Development of a protein microarray platform for the multiplex analysis of biomarkers associated with Rheumatoid arthritis

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DECLARATION

I declare that I wrote this thesis "Development of a protein microarray platform for the multiplex analysis of biomarkers associated with Rheumatoid arthritis" with the help indicated and only handed it in to the faculty of science of the University of Basel and to no other faculty and no other university to the dean's office.

Teresa Urbanowska

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ABSTRACT

Currently in the drug development process there is a growing awareness of the need to utilise a biomarker strategy which would allow compounds to be developed in a more efficient way with improved safety and pharmacology. Technologies which can evaluate, validate and monitor biomarkers in a cost effective and efficient manner are a necessity if such a biomarker strategy is to be properly implemented. In this thesis the development, validation and implementation of a protein microarray for quantitative and simultaneous analysis of proteins is described. In order to demonstrate the feasibility of this approach, Rheumatoid arthritis (RA) was chosen as a model for proof of concept. Based on the current literature, seven proteins thought to be associated with the development and progression of RA were selected. Initially, a protein microarray was developed on a glass chip treated either with a self assembled monolayer (SAM) of octadecyl phosphoric acid ester (ODP) or with poly-L-lysine. SAM showed its superiority over poly-L-lysine by generating more homogenous and less variable spots. However, the process of coating the chip with the SAM was time consuming and expensive. Moreover, assay processing was entirely performed manually and could not be automated without a significant investment of time and resources. As a result, high inter-chip variability was observed preventing sensitive, quantitative and reproducible analysis to be performed. An attempt was, therefore, undertaken to develop an alternative microarray platform. The appearance on the market of long neck tips for antibody printing devices, provided the option of using established polystyrene 96-well plates as the solid support for developing a fully automated microarray format. The development process involved reagent selection, printing protocol optimization, matrix investigation, assay protocol establishment, and detection system evaluation. The robustness and reproducibility of the methodology was investigated using the Food and Drug Administration (FDA) regulatory guidelines for pharmacokinetic assay validation, in which a spike-recovery validation test was elaborated and run over 3 days. The method was shown to be both quantitative and reproducible, with an assay accuracy between 70-130%, and assay precision less than 30%. Importantly, the working range for each assay covered the relevant physiological concentrations. In addition, protein microarray performance was compared with the classical ELISA approach. Sera collected from a total of 78 individuals representing either rheumatic or healthy patients were measured using

both approaches. Correlation coefficients (R^2) between the two technologies was calculated for each analyte giving: 0.90 for A, 0.60 for B, 0.93 for C, 0.96 for D, 0.94 for E and 0.95 for F. Finally, the developed protein microarray was used to compare the analyte concentration levels between patients with RA and other rheumatic diseases. Significant differences in the serum concentration of B ($p<0.0022$), C ($p<0.0107$), E ($p<0.0024$) and F ($p<0.0057$) between RA and other arthritic patients were observed. In conclusion, the obtained results demonstrate the applicability of the developed protein microarray for quantitative and simultaneous analysis of the selected RA-related proteins in clinical samples. It is anticipated that miniaturized and multiplexed immunoassays which allow for the rapid evaluation of multiple analytes in a single sample, will represent a valuable tool for validating and monitoring biomarkers in the drug development process.

1 General Introduction

The completion of the human genome project, and sequencing of additional genomes served to drive the development of new innovative technologies for genome-wide expression profiling (Kumble 2003). These so called “-omic” technologies, such as genomics and proteomics, have made it possible to follow transcriptional and translational events of genes and even the entire genome in response to biological processes (Hegde, White, & Debouck 2003). The “-omic” technologies are being increasingly used in the pharmaceutical industry at all levels of drug discovery and development, and commonly applied for the discovery of new drug targets, identification of drug efficacy and toxicity biomarkers, investigations into mechanisms of drug action and toxicity, and prioritization of new drugs (Colburn 2003; Guerreiro et al. 2003). This is exemplified by DNA microarray technologies (genomics) that allow for the high throughput quantitative comparison of the transcriptional activity of potentially thousands of genes in a single experiment (DeRisi, Iyer, & Brown 1997). Complex molecular perturbations occurring during the onset of disease and progression, and therapeutic intervention can be thoroughly interrogated from a multitude of target organs and cells, thereby, providing a more holistic approach to biological responses.

It is now accepted, however, that changes at the mRNA level are not necessarily proportional to changes at the protein level because of differences in rates of protein translation and degradation (Griffin et al. 2002). Furthermore, nucleotide screens are unable to provide information on the post-translational modifications of a protein, which may be critical for a protein's function (Kumble 2003). After all, it is the protein and not the mRNA that is the ultimate, biologically functional entity, providing the cellular function, whether it be for communication, metabolism or building cellular architecture. Therefore, there is a clear need to analyse the proteome activity of a biological sample and to complement this with genomics in order to obtain a higher level of understanding regarding the function of highly complex cellular networks.

Proteomics is a complementary approach to genomics, and encompasses protein analysis technologies which deals with the global separation, quantification and

functional characterisation of expressed proteins. Techniques like mass spectrometry in combination with separation tools such as two-dimensional gel electrophoresis and multidimensional liquid chromatography, allow for the parallel analysis of hundreds of proteins and peptides (Aebersold & Mann 2003). Although powerful, these techniques, however, are low throughput and require a high degree of technical skill. In particular, they currently lack the sensitivity and dynamic range to detect very low abundant proteins in complex biological samples (Sydor & Nock 2003). This is especially the case for body fluids such as serum, plasma which are exceptionally difficult to analyse due to a dynamic range of more than 10 orders of magnitude in concentration that separates the highest and lowest abundant proteins (Liotta et al. 2003).

Limitations of existing proteomic technologies have consequently driven the development of novel tools for the investigation of proteomes. An emerging technology is the protein microarray (Kodadek 2001; Templin et al. 2002; Wilson & Nock 2003; Zhu & Snyder 2001) which is developed using the concept of the current DNA microarray for mRNA expression profiling (Schena et al. 1995), but to profile protein expression (Templin et al. 2004). However, the biochemical diversity, and the sheer number of proteins are such that an equivalent analysis is much more complex and thus difficult to accomplish. Instead, low density antigen and antibody microarrays can be used as a high-throughput multiplex screening tool to measure specific target proteins associated with a disease process or therapeutic intervention (MacBeath 2002). They have a wide range of potential applications, especially in the drug development process. Antibody microarrays could potentially revolutionize the area of biomarker discovery, validation and monitoring of markers of disease onset and progression, drug efficacy and toxicity (Kodadek 2001). Most importantly, protein microarrays have the advantage of scalability, being amenable to automation, multiplexing as well as low sample volume requirements while giving a high degree of sensitivity and broad dynamic range.

This introduction is in two parts. The first part provides an overview of the protein microarray concept, the current strategies used to generate protein microarrays, and the technical aspects. The challenges and shortcomings of these approaches, as well as the potential applications of protein microarrays will be discussed. The second part

provides and overview of the pathogenic complexity of rheumatoid arthritis. Rheumatoid arthritis in this thesis was chosen as a disease model for proof of concept to demonstrate the feasibility of a microarray platform for monitoring the expression levels of selected proteins described to be associated with RA.

1.1 Protein microarray technology and concept

The protein microarray is based on the technology of coupling the capture molecules, either antibodies, proteins, antigens or ligands, down onto a support in an array format for multiplexed analysis (Haab, Dunham, & Brown 2001; MacBeath 2002; Zhu & Snyder 2003). As the concept is, that each of the capture molecule-protein analyte pairs is mutually exclusive, that is, each capture molecule only binds to a single cognate protein analyte and vice versa, a single microarray comprising multiple capture molecules can be used to assay the presence of different protein analytes in a complex mixture. There are two major technologies that are being utilized for multiplex measurement: spot-based arrays, and bead-based fluidic arrays. An example of the fabrication of these two technology platforms using antibodies as the capture agents is shown in Figure 1.

Spot-based arrays are primarily produced by direct printing of capture molecules onto the planar support surfaces (Huang 2001; Moody et al. 2001; Wiese et al. 2001). Planar surfaces involve glass slides which can be treated with numerous chemistries to improve binding capacity, plastic plates and membranes (Huang 2003). Deposition of subnanolitre volume of capture molecule solution is achieved by printing technologies. Detection of captured protein is usually accomplished by fluorescence or chemiluminescent system. Imaging in these cases is accomplished with a CCD camera or laser scanner (Seong & Choi 2003). Bead-based fluidic arrays utilize beads with colour and/or size differentiation of which capture molecules are conjugated to. Captured molecules on the beads are detected by fluorescence labelling and the signal is analyzed by flow cytometry (Bellisario, Colinas, & Pass 2001; Dunbar et al. 2003). This chapter will primarily focus on spot-based array technology.

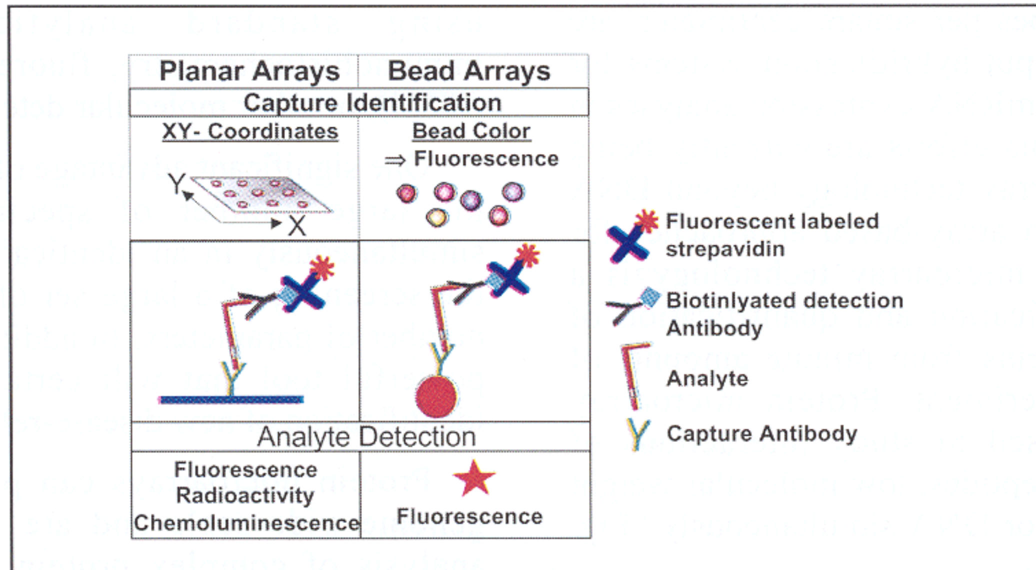

Figure 1: Different types of microarrays, either planar microarrays or bead-based microarrays can be employed for multiplexed microarrays. The figure was taken from (Templin et al. 2004).

1.1.1 Assays on a microspot: fundamental principles of spot-based arrays

The ability to miniaturize microarrays is of fundamental importance, especially with regard to assay sensitivity and sample amount needed. The basic principles of miniaturizing microarray assays were described by Ekins and coworkers (Ekins 1989; Ekins 1998). This theory demonstrates that the analysis of the same amount of capture agent in a small area is more sensitive than in a larger area (Figure 2). The sensitivity of the system is increased because the binding reaction occurs at the highest possible concentration, and the capture-detection complex is localized in the microarray spot. This occurs because the target molecule is not the limiting factor in a small spot. Signal intensity will remain virtually constant below a certain spot size (Ekins 1989), even if there is a further reduction in spot size. As the spot size increases, for a given amount of capture agent, the signal intensity subsequently decreases (Espina et al. 2003).

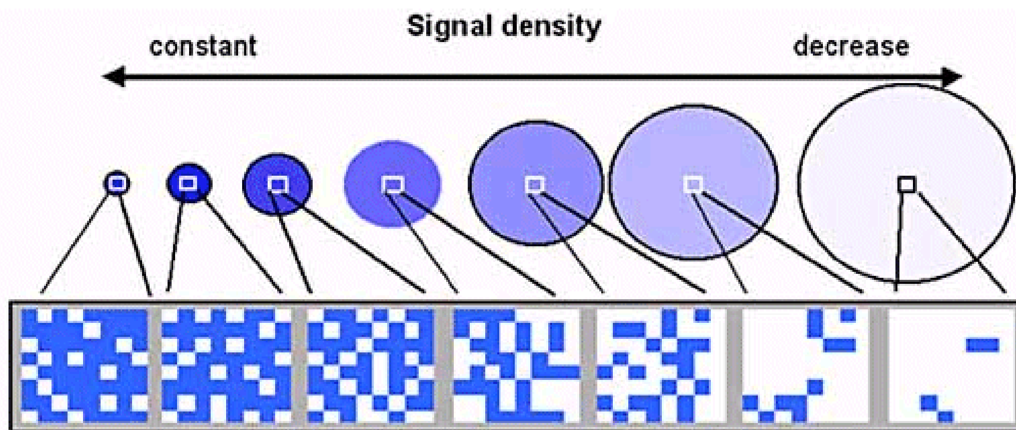

Figure 2: As the arrayed spot size increases for a given analyte concentration, the density of the spot reaches a maximum. In other words, for a given concentration of analyte occupying increasing spot diameters, the intensity (density) of the spot will decrease as the spot diameter increases. This is due to the same number of target molecules occupying an increasingly larger area. In contrast, as the spot size decreases, the density increases for a decreasing concentration of analyte. There is a point at which the spot intensity will remain constant for decreasing spot size. Incorporation of a sample dilution curve into the printed array format permits the analysis of each analyte in its linear dynamic range without limitations due to the unmatched antibody association/ dissociation constants. This figure was adapted from (Stoll et al. 2002).

1.1.2 Types of spot-based array formats

Depending on the desired application, different microarray designs can be constructed. Currently there are two types of protein microarray set-ups which have been defined: reverse phase arrays (RPAs) and forward phase arrays (FPAs) (Liotta et al. 2003) (Figure 3).

In RPAs a small amount of a tissue or cell sample is immobilized on each array spot, such that an array is composed of different patient samples or cellular lysates (Espina et al. 2003; Paweletz et al. 2001; Petricoin et al. 2002). In the RPA format, each array is incubated with one detection protein (e.g., antibody), and a single analyte endpoint is measured and directly compared across multiple samples (Figure 3).

In FPAs, capture agents, usually an antibody or protein antigen, are immobilized onto the surface and act as a capture molecule. Each spot contains one type of immobilized antibody or capture protein. Each array is incubated with one test sample, and multiple analytes are measured at once (Figure 3).

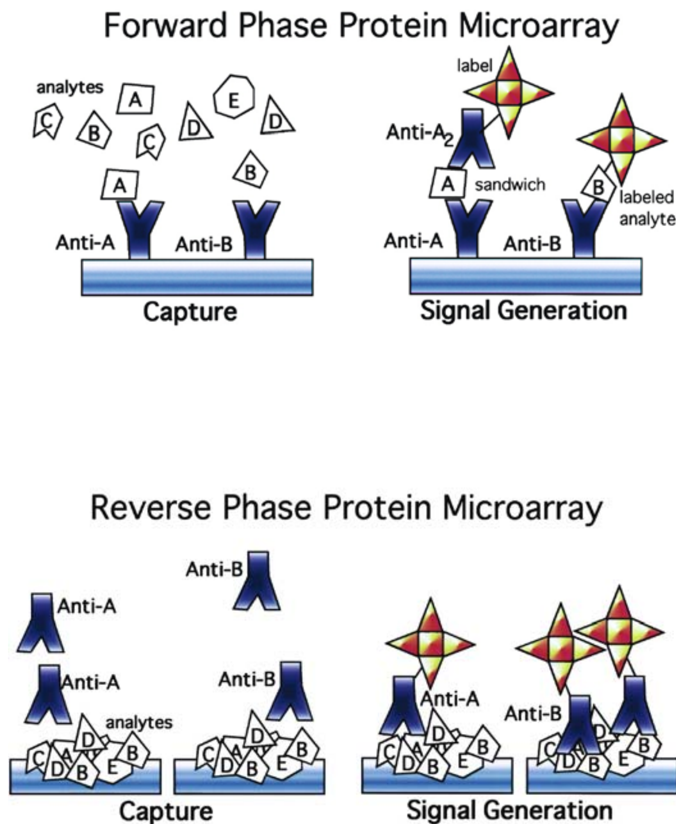

Figure 3: Types of protein microarray platforms. Forward phase arrays (top) immobilize a capture molecule such as an antibody designed to capture specific analytes with a mixture of test sample proteins. The bound analytes are detected by a second sandwich antibody or by labeling the analyte directly (upper right). Reverse phase arrays immobilize the test sample analytes on the solid phase. An analyte-specific ligand (e.g., antibody; lower left) is applied in solution phase. Bound antibodies are detected by secondary tagging and signal amplification (lower right). The figure was taken from (Liotta et al. 2003).

Antibody microarray is one form of FPAs. Antibody microarrays are usually produced in two forms, either by a sandwich assay or by direct labelling approach (Zhou et al. 2004). The sandwich assay approach to antibody arrays is simply a multiplexed version of standard ELISA immunoassays. This approach utilizes a matched pair of antibodies specific for every protein. One antibody is immobilized on a solid support and captures its target molecule from the sample. Using the appropriate detection system, the labelled second antibody detects the bound targets. The main advantage of the sandwich assay is its high specificity, accuracy and sensitivity (Templin et al. 2004). High sensitivity is achieved by a dramatic reduction of background yielding a high signal-to-noise ratio. This is primarily due to the high specificity accomplished through the combination of two analyte-specific antibodies

(Maiolini & Masseyeff 1975). Quantitative information of the abundance of proteins can also be derived from the sandwich assay approach (Huang et al. 2001; Schweitzer et al. 2002). The main drawback of this approach is the requirement for a pair of validated antibodies to be available for each analyte being analysed, therefore, limiting the scalability of this format.

As an alternative to the sandwich assay, protein-containing samples can be labelled with fluorescent tags followed by direct detection after capture. Other haptens such as radioactive isotope can also be used as label, followed by detection with an anti-hapten antibody conjugated to a fluorescent dye or to an enzyme such as horse-radish peroxidase (Zhou et al. 2004). An adaptation of the direct labelling approach was described by Haab and co-workers (Haab, Dunham, & Brown 2001) (Figure 4). In their approach, proteins from two different biological samples are labelled with either Cy3 or Cy5 fluorophores. These two labelled samples are then equally mixed together and applied to an antibody microarray. In this case, one compares the relative fluorescence intensity at each antibody feature so as to identify differences in protein levels between the two samples, for example treated vs. untreated or diseased vs. healthy. This approach has successfully been used to study cancer markers (Miller et al. 2003) and to protein profiling in cancer tissues (Knezevic et al. 2001).

The main advantages of direct label arrays are that detection antibodies are not necessary and incubation time of the assay procedure is relatively short (Li, Nath, & Reichert 2003). A major limitation to this approach is the poor signal-to-noise ratio often obtained, meaning that the sensitivity is generally not very high. Since potentially all proteins in a sample are labelled, proteins typically present in high amounts such as albumin in serum can non-specifically bind or adsorb to the antibodies or to the microarray surface leading to the measurable interference (Haab 2003). The background binding would subsequently reduce detection sensitivity or data accuracy. In addition direct labelling procedures can reduce the solubility and structural integrity of the analysed proteins (Phelan & Nock 2003) and labelling efficiency is often variable which would then compromise reproducibility (Phelan & Nock 2003). Another problem with this approach is that it does not quantify the protein antigens, but merely states relative abundance between samples.

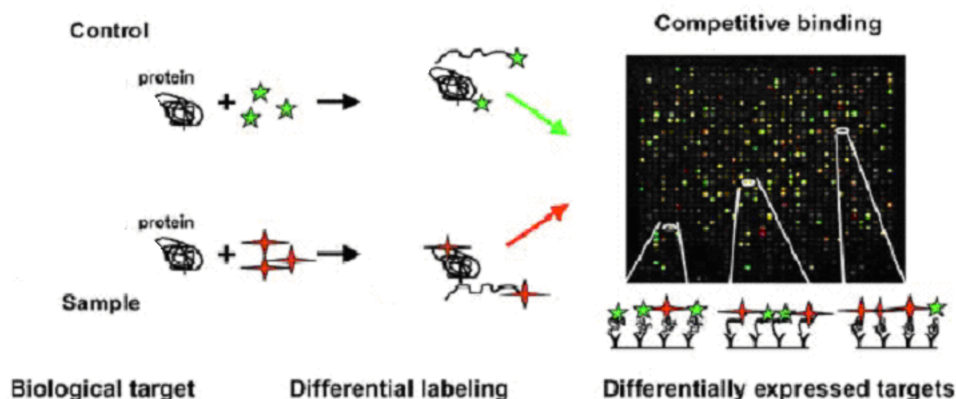

Figure 4: Comparative expression analysis using direct labelling. Molecules from controls and samples are isolated and labelled with two different fluorophores. Equal amounts of the samples are mixed and incubated simultaneously on the capture microarray. Labelled target molecules bound to their immobilized capture molecules are detected using dual colour modes. The signal intensities between the control and test samples are quantitatively compared and expression differences identified. The figure adapted from (Templin et al. 2004).

1.2 Protein microarray development: critical factors and challenges in spot-based arrays

In the development of protein microarrays many critical factors and analytical challenges are encountered. One of the most critical factors is the careful selection of capture molecules to avoid cross-reactivity. The capture molecule in the multiplex array must specifically recognize a single protein in a complex mixture and not cross-react with any others, this is referred to as specificity (Phelan & Nock 2003; Templin et al. 2003; Zhu & Snyder 2003). Another major challenge concerns the wide range of analyte concentration to be detected. Proteins of interest may exist in a broad dynamic range (up to a concentration of factor 10^{10}) (Espina et al. 2003), especially in body fluids. Consequently, the assay may need to simultaneously detect proteins present at very different concentrations on a single array. It is, therefore, important to identify capture agents which are highly specific for the protein of interest, with an affinity sufficient to effectively capture proteins at various concentrations (Phelan & Nock 2003). Another critical factor involves the immobilization of capture agents onto the solid support. It is crucial that capture agent are stable and remain in a functional state once immobilised. Among the other technical factors which are critical for the

successful development of protein microarray, there are the solid supports and chemical surfaces, printing methods, assay matrix, signal generated and signal detection (Haab, Dunham, & Brown 2001; Huang 2003; Templin et al. 2003; Zhu & Snyder 2003). These will be discussed in more detail in the following sections.

1.2.1 Capture agents

A capture agent is referred to as the molecule which is immobilized onto a microarray solid support and which is specific for a target molecule (Phelan & Nock 2003). Different types of molecules can serve as possible capture agents, and these are summarized in Table 1.

Table 1: Classes of capture molecules.

Capture molecule	**Reviewed in**
mAb	(Goldman 2000; Kohler & Milstein 1975)
Polyclonal sera	(Valle & Jendoubi 2003; Zhang 2003)
Antibody fragments (scFv/Fab)	(Gao et al. 1999; Knappik et al. 2000)
Aptamers (DNA/RNA/peptide)	(Brody & Gold 2000)

mAb - monoclonal antibody

Fab - antigen-binding fragment

scFv - single-chain variable region fragment

Highly specific capture molecules which exhibit high affinity to their target molecules are a prerequisite for the establishment of protein microarrays used for the identification and quantification of target proteins. Antibodies are particularly well suited to the task of protein profiling on a microarray, and represent the most common capture agents used for protein profiling microarrays (Huang 2003). Highly specific monoclonal antibodies can be generated by mouse immunization and continuous culture of hybridoma cells (Goding 1980). Importantly, this represents a potentially unlimited supply of uniform and pure binding molecules. Polyclonal antibodies, on the other hand usually contain multiple epitope specificities and are limited in quantity to the amount of serum that can be obtained from the immunized animal. Moreover, their performance in a protein microarray format may be impaired by a

decreased density of specific binding sites due to the presence of antibodies that do not recognize the protein of interest. Thus, monoclonal antibodies are preferred as capture agents for microarray fabrication. However, due to the lengthy, labour-intensive and thus expensive nature of monoclonal antibody production efforts have been undertaken to develop alternative technologies (Phelan & Nock 2003). One attractive approach is to use phage display techniques combined with highly diverse synthetic libraries. These libraries can be used to isolate antibody fragments against target proteins in a significantly shorter time frame than it is possible with immunization-based methods (Li 2000). Phage-display libraries of antibody fragments, therefore, offer the potential for antibody production in a large scale.

A major challenge encountered with the development of protein microarrays is the requirement for high affinity capture agents. Capture agents need to exhibit an affinity to the target molecule which is relevant to effectively capture target molecules. The binding of protein to capture molecule can be quantified by dissociation rate constant (K_d) (Wild 2001). The dissociation constant of monoclonal antibodies for their cognate proteins varies from 10^{-7} to 10^{-12} M. These values often define the lower limit of assay detection, thus detection sensitivity increases with the decreased K_d for capture antibody (Varnum, Woodbury, & Zangar 2004). For protein microarrays the dissociation constant for a useful antibody is generally at least in the single-digit nanomolar range (Wilson, Phelan, & Nock 2005). Lower affinities are usually associated with rapid ($>10^{-3}$M/sec) dissociation rates and a loss of antibody- antigen complexes during the wash and incubation steps (Wilson, Phelan, & Nock 2005).

For accurate quantitative measurements a relationship must be obtained between signal intensity and analyte concentration (Wild 2001). The affinity constants, however, constrain the range of the assay (Wild 2001). The detection range can only be attained if the concentration of the protein analyte and antibody are properly matched to the affinity. That is why it is important to select the capture and detection antibodies with the affinities adequate to measure the required concentrations of each analyte of interest belonging to the multiplexed array.

1.2.2 Solid support and surface chemistry

Protein microarrays may be fabricated on glass and plastic slides treated with various surface chemistries, plates or membranes. An optimal solid support or surface chemistry for protein microarrays should have the following characteristics: high binding capacity, preservation of capture agent functionality after immobilization, ease of manufacture, high reproducibility, high signal-to-noise ratio, and reasonably easy to manipulate (Kumble 2003).

An important issue during the microarray preparation is that the capture agent remains in a functional state once immobilized onto a solid surface. For maintaining functional integrity, buffers with similar properties to the physiological fluid are typically used for the preparation of capture agents solutions prior to spotting. For instance, the addition of stabilizers such as (30-40%) glycerol (MacBeath & Schreiber 2000; Zhu & Snyder 2003) can help maintain the native properties of antibodies. It is also advantageous to have the antibodies in a solution with the physical characteristics similar to physiological properties, (e.g., viscosity), to ensure uniformity between the different antibody spots.

A wide variety of surface substrates and attachment chemistries have been evaluated for the immobilization of capture agents on protein microarrays. Hydrophobic plastics such as polystyrene are commonly used surfaces. Most proteins physically adsorb onto polystyrene surfaces by van der Waals, hydrophobic and electrostatic interactions (Espina et al. 2004). This type of physical adsorption is generally used to immobilize antibodies onto polystyrene 96-well plates (Moody et al. 2001). The advantage of this type of immobilization is that it is relatively simple to perform, since it does not require any modifications of the protein for its attachment to the surface. The disadvantage is that the immobilized proteins often becomes denatured due to multiple uncontrolled interactions between the protein and the surface material (Morozov 2005). The physical adsorption of proteins onto surfaces also tends to be heterogeneous, with proteins clustering together in patches (Sydor & Nock 2003). Adsorption of proteins onto surfaces can also lead to problems with protein desorption during the assay, which can lead to signal loss. Similar effects are observed on other surfaces used for non-covalent protein adsorption, such as poly-lysine coated glass

which is hydrophilic and positively charged (Haab, Dunham, & Brown 2001) or hydrophobic nitrocellulose.

An alternative method for attachment of protein to surfaces is by covalent binding via amines or other amino acid side chains on chemically coated surfaces, such as aldehyde (Angenendt et al. 2002; MacBeath & Schreiber 2000; Peluso et al. 2003). The advantage of covalent binding is its stability, and the capture agents are immobilized at very high densities (Lahiri et al. 1999). This directly translates to highly sensitive detection. A drawback, however, is the possibility of protein denaturation.

