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An 8-minute colorimetric paper-based reverse phase vertical flow serum microarray for screening of Hyper IgE syndrome

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Abstract

Reverse phase microarrays are useful tools for affinity-based detection in hundreds of samples simultaneously. However, current methods typically require long assay times and fluorescent detection. Here we describe a paper-based Vertical Flow Microarray (VFM) assay as a rapid 8-minute colorimetric alternative for reverse phase microarray analysis. The VFM platform was optimized for detection of IgE with a detection limit of 1.9 µg/mL in whole serum. Optimized conditions were then used to screen 113 serum samples simultaneously for hyper IgE syndrome (hIgE), a rare primary immunodeficiency characterized by elevated levels of IgE. The same set of samples were then analysed with a conventional planar microarray with fluorescent detection for head-to-head testing. Both assays found elevated levels in three out of four hIgE patient samples, whereas no control samples displayed elevated levels in either method. The comparison experiments showed a good correlation between the two assays, as determined from a linear correlation study (Pearson's r=0.76). Further, the assay-time reduction and reproducibility (intra assay CV = 12.4 ± 4.11 %) demonstrate the applicability of the VFM platform for high throughput reverse phase screening.

Introduction

Protein microarrays are affinity proteomic tools used for highly multiplexed protein analysis with wide applications within areas such as protein detection, protein-protein interactions and screening for enzymatic properties.[1] There are two main types of protein abundance microarrays available, the forward phase capturing array often used for detecting multiple analytes in one sample and the reverse phase protein array typically used for detecting one analyte in multiple samples. Both array types consist of microspots used for protein detection. The microspots of forward phase arrays consist of capture molecules, each spot comprising of one specific capture molecule type such as antibodies or other affinity molecules, allowing one sample to be investigated with regards to several analytes. Reverse phase arrays on the other hand consist of miniature spots of immobilized complex sample allowing multiple samples to be probed for one or a few analytes at a time and have been widely used for analysis of cell and tissue lysates.[2-4] Reverse phase arrays have also been successfully used for analysis of body fluids such as serum and plasma.[5-9] Serum arrays have traditionally been printed on nitrocellulose coated glass (*e.g.* FAST slides Whatman/Schleicher & Schuell, ONCYTE slides Grace Biolabs, UniSart slides Satorius), where a coating efficiency of 40-100 µg/cm^2 can be achieved.[10] Nitrocellulose polymers form a 3D-like structure, allowing high protein binding through electrostatic non-specific interactions. Nitrocellulose membranes have also shown high protein stability over time in dried blood spots.[11] Due to the low sample volumes deposited on reverse phase protein arrays and the low surface density of target protein that follows, there is an intrinsic limitation in sensitivity and dynamic range, allowing reverse phase arrays to be used only for medium and high abundant proteins without sophisticated amplification strategies.[12, 13] Limit of detection for reverse phase arrays has previously been reported in the mid ng/mL to low µg/mL for serum biomarkers.[6, 14] Detection on microarrays has mainly been performed either by direct labelling with fluorophores or primary/secondary immunofluorescence staining. However, labelling with chemiluminescence, radioisotopes, gold nanoparticles, quantum dots and surface enhanced Raman scattering as well as label-free techniques have also been demonstrated.[15, 16]

Previously, a vertical flow microarray (VFM) platform has been developed in our group for allergen specific IgE detection.[17] The platform consists of a paper-based microarray where the reagents are transported to the array vertically by means of convective flow for forward phase detection with conjugated gold nanoparticles. Vertical addition of antibody conjugated gold nanoparticles enable rapid detection compared to traditional techniques that rely on diffusion. Other vertical and cross flow microarray on nitrocellulose assays have also been proposed.[18-21] In this study, a VFM platform was developed for reverse phase detection of total IgE in serum samples, screening for potential hIgE patients at much shorter assay times than traditional techniques and with less advanced equipment. The workflow of the reverse phase serum array used in this article is presented in Figure 1.

