HybriChip: an antigen microarray based screening tool, designed for a high-throughput production platform of mouse derived monoclonal antibodies.

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Abstract

The incapacity to generate a sufficient number of mAbs to conduct large-scale analyses remains one of the most serious limitations in the field of functional genomics. Current methods of mAb production are very labour intensive and disappointingly low throughput, with a per capita production level of about 20 antigens per year. Here we present a modified hybridoma production method incorporating a novel screening assay which can be scaled up to generate antibodies in sufficient quantities for proteomics-scale analysis. Our method circumvents previous obstacles to increasing the throughput level of mAb production, in two ways. First, by immunizing a single mouse with multiple target antigens, we dramatically reduce the number of tissue culture operations normally necessary for performing multiple fusions simultaneously using only one antigen per animal. This minimises tissue culture operations and results in hybridomas that secrete antibodies specifically recognizing each of the target antigens. Second, we developed a novel antigen microarray assay (HybriChip) to screen supernatants generated by large-scale production. In this assay, an antigen chip is generated by coating an aminosilane treated slide with a single target antigen. Hybridoma culture supernatants from a fusion are consolidated and spotted as a microarray onto the antigen chip. After probing with a suitable fluorescently labelled secondary antibody, positive hybridomas are identified in a microarray scanner. The isotype of the bound antibody can be concomitantly determined by probing the antigen chip with mixtures of isotype-specific secondary antibodies, such as Cy5 -conjugated anti-mouse IgM and Cy3-conjugated antimouse pan-IgG (recognizing all mouse IgG isotypes). Different antigen chips can simultaneously be spotted in parallel with the same hybridoma culture supernatants, allowing rapid automated assay of multiple antibodies against many target antigens.

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Scientific

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This thesis is my own work, except where stated above and in the main body of the text.

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1 Introduction

1.1 Proteomics

1.1.1 Conventional proteomic analysis tools

When the journal Science published, in 1995, the first paper describing the use of chemically modified glass slides to immobilse several different species of cDNAs (Schena et al., 1995), a new era began for biological research. While DNA microarrays were becoming common tools in genomic research, for example aimed at the study of differential gene expression, the field of proteomics had no such high-throughput analysis tools for the study of proteins at a global level. However, the combination of two dimensional gel electrophoresis (2D PAGE), mass spectrometry (MS) and yeast-two-hybrid (Y2H) approaches for the study of protein-protein interactions and protein profiling, together with other novel methods for the isolation and analysis of native protein complexes, such as the Tandem Affinity Purification (TAP) (Rigaut et al., 1999), had demonstrated the impact of proteoomic analyses in the post-genomic era (Naaby-Hansen et al., 2001).

2D PAGE is being extensively used as a primary tool for the separation and visualization of cellular protein pools. Carrying out a double separation of a pool of proteins by first a separation (in the horizontal dimension) based on isoelectric focussing, followed by a standard SDS-PAGE (O'Farrell et al., 1977), 2D PAGE allows for the separation of thousands of proteins. This technique allows for comparative proteomic experiments such as studying variations of protein levels in "healthy" and "diseased" tissues. This can be done using double-labelling systems, such as for isotope-coded affinity tags (I-CAT), or a Cy3/Cy5 combination of fluorophores for the two samples to be analysed (Gygi et al., 1999, 2000; Naaby-Hansen et al., 2001), or by direct superimposition of the 2D PAGE profiles. However, 2D PAGE can not be defined as a "user friendly" technique since it is time consuming, non-trivial to carry out, not 100% reproducible, and is not truly compatible for insoluble or low-abundant proteins.

Mass spectrometry has been widely used for the identification of proteins and for the study of protein-protein interactions. Starting from the 1980s and, more significantly during the 1990s, MS has become a crucial tool in biological research and is used in a wide range of applications, from protein/peptide sequencing (MS/MS) to the identification of single protein species in heterogeneous samples . Prior to the MS analysis, the proteins are usually cleaved using endoproteases, such as trypsin, to obtain peptides. These resulting fragments are electrically charged and brought to a gaseous phase by the mass spectrometer. Charged peptides are directed to a detector via an electric field and their time of flight (TOF) is this measured. Peptides with a lower mass-over-charge ratio (m/z) will travel faster than peptides with higher m/z, thus allowing for an identification of the mass of each single peptide when correlated to standard calibration peptides. Other detection methods that use the charge, or other physical properties of the peptides to determine the m/z value of each protein fragment are available and the combination of different methods in single MS machines allow for the design and execution of a great variety of experiments. The identification of proteins is done by analysing either sets of detected peptide masses (peptide fingerprint) and/or from partial sequence information, using bioinformatics tools and databases, such as MASCOT/MOWSE (`http://www.matrixscience.com/`)(Yates, 1998; Godovac-Zimmermann and Brown, 2001; Mann et al., 2001).

The coupling of techniques such as 2D PAGE and protein separation methods (like the TAP strategy and/or HPLC systems), has facilitated significant advances in the field of proteomic analysis. For example, it has been possible to isolate and identify large complexes and protein networks and to identify novel or differentially regulated proteins (Gavin et al., 2002). However, even though such advances have allowed for an increased throughput in proteomics, the currently available techniques are difficult to implement, since they require highly specialised researchers. Therefore, fast, cheap and efficient techniques, which could facilitate a high-throughput protein analysis are needed as alternatives to the current available analysis tools.

1.1.2 Protein microarrays

The concept of "protein microarray", and more precisely "antibody microarray", was first introduced by Roger Ekins when he proposed the idea of miniaturizing ligand binding assays to enable a "[...] simultaneous ultrasensitive measurement of many analytes in the same sample [...]" (Ekins, 1998). This report presented a precise description of how an antibody microarray would be generated and analysed, laying the building foundations for this novel technology.

Ekin's theories, combined with the potential impact that protein arrays could have in the fields of bio- and pharmacological research, lead several labs in the development of protein microarrays. Initially, proteins were arrayed onto membranes, or microtiter plate-based assays were developped to generate low-density protein arrays (Cahill, 2001). These developments allowed researches to perform large scale protein screens (usually around 4-500 proteins at a time) in a single-step procedure (Joos et al., 2000; de Wildt et al., 2000; Lueking et al., 1999). The possibility of using glass slides for arraying proteins was being heavily considered. However, the common belief was that proteins were not compatible with a DNA microarray equivalent because of their assumed instability and degradation if attached on a glass slide.

This view predominated until September 2000, when the journal Science published the first report of a functional protein microarray, in which small sets of proteins were attached and detected on a dry solid support (MacBeath and Schreiber, 2000). The authors presented several experiments where antibodies, antigens or substrates were attached to glass slides. The interactions of the immobilized samples with their binding partners were detected using a strategy similar to the one used for DNA microarrays. Soon after, Haab and coworkers (Stanford, USA) showed that, similarly to DNA microarrays, arrays of 115 immobilized antigens or antibodies could be made to specifically detect their cognate ligands in heterogeneous solutions (Haab et al., 2001).

Taken together, these reports opened the field of using microarrays for proteomic projects. From that point, an increasing number of possibilities have emerged for the

use of microarrays, including diagnostic applications, antibody screening and protein-protein interactions (Zhu et al., 2001). Figure 1 gives an overview of the currently reported applications of protein microarrays. Dependent on the biological questions to be addressed, microarrays can present immobilized molecules such as antibodies, peptides, carbohydrates or small molecules. The applications, advantages and disadvantages of the different types of microarray approaches are summarised in Table 1 (Zhu et al., 2003).

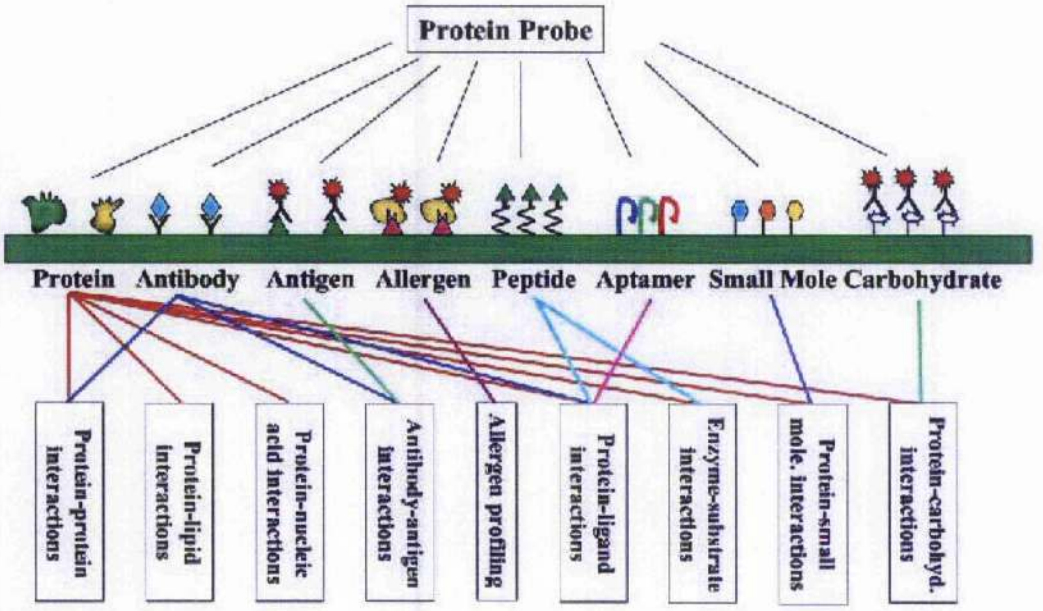

Figure 1. Applications for protein microarrays.
Ligands, such as proteins, peptides, antibodies, antigens, allergens and small molecules are immobilized in high density on modified surfaces to form functional and analytical protein microarrays. These protein microarrays can also be used for various kinds of biochemical analyses.

Approach	Application	Advantage	Disadvantage
Yeast two-hybrid	Protein-protein interactions, protein-DNA interactions	High-throughput and systematic to reveal protein interactions	No control over interaction condition; interactions are usually in the nucleus
Affinity tagging/MS	Dissecting protein complexes	In vivo interactions that involve multiple parameters	May miss transient or weak interactions, hard to identify false positives
Antibody array	Protein profiling, protein detection, clinical diagnostics	Very sensitive and low sample consumption, great potential in biomarker and drug development	Highly restricted by the quantity and quality of available antibodies; semiquantitative protein detection
Functional protein array	Diverse, e.g., protein-protein, protein-lipid, protein-small molecule, enzyme-substrate interactions as well as drug discovery and post-translational modifications	Great potentials for analyzing biochemical activities of proteins and high-throughput drug and drug target screening	In vitro assays
Peptide array	Enzyme-substrate interaction and drug discovery	Sensitive and straightforward way to identify epitopes	Expensive to fabricate; in vitro assays
Carbohydrate array	carbohydrate-mediated molecular recognition and anti-infection response	A new sensitive way to study carbohydrate-mediated molecular events	In vitro arrays; tough to acquire carbohydrate molecules in pure forms
Small molecule array	Protein-small molecule interactions, drug discovery, enzyme specificity profiling	Minimum small molecule consumption and high sensitivity	In vitro assays; necessary to improve throughput to cover 10^6 molecules in a normal combinatorial chemistry library

Table 1. Comparison of different technologies for interaction proteomics

1.1.3 Advantages of protein microarrays

Compared to other protein analysis tools, protein microarrays present several advantages. First of all, the possibility of immobilizing several thousands of molecules in defined areas of a small solid support allows for the design of complex experiments that would have been unrealistic to carry out using conventional methods. This facilitates large scale proteomics experiments where extensive libraries of proteins are simultaneously probed in one single experiment. This advantage was demonstrated by Snyder's group (Yale, USA), which identified novel calmodulin and phospholipid binding proteins using a microarray containing an almost complete yeast proteome (Zhu et al., 2001). The idea behind these two experiments was to express and purify the whole yeast proteome, immobilise each protein on a modified microscope glass slide and probe this array with labelled calmodulin and phospholipids. This group used an identical strategy to analyse the properties and substrates of several kinases (Zhu et al., 2000). In this experiment, phosphorylation by the kinases were detected by adding $^{32}\gamma$ATP into the kinase reaction buffers and detecting the incorporation of radioactive phosphate into the immobilized substrates using a phosphoimager. Since the microarrays contained 4800 proteins in duplicate, such experiments could not have been performed using conventional methods.

Another important advantage of protein microarrays is the low amounts of reagents and sample required for the production of reliable data. It has been shown that immobilized antigens and antibodies can be accurately detected via their specific interaction partners at concentrations as low as 1.6 and 0.34 μg.ml^{-1}, respectively (Haab et al., 2001). Microarray spotters are able to dispense minute volumes of reagents per spot (in the range of hundreds of picoliters) and an entire microarray can be hybridized with a total volume as low as 25 μl. Therefore, the absolute amounts of sample required to carry out a microarray experiment are very low. This particular property of protein microarrays is crucial, especially in experiments aimed at studying low abundance or difficult-to-produce proteins.

Protein microarrays allow for multiplexed experiments, where multiple samples are analysed simultaneously. Similarly to DNA microarrays, where it is possible to analyse in a genome wide manner the patterns of gene expression, protein microarrays can be used to analyse variations in cellular protein composition. This can be done by differentially labelling sets of samples derived from tissues grown under different conditions or from different cellular compartments. These differentially labelled samples can then be studied in parallel, and the microarray is analysed under different wavelengths, specific for each label used. This approach could prove essential in the study of cellular events following treatment of tissues with specific stimuli or under different pathological conditions. However, such a comparative analysis is still hampered by the lack of suitable hardware. The conventional tools used to produce and analyse protein microarrays are usually those designed for DNA microarray experiments, where usually only two fluorophores (Cy3 and Cy5) are used. Fortunately, "new-generation" multi-laser and multi-filter scanners (for example the LS400 from Tecan and the GenePix4200 series from Axon Instruments) are offering the possibility to detect several fluorophores in single experiments, because of the incorporation of multiple excitation lasers and emission filters.

The use of protein microarrays as diagnostic tools, especially in the fields of cancer and immune diseases, is an example of applications benefitting from of all of the advantageous features of protein microarrays as outlined above. The immobilization of several antibodies specific against defined disease markers, has allowed for a one-pass profiling of tissue samples with a high sensitivity and specificity, showing that the sensitivity and multiplexing nature of protein microarrays can be successfully applied for the detection of diseases (Paweletz et al., 2001; Robinson et al., 2002; Joos et al., 2000; Lin et al., 2003; MacBeath, 2002). A significant feature, from a clinical point of view, is that diagnostic microarrays require very low sample amounts (a few microliters of blood are usually sufficient), whereby the physical and psychological stress on patients to be diagnosed can be dramatically reduced.

1.1.4 Disadvantages of protein microarrays

One of the major bottlenecks in the use of protein microarrays in global protein analysis is the availability of samples. Whether it is proteins, peptides, antibodies or small chemical compounds, the availability of ready-to-use molecules is a major problem. For example, the majority of the commercially available reagents are not compatible with protein microarray applications: purified proteins often come already bound to beads, coupled with detection reagents or diluted in specific buffers. Alternatives to commercially available products are self-produced samples. However, self production of proteins/peptides/antibodies is a long and tedious process that usually requires specialised staff, working full-time on such projects. Difficulties in obtaining the required samples for microarray applications is a serious limiting factor for large scale proteomic projects. As an example, Snyder's group (Yale, USA) spent between 4 and 5 years to clone, express and purify the yeast proteome (Snyder, personal communication). The problems involved in obtaining the required number of samples leads to increasing difficulties in the design and implementation of large scale microarray projects. Under the guidance of the Human Proteome Organisation (`http://www.hupo.org`) and other Societies, several consortia are now being established in order to overcome these limitations.

The stability and activity of the attached molecules is an inherent issue of concerns when using protein microarrays, since the conditions for an attached protein to remain native might differ from those of its neighboring samples. Fortunately, it appears that proteins are able to maintain a good level of folding even after drying on the slide (MacBeath personal communication and my personal observations). However, the activity of proteins is more difficult to maintain, since the experimental conditions in protein microarrays applications are far from being physiological (immobilized molecule on a dry glass surface). In addition, due to the high density, the heavy miniaturization and the low sample amounts of the array, it is not possible to analyse the state of each immobilized sample.

A major disadvantage of protein microarrays is the inability to identify the proteins captured by immobilized interaction partners from a mixture of unknown molecules. Thus, protein microarray analyses are still limited to known samples and events. It is for instance impossible to identify interaction partners of immobilized proteins and peptides within a cell lysate. For this to be possible, the porting of MS technology to microarrays is required. One such technology is currently available. Ciphergen has produced the ProteinChip/SELDI-TOF system, which is based of an array of 8 spotted samples. Following binding of interaction partners, this array directly serves as a platform for ionization in the SELDI-TOF, allowing for the bound analytes to be identified. This technology has been very useful for protein profiling in cancer, but it still limited to a few samples per experiment (Fung et al., 2001; Petricoin et al., 2002). There is a current drive into developing novel protein microarrays, which can be directly coupled to MALDI-MS (Scrivener et al., 2003), but this field is still in an exploratory phase.

1.1.5 Labelling and detection strategies in protein microarrays analysis

One major aspect for the successful application of protein microarrays is the strategy adopted to detect an interaction between an immobilized unit and its interaction partner. This is done by the labelling of the hybridisation probe with fluorophores or other signal emitting compounds. Because of the varied properties of proteins and of their interactions, different labelling and detection strategies have been designed. The four most used strategies are discussed in this chapter (Fig 2).

First, detection based on the direct labelling of the probe is a very common strategy. This requires the prior attachment of labelling reagents, such as fluorophores or radioactive compounds, to the probe (Fig 2 A). This method allows for the direct detection of the bound probe to its immobilized interaction partner (Haab et al., 2001; MacBeath and Schreiber, 2000; Martin et al., 2003). In case of the probe being a purified molecule, this method can, in addition, generate semi-quantitative read-outs. However, this does not apply when complex mixtures of proteins are labelled, since

each probe in the mixture presents different biochemical properties, resulting in different numbers of labelling moieties per probe. Therefore, unless each molecule in the solution is individually analysed, it would be impossible to quantify the levels of each component within the mixture, thus making a quantitative, or semiquantitative, analysis impossible. Detection strategies based on direct labelling of the probe present some potential drawbacks, since the labelling reaction is done at the level of single amino-acids. For example, there is an intrinsic risk of incorporating a label molecule into the protein's binding domain, or catalytic region, reducing the interaction's affinity with the immobilized sample.

A second widely used method is indirect labelling of the interacting protein pair. This strategy is similar to a Western blot procedure in which the probe, bound to its immobilized interaction partner, is detected by a specific antibody labelled with a detection reagent (HRP, fluorophore, etc) (Fig. 2 B). In this experimental setup, no labelling reagents can interfere with the binding reaction. It requires, however, the availability of highly specific antibodies.

A third strategy, the "sandwich assay", is often applied for the specific detection of target proteins (Fig. 2 C). In this method, an array of antibodies is hybridised with an antigen and this antibody-antigen interaction is detected by a labelled second antibody, which is specific for a different epitope of the antigen. This approach requires the availability of two high affinity antibodies that are specific for different epitopes in the antigen. This system is widely used in diagnostics such as cytokine profiling, since this field benefits from the availability of such specific antibody pairs (Huang et al., 2001; MacBeath, 2002).

A fourth detection method, which combines several of the advantages of the previous methods is shown in figure 2 D. In this set-up, the screening probe is presented as a fusion protein, for example coupled to a biotin molecule. After the binding reaction is achieved, a labelled streptavidin molecule is then allowed to bind the biotin associated with the hybridisation probe. This strategy allows for a highly specific detection (since the biotin-avidin interaction is highly specific and strong) and has been used to identify

the interactions of calmodulin against the complete yeast proteome (Chaiet and Wolf, 1964; Zhu et al., 2001). It has also been applied in the study of cytokines (Lin et al., 2003). The fusion of light emitting proteins such as GFP to the probe, has also been used as a detection system in cell microarray experiments (Ziauddin and Sabatini, 2001).

Other methods for the labelling and detection of probes have been proposed, but seem to be limited to highly specific applications. It has been proposed, for example, to label proteins with Europium chelates that can be detected using specific scanners (Scorilas et al., 2000). Also, novel reagents have been developed to detect phosphorylated substrates in microarray based kinase assays. For example, a fluorescent Pro-Q Diamond phosphosensor dye technology has been developed by Molecular Probes (USA). This dye allows for the specific labelling of phosphorylated proteins, thus eliminating the requirement of γ^{32} and γ^{33}-ATP from kinase experiments (Martin et al., 2003).

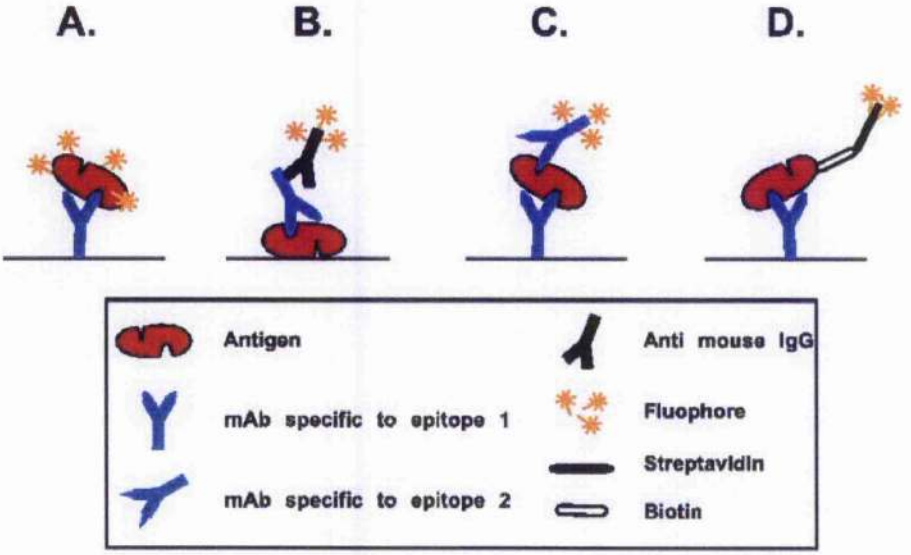

Figure 2. Detection approaches in protein microarrays
A - Direct detection
B - Indirect detection
C - Sandwich assay
D - Affinity detection

Detection strategies for the analysis of protein interactions is fundamental in all fields of the life sciences, including biomedical research and proteomics. There is therefore a high requirement for specific, high affinity detection reagents. Monoclonal antibodies are particularly valuable reagents for these experiments. Since their first description in 1975 (Köhler and Milstein, 1975), monoclonal antibodies have proven to be very powerful reagents for the detection of proteins.

1.2 Monoclonal Antibodies

The technique for the production of monoclonal antibody secreting hybridomas (Köhler and Milstein, 1975), has provided the life-sciences community with a revolutionary tool for the characterization, localization and manipulation of proteins and protein-complexes. With the advent of functional genomics and proteomics, the need for detection reagents, such as monoclonal antibodies, has spectacularly increased. Unfortunately, conventional monoclonal antibody production methods have failed to keep up with such a demand, and the lack of monoclonal antibodies is now a significant bottleneck in all fields of biomedical research. The protocols used to generate monoclonal antibodies have hardly been modified since their first publication, 29 years ago. Monoclonal antibody production is comprised of three distinct steps:

1. Immunization of (usually) a mouse with an antigen
2. Cell culture: somatic fusion of harvested splenocytes from the donor animal to a myeloma cell-line using the fusogen polyethylene glycol (PEG) and subsequent tissue culture
3. Screening of the produced culture supernatants against the target protein. Positive clones are then subject to rounds of subcloning at limiting dilution to ensure monoclonality.

The main limiting factors in this procedure are the cell culture steps and the screening of the hybridoma library generated at each fusion. Cell culture routines, including the fusion step, are extremely time consuming and require a lot of handling and are prone to human error. Generally, one person can generate highly specific, high-affinity monoclonal antibodies against 25 - 30 antigens per year. The conventional screening of the hybridoma libraries is generally performed by ELISA. This method is not a highly efficient screening platform, since it is time consuming and requires a lot of handling and reagents. In order to screen large libraries, containing thousands of samples, extensive automation is required to perform such a high scale range of ELISA screens. Thus, conventional monoclonal antibodies production methods are not suitable for high-throughput applications.

It has been recently stated that " **...Antibodies are the most prominent capture molecules used to identify targets. Owing to the labour intensive nature of monoclonal antibody production, however, the development of other alternatives has become crucial...**" (Templin et al., 2002). Other techniques which are truly high-throughput in their approach, such as phage-display, suffer from qualitative limitations, most notably low binding affinity. Antibodies generated by phage-display often require further molecular engineering to produce multi-use reagents, and are therefore not currently amenable to high throughput approaches (Irving et al., 1996; D'Mello and Howard, 2001; Gram et al., 1992). Alternatively, novel detection reagents have been developed and implemented in current research, such as aptamers, recombinant antibodies and affibodies (Rönnmark et al., 2002a; Nord et al., 2001; Gunneriusson et al., 1999; Hansson et al., 1999; Eklund et al., 2002; Brody et al., 1999; Bock et al., 1992; Knappik et al., 2000; Rönnmark et al., 2002b). However, these alternatives present limitations in the form of long selection processes and molecular engineering.

The establishment of ameliorated and more efficient protocols for the generation and screening of monoclonal antibodies is an important requirement in current research, and would allow for the generation of more complex collections of monoclonal antibodies. This would bring a new level of biomedical research, allowing for the establishment

of large scale proteomics projects.

1.3 Aims of project

1.3.1 Establishing a protein chip platform

At the beginning of this PhD project, protein microarray technology was a novelty, as only two papers reporting the use of protein microarrays on glass surfaces had been published (MacBeath and Schreiber, 2000; Haab et al., 2001). The first part of this project was aimed at establishing protocols and procedures that would allow the production of functional and reliable protein microarrays. The main factors that had to be established in order to obtain such a platform were:

- Substrate chemistry: which chemical modifications of the glass slide were most suitable for the attachment of proteins?
- Spotting conditions: which physical parameters should be used to successfully array sets of proteins?
- Labelling and detection strategies: which strategies would be more suitable for the applications we wanted to design?

The initial aim was to establish these basic required conditions for the generation of functional protein microarrays. Having done this, the following goal was to design novel biological applications using protein microarrays, demonstrating the impact of this technology in proteomic research.

1.3.2 Development of a high-throughput screening method for the detection of monoclonal antibodies

Concurrently with the beginning of this PhD project, the EMBL had established a Monoclonal Antibodies Core Facility (MACF). This facility was set up to meet the increasing

need for the establishment of novel and more efficient protocols for the production and screening of monoclonal antibodies, in order to accelerate the setup of large scale proteomics projects.

The initial strategy of this facility was to test the possibility of implementing multiplexing immunizations (ie, the simultaneous injection of several unrelated antigens into a single mouse), automated fusion and cell culture procedures and, as a screening technology, an automated ELISA procedure, using a dedicated custom built robotic solution. However, this screening approach was early on found to be complicated and not economically viable. Therefore, the MACF needed to develop a novel screening method that would allow for an increased throughput and lower costs, compared to the planned automated ELISA platform.

Together with the MACF, I sought to devise a novel hybridoma screening approach using protein microarrays. The aim was to develop a rapid, efficient and cost effective way to screen thousands of hybridomas for specificity, within a single experiment.

1.3.3 Development of a protein microarray-specific analysis software

After having established the protein microarray platform and designed a novel monoclonal antibodies screening platform, it became clear that the available chip analysis software available was not satisfactory for the analysis of protein microarrays. Because of the short history of protein microarrays, all data analysis involving this technology is currently performed using software developed for the analysis of DNA microarrays. Such software packages are optimised for DNA microarrays experiments, and focus on statistical analyses of gene expression patterns, whereas only a few of the present features by these applications are useful for protein microarrays. Therefore, a third aim of this project was to write an analysis software, which specifically analyses those parameters needed for protein microarrays.

The specific goal was to create an analysis software that would allow for:

- Creation of unique sample names to each sample in the array
- Mapping of the coordinates of each sample from its location in the cell culture plates
- Retrieval of meaningful data from the chip analysis
- Normalization of the data
- Analysis of the data and identification of positive samples
- Cross-experiment filtering (removal of false positives and cross reactive species)
- Population of a LIMS database (Laboratory Information Management Systems), where all data relative to each experiment is stored

2 List of abbreviations

2.1 Buffers and Chemicals

- PBS: Phosphate Buffered Saline
- PBS-T: Phosphate Buffered Saline - Tween20
- TPBS: PBS, 0.1% TritonX100
- BRB80: Brinkley BR buffer 1980
- PFA: Paraformaldehyde
- GA: Glutaraldehyde
- AH: Azaserine Hypoxanthine
- HCF: Hybridoma Cloning Factor

2.2 Terminology

- mAb(s): Monoclonal antibody(ies)
- HS(s): Hybridoma supernatant(s)

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals

- poly-l-Lysine (P8920); Casein (C3400); BSA (A3803); Sodium Azide (S8032), Ribi (M6536), Azaserine Hypoxanthine (A9666), OPI media supplement (O5003), p-nitrophenyl phosphate (N7653): Sigma Aldrich
- Cy3-Cy5 (PA25001), ECL (RPN2209): Amersham
- SuperSignal West Femto Maximum sensitivity substrate (34095): Pierce
- Alum (77161T): Pierce
- Immuneasy (303101): Qiagen
- FBS (SH30070.03): Hyclone
- Hybridoma Cloning Factor (ECO1021N): Euroclone

3.1.2 Microarray Slides

- GoldSeal (3010): Gold Seal Products
- Standard Glass Slides: Menzel-Glaser
- FAST (10484182); CAST (10484181): Schleicher & Schuel
- HydroGel (6050017): Perkin Elmer
- SuperAmine (SMM); SuperAldehyde (SMA): Telechem Array-it

- CodeLink (300011-4PK): Amersham
- PicoSlides (PS-ITC-20): PicoRapid
- eSurf (MA0110): LifeLineLab

3.1.3 Antibodies

- α-Tubulin Clone DM1A (T9026); Protein A Clone SPA-27 (P2921); FLAG M1 (F3040): Sigma Aldrich
- IκBε(sc-7275); IκBα (H4) (sc-1643); IκBα (C15) (sc-203); Mip1α (sc-1381); GST (sc-138); Fractalkine (sc-7226); His-Tag (sc-8036); p52 (sc-7386); p65 (sc-372); p53 (sc-6243); IκB (sc-9130): Santa Cruz Biotechnologies
- Cy3-conjugated goat anti-mouse IgG (115-165-164); Cy5-conjugated goat anti-mouse IgM (115-175-020), Alkaline Phosphatase goat anti-mouse IgG+IgM (115-055-044): Jackson Immuno Research

3.1.4 Purified Proteins

- IκBε(sc-4328 WB); c-Rel (sc-4030); p52 (sc-4095); H1 (sc-8030): Santa Cruz Biotechnologies
- Protein A (539203): Calbiochem
- Protein G (P5170); Human IgG (I2511): Sigma Aldrich
- All antigens received from internal and external research laboratories

3.1.5 Consumables

- Micro Bio-Spin Chromatography Columns: BioRad
- Microcon 10 Centrifugal Filter Units: Millipore

3.1.6 Robots and electronic equipment

- **Spotters**: GMS416 (Affimetrix); Omnigrid (GeneMachines) ; MicroGrid (Apogent-Discoveries)
- **Scanners**: GMS418 (Affimetrix); GenePix 4.1 (Axon Genomics); LS400 (Tecan); Biomolex (Biomolex); Magellan (Tecan)
- **Robots**: Biomek FX (Beckman); TECAN Custom Setup
- **Others**: Lab-on-a-Chip (Agilent)

3.1.7 Buffers

- Cleaning Buffer A: 35 g NaOH, 140 ml H2O, 210 ml 96% Ethanol
- poly-L-lysine solution: 25 ml poly-L-lysine, 280 ml ddH2O, 25 ml PBS
- BRB80: As much as 80 mM of K-PIPES (pH 6.8), 1 mM MgCl, and 1 mM EGTA, titrated to pH 6.8 with KOH.
- HM20: DMEM, 20% FBS, Gentamycin, L-Glutamine
- Hybridoma Selection Medium: HM20, 10% HCF, 2% AH, 1% OPI
- Hybridoma Growth Medium: HM20, 10% HCF

3.1.8 Blocking Solutions

- 3% Skimmed Milk in PBS-T (0.1%)
- 3% Skimmed Milk, 0.02% Sodium Azide in PBS-T (0.1%)
- 1% BSA in PBS
- 3% BSA in PBS
- 2.5% Casein in PBS

3.2 Methods - initial experiments

3.2.1 Poly-L-Lysine coating

Poly-L-lysine coating of slides was performed according to the Stanford protocol (`http://cmgm.stanford.edu/pbrown/protocols/1_slides.html`).

Gold Seal slides were cleaned in Cleaning Buffer A overnight, with constant shaking. Slides were rinsed in ddH_2O to remove traces of ethanol and NaOH. Clean slides were then incubated in the poly-L-lysine solution for 2 hours, at RT. After a further round of washes, the coated slides were centrifuged at 650 rpm for 5' to remove traces of liquid and then baked at 45^0C for 10'.

3.2.2 Aminosilane coating: EMBL protocol

Protocols and guidelines for the production of EMBL's aminosilane proprietary slides can be obtained from the patent application "Immobilisierung und Markierung von Biopolymeren" (DE10016073A1, publication date: March 1st, 2001).

The following protocols ("Preparation and spotting of baits", "labelling of probes" and "hybridization") were obtained from previously published data (MacBeath and Schreiber, 2000; Haab et al., 2001).

3.2.3 Preparation and spotting of baits

All samples to be spotted were transferred into a glycerol free PBS solution using BioRad BioSpin P6 columns. Samples were spotted either manually (0.2 μl) using a Gilson pipette or robotically, using a microarrayer. The location of the spots was marked on the back of the slide using a diamond pencil, since once dried, the spots tend to become invisible. Dried chips were stored at 4^0C in a sealed box. Before usage of the spotted arrays, these were quickly rinsed in a 3% skimmed milk in PBS-T(0.1%)

solution to remove unbound material. After this washing step, the slides were blocked in any of the blocking solutions (see 3.1.8) by incubation at RT for 90'. Excessive blocking solution was removed by washing the slides 3 x 1' in PBS and leaving the slides in fresh PBS until application of the probe solution.

3.2.4 Labelling of probes

Homo- or heterogenous protein solutions were prepared in 0.1 M sodium carbonate buffer pH 8.0. Cy3 and Cy5 powders were brought in solution, each in 0.1 M sodium carbonate pH 8.0. Protein and dye solutions were mixed together in order to obtain a final mix containing 0.2 - 2 $mg.ml^{-1}$of protein and a final dye concentration of 100 - 300 μM. This mix was allowed to react in dark for 45' at RT, and was quenched by addition of a tenth volume of 1 M Tris pH 8.0 (500 fold molar excess of quencher). The mix was brought to a volume of 0.5 ml with PBS, loaded into a microconcentrator spin column (Amicon Microcon 10) with a 10 kDa MWC and the volume was reduced to ~ 10 μl by centrifugation (~20', 15000 rpm in an Eppendorf bench centrifuge).

The dye was blocked with the addition of 25 μl of a 3% skimmed milk solution in PBS (60' at RT) and the volume was brought up to 0.5 ml with PBS. The blocked sample was loaded into a fresh Microcon 10 cartridge and concentrated to 10 μl. The sample was brought up to 25 μl with PBS and filtered by centrifugation in a 0.45 μm spin filter (Millipore) at 2000 rpm in an Eppendorf bench centrifuge. After filtration, the sample was ready for hybridization.

3.2.5 Hybridization

The washed slides were removed from the PBS bath and the excess liquid was shaken off. Without allowing the array to dry, we applied 25 μl of the dye labeled protein solution to the surface of the array. The slide was then covered with a coverslip to ensure uniformity of the probe solution and to minimize evaporation. Covered slides were stored in a humid chamber at RT for 60' (or ON at 4^0C). After this incubation, the

coverslip and the protein solution were removed by gently dipping the slides in PBS, and the slides were washed 2 x 5' in PBS-T (0.1%), 2 X 5' in PBS and 2' x 5' in ddH_2O at RT. The slides were then spun dry and scanned using a microarray laser scanner.

3.3 Methods - HybriChips

Immunizations, cell culture and ELISAs were performed by Alan Sawyer, Pieranna Chiarella and Heike Wilhelm of the Monoclonal Antibodies Core Facility at the EMBL.

All immunizations were performed using 20 μg of antigen for the primary injection, followed by boosts of 10 μg each.

