

Fluorescence immunoassay using an optical fiber for determination of *Dermatophagoides farinae* (*Der f1*)

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Abstract Immunoassay methods are generally used for measuring of allergenic substances. However, they need special facilities, skilled handling, and time-consuming procedure. In this work, a fiber-optic immunoassay system which could mea-

sure allergen by fluorescent intensities of immune complexes formed by allergens and fluorescently labeled antibodies was established. Immune complexes absorbed on the optical fiber probe surface, and excitation light was injected into the probe, then evanescent field is created in the proximity of the probe. The fluorophores were excited by the evanescent light, and fluorescence was detected by a photo diode. The target allergen detected by our system was *Der f1* derived from *Dermatophagoides farinae* that is one of the house dust mite and major source of inhaled allergens. The fluorophore used labeling on detecting antibody was cyanine 5. The system enabled to detect and quantitatively determine of *Der f1*. The measurement range was from 0.24 to 250 ng/ml, and the result competes with ELISA. The measurement time was 16 min/sample. The immunoassay system was applied to measurement of *Der f1* from actual dust samples. Calculated values of *Der f1* showed good correlations between the fiber-optic fluoroimmunoassay and ELISA.

Keywords Optical fiber · Evanescent wave · *Dermatophagoides farinae* · *Der f1* · Immunoassay

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Introduction

Allergies such as bronchial asthma and allergic rhinitis are serious global health problem

(Richard and ISAAC 1998; Janson et al. 2001; Togias 2003; Bousquet et al. 2001; Tattersfield et al. 2002). Particularly, a number of people living in the industrial countries have these allergies. With lifestyles changing, the prevalence and morbidity of allergy has been increasing progressively in many places of the world. For example, air pollution caused by factories and cars, hypoventilation caused by super-insulated houses, and suppression of the immune system caused by ingestion of a protein-rich or energy-rich diet can cause allergy (De Swert 1999; Wardlaw 1993; Schwela 2000; Carrer et al. 2001; Platts-Mills et al. 1997; Gergen et al. 1988). The first step of treatment for allergies is elimination and avoidance of environmental allergens. To show the current pollution caused by them and to improve the environment, we must have the indicator which can evaluate the level of contamination and the effect of environmental improvement.

Currently, immunoassay methods are generally used for measurement of allergenic substances in environment (Asturias et al. 1999; Neuner-Plattner et al. 1999; Chapman et al. 1987a, b; Luczynska et al. 1989). In particular, enzyme-linked immunosorbent assay (ELISA) is major methodology for allergen analysis. The ELISA is established method for its quantitative characteristic and reliability. However, the ELISA requires technical skill and time-consuming manipulation for enzymatic reaction (Curry et al. 1979; Kalhola et al. 1985; Eskola et al. 1985; Sohail et al. 2000) and still lack the ability to detect objects in real time.

In recent years, novel techniques for detection of allergens have been designed. Fluorescent multiplex array technology is one of those techniques (Earle et al. 2007). This technique enabled to measure multiple allergens simultaneously at the same time. Compared with ELISA, this technology requires less total assay time, fewer procedural steps, and a small sample volume. However, it also lacks the ability to detect objects in real time. The fiber-optic fluorescent immunoassay system, designed by Canon Chemicals Inc., is also one of novel immunological measurement techniques using evanescent wave (Golden et al. 1997; Leskinen et al. 2009). The principle of operation for the system is a sandwich immunoassay that uses tapered fiber-optic probes coated with

capture antibodies and fluorescent dye-labeled antibodies. The system holds potential to meet the need for simple, rapid, sensitive, and antigen-specific real-time detection for its structural design and assay principle. Several investigations have already been demonstrated using the same principle of the fluorescent immunoassay system, such as *Escherichia coli* O157:H7 (Geng et al. 2006; DeMarco and Lim 2001), *Salmonella* (Valadez et al. 2009), and *Listeria monocytogenes* (Geng et al. 2004). However, to our knowledge, there has been no report about detection of environmental allergens using the fluorescence immunoassay system.

