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"Method development for a DNA-microarray based high-multiplex mutation analysis"

Diplomarbeit zur Erlangung des akademischen Grades Diplomingenieur der Lebensmittel- und Biotechnologie an der Universität für Bodenkultur Wien

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ZUSAMMENFASSUNG

In den vergangenen Jahrzehnten wurde die genetische Ursache vieler Erbkrankheiten definiert. Größenteils sind Punkt-Mutationen für die fehlerhafte Funktion der Genprodukte verantwortlich. Mutationsscreenings zur Bestätigung der Verdachtsdiagnose sowie der Nachweis des Überträgerstatus sind eine wesentliche Aufgabe der humangenetischen Diagnostik. Der Mutationsnachweis hilft Krankheiten richtig zu diagnostizieren und die richtigen therapeutischen Maßnahmen für Patienten einzusetzen.

Der „Gold-Standard“ in der Mutationsanalyse ist derzeit die DNA-Sequenzierung, mit welcher einzelne Genbereiche von einigen hundert Basen analysiert werden können. Die zur Diagnostik von Krankheiten oft notwendige Analyse von mehreren Genbereichen ja mehrerer ganzer Gene ist mit diesem Verfahren sehr aufwändig. Gerade die zeitgleiche Analyse vieler Gene bzw. Genbereiche wäre von beproberem Vorteil. In den letzten Jahren wurden chip-basierte Methoden zur Resequenzierung spezifischer Genbereiche entwickelt. Chip-basierte Methoden ermöglichen es, viele Gene parallel zu analysieren und sind eine kostengünstige und zeitsparende Alternative. Allerdings sind die Kosten zur Herstellung und Validierung von Resequenzierungs-Chips sehr hoch, sodass der Aufwand gegenüber der DNA-Sequenzierung nur selten gerechtfertigt ist.

Diese Arbeit hatte zum Ziel, die Eignung des mutations-spezifischen MutS Proteins für eine neue DNA-microarray basierte hochmultiplexe Mutationsdetektions-Methode zu evaluieren.

In vivo ist MutS Teil des „mismatch repair systems“. Das Protein erkennt DNA - Punktmutationen sowie Insertionen und Deletionen von bis zu vier Basenpaaren und ist daher für den Einsatz zur Mutationsanalyse geeignet. Zudem kann das MutS-Protein in *E.coli* günstig produziert werden. Durch Transformation mit einem His_6-tag und GFP sind auch die Aufreinigung sowie die Visualisierung des Proteins am Chip günstig durchführbar. Diese Arbeit umfasste alle Schritte von der MutS-Proteingewinnung, über

das Chip-Design und die Herstellung eines Kontrolltests für die Evaluierung, als auch die Hybridisierungsexperimente. Letzten Endes musste festgestellt werden, dass das MutS-Protein für die chip-basierte Mutationsdetektion ungeeignet ist. Dennoch konnten alle Methodenparameter soweit optimiert werden, dass diese Arbeit die Basis für weitere alternative Lösungsansätze darstellt.

ABSTRACT

During the past decades the genetic reasons for many hereditary diseases were defined. Mainly point mutations are causing incorrect functions of gene products. Mutation screenings for confirmation of suspected diagnoses and for verification of carrier status are an essential part of humangenetic diagnostics. Mutation analysis helps for accurate diagnosis of diseases and is furthermore helpful concerning the correct choice of therapeutical actions.

The to-date “gold standard” in mutation analysis is direct DNA sequencing, that allows analysis of single gene areas up to lenghts of several hundred bases. Analysis of many gene areas or several complete genes for diagnostic reasons is very labour-intensive with this method. Especially the analysis of many genes and gene areas at the same time would be a big advantage. During the last few year chip-based methods for re-sequencing of specific gene areas were developed. Chip-based methods enable analysis of many genes at a time and furthermore they are a relatively cheap and timesaving alternative. However, the costs for production and validation of re-sequencing chips are still very high, what makes it somehow difficult to defend their application rather than usage of direct sequencing.

The aim of this work was to evaluate the suitability of the mutation-specific MutS protein for a new DNA-microarray based highly-multiplexed mutation detection method.

In vivo MutS is part of the “mismatch repair system”. The protein recognizes single point mutations, as well as insertions and deletions of up to four base pairs and is thus usable in mutation analysis. Furthermore the MutS protein is easy to produce in *E.coli*. By transformation with a His_6-tag and GFP, the protein is furthermore simple to purify and to visualise on the chip. This work included all steps, starting with MutS protein production, chip-design and development of a control test system, as well as hybridization experiments. Finally is was found out, that the MutS protein is not applicable in chip-based mutation

detection. Nonetheless it was possible to optimize the method parameters so this work could be the basis for further approaches.

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CHAPTER 1

INTRODUCTION

1.1 Preface

It was found out during the past few decades, that various genetic diseases are caused by single nucleotide mutations or small deletions and insertions in the patients genome. The way of how these mutations manifest in the body are versatile – they can be passed on from parents to offspring, occur *de novo* or they might as well be aquired somatically (Wagner *et al*, 1995).

According to the "Online Mendelian Inheritance in Man®" database, OMIM® (accessed on 19/01/2009), there are currently 2459 genetic diseases with known phenotype and known molecular basis identified. These numbers are also part of the "Human Gene Mutation Database®", HGMD® (accessed on 19/01/2009), which gives information about the type of

mutations that give rise to the disorders. Table 1.1 represents the variety of mutations that can be possible starting points of many diseases. Compared to the number of diseases with known genetic background and phenotype, this table reflects how little we know about most identified mutations and how much work is left to be done.

Table 1.1 *Types of mutations that cause most of the known genetic diseases. The number of genetic diseases with known genetic background and known phenotype is 2459 according to the OMIM database.*

(Source: HGMD database, accessed 19/01/2009)

Type of mutation	Number of entries
Missense/ nonsense	48343
Splicing	8219
Regulatory	1400
Small deletions	13628
Small insertions	5567
Small indels	1244
Gross deletions	5158
Gross insertions	1003
Complex rearrangements	736
Repeat variations	260

Due the the major impact that those mutations have in the development of diseases, there is a need to analyze them and also their effects on the human body. Mutation analysis is very important in cancer research because it is influencing the correct choice of treatment methods. Roses (2001) illustrates the importance of genetics with respect to treatment outcomes ("Pharmacogenetics"). According to Roses it is very important and helpful to know about the molecular background of syndromes, because the effects and also the

adverse reactions are very strongly connected to the genetic background of the patient. This makes drugs not only safer, but also more effective and economic.

The problem that remains unsolved, though, is the availability of methods that allow "high-throughput" mutation analysis on high standard and for acceptable costs. The enormous amount of genes that need to be screened leads to many problems with the currently available methods. According to Gödde *et al* (2006) the different methods for mutation analysis are based on either:

- Gel- and/or capillary-electrophoresis (e.g. direct DNA sequencing, SSCP, DGGE/TGGE SSCP-CE, chip based CE, CE-MS) and
- DNA-hybridization (e.g. DNA-chips, DotBlot, Lineprobe arrays, etc.)

Another method, that will certainly gain a lot of interest during the next few years, is "next generation sequencing", also called pyrosequencing. Based on the sequencing-by-synthesis principle, this method is best suited for DNA sequencing in a cost-effective and simple manner and is furthermore known to be a robust and accurate method (Doostzadeh *et al*, 2008).

Gel-electrophoresis based methods are applied since more than 35 years in everyday laboratory routine and are still very popular. Also the up-to-date "gold standard" in mutation analysis, direct DNA sequencing, relies on this method. Invented by Frederick Sanger around 1975 this method is still the preferred tool of choice in mutation analysis and applied in many laboratories (Gödde *et al*, 2006). For performing the "Sanger-method" the DNA, that needs to be analyzed is preamplified by PCR and subsequently denaturated. A primer anneals to the strand and a polymerase syntesizes new strand fragments complementary to the original DNA strand. The obtained PCR product is then divided into four vessels and additionally to the deoxy-NTPs (dNTPs) one type of labelled dideoxy-NTP (ddNTP) is mixed with the other components in each of the four vessels. The ddNTPs lack the 3´- OH group, so if they are introduced to the newly built strand, polymerisation is determined because no other nucleotides can bind. After proceeding the reaction there are

as many fragments present in the mixture as corresponding bases to the added ddNTP are existing in the strand. Finally all four samples are run on a polyacrylamidegel with single base resolution. Resulting in four different lanes – one for each type of ddNTP – one starts with the smallest fragment determining the corresponding base and continues until the highest base, the end of the DNA sequence (Reineke, 2004). Although the Sanger-method might be very reliable indeed, it has to be mentioned that it is an expensive and time-consuming option to screen for mutations of many different gene loci in parallel.

Attempts to minimize efforts for screening resulted in methods like single strand conformation polymorphism analysis (SSCP) and denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE), that include pre-treatment of the DNA amplicons. The variety of different PCR products that needs direct sequencing can be dramatically reduced with these methods, but still also these methods are not very robust and not applicable for sequencing of many gene loci at the same time. Advantages of SSCP on the other hand are it´s simple application, time- and money-saving performance and the fact that mutations are detectable at any position of a DNA fragment (Orita *et al*, 1998*)*. SSCP furthermore allows to look for unknown mutations in a large amount of samples (Gödde *et al*, 2006). SSCP analysis is currently applied in mutation- and SNP-analysis as well as in genotyping. Already in 1989 Orita *et al* suggested that this method could be helpful in discovering the genetic background of diseases and for detection of DNA defects that possibly induce cancer. The principle that lies behind this method is quite simple: The migration patterns of a wildtype and a mutant ssDNA in a non-denaturating electrophoresis are different. Even substitution of one single base in a certain sequence already results in a band mobility shift. By obtaining different bands for wildtype and mutant DNAs the mutations can be elucidated but have to be always confirmed by direct sequencing. Problems with this method, though, are the common issued with PAGE and resolving mutant DNA bands (Orita *et al*, 1989; Dong & Zhu, 2005; Gupta *et al*, 2005). The role of SSCP, however, has already been diminished by newly arising “high-throughput” methods, like chip analysis (Gödde et al, 2006).

Additionally to direct DNA sequencing and SSCP also denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are applied in mutation detection experiments. Also here the analysis needs a sequencing step for confirmation (Gödde *et al*, 2006). The separation mechanism of the samples is similar in both methods and relies on dissolving of DNA fragments. Even sequences that differ in only one single base have different migration patterns and thus can be distinguished (Myers *et al*,1985 a,b). DGGE and TGGE differ in the method, that leads to this DNA dissolving process. In DGGE it is induced by an rising urea-concentration in the gel and in TGGE the DNA starts melting with increasing temperature. The gradient can be applied between 10°C and 80°C and samples are applied vertically to the gradient. Advantages of the method are its stability and the good reproducibility of results. These methods allow discrimintation between two sequences that differ in only one base (Rosenbaum & Riesner, 1987). Especially in investigations of mutations in tumour cells (Kumar *et al*, 2005) these methods are very often applied for screening procedures.

However, the fact that all these methods are time-consuming and need validation by sequencing leads to the assumption, that gel-based methods will finally fall back in the competition with newer methods. Already very soon capillary electrophoresis systems will displace gel-systems (Gödde *et al*, 2006). The samples are thus no longer separated in gels but in little capillaries, which are filled with an electrolyte. Advantages of capillary electrophoresis are saving of time in comparison to PAGE/horizontal-gel based methods, due to reduced work and also quicker run times. Furthermore many samples can be analyzed with paralleled capillaries and the method is also more sensitive (Mitnik *et al*, 2001).

Hybridization based techniques are so far only applied when the mutation is already known. They are not yet used in many laboratories due to different reasons. First, there is still the problem with uncertainty of results that has to be overcome, second the costs plays a major role – especially the costs for the chip design but also all necessary tools for analysis of results are still quite expensive. Still – microarrays are the future hope in highly paralleled mutation analysis and will be used braodly in this area very soon (Gödde *et al*, 2006).

1.2 DNA-arrays

As described before DNA-arrays raise high expectations in developing highly paralleled mutation detection methods. The “high-throughput” characteristic is their major advantage compared to other biological methods. Still, there are many problems that need to be overcome and it remains an open question if the chip technology will enter routine mutation detection. Problems are for instance sensitivity and specificity (Preininger *et al*, 2005).

The chips themselves are actually quite simple tools. Except for increased hardness, DNA array slides are normal glass microscope slides at a size of 25 mm x 76 mm x 0.96 mm. The glass slides are covered with different surface materials, dependent of the type of experiment to be performed. On this surface material the so-called “DNA-probes” (i.e. oligonucleotides with known sequence) are spotted in a grid (Preininger & Sauer, 2003).

1.2.1 DNA-array coatings

The array surface has a big impact on binding capacities of DNA probes to the array and thus it is a very important factor in concerning the success of an experiment. Many different types of array coatings are commercially available. These coatings present for example amino-, aldehyde-, epoxy- or mercapto-groups on the chip surface and thus assure the covalent binding of probes to the array. The density of reactive groups is about 5×10^{12} groups /mm^2 (www.anapoli.com).

It has to be mentioned, though, that the surface coating (substrate) can also have negative influences on the fluorescence background signal, a problem called “substrate noise”. These include all things that are part of the background reading, but not caused by the sample or the instruments, usually it is very low but still has to be considered in some cases. Substrate noise can be both, intrinsic fluorescence or reflection off the coated surface. It is the signal obtained before a sample is put on the surface (www.anapoli.com).

1.2.1.1 Epoxy- and Aldehyde-coatings are commonly used for DNA-microarrays

Epoxy- and aldehyde-groups allow covalent binding between the chip surface and the spotted DNA. This means, that harsh washing steps are not affecting the binding strenght and subsequently better background and higher sensitivity can be obtained. Also nucleases are removed from the slides which is very important for nucleic acid chip hybridizations.

EPON™ Resin SU-8 is one example for epoxy-coatings and Figure 1.1 illustrates the binding mechanism between the slides and the DNA-probe.

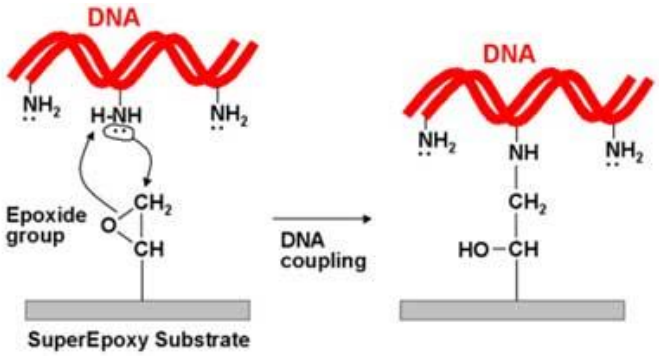


Figure 1.1 *Epoxy binding between the chip substrate and DNA. The covalent bond is actually built between two free electrons and one CH_2 group (Source: www.anapoli.com).*

The primary amine groups of the A, G and C residues of the oligonucleotide have two separate electrons each. These electrons bind the carbon in the CH_2 of the epoxy group and a covalent bond between the two reaction partners – the substrate on the chip and the DNA – is subsequently built. Although DNA is bound very thightly to this kind of surface a good hybridization capability is retained.

Another reactive group is aldehyde, e.g. Superaldehyde by Telechem (Figure 1.2).

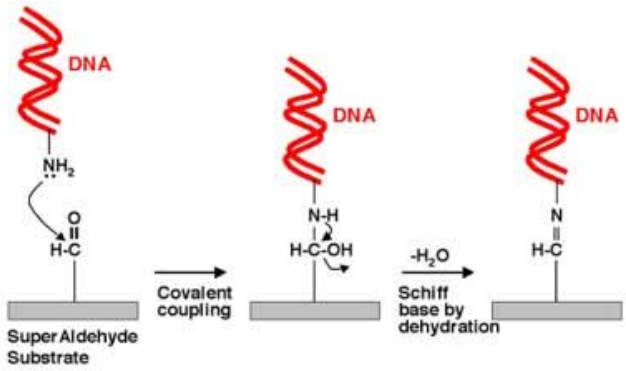


Figure 1.2 *Aldehyde binding between the chip substrate and DNA. The mechanism relies on the Schiff base reaction. (Source: www.anapoli.com)*

In this case primary aldehyde groups are covalently bound to the glass surface of the slide onto which NH_2 groups of the DNA are bound covalently. The following dehydration forms the main part of stabilizing the binding capacity, by leading to a Schiff base formation (www.anapoli.com).

The aldehyde surface is applied to the glass slides by a method called “inking”. This means, that the slides are dunk into the liquid and coated on both sides.

1.3 Mutation detection with proteins

Mutation detection is possible with different kinds of proteins. For examples CEL1 endonuclease, a member of the S1 superfamily, is able to recognice mutations and polymorphisms. CEL1 cleaves heteroduplexes at the mismatch position and this quality in turn enables mismatch detection. The protein is obtained by extraction from celery. It is very stable and cleaves mutated DNA strands in a pH range from 6.0 to 9.0 (Oleykowski *et al*, 1998). Goltz *et al* (1997) applied CEL1 endonuclease successfully in a mismatch detection assay.

In 1998 Del Dito *et al* developed a method called “Enzymatic Mutation Detction” (EMD) that uses the bacteriophage resolvase T4 endonuclease VII for mismatch detection. The assay comprises four steps: DNA amplification, forming of heteroduplex DNA, detection of the heteroduplex DNA by the help of the enzyme and gel-electrophoresis as the final analytical tool. With this method it is possible to detect mutations and polymorphisms. An advantage is also the time – the procedure (without electrophoresis) doesn´t even take one hour. Also the values for specificity, sensitivity and the reproducibility of results are very promising.

The most important DNA mismatch detection protein *in vivo* is the MutS protein complex. In the following paragraphs this protein and the possibilities it offers in terms of mutation detection are explained in more detail.

1.3.1 The MutS-Protein

1.3.1.1 MutS in eukaryotes

In humans the MutS protein is active as a heterodimer of about 100 kDa in size. Six different MutS homologues (MSH1, MSH2, MSH3, MSH4, MSH5 and MSH6) have been identified so far. The difference to prokaryotic MutS is, that in eukaryotes each MutS homologue has a specific function. MSH2, MSH3 and MSH6 are involved in mismatch recognition and the rest of the MutS homologues are parts of other cellular mechanisms (Stanislawska-Sachadyn & Sachadyn, 2005).

Depending on the binding ability to different mismatch types and the size of bound insertions and deletions in the DNA, two different MutS heterodimers can be discriminated: MutSα and MutSβ, respectively. MutSα is built of the two MutS homologues MSH2 and MSH6 and is responsible for detection of SNPs as well as small deletions and insertions. Bigger mutations are recognized by the MutSβ heterodimer, consisting of the two MutS homologues MSH2 and MSH3 (Lamers *et al*, 2000). Only one domain of either the MSH6 and MSH3 monomer is eventually responsibe for mutation detection. This domain is the

so-called N-terminal mismatch recognition domain and according to Sixma *et al* (2001) it is assumed, that this mismatch recognition structure is very similar to the mismatch recognition monomer of the MutS protein in *E.coli.*

1.3.1.2 MutS in prokaryotes

Also in *E.coli* the MutS protein is present as a heterodimer and, similar to MutS in eukaryotes, only one of the monomers is actually responsible for mismatch detection (see Figure 1.3). Figure 1.3 furthermore illustrates the modular characteristic of the protein (Lamers *et al*, 2000).

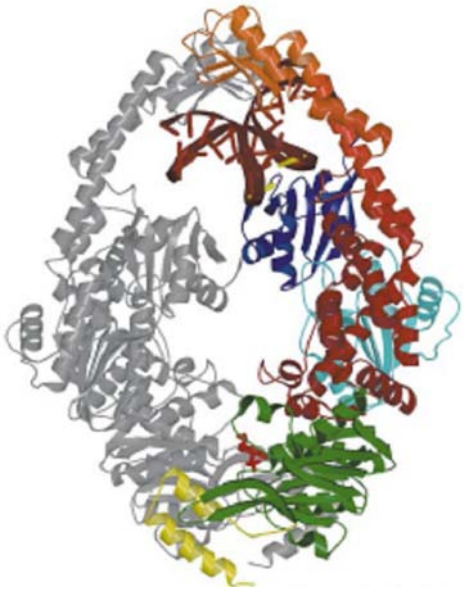


Figure 1.3 *E.coli MutS – the coloured monomer is responsible for mismatch detection. Dark blue – mismatch recognition domain, light blue – connector domain, red – core domain, orange – clamp, green with red ADP – ATPase domain, yellow – helix-turn-helix domain, DNA is shown in dark red with a yellow mismatch.*

(Source: Sixma, 2001)

In *E.coli* the MutS protein has a different affinity to different types of mismatches. Combination of results obtained by Cho *et al* (2006) and Brown *et al* (2001) shows

following order concerning the mismatch binding affinity from strongest to lowest: ΔT > GT ~ GG ~ CA ~ AA > CT > TT ~ TC ~ AG > CC >AT. The below-mentioned graph (Figure 1.4) shows the *E.coli* MutS protein with a bound G:T-mismatch DNA molecule.

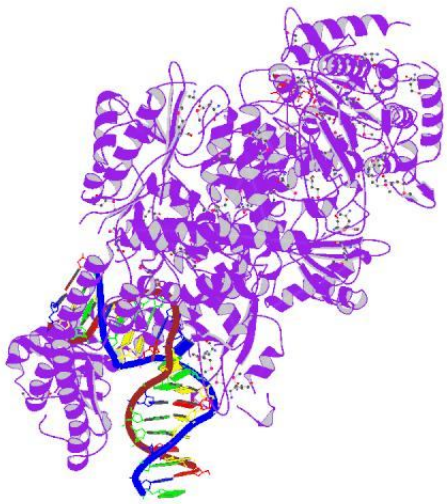


Figure 1.4 *The crystal structure of E.coli MutS binding to DNA containing a G:T mismatch.*

(Source: RCSB Protein Data Bank – http://www.rcsb.org/pdb/home/home.do; accessed on 15/01/2009)

1.3.1.3 MutS is part of the DNA mismatch repair system

The DNA mismatches repair system (MMR) is a very important system in the cells as it is responsible for correction of wrongly matched bases. The MMR system is highly conserved throughout all evolutional stages (Yang, 2000). In *E.coli* and other prokaryotes the MutS protein is responsible for mismatch detction and in eukaryotes this function is performed by several MutS homologues (see section 1.3.1.1) (Jacobs-Palmer & Hingorani, 2007).

In *E.coli* the mismatch binding mechanism of MutS does not only rely on one single protein, but MutS is part of a complex comprising three distinct Mut-proteins - MutS, MutH and MutL – forming the MutHLS system. As stated before, MutS is responsible for mismatch detection. After MutS has bound to the mismatch MutL is recruited. MutL

protein, though, is actually not participating in this process with any enzymatic function but its binding to MutS is a requirement for MutH attachment and activity (Aronshtam & Marinus, 1996). Finally it is the MutH protein, that introduces a nick in the heteroduplex target strand with its activity as an endonuclease and consequently the wrong base can be substituted (Yang, 2000).

Concerning its quality for mismatch detection Stanislawska-Sachadyn *et al* (2005) suggested, that the *E.coli* MutS protein could be very well suited as a tool for SNP detection.

1.3.2 MutS based methods for DNA mismatch detection

Ellis *at al* (1994) decribed a method, called "MutEx", for SNP detection with MutS. The method is based on the fact, that MutS bound to heteroduplex DNA protects this part of the DNA from exonuclease digestion. The degradation process stops, as soon as the mutated basepare is reached by the exonuclease and this allows mismatch detection. The obtained signal to noise ratio was very good, but the dependence of MutS binding intensity to the mismatch type led to false negative results in some cases.

Another approach was made by MutS immobilization on nitrocellulose, nylon, PVDF and other solid surfaces. In contrast to mismatch binding proteins in solution, that offer a ratio between binding of the protein to a G:T mismatch and homoduplex DNA of 5:1, the immobilized protein was able to distinguish mismatch from non-mismatch DNA much more efficient (ratio between binding to a G:T mismatch and homoduplex DNA is 1000:1). All mismatches, except for the C:C have been detectable by this method (Wagner *et al*, 1995). The immobilisation theory was further applied by Han *et al* (2002), who immobilized the *E.coli* protein onto an Au electrode for mismatch detection. DNA is electrochemically inactive. When the mismatch containing DNA and MutS were interacting this was "monitored by redox peak currents on cyclic voltammogram of redox-active species". After mismatch containing DNA was added to the sample solution, a significant

reduction of peak currents of ferrocyanide/ferricyanide redox couple was detectable. Also this study suggests that usage of MutS is a good tool for mutation detection.

Additionally to these experiments a sensor-methods for investigation of the MutS mismatch binding competencies was performed. Geschwind *et al* (1996) used MutS coupled to a biotinylated peptide for SNP detection with avidin in the KCNA1 gene. MutS was furthermore applied in combination with eg. quartz crystal microbalance for detection of point mutations and insertions by Su *et al* (2004). Other approaches combined MutS with representational difference analysis (Gotoh *et al*, 2000) and atomic force microscopy (Tanigawa *et al*, 2000) for analysis and imaging of small DNA mutations.

