MO). CRP-free serum and CRP calibrator were purchased from HyTest (EuroCity, Turku, Finland) and Wako (Richmond, VA), respectively. Human CRP came from Life Diagnostics (West Chester, PA) and was used as an immunogen. Sephadex G25 and activated Alexa Flour 647 were obtained from Amersham Pharmacia Biotech (Piscataway, NJ) and Molecular Probes (Eugene, OR), respectively. Nitrocellulose membrane (NC) was from Millipore (Watertown, MA), and sample pad and the absorption pads were obtained from Schleicher and Schuell (Keene, NH).

### 2.1. Production and purification of anti-CRP-mAbs

CRP from human plasma was used as an immunogen for the production of mouse monoclonal antibody (mAb). Immunizations, cell fusion, and screening of hybridoma cells producing anti-CRP-mAb were conducted according to a standard method [16]. In brief, 6–8-week-old Balb/c mice were immunized with 0.5 ml of CRP (50 µg) mixed with an equal volume of complete Freund's adjuvant. The first injection was followed by three or four booster injections of the same amount of immunogen mixed with incomplete Freund's adjuvant at 3–4-week intervals. Serum was

taken from the tail of the mouse and tested for antibody titer usually after the third injection. The cell fusion was completed by dropping 1 ml of 50% PEG 1500 onto a mixture of spleen cells and SP2/0-Ag-14 myeloma cells. We selected the fused cells in a hypoxanthine–aminopterin–thymidine medium for 2 weeks, collected the culture supernatants, and then screened them against CRP antigen by ELISA. The positive clones were further selected through two successive limiting dilutions. Ascitic fluids were generated for a large-scale production of mAbs by injection of hybridoma cells into the peritoneal cavity of mice. The mAbs were purified from ascitic fluids through three purification steps: (1) membrane ultrafiltration, (2) ammonium sulfate precipitation, and then, (3) protein G column. The eluted mAbs with 100 mmol/l glycine–HCl (pH 2.5) were neutralized with 0.1 vol. of 1 mol/l Tris (pH 8.0) and stored at  $-70\text{ }^{\circ}\text{C}$  until ready to use.

## 2.2. ELISA

For the screening of positive hybridomas, we incubated 96-well microtiter plates with 25  $\mu\text{l}$  (50 ng/well) of CRP (20 mg/l) in 50 mmol/l carbonate buffer (pH 9.6) overnight at  $4\text{ }^{\circ}\text{C}$ . After washing the plates extensively with PBST, we blocked them with 50  $\mu\text{l}$  of BSA (10 g/l) in PBST for 1 h, incubated with 100  $\mu\text{l}$  of culture supernatants for 1 h at  $37\text{ }^{\circ}\text{C}$ , and then probed with 50  $\mu\text{l}$  of HRP-conjugated goat anti-mouse IgG (1 mg/l). Following a final rinse with 50  $\mu\text{l}$  of PBST, we initiated the color reaction of antigen–antibody with 50  $\mu\text{l}$  of TMB substrate solution (10 g/l) and stopped it by adding 50  $\mu\text{l}$  of 10 mmol/l  $\text{H}_2\text{SO}_4$ . The absorbance was measured at 450 nm in an automatic ELISA reader (Bio-Rad model 550).

## 2.3. Labeling of detector antibodies

For labeling of the anti-CRP-mAb with FL, 10  $\mu\text{l}$  of a 1 mol/l sodium bicarbonate buffer (pH 8.3) was mixed with 100  $\mu\text{l}$  of purified anti-CRP-mAb (1 mg/l) in PBS. Next, we added 1  $\mu\text{l}$  of activated Alexa Fluor 647 (10 g/l) to the mixture, and then incubated it overnight at  $4\text{ }^{\circ}\text{C}$ . The mixture was applied onto a Sephadex G25 chromatography column to separate the free Alexa dye from conjugates. The mAb-fluo-

rescence conjugate (mAb-FL) was collected as elutes after centrifugation of the column at 2,500 rpm for 2 min. The conjugation of Alexa Fluor 647 to anti-RIgG and purification of conjugates was conducted as noted above. The FL labeled anti-CRP-mAb and anti-RIgG was mixed with PBS and kept at  $4\text{ }^{\circ}\text{C}$ .

