

Arbeiten aus dem Paul-Ehrlich-Institut
11th International Paul-Ehrlich-Seminar, Bad Homburg 2005
Verlag Chmielorz

Multiplex Technology for Allergen Detection and Immunodiagnosics

Martin D. Chapman, Amy Tsay, Branka Saric, Rebecca Godbout, Kerry G. Oliver

Introduction

Reliable methods for assessing IgE mediated sensitization and exposure to specific allergens are crucial for the investigation and diagnosis of allergic diseases, including seasonal or perennial rhinitis, asthma, food hypersensitivity, and respiratory diseases caused by molds [1,2]. Allergy diagnosis is routinely carried out by skin prick testing or using *in vitro* tests, such as fluorescent enzyme immunoassay (e.g. ImmunoCAP) or radioallergosorbent test (RAST). Aerobiological techniques (pollen or mold spore counts) are widely used for assessing outdoor allergen exposure, whereas enzyme immunoassay (ELISA) of dust or air samples has been the gold standard for assessing exposure to indoor allergens [3]. The use of skin testing, *in vitro* IgE assays and ELISA based allergen detection systems has been beneficial for both diagnostic and research purposes. However, all of these methods have a central flaw: Tests for each antibody or allergen have to be performed separately. The necessity to perform separate tests for each analyte is time consuming, costly, and prone to technical errors. This is especially true for birth cohort studies, large population surveys, or allergen avoidance studies, which may require antibody measurements or exposure assessments on hundreds or thousands of samples. The limitations of ELISA procedures are an impediment for large studies of exposure assessment and for prospective studies to be carried out over several years. *In vitro* testing uses significant amounts of serum, which is a further limitation, especially for pediatric studies.

This presentation describes the applications of multiplex technologies, which allow the simultaneous measurement of multiple analytes (allergens or IgE antibodies) in a single test. These technologies can be quantitative laboratory based tests, or may be screening or point of care tests that are designed for use by consumers. Multiplex technologies include fluorescent suspension arrays, microarrays, and rapid lateral flow chromatographic tests. The power of multiplex or microarray technologies is that they are high throughput systems that provide data covering 10, 20 or more measurements, simultaneously, under defined conditions.

Fluorescent multiplex suspension array technology

A novel, highly sensitive fluorescent multiplex suspension array platform, the xMAP system (Luminex Corporation, Austin, TX) has been used for measuring multiple analytes, including cytokines, growth, and cell signaling factors, kinases and bacterial pathogens [4-7]. The principle of the xMAP system relies on using polystyrene microspheres that are internally dyed with two spectrally distinct fluorochromes. Precise concentrations of each of the two fluorochromes are used to create an array of 100 unique fluorescently “bar-coded” microspheres (Figure 1). Each microsphere set can be coupled covalently with a different reactant on its surface and, theoretically, up to 100 different analytes can be detected simultaneously in a single microplate well. The reactions are read in a flow cytometer that uses microfluidics to align the microspheres in single file and lasers to illuminate the fluorochromes inside and on the surface of each microsphere. The fluorescent signals are captured and digital signal processing translates the signals into real-time, quantitative data for each reaction. The fluorescent “bar-code” identifies the reaction specificity throughout the assay; whereas, a third, spectrally-distinct fluorochrome coupled to a reporter molecule (detector antibody) quantifies the reaction that occurred at the microsphere surface (Figure 2).