In 2003 Bi *et al* published a paper, where they discribed the application of the mismatch binding protein MutS on a chip for mutation detection. The researches used immobilized MutS protein for binding of DNA heteroduplices. The principle of this experiment is illustrated in Figure 1.5. The outcome of the experiments were quite primising, as it was possible to detect all mismatches as well as insertions and deletions up to four basepairs with this system. The only problem was, that some unspecific binding of the protein to complementary DNA took place as well, but it is thought, that this can be overcome by an additional washing step. Another thing, that has to be considered concerning the results, is, that although different mismatches have different attraction for MutS binding there was no signal difference according to the mismatch type (only CC mismatch led to a very decreased signal).

Two years later Bi *et al* (2005) tried to apply the MutS protein in a DNA-chip system rather than on a protein-chip. For this approach not the protein was coated on the array, but oligo-probes with known sequence were spotted. After the target DNA was hybridized the mismatch detection was eventually performed by protein detection. The principle of this experiment is shown in Figure 1.6.

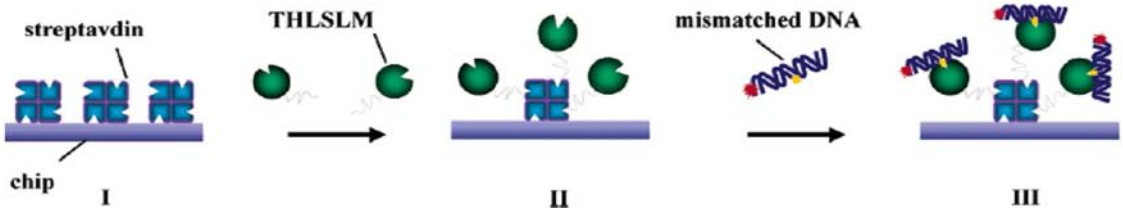


Figure 1.5 *Protein chip. The MutS protein is attached to streptavidin molecules on the chip-surface and subsequently labelled mismatch DNA is hybridized and bound by the protein. I: Chip with attached streptavidin molecules; II: Protein is bound to streptavidin particles through a Strept-tag II; III: Cy3-labelled mismatch-DNA is binding to the protein complex. (THLSLM = Trx-His6-Linker peptide-Strep-tagII-Linker peptide-MutS).*

(Source: Bi et al, 2003)

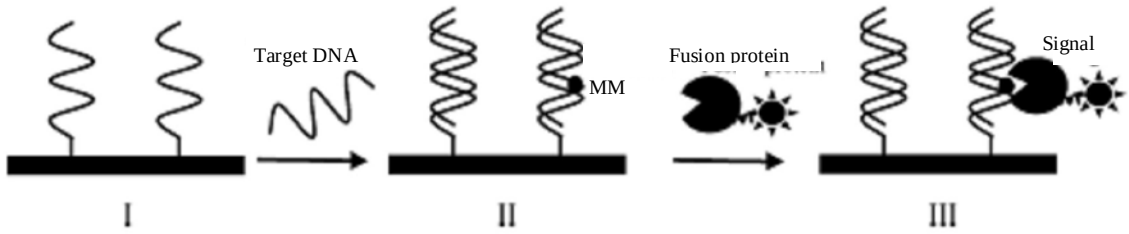


Figure 1.6 *DNA-chip. Target DNA is hybridized to DNA-probes on the chip and detection is performed with MutS. I – chip with vertically applied DNA probes; II: target DNA has bound to the chip probes resulting in one normal dsDNA and one dsDNA with a central mismatch; III: the fusion protein (mismatch detection protein with fluorescent label) binds the mismatch. (MM = mismatch)*

(Source: Bi et al, 2005)

For the DNA-chip approach three recombinant MutS-fusion proteins were tested for their efficacy and costs. The different constructs were: Trx-His6-GFP-(Ser-Gly)6-MutS (THGLM), Trx-His6-(Ser-Gly)6-Strep tagII-(Ser-Gly)6-MutS (THLSLM) and Trx-His6-(Ser-Gly)6-MutS (THLM). All the different protein constructs led to very good results and it was possible to detect all mismatched bases and insertions and deletions up to four

basepairs in the *rpoB* gene of *M. tuberculosis*. The highest signal to noise ratio (15:1) was obtained with the THLM construct labeled with Cy3, but this approach was also the most expensive one. The cheapest protocol was the one using THLSLM but a signal to noise ratio of only 6:1 was achieved with this protocol (Bi *et al*, 2003).

1.3.2.1 GFP-MutS fusion protein

Green-fluorescent protein (GFP) has proven its quality as a reporter gene in many experiments already. The main advantages of this reporter gene are its very small size (238 amino acid residues), the fact, that it does not need any substrates or co-factors and its stability in different buffer systems and at different temperatures (Stanislawska-Sachadyn *et al*, 2006). *E.coli* MutS on the other hand is known to bind efficiently to SNPs and is thus useful in mismatch detection.

All these facts make those two components an optimal couple for mutation detection on DNA-arrays. The GFP-MutS chimeric protein was firstly cloned and purified by Stanislawska-Sachadyn *et al* in 2006. The fusion protein, comprising GFP and also a His_6-tag, has a size of about 123 kDa. Very important concerning the GFP fluorescence, is the order of the different compounds in the vector. For example the proteins fluorescence is extremely diminished when the His_6tag is first, followed by MutS and GFP. On the contrary the fluorescent signal is comparable to the signal of free GFP, when following order is applied: His_6tag-GFP-MutS (Figure 1.7).

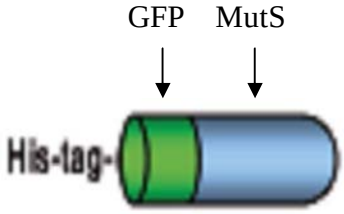


Figure 1.7 *His_6tag-GFP-MutS fusion protein (~123 kDa).*

(Source: Stanislawska-Sachadyn & Sachadyn, 2005)

1.4 The RET proto-oncogene (RET)

The RET proto-oncogene is part of the cadherin superfamily. RET encodes for a receptor tyrosin kinase, a cell-surface molecule, that is responsible for cell growth and differentiation directing signal transduction. It is very important in development of nerve cells. The RET proto-oncogene is mapped to chromosome 10q11.2 near the centromer (Figure 1.8).

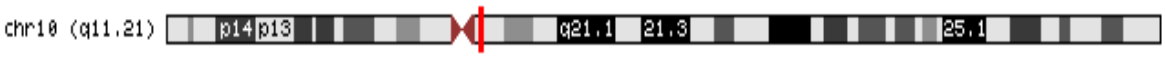


Figure 1.8 *Location of the RET proto onco-gene (red bar) on chromosome 10.*

(Source: USCS Genome Browser)

Three isoforms are encoded by the RET proto-oncogene, namely RET9, RET51 and RET 43. The three isoform differ concerning their C-terminal end after amino acid position 1062. The difference in binding characteristics to downstream adapter molecule suggests that all three isoforms have distinct functions. In transgenic mice studies it was discovered that RET9s function is limited to early developmental stages of e.g. the kidneys and the enteric nervous system and it is supposed that RET51 contributes to kidney development in later stages. It seems, as if each RET isoform has an individual function in normal RET, due to high conservation of isoforms in different species (Lee *et al*, 2002; Carter *et al*, 2001).

The RET proto-oncogene is about 55,000 bp in lenght and consists of 21 exons (de Groot *et al*, 2006).

1.4.1 Multiple endocrine neoplasia syndromes are linked to RET-mutations

Activating mutations in the RET proto-oncogene lead to neoplasia MEN2A and medullary thyroid carcinoma of endocrine glands. Multiple endocrine neoplasia (MEN) syndromes are cancer predisposition syndromes and results in hyperplasia and hyperfunction, as well as they can affect dispersed endocrine cells neurons and their supporting elements.

All MEN syndromes are autosomal dominant disorders and generally two different types of syndromes can occur. They differ in the type of clinical symptoms and also affected organs and are named MEN 1 and MEN2, respectively. Although the two types (MEN1 and MEN2) are clinically related their genetic background is quite different. MEN1 is caused by mutations in the so-called MENIN-gene on chromosome 11 and MEN2 as well as the hereditary type of medullary thyroid carcinoma (FMTC) are associated with mutations in the RET proto-oncogene (Niederle & Haas, 2002) and are described in the following paragraphs. The MEN2 syndrome can be classified in MEN2A, MEN2B and the familial medullary thyroid carcinoma.

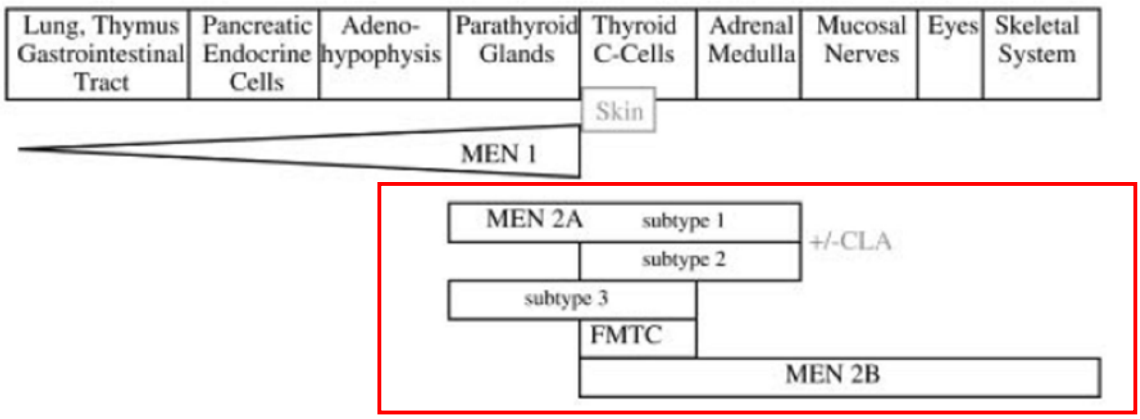


Figure 1.9 *This figure illustrates which organs are affected by the different MEN syndromes. Red box: syndromes caused by activating RET-mutations.*

(Source: De Lellis, 1995)

1.4.1.1 Multiple endocrine neoplasia 2 (MEN2) and familial medullary thyroid carcinoma

As mentioned before the MEN2 syndrom itself can furthermore be categorized in different subclasses. The three syndroms caused by activating RET mutations are MEN2A, MEN2B and the familial medullary thyroid cancer (FMTC) (Brandi *et al*, 2001). So is e.g. MEN2A characterized by medullary thyroid carcinoma (MTC), pheochromocytoma and hyperparathyroidism. MEN2B also shows MTC and pheochromocytoma, but also characteristic stigmata and finally FMTC is characterized by MTC only (Eng *et al*, 1996).

About 5% – 10% of thyroid carcinoma belong to the medullary type and 7% of all MTCs in turn are inherited. Independent of the MEN2 subtype, the risk of developing MTC is very high (up to 90% of patients) for carriers of the mutation (Brandi *et al*, 2001).

According to Brandi *et al* (2001) the MEN2A syndrome accounts for more than 75% of MEN2. Also Table 1.2 confirms that the MEN2A subtype occurs more often than MEN2B.

Table 1.2 *Specific mutations in Exons 10, 11 and 13 to 16 of the RET proto-oncogene are associated with MEN2. (SMENA = Study Group Multiple Endocrine Neoplasia Austria)*

(Source: Niederle & Haas, 2002)

Exon	Affected	Clinical syndrome	% of all MEN2-mutations*	SMENA (%)
10	609	MEN2A/FMTC	0–1	18
	611	MEN2A/FMTC	2–3	
	618	MEN2A/FMTC	3–5	
	620	MEN2A/FMTC	6–8	
11	630	MEN2A/FMTC	0–1	26
	634	MEN2A	80–90	
13	768	FMTC		22
	790	MEN2A/FMTC		
	791	FMTC		
14	804	MEN2A/FMTC		24
15	883	MEN2B		6
	891	FMTC		
16	918	MEN2B	3–5	4

All three phenotypes, MEN2A, MEN2B and FMTC, respectively, are caused by mutations that activate the RET proto-oncogene. These mutations take preferably place in exons 8, 10, 11, 13, 14, 15 and 16. Dependent in which exon a mutations occurs, the phenotype of the disease, the expected age when the disease occurs and also the aggressiveness are determined (Brandi *et al*, 2001). For instance the most common mutation in MEN2A is in codon 634, occurring in 80% of patients. For this phenotype the exons 10 and 11 are the most important ones. Also exon 13 and 15 may contribute to MEN2A a little, but their "main" phenotype is FMTC. The codon most frequently associated with MEN2B is a codon 918 mutation in exon 16 of the RET proto-oncogene (Sippel *et al*, 2008).

1.4.1.2 Hirschsprung disease (HSCR)

RET proto-oncogene inactivating mutations result in the so-called "Hirschsprung disease", a disorder that affects about one in 5000 newborns. Male babies outnumber female ones by far concerning the susceptibiliy to fall ill with this disease (Passarge, 1967). In kids with this disorder, germline mutation analysis of Exon 10 of the RET proto-oncogene is indicated (Brandi *et al*, 2001). According to the UniProt database the polymorphism Cys982 may be linked to increased risk for Hirschprung disease. But it is not only the RET proto-oncogene that causes this disorder. Additionally to mutations within the RET proto-oncogene, also mutations in two more loci may contribute to this disease. Firstly germline mutations in the endothelin receptor B gene (EDNRB) on chromosome 13q22 have been associates with Hirschsprung´s disease. And secondly homozygous germline mutations in the endothelin-3 gene (EDN-3) are thought to be involved in this disease (Eng & Mulligan, 1997).

1.4.1.3 The RET proto-oncogene is applied as a diagnostic and therapeutic target

Especially in investigations concerning the hereditary form of medullary thyroid carcinoma (hMTC), analysis of the RET proto-oncogene serves as a very good diagnostic tool. Patients and also their family members are tested for germline mutations located in exons 8, 10, 11 and 13 to 16 (Weinhäusel *et al*, 2008). Testing of at-risk family members, whose chances of carrying the mutations are 50%, gives insight in possible genetic predisposition for thyroid cancer and so the development of the disease can be prevented by prophylactic treatment or thyroidectomy (Sippel *et al*, 2008). This makes the RET proto-oncogene a very important and helpful marker in cancer prevention (Lips *et al*, 1994)

Medullary thyroid carcinoma can also occur sporadically (sMTC). In this case several single nucleotide polymorphisms (e.g. 691 in exon 11, 769 in exon 13, 836 in exon 14 and 904 in exon15) are thought to be part of induction of the disease, which was illustrated by earlier studies (Weinhäusel *et al*, 2008).

The malignant transformation of RET is provided by only a single point mutation, due to it´s nature of a proto-oncogene. Germline mutations occur in about one-fifth of all MTC patients, and even in sporadic MTC the chance of a germline mutation is 6% - 10%. That makes genetic testing of patients and also at-risk family members very important (Sippel *et al*, 2008).

According to Lips much more sensitive results are obtained with DNA testing compared to the previously used C-cell stimulation tests (Lips, 1998). That is maybe why RET proto-oncogene analysis has lately substituted the routine calcitonine testing for MEN2 carrier status diagnosis (Brandi *et al*, 2001). Brandi *et al* (2001) point out the advantages of RET analysis: efficacy and especially its general availabililty. The certainty about the RET proto-oncogene reliance is furthermore given by the fact, that in 98% of MEN2 patients a RET mutation was found, whereas MEN2 negative families do not show mutations in this gene at all.

The exons, that should be routinely tested for RET mutations are exons 8, 10, 11, 13, 14, 15 and 16. Only if these exons don´t show any mutations the other 15 exons have to be tested additionally (Brandi *et al*, 2001).

The intensity in aggressiveness depends on the type of mutation. Three stages (3-worst, 1-weakest) are so far distinguished and refer to the recommended type of treatment. Stage 3 affects the codons 883, 918 and 922 and by presenting these mutations the patient should achieve thyroidectomy in the first months of life. Level 2 RET mutations (codon 611, 618, 620 and 634) still resulting in high risk for patients and thyroidectomy should be performed in the first few years of life. Level 1 mutations affect the codons 609, 768, 790, 791, 804, and 891. The chance to develop MTC is still high, but the disease may occur later in life about age of 10 years and won´t be as aggressive as the other two types (Sippel *et al*, 2008).

1.5 Rationale - Aims and Objectives

As high-throughput mutation analysis is required for investigation and therapy of many diseases, like genetic disorders and cancer DNA-chip-based methods gain more and more importance in this area. Many methods are already available, but most of them are not suited for high-paralleled and high-throughput application or have other disadvantages, like high costs, low flexibility and time-consuming processing.

The aim of this project was to establish and optimize a DNA-microarray based high-multiplex mutation analysis system, especially for single nucleotide mutations. The advantages of the system should be relative low costs, simple application and high flexibility, but also the possibility of screening many marker genes at the same time should be provided by this system.

The idea was, that the MutS-protein, which is part of the mismatch repair system, would bind to DNA heteroduplexes formed by hybridization on a chip surface. Either fluorescent labelled MutS or the introduced GFP molecule would allow detection of the spots where the protein bound the DNA mismatches. The whole process, starting with bacterial culture, producing and purification of the protein, chip design and the hybridization experiments and optimization were part of this project.

CHAPTER 2

MATERIAL & METHODS

2.1 Materials

2.1.1 Chemicals, antibiotics and buffers

Table 2.1 *List of chemicals*

Material	Provider
Agar	“Fluka”
Albumin, bovine	“Sigma”
Ampicillin trihydrate	“Serva”

Boric acid	“Merck”
Chloramphenicol	“Serva”
DIGeasy Hybridization buffer	“Roche”
Disodiumydrogenphosphate	“Merck”
DYN PCR buffer	“Finnzymes”
ExpressHyb Hybridization Solution	“BD Bioscience”
Formaldehyde	“Sigma”
Formamide	“Merck”
GeneRuler™ DNA Ladder Plus, SM 0322	“Fermentas”
Glacial acetic acid	“Merck”
Glycerol	“Sigma”
HotStar Taq DNA Polymerase	“Qiagen”
Imidazole	“Sigma-Aldrich”
Isopropanol (2 – Propanol puriss.)	“Riedel – de Haën”
Isopropyl- 946-D-thiogalactopyranoside	“Serva”
IMAC Ni-charged resin Profinity™ 50%-solution	“Bio-Rad”
LDS loading buffer	“Invitrogen”
10x Phosphate buffered saline (PBS), pH 7.2	“Gibco”
Potassium chloride	“Merck”
Sodium chloride	“Merck”
10% Sodium dodecylsulfate (SDS)	“Ambion”
20% Sodium dodecylsufate (SDS)	“Fluka”
20x Standard saline citrate (SSC)	“Invitrogen”

Streptavidin – Cy3	"Caltaq"
Streptavidin – Cy5	"Caltaq"
10x Tris-borate-EDTA buffer (TBE)	"Gibcobre Life Technologies"
Tris(hydroxymethyl)-aminomethane	"Merck"
Triton-X100	"Sigma"
Tween-20	"Sigma"
Tryptone enzymatic digest from Casein	"Fluka"
Yeast extract	"Fluka"

All buffers produced in the lab are listed in Appendix II.

2.1.2 Devices

Table 2.2 *List of devices*

Device	Provider	Typus
Balance (µg)	"Sartorius"	1205 MP
Balance	"Sartorius"	
Centrifuge	"Sorvall"	RC6
Centrifuge	"Heraeus Sorvall"	Multifuge 3 S-R
Centriprep Filter Device	"Amicon"	
Fast Prep®	"MP Biomedicals"	FP120, Bio101, Thermo Savant
Hotplate/stirrer	"Heidolph"	MR 3002
Incubator (65°C)	"Memmert"	

Incubator (42°C)	"Memmert"	
Magnetic stirrer	"Variomag Poly"	Komet
Microarray Hybrid Chamber	"BioRad"	Camlab
Microcon Filter Device	"Amicon"	
NanoDrop®	"NanoDrop"	ND-1000 spectophotometer
PCR thermocycler	"Biometra"	T-3000 thermocycler
pH-meter	"Mettler Toledo"	SevenEasy InLab413
Photometer	"Beckmann"	DU 640 Spectrometer
Profinity IMAC Resins	"Bio-Rad"	
Scanner	"Axon Instruments"	GenePix 4000 A
SDS-polyacrylamide-gel	"Invitrogen"	NuPAGE 4-12% Bis-Tris Gel
Sonication	"Transsonic Digitals"	Elma
Spotter	"Newport GeneMachines"	OmniGrid
Syringe filter	"Asahi Techno Glass Co"	
Table-centrifuge	"Heraeus"	Biofuge fresco
Thermomixer	"Eppendorf"	Thermomixer comfort
Thermomixer	"Labnet Int. Inc."	AccuBlock Digital DryBath
Vortex	"IKA®"	MS2 Minishaker

2.1.3 *Escherichia coli* cells

E.coli cells expressing a His_6-tag MutS protein were kindly provided by Li-Jun Bi from the Chinese Academy of Sciences. The cells carried an ampicillin and a kanamycin resistance.

E.coli M15: pQE30-MutS

The GFP-MutS protein was extacted from transformed *E.coli* BL21(DE3)pLysS cells (Novagen).

Genotype: F-ompT hsdS_B(r_B^- m_B^-)*galdcm*(DE3)pLysS(cam^R).

The plasmid for transformation (pUET1-TTh-GFP-MutS) was kindly provided by Dr. Anna Stanislawska-Sachadyn and Pawel Sachadyn from the Gdansk University of Technology.

2.1.4 DNA-Chips

The utilized chips in this project were designed, produced and processed in-house (see section 2.2.3).

2.2 Methods

2.2.1 Cell biological methods

2.2.1.1 Bacterial culture

E.coli cells were cultivated with the commonly used LB broth. Table 2.3 shows the exact composition of this medium.

Table 2.3 *LB-broth ingredients*

Reagent	Amount
NaCl	10 g
Tryptone enzymatic digest from Casein	10 g
Yeast extract	5 g
Agar (for plates only)	10 g

Tryptone, NaCl and yeast extract were dissolved in 800 ml deionized water and the pH-value was set to 7.4 with 1 N NaOH. For production of plates also 10% (w/v) Agar was added to

the mixture before the volume was completed to 1 l with water. Finally the medium was autoclaved. 10 µl of ampicillin (5 mg/ml) and 1 µl of kanamycin (50 mg/ml) were added after autoclaving per ml media and plate, respectively.

First of all a preparatory culture was created by inoculating the *E.coli* strain in 2 ml media and growing it over night at 180 rpm. The temperature was set to 37°C for cultures without GFP and 30°C for GFP-MutS cultures. After this 100 µl of the over night culture were plated on a LB-plate (Amp^+, Kan^+) and incubated again over night to achieve single colonies. 2 ml cluture medium were subsequently inoculated with one single colony and grown for 24 hours. The 2 ml bacterial culture were consequently transferred into 298 ml of preheated (37°C/30°C) fresh LB-medium and grown again over night at 180 rpm and 37 and 30°C according to the culture type. During the following day OD_{600} measurements were performed with the culture until an OD value of 0.5 was reached. Then the culture was induced with 1 mM IPTG or 1 mM lactose.

The cell culture growth was stopped four hours after induction and a final OD_{600} measurement of the growth curve followed. The culture was then split into centifugation beakers for the Sorvall-centrifuge and centrifuged for 15 minutes at 4000 rpm and 4°C. Subsequently the supernatant was discarded. Then the cell pellet was washed once with 1x PBS and then it was centrifuged again for 5 more minutes at 4000 rpm and 4°C. PBS was discarded and the obtained pellet was weighed. 1 ml of lysis buffer per 0.1 g pellet for further treatment with sonication and 2 to 3 ml lysis buffer per 1 g pellet for French Press- and FastPrep® dissociation, respectively, were added to the cells. Finally the cell suspensions were stored at -20°C until cell lysis.

2.2.1.2 Induction with IPTG and Lactose

E.coli cells were induced with IPTG when reaching an OD_{600} of about 0.5. IPTG was added to a final concentration of 1 mM.

Instead of IPTG also lactose was used for induction of protein expression in some experiments. In this case lactose was added to a final concentration of 1 mM also after the bacterial culture had reached an OD_{600} of 0.5.

2.2.1.3 Transformation of competent cells

The competent *E.coli* BL21(DE3)pLysS Singles™ cells were used for transformation experiments. A plasmid containing a GFP-MutS fusion gene construct (pUET1-TTh-GFP-MutS), was introduced into the cells by $CaCl_2$ transformation.

That for two tubes of 50 µl competent cells each were defrosted slowly on ice for about 4 minutes and then mixed gently before 1 µl of DNA solution was added to one tube and 2 µl of DNA solution were added to the other tube. The cell suspensions were then mixed gently before the tubes were placed on ice for 5 more minutes. Afterwards the tubes were heated up for exactly 30 seconds at 42°C and then the tubes were placed on ice for 2 minutes.

