Protein Microarrays and Multiplexed Sandwich Immunoassays: What Beats the Beads?

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Abstract: Protein microarray technology allows the simultaneous determination of a large variety of parameters from a minute amount of sample within a single experiment. Assay systems based on this technology are currently applied for the identification, quantitation and functional analysis of proteins. Protein microarray technology is of major interest for proteomic research in basic and applied biology as well as for diagnostic applications. Miniaturized and parallelized assay systems have reached adequate sensitivity and hence have the potential to replace singleplex analysis systems. However, robustness and automation needs to be demonstrated before this technology will finally prove suitable for high-throughput applications. Miniaturized and parallelized sandwich immunoassays are the most advanced assays formats among the different protein microarray applications. Multiplexed sandwich immunoassays can be used for the identification of biomarkers and the validation of potential target molecules. In this review an overview will be given on the current stage of protein microarray technology with a special focus on miniaturized multiplexed sandwich immunoassays.

1 INTRODUCTION

The basic principles of protein microarray technology were described in the early 1980s by Roger Ekins [1]. The ambient analyte theory describes that small amounts of capture probes are the mandatory prerequisites for the detection of the correct analyte concentrations with the highest possible accuracy. In addition to the miniaturized format, an important advantage of microarray assays is their ability to quantify different analytes simultaneously without significantly changing the analyte concentration of the sample. With the further development of DNA chip technology, the interest in microarray-based assays has increased enormously. The possibility of determining thousands of different binding events in a massively parallel fashion in a single experiment suited the needs of genomic approaches in biology perfectly [2]. Currently, DNA-microarrays, some of them built from tens of thousands of different oligonucleotide probes per square centimeter, are well-established high-throughput hybridization systems for the generation of huge sets of mRNA expression analyses in a single experiment. Enormous efforts are currently being undertaken to expand microarray technology beyond DNA chips and to establish protein array-based approaches for proteomic research. Protein microarray technology is a powerful tool for the identification and quantification of large numbers of target proteins from minute amounts of sample within a single experiment. Protein microarray interaction assays can be used to study interactions of proteins with other proteins, peptides, low molecular weight compounds, oligosaccharides or DNA simultaneously "Fig. (**1**)". Low- and high-density protein arrays have been used to investigate binding properties of DNA, RNA, small chemical ligands, and proteins. Microarray-based substrate-enzyme assays have been performed for restriction enzymes, phosphatases, peroxidases, and phosphokinases (recently reviewed in [3, 4]).

An alternative protein microarray set-up is the so-called reverse-phase microarray, a method in which small amounts of a tissue or cell sample are immobilized in a microarray format onto a solid support [5-7]. Different antibodies or patient sera are used to screen these microarrays for the presence of absence of distinct target proteins and for the identification of auto-antibodies. Tissue microarrays can be regarded as another type of a reverse-phase microarray approach. A microarray of tissue samples which contains hundreds of tissue specimens can be screened for the presence or absence of DNA of RNA molecules or proteins using standard analytical methods such as immunohistochemistry, fluorescence *in situ* hybridisation (FISH), or other molecular detection methods [8-10].

One significant advantage of reverse-phase microarrays is the large number of specimens that can be treated simultaneously in an identical manner. This set-up allows the screening of a large set of patient samples for a large number of parameters. In addition, this makes the method a powerful tool that will certainly add to the accelerated identification of new disease-related markers.

Protein microarrays can provide functional data on a genome-wide scale and are required for the functional analysis of complex protein networks within biological systems. There are two major protein microarray-based approaches that can be used to study the expression of proteins: "array-based proteomics," and the more focused protein-profiling approach using "miniaturized and parallelized sandwich immunoassays".

