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Antibody profiling by proteome microarray with multiplex isotype detection reveals overlap between human and Aotus controlled malaria infections

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Abstract

The development of vaccines against malaria and serodiagnostic tests for detecting recent exposure requires tools for antigen discovery and suitable animal models. The protein microarray is a high throughput, sample sparing technique, with applications in infectious disease research, clinical diagnostics, epidemiology, and vaccine development. We recently demonstrated Qdot®-based indirect immunofluorescence together with a portable optical imager ArrayCAM® using single isotype detection could replicate data using the conventional laser confocal scanner system, opening up the opportunity for deployment of protein microarray-based immunoassays in more laboratories worldwide. We developed a multiplexing protocol for simultaneous detection of IgG, IgA and IgM and compared samples from a controlled human malaria infection model with those from controlled malaria infections of *Aotus nancymaae*, a widely-used non-human primate model of human malaria. IgG profiles showed the highest concordance in number of reactive antigens. Thus, of the 139 antigens recognized by human IgG antibody, 111 were also recognized by *Aotus* monkeys. Interestingly, IgA profiles were largely non-overlapping. Finally, on the path toward

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Conflict of interest statement

Authors have no conflict of interest to declare.

wider deployment of the portable platform, we show excellent correlations between array data obtained in 5 independent laboratories around the U.S. using the multiplexing protocol (R^2: 0.60 – 0.92).This study supports the use of this platform for wider deployment, particularly in endemic areas where such a tool will have the greatest impact on global human health.

Keywords

Antibody isotype; Diagnostics; Immunoassay; Multiplex; Protein microarray; Quantum dots

2.0 Introduction

Detection of specific antibodies in human serum and other fluids produced in response to infectious disease agents underlies many current diagnostic tests for exposure to pathogens.[1] Antibodies are detectable after clearance of infectious agents and provide a much longer diagnostic window than tests based on detection of the organism or its nucleic acids. Antibodies are also useful biomarkers for monitoring vaccine and drug efficacy, and to diagnose disease in immune-compromised or autoimmune patients.[2–4] Common immunoassays to characterize antibody-antigen interactions include, ELISA, Biacore, flow cytometry, Western blot, immunoprecipitation, and indirect immunofluorescence (IFA). In general, antibody-based detection assays require large quantities of valuable target antigens, and antibodies. One prevailing practical concern from clinical research scientists is the quantity of serum required per serological assay. Finger sticks collect less than 0.2 ml of blood, resulting in less than 100 μL of sera. More invasive venous blood draws typically collect 10-ml volumes, but is impractical for children and infants. Archival samples, samples collected from remote endemic areas and from costly clinical trials are finite and invaluable resources for future research. Thus, there is considerable interest in sample sparing assays to obtain maximal biological information from irreplaceable samples.

Protein microarrays are an inherently sample-sparing and high-throughput immunoassay platform, which, in contrast to ELISA, allows multiple antigens and/or antibodies to be investigated simultaneously in a single assay. For example, an array matrix of 17 × 17 with 200μm-diameter features yields 289 different immunoassays with 1μL of sera, in the same surface area as a one well of a standard 96-well microtiter plate, single-antigen ELISA. Protein arrays are highly scalable; larger arrays correspondingly have more serodiagnostic ability for only modest increases in sample use. Most strikingly, the microarray analysis requires only 1.0 ng antigen per spot. Protein microarrays are a high-throughput, highly reproducible platform that save time and primary samples, while also providing more information than conventional, single-antigen immunoassays.[5]

With the aim of adapting protein microarrays for wider deployment worldwide, we previously showed that quantum dot (Qdots®) can be used in place of organic dyes such as Cy3 and Cy5 fluorophores thereby enabling the use of a benchtop optical imager (ArrayCAM®) in place of the conventional confocal laser scanner.[6] To expand the utility of the platform, and further reduce sample quantities, we developed and optimized a robust triple-isotype detection system for simultaneous detection of IgA, IgG and IgM antibodies

on the same array. To assess the reproducibility of the new triple-isotype assay, we compared assay results obtained from five different laboratories in the United States using a *Plasmodium falciparum* proteome array following a standard operating procedure. We also used the triple-isotype detection method to compare antibody isotype profiles of two different *P. falciparum* challenge models performed in humans [7,8] and *Aotus nancymaae* monkeys to evaluate the similarities in the antibody profiles. Overall, this study adds wider functionality to the protein microarray platform, and wider deployment in research or diagnostic laboratories and is readily achievable without compromising the quality of the data.

3.0 Materials and methods

3.1 Ethic statement

Human sera were obtained from a controlled human malaria infection (CHMI) trial conducted (where?) as described.[7,8] The trial was conducted in compliance with the Declaration of Helsinki. All volunteers signed an informed consent form after hearing a detailed explanation of the study and passing a written examination designed to ascertain if they understood the risks of malaria infection. Study protocols were reviewed and approved by institutional review boards of the University of Maryland and the National Institute of Allergy and Infectious Diseases/Division of Microbiology and Infectious Diseases. The trial was monitored by PPD, Inc. (Wilmington, NC). Further details can be found at ClinicalTrials.gov (identifier NCT00744133).

Adult *A. nancymaae* (Owl monkeys) were housed at the Centers for Disease Control (CDC) Roybal campus primate facility, fully-accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Animal studies were reviewed, approved and conducted in compliance with the Institutional Animal Care and Use Committee (IACUC) of the CDC. Malaria naïve *A. nancymaae* were pair housed, with cage space and environmental recommendations for small New World Primates set forth by the Care and Use of Laboratory Animals, NIH. Monkeys were fed a diet that was shown to provide adequate nutrition and calories in captive *Aotus* spp. monkeys used in malaria research. Animals were weighed at weekly intervals and treated for veterinary problems by an attending veterinarian as they arose. All observations were recorded and entered into a computer database.

