

High-throughput fluorescent multiplex array for indoor allergen exposure assessment

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Background: Current enzyme immunoassay methods for detection of common indoor allergens in environmental dust samples are labor-intensive and time consuming.

Objective: To develop and validate a fluorescent multiplex array to measure 6 (Der p 1, Der f 1, Der p 2, Fel d 1, and Can f 1) indoor allergen levels simultaneously.

Methods: A multiplex array for 6 allergens, using mAbs covalently coupled to fluorescent microspheres, was developed using a single universal standard composed of purified natural allergens. The multiplex array was validated by comparing the measured dust mite, cat, and dog allergen levels in household dust samples to those obtained by standard ELISA methods.

Results: Linear regression analysis showed a highly significant quantitative correlation between the multiplex array and ELISA for dust mite, cat, and dog allergens: R^2 values ranging from 0.90 to 0.99 ($P < .001$). In addition, the sensitivity, limit of detection (<0.1 ng/mL), reproducibility, intra-assay coefficient of variance ($<5\%$), and interassay coefficient of variance ($<25\%$) of the fluorescent multiplex array were shown to be equal to or better than the ELISA method.

Conclusion: A multiplex array has been developed to measure simultaneously 6 indoor allergens from a single sample. The array will facilitate epidemiologic studies and indoor air quality assessments and can, in principle, be expanded to include other allergens and biologics.

Clinical implications: The multiplex array lends itself to clinical studies, population-based environmental surveys, and allergen avoidance studies comparing allergen exposure in large populations over several time points. (*J Allergy Clin Immunol* 2007;119:428-33.)

Key words: Indoor allergen, multiplex array, environment, exposure, asthma, indoor air quality

Sensitization to indoor allergens (dust mite, animal allergens, cockroach and fungi) is an important risk factor for the development of asthma.¹ Environmental measurements of exposure to these allergens, in dust or air samples, are routinely performed using separate ELISAs for each allergen. The ELISA provides a quantitative exposure assessment, but the requirement to perform separate tests for each allergen is time consuming, costly, and prone to technical errors. This is especially true for large-scale clinical studies, population-based environmental surveys, or allergen avoidance studies, which may require measurements of multiple allergens on hundreds or thousands of samples. The ELISA is currently the gold standard for indoor allergen analysis. The ELISAs either use pairs of mAbs directed against nonoverlapping epitopes on the allergen molecule or capture mAb and polyclonal rabbit antibody for detection. The antibodies used in these systems have been well defined and used for many years.²⁻⁹ However, the limitations of ELISA procedures are an impediment for large studies of exposure assessment, for prospective studies and, especially, for studies involving multiple allergens.

In recent years, fluorescent multiplex array technology has been developed for simultaneous measurement of multiple analytes in a single test.¹⁰ Multiplex technology uses polystyrene microspheres that are internally dyed with specific ratios of 2 spectrally distinct fluorophores, to create as many as 100 distinctly coded bead sets. Capture antibodies can be covalently coupled to different beads and used to develop quantitative immunoassays using biotinylated detector antibodies and a reporting fluorophore. This system of multiplex technology has been applied extensively to measure cytokines,¹¹⁻¹⁶ antibodies,¹⁷⁻²¹ nucleic acids,^{22,23} and bacterial pathogens.²⁴⁻³⁰ Typically, 5 to 30 proteins can be measured in a single test, and panels of different tests are commercially available.

This article describes the development and validation of a fluorescent multiplex array for detection of Der p 1, Der f 1, Der p 2, Der f 2, Fel d 1, and Can f 1 in a single quantitative test. The array uses the same (or equivalent) antibody combinations used in ELISA. The array is standardized by using purified natural allergens to form a universal allergen standard. These allergens were produced in accordance with criteria established as part of the European Union project on Certified Reference Materials for Allergenic Products (CREATE).³¹ The multiplex array

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Abbreviations used

CREATE: Certified Reference Materials for Allergenic Products
CV: Coefficient of variance

was validated by comparison with ELISA on a panel of environmental dust samples.

