DEVELOPMENT OF SANDWICH TYPE NUCLEIC ACID ARRAY PLATFORM FOR THE DETECTION OF MICRORNAs IN BREAST CANCER

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DEVELOPMENT OF SANDWICH TYPE NUCLEIC ACID ARRAY PLATFORM FOR THE DETECTION OF MICRORNAs IN BREAST CANCER

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ABSTRACT

DEVELOPMENT OF SANDWICH TYPE NUCLEIC ACID ARRAY PLATFORM FOR THE DETECTION OF MICRORNAS IN BREAST CANCER

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MicroRNAs are small non-coding RNAs that are involved in important regulatory pathways such as differentiation, development, metabolism, cell proliferation, and cell death. Several recent research show that deregulated expression of miRNAs has crucial roles in disease pathologies, mainly in cancer. Therefore, it is likely that the usage of miRNAs as diagnostic and prognostic biomarkers in patients and the development of various techniques for the detection of microRNA in clinical research will become widespread. In this study, we aimed to develop an alternative to existing tools for the detection of miRNAs using an array platform based on sandwich hybridization. The sandwich hybridization method is one of the techniques developed for faster, more sensitive and reliable detection. This method constitutes target miRNA to be detected and two oligonucleotide probes which are half complementary to target miRNA. In this method, the capture probe (P1) which is fixed to the glass surface with the help of cross-linkers, is specific to one part of the target molecule.

And then, target molecule binds to the capture probe and the existence of target molecule is defined with the binding of signal probe (P2). Sandwich hybridization system allows direct use of RNA and does not require any labelling or cDNA synthesis steps which are necessary in the current sequence systems. In order to construct platform with sandwich hybridization, glass slides coated with poly-l-lysin were used. The sequences of probe 1 were attached to the surface by using different cross linkers such as Sulfo-EMCS and $SM(PEG)_n$. After the determination of cross linker which fixed probe 1 to surface, several parameters were tried to determine blocking conditions, hybridization conditions like temperature, duration, oligonucleotide type, signal probe, probes' concentration, washing conditions, and the sensitivity of platform and the optimization experiments were conducted. These experiments were conducted first for miR-21 and optimum hybridization signal was obtained with $SM(PEG)_2$ as cross linker, 20 µM capture and 20 µM signal probe concentrations, and 0.1 µM platform sensitivity at 30 ^{0}C. Then, these conditions were used for synthetic miRNA sequences and optimized for all miRNA probe sets. After these, the developed platform was tested whether it is unique for the targeted sequence or not by applying target sequence, mixed with several signal probes, to surface involving different capture probes. According to our results, optimum hybridization signal was obtained with 10 µM capture and 10 µM signal probe concentrations and 0.01 µM platform sensitivity at 45 ^{0}C. The potential of platform workability with total RNAs obtained from different cell lines was tested. Finally, the comparison was made from the point of miRNA expression levels and signals between normal and cancer cells. Later, the obtained signals were compared with results taken from RT-PCR. The results indicated that the developed platform is specific enough to detect target miRNA sequences.

Keywords: micro RNA, probe, sandwich hybridization, breast cancer

ÖZ

MEME KANSERI TESPITI IÇIN SANDVIÇ TABANLI NUKLEIK ASIT DIZI PLATFORMUNUN GELIŞTIRILMESI

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MikroRNA'ların gelişim ve farklılaşma yolakları, metabolizmanın düzenlenmesi, hücre döngüsü ve hücre ölümü gibi önemli mekanizmalarda kritik görevleri olduğu ve ifadeleri bozulan miRNA'ların kanser başta olmak üzere pek çok hastalık patolojisinde önemli rolleri olduğu son yıllarda yapılan birçok araştırmada belirtilmektedir. Bu durum, mikro RNA' ların hastalarda diagnostik ve prognostik biyo-belirteçler olarak kullanılmasını gündeme getirmiş ve hem temel araştırmalarda hem de klinikte mikro RNA belirlemeye yönelik teknikler geliştirilmesi yaygınlaşmıştır. Bu çalışmada, mevcut sistemlere alternatif oluşturubilecek sandviç hibridizasyona dayalı bir miRNA dizi platformunun geliştirilmesi üzerine çalışılmıştır. Sandviç hibridizasyon metot daha hızlı, daha hassas ve daha güvenilir tespit için geliştirilmiş birçok teknikten biridir. Bu metot, tespiti amaçlanan hedef mikro RNA ve buna yarı eşlenik olan iki oligonükleotid probdan oluşmaktadır.

Bu sisteme göre hedef mikro RNA dizisinin bir kısmına eşlenik şekilde tasarlanmış olan yem prob (P1) cam yüzeye çapraz bağlayıcılar yardımıyla sabitlenmekte ve hedef mikroRNA'nın hibridizasyon yoluyla prob 1'e bağlanmasıyla birlikte adaptörün (hedef mikro RNA) varlığı yarı eşlenik olan sinyal probunun (P2) da sandviç sisteme bağlanması ile belirlenmektedir. Sandviç tabanlı mikro RNA dizi platformu RNA'nın direkt olarak kullanılmasına olanak sağlayarak, mevcut dizi sistemlerinde gerekli olan komplementer DNA sentezi ya da işaretlemeye gerek olmadan çalışabilmektedir. Sandviç hibridizasyon metotla platformun oluşturulabilmesi için poli-l lizin kaplı cam slaytlar kullanılmıştır. SM(PEG)n ve Sulfo-EMCS gibi farklı çapraz bağlayıcılar kullanılarak Prob 1 dizileri cam slayt yüzeyine tutturulmaya çalışılmıştır. Yem probu (Prob 1) yüzeye sabitleyecek çapraz bağlayıcı belirlendikten sonra bloklama koşullarını, sıcaklık, süre gibi hibridizasyon koşullarını, oligonükleotid tipini, probların konsantrasyonunu, washing koşullarını ve platformun hassasiyetini belirlemek amacıyla çeşitli parametler denenmiş ve optimizasyon deneyleri yapılmıştır. Bu deneyler ilk olarak miRNA 21 için denenmiş, neticesinde elde edilen sonuçlara göre en iyi sinyal 30 ^{0}C'de, çapraz bağlayıcı olarak SM(PEG)2 kullanılarak, 0.1 μM platform hassasiyetinde, 20 μM P1 ve 20 μM P2 konsantrasyonunda elde edilmiştir. Daha sonra bu koşullar, sentetik miRNA dizileri ile çalıştırılmış ve koşullar miRNA prob setlerinin tümü için iyileştirilmiştir. Geliştirilen platformun hedeflenen diziye spesifik olup olmadığı bir çok sinyal probuyla karıştırılmış hedef mikro RNA'yı farklı yem probları içeren yüzeye uygulayarak test edilmiştir. Elde edilen sonuçlara göre en iyi sinyal 45 ^{0}C'de, çapraz bağlayıcı olarak SM(PEG)2 kullanılarak, 0.01 μM platform hassasiyetinde, 10 μM P1 ve 10 μM P2 konsantrasyonunda elde edilmiştir. Elde edilen koşullar ile platformun total RNA'lar ile çalışabilme potansiyeli farklı hücre hatlarından elde edilen total RNA örnekleri ile test edilmiş, normal ve kanserli hücrelerdeki mikro RNA ifadeleri ve platformda elde edilen sinyaller karşılaştırılmıştır. Daha sonra bu sinyallerle RT-PCR'dan elde edilen sonuçlar karşılaştırılmıştır. Sonuç olarak, geliştirilen sistemin hedeflenen diziye spesiifik olduğu gözlemlenmiştir.

Anahtar Kelimeler: mikro RNA, prob, sandviç hibridizasyonu, meme kanseri

To my parents Havva & Mehmet ATILGAN

for their unconditional love and support…

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LIST OF ABBREVIATIONS

miRNA	Micro Ribonucleic Acid
UTR	Untranslated Region
mRNA	Messenger Ribonucleic Acid
premiRNA	Precursor miRNA
dsRNA	Double-stranded RNA
Exp-5	Exportin-5
DGCR8	DiGeorge syndrome critical region gene 8
TRBP	Transactivating response RNA-binding protein
RISC	RNA induced silencing complex
DB	Diabetes Mellitus
cDNA	Complementary deoxyribonucleic acid
Ago	Argonaute
C. elegans	*Caenorhabditis elegans*
D. melanogaster	*Drosophila melanogaster*
PB	Processing bodies (P bodies)
CNS	Central Nervous System
SCI	Spinal Cord Injury
TBI	Traumatic Brain Injury
AD	Alzheimer' disease

PD	Parkinson’ disease
N/A	Nonapplicable
β cell	Beta cell
Cy3	Cyanine dye 3
Cy5	Cyanine dye 5
UV light	Ultraviolet light
B cell	Bursa-derived cell
T cell	Thymus-cell
MS	Multiple Sclerosis
DNA	Deoxy ribonucleic acid
RNA	Ribo nucleic acid
TPM1	Tropomyosin 1
PDCD 4	Programmed cell death protein 4
EGFR	Epidermal growth factor receptor
EGF	Epidermal growth factor
TGF-α	Transforming growth factor-α
PCR	Polymerase chain reaction
qPCR	Real time polymerase chain reaction (quantitative)
RT-PCR	Reverse transcription polymerase chain reaction
EtBr	Ethidium Bromide
GTP	Guanosine-5'-triphosphate
SNP	Single nucleotide polymorphism

rRNA	Ribosomal Ribonucleic acid
daf-4	Decay accelerating factor-4
BRCA 1/2	Breast cancer ½
RNase	Ribonuclease
SUC 2	Sucrose transport protein 2
FAM	Fluorescein
DEPC	Diethylpyrocarbonate
BSA	Bovine serum albumin
DMSO	Dimethyl sulfoxide
PBS	Phosphate saline buffer
SSC	Saline sodium citrate
SDS	Sodium dodecyl sulfate
TCEP	tris(2-carboxyethyl)phosphine
SM(PEG)n	(succinimidyl-[(N-maleimidopropionamido)-(n)ethyleneglycol] ester)
Sulfo EMCS	((N-(ε-Maleimidocaproyloxy) sulfosuccinimide ester))
T_m	Melting Temperature
LNA	Locked Nucleic acid
P1	Probe 1 (Capture probe)
P2	Probe 2 (Signal probe)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
EDTA	Ethylenediaminetetraacetic acid

DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum

CHAPTER 1

INTRODUCTION

1.1 MicroRNA

The regulations of transcription and translation mechanisms for eukaryotes are very complex. With the recognition of micro RNAs, the complexity has increased. Before the 1990s, miRNAs were seen as unvalued product and had roles in nonmammalian species. In 1993, a study on the gene *lin-14* in *C. elegans* development performed by Victor Ambros, Rosalind Lee and Rhonda Feinbaum changed this view and indicated that a short RNA molecule, encoded by the *lin-4* gene, regulates the formation of *lin-14* protein by downregulating the expression [Ambros et al., 1993]. Until the discovery of *let-7*in *C.elegans* that caused the repression of expression in *lin-41*, *lin-14*, *lin-28*, *lin-42*, and *daf-12* during developmental stage [Basson, 2000]. Further studies demonstrated that miRNAs are conserved in many species and homologs of it are present in many organisms such as plants, birds, rodents, fish, worms, flies, viruses, and humans [Bartel, 2004]. Today, there are more than 1600 miRNAs in plants, animals, and viruses and 800-1000 of them may be found in only human genome [Lim et al, 2003].

1.2 Biogenesis of miRNA

MicroRNAs are RNA molecules controlling many developmental and cellular processes that are ~22 nucleotides in length. These evolutionary conserved RNA molecules prevent the translation of mRNA and result in the destruction of mRNA in some cases by binding the 3'UTR region of mRNAs [Abigail et al., 2010]. The location of miRNA genes may become introns and exons of other genes such as protein-coding and non-coding genes. Consequently, it can be said that the transcription of miRNA genes is regulated through the promoters of these genes.

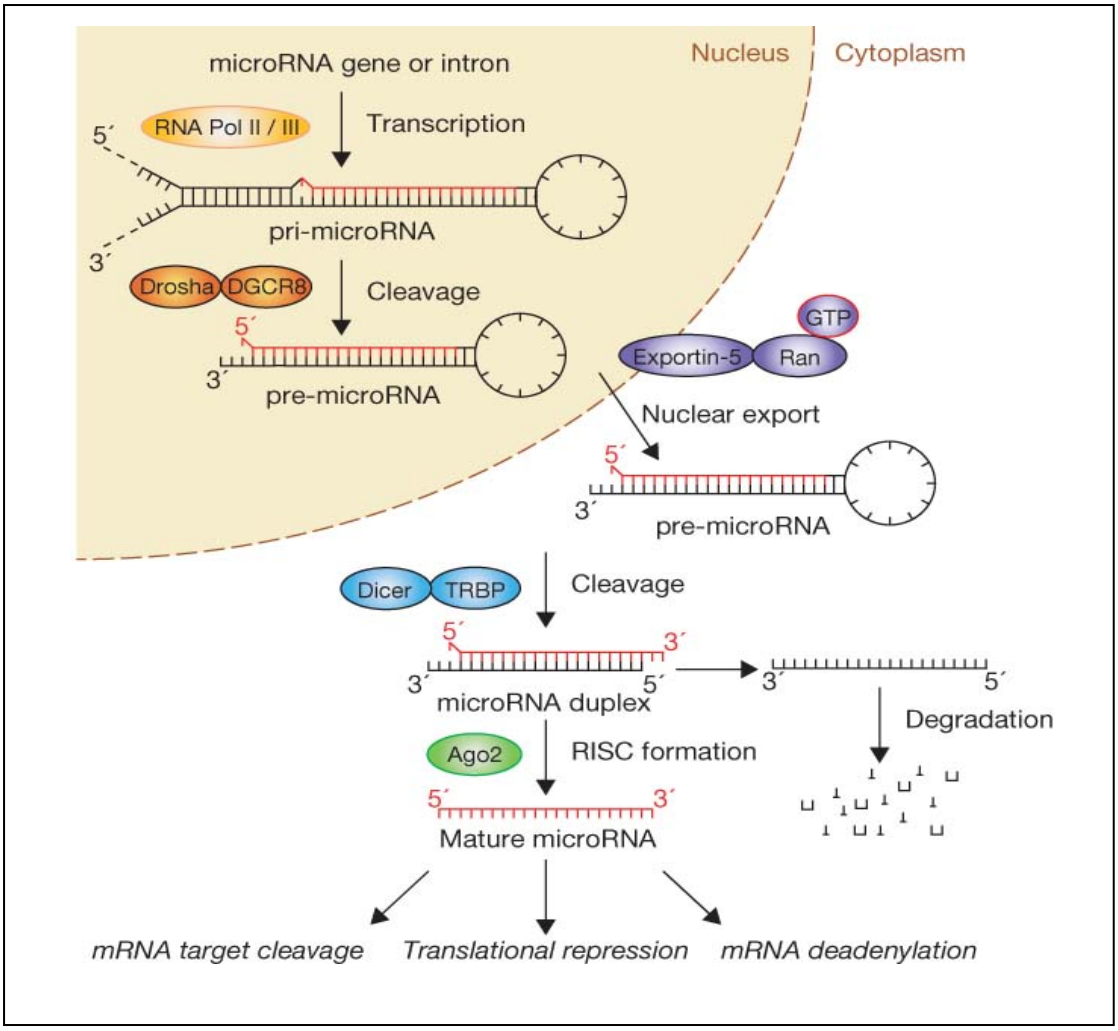

Figure 1.1 microRNA biogenesis pathways and their regulation [Winter et al., 2009]

MiRNAs are initially processed from precursor molecules as long and polyadenylated pri-miRNAs with the help of RNA polymerase II [Lee et al., 2004]. The hairpin loops of pri-miRNAs are formed with the RNA polymerase II binding and this hairpin becomes a substrate for enzymes such as Drosha and Dicer which are from RNAase III famiy (Figure 1.1).
The cleavage process occurs that these pri-miRNAs which may contain many sequences returned into shorter precursor miRNAs called as pre-miRNA, resulting ˜70 nucletides long product [Okamura et al., 2007]. This process takes place in nucleus was performed by a protein complex known as Microprocessor which contains Drosha and DGCR 8 Protein. The ds-RNA structure of these hairpins in a pri-miRNA is recognized by a DGCR8 [Gregory et al., 2006].