3.2 Sera and antibodies

Human sera were obtained from a controlled human malaria infection (CHMI) trial conducted [where?] designed to determine the minimum number of infected mosquito bites required to reliably give volunteers a case of malaria.[7,8]

The Owl monkey sera used in this study came from a malaria blood stage vaccine trial that included an adjuvant control group and two groups immunized with recombinant merozoite surface protein antigens. [9] For the first challenge infection, 50,000 ring stage parasite infected erythrocytes (iRBC) of the FVO strain of *P. falciparum* were administered via intravenous injection of the femoral vein. A second challenge infection of 500,000 iRBC of

the Malayan Camp strain was given approximately 5 months after the first challenge. The post-infection serum specimens used in the present study were collected 14 days after the second challenge infection when animals were parasitemic. Three days after challenge infection, Giemsa-stained blood smears were made to establish the number of parasites per μL blood by quantitative thick film or by thin film. Blood collections of no more than 5% of a monkey's total blood volume (~3ml.) were made by venipuncture and taken biweekly before and after challenge infections. Persons responsible for handling the monkeys and reading smears for parasitemia did not know the group to which any particular animal was assigned. Monkeys that developed high-density parasitemia (>200,000 parasites/μl of blood) or anemia (hematocrit below 20%) were cured with an antimalarial drug (mefloquine) and treated by iron supplementation and transfusion of whole blood as needed.

Affinity-purified goat anti-human IgA, goat anti-human IgG, and goat anti-human IgM conjugated to Qdot®585, Qdot®800 and Qdot®655, (Catalog numbers 110620, 110610 and 110630 respectively), were provided by Grace Bio-Labs, Inc., (Bend, OR). A schematic of the probing protocol is shown in Figure 1A. The particular Qdots® used to develop the multiplex system were selected on the basis of expected non-overlapping emission spectra (Figure 1B).

3.3 *Plasmodium falciparum* array composition

Plasmodium falciparum (*Pf*) protein microarrays were produced as described previously.[10–12] Briefly, exons were amplified from genomic *Pf* DNA (strain 3D7) and cloned into a T7 expression vector using homologous recombination. Exons larger than 3kb were cloned as multiple overlapping fragments. For protein expression, plasmids were expressed at 24°C for 16h in *E. coli*-based *in vitro* transcription/translation (IVTT) reactions (Expressway Maxi kits from 5 Prime, Gaithersburg, MD). For printing, 10 μl of the reaction mixture was mixed with 3.3 μl 0.2% Tween 20 to give a final concentration of 0.05% Tween 20, and printed onto nitrocellulose coated glass AVID slides (Grace Bio-Labs, Inc., Bend, OR) using an Omni Grid 100 microarray printer (Genomic Solutions). Protein expression was >99% using antibodies against N-terminal poly-histidine (His) and C-terminal influenza hemagglutinin (HA) epitope tags genetically engineered into each protein. Different arrays were used in the course of this study with different numbers of expression products, namely Pf200, Pf250 and Pf900 that comprised of 192, 251 and 823 exon products, respectively. All expression products were down-selected from seroreactive antigen lists from larger microarray studies.[11–13]

3.4 Protein array probing

Serum samples were diluted 1:100 in protein array blocking buffer (Maine Manufacturing, Sanford, ME) supplemented with *E. coli* lysate (GenScript, Piscataway, NJ) to a final concentration of 10 mg/ml, and pre-incubated at room temperature (RT) for 30 min. Concurrently, arrays were rehydrated in blocking buffer (without lysate) for 30 min. Blocking buffer was removed, and arrays were probed with pre-incubated serum samples in sealed chambers. Arrays were incubated overnight at 4°C with gentle agitation. Slides were then washed at RT six times with TBS-0.05% Tween 20 (T-TBS), followed by incubation with individual or mixtures of secondary antibodies directly conjugated to Qdots® at ½00

dilution. After incubation, arrays were washed three times with T-TBS, and once with water. Chips were air dried by centrifugation at 1,000 rpm for 5 min, and images acquired using an ArrayCAM® Imaging System from Grace Bio-Labs (Bend, OR). For each type of Qdot®, imager settings were set at gain 50, and the exposure time in milliseconds (ms) was adjusted for to achieve saturation for 5–10% of the protein spots on the array.

3.5 IgG removal

Goat anti-human IgG Fc reagent for removal of IgG from sera was purchased from Meridian Life Sciences (Memphis, TN; Catalog #L15406G). For depletion of IgG, 1μl of serum was diluted in 100 μl protein array blocking buffer supplemented with *E. coli* lysate and 3 μl IgG removal reagent and processed as described above. A non-depleted control was processed simultaneously along with depleted one and was subjected to multiplex Ig isotype detection.

3.6 Data handling

Protein microarray spot intensities were measured using ArrayCAM® Software (Grace Bio-Labs) using automatic local background subtraction for each spot to give raw data. The median of sample-specific *Escherichia coli* background spots, i.e., signals obtained from IVTT reactions without DNA template (“no DNA controls”), were then subtracted from IVTT raw data for each sample and used to generate the figures. Scatter plots and bar charts were generated in Microsoft Excel. For each antigen, the average of pre-immune signals were subtracted from their post immune counterparts, ranked by the average signal of the human challenge group and plotted as bar charts. A separate cut-off for each Ig isotype was defined as the mean+2SD of the total human and monkey pre-immune antigen signals. Venn diagrams were generated using the BioVenn web application (http://www.cmbi.ru.nl/cdd/biovenn/index.php). Hypergeometric p values were calculated in R statistical environment (http://www.r-project.org).

4.0 Results

4.1 Optimization of reagents

To develop a multiplex protocol for simultaneous detection of IgA, IgM and IgG isotypes, three Qdots® with non-overlapping emission spectra were selected for conjugation to the secondary antibodies: Qdot® 525, Qdot® 655 and Qdot® 800 respectively (with the numbers referring to the peak emission wavelength in nm). In practice, Qdot® 525 emission was found to be in the nitrocellulose autofluorescence spectrum, making Qdot® 525 undetectable above background on the nitrocellulose substrate. A low level of Qdot® 585 (Figure 1) cross-talks was observed through the 655 nm filter. This issue was resolved by narrowing the width of the Qdot® 655 bandpass filter from 40 nm to 15 nm. Narrowing bandpass filter width successfully blocked the cross-talk, but the signal intensity passing through the 655 nm filter (IgM) was slightly reduced. This was compensated by using longer exposure time during image acquisition.

