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ORIGINAL PAPER

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# Double-chip protein arrays: force-based multiplex sandwich immunoassays with increased specificity

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**Abstract** Protein assays provide direct access to biologically and pharmacologically relevant information. To obtain a maximum of information from the very smallest amounts of complex biological samples, highly multiplexed protein assays are needed. However, at present, cross-reactions of binding reagents restrict the use of such assays to selected cases and severely limit the potential for up-scaling the technology. Here we describe a double-chip format, which can effectively overcome this specificity problem for sandwich immunoassays. This format consists of a capture array and a reference array with fluorescent labeled detection antibodies coupled to the reference array via DNA duplexes. This format allows for the local application of the labeled detection antibodies onto their corresponding specific spots on the capture array. Here we show that this double-chip format allows for the use of cross-reactive antibodies without generating false positive signals, and an assay for the parallel detection of seven different cytokines was set up. Even without further optimization, the dynamic range and the limit of detection for interleukin 8 were found to be comparable to those obtained with other types of multiplexed sandwich immunoassays.

**Keywords** Sandwich immunoassay · Protein array · Cytokine · Specificity · Cross-reactivity · Multi-analyte

## Introduction

Today, it is widely accepted that the parallel analysis of proteins, their abundance, their modifications, and their interactions, reveal unique insights into complex biological systems, such as immunology and cell signaling [1, 2]. An ideal assay to answer both the questions posed in the drug development process and those posed in diagnosing disease should be able to measure many proteins in a small amount of sample with high specificity and sensitivity. Two formats are typically used: microarrays and sandwich immunoassays. Microarray formats are employed for the parallel measurement of proteins [3, 4, 5, 6, 7], and are well suited for the analysis of small sample volumes, whereas sandwich immunoassays have the potential for the specific detection of proteins [8], even at low concentrations. Both technologies are well established, and different groups have shown encouraging proof of principle experiments, which combine the two formats [9, 10, 11, 12, 13, 14, 15, 16].

One of the most serious drawbacks of multiplexed sandwich immunoassays is lack of specificity caused by antibody cross-reactions. In conventional sandwich-ELISAs the secondary detection antibody (detAb) improves the specificity of the assay [17]. However, in a multiplexed microarray format the use of a detAb is actually an additional source of false positive signals. When a cocktail of detAb is incubated on the array, each detAb can interact with any antigen bound somewhere on the surface of the array [9, 10, 13, 18]. As a result, the chance of false positives increases geometrically with the number of spots on a protein array [19]. For this reason it is no coincidence that all published capture array formats that employ a sandwich format measure cytokines [9, 10, 11, 12, 13, 14, 15, 16]. Cytokines are the only group of molecules for which sets of cross-reactivity optimized antibodies are commercially available from different suppliers [4, 8].

A common strategy to overcome this specificity problem is prescreening of antibodies for cross-reactivity [9, 10, 13, 14, 15, 16]. Alternatively, one can optimize assay conditions (e.g., buffers, blocking, use of detergents, and concentrations of detection molecules). These approaches can improve the specificity of the assay; however, they are time-consuming and expensive. Another approach is the use of more specific capture reagents [20], such as re-

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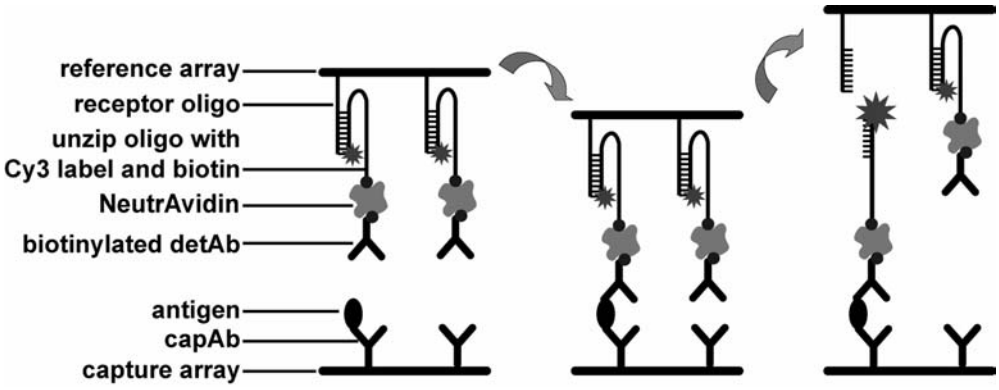



**Fig. 1** Principle of a double-chip assay. CapAbs are immobilized on the capture array by random coupling via their amino groups. The detAbs are immobilized on the reference array via DNA force sensors hybridized in unzipping geometry and carrying a Cy3 label. NeutrAvidin is used to connect the biotinylated detAb to the DNA force sensor. The assembly of the DNA force sensor NeutraVidin and the biotinylated detAb results in the force sensor complex. The two arrays are brought into contact to allow binding of the detAb to the antigen. If the antigen is present and the detAb can bind this antigen, the DNA duplex opens, and the detAb and the unzipping oligo (including the Cy3 label) are transferred onto the capture array

combinant antibodies [21, 22], affibodies [23], or photo aptamers [24]. But in this approach, too, the entire process of prescreening the capture reagents and optimizing assay conditions has to be carried out for each new capture reagent.

