

# Fluorescence Immunoassay of Human D-dimer in Whole Blood

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**Background:** D-dimer is a widely used biomarker for the initial clinical assessment of suspected deep vein thrombosis and pulmonary embolism. Here, we presented a new fluorescence (FL) D-dimer assay system, which was developed with a platform of point-of-care test (POCT) for clinical applications. **Methods:** Whole blood was mixed with FL-labeled anti-D-dimer detector antibody, and then loaded onto a disposable cartridge. After 12 min of incubation, the FL intensity was acquired by scanning of test cartridge and converted as level of D-dimer in a laser FL scanner. The analytical performance of FL immunoassay was evaluated by linearity, recovery, and precision tests. The comparability of the devel-

oped assay was examined with automated reference methods. **Results:** The FL assay system showed a good linearity, and the analytical mean recovery of control was 103% in a dynamic working range. The imprecision of intra- and inter-assay of coefficient of variations from assay system was less than 8%. The developed FL assay system showed strong correlation with two automated reference assays, Vidas D-dimer ( $r = 0.973$ ) and Stalio D-dimer ( $r = 0.971$ ). **Conclusion:** The new FL immunoassay for D-dimer is a user-friendly, precise, and reproducible platform of POCT in whole blood. J. Clin. Lab. Anal. 28:294–300, 2014. © 2014 Wiley Periodicals, Inc.

**Key words:** fluorescence; immunochromatographic assay; point-of-care test (POCT); D-dimer; whole blood

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## INTRODUCTION

D-dimer is a mixture of cross-linked fibrin degradation products, which is formed after fibrin clots are degraded by a fibrinolytic enzyme called plasmin. D-dimer reflects global activation of platelet coagulation pathway and the degree of thrombin turnover (1, 2). Thus, D-dimer levels have been the most popularly used parameter for the frontline assessment of suspected deep vein thrombosis (DVT) or pulmonary embolism (PE) for almost 30 years (3, 4). In general, commercial D-dimer assays for blood show a high sensitivity up to 95% but have poor specificity, making it difficult to prove the presence of DVT or PE instead of another clotting complication. Additionally, elevated levels of D-dimer have been reported in pregnancy, cancer, inflammation, surgery, the elderly, and remain a confounding factor limiting its use (5–9). However, the negative predictive values are nearly 100% accurate, with negative D-dimer test results of indication of a lack of DVT or PE. Hence, the main clinical application of D-dimer assay is to safely rule

out DVT, PE, or disseminated intravascular coagulation (10, 11).

Among the commercially available assay systems for D-dimer, enzyme-linked fluorescence (FL) immunoassays and microlatex immunoturbidimetric assays are popularly used (12, 13). Although their performances satisfy the requirements for D-dimer assays in terms of high diagnostic sensitivity and negative predictive values, these assays have some limitations in application during emergency situations or in analyzing a single sample (14, 15). Thus, we aimed to develop a new D-dimer assay that was

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cost-effective and reliable, and could work with whole blood on a point-of-care test (POCT) platform.

Here, we introduce a new D-dimer assay system that provides results in 12 min, has a POCT platform, and a reliable analytical performance compared to automated reference analyzers. This newly developed D-dimer assay system takes advantage of a simple lateral flow immunochromatographic assay (ICA) basis, adds a sandwich-type antibody scheme, and employs FL dye as a tracer to detect the level of D-dimer in samples. The FL-ICA D-dimer assay system consists of a monoclonal antibody coated (mAb) strip in a disposable cartridge, a detection buffer containing FL-labeled mAb, and biotin-bovine serum albumin (BSA), a chip for the calibration curve, and a laser FL scanner.

## MATERIALS AND METHODS

### Materials

Human D-dimer and mAb were purchased from Hytest Ltd. (Turku, Finland), and both streptavidin and biotin-BSA were purchased from Sigma-Aldrich (St. Louis, MO). Sephadex G25 and activated Alexa Fluor 647 were obtained from Amersham Pharmacia Biotech (Piscataway, NJ) and Molecular Probes (Eugene, OR), respectively. Nitrocellulose membrane (NC) was purchased from Millipore (Watertown, MA), sample pad and the absorption pads were obtained from Schleicher and Schuell (Keene, NH).