After the formation of pre-miRNAs, these pre-miRNAs are exported to cytoplasm from nucleus by folding into mini-helical structure. This structure allows the pre-miRNAs recognized by Exportin 5 protein which is a member of the karyopherin family [Yi et al., 2003]. This translocation is happened by using GTP bound Ran protein [Murchison & Hannon, 2004].

After the transportation, the pre-miRNAs are cleaved by an enzyme called as Dicer which is a member of RNase III family. The reaction is happened by Dicer similar with the reaction happened by Microprocesser, that is, Dicer cleavage occurs in a complex comprising TRBP which includes three dsRNA –binding domains and stabilizes the interaction between Dicer and pre-miRNA [Forsteman et al., 2005].

The product of Dicer cleavage is 19-25 nucleotide miRNA: miRNA duplex. One strand of this duplex is mature miRNA and other strand which has lower base pairing stability is called as guide strand. This guide strand is sometimes functional, but usually degraded [Lau et al., 2001]. MiRNA:miRNA duplex is separated and the mature strand is integrated to RISC that the interaction between miRNA and its target mRNA occurs. The RISC forms a complex by interacting with the Ago which is a key for RISC function. Ago binds to mature miRNA and adjust it for the reaction

with a target mRNA [Krol et al., 2010]. The formed mature miRNA has a role to intervene gene silencing by translational repression and degradation of target mRNAs via mRNA deadenylation and mRNA target cleavage.

1.3 Functions of microRNA

The organization and control of many genes are necessary to produce specific types of cells by multicellular organisms during development. These organization and control can be done with the help of environmental and cellular signals. The roles of regulatory RNA molecules in the development begin to emerge recently while the functions of proteins like gene regulatory are known for a long time. MicroRNAs, a member of small regulatory RNA family, were discovered in *C.elegans* at first time when they do not control the time for cell fate switches during development while they can [Carrington & James, 2003].

MicroRNAs are small RNAs that have roles in cell death, cell proliferation, and development by regulating the mRNA expressions [Ambros, 2004]. Target includes generally more than one binding region. In addition to this, one miRNA can control more than two thousand mRNAs which have different functions, that is, one miRNA is complementary to one or more mRNAs' parts [Krek et al., 2005]. MiRNAs in plants are usually complementary to coding regions of mRNAs while in animals, complementary to a part in the 3' UTR [Ambros, 2004].

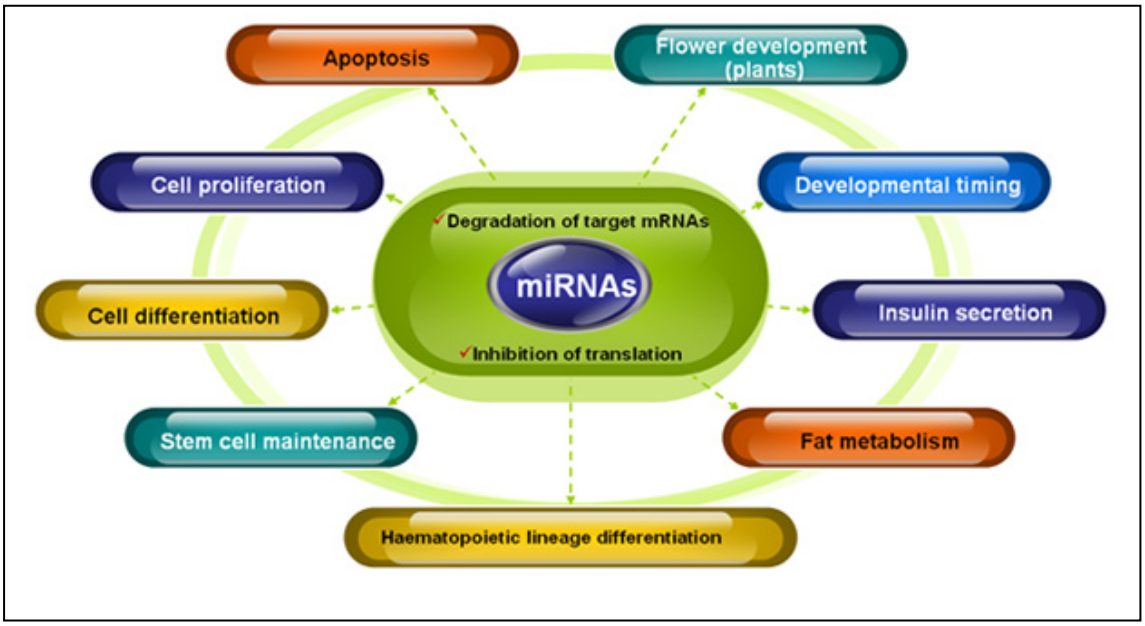

Figure 1.2 The main functions of miRNAs are degradation of mRNAs and inhibition of translation [URL 1]

MiRNAs regulate target gene expression post tarnscriptionally through the cleavageof mRNA or repression of translation. miRNA expression is controlled by RNA polymerase II. Moreover, the transcription of DNA methylation which causes the silence of genes is is done by RNA polymerase II. Hence, it can be said that DNA methylation regulates miRNA expression in human cells (Figure 1.3) [Han et al., 2007]. MiRNAs have a key role in the evolutionarily conserved system of RNA-based gene regulation. Their main function in the cell is the regulation of gene expression during development and they also result in the downregulation by many ways such as mRNA cleavage, translational depression, and deadenylation causing the degradation of mRNA (Figure 1.2) [Eulalio et al., 2009].

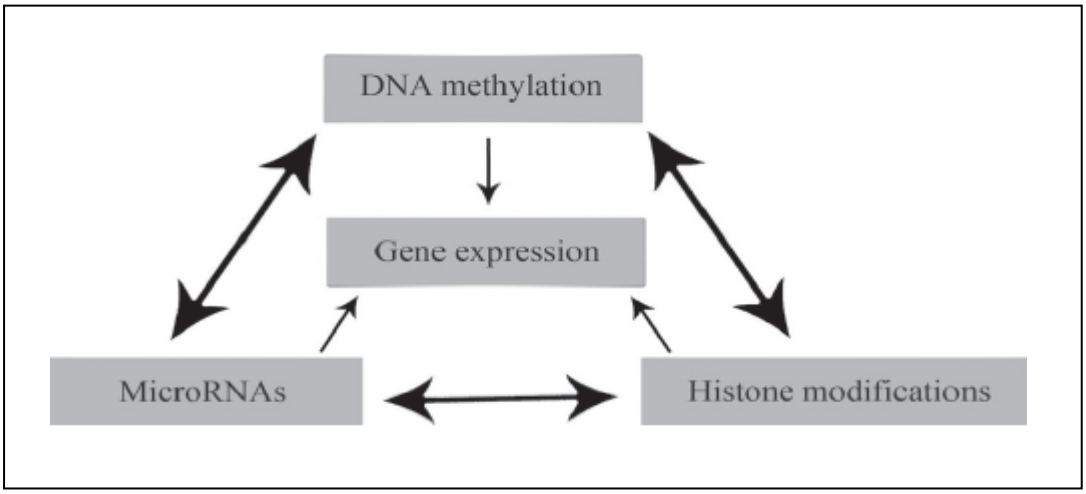

Figure 1.3 Relation between genomic methylation and miRNAs in the gene expression [URL 2]

In plants and animals, the mRNA encoding factors expressions which help for developmental timing, stem cell maintenance, and other developmental and physiological processes are prevented by miRNAs. Furthermore, it can be said that miRNAs affect regulation negatively by inhibiting protein synthesis in animals and degrading mRNAs [Carrington & James, 2003].

1.4 miRNA in diseases

MiRNAs inhibit the translation and cause the degradation of target mRNAs, that is, miRNAs affect gene expression negatively. Only one miRNA can provide the regulation in the expression of multiple target genes which have roles in many functions. According to this, it can be said that one miRNA has effect on a whole gene network and because of this; one miRNA can lead to many diseases.

Before the discovery of miRNA, junk DNA with no function is seen because a large part of the genome is not translated into proteins. Since discovery of miRNA, it is known that this phenomenon is related to this coding DNA in the gene expression

regulation. Many studies show that abnormal miRNA expression causes diseases (Figure 1.4).

miRNA	Target	Function	Reference
miR-15/miR-16	Bcl2	Apoptosis	Cimmino, et al
miR-1	GJA1/KCNJ2	Cardiac Arrhytmia	Yang, et al
miR-146	IRAK1/TRAF6	TLR-NFB	Taganov, et al
miR-520h	ABCG2	Stem Cell Differentiation	Liao, et al
miR-106a	Rb1	Cancer Pathogenesis	Volinia, et al
miR-let7	Multiple	Cell Cycle Regulation	Rodriquez, et al
mir-155	-	Adaptive Immunity	Johnnidis, et al
mir-223	-	Granulocyte Regulation	Johnnidis, et al
mir-208	-	Stress Response (Heart)	Van Rooij, et al

Figure 1.4 Examples of the relation between miRNAs and diseases

MiRNAs have role in the cells in normal functioning accordingly, it can be said that the defects in miRNAs cause diseases. For example, the bantham mRNA and miR-14 are necessary to control growth, through the programmed cell death in *D. melanogaster* (Figure 1.5) [Brennecke et al., 2003].

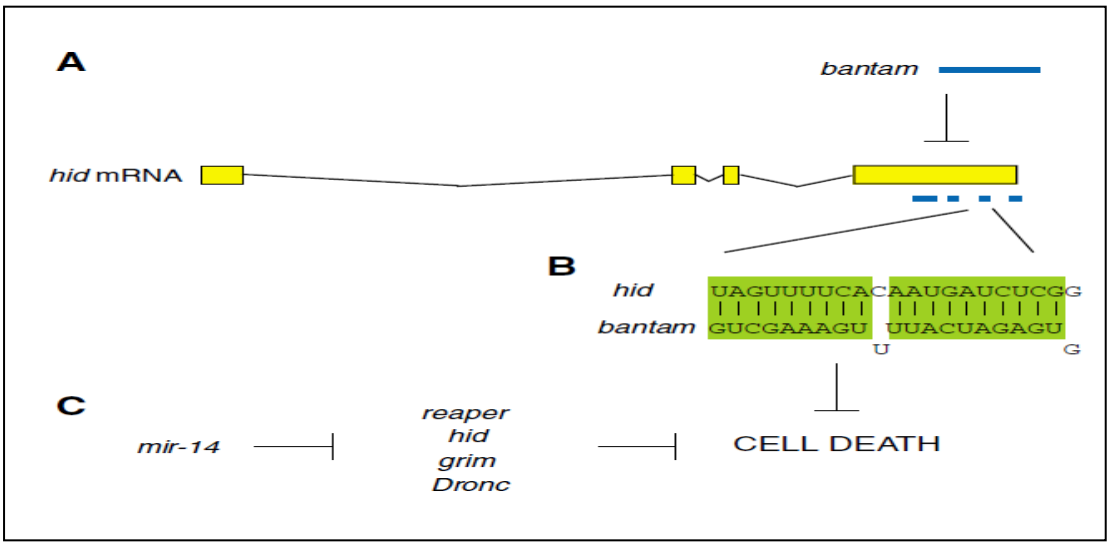

Figure 1.5 The inhibition of programmed cell death by miRNA bantam and miR-14. **A.** The mRNAof the pro-apoptotic gene hid (yellow) has five predicted target sites for the bantam miRNA (blue) in the 3_UTR. **B.** The alignment of the bantam miRNA with one of these sites is shown in green. **C.** miR-14 also inhibits programmed cell death by regulating several pro-apoptotic genes such as reaper, hid/wrinkled, grim, and Dronc [Alvarez-Garcia & Miska, 2005].

MiRNAs, partly complementary to target mRNA, have inhibitory role in gene expression by binding to 3' untranslated region (UTR) of target mRNAs. According to studies, it can be said that miRNAs were involved in various diseases like cancer, infectious diseases, diabetes, heart failure [Zhang et al., 2008].

Firstly, the incapability of the heart to pump sufficient blood correctly to the organisms is known as heart failure. With the activation of a number of intracellular signaling pathways and transcriptional mediators which lead to myocyte hypertrophy, remodeling of the extra cellular matrix, and reexpression of an embryonic gene program, the injury or hemodynamic overload happen in adult heart [Olson et al., 2008]. These result in the progressive myocardial fibrosis and ventricular dilation, and consequently, heart failure [Rooij et al., 2007]. MiRNAs emerge in a broad range of diseases, also in cardiovascular disease. MiRNAs can be said to be involved in gene expression, they take part in the cardiovascular disease. According to many studies, in the failure of heart and hypertrophy, dysregulation of

miRNAs is observed [Cheng et al., 2007]. Even though miR-1, downregulation becomes in miR-29, miR-30, miR-133, and miR-150, upregulation in miR-21, miR-23a, miR-125, miR-195, miR-199, and miR-214 is seen during hypertrophy (Figure 1.6) [Olson et al., 2008].

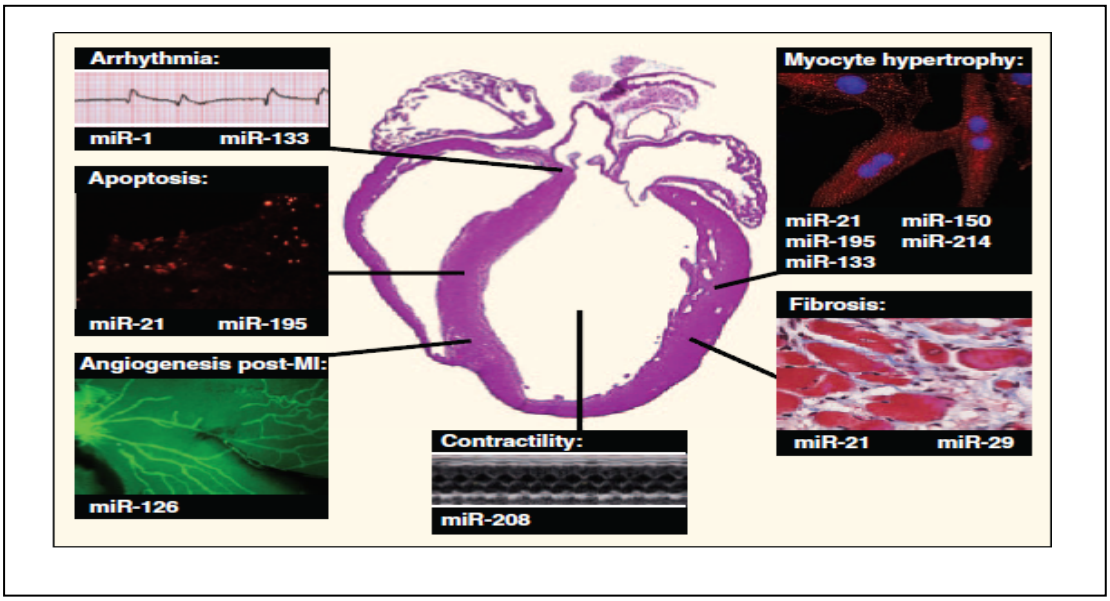

Figure 1.6 MiRNA function during heart disease [Olson et al., 2008].

Secondly, nervous system participates in many physiological processes in the organism and controlled by a number of genetic regulatory mechanisms. MiRNAs are an extensive class of small noncoding RNAs that regulate expression of a large number of protein-encoding genes so they control many important biological processes as in nervous system [Zeng, 2009]. MiRNAs are highly expressed in neurons that they have functions during neuronal differentiation, growth, proliferation, apoptosis, synaptic development, synapse maturation, and plasticity [Fiore et al., 2011].

