DEVELOPMENT OF AN OLIGONUCLEOTIDE BASED SANDWICH ARRAY PLATFORM FOR THE DETECTION OF TRANSGENIC ELEMENTS FROM PLANT SOURCES USING LABEL-FREE PCR PRODUCTS

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submitted by **FATMA GÜL** in partial fulfillment of the requirements for the degree of **Master of Science in Biotechnology Department, Middle East Technical University by,**

Prof. Dr. Canan Özgen
Dean, Graduate School of **Natural and Applied Sciences** _____________________

Prof. Dr. İnci Eroğlu
Head of Department, **Biotechnology** _____________________

Prof. Dr. Hüseyin Avni Öktem
Supervisor, **Biology Department, METU** _____________________

Assoc. Prof. Dr. Füsun İnci Eyidoğan
Co-Supervisor, **Education Faculty, Başkent University** _____________________

Examining Committee Members

Prof. Dr. Meral Yücel
Biology Department, METU _____________________

Prof. Dr. Hüseyin Avni Öktem
Biology Department, METU _____________________

Prof. Dr. Ufuk Bakir
Chemical Engineering Department, METU _____________________

Assoc. Prof. Dr. Füsun İnci Eyidoğan
Education Faculty, Başkent University _____________________

Assist. Prof. Dr. A. Elif Erson Bensan
Biology Department, METU _____________________

Date: _____________________

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name : Fatma GÜL

Signature :

ABSTRACT

DEVELOPMENT OF AN OLIGONUCLEOTIDE BASED SANDWICH ARRAY PLATFORM FOR THE DETECTION OF TRANSGENIC ELEMENTS FROM PLANT SOURCES USING LABEL-FREE PCR PRODUCTS

Fatma GÜL

M.Sc., Department of Biotechnology

Supervisor: Prof. Dr. Hüseyin Avni ÖKTEM

Co-supervisor: Assoc. Prof. Dr. Füsun İnci EYİDOĞAN

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Advances in DNA micro and macroarray technologies made these high-throughput systems good candidates for the development of cheaper, faster and easier qualitative and quantitative detection methods. In this study, a simple and cost effective sandwich hybridization-based method has been developed for the rapid and sensitive detection of various unmodified recombinant elements in transgenic plants. Attention was first focused on the optimization of conditions such as time, concentration and temperature using commercial ssDNA, which in turn could be used for real sample detection.

In this sandwich-type DNA chip platform, capture probes complementary to the first half of recombinant element (target adapter) were immobilized onto poly-L-lysine covered conventional microscope slides. PCR-amplified un-purified target adapter and biotin labeled detection probe, which is complementary to the second half of target adapter, were hybridized in solution-phase to complementary capture probes to create a sandwiched tripartite complex. Later, hybridization signal was visualized by the attachment of streptavidin conjugated Quantum Dot to the sandwiched complex under UV illumination. Sandwich based array system that has been developed in this study allows multiplex screening of GMO events on a single DNA chip platform. 35S promoter, NOS terminator, CRY1Ab and BAR target sequences were successfully detected on the same DNA chip platform. The platform was able to detect unlabeled PCR amplified DNA fragments of CaMV 35S promoter sequence and NOS terminator and BAR transgene sequences from transgenic potato plants and NK603 Certified GMO Reference material, respectively.

The DNA-chip platform developed in this study will allow multiple detection of label-free PCR-amplified transgenic elements from real GMO samples on a single slide via a cost effective, fast, reliable and sensitive sandwich hybridization assay.

Key words: DNA chip, Macroarray, Sandwich hybridization, Genetically Modified Organisms (GMOs)

ÖZ

İŞARETLENMEMİŞ PZR ÜRÜNLERİNİ KULLANARAK BİTKİLERDEN TRANSGENİK ELEMENTLERİN TESPİTİ İÇİN OLİGONUKLEOTİT TABANLI SANDVİÇ DİZİ PLATFORMUNUN GELİŞTİRİLMESİ

Fatma GÜL
Yüksek Lisans, Biyoteknoloji Bölümü
Tez Yöneticisi: Prof. Dr. Hüseyin Avni ÖKTEM
Ortak Tez Yöneticisi: Doç. Dr. Füsun İnci EYİDOĞAN

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DNA mikro ve makrodizi teknolojilerindeki ilerlemeler bu yüksek çıktılı sistemleri daha ucuz, hızlı ve basit nicel ve nitel tespit yöntemlerini geliştirmede aday kılmıştır. Bu çalışmada işaretlenmemiş çeşitli rekombinat elementlerin genetiği değiştirilmiş bitkilerden hızlı ve hassas bir şekilde tespiti için basit ve ucuz sandviç hibridizasyonuna dayalı bir yöntem geliştirilmiştir. Çalışmamız daha sonra gerçek örneklerin tespitinde kullanılmak üzere, tek sarmal DNA kullanarak inkübasyon zamanı, konsantrasyon ve sıcaklık gibi parametrelerin optimizasyonu ile başlamıştır.

Bu sandviç tipi DNA çip platformunda, tespiti hedeflenen DNA örneğinin ilk yarısına eşlenik olan yüzey probu (capture probe) poly-l-lizin kaplı mikroskop camına sabitlenmiştir. PZR ile çoğaltılmış fakat saflaştırılmamış hedef DNA ve hedef DNA' nın diğer yarısına eşlenik olan biotinle işaretlenmiş tespit probu sıvı fazda yüzey probu ile üçlü kompleks oluşacak şekilde hibridize olmuşlardır. Daha sonra streptavidin ile konjuge edilmiş kuantum noktacıklarının bu sandviç

kompleksine dahil olası ile birlikte ultraviyole ışığı altında sinyal elde edilmiştir. Bu çalışmada geliştirilen sandviç tabanlı dizi sistemi tek bir DNA çip platformunda GDO elementlerinin çoklu taranmasını sağlamaktadır. 35S promotor, NOS terminatör, CRY1Ab ve BAR hedef sekansları başarılı bir şekilde aynı platform üzerinde tespit edilmiştir. Platform ayrıca transgenik bitkiden elde edilen 35S promotoru ile sertifikalı % 5 GDO içeren referans materyalden çoğaltılan NOS terminatörünü ve BAR transgenini tespit edebilmiştir.

Geliştirilen DNA çip platformu ucuz, hızlı, güvenilir ve hassas sandviç yöntemi sayesinde tek bir platform üzerinde PZR ile çoğaltılmış ve işaretlenmemiş çoklu transgenik elementlerinin tespitini sağlayacaktır.

Anahtar Kelimeler: DNA çipi, Makrodizi, Sandviç Hibridizasyonu, Genetiği Değiştirilmiş Organizmalar (GDO)

To My Lovely Grandfather;

Hasan Kılbacak

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BSA	Bovine Serum Albumin
CTAB	Hexadecyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
GMO	Genetically modified organism
LB	Luria broth
PBS	Phosohate Buffered Saline
PCR	Polymerase chain reaction
QD	Quantum Dot
SDS	Sodium dodecyl sulfate
SSC	Saline-Sodium Citrate
ssDNA	Single stranded deoxyribonucleic acid

CHAPTER 1

INTRODUCTION

1.1 Microarray

Microarray is defined as a small chip (chemically coated glass, gold, nylon membrane or silicon) onto which thousands of DNA molecules are attached at defined positions. This enables simultaneous analysis and monitoring the expression levels of thousands of genes in parallel.

The first published paper on microarray technology was quantitative monitoring of gene expression patterns with a complementary DNA microarray (Schena *et al.*, 1995). They developed a high-capacity system to monitor the expression of 45 *Arabidopsis* genes in parallel on glass surface by means of simultaneous, two-color fluorescence hybridization. In 1996, commercial arrays were introduced into the market by Affymetrix After that Lashkari et al. placed complete eukaryotic genome on microarray and analyzed total mRNA expression (cDNA) in *S. cerevisia* (yeast), examining the effects of heat and cold-shock, and and culture in glucose vs. galactose on global gene expression profiles (Lashkari *et al.*, 1997). Identification of the specific subgroups of breast carcinomas (Perou *et al.*, 2000) and the completion of Human Genome Project in the following years lead an enormous increase in the microarray studies and new microarray methods.

By means of the quickly developing new technologies, in the present day, make it possible to use better surface technology, more powerful robots for arraying, new nucleic acid dye labeling methods, improved computational power and automated analyzer in complex microarray studies. Figure 1.1 shows a general microarray study.

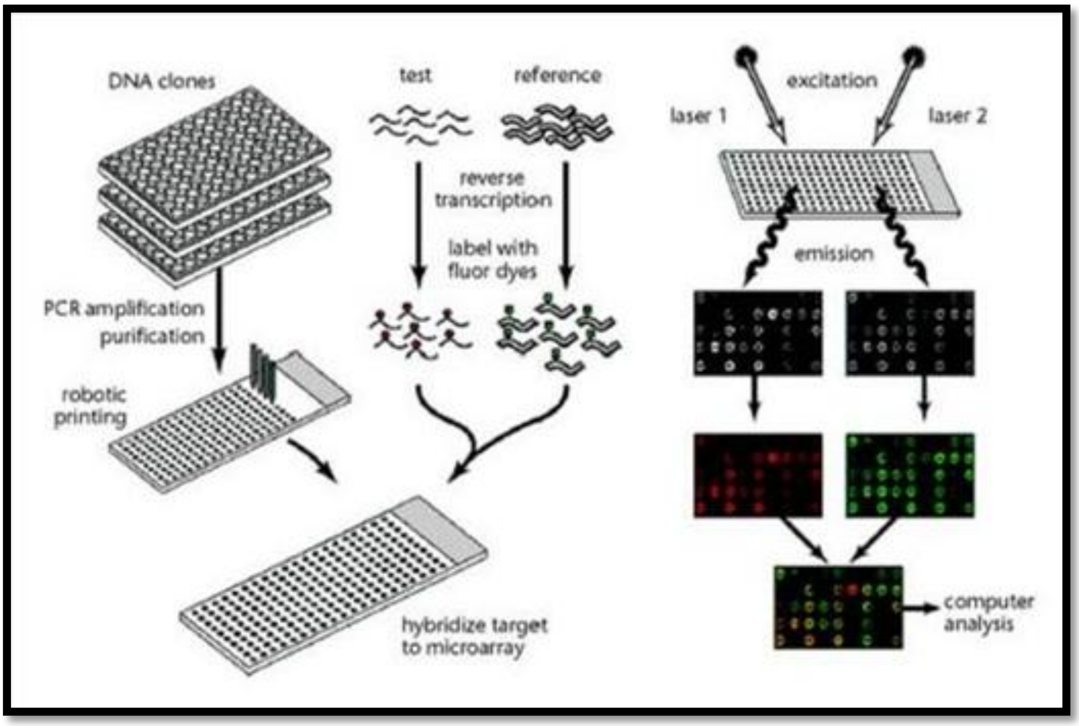

Figure 1.1 The representative figure of microarray (Duggan *et al.*, 1999)

1.2 DNA Microarray Technology

DNA microarray technology is an important tool for specific research applications including genomics, proteomics, and cellular analysis. This technology provides researchers to survey and address issues in life science research by permitting to gather molecular data at an unprecedented rate because it is a rapidly evolving technology. Advances in this technology create an improvement in the sensitivity and selectivity and make microarrays more economical research tool.

1.3 Applications of Microarray

1.3.1 Gene Expression and Discovery

With the advances in microarray technology, it is now possible to successfully identify new genes, study how they function and know about expression levels under different conditions. Because expression profile make it possible to find subset of gene transcripts or mRNA expressed in a cell or tissue, microarrays can help to characterize the functions of new genes, understand the role of genes in critical regulatory pathways and analyze genetic variation.

Differential expression measurements of 45 *Arabidopsis* genes were made by Schena, Shalon et al. 1995. This was the first gene expression measurement studied with microarray in the literature. They prepared flourescent probes from total *Arabidopsis* mRNA and then analyzed a transgenic line overexpressing the single transcription factor HAT4 with a simultaneous two color hybridization scheme. They got an intense hybridization signal from HAT4 cDNA position on the microarray. It is an important study for the detection of rare mRNA transcripts from plant *Arabidopsis thaliana.*

In another study, scientist used DNA microarray to characterize the changes in gene expression that take place during carbon metabolism, protein synthesis, and carbohydrate storage. Open reading frames of *Saccharomyces cerevisia* (from exponential growth culture) were amplified with PCR. Distinct DNA sequences printed on glass surface and fluorescently labeled cDNAs were hybridized to microarray. According to the results, during exponential growth, gene expression is stable. However when glucose was depleted from the culture medium, there were significant changes in the expression of genes investigated. Some of the genes were induced by a factor at least 2, and some of them declined by a factor 2. The responses of these previously unknown genes to the diauxic shift provided the first small clue about their possible roles (DeRisi *et al.*, 1997).

Hughes et al. (2000) constructed a reference database through which they were able to monitor hundreds of different cellular functions simultaneously on a single assay. They studied expression profiles of 300 diverse mutations and chemical treatments in *S. cerevisiae*. 276 deletion mutants, 11 tetracycline-regulatable alleles of essential genes and 13 well-characterized compounds were successfully profiled. According to their database different mutants or treatments that affect similar cellular processes displayed similar expression profiles (Hughes *et al.*, 2000).

Kuninger and co-workers identified a relatively small number of transcripts that were specifically induced in skeletal myoblasts by either IGF-I or PDGF through analysis of high density oligonucleotide arrays with a genome-wide transcriptional profiling approach (Kuninger *et al.*, 2004).

1.3.2 Genotyping

Microarray-based genomic analysis has led to determination of the genes by examining DNA sequences. Especially completion of the Human Genome Project made it possible to determine the single nucleotide polymorphism (SNP) and genomic variations. For example, Tsang and co-workers developed an electronic microarray to detect genetic variation in research and clinical diagnosis. This multiplex electronic microarray is specific for single nucleotide polymorphism (SNP), genotyping and mutation detection. In the assay, two SNP markers were typed on 18 inbred mouse strains. Strains were amplified with PCR primers tailed with M13 universal adaptor sequences. After amplification reaction, PCR products were immobilized on the microarray via capture oligonucleotides complementary to the universal adapter sequence. SNP marker sequences were simultaneously captured on the same test site as the detecting strands (Tsang *et al.*, 2004).

Microarray is a highly specific method that provides the detection of mutations in genes. The detection of disease- causing mutations involved in a neurological disorder called Charlot-Mary-Tooth disease was simply performed with a specific microarray method (Baaj *et al.*, 2008).

Microarrays also provide the detection of multiple mutations in the same gene. In a study, sensorineural hearing loss was detected by a comprehensive genetic testing. This microarray based test can be applied for newborn hearing screening and provides the detection of a genetic etiology in older children and adults (Gardner *et al.*, 2006).

1.3.3 Drug Discovery and Developments

In conventional drug discovery strategy, single end point screens were used. This method was very time consuming and requires comprehensive prior characterization of the gene product for assay development. At this point, microarray technology presents a new way for drug discovery and development because it is extensively used in pharmacogenomics in order to gain insight into new therapeutic targets.

Genes from a diseased and a normal cell are analyzed comparatively in microarray assay to find the proteins synthesized by the diseased genes. According to this information, it is possible to synthesize new drugs that block these proteins and weaken their effect. Figure 1.2 shows the microarray applications at various stages of drug discovery and development.

Microarrays help to investigate the mechanism of drug action which in turn provides to identify and validate novel therapeutics (Debouck and Goodfellow, 1999).

Some scientists compared the gene expression patterns of pancreatic cancer cell lines growing in tissue culture with those of normal pancreas using cDNA microarray analysis. They explored the variations in gene expression on a genomic scale. The identification of overexpressed genes using cDNA microarray provides a greater potential as targets for the development of small molecule inhibitors which in turn can be used as potential candidates for targeted drug development (Han *et al.*, 2002).

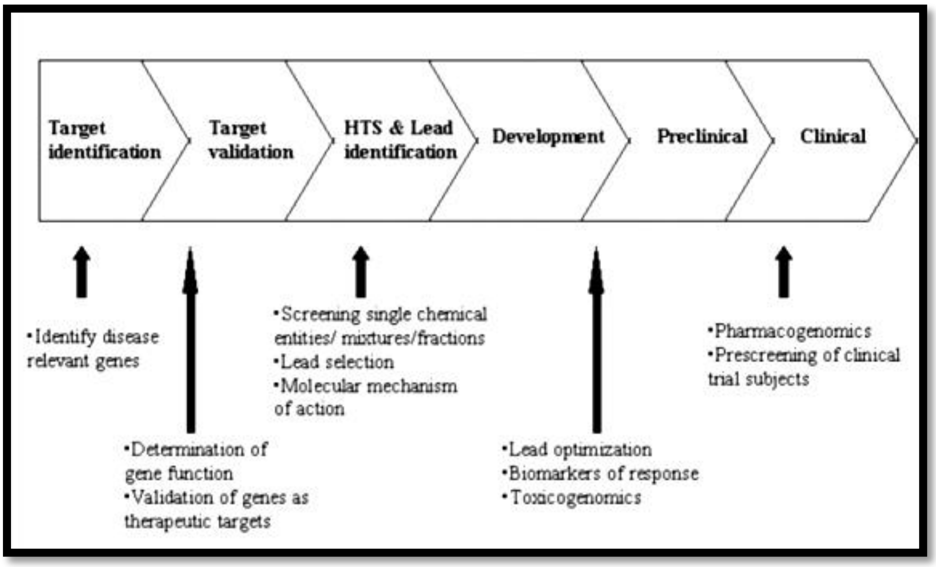

Figure 1.2 DNA microarray: applications in natural product drug discovery and development (Chavan *et al.*, 2006).

In a study, in vivo anticancer activity was assessed with a cell-based tissue microarray. Peripheral blood mononuclear cells (PBMC) were used as a tumor surrogate and analyzed for protein hyperacetylation in response to treatment with the histone deacetylase inhibitor SNDX-275 in Phase I clinical trials. Study showed that the cell microarray can be used to measure drug response in a high throughput manner, allowing analysis of an entire trial on one or two glass slides (Lee *et al.*, 2007).

1.3.4 Diagnostic Applications

DNA microarray profiling provides researchers to learn more about different kinds of diseases such as heart diseases, mental illness, infectious disease and cancer. Although cancer types have been classified according to the organs in which the tumors develop, now it is possible to categorize different kinds of cancer according to the patterns of gene activity in the tumor cells with microarray technology.

The sensitive detection of infectious diseases can be made by microarray methods. For example, the relationships between viral amplification efficiency, hybridization signal, and target-probe annealing specificity was investigated with a customized microarray platform. Scientists were able to detect 35 different pathogens with 100 % specifity through microarray technology in clinical diagnostics (Wong *et al.*, 2007).

Recently, the diagnosis of most common cancer types has gained more importance. In a study, the determination of the methylation state of the PITX2 promoter (prognostic biomarker for breast and prostate cancers) was achived by using a microarray called Epichip PITX2. The developed microarray technique provided an opportunity for accurate assessment of prognosis in prostate cancer patients who underwent radical prostatectomy (Schatz *et al.*, 2010).

It is also possible to diagnose herpetic encephalitis in children by detecting some human herpes viruses using PCR-microarray technology. This is a good example for rapid, accurate, and specific etiological diagnosis of herpetic encephalitis in children (Shi *et al.*, 2010).

1.4 Types of Microarray

1.4.1 According to Target Detection

According to be detected target sample, microarrays can be called tissue microarrays, cell microarrays, carbohydrate microarrays, protein microarrays and oligonucleotide microarrays.

1.4.1.1 Tissue Microarray

Tissue microarray is a recent technique used in the field of pathology. They are paraffin blocks produced by extracting cylindrical tissue cores from different paraffin donor blocks and placing these cores into a single recipient (microarray) block at defined array coordinates (Jawhar, 2009). This technique was found by a scientist and he called it as "sausage" block technique (Battifora, 1986). It was further developed by Kononen and co-workers. The technique became an array-based high-throughput technique that facilitates gene expression and copy number surveys of very large numbers of tumors. They found that detection of six gene amplifications as well as p53 and estrogen receptor expression in breast cancer demonstrates the power of this technique for defining new subgroups of tumors (Kononen *et al.*, 1998).

After the popularization of this technique, so many papers about this subject has become appearing in literature (Table 1.1).

In literature, it was emphasized that tissue microarrays can help to identify new prognostic markers and to define protein biomarkers that can be used in diagnosis (Pallares *et al.*, 2009)

For example in a recent study, the oral squamous cell carcinoma tumor behavior based on the expression of p53, Bcl-2 and E-cadherin was assessed with tissue microarray. Among the three biomarkers that were evaluated, Bcl-2 was found as the most frequently expressed biomarker. The expression of Bcl-2 was inversely related to the degree of differentiation Scientists concluded that the molecular data obtained from tissue microarray will enhance diagnosis, provide better prognostication and will improve cancer treatment for individual patients (Solomon *et al.*, 2010).

Table 1.1 The distribution of studies in the literature about tissue microarray (Aktas, 2004).

Cancer Type	Topics	% of manuscripts
Breast carcinoma	Estrogen, progesterone, Her2/neu, telomerase, cyclin D1, erbB2, BRCA1-2, mamoglobin, snoN, KIT, MCSF-1, minichromosome	18.4
Skin and malignant melanoma	Tirozin kinase, CD117, Act trans factor	17.6
Prostate carcinoma	CD10, PTEN, NCAM, Sindecan	14.4
Lymphoma/leucemia	Cyclin E, CD20, Pax 5, P18IN4C, CD44, SHP	5.5
Transitional carcinoma	FGFs, EMS1, erbB2, Her2/neu	5.5
Colorectal carcinoma	P 53, Mismatch repair, c-myc	4.7
Renal cell carcinoma	Keratin, steroid hormones, muc-1, cyclins, p53, VEGF	4.5
Lung Carcinoma	EGF, e-cadherin, her2/neu	4.5
Gastric carcinoma	Gastrin, cyclooxigenase 2,c-met	3.6
Ovarium	Erbb2, p53, A103, HIF	2.8
Hepatic tumors	P53, vimentin, hepatitis B	2.8
Endometrium	STK15, mismatch repair	1.2
Testis tumors	NKX3-1, Oct 3 / 4	1.2
Surrenal tumors	A103, Chromogranin	0.8
Review articles		3.2
Technical reports		6.7
Others	Tiroid, paratiroid, Synovial sarcoma, osteosarcoma, MFH, histiositosis	2.4

Mollerstrom and co-workers characterized the expression of the corresponding proteins in breast carcinoma and determined their correlation with clinical outcome by immunohistochemistry using tissue microarray. Protein expression was evaluated in 144 samples to assess whether the new markers predict the survival status of the patients better than the currently used markers. BTG2 expression was demonstrated in a significantly lower proportion of samples from dead patients compared to alive patients, both in overall expression and cell membrane specific expression whereas neither ADIPOR1, ADORA1 nor CD46 showed differential expression in the two survival groups. They conclude that high-level BTG2 protein expression correlates with prolonged survival in patients with breast carcinoma. (Mollerstrom *et al.*, 2010)

1.4.1.2 Protein Microarray

Protein microarrays are tools that can be used in many different areas of research, including basic and translational research. Protein arrays can take on many different formats and can be used to do more than simple expression profiling of samples. There are three formats of a standard protein microarray. The following Figure 1.3 shows these formats.

