Clinical Chemistry 60:9
000–000 (2014)

Point-of-Care Vertical Flow Allergen Microarray Assay: Proof of Concept

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BACKGROUND: Sophisticated equipment, lengthy protocols, and skilled operators are required to perform protein microarray-based affinity assays. Consequently, novel tools are needed to bring biomarkers and biomarker panels into clinical use in different settings. Here, we describe a novel paper-based vertical flow microarray (VFM) system with a multiplexing capacity of at least 1480 microspot binding sites, colorimetric readout, high sensitivity, and assay time of <10 min before imaging and data analysis.

METHOD: Affinity binders were deposited on nitrocellulose membranes by conventional microarray printing. Buffers and reagents were applied vertically by use of a flow controlled syringe pump. As a clinical model system, we analyzed 31 precharacterized human serum samples using the array system with 10 allergen components to detect specific IgE reactivities. We detected bound analytes using gold nanoparticle conjugates with assay time of ≤10 min. Microarray images were captured by a consumer-grade flatbed scanner.

RESULTS: A sensitivity of 1 ng/mL was demonstrated with the VFM assay with colorimetric readout. The reproducibility (CV) of the system was <14%. The observed concordance with a clinical assay, ImmunoCAP, was $R^2 = 0.89$ (n = 31).

CONCLUSIONS: In this proof-of-concept study, we demonstrated that the VFM assay, which combines features from protein microarrays and paper-based colorimetric systems, could offer an interesting alternative for future highly multiplexed affinity point-of-care testing.

Immunoassay-based affinity proteomic analysis of clinical samples may complement conventional mass spectrometry–based proteomic methods in improving diagnostic accuracy and clinical management in, for instance, autoimmunity *(1)*, oncology *(2)*, and clinical microbiology *(3)*. Recent efforts to create libraries of high-quality peptide affinity binders with the human proteome as a template have allowed researchers to discover novel protein biomarkers that can help distinguish between certain disease conditions with high accuracy *(4)*. Antigen/allergen microarrays, peptide-fragment microarrays, and antibody arrays have been used to evaluate variations in the plasma proteome *(5)* and determine detailed antibody signatures arising from infections *(6)* or autoimmune/allergic disorders *(7)*. However, although multiplexed affinity proteomic tools such as glass slide microarrays and suspension bead arrays can provide excellent sensitivity and reliability, their operation typically requires sophisticated laboratory equipment and settings, skilled operators *(8, 9)*, and long assay times. In clinical and near-patient settings such requirements cannot be met *(10)*. As a consequence, research in paper-based assays has expanded substantially, resulting in improved reagent delivery, dilution, and sample handling *(11)*. The potential benefits of lateral flow paper-based assays include low cost, robust operation in different environments, flexibility, and ease of use *(12)*. However, paper-based assays have typically had low sensitivity and high variability as well as a lack of multiplexing ability. Furthermore, in multiplexed lateral flow assays such as lateral flow microarrays, microspots often need to be positioned with care so that upstream spots do not alter the sample in a way that affects the detection on a downstream spot *(13)*. To address these concerns, researchers have recently developed flow-through assays in which reagents are applied vertically *(14–16)*. Further, Xu and Bao have reported a multistacked filtration-based protein microarray assay that uses nitrocellulose membranes to detect a variety of fluorescently labeled analytes *(17)*, and Dhar et al. have investigated an alternating cross-flow strategy for small-molecule covalent conjugation to biomolecules *(18)*.

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Received February 10, 2014; accepted June 9, 2014.
Previously published online at DOI: 10.1373/clinchem.2014.223230

