

Serum Microarrays for Large Scale Screening of Protein Levels^{*S}

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There is a great need for comprehensive proteomic analysis of large patient cohorts of plasma and serum samples to identify biomarkers of human diseases. Here we describe a new antibody-based proteomic approach involving a reverse array format where serum samples are spotted on a microarray. This enables all samples to be screened for their content of a certain serum protein in a single experiment using target-recognizing antibodies and fluorescently labeled secondary antibodies. The procedure is illustrated with the analysis of the IgA levels in 2009 spotted serum samples, and the data are compared with clinical routine measurements. The results suggest that it is possible to simultaneously screen thousands of complex clinical serum samples for their content of the relative amount of specific serum proteins of clinical relevance. *Molecular & Cellular Proteomics* 4:1942–1947, 2005.

The development of DNA microarrays has had an enormous impact in the research field of functional genomics where it has enabled large scale global analysis of whole genomes and transcriptomes. The developed methodology and technology have now become an established and relatively mature technique in terms of available instrumentation for production and analysis as well as commercially available premade microarrays, robust experimental protocols, and the long range of available tools and software for data analysis. Naturally there have been large efforts to continue the development of the microarray format with similar approaches for global protein analysis where great potential can be envisioned.

The microarray technology makes it possible to analyze a large number of proteins within a small sample volume in a single experiment or with a reverse set-up analyze a limited number of proteins in many samples. The basic principles for highly sensitive “microspot” ligand binding assays were described by Ekins (1) and Ekins and Chu (2), who showed that small amounts of capture molecules in microspots can detect

low concentrations of the analyte with high accuracy and sensitivity.

The protein microarray applications can be divided into two categories: functional protein arrays and protein profiling arrays. Functional protein arrays can effectively screen large quantities of proteins for biochemical activity, protein-protein interactions, protein-lipid interactions, protein-nucleic acid interactions, and protein-small molecule interactions. The arrays have been used to study activity of uncharacterized proteins (3), antibody specificity profiling (4), and immune response profiling. Protein abundance arrays are used to measure protein abundance and/or alterations (5). Lately there has been progress in microarrays printed with antigen and used for detection of circulating antibodies in clinical specimens (6–8). The protein profiling microarrays have also been used to measure the binding specificity of protein expression libraries (9, 10) and for protein profiling in cancer tissue (11).

Currently there are two main types of profiling microarrays: forward phase arrays and reverse phase arrays. In the forward phase array, the bait molecule is normally an antibody or antigen, and each spot will represent one type of bait molecule. In this format, one test sample is analyzed for several different analytes of interest, and the analytes are captured from the solution. The protein captured by the bait molecule can be detected by a secondary tagged molecule or by direct labeling of the analyte. The second format is a reverse phase protein (RPP)¹ array where the analytes are immobilized on the substrate. Each spot will then represent one test sample containing multiple analytes, and therefore many samples can be analyzed simultaneously under identical conditions, for any given analyte, using low sample volumes. A single detection molecule is used, and a single analyte is measured in each sample. This allows multiple samples to be analyzed under the same experimental conditions.

RPP microarrays have been used previously for profiling proteins in cancer and were initially described by Paweletz *et al.* (12) and Grubb *et al.* (13) for measurements of proteins relevant to apoptosis in malignant and normal prostate tissue. The technology has also been used to investigate defects in signaling in ovarian cancer tissue (14) and to profile proteins in

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¹ The abbreviations used are: RPP, reverse phase protein; IgAD, IgA deficiency; CV, coefficient of variation; C3, third component of complement.

cancer cells to screen compounds for anticancer activity (15, 16). RPP microarrays do not demand direct labeling of the analyte and do not utilize the two-site antibody sandwich. This decreases the experimental variability of the assay, but the biological variation within a complex sample must be taken into consideration. There are so far no publicly available reports, at least to our knowledge, where serum samples have been spotted for large scale screening of protein levels.

IgA deficiency (IgAD) (serum IgA concentration of <0.05 mg/ml) is the most common primary immunodeficiency in Caucasians with an estimated prevalence of one in 600 (17). The patients suffer from recurrent mucosal infections, allergies, and autoimmune diseases. In most immunodeficiency diseases, IgAD is included in the phenotype. Current techniques for quantification of IgA in serum are not suitable for large scale screening. To identify IgAD in a large number of samples, there is a need for a high throughput technique for measurement of IgA levels. We have therefore developed a novel serum microarray for screening of levels of IgA and other proteins in thousands of serum samples.