3.3.1 Immunization protocols

3.3.1.1 Alum protocol Antigens were mixed 1:1 with Alum adjuvant in combination with the oligonucleotide CpGDNA (10 nmols/mouse). Samples were mixed at RT for 30 minutes and then injected half intraperitoneal and half subcutaneous (2 subcut). Last boost (pre-fusion boost) has to be done at least 3 weeks after the previous boost. Animals were boosted every three weeks and bled 10 days after the boost. Blood was collected in separator tubes and spun down at 3000 rpm for 4 minutes in order to isolate the serum. Serum was then tested against the target antigen by ELISA in serial dilutions (1:500, 1:2500, 1:10000 and 1:50000)

3.3.1.2 Ribi injection protocol Mice were injected every 3 weeks with the antigen mixed 1:1 with Ribi adjuvant. Half of the sample was injected subcutaneous and half intraperitoneal. 10 days after the boost, blood was collected in separator tubes and spun down at 3000 rpm for 4 minutes in order to isolate the blood serum. Serum was then tested against the antigen by ELISA in serial dilutions (1:500, 1:2500, 1:10000 and 1:50000)

3.3.1.3 Details of Ribi adjuvant This adjuvant (MPL+TDM) is a stable oil-in-water emulsion which can be used as an alternative to the classical Freund's water-in-oil emulsion. It has been proven to be a powerful immunostimulant (Thompson et al., 1998; Ryll et al., 2001). MPL: Monophosphoryl LipidA (highly refined non-toxic LipidA isolated from re-mutants of *Salmonella minnesota*) TDM: synthetic Trehalose Dicorynomycolate (analogous of trehalose dymicolate from the cord factor of *Mycobacterium tuberculosis*).

3.3.1.4 Immuneasy injection protocol Antigens were mixed with a certain amount of Immuneasy adjuvant (Qiagen) according to the manufacturer's instruction, mixed at RT for 5-10 minutes and then injected with a 2 weeks interval. Half of the sample was injected subcutaneous and half intramuscular. The animal serum was collected 10 days after the boost and tested against the antigen by ELISA in serial dilutions (1:500, 1:2500, 1:10000 and 1:50000). The last boost (pre-fusion boost) has to be done two to eight weeks after the previous injection. The short protocol we used in the 80 Antigens trial consisted of 1 injection and 1 boost which coincides with the pre-fusion boost.

3.3.1.5 Details of Immuneasy adjuvant and importance of using CpGDNA Immuneasy Mouse adjuvant contains CpGDNA, short oligonucleotides that contain unmethylated cytosine-guanine dinucleotides within a certain base context. The mammalian immune system has evolved to recognize these sequences, which are found naturally in bacterial DNA, as a sign of infection. Exposure to CpGDNA results in very rapid and strong immune activation and, when applied with an antigen, CpGDNA produces high titers of antigen specific antibodies. Since Immuneasy mouse adjuvant induces high antibody titers in a short period of time, boosting can be performed earlier than with other adjuvants. The amount of CpGDNA used in the preparation of the samples is unknown.

3.3.2 Fusion protocols

All steps were performed under sterile or aseptic conditions in a laminar flow hood. Spleens were rendered into single-cell suspensions by mechanical disruption between two frosted-end glass microscope slides. The suspensions were filtered into 50ml barcoded conical-bottomed tubes (BD Falcon) through 70 m nylon cell strainers (BD Falcon) and transferred to the robotic system. Separately, SP2 myeloma fusion partners (ATCC) were cultured for five days prior to fusion in HM20 (DMEM, 20% Defined fetal bovine serum (Hyclone Defined), 10mM L-Glutamine, 50 M Gentamicin) and on the day of the fusion were transferred to HM20/HCF/2xOPI (HM20 containing 10% Hybridoma Cloning Factor (Origen) and 2%OPI cloning supplement (Sigma)) for at least one hour at 37^{0}C in a 5% CO2 incubator. The tubes were centrifuged at 100 g for 10' at RT and the cells were resuspended in 5 ml Red Cell Lysis Buffer (Sigma) for 9 minutes at RT. HM20 was added to reach a final volume 50ml and again centrifuged for 10 min at RT with no brake. The supernatant solutions were aspirated to waste and the cells resuspended in DMEM preheated to 37^{0}C. Cells were washed twice more by steps of centrifugation and resuspension. 50 μl of cell suspension were robotically pipetted to 1.5 ml microcentrifuge tubes and counted using a haemocytometer counting chamber. Simultaneously the SP2 cells were washed three times in a similar fashion and a similar aliquot (50 μl) was removed to a 1.5 ml tube for haemocytometric counting. SP2 myelomas and spleen cells were mixed at a ratio of 1:5 (SP2:Spleen) and again centrifuged at 100 g for 10 min with no brake. The supernatants were entirely aspirated to waste and Polyethyleneglycol 1500 in 50% HEPES (PEG: Roche Molecular Biochemicals) pre-heated to 37^{0}C was robotically pipetted smoothly and progressively over 1 min with rotation at 450 rpm on a Te-shake shaker (Tecan AG) to ensure even mixing. The cell/PEG mixtures were incubated for 1 min at 37^{0}C with gentle agitation. 1 ml of DMEM was similarly added over 1 min at 37^{0}C with similar agitation. The mixture was incubated for 1 min at 37^{0}C with gentle agitation. A further 1 ml of DMEM was robotically added over 1 min at 37^{0}C with gentle agitation and incubated similarly for

a further minute. 7 ml of HM20 were robotically added over 3 min at 37°C with gentle agitation. The tubes were then spun at 90 g for 5' with brake. The supernatant was aspirated to waste and the pellet manually resuspended in 20 ml of HM20/HCF/OPI/AH (HM20/HCF/OPI plus 10% Azaserine Hypoxanthine (Sigma)). The conical tubes were again placed on the robot workdeck and the post-fusion cell slurry was aspirated by each of the 8 wide-bore pipette tips of the liquid handling arm of the robot. 200 μl of the cell slurry was then pipetted into each well of a 96-well deep well plate (Greiner Masterblock). The deep-well plate was then robotically transferred to a TeMo 96-well pipetting robot integrated onto the Genesis work-deck and used as a source plate to plate out into the 20 sterile 96-well tissue culture plates. The post-fusion mixture was then robotically plated out into 20 X 96-well sterile plates (Nunc) sourced from a carousel attached and integrated to the robot at 100 μl/well and robotically transferred to an integrated 37°C incubator with 10% CO_2 through the integrated airlock.

3.3.3 Cell culture

On the third day after the fusion, cells were robotically transported from the incubator to the work deck and a further 100 μl HM20/HCF/OPI/AH was robotically added. On day 7 the plates were once again similarly transported from incubator to work deck and 200 μl/well of the culture supernatants was aspirated to waste and replaced with 150 μl fresh HM20/HCF. On day 11 the plates were again robotically transported to the work deck and 40 μl of supernatant was collected from each well (Temo head: Tecan Inc.) and transferred to 384-well plates (Greiner) supplied to the workdeck by a carousel plate stacker (Tecan Inc).

3.3.4 Enzyme-Linked Immunoadsorbent Assay (ELISA)

96 well plates were coated with 4 μg/ml of antigen and incubated overnight at 4°C. Plates were washed in PBS-T and blocked with 3% BSA in PBS for 1 hour at RT. 50 μl of hybridoma supernatant was added to each well and incubated for 1 hour at RT. After 4 washes in PBS-T, plates were incubated for 1 hour at RT with alkaline

phosphatase conjugated anti-mouse secondary antibody, diluted 1:5000 in PBS. Plates were washed in PBST and incubated with p-nitrophenyl phosphate for 10-15 min at RT. Reaction was stopped by adding 50 μl of 2 M NaOH and the optical density was spectrophotometricly determined at 405 nm.

3.3.5 HybriChip preparation

Aminosilane modified microscope slides (EMBL, Heidelberg) were homogeneously coated with 5 μg of antigen in 50 μl PBS using a 24x60 mm coverslip. Slides were incubated in a humid chamber at RT for 60 minutes, the coverslip removed and subjected to three five-minute washes in PBS. Slides were blocked in a 3% BSA solution in PBS for 60 minutes at RT. After five, 5 minute washes in PBS, the slides were dried by centrifugation.

Hybridoma supernatants were spotted onto the slides using a MicroGrid II 600 arrayer (ApogentDiscoveries, UK), using 32 MicroSpot 2500 pins in an 8x4 array (ApogentDiscoveries, UK). Humidity and temperature are maintained at 40% and 24°C respectively. Slides were then left to incubate in the arrayer for a further 60 minutes. The microarrays were washed five times 5 min in PBS and incubated with 40 μl of a mix of Cy3-conjugated goat anti-mouse IgG and Cy5-conjugated goat anti-mouse IgM (Jackson Immunoresearch, West Grove, PA) both diluted 1:1000 in 3% BSA-10% Glycerol (60 minutes, RT, humid chamber). Microarrays were then washed twice for 5 min in PBS-T, twice 5 min in PBS and finally rinsed in ddH_2O. Microarrays were dried by centrifugation and scanned in an LS400 Scanner (Tecan, Austria), using 633 and 543 nm lasers respectively for Cy5 and Cy3 excitation and 670 and 590 nm emission filters.

3.3.6 HybriChip analysis

Image analysis was performed using the GenePix Pro 4.1 software package (Axon Instruments, USA). Spots for which the diameter is not included in a fork of 80 - 150 μm or of bad quality (scratches, heavy background, dust, etc) were ignored. For each

remaining sample, we retrieved the median of the medians of the intensities of each group of replicates (MR). Each value was then normalised against the median value of all the MR of the chip [MR/(median of total MR)]. Samples showing a normalised value inferior to 2 were considered negative. Values between 2 and 20 were considered putative positives, while all samples having a normalised value equal or over 20 were considered positives. Data analysis was performed using a proprietary software application, HyCAT (Hybridoma Chip Analysis Tool).

3.3.7 Immunofluorescence

Coverslip preparation 12 mm glass coverslips were soaked in a 60:40 (vol:vol) Ethanol-HCl solution for 60' and extensively washed with ddH_2O. Clean coverslips were then incubated in a water solution containing 0.1 $mg.ml^{-1}$ poly-L-lysine (70 kDa or higher) for at least 2 hours. After washing and drying, the coverslips were sterilised under UV light.

Treatment of cells The prepared coverslips were overlayed with 10^6 XL117 cells (*Xenopus laevis*). The cells were allowed to recover and attach to the coverslips overnight. Fixation of the cells was done using two methods:

1. Paraformaldehyde: Coverslips were placed into a 12 well plate and overlayed with 1 ml of filtered 4% PFA, 0.1% GA, 0.5% TritonX100 in 1x BRB80, for 15 to 20 minutes. The fixation solution was gently removed by aspiration and replaced with a PBS solution containing 1 $mg.ml^{-1}$ $NaBH_4$. Samples were left in this solution for 10 minutes.
2. Methanol: Coverslips were dipped into a methanol container at -20^{0}C for 10'.

Immunostaining After fixation of the cells, the coverslips were allowed to equilibrate in TPBS. The samples were incubated for 20 minutes with a mixture of undiluted hybridoma supernatants and an anti-αTubulin polyclonal antibody (1:200 in TPBS) and

washed twice with TPBS. The cells were subsequently incubated with a mixture of anti-mouse IgG and anti-rabbit IgG, respectively coupled to Alexa468 and Alexa588 fluorophores at 1:1000 dilution in TPBS. After washing with TPBS, the DNA was stained with 5 μg.ml ^{1} Hoechst dye in TPBS and the coverslips were mounted in Mowiol.

Image acquisition and analysis Samples analysis was carried out on a Zeiss Axioscope fluorescence microscope. Image acquisitions were carried out using Image Sis F-View digital camera controlled by Analysis software.

4 Results

4.1 Establishment of a protein chip platform

When the project was initiated, only two reports of protein microarrays on glass supports had been published (MacBeath and Schreiber, 2000; Haab et al., 2001). Therefore, because of the novelty of this technology, all aspects regarding the production and generation of protein microarrays had to be established *ab initio*, starting from EMBL's extensive knowledge and experience in DNA microarrays and from the mentioned papers from Macbeath and Haab.

4.1.1 Determination of a functional surface chemistry for protein microarrays

The first aspect that had to be investigated was the determination of the appropriate surface chemistry (substrate), to be used for an efficient attachment of proteins to a glass slide. In Haab's report, 115 proteins were successfully attached and detected onto poly-L-lysine coated microscope slides. It seemed then, that this substrate would allow for a specific immobilization of an extensive range of proteins, thus being a "proteome wide" substrate. We decided to test this solution and try to reproduce some of the published observations. Two different substrates were tested in parallel: poly-L-lysine and a proprietary modified aminosilane chemistry, which is currently used in our laboratory for the production of DNA microarrays (EMBL surface chemistry). These very first experiments were carried using the well characterized IgG-ProteinG (Björck and Kronvall, 1984) and IgG-Protein A interactions (Forsgren and Sjöquist, 1969).

Human IgG was manually spotted onto both chemically modified slides. The microarrays were hybridised with a solution containing Cy3 labelled Protein A and Cy5 labelled protein G. The slides were scanned using both 635 and 533 nm lasers (Cy5 and Cy3 respectively). Figure 3 shows that while no signal was obtained by the poly-L-lysine

(left image), both Protein A and Protein G were able to bind to the immobilized hIgG on the EMBL chemistry slides. It appeared that the spotted hIgG was not retained onto the poly-L-lysine slide, thus was washed off during the array processing. From this observation, it was decided to base all further experiments on the EMBL surface chemistry.

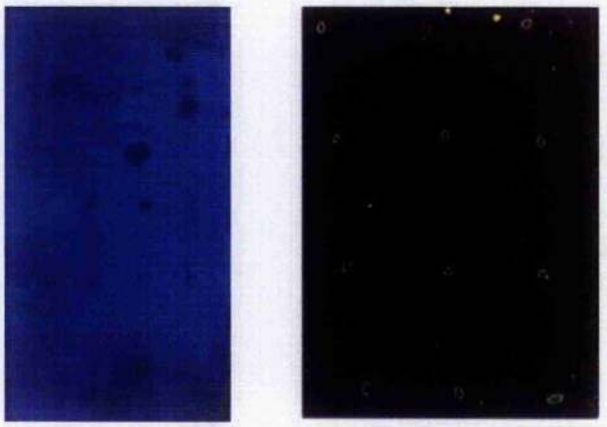

Figure 3. Surface chemistry analysis: poly-L-lysine vs EMBL chemistry
A - Poly-L-lysine coated slide: no signals can be detected after hybridisation and scanning of the slide
B - Aminosilane coated slide: processed exactly as the poly-L-lysine, this chemistry appears to allow for the attachement and detection of proteins on the glass surface

4.1.2 Can immobilized proteins be detected by specific interaction partners in a microarray experiment?

Having established that the EMBL surface chemistry allowed for the attachment of hIgG, we wanted test the detection specificity of the arrays. We prepared a chip containing a set of different antibodies specific for proteins or expression tags and where Protein A and Protein G were used as negative controls. The layout of this manually spotted chip can be seen in Table 2.

His	His	GST	GST	IκBβ	IκBβ	IκBα	IκBα	Fractalkine	Fractalkine
His	His	GST	GST	IκBβ	IκBβ	IκBα	IκBα	Fractalkine	Fractalkine
ProtG	ProtG	FLAG	FLAG	ProtA	ProtA	IκBα-H4	IκBα-H4	Mip1α	Mip1α
ProtG	ProtG	FLAG	FLAG	ProtA	ProtA	IκBα-H4	IκBα-H4	Mip1α	Mip1α

Table 2. Layout of the antibody microarray
All samples were spotted manually, where each spot contained 100 ng of sample. All immobilised samples are antibodies raised against the stated protein or expression tag. Protein G (ProtG) and Protein A (ProtA) are the full length proteins, and are used as negative controls. IκBα-H4 is an antibody specific for the N-terminus of IκBα

In order to determine the specificity of our system, we hybridised this chip using a solution containing two sets of differentially labeled antigens (Cy3 and Cy5). The first set, labeled with Cy5, contained proteins that included an expression tag (His, GST, FLAG) or a post-translational modification (biotinylation). The second set (Cy3) contained some of these same proteins, expressed without tags. Table 3 shows the exact contents of these mixtures.

Protein contents of the hybridisation solution	**Labelling Reagent**
Fractalkine IκBα IκBβ Mip1α	**Cy3**
GST-IκBα(1-54) GST-MAP3K2 GST-MAP3K3 FLAG-Mip1α FLAG-Fractalkine Biotin-IκBα His-IκBα	**Cy5**

Table 3. Hybridisation solution for the antibody microarray
Cy3 labelled proteins and Cy5 labelled tagged proteins were pooled into a single hybridisation solution. Each set contained a total of 20 ng of sample

Figure 4 shows a graphical description of the expected results (table) together with the obtained results (image). From the scanned image, we can appreciate that the negative controls did not show any signal. This was the expected result since Protein A and

protein G could only bind to IgG molecules, which were not present in the hybridisation solution. We could also observe a positive detection of the anti-FLAG, anti-IκBα-H4, IκBβ and anti-GST antibodies. However, the anti-FLAG antibody also showed to have some degree of unspecific binding, since it appears that Cy3 labelled sample was able to interact with that antibody. There is no signal for the anti-Fractalkine, anti-Mip1α and anti-IκBα antibodies, while the signals are very low for the anti-His sample. Considering that these antibodies were never tested on a microarray format, we could not speculate any further on these results, other than saying that there is some level of specificity in detection.

His	His	GST	GST	IκBβ	IκBβ	IκBα	IκBα	Fractalkine	Fractalkine
His	His	GST	GST	IκBβ	IκBβ	IκBα	IκBα	Fractalkine	Fractalkine
ProtG	ProtG	FLAG	FLAG	ProtA	ProtA	IκBα-H4	IκBα-H4	Mip1α	Mip1α
ProtG	ProtG	FLAG	FLAG	ProtA	ProtA	IκBα-H4	IκBα-H4	Mip1α	Mip1α

Figure 4. Manually spotted antibody microarray
Pictorial result of the antibody microarray. The top table gives an overview of the expected results

Following this experiment, a "reverse" microarray was generated. The antigens previously contained in the hybridisation solution were spotted onto an EMBL slide (Table 4), and their specific antibodies were used for hybridisation (Table 5). Differently from the antibody chip, Protein A and Protein G were here used as positive controls. A phage protein, T4E7, was used as negative control.

MAP3K2-GST	Frac-FLAG	IκBα-His	GST-IκBα-NT	T4E7	Mip1α	Frac
MAP3K2-GST	Frac-FLAG	IκBα-his	GST-IκBα-NT	T4E7	Mip1α	Frac
MAP3K3-GST	Protein A	Mip1α-FLAG	IκBα-Biotin	T4E7	IκBα	IκBβ
MAP3K3-GST	Protein G	Mip1α-FLAG	IκBα-Biotin	T4E7	IκBα	IκBβ

Table 4. Layout of the antigen microarray
All samples were spotted manually, where each spot contained 100 ng of sample. Protein G (ProtG) and Protein A (ProtA) are used as negative controls (binding of IgG molecules). T4E7 is a X-resolvase from T4 phages, here used as a negative control. All samples were hand-spotted in 0.2 μl drops containing 100 ng of sample

Antibody contents of the hybridisation solution	**Antibody type**	**Labelling Reagent**
FLAG	Monoclonal	**Cy3**
GST	Monoclonal	
His	Monoclonal	
IκBα-H4	Monoclonal	**Cy5**
IκBα	Polyclonal	
IκBβ	Polyclonal	
Fractalkine	Polyclonal	
Mip1α	Polyclonal	

Table 5. Hybridisation solution for the antigen microarray
Cy3 labelled antibodies and Cy5 labelled antibodies were pooled into a single hybridisation solution. Each set contained a total of 20 ng of sample

Figure 5 shows a graphical description of the expected results (table) together with the obtained results (image). Both positive controls, Protein A and Protein G had been detected by the IgG molecules contained in the hybridisation solution. The negative control T4E7 seemed to generate a positive signal on one of its locations. However, this could have been caused by the background signal present on that area of the slide. A clear positive signal from the anti GST, Fractalkine, and IκBα antibodies was also observed. Of the immobilized samples that should have shown a double recognition (Fractalkine-FLAG, Mip1α-FLAG, IκBα-His and GST-IκBα-NT), only GST-IκBα-NT shows the expected signal (yellow). As for the negative controls, the "top" area of this chip presented some background on the Cy3 channel, therefore we can not postulate

whether the orange signal detected for the two upper spots for FLAG-Fractalkine and His-IκBα were due to the correct binding of the anti-FLAG and anti-His antibodies. Furthermore, we can see that the anti-Mip1α antibody did not recognise its antigen. A very weak signal is seen from the un-tagged form of IκBα, while no signal is seen for its biotinylated form.

We could conclude that these two experiments showed that proteins immobilized onto an aminosilane coated glass slide could be specifically detected by their interaction partners. However, some of the samples could not be detected and a little cross-reactivity was detected. This could have been caused by the unprecise spotting procedure and/or by the instability of certain proteins under these experimental conditions.

MAP3K2-GST	Frac-FLAG	IκBα-His	GST-IκBα-NT	T4E7	Mip1α	Frac
MAP3K2-GST	Frac-FLAG	IκBα-His	GST-IκBα-NT	T4E7	Mip1α	Frac
MAP3K3-GST	Protein A	Mip1α-FLAG	IκBα-Biotin	T4E7	IκBα	IκBβ
MAP3K3-GST	Protein G	Mip1α-FLAG	IκBα-Biotin	T4E7	IκBα	IκBβ

Figure 5. Manually spotted antigen microarray
Pictorial result of the antibody microarray. The top table gives an overview of the expected results

4.1.3 Upscaling of the system

All initial experiments were performed by manually spotting the proteins onto the glass slides and, as mentioned, this technique does not allow for a precise deposition of the sample. It was decided to reproduce the previous experiments using a robotic spotter. Other than having qualitative advantages, the use of such a robot would allow us to

create titered arrays where different conditions, such as sample concentration and volume deposited on each spot, could be tested simultaneously. It is necessary to stress how the spotting hardware and protocols play a major role for the quality of the results to be expected. Using low quality spotting pins and non-optimal climatic conditions (air humidity < 36% or >50%; temperature < 30^{0}C) could lead to loss of sample functionality. These initial experiments were carried on an old GMS417 spotter, mounted with a pin-and-ring printing head. Depending on pin straightness, fluid viscosity and climatic conditions, more or less sample will be uploaded/printed thus leading to potential losses or increases of spot intensities.

A new antigen chip was produced, where the same antigens used for the prior experiments would be immobilized onto the slide at different concentrations (0.1 and 0.05 mg.ml^{-1}). Additionally, each sample was spotted in three distinct sets, where the pin dispensing the solution would hit the same location 1, 3 and 5 times. The aim of this layout was to determine the optimal spotting conditions in terms of sample amounts. Table 6 illustrates the layout of this chip setup.

MAP3K2-GST	IκBα	MAP3K3-GST	p53	IκBα-His	Fractalkine	Protein A	GST-IκBα-NT	Mip1α	IκBα-Biot	IκBβ	Mip1α-FLAG	Protein G	Fract-FLAG	
1 hit 3hits 5hits							1 hit 3hits 5hits							0.1 mg/ml
1 hit 3 hits 5 hits							1 hit 3 hits 5 hits							0.05 mg/ml

Table 6. Layout of the robotically spotted antigen microarray
Samples were spotted at decreasing concentrations (0.1 and 0.05 mg.ml^{-1} and with increasing volumes (1x 3x 5x the normal spot volume onto each spot)

The chip was hybridised with 40 μg of differentially labelled antibodies (Cy3: anti-GST, anti-p53, anti-Fractalkine, anti-IκBα-H4; Cy5: anti-IκBα, anti-HisTag, anti-Mip1α, anti-IκBβ, anti-FLAG). Similarly, we produced an antibody array, where the antibodies were

spotted at different concentrations (0.5, 0.25, 0.1, 0.05 and 0.01 mg.ml^{-1}) and number of hits (1, 3 and 5) (Table 7). The chip was hybridized with 40 μl of a solution containing labelled tagged and non-tagged antigens (Cy3 and Cy5 respectively). Furthermore, Protein G and Protein A were also added to the Cy3 and Cy5 mixtures, as controls for the immobilized hIgG and ant-Protein A antibody.

Figure 6 shows the scanned imaged of the antigen microarray. As expected, both Protein A and Protein G did detect and bind the IgG antibodies. The results for the detection of the immobilized antigens showed a good overall specificity. However, some expected interaction were only detected under precise spotting conditions, such as the GST-IκBα-NT interaction with the anti-GST (or anti-IκBα-NT) antibody which was only seen when more than 0.25 μg of antigen was immobilized onto the slide (3 x 0.1 and 5 x 0.05 mg.ml^{-1}). On the other hand, some specific interactions were seen when the amounts of antigen were superior to 0.5 μg (Mip1α and IκBα). Some of the predicted interactions were however not detected. For example, IκBα and His-IκBαshould have been detected by antibodies present in both labelled solutions, thus generating a yellow signal. However, we can only see a red signal being generated. This could be caused by an improper presentation of the immobilised eptiope for the monoclonal anti-IκBα-H4 anitbody (Cy3 labelled). Alternatively, that particular monoclonal might not be compatible with such an assay format. The first cause can be used as an explanation for the lack of signal generated from the anti-FLAG antibody, since this particular reagent failed to generate results in the previous "hand-spotted" antigen microarrays. When immobilised onto the array (Figure 4 and 7), this antibody seems to be able to detect FLAG containing proteins, suggesting that the problem is arising from the spotted antigens. Finally, as previously mentionned, some of the missing signals might be caused by non-optimal spotting conditions, thus unavailability of immobilised samples.

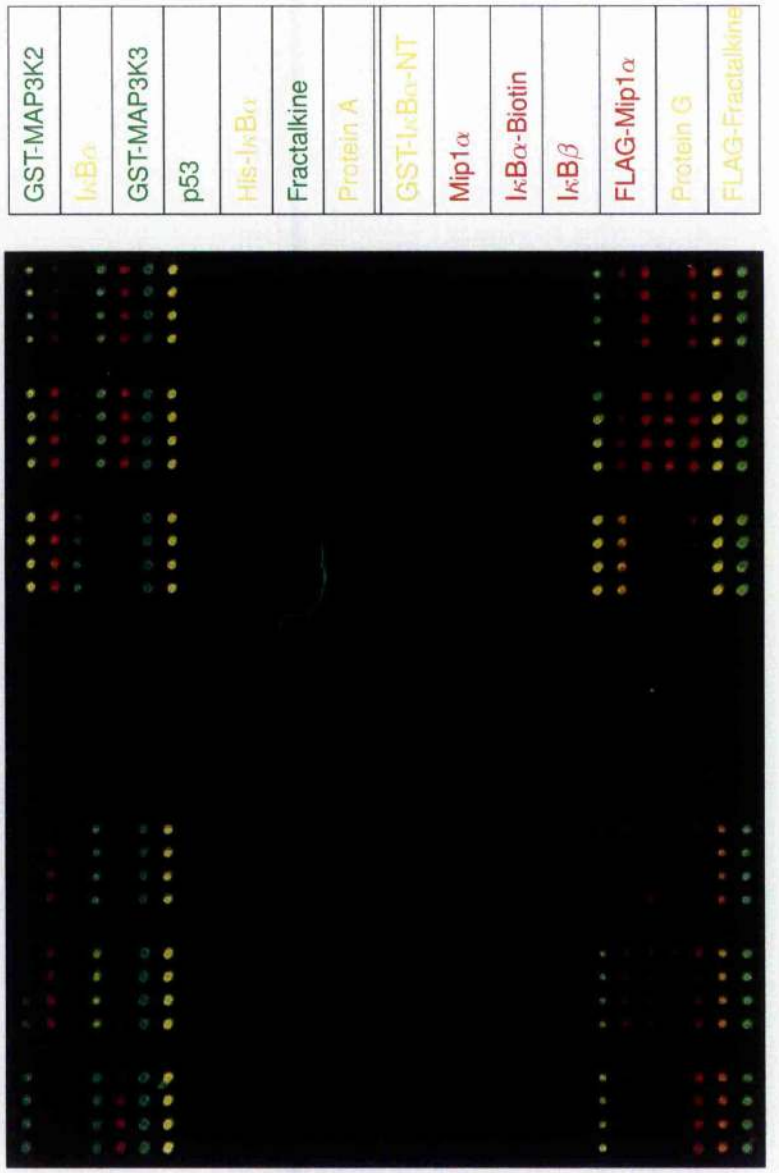

Figure 6. GMS spotted antigen microarray
Pictorial result of the antigen microarray. The top table gives an overview of the expected results

Figure 7, shows the scanned imaged of the antibody microarray. The immobilized hIgG showed a positive binding to both Protein A and Protein G (yellow signal) and the anti-Protein A antibody showed a specific binding to Protein A only (red signal). As for the antigen microarray, a good correlation with the expected results (Table in Figure 7) could be observed. Furthermore, we could appreciate the effect of the different spotting conditions for each immobilized antibody. Several of the samples can only be

detected after their amounts on the slide are higher than specific thresholds, which are different from sample to sample. However, like for the previous experiment, some of the expected dual-binding signals were not detected. In this case, the most plausible cause for this lack of signal could be the labelling of the antigens. The molecules used to label the proteins could be interfering with the epitopes recognised by the immobilised antibodies, thus dramatically lowering the affinity of the antigen-antibody recognition or, most probably, inhibiting such interactions.

We could then conclude that both antigens and antibodies could be specifically detected after immobilization and drying on a chemically modified glass surface. However, it appeared that spotting concentration plays a major role, especially for antibodies. Antibodies should also be tested for microarray compatibility before usage. As previously discussed, some of the tested samples did not produce the expected signals and this could be caused by problems in epitope presentation, compatibility with this assay format or, possibly, by the blocking of binding sites by fluorophore molecules.

Block 1a	Block1b	Block 1c	Block 2a	Block 2b	Block 2c	Hits
0.5	0.1	0.01	0.5	0.1	0.01	1
						3
						5
0.25	0.05		0.25	0.05		1
						3
						5

Table 7. GMS spotted antibody chip layout
Samples were spotted at decreasing concentrations (from 0.5 to 0.01 mg.ml) and with increasing volumes (1 - 5 hits per spot). Block 1 contains anti IκBα-H4, FLAG, Fractalkine, hIgG and Mip1α antibodies and Block 2 contains IκBβ, IκBα, GST, Protein A and HisTag antibodies.

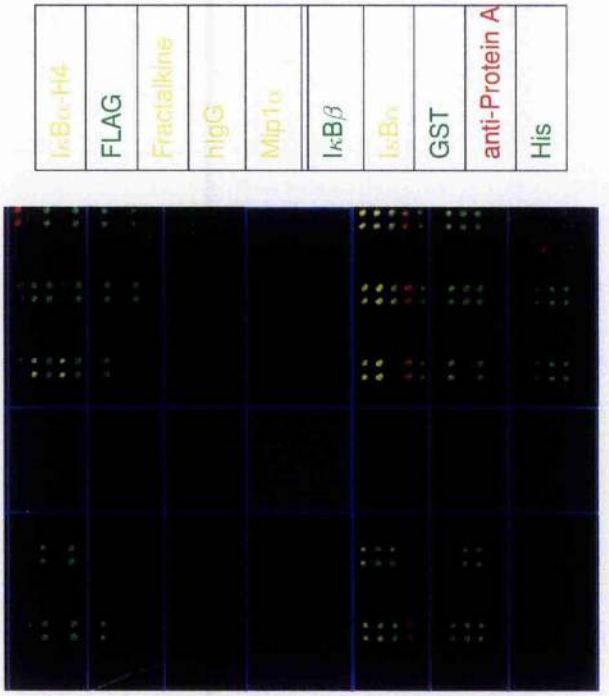

Figure 7. GMS spotted antibody microarray
Pictorial result of the antibody microarray. The top table gives an overview of the expected results. Antibodies were spotted at different conditions (see previous table) These different areas are separated by blue delimiters.

4.1.4 Do proteins maintain a correct folding after immobilization?

Having determined the basic protocols for the immobilization and detection of proteins onto glass slides, we needed to better assess the stability of proteins after their attach-

ment. All the previous experiments were done using antigen/antibody pairs. Antibodies are very stable proteins and can recognise linear epitopes resulting from unfolded proteins. This characteristic of antibodies could not allow us to make conclusions about the stability of the attached proteins.

To answer this question we used a combination of two proteins, TAP and p15. TAP is a mRNA transporting protein, which requires dimerization with p15 in order to be functional (Fribourg et al., 2001). We obtained purified TAP and p15, together with a mutant ΔTAP which lacked the p15 binding domain (Izaurralde, EMBL). All three proteins were spotted, together with Protein G (positive control). The microarray was then hybridised with a mixture of Cy3-p15, Cy5-TAP and Cy5-hIgG. If the spotted proteins are structurally stable, we should obtain a specific green signal on the immobilized TAP, a red signal for p15 and Protein G and no signal for the ΔTAP protein (no binding to p15). Figure 8 shows the result of this experiment.

Figure 8. Protein-protein interaction study: TAP/p15 binding analysis
TAP (green box), p15 (yellow box) and ΔTAP (blue box) were immobilised onto the slide, together with Protein G (positive control, red box). Pre-labelled TAP and IgG bind to their partners, p15 and Protein G. Pre-labelled p15 binds to the immobilised TAP but not to ΔTAP. This results shows that proteins can maintain a correct folding after immobilisation onto a glass slide

TAP and p15 are able to detect and bind their specific partners, while there is no signal (detection) from the immobilized ΔTAP. This showed that the results were obtained from a correct folding of the immobilized samples. If the immobilized proteins would have lost their structural features, these TAP/p15 interactions would not have been possible. We could therefore conclude that proteins were structurally stable once immobilized and dried onto a glass surface.

With these experiments, we had shown that we were able to produce functional protein microarrays. We were able to specifically detect antibody/antigen, antigen/antibody and protein/protein interactions in a solid phase experiment. Having determined the

feasibility of protein microarrays, we could start designing and executing experiments based on this technology.

4.2 HybriChips

4.2.1 Design of a monoclonal antibodies screening platform

After having considered several screening strategies (see Section 6.2), we decided to design a novel method, in which a library of hybridoma supernatants (HSs) would be spotted onto a layer of purified antigen, homogeneously attached onto a chemically modified slide (Figure 9 A). These arrayed spotted HSs would be hybridised with a mixture containing Cy3 labelled anti-mouse IgG and Cy5 labelled anti-mouse IgM (Figure 9 B). This system would allow for the detection of those hybridomas expressing the specific anti-antigen monoclonal antibodies, together with a direct isotyping of these detected antibodies (Figure 9 C).

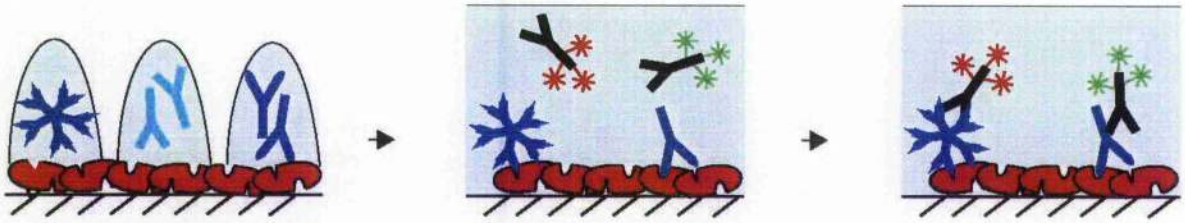

Figure 9. Hybridoma screening strategy
A - A chemically modified slide is homogeneously coated with purified antigen. Hyrbidoma supernatants are succesively spotted onto this antigen layer.
B - The microarray containing the spotted hybridoma supernatants is hybridised with a solution containing Cy3 labelled anti-mouse IgG and Cy5 labelled anti-mouse IgM.
C - Isotype specific signals (green: IgG, red: IgM) are detected from the spots presenting hybridoma supernatants containing monoclonal antibodies specific for the coated antigen

4.2.2 Coating of slide with antigen

The first step for validating our approach, was to test whether the coating of a slide with a homogeneous layer of antigen could be done. 20 μl of PBS containing 3.2 μg of Cy5 pre-labelled IκBα, were dispensed onto an aminosilane modified glass slide. After a 60' incubation at RT in a humid chamber, the slide was dried and scanned (Figure 10 A). The slide was subsequently blocked in a 3% Skimmed Milk solution

in PBS-T, washed 2 x 5' in PBS-T and scanned again (Figure 10 B). Following this blocking step, the slide was extensively washed and scanned for a final time (Figure 10 C). The three resulting images were analysed in order to determine the amounts of antigen lost and retained onto the slide surface after blocking and washing. From the data analysis (Figure 11), we can observe that even though a large amount of material is lost following the washing and blocking steps, there is a good retention of material onto the slide. However, it is not possible to absolutely confirm this observation since, because of the various scanning procedures, the fluorophore used for this experiment might have been subjected to intensive laser-induced photobleaching, thus reducing the signal intensity of the coated surface. Another important observation from this experiment is the apparent lack of coating uniformity. It is quite evident that there is more signal emitted from the central area of the slide, compared to the peripheral regions. This factor might result in a increased background signal and, possibly, will lower the selection stringency in that central area. This is due by the higher number of antigen molecules present in the central area, which allows for a higher number of epitopes to be presented to the screened antibodies, thus lowering the required affinity for proper binding to occur. It could be also possible that the increased signal in the central part of the slide is due by the free fluorophore molecules present in the antigen solution. Considering a normal tendency for peripheral evaporation, an increasing concentration of soluble fluorophores should be found in the liquid phase present in the less exposed area of the slide.

