

ASSAY DEVELOPMENT

A multiplexed immunoassay for the rapid detection of specific IgE in allergy diagnosis

Detecting allergic disease through multiplexing can produce more test data while reducing the burden on clinical laboratories.

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The prevalence of allergic disease has increased considerably over the last 20–30 years, particularly in the more developed regions of the world. As a result, it is rapidly becoming a major health problem, resulting in significant economic losses. In the United States alone, allergies are the sixth-leading cause of chronic disease, costing the healthcare system more than \$18 billion annually.^{1a–3} Genetic factors alone cannot explain the increasing prevalence, nor can they explain the large differences in prevalence among regions of similar ethnic backgrounds. Thus, environmental factors must also play a major role in the development and expression of allergic disease. Childhood infections, vaccination programs, and changes in life-style, including in indoor conditions, diet, and microbial gastrointestinal flora, have all been suggested as causes for the increase in cases.^{4a–12}



A lab technician runs an allergy microarray assay using experimental equipment.

Allergic disease is characterized by the involvement of specific IgE toward a multitude of offending allergens. Allergens binding to IgE molecules on different cells cause the release of certain mediators, leading to the various symptoms an allergic patient may experience. As a result, specific IgE has become an established marker for the disease. Treatments include antihistamines, steroids, allergen-specific immunotherapy, and anti-IgE, as well as both general and specific medical remedies. The wide range of therapies for allergies warrants not only the diagnosis of the general presence of allergic disease, but also the need for specific diagnostic methods.

The ImmunoCAP system by Pharmacia Diagnostics AB (Uppsala, Sweden) is frequently used for determining specific IgE. To allow for the simultaneous analysis of a large number of different allergens, Pharmacia has established an experimental protein microarray assay that can detect specific IgE antibodies in serum. The primary attraction of multiplexed allergy testing is the vast amount of serological information that it produces, at the same time, from a small volume of sample.^{12,14}

Assay Prerequisites

Due to their low background signals, which can help compensate for the lower capacity, activated glass surfaces are often used as a solid phase in microarray development. However, when a mixture of proteins is analyzed, the capacity of the glass surface is typically insufficient.^{15a–17}

The ImmunoCAP assay is made up of microspots arrayed on a capillary-flow membrane in a three-dimensional structure. Such an arrangement has two major benefits. First, it creates a large inner surface area and an enlarged capacity, which is beneficial for detecting low-concentration analytes. Second, it enables a liquid flow driven by capillary force, at the location of sample and reagent application through the reaction zone. This allows for the design of a simplified and robust instrument. The expanded inner surface area is also necessary for the detection of critical, low-content components in natural allergen extracts.

Membrane Selection. The choice of a capillary-flow membrane also simplifies instrument development for reagent management and detection. Fully automated systems tend to take a complex approach to the critical design of liquid handling. By using capillary forces to transport the liquid, fewer pumps and vacuum systems need to be used. The cost of capillary-flow membranes also tends to be significantly lower than most other solutions.

The assay time is directly dependent on the flow rate, which is determined by the pore size of the membrane. Decreased pore size slows the flow rate, leading to a longer assay time and an increased binding of IgE to allergens. This results in an improved signal-to-noise ratio and better test results.

The ratio between the width of the membrane and the volume of the reagents also affects the flow characteristics. The width of the ImmunoCAP flow membrane is 5 mm, which provides an optimal flow rate and a surface large enough for the required number of assay spots. However, due to the nature of the membrane, the area closest to the membrane edges have different flow characteristics from the rest of the surface and is not applicable for spots. Therefore, decreasing membrane width will create a proportional increased area that is not appropriate for spotting.

The capillary-flow membrane is attached to ordinary microscope glass slides measuring 76 Å—26 mm. These dimensions suit the instrumentation. The detection instrument has a narrow depth of focus. This means that the backing of the flow membrane must be entirely flat so that it does not affect the signal. To increase the throughput of the assay, then, the two flow membranes must run parallel to each other and be attached to the same microscope glass slide.