4.2. Comparing singleplex with multiplex Ig isotype detection

To ascertain the effect of multiplexing secondary antibodies, we probed an array containing 200 P falciparum proteins (Pf200) with a single CHMI serum followed by the secondary

anti- IgG, -IgA and -IgM antibodies in all possible singleplex, duplex and triplex combinations (n=7). The probing plan is shown in Figure 2A. The 8th pad was probed with the mixture of three secondary antibodies only (the secondary Antibodies control). The arrays were imaged for IgA (Figure 2B), IgG (Figure 2C) and IgM (Figure 2D) using the appropriate bandpass filters to pass specific spectral profiles with peaks at 585 nm, 800 nm and 655 nm, respectively. The images were captured as .tiff files and the signal intensity of the spots quantified using ArrayCAM® software. No signal spillover was observed between the filters, and all of the filters specifically pass through only the signals within their expected range. Also, in the serum-free pad, no signal was observed from antigen spots using any of the three filters. These observations confirm that all signals were specific to the antibody isotype. Raw data were subtracted of the average *E. coli* background signals on each pad and the subtracted data from duplex and triplex experiments plotted against singleplex for each antibody isotype (Figure 3). In all pairwise comparisons, we observed no loss of signals compared to use of the secondary antibodies used alone. Moreover we observed excellent correlations between the data sets. (R^2: 0.76 – 0.97). These observations indicate that duplexing or triplexing the secondary antibodies does not lead to interference between the secondary antibodies when combined.

4.3. Competition between primary sera Ig isotypes

The previous experiment indicates the secondary antibodies may be combined without interference with each other, thus, allowing multiple isotypes to be probed on a single array. However, it is possible this advantage is negated if there is competition between isotypes within the primary serum sample. Indeed, it is understood that measurement of IgM via ELISAs is often overwhelmed by IgG in the sample competing for the same antigen, thereby necessitating the removal of IgG by using an IgM capture or sandwich ELISA format. Therefore, we repeated the multiplexing experiment in the presence and absence of IgG removal reagent (Figure 4). All IgG signals were completely abolished in the presence of IgG removal reagent, whereas most IgA or IgM signals were retained (slope: 0.691, R^2: 0.7058 for IgA; slope: 0.6178, R^2: 0.7232 for IgM). The results indicate that the IgA or IgM detection was not compromised by the potential competition of abundant IgG in the sample. This observation further provides the validity of the multiplex detection offered by the combination of Qdots®.

4.4 Comparisons between Aotus and human IgA, IgG and IgM profiles

The New World primate *A. nancymaae* is susceptible to infection with *P. falciparum* and is a useful model for immunological investigations, pathology studies and the development of malaria vaccines.[14] To further test the utility of the multiplex protocol for vaccine design and monitoring antibody responses during malaria infections, we generated IgG, IgA and IgM profiles from 11 human and 8 *A. nancymaae* samples before and after challenge with *P. falciparum* parasites using the multiplex format. In a pilot experiment, we established that the anti-human secondary antibodies used for the human samples also detect *Aotus* antibodies (not shown), consistent with earlier studies.[15] The experiment was then repeated so that all human and *Aotus* samples were probed simultaneously. For each human and *Aotus* donor, the day 0 (pre-immune) signal was subtracted from the post-challenge signal for each antigen. The average mean fluorescence intensity (MFI) for each antigen was then

plotted as bar charts for the human and *Aotus* samples (Figure 5A). In each chart the antigens have been ranked by the average signal of the human samples. A separate cut-off for each Ig isotype was defined as the mean+2SD of the total human and monkey pre-immune antigen signals.

Of the 139 IgG targets recognized by humans, 111 were also recognized by *Aotus* (p=3.317E-32; Figure 5B). Similar to the IgG profile, there was also significant overlap of the IgM response. The *Aotus* sera recognized 23 of 25 IgM targets that was also recognized by human (p=6.038E-06). In addition to the overlap between the human and *Aotus* profiles, the *Aotus* samples recognized a significant number of antigens that were not recognized by the human samples (Figure 5B). Specifically, 181 antigens were recognized by IgG, and 386 antigens were recognized by IgM with the *Aotus* sera but not with the human samples (Figure 5B). Also, the magnitude of the signals for each antigen recognized by IgG and IgM was typically increased compared to the human samples (Figure 5A).

In stark contrast to the IgG and IgM profiles, the IgA response between the *Aotus* and humans was different. The intensity of the IgA response in the human samples was greater than the *Aotus* samples (Figure 5A). Moreover the *Aotus* sera recognized a total of 21 antigens with the human serum recognizing a total of 102, and there was minimal overlap between the IgA profiles of the two species (Figure 5B). The vigorous IgA response by humans undergoing CHMI was unexpected, and to our knowledge has not been reported previously. The IgA response by *Aotus* was confined to a single 'outlier' animal which recognized 120 target antigens. Of these 75 were also recognized by humans (p=1.47E-06) (data not shown).

4.5 Cross-laboratory comparison

We hypothesized that owing to the familiarity of most diagnostic laboratories with indirect immunofluorescence techniques, the multiplex platform developed here should be readily transferable with minimal training. To test the reproducibility of the multiplex probing protocol in different laboratories, we transferred identical materials, Standard Operating Procedure (SOP) and ArrayCAM® imager to four different laboratories across the United States: Case Western University (CW); University of California San Francisco (UCSF); University of Maryland Baltimore (UMB); Grace Bio-Labs (GBL) and compared the data with data generated at University of California Irvine (UCI) by linear regression analysis. All collaborators first scanned a pre-probed slide identically produced at UCI for familiarization with the imager. Each group then probed Pf200 arrays with an aliquot of the same serum sample in triplicate according to the SOP and probing plan depicted in Figure 2. Pairwise comparisons of the labs were performed by linear regression analysis. The correlation coefficients (R^2) from those comparisons are shown in Table 2. R^2 values (0.60–0.92) indicate that the data was reproducible in all four labs, and supporting wider deployment of this robust platform.

5.0 Discussion

In this study we have developed a simple protocol for simultaneous detection of three Ig isotypes from a single sample in a protein microarray immunoassay. Commercially available

secondary antibodies conjugated to Qdots® with peak emissions at 585, 655 and 800nm were used to probe a signature human sample known to have *P. falciparum*-specific IgG, IgA and IgM. We saw no cross-talk between channels (Figure 2). Thus, the system makes simultaneous detection of three isotypes feasible.

