Papers in Press. Published July 8, 2014 as doi:10.1373/clinchem.2014.223230
The latest version is at http://hwmaint.clinchem.org/cgi/doi/10.1373/clinchem.2014.223230

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

Thiruppathiraja Chinnasamy,[1] Loes I. Segerink,[2] Mats Nystrand,[3] Jesper Gantelius,[1] and Helene Andersson Svahn[1]*

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.

© 2014 American Association for Clinical Chemistry

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)*.

[1] Division of Proteomics and Nanobiotechnology, Science for Life Laboratory, KTH-Royal Institute of Technology, Stockholm, Sweden; [2] BIOS Lab on a Chip group, MESA+ Institute for Nanotechnology, University of Twente, Enschede, The Netherlands; [3] Thermo Fisher Scientific, Uppsala, Sweden.
* Address correspondence to this author at: Division of Proteomics and Nanobiotechnology, Science for Life Laboratory, KTH-Royal Institute of Technology, P-Box 1031, 171 21 Solna, Stockholm, Sweden. Fax +46-8-52481425; e-mail helene.andersson.svahn@scilifelab.se.
Received February 10, 2014; accepted June 9, 2014.
Previously published online at DOI: 10.1373/clinchem.2014.223230

Copyright (C) 2014 by The American Association for Clinical Chemistry

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.

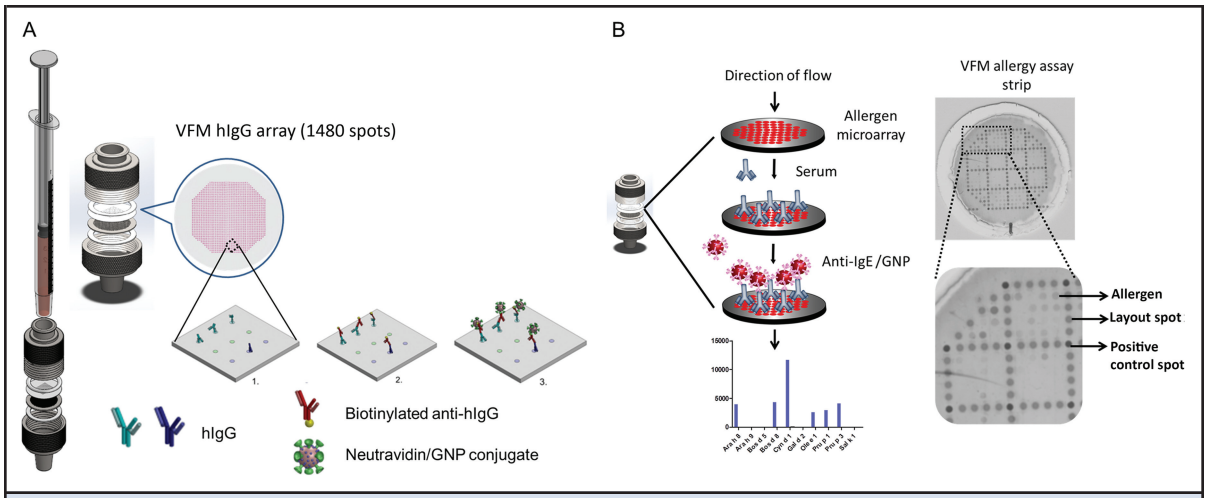


Fig. 1. VFM and paper-based VFM allergy assay.

(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

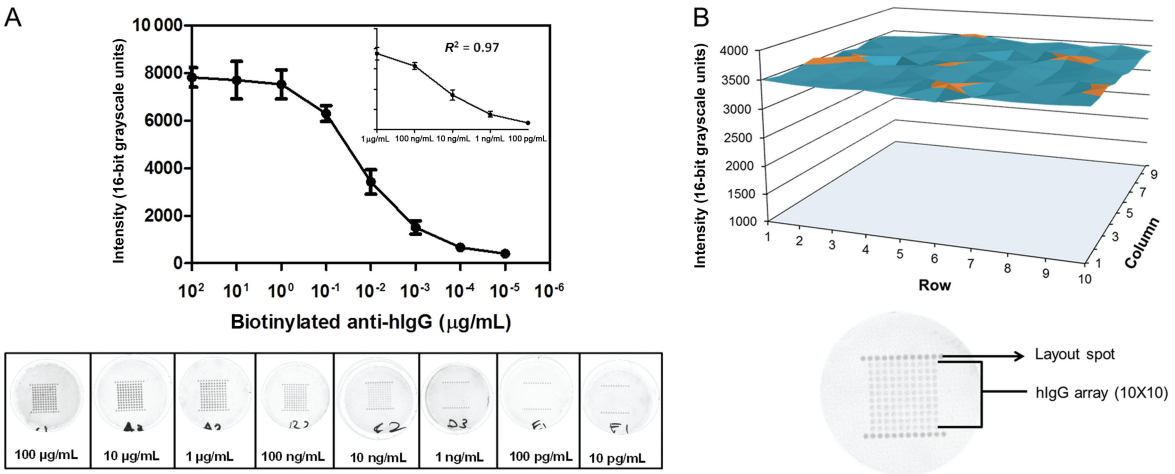


Fig. 2. Efficiency of paper-based analysis and flow profile analysis.

