

A highly sensitive quantitative immunochromatography assay for antigen-specific IgE

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Abstract

We have developed a highly sensitive quantitative enzyme immunochromatography system for antigen-specific IgE, which is clinically important for the diagnosis of allergic diseases. The system uses alkaline phosphatase-labeled anti-IgE antibody (ALP–anti-IgE) and immobilized target antigen, for example cedar pollen antigen, on a membrane. Antigen-specific IgE present in the serum binds the immobilized antigen after complexing with the ALP–anti-IgE. Subsequently, the enzyme substrate migrates to the complex on the antigen line, which is stained blue. The intensity of the staining was analyzed by a quantitative detector, but can also be assessed directly by the naked eye. This system was able to detect 0.2 U/ml of IgE specific for Japanese cedar pollen. The results correlated well (Spearman's rank correlation coefficient, 0.95) to those in AlaSTAT system, which is a reliable enzyme-linked immunosorbent assay (ELISA) method. This antigen-specific IgE assay system is suitable for point-of-care testing.

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1. Introduction

The number of allergic patients has been increasing recently, requiring the development of a simple and inexpensive method to assay antigen-specific IgE. Since the radioallergosorbent test (Wide et al., 1967) was developed, many types of assay system for

antigen-specific IgE have been devised, such as CAP® (Pharmacia Diagnostics, Uppsala, Sweden) and AlaSTAT® systems (Diagnostic Products Corporation, Los Angeles, CA, USA). Although certain assay systems, including the two mentioned above, provide reliable quantitative results (Plebani et al., 1998), they involve rather complicated procedures and require costly instrumentation. In contrast, the immunochromatography assay is simple, and can be used for point-of-care testing. Although most available immunochromatography systems are for qualitative evaluation, there are some systems suitable for quantitative analysis. For example, serum concentration of some drugs can be monitored by the AccuLevel system (Syntex Medical Diagnostics, Palo Alto, CA, USA) in which the length of the line stained with

Abbreviations: ALP–anti-IgE, alkaline phosphatase-labeled anti-IgE antibody; BSA, bovine serum albumin; CJ, *Cryptomeria japonica*; CV, coefficient of variation; EICA, enzyme immunochromatography assay; ELISA, enzyme-linked immunosorbent assay; P%R, peak % reflectance; SBP, Sugi basic protein.

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enzyme substrate on the membrane is proportional to the concentration of analyte (Fairchild et al., 1991). Another immunochromatography system has recently been released by Roche Diagnostics (East Sussex, UK) for the quantification of serum proteins such as troponin T. In this system, the concentration can be quantified by measuring the density of colloidal gold in terms of color intensity (Müller-Bardorff et al., 1999).

In the field of allergy, there are some immunochromatography tests for serum total IgE such as EasiLine™-Immunoglobulin E (Nubenco Diagnostics, Paramus, NJ, USA). However, to our knowledge, there is no commercially available kit or report on quantitative immunochromatography system for antigen-specific IgE. Therefore, we tried to establish a simple immunochromatography method for this purpose. In the present experiments, we used a protein fraction of Japanese cedar pollen (*Cryptomeria japonica*, CJ) as the antigen. CJ pollen is one of the major causative agents of allergic rhinitis, conjunctivitis, and bronchitis in Japan, and the prevalence rate in the Japanese population is estimated by random sampling to be as high as 13% in the year 2001 (Okuda et al., personal communication).

2. Materials and methods

2.1. Target antigen for immunochromatography

A protein fraction of CJ pollen termed Sugi basic protein (SBP) was obtained according to the methods described previously (Yasueda et al., 1983). It was confirmed that the fraction contained both Cry j 1 and 2, which are considered to be two major allergens in CJ pollen (Hashimoto et al., 1995).

2.2. Antibody

Alkaline phosphatase-labeled anti-IgE antibody (ALP-anti-IgE), mouse monoclonal IgG antibody, was supplied by Diagnostic Products Corporation.