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1386-2073/04 $45.00+.00

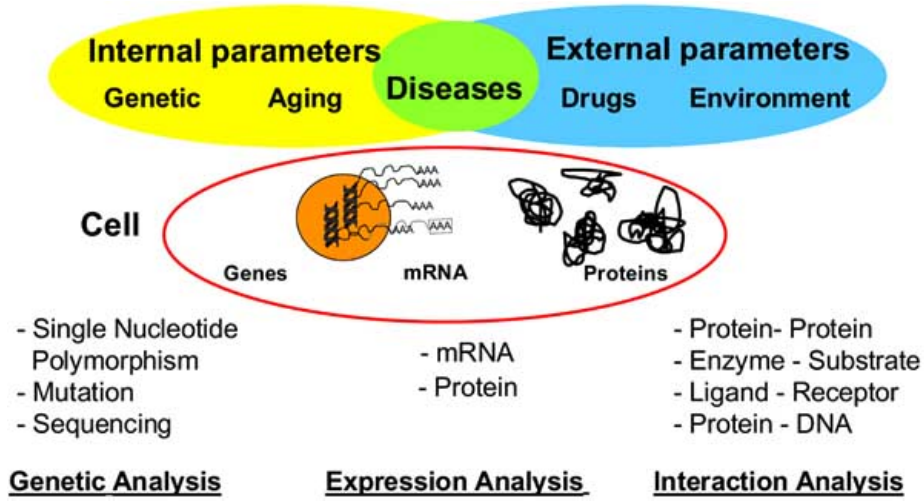

Fig. (1). Microarrays for genomics and proteomics. The physiological state of a cell is influenced by external and internal parameters. Microarray technology allows to monitor changes of genes, mRNA and proteins. DNA-Microarrays are used for genetic analysis as well as to study global mRNA expression. Protein microarrays are used for expression analysis of proteins and to study the function of proteins using protein interaction assays.

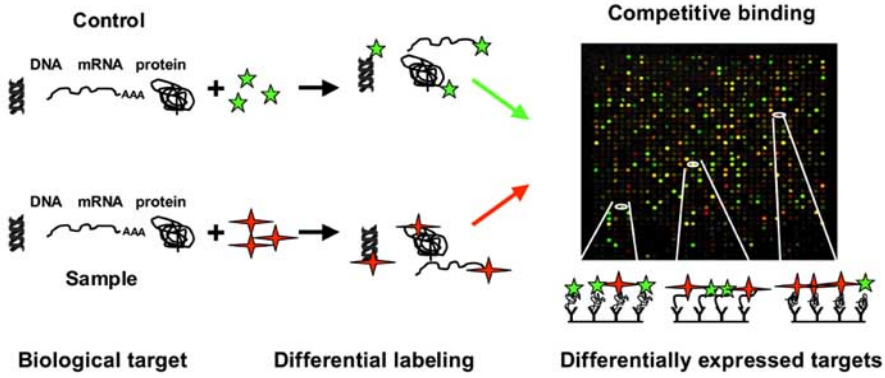

Fig. (2). Microarrays for comparative expression analysis. Principles of differential capture microarray assays: Molecules from controls and samples are isolated and labeled with two different fluorophores. Equal amounts of the samples are mixed and incubated simultaneously on the capture microarray. The labeled target molecules will bind to their immobilized capture molecules. The dual-color detection of bound target molecules reveals the differences in expression between control and sample directly.

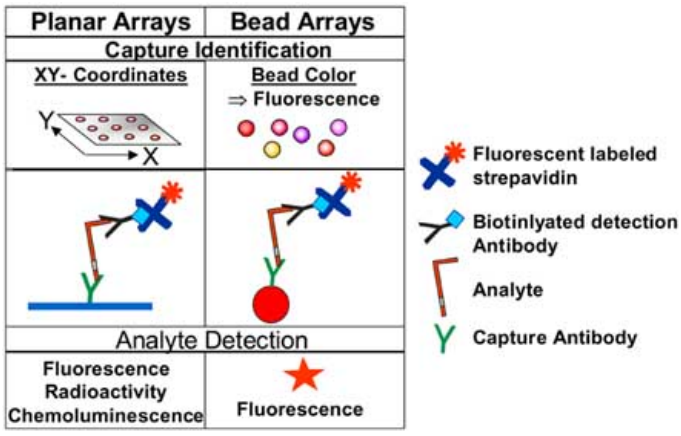

Fig. (3). Different types of microarrays, either planar microarrays or bead-based arrays can be employed for multiplexed ligand-binding assays. Planar microarrays can be generated with hundreds and thousands of different capture spots whereas multiplexing in bead-based arrays is limited to the number of distinguishable beads. The separation of beads is performed via color- (Luminex) and/or size-coding (BD) of capture beads. In planar arrays analyte spots are easily distinguishable by their xy-coordinates in the array. Detection on planar arrays is performed using either chemoluminescence, radioactivity, mass spectrometry, or fluorescence. The latter is the preferred detection method for bound analytes in bead-based microarray assays.