However, it appears quite clearly that it is indeed possible to coat a microarray slide with a layer of purified antigen. This allowed us to proceed with the establishment of this novel hybridoma screening platform.

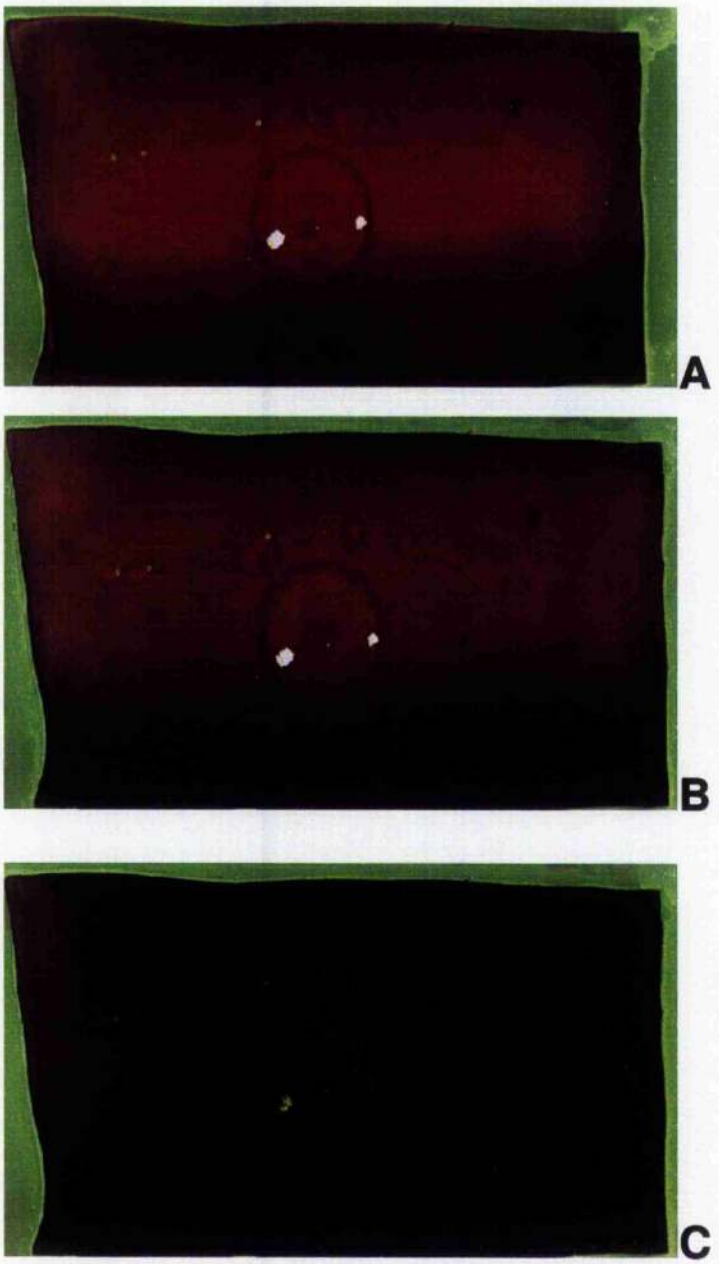

Figure 10. Slide coating with homogeneous layer of antigen
A) Initial coating of a slide: an aminosilane modified slide was overlayed with a homogeneous and Cy5 labelled IκBα solution, incubated for 60', dried and scanned
B) Determination of the amounts of antigen available to the hybridoma supernatants prior to spotting: the slide was washed in PBS-T, blocked with a 3% skimmed milked solution in PBS, dried and scanned
C) Amounts of antigen retained after the full processing of the microarray: after a 60' wash in PBS-T, the slide was scanned for a final time
Coating area was delimited by a hydrophobic barrier, applied with a hydrophobic marker (PapPen)

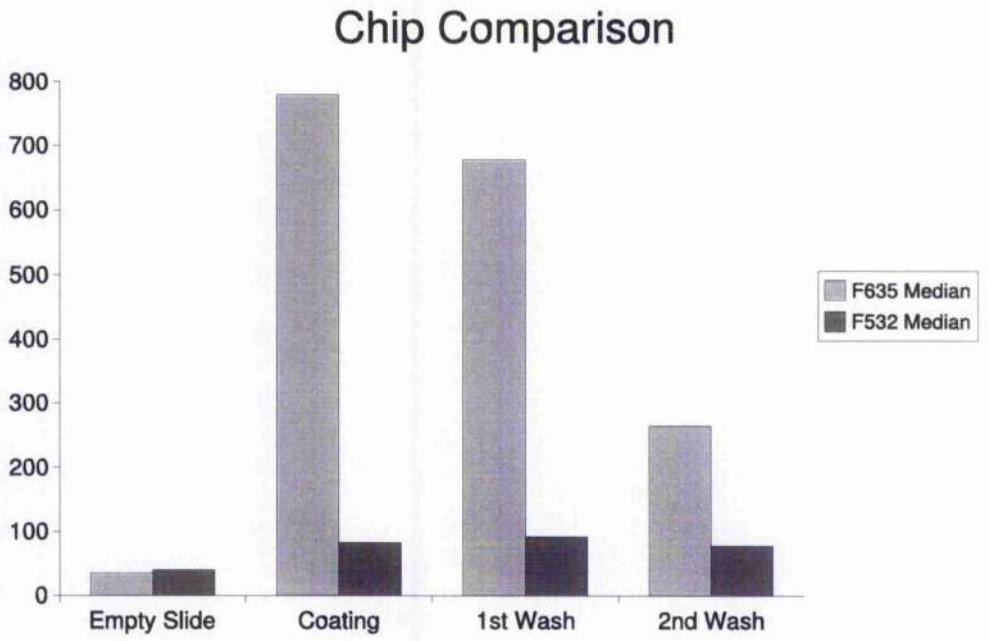

Figure 11. Chip coating experiment: data analysis
Each chip was analysed using the GenePix 3.0 software. For each slide, we retrieved the median of intensities for each channel. The histograms show the data relative to each step of the experiment. The loss of signal after the washing steps is quite evident on the Cy5 channel (F635), while there is no major change for the Cy3 channel (F532).
Empty slide: no antigen coating; Coating: antigen dispensed, no wash; 1st Wash: chip analysed after blocking and 2x5' min wash procedure; 2nd Wash: chip washed 3x10' in PBS-T, 3x10' in PBS, 1x10' in ddH2O.

4.2.3 Detection of Coated Protein by Antibodies

Having determined that we could uniformly coat a slide with an antigen, the next step was to test whether it would be possible to detect monoclonal antibodies recognising and binding to the antigen layer.

We tested this approach using an antigen-antibody pair, which had been determined to be functional on a protein microarray experiment (see section 4.1.2). We coated a slide with 3.2 μg of IκBα as previously described, and blocked the chip with 3% Milk in PBS-T. After drying of the chips, a Cy5 labelled rabbit polyclonal antibody specific for the C terminus of IκBα (IκBα-H4) was manually spotted onto the the coated slide.

A mixture of Cy3 labelled antibodies[1] was similarly spotted onto the same slide, to act as negative controls. Figure 12 A shows that both samples are clearly visible after spotting, with the red and green signal showing the anti-IκBα antibody and the negative control mixture respectively. Figure 12 B shows that only the signal from the anti-IκBα is detected after washing of the slide.

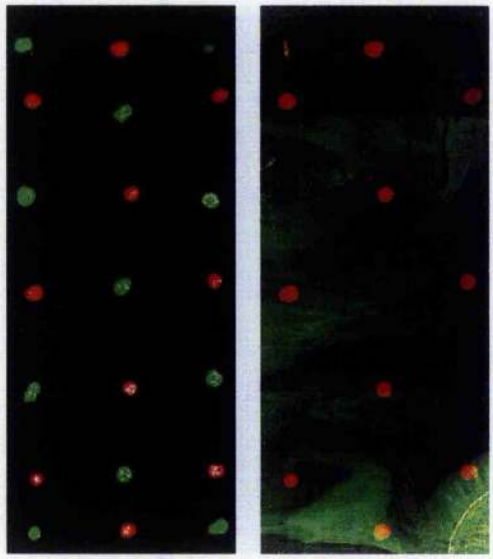

Figure 12. Detection of specific antibodies on an antigen coated slide
Cy5 labelled anti-IκBα and Cy3 labelled monoclonal anti-GST, polyclonal antibodies anti-Mip1$\alpha$and anti-Fractalkine were spotted onto an IκBα coated slide.
A - Scanned chip before washing: all spotted samples are visible
B - Scanned chip after washing: only the Cy5 labelled anti-IκBα antibody is detected. In order to avoid the appearance of false-negative results for the negative control spots, the scanning parameters were modified at run-time. The the scanning procedure was initiated using the same parameters as of figure A (laser: 100%, PMT: 450V) and terminated with a PMT value of 900V. This resulted in a clearly visible increase of background for the second half of the chip

Having obtained a specific detection of the attached antigen to the slide (no negative controls were detected after processing of the slide), we had to address whether this observation could be reproduced using hybridoma supernatants (HS - unpurified antibody) and whether this could be done by using am automated spotter, thus detecting sub-picoliter quantities of antibody. The EMBL's Monoclonal Antibodies Core Facility (MACF), with which this project was carried out, provided nine hybridoma lines secret-

[1] monoclonal anti-GST (mouse), polyclonal antibodies anti-Mip1α and anti-Fractalkine (goat)

ing monoclonal antibodies raised against human TPX2 protein. We coated a glass slide with purified TPX2 (1 μg) and the nine HSs were spotted onto this slide. A set of negative controls, consisting of monoclonal antibodies against IκBα and p52 together with 20% FBS in DMEM (negative control for the culture medium) was spotted onto this TPX2 layer. Figure 13 shows that following hybridisation with a labelled anti-mouse IgG antibody (all spotted antibodies were mouse IgGs), only the anti-TPX2 hybridoma supernatants were detected. No signal originated from the negative controls. This demonstrates that the system is able to specifically detect only those antibodies that were raised against the coated antigen.

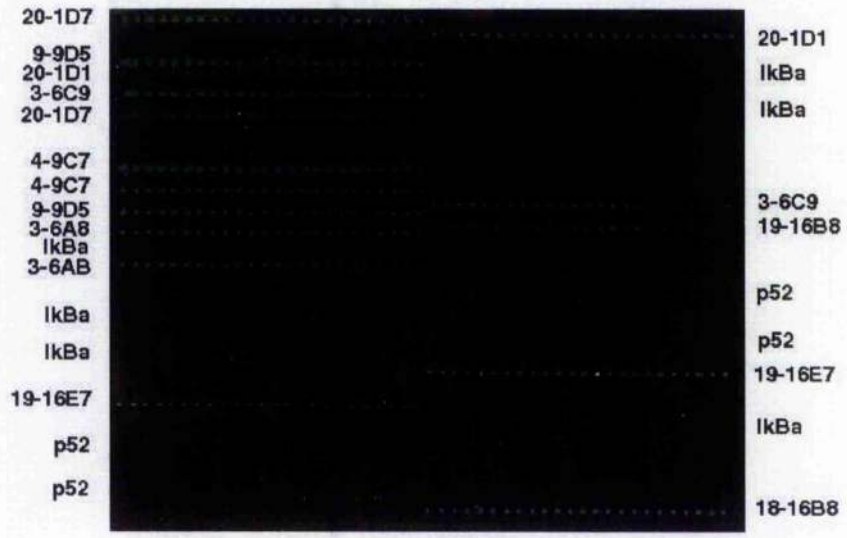

Figure 13. Detection of characterised monoclonal antibodies on an antigen coated slide
Nine hybridoma supernatants raised against hTPX2 were spotted onto a TPX2 coated slide. Two mAbs specific for IκBα and p52, together with DMEM, where used as negative controls. After incubation with the spotted antibodies, the slide was washed and hybridised with a Cy3 labelled anti-mouse IgG secondary antibody (1:1000, vol:vol). There is a clear signal from all spoted anti-TPX2 monoclonals, while no signal is detected from the negative controls

4.2.4 Screening of Hybridoma Library (1 antigen)

We were then able to show that we could coat a slide with a homogeneous solution of

antigen and that we could spot and selectively detect monoclonal antibodies against that antigen. However, the latter experiment was carried out with previously characterised hybridomas and known negative controls. We needed to test whether this system could be used to specifically and quickly detect and select specific antibodies from a library containing thousands of uncharacterised hybridomas. While testing five different immunization strategies, the MACF generated a library of 9600 hybridomas raised against TPX2. This library was tested against a TPX2 coated slide, using the protocols established in the previously described experiment (section 4.2.3). The nine positive hybridomas used for the first test were spiked into this library in locations unknown to me (blind positive controls). To allow for a direct isotyping of the detected monoclonal antibodies, the spotted HSs were hybridised with a solution containing both Cy3 and Cy5 conjugated anti-mouse IgG and IgM antibodies. To obtain a solid set of data to perform statistical analysis on, the supernatants were spotted singly, onto three antigen coated slides. This was necessary since it was not possible to spot in triplicate such a high amount of samples onto a single slide. 60 positive HSs, of which 45 IgG and 15 IgM, were selected form the analysed arrays (Figure 14). These selected HSs, together with randomly selected chip-negative samples, were tested by a conventional ELISA screen, in order to confirm their positive recognition of TPX2.

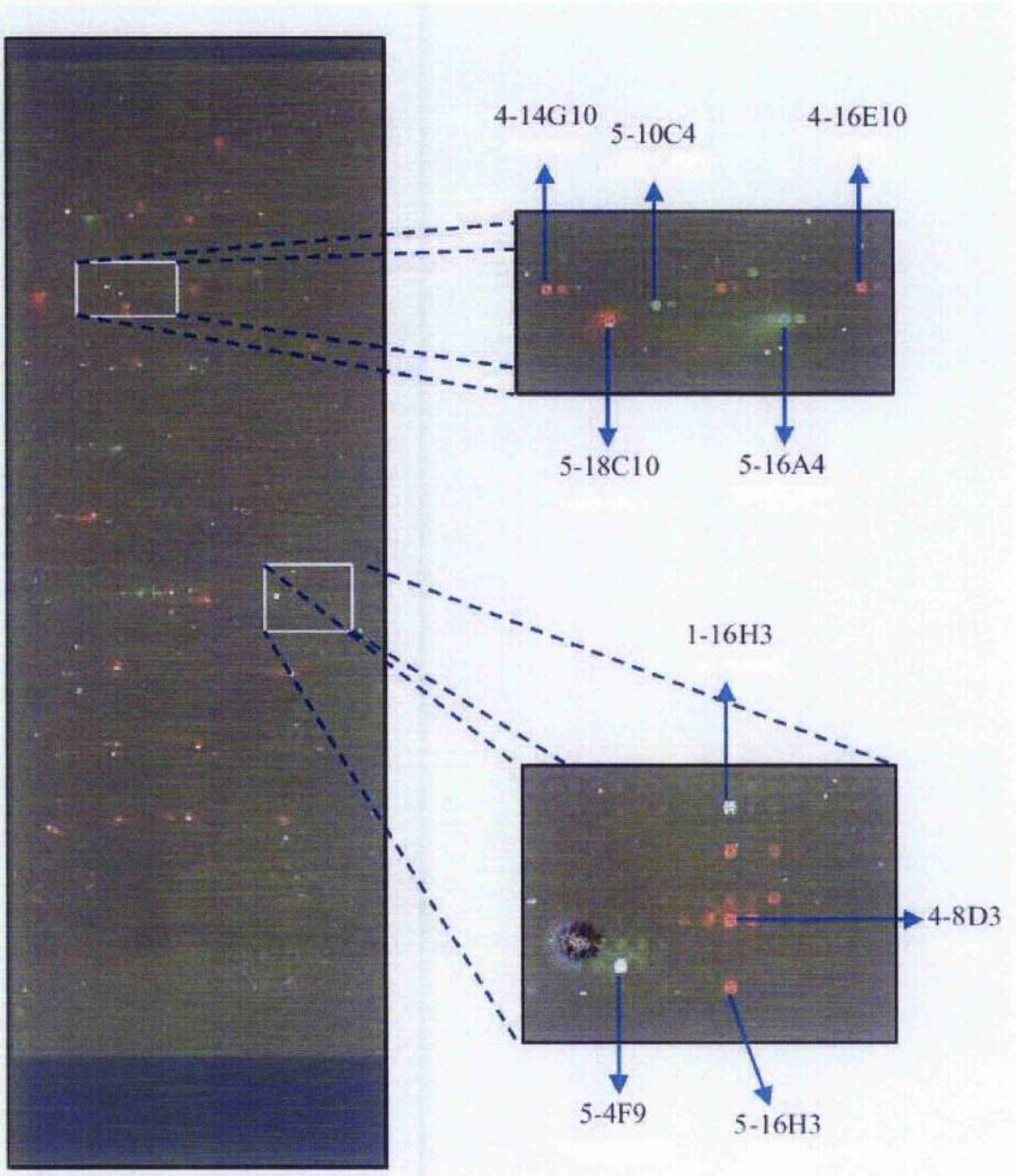

Figure 14. Screening of 5 TPX2 fusions:by antigen coated microarrays
Hybridoma supernatants from five fusions generated from mice immunized against TPX2 were spotted onto a TPX2 coated slide. The hybridoma supernatants were hybdridised with a mxture of Cy3 and Cy5 labelled anti-mouse IgG and IgM respectively.
Close-ups show magnified areas of the microarray were antibodes have been detected and directly isotyped (IgG: Green signal - IgM: Red signal). Blue arrows point at the hybridoma's ID

The ELISA confirmed the positivity of the IgG and IgM monoclonal antibodies detected with the microarray screen and showed that all nine "blind" positive controls had been

detected by the assay (Figure 15).

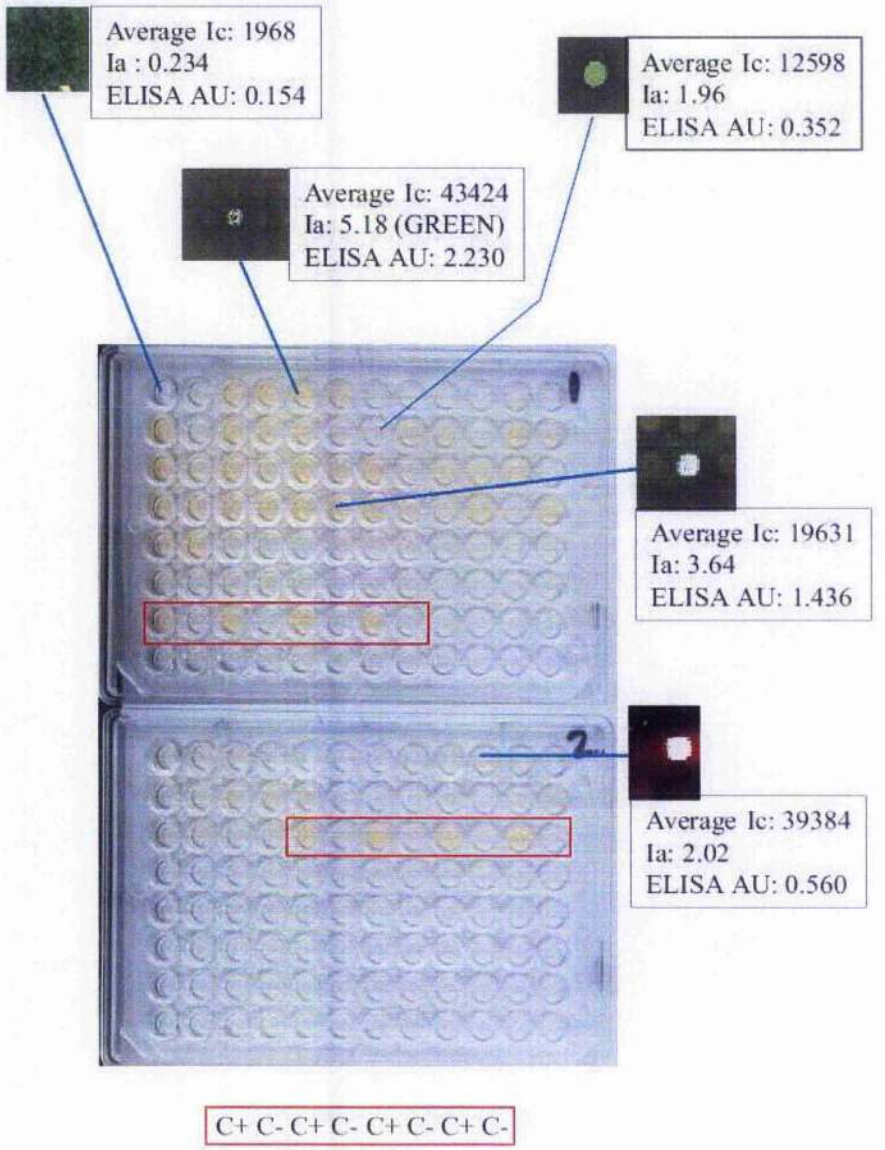

Figure 15. ELISA screening of chip-identified hybridomas
IgG (plate 1) and IgM (plate 2) monoclonal antibodies detected by the chip analysis were tested by ELISA. Of the 45 slected IgGs, 9 were identified as being the "blind positive controls" spiked into the spotting plates and were used as positive controls for the ELISA. Bound areas in the ELISA plates represent positive (C+) and negative (C-) controls. Microarray image of a negative and four positive samples are also shown. "Average Ia" and "Ic" values are explained in Section 5.1.1

4.2.5 Screening of library of antibodies raised against more antigens

We had demonstrated that we could use an antigen coated microarray based screen to analyse a extensive number of hybridomas in a fast and specific manner. However, since the goal of the project was to generate a high throughput method for the generation of monoclonal antibodies, a fundamental point of this was the possibility of using multiplexing immunization protocols (several distinct antigens injected in a single animal) combined with a highly selective screening method. We therefore needed to test whether our screening strategy could be used to detect and select specific samples from a library of hybridomas generated from a multiple antigen immunization. An experiment was designed where a single mouse was immunized with two unrelated antigens: *Xenopus laevis* γ-Tubulin (C-terminal 15 amino acids - AATRPDYISWGTQDK) and the Ket94 domain of *Drosophila megalonaster* muscle protein Titin (Ket94, 23kDa protein). The resulting hybridoma library was spotted onto two separate chips, coated with Ket94 and γ-Tub.

The analysis of the two microarrays was initially done visually and subsequently repeated using a method based on the mathematical analysis of the intensities of each spot. We used a simple algorithm, which determines the relative contribution of each spot to the total intensity if each chip. From the raw data, we calculate the average intensity of each set of spot replicates, and each value is divided by the total intensity of the chip. The resulting value represents the percentile contribution of that particular sample to the complete chip (Equation 1, in section 5.1.1).

From the visual analysis of the microarray results, 8 and 4 positive hybridomas for Ket94 and γ-Tubulin were selected. These selected hybridomas were further tested by Western blot analysis (Figure 16) and by ELISA (not shown), where the selected samples were proven to be positive. This showed that we could discriminate between hybridomas secreting monoclonal antibodies against different antigens from a unique library. Interestingly, the same samples were selected when using the mathematical

approach, which suggested that we could implement such a numerical approach for the selection of positive hybridomas.

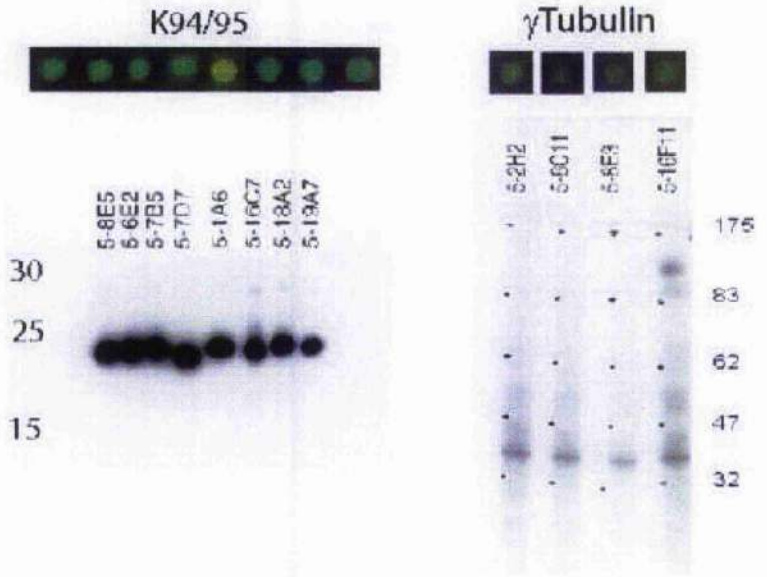

Figure 16. Western blot analysis of selected monoclonal antibodies
The selected monoclonal antibodies against Ket94 and γTubulin were tested by Western blot against a purified Ket94 antigen and the γTubulin's C-terminal 15 amino acids peptide, expressed with a GST-tag. All samples were positive in this analysis

In parallel with the ELISA and the Western blot analyses, the anti-Ket94 antibodies were screened by immuno-gold labelling on a Drosophila muscle fibre (Kevin Leonard and Sigrun Brendel, EMBL) and the γ-Tubulin hybridomas were screened by immunofluorescence on Xenopus XL177 cells (with the help of Andrei Popov, EMBL). These tests showed that only one Ket94 sample was positive on the immuno-gold labelling experiment, and two of the anti- γ-Tubulin antibodies gave a signal in the IF test (methanol fixation). These experiments showed that some of the samples selected via the microarray screen were able to detect their antigen in experiments where it is not purified and in a un-denatured, cellular environment (Figure 17).

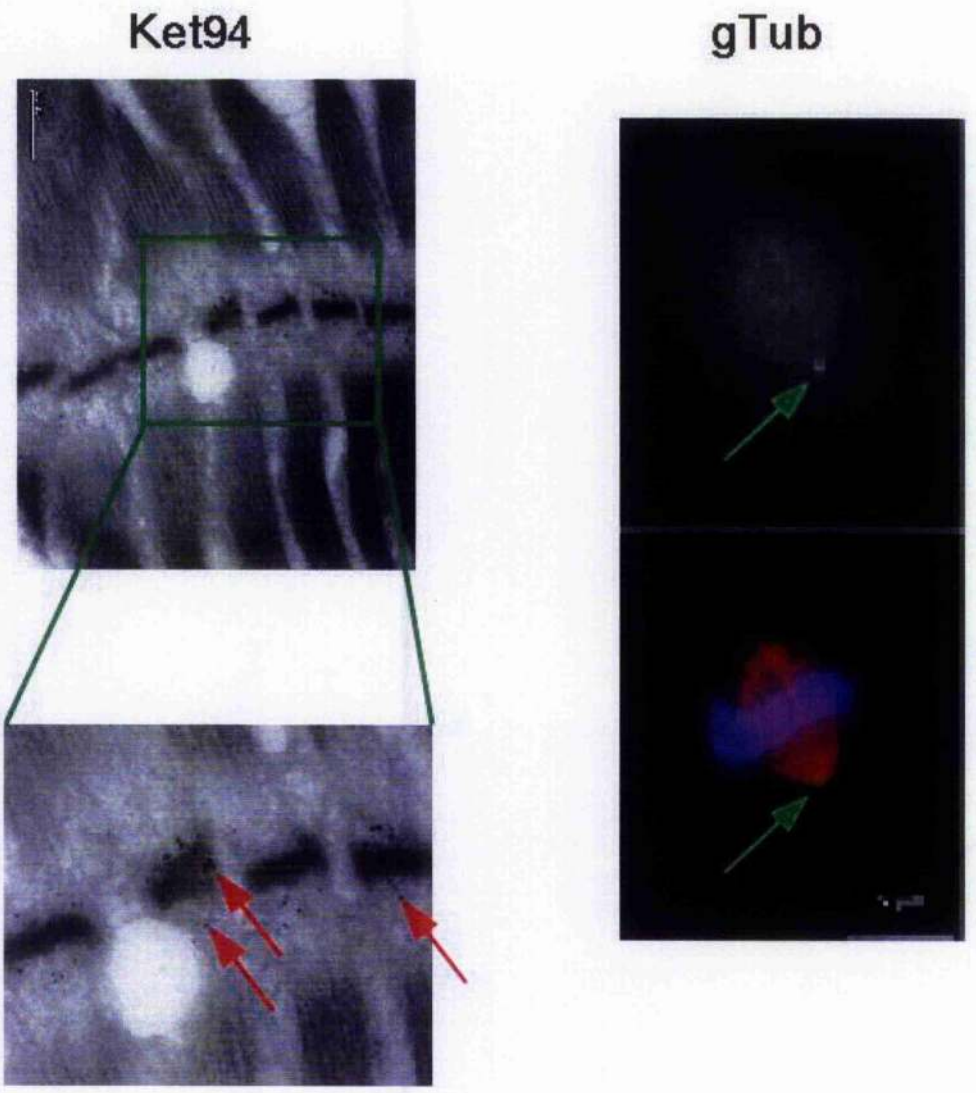

Figure 17. Immuno-gold labelling and Immunofluorescence analyses using antibodies identified by microarray screening
Selected monoclonal antibodies against Ket94 and γTub were tested by immuno-gold labelling (electron microscopy) and immunofluorescence.
The left panel shows a Drosophila's muscle fibre, where the Ket94 regions of the large Titin proteins have been labelled by one of the selected mAb (red arrows).
The right panel presents a mitotic XL177 Xenopus cell, where the γTubulin containing centrosome has been lebelled and visualised by one of the selected mAbs (green arrow; red: αtubulin; blue: DNA)

4.2.6 Upscaling of immunization multiplexing: five antigens per fusion

However, using two antigens per animal could not ensure the throughput we were seeking for this project. We needed to test this system with a higher number of anti-

gens in one single animal. We decided to test the procedure using five antigens. The five proteins listed in Table 8 were simultaneously injected in a single animal and, after three boosts at 10 day intervals, the fusion was performed. The hybridomas created were then screened by HybriChip and non-specific antibodies were eliminated by cross-referencing microarrays on different antigen chips. The positivity of all HybriChip selected IgG antibodies was confirmed by ELISA (Table 8). This experiment showed that it was possible to immunize one single animal with up to five antigens and that the screening of the resulting hybridoma library could be specifically done using our HybriChip platform.

Antigen Name	Total No. Positive Clones (HybriChip)	IgM secretors (HybriChip)	IgG secretors (HybriChip)	IgG secretors (ELISA)
Ago2	12	7	5	5
Mago JLY	17	8	9	9
KetB4	17	7	10	10
Tcnf2	29	9	20	20
Plg2Mut4	13	10	3	3

Table 8. Summary of results for a five antigen experiment.
Monoclonals were detected and obtained for all antigens tested. ELISA screens confirmed the positivity of all the IgG isotype monoclonals selected

4.2.7 Analysis of the correlation between HybriChip and ELISA results

At this point of the project, we had established that:

- We could coat a chemically modified slide with a homogeneous layer of an antigen
- We could specifically recognise the coated antigen with spotted antibodies
- We could screen a library of hybridomas raised against multiple antigens using this approach
- By screening the hybridoma library using differentially labelled detection antibodies, we could immediately determine the isotype of all positive antibodies
- All the HybriChip positive hybridomas were positive by ELISA and/or other applications

Some questions were however raised about this final point. All antibodies that were shown to be positive by ELISA and/or other methods, had been initially selected from the HybriChip analysis. Any information relative to the HybriChip negative antibodies was unavailable, which could result in the loss of potentially ELISA or Western blot positive antibodies (false negatives in HybriChip). In order to really validate our screen, we needed to carry a complete HybriChip/ELISA correlation study, were each and every hybridoma from a library would be tested by HybriChip and by ELISA.

This was accomplished by creating a hybridoma library from the immunization of five mice with a total of 9 antigens (Table 9). Contrary to the standard procedure, we decided to minimise the initial round of cell culture by at first growing the hybridomas on 96 well plates, without ensuring monoclonality and selecting, after a first round of HybriChip and ELISA screening, the five "best" samples per antigen (mother clones). The selected mother clones would then be subjected to monoclonality by a round of subcloning, where each mother clone is plated into 96 well plates at a concentration of 0.5 cells.well^{-1} (subclones).

A library of 5376 subclones was thus obtained and each of these hybridomas was screened by ELISA and by HybriChip. After processing and analysis of the experiments, the data obtained from each screening method was compared. Antibodies showing positive results on both assay formats would be defined as "positive", antibodies positive only on the HybriChip assay would be defined as "novel". The presence of HybriChip false negatives (positive by HybriChip could be explained by several reasons:

- Firstly, this result could be caused by a critical difference in epitope-presentation on both assays, making the eptiope unaccessible for a particular antibody.
- Secondly, the signal of a HybriChip false negative on an ELISA assay could be above the minimal threshold of 0.2 OD units, thus being selected as a positive binder merely based on that fact.
- Finally, and related to the previous point, ELISA assays generate single data points per antibody screened while each HybriChip experiment generates at least three. The risk of false positivity by ELISA might be statistically higher that the risk of false negativity in HybriChip, since one single data point can not be validated, or supported, by replicate values.

Table 9 shows the result of this comparison. From a first analysis of the results, we can see a random distribution of correlation values for the whole experiment. In order to get a real meaning of these results, we need to take the values generated by each antigen on the ELISA screens. Considering that the background noise in an ELISA experiment will have numerical values <0.2 OD, it is important to make a distinction between assays generating results close to these background values and those having values well over that threshold bar. We can divide our set of antigens into three groups: one containing KetB5, Ket95, HMG CoA and His-IPAPB (ELISA OD >> 0.2), a second with CSD SAP, 4950, IPAPBmid, IPAPBpep2 (ELISA OD < 0.6) and, finally, GST Hupf having a complete set of ELISA ODs below the 0.2 background mark.

Antigen Name	Fusion ID	ELISA Positives	ELISA Value Range	Chip/ELISA Matches	IgG	IgM	Total Correlation	Novel	Chip false negatives
KetB5	540715	53	0.6052 - 1.6616	51	48	3	96.22%	4	2
Ket94	540715	15	0.7615 - 1.9997	13	13	0	86.67%	8	2
HMG CoA	1520	69	0.5094 - 1.9562	53	51	2	76.81%	0	16
CSD SAP	1520	3	0.2515 - 0.6053	2	1	1	66%	0	1
4950	540712	3	0.2508 - 0.4096	2	1	1	66%	1	1
GST-Hupf	540710	0	All < 0.2	0	0	0	100%	0	0
His-IPAPB	540710	7	0.3042 - 1.2849	6	2	4	85.71%	0	6
IPAPB Pep2	540713	2	0.4048 - 0.4188	1	0	1	50%	0	1
IPAPB Mid	540713	19	0.1927 - 0.57	11	11	0	57.89%	0	8

Table 9. Correlation between HybriChip and ELISA results
Overview of the results of this HybriChip/ELISA correlation analysis. The number of positive samples, the range of ELISA ODs, and correlation data for IgG antibodies are presented for each antigen. Novel samples are those antibodies (IgGs) which were positive by HybriChip, but were negative in the ELISA screen

For the first group of antigens, our correlation ranges from 76.81% to 96.22%. Even though the number of positives for each antigen is variable (from 7 to 69), we can appreciate that the ELISA OD values for these antigens are all over the 1.0 OD bar. On the other hand the second group of antigens, which present much lower ELISA OD values, show a lower correlation with the chip experiment, ranging from 50% to 66%. The values are near the background mark and the selection of positive samples is quite subjective. Very few samples are selected in both experiments and the chance of having IgM positives seems to be higher. It is interesting to notice how a good number of IPAPBmid positive hybridomas were selected by ELISA (19) even though their ELISA ODs were still close to the 0.2 cut-off value and yet, we isolated 11 positives by chip. We have to point out that since this antigen was a peptide, the adsorbance efficiency for this antigen might have been quite limited. Finally, we have the last antigen CSD-Hupf, which was completely negative in both the ELISA and the microarray based screening assay. We can consider that this antigen showed a 100% correlation between the ELISA and the chip screening (no false positives).

It is possible to conclude that the HybriChip results show a high correlation with a conventional ELISA screening of hybridoma libraries. However, the lower the intensities

of the ELISA screen, the lower the correlation. The dynamic range of the HybriChips is greater that for the ELISA, thus making the detection of positive samples showing ELISA intensities near background levels more difficult and subjective to be made (Figure 18).

