A multiplexed antibody-based approach for analysis of denatured cellular proteins -Development of western-MAP

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TABLE OF CONTENTS

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Anette Lie Christensen

ABSTRACT

This thesis describes a new method for multiplexed analysis of cellular proteins. The cellular proteins were biotinylated and fractionated by gel-electrophoresis using a device (Gelfree 8100), which yields liquid fractions containing proteins of different sizes. Color-coded microspheres with antibodies to cellular proteins were added to the fractions. After incubation with the microspheres, captured proteins were labelled with fluorescent streptavidin and detected by flow cytometry. The results obtained with the assay resemble those obtained with standard western blotting. However, while western blotting is used to detect one or a few proteins at a time, the array-based assay developed here can be used to measure thousands of proteins simultaneously. This approach is hereafter referred to as western-MAP (Microsphere-based Affinity Proteomics).

Variables that were found to affect performance of western-MAP included sample loading, choice of protein label, type of gels, addition of detergents and removal of free SDS from the fractions. The assay performance was optimal when the gels were loaded with 230µg of protein. Combined labelling with amine- and thiol-reactive biotin was superior to either reagent used alone. Protein detection was enhanced when SDS in the protein fractions was removed by potassium chloride (KCl) precipitation, and further enhanced by the addition the non-ionic detergent Tween 20. The most useful gels contained 8% and 10% acrylamide.

The microsphere-based arrays that were used in this study contained thousands of antibodies. Among these, 537 were found to capture a protein with a size compatible with that of the intended target. For 89 antibodies we had access to western blotting data obtained with the same cell lysates. A total of 76.4% of these antibodies showed comparable results between standard western blotting and the method developed in this thesis. Thus, the work resulted in successful multiplexing of western blot, which is one of the most widely used assays in protein research.

SAMMENDRAG

Denne avhandlingen beskriver en ny metode for multipleksanalyse av cellulære proteiner. De cellulære proteinene ble biotinylert og fraksjonert ved gel-elektroforese ved hjelp av et instrument (Gelfree 8100), som gir væskefraksjoner med proteiner av forskjellige størrelser. Fargekodede mikropartikler med antistoffer mot cellulære proteiner ble tilsatt fraksjonene. Proteiner bundet av antistoff ble merket med fluorescerende streptavidin og detektert ved flowcytometri. Resultatene fra denne metoden ligner de man får med tradisjonell western blotting, men til forskjell fra sistnevnte som kun kan analysere ett protein av gangen, kan metoden denne avhandlingen beskriver analysere flere tusen proteiner samtidig. Denne metoden blir heretter kalt western-MAP (Microsphere-based Affinity Proteomics).

Variabler som ble funnet å påvirke ytelsen til western-MAP var proteinkonsentrasjonen i prøven, valg av proteinmerking, type geler, tilsetning av detergent og fjerning av fritt SDS fra fraksjonene. Ytelsen var optimal når gelene ble lastet med 230µg protein. Kombinert merking av proteinene med både amin- og thiolreaktivt biotin var bedre enn å anvende en av disse reagensene alene. Fjerning av fritt SDS ved kaliumklorid (KCl) presipitering forbedret deteksjonen av proteinene. Når fraksjonene ble tilsatt den ikke-ioniske detergenten Tween 20, resulterte dette i lavere bakgrunn, og forbedret påvisning av membranproteiner. De mest nyttige gelene inneholdt 8 % og 10 % akrylamid.

De partikkel-baserte arrayene som ble brukt i denne avhandlingen inneholdt tusenvis av antistoffer. Blant disse, ble 537 funnet å binde et protein med en størrelse tilsvarende proteinet antistoffet var rettet mot. For 89 antistoffer hadde vi tilgang til western blott data fra de samme cellelysatene. Totalt 76,4 % av disse antistoffene viste lignende resultater i standard western blotting og metoden som ble utviklet i denne avhandlingen. Således, resulterte arbeidet i en multipleks versjon av western blot, som er en av de mest brukte metodene i proteinstudier.

1.0 INTRODUCTION

The overall aim of the work presented in this thesis was to develop an improved technology for large-scale analysis of cellular proteins. Studying proteins is fundamental for understanding cellular processes, as proteins are the functional components of all biological systems [1].

Western blotting is one of the most widely used methods to study proteins in biochemical research. The technique is relatively simple to perform and has the advantage that it discriminates intended antibody targets from cross-reactive proteins. An important limitation is that the technique is limited to measuring one protein at a time.

During the past decades there has been a tremendous development in the field of large-scale protein analysis, or proteomics. This is largely due to advances in the field of mass spectrometry (MS). With modern MS it is possible to detect thousands of proteins in one sample. Studies based on the use of MS have greatly increased our understanding of cellular proteins. However, the protocols are complex and very time-consuming [2, 3].

Antibody array analysis may provide a high throughput alternative to MS. Many attempts have been made to develop protocols for antibody array analysis [4-9]. In this assay format, the proteins in the sample are labelled with fluorescent dyes or haptens. Antibodies to proteins of interest are spotted onto predefined locations on a slide or bound to microspheres with fluorescent colour codes. The immobilized antibodies are used to capture labelled sample proteins. A limitation of the assay format is that the specificity is determined by the capture antibody alone. This is an important limitation since antibodies often bind more than one target. The issue is further complicated by the fact that many cellular proteins occur in a variety of multi-molecular complexes. Thus, even a mono-specific antibody can bind more than one protein. A review from 2002 describes antibody array analysis as a western blot where all the bands in the lane are compressed into one[10]. Thus, all cross-reactive binding contribute to the signal.

The goal for the work performed in this thesis was to develop an "antibody array western blot". This could be achieved by fractionating biotinylated cellular proteins by gel-electrophoresis. However, rather than blotting the proteins from the gel over to a membrane, they could be eluted into liquid fractions, using the Gelfree 8100 fractionation system from Expedeon Inc. The fractions will contain proteins with a narrow size distribution, and when a series of fractions is

analysed by antibody arrays, the results should be similar to those obtained by western blotting, except that thousands of antibodies could be used in parallel. We call this approach Western-MAP (microsphere-based affinity proteomics).

The primary goal for this thesis was therefore:

To develop a method for large-scale analysis for cellular proteins using microsphere-based affinity proteomics (MAP) combined with a method for size fractionation of denatured proteins.

To achieve this goal I first investigated if the Gelfree fractionation system is suitable for fractionating proteins prior to analysis with MAP. Next, I optimized the conditions to get the best results possible, before the new approach was compared to traditional western blotting.

2.0 BACKGROUND

2.1 HUMAN GENOMICS

The human genome consists of all the genomic information in humans. The first draft of the human genome (HUGO-project) identified the presence of between 30.000 and 40.000 protein coding genes [11], but later studies have showed that the correct number is closer to 20.500 [12]. The genes provide a blueprint for all the building blocks that constitute the human organism: the proteins. Understanding the genes is therefore essential for understanding biology and disease.

There are a number of methods that allow genome-wide analysis. Microarrays have been available for more than two decades. The arrays consist of planar slides where fragments of DNA are spotted to predefined locations (Fig. 1). The DNA, cDNA or RNA in the sample to be analysed is labelled with fluorescence, and applied onto the slide. The fragments that are complementary to the fragments on the slide will hybridize, and the signal can be detected with a fluorescence scanner [13]. DNA microarrays provided the first possibility to perform genome-wide analysis.

For the last approximately 35 years the Sanger method has been used for sequencing genomes [14]. This is a resource-demanding method, and whole genome sequencing has therefore been limited to large sequencing centres. Almost ten years ago the first next-generation sequencing technology was commercialized. In the recent years these methods have evolved, and whole-genome sequencing can now be done in a matter of days. This has opened completely new possibilities for studying the human genome. The challenge today is not sequencing the genes, but analysing the tremendous amount of data acquired.

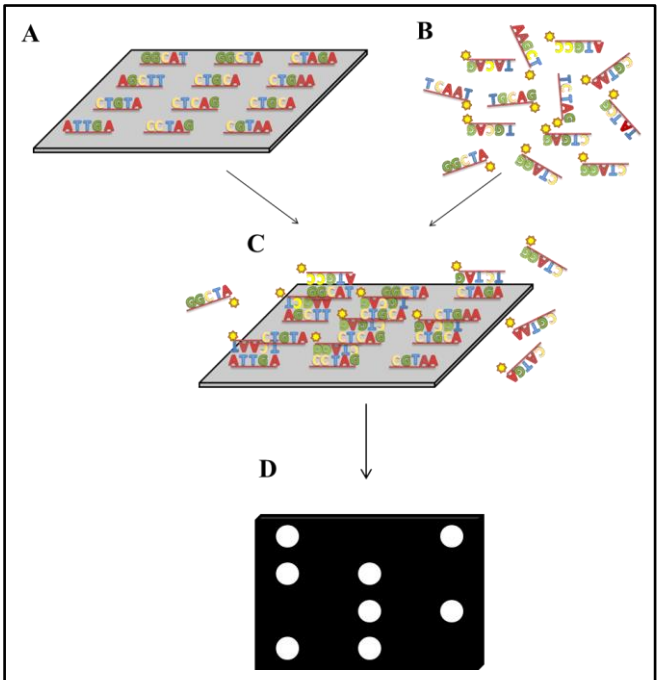

Fig. 1. DNA microarray. This is a schematic presentation of DNA microarray. **A:** Fragments of DNA is spotted onto predefined locations on a planar slide. **B:** The sample DNA or RNA are labeled with a fluorescent tag. **C:** The labeled sample DNA or RNA is applied to the slide, and fragments that are complimentary to fragments on the slide will hybridize. **D:** The fluorescence signal from the hybridized sample fragments can be detected.

2.2 HUMAN PROTEOMICS

Genomics provide information about the primary structure of proteins. However, proteins are more complex. Eukaryotic genes have both protein coding exons and non-coding introns, and the introns must be removed from the mRNA transcripts before translation. This process is called RNA-splicing, and a given mRNA can often be spliced in different ways to provide multiple products. This is called alternative splicing and results in that a given gene can give rise to more than one protein species. About 95% of multiexon human genes are subject to alternative spicing[15].

After translation the proteins can undergo post translational modifications (PTM). Over 300 such modification exists, and new modifications are discovered regularly [16]. The most important PTMs are phosphorylation, glycosylation, formation of disulphide bridges or cleaving of a pro- peptide.

Due to alternative splicing, there are more protein species than protein coding genes, and PTMs give rise to an even greater diversity (Fig. 2). It has been suggested that the human genome potentially can produce 1.8 million different protein species [16]. Today the Uniprot database has more than 23.000 reviewed entries for human proteins [17].

The genome is relatively stable, whereas the transcriptome and the proteome are constantly changing. The transcriptome is all the mRNAs in an organism, a cell or a population of cells at a

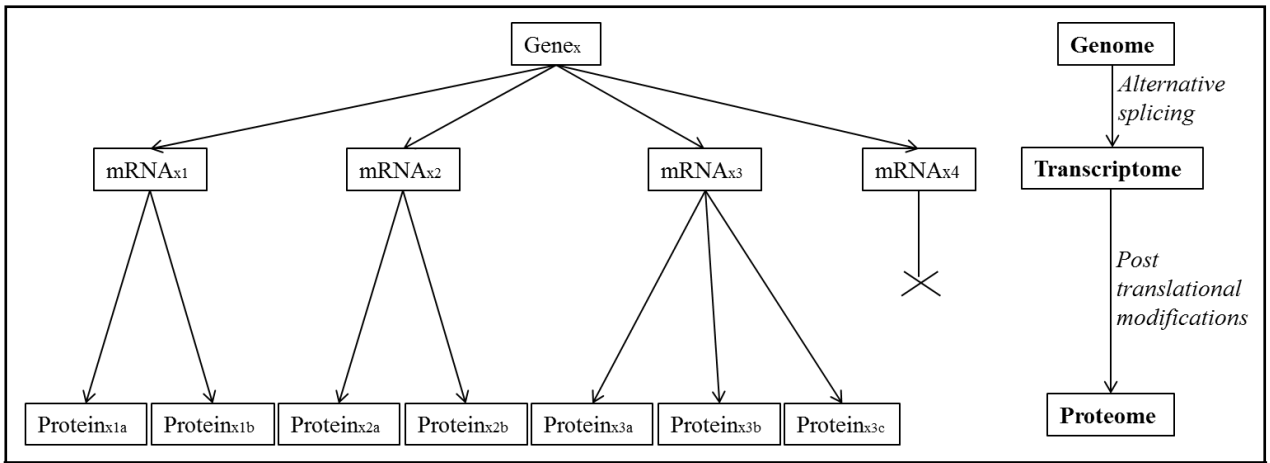

Fig. 2. The complexity increases from the genome to the proteome. Many transcripts (mRNAs) can be produced from one single gene due to alternative splicing, and not all mRNAs are translated into proteins. Post translational modifications can result in several different protein species from the same mRNA. As a consequence of this, humans have a greater number of proteins than the number of protein coding genes. The exact number of proteins that make up the human proteome remains unknown, but up to 1,8million protein species has been suggested [(15)].

given time, under given circumstances, and the proteome is the equivalent for proteins. The transcriptome can to a certain extent be used to predict protein levels, however not all mRNA is translated, and different mRNA is translated and degraded at differential rates. It is possible to envision the genome as what the cell knows how to do, the transcriptome as what the cell is thinking about doing and the proteome as what it is currently doing. It is fair to say that while genes encode biological systems, the proteins are biological systems. It is therefore essential to study proteins to understand the cellular systems and processes.

Initiatives similar to the human genome project have now been taken to characterize the human proteome. This project is called the human proteome project (HPP). This project may seem like a logical extension of the HUGO-project, but it has been questioned if the current technology is ready to handle such an immense task [18]. The experimental strategy suggested for the human proteome project is to use three working pillars: MS, antibody capture and immunohistochemistry and bioinformatics [19]. Even though there has been a tremendous development in all these fields, the technology is not nearly as robust as those used to sequence the genome.

2.3 PROTEIN SEPARATION

The proteome is highly complex, and identifying individual proteins in complex biological samples is therefore a difficult task. To simplify the task, the samples are often fractionated to reduce sample complexity. Fractionation methods are therefore fundamental for protein analysis. The proteins molecular mass is a highly predictable parameter for fractionation, since it is usually unaffected by sample or solvent conditions.

2.3.1 ELECTROPHORESIS

Polyacrylamide gel electrophoresis (PAGE) is a widely used approach for fractionating proteins according to their molecular weight (MW). Electrophoretic fractionation is based on the principle that charged molecules migrate under the influence of an electric field. PAGE is carried out by adding the samples to wells on the polyacrylamide gel and applying an electrical current over the gel. The migration of the molecules in the gel is influenced by the size, shape and net charge of the molecules [20, 21]. The difference in migration results in molecules with similar properties ends up at the same place in the gel, called a band.

Gel electrophoresis can be carried out with both native and denatured proteins. When performing gel electrophoresis with native proteins, it is not possible to distinguish between the effects of the size, shape and net charge to the migration speed through the gel. This means that proteins with different MWs can have the same mobility in the gel, and end up in the same band.

This limitation with native-PAGE is overcome in SDS-PAGE. Here the proteins are heated with the anionic detergent sodium dodecyl sulphate (SDS) prior to electrophoresis. SDS binds most proteins, and provides them with a negative charge. Binding of SDS also causes denaturation of proteins by disrupting all the non-covalent protein bonds. The results is that all proteins migrate towards the anode, and the migration speed will only depend on the size of the protein [20].

SDS disrupts all non-covalent protein bonds, but it is also desirable to break the disulphide bonds. Disulphide bonds are covalent connections that can form between thiol-groups on two cysteine-residues in a protein, or between two cysteine-residues on separate proteins. Disulphide bonds are broken by treating them with reduction agents. The most widely used reduction agents are DTT (dithiothreitol) and β-mercaptoethanol, but in the last decade TCEP (tris(2-carboxyethyl)phosphine) has been available [22]. An advantage of TCEP is that the agent does not contain thiols and is therefore compatible with thiol-reactive protein labels [22].

2.3.2 TWO DIMENSIONAL GEL ELECTROPHORESIS (2DE)

To fractionate the samples into even simpler factions two dimensional electrophoresis (2DE)[23] is used. In 2DE the proteins are first fractionated by isoelectric focusing (IEF) in the first dimension. Typically IEF occurs in a gel strip containing a pH gradient. The proteins will stop migrating in the gel when it reaches the pH value that corresponds to their isoelectric point (p*I*). p*I* is the p*H* where the net charge of the protein is zero. The charge of the protein depends on the residuals in the protein, as well as the p*H* of the environment. After the first dimension the IEF strip is layered over an SDS-PAGE to fractionate proteins by MW (Fig. 3.). The proteins in the gel may be detected by silver staining or fluorescent labelling [24].

While 2DE is highly useful to fractionate proteins, identification relies on other methods. The most commonly used detection method for proteins fractionated with 2DE today is MS. To analyse the proteins in the gel with MS or other detection methods, the protein spots have to be cut out of the gel and treated with a proteolytic enzyme, usually trypsin, to form peptides that elute from the gel [24].

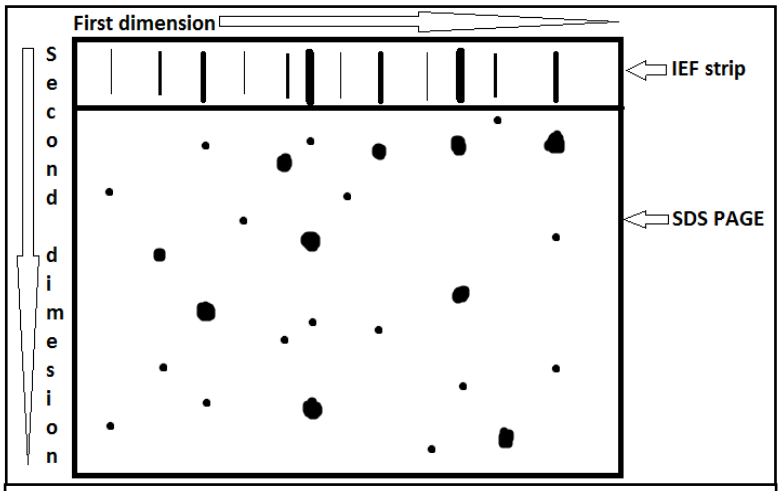

Fig. 3. Two dimensional electrophoresis (2DE). This is a schematic presentation of 2DE. In the first dimension the proteins are fractionated using a strip with a pH gradient, witch fractionates the proteins according to p*I*. This is called isoelectric focusing (IEF). After IEF the strip is layered over an SDS-PAGE, and the proteins are fractionated according to molecular weight in the second dimension.

Two dimensional electrophoresis followed by in-gel protein digestion and elution is a slow and laborious method. Problems are also associated with elution and solubility, especially with hydrophobic proteins [25], even though detergents [26, 27] have increased the representation of membrane proteins in the results. Moreover, the resolution provided by the electrophoretic fractionation step is limited. Proteins with very high or very low MW and proteins with extreme p*I*s are usually not detected using standard 2DE, but improvements have been obtained using wide p*H* gradients [28] and different buffer systems [29]. The last limitation with 2DE is the ability to detect low abundance proteins. The dynamic range of 2DE is about 10^4, but the protein expression in human cells is estimated to be 7-8 orders of magnitude.

2.3.3 CHROMATOGRAPHY

Chromatography is a widely used approach for fractionation of native proteins. In chromatography, proteins in solution are fractionated by their migration pattern across an immobile matrix. The solution is referred to as the mobile phase and the immobile matrix as the stationary phase. The basis for the fractionation is that molecules migrate at differential rates depending on the affinity for each of the two phases.

There are many types of chromatography, and the various approaches use different properties of the peptides or proteins for fractionation. Hydrophobic interaction chromatography (HIC)

fractionates peptides or proteins according to their polarity, while in ion exchange chromatography (IEC) the proteins are fractionated and purified on the basis of ionic interactions. Affinity chromatography fractionates proteins on the basis of reversible biological interactions, such as antibody-antigen interactions.

