DEVELOPMENT OF A SANDWICH-TYPE DNA ARRAY PLATFORM FOR THE DETECTION OF LABEL-FREE OLIGONUCLEOTIDE TARGETS

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A SANDWICH-TYPE DNA ARRAY PLATFORM FOR THE DETECTION OF LABEL-FREE OLIGONUCLEOTIDE TARGETS

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ABSTRACT

DEVELOPMENT OF A SANDWICH-TYPE DNA ARRAY PLATFORM FOR THE DETECTION OF LABEL-FREE OLIGONUCLEOTIDE TARGETS

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DNA arrays have become a major bioanalytical method as they enable high-throughput screening and they can be manufactured on different surfaces depending on the nature of diagnostic purpose. However, current technologies to produce and detect arrays of DNA probes are expensive due to the requirement of specialized instrumentation. In this study we have established an array platform in sandwich hybridization format for the detection of label-free nucleic acid targets.

Unlike direct hybridization, which is the main microarray hybridization principle, sandwich assay enables unlabeled target detection, lowering the cost and assay time. To this end, sequence specific signal development was achieved by a sandwich complex which is composed of a surface immobilized capture DNA probe (Probe1) and a fluorescein-tagged signal DNA probe (Probe 2), which are partially complementary to the sequence to be analyzed (target oligonucleotide).

As the solid support of the array platform both 3-aminopropyl-3-methoxysilane (APTMS) activated and commercially purchased poly-L lysine coated glass slides were used and due to the less background noise property the latter one was preferred. Similarly, for the immobilization of the capture Probe (P1) onto the solid support two different methods were tried; heat immobilization and immobilization via a heterobifunctional cross-linker (HBCL). In regard to the experiments, it is observed that using a cross-linker instead of heat immobilization reduces the ratio of false negative control results in a significant manner.

Following the solid support and immobilization method choice comparative optimization studies which include cross-linker type, probe concentration, sensitivity of the platform and hybridization conditions (sequence, temperature and duration) were conducted. Optimum hybridization signal was obtained with a 32.5 Å cross-linker, 10 µM capture and 20 µM signal probe concentrations at 35°C.

Finally, in order to evaluate the specificity of the array system a cocktail of target DNA were applied to the system on which different capture probes of those target DNAs were immobilized. The results indicated that the optimized array system is specific enough to detect only the target oligonucleotide yet the unspecific ones.

Keywords: biosensor; DNA array; sandwich hybridization; genotyping; fluorescence

ÖZ

İŞARETSİZ HEDEF OLİGONÜKLEOTİDLERİN TAYİNİ İÇİN SANDVİÇ TABANLI DNA DİZİ PLATFORMUNUN GELİŞTİRİMESİ

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DNA tabanlı mikrodiziler günümüzde sıklıkla kullanılan biyoanalitik yöntemlerin başını çekmeye adaydır. Genetik hastalıkları tayin etmek için kullanılan DNA tabanlı mikrodizi platformları, değişik yüzeyler kullanılarak oluşturulmaktadır. Günümüzde tanı amaçlı kullanılan aletlerin çok pahalıya malolmasının sebebi kendine özel donanıma sahip olmasıdır. Bu çalışmada, işaretsiz hedef nükleik asitleri tespit etmek amacıyla sandviç hibridizasyonu formatında dizi platformu geliştirilmesi üzerine çalışılmıştır.

Sandviç hibridizasyonu direk hibridizasyondan farklı olarak, işaretlenmemiş hedef oligonükleotidi tespit edebilmekte, tanı maliyetini azaltmakta ve tanı süresini kısaltmaktadır. Sekans spesifik sinyalini geliştirmek için sandviç hibridizasyon dizi platformu, yüzeye sabitlenmiş birinci DNA dizisinden (prob1) ve florasan ile işaretlenmiş olup, hedeflenen oligonükleotitin kısmen tamamlayıcı bazları bulunmakta olan ikinci DNA parçacığından (prob2) oluşmaktadır.

Mikrodizi platformunda sekansların yüzeye sabitlenmesi için hem 3-aminopropil-3 metoksi silan(APTMS) hem de piyasada satılan poli-L- lizin kaplı cam

slaytlar(lamlar) kullanılmıştır. Poli-L-lizin kaplı cam slaytlar(lamlar) kullanıldığında daha az arka plan sinyali elde edildiğinden bu yüzey tipi tercih edilmiştir.

İlk DNA dizisini (Prob 1) yüzeye sabitlemek için iki farklı yöntem kullanılmıştır. Birinci yöntemde yüksek ısı kullanılarak DNA dizileri yüzeye tutturulmaya çalışılırken, ikinci yöntemde çapraz bağlayıcı yardımı ile diziler yüzeye sabitlenmeye çalışılmıştır. Yapılan deneylere ışığında çapraz bağlayıcı yöntemi ile DNA dizilerini yüzeye sabitlemenin negatif kontrollerdeki pozitif sonuçları ortadan kaldırmak suretiyle daha etkili bir yöntem olduğu belirlenmiştir.

Yüzey seçimi ve DNA parçaçıklarını yüzeye bağlama methodu seçildikten sonra çapraz bağlayıcı çeşidi, DNA problarının konsantrasyonu, platformun hassasiyeti ve hibridizasyon koşullarını (sıcaklık, sekans sırası ve süre) belirlemek amacıyla karşılaştırmalı optimizasyon deneyleri yapılmıştır. Bu deneyler sonucunda, elde edilen verilere gore en iyi hibridizasyon sinyali 35° C'de, 32,5 Å çapraz bağlayıcı uzunluğunda, 10 μM platform hassasiyetinde ve 20 μM sinyal prob konsantrasyonunda elde edilmiştir. Son olarak, çip sisteminin hedeflenen DNA dizisine özel olup olmadığını test etmek için farklı dizideki DNA sekansları platform uygulandı. Sonuç olarak geliştirilen makrodizi sisteminin sadece hedeflenen DNA dizisine spesifik olduğu gözlemlenmiştir..

Anahtar Kelimeler: biyosensör; DNA mikrodizisi; sandviç hibridizasyonu; genotipleme;fluoresans

To my parents Nevin & Mehmet CANSIZ,
for their unwavering support and endless love…

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3- Aminopropyl-3-methoxysilan	APTMS
Adapter	A
Bovine Serum Albumin	BSA
Cauliflower Mosaic Virus	CaMV
Dimethyl sulfoxide	DMSO
Heterobifunctional Cross-linkers	HBC
Phosphate Buffered Saline	PBS
Poly-L lysine	PLL
Probe 1	P1
Probe 2	P2
Saline-Sodium Citrate	SSC
Sodium Dodecyl Sulfate	SDS
Tris(2-Carboxyethyl) phosphine Hydrochloride	TCEP
Uncomplimentary Adapter	unA

CHAPTER 1

INTRODUCTION

1.1 Biosensors

In 1962, a study performed by Clark and Lyons analyzed and indicated the blood glucose concentration by using the enzyme glucose oxidase via an oxygen electrode. Indeed, this research; quantification of analytes in blood via an oxygen electrode lead to the development of biosensor concept (Clark Jr and Lyons, 1962). Due to the several advantages of biosensing devices like being simple, economical, safe, highly sensitive and easy to handle as well as making precious and accurate measurements, many researchers turned their attentions to biosensor applications for the detection and identification of several biological substances. Today, a biosensor can be defined as "an analytical device that combines the specificity of a biological sensing element with a transducer to produce a signal proportional to target analyte concentration" (Zhai et al., 1997). Figure 1.1 indicates working principle of a basic biosensor.

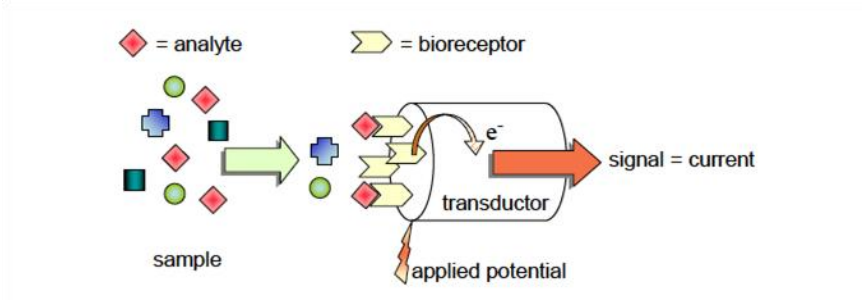

Figure 1.1 Schematic depicting for the basic working principle of a biosensor (Belluzo et al., 2008)

Mainly, biosensing applications can be categorized in two different groups according to their target biological analytes, namely catalytic and noncatalytic (affinity) biosensors. In the case of catalytic biosensors the biological analytes to be screened are enzymes, microorganisms whereas antibodies, oligonucleotides, cells *etc.* can be detected and analyzed by the affinity biosensors.

1.2 DNA Biosensors

DNA biosensors or in a different saying genosensors are analytical devices that composed of a sequence-specific DNA probe and a signal transducer. These biosensors are highly sensitive and selective when detecting a specific DNA sequence, since the solid surface of the sensor can be immobilized with a specific probe which is complementary to target sequence (Teh et al., 2005). There are various techniques for the interpretation of signal coming from the system. Among them, optical, electrochemical and piezoelectric biosensors are the most widely used methods for their simplicity, economical instrumentation and enabling real-time and label-free sensing (Lucarelli et al., 2008; Sassolas et al., 2008; Yao et al., 2006).

More than a decade DNA-based diagnostic tests have been taking attention to researchers. As can be inferred from Fig 1.1, there is a significant increment in the number of studies related with DNA arrays and DNA biosensors. As a result, this growing research area of biosensors leads in to the development of various applications in various fields: like DNA diagnostics, genotyping, rapid monitoring of biological warfare agents and forensic applications. In addition, screening of genetic mutations like point mutations result in performing reliable diagnostics before any symptom of a disease arises (Wang, 2000).

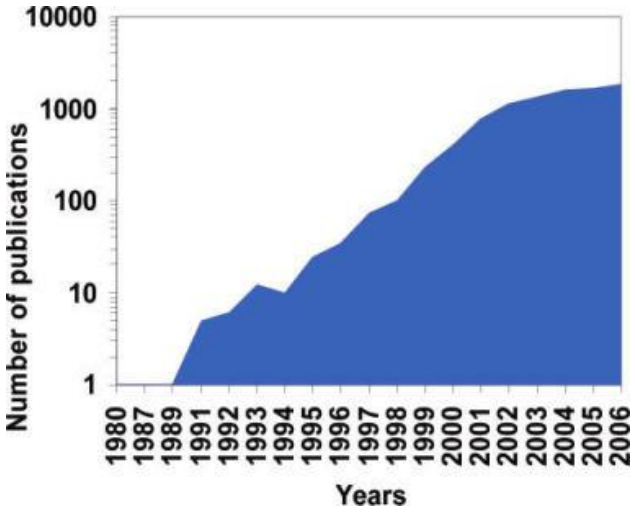

Figure 1.2 Annual trends in the number of publications for DNA biosensors and microarrays (Sassolas et al., 2008).

Indeed, several DNA detection systems based on the hybridization between a DNA target and its complementary probe, which is either in solution or on a solid support are present. These formerly developed assays are allowing the determination of DNA sequences. On the other hand, they do not provide, economical screening and could not be handled easily. DNA biosensors and DNA chips are better alternatives to these methods because they allow rapid, selective, continuous and reproducible detection of DNA hybridization. These systems can be based on optical (Wua et al., 2006) or electrochemical (Castañeda et al., 2007) detection.

Hybridization biosensors rely on the immobilization of a single stranded (ss) DNA probe onto the transducer surface. The hybridization duplex arrangement can be detected by means of a hybridization signal or by other changes obtained as a result of the hybridization event (Wang, 2000).

There are two important points when developing hybridization DNA arrays and biosensors: the sensitivity and the selectivity.

It is important to be able to detect low DNA concentrations and to detect a minute amount of differences in a given target gene like in the case of point mutations. Two types of systems can be developed: systems for DNA hybridization and systems for detection of DNA damage(Palecek et al., 1998). In the case of DNA hybridization systems a 100 % match in the target sequence produces very stable double-stranded DNA (dsDNA), whereas in the case of systems for detection DNA damage, one or more base mismatches decreases the stability, causing a signal modification and thus enabling detection of the mutation.

1.3 Microarray Technology

Over the past decade, several eukaryotic and prokaryotic genomes have been analyzed. Especially, after completion of the Human Genome Project, vast amount of data arose along with the necessity to analyze and manipulate this newly formed information pool. Microarrays, in which thousands of hybridization reactions are carried out in parallel and concurrent manner are a remedy for this huge information pool because they can used for pointing out basic questions in biology and used for the characterization of mutations up to whole-genome scale (Levicky and Horgan, 2005). For example, the genetic structure of complex disease, such as diabetes, asthma, and inflammatory bowel disease, were difficult to detect since very recently(Grant and Hakonarson, 2008; Martel et al., 2005). On the other hand, with the help of microarray technology, it is much more possible and simple to identify the mutations causing these complex diseases. In brief it can be said that arrays have become an increasingly different set of tools for biological studies and their use continues to expand rapidly.

A microarray experiment makes use of common assay systems such as micro-plates and/or standard hybridization membranes. The sample spot sizes are nearly less than 250 micro meters in diameter and the solid support usually contains tens of thousands of spots. Generally, arrays have composed of collections of different capture molecules typically cDNAs or oligonucleotides immobilized to the solid support usually a glass slide at predefined locations within a arrayed pattern.(Dufva,

2005; Wodicka et al., 1997) Moreover, the successful applications of microarray technologies depend on the development of many methods and techniques for producing the microarrays, spotting the probes, performing and screening the hybridization reactions, and informatics for analyzing the signal data.

Despite the fact that, there are several techniques when constructing a microarray, all arrays share a common feature: They are designed for multiplexed analyses. Arraying systems allow multiple tests to be performed simultaneously. This is applicable both when many analytes are measured concurrently in an individual sample and also when many samples are tested at one time for an individual analyte. For instance, DNA arrays can be used to determine the expression levels of thousands of genes in an individual biological specimen (Duggan et al., 1999), while tissue arrays can be used to determine the presence of a specific antigen in hundreds of specimens in a single experiment.(Fejzo and Slamon, 2001)

The classes of capture molecules used in arrays include not only DNA, but also proteins(Schweitzer and Kingsmore, 2002), carbohydrates(Love and Seeberger, 2002), drug-like molecules (Lam and Renil, 2002), cells (Wu et al., 2002), tissues (Fejzo and Slamon, 2001) and the like.

1.3.1 Protein Microarrays

Protein microarrays which are actually protein analogs of DNA chips are one of the successful alternatives for profiling proteins and examine hundreds to thousands of them in a parallel and concurrent manner(Albala, 2007). This technology based on predefined gridding to identify a pool of capture agents arrayed on the chip surface (Zhou et al., 2001)

Protein arrays were not used and developed as DNA microarrays mainly because of the complexity of proteins. While DNA microarrays have become the tools of choice for characterizing patterns of gene expression, two dimensional gel electrophoresis remains the standard method for protein finger-printing. (Hackler et al., 2003)

Protein microarrays can be generally categorized into two different classes: protein profiling microarrays, functional protein arrays. Protein profiling arrays typically are arrays of antibodies used to compare two different cell types or disease states for novel biomarker identification, whereas functional protein arrays are generally consist of recombinant proteins used for identification of protein-protein or protein-DNA interactions or other biochemical activities. Multiplexed immunoassays are the most novel application for protein arrays. One is the miniaturization and multiplexing of the standard enzyme linked immunosorbent assay (ELISA), in which capture antibodies are arrayed onto slides or microtiter plates. A different format of this method that needs to only a single antibody for each target antigen is to tag the proteins with one fluorochrome and the proteins in a reference sample with a second fluorochrome. The differentially labeled samples are mixed and incubated with an antibody microarray which is scanned. The ratio of the two fluorescent dyes at each spot in the array corresponds to the relative concentration of each protein in the two samples (Haab et al.).Developments in sensitivity and signal to noise ratio will be required for this technique to become useful for measuring protein changes in biological samples. In a different strategy, which may especially be useful for diagnostic assays is to prepare arrays of antigens. Such arrays allow samples to be tested for the presence of antibodies to particular antigens (Schweitzer and Kingsmore, 2002). This approach is used for identification for certain autoimmune diseases and for exposure to infectious agents. In brief, it can be said that different types of protein arrays will be required for arranging the proteome, detecting differences in expression, and for detecting compounds. (Cai et al., 2005)

There are several researchers which assigning protein arrays in their studies. For instance, in the study conducted by Albala *et al* a protein interaction microarray was generated to identify protein-protein and protein-DNA interactions for the Rad51 protein which assist in repair of DNA double strand breaks. They prepared protein microarrays containing various DNA repair proteins, histones, nucleosomes, DNA and several antibodies. These analytes were immobilized onto functionalized solid surface on which Rad51B and Rad51C were applied to detect protein-protein interactions for the ones involving in DNA double strand break repair (Albala, 2007).

1.3.2 DNA Arrays

Basically, a DNA array is an orderly arrangement of samples where matching of known and unknown DNA samples is done based on base pairing rules (Lockhart DJ et al., 1996; Schena et al., 1995). This technology has become a common tool in biological studies with applications in gene expression monitoring, sequencing, and drug discovery, all benefiting greatly by the simultaneous detection principle of the technique. One of the most distinguishing properties of a DNA array is the availability of the single-stranded capture probes for hybridization with the target sequence (Moiseev et al., 2006).

1.3.2.1 Immobilization of DNA on Glass Surface

In microarray technology, surfaces must be designed and prepared to optimize the immobilization of probe sequences, but also they should be selective enough to prevent non-specific binding of target species.(Hanson et al., 2005)

DNA immobilization on solid surfaces can be succeeded by several techniques. An ideal immobilization method should enable the immobilized nucleic acids to mimic their solution phase state. For example, immobilized molecules located away from the solid support are closer to the solution state and thus are more available for contact with dissolved analytes (Moiseev et al., 2006). Also the technique should be effective enough as well as being specific, reproducible and reusable. (Balamurugan et al., 2008)

While designing a DNA immobilization there are several factors like type of immobilization (covalent, non-covalent *etc.*), point of attachment, linker length and linker properties that should be taken into consideration. (Bajaj, 2000) In addition, choice of solid support that constructs the backbone of the array system is also important. Glass is the most widely used substrate for DNA arrays as it is flat, transparent, resistant to high temperatures, easy to handle, and has low fluorescence.

Techniques for modifying glass substrates are also well developed (Carrillo et al., 2005).

Mechanisms of immobilization can be divided into two major categories: adsorption, which is actually based on non-covalent interactions (mainly electrostatic, van der Waals, and dehydration of hydrophobic interfaces) and covalent binding of functional groups on the biomolecule or probe onto functionalized surfaces. The former mechanism has a physical nature and therefore displays changing levels of reversibility, whereas covalent binding, as its names implies, involves the formation of effectively irreversible chemical bonds between biomolecule and functionalized surface (Hanson et al., 2005; Misra and Dwivedi, 2007).