### Labeling of Antibodies With Fluorescent Dye

For conjugation of the anti-D-dimer-mAb with a FL-dye, 10  $\mu$ l of a 1 mol/l sodium bicarbonate buffer (pH 8.3) was mixed with 100  $\mu$ l of anti-D-dimer-mAb (1 mg/l) in phosphate-buffered saline (PBS) and followed by the addition of 1  $\mu$ l of activated Alexa Fluor 647 (10 g/l) to the mixture. After overnight incubation at 4°C, the mixture was applied onto a Sephadex G25 column to remove the free dye, and FL-labeled mAb conjugates were collected as elutes after centrifugation of the column at 2,500 rpm for 2 min. The biotin-BSA complex was similarly conjugated with Alexa Fluor 647, and FL-labeled biotin-BSA conjugates were purified with same processes as noted above, and used as an internal control for the assay system. The FL-labeled mAb and the FL-labeled biotin-BSA were mixed together with the assay buffer to form the detection buffer and were kept at 4°C until use.

### Immunoassay Strip, Cartridge, and Scanner

The FL-ICA test strip was fabricated in-house to fit into a disposable cartridge and a laser FL scanner. The sample pad and the absorption pad were cut to a size of 4 ×

20 mm and assembled with mAb- and streptavidin-coated NC onto a polystyrene-backing card. The capture mAb and streptavidin were dispensed as 1-mm-wide lines at the test line and the control line, respectively, using a BioJet dispenser (BioDot, Irvine, CA). The assembled strip was kept in a dry vacuum chamber overnight before being placed into a cartridge (15 × 90 mm), which was designed to fit into the holder of the laser FL scanner. The cartridge was then sealed in a foil pouch containing a desiccant and stored at room temperature. **Because the appearance of the test cartridge was unique, a laser FL scanner called *i*-CHROMA™ (Boditech Med, South Korea) was used to measure the distribution of FL intensity along the strip of cartridge. The principle of the *i*-CHROMA™ FL scanner was previously described in detail (16).**

### Assay Procedure

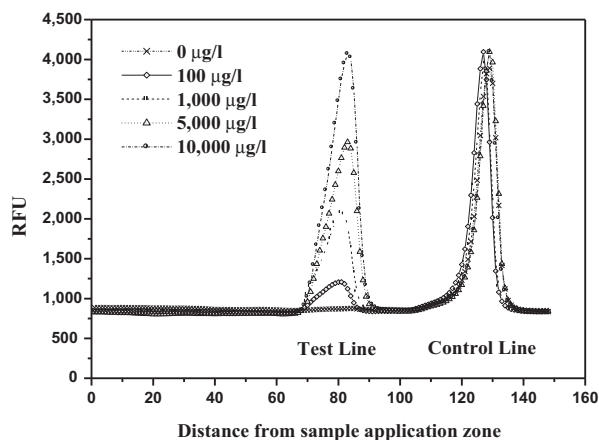
The detection buffer was a mixture of FL-labeled mAb (detector Abs) and FL-labeled biotin-BSA (internal control) in PBS. A quantity of 10  $\mu$ l from whole blood (plasma and serum) was added to 150  $\mu$ l of detection buffer, and 75  $\mu$ l of the mixture was then loaded onto the sample well of cartridge. After 12 min of incubation for immune reactions, the cartridge was inserted into the laser FL scanner for detection of FL intensity. The scanner converts FL intensity to numeric data, calculates the relative amount, and displays the level of D-dimer in the sample as ng/ml on the screen.

### Blood Sample

Blood samples were obtained from individuals who visited the Kangwon National University Medical Center in Chuncheon, South Korea. Informed consent was obtained from volunteers before their participation in the study. The ages of all adult female and male participants were between 32 and 80. Venous blood was collected in 5 ml vacuum tubes (Becton-Dickinson, Franklin Lakes) containing sodium citrate, K<sub>2</sub>-EDTA, or sodium/lithium heparin, and was centrifuged at 3,600 × *g* for 10 min at 4°C to obtain plasma samples. The blood samples were generally tested within 30 min of collection; otherwise, plasma or serum samples were aliquoted in small volumes and frozen at −70°C before analysis.