Hyper IgE syndrome (hIgE), also known as Job's syndrome, is a primary immunodeficiency disorder characterized by elevated serum IgE levels, eczema and recurrent staphylococcal skin abscesses and pneumonia among other symptoms.[22-25] There is currently no available cure for hIgE patients and treatment is given in form of prevention and management of infection.[25] hIgE is rare with a prevalence of around 1:1000 000 worldwide and can be caused by

mutations in the genes of either STAT3 or DOCK8.[26-29] Total IgE levels are traditionally measured through Fluoro Enzyme Immunoassays (FEIA) and IgE serum levels in these patients have been reported in international units per litre (IU/L). Hyper IgE patients typically present with IgE-levels of 2000-100 000 IU/mL, whereas normal IgE serum levels are generally <130 IU/mL for adults.[30, 31] The unit IU/L can be multiplied with 2.4 to achieve the level expressed in µg/L.[32, 33] By the latter unit, serum IgE levels in hIgE patients typically amount to 4.8-2400 µg/mL, and consequently it appears feasible to screen for hIgE with reverse phase serum arrays.

Experimental information

Vertical Flow Microarrays

Array printing

In this work, serum microarrays were deposited on nitrocellulose paper membrane Protran BA79 0.1 µm (Whatman) using a Nano-Plotter NP 2.1 (GeSim) robotic printer. The arrays were printed with 5 droplets per spot giving an approximate printing volume of 2 nL per spot and resulting in 200 µm diameter spots. After printing, the membranes were left to dry overnight in room temperature before running the vertical flow assay.

Vertical flow assay

After drying overnight, the serum array membrane was placed inside a XX3001200 Swinny Filter Holder 13 mm (Merck Millipore). Assay buffer consisting of 29 mM sucrose, 0.44 mM BSA, 0.45 M NaCl, 0.5 % w/v Tween20 in 0.1 M Phosphate buffer, pH 7.4 was used for all VFM assays both as blocking buffer, washing buffer and for dilution of gold nanoparticles. Anti-IgE conjugated 40 nm gold nanoparticles with OD 20 were supplied by Thermo Fisher Scientific. Both assay buffer and diluted gold nanoparticles were applied vertically onto the array with controlled flow using a PhD2000 ultrasyringe pump (Harvard Apparatus). After running the vertical flow assay, the membrane was left to dry for 5 minutes before being scanned during less than one minute in a flatbed scanner CanoScan 9000F Mark II (Canon) in 16 bits grayscale and saved as a TIFF-file.

IgE dilution

Purified IgE and IgE negative serum were supplied by Thermo Fisher Scientific. High concentration IgE was spiked into IgE negative serum, starting from 250 µg/mL. To make the IgE dilutions comparable to the PBS-diluted serum samples later used, the spiked samples were also diluted in PBS. Each point in the dilution series was diluted between zero and 1:10 times in 1x PBS. For the array printing, 40 nm gold nanoparticles were printed as a visual grid on the nitrocellulose membrane. The dilutions were subsequently spotted onto the membrane as triplicate spots. The layout of the microarray is shown in Supplementary Information Figure 1. After drying over night, the membrane was put inside the filter holder. Next, 0.5 mL of assay buffer was added to block the membrane by the pump driven syringe at a flow rate of 1 mL/min. Following blocking, 0.5 mL anti-IgE conjugated gold nanoparticles diluted in assay buffer were added at flow rates between 0.75 mL/min and 1.25 mL/min. Lastly, 1 mL of assay buffer was added at 1 mL/min to wash. An experiment using optimized assay conditions with a flow rate of 1 mL/min and a gold nanoparticle dilution of 1:4 was also performed

with total assay times of 8, 11 and 16 minutes while the blocking and washing steps were kept constant. Here 0.5 mL, 3.5 mL and 8.5 mL of conjugated gold nanoparticle were added to the 8, 11 and 16-minute assay respectively.

Serum array

A serum array consisting of 113 samples, including 4 diagnosed hIgE cases and 109 controls samples was diluted 1:10 in 1x PBS and printed in quadruplicate spots onto the nitrocellulose membrane together with control IgE spots of high concentration IgE spiked into undiluted IgE negative serum. Layout of the microarray can be seen in Figure 2A. The serum array consists of 1208 spots in total including 328 layout spots giving a maximum of 880 spots available for reverse phase screening. After printing and leaving the array to rest over night, the vertical flow assay was run under optimized conditions, *i.e.* applying 0.5 ml anti-IgE gold nanoparticles diluted 1:4 in assay buffer at 1 mL/min, giving a 8 minute total assay time including 0.5 minutes blocking,1 minute washing, 5 minute drying and 1 minute for scanning of the array for signal acquisition.