Non-covalent immobilization methods like immobilization on nitrocellulose membranes (Southern, 1975) and association on lipid bilayer (Kwok, 2001) generally result in improper probe strand orientations, low immobilization densities, low mobilities and regions of the nucleic acid sequence being away for hybridization due to the immobilization deficiencies. Gel entrapment of DNA (Kwok, 2001) leads to excessive diffusion limitations for the target DNA and thus the hybridization kinetics are slow. Also these cases, the DNA molecules are prone to removal from the surface under high salt or high temperature conditions. Moreover, non-covalent immobilization either through the backbone or the bases increases the chances of non-specific attachment due to poor availability of the entire sequence for hybridization. It has been found that DNA could become totally inaccessible for hybridization when only 3% of its bases are involved in the non-covalent linkage (Kwok, 2001). The hybridization rates on the surface are much slower than those in solution. By having a long enough linker to distance the molecule from any interactions with the surface, it is possible to have a sequence mimic its solution phase behavior even while being immobilized (Beaucage, 2001).

The covalent binding of biomolecules allows for very strong attachment and a positional linking at one end of a biomolecule. A variety of side groups are easily used for covalent binding, most commonly amino, carboxy, hydroxy, and thiol

groups (Chrisey et al., 1996; Fixe et al., 2004). Therefore it can be said that, covalent attachment provides far more stable situation for the experimental conditions employed for hybridization.

Covalent attachment of probes to glass surfaces functionalized with amino groups is among the most widely used methods for immobilizing probes onto glass substrates. Glass slides can be silanized by immersing them in a 2, 5, 10% solution of 3–aminopropyl–triethoxysilane (APTES) or 3–aminopropyl–trimethoxysilane (APTMS) in acetone for a defined amount of time at room temperature followed by several acetone washes (Joos et al., 1997). Alternatively, DNA probes can be attached to poly- L lysine-coated glass substrates. This technique makes use of both the adsorption (Graves, 1999) and covalent attachment of DNA via a cross linker (Sawant and Nicolau, 2006). Table 1.1,which was taken from a valuable work of Bajaj (Bajaj, 2000) summarizes current techniques used for DNA immobilization.

1.3.2.2 DNA Surface Hybridization

Solid-phase or in a different saying surface hybridization forms the bases of contemporary DNA array and biosensing technologies widely used in applied genomics for genotyping, drug discovery, gene expression profiling, based on measurement of genetic information (Gong and Levicky, 2008; Graves, 1999; Schena et al., 1998). The relationship between two complementary probe and target strand is studied well in solution phase. On the other hand, this knowledge is not sufficient to understand solid- phase hybridization.

Table 1.1 Comparison of various immobilization chemistries (Bajaj, 2000)

Group	Immobilization Chemistry	Substrate	Probe / Target, Length	Probe Density Molecule/cm²	Density Control	Hybrid. Time	Hybridization Conditions
Kumar et al.	Silanized DNA	Glass slide	20 nt / 20 nt	$2x10^{13}$	No	30 min – 12 h	37 °C, 20 nM to 1 µM oligo, 750 mM NaCl, 125 mM Na citrate, 0.1% Tween
McGall et al.	OH-silane + oligo synthesis	Glass slide	20-25 nt / PCR product	-	No	4 h	35-40 °C, 6x SSPE, 0.001% Triton X-100
Shalon et al.	Poly-lysine + PCR product	Glass slide	PCR / PCR products	-	No	14-18 h	65 °C, 0.5 M NaCl, 0.05 M Na citrate, 0.3% SDS
Piunno et al.	Glycidoxy propyl silane + DMT-HEG linker + oligo synthesis	Fused silica	20 nt / 20 nt	$9x10^{10}$ to $4.6x10^{12}$	Some	40 min	90 °C, 0.62 µM oligo, 1 M NaCl, 50 mM NaH_2PO_4
Shchepinov et al.	Glycidoxy propyl silane + linker + oligo synthesis	Glass slide	12 nt / 12 nt	$6x10^{12}$	Some	2 h	30 °C, 3 nM oligo, 0.1 M NaCl
Guo et al.	NH_2 propylsilane + PDC + 5' NH_2-PCR product	Glass slide	157 nt / 182 nt	$6x10^{12}$ to $3x10^{13}$	Some	3 h	30 °C, 20-50 nM PCR product, 5x SSPE, 0.5% SDS
Graves et al.	NH_2 propylsilane + PDC + 5' amino oligo/ PCR product	Glass slide	15 nt / 15 nt	$1x10^{12}$ (appx.)	Some	3-6 h	46 °C, 2 µM oligo, 0.9 M NaCl, 0.06 M NaH_2PO_4, 6 mM EDTA, pH 7.4
Chrisey et al.	NH_2 silane + SMPB + 3' SH-oligo	Si/SiO_2, Fused silica	20 nt / 20 nt	$1.2x10^{13}$	Some	2 h	25°C, 1µM oligo, 10 mM HEPES, 5 mM EDTA buffer
Strother et al.	(NH_2-decene + dodecene) + SSMCC + SH-oligo	Si (001)	16 nt / 16 nt	$2.3x10^{12}$	Yes	30 min	25 °C, 2 µM oligo, 2x SSPE, 0.2% SDS
Cavic et al.	(SH-silane + Alkane silane) + BMH + 3' SH-oligo	Si/SiO_2	25 nt / 25 nt	$2x10^{13}$	Yes	1.5 h	25 °C, 20 µM oligo, 10 mM Tris-HCl, 1 mM EDTA, 1.5 M NaCl
Tarlov et al.	(5' SH-oligo + mercapto hexanol)	Au-coated silicon	25 nt / 25 nt	$5.7x10^{12}$	Yes	90 min	24°C, 1µM oligo, 10mM Tris-HCl, 1 mM EDTA, 1 M NaCl
Our system	(OH-silane + alkane silane) + oligo synthesis	Si/SiO_2, Glass slide	12 nt / 12 nt	$\sim 3x10^{13}$ (optim.)	Yes	24 h	4°C, 0.5 µM oligo, 1 M NaCl, 0.1 M Na citrate, 0.1% SDS

Hybridization on surfaces is influenced by factors such as surface chemistry and surface probe density (Gong and Levicky, 2008; Levicky and Horgan, 2005; Peterson et al., 2001) as well as those factors that influence hybridization in solution(Bajaj, 2000; Gong and Levicky, 2008): pH, salt concentration, temperature,

solvent properties, GC content, and DNA length. Higher GC content increases the stability of DNA duplexes because a GC base pair produces three hydrogen bonding resulting in greater stability than AT base pairing that produces just two hydrogen bonds. In addition, the addition of salt stabilizes DNA due to the charges between two ionic oligonucleotide strands. "Furthermore, higher salt concentration helps in improving the kinetics of the process, but at the same time reduces the stringency of discriminating between mismatch sequences"(Bajaj, 2000). Higher temperatures provide better dissociation between mismatches by denaturing the less stable mismatched sequences. On the other hand, higher temperatures may also cause the dissociation rate to increase and thus lead to a decrease hybridization amount.

Some detergents and surfactants like Sodium Dodecyl Sulphate (SDS) help in improving the signal-to-noise ratios from hybridization experiments by reducing non-specific adsorption events. However, the addition of too much surfactant can inhibit the hybridization process by interacting with the charged DNA.(Herzer and Englert, 2002) In numerous studies SDS and Bovine Serum Albumin (BSA) were included when developing the array systems (Fixe et al., 2004; Guo et al., 1994; Hackler et al., 2003).

The impact of probe surface coverage on target hybridization is another significant factor when dealing with surface hybridization. Generally, it is desired that the number of surface-attached DNA probes be considerably higher than the amount of targets in solution in order to succeed high amount of hybridization signals even in the case of low target concentrations. On the other hand, too much surface probe densities can limit the binding possibility of the incoming target DNA molecules by providing steric as well as electrostatic repulsion to the target molecules (Gong and Levicky, 2008; Peterson et al., 2001; Steel et al., 1998).

1.4 Sandwich Hybridization

Hybridization assays which are derived from unique and powerful interaction among two complementary nucleic acid strands according to the Watson-Crick base pairing

of adenine with thymine (A-T) and cytosine with guanine (C-G) are one the fundamental tools in molecular genetics and used by various researchers for several reasons. For example, DNA hybridization technique is employed for analyzing gene expression levels of cancers cells (Todd and Wong, 2002) or discovering expression patterns of genes (Brown and Botstein, 1999; Duggan et al., 1999). In addition, they may be used to determine polymorphisms (Kwok, 2001), to detect pathogens (Lazcka et al., 2007) (Sapsford et al., 2008) and to carry out transcript profiling of tumors (Dhanasekaran et al., 2001).

When performing nucleic acid hybridization assays, two different strategies can be employed namely; direct hybridization or sandwich hybridization. Direct hybridization method which is actually the standard microarray hybridization method can be used where the immobilized DNA probe hybridizes with the labeled DNA target. On the other hand, as its name depicts a sandwich type hybridization assay can be formed by partially hybridization of capture DNA probe to a part of the target whereas the other part of the target is complementary to a signaling DNA sequence which actually the reporter probe of the system.

The sandwich hybridization for the analysis of nucleic acid sequences was first used in 1977 (Dunn and Hassell, 1977) for transcript mapping purposes. However, its potential as a diagnostic assay was not realized until 1983, when it was applied to the detection of adenovirus DNA in nasopharyngeal aspirates from children with acute respiratory infection (Ranki et al., 1983). Since then, this technique has been used by various researchers, mostly for detection of pathogens, bacteria, viruses *etc* (Bavykin et al., 2001; Dankbar et al., 2007; Palva, 1985; Seidel and Niessner, 2008). Neubauer and his group includes sandwich hybridization assay into their works frequently. In the study which they perform in 2003 they used sandwich hybridization method for quantification of RNA for measuring levels of specific RNAs in yeast cells as an informative tool for the state and control analysis of bioprocesses. They evaluated the developed method by measuring the levels of *Saccharomyces cerevisae carlsbergensis* 18S rRNA and SUC2 mRNA in shake flask experiments. Actually, the developed assay is based on hybridization of RNA target

molecules with a biotin labeled capture probe and a digoxigenin labeled detection probe where alkaline phosphatase enzyme is attached to the detection probe. After hybridization, the enzyme amplifies a fluorescence signal used for quantification. (Rautio et al., 2003)

In another study of the same group, they developed a sandwich hybridization assay for detecting *Legionella* bacteria in aquatic environments using two genus-specific oligonucleotide probes targeting the 16S rRNA. First, the target molecule is hybridized to the capture and detection probes. Following this, the target probe complex (sandwich) is bound to streptavidin coated magnetic beads via the biotin labeled capture probe. The detection of the hybridization complex is performed by means of anti-DIG-alkaline phosphatase, which binds to the DIG-labeled signal probe. The screening was performed by fluorescence readout of the signal produced by the enzymatic substrate reaction of the alkaline phosphatase (Leskela et al., 2005). The extension of the described study was performed by Huhtamella and co-workers. Their main intention was to developed a simple and economical method to indicate the capacity of the 16S rRNA targeting bead based sandwich hybridization assay combined with electrochemical and fluorescence signal readout systems for the group-specific detection of various beer spoiling *lactobacilli* and *pediococci* in samples containing high amounts of yeast (Huhtamella et al., 2007).

Similarly, Thieme *et al* present an improved solution-based sandwich hybridization method for the fast quantitative analysis of transcripts in bacteria. With this method, they made possible to measure absolute transcript levels in crude cell extracts without prior mRNA isolation. In fact, the study is a proof of concept and determines the responses of three *E. coli* genes involved in copper homeostasis to copper ions due to the fact that high concentrations of copper cations are lethal *to E. coli.* Consequently, specialized mechanisms in this bacterium and in others have evolved to counter the effects of copper toxicity.(Thieme et al., 2008)

In another research, sandwich DNA hybridization was carried out by mixing the target DNA with the magnetic bead-captured probe and the biotinylated signal probe,

followed with the biotin-streptavidin reaction with conjugated Horseradish peroxidase (HRP). The target is a DNA sequence related to the avian influenza A H1N1. In addition, single-base mismatched DNA sequences detection was studied by increasing the hybridization temperature. (Li and He, 2009)

1.5 Sandwich Type DNA Arrays

Compared to the direct hybridization, which is actually the main hybridization principle of a standard microarray, sandwich hybridization offers a number of advantages. First, target sequence does not require any labeling. In fact, sandwich hybridization is considered as the most useful technique to the determination of unlabeled target DNA in crude cellular extracts (H. L. Chiu and Peltier, 1998). In addition, no requirements for target to be labeled provide flexibility in the signal development strategy. A signal probe can be labeled in a variety of ways (gold-silver, fluorescence, radiolabelling, biotin, etc). Furthermore, sandwich hybridization is more specific than direct hybridization assays because two hybridization events must occur in order to generate a signal. Figure 1.3 compares direct and sandwich hybridization schematically.

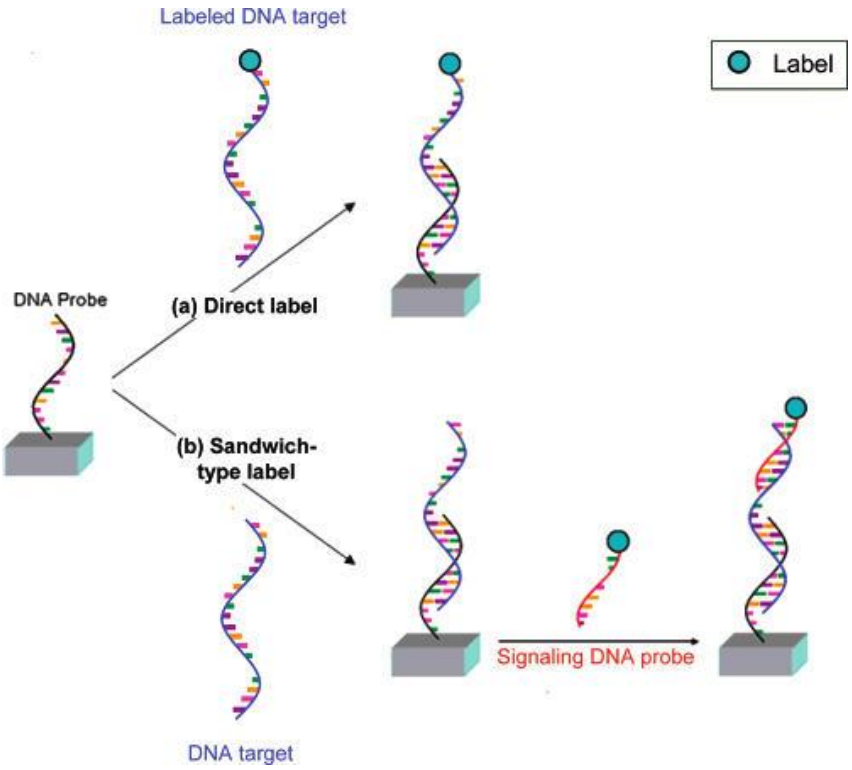

Figure 1.3 Schematic representation of the principal constructions of DNA microarrays and biosensor: (a) direct (binary) hybridization of single stranded (ss) DNA probe with labeled DNA target; (b) sandwich hybridization assay formed after hybridization of ss DNA probe, label-free ss target DNA and labeled ss signaling DNA probe. (Sassolas et al., 2008)

In a study conducted by Taton *et al* a nanoparticle-based array detection system was developed that uses two gold particle probes with covalently bound oligonucleotides that are complementary to a target of interest which is actually a sandwich type array study. This sequence-selective DNA detection method employs oligonucleotide modified gold nanoparticle probes. According to the study, labeling oligonucleotide targets with nanoparticle rather than other signaling molecules like florophore probes changes the melting behaviors of the target sequences from an array substrate. This difference leads to a distinction of a 100% complementary target sequence from targets with single nucleotide mismatches. Thus, as they stated the mismatch

sequences can be discriminated from the perfectly complementary target sequences by silver enhancement method of gold nano-particles easily by using the advantages of sandwich hybridization assay principle (Taton et al., 2001; Taton et al., 2000).

In a similar study performed by Zhou *et al*, an ultrasensitive DNA analysis assay with a 0.8 fM detection limit was developed using a bioconjugated nanoparticle-based sandwich hybridization assay. They synthesized "organic dye-doped silica nanoparticles" which are highly fluorescent and extremely photostable for bioanalysis. In addition, the nanoparticle based DNA bioanalysis assay can effectively discriminate one-base mismatched DNA sequences (Zhao et al., 2003).

1.6 Aim of the Study

In this study, it is described that the construction of sandwich-based macro-array platform for the detection of label-free PCR-amplified target DNA sequences. A part of *Cauliflower Mosaic Virus* (CaMV) 35S promoter was used as the test sequence and designed partially hybridizing capture and fluorescein-tagged signal probes to form the sandwich complex on 3-aminopropyl-3-methoxysilane (APTMS) and poly-L-lysine coated glass slides. Slide scanning and fluorescence signal detection were performed using a confocal laser scanning microscope. Various parameters such as glass surface functionalization, crosslinker length, capture and signal probe concentrations were investigated and comparative results were reported. It is also studied that the effect of probe/target hybridization sequence, temperature and duration on the hybridization signals. Since the described platform does not require specialized arraying hardware such as probe printing robots and slide readers, it offers an economical and flexible label-free oligonucleotide detection platform in genotyping applications.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

All chemicals were analytical grade, purchased from Sigma-Aldrich or Merck (Darmstadt, Germany) unless otherwise specified. Each solution used in experiments was prepared with Milli-Q water.

2.1.2 Support Materials

As a support material standard sized poly-L lysine coated microscope slides were purchased from the company Menzel Gläser (Braunschweig, Germany). For the silanization experiments standard sized, uncoated microscope slides were bought from the company Marienfield GmbH & Co. (Lauda-Königshofen, Germany).

2.1.3 Heterobifunctional Cross-linkers and Oligonucleotides

All cross-linkers were purchased from the company Pierce (IL, USA). Similarly, all oligonucleotides, modified or unmodified, were bought from the company Integrated DNA Technologies (Coralville, IA, USA). A part of *Cauliflower Mosaic Virus* (CaMV) 35S promoter was used as the test sequence and designed partially hybridizing capture and fluorescein-tagged signal probes to form the sandwich complex on glass slides (Table 2.1).

Table 2.1 Oligonucleotides used in the sandwich assay

Name	**Sequence**
Capture Probe (Probe 1)	**5'/5ThioMC6D**/AAAAAAAAAGCATCTTCAAC GATGGCCTTTCCTTT3'
Signal Probe (Probe 2)	5'TCGCAATGATGGCATTTGTAGGAGCAAAA AAAAA**/6-FAM/-3'**
Adapter (Target)	5'GCTCCTACAAATGCCATCATTGCGATAAAG GAAAGGCCATCGTTG AAGATGC 3'
Uncomplimentary Adapter (Negative Control)	5'ACCCTGTAAACGATCATCCCCATTTTTTAC GGCCAATTGGAGGCC TCCCAAT 3'
Capture Probe with 6-FAM (Positive Control)	5'/5ThioMC6D/AAAAAAAAAGCATCTTCAACG ATGGCCTTTCCTTT/6-**FAM/-3'**

2.2 Methods

2.2.1 Construction of Array Platform

2.2.1.1 Cleaning the Glass Slides

Before the coating step, in order to increase the surface density of the OH groups and get rid of surface dust and debris the slides were soaked into Piranha solution (1:3 30 %H_2O_2/ 96%H_2SO_4) for 2 hours. Following this, they were rinsed with several liters of MilliQ water and spin dried.