(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

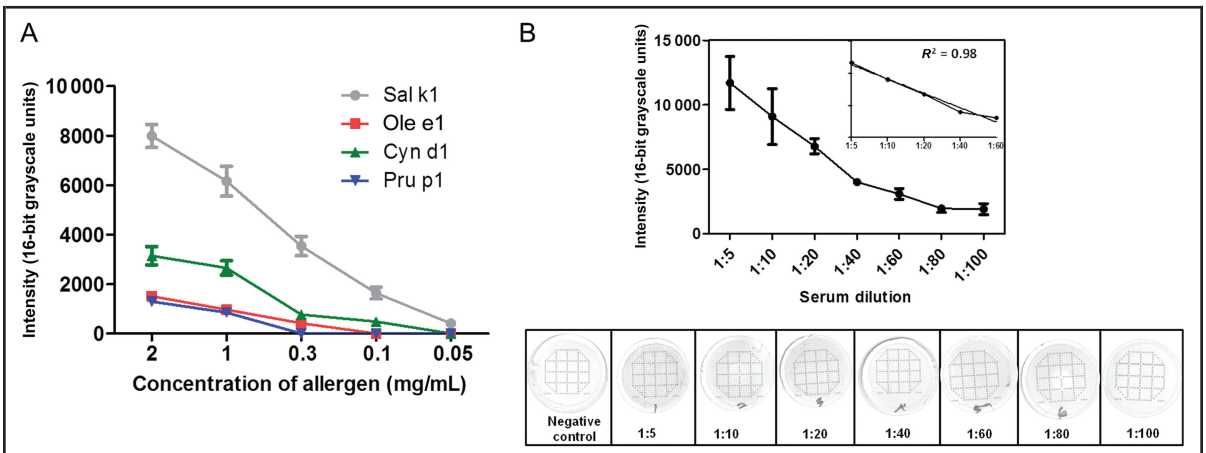


Fig. 3. Optimization of allergen concentration and efficiency of VFM allergy assay.

(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.

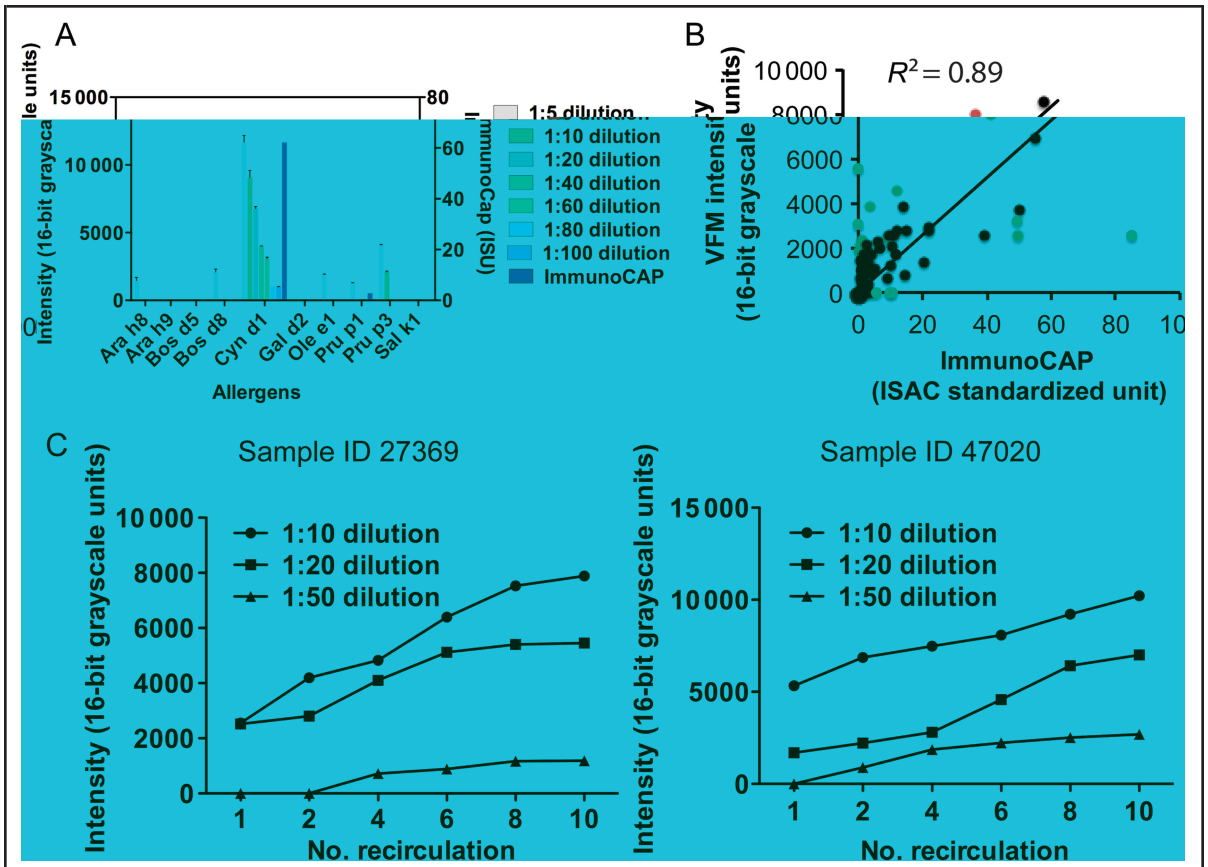


Fig. 4. Analysis of VFM allergy assay, comparison between VFM and ImmunoCAP, and effect of recircluation of serum sample.