2.3. Serum samples

Serum samples were obtained from 70 individuals including patients with CJ pollinosis from whom informed consent was obtained after the nature and

possible consequences of the studies had been fully explained. These sera were stored at -20°C until use. Concentrations of CJ pollen-specific IgE in these sera were first assayed by the AlaSTAT system using the CJ pollen-antigen reagent provided in the kit. AlaSTAT is an ELISA system. In this system, patient antibody reacts first to biotinylated antigen in the fluid phase, the complex binds to biotinylated solid phase by the addition of avidin, and then the presence of antigen-specific IgE is detected by enzyme-labeled anti-IgE antibody. Sera without detectable CJ pollen-specific IgE were used as negative controls. Reference samples containing CJ pollen-specific IgE were also prepared by dilution of the serum strongly positive for the antibody with the pooled negative sera. These samples were prepared to contain 0.20, 0.35, 0.70, 3.50, 17.50, and 52.50 U/ml of CJ pollen-specific IgE in terms of the AlaSTAT system with reference to the WHO Second International Reference Preparation for Human Serum IgE, 75/502.

2.4. Enzyme immunochromatography assay system

IgE in the test serum first reacts with the ALP-anti-IgE. During migration through the immunochromatography membrane, ALP-anti-IgE bound to SBP-specific IgE is captured by SBP immobilized in a line on the membrane. The alkaline phosphatase substrate, a mixture of 5-bromo-4-chloro-indolyl-phosphate and nitroblue tetrazolium (BCIP/NBT Phosphatase Substrate; Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA), migrating from the substrate reservoir pad into the membrane, then reacts with the ALP-anti-IgE to stain the SBP line. We refer to this system as the enzyme immunochromatography assay (EICA) in this report.

2.5. Immobilization of SBP on membrane

The SBP preparation was dialyzed against 5 mM borate buffer (pH 8.5), and diluted to a concentration of 2 mg/ml. The protein concentration was determined by Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL, USA) with reference to bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA).

SBP was sprayed onto an Immunodyne ABC membrane, BC300H5R, (50 × 300 mm; Pall Europe,

Portsmouth, UK) using BioJet 3000™ (BioDot, Irvine, CA, USA) in a 1-mm-wide line. After drying at 37 °C for 2 h, the membrane was treated for 15 min at room temperature with 5 mM phosphate buffer (pH 7.5) containing 0.5% (w/v) BSA, 0.02% (w/v) polyvinyl alcohol (Wako, Osaka, Japan), and 0.2% (v/v) Triton X-100 (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was then dried at 37 °C for 2 h, and was kept in a desiccator at room temperature until use.

2.6. Preparation of conjugate pad with ALP–anti-IgE

ALP–anti-IgE was diluted to a concentration of 2 µg/ml with 5 mM phosphate buffer (pH 7.5) containing 10% (w/v) trehalose (Merck, Darmstadt, Germany), 4% (w/v) BSA, 0.02% (w/v) polyvinyl alcohol, 0.2% (v/v) Triton X-100, 1 mM MgCl₂, and 0.1 mM ZnCl₂. Twenty microliters of the ALP–anti-IgE solution was deposited on a polyvinyl pad (5 × 8 mm, BELL-ETA, Kanebo Chemical Industries, Osaka, Japan). The pad was dried on a stainless steel wire gauze at 37 °C for 2 h, and kept in a desiccator at room temperature until use.

2.7. Preparation of the immunochromatography strip

Cellulose sheets, WF1.5 (Whatman, Fairfield, NJ, USA) and AP25 (Millipore, Bedford, MA, USA), were cut into sizes of 30 × 300 mm for the absorbent

sheet and 20 × 300 mm for the substrate reservoir sheet, respectively. Both sheets were assembled with the SBP-immobilized membrane on a laminate card kindly provided by Millipore. The assembled sheet was cut into strips 5 mm in width using Cutter 3000™ (BioDot), and the ALP–anti-IgE conjugate pad was located on the center of the membrane. The assembled strip is shown in Fig. 1.