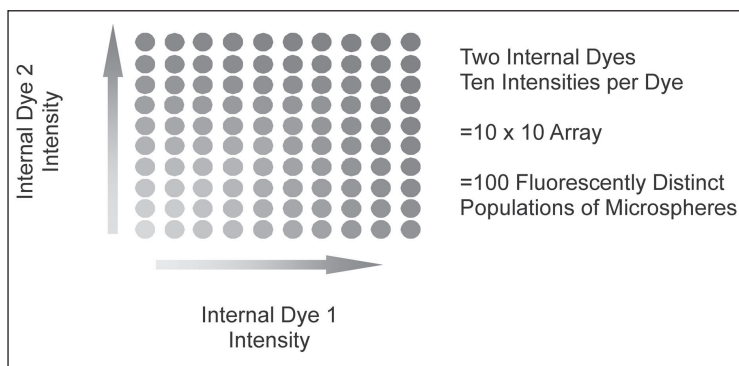


Fig. 1. Principle of the Luminex xMAP fluorochrome array system

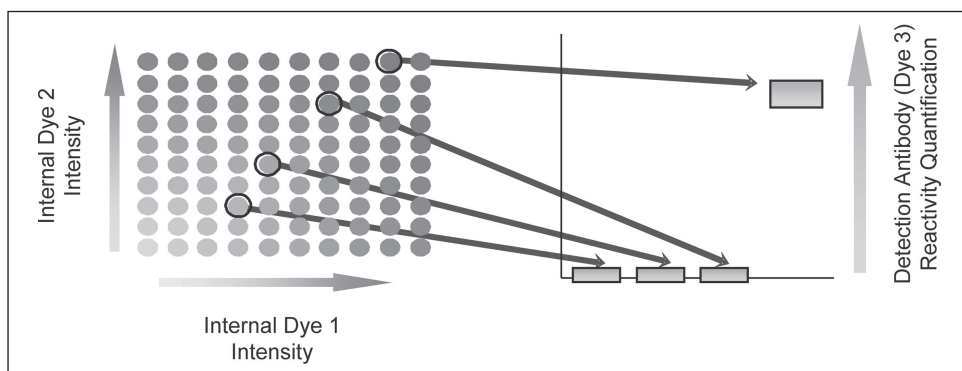


Fig. 2. Internal dyes (dye 1 & dye 2) identify specificity; Detection antibody (dye 3) quantifies reaction

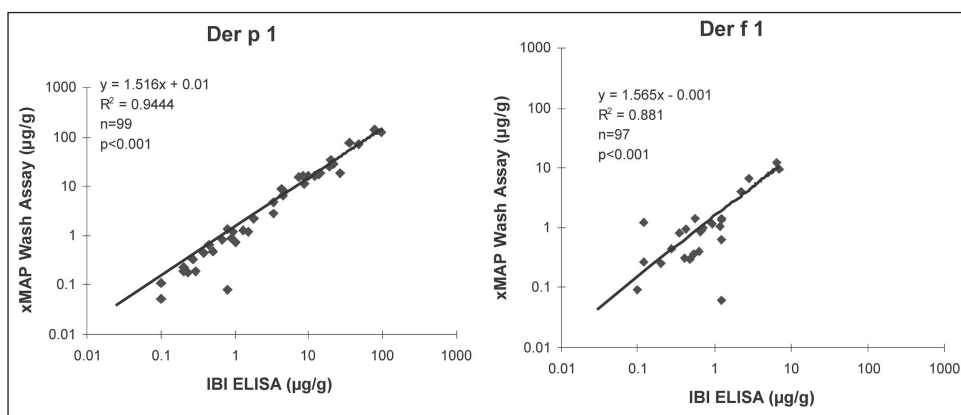
Unlike most other immunoassay platforms (e.g. ELISA, two dimensional flat arrays) that rely on nonspecific absorption of the capture molecule to the reaction surface, xMAP microspheres are covalently coupled through free carboxyl groups on the microsphere surface to free amines on the capture molecule. Covalent attachment of capture molecules ensures stability of the reaction surface as well as directed orientation of the capture molecules, if required. The buoyant density of xMAP microspheres allow them to remain in suspension during the assay procedure, which produces an assay that approaches near fluid phase reaction kinetics. The power of the xMAP system allows for sample reagent, and performance controls to be added into each sample as additional microsphere sets. Since each assay of the xMAP system is produced individually and then multiplexed with other arrays, the introduction of new members to the array requires only the addition of a new microsphere set coupled with a new capture molecule. This capability to add or remove assays depending upon need rather than remapping a new array provides additional flexibility and versatility to the platform.

Multiplex array for indoor allergen detection

Our aim was to transfer the strengths of the monoclonal antibody (mAb) ELISA for allergen detection to the xMAP system to enable simultaneous detection of multiple allergens in dust or air samples, and in allergenic products. A multiplex array that measures five indoor allergens (Der p 1, Der f 1, Der p 2, Der f 2, and Fel d 1) simultaneously has been developed. Multiplexed microspheres, each conjugated with an allergen specific capture antibody, were incubated with dust or allergen extract. Following the capture phase, a biotinylated allergen specific detector antibody was added and the samples were analyzed on a Luminex 100 flow cytometer. Real-time analysis of individual microspheres allowed each set to be classified into the appropriate allergen specificity. The median fluorescence intensity of at least 100 microspheres was recorded for each allergen. Each microsphere set contained a unique fluorochrome to identify the allergen (Table 1). The assay was calibrated using a “universal” standard containing defined concentrations of purified natural allergens. The multiplex allergen assay has a sensitivity of 100 – 500pg/ml, is repro-