Allergic diseases and atopic disorders such as asthma are on the rise *(19)*, and testing of multiple specific IgE reactivities can improve the diagnostic accuracy and management of patients with these conditions *(20, 21)*. The clinical methods for specific IgE reactivity include single-plexed affinity chromatographic ImmunoCAP system and multiplexed array-based ImmunoCAP Immuno Solid-phase Allergy Chip (ISAC)[4] (Thermo Fisher Scientific); both require considerable investment in instrumentation and are relatively costly. Efforts have been made to develop more sensitive IgE detection methods such as silver nanoparticle–based metal-enhanced fluorescence analysis *(22)*. Further, a superparamagnetic nanoparticle–based lateral flow immunoassay for rapid detection of IgE sensitivity toward shellfish major allergen tropomyosin has been reported *(23)*. Teste et al. have reported that microchip integrated core-shell magnetic nanoparticles could be used to detect specific serum IgE at a concentration of 1 ng/mL with a small sample volume. An allergen functionalized magnetic nanoparticle–based diagnostic assay was developed to detect peanut IgE reactivity in serum samples with sensitivity close to that of the ImmunoCAP assay *(24)*. Recently, our group reported a dual-labeled gold nanoparticle–based lateral flow microarray immunoassay for sensitive detection of 15 specific IgE reactivities with assay performance comparable to that of the ImmunoCAP assay *(25)*.

Here we present a novel assay framework inspired by flow-through assays, planar microarrays, and nanoparticle-based colorimetric detection. The vertical flow microarray (VFM) presented here uses a carefully chosen nitrocellulose membrane patterned with a high-density microarray and uses cross-flow for delivery of sample, buffers, and detection reagents.

Methods

PAPER-BASED VFM ASSAY

We developed the paper-based VFM assay using dense meshed nitrocellulose membranes as microarray substrates. We investigated nitrocellulose membranes with 3 different pore sizes, Whatman Protran BA 79 (0.1 μm), BA 83 (0.2 μm), and BA 85 (0.45 μm). Microarrays were printed onto the membrane by use of a Nanoplotter 2.0 (Gesim) robotic printer, depositing approximately 300 pL $\times$ 10 droplets per spot, creating 120-μm-diameter spots. The membrane was subsequently assembled on a 13-mm-diameter stainless steel syringe filter device (Swinny, Millipore). A sandwich-based immunoassay was performed with all reagents and buffers transported vertically through the membrane by use of a flow-controlled syringe pump (PHD ultrasyringe pump, Harvard Apparatus), as well as by manual pressure (Fig. 1A). For the sensitivity and flow characterization, initially 1 mL assay buffer (see Supplemental Data, which accompanies the online version of this article at http://www.clinchem.org/content/vol60/issue9) was flowed through on the human IgG (hIgG)-spotted membrane (1 g/L hIgG with 100 replicate spots) to block the membrane. Subsequently, 1 mL biotinylated antihuman IgG in a dilution series was applied, and finally the membrane was developed colorimetrically with Neutravidin-conjugated gold nanoparticles and sequential washing procedures (see online Supplemental Data). The assay procedure could be completed within 10 min. Finally, the device was dismantled, and the membrane was imaged within 5 min after the final assay step (no active drying) by use of a consumer-grade tabletop scanner (CanoScan 9000F mark II) with VueScan 9 (Hamrick Software), with 16-bit grayscale settings and 2400-dpi resolution exported into an uncompressed TIFF file format. The resulting image was inverted and imported into GenePix 5 (Axon Laboratories), where spots were aligned to a microarray grid for data extraction. Data processing and statistical analysis were performed by use of the R environment for statistical computing (version 3.01).

DEVELOPMENT OF A MULTIPLEXED VFM ALLERGY ASSAY

We developed the VFM allergy assay using different allergens spotted on 0.1-μm pore size nitrocellulose membrane followed by a sandwich-based immunoassay with in-house–prepared antihuman-IgE–conjugated gold nanoparticles (see online Supplemental Fig. 1). As shown in Fig. 1B, the octagonal array layout was divided into 4 segments by layout spots (to guide automatic array alignment in postprocessing), 10 different allergens including a series of 3 different concentrations for each allergen (1 g/L, 300 μg/mL, and 100 μg/mL were spotted on the membrane with 4 replicate spots each, spread symmetrically in 2 mirror planes horizontally and vertically to the array center). As a positive control, human IgE was spotted on each corner of the segments, which also allowed an estimation of the flow profile through the membrane (see online Supplemental Fig. 2). All steps of the VFM assay were performed by serially pumping samples and reagents through the reaction cartridge. First, assay buffer was introduced to reduce nonspecific binding, followed by application of 1 mL serum sample at a predefined dilution rate. Second, the membrane was exposed to anti-hIgE–conjugated gold nanoparticles followed by successive washing procedures, and then the membrane was removed from the device for imaging and data processing.

