

Chapter 7

Detection of Known Allergen-Specific IgE Antibodies by Immunological Methods

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Summary

An increasing number of patients are suffering from allergic diseases such as rhinoconjunctivitis, atopic eczema, urticaria, anaphylaxis, and food and drug allergies. Although it is possible to measure a multitude of allergen-specific IgE antibodies by radio or enzyme immunoassays in the patients' blood, these tests are expensive, time-consuming, and usually need a rather high volume of reagent solutions (allergens and blood). Protein microarrays offer the possibility to circumvent these limitations. The described in vitro allergy testing system is based on microscopic glass slides activated with glycidyloxypropyltrimethoxysilane. Allergen solutions (allergen extracts and/or purified allergens; approximately 10 nL) are printed on the activated glass surface with a piezoelectric spotting machine. The protein components of the allergen solutions are immobilized on the modified glass surface via hydrophobic interaction and/or covalent binding. After a blocking step, the slides are incubated with the respective diluted serum sample (approximately 25 µL serum required) and bound IgE antibodies are detected with a secondary horseradish peroxidase (HRP) labelled anti-human-IgE antibody via chemiluminescence. The measurement can be performed automatically with the so called *PASA system*. Test results are directly visualized with a CCD-camera. Analytical and clinical data have shown that the microarray-based test format offers significant advantages in time and costs compared with traditional test formats. The described allergen microarray demonstrated a sufficient qualitative reproducibility and enabled the distinction between allergic and non-allergic patients. Detection limits of 0.35 kU/L (r Bet v1), 0.16 kU/L (PLA₂), 1.9 kU/L (Der p1), and 41 kU/L (total IgE) were achieved.

Key words: Serum IgE, In vitro allergy diagnosis, Immunoassay, Recombinant allergens, Purified allergens (Der p1, Der f1, PLA₂), Allergen extracts, Chemiluminescence, Protein microarrays, Protein chips.

1. Introduction

Elevated levels of immunoglobulin E (IgE) play a key role in the development of allergic diseases. IgE was identified in the mid sixties of the last century by Ishizaka and Johansson et al.

(1, 2). After contact with the allergen the immune system starts to produce IgE antibodies against the specific allergen. These antibodies bind to receptors on the surface of mast cells. The cell degranulation is triggered by the bridging of cell-bound IgE antibodies via the specific allergen. This results in the release of chemical substances such as leucotrienes, histamine, and prostaglandins that cause smooth muscle contraction, itching, swelling, and transmucosal leakage of extracellular fluids. The most common clinical manifestations are hay fever, asthma, hives, and anaphylactical shock (3).

The majority of allergenic substances consist of water-soluble proteins with molecular masses of 5–70 kDa. Aqueous extracts are prepared by suspending the raw material (e.g. pollen, fruits, and nuts) in physiological buffer solutions while continuously shaking (4). Extracts are mixtures of different allergenic and non-allergenic proteins, and the quality (raw material used, contaminations and storage conditions) of the extract strongly influences the biological activity (5). The combination of two-dimensional gel electrophoresis together with immunoblotting techniques allowed the determination and purification of the major allergenic compounds of several allergen mixtures (6). Recombinant DNA technology offered the possibility to isolate allergen-encoding cDNAs. In eucaryotic or prokaryotic expression systems different recombinant allergenic proteins were produced (7). As a result most of the important allergens from trees, grass pollens, mites, animal epithelia, insect venoms, and foods have been cloned, sequenced, and expressed in the past (8–10). It was shown by further studies that recombinant allergens are useful for the diagnosis and therapy of allergic diseases (11).

The diagnosis of allergic disease is performed either *in vivo* or *in vitro* (12). For the *in vivo* diagnosis the patient is brought into direct contact with the allergenic substance using different techniques, e.g. prick or challenge tests. *In vivo* tests bear the risk of resulting in an anaphylactical reaction, which could be very dangerous for the patient. For that reason, *in vitro* immunoassays are a safer alternative.

Since 1967, when the first assay (RAST) for serum immunoglobulin E (IgE) and allergen-specific antibodies in serum was described (13), different immunoassay-based procedures (e.g. modified labels, surfaces) were tested (14–18). Radioactive labels were replaced by chromogenic (EIA) or fluorescent (FIA) detection labels. In clinical routine the most common immunoassay is the “CAP system-specific IgE FEIA test” (Pharmacia Upjohn, Uppsala Sweden) for total and allergen-specific IgE. The CAP system consists of a cellulose polymer densely loaded with allergen extracts or recombinant allergens (6). The results are expressed in arbitrary units (kU/L) or specific IgE classes as shown in [Table 1](#).