Here, we employ a double-chip format [25, 26], which can effectively overcome the specificity problems of multiplexed sandwich immunoassays. The concept relies on using a conventional capture array coupled with a reference array, which is used to locally apply the detAbs. The detAbs are coupled to the reference array via DNA duplexes which serve as molecular force sensors. After the antigens are bound to the capture array, the reference array (with the detAbs arranged such that each detAb is right opposite the corresponding capAb) is brought into contact with the capture array. If the specific antigen is present, the detAb will bind, and the DNA duplex will open when the two chip surfaces are separated. As the detAb carries a fluorescence label, the transfer of this label onto the capture array is finally measured.

By using this format, we demonstrate that cross-reactive capAbs do not lead to false positive results and that commercially available antibody sandwiches can be used for a multiplex assay without any previous antibody testing. Finally, we give an example which demonstrates that both the dynamic range and the limit of detection (LOD) of this new format are comparable to that obtained by other sandwich immunoassays. For better comparison of the data with other types of assays, all experiments were carried out with cytokine antibodies.

## Experimental

Three different series of double-chip experiments have been carried out using the same assay principle, which is illustrated in Fig. 1. The preparation of capture and reference arrays follows the same procedure for the different experiments. Briefly, the capture array was prepared by spotting solutions of capture antibodies onto a glass microarray slide. After blocking, the slide was incubated with a sample containing the antigens. The reference array was prepared in a two-step procedure. First, the components of the force sensor complex (including the detAbs) were bound sequentially to 10 mm×10 mm pads of poly(dimethylsiloxane) (PDMS), covering the whole surface homogenously. Second, disks were punched out of the different PDMS pads (each containing one of the detAbs) and assembled as an array such that the detAbs were right opposite to their corresponding cabAbs during the contact process. Both arrays were aligned and brought into contact using the contact device shown in Fig. 2A. Finally, the fluorescence on the slide was measured using a microarray scanner. In the following sections the description of one series of experiments (cross-reactive model system) is given in detail. For the other series only the differences are described.

### Materials

All monoclonal antibodies used as capAbs as well as the biotinylated monoclonal antibodies used as detAbs were commercially available (see Table 1 for details). Purified human cytokines were purchased from the following suppliers: interleukin 2 (IL-2), interleukin 12 (IL-12), and monocyte chemoattractant protein-1 (MCP-1) from BD Biosciences Pharmingen (Heidelberg, Germany); tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), and interleukin 8 (IL-8) from Perbio Science (Bonn, Germany); and interleukin 5 (IL-5; human and mouse) from R&D Systems (Wiesbaden, Germany). β-Galactosidase (β-gal) was purchased from Roche Diagnostics (Mannheim, Germany). Lyophilized antibodies and antigens were reconstituted as recommended by the supplier. Antibody solutions were divided into aliquots and stored as recommended by the supplier. Bovine serum albumin (BSA) was purchased from Roth (Karlsruhe, Germany), Perfect-Block from MoBiTec (Göttingen, Germany), and fetal calf serum (FCS) from Biochrom (Berlin, Germany). Unless stated otherwise, chemicals for the modification of the surfaces were purchased from Sigma (Taufkirchen, Germany).

### Preparation of antibody spotting solutions

Most capAbs were supplied in phosphate-buffered saline (PBS) without any additives (see Table 1 for details). These antibody stock solutions were diluted to a concentration of 200 µg mL$^{-1}$ in 10% glycerol to obtain the spotting solution. The anti-MCP-1 and anti-IFN-γ antibodies were purified by using magnetic protein G Beads (Dynabeads; Dynal Biotech, Hamburg, Germany), as they