Unisart Reference Array

Unisart serum array

As a reference comparison assay, the 113 serum samples including both hIgE patients and healthy controls were printed together with a dilution of high concentration IgE in IgE negative serum. All samples were diluted five times in 1x PBS with 0.1% w/v Tween20 and 40% w/v glycerol and printed onto a 16 pad UniSart 3D Nitro membrane slide (#2UNY2GW00600616G by Sartorius Stedim Biotech) using a Marathon Inkjet Microarrayer (Arrayjet Ltd) using 3 droplets per spot and a total volume of about 300 pL per spot. After printing, the slides were dried in 38°C for 5 hours and stored at 4°C. Just before use, the slides were soaked in PBS-T 0.1% w/v and mounted into a Chip Clip slide holder (Kerafast) while the membranes were still wet. The membrane was then incubated in duplicate with a 1:1000 dilution of monoclonal Anti-Human IgE antibody produced in mouse (#I6510, Sigma-Aldrich) in PBS-T. The primary incubation took place for 1 hour at 150 rpm in room temperature on a tabletop shaker before the membrane was washed 3 x 5 minutes with PBS-T at 110 rpm. After washing, a secondary antibody goat anti-Mouse IgG Alexa Fluor 647 conjugate (#A-21235, Life technologies) was diluted 1:60000 in PBS-T and added to the array, incubated for 1 hour in a container protected from light from light at room temperature at 150 rpm on a table top shaker. After the secondary incubation, the slides were washed 3 x 5 min in PBS-T 0.1% at 110 rpm and were then rinsed with deionized water. After rinsing, the slides were centrifuged dry before being scanned as a TIFF-file in LuxScan HT24 (CapitalBio).

Data analysis

After running the microarray assays, the image analysis of the TIFF-files was performed with GenePix Pro 5.1 (Axon Instruments). The median intensity of each spot was calculated from GenePix and the sample spot data were normalized by subtracting the median intensity of the sample specific blank. For the serum array and the IgE dilution experiments, the sample specific blank was IgE negative serum. For the dilutions of IgE in serum and PBS, the blank was defined as the IgE negative serum diluted in PBS to the same extent. All

statistical analysis and plots were made in R (http://www.r-project.org/) using the add-on R-packages; Hmisc, beeswarm, pracma and ROCR.

Results and discussion

In this paper we describe an 8-minute reverse phase vertical flow array capable of high throughput screening for the rare primary immunodeficiency hIgE. Of the 113 samples used for testing the platform, four samples had been drawn from patients previously diagnosed with hIgE. The serum array was further characterized using dilutions of IgE spiked into serum and then compared with a traditional reference serum array. The VFM assay was optimized using purified IgE spiked into IgE negative serum and optimized conditions were used to estimate limit of detection for the reverse phase VFM assay

Optimization of VFM assay parameters

Assay parameters for the VFM serum array were determined using high concentration IgE spiked into IgE negative serum and evaluated as median colorimetric intensity (MCI) with VFM. Dilutions of gold nanoparticles in assay buffer were applied to the VFM IgE dilution array to evaluate the optimal concentration and flow rate for applying the detection gold nanoparticle. As presented in Figure 3, 1:4 of conjugated gold particle dilution resulted in the highest MCI and highest signal to noise compared to the other dilutions and shows improved performance of the particles when diluted in assay buffer. As seen in Figure 3C and Figure 3D, a flow rate of 1 mL/min for detection gold particles resulted in the highest MCI as well as the highest signal to noise ratio. The 1:4 dilutions also show the lowest variability, as presented in Supplementary Information Figure 2. The results from the experiment using 11 and 16 minutes addition of gold nanoparticle compared to the 8-minute assay can be seen in Supplementary Information Figure 3-4. While a longer assay time with more detection reagent appears to yield higher MCI signals, the background also increases making the signal to noise levels lower in these samples with longer assay time as compared to the 8-minute VFM assay.