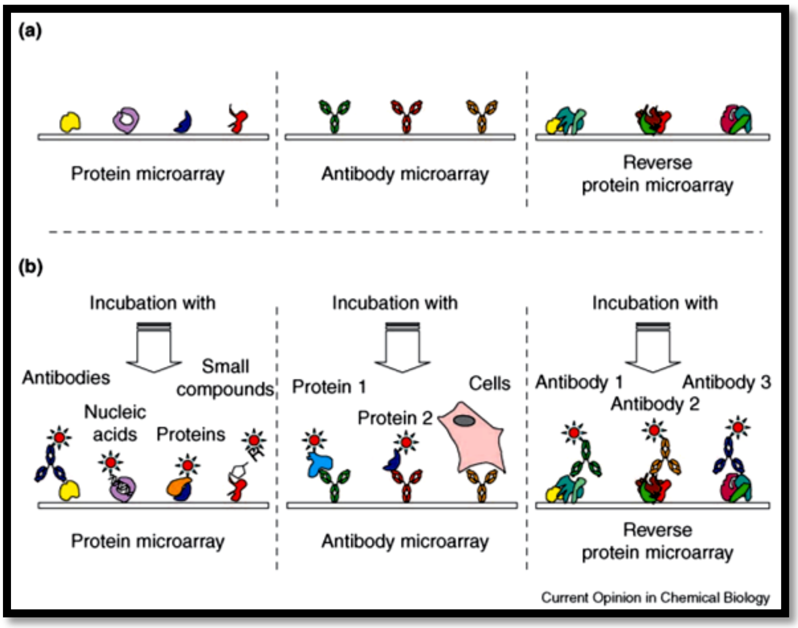

Figure 1.3 Types of protein microarrays and their applications. (a) protein microarray, antibody microarray and reverse protein microarray (b) Screening applications of the three array types with known or putative directly labeled interaction partners (Hultschig *et al.*, 2006)

The first study on protein microarray was about for the determination of antibody specifities using arrays of denatured recombinant proteins. Lueking et al. (1999) identified putative protein expressors at 250 amol or 10 pg of a test protein (GAPDH) on microarray by using bacterial lysates of 92 human cDNA clones expressed in a microtiter plate (Lueking *et al.*, 1999). In the following years, this method was extended to screening monoclonal and polyclonal antibody specifities by using whole proteome of yeast (Michaud *et al.*, 2003).

An example of using protein microarray in diagnostic application is the identification of disease specific proteins. In a study, immune responses to phase-variable expressed proteins from *Neisseria meningitides* were detected. In addition, OpaV protein was found as a disease marker in the diagnosis of meningitis (Steller *et al.*, 2005).

Recent advances in array production and assay performance combined with high-throughput generation of protein targets and ligands, increases the number and variety of applications of protein microarrays. It is now possible to detect antigens of sexually transmitted diseases, breast cancer-related antigens through cDNA phage, to profile antibody responses of novel vaccines and to purify proteins in high quality ((Tang *et al.*, 2010); (Bi *et al.*, 2010); (Crompton *et al.*, 2009); (Kwon *et al.*, 2009))

1.4.1.3 Oligonucleotide Microarray

Oligonucleotide arrays are miniaturized DNA microarrays and contain oligonucleotides not the target itself. They are becoming increasingly useful tools in the detection of gene expression and single nucleotide polymorphism. They have advantages over cDNA microarrays. It is possible to immobilize large number of sequences within an oligonucleotide microarray. Also they provides more controlled specificity of hybridization (LaForge *et al.*, 2000) and screening whole genomic regions for gene discovery (Hughes and Shoemaker, 2001; Shoemaker et al., 2001).

Oligonucleotide array−based detection of known genomic DNA sequence variations was first published in 1989. Onto nylon filters probes complementary to six *HLA−DQA* alleles as well as nine mutations in *HBB* (encoding β−globin) were spotted. Biotin labeled PCR products were hybridized. Genotype was determined with the acquired hybridization signal intensity produced by a colorimetric assay at each allele−specific probe demonstrating the application of the 'reverse dot blot' approach to a simple system relevant to medical genetics (Saiki *et al.*, 1989).

Some scientists were tried to show the relationship between human papillomavirus (HPV) and cervical neoplasia in cervical cancer which is a common cancer type in women. They established a new HPV genotyping method using an oligonucleotide microarray-based system to detect HPV specific oligonucleotide sequences. Samples were collected from women who had previously undergone HPV testing Hybrid Capture II assay. Because there are 35 distinct types of HPV, this method was designed to detect double and triple high risk HPV infections on the same microarray. According to the results, it is possible to detect infection with multiple types of HPV. In clinical practice, for the detection and genotyping of HPV, this new oligonucleotide microarray method provides a reliable management of cervical neoplastic lesions (Kim *et al.*, 2003).

1.4.2 According to Hybridization Type

In microarray studies, there are two main hybridization strategies. These are direct and sandwich hybridization.

1.4.2.1 Direct Hybridization

In this type of hybridization method in microarrays, the target DNA must labeled chemically by fluorescent or other types of signal molecules prior to hybridization with capture probes.

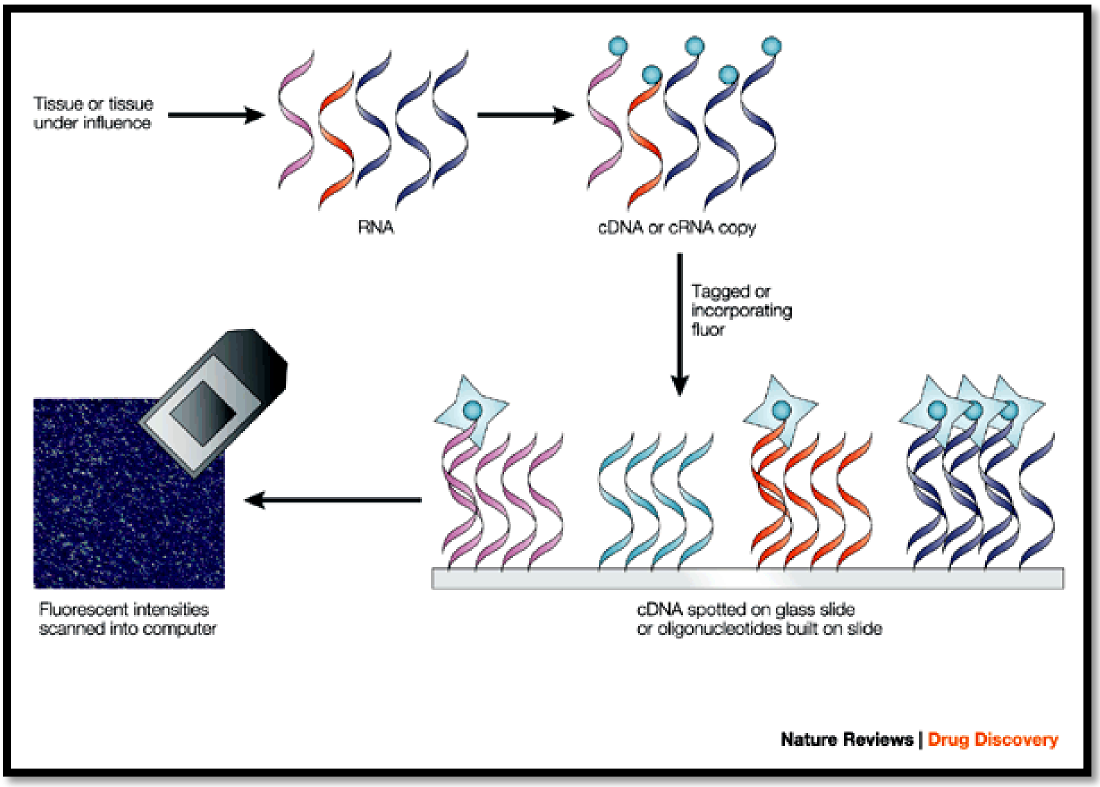

Figure 1.4 Schematic illustration of direct hybridization (Butte, 2002)

Giraud and co-workers were studied the lifetime imaging of Quantum Dot labeled DNA microarray. In the study, they showed that human cytomegalovirus and hepatitis C virus can be labeled with quantum dots and detected on epoxy silane coated glass substrates through a DNA array (Giraud *et al.*, 2009).

Although this direct hybridization is used commonly, there are several advantages faced during experiment. Labeling the DNA is time consuming procedure and also it needs to purification from excess labels and un-labeled DNA. Because of these reason sandwich hybridization method is seems as an alternative method in DNA microarray studies even it requires an extra hybridization step.

1.4.2.2. Sandwich Hybridization

A method based on three-DNA-component is called as sandwich hybridization. Sandwich hybridization is achieved by hybridization of three sandwiched nucleic acid sequences and provides sensitive detection and quantification of specific genes. This method is considered as the most useful approach when it is compared with direct hybridization strategy in which target adapter must be chemically labeled before detection (Zhao *et al.*, 2005). Another advantage of sandwich hybridization from direct hybridization is that it is more specific because two hybridization events must occur in order to generate a signal (Chiu *et al.*, 1998).

This type of hybridization was first described in 1977. In the study, two-step hybridization procedure was carried out. The extracted RNA from adenovirus infected cells was hybridized to restriction endonuclease fragments of adenovirus type 2 (Ad2) DNA immobilized on nitrocellulose filters. RNAs containing both Ad2 and SV40 sequences formed duplexes through their Ad2 sequences, leaving their SV40 sequences as protruding tails. Annealing with 32P-labeled SV40 DNA caused these tails to become labeled This permited the autoradiographic identification of the sequences of Ad2 DNA which are homologous to the RNA (Dunn and Hassell, 1977). However the potential of this sandwich hybridization method as a diagnostic tool was not understood until Ranki and coworkers used this method in the detection of adenovirus DNA in nasopharyngeal aspirates from children with acute respiratory infection (Ranki *et al.*, 1983).

The following Figure 1.5 shows a general scheme for sandwich type hybridization studies.

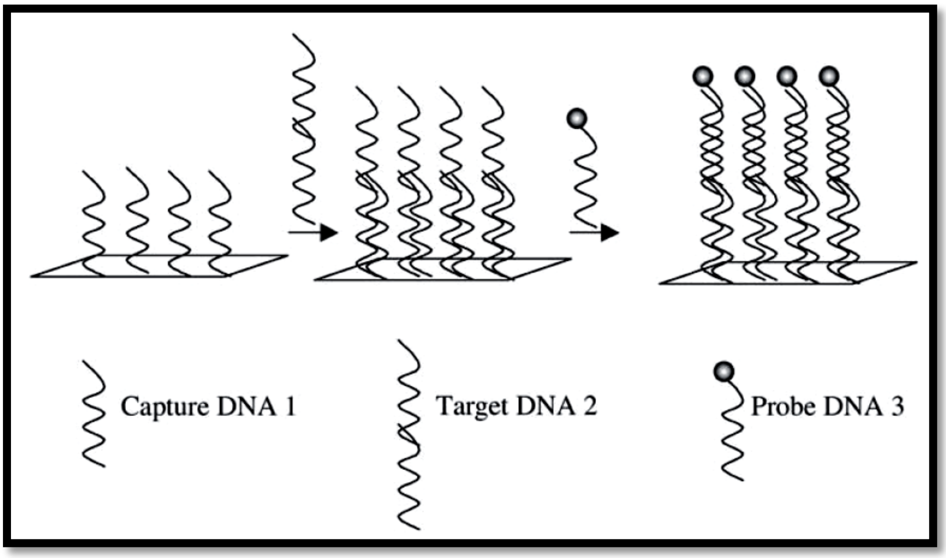

Figure 1.5 The representative figure of sandwich DNA array (Zhao et al., 2005).

1.5. Sandwich Hybridization Based Detection

Sandwich hybridization method can be used in many research areas such as biochemistry, microbiology, virology, plant sciences, oncology, molecular biology and so on. That is to say sandwich hybridization is such a method that can be applied in different areas to detect specific desired molecule such as DNA, cell, protein, etc. In addition, the surface for sandwich hybridization method can be a magnetic bead, a microscope slide or a membrane.

For example, scientists have developed a sandwich hybridization assay on a magnetic bead to detect and quantify a single mRNA of *Sacchoromyces cerevisiae*. The target RNA sensitivity for this assay was determined as 2 fmol (Rautio *et al.*, 2003).

Zhen and co-workers detected harmful algal blooms (HABs), which have serious impacts on fish resources and the marine environment, with integrated sandwich hybridization and nuclease protection assay. This assay detects 12 harmful algal species in marine environment qualitatively and quantitatively (Zhen *et al.*, 2009).

In a very recent study, sandwich hybridization method was used to detect Staphylococcal genomes in positive blood cultures by polymeric enzyme detection (PED) approach. This approach was increased the sensitivity of the assay because for a single biotin molecule per detection probe, multiple streptavidin conjugated enzymes bound. This provided enhanced signal production. PED approach improved the lower limit of detection as 1 fmol/L target DNA (Klonoski *et al.*, 2010).

1.6 Aim of the Study

In this study, the aim is to develop a sandwich-based DNA array for the detection of unlabeled DNA from unpurified PCR mixture. In the frame of this purpose, different transgenes was used as DNA sample in the developed platform.

Although the modification of genetic material of food crops aims to obtain these crops insect, virus, herbicide, salt or drought resistant, there is an increasing public concern about the safety of GM foods. In addition, it is not possible to know which product contains GMO because according to European Union labeling regulations in April 2004, only food and feed containing GM crops must be labeled if the level is higher than 0.9 % (Arvanitoyannis *et al.*, 2006). It means the level below this percentage does not need any labeling. At this point, it is important to detect low levels of GMOs from their source. Therefore we have developed a DNA chip platform which easily and specifically detects the unmodified transgenes from PCR mixture.

CHAPTER 2

MATERIALS & METHODS

2.1. MATERIALS

2.1.1. Chemicals

In this study all chemicals were purchased from Sigma-Aldrich Chemical Company, Merck Chemical Company or AppliChem Chemical Company. All solutions were prepared with ultrapure water which has 18 M Ohm cm^{-1} resistance.

2.1.2 Buffers and Solutions

Preparations and composition of buffers and solution were given in Appendix E.

2.1.3 Synthetic Oligonucleotides

Synthetic oligonucleotides used in this study were designed specifically for CaMV 35S Promoter, NOS Terminator and BAR transgenes and purchased from Integrated DNA Technology (USA) in lyophilized form with purification by standard desalting. Capture and detection probes were partially complementary to their targets. All capture probes were C6 amino-terminated. Also capture probes of CaMV 35S Promoter have a 9-mer poly-dA space. The sequences of commercial synthetic oligonucleotides were listed in Appendix A.

2.1.4 Primer Sequences

Sense and antisense primers specific to CaMV 35S Promoter region were yielded a 52, 72, 92 and 200 base paired products after 45 cycles PCR amplification. Sense and antisense primers specific NOS Terminator and BAR region were yielded 62 and 59 base paired products after 45 cycles PCR amplification, respectively. The sequences of sense and antisense primers are listed in Appendix B.

2.1.5 DNA Samples

Competant *E.coli* strain DH5α with pCAMBIA plasmid containing CaMV 35S promoter was kindly provided by Hamdi Kamçı, from Department of Biological Sciences, METU, Ankara, Turkey. A transgenic potato line (S3) transformed with a CAMBIA vector carrying Myb4 gene under the control of CaMV 35S promoter was used in DNA chip studies to obtain different lengths of transgenic DNAs.

Wild type potato was used as a negative control because it lacks CaMV 35S promoter. These transgenic and wild type potatoes were kindly provided by Gülsüm Kalemtaş, from Department of Biological Sciences, METU, Ankara, Turkey.

In addition, Certified Reference Materials (CRM) NK603 containing 5 % NOS terminator, BAR gene and CaMV 35S promoter was kindly provided by Remziye Yılmaz, from Central Laboratory, METU, Ankara, Turkey. Plasmid DNA was isolated by miniprep plasmid DNA isolation method and explained in section **2.2.3.1.** Genomic DNAs were isolated by CTAB method and explained in section **2.2.3.2.** Plasmid map of pCAMBIA and pSA-MYB4 with 35S promoter are given in Appendix C and D, respectively.

2.1.6 Support Material

In this study, 75x25x1 mm sized Poly-L-Lysine covered glass microscope slides were used and purchased from Menzel Gläser (Braunschweig, Germany).

2.1.7 Crosslinker and Reducing Agent

Tris (2-carboxyethyl) phosphine (TCEP) is a reducing agent and Sulfo-EMCS is a crosslinker (CL-18). They were purchased from Pierce, Thermo Scientific Corp., USA.

2.1.8 Quantum Dots

Quantum Dots were purchased as a kit containing streptavidin conjugated-QD and QD buffer from Invitrogen Corp., California.

2.2. METHODS

2.2.1. Construction of DNA Chip Platform

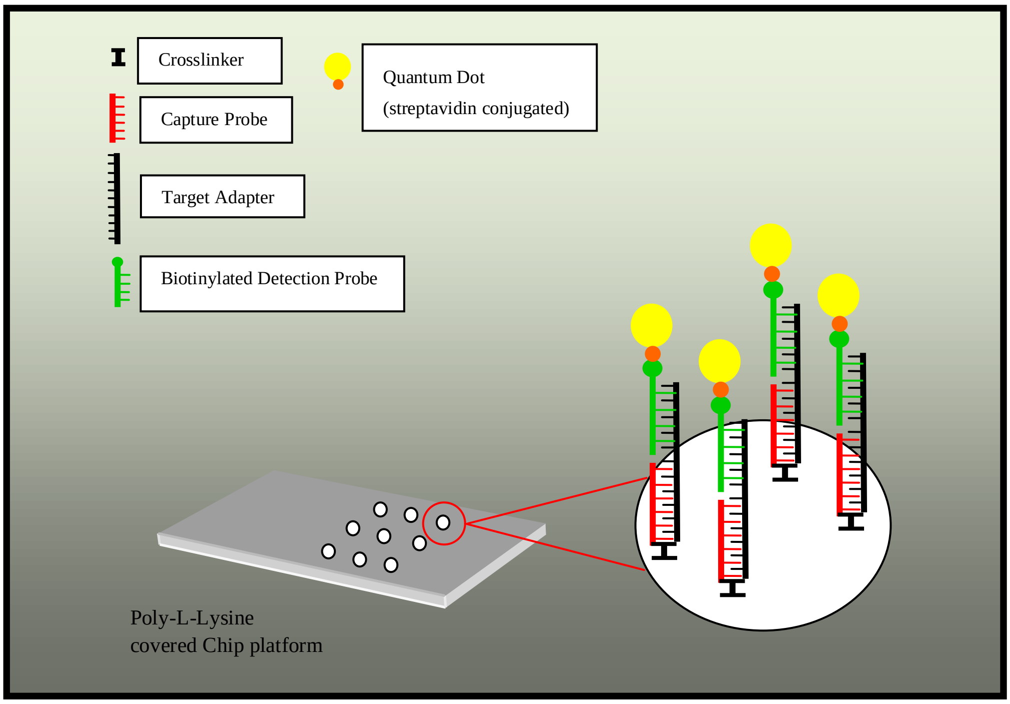

Figure 2.1 Representative figure of the construction of the DNA chip platform

2.2.2 Preparation of Oligonucleotides and Primers

Commercial synthetic oligonucleotides and primers were dissolved in sterile ultrapure water in order to reach a final 100 µM (pmole/µL) concentration and stored in (-) 20 °C until use.

2.2.3 DNA Isolations

2.2.3.1 Plasmid DNA Isolation from *E.coli* cells

Plasmid DNA isolation was done by GeneJet Plasmid Miniprep kit of Fermentas. According to the protocol, 1.5 mL *E.coli* culture grown in liquid LB medium for 12-16 hours was transferred to a microfuge tube and spinned at 6800 g for 2 minutes at room temperature. Supernatant was discarded and pellet was resuspended with 250 µl of the resuspension solution. Immediately, 250 µl of the lysis solution was added and mix thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear. 350µl of the neutralization solution was added and mixed immediately by inverting the tube 4-6 times. Cell debris and chromosomal DNA was centrifugated at 12000g for 5 minutes. Supernatant was transferred to the supplied spin column by pipetting. It was centrifugated at 12000g for 1 minute. Flow-through was discarded and the column was placed back into the same collection tube. 500µl of the wash solution (diluted with ethanol prior to first use) was added to the spin column. It was centrifugated at 12000g for 30-60 seconds. Flow-through was discarded and the column was placed back into the same collection tube. This washing and centrifugation steps were repeated again. After that one additional centrifugation at 12000g for 1 minute was performed. Spin column was transferred to a new clean microfuge tube and 30 µL of sterile distilled water was added. It was incubated for 2min at room temperature and centrifuged for 2min. Column was discarded and the purified plasmid DNA was stored at -20°C until use.

2.2.3.2 Genomic DNA Isolation from CRM and Transgenic Potato Plant

The genomic DNA was isolated from leaves of transgenic potato plant and from certified reference material (CRM) NK603 with CTAB DNA extraction method of Saghai-Maroof (Saghai-Maroof *et al.*, 1984).

Transgenic potato and reference materials were used in the genomic DNA isolation according to the same CTAB method. 0.1 g of fresh plant material and reference materials were ground separately to a fine powder with a pestle and mortar in liquid nitrogen.

Approximately 700 μl of preheated (65 °C) CTAB buffer was added to powders and mixed gently. 7 μL RNAse (10mg/mL) was added and mixed. This CTAB/plant extracts were transferred to centrifuge in microfuge tubes. They were incubated at 65 °C for 30-45 minutes with occasional swirling.

After incubation, microfuge tubes were centrifugated at 13000 rpm 4 °C for 10 min to spin down cell debris. The supernatants were transferred to clean microfuge tubes. Phenol: Chloroform : Isoamylalcohol (25:24:1) was added to tubes as 0.8 volume of supernatants and mix by gentle inversion. After mixing, tubes were spinned at 13000 rpm 4 °C for 10 minutes. The upper aqueous phases were transferred to clean microfuge tubes and chloroform : isoamylalcohol (24:1) was added as 0.8 volume of supernatants. After gentle mixing, tubes were spinned at 13000 rpm 4 °C for 10 minutes. The supernatants were transferred to clean microfuge tubes and isopropanol was added as 0.8-1 volume of supernatants. After gentle mixing, tubes were placed to -20 °C for 2 hours or overnight for the precipitation of gDNA. After this, the tubes were centrifuged at 13000 rpm 4 °C for 5 minutes and supernatants were discarded. The pellets were washed with 70 % cold ethanol at 13000 rpm 4 °C for 5 minutes. Supernatants were discarded and pellets were dried at room temperature. Pellets were dissolved with 30 μL nuclease free water and stored -20 °C.