Competition of serum IgG and IgA for the same antigen could potentially affect detection of either isotype in multiplex or singleplex assays. However, both are the product of class-switching, and both undergo affinity maturation through somatic hyper mutation.[16–18] Thus any competition between IgG and IgA is unlikely to significantly affect the detection of either; examples of asymmetrical competition between IgG and IgA are also scant.[19] In contrast, IgM, which is produced prior to class switching and affinity maturation, is usually out-competed for binding to antigen by other Ig isotypes at higher titer and affinity, such as IgG remaining from a previous exposure. This is resolved in the ELISA format by using an IgM capture (MAC ELISA) or sandwich assay, which is the assay of choice for serodiagnosis of several different acute virus infections,[20–25] or by removal of IgG with an anti-IgG reagent. For the array format used here, we have used an anti-IgG reagent to block potential competition from IgG. For the particular sample tested (Figure 4), removal of IgG reduced non-specific IgM and IgA background reactivity against many antigens while detecting IgM and IgA. IgG binding to antigens may provide a scaffold for IgM and IgA binding. Thus, this reagent can be used routinely to reduce background and improve recognition of IgM and IgA specific antigens. Interestingly, out-competition of IgM by IgG or IgA may be associated with specific protein or peptide antigens. For example, antibodies to glycans appear to behave differently, with IgM out-competing IgA and IgG in a microarray assay, possibly through the enhanced avidity provided by pentameric IgM.[26]

Nonhuman primates (NHPs) are ideal animal models for the study of malaria due to their genetic and physiological similarities with humans and ability to recapitulate pathology commonly seen in malaria patients.[27–29] New World monkeys are particularly useful for such studies because they can be infected with human malaria parasites such as *P. falciparum* and *P. vivax*, which makes these NHPs ideal models for evaluating vaccine candidates as well as confirming or evaluating specific observations made from human studies related to immunity.[9,14,30,31] Here, the protein microarray immunoassay was utilized to evaluate and compare the response of *Aotus* and humans after infection with *P. falciparum*. Overall, the results support that this platform is useful for the simultaneous evaluation of antigen-specific IgG, IgM, and IgA antibody responses when using *Aotus* monkeys for malaria research. One of the drawbacks of using New World Monkey models of malaria is the paucity of commercially-available assays and reagents; thus, the development of this assay enhances the utility of this animal model. This assay is an excellent addition to the armamentarium of immunological tools available for evaluating serological responses in *Aotus* monkeys for immunological investigations as well as for vaccine studies.

There was significant overlap of the antigens recognized by IgG and IgM in both the human and *Aotus* samples, demonstrating that the *Aotus* response was similar to the antibody response of humans infected with *P. falciparum*. Despite overlap, the *Aotus* recognized more antigens and with greater intensity for both IgG and IgM compared to the sera from the CHMI. The difference in the intensity and breadth of the IgG and IgM response is likely due

to two reasons. First, the *Aotus* samples used in this study were from a vaccine trial. The animals were immunized and then challenged with FVO strain of *P. falciparum* and, then, re-challenged with the virulent Malayan CAMP strain. Therefore, they had already experienced a prior infection, which would result in the formation of immunological memory that would respond to the subsequent infection with a heterologous strain of the parasite. Secondly, in contrast to individuals in the CHMI study, the *Aotus* were parasitemic when the samples were collected. Thus, the lower number of targets recognized by IgM in human samples could be due to the lack of parasitemia and shorter half-life of IgM in the plasma following exposure compared to other isotypes.

Interestingly, the IgA profiles were strikingly different between humans and NHPs. IgA is produced after class-switch recombination, and there are specific signals such as cytokines and receptor-ligand interactions that stimulate class switching to IgA.[29] The signals that direct class-switching are influenced by the location where a pathogen exposure and/or replication occur. For example, infections that occur at mucosa or in the skin tend to produce an IgA oriented response against pathogens whereas a systemic infection typically is skewed towards IgG. Taking this information into account, the differences in the IgA profiles between the humans and *Aotus* could be due to differences in the challenge protocols utilized in each study. The *Aotus* were inoculated intravenously with iRBC whereas the CHMI challenge was carried-out by mosquito bite. We speculate that while the mosquito is probing in the dermis, the release of salivary gland proteins, commensal microflora, etc., could result in signals to class-switch to IgA whereas a systemic initiation via inoculation of infected red blood cells results in class switching to IgG. Alternatively, challenging with sporozoites versus iRBCs may also influence the dynamics of class-switching. Nonetheless, the factors that influence class-switching during malaria should be further investigated in the future to inform vaccine design and understanding of antibody responses.

Protein microarrays are a useful tool to identify immunodominant and serodiagnostic antigens against infectious agents in humans and animals.[32–34] In human clinical trials protein microarrays have identified vaccine antigen candidates associated with protective immunity,[35–38] and they have also been used to quantify worldwide seroprevalence of infectious agents.[13,39–41] Although the specimens for these studies come from dozens of laboratories and clinical sites the world over, probing, quantification and analysis of them has been done in a few specialized labs with instrumentation resources and analysis experience. We have published results showing that probing of specimens can be done reproducibly with different people at clinical locations wherever the samples are collected by following a simple protocol, so probing is not a bottleneck.[38,42] More widespread deployment of the platform is limited by the lack of a robust and affordable instrument and accessible analysis software. In order to bridge the technology gap, we investigated the application of Qdot®-labeled secondary antibodies together with a CMOS (complementary metal-oxide semiconductor) camera microarray imager, and confirmed high levels of reproducibility of the platform in five independent laboratories in a cross-validation experiment.