In this paper, we present data on the detection of *Der f1*. *Der f1* is derived from *Dermatophagoides farinae*, so-called house dust mite, and it is one of the most important indoor allergen (Chapman et al. 1987a, b; Platts-Mills and Chapman 1987; Platts-Mills et al. 1992; Platts-Mills and Weck 1989; Voorhost et al. 1964; Stephenson 1991; Frankland 1972; Cunningham 1971; Takeda et al. 1993). The immunoassay method for *Der f1* using the fiber-optic fluorescence immunoassay system was established and enabled to measure *Der f1* with rapidity and sensitivity.

Experimental

Optical fiber pretreatment

The polystyrene optical fiber probes (4 cm in length and 0.78 mm in diameter) were contributed by Canon Chemicals Inc. Capture antibodies were absorbed onto the polystyrene optical fiber probes. Commercial monoclonal antibodies (Anti-*Der f1* mAb 6A8, Lot 2828, 2 mg/ml, INDOOR Biotechnologies Ltd.) were used as a capture antibody. Probes were immersed into capture antibody solution (100 μ l) in General, 10 μ g/ml antibodies in 80 mmol/l carbonate-bicarbonate buffer (pH 9.5). After overnight incubation at 4°C, probes were rinsed with phosphate-buffer (PB; pH 7.4) and then incubated with PB containing 1% bovine serum albumin (BSA; albumin, from bovine serum, Lot 401-044, 10 g, ITOHAM FOODS Inc.) to block nonspecific binding sites on the probe.

Preparation of Cy5-labeled antibody

Cyanine reagent is known to be useful as fluorescent labels for biological compound (Mujumdar et al. 1993; Southwick et al. 1990). Commercial monoclonal antibodies (Anti-*Der f1* mAb 4Cl, Lot 2469, 2 mg/ml, INDOOR Biotechnologies Ltd.) were used for fluorescent-labeled antibody. A labeling kit (Cy5 Ab Labeling Kit, PA 35000, GE Healthcare UK Ltd.) was used for labeling antibodies with cyanine 5 (Cy5) dye according to manufacturer's instructions. Cy5-conjugated antibodies were prepared by 0.6 mg of protein (1 mg/ml) in 1 mol/l sodium carbonate buffer, pH 9.3 with one vial Cy5 bisfunctional dye ($\lambda_{\text{ex}} = 649 \text{ nm}$, $\lambda_{\text{em}} = 670 \text{ nm}$) for 30 min with mixing approximately every 10 min at room temperature in the dark. Then, the labeled protein was separated from un-conjugated dye by gel filtration column. Final dye-to-protein (D/P) ratio was 4.77 (Cy5 molecules per IgG) calculated based on the absorption at 280 and 650 nm. The labeled antibody was diluted with PB containing 1% BSA and 0.1% Tween 20 and stored at 4°C until used.

Other reagents

Purified protein of *Der f1* derived from *D. farinae* (*Der f1* standard, Lot 2761, 2500 ng/ml) was purchased from INDOOR Biotechnologies Ltd. (Manchester, UK). *Der f1* was diluted from 0.244 to 250 ng/ml with PB containing 0.05% Tween 20 (PBT). Wash buffer of probes used after the immersion into capture antibodies was PB, and that used at the immunoassay process performed by the fiber-optic immunoassay system was PBT.

Principle and process of the fluorometric immunoassay

The measurement was performed using The PU6102 that is robotic fluorometric assay system designed by Canon Chemicals Inc. The assay principle is based on a sandwich immunoassay, using optical fiber probes coated with capture antibodies and Cy5-labeled antibodies for fluorometric detection. A laser diode that contained exciting

wavelength of Cy5 was launched into the optical fiber probe through the lens attached on the end of the probe. The evanescent field is created along the surface of the probe, and the evanescent light excited the Cy5 labeling antibodies specifically bound antigens. The fluorescent light recoupled into the probe was collected by a photodiode through an optical filter rejecting reflected laser light (Fig. 1). The signal was saved to disc on a computer in picoamps.