Size exclusion chromatography (SEC) is used to fractionate proteins according to molecular weight. The resin in the column consists of porous particles. Large molecules cannot enter into the pores and elute first, while small molecules pass in and out of the pores through the column, and elute later. While SDS-PAGE is used to separate denatured proteins, SEC is typically performed under native conditions. A given protein species may therefore elute in non-overlapping fractions depending on whether it occurs as a monomer or in one or more complexes. SEC is therefore often used to analyse protein complexes.

2.3.4 CONTINUOUS ELUTION TUBE GEL ELECTROPHORESIS

In continuous elution tube gel electrophoresis, proteins are separated by gel-electrophoresis, but are finally eluted into liquid fractions. Several such approaches have been developed and used in research (for example [30-34]). The utility of this method has been limited by the fact that it has been biased towards the lower MW range. Moreover, the fractions are subject to large dilution, especially with the fractions containing proteins with high MW, and the fractionation is time consuming.

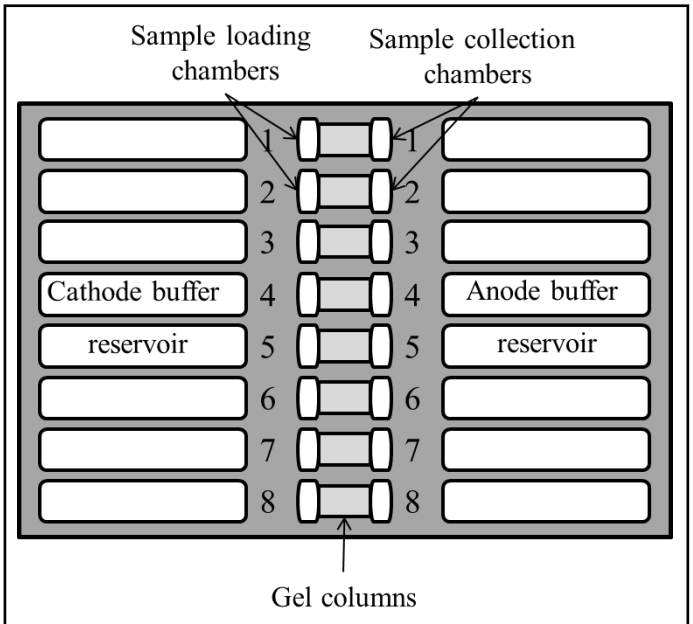

Fig. 4. Gelfree cartridge. This is a schematic presentation of a Gelfree cartridge. The gel columns, the cathode and anode buffer reservoir, the sample loading and the sample collection chamber make up the five main parts. The electrodes are inserted into the cathode and the anode buffer reservoir, the sample is loaded into the sampled loading chamber and electrical current is applied. The proteins will now migrate through the gel column and end up in the sample collection chamber, where fractions can be collected at predefined intervals. The smallest proteins will migrate fastest through the gel, and end up in the first fractions, while the larger proteins will end up in the later fractions.

To overcome the limitations of traditional techniques, Tran and Doucette developed a device that uses a short gel column for protein fractionation where the proteins

ultimately are eluted from the column, and can be collected in solution. The separation technique was named Gelfree for Gel-Eluted Liquid FRaction Entrapment Electrophoresis[35]. The Gelfree fractionation cartridges consist of five main parts: a cathode and an anode buffer reservoir, a sample loading and a sample collection chamber and the gel column (Fig. 4). The anode, cathode and sample collection chamber are filled with running buffer and the sample are loaded onto the sample loading chamber. When electrical current is applied the proteins will migrate through the gel column, and are fractioned according to MW, before they finally elute into the collection chamber. The fractions are then collected from the collection chamber at defined time intervals [35-37]. The short column makes the separation more rapid [37], and the protein recovery is high [35].

Botelho et al. performed a MS experiment to compare the Gelfree fractionation system to a more traditional approach involving proteolytic digestion of proteins in bands cut out from SDS-PAGE gels, and concluded that the two methods yield comparable results in both type and number of proteins identified [38]. The Gelfree system has also been used to prepare samples for MS with good results in a number of other studies [38-46].

2.4 ANTIBODY-BASED METHODS

Antibodies are widely used to detect proteins. These reagents provide means to detect proteins in complex samples with high throughput and precision. As explained below, however, antibody-based methods are largely limited to detecting one or a few proteins simultaneously.

2.4.1 ANTIBODIES

Antibodies are glycoproteins produced by B-cells and are part of our immune system. B-cells carry antibodies on their surface, while activated B-cells, called plasma cells secrete soluble antibodies. Antibodies bind antigens specifically. Antigens are molecules which stimulate B-cells to antibody production, and the binding site is referred to as an epitope. A linear epitope is a result of a contiguous amino acid sequence (Fig. 5), whereas a conformational epitope consists of non-neighboring amino acids brought in proximity by protein folding. Conformational epitopes will be destroyed by denaturation, while some linear epitopes can be masked when the protein is in its native form and can therefore only be detected when the protein are denatured.

Antibodies consist of four polypeptide chains that are covalently linked together with disulphide bonds (Fig. 6). The four polypeptide chains are divided into two heavy and two light chains. There are two types of light chains called κ and λ, and five types of heavy chains called α, μ, γ, δ and ε. The five different types of heavy chains, give rise to five classes of antibodies: IgA, IgM, IgG, IgD and IgE. These five different classes have different structural and functional properties, and IgG is the class most often used in antibody-based methods, since it has the highest affinity for their antigens.

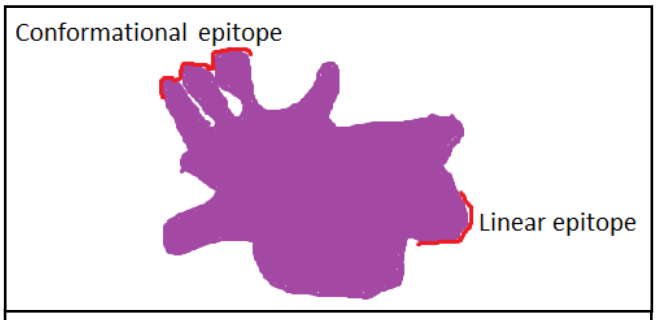

Fig. 5. Conformational and linear epitopes. This is an illustration of an antigen. Epitopes are the precise parts of an antigen where the antibody binds. Epitopes can be conformational or linear. Conformational epitopes is made as a result of the folding of the amino acid chain, while linear epitopes consist of a contiguous sequence of amino acids.

Antibodies used in antibody-based methods can be divided into two major groups: polyclonal and monoclonal antibodies (Fig. 6). Polyclonal antibodies are obtained from serum of immunized animals, and may recognize different epitopes on the same antigen. Monoclonal antibodies have specificity for only one epitope, and are made by hybrid cells, which are generated by a fusion of B-cells and myeloma cells. The fusion gives rise to cells that have the antibody specificity of the B-cells, and self-renewing capacity of the myeloma cell. These cells can be grown in large quantities, and the technology is called hybridoma technology. In 1984 Milstein and Kohler received the Nobel Prize for the discovery and production of monoclonal antibodies [47-49].

Production of antibodies by immunization of animals is time-consuming and expensive. Hybridoma technology has for the past 35 years enhanced research and diagnostics by providing monoclonal antibody reagents. In 1990 McCafferty described a technique for *in vitro* production of monoclonal antibodies [50]. In this technique the variable immunoglobulin genes from B-cells are

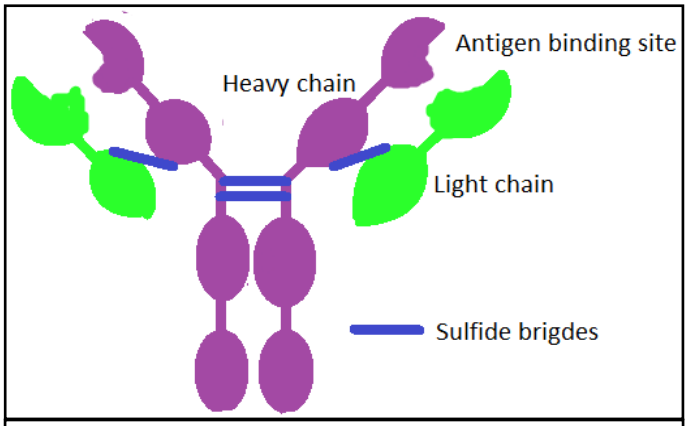

Fig. 6. Antibody. An antibody consists of four polypeptide chains, two heavy and two light chains, which are linked together with disulphide bonds.

amplified by polymerase chain reaction (PCR), and expressed on the surface of bacteriophages. Phage particles with inserts encoding specific binding domains are selected by using a chromatography column with immobilized antigen. This technic permits control over selection and screening conditions, and therefore allows production of antibodies against defined epitopes. With *in vitro* display techniques it is possible to target molecular structures that are not easily recognized during an immune response in vivo [51-56].

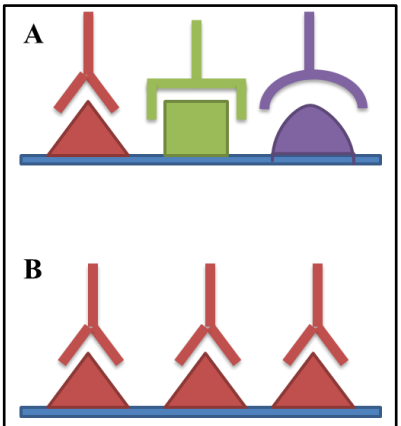

Figure 7. Polyclonal and monoclonal antibodies. This is an illustration of the difference between polyclonal and monoclonal antibodies. **A:** Polyclonal antibodies bind different epitopes on the same antigen. **B:** Monoclonal antibodies have specificity for only one type of epitope.

2.4.2 WESTERN BLOTTING

One of the most widely used antibody-based methods today is western blotting. This technique was developed about 30 years ago [57], and is applicable for a wide range of samples including serum, tissue culture supernatants, cell and tissue extracts.

The first step of western blotting is SDS-PAGE or native-PAGE where the proteins are fractionated by MW. Then the proteins in the PAGE-gel are transferred, or blotted, onto a membrane. During transfer the gel and the membrane are placed on top of each other in a buffer, and an electrical current is applied to cause the proteins to migrate. After transfer, the membrane is treated with agents that block non-specific binding sites, before it is labelled with antibodies. In a typical experiment, binding of the primary antibody to the proteins on the membrane is detected using a secondary antibody, which is conjugated to an enzyme such as horse radish peroxidase. A substrate is added, and the enzyme generates a product that gives a signal, usually chemiluminescence (Fig. 8).

Antibody reactivity on western blots is observed as bands, and in many cases there are many antibody stained bands on the membrane. The position of the band corresponding to the intended target, however, is predictable from the size of the protein. Moreover, the specific binding is usually more consistent among different samples than the cross-reactivity. The relative intensity of bands from different samples provides semi-quantitative information about the amount of the protein. The limitation with western blotting is that the technique can only be used to detect one

or a few proteins at a time.

2.4.3 FLOW CYTOMETRY

Flow cytometry is used to analyse cells in suspension. In diagnostics flow cytometry is commonly used to count and characterize subsets of leucocytes. This is carried out with supravital stained cells that are introduced to the flow cytometer by a fluidics that focus the cells into a capillary. The capillary is intersected by one or more lasers, and detection of scattered laser light is used to estimate cell shape, cell size, cellular granularity, nuclear lobularity and cell surface structure.

The cells can also be labelled with fluorochrome-conjugated antibodies. Multiple antibodies with different fluorescent probes can be used at the same time [58]. The lasers excite the fluorochromes on the antibodies, and the emitted light is collected through lenses and guided via fibre optics to detectors. Fluorescent dyes that are excited by the same laser and emits light at different wavelengths are discriminated by the use of optical filters. Dyes with similar emission, but different excitation can also be discriminated since they pass the lasers at different time points, and the instrument can resolve the time difference.

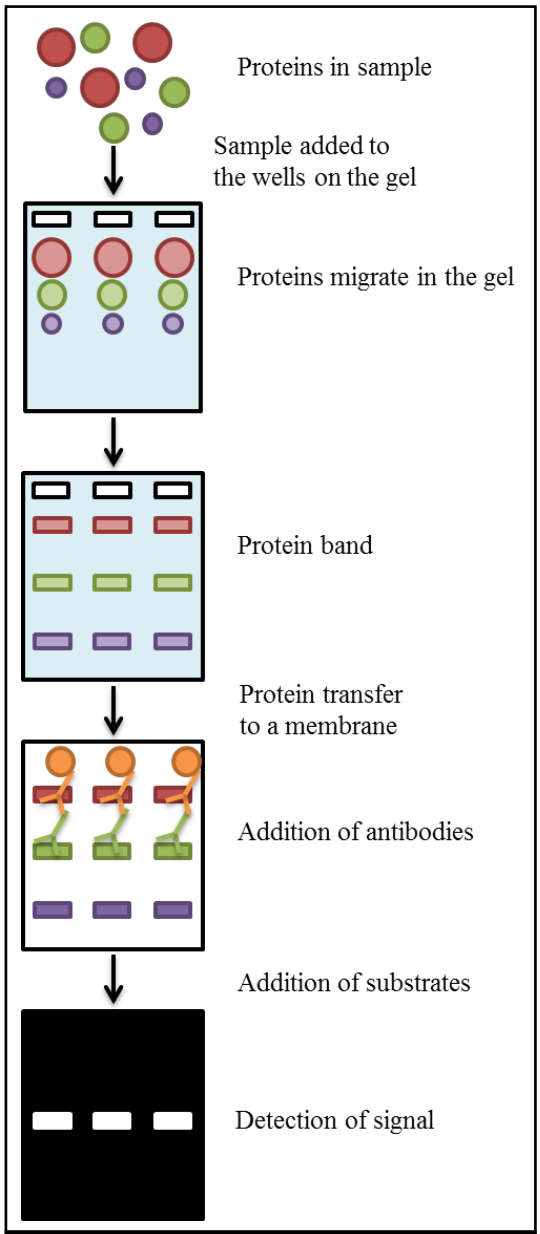

Fig. 8. Western blotting. This is a schematic presentation of the work flow in western blotting. First the proteins are applied to wells on a PAGE gel and electrical current is applied. The small proteins will migrate faster than bigger proteins, resulting in fractionation of the proteins according to MW. After PAGE the proteins are transferred to a membrane, and antibodies are added. The primary antibody binds the target and a secondary antibody conjugated with an enzyme targets the primary antibody. A substrate, which is converted to a chemiluminescent compound by the enzyme, is added, and the signal can be detected.

2.4.4 ANTIBODY SANDWICH ASSAYS

All the methods described so far in this thesis are used to detect immobilized proteins. Antibody sandwich assays detect proteins in solution. The term ELISA (Enzyme-linked immunosorbent assay) is used to describe all forms of enzyme immunoassays with colorimetric detection principle. When performing ELISA, capture antibodies are immobilized on a solid matrix, typically a microtiter plate, the samples are added and the proteins from the sample bind to the capture antibodies. A second antibody, binding a different epitope on the same protein, is used for detection. Typically this antibody is conjugated with an enzyme such as peroxidase, and the signal is detected using a substrate that is converted to a coloured or chemiluminescent compound. The ELISA format provides dual specificity since the capture and detection antibodies target two distinct epitopes. This makes this method less affected by cross-reactivity.

There are a large number of different variants of antibody assays with different types of solid matrix, different types of antibody arrangement and different types of detections methods. Different immunological assays are widely used both in diagnostics and in research. The solid matrix can be covered with antibodies or antigens, and can therefore bind antigens or antibodies in solution. The solid matrix can be different types of beads or slides instead of a microtiter plate. The beads can have diverse features such as being paramagnetic or have the ability to be coloured with fluorescents labels, giving the advantage that the beads can be coloured differently, and several antibodies can be used at the same time. Paramagnetic beads are often used in automated diagnostic methods to bypass the need for centrifugation.

2.4.5 LIMITATIONS WITH ANTIBODY-BASED METHODS

Antibody-based methods are inexpensive, rapid and simple to preform, but some important limitations apply. The assays are largely limited to detecting a small number of proteins at a time. Equally important is the fact that well characterized reagents mainly cover a relatively low number of proteins that have been studied extensively over many years.

An initiative for producing antibodies towards all human proteins has been taken [59]. This is called the HUPO antibody initiative. It has been suggested that an objective for antibody-based proteomics should be to generate renewable, preferably monoclonal, paired antibodies towards all human proteins [59]. Paired antibodies are two or more antibodies recognizing distinct and non-overlapping epitopes on the same antigen (Fig. 9).

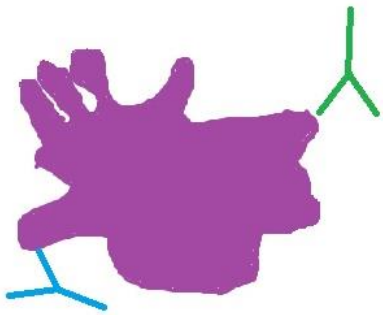

Fig. 9. Paired antibodies. Cross-reactivity can be minimized when using paired antibodies in antibody arrays. Paired antibodies mean two or more antibodies that recognize distinct and non-overlapping epitopes on the same antigen.

Antibodies may bind other proteins than the one used for immunization. This is commonly referred to as cross-reactivity or off-target binding. Antibodies can react with similar, related epitopes on proteins from the same gene family, but also to completely unrelated epitopes [60, 61]. This gives results that are difficult to interpret. Cross-reactivity is a big problem when using polyclonal antibodies, but can potentially be a problem when using monoclonal antibodies too. A way of limiting cross-reactivity is to use paired antibodies.

Exactly how frequent cross-reactivity occurs is difficult to say, but in a study performed by Schwenk et.al.[62] only 531 out of 11,000 antibodies showed a single band in western blotting. In another study the proteins captured by anti-TSP1 was analysed by MS [63]. The study showed that TSP1 (thrombospondin-1) was the major target, but other proteins were also detected.

Antibody performance is application-dependent. To some extent this is because the proteins are denatured by detergents or formalin in many applications, meaning that conformational epitopes are destroyed. In these types of applications antibodies directed against linear epitopes should be used. For applications where the proteins are in their native form, the epitopes can be masked due to the folding of the protein, or due to protein-protein interactions. A study of more than 5000 antibodies from over 50 commercial providers showed that as many as 50% of the antibodies where non-functional in the immunohistochemistry application used [64].

In all the methods except for ELISA, there are no standardized measurements for quality control. Researchers must therefore frequently perform their own evaluation [65]. As a part of the HUPO antibody initiative a web portal called Antibodypedia has been developed [59, 64]. Antibodypedia is

a portal where scientists can share information about validation of antibodies. Today Antibodypedia contains 810,580 reviewed antibodies from 43 providers, covering gene products from 18,831 protein coding genes, which is about 91% of all human genes [66]. The aim of Antibodypedia is to make a resource for validated antibodies towards all human proteins [64].

2.5 MASS SPECTROMETRY

Mass spectrometry provides means to measure the mass and charge of peptides and peptide fragments. Mass spectrometers can examine peptides of up to 50 amino acids in length, but since most proteins are longer than this, the proteins are treated with a proteolytic enzyme prior to analysis. The standard approach is to digest the proteins with a sequence specific protease, such as trypsin. Trypsin cleaves the proteins immediately after arginine or lysine residues, and since the amino acid sequences of all human proteins are generally known from HUGO-project, digestion of any protein will result in a predictable set of peptides with known mass. The peptide masses obtained from a MS experiment can therefore be used to search different bioinformatics databases to identify the protein.

Mass spectrometric measurements are carried out on ionized analytes in the gas phase. Protein and peptides are large non-volatile molecules, but there are two approaches to make gas phase ions from proteins: matrix-assisted laser desorption/ionization coupled to time-of-flight analysers (MALDI-TOF)[67] and electrospray ionization (ESI) [68]. When using MALDI-TOF the proteins are mixed with a matrix, which contains small organic solvents and is responsible for ionization of the proteins. The matrix is dried out, and energy from a laser makes the peptides in the matrix go into gas phase. Then the mass-charge ratio is measured. This is done by measuring the time a peptide uses from the place of ionization to the detector (Time-of-flight, TOF).