### Method of Comparison and Statistics

The concentrations of D-dimer in the blood samples were measured with *i*-CHROMA™ and compared with the concentrations of D-dimer obtained with bioMerieux Vidas assay, bioMerieux Diagnostics, Marcy l'Etoile, France, Diagnostica Stago Stalia assay, Diagnostica Stago Inc., Parsippany, NJ, and Nycocard assay, Axis-Shield,



**Fig. 1.** The scanning profiles of RFUs in the FL-ICA system for D-dimer. The RFUs at the test and the control lines are plotted on the Y-axis. An arbitrary distance from the sample well of cartridge is plotted on the X-axis to generate two peaks to measure test and control concentrations. The RFUs of the test lines increase as concentrations of D-dimer in the samples increase. In contrast, the RFUs of the control lines remain same at different concentrations of D-dimer, indicating that FL-labeled biotin-BSA and streptavidin in assay system work properly as an internal control.

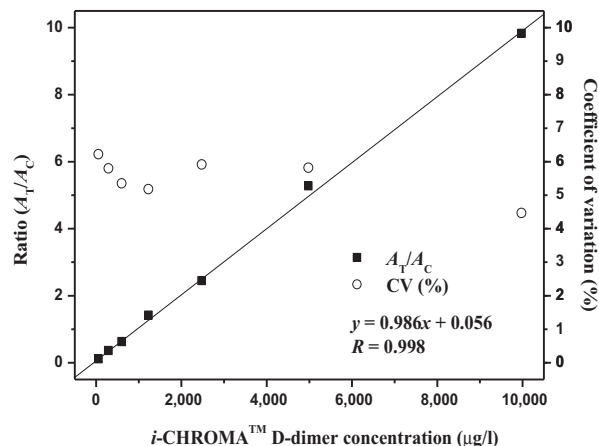
Oslo, Norway. MedCalc version 7.6 software (Mariaekerke, Belgium) and Microsoft Excel 2010 (Redmond, WA) were used for analysis and comparison of test results. Pearson correlation coefficients ( $r$ ) and linear regression with the least-squares method were used to evaluate correlations between methods.  $P$  values  $<0.05$  were considered significant.

## RESULTS

### Characterization of FL Immunoassay for D-dimer

The developed FL-ICA system was tested over a wide range of D-dimer concentrations (0–10,000  $\mu\text{g/l}$ ). Figure 1 shows a profile of relative fluorescence units (RFUs) at the test and control lines from the scanned test cartridges. The RFUs of the test lines (left peak) increase gradually as the concentrations of D-dimer increase. Conversely, the RFUs at the control lines (right peak) remain constant at different concentrations of D-dimer, indicating that the interaction between FL-conjugated biotin-BSA and streptavidin is independent of D-dimer concentrations in samples and they thus function as a good internal standard.

For the calibration curve, the RFUs displayed at the test and control lines were converted into the area values (test:  $A_T$ , control:  $A_C$ ), and the area ratios ( $A_T/A_C$ ) were plotted against the concentrations of D-dimer. A reliable correlation coefficient ( $r$ ) was observed between the area ratio value and the D-dimer concentration ( $r = 0.998$ ), and the expected linearity was displayed throughout the



**Fig. 2.** The calibration curve obtained from the area ratio ( $A_T/A_C$ ) against the concentration of D-dimer. It demonstrated the linear coefficient correlation ( $r$ ) between the area ratio ( $A_T/A_C$ ) and D-dimer concentration, and CVs of the area ratio at various D-dimer concentrations. The spiked points for calibration curves (■) and for CVs (○) were obtained from the mean values of ten independent experiments at each D-dimer concentration.

entire tested D-dimer range, as shown in Figure 2. The coefficient of variation (CV) was  $<7\%$  for ten independent experiments at different concentrations of D-dimer. The limit of detection for the assay system was 30  $\mu\text{g/l}$  of D-dimer, calculated as mean value plus 3 SD of a zero calibrator, and was comparable to those of other reported assay methods (17). Additionally, the limit of quantification, which was the lowest concentration measured with a CV  $<10\%$ , for the FL-ICA D-dimer system was 50  $\mu\text{g/l}$ . The FL-ICA system did not show a “hook effect” even at a D-dimer level of 10,000  $\mu\text{g/l}$ , and the working dynamic range of the system was determined to be 50–10,000  $\mu\text{g/l}$ . These together demonstrate that the new FL-ICA system successfully quantifies blood concentrations of D-dimer in a clinically relevant range and can be used as a tool for the quantification of D-dimer in samples.