2 ARRAY-BASED PROTEOMICS

The "array-based proteomics" approach transfers the achievements of DNA chip technology directly to the world of proteins “Fig. (**2**)” [11]. However, the design of specific protein capture arrays is based on the knowledge about the target proteins and on the availability of highly-specific capture agents against them. The limited availability of highly-specific capture molecules and their functionality is

Table 1. Examples of Commercially Available Tools, Kits and Services Within the Field of Miniaturized and Parallelized Protein Assays. Planar Protein Arrays are Offered for Comparative Protein Profiling, Reverse Screening, Cytokine Profiling, and Protein Interaction Studies

Company	Product	**Applications**	Web link
Planar Protein Arrays			
BD Biosciences	BD Clontech™ Ab Microarray	Comparative protein analysis	www.bdbiosciences.com
Hypromatrix, Inc.	Signal Transduction AntibodyArray™	Protein-protein Interactions and Protein Phosphorylations	www.hypromatrix.com/
Grace Bio-Labs Inc	ProPlate™ Multiarray Slide System	Integration of microplate and biochip	www.gracebio.com
Molecular Staging Inc.	Rolling circle amplification technology (RCAT™)	Multiplexed protein profiling	www.molecularstaging.com
Pierce Biotechnology Inc.	SearchLight™ Arrays	Cytokine profiling	www.searchlightonline.com
Protometrix, Inc.	The Yeast ProtoArray™	Services protein interaction studies	www.protometrix.com
RayBiotech Inc.	RayBio ™ Cytokine Arrays, Custom Ab Arrays	Cytokine and protein profiling	www.raybiotech.com
Schleicher & Schuell Bioscience	FAST® Frame Multi-Slide Plate, Provision™ HCA	Throughput Processing of Protein Microarrays Cytokine profiling	www.schleicher-schuell.de/bioscience
SIGMA-ALDRICH Co.	Panorama™ Ab Microarray Cell Signalling Kit™	Comparative protein analysis	www.sigmaaldrich.com
Zeptosens AG	ZeptoMARK™ CeLyA Cell Lysate Arrays	Reverse Screening	www.zeptosens.com
Zyomyx Inc.	Zyomyx Protein Profiling Biochip System	Cytokine profiling	www.zyomyx.com
Bead Based Systems			
Luminex Corporation	Luminex 100™	xMAP®	www.luminexcorp.com
	Bender Medsystems	Fluorescent bead immunoassays (FBIs)	www.bendermedsystems.com
	Bio-Rad Laboratories GmbH	Bio-Plex™ Array	www.bio-rad.com
	BioSource International	Multiplexed antibody bead kits	www.biosource.com
	INOVA Diagnostics Inc.	QUANTA Plex™	www.inovadx.com
	LINCO Research, Inc.	LINCOplex™ cytokine arrays	www.lincoresearch.com
	Qiagen	Liquichip	www.qiagen.com
	Radix BioSolutions	Customized assay products for research	www.radixbiosolutions.com
	R&D Systems	Fluorokine® MAP arrays	R&D Systems
	Rules Based Medicine Inc.	Species-specific multi-analyte profiles	www.rulesbasedmedicine.com
	Upstate Biotechnology	Beadlyte®	www.beadlyte.com
BD Biosciences	Cytometric Bead Array (CBA)		www.bdbiosciences.com

Bead based systems are used for focused protein expression profiling like cytokines and cell signaling molecules. So far, most of the commercially available bead based protein microarrays are based on the Luminex™100 platform.

the main difference between DNA and protein microarray technology. DNA is a rather uniform molecule and binds its complementary targets according to the well-defined base-pairing principle. Based on the primary sequence of the target DNA, it is very easy to predict highly selective and specific DNA capture sequences. In addition, high-throughput oligonucleotide synthesis facilities or PCR-based approaches enable the fast and cost-effective generation of any type of DNA capture agents. Unfortunately, proteins are not as easy to handle: It is impossible to predict high-affinity capture molecules for proteins on the basis of their primary amino acid sequence alone. Proteins exhibits very diverse and individual tertiary molecular structures and can interact via strong electrostatic forces, hydrogen bonds or weak hydrophobic van der Waals interactions or all in combination. In addition, proteins appear in complexes and interact with different binding partners simultaneously. Steady or dynamic post-translational modifications such as glycosylation or phosphorylation also have an enormous additional influence on protein interactions. Therefore, each capture molecule must be generated individually following the screening of large sets of candidate capture molecules against their individual target proteins. For proteins, there are no PCR equivalents available either. The development of methods for the cost-effective, fast high-throughput generation of highly-specific, high-affinity protein capture molecules and protein targets comes first on the cumbersome path to establishing array-based proteomic approaches. Last but not least, to retain their functionality, proteins must be immobilized without damaging their tertiary structure. This is, of course, much more difficult than attaching oligonucleotides or PCR fragments onto a solid support. However, microarray surfaces have been optimized to accept functional proteins, protein-labeling technologies have been improved and our knowledge on additives to make the proteins more stable has increased. The successful application of protein microarray technology has already been demonstrated for a variety of assay systems [3, 12].