2.8. Assay procedure

First, 25 µl of the serum to be assayed was applied to the ALP–anti-IgE conjugate pad of the assembled strip. Soon after application, 150 µl of the enzyme substrate mixture mentioned above was loaded onto the substrate reservoir pad. Twenty minutes after serum application, the color of the SBP-immobilized line on the membrane turned blue if the serum contained SBP-specific IgE. The color intensity of the stained blue line was confirmed by observation with the naked eye, and quantified by an immunochromatography detector, QuadScan (BioDot), measuring the reflectance of red light (586–660 nm) according to the manufacturer's instructions. Results are expressed as peak % reflectance (P%R), which is the difference of detection line reflectance and background reflectance.

2.9. Statistical analysis

Detection limits were evaluated by one-sided *t*-test. Correlation of class scores was analyzed by Spear-

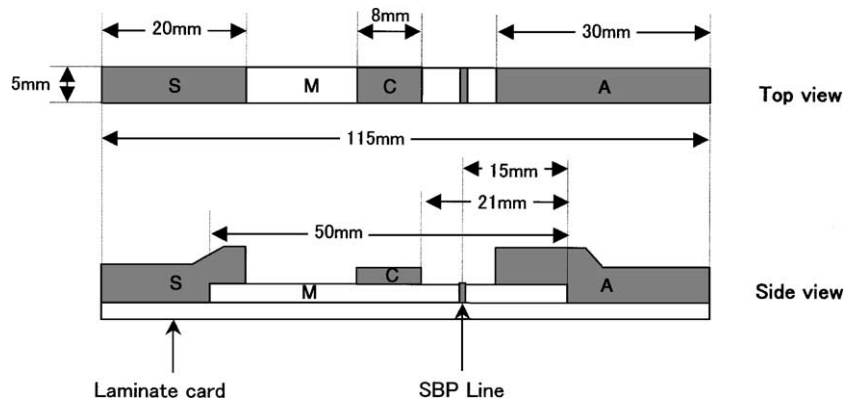


Fig. 1. Structure of EICA strip. S: substrate reservoir pad, M: SBP-immobilized membrane, C: conjugate pad with ALP–anti-IgE, A: absorbent pad.

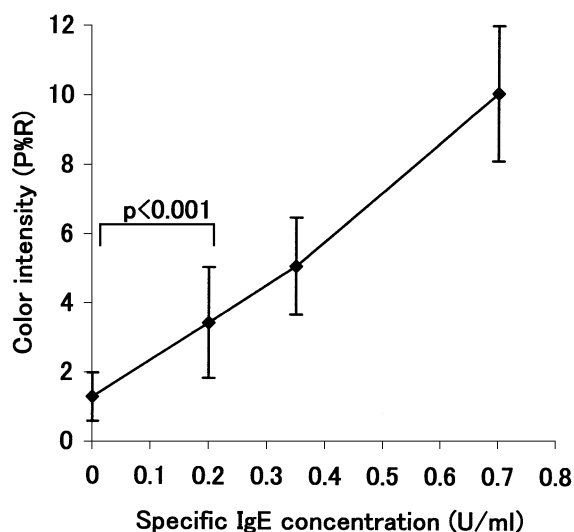


Fig. 2. Detection limits. Negative serum and three reference samples (0.20, 0.35, 0.70 U/ml) were assayed in EICA five times each. SBP line color intensity in EICA was expressed as P%R. Concentration of the specific IgE of the negative serum was expressed as 0 U/ml. Data are presented as the average $\pm$ 2S.D. of five measurements.