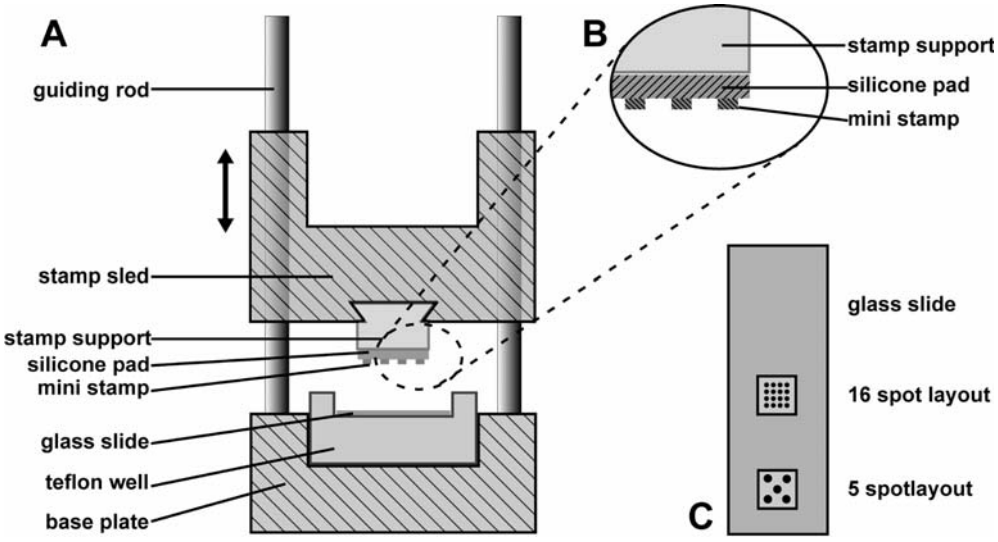



**Fig. 2A–C** Schematic of the contact device and the array layout. **A** The contact device consists of an element to position a glass slide (containing the capture arrays) and a unit, which holds the reference array and allows for the contact of the arrays. A Teflon well, which can be filled with buffer solution, containing the glass slide is adjusted on the base plate. The reference array is put onto the stamp support. This assembly is mounted onto the stamp sled, which runs on two guiding rods. The contact device has two stamp sleds, which can be used in parallel. **B** Detailed view of the reference array. The reference array consists of 5 or 16 individual mini stamps, which are assembled on a plain silicone pad in a pre-defined layout. To maintain an appropriate pressure, the diameter of one mini stamp is 2 mm for the 16-spot layout and 3.2 mm for the 5-spot layout. These mini stamps are manually positioned on the stamp support. The silicone pad between the mini stamps and the stamp support is necessary to compensate for local unevenness. **C** Layout of capture and reference array. One glass slide contains two capture arrays, which are either spotted with 16 or 5 spots. The reference array uses exactly the same layout to ensure an overlap of the corresponding spots during the contact process

were supplied in Tris or BSA, which can react with an amino-reactive surface and influence the coupling efficiency. Binding and washing steps were carried out as recommended by the supplier. Bound antibodies were eluted with 10 mM citrate buffer, pH 2.5, and neutralized to pH 7 with NaOH. Finally, the antibody was diluted to 200 µg mL$^{-1}$ in an aqueous solution of 10% glycerol.

### Preparation of capture arrays

For the cross-reactive model system CSS aldehyde slides (Genetix, Hampshire, UK) were incubated with 6 mM HCl·$NH_2$-PEG-COOH (3,400 g mol$^{-1}$; Shearwater Polymers, Huntsville, AL) for 1 h under a 24 mm×60 mm cover slip (300 µL) and rinsed with $ddH_2O$. The Schiff bases were reduced in 1% aqueous $NaBH_4$ for 30 min and again rinsed with $ddH_2O$. For the activation of the carboxy groups, the slides were treated with 50 mM each of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) for 30 min under a 24 mm×60 mm cover slip in a humid atmosphere at room temperature (RT). The slides were rinsed with $ddH_2O$ and dried. The freshly prepared antibody spotting solution of the anti-IL-5 capAb was manually spotted (1 µL spot$^{-1}$) onto the slides using a standard 10-µL pipette. The 5-spot layout of Fig. 2C was used. After 1 h incubation in a humid atmosphere at RT the spots were removed by aspiration, and the slides were washed in PBS+0.05% Tween 20 (PBST)+1% PerfectBlock for 3 min. Finally, the slides were blocked in PBS+3% PerfectBlock+1% BSA at 4°C overnight.

**Table 1** Capture and detection antibodies used for the protein array experiments[a]

| Sandwich | Capture | | | | Detection | | |
|---|---|---|---|---|---|---|---|
| | Supplier | Conc. (mg ml$^{-1}$) | Buffer | Purified | Supplier | Conc. (mg ml$^{-1}$) | Buffer |
| Interferon γ | Calbiochem | 5.06 | PBS+BSA | Protein G | BD | 0.5 | PBS |
| Interleukin 2 | BD | 0.5 | PBS | No | BD | 0.5 | PBS |
| Interleukin 5 (hu) | R&D | 1.0 | PBS | No | R&D | 0.5 | TBS+BSA |
| Interleukin 5 (mu) | | | | | R&D | 0.5 | TBS+BSA |
| Interleukin 8 | Perbio | 1.0 | PBS | No | Perbio | 0.21 | PBS+BSA |
| Interleukin 12 | BD | 1.0 | PBS | No | BD | 0.5 | PBS |
| TNF-α | Perbio | 1.0 | PBS | No | Perbio | 0.5 | PBS+BSA |
| MCP-1 | BD | 0.5 | TBS | Protein G | BD | 0.5 | PBS |
| β-Galactosidase | Biotrend | 10 | PBS | No | Dunn | 0.2 | PBS+BSA |

All capture antibodies listed are specific for human cytokines, except the anti-IL5 capture antibody, which is specific for human and mouse IL-5. All detection antibodies are specific for human cytokines, except one IL-5 antibody, which is specific for the murine antigen. *R&D* R&D Systems; *BD* BD Biosciences Pharmingen; *Perbio* Perbio Science; *Dunn* Dunn Labortechnik, Asbach, Germany; *Biotrend* Biotrend, Köln, Germany