Table 1
Specific IgE classes

Specific IgE class	Specific IgE ^a (kU/L)
0	<0.35
1	0.35–0.69
2	0.70–3.49
3	3.50–17.50
4	17.50–52.5
5	52.5–100
6	>100

^a1 IU/L is equivalent to 2.4 ng IgE/L

Other commercially available methods are based on either liquid phase inhibitor assays (e.g. ALASTAT, DPC Biermann, Los Angeles, USA) or multi-allergen coated nitrocellulose strips (e.g. IgEquick, Teomed AG, Greifensee, Switzerland; CMG Immunodot, Trimedal AG, Brütisellen, Switzerland). Certain characteristics of these assays, notably large sample volume in combination with time-consuming and intensive protocols, render them unsuitable for diagnostic tests requiring the ability to determine hundreds of allergenic substances in a timely and cost-effective manner in a single run.

Multi-allergen dipstick tests based on nitrocellulose strips were a first step of miniaturisation and cost saving, but mostly lack automatisation. Microdeposition technologies enabled the defined immobilization of small protein volumes in the low nanoliter range on a modified surface (19). Twenty years ago, Ekins proposed the application of the microarray technology in the field of medical diagnosis (20). However, the lack of necessary instrumentation delayed the realization of miniaturized immunoassays. Therefore, the first experimental microarray system for allergy diagnosis was not reported before 2000 (21). Recently, a fluorescence-based microarray technique was published (22).

Here, the authors describe a method for the development of chemiluminescence-based microarrays for the simultaneous detection of serum IgE antibodies to various common allergen extracts and recombinant allergens using the so-called *PASA system* (23).

2. Materials

2.1. Equipment

1. Piezoelectric spotting machine (GeSIM, Großerkmannsdorf, Germany): The spotting system consists of a three-dimensional stage, piezoelectric nanoliter pumps (standard micro pipette SPIP, manufactured by silicon etching), and a liquid handling system (syringe pumps) for priming and washing the silicon pumps. A standard 96-well microtiter plate was used as reagent reservoir for the diluted solutions.
2. Equipment for on-line measurement (*PASA system*, [Fig. 1](#)):
 - (a) Flow chamber: 4.5 cm × 5.5 cm × 2.0 cm, PEEK painted black; reaction area: 4.5 cm × 2.0 cm × 0.1 cm.
 - (b) Sealing (0.5-mm silicon).
 - (c) Flow system: air trap bubble trap (Trace-trap, Trace, Braunschweig, Germany); XL smart valve 3+ (Cavro Scientific Instruments, Sunnyvale, CA, USA); XL 3000

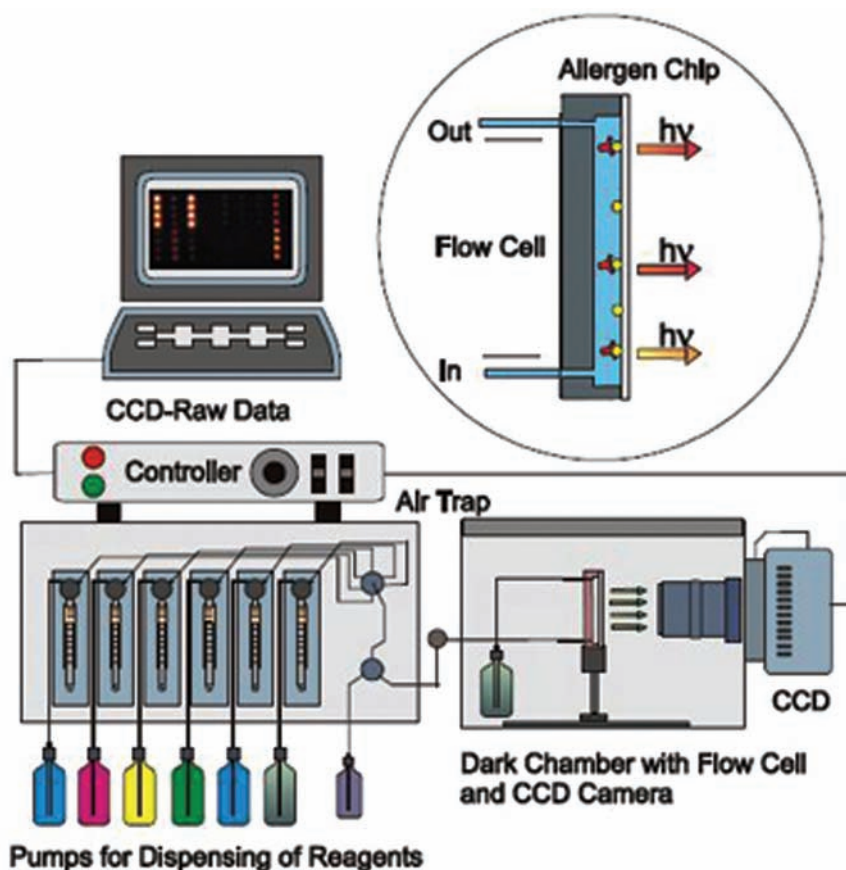


Fig. 1. General setup of the parallel-affinity-sensor-array (PASA) system including close-up of the flow cell.

modular pump (Cavro); Teflon tubing 1/16 in. $\times$ 0.25 mm (Upchurch Scientific, Postnova Analytics, Landsberg, Germany).