The probes coated with capture antibodies, containers of Cy5-labeled antibodies (100 μl), and containers of *Der f1* solutions (100 μl) were placed into discrete chambers of the immunoassay system. At the beginning of the assay, the optical fiber probe with capture antibody was rinsed with PB, and established a initial value (step I). The probe was incubated in Cy5-labeled antibody for 5 min to obtain a background signal generated by nonspecific binding of the fluorescent antibody (step II). The probe was then rinsed with wash buffer PBT and incubated with *Der f1* (0.244 to 250 ng/ml) for 5 min as primary antigen-antibody reaction (step III). The probe was then rinsed with PBT to eliminate unbound *Der f1* and incubated with Cy5-labeled antibody for 5 min again as secondary antigen-antibody reaction (step IV; Fig. 2). The probe was moved to the wash-and-read position after each assay reaction in the each specific chamber, and the fluorescence of the probe was read for 20 s after washing with PBT for 15 s. The entire assay time, including all wash steps,

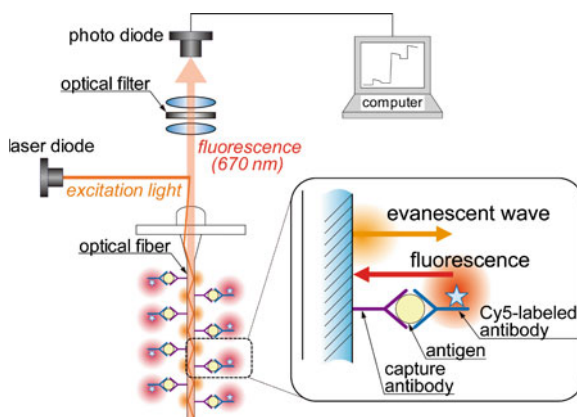
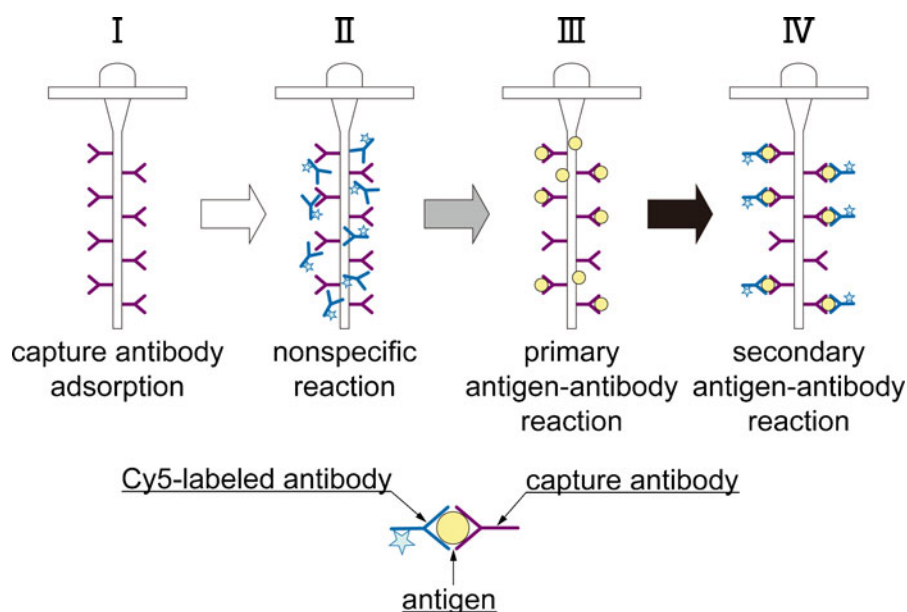


Fig. 1 Fluorescent immunoassay system using optical fiber probe

Fig. 2 Process of the *Der f1* measurement by the fluorescence immunoassay system



was completed in about 16 min. The fluorescence of each step was average value calculated by final 5 s pA of each 20 s reading.