### Immobilization of the force sensor complex on the PDMS pads

PDMS (poly(dimethylsiloxane), Sylgard 184, Dow Corning, Wiesbaden, Germany) was prepared according to the manufacturer's instructions. For casting, a 4" microstructured silicone wafer glued to a glass plate (NMI Natural and Medical Sciences Institute, Reutlingen, Germany), a 1-mm spacer ring with a casting gap, and a plain glass plate were clamped together to form a mold. This mold was placed vertically, filled with the degassed elastomer/curing agent mixture (10:1), and left at RT for 24 h. The resulting PDMS slab was transferred to a plastic dish and allowed to terminate polymerization for 24 h at 50°C. Pads of 10 mm×10 mm, a suitable size for efficient binding and hybridization processes, were cut out manually. The PDMS surface microstructure was composed of 150-µm squares separated by channels of 50-µm width and 5-µm depth. These 10 mm×10 mm PDMS pads were cleaned ultrasonically in 50% abs. ethanol for 3 min, and rinsed with ethanol and $ddH_2O$. Surface activation was carried out by water plasma treatment. The pads were put into a plasma cleaner (Harrick Scientific Corporation, Ossining, NY) together with a glass petridish filled with ice. The chamber was evacuated until constant pressure was obtained. The plasma was applied at "low" for 60 s. The pads were immediately transferred to an ethanolic solution of 2% 3-aminopropyldimethylethoxysilan (ABCR, Karlsruhe, Germany)+10% $ddH_2O$. After 30 min incubation at RT, the pads were rinsed with ethanol and $ddH_2O$ and dried under $N_2$. An aqueous solution (25 µL) of 18 mM NHS-PEG-NHS (MW=3,000 g mol$^{-1}$; Rapp Polymere, Tübingen, Germany) was incubated for 1 h on the pads under a cover slip in humid atmosphere at RT, rinsed with $ddH_2O$, and dried. The amino-labeled receptor oligonucleotide (5′-$NH_2$-AAA AAA AAA AAT CTG TCT CCG GCT TTA CGG CGT AT-3′; metabion, Martinsried, Germany, 1.5 µM) and 50 mM EDC were bound to the PEG surface under a cover slip in humid atmosphere at RT for 1 h. The pads were washed (2×15 min) with saline-sodium citrate buffer (1×SSC) containing 0.5% sodium dodecyl sulfate (SDS). The pads were blocked in an aqueous solution of 2% BSA for 1 h at RT to reduce non-specific binding. Cy3-labeled unzip oligonucleotides (5′-Cy3-ATA CGC CGT AAA GCC GGA GAC AGA TAA GAC GCT ACA TGA AAA AAA AAA AA-biotin-3′) were diluted to 0.5 µM in 5×SSC and hybridized under a cover slip (15 µL) in humid atmosphere at 4°C overnight. The pads were washed (3×5 min) in 1×SCC+0.1% SDS, and rinsed with PBS. NeutrAvidin (Perbio Science) was used as linker between the 3′-biotinylated unzip oligonucleotide and the biotinylated detection antibody. The pads with the attached DNA were incubated in 4 mL of 2 µg mL$^{-1}$ NeutrAvidin in PBS containing 0.4% BSA (PBS-BSA) for 1 h at RT and washed with PBST and PBS. The biotinylated detAbs against human IL-5 (huIL-5) or murine IL-5 (muIL-5) were diluted to a concentration of 2 µg mL$^{-1}$ in PBS-BSA. Each PDMS was incubated with 150 µL of one of the detAb solutions, rinsed with PBST, PBS, and dried.

### Sample incubation and reference experiment

The capture array was either incubated with a solution of 10 nM huIL-5, 10 nM muIL-5, or a mixture of both in PBS-BSA (10 nM of each antigen). Each slide was gently shaken with 4 mL of one of the sample solutions for 1 h at RT and washed with PBST and PBS immediately before the contact process.

For the reference experiment using a conventional sandwich setup the capture arrays were prepared, and the incubation of the antigens was carried out exactly as described above. A cocktail of detection antibodies (2 µg mL$^{-1}$ of each biotinylated anti-IL-5 antibody) in PBS-BSA was applied to the capture arrays. This step was followed by incubation with Cy3-streptavidin.

### Assembly of the reference arrays and contact process

Since conventional spotting was found to be poorly reliable on the PDMS surface, an alternative method was employed. After the force sensor complexes had been immobilized on pads of PDMS homogenously, as described above, disks were cut out of these 10 mm× 10 mm pads and reassembled in the desired order (see Figs. 2B and C). For this process, the shaft of a disposable surgical biopsy punch (Stiefel Laboratorium GmbH, Offenbach, Germany) was hollowed out with a drill, and its back end was attached to a conventional disposable 5-mL syringe. With mild pressure, disks (mini stamps) of 3.2-mm diameter were punched out of one freshly coated 10 mm× 10 mm PDMS pad. Deposition onto plain silicone pads was effected by an air pressure pulse (i.e., by pressing the syringe).

Two pre-assembled reference arrays (each consisting of 5 mini stamps) were then positioned on the two stamp supports of the contact device (see Fig. 2A). For each of the two reference arrays, two mini stamps containing the anti-muIL-5 and two mini stamps containing the anti-huIL-5 antibody were used. The fifth mini stamp in the middle contained either anti-muIL-5 or anti-huIL-5. The slide containing the two capture arrays was positioned in the Teflon well under pre-cooled PBS. The stamp sled was cranked down slowly onto the slide until its weight resulted in a pressure of 1.6 N cm$^{-2}$ in the contact areas. After 10 min the arrays were separated carefully, and the slide was rinsed with $ddH_2O$ and dried.

### Multiplexing experiment (7 cytokines)

For the multiplexing experiment a different protocol was used to prepare an amino-reactive surface for the spotting of the capAbs. QMT aldehyde slides (Quantifoil Microtools GmbH, Jena, Germany) were oxidized in a 0.5% solution of $KMnO_4$ in 150 mM $Na_2HPO_4$, pH 9.1 at 70°C for 20 min and dried. The generated carboxy groups were activated with EDC/NHS and used for spotting immediately. In a 16-spot layout the spotting solutions of 8 different capAbs were applied (0.3 µL spot$^{-1}$) as shown in Fig. 4A. The reference arrays were prepared as described using the 16-spot layout. The diameter of individual mini disks was reduced to 2 mm to maintain the pressure in the contact area in the appropriate range.

