



An automated microfluidic-based immunoassay cartridge for allergen screening and other multiplexed assays

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ABSTRACT

A microfluidic cartridge and system for multiplexed immunoassays is described. The passive microfluidic cartridge was composed of three layers of injection molded plastic sealed together using a thermal staking technique. Using this platform technology, a specific immunoglobulin E (IgE) panel assay was constructed. Allergen extract targets, positive and negative controls, and IgE calibration standards were immobilized within the cartridge as a microarray. A computer-controlled solenoid array provided the necessary actuation force for pumping reagents within the cartridge to perform an automated, chemiluminescent indirect immunoassay. A 20-target allergen extract panel was demonstrated on the device with a total analysis time of 27 min. Allergen screening results showed 84% agreement for 3 house dust mites ($N = 300$) compared with a commercial test and 80% agreement overall ($N = 978$). Average coefficients of variation ($N = 80$) were measured as 20.5% for low/medium levels and 20.4% for medium/high levels. The average limit of detection ($N = 160$) was measured at 0.535 AU, and cutoff levels of 1.0 AU were estimated at less than 1 IU/ml (2.4 ng/ml). Such a system has potential applications in decentralized allergen screening as well as in other near-patient diagnostic immunoassays where multiplexed analysis, ease of use, and short analysis time are critical.

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For more than 30 years, in vitro testing of allergen-specific immunoglobulin E (IgE)¹ has been used along with, or as an alternative to, invasive skin prick testing [1–3]. Such tests allow allergists to accurately monitor immunotherapy techniques as well as to screen adults, infants, and small children for atopic allergic sensitivities. Clinically, the most common in vitro technique today is the ImmunoCAP System (Phadia, Uppsala, Sweden) [4], but other tests are becoming more widely used, including the CLA Allergy Test (Hitachi Chemical Diagnostics, Mountain View, CA, USA) [5,6], AlaSTAT and IMMULITE (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA) [7,8], and HY-TEC (Hycor Biomedical, Garden Grove, CA, USA) [9,10], and a variety of multiple allergen-coated nitrocellulose strip tests [11]. Of these, several tests are available as quantitative assays (e.g., ImmunoCAP, IMMULITE, HY-TEC), where specific IgE response is quantified using a nonspecific IgE calibration curve and results

are expressed as international units (IU) per milliliter (IU/ml, where 1 IU is equivalent to 2.4 ng). Because currently there are no specific IgE standards available, it has been noted that specific IgE results can vary from test to test due mainly to differences in allergen source materials [12]. Other tests (e.g., CLA) are available as semiquantitative, and results are reported on a continuous scale using arbitrary units. In both cases, results are typically separated into class scores.

Recently, there has been increased interest in the use of microarrays for the analysis of specific IgE levels in serum. The advantages of microarrays for allergen screening are the ability to provide a multitarget screening technique (50+) while using minimal serum (25–100 μ l). This is even more critical for allergen screening in small children, where large blood draws are difficult and serum supply is limited. Schweitzer's group used microarrays and rolling circle amplification to detect specific IgE [13,14]. Signal amplification using streptavidin–horseradish peroxidase (HRP) conjugate and a fluorescent substrate was later employed in a seven-allergen panel using a microarray [15]. Cutoff levels achieved on the microarray were 1 kIU/L. A house dust mite and food extract microarray was also demonstrated using a streptavidin–cyanine-3 (Cy3) conjugate with clinical limits of detection (LODs) near 1 kIU/L [16]. Recombinant proteins have also been demonstrated on microarrays for grass and tree pollen specific IgE detection using a

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¹ Abbreviations used: IgE, immunoglobulin E; IU, international units; HRP, horseradish peroxidase; Cy3, cyanine-3; LOD, limit of detection; RT-PCR, reverse transcription polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; CCD, charge-coupled device; ABS, acrylonitrile, butadiene, and styrene; CV, coefficient of variation; AU, arbitrary units; SD, standard deviations.

fluorescently labeled anti-IgE conjugate for detection [17]. Typical incubation times for specific IgE detection on microarrays is 60 min per step. Therefore, total reaction times for these assays were between 2 and 3 h. Additional time is required for washing the arrays between steps and scanning the arrays in a fluorescent scanner. Chemiluminescence detection has also been reported in microarray analysis of specific IgE using a flow cell [18]. Total analysis times were reduced to 1 h plus washing by shortening incubation times to 30 min. In the case of chemiluminescence, LODs for both allergen extracts and recombinant proteins were similar to those for the other fluorescence-based methods. A high-sensitivity colorimetric-based microarray was used to test allergic response to mold, Timothy grass, and dust mite allergies with analysis times between 2 and 3 h [19].

Although progress has been made in reducing reaction times for allergen microarray analysis, little has been done to improve the automation of such analysis outside the employment of large robotic workstations. Microfluidic devices provide much promise in the area of automation for microarray analysis and have potential to speed up analysis times due to high surface-to-volume ratios and active mixing. Such devices have performed HIV subtyping using reverse transcription polymerase chain reaction (RT-PCR) followed by microarray analysis [20], performed DNA purification and real-time PCR with the TaqMan probe for infectious disease detection [21], and performed DNA amplification followed by capillary electrophoresis analysis [22,23]. In the area of immunoassays, compact disk-like microfluidic platforms have been developed to carry out automated enzyme-linked immunosorbent assay (ELISA) reactions in the laboratory [24] and in clinical settings [25]. Platforms such as these offer parallel analysis of many samples on the same disk and may be advantageous for a variety of clinical immunoassays. Multiplexed bead-based immunoassays have been in use for years [26], and the use of microfluidics has been proposed to miniaturize these assays as well [27]. The challenge for microfluidic devices in protein-based diagnostics is ensuring that the benefits outweigh the extra cost. Many diagnostic applications already have a rigorous cost structure that makes it difficult to justify the additional cost of microfluidic devices.

