

Expanding Assay Dynamics: A Combined Competitive and Direct Assay System for the Quantification of Proteins in Multiplexed Immunoassays

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BACKGROUND: The concurrent detection and quantification of analytes that vary widely in concentration present a principal problem in multiplexed assay systems. Combining competitive and sandwich immunoassays permits coverage of a wide concentration range, and both highly abundant molecules and analytes present in low concentration can be quantified within the same assay.

METHODS: The use of different fluorescence readout channels allows the parallel use of a competitive system and a sandwich configuration. The 2 generated assay signals are combined and used to calculate the amount of analyte. The measurement range can be adjusted by varying the competitor concentration, and an extension of the assay system's dynamic range is possible.

RESULTS: We implemented the method in a planar protein microarray-based autoimmune assay to detect autoantibodies against 13 autoantigens and to measure the concentration of a highly abundant protein, total human IgG, in one assay. Our results for autoantibody detection and IgG quantification agreed with results obtained with commercially available assays. The use of 2 readout channels in the protein microarray-based system reduced spot-to-spot variation and intraassay variation.

CONCLUSIONS: By combining a direct immunoassay with a competitive system, analytes present in widely varying concentrations can be quantified within a single multiplex assay. Introducing a second readout channel for analyte quantification is an effective tool for spot-to-spot normalization and helps to lower intraassay variation.

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Protein microarrays provide a convenient format for multiplexing immunoassays. These miniaturized assay systems offer the possibility of monitoring the abundance of larger sets of proteins in a single reaction while keeping the required amount of sample material low. Different configurations of array-based immunoassays are in use, but the 2 most common configurations are (a) reversed-phase protein microarrays and (b) forward-phase assay systems (1). In reversed-phase assay systems, arrays of samples are generated, and up to several hundred immobilized samples are probed in parallel with one analyte-specific detector molecule on the array. Probing a larger number of arrays with sets of specific antibodies can produce a complex picture of the proteins and their modified forms that are present in sets of samples (2–4).

Forward-phase assays use protein microarrays with up to hundreds of immobilized capture molecules that interact specifically with various analytes in a single sample (5, 6). Signals are generated on the spots of capture molecules by labeling the protein in the analyzed sample during sample preparation and detecting the captured analyte directly on the protein microarray (7, 8). The most common approach uses a mixture of labeled secondary antibodies to generate detectable signals. Because of the cross-reactivity between capture and detection antibodies, matched antibody pairs are required for this parallelized and miniaturized sandwich ELISA, and the degree of multiplexing appears to be limited to approximately 40 analytes (9). However, properly implemented micro-ELISAs of this type have been shown to be highly specific and sensitive tools for analyte quantification (10) and are being developed into important assay systems for basic and clinical research.

The multiplex detection of autoantibodies with miniaturized immunoassays (11, 12) was developed

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into the first commercially available products based on microarray technology, and different systems have been introduced into the marketplace (BioPlex 2200 ANA Screen, Bio-Rad Laboratories; AtheNA Multi-Lyte System, Zeus Scientific; CombiChip, Whatman). These systems benefit from increased throughput and from the potential for testing a wide variety of autoantigens in a single assay, which lowers assay times and costs. Nevertheless, assay systems that give diagnostically relevant information are very demanding, and although several interesting approaches have recently been developed (13–15), standardization and validation are still required before such assays can be used in clinical diagnostics.

One of the obvious problems for multiplexed assays is that analytes can occur over large concentration ranges in biological samples. Serum concentrations can vary from the grams-per-liter range for such proteins as albumin and IgG to <1 ng/L for important effector molecules such as cytokines (16). This enormous range of more than 10 orders of magnitude presents a challenge. Depending on the sample dilution, analytes of interest may be present at concentrations below the limit of detection or, conversely, be so high as to saturate the detection system. Only through the use of sets of dilutions can measurement ranges of this magnitude be covered.

To address this problem, we set up a novel assay format that combines the principles of competitive and direct immunoassays into one system. Molecules present in high concentrations as well as those occurring at low concentrations can be quantified within the same assay. To obtain information on the performance of the system, we implemented a multiplexed autoimmune assay on a planar microarray platform in which we simultaneously monitored the reactivities against 13 different human autoantigens and quantified the amount of human IgG present in the same sample.