EXPERIMENTAL PROCEDURES

Generation of Serum Slides—The system was initially optimized with dilution series of serum, primary antibody (rabbit anti-human IgA; DAKO), and secondary antibody (Alexa Fluor 555 goat anti-rabbit IgG; Molecular Probes) using three serum samples with known IgA concentrations (0.07, 0.20, and 1.04 mg/ml). All three samples were diluted in 10-fold steps (1:10, 1:100, 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000) in $1\times$ PBS and loaded onto a 96-well plate in volumes of 50 μ l/well. The samples were subsequently arrayed onto 25×75 -mm SuperEpoxy slides (ArrayIt, Telechem) using an eight-pin and ring GMS 427 arrayer (Affymetrix). The spot to spot distance was set to 500 μ m, and the resulting spot diameter was ~ 200 μ m. Twelve identical blocks were printed on each slide with each block containing all three samples in all dilutions.

Slides were subsequently blocked with SuperBlock solution (Pierce) for 30 min and subsequently washed in $1\times$ PBS for another 5 min before being spun dry in a table centrifuge. A 16-well adhesive silicone mask (Schleicher & Schuell BioSciences) was attached to the slides, the primary antibody (4.6 mg/ml) diluted 1:5,000, 1:10,000, 1:20,000, 1:40,000, 1:60,000, and 1:100,000 in PBS, 0.1% Tween 20 was applied to adjacent wells, and the mask was sealed with a plastic lid. The mask was removed after 60 min, and the slides were washed for 10 min in PBS, 0.1% Tween 20 followed by two washes in PBS for 10 min each. Slides were subsequently incubated with the secondary antibody (2 mg/ml) diluted 1:5,000, 1:10,000, 1:20,000, 1:40,000, 1:60,000, and 1:100,000 in PBS, 0.1% Tween 20 for 60 min (one dilution per slide). The slides were then immersed in a wash bath (conducted under dark conditions to protect the light-sensitive fluorophore), and the same washing procedure followed as for the first incubation. The detection procedure was performed at room temperature, and all washes and incubations were carried out under gentle agitation. Slides were scanned in a G2565BA array scanner (Agilent) with the photo multiplier tube set to 100% for both channels and scan resolution 10 μ m.

IgA Screening—2009 serum samples with known IgA concentrations (using nephelometry) collected during 1999–2002 (determined at the department of Clinical Immunology, Karolinska Hospital, Huddinge, Sweden) were printed. The samples had been referred from patients with a history of increased susceptibility to infections. 1191

samples had normal IgA levels (0.7–3.65 mg/ml), 153 had increased IgA levels, and 665 had low IgA levels including 99 IgA-deficient samples (<0.05 mg/ml). All samples were kept at -20°C until used. The protocol used for optimization was also used in this screening. The serum samples were diluted 1:10 in PBS, the primary antibody was diluted 1:100,000 to a concentration of 46 ng/ml, and the secondary antibody was diluted 1:60,000 to a concentration of 33 ng/ml. To establish whether freezing and storage of serum samples could affect the results, we reanalyzed 32 from 1999 samples using nephelometry.

Third Component of Complement Screening—Thirty-five serum samples were collected at the department of Clinical Chemistry, Karolinska Hospital, Stockholm, Sweden during 2004 and were analyzed for the third component of complement (C3). Rabbit anti-human C3 complement (DAKO) (11.5 mg/ml) was used as primary antibody (dilution, 1:100,000 to 115 ng/ml), and Alexa Fluor 555 goat anti-rabbit IgG (Molecular Probes) (2 mg/ml) was used as secondary antibody (dilution, 1:60,000 to 30 ng/ml). The protocol developed for IgA was also used for detecting C3.

Data Analysis—The image analysis was performed with GenePix-Pro 5.1 (Axon Instruments) using the non-circular feature alignment. The median feature intensities were used, and the median of the intensities for all 2009 samples was utilized to scale the array intensities versus the measured IgA concentrations. A scaling factor was calculated individually for each replicated set of samples as the ratio between the median of the array intensities with the median of the concentrations. The average of the scaled values for 12 measurements, four slides with three replicates, is presented.