2.2.1.2 Aminosilane Coating of the Slides

Aminosilane coating was achieved using 1, 5 and 10% aminopropyl-3-methoxysilan (APTMS) containing acetone solution for one hour. Afterwards, slides were washed 3 times with pure acetone, rinsed with MilliQ water and spin dried. The last step of coating procedure is baking slides at 110 °C for 30 minutes.

2.2.1.3 Labeling Slides

Both aminosilane coated and commercially purchased poly-L lysine coated slides were labeled with a diamond tipped glass cutter, washed in MilliQ water and spin dried before activation & immobilization step.

2.2.1.3.1 Type 1 Labeling Design

Type 1 design includes one positive control, four different negative controls and the sandwich hybridization assay column on which three replicas for each. This design type allows observing both negative controls and sandwich assay at the same slide.

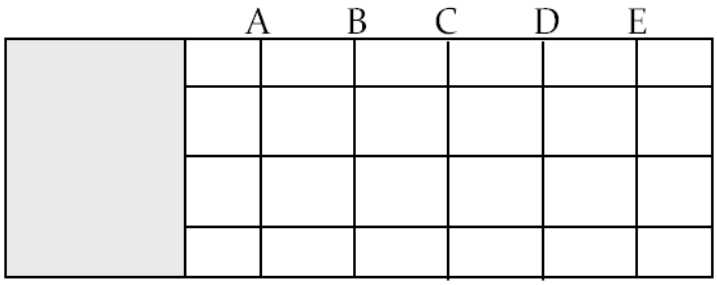

Figure 2.1 Type 1 Labeling Design. Capture probes were immobilized on to the intersection points of vertical and horizontal lines. A Positive Control: (Capture Probe with 6-FAM-P1-F) B Negative Control: (Capture Probe-P1)+(Signal Probe-P2) C Negative Control: (Capture Probe-P1)+(Uncomplimentary Adapter-unA)+ (Signal Probe-P2) D Negative Control: (Signal Probe-P2) E Sandwich Assay: (Capture Probe-P1)+(Adapter-A)+ (Signal Probe-P2)

2.2.1.3.2 Type 2 Labeling Design

Unlike Type 1 design, in type 2 labeling only sandwich assay or one of the negative controls can be carried out. There are nine replicas per slide.

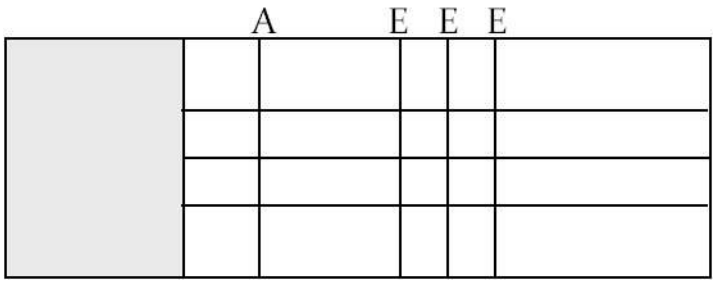

Figure 2.2 Type 2 Labeling Design. Capture probes were immobilized on to the intersection points of vertical and horizontal lines. A Positive Control: (Capture Probe with 6-FAM-**P1**-**F**) E Sandwich Assay: (Capture Probe-**P1**)+(Adapter-**A**)+ (Signal Probe-**P2**)

2.2.1.4 Surface Activation

Both aminosilane coated and commercially purchased poly-L lysine coated slides were activated using 2 mM heterobifunctional cross-linkers. Detailed information about cross-linkers used in experiments is given in Table 2.2. Except for Sulfo-EMCS all other cross-linkers were suspended in Dimethyl sulfoxide (DMSO) and stored (-20) °C as aliquots. However, sulfo EMCS was prepared freshly before each experiment in 10mM Phosphate Saline Buffer (PBS), pH: 7.2.

Table 2.2 Heterobifunctional crosslinkers used for the surface activation

Name	**Spacer Arm Length**
(N-[ε-maleimidocaproyloxy]sulfosuccinimide ester) **(Sulfo_EMCS)**	9.4 Å
(succinimidyl-[(N-maleimidopropionamido)-diethyleneglycol]ester) **$SM(PEG)_2$**	17.6 Å
(succinimidyl-[(N-maleimidopropionamido)-hexaethyleneglycol]ester) **$(SM(PEG)_6$**	32.5 Å
(succinimidyl-[(N-maleimidopropionamido)-dodecaethyleneglycol]ester) **$SM(PEG)_{12}$**	53.4 Å

2.2.1.5 Capture Probe Immobilization

Reduction of disulfide bonds of capture probe, activation of Poly-L lysine and/or APTMS coated glass slides and immobilization of the capture probe was performed at one step. Specified concentrations of P1, specified heterobifunctional cross-linker with a final concentration of 2mM and TCEP with a final concentration of 0.1mM mixed in 10mM Phosphate Buffered Saline (PBS) printing buffer. For the reduction of disulfide bonds, the mixture is incubated for 30 minutes at room temperature. Following the incubation, the immobilization solution was spotted on the glass slides with a volume of 0.5 µL/per spot at 4°C (cold room) by means of micropipette.

Spotted slides were incubated at 30°C for 1.5 hours in a humidifying chamber which is a petri dish containing little amount of water. At the end of incubation, slides were washed with 5 X Saline-Sodium Citrate (SSC) buffer containing 0.1% Sodium Dodecyl Sulfate (SDS), water and spin-dried.

2.2.1.6 Surface Blocking

Blocking was achieved using 2% Bovine Serum Albumin (BSA) in 10 mM PBS for 2 hours. After the immobilization of Probe 1 on to glass surfaces the slides are dipped in to blocking solution at least for 2 hours. Blocking step was performed at 4°C.

2.2.1.7 Hybridization

Hybridization solution containing specified concentrations of adapter and signal probe in 0.6X SSC, 0.5% SDS and 2% BSA was applied to the sample application part of the slide and covered with a cover slip or spotted on the immobilized capture probe as 5 µL/per spot with a micropipette. Slides were then incubated at a specified temperature and duration in humidifying chambers in a dark incubator. Post-hybridization wash was performed at three stages and included 1X to 0.1X SSC, 0.1% SDS and water washing steps. Before scanning slides were dried with a spin drier. Unless specified otherwise, standard hybridization conditions (duration: 5 hour, temperature: 35°C, [capture probe]: 10 µM, [signal probe]: 20 µM) were used.

2.2.2 Determination of Surface Coating Agent

For the determination of most compatible surface coating in order to obtain best capture probe immobilization and hybridization signal, aminosilane coating and poly-l lysine (PLL) coating were compared. In the case of aminosilane coating aminopropyl-3-methoxysilan (APTMS) was used as the silanization agent and coating of the slides was performed as different concentrations of APTMS (1, 5 and 10%) coating as described above. In the case of PLL coating, commercially

purchased PLL coated standard microscope slides were used. The results are evaluated in terms of background noise intensity and signal-noise ratio of spotted area.

2.2.3 Determination of Capture Probe Immobilization Technique

Attachment of capture probe on the coated glass slide was implemented in two different manners; by baking and by using heterobifunctional cross-linkers. The results are evaluated in terms of the ratio of false positive negative control ratio. Moreover, in order to obtain best hybridization signal, four different cross-linkers with different arm lengths were compared according to their signal/noise values.

2.2.4 Capture Probe Optimization

After determining immobilization technique, in order to find out the most optimized capture probe concentration, a sandwich hybridization assay with different concentrations of was P1 constructed. Tested concentrations of P1 are 1, 10, 20 µM each of which was immobilized on to PLL coated glass slides via heterobifunctional cross-linker $SM(PEG)_{2.}$ Signal Probe (P2) concentrations of each corresponding P1 was kept in a constant ratio; 2, 20, 40µM respectively.

2.2.5 Signal Probe Optimization

In this experiment, the effect of different concentrations of signal probe (P2) on signal intensity was studied. The concentrations of P1 and Target sequence (Adapter) were kept constant as 10 µM and 20 µM, respectively. The experiment was performed by using PLL coated slides activated with the cross-linker $SM(PEG)_2$ where the test concentrations for the P2 are 20, 40 and 50 µM.

2.2.6 Optimization of Hybridization Conditions

A serious of experiments on hybridization temperature, duration and sequence was carried out in order to determine the optimum hybridization conditions for the sandwich assay. For the determination of optimum hybridization temperature, three different temperatures (35, 40, 45°C) were chosen as regard to Tm values of the sequences and evaluation was performed according to signal/noise ratio of each parameters. In the case of hybridization duration, the variation of signal intensity by increasing the duration of the hybridization was observed. Test parameters for the experiment are as follows; 2.5, 5, 12, 24 hours. Finally, the effect of probes- adapter application sequences was studied. In the first test, P1, P2 and Adapter were applied on to system and incubated for 10 hours at the same time without a pre-incubation step. In the second one, hybridization was performed gradually. At first step P1 and Adapter were incubated for 5 hours. Following this, slides were washed to remove unbound Adapter sequences. After washing, P2 was applied onto system and incubated for a second 5 hours. In the case of third test, Adapter and P2 were pre-incubated before the application on slide at hybridization temperature for 5 hours. Following this, the pre-incubated Adapter-P2 complex was applied on to system and incubated for another 5 hours.

All hybridization experiment were performed with PLL coated slides by using HBC $SM(PEG)_2$. Capture and signal probes were kept constant, 10 and 20 µM respectively.

2.2.7 Determination of Sensitivity

In order to determine sensitivity of the constructed platform, under optimized standard experimental conditions the assay was performed with different concentrations of target sequence, in a range 0.001 µM through 10 µM, including 0.01, 0.05, 0.1, 1 and 10 µM. The ratio between capture probe and adapter as well as adapter and signal probe were kept constant. The results are evaluated according to net signal intensity of the spots.

2.2.8 Multiplex Assay

In order to confirm selectivity of the platform a multiplex assay was designed. In this respect, in addition to the the regular set of sequences used for the construction and optimization of the platform (P1, A/unA/ P2), three more sequence sets were designed. See Table 2.3. On the PLL coated slides all capture probes from four different sets were immobilized in a regular order. In hybridization step, a mixture solution containing all target sequences as well as their corresponding signal probes were applied on to slide concurrently and let them incubate under coverslip. In order to test selectivity, one of the defined target sequences removed from the mixture and applied on to system in the presence of its corresponding signal probe. This experiment was carried out for every single test sequence and the results are evaluated in terms of presence or absence of the signal. The multiplex assay was performed under standard experimental conditions; 10 µM capture probe, 20 µM signal probe, 35°C hybridization temperature for 5 h with a hybridization sequence P1+(A+P2).

Table 2.3 Oligonucleotides used in the Multiplex Assay

Name	**Sequence**
35S Capture Probe	5'/5ThioMC6D/GCATCTTCAACGATGGCCTTTCCTTT 3'
35S Signal Probe	5'TCGCAATGATGGCATTTGTAGGAGC/6-FAM/-3'
35 S Adapter (Target)	5'GCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTTG AAGATGC 3'
NOS Capture Probe	5'/5ThioMC6D/AATGTATAATTGCGGGACTCTAATC 3'
NOS Signal Probe	5'ATAAAAACCCATCTCATAAATAACG/6-FAM/-3'
NOS Adapter (Target)	5'GCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATAC 3'
BAR Capture Probe	5'/5ThioMC6D/CCGATGACAGCGACCACGCTCTTGA 3'
BAR Signal Probe	5'AGCCCTGTGCCTCCAGGGACTTCAG/6-FAM/-3'
BAR Adapter (Target)	5'ACCTGCTGAAGTCCCTGGAGGCACAGGGCTTCAAGAGCGTGGTCGCTGTCATCGGGCTG 3'
CRY Capture Probe	5'/5ThioMC6D/CCGCCTTTTGTGCTCTTTCTAAATC 3'
CRY Signal Probe	5'ATATTCTGCCTCAAAGGTTACTTCT/6-FAM/-3'
CRY Adapter(Target)	5'TCCGGCAGAAGTAACCTTTGAGGCAGAATATGATTTAGAAAGAGCACAAAAGGCGGTGAATG 3'

2.2.9 Slide Scanning and Image Analysis

Slides were scanned on Zeiss LSM 510 confocal laser scanning microscope (Jena, Germany) using a low magnification (4X, NA 0.1) objective at 1024x1024 resolution and 12-bit dynamic range. Experiments were conducted in duplicates. Net fluorescence intensity values were calculated by subtracting background intensity from spot intensity using Image J(Abramoff et al., 2004) software. Figure 2.3 summarizes the scanning and analysis process.

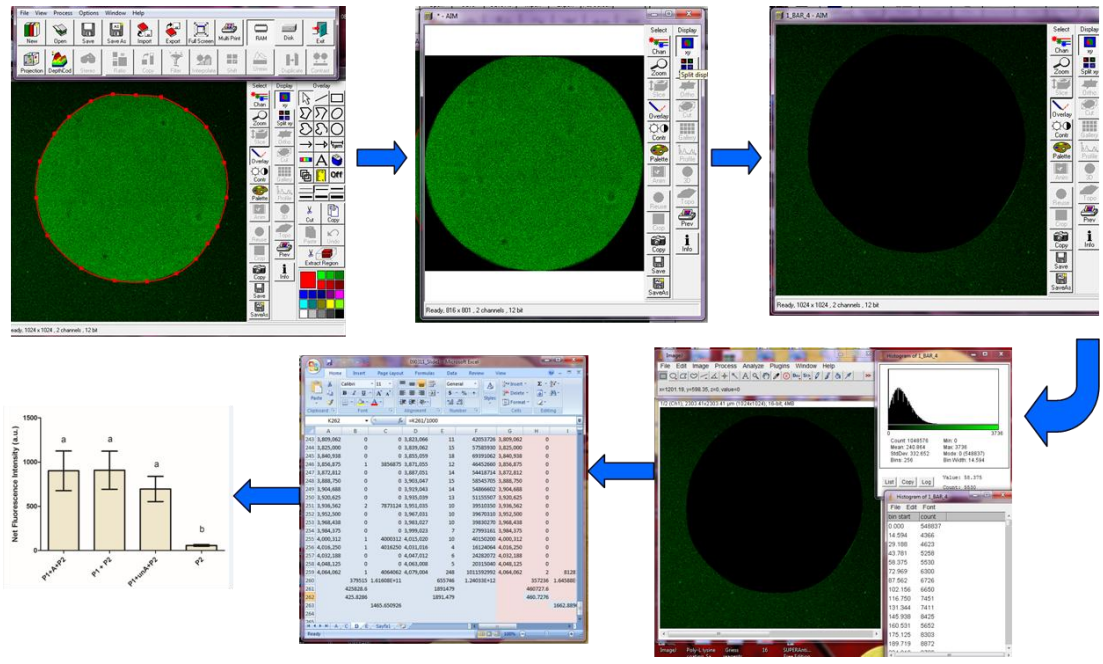

Figure 2.3 Schematic representation of scanning and analysis of the spots

2.2.10 Statistical Analysis

For the statistical analysis of the color intensity the GraphPad Prism software was used. The mean values and standard error of means (SEM) of replicates were

calculated and the variance in mean values of different treatments was evaluated in one way analysis of variance (ANOVA) at 95 % confidence interval.

CHAPTER 3

RESULTS AND DISCUSSION

In this study, a DNA array platform has been developed which comprised of a glass surface on which capture oligonucleotide probe (P1) was immobilized, an unlabeled target nucleic acid (Adapter) which is a part of 35S promoter of Cauliflower Mosaic Virus (CaMV) and a fluorescein-tagged signal oligonucleotide probe. Both capture and signal probes were designed to partly hybridize to the target sequence in a sandwich assay format. The platform was optimized in terms of surface coating agents, cross-linkers for the immobilization of capture probe, and hybridization conditions. Moreover, standardization of probe concentrations, and the platform's sensitivity and selectivity were also studied.

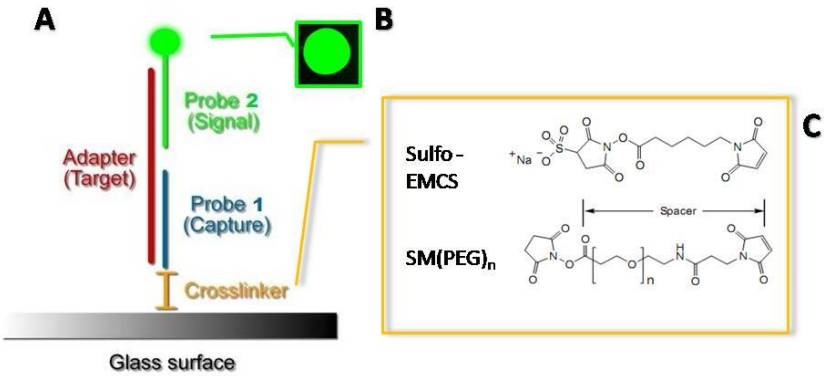

Figure 3.1 Schematic representation of the DNA array platform A- Sandwich platform consists of a surface immobilized oligonucleotide (Probe 1, 25mer) which is designed to partly hybridize to an unlabeled target sequence (Adapter, 52mer) which in turn is detected by hybridization to a second labeled probe (Probe 2, 26mer) B- A typical spot morphology acquired with the laser scanning microscope at 4X magnification. C- Heterobifunctional crosslinkers used in surface activation.

3.1 Determination of Solid Support

In this study, glass slides were chosen as the solid support. Essentially, glass supports have been employed by many research groups (Fodor et al., 1991), (Guo et al., 1994) in the fabrication and use of peptide and oligonucleotide arrays. These groups also showed that the glass supports are well-suited for the fluorescence detection, which is essential and important in this study. In addition, glass slides are easy to obtain and inexpensive support media, possessing a relatively homogeneous chemical surface which is amenable to chemical modification using very versatile and well developed silanization chemistry.

3.2 Surface coating agent choice

In the platform desing, determination of DNA (capture probe, P2) attachment strategy on the solid support (glass slide) was a crucial step. There are mainly two strategies that can be employed: Adsorption or Covalent immobilization. Among them the former is the simplest one as it eliminates possible nucleic acid alterations. In fact, the attachment of the probe to the surface is based on the ionic interaction between negatively charged DNA and positively charged surface coating (Sassolas et al., 2008) (Sassolas et al., 2008). On the other hand, covalent immobilization of DNA molecules on a surface is a more commonly used technique. The main reason for this widespread use is the more proper distribution of the immobilized nucleic acids on the surface (Du et al., 2005). An improper localization of the capture probe on a solid surface will prevent further binding of target sequences or ligands. We determined covalent immobilization as the attachment strategy for the capture probe which requires chemical modification of the solid surface. In this study, two different surface coating agents were tried: 3- Aminopropyl-3-methoxysilan (APTMS); poly L-lysine.

Silanes are monomeric silicon chemicals which can coat mineral components like glass, mica surfaces through their hydroxyl groups by means of a hydroxylation reaction called silanization. In addition, there is a subgroup of silane compounds

called, organo-functional silanes which are molecules carrying two different reactive groups one of which is an organo-functional group, such as epoxy, amino, on their silicon atom as a result; they can react and couple with different materials.(Buyl, 2002)

Inserting amine groups onto the surface is the most common technique in order to functionalize solid supports with biological molecules (Fixe et al., 2004). Therefore, constructing of a two dimensional array surface usually involves treatment with an aminosilane reagent to the glass or silicon surface leading to a uniform layer of primary amines. (Guo et al., 1994) (Lamture et al., 1994)

In accordance with these findings, 3-Aminopropyl-3-methoxysilan (APTMS) was chosen as the silanization agent of the platform. Figure 3.2 indicates the reaction between APTMS and glass surface schematically.