When using ESI for ionization a strong electrical field is applied, under atmospheric pressure, to a liquid passing through a capillary tube. This leads to highly charged droplets, which in turn causes the ions to be separated from the solvent. When using ESI the samples start out as liquid, so this approach is often combined with different types of liquid chromatography (LC-MS). There are a number of different mass analysers. Along with TOF, which is already mentioned, the ion trap, quadrupole and Fourier transform ion cyclotron are currently used in research.

A tandem mass spectrometer or MS/MS consists of two mass analysers separated by a collision cell. The first mass analyser is set to only allow a single type of peptide with a specific mass to charge ratio to continue into the collision cell. In the collision cell, the peptide is fragmented even further. This fragmentation occurs primarily at peptide bonds, and gives therefore ions that only differ by a single amino acid in mass. Since almost all amino acids have different mass, the recorded masses can be used to determine the amino acid sequence of the protein.

MS-based proteomics has been very important for much of our knowledge regarding proteins and protein activity. Among others the approach has been used to determine the proteome of organelles, the protein composition of a cell, members of protein complexes and post translational modifications [69, 70].

In "shot-gun" or "discovery" proteomics, the aim is to identify all the proteins in a sample. This is challenging, since the number of peptides that are formed when complex samples, such as serum or cell lysates, are digested with enzymes by far exceeds the number that the mass spectrometer can resolve. Extensive fractionation is therefore necessary prior to analysis.

MS is still a technically difficult method which requires a high level of expertise by the users. This is well illustrated by a study where 27 laboratories received a test sample containing a mixture of 20 purified proteins. Only 7 laboratories were able to identify all proteins in this simple mixture [3]. In another study more than 1000 proteins were detected reproducibly in serum samples from patients with cardiovascular disease [71]. This was achieved after depletion of 12 abundant proteins and extensive fractionation of the remaining protein mixture, using 2800 hours of instrument time. While it is impressive to detect such a large number of proteins by MS, the actual number of proteins is likely to be at least ten-fold higher. No proteome has yet been completely analysed, and it will be difficult to determine when that milestone has been achieved, since a suitable reference does not exist [72].

2.6 ANTIBODY ARRAY ANALYSIS

To overcome limitations with antibody-based assays, attempts have been made to develop platforms similar to those used in genomics [4-8, 10, 73-76]. Collectively, these can be referred to as antibody array analysis.

Antibody arrays can be broadly divided into two categories. The first category is an assay format where immobilized antibodies are used to capture labelled sample proteins (Fig. 10A) [4, 77]. Another approach is to use dual specificity sandwich arrays, where the proteins first are captured by one antibody and then detected using another antibody against a different epitope on the same target (Fig. 10B) [78-80]. It is possible to label the detection antibodies directly or using two different antibodies for detection: one detection antibody that binds the target and one read-out antibody that binds the detection antibody.

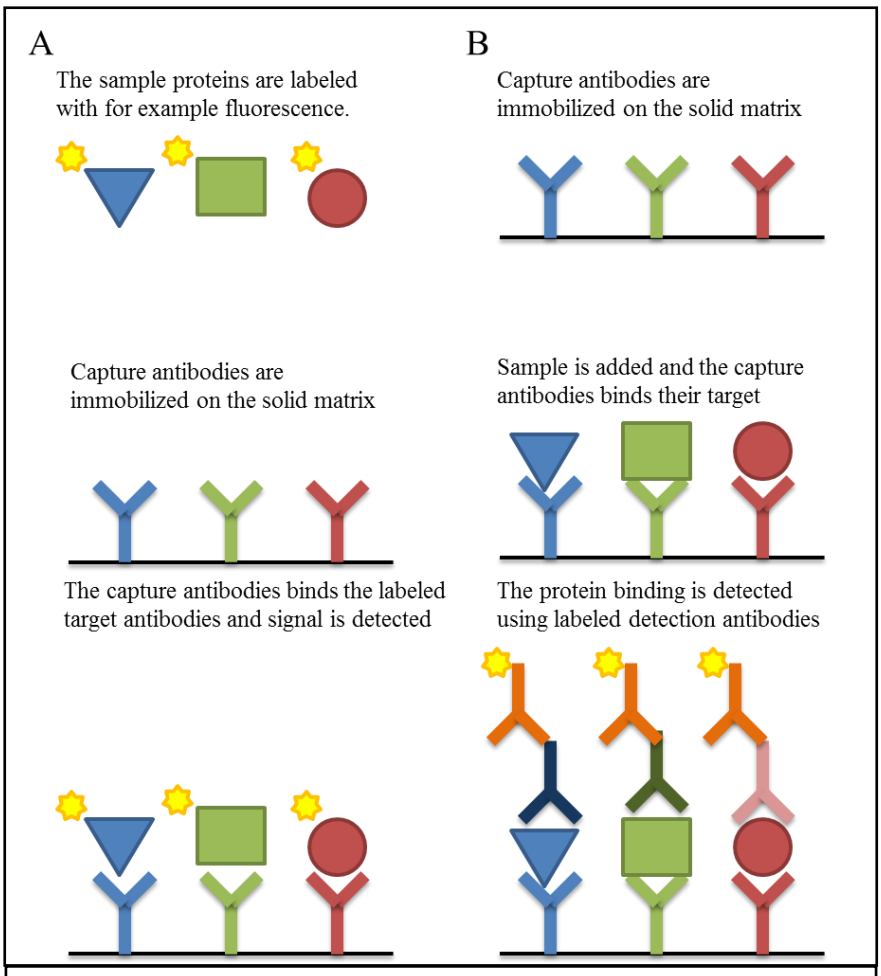

Fig. 10. Different experimental formats for antibody microarrays. This is a schematic presentation of two different antibody assay formats. **A:** In direct labelling, single-capture antibody array experiments the proteins in the sample is labelled prior to analysis. The capture antibodies are immobilized on the solid matrix and the samples are applied. The capture antibodies bind the labelled proteins and the signal is detected. **B:** This shows dual-antibody sandwich microarrays. The proteins from the sample are captured by the captures antibodies which is immobilised on the solid matrix. Detection antibodies, which provide dual specificity to the target protein, are added. Binding of the detection antibody to the target is detected using a labelled read-out antibody.

Direct labelling single capture antibody assays are simple to perform, and the number of proteins that can be analysed is nearly unlimited, but there are problems associated with obtaining specificity [4, 10] Assays where immobilized

antibodies are used to capture labelled sample proteins are reliable when each capture reagent binds a single target only. However, mono-specific antibodies are exceedingly rare. Dual-antibody sandwich immunoassays have a much higher specificity, as the target is recognized by two different antibodies. On the other hand it can be difficult to find paired antibodies for the microarray. This type of array is also limited to analyse about 50 targets simultaneously[74], because the possibility for cross reactivity between the different detection antibodies increases with higher numbers of analytes [81].

Different types of solid matrixes can be used in microarrays, but planar glass or silicon arrays or microbead arrays are most common. In planar glass [4, 79, 80] or silicon [6] arrays the capture reagents are spotted onto slides at predefined locations, as in DNA microarray. This approach requires access to sophisticated printing devices. An alternative is to use microspheres with fluorescent colour codes [7, 9]. These are typically analysed by flow cytometry, and the assays can be extensively multiplexed by using microspheres with different fluorescent labelling and different sizes.

The limitations associated with antibody microarrays compared to MS are that the assays depend on the availability of affinity probes. In antibody microarray it is also necessary to decide which proteins that are going to be studied prior to the experiments, MS allows unbiased analysis.

2.6.1 PROTEIN LABELLING

While sample proteins can be labelled directly with reactive forms of fluorescent dyes, earlier studies have shown that indirect labelling with biotin and streptavidin is superior [82]. Biotin is a small molecule that binds to streptavidin with high affinity. Streptavidin can in turn be conjugated to detectable molecules such as fluorescent dyes.

The two most commonly used forms of biotin react with amine- and thiol groups in proteins. N-Hydroxysuccinimide (NHS) esters react with primary amino groups (-NH2). Primary amines are found in the side chain of lysine residues and the N-terminus of the polypeptide.

Maleimide derivatives react with thiols, found in cysteine residues. Cysteines are unstable in non-reducing environments where they form di-sulphide bonds with each other. For extracellular proteins, which occur in non-reducing environments, this is an important mechanism for protein

folding. When cysteines are reduced and reacted with maleimides, they can no longer form disulphide bonds. Thus, this type of labelling prevents refolding of denatured proteins. This is advantageous for the approach used in this thesis, where the aim is to detect and analyse denatured proteins. On the other hand, most proteins contain fewer cysteines than lysines, and approximately 4% of proteins lack cysteines.

2.6.2 WESTERN-MAP

In this thesis the aim was to develop an approach for multiplexed large scale analysis of cellular proteins where the results should resemble the results obtained with western blotting. The development of this approach was based on a previously published approach called SEC-MAP (Size-Exclusion Chromatography – Microsphere-based Affinity Proteomics) [9, 83-85]. In SEC-MAP the samples are fractionated with SEC prior to detection and analysis with antibody microsphere arrays. Microspheres give the opportunity to use microtiter plates with samples, and this gives a higher sample throughput compared to microarray slides.

The experimental format for the antibody microsphere array used in SEC-MAP is direct labelling single capture antibody. As already discussed direct labelling single capture antibody arrays have potential to detect and analyse far more proteins than the other antibody microarray approaches mentioned.

When using direct labelling single capture antibody arrays it is necessary to have a way of controlling the specificity. In western blotting the samples are fractionated according to MW prior to detection and analysis with antibodies. This separation makes it possible to distinguish unspecific bands from the intended targets. This approach can be adapted to microarrays, and is precisely what is done when preforming SEC-MAP. The sample fractionation, along with several antibodies against the same target in the same array, makes it possible to identify specific binding of proteins, and rule out cross-reactive binding [85].

In traditional western blotting the proteins are denaturated with heat and reduction agents prior to fractionation. This gives a different fractionation pattern to what is obtained with SEC, witch fractionate native proteins. To develop an approach that would resemble western blotting it was therefore necessary to investigate if SEC could be replaced by another fractionation approach that fractionated denaturated proteins.

Fractionation with denaturated proteins could give several benefits. First of all protein complexes are disrupted during denaturation, meaning that all proteins will mainly occur an in monomeric form. The proteins´ MW are also the only factor affecting the protein fractionation, as opposed to fractionation with native proteins where shape and surface charge also can have an influence. This makes the results easier to interpret. Many antibodies are developed for use in western blotting, as this is a widely used application. A study showed that antibodies developed for western blotting performed better when the samples were treated with heat [62]. A limitation with denaturation is that the anionic detergent SDS is commonly used for this purpose, an in microarrays SDS has been shown to affect the results obtained [83, 86]. Antibodies directed at

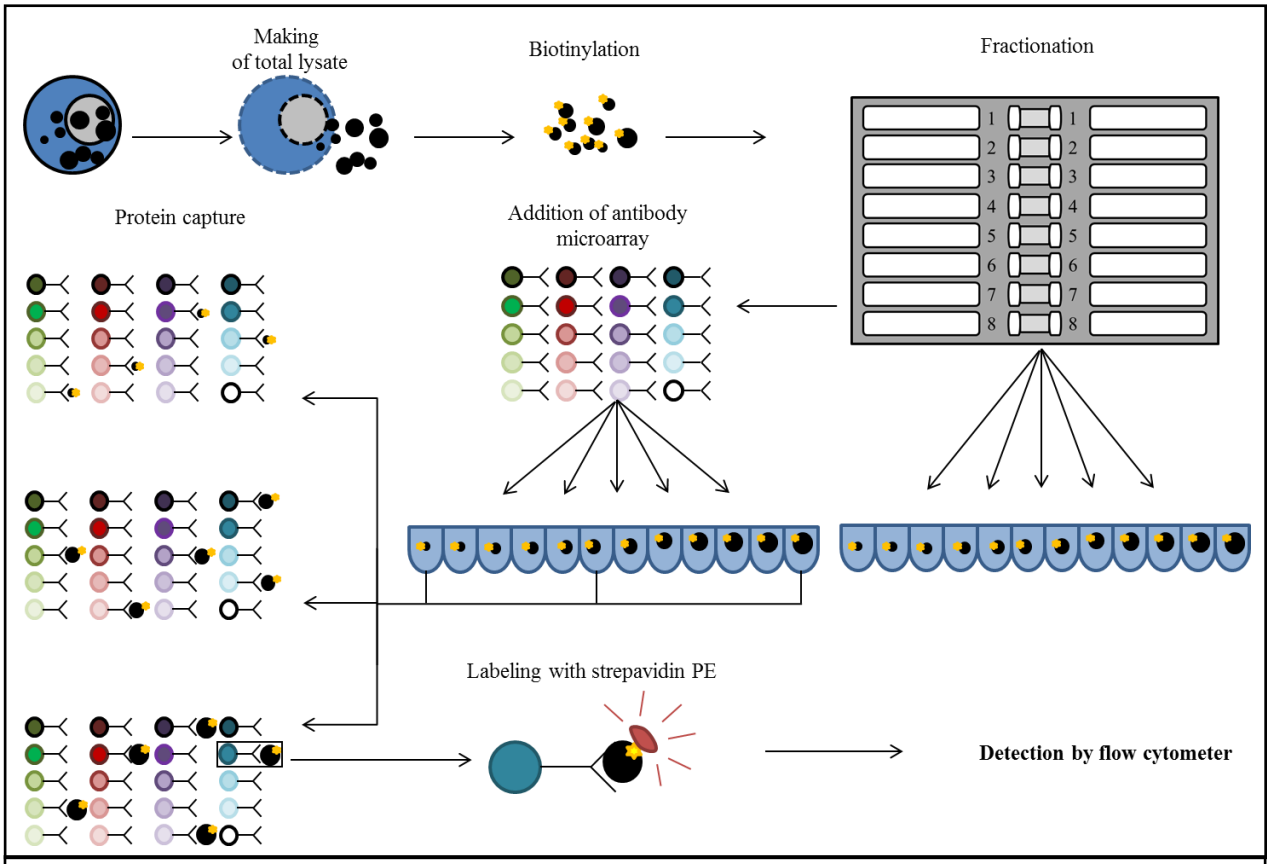

Fig. 11. Western-MAP work flow. The approach for large scale analysis of cellular proteins developed in the thesis is called Western-MAP. To preformed western-map first lysates is made from the sample cells. The proteins in the sample are the labelled with biotin, and then fractionated. The three fractionation systems examined in this thesis are the Gelfree 8100 fractionation system from Expedeon (shown), the whole gel eluter from Biorad and Size-exclusion chromatography with denaturated proteins. After fractionation the antibody arrays is added to each fraction. The antibody array consists of antibody covered beads witch are coloured with fluorescent dyes. Each type of antibody had its microspheres coloured with a unique colour-code and/or size, and thereby the fluorescent colouring of the microspheres together with the microsphere size works as a barcode for the type of antibody bound. The antibody in the arrays then binds their target proteins from the fractionated sample. The antibody array microspheres are then collected, and the captured protein is labelled with streptavidin-PE. A flow cytometer is used to detect the PE-signal of any captured protein and the size and colour-code of the microspheres to identify the protein bound.

conformational epitopes will also be non-functional when the proteins are denatured

If SEC could be replaced with a fractionation approach that fractionated denaturated proteins, this would theoretically be a multiplexed approach that gave the same results as western blot (Fig. 11). This approach will be called western-MAP.

3.0 MATERIALS

3.1 CELL LINES

HeLa (Human epithelial carcinoma cell line)	(Abcam, Cambridge, United Kingdom)
Jurkat (Human T-cell lymphoblast-like cell line)	(Abcam, Cambridge, United Kingdom)

3.2 REAGENTS AND CHEMICALS

Acetic Acid	(Acetic acid (glacial) 100%, cat.no: 1000632500, Merck Millipore, Darmstadt, Germany)
Albumin	(Albumin 40 mg/ml, cat.no: 478198, octapharma, Lachen, Switzerland)
β-mercaptoethanol	(2-Mercaptoethanol, cat. no: M7522 Sigma, Sigma-Aldrich, St. Louis, Missouri, USA)
Coomassie Brilliant Blue R-250	(cat.no: 161-0400, Bio Rad, Hercules, California, USA)
DMSO	(Dimethyl sulfoxide, cat.no: D4540 SIGMA, Sigma-Aldrich, St. Louis, Missouri, USA)
DTT	(Dithiothreitol (DTT), cat.no: D-1532, Eugene, Oregon, USA)
EDTA	(Titriplex III, cat.no: 108418, Merck Millipore, Darmstadt, Germany)
Foetal bovine serum	(cat.no: F6178 SIGMA, Sigma-Aldrich, St. Louis, Missouri, USA)
Glycerol	(Glycerol solution, cat.no: 49782, Sigma-Aldrich, St. Louis, Missouri, USA)

HEPES	(cat.no: H3375 SIGMA, Sigma-Aldrich, St. Louis, Missouri, USA)
KCl	(cat.no:104936, Merck Millipore, Darmstadt, Germany)
Lauryl maltoside (LM)	(*n*-Dodecyl β-D-maltoside, cat.no: D4641 Sigma**,** St. Louis, Missouri, USA)
Maleimide-biotin	(EZ-Link Maleimide-PEG2-Biotin, cat.no: 21901 Thermo Fisher Scientific, Rockford, Illinois, USA)
Methanol	(cat.no: 32213 Sigma-Aldrich, Sigma-Aldrich, St. Louis, Missouri, USA)
$MgCl_2$	(cat.no: 63065 Fluka, Sigma-Aldrich, St. Louis, Missouri, USA)
NaCl	(cat.no: 106404, Merck Millipore, Darmstadt, Germany)
NaF	(cat.no:S7920 Sigma-Aldrich, Sigma-Aldrich, St. Louis, Missouri, USA)
$Na_2HPO_4*12H_2O$	(cat.no: 106579, Merck Millipore, Darmstadt, Germany)
$NaH_2PO_4*H_2O$	(cat.no: 106346, Merck Millipore, Darmstadt, Germany)
NA_3VO_4	(Sodium orthovanadate, cat.no: S6508 Sigma**,** Sigma-Aldrich, St. Louis, Missouri, USA)

NHS-biotin	(EZ-Link NHS-PEG_4-Biotin, cat.no:21363, Thermo Fisher Scientific, Rockford, Illinois, USA)
PMSF	(Phenylmethanesulfonyl fluoride**,** cat.no: P7626 Sigma**,** Sigma-Aldrich, St. Louis, Missouri, USA)
SDS	(Sodium dodecyl sulphate, cat.no: L3771 SIGMA, Sigma-Aldrich, St. Louis, Missouri, USA)
Sephadex G25 Fine	(cat.no: 17-0032-01, GE Healthcare, Uppsala, Sweden)
Sigma protease inhibitor cocktail	(cat.no: P8340 Sigma, Sigma-Aldrich, St. Louis, Missouri, USA)
Streptavidin-Phycoerythrin	(R-phycoerythrin conjugated with streptavidin, cat. no: 016-110-084, Jackson Immunoresearch, West Grove, Pennsylvania, USA)
TCEP	(Tris(2-carboxyethyl)phosphine hydrochloride, cat. no: C4706 Aldrich, Sigma-Aldrich, St. Louis, Missouri, USA)
Tween 20	(cat. no: P1379, Sigma-Aldrich, St. Louis, Missouri, USA)

3.3 COMMERCIAL SOLUTIONS

Casein blocker in PBS	(cat.no: 37582, Thermo Fisher Scientific, Rockford, Illinois, USA)
Gelfree 8100 HEPES Running Buffer	(cat.no: 42202, Expedeon, Cambridgeshire,

	United Kingdom)
Gelfree 8100 Tris Acetate Sample Buffer	(cat.no: 42302, Expedeon, Cambridgeshire, United Kingdom)
Precision Plus Protein Dual Colour Standard	(cat.no: 161-0374, Bio Rad, Hercules, California, USA)
RPMI-medium	(RPMI 1640 Medium, GlutaMAX, cat.no: 61870-010, Life Technologies, Paisley, UK)
RunBlue LDS Sample Buffer	(cat.no: NXB31010, Expedeon, Cambridgeshire, United Kingdom)
RunBlue SDS Run Buffer	(cat.no: NXB50500, Expedeon, Cambridgeshire, United Kingdom)

3.4 SOLUTIONS PREPARED IN THE LAB

Coomassie blue staining solution

0.5g Coomassie blue R-250

800mL Methanol

140mL Acetic Acid

Deionized water (dH_2O) up to 2.0L

The components were mixed together, and the solution was stored at room temperature (RT).