### Performance Tests of the FL Immunoassay for D-dimer

The recovery, precision, and interference tests described below were carried out to evaluate the technical performance of the new FL immunoassay system for D-dimer. Samples were prepared from two serum pools for a recovery test. The high pool was prepared using a combination of spiked samples with an adjusted D-dimer concentration of 4,000  $\mu\text{g/l}$ , and the low pool was a D-dimer-free serum. 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%. Twenty replicates in one analytical run were tested at each D-dimer level. Table 1 shows the test result of

TABLE 1. Recovery of the FL-ICA System for D-Dimer

	<i>i</i> -CHROMA™ D-dimer (μg/l)		
	Measured concentration <sup>a</sup>	Expected concentration	Recovery (%)
100%	4,113.76	4,000	102.8
50%	2,097.22	2,000	104.9
25%	1,057.28	1,000	105.7
12.5%	529.67	500	105.9
6.25%	247.85	250	99.1
3.12%	123.14	125	98.5
0%	0.00	0	—

<sup>a</sup>Mean value of 20 replicates.

TABLE 2. Precision of the FL-ICA System for D-Dimer

Concentration (μg/l)	Whole blood					
	Intra-assay			Interassay		
	Mean <sup>a</sup>	SD	CV	Mean <sup>a</sup>	SD	CV
100	100.28	7.43	7.41	99.21	7.82	7.88
1,000	1,017.32	76.13	7.48	995.79	74.92	7.52
5,000	5,048.26	333.33	6.60	4,922.66	332.01	6.74
10,000	9,429.85	524.59	5.56	9,338.35	542.31	5.81

<sup>a</sup>Mean value of 20 replicates.

the comparison between the measured and the expected values of D-dimer. A mean recovery of control was 103% with a linearity maintained throughout the measuring range ( $r = 0.992$ ), which was consistent with the result obtained from the calibration curve of Figure 2.

The precisions of the intra- and interassay were evaluated to determine the accuracy of the FL-ICA system. The intra-assays were performed on 20 replicate tests for each D-dimer concentration of 100, 1,000, 5,000, and 10,000 μg/l. The interassays were carried out on ten sequential days, two runs per day, with 20 replicates at each concentration. The intra- and interassay CVs in the FL-ICA assay system were, respectively, 7.41% and 7.88% at 100 μg/l, 7.48% and 7.52% at 1,000 μg/l, 6.60% and 6.74% at 5,000 μg/l, and 5.56% and 5.81% at 10,000 μg/l, as shown in Table 2. These results suggest that the FL-ICA system for D-dimer is comparable to other automated reference assays with CVs <10% in the dynamic range (18). Specifically, CVs <8% for all tested values in intra- and interassays indicated that the FL-ICA assay could be applied for the exclusion of DVT or PE; assay imprecisions with CVs <10% in automated or semi-automated immunology analyzers have been proposed as acceptable tools to determine risk stratification (19).

Even though the FL-ICA system for D-dimer quantification was developed for on-the-spot testing using whole blood from a fingertip or venous blood, it can addition-