ELISA procedure

As a popular method of conventional immunoassay, ELISA was demonstrated to detect *Der f1*. The ELISA was performed according to the manufacturer's recommended protocol. First, capture antibodies (2 µg/ml) were bound to a microtiter plate wells (polystyrene microplate, 96-well, greiner bio-one Co., LTD.) to create the solid phase. Unbound antibodies were removed by washing the plate (PBT) and blocking reagent (1% BSA-PBT) was added. Following washing the plate, *Der f1* standards (0.244 to 250 ng/ml) were then added and incubated with the solid phase antibodies. After washing away unbound *Der f1*, biotin conjugated detection antibodies (2 µg/ml; biotinylated Anti-*Der f1* mAb 4C1, Lot 2401, INDOOR Biotechnologies Ltd.) were added. Binding these detection antibodies to *Der f1*, the sandwich immune complexes were completed. Following wash to remove unbound detection antibodies, detection reagent of streptavidin-peroxidase (0.25 µg/ml; Streptavidin-

Peroxidase from *Streptomyces avidinii*, S5512, Sigma-Aldrich, Inc.) was added. The plate was washed, a substrate solution (1 mmol/l 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonate acid) diammonium salt (ABTS) in 70 mmol/l citrate phosphate buffer; ABTS, A1888, Sigma-Aldrich, Inc.) was added and color developed in proportion to the amount of bound *Der f1*. The absorbance of the well at 405 nm was read by a microplate reader (SH-1000 Lab, CORONA ELECTRIC Co., LTD.).

Dust sampling procedure and allergen detection

Der f1 levels in actual dust samples were evaluated using the fiber-optic fluoroimmunoassay system and ELISA. A disposable dust bag was attached to a tube of a vacuum cleaner (Toshiba, VC-PX8F(P)), and areas in home, such as living room or bedroom floor carpet, and hallway or closet floor, were vacuumed at a rate of 1 m²/min. Dust samples were weighed and suspended in phosphate buffer containing 0.1% Tween 20 (1:10 weight/volume for dust samples). After centrifuging the dust suspension (2,000 × g, 10 min), the supernatant was measured with the fluoroimmunoassay system and ELISA.

Results and discussion

Optimization of the fiber-optic immunoassay system

To reduce nonspecific background and improve sensitivity for detection of *Der f1* by the fluorometric immunoassay system, diluting buffer of capture antibody and concentration of the capture antibody solution were optimized. First, appro-

prate diluting buffer was selected. In general, it is said that more alkaline protein solution than the isoelectric point is suited to coat the solid phase. Carbonate–bicarbonate buffer was thus selected for diluting capture antibodies. First, the pH of the carbonate–bicarbonate buffer was selected and decided to use at 9.5 (data not shown). Figure 3a shows the optimum concentration of the carbonate–bicarbonate buffer (pH 9.5) for diluting capture antibody. At the concentration of 80 mmol/l carbonate–bicarbonate buffer, the strongest fluorescent signal enhancement after secondary antigen–antibody reaction was obtained. Figure 3b shows the optimum concentration of the capture antibody solution. At the concentration of 10 $\mu\text{g/ml}$ capture antibody, the strongest fluorescent signal enhancement after secondary antigen–antibody reaction was obtained, and at the higher concentration over 10 $\mu\text{g/ml}$, the fluorescence was progressively decreasing. Figure 4 shows the difference of fluorescence intensity between (1) unoptimized condition (2 $\mu\text{g/ml}$ capture antibody in PB (10 mmol/l, pH 7.4)), (2) after optimization of the concentration of carbonate–bicarbonate