[4] Nonstandard abbreviations: ISAC, Immuno Solid-phase Allergy Chip; VFM, vertical flow microarray; hIgG, human IgG.

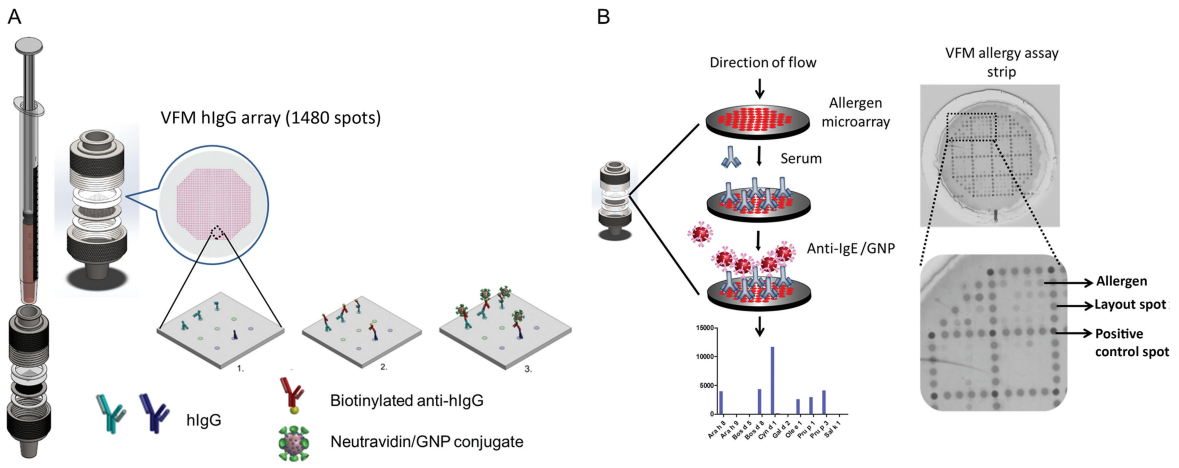

Fig. 1. VFM and paper-based VFM allergy assay.

(A), Schematic illustration of VFM device and assay. The cartridge harboring the metal mesh and paper microarray was connected to a syringe with flow actuation accomplished by means of an electromechanical pump or by manual pressure. An array density of at least 1480 microspots could readily be achieved, here patterned with 100 μg/mL hIgG and developed colorimetrically with biotinylated anti-IgG followed by Neutravidin-conjugated gold nanoparticles. (B), Schematic representation of a paper-based VFM allergy assay. IgE antibodies from applied clinical sera were bound specifically to component allergens in the array microspots (420 microspots per array). Gold nanoparticles conjugated to anti-IgE antibodies were subsequently applied to colorimetrically reveal the bound IgE-antibodies. After the scanning of the microarray, the color intensity of microspots was quantified. GNP, gold nanoparticles.

DEVELOPING A VFM FOR SPECIFIC IgE DETECTION

We used the VFM immunoassay to assess multiplexed IgE sensitization profiles in human serum samples. In the present study, we used a panel of 10 allergens and 31 clinical samples provided by Thermo Fisher Scientific. We present results from VFM assay performance optimization for specific IgE detection by various assay parameters including concentration of printed allergens, optimum serum dilution, and sample recirculation. The assay reproducibility was also estimated. Finally, we used the VFM assay in the analysis of the full cohort of clinical samples with a variety of specific IgE sensitizations and compared the results to ImmunoCAP data.

