

Allergen Microarrays

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Summary

Allergy affects more than 25% of Western populations (1) and is estimated to be the sixth leading cause of chronic disease in the United States and Western Europe. The complexity of the condition is such that hundreds of common allergens have been described, and in order to maximize diagnostic efficiency there is an urgent clinical requirement for assays to provide multiple-allergen determination in a timely and cost-effective manner. Miniaturized immunoassays that utilize protein microarray technology now offer the possibility of circumventing most of the current limitations in the serodiagnosis of allergic disease. The heterogeneous nature of allergens presents many challenges in all aspects of developing such arrays, from immobilization of the capture molecule to detection of the bound ligand. In addition, there is no simple method of protein amplification (such as PCR for nucleic acids), and stabilization is yet a further major consideration. Notwithstanding these challenges, protein microarrays have been developed for the serodiagnosis of allergies and other complex clinical conditions. These assays exhibit good analytical and clinical performance and deliver significant advantages in convenience and cost compared with traditional ELISA test formats. This chapter details the techniques employed in the construction and processing of an allergen array specific for the serodiagnosis of allergic disease. An overview of protein microarray technology is provided and the principles that underpin the suitability for use of this technology in the identification and measurement of particular proteins in patient sera (serum profiling) are discussed.

Key Words: Protein microarray; immunoassay; serodiagnosis; allergy; signal amplification.

1. Introduction

1.1. Protein Microarrays and Their Use in Serum Profiling

The term *microarray* refers to the deposition of minute volumes of aqueous biomolecules onto a solid surface in an ordered and highly dense manner. Microarray technology represents the culmination of novel technological advances that together permit the simultaneous, parallel analysis of thousands of

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parameters within a discrete microenvironment. With its innate capacity for miniaturization, the microarray format overcomes the spatial, logistical, and economic constraints that have previously made such wide-scale, truly parallel investigations impossible. Interest in the microarray platform has grown rapidly since its inception. Following the initial development of DNA-based arrays (2), the number of publications documenting protein-based (3,4) and more recently carbohydrate-based (5,6) microarray technology has increased exponentially.

Protein microarrays can be conveniently divided into two classes on the basis of their application. Broadly speaking, these categories constitute those arrays that are functional and those that are analytical in their nature (7). Functional microarrays are used to assess proteins for a variety of biochemical activities and provide valuable insight into the function of specific proteins. The high-throughput capacity of such arrays permits the simultaneous screening of multiple proteins, such that global functional analysis at almost the entire proteome level has recently been demonstrated (8). These high-density functional arrays have huge applications in protein characterization as well as in drug and drug target identification. Future improvements in protein expression technology will enormously benefit the ability to scale up these arrays (9). Analytical arrays, on the other hand, are implicitly designed to detect the presence and measure the concentration of particular proteins of interest (target proteins) from a complex protein mixture. Here immobilized capture molecules (typically antibodies or antigens) with characterized affinity for target protein(s) are used to screen sera or lysate for the presence of the target protein(s). Analytical arrays are typically of low to medium density owing to limited numbers of characterized capture molecules and have application in many fields including diagnostics, prognostics, and protein expression profiling (10).

Key products for functional and analytical applications for protein arrays are whole proteome array slides, slides for diagnostic applications, and slides for ligand and/or small molecule screening. All applications have the same general requirements;

1. Immobilization of a capture molecule on the slide.
2. Getting the target molecule to the capture site.
3. Detection of the target molecule when bound.

The technology for printing high-density protein arrays has been lifted almost entirely from DNA chip construction. There are numerous attachment strategies and many commercial suppliers for these products (11). The capture surface plays a critical role in the quality of the microarray since it influences the efficiency of antibody attachment, the degree of nonspecific binding, and the accessibility of the capture molecules to the target proteins. Glass slides have been adopted as the substrate of choice owing to their ease of use, compatibility with laboratory equipment, and robustness. However, many new surfaces such as hydrogels and