The principals of array-based proteomics approaches have already been demonstrated "Fig. (**2**)" [13-15]. Similar to the dual-color labeling approach used for the visualization of differential mRNA expression, proof-of-concept studies were performed for differential display protein analysis. In these studies, antibody-antigen microarrays were used in which two different protein samples were labeled with two different fluorophores. Equal amounts of the differently-labeled protein samples were mixed and incubated on the antibody microarray [13]. The differences in the concentrations of the target proteins in each sample on each capture spot were visualized using a confocal biochip reader through dual wavelength fluorescence. But the results have to be handled carefully: Proteins are often assembled to multiprotein complexes. Thus, a strong signal can either result from a large amount of target protein or from the capture of a huge complex of different proteins bound to the captured target. Sreekumar *et al.* [15] used antibody microarrays to investigate the changes in the protein expression level of LoVo Colon carcinoma cells after ionizing radiation treatment. In a similar experiment, Knecevic *et al.* [14] were able to show cancer-specific alterations in the expression of proteins. Both research groups detected more than ten proteins which altered their expression level in response to the ionizing radiation treatment or in correlation to tumor progression, respectively. Meanwhile, the first commercial antibody microarrays constituted of several hundred monoclonal antibodies have become commercially available "Table (**1**)"

An alternative antibody microarray approach to profile the expression of proteins was described by Belov *et al.* [16]. These authors have developed antibody microarrays for the characterization of leukocytes for their pattern of cell surface molecule expression. An antibody microarray containing more than 50 CD-specific immobilized antibodies was used to identify the expressed CD pattern on leukocytes or leukemia cells in a single experiment. A cell suspension instead of a cell lysate was applied to the antibody microarray. Cells only bound to CD-specific antibody microspots against which they express the corresponding CD antigen. Different patterns of cell binding have been obtained for normal peripheral blood leukocytes and different types of leukemia. This type of microarray can be used to perform immunophenotyping. In addition, intact cells which are captured on antibody microspots can be further characterized using soluble, fluorescently-labeled antibodies. Whether such assays can compete with the advanced FACS technology still needs to be demonstrated. Nevertheless, the different types of antibody arrays demonstrate the potential of this technology for the simultaneous analysis of protein expression.

3 MINIATURIZED AND PARALLELIZED SANDWICH IMMUNOASSAYS

Miniaturized and parallelized sandwich immunoassays are of general interest for all proteomic and diagnostic approaches in which several parameters have to be determined simultaneously from a limited amount of sample material and when the number of parameters to be determined is rather low "Fig. (**3**)". Solid phase-bound sandwich immunoassays are one of the major achievements within the field of protein quantitation. The use of two different specific antibodies has led to a dramatic improvement in assay performance and high-throughput capacities [17, 18]. Sandwich immunoassays involve two different antibodies that are presented to two different and spatially separated epitopes of the antigen or target molecule. One antibody is immobilized on a solid support and captures its target molecule from the sample. Using an appropriate detection system, the labeled second antibody is used to detect the bound targets. Such assay types can exhibit an extraordinary specificity and sensitivity to identify analytes in the fM concentration range. This is achieved by a dramatic reduction of background, due to the high specificity of the combination of two analyte-specific antibodies and due to the fact that only a minimal amount of labeled detection antibodies are applied in contrast to the huge amount of labeled sample proteins used in the whole protein labeling approach. The sandwich immunoassay format can easily be adapted to the field of microarray technology. Different types of capture molecules, immobilized at distinct locations, allow the performance of multiplexed sandwich immunoassays "Fig. (**3**)". The feasibility of highly-sensitive multispot and multianalyte immunoassays was already demonstrated more than a decade

ago, and an enormous potential for diagnostic applications was foreseen [1, 19]. Finckh *et al.* developed a very sensitive microarray-based analytical method and were able to prove the high sensitivity of the miniaturized assay. With their microspot system, analytes such as thyroid stimulating hormone (TSH) or Hepatitis-B surface antigen (HbsAG) could be accurately quantified down to the femtomolar concentration range (corresponding to 10^6 molecules/ml) [20]. Different groups have applied sandwich immunoassays to the protein microarray format and have been able to identify and quantify up to 24 different cytokines from conditioned media and/or patient sera [8, 21-25].