METHODS

Dust sample preparation

House dust samples collected from bedding, carpet, and furniture were previously obtained using the Mitest dust collector system (Mitest Limited, Dublin, Ireland).^{32,33} The dust was removed from the filter insert, and 100 mg fine dust was extracted with 2.0 mL PBS 0.05% Tween, pH 7.4. After extraction for 2 hours at room temperature, the extract was centrifuged at 2500 rpm for 20 minutes to pellet the dust particles, and the supernatant was removed and stored at -20°C before analysis.

Universal allergen standard

To ensure comparability of the ELISA and fluorescent multiplex array, a single universal standard was prepared containing all allergens to be measured. Mite group 1 and group 2 allergens were purified from spent culture media by affinity chromatography and size exclusion HPLC. The mite allergens were purified and quantified according to the protocols established as part of the European Union CREATE project.³¹ Cat allergen, Fel d 1, was purified from house dust extract as previously reported.⁴ Dog allergen, Can f 1, was purified from dog hair and dander extract.³⁴ Protein concentrations were determined by amino acid analysis, advanced protein assay, or A₂₈₀ measurements. A stock solution of the universal allergen standard was prepared using purified natural allergen (1.0-3.0 mg/mL) and diluted to final concentrations of 1000 to 5000 ng/mL in 1% BSA PBS 0.05% Tween, pH 7.4, containing 50% glycerol.

ELISA analysis

The Der p 1 ELISA used mAbs 10B9 for allergen capture and biotinylated 5H8 for detection. The Can f 1 ELISA was modified from the original monoclonal/polyclonal assay to use 2 mAbs, clone 10D4 for capture and biotinylated 6E9 for detection. All other assays used the same mAb combinations as described previously.²⁻⁵ Immediately before analysis, the dust samples were equilibrated to room temperature and then centrifuged at 3000g for 2 minutes to settle any remaining solid dust particles in the tube. The universal standard was diluted 1/10 and then serially diluted to 1/5120 by 2-fold dilutions. The dust samples were analyzed at 4 doubling dilutions from 1/10 to 1/80. After all reagent incubations, plates were read when the optical density at 405 nm was between 2.0 and 2.4.

The Der p 1, Der f 1, and mite group 2 ELISA coefficients of variance (CVs) were previously reported for the European Union CREATE allergen standardization project.³⁵ The intra-assay CVs for the Fel d 1 and Can f 1 ELISA were determined from analysis of 18 and 12 dust samples, respectively, measured in triplicate in a single assay. The interassay CVs for the Fel d 1 and Can f 1 ELISA were determined from analysis of 49 and 36 dust samples run on 3 separate days.

Fluorescent multiplex array for indoor allergens

Coupling of mAb to fluorescent microspheres. Carboxylated fluorescent microsphere bead sets (Luminex xMAP system; Luminex

TABLE I. Formulation of a universal allergen standard for immunoassays

Allergen	Lot no.	Concentration (mg/mL)	Amount (μL) in 20 mL	Final allergen concentration (ng/mL)
Der p 1	2546	3.0*	17	2500
Der f 1	2547	1.4*	36	2500
Der p 2	2551	1.7*	30	2500
Fel d 1	2609	2.0†	10	1000
Can f 1	29045	2.0†	50	5000

*Protein content based on amino acid analysis.

†Protein content based on extinction coefficient at 280 nm.