Materials and Methods

PRODUCTION OF MICROARRAYS

Human autoantigen protein microarrays were generated as described by Kramer et al. (17). The array substrate was aldehyde-coated glass slides (CEL Associates). Thyroglobulin, thyroid peroxidase, glutamic acid decarboxylase, centromere antigen B, a 100-kDa autoantigen associated with polymyositis and scleroderma (PmScl100), a 70-kDa antigen associated with scleroderma (Scl70), double-stranded DNA, Sjögren syndrome antigen B, ribonucleoproteins A and C, Sjögren syndrome antigen A proteins 52 and 60, and Sm autoantigens were kindly provided by Phadia (Freiburg, Germany). Goat antihuman IgG was purchased from Dianova. These autoantigens were each

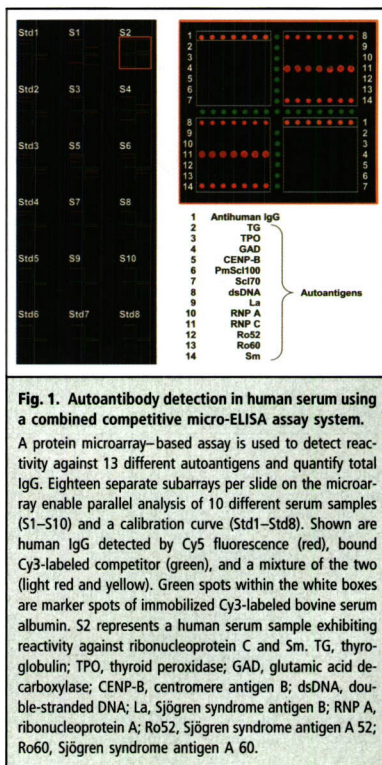
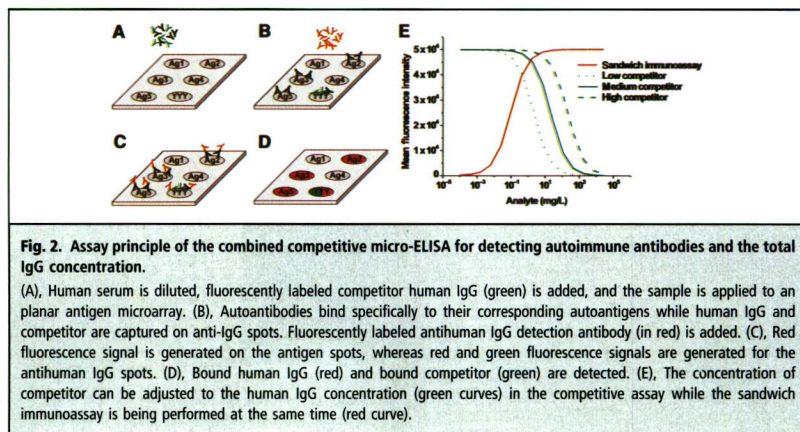


Fig. 1. Autoantibody detection in human serum using a combined competitive micro-ELISA assay system.

A protein microarray-based assay is used to detect reactivity against 13 different autoantigens and quantify total IgG. Eighteen separate subarrays per slide on the microarray enable parallel analysis of 10 different serum samples (S1–S10) and a calibration curve (Std1–Std8). Shown are human IgG detected by Cy5 fluorescence (red), bound Cy3-labeled competitor (green), and a mixture of the two (light red and yellow). Green spots within the white boxes are marker spots of immobilized Cy3-labeled bovine serum albumin. S2 represents a human serum sample exhibiting reactivity against ribonucleoprotein C and Sm. TG, thyroglobulin; TPO, thyroid peroxidase; GAD, glutamic acid decarboxylase; CENP-B, centromere antigen B; dsDNA, double-stranded DNA; La, Sjögren syndrome antigen B; RNP A, ribonucleoprotein A; Ro52, Sjögren syndrome antigen A 52; Ro60, Sjögren syndrome antigen A 60.

immobilized in 14 replicate spots per array. Bovine serum albumin labeled with the fluorescent cyanine dyes Cy3 and Cy5 was included in the array as marker spots to facilitate orientation (Fig. 1). We generated 18 subarrays per slide in a layout that allowed an adapter (Microarray Microplate; TeleChem International) to separate the subarrays. This device adapts the generated microarrays to the microtiter plate format to facilitate the use of liquid-handling robots.