250 µl of SOC medium, supplied togther with the competent cells from Novagen, were added to the cell mixture which was still kept on ice. Prior to plating the cell culture, the suspensions were incubated on a shaker at 250 rpm for 1 hour at 37°C. Finally 50 µl of the transformed cells were plated on ampicillin and chloramphenicol selection plates. One plate contained the transformed *E.coli* with 1 µl plasmid DNA and one plate contained the 2 µl plasmid DNA. The plates were incubated over night at 37°C. A positive control which was supplied with the kit was run in parallel to the test samples.

2.2.1.4 Cryopreservation

Bacteria cells were frozen down to -80 °C in 75% sterile gycerin in a 1:1 ratio.

2.2.2 Protein biological methods

2.2.2.1 Protein isolation from E.coli cells

To extract the protein from the *E.coli* cells the first step was to break up the cells. That for three different methods were tested concerning their efficacy. These methods were French Press disruption, sonication and application of the FastPrep® device relying on a bead mill principle. All of these methods are based on mechanical disruption of cells and are explained in the following.

- French Press

For French Press disruption the cell suspension is put in a centered hole of a steel cylinder and, by the help of a big hydraulic plunger, high mechanical pressure is applied to the sample. The cells in suspension are subsequently squeezed through a little valve at the bottom of the device and the cells are exposed to such high pressure that they break open immediatly.

The French Press device for this project was kindly provided by the “Abteilung für Lebensmittel-Biotechnologie at the Universität für Bodenkultur,Wien”.

- Ultrasound/Sonication

By applying ultrasound to the sample the cells can be disrupted in a quite simple manner. Electric energy enables high-frequency and mechanical energy is tranferred to the sample with a metal stick that swings with high-frequency. The metal stick is simply put inside the sample-containing vial and following parameters were used for sonication of the sample:

- Cycles: 50
- Power: ~75%
- Pulses: three times 30 to 40 pulses

The sonication device was kindly provided by the “Abteilung für Lebensmittel-Biotechnologie at the Universität füt Bodenkultur,Wien”.

- FastPrep®

FastPrep® is a device that that utilizes different matrices for cell disruption. Two types of matrices were tested in this project – the so-called matrices B and E.

Matrix B is optimized for RNA and protein isolation from gram + and gram - bacteria and spores. This matrix is a white powder. Matrix E is optimized for DNA and RNA isolation from soil, sediments, water and feces. The matrix contains many little bullets and one big bullet.

First of all 2 ml of cell suspension were added to each vial, filled with the according matrix material. Afterwards the vials were put on the FastPrep® machine and it started rotating very quickly, what enables the matrix to break up the cells. After three times 20 seconds at a speed of 4.0 m/sec the obtained cell suspension was very cloudy and needed to be cleaned. All other substances than protein were therefore removed from the sample by centrifugation. The obtained supernatant was the so-called crude lysate that was used for all further experiments. 50 µl crude lysate of each sample were frozen down as a stock.

After harvesting the cells and before disruption the cells were kept in a lysis buffer. MutS samples were kept in a phosphate lysis buffer (see Appendix II) and GFP-MutS protein was kept in a Tris-HCl lysis buffer (see Appendix II).

2.2.2.2 Protein purification

The protein was purified with chromatography empty spin columns from Bio Rad (Micro-Bio-Spin®). Purification was processed with a His_6-tag, which was cloned into the protein. For purification the principle of IMAC (immobilized metal ion affinity chromatography), a special form of affinity-chromatography, was used. The proteins His_6-tag bound the nickel-beads in the column-material. Other proteins and contamination were washed off the column

finally the MutS protein was eluted off the column with an increased Imidazole concentration in the buffer.

First the column was placed in an Eppendorf-tube and 2 ml of column material were filled into the column. Centrifugation for 30 seconds at 1000 rpm followed and the flow-through (mainly ethanol) was discarded. Afterwards the column was equilibrated with different washing steps. First it was washed with 5 ml destilled wated. The water was added and mixed well, followed by centrifugation of the resin for 30 seconds at 1000 rpm. Then the column was washed once with the according elution buffer (B) for each type of protein (see Appendix II) at the same conditions as before. The washing step with elution buffer was repeated two additional times. After equilibration the main aim was the protein binding to the Ni-beads. Thus before the column was filled with the sample it was closed below with a plug to keep the sample inside the column. The whole sample volume mixed with lysis buffer (together ~ 800 µl) was applied to the column and incubated for 5 minutes at 37°C. Finally the plug was removed from the column and the flow-through was collected and applied two additional times to the column ("gravity flow"). Finally the column was centrifuged for 1 minute at 1000 rpm and the flow-through was collected. To remove protein that had not bound the column material it was washed twice with 5 ml of the according lysis buffer (B). Before centrifugation (1 minute, 1000 rpm) the buffer was incubated for 5 minutes on the column. The flow-through was collected together with the flow-through from before (flow-through Elu1). The protein was eluted by using lysis buffer C (eluate1, Elu1). After adding 2 ml of buffer (C) eluate 2 (Elu2) was obtained. Finally the obtained samples were transferred into 50 ml vials and stored at 4°C for further analyzes. Before purification of the next sample the column needed to be cleaned. 4 ml aqua dest. were added twice followed by two times 4 ml of lysis buffer (B). For storage the column was washed once with 4 ml of 1M NaOH and twice with 4 ml of aqua dest. and finally it was filled with 20% ethanol.

After the protein elution off the purification column the samples needed to be desalted and also a buffer change from the elution buffer containing 500 mM Imidazole to PBS was necessary. That for Zeba™ Desalt Spin Columns (5 ml) were used according to

manufacturer´s instructions (Prodecure for Buffer Exchange) for high volume samples. With this protocol it is possible to realize both needs at once.

First of all the column was opened in the bottom and placed on a collection tube. The storage solution in the column was removed by centrifugation (2 minutes, 1000 g) and the side where the packing material was higher in the columns was marked. This mark had to be facing outward in all further centrifugation steps (all centrifugation steps were performed at 4°C). 2.5 ml of the desired buffer were added to the column and subsequently removed by centrifugation (2 minutes, 1000 g). This step was repeated three times and the flow-through was always discarded. Finally the column was placed in a new collection tube and the sample could be added to it (2 x 1.5 ml). After afresh centrifugation at 1000 g for 2 minutes the sample was present in the new buffer and ready to use in detection experiments on the chip.

For small volume samples the buffer change was performed by ultrafiltration using Ultracel YM-30 Microcon columns. This process also enables an enrichment of the samples. The sample was therefore applied to the Microcon spin cartridge and centrifugation at 4°C and 11,000 rpm followed for 10 minutes. The centrifuged samples were then merged to a volume of about 100 µl and 150 µl of assay buffer (see Appendix II) were added. Afterwards the protein sample was centrifuged for about 15 minutes at 11,000 rpm and 4°C until the membrane of the column was almost dry. Adding buffer and centrifugation was performed two additional times. Finally 10 times more µl assay buffer than present sample volume were added to the membran and carefully mixed. The spin cartridge was inverted, placed in an Eppendorf tube and carefully centrifuged. The obtained protein was present in a 10x concentration and stored at 4°C.

2.2.2.3 Protein assay

To calculate the protein amount in the crude lysate-samples and in the purified protein samples an OD-measurement was performed using the “DC Protein assay”. This method enables simple determination of protein concentrations by measuring the absorbance of the

protein solution at 750 nm. The actual protein concentration is obtained by comparison of the measured value to a standard curve

85 µl solution A´ (= 1 ml solution A + 20 µl solution S), 17 µl protein sample and 700 µl solution B were mixed and incubated for 15 minutes at room temperature in the dark. Afterwards the samples were measured at 750 nm in relation to a previously performed standard curve. The standard curve was built of values for 0, 0.25, 0.5, 1 and 2 µg/ml BSA. Crude lysate-samples were applied in a 1:10 dilution (in aqua dest.) and the purified protein was applied undiluted in the test.

2.2.2.4 Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis

This method is a very helpful instrument, when it come to analysis of protein mixtures and also it helps for detection the molecular mass of proteins. The reason why the protein size can be determined is, that SDS from the gel binds to the protein in a 1.4:1 ratio, so that the protein subsequently carries a negative charge. That means, that the protein-SDS complex migrates to the anode in an electric field, and furthermore it means that the intensity of charge correlates to the protein size and the electrophoresis separates the proteins exclusively dependent on their mass. Smaller proteins migrate faster and are found on the lower part of the gel. With the help of an molecular weight marker the approximate size of the samples can be determined.

15 µl of each protein sample and 5 µl of LDS sample loading buffer were mixed and the protein was consequently denatured by heating it up to 95°C for 3 minutes. Then all 20 µl of each sample were loaded per lane of the gel. Electrophoresis was performed at 200 V and the process was stopped when the first band reached the end of the gel. The utilized moecular weight marker was the Fermentas PageRuler.

2.2.2.5 MutS-staining with the Alexa Fluor 555 Protein Labeling Kit

To have another possibility than GFP for MutS detection on the chip, the MutS protein without GFP, was stained with a fluorescent dye.

First of all the supplied sodiumbicarbonate powder was diluted in aqua dest. to a 1 M solution. 0.5 ml protein sample and 50 µl of the 1 M sodiumbicarbonate solution were mixed and the reactive dye was warmed up to room temperature. Then both solutions were mixed and incubated in the dark for one hour at room temperature on a stirrer. In the meantime the column was build up and the elution buffer was diluted to a 1x solution. With the help of a funnel the purification resin solution was filled into the plastic column until 3 cm below the upper edge. The buffer was dripped trough and finally the sample was applied to the column. The sample containing vial was washed with 100 µl 1x elution buffer and also this solution was applied to the column. After the whole sample had entered the column material 2 ml of elution buffer were added. Now it was very important to protect the column from light and thus a piece of foil was put around it. After a while two bands were visible in the column, from which the lower one was the stained protein and the upper one was non-bound fluorescent dye.

After the labelling it is usually necessary to do a buffer change, because the sample is diluted in the elution buffer of the labelling process. Here the desired buffer was the assay buffer (see Appendix II) and so first of all the collected, labelled fractions were concentrated with the help of a Microcon column and afterwards they were merged. After afresh concentration to a volumn of about 200 µl, 200 µl of assay buffer were added to the column. This process was performed two additional times and after this the sample was centrifuged until the membrane was almost dry. Finally 200 µl of assay buffer were added to collect the sample and it was stored at 4°C.

2.2.2.6 Gel-shift assay

Gel-shift assays provide the possibility to investigate DNA-protein binding properties. That for a certain amount of DNA is incubated together with the protein of choice under chip hybridization conditions. Then the hopefully built DNA-protein-complex is applied in a normal agarose gel electrophoresis (2% agarose gel). Additionally protein and DNA alone, respectively, are run on the gel so that the formed complex can be seen compared to the non-bound samples.

MutS, in particular, is supposed to bind SNPs. That means that a dsDNA sample containing one mismatch was used in the experiments and dsDNA without mismatch was used as the negative control.

First the fw-wt 691 (Exon 11) primer and the re-wt 691 (Exon 11) primer were utilized for obtaining the non-mismatch DNA strand. Also the fw-wt 691 (Exon11) primer and re-poly 691 (Exon11) primer were mixed resulting in a dsDNA strand containing a GT-mismatch. The experiment was also performed with the 863 fw-poly and re-poly primers, and the 863 fw-poly and re-wt primers for obtaining the heteroduplex DNA. Also these annealing resulted in a G:T mismatch. This mispairing comes second in the order of the strongest signals given after MutS binding. A stronger signal can only be obtained with an unpaired T base (Brown *et al*, 2001; Cho *et al*, 2006; Joshi & Rao, 2001; Su & Modrich, 1986).

4 µl of each primer were used to produce the dsDNA strands. The obtained 8 µl primer samples were incubated on a thermocylcer for 5 minutes at 95°C. Afterwards the samples were cooled down for 0.2°C/sec until reaching 15°C. The mixture of DNA, protein and glycerin was initially 1 µl dsDNA, 5 µl of protein and 3 µl glycerin. Later this ratio was modified and many different combinations were tried out and the results are shown in chapter 3. The three components were mixed and incubated at three different conditions: 1 hour at 4°C, 1 hour at room temperature and overnight at 4°C. Afterwards the samples were run on an 2% agarose gel for 15 minutes at 170 V. The utilized running buffer was TBE in the first few experiments but it was changed to Tris-borate buffer later (see Appendix II).

2.2.3 DNA-Chips

2.2.3.1 Chip design and production

Chips were spotted on common glass microscope slides using the "OmniGrid" Spotter by "Newport GeneMachines". The chip-layout is roughly shown in figure 2.1 and in more detail in Table 2.4 and 2.5, respectively. Three replicas of 20 oligos were spotted per block and this was performed in three repeats (blocks I, II, III). The oligo chips were designed to test different lenghts of chip probes. Row A were the 50-mer oligos, row B contained the 30-mer oligos (sequences shown in Appendix III) and in row C the self-produced PCR products (section 2.2.3.3) were spotted on the chip. A biotinylated probe served as positive control and the negative control was spot buffer only.

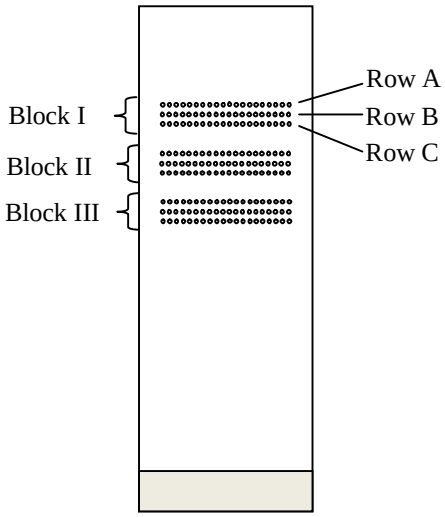


Figure 2.1 *Oligochip design I*

For spotting of the chips a 396-well plate containing the chip probes had to be prepared. This plate was then placed on the spotting machine and served as a template for the chips. Table 2.4 shows the exact plate pipetting scheme.

3.5 µl of 10x spot buffer, 3.5 µl sample and 28 µl distilled water were mixed in a tube and 30 µl of the resulting mixture were subsequently pipeted in each well. For the negative control 3 µl of 10 x spot buffer and 27 µl of aqua dest. were filled in the according wells.

The recipe for the spot buffer is shown in Appendix II.

Table 2.4 *Pipeting scheme of plate for chip spotting (B = buffer, - = negative control, + = positive control)*

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
A	+	-	50-mer oligos																-	+
B	+	-	30-mer oligos																-	+
C	+	-	PCR-products			B	PCR-products			B	PCR-products			B	PCR-products			B	-	+

Table 2.5 *Details of probes used for chip spotting*

well	**sample**	**well**	**sample**	**well**	**sample**
A1	BIO_691-fw-wt-G	B1	BIO_691-f-wt-G	C1	BIO_691-f-wt-G
A2	negative control	B2	negative control	C2	negative control
A3	691-fw-wt-G	B3	691-fw-wt-G	C3	11-1493
A4	691-fw-poly-A	B4	691-fw-poly-A	C4	11-1517
A5	691-re-wt-C	B5	691-re-wt-C	C5	11-1494
A6	691-re-poly-T	B6	691-re-poly-T	C6	buffer
A7	769-fw-wt-T	B7	769-fw-wt-T	C7	13-1517
A8	769-fw-poly-G	B8	769-fw-poly-G	C8	13-1493
A9	769-re-wt-A	B9	769-re-wt-A	C9	13-1494
A10	769-re-poly-C	B10	769-re-poly-C	C10	buffer
A11	836-fw-wt-C	B11	836-fw-wt-C	C11	14-1517

A12	836-fw-poly-T	B12	836-fw-poly-T	C12	14-1201
A13	836-re-wt-G	B13	836-re-wt-G	C13	14-1494
A14	836-re-poly-A	B14	836-re-poly-A	C14	buffer
A15	904-fw-wt-C	B15	904-fw-wt-C	C15	15-1493
A16	904-fw-poly-G	B16	904-fw-poly-G	C16	15-1517
A17	904-re-wt-G	B17	904-re-wt-G	C17	15-1494
A18	904-re-poly-C	B18	904-re-poly-C	C18	buffer
A19	negativ control	B19	negativ control	C19	negativ control
A20	BIO_691-fw-wt-G	B20	BIO_691-fw-wt-G	C20	BIO_691-fw-wt-G

Positive controls (BIO-691-fw-wt) were supposed to show positive results after Streptavidin/Cy3 and Streptavidin/Cy5 staining, respectively.

After the chips were spotted the slides were treated with UV-light for a few minutes and then they were “baked” in the incubater at 65°C for 2 hours. A little mark on the front side of the chip helped to identify the right side for hybridization experiments.

The procedure was the same for SU-8- and aldehyde-arrays.

2.2.3.2 Chips for spot buffer experiments

For experiments to investigate the effects of different spot buffers, special chips needed to be spotted. Table 2.6 illustrates the spot layout for these arrays.

Following spot buffers were tested:

1) Sodium-phosphatebuffer + 0.01% SDS
2) PBS + 10% Glycerin
3) 10 mM NaOH + 70% Formamid + 0.01% SDS

4) 3xSSC +1.5 M Betain + 0.01% SDS

5) PBS + 50% Formamid + 0.01% SDS

6) 10 mM NaOH + 70% Formamide + 10% Glycerin

Each well contained 18 µl of the according buffer and 2 µl of the oligo. As positive control served again the biotinylated 691-fw-wt probe. It was applied in two different concentrations: 5 mM for the Sodium-phosphate buffer + 0.01% SDS, and 1 mM for all other tested buffers. Water was used as a negative control. Only 50-mer oligos and 30-mer oligos were spotted on these arrays, PCR-products were left out.

Table 2.6 *Oligochip design II: spot buffer test array, += positive control, - = negative control)*

Row	**Probes A3 – A18**	**Probes B3 – B18**	**Probes A3 – A18**	**Probes B3 – B18**
1	probes in buffer 1		probes in buffer 4	
2	probes in buffer 2		probes in buffer 5	
3	probes in buffer 3		probes in buffer 6	
4	+/- all buffers	+/- all buffers	+/- all buffers	+/- all buffers

This array was spotted on the same slides above the other array.

2.2.3.3 PCR products – chip probes

The exons 11, 13, 14 and 15 of the RET-proto-oncogene were used as chip probes (row C). That for those exons were amplified using polymerase chain reaction (PCR). Table 2.7 illustrates the product lenghts of each exon and the utilized PCR program for amplification is shown in Table 2.8 and 2.9.

Table 2.7 *Exons - details*

	Exon 11	**Exon 13**	**Exon 14**	**Exon 15**
Product lenght	379 bp	295 bp	327 bp	294 bp
PCR program	1	1	2	1

Table 2.8 *PCR program 1 for amplification of exons 11, 13 and 15*

Temperature	**Time**	**Cylces**
95°C	15 min.	1
95°C	20 sec.	35
63°C	20 sec.	
68°C	1 min.	
72°C	7 min.	1
4°C	∞	1

Table 2.9 *PCR program 2 for amplification of Exon 14*

Temperature	**Time**	**Cycles**
95°C	5 min.	1
95°C	40 sec.	34
68°C	20 sec.	
72°C	40 sec.	
72°C	7 min.	1
4°C	∞	1

Following mastermix was used for the PCR experiments (Table 2.10).

Table 2.10 *PCR-Mastermix*

Reagent	Volume
dNTPs (2 mM)	100 µl
Q10 PCR buffer	125 µl
Aqua dest.	880 µl
HotStar Taq Polymerase	7.5 µl

275 µl mastermix were transferred in each of four vials and to each of them the according primer pair (2 µl forward and 2 µl reverse primer) was added. Then the amplification was performed on a thermocycler. Each exon was amplified using several different human DNAs, known from DNA sequencing to be either wildtype heterozygous or homozygous for different SNPs.

The PCR products were purified and used as chip probes. The genotyped DNAs served as control DNAs and were homozygous and heterozygous to the according exon (Table 2.11).

Table 2.11 *Array probes – PCR products*

Chip probe	Control DNA	Status	SNP
Exon 11	1493, 1517	homozygous	G691S
Exon 11	1494	heterozygous	G691S
Exon 13	1493, 1517	homozygous	L769L
Exon 13	1494	heterozygous	L769L
Exon 14	1201, 1517	homozygous	S836S
Exon 14	1494	heterozygous	S836S
Exon 15	1493, 1517	homozygous	S904S
Exon 15	1494	heterozygous	S904S

1 µl of the PCR product from before was mixed with 19 µl mastermix (Table 2.10) and then the PCRs were performed under the same conditions as before with the according PCR-program for each exon. The utilized primers are biotinylated at the 5´-end and are shown in Table 2.12.

Table 2.12 *RET-primer sequences*

Primer	Sequence (5´ - 3´)
bio-RET-11-fw	ccagtggtgccgagcctct
bio-RET-11-re	ctggcctccctccctggaa
bio-RET-13-fw	gcaggcctctctgtctgaactt
bio-RET-13-re	ggaggacagggctgtatgga
bio-RET-14-fw	aagacccaagctgcctgac
bio-RET-14-re	gtggtgggtcagggtgtgg
bio-RET-15-fw	acaccacccctctgctggtc
bio-RET-15-re	ctaggcttcccaagggcactg

PCR-products were purified with an Invitec-column according to manufacturer´s instruction – only the elution step was a little modified: samples were not eluted once with 30 µl of elution buffer but twice with 15 µl water (preheated to 37°C).

After purification the DNA amount of the samples was measured with the NanoDrop device. As the results were not satisfying due to too little DNA in the samples (initially an amount of 100 ng/µl was expected) the equivalent samples were merged and the volume was furthermore decreased by using a Speed-Vac to raise the DNA content of the sample. The fact that this high purity was not obtained forced standardization of the samples and so the following plate pipeting scheme was worked out (Table 2.13).

3.5 µl of spot buffer plus the amounts of water and sample shown in table 2.13 were mixed. Finally 30 µl of this solutions were transferred into the according wells of the spotting plate and the chips were manufactured.

Table 2.13 *Standardization of PCR-probes*

ID	Lengt [bp]	ng/µl	V [µl]	ng	ng/bp		µl standardized	µl AD
11-1493	379	37.6	12	451	1.19050132	total	12.0	19.5
11-1517	379	71.8	26	1867	4.92559367	1.0052232	25.9	5.6
11-1494	379	67.5	18	1215	3.20580475	total	18.0	13.5
13-1493	295	56.8	26	1477	5.00610169	1.02165341	25.4	6.1
13-1517	295	72	24	1728	5.85762712	1.19543411	20.1	11.4
13-1494	295	81	26	2106	7.13898305	1.45693532	17.8	13.7
14-1201	327	98.4	24	2362	7.22201835	1.4738813	16.3	15.2
14-1517	327	113.7	18	2047	6.2587156	1.2772889	14.1	17.4
14-1494	327	115	14.5	1668	5.09938838	1.04069151	13.9	17.6
15-1493	294	79	20	1580	5.37414966	1.09676524	18.2	13.3
15-1517	294	49.3	22	1085	3.68911565	total	22.0	9.5
15-1494	294	101	20	2020	6.8707483	1.40219353	14.3	17.2

2.2.3.4 Spotcheck

To check the presence of spotted probes on the micro-array a commercial spotcheck protocol was performed.

A 15 µl aliqot of the supplied spot-check solution was thawn. Then the solution was heated up to 95°C for 5 minutes. In the meanwhile a hybridization chamber containing 3x SSC was

prepared. The hot solution was now put on ice for 2 minutes before it was spun down and finally applied toto the chip. The solution was hybridized for 20 minutes at room temperature in the dark. Afterwards the slide was washed according to the following protocol:

- 1x SSC + 0.2% SDS for 5 – 10 minutes on a magnetic stirrer
- 1x SSC for 5 minutes on a magnetic stirrer
- 1x SSC for 5 minutes on a magnetic stirrer
- 0.1x SSC for 30 seconds on a magnetic stirrer

Finally the slides were spin-dried in a centrifuge at 900 rpm for 1 minute and scanned.

2.2.4 Hybridization experiments on the chip

The high amount of different process steps in a chip hybridization protocol has its disadvantages. Thus many different parts of such a protocol have to be optimized. An ordinary hybridization protocol comprises following steps:

1) DNA sample preparation for hybridization
2) Slide blocking I
3) Hybridization of DNA samples
4) Slide blocking II
5) Detection
6) Analysis of results

In the course of this project each of the following 11 points was optimized:

1) Chip coating

Two different coatings, called “SU-8” and “aldehyde” were used in the course of this thesis. The dissimilar coatings differ in the kind of how the probes are bound to the chip surface and thus they can increase or decrase the affinity (see section 1.2.1.1).

2) Spot buffer

Six different types of spot buffers were tested concerning their effects on the spot quality but also with respect to DNA binding capacities. The different buffers were:

- Sodium-phosphate buffer + 0.01% SDS
- 3x SSC + 1.5 M Betain + 0.01% SDS
- PBS + 10% Glycerin
- PBS + 50% Formamide + 0.01% SDS
- NaOH 10 mM + 70% Formamide + 0.01% SDS
- NaOH 10 mM + 70% Formamide + 10% Glycerin

3) Chip blocking prior to hybridization

Different chip blocking possibilities are available for different chip surfaces. Four of them were applied in this project: the standard blocking method for SU-8-chips is the DIGeasy blocking (see page 58) but also the effects of the Cyanoborhydrid blocking (see page 62) and Tris-blocking (see page 67) were tested on these chips. For Aldehyde-chips mainly the Cyanoborhydrid-blocking method was used. Finally the Ethanolamin-blocking (see page 63) was also introduced for both types of slides.