2.2.4 Preparation of PCR Products

2.2.4.1 PCR Conditions for CaMV 35S Promoter Primers

PCR was carried out in a total reaction mixture of 25 µL. All of the components of PCR were kept in ice before usage. Optimized PCR conditions for 35S promoter region, 5'- GCTCCTACAAATGCCATCAT -3' sense and 5'- GCATCTTCAACG ATGGCCTT -3' antisense primers are listed as follows;

Table 2.1 Optimized conditions of PCR to amplify CaMV 35S Promoter

Ingredients	Stock Concentration	Amount	Final Concentration
dH_2O		12.7 µl	
Reaction Buffer $NH_4(SO_4)$	10X	4 µl	1.6 X
$MgCl_2$	25 mM	2.5 µl	2.5 mM
Sense primer	5µM	3 µl	0.6 µM
Antisense primer	5µM	3 µl	0.6 µM
dNTP	2 mM	4 µl	0.32 mM
DNA	400 ng/µL	0.5 µl	8 ng/µL
Taq polymerase	(5U/µl)	0.3 µl	0.06 U/µL

Table 2.2 PCR cycling conditions to amplify CaMV 35S Promoter

Steps	35S Promoter PCR Parameters
Denaturation	95 °C 5 minutes
Amplification	95 °C 30 seconds 52.1°C 30 seconds 72 °C 30 seconds
Number of cycles	45
Final extension	72 °C 5 minutes

2.2.4.2. PCR Conditions for NOS Terminator Primers

PCR was carried out in a total reaction mixture of 25 µL. All of the components of PCR were kept in ice before usage. Optimized PCR conditions for NOS terminator region, 5'- GCATGACGTTATTTATGAGATG -3' sense and 5'-GTATTAAAT GTATAATTGCGGGAC -3' antisense primers are listed as follows;

Table 2.3 Optimized conditions of PCR to amplify NOS terminator

Ingredients	Stock Concentration	Amount	Final Concentration
dH_2O		12.7 µl	
Reaction Buffer $NH_4(SO_4)$	10X	4 µl	1.6 X
$MgCl_2$	25 mM	2.5 µl	2.5 mM
Sense primer	5µM	3 µl	0.6 µM
Antisense primer	5µM	3 µl	0.6 µM
dNTP	2 mM	4 µl	0.32 mM
DNA	400 ng/µL	0.5 µl	8 ng/µL
Taq polymerase	(5U/µl)	0.3 µl	0.06 U/µL

Table 2.4 PCR cycling conditions to amplify NOS terminator

Steps	NOS Terminator PCR Parameters
Denaturation	95 °C 5 minutes
Amplification	95 °C 30 seconds 45 °C 30 seconds 72 °C 30 seconds
Number of cycles	35
Final extension	72 °C 5 minutes

2.2.4.3 PCR Conditions for BAR Primers

PCR was carried out in a total reaction mixture of 25 µL. All of the components of PCR were kept in ice before usage. Optimized PCR conditions for BAR gene region, 5'- ACCTGCTGAAGTCCCTGGAG -3' sense and 5'- CAGCCCGATGACAGC GAC -3' antisense primers are listed as follows;

Table 2.5 Optimized conditions of PCR to amplify BAR transgene

Ingredients	Stock Concentration	Amount	Final Concentration
dH_2O		12.7 µl	
Reaction Buffer $NH_4(SO_4)$	10X	4 µl	1.6 X
$MgCl_2$	25 mM	2.5 µl	2.5 mM
Sense primer	5µM	3 µl	0.6 µM
Antiense primer	5µM	3 µl	0.6 µM
dNTP	2 mM	4 µl	0.32 mM
DNA	400 ng/µL	0.5 µl	8 ng/µL
Taq polymerase	(5U/µl)	0.3 µl	0.06 U/µL

Table 2.6 PCR cycling conditions to amplify BAR transgene

Steps	BAR Region PCR Parameters
Denaturation	95 °C 5 minutes
Amplification	95 °C 30 seconds 54 °C 30 seconds 72 °C 30 seconds
Number of cycles	35
Final extension	72 °C 5 minutes

2.2.4.4 Agarose Gel Electrophoresis of PCR Products

50 mL 1 % agarose gel buffered with 1X TAE containing 5 µL of ethidium bromide (10 mg/ml) was prepared to run PCR products. The microwave oven-melted gel solution was poured into an electrophoresis gel tray and the comb was placed to form wells. After removal of the comb and placing of the gel in 1X TAE buffer-filled electrophoresis tank, PCR samples and molecular weight size markers (100 bp DNA ladder, MBI Fermentas) were loaded into wells by mixing with 6X loading buffer (Fermentas) at a final concentration of 1X. Power supply was adjusted to 100 V and the gel was run for about 1 hour. Then the bands were visualized under UV light.

2.2.4.5 Determination of dsDNA Concentration of PCR Products

Spectrophotometric reading was done with Shimadzu UVmini 1240 at A_{260nm} after auto-zeroing with the blank solution (ultrapure water). The measured value at A_{260nm} was recorded. The concentration of pure double-stranded DNA with an A_{260nm} of 1.0 is 50 µg / ml. Because PCR products were double-stranded we should use the following formula (1) to determine the concentration of PCR products.

$$\text{Unknown} (\mu g / mL) / \text{Measured } A_{260nm} = 50 (\mu g / mL) / 1.0 \, A_{260nm} \qquad \textbf{(1)}$$

There is a linear relationship between absorbance and DNA concentration. Therefore the following formula (2) regenerated from formula (1) can be used to calculate concentration of PCR product.

$$\text{Unknown } \mu g / mL = 50 \, \mu g / mL \times \text{Measured } A_{260nm} \times \text{dilution factor} \qquad \textbf{(2)}$$

2.2.5 Preparation of DNA Chip

2.2.5.1 Cleaning of Microscope Slides

Poly-L-Lysine covered glass microscope slides were cleaned with ultrapure water (ELGA) for 15 seconds two times at 130 rpm in a coplin jar. They were dried immediately by means of the spinner at maximum speed for 10 seconds.

2.2.5.2 Design of Microscope Slides

During chip experiments, sandwich hybridization assay was constructed through 9 sample spots on PLL slides. These 9 spots applied at the intersection of lines on the slide surface as following. Schematic illustration of the mask slide is shown in Figure 2.2.

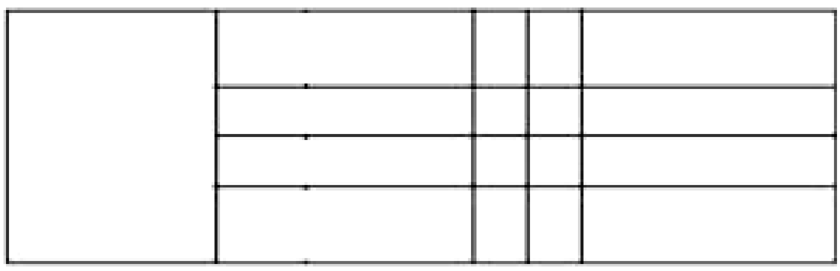

Figure 2.2 Schematic representation of the design of the mask slide.

2.2.6 Establishment of Sandwich Hybridization Platform

2.2.6.1 Preparation of Capture Probe Solution

5’ Thiol modified Probe-1 is called capture probe of the array platform. In capture probe solution, the disulfide bond of this capture probe was reduced to thiol (–SH) with TCEP and CL-18, 18 Å length, was attached to –SH end of probe. For all capture probes of transgenes, solution preparation was the same as following:

Table 2.7 Preparation of capture probe solution

Ingredients	Stock Concentration	Amount	Final Concentration
Capture Probe	25 μM	6 μL	10 μM
1X PBS	10 mM	6.6 μL	4.4 mM
CL-18	33.3 mM	0.9 μL	0.2 mM
TCEP	1 mM	1,.5 μL	0.1 mM
Total Volume		15 μL	

This capture probe solution was allowed to go to reduction for 30 minutes at room temperature.

2.2.6.2 Surface Probe Immobilization

Take 0.5 μL from capture probe solution by a micropipette and apply it to each spot of positive and negative control slides. Then immediately incubate the slides at 30 °C in humidifying chamber for 1.5 hours.

2.2.6.3 Post-Immobilization Washing and Drying of the Slides

For the removal of unbound or weakly bound capture probe, slides were washed with 5X Saline Sodium Citrate (SSC) buffer containing 0.1% SDS at room temperature with agitation (130 rpm) for 15 minutes in a coplin jar. Then slides washed three times with distilled water at room temperature with agitation (130 rpm) for 5 minutes in a clean coplin jar. They were dried immediately by means of the spinner at maximum speed for 10 seconds.

2.2.6.4 Blocking of the Slides

Dried slides were placed in a clean coplin jar containing 2 % Bovine Serum Albumin in 1X Phosphate Buffered Saline (PBS) at pH 7.2. It was used for blocking to inactivate the reactive groups on the surface of poly-l-lysine covered slides. Blocking was carried out at room temperature for 1 hour. It provides that background noise decreases while signal intensities remain unchanged.

2.2.6.5 Post-Blocking Washing and Drying of the Slides

Post blocking washing was done two times with 0.1X SSC (pH 7.0) for 15 seconds and two times with ultrapure water at 130 rpm in a coplin jar. This step prevents the non-specific binding of the probe and DNA molecules during hybridization. After washing, slides were dried immediately by means of the spinner at maximum speed for 10 seconds.

2.2.6.6 Preparation of Hybridization Solution

2.2.6.6.1 Hybridization Solution for ssDNA

The composition of hybridization solutions for ssDNA used in DNA chip optimization studies was as following. Hybridization solution was prepared with 52-mer length ssDNA for both positive and negative controls of sandwich hybridization assay as 25 μL total volume. This hybridization solution should be prepared before hybridization step at least 30 minutes early.

Table 2.8 Preparation of hybridization solution for ssDNA (positive control)

Positive Control Solution	**Stock Concentration**	**Amount (μL)**	**Final Concentration**
ssDNA (Target)	100 μM	5	20 μM
Detection Probe	100 μM	5	20 μM
BSA	10 %	5	2 %
SDS	10 %	1.25	0.5 %
SSC	5X	3	0.6X
Distilled water		5.75	

Table 2.9 Preparation of hybridization solution for ssDNA (negative control)

Negative Control Solution	**Stock Concentration**	**Amount (μL)**	**Final Concentration**
ssDNA (Un-complementary Target)	100 μM	5	20 μM
Detection Probe	100 μM	5	20 μM
BSA	10 %	5	2 %
SDS	10 %	1.25	0.5 %
SSC	5X	3	0.6X
Distilled water		5.75	

2.2.6.6.2 Hybridization Solution for multiplex assay

Hybridization solution of multiplex assay was prepared as 100 μL for one chip platform.

Table 2.10 Preparation of hybridization solution for multiplex assay

	NOS	BAR	CRY1Ab	35S
ssDNA	5 µl (100 µM)	5 µl (100 µM)	5 µl (100 µM)	5 µl (100 µM)
Detection Probe	5 µl (100 µM)	5 µl (100 µM)	5 µl (100 µM)	5 µl (100 µM)
BSA	5 µl (10 %)	5 µl (10 %)	5 µl (10 %)	5 µl (10 %)
SDS	1.25 µl (10 %)	1.25 µl (10 %)	1.25 µl (10 %)	1.25 µl (10 %)
SSC	3 µl (5X)	3 µl (5X)	3 µl (5X)	3 µl (5X)
Distilled water	5.75 µl	5.75 µl	5.75 µl	5.75 µl
Total Volume	100 µL			

2.2.6.6.3 Hybridization Solution for dsDNA

Hybridization solutions were prepared as 5 µL and contain PCR-amplified dsDNA. Their amounts and final concentrations in the solution are as following:

Table 2.11 Preparation of hybridization solution for kit-eluted PCR product

Positive Control Solution	Stock Concentration	Amount (uL)	Final Concentration
Kit-eluted PCR Product	2.72 µM	13.75	1.5 µM
Detection Probe	100 µM	5	20 µM
BSA	10 %	5	2 %
SDS	10 %	1.25	0.50 %

Table 2.12 Preparation of hybridization solution for un-eluted PCR product

Positive Control Solution	Stock Concentration	Amount (uL)	Final Concentration
Un-eluted PCR Product	18.18	13.75	10 µM
Detection Probe	100 µM	5	20 µM
BSA	10 %	5	2 %
SDS	10 %	1.25	0.50 %

2.2.6.7 Hybridization

2.2.6.7.1 Hybridization of ssDNA

Slides were placed on mask slides. 25 µL previously prepared positive and negative control hybridization solution applied to 9 spots area of positive and negative control slides, respectively. By a coverslip, hybridization solution was distributed uniformly throughout this area of slides. Then, these slides were incubated at 40 °C for 1 hour.

2.2.6.7.2 Hybridization of dsDNA

The positive and negative control hybridization solutions prepared previously in eppendorf tube were put in a water bath at 90 °C for 10 minutes to denature PCR-amplified dsDNA. Slides were placed on mask slides. 25 µL positive and negative control hybridization solution immediately applied to 9 spots area of positive and negative control slides after denaturation process. By a coverslip, hybridization solution was distributed uniformly throughout this area of slides. Then, these slides were incubated at 40 °C for 1 hour.

2.2.6.8 Post-Hybridization Washing and Drying of the Slides

Slides were placed in a coplin jar containing ultrapure water for the removal of coverslips from slide surface. Then these slides were put in another coplin jar containing 75 mL 10 mM SSC and 0.1% SDS and washed at room temperature for 5 minutes with agitation (130 rpm). This solution was poured in a waste container. After that, slides were washed with 75 mL 0.1X SSC for 5 minutes at room temperature with agitation (130 rpm). This solution was poured in a waste container. Then slides washed three times with distilled water at room temperature with agitation (130 rpm) for 15 minutes in a clean coplin jar. They were dried immediately by means of the spinner at maximum speed for 10 seconds.

2.2.6.9 Quantum Dot Application

Quantum dots are cadmium selenide nanocrystals composed of a semiconductor core including group II-VI or group III-V elements encased within a shell comprised of a second semiconductor material. Their diameters range to 10 nm containing roughly 200 to 10,000 atoms. They have unique optical and electronic properties such as size- and composition-tunable light emission, improved signal brightness, resistance to photobleaching and simultaneous excitation of multiple fluorescence colors (Peng and Li, 2010). Also, different colors of quantum dots can be simultaneously excited with a single light source, with minimal spectral overlapping, which provides significant advantages for multiplexed detection of target molecules (Gokarna et al., 2008; Marchal et al., 2008; Michalet et al., 2005; Weng et al., 2008; Zhou and Ghosh, 2007).

The emission wavelength of QD used in this study is 565 nm. The excitation wavelength is between 405-525 nm. Absorption of a photon causes an electron to move from the semiconductor valence band to the conductance band, creating an exciton (electron-hole pair). Absorption occurs as long as the energy of the incident photons is higher than the semiconductor bandgap energy; thus, excitons can be created over a wide range of energies within the nanocrystal core (Invitrogen, 2010).

In this step, streptavidin conjugated QDs were used as 2 nM. To reach that concentration, it was taken 0.5 µL from 2 µM QD stock and mixed with 49.5 µL QD buffer. This 50 µL QD solution was used for one positive and one negative control and applied as 25 µL to 9 spots area of each platform. After application, a coverslip was used to spread the QD solution uniformly throughout the spots. Slides were placed in humidifying chamber and incubated at 30 °C for 1 hour.

2.2.6.10 Post-Quantum Dot Application Washing

Slides were placed in a coplin jar containing ultrapure water for the removal of coverslips from slide surface. Then these slides were put in another coplin jar containing 75 mL 10 mM SSC and washed at room temperature for 5 minutes with agitation (130 rpm). This solution was poured in a waste container. After that, slides were washed with 75 mL 0.1X SSC for 5 minutes at room temperature with agitation (130 rpm). This solution was poured in a waste container. Then slides washed three times with distilled water at room temperature with agitation (130 rpm) for 15 minutes in a clean coplin jar. They were dried immediately by means of the spinner at maximum speed for 10 seconds.

2.2.7 UV Imaging

Slides were placed under UV illuminating instrument. In this study, Vilber-Lourmart Infinity 1000 was used as a UV light source. When spots are clearly visualized, images were taken in JPEG format.

2.2.8 Fluorescence Intensity Analysis

Fluorescence intensity obtained under UV light was analyzed by NANObiz Color Master Software. After UV illumination, the image of established sandwich platform was photographed at 8 bit dynamic range (256 levels). In NANObiz Color Master Software, there are 10 boxes which are appropriate dimension for the analysis of spots. First 9 values of all are for 9 spots area on the platform. The last one (10^{th}) is to get background intensity of the platform. Figure 2.3 shows the signal intensity analysis of spots.

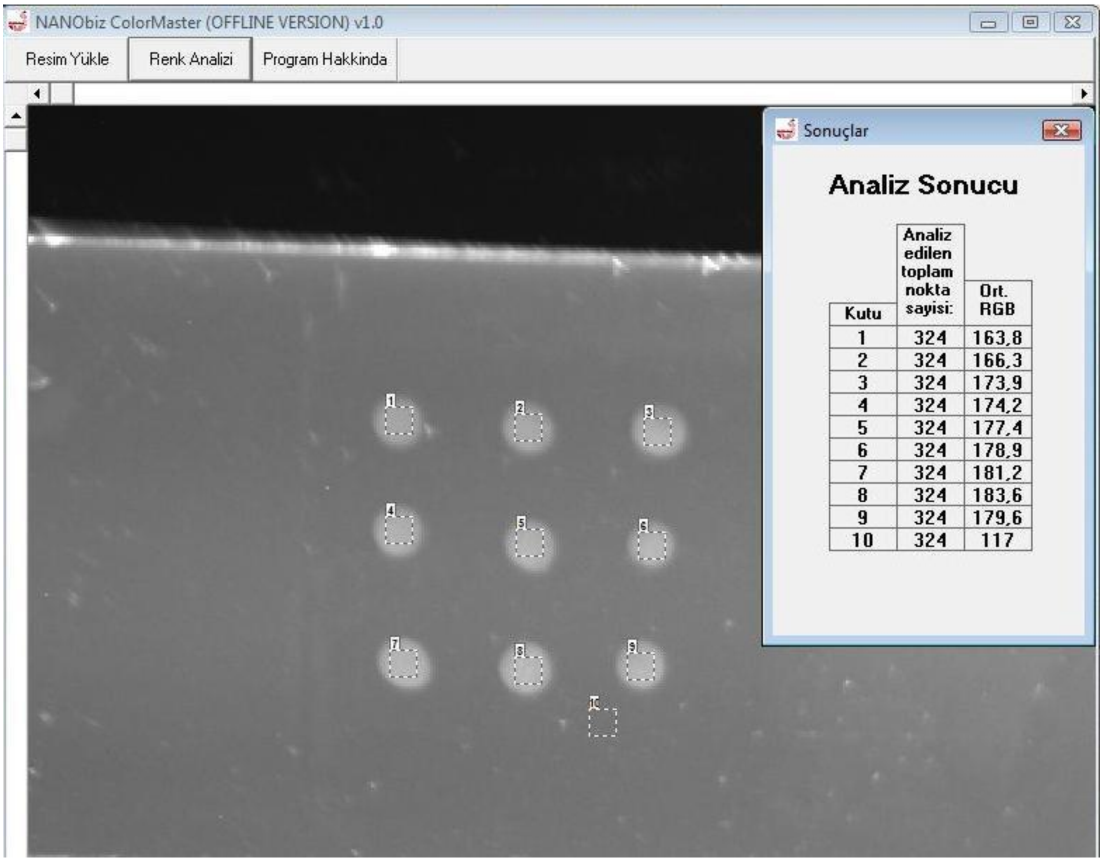

Figure 2.3 Signal intensity analysis performed by NANObiz Color Master Software.

9 boxes were placed on the 9 spots and the last one was placed the out of 9 spot-area. Then analysis was done. Background intensity value was subtracted from each spot intensity value. Because each experiment was carried two times, the same process was done for the other experiment. At the end of the subtraction process, all 18 spot intensity were averaged and divided to the number of analyzed dot (324). The formula is as follows;

$$\text{Signal Intensity Ratio} = \frac{\text{Signal Intensity-Background Intensity}}{\text{324 (number of analyzed dot)}}$$

2.2.9 Statistical Analysis

The statistical analysis for spot intensity ratios was carried out by using the Minitab 15.0 software package. 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 for independent samples more than 2. For 2 independent samples, T-test analysis was done at 95 % confidence interval.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 DNA Samples and PCR Products

3.1.1 pCAMBIA Plasmid DNA and PCR Products

pCAMBIA plasmid DNA was used as a template for CaMV 35S promoter in PCR analysis. Amplification of pCAMBIA was achieved by the transformation of plasmid DNA to competent *E.coli* cells. Transformed bacterial colonies were selected on Spectinomycin (50mg/L) and Streptomycin (50mg/L) containing LB agar medium. These selected colonies were amplified by culturing in liquid LB medium. Miniprep isolation of pCAMBIA plasmid DNA is illustrated in Figure 3.1.

Isolated plasmid DNA was used in PCR in order to confirm the presence of the expected sized PCR product with respect to standard molecular markers. The corresponding CaMV 35S promoter PCR product band is shown in the following Figure 3.2.

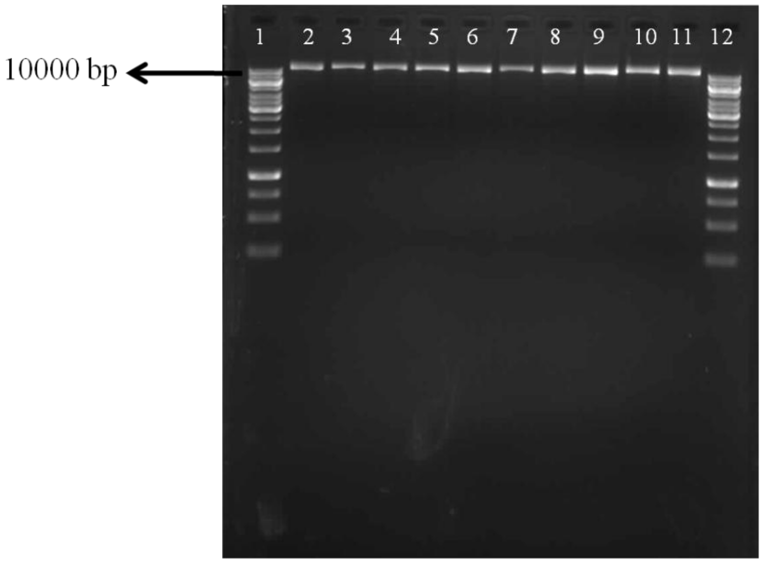

Figure 3.1 1 % Agarose gel electrophoresis (in 1X TAE buffer) of miniprep isolated pCAMBIA plasmid DNA. Lane 1 and 12: 1 kb DNA ladder; Lane 2-11: pCAMBIA plasmid DNA.