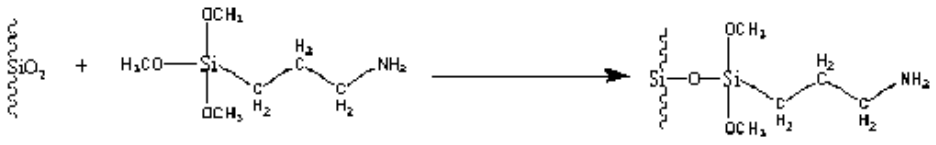

Figure 3.2 Glass surface modifications with 3-Aminopropyl-3-methoxysilan (APTMS).

Based on previous studies [(Guo et al., 1994), (Oh et al., 2002), (Goddard and Erickson, 2009)] a general coating procedure was established, as explained in materials and methods chapter. The effect of different concentrations of APTMS coating on localization of capture probe and thus sandwich hybridization was compared. Below figures indicate the first experiment results of APTMS coating.

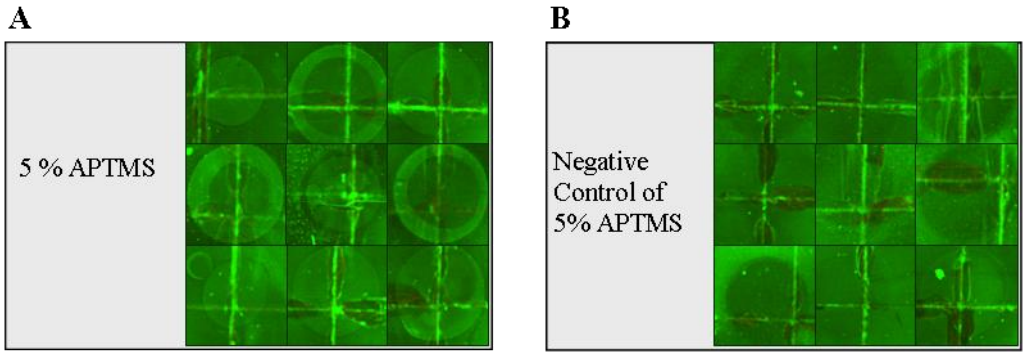

Figure 3.3 Confocal laser scanning results of sandwich hybridization assay on 5% APTMS coated slides: **A**. Nine spots of sandwich hybridization assay performed with target sequence (Adapter) each of which scanned separately. **B.** Negative control of the experiment. Nine spots of sandwich hybridization assay performed with an uncomplimentary sequence instead of Adapter each of which scanned separately.

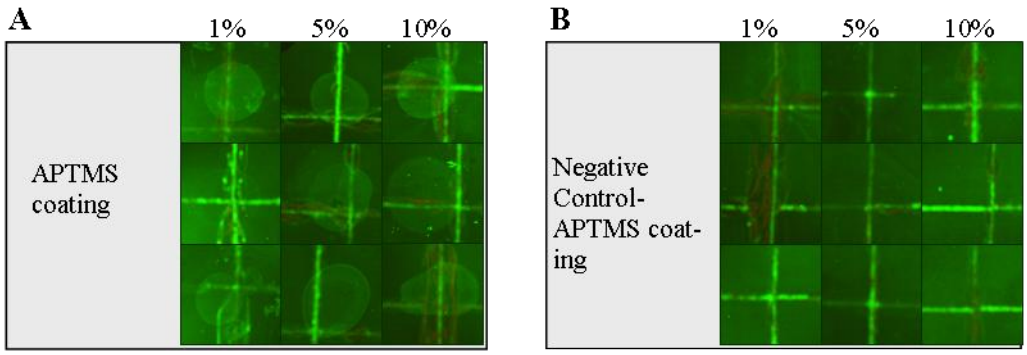

Figure 3.4 Confocal laser scanning results of sandwich hybridization assay on APTMS coated slides with different concentrations: **A**. Each column with three spots represents different APTMS coating concentrations on which sandwich hybridization assay constructed with Adapter. Each spot was scanned separately. **B.** Negative control of the experiment. Each column with three spots represents different APTMS coating concentrations on which sandwich hybridization assay constructed with an uncomplimentary sequence instead of Adapter. Each spot was scanned separately.

Results of APTMS coating clearly demonstrated that there was a significant background signal noise problem. This was most probably due to the uneven distribution of the aminosilane molecules and insufficient post-coating washing steps. APTMS is a commonly used silanization agent and considered as compatible with many of the materials used in a biological researches (Xu et al., 1997; Zhang et al., 2007). Therefore, improving the coating and application procedure of APTMS on glass slides was preferred instead of changing the chemical. First, the application of Piranha Solution, as described in chapter three, was renewed. The composition of the solution was not changed yet the application time and incubation temperature were increased in order to increase the reactive hydroxyl groups on the surface. In addition to this, slides were washed with acetone after APTMS coating in order to remove cloudy appearance on the surface. Beyond these, as described before, labeling of slides had been performed by scratching the back surface of the glass by a diamond tipped glass cutter. These scratches could create very significant amount of background signal which might interfere with the fluorescent signal.

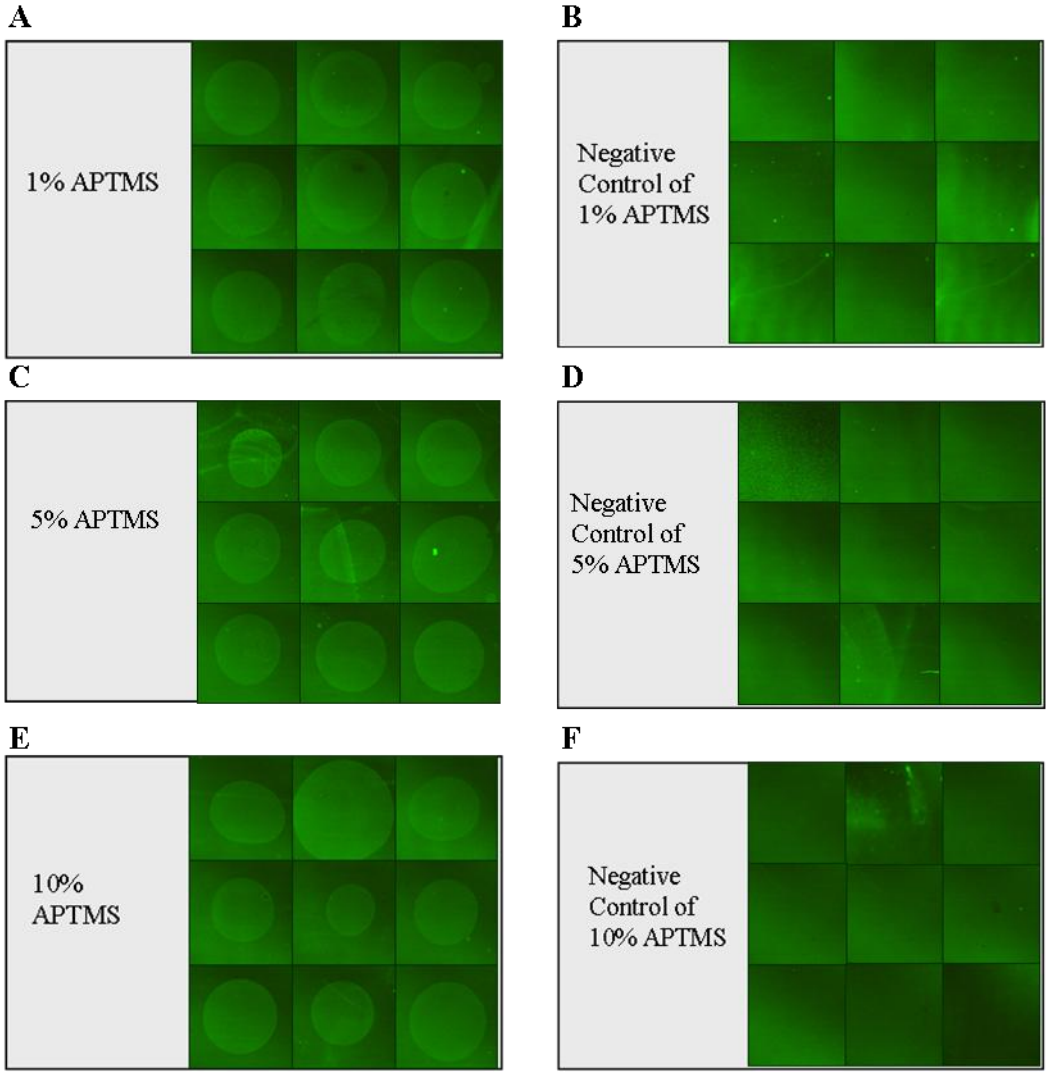

Figure 3.5 Confocal laser scanning results of sandwich hybridization assay on APTMS coated slides with different concentrations: **A,C,E** Nine spots of sandwich hybridization assay performed with target sequence (Adapter) each of which was scanned separately. APTMS coating concentrations of the glass slides are 1, 5, and 10 %, respectively. **B,D,F** Negative control of the corresponding experiment. Nine spots of sandwich hybridization assay performed with an uncomplimentary sequence instead of Adapter each of which scanned separately. APTMS coating concentrations of the glass slides are 1, 5, and 10 %, respectively.

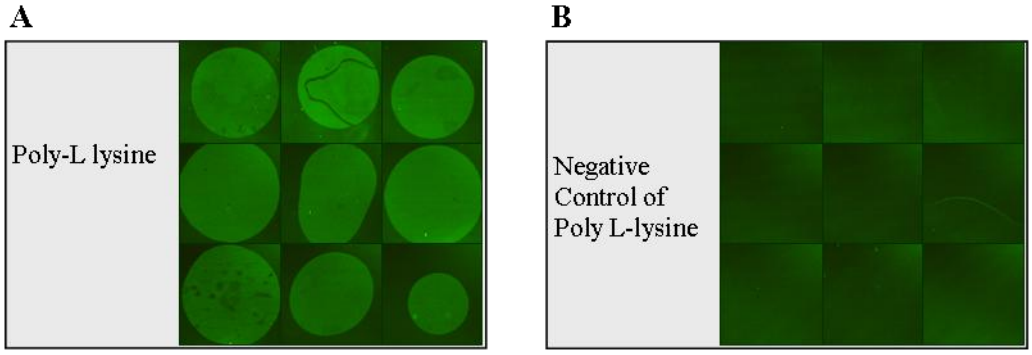

Figure 3.6 Confocal laser scanning results of sandwich hybridization assay on commercially purchased Poly-L lysine coated slides. **A**. Nine spots of sandwich hybridization assay performed with target sequence (Adapter) each of which scanned separately. **B.** Negative control of the experiment. Nine spots of sandwich hybridization assay performed with an uncomplimentary sequence instead of Adapter each of which scanned separately.

Like aminosilane coated surfaces, poly-L lysine (PLL) coated glass slides are widely used for microarray analysis (Diehl et al., 2001). By poly-L lysine coating similar to the case of APTMS, the slides become covered with free primary amines. Former microarray studies used PLL coated glass microscope slides for initial ionic attachment of the negatively charged phosphate groups in the DNA backbone which is actually an ionic interaction rather than immobilization. In accordance with, there are several studies in literature which claim that aminosilane coating is much more chemically stable and has much less background noise than PLL coating (Du et al., 2005). On the other hand, in this study as inferred from the Figure 3.7, PLL has a better signal intensity over APTMS coating with different concentrations. Similarly, in the research carried out by Benters and coworkers a novel dendritic linker system for the immobilization of probes on DNA microarray platforms was studied (Benters et al., 2002) They have proved PLL coating slides to have more effective immobilization profile than aminosilane coated ones both of which were used as a control for the novel system. In the light of this information it could be said that,

depending on the application procedure and conditions, one type of coating may predominate the other one. In this study, in addition to better signal intensity profile of PLL coating, because aminosilane coating is a more time consuming procedure and it has more difficult operating steps like dealing with hazardous solutions, such as Piranha Solution, PLL coating was preferred as the coating agent of glass surfaces.

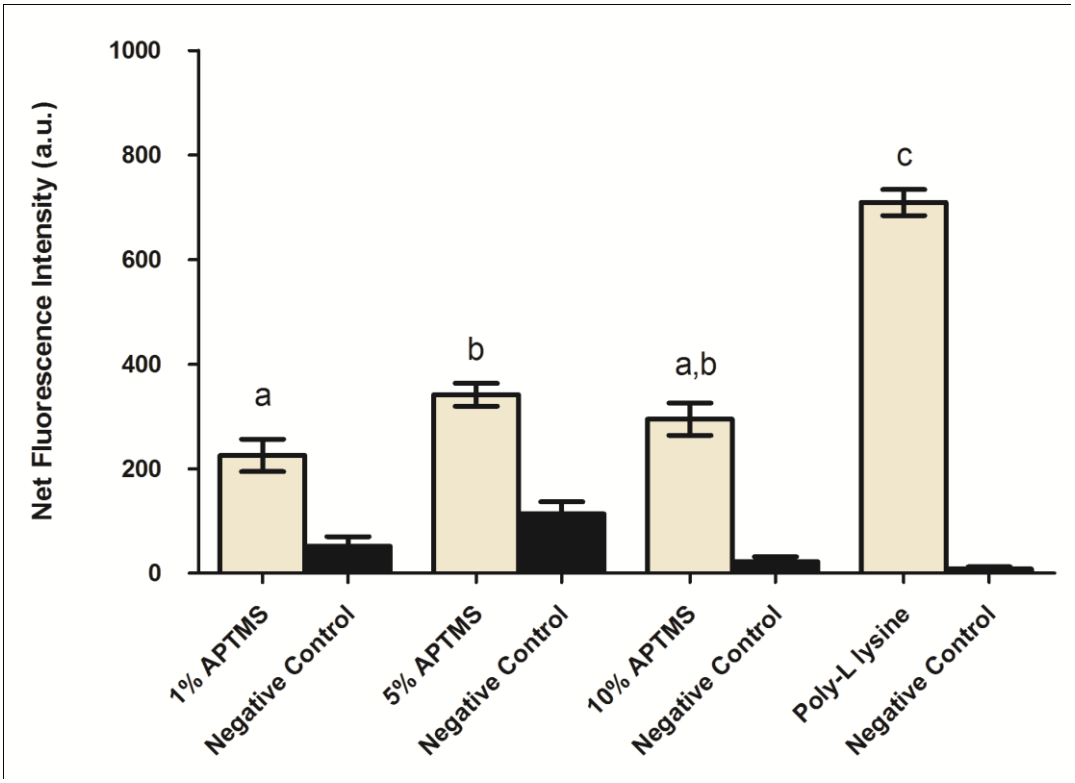

Figure 3.7 Signal intensity comparisons of Poly-L lysine coating and APTMS coating with different concentrations. The height of the histograms represents the average fluorescence pixel intensity of all spots. Net intensity values were calculated as described in materials and method section. Error bars represent standard error of mean calculated from 18 spots. Different letters indicate significant difference at $p < 0.05$.

3.3 Determination of Capture Probe Immobilization

Immobilization of bio-molecules onto solid surfaces is a very crucial step for the production of biochips and biosensors(Xiao et al., 2004). As stated previously the covalent bonding of DNA on the slide surface usually provides good stability and reproducibility. On the other hand, physical attachment of oligonucleotides onto a solid surface has some advantages. First, this method does not require modifications of DNA probes. It is easy to handle and cost effective.

Attachment of DNA probes on to solid surfaces by using heat (baking) is a conventional method used from 1975 (Southern, 1975). In fact, it is a fixation method, performed with baking nucleic acids at high temperature leading to the formation of strong ionic interactions between the surface and the DNA molecules. Besides advantages, baking are nonspecific fixation techniques, leading to a variety of unpredictable orientations of immobilized nucleic acids on the glass surface. This often results in immobilization of an unnecessary fraction of bio-molecules with improper orientation, thus hindering their binding with ligands and causing problems with other downstream biological assays (Du et al., 2005). In the given study fixation of capture probe on glass surface was performed by incubation the spotted slides at 80° C for 1 hour. As well as sandwich hybridization (P1+A+P2), three different negative controls are included into experiment. One of them is sandwich hybridization constructed with an uncomplimentary target sequence (P1+unA+P2). In another negative control the interaction of capture probe and signal probe was evaluated (P1+P2). In addition, to determine any interaction between signal probe and surface P2 was applied on the system alone.

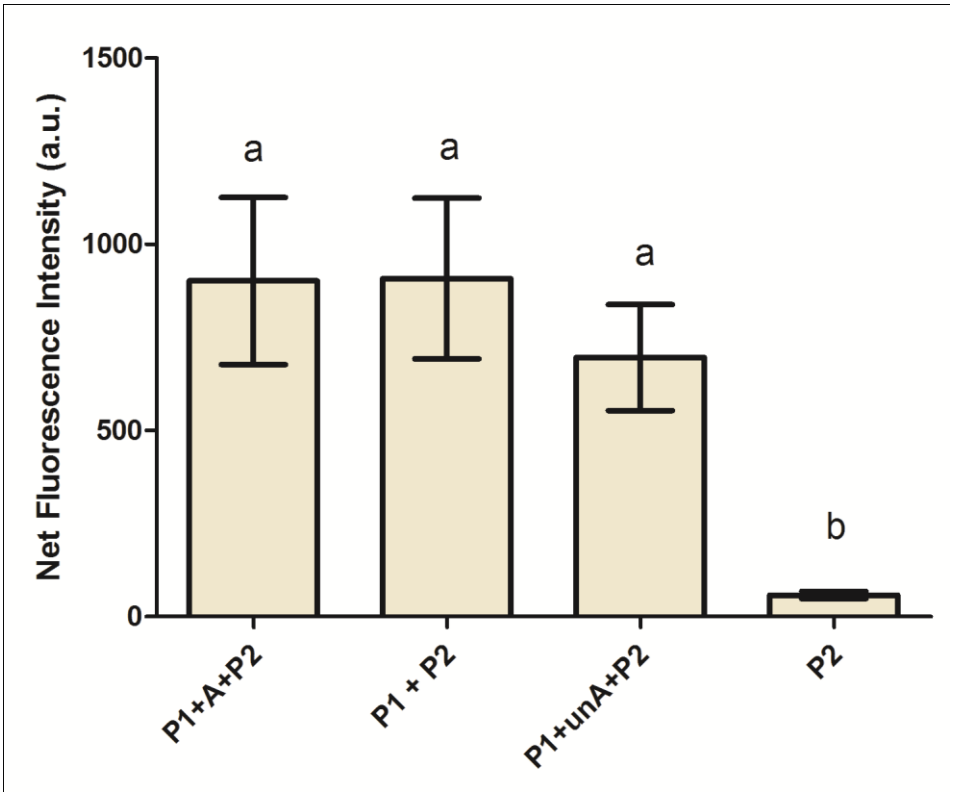

Figure 3.8 Signal intensity comparisons of Sandwich hybridization with negative controls where capture Probe P1 was attached to surface via baking method. The height of the histograms represents the average fluorescence pixel intensity of all spots. Net intensity values were calculated as described in materials and method section. Error bars represent standard error of mean calculated from 18 spots. Different letters indicate significant difference at $p < 0.05$.