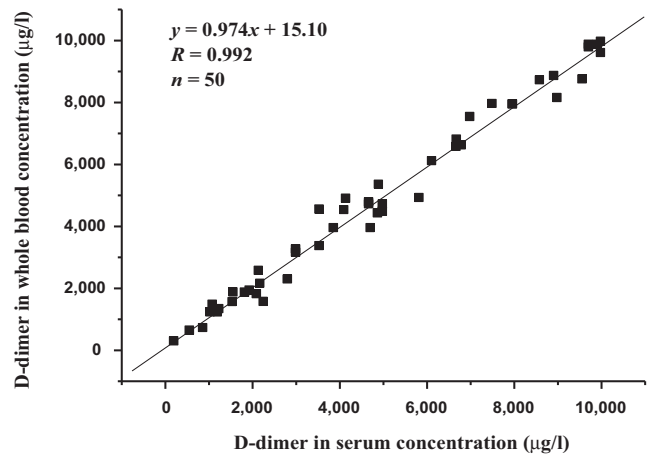
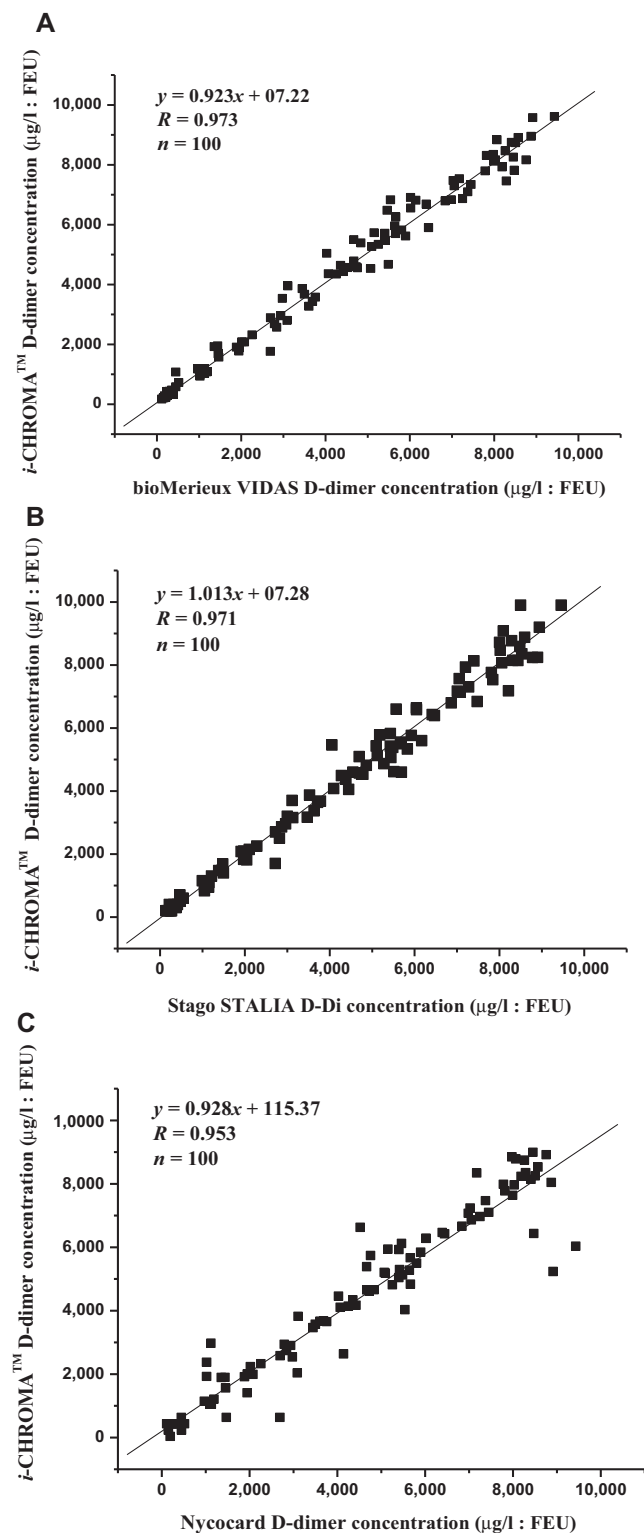


Fig. 3. Comparison of the D-dimer levels in serum and whole blood from the new FL-ICA system. The 50 samples were divided into two tubes, and one was used for measuring the D-dimer level of whole blood and the other for measuring the D-dimer level of serum in the FL-ICA system. Concentrations of D-dimer in 10 μl of serum were compared and plotted against those of 10 μl of whole blood. The coefficient correlation ( $r$ ) between them was 0.992 ( $P < 0.001$ ).

ally accept serum and plasma because the three different calibration curves for these media are provided depending on the cartridge batches. The correlation of D-dimer concentration between serum and whole blood from the same individual was evaluated. As shown in Figure 3, the correlation coefficient ( $r$ ) obtained from 50 samples was 0.992, suggesting that the levels of D-dimer for serum and whole blood were in good agreement in the FL-ICA system.

### Comparability of FL Immunoassay to Other Reference Assays

The developed FL-ICA system for D-dimer was compared with several automated and semi-automated assay analyzers that are widely being used: the enzyme-linked FL assay on Vidas (bioMérieux), the chromogenic and immunological assay on Stalia (Diagnostica), and the immunoturbidimetric assay on Nycocard. The levels of D-dimer from plasmas of 100 blood donors were measured at the same time and analyzed in each system according to each specific instruction manual. The results of all comparison plots between the *i*-CHROMA™ D-dimer assay method and the other D-dimer assay methods are shown in Figure 4. There were highly significant correlations between the assay methods of Vidas, Stalia, or Nycocard and the FL-ICA with  $r = 0.973$ ,  $r = 0.971$ , and  $r = 0.953$ , respectively. Other articles also reported that the compared D-dimer assay methods against the *i*-CHROMA™ D-dimer assay agreed well with other D-dimer immunoassay methods (18). Therefore, these test results demonstrate that the FL-ICA system for D-dimer



**Fig. 4.** Comparisons between (A) bioMérieux Vidas, (B) Diagnostica Stalia, and (C) Nycocard (X-axis), and *i*-CHROMA™ (Y-axis) for D-dimer concentration in samples. A total of 100 samples were analyzed for D-dimer levels side by side and simultaneously. FEU, fibrinogen equivalent units.

provided a confident performance that parallels other current systems in use.