Results and Discussion

DESIGN AND OPTIMIZATION OF THE VFM ASSAY

The VFM concept overcomes a limitation of multiplexed lateral flow assays in which downstream binding surfaces may be affected by upstream ones *(13)*; all spots are separate and independent of each other. The VFM assay format consists of a syringe filter commonly used in other applications to filter large volumes of liquids on laboratory scale. In this system, the ability for high fluid flow allows the user to experimentally match the maximum binding rate of the microspot to the convective flux determined by the flow velocity and expected analyte concentration, effectively reducing the time needed to reach equilibrium. Therefore, it is possible to achieve the shortest assay time possible given the limitations of the included affinity interactions. Furthermore, the high flow rate allows a large range of sample volumes to be processed, spanning at least the range 10 μL to 10 mL at assay times of $<$10 min, not including imaging and data analysis. Binder molecules such as purified allergen components are spotted by means of a microarray printer onto the porous membrane so that all reagents included in the assay are applied vertically by use of a flow-controlled syringe pump (Fig. 1A) or by manual pressure (the effect of different flow-control options is presented in online Supplemental Table 2).

After the assembly of the device, a sandwich-based immunoassay was performed so that analytes were bound on capture molecules spotted in the nitrocellulose membrane. Finally, the microarray spots were colorimetrically developed by vertical flow with affinity-labeled gold nanoparticles.

SENSITIVITY OF THE VFM ASSAY IN ANTI-IgG DETECTION

The VFM assay sensitivity was investigated by examining parameters including membrane pore size and fluid flow speed (see online Supplemental Table 2). Various concentrations of biotinylated anti-hIgG from 10 pg/mL to 100 μg/mL were analyzed by use of 1 g/L hIgG spotted in the membrane. In this sandwich

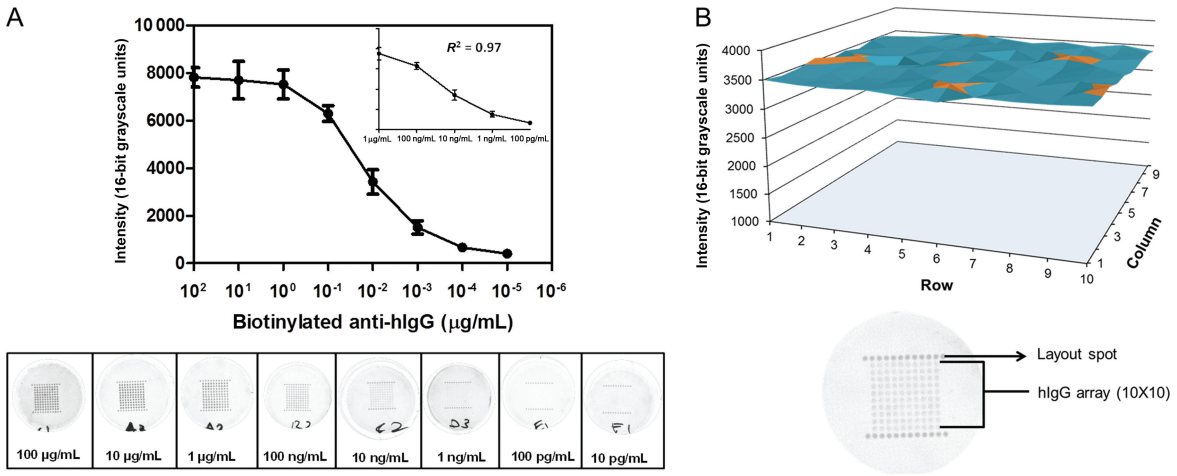

Fig. 2. Efficiency of paper-based analysis and flow profile analysis.

(A), Efficiency of paper-based vertical flow analysis for the detection of human IgG (1 g/L hIgG was spotted on the membrane). The presented values are means of triplicates (n = 3). The mean spot intensity with subtracted local background was acquired from the 16-bit scanned grayscale image. (B), Flow profile analysis of VFM assay and its corresponding image. In the immunoassay, 10 ng/mL biotinylated anti-IgG was analyzed on 1 g/L hIgG spotted membrane. The mean spot intensity with subtracted local background was acquired from the 16-bit scanned grayscale image.