The protein microarray platform allows the screening of immune sera for vaccine and serodiagnostic antigens at the proteomic level that is cost-effective, high throughput and

sample-sparing. Currently, routine serological tests such as ELISAs are concerned only with a very narrow window into the potential pathosphere and are an inefficient use of patient samples. Yet, serodiagnostic panels for multiple pathogens exist, such as a ‘fever panel’, ‘rash panel’, or ‘diarrhea panel’, and can be custom designed using any combination therein. The broader concept of routinely interrogating the whole immunome is currently an unmet opportunity, limited only by the technology used. Gene expression arrays of thousands of genes are already familiar tools for transcriptomics and are making inroads into diagnostics in cancer,[43] neuropathies and autoimmune disease,[44,45] while point-of-care (POC) qPCR-based diagnostic devices are becoming reality (UbiquitomeBio.com). We envision a similar paradigm shift in antibody-based diagnostic practice, from single tests or panels of tests, to a true multiplex platform in which serological data for hundreds or thousands of infectious agents, or biomarkers of autoimmune disease, cancers and other ailments, will be obtained simultaneously from a single sample. For this to happen, serological tests have to be multiplexed and miniaturized. Thus, where the ELISA obtains information on antibodies to a single antigen, the microarray can reveal antibody levels to hundreds or thousands of antigens simultaneously from the same volume of sample (0.5–1μl serum/test). Increasing the number of pathogens screened by conventional tests requires a separate test for each pathogen. In contrast, arrays are infinitely scalable; larger arrays can be produced with correspondingly more diagnostic power within the same, single test. Here we have described a simple protocol for the simultaneous detection of three human Ig isotypes from the same sample on a single array. This effectively triples the data generated with no increase in resources (serum sample or arrays) used. Combined with the ArrayCAM® imager, which replaces the cumbersome and expensive confocal laser scanners used previously, the array platform can now be deployed in any lab worldwide, particularly in endemic areas where multi-pathogen arrays could be applied to defining seroprevalence in populations, monitoring disease outbreaks and monitoring the effect of control measures such as vaccines, as well as for multi-pathogen POC screening.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

CDC	Centers for Disease Control and Prevention
CHMI	controlled human malaria infection

Ig	immunoglobulin
iRBC	infected Red Blood Cells/ erythrocytes
IVTT	in vitro transcription/translation
NHP	Nonhuman primates
Pf	*Plasmodium falciparum*
POC	point-of-care
Qdot®	quantum dots
RT	room temperature
T-TBS	TBS-0.05% Tween 20

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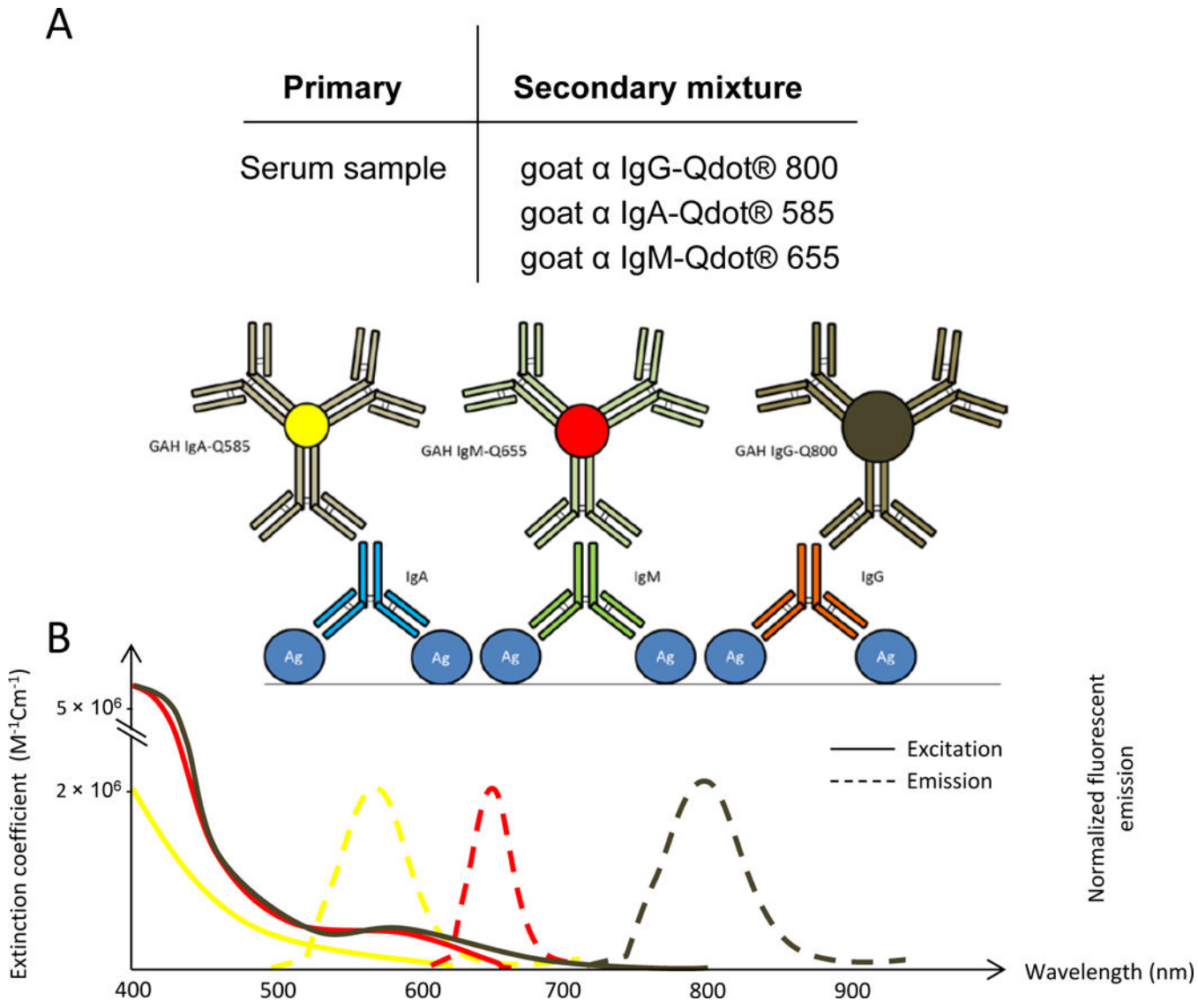

Figure 1. Multiplex antibody isotype profiling using Quantum dot immunofluorescent protein microarrays.

(A) Antigen-reactive immunoglobulin A, G and M in the serum (diluted 1:100 in blocking buffer) on the proteome microarray chip is specifically detected by Qdot®-labelled secondary antibodies against IgA (GαH IgA-Qdot® 585), IgG (GαH IgG-Qdot® 800) and IgM (GαH IgM-Qdot® 655). Several secondary antibodies may bind to each of Qdot® particles; the image shows approximate three of antibodies per particle (ThermoFischer Scientific). (B) Schematic representation of excitation and emission profile for the three Qdots® (Qdot® 585, Qdot® 655 and Qdot® 800) used in the multiplex study.