## DISCUSSION

As diagnostics for D-dimer are being increased at healthcare centers as well as emergency departments, a new trend toward the assay of whole blood instead of serum or plasma is also increasing (20, 21). Many commercial automated or semi-automated analyzers that employ immunological methods have limitations on sample application (e.g., plasma, serum, or whole blood only) or quantitative single sample analysis (14, 15). Thus, the goal of this study was to develop a new FL immunoassay system for D-dimer that met a platform of POCT by using whole blood for the clinical application. The developed FL-ICA system was different from traditional ICA by capillary action in that the FL chromogen was adopted for the detection of D-dimer, FL-conjugated molecules were integrated into the detection buffer, and the level of antigen in the sample was measured quantitatively from the intensity of FL in a laser FL scanner.

In the present study, the FL immunoassay system for D-dimer offers rapid analysis within a 12 min turnaround time and exhibits a competent accuracy when compared to automated reference analyzers, as shown in Table 2 and Figure 4. The dynamic range of 50–10,000  $\mu\text{g/l}$  and cut-off value of 500  $\mu\text{g/l}$  in the FL immunoassay system are comparable with other automated analyzers and allow the users to detect not only low D-dimer concentrations in healthy people, but also high D-dimer levels in individuals suffering from DVT, PE, or cardiovascular disease (22). It meets the criteria of POCT, including affordable cost, a disposable device, and requiring minimum maintenance to perform test (23). In addition to a short turnaround time, the assay has the advantages of using serum/plasma as well as whole blood and as little as 10  $\mu\text{l}$  of blood, which may be easily drawn from a fingertip (Fig. 3). Triage D-dimer (Alere, San Diego, CA) is the only available D-dimer assay on the POCT platform using whole blood, but it requires 250  $\mu\text{l}$  of blood for measurement (24).

The majority of automated immunology analyzers for D-dimer currently available use plasma. In case of plasma, they recommend to use the sample that has been stored with a specific anticoagulant since the levels of D-dimer are not consistent among the presence of different anticoagulants, presenting a potential problem for comparing values (25). Triage also recommends that blood samples stored only in EDTA-treated tube are used. When D-dimer levels from plasma samples ( $n = 70$ ) treated with heparin, EDTA, or sodium citrate were compared with those from whole blood, they showed strong correlation coefficients between them, indicating that different anticoagulants did not influence D-dimer levels of samples in the



FL immunoassay (Supplementary Information Data 1). The FL-ICA system for D-dimer also exhibited no significant interference from common interfering serum. Five interfering substances were tested at concentration beyond physiological levels: bilirubin (150 mg/l), hemoglobin (10 g/l), glucose (1,200 g/l), L-ascorbic acid (300 mg/l), and albumin (100 g/l). None had a significant impact on the results of the assay when compared to blood without interfering substances (CV <5%, data not shown).

It is important to note that the results obtained from different D-dimer immunoassays are not interchangeable. Variations among immunoassays largely reflect the specificity of different antibodies, reagent specificity, and differences in assay methods. Thus, it is impossible to compare directly between clinical trials using different D-dimer assays due to lack of standardization and each assay should be validated and compared to a clinical gold standard. **We have compared *i*-CHROMA™ D-dimer assay to assays by Vidas, Stalia, and Nycocard, and believe that this assay accurately reflects actual D-dimer concentrations in blood, serum, and plasma.** And the slight variability observed from strip to strip at the same level of D-dimer may be attributed to differences in membrane properties, component aging, humidity effects, and many other factors (26, 27). Thus, setting an internal standard is essential to minimize this variability.

In conclusion, the *i*-CHROMA™ D-dimer assay method demonstrates excellent performances in precision, dynamic working range, and results compared to other currently available commercial immunology analyzers. The developed FL-ICA system for D-dimer with POCT platform is an easy, fast, and reliable method for measurement of D-dimer concentration in whole blood.

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