immunoassay, 25-nm-sized Neutravidin-conjugated gold nanoparticles were used to visualize the spots. The signal intensities for various concentrations of anti-hIgG are shown in Fig. 2A. It was found that the color intensity of the VFM assay was linearly proportional to the anti-hIgG concentration over the range 100 pg/mL to 1 μg/mL (r^2 = 0.97) with a detection limit of approximately 1 ng/mL. The limit of detection was defined as the mean background signal plus 3 times the SD. Furthermore, the surface plot of the spot intensities of the VFM

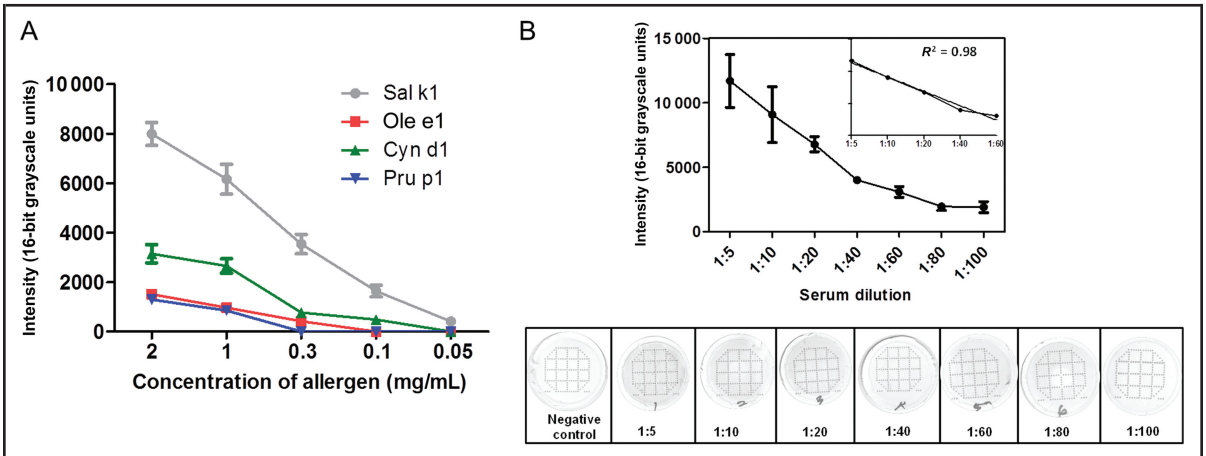

Fig. 3. Optimization of allergen concentration and efficiency of VFM allergy assay.

(A), Optimization of allergen concentration spotted on the membrane for detection of IgE sensitivities on serum ID 47020. The presented values are a mean of triplicates (n = 3). The mean spot intensity with subtracted local background was acquired from the 16-bit scanned grayscale image. (B), Efficiency of VFM allergy assay in detecting IgE sensitivities toward allergen Cyn d1 over a range of dilutions of serum ID 45359. The presented values are a mean of triplicates (n = 3). The mean spot intensity with subtracted local background was acquired from the 16-bit scanned grayscale image.