	ELISA:		xMAP	
Allergen	Capture	Detector	Capture/Bead No	Detector
Der p 1	10B9	5H8 ^B	10B9 (33)	5H8 ^B
Der f 1	6A8	4C1 ^B	6A8 (52)	4C1 ^B
Der p 2	1D8	7A1 ^B	1D8 (56)	7A1 ^B
Der f 2	1D8	7A1 ^B	1D8 (56)	7A1 ^B
Fel d 1	6F9	3E4 ^B	6F9 (58)	3E4 ^B

Table 1. Monoclonal antibody combinations using in ELISA and xMAP system

Fig. 3. Linear regression analysis of dust samples analyzed by xMAP and ELISA

ducible, and can be completed within three hours. The assay was validated using ~100 dust samples that had been assayed by ELISA using the same universal allergen standard. These samples had been selected to contain a range of dust mite and cat allergen levels from $<1\mu\text{g/g}$ dust to $>100\mu\text{g/g}$.

Overall, there was a very good quantitative correlation between the ELISA and multiplex data (Figure 3). There was a highly significant quantitative correlation between the xMAP and ELISA results for all of the allergen assays, with R^2 values ranging from 0.79 – 0.94 [8]. The strongest correlations were observed for Der p 1 and Der f 1 and the Group 2 allergens. The overall strength of the correlation between ELISA and the multiplex array was very encouraging. The results cover a 2-3 log range of allergen concentration which encompasses over 90% of the allergen levels that are measured in environmental studies. Approximately 20% of samples were above the limits of detection in the xMAP system and below the detection limit in ELISA, suggesting that the xMAP is more sensitive.

Arrays in allergy diagnostics

The application of suspension or flat arrays to allergy diagnostics raises some theoretical problems, such as whether sufficient quantities of allergen can be coupled to the arrays and whether IgG antibodies will inhibit IgE antibody binding. Arrays have relatively low capacity for allergen when compared with CAP or RAST assays. These potential problems do not appear to have been borne out in practice. Flat arrays spotted with purified allergens have been produced on glass slides and are capable of measuring IgE antibodies to over 50 allergens [9,10]. The microarray results correlate with IgE antibody measurements made by CAP/RAST [10]. Recently, microarrays coupled with peptides derived from three major peanut allergens have been used to map IgE epitopes and the results suggested that the peptides could be used to define clinically relevant epitopes [11]. The repertoire of IgE responses to recombinant cockroach allergens has been assessed by multiplex assay and using a streptavidin CAP system. The results showed an excellent correlation

between both methods, suggesting that suspension arrays could be applied to allergy diagnostics using purified allergens [12].

Allergy screening tests for consumers

Point of care tests are increasingly used as a first step to provide consumers with basic health care information and to enable them to make a reasonably informed decision about their medical needs. Pregnancy tests are the classic example, but point of care tests are now used to monitor diabetes, AIDS infection, drug tests, and in cancer screening. Many of these tests use lateral flow chromatographic strips and gold-labeled detector antibodies. Rapid tests for dust mite Group 2 allergens have been developed and can be used to screen for allergen exposure in the home, in workplaces or public buildings [13,14]. We are currently developing a “multiplex” version of these tests that will enable mite, cat, dog, and cockroach allergens to be detected at the same time. This test will be approximately the size of a credit card and will be similar to a multiple drug-screening test. Preliminary results demonstrate a significant correlation ($p < 0.001$) between rapid tests results for mite Group 1 and Group 2 allergen, Fel d 1 and Can f 1, and ELISA [15]. Overall, there was 85% agreement between the results of the two tests. The allergen-screening test is expected to provide consumers, allied health care personnel and indoor air quality specialists with an objective method for detecting the presence of allergen in indoor environments.

Recently, a lateral flow test for detecting IgE antibodies to 10 allergen extracts has been developed and shown to correlate with CAP assay [16]. This test appears suitable for use by consumers and in allergy clinics and other health care facilities. The availability of rapid tests for allergy diagnosis and allergen exposure screening is complimentary and should enable consumers to make informed decisions to consult with allergists and other physicians about allergic symptoms.