Both adsorption and covalent binding approaches, however, attach proteins to the surface in a random fashion. As a consequence, this may alter the native conformation of proteins, reduce the activity of proteins or make them inaccessible to the binding agent (Zhu & Snyder 2003). An alternative approach is the utilization of site-oriented immobilization methodologies (Templin et al. 2002; Zhu et al. 2000). Nock et al. (Peluso et al. 2003) found that 90% of Fab' fragments attached to a streptavidin-coated surface through biotinylation of the thiol group of antibody Fab' fragment are active, while randomly attached Fabs have up to sixfold lower activity. The benefit of orienting full-length antibodies is, however, by far not as pronounced. This is reflected in the fact that a wide variety of substrates have been used successfully for antibody microarray applications (Nielsen & Geierstanger 2004). The disadvantage of the oriented immobilization is the possible loss of the capture agent functionality.

1.2.3 Arraying devices for immobilisation of capture agents

One of the main technical challenges of microarray measurements is the production of the microarray themselves. In order to carry out reproducible and reliable assays on a protein microarray, it is necessary to print the capture agent in a way that results in efficient deposition of functional capture agent. Commercially available array printers, providing different types of printing methods, can be used for the printing of micrroarrays in a variety of configurations (Schena 2000). Each printing method has its advantages and disadvantages depending on the type of sample to be printed,

sample volume and microarray density. Printing technologies currently exist in two forms: contact and non-contact arrayers.

1.2.3.1 Contact printing devices

Contact printing is accomplished by direct contact between a metal pin head and the solid surface. Contact printing devices come in three main formats, solid pin, quill, and pin and ring.

A solid pin printing assembly is composed of a solid pin with a flat end. Submerging the pin in a liquid sample transfers a sub-nanolitre volume of sample to the tip of the pin. Direct contact of the pin with the surface delivers the fluid to the surface. The pin diameter and fluid properties determine the volume of fluid deposited, and thus the spot size. The solid pin format does not lend itself to duplicate or triplicate printing due to the necessity of the pin to be re-submerged in the sample for each spot printing (Espina et al. 2003; Schena 2000).

A quill type printing assembly consists of a flat pin head with a defined hollow bore, similar to quill-style writing instruments. Sample fluid wicks into the hollow space and is deposited on the solid surface when the pin head touches the surface. The quill style formats allow multiple spot printing from each sample (Schena 2000).

Pin and ring assemblies are a combination of a ring that holds microliter quantities of sample and a flat head pin. The pin travels through the fluid retained in the ring and deposits the sample on the solid surface. The pin and ring assembly is capable of replicate spot printing (Schena 2000).

The dispensed volume of printing solution during the contact printing process ranges from 0.3-2 nL per drop (Espina et al. 2003). The main disadvantage of contact printing is the danger that proteins might by denatured while the pin touches the surface. The protein can also be deactivated or lose its functionality because of direct contact with the metal pin during printing. Moreover, proteins might remain attached to the pin heads following washing leading to cross-contamination of samples. Lastly

contact printing might result in less homogenous protein spotting causing assay variability.

1.2.3.2 Non-contact printing devices

Non-contact printing devices utilize a sensor for depositing printing solution (Espina et al. 2003) above the surface. This sensor may be either a piezoelectric crystal or a solenoid.

Piezoelectric devices consist of a glass capillary tube surrounded by a deformable piezoelectric material (Espina et al. 2003; Schena 2000). Piezoelectric material is typically a ceramic that changes form in the presence of an electrical charge. The deformation induced by electric charge of the piezoelectric material provides pressure on the glass capillary containing the sample, causing fluid to be dispensed from the tip of the glass capillary. Picoliter quantities of fluid may be dispensed with a piezoelectric tip. Typical sample delivery volumes for these devices are 0.1–0.3 nL.

Syringe solenoid systems utilize pressure supplied by a syringe to aspirate fluid into a sample tip. Opening the solenoid valve allows droplets of fluid to be ejected from the tip. The dispensed droplet volume is 4–8 nL (Schena 2000).

Non contact printing is generally believed to yield the lowest spot to spot variability in the amount of sample deposited. In addition, non contact printing could cause less harm to the protein structure because the capillary does not touch the surface, but the shear forces produced during droplet delivery may have a negative effect on protein structure (Morozov 2005).

1.2.4 Signal generation and signal detection

The binding between capture molecule and protein analyte can be monitored by quantifying the signal generated from each pair using various detection techniques (MacBeath 2002; Zhu & Snyder 2003). Detection of captured protein is usually accomplished by fluorescence or chemiluminescent labeling (Espina et al. 2004).

In chemiluminescence detection, the signal is generated with secondary antibodies conjugated for instance to alkaline phosphatase or horseradish peroxidase. The enzymatic oxidation of a substrate, such as luminol, produces a prolonged emission of light, which is captured with phosphor imager or a CCD camera. The sensitivity of chemiluminescence can also be increased by performing the oxidation of luminol by horseradish peroxidase in the presence of chemical enhancers such as phenols. This has the effect of enhancing the light output by approximately 1000-fold and extending the time of light emission, consequently increasing the sensitivity. Chemiluminescence detection has been used to detect proteins on membranes (Joos et al. 2000), glass arrays (Arenkov et al. 2000) and 96-well plates (Mendoza et al. 1999; Moody et al. 2001). Chemiluminescence, although highly sensitive, has drawbacks in terms of the potential for low feature resolution due to signal bleeding and limited dynamic range.

Fluorescence is the most commonly used method to detect proteins on microarray formats (Templin et al. 2002). This popularity is mainly for reasons of simplicity, stability and availability of fluorescent scanners tailored for microarray use. Fluorescent molecules absorb photons of light energy from an external light source. This causes an excitation of electrons within the molecule and an emission of light at a different wavelength than the incident light. The Cy3 and Cy5 fluorescent dyes are commonly used for fluorescent detection, and have been widely utilized in DNA microarray applications (Kumble 2003). Fluorescence can be directly labeled onto proteins (Haab, Dunham, & Brown 2001) or conjugated onto other detection molecule such as streptavidin. Since there is low auto-fluorescence in glass slides, protein arrays are normally constructed on glass when using fluorescence detection. However, the sensitivity of fluorescence detection is usually lower than chemiluminescence, and may be insufficient in some cases to measure proteins present at very low concentrations.

To address this limitation, a powerful signal-enhancement methods (e.g. rolling circles amplification (RCA) (Schweitzer et al. 2002) and thyramide signal amplification (TSA) (Woodbury, Varnum, & Zangar 2002)) have been developed. In RCA, an oligonucleotide-conjugated antibody binds to a hapten (such as biotin) common on all antigen-specific secondary antibodies. A circular DNA molecule then

hybridizes to the oligonucleotide and replicates using DNA polymerase, thereby amplifying the signal. Tyramide signal amplification (TSA) uses horseradish peroxidase (HRP) to catalyze biotin accumulation from biotinyl–tyramide, an HRP substrate. The "amplified" biotin localized at the reaction site can be detected using strepatvidin-HRP in conjunction with an HRP substrate that produces a fluorescent or chemiluminescent product.

The binding to capture agents of protein analytes in a sample is detected by scanning the array, using either a scanning confocal laser or a charged coupled device (CCD) camera-based reader (Seong & Choi 2003). Once the array image has been generated, sophisticated software is used to obtain signal intensity values for each of the spots after gridding the elements of the array.

1.2.5 Matrix to prepare calibrators for protein expression microarrays

As in any other immunoassay, standards curves are required for the quantitative application of protein expression microarrays. Standard curves are used to calculate the concentration of analytes in the measured samples. Calibrators can be prepared using different matrix: e.g. serum, plasma, culture medium or buffer. According to the Food and Drug Administration (FDA) guidance for pharmacokinetic assays (Food and Drug Administration 2001) (Findlay et al. 2000), calibrators should be prepared preferentially in the same matrix as the tested samples. However, using the same matrix can complicate assay development and validation. This is because of the presence of the endogenous molecules of interest. As a consequence the blank calibrators can not be generated which perturb the production of the quantitative standard curves. In addition, other molecules such as soluble receptors, anti-cytokine antibodies, autoantibodies or heterophilic antibodies present in the matrix can potentially interfere with the measurements (Hennig et al. 2000). As a consequence, one of the strategies that can be used to limit or eliminate interference from endogenous molecules involves preparing the calibrators in a heterologuos biological matrix (e.g. biological matrix of another species) that is lacking the measured human analytes (Findlay et al. 2000). However, heterologuos matrix for standard curve generation still needs to be verified for the presence or absence of any cross reactivity between the heterologuos matrix and assay regents. Another strategy is to prepare a

standard curve in an analyte free protein based buffer (Findlay et al. 2000). Nevertheless this approach could also provide the false measurements as for instance functionality of some proteins depends on the presence of other naturally occurring matrix components e.g. protein (Zhu & Snyder 2003).

1.3 Application of protein microarrays

Protein microarrays can be classified into two major categories according to their applications. These are defined as protein expression microarrays, and protein function microarrays (Kodadek 2001). Protein expression microarrays mainly serve as an analytic tool, and are used to detect and quantify proteins, antigens or antibodies in a biological fluid. Protein function microarrays on the other hand are used to study protein activities such as protein-protein, enzyme-substrate and small molecule-protein interactions. (Huang 2003). Depending on the microarray type, different capture agents are immobilized onto the microarray surface. The different protein microarray applications are shown in Figure 5, and are described in the following sections.

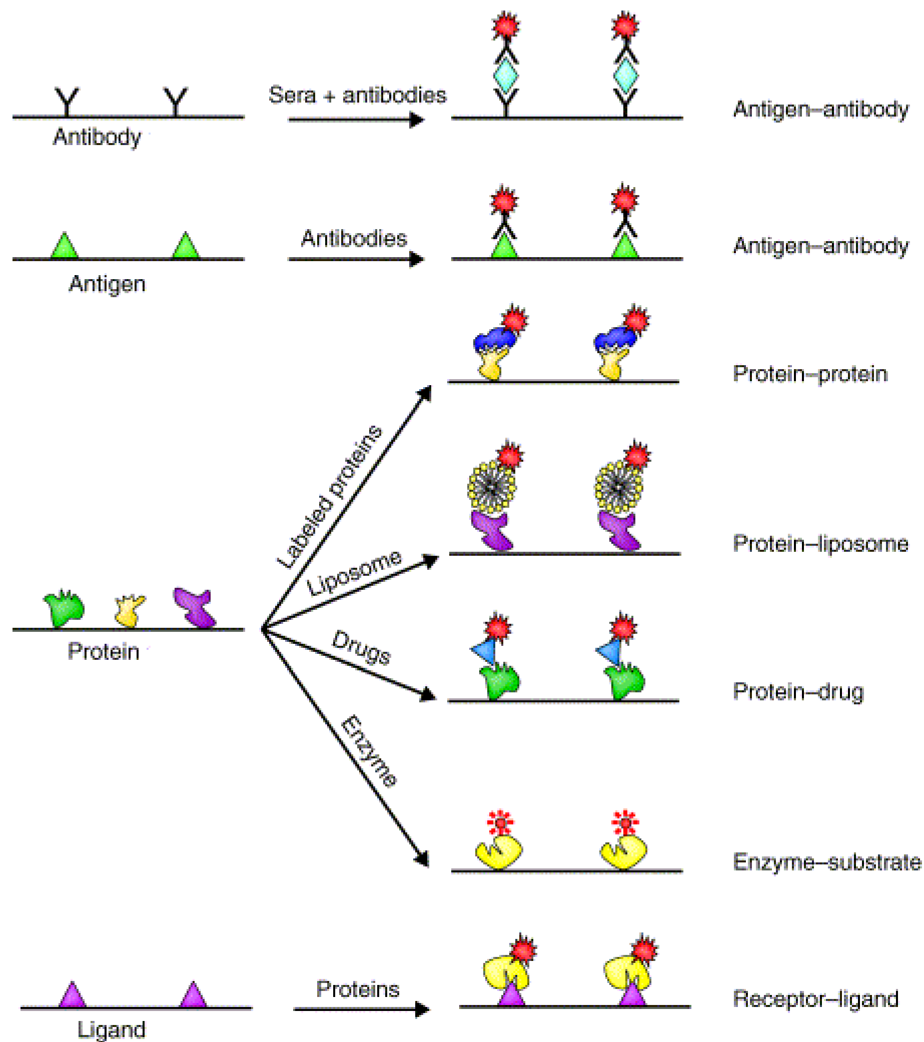

Figure 5: Applications of protein microarrays. Figure adapted from (Zhu & Snyder 2003)

1.3.1 Protein expression microarrays

Protein expression microarrays involve the profiling of protein levels in a complex mixture. For instance, they can be used to screen samples for the presence of antibodies or antigens associated with a disease state. The examples of protein expression microarrays to measure antibodies are described below.

Miniaturized and multiplexed immunoassays were described for the use to screen sera for the presence or absence of a large number of different types of autoantibodies (Joos et al. 2000; Robinson et al. 2002). Autoantigens used as diagnostic markers for autoimmune diseases were immobilized in a microarray format. Consequently,

different types of autoantibodies could be accurately determined from patient serum (Joos et al. 2000). This reflects the enormous potential of protein microarrays for investigating the humoral response against a large number of antigens.

Allergy diagnostics might find protein microarrays quite a suitable format as a sufficiently high number of parameters must be determined. In general, allergies are being diagnosed by provocation testing and employing IgE serology which makes use of allergen extracts or complex mixtures of different potential allergens (Harwanegg et al. 2003). The identification of the disease-causing allergenic molecules is a costly and time consuming procedure. Miniaturized and multiplexed ligand-binding assays can avoid most of the current limitations. Hiller et al. (Hiller et al. 2002) used 78 recombinant and 16 purified allergen molecules to generate a microarray. The individual IgE reactivity towards the microarrayed allergenic components reflected the clinical sensitivity of the individual patients to the different allergens.

1.3.2 Protein function microarrays

Functional protein microarrays are constructed by immobilizing large numbers of purified proteins onto a solid surface. Protein function microarrays have the potential in assaying for a wide range of biochemical activities, including protein–protein, protein-small molecule and enzyme–substrate interactions.

Many biological events are mediated by protein-protein interactions. By identifying protein interaction partners of known function with those whose role is uncharacterised, it is often possible to infer the biological process and/or activity of the uncharacterised protein. Moreover, novel functional activities in additional to those currently identified for known proteins can be uncovered. In protein-protein interaction microarrays the recombinant or purified proteins are spotted onto the array support. Other purified proteins are then applied to the array and analyzed for their interactions. For example, Zhu et al. (Zhu et al. 2001) created a protein microarray containing 5,800 recombinant proteins. Using this proteome microarray 39 calmodulin-interacting proteins were identified by adding biotinylated calmodulin to the microarray. Within this set, several expected calmodulin-binding proteins as well as 33 novel calmodulin-interacting partners were revealed.

The ability to screen whole proteomes for protein-binding molecules (or drugs) has important implications for the drug discovery and development process. For many drugs identified by cell-based screening, the mode of action is often unknown. By screening a protein array with drugs, candidate protein targets can be determined in a single experiment. MacBeath and Schreiber demonstrated the utility of this approach for screening labelled ligands for potential binding partners on protein microarrays in a high-throughput manner (MacBeath & Schreiber 2000). Specifically, they were able to show binding of the FK506 binding protein, FKBP12, with several compounds. Alternatively, small molecules that are produced by combinatorial chemistry or potential ligands immobilised onto arrays could also be used to identify protein targets.

Enzyme-substrate interaction microarrays can be used for the identification of novel enzyme activities. Protein kinases, in particular, represent a class of proteins that are of great interest for pharmaceutical development. A comprehensive array of kinases would have utility for development of new kinase inhibitors as well as for profiling the specificity of existing inhibitors. For example, yeast protein kinases (119 of the 122 known or suspected protein kinases from *Sacharomyces cerevisiae*) were arrayed onto microwell plates and used to characterise substrate specificities with 17 different substrates in microwell-type arrays (Zhu et al. 2000). As a result, novel aspects of protein kinase selectivity were uncovered. Clearly, the next step is to create a human kinase array in a similar manner.

1.4 Protein Microarray for protein biomarker screening

A protein biomarker is defined as a protein that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Frank & Hargreaves 2003). In clinic, biomarkers of efficacy or mode of action, are especially valuable as they can offer both sensitive and specific measurable endpoints to better monitor and predict patient responses to drug treatment. Toxicity and safety markers are indicators of adverse drug effects. Ideally, such biomarkers would act as a warning sign to signal the beginning of an adverse drug response at a much earlier timepoint prior to the

manifestation of a pathological condition. Another type of biomarker is a surrogate marker (a substitute for a clinical endpoint), which is often related to disease progression or regression. Biomarkers which are measured in readily accessible body fluids are the most valuable for disease detection and treatment monitoring, as only non invasive techniques are required for sample collection (Colburn 2003). Therefore, efforts at discovering disease markers have concentrated on identifying proteins in body fluids, such serum, plasma, urine, synovial fluid, cerebrospinal fluid (CSF), saliva and nipple aspirate.

The usual 'single biomarker' approach to disease diagnosis and prognosis, is undergoing a paradigm shift as it is becoming increasingly clear that single markers do not show enough specificity and sensitivity to be applied to all patients due to inter-individual variability. Panels of biomarkers, which offer higher sensitivity and specificity for disease are proposed for better patient stratification and management in clinic (Seong & Choi 2003).

One of the driving forces of the "–omic" technologies in the drug discovery and development pipeline is the identification and utilization of sensitive and specific biomarkers to advance clinical diagnostics and therapeutic monitoring. These "–omic" technologies have led to the discovery of a vast amount of biomarker candidates for disease, drug efficacy and toxicity. This situation, however, has created new bottlenecks in drug development. Once a biomarker candidate has been defined, a statistical validation across many samples has to be performed, and there is an immediate need for tools that help to both validate these potential biomarkers and to screen them once in clinic (Bodovitz & Joos 2004; Ilyin, Belkowski, & Plata-Salaman 2004).

Currently, the conventional ELISA, radioimmunoassay and western blotting techniques are used for biomarker validation and screening (MacBeath 2002). However, ELISA allows for the analysis of one analyte per assay only, thus many assays are required for many analytes. Substantial savings could be made both in terms of time, cost, sample volume and precious reagents by multiplexing and miniaturizing these assays. Moreover, simultaneous measurement of analytes of interest would allow more information to be obtained from the same sample volume.

This is especially important when the amount of sample is limiting (i.e. in preclinical studies or "rare" matrix's such as CSF and synovial fluid). Therefore, this is an area whereby protein microarrays could potentially improve biomarker validation, therapeutic monitoring and diagnostics by offering a novel format to perform immunoassays in a high-throughput multiplexed fashion (Figure 6) (Cahill 2001; Kodadek 2001; Wilson & Nock 2003).

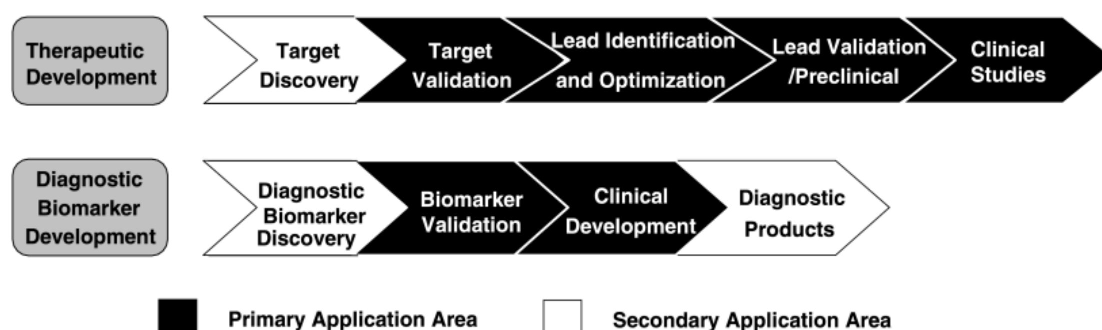

Figure 6: The use of protein microarrays for the development of drug and diagnostic biomarkers. Figure taken from (Wilson & Nock 2003).

1.5 Rheumatoid arthritis

1.5.1 Introduction

Rheumatoid arthritis (RA) is an inflammatory, autoimmune, systemic disease of unknown pathogenesis. Rheumatoid arthritis affects about 1% of the Caucasian population in a female to male ratio of 2.5/1 (Lee & Weinblatt 2001). The prevalence increases with age, and sex differences diminish in the older age group (Sweeney & Firestein 2004).

The main feature of RA is persistent inflammatory synovitis usually involving peripheral joints in a symmetrical distribution. The joints typically affected are those of the hands, wrists, knees and feet. Synovial inflammation causes pain, warmth, swelling, tenderness, stiffness and limitation of motion. The potential of the synovial inflammation to cause cartilage destruction, bone erosions and joint deformities is the hallmark of the disease (Lee & Weinblatt 2001; Sweeney & Firestein 2004). Extra-articular involvement is another hallmark of RA, and this can range from rheumatoid

nodules occurring most commonly over bony prominences to life-threatening heart nodules and vasculitis (Smolen & Steiner 2003).

There is no pathognomonic symptom or sign of RA. Diagnosis is based on the use of the following diagnostic criteria recognized by The American Rheumatism Association (Arnett et al. 1988):

1) morning stiffness in and around joints lasting at least 1 hour before maximal improvement;
2) soft tissue swelling (arthritis) of 3 or more joint areas observed by a physician;
3) swelling (arthritis) of the hand joints;
4) symmetric swelling (arthritis);
5) rheumatoid nodules;
6) elevated levels of serum rheumatoid factor (RF);
7) radiographic changes in hand and/or wrist joints.

At least 4 of the 7 criteria must be present for a minimum of six weeks before a diagnosis of RA can be made. Rheumatoid factor is defined as an IgM and/or IgG autoantibody reactive against the Fc region of the IgG molecule (Corper et al. 1997). These antibodies have low disease specificity and can be detected in sera from healthy individuals, and patients with other autoimmune disorders or chronic infections (Williams DG 1998). In recent years, anticyclic citrullinated peptide antibody (anti-CCP) has been characterized as a novel RA-specific antibody that can be detected very early in the disease (Bas et al. 2003).

1.5.2 Etiology of Rheumatoid arthritis

The etiology of RA is unknown, although it appears that genetic, infectious, environmental and hormonal factors are involved in complex, interrelated ways (Smith & Haynes 2002).

In genetic studies, RA is strongly linked to the major-histocompatibility-complex (MHC) class II antigen HLA-DR genes that participate in antigen presentation to CD4+ T cells (Lanchbury 1992). It has been found that approximately 70% of Caucasian patients with classic rheumatoid arthritis, compared with 28% of patients

with non-rheumatoid arthritis, expressed HLA-DR4. This association with HLA-DR4 has been demonstrated in most populations studied. Some non Caucasian populations exhibit a different association. For instance, RA in the Native American population is associated with HLA-DR9 (Stastny 1978).

The greater prevalence of RA among women suggests that sex hormones are implicated in the development of the disease. This is further endorsed by the observation that pregnancy has improving an effect on RA, and patients with RA are more likely to be nulliparous before disease onset when compared with healthy patients (Hazes 1991).

It has long been speculated that RA could be triggered by infectious agents. There have been a large number of infectious agents implicated in RA, including Epstein–Barr virus and parvovirus, as well as other agents, including bacteria such as Proteus and Mycoplasma, but proof of this is still lacking (Silman & Pearson 2002). The immune system may overreact to the infectious agent, and continue to attack the infected area even after the infection has been eliminated.

Heat shock proteins (HSPs) are a family of proteins produced by cells of all species in response to stress. These proteins have conserved amino acid sequences, that is, there is a sequence homology. Certain human HSPs and *Mycobacterium tuberculosis* HSPs have 65 per cent sequence homology (Kaufmann 1990). A potential hypothesis is that antibodies and T-cells exist that recognize epitopes shared by the HSP of both the infectious agents and host cells, triggering an immunological reaction. This is referred to as molecular mimicry.

1.5.3 Pathogenesis of Rheumatoid arthritis

Normal synovial tissue consists of the synovial lining (comprising of one to three cell layers) and the synovial sublining, which merges with the joint capsule (Figure 7a). The synovial lining consists of two major cell types: macrophages and fibroblast like cells. Usually the synovial sublining is relatively acellular. In the early stages of the disease, the most noticeable feature is tissues oedema. Vessel proliferation and new vessel formation (angiogenesis) is also observed. Further, synovial lining hyperplasia

begins to develop (Figure 7b). As the disease enters a more chronic phase, synovial lining hyperplasia extends to a depth of over 10 cells. The cells consist of type A (macrophage-like) and also type B (fibroblast-like) synoviocytes. The sublining also evolves with the disease progression (Firestein 1991). Most noticeably there is a exuberant infiltration with mononuclear cells comprising T-cells (predominantly CD4+ helper T-cells), B-cells, macrophages and plasma cells (Figure 7b) (Smolen & Steiner 2003). New blood vessel formation continues and both the degree and content of the cellular infiltrate changes. Generally, an inflammation process exhibits an infiltration similar to that described above. Another feature includes the ability of the synovium of RA to become locally invasive at the synovial interface with cartilage and bone. This result in the formation of a mass of tissue called pannus (Figure 7b). The cells of the pannus produce destructive proteins called matrix metalloproteinases (MMPs) that cause joint erosion (Smith & Haynes 2002).

How environmental and genetic factors induce RA is not yet understood. One view is that the inflammatory process in the tissue is driven by T cells (Fox 1997). It is often assumed that the initiation phase of the disease is marked by localization of an arthrotropic agent in the joint followed by antigen presentation and specific T cell activation (Firestein 1991). Stimulated T cells would subsequently generate cytokines, including IFN-γ, that activate macrophages, other T cells, B-cells (which produce rheumatoid factor), and endothelial cells. Activation of the vascular endothelium by cytokines induces adhesion molecules and recruits new cells that express the appropriate counterreceptors into the joint. The accumulation of T cells would ultimately result from nonspecific infiltration of the synovium with cells from the blood as well as local proliferation of lymphocytes in the synovium that recognize their specific antigen in the context of MHC molecules.

1.5.4 Soluble mediators of inflammation and join damage in RA

Monocytes, macrophages, fibroblasts, and T cells release numerous cytokines on stimulation (Firestein 1991). Cytokines are small soluble proteins that mediate intercellular communication between cells involved in immune responses. They affect cell division, differentiation, and chemotaxis, as well as proinflammatory or anti-inflammatory actions. Quantitative analyses suggest that there are few T-cell-derived

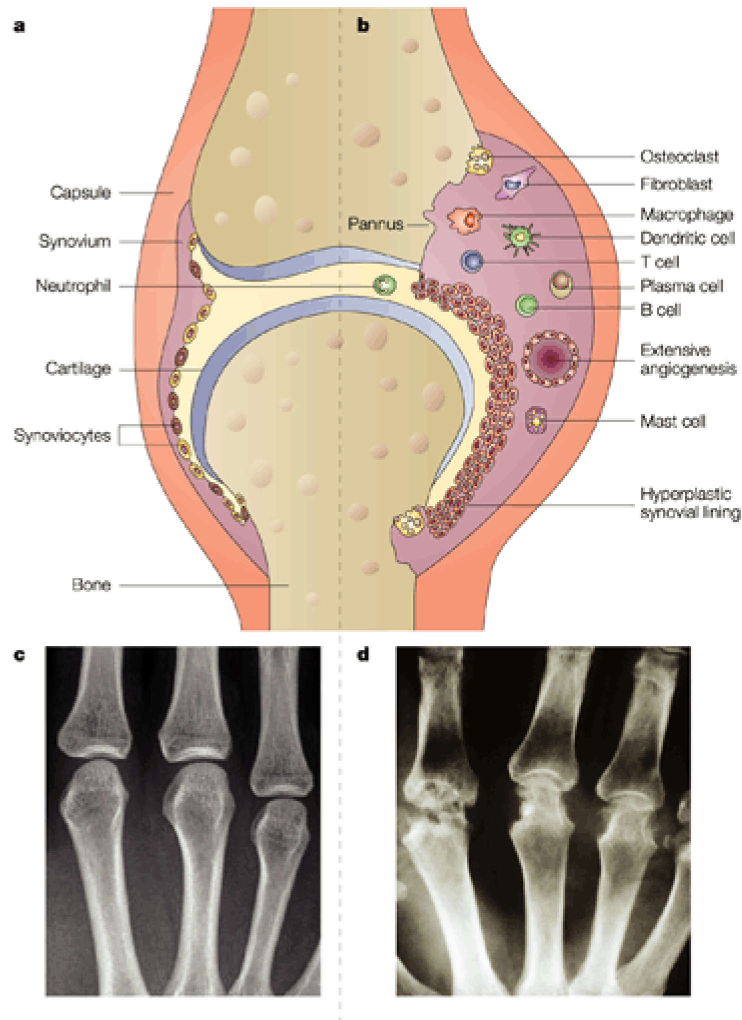

Figure 7: Schematic and radiographic view of a normal joint (a and c) and its changes in rheumatoid arthritis (b and d). This figure is taken from (Smolen & Steiner 2003).

cytokines such as interleukins 2 (IL-2) and 17 (IL-17), and interferon gamma (IFN-γ) in the inflamed synovial tissue. However, many other cytokines are also present in moderate to high concentrations in RA. Tumour necrosis factor (TNF-) and interleukin 1 (IL-1) are both present in large quantities in affected synovial fluid and synovial tissue (Lee & Weinblatt 2001) (Figure 8). Both TNF- and interleukin-1 are likely to have primary roles in the pathogenesis of rheumatoid arthritis. The serum and synovial concentrations of both cytokines are high in patients with active rheumatoid arthritis (Saxne et al. 1988). Furthermore, TNF- and interleukin-1 are potent stimulators of mesenchymal cells, such as synovial fibroblasts, osteoclasts, and chondrocytes, that release tissue-destroying matrix metalloproteinases (Shingu et al.