(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.

References

1. Maecker HT, Lindstrom TM, Robinson WH, Utz PJ, Hale M, Boyd SD, et al. New tools for classification and monitoring of autoimmune diseases. Nat Rev Rheumatol 2012;8:317–28.
2. Taguchi A, Hanash SM. Unleashing the power of proteomics to develop blood-based cancer markers. Clin Chem 2013;59:119–26.
3. Kunnath-Velayudhan S, Salamon H, Wang H-Y, Davidow AL, Molina DM, Huynh VT, et al. Dynamic antibody responses to the Mycobacterium tuberculosis proteome. Proc Natl Acad Sci U S A 2010;107:14703–8.
4. Li J, Zhang Z, Rosenzweig J, Wang YY, Chan DW. Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. Clin Chem 2002;48:1296–304.
5. MacBeath G. Protein microarrays and proteomics. Nat Genet 2002;32:526–32.

6. Mezzasoma L, Bacarese-Hamilton T, Di Cristina M, Rossi R, Bistoni F, Crisanti A. Antigen microarrays for serodiagnosis of infectious diseases. Clin Chem 2002;48:121–30.
7. Gelfand EW. Antibody-directed therapy: Past, present, and future. J Allergy Clin Immunol 2001; 108:S111–S6.
8. Pomponi D, Bernardi ML, Liso M, Palazzo P, Tuppo L, Rafaiani C, et al. Allergen micro-bead array for IgE detection: a feasibility study using allergenic molecules tested on a flexible multiplex flow cytometric immunoassay. PloS One 2012;7: e35697.
9. Wiltshire S, O'Malley S, Lambert J, Kukanskis K, Edgar D, Kingsmore SF, Schweitzer B. Detection of multiple allergen-specific IgEs on microarrays by immunoassay with rolling circle amplification. Clin Chem 2000;46:1990–3.
10. Yager P, Domingo GJ, Gerdes J. Point-of-care diagnostics for global health. Annu Rev Biomed Eng 2008;10:107–44.
11. Martinez AW, Phillips ST, Whitesides GM, Carrilho E. Diagnostics for the developing world: microfluidic paper-based analytical devices. Anal Chem 2009;82:3–10.
12. Gantelius J, Bass T, Sjöberg R, Nilsson P, Andersson-Svahn H. A lateral flow protein microarray for rapid and sensitive antibody assays. Int J Mol Sci 2011;12:7748–59.
13. Gantelius J, Hamsten C, Neiman M, Schwenk JM, Persson A, Andersson-Svahn H. A lateral flow protein microarray for rapid determination of contagious bovine pleuropneumonia status in bovine serum. J Microbiol Methods 2010;82:11–8.
14. Ramachandran S, Singhal M, McKenzie KG, Osborn JL, Arjyal A, Dongol S, et al. A rapid, multiplexed, high-throughput flow-through membrane immunoassay: a convenient alternative to ELISA. Diagnostics 2013;3:244–60.
15. Oh YK, Joung H-A, Kim S, Kim M-G. Vertical flow immunoassay (VFA) biosensor for a rapid one-step immunoassay. Lab Chip 2013;13:768–72.
16. Desmet C, Le Goff GC, Brès JC, Rigal D, Blum LJ, Marquette CA. Multiplexed immunoassay for the rapid detection of anti-tumor-associated antigens antibodies. Analyst 2011;136:2918–24.
17. Xu Y, Bao G. A filtration-based protein microarray technique. Anal Chem 2003;75:5345–51.
18. Dhar TK, Dasgupta S, Ray D, Banerjee M. A filtration method for rapid preparation of conjugates for immunoassay. J Immunol Methods 2012;385:71–8.
19. Scala E, Pomponi D, Giani M. Allergen microbead arrays: the future of allergy diagnostics? Exp Rev Clin Immunol 2013;9:1–3.
20. Scala E, Alessandri C, Bernardi M, Ferrara R, Palazzo P, Pomponi D, et al. Cross-sectional survey on immunoglobulin E reactivity in 23 077 subjects using an allergenic molecule-based microarray detection system. Clin Exp Allergy 2010; 40:911–21.
21. Chapman MD. Lateral flow tests for allergy diagnosis: point-of-care or point of contention? Int Arch Allergy Immunol 2010;152:301–2.
22. Wei X, Li H, Li Z, Vuki M, Fan Y, Zhong W, Xu D. Metal-enhanced fluorescent probes based on silver nanoparticles and its application in IgE detection. Anal Bioanal Chem 2012;402:1057–63.
23. Shi L, Wang XC, Liu Y, Lu Y. Rapid detection of shellfish major allergen tropomyosin using superparamagnetic nanoparticle-based lateral flow immunoassay. Adv Mat Research 2011;311:436–45.
24. Teste B, Malloggi F, Siaugue J-M, Varenne A, Kanoufi F, Descroix S. Microchip integrating magnetic nanoparticles for allergy diagnosis. Lab Chip 2011;11:4207–13.
25. Chinnasamy T, Segerink LI, Nystrand M, Gantelius J, Andersson-Svahn H. A lateral flow paper microarray for rapid allergy point of care diagnostics. Analyst 2014;139:2348–54.
26. Wang J, Lin J, Bardina L, Goldis M, Nowak-Węgrzyn A, Shreffler WG, Sampson HA. Correlation of IgE/IigG4 milk epitopes and affinity of milk-specific IgE antibodies with different phenotypes of clinical milk allergy. J Allergy Clin Immunol 2010;125:695–702.
27. Mita H, Yasueda H, Akiyama K. Affinity of IgE antibody to antigen influences allergen-induced histamine release. Clin Exp Allergy 2000;30: 1583–9.
28. King E-M, Vailes LD, Tsay A, Satinover SM, Chapman MD. Simultaneous detection of total and allergen-specific IgE by using purified allergens in a fluorescent multiplex array. J. Allergy Clin Immunol 2007;120:1126–31.