Corp, Austin, Tex) were carefully resuspended with vortexing and sonication before coupling. In a typical coupling, 5.0 × 10⁶ carboxylated microspheres were placed in a microcentrifuge tube, washed with deionized water, and resuspended in 100 mmol/L monobasic sodium phosphate, pH 6.2. The bead suspension was activated with 50 mg/mL N-hydroxysulfosuccinimide sodium salt, followed by 50 mg/mL N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide for 20 minutes. The beads were washed twice with 50 mmol/L 4-morpholine-ethanesulfonic acid buffer, pH 5.0, to remove any excess activating agents, and then resuspended in 50 mmol/L 4-morpholineethanesulfonic acid, pH 5.0. Monoclonal capture antibodies were added to each bead suspension and incubated for 2 hours with mixing by rotation. The beads were washed and resuspended in storage buffer (PBS, 0.1% BSA, 0.02% Tween-20, 0.05% azide, pH 7.4) and incubated for 30 minutes with mixing by rotation. The beads were washed twice and then resuspended with storage buffer for storage in the dark at 2 to 8°C.

Multiplex assay. Dust samples were equilibrated to room temperature and centrifuged at 3000g for 2 minutes to settle any remaining solid dust particles. Aliquots of the supernatant were diluted 1/100 in assay buffer (PBS, 1% BSA, 0.02% Tween-20, pH 7.4) and serially diluted 1/10 to give a 1/10,000 final dilution. Dust samples were assayed at 1/100 and 1/10,000 dilutions.

The 96-well filter plates were prewet with 100 μL/well assay buffer, and the buffer was removed by vacuum filtration. For each microtiter plate, 15 μL of each mAb-coupled microsphere bead set (1 × 10⁷ beads/mL) was added to 5.5 mL assay buffer. The solution was thoroughly mixed to assure that the microspheres were homogeneously dispersed throughout the solution. For the multiplex assay, 50 μL microsphere solution (1500 microspheres each bead set/well) was thoroughly mixed with 50 μL dust extract, universal allergen standard, or control and incubated for 60 minutes in the dark. The universal standard was diluted 1/20 and then serially diluted to 1/40,960 by 2-fold dilutions. The controls contained only assay buffer with no allergen present. The wells were washed twice by vacuum filtration, and beads were incubated with 100 μL biotinylated detection mAb diluted 1/250 to 1/1000 in assay buffer for 60 minutes in the dark. After further washing, microspheres were incubated with 100 μL streptavidin R-phycoerythrin (1 mg/mL) diluted 1/250 in assay buffer for 30 minutes in the dark. The wells were washed twice, and the microspheres were resuspended with 100 μL assay buffer. The plate was read in a Bio-Plex fluorescent suspension array reader (Bio-Rad Laboratories, Hercules, Calif), which consists of Luminex xMAP instrumentation (Luminex Corp) supplied with Bio-Rad proprietary software.

Data analysis. The fluorescent signal for each of at least 100 beads from each bead set was quantified, and the median fluorescent intensity from the 100 measurements for each bead set was calculated and used to establish the standard curve or calculate the concentration of each allergen present in the sample. The equation for the standard curve was determined by using a Logistic-5PL curve fit developed by Brendan Technologies (Carlsbad, Calif).