FCS was used as a matrix for three different "sample solutions". A single, partial, or complete mixture of all 7 antigens was prepared by diluting the stock solutions in 20% heat-inactivated FCS in PBS (20% FCS) to a final concentration of 10 ng mL$^{-1}$. The complete mixture contained all 7 cytokines, namely, IFN-γ, IL-2, IL-5, IL-8, IL-12, TNF-α, and MCP-1. (The β-gal antibody sandwich served as a negative control.) The partial mixture contained IL-8, TNF-α, and MCP-1, the "single mixture" only IL-8.

### Dilution series of interleukin 8

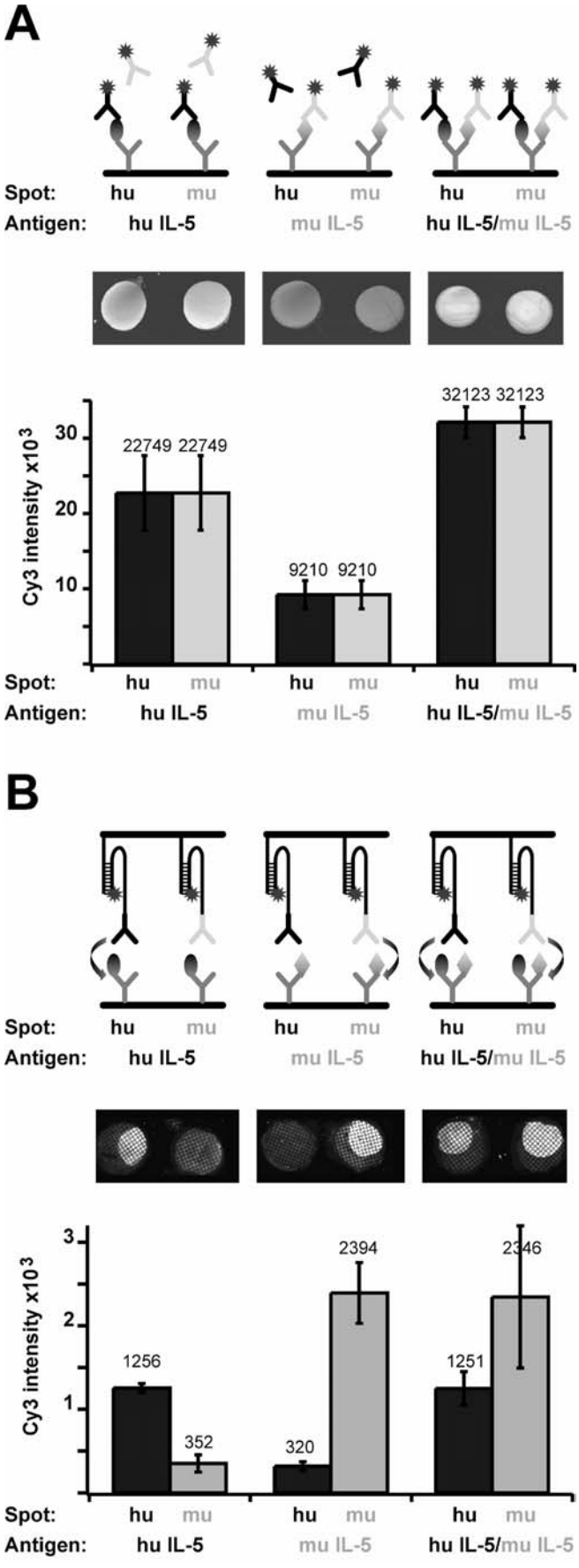
Dynamic range and limit of detection were determined for one of the cytokine sandwiches (IL-8) in the 5-spot layout. On the capture array (based on QMT slides), 4 identical spots (1 µL) of the capAb were applied in the corner positions, and the center was left empty. The reference arrays were prepared with 5 mini stamps, as described above using only anti-IL-8 detAbs.

Seven identical capture arrays were incubated in 4 mL IL-8 solution of 0, 0.1, 1, 10, 100, 1,000, or 10,000 pg mL$^{-1}$ (diluted in PBS-BSA) at RT for 1 h. The slides were stored at 4°C and washed with PBST and PBS one by one immediately before the contact process.

A reference experiment was carried out as described for the cross-reactive model system using the biotinylated anti-IL-8 antibody and Cy3-streptavidin.

### Fluorescence measurement and data evaluation

The capture array slide was transferred to a GenePix 4000B microarray reader (Axon Instruments, Foster City, CA, USA) and measured in the Cy3 channel. Mean fluorescence transfer was determined with the NIH Image (NIH Bethseda, MD, USA) analysis software. Mean background fluorescence, measured in the grid between the printed microstructure squares, was subtracted from the mean value of the squares to obtain the mean fluorescence transfer.