A point of care vertical flow allergen microarray assay: a proof of concept

Thiruppathiraja Chinnasamy[1], Loes I. Segerink[2], Mats Nystrand [3], Jesper Gantelius[1] and Helene Andersson Svahn[1]*

Materials and Methods

Reagents and samples

Chloroauric acid ($HAuCl_4$), trisodium citrate, Neutravidin, Tween20, bovine gamma globulin, bovine serum albumin (BSA), sucrose, trehalose, and NaCl were purchased from Sigma Aldrich. Nitrocellulose membranes with 0.1 (Whatman Protran BA 79), 0.22 (Whatman Protran BA 83) and 0.45 µm (Whatman Protran BA 85) pore sizes were obtained from Sigma Aldrich. All used chemicals were analytical grade and were applied without further purification.

The complete VFM assay was performed using assay buffer containing 0.5% Tween20, 0.45M NaCl, 0.05% bovine gamma globulin, together with 3% Bovine serum albumin (BSA), 0.1% PEG 20000 and 1% sucrose in 0.1 M phosphate buffer (PB) at pH 7.4. Finally, other buffers such as storage buffer (1% BSA, 5% sucrose, 5% trehalose in 0.1 M PB) and washing buffer (0.1% BSA, in 0.1 M PB pH 7.4) were used in this study.

All human sera used in this study (n=31) were selected for good sensitization profile coverage and provided by Thermo Fisher Scientific (Uppsala, Sweden-division, formerly Phadia AB) with corresponding ImmunoCAP data (Supplementary table 1). Validated purified allergen components (Ara h 8, Ara h 9, Bos d 5, Bos d 8, Cyn d 1, Gal d 2, Ole e 1, Pru p 1, Pru p 3, Sal k and monoclonal anti-human IgE were also provided by Thermo Fisher Scientific.

Data analysis

The membrane was scanned within 5 minutes after the final assay step (room temperature, no active drying) in grayscale (1200 dpi) using a CanoScan 9000F (Canon) and Vuescan 9 software (Hamrick). The scanned image was inverted and loaded in Genepix 5.0 (Axon laboratories) to analyze intensities of all spots (spot size 120 µm). The mean intensity of each allergen spot was subtracted by local background intensities and further data analysis was performed using Graph pad 5 and R-Studio.

Synthesis of gold nanoparticles

The 25 nm Gold nanoparticles (GNPs) were prepared using standard citrate reduction method (1). In brief, 50 mL of 0.01% aqueous $HAuCl_4$ solution was heated to 100 ºC under refluxing conditions. During vigorous stirring, 750 µL of 1% sodium citrate was rapidly added, such that the yellow solution became finally dark red indicating the end point. The mixture was then kept at 100 ºC for 15 minutes, followed by continuously stirring at room temperature until cool. The prepared nanoparticles were characterized by UV–VIS spectral analysis (The SpectraMax® M5 Multi-Mode Microplate Reader) and High Resolution Transmission Electron Microscopy (HR-TEM, JEM-2100F instrument).

Preparation of nanoconjugates

The gold nanoparticles conjugates were prepared according to a modified procedure (2). In brief, 1 mL of (0.4 A530 units/mL and pH 8.2) GNPs was mixed with 100 µL of 100 µg/mL Neutravidin and kept at room temperature for 2 hours on gentle stirring. Subsequently, the gold nanoparticle conjugate was blocked with 10% BSA in MilliQ for an hour with gentle stirring. The conjugate suspension was then centrifuged at 10,000 rpm at 4 ºC for 10 minutes and the supernatant was discarded. The pellet was resuspended in washing buffer and centrifuged at

10,000 rpm at 4 ºC for another 10 minutes. The washing and centrifugation procedure was repeated twice. Finally, the supernatant was removed and the end volume adjusted to 500 µl with storage buffer and the conjugate suspension was kept at 4 ºC prior to use. The same procedure was followed to prepare the anti-human IgE conjugated gold nanoparticles used in this study.

Characterization of GNP conjugates

Spectrophotometric analysis of GNPs before and after coupling with biomolecules indicated that the absorption maximum shifted from 530 nm to 540 nm upon coupling (Supplementary figure 1A). Hence, UV–spectral analysis confirms the coupling of the biomolecules on gold nanoparticles surface. The FE-TEM image shows that the GNPs have an average diameter of 26 ± 0.2 nm and after the biomolecules coupling the average diameter was 29 ± 0.2 nm (Supplementary figure 1B & C). Also grayish halos around the modified GNPs were observed, indicating that biomolecules were bounded on the GNPs surface (2). From GNP conjugation optimization it was found that a concentration of 6 µg/mL of biomolecule could be fully conjugated on 1 ml of GNPs.