## 2.4. Fluorescence immunochromatographic assay strip

A  $4 \times 60$  mm assay strip was fabricated in-house to fit into a disposable cartridge. It was composed of the following: a NC membrane, a sample pad, an absorption pad, and a backing card. The backing polystyrene card was placed at the bottom to support other components of the strip. The control line on the NC was dispensed with rabbit IgG (1 g/l) as an internal standard, and the test line was coated with anti-CRP-mAb 1C1 (capture Ab, 1.5 g/l) to capture CRP in sample. The assembled strip was placed into a plastic disposable cartridge ( $15 \times 90$  mm), which was designed to fit to the holder of the laser FL scanner.

## 2.5. Instrumentation

We constructed a one-dimensional FL scanner to quantify the distribution of the FL intensity along the strip. We used a semiconductor laser, WLM-6305NH from Lanics, as an illumination source. A focusing lens was used to bring the laser through an excitation filter (D640/20x, Chroma Tech, VT) to get the best focus on the sample strip. Since the strip was opaque, most of FL light, as well as the diffusely scattered laser light, directed toward the ellipsoidal mirror. This mirror was positioned in such a way that the focus of the laser on the strip was poised on one of the two foci of the mirror so that any beam emanating from that focal point would eventually come to another focal point. FL energy that passed through the emission filter was detected by a PM tube, H5784-01 (Hamamatsu Photonics, Hamamatsu, Japan), and was converted to a digital-readout signal. The on-board computer controls each operation step and measures signals via preprogrammed instructions to generate statistically analyzed results. The system components and the principle of the laser FL scanner are described in detail elsewhere [17].

## 2.6. Fluorescence immunochromatographic assay

The FL antibody detector buffer was a mixture of Alexa 647 labeled anti-CRP-mAb 18C2 (detector Ab, 2.4 mg/l) and anti-RiGg (internal-control detector Ab, 0.16 mg/l) in PBS. A 10  $\mu$ l of whole blood was added to 70  $\mu$ l of detector buffer, and the mixture was loaded onto the sample well of the cartridge. After a 10-min incubation, the cartridge was inserted and scanned with the laser FL scanner. We collected the scanning result, and converted the relative FL units at the test and the control line to the area value (test:  $A_T$ , control:  $A_C$ ). The area ratio of  $A_T/A_C$  was plotted against the concentration of CRP. The equation for the calibration curve and the correlation of coefficient ( $r$ ) were obtained using a Microsoft Excel program.

## 2.7. Method comparison

Serum samples for the comparability of the assay systems were obtained from 100 random individuals visiting for their annual health check-up at Hallym University Medical Center in Chuncheon, Korea. They were collected from all adult females and males between the ages of 25 and 75. Among them, 10 serum samples that differed from the normal range of various chemistry profiles, such as cholesterol, bilirubin (direct), uric acid, AST/ALT, and calcium, were discarded. The serum sample from same individuals was run in Hitachi 911 and in laser FL scanner, and the level of CRP was compared.

## 3. Results

### 3.1. Monoclonal antibodies to CRP

To select specific mAbs, we screened hybridoma cells against CRP by ELISA and were able to select more than 20 positive clones. Among them, six clones showing a high positive signal were further evaluated to match sandwich pairs. Each of these six clones recognized a unique epitope on CRP, but they did not show cross-reactivity with its structural homologue, serum amyloid P component (data not shown). After testing numerous combination pairs, we finally selected three sandwich pairs including anti-CRP-mAb 1C1 and anti-CRP-mAb 18C2. Since CRP has

known to bind to a variety of substrates in a  $\text{Ca}^{2+}$ -dependent manner [18], we examined the binding kinetics of antibody pairs to CRP in the presence of 5 mmol/l  $\text{Ca}^{2+}$  or 10 mmol/l EDTA. Anti-CRP-mAb 1C1 and anti-CRP-mAb 18C2 showed high specificity and sensitivity in either 5 mmol/l  $\text{Ca}^{2+}$  or 10 mmol/l EDTA (data not shown). Thus, anti-CRP-mAb 1C1 and anti-CRP-mAb 18C2 pair was used as a capture antibody and a FL-labeled detector antibody, respectively, for this study.