Heterobifunctional cross-linkers (HBC) are reagents modified with two hetero reactive groups that connect two molecules with different functionalities. Immobilization of nucleic acids by means of heterobifunctional cross-linkers (HBC) on solid support was first established by Chrisey and coworkers(Chrisey et al., 1996). They used HBC for the attachment of thiol modified DNA on to the aminosilane coated film and compared the results with UV crosslinking which is one of the conventional DNA fixation methods. In this study, similar to the described research,

coupling of the cross-linker to surface and immobilization of Probe 1 is carried out in a similar fashion. The chemistry lying under the reaction can be described as fallows; the NHS ester end of the crosslinker couples with primary amines on the surface at pH 7-9 to form stable amide bonds. Concurrently, maleimide group reacts with -SH groups on the Probe 1 at pH around 6.5-7.5 and forms stable thio-ether linkages. In this respect, an oriented coupling between the modified surfaces and the reactant that has to be immobilized is guaranteed (Heise and Beier 2006).

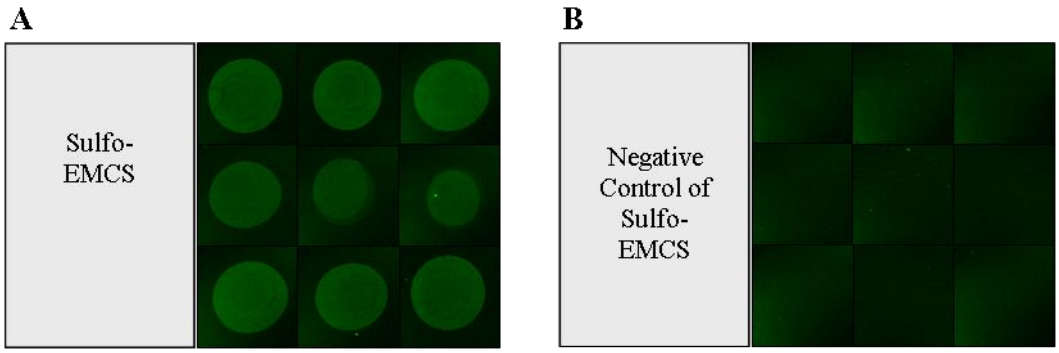

Figure 3.9 Confocal laser scanning results of sandwich hybridization assay performed with crosslinker Sulfo-EMCS. **A**. Nine spots of sandwich hybridization assay performed with target sequence (Adapter) each of which scanned separately. **B.** Negative control of the experiment. Nine spots of sandwich hybridization assay performed with an uncomplimentary sequence instead of Adapter each of which scanned separately.

In this part, four different heterobifunctional cross-linkers were used for the immobilization of capture probe on to surface. They are namely, Sulfo-EMCS, SM(PEG)2, SM(PEG)6, SM(PEG)12 .

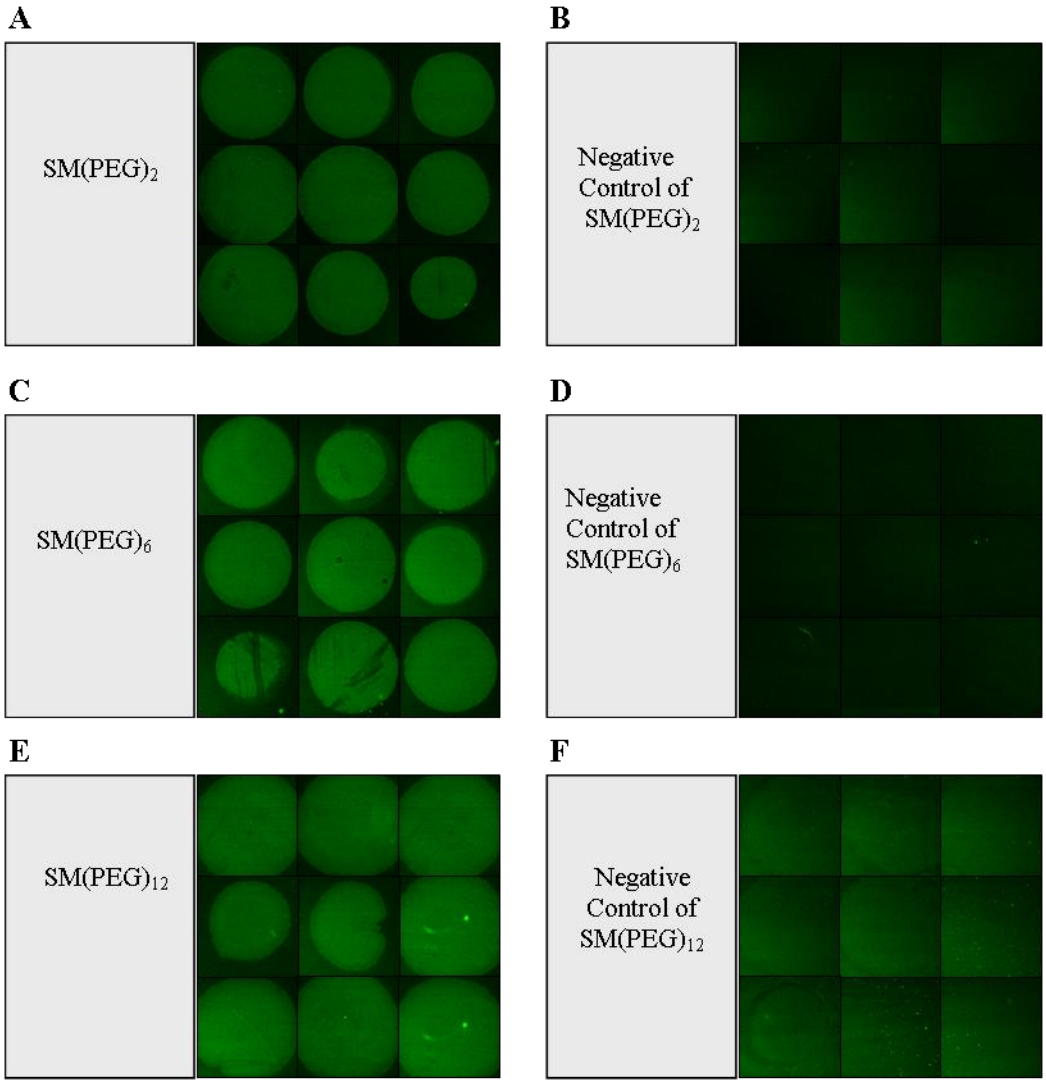

Figure 3.10 Confocal laser scanning results of sandwich hybridization assay performed with different lengths of SM(PEG)n type crosslinkers: **A,C,E** Nine spots of sandwich hybridization assay performed with target sequence (Adapter) each of which was scanned separately. Crosslinkers that are used for the immobilization P1 are $SM(PEG)_2$, $SM(PEG)_6$, and $SM(PEG)_{12}$, respectively. **B,D,F** Negative control of the corresponding experiment. Nine spots of sandwich hybridization assay performed with an uncomplimentary sequence instead of Adapter each of which scanned separately. Crosslinkers that are used for the immobilization P1 are $SM(PEG)_2$, $SM(PEG)_6$, and $SM(PEG)_{12}$, respectively.

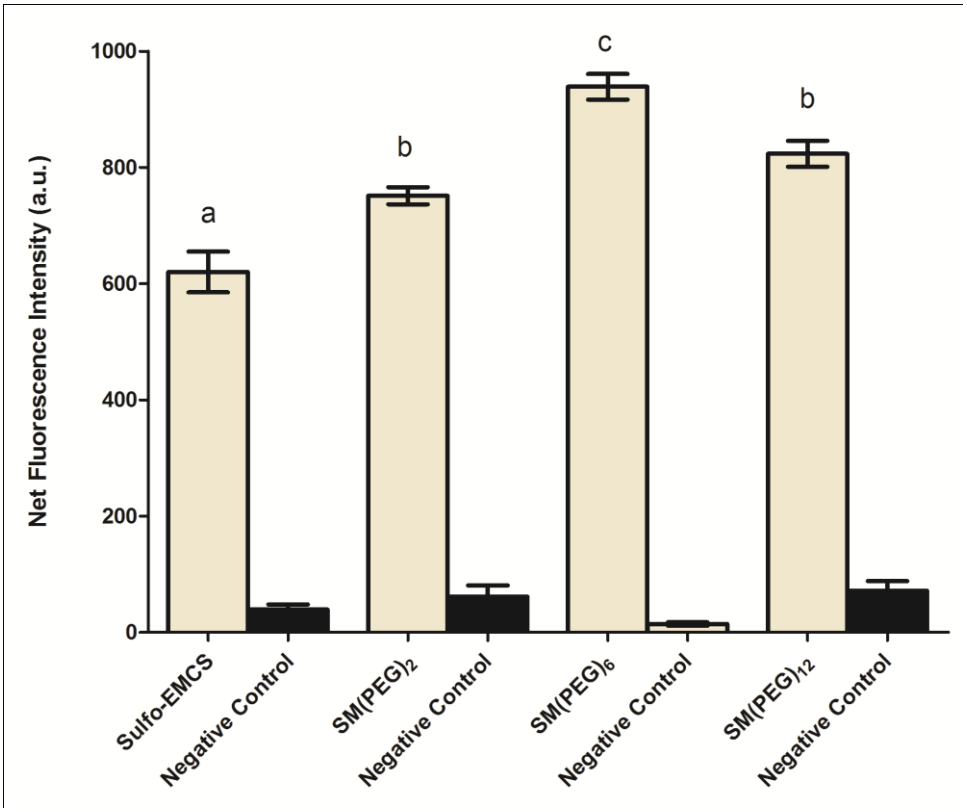

Figure 3.11 Signal intensity comparisons of heterobifunctional crosslinkers with different arm-lengths. The height of the histograms represents the average fluorescence pixel intensity of all spots. Net intensity values were calculated as described in materials and method section. Error bars represent standard error of mean calculated from 18 spots. Different letters indicate significant difference at $p < 0.05$.

Surface immobilization of capture probe was achieved by two methods; baking and heterobifunctional crosslinker. Following the washing steps and hybridization with the adapter and signal probe, slides were scanned for signal comparison.

As inferred from the Figure 3.8 a significant amount of false positive results were obtained from the negative controls of the experiment. This was an expected result since the baking method covalently links the oligonucleotides to the surface from their ends or sides which may result in probe orientations unfavorable for the

hybridization. On the other hand, a crosslinker can only link the probe from its thiolated end resulting in a more uniform immobilization and higher hybridization efficiency.

Surface distance of capture probes has been shown to play an important role in hybridization efficiency (Bajaj, 2000; Shchepinov et al., 1997; Southern et al., 1999). Therefore, comparison of hybridization efficiency of capture probe (P1) is an important parameter. Four types of cross-linkers with 9.4, 17.6, 32.5 and 53.4 Å arm-lengths were used for the comparison. As shown in Figure 3.11, increasing probe-surface distance also increased the hybridization signal up to a point. As the distance was further increased, a loss in signal strength was observed. Among all the crosslinkers, SM(PEG)6 (32.5 Å arm length) provided the best hybridization signal. Increasing hybridization signal as a function of the probe surface distance can be attributed to higher probe flexibility and consequent increase in target-probe hybridization. On the other hand, decreased signal as a result of additional probe-surface distance as in the case of 53.4 Å arm-length cross-linker may be due to molecular motions which do not favor probe-target complex formation such as bending towards slide surface or probe-probe entanglement.

3.4 Capture Probe (P1) concentration optimization

The density of the capture probe on the array surface is one of the most critical parameters determining the hybridization efficiency and array sensitivity (Bajaj, 2000; Diehl et al., 2001; Zhou et al., 2004). Increasing the probe density on the surface also increases the amount of target sequence captured on the slide. However, further increase in the density may reduce the hybridization efficiency due to steric factors. Therefore, we investigated the effect of probe concentration on the signal strength by spotting 0.5 µL of 1 µM, 10 µM and 20 µM capture probe solutions on the slides.

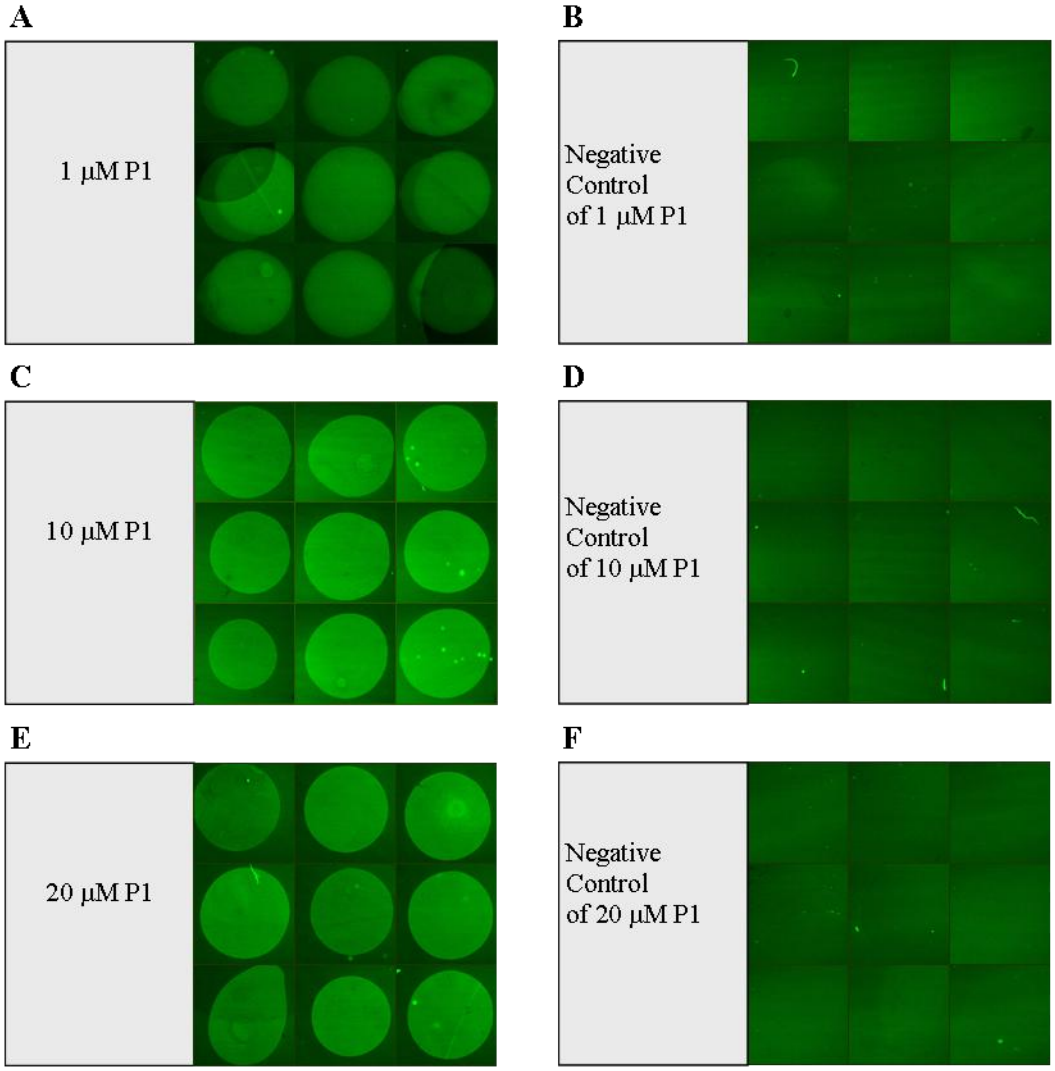

Figure 3.12 Confocal laser scanning results of sandwich hybridization performed with different concentrations of capture probe (P1) **A, C, E** Nine spots of sandwich hybridization assay performed with target sequence (Adapter) each of which scanned separately.P1 concentrations of the slides are 1, 10, 20 µM, respectively. **B, D,F** Negative control of the experiment. Nine spots of sandwich hybridization assay performed with an uncomplimentary strand each of which scanned separately. P1 concentrations of the slides are 1, 10, 20 µM, respectively.

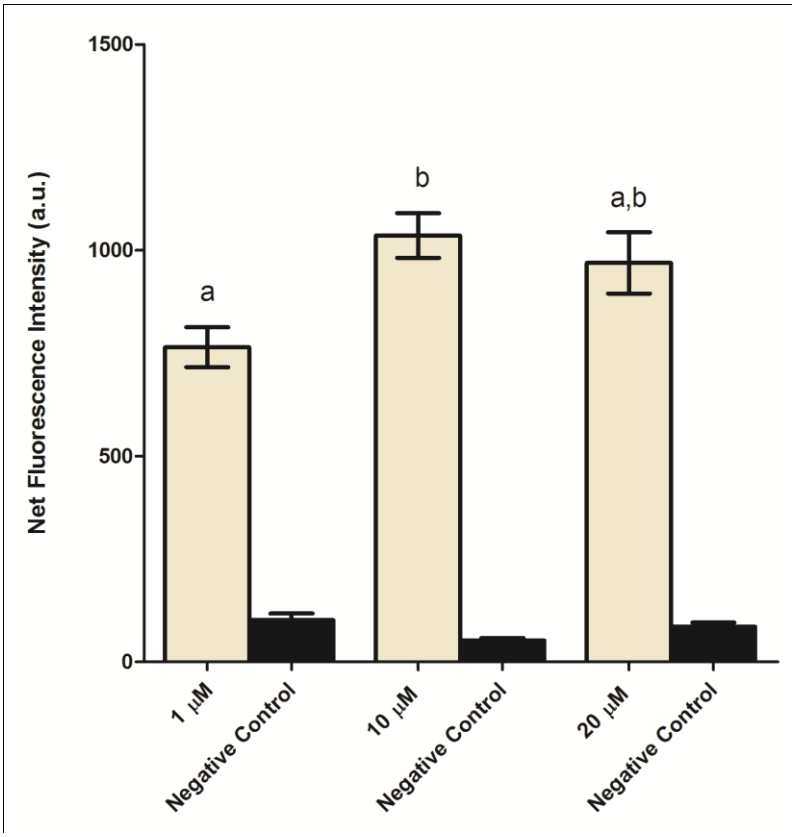

Figure 3.13 Hybridization signal intensity as a function of capture probe (P1) concentration. The height of the histograms represents the average fluorescence pixel intensity of all spots. Net intensity values were calculated as described in materials and methods chapter. Error bars represent standard error of mean calculated from 18 spots. Different letters indicate significant difference at $p < 0.05$.

Immobilization was achieved via the $SMPEG_2$ cross-linker. The results summarized in Figure 3.13 indicated that increasing the spotted probe concentration above 10 µM has no significant effect on the hybridization signal strength. In fact, increasing the density of capture probe on the surface may negatively affect the hybridization efficiency (Bajaj, 2000). For this reason, 10 µM P1 was selected as standard capture probe concentration for the developed platform. Various experimental studies report

a drop in hybridization efficiencies with increase in probe density. (Peterson et al., 2001; Shchepinov et al., 1997; Steel et al., 1998)

They also indicate the existence of an optimal probe density to achieve maximum hybridization signal for a given experimental setup. In a similar manner it can be concluded that 10 µM P1 was selected as standard capture probe concentration for the developed platform.

3.5 Signal Probe (P2) Concentration Optimization

Concentration of the signal probe in the hybridization solution is another important factor determining the strength of the acquired signal. In accordance with, the signal intensities obtained from the hybridization solutions containing 20, 40 and 50 µM 6-FAM tagged signal probe was compared. As shown in Figure 3.15, hybridization signal increased as the signal probe concentration changed from 20 to 50 µM. Since the difference between 40 and 50 µM was not significant, we selected the former value as an ideal signal probe concentration in the sandwich assay where the capture probe concentration was 10 µM.