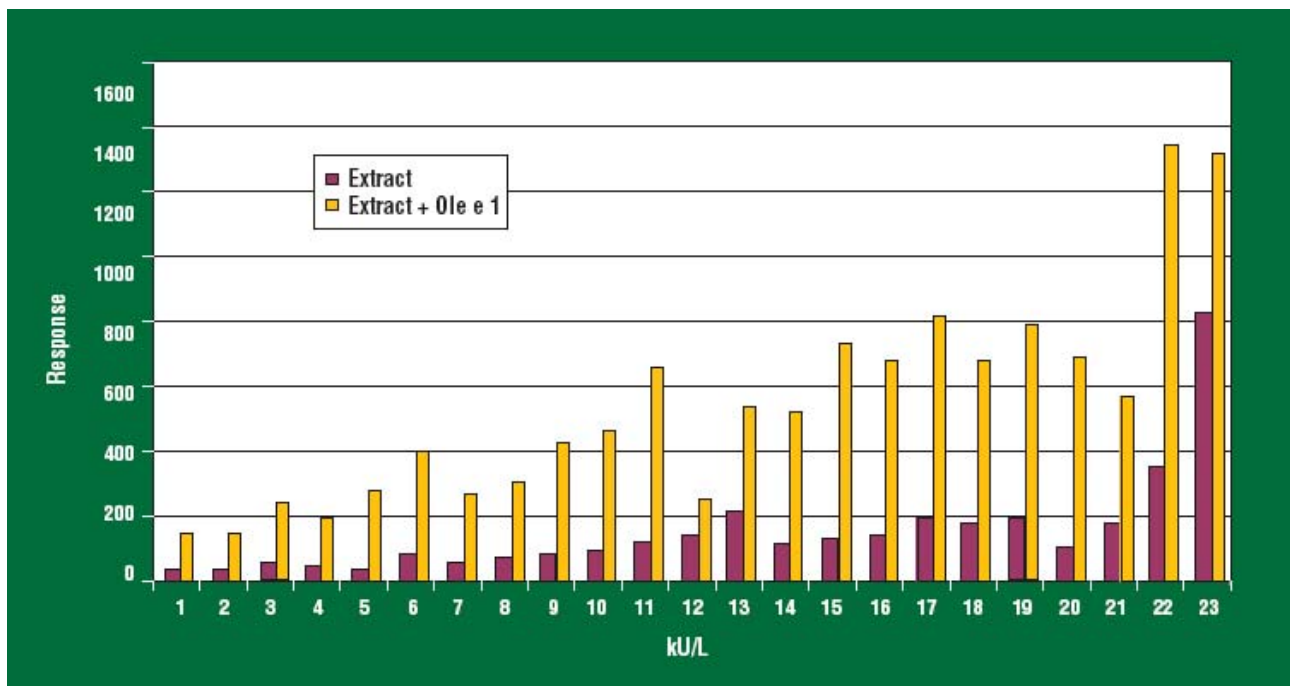


Figure 1. (click to enlarge) An example of allergen preparations in which an increased sensitivity was achieved while maintaining specificity. The original extract was spiked with purified Ole e 1, a major IgE binding component in olive pollen.

Antigen Preparation. As the solid phase in a microarray is limited, the preparation of the allergen is critical in order to obtain the required sensitivity and specificity. To achieve the optimal allergen preparation, purified original extract should be spiked with the critical allergen components (see Figure 1). Because natural allergen extracts contain a mixture of chemical compounds, allergenic proteins, and nonrelevant proteins, in addition to other nonrelevant chemical compounds, the proportion between allergenic proteins and nonrelevant compounds varies between different allergens. Each allergen may contain up to at least 10 different IgE binding compounds. In addition, each patient possesses an individual pattern of IgE antibodies.

Antibody Selection. The IgE-detection antibody used in the micro-array is a monoclonal anti-IgE antibody, which is covalently bound to a fluorophore. The most widely used fluorescent probes for microarray imaging are the fluorophores Cy3 and Cy5. The affinity and the kinetics of the antibodies are important for achieving enough sensitivity in the test system. For this array, more than 70 anti-IgE antibodies were screened and tested.

Method

The microspots contain natural allergen extracts as well as purified and/ or recombinant allergens, which are covalently immobilized. To achieve optimal assay conditions, the allergenic components are coupled through a spacer linked to the solid phase. This minimizes the risk of steric interferences and the coupling process influencing the three-dimensional structure of the allergen components. Each allergen has its own specific buffer composition when spotted. This variety maximizes the amount coupled and maintains the binding ability of the IgE in the patient sample.

The allergen reagents are spotted on the membrane at a density of about 100 spots per cm² with a spot diameter of approximately 250 Åµm. Using the current flow membranes, it is possible to have 150–200 spots on each. The spots are deposited with a Nano-Plotter Type 2.0 system by Gesellschaft für Silizium-Mikrosysteme mbH (GeSim; Göttingen, Germany), which uses a contact-free dispenser with a piezoelectric pipette. This technique allows a droplet size of less than 1 nl with a reproducibility of 2–4%. This precision is required to achieve a quantitative assay comparable to traditional laboratory immunoassays.

After the coating procedure is complete, the membranes are dried and stored at 2–8°C in low-humidity conditions. Preliminary data indicate that in this environment, a shelf life of at least one year is obtainable.