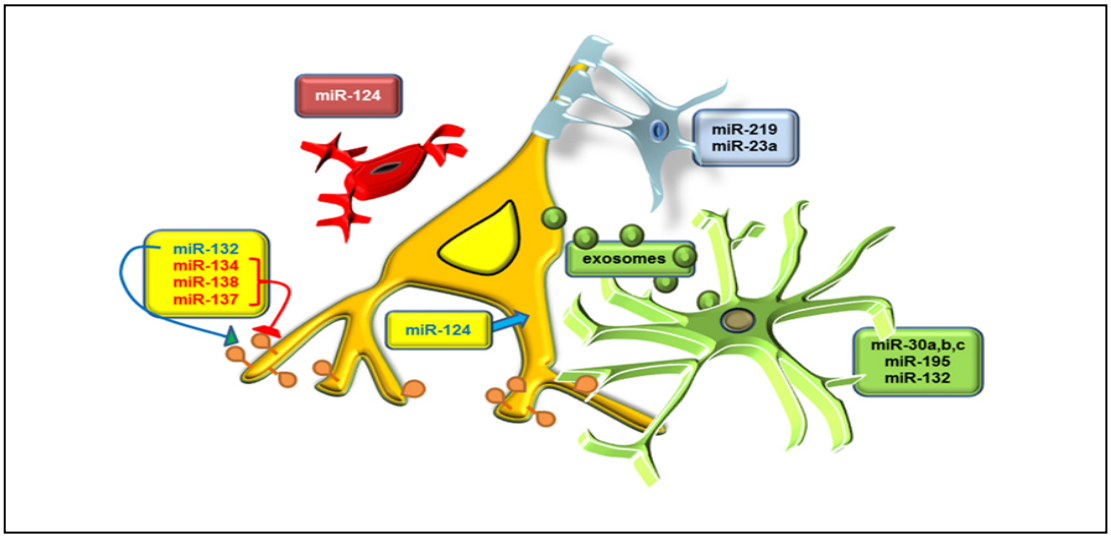

Figure 1.7 The neuronal and glial functions are regulated by miRNAs [Mellios & Sur, 2012].

While miR-132 activates, miR-134, miR-137, and miR-138 inhibits the spine growth in neurons shown in yellow. The existence of miR-132 expression in astrocytes, shown in green, stimulates expression of miR-195, miR-30a, miR-30b, and miR-30c. MiR-219 and miR-23a are expressed in a high level by oligodendrocytes which provide protection for neurons and insulation to axons, and with the expression, oligodendrocytes regulate their differentiation (Figure 1.7).

The lack of miRNAs can cause a variety of neurological disorders. Some miRNAs are found in neurological disorders such as traumatic CNS injuries, neurodegenerative diseases, AD, PD, Huntington's disease, Fragile X syndrome, etc [Liu, Xu, 2011]. According to these, it can be said that miRNAs, regulating entire set of genes, participates in both developmental and functional mechanisms in nervous system.

Moreover, miRNAs play significant roles in insulin production and secretion, pancreatic islet development, β-cell differentiation and insulin resistance, and adipocyte differentiation, that is, they are involved in both glucose homeostasis and lipid metabolism [Dehwah et al., 2012]. For example, miR-375 which found in islet

cells in a very high amount regulates pancreatic insulin secretion that the overexpression in the miR-375 results in the inhibition of glucose-induced insulin secretion [Poy et al., 2004]. Furthermore, miR-9 controls the expression of key components required for insulin-secreting cells and participates in β cell specificity and regulation of β cell death [Plaisance et al., 2006]. An increase in the amount of miR-9 suppresses insulin secretion by inhibiting the transcription factor onecut-2 which elevates the granuphilin/Slp4 level known as negative regulator of insulin release.

It can be said that miRNAs are found in glucose metabolism in the very beginning of the mechanism. The study carried out by *Lynn et al*. shown that the removal of Dicer in mice causes blocking of miRNA process that results in the problem in the development of endocrine pancreatic cells, especially β-cells [Lynn et al., 2007]. On the other hand, in adipose tissue, the dysregulation is observed in miRNAs. During adipogenesis, miRNAs have a role that they can hasten or prevent adipocyte differentiation [Williams & Mitchell, 2012]. Consequently, it can be said that miRNAs regulate fat cell development. Moreover, miRNAs can control fat cell numbers by regulating the adipogenic lineage [Dehwah et al., 2012]. For example, according to study applied by Trajkovski in 2011, the upregulation happens in the expression of miR-103/107 in obese mice and obesity is observed but with the silencing of these miRNAs, glucose homeostasis becomes better and obesity becomes getting lost [Trajkovski et al, 2011].

Furthermore, a mutation mechanism which affects miRNA function can cause human genetic disorders. The main types of these mechanisms are mutation which have effect on miRNA sequences resulting from duplications or deletions, mutation occurs in the recognition sites for miRNAs, and mutations in genes which have function in the general process and function of miRNA (Figure 1.8).

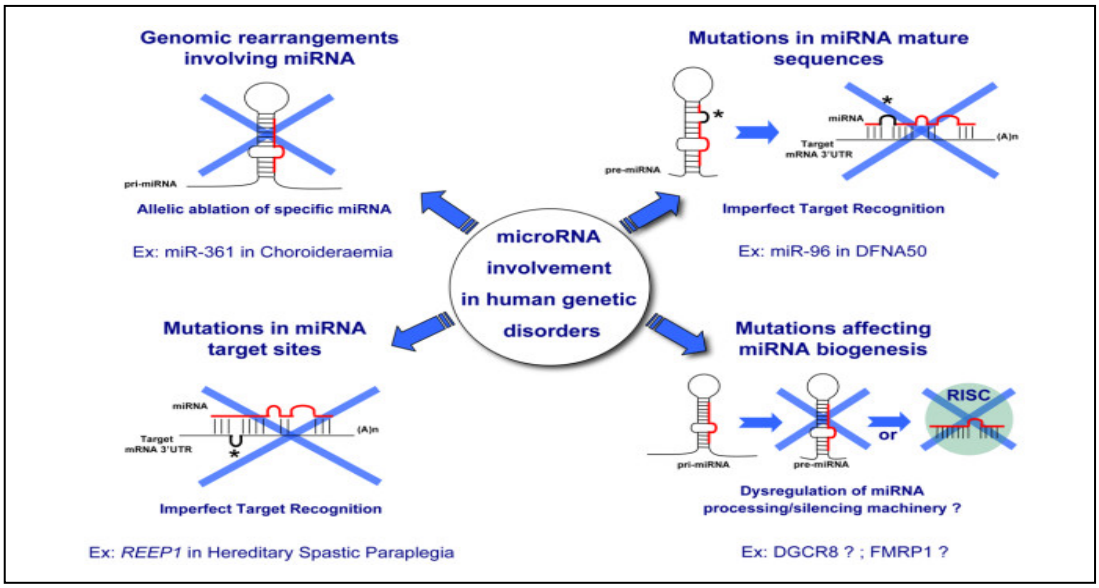

Figure 1.8 Schematic diagrams for the main types of miRNA mutations in disorders [Meola et al., 2009]

For instance, a mutation in miR-96 leads to hereditary hearing loss and a mutation in miR-184 hereditary keratoconus with cataract [Mencia et al., 2009; Hughes et al., 2011]. Moreover, the skeletal and growth defects are resulted from the deletion of the miR-17.

In addition to these, miRNAs have roles in the control of immune cell development and function (Figure 1.9). They are found in T cell differentiation in the thymus and B cell in bone marrow. For example, miR-150 is involved in B cell differentiation [Xiao et al., 2007] while miR-125b has a function in innate immune response and TLR signaling [Tili et al., 2007].

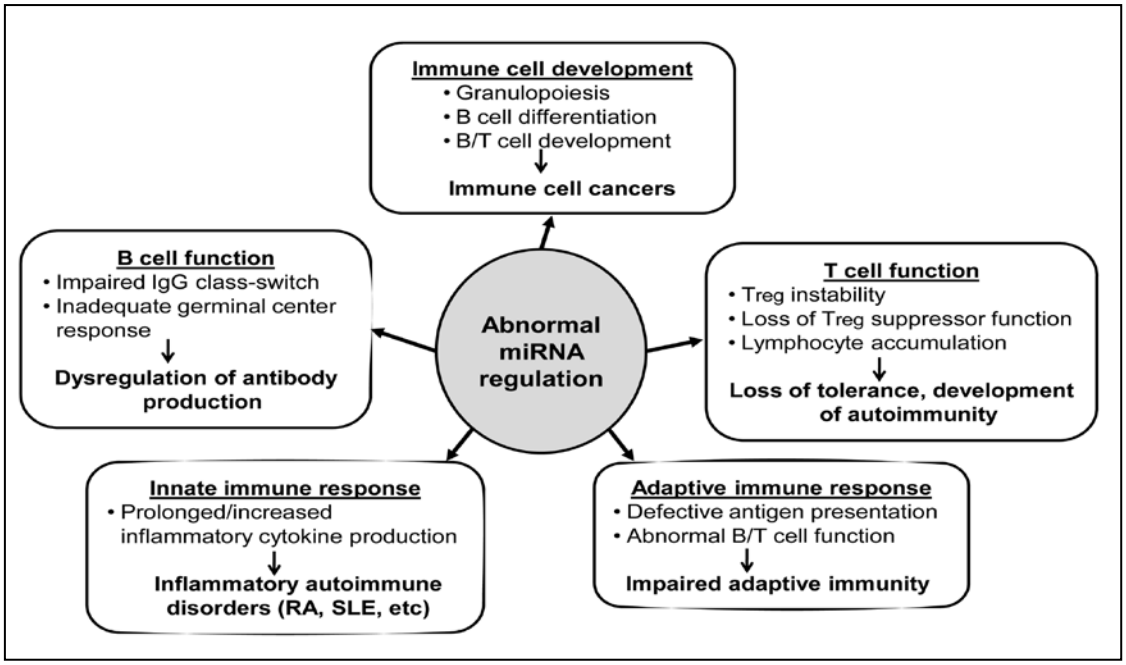

Figure 1.9 Summary of potential consequences of abnormal miRNA regulation in immune functions [Pauley et al., 2009]

When the miRNAs are deregulated in immune responses, these cause autoimmune diseases such as MS, and rheumatoid arthritis [Pauley et al., 2009].

1.5 The relationship between miRNAs and Cancer

MiRNAs have function in many gene regulation mechanisms and so altered expression of miRNAs leads to a number of diseases, including cancer which arises from the uncontrolled growth [Erson & Petty, 2008]. The idea that the dysregulation of miRNAs results in the cancer comes from the existence of miRNAs which are differentially expressed in cancer related tissues when compared to normal tissues [Volinia et al., 2006]. The lessened amount of mature miRNAs exists often in tumors that result from genetic deletion, epigenetic silencing, mutation, and any defects in their biogenesis pathway [Lu et al., 2005]. For example, considered to biogenesis pathway, a decrease in the expression level of DICER has been found in human tumors [Karube et al., 2005]. The idea that miRNAs have a key role in cancer

formation rises at the first time, with discovery of miR-15aand miR-16a genes which are located at 13q14 region and deleted and/or downregulated in patients with B cell chroniclymphocytic leukemia [Calin et al., 2002]. When the genomic instability regions in cancer cells are examined, it was seen that miRNAs are found in these regions in very concentrated values [Calin et al., 2004].

MiRNAs can act as tumor suppressor and oncogene (Figure 1.10). MiRNAs can act as tumor suppressors when they lose their function, in these cases; they contribute to malignant transformation in normal cell. Moreover, they can act as oncogene that they target mRNAs encoding tumor suppressor proteins [Rothschild, 2014].

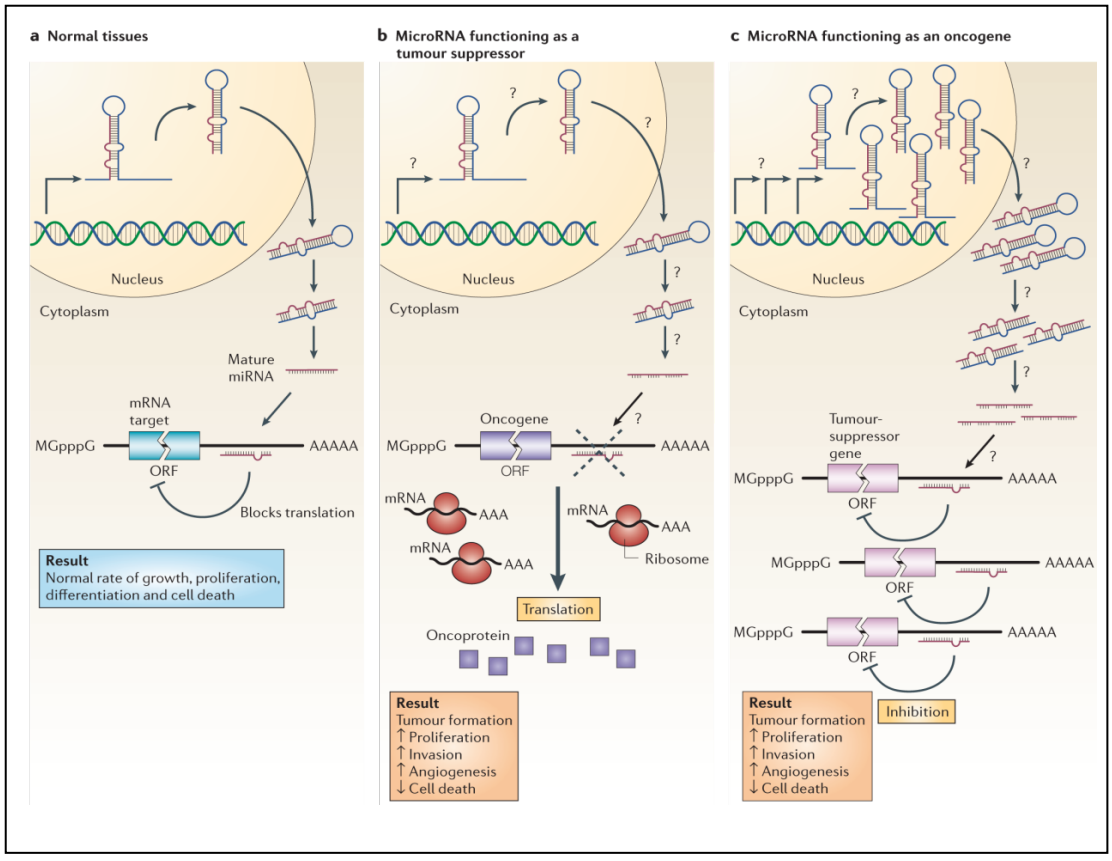

Figure 1.10 MicroRNAs can function as tumor suppressors and oncogenes [URL 3]

As seen in the Figure 1.10, in normal tissues, miRNA biogenesis takes place in which the repression of target gene expression occurs with the blocking in protein translation. As a result of this, normal rate of growth, proliferation, differentiation,

and cell death are observed (a). A defect in any part of the miRNA biogenesis may cause a reduction in miRNA levels and this may lead to improper expression of the miRNA target oncoprotein. This may result in the formation of excess oncogenic proteins, shown in purple, and cause tumour formation (b) [Palmero et al., 2011]. With the overexpression of miRNA, miRNA act as an oncogene and causes tumor formation. An increase in miRNA levels results in the elimination of the expression of a miRNA target tumor suppressor gene shown in pink and cancer progression exists (c).

Breast cancer is found in the second rank for the cause of cancer deaths in the world and is seen commonly in women [Bonev et al., 2011]. The down regulation and upregulation of 13 miRNAs found have roles in the breast tumors formation. For instance, an increase in the expression of miR-520c and miR-373 decreases the CD44 protein amount which functions in the adhesion and mobility of breast cells and consequently, this situation can confer metastatic property to breast cancer cell [Huang et al., 2008]. Another example is that as a result of overproduction of miR-21, it is seen that the levels of proteins such as PDCD4 that has function in apoptosis and TPM1 which binds to actin filaments, decrease [Frankel et al., 2008; Zhu et al., 2007].