Destaining solution

300mL Methanol

100mL Acetic acid

dH_2O up to 1.0L

The components were mixed together, and the solution was stored at RT.

Lysis buffer

20 mM HEPES, pH8.0

2 mM $MgCl_2$

1 mM EDTA

1mM NaF

1mM Na_3VO_4

140mM NaCl

1% Lauryl Maltoside

1mM TCEP

1mM PMSF

1mM Sigma protease inhibitor cocktail

The components were dissolved in dH_2O, and stored at 4°C.

Maleimide-biotin (10mg/mL)

10mg dissolved in 1mL DMSO and stored at -20°C.

NHS-biotin (60mg/mL)

60mg/mL NHS-biotin was dissolved in 1mL DMSO

Phosphate buffered saltwater (PBS) x25

4.48g $NaH_2PO_4*H_2O$

48.45g $Na_2HPO_4*12H_2O$

204.5g NaCl

The components were dissolved in 1liter of dH_2O, and stored at RT. PBS was made from the 25xPBS stock by taking 40mL stock and diluting it to 1liter in dH_2O.

PBS-1%tween

40mL PBS

10mL Tween 20

The components were dissolved in 1L of dH2O, and stored at RT.

Sephadex G25 solution

7g of Sephadex G25 was added 50mL dH_2O, and stored at RT.

Tween 20 (10%)

1mL Tween 20 was dissolved in 9mL dH_2O, and stored at 4°C.

3.5 ANTIBODIES

Goat gamma globulins	(cat. no: 005-000-003 Jackson Immunoresearch, West Grove, Pennsylvania, USA)
Mouse gamma globulins	(cat. no: 015-000-003 Jackson Immunoresearch, West Grove, Pennsylvania, USA)
Peroxidase-conjugated goat anti-mouse IgG	(cat. no: 115 035 146, Jackson Immunoresearch, West Grove, Pennsylvania, USA)
Peroxidase-conjugated goat anti-rabbit IgG	(cat. no: 111 035 144, Jackson Immunoresearch, West Grove, Pennsylvania, USA)

3.6 GELS

Criterion Tris-HCl Gel, 4–20% polyacrylamide	(cat.no: 345-0033, Bio Rad, Hercules, California, USA)
Criterion Tris-HCl Gel, 7.5% polyacrylamide	(cat.no:345-0005, Bio Rad, Hercules, California, USA)
Gelfree 8100 Cartridge - 5% tris-acetate	(cat.no: 42402, Expedeon, Cambridgeshire, United Kingdom)
Gelfree 8100 Cartridge - 8% tris-acetate	(cat.no: 42403, Expedeon, Cambridgeshire, United Kingdom)

Gelfree 8100 Cartridge - 10% tris-acetate	(cat.no: 42404, Expedeon, Cambridgeshire, United Kingdom)
Gelfree 8100 Cartridge - 12% tris-acetate	(cat.no: 42405, Expedeon, Cambridgeshire, United Kingdom)

3.7 VARIOUS EQUIPMENT

5mLtube	(Sarstedt, Nümbrecht, Germany)
50mL tube	(Corning incorporated, New York, USA)
Barseal	(Thermo Fisher Scientific, Rockford, Illinois, USA)
BD HTS Option for BD LSR II	(BD Biosciences, San Jose, California, USA)
BD LSR II	(BD Biosciences, San Jose, California, USA)
Canon EOS 450D	(Canon, Tokyo, Japan)
Criterion Cell	(Bio Rad, Hercules, California, USA)
Direct Detect Spectrometer	(Merck Millipore, Darmstadt, Germany)
Direct Detect Assay-free Cards	(Merck Millipore, Darmstadt, Germany)
Centrifuge 5810R,	(Eppendorf, Hamburg, Germany)
Gelfree 8100 Fractionation Station	(Expedeon, Cambridgeshire, United Kingdom)
Heat block	(DRI-block, Techne, Staffordshire, United Kingdom)
Microtube	(Sarstedt, Nümbrecht, Germany)

Microtube centrifuge	(Mikro 22R, Hettich zentrifugen, Tuttlingen Germany)
Microtitre plate 96 well/v-bottom non sterile	(Sterilin, Newport, UK)
Mix Mate	(Eppendorf, Hamburg, Germany)
Omicron-Laserage Laserprodukte GmbH	(Omicron-Laserage, Rodgau, Germany)
PCR tube	(Axygen, Corning incorporated, New York, USA)
Liquid handling robot	(Zephyr Compact, Caliper LifeScienses, Hopkinton, Massachusetts, USA)
PowerPac Basic Power Supply	(Bio Rad, Hercules, California, USA)
Rotator	(Rotator SB3, Stuart, Staffordshire, United Kingdom)
Sapphire561 DS	(Coherent, Santa Clara, California, USA)

3.8 SOFTWARE

Cluster 3.0 [87]	(Stanford University, Stanford California, USA)
Excel 2003	(Microsoft, Redmond, Washington, USA)
FACS Diva software	(BD Biosciences, San Jose, California, USA)
Java TreeView [87]	(Stanford University, Stanford California, USA)

4.0 METHODS

4.1 PREPARATION OF WHOLE CELL LYSATES

For the cellular proteins to be accessible for analysis, the cells have to be lysed. In this thesis whole lysates, containing both cytosolic-, nuclear-, organelle - and membrane proteins, were used. This lysate was prepared by suspending the cells in a solution containing NaCl and lauryl maltoside (LM). LM is a strong detergent which dissolves all membranes, and the proteins from organelles, cytosol and membranes will be solubilised. Recently obtained results from our laboratory have shown that a large number of nuclear proteins are released when cells are lysed in buffers containing 140mM NaCl (no yet published).

The cells were cultured in RPMI-medium with 5% foetal bovine serum. When preparing whole cell lysates the cells were first transferred to a 50mL tube and pelleted by centrifugation at 300g for 3 minutes at 4°C. The cells were washed twice in PBS to remove residual protein from the medium.

Approximately $5x10^7$ cells were suspended in 700ul of prepared lysis buffer. The microtube was placed in a 50mL tube prefilled with wet ice and rotated for 30 minutes. After incubation the sample was centrifuged at 24100g at 4°C for 5 minutes. The supernatant was aliquoted to microtubes, and stored at -70°C.

4.2 MEASURING PROTEIN CONCENTRATION

To measure protein concentration, Direct Detect from Millipore was used. This is an infrared-based system that measures the amide bonds in the protein chains and therefore does not rely on the amino acid composition of the proteins, as traditional Bradford assay does [88, 89]. Detergents, such as SDS, would interfere with the results obtained with Bradford assays [89], and this would be a problem in the experiments described in this thesis.

The sample was applied to the Direct Detect Assay-free Cards. A total volume of 2µL sample was applied to the spots on the card, and the samples where spotted in triplicates. The lysis buffer was used as a blank. The cards were put in the Direct Detect spectrometer, and the proteins concentration was measured.

4.3 LABELLING OF SAMPLE PROTEINS

Maleimide - and NHS-biotin bind thiols and free amines, respectively. In this thesis the two were used separately and in combination to compare effects on protein detection.

Whole cell lysates stored at -70^{o}C were thawed. Biotin derivatives dissolved in DMSO were added to a final concentration of 2mg/mL of the NHS-biotin and 0.5mg/mL for Maleimide-biotin. The labelling reagents were used both separately and in combination in the optimization experiments. The samples were incubated with biotin on ice for 20 minutes, and then passed over a Sephadex G25 spin column. The latter step was performed to remove salts that interfere with protein fractionation in the Gelfree device. However, this step also removes residual biotin from the samples.

4.4 FRACTIONATION WITH GELFREE 8100

As already discussed, unspecific binding can be a major problem when using immunological detection methods. To avoid this problem, fractionation of proteins prior to detection and analysis could provide a solution. In this thesis the Gelfree 8100 fractionation system was examined for fractionating the proteins according to MW before using antibody microspheres arrays for detection.

The desalted sample (120µL) was transferred to a PCR tube prefilled with 30µL of sample buffer. Attempts were also made using the Gelfree 8100 HEPES Running Buffer with addition of 0.3% SDS as sample buffer. Unless indicated, the sample buffer was supplemented with 1mM TCEP. The sample was heated to 90°C on the heat block for 10minutes, and then brought to 22^{o}C.

There are four different cartridges for the Gelfree instrument. These contain gels with different percentages of acrylamide (5, 8, 10 and 12%). The different cartridges have different separation ranges. The cartridges was loaded and run as described by elsewhere [36].

The run was paused at preprogramed intervals. During the first pause 2mL of running buffer was added to the cathode chamber to a total of 8mL. The sample loading chamber was washed twice with 200µL of running buffer, and then added 150µL running buffer. In one of the optimization experiments an extra pause prior to this was made, to wash the sample loading chamber and in that way remove any accumulated SDS.

In the later pauses the fractions were collected from the sample collection chamber to a microtiter plate. Pipetting up and down twice before collecting the fractions maximizes the protein recovery. After collecting the fractions the sample collection chamber was washed twice with 200µL of running buffer before the process was continued. Approximately every hour the running buffer in the cathode, anode and sample loading chamber was changed. The running time and voltage for the different intervals varied for the different cartridges (Table 1 to 4).

Table 1. Intervals and voltage for 12% acrylamide Gelfree fractionation cartridge. This table shows the fraction intervals, voltage and actions for every step when fractionate proteins with a 12% acrylamide Gelfree fractionation cartridge.

Interval	1	2	3	4	5	6	7	8	9	10	11	12	13
Fraction interval (min)	16	44	3	3.6	4	2.3	2.5	2.8	3.3	4	7	10	18
Voltage (V)	50	50	50	50	50	85	85	85	85	85	85	85	85
Action/ collect fraction number	Add running buffer to cathode and anode chamber. Wash sample loading chamber	Change running buffer/1	2	3	4	5	6	7	8	Change running buffer/9	10	11	12

Table 2. Intervals and voltage for 10% acrylamide Gelfree fractionation cartridge. This table shows the fraction intervals, voltage and actions for every step when fractionate proteins with a 10% acrylamide Gelfree fractionation cartridge.

Interval	1	2	3	4	5	6	7	8	9	10	11	12	13
Fraction interval (min)	16	36	2	3	4	3	4	5	7	10	15	20	35
Voltage (V)	50	50	50	50	50	100	100	100	100	100	100	100	100
Action/ collect fraction number	Add running buffer to cathode and anode chamber. Wash sample loading chamber	1	2	Change running buffer/3	4	5	6	7	8	Change running buffer/9	10	Change running buffer/11	12

Table 3. Intervals and voltage for 8% acrylamide Gelfree fractionation cartridge. This table shows the fraction intervals, voltage and actions for every step when fractionate proteins with an 8% acrylamide Gelfree fractionation cartridge.

Interval	1	2	3	4	5	6	7	8	9	10	11	12	13
Fraction interval (min)	16	41.5	2	2	3	2	2	3	5	7	10	15	20
Voltage (V)	50	50	50	50	50	100	100	100	100	100	100	100	100
Action/ collect fraction number	Add running buffer to cathode and anode chamber. Wash sample loading chamber	1	Change running buffer/2	3	4	5	6	7	8	9	Change running buffer/10	11	12

Table 4. Intervals and voltage for 5% acrylamide Gelfree fractionation cartridge. This table shows the fraction intervals, voltage and actions for every step when fractionate proteins with a 5% acrylamide Gelfree fractionation cartridge.

Interval	1	2	3	4	5	6	7	8	9	10	11	12	13
Fraction interval (min)	12	44	4	4	8	6	8	10	12	15	18	25	30
Voltage (V)	50	50	50	50	50	100	100	100	100	100	100	100	100
Action/ collect fraction number	Add running buffer to cathode and anode chamber. Wash sample loading chamber	Change running buffer/1	2	3	4	5	Change running buffer/6	7	8	9	Change running buffer/10	11	12

4.5 SDS REMOVAL

Free SDS can affect the antibody binding [83, 86]. The fractions obtained from the Gelfree 8100 fractionation system contain SDS, and it was therefore determined if removal of SDS would improve the results obtained in western-MAP. This can be achieved by the addition of potassium chloride (KCl) which forms insoluble complexes with SDS that precipitate in cold solution [90].

Fractions obtained from the Gelfree 8100 were supplemented with indicated amounts of KCl. The fractions were placed on ice for 15minutes and precipitates were pelleted by centrifugation at 2000g at 4°C for 4minutes. The supernatants were collected and used immediately, or stored at -70°C.

4.6 ANTIBODY ARRAY ANALYSIS (MICROSPHERE-BASED AFFINITY PROTEOMICS, MAP)

The production and use of bead-based antibody arrays was not a part of this thesis, and has been described earlier [9, 83-85]. In brief, the arrays consist of microspheres with fluorescent bar codes that can be discriminated by the flow cytometer. By using five fluorescent dyes, it is feasible to generate as many as 2,300 different colour codes. Each microsphere subset has a different antibody. Mixtures of beads are added to the sample fractions, and the proteins are labelled with streptavidin for detection of captured sample proteins.

Aliquots of the antibody microsphere arrays were stored at -70°C, and had to be thawed before use. The microspheres were pelleted by centrifugation, and resuspended in Casein blocker in PBS with 40µg/mL of mouse and goat gamma globulins and incubated at RT at the rotator for 15 minutes to block non-specific binding of proteins to the microsphere surface.

The fractions to be analysed were thawed, and 50µL was transferred to a microtiter plate. If nothing else is indicated 20µL Tween 20 (10%) was added to the fractions together with 10µL of the bead suspension, and the volume adjusted to 200µL with PBS. The 96-well microtiter plate was sealed with plastic, and rotated over night at 4°C in the dark.

After incubation the microspheres were pelleted by centrifugation. The supernatant was removed by using a liquid handling robot, and stored at -70°C. The supernatant still contains most of the proteins, and can therefore be reanalysed later.

The beads were washed by adding 180µL PBS supplemented with 1 % Tween 20 (PBT), and pelleted again. The supernatant was discarded, and 20µL of streptavidin-R-Phycoerythrin (SA-PE) (2µL/mL in PBS with 1% albumin) was added. The beads were incubated with SA-PE for 20minutes with constant shaking. After incubation, the beads were washed again, and resuspended in 110µL PBT, and analysed by flow cytometry.

4.7 FLOW CYTOMETRY

The flow cytometer was used to detect the size and colour-coding of the microspheres, and to detect the fluorescent signal emitted by the SA-PE on the captured antibodies.

The flow cytometer used for analysis was an LSRII instrument equipped with three lasers that emit violet (405nm), blue (488nm) and red (632nM) light. The instrument is also equipped with a device that harvests samples directly from microtiter plates. (BD HTS Option for BD LSR II). For detection of fluorescence we used the following optical filters: Pacific Blue: (ex 405 nm, 450/50 bandpass), Pacific Orange (ex 405 nm, 537/26 bandpass), Alexa 488 (ex 488 nm, 530/30 bandpass), Alexa 647 (ex 633 nm, 660/20 bandpass), Cy7 (ex 633 nm, 780/60 bandpass), and R-Phycoerythrin (ex 561 nm, 586/15).

4.8 DATA ANALYSIS

The acquired data were exported from FACS Diva software in FCS 3.0 format. The files were processed using custom-made software based on the R-bioconductor platform [84]. The software identifies microsphere subsets semi-automatically and exports signal values from bound protein (i.e. SA-PE fluorescence intensity) in a tabular format. The rows in the table correspond to the microsphere subset (i.e. antibody) and the columns correspond to the fractions.

Signal values were processed further using Microsoft Excel. The operations included subtraction of background signal from beads coupled with non-immune IgG and a linear smoothing function. Excel was next used to generate line plots where signal intensity (y-axis) was plotted against the fraction number (i.e. protein size). To a large extent validation of antibodies were based on visual inspection of these line plots. Antibodies that showed sharp and discrete peaks in fractions compatible with the size of the intended antibody target were selected for further analyses.

4.8.1 HEAT MAPS

In this thesis I make extensive use of heat maps. Heat maps are useful to display large amounts of data in a single figure. Commonly, heat maps are used to show data that have been subjected to hierarchical clustering. In this thesis, however, I used Excel to sort data (Fig. 12), and the clustering program (Cluster 3.0)[87], was only used to format data, not to for clustering. Data formatting in all figures is the same. Each antibody is associated with 12 signal values collected by measuring the fractions from the Gelfree 8100 instrument. The Cluster 3.0 program subtracts

12	432	302	165	236	1488	3307
11	596	407	196	830	3057	468
10	400	395	254	2240	2576	362
9	237	242	232	1960	264	112
8	263	212	369	612	231	120
7	139	206	869	174	214	109
6	159	236	1691	178	198	122
5	199	275	1331	166	196	110
4	261	565	327	144	214	118
3	1365	1144	162	147	240	127
2	2625	850	143	127	203	110
1	301	137	134	103	136	111
Protein size	12	22	41	56	87	104
antibody	5948	275	381	2650	121	3070
TARGET	B2M	CDKN1B	MAPK1	AKT1	STAT1	PKN1

Fig. 12. Sorting of data for generating of heat maps. The values had to be sorted in Excel, prior to generating heat maps with Cluster 3.0. Antibody targets were sorted by size (increasing from left to right, numbers in the blue cells correspond to mass in kDa). The fraction numbers is in the left column (green), and represent the y-axis in the later generated heat map. The numbers in the white columns correspond to the signal intensity measured with the appropriate antibody in each fraction. Numbers that are above the median value are indicated as red pixels in the heat map. Thus, antibodies to small proteins such as CDKN1B (Cyclin-dependent kinase inhibitor 1B) and B2M (beta-2-microglobulin) captured targets in fractions 1-3. These fractions will therefore be indicated with red pixels in the heat map. By contrast antibodies to the large proteins STAT1 (Signal transducer and activator of transcription 1-alpha/beta) and PKN1 (Serine/threonine-protein kinase N1) captures proteins from the last two fractions. This presentation format is essentially the same as that of a western blot, where antibody binding is detected as a band of antibody reactivity. The position of the correct band is predictable from the size of the protein. Thus, if the antibodies capture mainly their intended target, the position of the "bands" will move upwards from left to the right of the heat map since the antibody targets were sorted according to their molecular weight.

the median value from the series and normalizes the values. The output from cluster 3.0 is a clustered data table. This file is read by Java TreeView[87] to visualize the data as a heat map.

While, most heat maps use red and green pixels to indicate values above and below the median, respectively, I chose to use red for positive values (i.e. reactivity peaks) and black for negative and zero values. Thus, red pixels are to a large extent comparable to bands on a western blot.

4.9 ELECTROPHORESIS

To validate the performance of the Gelfree 8100 fractionation system, the fractions obtained were subjected to SDS-PAGE and visualized by staining of the gels with Coomassie blue.

The twelve fractions obtained by Gelfree fractionation were mixed with 5µL RunBlue LDS Sample Buffer to 15µL fraction. The whole cell lysate was used as a control, using 5µL whole cell lysate diluted with 10µL of dH_2O and added 5µL of sample buffer. The samples were heated for 5 minutes at 90°C on the heat block.

The Criterion Tris-HCl Precast gel was put in the electrophoresis chamber (Criterion Cell), and approximately 0.5L of SDS running buffer (RunBlue SDS Run Buffer) was added. For the fractions obtained by the 8%, 10% and 12% acrylamide Gelfree cartridges a Criterion Tris-HCl Gel with 4–20% polyacrylamide was used for visualization, while the Criterion Tris-HCl Gel

with 7.5% polyacrylamide were used for the fractions obtained by the 5% acrylamide Gelfree cartridge.