Limit of detection

To further characterize the reverse phase VFM platform, limit of detection (LOD) experiments were conducted under optimal assay conditions, *i.e.* 1:4 dilution of detection gold nanoparticles at 1 mL/min. LOD in this experiment was defined as the mean intensity of the non-spiked IgE depleted undiluted serum plus three times the standard deviation of the non-spiked IgE depleted undiluted serum.[34-38] IgE was spiked into IgE negative sera starting from 250 µg/mL and diluted in PBS up to 1:10 (Supplementary Information Figure 5). The MCI decreased with the dilution rate and LOD for whole serum was estimated to 1.9 µg/mL for IgE spiked into undiluted serum in VFM and 0.9 µg/mL for IgE in serum diluted 1:5 for the Unisart array, see Supplementary Information Figure 2C, 6 and 7. The VFM with colorimetric detection thereby shows slightly inferior LOD compared to the Unisart array. However, both obtained LODs are lower than the lowest clinically relevant levels for hIgE with a threshold of approximately 4.8 µg/mL. Furthermore, both LODs are comparable to previously reported LODs for serum biomarkers using fluorescent detection and 1-2 hour incubation time. [6, 8, 14]

Head-to-head testing of reverse phase serum arrays

To demonstrate the capacity for reverse phase screening with VFM, serum samples from 109 healthy controls and 4 diagnosed hIgE patients were printed onto the array and screened for total IgE levels. A layout and image scan of this array can be seen in Figure 2B. The results from image analysis were then compared to a traditional Unisart reverse phase array, using the same set of serum samples.

Figure 4 shows box plots displaying the median intensity, upper and lower percentile of the IgE protein profiles from both platforms for the same serum samples. In both platforms, the median intensity is elevated in the hIgE group and the same three hIgE patients show elevated levels for IgE. The remaining hIgE sample shows IgE levels close to the median intensity of the healthy control group in both platforms, suggesting that this patient sample may have normal levels of IgE. The IgE levels of this sample had previously been measured using FEIA to be 520-2200 IU/mL corresponding to 1.2-5.28 µg/mL, which is lower than typical hIgE levels of >2000 IU/mL. IgE levels of hIgE patients have been shown to be elevated in 97% of the cases and the remaining 3% may be explained by IgE levels being variable with age and may decrease to normal levels in adulthood.[25, 31] This could be one explanation to the low FEIA level and why this sample also showed low intensity signals in both the VFM and Unisart assay.

Intra and inter assay coefficient of variations (CVs) for the VFM experiments can be seen in Table 1.The intra assay CV was 12.3% and 13.4% for the hIgE and control samples respectively and are similar to other previously reported intra assay CVs for other traditional reverse phase serum arrays[5, 6, 9]. Intra assay CVs of reverse phase arrays are traditionally higher compared to those of forward phase arrays, most likely due to the small immobilized sample volumes. Forward phase approaches could determine IgE levels in one sample and would conceivable yield lower CVs and LOD but would not allow the number of samples necessary for high throughput screening screening. Intra assay CV for the Unisart experiment was 3.7%. Inter assay CVs were higher, around 30% for both the hIgE and control serum samples. CVs for the spiked IgE dilutions were lower than for the serum array in both intra and inter assay CV. The high inter assay CVs is most likely a result of unreliable printing performance and variability between print batches, a factor that was deemed outside the scope of this paper to optimize further.

Receiver operating characteristic (ROC) curves were generated for both the VFM and Unisart assay, shown in Supplementary Information Figure 8. Analysis of the ROC curves showed an Area Under Curve (AUC) of 0.86 and 0.87 for the VFM and the nitrocellulose coated glass slide respectively. To compare the two methods further, Pearson's correlation coefficient r of 0.76 was calculated for the linear regression between the two assays in Figure 5.

The colorimetric detection technique could conceivably enable the VFM assay results to be stored without the risk of photo bleaching and the need for light protection in case of follow up analysis is needed. In addition to the colorimetric stability, it is likely that VFM printed serum arrays could be stable for extended periods of time before running the assay, similar to the stability of samples stored with the dried blood spot technique that is also paper-based[11]. Long-term stability of serum arrays on nitrocellulose could enable later use for research purposes, quality control and for additional printing.