The detection system is based on fluorescence, a technique that is sensitive enough to detect IgE molecules measuring approximately 10¹⁷ moles. The detector used is the GenePix 4000B microarray scanner by Molecular Devices Corp. (Sunnyvale, CA), which simultaneously scans the microarray at two wavelengths using a dual-laser system. The laser excites the fluorophore, and the fluorescence emitted is directly proportional to the amount of specific IgE bound in each spot, which corresponds to the relative concentration of IgE in the sample. The total depth of focus is 64 Åµm while the thickness of the flow membrane is about 150 Åµm. To maximize the signal, the focal point of the laser is approximately 30 Åµm below the surface of the membrane.

Assay Procedure

The assay sequence begins by applying a 20-ml serum sample, followed by 30 ml of the detecting antibody conjugated to the fluorophore. This is followed by 60 ml of a washing buffer. The optimal total assay time is approximately 30 minutes, sufficient for achieving adequate sensitivity and signal-to-noise ratio.

The entire assay is performed in the open air at room temperature. After the assay process is complete, the fluorescence is quantified by transferring the device into the GenePix laser scanner. The procedure for scanning one slide takes about 1–2 minutes.

GenePix Pro 5.0 software is then used to analyze the microarray data. Aside from its primary function of quantifying the light emitted from the fluorophore, the software must also compensate for any spot irregularities, background reductions from components (e.g., the flow membrane), and differences between patient samples.

Results

Patient serum was selected based on the values derived from the ImmunoCAP system. As the cutoff in normal clinical practice is 0.35 kUA/L, this value was also used for the allergens tested with the new assay. The detection limit can vary between allergens depending on the composition of the different extract components. Some allergens may consist of one or a few allergenic components in a high ratio against nonallergenic components. Other allergens may contain several components, and the ratio of allergenic components to nonallergic components could be very low. It is possible to improve the integrity of the extracts either by purification to eliminate nonallergenic components or by the enrichment of the important components as described earlier. For allergens that are sufficiently optimized, detection limits of 0.04 kUA/L or lower can be obtained.

ImmunoCAP was also used as a reference to ensure that the treatment of the extracts did not affect the clinical performance. A comparison study of the microarray assay and ImmunoCAP showed an agreement of more than 80% between the two technologies for the allergens tested. This level of assay concordance was fully acceptable considering the limited optimization of the allergens.

The precision of the test system was calculated as intraassay variation, using pooled values from 20 of the devices with four replicates on the same device. The precision was found to be in the range of 5–8% depending on the allergen. The interassay variation, analyzed by repeated analysis of samples using the same lot for all reagents, was found to be 10–13%.

Of the total amount of IgE in the patient sample, only a fraction is specific to a certain allergen. The proportion between specific IgE and total IgE can vary widely, from a high of 1:3 to a low of 1:30,000. The microarray setup must be able to distinguish between the specific and nonspecific IgE under these conditions.

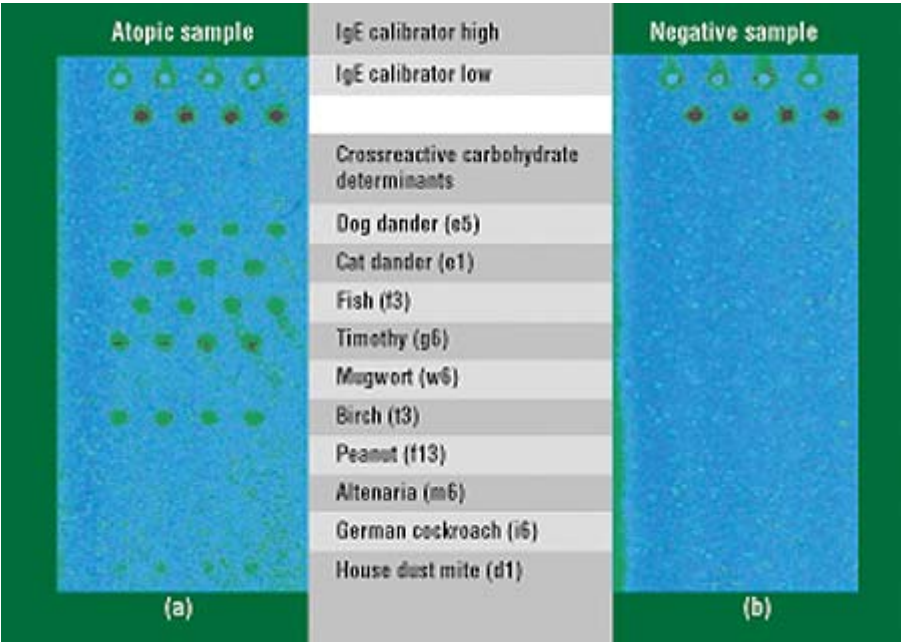


Figure 2. (click to enlarge) An example of the pattern from an atopic patient sample containing IgE antibodies (a) and a negative sample (b) after running the microarray assay. The color intensity is directly proportional to the specific IgE concentration of the sample (i.e., white > red > yellow > green). Each row contains four spots of the same allergen.