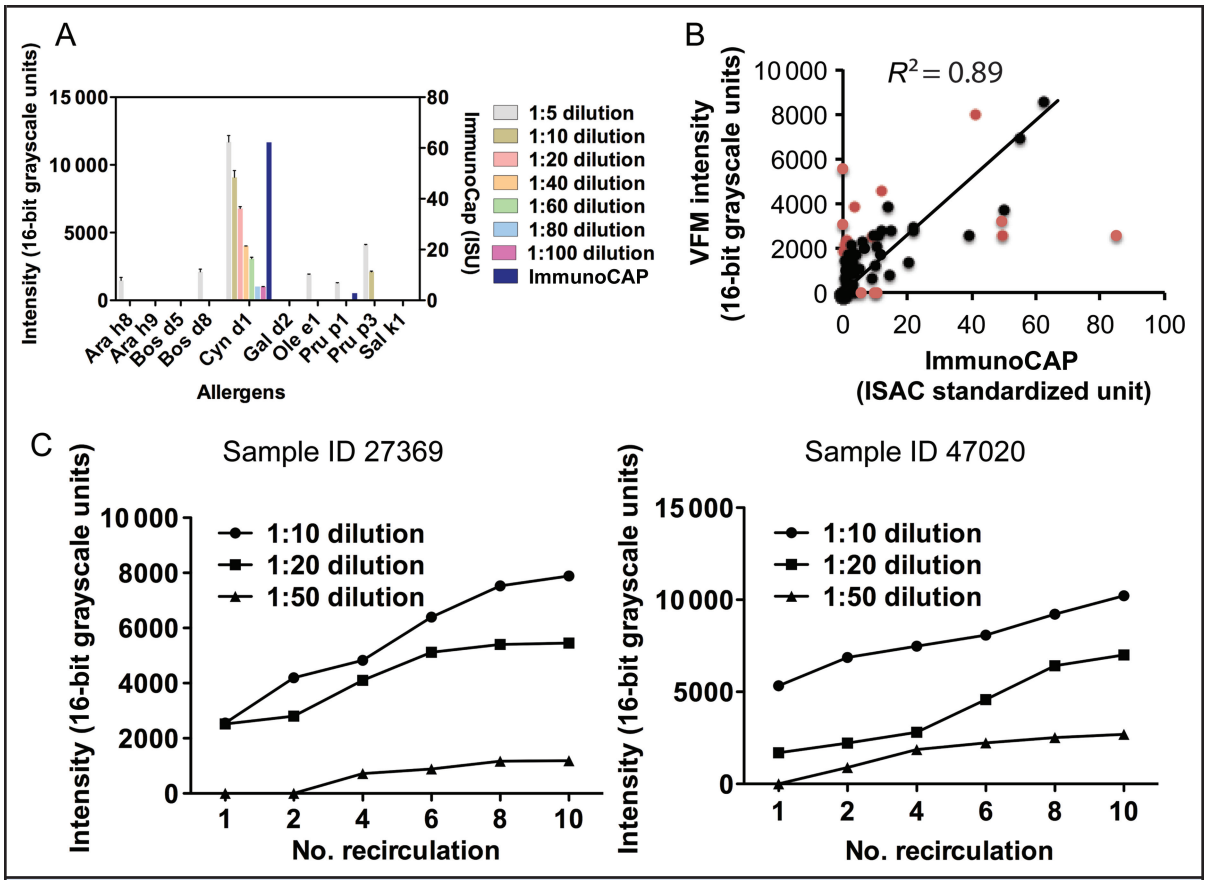

Fig. 4. Analysis of VFM allergy assay, comparison between VFM and ImmunoCAP, and effect of recircluation of serum sample.

(A), Analysis of the performance of the VFM allergy assay by use of various serum dilutions compared with the ImmunoCAP assay. Serum ID 45359 and the corresponding allergen Cyn d1 were used for the presented graph, and the values are means of triplicates. The mean spot intensity with subtracted local background was acquired from the 16-bit scanned grayscale image. ISU, ISAC standardized unit. (B), Comparison between VFM and ImmunoCAP for a cohort of 31 clinical samples and 10 component allergens revealed a good overall concordance in linear regression analysis (R^2 = 0.89). Two allergens, Ara h8 and Pru p1, demonstrated low individual coefficients of determination (0.41 and 0.32, respectively), and their associated data points are colored red. The mean spot intensity with subtracted local background was acquired from the 16-bit scanned grayscale image. (C), Effect of recirculation of the serum sample for the detection of specific IgE by use of the VFM allergy assay. Serum ID 27369 and its corresponding allergen Pru p1 and serum ID 47020 and its corresponding allergen Sal k1 were used for the assay. The mean spot intensity with subtracted local background was acquired from the 16-bit scanned grayscale image.

assay indicates that the flow profile of the reagents applied on the membrane was largely uniform, which can be seen in the low mean CV (9%) (Fig. 2B).