Unlike the dried blood spot technique, both VFM and other reverse phase microarray methods need to be printed in a laboratory from separately collected samples before analysis. Reverse phase VFM arrays have shorter assay time compared to traditional reverse phase arrays using fluorescent detection although both methods are based on microarray printing, intrinsically a time consuming process. However the total turnaround time for VFM arrays are shortened by the rapid assay time compared to traditional serum microarrays. Further, it is likely that the total turnaround time would not increase particularly if the same samples used for printing were to be tested for additional protein markers, linked for instance to other primary immunodeficiencies.

It has been suggested that reverse phase arrays have the greatest application in screening across many samples for well-known biomarkers followed by more detailed and quantitative follow-up analysis using other methods.[12] VFM reverse phase arrays therefore are most suited to use for high throughput sample analysis that fit the following criteria; detecting a medium to high abundant protein biomarker without too narrow time constraints regarding turnaround time. For a large hospital with access to a microarray printer, the screening analysis of large number of samples for biomarkers giving information on semi-urgent conditions and diseases is therefore possibly a useful application for the presented array assay. Neonatal screening of hIgE is most likely not possible with current reverse phase array technologies since IgE levels in cord blood are lower than the current detection limits. However, an IgE level of 10-times above the 95 percentile of the normal age specific norm could arguably be used for screening younger children for elevated IgE levels.[25, 33] Additionally, other conditions are also linked to elevated levels of IgE >1 µg/mL such as asthma and allergy parasitic infections, as well as other primary immunodeficiencies such as Wiskott-Aldrich syndrome among others.[33] Further clinical data as well as genetic information about STAT3 and DOCK8 mutations could potentially be used together with the total IgE-levels when making a hIgE diagnosis.[31]

Conclusions

In this study, we present a rapid reverse phase VFM screening platform for screening of the rare primary immunodeficiency hIgE in 113 serum samples. Head-to-head testing between the VFM assay and a conventional array show similar performance with regards to LOD, relative IgE intensity levels in patient samples and AUC when discriminating between hIgE cases and controls. While the conventional assay is performed with two hours of antibody incubation time and a half hour washing, the presented VFM assay can be finished within 8 minutes. Further, the VFM assay only uses a flatbed scanner for detection, avoiding the need for advanced instrumentation such as a fluorescent scanner. The assay reduction time, the need for less instrumentation as well as being a paper-based method shows the competitiveness of the VFM platform for low-cost reverse phase screening. Further, other applications in which detecting high to mid abundant biomarkers in a multitude of samples could be envisioned for the VFM reverse phase platform such as screening for primary immunodeficiency disorders involving elevated or decreased levels of IgA, IgG, IgM deficiencies or complement proteins. Additionally, the reverse phase VFM platform could also prove useful in detection of biomarkers in complex samples other than serum such as cell and tissue lysates.