### 3.2. Calibration curve of fluorescence hs-CRP immunoassay

We set up experiments for the calibration curve with different concentrations of standard CRP, from 0 to 10 mg/l, diluted in CRP-free serum. Fig. 1A shows a profile of relative FL units graphed from the scanned raw data. The first peak (a) and the second peak (b) displayed the relative FL units on the internal standard and on the test line, respectively, as plotted in  $Y$ -axis. An arbitrary distance from the sample well of cartridge was plotted in  $X$ -axis. The FL intensity on the control lines was almost constant at different levels of CRP in sample as shown in first peak (a) of Fig. 1A. The result indicated that the FL intensity from rabbit IgG and anti-RiGg interaction is independent of CRP concentration in sample, and thus it is functioning as a good internal standard. The variability observed from strip to strip at the same CRP concentration attributes to variations in membrane properties, component aging, humidity effects, and many other factors. Thus, an internal standard is essential for setting to minimize this variability. The relative FL units on the test line increased gradually as the concentration of CRP in sample went up. The profiles of relative FL units in CRP concentration, 1, 3, 5, 7, and 9 mg/l, were omitted in Fig. 1A for the clarity of graph. This result demonstrated that the FL immunochromatographic assay system works accordingly and can be used as a tool for the quantification of hs-CRP in whole blood.

The calibration curve was obtained from area ratio ( $A_T/A_C$ ) and CRP concentration in sample. The profile of relative FL units was converted to  $A_T/A_C$  and plotted at the  $Y$ -axis against CRP concentration at the  $X$ -axis. A reliable correlation of coefficient ( $r$ ) was observed between them ( $r=0.998$ ), and a good linearity was displayed throughout the entire measur-