EFFECT OF BINDER CONCENTRATION ON ASSAY SENSITIVITY

Various concentrations (2 g/L, 1 g/L, 300 μg/mL, 100 μg/mL, and 50 μg/mL) of 4 different allergens were spotted, followed by analysis with a clinical serum sample (ID 47020). The immunoassay was performed by use of anti-IgE–conjugated gold nanoparticles. As expected, the signal intensity of the spot increased in a largely linear fashion with the concentration of the allergens spotted on the membrane. As shown in Fig. 3A, it was found that spots printed with 2 and 1 g/L of allergens could successfully detect even the lowest IgE concentrations present in the sample (5.64 ISAC standardized unit), whereas with spots printed at 300, 100, and 50 μg/mL, the sensitivity deteriorated.

EFFECT OF SAMPLE DILUTION FACTOR

To evaluate the sensitivity of the developed assay in a complementary way and investigate the effect of sam-

ple dilution on assay performance, a limit-of-dilution strategy was tested. A series of serum dilutions (1:10, 1:20, 1:30, 1:40, 1:60, 1:80, and 1:100 [1:10 indicates 1 part plus 9 parts; other dilutions follow the same pattern]) was created and analyzed. The VFM spot intensities for the allergen Cyn d1 for different serum dilutions (sample ID: 27369) are shown in Fig. 3B. It was found that the signal intensity of the spot decreased as expected with increased serum dilution, displaying a linear response from 1:5 to 1:60 ($r^2 = 0.98$). In addition, the specificity of the VFM allergy assay was validated by use of a negative control serum sample (provided by Thermo Fisher Scientific) that was found to present insignificant signals on the membrane. Further, the performance of the VFM allergy assay for the serum sample ID 27369 was compared with the ImmunoCAP assay results, and it was found that the sensitivity of the 2 assays was similar (Fig. 4A). When comparing the multiplexed IgE-sensitivity response between the ImmunoCAP and VFM results for sample ID 27369, it was found that at a dilution of 1:5, in addition to those reactivities in Cyn d1 and Pru p1 found by ImmunoCAP, signals were seen for allergens Ara h8, Bos d8, Ole e1, and Pru p3. When the dilution factor was increased to 1:10, 1:20, and 1:40, these additional signals gradually disappeared and the expected signal decreased in intensity. The maximum concordance with ImmunoCAP data was found at a 1:10 dilution factor. Given the low tendency for nonspecific signals experienced in the optimization, we suggest that although these additional signals could result from a nonspecific binding of serum proteins, it is also appears possible that the rapid procedure of the presented assay does not allow much time for dissociation of affinity binder complexes. This could result in the detection of a range of low- to medium-affinity IgE species that cannot presently be detected in conventional endpoint assays with longer durations. Both the existence and clinical significance of IgE species with different affinities have been discussed *(26, 27)*, and we suggest that in future studies, it could be valuable to investigate and validate these findings with, for instance, affinity kinetic biosensor experiments (via BIAcore or other platforms). If the findings are validated, the ability to tune the affinity range by varying the dilution factor, flow speed, and printed allergen concentration could allow the VFM to offer an important novel avenue of clinical investigation.

Table 1 Reproducibility of the VFM allergy assay.

Sample ID and allergen	Interassay same-day CV, %	Interassay different-day CV, %
31464		
Ara h8	3.6	5.1
Cyn d1	1.3	2.8
Gal d 2	3.3	1.7
Pru p1	12.0	8.3
47177		
Ara h9	10.3	13.7
Bos d5	7.0	11.8
Bos d8	1.7	7.8
Gal d2	11.2	9.1
57028		
Pru p3	7.3	4.1
Sal k1	13.1	11.9
63000		
Ara h9	14.0	8.8
Bos d8	4.2	8.3
Gal d2	6.3	12.1
Pru p3	9.3	7.4