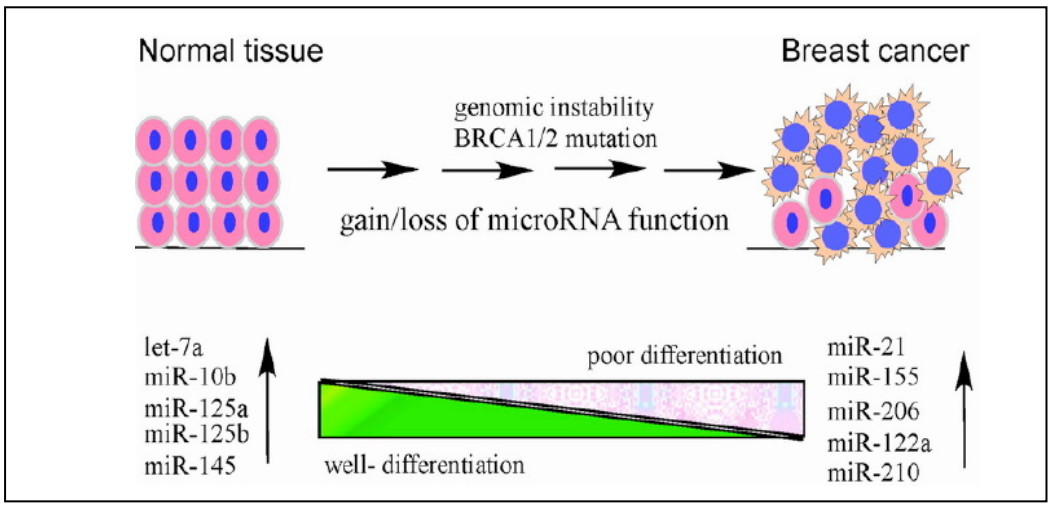

Figure 1.11 MicroRNAs regulations in normal tissue and breast cancer [Wang & Wu, 2007]

The BRCA1/2 mutations and gain or loss of miRNA functions have impact on the formation of breast cancer (Figure 1.11). When compared to normal tissue and breast cancer tissue, different expression levels in miRNAs are seen that while let let-7a, miR-10b, miR-125a, miR-125b, and miR-145 are higher in normal cells relative to breast tumor cells miR-21, miR-155, miR-206, miR-122a, and miR-210 are higher in breast tumor cells.

Furthermore, some types of miRNAs gene expressions are related to especially some tumor types and tumors in which miR-30 expression levels decrease are specified as estrogen and progesterone receptor negative tumors [Iorio et al., 2005].

1.6 miRNAs as Diagnostic Biomarkers

As indicated before, miRNAs are short, approximately 22 nucleotide length RNAs that perform gene expression regulation by the degradation of mRNA or inhibition of translation. The dysregulation in the expression of miRNAs in various tissues results in the variety of diseases. The understanding of function of miRNAs is very important to realize the role of miRNAs in the formation of disease. Also, the detection of expression levels of them is necessary to realize the function of them.

The existence of miRNAs in serum and plasma stable form, often their tissue and disease specific expressions, and the measurement of them with high sensitivity and specificity bring the idea of using them as diagnostic markers. The ideal biomarker for any disorder must be accessible by using noninvasive methods, economic, specific and sensitive to disease of interest, allowing early detection, a long half-life within the sample, rapid, and reliable [Tijsen et al., 2012]. In terms of these properties, it can be said that miRNAs which are abundant and stable in plasma, serum, and peripheral blood, protected from the effects of RNase because of their small size, are very suitable to become diagnostic marker. For example, miRNs as biomarkers are more efficient than protein based biomarkers. In protein based

biomarkers, bottlenecks at the point of specific antibody generation exist due to posttranslational modifications, complex protein composition, and the low abundance of many proteins in plasma and serum [Mitchell, 2008]. Because of these, miRNAs are utilized in the diagnosis of diseases especially in cancer (Figure 1.12).

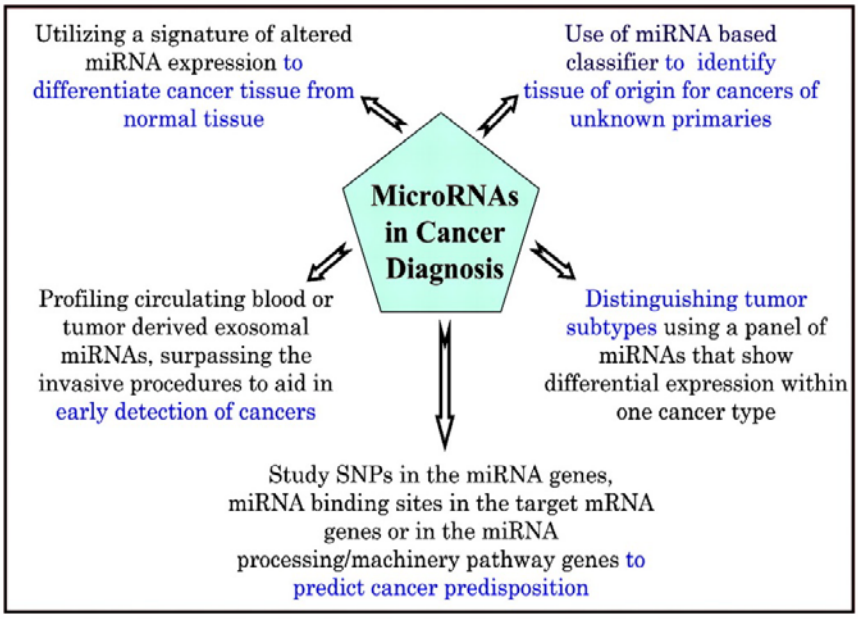

Figure 1.12 MicroRNAs usage in the diagnosis of cancer [URL 4]

Consequently, the detection of miRNAs in both basic research and clinical applications is gaining importance. Therefore, for the detection of miRNAs, various techniques are developed.

1.7 Detection Methods for miRNA Expression in Biological Samples

As indicated before, miRNAs are a type of small, non-protein coding RNA molecules found in many organisms. Since 2001, understanding of the regulatory role of miRNAs which expressed in both plants and animals becomes very popular [Wark et al., 2008]. Therefore, the development of new methods for detection of miRNAs starts to gain importance.

1.7.1 Northern Blotting for miRNAs

Northern Blot is a widely used method in order to detect miRNAs since it is a rutine method for a molecular laboratory which does not require any special equipment and technical knowledge. The method detects specific RNA molecules among a mixture of RNA and can be used to measure the expression of RNA during morphogenesis, differentiation, and disease in particular tissue or cell [Schlamp et al., 2008].

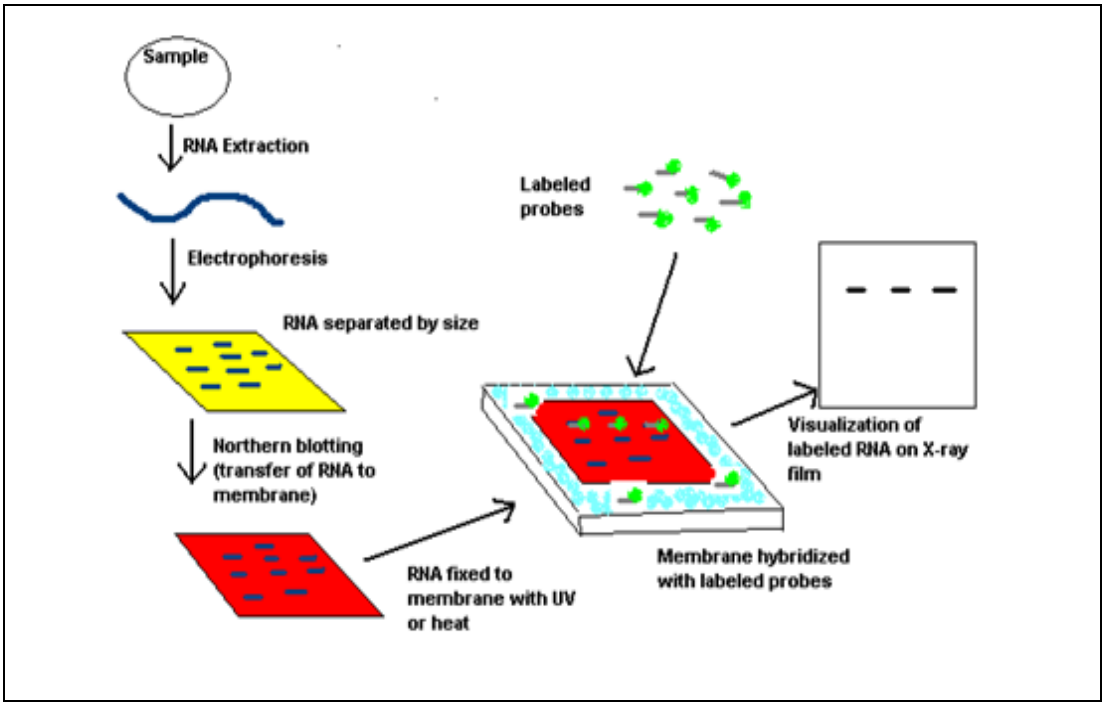

Figure 1.13 The steps used in Northern Blot analysis [URL 5]

In Northern blotting, the utilization of electrophoresis for the separation of RNA samples in terms of size, the detection with a hybridization probe which is complementary to gene partly or entirely, and the transfer of RNA from gel to membrane are found.

Northern Blot starts with the isolation of RNA from sample and purification (Figure 1.13). The denaturation is important that the transformation of the RNA within the sample into single strands, which ensures that the strands are unfolded and that there is no bonding between strands. After isolation and purification, RNA is loaded to electrophoresis gel and an electric current is passed through a gel and RNA is moved to positive electrode. The RNA is separated with regard to sizes by using electrophoresis. After electrophoresis, it is transferred onto a blotting membrane and all of the RNA bands are transformed originally to membrane in the presence of UV or heat. Following transfer, the membrane with a bound RNA is incubated with a radioactive or fluorescent DNA/RNA or oligonucleotide probe which is designed partly or entirely complementary to sequence of RNA with a minimum 25 complementary bases, that is, can hybridize to any RNA on the membrane. Furthermore, the probe is designed with a label like a radioactive atom or a fluorescent dye in order to detect. After incubation, washing step is applied to remove nonspecific binding probes and waited for drying of blot. In order to visualize, different methods are used such as autoradiography for radioactive probe and light of a specific wavelength for fluorescent probe.

Northern Blot method can be carried out to observe cellular control and function of genes by the determination of gene expressions. This provides the comparence of gene expressions by one cell to other cell and cells in normal conditions and diseased states. In other words, the changes in gene expressions can be observed between cells, in developmental stages, in pathogen infection, during environmental stress, and with the treatment [Baldwin et al., 1999]. For example, the finding of new miRNAs which have function in the viral infectious cycle and understanding of the alteration of host miRNAs with the presence of viral infection have been provided

with the Northern Blot analysis in the study carried out by McClure and friends (2011). In this study, Northern Blot analysis was tried to be optimized for the detection of viral miRNAs from cells which undergone a lytic infection from the members of the Herpes and Polyoma virus families [McClure et al., 2011]. This technique can be utilized also for the detection of the alterations in gene expression of RNA in cancer cells [Michalski et al., 2009].

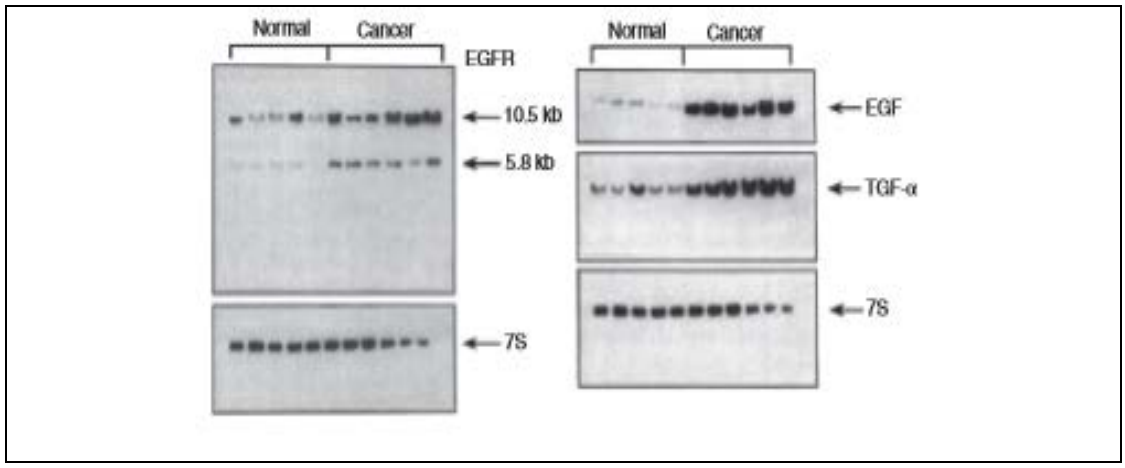

Figure 1.14 Northern blot analysis for EGFR, EGF and TGF-a in the normal and pancreatic cancer cells [Michalski et al., 2009]

According to this figure 1.14, when compared with the normal pancreatic cell, RNA levels encoding the EGFR, EGF, and TGF-α increase in very high amounts in pancreatic cells with cancer state. It can be said that the overexpression in the EGFR, EGF, and TGF-α can be used as a diagnosis marker in the pancreatic cancer.

Northern Blot analysis has many advantages such as the simplicity of method, the high specificity, the capability of detection of RNA size, low cost, the storage of membranes for several years and usability again, the possibility of visible RNA splicing, the utility of sequences with partial compability as probes, and easy verifying for the quantity and quality of RNA after electrophoresis [Michalski et al., 2009]. Although Northern Blot method is used commonly, it has many disadvantages like the risk for the degradation of RNA during electrophoresis which affects the

quality and quantity of RNA, high doses of formaldehyde and radioactivity to label probes for environment, the difficulty of detection with multiple probes, the use of ethidium bromide, DEPC, and UV which need usage knowledge, and the sensitivity which is low when compared to RT-PCR.

1.7.2 Real-Time PCR & RT-PCR

PCR is used for the amplification of a single sequence in DNA. PCR is consist of 3 steps such as denaturation in which the double strand transforms to single stranded DNA by melting at high temperatures as 94°C, annealing that polymerase attach and copies the template, and extension that synthesis of DNA occurs (Figure 1.15).