In this article, we report the development of a microfluidic cartridge for the automated analysis of specific IgE using allergen extracts. The device was entirely injection molded, and protein was bound to the surface using an inexpensive nitrocellulose coating. The material cost of the device was less than one-tenth that of the activated slide typically employed in microarray experiments. Furthermore, the cartridge was driven by a low-cost analyzer composed of a solenoid actuator array and a low-resolution cooled charge-coupled device (CCD) camera for chemiluminescence detection. The total analysis time for allergen screening on the cartridge was less than 30 min. This system has potential for decentralized allergen screening and near-patient testing where short analysis times, ease of use, and low instrumentation cost are critical.

Materials and methods

Cartridge design

The microfluidic cartridge for allergen screening incorporated five reagent delivery channels consisting of storage tanks and unidirectional pumps, a single reaction zone in which allergen extracts are immobilized, and a waste tank to contain all reaction by-products. Fig. 1 shows a photograph of the cartridge. The tank size was determined by the function of that particular reagent. The cartridge was constructed from three injection molded plastic parts. Transparent general-grade polycarbonate was used for the upper

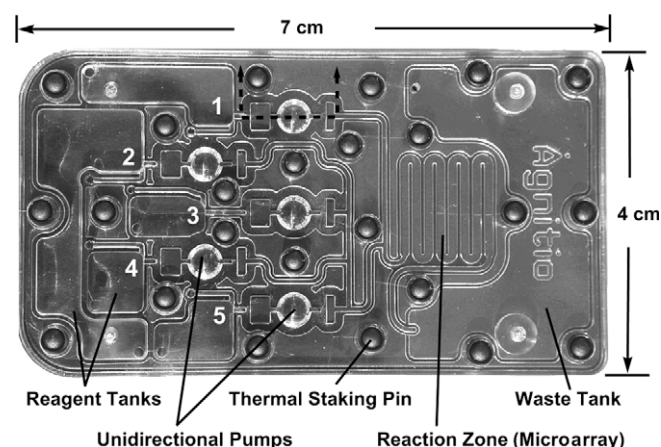


Fig. 1. Injection molded microfluidic cartridge for automated immunoassays. The top view photograph shows five reagent delivery channels and a single reaction zone.

part. Polycarbonate was chosen due to its better stability during thermal assembly processes. The upper part was 3 mm thick. The middle layer was composed of reaction injection molded silicone rubber that was 0.5 mm thick with a hardness of 40 Shore A. Black was the preferred color of this layer due to lower background during chemiluminescence detection. White color additives were found to contain a high percentage of slow-decaying phosphorescent particles that interfered with signal detection. The 3-mm-thick bottom layer was constructed from a copolymer of acrylonitrile, butadiene, and styrene (ABS).

Fig. 2 shows a schematic of the pump structure constructed from these three plastic layers. The unidirectional pump was composed of two passive check valves and a pumping chamber. An external linear actuator was required to displace the silicone diaphragm of the pumping chamber. On displacement, the check valve limits flow in one direction only. The check valves were of different sizes due to the different displacement mechanisms of the pump diaphragm. Because positive displacement forces were provided by the linear actuator, high positive pressures could be achieved and, therefore, the forward check valve was smaller and "tighter." The returning force, however, was due only to the stretching of the silicone diaphragm itself and, therefore, the neg-

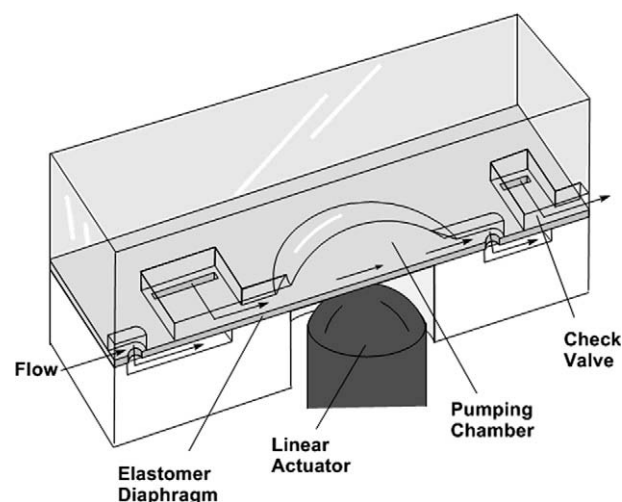


Fig. 2. Cross-sectional schematic of the pump structure corresponding to the dotted line in Fig. 1.

ative pressures achieved were much lower. This necessitated a larger and “looser” check valve in this direction.

All five reagent delivery channels were connected to a single reaction zone in which protein–protein interaction occurred with immobilized targets. The channel in the reaction zone was 1.1 mm wide with a height of 0.49 mm. Wider channels resulted in the trapping of air within the 180° turns. Finally, the reaction zone was connected to a waste tank that contained all of the liquid wastes within the cartridge itself.