RECIRCULATION OPTION FOR IMPROVED ASSAY SENSITIVITY

A benefit of the VFM assay format is an ability to improve the sensitivity of the assay by recirculating a serum sample that is limited in volume in the same immunoassay. In the recirculation assay, after the serum has been introduced to the device, it can be collected at the outlet and reapplied into the device multiple times. Two different serum samples (ID 27369 and 47020) at different dilutions (1:10, 1:20, and 1:50) were recirculated up to 10 times. The performance of the recirculation assay for the allergen Pru p1 and Sal k1 is shown in Fig. 4C. As expected, the spot intensity at first increased with a linear tendency before reaching a plateau after 6–10 rounds. In addition, a similar observation was made in the performance of the recirculation assay over a range of concentrations of spotted allergens (see online Supplemental Figs. 4 and 5). However, on recirculating the sample >3 times, signals increased on the membrane surface in the microspot local background. In contrast, the VFM assay specificity did not appear to be affected by recirculation, as seen from negative control spots being unaffected. The sample recirculation option is unavailable in most low- and high-multiplexed methods such as lateral flow tests or planar microarray systems. We suggest that the VFM with enabled recirculation protocol could offer an interesting option for high-sensitivity assays, particularly when the available sample volume is small such as in pediatric/neonatal diagnostics and biobank studies.

Table 2 Correlation between ImmunoCap and VFM allergy assay for individual allergens in the analysis of 31 clinical samples.

	Ara h8	Ara h9	Bos d5	Bos d8	Cyn d1	Gal d2	Ole e1	Pru p1	Pru p3	Sal k1
R^2	0.41	0.86	0.98	0.76	0.95	0.76	0.84	0.32	0.74	0.95

REPRODUCIBILITY OF THE VFM ASSAY

To determine the reproducibility and precision of the allergen VFM method, triplicate assays were run on the same day and on 3 different days with 2 different serum samples. As shown in Table 1, the interassay CV achieved from performing replicate assays on the same day and different days varied from 1.3% to 14% and 1.7% to 14%, respectively.

EVALUATION OF A VFM FOR SPECIFIC IgE DETECTION IN A CLINICAL COHORT

We compared VFM results from the analysis of the clinical sample cohort (n = 31) by use of 10 allergen components with ImmunoCAP data provided by Thermo Fisher Scientific. A largely linear relation between the ImmunoCAP values and VFM allergy assay results can be seen in Fig. 4B, and coefficients of determination are presented for each individual allergen in Table 2. The concordance between the ImmunoCAP and VFM results varied as expected for the different allergens, with 8 of the 10 allergens displaying a good correlation with ImmunoCAP (between 0.74 and 0.98). Two of the allergens (Ara h8 and Pru p1, indicated as red dots in the scatter plot in Fig. 4B) presented lower R^2 values (0.40 and 0.32), which may be explained by the differences in assay principles and immobilization strategy between the VFM assay and ImmunoCAP. A fraction of affinity binders used in a high-throughput immunoassay like the VFM will need additional optimization steps to achieve the best performance, such as attachment to nanoparticle scaffolds and adjusting printing buffers, although this was deemed outside the scope of the present work. It is also possible that some affinity binders, because of low kinetic association rates or tendency for nonspecific interactions, may work well only in equilibrium-based assays with long assay durations. Future attempts to incorporate a major share of allergens used in clinical assays into the VFM system will likely reveal such patterns. A considerable difference in performance between allergens has further been reported in other comparative studies, such as in a recently presented microarray assay with fluorescence-based detection *(28)*.

Conclusion

Our study demonstrates a proof of concept for a paper-based VFM affinity assay that can incorporate at least 1480 unique binders with an assay time of <10 min and gold nanoparticle–based colorimetric readout amenable to imaging with a consumer-grade flatbed scanner. We demonstrate that it is possible to achieve an even flow rate over the full array surface and adequately low variability for diagnostic immunoassays. The assay had a sensitivity of <1 ng/mL when detecting purified IgG, a dynamic range of around 3 orders of magnitude, reproducibility (CV) of <14%, and a good concordance (R^2 = 0.89) with the clinical assay, ImmunoCAP, by use of samples from 31 individuals with 10 printed component allergens. The presented assay format may offer an interesting option for future comprehensive affinity proteomic point-of-care tests in a variety of clinical conditions.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: L.I. Segerink, KTH; H. Andersson-Svahn, financial support to the Division of Nanobiotechnology from the Swedish Government Agency for Innovation Systems (VINNOVA) via the ProNova VINN Excellence Centre for Protein Technology.
Expert Testimony: None declared.
Patents: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

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