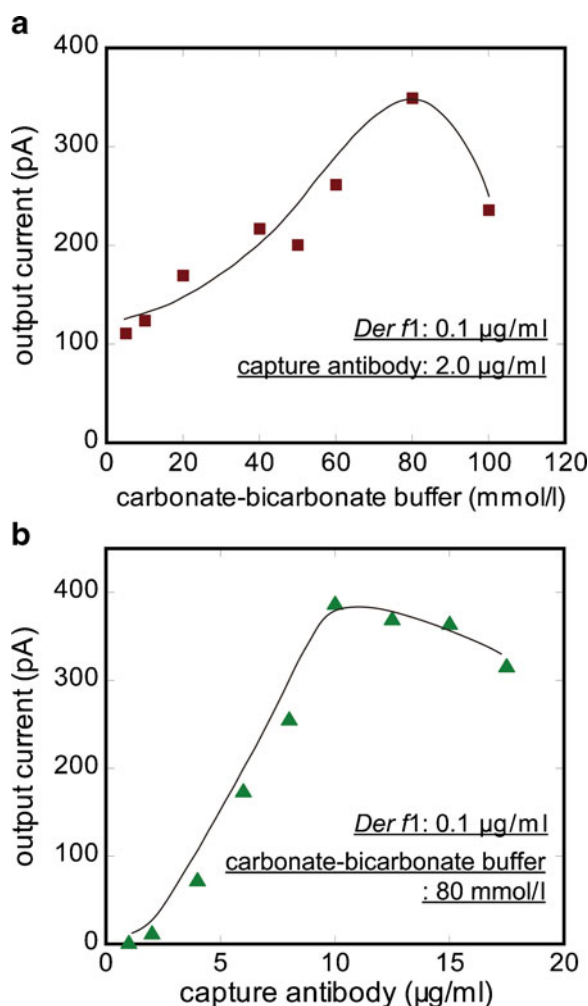


Fig. 3 Optimization of the immunoassay system. **a** Optimization of the concentration of carbonate–bicarbonate buffer for diluting capture antibody. **b** Optimization of the concentration of capture antibody to immobilize on the probe surface

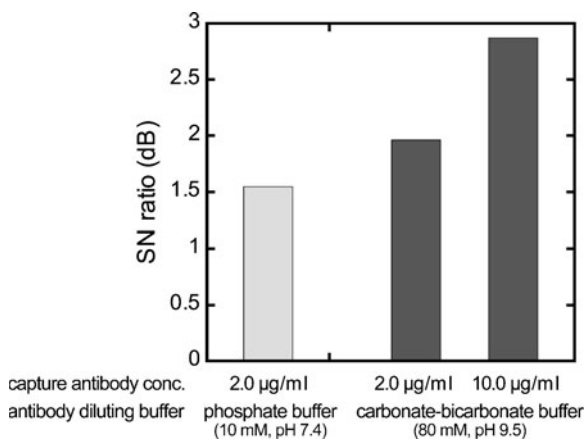


Fig. 4 Difference of fluorescence intensity between (1) the unoptimized condition (2 $\mu\text{g/ml}$ capture antibody in PB (10 mmol/l, pH 7.4)), (2) after optimization of the concentration of carbonate–bicarbonate buffer (80 mmol/l, pH 9.5), and (3) after optimization of the concentration of capture antibody (10 $\mu\text{g/ml}$) diluted carbonate–bicarbonate buffer (80 mmol/l, pH 9.5)

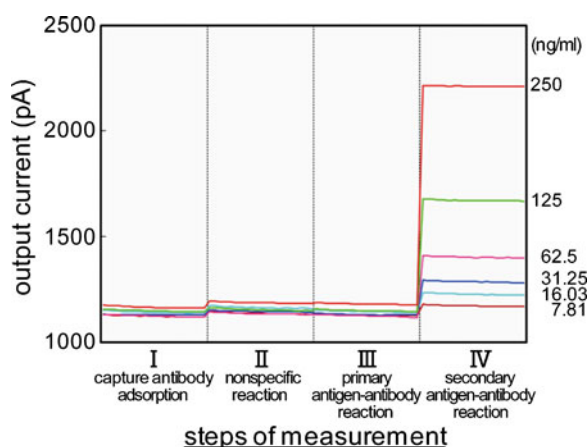


Fig. 5 Signal responses to *Der f1* measured by the fluorescence immunoassay system. Step I is initial value of the probe, step II is checking nonspecific reaction on the probe, step III is primary antigen-antibody reaction's current value and step IV is secondary antigen-antibody reaction's current value. Measurement time at each step is 20 s