The fractions were loaded to the wells, with 10µL Precision Plus Protein Dual Colour Standard on each side of the samples as a standard. Electrical current was applied (PowerPac Basic Power Supply), and the gel was run for approximately 55 minutes at 150V.

After electrophoresis, the gel was stained with Coomassie blue for visualization of the proteins. For one gel 15mL of Coomassie blue staining solution was applied and incubated for 1 hour with constant shaking. Afterwards the gel was rinsed with dH_2O, and then with destaining solution for 20 seconds. The gel was left in fresh destaining solution for at least one hour, preferably overnight. A picture of the gel was taken with Canon EOS 450D.

5.0 RESULTS

5.1 THE GELFREE 8100 INSTRUMENT EFFECTIVELY FRACTIONATES PROTEINS ACCORDING TO THEIR SIZE

The fractions obtained with the Gelfree 8100 fractionation system were separated with standard SDS-PAGE, and visualised by Coomassie staining to validate the fractionation (Fig. 13). Four different fractionation cartridges, with four different percentages of acrylamide are available. Our experiments showed a good fractionation of proteins with a size spanning from 75kDa to over 250kDa for the 5% acrylamide cartridge. The 8% acrylamide cartridge showed a resolution from around 30kDa and up to 125kDa, whereas the 10% cartridge fractionated proteins with a size of 18kDa to 100kDa. Thus, the results obtained with these cartridges were in agreement with those reported by the manufacturer. The 12% cartridge is claimed to fractionate proteins with a MW between 10kDa and 50kDa, but our experiments showed that the resolution was slightly lower and proteins with a size between 13kDa and 37kDa were resolved (Fig. 13).

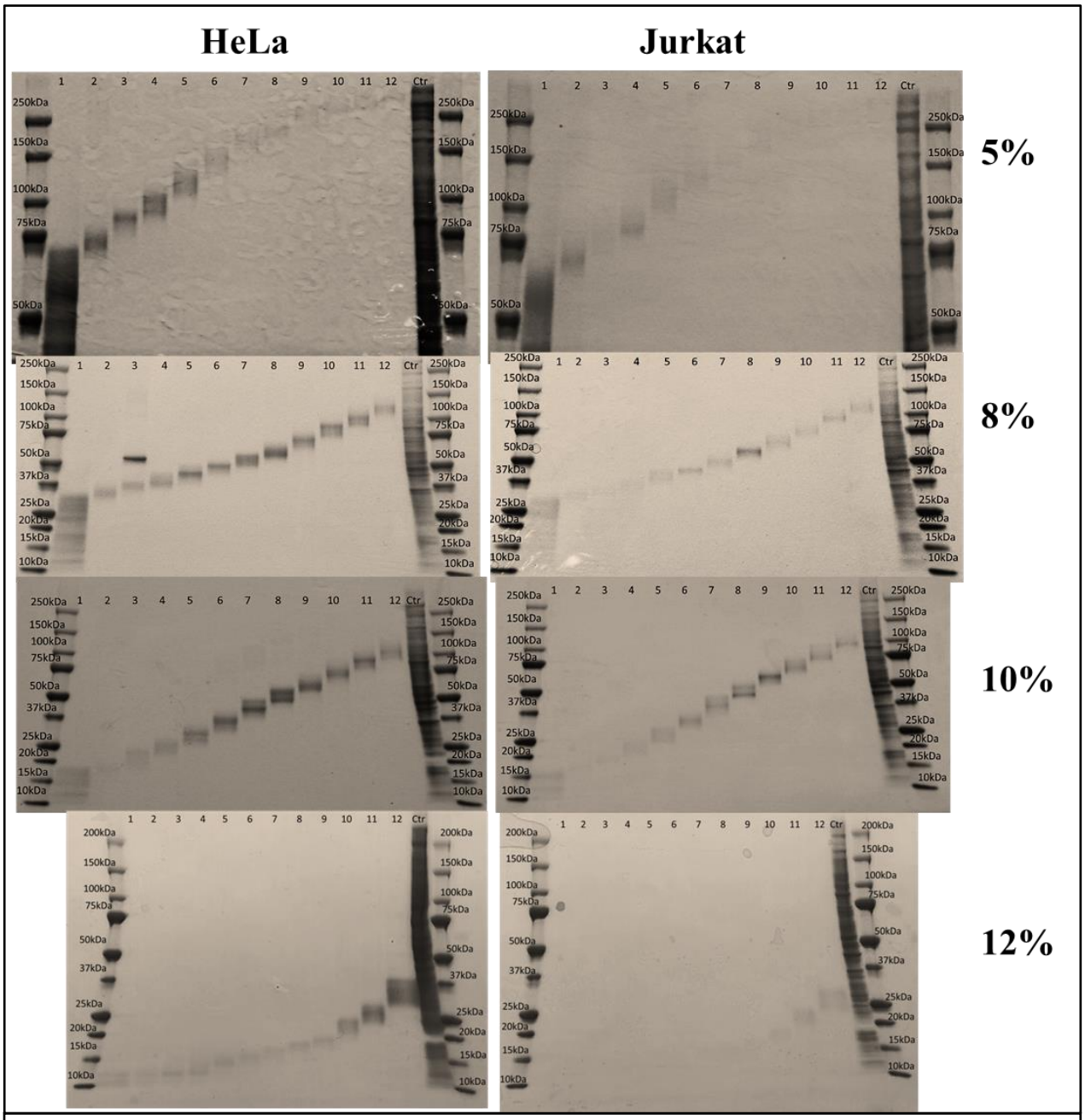

Fig. 13. Protein fractionation. Comassie blue stained gel images of fractions obtained from the Gelfree 8100 fractionation system. The samples where first fractionated with the Gelfree 8100 fractionation system, the fractions obtained were run on a SDS-PAGE gel and then stained with Comassie blue. The proteins with the smallest MW were in fraction number 1 and the largest in fraction 12. The control samples (Ctr) were the whole cell lysate initially used for fractionation, diluted 1:3 in dH_2O. The left column shows HeLa samples, and the Jurkat samples are to the right. The protein concentration in the HeLa samples were 7.0mg/mL, and the proteins concentration in the Jurkat samples were 2.4mg/mL.

5.2 PROTEINS FRACTIONATED BY GELFREE 8100 CAN BE DETECTED BY ANTIBODY ARRAY ANALYSIS

In the initial experiments executed in this thesis I was able to detect a few proteins when using the Gelfree fractionation system in combination with MAP (western-MAP) (Fig 14). While these results were encouraging, the number of antibodies that captured their intended target was only between 3-8%. Moreover, the results varied considerably between experiments (not shown). Optimization was therefore necessary, and a number of factors where investigated to improve the results.

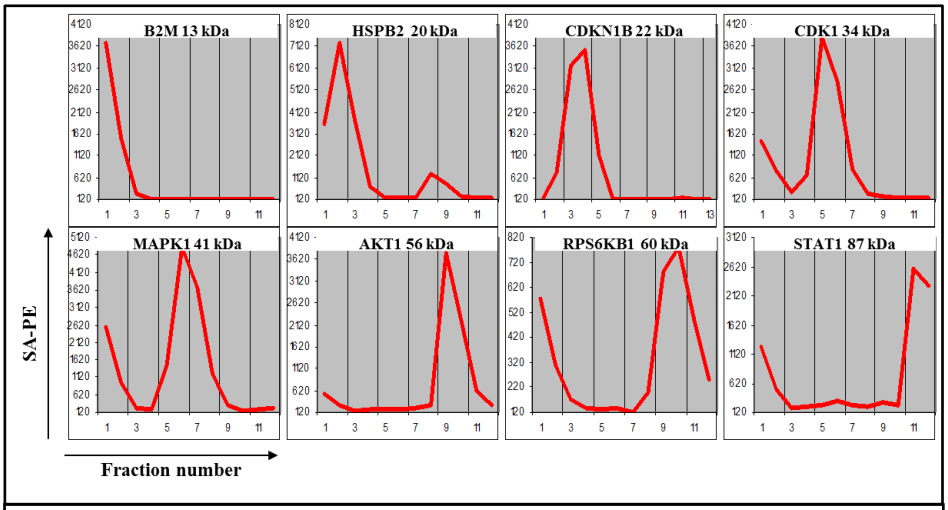

Figure 14. Protein detection with western-MAP. Graphs showing Gelfree fraction number on the x-axis and fluorescent PE-signal on the y-axis. These graphs show eighth different proteins (from the upper left: Beta-2-microglobulin, Heat shock protein beta-2, Cyclin-dependent kinase inhibitor 1B, Cyclin-dependent kinase 1, Mitogen-activated protein kinase 1, RAC-alpha serine/threonine-protein kinase, Ribosomal protein S6 kinase beta-1 and Signal transducer and activator of transcription 1-alpha/beta) recognized by different their corresponding antibodies. The fractions were added antibody microsphere arrays directly after fractionation, without any further manipulation. A few proteins that were recognized in the predicted fractions are shown. The samples were Jurkat whole cell lysate (4.4mg/mL protein) fractionated with an 8% acrylamide Gelfree cartridge

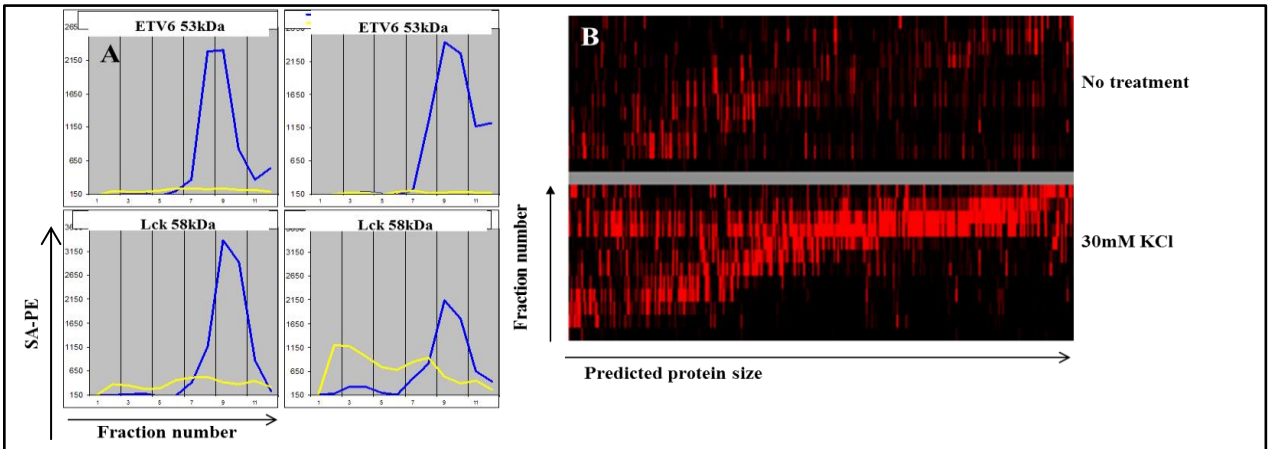

Fig. 15. Removal of SDS, 30mM KCl versus no removal. A: Graphs showing Gelfree fraction number on the x-axis and fluorescent PE-signal on the y-axis. These graphs show two different proteins (from the top: Transcription factor ETV6 and Tyrosine-protein kinase Lck) recognized by different antibodies. One sample (blue) was precipitated with 30mMKCl for SDS removal, and the other (yellow) was added microspheres directly after fractionation, without any further manipulation. The proteins shown were not recognized by their antibodies in the sample with free SDS present, and there was some unspecific binding. When SDS was removed, the proteins were detected. This was typically observed with most antibodies. **B:** Heat maps showing the same two sample preparation. The upper heat map shows results obtained with fractions that were only diluted in PBS prior to contact with the arrays. The lower map shows results obtained in fractions treated with 30mM KCl to precipitate SDS. The intensity of nearly all bands is higher in the lower heat map, and to a large extent the bands were observed at the expected position, but extra bands were also observed. This is particularly evident in the left part of the map where a number of bands are seen in the higher fraction numbers. The samples were Jurkat whole cell lysate (4.4mg/mL protein) fractionated with an 8% acrylamide Gelfree cartridge.

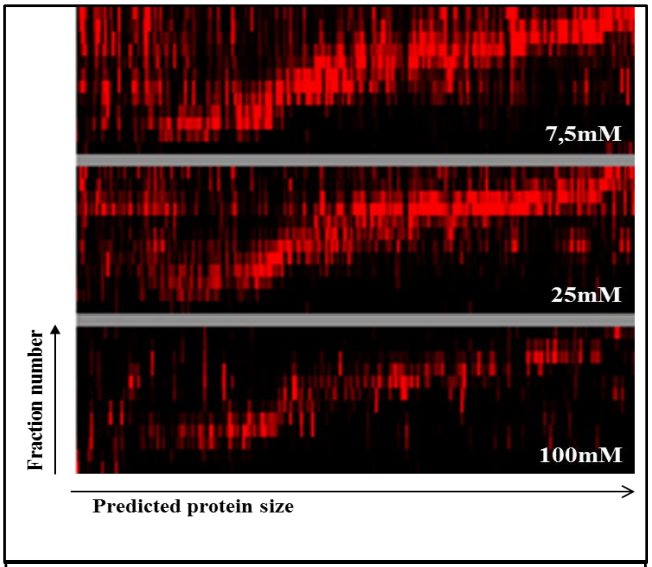

Fig. 16. Removal of SDS with different concentrations of KCl. Heat maps of three samples fractionated on the Gelfree fractionation system. Three different concentrations of KCl were used for removal of SDS. The signal intensity increased with lower concentrations of KCl. However, when only using 7,5mM KCl the bands were broader, and cross-reactivity was more extensive. The samples were Jurkat whole cell lysate (4.4mg/mL protein) fractionated with an 8% acrylamide Gelfree cartridge.

5.3 OPTIMIZATION OF WESTERN-MAP

5.3.1 REMOVAL OF SDS ENHANCES ASSAY PERFORMANCE

The fractions contain the detergent SDS, which may interfere with antibody binding. Attempts were therefore made to remove SDS by using KCL perspiration (Fig.15). When free SDS was present in the fractions, the results generally showed that the antibodies did not bind their target. Often the predicted peaks were missing, or the peaks were low. When free SDS was removed

more antibodies recognized their intended target, and the peaks observed in the presence of SDS were taller. Cross-reactivity was also reduced. However, extra bands were still observed particularly for the smaller proteins (Fig. 15).

Different concentrations of KCl were tested. The signal intensity increased with lower concentrations of KCl. However, with the lowest concentration the bands were broader and cross-reactivity was extensive (Fig. 16).

5.3.2 ADDITION OF THE DETERGENT TWEEN 20 ENHANCES DETECTION AND REDUCES CROSS-REACTIVITY

Proteins that have undergone denaturation are often less soluble. Removal of detergents would therefore be expected to result in precipitation of proteins and formation of protein aggregates. Therefore supplementing the fractions with a non-ionic detergent such as Tween 20 might be beneficial when added after KCl precipitation. First of all the detergent would increase protein solubility. In addition, residual SDS remaining after KCl precipitation would be solubilized into

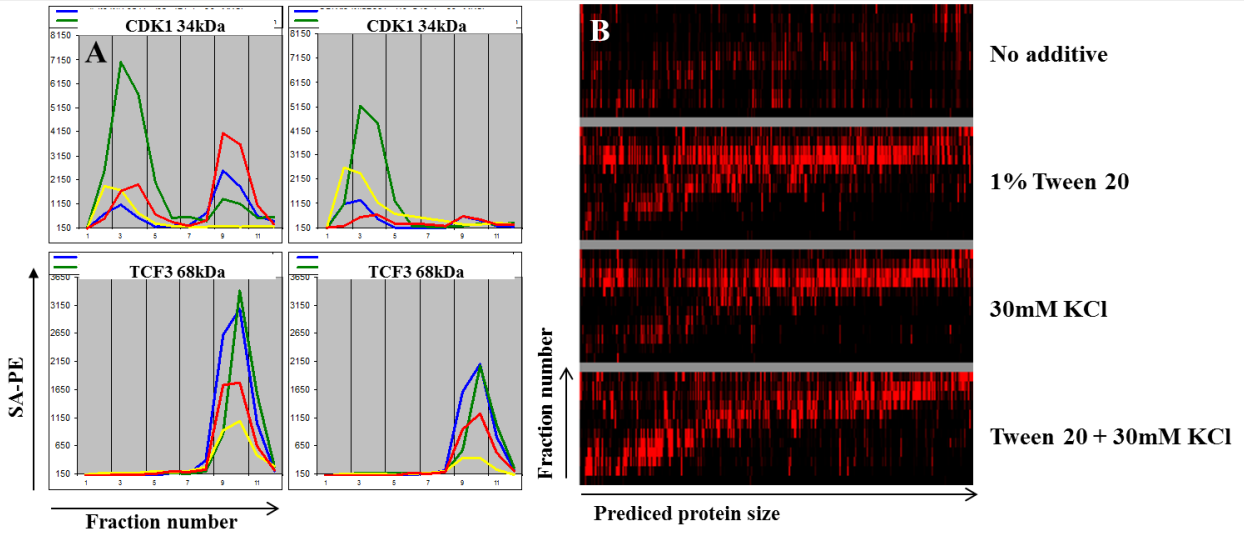

Fig. 17. Addition of the detergent Tween 20. A: These graphs show two different proteins (from the top: Cyclin-dependent kinase 1 and Transcription factor 7-like 1) recognized by different antibodies. Four samples with different sample preparations are shown. One sample (blue) was precipitated with 30mM KCl for free SDS removal before incubation with the antibody microsphere arrays. Another sample (red) was added the detergent Tween 20 prior to incubation. The third sample (green) was both added 1% Tween 20 and free SDS was removed with 30mM KCl, and the last sample (yellow) was added the antibody microsphere arrays directly after fractionation, without addition of Tween 20 or SDS removal. The majority of the validated antibodies showed the best results when the combination of free SDS removal and addition of Tween 20 was used. This was particularly evident with the smaller proteins. **B**: Heat maps showing the same four sample preparations. When using no additive the signal was low and the cross-reactivity excessive. With addition of 30mM KCl or 1% Tween 20 the signal was enhanced, but the specificity was still low. When using a combination of 30mM KCl and 1% Tween 20 the specific signal was further enhanced and the cross-reactivity reduced. The samples were Jurkat whole cell lysate (4.4mg/mL protein) fractionated with an 8% acrylamide Gelfree cartridge.

mixed detergent micelles. The addition of Tween 20 to the fractions has also the added advantage of preventing aggregation of the bead-based arrays.

The effect of adding Tween 20 was examined both in fractions where free SDS was not removed, and in combination with removal of free SDS with 30mM KCl. In general the addition of Tween 20 was beneficial for the results (Fig. 17). Similar results were observed in more than five independent experiments, and in all cases, the combination of 1% Tween 20 and 20-30mM KCl provided the best results. Addition of detergent was particularly beneficial for detecting and analysing membrane proteins. Membrane proteins were also often detected in later fractions than predicted (Fig. 18).