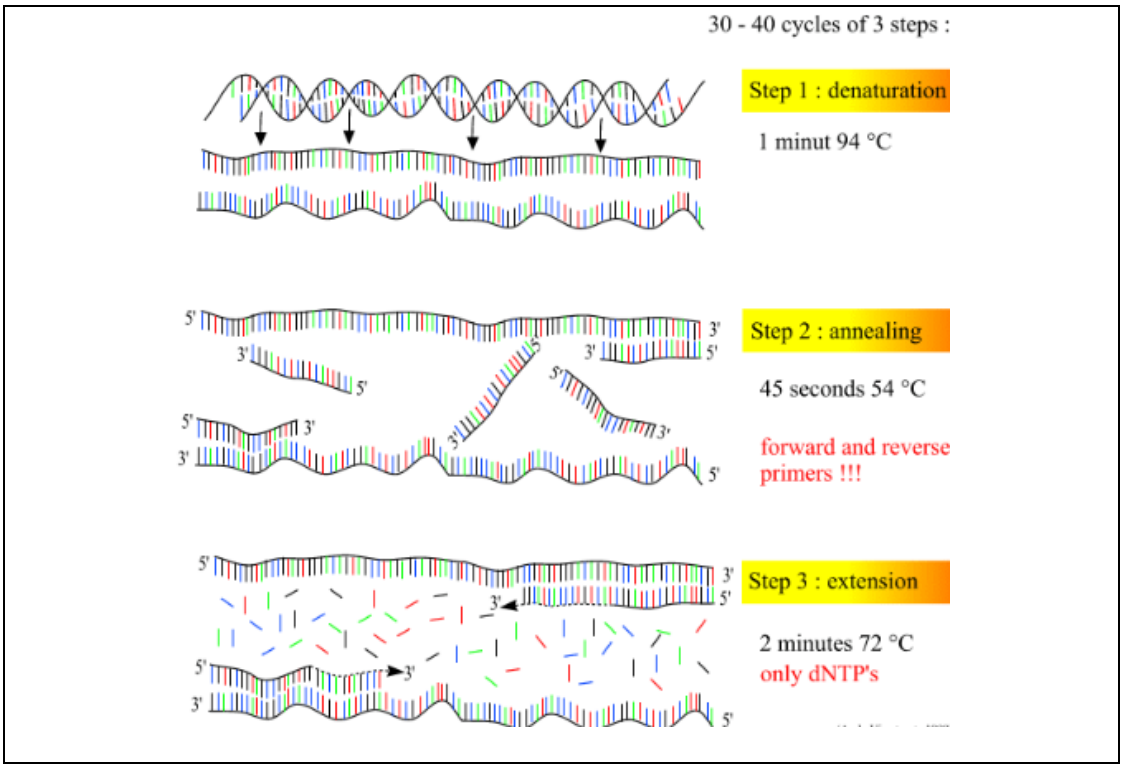

Figure 1.15 The steps in PCR [URL 6]

The detection of products is carried out by using agarose gel electrophoresis. The products are stained with EtBr and loaded onto gel. And, gel is visualized under UV light. The result is not indicated with numbers, products are seen only as bands.

On the other hand, real-time PCR can detect the amplification of products during the early phases of the reaction by using fluorescent dye system and express results as numbers.

qPCR is one of the most sensitive gene analysis techniques that is utilized for many areas such as quantitative gene expression, genotyping, SNP analysis, pathogen detection, mutation detection, drug target validation, allelic discrimination, and the measurement of RNA interference (Figure 1.16).

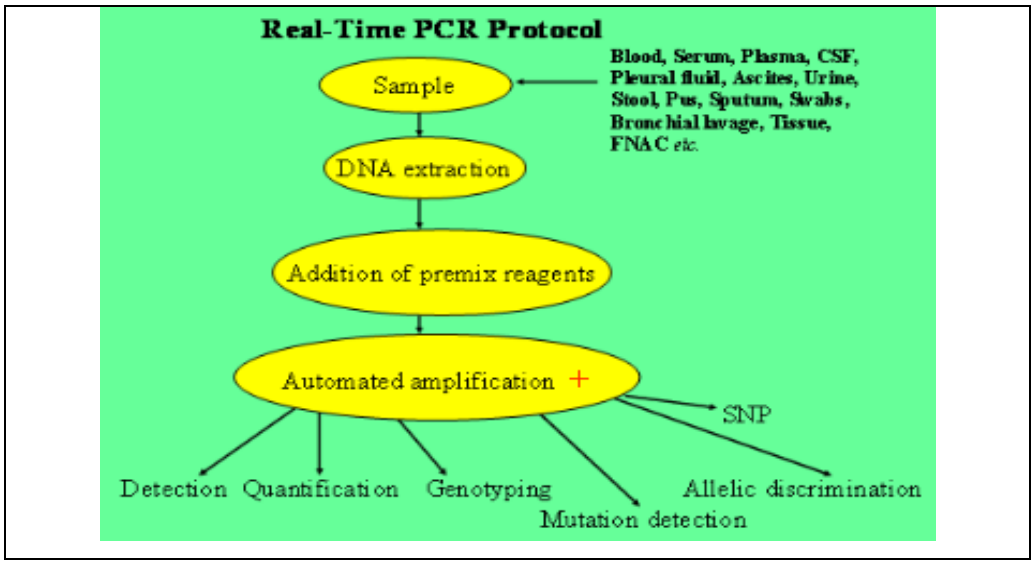

Figure 1.16 The areas usually used qPCR and usuaaly used steps [URL 7]

Development of PCR technologies depends on the reverse transcription and fluophores allow measuring the DNA amplification during PCR while the reaction is progressing in real time. Two common methods such as non-specific fluorescent dyes intercalating with any double-stranded DNA and sequence specific DNA probes including oligonucleotides which are labelled with a fluorescent that allows detecting only after hybridization of the probe with its complementary sequence to survey mRNA and non-coding RNA in cells are used to detect products.

qPCR and RT-PCR are separate and distinct techniques. The usage of RT-PCR is for the detection of gene expression through creation of cDNA transcripts from RNA

whereas qPCR is utilized to quantitatively measure the amplification of DNA using fluorescent probes.

In RT-PCR method, the conversion of RNA template to cDNA is happened by using a reverse transcriptase [Nolan et al., 2006]. Then, the cDNA is utilized as a template for amplification using PCR and the detection and quantification of products in real time happens (Figure 1.17).

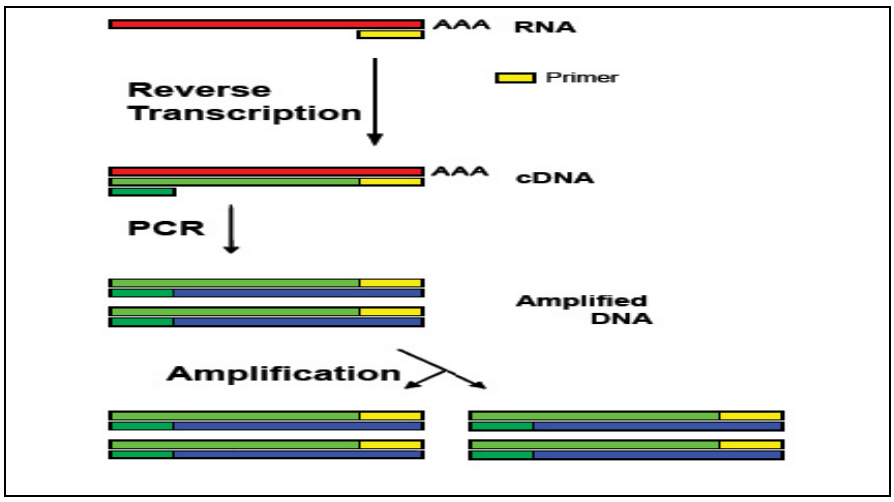

Figure 1.17 The steps of RT-PCR [URL 7]

The measurement of miRNA expression level is an important to observe the regulation of biological functions and their usage as diagnostic biomarkers for diseases. The amplification of miRNAs with PCR is difficult because of involving a stable hairpin and having a short length which is close to the size of PCR primer [Chen et al., 2011]. Therefore, qPCR and RT-PCR which is formed with the combination of qPCR and reverse transcription for the amplification of the messenger RNA (mRNA) and microRNA (miRNA) in cells are developed. For example, according to study applied by Cubedo and friends, the expression of 156 mature miRNA in colorectal cancer was investigated by using real-time PCR and a group of 13 miRNAs that expressions change significantly are determined [Cubedo et al, 2006].

Due to several powerful advantages such as high sensitivity, repeatability, detection of amplified DNA during reaction, having high throughput by processing large amounts of samples in a short time, and sufficiency of lower amounts for reaction, qPCR can be applied in a wide range like molecular diagnosis, detection and quantification of microorganisms causing diseases.

1.7.3 Microarray

Microarray is a method in which the hybridization of a target as nucleic acid sample to a very large set of oligonucleotide probes which are attached to a solid support such as a membrane or a glass microscope slide to determine sequence or to detect commonly variations in gene expressions exists. This method permits to simultaneous profiling of tens of thousands genes in parallel in a single experiment [Liu et al., 2008] and is used to point out basic questions in biological research [Auer et al., 2009]. Thanks to microarray technology, it can be determined which genes are active or inactive in different cell types and which function normally and how they are affected when genes do not function properly. For example by using this method, many complex diseases such as cancer, heart disease, inflammatory disease, and diabetes can be detected. That is, due to these advantages, this method can be utilized in many studies and their usage becomes widespread.

In this technique, RNAs are isolated from two samples and these RNAs are converted to cDNA by using reverse transcriptase enzyme and then tumor and normal samples are labelled with two different fluorescent dyes like green Cy3 and red Cy5. After labelling, these are hybridized to a solid support as micro-plates and membranes and bind to their synthetic complementary DNAs which are attached on the support. After hybridization, the intensity of fluorescent signals for each spot that is proportional to transcript levels in samples is measured at given wavelengths by using special scanner.

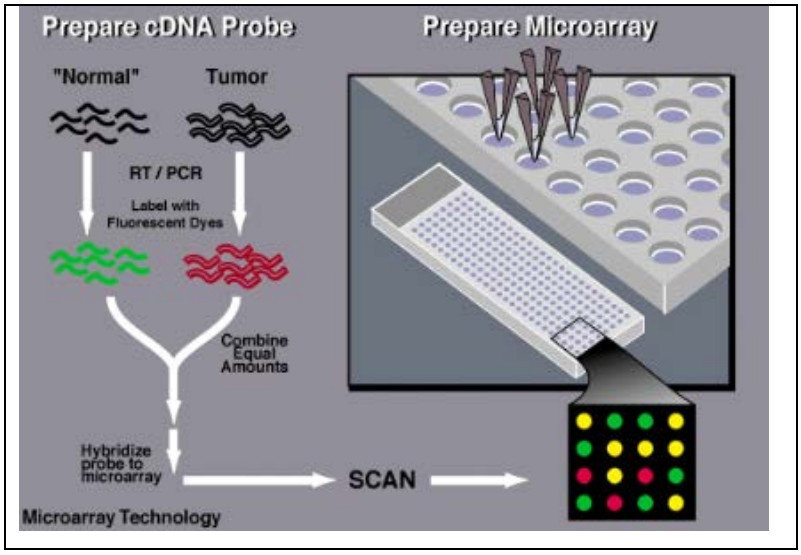

Figure 1.18 The example for microarray applications [URL 7]

If a particular gene is very active, it produces many molecules of messenger RNA, thus, more labeled cDNAs, which hybridize to the DNA on the microarray slide and generate a very bright fluorescent area. According to figure, if the spot is red, this means that gene is more expressed in tumor than in normal, in other words, it is up-regulated in cancer (Figure 1.18).

Microarray is used not only for DNA, but also proteins [Schweitzer & Kingsmore, 2002], carbohydrates [Love & Seeberger, 2002], cells [Wu et al., 2002], tissues [Fejzo & Slamon, 2001], drug like molecules [Lam & Renil, 2002], and miRNAs [Liu et al., 2008].

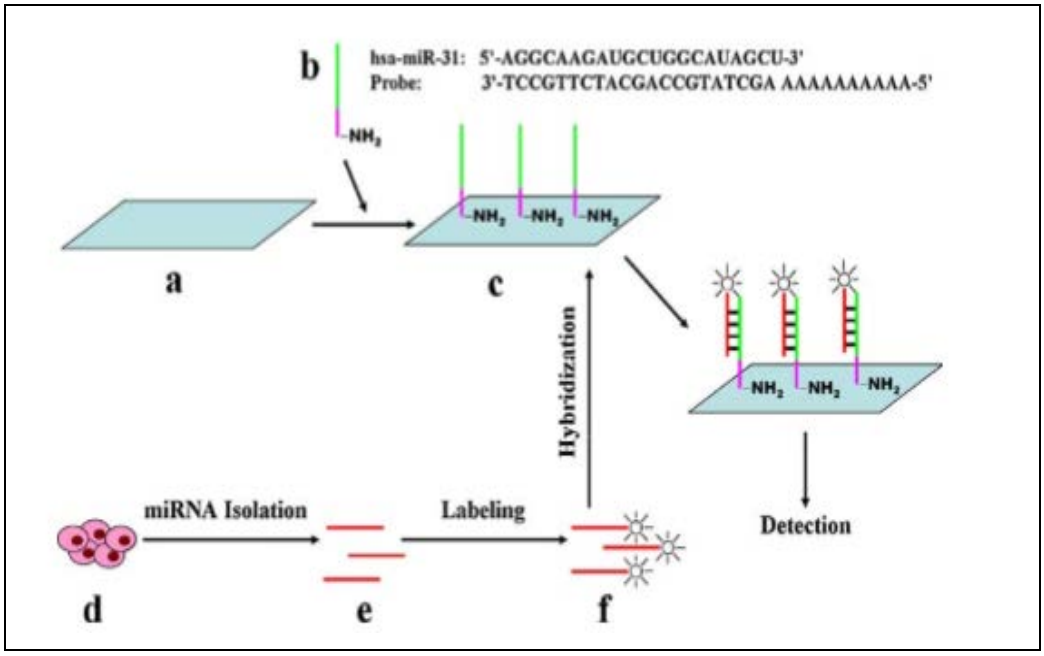

Figure 1.19 The principle of microarray for profiling miRNA. **A.** Amine-reactive glass slides. **B.** Amine-modified miRNA probes involving linker sequences in purple and capture sequences in green. **C.** Probes immobilized onto platform. **D.** Samples. **E.** Isolated miRNAs. **F.** miRNAs labeled with fluorescence dye. [Li & Ruan, 2009]

MiRNA microarray depends on the nucleic acid hybridization between target molecules and their complementary probes. MiRNA oligonucleotide probes which are amine-modified in 5'terminus are immobilized onto glass platform via covalent crosslinking. Then, the hybridization occurs between isolated miRNAs which labeled with fluorescent dye and probes. By analyzing the fluorescence signals, the detection of miRNAs can be realized (Figure 1.19).

1.7.4 Hybridization Assay

Hybridization assay method depends on the microarray principle that two kinds of probes bind to different sites on a target molecule. This method has many advantages such as high specificity and applicability of different labelling techniques [Ueno & Funatsu, 2014] and can be utilized to identify polymorphism to detect virus,

pathogens [Rautio et al., 2003] and detect cancer by analyzing gene expression levels in cells [Sarosdy et al., 2006].

Two different strategies as direct hybridization and sandwich hybridization can be used when hybridization assay is applied. In direct hybridization method, the hybridization principle of microarray that the occurrence of hybridization of immobilized DNA probe with the labeled DNA target exists is utilized. However, in sandwich hybridization method depends on the recognition of a specific DNA/RNA sequence, called as a target, by two probes. In other words, in the platform, the hybridization of capture probe, partly complementary to target, to target and signal probe to a part of the target exist (Figure 1.20).

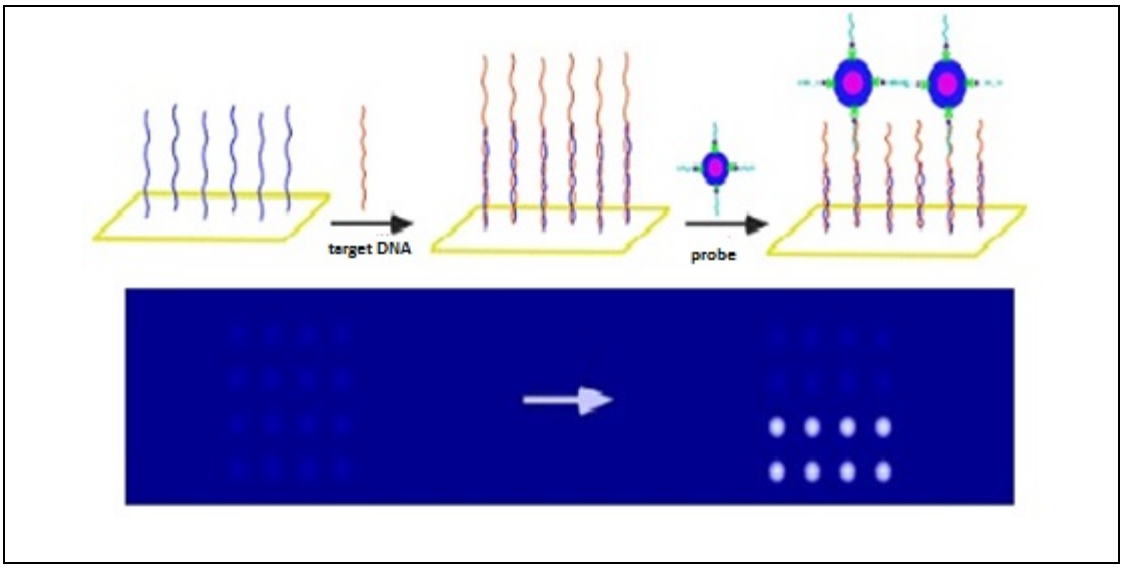

Figure 1.20 miRNA array platform in sandwich hybridization format (URL 8)

The sandwich hybridization method is a good alternative for the quantification of RNAs. The utilization of sandwich hybridization assay for the analysis of nucleic acid sequences at first time was in 1977 [Dunn & Hassell, 1977]. On the other hand, it was not used for diagnosis purposes till 1983 and with the utilization of this

method for the detection of adenovirus DNA of children in nasopharyngeal aspirates, its potential was understood for diagnosis of diseases [Ranki et al., 1983].