1993). Interleukin-1 and TNF- also inhibit the production of tissue inhibitors of metalloproteinases by synovial fibroblasts (Shingu et al. 1993). These dual actions are thought to lead to joint damage. TNF and IL-1 seem to function synergistically in inducing effector function.

Investigations on animal models have also suggested a central role for TNF- and interleukin 1 in the process of synovitis and joint destruction. Addition of exogenous interleukin 1 or TNF into experimental models of arthritis induces or exacerbates synovitis. Furthermore, mice transgenic for TNF-, and mice with dysregulated TNF- production develop arthritis (Keffer et al. 1991; Taylor et al. 1996).

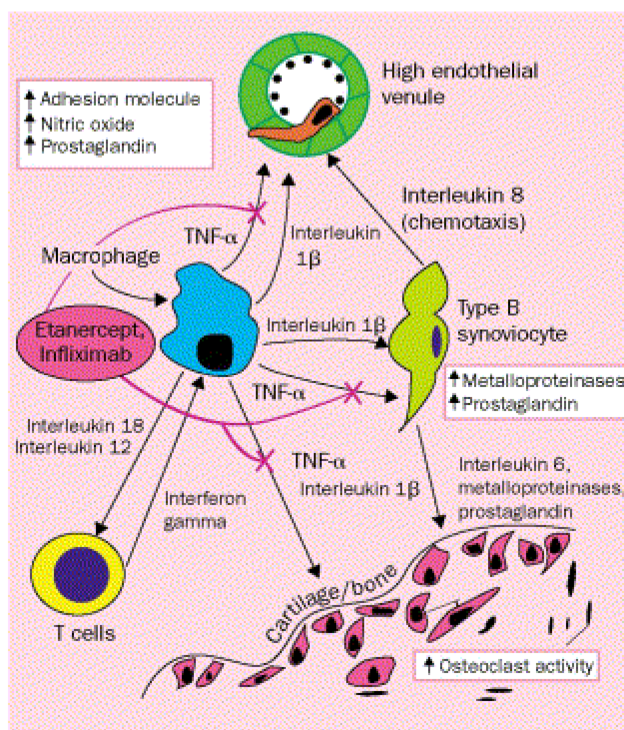

Figure 8: Simplified schematic representation of cytokine network in rheumatoid arthritis. Black arrows indicate upregulatory effects. Red crosses represent pathways blocked by anti-TNF drugs. Figure taken from (Lee & Weinblatt 2001).

A subclass of cytokines and cytokine receptors are thought to exert anti-inflammatory activity in the synovium. There are two TNF receptors (p55 and p75), both of which occur naturally the soluble form in synovial fluid (Cope et al. 1992). They inhibit TNF- activity by competing with cell-surface receptors for binding. Similarly, the two interleukin 1 receptors, IL-1R1 and IL-1R2 also occur in the soluble form in synovial fluid. These receptors are capable of binding interleukin 1, thus forming competition

for cell-surface receptors (Arend 2002). Additionally, a naturally occurring competitive inhibitor for interleukin 1 at the IL-1 receptor (IL-1 receptor antagonist [IL-1ra]) is also present in rheumatoid arthritis synovial fluid (Arend 2002). This member of the interleukin 1 family binds to IL-1R1 without transducing a signal, thus blocking the receptor binding ability of IL-1.

To perform the proof of concept studies described in this thesis, prteins that are associated with RA were chosen. They are marked as A, B, C, D, E, F. In addition, the inflammatory related protein, serum amyloid A (SAA), was also chosen.

1.5.5 Treatment of Rheumatoid arthritis

Medical management of RA involves five general approaches. The first is the use of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) and simple analgesics to control the symptoms and signs of the local inflammatory process. These agents are rapidly effective at stopping signs and symptoms, but they appear to exert minimal effect on the progression of the disease. Recently, specific inhibitors of the isoform of cyclooxygenase (Cox) that is upregulated at inflammatory sites (Cox-2) have been developed. Cox-2-specific inhibitors have been shown to be as effective as classic NSAIDs (which inhibit both isoforms of Cox), but cause significantly less gastroduodenal ulceration. The second line of therapy involves the use of low-dose oral glucocorticoids. Although low-dose glucocorticoids have been widely used to suppress signs and symptoms of inflammation, recent evidence suggests that they may also retard the development and progression of bone erosions. Intraarticular glucocorticoids can often provide transient symptomatic relief when systemic medical therapy has failed to resolve inflammation. The third line of agents includes a variety of agents such as methotrexate (MTX) that have been classified as the disease-modifying or slow-acting antirheumatic drugs (DMARDs). These agents appear to have the capacity to decrease elevated levels of acute-phase reactants in treated patients and, therefore, are thought to modify the inflammatory component of RA and reduce its destructive capacity. Recently, combinations of DMARDs have shown promise in controlling the signs and symptoms of RA. A fourth group of agents are the TNF- neutralizing agents, which have been shown to have a major impact on the signs and symptoms of RA. A fifth group of agents are the immunosuppressive and

cytotoxic drugs that have been shown to ameliorate the disease process in some patients.

The pro-inflammatory role of cytokines, and the involvement of different cell types and their surface molecules in the pathogenesis of RA, provides the rationale for the development of highly specific therapeutics to target these molecules so called biologic DMARDs. Targeting of pro-inflammatory cytokines can be achieved by several strategies. First, monoclonal antibodies, soluble receptors, binding proteins or receptor antagonists can bind to pro-inflammatory molecules or their receptors and interfere with receptor ligation and its consequences. Second, anti-inflammatory cytokines, such as IL-1ra, IL-4, IL-10 or IL-13, can antagonize the production or action of the pro-inflammatory cytokines. Third, monoclonal antibodies targeted against differentiation- or function-associated cell-surface antigens can lead to either elimination of the targeted cells or interference with the cell's function. Table 3 shows the approved therapies that prevent pro-inflammatory cytokines, in particular TNF-α and IL-1, from interacting with their receptors.

Table 2: Approved biological DMARDs and related drug development.

Agent	Status‡	Properties	Comments
Anti-TNF therapies			
Infliximab	Approved (RA: 1999)	Chimaeric monoclonal antibody to TNF	First biological DMARD to be clinically tested; trials showed that TNF blockade is clinically effective short- and long-term
Etanercept	Approved (1998)	Construct of TNF-RII and the Fc portion of IgG1	Efficacy in RA comparable to infliximab used as a monotherapy or combination therapy
Adalimumab	Approved (2002)	Human monoclonal antibody to TNF	Efficacy in RA comparable to infliximab used as a monotherapy or combination therapy
CDP870	In study	PEGylated Fab fragment of CDP571, a humanized antibody to TNF	
PEG-TNF-RI	In study	PEGylated form of soluble TNF-RI	
IL-1-blocking agents			
Anakinra	Approved (2001)	IL-1R antagonist	Clinically effective as a monotherapy and in combination with MTX
IL-1 trap	Phase I	Construct of two IL-1R chains with an IgG-Fc domain	

AIM OF THIS THESIS

The emerging field of biomarkers is proposed to play a crucial activity in the drug development process. Biomarkers have applications in the monitoring of clinical responses to therapeutic intervention such as efficacy determination and safety monitoring, in the diagnosis, prognosis and monitoring of disease progression, as well as in patient population stratification. The drug development plan which makes effective use of a biomarker strategy will allow compounds to be developed in a more efficient way by reducing drug attrition rates, improving patient management and therapy assessment in clinic, and facilitating regulatory decision making. Ultimately, biomarkers will provide the possibilities to get safer and more effective drugs to market faster.

Recently, proteomic and genomic technologies have dramatically accelerated the discovery of a large number of potential biomarkers. Once a biomarker is identified, an assay is developed and the biomarker is extensively tested and validated in both preclinical and clinical trials. To keep pace with the discovery rate of potential biomarkers, high-throughput and/or high-output technologies are needed. Moreover, the traditional 'single biomarker' approach to disease diagnosis and prognosis, is undergoing a paradigm shift as it is becoming increasingly clear that single biomarkers do not exhibit enough specificity and sensitivity to be applied to all patients because of inter-individual characteristics. Panels of biomarkers are proposed for better patient stratification and management in clinic. Therefore, there is also a need for the development of screening technologies which would simultaneously measure several parameters in a multiplex format.

Enzyme-linked immunosorbent assay (ELISA) and Western Blot have for many years been the mainstay techniques in clinical samples analysis. These techniques, however, are only suited for the measure of a single analyte per assay. Substantial savings could be made in terms of cost, time and sample volume by multiplexing and miniaturizing these assays. Subsequently, novel technologies such as protein microarray and flow cytometric bead-based assay platforms are currently being developed. These technologies allow for: the evaluation of multiple analytes in a single sample; the

utilisation of minimal sample volumes to obtain data; and a more rapid evaluation of multiple analytes in a single platform.

The aim of this thesis was to examine whether a protein microarray platform can be applied to the drug development process for monitoring biomarkers in both preclinical and clinical studies. In order to demonstrate the feasibility of this approach, rheumatoid arthritis (RA) was chosen as a model for proof of concept. Several proteins which were described in the literature to be associated with RA were selected, and a protein microarray platform for the simultaneous measurement of these proteins in serum samples was subsequently developed. The robustness of this protein microarray was then assessed and finally validated with real sample measurements in RA patients.

2 Materials and methods

In this chapter general regents, equipment, and software are listed. The specific methods are explained in the individual chapters.

2.1 General reagents

Table 3: General reagents

Reagents	**Manufacturer**
PBS buffer	10 X PBS buffer, Roche Diagnostic, Indianapolis, IN, USA
Tween® 20	Fluka Chemie Sarl, Buchs, Switzerland
Tris	Trizma®, Sigma®, Sigma-Aldrich Steinheim, Germany
SuperBlock™	Pierce Biotechnology, Inc., Rockford, IL, USA
Albumin bovine (fraction V)	Sigma®, Fluka Chemie Sarl, Buchs, Switzerland Buchs, Switzerland
SuperSignal® ELISA Femto Maximum Sensitivity Substrate	Pierce Biotechnology, Inc., Rockford, IL, USA
ImmunoPure® Streptavidin, Horseradish Peroxidase Conjugated	Pierce Biotechnology, Inc., Rockford, IL, USA

Table 4: Buffers

Buffer	Buffer Content
Wash buffer	PBS 1X containing 0.05% Tween
Assay buffer	PBS 1X containing 0.05% Tween, 3% BSA

2.2 Software

Table 5: Sofware

Software	**Manufacturer**
Quantity One Version 4.2.1	Bio-Rad, Hercules, CA, USA
Aida Version 5.0	Raytest GmbH, D 75339 Straubenhardt, Germany
ImaGene™ Version 5.0	BioDiscovery, Inc, El Segundo, CA, USA
Array Vision™ Version 8.0	Imaging Research Inc., Ontario, Canada
SOFTmax® PRO Version 3.1.1	Molecular Devices Corp., Sunnyvale, CA, USA

2.3 Laboratory equipment

Black plate	Maxisorp™ 96-well plate (Nalge Nunc International, Rochester, NY, USA).
Transparent plate	Maxisorp™ 96-well plate (Nalge Nunc International, Rochester, NY, USA).
Plate's shaker	Wesbart (IS89), Fischer scientific, Wohlen, Switzerland
Washer	Embla, Molecular Devices, Bucher Biotec AG, Basel, Switzerland

3 Development of a protein microarray in a glass chip format

3.1 Introduction

The protein microarray development process utilises a combination of technologies for protein deposition, assay generation procedures, signal detection and data analysis. Many attempts have been undertaken to develop protein microarrays using different supports, liquid handling and detection systems (Kodadek 2001; Stoll et al. 2002; Templin et al. 2003).

In this chapter the development of a sandwich protein microarray on a glass chip is described. The development covered the following steps:

- comparison of the treatment of a glass chip with two surface chemistries: Poly-L-lysine versus self-assembled monolayer of octadecyl phosphoric acid ester
- comparison of contact and non contact piezoelectric arrayers for antibody deposition.
- comparison of different concentration of coating antibodies
- comparison of different detection systems: CCD camera versus fluorescent scanner.

The results were described in the article: “Development of protein microarray technology to monitor biomarkers of rheumatoid arthritis disease”, published in “Cell Biology and Toxicology” 2003; 19: 189 – 202 journal. This publication is included in the next chapter.

3.2 Development of protein microarray technology to monitor biomarkers of rheumatoid arthritis disease.

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Development of protein microarray technology to monitor biomarkers of rheumatoid arthritis disease

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Abstract

Most biological processes are mediated by complex networks of molecular interactions involving proteins. The analysis of protein expression in biological samples is especially important in the identification and monitoring of biomarkers for disease progression and therapeutic endpoints. In this paper, the development of a protein microarray format for multiplexed quantitative analysis of several potential markers for rheumatoid arthritis (RA) is described. Development of a high-performance protein microarray system depends on several key parameters such as surface chemistry, capture agents, immobilization technology, and methods used for signal detection and quantification. Several technical possibilities were investigated and compared: poly-L-lysine versus self-assembled monolayer of octadecyl phosphoric acid ester for surface chemistries; noncontact piezoelectric versus contact printing technology for antibody deposition; CCD camera capture versus fluorescent scanning for image detection; and the concentration of coating antibody. On the basis of reproducibility, signal-to-noise ratio, and sensitivity we have selected self-assembled monolayer, noncontact piezoelectric printer, and high-read-out fluorescence scanning for our microarray format. This format was used to perform multiplexed quantitative analysis of several potential markers of disease progression of rheumatoid arthritis: IL-1β, IL-6, IL-8, MCP-1, and SAA. Some assays, such as MCP-1, provided a working range that covered physiologically relevant concentrations. Other assays, such as IL-6 and SAA, lacked sensitivity or were too sensitive for measuring biological concentrations, respectively. The results described demonstrate the applicability of protein microarrays to monitor RA markers; however, sandwich assay methodologies need to be further optimized to measure the appropriate biological ranges of these markers on one chip.

Abbreviations: CCD, charge-coupled device; CY5, cyanine 5; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-6-CY5, interleukin-6 CY5-conjugated; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; SAA, serum amyloid A; SAM, self-assembled monolayer; NHS, *N*-hydroxysuccinimide

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Introduction

Understanding biological processes, and more specifically disease mechanisms, pharmacological activities, and toxic effects, requires a holistic approach to the investigatation of molecular events. In the postgenome era, triggered by the sequencing of the human genome (Lander et al., 2001; Venter et al., 2001) the amount of information necessary to describe physiological situations is increasing exponentially. This global approach to biological processes requires very efficient and precise analytical systems. For instance, DNA microarray technologies are able to analyse the transcript levels of thousands of genes simultaneously (Lockhart et al., 1996). However, gene expression limited to messenger RNA (mRNA) provides only a partial picture of the biological events. The amount of protein synthesized from mRNA cannot yet be fully predicted from transcriptional activity alone (Gygi et al., 1999; Lian et al., 2002). In addition, post-translational modification (PTM) events cannot be predicted from genomics data only. PTM events are known to play a major role in the biological activity and function of many proteins (Han and Martinage, 1993; Daniels et al., 2002). From a functional point of view, biological events are mostly mediated by molecular interactions involving protein activity (Schwikowski et al., 2000; Ideker et al., 2002). Subsequently, proteomics using two-dimensional gel electrophoresis coupled with mass spectrometry has emerged as a reference methodology for quantitative protein analysis in biological samples (Corthals et al., 2000; Shevchenko et al., 2000). However, the limitations of this approach in terms of throughput and analyzable protein range have elicited the development of other proteomics approaches for protein expression profiling (Griffin et al., 2001). The development of microchips, in a manner analogous to the current use of DNA arrays for mRNA expression analysis but involving proteins, has accelerated within the past few years (MacBeath and Schreiber, 2000; Haab, 2001; Haab et al., 2001). High-throughput profiling technologies using protein arrays hold much promise in diagnostic and prognostic monitoring of diseases, and in drug discovery and development.

Rheumatoid arthritis (RA) is a destructive inflammatory joint disease, in which a broad range of extra-articular manifestations may occur, and in which a number of cellular and humoral abnormalities have been observed. The underlying mechanism of the disease process has remained illusive (Lipsky, 1998). Monitoring the progression of RA or the efficacy of pharmacological treatment requires the simultaneous analysis of several proteins such as interleukins, chemokines, and other markers of inflammation (Choy and Panayi, 2001). Traditionally, the amount and availability of samples (e.g., synovial fluid) has limited the individual analysis of each of these markers in biological fluids. Moreover, analyzing a set of markers from each patient using conventional ELISA would also dramatically increase the complexity and cost of diagnostics in patient monitoring. Therefore, there is an increasing need to implement multiplexed analysis technologies, such as protein microarrays, for the quantification of several markers within a single microarray in an efficient and cost-effective manner (Kodadek, 2001).

The generation of protein microarrays requires a much more substantive effort than that for DNA microchips, largely owing to the chemical heterogeneity of proteins and difficulty in maintaining their specificity and activity. For protein microarrays, capture molecules (either proteins or antibodies) are immobilized onto a solid support. Further steps, such as sample and secondary reagent incubations, are required for the simultaneous analysis of several proteins in one sample. The key components of a protein microarray system are surface chemistry, deposition tech-

nology, capture elements, and detection methods (Stoll et al., 2002). The analytical performance of this technology needs to provide reliable information in a high-throughput mode. The reproducibility and the precision of the technology should correspond to reference methods already available (Bowsher and Smith, 2002). The sensitivity of the assays should fit with the working range of the expected concentrations of these proteins in biological fluids. Today, technologies for simultaneously analyzing several proteins in patient monitoring are applied only in limited areas.

A prototype for multiplexed analysis of proteins based on a Novartis technology (Neuschäfer et al., 2003) is being developed. This paper describes the different steps and requirements for construction of a protein microarray based on antibodies. As an example, we have chosen different proteins involved in RA: IL-1β, IL-6, IL-8, MCP-1, and SAA (De Benedetti et al., 1999; Charles et al., 1999; Choy and Panayi, 2001). Several chemical surface preparations, antibody printing procedures, and signal detection methods were investigated to evaluate the possibility of simultaneously measuring these markers in their respective working ranges.

Materials and methods

Cleaning of the chip surface

All experiments were done on Novartis proprietary glass chips (Neuschäfer et al., 2003). These chips were first assembled in a metal rack (Zuehlke, Basel, Switzerland) and placed in a glass container for cleaning. Approximately 200 ml of 2-propanol solution was added and the chips were sonicated in a water bath for 15 min at 24°C. Sonication was repeated twice with 200 ml additions of 2-propanol solution. The chips were then dried under a stream of nitrogen and cleaned using a UV cleaner (Boekel Industries Inc., Pennsylvania, PA, USA) for 20 min at 24°C.

Surface chemistry preparation

Glass chips were treated with two different surface chemistries: a self-assembled monolayer (SAM) of octadecyl phosphoric acid ester (ODP), or poly-L-lysine. A solution of ODP for SAM formation was prepared according to previously described methods (Brovelli et al.,). For poly-L-lysine, a solution was prepared according to a previously described method (Haab et al., 2001) with some modifications. Briefly, 60 ml of poly-L-lysine (Sigma Diagnostics Inc., St. Louis, MO, USA) was mixed with 500 ml of deionized water and 68 ml of sterile phosphate-buffered saline (PBS) (Roche Diagnostic GmbH, Mannheim, Germany) at 24°C. Clean chips were placed in either ODP solution or poly-L-lysine solution for 48 h to allow the chemistry layer to assemble onto the chip surface. Chips were removed from their respective chemical solutions and sonicated for 5 min in 2-propanol to remove any chemical excess immediately prior to printing.

Immobilization of the antibody

Monoclonal antibodies for anti-hirudin (Novartis Pharma, Basel, Switzerland), IL-1β, IL-6, MCP-1 (R&D Systems Inc., Minneapolis, MN, USA; MAB601, MAB206, MAB679, respectively), IL-8 (Endogen, Woburn, MA, USA; M-801-E), and SAA (Antigenix America, NY, USA), were diluted in PBS (pH 7.4) to a concentration ranging from 50 to 300 μg/ml. Biotin-labeled albumin (A8549; Sigma Diagnostics) was diluted to concentrations of 5 and 10 μg/ml in PBS. Particulate matter was removed by centrifugation for 5 min at 10 000*g* and the supernatant was collected in fresh tubes. The antibody solutions were deposited onto the chips, using two different printing procedures. A noncontact dispenser

(Microdrop, Hamburg, Germany) was used to deposit 500 pl droplets of antibody solution, and a contact dispenser (Genetic Microsystems Inc., Woburn, MA, USA) was used to deposit approximately 50 pl droplets. The printed chips were incubated overnight at 4°C in a humidity chamber. Following incubation, the chips were dried for 30 min at ambient temperature and then dried for 20 min at 37°C under vacuum.

Sandwich assay on chips

A typical sandwich assay was performed on the chips for analyte capture. Briefly, blocking buffer (PBS containing 10% BSA) was applied onto the chip surface to preclude nonspecific binding of the analytes. The chip was incubated for 2 h at 37°C with a mixture of all the recombinant human proteins prepared in assay buffer (PBS containing 0.4% BSA) at concentrations of 0, 0.05, 0.1, 0.5, 1, 10, and 100 ng/ml for IL-1β, IL-6, MCP-1 (R&D Systems; 201-LB, 206IL, 279-MC), and IL-8 (R-IL-8-10; Endogen). For SAA (Antigenix America), the following concentrations of protein were used: 0, 0.01, 0.02, 0.1, 0.2, 2, and 20 μg/ml in assay buffer. Recombinant human hirudin (Novartis Pharma) at a constant amount of 100 ng/ml was added to each sample. Unbound proteins were washed from the chip surface with washing buffer (PBS containing 0.05% Tween 20 (Merck, Darmstadt, Germany)). Afterwards, a reaction mixture prepared in PBS containing biotinylated secondary antibodies in concentrations recommended by the manufacturer [hirudin (Novartis Pharma); IL-1β, IL-6, MCP-1 (R&D Systems; BAF201, BAF206, BAF279); IL-8 (Endogen; M-802-B); SAA (Antigenix America)] was added to the chip and the reaction was allowed to proceed for 2 h at 37°C. For SAA, the secondary antibody was biotinylated with NHS esters of Biotin (Pierce, Rockford, IL, USA) according to the product protocol. Following the sandwich reaction, signal was detected after a 30 min incubation at 37°C with 0.2 μg/ml of streptavidin CY5 fluorescent dye (Amersham Pharmacia Biotech) prepared in PBS. The experiments were performed at least in triplicate using flow chambers (Zuehlke, Basel, Switzerland) consisting of two parts between which the chip was fixed to create a chamber of 7 μl volume. A 200 μl sample was then manually pumped through the chamber with a syringe.

Detection and image analysis

Signal was detected either with a fluorescence scanning device (Genetic Microsystems) set at a wavelength of 635 nm, or with a CCD camera (Astrocam EEV 30/11 built in house). Images were digitized as 16-bit tiff files and features were quantified (spot volume) with commercially available software (Quantity One version 4.2.1 from Bio-Rad, Hercules, CA, USA). Data were also exported to Excel software for further processing and signal intensities were defined in arbitrary units.

Normalization procedure

The average background value from the chip was subtracted from each spot. The resulting value was divided by the average signal obtained from hirudin spots.

Results

Evaluation of surface chemistry

The signals emitted from the spotted regions on both SAM- and poly-L-lysine-coated chips were evaluated. Spots obtained with SAM had a diameter of about 100 μm, and were smaller in size compared to spots obtained with poly-L-lysine (∼200 μm). In addition, spot signals obtained from poly-L-lysine-treated chips

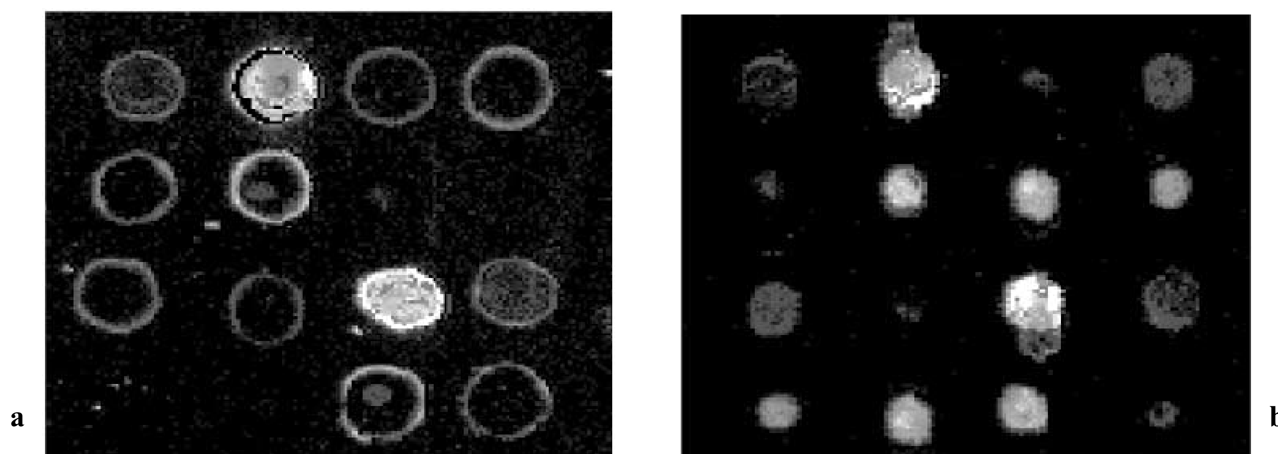


Figure 1. Comparison of surface chemistry. Anti-hirudin, anti-IL-1β, and anti-IL-8 antibodies and biotin-labeled albumin were spotted onto either poly-L-lysine-treated (a) or SAM-treated (b) surfaces in the following format. First row: biotinylated albumin (5 and 10 μg/ml of coating solution) and anti-IL-8 (100 and 200 μg/ml of coating solution). Second row: anti-IL1-ß (100 and 200 μg/ml of coating solution) and anti-hirudin (300 and 200 μg/ml of coating solution). Third row: anti-IL-8 (200 and 100 μg/ml) and biotinylated albumin (10 and 5 μg/ml). Forth row: anti-hirudin (200 and 300 μg/ml) and anti-IL1-ß (200 and 100 μg/ml). The spots obtained with poly-L-lysine treated chips showed a characteristic edge effect along the perimeter creating a ring. Spots produced on poly-L-lysine were 200 μm in size while the diameter of spots from SAM were 100 μm in size. Spots obtained on SAM were more homogenous within the spot area. Hirudin was less performant on poly-L-lysine compared to SAM.

showed heterogeneity with a characteristic edge along the spot perimeter creating a "ring" effect whereas spot signals from SAM were more homogenous along the spotted region (Figure 1). Subsequently, signal intensities obtained with SAM-coated chips were less variable between triplicates of the same measurements performed on different chips when compared with poly-L-lysine (Table 1). In addition, on average nearly 300 times less background signal was observed with SAM than with poly-L-lysine-coated chips across the different analyte concentrations (Table 1). Owing to interchip variability, signal intensities had to be normalized using a signal provided by an external control spiked in a constant amount in all samples. Hirudin was chosen for this normalization, mainly because of the absence of this polypeptide in human serum. Clear differences in the immobilization efficiency of coating anti-hirudin antibody to SAM and poly-L-lysine were also observed (Table 1). These findings demonstrated SAM as the superior surface chemistry. Subsequent experiments were performed on chips coated with SAM.