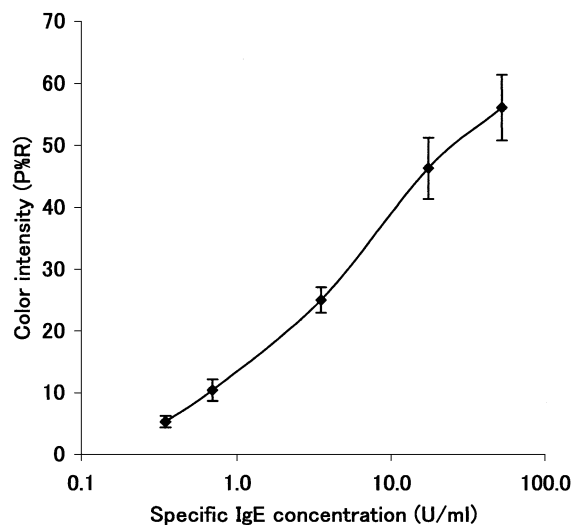


Fig. 3. IgE-dose dependent reactivity. Five reference samples (0.35, 0.70, 3.50, 17.50, 52.50 U/ml) were assayed in EICA. The color intensity in EICA was expressed as P%R. Data are presented as the average $\pm$ 2S.D. of four measurements. The abscissa is logarithmic.

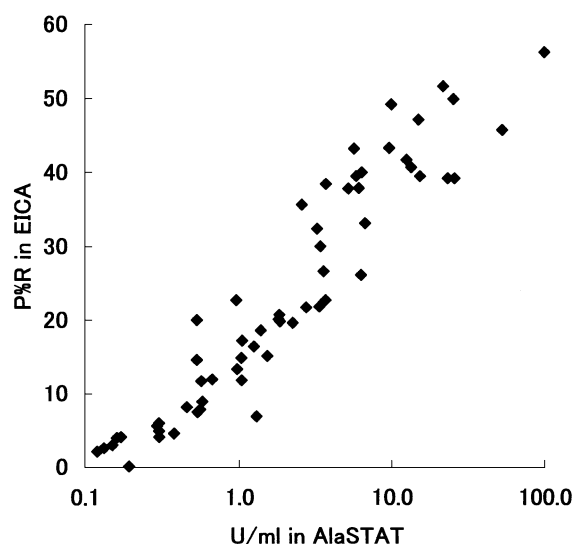


Fig. 4. Correlation between EICA (P%R) and AlaSTAT (U/ml). The abscissa is logarithmic, and results lower than 0.1 U/ml in AlaSTAT and 2 P%R in EICA ($N=10$) are not shown.

man's rank order correlation coefficient testing. Assay reproducibility was evaluated by calculating the coefficient of variation (CV).

3. Results

3.1. Detection limits in EICA

Reference samples containing three different concentrations of CJ pollen-specific IgE (0.20, 0.35, and 0.70 U/ml) and the sample without detectable IgE in terms of AlaSTAT system were assayed five times each by EICA. The color intensity of the stained SBP

Table 1
Classification of results in EICA and AlaSTAT systems

EICA		AlaSTAT	
P%R	Class	U/ml	Class
<5.0	0	<0.35	0
5.0–9.9	1	0.35–0.69	1
10.0–24.9	2	0.70–3.49	2
25.0–44.9	3	3.50–17.49	3
45.0 $\leq$	4	17.50–52.49	4
		52.50–99.99	5
		100 $\leq$	6

EICA class score	4				2	4
	3			3	13	2
	2		4	14	1	
	1	2	4	1		
	0	19	1			
		0	1	2	3	4–6
		AlaSTAT class score				

Fig. 5. Correlation between class scores in EICA and AlaSTAT ($N=70$). Classification of the results in EICA is defined in Table 1. Class 4, 5, and 6 in AlaSTAT were treated as one class score. Spearman's rank correlation coefficient was 0.95.

line was analyzed by QuadScan and also assessed by the naked eye.