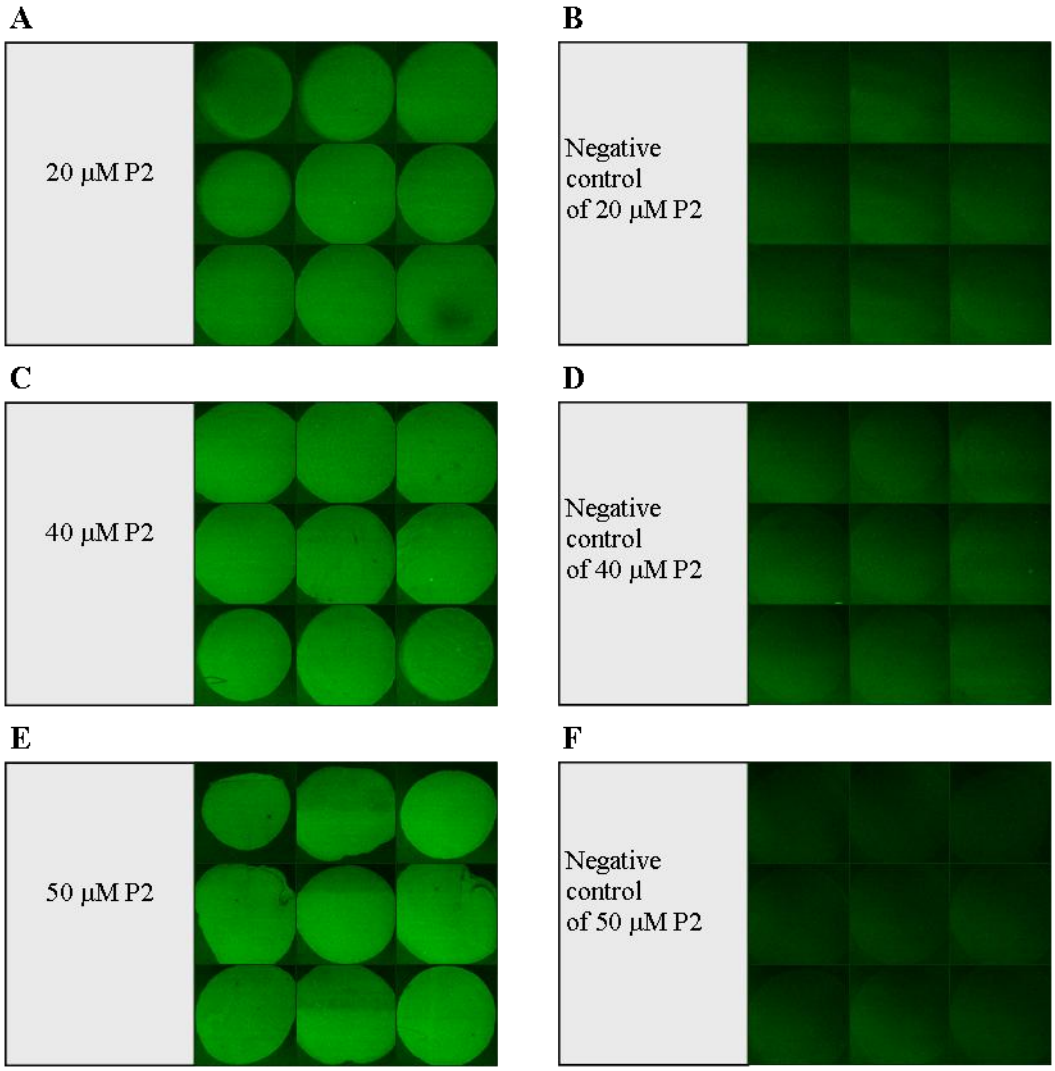

Figure 3.14 Confocal laser scanning results of sandwich hybridization performed with different concentrations of signal probe (P2) where the P1 concentration were kept fixed(10 µM) **A, C, E**. Nine spots of sandwich hybridization assay performed with target sequence (Adapter) each of which scanned separately. P2 concentrations of the slides are 20, 40, 50 µM, respectively. **B,D, F.** Negative control of the experiment. Nine spots of sandwich hybridization assay performed with an uncomplimentary strand each of which scanned separately. P2 concentrations of the slides are 20, 40, 50 µM, respectively.

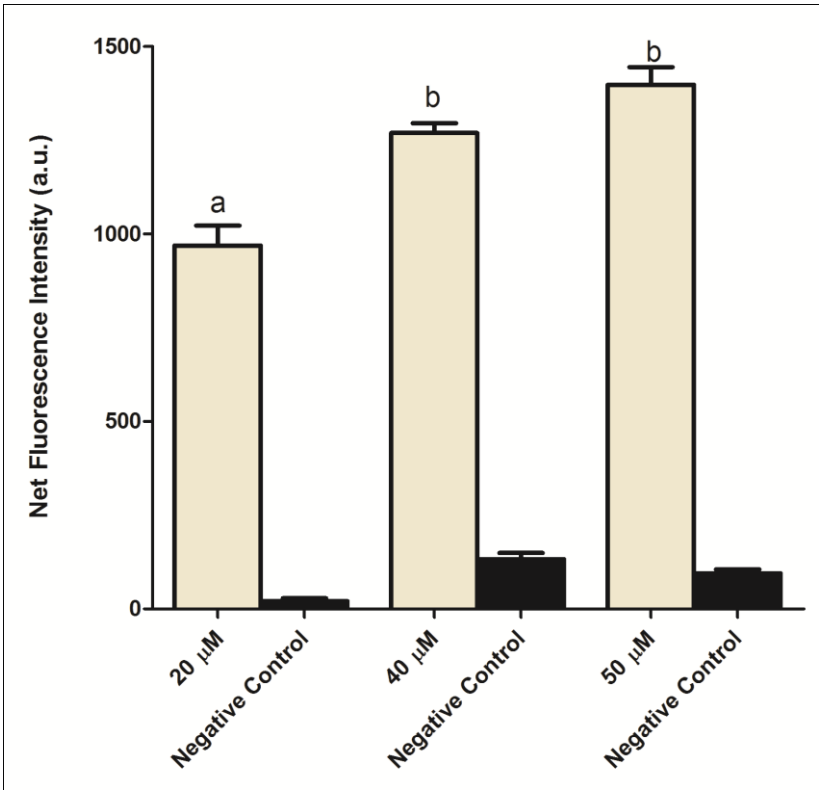

Figure 3.15 Hybridization signal intensity as a function of signal probe (P2) concentration. Standard experimental protocol was conducted at 10 μM capture probe concentration. The height of the histograms represents the average fluorescence pixel intensity of all spots. Net intensity values were calculated as described in materials and method section. Error bars represent standard error of mean calculated from 18 spots. Different letters indicate significant difference at $p < 0.05$.

3.6 Optimization of Hybridization Conditions

Unlike direct hybridization assay formats, which is formed by two components (probe and target), a sandwich construct has three components forming two duplexes. For this reason optimizing hybridization parameters is a very crucial step for the construction of the array systems. Mainly, three parameters were evaluated for the

optimization studies: Hybridization temperature, duration, probe- adapter application sequences.

3.6.1 Optimization of Hybridization Duration

Another important hybridization parameter is the duration of probe-target incubation. To determine the optimum hybridization duration, a serious of experiment carried out at 2.5, 5, 12 and 24-hour time periods. The highest net fluorescence was obtained after 12-hour incubation. Reducing the hybridization duration from 12 to 2.5 hours results in only 50% signal loss. Surprisingly, a significant decrease in the signal is observed as the duration was extended to 24 hours. This may be due to the stability of fluorescent tag under experimental conditions.

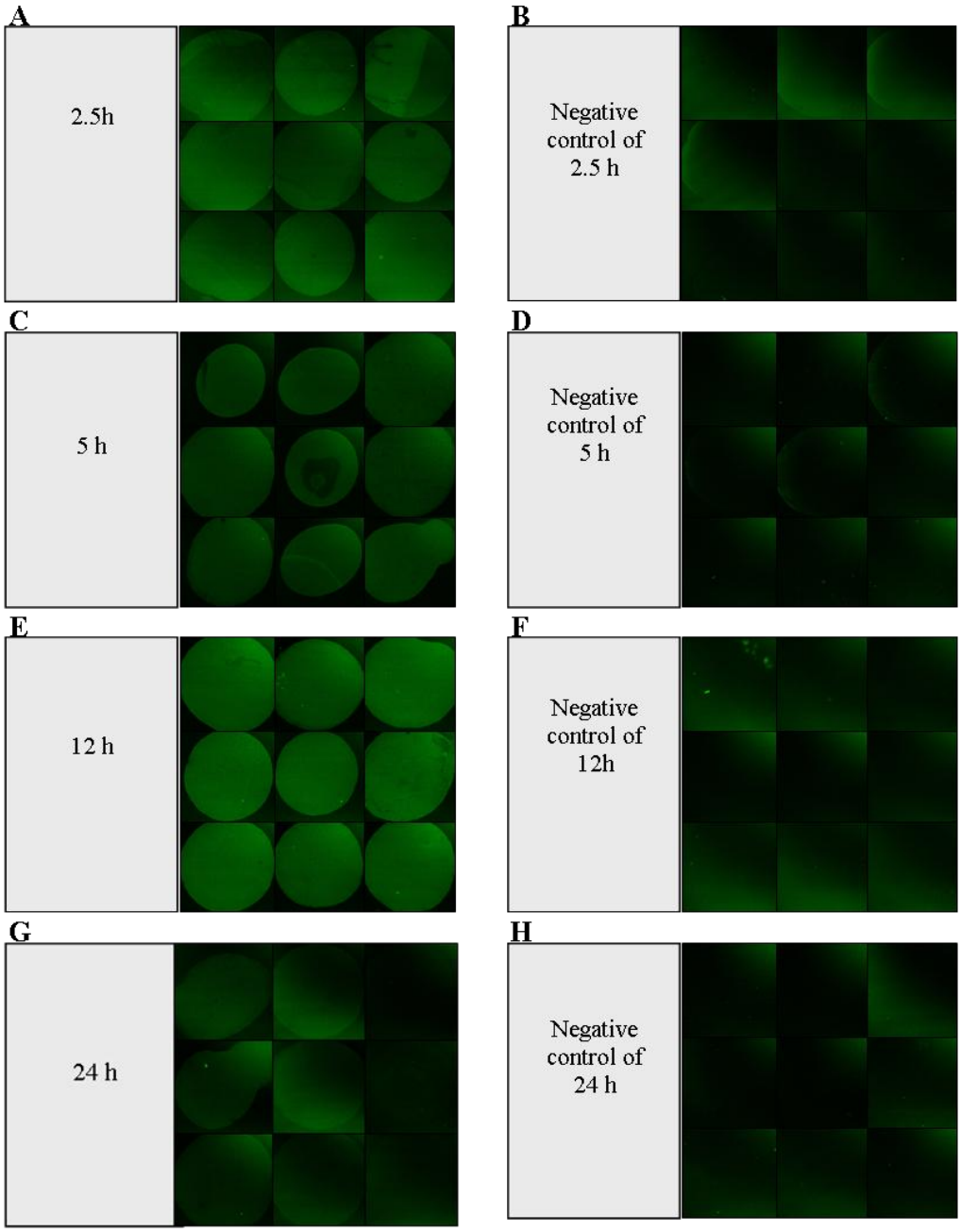

Figure 3.16 Confocal laser scanning results of sandwich hybridization performed with different hybridization durations **A, C, E, G**. Nine spots of sandwich hybridization assay performed with target sequence (Adapter) each of which scanned separately. Hybridization duration of the slides are 2.5, 5, 12, 24 hours, respectively. **B, D, F, H.** Negative control of the experiment. Nine spots of sandwich hybridization assay performed with an uncomplimentary strand each of which scanned separately. Hybridization duration of the slides are 2.5, 5, 12, 24 hours, respectively

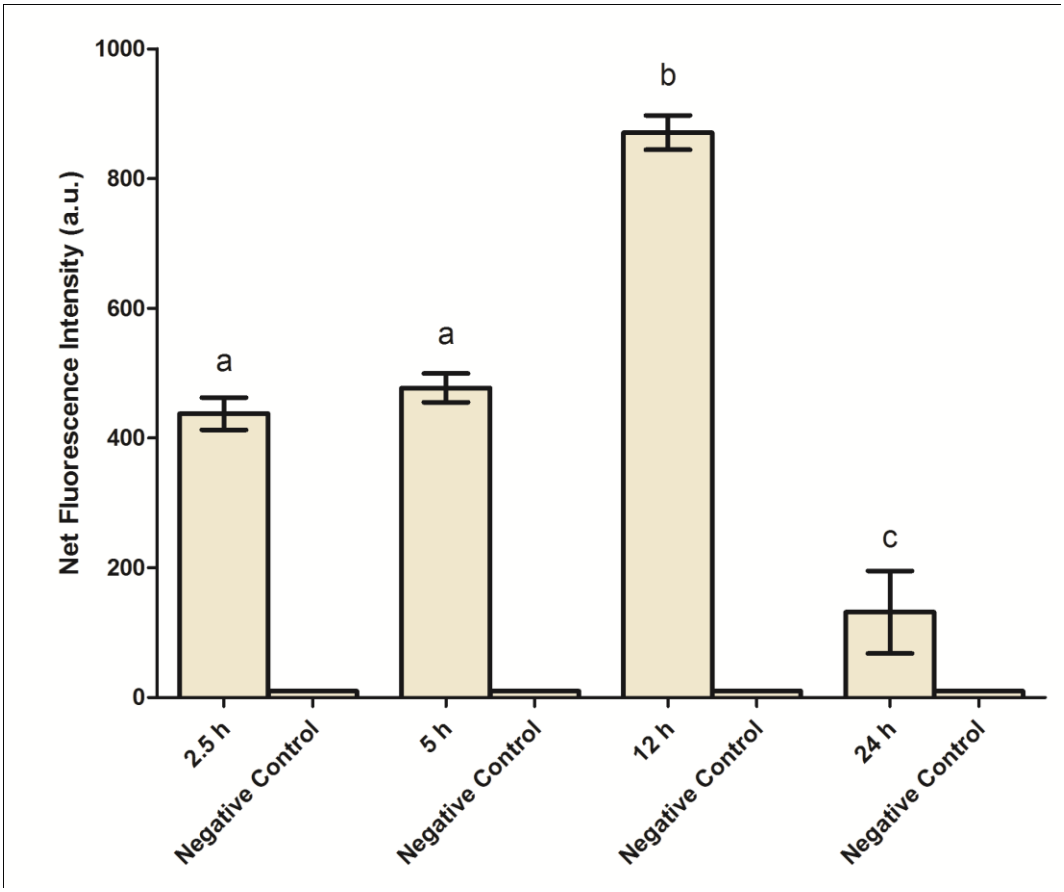

Figure 3.17 Hybridization signal intensity as a function of hybridization duration. The height of the histograms represents the average fluorescence pixel intensity of all spots. Net intensity values were calculated as described in materials and method section. Error bars represent standard error of mean calculated from 18 spots. Different letters indicate significant difference at $p < 0.05$.

3.6.2 Optimization of Hybridization Temperature

In microarray studies, low stringency hybridization temperature is typically 20°C below the melting temperature (Tm) of the duplex. As the temperature is elevated towards the Tm value, hybridization stringency also increases. We computationally determined the average Tm value of the sandwich complex as 55°C and conducted

temperature experiments at 35, 40 and 45°C. Experiment results indicate that the best signal intensity was obtained from the hybridization carried out at 35 °C. By increasing the temperature of the hybridization, that is when hybridization temperature becomes closer to Tm, selectivity of the binding increases and thus we observed a decrease in signal intensity.

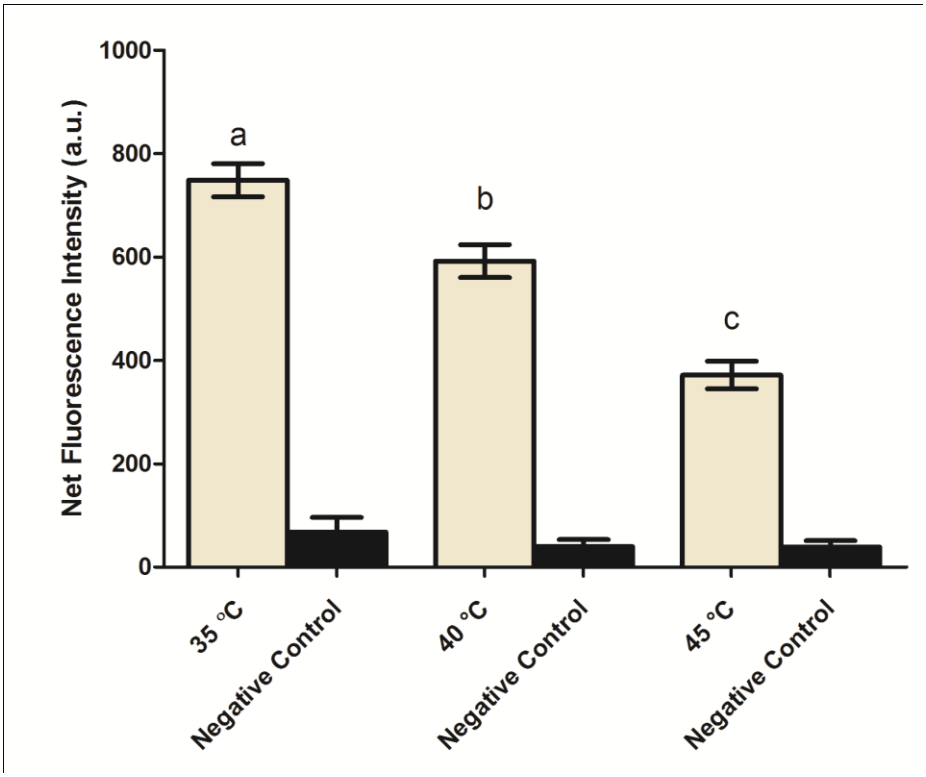

Figure 3.18 Hybridization signal intensity as a function of hybridization temperature. The height of the histograms represents the average fluorescence pixel intensity of all spots. Net intensity values were calculated as described in materials and method section. Error bars represent standard error of mean calculated from 18 spots. Different letters indicate significant difference at $p < 0.05$.