Results

VFM assay - influence of pore size and fluid flow

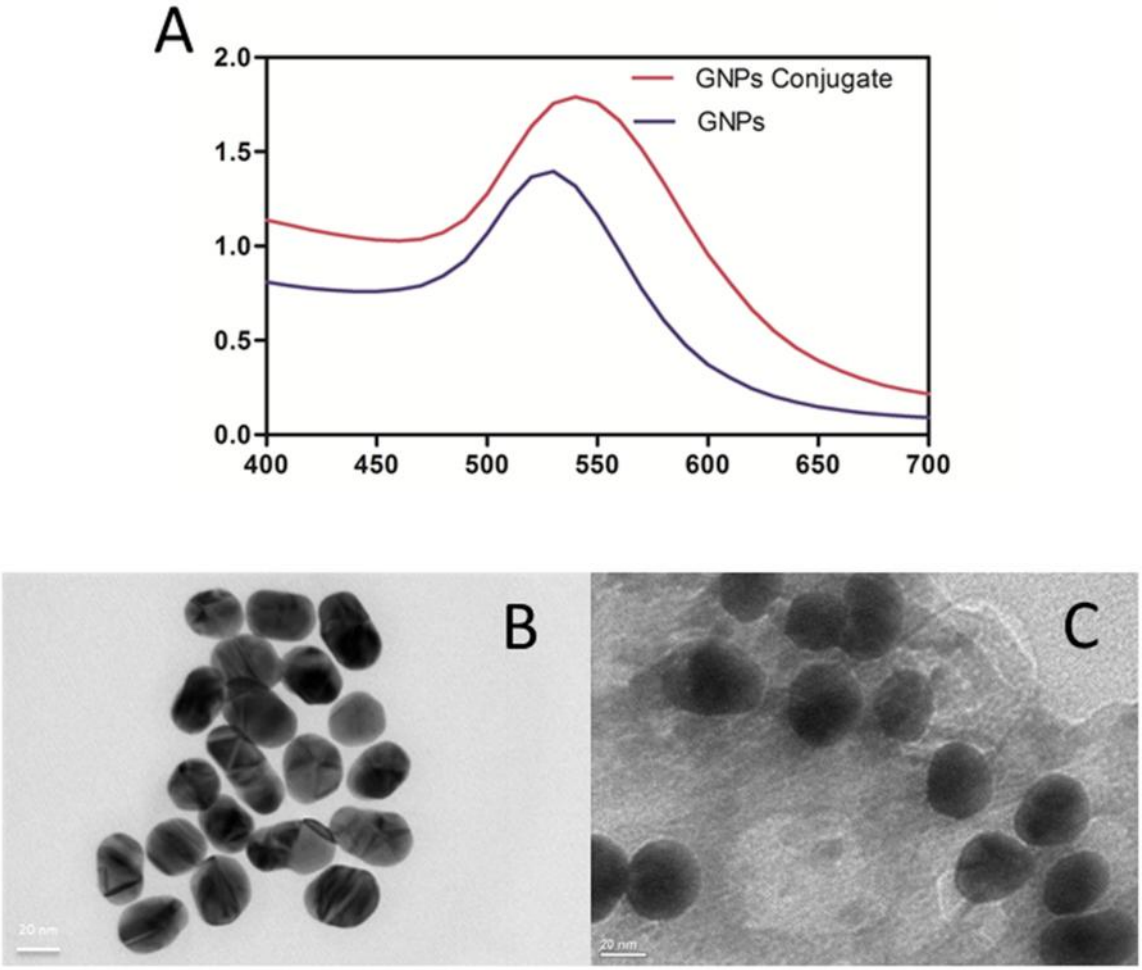
The influence of the membrane pore size and fluid flow on performance of the VFM assay was investigated. In total three membranes of different pore size (0.1 µm, 0.2 µm and 0.45 µm) were examined with different flow rates (500 µL/min, 1000 µL/min, 1500 µL/min, 3000 µL/min and manual pressure-approximately 4000 µL/min) were used and analyzed to ensure optimum choice of membrane and rate of fluid flow. The performance of the VFM assay for different membrane pore sizes and varying flow rates is shown in supplementary table 2. Average mean

intensities of the 100 spots were calculated and the coefficient of variation was calculated. As expected, the spot intensity decreased after increasing the flow rate above a threshold, which may be explained by a limitation in the maximum association rate of the affinity reaction. Further, it was observed that spot intensity increased when reducing the pore size of the membrane while flow speed was held constant (Supplementary figure 2). However, as also shown in Supplementary figure 2, a high background noise was observed at 0.1 µm pore at a fluid flow rate of 500 µl/min but not at flow rates from 1500 µL /min and up. This effect could be explained by the reduction in pore sizes at the same time a) providing a larger surface area for specific analyte-binding as well as non-specific binding of unrelated proteins and detection reagents and b) resulting in higher fluid shear rates within the pores that would conceivably work to reduce particularly the non-specific adherence of nanoparticle reagents. Consequently, it can be argued that the best signal to noise performance can be found experimentally at a balanced choice of pore-size and flow rate. Further, it was also observed that the higher CV values (CV>0.85) were obtained for the larger-pore (0.45 and 0.22 µm) membranes over the different flow rates. Conversely, very low CV values (CV>0.09) were obtained with 0.1 µm membranes. Due to that a) the variability in the larger-pored membranes arose from both high variation between runs and from intra-array variations, and b) inspection of arrays printed with fluorescently-labelled BSA (data not shown) revealing homogeneous printing patterns, we conclude that the variability occurred due to unreliable flow profiles and not through variability in the printing efficiency. It is possible that large pores and high flow speeds result in a higher susceptibility for deformation of the membrane mesh, resulting in an inhomogeneous flow resistance. From these findings, we decided to perform subsequent experiments with the 0.1 µm membrane at flow rate 1 mL/min.