## Results

### Cross-reactive model system

The following system was chosen to investigate and compare the effects of a cross-reactive capAb in a conventional protein array experiment and in a double-chip assay. An antibody binding both murine (mu) and human (hu) IL-5 was used as capAb. While this antibody was 100% cross-reactive for both antigens, the detAbs were specific for either muIL-5 or huIL-5. The specificity was tested in simple binding experiments (data not shown). Spots for the detection of either huIL-5 or muIL-5 were defined by their *x*,*y* position on the capture array. Two spots were defined as anti-mu and two spots were defined to be anti-hu. The capture array was incubated with a solution of muIL-5, huIL-5, or a mixture of both in a concentration tested to be below saturation of the IL-5 capAb. Bound antigens were detected using a cocktail of both detAbs. The results are shown in Fig. 3A. If only muIL-5 was incubated on the array, it could be detected on the anti-mu and on the anti-hu spots with the same intensity. This was also true if only huIL-5 was used. For the mixture of the antigens, again the same signal was measured on the anti-mu and on the anti-hu spots. Here, the signal was the sum of the signals generated when only one antigen was present. With this setup, it was not possible to discriminate between the two antigens resulting in false positive signals. In addition, the signal of one particular spot was not correlated to the concentration of the antigen to be measured on this spot, but to the total amount of all antigens bound to this spot, making a precise quantification of the different antigens impossible.

By using the double-chip format to detect the antigens, each capture spot was brought into contact with only one sort of detAb. The anti-mu capture spot was opposite to an anti-mu detAb, and similarly, the anti-hu spot was

**Fig. 3** Cross-reactive model system. Spots for the detection of either huIL-5 or muIL-5 were defined by their *x*,*y* position on the capture array. The capAb at the different positions was cross-reactive between the human and the murine antigen. **A** If this capture array is incubated with huIL-5 (*left*), muIL-5 (*middle*), or a mixture of both antigens (*right*) and a cocktail of detAbs is used as in a traditional format, a signal is measured on the anti-hu and anti-mu spots. The scan shows that discrimination between the two antigens is not possible, as each detAb will interact with its antigen, regardless of where it is bound. The resulting signal is not dependent on the species and amount of antigen in the sample. Mean fluorescence intensities of 4 spots for each antigen are summarized in the diagram. **B** If the double-chip format is used, where the detAbs are immobilized at defined positions on the reference array, labeling of the antigen will only take place at the allocated spot on the capture array. The measured signals where detAb and capAb do not form a specific sandwich (intensity of 352 or 320) represent non-specific transfer which is also measured when a detAb is brought into contact with the blocked capture array containing no antigen (data not shown). In addition, precise quantification of antigen is now possible. Both antigens are measured independently when the second chip is used for specific encoding. The diagram shows the mean fluorescence intensities of four spots