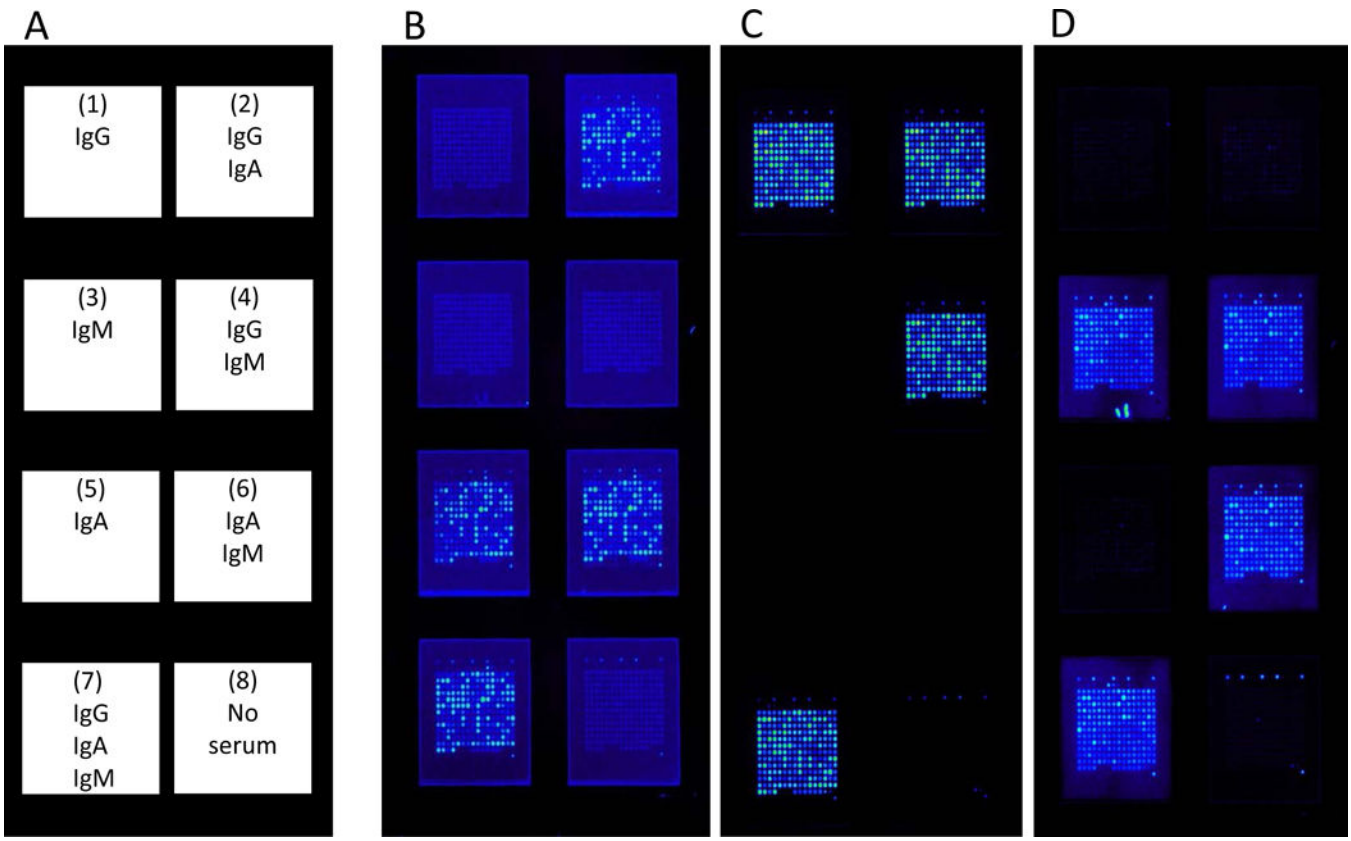

Figure 2. Detection of IgA, IgG and IgM immune response in an individual serum specimen participated in controlled malaria challenged study [7,8] in singleplex, duplex and triplex combinations.

Plasmodium falciparum array containing 192 antigens (Pf200) was used in this study. Goat anti human IgA labelled with Qdot® 585, goat anti human IgG labelled with Qdot® 800 and goat anti human IgM labelled with Qdot® 655 were used according to the plan (A), to capture the bound IgA, IgG and IgM, respectively. Detection was carried out by conjugated dyes. Images acquired using ArrayCAM® with 435±15 nm diode laser as excitation source by using different bandpass filters to pass specific spectral profile max at 585 nm (B), 800 nm (C) and 655 nm (D), respectively. Optimal image acquisition setting was used for each type of Qdot®. All filters pass distinct spectrum and no spillover was observed.