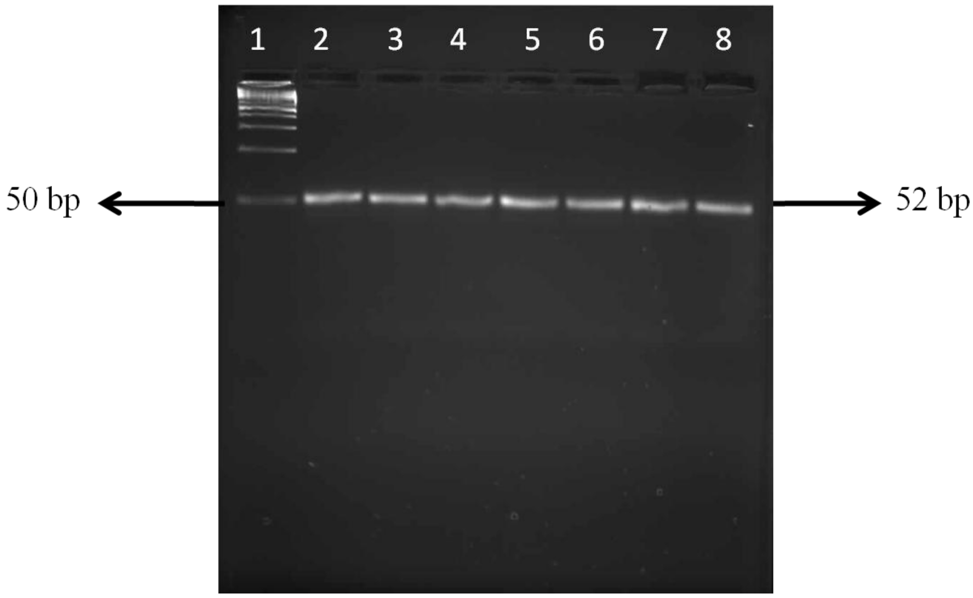

Figure 3.2 4 % Agarose gel electrophoresis (in 1X TAE buffer) of CaMV 35S promoter PCR product amplified from pCAMBIA plasmid DNA. Lane 1: 50bp DNA ladder, Lane 2-8: 52 bp CaMV 35S promoter PCR product.

3.1.2 Genomic DNAs and PCR Products

Genomic DNA was isolated from wild type potato plant, transgenic potato plant and reference material NK603 by using CTAB method. Wild type genomic DNA was used as a negative control for DNA chip studies. Agarose gel electrophoresis results of genomic DNA from wild type-transgenic potato plant (Figure 3.3) and NK603 CRM (Figure 3.6) are illustrated, respectively. Also the corresponding PCR product bands are shown in Figure 3.4, 3.5 and 3.7.

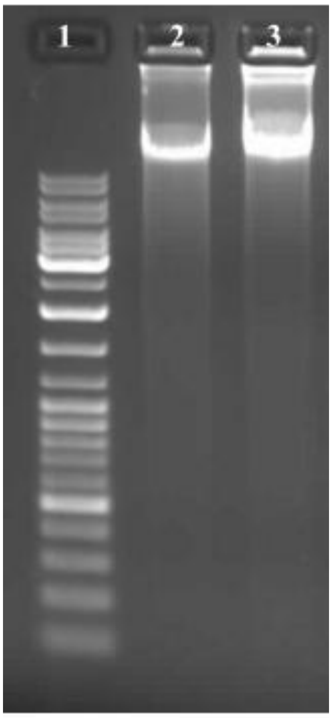

Figure 3.3 1 % Agarose gel electrophoresis (in 1X TAE buffer) of wild type and transgenic potato genomic DNAs Lane 1: 1 kb DNA ladder, Lane 2: genomic DNA of transgenic potato plant and Lane 3: genomic DNA of wild type potato plant.

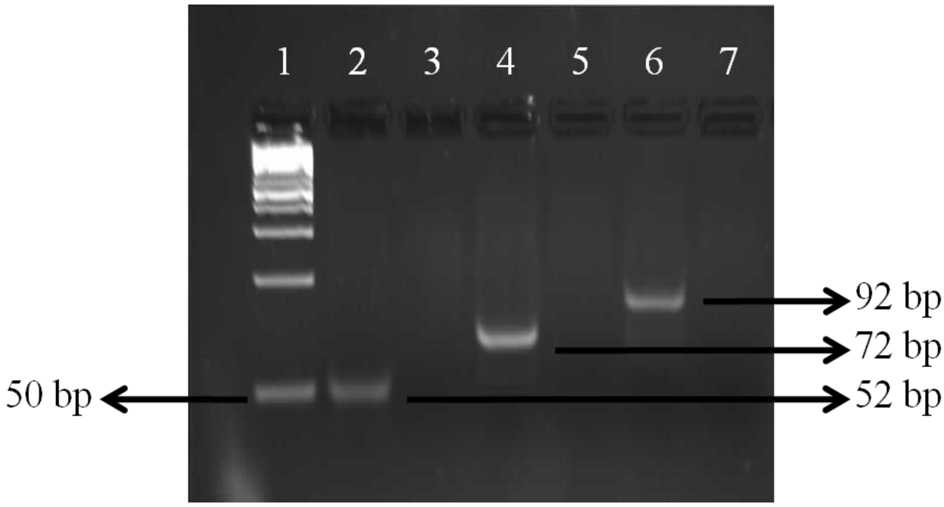

Figure3.4 4 % Agarose gel electrophoresis (in 1X TAE buffer) of CaMV 35S promoter PCR Product amplified from transgenic potato genomic DNA. Lane 1: 50bp DNA ladder, Lane 2: 52 bp CaMV 35S promoter PCR product, Lane 4: 72 bp CaMV 35S promoter PCR product, Lane 6: 92 bp CaMV 35S promoter PCR product, Lane 3-5-7: Negative controls for 52-72-92 bp from wild type DNA.

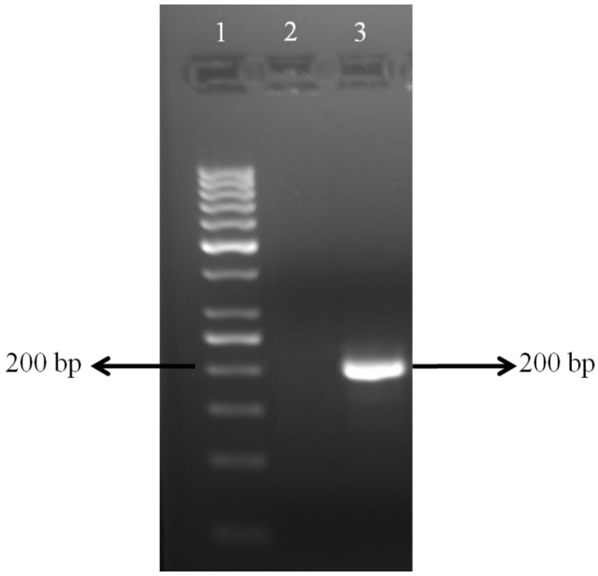

Figure 3.5 4 % Agarose gel electrophoresis (in 1X TAE buffer) of CaMV 35S promoter PCR Product amplified from wild type and transgenic potato genomic DNA . Lane 1; 50bp DNA ladder, Lane 2; Negative control for 35S promoter from wild type potato, Lane 3; 200 bp CaMV 35S promoter PCR Product amplified from transgenic potato .

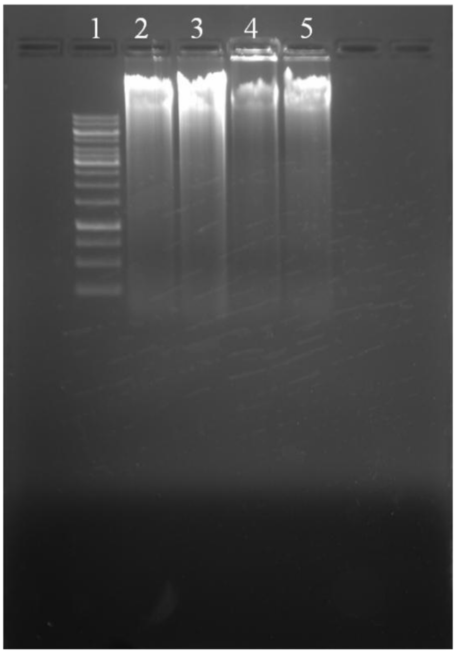

Figure 3.6 1 % Agarose gel electrophoresis (in 1X TAE buffer) of genomic DNA isolated from NK603 CRM containing 5 % GMO. Lane 1: 1 kb DNA ladder, Lane 2-5: NK603 genomic DNA.

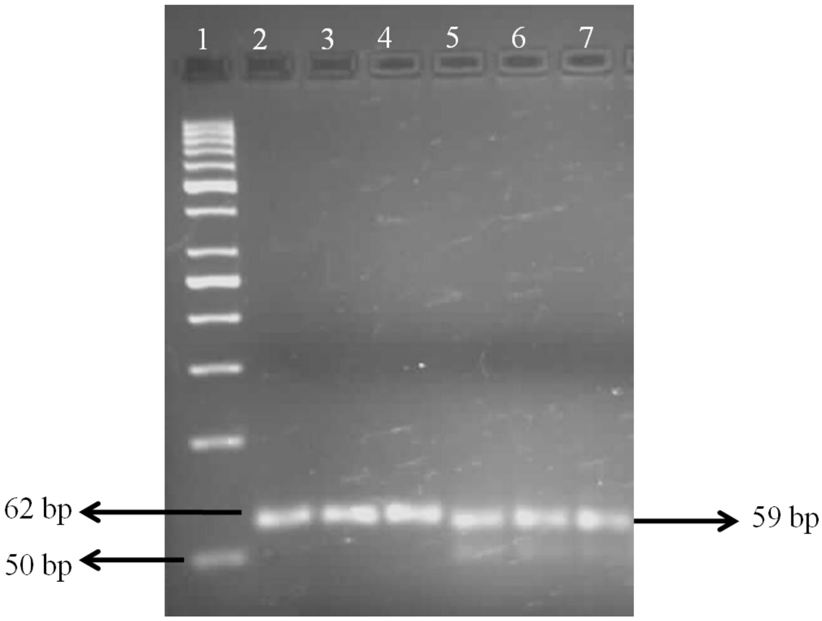

Figure3.7 4 % Agarose gel electrophoresis (in 1X TAE buffer) of NOS terminator and BAR gene PCR product amplified from reference material NK603genomic DNA. Lane 1: 50bp DNA ladder, Lane 2-4: 62 bp NOS terminator and Lane 5-7: 59 bp BAR gene.

3.2 DNA Chip Optimization Studies

In proposed method, capture probe was firstly spotted onto the chip platform and immobilized. This immobilized capture probe was complementary to the first half of target adapter while the second half of target adapter was complementary to the detection probe that has been labeled with biotin molecules. After the hybridization of the target adapter and the probe, streptavidin coated Quantum Dots (QD) were applied on the chip platform. Once QD bound to the biotinylated probe, it gave a detectable signal corresponding to the amount of hybridized target DNA.

In this study, 0.5 µl of capture probe was spotted onto the chip surface via hand-pipetting. Due to the intensity difference of the hybridization signal within spots, capture probe was spotted as 9 replicas on a single platform and the intensities from these 9 spots were averaged for each experiment.

We began the design and development of DNA chip platform with a series of optimization studies. Through these studies we aimed to get spots that were homogenous and yielding high hybridization density with low background. In addition, the immobilized DNA probe should specifically capture the target DNA.

The sandwich hybridization method was optimized to detect a section of CaMV 35S Promoter from GMOs with single stranded DNA.

We have set standard experimental conditions for this platform using available literature. To optimize different parameters in the platform, we have changed various conditions from the standard protocol such as capture probe concentration, blocking time, adapter and detection probe concentration, hybridization temperature and time and quantum dot incubation time. In these optimization studies, single stranded 52 base long commercial DNA was used. At the end of optimization studies, sandwich hybridization on DNA chip platform were carried out with real GMO samples (dsDNA from transgenic potato) by using optimized parameters. These assayed conditions as well as main steps are given in Table 3.1.

Table 3.1 Standard experimental conditions for DNA chip platform

Main Steps	Standard Conditions
Capture Probe Concentration	10 µM
Capture Probe Immobilization Time & Temperature	1.5 hours & 30 °C
Post-Immobilization Washing	5X SSC+1 % SDS/5 min dH_2O/5 min/3 times
Blocking Time & Temperature	1 hours & RT
Post-Blocking Washing	0.1X SSC / 30 sec/2 times dH2O/rinse
Target Adapter Concentration	20 µM for ssDNA (52-mer)
Detection Probe Concentration	20 µM
Hybridization Time & Temperature	1 hour at 40 °C
Post-Hybridization Washing	1X SSC + 0.1% SDS / 5 min 0.1X SSC / 5 min dH2O/ 3min
Quantum Dot Concentration	20 nM
Quantum Dot Attachment Time & Temperature	1 hour at 30 °C
Post- QD Application Washing	1X SSC / 5 min 0.1X SSC / 5 min dH2O/ 3min

3.2.1 Optimization of Capture Probe Concentration

Successful immobilization of capture probe on the DNA Chip platform is the first and one of the most critical step. Its density has been shown to affect the hybridization efficiency and array sensitivity (Zhou *et al.*, 2004)). Up to a level, increasing capture probe concentration leads to an increase in the hybridization efficiency. Earlier reports show that the efficiency of hybridization is low for high capture probe density (Southern *et al.*, 1999). Therefore, we studied only 5 and 10 µM capture probe concentrations on the glass platform.

The efficiency of capture probe immobilization was tested with standard experimental conditions through sandwich hybridization assay. Figure 3.8 and 3.9

show sandwich hybridization results with 5 μM and 10 μM probe concentration in terms of spot images and spot analysis, respectively.

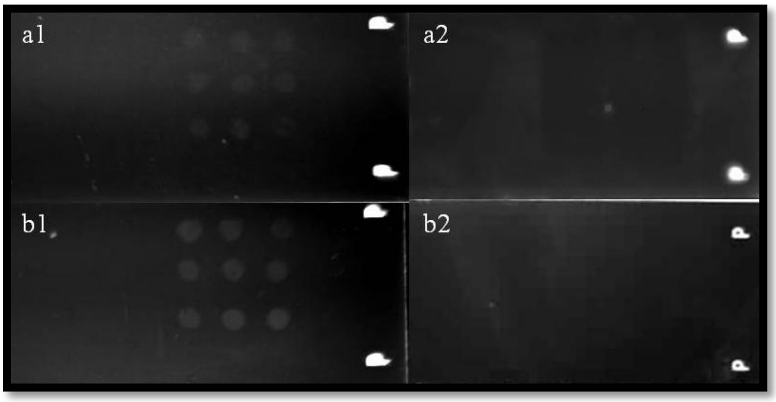

Figure 3.8 Images of DNA chip platform upon immobilization with different concentrations of capture probe. Standard experimental conditions were used at 5 μM capture probe concentration for positive (a1) and negative (a2) controls and at 10 μM capture probe concentration for positive (b1) and negative (b2) controls.

When the concentration of capture probe was 5 μM, a decrease was observed in the signal strength (Figure 3.8). However hybridization signal increased as the capture probe concentration changed from 5 to 10 μM. Immobilized probes at 10 μM concentration were able to capture solution phased targets. This result was supported quantitatively by intensity analysis (Figure 3.9). According to T-test analysis, the difference between two concentrations is considered to be very statistically significant at $p < 0.05$. Our results have indicated that 10 μM capture probe concentration is appropriate for DNA chip platform for further studies.

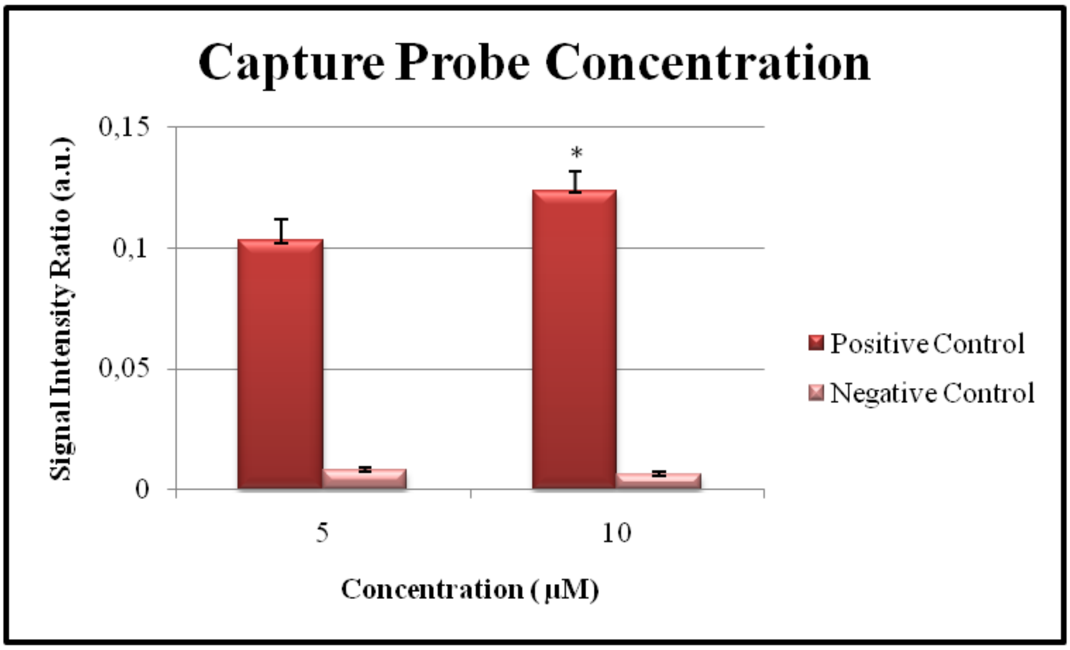

Figure 3.9 The analysis of signal intensity ratio as a function of capture probe concentration for DNA chip platform under standard experimental conditions. Error bars represent standard error of mean (SEM) calculated from 18 spots. * indicates significant difference ($p < 0.05$).

3.2.2 Optimization of Blocking Time

Blocking of the glass microarray surface after the immobilization of the capture probe prior to target adapter hybridization is an important step to obtain a low background. Low background increases the hybridization sensitivity of microarrays (Jayaraman *et al.*, 2006) and therefore, the influence of blocking time on DNA chip platform was investigated. Generally, in microarray studies, 45 and 60 minutes blocking times are ideal to reduce background signal (Diehl et al., 2002; Taylor et al., 2003; Tran et al., 2002). In this part, all standard experimental conditions remained unchanged except the blocking time: 15, 30, 60 and 120 minutes.

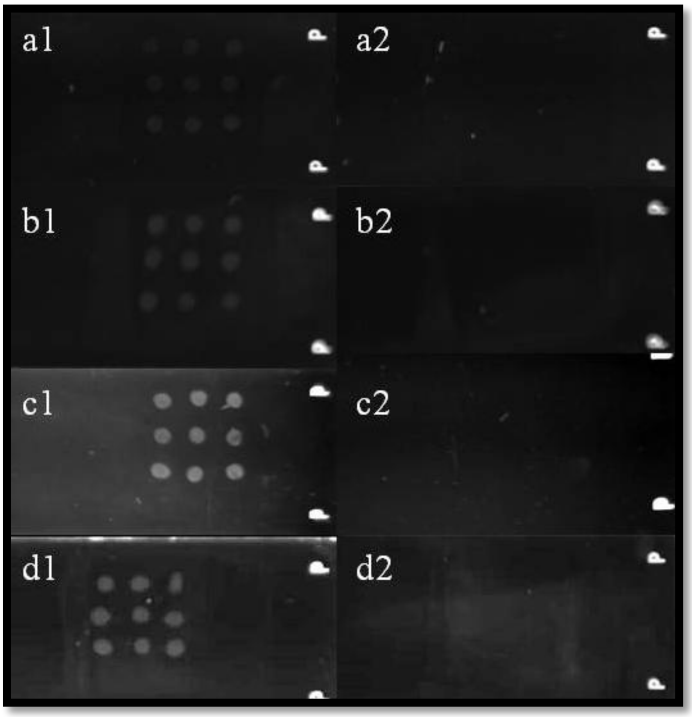

Figure 3.10 Images of DNA Chip platform at different blocking durations. Standard experimental conditions were conducted at 15 minutes blocking time for positive control (a1), at 30 minutes blocking time for positive control (b1), at 60 minutes blocking time for positive control (c1) and at 120 minutes blocking time for positive control (d1). (a2), (b2), (c2) and (d2) are the negative controls, respectively.

Our studies showed that, at short periods of blocking time as in 15 and 30 minutes, spot's morphology and size were affected (Figure 3.10.) Spots were also faint and hard to observe. As the blocking time increases, spots had higher signal intensities, good spot morphology and optimal size.

Our results also indicated a low signal intensity ratio at blocking times under 60 minutes. This result can be explained through non-specific bindings of hybridized target adapter and detection products in solution to poorly blocked amine surface of the chip platform. After stringent post-hybridization washing, however, these weakly bound target and detection probe washed away from platform. As a result, the amount of sandwich hybridization of the target was low resulting a low signal intensity (Figure 3.11).