a variety of membranes are in development for specific applications. The accurate and precise high-density spotting of proteins is performed by robotic arrayers mainly based on either contact or ink-jet printing techniques (12,13). The binding of the target molecules to the capture surface is performed using traditional solid-phase immunoassay techniques such as, enzyme-linked immunosorbent assay (ELISA). The key consideration is to bind the target molecules specifically and avidly and to minimize nonspecific binding. Nonspecific binding slows the equilibration of the target molecules with the capture surface and is one of the major constraints on the sensitivity of the system. The glass slide allows for good washing efficiency since most of the protein array assays developed to date are of the "heterogeneous" type in which various wash steps are performed to remove the unbound label (14). At least one "homogeneous" assay format incorporating the optical phenomenon of evanescent waves is also in development (15). Other "homogeneous" assay formats, e.g., using microcantilever technology, have also been described (16). The main detection method used in both DNA and protein array technology is fluorescence. This is because fluorophores are in the main small molecules that produce high specific affinity labels (i.e., high dye: protein incorporation ratios), and fluorescence offers a large dynamic range and lends itself to scanning techniques. Fluorescence is detected by either confocal microscopy or by cameras incorporating charged coupled devices (CCDs) (17). Other detection methods described include chemiluminescence, colorimetry, mass spectrometry and surface plasmon resonance (18-21).

In recent years, much interest has focused on the use of protein microarrays for immunoassay development. Ekins developed the theoretical background for microarray-based ligand-binding assays in the late 1980s (22-24). He formulated the concept of the ambient analyte immunoassay, which he predicted would not only allow simultaneous detection of an analyte panel but would also have a sensitivity equal to, or even greater than, standard immunoassay techniques. Validation of these theories has had to await the advent of robotic arrayers and fluorescent scanners, but they are now widely accepted and are the basis of all microarray assay applications.

The profiling of serum is fundamental to both diagnostic clinical procedure (through identification of serum antibodies that suggest the presence of disease agents) and prognostic clinical procedure (using biomarker and surrogate levels in serum to predict the risk of disease and monitor disease progression/regression during treatment). However, the assays currently used commercially (e.g., ELISA [25]) fail to meet the clinical demand for high-throughput and low sample requirements (Tables 1 and 2). Protein microarrays can overcome these limitations of current clinical assays (26) and facilitate the simultaneous, parallel determination of hundreds of target proteins within patient sera. In recent years huge progress has been made in the development of microarray immunoassays intended for the profiling of human serum. Microarrays have been developed

Table 1
Comparison of Microarray and ELISA for Serum Reactivity^a

Allergen	ELISA		Microarray			
	Positive	Negative	Positive	Negative	Sensitivity %	Specificity %
<i>Dermatophagoides pteronyssinus</i>	11/22	11/22	13/22	9/22	90.9	72.7
<i>Dermatophagoides farinae</i>	8/16	8/16	9/16	7/16	87.5	75
<i>Olea europaea</i>	7/22	15/22	8/22	14/22	71.4	80
<i>Altemaria altemata</i>	3/20	17/20	4/20	16/20	100	94.1
<i>Artemisia vulgaris</i>	2/22	20/22	2/22	20/22	50	95
<i>Dactylis glomerata</i>	6/22	16/22	7/22	15/22	83.3	87.5

Clinical sensitivity is calculated by expressing the number of samples that are positive by microarray immunoassay as a percent of the number of samples positive for the disease (i.e., positive by ELISA). Clinical specificity is calculated by expressing the number of samples that are negative by the microarray immunoassay as a percent of the number of samples negative for the disease (i.e., negative by ELISA). As an example, for *Dactylis glomerata*:

	Test positive	Test negative
Disease present: 6	5	1
Disease absent: 16	2	14

Therefore sensitivity = $5/6 = 83.3\%$ and specificity = $14/16 = 87.5\%$.

^a For serodiagnosis of allergy.

From ref. 27.

that permit the detection of antibodies directed against antigens implicated in allergy (1,18,27), autoimmune disease (28,29), and parasitic/viral infections (30). Similarly, protein microarrays have been developed that permit the detection and accurate quantification of prognostic biomarkers from human serum (31,32).