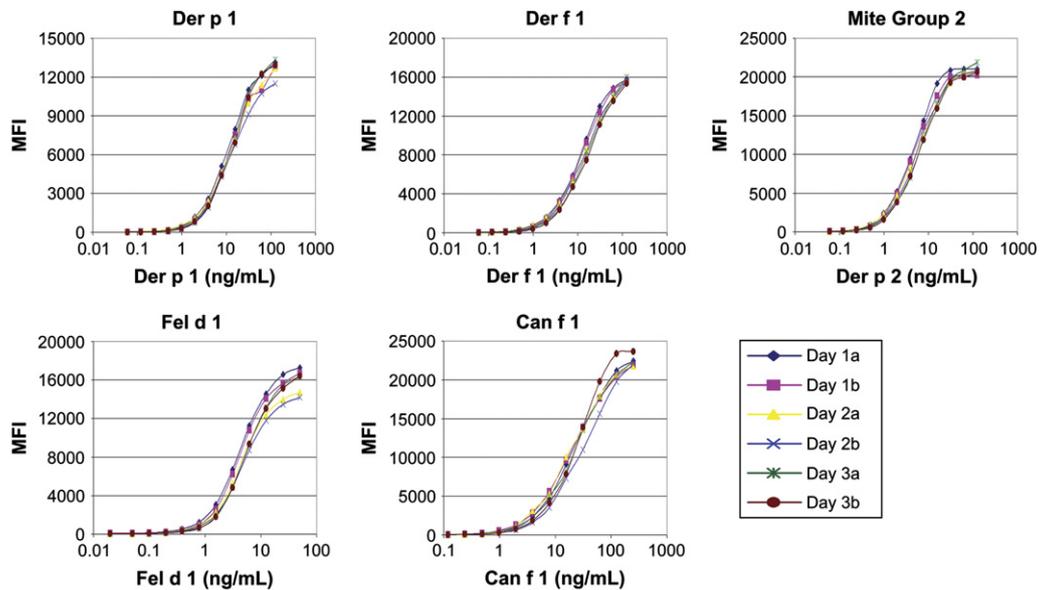


FIG 1. Control curves for 5 allergens measured in 6 replicates over a period of 3 days (2 plates/d).

TABLE II. mAb pairs used in the fluorescent multiplex assay

Allergen	Capture mAb	Detection mAb	Multiplex bead set #
Der p 1	10B9	5H8	33
Der f 1	6A8	4C1	51
Mite group 2	1D8	7A1	53
Fel d 1	6F9	3E4	58
Can f 1	10D4	6E9	20

Sensitivity and reproducibility. To determine the sensitivity and reproducibility of the fluorescent multiplex array, samples were analyzed both for interassay and intra-assay variation. Over a period of 3 days, 70 dust samples containing varying amounts of Der p 1, Der f 1, mite group 2, and Fel d 1 along with 32 dust samples containing Can f 1 were measured at 2 dilutions. The standard curves from each microtiter plate were compared to ensure reproducibility in the assay. The concentrations of each allergen in the samples were measured, and an interassay CV was calculated from the 6 replicates. To determine the intra-assay CVs for each allergen, 12 dust samples were measured at 2 dilutions, run in triplicate on a single plate.

Correlation of the multiplex array to ELISA for Der p 1, Der f 1, mite group 2, and Fel d 1 was accomplished by analysis of 70 house dust samples. The Can f 1 assay was correlated by analysis of 170 house dust samples. Correlation values, R^2 , were determined by least-squares linear regression analysis.

RESULTS

Universal allergen standard control curves

A single universal allergen standard containing purified natural Der p 1, Der f 1, Der p 2, Fel d 1, and Can f 1 was formulated to minimize nonspecific interactions in the array. The purified allergens showed >90% purity (data not shown) in SDS-PAGE, and the protein content was based on either amino acid analysis or extinction

coefficient (Table I). Der p 2 was used as the standard for mite group 2 allergens because Der p 2 and Der f 2 are highly cross-reactive allergens.³

The reproducibility of the universal allergen standard in the multiplex array was assessed by comparing control curves for 5 allergens on 3 separate days. The results showed sigmoidal dose response curves over a 2 to 3 log range, with CVs ranging from 10% to 23% over the linear portion of the curve (Fig 1). These results also showed no interference when measuring 5 allergens simultaneously.

Quantitative comparison of ELISA and multiplex array

House dust samples from bedding, carpet, and furnishings were analyzed for dust mite, cat, and dog allergens by fluorescent multiplex array and ELISA by using the universal standard and standard antibody pairs (Table II). The dust samples were derived from more than 70 extracts in an in-house dust bank that contained a 2 to 3 log range of allergen levels. There was excellent agreement between the concentrations measured by ELISA and multiplex array. Linear regression analysis showed a highly significant correlation between the 2 methods with R^2 values ranging from 0.90 to 0.99 ($P < .001$; Fig 2). The allergen concentrations in the samples ranged from <1 to 1000 $\mu\text{g/g}$. Approximately 14% of the samples were detected as positive with the fluorescent multiplex array, but were below the detection limit of the ELISA.