4) Chip pre-treatment to hybridization

Chips were treated for denaturation and opening of secondary structures of spotted chip probes with water or NaOH (30 mM, 300 mM) at different temperatures (room temperature, 70°C, 96°C) and with or without addition of formaldehyde to see if pre-treatment leads to decreased background signals or other positive effect on chip coatings.

5) Type of hybridization buffer

Six different hybridization buffers were tested in the course of this project to study their effects on DNA binding and chip background signals.

6) Hybridization conditions

This point may be the one that offers most and very simple variation possibilities. For example the hybridization temperature and duration can be easily modified.

7) Hybridized oligos, obtained mismatches and enzymatic digestion of overhangig DNA fragments

The choice of the applied oligos can give information about the spot quality on the chip and shows how specificly the protein binds to different mismatches. Also the effects of a Mung-Bean nuclease digestion after DNA-hybridization step was part of some experiments.

8) Chip blocking prior to detection

Before the protein is incubated on the chip the chip can be blocked with BSA to avoid unspecific binding of MutS. The blocking can also be left out to see the difference between the two options.

9) Applied protein type

In this work differnet types of protein-modifications were used for signal detection. For example a GFP-MutS and a fluorescent labelled MutS (Cy3) protein were tested. This allows different detection mechanisms, as scanning or using a fluorescent microscope. Further more a commercial MutS protein (Cy5 labelled) was used for some experiments so that the quality of the self-produced ones could be tested.

10) Protein concentration and applied additives

The concentration of the applied protein to get a signal was varied in the different experiments and also other substances, like ATP or $MgCl_2$, were added to the protein solution to check for positive changes.

11) Signal detection

The obtained signal on the chip was either detected with the scanner or with the help of a fluorescent microscope.

In Table 2.14 an overview about all necessary steps in a hybridiaztion experiment is illustrated.

Table 2.14 *Experimental scheme of a hybridization experiment*

Step	Procedure
1	Initial slide blocking
2	Wash step
3	Target DNA hybridization
4	Wash step
5	Slide blocking prior to protein detection
6	Wash step
7	Incubation with protein
8	Wash step
9	Scanning of slides
10	Analysis of results

After step 10 follows repetition of steps 7 to 10 with labelled streptavidine for positive controls

2.2.4.1.1 *<u>Protocol 1(SU-8 slides)</u>*

This first experiment was performed with the GFP-MutS protein sample. Only 50-mer wt-oligos were used for hybridization on the chip and sample 691 fw-wt was substituted with a biotinylated positive control (BIO-691 fw-wt). That for 2 µl of each of the following oligos (100 µM) and 184 µl aqua dest. were mixed to obtain a 1 mM concentration for each primer

in the hybridization mixture: 691-fw-wt_BIO, 691-re-wt, 769-fw-wt, 769-re-wt, 836-fw-wt, 836-rw-wt, 904-fw-wt, 904-re-wt (= oligo-mix 1).

In this first protocol different hybridization buffers were tested (see recipies in Appendix II). These were:

- DIGeasy
- PCR-buffer (1x DYN)
- Na-Phosphate-buffer
- PBS-T (PBS + 0.1% Tween-20)
- TBS-T (TBS + 0.1% Tween-20)

Before the Oligos were put on the chip the chips were blocked with DIGeasy. 14 – 17 µl of the DIGeasy solution were put on a coverslip and put on the chip. Then the chips were transferred into hybridization chambers and incubated for 30 minutes at 42°C. Afterwards the coverslips were removed by dipping the chips in wash buffer 2 (Table 2.4) and then the chips were washed in this solution for 5 minutes. Finally the chips were dipped into water 5 times and blow-dried in the centrifuge.

2 µl of the Oligo-mix and 18 µl of hybridization buffer were mixed and heated upto 95°C for 5 minutes on the thermocycler. Afterwards the samples were cooled down again to 42°C und 7.5 µl of the sample were then applied to the chip. The hybridization was performed for 1 hour at 42°C. After 1 hour the coverslips were removed in PBS-T and the slides were washed in PBS-T for 5 more minutes. Finally the chips were blow-dried in the centrifuge.

Before the detection step with GFP-MutS the chips were blocked to avoid unspecific binding of the protein. Following blocking solution was used (Table 2.15).

Table 2.15 *Blocking solution recipe*

Reagent	Concentration
Assay buffer	1x
BSA	3% (w/v)
Tween-20	0.5%

The recipe for the assay buffer is shown in Appendix II.

17 µl of blocking buffer were applied to the chip and incubated 20 minutes at room temperature in a hybridization chamber. Afterwards the coverslips were romoved by dipping the chip in a solution of assay buffer with 0.1% Tween-20. Before the detection step the slides were washed in assay buffer with 0.1% Tween-20, dipped three times in fresh assay buffer with 0.1% Tween-20 and finally spun dry in the centrifuge.

For the detection step 7.5 µl of GFP-MutS protein solution (concentration 5 mg/µl; diluted 1:5 in assay buffer) were incubated on the chip for 1 hour at 4°C in the dark. Afterwards the coverslips were removed in assay buffer with 0.1% Tween-20 and the chips were washed also in this solution for 5 more minutes, dipped into a fresh assay buffer solution with 0.1% Tween-20 three times and spun dry and scanned.

Afterwards the chips were detected with a Streptavidin/Cy3 solution to check the positive and negative controls. First of all the slides were washed in PBS-T for 5 minutes and then 7.5 µl of the Streptavidin/Cy3 solution (diluted 1:400 in PBS-T) were applied to the chip and incubated for 30 minutes at room temperature in the dark. The coverslip was subsequently removed in PBS-T and the chips were washed for 5 minutes in PBS-T before they were blow-dried in the centrifuge and scanned.

All washing steps were performed in jars on a magnetic stirrer.

This protocol was the main body for all further hybridization experiments. In the protocols 2 to 18 only the changes compared to this protocol are listed.

2.2.4.1.2 Protocol 2 (SU-8 slides)

→ *protein concentration*

In this protocol the GFP-MutS was applied to the chip in a 1:1 rather than a 1:5 dilution.

2.2.4.1.3 Protocol 3 (SU-8 slides)

→ *protein type*

In this protocol the GFP-MutS was substituted with the fluorescently marked MutS/Cy3 sample. The protein was applied undiluted on the chip.

2.2.4.1.4 Protocol 4 (SU-8 slides)

→ *reduced number of hybridization buffers; protein type and concentration; additional washing step after detection*

The aim of this protocol was to achieve a reduced background signal. The number of hybridization buffers was reduced to two (DYN and TBS-T). Detection was again performed with the MutS/Cy3 sample and the protein was applied undiluted to the chip. After the detection with 7.5 µl of MutS/Cy3 for 1 hour at 4°C an additional washing step was introduced into the protocol:

1. The coverslip was removed in assay buffer + 0.1% Tween-20 and the slides were washed for 5 minutes in the same solution
2. Slides were spin-dried
3. 7.5 µl of PBS-T were incubated on the chip for 30 minutes at room temperature
4. Slides were washed in PBS-T for 5 minutes at room temperature
5. Slides were dipped in PBS-T three times
6. Slides were spin-dried

Table 2.16 illustrates the actual protocol for three types of slides.

Table 2.16 *Applied steps in the protocol for different chips (nc = negative control)*

Test	NC I	NC II
1	-	1
2	-	2
3	-	-
4	4	4
5	5	5
6	6	6

2.2.4.1.5 *Protocol 5 (SU-8 slides)*

→ *reduced number of hybridization buffers; no BSA blocking*

In this protocol the blocking step with BSA prior to protein detection was left out completely. Again TBS-T and DYN buffers were used for hybridization and for each type of buffer two slides were prepared – one slides was subsequently treated without the BSA blocking step and one with BSA as a control to see the difference between the slides.

2.2.4.1.6 *Protocol 6 (SU-8- and aldehyde-slides)*

→ *reduced number of hybridization buffers; protein type and concentration; detection step temperature; BSA blocking +/-*

The GFP-MutS protein sample was used for SU-8 slides and MutS/Cy3 was tested on both types of arrays in this experiment. All protein samples were used undiluted on all slides. In this protocol the main difference to before was the change of temperature for the detection step (4°C → room temperature). Also the need of a BSA blocking prior to protein detection

was tested again as described in protocol 5. Hybridization was performed in PBS-T buffer for all slides.

2.2.4.1.7 *Protocol 7 (SU-8- and aldehyde-slides)*

→ initial blocking; new hybridization buffer; hybridization temperature; different blocking procedures prior to protein detection; final wash step

The initial blocking for all aldehyde slides was not the DIGeasy blocking as before, but the cyanoborohydride method. That for the slides were kept in cyanoborohydride solution (see Table 2.17) for 30 minutes and afterwards the slides were quickly dipped into water and blow-dried in the centrifuge.

Table 2.17 *Cyanoborohydride solution*

Reagent	Concentration
$NaH_2PO_4.H_2O$	20 mM
Na_2HPO_4	20 mM
NaCl	200 mM
Sodiumcyanoborohydride	3 g/l

SU-8 slides were blocked with DIGeasy as usual. For half of the SU-8 slides the previously used TBS-T buffer was used for hybridization, for the other half and all aldehyde slides DIGeasy + 10% formaldehyde was used for this purpose. After hybridization for one hour at room temperature two slides were blocked as before, two slides were blocked with milk powder dissolved in PBS and for two slides the blocking step was left out. Blocking was performed for one hour at room temperature. For all chips the MutS/Cy3 protein sample was used undiluted for mismatch detection. Also the final wash step was varied: assay buffer + 0.1% Tween-20 and PBS-T, respectively, were used in this protocol.

2.2.4.1.8 Protocol 8 (SU-8 slides)

→ *reduced number of hybridization buffers; Hybridization +/-; detection temperature; protein type, concentration and additives*

The chosen hybridization buffer for this experiment was TBS-T. Two slides were only hybridized with TBS-T and no DNA. Detection with protein was carried out at room temperature for one hour with undiluted MutS/Cy3 protein and GFP-MutS, which were applied in mixtures with:

1) + 0.01% Triton X-100
2) + 0.1% Tween-20
3) + 0.01% Triton X-100 + 0.1% Tween-20

The DNA negative chips were detected with protein and for two normally hybridized chips the detection step was performed without protein, but only assay buffer.

2.2.4.1.9 Protocol 9 (SU-8 slides)

→ *initial blocking method; new hybridization buffers; hybridization conditions; protein type*

In comparison to DIGeasy blocking ethanolamine blocking was introduced here. The slides were therefore incubated in following solution (Table 2.18) for 30 minutes at 50°C.

Table 2.18 *Ethanolamine blocking – blocking solution (V = 250 ml)*

Reagent	Concentration	Volume
Tris	1 M	50 ml
Ethanolamine		1.5 ml
Aqua dest.		193 ml
SDS	10%	2.5 ml

Afterwards the solution was discarded and the slides were rinsed twice with deionized water. Then the slides were washed in washing solution (Table 2.19) for 30 minutes at 50°C.

Table 2.19 *Ethanolamine blocking – washing solution (V = 250 ml)*

Reagent	Concentration	Volume	Final concentration
SSC	20x	50 ml	4x
SDS	10%	2.5 ml	0.1%
Aqua dest.		197.5 ml	

The blocking solution was discarded and the slides were rinsed with deionized water before they were blow-dried in the centrifuge.

Not only the blocking, but also the hybridization step was modified in terms of additives to the buffer and the hybridization temperature. Following conditions were tested:

1) TBS-T + 25% formalin, 42°C
2) TBS-T, 52°C
3) TBS-T, 42°C
4) TBS-T + 2 mM ATP, 42°C
5) DIG + 10% formalin, 52°C

2.2.4.1.10 *<u>Protocol 10 (SU-8 slides)</u>*

→ *SU-8 initial blocking; reduced number of hybridization buffers; Hyb+/-, protein plus BSA*

Cyanoborohydride blocking was used here for SU-8 slides to compare it with DIGeasy blocking.

Furthermore the specificity of the protein was checked with following experiment: At a time two slides were treated as follows (Table 2.20):

Table 2.20 *Experiment scheme*

Hybridization	**Detection**
Positive	Positive
Negative	Positive
Positive	Negative

Hybridization was performed in TBS-T and 3% BSA were added to the MutS/Cy3 solution prior to the detection step.

2.2.4.1.11 Protocol 11 (SU-8-slides)

→ *new initial blocking buffer; reduced number of hybridization buffers; hybridization buffer additives*

Half of the slides were blocked with DIGeasy and half of it with ethanolamine. Following buffer-additive combinations were used for hybridization:

1) TBS-T
2) PBS-T
3) TBST + 5 mM ATP
4) PBST + 5 mM ATP
5) DIG + 10% formaldehyde

2.2.4.1.12 Protocol 12 (SU-8- and aldehyde-slides)

→ initial chip blocking; chip pre-treatment; reduced number of hybridization buffers; new hybridization-mix; protein type and detection temperature

For the initial blocking in this experiment the ethanolamine-blocking was used for SU-8 slides and the cyanoborohydride-blocking was used for aldehyde-slides. Before hybridization the slides were pre-treated with the following procedures:

1) Aqua dest.: 70°C and 100°C, respectively
2) NaOH (300 mM): room temperature and70°C, respectively
3) NaOH (30 mM): room temperature and70°C respectively
4) NaOH (30 mM) + 70% formaldehyde: room temperature and 70°C, respectively

After these treatments the slides were dipped in ice-cold isoproanol and blow-dried in the centrifuge. For each type of pre-treatment four slides were prepared. To evaluate if the condition of the slides after pre-treatment has any influence on the results the wo better looking slides were applied in this protocol and the two worse looking slides were applied in protocol 13.

Only two oligos were hybridized on the chip (= oligo mix 2). 2 µl of 691-fw-wt_BIO (100 µM) and 2 µl of 691-re-wt (100 µM) were mixed with 98 µl aqua dest. and applied to the chip in TBS-T.

The utilized protein was undiluted MutS/Cy3 at room temperature for one hour.

2.2.4.1.13 Protocol 13 (SU-8 slides)

→ initial blocking; reduced number of hybridization buffers; detection step conditions

Additionally to DIGeasy- and ethanolamine-blocking the new method of Tris-blocking was introduced here. Following solution was incubated on the chips for one hour at room temperature:

Tris-HCl	20 mM
KCl	50 mM
$MgCl_2$	5 mM
Tween-20	0.01%
BSA	3%

After incubation the slides were washed in TBS-T for 5 minutes at room temperature and blow-dried in the centrifuge.

Hybridization was performed in TBS-T for one hour at 42°C. The detection step was extended from one to two hours and performed at room temperature. The utilized protein type was the purchased MutS/Cy5 sample. After detection the slides were washed for 5 minutes in following solution:

Tris-HCl (pH 7.6)	20 mM
KCl	50 mM
$MgCl_2$	5 mM
Tween-20	0.01%
BSA	1% (w/v)
DTT	0.1 mM

2.2.4.1.14 Protocol 14 (SU-8 slides)

→ *modified DIGeasy blocking; chip pre-treatment; reduced number of hybridization buffers; hybridization DNA+/- and duration*

Alternatively to the previously described DIGeasy blocking in this case the slides were simply put into a jar filled with DIGeasy and kept there for 30 minutes at room temperature. Also slides from earlier experiments blocked with Tris- and ethanolamine were used.

Following slide pre-treatments were applied in this protocol:

1) Aqua dest.: 70°C and 100°C, respectively
2) NaOH (30 mM): room temperature and70°C respectively
3) NaOH (30 mM) + 70% formaldehyde: room temperature

For hybridization the slides were split in two groups. The slides were hybridized in DIGeasy + 10% formaldehyde for two hours at 42°C. One group of slides was incubated without DNA in the sample, the other group was hybridized with the previously used wt-oligo mix (oligo mix 1).

2.2.4.1.15 Protocol 15 (SU-8 slides)

→ *initial blocking; chip-pre-treatment; reduced number of hybridization buffers*

The initial blocking was Tris-blocking for half of the slides and DIGeasy blocking for the other half. The applied pre-treatments were:

1) 100°C H_2O for 1 minute → isopropanole on ice
2) 100°C H_2O for 1 minute → 10 mM NaOH on ice

For detection the purchased MutS/Cy5 was used and was incubated on the chip for 2 hours at room temperature.

2.2.4.1.16 Protocol 16 (SU-8- and aldehyde-slides)

→ *slide type; initial blocking; chip-pre-treatment; reduced number of hybridization buffers; hybridization conditions; protein type; protein plus additives; detection buffer*

This was the first experiments in which the spot-buffer check slides were used. The initial blocking for SU-8 slides was the Tris-blocking and for aldehyde slides the cyanoborohydride-blocking method was used. The chips were furthermore pre-treated in 100°C H_2O for 1 minute and finally dipped in ice-cold isoproanol before they were blow-

dried in the centrifuge. No hybridization was performed at all. The MutS/Cy5 detections step was modified by adding different concentrations of $MgCl_2$ (0, 2.5 and 5 mM) to the buffer. The protein was thus kept in following buffer for the detection:

Tris-HCl (pH 7.6)	20 mM
KCl	50 mM
Tween-20	0.01%
BSA	1% (w/v)
DTT	0.1 mM
$MgCl_2$	0; 2.5; 5 mM

Detection was performed for two hours at room temperature and for each $MgCl_2$ concentration one slides was detected with 5 mM ATP in the protein sample and one without.

2.2.4.1.17 Protocol 17 (SU-8- and aldehyde spot buffer-check slides)

→ *slide type;initial blocking conditions; reduced number and new hybridization buffer and conditions; MungBean nuclease step; protein plus additives*

Both types of slides were blocked following the Tris-block protocol. The hybridization step was performed for 4 hours at 42°C and52°C, respectively, with two types of buffers (TBS-T and ExpressHyb) for each condition. Two slides of each type were hybridization negative, one of them was MungBean nuclease positive and the other one was only incubated with the enzyme buffer.

The wash protocol after hybridization was as follows:

- Wash1: 5 minutes
- Wash 2: 5 minutes

- Wash 3: 3x dip
- Wash "Tris", 5 minutes: 20 mM Tris-HCl
  50 mM KCl
  5 mM $MgCl_2$
  0.01% Tween-20
  0.1 mM DTT

After hybridization the overhangig DNA fragments were digested by using MungBean nuclease (10 U). The enzyme was incubated for 30 minutes at 30°C on the chips and afterwards the slides were washed in Wash 2 buffer (see Appendix II) for 5 minutes followed by a wash step in "Tris"-solution for 5 more minutes.

Detection was performed with the purchased MutS/Cy5 (5 ng/µl), with 5 mM $MgCl_2$ in the buffer, for 2 hours at room temperature. Afterwards the slides were washed in "Tris"-solution for 5 minutes at room temperature.

2.2.4.1.18 Protocol 18 (SU-8- and aldehyde spot buffer-check slides)

→ *slide type; initial blocking conditions; chip pre-treatment; hybridization buffer and conditions; MungBean nuclease step; protein plus additives; detection conditions*

SU-8 and aldehyde slides were blocked with two methods: Tris and DIGeasy blocking. After the Tris blocking the slides were washed in TBS-T for 5 minutes at room temperature and following the DIGeasy blocking the slides were washed in Wash 2 for 5 minutes, dipped three times in aqua dest. and finally blow-dried in the centrifuge.

Chip pre-treatment was performed in aqua dest. at 100°C for 1 minute and then the slides were kept in 10 mM NaOH on ice.

Hybridization was performed over night at 42°C with PBS-T and DIGeasy + 10% formaldehyde with and without DNA (negative control).

Detection was performed with MutS/Cy3 with 5 mM $MgCl_2$ for 2 hours at room temperature.

In parallel some chips were hybridized over night at 52°C using the buffers TBS-T and ExpressHyb. For those slides three different protein types were used for the detection step (all proteins were incubated for two hours at room temperature together with 5 mM $MgCl_2$):

- MutS/Cy5
- MutS/Cy3
- GFP-MutS/Cy3

Finally the chips were washed in “Tris”-solution (see page 70) for 5 minutes at room temperature.

Eventually all slides of all experiments were scanned with the GenePix™ 4000A Scanner by Axon Instruments. The GenePix Pro 3.0 program was used for analysis and filing of results.

The chips had to be completely dry when put into the scanner. They were fixed inside the scanner upside down and then a pre-scan was performed to localize the array on the glass slide. Then the best contrast value (photomultiplier tube, PMT, usually a value about 700 PMT) is set for the fluorescent dye wavelenghth (635 nm - red signal and 532 nm – green signal).

After opening the according “gal-file” for each array layout, the obtained grid was layed over the scanning picture. This grid tells the specific probe names, which helps to identify the probes on the array and which is needed for analysis. Eventually the results with spot intensity values are saved as a “gpr.file”.

CHAPTER 3

RESULTS

3.1 Bacterial culture

3.1.1 Cell disruption

Three different methods of mechanical cell disruption we investigated in the course of this project. Following results (Table 3.1) were obtained with the FastPrep® device.

It was found out, that induction with lactose generally resulted in a higher pellet weight than induction with IPTG. On average the obtained cell pellets weighted 2.16 g/ 300 ml cell culture for the lactose-induced cultures and 0.57 g/ 300 ml cell culture for the IPTG-induced bacterial culture.

Table 3.1 *Pellet weight for FastPrep® cell disruption with different lysis matrices. MutSA as well as MutSB and GFP-MutS1 as well as GFP-MutS2, respectively, were four different bacterial cultures, obtained from four single colonies on the agar plate.*

Sample ID	***E.coli* strain**	**Induction**	**Pellet weight [g/300 ml]**	**Lysis matrix**
1	MutS B	Lactose	2.03	E
2	MutS A	Lactose	1.8	
3	GFP-MutS 2	Lactose	2.18	
4	GFP-MutS 1	Lactose	2.64	
5	MutS B	IPTG	0.75	B
6	MutS A	IPTG	1.09	
7	GFP-MutS 1	IPTG	0.70	
8	GFP-MutS 2	IPTG	0.53	
9	GFP-MutS 1	IPTG	0.77	E
10	GFP-MutS 2	IPTG	0.53	

The pellets that were broken open with French Press weighted 1 mg twice and one time 2 mg and were obtained from 300 ml bacterial culture each. The cell pellet for sonication weighted 2.79 mg and was obtained from 300 ml of bacterial culture as well. However, the smallest amount of extracted protein in the crude lysate (1.39 mg/ml) was obtained with sonication and the highest protein concentration in the crude lysate (2.37 mg/ml) was obtained after disruption with FrenchPress.

3.2 Protein

3.2.1 Protein concentrations

After bacterial lysis and centrifugation the protein was isolated from the cells and concentrations were measured in the 14 clarified crude lysate samples. It is thought that about 20% of the protein amount in the crude lysate represent the desired protein type. After IMAC-purification of the protein, equal samples were merged and the protein concentration was measured again. Figure 3.1 illustrates how much protein eventually was obtained.

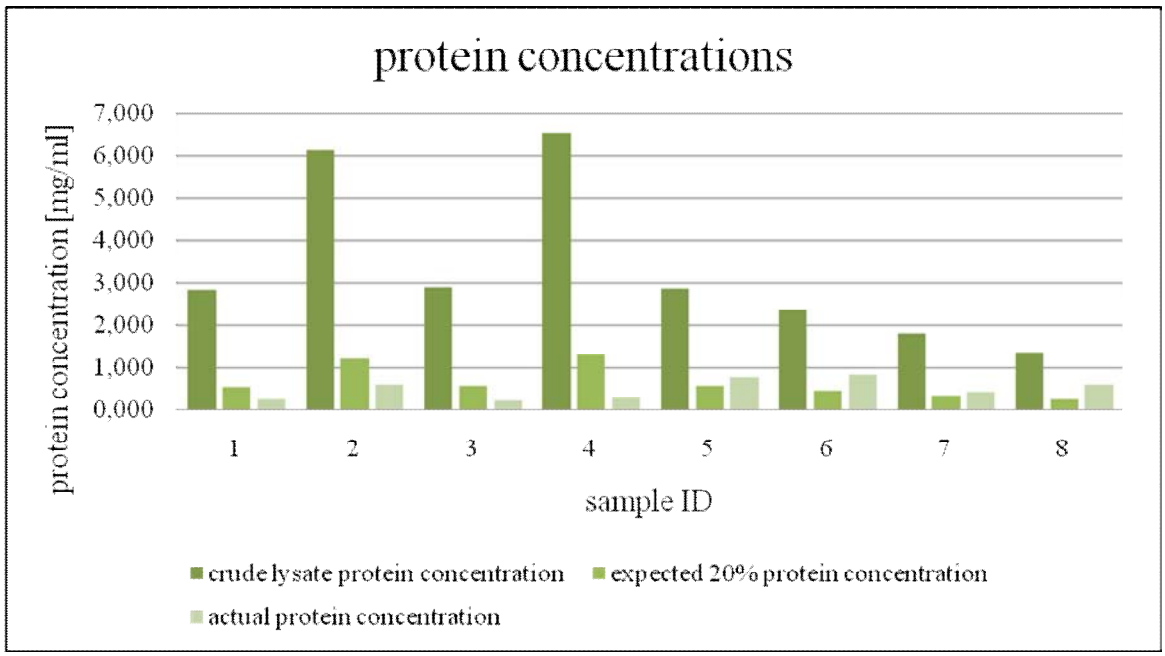


Figure 3.1 *Illustration of protein concentration from crude lysate to the purified protein derived from 300 ml bacterial cultures (1: samples 1+2, 2: samples 3+4, 3: samples 5+6, 4: samples 7+8+9+10, 5: sample A, 6: sample B, 7: sample C, 8: sample D). To obtain the actual protein concentration the sum of all fractions of the particular sample was calculated.*

The distinct concentrations of each fraction from each sample are shown in Table 3.2.