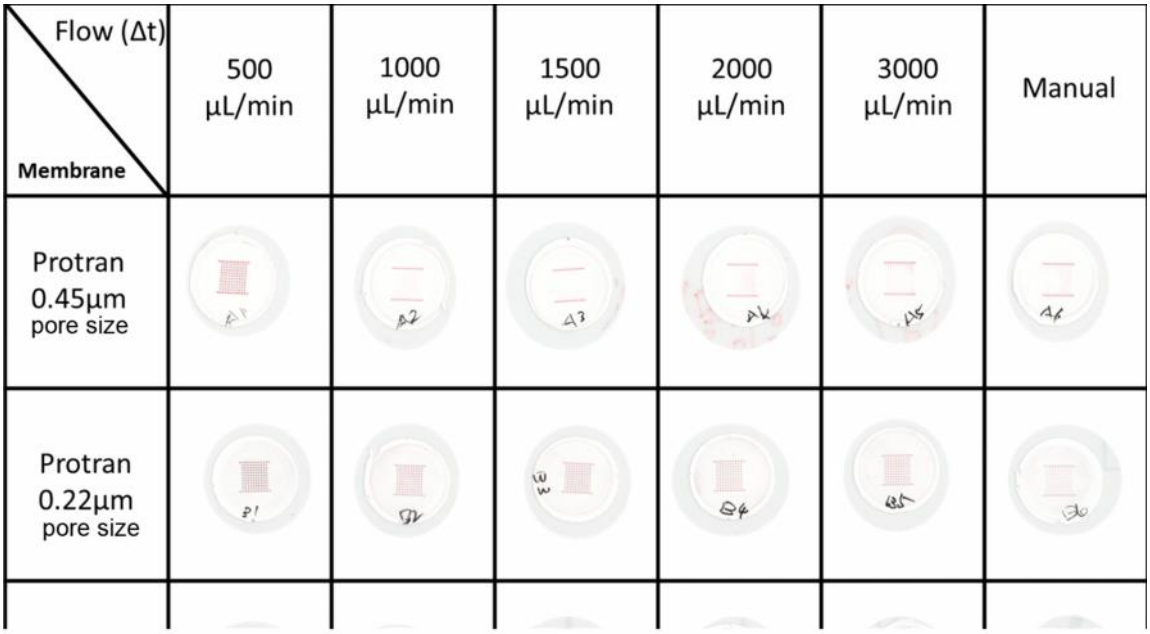
Supplementary table 1. ImmunoCAP values (ISAC standardized units, ISU) for the serum samples and the corresponding allergens provided by the Thermo Fisher Scientific, Uppsala, Sweden.

Sample ID	Ara h 8 ISU	Ara h 9 ISU	Bos d 5 ISU	Bos d 8 ISU	Cyn d 1 ISU	Gal d 2 ISU	Ole e 1 ISU	Pru p 1 ISU	Pru p 3 ISU	Sal k 1 ISU
6650	0	0	0	0	0	0	0	0	0	0
13694	0.91	0	0	0	10.91	0	0	1.72	0	0
27369	49.53	0	0	0	0.3	0	2.54	85.02	0	0
27430	11.71	1.13	0	0	0.74	0	0		0	0
28938	6.67	0	0	0	1.22	0	0	4.18	0	0
29140	0	0	0	0	0	0	0	0	0	0
31464	0.48	0	0	0	15.22	49.96	0	0.42	0	0
31912	0	0	0	0	1	1.17	0	0.34	0	0
39724	9.04	0	0	0	2.28	0	0	10.56	0	0
43109	0.31	0	0	0	0.26	0	0	0	0	0
44789	0	0	1.15	0.92	0	0	0	0.69	0	0
45359	0	0	0	0	62.24	0	0	2.81	0	0
47020	0	0	0	0	21.83	0	5.64	9.81	0	54.92
47177	0	9.01	5	41.14	0.2	12.2	0	0	0	0
50368	0	0	0	1.1	0.18	0	0	0	0	0
51083	0	0	0	0	0.81	0	0.99	0	0	1.18
51111	1.12	0	0	0	3.52	0	0	1.13	0	0
51113	0	0	0	0	0	0	0	0	0	0
51255	0	0	0	0	0.48	0	2.6	0.92	0	9.7
51287	0	0	0	0	14.74	0	39.26	0	0	0
52005	0	0	0	0	0	0	0	0	0	0

52282	0	0	0	0	0	0	0	0	0	0
52508	0	0	0	0	0	0	1.3	0	0	0
54350	0	0	0	0	0	0	0	0	10.56	0
54352	0	0	0	0	0	0	3.66	0	1.15	0
54358	0	0	0	0	0	0	10.01	0	0	0
56714	0	0	14	3.42	0	0	0	0	0	0
57028	0	0	0	0	0	0	0	0	49.43	0.72
62474	0	0	0	0	2.96	0	0.35	0	0	0
63000	0	3.19	0.65	12.21	0.1	3.23	0	0	5.99	0
63734	0	21.8	0	0	0	0	0	0	20.5	0