In our laboratory, the main application of protein arrays is for serodiagnosis of disease. Such assays, because of their ability to detect in parallel many antibodies with different specificities, have great potential in epidemiological research and vaccine development as well as in the diagnosis of complex clinical conditions such as allergy and autoimmune and infectious diseases. In the serodiagnosis of autoimmune, infectious diseases, IgG and IgM immunoglobulins are the antibody responses determined in patient sera. These antibodies circulate at $\mu\text{m/mL}$ concentrations, and their determination is made directly, using fluorescently labeled anti-IgG and anti-IgM reagents. However, the diagnosis of allergy necessitates the measurement of immunoglobulin IgE, which circulates at ng/mL concentrations in human serum. Consequently, techniques have been developed to increase the sensitivity of protein arrays for such purposes.

Table 2
Comparison of Microarray and ELISA Test Protocols^a

	ELISA	Microarray
Sample		
Dilution	1:300	1:200
Volume (μ L)	100	80
Incubation (min)	60	15
Temperature	37°C	Room temperature
Wash (μ L)	1050	5000
Conjugate		
Volume (μ L)	100	80
Incubation (min)	30	5
Temperature	37°C	Room temperature
Wash (μ L)	1050	5000
Substrate/Stop		
Volume (μ L)	100 + 100	None
Incubation (min)	10	None
Temperature	37°C	N/A
Read time (min/sample)	2	2
Signal stability	15 min	>3 mo
Total time (min)		
1 parameter	110	20
10 parameter	1100	20
Estimated cost (US\$)		
1 parameter	1.5	5
10 parameter	15	5

^a For serodiagnosis of infectious disease. NA, not applicable.
 From ref. 30.

One such technique is tyramide signal amplification (27); another uses rolling circle amplification (18).

1.2. Arrays for Serodiagnosis of Allergy

Allergy is a state of immune dysregulation that results in an overproduction of IgE (33). Identification of the causative agents that provoke allergies in individuals is a challenging task owing to the large numbers of such agents. Traditionally, techniques such as skin prick testing and immunoassay determination of single allergens at a time have been performed to identify these causative agents (34). Skin prick testing is uncomfortable for the patient and requires observation in case the patient develops anaphylactic shock; traditional

ELISAs do not address the full spectrum of allergens because of the quantities of blood required and the expense of the kits. Allergen arrays offer the possibility of determining and monitoring the IgE reactivity profile of allergy sufferers to very large numbers of disease-causing allergens simultaneously and use minute volumes of serum. This is particularly important for the diagnosis of allergy in children, in whom taking a blood sample can be difficult. The following sections detail our experiences in the development of such arrays.

2. Materials

2.1. Equipment

1. Computer-controlled high-speed robot (Total Array System, Biorobotics, www.biorobotics.com).
2. S5000. Scanner including ScanArray™ and QuantArray™ software (Perkin Elmer).
3. Equipment for ELISA:
 - a. Microtiter plate washer: Autura Plate Washer (Mikura, www.mikura.co.uk).
 - b. Microtiter plate reader: Kinetic Microplate Reader, v max (Molecular Devices).
4. Spectrophotometer: DU 640 (Beckman Coulter).
5. Silanized glass microscope slides (CEL Associates, www.cel-1.com).
6. 96- or 384-Well microtiter plates (Thermo Life Sciences, www.thermols.com).
7. Gene-frames (65 μ L, 1.5 $\times$ 1.6 cm) (Abgene, www.abgene.com).

2.2. Reagents

1. Allergen extracts: *Dermatophagoides pteronyssinus* (D1), *Dermatophagoides farinae* (D2), *Alternaria alternata* (M6), *Olea europaea* (T9), *Artemisia vulgaris* (W6), *Dactylis glomerata* (G3), and house dust (H2; Allergon AB, www.allergon.com).
2. Human IgE purified from myeloma plasma (Calbiochem).
3. Horseradish peroxidase-streptavidin conjugate (0.1 mg/mL), Alexa 546-tyramide conjugate, amplification buffer, and 300 mL/L hydrogen peroxide as provided in the TSA™ kit # 23 (Molecular Probes).
4. Biotinylated anti-human IgE at a final concentration of 1 mg/mL (KPL).
5. Slide blocking solution: 20 g/L bovine serum albumin (BSA) in 1X phosphate-buffered saline (PBS).
6. Signal control: Alexa 546 dye (Molecular Probes.) in 2X PBS containing 0.1 mL/L Tween-20.
7. Negative control: 10 g/L BSA in 2X PBS containing 0.1 mL/L Tween-20.
8. Dilution buffers.
 - a. Human IgE, allergen and slide blocking solution diluent: Phosphate-buffered saline (PBS) 1X (0.2 g/L KCl, 1.44 g/L Na₂HPO₄, 0.24 g/L KH₂PO₄, 8 g/L NaCl, pH 7.4).
 - b. Serum sample, horseradish peroxidase-streptavidin conjugate and conjugated antibody diluent: 2X PBS containing 10 g/L BSA and 0.1 mL/L Tween 20.
9. Wash buffer slide wash buffer: 1X PBS with 0.1 mL/L Tween-20.