RESULTS

Optimization of the Method—To establish suitable concentrations of the primary rabbit anti-human IgA antibody and the secondary fluorescently labeled goat anti-rabbit antibody for the analysis of a wide range of IgA concentration, a criss-cross titration of serum and primary and secondary antibodies was performed. Sera from three patients, with IgA concentrations of 0.07, 0.2, and 1.04 mg/ml, were spotted in dilution series. Relatively high concentrations (in the range of 1:5,000–10,000 dilutions) of both primary and secondary antibodies were needed if serum dilutions higher than 1:10–1:100 should give significant fluorescence intensities, but then there was also a need for extensive washing procedures to avoid fluorescence cross-talk between spots. Despite extensive washing, however, the spot morphology was disturbed to such a degree that it would prevent high density spotting. Furthermore the 1:10 dilution showed the most prominent difference in intensity between high and low levels of IgA, and therefore subsequent screening was performed using this dilution. The influence of various combinations of primary and secondary antibody concentrations on 1:10 diluted sera is shown in Fig. 1, and the results indicate that a wide range of antibody dilutions can be applied to the protocol without compromising the result for detection of samples with low levels of IgA.

Limits of Detection—To establish the approximate limit of detection using the chosen serum microarray set-up, dilution series of the above mentioned three patient sera were spotted in a low density pattern, enabling the use of high concentrations of both primary and secondary antibodies. The resulting

FIG. 1. The effect of different combinations of dilutions of the primary and secondary antibodies. Sera from three patients were spotted in different dilutions, and the resulting fluorescence intensities are shown from sera diluted 10 times with various combinations of primary and secondary antibody concentrations. The IgA concentrations measured by nephelometry in the three patient sera were 0.07, 0.2, and 1.04 mg/ml, respectively for Patients 1, 2, and 3. Ab, antibody.

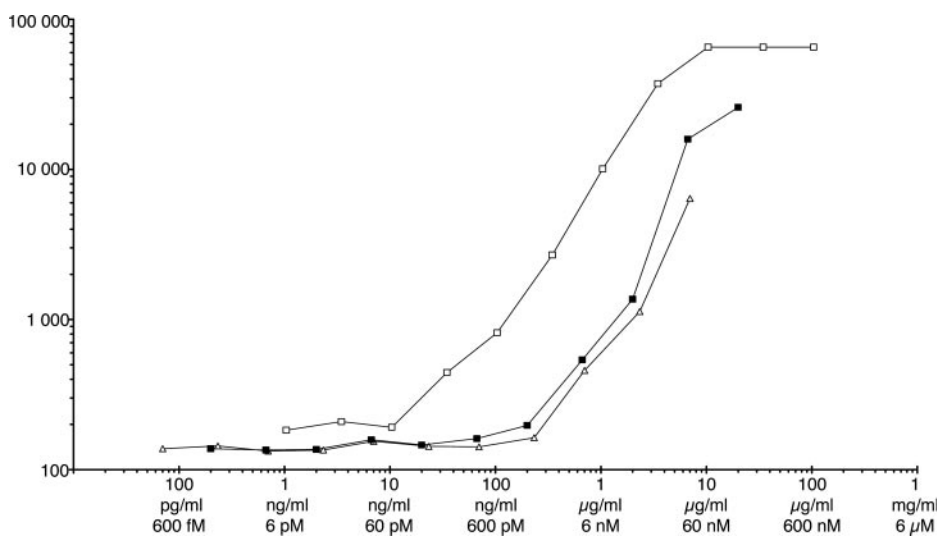
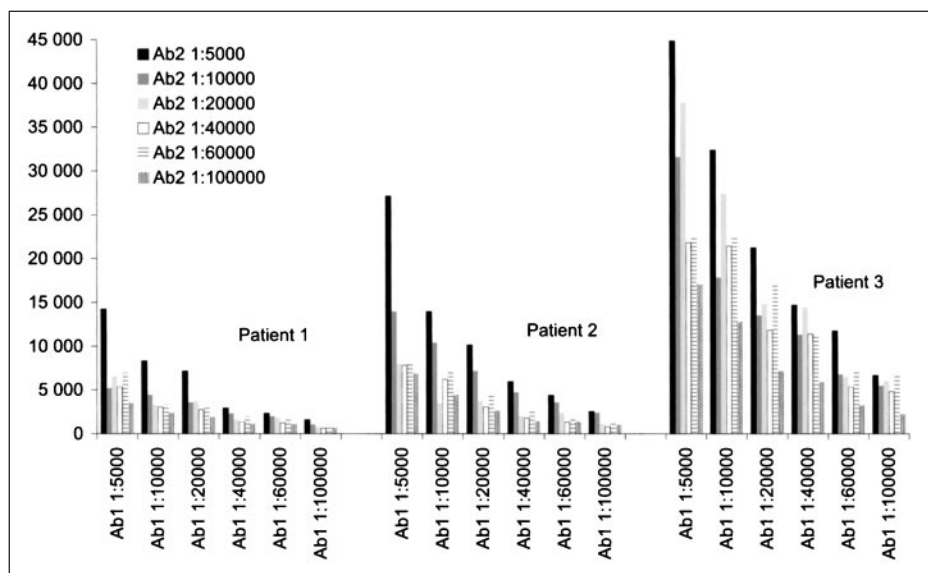