Cartridge preparation

The raw injection molded parts were first inspected and cleaned using a two-step wash of 70% ethanol for 10 min and 95% ethanol for 1 min. The parts were then dried in an oven at 40 °C for 1 h. A thin porous nitrocellulose layer was sprayed onto the surface of the silicone rubber using an ultrasonic nozzle (8700–48H, SonoTek, Milton, NY, USA) and low-pressure nitrogen (55 kPa). Nitrocellulose solution was prepared by dissolving nitrocellulose membranes (Pierce, Rockford, IL, USA) in a solution of 30% acetone and 70% isopropanol (IPA) at a concentration of 1% (w/v). The different vapor pressures of the solvents produced a resulting film that was porous. After spraying, the silicone rubber was dried in an oven at 40 °C for 24 h without any additional washing steps. Allergen extracts and other targets were arrayed onto the nitrocellulose-treated silicone rubber surface using a split pin (Point Technologies, La Aurora de Heredia, Costa Rica) mounted on an in-house arraying system. Arraying was performed under controlled conditions of 19 °C and 60% relative humidity. The sample plate was controlled at 12 °C, just above the dew point, using cooling water. The pin spotting produced spot sizes of less than 175 µm with estimated delivery volumes between 1.0 and 1.5 nl. Pin-to-pin variations were managed by printing all spots on each cartridge with only one pin and calculating results based on relative intensity to reference spots. All targets were printed in quadruplicate. Special holders were used to provide adequate alignment tolerances (± 50 µm). Vacuum was not necessary for securing the substrates. After printing the allergen targets, the substrates were dried in an oven at 37 °C for 30 min to reduce run-to-run variation. Cartridges were then assembled using an in-house, pneumatic, thermal staking machine capable of staking all pins in the cartridge simultaneously. A pin temperature of at least 110 °C and a pressure of 5 bar for 10 s was necessary to stake the cartridge completely. Thermal staking was localized to the staking pins only, and the temperature of the reaction zone and spotted protein was never above 37 °C. No detrimental effects of staking on the arrayed proteins were observed. After assembly, cartridges were stored in a dark dry box or vacuum-sealed in foil envelopes with desiccant prior to testing.

Analyzer system

Fig. 3 shows a schematic of the hardware setup used to actuate the cartridge, control the assay protocol, capture the resulting image, and perform the necessary image and data analysis. The dark chamber was composed of a solenoid actuator (300 g force maximum at 4 W) array for actuating the cartridge, a heater module for constant reaction temperature control, and a low-resolution cooled CCD camera (ME2, Finger Lakes Instrumentation, Lima, NY, USA). A 25-mm f/0.95 c-mount lens was used to image the chemiluminescence signals within the reaction zone onto the 768 × 512-pixel full-frame CCD sensor. A desktop computer controlled the reaction temperature, microfluidic actuation, image capture, and data analysis for the test using in-house software. Alignment marks spotted on the cartridge allowed automatic image alignment and spot identification. The image analysis was performed using a competitive seeded region-growing algorithm

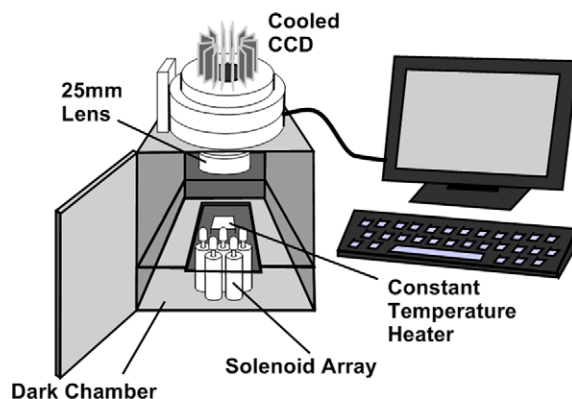


Fig. 3. Hardware setup for operation of the microfluidic cartridge. The entire immunoassay is automatically performed within the dark chamber after reagents are loaded onto the cartridge.

[28] bounded by a rectangle of interest to determine foreground regions. This technique allowed for some variation in spot position due to alignment tolerances. Foreground intensities were calculated as average pixel values within the foreground region. Background intensities were calculated as average pixel values within the 5th and 30th percentiles of the histogram of the rectangle of interest. The use of the histogram technique reduces background dependence on the foreground intensity and random noise. Fig. 4 shows chemiluminescence images of four assay results from pooled allergenic plasma samples.

Microfluidic characterization

Average pump speeds were measured on the cartridge using 1-Hz actuation frequencies by loading deionized water into the reagent tanks and measuring the distance that the water was pumped within the reaction zone between cycles. Pump speeds were then calculated based on the cross-sectional area of the reaction zone channel. Some discrepancies may occur because the channel cross-sectional area may differ slightly after thermal staking. Cartridge-to-cartridge variation was determined by calculating the pumping speed coefficient of variation (CV) from five identical cartridges. Tank volumes were verified after thermal staking by injecting known volumes of water with a micropipette. Maximum backpressure of the pumps was measured using a digital pressure meter (GS521, Koganei, Tokyo, Japan) by connecting 1.6-mm OD tubing to the pump outlet and sealing the downstream micro channels with silicone rubber.