Capture molecules were printed with a noncontact spotting system (BioChip Arrayer; PerkinElmer) under clean room conditions with controlled temperature (20 °C) and humidity (65%). Approximately 300 pL of liquid was deposited per spot (spot diameter, 130–210 μ m). Differences in size were due to the use of different buffers to dissolve the arrayed proteins. The pitch from



spot to spot on the x-axis and y-axis was set to 300 μm . After printing, we left the slides in a humid chamber overnight and in the morning blocked the slides with blocking buffer [15 g/L bovine serum albumin, 50 g/L low-fat dried milk, and 1 mL/L polysorbate 20 (Tween 20; Merck) in PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 11.9 mmol/L PO_4 , pH 7.4; Fisher Scientific)]. We nebulized the buffer as previously described (18) and left the slides to dry at room temperature. Slides were stable for several months when stored dry at 4 $^{\circ}\text{C}$.

MICROARRAY ASSAY

For the assay, slides were mounted to the TeleChem International Microarray Microplate adapter and rehydrated for 1 h with shaking in a MTP plate shaker (Thermomixer; Eppendorf) at 650 rpm at 25 $^{\circ}\text{C}$. We hydrated each subarray by adding 50 μL of assay buffer [15 g/L bovine serum albumin and 25 g/L low-fat dried milk in PBS containing 1 mL/L Tween 20 (PBS+T)³] and performing a short washing step with 100 μL PBS+T.

We diluted calibrators and samples in assay buffer. For the combined competitive assay, we added Cy3-labeled human IgG or Cy5-labeled human IgG (labeling density, 3–4 fluorophores/antibody; Dianova) as the competitor to the assay buffer at a final concentration of 25 mg/L. We used human IgG (Dianova) as the calibrator and prepared 8 dilutions (calibrators 1–8) from 972 mg/L to 440 $\mu\text{g/L}$. We diluted previously

characterized serum samples (kindly provided by Phadia) 200-fold in assay buffer and incubated a 30- μL aliquot by shaking at 650 rpm for 45 min at 25 $^{\circ}\text{C}$ (Fig. 2A). We then washed each subarray 8 times with 100 μL PBS+T, added 30 μL of a 3-mg/L solution of Cy5-labeled goat antihuman IgG or Cy3-labeled goat antihuman IgG (Dianova) in assay buffer (Fig. 2B), and incubated with shaking at 650 rpm for 45 min at 25 $^{\circ}\text{C}$ (Fig. 2C). The slides were then washed 8 times with 100 μL PBS+T, demounted, rinsed once with PBS+T and twice with doubly distilled water, and dried by centrifugation at 1500g with a slide spinner (Abimed).

IMMUNOASSAYS

We quantified IgG in human plasma with a commercial ELISA kit (Immunoglobulin G ELISA Kit; Immunodiagnostik) according to the manufacturer's instructions and used the Varelisa test system (Phadia) to assay human sera for reactivity against the autoantigens listed above.

DATA ANALYSIS

Arrays were scanned with a dual-laser confocal scanner (ScanArray Express; PerkinElmer) with fluorescence excitation at 532 nm and 635 nm. We set the laser power to 90% and adjusted the photomultiplier gain to avoid signal saturation. The resolution of the resulting 16-bit TIFF images was 10 μm . Images were analyzed with ScanArray Express software (PerkinElmer), and the mean signal intensity was calculated from the 14 replicate spots. We designated test results as positive

³ Nonstandard abbreviations: PBS+T, PBS containing 1 mL/L Tween 20.

when signal intensities exceeded the signals of the negative-control sera by a factor of 3.