FIG. 2. Limits of detection. Spotted dilution series of sera from three patients were analyzed with high concentrations of primary and secondary antibodies. Plotted on the x axes are the concentrations of IgA in the diluted sera, and the detected fluorescence intensity is plotted on the y axes, both in log₁₀ scale. The clinically measured IgA concentration of Patient 1 (triangles) was 0.07 mg/ml, of Patient 2 (filled squares) was 0.2 mg/ml, and of Patient 3 (open squares) was 1.04 mg/ml.

fluorescence intensities for the three different sera are shown in Fig. 2, and the response curves indicate that the present limit of detection in spotted complex serum is below 1 µg/ml (approximately corresponding to 1 nM). It is noteworthy that one of the samples, for unknown reasons, displays a 10-fold higher sensitivity than the other two.

Influence of Freezing and Storage on IgA Levels—The normal range of serum IgA in adults is 0.7–3.65 mg/ml, and IgA deficiency is defined as IgA concentration of <0.05 mg/ml. The IgA concentrations of all 2009 clinical serum samples that were spotted onto the arrays had been determined by clinical routine nephelometric analysis (lower detection limit, 0.07 mg/ml) and radial immunodiffusion as described by Mancini *et al.* (18) was used for analysis of low level IgA samples (levels <0.07 mg/ml).

As the array was based on frozen serum samples, a reanalysis of 32 samples was performed with nephelometry to es-

timate variation in the analysis method for samples that had been exposed to one freeze/thaw cycle. The deviation was on average 12% for samples in the range of >0.07–4.9 mg/ml ($n = 22$), 31% for samples >5 mg/ml ($n = 3$), and 56% for samples with concentrations ≤0.07 mg/ml ($n = 7$). The large deviation for low concentrations is explained by the different limit of detection using the two routine analyses (0.02 versus 0.07 mg/ml). The linear regression coefficient (R^2) for the correlation between the two measurements for samples >0.07 was 0.89.

Screening of IgA Levels—After having established a relation between the fluorescence intensities and the serum and antibody concentrations and that the limit of detection is far below the level defining IgA deficiency (<0.05 mg/ml), screening of IgA levels in clinical samples was initiated. The 2009 serum samples were spotted in triplicate on epoxy-coated microarray slides as visualized in Fig. 3. The variation in neph-