- (d) CCD system: Objective (50 mm, 1:1.4D, AF Nikkor, Nikon Precision Europe, Langen, Germany); Software Winview (Version 1.6.2, Spectroscopy & Imaging GmbH, Erwitte, Germany); TE/CCD – 1100PB/VISAR/1 CCD-detector: grade 1, back-illuminated, VIS-to-NIR coating; 1,100 $\times$ 300 pixels (Spectroscopy & Imaging).
- 3. Equipment for off-line measurement: Hybri-slips: 22 mm $\times$ 40 mm (Sigma-Aldrich, Deisenhofen, Germany), Hybri-well chamber slides (180–200 μ L, Sigma-Aldrich).
- 4. Equipment for ELISA:
 - (a) Microtiter plate washer: Columbus washer (SLT, Crailsheim, Germany).
 - (b) Microtiter plate reader: eight-channel photometer (TR 340, SLT, Overath, Germany).

2.2. Common Reagents and Materials

1. Raw pollen material: *Alnus glutinosa* (black alder), *Betula verrucosa* (white birch), coryllus, avellana (hazel), *Secale cereale* (rye), *Phleum pratense* (timothy), *Triticum aestivum* (wheat), *Ambrosia artemisiifolia elatior* (ragweed common), *Malus domestica* (apple), *Felis domesticus* (cat hair), *Canis familiaris* (dog hair), *Cladosporium herbarum* (mould), *Apis mellifera* (honey bee), from Allergon, Ängelholm, Sweden; Peanuts, unroasted (local Chinese food store, Munich, Germany).
2. Purified allergens: bee venom, phospholipase A₂ (PLA₂), casein, gliadin, and BSA (Sigma-Aldrich), affinity purified Der p1 and Der f1 (Indoor Biotechnologies, Cardiff, UK).
3. Allergen extracts: prick tests for apple, celery, cat epithelia, and mould allergens (*Aspergillus fumigatus*, *Penicillium notatum*) from Allergopharma, Reinbek, Germany.
4. Recombinant allergens: r Cor a1, r Aln g1, r Phl p1, r Api g1, r Mal d1, r Bet v2, r Alt a1 (Biomay, Linz, Austria); r Fel d1 (Indoor Biotechnologies).
5. Immunoglobulins: Myeloma-IgE and purified IgG (Calbiochem-Novabiochem, Bad Soden, Germany).
6. Anti-human-IgE antibodies: monoclonal rat-anti-human-IgE antibody (clone LO-HE-10), Technopharm, Paris, France; polyclonal affinity-purified horseradish peroxidase (HRP)-labelled goat-anti-human-IgE antibody (Dunn, KPL, Reinbek, Germany).
7. Antibodies against allergenic proteins: polyclonal rabbit-anti-Betv1 antibody and monoclonal mouse anti-Betv1 antibody from ALK-Abelló, Horsholm, Sweden. For the detection

the following HRP-labelled antibodies were used: goat-anti-rabbit-IgG (Sigma-Aldrich); goat-anti-mouse-IgG (Vector Laboratories, Burlingame, NY, USA).

8. Protein tests: Bicinchonic acid (BCA) assay from Sigma-Aldrich; Micro-BCA assay (Pierce, Rockford, IL, USA).
9. Chemiluminescence reagent: SuperSignal ELISA Femto Maximum Sensitivity substrate (Pierce, Rockford, IL, USA); SuperSignal West Pico Chemiluminescence substrate (Pierce).
10. Human sera: 19 patients according to the reference measurement with Pharmacia CAP system (CAP-RAST-FEIA, Pharmacia Upjohn, Uppsala, Sweden) performed at the hospital "Biederstein". Munich was selected. Samples with and without specific IgE antibodies have been used for the determination.
11. Water HPLC grade: Millipore, Milli-Q Plus 185.
12. Washing solution: 60 mM KH_2PO_4 , 420 mM K_2HPO_4 , 870 mM NaCl, 0.3% Tween-20.
13. Printing carbonate buffer: 15 mM Na_2CO_3 , 25 mM NaHCO_3 , pH 9.8, 1% glycerol.
14. Antibody dilution buffer: PBS, 0.1% BSA, 0.01% Tween-20.
15. Ammonium hydrogen carbonate solution contains 0.015 M sodium azide, 20 mM EDTA, 5 mM ϵ -amino-caproic acid (EACA), pH 7.5.
16. Coupling buffer: 300 mM Na_2HPO_4 , pH 8.5, 0.5% trehalose, 1 mM EDTA.
17. Blocking solution: 1% BSA, 0.5% glycine in Tris-HCl, pH 8.3.
18. Microtitre plates (MTPs): Microlon MTPs (Greiner, Nürtingen, Germany); NHS amine – binding plates ("NOS", Corning, Cardiff, UK).
19. Slide-A-Lyzer dialysis cassettes, cut off: 10 kDa, Pierce.
20. Microscopic glass slides: Merck, Darmstadt, Germany.
21. Filter: cellulose acetate, 0.45 μm , Sartorius, Göttingen, Germany.