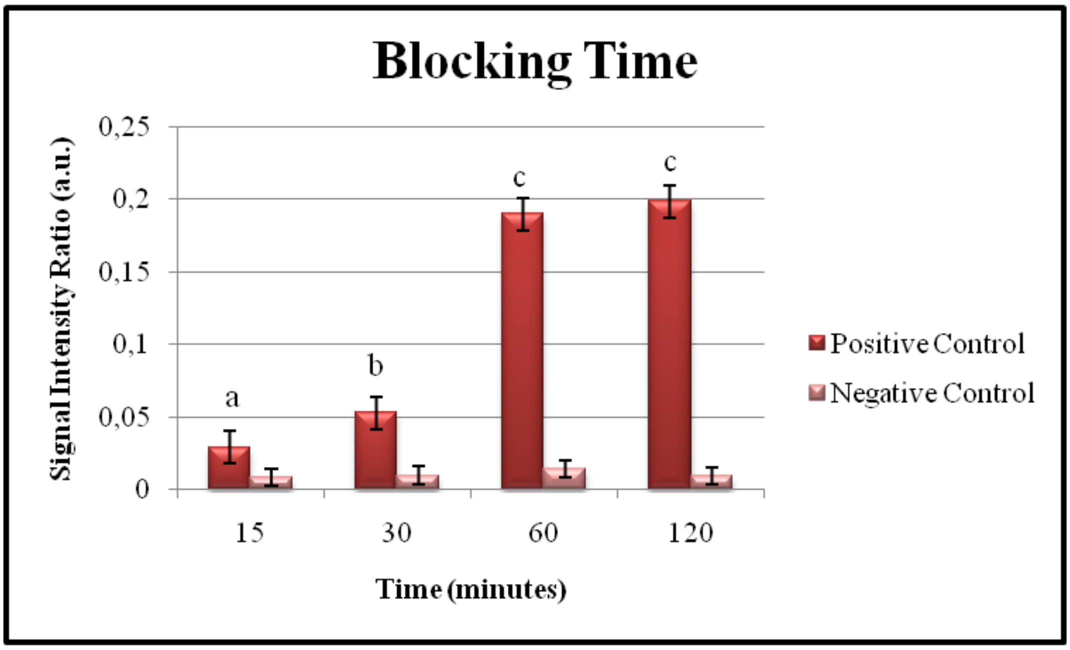

Figure 3.11 The analysis of signal intensity ratio as a function of blocking time for DNA chip platform under standard experimental conditions. Error bars represent standard error of mean (SEM) calculated from 18 spots. Different letters indicate significant difference at $p < 0.05$.

In addition, high background intensity was also obtained due to the insufficient blocking. In our signal intensity analysis, the background intensity is subtracted from signal intensity and this value is divided to the analyzed spot area. Insufficient blocking, therefore, results in low signal intensity ratio due to high background intensity.

When we increased blocking times to 60 and 120 minutes, signal intensity ratio was also increased. Our results summarized in Figure 3.5 indicated that the lowest possible blocking time was 60 minutes as lower blocking times caused higher background intensities. . Increasing the blocking time above 60 minutes had no significant effect on the hybridization signal strength, and therefore, 60 minutes blocking time was preferred for further studies. As also observed in our experiments generally, in microarray studies, 45 and 60 minutes blocking times are ideal to reduce background signal (Diehl et al., 2002; Taylor et al., 2003; Tran et al., 2002).

3.2.3 Optimization of Adapter Concentration

To assess the sensitivity of this platform, various concentrations of target adapter were investigated by using commercially available single stranded 52 base long section of CaMV 35S promoter region at a concentration range of 20 µM-0,001 µM. Experimental results are given in Figure 3.12.

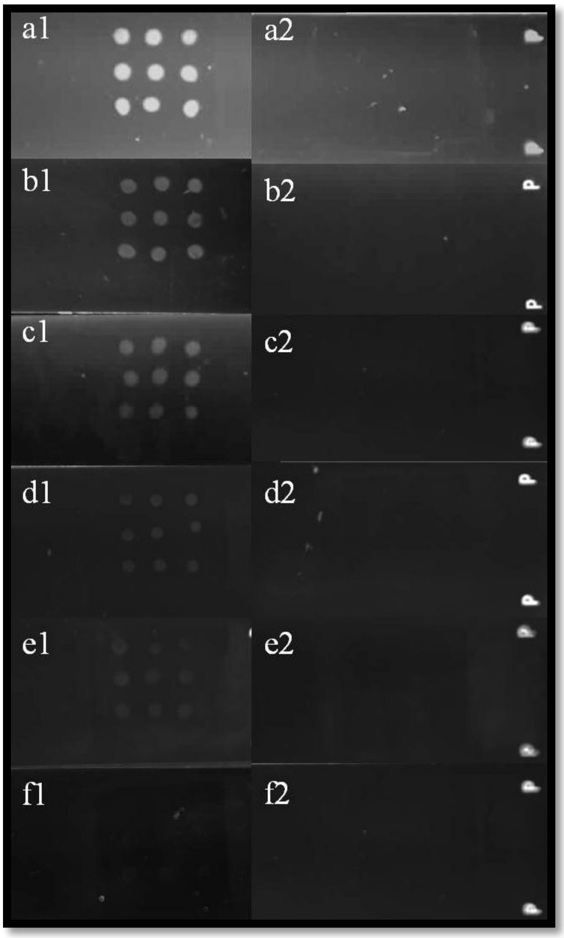

Figure 3.12 Images of DNA Chip platform at different target adapter concentrations. Standard experimental conditions were conducted at 20 µM adapter concentration for positive control (a1), at 10 µM adapter concentration for positive control (b1), at 0,5 µM adapter concentration for positive control (c1), at 0,1 µM adapter concentration for positive control (d1), at 0,05 µM adapter concentration for positive control (e1) and at 0,001 µM adapter concentration for positive control (f1). (a2), (b2), (c2), (d2), (e2) and (f2) are the negative controls, respectively.

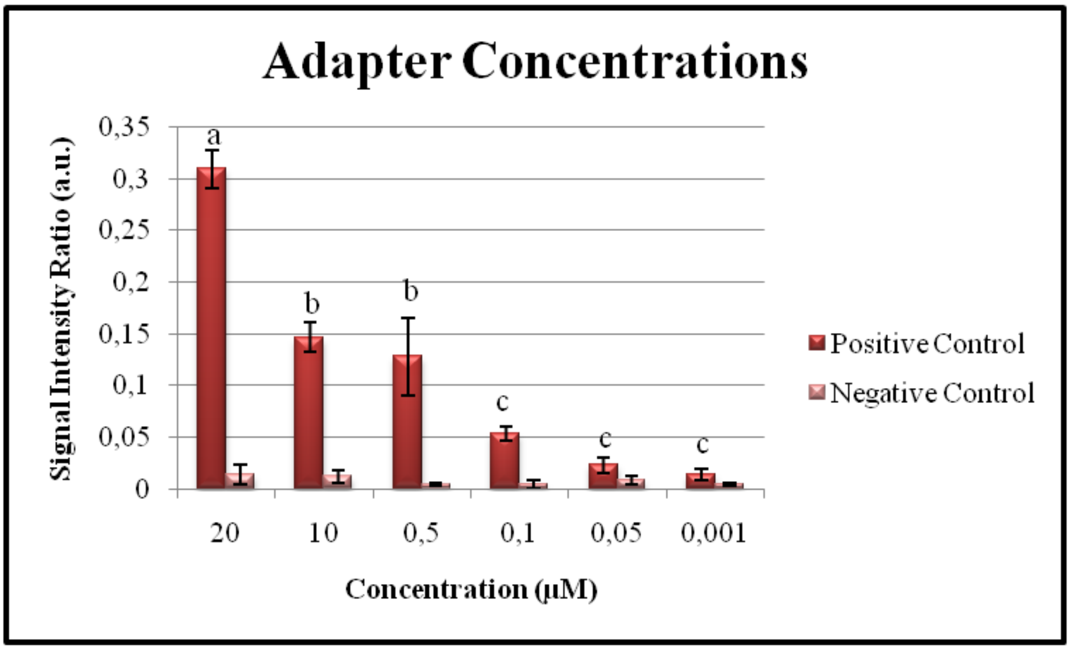

Figure 3.13 The analysis of signal intensity ratio as a function of adapter concentrations for DNA chip platform under standard experimental conditions. Error bars represent standard error of mean (SEM) calculated from 18 spots. Different letters indicate significant difference at $p < 0.05$.

The lowest tested concentration of target adapter was 0.001 µM (1nM) and it gave an averaged 0.014129 signal intensity ratio. A significant decrease was observed due to the decreased adapter concentration from 20 µM to 0.001 µM (Figure 3.13)

In the Figure 3.12, f1 and f2 labeled slide images represent 1 nM target adapter concentration for positive and negative controls, respectively. This concentration gave a very poor signal intensity due to inadequate sandwich hybridization, so concentrations below 1nM were not further studied. The detection limit for DNA chip platform was recorded at nanomolar level as 1 nM. With respect to sensitivity, there are better methods in literature which are capable of detect targets at femto even at atto levels (Angenendt et al., 2003; Klonoski et al., 2010). However, ease of use and having multiple targets on one DNA chip are preferred in our studies over the detection limit.

The sensitivity of this method was evaluated according to the images obtained after UV illumination. We preferred UV instrument because it is inexpensive and it is very easy to simultaneously monitor all spots from a single experiment.

Incubation time for the hybridization is another parameter that affects the sensitivity. To achieve a higher sensitivity, the hybridization time can be increased from 1 hour as we used in our studies to several hours. However, increased incubation period is not desired for our purpose to have a fast and easy to use assay platform. The content of hybridization solution is yet another factor that affects the sensitivity of the assay. It can be carried out by slightly decreasing the concentration of BSA, SDS and SSC and hybridization is maintained under low stringent conditions to decrease the detection limit of chip platform.

Although the sensitivity of our DNA chip platform was lower than other methods reported in literature, it could be improved up to a level by enhancing the signal intensity with bi-labeling of the detection probe (Korn *et al.*, 2003).

3.2.4 Optimization of Detection Probe Concentration

Detection probe used in this study is commercially biotinylated. Signal was obtained under UV illumination after the binding of streptavidin conjugated QD to biotinylated detection probe. Therefore the strength of the signal is related with the integration of detection probe to the DNA Chip platform. In the study, 5, 20 and 40 μM concentrations of detection probe were tested. The response of chip platform to these concentrations and signal intensity analysis are shown in Figure 3.14 and 3.15.

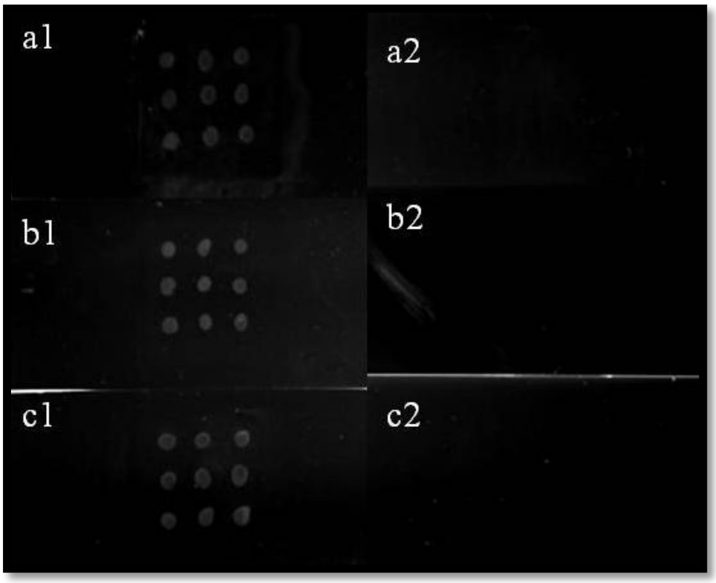

Figure 3.14 Images of DNA Chip platform upon hybridization with biotinylated detection probe at different concentrations. Standard experimental conditions were used at 5 µM Probe-2 (a1) for positive control, 20 µM Probe-2 (b1) for positive control and 40 µM Probe-2 (c1) for positive control. (a2), (b2) and (c2) are the negative controls, respectively.

At all these concentrations, spots were visible (Figure 3.14). However a low signal intensity ratio was obtained at 5 µM capture probe concentration. It increased as the detection probe concentration changed from 5 to 40 µM. Although a significant increase was observed from 5 to 20 µM probe concentration, there was not any significant difference at the 0.05 level between 20 to 40 µM concentrations (Figure 3.15). Their signal intensity ratio was found so close to each other. Therefore to reduce the cost of the assay, the optimum detection probe concentration was chosen as 20 µM for further studies.

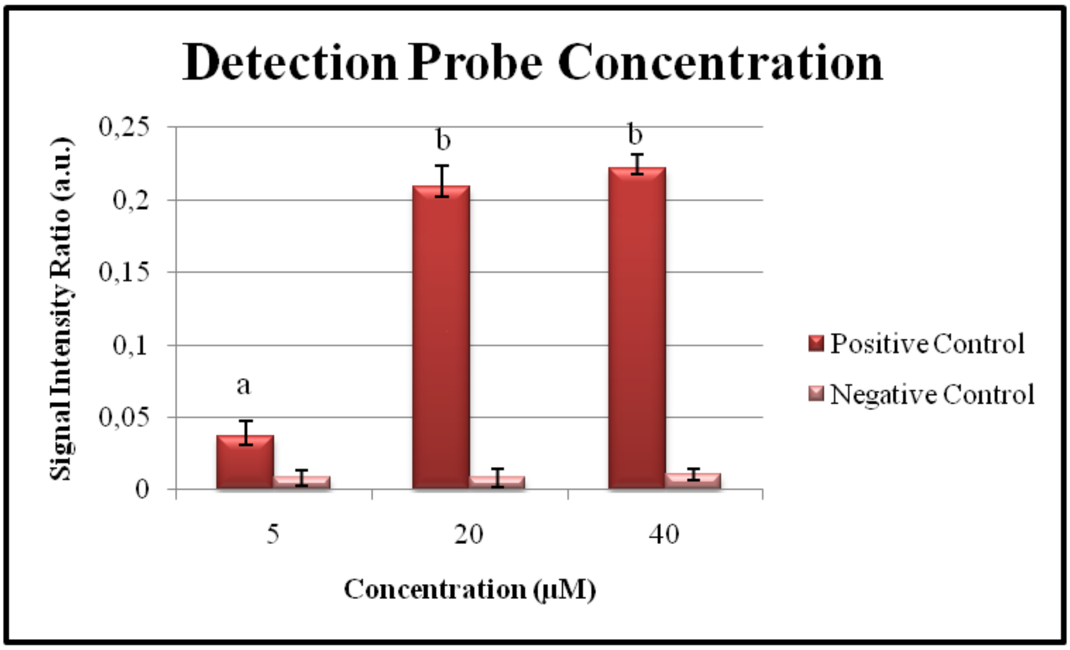

Figure 3.15 The analysis of signal intensity ratio as a function of detection probe concentration for DNA chip platform under standard experimental conditions. Error bars represent standard error of mean (SEM) calculated from 18 spots. Different letters indicate significant difference at $p < 0.05$.

3.2.5 Optimization of Hybridization Time

Hybridization time is another important factor in microarray studies. In order to observe the effect of hybridization time on DNA chip platform, hybridization solution was incubated with the immobilized capture probe at different periods of time under standard experimental conditions. 0,5, 1, 2,5 and 5-hour time periods were examined. Figure 3.16 shows the result of different hybridization time on chip platform.

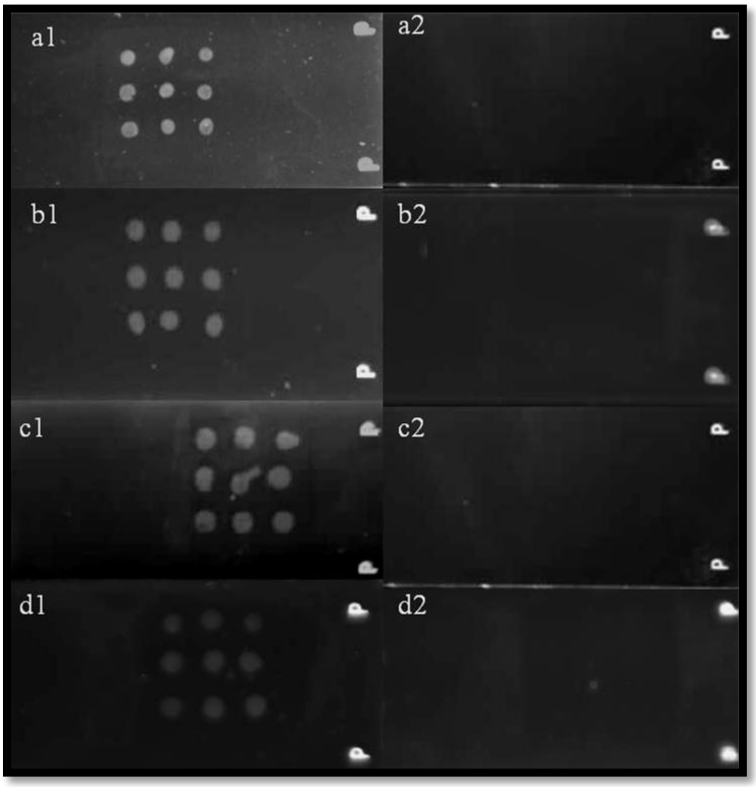

Figure 3.16 Images of DNA Chip platform at different hybridization durations. Standard experimental conditions were conducted at 5-hour hybridization time for positive control (a1), at 2.5-hour hybridization time for positive control (b1), at 1-hour hybridization time for positive control (c1) and at 0.5-hour hybridization time for positive control (d1). (a2), (b2), (c2) and (d2) are the negative controls, respectively.

An increased specific hybridization signal observed with increasing hybridization time (Figure 3.16). The highest net signal intensity ratio was obtained at 5-hour incubation. The strength of signal intensity at 2.5-hour hybridization period was lower than 5-hour period. As the hybridization time was decreased, a decrease in signal strength was observed. According to the analysis in Figure 3.17, a significant difference in the signal intensity ratio was recorded when the duration of hybridization decreased 1-hour to 0.5-hour.

Longer hybridization times such as 20, 16 and 12 hours were used in various microarray studies (Diehl *et al.*, 2002; Tran *et al.*, 2002; Zhou *et al.*, 2004).

However, it is also shown that longer hybridization period could generate substantial nonspecific background (Zhen Guo, 2002). Therefore, in the optimization of hybridization time, we did not study above 5-hour durations.

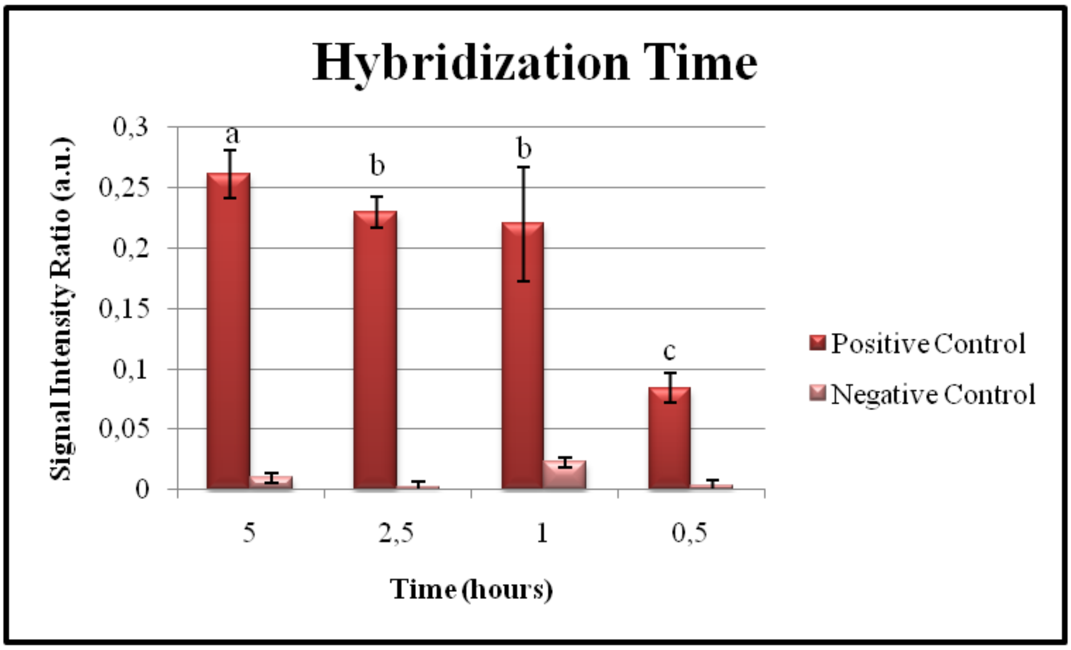

Figure 3.17 The analysis of signal intensity ratio as a function of hybridization time for DNA chip platform under standard experimental conditions. Error bars represent standard error of mean (SEM) calculated from 18 spots. Different letters indicate significant difference at $p < 0.05$.

Although signal intensity ratio in 5-hour hybridization time was the highest, it was not preferred for chip studies due to the long assay time. Moreover, because there was not a big signal intensity difference between 2.5 and 1-hour incubation time, 1-hour hybridization time was chosen for further studies to reduce the time of hybridization experiments. The same hybridization time was used by Lee et al. (2010) for the rapid analysis of the spoOA gene of Bacillus species with flow cytometric detection method based on DNA sandwich hybridization. (Lee *et al.*, 2010).

3.2.6 Optimization of Hybridization Temperature

Hybridization temperature has a crucial impact on signal strength in microarray studies. Melting temperature (T_m) of the duplex is determined by the length of duplex. Detection probe and target DNA were previously hybridized in an eppendorf containing hybridization solution. Therefore, hybridization temperature was related with T_m of the capture probe and target DNA-detection probe duplex. Because in all optimization studies, 52 base ssDNA was used, T_m was calculated accordingly to the capture probe of this target DNA. T_m of the 26 base-paired complementary duplex in this study was computationally found as 55 °C. In literature, the common approach is to use 15-20 °C below T_m for the determination of hybridization temperature. Based on this data, 35 and 40 °C were studied to determine the hybridization temperature.

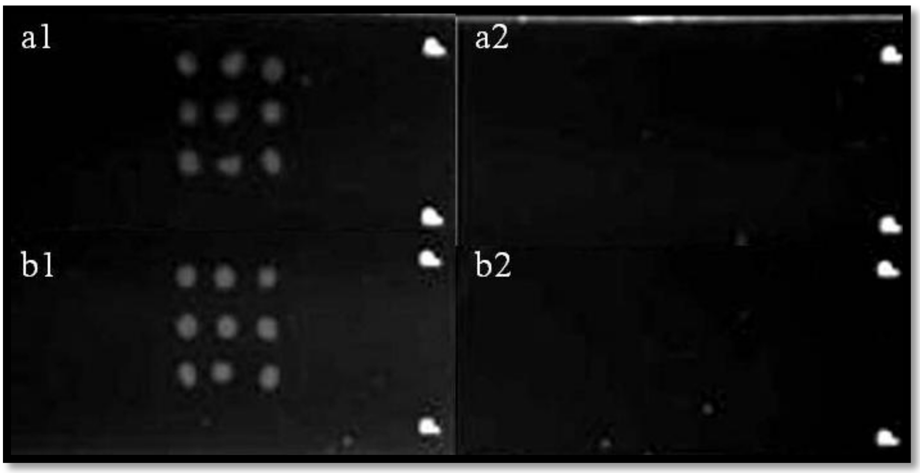

Figure 3.18 Images of DNA Chip platform at different hybridization temperatures. Standard experimental conditions were conducted at 35 °C hybridization temperature for positive control (a1), at 40 °C hybridization temperature for positive control (b1). (a2) and (b2) are the negative controls, respectively.