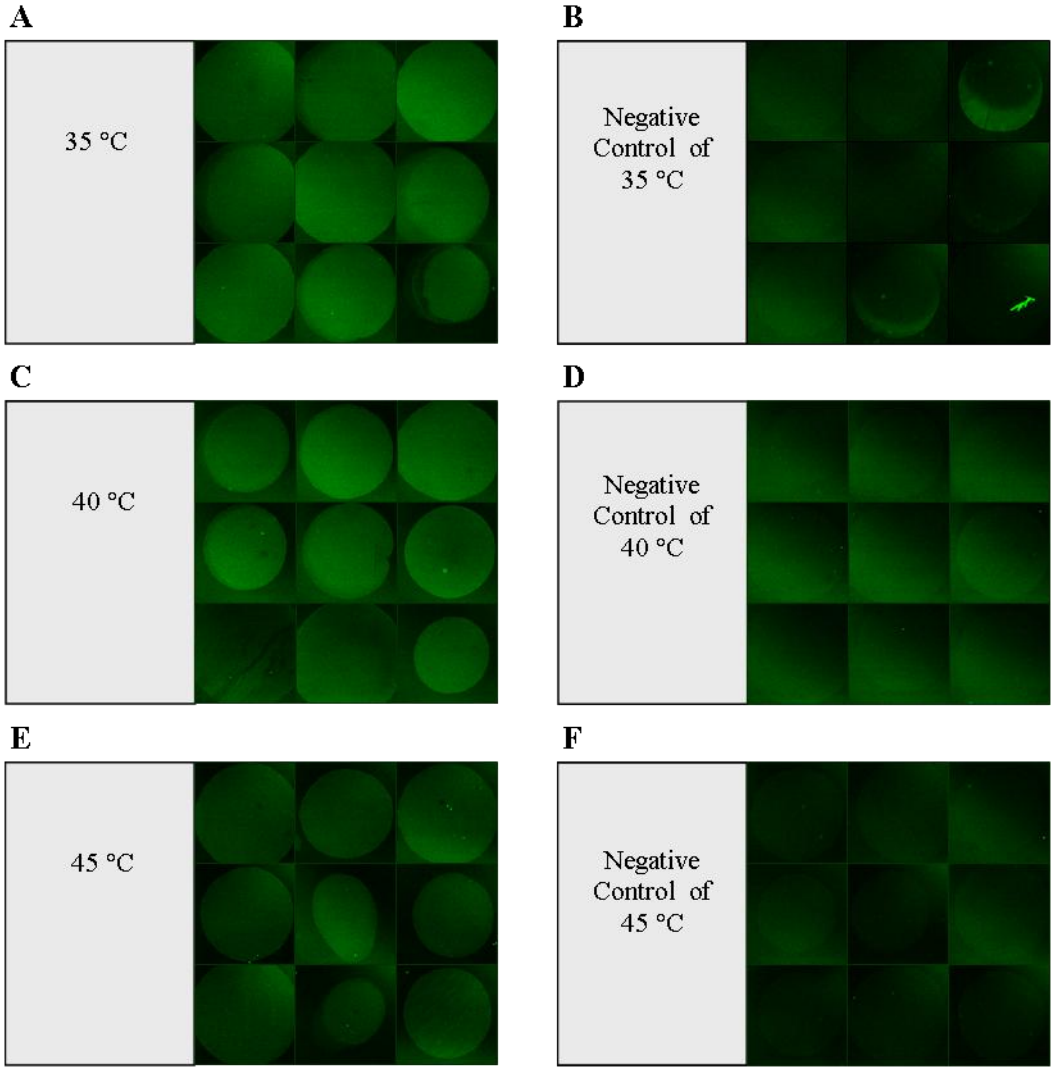

Figure 3.19 Confocal laser scanning results of sandwich hybridization performed with different hybridization temperatures where the other parameters were kept constant. **A, C, E**. Nine spots of sandwich hybridization assay performed with target sequence (Adapter) each of which scanned separately. Hybridization Temperature of the slides are 35, 40, 45°C, respectively. **B, D, F.** Negative control of the experiment. Nine spots of sandwich hybridization assay performed with an uncomplimentary strand each of which scanned separately. Hybridization Temperature of the slides are 35, 40, 45°C, respectively.

3.6.3 Optimization of Hybridization Sequence of the Probes

Unlike direct hybridization assay formats, which is formed by two components (probe and target), a sandwich construct has three components forming two duplexes. This brings up the question of hybridization efficiency as a function of the sequence of the application of probes and adapter in which the duplexes of capture probe-target and target-signal probe form. Three sets of hybridization sequence experiments were conducted to answer this question. In the first set, the target was hybridized to before the addition of signal probe to the hybridization mixture for an additional 5 hour incubation ([P1+A]+P2). The next set was carried out by mixing and incubating all three components for 10 hours (P1+A+P2). Finally, target and signal probes were pre-incubated for 5 hours before applying the mixture on the slide surface for an additional 5 hours in the last set (P1+[A+P2]). Results summarized in Figure 3.22 showed that there was no difference in hybridization signal when capture probe and target had been pre-incubated. However, signal strength slightly increased when the target sequence was pre-incubated with the signal probe (P1+[A+P2]). The main reason for this difference might be related to the secondary structure of the Adapter sequence. Pre-incubation of Adapter with P2 results in hybridization of two strands thus making the other half of the Adapter which is complementary to P1 more available. The same situation is not valid for (P1+A)+P2 case because after incubation of Adapter sequence on P1 immobilized surface a mild washing step was applied to remove unbound Adapter and excess hybridization solution. Most probably, this washing step would result in a loss of adapter-P1 hybrid.

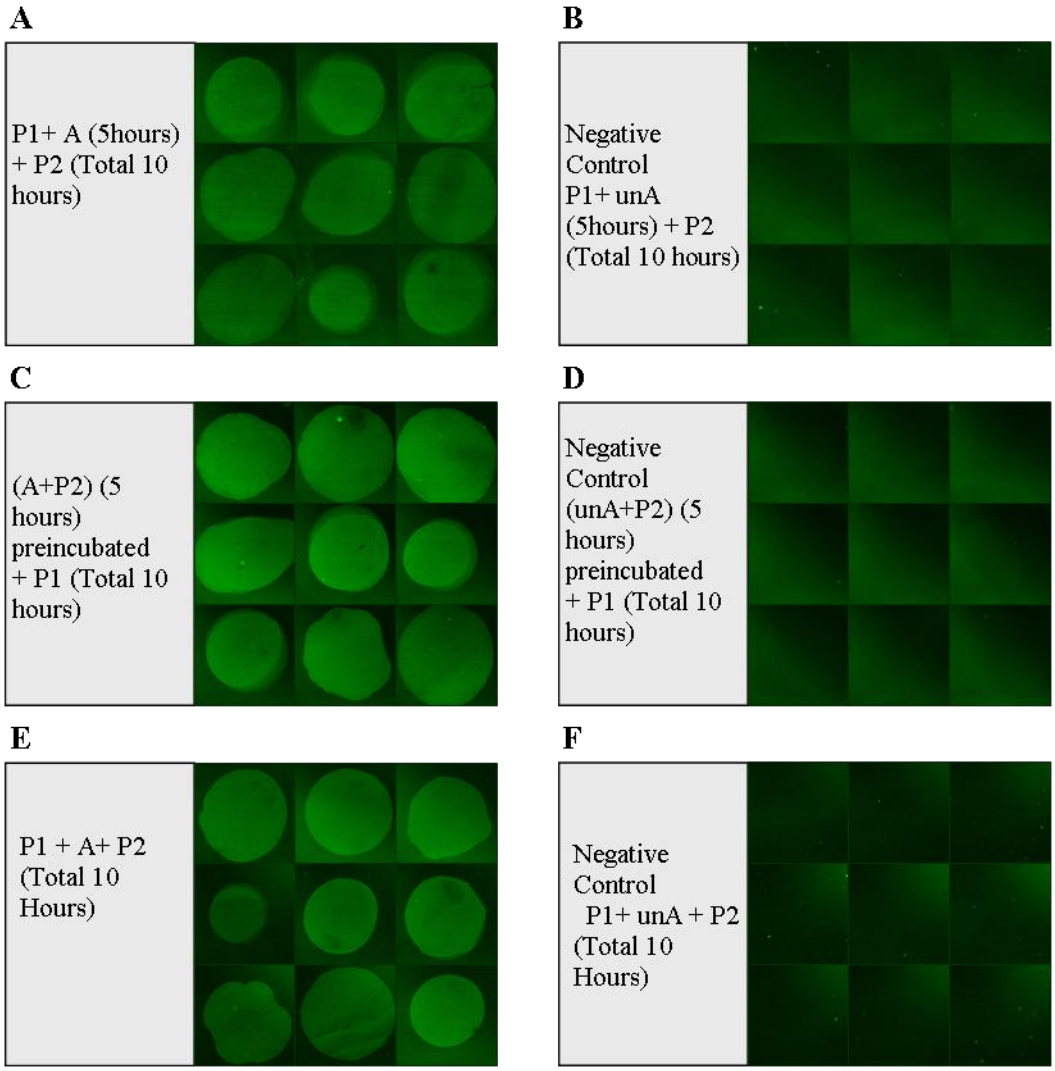

Figure 3.20 Confocal laser scanning results of sandwich hybridization performed with different probes-target hybridization sequence strategies where the other parameters were kept constant. **A, C, E**. Nine spots of sandwich hybridization assay performed with target sequence (Adapter) each of which scanned separately. Hybridization sequences of the slides are ([P1+A]+P2),(P1+[A+P2]) and (P1+A+P2), respectively. **B, D, F.** Negative control of the experiment. Nine spots of sandwich hybridization assay performed with an uncomplimentary strand each of which scanned separately. Hybridization sequences of the slides are ([P1+unA]+P2),(P1+[unA+P2]) and (P1+unA+P2), respectively.

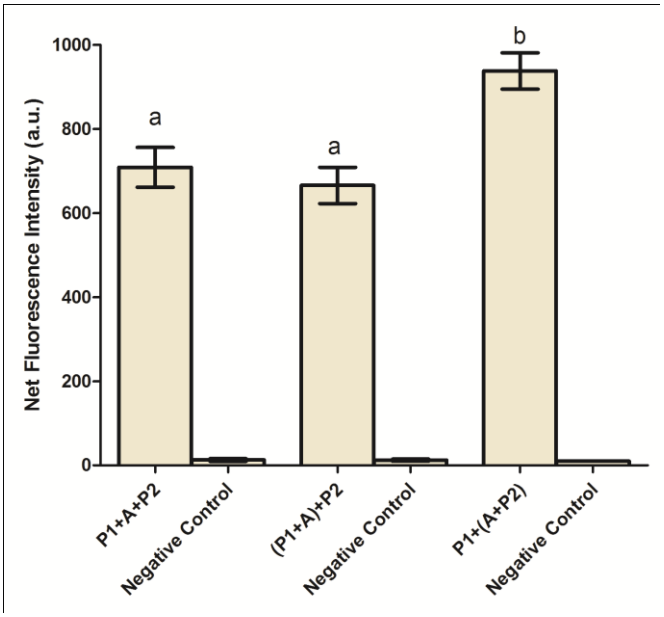

Figure 3.21 Hybridization signal intensity as a function of hybridization sequence of the probes and the target. The height of the histograms represents the average fluorescence pixel intensity of all spots. Net intensity values were calculated as described in materials and method section. Error bars represent standard error of mean calculated from 18 spots. Different letters indicate significant difference at $p < 0.05$.

3.7 Sensitivity of the Array Platform

Following the optimization of the platform, its sensitivity was investigated in the 0.001 to 20 µM target DNA range (Figure 3.23-24). Since we could not observe a detectable signal below 0.01 µM, we reported the sensitivity of the assay at nanomolar level. In the literature various array studies with femtomolar sensitivity were reported.(Santra et al., 2001) However, since our platform is PCR-dependent, the level of sensitivity is considered acceptable for detection of an amplified DNA sequence. Also, for more demanding applications, various signal amplification

strategies can be employed to increase the sensitivity of the platform(Zhao et al., 2003).

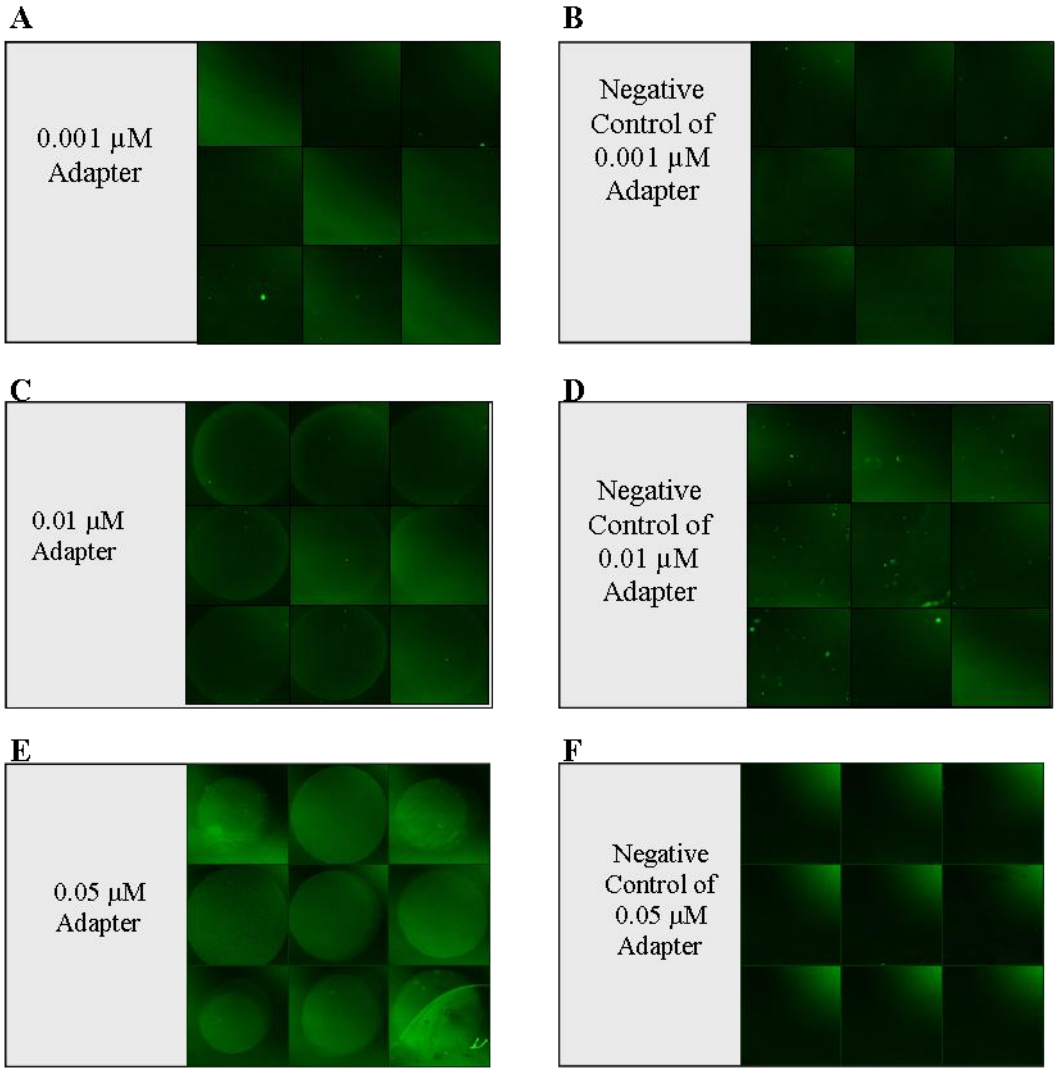

Figure 3.22 Confocal laser scanning results of sandwich hybridization performed with different target sequence (Adapter) concentrations where other parameters were kept constant. **A, C, E**. Nine spots of sandwich hybridization assay performed with target sequence (Adapter) each of which scanned separately. Adapter concentrations of the slides are 0.001, 0.01, 0.05µM, respectively. **B, D, F.** Negative control of the experiment. Nine spots of sandwich hybridization assay performed with an uncomplimentary strand each of which scanned separately. Uncomplimentary Adapter concentrations of the slides are 0.001, 0.01, 0.05µM, respectively

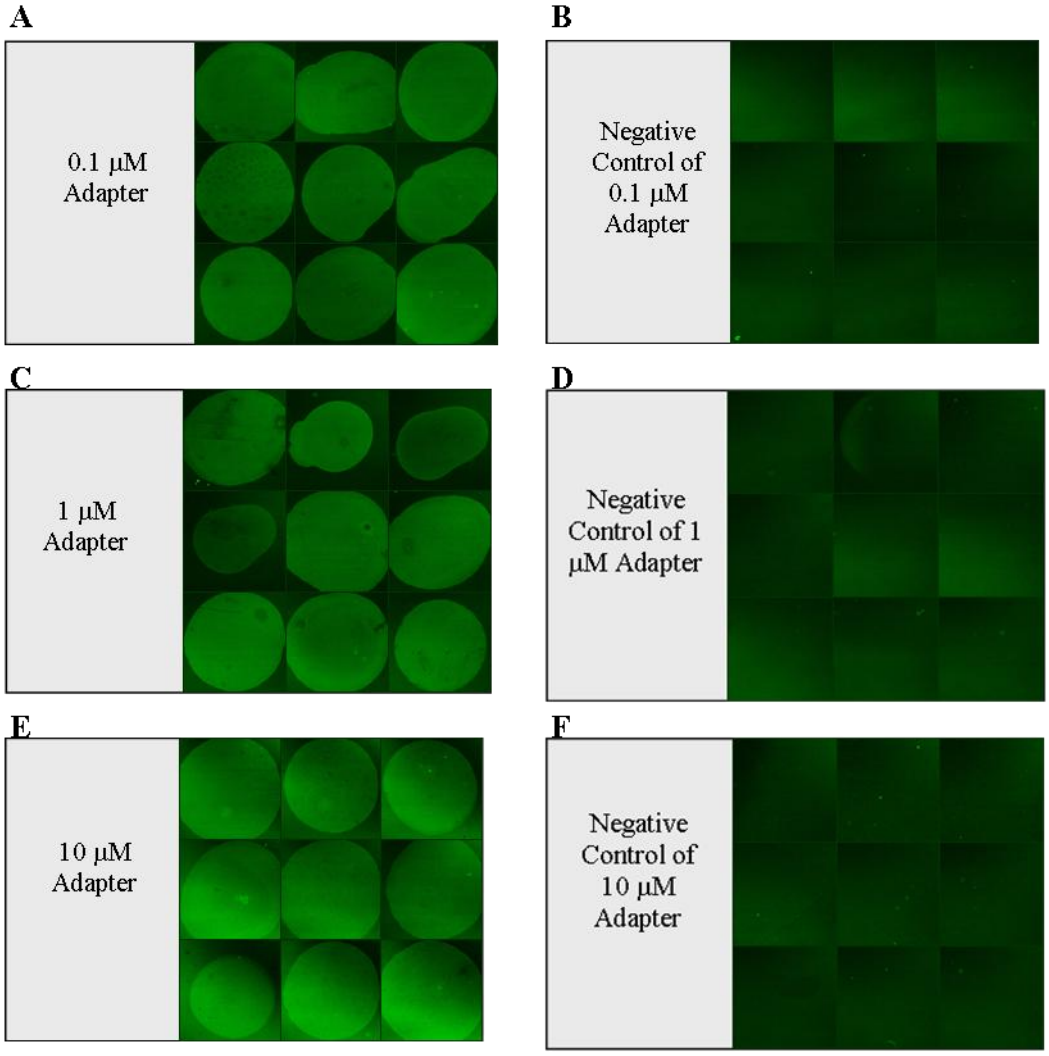

Figure 3.23 Confocal laser scanning results of sandwich hybridization performed with different target sequence (Adapter) concentrations where other parameters were kept constant. **A, C, E**. Nine spots of sandwich hybridization assay performed with target sequence (Adapter) each of which scanned separately. Adapter concentrations of the slides are 0.1, 1, 10µM, respectively. **B, D, F.** Negative control of the experiment. Nine spots of sandwich hybridization assay performed with an uncomplimentary strand each of which scanned separately. Uncomplimentary Adapter concentrations of the slides are 0.1, 1, 10 µM, respectively.