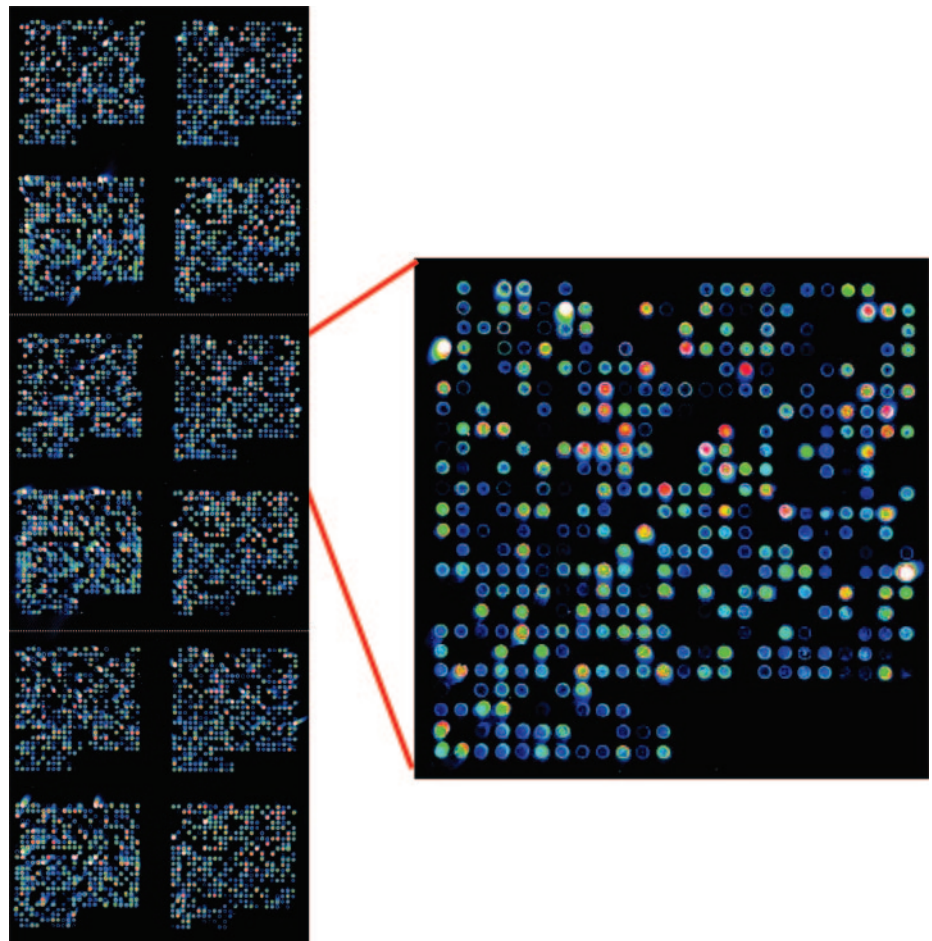


FIG. 3. Visualization of the fluorescence intensities of the serum array with 3×2000 spotted serum samples. The enlarged area corresponds to ~ 500 patient samples.

elometric determination of IgA concentrations increases significantly for high levels of IgA, and because those samples also are of less clinical relevance, the 45 samples with IgA concentrations >5 mg/ml were excluded from further analysis. To determine the coefficient of variation (CV) in general and to have a proper number of replicates, four different slides were analyzed, resulting in up to 12 individual measurements. The distributions of CV values for all samples are plotted in Supplemental Fig. 1. The variance was found to be higher for samples with concentrations ≤ 0.07 mg/ml (median CV was 29% ($n = 424$)) than for the rest (0.08–4.93 mg/ml) where the median CV was 10% ($n = 1540$) and relatively even distributed throughout the given concentration range. The median CV for all samples was found to be 15%, and the average was 19%.

To be able to compare the fluorescence intensities obtained from the serum microarrays with the IgA concentrations obtained from the routine clinical nephelometric analysis, the array data were scaled (see “Experimental Procedures”). A visual comparison of the data for the first 100 samples, in chronological order, is shown in Supplemental Fig. 2. The same high level of correlation was observed throughout the material. The results for all samples are visualized in Fig. 4

where the deviation between the two methods is plotted as the \log_2 ratio between the measurements versus the IgA concentration. There are in total 65 samples (3.3%) where the deviation is larger than 5-fold; 34 of these have higher values in the nephelometric analysis than on the array, and these samples displayed a significant higher variation (median CV of 29%) than the average. The 31 samples with higher values on the array all have a very low concentration (0.02 mg/ml as median value). The median CV for the array determination of those samples was 24%, which is lower than that for the samples with low concentration in general.

Screening of C3 Levels—To establish whether this method can be used for detecting proteins other than IgA, we analyzed the concentration of C3. C3 is the most abundant complement protein in serum (1–2 mg/ml) and plays an important role in mediated clearing of infections. C3 was chosen because its serum concentration is in the same range as that of IgA. 35 samples with known C3 levels were measured using the same procedure as for IgA. Rabbit anti-human C3 antibodies were used as primary antibody, and fluorescently labeled goat anti-rabbit IgG were used as secondary antibody. The same dilutions of serum and primary and secondary antibodies as when measuring IgA were used. The amount of