We measured the amount of human IgG by combining the signals of the 2 fluorescence readout channels (Fig. 2D). The assay signal generated by the binding of the fluorescently labeled human IgG competitor molecule was detected at 570 nm, and the sandwich immunoassay signal was detected at 670 nm. The sandwich immunoassay signal consists partly of a signal generated by the human IgG competitor and partly of a signal that represents the amount of human IgG in the sample. IgG is quantified by calculating the ratio of the fluorescence signals measured at 670 nm and 570 nm for each individual spot:

$$\text{Ratio}_{670\text{ nm}/570\text{ nm}} = \frac{\text{Mean}_{670\text{ nm}}}{\text{Mean}_{570\text{ nm}}} = \frac{[\text{Competitor}] + [\text{Analyte}]}{[\text{Competitor}]}$$

Calibration curves were prepared from purified human IgG and used to quantify the serum concentration of IgG. We analyzed the data and calculated concentrations with the 4-parameter logistic-fit method included in the Origin data-analysis package (version 6.0; MicroCal).

Results

QUANTIFICATION OF HUMAN IgG

We added fluorescently labeled human IgG to human serum samples to serve as the detectable competitor and applied the diluted serum samples to protein microarrays containing immobilized antihuman antibodies as capture molecules. We used 2 different readout channels for detection (Fig. 2E): The Cy5 channel (635 nm excitation) detected the activity of serum against spotted autoantigens, and the Cy3 channel (532 nm excitation) recorded the signal of competitor Cy3-labeled human IgG for quantifying total IgG. Because Cy5-labeled goat antihuman IgG detection antibodies bind both competitor and autoantibodies, both Cy3 and Cy5 fluorescence signals are generated on the antihuman IgG spots. For the serum sample in Fig. 1 (serum dilution 200-fold and a competitor concentration of 25 mg/L), the antihuman IgG capture spots are fully occupied with IgG, producing a maximal Cy5 signal. Therefore, the differences in the sandwich signal on antihuman IgG spots reflect the binding capacities of the spots. These differences are also apparent for the Cy3 signal, because the amount of competitor depends on the binding capacity of the spot. The ratio of the 2 signals should stay constant, however, and the differences caused by spot-to-spot variation should be eliminated. For that reason, concentration measurements for the competitive system (Cy3 signal originating

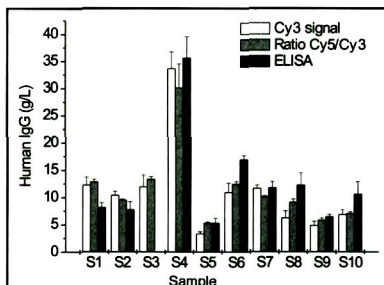


Fig. 3. Quantification of human immunoglobulins with different assay systems.

The concentration of human IgG was quantified in 10 serum samples with a competitive micro-ELISA on the microarray (white bars), with the combined competitive assay system on the microarray (gray bars), and with a commercial sandwich ELISA as a reference. Data are presented as the mean (SD) of 4 independent measurements.

from the labeled competitor) and the combined competitive assay (Cy5/Cy3 signal ratio) were compared.

To evaluate the method, we measured total IgG in 10 serum samples with a set of identical slides. We applied each of the serum and IgG standards at 8 different concentrations to separated arrays on the same slide (Fig. 1). We then detected the Cy3 and Cy5 signals, generated calibration curves with the Cy3 signal only (competitive assay) and with the Cy5/Cy3 signal ratio (combined competitive assay), and then calculated IgG concentrations. We measured serum IgG in the same samples with a commercially available kit based on a standard sandwich ELISA. The results for the ELISA and the 2 competitor systems are shown in Fig. 3. A comparison of the combined competitive assay and the commercial ELISA (19) produced a concordance correlation coefficient (ρ_c) of 0.9124 [Pearson correlation coefficient (ρ), 0.9468; bias correction factor (C_b), 0.9636], indicating a good correspondence between the 2 different assay systems.