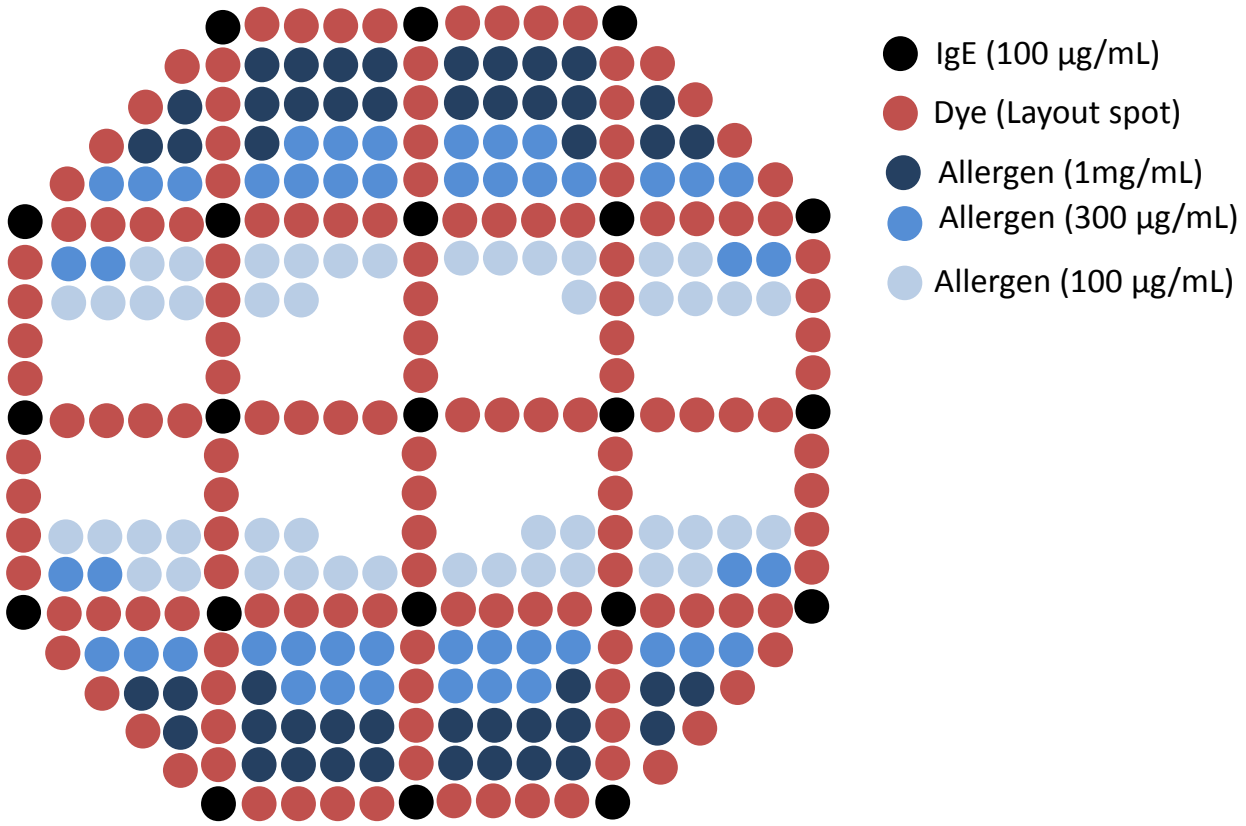
Supplementary figure 1 (A), UV spectral analysis yielding absorption maximum of GNPs (530 nm) and dual labelled gold conjugates (540 nm). (B), the FE-TEM images of GNPs (26 ± 2 nm) and (C), biomolecule conjugated GNPs (29 ± 2 nm). The scale bar is 20 nm.