Antibody printing

Antibodies were immobilized using two different methods: contact dispensing and noncontact piezoelectric dispensing. For contact printing technology, the rigid pins are dipped into the sample solution and subsequent touching of the pins onto the chip surface leaves a spot. Piezoelectric printing technology uses a piezoelectric crystal. The sample is drawn up into the reservoir and the pin is biased with a voltage, which results in the ejection of fluid from the tip (Schena, 2000). CY5-labeled IL6 antibody and PBS were printed using both dispensing methods. Contact dispensing was found to be more variable than noncontact piezoelectric printing, generating a CV of 18.9% compared with a CV of 2.5% for noncontact piezoelectric printing. Moreover, some

Table 1. Comparison of surface chemistry. Sandwich assays for IL-1β and IL-8 were performed in triplicate on poly-L-lysine-treated and SAM-treated chips. Nonnormalized signal intensities provided by SAM-coated chips were less variable between triplicate measurements when compared with poly-L-lysine. Background generated from SAM-coated chips was lower compared to poly-L-lysine. Hirudin showed a very weak performance on poly-L-lysine. The signal was defined using arbitrary units as a measure for mean signal between triplicates ± SD

	Analyte (ng/ml)		
	0	0.5	1
Poly-L-lysine			
IL-1β	2.55 ± 1.86	145.15 ± 52.9	176.19 ± 49.37
IL-8	0.29 ± 1.28	80.88 ± 35.67	121.49 ± 39.86
Controls			
Hirudin	2.58 ± 2.47	–2.37 ± 1.97	–0.87 ± 1.3
Biotin-labeled albumin	44.98 ± 19.09	75.05 ± 24.00	59.64 ± 24.85
Background noise	3.29 ± 6.29	6.4 ± 11.44	5.54 ± 11.04
SAM			
IL-1β	0.02 ± 0.03	121.86 ± 10.02	237.29 ± 17.64
IL-8	0.00 ± 0.00	12.67 ± 1.1	31.68 ± 6.06
Controls			
Hirudin	82.01 ± 2.86	62.41 ± 21.76	45.73 ± 1.77
Biotin-labeled albumin	4.98 ± 0.44	5.87 ± 4.66	3.85 ± 6.06
Background noise	0.007 ± 0.04	0.014 ± 0.06	0.07 ± 0.43

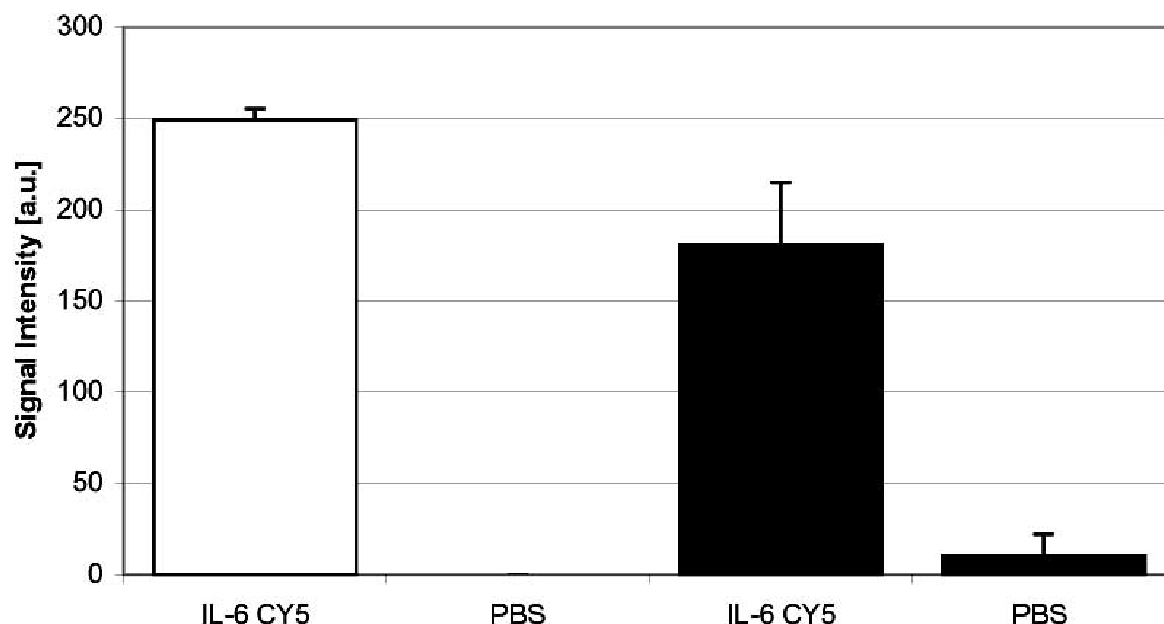


Figure 2. Evaluation of cross-contaminations. IL-6-CY5 and PBS were alternatively printed on a SAM-treated surface using either a noncontact piezoelectric dispenser (white bars) or a contact dispenser (black bars). Interchip variability between four chips for the noncontact dispenser and six chips for the contact dispenser was 2.5% and 18.9%, respectively. The cross-contamination for PBS spots printed with both technologies was calculated by dividing the signal obtained from PBS spots by the signal from IL-6-CY5 spots. Cross-contamination with the contact dispenser was 5.7%, while no detectable cross-contamination was found with the noncontact piezoelectric system.

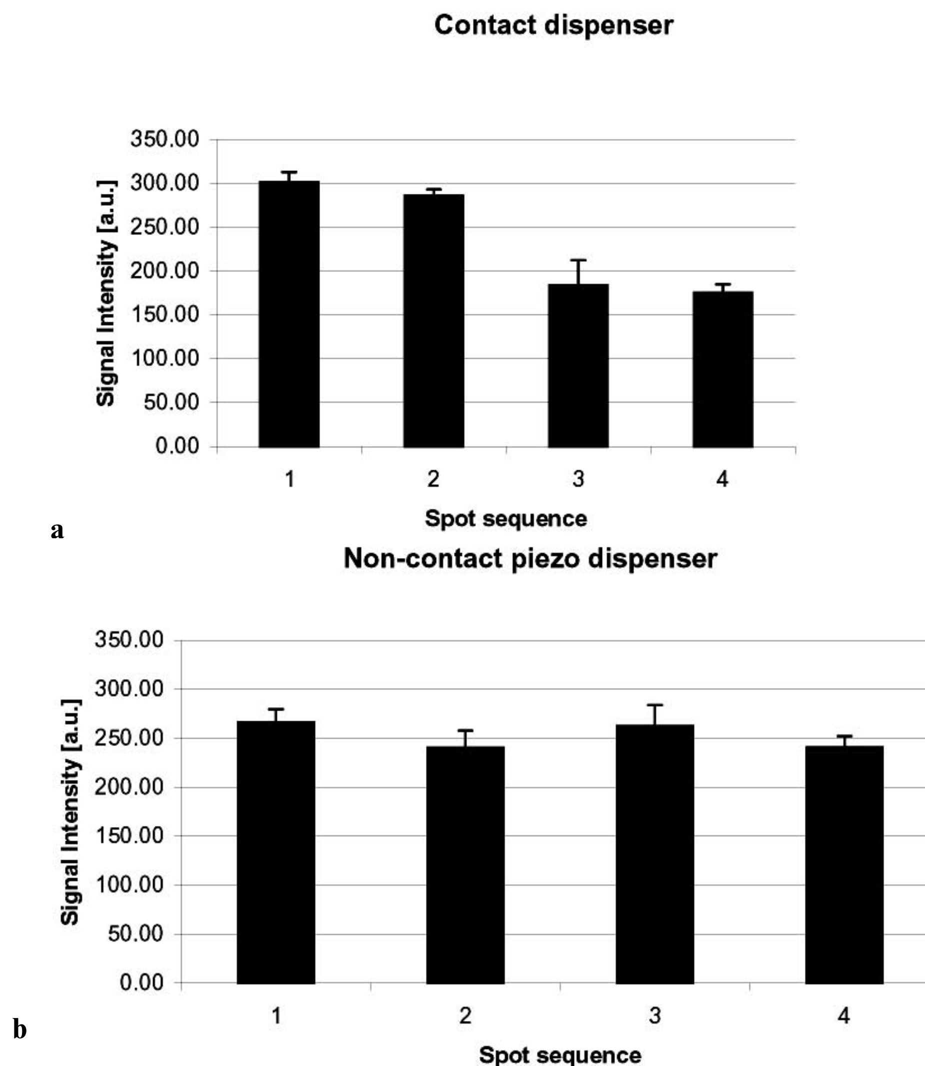


Figure 3. Reproducibility of contact dispensing. (a) Four sequential spots of IL-6 CY5 were printed on a SAM-treated surface using a contact dispenser. A 40% loss of signal was observed in the third and fourth positions of the sequence. (b) Reproducibility of noncontact piezoelectric dispensing. IL-6 CY5 was printed in sequential positions on a SAM-treated surface using a noncontact dispenser. No significant drift was observed between spot intensities.

cross-contamination (5.7%) was observed on chips printed with the contact dispenser (Figure 2). This cross contamination was calculated by dividing the signal emitted from PBS spots by the signal emitted from IL6-CY5 printed spots, giving a value of 5.7%. In contrast, no detectable cross-contamination was observed from chips printed with the noncontact piezoelectric technology. A 40% loss of signal between sequential spots within the one chip was observed in arrays printed with the contact dispenser (Figure 3a), while no loss of signal was noted in the experiments performed with the noncontact dispenser (Figure 3b). On the basis of these findings, the noncontact piezoelectric dispenser was chosen as the preferred printing technology.

Different concentrations of coating anti-IL-1β and anti-MCP-1 antibodies, ranging from 50 (data not shown) to 300 μg/ml in PBS, were printed for the selection of the concentration that provides the best signal intensity. A threshold effect which differed for IL-1β and for MCP-1 was observed across the different concentrations of coating antibody. A signal-to-noise ratio of 59.6 was measured from spots printed with concentrations of 100 μg/ml of coating anti-IL-1β, while a ratio of 1920 was observed with concentrations of 200 μg/ml. For MCP-1 assay, higher concentrations of coating antibody were required to reach signal intensities comparable to that of IL-1β assay. A signal-to-noise ratio of 16.6 was measured using 200 μg/ml of coating anti-MCP-1, compared with a ratio of 239 for concentration of 300 μg/ml (Figure 4).

The effects of storing chips at –20°C and –80°C (data not shown) were investigated. A significant decrease in MCP-1 signal intensity was observed from chips stored for 2 weeks at –20°C (Figure 5a) and –80°C (data not shown). However, after normalization of MCP-1 signal with hirudin, signal ratios remained similar (Figure 5b), indicating that storage had a constant diminishing effect on signal intensities.

Signal detection

Two different detection systems were evaluated. Signal intensities from hirudin sandwich assays were measured using both a fluorescence scanner and CCD camera detection system. The mean signal, background, and signal-to-noise ratio values are shown in Table 2. A large difference in signal-to-noise ratio was observed between the two detection systems, with the fluorescence scanner exhibiting more favorable ratios of 484 compared to a ratio of 3 from images captured with the CCD camera.

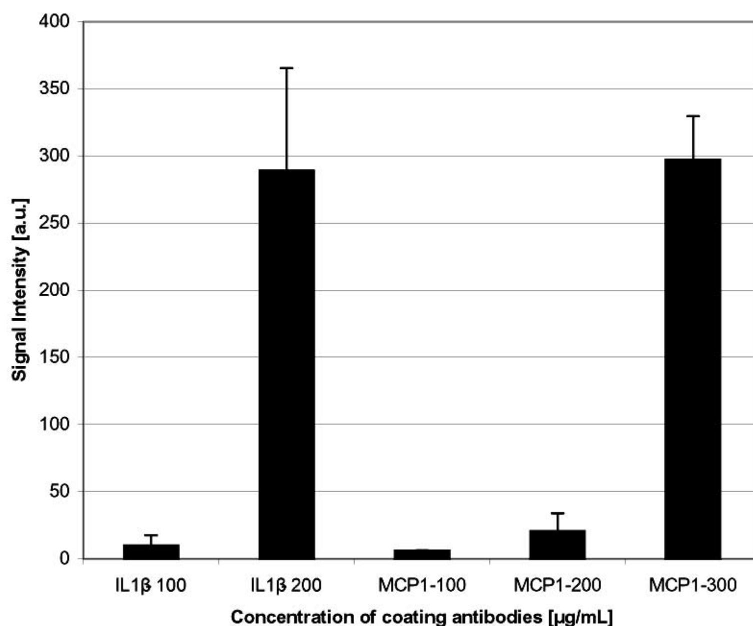


Figure 4. Evaluation of threshold effect. IL-1β and MCP-1 assays were performed using fixed conditions except for the coating antibody concentration. There was a strong difference in IL-1β signal intensity for 100 and 200 μg/ml of coating concentration. For MCP-1 assay, a similar difference was observed for 200 and 300 μg/ml coating concentration.

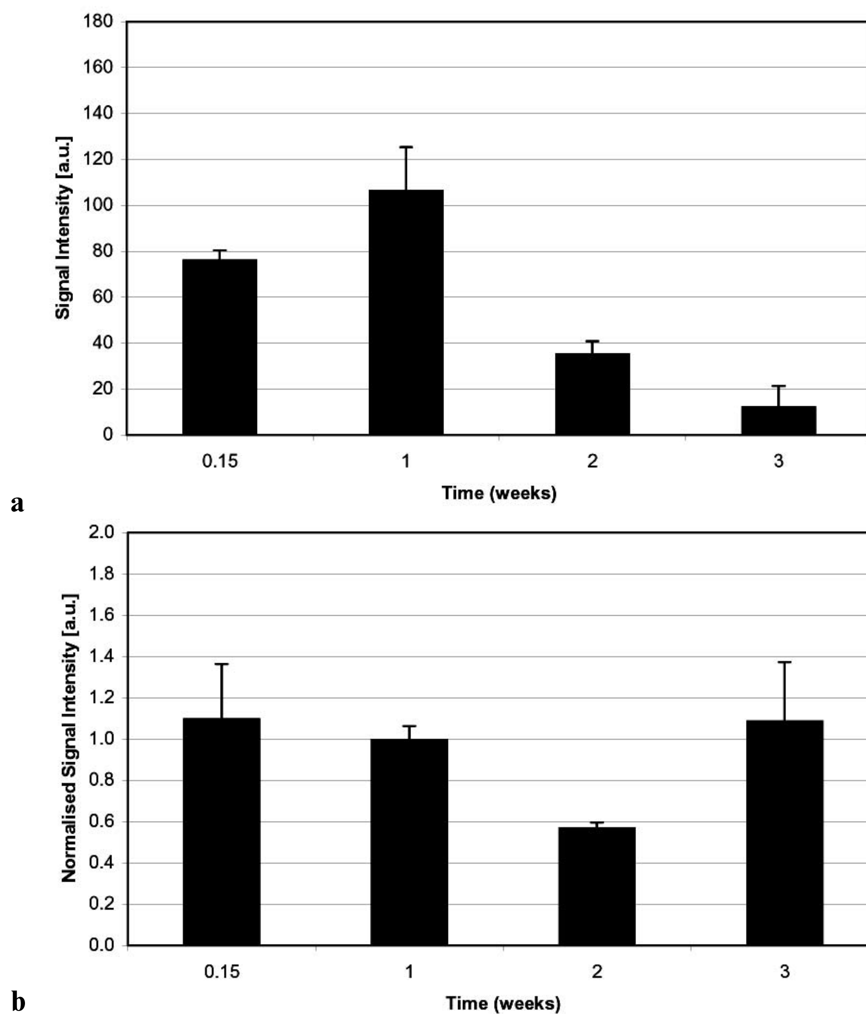


Figure 5. Evaluation of the chips after storage. Printed chips were either processed immediately or stored at –20°C for 1, 2, and 3 weeks before processing. (a) Results for MCP-1 sandwich. After the second week of storage signal intensity had diminished substantially. (b) Same results after normalization with hirudin. Signal ratios remained similar.

Table 2. Comparison between CCD camera and fluorescent scanner for hirudin sandwich assay. The signal intensity of mean and background ± CV% in arbitrary units was measured on the same chips successively with both detection systems. The signal/background ratio is much more favorable with the scanner

	CCD camera	Fluorescent scanner
Hirudin	300.6 ± 60.2%	198.5 ± 25.3%
Background noise	97.7 ± 1.1%	0.41 ± 99%
Signal/background noise ratio	3	484

Assay performance

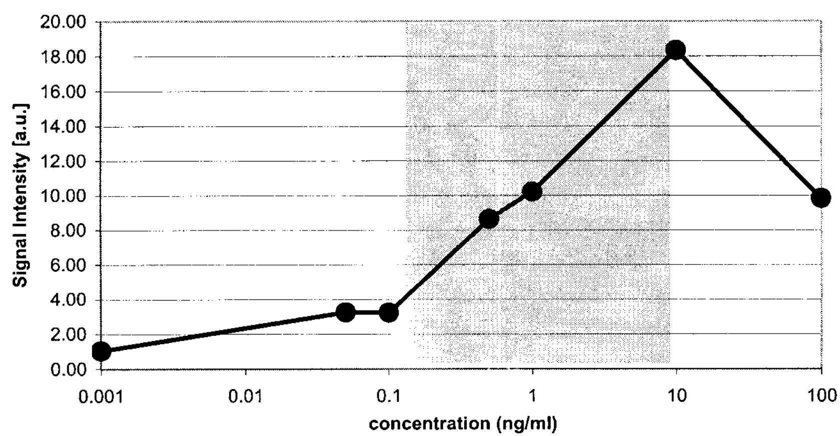
Several assays of different working ranges relevant to the biological concentrations were performed on the chosen microarray format. For MCP-1, a linear relationship between signal and concentration was observed from 100 pg/ml to 10 ng/ml (Figure 6a). For IL-6 and SAA, linear detection was observed in the concentration range from 500 pg/ml to 10 ng/ml (Figure 6b) and from 10 to 100 ng/ml (Figure 6c), respectively.

Discussion

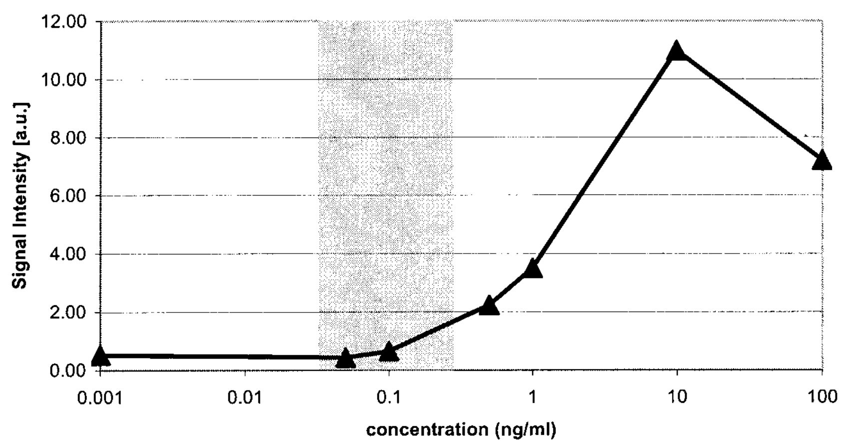
The glass substrate of the chip was pretreated in order to adsorb proteins onto the surface. Two treatments were tested to induce either hydrophobic characteristics or electrostatic charges. The hydrophobic properties were given by SAM of octadecyl phosphoric acid ester. The electrostatic charges were provided by poly-L-lysine. The character of the surface in terms of electrostatic charges or hydrophobicity had a strong influence on the printing quality, signal generation, background noise, and the variability of the assays. The hydrophobicity conferred by SAM was strong, and this likely contributed to the smaller diameter and homogeneity of the spot features compared to those obtained with poly-L-lysine. The strong hydrophobic adsorption of antibody spotted onto the SAM would make the possibility of diffusion rather weak. This would result in spot diameters close to the estimated diameter of the antibody solution immobilized (about 100 μm). The rather small size of the spot would provide a higher potential to increase the microarray density. Poly-L-lysine-treated chips had hydrophilic properties. It is possible that in such an environment the printed antibody would have more freedom to diffuse along the chip surface. This would have two consequences: a larger spot diameter (200 μm) and an accumulation of antibodies at the edge of spots, creating heterogeneity within the spot region such as the "ring" effect.

The activity of spotted antibody could be altered in different ways depending on the surface properties. Some antibodies, such as anti-IL-1-β, performed well on both surfaces. Others were more proficient on one surface than the other. For instance, anti-IL-8 worked better on poly-L-lysine, while anti-hirudin was more effective on SAM. Antigen–antibody binding is based on different kinds of interactions such as hydrogen-bonding, electrostatic, hydrophobic, or van der Waals forces. The nature of the physicochemical properties that maintain the antibody on the chip surface should have a strong impact on its binding activity. For instance, if the nature of the antigen–antibody interaction is based mainly on electrostatic charges, the binding site of the antibody will contain some electrostatic charges. In this case the adsorption of this antibody onto a poly-L-lysine surface could orientate the molecule in a way that may affect antigen accessibility to the binding site. On the other hand, when the antigen–antibody interaction is based mainly on hydrophobic interactions, its adsorption onto a SAM surface, which is highly hydrophobic, will orientate the molecule in a way that would reduce the binding capacity of antibody to antigen. Most of the time the antibody orientation is unpredictable, which means that all antibodies need to be individually tested on each surface.

Figure 6 (opposite). Assay performance. The range of biologically relevant concentrations is marked in grey: 0.15–10 ng/ml for MCP-1; 0.03–0.2 ng/ml for IL-6 and 5 μg/ml–1.5 mg/ml for SAA. The protein microarray allowed for measurement of biologically appropriate concentrations for MCP-1 (a). For IL-6 (b), the system lacked the sensitivity to detect the concentrations of IL-6 similar to concentrations found in biological fluids. For SAA (c), the system measured concentrations lower then those usually found in biological fluids.



a



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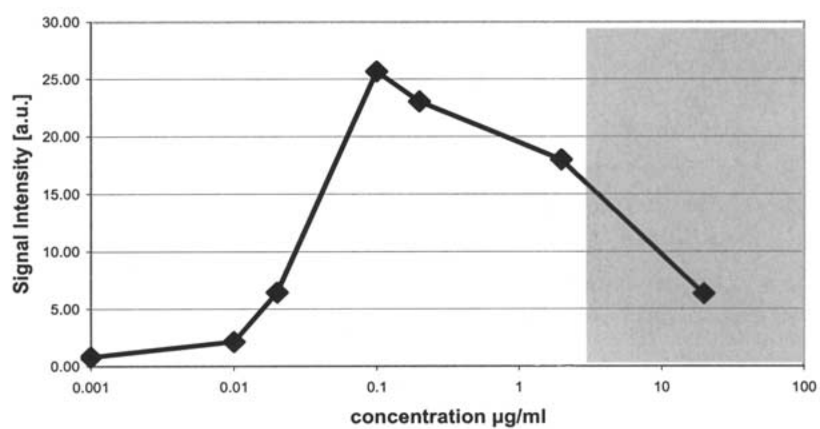

c

The background noise obtained on SAM was considerably lower than that measured on the poly-L-lysine surface. A possibile explanation for this could be the strength of the hydrophobic interaction induced by SAM. The blocking components would be strongly bound onto the surface, and not easily desorbed. The efficient blocking would prevent further reagent from binding onto the surface, keeping the background noise rather low. By contrast, the electrostatic charges of poly-L-lysine could provide a relatively weak interaction. After blocking, the bound proteins could be released during the washing steps. Thus, further reagents could bind onto the surface, increasing the background level. The high variability observed between signals produced on poly-L-lysine could have a similar explanation. The weak interactions could affect all the steps of a sandwich assay, and the immunological complex could be released in a variable manner. The lower background and variability provided by SAM had the potential to improve assay sensitivity. On the basis of these findings, SAM was selected as the surface for further experiments.

Contact printing and noncontact piezoelectric dispensing technologies were compared. Contact printing posed two major concerns: the reproducibility of the spots and the cross-contamination between spots. A possible reason was that the pins used by contact printing could take only a fixed and limited volume of antibody solution. The volume of reagent deposited by the contact printer would probably decrease with the number of applications, resulting in a decrease in signal after the third application. In contrast, no loss in signal was observed between spots printed with the noncontact dispenser. The tips used for piezoelectric dispensing are glass capillaries filled with a relatively large volume of antibody solution. The printer delivers a fixed and precise volume of antibody solution. The spotting is consequently much more accurate and precise. In addition, the washing of the tips or pins is a key factor in avoiding cross-contamination between different antibodies. Under our conditions, the washing of pins for contact printing was never properly achieved. However, the washing procedure of the noncontact printing tips was notably much more efficient, and negligible cross-contamination was observed. On the basis of these results, the noncontact piezoelectric dispensing technology was selected for the preparation of the arrays.

When all conditions were kept fixed for the assays (tracer and antigen concentrations), except for the concentration of spotted antibody, a threshold effect was observed between the concentration of the antibody spotted and the signal detected. The most likely explanation for this was that, at a concentration lower than the threshold, most antibodies adsorbed onto the chip surface were denatured by the strong hydrophobic interactions. When the antibody concentration is higher than the threshold, the hydrophobic surface is partly re-covered by hydrophilic proteins (antibodies). This would induce a protective environment, which would facilitate further adsorption of antibodies onto the surface. The concentration corresponding to this threshold effect differs from one antibody to another. For IL-1β this limit was between 100 and 200 μg/ml. For MCP-1 the limit was between 200 and 300 μg/ml. A critical step in microarray optimization is the evaluation of this effect for each of the antibodies that are to be spotted.

One of the key elements that drive the assay sensitivity is the signal-to-noise ratio. An efficient detection system should provide a signal as high as possible and have a background noise as low as possible. Two detection technologies, fluorescent scanning and CCD camera imaging, were tested. No major differences in signal intensity between arrays measured with the two detection systems were observed. However, images detected with the

scanner produced a much higher signal-to-noise ratio. In addition, the signals measured from the same spots showed much lower variability using the fluorescent scanner. On the basis of these results, the fluorescent scanner was selected for evaluation of the protein microarrays.

Storage time had a significant effect on signal intensity. For instance, after 3 weeks at –20°C absolute signal intensities decreased by about 90%. The results showed a relatively high instability of the chips after preparation. The chips were not stable for more than one week at –20°C. Without protective agents on the surface of the chip the antibody was probably denatured very rapidly. However, following normalization, relative signal intensities remained constant over the 3-week storage period, indicating that storage had a proportional effect on different antibodies. Although sensitivity had clearly decreased during the storage period, normalization was shown to play a key role following protein microarray storage. However, for optimal results we decided to use chips immediately after printing.

The conditions defined from these experiments for the microarray format were applied to different concentrations of IL-1β, IL-6, IL-8, MCP-1, and SAA to measure the performance of the microarray platform. The results showed three different cases of performance. In the first case, MCP-1 assay showed a linear relationship that covered the anticipated working range of MCP-1 in serum: 0.15–0.86 ng/ml in healthy volunteers and 0.36–6.5 ng/ml for RA patients (De Benedetti et al., 1999). In the second case, IL-6 assay showed a linear relationship at a concentration range too high to be biologically relevant, since serum levels of IL-6 in healthy individuals are usually less than 0.005 ng/ml and between 0.027 and 0.096 ng/ml in patients with RA (Arvidson et al., 1994). In the third case, SAA showed a linear relationship in a concentration range lower than the anticipated concentration in human serum samples. Human SAA is associated with inflammatory responses and is found in a normal range of approximately 1–5 μg/ml, while in RA conditions serum SAA is observed at levels from 0.3 mg/ml (Charles et al., 1999) up to 1.5 mg/ml (Cunnane et al., 2000).