Table 3.2 *Protein concentrations after purification out of cell pellets derived from 300 ml bacterial cultures. ID corresponds to table 3.1. The Flow-through fractions are not included in Table 3.1.*

Sample	Concentration (mg/ml)	Volume (ml)	Protein (mg/V)
1+2 Elu1	0.2707	3	0.81
1+2 Elu2	negative	-	-
3+4 Elu1	0.5587	3	1.68
3+4 Elu2	0.0532	2	0.11
5+6 Elu1	0.2219	3	0.67
5+6 Elu2	0.0057	2	0.01
7 to 10 Elu1	0.1912	3	0.57
7 to 10 Elu2	0.1032	2	0.21
7 to 10 FT-Elu1	2.5133	3	7.54
A Elu1	0.6575	3	1.98
A Elu2	0.1268	2	0.25
A FT-Elu1	1.1879	3	3.56
B Elu1	0.8076	3	2.42
B Elu2	0.0394	2	0.79
C Elu1	0.4037	3	1.21
C Elu2	0.0388	2	0.078
C FT-Elu1	1.4031	3	4.21
D Elu1	0.6091	3	1.83
D Elu2	negative	2	-

Table 3.2 illustrates how much protein was not bound by the Ni-beads in the IMAC column, but washed out with the flow-through. Certainly not all of this non-bound protein was MutS protein, but for sure some parts of MutS have been lost here.

The values for eluate 1 samples are generally higher than Eluate 2, what was expected. Eluate 2 samples contain protein only in very low concentration. The overall protein yield of all purified samples was low with respect to how much bacterial culture was applied in the purification process.

3.2.2 Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

An SDS-PAGE gives information about the proteins purity in the sample and furthermore informs about the protein´s size. Also it is possible to draw conclusions about the concentration ratios between the different samples by the help of band intensities.

Results of the performed SDS-PAGE are shown below in Figure 3.2. The applied samples were the protein samples (MutS and GFP-MutS, respectively) after purification in the IMAC column.

MutS protein lacking GFP (lanes 4, 5, 6, 8, 9, 12, 17) should be detectable as a band at the size of 100 kDa and a band at this size is seeable for all these samples. The GFP-MutS protein (lanes 1, 2, 3, 10, 11, 13, 14, 15 and 16) should give the signal at the size of about 130 kDa and also here it is possible to detect a band at this size for each sample. The molecular weight marker was applied in the middle of the gel in lane 7.

Furthermore the band intensities correlate with the measured protein concentration values from Table 3.2.

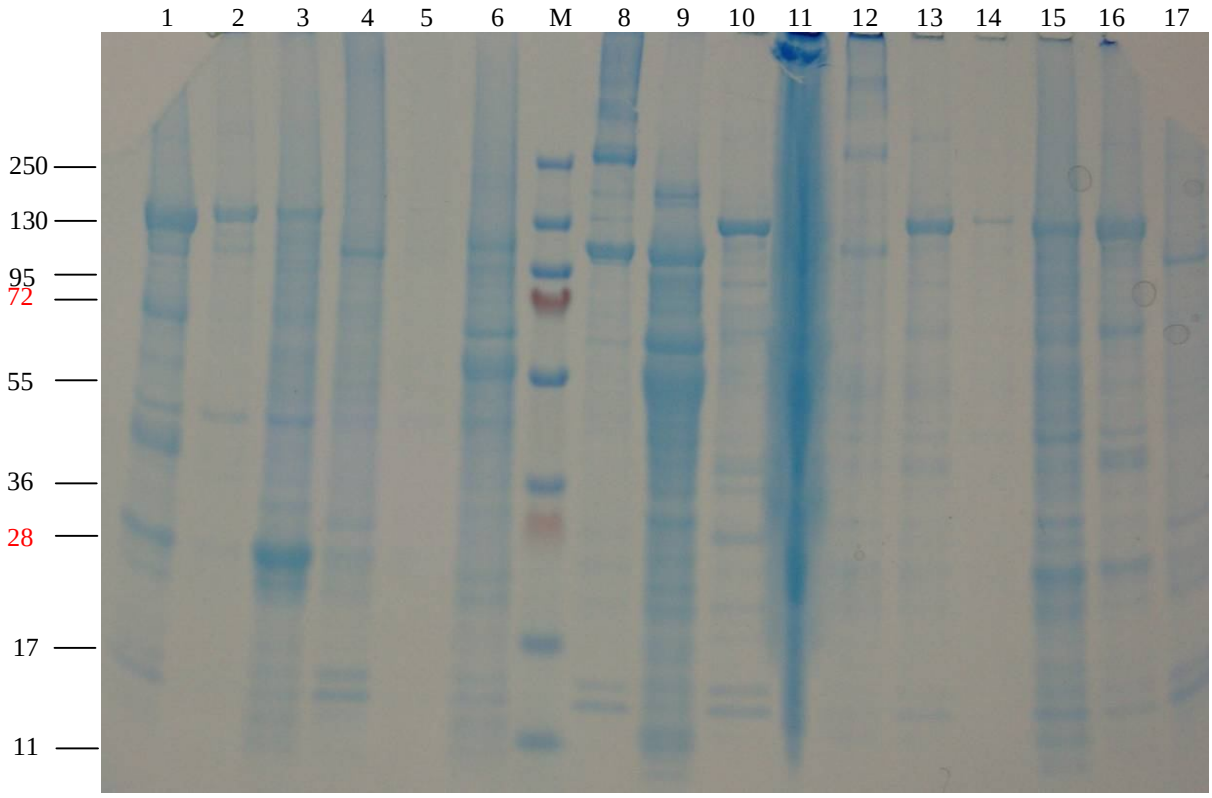


Figure 3.2 *Results of the SDS-PAGE; GFP-MutS ~ 130 kDa, MutS ~100 kDa. (1:sample A, Elu1; 2:sample A, Elu2; 3: sample A, flow-through Elu1; 4:sample C, Elu1, 5: sample C, Elu2; 6:sample C, flow-through, Elu1; M: Marker, 8: sample 1+2, Elu1; 9: sample 1+2, flow-through Elu1; 10: sample 3+4, Elu1; 11: sample 3+4, flow-through Elu1; 12: sample 5+6, Elu1; 13: sample 7-10, Elu1; 14: sample 7-10 Elu2; 15: sample 7-10, flow-through Elu1, 16: sample B, Elu1; 17: sample D, Elu1)*

3.2.3 Gel-shift assays

Gel-shift assays turned out to be very difficult to optimize and thus unfortunately it was not possible to reproduce the protein-mismatch binding successfully in the course of this project. Only one experiment showed the expected complex, formed between the GFP-MutS protein and mismatched DNA (Figure 3.3) Only a very little amount of mismatched DNA is detectable on the gel in lane 4, the rest of the DNA is bound to the protein. In this case the utilized DNA was containing a G:T mismatch (Table 3.3). MutS/Cy3 and also the native MutS sample, that was not treated at all before, did not lead to the expected results.

Table 3.3 *Sequences of utiliezed primers in gel-shift assay*

Primer	Sequence (5´→ 3´)	Obtained mismatch
691-fw wildtype	gct act cct ctt ccg gtg ccc gcc ggc cct	no mismatch (n DNA)
691-re wildtype	agg gcc ggc ggg cac cgg aag agg agt agc	
691-fw wildtype	gct act cct ctt ccg gtg ccc gcc ggc cct	G:C mismatch (m DNA)
691-re polymorph	agg gcc ggc ggg cac Tgg aag agg agt agc	

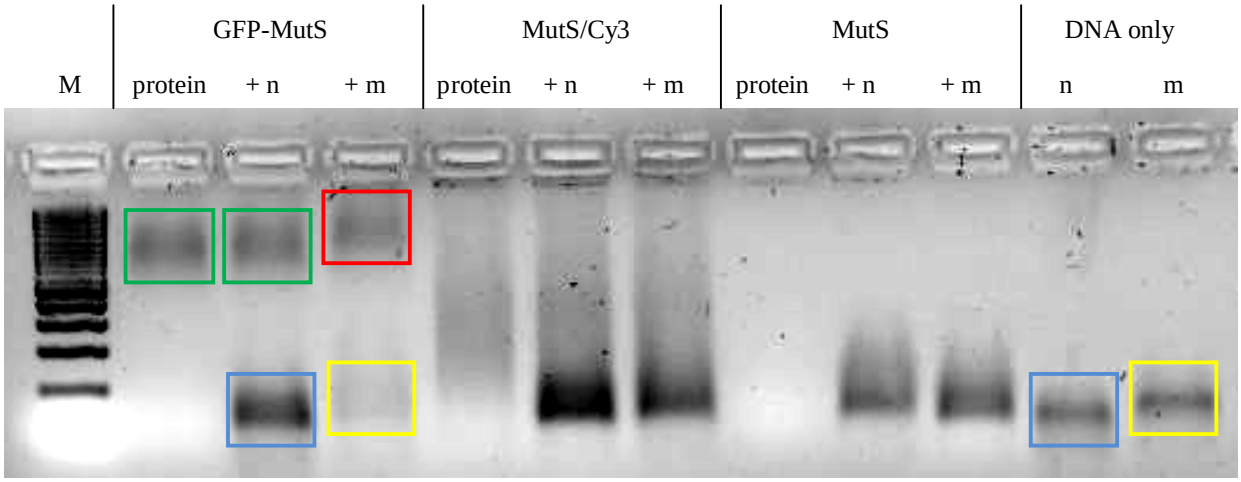


Figure 3.3 *Gel-shift assay. 83 ng DNA and 1.87 µg protein were incubated under array-hybridization conditions and subsequently applied to the gel. (green boxes: protein, blue box: DNA without mismatch, yellow boxes: G:T mismatch DNA, red box: [DNA-protein-complex], M = marker, n = normal DNA without mismatch, m = DNA with mismatch)*

3.3 Array-design

3.3.1 PCR-probes for array spotting

In row A and B primer oligos were spotted on the arrays. In row C though, self-produced PCR-probes were spotted. These PCR probes were obtained from PCR on exons 11, 13, 14 and 15. For each exon two homozygous and one heterozygous PCR probes were spotted on the array to have a control system on the array.

After amplification the PCR-products were purified using the Invisorb® Spin PCRapid Kit for purifiaction of PCR-fragments (Invitek) according to manufacturer´s recommendations, measured with the NanoDrop and mixed with spot buffer as explained in Table 2.13.

Amplification of the exons 11, 13 and 15 worked very well as seeable in Figure 3.4. Only exon 14 showed a double band after gel-electrophoreses but this was subsequently overcome by using a PCR-program with a higher annealing temperature (Figure 3.5).

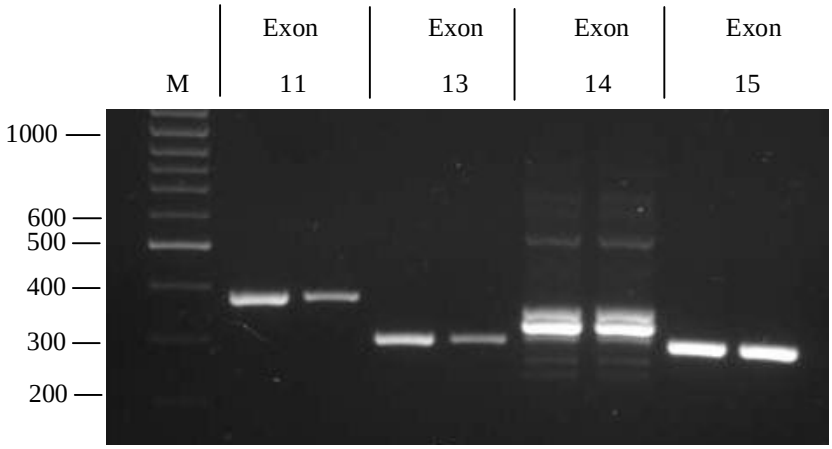


Figure 3.4 *PCR on exon 11 (379 bp fragment), exon 13(295 bp fragment), exon 14 (327 bp fragment) and exon 15 (294 bp fragment).*

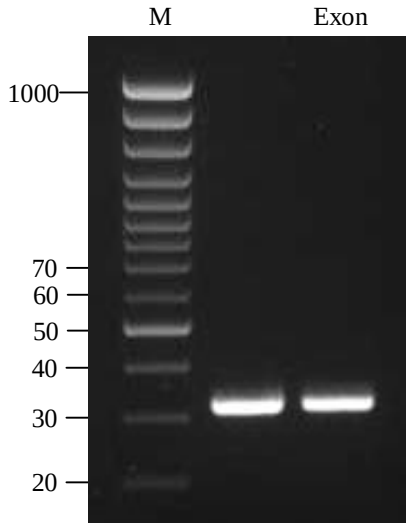


Figure 3.5 *PCR on exon 14 (327 bp fragment).*

For each exon three PCR-products, either homozygous or heterozygous, were amplified. Figure 3.6 shows the obtained results for every single probe.

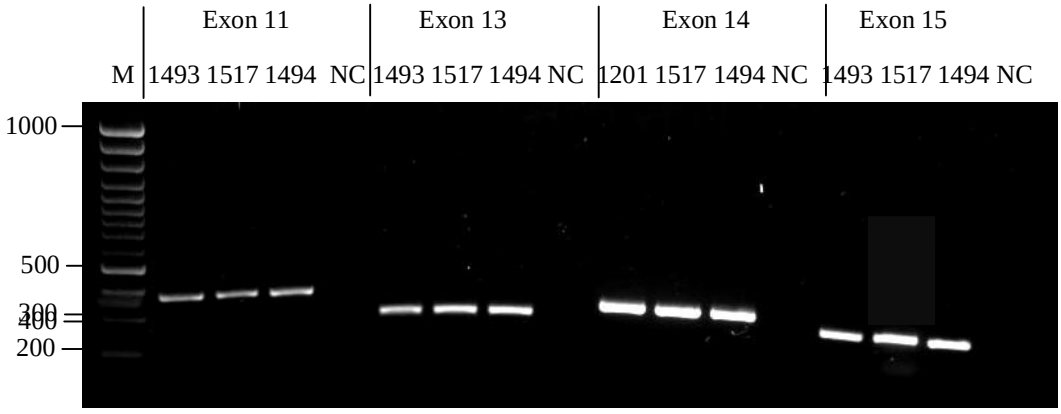


Figure 3.6 *PCR products of row-C chip probes: exon 11 (379 bp), exon 13(295 bp), exon 14 (327 bp) and exon 15 (294 bp) (NC = negative control)*

3.3.2 Spotcheck

The spotcheck can be performed after spotting of each batch of slides. For the first batch of spotted slides, that was used in protocols 1 to 15, probe B4 (red circle in Figure 3.7 A) was

negative due to a spotting error. All the other spots were detectable on the array. Figure 3.7 illustrates the spotcheck results of the slides with both arrays. It is obvious that only the sodium-phosphate buffer was successfully applied in the spot buffer test (Figure 3.7, C,D).

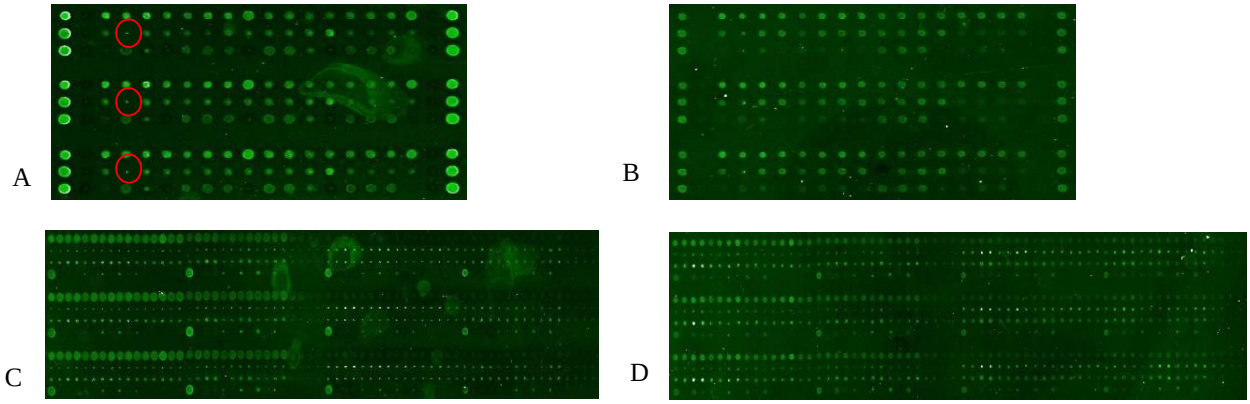


Figure 3.7 *Spotcheck results (A: SU-8 slide array I, B: aldehyde slide array I, C: SU-8 slide array II, D: aldehyde slide array II).*

3.4 Hybridization experiments

Results of the hybridization experiments were obtained after scanning the slides with the GenePix 4000A Scanner by Axon Instruments. According to the hybridized oligo-mix different signal combinations were expected depending of heteroduplex or homoduplex formation and mismatch-specific MutS binding.

Table 3.4 illustrates the expected signals after wildtype-DNA hybridization was performed (see oligo-mix 1 on page 58 and oligo-mix 2 on page 66).

Because a wildtype-oligo mix was hybridized in all experiments it was expected, that all polymorphic probes and also the heterozygous PCR-products would give positive signals after MutS binding. These results were the expected outcome for all protocols, except for protocol 12, because in this experiment another oligo-mix (= oligo-mix 2) was used. The difference in results was, that for this experiment only the polymorphic probes of Exon 11

should give positive signals and all other polymorphic probes on the array (exons 13, 14 and 15) should be negative after MutS detection.

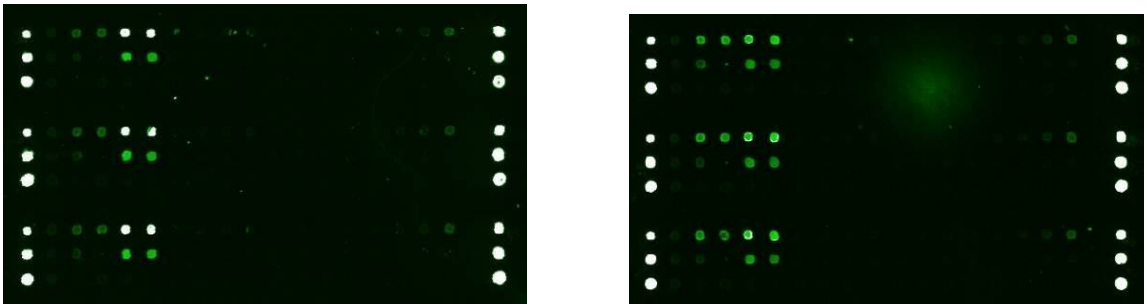
Furthermore it was expected, that after streptavidin/Cy3 and streptavidin/Cy5 detection, respectively, all positive controls should give positive signals and also the reverse-probes of Exon 11 should be positive. This is due to the binding of the biotinylated Exon 11 fw-primer in both oligo-mix samples, which means that these results are expected to be the same for all 18 hybridization experiments. A weak signal after Streptavidin/Cy3 and Streptavidin/Cy5 detection, respectively, could also be obtained with both Exon11-forward probes, due to sequence similarities and thus binding of the biotinylated forward-oligo to the probes.

Table 3.4 *Expected results of wildtype-DNA-hybridization. Green: expected positive signal after MutS-detection, red: expected positive signal after streptavidin/Cy3 and streptavidin/Cy5 detection, respectively. (fw = forward, re = reverse, wt = wildtype, poly = polymorph, hom = homozygote, het = heterozygote).*

			Exon 11 probes				**Exon 13 probes**				**Exon 14 probes**				**Exon 15 probes**					
50-mer probes	pc	nc	fw-wt	fw-poly	re-wt	re poly	fw-wt	fw-poly	re-wt	re-poly	fw-wt	fw-poly	re-wt	re-poly	fw-wt	fw-poly	re-wt	re-poly	nc	pc
30-mer probes	pc	nc	fw-wt	fw-poly	re-wt	re poly	fw-wt	fw-poly	re-wt	re-poly	fw-wt	fw-poly	re-wt	re-poly	fw-wt	fw-poly	re-wt	re-poly	nc	pc
PCR products	pc	nc	hom	hom	het	-	hom	hom	het	-	hom	hom	het	-	hom	hom	het	-	nc	pc

Results – Protocol 1

This protocol was performed to get a very first impression about how the system was working at all. Five different hybridization buffers were tested for their usability on the array and oligo-mix I was hybridized. The different hybridization buffers can be split in two groups concerning the outcomes. DYN and TBS-T led to stronger signals than the rest (Figure 3.8). The obtained signals for the positive controls were as expected and also the background signal was good after streptavidin/Cy3 detection.



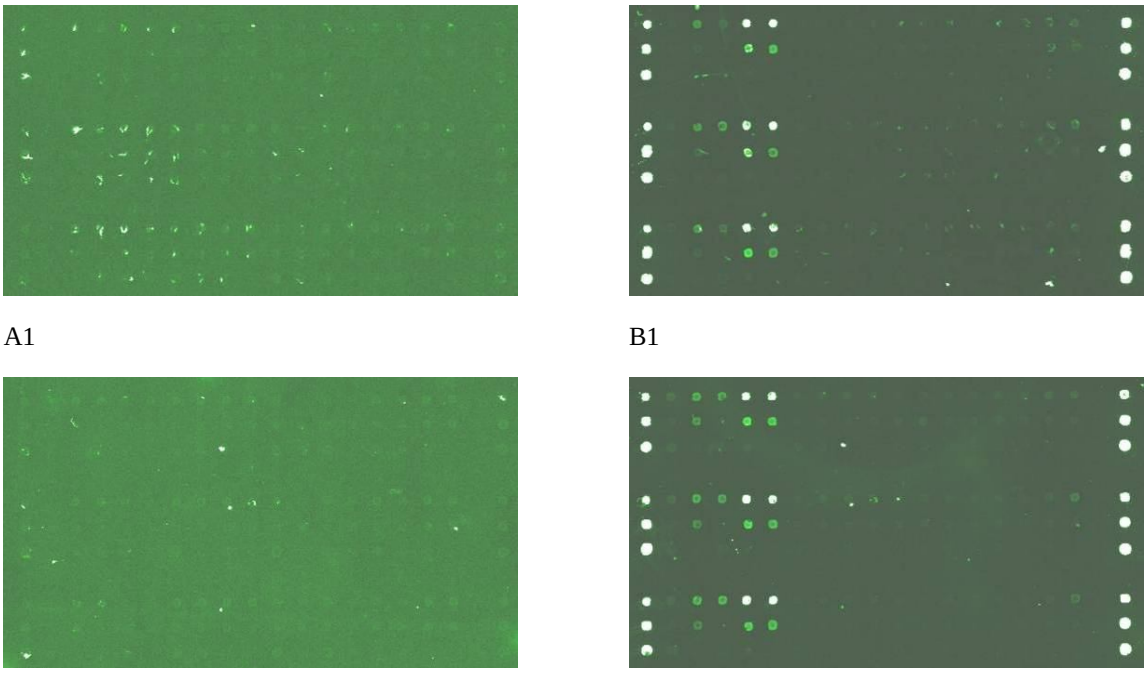
A B

Figure 3.8 *Results obtained with protocol 1. DNA-arrays are shown after Streptavidin/Cy3 detection, A: hybridization buffer: 1x DYN (result comparable to experiment with TBS-T hybridization buffer), B: hybridization buffer: PBS-T (results comparable to experiments with Na-phosphate- and DIGeasy hybridization buffer).*

Results – Protocol 2

This experiment was the same as experiment one, but this time GFP-MutS was applied undiluted on the chip. The results of this experiment were quite dissapointing. No specific GFP-MutS binding to the probes was noticable. The most promising picture in Figure 3.9 out of the bunch was maybe the one, when DIGeasy was used as the buffer for hybridization. The spots, though, showed not the common round form but a more lacerated structure.

The rest of the slides showed no signals at all, as Figure 3.9 A2 represents. Furthermore the background signal was worse than in protocol 1. It has to be mentioned too, that for obtaining the GFP-MutS slide results the scanner was set to 1000 PMT, whereas the signals after Streptavidin/Cy3 staining were obtained with only 700 PMT. This means, that the obtained signal with GFP-MutS was significantly weaker than Streptavidin/Cy3 signals.