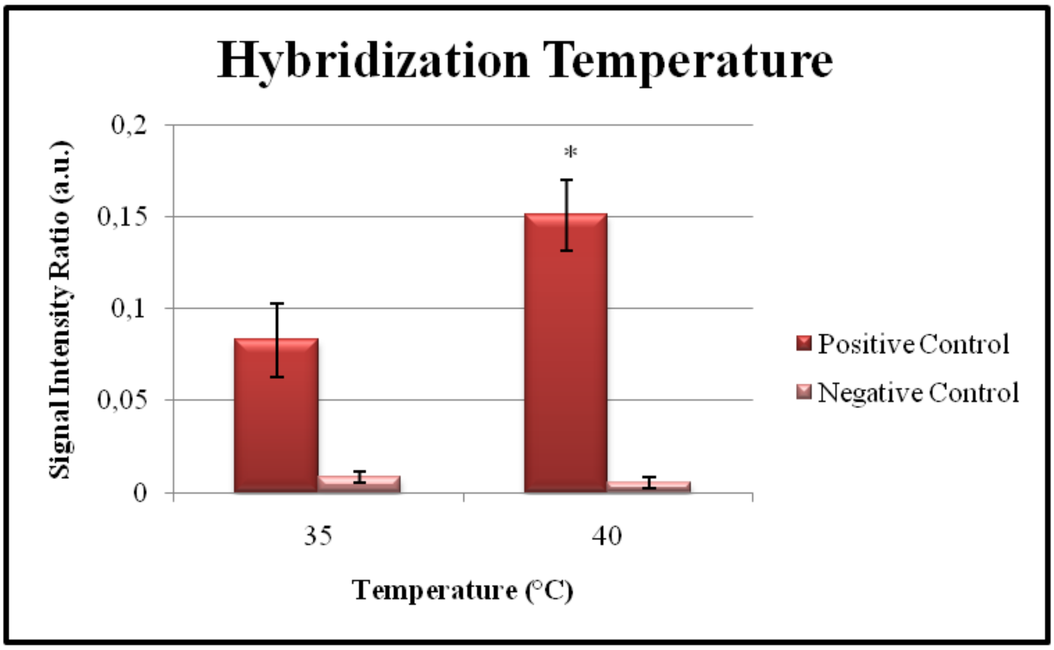

Figure 3.19 The analysis of signal intensity ratio as a function of hybridization temperature for DNA chip platform under standard experimental conditions. Error bars represent standard error of mean (SEM) calculated from 18 spots. * indicates significant difference ($p < 0.05$).

According to T-test analysis of the collected data, the difference between two hybridization temperatures was considered to be statistically significant at p 0.05 level. At 40 °C hybridization temperature, DNA chip platform yielded stronger signal than 35 °C. This is not observable when the images regarding to two temperatures. Spots of both experiment seemed to be the same (Figure 3.18). However, the signal intensity ratio analysis showed that, the higher numerical value was obtained when the hybridization of capture probe and target-detection probe duplex was occurred at 40 °C hybridization temperature (Figure 3.19).

Hybridization temperatures for DNA:DNA hybrids are mostly carried out at around 40 °C. If hybridization buffer contains 50 % formamide (reduces the melting temperature of the DNA:DNA hybrid and permits users to reduce hybridization temperatures), hybridization takes place generally at 42 °C even long capture probes are used (Diehl *et al.*, 2002). Also when the same length capture probe as in our study was used, 40 °C hybridization temperature was chosen in the detection of DNA hybridization electrochemically (Flechsig and Reske, 2007).

3.2.7 Optimization of Quantum Dot Incubation Time

Another critical parameter in this sandwich hybridization assay is the required time for the attachment of streptavidin conjugated QD to biotinylated detection probe, as it accounts for the strength of the signal. Although streptavidin has a high affinity for biotin, they need to be incubated for a certain time for a complete attachment.

In this optimization study, incubation time for QD attachment was investigated at 3 different incubation times. Figure 3.20 and 3.21 show the signal images and analysis, respectively.

Figure 3.20 shows the spot images when the QD incubation time is 5, 15 and 60 minutes. At 5 and 15 minutes, streptavidin conjugated QDs were bind to biotinylated detection probe of sandwich complex but binding did not reach maximum level. These durations were not enough to saturate the detection probe because spots had poor signal intensity.

However 60 minutes of QD incubation time significantly increased the signal intensity (Figure 3.21), and therefore selected for further studies. Yet for qualitative and practical analysis it is possible to get a signal at 30 min signal probe incubation time.

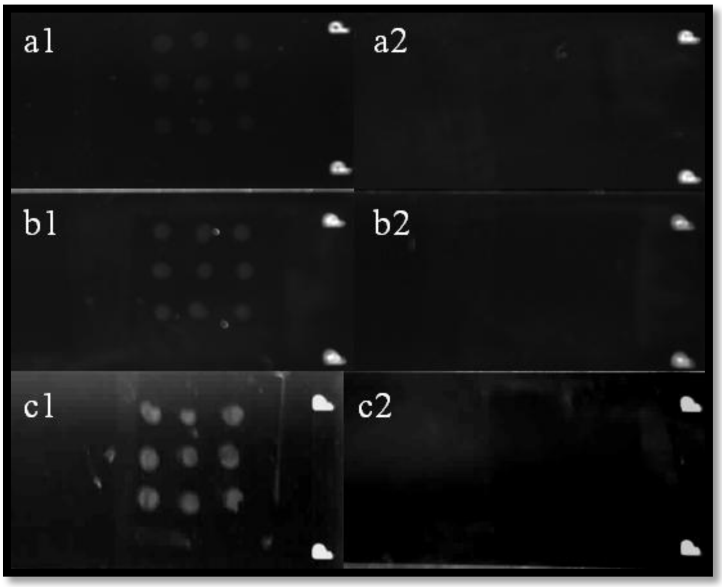

Figure 3.20 Images of DNA Chip platform at different QD incubation times. Standard experimental conditions were conducted at 5 minutes incubation of QD for positive control (a1), at 15 minutes incubation of QD for positive control (b1) and at 60 minutes incubation of QD for positive control (c1). (a2), (b2) and (c2) are the negative controls, respectively.

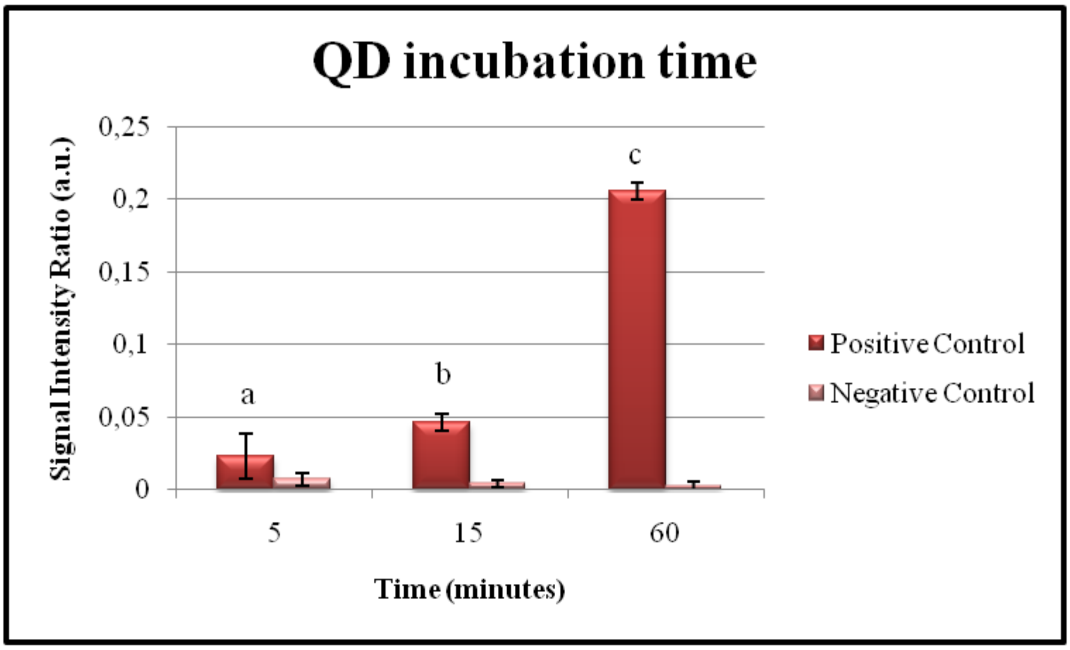

Figure 3.21 The analysis of signal intensity ratio as a function of QD incubation time for DNA chip platform under standard experimental conditions. Error bars represent standard error of mean (SEM) calculated from 18 spots. Different letters indicate significant difference at $p < 0.05$.

3.3 Different Spot Size on Chip Platform

In this step, the relationship between signal intensity and spot size was assayed. Thiol modified capture probes were immobilized on DNA Chip platform at different sizes. This step was followed by hybridization of single stranded 52 base long target DNA under standard experimental conditions. Spot volumes ranged from 3 µL to 0.1µL.

The apparent capture probe density for DNA chip platform was calculated as 4pmol/µL and 630fmol/mm^2 (r=1 mm area=3.14 mm^2 (0.5 µL spot)). According to the different spotted volumes in this study, this density was between 12 pmol-0.4 pmol. The amount and density of capture probe are listed in the following Table 3.2

Table 3.2 The amount and density of capture probe according to the spotted volume on DNA chip platform.

Spot Volume (µL)	3	2,5	2	1,8	1,5	1,2	1	0,5	0,3	0,1
Amount of capture probe (ng)	132	110	88	79.2	66	52.8	44	22	13,2	4,4
Density of capture probe (pmol/ µL)	12	10	8	7,2	6	4,8	4	2	1,2	0,4

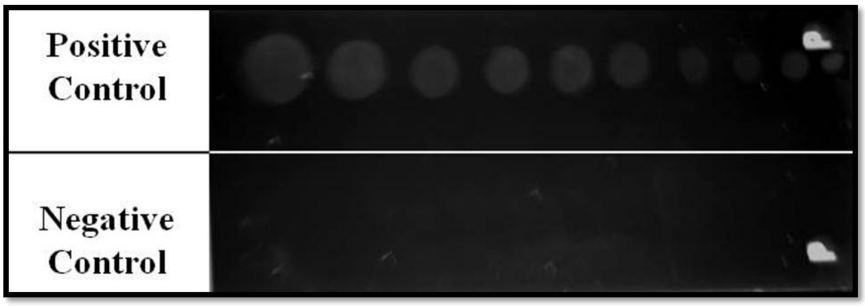

Figure 3.22 Different spot sizes on a single chip platform. The upper part of slide designed as positive control and the bottom part as negative control. The numbers in boxes above the figure represents the volume, amount and density of spotted capture probe solution.

In all DNA chip studies, capture probe solutions were spotted by hand-pipetting. During previous studies, 0.5 µL capture probe solution was spotted on the platform. Here we compared spot sizes in a single experiment. The minimum volume was recorded 0.1 µL. It is low from the volume that was used in the study of Kato et al. (2004). They were able to manually spot capture probes as 0.2 µL volume in a DNA microchip that combines polymerase chain reaction/ligase detection reaction (PCR/LDR) with ‘‘zip-code’’ hybridization for multiplex detection of mutations in cancer (Kato *et al.*, 2004).

Nearly in all microarray studies, capture probes are immobilized to the array surface via microspotting device or robotic systems to catch nanoliter level. For example, in a study, it was possible to deliver 32 capture probe sample simultaneously with a deposition of 1 nL volume (Lemieux *et al.*, 1998). However for immobilizing capture probe solution in small volumes like this, it is necessary to have microspotting or robotic array. In the absence of such expensive devices, spotting can be done by a micropipette as 0.1 µL (Figure 3.22). In addition, as spot sizes down to nanoliter level, humidity control becomes the key factor for spot homogeneity. At this nanoliter level, relative humidity should be much more than 70 %. If this humidity level cannot be met then capture probes condense at the edge of the spot. This situation brings low homogeneity and low quality to microarray and also cause poor hybridization signal (Pirrung, 2002).

Another disadvantage of using capture probe solution in very small volume is that any small dusts on spot decreases hybridization efficiency and cause false negative result. In such a case, it is important to use dust-free rooms for microarray manufacturing.

The reasons (expensive equipment, humidity control and dust-free environment) mentioned above is a problem in manufacturing of microarrays for an standard research laboratory. Therefore, spotting capture probe solution by a micropipette eliminates these problems easily.

3.4 Shelf Life of DNA Chip

Shelf life is another important parameter to be tested and validated for our main purpose which is to develop an assay system. Preimmobilized microarrays can expire long before all samples are collected and processed. Therefore it was crucial for us to investigate the stability of our pre-immobilized slides under various conditions.

We investigated the shelf life of our DNA chip platform in two groups:

-In the first group, capture probe was immobilized on slides, and these slides were kept in refrigerator (at 4°C) under dry conditions after 2% BSA blocking step.

-In the second group, capture probe was immobilized on slides, and these slides were kept in refrigerator (at 4 °C) in dry conditions without blocking. These slides were blocked at the day of the experiment.

All other experimental parameters kept unchanged during the stability studies. Positive and negative controls of capture probe solution were spotted onto the same slide. The following figure (Figure 3.23) shows the design of the slide used in these shelf life studies.

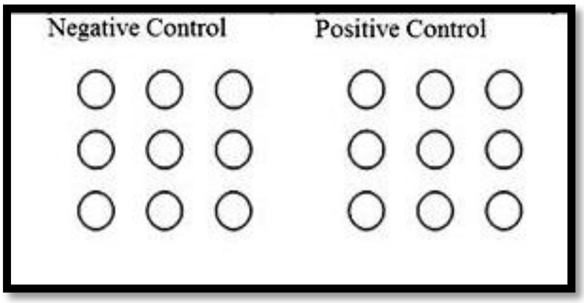

Figure 3.23 Schematic representation of positive and negative controls on the same glass slide for shelf life experiments.

Until two months, blocked and unblocked preimmobilized slides showed a similar stability. For these slides sandwich hybridization assays were formed and good signal intensities were obtained under UV illumination (Figure 3.24). Brightness of spots for both groups was nearly identical at the time of the experiment.

In first two slides corresponding to the first and the second week time points, spots were not in good morphology and quality, therefore we obtained faint signal from them (Figure 3.24). However for the following time points, better results from both blocked and unblocked slides were obtained as displayed in Figure 3.24. This observation was also supported with high signal intensity ratios. We were expecting a gradual decrease in signal intensity ratio as shelf time increased. There was a slight increase in spot intensity and intensity ratios after starting from the slides belonging to the third week time point, and later a slight decrease along with the time point assayed as expected both for the blocked and unblocked slides (Figure 3.24, Figure 3.25 and Figure 3. 26). This unexpectedly lower signal ratios for the first two time points was probably due to an experimental error and can be repeated in the next experiments.

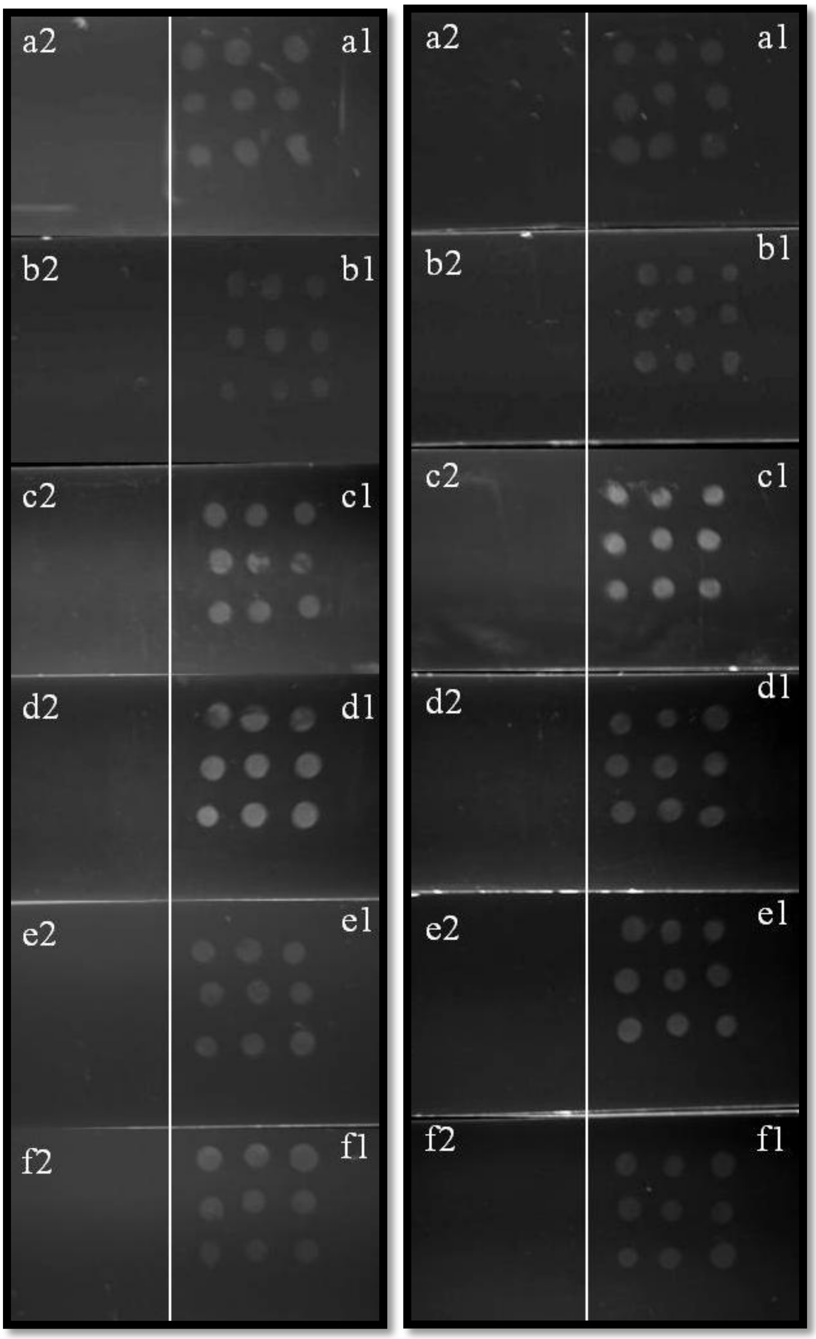

Figure 3.24 Images of DNA Chip platform in shelf life studies. Blocked (left side) and un-blocked (right side) slides were compared. Standard experimental conditions were conducted at time 0 (day of experiment) for positive control (a1), at 1 week for positive control (b1), at 2 weeks for positive control (c1), at 4 weeks for positive control (d1), at 6 weeks for positive control (e1) and at 8 weeks for positive control (f1) for both blocked and unblocked slides. (a2), (b2), (c2), (d2), (e2) and (f2) are the negative controls of blocked and un-blocked slides, respectively.

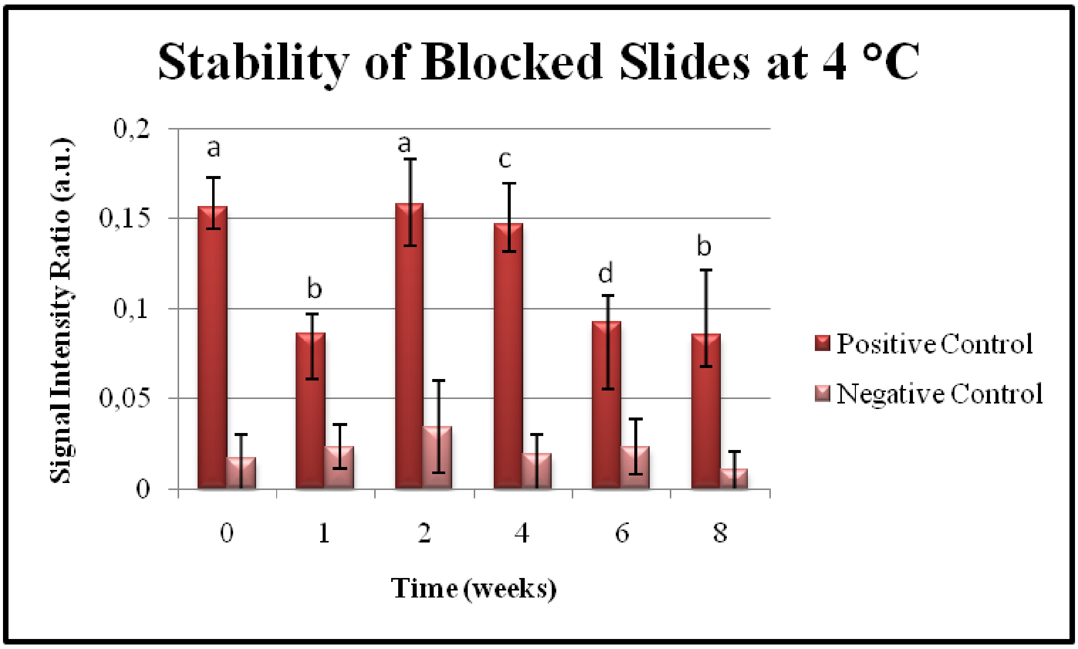

Figure. 3.25 The analysis of signal intensity ratio as a function of blocked slides of shelf life experiments for DNA chip platform under standard experimental conditions. Error bars represent standard error of mean (SEM) calculated from 18 spots. Different letters indicate significant difference at $p < 0.05$.

Antibody containing microarray slides stored at 4 °C have already been shown to be stable for at least 6 months (Kusnezow *et al.*, 2003). Stability of microarrays (as enabled on glass slides) have also been shown to be 2 to 6 months depending upon receptor content was shown for high-throughput multiplexed drug discovery in a recent study (Hong *et al.*, 2009).