To evaluate the robustness of the assay system, we performed dye-swap experiments. The fluorescent dyes and the corresponding readout channels were exchanged, but the basic setup of the assay was left unchanged. We generated calibration curves with purified human IgG in parallel for Cy3- and Cy5-labeled competitors and analyzed assay performance by comparing assay characteristics with a linear fit in the concentration range of 1.3–324 mg/L. Both approaches

produced a linear response and good correlation for both competitors ($r^2 = 0.998$, Cy3 label; $r^2 = 0.998$, Cy5 label), indicating that assay performance is independent of the sequence.

DETECTION OF AUTOANTIBODIES

We used the same microarrays for autoantibody detection and detected reactivity to the immobilized recombinant antigens with a direct ELISA. Bound autoantibodies were detected with a Cy5-labeled antihuman IgG antibody (Fig. 4). We analyzed 13 different autoantigens consisting of antinuclear antigens (centromere antigen B, PmSc100, Scl70, double-stranded DNA, Sjögren syndrome antigen B, ribonucleoproteins A and C, Sjögren syndrome antigen A proteins 52 and 60, and Sm), thyroglobulin, thyroid peroxidase, and glutamic acid decarboxylase. The patient sera had previously been analyzed for autoantibodies against most of the autoantigens by means of the established Varelisa assay (Phadia). We obtained similar positive and negative autoantibody results in both assays for the small number of samples analyzed ($n = 10$; Fig. 4A). We observed additional autoantibody reactivity in the microarray experiments for some of the serum samples, which had been tested only for a subset of autoantigens in the Varelisa assay.

The results for the combination assay allow referencing of antibody titers to total IgG concentration (Fig. 4B), an assay feature that provides extra clinical data to an autoantigen pattern.

EXPANSION OF THE DYNAMIC RANGE AND IMPROVED REPRODUCIBILITY

One of the potential advantages that our system shares with classic competitive assays is the possibility of shifting the measurement range by varying the amount of added competitor (Fig. 2E). We investigated this potential feature with a dilution series of the IgG calibrator and different added amounts of Cy3-labeled human IgG competitor (0–10 mg/L). Fig. 5 shows the results for the Cy5-sum signal, the Cy3 competitor signal, and the normalized Cy3 signal. The sandwich signal on the Cy5 channel approaches saturation for IgG concentrations > 1 mg/L. Therefore, IgG quantification with a sandwich assay requires dilution to 0.001–1 mg/L (dilution factor, $> 1:10\,000$) to get results within the calibration curve. The addition of the competitor pushes the detection range toward higher concentrations; thus, combining competitive and direct immunoassays creates a tool that allows the quantification of analytes that differ in concentration by orders of magnitudes. A convenient side effect when normalizing the Cy3 signal with the Cy5/Cy3 signal ratio is the back conversion from an inverted competitive calibration curve to a conventional ascending curve.

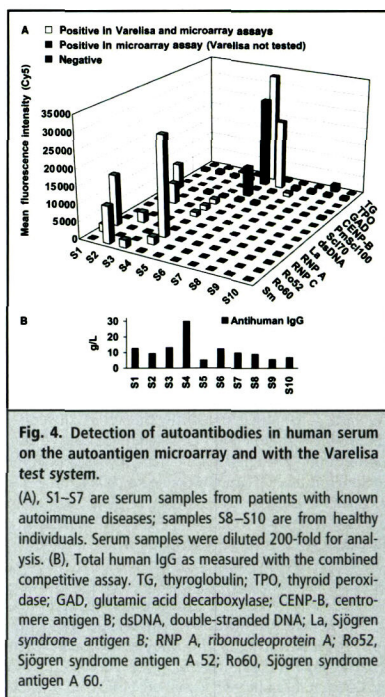


Fig. 4. Detection of autoantibodies in human serum on the autoantigen microarray and with the Varelisa test system.

(A), S1–S7 are serum samples from patients with known autoimmune diseases; samples S8–S10 are from healthy individuals. Serum samples were diluted 200-fold for analysis. (B), Total human IgG as measured with the combined competitive assay. TG, thyroglobulin; TPO, thyroid peroxidase; GAD, glutamic acid decarboxylase; CENP-B, centromere antigen B; dsDNA, double-stranded DNA; La, Sjögren syndrome antigen B; RNP A, ribonucleoprotein A; Ro52, Sjögren syndrome antigen A 52; Ro60, Sjögren syndrome antigen A 60.