A1 B1

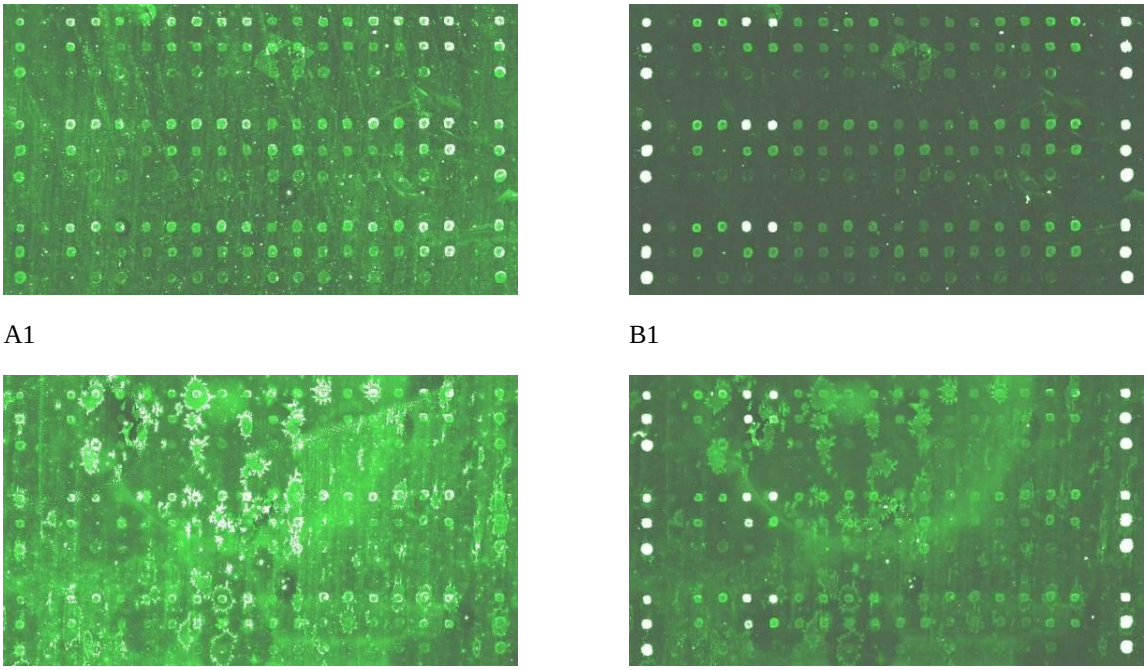
A2 B2

Figure 3.9 *A1 and A2 (after GFP-MutS detection) should lead to signals as shown in the table with the expected results (Table 3.3) in green, which is not the case. A2 and B2 (after streptavidin/Cy3 detection) show the expected signals according to Table 3.3 (red signals). A1, B1: DIGeasy hybridization buffer was used, A2, B2: TBS-T hybridization buffer was used (results are comparable to all other hybridization buffers tested).*

Results – Protocol 3

In this protcol the utilized protein was not GFP-MutS but the Cy3 labelled MutS sample. The protein was applied undiluted to the array. The first thing to be mentioned is, that the

background signal of the slides was very strong and irregular. After the washing step and subsequent streptavidin/Cy3 detection the intense background signal was reduced, but results were still not satisfying. Figure 3.10 A1 showed structures like ice cristals on the surface, maybe due to precipitate-formation upon hybridization at 4°C. The best and worst results of this experiment are illustrated in Figure 3.10 and Figure 3.11 gives an impression about signal intensities. Probes 2, 4, 6, 8, 10, 12, 14 and 16 should have shown much stronger signals than the rest of the probes.



A1 B1

A2 B2

Figure 3.10 *Results obtained after detection with MutS/Cy3. Usage of TBS-T hybridization buffer led to the best results (A1, B1). DIGeasy hybridization (A2, B2) led to cristal strucutre on the chip and very strong background signal (A1, A2: after MutS/Cy3 detection, B1, B2: after Streptavidin/Cy3 detection).*

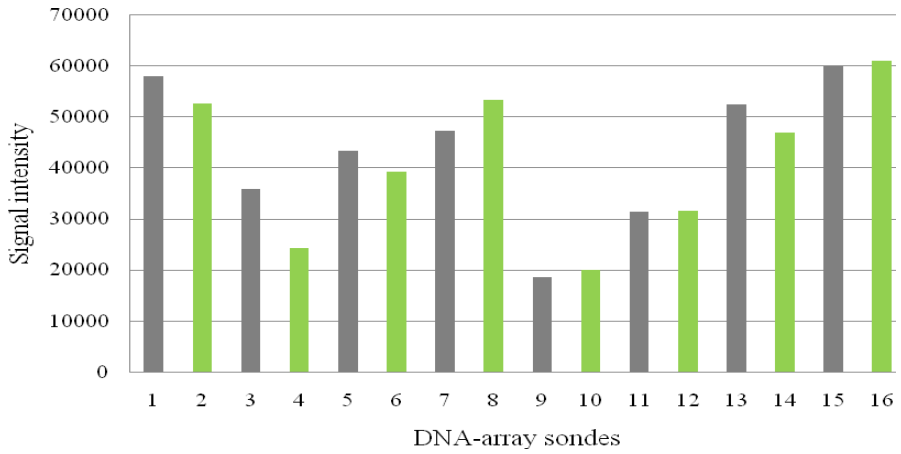
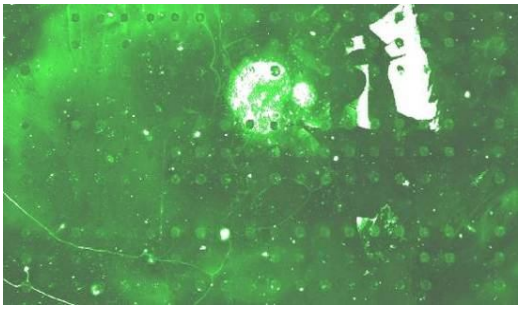


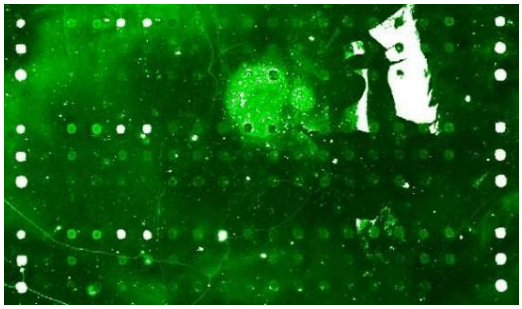
Figure 3.11 *Signal intensities obtained after MutS detection. The wt-mix hybridization should lead to increased signals of all polymorphic probes (2, 4, 6, 8, 10, 12, 14 and 16), which is clearly not the case. Results are mean values of triplicates of the 50-mer probe signals.*

Results – Protocol 4

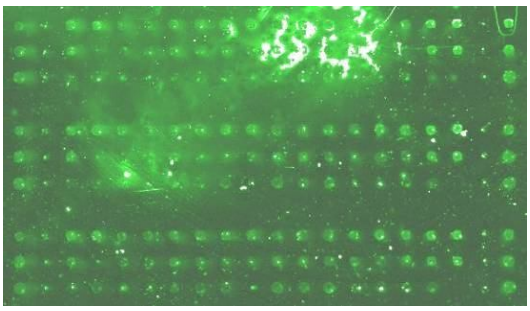
Three different types of experiments, differing in the number of washing steps after detection with the protein, were performed to investigate the strong background signal from the former protocol in more detail. Unfortunately this experiment did not reveal the desired results and it can be stated that the background signals remained very strong and irregular independently of the protocol type (Figure 3.12). No difference was seen between DYN-hybridized and the TBS-T hybridized chips. No expected signals after MutS/Cy3 staining were detectable at all; signals after Streptavidin/Cy3 staining were OK.



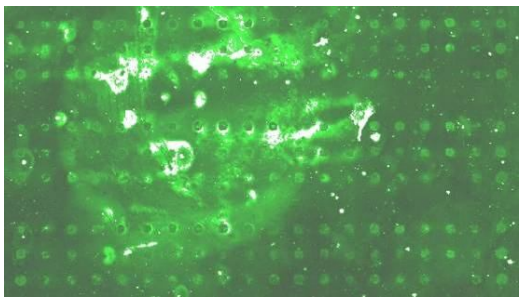
A1



B1



A2



A3

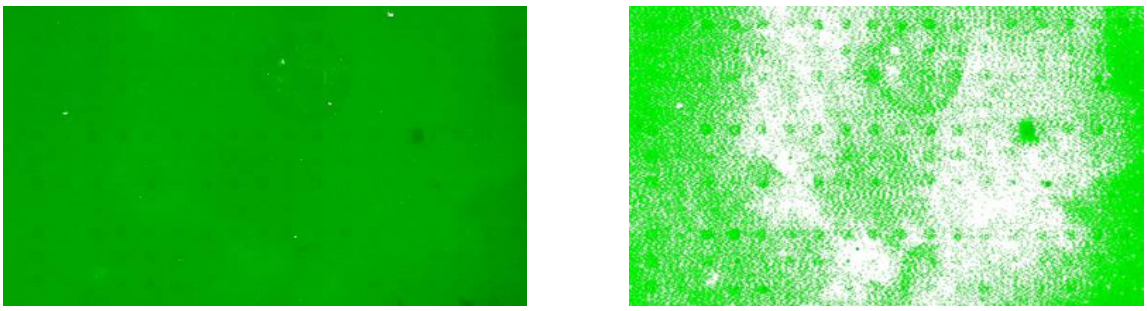
Figure 3.12 *Results of protocol 4 are shown on the example of TBS-T hybridized chips. A1, B1: protocol A; A2: protocol B; A3: protocol C. A1, A2, A3: after MutS/Cy3 detection, B1: after Streptavidin/Cy3 detection.*

Results – Protocol 5

In this protocol the blocking step with BSA prior to MutS detection was left out for some of the slides. The background signal was very strong for all slides even after Streptavidin/Cy3 staining, which has definitely been much better before. The chip, that was hybridized with TBS-T and obtained the BSA blocking, led to the cristal structures on the surface as it happened before in protocol 3 with the DIGeasy hybridized chip (Figure 3.10 A2).

Results – Protocol 6

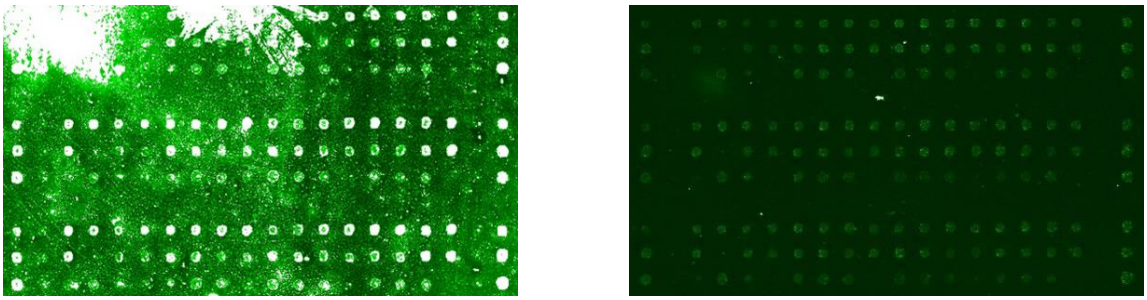
This was the first experiment in which also aldehyd slides were utilized. Unfortunaltely these slides showed no signals at all. Even the spots themselves were not seeable on the chips after MutS/Cy3 detection. Figure 3.13 shows one example obtained with the aldehyde surface that is comparable to other aldehyde arrays.



A B

Figure 3.13 *Aldehyde slide treated with BSA blocking and hybridized at room temperature, A: afterMutS/Cy3 detection, B: after Streptavidin/Cy3 detection.*

SU-8 slides looked all very similar but compared to the aldehyde slides they showed much better signals. Two different protein types were applied and the protein detection step was performed at room temperature rathern than at 4°C. The difference between the two proteins, MutS/Cy3 and GFP-MutS, was significant (Figure 3.14). Concerning the background, GFP-MutS slides were better, but regarding the signal intensity MutS/Cy3 samples showed better results.

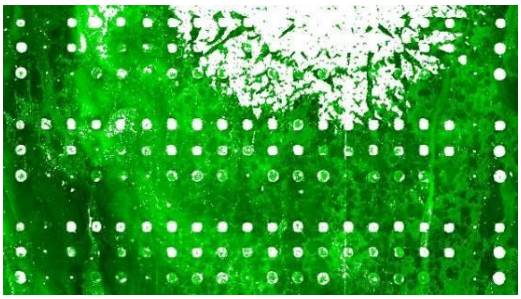


A1 B

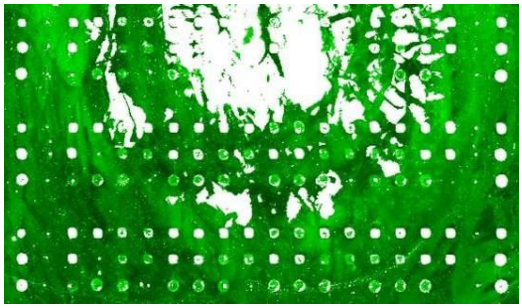
Figure 3.14 *Results of SU-8-slides after BSA blocking and hybridization at room temperature. A: MutS/Cy3 detection, B: GFP-MutS detection.*

Results – Protocol 7

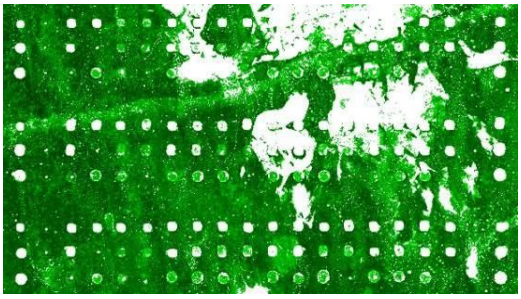
Cyanoborohydride blocking was introduced in this protocol. Also the hybridization buffer and temperature were modified and different blocking procedures prior to protein detection were performed. Once again all aldehyd slides showed very bad results. After streptavidin/Cy3 detection the background was blurred and the worst obtained result so far. Unfortunately also the different blocking methods did not lead to different results. For the SU-8 slides there was no difference seeable between non-blocked slides, slides blocked with blocking solution from protocol 1 and slides blocked with milk powder in PBS (Figure 3.15). Also the two different initial blocking methods did not lead to different results. Furthermore it was still not possible to achieve a good background signal but positive detection with streptavidin/Cy3 confirmed that the arrays were ok.



A



B

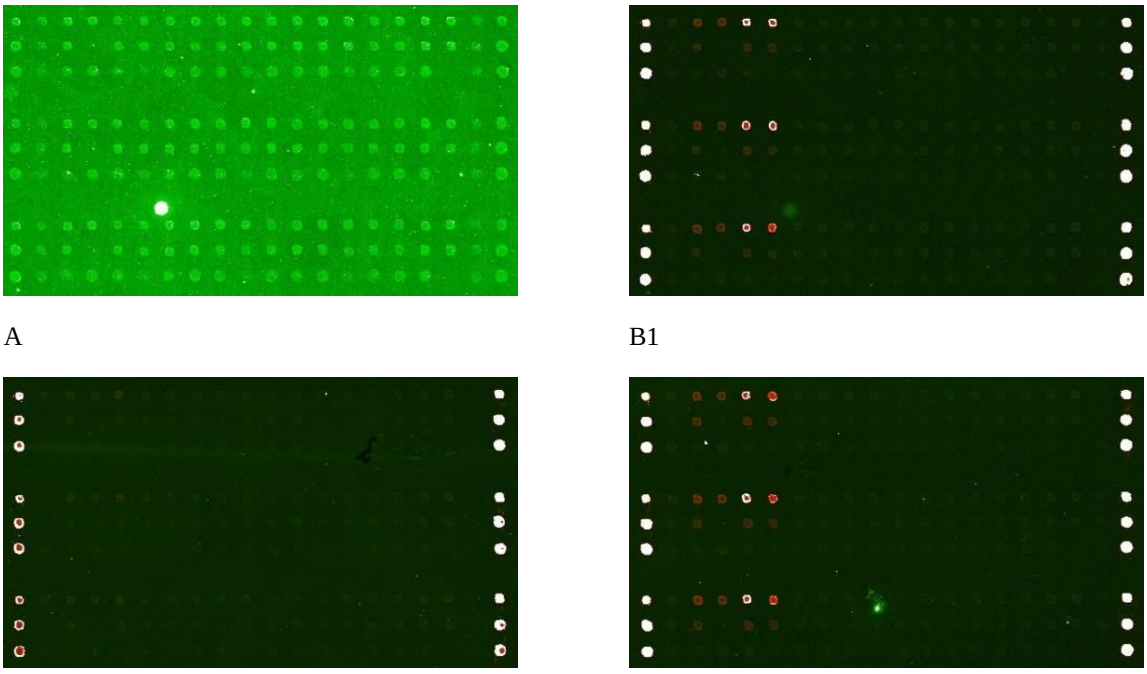


C

Figure 3.15 *A: no blocking step prior to protein detection, B: blocked with blocking solution prior to protein detection, C: blocked with milk powder in PBS prior to protein detection.*

Results – Protocol 8

Different additives in the protein samples were tested in this protocol and the signal intensities between slides with and slides without hybridization were tested. In this protocol Streptavidin/Cy5 was used for detection of the positive controls. The detection with GFP-MutS did not work at all. There was no difference of signals when no DNA was hybridized and also if the detection step was performed without protein. After Streptavidin/Cy5 detection the slides showed the expected signals, so it can be excluded that the slides were not working. For MutS/Cy3 protein detection the results were not much better. Little differences were noticable between the three slides. The best result, however, was obtained when both additives – Triton X-100 and Tween-20 – were present in the protein sample (Figure 3.16).



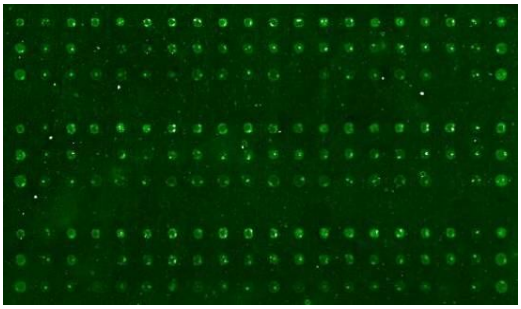
A B1

B2 B3

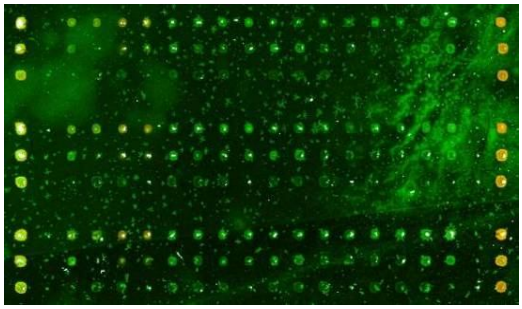
Figure 3.16 *A: Hhybridization and detection positive slide after MutS staining. B: slides after Streptavidin/Cy5 detection. B1: hybridization and detection positive, B2: hybridization negative, detection positive, B3: hybridization positive, no detection with protein.*

Results – Protocol 9

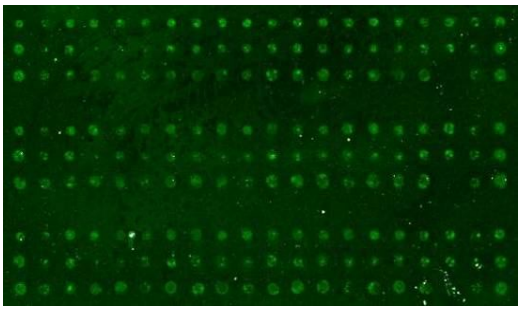
The new initial blocking method (ethanolamine blocking) showed no improvement concerning the specificity and background signal, compared to the DIGeasy blocking method from before. Worst results were obtained after hybridization at 42°C without additives in the hybridization buffer. It was also found out, that, at least in a concentration of 2 mM, ATP seems to have no impact on protein binding capacities. The signal after Streptavidin/Cy5 detection, which was quite good before, was generally very weak in this protocol. The best backgrounds were obtained with DIGeasy blocked slides hybridized with TBS-T + 25% formalin at 42°C and TBS-T at 52°C as well as ethanolamine-blocked slides, which were hybridized with TBS-T + 25% formalin at 42°C (Figure 3.17).



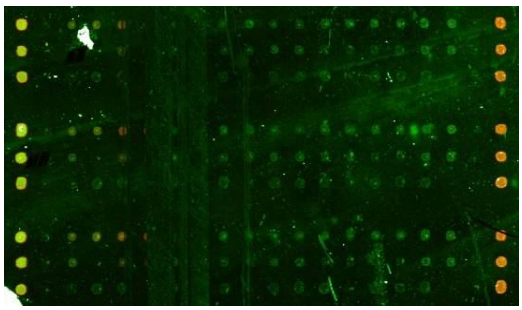
A1



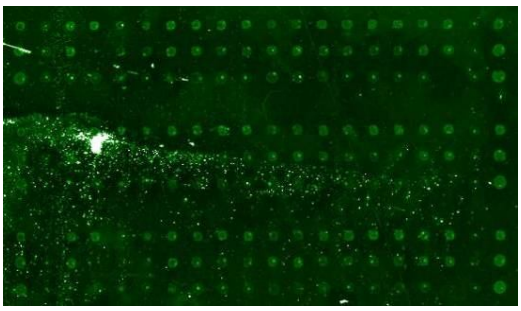
B1



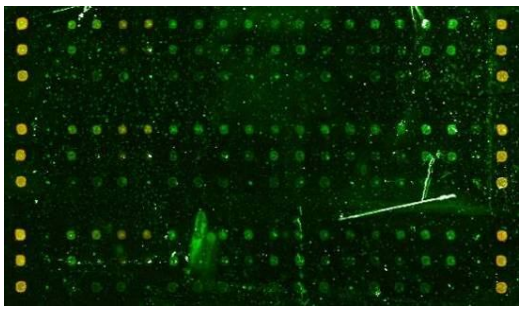
A2



B2



A3

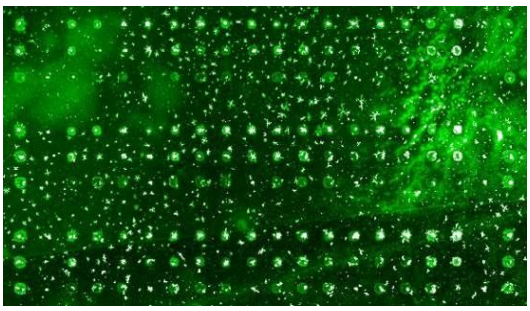


B3

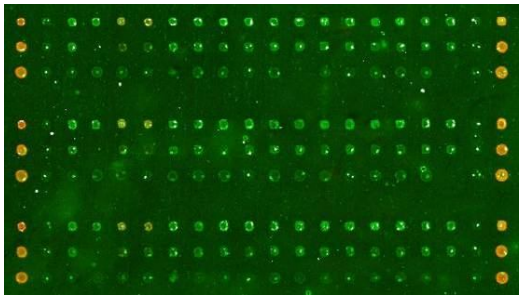
Figure 3.17 *A1, B1: DIGeasy blocked, hybridization with TBS-T + 25% formalin at 42°; A2, B2: DIGeasy blocked, hybridization with TBS-T at 52°C; A3, B3: ethanolamine blocked, hybridization with TBS-T + 25% formalin at 42°C; A: after MutS/Cy3 detection, B: after Streptavidin/Cy5 detection.*

Results – Protocol 10

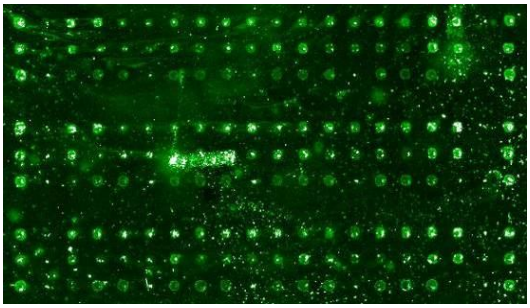
In this protocol different initial blocking methods were tested. Also the difference of hybridization/detection negative and hybridization/detection positive slides was investigated again and BSA was added to the protein samples for detection. DIGeasy blocking resulted in a little bit better background than the cyanoborohydride blocked slides– still it was not very good. Slides showed quite strong signals, even if no protein was incubated on them. The difference between the three types of slides was very weak (Figure 3.18).