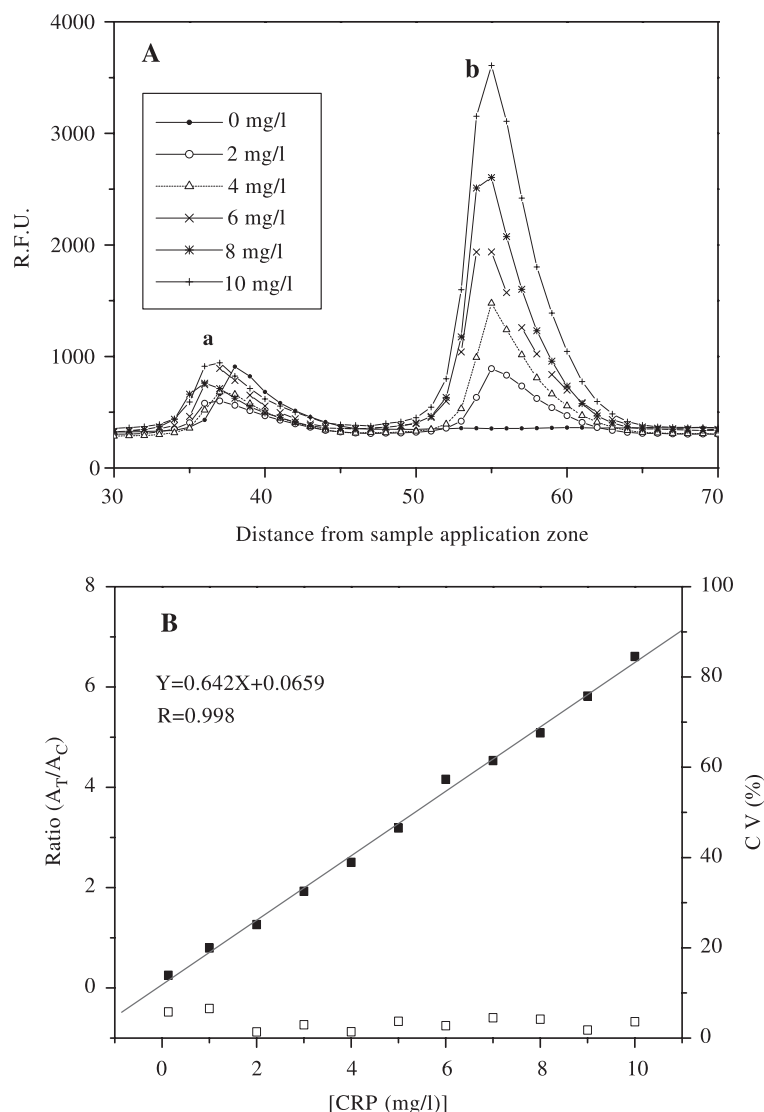


Fig. 1. The scanning profile of relative fluorescence unit with the laser fluorescence scanner (A) and calibration curve obtained from the ratio of  $A_T/A_C$  against the concentration of CRP in sample (B). A shows the fluorescence unit changes on the control line and the test line in the sample containing 0 to 10 mg/l of CRP. B demonstrates the linear coefficient of correlation ( $r$ ) between the area ratio ( $A_T/A_C$ ) and CRP concentration, and CV% of the area ratio at various CRP concentrations.

ing range (Fig. 1B). The coefficient of variations (CVs) from the area ratio ( $A_T/A_C$ ) of 10 independent experiments at each concentration was below 10%, as spiked in Fig. 1B. LOD of the assay system was 0.133 mg/l of CRP, calculated as mean value plus 3 SD of a zero calibrator. The LOD of the assay system was comparable to those of other improved methods

reported [12,15]. When samples with very high CRP concentrations at 10–50 mg/l were analyzed, they displayed a high-dose hook effect for the method. In cases showing CRP level above 10 mg/l, samples were thus diluted with CRP-free serum to bring CRP level range to 0–10 mg/l, which demonstrated a good linearity in the FL hs-CRP immunoassay.

### 3.3. Reproducibility of the immunoassay system

We conducted recovery and imprecision tests to evaluate the technical performance of the new assay system. Seven samples were prepared from two serum pools for a recovery test. The high pool was prepared using a combination of patient samples with adjustment to hs-CRP concentration of  $\sim 8$  mg/l, and CRP free-serum was used as the low pool. The high pool (100%) was diluted with the low pool (0%) to the following final percentages: 100%, 50%, 25%, 12.5%, 6.25%, 3.12%, and 0%. We assayed five replicates in one analytical run at each CRP level. Table 1 shows the test result of comparison between the measured CRP concentrations and the expected values. A mean recovery of control was 105.2% with a good linearity within the whole measuring range, which was well consistent with Fig. 1B. We also evaluated the imprecision of the intra- (within day) and inter-assay (between days) variations to determine the accuracy of the new assay system. The intra-assays were performed on 10 replicate tests for each CRP concentration of 0.5, 2, 4, 6 mg/l. The inter-assays were performed on 5 sequential days, two runs per day, with 10 replicates at each concentration. The intra- and inter-assay CVs in the new hs-CRP assay system were, respectively, 4.12% and 5.33% at 0.5 mg/l, 4.47% and 6.38% at 2 mg/l, 5.91% and 6.14% at 4 mg/l, and 5.70% and 8.02% at 6 mg/l (Table 2). This result suggested that the new fluorescence hs-CRP assay was comparable to other automated reference assays [19,20] with CVs  $< 10\%$  in the dynamic range. Specifically, CVs  $< 6\%$  at 0.5 mg/l in both the intra- and inter-assays indicated that the new assay can be

Table 1  
Recovery of fluorescence hs-CRP immunoassay system

	Measured concentration (mg/l) <sup>a</sup>	Expected concentration (mg/l)	Recovery (%)
100%	7.84	8.0	97.8
50%	4.37	4.0	109.2
25%	2.01	2.0	100.2
12.5%	1.11	1.0	111.2
6.25%	0.54	0.5	108.4
3.12%	0.26	0.25	104.6
0%	0.01	0	–

<sup>a</sup> Mean value of five replicates.