Allergenic test samples

Allergenic human plasma and serum were purchased from PlasmaLab International (Everett, WA, USA), collected with written informed consent, and also acquired in cooperation with Mackay Memorial Hospital (Hsinchu, Taiwan) and Min-Sheng Hospital (Taoyuan, Taiwan). Compatibility of plasma was confirmed using three matched plasma/serum sets donated at the same time. In all cases, serum results were slightly higher than plasma, consistent with the slightly lower yield of serum from whole blood. Samples were analyzed by ImmunoCAP and CLA using a local testing service. Plasma samples were stored at –20 °C until use. Serum samples for screening use were stored after collection at 4 °C for no longer than 1 week prior to testing. Serum samples for CV analysis were aliquoted and stored at –20 °C until use. All frozen samples were thawed at 4 °C and vortexed prior to use. Human samples should be handled with caution even if they are tested negative for infectious diseases.

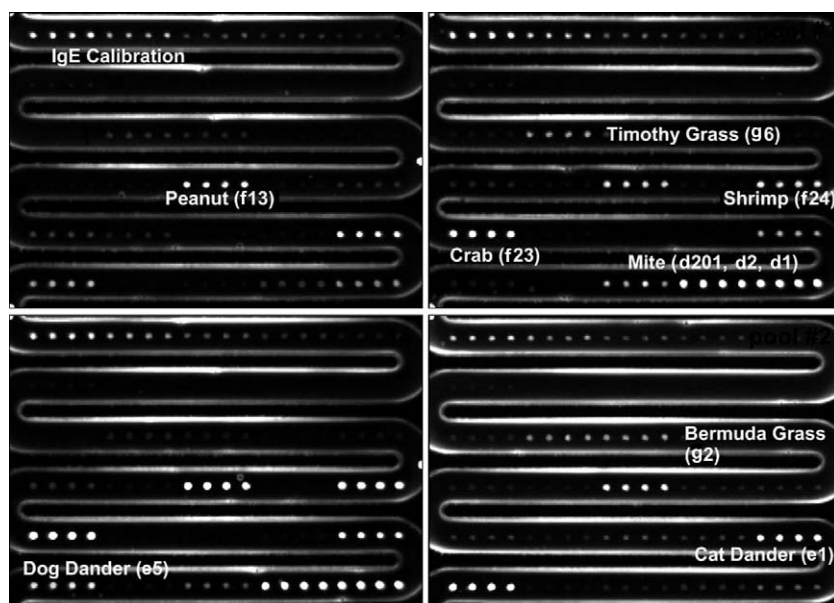


Fig. 4. Chemiluminescence images of four allergen screening results of plasma samples on the microfluidic cartridge. All allergens are arrayed in quadruplicate.

Allergen materials and reagents

Freeze-dried *Dermatophagoides pteronyssinus* (d1), *Dermatophagoides farinae* (d2), *Blomia tropicalis* (d201), German cockroach (i6), cat dander (e1), dog dander (e5), egg white (f1), milk (f2), codfish (f3), wheat (f4), peanut (f13), soybean (f14), almond (f20), crab (f23), shrimp (f24), Bermuda grass (g2), Timothy grass (g6), *Aspergillus fumigatus* (m3), *Candida albicans* (m5), and common ragweed (w1) extracts (with CCLS allergen codes shown in parentheses) were purchased from a commercial supplier. HRP-conjugated polyclonal goat anti-human IgE and human IgE myeloma serum were obtained from U.S. Biologicals (Swampscott, MA, USA). SuperBlock Blocking Buffer–Blotting in TBS, SuperSignal West Femto Maximum Sensitivity

Substrate, BupH Tris Buffered Saline Packs, BupH Phosphate Buffered Saline Packs, and Guardian HRP Conjugate Stabilizer were purchased from Pierce. Tween 20 was obtained from Merck (Whitehouse Station, NJ, USA). Triton X-100 and carbonate bicarbonate buffer capsules were obtained from Sigma–Aldrich (St. Louis, MO, USA). A 2× conjugate solution was prepared by diluting the conjugate 1:500 in conjugate stabilizer. The final 1× working solution was prepared by diluting 1:1 with SuperBlock containing 0.2% Tween 20, and 0.1% Tween 20 in TBS was used as the wash buffer.

Extracts were reconstituted using deionized water to a protein concentration of 5 mg/ml (Bradford method). Precipitates were spun down using light centrifugation, and stock solutions were separated into 10-μl aliquots and stored at −20 °C. Working solutions were then prepared by diluting the stock with the appropriate immobilization buffer. Working solutions were stored at 4 °C for a maximum of 1 week.

Table 1

Automated microfluidic protocol for allergen screening assay.

Active channel ^a	Time ^b (s)	Description
—	120	Temperature stabilization
2	10	Buffer preinse
3	14	Sample
—	600	First hybridization
4	3	Air
2	4	Buffer
4	3	Air
2	4	Buffer
4	3	Air
2	4	Buffer
4	3	Air
2	4	Buffer
3	16	Anti-IgE antibody–HRP conjugate
—	600	Second hybridization
1	2	Substrate
2,4	28	Reaction zone wash protocol
2	6	Buffer
1,2,4	2	Three channels simultaneously
2,4	2	Two channels simultaneously
2	10	Buffer
1	13	Substrate
—	30	Substrate reaction
—	120	CCD exposure

^a Pumping frequency was 1 Hz.

^b Total time was 26.7 min.

Extract inhibition testing

The efficacy of extract materials was verified by ELISA inhibition testing. Positive allergenic plasma was spiked with extract concentrations in the range of 0–100 μg/ml, and quantitative specific IgE response was measured using a commercial test. Negative “sham” controls of non-cross-reactive extracts were used to verify results.