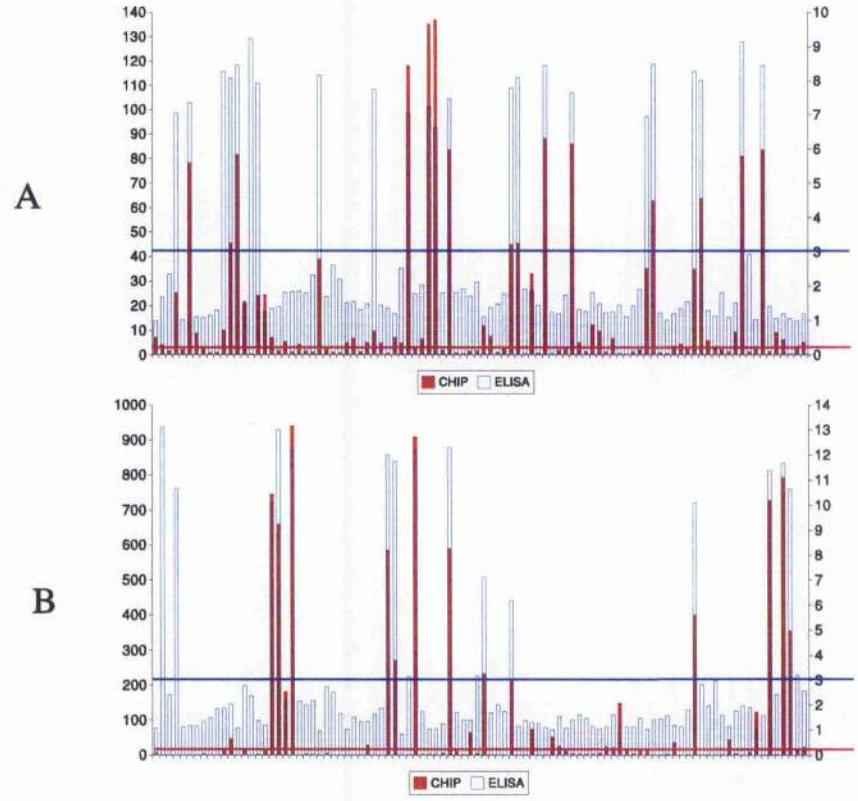

Figure 18. HybriChip/ELISA correlation: graph
Example of the correlation between HybriChip (IgG results only) and ELISA (IgG and IgM) for one KetB5 (A) and Ket94 (B) plate. Normalised values for each sample in the plates, from the chip and the ELISA analysis were plotted in a sample by sample manner. HyrbiChip and ELISA normalised values are shown on the left and right axes respectively.

4.2.8 Upscaling of the whole platform: 80 antigens trial

Having validated both the multiplexed immunization protocols and the microarray based assay, we wanted to try to scale up the platform. For this "scaling up" experiment, we immunized 8 mice with 10 antigens each (total of 80 antigens). Considering the high number of antigens injected into each mouse, it was essential to try to minimise

immuno-focussing and immuno-dominance (Harlow and Lane, 1998) and to generate a wide spectrum of immune response in the animals. Therefore, after primary immunization the animals were boosted only once on day 14, and the spleens were harvested on day 18. Fusion was robotically accomplished using standard protocols and individual hybridoma culture supernatants were subsequently harvested and screened by HybriChip. Table 10 gives a summary of the HybriChip selected clones in each fusion, together with the results of their screening by ELISA and WB.

Mouse ID	Antigens	Chip Positives	IgM Chip	IgG Chip	ELISA Positives	WB Positives
1	10	32	13	19	9	3
2	10	26	6	20	14	4
3	10	45	15	30	9	9
4	10	39	12	27	6	9
5	10	32	1	31	21	19
6	10	45	11	34	5	9
7	10	43	14	29	13	5
8	10	27	6	21	4	3
Total	*80*	*289*	*79*	*211*	*91*	*51*

Table 10. 80 antigen experiment: selected antibodies per fusion

As shown in Table 11, mAbs were raised against 67 of the 80 antigens (83%) as tested by HybriChip. Of these, IgG secreting clones were raised against 61 (77.5%) of the targets and IgM-secreting clones were raised against 32 (40%) of the target antigens. Multiple hybridomas were generated from these IgG secretors, which recognised 32 (40%) of the target antigens by HybriChip and were positive in at least one further immunoassay. Of the 61 IgG secretors raised against the 80 original antigens and identified by HybriChip, hybridomas were isolated that recognised 15 of the target antigens by both western blot and ELISA; hybridomas isolated against 10 of the targets were positive by ELISA only and hybridomas raised against 7 of the targets were positive by Western blot only. Hybridomas positive in both the IgG and IgM channels were not taken into consideration since this effect might be mainly caused by a lack of monoclonality in the source cell-culture plate. These samples are regarded as negative and

are thus not selected for expansion.

Mouse ID	Chip Positives	Chip IgM	Chip IgG	ELISA Positives	WB Positives	Chip/ELISA/WB Positives	Chip/ELISA Positives	Chip/WB Positives	Chip Only positives	Negatives	Missing WB Data
1	9	4	7	5	2	2	3	0	4	1	2
2	6	2	6	3	1	1	2	0	3	4	0
3	10	6	8	2	2	2	0	0	8	0	3
4	9	6	9	4	5	4	0	1	4	1	1
5	8	1	8	5	6	3	2	3	0	2	2
6	8	4	8	1	4	1	0	3	4	2	3
7	9	6	9	4	1	1	3	0	5	1	4
8	8	5	6	1	1	1	0	0	7	2	0
Total	*67*	*34*	*61*	*25*	*22*	*15*	*10*	*7*	*35*	*13*	*15*

Table 11. 80 antigen experiment: antigen specific data
Summary of results for each mouse. The figures represent the number of target antigens against which antibodies were raised

4.2.8.1 80 Antigens experiment: example images Figures 19, 20 and 21 show typical HybriChip results.

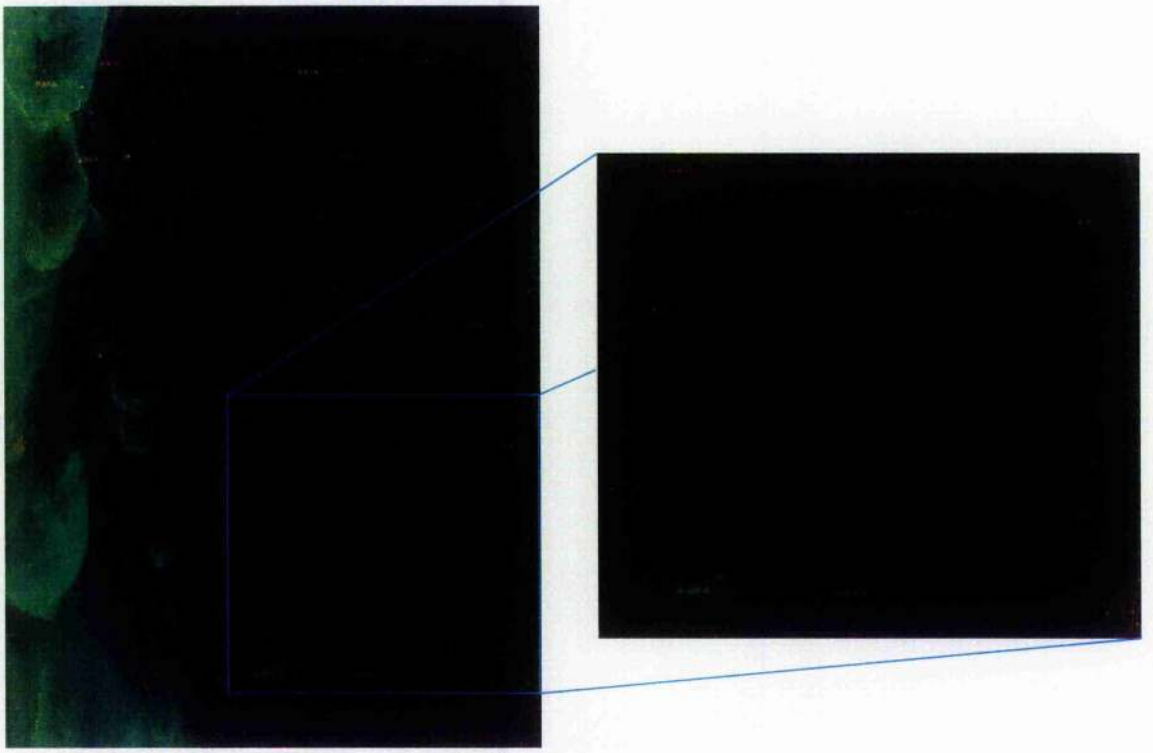

Figure 19. Antigen 1
Image resulting from the processing of antigen 1. The magnified area shows a detail of positive IgG and IgM samples

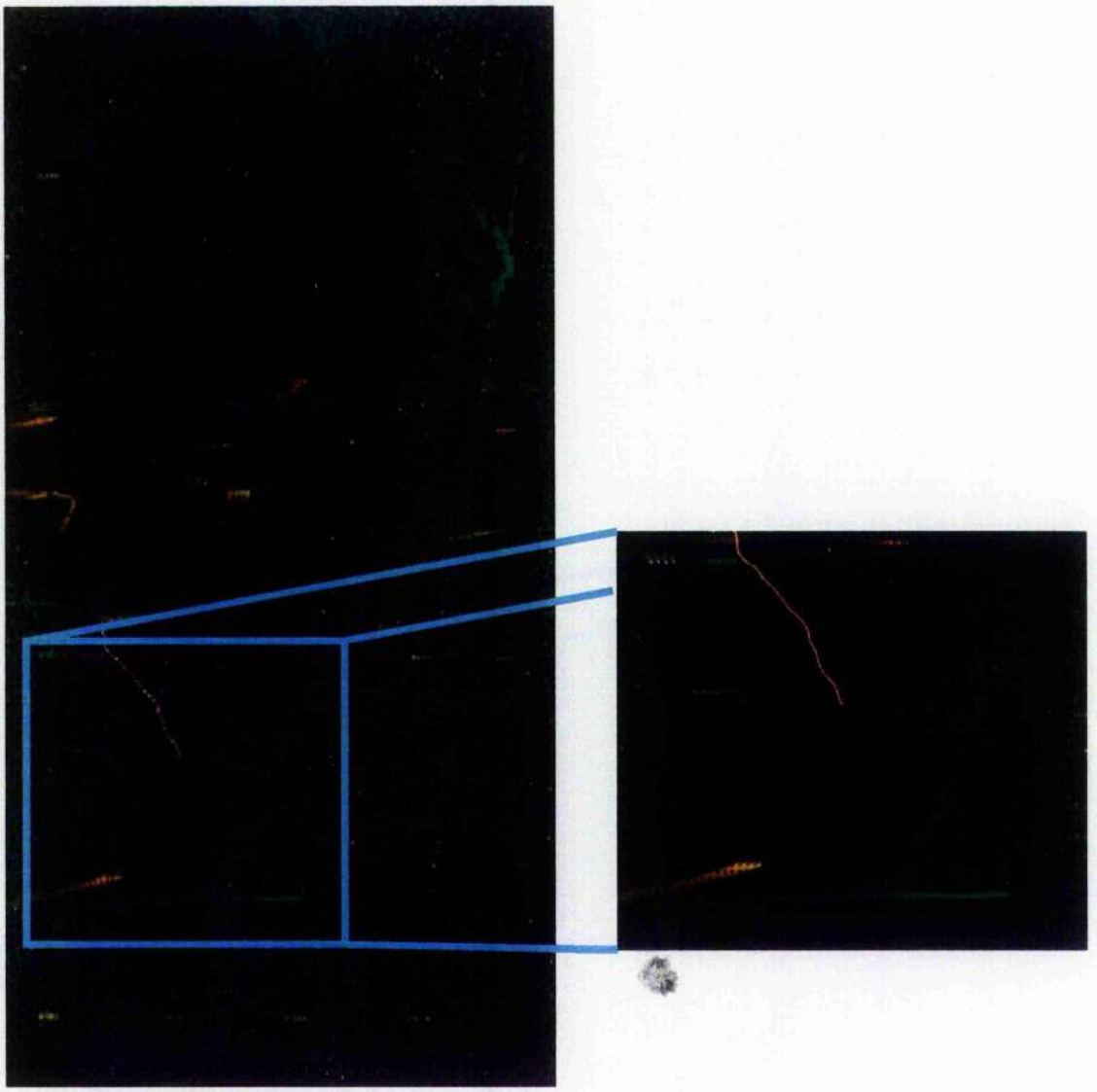

Figure 20. Antigen 2
Image resulting from the processing of antigen 1. The magnified area shows a detail of positive IgG and IgM samples

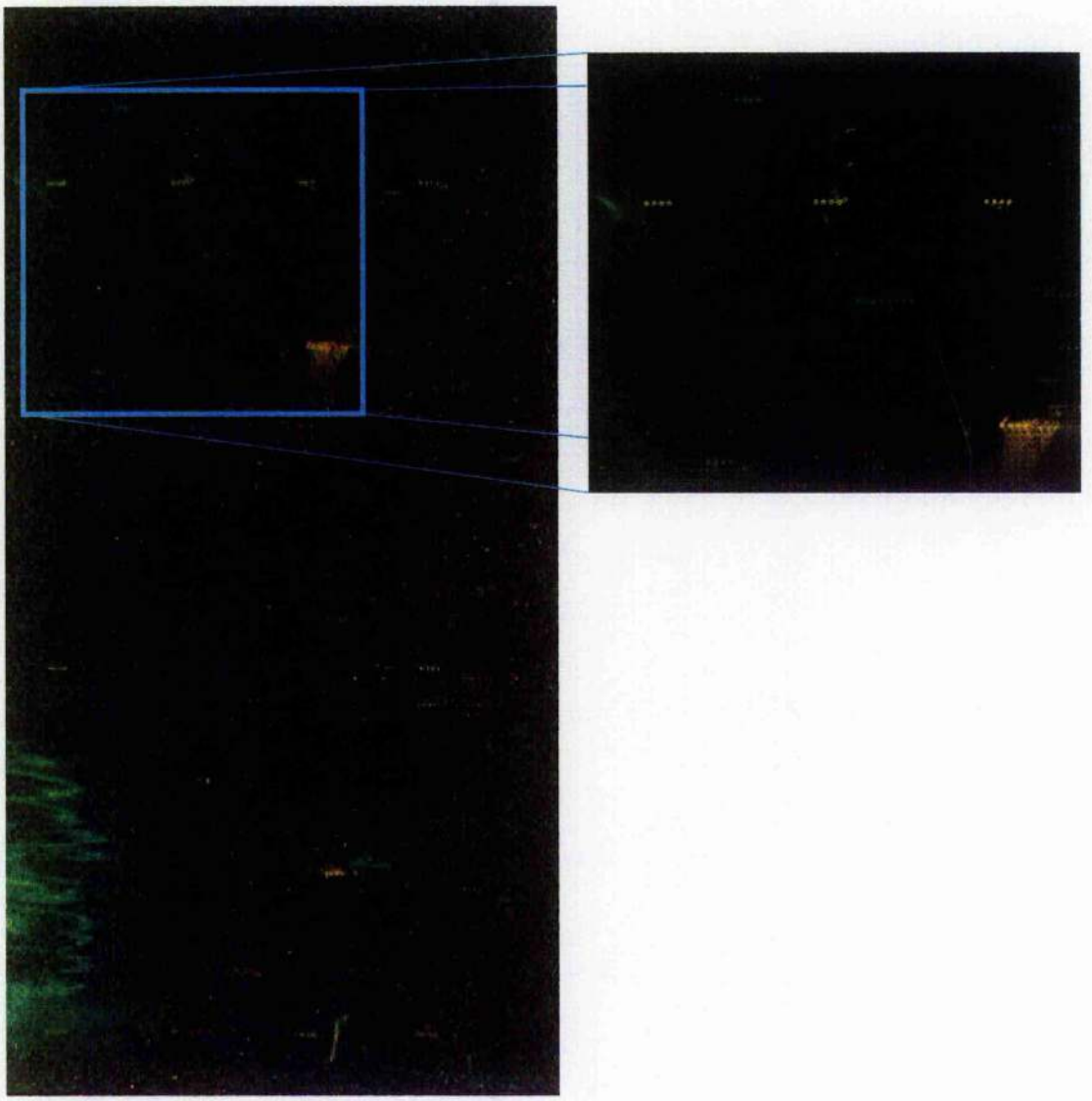

Figure 21. Antigen 3
Image resulting from the processing of antigen 1. The magnified area shows a detail of positive IgG and IgM samples

4.2.9 Determination of the most suitable chip substrate for the HybriChip platform

All the experiments were carried using "home-made" slides (Drzonek, EMBL), which presented extremely high quality features. However, these slides lacked a proper quality control and, depending on uncontrollable events such as climatic conditions and human handling factors, the uniformity of quality could not be guaranteed from batch

to batch. Having validated and upscaled the complete platform, a large scale analysis of available commercial surface chemistries was performed. EMBL slides and seven commercial chips were used for this comparison (Table 12).

All slides were treated and processed similarly. Because of their physical entrapment of the spotted samples into the substrate, the FAST and HydroGel slides did not show compatibility with our assay format. All the spots emitted a signal, and this could be caused by the impossibility of releasing the unbound hybridomas form the coating polymer. Alternatively, it is possible that the coated antigen has a low penetration into the polymer, thus making the assay un-feasible. Similarly, the eSurf slides also showed signals emitted from all spotted samples. The explanation for this outcome using these slides is still unknown. Finally, the SuperAmino and SuperAldehyde microarrays showed very disappointing results, where the background was quite high on the Cy3 channel and the spot intensities were quite low.

On the other hand, the Ansorge, PicoSlide and CodeLink slides showed interesting results (Figure 22). Based on the visual analysis of the chips, it was observed that:

- The EMBL slides had the highest spot intensity: signals from positive samples are very high compared to the other slides. The experiments could be performed with lower amounts of secondary antibodies, since it appears that with our HybriChip protocols, high affinity hybridomas create a smear of label onto the slide.

Slide	Producer	Chemistry	Interaction
Ansorge	EMBL	Aminosilane	Electrostatic/Adsorption
PicoSlides	PicoRapid	Proprietary	Covalent
CodeLink	Amersham	Proprietary	Covalent
SuperAmine	Telechem	Aminosilane	Electrostatic/Adsorption
SuperAldehyde	Telechem	Aldehyde	Electrostatic/Adsorption
FAST	Schleicher & Schuell	Nitrocellulose	Immobilisation/Trapping
HydroGel	Perkin Elmer	Polyacrylamide	Immobilisation/Trapping
eSurf	LifeLineLabs	Proprietary Polymer	Covalent

Table 12. Comparison of substrate chemistries
Properties of the different modified slides used for the selection of the most suitable solution for the HybriChip platform

- PicoSlide and EMBL slides showed very low background: this is an advantage for the identification of positive hybridomas, since a low background creates a clear division between a spot and its environment.

- CodeLink slides present the best spot geometry: while for the other two slides spots tend to present variable diameters and, in several cases, to be characterised by a "doughnut" shape, spots in the CodeLink chemistry are very consistent in diameter and shape (not shown).

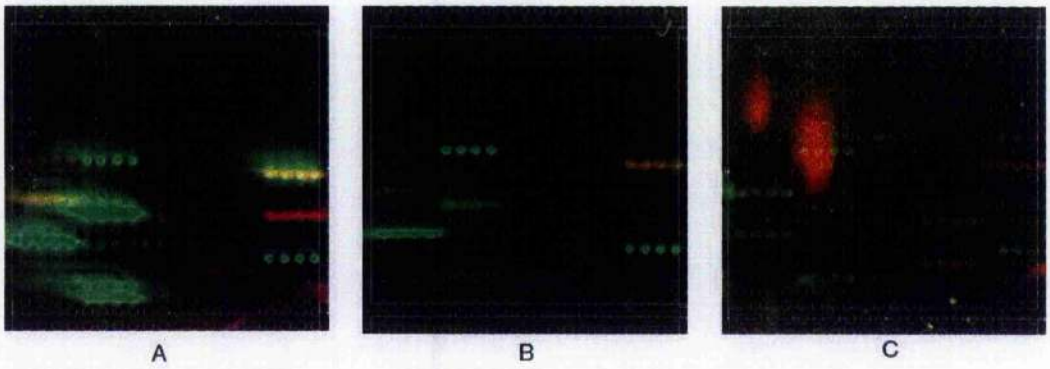

Figure 22. Comparison of substrate chemistries
All slides were coated with 5 μg of an antigen and blocked with 2.5% BSA in PBS. Supernatants were spotted onto each slide and the generated microarrays were processed with the standard protocols. Each slide was scanned using the the same laser parameters 100% power, 130 and 140 pmt gain for Cy3 and Cy5 respectively.
A - EMBL chemistry
B - PicoSlide
C - CodeLink

It was then decided to use both the EMBL and the PicoSlide chemistries for our platform, where the EMBL slides are commonly used and, for projects involving critical antigens, both solutions are used in parallel. This decision was based on economical reasons, since the Ansorge slides are obtained free of charge (or at production costs) while the PicoSlides are quite expensive (~15 €/slide).

A similar analysis had been previously performed, whereby several trimethoxysilane based substrates were determined as being the "best" solutions for arraying proteins (Kusnezow et al., 2003). It is difficult to compare both experiments, mainly because of

the assay formats. While the previous study was performed using standard antibody microarray protocols (antibodies spotted directly onto the surface), we are performing "reverse phase" antibody screening, therefore chemical interactions and properties might be different. However, we can suggest that, generally, silane based chemistries can be considered as the most suitable substrates for protein arraying.

5 Hy-CAT©

Protein microarrays are a technology arising from the well established field of DNA microarrays. Because of this, the technology used to produce DNA chips, such as surface chemistry, liquid handling robots, spotters, hybridisation tools and image acquisition software, can be easily adapted to generate and analyse protein based microarray experiments. Considering that DNA microarray analyses require several statistical methods, normalisations and clusterings to obtain relevant information about the differences in gene expression patterns, analytical software has proved to be "bulky" for the analysis of protein microarray data.

Contrary to DNA microarray experiments, not all samples on a protein microarray will generate a signal. Therefore, as a first procedure, samples not emitting signals must be removed from the data set. The remaining samples need to be analysed and selected in order to remove all false positives, low affinity species and cross reactive antibodies. This analysis procedure requires a specific set of algorithms in order to be routinely performed. We have therefore written a software application, Hybridoma Chip Analysis Tools (Hy-CAT), which is able to quickly perform all these functions and to quickly and efficiently identify all positive monoclonal antibodies in a screened hybridoma library.

5.1 The Chip analysis process

The analysis of HybriChips using the HyCAT application is divided into two steps:

1. Gathering of the general chip information and selection of normalisation and filtering parameters: via a graphical user interface (GUI), the user enters general information about the chip, such as the name of the antigen (antigenID), the fusion identifier (fusionID) and the number of replicates for each spot. Via the same GUI, the user can select the normalisation method to be used, together with the range of spot diameters to be used for the spot selection procedure.

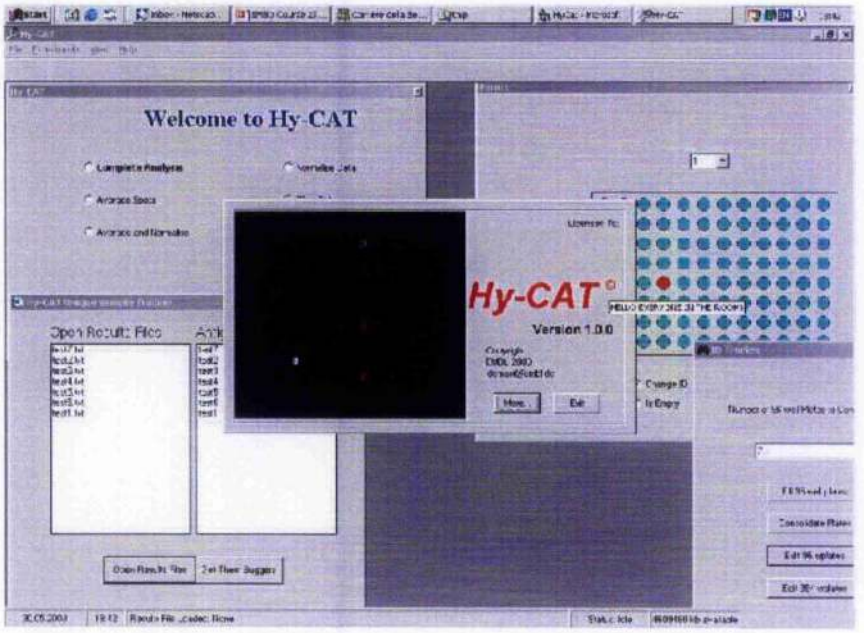

Figure 23. HyCat

2. Gathering of the chip data for analysis: using conventional microarray analysis applications, information such as "spot total intensity", "background", "ratios", "standard deviations" and other statistical data will be retrieved for each single spot, generating a massive amount of data. As previously mentioned, the analysis of HybriChips does not require all of this data, therefore a method for the selection of the relevant data for each sample is necessary. HyCAT's first procedure is to facilitate this selection, via a easy-to-use GUI. The user loads a data file into the application and selects the relevant data fields for the analysis. HyCAT will analyse the chip data using the parameters entered in point 1.

These two steps being completed, HyCAT will perform the complete analysis of the microarray data, with the following sequence of events:

- Filter-out all samples that do not fit in the diameter fork: all spots having a diameter below or above the diameter range will be removed from the analysis. Dust, scratches, heavy background can cause unspecific signals onto the slide, therefore these have to be eliminated to avoid selecting false positives.

- Normalise all replicate spots to generate a single value per sample: one single parameter per HS has to be used for the analysis
- Normalise all spots: a relevant statistical set of data has to be used for the analysis of the arrays
- Determine the distribution of spots by intensity ranges
- Graphically display this intensity distribution and prompt the use to select a filter value: a binomial distribution should be obtained from the analysis where the high-intensity peak represents the distribution of intensities from spots containing positive hybridomas. The user is prompted to enter the normalised intensity value (threshold) to be used for the retrieval of these positive hybridomas (Figure 5.1)

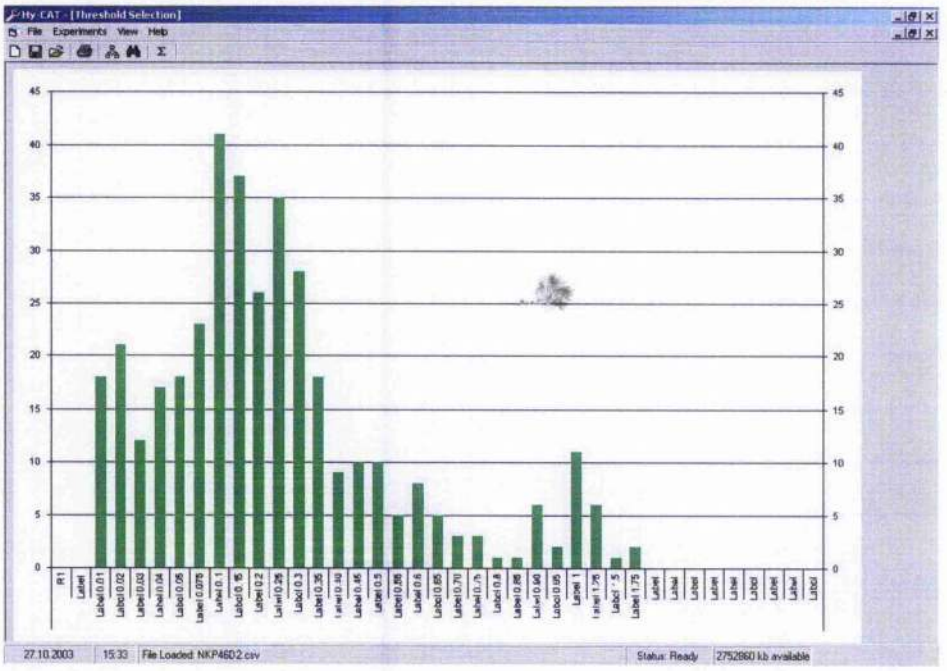

Figure 24. Distribution of intensities

- Filter-out false-positives, ie those samples belonging to the wrong fusionID: see section 5.1.2

- Retrieve and display all IgG and IgM samples having a normalised intensity value equal or greater than the user-selected threshold (Figure 25)

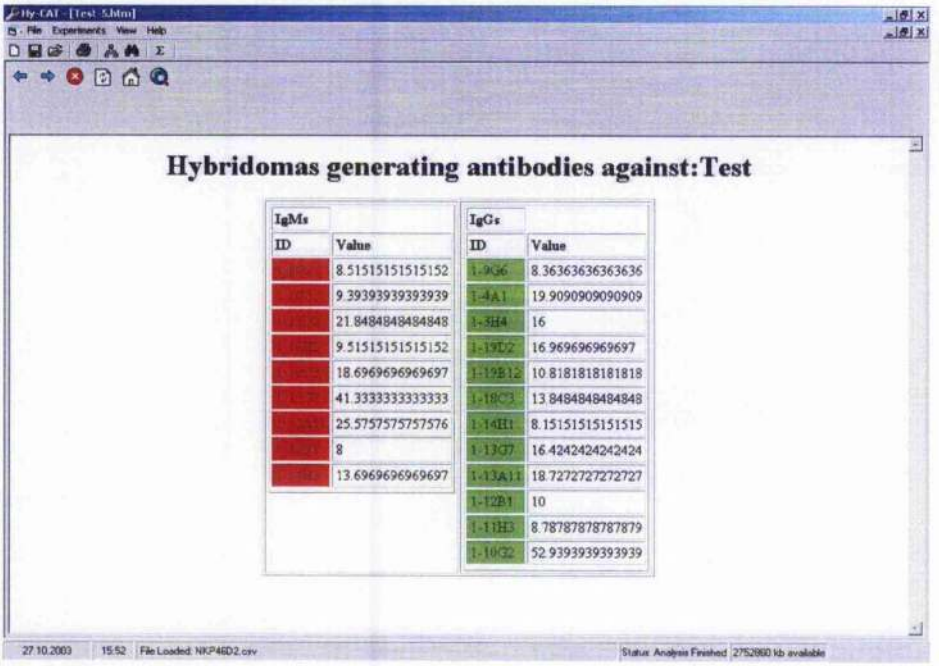

Figure 25. Results table - single antigen

- Generate a text string ready for import into a LIMS SQL database.

5.1.1 Normalisation methods

The most important steps into the chip analysis are the normalisations of the replicate samples and of the complete chip data. Two statistical methods are used for these key steps. The first one is based on the calculation of the percentile contribution of each spot to the total intensity of the chip. Replicate intensities are averaged, to obtain an average intensity for each sample (I_R) and each I_R value is divided by the sum of the intensities of the chip (Equation 1).

The second, which is the default method, calculates the median of the median intensities of each set of replicates (I_R) and then divides this resulting value by the median of all the microarray's I_R values (Equation 2).

$$I_R = \frac{\sum_1^n I}{n}$$

$$H = \frac{I_R}{\sum_1^N I_R} x\,100$$

Equation 1. Percentile contribution normalisation
where I_R is the average of the intensities for each set of replicates, H is the normalised value of the sample, I is the total intensity of each spot, n is the number of replicates and N is the number of spotted hybridomas

While the first method is fast and reliable when we analyse a "single antigen" experiment, it becomes less solid in case of several antigens being used to immunize an animal. Each microarray will produce a different "total intensity", thus the results of each microarray will need to be re-normalised against the whole set of results. One array could have a set of positives lying at the 1-2% range, while the next, with more intense signals, could present positives in a range of 5-7%. With this method, it is not possible to determine a general rule by which the positivity of one sample can be assessed directly from its normalised intensity. Furthermore, results between slides can not be directly compared to each other.

Another limitation of this method is that arithmetic averages are sensitive to single events. This limitation is of particular relevance for the normalisation of the spotted replicates. In the case of a missing spot (caused by a spotting error, for example), the final value of that set of replicates will be dramatically modified. For example, a set of 4 replicates with values {1, 1.5, 1, 2}, will have an averaged intensity of 1.375. By insertion of a spotting error, the values of the same set of replicates could become {1, 1.5, 0, 2}, resulting in a new averaged value of 1.125, thus modifying the properties of

$$I_R = median_1^n I$$

$$H = \frac{I_R}{median_1^N I_R}$$

Equation 2. Median Intensities Normalisation
where I_R is the median of the median intensities for each set of replicates, I is the median intensity value of each spot, H is the normalised value of the spot, n is the number of replicates, N is the number of spotted hybridomas

the particular sample. However, the same set of replicates will have a median value of 1.25. By applying again the same spotting error, thus having a zero value for the third replicate, the median value for this modified set would still have a value of 1.25, thus maintaining the properties of the sample.

Another motivation for using the "median of medians" normalisation method is that it can allow for a fast comparison of results between different chip results. Since all samples are normalised against the median value of a microarray, all samples having an intensity value close to the median, will have a normalised value of ~1. All chips will therefore have a normalised intensity value of 1 for all samples showing average intensities, thus showing values much greater than 1 for high intensity samples, ie positive monoclonal antibodies. Since the method is independent of a chip's total intensity calculation, we can thus consider that once normalised, each normalised value will represent the same "sample quality" in each analysed chip. This allows for the determination of value boundaries for the characterisation of samples. We have postulated that all samples showing a I_R value lower than 3 are negative; if the I_R is between 3 and 20, the sample is "likely to be positive", meaning that the antibody might be functional in ELISA and/or in Western blot; if the I_R is greater than 20, we can consider the sample to be a "sure positive", ie the sample will be functional in ELISA and/or Western blot.

However, this normalisation procedure has its own limitation. When a chip contains a large number of values equal or very close to 0, the median of that particular chip will, in its turn, be equal or close to 0. When we then divide each spot's value by the chip's median (~0), the normalised values of the totality of spots will be too high, or impossible to determine (division by zero), to be analysed. To overcome this problem, the 80-90th percentile value is used as the normalisation factor, instead of the median of the chip.

5.1.2 Filtering of false positives

This quick procedure became necessary with the aim of analysis automation. Since, when spotting more fusions onto a single slide, it was common that samples from the wrong fusion would appear as positives in the final results list (heavy background on parts of the slide, scratches, dirt, cross reactive antibodies, etc), it soon became necessary to remove those samples from the displayed results.

The system used in HyCAT is quite simple and consists in using the antigen's fusionID (see section 5.1, page 82). When the selection of positive samples has been done by the analysis process, all samples not having the correct "fusionID" in their unique identifier (sampleID, see Appendix 7.1) will be discarded.

5.2 Unique samples selection: elimination of cross-reactive species

One of the most important requirements of our screening system is to isolate those hybridomas that are specific for one and only one antigen. Unfortunately, several samples might contain elements that are able to generate a signal on several or all of the microarrays. Therefore, these samples might appear to be good positive monoclonals while we analyse the chips one at a time, but are then found to be cross-reactive. It is then important to cross reference each set of hybridomas against all chips, and to filter out only those that are specific for one antigen only.

HyCAT has been implemented with a tool that is able to quickly and specifically achieve this task. By loading a set of text files containing the sampleID of each positive antibody isolated for each antigen (1 file per antigen), HyCAT will generate a list of "uniques" that will then be used to select the specific positive hybridomas from the cell-culture plates. For each file loaded into the program, each sampleID will be compared with the sampleIDs present in the other files. If no matching sampleID is found, this sample will be stored as a "unique" sample.

5.3 HyCAT's future prospects

Even though HyCAT is currently used as the standard data analysis tool for the monoclonal antibodies production platform, there are still some features that should be implemented. Some can be defined as non-indispensable, such as:

- Direct conversion of results files to comma delimited file, ready to use (removing scanner specific file headers).
- Replacement of the FusionID number with the antigen name
- More normalisation procedures, such the mentioned 80-90th percentile method
- Faster code, to decrease calculation time
- Help manual

Other features are, however, highly required, such as:

- Spot image extraction, to cross-link with the results table. This is necessary to validate each positive sample, since it might happen that a particular "high intensity" sample is nothing else than an unspecific signal that matched the filtering parameters
- Database functionality, for the storage of all analyses and results, and which could be directly linked to the LIMS system
- Direct communication with the cell-culture robot, to allow for a direct cherry-picking of the positive hybridomas

However, these last implementations would require a more extensive knowledge of the programming language (Visual Basic 6.0) and the time required to code these features would be too long. Therefore, it was decided to leave the application as it is, until all of these extra tools could be easily implemented.

6 Discussion and Conclusions

6.1 Novel protocols for the production of monoclonal antibodies

The importance of detection reagents, and especially of monoclonal antibodies in biomedical research, has been driving several laboratories towards the design and implementation of novel protocols for their production. A common feature that has characterised these efforts is the need for a truly high-throughput method, which could alleviate production facilities from heavy labour, time and costs. With the opening of a Monoclonal Antibodies Core Facility in the year 2000, the EMBL decided to participate in this particular area. In order to obtain a high-throughput method for the production monoclonal antibodies, it had been decided to specifically focus on those areas of monoclonal antibody production that were identified as major bottlenecks in the standard protocols. As described in the introduction (page 26), these bottlenecks were identified as being the fusion and cell culture steps, together with the hybridoma screening methods. It was also decided to develop novel immunization protocols to optimise the immune responses of the host animals, thus minimizing the timeline of antibody generation. A general working plan was formulated, which comprised three steps:

1. Immunization protocols: we considered that if one mouse could respond to one antigen, it should specifically respond to more. This was an obvious consideration since it had already been common practice to immunize animals with tissue slurries, or with predigested proteins, thus technically having a mixture of antigens. These can however be considered as mixtures of "unknown" antigens (slurries) or as a unique antigen (digested peptides). We therefore envisaged using cocktails of known antigens (independent of their origin) to immunize each mouse. Our goals were to be able to immunize each animal with at least 10 different antigens.

2. Fusion and Cell Culture methods: since all the tissue culture steps are long, repetitive and error prone, we considered modifying a commercial robotic platform

to perform the relevant steps of hybridoma culture. Such an automated approach would be able to perform the fusion process of splenocytes to myelomas and to generate and maintain the single cell cultures derived from such a fusion.