Table 2  
Precision of fluorescence hs-CRP immunoassay system

Sample	Intra-assay precision			Inter-assay precision <sup>a</sup>		
	Mean (mg/l) <sup>b</sup>	SD (mg/l)	CV%	Mean (mg/l) <sup>b</sup>	SD (mg/l)	CV%
Very low (0.5 mg/l)	0.52	0.02	4.12	0.51	0.03	5.33
Low (2 mg/l)	1.96	0.08	4.47	1.93	0.12	6.38
Medium (4 mg/l)	4.07	0.24	5.91	3.95	0.24	6.14
High (6 mg/l)	6.12	0.35	5.70	6.11	0.49	8.02

<sup>a</sup> Assay was performed on 5 sequential days, two runs per day, with 10 replicates at each concentration.

<sup>b</sup> Mean value of 10 replicates.

applied for long-term, risk assessment of CVD since assay imprecision with CVs  $< 10\%$  at 0.2–0.5 mg/l was proposed as acceptable methods for risk stratification [19,21].

### 3.4. Comparison of CRP concentration in whole blood and serum

To determine correlation of CRP concentration between serum and whole blood from the same individual, we collected 27 samples, including 15 patients who had been hospitalized resulting from car accidents and who had waist disks. The CRP level

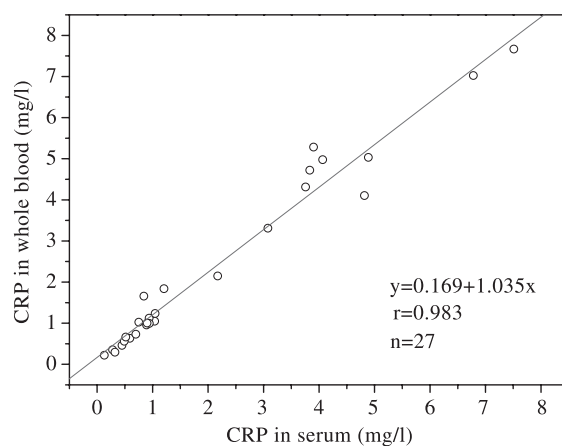


Fig. 2. Comparison of CRP level of serum and whole blood in the new fluorescence-immunochromatographic assay system. Concentration of CRP from the same sample was compared between 5  $\mu$ l of serum and 10  $\mu$ l of whole blood.

in 10  $\mu$ l of whole blood was compared with those in 5, 10, 15, and 20  $\mu$ l of serum with the laser FL scanner (data not shown). The CRP level in 10  $\mu$ l of whole blood corresponded well to that in 5  $\mu$ l of serum, as shown in Fig. 2. The correlation of coefficient ( $r$ ) obtained was 0.983, and the slope of calculated equation for calibration curve was 1.03. This result demonstrated that the new system could be applied as a platform of point-of-care testing (POCT) with whole blood that needs no accessory equipment to separate serum.

### 3.5. Comparability to other immunoassay methods

We set out to compare the fluorescence immunoassay with an automated assay that is widely used as a reference method. We studied the correlation between the new assay system and an immunoturbidimetric assay on a Hitachi 911 with 90 serum samples from apparently healthy individuals. There was a highly significant correlation ( $r=0.955$ ,  $p<0.0001$ ) between the two assays (Fig. 3). Reportedly, the Hitachi 911 performed and agreed well with other immunonephelometric assays including Dade Behring [19]. Thus, these findings suggested that the fluorescence immunochromatographic assay system for hs-CRP gave a confident performance compared to other well-known assay systems.