10. Print buffer human IgE print buffer: 1X PBS containing 0.1 mL/L Tween-20 and 1 g/L sodium dodecyl sulfate (SDS).

3. Methods

3.1. Construction of the Array

3.1.1. Preparation of the Slide Surface

Commercial aminosilane-coated slides provided by Cel Associates are routinely used in our laboratory. Some commonly used silanization methods have been documented (35).

3.1.2. Preparation of Biomolecules for Printing

1. The arrays employed in our investigations are usually designed as 7×7 matrices. This format incorporates human IgE antibodies, allergens, and negative controls (**Fig. 1A**; see Color Plate 18 following p. 178). Human IgE antibodies included in the array are printed at known concentrations and serve as internal calibration curves.
2. Human IgE, provided by Calbiochem, is diluted in 1X PBS to a concentration of 100 $\mu\text{g/mL}$ and stored at -20°C .
3. According to the manufacturer's instructions, the resulting solution should not undergo freeze/thaw cycles; to avoid this problem, the solution is aliquoted prior to storage.
4. The IgE calibration curve (2–200 ng/mL) is prepared by diluting the human IgE preparation in 1X PBS containing Tween-20 (0.1 mL/L) and SDS (1 g/L). It is our experience that once diluted in its printing buffer, the human IgE should not be stored and reused for further print runs.
5. Importantly, 0.01% Tween-20 is routinely added to all print solutions (including allergen preparations; see **Note 1**).
6. Allergens are obtained as 1-g samples, reconstituted in 1X PBS, pH 7.2, to a concentration of 10 mg/mL and stirred overnight at room temperature.
7. After centrifugation at 503g for 30 min at 10°C , the allergen extracts are filtered in four stages: first through Whatman paper (125-mm diameter, cat. no. 1001125), then through 5- μm filter disks (Pall, cat. no. 4199; www.pall.com), then through 0.45- μm filter units (Schleicher & Schuell, cat. no. 10462 450; www.S-and-Side), and finally through 0.2- μm filter units (Schleicher & Schuell, cat. no. 10462 200).
8. Extracts are stored at -20°C as 1.5-mL aliquots.
9. A solution of 10 g/L BSA in 2X PBS containing 0.1 mL/L Tween-20 is employed as a negative control for accurate, parallel assessment of signal specificity within the array. A preparation of Alexa 546 dye (described in **Subheading 2.2.**) can also be employed as a signal control. It should be noted that such a control is not included within the array depicted in this chapter. Alexa 546 is chosen for use as the signal control because the signal amplification technique used to process the slides makes use of this same fluorophore coupled to the tyramide (see **Note 2**).