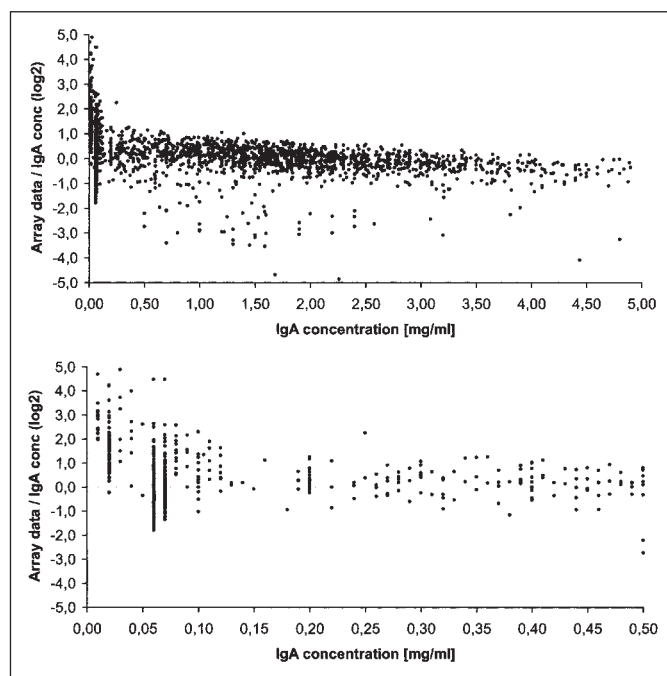


FIG. 4. **The correlation between serum microarray data and clinically measured IgA concentrations.** *a*, the \log_2 ratios between the scaled array data and the IgA concentrations are plotted versus the IgA concentrations. *b*, enlargement of the ratios for the lowest IgA concentrations.

C3 in the samples was measured previously using nephelometry. The correlation between the results obtained using protein microarrays and those obtained using nephelometry is shown in Supplemental Fig. 3.

DISCUSSION

To our knowledge no reports concerning reverse phase protein microarrays with spotted serum samples are publicly available. The potential of such a system, as described here, is huge because of the existing need for sensitive and robust methods for large scale screening of protein levels in serum. Here it has been shown that serum microarrays can detect IgA levels at least as low as $1 \mu\text{g/ml}$ when using the antibody dilutions applied, and even lower protein levels could probably be detected with this method if undiluted or concentrated samples and higher concentrations of primary and secondary antibodies were used. There are also several other options to further increase the sensitivity. If the sera were depleted of serum albumin, IgG, and other highly abundant serum proteins, the sensitivity would potentially increase at least a magnitude. The main issue would then be to develop an automated and robust sample preparation procedure. Alternative detection systems such as those based on thin film planar waveguides (19) and various signal amplification procedures would also increase the possibility to utilize the serum microarray format for analysis of low and very low abundance serum proteins.

Here we have simultaneously analyzed 2000 samples in triplicate on one single array slide, and the capacity for simultaneous analysis of a very large amount of serum samples in an array format can be extended beyond those 6000 spots to at least double the amount. This means that an even further optimized system has the potential to enable large scale screening of huge clinical sample collections. One possible application is to analyze the IgA levels in newborns. IgA deficiency is included in the phenotype in a majority of immunodeficiencies, and screening of IgA at birth could be used for early identification of most of the known immunodeficiency diseases.

A relevant concern with different immobilization chemistries for complex samples is whether the proportion of all proteins is kept constant compared with when they are in solution. An advantage in that context is to use a reactive surface that binds proteins covalently in several different ways, such as the epoxide, which can utilize both primary amines and hydroxyl- and thiol-based attachment. Such general coupling reduces the risk of introducing bias in the protein composition proportions, which could occur if the surface has a preference for certain competing proteins. However, further investigations are needed to analyze the degree of bound proteins compared with the applied amount, but here there are no indications that this would be a problem, and as long as the immobilization efficiency is equal for all samples it will not be an issue that could not be handled with a proper data normalization and standard curve calibration.

The relatively low overall median CV of the array screened samples at 15% and only 10% for the samples with IgA concentrations above the deficiency range indicates a satisfying robustness comparable to other methods. The average CV for nephelometry is 5–10%, and antibody-based immunoassays in general have an average CV of 10–15%. The samples with the highest deviation between the previously determined concentrations and the array results have in general either a very low concentration or a significantly higher variation between replicates. There are no obvious reasons for the high variation and thereby high deviation for some samples. Further investigations are needed to clarify whether the compositions of those samples deviate from the average. To determine concentrations in subsequent studies where the concentrations are unknown, there will be a need to establish proper standard curves and also to introduce a quality filtering based on variance.

By comparison with nephelometry, a routine fluid phase immunoprecipitation technique currently used for quantification of serum proteins, and the radial immunodiffusion method used for low level analysis that is a slow and relatively costly technique not suitable for large scale screening, the serum microarrays offer several major improvements. Only a few microliters, depending on spotting equipment, of serum is adequate for hundreds of microarrays, and each slide can then be used to screen thousands of samples for the content

of a specific serum protein. Furthermore the reagent cost is drastically reduced because of the very large amount of samples analyzed simultaneously. In conclusion, the serum microarray format has, as shown here, great potential to become a very important tool for serum screening. If the serum microarray format is designed to contain sera from patients with certain diseases as well as normal sera and is combined with a program for high throughput generation of antibodies, great possibilities arise for large scale biomarker discovery strategies.