To test the reproducibility of internal spot normalization, we prepared 24 replicates from one diluted serum sample and incubated the replicates on 4 slides, each with 6 arrays. We used the Cy3 signal and the Cy5/Cy3 signal ratio to evaluate the CVs of 14 replicate spots present in each array and grouped the 6 CVs for each array (Fig. 6). These box-plot diagrams show CVs of $< 10\%$ when internal spot normalization is used and demonstrate the clear improvement over the Cy3 competitive assay system.

Discussion

The concurrent detection of analytes that exhibit widely different concentration ranges is a principal problem of multiplexed assay systems. The possibility of measuring dozens to hundreds of analytes in the same reaction points to the fact that the dynamic range of the assay is an important variable and determines the

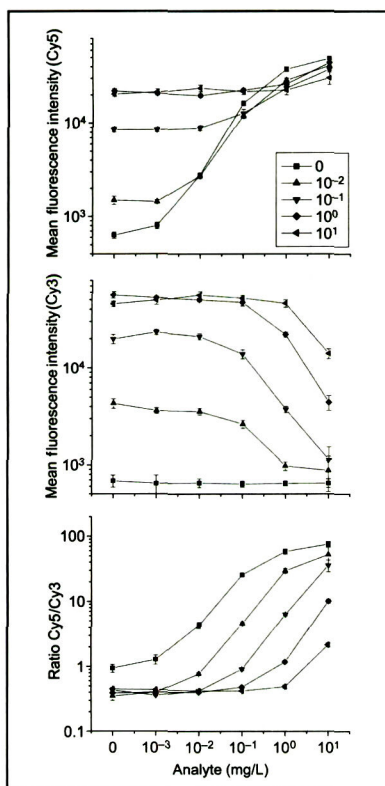


Fig. 5. Assay dynamics and detection window for the sandwich immunoassay, the competitive immunoassay, and the combined competitive immunoassay.

The detection window was adjusted by incubating protein microarrays containing antihuman IgG antibody spots with calibrator solutions containing human IgG and different concentrations of Cy3-labeled IgG competitor (concentrations in milligrams per liter indicated by boxed values). The fluorescence measured in the Cy5 channel (top) corresponds to the sandwich immunoassay, the fluorescence measured in the Cy3 channel (middle) represents the competitive immunoassay, and the Cy5/Cy3 ratio of signal intensities (bottom) represents the combined competitive immunoassay. The values represent the mean of 14 replicate spots, and the bars indicate the CVs of the replicates.

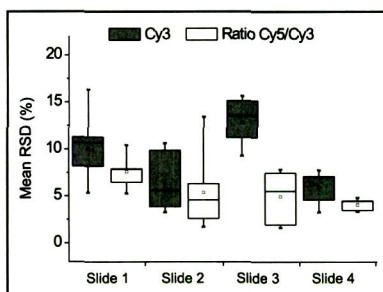


Fig. 6. Assay reproducibility in the competitive and the combined competitive assay modes.

Serum samples were diluted 200-fold with assay buffer before analysis. Intraarray imprecision was measured by plotting the residual SD (RSD) for 84 replicate spots on 6 subarrays in a box-plot diagram. Data represent the results of 4 different experiments.

The data are presented as standard box-and-whisker plots; open squares within each plot indicate mean values. The median is very close to the upper quartile in 3 of the plots and thus is not apparent.

range of detectable concentrations. The fluorescence- or chemiluminescence-based readout systems commonly used in protein microarray-based assay systems possess excellent dynamic ranges. Nevertheless, the large differences in concentration that exist between analytes require either the use of several dilutions or assay systems capable of adjusting for these differences. We addressed this problem by developing a combined competitive assay system. As in standard competitive assays, we added a labeled competitor to the analyzed sample. The array signal generated on the microspots is inversely proportional to concentration of the analyte. A sandwich-type immunoassay is performed at the same time, but a different readout channel is used for this signal. Creating 2 readout channels is most easily achieved through the use of 2 different fluorophores as labels. The 2 different signals generated from a microspot readout are used to calculate a combined value, and analyte concentration is calculated from a calibration curve. As with competitive systems, the optimal concentration range is adjustable (Fig. 2E), and analytes that occur at widely different concentration ranges can be quantified in a single assay. Lower intra-array variation is achieved at the same time because in principle 2 independent assay signals are used to calculate 1 concentration. Therefore, this calculation can be viewed as a normalization strategy.