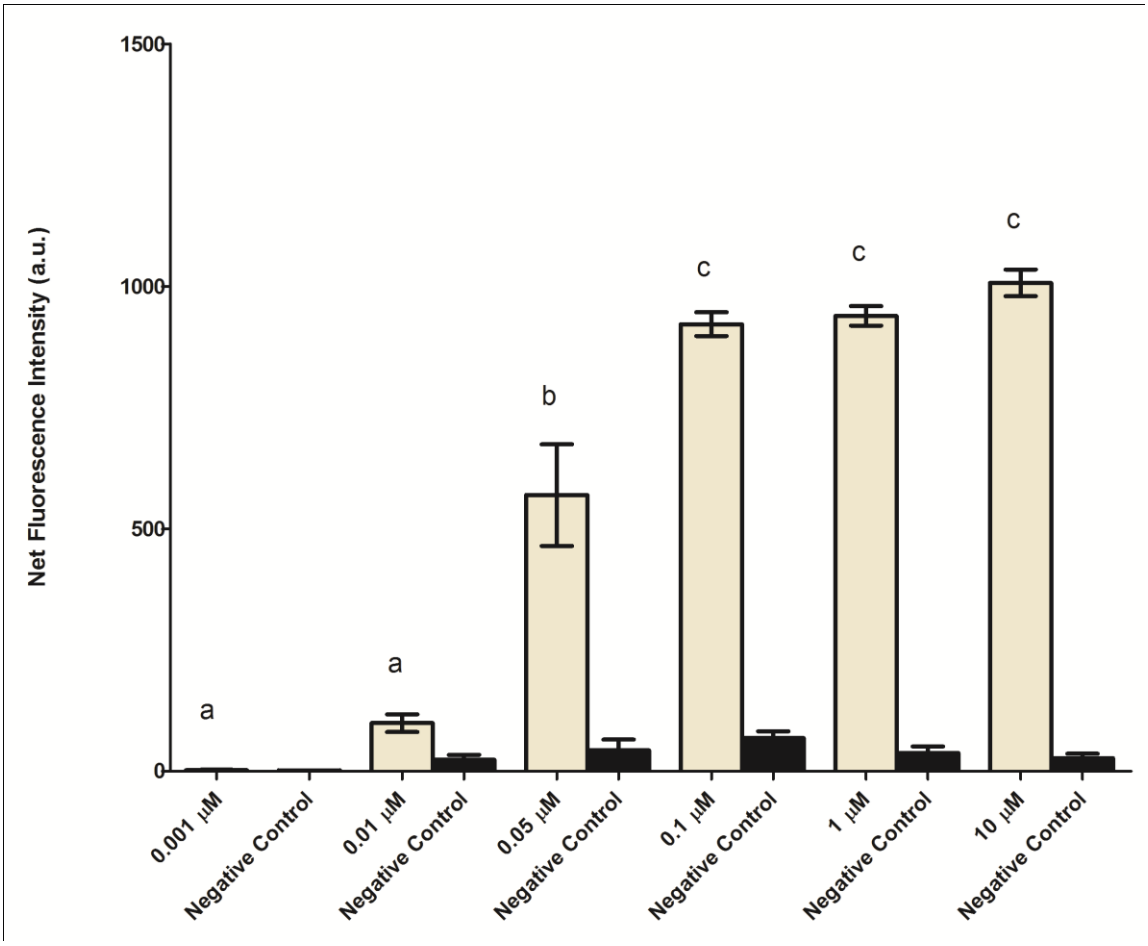

Figure 3.24 Sensitivity of the sandwich assay**.** The height of the histograms represents the average net fluorescence pixel intensity of all spots. Net intensity values were calculated as described in materials and method section. Error bars represent standard error of mean calculated from 18 spots. Different letters indicate significant difference at $p < 0.05$.

3.8. Selectivity of the Array Platform

Today, it is important to develop new assays which are able to detect multiple target DNA sequences simultaneously. These assays allow the parallel testing for a wide variety of features in a single assay, and therefore, offer an advantage over

established single analyte detecting systems(Li et al., 2010). Like in the case of microarray, by means of multiplex array systems different sequences can be immobilized on the solid support, and thus a parallelized and simultaneous screening for a wide variety of targets can be achieved(von Götz, 2010).

In order to succeed the multiplex target detection in a concurrent manner, the developed systems should be reliable and efficient and show high levels of sensitivity and selectivity. In recent years, fluorescent multiplex array technology has been developed for simultaneous measurement of multiple analytes in a single test (Earle et al., 2007) (Vignali, 2000). The technology utilizes microspheres as the solid support for a conventional immunoassay, affinity assay or DNA hybridization assay which is analyzed on a flow-cytometer.

In this study, following the construction and optimization of the array platform, the selectivity of the platform was observed by means of a multiplex assay experiment. In addition to the sequences which are used for the optimization studies, three different probes-adapter sets (35S, Nos, Bar and Cry) were used for the experiment. Like a standard multiplex array principle, the capture probes of each set were immobilized on the glass surface in a regular format with three replicas for each (Figure 3.26). Following the immobilization, a hybridization cocktail containing all target and corresponding signal probes was prepared and applied onto the same surface for hybridization. We detected considerable amount of signal from each target sandwich hybridization assay (Figure 3.26). In order to observe any background signal which might have been derived from the capture and signal probes interaction, an control experiment was performed in the absence of target sequences -only signal probes were applied on capture probe immobilized surface. As indicated in Figure 3.27, in the absence of target sequences sandwich hybridization could not formed and thus no signal can be detected..

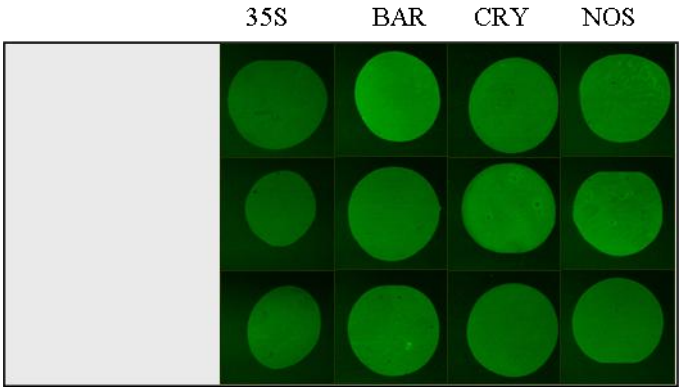

Figure 3.25 Confocal laser scanning results of sandwich hybridization constructed with four different probes-target systems. Each column with 3 replicas represents an independent sandwich assay with corresponding target-probe constructs performed concurrently**.**

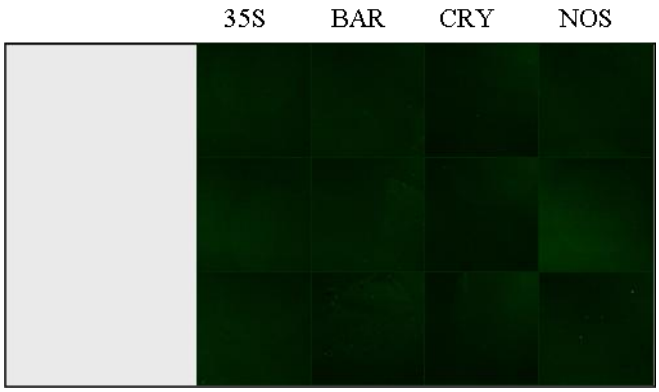

Figure 3.26 Negative Control for the assay. Confocal laser scanning results of sandwich hybridization assay constructed without target sequences. Each column with 3 replicas represents an independent sandwich assay performed concurrently in the absence of target sequences.

In order to detect the selectivity of the platform, a multiplex assay was designed. Four different hybridization cocktail were prepared each missing one target sequence. After the application of the mixtures, no signal was obtained from the column corresponding to the missing target sequence, as expected(Figures3.28-29-30-31). These results showed that our platform is selective for multiple analysis under conditions assayed.

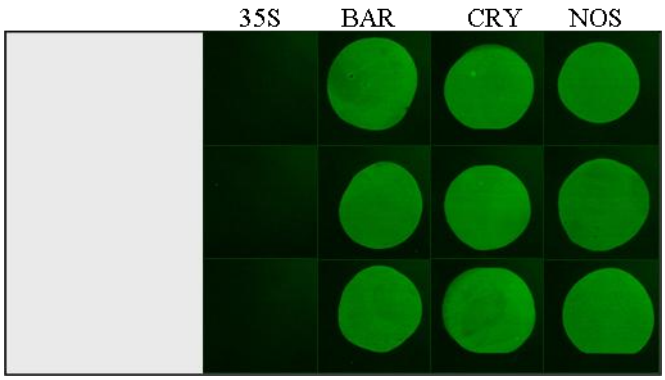

Figure 3.27 Confocal laser scanning results of sandwich hybridization assay constructed with different probes-target systems. Except for the 35S sequence, each column with 3 replicas represents an independent sandwich assay with corresponding target-probe constructs performed concurrently. The system contains all target sequences but the 35S.

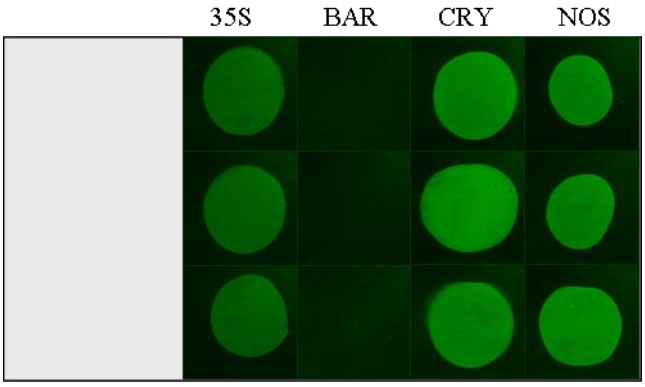

Figure 3.28 Confocal laser scanning results of sandwich hybridization assay constructed with different probes-target systems. Except for the Bar sequence, each column with 3 replicas represents an independent sandwich assay with corresponding target-probe constructs performed concurrently. The system contains all target sequences but the Bar**.**

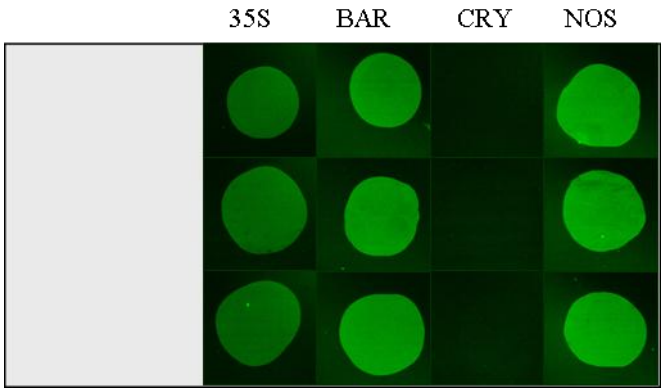

Figure 3.29 Confocal laser scanning results of sandwich hybridization assay constructed with different probes-target systems. Except for the Cry sequence, each column with 3 replicas represents an independent sandwich assay with corresponding target-probe constructs performed concurrently. The system contains all target sequences but the Cry.

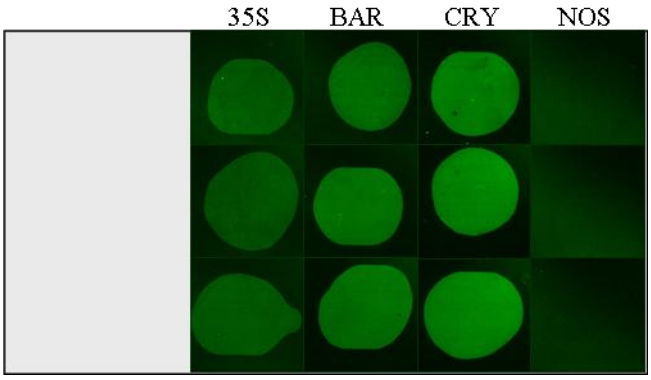

Figure 3.30 Confocal laser scanning results of sandwich hybridization assay constructed with different probes-target systems. Except for the Nos sequence, each column with 3 replicas represents an independent sandwich assay with corresponding target-probe constructs performed concurrently. The system contains all target sequences but the Nos.

CHAPTER 4

CONCLUSION

Ability to detect an unlabeled oligonucleotide target and flexibility in signal development are the main advantages of the sandwich format. In this study, a DNA macroarray platform is constructed by using sandwich hybridization assay which comprised of a glass surface on which capture oligonucleotide probe (P1) was immobilized, an unlabeled target nucleic acid (Adapter) which is a part of 35S promoter of *Cauliflower Mosaic Virus* (CaMV) and a fluorescein-tagged signal oligonucleotide probe.

Before construction of the platform, determination of coating of the solid support which is a glass slide in this case, is a very crucial step. Accordingly, two different surface coating agents are employed, namely 3-Aminopropyl-3-methoxysilan (APTMS) and Poly-L lysine. Among them, poly-L lysine was selected as the coating agent. Main reasons of this selection are less background noise and thus high signal/noise intensity of the assay on PL slides compared to APTMS coated ones. In addition, using PL slides eliminates the handling of dangerous chemicals.

Immobilization of capture probe on PL coated surface is another important criterion for the construction of macroaaray platform. Similarly, two different immobilization techniques were used for the attachment of capture probe on the surface; heat immobilization and immobilization via a heterobifunctional cross-linker. Among them the latter one was selected because covalent immobilization of capture DNA leads to a more proper attachment of P1 and thus eliminates the false positive results of negative controls.

In addition to determine optimum distance from the surface, immobilization was performed with several cross-linkers with different armlenghts. SM(PEG)$_6$ (32.5 Å arm length) heterobifunctional cross-linker provided the best hybridization signal.

Surface hybridization is directly related with the amount of immobilized DNA. In order to determine best signal intensity different concentrations of capture and signal probes were evaluated. Among them 10 μM capture probe was selected as the optimum concentration of the developed platform.

Hybridization conditions were optimized by means of optimizing hybridization time, temperature and sequence parameters. The results indicate that acceptable hybridization signal can be acquired with short (2.5 hours) hybridization duration at 35°C. In addition, pre-incubation of signal probe with target sequence results in better signal intensities.

With the described experimental protocols the platform generates reproducible results at nanomolar target nucleic acid concentration range and selective enough to determine corresponding target sequence in a multiplex assay experiment.

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APPENDIX A

PREPARATION OF SOLUTIONS AND BUFFERS

100mM (10X) Phosphate Buffered Saline
(1370 mM NaCl, 100 mM Phosphate, 27 mM KCl)

80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 were dissolved in 1L of distilled H_2O and pH was adjusted to 7.2. Sterilization was performed by autoclaving and the buffer was stored at room temperature.

5X Saline-Sodium Citrate Buffer
(75 mM sodium citrate, 750 mM sodium chloride, pH 7.0)

22.06 g Sodium Citrate, 43.8 g Sodium Chloride were dissolved in 1L of distilled H_2O and pH was adjusted to 7.0. Sterilization was performed by autoclaving and the buffer was stored at room temperature.

10% SDS

10 g SDS was dissolved in 100mL of distilled H_2O.Sterilization was performed with filter sterilization. (Filter size: 0.4 µm) and the solution stored at room temperature

2% BSA in 10 mM PBS

2 g BSA was dissolved in 100mL of 10mM PBS Buffer and filter sterilized (Filter size: 0.4 µm). The solution was stored at +4°C

1 mM TCEP

0.00286 g of TCEP was dissolved in 1 mL distilled H2O. A dilution was carried out by mixing 100 µL of 10 mM TCEP with 900 µL dH2O and 40 aliquotes x 25 µL was stored at (-20)°C for maximum 2 months.

.

APPENDIX B

TABULATED VALUES OF NET FLUORESCENCE INTENSITIES

Table B1. Mean values and SEM for Figure 3.7

APTMS vs PL	**Net Fluorescence Intensity (a.u.)**	
	Positive Control	**Negative Control**
1 % APTMS	225.62 ± 92.97	51.94 ± 30.76
5 % APTMS	341.56 ± 66.17	113.75 ± 68.67
10 % APTMS	294.81 ± 92.84	22.47 ± 13.56
Poly-L lysine	709.30 ± 75.17	12.31 ± 8.02

Table B.2. Mean values and SEM for Figure 3.8

Heat Immobilization	**Net Fluorescence Intensity (a.u.)**
P1 + P2	908.22 ± 610.01
P1+A+P2	901.64 ± 672.81
P1+unA+P2	696.11 ± 426.50
P2	58.184 ± 29.911

Table B.3. Mean values and SEM for Figure 3.11

Crosslinker Type	**Net Fluorescence Intensity (a.u.)**	
	Positive Control	**Negative Control**
Sulfo-EMCS	609.372 ± 107.13	36.784 ± 27.212
$SM(PEG)_2$	750.445 ± 46.77	49.898 ± 48.159
$SM(PEG)_6$	938.928 ± 70.945	14.996 ± 9.430
$SM(PEG)_{12}$	819.510 ± 70.194	71.727 ± 53.355

Table B.4. Mean values and SEM for Figure 3.13

P1 Concentration	**Net Fluorescence Intensity (a.u.)**	
	Positive Control	**Negative Control**
1 μM	764.14 ± 145.76	101.56 ± 48.30
10 μM	1035.63 ± 163.1911	51. 44 ± 19.63
20 μM	969.07 ± 223.38	85.11 ± 32.17

Table B.5 Mean values and SEM for Figure 3.15

P2 Concentration	**Net Fluorescence Intensity (a.u.)**	
	Positive Control	**Negative Control**
20 μM	969.41 ± 159.27	20.78 ± 2.58
40 μM	1269.45 ± 78.25	132.44 ± 5.79
50 μM	1398.65 ± 139.36	95.22 ± 3.94

Table B.6. Mean values and SEM for Figure 3.18

Hybridization Duration	**Net Fluorescence Intensity (a.u.)**	
	Positive Control	**Negative Control**
2.5 hours	437.64 ± 74.828	10.00 ± 0.00
5 hours	477.216 ± 66.81	10.00 ± 0.00
12 hours	870.87 ± 79.412	10.00 ± 0.00
24 hours	131.57 ± 168.38	10.00 ± 0.00

Table B.7 Mean values and SEM for Figure 3.19

Hybridization Temperature	**Net Fluorescence Intensity (a.u.)**	
	Positive Control	**Negative Control**
35 °C	748.60 ± 95.62	68 ± 8.70
40 °C	592.06 ± 89.29	39.67 ± 4.57
45 °C	371.55 ± 80.01	38.67 ± 3.05

Table B.8 Mean values and SEM for Figure 3.22

Hybridization Sequence	**Net Fluorescence Intensity (a.u.)**	
	Positive Control	**Negative Control**
P1+A+P2	708.87 ± 142.04	12.89 ± 9.59
(P1+A)+P2	665.73 ± 129.21	12.33 ± 7.62
P1+(A+P2)	938.12 ± 129.83	10.00 ± 0.00

Table B.9. Mean values and SEM for Figure 3.23

Sensitivity	**Net Fluorescence Intensity (a.u.)**	
	Positive Control	**Negative Control**
0.001 μM	2.43 ± 3.58	1.024 ± 1.093
0.01 μM	99.33 ± 55.10	23.54 ± 30.80
0.05 μM	569.58 ± 314.78	43.15 ± 67.40
0.1 μM	922.16 ± 73.90	68.57 ± 41.08
1 μM	939.20 ± 59.97	37.64 ± 40.85
10 μM	1007.3 ± 82.098	26.87 ± 28.28