buffer (80 mmol/l, pH 9.5), and (3) after optimization of the concentration of capture antibody (10 μ g/ml) diluted with carbonate-bicarbonate buffer (80 mmol/l, pH 9.5). At the condition of (3), the strongest fluorescence intensity was obtained all over the three conditions investigated. Consequently, the capture antibody solution was diluted to 10 μ g/ml in carbonate-bicarbonate buffer (80 mmol/l, pH 9.5) and used later in all experimentation.

Der f1 measurement by the immunoassay system

Figure 5 shows variation of fluorescent intensity corresponding to *Der f1* concentrations at each measurement step. The nonspecific reaction was hardly confirmed at step II, and stable current values at every four steps were obtained. *Der f1* concentration of 250 ng/ml gave the strongest signal enhancement after secondary antigen-antibody reaction, and the signal strength decreased as the *Der f1* concentration decreased. The reaction time in the Cy5-labeled antibodies and *Der f1* standards was 5 min, respectively, and the measurement time of each step was 20 sec. Therefore, the total measurement time of the immunoassay system was about 16 min and 20 sec. The immunoassay system was less time-consuming to

measure allergen than ELISA, which needed long reaction time for each reagent (such as samples, detection antibodies and substrate solution). Figure 6 shows sigmoidal calibration curves measured by the immunoassay system and ELISA. The values measured by the system were the differences between step III (primary antigen-antibody reaction) and step IV (secondary antigen-antibody reaction), and the values measured by ELISA were absorbance at 405 nm. The sigmoid curves were fitted using nonlinear regression analysis. The correlation coefficients of the curves by the fiber-optic immunoassay system and ELISA were 1.000 and 0.999, respectively. The equations were as follows:

$$\text{output current (pA)} = -293.3 + 1116.1 / (1 + \exp(-([Der f1 \text{ (ng/ml)}] - 57.5)/57.6))$$

$$\text{absorbance} = -1.9 + 8.8 / (1 + \exp(-([Der f1 \text{ (ng/ml)}] + 2.78)/40.4))$$

The lowest allergen concentration that gave a positive signal compared to a control (no *Der f1*) was 0.24 ng/ml and was considered to be the lower detection limit for the system and ELISA. The upper limit of ELISA was 250 ng/ml, but the value

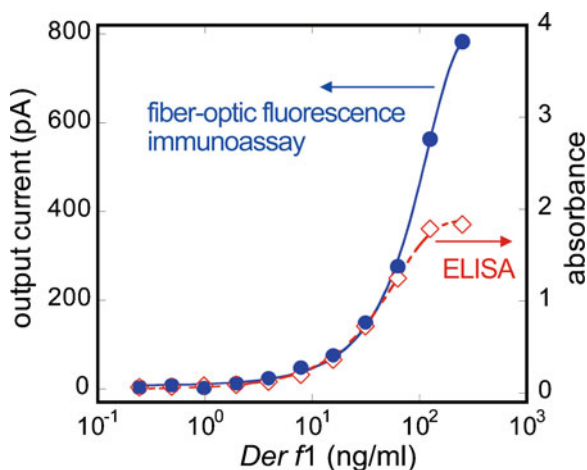


Fig. 6 Calibration curve for *Der f1* measured by the immunoassay system and ELISA. The value measured by the system is the differences between step III (primary antigen-antibody reaction) and step IV (secondary antigen-antibody reaction), and the value measured by ELISA is absorbance at 405 nm

at the same *Der f1* concentration measured by the system was not saturation point yet. Therefore, it suggested that the immunoassay system has larger dynamic range than conventional method of ELISA.