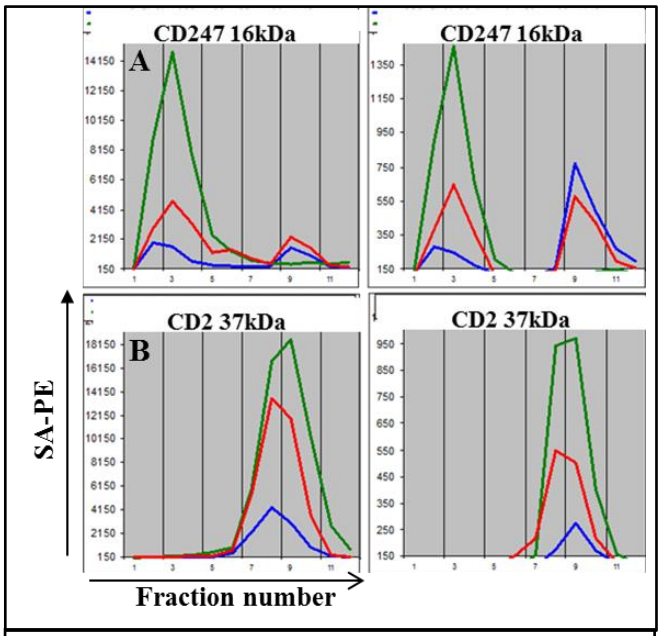

Fig. 18. Membrane proteins and addition of the detergent Tween 20. These graphs show two different membrane proteins (**A:** T-cell surface antigen CD2 and **B**: T-cell surface glycoprotein CD3 zeta chain) recognized by different antibodies. Three samples with different sample preparations are shown. One sample (blue) was precipitated with 30mM KCl for removal of free SDS before incubation with the antibody microsphere arrays. Another sample (red) was added the detergent Tween 20 prior to incubation. The third sample (green) was both added Tween 20 and free SDS was removed with 30mM KCl. **A**: The majority of membrane proteins were best recognized when the combination of free SDS removal and addition of detergent was used. Addition of Tween 20 was highly favourable. **B**: Many of the membrane proteins were detected in later fractions than what was predicted from the proteins molecular weight. The samples were Jurkat whole cell lysate (4.4mg/mL protein) fractionated with an 8% acrylamide Gelfree cartridge.

5.3.3 REDUCTION OF PROTEINS WITH 1mM TCEP IS SUFFICIENT TO OBTAIN SATISFACTORY RESULTS

Different types of reducing agents were examined to investigate if these would improve the fractionation resolution or signal strength. TCEP (Tris(2-carboxyethyl)phosphine hydrochloride), dithiothreitol (DTT) and β-mercaptoethanol were examined. The three different reducing agents gave similar results in Western-MAP, both in signal strength and resolution (Fig. 19).

Since TCEP has other beneficial properties, such as not inhibiting labelling with malemide derivatives, this reducing agent was chosen to be used in the later work. It was investigated what concentration of TCEP that should be used. Three different concentrations were examined and the best results were obtained when using 1mM TCEP. The highest concentration of 50mM gave additional peaks and the proteins seemed to migrate more slowly through the gel. A medium high concentration of 10mM TCEP showed similar results as to1mM, but with slightly lower signal strength (Fig. 20).

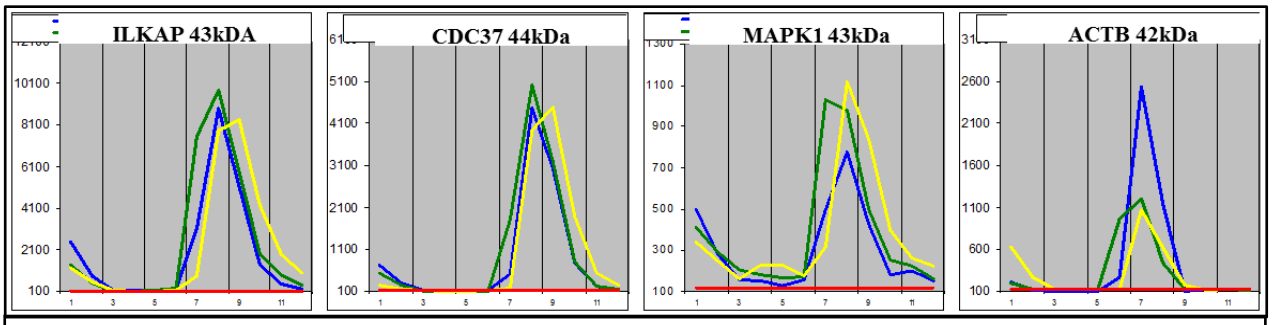

Fig. 19. Reducing agents, TCEP, DTT or β-mercaptoethanol. These graphs show four different proteins (from the left: Integrin-linked kinase-associated serine/threonine phosphatase 2C, Hsp90 co-chaperone Cdc37, MAP kinase kinase 1 and Beta-actin) recognized by their corresponding antibodies. Three different sample preparations are shown. One sample (blue) was reduced with TCEP (1mM) prior to fractionation, while the two other samples were reduced with 50mM DTT (green) or 3% β-mercaptoethanol (yellow). The results were similar with all the three different reducing agents. The samples were Jurkat whole cell lysate (4.4mg/mL protein) fractionated with an 8% acrylamide Gelfree cartridge.

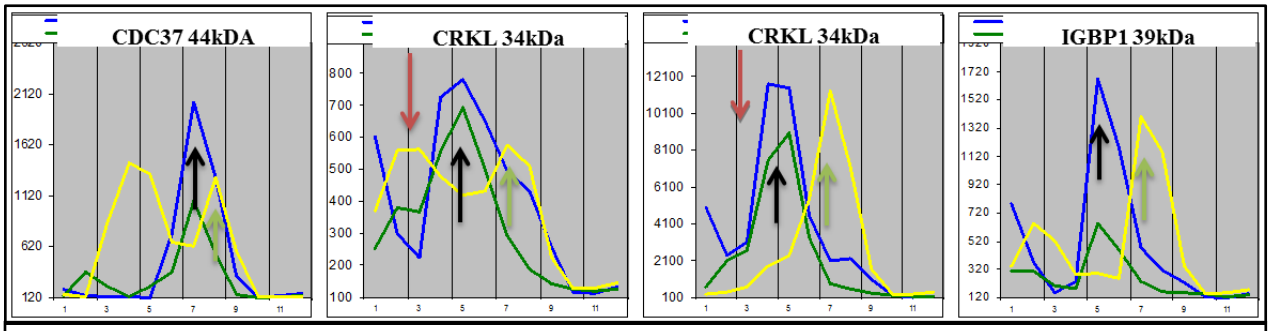

Fig. 20. TCEP concentration. These graphs show four different proteins (from the left: Hsp90 co-chaperone Cdc37, two different antibodies against Crk-like protein and Immunoglobulin-binding protein 1) recognized by their corresponding antibodies. Three different concentrations of TCEP were used in the sample preparations. One sample (blue) was reduced with 1mM TCEP prior to fractionation, while the two other samples were reduced with 10mM TCEP (green) or 50mM TCEP (yellow). When using 50mM TCEP in the sample preparation an extra peak (red arrow, **A** and **B**) was often observed. What this peak represents was uncertain, but if a comparison between picture **B** and **C** is done, it can indicate that this is cross-reactivity. Picture **B** and **C** shows two different antibodies against the same protein, and since one of the peaks (red arrow) is absent in picture **C**, while present in picture **B** this peak is most likely cross-reactivity. The peaks of the assumed intended target (green arrows) observed when using 50mM TCEP in the sample preparation are slightly to the right, compared to the corresponding peaks (black arrows) in the two samples with lower TCEP concentration. This indicates that the proteins migrated slower through the gel column during fractionation, when a high TCEP concentration was used. The samples were Jurkat whole cell lysate (5.4mg/mL protein) fractionated with an 8% acrylamide Gelfree cartridge.

5.3.4 COMBINED PROTEIN LABELLING WITH AMINE- AND THIOL-REACTIVE BIOTIN IS SUPERIOR TO LABELING WITH EITHER ALONE TO SINGLE LABELING

Many reducing agents compete with thiol reactive protein labels, such as maleimide, but TCEP is compatible with these types of labels. Since 1mM TCEP proved sufficient for obtaining good results with western-MAP it was examined if using a combination of NHS-biotin and maleimide-biotin for protein labelling would improve the results of western-MAP even further.

It is common to use N-hydroxy-succinimidyl esters (NHS) of haptens or fluorescent dyes for labeling of proteins prior to antibody array analysis. These binds free amines, which are present in the side chains of lysines and at the amino terminus of proteins. Lysines are more frequent in proteins than cysteines, and one would therefore expect amine-reactive biotins to provide stronger signals than the thiol-reactive maleimide derivatives. Indeed, the results obtained here show that the bands observed in samples labeled with maleimide-biotin were rather weak compared to those observed with biotin-NHS (Fig. 21). However, the maleimide appeared to act almost synergistically as a label. The increase observed when the two labels were used simultaneously was remarkable. Moreover, there was little apparent increase in non-specific

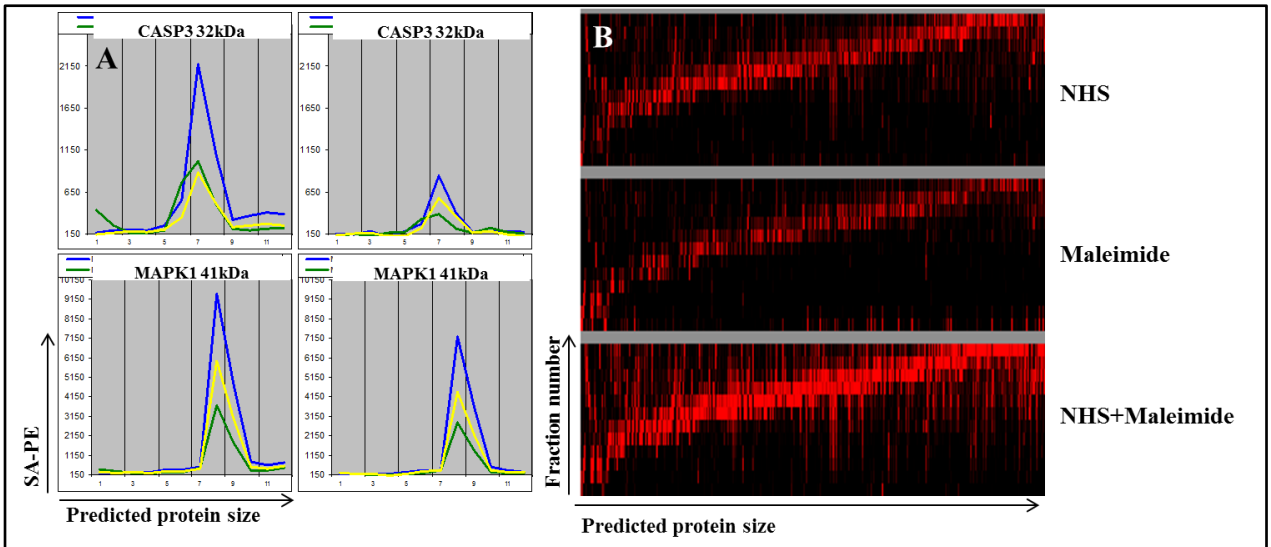

Figure 21. Protein labelling. A: These graphs show two different proteins (from A: Caspase-3 and Mitogen-activated protein kinase 1) recognized by different antibodies. Three differently labelled samples are shown. In one sample (blue) the proteins was labelled with both NHS-biotin and malemide-biotin, and the two others was labelled with only NHS-biotin (yellow) or malemide-biotin (green). Labelling with both NHS-biotin and malemide-biotin gave the best results for almost all the validated antibodies. **B:** Heat map showing the same three differently labelled samples. Combined labelling with both NHS-biotin and malemide-biotin gave considerable higher signal than either of the two labelling reagents used separately. A concentration of 2.0mg/mL NHS-biotin was used, and the malemide-biotin concentration was 0.5mg/mL. The samples was Jurkat whole cell lysate (4.4mg/mL protein) fractionated with a 8% acrylamide Gelfree cartridge

binding. The results shown are representative of three independent experiments, and in all cases, the combination showed better results than what was obtained when the labels were used alone.

5.3.5 PROTEIN CONCENTRATION

A high protein concentration can possibly reduce resolution when using electrophoresis as a fractionation method. When using an immunological detection principle, such as MAP, a high protein concentration is beneficial. Therefore it was examined how the protein concentration in the samples affected the results in western-MAP.

Two separate experiments were done to investigate which protein concentration that gave the best final results in western-MAP. Three different amounts of proteins were loaded onto the gel in these experiments: 700µg, 230µg and 78µg (Fig. 22). Surprisingly the resolution was not reduced with increasing loading. Instead, there was a reduction in the absolute signal intensity. In these experiments we did not see a clear increase in signal upon further dilution of the samples.

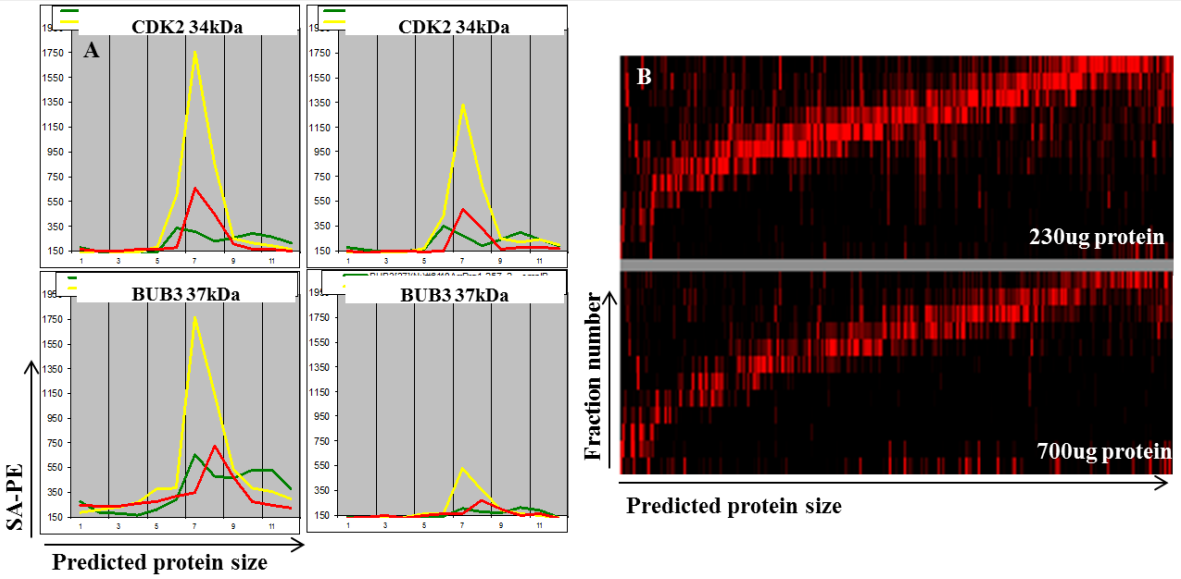

Figure 22. Protein loading, comparison between loading 700µg, 240µg and 78µg protein onto the Gelfree cartridge. A: These graphs show two different proteins (from the top: Cdk2 and Mitotic checkpoint protein BUB3) recognized by their corresponding antibodies. Three different protein concentrations were used to load the Gelfree cartridge: 700µg, 240µg and 78µg. For the majority of the validated antibodies a protein concentration of 240µg gave the best results. **B**: Heat maps showing the signal obtained when loading the gel with 700µg and 230µg. Surprisingly the resolution was not reduced by an increased loading of protein, and a lower loading concentration of 230µg showed the highest signal intensity. The sample used was HeLa whole cell lysate with a starting concentration of 7.0mg/mL that was diluted to achieve the correct concentrations. The samples were fractionated with a 10% acrylamide Gelfree cartridge.

5.4 PERFORMANCE OF WESTERN-MAP

When proteins are fractionated with the Gelfree 8100 fractionation system the proteins with the same MW will end up in the same fraction, and smaller proteins will end up in earlier fractions than proteins with larger MWs. In the experiments done in this thesis the proteins distribute mainly as expected throughout the fractions (Fig. 23 and 24). The proteins with the smallest MW were observed in the first fractions and the largest in the latest fractions. The 12% acrylamide cartridge detected the smallest proteins and the 5% the largest.

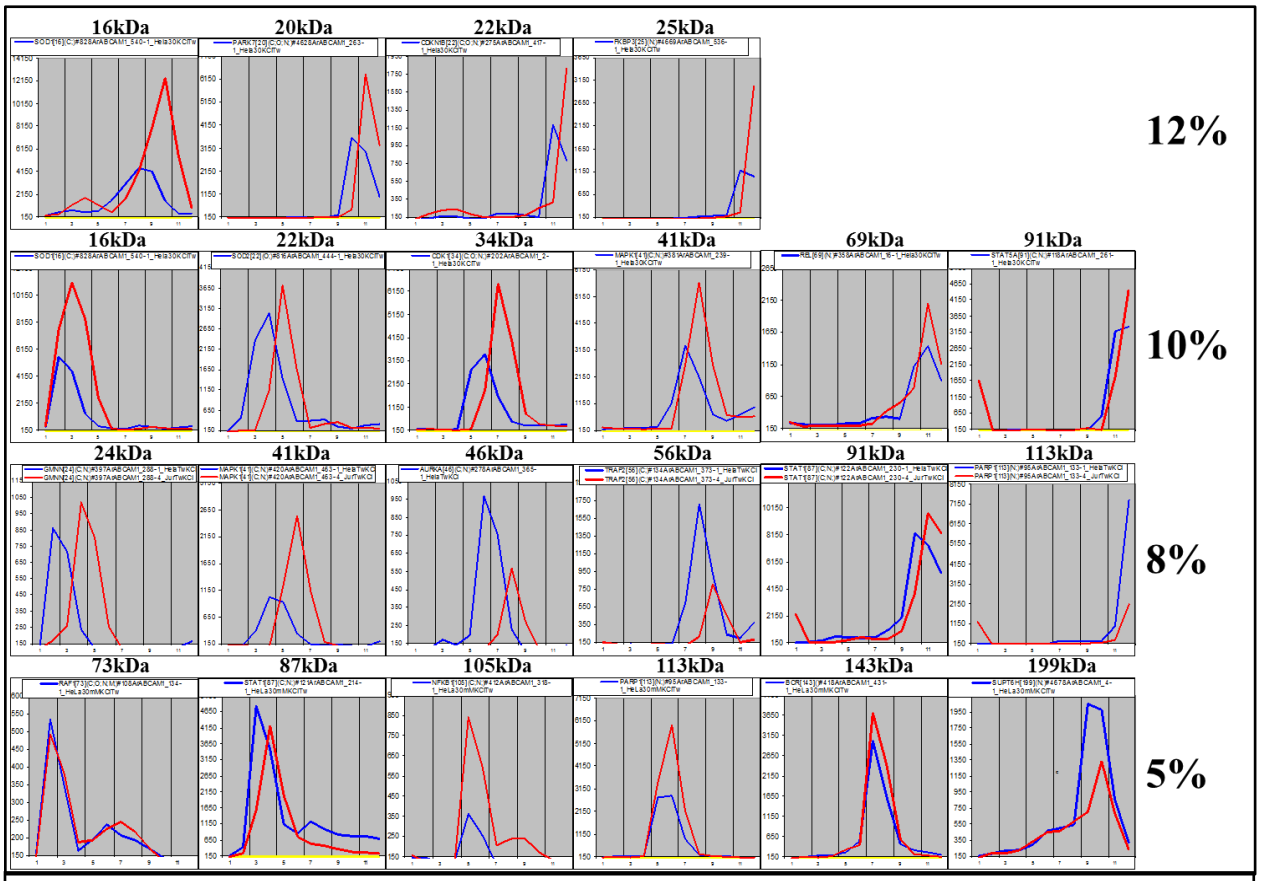

Figure 23. Protein distribution. Graphs showing Gelfree fraction number on the x-axis and fluorescent signal on the y-axis. These graphs are describing different proteins with different predicted molecular weight. The molecular weights are indicated above the graphs. Examples from all the four different Gelfree fractionation cartridges are show (from the top row: 12% acrylamide, 10% acrylamide, 8% acrylamide and 5% acrylamide cartridge at the bottom row). The proteins distributes well throughout the fractions, as the smallest proteins was detected in the first fractions, and the largest in the latest fractions. When using the cartridge with the highest tris-acetate percentage (12%) the smallest proteins was detected and the biggest proteins were detected when using the cartridge with the lowest tris-acetate percentage (5%). The samples were fractionated HeLa whole lysate (blue) and Jurkat whole cell lysate (red). The protein concentrations for the samples were 7.0mg/mL for the HeLa whole cell lysate, and 2.4mg/mL for the Jurkat whole cell lysate. The samples are fractionated with an 8% tris-acetate cartridge.