In conclusion, the preparation of protein microarrays based on antibodies is not trivial or straightforward. One needs to investigate all assays individually and find an acceptable compromise of experimental conditions in order to cover all antigen concentration ranges corresponding to all biological samples. The protein microarray format needs to be adapted to measure simultaneously a broad range of biomarker concentrations (pg/ml up to mg/ml) related to the concentrations found in the progression of the disease. For instance, improvements in sensitivity can be achieved with lower background noise by using a more efficient blocking surface step. Sensitivity can be also improved by employing alternative signal generation methodologies such as secondary antibodies with a higher affinity, and/or by using chemiluminescence instead of fluorescence (Bates, 1989). On the other hand, oversensitivity could be solved by using an excess of non biotinylated secondary antibody.

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3.3 Assay optimization

In addition to the results of the protein microarray development on glass slide described in the previous chapter, additional investigations were undertaken to further optimize the assay. The goal was to explore whether adding glycerol to the coating solution could potentially help to stabilize the monoclonal antibody used as capture agent. In addition glycerol and its viscous properties could help to keep the spots more compact, consequently making image analysis more precise, and reducing spot to spot variability. The influence of adding glycerol to a printing buffer was investigated on the MCP-1 assay. Antibodies against MCP-1 were diluted in PBS alone and in PBS with the addition of 10, 20, 30, 40 and 50% of glycerol. Signal generated during sandwich assay for the concentration of 5 ng/mL of MCP-1 was plotted against different amounts of glycerol added to a coating solution (Figure 9). The results showed that the signal intensity decreased when 10% of glycerol was added to a coating solution. Whereas with the addition of 20-50% of glycerol the generated signals was comparable to PBS alone. However the variability between the spots (n=16) increased considerably.

The main concern however, was the difficulty associated with printing coating solution containing glycerol. The tips easily became clogged preventing the proper dispensing of coating solution. On the base of these findings no glycerol was added to the coating solution.

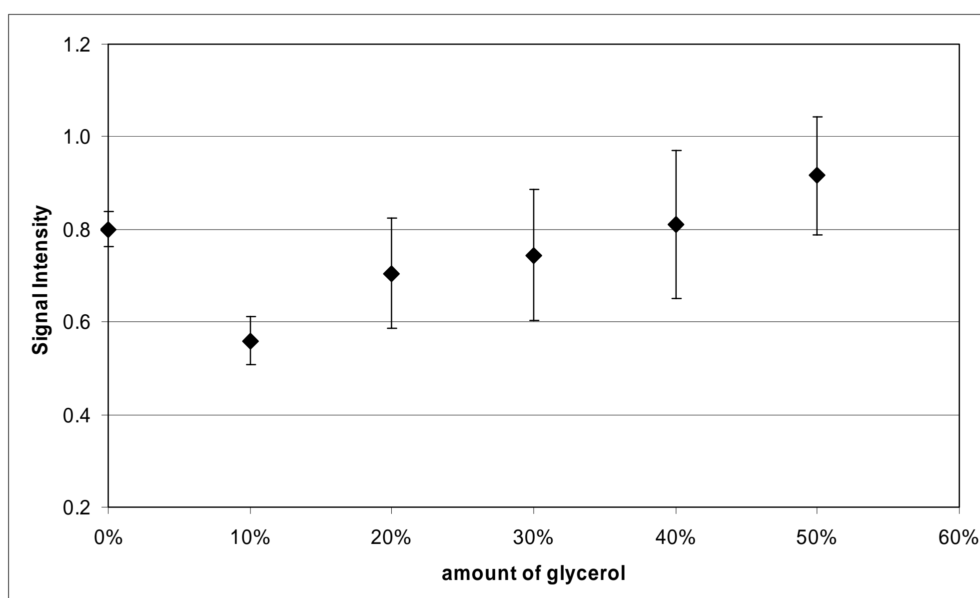


Figure 9: Addition of glycerol into a coating solution for MCP-1 assay.

4 Development of a protein microarray in 96-well plate format

4.1 Introduction

In chapter 3.2, the protein microarray was developed on a glass surface. For development, a number of components were evaluated, including the surface chemistry for glass treatment, the concentration of coating antibody, the arrayer for antibody deposition, and the detection system. Based on the obtained results, the following conditions were chosen for the preparation of the microarray: SAM of ODP for glass treatment; deposition of coating antibody at the concentration of 200 µg/mL using a non-contact piezoelectric printer, and signal detection by fluorescent scanner.

However, the time consuming and complicated process of coating the chip with the surface chemistry, together with the lack of robotic automation for assay processing, low sensitivity and the high inter-chip variability prompted other alternatives to be looked at which would provide less complicated and more robust options for protein microarray fabrication. Consequently, an attempt was undertaken to develop a similar microarray, but instead of using glass slides, the polystyrene surface of a 96-well plate was used as the solid support.

Polystyrene plates in a 96-well format are strongly established solid supports used for immunoassays, and are widely utilized in the classical ELISA technique (Angenendt et al. 2002). The surface chemistry is standardized and optimized for antibody binding, and the assay processing step is fully automated. Therefore, the possibility to adapt this well characterized technology to the microarray format could offer many advantages over other solid supports.

The availability of long neck tips (from PerkinElmer, UK) compatible with the Biochip Arrayer (BCA) printing device provided the option of using a 96-well plate as the solid support. Importantly, these long neck tips allow the antibodies to be correctly spotted onto the bottom of a 96-well plate (Moody et al. 2001). The development process of an antibody microarray to measure serum samples in a 96-well format is described in this chapter. The development process involved reagent selection, printing protocol optimization, matrix investigation, assay protocol establishment, and detection system evaluation.

4.2 Materials and methods

4.2.1 General reagents

- Serum

  dog serum, human serum, calf serum – (Novartis Pharma, Basel - internal blood bank)

4.2.2 Instruments

- Printing instrument

  Biochip Arrayer (BCA) (PerkinElmer, Boston, MA, USA)

  Instrument operated with Biochip BCTable Version 3.2

  Long-neck tip – Part nr. 7402251, PerkinElmer, Boston, MA, USA
- Cameras

  LAS 1000 – Fuji, Raytest GmbH, D 75339 Straubenhardt, Germany

  LAS 3000 – Fuji, Raytest GmbH, D 75339 Straubenhardt, Germany

4.2.3 Printing system set-up

The microarray was fabricated using the piezoelectric Biochip Arrayer. The piezoelectric system was set to 110-200 volts per tip. The distance between the tip and bottom of the well was 0.5 mm. The antibodies were dispensed in duplicates with a 1 mm distance spots in a 4 x 4 array. The layout of the dispensed antibodies is shown in the Figure 10. The dispensing process was programmed and operated with the use of BCTable software.

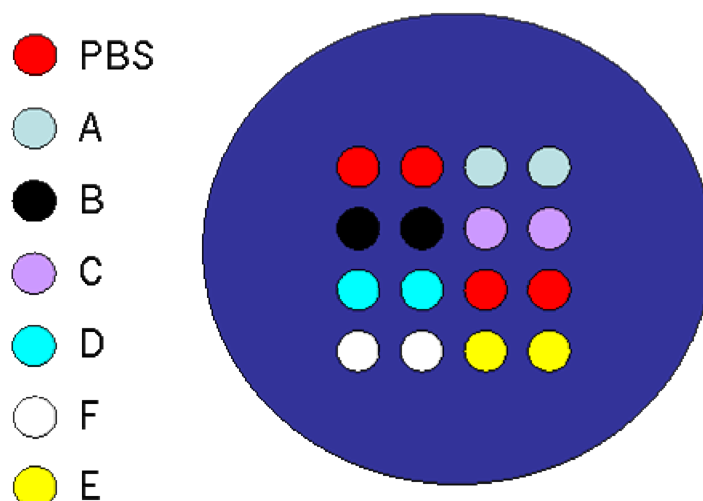


Figure 10: Layout of the printed antibodies. The antibodies were printed in duplicate. Spots are marked with different colours. Each colour corresponds to the specific pair of antibodies.

4.2.4 Assay protocol

Assay development process in a 96-well format utilised a sandwich assay approach. The monoclonal capture antibodies were spotted on the bottom of the well with a non-contact, robotic dispensing instrument – BCA. 100 µL of calibrated standard cocktails or samples were pipetted into pre-designated standard wells of a pre-printed microplate. The plate was incubated for 2 hr at 25º C with shaking. Following incubation, the plate was subjected to a wash step (300 uL, 3 times with wash buffer) to remove unbound material, and 100 uL of biotinylated detection antibody cocktail was added to each well. The plate was incubated for 2 hr at 25º C, and subsequently subjected to a wash step. A 100 uL streptavidin-horseradish peroxidase conjugate solution was added, and the plate was incubated for 30 min at 25º C. Following a wash step, chemiluminescent substrate (Super Signal ELISA Femto Substrate) was added. Generated chemiluminescent signal was digitized my the immediate imaging of the entire plate with the CCD camera. Analysis software was utilized to locate and quantify each spot and to generate standard curves. Signal intensities of a sample spot were compared to signal intensities from the respective calibrated standard curve to enable protein quantification.

4.3 Results

4.3.1 *Antibody selection*

The reagents used for the antibody 96-well plate development were commercially available ELISA antibody match pairs against different epitopes, and corresponding antigens. The panel of biomarkers chosen for the microarray development was A, B, C, D, E, F and SAA.
In order to check the applicability of the antibody match pairs in the multiplex format, any potential cross-reactivity was first investigated. All capture antibodies were spotted onto the bottom of each well and incubated with PBS spiked with a single recombinant cytokine (0.64 ng/mL of A, C, D, 0.58 ng/mL of B, 0.8 ng/mL of E and 0.038 ng/mL of F) and a cocktail of biotinylated antibodies against all antigens. All analytes levels that were not spiked in the sample were not detectable. These findings allowed to conclude that the selected antibodies match pairs were specific and did not cross react with other then analytes of interest target proteins in this microarray format.

4.3.2 *Reagents condition optimization*

4.3.2.1 *Titration of reagents*

The reagents titration included: coating antibody, detection antibody and streptavidin-HRP concentration.

In order to select the concentration of coating antibody that would produce the most optimal signal-to-noise ratio, antibodies against A, C and D were diluted in PBS to four different concentrations: 25, 50, 100 and 200 µg/mL and printed in the bottom of each well. Other reagents concentrations were set to the standard conditions recommended by the manufacturer: biotinylated Ab – 500 ng/mL; streptavidin-HRP – 0.2 µg/mL. After performing the assay, signal generated for each analyte was divided by the background value. Background was quantified from the well where no analyte was added (blank). The signal-to-noise ratio was calculated for the concentration of 10000, 1000 and 100 pg/mL for each analyte. In the Figure 11 the signal-to-noise ratio for the analyte concentration of 100 pg/mL is shown. For A a signal-to-noise

ratio of 1.5, 19.5, 92.9 and 129.2 was measured for 10, 50, 100 and 200 µg/mL of the concentration of coating antibody. For C - 1.0, 106.9, 113.9, 116.5 and for D - 2.1, 59.7, 155.1, 157.8, respectively. (Figure 11). The highest signal-to-noise ratio for all analytes was obtained when the concentration of 200µg/mL of coating antibody was used, as a result this concentration was chosen for microarray fabrication.

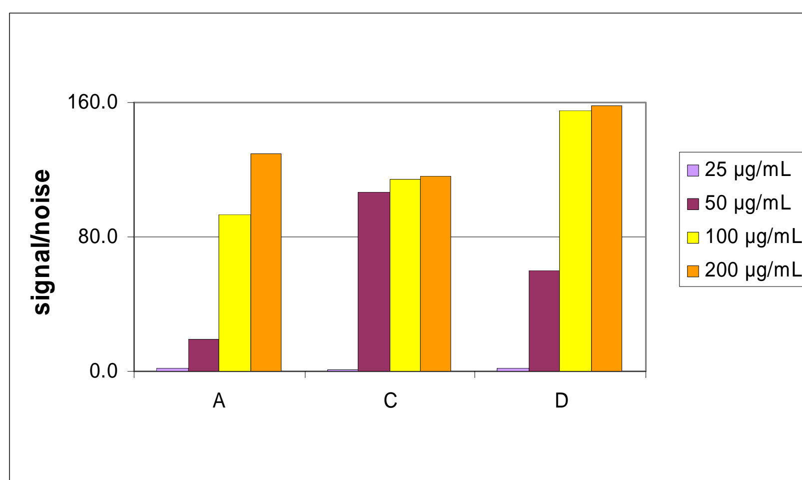


Figure 11: Coating antibody titration. Different concentrations of coating antibody against A, C and D were printed. The best signal-to-noise ratios were obtained using coating antibody concentrations of 200µg/mL for all the measured analytes.

Different concentrations of detection antibody were also tested in order to select the concentration which gave the optimal signal-to-noise ratio. Capture antibodies against A, C and D were printed at the concentration of 200 µg/mL. Respective biotinylated antibodies were diluted to 62.5, 125, 250 and 500 ng/mL. The used concentration of strepatividin-HRP was 0.2 µg/mL. The signal generated for each dilution of biotinylated antibody was divided by the corresponding background. The highest signal-to-noise ratio was obtained when the concentration of 500ng/mL of coating antibody was used for each analyte. The example of signal-to-nose ratios generated for D assay with different concentration of biotinylated Ab is shown in Figure 12. Consequently, a concentration of 500 ng/mL of detection antibody was chosen for microarray fabrication.

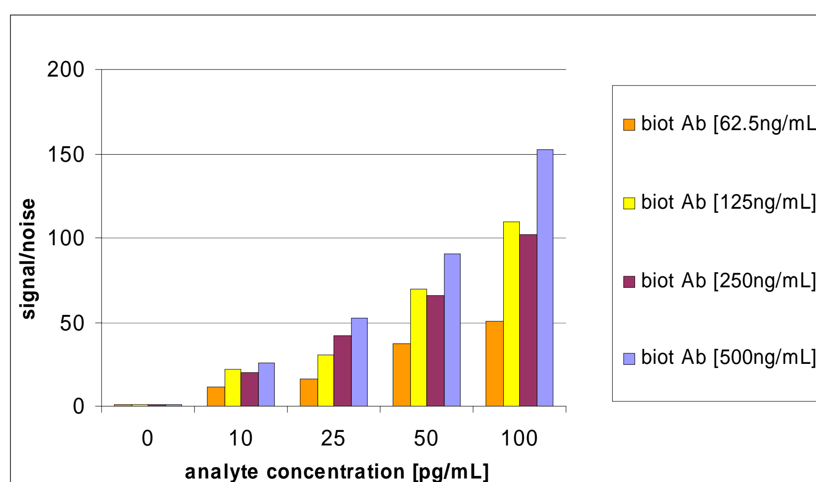


Figure 12: Assay was performed using different concentrations of biotinylated antibody. The best signal-to-noise ratio was generated with the concentration of 500ng/mL of biotinylated Ab for each analyte. The signal-to-noise ratios showed in the figure were generated for D assay.

Two different concentrations (0.2 and 0.1 µg/mL) of strepatavin-HRP in assay buffer were evaluated (data not shown). There was no significant difference in the assay performance comparing these two conditions. Consequently the 0.2 µg/mL concentration of streptavidin-HRP was chosen for microarray fabrication.

4.3.2.2 Volume of coating solution per spot

Different volumes of dispensed coating antibody were tested to check whether this parameter can improve the assay performance.

Antibodies were printed onto the surface of each well in quadruplicate. Each standard curve concentration was dispensed into two wells. Consequently 8 spots per analyte were generated. The antibodies solutions were dispensed at a volume of ~0.333 pL per drop. Four different cases of total spot volume were investigated: 20nL (60drops), 10nL (30drops), 3nL (9drops) and 0.333 nL (1drop). The coefficient of variation (CV%) on the signal provided by the spots after performing an assay, was calculated for each dispensed volume. When 20 nL per drop was dispensed the size of the spots was relatively big resulting in spot overlap and subsequent inaccuracies in spot assaying. In three other cases CV% of signal intensities generated from each spot was calculated for all standard curve concentrations: 10, 25 50 100 and 1000 pg/mL. The best sensitivity and CV% was obtained when the volume of 3 nL per spot was

dispensed. The CV% range over the standard curve concentration is shown in (Table 6). As a result the volume of 3nL /spot was chosen for microarray fabrication.

Table 6: Dispensed spot volume. Different volumes of coating antibody solution per spot were dispensed for each analyte. CV% was calculated on signal densities generated from each spot (n=8) using the formula: (stdev/mean*100). The CV% was the best when 3nL / spot volume was dispensed.

	A	C	D
Spotted volume	CV%		
0.333 nL	35.4 – 76.8	33.4 – 54.6	11.5 – 105.4
3 nL	4.7 – 13.3	10.3 – 37.6	6.1 – 12.0
10 nL	18.2 – 25.2	22.3 – 53.3	11.1 – 30.4

4.3.3 Matrix evaluation

As recommended by the FDA guidelines for pharmacokinetic immunoassays (Food and Drug Administration 2001) standard curve should be prepared in the same matrix as the analyzed samples. Alternatively, other species matrixes or analyte free buffer can be used. During the microarray development, quantitative curves were produced for A and C assays. D quantification, however was not possible when the standard curve was prepared in human serum. In order to produce a standard curves that would allow for the simultaneous measurement of all analytes of interest; dog, calf, human serum pool and analyte free protein based buffer were evaluated as potential matrix's for the standard curves preparation.

4.3.3.1 Human, dog and calf serum evaluation

Standards curves for A, C and D were prepared in single dog, human, and calf serum. Standard curves generated for D, did not provide the linearity sufficient to perform quantitative D measurement (Figure 13). For instance, when human and dog serum matrix's were used for standard curve preparation, D signal generated for the blank samples was about 70 times higher compared to the C blank signal. When calf serum was used to generate a standard curve this difference was even 700 times higher.

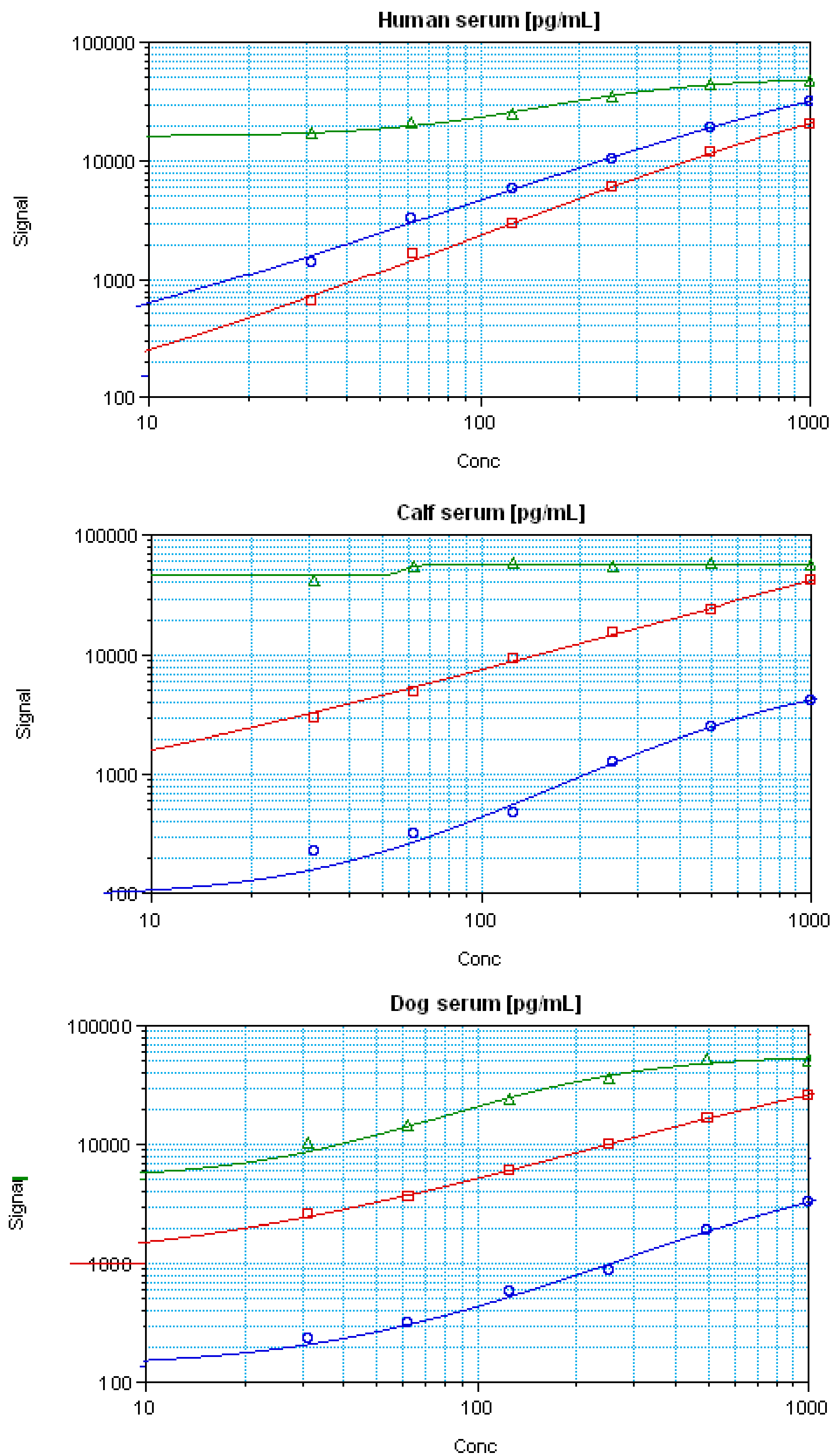


Figure 13: Standard curves for A, C, D generated in dog, human and calf serum. Local background from each well was subtracted from the signal intensities before plotting.

□ A
△ D
○ C

4.3.3.2 Buffer evaluation

Standard curves for A, C and D were prepared in assay buffer used for standard curve generation in commercially available ELISA kits (TCM sample diluent – ENDOGEN). The obtained standard curves are shown in Figure 14. Human serum pool was spiked with recombinant proteins at various concentrations. These samples were analysed on the produced standards. The found concentration did not meet the spiked amount of each analyte. For instance results obtained for A gave half of the values (accuracy ~ 50%) compare to the spiked amount of A (Table 7).

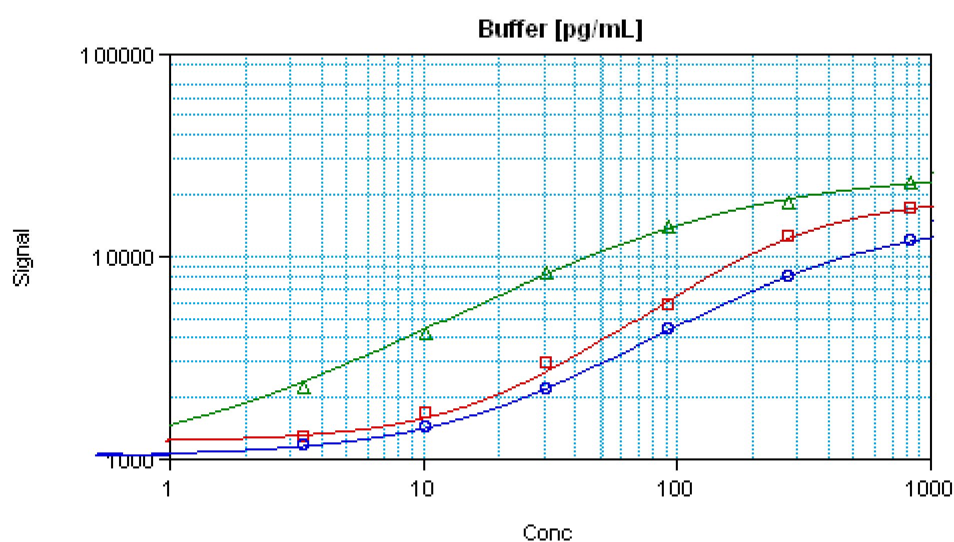


Figure 14: Standard curves obtained for A, C, D prepared in assay buffer.

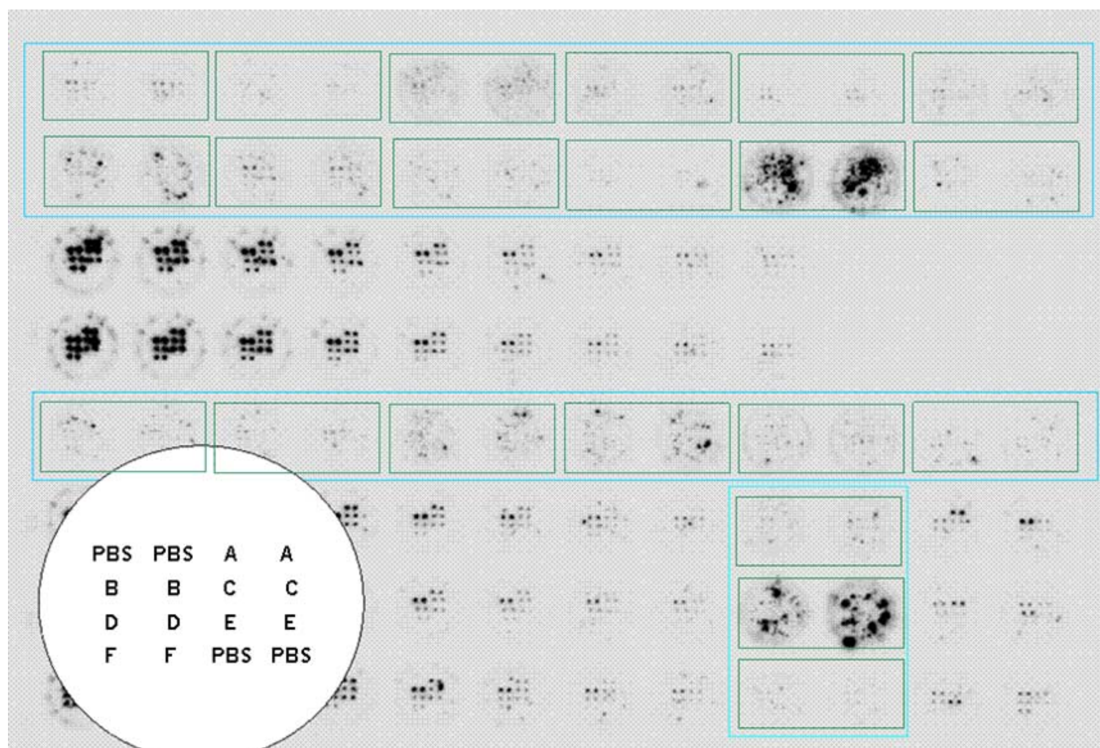
Table 7: Accuracies [(spiked analyte concentration/found analyte concentration)*100] calculated for human serum samples spiked with A. The accuracies were calculated on A standard curve prepared in buffer. The obtained values gave half of the values compared to the spiked amount of A

Spiked analyte concentration	Found analyte concentration	Accuracy
ng/mL	ng/mL	%
1.000	0.489	48.9
0.400	0.193	48.3
0.160	0.074	46.5
0.064	0.031	48.3
0.026	0.014	52.8

4.3.3.3 Different human serums screening

In order to select the human sera that contain only low levels of A, B, C, D, E, F, antibodies against them were printed onto the surface of each well. Different batches of human sera were applied onto an array (Figure 15a). Sera that did not provide the signal or these for which the generated signal was very low for the investigated analytes were selected for pooling (Figure 15b). The amount of endogenous analytes in these samples was measured with the use of commercially available ELISA kits in order to know to which level do they correspond in the individual samples and in the serum pool. These data was needed to use ELISA in the future to screen the sera prior pooling, and consequently to prepare the pool itself. In total 36 batches of human sera were screened out of which 12 were selected and pooled. Levels of analytes of interest in the pool are shown in Table 8.

a)



b)

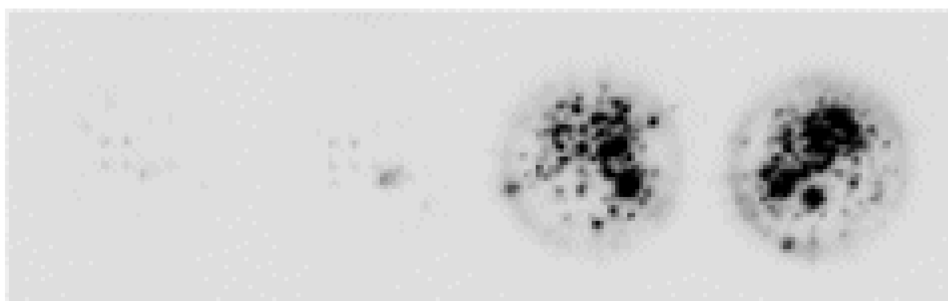


Figure 15: Screening of different batches of human sera**.** Different batches of human sera were applied into each well (marked in blue –(a)) . Each batch was analyzed in duplicate (green (a)). Sera that gave high signal for the printed analytes were rejected. The example of the rejected serum is shown in (b) - 2 wells on the right site.