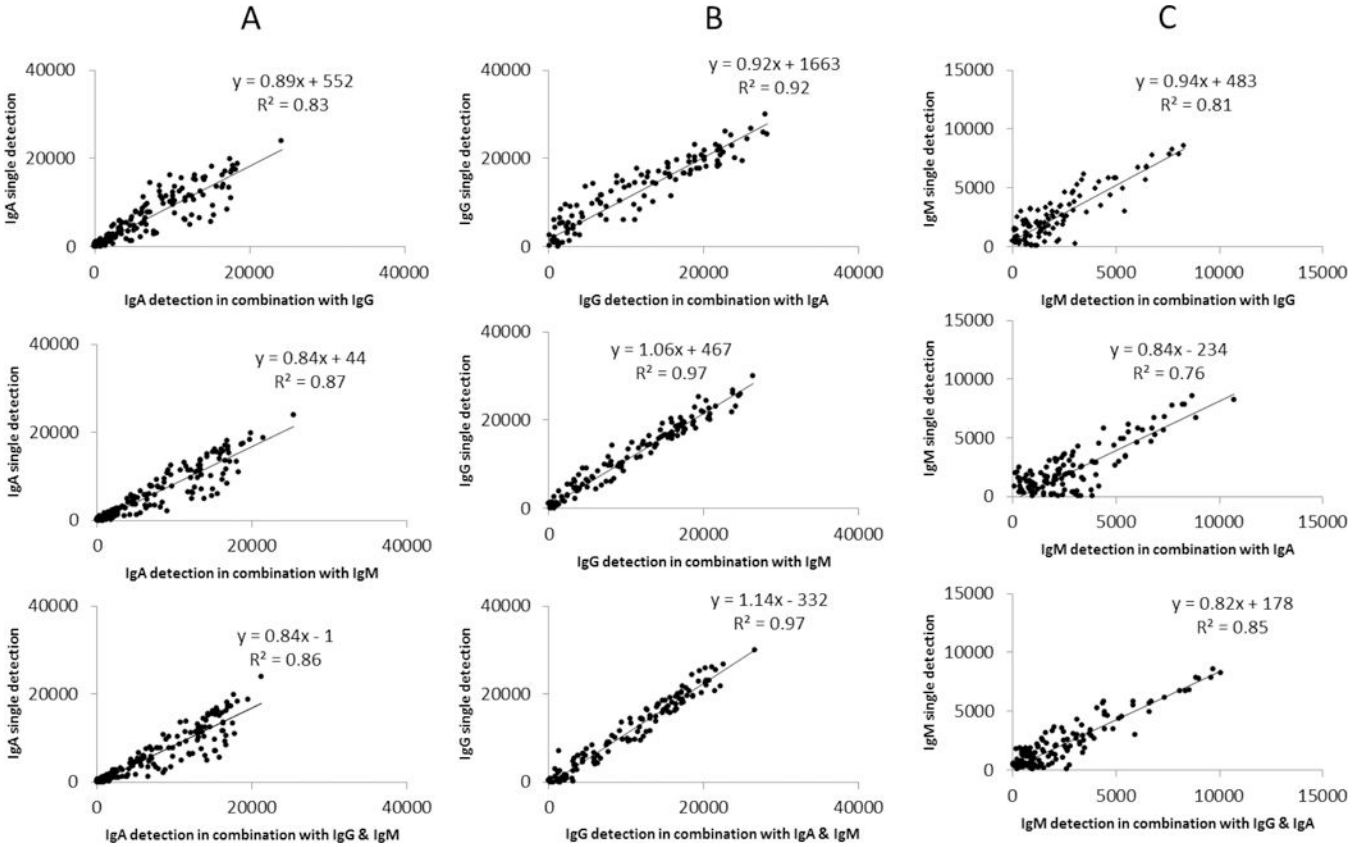

Figure 3. Comparison of antibody isotype detection in singleplex versus duplex and triplex combination with other antibody isotypes.

All detection combinations were carried out as described in figure 2. Scatter plots were generated by plotting data obtained from singleplex vs duplex and triplex combination with other isotypes for IgA (A), IgG (B) and IgM (C). Data are raw signal values with *E. coli* background signals subtracted.

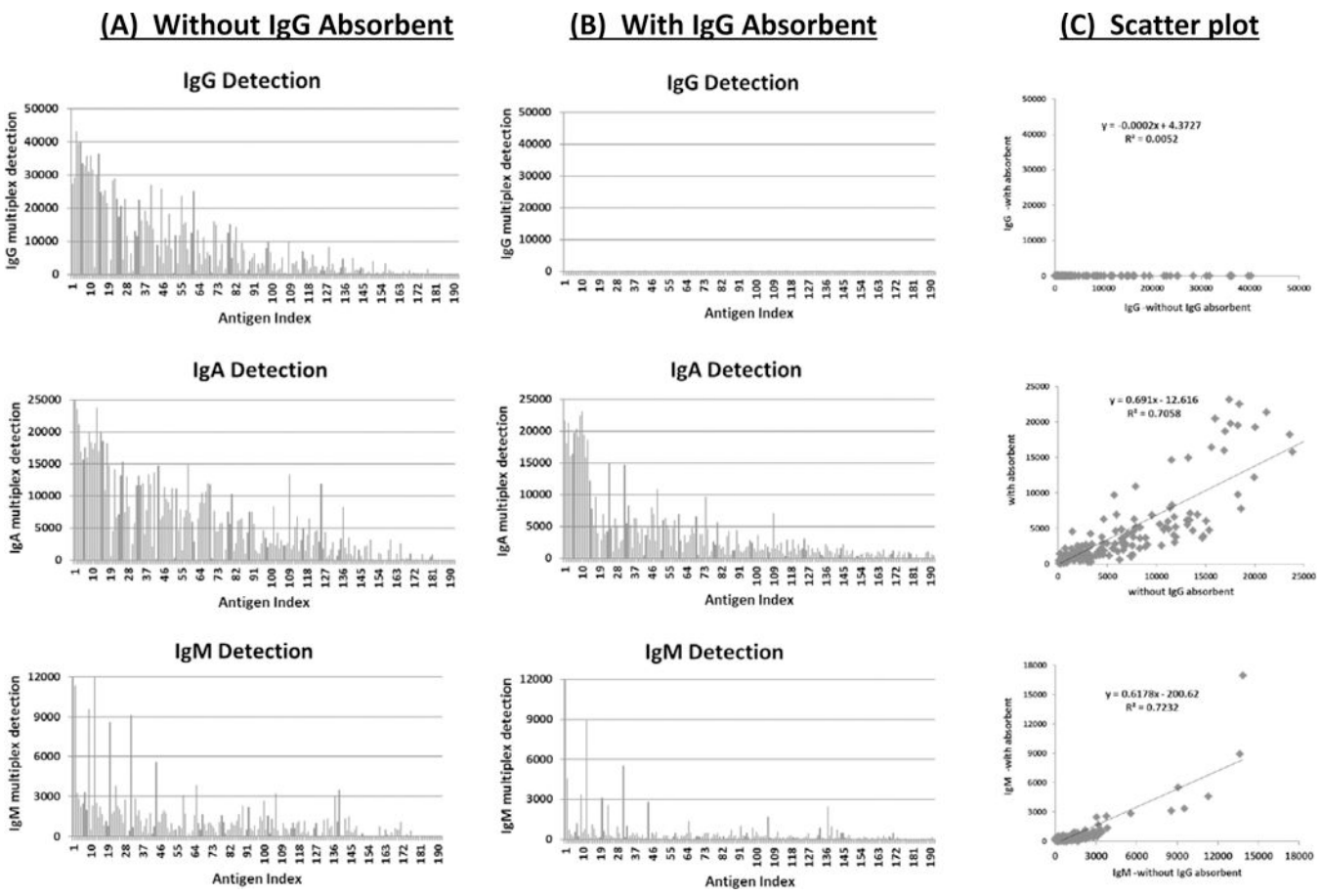

Figure 4. Comparison of IgG, IgA and IgM detection in triplex in the presence and absence of IgG removal reagent.