3. Hybridoma screening: the initial idea for the improvement of the screening method was based on an automated ELISA platform, using the aforementioned robotic platform. However, this solution presented heavy limitations, and of speed and costs. Large scale ELISA screens would have been time-consuming, even with automation, and the quantities of plastic-ware and reagents would have been enormous. Having considered several alternatives or solutions for this problem, we decided to test a microarray approach, which could enable us to screen several thousands of hybridoma supernatants quickly and using less materials.

Figure 26 compares the standard protocols of antibody generation with our designed strategy.

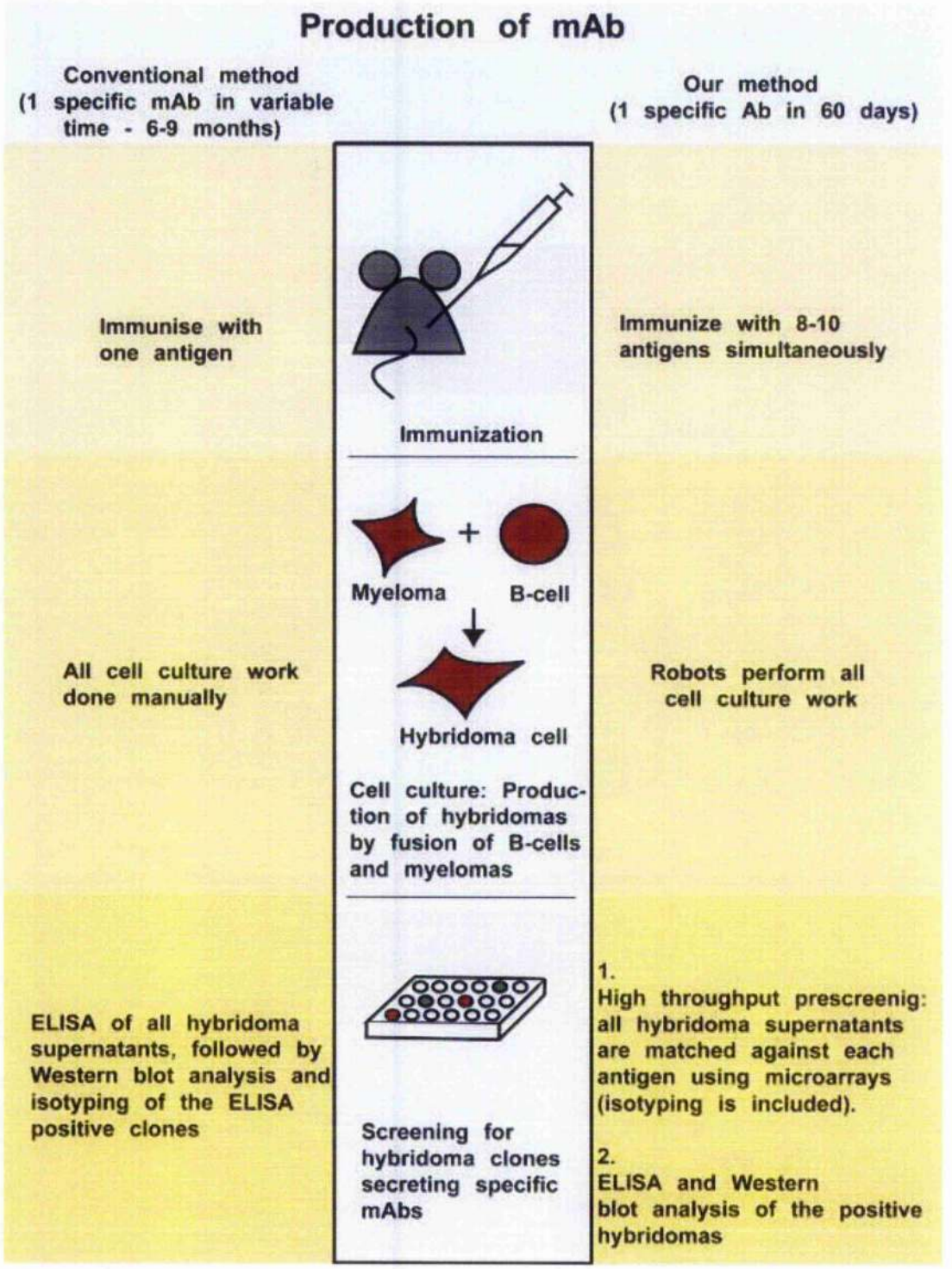

Figure 26. Production of monoclonal antibodies

6.2 HybriChips: establishment of a screening platform

The experience gained in the development and optimization of a protein microarray platform allowed us to design a screening tool that could be used for the analysis of thousands of samples obtained from the process of creating monoclonal antibody secreting hybridomas. Our goal was to replace a conventional ELISA screen with a microarray based assay in order to accelerate the selection of monoclonal antibodies for an extensive library of hybridomas. Several possibilities for designing our screening method were available (Haab, 2003; Templin et al., 2002). We could perform a sandwich assay, which involves the spotting of the hybridoma supernatants (HS), followed by the hybridisation of the array with the purified antigen. The binding of the antigen to an attached monoclonal antibody would be then detected by hybridizing the array with a second monoclonal against the specific antigen (Figure 27 A). While this approach appeared to be promising, we determined that it presented two important flaws. First, we might have been trying to generate a novel monoclonal antibody against an antigen for which there are no existing recognition molecules. It would then be impossible to perform a sandwich assay. Secondly, we might have generated a certain number of monoclonals that recognise the same epitope as the sample we would use for the detection of the interaction. This would mean that these samples could not be detected because of the non-availability of the binding domain. We decided that this strategy would be of no use for our project .

A second possibility for our screen would involve again the spotting of the HS library and the screening using a previously labelled antigen. This could be done using standard Cy3 or Cy5 labelling or by biotinylation of the antigen and detection with labelled streptavidin (Figure 27 B). However, we again considered that this approach could have presented some problems. The direct labelling of proteins with fluorophores is a long process and the labelling efficiency is sequence dependent (Mujumdar et al., 1993). Due to the binding of the fluorophore molecules to primary amines on the antigen, different antigens would be heterogeneously labelled. Furthermore, the fluorophore

molecules might have been attached to a specific epitope, thus lowering the affinity or, in some cases, inhibiting the interaction with the antibody through steric hindrance. These effects could also be seen in the case of the biotinylation of the antigen .

The final option we identified for the design of the array is based on the spotting of the antigen onto the slide and the hybridisation of this array with the HSs. The detection of the interaction between an antibody and the immobilized antigen would be done using a commercially available labelled secondary anti-mouse Ig monoclonal (Fig 27 C). This strategy gave us the idea that we could perform direct isotyping of the monoclonal antibodies by using differentially labelled (Cy3 and Cy5 for example) anti-mouse IgG and anti-mouse IgM secondary antibodies. However, this design presents a major problem, which is that we might have a maximum of 10 antigens per mouse, and a total of 1920 hybridomas per fusion. Therefore, we would create a relatively small array with 10 samples, and would need an extremely large number of chips in order to screen all the hybridomas. .

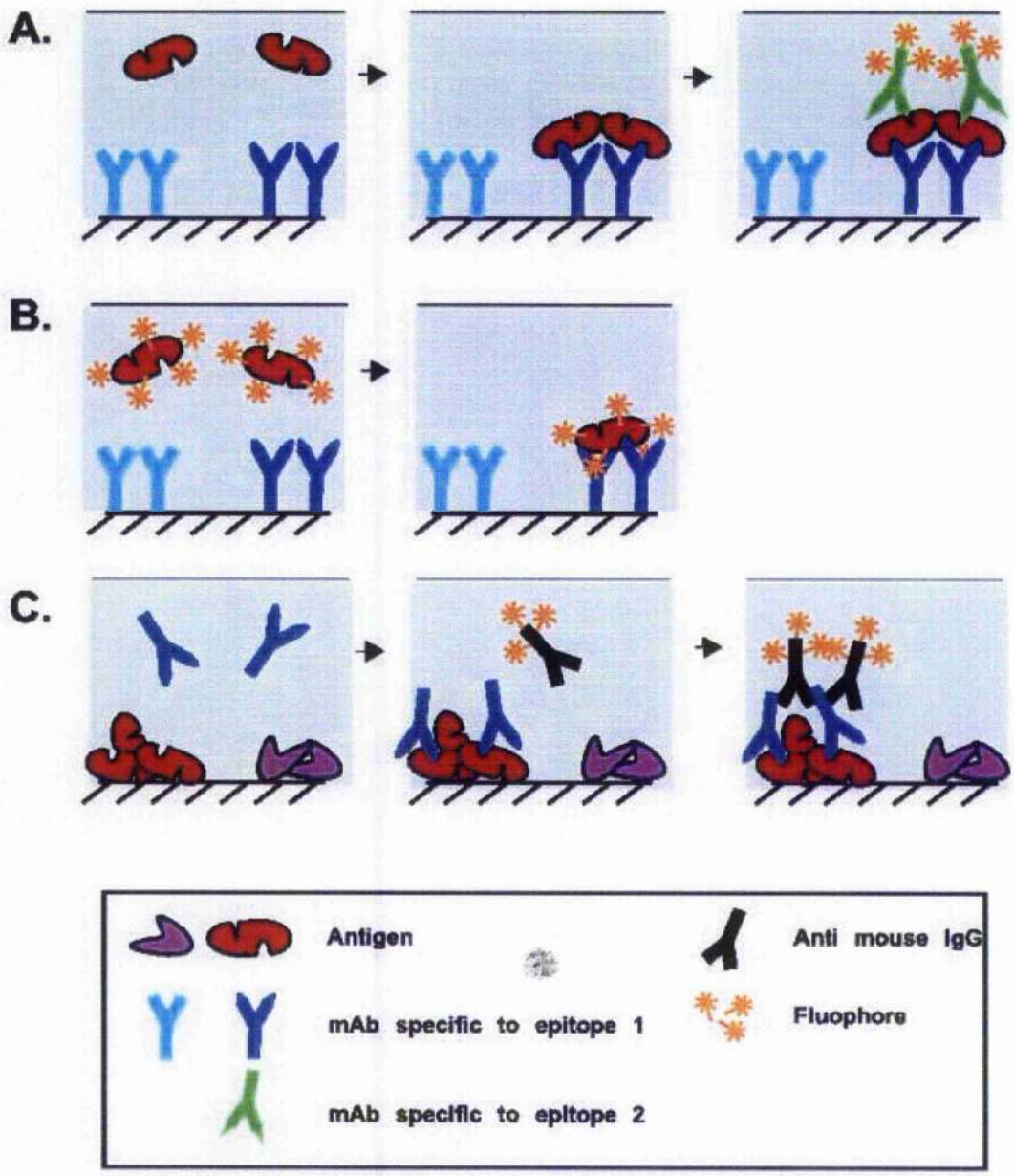

Figure 27. Possible strategies for a microarray based screening platform
A - Sandwich assay: hybridoma supernatants are spotted onto the modified glass. The arrayed supernatants are hybridised with the target antigen and the resulting interaction is detected with a paried specific antibody.
B - Direct labelling of the specific antigen: same procedure as in point A, whereby detection of the antibody/antigen binding is done by directly labelling the antigen.
C - Spotting of the antigens used for immunization. Hybridisation with single hybridoma supernatants. Detection by labelled secondary anti-mouse IgG antibody

We were then faced with the problem of having to design a novel microarray strategy. By immobilizing the HSs on the slide, we could screen extensive libraries of hybridomas in one single experiment, while with the immobilization of the antigens we could perform direct isotyping of the positive samples, directly in the same screening experiment. Combining these two properties, we developed a novel antigen microarray screening method (HybriChip). In this assay, an antigen chip is generated by coating an aminosilane treated slide with a single target antigen. Hybridoma culture supernatants from a fusion are spotted as a microarray onto the antigen chip. After probing with a suitable fluorescently labelled secondary antibody, positive hybridomas are identified in a microarray scanner. The isotype of the bound antibody can be concomitantly determined by probing the antigen chip with mixtures of isotype-specific secondary antibodies, such as Cy5-conjugated anti-mouse IgM and Cy3-conjugated anti-mouse pan-IgG (recognising all mouse IgG isotypes). Different antigen chips can simultaneously be spotted in parallel with the same hybridoma culture supernatants, allowing for a rapid automated assay of multiple antibodies against many target antigens. (Figure 28).

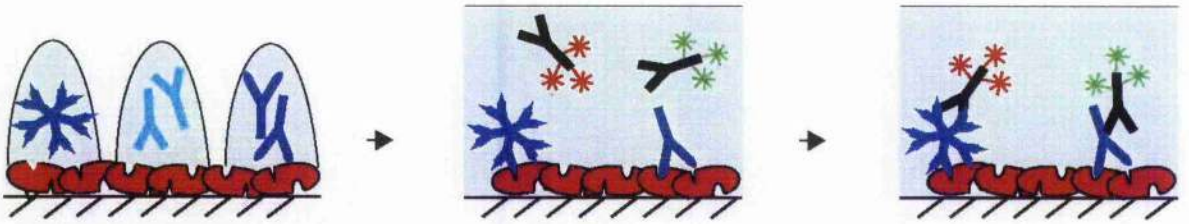

Figure 28. Hybridoma screening strategy
A - A chemically modified slide is homogeneously coated with purified antigen. Hyrbidoma supernatants are succesively spotted onto this antigen layer.
B - The microarray containing the spotted hybridoma supernatants is hybridised with a solution containing Cy3 labelled anti-mouse IgG and Cy5 labelled anti-mouse IgM.
C - Isotype specific signals (green: IgG, red: IgM) are detected from the spots presenting hybridoma supernatants containing monoclonal antibodies specific for the coated antigen

6.3 HybriChips - the results

The validity of the described screening platform has been established experimentally. The first part of the project was focussed on feasibility experiments, where we demonstrated that:

1. Coating of a chemically modified glass slide with a homogenous solution of antigen: using the chemical properties of the aminosilane chip chemistry, it is possible to apply an antigen layer onto the slide (Section 4.2.2).

2. Detection of spotted antibodies: because of the screening format we wanted to use, it was crucial that only antibodies specific for the coated antigen were detected on the microarray. This means that antibodies should not be able to unspecifically bind onto the antigen, or to free aminosilane active sites. Experiments involving characterized antibodies and hybridoma libraries have shown that antibodies are able to specifically recognize the coated antigen. Furthermore, no cross-reactivity or direct binding of antibodies to the aminosilane coating can be detected (Sections 4.2.3 and 4.2.4).

3. Detection of antibodies from hybridoma libraries generated by multiplexed immunizations: in order to obtain a high-throughput monoclonal antibody production platform, a fast, specific and highly sensitive screening method is required. We have shown that using a microarray assay, it is possible to select monoclonal antibodies specific for single antigens, from a unique hybridoma library generated from a multiplexed immunization (Sections 4.2.54.2.6).

4. Direct isotyping of the detected monoclonal antibodies: because of the format of the assay, direct isotyping of the monoclonal antibodies could have been possible by using, as detection method, differentially labelled secondary antibodies. We used Cy3-anti-mouse IgG and Cy5-anti-mouse IgM antibodies. The selected positive antibodies were confirmed by a second round of isotyping, in an

ELISA format and using a commercially available strip assay (McDougal et al., 1983)(Section 4.2.4).

These results show that the designed assay format can be used to screen extensive hybridoma libraries. All HybriChip selected IgG monoclonal antibodies in these initial experiments were proven to be positive by ELISA and/or Western blot. Furthermore, antibodies selected with this screening have been shown to be functional in more demanding experiments, where for example, the antigen needs to maintain a native conformation. These results validated our strategy and established the use of the HybriChips as a powerful primary hybridoma screening procedure.

However, before using this strategy as a routine primary screen, an higher level of validation was needed. In the first part of the project, only those IgG monoclonal antibodies that were selected by HybriChip were further analysed by ELISA and Western blot. No further information was available for IgM antibodies and for HybriChip negative samples. In order to eliminate the risk of "missing" false negatives, a large comparison between HybriChip and ELISA had to be performed to determine the correlation between both assays. From this experiment (Section 4.2.7), we were able determine that both assays are able to identify the same sets of antibodies. Correlation values were elevated (>75%) in the case of antigens were a statistically relevant number of antibodies where generated and their respective ELISA OD values were well above the background levels (0.2 OD). Other antigens, for which few antibodies were selected, seemed to have lower correlation values. Due to the low sample size however, statistical significance cannot be established for this data. It is important to point out that even though there are few antibodies against these antigens, we were still able to specifically detect them by HybriChip and ELISA (Table 9). We can then conclude, from these results and considerations, that the HybriChip approach can be used as an effective, fast and high-throughput primary screen.

To test the complete high-throughput monoclonal antibody production platform, a large scale experiment was carried out. Eight mice were immunized each with 10 antigens

(Section 4.2.8). The fusions were robotically performed and the complete hybridoma library (15320 samples) was screened by HybriChip. ELISA and Western blot analyses of the selected IgG antibodies were performed to validate the HybriChip results. Monoclonal antibodies were selected against 67 of the antigens. Of these, 25 were positive by ELISA and 22 by Western blot (Table 11). We achieved an overall success rate of 40% for antibodies identified with HybriChip that tested positive at least one other immunoassay, and 28% for antibodies that were functional in at least one immuno-application. The disparity between HybriChip and ELISA in this large scale production of monoclonal antibodies partially reflects the increased sensitivity of the HybriChip (Tonkinson, J.L., `http://www.devicelink.com/ivdt/archive/03/03/001.html`). In this experiment, the threshold for HybriChip-positive clones was set too low; the correlation between HybriChip and ELISA approached 100% when the threshold was increased. Thus, clones testing negative by ELISA due to factors such as low antibody concentration in the supernatant or the presence of an antibody of low binding affinity (both are plausible causes considering the short immunization protocol), gave a positive result by HybriChip. In addition, the conformation of proteins on the HybriChip substrate may render available antibody-binding sites that are masked by adsorption onto the polycarbonate substrate used in ELISA. In support of this possibility, several of the proteins negative by ELISA were subsequently found positive by Western blot. Because of the lack of Western blot data for 15 of the antigens, it is not possible to speculate about the correlation between HybriChip and Western blot.

6.4 Conclusions

We have developed a fast, economical, high-throughput, method of hybridoma generation which with a simple scale-up could help to alleviate the current bottleneck in mAb production. Comparison with similar studies is difficult as others have focused on the production of antibody serum titre (Chambers and Johnston, 2003; Field et al., 1998; Pizza et al., 2000) as opposed to the isolation and production of hybridomas.

Compared to the best of these serum-titre studies (Chambers and Johnston, 2003), our method is about half as successful as the 80% success rate achieved there. Improvements may be possible by lengthening the immunization protocol or using genetic immunization, which may be more effective in attaining a suitable response against a higher proportion of the target antigens (Chambers and Johnston, 2003).

Our method represents a qualitative improvement over the ELISA, since clones secreting antibodies with different isotypes can be selected at the primary screen. IgM isotypes are often more useful to investigators for fluorescence-based assays such as FACS analysis or immunofluorescence, but this isotype is almost useless for assays involving protein-A as a detection reagent, most notably immuno-gold labelling (electron microscopy) and immunoprecipitation, due to their lack of affinity for protein-A or protein-G. IgM isotype antibodies are also notoriously difficult to purify and in these two cases IgG isotypes are more desirable.

Moreover, the ability to assay supernatants from the same fusion against multiple targets simultaneously enables us to perform limited epitope mapping on-the-fly at the primary screening stage. This level of differentiation in the primary screening assay facilitates the differentiation and isolation of hybridomas secreting mAbs specific for (for example) phosphorylated and non-phosphorylated peptide antigens.

6.5 HybriChips: future prospects

6.5.1 Improvements to the platform

This presented novel platform for the production of monoclonal antibodies is now used as a well established method by the MACF at the EMBL. Other than serving the internal demands of the institute, it also functions as a commercial entity (HybriCore) for non-EMBL laboratories. However, this method can still be improved and accelerated:

1. Immunization protocols: immunization protocols could be optimized in order to increase the efficiency of the immune response into the hosts. We obtained mon-

oclonal antibodies specific against 67 of 80 antigens with a 24 days immunization protocol. It should be possible, by testing different host lines or finding standard parameters for the contents of the antigen mixture to increase this response so that it approachesto 100%. Genetic immunisation (Chambers and Johnston, 2003) should also be tested.

2. Fusion procedure: the possibility of performing the fusions of B cells with myelomas using a robotic method allows for 8 fusions to be done simultaneously. However, it has been seen that the fusion efficiency of the robot cold be increased (still lower than a manual fusion). This improvement to the procedure could allow for the generation of a higher number of monoclonal antibody-secreting hybridomas.

3. Automation of the production of the HybriChips: manual preparation of the microarrays is time consuming and is influenced by external factors, such as room temperature, humidity and labware calibration. Automation of this step could remove some of the variations in the coating efficiency and accelerate the complete procedure. The slides could for example be located in a fixed position, where different solutions and reagents could be dispensed onto them. Furthermore, if done into a climatically controlled location, binding efficiencies should be constant.

4. Screening of the microarrays: it would be very important to use the potentiality of the available scanning hardware (four lasers) to extend the isotyping screen. It could be possible to use differentially labelled secondary antibodies specific for the various IgG subclasses. However, the availability of such secondary antibodies is currently very limited.

5. Selection of positive antibodies: Hy-CAT should be implemented with more normalization methods and faster algorithms. The possibility of sending the list of selected antibodies directly to the robot controlling software for the cherry-picking of the clones is a necessary feature to be implemented into the software.

6.5.2 Projects

Large-scale proteomic projects have so far been hampered by the lack of complete libraries of monoclonal antibodies raised against complete cellular proteomes. The generation of such libraries would be a very committing and financially challenging project if performed using conventional methods of antibody production. At optimal conditions, our platform could generate antibodies specific against 3000 antigens per year. Currently, the MACF is processing 600-700 antigens per year. Because of this truly high-throughput method, the MACF is becoming a major player in an all-European consortium, the European Proteomics Initiative (EPI). The aim of this consortium is to establish a pipeline of monoclonal antibodies specific for the human proteome. It is hoped that, with a unified effort and combination of various expertise, such a project would be feasible. The scientific community would be presented with the opportunity to have unlimited availability of detection reagents for their projects and, hopefully, a full human proteome analysis could be done.

7 Appendix

7.1 Array definition: automated generation of sampleID and sample coordinates

Hy-CAT contains a feature that allows one to easily create the files needed by the spotters to generate an array list. For the user to be able to track his samples on the chip, a grid definition file is created, where all samples are listed relative to their geometrical position. In order to create such a file, the spotter software requires a listing of all the samples present in each well of the plates used for the spotting procedure. Considering that the source plates are standard 96 wells and that the spotter uses 384 wells plates, the mapping of the samples in the plates might not be easy. The transfer of samples from one plate format to the other dramatically changes the layout of the IDs presented to the spotter. With a simple procedure, this can be easily achieved by Hy-CAT.

The first step done by this feature is to populate the source 96 well plates with unique IDs. After the users enters the number of plates used, each sample in each well will be identified in the following manner:

Fusion#-Plate#Row#Column#

where the Fusion# is determined by the number of plates used. Each fusion procedure will generate twenty 96 well plates. Therefore, the first 20 plates will be Fusion1, plates 21 - 40 will be Fusion 2 and so on. So, as an example, the fist sample will be identified as 1-1A1 and the second will be 1-1A2. A graphical interface can be loaded to modify these IDs or to mark empty wells. Transfer of samples from 96wp to 384wp is done following the zig-zag method, where samples from plate 1 will start from well A1, plate 2 will start at well A2, plate 3 at B1 and plate 4 at B2 (Figure 29)

This procedure will output a comma delimited file (*.csv) containing the following structure:

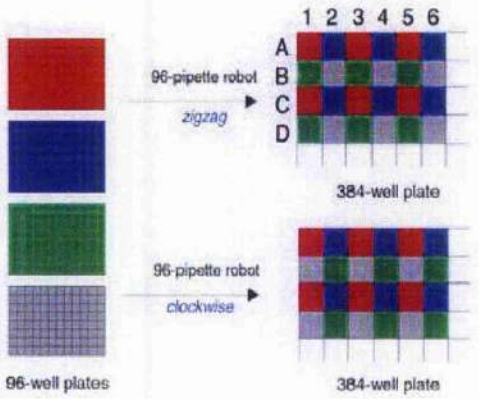

Figure 29. Plate transfer methods
Each 384 wells plate contains four 96 wells plates. Two methods of transferring 96 wells plates into the 384 wells plate exist:
- The zigzag method
- The clockwise method

Plate	Row	Column	ID	Name
1	1	1	1-1A1	
1	1	2	1-2A1	
1	2	1	1-3A1	
1	2	1	1-4A1	
...	...	...	...	
5	1	1	2-1A1	
...	...	...	...	
Last Fusion Plates	12	24	Last Fusion ID-20H12	

Table 13. Output format for sample identification
The "Name" column is empty, for the user can add sample specific comments, such as "Positive Control" or other

This file is then loaded into the spotter software, where it will be used to generate a map of the spotted samples that follows the specific spotting procedure used, and which will be used for the identification of the spots at analysis time.

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(54) Title: METHOD FOR PRODUCING MONOCLONAL ANTIBODIES

(57) Abstract: The present invention relates to methods for producing monoclonal antibodies. In particular, the invention relates to high throughput methods for producing and screening monoclonal antibodies more rapidly than conventional methods.

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Method for producing monoclonal antibodies

The present invention relates to methods for producing monoclonal antibodies. In particular, the invention relates to high throughput methods for producing monoclonal antibodies more rapidly than conventional methods.

The development of monoclonal antibody-producing cell lines by somatic fusions of B lymphocytes with myeloma cells was first described by Kohler and Milstein over 25 years ago (Kohler & Milstein, 1975). Since then, monoclonal antibodies have played a central role in the exponential growth of our understanding of human physiology, biochemistry and genetics. Monoclonal antibodies are versatile and sensitive tools for detecting and localising specific biological molecules. Monoclonal antibodies can be made against any cell molecule, enabling that molecule to be identified, localised and purified. In some cases, monoclonal antibodies also help identify the function of the molecules to which they bind.

The diagnostic and therapeutic potential of monoclonal antibodies was also quickly realised after the hybridoma technique allowed their development in the mid 1970s. Monoclonal antibodies have become key components in a vast array of clinical laboratory diagnostic tests. In addition, a large number of licensed drugs contain monoclonal antibodies and vast numbers of drugs in development are monoclonal antibodies. The clinical use of monoclonal antibodies has been improved by the development of chimeric and fully humanised monoclonal antibodies which minimise side-effects in patients.

However, despite the central role of monoclonal antibodies in these developments in medicine and molecular biology, the process for producing and screening monoclonal antibodies has changed little since it was first developed by Kohler & Milstein in the mid-1970s. The approach most often used to produce a monoclonal antibody against a specific antigen requires a series of immunisations of mice or rats with an antigen over the course of several weeks to enhance the activation and proliferation of mature B cells producing antigen-specific antibodies. Multiple mice are generally immunised and are tested periodically for the presence of relevant serum immunoglobulin titres prior to somatic fusion. Following fusion, the supernatants of hybridomas are screened using immunoassays to identify monoclonal antibodies with a high specificity for the antigen. The time frame required for developing a monoclonal antibody using this approach is generally 3 to 9 months.

The development of RIMMS (repetitive, multiple site immunisation strategy) has enabled somatic fusions to take place just 8-14 days after the initiation of immunisation (Kilpatrick *et al*, 1997). The supernatants of the hybridomas produced can then be screened using standard immunoassays, allowing a monoclonal antibody against a specific antigen to be isolated much more quickly

However, even using RIMMS, the production and screening of monoclonal antibodies against large numbers of different antigens requires considerable time and resources. As more and more novel proteins are discovered, there is a need for faster and more efficient methods for producing and screening monoclonal antibodies against these proteins, in order to allow their further characterisation.

Summary of the invention

Accordingly, the invention provides a method for producing a monoclonal antibody, said method comprising the steps of:

a) introducing at least one candidate antigen into an animal;
b) recovering antibody-producing cells from said animal and rendering these cells into a single cell suspension;
c) generating an immortalized cell line from said single cell suspension;
d) screening the supernatant of said immortalized cell line against a protein chip on which the candidate antigen is displayed; and
e) selecting as said monoclonal antibody, an antibody that binds to said candidate antigen.

The method of the invention has considerable advantages over the methods of producing monoclonal antibodies that are currently available. As already discussed, current methods for producing monoclonal antibodies against more than one antigen involve laborious immunisation and isolation protocols for each individual antigen. In contrast, in the method of the invention, the animal may be injected with multiple antigens resulting in the simultaneous production of monoclonal antibodies against multiple antigens and increasing the speed and efficiency of monoclonal antibody production. The use of a protein chip in the method of the invention accelerates the process of screening to detect monoclonal antibodies that bind to the antigen or antigens with which the animal has been injected. In addition, the protein chip is more sensitive than conventional screening assays, such as enzyme linked immunosorbent assays (ELISAs), resulting in an improved detection rate for slow secreting hybridoma cells which would be missed using conventional screening

methods. Additionally, the use of a protein chip in the method of the invention enables each supernatant to be screened multiple times against an antigen and uses only a fraction of the amount of antigen required for a single screening in a conventional screening assay such as an ELISA. For example, each supernatant can be screened in duplicate, triplicate or quadruplicate against an antigen.

The animal in step a) of the method of the invention may be any non-human mammalian animal. Preferably, the animal is a mouse, rat, rabbit, hamster or guinea pig. Preferably, the animal is a mouse.

The candidate antigen in step a) is preferably a purified candidate antigen. Either a purified candidate antigen or a mixture of purified candidate antigens may be introduced into the animal. By "purified candidate antigen" is meant that the antigen is a homogenous preparation of antigen that is substantially free from any other components. By "a mixture of purified candidate antigens" is meant that more than one purified antigen is present in the composition used for immunisation, but that the preparation is free from contaminating components for which there is no intention to elicit the production of antibodies. For example, although using conventional procedures, an animal may be immunised with multiple antigens simply by immunisation with homogenised tissue, such immunisation does not represent immunisation with purified candidate antigens as this is defined herein, since the antigens would be contaminated with cellular debris.

Any number of purified candidate antigens may be introduced into the animal. Preferably, between 1 and 50 purified candidate antigens are introduced into the animal. Preferably, more than one purified candidate antigens are introduced into the animal. For example, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50 or more than 50 purified candidate antigens may be introduced into the animal. The antigens may be introduced simultaneously, in the sense that they are all mixed together. Alternatively, the antigens may be introduced separately one after the other. The introduction of different antigens may be separated by a time period of days. Preferably, the period separating the introduction of different antigens is less than 48 hours, preferably less than 24 hours. Preferably, the method of introduction involves injection of the antigen(s) into the animal.

By the term "candidate antigen" is meant any substance capable of inducing an immune response in an animal when that candidate antigen is introduced into the animal. The term therefore includes proteinaceous substances and non-proteinaceous substances.

Proteinaceous substances which are antigens include proteins and derivatives thereof, such as glycoproteins, lipoproteins and nucleoproteins and peptides. Fragments of such proteinaceous substances are also included within the term "antigen". Preferably, such fragments consist of or comprise antigenic determinants. Non-proteinaceous substances which are antigens include polysaccharides, lipopolysaccharides and nucleic acids. In particular, the term "antigen" includes nucleic acid molecules that induce an immune response against the proteins they encode. Fragments of such non-proteinaceous substances are also included with the term "antigen". The term "antigen" further includes proteinaceous or non-proteinaceous substances linked to a carrier which are able to induce an immune response, such as lipids or haptens upon which antigenicity is conferred when they are linked to a carrier. The antigens of the invention may be naturally occurring substances or may be synthesised by methods known in the art.

Where one purified antigen is used for immunisation, the antigen is preferably a proteinaceous substance or a nucleic acid molecule. Where the purified antigen is a proteinaceous substance, it may be introduced alone or in the form of a fusion protein. In particular, the invention provides that the antigen may be in the form of a fusion protein expressed on the surface of a recombinant virion with the animal being injected with the recombinant virion. The production of such recombinant virions using a nucleic acid sequence encoding the proteinaceous antigen, is described in Lindley et al, 2000.

Where more than one purified antigen is used for immunisation, any combination of purified antigens may be used. The animal may be injected with only proteinaceous antigens, only non-proteinaceous antigens, or a mixture of both. According to one embodiment of the invention, the purified antigens are all proteinaceous. The purified antigens may be fragments derived from the same protein or different proteins. The purified antigens may be recombinant virions derived from a cDNA library, each recombinant virion expressing a protein encoded by a cDNA from the library on its surface.

According to a further embodiment, the invention provides that multiple purified antigens are introduced into the animal in the form of nucleic acid molecules encoding proteins against which it is desired to produce monoclonal antibodies. The nucleic acid molecules may be in DNA molecules, cDNA molecules or RNA molecules. Preferably, in this aspect of the invention, a cDNA library may be introduced into the animal. It is therefore possible

to inject the animal with nucleic acid molecules encoding a protein of unknown identity and as described below, cell chips may be used to isolate an antibody against the protein which in turn allows the protein to be purified.

Where the purified antigen is a nucleic acid molecule, it preferably consists of or comprises a DNA, cDNA or RNA sequence encoding a protein against which an immune response is to be induced. The nucleic acid molecule may be a naked nucleic acid molecule or it may be in the form of a vector.

The vector may be a viral vector, preferably a retroviral, adenoviral, adeno-associated viral (AAV), herpes viral, alphavirus vector or any other suitable vector as will be apparent to the skilled reader. Alternatively, the nucleic acid molecule may be in the form of a non-viral vector, preferably a plasmid vector. Many such vectors are known and documented in the art (see, for example, Fernandez J.M. & Hoeffler J.P. in Gene expression systems. Using nature for the art of expression ed. Academic Press, San Diego, London, Boston, New York, Sydney, Tokyo, Toronto, 1998). Such vectors may additionally incorporate regulatory sequences such as enhancers, promoters, ribosome binding sites and termination signals in the 5' and 3' untranslated regions of genes, that are required to ensure that the coding sequence is properly transcribed and translated.

Alternatively, the nucleic acid molecule may be in the form polycationic condensed DNA linked or unlinked to killed adenovirus alone, for example see Curiel (1992) *Hum Gene Ther* 3:147-154, or ligand linked DNA, for example see Wu (1989) *J Biol Chem* 264:16985-16987. The nucleic acid molecule may also be in the form of DNA coated latex beads. Alternatively, the nucleic acid molecule may be encapsulated in liposomes as described, for example, in WO95/13796, WO94/23697, WO91/14445 and EP-524,968. *SA* 91(24):11581-11585.

Antigen may be introduced into the animal by any suitable means. Preferably, the method of introduction involves injection. The animals may be immunised with the purified antigen or antigens intrasplenically, intravenously, intraperitoneally, intradermally or subcutaneously or by any other suitable means. The animals may be immunised with the purified antigen or antigens via more than one of these routes. For example, some of the purified antigen or antigens may be injected intraperitoneally and the rest subcutaneously. The means of injection will depend on the antigen or antigens being injected. For example,

in the case of injection with a nucleic acid molecule a hand-held gene transfer particle gun, as described in US 5,149,655 can be used to inject the nucleic acid molecule.

In the case of a proteinaceous antigen, the dose of each antigen should preferably be in the range of between 0.01 and 1000 micrograms.

Preferably, the method of the invention comprises the additional step of supplying the animal with a booster dose of some or all of the antigens which are introduced into the animal prior to the recovery of the antibody-producing cells. The animals may be given a booster 1-365 days after the first injection. Preferably, the animals are boosted 1 to 20 times.

Preferably, the immunisation protocols used in the methodology of the present invention are short where more than one antigen is used in order to prevent one antigen becoming immunodominant. Preferably, where the animal is immunised with more than one antigen, it is injected with a booster of each antigen or combined booster of more than one antigen 3 days after the first injection and a further booster 5 days after the initial injection with spleen tissue or lymph nodes being removed between day 6 and day 15. Where a longer immunisation protocol is desired, the animal may be injected, for example, with a booster of each antigen or combined booster of more than one antigen 21 days after the first injection, with the spleen tissue or lymph nodes being removed on day 26.

Immunisation of the animal may be carried out with or without pharmaceutical carriers. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Additionally, these carriers may function as immunostimulating agents ("adjuvants"). Immunisation of the animal may be carried out with or without adjuvants in addition to the pharmaceutical carriers.

Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (1) aluminium salts (alum), such as aluminium hydroxide, aluminium phosphate, aluminium sulfate, *etc*; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59™ (WO90/14837; Chapter 10 in *Vaccine design: the subunit and adjuvant approach*, eds. Powell & Newman, Plenum Press

1995), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing MTP-PE) formulated into submicron particles using a microfluidizer, (b) SAF, containing 10% Squalene, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); (3) saponin adjuvants, such as QS21 or Stimulon™ (Cambridge Bioscience, Worcester, MA) may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes), which ISCOMS may be devoid of additional detergent *e.g.* WO00/07621; (4) Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA); (5) cytokines, such as interleukins (*e.g.* IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12 (WO99/44636), *etc.*), interferons (*e.g.* gamma interferon), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), *etc.*; (6) monophosphoryl lipid A (MPL) or 3-O-deacylated MPL (3dMPL) *e.g.* GB-2220221, EP-A-0689454, optionally in the substantial absence of alum when used with pneumococcal saccharides *e.g.* WO00/56358; (7) combinations of 3dMPL with, for example, QS21 and/or oil-in-water emulsions *e.g.* EP-A-0835318, EP-A-0735898, EP-A-0761231; (8) oligonucleotides comprising CpG motifs (Roman *et al., Nat. Med.*, 1997, 3, 849-854; Weiner *et al., PNAS USA*, 1997, 94, 10833-10837; Davis *et al., J. Immunol.*, 1998, 160, 870-876; Chu *et al., J. Exp. Med.*, 1997, 186, 1623-1631; Lipford *et al., Eur. J. Immunol.*, 1997, 27, 2340-2344; Moldoveanu *et al., Vaccine*, 1988, 16, 1216-1224, Krieg *et al., Nature*, 1995, 374, 546-549; Klinman *et al., PNAS USA*, 1996, 93, 2879-2883; Ballas *et al., J. Immunol.*, 1996, 157, 1840-1845; Cowdery *et al., J. Immunol.*, 1996, 156, 4570-4575; Halpern *et al., Cell. Immunol.*, 1996, 167, 72-78; Yamamoto *et al., Jpn. J. Cancer Res.*, 1988, 79, 866-873; Stacey *et al., J. Immunol.*, 1996, 157, 2116-2122; Messina *et al., J. Immunol.*, 1991, 147, 1759-1764; Yi *et al., J. Immunol.*, 1996, 157, 4918-4925; Yi *et al., J. Immunol.*, 1996, 157, 5394-5402; Yi *et al., J. Immunol.*, 1998, 160, 4755-4761; and Yi *et al., J. Immunol.*, 1998, 160, 5898-5906; International patent applications WO96/02555, WO98/16247, WO98/18810, WO98/40100, WO98/55495, WO98/37919 and WO98/52581) *i.e.* containing at least one CG dinucleotide, with 5-methylcytosine optionally being used in place of cytosine; (9) a polyoxyethylene ether or a polyoxyethylene ester *e.g.* WO99/52549; (10) a polyoxyethylene sorbitan ester surfactant

in combination with an octoxynol (WO01/21207) or a polyoxyethylene alkyl ether or ester surfactant in combination with at least one additional non-ionic surfactant such as an octoxynol (WO01/21152); (11) a saponin and an immunostimulatory oligonucleotide (*e.g.* a CpG oligonucleotide) (WO00/62800); (12) an immunostimulant and a particle of metal salt *e.g.* WO00/23105; (13) a saponin and an oil-in-water emulsion *e.g.* WO99/11241; (14) a saponin (*e.g.* QS21) + 3dMPL + IL-12 (optionally + a sterol) *e.g.* WO98/57659; (15) other substances that act as immunostimulating agents to enhance the effectiveness of the composition. Additional examples of adjuvants that may be used include Montanide ISA 50, Hunter's TiterMax, and Gerbu Adjuvants.

Preferred antibody-producing cells for use in the invention include B cells, T cells and stem cells. These antibody-producing cells for use in the invention may be recovered by removal of any suitable cellular components of the immune system from the animal. Preferably, antibody-producing cells are recovered from the animal by removal of the spleen, lymph nodes or bone marrow or portions thereof. These may be rendered into a single cell suspension according to step b) of the method of the invention via any suitable means. Preferably, spleen tissue, lymph nodes or bone marrow removed from the animal are rendered into a single cell suspension by mechanical disruption or enzymatic digestion with proteases. Red cells may be removed from the cell suspension by hypotonic lysis.

Preferably, the immortalized cell line specified in step c) of the method of the invention is a hybridoma cell line produced by somatic fusion of the cells in the single cell suspension to myeloma cells. Cells in the single cell suspension are fused to myeloma cells with a fusogen. Examples of myeloma cells which may be used include SP2, NS1 and NS0. Preferably, the fusogen is PEG, a virus or a method of electrofusion (Zimmermann et al. 1990).

The hybridoma cells produced by the fusion of the single cell suspension with the myeloma cells should be cultured. Preferably, the hybridoma cells are initially cultured in a selective media, such as Azaserine hypoxanthine or Hypoxanthine aminopterin thymidine, and are then transferred to a non-selective media. Preferably, the hybridoma cells are cultured on selective media for 7 days and are then transferred to a non-selective media for 3 days. This ensures that the growth rate of the cells increases prior to the screening step. Preferably, the steps involved in hybridoma production are conducted robotically in order

to speed up the process. The Examples set out one way of conducting the steps involved in hybridoma production robotically.

Alternatively, the immortalized cell line may be a cell line generated by infection of cells in the single cell suspension with an immortalizing virus. Preferably, the immortalizing virus is Epstein-Bar virus (see, for example, Epstein Barr Virus Protocols, Eds. Wilson and May, Humana Press; ISBN: 0896036901).

Step d) of the method of the invention comprises screening the supernatant of the immortalized cell line, preferably a hybridoma cell line, against a protein chip comprising a candidate antigen with which the animal was immunised. As used herein, the term "protein chip" is used to encompass any microarray made up of a supporting means to which a candidate antigen has been anchored.

Where just one purified antigen has been introduced into the animal, that purified antigen and no additional antigens may be anchored on the protein chip. Where more than one purified antigen has been introduced into the animal, each purified antigen may be displayed at a different position on the protein chip, preferably at a predetermined position. Each position on the protein chip may thus display a different antigen. Alternatively, the same antigen may be anchored to each position in a row or column of a protein chip with a different antigen being displayed in each row or column. In some cases, it may be desirable, even when the animal has been immunised with more than one purified antigen, to have a different chip for each antigen. A protein chip may have a large number, such as between 1 and 1000 purified antigens, anchored at predetermined positions on a chip.

Any type of protein chip known in the art may be used in the method of invention. For example, the protein chip may be a glass slide to which the purified antigen or antigens are anchored. Where only one antigen is being tested, such a slide may be prepared simply by coating glass microscope slides with aminosilane (Ansorge, Faulstich), adding an antigen-containing solution to the slide and drying. Slides coated with aminosilane may be obtained from Telechem and Pierce for coating with the purified antigen. Preferably, such a glass slide may be coated with (6-aminohexil) aminosilane.

Other types of protein chips which may be used in the method of the invention include a 3D gel pad (Arkenov *et al*, 2000) and microwell chips. As will be apparent to the skilled reader, types of protein chips that have not yet been conceived but which are devised in the future may well prove to be suitable for use in accordance with the present invention.

The term "protein chip" also includes microarrays of cells expressing defined cDNAs (Ziauddin *et al*, 2001) referred to herein as "cell chips". In this technique, mammalian cells are cultured on a glass slide printed in defined location with different cDNAs. Cells growing on the printed locations take up and express the cDNAs. Cell chips are particularly useful when the animal has been injected with a cDNA or a cDNA library or with a recombinant virion or virions produced from a cDNA library, as described above. In such cases, the proteins encoded by the cDNA sequences may not have been isolated. By injecting the animal with cDNAs encoding the proteins or recombinant virions expressing the cDNAs, it is possible to produce monoclonal antibodies against the proteins expressed by the cDNAs. If the same cDNAs are expressed using a cell chip, these antibodies will bind and the binding may be detected as described below. Providing that a nucleic acid sequence encoding the protein is available, the invention in this manner enables the detection and isolation of a monoclonal antibody against that protein which may be used to purify the protein itself.

For selection of antibodies, or selection of immortalised cell lines producing such antibodies, the supernatant from the immortalised cell line or cell lines is spotted onto the protein chip or protein chips at defined positions on the chip. Spotting of supernatants is preferably done robotically, for example with a Genemachines Ominigrid arrayer using Telechem pins. Preferably, the supernatants spotted onto the protein chip or protein chips contain glycerol to minimise drying and fixing of the antibodies on the slide. For example, 0 to 99.9% glycerol may be used. The chip is then washed to remove any unbound supernatant. At this stage, any monoclonal antibody produced by the immortalized cell line and hence in the supernatant may be bound to an antigen on the chip.

By using different elution conditions, the method allows the approximate quantification of the binding affinity of the monoclonal antibody for its binding partner. Elution agents that may be used include chaotropic agents such as guanidine hydrochloride or urea at concentrations between 10 μmolar and 8 molar or ethylene glycol in an aqueous solution of 0.01% to 100% w/v. Elutions may also be carried out using aqueous or non-aqueous solutions of glycine at concentrations of between 0.01molar and a saturated solution (preferably 200mM), at a pH of between pH9 and pH1, preferably pH3.2. High pH elutions may be carried out using aqueous or non aqueous solutions of triethylamine between 1μmolar and a saturated solution, preferably 100mM, at a pH of between pH8 and pH13, preferably pH 11.5.

Step e) of the method of the invention involves selection of a monoclonal antibody that binds to the antigen. Preferably, this step incorporates a detection step, such as by adding a marker which will bind to bound monoclonal antibody and indicate its presence. Preferably, the marker is labelled with a label such as an enzymatic or fluorescent label that enables its presence to be detected. For example, the marker may be labelled protein A or labelled protein G. Protein A or protein G may be labelled with a fluorescent label such as Cy3 or Cy5. Alternatively, protein A or protein G may be labelled with an enzymatic label such as biotin, the presence of which can be detected by the binding of labelled streptavidin or avidin.

Preferably, the marker is an antibody that will bind to the first antibody. Preferably this antibody is labelled with a label such as an enzymatic or fluorescent labels. Preferably this antibody is labeled with fluorescent labels as this enables equipment developed for scanning of DNA chips to be used for detection.

Preferably, the step of detecting a monoclonal antibody bound to the antigen further comprises isotyping the monoclonal antibodies. Preferably, this step of detecting and isotyping the monoclonal antibodies comprises adding isotype-specific anti-immunoglobulin antibodies to said protein chip, wherein each anti-immunoglobulin antibody having a different isotype specificity has a different label, and detecting the presence of said labels. This method enables the simultaneous detection of the monoclonal antibody and determination of its isotype.

It will be appreciated that the method may employ as many different isotype-specific anti-immunoglobulin antibodies, each with a different label, as there are antibody isotypes in the animal which has been immunised. For example, if a mammal, such as a mouse, has been injected with an antigen, the step of detecting and isotyping monoclonal antibodies bound to the antigen may comprise adding an anti-IgA antibody labelled with a first label, and/or an anti-IgD antibody labelled with a second label, and/or an anti-IgE antibody labelled with a third label, and/or an anti-IgG1 antibody labelled with a fourth label, and/or an anti-IgG2a antibody labelled with a fifth label, and/or an anti-IgG2b antibody labelled with a sixth label, and/or an anti-IgG3 antibody labelled with a seventh label, and/or an anti-IgG4 antibody labelled with a eighth label, and/or an anti-IgM antibody labelled with a ninth label. Alternatively, the step of detecting and isotyping monoclonal antibodies bound to the antigen may comprise adding isotype-specific anti-immunoglobulin

antibodies that bind to a subset of the possible isotypes. Preferably, the isotype-specific anti-immunoglobulin antibodies comprise an anti-IgM antibody labelled with a first label and an anti-IgG antibody labeled with a second label. Preferably, the labels are fluorescent labels.

Detection of the label indicates the presence of a monoclonal antibody bound to an antigen and is preferably done robotically. Where the label is a fluorescent label, detection of the label and hence the presence of the monoclonal antibody may be done using equipment available for scanning protein chips. For example, scanning of the chips may be done with a GenePix 4000B scanner (Axon Instruments, Inc.) or with a Tecan LS200 or LS400 scanner. Scanning may be carried out with between 1 and 4 lasers and combinations of filters to enable visualisation of multiple fluorescent labels. Preferably, visualisation of multiple fluorescent labels is carried out simultaneously although it may be carried out sequentially.

Images may be obtained and analysed using appropriate software such as the GenePix Pro software (Axon Instruments, Inc.), Chipskipper software (Schwager, Ansorge) or Tecan LS200 or LS400 software.

In order to ensure that the detection of a monoclonal antibody is reliable, the screening method preferably employs various controls. For example, in the case of a protein chip coated with one antigen, not only will the supernatants from the immortalized cell lines produced by the method of the invention be spotted onto the protein chip but so will positive and negative controls.

Positive controls may be in the form of previously tested monoclonal antibodies or commercially available polyclonals. Alternatively, positive controls may consist of diluted or undiluted serum previously collected from the immunized mouse either a suitable period after the boost or at the moment the animal is sacrificed for the collection of the source of B-cells.

Negative controls may be in the form of mock supernatants at defined positions. Another level of control is determined by the fact that each supernatant is screened against several antigens. Signals obtained against only one antigen are considered to be potential positive monoclonal antibody containing supernatants.

Positive signals on the protein chip can be traced back to a particular immortalised cell line enabling the monoclonal antibody to be isolated according to step e) of the method of the invention. Further characterisation of the antibodies identified can then be carried out.

Methods for carrying out further characterisation of the antibody may include, for example, the further step of determining the specificity of the monoclonal antibodies identified. For example, a monoclonal antibody identified by the method of the invention may be used to scan a second protein chip having a large number of different proteins anchored to its surface to establish if the monoclonal antibody binds specifically to one antigen. The scanning methods described above in the initial identification of the protein may be used to scan for its specificity. The binding specificity and affinity of the monoclonal antibodies produced by the method of the invention may be further characterised by altering the concentrations of antigen on protein chips or altering the stringency of eluting conditions, as described above.

According to a further embodiment of the first aspect of the invention, there is provided a method for producing an immortalised cell line that produces a monoclonal antibody of interest, said method comprising the steps of:

a) introducing at least one candidate antigen into an animal;

b) recovering antibody-producing cells from said animal and rendering these cells into a single cell suspension;

c) generating an immortalized cell line from said single cell suspension;

d) screening the supernatant of said immortalized cell line against a protein chip on which the candidate antigen is displayed; and

e) selecting as said immortalised cell line, that which produces a supernatant containing an antibody that binds to said candidate antigen.

Immortalised cell lines produced by such a method are of immense utility in the generation of antibodies with tailored specificities.

According to a particular embodiment of the first aspect of the invention, there is provided a high-throughput method for producing a plurality of monoclonal antibodies, each of which binds to a different candidate antigen, comprising the steps of:

a) introducing a plurality of candidate antigens into an animal;

b) recovering antibody-producing cells from said animal and rendering these cells into a single cell suspension;

c) generating immortalized cell lines from said single cell suspension;

d) screening the supernatant of said immortalized cell lines against one or more protein chips on which the candidate antigens are displayed; and

e) selecting as said monoclonal antibodies, antibodies that bind to said candidate antigens.

The candidate antigens are preferably purified candidate antigens, as described above. Suitable procedures for introducing the candidate antigens into the animal, recovering antibody-producing cells, generating immortalized cell lines and screening the supernatants of the immortalized cell lines are described above.

Prior art methods involve laborious and time-consuming procedures to generate and screen for a monoclonal antibody against a single antigen. In contrast, this method enables the generation and high-throughput screening of monoclonal antibodies against a plurality of antigens simultaneously. The use of a protein chip to conduct high-throughput screening of the antibodies is more efficient and more accurate than the use of conventional assays and requires less candidate antigen.

Preferably, step e) of this embodiment further comprises isotyping the monoclonal antibodies, as described above. This provides an additional advantage over the prior art methods which do not disclose simultaneous detection and isotyping of monoclonal antibodies.

According to a second aspect of the invention, there is provided a monoclonal antibody produced by a method of the invention. The invention may also be used to generate a bank of antibodies, for example, with specificities encompassing an entire organismic proteome. Such a bank of antibodies represents a further aspect of the invention.

According to a third aspect of the invention, there is provided an immortalized cell line, preferably a hybridoma cell line, which produces a monoclonal antibody according to the second aspect of the invention. This aspect of the invention also includes a bank of immortalized cell lines, preferably a bank of hybridoma cell lines. The invention may also be used to generate a bank of hybridoma cell lines, for example, that produce antibodies encompassing an entire organismic proteome.

According to a fourth aspect of the invention, there is provided a method for producing a plurality of monoclonal antibodies, each of which binds to a different purified candidate antigen, comprising introducing a plurality of purified candidate antigens into an animal,

Preferably, each candidate antigen is derived from a different source. By this is meant that each antigen is derived from a different protein, a different nucleic acid and so on. It is intended that methods of antibody production that involve injecting an animal with different fragments of the same protein are excluded from the scope of this aspect of the invention. For example, the purified candidate antigens may all be proteinaceous substances provided that they are not all fragments of the same protein.

This method has an advantage over methods disclosed in the prior art in that it enables the simultaneous production of more than one monoclonal antibody, each of which binds to a different purified candidate antigen.

The animal may be injected with the purified candidate antigens using any of the techniques described herein. For example, the method of this aspect of the invention may further comprise the steps of recovering antibody-producing cells such as B cells, T cells and stem cells from an immunised animal, such as by removing spleen tissue, lymph nodes or bone marrow, and rendering them into a single cell suspension. The method may further comprise generating immortalized cell lines, preferably hybridoma cell lines, from the cells the single cell suspension. Preferably, the method of this aspect of the invention comprises these steps and additionally comprises the steps of screening the supernatants of the immortalized cell lines, preferably hybridoma cell lines, against a protein chip or protein chips on which the candidate antigens are displayed; and selecting monoclonal antibodies that bind to the antigens and preferably isolating these and/or the immortalized cell lines that produce the monoclonal antibodies. Suitable procedures for generating the immortalized cell lines and subsequent screening of the supernatants are the same as those described in above in connection with the method of the first aspect of the invention. In particular, the step of detecting the monoclonal antibodies may involve simultaneous detection of the monoclonal antibodies and determination of this isotype, as described above. In addition, the method may comprise further characterisation of the monoclonal antibodies, as described above.

The invention also provides a monoclonal antibody produced by a method of this aspect of the invention. Again, this aspect of the invention may be used to generate a bank of antibodies, for example, encompassing antibodies with specificity for protein in an entire organismic proteome. Such a bank of antibodies represents a further aspect of the invention.

The invention also provides an immortalized cell line, preferably a hybridoma cell line, which produces a monoclonal antibody as described above. The invention may also be used to generate a bank of immortalized cell lines, preferably a bank of hybridoma cell lines, for example, that produce antibodies encompassing an entire organismic proteome.

According to a fifth aspect of the invention, anti-idiotype antibodies may be generated that bind to a monoclonal antibody according to the second aspect of the invention. Anti-idiotype antibodies are useful as they have a variable region that mimics the shape of the molecule to which the original antibody was raised. Anti-idiotype antibodies may therefore be useful in therapy as replacements for the molecules against which the original antibody was raised. An anti-idiotype antibody may be produced by employing the method of the first aspect of the invention or the fourth aspect of the invention using a monoclonal antibody according to the second aspect of the invention as the purified candidate antigen.

Accordingly, this aspect of the invention provides a method of producing an anti-idiotype antibody that binds to a monoclonal antibody according to the second aspect of the invention, the method comprising using a monoclonal antibody according to the second aspect of the invention as a purified candidate antigen in a method of the first aspect of the invention or the fourth aspect of the invention. The invention also includes anti-idiotype antibodies generated by such methods.

According to a sixth aspect of the invention, anti-anti-idiotype antibodies may be generated that bind to an anti-idiotype antibody produced according to the fifth aspect of the invention. Such anti-anti-idiotype antibodies may be produced by employing the method of the first aspect of the invention or the fourth aspect of the invention using an anti-idiotype antibody as described above as the purified candidate antigen. This aspect of the invention thus provides a method of producing an anti-anti-idiotype antibody that binds to an anti-idiotype antibody generated according to the fifth aspect of the invention, the method comprising using an anti-idiotype antibody as described above as a purified candidate antigen in a method of the first aspect of the invention or the fourth aspect of the invention.

Various aspects and embodiments of the present invention will now be described in more detail by way of example. It will be appreciated that modification of detail may be made without departing from the scope of the invention.

Brief description of the Figures:

Figure 1: Whole image of scanned chip, where green and red spots represent positive IgG and IgM producing supernatants respectively. Close ups are to show details of specific areas of chip where good spots are to be found.

Figure 2: Comparison between chip analysis and ELISA screen. First image is negative sample (Ia <0.5), while others are positive. Average Ic: Average total intensity of spot on 3 chips. Ia: Average contribution of spot to sum of intensities over three chips (%).

Figure 3: Comparison of contribution to total intensity (%) of culture supernatants screened by ELISA (■) or by chip analysis (□) for binding to B5 antigen (Figure 3A). The background values are shown in Figure 3B. A number of positive supernatants identified by chip analysis and/or ELISA are shown.

Figure 4: Comparison of contribution to total intensity (%) of culture supernatants screened by ELISA (■) or by chip analysis (□) for binding to B5 antigen (Figure 4A). The background values are shown in Figure 4B. A single positive supernatant identified by chip analysis and ELISA is shown.

Figure 5: Comparison of contribution to total intensity (%) of culture supernatants screened by ELISA (■) or by chip analysis (□) for binding to Ket94/95 antigen (Figure 5A). The background values are shown in Figure 5B. A number of positive supernatants identified by chip analysis and/or ELISA are shown.

Figure 6: Comparison of contribution to total intensity (%) of culture supernatants screened by ELISA (■) or by chip analysis (□) for binding to Ket94/95 antigen (Figure 6A). The background values are shown in Figure 6B. No positive supernatants were identified by either ELISA or chip analysis.

Examples

Example 1: Immunization with 10 protein antigens

Immunization

An 8-week old female Balb/c mouse was injected intrasplenically with 10 µg of each of 10 protein antigens in 100µl phosphate buffered saline (PBS). On the third day following (day 3) the mouse was injected intraperitoneally with 1ug of each of the same 10 protein antigens in 100µl PBS. On day 5, the same mouse was injected intravenously with 0.1µg

of each of the same 10 protein antigens. On Day 8 the mouse was killed by cervical dislocation and the spleen removed and collected into Dulbecco's Modified Eagle's Medium (DMEM: Life Technologies Inc.).

Fusion

All steps are performed under sterile or aseptic conditions in a laminar flow hood. The spleen was rendered into a single-cell suspension by mechanical disruption between two frosted-end glass microscope slides. The suspension was filtered into a 50ml bar-coded conical-bottomed tube (BD Falcon) through a 70µm nylon cell strainer (BD Falcon) and transferred to the robotic system.

Separately, SP2 myeloma fusion partners (ATCC) were cultured for five days prior to fusion in HM20 (DMEM, 20% Defined foetal bovine serum (Hyclone Defined), 10mM L-Glutamine, 50µM Gentamicin) and on the day of the fusion were transferred to HM20/HCF/2xOPI (HM20 containing 10% Hybridoma Cloning Factor (Origen) and 2%OPI cloning supplement (Sigma)) for at least one hour at 37°C in a 5% CO_2 incubator.

The bar codes were read by a bar code reader and the 50ml Falcon tube loaded into the rotor by the RoMa arm on the genesis Freedom system (Tecan). The rotor was loaded into the centrifuge through the workdeck.

The tube was centrifuged at 100g for 10 mins at room temperature (RT) and the rotor extracted from the centrifuge. The tube was extracted from the rotor and the bar code was again read to distinguish from the balance tube. Cells were resuspended in 5ml Red Cell Lysis Buffer (Sigma) for 9 minutes at RT. HM20 was added to 50ml and the tube once again centrifuged for 10 min at RT with no brake. The supernatant solution was aspirated to waste and the cells resuspended in DMEM preheated to 37°C. Cells were washed twice more by steps of centrifugation and resuspension. 50µl of cell suspension were robotically pipetted to a 1.5ml microcentrifuge tube. Cells were counted using a haemocytometer counting chamber.

Simultaneously the SP2 cells were washed three times in a similar fashion and a similar aliquot (50µl) "handed off" to a 1.5 ml tube for haemocytometric counting. SP2 myelomas and spleen cells were mixed at a ratio of 1:5 (SP2:Spleen) and again centrifuged at 100g for 10 min with no brake.

The supernatant solution was entirely aspirated to waste and Polethyleneglycol 1500 in 50% HEPES (PEG: Roche Molecular Biochemicals) pre-heated to 37°C was robotically pipetted smoothly and progressively over 1 min with rotation at 450rpm on a Te-shake shaker (Tecan AG) to ensure even mixing. The cell/PEG mixture was incubated for 1 min at 37°C with gentle agitation. 1 ml of DMEM was similarly added over 1 min at 37°C with similar agitation. The mixture was incubated for 1 min at 37°C with gentle agitation. A further 1ml of DMEM was robotically added over 1 min at 37°C with gentle agitation and incubated similarly for a further minute. 7mls of HM20 were robotically added over 3 min at 37°C with gentle agitation. The Tube was then spun at 90g for 5 min with brake. The supernate was aspirated to waste and the pellet resuspended in 20ml of HM20/HCF/OPI/AH (HM20/HCF/OPI plus 10% Azaserine Hypoxanthine (Sigma).

The conical tube was again placed on the robot workdeck and the post-fusion cell slurry was aspirated by each of the 8 wide-bore pipette tips of the liquid handling arm of the robot. 200µl of the cell slurry was then pipetted into each well of a 96-well deep well plate (Greiner Masterblock).

The deep-well plate was then robotically transferred to a TeMo 96-well pipetting robot integrated onto the Genesis work-deck and used as a source plate to plate out into the 20 sterile 96-well tissue culture plates.

The post-fusion mixture was then robotically plated out into 20 96-well sterile plates (Nunc) sourced from a carousel attached and integrated to the robot at 100µl/well and robotically transferred to an integrated 37°C incubator with 10% CO_2 through the integrated airlock. Plates were stored in a carousel contained with the incubator.

Cell Culture

On the third day following the fusion cells were robotically transported from the incubator to the work deck and a further 100µl HM20/HCF/OPI/AH was robotically added. The plates were then robotically returned to the incubator.

On day 7 the plates were once again similarly transported from incubator to work deck and 200µl/well of culture supernates was aspirated to waste and replaced with 150µl fresh HM20/HCF.

On day 11 the plates were again robotically transported to the work deck and 30µl of supernate was collected from each well (Temo head: Tecan Inc.) and transferred to 384-well plates supplied to the workdeck by a carousel plate stacker (Tecan Inc).

Microarray screening

Aminosilane coated glass slides were homogeneously coated with purified antigen by dropping 40µl of ddH_2O containing 1-5 µg of antigen and covering with a 22*60mm coverslip for 60min in a humid chamber at RT.

Coated slides were rinsed briefly in PBS and blocked for 60' in 5% milk in PBS, 0.1% Tween, then washed for 10' in PBS. The chips were then dried by centrifugation, 10" at 2000 rpm.

Culture supernatants were consolidated into 384 well plates using a Beckman Biomek FX robot.

Culture supernatants were printed singularly onto three identical antigen-coated slides at a density of 9600 spots per chip and a spot size of ~120µm using a GeneMachines OmniGrid microarray printer.

The microarray chips were incubated in a humid chamber for 60', at RT and then washed 5 x 5' in PBS-0.1% Tween (PBST)

40µl of Cy3 conjugated goat anti-mouse IgG-specific and Cy5 conjugated goat anti-mouse IgM-specific antibodies were diluted 1:1000 in PBST, mixed and applied uniformly to the chips and covered with 22x60 mm coverslips and incubated in a humid chamber, for 30' at RT. The chips were then washed 2 x 10' in PBST, 2 x 10' in PBS and 1 x 10' in ddH_2O. The chips were dried by centrifugation at 2000 rpm for 10".

Hit-Picking

Chips were scanned with a GenePix 4000B scanner (Axon Instruments), at a resolution of 10 um.pixel^{-1}. PMT voltages were 540V and 610V, for the Cy3 and Cy5 channels respectively. Both lasers were set at 100% intensity. Each scanned chip was assigned a fitted grid, and all spots were analysed by the GenePix Pro 3.0 (Axon Instruments).

All chips were analysed by the GenePix Pro 3.0 software and we collected, for each chip, the information relative to the corrected intensity (I_c) of each spot (Median of intensities – Background), for the Cy3 and Cy5 channels. From this data, we obtained, for each chip,

the contribution of each spot to the total Corrected Intensity of each channel ($\left(I_c / \sum_1^{9600} I_c\right) \times 100$). The three values of each channel were averaged for each spot, and this Average Corrected Intensity (I_a) was used for the final analysis of the dataset. We considered as "likely to be positive" samples the ones that have a value above 0.5% and as "sure positives", all the samples showing a value superior to 1.5%.

Post Screening Processing

Cells from positive wells were resuspended in the well and 20µl transferred to a 96-deep-well plate previously filled with 1.5mls of HM20/HCF and returned to the incubator for 48hrs at 37°C, 5%CO_2.

1.4ml of culture each supernate was transferred to another deep well plate and the remaining 100µl used to resuspend the cells which were then transferred to a freezing vial containing 90% Foetal bovine serum/10%DMSO (Sigma) and transferred to -80°C for 2hrs and from there to liquid nitrogen store. Culture supernate was then used for evaluation and further characterization of the generated monoclonal antibody.

Results:

The results of microarray screening are shown in Figure 1. This Figure demonstrates that a number of positive monoclonal antibodies were detected as binding to candidate antigens on the slide. The green spots are IgG monoclonal antibodies which bind to the candidate antigens while the red spots are IgM monoclonal antibodies which bind to the candidate antigens. Figure 1 thus demonstrates that the method of the invention can be used to simultaneously identify monoclonal antibodies that bind to the candidate antigens and the isotypes of those monoclonal antibodies.

When a comparative experiment was conducted using ELISA in the place of a protein chip, a number of monoclonal antibodies identified as binding to candidate antigens in the microarray screening were not identified as binding to candidate antigens using ELISA, as shown in Figure 2 (see comparison of microarray results with ELISA results). A supernatant which was found to be negative using microarray screening (Ia:0.234) was also found to be negative using ELISA. However, of four supernatants found to be positive using microarray screening (Ia: 5.18, 1.96, 3.64 and 2.02), only two were found to be positive using ELISA (see Figure 2). It is important to note how the supernatants that were

negative in the ELISA experiment can be actual positive samples as detected by the more accurate and sensitive microarray approach.

Example 2: Immunization with nine antigens

Immunization:

A mouse was injected with 25μg of nine antigens, including 25μg of a fusion of the antigens B5 and Ket94/95, each antigen being mixed with 10μg CpG DNA and adsorbed onto alum adjuvant (Imject Alum from Pierce). Half of each antigen was administered intraperitoneally and half subcutaneously.

The mouse was boosted 21 days later with 10μg of each antigen mixed with 10μg of CpG DNA and adsorbed onto alum adjuvant, half of which was administered intraperitoneally and half subcutaneously.

Five days after the boost, the spleen was removed.

Fusion and cell culture

The fusions and cell culture were performed as described in Example 1.

Microarray screening of antibodies against B5 and Ket94/95

An aminosilane glass slides was homogenously coated with purified B5 by dropping 40μl of ddH2O containing 5μg of purified B5 and covering with a 22*60mm coverslip for 60 min at room temperature. The same procedure was used to produce an aminosilane glass slide homogenously coated with purified Ket94/95.

The coated slides were rinsed, blocked, washed and dried, as described in Example 1, except that 3% BSA in PBS was used in place of 5% milk in PBS to block the slide.

Culture supernatants were consolidated into 384 well plates, as described in Example 1 and were printed in triplicate onto the slide coated with B5 and the slide coated with Ket94/95 at a density of around 16000 spots per chip and a spot size of ~150μm using a Microgrid II 610 microarray printer (ApogentDiscoveries).

The microarray chips were incubated and Cy3 conjugated goat anti-mouse IgG-specific and Cy5 conjugated goat anti-mouse IgM-specific antibodies were applied to the chips as described in Example 1.

methods. Additionally, the use of a protein chip in the method of the invention enables each supernatant to be screened multiple times against an antigen and uses only a fraction of the amount of antigen required for a single screening in a conventional screening assay such as an ELISA. For example, each supernatant can be screened in duplicate, triplicate or quadruplicate against an antigen.

The animal in step a) of the method of the invention may be any non-human mammalian animal. Preferably, the animal is a mouse, rat, rabbit, hamster or guinea pig. Preferably, the animal is a mouse.

The candidate antigen in step a) is preferably a purified candidate antigen. Either a purified candidate antigen or a mixture of purified candidate antigens may be introduced into the animal. By "purified candidate antigen" is meant that the antigen is a homogenous preparation of antigen that is substantially free from any other components. By "a mixture of purified candidate antigens" is meant that more than one purified antigen is present in the composition used for immunisation, but that the preparation is free from contaminating components for which there is no intention to elicit the production of antibodies. For example, although using conventional procedures, an animal may be immunised with multiple antigens simply by immunisation with homogenised tissue, such immunisation does not represent immunisation with purified candidate antigens as this is defined herein, since the antigens would be contaminated with cellular debris.

Any number of purified candidate antigens may be introduced into the animal. Preferably, between 1 and 50 purified candidate antigens are introduced into the animal. Preferably, more than one purified candidate antigens are introduced into the animal. For example, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50 or more than 50 purified candidate antigens may be introduced into the animal. The antigens may be introduced simultaneously, in the sense that they are all mixed together. Alternatively, the antigens may be introduced separately one after the other. The introduction of different antigens may be separated by a time period of days. Preferably, the period separating the introduction of different antigens is less than 48 hours, preferably less than 24 hours. Preferably, the method of introduction involves injection of the antigen(s) into the animal.

By the term "candidate antigen" is meant any substance capable of inducing an immune response in an animal when that candidate antigen is introduced into the animal. The term therefore includes proteinaceous substances and non-proteinaceous substances.

It is clear from these results that microarray screening is at least as effective as ELISA at identifying monoclonal antibodies that bind a specific antigen. Indeed the identification of positive supernatants not identified by ELISA suggests that microarray screening is more sensitive than ELISA. Microarray screening further had the significant advantage that it allowed simultaneous determination of the IG or IgM isotype of the monoclonal antibodies identified.

Figure 3A shows the normalised values of percentage contribution to total intensity for each culture supernatant in an ELISA plate (■) containing positive samples that bind B5 compared to the normalised values of percentage contribution for the same culture supernatants obtained by microarray screening of a B5-coated slide (□). Figure 3B shows the level of background noise in these experiments. It can be seen that positive supernatants showed a greater percentage contribution to total intensity using microarray screening compared to ELISA. As a result, there was a greater difference between background noise and a positive supernatant in microarray screening compared to ELISA, enabling positive supernatants to be identified more easily and more accurately.

Figure 4A compares the normalised values of percentage contribution to total intensity for each culture supernatant in an ELISA plate (■) containing a single positive sample that binds B5 compared to the normalised values of percentage contribution for the same culture supernatants obtained by microarray screening on a B5-coated slide (□). The level of background noise is shown in Figure 4B and it can be seen that positive sample was more readily detectable above the background noise using microarray screening compared to ELISA.

Figure 5A compares the normalised values of percentage contribution to total intensity for each culture supernatant in the ELISA plate found to contain positive supernatants that bind KET94/95 (■) compared to the normalised values of percentage contribution for the same culture supernatants obtained by microarray screening on a KET94/95-coated slide (□). The positive supernatants were more readily detectable above the background noise using microarray screening compared to ELISA, as shown in Figure 5B.

Figures 6A compares the data obtained from an ELISA plate (■) in which there were no positive supernatants to data obtained using microarray screening (□) of the same culture supernatants. As shown in Figure 6B, the readings in both cases were due to background noise.

These results demonstrate that the method of the invention can be used to simultaneously identify monoclonal antibodies against more than one antigen. The use of microarray screening in the method of the invention is quicker, cheaper and more accurate than the use of conventional antibody screening methods, such as ELISA.

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Claims

1. A method for producing a monoclonal antibody, said method comprising the steps of:
   a) introducing at least one candidate antigen into an animal;
   b) recovering antibody-producing cells from said animal and rendering these cells into a single cell suspension;
   c) generating an immortalized cell line from said single cell suspension;
   d) screening the supernatant of said immortalized cell line against a protein chip on which the candidate antigen is displayed; and
   e) selecting as said monoclonal antibody, an antibody that binds to said candidate antigen.
2. The method of claim 1 wherein said animal is a mouse, a rat, a guinea pig or a rabbit.
3. The method of claim 1 or claim 2 wherein said candidate antigen is a purified candidate antigen.
4. The method of claim 3 wherein between one and fifty different purified candidate antigens are introduced into the animal.
5. The method of claim 4 wherein between 0.001 and 1000 micrograms of each antigen is introduced into the animal.
6. The method of any one of claims 1 to 5 comprising the additional step of supplying the animal with a booster dose of some or all of the antigens which were introduced into the animal prior to the removal of antibody-producing cells.
7. The method of any one of claims 1 to 6 wherein the antibody-producing cells are B cells, T cell or stem cells.
8. The method of any one of claims 1 to 7 wherein the antibody-producing cells are recovered by removal of spleen tissue, lymph nodes or bone marrow of the animal.
9. The method of any one of claims 1 to 8 wherein the immortalized cell line is a hybridoma cell line produced by somatic fusion of the cells in the single cell suspension to myeloma cells.
10. The method of any one of claims 1 to 9 wherein said protein chip is a plain-glass slide, a 3D gel pad chip, a microwell chip or a cell chip.