Microfluidic cartridge allergen test

Using a micropipette, 90 μl of undiluted plasma or serum, 450 μl of wash buffer, 120 μl of premixed substrate, and 120 μl of a 1:1000 dilution of HRP conjugate were added to the appropriate tanks within the cartridge prior to use. Table 1 shows the protocol used to operate the microfluidic cartridge to perform allergen testing. A two-step, 10/10-min reaction ELISA for detecting allergen-specific IgE was employed with a final luminol substrate addition. Washing efficiencies were enhanced using a combination of air and wash buffer. Reaction temperature was controlled at 32 °C. Total analysis time was 26.7 min. Cartridges were discarded after use.

To determine optimal allergen concentrations as well as immobilization buffer, dilutions of allergen stock solutions in four immobilization buffers were arrayed and tested with several known

positive and negative samples. Immobilization buffers were chosen based on signal strength. Serial dilutions of human IgE were used for internal calibration as well as for semiquantification. Optimized conditions were empirically determined based on signal strength and correlation, similar to those described previously [16].

The screening panel was composed of all 20 allergen extracts, the human IgE serial dilution curve, and negative controls. All targets were arrayed in quadruplicate. Target intensities were calculated by removing the spot furthest from the mean, averaging the remaining three spots, and subtracting the negative control intensity. Semiquantitative results were generated by linear interpolation of target intensities against the human IgE serial dilution curve to determine specific IgE response in arbitrary units (1–32 AU) [16]. Class scores from 0 to 6 were determined by the following ranges: less than 1 AU, 1 to 2 AU, 2 to 4 AU, 4 to 8 AU, 8 to 16 AU, 16 to 32 AU, and more than 32 AU, respectively.

Limit of detection

The LOD for allergen-specific IgE was estimated by analysis of 20 repeats of two negative serum samples over four lots of microfluidic cartridges. LOD values were then determined by extrapolation of the mean plus 2 standard deviations (SD) on the internal IgE calibration curve.

Precision

Assay precision was evaluated using two serum pools representing allergenic samples of low level and medium level. CVs were determined over a 20-day period testing each serum pool in duplicate twice daily ($N = 80$). Outliers greater than 2 SD from the mean were removed.

Results

Microfluidic performance

Backpressure measurements showed a maximum backpressure of 16.2 kPa at a 10-Hz actuation frequency for air pumping. Table 2 summarizes the measurements of channel-to-channel and cartridge-to-cartridge variation in pump speeds. Within the same cartridge, pumps showed an average variation of 14.3%. Between cartridges, channel variation was less than 10%. The pump chamber volume was approximately 12 μ l, leading to a pump efficiency of approximately 76%. Because reactions were performed under static conditions within the cartridge, pump speed is not critical to the performance of the device. However, tank volumes must be in enough excess of the reaction zone volume to compensate for these variations in pump speed. Air bubbles were sufficiently reduced by adjusting the solenoid positions for maximum compression of the pumping chamber.

Table 2
Channel design and performance parameters of microfluidic cartridge.

Channel ^a	Tank volume ^b (μ l)	Pump speed ^c (μ l/s)	Pump speed CV ^d (%)	Function
1	200	7.98	9.3	Substrate
2	434	9.82	4.7	Wash buffer
3	92	7.92	3.9	Sample
4	101	10.8	3.9	Air
5	120	8.88	9.5	Conjugate

^a Numbering 1 to 5 corresponds to pumps in Fig. 1 from top to bottom.

^b Reaction zone volume was 53 μ l, and waste tank volume was 884 μ l.

^c Pump actuation frequency was 1 Hz.

^d CV was measured using five identical cartridges.

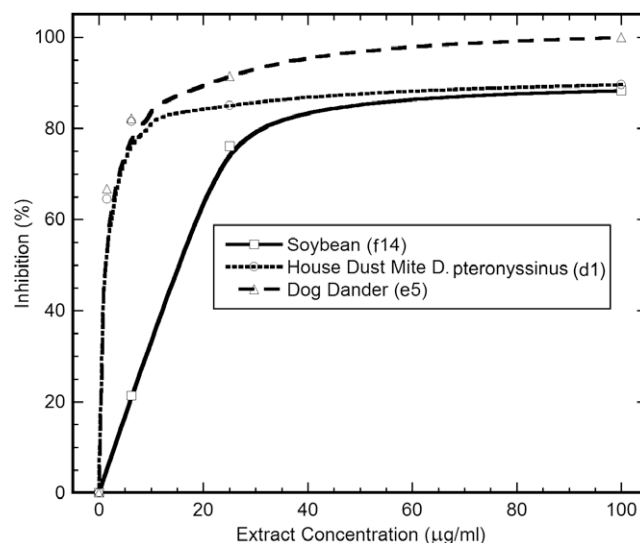


Fig. 5. Representative inhibition curves for soybean (f14), house dust mite *D. pteronyssinus* (d1), and dog dander (e5) extracts.

Allergen material optimization

Fig. 5 shows representative inhibition curves for three allergen extracts. Typically, the extracts show a steep inhibition curve with a long tail such as is shown for d1 and e5. Some extracts also show inhibition such as is shown for f14 where the curve is not as steep. The higher the slope of the curve, the higher the potency of the extract. The long tail and variations in the ultimate limit of the inhibition are most likely due to concentration differences of certain allergenic proteins in the extract compared with the specific IgE test that was inhibited. The plasma samples used to generate the inhibition curves may also play a role if they show a high response to minor allergenic proteins.