In addition, variance of the fiber-optic immunoassay using different optical fiber probes ($n = 5$) at the same *Der f1* concentration (31.25 ng/ml) was investigated. The probe was made from polystyrene and usually used as disposable. Therefore, output current was examined when different fibers measured the same concentration solution of *Der f1*. The result showed good reproducibility and coefficient of variation was 6.98% (data not shown).

D. farinae is one of the major components of house dust. In particular, it has been reported that the protein in mites' deposits and shells causes allergy like bronchial asthma and allergic rhinitis. Therefore, rapid and sensitive methods are required to detect these allergenic substances. However, conventional methods are time-consuming. Several immunological methods are sensitive and specific but often expensive and cumbersome. Rapid method such as immune chromatographic assay cannot indicate accurate concentration of allergen.

In this study, we constructed a fiber-optic immunoassay system for *Der f1*. Commercial monoclonal antibodies were used as capture antibodies and Cy5-labeled antibodies. Preparation for the probe and measuring system avoided to do complicated procedures.

The measurement range for *Der f1* diluted with PB was from 0.24 to 250 ng/ml. The system could perform every measurement of sample within about 16 min. In the ELISA for detection of *Der f1*, while the detection limit is similar to the immunoassay system, long time is required to get results more over 2 to 3 h. This fiber-optic immunoassay system could be one of the best methods to meet the need for rapid, sensitive, and simple detection system.

Der f1 detection from actual dust samples using the immunoassay system

The fiber-optic immunoassay was applied to testing of actual dust samples. The result is shown in

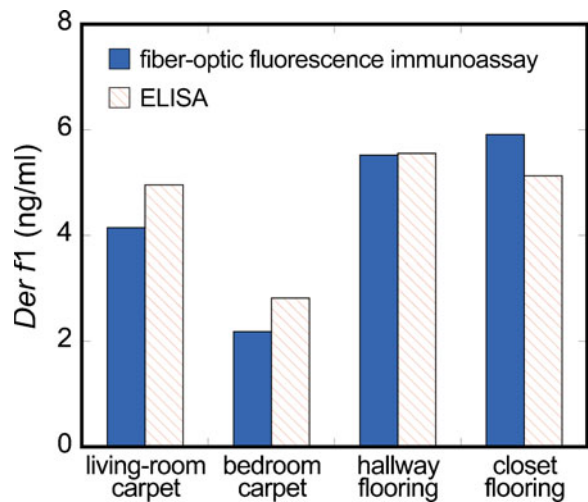


Fig. 7 Correlation between the fluorescent immunoassay system and ELISA about detecting *Der f1* in actual dust samples collected from house

Fig. 7. Concentrations of *Der f1* in each sample were calculated by current values or absorbance values using calibration equations mentioned above. Calculated values of *Der f1* showed good correlations between the fiber-optic fluoroimmunoassay and ELISA. This investigation showed that the fluoroimmunoassay system was usable to detect *Der f1* from actual house dust samples.

Conclusions

Fluorescence immunoassay methods using evanescent wave recently have found attention. These methods, in general, possess high sensitivity and need only small sample volume.

In this work, the fiber-optic fluorescence immunoassay method for detection of *Der f1* was established. The measurement range for *Der f1* diluted with PB was from 0.24 to 250 ng/ml. For improving the sensitivity of the immunoassay, experimental conditions was optimized. Consequently, a quick and stable response and high reproducibility were acquired in the optimized immunoassay. Measurement time for every sample was about 16 min. The fiber-optic fluorescence immunoassay has been proved to be much faster than that of conventional method. The immunoassay system was applied to detection of *Der f1*

from actual house dust samples. Concentrations of *Der f1* calculated by regression equations showed good correlations between the fiber-optic fluoroimmunoassay and ELISA. Therefore, the immunoassay system was useful for analysis of actual samples containing various contaminating substances.

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