11. The method of any one of claims 1 to 10 wherein the step of detecting the monoclonal antibodies bound to the antigens further comprises isotyping the monoclonal antibodies.

12. The method of claim 11 wherein said step of detecting and isotyping the monoclonal antibodies comprises adding isotype specific anti-immunoglobulin antibodies to said protein chip, wherein each anti-immunoglobulin antibody having a different isotype specificity has a different label, and detecting the presence of said labels.

13. The method of any one of claims 1 to 12 further comprising assessing the specificity with which each isolated monoclonal antibody binds to an antigen using a protein chip comprising said antigen.

14. A high-throughput method for producing a plurality of monoclonal antibodies, each of which binds to a different candidate antigen, comprising the steps of:

a) introducing a plurality of candidate antigens into an animal;

b) recovering antibody-producing cells from said animal and rendering these cells into a single cell suspension;

c) generating immortalized cell lines from said single cell suspension;

d) screening the supernatant of said immortalized cell lines against one or more protein chips on which the candidate antigens are displayed; and

e) selecting as said monoclonal antibodies, antibodies that bind to said candidate antigens.

15. A method according to claim 14, which further comprises any of the steps recited in any one of claims 1 to 13.

16. A method for producing an immortalised cell line that produces a monoclonal antibody of interest, said method comprising the steps of:

a) introducing at least one candidate antigen into an animal;

b) recovering antibody-producing cells from said animal and rendering these cells into a single cell suspension;

c) generating an immortalized cell line from said single cell suspension;

d) screening the supernatant of said immortalized cell line against a protein chip on which the candidate antigen is displayed; and

e) selecting as said immortalised cell line, that which produces a supernatant containing an antibody that binds to said candidate antigen.

17. An immortalised cell line isolated by the method of claim 16.

18. A method for producing a plurality of monoclonal antibodies, each of which binds to a different purified candidate antigen, comprising introducing a plurality of purified candidate antigens into an animal, each purified candidate antigen being derived from a different source.

19. A method according to claim 18 which further comprises any of the steps recited in any one of claims 1 to 13.

20. A monoclonal antibody isolated by the method of any one of claims 1 to 16 or 18 to 19.

21. An antibody according to claim 20 which is an anti-idiotype antibody.

22. An antibody according to claim 21 which is an anti-anti-idiotype antibody.

23. An immortalized cell line producing a monoclonal antibody of claim 20, claim 21 or claim 22.

24. An immortalized cell according to claim 23 which is a hybridoma cell line.

25. A bank of antibodies according to claim 20, claim 21 or claim 22.

26. A bank of immortalized cell lines according to claim 15, claim 21 or claim 22.

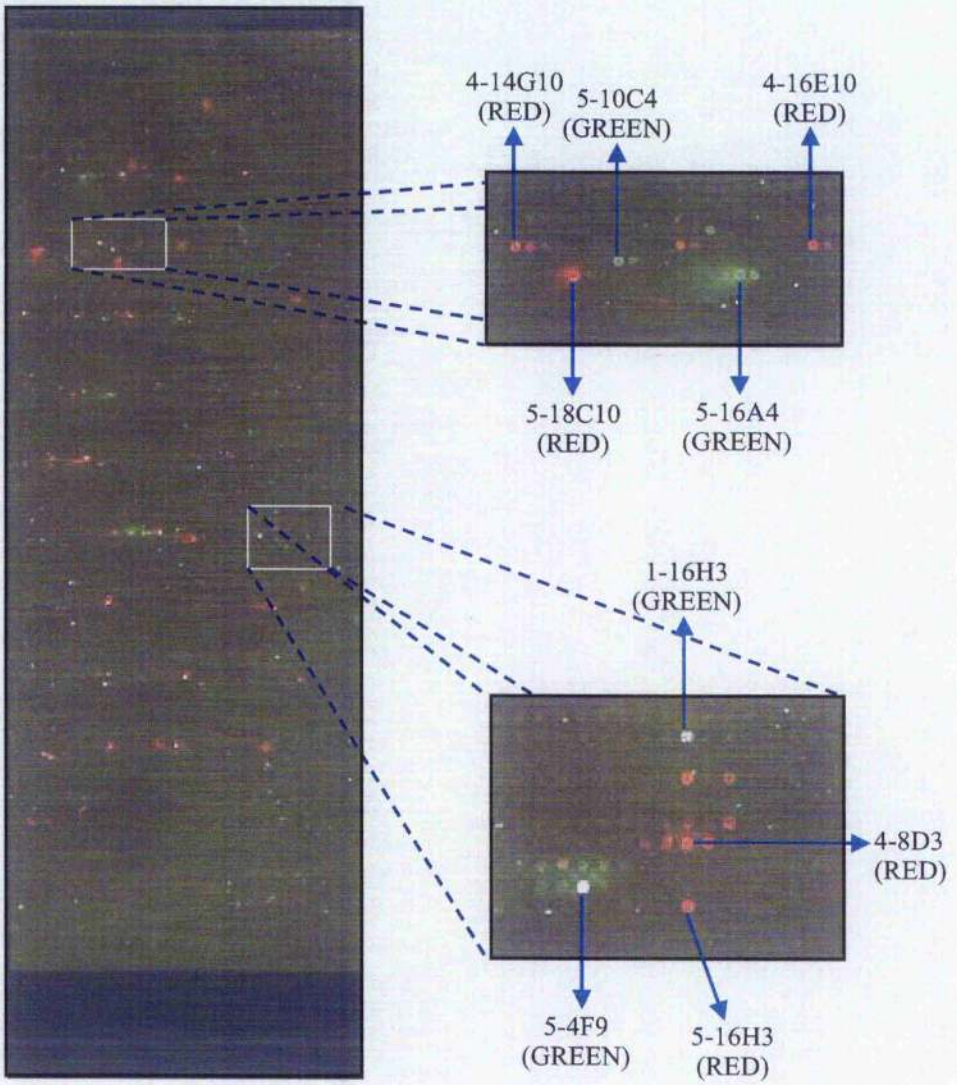
29

Abstract

The present invention relates to methods for producing monoclonal antibodies. In particular, the invention relates to high throughput methods for producing and screening monoclonal antibodies more rapidly than conventional methods.

1/10

FIG. 1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(RED)
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(GREEN)
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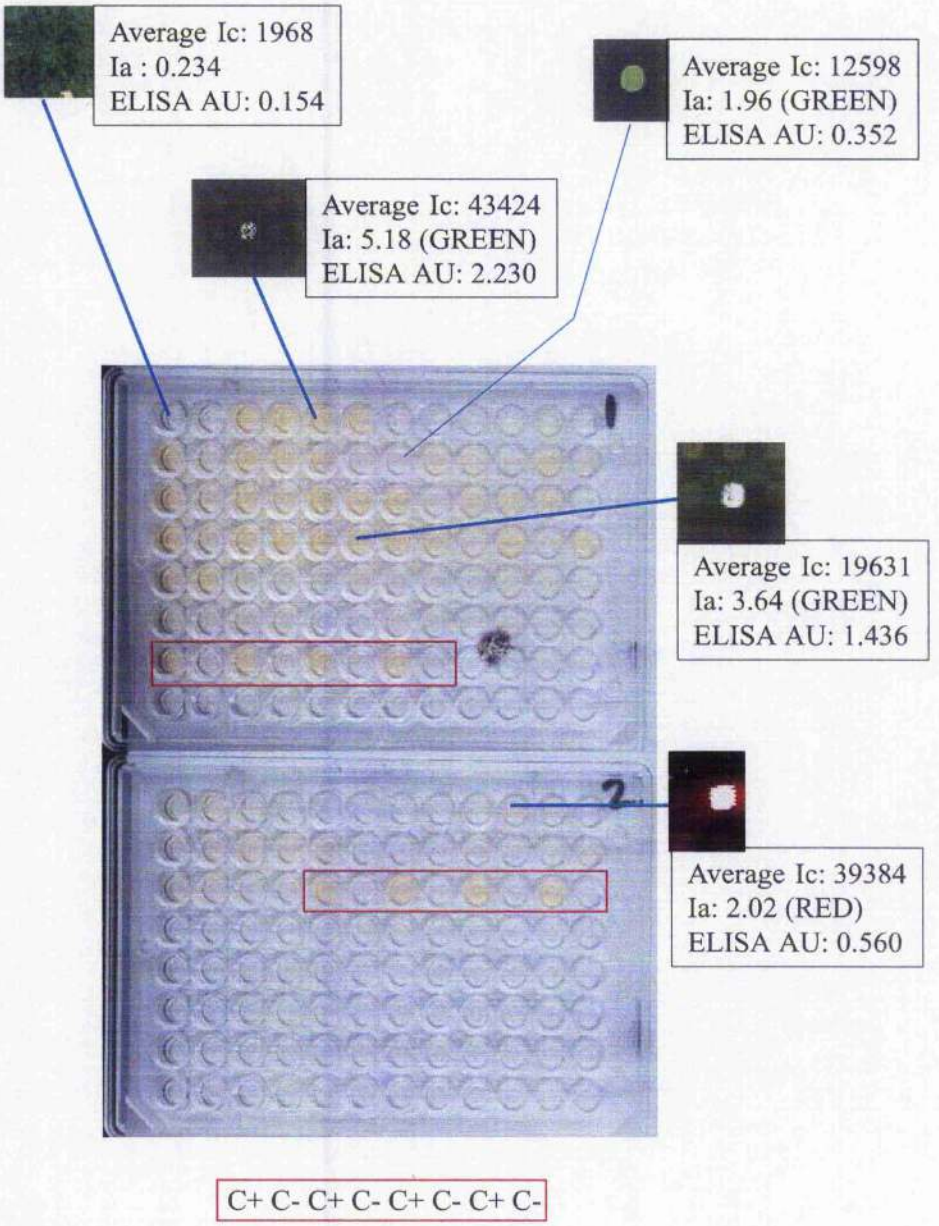
(GREEN)
5-16H3
(RED)

2/10

FIG. 2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Average Ic: 12598
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ELISA AU: 0.352
Average Ic: 43424
Ia: 5.18 (GREEN)
ELISA AU: 2.230
Average Ic: 19631
Ia: 3.64 (GREEN)
ELISA AU: 1.436
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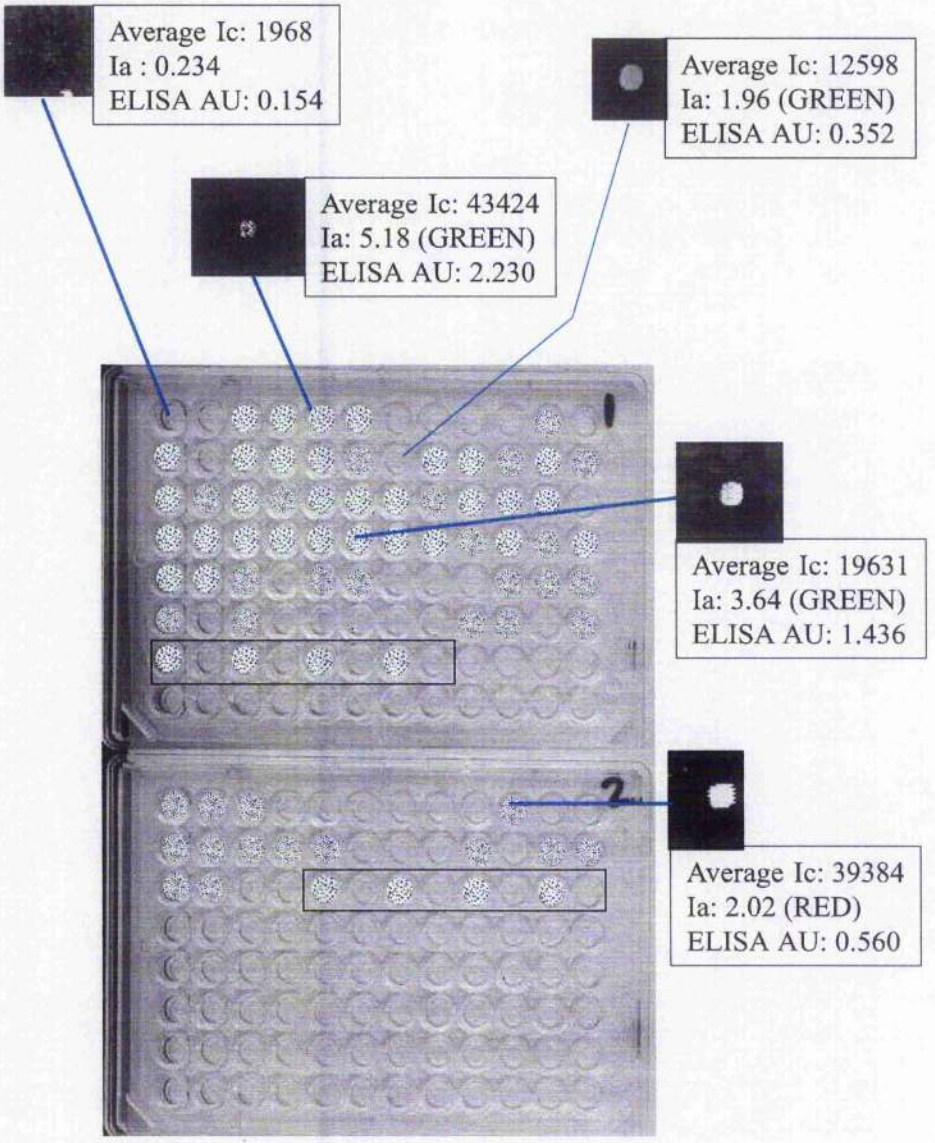
Ia: 2.02 (RED)
ELISA AU: 0.560
C+ C- C+ C- C+ C- C+ C-

FIG. 2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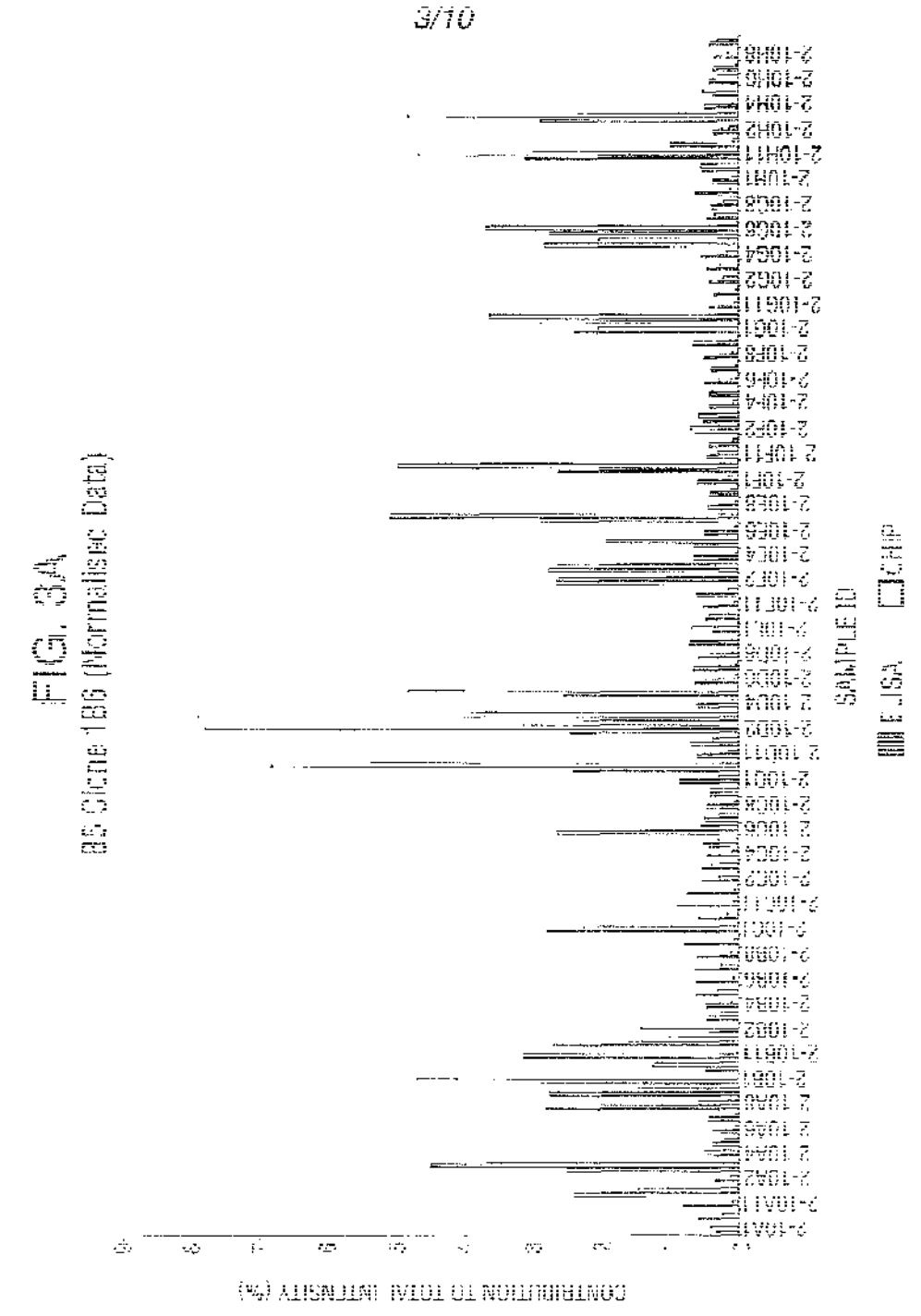
3/10
FIG. 3A
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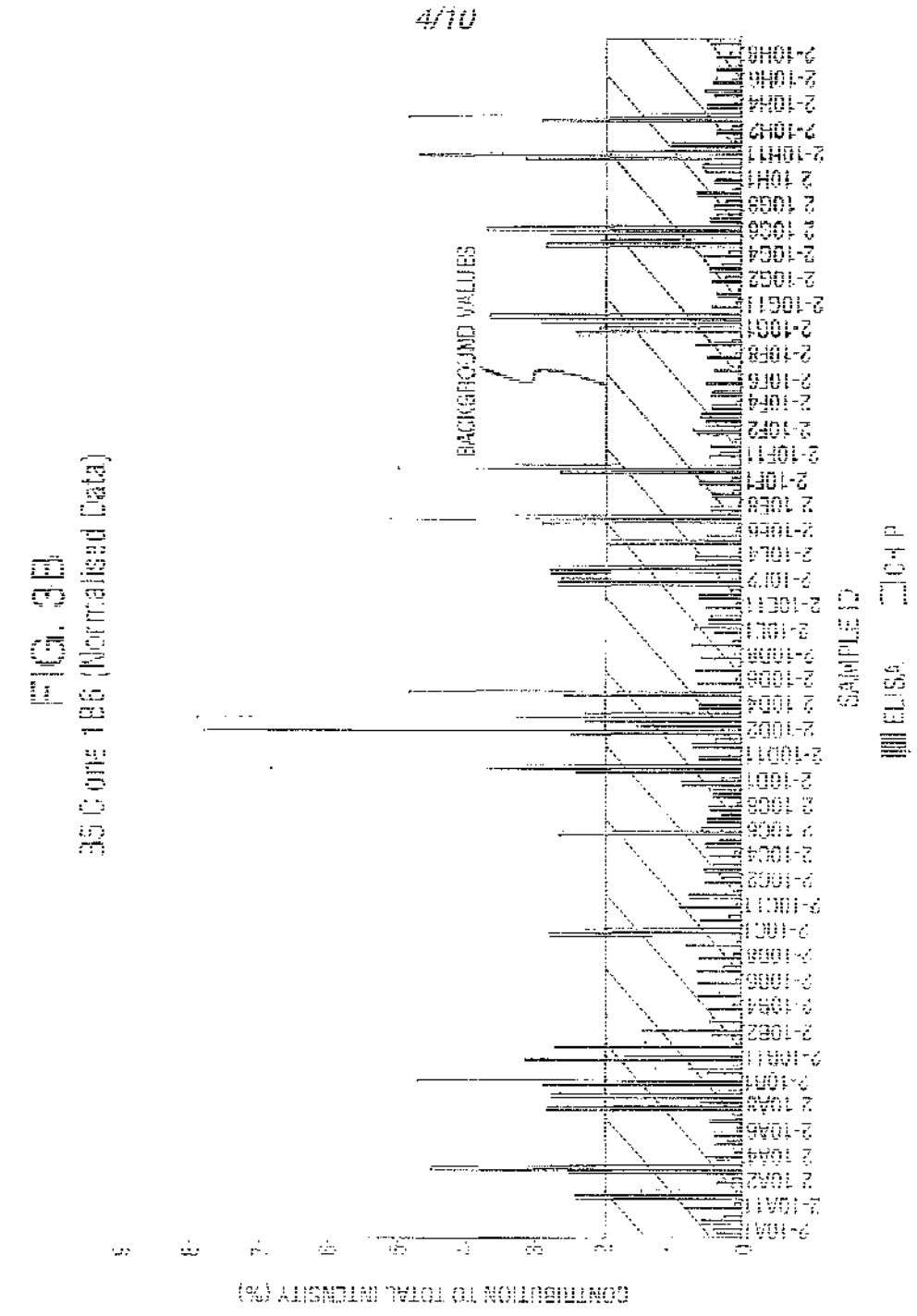
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FIG. 3E
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BACKGROUND VALUES
SAMPLES
ELISA
CONTRIBUTION TO TOTAL INTENSITY (%)

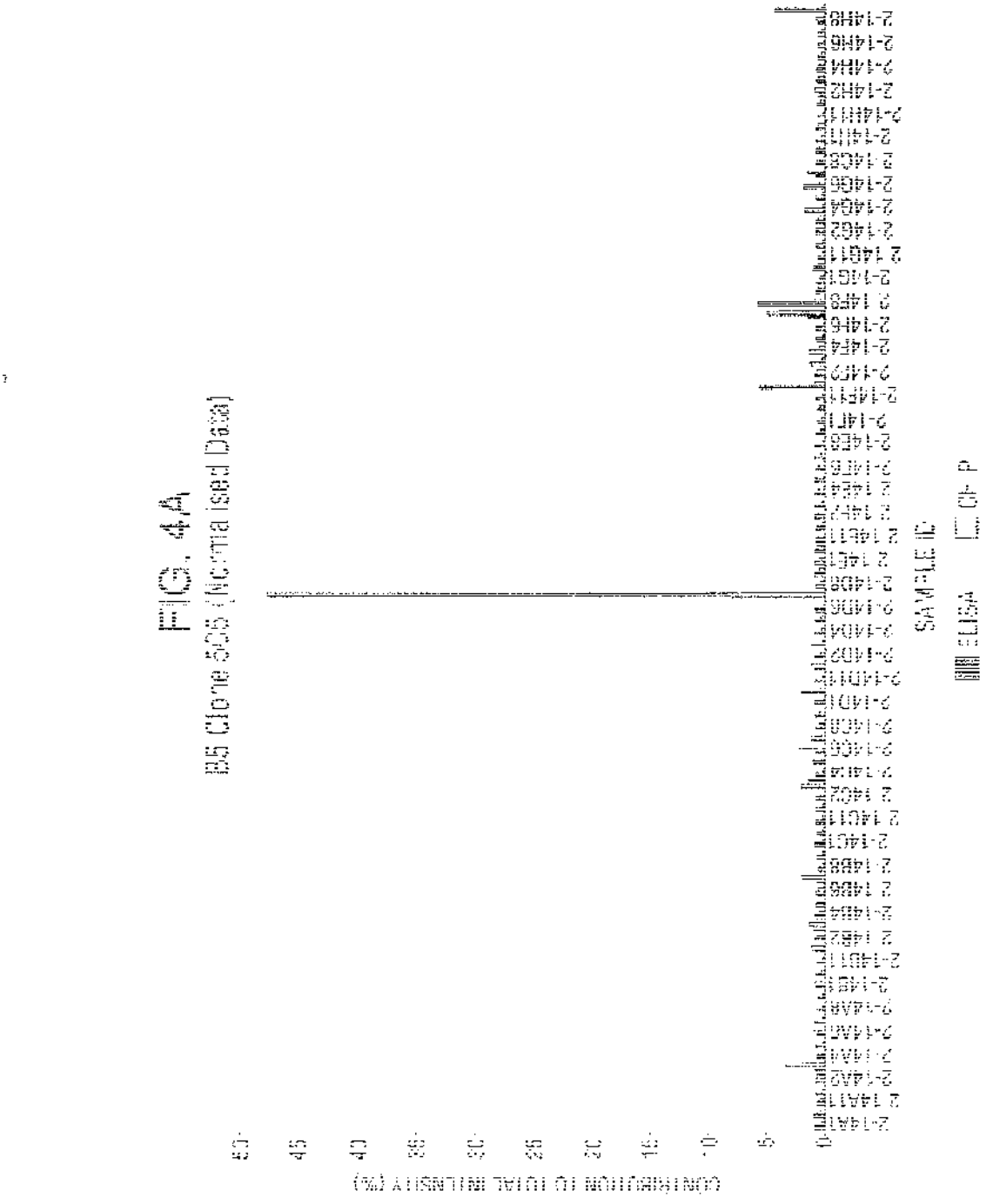
FIG. 4A
B5 Clone 525 (Neutralized Data)
SAMPLE ID
ELISA
CONTAMINATION TO TOTAL MELANIN (%)
50
45
40
35
30
25
20
15
10
5
2-14A1
2-14A11
2-14A2
2-14A7
2-14A4
2-14AC
2-14AB
2-14AE
2-14D11
2-14B2
2-14B4
2-14B6
2-14B8
2-14D1
2-14C11
2-14C2
2-14C4
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6/10

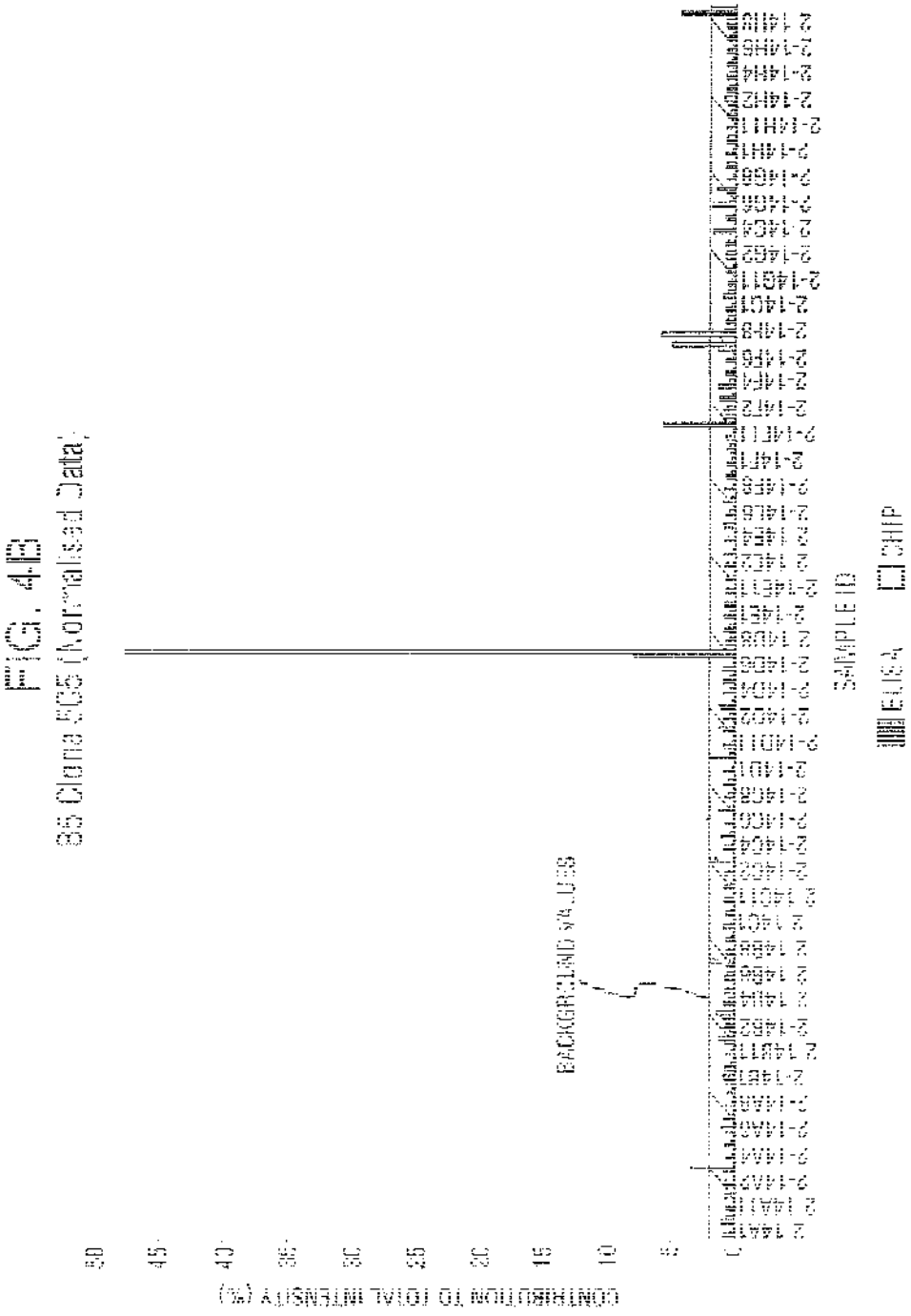
FIG. 4B
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SAMPLE ID
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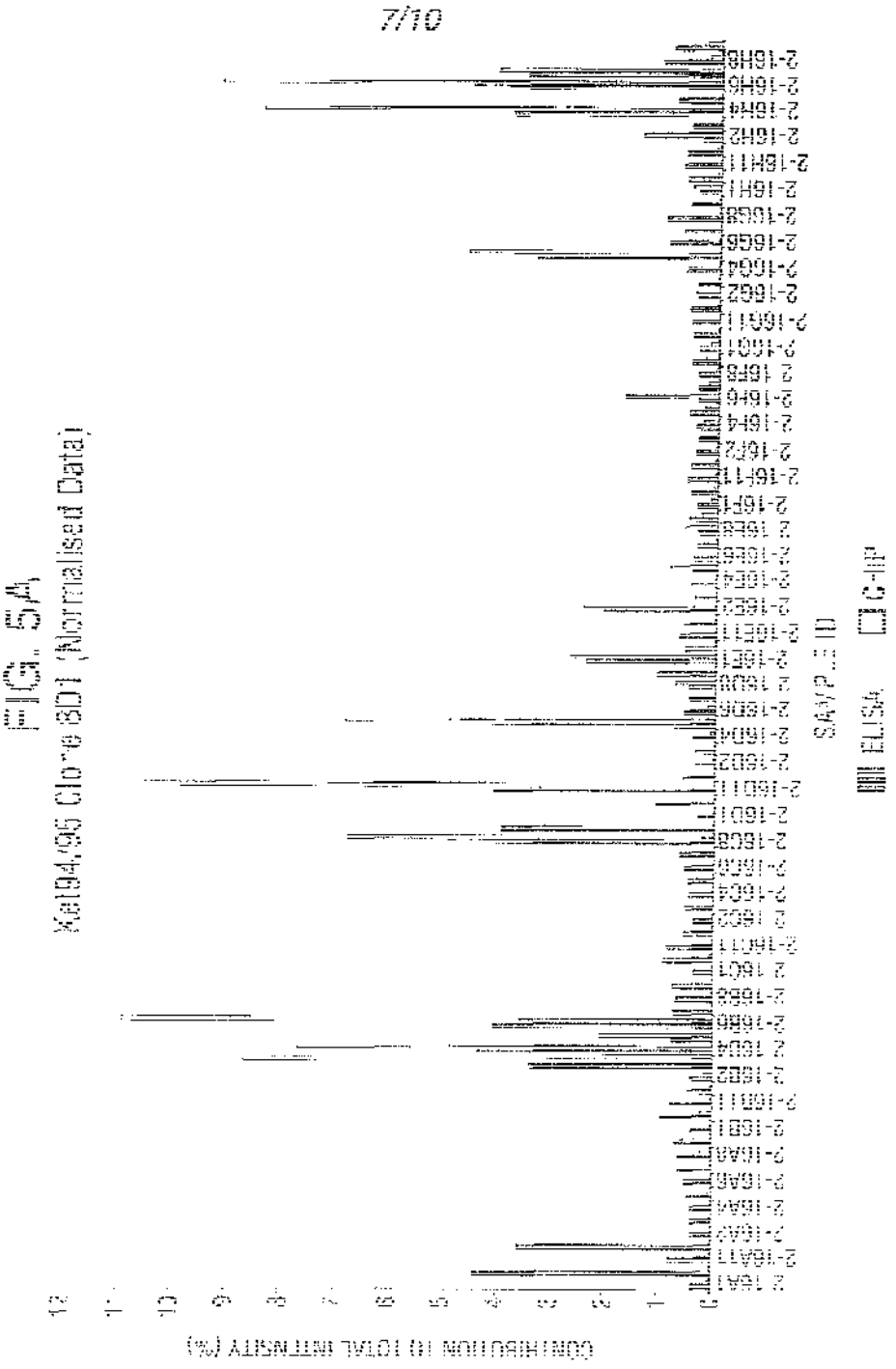
CONTRIBUTION TO TOTAL INTENSITY (%)


7/10
PLOT 5A
Xe194/95 Clone 8D1 (Normalised Data)
SAVP - ID
ELISA
CTC-HP
CONTRIBUTION TO TOTAL INTENSITY (%)