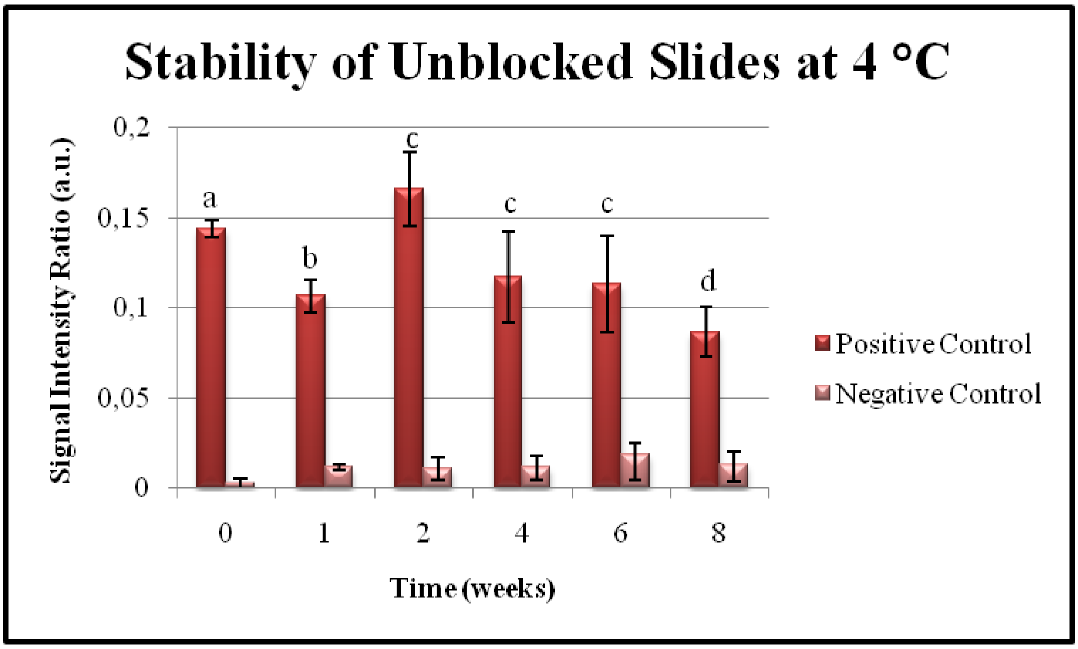

Figure. 3.26 The analysis of signal intensity ratio as a function of un-blocked slides of shelf life experiments for DNA chip platform under standard experimental conditions. Error bars represent standard error of mean (SEM) calculated from 18 spots. Different letters indicate significant difference at $p < 0.05$.

In this part, we have shown that both blocked and unblocked DNA chip platforms which were previously immobilized with capture probe showed good stability for at least 2 months. Shelf life studies were carried out until 2 months due to time limitations, and therefore their stability for longer period of time should be further assessed.

3.5 PCR Studies

Optimized parameters later were used to integrate double stranded genomic DNA to the chip platform. For this reason, PCR studies were first started using 35S Promoter in a pCAMBIA plasmid from *E.coli*. PCR amplified 52 base pair double stranded DNAs were used either directly as PCR products or as purified PCR products. Real transgenic potato plants were also used as a DNA template to test DNA chip platform with different length dsDNAs.

3.5.1 Plasmid

The isolated plasmid DNA was PCR amplified in 45 cycles. Length of the PCR product was 52 base pair . During the purification of PCR product, DNA loss is imminent and especially if the starting material is low then the purified DNA concentration can be too low to obtain a good result from the DNA chip. Therefore we first compared purified and un-purified PCR product on our array system and showed that un-purified PCR products work successfully and even with a better signal ratio. (Figure 3.27 and 3.28).

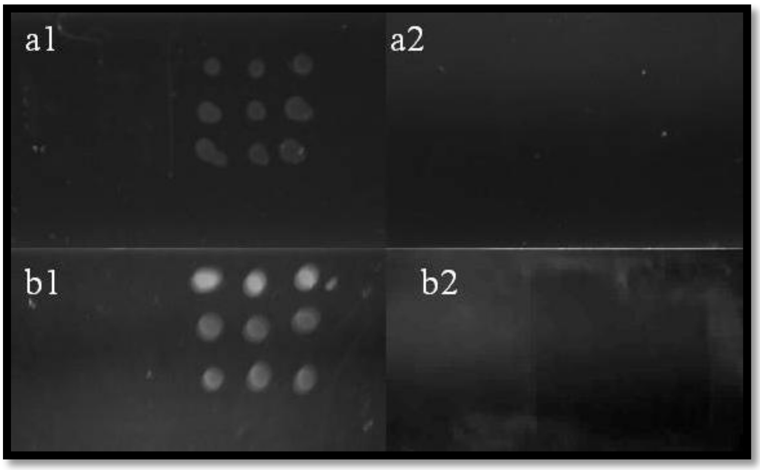

Figure 3.27 Images of DNA Chip platform when the amplified 35S promoter plasmid DNA was used as target adapter. Standard experimental conditions were conducted with kit-eluted 52 base paired PCR product (a1) and un-eluted 52 base PCR product (b1). (a2) and (b2) are the negative controls, respectively.

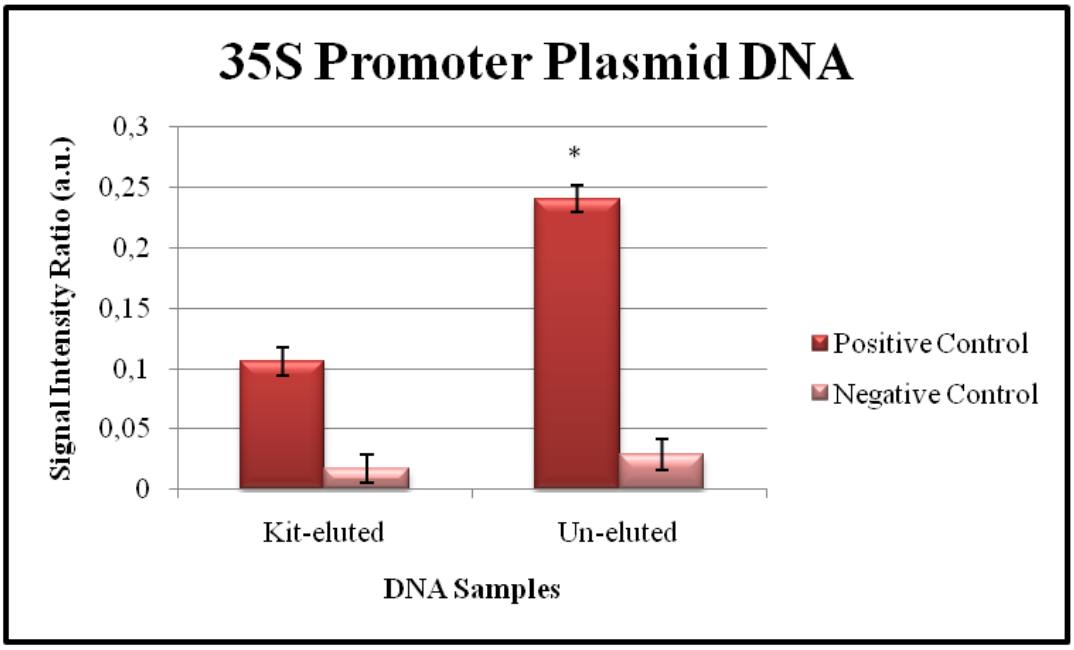

Figure 3.28 The analysis of signal intensity ratio as a function of kit eluted and un-eluted PCR products for DNA chip platform under standard experimental conditions. Error bars represent standard error of mean (SEM) calculated from 18 spots. * indicates significant difference ($p < 0.05$).

According to the T-test analysis, the difference of signal intensity ratio between purified and un-purified DNA samples was considered to be statistically significant at the 0.05 level. Hence, we preferred to study DNA samples as un-purified PCR product for further studies.

3.5.2 Detection of Transgenic Plants

The major challenge in sandwich-type array studies is to integrate transgenic elements from genomic DNAs obtained from transgenic plants. In this study, we used genomic DNA from a transgenic potato. The transgenic nature of the transgenic line that has been used in our study was previously evaluated in our laboratory by using southern and northern blotting data.

As expected we were able to detect the 52 base pair length 35Spromoter of transgenic potato plant (Figure 3.29). Because recent efforts in the detection of transgenes with microarrays have focused on the detection of longer and different amplicon sizes (Schmidt *et al.*, 2008), in the next experiment DNA chip platform was studied with longer PCR amplified 35S promoter DNA fragments.

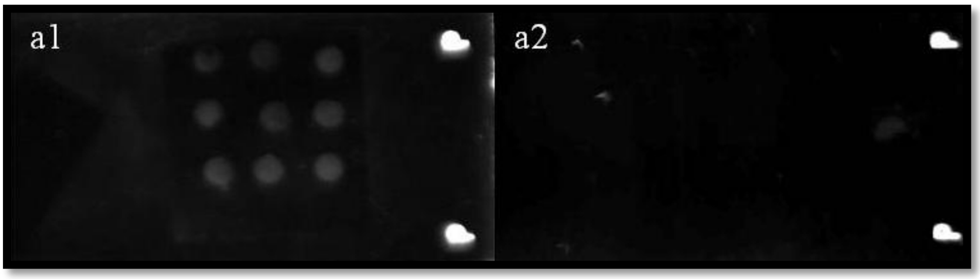

Figure 3.29 Images of DNA Chip platform when the amplified 35S promoter transgenic genomic DNA was used as target adapter. Standard experimental conditions were conducted with 52 base paired un-eluted PCR product (a1) with transgenic real sample. (a2) is the negative control.

3.5.3 dsDNAs in Different Length

So far the dsDNA sample from plasmid and real sample was 52 base-pair in length. To further understand the effect of DNA length on the chip performance we compared different lengths of CaMV dsDNAs amplified from transgenic potato.

Figure 3.30 displays the images of 4 different lengths of 35S promoter amplified from transgenic potato. It is clearly evident that, with different lengths of PCR amplicons the platform can exhibit a good performance.

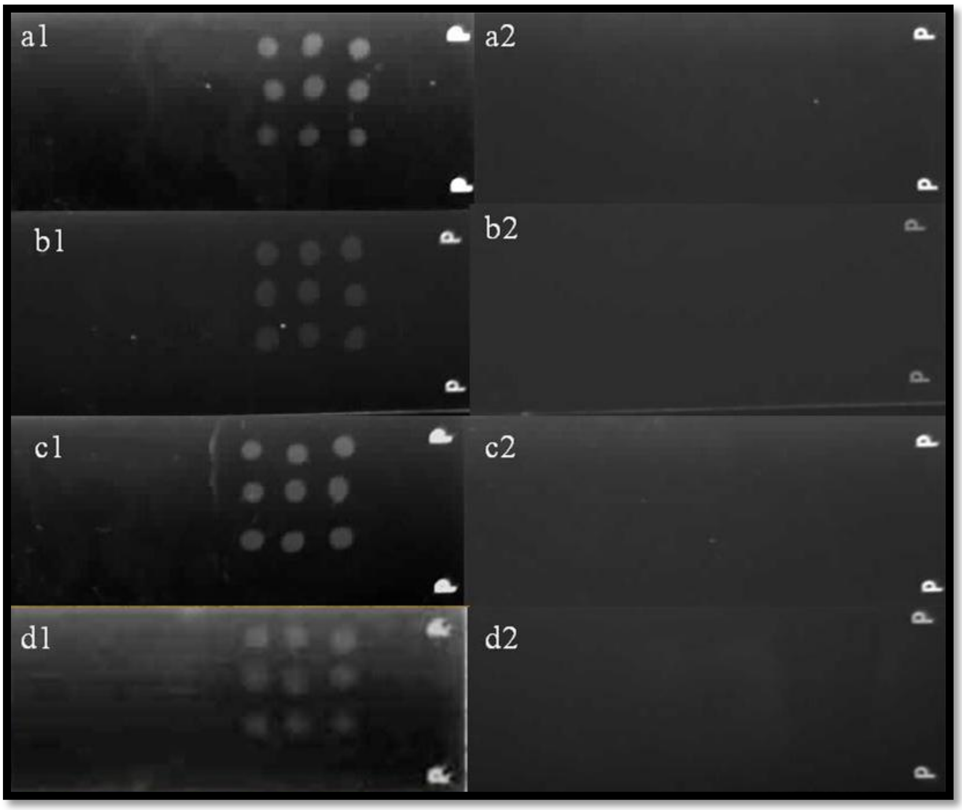

Figure 3.30 Images of DNA Chip platform when it was studied with different length dsDNAs. Standard experimental conditions were conducted with 52 base dsDNA for positive control (a1), with 72 base dsDNA for positive control (b1), with 92 base dsDNA for positive control (c1) and with 200 base dsDNA for positive control (d1). (a2), (b2), (c2) and (d2) are the negative controls, respectively.

In Figure 3.31, it was clear that the length of 35S DNA increased from 92 to 200 bases, no decrease in signal intensity ratio but rather a slight increase was observed. The reason for this is that we used 3 more internal biotinylated detection probes for 200 base length 35S DNA for signal amplification and to prevent the folding of target DNA during hybridization. Therefore, in this case more signal intensity ratio was obtained.

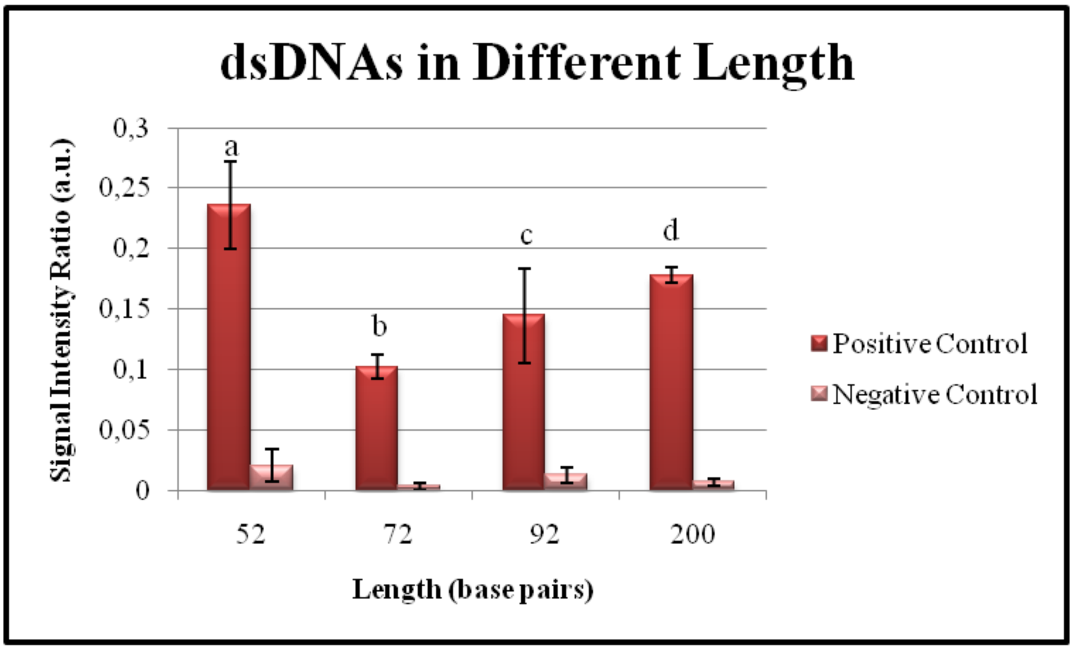

Figure 3.31 The analysis of signal intensity ratio as a function of different length of dsDNA for DNA chip platform under standard experimental conditions. Error bars represent standard error of mean (SEM) calculated from 18 spots. Different letters indicate significant difference at $p < 0.05$.

3.6 GMO Detection on the Chip platform

To study the specificity of the DNA Chip platform, both ssDNA and dsDNA of transgenes were used. In the first part, studies were carried out with 3 different ssDNA recombinant elements (NOS, BAR, CRY) except 35S promoter. Then genomic DNAs (gDNA) from Reference Materials were studied by using PCR amplified NOS terminator and BAR genes.

3.6.1 Multiple Target Assay

Although GMOs have been developed to improve agricultural crops quality and cope with the problems faced during cultivation, there is an increasing concern about different kinds of transgenes that are found in crops and foods. Demands for testing transgenes lead to developments of new detection methods. One of them is the detection of different transgenes on a single platform.

After the establishment of DNA chip platform with 35S promoter from transgenic plant, further studies were performed to evaluate the ability of the platform for simultaneous detection of multiple transgenic elements from a complex mixture. In this study, 4 different ssDNA transgenic elements were studied on the same platform. There were five replicates for each target transgenes. The following figure shows the slide design.

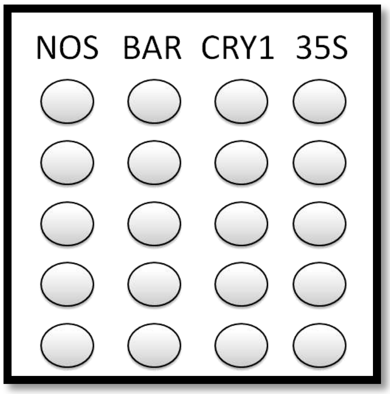

Figure 3.32 Schematic representation of slide design with 4 different transgenes.

The target adapter composition of each hybridization solution for each platform was as follows:

Table 3.3 Transgene composition of hybridization solutions for each slide.

	NOS	BAR	CRY1ab	35S PRO
Hybridization Solution-1 for 1st Slide	+	+	+	+
Hybridization Solution-2 for 2nd Slide	-	+	+	+
Hybridization Solution-3 for 3rd Slide	+	-	+	+
Hybridization Solution-4 for 4th Slide	+	+	-	+
Hybridization Solution-5 for 5th Slide	-	-	-	-

Such an experimental design allowed us to test the performance of the platform with a mixture of target sequences. This study showed us that the detection of multiple transgenes on a single platform could successfully and practically be achieved by using ssDNA in our platform (Figure 3.33).

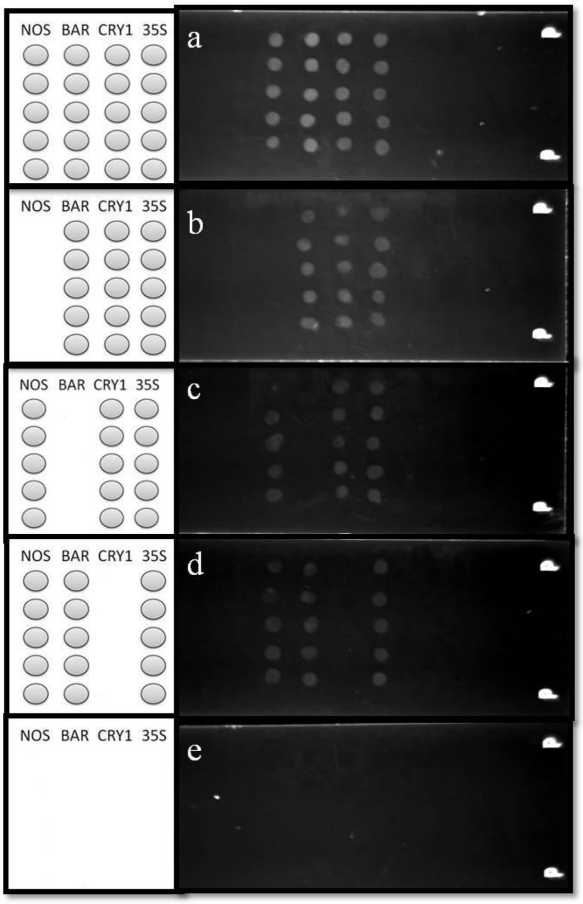

Figure 3.33 Images of DNA Chip platform when it was studied with different transgenes. Standard experimental conditions were conducted with hybridization solution contains all ssDNA transgenes; NOS terminator, BAR gene, CRY1Ab gene and 35S promoter (a), contains all ssDNA transgenes except NOS terminator (b), contains all ssDNA transgenes; except BAR gene (c), contains all ssDNA transgenes; except CRY1Ab gene (d). (e) is the negative control contains no transgene in the hybridization solution.

3.6.2 Reference Material Studies

Overall our findings suggest that when capture and detection probes are designed properly according to the 35S promoter, sandwich hybridization occurs under optimum conditions. Thus the same approach can be applied for the detection of different transgenes amplified from reference materials. NK603 5% GMO reference material was used as a DNA sample. It contains 35S promoter, NOS terminator, BAR gene and CP4 EPSPS gene. Our preliminary results showed the potential of the platform to identify GMO related transgenes in certified reference material (Figure 3.34 and 3.35). This result exhibited the potential of the platform to be used as a powerful tool in identifying multiple GMO events in unknown samples.

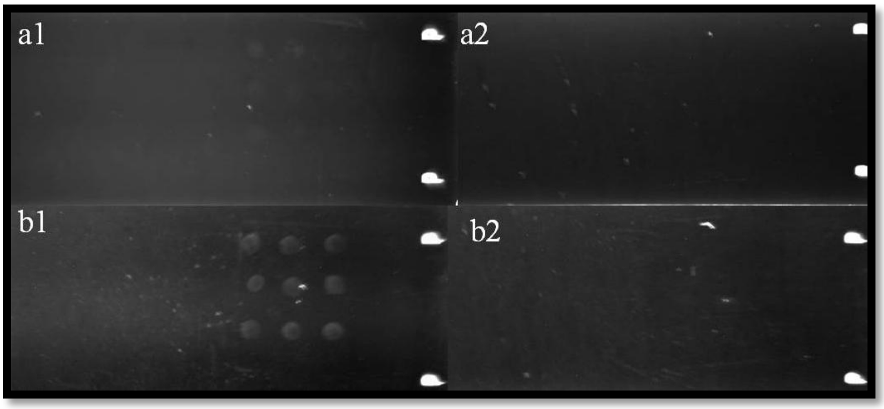

Figure 3.34 Images of DNA Chip platform when it was studied with two different types of transgene from NK603 5 % reference material. Standard experimental conditions were conducted with NOS terminator for positive control (a1) and with BAR gene for positive control (b1). (a2) and (b2) are the negative controls, respectively.

In this study, two experiments were carried out simultaneously. NOS and BAR genes were integrated to DNA chip platform separately (Figure 3.34) and multiplex form (Figure 3.35). Because they studied at the same time, the hybridization temperature of them was the same in the same incubator. Hybridization temperature of this experiment was set according to the melting temperature of BAR gene. It was found as 50 °C for BAR gene. However this temperature was so high for NOS terminator DNA because it has 40 °C hybridization temperature. As it seen from the Figure 3.34, DNA chip platform studied with NOS DNA yielded poor signal strength. BAR DNA at this hybridization temperature was able to hybridize with its probes and give brighter signal intensity. The result obtained from Figure 3.35 was also confirmed in multiple assay on a single platform.