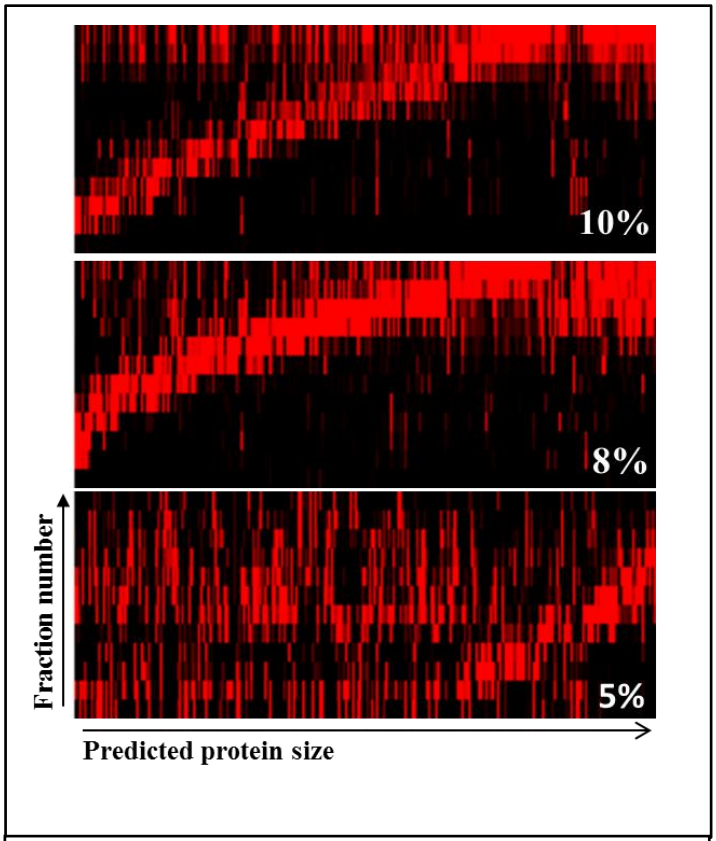

Figure 24. Performance of the different Gelfree cartridges. Heat maps showing the fractionation obtained with three of the Gelfree cartridges. The 10% and 8% acrylamide cartridge showed good resolution with proteins spanning from about 15kDa an up to a 100kDa. The results obtained with the 5% acrylamide cartridge were less optimal. However the 5% gel appears to be useful for detection of several large proteins, but the large number of bands observed with antibodies to smaller proteins is clearly a reason for concern. The sample used was HeLa whole cell lysate protein concentration of 7.0mg/mL.

The four types of gel cartridges available for the Gelfree8100 fractionation system should collectively, fractionate proteins within a very broad size range (10-500kDa). Most of the results shown so far in this thesis were obtained with gels that contain 8% or 10% acrylamide. These two types of cartridges generally showed the best fractionation pattern, and proteins between 15kDa and about 100kDa were successfully detected. The results obtained with the 5% acrylamide gels were less optimal. While there is correlation between the position of the band and the protein size between 70kDa and 150kDa, antibodies to proteins of smaller sizes showed considerable binding (Fig. 24). Currently, it is not clear whether this represents cross-reactivity or binding of fragments from the intended targets. The cartridge appears to be useful for detection of several large proteins. However, the large number of bands observed with antibodies to smaller proteins is clearly a reason for concern.

A total of 537 different antibodies were validated using western-MAP in this thesis. An antibody was validated if it showed peaks in the predicted fractions or if multiple antibodies against the same target showed similar peaks. The validated antibodies recognized a total of 350 proteins. Table 5 shows an overview over the MW of the proteins detected after fractionation of HeLa whole cell lysate with the different types of Gelfree cartridges.

Table 5. Protein distribution. This table shows the molecular weight of the proteins observed in the various fractions when HeLa whole cell lysate (7.0mg/mL) was fractionated with the different Gelfree fractionation cartridges. The values shown are approximates, and the values are more unsure for the smallest and the biggest molecular weights, since we had fewer antibodies against proteins in more extreme molecular ranges. None antibodies that had targets below 14kDa or above 199kDa were validated.

	Fraction 1	Fraction 2	Fraction ‘3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Fraction 8	Fraction 9	Fraction 10	Fraction 11	Fraction 12
12%							14-16 kDa	16-17 kDa	18-19 kDa	20-22 kDa	22-28 kDa	29-35kDa
10%		12-17 kDa	18-22 kDa	20-25 kDa	24-32 kDa	31-37 kDa	35-46 kDa	44-49 kDa	48-60 kDa	56-70 kDa	67-83 kDa	84-119 kDa
8%		17-32 kDa	31-37 kDa	34-40 kDa	37-46 kDa	46-49 kDa	49-56 kDa	53-60 kDa	59-73 kDa	70-88 kDa	65-94 kDa	92-119 kDa
5%		69-73 kDa	70-88kDa	85-94 kDa	94-106 kDa	100-133 kDa	123-145 kDa	141-162 kDa	159-199 kDa			

One recurring deviation with all four types of cartridges in western-MAP was that there was rarely a peak in the first fraction, although the gel images show plenty of proteins here (Fig. 13). The peaks were absent even with antibodies that were proven to work with other types of cartridges (Fig. 25). Several experiments were executed to try to overcome this problem. It was possible that a high SDS concentration in first fraction caused this lack of detected proteins. This high SDS concentration could be a result of either the high concentration of SDS in the sample buffer or due to accumulation of SDS during the first running interval of the cartridge. Attempts were therefore made to use a sample buffer with lower SDS concentration, and to change the buffer in the sample

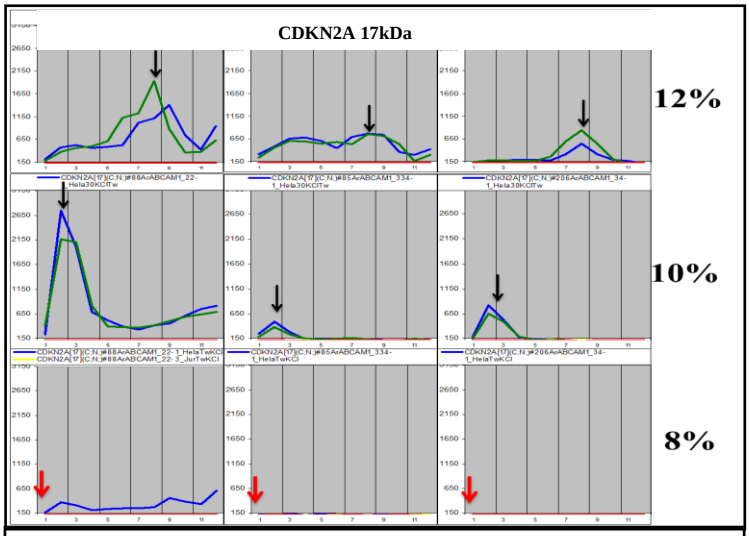

Figure 25. Expected protein peaks absent in the first fraction. Graphs showing Gelfree fraction number on the x-axis and fluorescent signal on the y-axis. These graphs show Cyclin-dependent kinase inhibitor 2A (CDKN2A) recognized by three different antibodies (from left to right). The samples are fractionated using Gelfree cartridges with three different acrylamide concentration (from the top: 12%, 10% and 8% at the bottom). When fractionating the samples with 12% - or 10% acrylamide cartridges, peaks were observed in the expected fraction (black arrows). When fractionating with an 8% acrylamide cartridge it was expected to observe a peak in the first fractions, but this was absent (red arrows). The samples were HeLa whole cell lysates with a protein concentration of 7.0mg/mL. The samples were run in duplicates, with the exception of the sample fractionated with the 8% acrylamide cartridge.

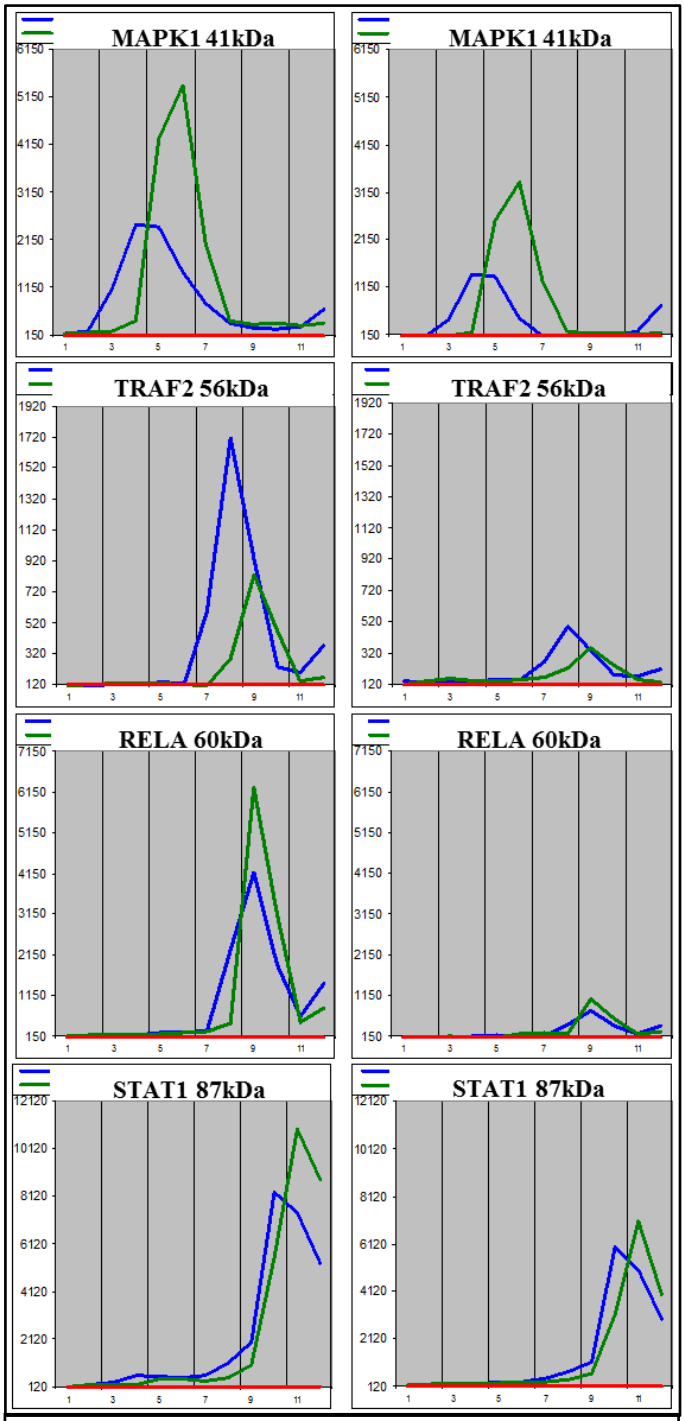

Fig. 26. Different antibodies recognize the same protein. Graphs showing four different proteins (from the top: mitogen-activated protein kinase 1, TNF receptor-associated factor 2, transcription factor p65 and signal transducer and activator of transcription 1-alpha/beta) recognized by different antibodies. The different antibodies directing against the same protein, recognize proteins in the same fraction. The samples were HeLa whole lysate (blue) with a protein concentration of 7.0mg/mL

collection chamber 10 minutes prior to collecting the first fraction. None of these attempts improved the results.

One way of validating that the antibodies recognize the intended target is to have two or more antibodies directed against the same target. Different antibodies do not often cross-react equally and cross-reactivity can therefore be distinguished from binding of the intended target. If different antibodies recognize a target in the same fractions, this is probably the correct target, while targets that are only recognized by one type of antibody are probably unspecific (Fig. 26 and 27).

A new method, such as western-MAP, should be compared to a traditional method to be fully validated. The results obtained with western-MAP were therefore compared to traditional western blotting. The western blotting result used in this thesis was obtained by Abcam.

Western-MAP showed comparable results to traditional western blotting with 68 out of 89 antibodies available (76.4%) (Fig. 28-31) (Appendix). All the antibodies used in these experiments are polyclonal, so some of the antibodies show extensive cross-reactivity.

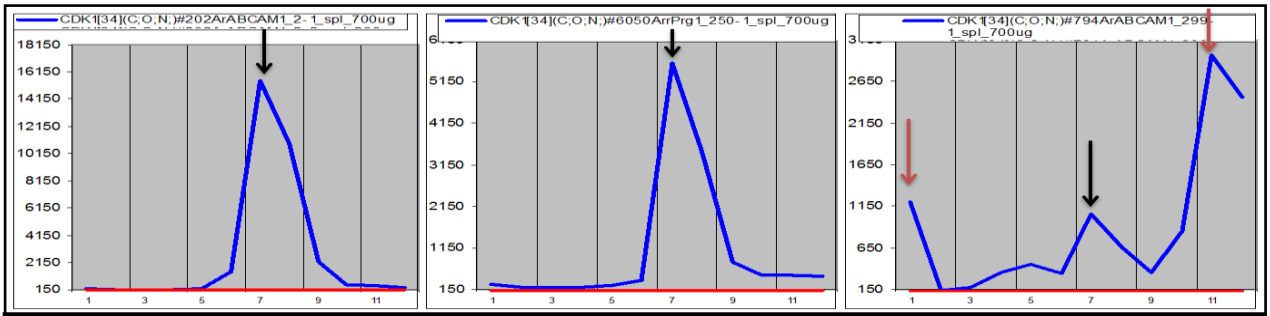

Fig. 27. Example of cross-reactivity. Graphs showing Gelfree fraction number on the x-axis and fluorescent PE-signal on the y-axis. These graphs show CDK1 recognized by three different antibodies. The black arrows indicate the peaks of the intended target. These peaks are consistent with all three different antibodies. Some peaks only occur with one of the antibodies, indicating that this probably is cross-reactivity (red arrows). The sample was HeLa whole cell lysate with a protein concentration of 7.0mg/mL fractionated with a 10% acrylamide cartridge.

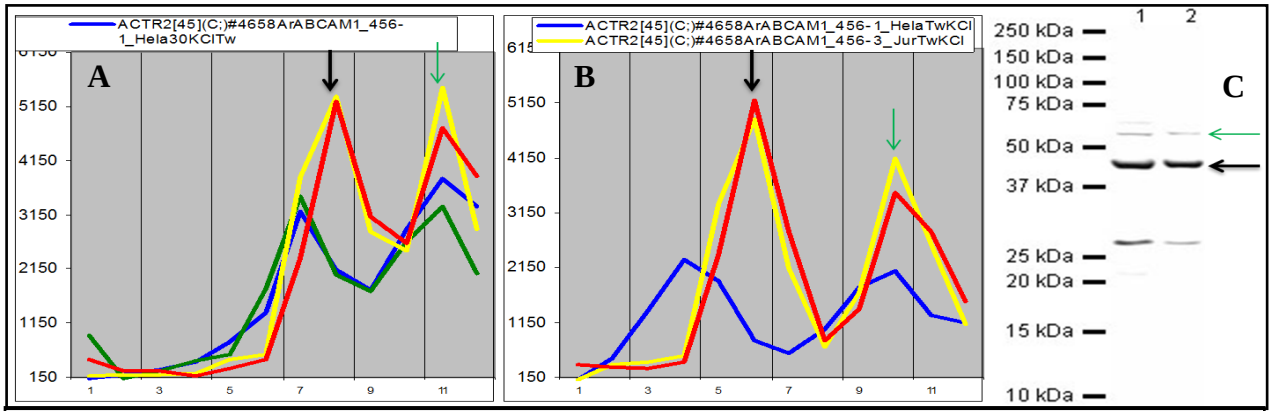

Fig. 28. Comparison between traditional western blotting and western-MAP, anti-actin-related protein 2 (ACTR2). Graph showing ACTR2 recognized by one antibody in HeLa (blue and green) and Jurkat whole cell lysates (yellow and red) fractionated on the Gelfree 8100 fractionation system. The samples where run in duplicates. **A:** The samples were fractionated using a 10% acrylamide cartridge. Two peaks were observed, one (black arrow) corresponding to the predicted molecular size of the protein (45kDa) and one peak (green arrow) representing a bigger target. **B:** When the samples were fractionated with an 8% acrylamide cartridge the same two peaks were observed. The peaks were observed slightly to the left compared to the 10% acrylamide cartridge fractionation, as expected. **C:** Traditional western blotting with HeLa (lane 1) and Jurkat (lane 2) whole cell lysate. Three bands were observed: 60kDa, 45kDa and 27kDa. The bands at 60kDa and 45kDa correspond to the two peaks observed in western-MAP.
Picture of western blot taken from: *http://www.abcam.com/arp2-antibody-ab47654.html*

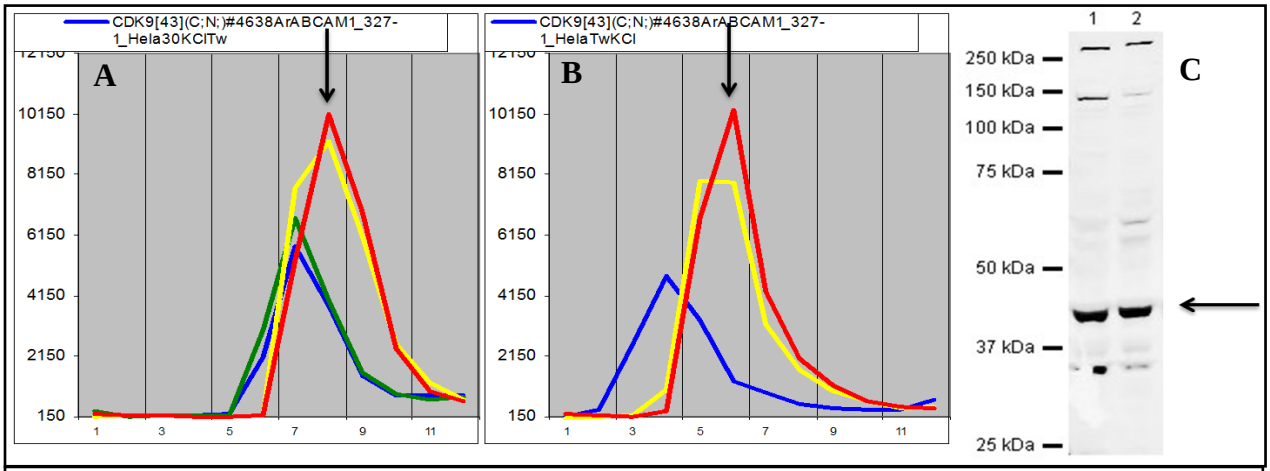

Fig. 29. Comparison between traditional western blotting and western-MAP, anti-CDK9. Graph showing CDK9 recognized by one antibody in HeLa (blue and green) and Jurkat whole cell lysates (yellow and red) fractionated on the Gelfree 8100 fractionation system. The samples where run in duplicates. **A:** The samples were fractionated using a 10% acrylamide cartridge. One peak were observed corresponding to the predicted molecular size of the protein (43kDa). **B:** When the samples were fractionated with an 8% acrylamide cartridge the same peak was observed. The peak was observed slightly to the left compared to the 10% acrylamide cartridge fractionation, as expected. **C:** Traditional western blotting with HeLa (lane 1) and Jurkat (lane 2) whole cell lysate. Three bands were observed: 41kDa, 140kDa and 260kDa. The band at 41kDa corresponds to the two peaks observed in western-MAP.
Picture of western blot taken from: *http://www.abcam.com/cdk9-antibody-ab38840.html*

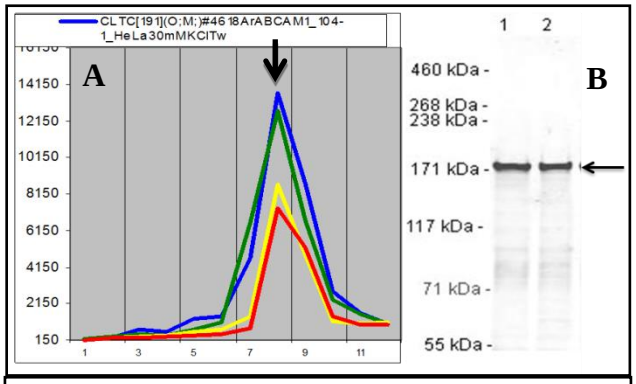

Fig. 30. Comparison between traditional western blotting and western-MAP, anti-Clathrin heavy chain 1 (CLTC). A: Graph showing CLTC recognized by one antibody in HeLa (blue and green) and Jurkat whole cell lysates (yellow and red) fractionated on the Gelfree 8100 fractionation system, with a 5% acrylamide cartridge. The samples were run in duplicates. One peak was observed, and this peak corresponds to the predicted molecular size of the protein (191kDa). **B:** Traditional western blotting with HeLa (lane 1) and Jurkat (lane 2) whole cell lysate. One band was observed at about 180kDa, which corresponds to the peak observed in western-MAP.
Picture of western blot taken from:
http://www.abcam.com/clathrin-heavy-chain-antibody-ab21679.html

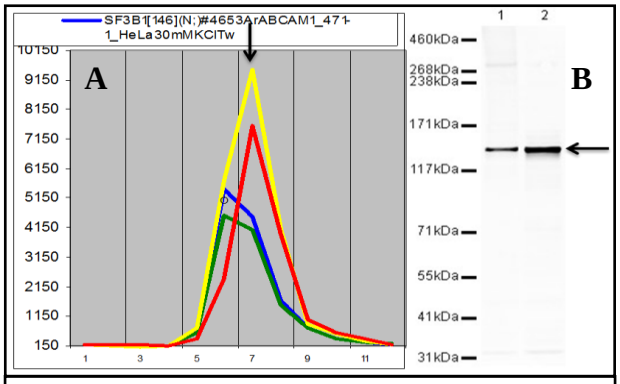

Fig. 31. Comparison between traditional western blotting and western-MAP, anti-Splicing factor 3B subunit 1 (SF3B1). A: Graph showing SF3B1 recognized by one antibody in HeLa (blue and green) and Jurkat whole cell lysates (yellow and red) fractionated on the Gelfree 8100 fractionation system, with a 5% acrylamide cartridge. The samples were run in duplicates. One peak was observed, and this peak corresponds to the predicted molecular size of the protein (146kDa). **B:** Traditional western blotting with HeLa (lane 1) and Jurkat (lane 2) whole cell lysate. One band was observed at about 150kDa, which corresponds to the peak observed in western-MAP.
Picture of western blot taken from:
http://www.abcam.com/sap155-antibody-ab39578.html

6.0 DISCUSSION

6.1 ADVANTAGES OF WESTERN-MAP

The results presented in this thesis show that it is feasible to perform antibody array analysis of cellular proteins that have been separated by SDS-PAGE. Western-MAP combines the specificity of western blotting with the multiplexing capacity of antibody array analysis. This opens a wide range of new possibilities.