Following inhibition testing, each extract was optimized for correlation and repeatability. An appropriate immobilization buffer was determined for each extract, and the concentration was titrated to find the best operating point. The following four buffers were used to immobilize the extracts: phosphate-buffered saline (pH 7.4) with 0.01% Tween 20 or 0.01% Triton X-100 and carbonate/bicarbonate (pH 9.6) with 0.01% Tween 20 or 0.01% Triton X-100. All concentrations were between 20 μ g/ml and 0.5 mg/ml. In many cases, extracts showed good correlation at more than one concentration (typically at a low level and a very high level). In those cases, the lower concentration was preferred due to cost considerations, lower nonspecific binding, and typically better repeatability.

Limit of detection

Two negative serum samples for e1, f1, f3, and f13 were used for determining the LOD. The mean response plus 2SD values were measured as 0.51, 0.52, 0.48, and 0.63 AU, respectively. The cutoff for the assay is 1.0 AU, and this value has been estimated to be less than 1 IU/ml from dilution experiments.

Precision

The precision results for low- and medium-sensitivity serum pools are shown in Table 3. The average CV value for the low/medium serum pool A was 20.5%, and the average CV for the medium/high serum pool B was 20.4%. All CV values were found to be less than 25% with the exception of f4 for serum pool B. Many values were in the 15 to 20% range. This is comparable to reports in the

Table 3

Precision evaluation of the microfluidic cartridge using two serum pools.

Allergen ^a	Serum pool A		Serum pool B	
	Response (AU)	CV ^a (%)	Response (AU)	CV ^b (%)
d1	4.69	17.7	14.37	20.7
d2	4.26	19.8	12.57	22.4
d201	2.12	19.2	2.73	17.4
i6	2.11	20.0	4.09	17.1
e1	2.56	21.6	7.53	21.2
e5	—	—	3.71	16.9
g2	20.69	21.2	—	—
g6	5.35	20.1	19.4	23.8
f1	—	—	4.82	17.0
f2	3.05	23.3	12.54	24.5
f3	—	—	2.68	16.5
f4	3.32	23.8	12.56	26.3
f13	—	—	5.45	17.1
f14	—	—	5.42	22.0
f20	5.17	22.4	11.4	23.0
f23	—	—	2.91	15.6
w1	5.63	22.2	22.44	24.3
m3	4.06	14.2	—	—
Average CV		20.5		20.4

^a Allergens designated by CCLS codes (see text).^b CV defined as SD/mean expressed as a percentage.

literature for specific IgE tests [29] but might not be quite as good as more recent quantitative assays. CV values for raw intensity were on average 4 to 5% lower than the converted AU values.

Allergen screening

Allergen screening was performed using 100 adult serum samples. Because many of the allergens in the general adult population, with the exception of house dust mites, showed little positive response on either the microfluidic cartridge or the reference method, a set of 15 highly allergenic plasma samples was also screened. Table 4 shows the results of the serum screening in two groups: house dust mites and all allergens. For the mites d1, d2, and d201, the total data points were 300 and approximately

Table 4

Comparison of microfluidic cartridge with ImmunoCAP.

	Mite results (d1, d2, d201)	All results ^a
N	300	978
Sensitivity ^b (%)	89	81
Specificity ^c (%)	80	80
Total agreement ^d (%)	84	80
One-class agreement ^e (%)	85	89

^a At least 24 data points per allergen with the exception of almond (f20) and Timothy grass (g6), which were not available for ImmunoCAP comparison.^b Sensitivity defined as percentage of true positives among all reference method positives.^c Specificity defined as percentage of true negatives among all reference method negatives.^d Total agreement defined as percentage of true positives and true negatives among all data points.^e One-class agreement defined as percentage of results within one class score of the reference method.

one-half of the samples showed a positive response to house dust mites. Sensitivity compared with ImmunoCAP was 89%, and specificity was 80%. One-class agreement was 85%. The overall results for 18 allergens (f20 and g6 were not available for comparison) showed a sensitivity of 81% and a specificity of 80% compared with ImmunoCAP. One-class agreement was 89%.

Table 5 shows the screening results for 15 samples of highly allergenic plasma for the microfluidic cartridge, ImmunoCAP, and CLA. Overall, the microfluidic cartridge showed a 74% agreement with CLA ($N = 270$), a 70% agreement with ImmunoCAP ($N = 300$), and an 85% agreement with either CLA or ImmunoCAP ($N = 270$). In comparison, CLA and ImmunoCAP showed a 78% agreement between each other ($N = 270$). It should be noted that the CLA combination panel does not contain *B. tropicalis* (d201) or almond (f20) and that the allergens German cockroach (i6), Timothy grass (g6), and common ragweed (w1) are present only as part of allergen mixes.

Discussion

The differences of agreement between specific allergen targets among the three assays presented are likely attributed to differ-

Table 5

Class score comparison for microfluidic cartridge, ImmunoCAP, and CLA for 15 highly allergenic samples.