Schweitzer *et al.* have described the most complex multiplexed sandwich immunoassay so far. This assay allowed the quantification of 75 different cytokines [26]. However, it was not possible to perform a 75-plex sandwich immunoassay within a single microarray. This might have been due to cross-reactivity of some of the detection antibodies with immobilized capture antibodies or with the non-specific analytes. To separate the different cross-reacting antibodies, two sets of multiplexed sandwich immunoassays were generated containing 38 or 37 distinct features in a single microarray, respectively. These microarrays were used to study the expression of target protein present in stimulated and non-stimulated human dentritic cells. The highly-sensitive isothermal rolling circle amplification method was used for detection purposes. Different cytokines could be detected in the femtomolar concentration range. In principle, it should be feasible to detect single binding events on microspots using this amplification method [27]. Molecular Staging Inc. (www.molecularstaging.com) uses this technology to offer a service for multiplexed protein analysis designed to yield a comprehensive insight into the biology of the underlying disease and drug response.

The aspect of automation for miniaturized and parallelized sandwich immunoassays was already addressed several years ago. A fully-automated, microarray-based approach for high-throughput, enzyme-linked immunosorbent assays adapted to the microtiter plate format was already described by Mendoza *et al.* [28]. This system consisted of an optically flat glass plate with 96 wells separated by a Teflon mask. A microarray containing more than a hundred different types of capture molecules could be immobilized in each well. Sample incubation, washing, and fluorescence-based detection was performed with an automated liquid pipettor. The microarrays were quantitatively imaged with a scanning charge-coupled device detector. Thus, the feasibility of multiplex detection of arrayed antigens in a high-throughput fashion using marker antigens could be successfully demonstrated. However, this approach could not be transferred into research laboratories, probably due to the fact that most of the current microarray equipment is adapted to the microscope-slide format. Any new format would need an adjustment of the arrayer and the biochip reader before they can handle the new format. Several companies have started to offer a microtiter plate format that is adapted to the microscope-slide format (Table 1). A grid corresponding to two rows of a 96 well microtiter plate is placed onto a single microscope slide containing 16 identical arrays. Four slides are used to fill a frame of a standard microtiter plate. This kind of set-up allows the performance of miniaturized and multiplexed assays manually or using standard pipetting systems. For the readout, the grid is removed and the microscopic slides analyzed in a standard biochip reader. This allows each individual research facility to increase their throughput. Besides this, companies also offer miniaturized multiplexed sandwich immunoassays in a microtiter plate format "Table (**1**)".

Other platforms for planar microarrays have been developed by several companies. Randox (www.randox.com), for example, has launched the automated "Evidence" biochip array system along with prefabricated microarrays that can be used to test for drug abuse. Zyomix (www.zyomyx.com) has developed a protein microarray system on the basis of an appropriate surface chemistry that will allow the attachment of proteins in their functional state and defined orientation. Zeptosens AG (www.zeptosens.com) offers a highly sophisticated protein microarray system which is coupled to a robust and very sensitive detection system based on planar waveguide technology.

Besides the planar microarray-based systems, which are perfectly suited to screen for a large number of target proteins, bead-based assays are a very interesting alternative, especially when the number of parameters of interest is comparably low "Fig. (**3**)". Bead-based assay systems employ different color-coded or size-coded microspheres as the solid support for the capture molecules. A flow cytometer, which is able to identify each individual type of bead and quantify the amount of captured targets on each individual bead is also required. In a first step, antigen-specific capture antibodies are immobilized on the individual bead type. Different bead types are combined and incubated with the sample of interest. A labeled secondary antibody detects captured analytes and is visualized with a fluorescent reporter system. Sensitivity, reliability, and accuracy are similar to those observed with standard microtiter ELISA procedures. Color-coded microspheres can be used to perform up to a hundred different assay types simultaneously. The flow cytometers can identify several thousand of beads in a second, hence allowing the quantitation of the amount of captured analytes "Table (**1**)" [29-33]. For example, microsphere-based systems have been used to simultaneously quantify cytokines or auto-antibodies from biological samples [30, 31, 34-37]. Bellisario *et al.* have used this technology to measure antibodies HIV-1 antigens from newborn dried blood-spot specimens simultaneously [37]. Opalka and collegues have used a bead assay system to quantitate neutralizing antibodies directed against the papilloma virus simultaneously. Using a competitive immunoassay, these researchers were able to measure the titers of polyclonal antibodies in serum directed against conformational sensitive, neutralizing epitopes on the respective virus-like particles accurately. The competitive bead-based immunoassay proved to be as sensitive, accurate, and precise as a competitive radio immunoassay [38].