3. Methods

3.1. Cleaning of the Microscopic Glass Surfaces

The microscopic glass slides are cleaned using a 1:1 mixture of dry methanol and HCl (36%) for 30 min at ambient temperature, washed with water (HPLC grade), and immersed in H_2SO_4 (98%) for 30 min. The glass slides are rinsed with water, dry methanol and dried under a nitrogen stream.

3.2. Activation of Glass Surface with 3-Glycidyloxypropyltrimethoxysilane (3-GOPS)

Activation of the glass slides is achieved with a solution of 1% 3-GOPS in dry toluene for 18 h at ambient temperature. After washing steps with toluene and methanol, the glass slides are dried under a nitrogen stream and stored until use in a desiccator.

3.3. Extraction of Allergens

3.3.1. Raw Pollen Materials

1. For preparation of allergen extracts 10% raw pollen material (w/v) is extracted with either PBS buffer (pH 7.8) or 0.125 M ammonium hydrogen carbonate solution.
2. After suspending the material for 1 h, the medium is centrifuged ($14,000 \times g$, 10 min, 4°C). The supernatant is collected and the insoluble residue is extracted again for 1 h.
3. After the filtration step the supernatant is dialyzed (Slide-A-Lyzer dialysis cassettes) against water/PBS (10:1) for 24 h and then lyophilized.
4. The lyophilized samples can be stored at -20°C in small aliquots.

3.3.2. Prick Test Solutions

1. Prick test solutions are dialyzed (Slide-A-Lyzer dialysis cassettes) against water/PBS (10:1) for 24 h and then lyophilized.
2. The lyophilized samples can be stored at -20°C in small aliquots.

3.4. Preparation of Allergen Solutions

1. Ten milligrams of dry substance is dissolved in water/PBS (10:1).
2. To characterize the obtained allergen extracts the protein content is determined using the Micro-BCA assay.
 - (a) For this purpose the allergen extracts are diluted 1:100 to 1:1,000 with PBS.
 - (b) One-hundred fifty microlitres of this solution is added to a MTP.
 - (c) One-hundred fifty microlitres of a BSA solution (0.125–200 µg/L) is used for calibration.
 - (d) By addition of 150 µL of the Micro-BCA solution, a colour change to green-purple occurs.
 - (e) After an incubation time of 30 min at 36°C the purple colour is measured at 550 nm.

3.5. Printing and Immobilization of Protein Solutions

3.5.1 Preparation for Printing

1. Crude allergen extracts (total protein content 5–10 g/L, 1:40) and recombinant allergens (protein concentration 1–2 g/L, 1:100) are diluted with coupling buffer.
2. Positive controls: monoclonal anti-human-IgE antibody clone LO-HE-10 (1 g/L, 1:100 diluted with printing carbonate buffer + 0.01% Tween-20 protein grade), Myelome – IgE (1 g/L, 1:100 diluted with printing carbonate buffer), streptavidin-HRP (1:1,000 diluted with printing carbonate buffer).

3. Negative controls: human IgG (10 g/L, 1:1,000 diluted with printing carbonate buffer).

3.5.2. Printing and Immobilization

The printing solutions (antibodies/allergens) are prepared just before the start of the print run and transferred into a 96-well microtitre plate (Microton). The dispensing is done with a piezoelectric spotting system; five droplets; spot diameter approximately 200 μm , $U = 60 \text{ V}$, $f = 300 \text{ Hz}$. The coating is performed for 18 h at 4°C.