In the QuadScan analysis, the reference samples containing 0.20 U/ml or more of CJ pollen-specific IgE showed significantly positive signals ($p<0.001$) (Fig. 2). By the naked eye, we were able to observe stained blue lines in all five strips to which serum containing 0.20 U/ml of CJ pollen-specific IgE had been applied, although we did not observe any color development at all with the negative sera. We carried out similar experiments with similar results using three different sets of reference samples prepared from different positive sera (data not shown).

Table 2
Intra-assay reproducibility

Reference serum (U/ml)	Intra-assay reproducibility (mean $\pm$ S.D.)					Mean coefficient of variation (%)
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	
0.35	5.2 $\pm$ 0.8	5.2 $\pm$ 0.3	5.4 $\pm$ 0.5	5.0 $\pm$ 0.7	4.9 $\pm$ 0.8	12
0.70	10.1 $\pm$ 1.1	11.7 $\pm$ 1.3	10.7 $\pm$ 0.8	10.7 $\pm$ 1.0	10.1 $\pm$ 1.2	10
3.50	20.1 $\pm$ 1.7	19.7 $\pm$ 1.4	20.5 $\pm$ 1.4	21.7 $\pm$ 2.1	19.5 $\pm$ 1.4	8
17.50	52.8 $\pm$ 5.5	45.4 $\pm$ 3.1	54.1 $\pm$ 4.1	48.1 $\pm$ 6.1	50.4 $\pm$ 4.4	9

Four reference samples (0.35, 0.70, 3.50, 17.50 U/ml) were assayed in EICA four times each. The assay was repeated five times on five different days (Experiments 1–5). The mean coefficient of intra-assay variations was calculated using the average of each coefficient of variation of 5 days run.

These results indicate that our EICA system reproducibly detects CJ pollen-specific IgE with a sensitivity of at least 0.2 U/ml.

3.2. Specific IgE dose-dependent reactivity in EICA

The reference samples containing five different concentrations of CJ pollen-specific IgE (0.35, 0.70, 3.50, 17.50, and 52.50 U/ml in AlaSTAT) were assayed in our EICA system four times each, and the staining intensity was analyzed by QuadScan. The results are shown in Fig. 3. We carried out similar experiments using eight other different reference samples, and obtained similar results (data not shown).

These data show that the results obtained from EICA system correlate well to the concentrations of CJ pollen-specific IgE measured by AlaSTAT.

3.3. Correlation between the EICA and AlaSTAT

To confirm further the correlation between results obtained in EICA and AlaSTAT, the amount of antigen-specific IgE in the sera of 70 individuals including patients with CJ pollinosis were quantified by both systems. As shown in Fig. 4, when the results in EICA system were expressed in P%R and those in AlaSTAT in U/ml, the results obtained in the two systems correlated very well to each other.

3.4. Class score in EICA system

Data on antigen-specific IgE are commonly evaluated in terms of class score. Therefore, we scored the data in the EICA analysis and compared the results with those in the AlaSTAT system. The results expressed as P%R in QuadScan analysis were classified

Table 3
Inter-assay reproducibility

Reference serum (U/ml)	Inter-assay reproducibility (mean $\pm$ S.D.)	Coefficient of variation (%)
0.35	5.1 $\pm$ 0.2	4
0.70	10.7 $\pm$ 0.7	6
3.50	20.3 $\pm$ 1.0	4
17.50	50.1 $\pm$ 3.7	7

Coefficient of variation was calculated from the average of each intra-assay data.

into five class scores (class 0, 1, 2, 3, 4) as shown in Table 1. Class scores of 70 sera in EICA corresponded well to those in AlaSTAT when class scores 4, 5, and 6 in AlaSTAT were taken together to arrange the numbers of class score for both assay systems to same (Fig. 5). The Spearman's rank correlation coefficient was 0.95.