Transcript quantification techniques are usually based on the purification of mRNAs. In study carried out by Thieme and his coworkers, the sandwich hybridization assay was used for the quantification of mRNAs from E. coli without any preliminary preparation as RNA isolation.
This method provided rapid analysis and sensitivity in high degrees because of not negatively affecting by genomic DNA to detect transcript levels in crude cells extracts [Thieme et al., 2008].

Furthermore, in the study done by Rautio and his coworkers in 2003, the sandwich hybridization assay was used to measure levels of specific RNAs as 18S rRNA during cell growth and SUC2 under suppression and causative conditions form the applications of copper ions from *Saccharomyces cerevisiae*. This method was applied by the hybridization of target molecules with a biotin labeled capture probe and a digoxigenin labeled detection probe and the detection was provided by fluorescence signal created by alkaline phosphatase. According to results, it can be said that this method became suitable and fast to quantify the amounts RNAs, that is, it was successful [Rautio et al., 2003].

Sandwich hybridization method can be applied for both DNA and RNA and different signal methods such as electrochemical and optic types [Wang, et al., 2008; Du et al., 2009]. However, the detection which is based on the fluorescent is one of the most preferably mechanisms.

In order to quantify the transcripts from different organisms, several methods which have different properties such as time, expense, and steps have been developed. According to RNA assay methods as Northern Blot, sandwich hybridization method provides faster and more reliable results and also, it offers more economical solutions when compared to modern methods like RT-PCR which is more sensitive but more costly than sandwich hybridization assays [Thieme et al., 2008].

1.8 Aim of the Study

In this study, the aim is the development of sandwich type nucleic acid array platform for the detection miRNAs whose expression levels change in breast cancer. This platform becomes an alternative to existing tools to detect miRNAs. FAM labelled capture probe for miR-21, the DNA sequence of miR-21, and FAM labeled miR-21 were used as positive controls, in other words, test sequence, to optimize experimental conditions for the design of the sandwich hybridization platform on poly-L-lysine coated glass slides by using capture and signal probes. The visualization of slides and the detection of signals were performed with confocal laser microscope and Axon Genepix 4100A which is a microarray scanner system. Various parameters such as the types of probes, the types of signal, the concentration of probes and targets, hybridization temperature and duration, the type of cross linker, blocking solution, hybridization solution, and washing solution were studied and the results for the effects of these parameters alterations were shown relative to others. After optimization studies, RT-PCR was done. This method depends on the recognition of specific miRNAs by two probes and does not require any labelling or cDNA synthesis steps. This platform offers an economical and flexible detection system, can be used for different application areas such as the diagnosis and treatment of diseases by detecting the drug and impacts on diseases.

CHAPTER 2

MATERIALS AND METHOD

2.1 Materials

2.1.1 Chemicals

All chemicals were analytical grade, purchased from Sigma-Aldrich, Thermo Scientific, Invitrogen, Merck or AppliChem Chemical Company. Each solution used in experiments was prepared with Diethylpyrocarbonate (DEPC), used for the inactivation of RNase enzymes, treated Milli-Q water.

2.1.2 Support Materials

In this study, 25 x 75 x 1 mm sized poly-L-lysine coated glass microscope slides were used as a support material and bought from the Thermo Scientific Chemical Company.

2.1.3 Buffers and Solutions

Preparations and composition of the buffers and solution were given in Appendix A.

2.1.4 Heterobifunctional Cross-linkers and Reducing Agent

In this study, heterobifunctional cross-linkers were used to bind thiol modified oligonucleotides to amino groups on slides covalently. Tris (2-carboxyethyl)

phosphine (TCEP - $C_9H_{16}O_6PCl$), a thiol-free compound, was used to reduce protein and peptide disulfide bonds. The heterobifunctional cross-linkers and TCEP were purchased from ThermoScientific Chemical Company.

2.1.5 Synthetic Oligonucleotides

All oligonucleotides were purchased from the companies Integrated DNA Technologies" (C Leuven, Belgium) and Exiqon (Vedbaek, Denmark). Micro RNA 21 was used as the test sequences and designed partially hybridizing capture and signal probes to form the sandwich complex on glass slides. The sequences of synthetic oligonucleotides were listed in Appendix B.

2.1.5.1 Capture probe (P1)

The oligonucleotides, bound to poly-L-lysin coated glass surface with the help of cross-linkers and had a thiol modification on 5' end, was called as Probe 1 (P1). The capture probes used in the first stage of study were shown in Appendix B, B.1, and in the second stage of study in Appendix B, B.5.

2.1.5.2 Adapter (A)

Target micro RNA molecule, detected in the study, was called as Adapter (A). The adapters used in the first stage of study were shown in Appendix B, B.3, and in the second stage of study in Appendix B, B.7.

2.1.5.3 Non-conjugated Adapter (Ank)

The micro RNA, non-conjugated to Probe 1 and Probe 2, was used as a negative control and called as Ank.
The non-cojugated adapter used in the first stage of study was shown in Appendix B, B.3, and in the second stage of study in Appendix B, B.7.

2.1.5.4 Signal Probe (P2)

The oligonucleotide, half-conjugated to target molecule that was hold on the surface by P1 and had a 6-carboxyfluorescein (6-FAM) which was a fluorescent dye or a biotin ligand, was called as Probe 2 (P2). The signal probes used in the first stage of study were shown in Appendix B, B.2, and in the second stage of study in Appendix B, B.6.

2.1.5.5 Modified Probes

In the study, modified probes such as locked (locked nucleic acid-LNA) and 2'O-Methyl modified oligonucleotides were used in order to increase the selectivity of probes that recognized the target miRNAs and thermal stability of the hybrid.

Locked nucleic acids, nucleic acid analogs, had a methylene linkage between the 2'-O atom and 4'-C atom of locked nucleic acids, as shown in Figure 2.1. The nucleotides locked with methylene linkage remained optimally to bind other nucleotides; thus, they bound to half-conjugated nucleotide sequence much easier and quicker than non-modified oligonucleotides.

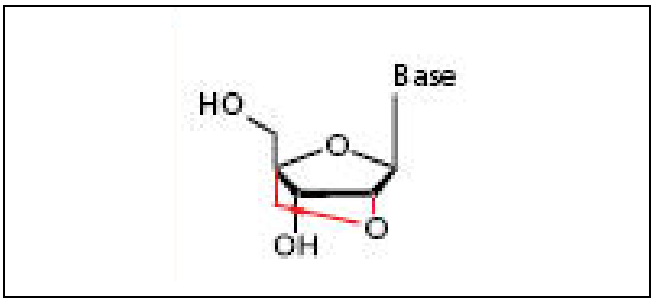

Figure 2.1 Locked nucleic acid monomers

2'O-Methyl modified oligonucleotides that had an alkyl modification on 2' position of ribose sugar were RNA nucleotides, as shown in Figure 2.2. As locked nucleic

acids, the presence of nucleotides with these modifications increases the probability of binding to target sequence (miRNAs) by affecting the kinetics of hybridization.

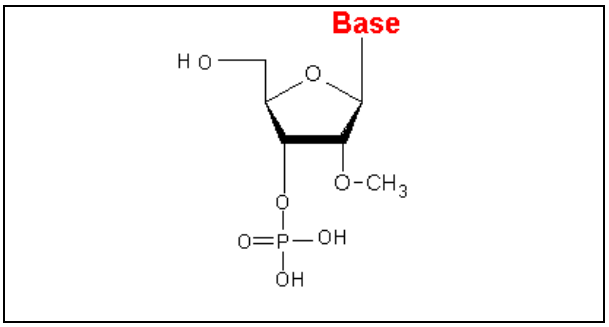

Figure 2.2 2' 0-Methyl modified nucleic acid monomers

2.1.6 Cancer Cell Lines

In this study, two breast cancer cell lines as MDA-MB-231 (metastasis positive) and MCF7 (metastasis negative) and two non-tumorigenic immortalized mammary cell lines such as MCF10A and MCF12A were utilized for investigation of platform availability and RT-PCR experiments. MCF10A, MCF12A, MCF7, and MDA-MB-231 were a kind of gift from Assoc. Prof. Dr. A. Elif Erson Bensan (METU, Ankara). In addition to these, total RNA samples taken from normal tissue and tissue with cancer were bought from Ambion and Clontech, respectively.

2.1.7 Probes in Real Time PCR (RT-PCR)

In order to perform RT-PCR, Taqman microRNA RT-PCR probes for miRNA sequences and U6, shown in Table 2.1, were obtained from Applied Biosystems and used according to instructions for the detection of expression levels of miRNAs. U6 was used as internal control for several cell lines.

Table 2.1 Taqman Probes in study

MiRNA	Taqman Probes
miR21	TaqMan ® MicroRNA Assays,Assay ID:000397
miR10b	TaqMan ® MicroRNA Assays,Assay ID:000388
miR125b	TaqMan ® MicroRNA Assays,Assay ID:000449
miR145	TaqMan ® MicroRNA Assays,Assay ID: 000467
miR200a	TaqMan ® MicroRNA Assays,Assay ID: 000502
miR200b	TaqMan ® MicroRNA Assays,Assay ID: 001800
miR200c	TaqMan ® MicroRNA Assays,Assay ID: 000505
U6	Taqman RNU6B assay : ID001093

2.2 Methods

2.2.1 Construction of Array Platform

The platform, used for the detection of miRNAs, was based on sandwich hybridization method. The sandwich hybridization method depends on the recognition of a specific DNA/RNA sequence, called as a target, by two probes. Therefore, the platform was formed from target and oligonucleotides probes which were half-conjugated to target. In this method, the capture probe (P1) which was fixed to the solid surface with the help of cross-linkers, specific to one part of the target molecule. Target molecule bonded to the capture probe by hybridization and the existence of target molecule was defined with the binding of signal probe (P2). The platform, used in the study, was shown in the Figure 2.3.

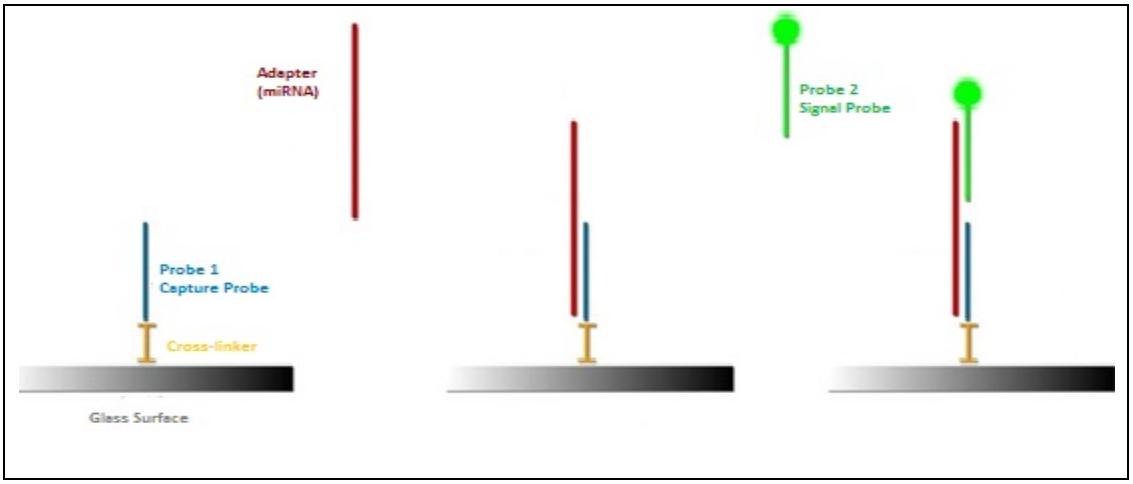

Figure 2.3 nucleic acid-based miRNA array platforms in sandwich format

2.2.1.1 Slide Design

2.2.1.1.1 Type 1

Type 1 design was used in the first stage of the project which was the development of sandwich hybridization based miRNA array platform. Type 1 design includes three positive controls and nine test spots, as shown in the Figure 2.4. As positive control, miR 21 probes labelled with FAM (miR-21-F) were used. Each slides had three positive controls, nine P2-A-P1 sandwich systems or nine P2-Ank-P1 negative controls. Therefore, in the first stage of the study each slide contained twelve spots.

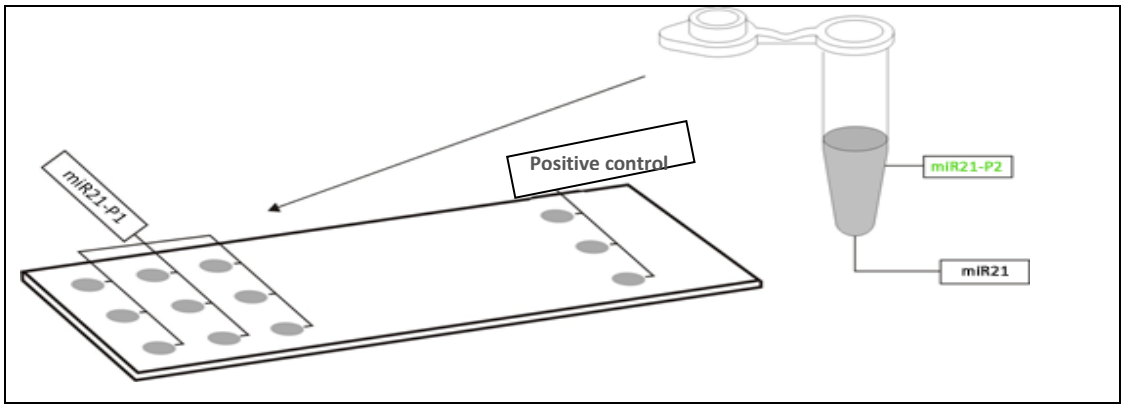

Figure2.4 Slide design in the first stage

2.2.1.1.2 Type 2

In the second stage of the project that is the recognition of different miRNAs on array platform, the slide design was used as shown in Figure 2.5.