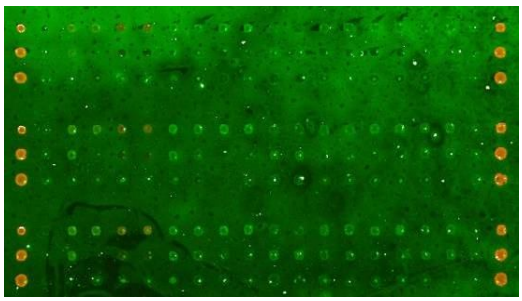
A1



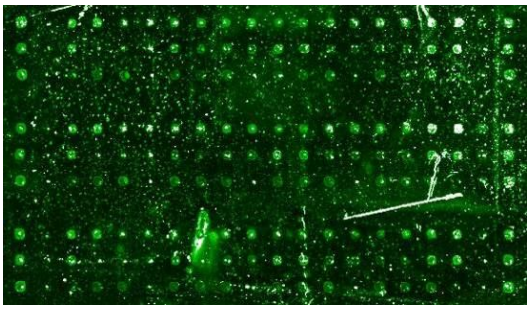
B1



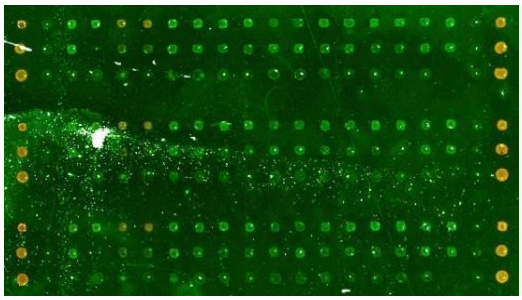
A2



B2



A3

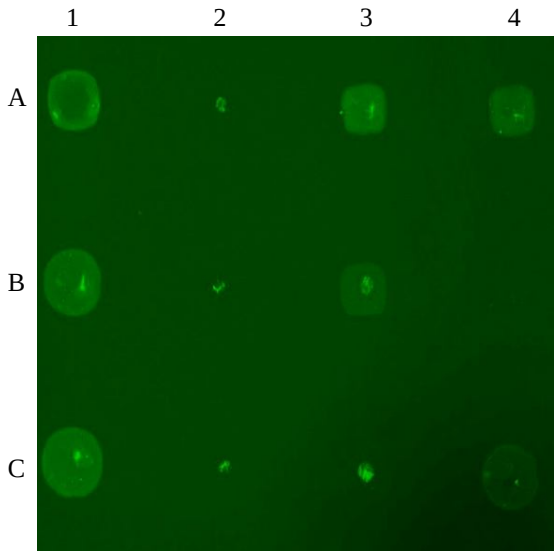


B3

Figure 3.18 *A1; B1: Hyb+/Det+, A2, B2: Hyb-/Det+, A3, B3: Hyb+/Det-; A: after MutS/Cy3 detection, B: after Streptavidin/Cy5 detection.*

Results – Protocol 11

The fluorescent microscope image (Figure 3.19) shows that the signal is much better for the 50-mer probes than for the 30-mer probes. The signal A4 should be more intense that A3, because the oligo mix 1 (containing all wt-probes) was used.

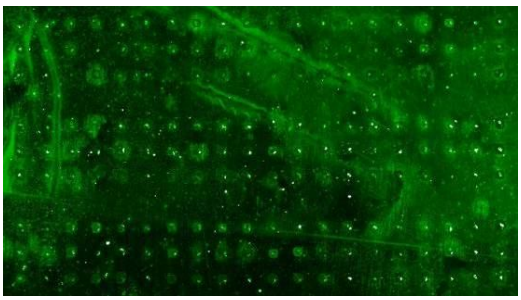


A1 = pc	A3 = 50-mer 691-fw-wt
B1 = pc	B3 = 30-mer 691-fw-wt
C1 = pc	C3 = 11-1493
A2 = nc	A4 = 50-mer 691-fw-poly
B2 = nc	B4 = 30-mer 691-fw-poly
C2 = nc	C4 = 11-1517

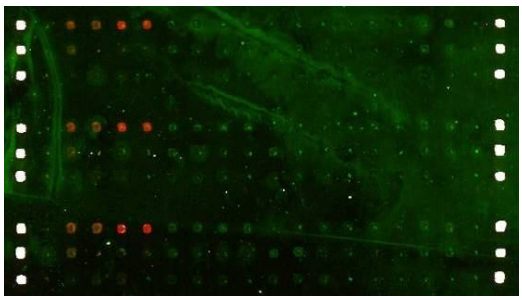
Figure 3.19 *Fluorescent microscope results of the hybridization with TBS-T + 5 mM ATP. Probe 4B is negative due to a spot error. (pc= positive control, nc = negative control)*

Results – Protocol 12

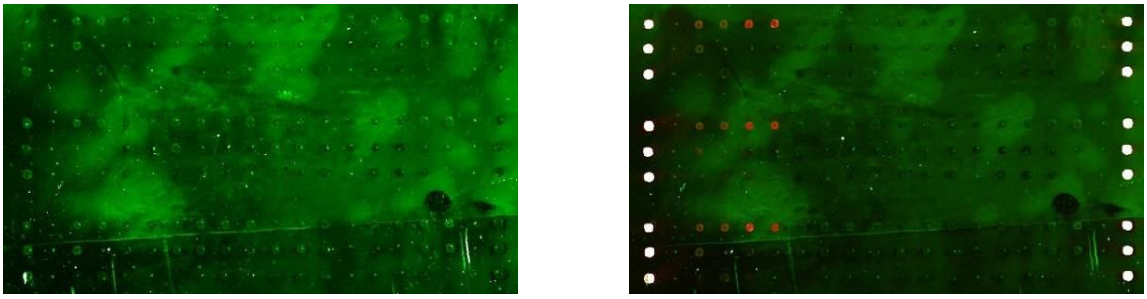
In this protocol eight different chip pre-treatments were tested. For SU-8-slides it can be stated that the different chip pre-treatments did show different results – but none of the methods led to a really good outcome. The treatment with NaOH generally seems to be too harsh, most of the spots were blurred after this pre-treatment. Subsequently also the streptavidin/Cy5 detection led to very weak results, which was never the case before. In general the background of the slides was very strong and irregular. Also the pre-treatment with water did not show better results.



A1



B1



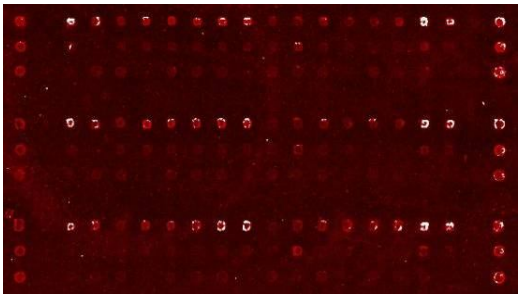
A2 B2

Figure 3.20 *A1, B1: H_2O, 70°C, A2, B2: NaOH room temperature, A: after MutS/Cy3 detection, B: after Streptavidin/Cy5 detection*

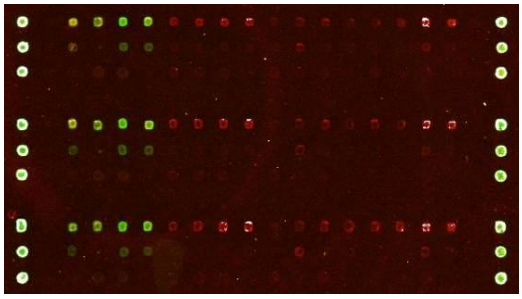
All aldehyde slides treated with NaOH showed no signals after the scanner. No spots could be seen on the slides, except for the slide treated with 70°C H_2O. Still, after Streptavidin/Cy5 detection signal was obtained with the slides treated at room temperature. Surprisingly, though, the only slide that showed signal after the 70°C pre-treatment was the one treated with 30 mM NaOH + 70% formaldehyde (Figure 3.20).

Results – Protocol 13

The assumed worse slides from protocol 12 were used here, but the final results were not worse than before. This means, that the obvious state of the slide, visible with the naked eye, has no influence on the actual chip quality. Again the different chip pre-treatments did show different results. The best obtained slides were the ones treated with H_2O at 70°C (Figure 3.21).



A

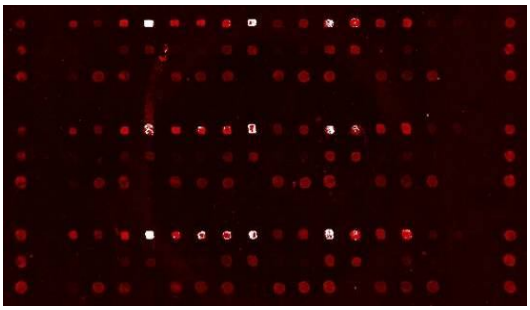


B

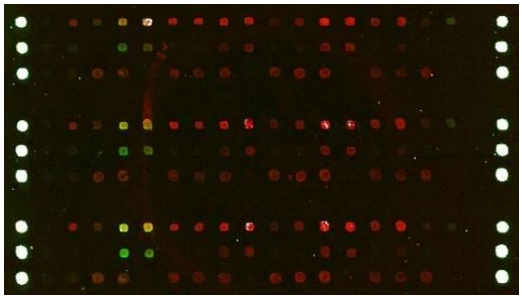
Figure 3.21 *Results after pre-treatment with H_2O at 70°C, , A: after MutS/Cy5 detection, B: after Streptavidin/Cy3 detection.*

Results – Protocol 14

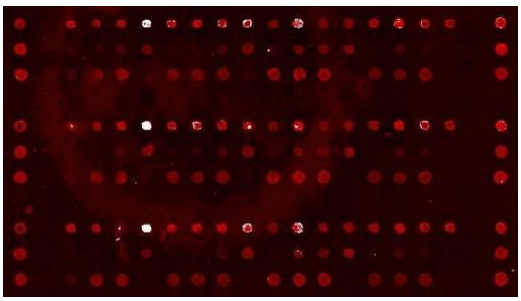
Pre-treatment with H_2O at 100°C was tried out in combination with all three types of array blocking here. Also some slides were hybridized without DNA in the buffer to see a difference between hybridization positive and hybridization negative slides but unfortunately there was not much difference seeable between the slides. The best results, however, were once more obtained with slides pre-treated with H_2O at 70°C (Figure 3.22).

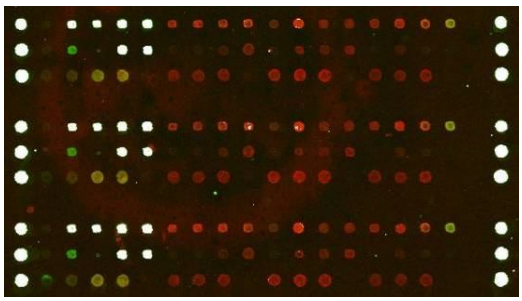


A1



B1

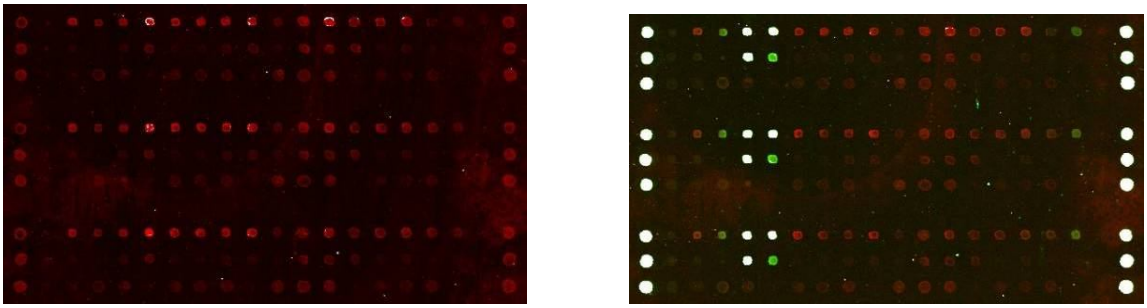




A2 B2

Figure 3.22 *Pre-treatment with H_2O at 70°C. A1, B1: Hybridization negative, A2, B2: Hybridization positive.*

Also the DIGeasy-blocked slides, that did not receive hybridization showed relatively strong signal after MutS/Cy5 detection (Figure 3.23).

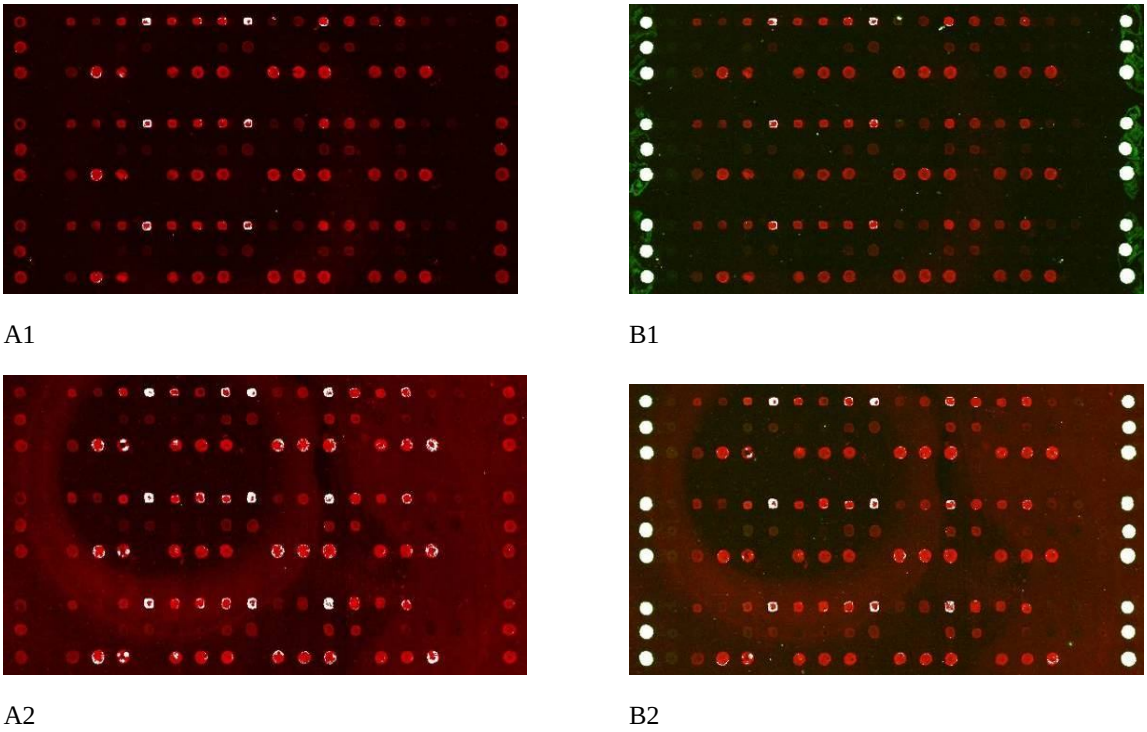


A1 A2

Figure 3.23 *Results of DIGeasy blocked slideswithout pre-treatment, that are hybridization negative. A1:after Mut/Cy5 detection, A2: after Streptavidin/Cy3 detection.*

Results – Protocol 15

Tris- and DIGeasy blocking were used here and the chip pre-treatment with H_2O at 100°C was investigated for it´s outcomes. This experiments revealed a much better background with the Tris-blocked slides than with DIGeasy-blocked arrays. Signal intensities, though, were better on the DIGeasy-blocked slides. The pre-treatment with H_2O (100°C) and subsequent treatment with 10 mM NaOH seemed to be very promising because background signal was reduced here compared to before and also the signals seemed to be more like expected (Figure 3.24).



A1 B1

A2 B2

Figure 3.24 *A1, B1: Tris blocked, A2, B2: DIGeasy blocked, pre-treatment all: 100°C H_2O - 10mM NaOH*

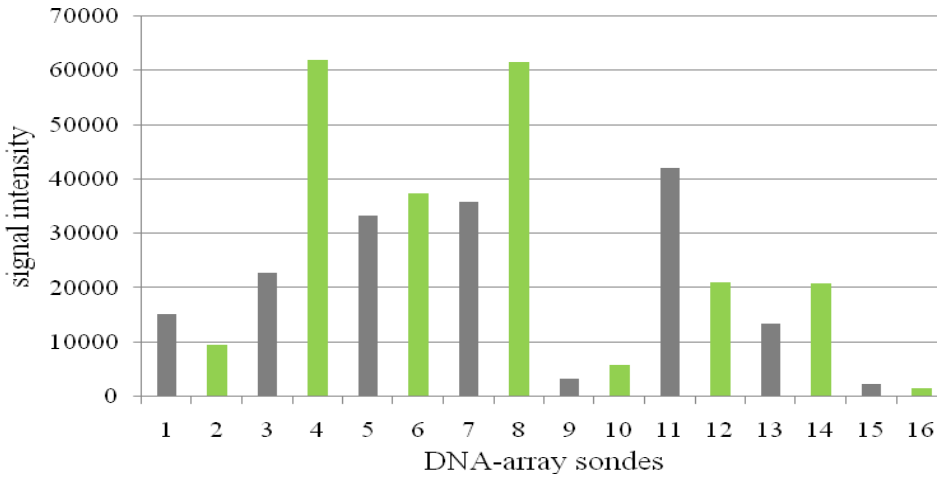


Figure 3.25 *Signal intensities of Tris-blocked slide that obtained pre-treatment: 100°C H_2O - 10 mM NaOH.*

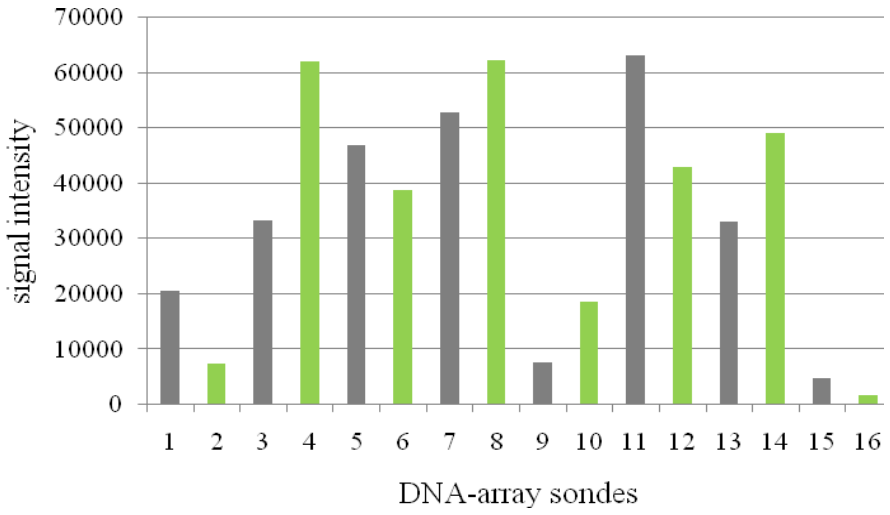
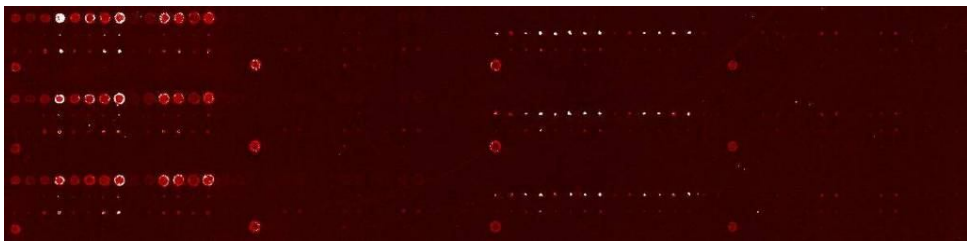


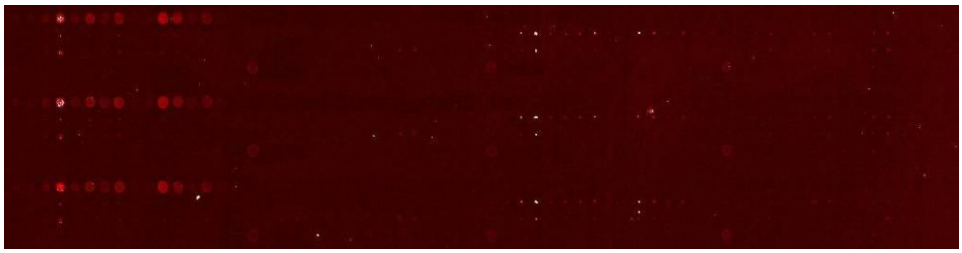
Figure 3.26 *Signal intensities of DIGeasy-blocked slide that obtained pre-treatment with 100°C H_2O → 10 mM NaOH.*

Results – Protocol 16

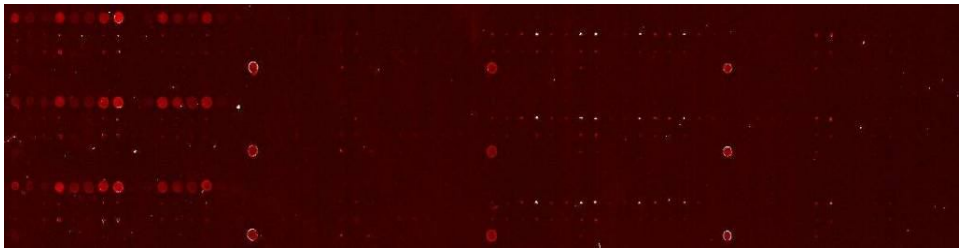
The spot-buffer test slides were applied here for the first time and also the effect of different concentrations of ATP and $MgCl_2$ in the protein buffer were investigated The first and very obvious results is that the 5 mM ATP in the protein sample did not lead to better results. Also the different concentrations of $MgCl_2$ in the protein sample did not have any effect on signal intensity or specificity. For the new slides it is to note, that spot buffer SSC + Betain was the only buffer, in which also the 50-mer probes did not show any signals. All other new buffers showed little signal but eventually the so far used spot buffer (Sodium-phosphate buffer) was thougth to be best suited. In comparison to the early experiments the aldehyde-slides worked better, but the background was still quite strong and not usable at all. For SU-8 slides the strong-background was almost removed, but the protein specificity remained a problem. Results after Streptacidin/Cy3 detection confimed the usability of the slides (Figure 3.27).



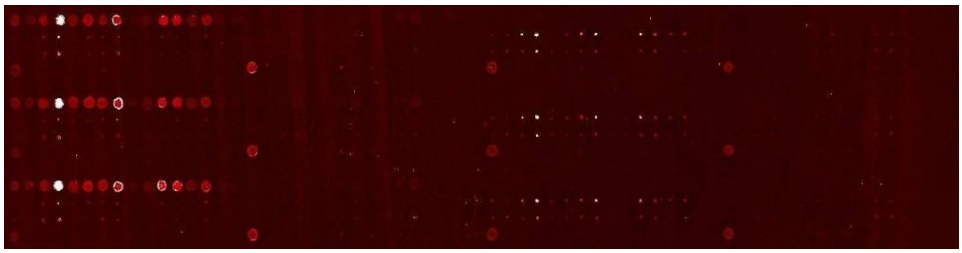
A



B



C

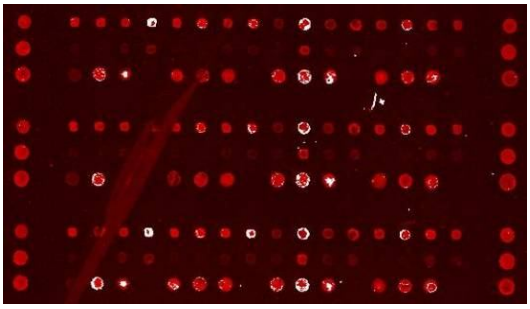


D

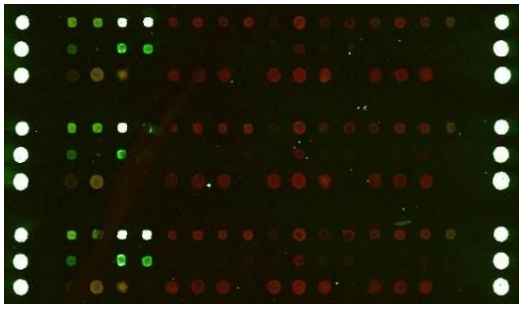
Figure 3.27 *SU-8 slidesA: 0 mM $MgCl_2$, 0 mM ATP, B: 0 mM $MgCl_2$, 5 mM ATP, C: 5 mM $MgCl_2$, 0 mM ATP, D: 5 mM $MgCl_2$, 5 mM ATP.*

Results – Protocol 17

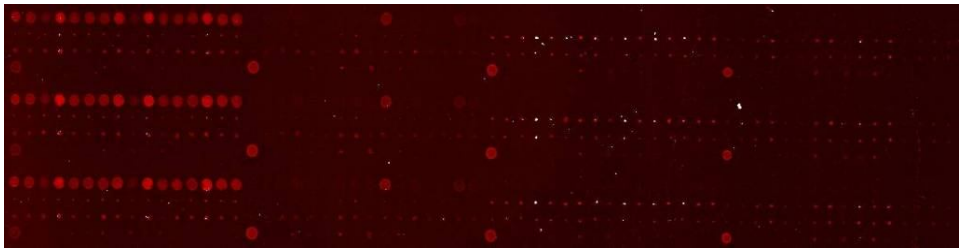
Two hybridization temperatures (42°C and 52°C, respectively) were tested. Also the use of a nuclease digestion after hybridization was investigated and again it was tried to increase the proteins mismatch binding specificity by adding $MgCl_2$ to the detection buffer. It is interesting, that on array 1 all 30-mer probe signals (except probe 10) were negative but on array 2 and by using the same spot buffer 30-mer probe signals were similar to 50-mer probe signals. Generally the signal was stronger without the nuclease step, but not specific. The background signal was OK. ExpHyb showed stronger signals than TBS-T, but no significant difference was seeable between 42°C and 52°C hybridization temperature. The strongest signals were obtained with ExpHyb at 52°C and nuclease negative conditions, the weakest signal with TBST at 52°C and nuclease positive conditions (Figure 3.28).