3.5. Assay reproducibility

Intra- and inter-assay variations in the data (P%R) obtained from EICA were assessed by the analysis of four reference samples containing 0.35, 0.70, 3.50, and 17.50 U/ml of CJ pollen-specific IgE. Each sample was assayed in quadruplicate, and the assay was repeated five times, once a day. The mean coefficient of intra-assay variation ranged from 8% to 12% (Table 2), and that of inter-assay variation from 4% to 7% (Table 3). Room temperature during the 5 days varied from 21 to 28 °C, and there was no significant correlation between the temperature and results obtained (data not shown).

We also evaluated the precision of the detector, QuadScan, by measuring each strip 10 times using four different strips with different concentrations of the CJ pollen-specific IgE mentioned above. The coefficient of variation ranged from 0.8% (17.5 U/ml specific IgE) to 3.7% (0.35 U/ml specific IgE), indicating that variation was greater for strips with lower color intensity.

4. Discussion

In the present study, we developed a highly sensitive quantitative immunochromatography assay system to detect antigen-specific IgE. In our EICA system, we

were able to assay at least as a low concentration of SBP-specific IgE equivalent to 0.20 U/ml CJ pollen-specific IgE. This was done by quantitative analysis with QuadScan, but the staining of the antigen line could also be confirmed by the naked eye. This level of sensitivity is almost comparable to prevalent assay systems such as CAP and AlaSTAT. We first explored the immunochromatography approach using colloidal gold-labeled anti-IgE antibody; however, the sensitivity of this system was not enough to detect allergen-specific IgE. The antigen line could be observed by the naked eye in this system only when 4 U/ml or more of CJ pollen-specific IgE was applied. Thus, the EICA system we developed showed about a 20-fold higher sensitivity than the immunochromatography system with colloidal gold. Immunochromatography using antibody conjugated with alkaline phosphatase has previously been applied to qualitative assays for hepatitis B virus surface antigen (ESPLINE HBsAg; Fujirebio, Tokyo, Japan), and was reported to be more sensitive than other systems using colloidal gold conjugates (Deguchi et al., 2000). There is also one report on a total IgE assay using immunochromatography with carbon black (Lönnberg and Carlsson, 2001) in which 0.13 IU/ml of total IgE could be assayed using 30 μ l serum.

Another important feature of our EICA system is its ability to assay antigen-specific IgE quantitatively. The results in the EICA system correlated well to those obtained by the AlaSTAT system, and could be classified into five scores from 0 to 4. In the present classification, all sera with 45 P%R or more of SBP-specific IgE were classified into one class, score 4, because the P%R value in QuadScan analysis did not increase in proportion to the concentration of CJ pollen-specific IgE when the serum contained more than 17.50 U/ml of the specific IgE. Most of these samples with a P%R value greater than 45 were classified into score 4, 5, or 6 in AlaSTAT. Results in AlaSTAT and CAP systems were previously classified into five scores (van Houte and Bartels, 1992), reported to be valuable for the diagnosis of allergic diseases (Foucard and Johansson, 1976).

The assay system of our EICA is different in principle from that of AlaSTAT as described in Materials and methods. In EICA, antigen in the solid phase captures the complex of antigen-specific IgE and anti-IgE antibody, whereas, in AlaSTAT, antigen binds to

patient antibody in the liquid phase and then the complex reacts to anti-IgE antibody in the solid phase. However, the results obtained in these different systems correlated well to each other in our present experiments.

In our system, we used SBP as the immobilized antigen on the membrane. SBP is partially purified protein from CJ pollen and contains both Cry j 1 and 2 (Sakaguchi et al., 1990). Hashimoto et al. (1995) reported that all their patients with CJ pollinosis possessed IgE specific for at least one of these two proteins, and more than 90% for both of them. The good correlation between the results in EICA and AlaSTAT with the patient sera tested here also suggests that Cry j 1 and 2 are major allergens in CJ pollen, and that SBP is an appropriate protein preparation to use for CJ pollen-specific IgE assay.