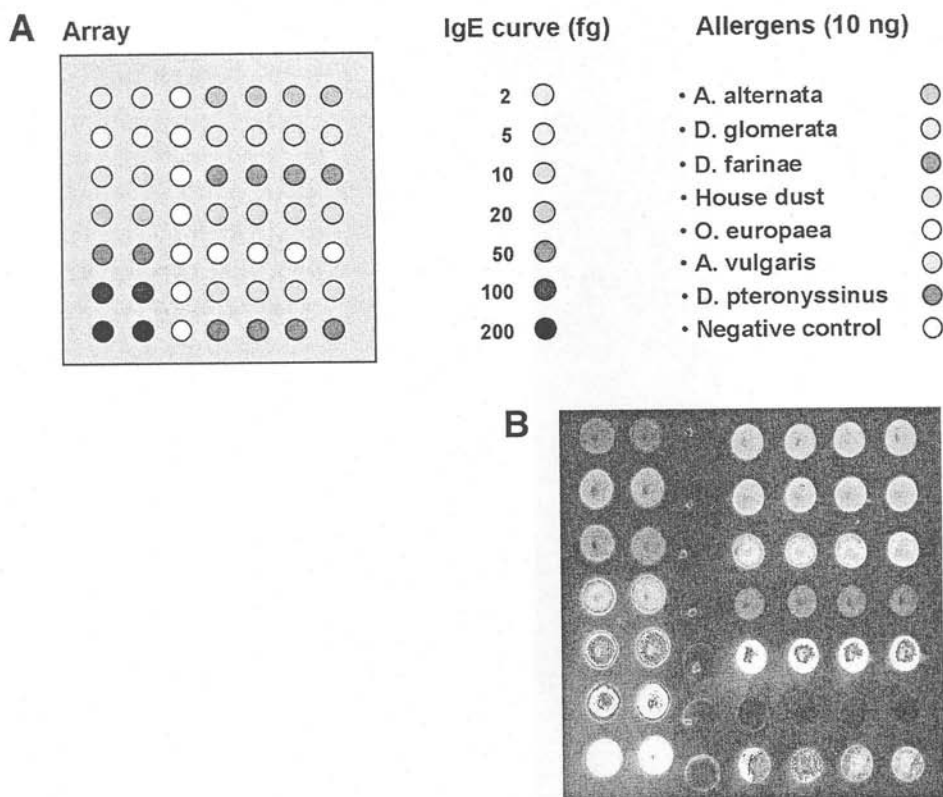


Fig. 1. Schematic array format and fluorescent scan for the allergen array (see Color Plate 18 following p. 178). **(A)** Schematic representation of the allergen array format. Arrays are printed on silanated glass slides using a robotic contact-arrayer. Printed arrays consist of various allergens printed in replicate alongside a standard curve of human IgE for quantitation and a negative control. **(B)** Fluorescent scan of allergen array incubated with serum and developed using the tyramide amplification protocol. For processing, the slide surface is blocked before the array is incubated with serum. Slides are then washed, and bound human IgE is revealed utilizing the tyramide single amplification system. Slides are incubated with biotinylated antihuman IgE, then with HRP-labeled streptavidin, and finally with activated Alexa 546-labeled tyramide. After a final washing step, slides are dried before being scanned with a confocal laser. The signal from the scanned digital image is then quantified.

3.1.3. Printing of Biomolecules

1. In order for printing of the array to take place, the solutions for printing (antibodies/allergens) must be transferred to the source plate (a 96- or 384-well microtiter plate). In our laboratory we commonly employ 384-well plates.

2. The solutions are prepared, and at least 50 μL of each are transferred, ensuring thorough mixing, to the source plate just prior to the start of the print run.
3. Depending on the number of replicate arrays required, a corresponding number of pins is loaded into the pinhead, and the appropriate number of wells is filled with each printing solution.
4. Biomolecules are contact-printed on silanized glass slides by means of computer-controlled high-speed robotics.
5. The samples are transferred from the microtiter plate to immobilized glass slides by use of stainless-steel solid pins (200 μm diameter). Each pin is estimated to transfer approx 1 nL of sample to the slide and produces spots with a pitch of 0.6 mm.
6. After deposition of each print solution, pins are washed first with distilled water and then with 70% ethanol and finally dried as part of the printing program.

3.1.4. Storage of Printed Slides

Once the printing process has been completed, the slides remain inside the robot printer cabinet for at least 12 h to "mature." This stable and controlled environment (approx 55% humidity and 25°C) is conducive to the binding of the antibodies and the allergens to the slide surface. Printed slides are stored in the dark in boxes containing silica gel bags. It is critical that the slides be kept dry; slides that have been exposed to moisture during storage give imprecise and varying signals.

3.2. Processing of the Printed Array for Determination of Serum IgE

A protocol incorporating signal amplification through tyramide reagentry has been identified (*see Note 3*) and is described here. Use of this type of chemistry results in significant amplification of the bound signal (50–100-fold). Amplification is required to increase test sensitivity since human IgE circulates at very low concentrations.