3.6. Determination of Immobilized Allergens

To specify whether a specific allergen is successfully immobilized on the chip surface, the protein could be detected with an allergen-specific antibody. For the detection of the major allergenic compound of Birch pollen (*Betula verrucosa*) the following procedure was successfully tested:

1. Preparation for printing: Birch pollen extracts (total protein content 5–10 g/L, 1:40) and recombinant r Bet v1 (protein concentration 1 g/L, 1:100) were diluted with coupling buffer.
2. Wash with washing solution.
3. Blocking of free surface groups: 1% BSA in PBS; 1 h at ambient temperature.
4. Incubation with diluted monoclonal mouse-anti-Bet v1 antibody (1:500 in antibody dilution buffer); 1 h at ambient temperature. Alternatively the detection could be done with the polyclonal rabbit-anti-Bet v1 antibody.
5. Incubation with diluted HRP-labelled polyclonal goat-anti-mouse-IgG antibody (1:1,000 in antibody dilution buffer), 30 min at ambient temperature. Alternatively the incubation could be done with the HRP-labelled polyclonal goat-anti-rabbit-IgG antibody.
6. Interpretation of test results: A positive signal demonstrates that the respective allergenic compound was successfully immobilized on the activated glass surface. Signal intensities for the recombinant protein are higher than the ones for the birch pollen extract. This clearly indicates that in the case of the pollen extract the available binding sites are also occupied by other proteins of the extract.

3.7. Reference Measurement: Determination of Allergen-Specific IgE on MTPs

1. Coating with allergen solution: recombinant allergens/allergen extracts (1:400/1:100 with PBS, pH 7.8 for Microton MTPs and 1:400 with Na_2HPO_4 , pH 8.5 for “NOS” MTPs), 100 μL , 18 h at ambient temperature.
2. Washing with washing solution: this step is repeated after each reaction and is not mentioned further.

3. Blocking of free surface groups: 1% BSA (*see Note 1*) in PBS (Microton MTP) or blocking solution (“NOS” MTP), 300 μ L, 2 h at ambient temperature.
4. Incubation with diluted human serum samples: 1:8 in antibody dilution buffer, 50 μ L, 1 h at ambient temperature.
5. Detection with anti-human-IgE antibody: 1:5,000 diluted HRP-labelled anti-human-IgE antibody (polyclonal, goat-anti-human, KPL), 100 μ L, 45 min at ambient temperature.
6. Measurement: The colour development (25-mL citrate buffer, 300 μ L of 1% TMB solution in methanol, 100 μ L 1% H_2O_2 solution) is stopped after 10 min with 5% H_2SO_4 solution. The absorbance is read at 450 nm with an eight-channel photometer.

3.8. Determination of Total and Allergen-Specific IgE on GOPS Slides (*see Note 2*)

1. *Washing step* with washing solution and HPLC grade water. This washing step is repeated after each reaction step and will not be mentioned further.
2. *Blocking of free surface groups* with blocking solution for 2 h at ambient temperature.
3. Incubation with human serum samples:
 - (a) Positive controls: serum samples tested positive against specific allergens using the reference measurement (Pharmacia CAP system) are diluted 1:8 in 200- μ L antibody dilution buffer and incubated in chamber slides (200 μ L, Sigma; *see Note 3*) for 30 min at 36°C. The incubation with the serum sample could also be done automatically in the *PASA system*. For this purpose a volume of 500- μ L diluted serum sample (1:8 diluted in antibody dilution buffer) is incubated for 30 min at ambient temperature.
 - (b) Negative controls: serum samples tested negative for allergen-specific IgE antibodies with the reference method (Pharmacia CAP system) or heat-denatured serum samples (serum samples treated for 2 h at 56°C; *see Note 4*) are used.
4. *Detection with anti-human-IgE antibody*: the antibody is diluted 1:500 in antibody dilution buffer; 250 μ L are applied with a cover slip and incubated for 30 min at ambient temperature.
5. *Detection of bound IgE antibodies* in the flow cell of the *PASA system*: 500- μ L chemiluminescence substrate is pumped into the flow cell.
6. *Evaluation of test results*: Light emission is accumulated for 60 s using a 2×2 binning. Data evaluation is performed with the software “Imagetool” (version 2.0, University of Texas health Science Center, San Antonio, TX, USA). A trimmed mean

(25%) in combination with a winsorized standard deviation is used to calculate robust statistical parameters (24). Using this method the standard error of the mean for the streptavidin-HRP signals on a single chip surface should be between 2.6 and 7.6%. In previous studies between 17 GOPS slides the standard error was 19.7% (mean) or 7.9% (trimmed mean, winsorized sample mean). Some outliers could explain these results.