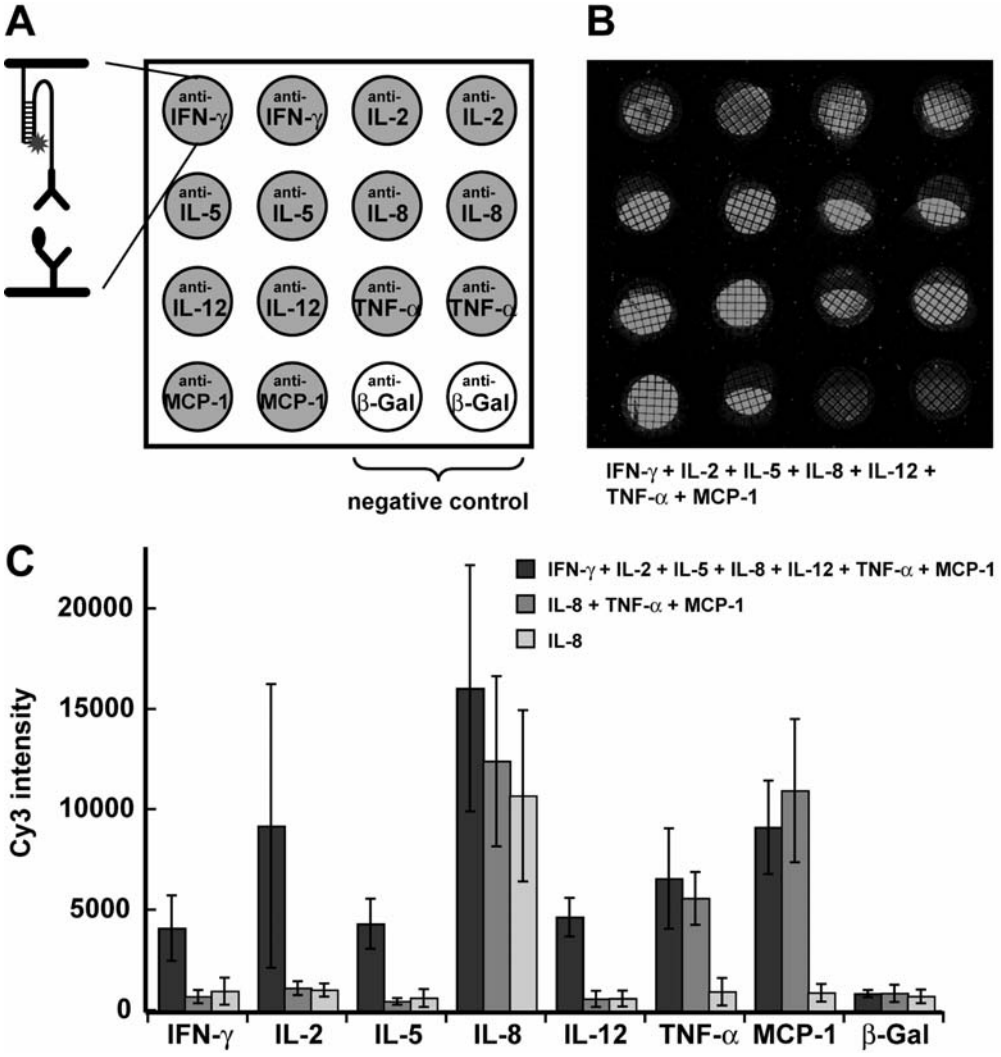



**Fig. 4A–C** Detection of 7 different cytokines with a 4×4 array. **A** Layout of one capture array. The capAbs against 7 different cytokines (IFN-γ, IL-2, IL-5, IL-8, IL-12, TNF-α, and MCP-1) were spotted in duplicate. The anti-β-gal antibody (*bottom right*) was used as a negative control. After the incubation of this capture array with a mixture of different cytokines, it was brought into contact with the reference array. The reference array contained detAbs for the respective antigens, which were assembled in a way such that each capture and detection antibody formed a specific antibody sandwich. **B** Fluorescence scan of one capture array. On this array a complete mixture of all 7 cytokines was incubated. All positions containing an anti-cytokine antibody are brightly illuminated (intensities between 16,052 for IL-8 and 4,106 for IFN-γ). Only a faint signal (max. intensity 860) is measured on the two negative control spots. **C** Diagram showing the fluorescence intensities determined for 3 different mixtures of cytokines. The mean values and standard deviations for each mixture were calculated from 12 spots (3 independent experiments with 2 identical capture arrays containing duplicates). The *black bars* show the intensities determined for a complete mixture of all 7 cytokines, the *dark gray bars* show a partial mixture with only IL-8, TNF-α, and MCP-1 and the *light gray bars* show the intensities when only IL-8 was incubated on the array

probed only with an anti-hu detAb. The results of this local application of detAbs are shown in Fig. 3B. Although it was possible for muIL-5 to bind to the anti-mu and the anti-hu spots, it was only detected if it was bound to the anti-mu spot. Similarly, the human antigen was only detected on the anti-hu spot. If both antigens were present, both types of capture spots were labeled; however, in this case, the signal on the anti-mu spot, for example, was not influenced by the presence of the human antigen, and vice versa.

## Multiplexing experiment

Six antibody pairs, which were optimized for sandwich ELISAs (IL-2, IL-5, IL-8, IL-12, TNF-α, and MCP-1) and one non-optimized pair (IFN-γ; capAb and detAb from different suppliers, see Table 1) were arbitrarily chosen from different suppliers, without considering possible cross-reactivities. All seven sandwiches were functional in our assay format and could directly be used for a multiplexing assay for the detection of 7 different cytokines in parallel. To investigate interferences of the cytokines with other proteins, the cytokines were used in a rather high concentration (10 ng $mL^{-1}$) and in a complex biological sample (20% FCS). The capAbs were spotted in duplicate, and an antibody directed against $\beta$-gal was used as a

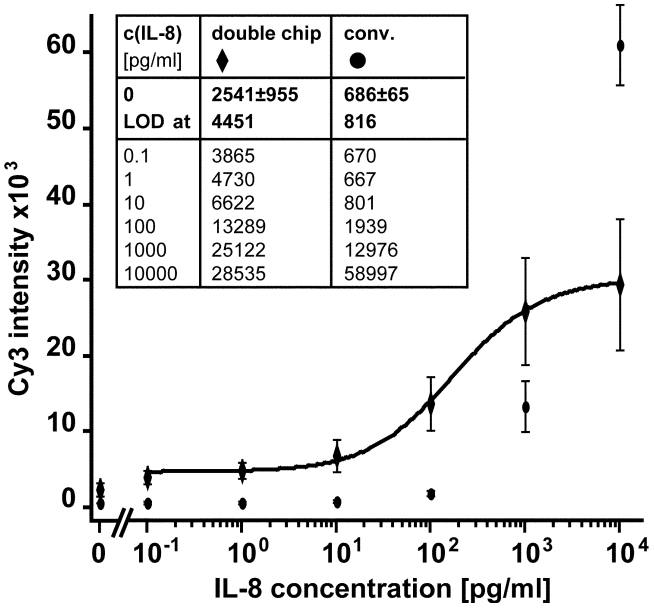



**Fig. 5** Standard curves for the IL-8 sandwich. The *table* summarizes the mean values of 16 spots for each concentration (2 independent experiments with 2 identical capture arrays containing 4 capture antibody spots). The limit of detection (LOD) was determined from the experimental data using the signal at zero analyte concentration incremented by two-fold standard deviation of this signal. For the double-chip experiment, the LOD is below 1 pg mL$^{-1}$ and for the conventional setup (conv.), the LOD is little above 10 pg mL$^{-1}$. The mean values and the standard deviations of the measured data (♦ double chip and ● conv.) were plotted and the double-chip data were fitted using 5PL logistic regression (–)

negative control. For each spot on the capture array, there was a corresponding detAb spot on the reference array. For a detailed layout of the assay see Fig. 4A. The capture arrays were incubated with 3 different mixtures of cytokines, brought into contact with the reference arrays, separated, and fluorescence intensities were recorded. The results are summarized in Figs. 4B and C. Figure 4B shows a fluorescence scan of a capture array where the complete mixture of all 7 cytokines was used. All spots show a signal significantly higher than the negative control (bottom right). In the diagram of Fig. 4C, the average fluorescence intensities of the three different mixtures are quantified. The black bars represent the complete mixture and again show that a signal significantly above the negative control is measured for all 7 cytokines. If only one (light gray) or three (dark gray) cytokines were present, only the corresponding capture spot(s) show(s) a high signal, while the other signals are in the same range as the negative control. For IL-8 in the different mixtures, the differences in fluorescence intensity are well within the respective error bars. The same is the case for MCP-1 and TNF-α. The results demonstrate that the presence of other proteins in the mixture does not interfere with the measurement of one of the cytokines and that all 7 cytokines can be detected specifically.