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§ The on-line version of this article (available at <http://www.mcponline.org>) contains supplemental material.

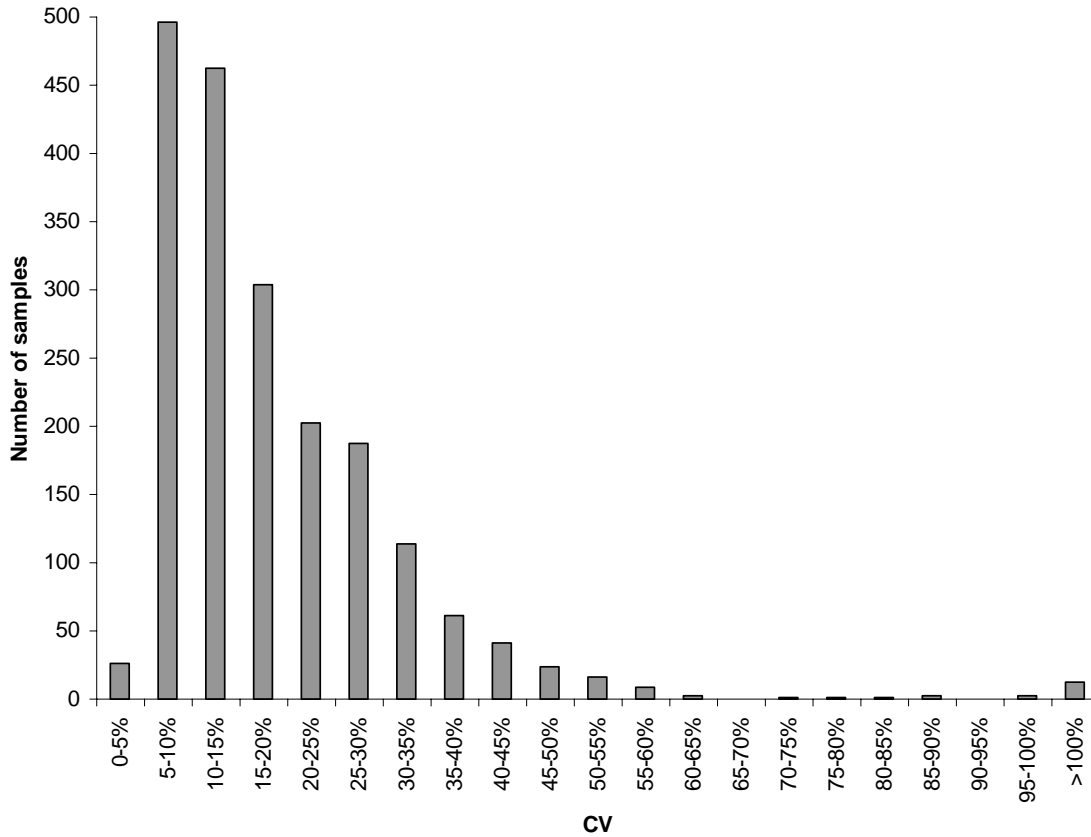
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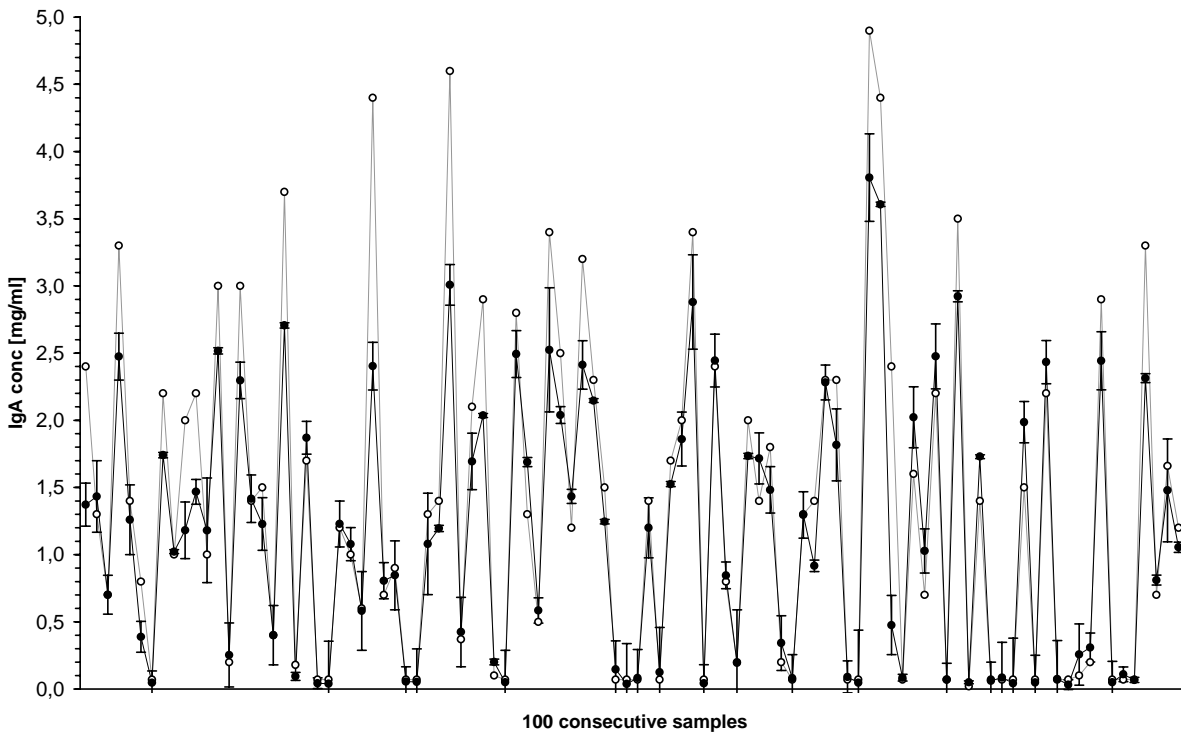
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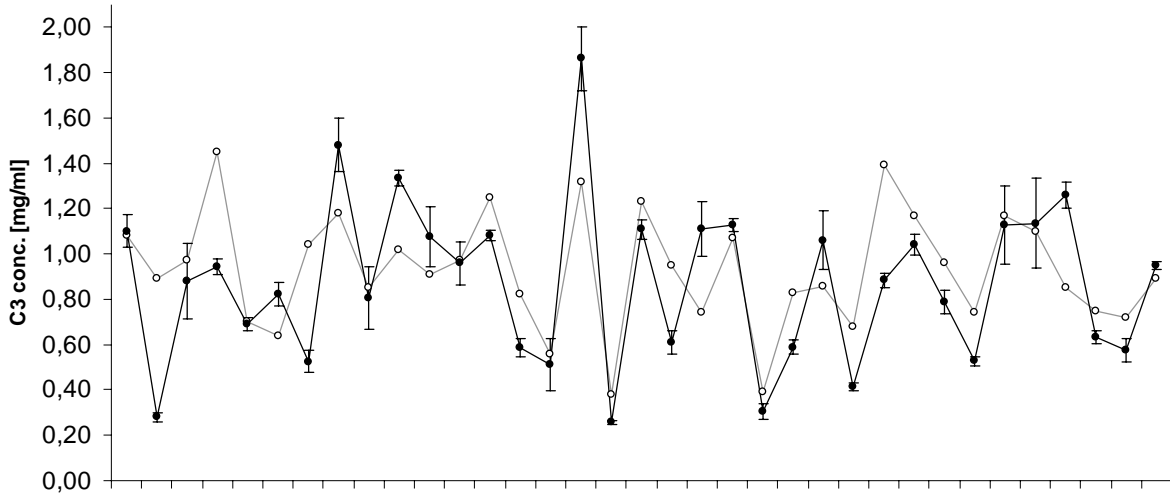
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Supplementary figure 1. The distribution of the coefficient of variation (CV) based on 12 individual measurements, i.e. four slides with triplicates, is plotted against the IgA concentration for 1964 samples (<5.0 mg/ml).



Supplementary figure 2. A comparison of the first 100 samples in consecutive order. The scaled array data are shown with filled circles and black line and standard deviation bars and the IgA concentrations are shown in grey with open circles.



Supplementary figure 3. Array results from the analysis of the levels of complement factor 3 (C3) in 35 spotted serum samples. The scaled array data are shown with filled circles and black line and standard deviation bars and the clinically measured C3 concentrations are shown in grey with open circles.