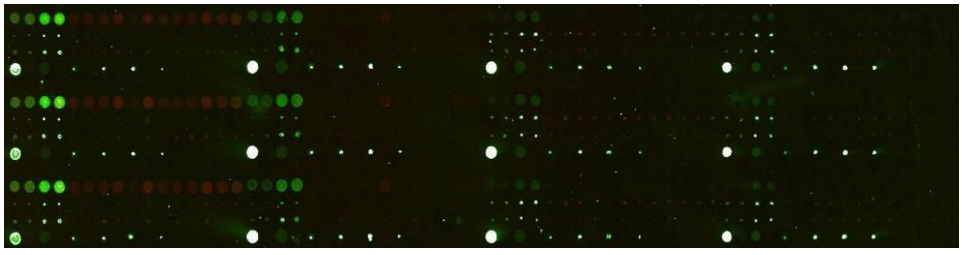
A1



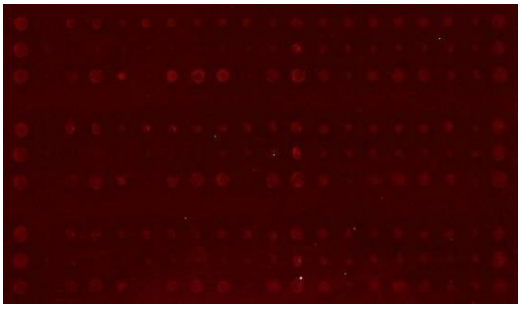
B1



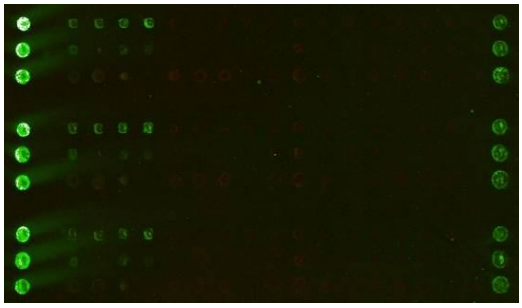
A2



B2



A3

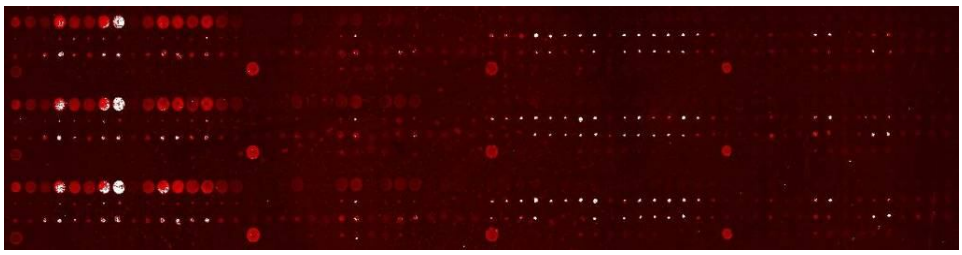


B3

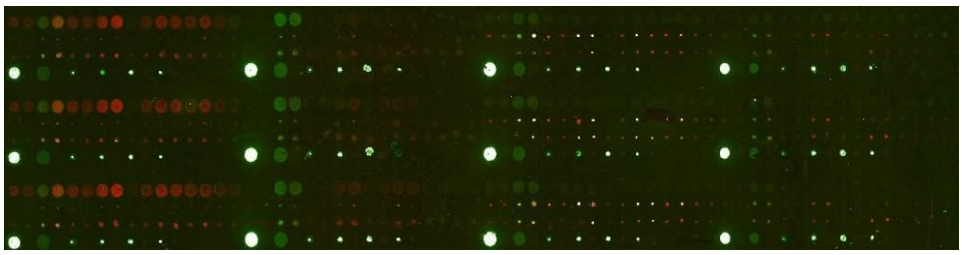
Figure 3.28 *A1, B1, A2, B2: Hybridization with ExpHyb at 52°C and without nuclease led to best results. A3, B3: Hybridization in TBS-T at 52°C with nuclease resulted in no signals. A: After MutS/Cy5, B: after Streptavidin/Cy3.*

Results – Protocol 18

In this final experiment Tris- and DIGeasy blocking were used. Again the chip-pretreatment in 100°C H_2O and subsequent dipping in ice-cold NaOH was applied. Hybridiaztion was performed at 42°C overnight with and without DNA. Only SU8 slides led to positive results, the aldehyde surface was again very bad. The weakest background signal was obtained with the Tris-blocking and hybridization in PBS-T. The overnight blocking resulted in complete disappearance of spots. The best obtained results are shown below in Figure 3.29.



A



B

Figure 3.29 *A1: Best results were obtained with the Tris-blocked array that was hybridized at 42°C in PBS-T, detection with Muts/Cy5+$MgCl_2$. A: after MutS/Cy5 detection, B: after Streptavidin/Cy3 detection.*

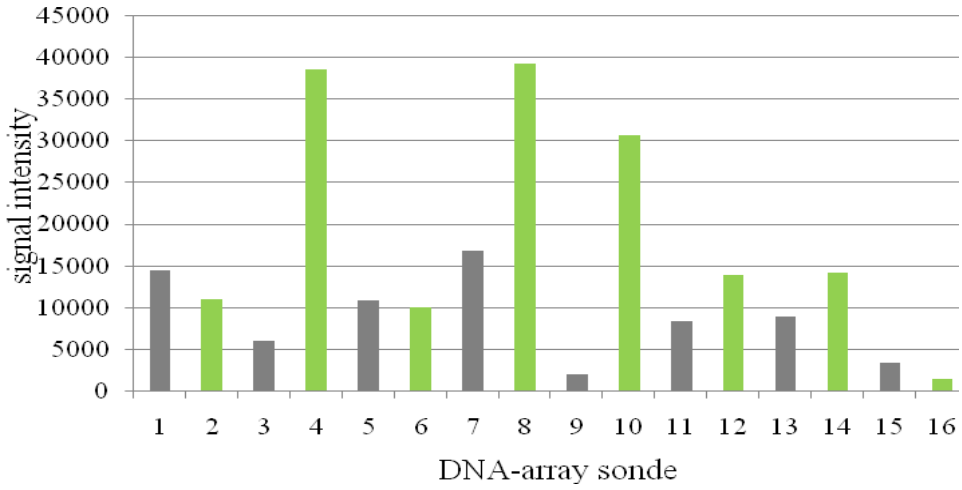


Figure 3.30 *Signal intensities after MutS/Cy5 detection. 5 of 8 probes pairs (wt-polymorph) show the expected signal ratios.*

CHAPTER 4

DISCUSSION

The goal of this thesis project was the development of a new DNA-array based system for detection of mutations and single nucleotide polymorphisms. The innovative technology of mismatch detection with the MutS-protein was suggested as a very promising tool for this kind of experiments (e.g. Bi *et al*, 2005; Stanislawska-Sachadyn *et al*, 2005).

MutS is raising very big expectations in mutation analysis. Already some companies included this protein into their product line and research. For example Nanogen Recognomics GmbH worked a lot with MutS and its application in a microelectronic protein-DNA chip format. Nanogen Recognomics GmbH developed an electronically controlled DNA-array for detection of point mutations in the p53 gene. The detection of SNPs and other DNA alteration was performed with fluorescently labelled MutS. Results were very promising and it was possible to confirm the results with direct sequencing

experiments. This led to the assumption that chip-technology together with MutS mismatch detection might be a helpful tool in genomics, especially for mutation testing (Behrensdorf *et at*, 2002).

Wagner *et al* (1995) performed their work with the MutS protein also in an industrial aspect for a company called GeneCheck, Inc.. Also in these experiments the obtained results after MutS protein application on chip-based systems were very promising. GeneCheck, Inc. offered a MutS based detection system before and is currently still selling the *E.coli* MutS protein. However, no mutation analysis method on MutS detection basis is commercially available at the moment (Stanislawska-Sachadyn & Sachadyn, 2005).

Those positive approaches from earlier experiments were encouraging to perform the at-hand thesis for evaluation of MutS based mutation detection on a microarray with immobilized DNA-probes and using MutS protein for mismatch detection

This thesis did not only cover the application of the mismatch binding protein on the chip and investigations on mismatch binding properties, but this work also incorporated all preparation steps to reach this aim – starting with protein production.

Some experimental problems had to be overcome concerning the bacterial culture. MutS growth was problematic in the very beginning, because the transformed cells did not continue to grow after the inoculation of the 2 ml culture into the bigger volume. Usually 37°C are the commonly used cultivation temperature for this type of cells. This has the advantage of short growing periods but on the other hand this temperature could possibly affect the protein´s activity in a negative way (Bi *et al*, 2005). Changing the incubation temperature from 37°C to 30°C for GFP-MutS strains, as suggested by Stanislawska-Sachadyn *et al* (2006) and Bi *et al* (2005) had two positive effects. First, it was possible to get the cell culture growing and second protein activity was increased. The positive transformation of the bacteria with the green fluorescent protein was checkable by the

intensive green colour of the obtained protein sample. However, induction of the culture had to be performed at lower OD values than 0.5 in some cases.

The next step after bacterial culture was the extraction of the protein from the cells. Three different mechanical disruption methods were tested and all worked quite well. Only 1.39 mg protein were extracted after sonication and it was possible to extract 2.37 mg of FrenchPress disrupted cells. This means, that FrenchPress was the most effective method – most protein was gained out of the smaller cell pellets. Subsequent protein purification worked well, although it has to be mentioned that a lot of protein was lost during purification. It was thought that about one fifth of the overall protein amount in the crude lysate was the one protein to be purified, but the actual rate of purified MutS protein was much lower (Figure 3.1). Some samples were furthermore still contaminated with other components after purification what was seeable on the SDS-PAGE (Figure 3.2).

Concerning the protein´s specificity for SNP binding on the chip the outcomes were very different depending on the different protein types. Eventually it was found out that the best results were obtained with the purchased MutS/Cy5 sample. Strangely enough, the GFP-MutS protein was not working on the array, because this protein type was the only one that could be successfully applied in the gel-shift assay.

Gel-shift assays generally led to very dissapointing results. Usually the mismatch binding protein should bind to the heteroduplex DNA strands and build a complex with it. Here, though, always two distinct band were seeable on the gel of samples containing mismatch DNA and MutS. It is very hard to say why this was so, because earlier gel shift assays performed by e.g. Lishanski *et al* (1994) and Stanislawska-Sachadyn *et al* (2005) were successful and confirmed the mismatch binding quality of the MutS protein in gel-shift assays. According to Stanislawska-Sachadyn (2005) the proportion between DNA and protein can vary a lot, but best results are obtained with a ratio of 100 ng DNA:2 µg protein. Similar values were also applied in the experiment in this project and one positive gel-shift assay result was obtained (Figure 3.3). The experiment included the GFP-MutS protein bound to a G:T mismatch. However, the result was not reproducable, neither with

this protein type, nor with another one. One reason for this could be the fact, that the complex seems to be quite unstable and the signal on the gel might even be lost if the running times on the gel are too long (Lishanski *et al*, 1994).

DNA-array spotting worked very well with 50-mer / 30-mer and PCR product probes. It´s noteworthy, that the different spot buffers had a huge influence on spot quality. Results of the spotcheck (Figure 3.7, C) illustrate, that the only suitable spot buffer was actually the sodium-phosphate buffer.

After successful protein purification and chip-production the next step were hybridization experiments. Regarding the coating type, it was found out very quickly, that the epoxy-surface could be determined as the well-suited one and aldehyde coating can be excluded for further approaches. Aldehyde slides did not show any usable signal throughout all experiments.

Regarding the probes it was clear, that the 50-mer probes showed much stronger signal than the 30-mer probes. The PCR products hardly showed any results and it was not possible to make out a difference between homozygous and heterozygous probes.

Positive controls worked on all slides and thus confirmed that the arrays worked well. Additionally to prostive controls, also all “50-mer Exon 11 probes” and “30-mer Exon 11-reverse probes” showed positive signals after Streptavidin detection. This is, because the reverse probes of Exon 11 were also bound by the biotinylated oligo in the hybridization mix. The forward probes should actually be negative, but maybe due to sequence similarities they are bound as well and lead to a signal after Streptavidin detection in some cases. All negative controls led to negative signals for the entire bunch of slides throughout all 18 experiments.

The most successful initial chip blocking was the Tris blocking method. DIGeasy blocking, the cyanoborohydride method and also ethanoleamine blocking did not lead to much difference in the results. It cannot be known if the specificity of the hybridized DNA was influenced by the blocking type. The only noticable difference was the intensity of the

background signal. However, the protein type seemed to have more influence on background signal than the initial chip blocking.

Array pre-treatment was performed to optimize the target DNA hybridization step. For example denaturating conditions are thought to help against building of secondary structures of the array probes. Too harsh treatment, though, can have negative effects on the slides. Pre-treatment with 300 mM NaOH for instance led to removement of the array spots. Dipping of slides into boiling water for one minute and chilling the slides in isopropanole on the other hand led sometimes to destruction of the SU-8 chip surface – the slides became completely milky after this treatment. The pre-treatment that was most successful was dipping the slides into boiling water for one minute and subsequent chilling of arrays in ice-cold 10 mM NaOH for a few seconds.

No particular hybridization buffer could be determined to be for subsequent MutS-detection. The hybridization buffers that seem to be at least best suited for this purpose are TBS-T, DIGeasy and PBS-T.

Background signals were quite strong throughout initial experiments. In general the signal after streptavidin detection was weaker than after the detection step with the protein. Maybe unspecific protein binding to the slide surface and also to all probes, either without mismatch or to heteroduplexes, is responsible for this. The reduced background signal after Streptavidin detection could also be due to the additional washing steps that the arrays obtained during this process and more specific binding of the Streptavidin samples with the biotinylated probes.

Mung bean nuclease was applied in some of the later experiments for digestion of overhangig DNA single strands after target DNA hybridization. It was thought, that the specificity of the protein towards the mismatch could be increased when all single strand DNA is removed from the array, but in fact the experiments lacking the enzymatic digestion step led to better results than the experiments where the nuclease was applied. Maybe the mung bean nuclease affected also the array probes or the bound DNA to the probes and the signal was decreased because of this.

Another thing that can be investigated in more detail is the target DNA lenght. Bi *et al* (2003) applied DNA fragments from 30 to 621 bp successfully on their protein chip. Target DNA fragments in this project were oligos of ~50 bp lenghth. Although unlikely longer target DNA could lead to better and more specific signals in DNA-chip experiments, but this was not tested.

The influence of the blocking step prior to protein detection can not be confirmed nor rejected. Mostly the results were quite similar between experiments including blocking steps and experiments without blocking. The different kinds of blocking, i.e. blocking with BSA and blocking with milk powder in PBS, did not lead to different results and thus this protocol step was maintained in the hybridization process from experiment 9 on.

Subsequently the influence of adding BSA directly to the detection buffer was tested but also without success. Additional BSA in the protein hybridization buffer did not affect the binding specificity of the protein.

Also it was thought that adding ATP to the protein sample would increase the binding capacities and specificity of MutS. Firstly, because when ATP is present in the protein solution it completely inhibits unspecific binding of MutS to homoduplexes. And secondly it was found out, that the affinity of MutS for binding mismatched DNA is hightened in the presence of ATP (Lebbink *et al*, 2006) .The influence of added ATP in the detection step, though, seems to be not significant. Both ATP-concentrations that were tested (2 mM and 5 mM, respectively) had no effect on binding specificity.

Furthermore the positive effects of $MgCl_2$ in the protein hybridization buffer were not confirmed during the course of this thesis, although it has to be mentioned that $MgCl_2$ was present in the buffer when the results of experiment 18 were obtained. At least this additive has no negative influence on the signals.

In our setting GFP-MutS fluorescent signals were very low and the laser wavelenght of the microarray scanner was not suitable for GFP. Thus the purchased MutS/Cy5 sample was used and led to good results. What has big impact on mismatch binding is the protein

concentration. In experiment 18 the applied protein concentration on the array was 5 ng/µl. This value seems to be well suited for SNP detection on this array.

From the first to the final protocol it was possible to achieve a very good optimization of the protocol. The proteins specificity was significantly increased in the last performed experiment compared to experiments that were performed in the beginning of this work. The following Table (4.1) shows the improvements that were achieved with respect to mismatch binding specificity.

Table 4.1 *Overview of improved signal accuracy. Polymorphic probes should lead to stronger signals than wildtype probes. In Experiment 3 only 2 polymorphic probes showed stronger signals than the wildtype probes. Eventually 5 polymorphic probes showed stronger signals than the wildtype probes and at the same time the number of false positive signals was reduced from 4 to 2.*

Signal accuracy	Experiment 3 of 18	Experiment 15 of 18	Experiment 18
Right signal	2	4	5
No difference	2	-	1
Wrong signal	4	4	2

In summary best conditions were achieved by initial Tris-blocking, hybridization in PBS-T + 5 mM $MgCl_2$ at 42°C and using MutS/Cy5.

The type of mismatch that has to be bound is influencing the signals too. As illustrated in the introduction (see section 1.3.1.2) MutS has different affinity to different mismatch types, with G:T being the mismatch with the highest and C:C the mismatch with the lowest affinity for MutS binding. In experiment 18 the one of the two wrong results was obtained with a C:C mismatch, which is thus not so surprising. On the contrary the five wrongly bound probe-pairs in experiment 3 were concerning all types of mismatches. This also shows that the specificity of results was significantly increased.

CHAPTER 5

CONCLUSION

The development of a mismatch detection system with the help of a protein was the aim of this project. Unfortunatley it was not possible to reach this goal.The development of a valid DNA-array based test system for SNP detection was not successful and MutS does not seem the be applicable for mismatch detection on standard DNA-arrays.

The question, why MutS-based mismatch detection on the DNA-array did not lead to the desired results might have different reasons. First, there is the probe-accessibility. Throughout all experiment in this work there were much stronger signals obtained with the 50-mer probes compared to 30-mer probes. Lengthening of probes by the insertion of a spacer fragment could be a solution to obtain better results.

Also the secondary structures of the immobilized probes could cause problems. If the probes bind themselves or among each other the traget DNA will not be bound and mismatch detection is impossible. In this case weak denaturationg conditions might denaturate the probes, which was tried out here too, but the results were still the same. Maybe denaturation was too weak.

Finally another array-coating could be better suited for this purpose. Maybe more porouse surfaces should be tested for providing accessibility of probes during hybridization and the MutS protein during detection.

The idea of this project is still very promising. Other proteins are supposed to have different properties than MutS concerning mismatch detction but might be applicable in future experiments.

Although this work did not reveal a new detection method for SNPs there was a significant progress achieved during the course of this project**.** All obtained results, positive and negative, are very helpful for further approaches – maybe when testing other chip surfaces and alternative proteins. This work might represent an important and helpful basis for future developments to find a system for highly paralleled mutation testing.

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APPENDIX

Appendix I: List of kits

Kit	Provider
Alexa Fluor 555 Protein Labeling Kit	"Invitrogen"
BL21(DE3)pLysS Singles Competent Cells	"Novagen"
DC protein assay	"Bio-Rad"
High Pure PCR Template Preparation Kit	"Roche"
Invisorb® Spin PCRapid Kit	"Invitek"
Zeba™ Desalt Spin Columns	"PIERCE"

Appendix II: Buffer recipes

- Phosphate lysis buffer (pH 7.0) for MutS

  Phosphate buffer 50 mM

  NaCl 300 mM

  Aqua dest.

- Tris-HCl lysis buffer (pH 7.9) for GFP-MutS

  Tris-Hcl 20 mM

NaCl	500 mM
Imidazole	5 mM
Triton-X100	0.1%
Aqua dest.	

- Elution buffer (pH 7.0) for MutS

Phosphate buffer	50 mM
NaCl	300 mM
Imidazole	0 mM (A)
	5 mM (B)
	500 mM (C)
Aqua dest.	

- Elution buffer (pH 7.9) for GFP-MutS

Tris-HCl	20 mM
NaCl	500 mM
Imidazole	0 mM (A)
	5 mM (B)
	500 mM (C)
Triton X-100	0.1%
Aqua dest.	

- Wash buffer 2

SSC (20x)	1x

SDS (20%)	0.2%
Aqua dest.	

- Sodium-Phosphate Buffer (pH 7.0):

Sodium-Phosphate buffer	50 mM
NaCl	300 mM

- PBS-T

PBS	1x
Tween-20	0.1%
$MgCl_2$	5 mM

- TBS-T

NaCl	3 M
Tris base	200 mM
Tween-20	0.5%
$MgCl_2$	5 mM

- Assay buffer

Tris-HCl (pH 7.6)	20 mM
$MgCl_2$	5 mM
DTT	0.1 mM
0.1 EDTA	0.01 mM

- Tris-borate running buffer

Tris-borate buffer (pH 8.3)	89 mM
$MgCl_2$	1 mM

- Spot buffer (sterile filtrated)

Na-phosphatebuffer (pH 8.0)	10 ml
SDS (20%)	50 µl

Appendix III: Oligo sequences

50-mer oligo sequences

All probes contain a 5'-C6-Amino motif.

- Exon 11: (poly 691 GGT>AGT)

691-fw_wt_G	ttcccggtca gctactcctc ttcc-ggt-gcc cgccggccct cgctggactc
691-fw_poly_A	ttcccggtca gctactcctc ttcc-Agt-gcc cgccggccct cgctggactc
691-re_wt_C	gagtccagcg agggccggcg ggc-acc-ggaa gaggagtagc tgaccgggaa
691-re_poly_T	gagtccagcg agggccggcg ggc-acT-ggaa gaggagtagc tgaccgggaa

- Exon 13: (poly 769 CTT>CTG)

769-fw_wt_T	tcagagaacg cctccccgag tgag-**ctt**-cga gacctgctgt cagagttcaa
769-fw_poly_G	tcagagaacg cctccccgag tgag-**ctG**-cga gacctgctgt cagagttcaa
769-re_wt_A	ttgaactctg acagcaggtc tcg-**aag**-ctca ctcggggagg cgttctctga
769-re_poly_C	ttgaactctg acagcaggtc tcg-**Cag**-ctca ctcggggagg cgttctctga

- Exon 14: (poly 836 AGC>AGT)

836-fw_wt_C gcagtggagg cagccgcaac tcc-agc-tccc tggaccaccc ggatgagcgg

836-fw_poly_T gcagtggagg cagccgcaac tcc-agT-tccc tggaccaccc ggatgagcgg

836-re_wt_G ccgctcatcc gggtggtcca ggga-gct-gga gttgcggctg cctccactgc

836-re_poly_A ccgctcatcc gggtggtcca ggga-Act-gga gttgcggctg cctccactgc

- Exon 15: (poly 904 TCC>TCG)

904-fw_wt-C tcccgagatg tttatgaaga ggat-**tcc**-tac gtgaagagga gccaggtgcc

904-fw_poly_G tcccgagatg tttatgaaga ggat-**tcG**-tac gtgaagagga gccaggtgcc

904-re_wt_G ggcacctggc tcctcttcac gta-**gga**-atcc tcttcataaa catctcggga

904-re_poly_C ggcacctggc tcctcttcac gta-**Cga**-atcc tcttcataaa catctcggga

30-mer oligo sequences

All probes contain a 5'-C6-Amino motif.

- Exon 11: (poly 691 GGT>AGT)

691-fw_wt_G gctactcctc ttcc-ggt-gcc cgccggccct

691-fw_poly_A gctactcctc ttcc-Agt-gcc cgccggccct

691-re_wt_C agggccggcg ggc-acc-ggaa gaggagtagc

691-re_poly_T agggccggcg ggc-acT-ggaa gaggagtagc

- Exon 13: (poly 769 CTT>CTG)

769-fw_wt_T	cctccccgag tgag-**ctt**-cga gacctgctgt
769-fw_poly_G	cctccccgag tgag-**ctG**-cga gacctgctgt
769-re_wt_A	acagcaggtc tcg-**aag**-ctca ctcggggagg
769-re_poly_C	acagcaggtc tcg-**Cag**-ctca ctcggggagg

- Exon 14: (poly 836 AGC>AGT)

836-fw_wt_C	cagccgcaac tcc-agc-tccc tggaccaccc
836-fw_poly_T	cagccgcaac tcc-agT-tccc tggaccaccc
836-re_wt_G	gggtggtcca ggga-gct-gga gttgcggctg
836-re_poly_A	gggtggtcca ggga-Act-gga gttgcggctg

- Exon 15: (poly 904 TCC>TCG)

904-fw_wt-C	tttatgaaga ggat-**tcc**-tac gtgaagagga
904-fw_poly_G	tttatgaaga ggat-**tcG**-tac gtgaagagga
904-re_wt_G	tcctcttcac gta-**gga**-atcc tcttcataaa
904-re_poly_C	tcctcttcac gta-**Cga**-atcc tcttcataaa