Table 8: Analyte level in the selected human serum pool.

Analyte	Result [ng/mL]	Analyte levels range in normal serum [ng/mL]
A	blq	blq
B	0.232	0.106 – 1.552
C	blq	0 - 0.149
D	0.049	0.001 – 0.016
E	blq	blq
F	0.282	0.200 – 0.722

Subsequently standard curves for all analytes were produced in a human serum pool. All standards showed satisfactory linearity. The example of A, C, D are shown in Figure 16.

Samples prepared in the same manner as during the buffer matrix evaluation (section 4.3.3.2, page 44) were measured with the human serum pool standards. The determined concentration for all analytes corresponded to the spiked concentrations gave the assay accuracy within 70% ≤ and ≤130%. As an example, results for A are shown in Table 9. Selected human serum pool was used for the assay validation.

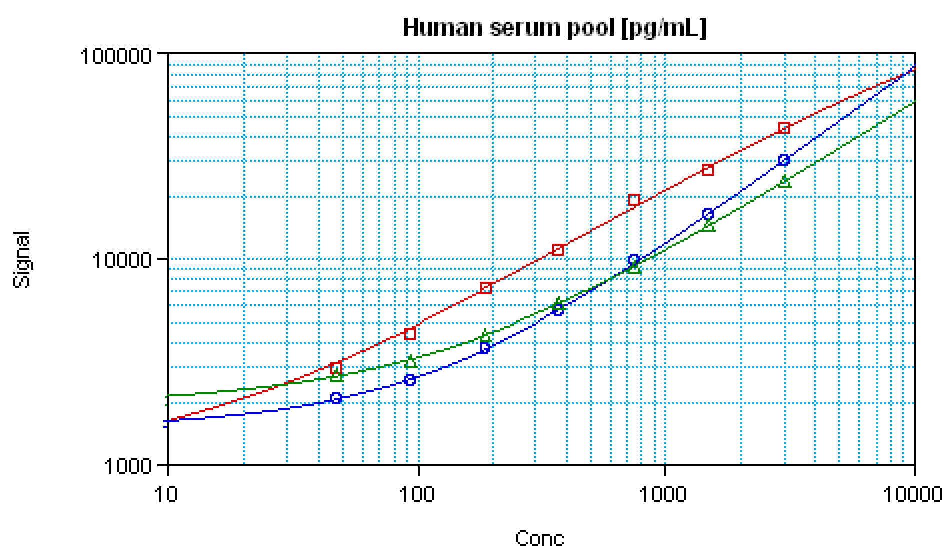


Figure 16: Standard curves for A, C, D generated in human serum pool. Local background from each well was subtracted before plotting the signal and concentration.

Table 9: Results for A assay. Analyte A human serum spiked samples were measured using a standard curve prepared with human serum pool. The concentrations were found to lie within the satisfactory accuracy (70% ≤ and ≤130%).

Spiked analyte concentration	Found analyte concentration	Accuracy
ng/mL	ng/mL	%
1.000	0.798	79.8
0.400	0.373	93.3
0.160	0.138	86.2
0.064	0.055	85.4
0.026	0.022	87.3

4.3.4 Assay condition optimisation

4.3.4.1 Addition of glycerol into a coating solution

The influence of glycerol for the assay performance was investigated. PBS both without and with, 5 or 10 % addition of glycerol was used as coating solution. The spotting device producer recommended the addition of 10% of glycerol as maximum amount of glycerol added into a coating solution, due to the technical issues. CV% and accuracy was calculated for all standard curve concentration points. The representative data for A is shown in Table 10. There was no major differences in CV% and Acc% in between the results obtained with the addition of glycerol. Addition of glycerol, however, was problematic from the technical point of view. The viscous glycerol properties caused the printing tips to clog. Therefore, based on these results it was decided not to add glycerol to PBS in preparing coating buffer for microarray production.

Table 10: Addition of glycerol into a coating solution.

A pg/mL	10 % glycerol		5 % glycerol		0 % glycerol	
	CV%	Acc%	CV%	Acc%	CV%	Acc%
7.8	7.4	105.1	13.8	99.7	11.3	84.0
15.6	17.6	108.4	19.7	102.8	26.6	128.9
31.2	15.3	92.4	12.5	111.5	24.4	119.8
62.5	16.0	88.4	37.1	83.5	27.1	107.3
125	19.7	99.9	34.0	94.6	24.0	92.8
250	16.0	98.9	28.4	106.0	16.2	102.2
500	8.7	108.4	44.8	108.5	20.3	99.5
1000	17.8	100.3	34.2	96.3	26.4	100.1

4.3.4.2 Assay format

The developed assay conditions were also applied to co-incubation assay format. In case of co-incubation, assay was performed in 1 step. Printed coating antibodies were incubated together with the mixture of sample and cocktail of biotinylated antibodies (Figure 17). Co-incubation approach could minimize the time difference between the sample application into the plate. Another advantage could be assay practicability – only one incubation step required, no washing steps in between. In order to evaluate the performance of sandwich (2steps) and co-incubation (1 step), the same experiment was carried out utilising both formats. The schematic design of both formats is shown in Figure 17. For 1 step format the two incubation steps were replaced with one 4h incubation of sample mixture (50 µL/well) and biotinyletd antibody (50 µL/well). Subsequent steps with strepatvidin-HRP and chemiluminescent substrate were the same for both formats. C standard curve represents the standard curves generated with the use of both assay formats (Figure 18). When 1 step format was performed the generated standard curve linear range augmented, showing the potential to increase assay sensitivity. Consequently 1 step protocol was applied for microarray fabrication.

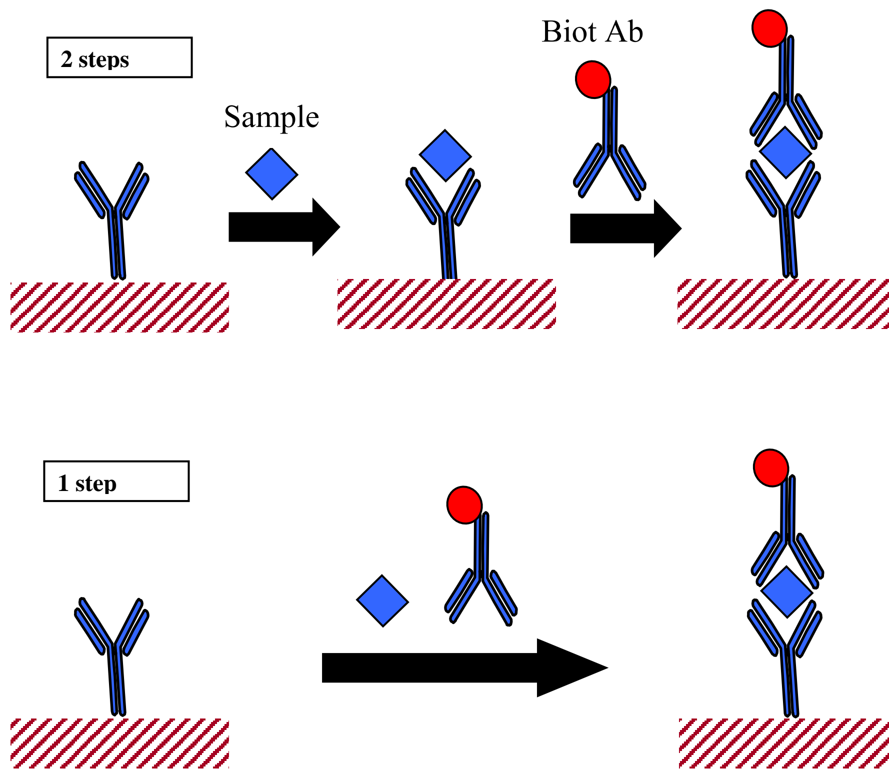
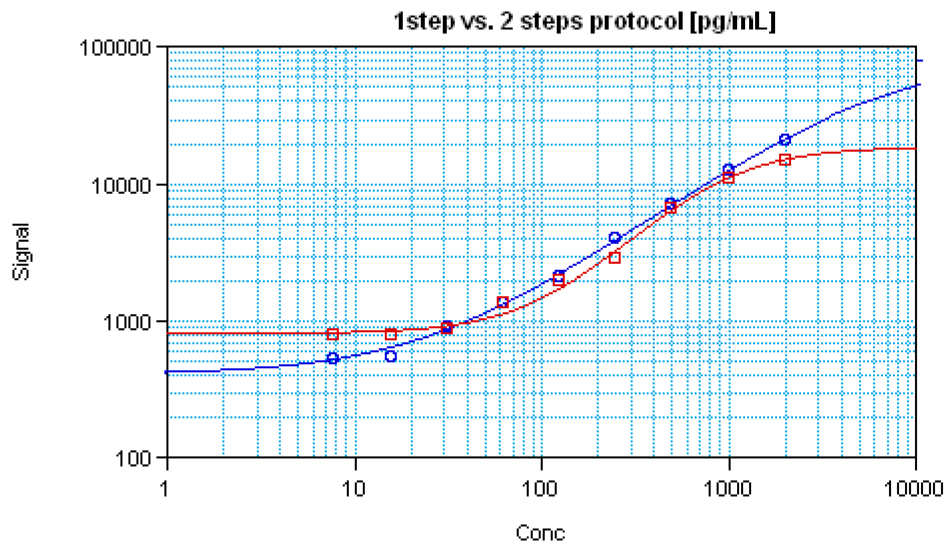


Figure 17: 2 and 1 step assay procedure. Two steps protocol consists of two incubation steps (2h each): sample is added and incubated with coated antibodies, after washing, assay is incubated with biotinylated antibodies. In co-incubation protocol, sample and antibodies are added together and incubated for 4 h. There is no washing step required.



○ 1 step
□ 2 steps

Figure 18: Representative C standard curve for an assay performed with two step and co-incubation protocol. The obtained results show that 1 step protocol can improve assay sensitivity.

4.3.4.3 SAA assay approach

According to literature SAA level in RA sera is observed at levels ranges from 300 µg/mL (Charles et al. 1999) up to 1500 µg/mL (Cunnane et al. 2000). The standard curve linear range generated for SAA was not sufficient to quantify the biological concentration for SAA found in RA patients. Following the hypothesis that the cold coating antibody could bind to the analyte (Figure 19) reducing the amount of analyte-biotinylated antibody complex that could bind to coating Ab, cold monoclonal antibody in the amount 20 and 80µg/mL was added into a sample. Theoretically the linear part of standard curve could be moved in a manner that the quantification of higher concentration of SAA would be possible. Only addition of 80 µg/mL of coating antibody showed shift of a standard curve range however the shift was not significant enough to measure SAA in biological undiluted samples (Figure 20). This approach already made an assay too expensive in comparison to single SAA ELISA kit. As a result SAA was removed from the panel of analytes.

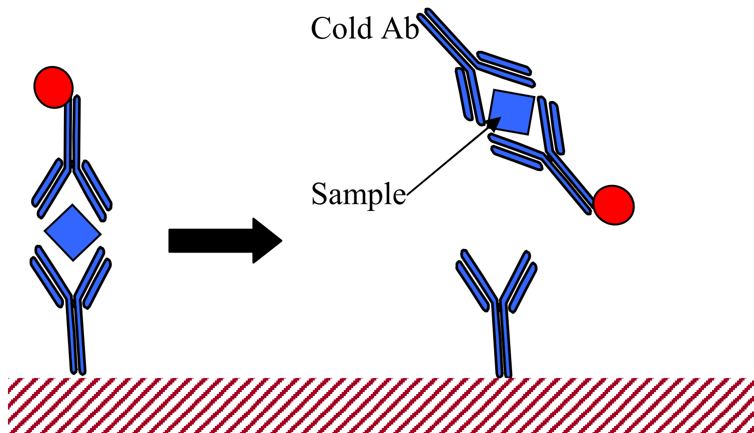


Figure 19: Approach of adding cold coating antibody into a sample. Cold antibody could bind to the analyte reducing the amount of analyte-biotinylated Ab complex bound to the capture Ab. This could help to shift SAA standard curve in a manner allowing to measure higher SAA concentrations.

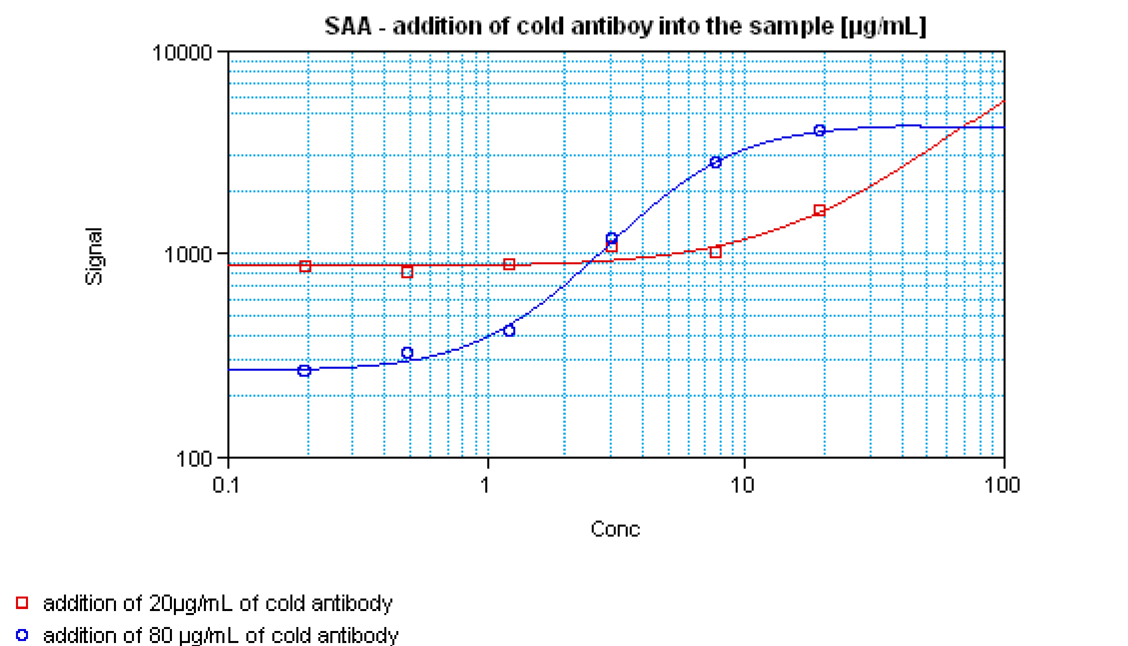


Figure 20: SAA standard curve range did not cover the biological level of SAA. Cold anti SAA monoclonal antibody in the concentration of 20 and 80µg/mL was added into a sample in order to optimize standard curve to meet SAA levels in biological samples. Only addition of 80µg/mL of an antibody shifted standard curve range.

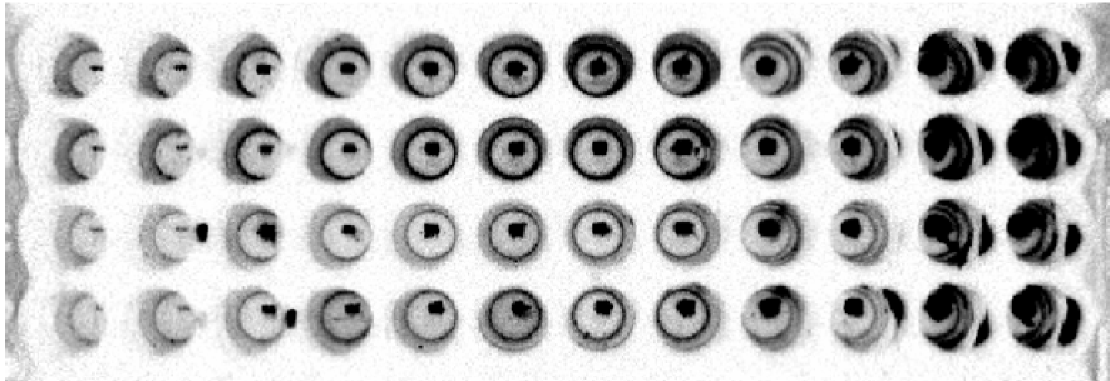
4.3.5 CCD camera selection and set up

Signal visualised using HRP-conjugated streptavidin and chemiluminescence was then imaged with the use of charge-coupled device (CCD) camera.

The choice of chemiluminescence and CCD camera combination was dictated by the 96-well format. At that time, there was no fluorescence scanner available for digitizing the signal from the bottom of 96-well pate. Two CCD cameras were utilized for imaging: LAS 1000 and LAS 3000. Figure 21a shows the image of 96-well plate digitized with LAS 1000, figure 21b with LAS 3000. The advantage of the second camera is a non-parallax tray option eliminating parallax in each of 96 well plate wells. The parallax effect causes that the image of the bottom of the well is deformed making the spot analysis inadequate. LAS 3000 also gave an option of high resolution binning mode which by decreasing the size of the spot makes microarray analysis easier and more precise (Figure 21c). In consequence second camera was chosen for microarray fabrication.

Various options are available for background measurement: local (area around individual spots) or global (area outside of the grid) background corrections, as well as user-defined values such as those from negative control data points contained within the array may all be employed for analysis. All these options for background correction were evaluated (data not shown). Background generated during the assay was stable across the all analyte concentrations for all wells of the plate. CV% between the background signal intensities from each well was 3.3%. The assay results with and without background subtraction were compared. There was no difference in assay performance in terms of sensitivity and variability. Based on these results in the developed assay there is no background correction performed for the produced signal intensities.

a)



b)

c)

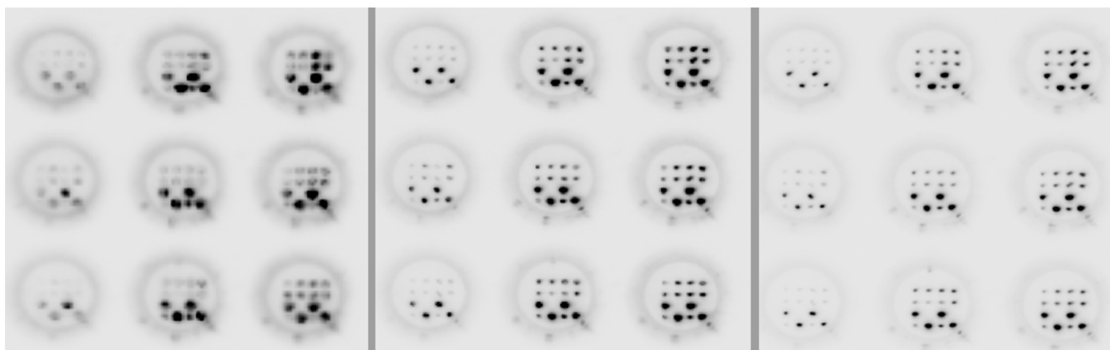


Figure 21: Images generated with two different CCD cameras: a – LAS 1000, b and c – LAS 300. The picture a) shows an image produced without non-parallax tray, created edge effect makes an image analysis imprecise. Picture b) and c) was taken with the non-parallax tray device. There is no more edge effect b). On the picture c) three different image modes are compared: high (left), standard (middle) and high resolution mode (right).

4.3.6 Quantification software

To quantify the produced signal many companies offer the specialized quantification software. The main issues raised with the signal quantification is analysis practicability and precision. Three of the commercially available quantification software were evaluated: Aida, ImaGene™ and ArrayVision™. ArrayVision™ package was superior over two other software because of the standard curve generation feature. Other programs required additional tools for standard curve generation. For instance, digitized signal values had to be exported into a Excel program to perform the variability calculations. Afterwards the data was again exported to Softmax® PRO software for standard curve generation. This was the main factor that caused the ArrayVision™ to be chosen as the software for antibody microarray fabrication. The comparison of the three software is shown in Table 11.

Table 11: Quantification software comparison.

	Aida	**ImaGene™**	**Array vision™**
Speed of analysis	+	+++	+++
Auto spot adjustment	-	+	+
Easy to use	+	+++	+++
Standard curve generation	-	-	+

4.3.7 Developed protocol for the miniaturized assay in 96-well format

As an outcome of all the development steps described above, the protocol for the microarray fabrication in a 96-well format to measure serum samples was created. This protocol is shown below:

Protocol for the microarray generation in a 96-well format

General reagents:

Plate	black plate
Coating buffer	PBS1X
Matrix	human serum pool
CCD camera	LAS-3000. High resolution binning mode.
Software	ArrayVision™

Assay Protocol:

Coating	- 200µg/mL of monoclonal Ab, - 3 nL/ spot - 2 hours drying in the Biochip arrayer enclosure - over night incubation at 4ºC - drying 30 minutes at RT - drying 10 minutes at 37ºC
Blocking	- 3 X 200 µL of SuperBlock/well - 100 µL of SuperBlock/well – 30 minutes incubation RT
Samples incubation	- 50 µL/well of sample - 50 µL/well of cocktail of biotinylated antibodies at 500 ng/mL - 4 hours incubation at room temperature on shaking platform - wash 3 times with wash buffer 300 µL/well
Streptavidin-HRP	- 100 µL/well at concentration 0.2 µg/mL - 30 minutes incubation at room temperature on shaking platform - wash 3 times with wash buffer 300 µL/well
Chemiluminescent substrate	
	- 100 µL/well – immediate readout
Imaging	- CCD camera exposure for 20 second - images digitized and saved as 16-bit tiff files
Signal quantification	- ArrayVision™

4.4 Discussion

The objective was to develop an efficient method to measure simultaneously serum biomarkers in the 96-well format. The main components, steps and conditions that make up a microarray fabrication were evaluated. Assessment covered the reagents titration, amount of coating antibody per spot deposition, matrix selection, signal detection, assay format and analysis software evaluation.

The concentration of 200 µg/mL of coating antibodies produced the most optimal signal-to-noise ratio. The reason why the lower concentrations investigated did not produce similar signal-to-noise could be caused by the antibodies denaturation during the printing process. When the concentration of coating solution was higher, probably the amount of Ab that remained in a functional state was sufficient for optimal assay performance. Another explanation is the nature of physical adsorption by which antibodies are immobilized on the polystyrene 96-well plate. Physical adsorption can lead to the protein desorption during the assay, which can lead to signal loss. Consequently, when the concentration of coating Abs was higher, even though some Abs desorped from the surface, the remained amount was still sufficient to perform an assay.

Addition of glycerol as a potential stabilizer to the coating solution could help Abs to maintain their functionality. Thus, the influence of glycerol in a coating solution on assay performance was investigated. However there was no advantage observed in terms of variability and accuracy when the glycerol was added. Maybe the fact that antibody spots were in the bottom of the well prevented the spots from drying out and created the microenvironment with the conditions that did not denature the mAb.

Existing guidance for pharmacokinetic immunoassays recommend to prepare standard curve for analyte measurement in the matrix of the same origin as the analyzed samples. Alternatively the heterologus animal matrix or analyte free buffer could be used. During the microarray development the linearity obtained for D standard curve prepared in human serum, did not allow for D quantification. Probably serum used to generate standard curve contained endogenous D that introduced bias in the analyte measurement. Precise D quantification was only possible in the selected pool of

human sera that contained low levels of endogenous analytes of interest. This approach however has its limitations. Might be that for a different panel of analytes this kind of pool could never be selected. In such situation the compromise has to be taken: either the problematic analytes should be excluded from the set of analyzed markers, or other matrixes such as analyte free buffer should be used as matrix. Choosing the second option though, the spike recovery investigations have to be performed and necessary corrections included in the results calculations. For instance for the panel of markers for which the microarray was developed in the above chapter the accuracy of spiked A was about 50 % when buffer was used as a matrix. This justify the need of assay performance validation and necessary results corrections.

The light produced in chemiluminescence reaction is emitted equally in all directions. That is why it is recommended to use black instead of clear plates for the chemiluminescence measurements in order to avoid so called light piping phenomenon. Light piping is simply the interference of the signal generated from each spot and well. Black plates however require the image to be taken from above the plate which creates the danger of introducing the parallax error. Therefore we compared two kinds of CCD camera as imaging tool for the antibody array. The non parallax tray option provided by LAS 3000 camera allowed for imaging the whole plate with no parallax effect. In the future, the availability of the scanner to image microarray in a 96-well format could allow changing to fluorescence signal generation. This could help to avoid light piping as well as stable, multi-readable signal production.

Described results demonstrate the microarray development steps and conditions. However the applicability of the platform to the real samples measurement, its reproducibility, and accuracy need to be validated. The individual validation steps are described in the following chapters.

5 Validation of Antibody microarray in 96-well plate

5.1 Introduction

The developed antibody microarray in 96-well plate is based on a sandwich ELISA procedure utilized in classical immunoassays (Wild 2001). Immunoassays are frequently applied for biomarkers quantification in clinical studies (Findlay et al. 2000). Thus, it is very important for the immunoassay to be accurate and precise. Accuracy, is defined as the closeness of the concentration value obtained by the method to the known true concentration value of the analyte. Precision is the closeness of individual measures of an analyte when the method is applied repeatedly to multiple aliquots of the same biological sample.

- Accuracy = (calculated concentration/nominal concentration)*100.
- Precision = (standard deviation/calculated mean concentration)*100

These key characteristics of any bioanalytical method are investigated during the process of validation. Validation of an analytical method identifies the sources and quantifies the potential errors in the method (Findlay et al. 2000; Shah et al. 2000). An assay validation describes in mathematical and quantifiable terms the performance characteristics of an assay. Classical immunoassays have to be validated in line with the FDA directives (Food and Drug Administration 2001). Such regulations do not exist for biomarkers and multiplex assays. Therefore, in order to validate the developed antibody microarray, the protocol that followed the FDA guidelines for industry for pharmacokinetic immunoassays validation (Food and Drug Administration 2001) was established. The validation covered the assessment of the methods robustness and reproducibility. A spike-recovery validation test was elaborated and run over 3 days.

5.2 Materials and methods

5.2.1 Validation protocol

The protocol adopted for validation of the Ab microarray was based on FDA directives for pharmacokinetic immunoassays (Food and Drug Administration 2001). The following section describes the process used to validate the Ab microarray. A modified protocol adopted for Ab microarray in a 96-well format that was implemented for the validation process is shown below:

Validation protocol of antibody microarray in 96-well plate:

Matrix

Matrix is used to describe a biological medium like plasma, serum, whole blood, urine, faeces, tissue, from which the analyte(s) is quantified.

All validation experiments were performed on a selected pool (n=12) of human serum containing low levels of the measured analytes.

Standard curve

The standard curve is a relationship between instrument response and known concentration of analyte.

- The linear part of standard curve should lie within the analyte concentration levels in the disease state. The standard curve was constructed using 8 different concentration levels. The samples were blanked with sample to which neither analyte(s) nor internal standard had been added.

Table 12: Concentrations of standard curve points.

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std9
ng/mL									
A	blank	0.023	0.047	0.094	0.188	0.375	0.750	1.500	3.000
B	blank	0.090	0.180	0.359	0.719	1.438	2.875	5.750	11.500
C	blank	0.023	0.047	0.094	0.188	0.375	0.750	1.500	3.000
D	blank	0.023	0.047	0.094	0.188	0.375	0.750	1.500	3.000
E	blank	0.117	0.234	0.469	0.938	1.875	3.750	7.500	15.000
F	blank	0.014	0.027	0.055	0.109	0.219	0.438	0.875	1.750

- Standards in matrix must be freshly prepared and run on each (n=3) validation day.
- Standards were prepared singly and analyzed in triplicates.
- Back-calculated values for the standards within the working range should give ≤30% deviation from the expected concentration (70% ≤ Accuracy ≤130%).
- Precision should be ≤ 30% at each concentration level
- Maximally 1/4 of the individual standards can be excluded.
- At least 50% of the values (spots) at each standard concentration must be within accuracy and precision range.

Quality control samples

Quality control sample (QC) is a spiked sample used to monitor the performance of a bioanalytical method and to assess the integrity and validity of the results of the unknown samples analyzed in an individual batch. QC is prepared in the same matrix as standard the curve.

- QC samples were prepared in six concentration levels for each analyte.

Table 13: Concentrations of quality control samples.