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Figures

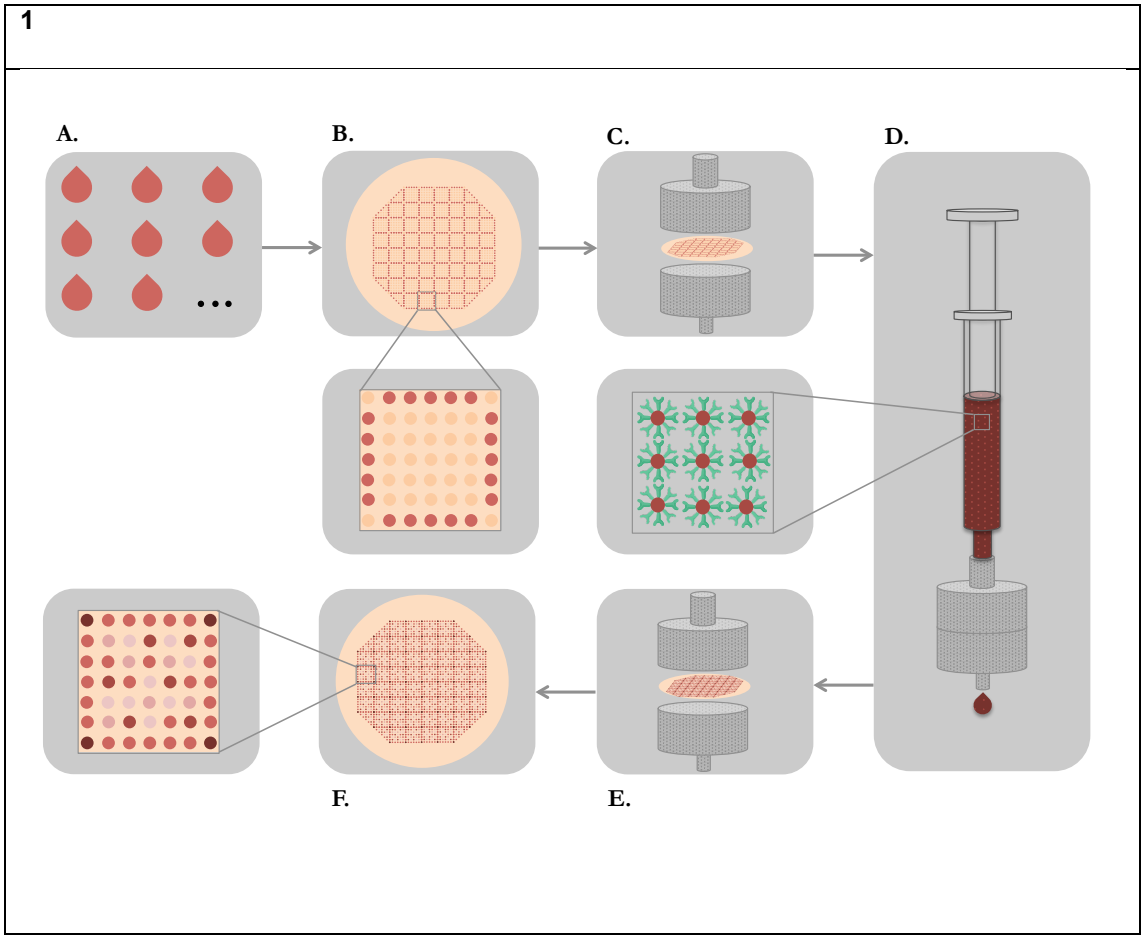
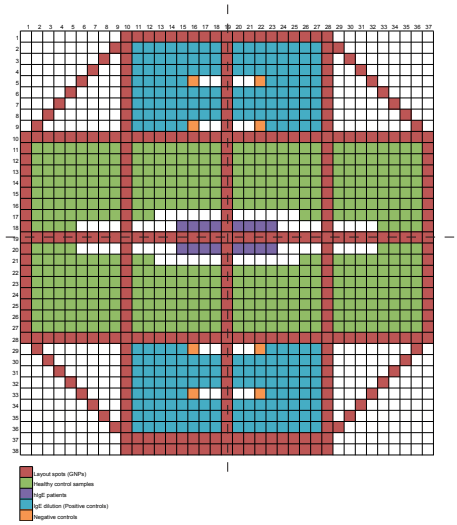


Figure 1. Concept picture
(A) Serum samples are diluted in PBS. (B) The serum samples are then printed onto a nitrocellulose membrane to a serum array with visual layout spots. (C) The membrane is placed inside a filter holder. (D) Subsequently, anti IgE gold nanoparticles employed as detection reagents are supplied onto the serum array in a vertical flow with a pump driven syringe. (E) Next, the membrane is removed from the filter holder. (F) The membrane is then left to dry before the colorimetric intensity is scanned for image analysis.

2A



2B

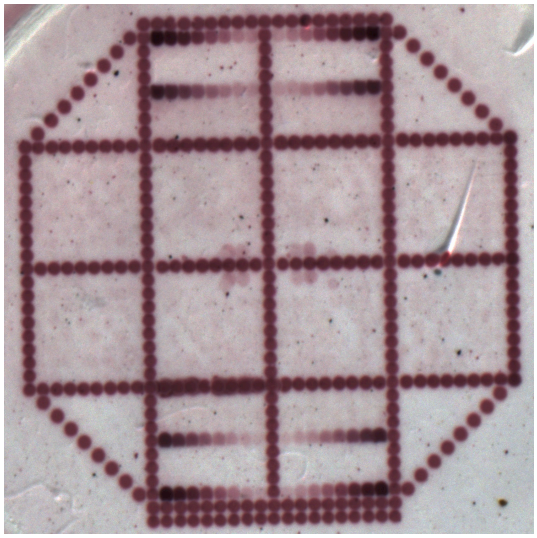


Figure 2. Reverse phase VFM with serum samples

To test the performance of the VFM assay under optimized conditions, 113 serum samples including 109 healthy controls and 4 hIgE patients were printed in reverse phase and run at optimal conditions.