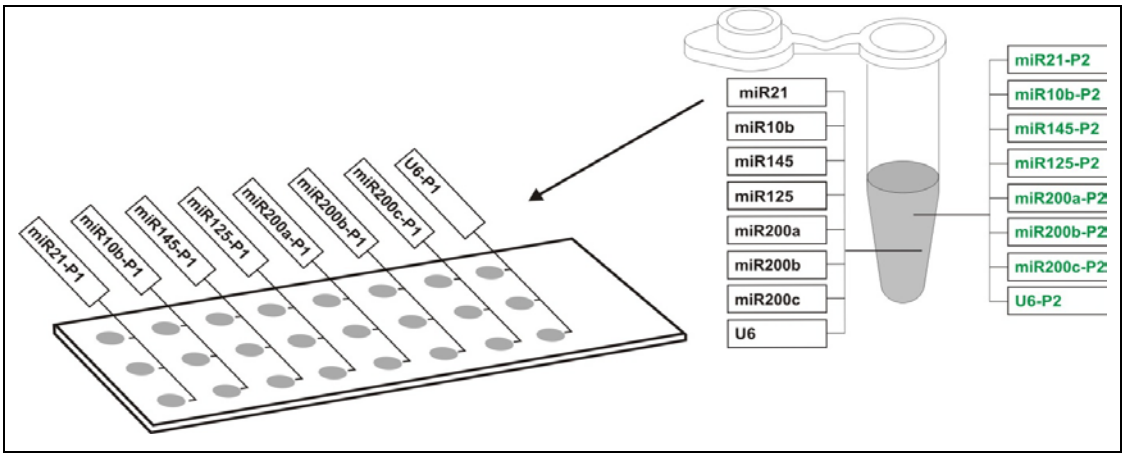

Figure2.5 Slide design in second stage

Accordingly, probe 1 was spoted to each line on hybridization area for one type miRNA and three repetition points were created for each type of miRNA. In the study, adapters such as miR21, miR10b, miR145, miR125, miR200a, miR200b, miR200c, and U6 and P2-Ls such as miR21-P2-L, miR10b-P2-L, miR145-P2-L, miR145-P2-L, miR200a-P2-L, miR200b-P2-L, miR200c-P2-L, and U6-P2-L were incubated together by forming complex and applied to the slide. Thus, the binding of a pair of P2-A to P1 at suitable hybridization temperature was provided.

2.2.1.2 Surface Activation

Poly-L lysine coated slides were activated using heterobifunctional cross-linkers. Heterobifunctional cross-linkers were used to bind thiol modified oligonucleotides to amino groups on slides covalently. The ester (NHS) groups of heterobifuncitonal cross-linkers on one side gave a reaction with primary amines and amide bond formed while maleimide groups of heterobifunctional cross-linkers on other side gave a reaction with sulfhydryl groups at pH 6.5-7.5 and stable thioether bond

formed. In this study, heterobifunctional cross-linkers such as Sulfo EMCS ((N-(ε-Maleimidocaproyloxy) sulfosuccinimide ester)), $SM(PEG)_2$ ((succinimidyl-(N-maleimidopropionamido)-diethyleneglycol)ester)), $SM(PEG)_6$ ((succinimidyl-(N-maleimidopropionamido)-hexaethyleneglycol)ester)), and $SM(PEG)_{12}$ ((succinimidyl-(N-maleimidopropionamido)-dodecaethyleneglycol)ester)), that have different lenghts were tried. The chemical structures of heterobifunctional cross-linkers used in experiments were given in the Figure 2.6.

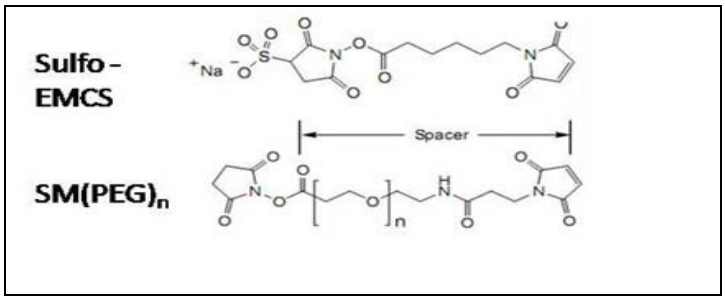

Figure 2.6 Chemical structures of heterobifunctional cross-linkers

Except for Sulfo-EMCS, all other cross-linkers were suspended in Dimethyl sulfoxide (DMSO) and stored at -20 ^{0}C as aliquots. However, Sulfo-EMCS was prepared freshly before each experiment in 10mM Phosphate Saline Buffer (PBS), pH: 7.2.

2.2.1.3 Capture Probe Immobilization

Reduction of disulfide bonds of capture probe (P1), activation of Poly-L lysine coated glass slides, and immobilization of the capture probe were performed at two steps. The first step was that specified concentrations of P1, specified heterobifunctional cross-linker with a final concentration of 2mM, and TCEP with a final concentration of 0.1 mM were mixed in 10 mM Phosphate Buffered Saline (PBS) buffer and the solution was incubated for 30 minutes at room temperature to perform the reduction of disulfide bonds. The immobilization mixture was spotted on the slides with a volume of 0.5 µL on ice with the help of micropipette. Then

immediately, spotted slides were incubated for 30 minutes at room temperature in humidifying chamber, constituted of a petri dish containing a little amount of water. In this way, the second step of immobilization was completed. The slides were washed with a solution, the mixture of 5X Saline-Sodium Citrate (SSC) and 0.1% Sodium Dodecyl Sulfate (SDS), for 15 minutes and DEPC treated water for 15 minutes in the coplin jar by using shaker (130 rpm) in order to remove weakly bound and unbound capture probes. At the end of washing, slides were dried by means of a spinner.

2.2.1.4 Surface Blocking

Blocking was done by using 2% Bovine Serum Albumin (BSA) in 10 mM PBS at pH 7.2 for 1 hour at room temperature. This step was done in order to inactivate the reactive groups on the surface of poly-L lysine coated slides. Before application, this solution was heated for 30 minutes at room temperature. After application of blocking solution for 1 hour, slides were washed with 0.1 X SSC for 15 seconds and DEPC treated water for 15 seconds in coplin jar. After washing, slides were dried by using a spinner. Blocking decreased the background noise to take signals effectively.

2.2.1.5 Hybridization

Hybridization solution was prepared by mixing specified concentrations of adapter and signal probe in 0.6 X SSC, 0.5% SDS, 2% BSA, and DEPC treated water. After preparation, this solution was heated at 45^0C in incubator for 30 minutes. Hybridization solution was applied to the slide and then, covered with a cover slip after incubation of solution. Slides were incubated at 45^0C for 30 minutes in humidifying chambers in a dark incubator. After 30 minutes, post hybridization washing was performed. Before washing, post hybridization buffers were preheated at hybridization temperature. Slides were placed in a coplin jar containing DEPC treated water for the removal of coverslips from the slides. After removal of coverslips from slides, post hybridization washing was done with a mixture consisting of 5X SSC and 0.1% SDS for 10 minutes, 1X SSC for 5 minutes, and 2 times with DEPC treated water for 2 minutes at 130 rpm by using shaker in a clean

coplin jar. They were dried with the help of spinner at maximum speed. Unless indicated, 5 µM capture probe, 5 µM signal probe, and 5 µM adapters (target molecule) were used in the study.

2.2.2 Determination of Capture Probe Immobilization With heterobifunctional Cross-linkers

In order to obtain more effective hybridization signal, four different cross-linkers were tried by using control probes which were hold thiol modification on 5’ end and 6-FAM signal on 3’ end (5’/5ThioMC6-D/AAA AAA AAA GCA TCT TCA ACG ATG GCC TTT CCT TT/6-FAM/-3’). They were compared according to their signal/noise values.

2.2.3 Determination of Oligonucleotide Type

In array platforms, selectivity and probe-adapter hybrid stability had very great importance. For this reason in this study, locked nucleic acid and 2’O-Methyl modified oligonucleotides which had advantages in terms of selectivity and thermal resistance were tested besides unmodified oligonucleotides within a separate set of experiments and the performance of platform was tried.

2.2.4 Capture Probe Optimization

After determination of heterobifunctional cross-linker, different capture probe (P1) concentrations were proven for finding of the most optimized capture probe concentration. Four different concentration values like 1, 10, 20, and 40 µM, immobilized on to Poly-L lysine coated slides with heterobifunctional cross-linker, were tested by keeping signal probe (P2) and adapter (A) concentrations constant as 40 µM.

2.2.5 Determination of Signal Probes

The sandwich hybridization system, tried to set, was very flexible in the sense of signals. In this study, signal probe was marked with two different markers such as FAM and Biotin.

2.2.6 Signal Probe Optimization

After identification of P1 probe concentration, different P2 concentrations were tested for sandwich system and attempted to determine the optimal concentration of P2. To this end, three different concentrations such as 20, 30, and 40 µM were tried by keeping capture probe (P2) as 20 µM and adapter (A) concentration as 40 µM.

2.2.7 Hybridization Conditions Optimization

In order to optimize hybridization conditions, different hybridization durations, temperatures, and washing conditions were applied to the system. Hybridization duration was very important parameter for the determination of sandwich system. Therefore, different durations such as 30 minutes, 1, 1.5, 5, and 18 hours parameters were tried for the hybridization incubation. Temperature affected the dynamics of hybridization dynamic, the pivotal factor. Four different hybridization temperatures like 30, 35, 40, 45 ^{0}C were chosen in terms of Tm values of the sequences. For the prevention of nonspecific bindings and reduction of background, washing conditions were very important. Optimal conditions for washing were worked to determine by trying out different solution concentrations, durations, and agitation speeds. Capture and signal probes and adapter concentrations were kept constant as 20 µM, $SM(PEG)_2$ like heterobifunctional and oligonucleotides marked with FAM were used for all hybridization optimization experiments.

2.2.8 Determination of Sensitivity

Minimum adapter concentration, the sensitivity of platform can be detected by developed miRNA array platform, was worked to detect in the experiments done with target miRNA sequences, had different concentrations, including 0.01, 0.1, 1, 10, and 20 µM. Capture and signal probe concentrations were kept constant as 20 µM, $SM(PEG)_2$ as heterobifunctional cross-linker and FAM marked signal probes were used in the sensitivity studies.

2.2.9 Total RNA isolation

In the final stage of study, in order to obtain RNA isolation, firstly, MCF10A, MCF12A, MCF7, and MDA-MB-231 cell lines were grown in mediums shown in Table 2.2.

Table 2.2 The mediums used in growing cell lines

Cell Line	Property	Medium
MCF 10A	Immortal , non-tumorigenic	DMEM/ Ham's F12, 20 ng/ml EGF, 0.5 ug/ml Hydrocortisone, 0.1 ug/ml Choleratoxin, 10 ug/ml insulin, %1 pen-strep, %5 horse serum
MCF12A	Immortal , non-tumorigenic	DMEM/Ham's F12, %10 FBS, 20 ng/ml EGF, 500 ng/ml Hydrocortisone, 0.01 mg/ml insulin, %1 non-essential aminoacid, %1 pen-strep
MCF7	Tumorigenic, metastasis negative	DMEM, %10 FBS ve %1 pen-strep
MDA-MB-231	Tumorigenic, metastasis positive	DMEM, %10 FBS ve %1 pen-strep

After the preparation of mediums given in Table 2.2, all cell lines were grown as monolayers and were incubated at 37°C with 95% humidified air and 5% CO2 and the cell lines were transfered into a T25 flasks involving mediums for overnight. After incubation, the cell lines were washed with Hanks' salts solution till reaching full confluency. Then, the cell lines were washed with Hanks' buffer and after washing, the washed cells were incubated in the solution involving trypsin/EDTA until all cells stand from the flasks with 5% CO2 at 37°C. The obtained cells were divided into two and cultured in fresh media. Every 2-3 days, the old medium was replaced with new medium.

When cell lines were confluent approximately 70%, trizol was added onto the grown cell lines in flasks and these were incubated at room temperature to allow the dissociation of nucleoprotein complexes completely; consequently, the cells were homogenized. After that, the phase separation process was applied that the chloroform was added into tubes and centrifugation was performed. The RNA remaining in the aqueous phase was transfered to a clean tube and incubated with isopropyl alcohol to precipitate the RNA. After that, the RNA pellet was washed with 70% ethanol and centrifuged again. After centrifugation, samples were dried and dissolved in RNase free water. These were incubated at 55-60°C for 10 minutes and these were stored at -20°C. After isolation, total RNA amounts were measured with NANODROP.

2.2.10 DNase Application

For DNase application, DNase was added onto RNA samples and incubated for 30 minutes at 37°C. After incubation, EDTA was added and incubated for 10 minutes at 65°C. After DNase application, RNA samples were measured with NANODROP. After this process, PCR was carried out for samples by using GAPDH primers. The conditions used in PCR were shown in Table 2.3 and 2.4.

Table 2.3 GAPDH PCR mix

10X PCR Buffer	1X	3 µl
MgCl2 (25 mM)	2 mM	2.4 µl
dNTP (2.5 mM)	0.25mM	3 µl
GAPDH F (5µM)	0.5 µM	3 µl
GAPDH R (5µM)	0.5 µM	3 µl
Template		1 µl
Taq polymerase (5U/µl)	0.04U/ µl	0.25 µl
dH2O		14.35 µl
Total		30 µl

Table 2.4 GAPDH PCR Conditions

Temperature	Duration	Cycle
95 ^{0}C	2 min	
95 ^{0}C	30 sec	35
56 ^{0}C	30 sec	
72 ^{0}C	30 sec	
72 ^{0}C	10 min	

The products obtained from PCR were loaded onto gel and scanned.

2.2.11 cDNA Analysis

cDNA samples were prepared via reverse transcription method by using obtained total RNA samples. In order to realize cDNA analysis, the conditions were used in Table 2.5. The microtubes that were prepared for each miRNA were hold on ice for 5 minutes and then programme was carried out that samples were waited at 16^{0}C for 30 minutes, 42^{0}C for 30 minutes, and 85^{0}C for 5 minutes. After this application, microtubes were hold on ice for 1-2 minutes and centrifugated. These samples were stored at -20^{0}C for use in further studies.

Table 2.5 The conditions used for reverse transcription analysis

Chemicals	%	
10X RT, buffer	1x	1.5 µl
dNTP (100mM)	1 mM	0.15 µl
RT enzyme (50µl/ml)	1µl/ml	1 µl
Rnase inhibitor		0.19 µl
Primer	5 µM	3 µl
Total RNA	100 ng	5 µl
dH2O		4.16 µl
Total		15 µl

2.2.12 RT-PCR

The existence of miRNA in different cell lines was confirmed with RT-PCR analysis and Taqman probes were used in these studies. The total RNA samples which were commercially available and obtained from normal tissues were utilized for calibration. Three replicates were prepared for each type cell line, U6 was used as reference material and the conditions used in Table 2.6.

Table 2.6 The conditions used in RT-PCR

Taqman universal mix	5 µl
20x taqman	1X
cDNA	0.665µl
dH_2O	3.835 µl
Total	10 µl

2.2.13 Slide Scanning

In the first period of study, signals were taken by scanning slides using confocal laser microscope. Signals were recorded as 1024x1024 resolution and 12 bit by stimulating slides with 488 nm argon lazer and using 4X magnification lenses and

long-pass LP505 filter set. In the rest of the study, microarray scanner system called as Axon Genepix 4100A was used for the analysis of slides.

CHAPTER 3

RESULTS AND DISCUSSION

In this study, array platform based on sandwich hybridization, was aimed to develop an alternative to existing tools to detect miRNAs, including glass surface, capture probe, and signal probe with fluorescein. In this method, the capture probe (P1), specific to one part of the target molecule, was fixed to the solid surface with the help of cross-linkers and target molecule bound to the capture probe and the existence of target molecule was defined with the binding of signal probe (P2), as shown in Figure 3.1. The platform was optimized according to heterobifunctional cross-linkers for the surface activation, surface blocking, oligonucleotide types, capture probe immobilization, and hybridization conditions. In addition to these, probes concentrations, the selectivity and sensitivity of platform were optimized.