7. *Calibration of the test results:* As internal standard, the intensities detected for the single allergen-specific IgE antibodies are standardized against the signal mean intensity obtained for immobilized myeloma-IgE.
8. *Example for the interpretation of test results:* The results obtained with the microarray format were compared with the reference method done with the Pharmacia CAP system. As seen in [Fig. 2](#) each serum sample showed its own signal pattern.
 - (a) Negative controls (serum samples without allergen-specific IgE antibodies): regarding the negative controls, no false positive signals for a single allergen have been obtained. The positive control (Myeloma-IgE) was consistently shown, but there was a variation in signal intensity present for the different microarrays tested. To be able to compare results of different microarrays, the signal intensity should be normalized against a test-independent standard (e.g. immobilized streptavidin-HRP). Nevertheless, it was possible to distinguish between patients with and without specific IgE.
 - (b) Positive results for allergen extracts: in case of allergen extracts, the immobilization of the main allergenic proteins could be difficult due to an unfavourable ratio of allergenic to non-allergenic proteins. In general, it is nearly impossible to predict which protein of the protein mixture will be immobilized on the chip surface. Positive signals in case of allergen extracts could be caused either by specific reaction between the allergen and specific IgE or by a “cross-reaction” of human IgE with other components of the allergen extract (e.g. lectins) (25). Homologous sequences of the major allergenic protein compounds [e.g. between Bet v1 (birch), Api g1 (celery), mal d1 (apple), Pru a1 (plum)] are also a reason for the cross-reactivity between birch, legumes, and fruits (26).
 - (c) Positive results for recombinant allergens: in case of recombinant allergens, a defined single protein is immobilized on the surface. Positive results are therefore directly related to the used allergen. Screening tests with recombinant allergens ([Fig. 2](#)) showed that recombinant or purified allergens such as r Bet v1 (birch), r Bet v2

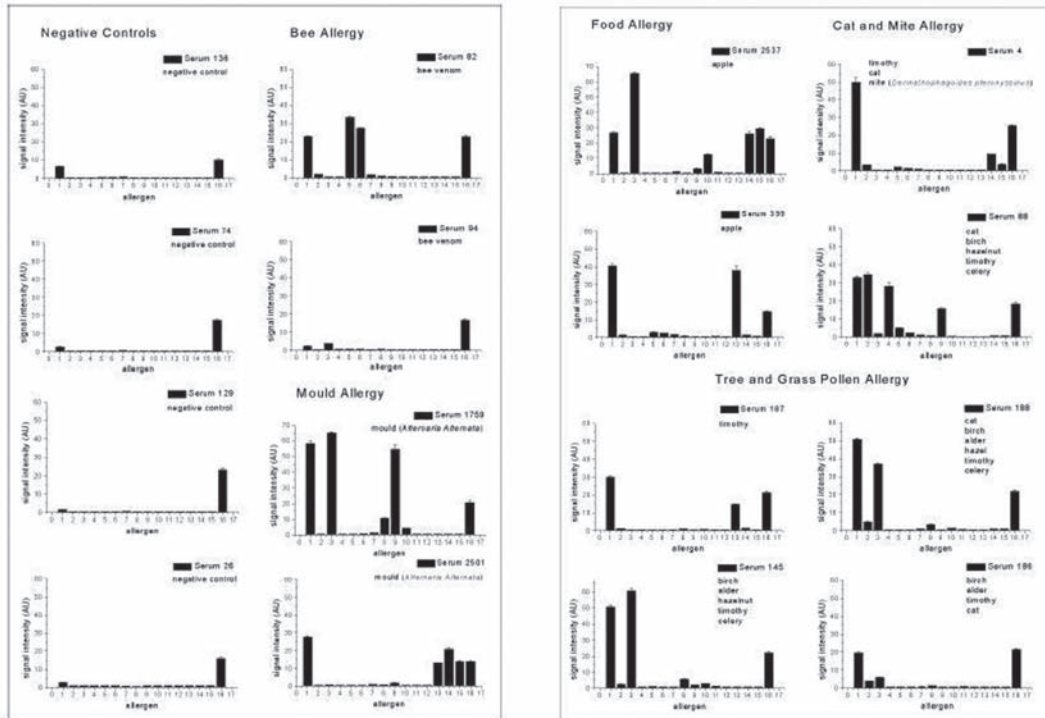


Fig. 2. Screening of single serum samples based on recombinant and purified allergens immobilized on GOPS – modified glass slides. Immobilized recombinant/purified allergens: 1 – anti-human IgE LO-HE-10, 2 – r Fel d1, 3 – r Bet v1, 4 – r Bet v2, 5 – PLA₂, 6 – bee venom, 7 – IgG, 8 – r Aln g1, 9 – r Phl p1, 10 – r Cor a1, 11 – r Api g1, 12 – r Mal d1, 13 – r Alt a1, 14 – Der p1, 15 – Der f1, 16 – streptavidin-HRP.

- (birch), r Phl p1 (timothy grass), r Aln g1 (alder), r Fel d1 (cat epithelia), Der p1 (mite), Der f1 (mite), r Alt a1 (mould), and PLA₂ (bee venom) could be used for the screening of allergen-specific IgE. Only weak signals were obtained for r Api g1 (celery), r Mal d1 (apple, [see Note 5](#)), and r Cor a1 (hazelnut).
- (d) Missing signals for allergen extracts and recombinant allergens: working with recombinant proteins, a lacking spot on the multi-allergen chip in the test could have different reasons: an insufficient immobilisation of the corresponding protein on the chip surface, sterical hindrance, a partial or complete denaturation of the immobilized protein, or the lack of allergen-specific IgE in the patient's serum sample. Another possible reason for false negative test results is that using MTPs or GOPS-modified glass surfaces, allergens can not be immobilized as densely as on the cellulose polymer surface of the Pharmacia CAP test ([see Note 6](#)).