An individual serum specimen was probed in the presence and absence of IgG absorbent on the array and each isotype was detected by the respective secondary Antibody–Qdot® conjugate in triplex combination as described in figure 2. Raw data signals from all antigens were plotted in the absence of IgG absorbent (A), and presence of IgG absorbent (B). Their scatter plots were also generated (C).

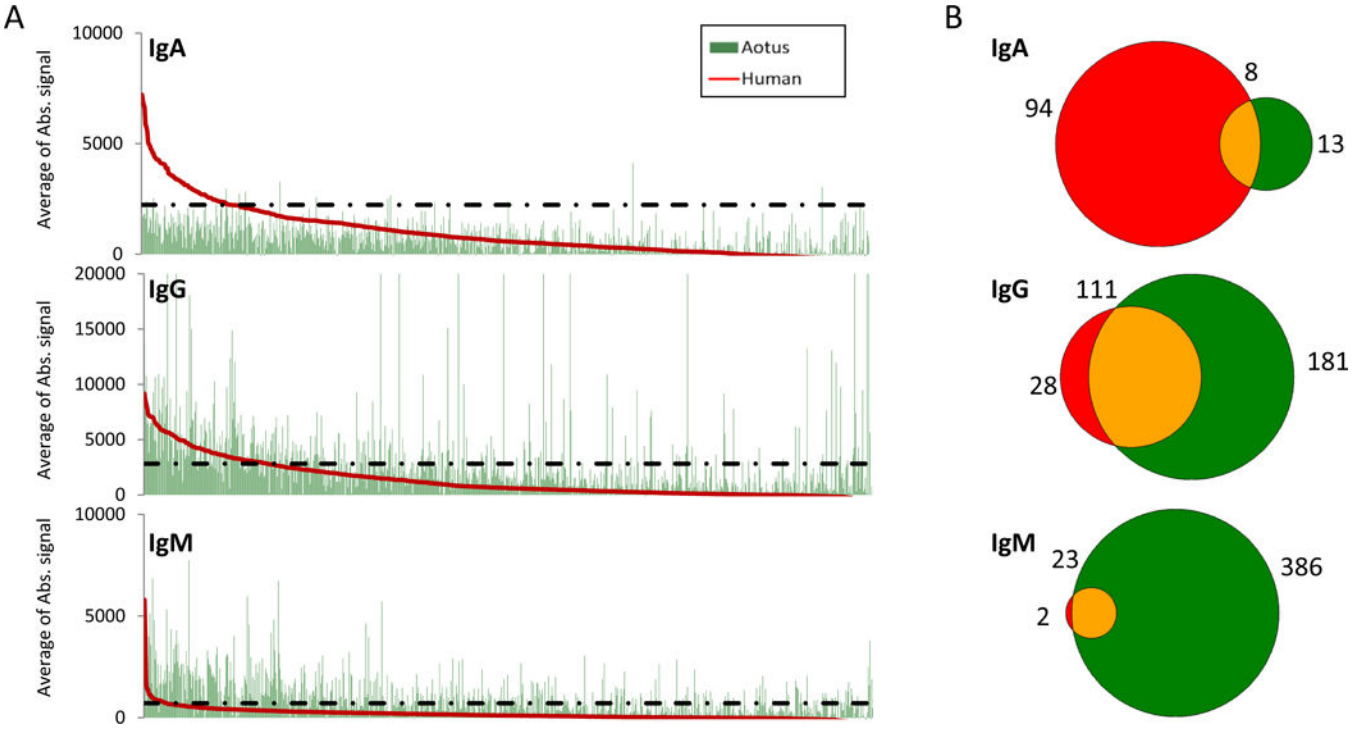

Figure 5. Demonstration of the antigen reactivity signals associated with human and *Aotus* malaria challenge study using multiplex protein microarray.

The *Plasmodium falciparum* arrays (Pf250 and Pf900) were probed with sera from two independent challenge studies on human (*n= 11*)[7,8] and Aotus (*n= 8*). Sera obtained from all individuals on pre-challenge and on 4-week post challenge. IgA, IgG and IgM were visualized by using a cocktail of direct immunofluorescence anti IgA, IgG and IgM antibodies in multiplex manner (see Materials and Methods). Array raw data were normalized by subtracting the median of 35 IVTT reaction protein spot intensity from antigen signals. Signals from pre-challenge samples were also subtracted from their post-challenge counterpart. (A) Average of absolute signals for each antibody isotype were plotted for each antigen and sorted from highest to lowest according to their reactivity with human sera. Average of total signals from pre-challenged samples in both human and aotus study plus two times of its standard deviation were considered as cut-offs for each isotype. (B) Venn diagrams were plotted to compare the number of reactive antigens in human with autos challenge studies. Numbers of exclusive markers as well as common ones were indicated for corresponding areas.

Table 1.

Specifications of ELISA assay compared to protein Microarray assay.

	ELISA	Microarray		
Format	**96-well**	**3-pad**	**8-pad**	**16-pad**
Number of antigens/assay	1	4500	1200	289
Total amount of antigen (ng/well or spot)	500	1	1	1
Working volume (μl/well or pad)	50	400	200	100
Serum volume (μl/assay) 1:100 dilution	1	4	2	1
Serum volume (μl/antigen)	0.5	0.0009	0.0017	0.0034
Approximate assay duration (h)	5	5	5	5
Average number of antigen studied per assay per hour	0.2	900	240	58.7

Note: the surface area of a well in 96-well plate and a pad from a 16-pad slide area are the same.

Table 2.

Cross-lab comparison validation study. The collected data were compared to the results obtained at UCI lab.

	CW	UCSF	UMB	GBL
	IgA	IgA	IgA	IgA
R^2	0.78 ± 0.02	0.90 ± 0.01	0.92 ± 0.01	0.91 ± 0.01
	IgG	IgG	IgG	IgG
R^2	0.60 ± 0.01	0.87 ± 0.09	0.81 ± 0.05	0.80 ± 0.05
	IgM	IgM	IgM	IgM
R^2	0.64 ± 0.01	0.89 ± 0.02	0.89 ± 0.01	0.81 ± 0.03