Assay sensitivity and reproducibility

The intra-assay reproducibility was excellent with CVs >10% for each allergen (Table III). The interassay CVs were found to be comparable to results for the ELISA, and in most cases, the reproducibility was slightly

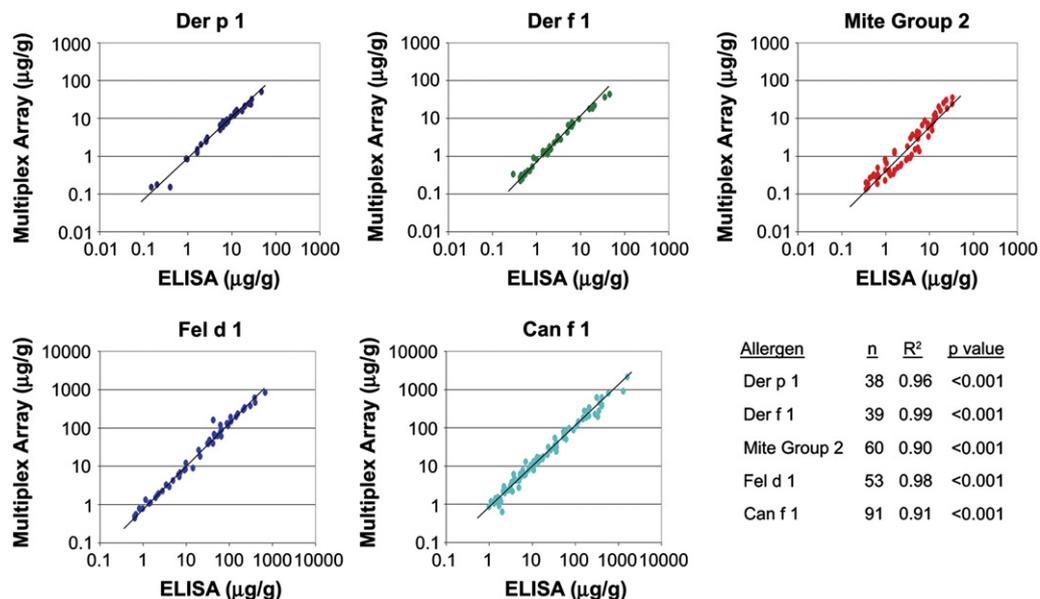


FIG 2. Correlation between the fluorescent multiplex array and ELISA data for Der p 1, Der f 1, Der p 2/Der f 2, Fel d 1, and Can f 1.

better for the fluorescent multiplex array. CVs were less than 15% for the dust mite and dog allergens and slightly higher but less than 25% for Fel d 1. It also should be noted that the limit of detection was an order of magnitude lower for the multiplex assay, dropping the lower limit of detection to less than 1 ng/mL for each of the allergens.

DISCUSSION

Over the period of the past 20 years, measurement of specific allergens by ELISA has been the gold standard for environmental exposure assessment. Most recently, ELISA was used to measure allergen exposure in the US National Survey of Allergens and Lead in Housing, in the National Institutes of Health Inner City Asthma Study, and in European Community Respiratory Health Survey of exposure in 22 centers across Europe.³⁶⁻³⁹ However, it is in these large studies, involving hundreds or thousands of samples, that the limitations of ELISA become apparent. Logistical and financial constraints may limit the number of allergens to be measured and affect study design. Large studies require several technicians, often working in different centers, to process samples and perform ELISA assays, which increases variability. Data may take months (or years) to accumulate because of the time constraints in performing ELISA for multiple allergens on large numbers of samples. These considerations reduce the scope for using ELISA in epidemiologic studies and for routine use in evaluating indoor air quality.