Theoretically, nonspecific IgE could possibly interfere with the results in EICA, should the test serum contain a large amount of it. However, the results on nine patient sera which contain more than 1000 IU/ml total IgE (maximum 1980 IU/ml) still correlated well to those in AlaSTAT, which is not affected even when the serum contains 30,000 IU/ml of total IgE (Alaba and El Shami, 1989). These findings indicate that our system contains sufficient amount of enzyme-labeled anti-IgE antibody not to be interfered by the usual concentration of nonspecific IgE, although we have no data at present on its performance with serum containing extremely high concentration of IgE.

The intra-assay CV value of our EICA (8–12%) was not as low as the prevalent assay systems including the AlaSTAT and CAP (intra-assay CV, 2.3–7.7%) (Plebani et al., 1998). However, it was almost identical to the available quantitative immunochromatography test system for troponin T (intra-assay CV, 9–14%) (Müller-Bardorff et al., 1999), indicating that the reproducibility of our assay system is practically permissible.

Taken together, these results indicate that the EICA system is useful for diagnosis of CJ pollinosis, though it remains to be studied whether results in EICA will correlate with clinical symptoms.

The EICA system has a number of advantages over other methods for antigen-specific IgE assay. First, it is possible to assay IgE antibodies against different antigens in a sample at the same time by the immobilization of antigens in different lines. In preliminary

experiments, we were able to assay IgE antibodies against two different kinds of antigens. Indeed, house dust mite antigen immobilized upstream of SBP line did not affect the color intensity of SBP line immobilized downstream. The color intensity of SBP line without the house dust mite line was 45.5 ± 2.8 (P%R, average $\pm$ 2S.D., $N=5$), and that of SBP with the upstream house dust mite line was 43.7 ± 2.5 ($p=0.06$, both sided t -test). A representative result of these experiments is shown in Fig. 6. There is already a test available to assay IgE antibodies against different antigens at the same time using cellulose thread (MAST™ system; Hitachi Chemical, Tokyo, Japan). However, this system requires an overnight incubation and some special instruments (Nakagawa et al., 2000).

Another advantage of our system is that only 25 μ l serum is sufficient to assay IgE antibodies for different antigens. The EICA system may therefore be suitable for the screening of causative agents of allergic disease in babies by using small amount of blood spotted onto a filter paper.

The third advantage of our EICA system is that it enables us to assess the concentration of antigen-specific IgE as class score by the naked eye when color intensity reference standards are provided. For medical institutions, our EICA system may make it easier to assay causative agents of allergy in a short time without the necessity for investing in any special equipment or outsourcing. A noninstrumented antigen-specific IgE test, the Quidel allergy screen (Fujir-

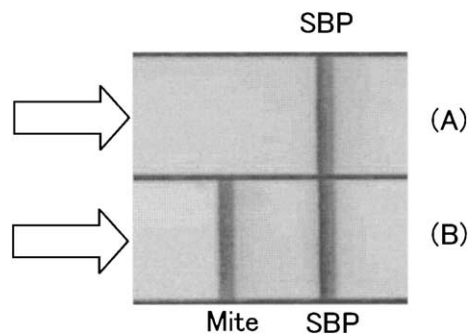


Fig. 6. No interference of upstream antigen with downstream antigen line in EICA. The color intensity of SBP antigen line was not significantly interfered by house dust mite line immobilized upstream of SBP line (B) compared to that of the SBP line immobilized alone (A). Arrows indicate the flow direction.

ebio, Tokyo, Japan), is now available. Quidel allergy screen is a dipstick system, which bears several sheets with different antigens. Although different kinds of antigen can be assayed on the dipstick, it requires more than five steps of manual operation and 19 h to obtain the result (Iwamoto et al., 1990).

We developed the EICA system to assay antigen-specific IgE with high sensitivity. The quantitative results showed a good correlation to those obtained by a conventional ELISA method. Because the assay procedure for this system is very simple, it may be useful for point-of-care testing for antigen-specific IgE.

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