1. First, undiluted serum samples are analyzed with a commercial ELISA (e.g., Radim). ELISAs are performed and samples categorized according to the manufacturer's instructions.
2. Slides are then prepared for processing by the attachment of an adhesive frame (Gene-Frame; *see Subheading 2.2.*) around the printed array. This frame serves two purposes: first, this ensures that samples and reagents are contained within the array area; second, the frame limits the development area to the direct vicinity of the printed array, thereby minimizing reagent use.
3. To block nonspecific binding to the activated slide surface, the printed slides are incubated for 60 min at room temperature (RT) with the slide blocking solution (*see Subheading 2.2.*). Staining jars with at least 50 mL of blocking solution are commonly used for this purpose.
4. Slides are then incubated with serum (150 μL) for 60 min at RT and then thoroughly rinsed with wash buffer (5 $\times$ 1 mL).

5. A 60-min incubation with a biotinylated antihuman IgE (150 μ L) at a final concentration of 1 mg/L in the appropriate diluent (*see Subheading 2.3.*) is then performed at RT.
6. After rinsing with wash buffer (5×1 mL), slides are incubated for a further 60 min at RT with a horseradish peroxidase-streptavidin conjugate diluted 1:100 in the appropriate diluent (*see Subheading 2.3.*).
7. Slides are rinsed again (5×1 mL), and the resulting streptavidin-biotinylated antibody complexes are detected by incubating the slides for 15 min at RT with Alexa 546-tyramide conjugate, diluted 1:100 with Molecular Probes diluent (namely, Amplification Buffer) containing 0.015 mL/L hydrogen peroxide.
8. Upon completion of the final processing step, slides are rinsed for the final time (5×1 mL), and the gene-frame is removed.
9. After drying at 37°C for 10–15 min, slides are then ready to be scanned.

3.3. Collection and Analysis of Array Data

3.3.1. Scanning of Processed Slides

Scanning is performed using ScanArray software, according to the manufacturer's instructions. Images (**Fig. 1B**) are optimized using the fine-tuning capabilities provided (notably, adjustment of laser/PMT settings) and stored as Bitmap files.

3.3.2. Quantitative Analysis of Image Data

Quantification is achieved using QuantArray software according to the manufacturer's instructions. Background signal is automatically subtracted, and data are stored as Excel files. Calibration curves incorporated in each protein array are fitted using the appropriate curve-fitting routine and provide a basis on which to correlate the signal generated against a concentration of the analyte under determination. Signals from printed antigens are then interpolated from these calibration curves.

4. Notes

1. Printing solutions for allergens: the heterogeneous nature of allergens demands careful optimization of print conditions for individual allergens. Useful additions include Tween-20 as the surfactant of choice; print quality can be much improved by addition of 0.01% Tween-20 to print solutions. SDS can be beneficial to the printing of certain allergens owing to its denaturing properties, which can enhance binding to the substrate and/or expose epitopes for binding to the serum antibody. Sucrose can be added to print solutions as a means of extending shelf life; sucrose retains a hydration shell around the immobilized material, thereby maintaining its 3D structure.
2. Signal generation: fluorescent end points are used as the signal generation option of choice. Other end points have been described (such as colorimetric

and chemiluminescent), but direct labeling of proteins with fluorophores to generate high specific activity and stable labels is easy to perform and instrumentation is widely available for the determination of the emitted signal. It is preferable to choose fluorophores with spectral properties >500 nm since many proteins in blood fluoresce at <500 nm, and background signals are much reduced at these wavelengths. The Alexa range of dyes (Molecular Probes) and the Cy dyes (Amersham Biosciences) can be recommended for this type of protein array immunoassay.

3. Signal amplification using tyramide reagentry: In the presence of hydrogen peroxide, horseradish peroxidase activates the dye-labeled tyramide derivatives, creating extremely reactive short-lived tyramide radicals that bind to nucleophilic residues on the slide surface. This results in significant amplification of the bound signal. Use of this type of chemistry can increase signal generation by 50- to 100-fold.

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