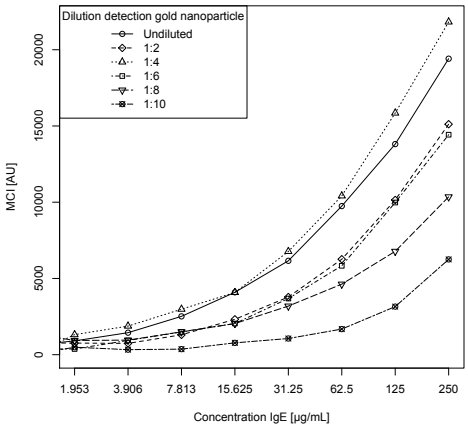
Figure 2A.

Layout of the VFM array where the red spots are layout spots, green are the healthy controls, purple hIgE samples, blue dilutions of IgE and orange is blanks (IgE negative serum). Empty space in the array is illustrated as white.

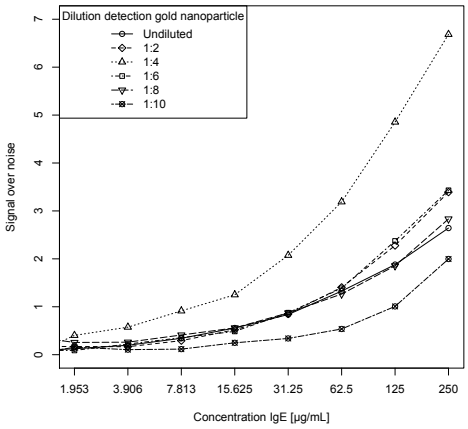
Figure 2B.

Image scanned in a flatbed scanner of the serum array after the VFM assay. The color intensity of each spot corresponds to the amount of anti-IgE conjugated gold nanoparticle that has been bound which in turn corresponds to the amount of IgE present in the spotted sample. For image analysis, the same array was scanned in black and white.

3A



3B



3C **3D**

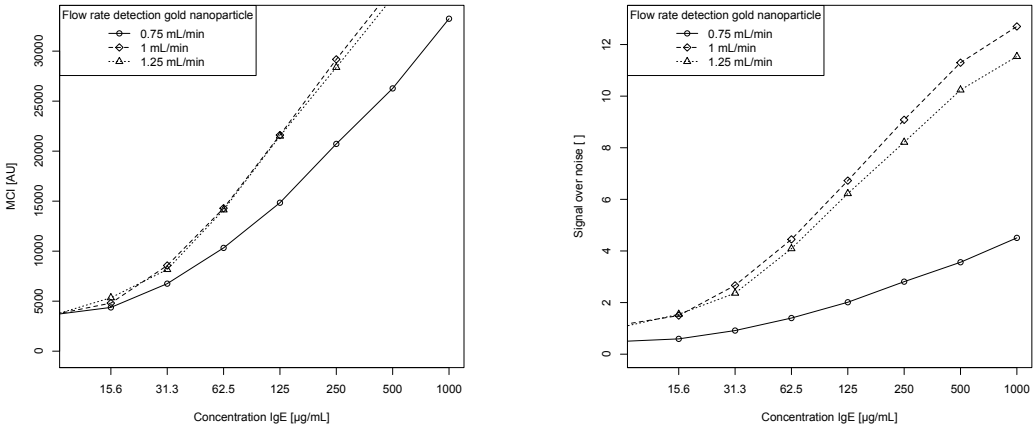


Figure 3. Optimization of reverse phase VFM assay
A dilution of IgE spiked into IgE negative serum was spotted onto a VFM membrane to determine the optimal dilution for the anti-IgE conjugated gold nanoparticles and flow rate for reverse phase IgE detection.
Figure 3A. *Median colorimetric intensity (MCI) for the dilution series of IgE for different gold nanoparticle dilutions used for detection.*
Fig 3B. *Signal to noise plot between the IgE spiked in signal over the unspiked IgE negative sample for different gold nanoparticle dilutions.*
Figure 3C. *Median colorimetric intensity (MCI) for the serum dilution of IgE at different flow rates of detection gold nanoparticle.*
Figure 3D. *Signal to noise plot between the IgE spiked in signal over the unspiked, IgE negative sample for the different flow rates.*