Allergen ^a	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
d1	0, 0, 0	6, 6, 4	3, 2, 0	6, 6, 4	4, 2, 0	4, 4, 4	5, 4, 4	0, 2, 0/1	0, 0, 1	0, 0, 0	6, 5, 4	6, 6, 4	6, 6, 4	6, 5, 4	3, 2, 1
d2	0, 0, 0	6, 6, 4	3, 2, 0	5, 5, 4	0, 1, 0	3, 4, 4	4, 4, 4	2, 2, 0	1, 0, 0/1	2, 1, 0/1	6, 5, 4	6, 6, 4	6, 6, 4	6, 6, 4	3, 2, 3
d201 ^b	2, 0	6, 5	0, 0	2, 4	0, 0	3, 1	0, 3	0, 2	0, 0	0, 0	3, 4	4, 4	3, 5	3, 4	0, 0
i6 ^c	0, 2, 0	5, 4, 4	0, 2, 0	0, 3, 4	0, 0, 0	1, 0, 0	0, 3, 1	1, 3, 0	0, 2, 1	0, 2, 0/1	0, 2, 4	5, 1, 2	4, 4, 4	0, 4, 4	0, 2, 0
e1	0, 0, 0	5, 4, 4	3, 3, 4	6, 5, 4	0, 1, 1	0, 2, 4	0, 1, 0	0, 0, 0	3, 3, 4	3, 3, 4	6, 5, 4	6, 5, 4	3, 4, 4	6, 5, 4	3, 3, 4
e5	0, 2, 0	6, 6, 4	2, 4, 0	6, 6, 4	0, 0, 0	1, 2, 1	0, 2, 0	0, 2, 0	0, 2, 2	2, 2, 2	6, 6, 4	5, 5, 4	2, 3, 2	6, 6, 4	1, 4, 3
f1	0, 0, 0	3, 3, 4	6, 5, 4	2, 2, 4	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 1	2, 0, 1	2, 2, 4	0, 0, 0	0, 2, 4	2, 3, 4	6, 5, 4
f2	0, 0, 0	1, 1, 4	6, 6, 4	0, 2, 2	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0/1	0, 0, 0/1	0, 0, 0	0, 2, 4	1, 2, 0	0, 1, 4	0, 2, 3	6, 6, 4
f3	0, 0, 0	6, 5, 4	1, 0, 4	5, 3, 4	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0/1	0, 0, 0/1	0, 0, 0/1	5, 3, 4	0, 0, 0	0, 2, 4	0, 2, 4	0, 0, 4
f4	1, 2, 1	3, 3, 4	6, 4, 4	3, 3, 3	0, 1, 0	0, 1, 0	1, 2, 1	3, 3, 1	0, 2, 3	2, 2, 2	3, 3, 3	0, 0, 0	6, 4, 4	0, 3, 4	6, 4, 4
f13	0, 2, 0/1	6, 6, 4	0, 2, 4	6, 5, 4	0, 0, 0	1, 2, 0	0, 2, 0	2, 3, 2	0, 3, 3	1, 3, 3	6, 5, 4	1, 1, 0	4, 4, 4	6, 5, 4	0, 2, 4
f14	0, 2, 1	6, 4, 4	0, 2, 4	3, 3, 4	0, 0, 0	0, 0, 1	0, 2, 2	2, 3, 2	1, 2, 3	3, 2, 3	3, 3, 4	2, 1, 2	5, 4, 4	2, 2, 4	0, 2, 4
f20 ^b	0, 2	5	0	1, 0	0	0	0, 2	3, 3	0	0	1	0	6	4	0
f23	1, 1, 0	2, 4, 4	4, 3, 4	5	2	1, 0, 0	0, 2, 0	2, 2, 0	0, 2, 0	0, 2, 0	5, 3, 4	6, 0, 0	2, 4, 4	6, 4, 4	4, 3, 4
f24	0, 1, 0	4, 4, 4	4, 2, 4	5, 3, 4	0, 0, 0	0, 0, 0	0, 2, 0	0, 2, 0	0, 1, 0	0, 1, 0	5, 3, 4	2, 1, 0	1, 4, 4	6, 4, 4	4, 2, 4
g2	3, 3, 4	4, 4, 4	0, 2, 0	3, 3, 4	0, 2, 0/1	0	3, 2, 2	4, 3, 3	3, 4, 4	3, 4, 4	4, 3, 4	0, 0, 0	6, 5, 4	3, 3, 4	0, 2, 0
g6 ^c	5, 4, 4	6	0	5, 5, 4	2, 3, 4	4	3, 3, 4	4, 3, 3	6, 5, 4	6	5	0, 2, 3	6	3	0
m3	2, 0, 0	0, 3, 1	0, 0, 0	0, 3, 0/1	0, 1, 0	0, 0, 0	1, 0, 0	0, 1, 0	0, 0, 0	0, 0, 0	0, 3, 0	0, 1, 1	1, 2, 3	1, 3, 3	0, 0, 0
m5	4, 0, 0	2	1, 3, 0	1, 3, 3	0, 1, 0	1, 0, 0	0, 0, 0	0, 2, 0	0, 1, 0	1, 1, 0	1, 3, 1	3, 3, 2	0, 2, 3	3, 3, 3	0, 3, 3
w1 ^c	2, 3, 4	5, 4, 4	0, 1, 0	0, 2, 1	0, 0, 0	0	2, 2, 2	3, 3, 3	1, 4, 4	2, 4, 4	0, 2, 0	0, 0, 0	6, 4, 4	0, 2, 1	0, 1, 0

Note. Microfluidic cartridge and ImmunoCAP class scores are 0, 1, 2, 3, 4, 5, and 6. CLA class scores are 0, 0/1, 1, 2, 3, and 4. Class scores shown are for microfluidic cartridge, ImmunoCAP, and CLA, respectively, separated by commas.