The experimental system we have tested is a single-assay format in which the competitive quantification of human IgG, as the highly abundant analyte, is combined with the detection of 13 different types of auto-immune antibodies. We chose a serum dilution of 200-fold, a dilution commonly used for the detection of autoantibodies. This range presents a good compromise between minimizing false-positive results and maintaining an adequate assay response (20). For directly quantifying human IgG in an array ELISA, we diluted serum samples 10 000-fold to define the linear range of the assay (Fig. 5). With the combined competitive ELISA, we have shown that it is possible to measure both autoantibodies and total IgG within a single assay, despite the fact that the optimal dilutions for detecting the single analytes differ by more than 2 orders of magnitude. The coverage of a wide concentration range is possible because proteins at low abundance are quantified via a direct ELISA while more abundant proteins are quantified competitively, as we have described (21). Adding different concentrations of competitor allows the measurement range of the assay system to be adjusted to the required concentration range, a feature typical of competitive assay systems. This adjustment is not equivalent to an expansion of the assay dynamics; it brings the detection window into the range that has been found to be optimal for the analyte of interest and often helps it to stay within the linear range of the calibration curve.

The use of multiple competitors produces a set of fluorescently labeled proteins that are present together within the assay and therefore may be a source of cross-reactivity and nonspecific background signals. Although this situation certainly applies for highly parallel assays, the amount of fluorophore introduced into systems of low to medium complexity (up to 20 analytes) is well below the amount of label found in systems that feature complete labeling of the sample (8, 15). The limitations we have observed may be comparable to those of multiplexed assays, and comparable assay performance is expected. As shown by Barry et al. (22), such multiplexed competitive antibody arrays are capable of measuring absolute concentrations of proteins instead of their relative abundance in 2 compared samples.

The use of 2 different readout channels within one assay directly addresses the problems of reproducibility in array-based protein-analysis methods. Although spotting techniques have matured and microarray surfaces have improved, spot-to-spot variation

caused by the unequal distribution of capture molecules in the microspots still represents a major problem. Reproducibility problems often occur when the spot morphology is irregular, and such problems can be caused by high protein concentrations, viscous buffers, and high salt or detergent concentrations. Inadequate blocking procedures and poor mixing of the reaction chamber also contribute to irregularities in microarray spots (23). Olle et al. (24) presented a spot-normalizing strategy that uses an antibody directed against the capture molecule. In the present study, we measured the binding signal of the normalization antibody with a second readout channel while detecting the specific signal by conventional methods. The normalization process now takes advantage of the ratio calculated from both signals and is capable of correcting spot-to-spot differences; however, this normalization strategy does not work under saturation conditions. Another implementation that takes advantage of 2 readout channels is the comparative assay system put forward by Kattah and colleagues (15). In this assay, the proteins from 2 different samples are covalently labeled with 2 different fluorophores; the two samples are then mixed and added to a microarray. Differences in abundance for the different analytes in the 2 samples are observed as changes in the ratio of the 2 fluorophores. For the combined competitive micro-ELISA, a defined amount of one or more competitors is added, and quantitative data are obtained from the 2 generated readout signals. The competitor signal depends on the amount of functional capture molecules in the spot, and the sum of the signals of the competitor and the analyte is also dependent on active capture molecules. Therefore, this type of normalization not only takes the presence of a capture molecule into account but also allows for the activity of the capturing reagent. This strategy improves the intraarray imprecision (CV, 5%–10%), whereas the CV range for replicate spots for the competitive assay alone is 5%–15%.

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