Previously, antibody arrays have been used to measure native proteins that have been separated by size exclusion chromatography (SEC-MAP) [9, 83-85]. While SEC offers the possibility to study protein complexes a "western blot format" has many advantages. Most important is the fact that the results are simple to interpret as the position of the peak corresponding to specific binding is predictable.

A second advantage with a "western blot format" is that a large number of antibodies against cellular proteins have been developed for use in western blotting. An earlier study showed that antibodies developed for western blotting performed better in applications where the proteins were denaturated prior to analysis [62]. Several of the antibodies used in this thesis have previously undergone testing for their ability to capture native proteins separated by SEC, but found to be of limited use. The western-MAP format therefore extends the potential of antibody array analysis.

Finally, a format for antibody array analysis that resembles western blotting may lower the threshold for implementation. Antibody array analysis was first described more than ten years ago, but the technique is yet to see in widespread use. Antibody array analysis has been described as a western blot were all the bands in one lane has been compressed into one [10], resulting in that all cross-reactive binding contribute to the signal. With the approach described in this thesis, users have the same type of intrinsic control they are used to from western blots.

6.2 OPTIMIZATION OF WESTERN-MAP

While early results showed that several antibodies could capture their targets from fractions obtained by the Gelfree 8100 fractionation system, the overall performance of the assay was generally poor. A high concentration of free SDS during incubation with the microsphere arrays is known to have an impact on the antibody array results [83, 86]. Potassium salts of dodecyl

sulphate (KDS) have a low solubility [90], and it was therefore investigated if precipitation of SDS with potassium chloride (KCl) prior to addition of the microsphere arrays would improve the results obtained with western-MAP.

The results obtained in this thesis showed that removal of SDS improved the results. A possible explanation to why a high SDS concentration not was beneficial for the results could be that SDS binds the proteins and alters the charge of the protein. The protein charge is probably effecting the antigen-antibody interaction. When using a high concentration of KCl the amount of protein detected decreased, and a medium concentration of 20-30mM showed to be optimal. A plausible explanation for this is that some of the proteins could precipitate together with KDS at high levels of KCl, or the proteins get less soluble without the SDS present and is therefore inaccessible for the antibody array.

Addition of the detergent Tween 20 prior to incubation with the antibody array showed beneficial for the results. A combination between SDS removal and Tween 20 was shown to be optimal. The detergent Tween 20 will make micelles with SDS and therefore reduce the concentration of free SDS in the sample. Detergents will also help solubilize the proteins and make them accessible for detection and analysis.

Addition of detergent was particularly favourable for detection of membrane proteins. Membrane proteins have hydrophobic regions, and will therefore often be less soluble without the presence of detergents. The membrane proteins were also often detected in later fractions than what was predicted from their MW. A plausible explanation for this could be poor solubilisation of the protein during fractionation, as reported in another study [46]. It is also possible that the hydrophobic proteins have bound detergents, such as lauryl maltoside, prior to fractionation. This could block SDS from binding the proteins properly or increase the molecule size. Less SDS bound means less negative charge on the proteins, which will lower the migration speed through the gel. An increased molecule size will also result in slower migration of the proteins.

Both removal of SDS and addition of Tween 20 can be performed while the fractions are in microtiter plates. Thus, the work on optimization resulted in the discovery of simple and inexpensive means to obtain far better signal to noise ratios.

How reducing agents affect the results was also examined. The three different reducing agents investigated (TCEP, DTT and β-mercaptoethanol) showed similar results, but since TCEP does not interfere with maleimide binding, and is more stable upon storage [91] this was the reduction agent of choice. Different concentrations of TCEP were also examined, and 1mM showed efficient to obtain good results.

The work on optimization of protein labelling led to an unexpected discovery. I found that thiol- and amine-reactive biotins have a synergistic effect on signal enhancement. Lysines are more frequent in proteins than cysteines. Thus, it seems unlikely that the effect is simply a result of adding more label molecules per protein. An explanation may be that alkylation of thiols by biotin-maleimide prevents refolding of proteins. This could provide better access to the epitopes for the antibodies or enhance exposure of labelled amino-acids and thereby providing better access for streptavidin. This possibility can be explored further by replacing biotin-maleimide with N-Ethyl-Maleimide.

One might expect that more extensive labelling could alter antibody reactivity. However, an earlier study showed that the levels of amine-reactive biotin used have little inhibitory effect on antibody binding[83]. This study confirms and extends these findings. While several epitopes are likely to contain lysine residues, the same residues may not be labelled in all molecules. It is also worth noting that most antibodies can be labelled with biotin with little loss in functionality.

The protein concentration in the samples fractionated with the Gelfree fractionation system seemed to have impact on the final results. We expected to see a reduced resolution with increased loading, as stated by the developers of the Gelfree fractionation system[37]. Instead, there was a reduction in the absolute signal intensity, whereas the resolution was not altered. In these experiments we did not see an increase in signal upon further dilution of the samples, and further studies are therefore needed to determine linearity and optimal loading.

6.3 PERFORMANCE OF WESTERN-MAP

For the sample fractionation to be helpful to rule out unspecific binding, it was important that the fractionation had a high resolution. The protein fractionation done in my experiments showed good resolution, as the MW of the proteins in the same fraction, differed less than 10kDa on average. Proteins with the same predicted MW was at most found in two different fractions. The

peaks obtained after processing the flow cytometric data was sharp and slim, indicating that the protein of interest was mainly detected in one or two fractions. These findings are supported by another study, which compared a chromatography approach to Gelfree fractionation prior to detection and analysis with MS [38]. Here 44% of the proteins were only detected in one fraction.

Almost no proteins were detected in the first fraction with western-MAP. This problem was tried overcome by using different sample buffers to lower the SDS concentration in the first fraction and eliminating substances that could interfere with the SDS removal. These attempts did not improve the results. The first fraction generally contains a high protein concentration and many more different protein species compared to the other 11 fractions, and as shown in the optimization experiments, a high protein concentration seemed not beneficial for the results.

Proteins with a MW spanning from 14kDa and up to about 200kDa was observed in the western-MAP results. As visualized with the standard SDS-PAGE experiments fractionation of proteins from about 13kDa an up to well over 250kDa was achieved using the four different cartridges in the Gelfree fractionation system. This means that it should be possible to detect and analyse bigger proteins than 200kDa, if using the appropriate antibodies in the arrays.

A total of 350 different proteins were detected reproducibly in this thesis by 537 different antibodies. This is considerably more than other multiplexed antibody arrays [4, 6-8, 76], but less than the large proteomic studies done by MS [2]. Compared to MS is western-MAP a simpler and rapider protocol, and with the correct antibodies a much larger amount of proteins could be detected in a short time using western-MAP.

It is important to compare new methods to traditional methods for validation. In this study traditional western blotting was not performed, but I had access to blots prepared using a panel of 89 antibodies. The lysates I used were from the same source as that used to prepare the blots. The results obtained in this study were to a large extent comparable to those on the blots, since as many as 76.4% of these antibodies captured a target with the appropriate size from Gelfree fractions (Appendix). This illustrates another use of western-MAP besides large scale analysis of cellular proteins. Since western-MAP gives similar results to traditional western blotting, western-MAP could be used to predict an antibody's performance in western blotting. Western-

MAP could be an efficient tool to screen for potential antibodies for western blotting applications.

It is clear that traditional western blotting has higher resolution than western-MAP, but this was expected. The resolution in SDS-PAGE correlates with the length of the gel, and the gels in the Gelfree cartridges are very short. It seems possible that the resolution could be enhanced by increasing the number of fractions that are obtained. It will also be possible to combine Gelfree separation with methods such as subcellular fractionation, which would result in a reduction in sample complexity and simultaneously provide biologically relevant information.

The journal Nature has recently published a series of editorials on the problems associated with the poor reproducibility in biomedical research [92]. The variable performance of commercially available antibodies is among one of several problems. The issue is further complicated by the fact that the antibodies are used one at a time. It is common to vary assay conditions to optimize detection of a given protein with a given antibody. Common variables include sample loading, antibody concentration and blot exposure times. All these issues can be effectively dealt with using an array-based approach. The antibodies are all used under the same conditions, there is no variation in antibody concentration, and importantly different antibodies serve as references for each other.

Access to searchable online data sets with information about the performance of antibodies should greatly simplify the task of finding the optimal antibody for a given application. Today, researchers are limited to relying on the information provided by the manufacturer or search published articles for images of western blots. There is rarely any side by side comparison of different antibodies to the same protein. Thus, the information that is available today is often inadequate to select the best affinity reagents. To a large extent, the variable performance of antibodies is a result of inadequate testing by the manufacturers. Antibody validation is a bottleneck in the production of new affinity reagents, and thorough validation is costly. The technique developed here has the potential to reduce these costs significantly. Thus, western-MAP is a step in the direction of better standardization and hopefully also better reproducibility of antibody performance.

The most important implication of the technology developed here is that it provides a better tool for protein discovery. Proteins act in networks of synergistic and antagonistic partners. It seems rational to believe that it is beneficial to analyse as many components of each network as possible simultaneously. While this is feasible by the use of MS, most biologists do not have access to this technique on a daily basis. The limitation of MS-based proteomics is therefore not the detection of proteins, but rather implementation of the technique in cell biology research laboratories.

Apart from providing the ability to use thousands of antibodies in parallel, western-MAP also eliminates some of the most time consuming and laborious steps in western blotting such as transfer of proteins from gels to membranes. Importantly, most of the steps in the procedure can be automated. Currently, the operation of the Gelfree instrument is manual, and the process requires harvesting of fractions at given time points. The manufacturer is, however, in the process developing a new device that allows automatic harvesting of fractions (Heikki Lancriet, Expedeon CEO, personal communication). This will greatly simplify procedures and also be cost-effective. Steps downstream of the fractionation can be performed using microtiter plates and liquid handling robots. Thus, western-MAP opens for streamlined and automated protocols for large-scale protein analysis that can readily be implemented in a large number of cell biology research laboratories.

6.4 FURTHER RESEARCH

This thesis provides proof of principle for western-MAP, but several issues should be addressed before the technology can be widely used. We found that the absolute signal was reduced when the amount of protein used to load the gel cartridges was increased from 230ug to 700ug. To investigate the reason for this surprising result, it seems necessary to measure the protein concentration in fractions obtained after loading of gels with different amounts of protein. Experiments on antibody array analysis should encompass a wider range of titrations to determine the linear range.

It should also be examined if using lysis buffers that do not contain any other detergent than SDS, would improve the results. If the membrane proteins end up in later Gelfree fractions than predicted because they bind other detergents prior fractionation, this could make the membrane proteins migrate in the gel as predicted.

It is clear that the reproducibility of the results should be investigated in further detail. The present study was largely limited to showing reproducibility of intra-experimental variable such as the effect of removal of SDS and use of protein labels. Studies where the same lysates are analysed repeatedly over time are needed.

Future studies should also address the problems we encountered when using the gels with 5% or 12% acrylamide. The results obtained after fractionation with these gels were unsatisfactory. This limits the utility of the approach to studies of proteins with a size range of 15-100kDa. It seems possible that the problem may be solved by the use of alternative gels or buffer systems. This work will have to be performed in close collaboration with the manufacturer of the gels. An alternative is to explore electro-elution of proteins from standard SDS-PAGE gels. While this procedure will involve time-consuming and elaborate dissection of gel pieces, it may provide the possibility to obtain a higher resolution and a wider separation range than what is possible with the Gelfree 8100 instrument.

It is worth noting that the Gelfree 8100 fractionation system was originally developed to obviate the need to dissect gel fragments and perform in gel digestion prior to MS. The fact that the same instrument is useful to prepare samples for antibody array analysis and MS opens possibilities for studies that compare the two techniques. In future studies, the fractions should be analysed by both methods. Results obtained by quantitative MS will be highly useful as a reference for antibody specificity. Simultaneously, antibody array analysis may uncover limitations in the detection of proteins by MS. Antibody array analysis is likely to be more reproducible, and one would expect that several proteins that go undiscovered in MS may be detected using antibodies. Such comparative studies open completely new possibilities for comparing protein detection by antibodies and MS and should ultimately bring platforms where the two technologies can be integrated seamlessly.

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APPENDIX

Cat.no	Gene name	Protein name	Mass target (kDa)	Western blott		Compatible results	Not compatible results
				Tested on HeLa	Tested on Jurkat		
1801	ACT	Actin - Loading Control	42	x			1
47654	ACTR2	Arp2	45	x	x	1	
28448	BCL11B	Ctip2	96		x	1	
37305	BCL9	BCL9	149	x	x	1	
23423	BIRC3	cIAP2	68		x	1	
4180	BUB3	Bub3	37	x		1	
39818	CALR	Calreticulin - ER Marker	46		x	1	
22595	CANX	Calnexin - ER membrane marker	65	x		1	
10478	CBX1	CBX1 / HP1 beta	21	x		1	
10480	CBX1	HP1 gamma	21	x		1	
66573	CDC42	CDC42	21	x	x	1	
38840	CDK9	Cdk9	43	x	x	1	
25734	CENPB	CENPB	65	x	x	1	
21679	CLTC	Clathrin heavy chain	191	x		1	
16051	CTNNB1	beta Catenin	85	x		1	
51668	CX3CR1	CX3CR1	40	x		1	
42547	DACT1	Dact1 / Dapper homolog 1	90		x	1	
24601	DDX17	DDX17	80	x	x	1	
40684	DDX6	DDX6	54		x	1	
64667	DKC1	DKC1	58	x			1
12286	DROSHA	Drosha - ChIP Grade	159	x			1
2900	EEA1	EEA1 - Early Endosome Marker	162	x		1	
32381	EIF2C2	Ago2 / eIF2C2 - ChIP Grade	97	x	x	1	
31217	eIF4A1	eIF4A1	46	x	x	1	
31218	eIF4A2	eIF4A2	46		x	1	
40688	EMD	Emerin	29	x	x	1	
64668	ENDOG	Endo G	28	x		1	
5821	FBL	Fibrillarin - Nucleolar Marker	34	x		1	
16654	FKBP3	FKBP25	25	x		1	
23683	FOXO3A	FOXO3A	71		x	1	
31581	GEMIN4	Gemin 4	120	x	x		1
31689	GEMIN5	Gemin 5	169	x	x		1
45953	GLRX	Glutaredoxin 1	12		x		1
37137	GSG2	Haspin	88	x			
25388	HEXIM1	HEXIM1 - ChIP Grade	41	x	x	1	
18255	histone2	Histone H2A - ChIP Grade	14	x			1
18256	HMGB1	HMGB1 - ChIP Grade	25	x	x	1	
10479	HPRT1	HPRT	24	x		1	
21685	HSPA5	GRP78 BiP	70	x		1	
26083	IKZF1	Ikaros	58	x		1	
31097	ITCH	ITCH/AIP4	103	x		1	
64575	JMJD6	JMJD6	46	x			1
17721	KDM1A	KDM1 / LSD1 - ChIP Grade	93	x	x	1	

37814	KIF11	Eg5	119	x	x	1	
47010	LDHA	LDHA	37	x	x	1	
16048	LMNB1	Lamin B1 - Nuclear Envelope Marker	66	x	x	1	
20663	MITF	MiTF	59	x		1	
45989	MTOR	mTOR	289	x	x	1	
66039	MTR	MTR	141	x		1	
45890	NAMPT	Visfatin	56		x	1	
39242	NODAL	Nodal	13	x			1
23426	NOTCH3	NOTCH3	240	x			1
33613	PA2G4	EBP1	44		x	1	
18257	PARK7	PARK7/DJ1	20	x	x	1	
34360	PAX7	PAX7	57	x	x		1
38338	POLD2	DNA polymerase delta p50	51	x	x	1	
21382	POT1	POT1	71	x		1	
41684	PPIA	Cyclophilin A	18	x	x	1	
16045	PPIB	Cyclophilin B	20	x		1	
10559	PTPRC	CD45	145		x	1	
10558	PTPRC	CD45	145		x	1	
18211	RAB5A	Rab5 - Early Endosome Marker	24		x	1	
31917	RAD6	Rad6	17	x	x	1	
32631	RCOR1	CoREST	53	x	x	1	
21635	REST	REST / NRSF	122	x			1
40820	RPS6	RPS6	29	x			1
23980	RUNX1	RUNX1 / AML1 - ChIP Grade	49		x	1	
31748	SAP18	SAP18	18	x	x	1	
39578	SF3B1	SAP155	146	x	x	1	
21633	SGOL2	Shugoshin	145	x			1
10546	SKP1	Skp1	19	x		1	
32551	SLC2A1	Glucose Transporter GLUT1	54	x			1
21583	SMC1	SMC1	143	x	x	1	
33033	SMG5	SMG5	114		x		1
3749	SNF2H	SNF2H - ChIP Grade	122	x			1
36593	SP5	Sp5	42	x	x		1
32820	SUPT6H	Spt6	199	x	x	1	
14106	TAGLN	SM22 alpha	22	x		1	
47517	TFAM	mtTFA	24	x	x	1	
66579	TNF	TNF alpha	26	x			1
65360	TNFSF13B	BAFF	31		x		1
4182	TRF2	TRF2	56	x		1	
4074	TUBA	alpha Tubulin - Loading Control	50	x			1
18251	TUBA1A	alpha Tubulin	50	x	x	1	
26320	TXN	Thioredoxin / TRX	12	x		1	
37483	U2AF2	U2AF65	53	x	x	1	
15895	VDAC1	VDAC1 / Porin - Mitochondrial Loading Control	31	x		1	
46154	VEGF	VEGF	24	x	x	1	
12148	YB1	YB1	36	x	x	1	
71842	ZYX	Zyxin	61	x		1	
					SUM	68	21
					Presentage	76,4	23,6