Dilution series of IL-8

The results from the previous experiments demonstrate that the specific detection of antigens is possible with this new assay format. However, in most protein assays a reliable measurement of different concentrations of an antigen is also important. Therefore, for one of the cytokine sandwiches (IL-8), the dynamic range and LOD were determined and compared to a conventional protein array setup. Figure 5 shows the measured data points for the double-chip and the conventional setup, where a biotinylated detAb and Cy3-streptavidin was incubated on the array. The LOD determined from the experimental data is below 1 pg mL$^{-1}$ (125 fM) for the double-chip assay and above 10 pg mL$^{-1}$ for the conventional setup.

The double-chip standard curve can be fitted by using logistic regression (5PL fit function), which is a standard curve-fitting algorithm for ELISA data [27, 28]. A dynamic range over 4 orders of magnitude (from 1 pg mL$^{-1}$ to 10,000 pg mL$^{-1}$) could be determined. Unfortunately, the standard curve for the conventional setup does not reach saturation and cannot be fitted with the above equation. Nevertheless, the measured data points clearly show that the performance of the double-chip assay is comparable to standard ELISA formats or conventional protein arrays, especially for low analyte concentrations.

## Discussion

Both the cross-reactive model system and the multiplexing experiment show that a multiplex sandwich immunoassay in the double-chip format can be easily set up without time-consuming prescreening of antibodies for cross-reactions and optimization of assay conditions. The problem of cross-reactive antibodies can be overcome by the local application of detAbs, which is achieved by the attachment of the detAbs in a complementary pattern on the reference array. In this format, the second chip surface provides a second dimension of specific encoding.

The detAbs are bound to the reference array via molecular force sensors consisting of DNA duplexes in unzipping geometry. Since the unzipping force is independent of the length of the duplex, the latter may be chosen such that the spontaneous off-rate of the duplex by far exceeds the time required for the assay [29]. In our assay, the unzipping force of this duplex serves as a reference for the discrimination between specific and non-specific binding [26]. This force threshold establishes stringent assay conditions, which are difficult to obtain using standard protocols, as only little modifications in wash stringencies can be applied for protein interactions [30].

Both experiments clearly demonstrate that commercially available antibody sandwiches can be easily integrated in a double-chip assay. If a desired antibody sandwich cannot be obtained from commercial sources and a new sandwich pair is needed, testing for an appropriate pair is necessary. However, it is only necessary to screen for a pair which performs well in a standard sandwich

ELISA format, meaning that both antibodies bind different epitopes with appropriate affinity constants for the analyte concentration of interest. Still, no screening for potential cross-reactions is necessary, which is otherwise most time-consuming and costly.

Not only could we demonstrate that our new assay format is highly specific, we could also provide an example which demonstrates that the LOD of the double-chip assay is at least comparable to a conventional protein array setup using the same capture array. In addition, the LOD and the dynamic range are comparable to those obtained in other cytokine assays [9, 11, 12, 13, 14, 15, 16, 31]. While many other assays use procedures for signal amplification, such as chemiluminescence [9, 11], RCA [15], or TSA [31], or complicated instrumentation [16], here the LOD is a direct consequence of the double-chip format itself. First, the detAbs are applied in a very high local concentration. Therefore, hindered diffusion and depletion of antibodies near the chip surface do not limit the sensitivity. Second, as the contact is made immediately after incubating the sample, there is no loss of bound antigen during further incubation steps (e.g., of the secondary antibody). Third, the concentration of the detAb, applied to the surface, is independent of the number of analytes to be detected. In a conventional assay, the total concentration of detAbs is increased with the number of analytes [19] resulting in an increase of the background signal. Here, the local application provides a constant amount of detAb, which can interact with the capture surface. As mentioned earlier, neither the spotting of the antibodies onto the arrays nor the contact process was done in an automated way. We believe that improvements in this field will greatly reduce the variability of the assay in the future and that even better values for the LOD or the limit of quantification (LOQ) can be obtained.

## Conclusions

In summary, specific and sensitive detection of different analytes is possible in a multiplexing format with this new double-chip assay. As a result of the local application of detAbs, cross-talk between the different spots is effectively eliminated and the complexity of a multi-analyte protein assay is reduced to the simplicity of single analyte ELISAs, where extensive screening for cross-reactivities is usually not necessary. Here, the performance of the assay is independent of the degree of multiplexing. One of the most promising applications of this new format will be protein arrays for the detection of structurally related proteins or disease markers, where multiple sets of optimized antibody sandwiches are not commercially available. Therefore, this new format will be extremely useful in all areas in which the analysis of patterns of markers creates additional information [32, 33, 34] for the drug development process and for biomedical diagnostics (e.g., cell signaling, early prediction of cancer, and differential cancer diagnostics).

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