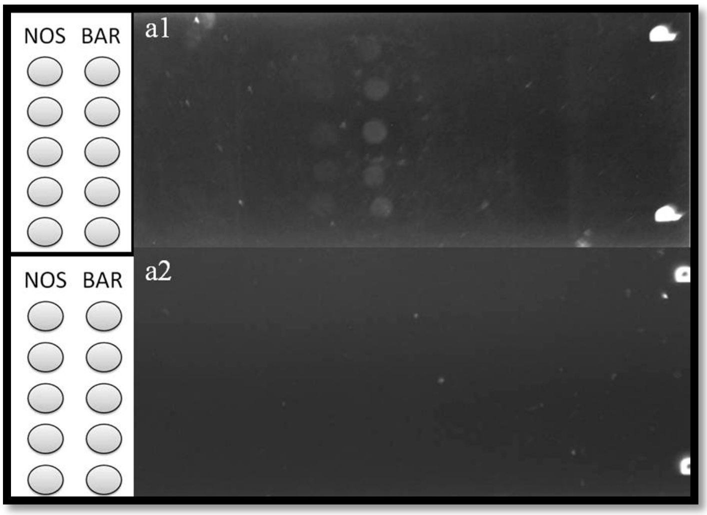

Figure 3.35 Images of DNA Chip platform when it was studied with two different transgenes from NK603 5 % reference material. Standard experimental conditions were conducted with hybridization solution contains transgenes; NOS terminator, and BAR gene for positive control (a1). (a2) is the negative control contains no transgene in the hybridization solution.

This study needs further optimization. An optimum hybridization temperature should be found to the integration of different source of transgenes on the same chip platform simultaneously.

CHAPTER 4

CONCLUSION

In this work, proof-of-concept for the DNA chip platform based on sandwich hybridization assay to detect unlabeled transgenes of genetically modified organisms (GMOs) from unpurified PCR mixture was presented.

The optimized conditions for sandwich hybridization-based DNA chip platform were as follows;

- 10 µM capture probe concentration
- 1 hour blocking time
- 20 µM target adapter concentration
- 20 µM detection probe concentration
- 1 hour hybridization time at 40 °C
- 1 hour Strepravidin-QD conjugate incubation time

Under these conditions it was possible to get a signal from PCR products in about 2 hours. The platform was able to identify a mixture of target sequences of 35S promoter, NOS terminator CRY1Ab and BAR genes.

With or without blocking, DNA chip platform shows good stability for at least 60 days. Pre-immobilized and blocked chips decreases the assay time from 6 to 2-3 hours.

Performance of the platform was also analyzed by PCR amplified target sequences from real GMO material. Although it requires improvement, the platform was able to identify transgene events in reference GMO material.

Ease of use, low cost, minimal equipment requirements and fast response time makes this DNA chip platform a very promising tool for development of molecular diagnostic kits.

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APPENDIX A

SEQUENCES OF COMMERCIAL OLIGONUCLEOTIDES

Table A.1. Sequences of commercial oligonucleotides for transgene targets

For 52 base Adapter	**Sequences (Specific to CaMV 35S Promoter)**
Capture Probe	5'- /5ThioMC6-D/AAA AAA AAA GCA TCT TCA ACG ATG GCC TTT CCT TT-3'
Target	5'GCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATGC 3'
Detection Probe	5'-TCG CAA TGA TGG CAT TTG TAG GAG CA AAA AAA AA/3Bio/ -3'
Un-Complementary Target	5- CGAGGATGTTTACGGTAGTAACGCTATTTCCTTTCCGG TAGCAACTTCTACG -3'
For 72 base Adapter	**Sequences (Specific to CaMV 35S Promoter)**
Capture Probe	5'- /5ThioMC6-D/AAA AAA AAA GTC GGC AGA GGC ATC TTC AAC GAT GGC CTT TCC TTT -3'
Target	5'- AAG GAA GGT GGC TCC TAC AAA TGC CAT CAT TGC GAT AAA GGA AAG GCC ATC GTT GAA GAT GCC TCT GCC GAC -3'
Detection Probe	5'- TCG CAA TGA TGG CAT TTG TAG GAG CCA CCT TCC TTA AAA AAA AA/3Bio/ -3'
Un-Complementary Target	5'- TTC CTT CCA CCG AGG ATG TTT ACG GTA GTA ACG CTA TTT CCT TTC CGG TAG CAA CTT CTA CGG AGA CGG CTG -3'
For 92 base Adapter	**Sequences (Specific to CaMV 35S Promoter)**
Capture Probe	5'- /5ThioMC6-D/AAA AAA AAA TGG GAC CAC TGT CGG CAG AGG CAT CTT CAA CGA TGG CCT TTC CTT T -3'
Target	5'- GAT AGT GGA AAA GGA AGG TGG CTC CTA CAA ATG CCA TCA TTG CGA TAA AGG AAA GGC CAT CGT TGA AGA TGC CTC TGC CGA CAG TGG TCC CA -3'
Detection Probe	5'- TCG CAA TGA TGG CAT TTG TAG GAG CCA CCT TCC TTT TCC ACT ATC AAA AAA AAA/3Bio/ -3'
Un-Complementary Target	5'- CTA TCA CCT TTT CCT TCC ACC GAG GAT GTT TAC GGT AGT AAC GCT ATT TCC TTT CCG GTA GCA ACT TCT ACG GAG ACG GCT GTC ACC AGG GT -3'

Table A.1 cont'd

For 200 base Adapter	Sequences (Specific to CaMV 35S Promoter)
Capture Probe	5'- /5ThioMC6-D/AAA AAA AGA CGT GGT TGG AAC GTC TTC TTT TTC CAC GAT GCT CCT CGT GGG GTG GGG -3'
Target	Commercial synthesis is not available for 200 base
Detection Probe	5'- AAT AAA GTG ACA GAT AGC TGG GCA ATG GAA TCC GAG GAG GTT TAA AAA AA/3Bio/ -3'
Un-Complementary Target	Commercial synthesis is not available for 200 base
For 62 base Adapter	**Sequences (Specific to NOS Terminator)**
Capture Probe	5'-/5ThioMC6-D/AAT GTA TAA TTG CGG GAC TCT AAT C/-3'
Target	5'-GCA TGA CGT TAT TTA TGA GAT GGG TTT TTA TGA TTA GAG TCC CGC AAT TAT ACA TTT AAT AC -3'
Detection Probe	5'-ATA AAA ACC CAT CTC ATA AAT AAC G/3Bio/-3'
Un-Complementary Target	5'- TTC CCA CCG AGG TTT ACA ACG CTA TTT CCT TTC CGG TAG CAA CTT CTA CGG AGA CGG CTG AT-3'
For 62 base Adapter	**Sequences (Specific to CRYIAb Gene)**
Capture Probe	5'-/5ThioMC6-D/CCG CCT TTT GTG CTC TTT CTA AATC/-3'
Target	5'-TCC GGC AGA AGT AAC CTT TGA GGC AGA ATA TGA TTT AGA AAG AGC ACA AAA GGC GGT GAA TG -3'
Detection Probe	5'-ATA TTC TGC CTC AAA GGT TAC TTC T/3Bio/-3'
Un-Complementary Target	5'- TTC CCA CCG AGG TTT ACA ACG CTA TTT CCT TTC CGG TAG CAA CTT CTA CGG AGA CGG CTG AT-3'
For 59 base Adapter	**Sequences (Specific to BAR Gene)**
Capture Probe	5'-/5ThioMC6-D/CCG ATG ACA GCG ACC ACG CTC TTG A/-3'
Target	5'-ACC TGC TGA AGT CCC TGG AGG CAC AGG GCT TCA AGA GCG TGG TCG CTG TCA TCG GGC TG-3'
Detection Probe	5'-AGC CCT GTG CCT CCA GGG ACT TCA G/3Bio/-3'
Un-Complementary Target	5'- TTC CCA CCG AGG TTT ACA ACG CTA TTT CCT TTC CGG TAG CAA CTT CTA CGG AGA CGG CTG AT-3'

APPENDIX B

PRIMER SEQUENCES SPECIFIC TO TRANSGENES

Table B.1. Primer sequences specific to transgenes

For 52 base Adapter	Primer Sequences (Specific to CaMV 35S Promoter
Sense Primer	5'-GCT CCT ACA AAT GCC ATC AT-3'
Antisense Primer	5'-GCA TCT TCA ACG ATG GCC TT-3'
For 72 base Adapter	**Primer Sequences (Specific to CaMV 35S Promoter**
Sense Primer	5'-AAG GAA GGT GGC TCC TAC AA-3'
Antisense Primer	5'-GTC GGC AGA GGC ATC TTC AA-3'
For 92 base Adapter	**Primer Sequences (Specific to CaMV 35S Promoter)**
Sense Primer	5'-GAT AGT GGA AAA GGA AGG TG-3'
Antisense Primer	5'-TGG GAC CAC TGT CGG CAG AG-3'
For 200 base Adapter	**Primer Sequences (Specific to CaMV 35S Promoter)**
Sense Primer	5'- AAA CCT CCT CGG ATT CCA TT -3'
Antisense Primer	5'- AGA CGT GGT TGG AAC GTC TT -3'
For 62 base Adapter	**Primer Sequences (Specific to NOS Terminator)**
Sense Primer	5’-GCA TGA CGT TAT TTA TGA GAT G -3'
Antisense Primer	5’-GTA TTA AAT GTA TAA TTG CGG GAC-3'
For 62 base Adapter	**Primer Sequences (Specific to CRYIAb Gene)**
Sense Primer	5’-TCC GGC AGA AGT AAC CTT TG -3'
Antisense Primer	5’-CAT TCA CCG CCT TTT GTG-3'
For 59 base Adapter	**Primer Sequences (Specific to BAR Gene)**
Sense Primer	5’-ACC TGC TGA AGT CCC TGG AG -3'
Antisense Primer	5’-CAG CCC GAT GAC AGC GAC-3'

APPENDIX C

pCAMBIA PLASMID DNA MAP

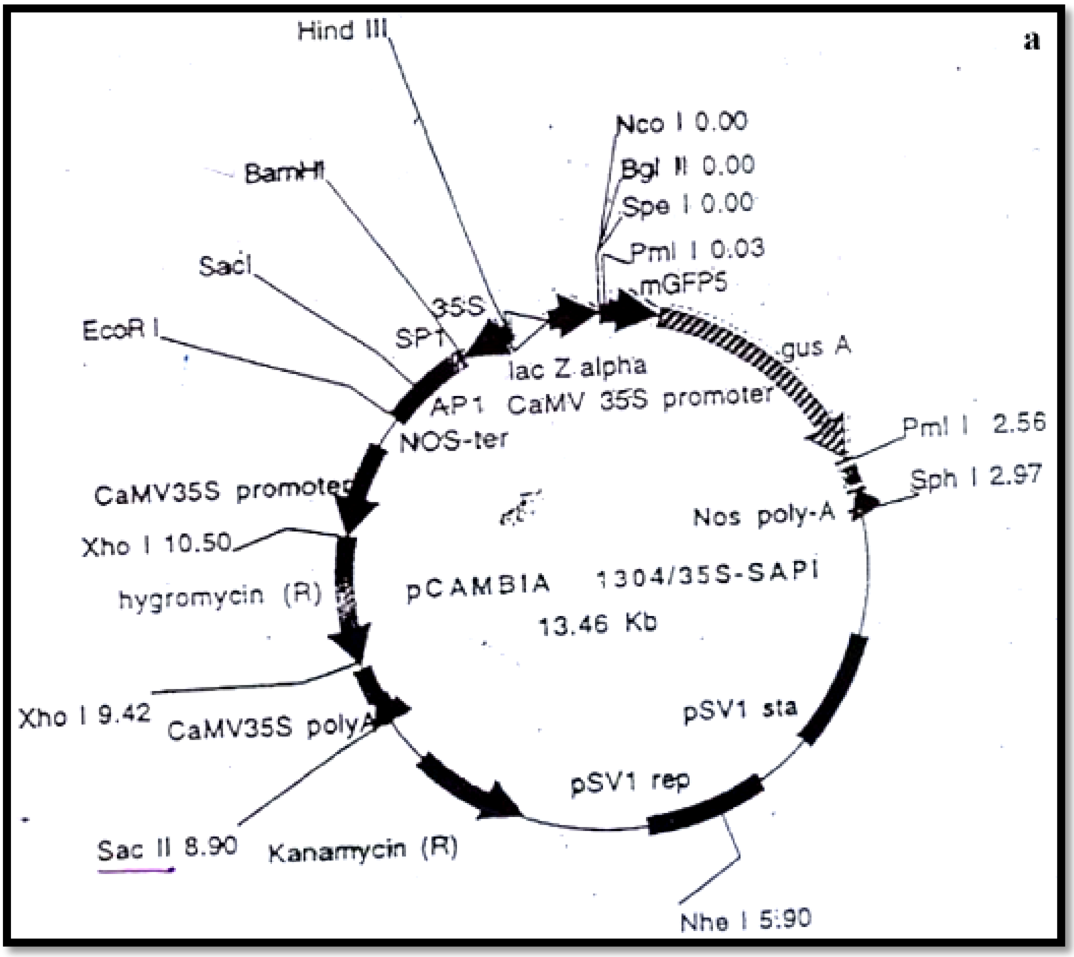

Figure C.1. pCAMBIA plasmid DNA map

APPENDIX D

pGA-MYB4 PLASMID MAP

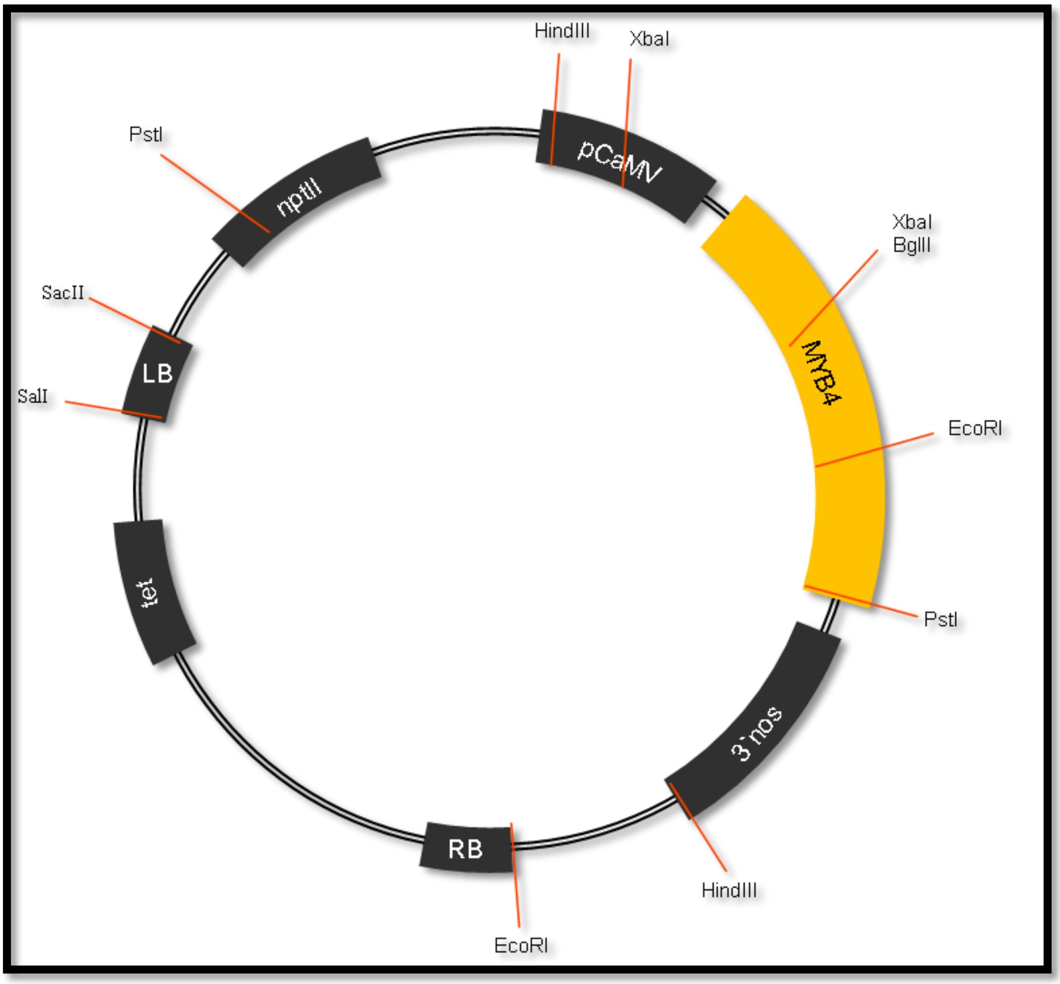

Figure D.1. pGA-MYB plasmid map

APPENDIX E

PREPARATIONS OF BUFFERS

I. 1X PBS

NaCl	8 g
KCl	0,2 g
Na_2HPO_4	1,44 g
KH_2PO_4	0,24 g

Dissolved in 900 mL of ultrapure water, adjusted the pH 7.2 completed to 1000 mL with ultrapure water, filtered and autoclaved and stored at RT.

II. 5X SSC

Sodium Citrate (MW: 294.1)	11,03 g
Sodium Chloride (MW: 58.4)	21,9 g

Dissolved in 400 mL of ultrapure water, adjusted the pH 7.0 completed to 500 mL with ultrapure water, filtered and autoclaved and stored at RT.

III. 1X SSC

100 mL from 5X SSC buffer was taken. 300 mL of ultrapure water was added and adjusted the pH 7.0 completed to 500 mL with ultrapure water, filtered and autoclaved and stored at RT.

IV. 0.1X SSC

10 mL from 5X SSC buffer was taken 400 mL of ultrapure water was added and adjusted the pH 7.0 completed to 500 mL with ultrapure water, filtered and autoclaved and stored at RT.

V. 10 % SDS

SDS 10 g

- 100 mL dH_2O

Dissolved in 100 mL of ultrapure water, filtered and stored at RT.

APPENDIX F

TABULATED VALUES OF GRAPHS

Table F.1. Mean values and SEM for Figure 3.9 (Effect of capture probe concentration on DNA chip platform.)

Capture Probe Concentration	**Signal Intensity Ratio (a.u.)**	
	Positive Control	Negative Control
5 μM	0.103 ± 0.009	0.0081 ± 0.001
10 μM	0.124 ± 0.0077	0.006 ± 0.001

Table F.2. Mean values and SEM for Figure 3.11 (Effect of blocking time on DNA chip platform.)

Blocking Time	**Signal Intensity Ratio (a.u.)**	
	Positive Control	Negative Control
15 minutes	0.029 ± 0.012	0.0081 ± 0.006
30 minutes	0.052 ± 0.013	0.01 ± 0.009
60 minutes	0.190 ± 0.02	0.014 ± 0.007
120 minutes	0.0201 ± 0.019	0.009 ± 0.008

Table F.3. Mean values and SEM for Figure 3.13 (Effect of adapter concentration on DNA chip platform.)

Adapter Concentration	Signal Intensity Ratio (a.u.)	
	Positive Control	Negative Control
20 μM	0.329 ± 0.021	0.0145 ± 0.009
10 μM	0.147 ± 0.014	0.0122 ± 0.0056
0.5 μM	0.128 ± 0.037	0.004 ± 0.001
0.1 μM	0.053 ± 0.007	0.005 ± 0.004
0.005 μM	0.023 ± 0.007	0.008 ± 0.003
0.001 μM	0.014 ± 0.005	0.004 ± 0.001

Table F.4. Mean values and SEM for Figure 3.15 (Effect of detection probe concentration on DNA chip platform.)

Detection Probe Concentration	Signal Intensity Ratio (a.u.)	
	Positive Control	Negative Control
5 μM	0.036 ± 0.107	0.008 ± 0.005
20 μM	0.209 ± 0.014	0.008 ± 0.006
40 μM	0.221 ± 0.012	0.011 ± 0.004

Table F.5. Mean values and SEM for Figure 3.17 (Effect of hybridization time on DNA chip platform.)

Hybridization Time	Signal Intensity Ratio (a.u.)	
	Positive Control	Negative Control
5 hours	0.237 ± 0.019	0.0108 ± 0.007
2.5 hours	0.230 ± 0.013	0.002 ± 0.001
1 hour	0.219 ± 0.049	0.002 ± 0.001
0.5 hour	0.084 ± 0.0125	0.0036 ± 0.0024

Table F.6. Mean values and SEM for Figure 3.19 (Effect of hybridization temperature on DNA chip platform.)

Hybridization Temperature	Signal Intensity Ratio (a.u.)	
	Positive Control	Negative Control
35 °C	0.083 ± 0.021	0.0085 ± 0.0047
40 °C	0.151 ± 0.0195	0.0053 ± 0.0038

Table F.7. Mean values and SEM for Figure 3.21 (Effect of QD incubation time on DNA chip platform.)

QD Incubation Time	Signal Intensity Ratio (a.u.)	
	Positive Control	Negative Control
5 minutes	0.023 ± 0.016	0.008 ± 0.005
15 minutes	0.209 ± 0.014	0.008 ± 0.006
60 minutes	0.221 ± 0.012	0.011 ± 0.004

Table F.8. Mean values and SEM for Figure 3.25 (Shelf life of blocked DNA chip platform.)

Stability of Blocked Slides	Signal Intensity Ratio (a.u.)	
	Positive Control	Negative Control
0	0.1559 ± 0.0169	0.0166 ± 0.0136
1 week	0.0856 ± 0.0116	0.0231 ± 0.0126
2 weeks	0.0158 ± 0.0450	0.0341 ± 0.0256
4 weeks	0.1465 ± 0.0431	0.0194 ± 0.0109
6 weeks	0.0919 ± 0.0151	0.0229 ± 0.0159
8 weeks	0.0848 ± 0.0365	0.0104 ± 0.0101

Table F.9. Mean values and SEM for Figure 3.26 (Shelf life of un-blocked DNA chip platform.)

Stability of Unblocked Slides	**Signal Intensity Ratio (a.u.)**	
	Positive Control	Negative Control
0	0.144 ± 0.0049	0.0029 ± 0.0027
1 week	0.1068 ± 0.0089	0.0118 ± 0.00179
2 weeks	0.166 ± 0.0252	0.0109 ± 0.0062
4 weeks	0.1179 ± 0.025	0.0118 ± 0.0075
6 weeks	0.113 ± 0.027	0.0184 ± 0.0139
8 weeks	0.0867 ± 0.0138	0.0131 ± 0.0095

Table F.10. Mean values and SEM for Figure 3.29 (Effect of plasmid DNA sample on DNA chip platform.)

35S Promoter Plasmid DNA	**Signal Intensity Ratio (a.u.)**	
	Positive Control	Negative Control
Kit-eluted	0.106 ± 0.0114	0.0172 ± 0.0112
Un-eluted	0.2406 ± 0.0100	0.0288 ± 0.0127

Table F.11. Mean values and SEM for Figure 3.31 (Effect of dsDNAs in different length on DNA chip platform.)

dsDNAs in Different Length	**Signal Intensity Ratio (a.u.)**	
	Positive Control	Negative Control
52 bp	0.2358 ± 0.0364	0.0199 ± 0.0133
72 bp	0.1021 ± 0.0102	0.0032 ± 0.0031
92 bp	0.1445 ± 0.0392	0.0126 ± 0.0062
200 bp	0.1778 ± 0.065	0.0065 ± 0.0025