The fluorescent multiplex array reported here allows 6 (or more) allergens to be measured at the same time in a single microtiter well. Thus the assay conditions are the same for each allergen and provide greater efficiency and reproducibility. Technically, the array offers several

TABLE III. Comparison of the sensitivity and reproducibility of ELISA and the fluorescent multiplex array

	Intra-assay %CV	Interassay %CV	Limit of detection (ng/mL)
ELISA			
Der p 1	6.4	15	0.5
Der f 1	7.1	15	0.5
Mite group 2	8.9	30	0.8
Fel d 1	8.3	19	0.8
Can f 1	6.1	13	4.0
Fluorescent multiplex array			
Der p 1	3.9	11	0.06
Der f 1	4.7	10	0.06
Mite group 2	4.2	13	0.06
Fel d 1	4.2	23	0.02
Can f 1	4.2	10	0.12

improvements compared with ELISA. Using a universal allergen standard of purified natural allergens improves the reproducibility and standardization of the assay over the long term. The ability to measure multiple analytes decreases the number of assays to be performed. The increased dynamic range of the multiplex array allows each sample to be tested at only 2 dilutions (1:100 and 1:10,000) to cover the full range of allergens found in homes. At these dilutions, the allergens can be reliably measured from 0.01 to 10,000 µg/g dust (compared with 0.1-100 µg/g in ELISA). We estimate that testing 6 allergens at 2 dilutions simultaneously results in an ~12-fold savings in microtiter plates and ancillary supplies. Clearly, the major advantage of the multiplex array compared with ELISA is the time savings achieved by analyzing multiple samples at once. The results show that

the multiplex also has comparable (or improved) sensitivity, accuracy, and reproducibility. The strong correlation between ELISA and multiplex array for allergens ($R^2 \geq 0.90$) is similar to correlations reported for other proteins, including cytokines.⁴⁰ Other important features of the fluorescent multiplex array system are that bead sets can be mixed in different combinations to provide customized allergen panels and that, as tests for new allergens are developed, they can be easily added to the system. We have recently developed bead sets for cockroach (Bla g 2) and rodent allergens (Rat n 1 and Mus m 1) that extend the multiplex to 9 allergens (abstract submitted for publication at the American Academy of Allergy, Asthma & Immunology's annual meeting, Feb 23-27, 2007, San Diego, Calif).

Comparison of the ELISA and multiplex array was dependent on having the universal allergen standard formulated using pure allergens. This standard proved suitable for calibration of both assays. However, the ELISA results may not be directly comparable to current ELISA kits that use allergen extracts as standards. The estimated amount of allergen present in the allergen extract standards may vary by 2-fold to 3-fold from the absolute amount of protein in purified allergen preparations determined by amino acid analysis, extinction coefficient, or advanced protein assay. Purified allergens are increasingly available for use in research, diagnostic, and therapeutic purposes. They can be standardized according to criteria established in the European Union CREATE study and through the development of international reference preparations.³¹ Thus, it is likely that exposure assessments will in the future be based on formulations of similar composition to the universal allergen standard described here, rather than current allergen extract standards.

The multiplex array should lend itself to large epidemiologic studies or population-based environmental surveys in which hundreds or thousands of samples will be collected and analyzed for multiple allergens. The array will benefit these studies by reducing the number of assays and amount of sample required and by increasing the speed and efficiency of producing data on allergen exposure. The array should be readily expandable to other indoor allergens, which should further increase the scope of these studies. In principle, the array can also be expanded to include panels of other important allergens, such as pollens, foods, and molds. The widespread use of multiplex assays for other immunologic assays in academia, government agencies, and industry should facilitate use of the allergen array for research purposes. The technology will also enable indoor air quality and environmental control companies to provide allergen exposure assessments more efficiently and cost effectively on a routine basis. These advances should benefit patients with allergy by providing better access to environmental allergen exposure assessments and education about objective measures to reduce harmful exposures.⁴¹

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