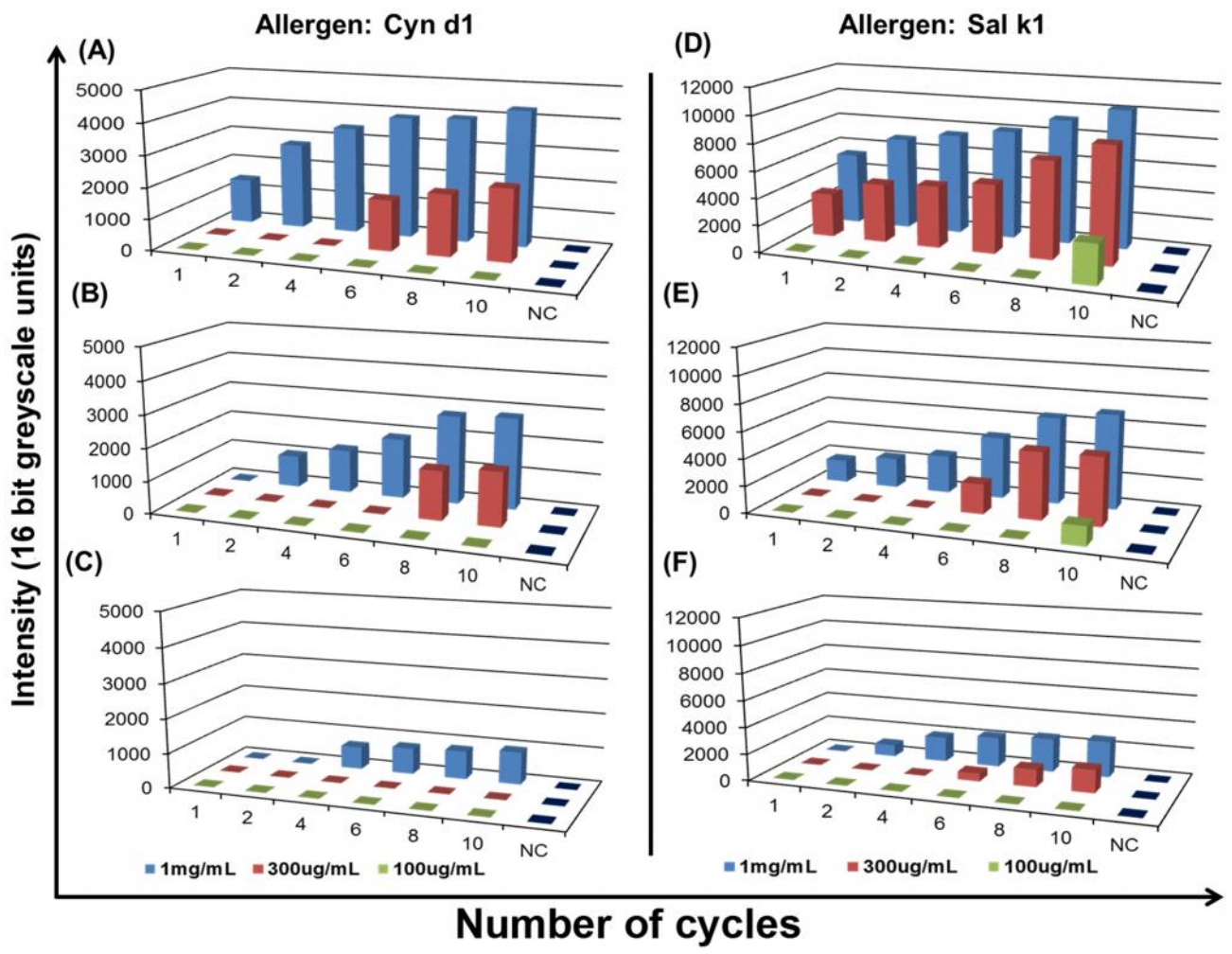
Supplementary figure 2. Investigation of optimal membrane and fluid flow for the development of the vertical flow microarray assay. 1 mg/mL of hIgG was deposited in all spots the membrane.

Supplementary table 2. Influence of membrane pore size and various flow rates on the VFM assay performance.

Membrane (pore size)	**Flow (Δt)**	**500 µL/min**	**1000 µl/min**	**1500 µl/min**	**3000 µl/min**	**Manually**
Protran 0.1 µm	Mean intensity	13182.36	16938.34	16580.89	13306.29	12603.73
	Background	19979.71	15039.96	10367.55	9646.01	8451.57
	CV	0.07	0.06	0.06	0.06	0.08
Protran 0.2 µm	Mean intensity	7651.18	13720.46	1505.51	8288.68	6865.99
	Background	4711.84	2876.67	1907.07	1790.01	1439.18
	CV	0.92	0.07	1.28	0.1	0.08
Protran 0.45 µm	Mean intensity	4526.08	4535.00	10519.63	4979.49	4020.78
	Background	4711.84	2876.67	1118.72	89.11	837.66
	CV	1.27	1.04	0.45	1.03	0.81



Supplementary figure 3. Spot layout of the allergen VFM. A frame of unconjugated gold nanoparticles (red) was used to guide alignment in post-processing. High-concentration pure IgE spots were interspersed in the frame as positive control for detection reagents and as aid in detecting heterogeneity in the flow profile (black). All allergens were printed in three concentrations (blue gradient spots) and positioned in two mirror planes in the array.


Allergen: Cyn d1
Allergen: Sal k1
(A)
(B)
(C)
(D)
(E)
(F)
Intensity (16 bit greyscale units)
Number of cycles
1
2
4
6
8
10
NC
1mg/mL
300ug/mL
100ug/mL

Supplementary figure 4. Effect on re-circulation protocol on VFM assay performance using different concentrations of allergens Cyn d1 and Sal k1 for the serum sample ID 47020. (A and D) 1:10 serum dilution, (B and E) 1:20 serum dilution and (C and F) 1:50 serum dilution. NC-Negative control. For each inset, the x-axis represents the number of recirculation cycles. The mean spot intensity with subtracted local background was acquired from the 16 bit scanned greyscale image.

Supplementary figure 5. Results from re-circulation study using different concentration of allergens Ara h8 and Pru p1 for serum sample ID 27369. (A and D) 1:10 serum dilution, (B and E) 1:20 serum dilution and (C and F) 1:50 serum dilution. NC- Negative control. For each inset, the x-axis represents the number of recirculation cycles. The mean spot intensity with subtracted local background was acquired from the 16 bit scanned greyscale image.

References

1. Frens G. Controlled nucleation for regulation of particle-size in monodisperse gold suspensions. Nature Phy Sci 1973; 241(105): 20-22.

2. Thiruppathiraja C, Kamatchiammal S, Adaikkappan P, Alagar M. An advanced dual labeled gold nanoparticles probe to detect *Cryptosporidium parvum* using rapid immuno-dot blot assay. Biosens Bioelectron 2011; 26: 4624-27.