- (e) Missing signals for immobilized food allergens: Direct ingestion of food allergens also stimulates the production of allergen-specific IgG antibodies. Concentration of allergen-specific IgG can be 10,000 times higher than the allergen-specific IgE level. Weak IgE signals could be caused by the competition between allergen-specific IgG and IgE for the limited binding sites. Therefore, allergens immobilized on ELISA MTPs or on microarrays may be blocked by specific IgG leading to an underestimation of IgE.

As shown in [Fig. 3](#) patients tested positive against birch pollen showed positive signals for the birch pollen extract and either r Bet v1 or r Bet v2 in the microarray format. A positive signal for

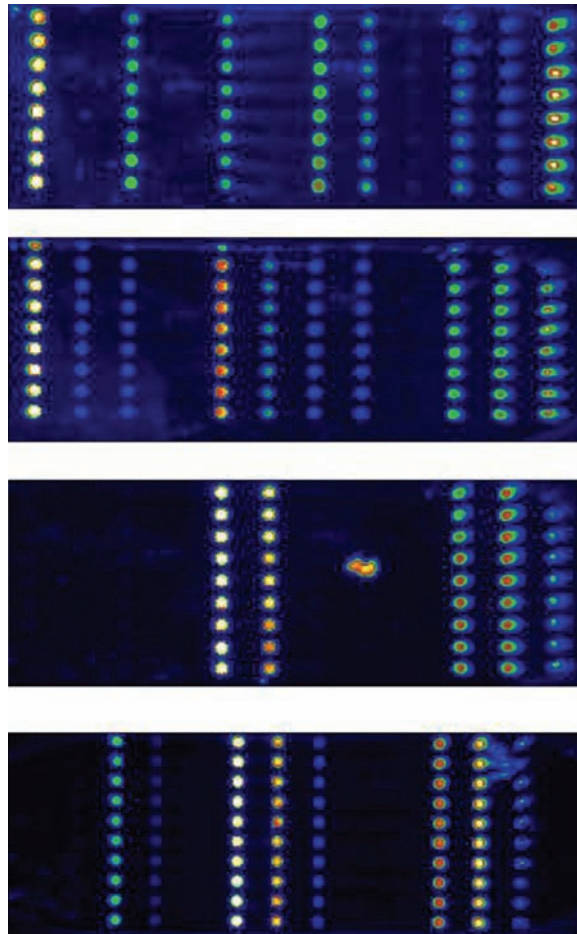


Fig. 3. Screening of serum samples of tree and grass pollen allergic patients using allergen extracts and recombinant allergens on the same chip surface. Immobilized recombinant and allergen extracts: 1 – r Bet v1, 2 – r Bet v2, 3 – birch extract, 4 – buffer, 5 – timothy extract, 6 – r Phl p1, 7 – alder extract, 8 – r Aln g1, 9 – r Mal d1, 10 – rye extract, 11 – wheat extract, 12 – myeloma-IgE.

the allergen extract (alder, timothy) correlated in most cases with a positive signal for the respective recombinant allergen (r Aln g1, r Phl p1). For some serum samples only positive signals for the allergen extracts were obtained, indicating that these patients have no allergen-specific IgE antibodies against the main allergenic proteins of the extract. The use of recombinant and purified allergens in combination with allergen extracts on the same microarray surface seems to be a good compromise to cover all possible cases.

3.9. Estimation of Allergen-Specific IgE Detection Limits on GOPS-Modified Slides

3.9.1. Test Performance

1. For the estimation of the detection limits for allergen-specific IgE antibodies, the corresponding proteins (e.g. r Bet v1, PLA₂, Der f1, LO-HE 10) are immobilized on different GOPS glass slides as described in [Subheading 3.3](#).
2. A serum sample with a known specific IgE concentration against the corresponding allergen extract, e.g. birch pollen positive serum sample in case of r Bet v1, is diluted 1:1 with a heat-denatured (2 h, 56°C; *see* [Note 4](#)) blank serum sample.
3. The obtained serum samples of different IgE concentrations are diluted 1:4 in antibody dilution buffer.
4. Incubation with serum sample and HRP-labelled anti-human-IgE antibody is performed as already described in [Subheading 3.4](#).
5. After a washing step the slide is placed in the flow cell of the PASA system ([Fig. 1](#)), and 500 µL of chemiluminescence substrate is filled into the flow cell.
6. Light emission is accumulated for 90 s using a 2 × 2 binning.