	QC1	QC2	QC3	QC4	QC5	QC6
ng/mL						
A	0.026	0.064	0.160	0.400	1.00	2.500
B	0.092	0.230	0.576	1.440	3.600	9.000
C	0.026	0.064	0.160	0.400	1.00	2.500
D	0.026	0.064	0.160	0.400	1.00	2.500
E	0.128	0.320	0.800	2.000	5.000	12.500
F	0.015	0.038	0.096	0.240	0.600	1.500

- Two QC sets were prepared from the same matrix independently for each QC concentration and analyzed in triplicates.
- The QC samples should cover the anticipated dynamic concentration range, with one QC at the anticipated lower limit of quantification (LLOQ); one within 3 times the LLOQ, one approximately between the high and low QC concentrations and one close to the anticipated upper limit of quantification (ULOQ).

  LLOQ is the lowest concentration of analyte in a sample that can be quantitatively determined with suitable precision and accuracy.

  ULOQ is the highest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy.
- 70% ≤ Accuracy ≤130% based on the mean value at each concentration level
- Precision ≤ 30% at each concentration level
- At least 2/3 of the individual QC concentration values must be within accuracy and precision range
- At least 50% of the values (spots) at each QC concentrations must be within accuracy and precision range.
- Standards and QC prepared from the same stock solution
- Inter-day variability: 3 validation runs performed on different days. A validation run should be rejected only if there is an analytical problem (e.g. an error in reagents preparation, instrument failure, pipetting error or if the data from a run are so erratic that the values could only have resulted from an unexplained analytical errors.)

Stability

Stability is a physico-chemical constancy of an analyte in a given solution or matrix under specific storage conditions for given time intervals.

- Short-term stability of spiked samples. Stability of the stock spiking human serum pool of analyte to be evaluated at RT for at least 6 hours. After completion of the desired storage time, the stability should be tested by measuring stored vs. freshly prepared samples. The stability will be tested on QC3 and QC4 (the most representative concentrations for the standard curve linear range).
- Long-term stability: The QC3 and QC4 prepared for inter-day variability validation will be analysed on first validation run, in 1 week interval and 1 month interval.

5.2.2 Calibrators and QC samples preparation

The same stock solution was used for the preparation of standards and QC samples for each analyte. Standards and QC samples were freshly prepared on each analysis day from the same pool of human serum. The concentrations ranges covered for standards and QC were as indicated in validation protocol.

5.2.2.1 Calibrators preparation

The pre sample solution was prepared in human serum pool using an analyte stock solution of 20 µg/mL. The stock solution was diluted two times (1:10) in human serum pool to the concentration 10 ng/mL. To prepare the target concentration of A, B, C, D and E the 100 ng/mL concentration was used. For F - 10 ng/mL. The cocktail of recombinant proteins in human serum was diluted 1:2 in human serum in serial dilutions to the target concentrations defined in the validation protocol. The standard curve was obtained by plotting the signal versus concentration, using logistic (ELISA) settings in ArrayVision™ for each analyte. The quality of the individual calibration lines was assessed from the accuracies and precision of the back calculated concentrations of the calibration standards. These accuracies were calculated with use of SOFTmax® PRO software.

Calculations	
Accuracy % and Precision %:	
Mean	of at least 50% of all spots (n=6) for each standard concentration
CV%	(SD of at least 50% of all spots for each standard concentration / Mean)*100%
Acc%	(observed concentration / expected concentration)*100% - based on the mean value at each concentration level.

5.2.2.2 QC sample preparation

The pre sample solution was prepared in human serum pool from the stock solution 20 µg/mL for each analyte. The stock solution was diluted two times (1:10) in human serum pool to the concentration 10 ng/mL. To prepare the target concentration of A, B, C, D and E the 100 ng/mL concentration was used. For F - 10 ng/mL. The cocktail of recombinant proteins in human serum was diluted 1:2.5 in serial dilutions to the target concentrations defined in the validation protocol. Calculating the inter-day accuracy and precision and the intra-day accuracy and precision of the QC samples analyzed together with calibration samples on each day (n=3) assessed the accuracy and precision of the method. QC were prepared in triplicates, 2 spots per well (n=12) for two independent QC sets. The accuracy and precision for QC samples were calculated with ArrayVision™ software using following formulas.

Calculations	
Intra-day accuracy % and precision %:	
Mean	of at least 50% of all spots (n=12) for each QC concentration
CV%	(SD of at least 50% of all spots for each QC concentration / Mean)*100%
Acc%	(observed concentration / expected concentration)*100% - based on the mean value at each concentration level.
Inter-day accuracy % and precision %:	
Mean	of at least 50% of all spots (n=36) from 3 validation days for each QC concentration
CV%	(SD of at least 50% of all spots from 3 validation days for each QC concentration / Mean)*100%
Acc%	(observed concentration / expected concentration)*100% - based on the mean value at each concentration level.

5.2.3 Stability sample preparation

Human serum pool was spiked with the recombinant proteins at the concentrations corresponding to QC3 and QC4 for each analyte. The samples were analyzed in triplicates for two sets prepared independently. Stability of spiked human serum was measured after storage for 8 h at RT on each validation day. Fresh samples were prepared every day and stored for 8 h at RT. In case of 1 week and 1 month storage at - 80ºC, samples were prepared on the first validation day. The stability was investigated in single experiments after particular storage time calculating the data of stored samples on the freshly prepared standard curve.

5.2.4 Assay procedure

Experiments on each validation day were performed according to the protocol described in section 4.3.7.

5.3 Results

5.3.1 Calibration curve

A calibration curve was established on each validation day (total of 3 days) using the concentration of calibrants as described in the validation protocol. The typical standard curves obtained are shown in Figure 22. For A, C, D and F precision and accuracy for all calibrants were within the acceptance criteria. For the B and E standards, accuracy and precision fulfilled the acceptance criteria for all samples except for the lowest calibration samples. The B lowest calibration sample, 0.090 ng/mL, gave an precision of 40.2% (average of the precision obtained on each validation day). The accuracy met the acceptance criteria though. The E lowest calibration sample, 0.117 ng/mL, gave an average accuracy and precision from 3 validation days of 198.3% and 38.5% respectively (Table 14 and Table 15).

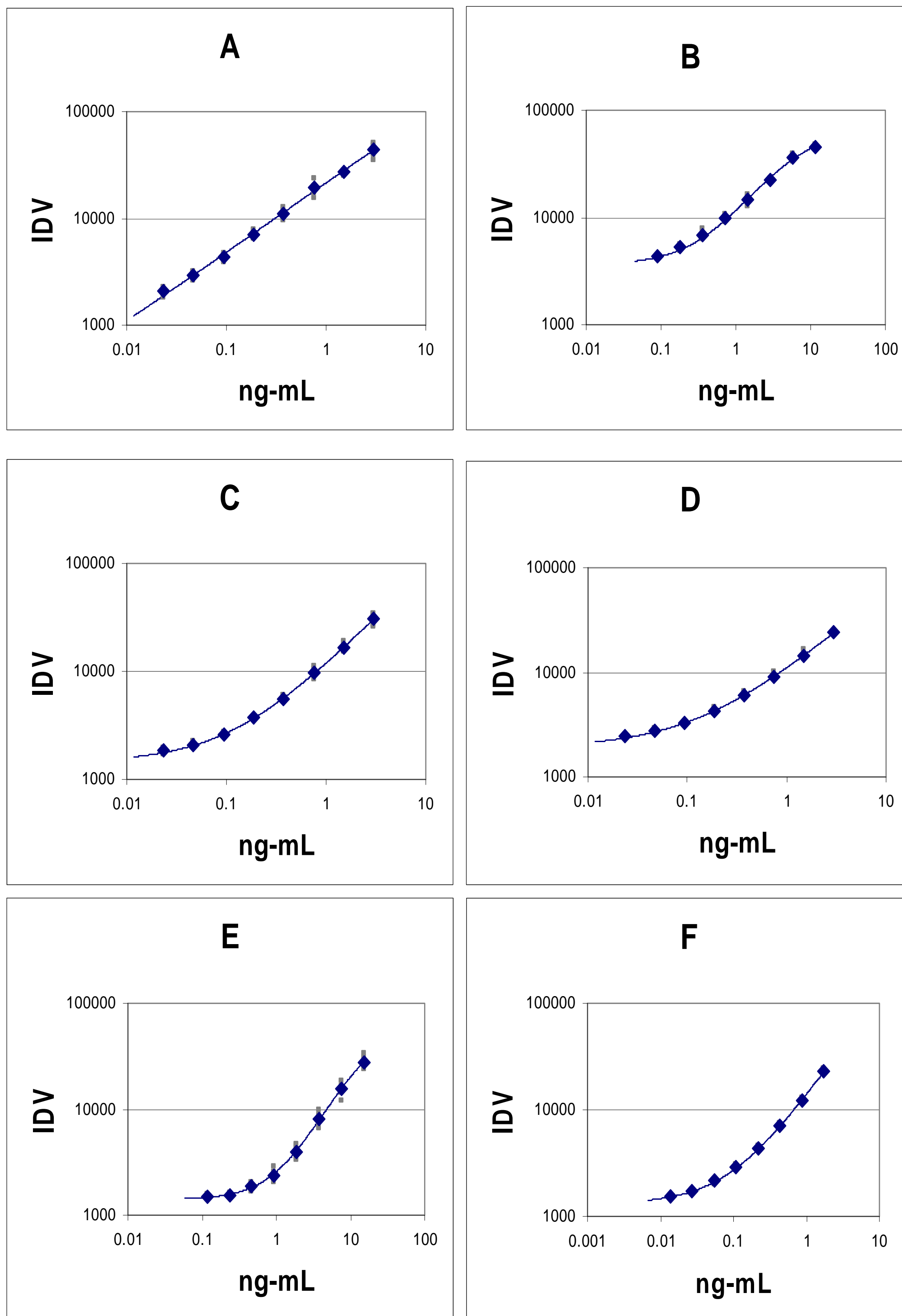


Figure 22: Typical standard curve and calibration results for each analyte. Standard curves were prepared in human serum pool on each validation day using the concentrations indicated in the validation protocol. The signal density is defined as the integrated data value (IDV). The signal was plotted against the analyte concentration using a logistic ELISA fit.

Table 14: Mean accuracy of back-calculated concentrations of calibration samples. The back-calculated values for each standard curve concentration was accessed with SOFTmax® PRO software. Mean accuracy was calculated on at least 50% of all spots (n=6) for each standard concentration.

A									
[ng/mL]		3.000	1.500	0.750	0.375	0.188	0.094	0.047	0.023
Accuracy (%)	Day 1	100.7	103.5	96.7	98.3	105.5	109.2	98.5	94.9
	Day 2	101	99.8	98.4	106.4	104.8	98.8	94.3	80.4
	Day 3	102.3	93.2	110.6	96.4	100.3	91.1	90.8	87.1

B									
[ng/mL]		11.500	5.750	2.875	1.438	0.719	0.359	0.180	0.090
Accuracy (%)	Day 1	96.2	107.7	97.0	99.0	102.6	108.6	121.9	106.9
	Day 2	97.9	105.3	94.3	96.9	114.2	107.7	127.6	103.9
	Day 3	97.8	107.6	92.7	98.9	108.2	113.1	113.1	93.7

C									
[ng/mL]		3.000	1.500	0.750	0.375	0.188	0.094	0.047	0.023
Accuracy (%)	Day 1	100.1	100.5	99.0	101.5	97.8	103.3	107.8	108.3
	Day 2	100.8	100.4	99.7	98.7	104.7	98.7	96.6	114.9
	Day 3	100.4	98.6	103.4	98.3	103.6	96.3	97.8	104.6

D									
[ng/mL]		3.000	1.500	0.750	0.375	0.188	0.094	0.047	0.023
Accuracy (%)	Day 1	100.2	101.7	97.6	97.9	103.9	112.4	109.4	76.3
	Day 2	100.0	100.7	98.6	99.8	103.2	100.4	101.6	100.2
	Day 3	100.0	100.0	100.1	101.2	99.2	96.0	100.9	110.0

E									
[ng/mL]		15.000	7.500	3.750	1.875	0.938	0.469	0.234	0.117
Accuracy (%)	Day 1	102.9	94.4	107.6	91.2	89.1	105.7	128.0	201.0
	Day 2	89.9	105.0	96.6	96.1	114.2	123.1	121.4	247.5
	Day 3	101.7	99.8	101.9	97.3	90.2	110.9	86.4	146.4

F									
[ng/mL]		1.750	0.875	0.438	0.219	0.109	0.055	0.027	0.014
Accuracy (%)	Day 1	100.1	100.3	99.1	99.2	102.4	115.2	106.9	93.1
	Day 2	100.2	100.2	100.8	94.1	110.8	107.1	101.1	126.5
	Day 3	100.3	98.9	101.2	101.3	104.5	103.9	99.9	85.8

Table 15: Mean precision of back-calculated concentrations of calibration samples. The back-calculated values for each standard curve concentration was assessed with SOFTmax® PRO software. Mean precision was calculated on at least 50% of all spots (n=6) for each standard concentration

A									
[ng/mL]		3.000	1.500	0.750	0.375	0.188	0.094	0.047	0.023
Precision (%)	Day 1	18.2	14.1	8.9	12.0	13.8	15.4	11.7	18.3
	Day 2	25.7	21.5	24.9	23.2	14.9	11.3	15.8	18.3
	Day 3	24.3	8.6	21.4	16.9	12.5	12.8	14.5	18.1

B									
[ng/mL]		11.500	5.750	2.875	1.438	0.719	0.359	0.180	0.090
Precision (%)	Day 1	6.5	21.1	19.7	17.3	18.7	17.6	27.6	31.3
	Day 2	9.7	7.3	5.5	11.0	8.5	15.9	22.0	61.5
	Day 3	11.3	13.4	4.6	11.5	9.5	16.3	17.5	27.8

C									
[ng/mL]		3.000	1.500	0.750	0.375	0.188	0.094	0.047	0.023
Precision (%)	Day 1	8.7	11.8	8.5	13.1	5.3	11.2	27.6	24.1
	Day 2	23.8	15.4	12.6	13.9	11.5	10.2	17.5	27.8
	Day 3	15.2	11.9	13.5	10.7	3.4	6.3	21.8	29.2

D									
[ng/mL]		3.000	1.500	0.750	0.375	0.188	0.094	0.047	0.023
Precision (%)	Day 1	15.0	15.0	18.4	17.5	14.2	27.7	20.0	27.1
	Day 2	5.0	9.2	6.9	5.4	6.0	11.8	8.4	28.2
	Day 3	3.4	9.7	9.5	9.1	12.3	9.9	22.9	25.7

E									
[ng/mL]		15.000	7.500	3.750	1.875	0.938	0.469	0.234	0.117
Precision (%)	Day 1	12.6	4.5	26.2	19.5	15.4	4.9	7.8	23.7
	Day 2	22.7	21.7	17.1	14.5	18.8	13.9	10.6	42.7
	Day 3	21.0	14.7	17.1	18.2	23.8	21.4	28.3	49.2

F									
[ng/mL]		1.750	0.875	0.438	0.219	0.109	0.055	0.027	0.014
Precision (%)	Day 1	4.4	6.9	10.7	6.3	5.3	9.2	10.5	28.3
	Day 2	6.6	8.8	6.3	10.7	12.7	11.3	17.7	9.5
	Day 3	4.7	2.9	4.6	7.2	8.0	10.2	11.6	27.7

5.3.2 Quality control samples

The results for intra-day accuracy and precision, and inter-day accuracy and precision are shown in Table 16 and Table 17 and Table 18 respectively. All QC samples met the acceptance criteria for intra and inter-day assay accuracy and precision.

Table 16: Intra-day Accuracy. Individual accuracy of calculated concentrations of each QC concentration for all analytes.

A			
QC level	Accuracy(%)		
[ng/L]	Day 1	Day 2	Day 3
2.500	96.0	81.8	96.6
1.000	97.6	103.4	112.7
0.400	93.4	108.4	101.2
0.160	85.9	94.7	86.7
0.064	93.3	86.7	83.7
0.026	89.4	93.6	103.8

B			
QC level	Accuracy(%)		
[ng/L]	Day 1	Day 2	Day 3
9.000	116.1	122.3	126.1
3.600	113.8	125.3	115.4
1.440	110.2	114.7	112.6
0.576	111.0	100.4	117.0
0.230	111.5	111.7	125.3
0.092	128.1	121.6	112.6

C			
QC level	Accuracy(%)		
[ng/L]	Day 1	Day 2	Day 3
2.500	99.2	94.7	102.0
1.000	100.4	97.8	112.5
0.400	103.7	101.8	113.7
0.160	101.7	97.7	107.9
0.064	111.2	93.5	102.4
0.026	115.9	79.8	112.2

D			
QC level	Accuracy(%)		
[ng/L]	Day 1	Day 2	Day 3
2.500	95.1	98.0	98.6
1.000	106.4	100.4	107.8
0.400	106.2	101.7	114.3
0.160	94.3	95.6	105.9
0.064	94.2	93.9	105.5
0.026	89.1	100.1	98.4

E			
QC level	Accuracy(%)		
[ng/L]	Day 1	Day 2	Day 3
12.500	105.5	98.3	104.9
5.000	89.1	81.5	107.5
2.000	75.5	82.3	92.4
0.800	78.8	85.0	94.8
0.320	85.5	105.7	106.6
0.128	127.4	127.2	112.3

F			
QC level	Accuracy(%)		
[ng/L]	Day 1	Day 2	Day 3
1.500	96.4	94.5	98.1
0.600	97.3	95.9	106.0
0.240	101.0	96.5	105.7
0.096	97.1	91.2	100.6
0.038	95.3	84.5	93.3
0.015	90.4	103.5	78.6

Table 17: Intra-day Precision. Individual precision of calculated concentrations of each QC concentration for all analytes.

A			
QC level	Precision(%)		
[ng/L]	Day 1	Day 2	Day 3
2.500	23.4	20.7	24.5
1.000	15.2	20.4	23.8
0.400	13.7	22.8	23.3
0.160	14.8	23.1	18.7
0.064	25.2	17.0	15.5
0.026	14.2	23.0	9.0

B			
QC level	Precision(%)		
[ng/L]	Day 1	Day 2	Day 3
9.000	23.4	21.2	12.4
3.600	15.2	13.9	13.7
1.440	13.7	17.8	15.7
0.576	14.8	25.5	20.8
0.230	25.2	22.0	28.5
0.092	14.2	23.7	24.8

C			
QC level	Precision(%)		
[ng/L]	Day 1	Day 2	Day 3
2.500	11.6	14.9	19.5
1.000	6.9	15.1	14.5
0.400	8.4	13.1	15.9
0.160	10.9	16.4	14.1
0.064	17.2	15.2	14.2
0.026	25.1	28.7	27.5

D			
QC level	Precision(%)		
[ng/L]	Day 1	Day 2	Day 3
2.500	12.2	10.3	9.5
1.000	12.0	6.8	9.6
0.400	12.5	10.7	14.0
0.160	17.7	14.8	17.5
0.064	23.6	17.8	26.8
0.026	28.1	26.3	21.7

E			
QC level	Precision(%)		
[ng/L]	Day 1	Day 2	Day 3
12.500	24.6	27.2	17.1
5.000	19.2	15.4	22.0
2.000	18.7	18.9	21.7
0.800	17.3	24.1	27.6
0.320	24.8	24.8	28.4
0.128	21.1	3.3	29.8

F			
QC level	Precision(%)		
[ng/L]	Day 1	Day 2	Day 3
1.500	9.5	10.0	8.6
0.600	8.5	8.6	7.6
0.240	13.3	11.2	8.8
0.096	12.8	11.6	17.3
0.038	13.3	17.1	17.4
0.015	24.5	22.8	29.4

Table 18: Inter-day accuracy and precision. The range of accuracy and precision over the all QC sample concentrations.

	Accuracy%	Precision%
A	87.9 – 104.6	17.5 – 24.3
B	109.5 – 121.1	14.7 – 26.3
C	98.6 – 106.8	14.0 – 28.9
D	96.0 – 107.4	10.3 – 28.9
E	83.4 – 122.1	21.8 – 28.3
F	89.2 – 101.1	9.4 – 27.9

5.3.3 Assay working range

The assay working range was determined between the LLOQ and the ULOQ (Table 19).

Table 19: Assay working range determined by between the LLOQ and the ULOQ.

Analyte	Assay working range
pg/mL	
A	26 - 2500
B	230 - 9000
C	26 - 2500
D	26 - 2500
E	320 – 12500
F	15 - 1500

5.3.4 Stability

All the analytes except for E were stable after 8 h at RT and after 1 week at -80°C storage. After 1 month storage at -80°C, the concentration of the spiked samples were three time less when compared to the spiked nominal value. These results are shown in Table 20 for RT storage determination, and in Table 21 for one week and 1 month storage at -80°C.

Table 20: Short-term stability. Accuracy of calculated concentration of each QC samples after 8h of storage at RT. Fresh samples were prepared on each validation day.

Analyte	QC Nominal value [ng/mL]	Day 1	Day 2	Day 3
		Accuracy %		
A	0.4	110.2	88.2	88.0
	0.16	80.2	79.9	71.5
B	1.44	98.7	102.4	87.6
	0.58	111.8	104.6	96.1
C	0.4	93.7	96.4	77.6
	0.16	95.6	97.6	84.9
D	0.4	112.8	111.9	88.6
	0.16	110.2	105.1	89.2
E	2	53.5	70.0	71.0
	0.8	50.9	60.6	70.3
F	0.24	106.3	92.0	80.4
	0.096	94.9	84.0	75.0

Table 21: Long-term stability of spiked samples. Accuracy of calculated concentration of each QC samples after 1 week storage at -80ºC and 1 month of storage at -80ºC.

Analyte	QC Nominal value [ng/mL]	After 1 week at -80ºC	After 1 month at - 80ºC
		Accuracy %	
A	0.4	94.6	35.7
	0.16	74.1	24.7
B	1.44	97.7	43.6
	0.58	94.6	35.0
C	0.4	101.7	57.3
	0.16	96.7	41.4
D	0.4	97.5	25.1
	0.16	87.2	blq
E	2	45.9	27.0
	0.8	23.6	41.4
F	0.24	89.7	53.7
	0.096	80.3	31.9

5.4 Discussion

Immunoassay as a technology used for biomarker quantification needs to be validated in terms of accuracy, precision and reproducibility (Findlay et al. 2000). The antibody microarray in 96-well format was validated according to the protocol created at basis of the Food and Drug Administration guidelines for pharmacokinetic assays (Food and Drug Administration 2001). The following parameters were investigated during the validation process: calibrants accuracy and precision based on back calculated values; intra and inter day accuracy and precision based on quality control samples; stability of antigen spiked human serum samples after 8h storage at RT as well as after 1 week and 1 month storage at -80ºC; assay WR based on LLOQ and ULOQ.

The obtained data showed that the assay working range met the disease concentrations for the measured analytes. Accuracy and precision for all calibration samples for A, C, D, and F analytes fulfilled the acceptance criteria. In case of E accuracy and precision and for B only precision for the lowest concentration of standard curve did not covered the acceptance criteria. That could be caused by recombinant proteins instability or precipitation. Could be that recombinant proteins are degraded by the serum components. Another reason might be the binding of the proteins to the soluble endogenous receptors in serum matrix or naturally occurring heterophilc antibodies (Hennig et al. 2000). However these samples were excluded during the assay working range determination. Overall the validation was successful because the assay working range covered the examined analytes levels in RA serums.

All samples were stable after 8 hours storage at RT determined on 3 validation days and after 1 week storage at -80ºC except for E. None of the samples were stable after 1 month storage at -80ºC. The inaccuracy of stored samples might be caused by antigen precipitation or degradation in the presence of endogenous proteins, as in case of E quality control samples were not stable after 8 h storage at RT as well as 1 week and 1 month storage at -80ºC. The above results raise the question if similar E instability would be found for naturally occurring E in real samples as well? This aspect could be investigated by measuring the E levels in stored real samples in different time intervals. Due to the lack of resources and time this experiments were

not covered by this thesis. The above findings indicate that quality control samples should be freshly prepared on each measurement day.

Validation results demonstrate that antibody microarray in 96-well plate is accurate, precise and reproducible within the determined assay working range. However the platform needs to be validated with the real samples.

6 Comparison of antibody microarray and ELISA technology

6.1 Introduction

ELISA is a standard method for analyzing protein levels in serum samples and has been broadly used for the detection of serum cytokines in clinical and microbiological research over the last decade (Klimiuk et al. 2002; Lloyd et al. 1991; Mangge et al. 1995). In this section the performance of protein microarray validated in Chapter 5 is compared with the ELISA technology. A, B, C, D, E and F levels in 78 distinct sera from rheumatic patients and healthy individuals were measured twice, once by the developed protein microarray and once by ELISA. Subsequently the results obtained with both methods were compared using linear regression analysis.

6.2 Materials and methods

6.2.1 Multiplex assay

The serum concentrations of A, B, C, D, E and F were measured with protein microarray validated in chapter 5.

6.2.2 ELISA assay

Sandwich ELISA was prepared with the same reagents that were used for microarray fabrication for antibodies matched pairs and recombinant proteins respectively. Also the same serum pool was used to prepare standard curves and QC samples with the concentration points identical as used to produce antibody microarray (Table 12 and Table 13 page 62 and 63). Briefly, the ELISA protocols for each analyte are summarized below.