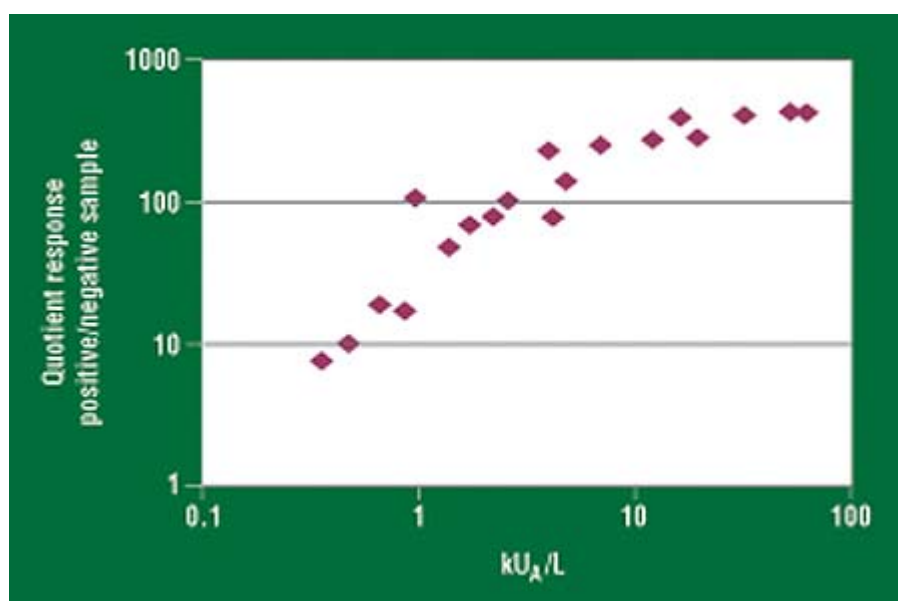


Figure 3. (click to enlarge) A graph showing the correlation between the signal and the concentration of IgE in patient samples sensitized to a common allergen, timothy grass pollen. The scale on the y-axis represents the response quotient between a positive and a mean value of negative samples. In theory, the detection limit would be a quotient of 1. However, when the variation in the test system is included, a quotient of 2.5 is more realistic. In this example, the quotient corresponds to a detection limit of approximately 0.1 kUA/L.

isolated missing spots, it could still be useful if two of three spots are correct. The software developed for the assay can automatically exclude these types of errors, thereby helping to increase yield and reduce cost.

The normal concentration of IgG, IgA, IgM, and IgD is between 0.5 and 15 mg/ml. However, for IgE, the concentration is about 0.0005 mg/ml (i.e., one-thousand-fold less). Even so, no cross-reactivity was detected at normal serum levels for the higher-concentration immunoglobulins. Results were not affected when a specific IgE-free sample was spiked with concentrations of 10,000 kU of nonspecific IgE per liter in the array assay. The results were measured as an increase in background signal as opposed to a sample without specific and nonspecific IgE (see Figures 2 and 3).

Manufacturing Considerations

Although the microarray assay described in this article is able to make 150–200 different determinations simultaneously, manufacturers need to take certain production steps to achieve maximum performance. For instance, it is best to spot two or three replicates of every allergen over the measuring area of the flow membrane. This reduces the number of determinations that can be performed in the same assay. In addition, applying replicates on each assay can reduce manufacturing errors. Even if a device has

The assay described reduces the amount of allergen necessary by a factor of 100–1000. As the allergen is the most critical test component, the need for adequate allergen component composition is important to obtain the same clinical value as the reference method. Therefore, a higher degree of purification is necessary, especially because the microarray has a limited solid-phase capacity. Increased purification yields less sample and increases costs. However, it is possible to obtain a balance, with a smaller amount of allergen required on the solid phase.

Conclusion

The microarray assay described has the analytical sensitivity sufficient to fulfill the requirements of a specific IgE assay. This tool is capable of generating a vast amount of data from a small sample volume. The major challenges ahead include the development of a sufficient range of suitable allergen reagents and the development of clinically meaningful ways to process and interpret the complex datasets. Rapid advances in microarray technologies and related computational analyses may someday provide new opportunities in the clinical laboratory.



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