McBride *et al.* [39] have developed a bead-based multiplexed sandwich immunoassay to detect a broad range of pathogens including virus, protein toxins, and bacterial spores. The assay performance of the multiplexed assay revealed excellent specificity and resulted in similar dynamic range and sensitivity as observed for the singleplex enzyme-

linked immunosorbent assays. The same group also described the integration of the bead-based system into a fully-autonomous pathogen detection system that is capable of monitoring the environment for airborne biological threat agents continuously. The system performs aerosol collection, multiplexed immunoassays, sample archiving, data reporting, and alarming. The complete system was successfully evaluated to detect virulent biological threat agents such as *Bacillus antracis* and *Yersinia pestis* [40].

Based on the bead-based Luminex technology several companies offer a steadily growing list of ready–to-use multiplexed sandwich immunoassays to quantitate cytokines, cell signaling molecules, for example, and to analyze kinase activities "Table (**1**)". Rules Based Medicine (www.rulesbasedmedicine.com) offers a special service to screen mouse or human serum samples for the presence or absence of approximately 90 mouse serum parameters and approximately 170 human serum parameters from minute amounts of sample volumes. Using the bead-based technology, thousands of samples can be screened within a very short time. Throughput and sample volume are no longer problems for the bead-based systems. Recently, the BD™ FACSArray Bioanalyzer (www.bdbiosciences.com) was launched as an alternative bead-based system (Cytometric Bead Array, BD™ CBA). This bead-based system can discriminate different bead sizes and can use a two-color detection, hence allowing the design of more complex assays.

The advantages of the liquid array-based assays over the planar assays include their flexibility, the robustness of the system, and the advanced state of automation. The basic technology for the liquid arrays, the FACS technology, is a highly developed and very robust technology. This technology has been routinely used in diagnostics for more than 20 years. In addition, the assays have already been adapted to the microtiter plate format, a format which is highly automated in routine diagnostic and screening applications. With regard of assay types, there are several companies that offer different panels for focused protein quantitation assays such as cytokine and growth factor panels for human, mouse, and rat, as well as cell-signaling molecules "Table (**1**)". However, it is also possible to set up one's own assays if no appropriate commercial assay is available. The bead-based system has the additional advantage that it is quite ease to integrate a simple quality control at every step of the process. A quality control step can be introduced after the immobilization of the capture antibodies on the bead surface in order to ensure that only those beads are mixed together which contain the capture molecules in a functional state. In addition, it is simple to keep the immobilized proteins in a functional state since they are always kept in solution. Additional parameters can easily be introduced to already existing panels.

4 OUTLOOK

Before it will be possible to apply microarrays for screening or diagnostic purposes, these miniaturized assay systems have to demonstrate excellent precision, sensitivity, and reliability. In addition, the system must be suitable for rapid analyses and be amenable for automation. At present, immunoassays used in clinical diagnostics or HTS systems used in the pharmaceutical industry are highly automated and extremely robust. Any new assay format for diagnostic applications or screening approaches has to compete with this highly robust technology - not only with regard to its performance but also with regard to its costs. New instruments in combination with novel assay formats will involve huge investments. As long as only a few additional parameters have to be analyzed from the same sample, microarrays are not competitive. It will be much more economical to increase the throughput of the currently available highly robust and automated diagnostic analyzers. However, with an increasing number of parameters (as they are already available in allergy diagnostics, for example) or with regard to the simultaneous screening of potential drug candidates for selectivity and cross-reactivity, the multiplexing approach is certainly a very promising alternative.

Miniaturized and multiplexed immunoassays will improve the throughput by greatly increasing the amount of information obtained from a single experiment. In addition, they will lower the expenditures by reducing the amount of material and reagents needed. The reduction of sample volume is of great importance for all those applications where only minimal amounts of samples are available (e.g. analysis of multiple tumor markers from a minimum amount of biopsy material). Therefore, protein microarray-based technology will be successfully applied to different aspects within the broad field of proteomics and diagnostics.

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Received: 07 March, 2004 **Accepted: 18 March, 2004**