3.9.2. Interpretation of Test Results

Table 2 shows the detection limits of IgE against r bet v1, Der p1, Dre f1, PLA₂, and total IgE. With the microarray format it is possible to distinguish between specific IgE class 0 (0.35 kU/L) and 1 (0.69 kU/L) for r bet v1, PLA₂, and Der p1. With the detection limit of Der f1 specific IgE, class 1 and 2 could be separated. In some cases, e.g. r Phl p1, sensitivity improvements may be necessary to reach lower specific IgE classes (*see* [Notes 7 and 8](#)).

3.10. Determination of Total and Allergen-Specific IgE and IgG on GOPS Slides

3.10.1. Test Performance

1. Crude allergen extracts and recombinant allergens are immobilized on the modified glass surface as described in [Subheading 3.3](#).
2. The measurement of allergen-specific IgE antibodies is performed according to [Subheading 3.4](#). Serum samples could be diluted 1:500 for the determination of allergen-specific IgG antibodies (a dilution of 1:8 was applied for the allergen-specific IgE test).
3. For the detection of allergen-specific IgG antibodies an HRP-labelled rabbit-anti-human-IgG antibody (Sigma-Aldrich,

Table 2
Detection limits for specific IgE using recombinant/purified allergens on a GOPS-modified glass surface

Allergen	Serum sample	IgE ($\mu\text{g/L}$)	LOD ($\mu\text{g/L}$) ($3s_g$ -method)
R bet v1	96	38.0	0.35
PLA ₂	82	54.6	0.16
Bee venom	82	54.6	1.03
Mellitin	82	54.6	4.80
Der p1	4	240	1.85
Der f1	4	240	4.62

The detection limit was determined by dilution of serum samples of known specific IgE class against the specified allergen extract (e.g. birch pollen positive serum sample in case of r Bet v1) with an IgE heat-denatured (2 h, 56°C) serum sample of a patient without specific IgE

1:1,000 in PBS/0.5% BSA/0.5% Tween-20) was used (30 min, ambient temperature).

3.10.2. Elimination of IgG Antibodies from Human Serum Samples

1. “Protein G” coated agarose beads (40 μL) are mixed with 20- μL human serum and 80- μL PBS buffer using a 1.5-mL Eppendorf tube.
2. As a control 20 μL of the same serum is diluted with 120- μL PBS and treated similar to the “agarose bead suspension”.
3. The content is mixed thoroughly with a vortexer and slightly mixed for 2 h at ambient temperature on a microtitre plate shaker.
4. The agarose beads are removed by centrifugation (Ultrafree C-Caps, 17,000 g, 5 min, 4°C).
5. The remaining serum solution is transferred into a new Eppendorf tube and diluted with 800- μL PBS.

3.10.3. Interpretation of Results

The treatment with *Protein G*-coated agarose beads influences the intensities for specific IgE antibodies only slightly. In opposition to this, the signals obtained for allergen-specific IgG antibodies could be reduced. For routine clinical testing, the elimination of IgG antibodies from human serum samples is too time and cost intensive. For this purpose, the IgE and IgG-specific antibodies should be measured with different microarrays. The clinical symptoms could thereafter be evaluated based on both test results.

4. Notes

1. It has to be considered that the patient could also be allergic against the blocking reagent (e.g. cow's milk casein – casein as blocking reagent should be avoided, sea block blocking reagent should be avoided for patients with a sea food allergy).
2. The test results of GOPS slides seemed to be closer to the results obtained with the hydrophobic Microlon MTPs than to the hydrophilic NHS activated MTPs, indicating that an adsorptive binding mechanism on the hydrophobic GOPS slide is predominant in relation to the covalent attachment of the protein's amino groups to the epoxy groups of the surface.
3. If a colorimetric detection of HRP is used, the measurement could also be performed off-line using chamber slides and coverslips in combination with a commercially available scanner.
4. Heating of blood samples over 56°C over a longer time period leads to the specific destruction of immunoglobulin IgE.
5. Some recombinant proteins, e.g. r Mal d1 (apple, 17 kDa, Bet v1 homologue) could neither be recognized on hydrophobic Microlon MTPs nor on GOPS-modified chips. Commercially available recombinant proteins such as r Mal d1 seem to be less reactive than its natural counterpart (27).
6. Summarizing all results for the samples tested so far, it was evident that for the microarray-based system false negative signals are more common than false positive ones.
7. For serum samples with higher IgE concentrations (>specific IgE class 3) a saturation of the chip surface could be reached. Appropriate dilution of serum samples could solve this problem.
8. For the simultaneous measurement of the total IgE and allergen-specific IgE (saturation level reached after different time points) the accumulation time of the chemiluminescence signal could be varied.

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