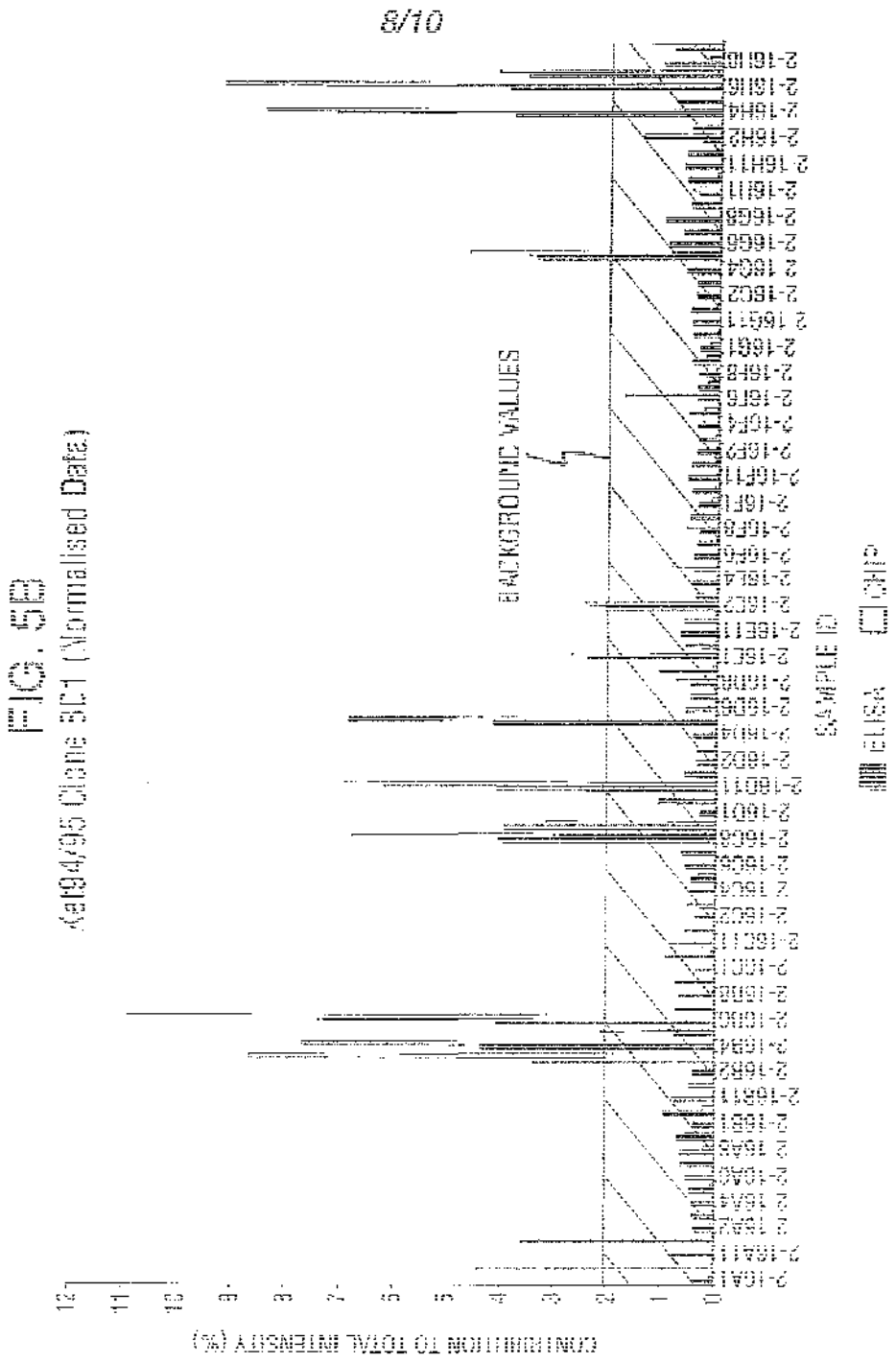


FIG. 5B
Ag194/95 Clone 5C1 (Normalised Data)
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SAMPLE ID
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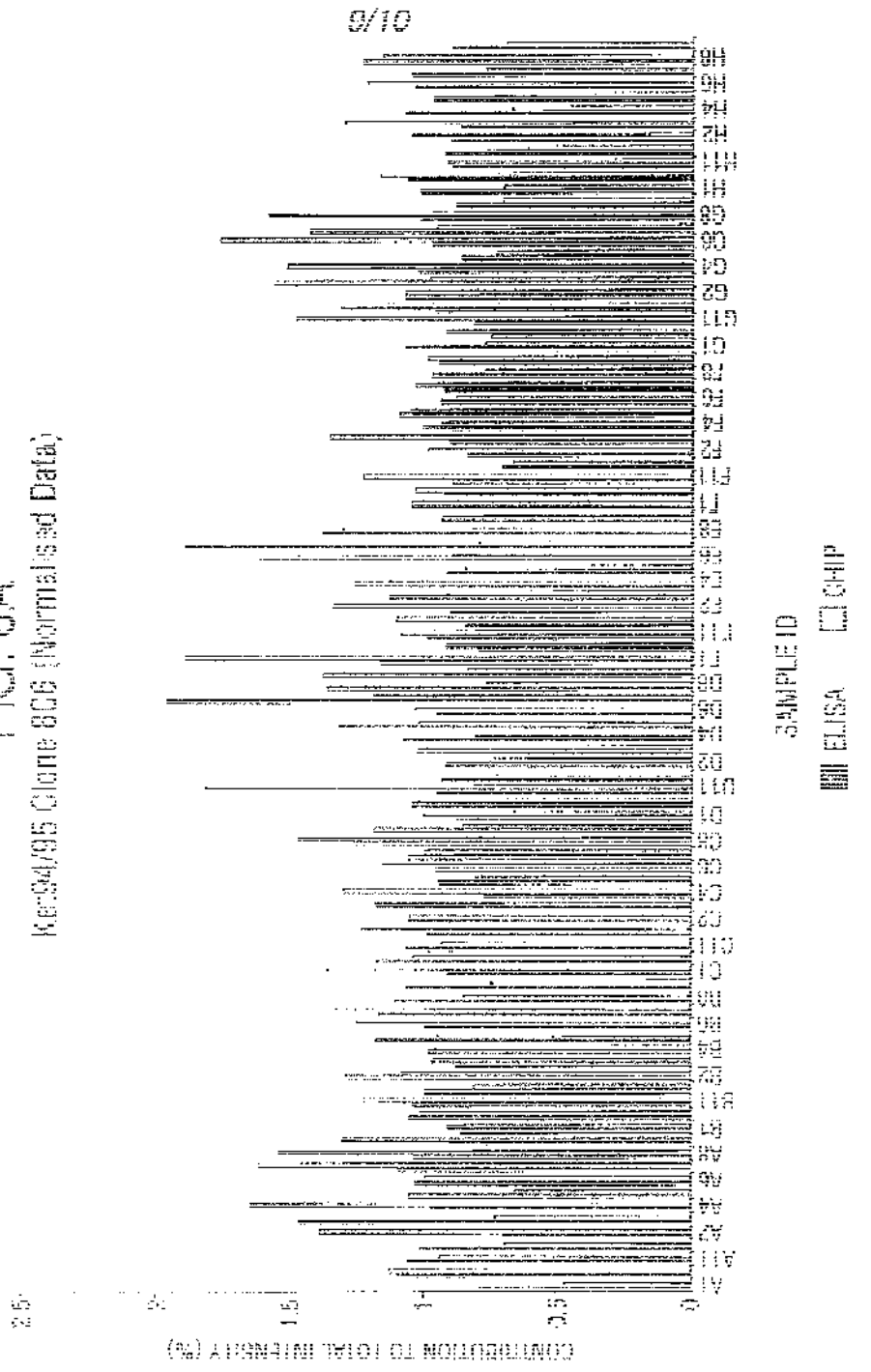
CUMULATIVE TO TOTAL INTENSITY (%)


9/10
PICA, 6A
Ker-94/95 Clone 6C6 (Normalised Data)
CONTRIBUTION TO TOTAL INTENSITY (%)
SAMPLE ID
ELISA
CHIP

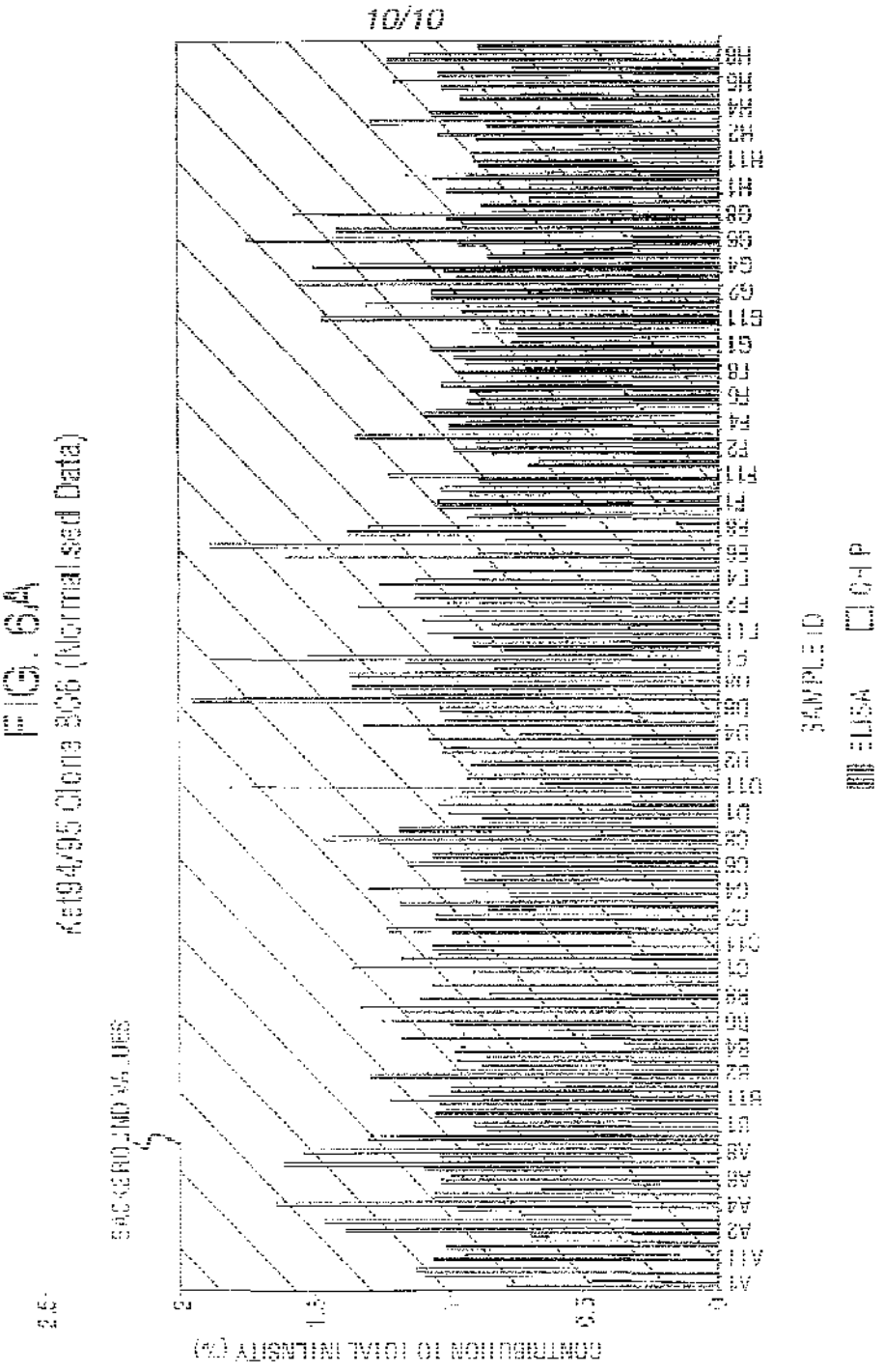

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FIG. 6A
Ke9/4/95 Clone 3C6 (Normalised Data)
BACKGROUND VALUES
CONTRIBUTION TO TOTAL INTENSITY (%)
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ELISA
C-I-P

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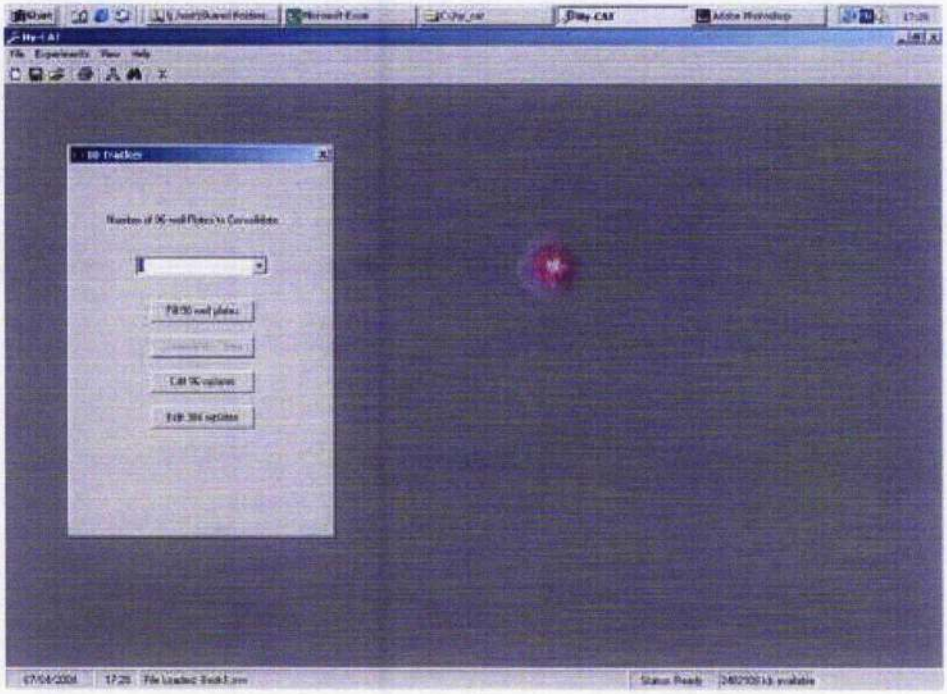
8 Contents of the enclosed CD

A CD containing a working version of Hy-CAT is enclosed with this manuscript. The software has been tested with Windows 98 and 2000. To use the software, copy all contents of the CD into a new directory (eg: c:\Hy-CAT) and launch Setup.exe

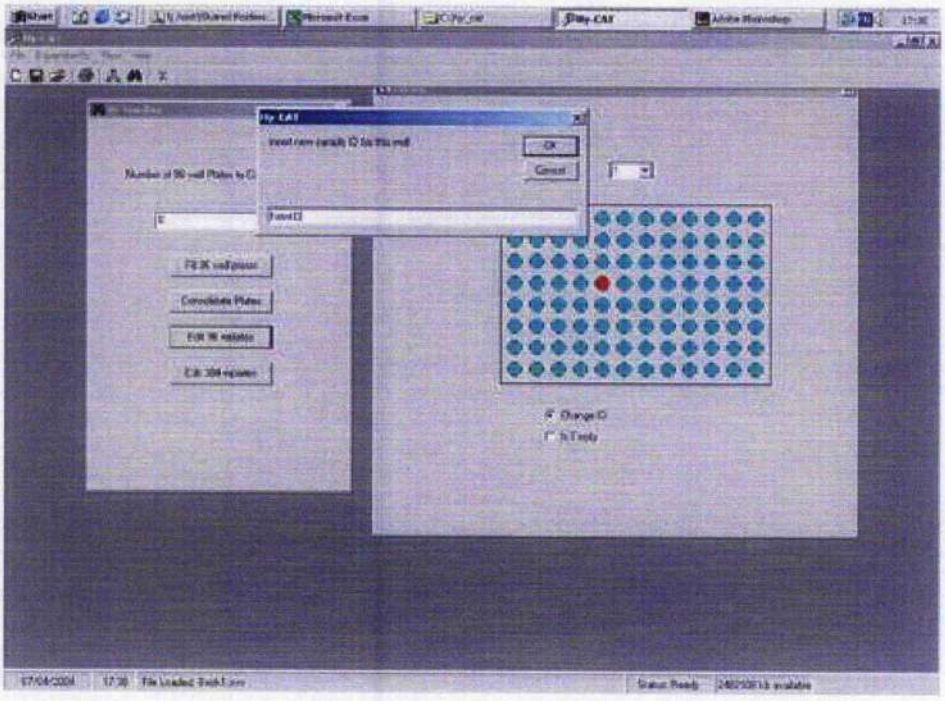
8.1 Preparation of the plate definitions

To prepare a new set of plate definitions, select "Experiment –> MakeIDs" from the menu bar:

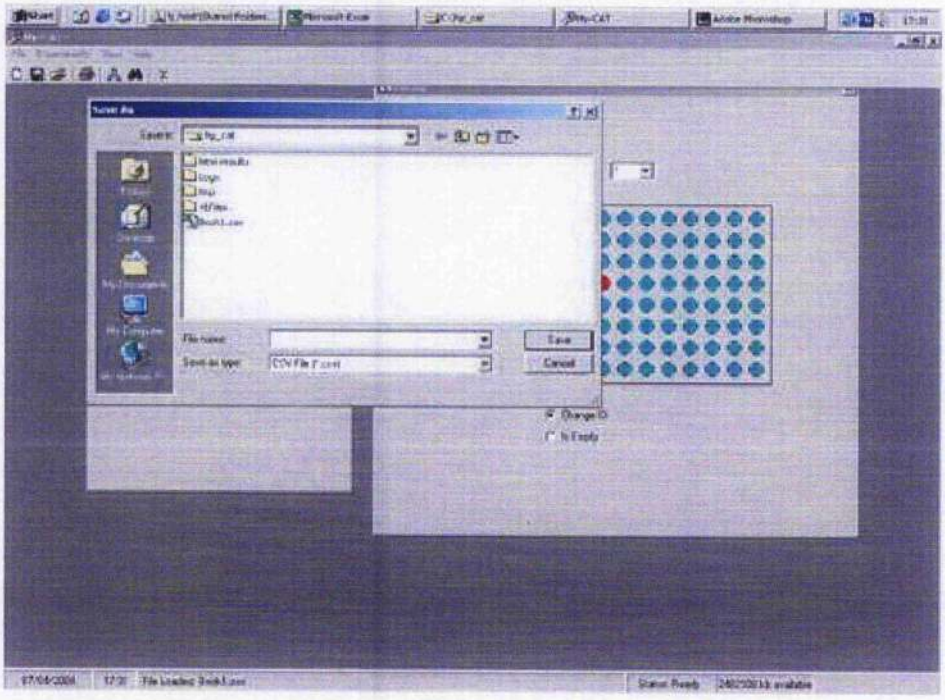
- Enter the number of 96 well plates to be consolidated into 384 well plates:



- Click on the "Fill 96 well plates" button. This will populate the table as described in Section 7.1 (page 103). It is possible to change the ID of individual wells (new ID or mark as "empty") by clicking onto the desired well:



- When ready, click on the "Consolidate Plates" button, enter a filename in the "Save file" dialog box. The created file will be ready to be used by any spotting software to generate a chip definition grid:



8.2 Chip analysis

Data analysis can be done by selecting the "Experiment –> New" menu item.

- Click next on the first screen:

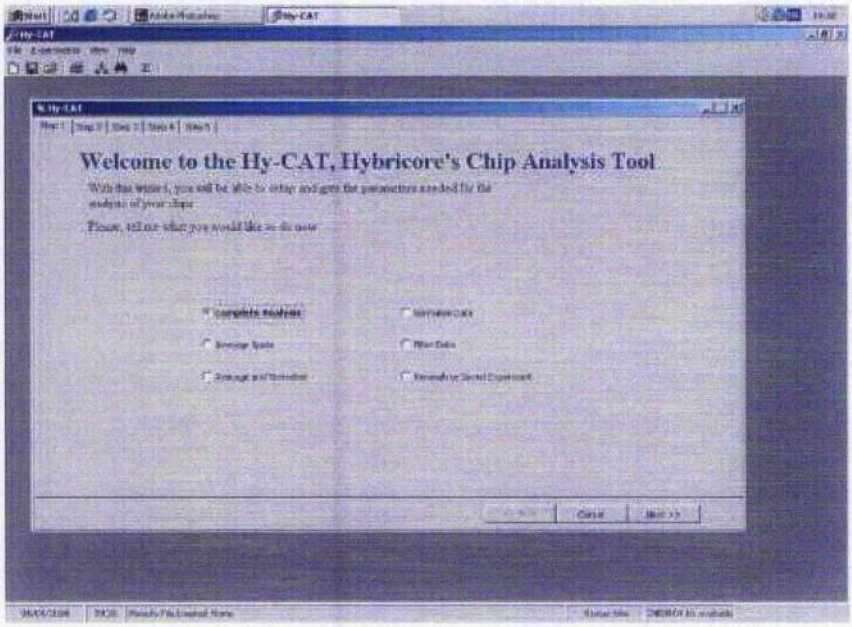
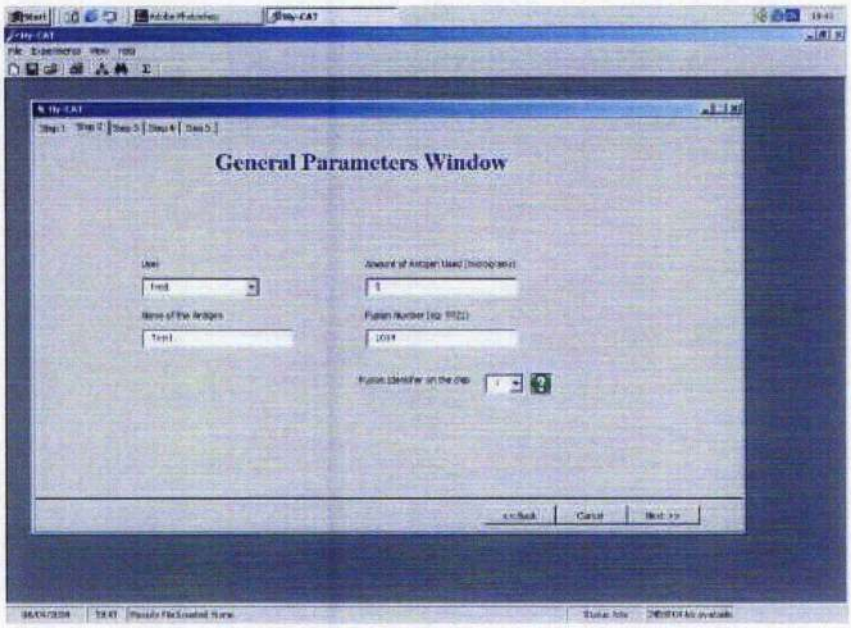
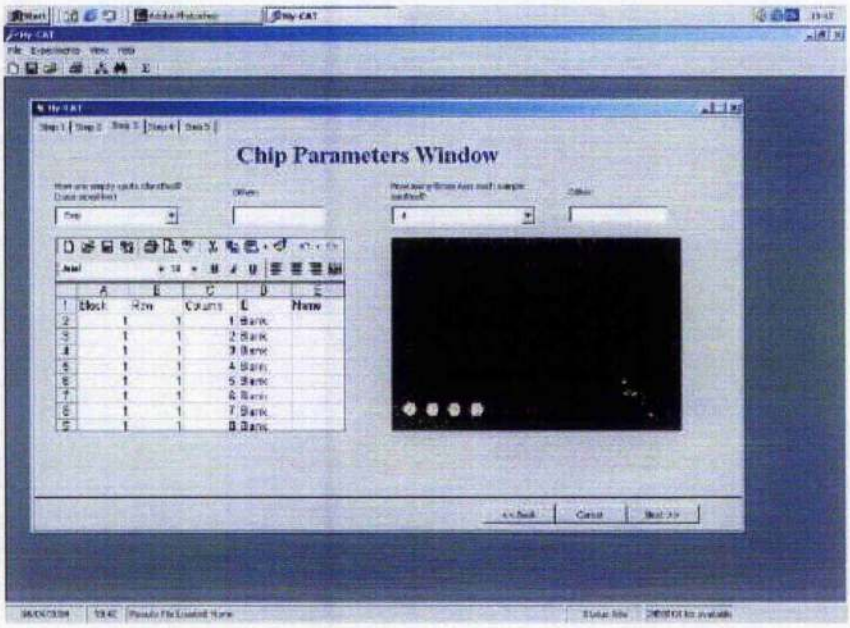


Figure 30. Data analysis: Step 1

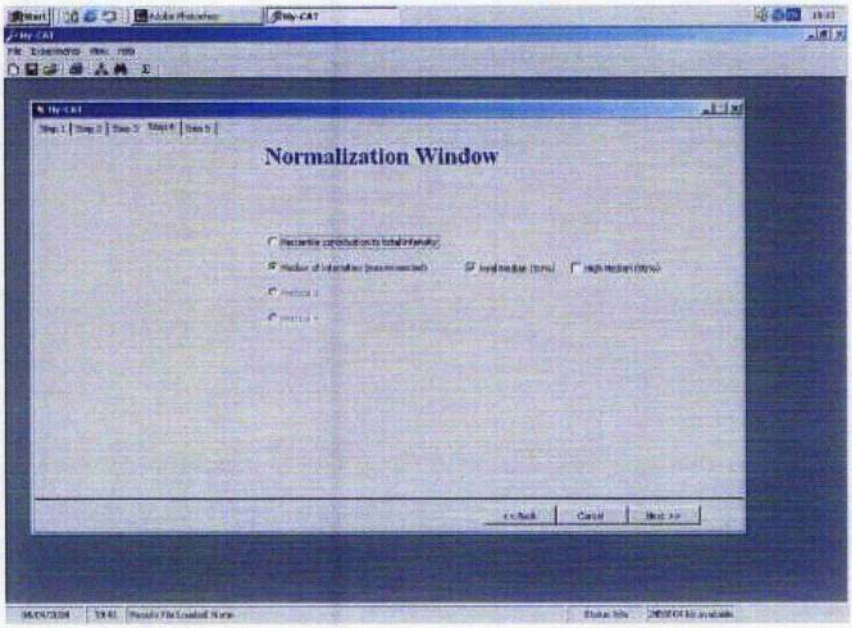
- Fill the "antigen description" fields:



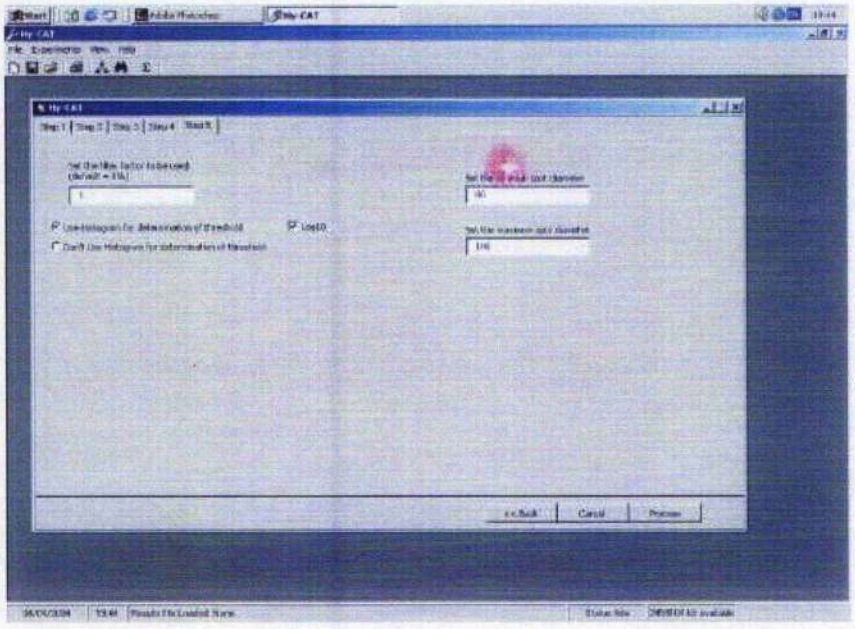
- Select the spotting parameters:



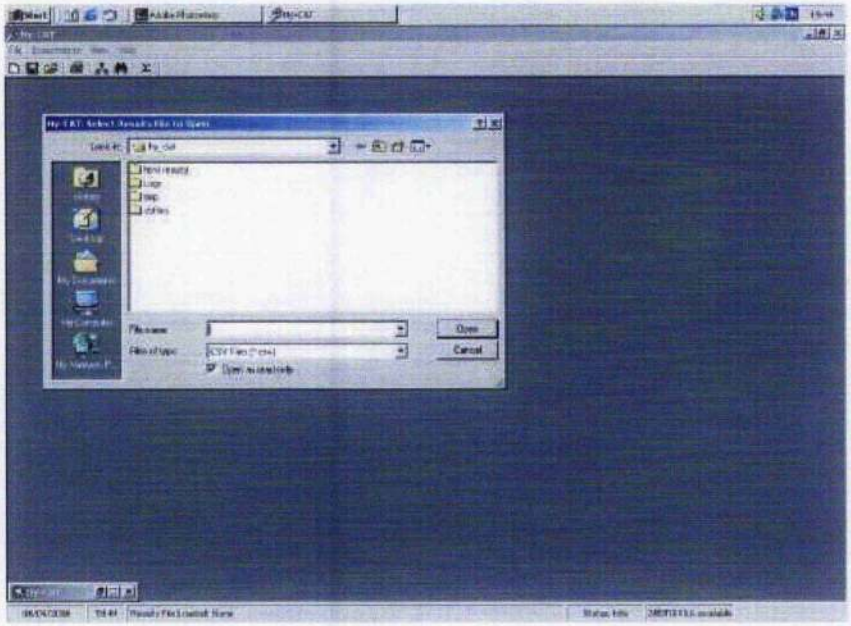
- Select the required normalisation protocol:



- Insert filtering parameters and selection methods:

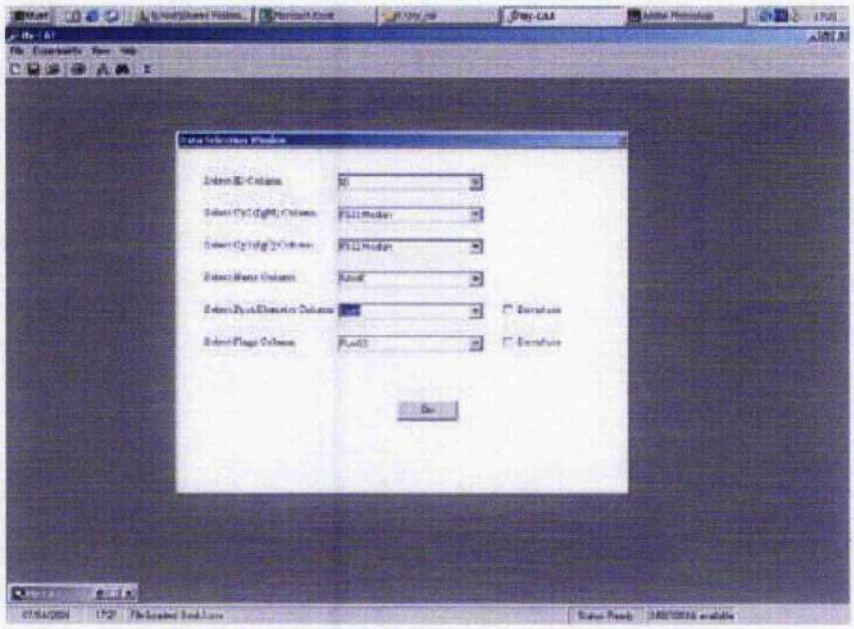


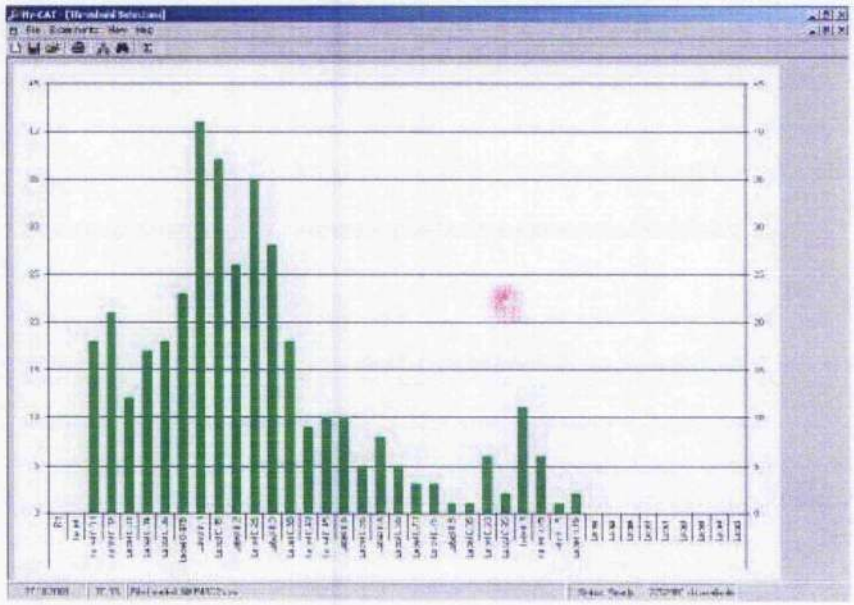
- Select and load data file:

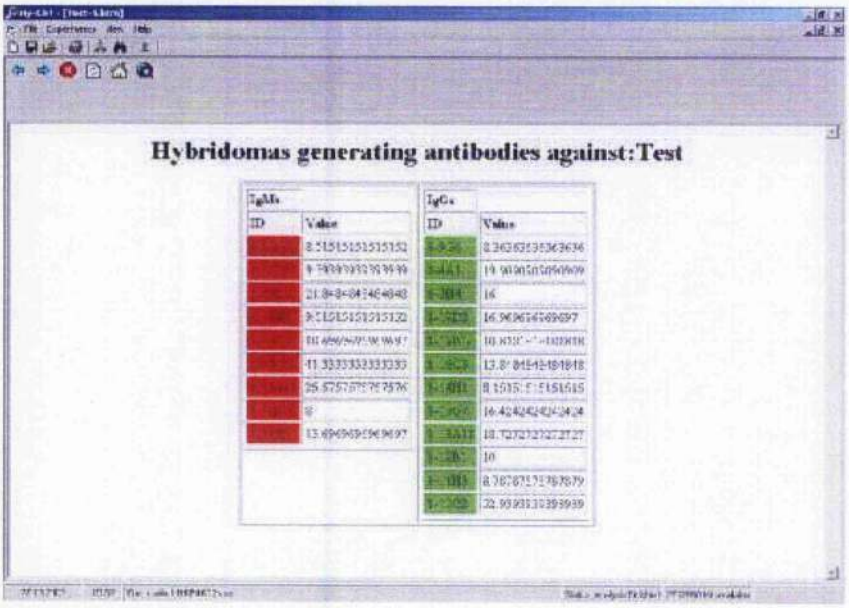


- Select the data fields for the analysis and click the "Go" button to start the calculations:

- When the calculations have been completed, a histogram of the distribution of intensities is presented. Click any histogram bar to use that value as a selection threshold and obtain the IDs of all hybridomas showing an intesity greater than the chosen threshold. These selected hybridomas are displayed in an HTML page, divided by isotype (IgM: Red, IgG: Green)



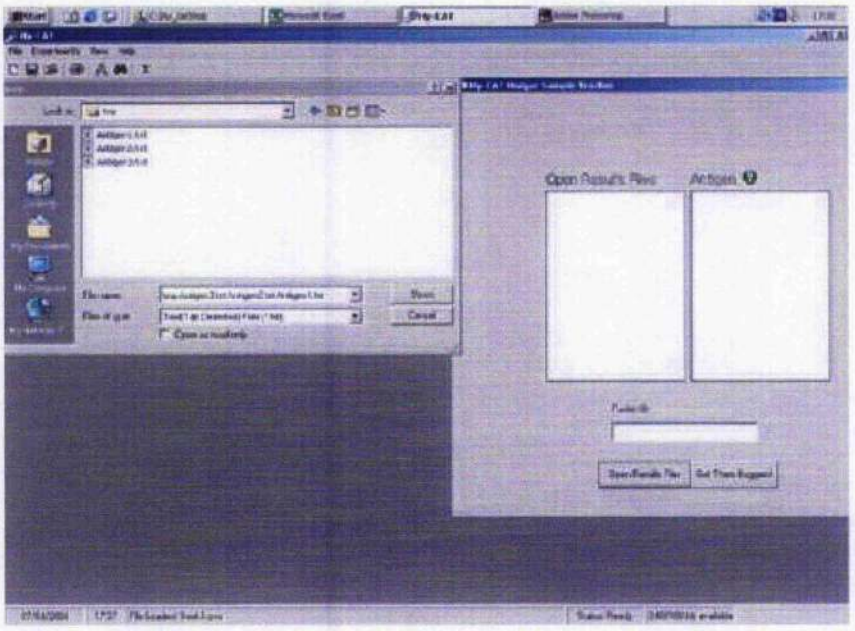




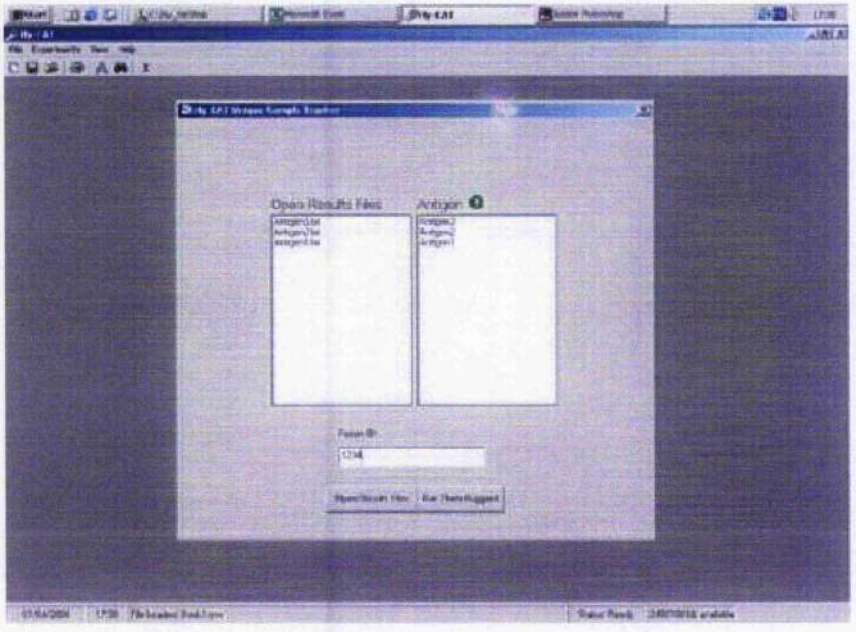
8.3 Selection of "Unique" samples

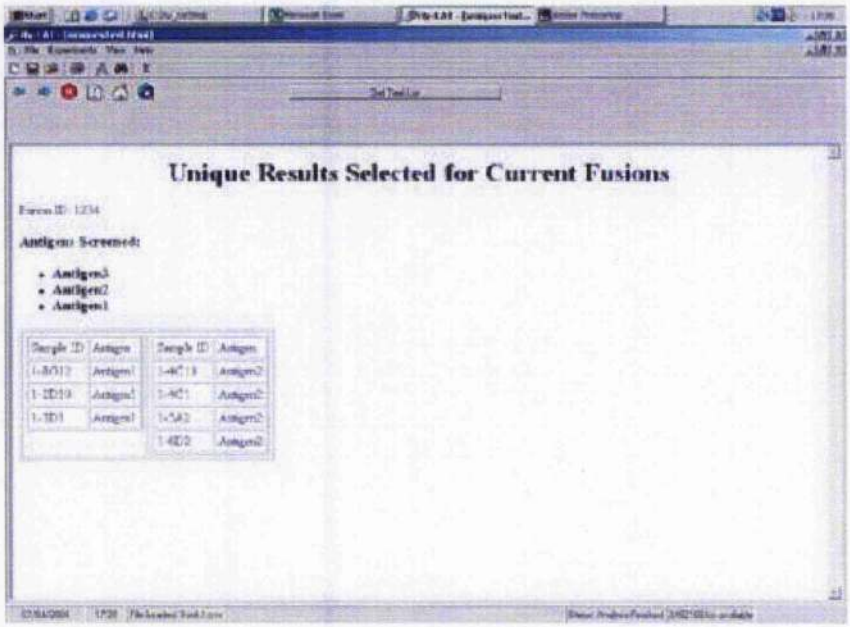
When more than one antigen have been screened in a single experiment, possible false positive, "sticky" or cross reactive antibodies have to be detected and eliminated from the result lists. For this purpopse, HyCAT generates text files containing the selected IgG and IgM antibodies after each screening procedure. This files are needed for the detection of unique samples. From the menu bar, select "Experiment –> FindUniques".

- Click on the "Load the results files" button and select the required files to be analysed:



- Enter a FusionID in the text box and click on the "Get them b...." button. All the "unique" samples will be displayed in antigen specific tables in an HTML file.





This Thesis was written using LyX 1.3.2 powered by TEX version (Web2C 7.4.5) 3.14159, running on a SuSe 9.0 platform.