A

1. Coating concentration 3µg/mL in 0.03M Sodium Carbonate/0.068 Sodium Bicarbonate, 100µL/well
2. Incubation at 25ºC overnight
3. Emptying the plate
4. Blocking 200 µL/well of PBS with 4%BSA,

5. Incubation (1h) at 25ºC
6. Wash, 300 µL/well of 50 mM Tris, 0.2% Tween-20, 3 times
7. 50 µL/well of standards and samples
8. Incubation (1h) at 25ºC
9. 50 µL/well of biotinylated Ab at 500ng/mL
10. Incubation (1h) at 25ºC
11. Wash like in 6.
12. Streptavidin-HRP (N100, Pierce, stock diluted 1:15000, 100 µL/well
13. Incubation (30 min) at 25ºC
14. Wash like in 6
15. TMB substrate solution (N301 Pierce), 100 µL/well
16. Incubation 30 minutes at 25ºC
17. Stop solution (0.18 M H_2SO_4), 100 µL/well
18. Absorbance measured at 450 nm minus 550 nm

B

1. Coating concentration 5µg/mL in PBS, 100µL/well
2. Incubation at 25ºC overnight
3. Emptying the plate
4. Blocking 200 µL/well of Super block, 3 times
5. Incubation (1h) at 25ºC
6. Wash, 300 µL/well of 50 mM Tris, 0.2% Tween-20, pH 8, 3 times
7. 50 µL/well of standards and samples
8. 50 µL/well of biotinylated Ab at 500ng/mL
9. Incubation (4h) at 25ºC
10. Wash like in 6.
11. Streptavidin-HRP (N100, Pierce, stock diluted 1:15000, 100 µL/well
12. Incubation (30 min) at 25ºC
13. Wash like in 6
14. TMB substrate solution (50-76-00, Kirkegaard & Perry), 100 µL/well
15. Incubation 8 minutes at 25ºC
16. Stop solution (1 M H_2SO_4), 100 µL/well
17. Absorbance measured at 450 nm minus 550 nm

C

1. Coating concentration 3µg/mL in PBS, 100µL/well
2. Incubation at 25ºC overnight
3. Emptying the plate
4. Blocking 200 µL/well of PBS with 4%BSA,
5. Incubation (1h) at 25ºC
6. Wash, 300 µL/well of 50 mM Tris, 0.2% Tween-20, 3 times
7. 50 µL/well of biotinylated Ab at 500ng/mL
8. 50 µL/well of standards and samples
9. Incubation (2h) at 25ºC
10. Wash like in 6.
11. Streptavidin-HRP (N100, Pierce, stock diluted 1:15000, 100 µL/well
12. Incubation (30 min) at 25ºC
13. Wash like in 6
14. TMB substrate solution (N301 Pierce), 100 µL/well
15. Incubation 10 minutes at 25ºC
16. Stop solution (0.18 M H_2SO_4), 100 µL/well
17. Absorbance measured at 450 nm minus 550 nm

D

1. Coating concentration 6µg/mL in PBS, 100µL/well
2. Incubation at 25ºC overnight
3. Emptying the plate
4. Blocking 200 µL/well of PBS with 4%BSA,
5. Incubation (1h) at 25ºC
6. Wash, 300 µL/well of 50 mM Tris, 0.2% Tween-20, 3 times
7. 50 µL/well of PBS with 4%BSA
8. 50 µL/well of standards and samples
9. Incubation (1h) at 25ºC
10. Wash like in 6
11. 100 µL/well of biotinylated Ab at 500ng/mL
12. Incubation (1h) at 25ºC
13. Wash like in 6.
14. Streptavidin-HRP (N100, Pierce, stock diluted 1:15000, 100 µL/well

15. Incubation (30 min) at 25ºC
16. Wash like in 6
17. TMB substrate solution (N301 Pierce), 100 µL/well
18. Incubation 30 minutes at 25ºC
19. Stop solution (0.18 M H_2SO_4), 100 µL/well
20. Absorbance measured at 450 nm minus 550 nm

E

1. Coating concentration 2µg/mL in PBS, 100µL/well
2. Incubation at 25ºC overnight
3. Wash, 300 µL/well of 50 mM Tris, 0.2% Tween-20, 3 times
4. Blocking 200 µL/well of Super block
5. Wash like in 3.
6. 50 µL/well of standards and samples
7. 50 µL/well of biotinylated Ab at 500ng/mL
8. Incubation (4h) at 25ºC
9. Wash like in 3.
10. Streptavidin-HRP (N100, Pierce, stock diluted 1:15000, 100 µL/well
11. Incubation (30 min) at 25ºC
12. Wash like in 3.
13. TMB substrate solution (50-76-00, Kirkegaard & Perry), 100 µL/well
14. Incubation 15 minutes at 25ºC
15. Stop solution (1 M H_2SO_4), 100 µL/well
16. Absorbance measured at 450 nm minus 550 nm

F

1. Coating concentration 6µg/mL in PBS, 100µL/well
2. Incubation at 25ºC overnight
3. Emptying the plate
4. Blocking 200 µL/well of PBS with 4%BSA
5. Incubation (1h) at 25ºC
6. Wash, 300 µL/well of 50 mM Tris, 0.2% Tween-20, 3 times
7. 50 µL/well of standards and samples
8. Incubation (1h) at 25ºC

9. 50 µL/well of biotinylated Ab at 500ng/mL
10. Incubation (1h) at 25ºC
11. Wash like in 6.
12. Streptavidin-HRP (N100, Pierce, stock diluted 1:15000, 100 µL/well
13. Incubation (30 min) at 25ºC
14. Wash like in 6.
15. TMB substrate solution (N301 Pierce), 100 µL/well
16. Incubation 30 minutes at 25ºC
17. Stop solution (0.18 M H_2SO_4), 100 µL/well
18. Absorbance measured at 450 nm minus 550 nm

6.2.3 Samples

Samples comprised of 52 sera of RA and other arthritic patients as well as 26 control human sera. In addition human serum pool was spiked with recombinant A, C, D and F and the concentration of 3, 1.500, 0.750, 0.375, 0.188, 0.094, 0.047, 0.023, 0.012 and 0.006 ng/mL, B (0.023, 0.047, 0.094, 0.188, 0.375, 0.750, 1.5, 3.0, 6.0, 12 ng/mL) and E and the concentration of 0.020, 0.039, 0.078, 0.156, 0.313, 0.625, 1.250, 2.5, 5.0, 10 ng/mL. The concentration were chosen in the manner to cover the standard curve range of microarray and ELISA used for cytokine level determination. Prior to analysis the spiked samples were frozen at - 80ºC.

6.3 Results

Multiplex assay performance was compared with sandwich ELISA assay. Overall 78 sera were measured using both technologies. In case of A there was only one value detectable by both technologies. Thus, in order to perform correlation analysis 10 human serum spiked samples was prepared. Subsequently levels of A, C, D and F were measured in the spiked samples. The results from spiked samples measurements were pooled with the results from the 78 sera analyzed previously and subjected to linear regression analysis. The results are shown in Figure 23. The correlation coefficients (R^2) for A, B, C, D, E, and F were 0.90, 0.60, 0.93, 0.96, 0.94 and 0.95 respectively.

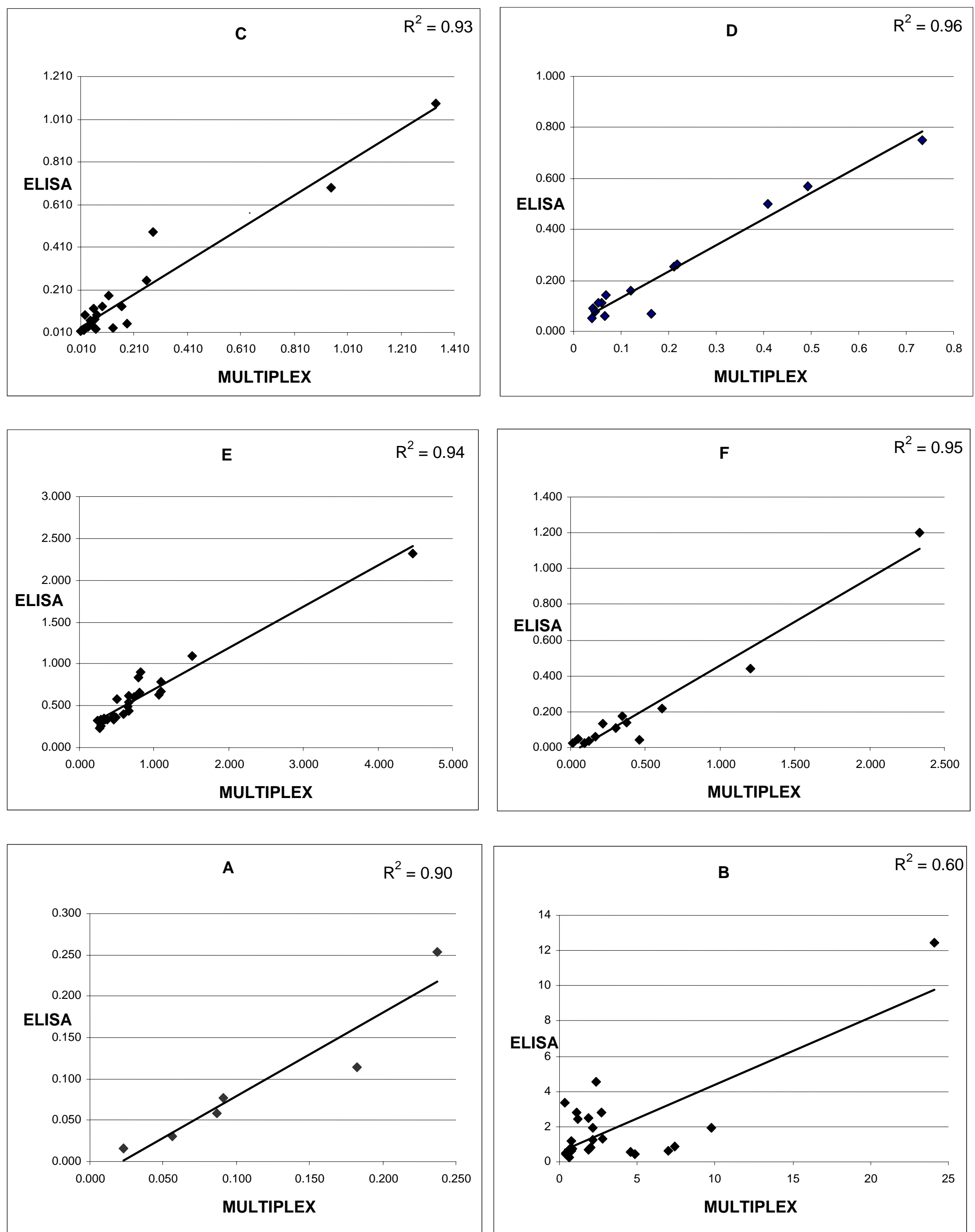


Figure 23: Correlation of antibody array and ELISA procedures. Cytokines in 78 sera and 10 spiked samples were quantified in parallel using either antibody microarray or ELISA. Data from these two analyses were plotted against each other and the correlation coefficients determined by linear regression analysis.

The correlation analysis was performed only when the values were detectable by both technologies. When the values were detectable by one technique only, they were omitted in the correlation analysis. Consequently the correlation was performed for 6, 26, 20, 14, 26 and 14 samples for A, B, C, D, E and F respectively (Figure 23).

6.4 Discussion

The multiplex assay for A, C, D, E and F showed very good correlation with individual ELISAs for the same analytes. The correlation coefficients were 0.90, 0.93, 0.96, 0.94 and 0.95 respectively. For B assay, despite the fact that Abs used in both cases were the same, the correlation with ELISA was lower (0.60). This could be explained by the problems often met with sandwich immunoassay and possibly magnified by assay multiplexing. For instance the interference of rheumatoid factors or naturally occurring human heterophilic antibodies often associated with autoimmune or inflammatory diseases, which can recognize animal and human immunoglobulin. Consequently false-positive or –negative results are generated (Hennig et al. 2000). Another reason could be the presence of soluble receptor. Maybe the binding affinity of B to this soluble receptor is biased by multiplex format. That can also cause false high or low cytokine level different for multiplex and single ELISA.

The results described demonstrate the applicability of protein microarray to monitor simultaneously several analytes per sample. Good correlation between protein microarray and ELISA shows its potential to replace ELISA as a cost-effective and high throughput screening tool. ELISA allows for the measurement of one analyte per sample only, while using the multiplex approach from the same amount of sample the information of many analytes can be obtained. However B assay indicates that the measurements performed with different technologies not always give the same results. Additional analysis is required to explain these findings for instance, spike-recovery investigations to access the influence of matrix on the measurements performed with both techniques.

7 Application of antibody microarray to evaluate analytes in rheumatic samples.

7.1 Introduction

The multiplex assay validated in chapter 5 was used to measure A, B, C, D, E and F serum concentrations in samples from the patients diagnosed with RA, other rheumatic diseases and control human sera. The goals were to investigate whether multiplexed microarray could be applied to real samples measurement and to conduct an exploratory study to assess whether those biomarkers could be relevant in diagnosis and monitoring of RA.

7.2 Materials and methods

7.2.1 Subjects and samples

The serum samples were obtained from Felix Platter-Spital and Kantonsspital Aarau. This study was approved by ethic Committee (Study protocol EKBB 212/01).
Studied samples consisted of 35 samples from patients diagnosed with RA and 13 samples from patients diagnosed with other rheumatic diseases such as polymylagia rheumatica (PMR) (n=3), juvenile rheumatoid arthritis (JRA) (n=1), connective tissue disease (CTD) (n=3), giant cell arteritis (n=1), vasculitis (n=2), undefined seronegative polyarthitis (n=1), low back pain (n=1), Seronegative Spondylarthropathie (n=1). Characteristics of the patients populations are shown in Table 22.

Table 22: Patients characteristics. Data presented as mean ± SD.

	RA (n=35)	Non RA (n=13)
Women/men	29/6	8/5
Age (years)	61.8 ± 15.1	58.6 ± 14.5
Disease duration (years)	9.7 ± 9.2	NA
RF positive patients	22/35	2/13
DMARDS treated patients	34/35	7/13

SD-standard deviation
RF-rheumatoid factor
DMARDS-disease modifying anti-rheumatic drugs
NA- not assessed

Information about patient's extraarticular manifestations (bone erosions, skin changes, pleuropulmonary manifestations, heart disease, Raynaud's phenomenon, Sjörgen's syndrome, rheumatoid vasculitis, osteoporosis and major joint replacement) were also collected (data not shown).

7.2.2 Multiplex assay

The serum concentrations of A, B, C, D, E and F were measured with the protein microarray validated in chapter 5. In case of A, C, F, for the values that were below limit of quantification (blq), the high sensitivity quantikine ELISA kit was used for samples measurements. The quantikine sensitivity was 0.125, 0.156 and 0.5 pg/mL for of A, C and F respectively.

7.2.3 Data analysis

Analysis of the data was performed using one way Anova analysis (Kempthorne O 1983). The results were considered significant when the p values was less then 5 % ($p<0.05$).

7.3 Results

Mean values for each of the six analytes were calculated for the RA and non RA patients. These data showed the difference for 5 analytes levels (B, C, D, E and F) between the two groups (Table 23).

Table 23: Serum concentrations of A, B, C, D, E and F for RA versus non-RA patients. Data is presented as mean and log (mean) and their SD respectively.

Analyte	RA				Non RA			
	MEAN [ng/ml]	SD [ng/ml]	LOG (MEAN)	LOG (SD)	MEAN [ng/ml]	SD [ng/ml]	LOG (MEAN)	LOG (SD)
A	0.0002	0.0005	-3.634	-3.585	0.0004	0.001	-3.394	-3.101
B	1.074	2.160	0.031	0.334	0.140	0.112	-0.853	-0.950
C	0.090	0.160	-1.045	-0.796	0.004	0.007	-2.447	-2.162
D	0.070	0.162	-1.157	-0.791	0.029	0.057	-1.539	-1.241
E	0.551	0.430	-0.259	-0.366	0.243	0.240	-0.615	-0.620
F	0.140	0.312	-0.853	-0.505	0.002	0.002	-2.621	-2.653

SD-standard deviation

Subsequently, the statistical Anova analysis included the comparison of three patient's groups: RA versus non RA, Rheumatoid factor (RF) positive versus RF negative, bone erosion positive versus bone erosion negative. The analysis was performed in regard to serum levels of six analytes of interest. The results of the comparison of RA and non RA rheumatic patients are shown in Table 24. The concentrations of B, C, E and F in serum were significantly higher in RA patients ($p<0.0022$, $p<0.0107$, $p<0.0024$ and $p<0.0057$ respectively).

Table 24: One way Anova analysis for RA versus non RA patients.

Column ID	Mean (non RA)	Std (non RA)	Mean (RA)	Std (RA)	p-value(RA)
log(A)	-3.7043	0.4199	-3.7741	0.2949	0.5207
log(B)	-0.9733	0.3361	-0.3847	0.6171	0.0022
log(C)	-2.8904	0.6490	-1.9610	1.1897	0.0107
log(D)	-1.7916	0.3407	-1.5526	0.4979	0.1182
log(E)	-0.8737	0.5469	-0.3988	0.4164	0.0024
log(F)	-2.7765	0.3848	-1.8638	1.1022	0.0057

The results from the comparison of RF positive versus RF negative patients are shown in Table 25. There was a significant difference between this two groups of patients for B, C and F ($p<0.0210$, $p<0.0325$, $p<0.0109$) respectively.

Table 25: One way Anova analysis for RF positive versus RF negative patients.

Column ID	Mean (non RF)	Std (non RF)	Mean (RF)	Std (RF)	p-value(RF)
log(A)	-3.7583	0.3472	-3.7401	0.3323	0.8038
log(B)	-0.8047	0.4094	-0.3316	0.6992	0.0210
log(C)	-2.6150	0.9245	-1.9542	1.2258	0.0325
log(D)	-1.7425	0.3755	-1.4801	0.5300	0.1174
log(E)	-0.6588	0.5103	-0.4005	0.4834	0.2099
log(F)	-2.5858	0.7778	-1.7294	1.1213	0.0109

Table 26 shows the results of the comparison between patients with erosions and patients without erosion. There was a significant difference for B and C between this two groups ($p<0.0105$ and $p<0.0220$).

Table 26: One way Anova analysis for patients diagnosed with erosion (E) versus non erosions patients (non E).

Column ID	Mean (non E)	Std (non E)	Mean (E)	Std (E)	p-value (Erosions)
log(A)	-3.7737	0.3331	-3.7351	0.3329	0.6906
log(B)	-0.7566	0.4674	-0.3132	0.6738	0.0105
log(C)	-2.5707	0.9646	-1.8236	1.2127	0.0220
log(D)	-1.6861	0.3879	-1.5425	0.5438	0.2946
log(E)	-0.6119	0.5004	-0.4356	0.4880	0.2235
log(F)	-2.3499	0.8622	-1.8513	1.1709	0.0979

7.4 Discussion

Statistical analysis performed for each individual analyte showed a significant difference in the serum concentration of B ($p<0.0022$), C ($p<0.0107$), E ($p<0.0024$) and F ($p<0.0057$) between RA and other arthritic patients. Additionally, when comparing RF positive with RF negative patient populations, a statistical difference was observed in the levels of B ($p<0.0210$), C ($p<0.0325$) and F ($p<0.0109$). Finally, a difference was also observed in the concentration levels of B ($p<0.0105$) and C ($p<0.0220$) in patients with and without bone erosions.

The obtained results demonstrated the applicability of the protein microarray to measure and compare the concentration levels of selected analytes within samples obtained from patient populations. Additionally, the differences observed in the serum levels of B, C, E and F demonstrates the feasibility of the protein microarray format as a tool for identifying, confirming or validating biomarkers in clinical samples. Although promising, these findings are, however, just exploratory. Additional experiments are required to further validate the discriminating power of selected panel of biomarkers. Additional analysis on a larger sample size are required to confirm whether B, C, E and F are valid biomarkers for the diagnosis or the monitoring of RA progression and treatment. A patent filing is ongoing. Thus, no additional results or analysis will be described in this thesis.

Additional analysis using learning algorithms on training sample sets to create models based on the expression profiles of the six biomarkers, followed by testing these models on blind samples would demonstrate the discriminating power of the combined biomarker approach for separating the patient groups (White, Chan, & Zhang 2004). Further blind testing of the model could then be performed on a larger sample size, and valuable information on the sensitivity and specificity of the combined biomarkers for identifying RA patients would be obtained. It could be that the best sensitivity and specificity is obtained with a model combining for instance five biomarkers rather than the full set of six. Alternatively, sensitivity and specificity could be further improved with the addition of other analytes to the current set of biomarkers on the microarray.

8 General discussion

The aim of this thesis was to examine whether protein microarrays can be applied in the drug development process to monitor biomarkers in preclinical and clinical studies. In order to demonstrate the feasibility of this approach, RA was chosen as a model for proof of concept. Several proteins (A, B, C, D, E, F) which were described in the literature to be associated with RA were selected. A protein microarray for the simultaneous measurement of the disease concentrations of those proteins was developed. The robustness of this protein microarray was then assessed and finally validated with real sample measurements.

The concept of protein microarrays was inspired by DNA microarrays, which enable the measurement of mRNA expression level of thousands of genes in a single experiment (Schena et al. 1995). There are, however, several reasons why DNA microarray approach cannot be similarly adapted for the development of protein microarrays. Firstly, DNA molecules are much more robust and easy to handle than proteins. Secondly, DNA molecules are physically and chemically similar, allowing unification of manufacturing and assay procedures, and finally DNA reactivity (hybridization) exploited in DNA microarrays is simple and depends only on nucleotide sequence. In contrast to DNA, protein binding depends not only on primary sequence, but also on tertiary structure, which is far more vulnerable to degradation than DNA (Wilson & Nock 2002). During the dispensing and immobilization process, the proteins must maintain the integrity of their three-dimensional structures. Despite these difficulties many research groups have worked on the development of various protein microarray platforms for a range of applications. This thesis describes the process of protein microarray development and validation for the monitoring of biomarkers in serum samples.

In chapter 3, the development of the protein microarray on a glass slide treated with ODP was described. This platform utilized a glass slide with an evanescent wave guide property, that for DNA arrays are known to provide 100 times signal amplification (Neuschafer et al. 2003). This feature presented the possibility to produce a sensitive tool for parallel protein measurements. The main drawback of using this approach was that surface treatment and all assay processing were

performed manually. This probably had a major contribution to the large variability observed between chips, and consequently the sensitivity level was inadequate to measure analytes of interest. The technique needed to be automated in terms of surface treatment and assay processing, however, at that time there was no robotic liquid handling systems for processing protein microarrays in the market. The existing systems for DNA microarrays fabrication utilized the plastic tubing equipment which could not be directly applied to proteins. The main reason for that was the 'sticky' nature of proteins (e.g. proteins clog the robots fluidic systems by sticking to the inside of the tubing) (Mitchell 2002). The proteins simply stayed attached to the inside tubing. Consequently, the lack of appropriate sensitivity, together with the time consuming process of chemically treating the surface, the complicated ODP synthesis procedure, and the lack of automation did not provide the required flexibility, reproducibility and robustness suitable for quantitative measurements. For all these reasons efforts towards the development of a protein microarray on a glass slide format were discontinued.

The appearance on the market of long neck tips for antibody printing devices, provided the option of using a 96-well plate format for developing a protein microarray. Polystyrene 96-well plates are strongly established solid supports used for immunoassays (e.g., ELISA technique). The surface chemistry is standardized and optimized for antibody binding, and the assay processing can be fully automated. Therefore, the decision to adapt a RA protein microarray to this well characterized format was taken. The main advantage of this approach is the utilisation of commercially available 96-well plate and standard laboratory equipment together with standard plate washers. This aspect is of particular importance in cases when the samples from drug trials need to be analyzed in different laboratories. On the other hand the 96-well plate format limits the number of analytes multiplexed per well. This fact predisposed the 96-well platform as a monitoring rather then discovery tool for a defined number of biomarkers to be measured in a large number of samples.

During the development of the microarray in a 96-well plate, issues commonly faced with the development of any immunoassay (such as antibody match-pair, matrix and assay format selection, cross reactivity, printing protocols and choice of signal detection system) were investigated. For the selection of matrix for calibrators preparation, FDA industry guidelines for pharmacokinetic immunoassays recommend immunoassay development in the same matrix as the analyzed samples (Findlay et al.

2000; Food and Drug Administration 2001). This approach is quite often problematic during immunoassay development because the presence of endogenous molecules of interest, which may interfere with measurements. In addition, other endogenous molecules such as soluble receptors and heterophilic antibodies bind to the assay reagents leading to false positive or false negative results (Hennig et al. 2000). To avoid endogenous molecule interference, commercially available ELISA kits use analyte of interest free buffers for standard curve preparation. The 96-well microarray in this thesis was developed to measure human serum samples. Consequently human serum was the first choice for a matrix to prepare the standard curve. However, because the linearity of standard curve for some analytes could not be obtained using human serum as matrix, in parallel standard curve was prepared in analyte free buffer. Though, when the buffer was used for standard curve preparation the recovery of spiked samples failed to fulfil the assay acceptance criteria. For instance, IL-1β assay accuracy of human serum spiked samples was ~50%. In general this approach, creates an artificial assay environment which does not contain the same molecules as when using authentic sample matrix. This could introduce measurement mistakes as the protein functionality often depends on partnership with other proteins (Colburn 2003; Zhu & Snyder 2003). Based on these results the assay was developed in the pool of human sera that contained low levels of the measured analytes. The question is what if such a pool can not be selected for all the analytes of interest? One alternative could be to select pools of sera from other species in which cross reactivity with the assay reagents is negligible.

Talking about the choice of the detection system, the reason for which chemiluminescent was selected, was dictated by the 96-well plate format. The scanner that would allow to image the fluorescent signal in 96-well plate at that time was not available on the market. Instead the CCD camera commonly used for many laboratory applications could be utilized for chemiluminescent detection in 96-well plate. However chemiluminescent detection has its drawback. Firstly the readout has to be done immediately after the addition of the chemiluminescent substrate, as the enzymatic reaction progresses with time. Otherwise, the signal generated from each spot could contaminate signals generated from neighbouring spots. In the future fluorescent detection mainly because it is easy to perform, stable, gives the

opportunity for multiple measurement over the time and is compatible with the signal amplification methods, could be adapted to the microarray in 96-well plate.

Currently, there are no existing guidelines for the validation of biomarker and antibody microarrays. Standardised guidelines would be crucial for reliable multiplex measurements, especially when samples are analysed in different study centres (Kumble 2003). Thus, in order to validate the robustness and reproducibility of the developed protein microarray, a protocol based on the existing FDA regulatory guidelines for pharmacokinetic immunoassays (Findlay et al. 2000) was created (Food and Drug Administration 2001). A spike-recovery validation test was elaborated and run over 3 days. This validation study demonstrated the microarray to be both quantitative and reproducible at assay working ranges covering the relevant physiological concentrations of the analytes, with an accuracy between 70-130%, and assay precision less than 30%. However, problems could arise if in future the FDA releases a guideline which does not match with the above set criteria. Consequently, all steps in microarray development should be optimized in order to easily meet any future FDA guidelines.

ELISA technique has been widely used for protein quantification since its introduction by Engvall and Perlaman in 1971 (Engvall & Perlman 1971). Subsequently, ELISA if often treated as a reference standard when investigating the performance of protein microarrays (Kellar et al. 2001; Mezzasoma et al. 2002; Prabhakar, Eirikis, & Davis 2002; Woodbury, Varnum, & Zangar 2002). The microarray developed and validated in this thesis was also compared with the single ELISA. Correlation coefficients between the two technologies was calculated for each analyte in sera collected from a total of 78 individuals representing either rheumatic or healthy patients. High correlation coefficients were obtained for five out of six analytes measured (0.90, 0.93, 0.96, 0.94 and 0.95 for A, C, D, E, and F respectively). Generally, with assays based on immunochemical methods, it is common to see variations in absolute quantification between methods, and improving their comparability is a recognizable challenge (Bidart et al. 1999; Woodbury, Varnum, & Zangar 2002). That is why results obtained with single ELISA and multiplex assays do not always agree. Despite using the same antibodies to prepare both microarray and ELISA methods, for B the correlation coefficient was only 0.60. B could bind to

the soluble receptor or its measurement could be biased by the heterophilic antibodies present in serum (Hennig et al. 2000). Perhaps, this events could occur differently in single compared to multiplexed set ups. In order to explore this matrix effect for both techniques, additional analysis of spike-recovery of samples is required.

When choosing between techniques used for analysing samples from clinical studies, it is important to consider sample amounts available and the quantity of information which can be generated from the same amount of sample. Single ELISA allows for the analysis of one analyte per assay, thus many assays are required for many analytes. This dramatically increases the cost and sample volume requirements for generating a single data point when compared to a multiplex ELISA approach. On the other hand there is a risk of cross reactivity between the antibody pairs and proteins other than the target proteins when using the multiplex approach. This could represent a potential limiting factor for multiplex assay application. Sometimes, however, depending on the application, it will not be necessary to develop and utilise a protein microarray approach. There could be cases where the application of a single ELISA assays to measure a certain number of samples will be more reasonable from an economical point of view. In other cases, if commercial microarrays to measure certain analytes are available, the microarray development process would not be worth the investment in time and cost.

The applicability of the developed protein microarray to analyze samples from a cohort of patients diagnosed with either RA or other rheumatic diseases was investigated in chapter 7. The results showed a significant differences in the serum concentration levels of B ($p<0.0022$), C ($p<0.0107$), E ($p<0.0024$) and F ($p<0.0057$) between RA and other arthritic patients. These findings, however, should be considered as exploratory only, due to the small sample population size. Differences in the measured levels of B, C, E and F between respective groups should be validated on a larger patient population. Nevertheless, the potential for the identification of a panel of biomarkers that could differentiate RA from other rheumatic diseases would revolutionize the RA diagnosis.

9 Conclusion and future perspective

In this thesis, a multiplexed assay for quantitation of A, B, C, D, E and F in human sera has been described and validated. It is proposed that miniaturized and multiplexed immunoassays will offer a cost effective and efficient manner for validating and monitoring biomarkers during the drug development process. In preclinical studies it could find its utility in the validation of biomarkers identified by genomics and proteomics. Microarrays consisting of validated panels of biomarkers can then be used in clinic for the diagnosis, prognosis and monitoring of disease progression, in the monitoring of clinical responses to therapeutic intervention such as in efficacy determination and safety monitoring, as well as in the stratification of patient populations. Another direction in which protein microarrays will continue to be developed is in the investigation of protein function. Protein function arrays will be extremely useful for investigating the activities and binding properties of native proteins, especially in the screening of protein-binding small molecules including potential drug candidates.

LIST OF ABBREVIATIONS

Ab	antibody
BCA	biochip arrayer
CCD	charge coupled device
CSF	cerebrospinal fluid
FDA	food and drug administration
HRP	horse radish peroxidise
HSPs	heat shock proteins
mAb	monoclonal antibody
MHC	major histocompatibility complex
ODP	octadecyl phosphoric acid ester
RA	Rheumatoid arthritis
RF	rheumatoid factor
SAM	self assembled monolayer

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