^a Allergen designations by CCLS code (see text).^b CLA assay does not contain d201 or f20.^c These allergens are present in the CLA assay only in the form of mixes with other related allergens.

ences in the extract source materials used by the different assays. It is well known that the composition of allergenic proteins among allergen extracts can vary significantly [30]. Likewise, specific IgE results from different assay systems have been shown to vary significantly, especially for food-specific IgE levels [12].

Because allergen extracts contain a very complex mixture of both allergenic and nonallergenic proteins, allergen-specific IgE assays typically rely on high surface areas for immobilizing such extracts. This is a major challenge for miniaturized assays. The microfluidic cartridge did, however, show sensitivities similar to those of other microarrays employing allergen extracts [15–18]. Improvements can be made by employing a higher capacity nitrocellulose surface, but there are limitations to this approach. The preferred method is to take better advantage of the capabilities of the microfluidics and perform reactions under dynamic conditions (active mixing or continuous flow) rather than static (stop flow) [20]. It is estimated that the current device uses an effective reaction volume of approximately 1 μ l per spot under static conditions. Under dynamic reaction conditions, effective reaction volumes could be increased several-fold and reaction rates may also increase during the process. The main disadvantage of continuous flow, however, is that more reagents are typically required. Another approach is the use of a circulatory design where reagents are continuously circulated through the reaction zone. This method would maintain low reagent volumes while increasing reaction rates [31].

Precision values obtained with the microfluidic assay are generally higher than current quantitative allergen assays on the market [29]. For assays such as these employing allergen extracts, CV values tend to be higher than those for assays using purified or recombinant proteins. Outside of the extracts, the other major factors influencing the CV values are the calibration method and the cartridge-to-cartridge wash efficiencies. The calibration method used in the microfluidic assay added more than expected variation (4–5%). It is reasonable to expect that improvements to the calibration method could improve CV values by at least 2%. Improving cartridge-to-cartridge variation would involve improving the pump and valve structures to perform more consistently within current injection-molding tolerances.

Cross-reactivity among allergen extracts has been an area of much research. It is a concern not only for both multiplexed and nonmultiplexed allergen assays but also for practitioners due to cross-allergenicity [32]. It is known that many extracts, especially within similar groups, can share common allergenic proteins and that IgE antibodies can exhibit cross-reactivities [33–35]. The inhibition assays performed during this study did show some unexpected cross-reactivity between the commercial assay and the extracts as follows: both common ragweed and Bermuda grass from the commercial assay with almond and mite *D. farinae* extracts and soybean from the commercial assay with Timothy grass extract. Further study is required to determine the extent and nature of these cross-reactivities and whether cross-reactivities exist between the extracts themselves or just with the commercial assay. It is important, however, to be aware of such cross-reactivities when interpreting results [32].

As an analytical technique, the use of class scores may be considered as outdated given that many newer generation allergen tests (e.g., ImmunoCAP, IMMULITE, HY-TEC) are quantitative specific IgE assays using heterogeneous calibration curves based on nonspecific IgE response [8]. Such calibration techniques are straightforward for single test analysis equipment where many tests are included within a single calibration run. They are, however, not as easily adapted to multiplexed assays. Still, it does remain feasible to calibrate the internal IgE curve on the microfluidic cartridge to a quantitative IgE response and employ the neces-

sary quality control methods to ensure accuracy with traceable IgE standards.

Regarding sample amounts, 90 μ l is considered a large amount of sample for even a traditional microarray experiment, let alone a microfluidic assay. However, at 4.5 μ l per target, it is an improvement over current commercial allergen assays, and it may be the only alternative when considering multiple allergen testing on blood draws taken from very small children or those taken from capillary blood. Amounts could be reduced, however, by lowering the channel heights and eliminating dead volumes as much as possible. Still, one must be careful not to adversely affect sensitivity when reducing reaction amounts.

A 20-target test is arguably on the lower end of market demand for a multiplexed allergen assay. Increasing the number of targets is possible by making better use of the CCD area and/or employing higher density printing.

The current system is suitable for decentralized testing where the test frequencies are approximately 16–20 per day. Some improvements are most likely necessary for true point-of-care operation of such a system. Currently, manual reagent handling and pipetting requires a technician to operate. Storage of reagents on the cartridge itself would be the ideal solution provided that the shelf life of the cartridge could be maintained long enough to be commercially feasible.

For large-volume test sites, more complex instrumentation, such as analyzing cartridges in a carousel format or simply in parallel, could provide higher throughput analysis. Such a system employing 10 cartridges could reasonably complete a run in 75 min. With six runs per day, this would be a three- to fourfold improvement over the current instrument without any change to the cartridge or method.

This study has demonstrated the performance of the microfluidic cartridge technology for the screening of allergen-specific IgE sensitivities in human serum. The microfluidic assay presented here provides an inexpensive multiplexed assay format that dramatically reduces the complexity of the analysis instrumentation while still providing rapid automated analysis for low-volume specific allergen testing. The platform is capable of providing a solution for multiple allergen testing on small blood draws in children and infants. However, improvements in cartridge design, such as employing dynamic reactions, using heterogeneous calibration curves, and increasing the number of allergen targets, would make the assay more comparable to current generation allergen-specific IgE tests. Results showed favorable comparisons with two commercial specific IgE assays.

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