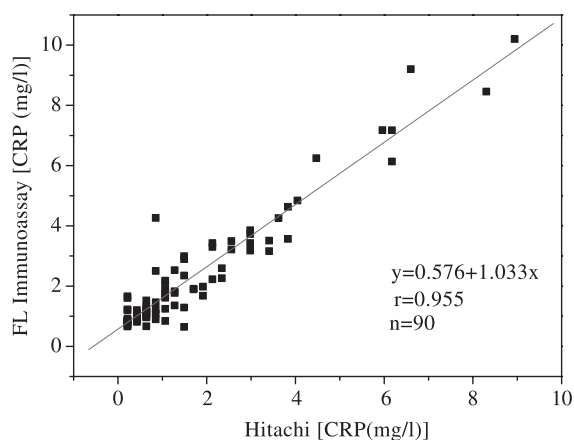


Fig. 3. Method comparison between the immunoturbidimetric assay on Hitachi 911(X-axis) and the fluorescence immunochromatographic assay (Y-axis) for CRP concentrations in serum samples.

## 4. Discussion

In this study, we demonstrated that the FL immunochromatographic assay system, a modified traditional lateral-flow immunochromatography, gave a confident analytical performance when compared to other assay methods for hs-CRP. The dynamic range, from 0.133 to 10 mg/l, allows the new assay to detect not only low CRP concentrations in healthy people, but also to detect high CRP levels in individuals during times of infection and inflammation. Several prospective epidemiologic studies with hs-CRP on healthy individuals have shown a reference interval: median values, 0.6 to 1.1 mg/l; 75th percentile values, 1.4 to 2.5 mg/l; and 90th percentile values, 3 to 5.4 mg/l [22–24]. Therefore, the FL hs-CRP immunoassay with demonstrated technical performance of dynamic range, recovery, and precision can be used as a method for predicting the risk of future cardiovascular events.

A previous study demonstrated a pioneering quantitative immunochromatographic assay for myoglobin [25]. The RAMP™ system is a rapid, quantitative, whole-blood immuno-chromatographic platform for POCT. In the assay, a membrane-based immunochromatographic device is read with a laser fluorescence scanner. Instead of fluorescence, a study has been reported using the combination of carbon black as an antibody label and a flatbed scanner as a quantitative test system [26]. Compared to a fluorescence scanner, the flatbed scanner is an inexpensive instrument with multiple uses, which also shows enough sensitivity to detect analyte at nanogram range.

Recently, several POCT CRP assays with extremely short turnaround times have been developed. For instance, the use of a kinetic microparticle approach combined with time-resolved fluorometry allows ultrasensitive quantification of CRP in whole blood in 2 min with a linear-assay range spanning more than 4 orders of magnitude [21]. Although this method has advantages of ultrasensitivity and a wide dynamic range, an experienced lab personnel is required to perform the analytical experiments. A 2-min reflectometric NycoCard™ CRP Whole-Blood test (Axis Shield) [27] and the turbidimetric, 3-min Quik-Read™ CRP whole-blood/serum/plasma assay (Orion Diagnostica) [28] also represent quantitative test systems suitable for extra-laboratory testing. In the flow-through NycoCard assay, the results are corrected with

an assumed Hct value of 0.40, and the assay needs visual interpretation of the results. Both assay procedures, however, require manual dispensing of the assay-specific reagents and the sample. Furthermore, analytical detection limits of the assays are too high, 10 mg/l for the NycoCard and 8 mg/l for the QuikRead, to be used for hs-CRP. Our assay is suitable for the lower range between 0.133 and 10 mg/l. The successful development of the new fluorescence immunochromatographic assay emphasizes the fast, convenient, and accurate method for quantification for hs-CRP with the same level of performances of automated popular assays commercially available. In addition, the new immunoassay is a platform for POCT using whole blood without centrifugation of the sample or trained expert.

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