References

1. Platts-Mills TAE, Vervloet D, Thomas WR, Aalberse RC, Chapman RD. Indoor allergens and asthma: Report of the Third International Workshop. *J Allergy Clin Immunol* 1997; 100: S1-S24.
2. Rosenstreich DL, Eggleston P, Kattan M. The role of cockroach allergy and exposure to cockroach allergen in causing morbidity among inner-city children with asthma. *N Engl J Med* 1997; 336: 1356-1363.
3. Chapman MD, Tsay A, Vailes LD. Home allergen monitoring and control: Improving clinical practice and patient benefits. *Allergy* 2001; 56: 604-610.
4. Fulton RJ, McDade RL, Smith PL, Kienker LJ, Kettman Jr JR. Advanced multiplexed analysis with the Flow Metrix™ system. *Clin Chem* 1997; 43: 1749-1756.
5. Kettman JR, Davies T, Chandler D, Oliver KG, Fulton RJ. Classification and properties of 64 multiplexed microsphere sets. *Cytometry* 1998; 33: 234-243.
6. Oliver KG, Kettman JR, Fulton RJ. Multiplexed analysis of human cytokines by use of the FlowMetrix system. *Clin Chem* 1997; 44: 2057-2060.
7. Dunbar SA, van der Zee CA, Oliver KG, Karem KL, Jacobson JW. Quantitative, multiplexed detection of bacterial pathogens: DNA and protein applications of the Luminex LabMAP system. *J Microbiol Methods* 2003; 53: 245-252.
8. Earle CD, Tsay A, Saric B, Godbout R, Oliver KG, Chapman MD. High Throughput Fluorescent Multiplex Array for Indoor Allergen Exposure Assessment. *J Allergy Clin Immunol* 2006; 117 (2, Suppl.): S28 (abstract No. 111).
9. Hiller R, Laffer S, Harwanegg C, Huber M, Schmidt WM. Microarrayed allergen molecules: Diagnos-

- tic gatekeepers for allergy treatment. *FASEB J* 2002; 16: 414-416.
10. Jahn-Schmid B, Harwanegg C, Hiller R, Bohle B, Ebner C, Scheiner O, Müller MW. Allergen microarray: Comparison of microarray using recombinant allergens with conventional diagnostic methods to detect allergen-specific serum immunoglobulin E. *Clin Exp Allergy* 2003; 33: 1443-1449.
 11. Shreffler WG, Beyer K, Chu TH, Burks AW, Sampson HA. Microarray immunoassay: Association of clinical history, *in vitro* IgE function, and heterogeneity of allergenic peanut epitopes. *J Allergy Clin Immunol* 2004; 113: 776-782.
 12. Satinover SM, Reefer AJ, Pomes A, Chapman MD, Platts-Mills TAE, Woodfolk JA. Specific IgG and IgE antibody binding patterns to recombinant cockroach allergens. *J Allergy Clin Immunol* 2005; 115: 803-809.
 13. Polzius R, Wuske T, Mahn J. Wipe test for the detection of indoor allergens. *Allergy* 2002; 57: 143-145.
 14. Tsay A, Williams L, Mitchell EB, Chapman MD. A rapid test for detection of mite allergens in homes. *Clin Exp Allergy* 2002; 32: 1596-1601.
 15. Tsay A, Franck C, Chapman A, Saric B, Vailes LD, Chapman MD. Multiple screening test for assessment of environmental allergen exposure. *J Allergy Clin Immunol* 2006; 117 (2, Suppl.): S218 (abstract No. 842).
 16. Jonsson A, Lindqvist A, Molander H. A new POC test for rapid detection of IgE antibodies. *Allergy Clin Immunol Int: J World Allergy Org* 2005; Suppl 1: Abstract #515.

Discussion

Barber: When do you think will this kind of multiarray system be available on the market for routine use? Because there are a lot patent issues. There are a lot of patented allergens or allergens that have a specific patented expression system, and you have to combine more than 100 molecules. Should we wait until the patents do expire in 20 years?

Chapman: I do not think we should worry about that. It is a good question, it is one of the problems with all recombinant allergenic products, whether it is diagnostics or therapeutics. One is constrained by the intellectual property issues. The advantage of this particular multiplex system is that you actually do not need very large quantities of allergen. And so you typically couple a series of beads with about 10 - 20 µg of allergen. And that makes it then very attractive for using purified natural products, because you do not have the same issues as regards yield that you may have with recombinants. But otherwise I agree, there are licensing issues that have to be resolved. But obviously, we ultimately expect to commercialize this technology.

Bufe: One question to the correlation between cockroach allergen on the microarray in your assay. I am a little bit concerned because it is not really a very good correlation. What do you think is the reason for that?

Chapman: Part of it may be because of limitations of these microspheres in their capacity for allergens. That is something where we have done some more recent studies with dust mite, and also cat and *Alternaria*, allergens coupled to the beads and we have seen a much lower sensitivity of the multiplex system. So, I think that that is a problem that has to be resolved. We have some ideas of what can be done, which we are going to test over the next few years. The multiplex system does not have the same capacity as, for example, the CAP system. But at the same time we think it is worth pursuing because of the power that using that multiplexing technology has.