4A **4B**

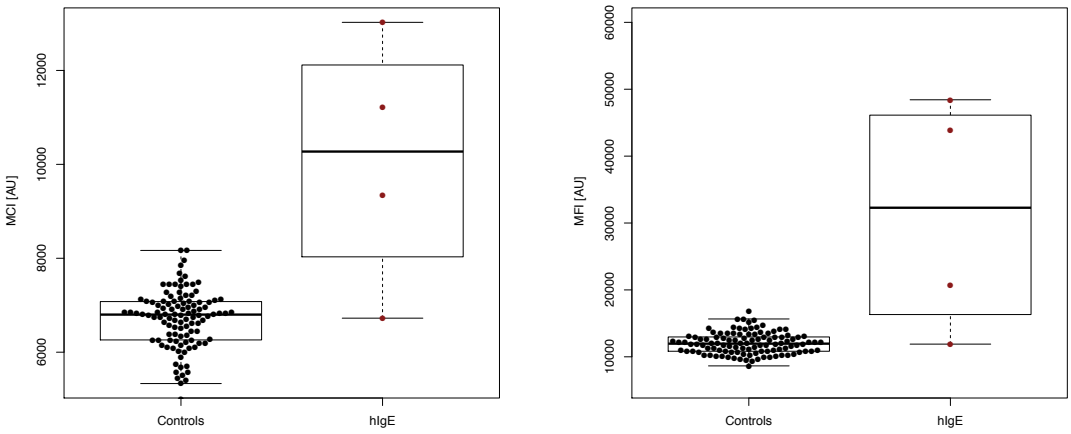


Figure 4. Boxplot of difference in colorimetric intensity between hIgE cases and healthy controls
Boxplots of the two sample groups where the black dots represent sample intensity of the healthy controls and red dots the hIgE patients. The horizontal line represents the median sample intensity, the box the upper and lower quantile and the whiskers 5 and 95% percentile.
Figure 4A. *Boxplot of sample groups from the serum serum array performed with VFM in MCI units.*
Figure 4B. *Boxplot of sample groups from the serum serum array performed with the Unisart array in MFI units.*

5

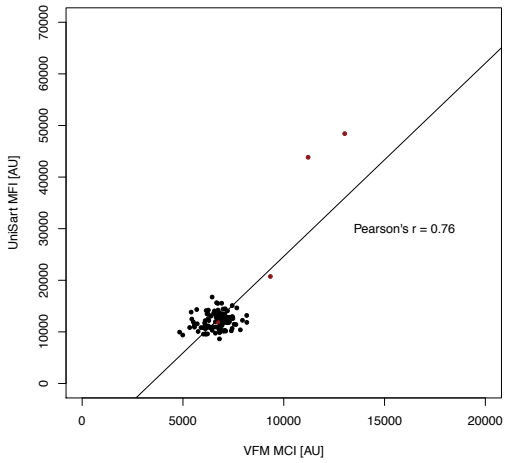


Figure 5. Comparison between VFM and traditional Unisart serum array
Scatterplot of the data from VFM and Unisart serum arrays in MCI and MFI units respectively. The intensities show a correlation of Pearson's r of 0.76.

Table 1. Coefficient of variance of reverse phase serum VFM
The table provides CVs for intra and inter assay variability for both serum samples and IgE spiked controls. The high IgE sample refers to concentrations between 250 and 62.5 µg/mL, median IgE 31.3-7.81 µg/ml and low 3.91-0 µg/ml.

		Mean CV (± SD CV) [%]	
Type	**Sample**	**Intra assay**	**Inter assay**
Serum samples	hIgE	12.3 (± 4.11)	30.6 (± 4.16)
	Control	13.4 (± 5.68)	30.5 (± 6.08)
IgE spiked serum	High IgE	2.043 (± 0.766)	15.1 (± 8.95)
	Median IgE	2.88 (± 0.683)	22.2 (± 6.81)
	Low IgE	7.86 (± 0.664)	2.80 (± 3.23)

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