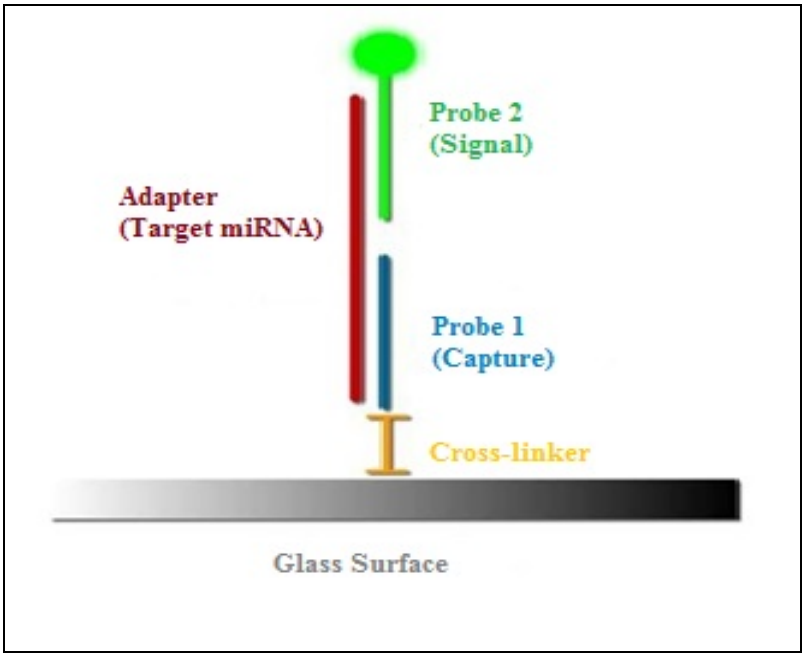

Figure 3.1 Schematic presentation of sandwich array platform-Sandwich platform is designed partly conjugated target sequence. Probe 1 is immobilized to surface with the help of cross-linker and probe 2 is bound to adapter and target is detected by labeled probe.

The most appropriate sequences for probes used in study such as P1 and P2 were determined by using software used in molecular biology studies. These sequences were tested according to hybridization which was targeted between P1 and P2 with adapter, unwanted hybridization between P1 and P2, and secondary structure, and hairpin formation.

In consequence of this analysis, it is emerged that there was not any problem arising from sequences from the point of targeted hybridization, unwanted hybridization, secondary structure, and hairpin formation.

In this study, three basic stages were targeted for completion in order to develop miRNA platform which was DNA-based. These stages were the development of sandwich hybridization based microRNA array platform, study of array platform

with different stages, and investigation of the workability of platform with total RNA samples.

3.1 Development of Sandwich Hybridization Based miRNA Platform

In the first part of study, the oligonucleotide probe was fixed to glass slides, coated with amino-functional groups by using poly-L-lysine, with the help of cross-linker from thiol-modified side and the sandwich formed between target miRNA which was hold on the surface by this probe and signal probe which was half-conjugated to target miRNA and this format was optimized.

3.1.1 The Determination of Cross-linker

The immobilization step is very important for the formation of hybridization and has many potential applications as microarrays, biosensors, chips production (Sun et al., 2006). The immobilization of molecules to surface by using cross-linkers is very simple and effective way. The first use of them for immobilization was done by Chrisey and coworkers. The cross-linkers are used for two purposes such as binding two distinct chemicals covalently that otherwise remain unreactive toward each other and being physical spacer which provides greater accessibility (Chrisey, et al., 1996).

In this study, heterobifunctional cross-linkers, modified with two hetero reactive groups, were used to bind thiol modified oligonucleotides to amino groups on slides covalently. The covalent bonding provides a good stability by binding probe from its thiolated end and the hybridization efficiency becomes better. The ester group (NHS) of cross-linkers gave a reaction with primary amines at pH 7-9 and amide bond was formed while the maleimide group gave a reaction with sulfhydryl groups at pH 6.5-7.5 and stable thioether bond was formed as a result of this reaction. In this study, heterobifunctional cross-linkers which had different lengths were tried. The chemical structures of cross-linkers were given in Figure 3.2.

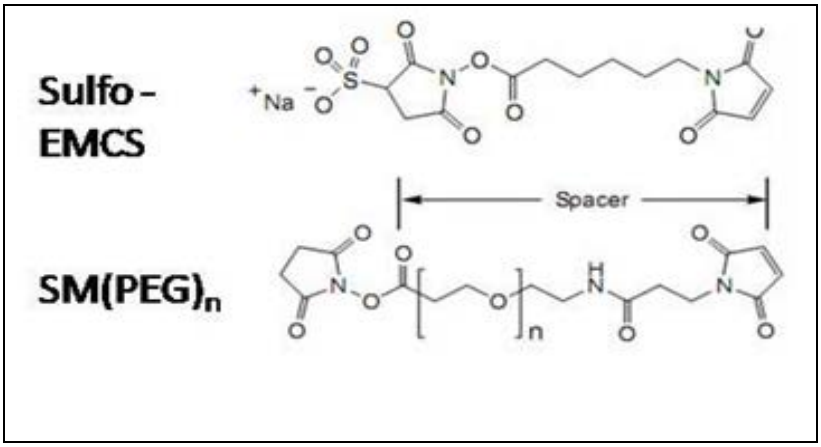

Figure 3.2 The chemical structures of heterobifunctional cross-linkers such as Sulfo-EMCS and SM(PEG)$_n$.

The activity of cross-linkers was tested by using control probes (5'-/5ThioMC6-D/AAA AAA AAA GCA TCT TCA ACG ATG GCC TTT CCT TT/6-FAM/3') which carries thiol modification on 5' side and 6-FAM signal on 3' side.

In order to test cross-linkers, control probe and heterobifunctional cross-linkers were conjugated and then, this conjugated mixture was applied onto the glass slide. In this study, four different heterobifunctional cross-linkers such as SM(PEG)$_2$, SM(PEG)$_6$, SM(PEG)$_{12}$, and Sulfo-EMCS were utilized (Figure 3.3).

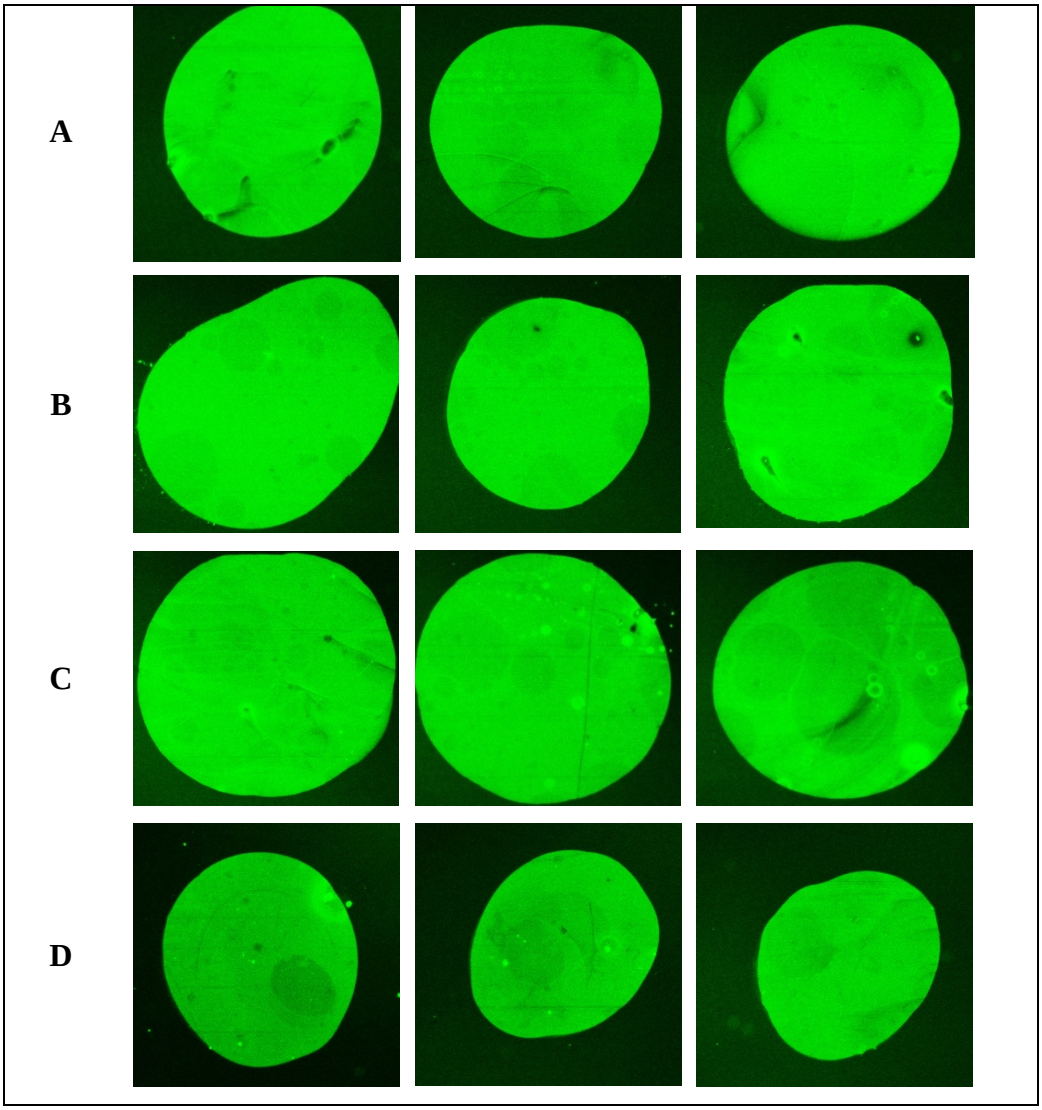

Figure 3.3 Confocal laser scanning results of sandwich hybridization assay performed with different cross-linkers. **A.** Three spots of sandwich hybridization assay performed with $SM(PEG)_2$. **B.** Three spots of sandwich hybridization assay performed with $SM(PEG)_6$. **C.** Three spots of sandwich hybridization assay performed with $SM(PEG)_{12}$. **D.** Three spots of sandwich hybridization assay performed with Sulfo-EMCS.

Following washing steps, in order to observe the efficiency of surface binding of cross-linkers, fluorescent signals were detected by laser scanner. Although positive results were obtained from all cross linkers, we observed that $SM(PEG)_n$ gave better results than Sulfo-EMCS. Furthermore, it was observed that in $SM(PEG)_n$ cross-

linkers of different lenghts, $SM(PEG)_2$ gave the best results and $SM(PEG)_2$ was used in platform.

3.1.2 Testing miRNA Analysis Platform in Nucleic Acid-Based Sandwich Format with DNA Adapters

First, the optimized conditions were tried with DNA probes because the optimization of miRNAs is more difficult. After the determination of optimized conditions, the experiments were conducted with miRNAs.

Probe 1, probe 2, adapter, and Ank sequences were used to test the sequence analysis platform in nucleic acid-based sandwich format and the conditions of this platform, these sequences shown in Table 3.1.

Table 3.1 The Probes and Adapter Sequences for test of the sandwich system

P/A	Sequences
P1	5'-/5ThioMC6-D/AAA AAA AAA GCA TCT TCA ACG ATG GCC TTT CCT TT -3'
P2	5'-TCG CAA TGA TGG CAT TTG TAG GAG CAA AAA AAA A/6-FAM/ -3'
Ank	ACC CTG TAA ACG ATC ATC CCC ATT TTT TAC GGC CAATTG GAG GCCTCC CAAT -3'
A	5'- GCT CCT ACA AAT GCC ATC ATT GCG ATA AAG GAA AGG CCA TCG TTG AAG ATG C -3'

In this study, P2 and A concentrations were kept constant as 20 µM and parameters of 1, 5, and 10 µM were tried for the P1 concentration (Figure 3.4).

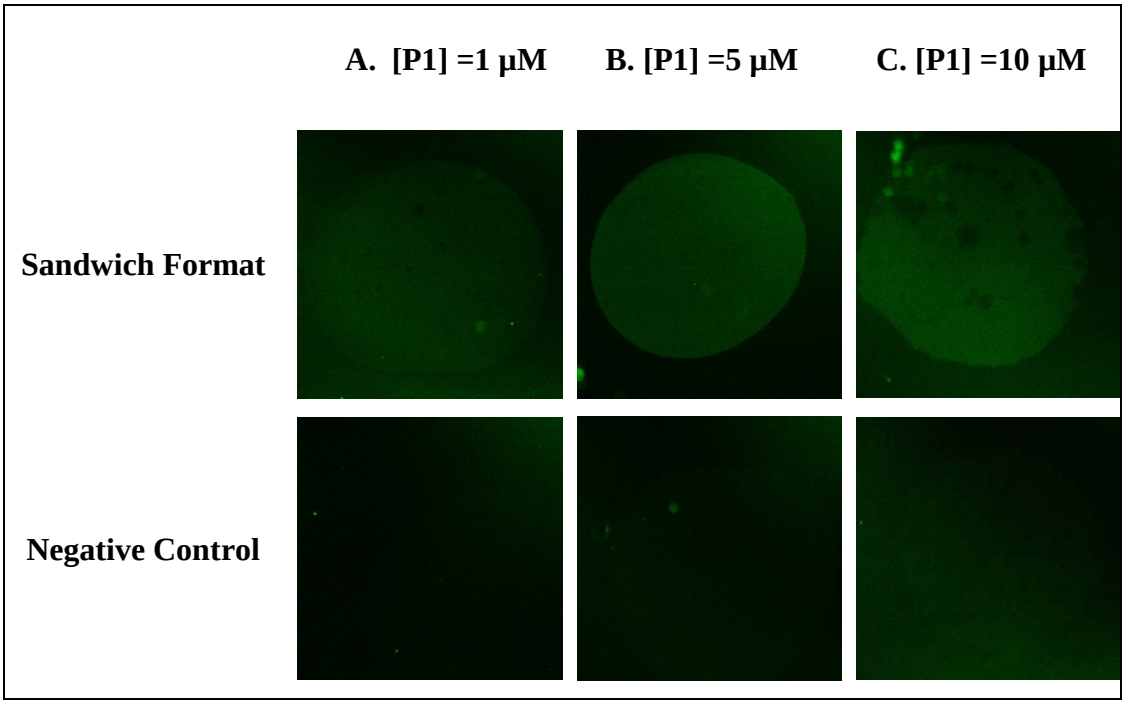

Figure 3.4 Confocal laser scanning results of sandwich hybridization assay performed with different probe 1 concentrations. **A.** P1 concentration equal to 1 µM and its negative control. **B.** P1 concentration equal to 5 µM and its negative control. **C.** P1 concentration equal to 10 µM and its negative control.

As a result of this study, it can be observed that the targeted platform in DNA-DNA format worked smoothly.

3.1.3 Optimization of Blocking and Hybridization Conditions

Before the target adapter hybridization, blocking of surface after the immobilization of the capture probe was occurred. It was important to prevent non-specific nucleic acid-surface interactions by closing the exposed reactive amino groups in order to obtain clean background in microarray studies and low background increases the hybridization sensitivity (Jayaraman et al., 2006). Consequently, two different

blocking solutions were used in this study: Blocking Solution I was consist of 5X SSC, 0.1% SDS, and 1% BSA and Blocking Solution 2 II was consist of 2% BSA.

Hybridization solution was one of the important factors affecting hybridization dynamic. In this study, two different hybridization solutions such as hybridization solution I including 5X SSC, 0.1% SDS, and 50% formamide and hybridization solution II including 0.6X SSC, 0.5% SDS, and 2% BSA were tested. The other conditions used in study to determine blocking and hybridization solutions shown in Table 3.2.

Table 3.2 The Conditions used in blocking and hybridization solutions.

Probe	Adapter	Mark	Cross Linker	Probe and adapter concentration	Hybridization Temperature	Hybridization Duration
DNA	miR21	FAM	$SM(PEG)_2$	P1 = 20 µM P2 = 20 µM A = 20 µM	30 ^{0}C	18 hours

In order to optimize blocking and hybridization conditions, type 1 slide design was used with nine test spots and the expectation from these experiments was nine green spots. As a result of studies, sandwich system with less background and a higher yield was obtained by using blocking solution II (2% BSA) and hybridization solution II (0.6X SSC, 0.5% SDS, and 2% BSA) (Figure 3.5). Therefore, 2% BSA as a blocking solution and the solution, including 0.6X SSC, 0.5% SDS, and 2% BSA, as a hybridization solution were used in other stages of studies.

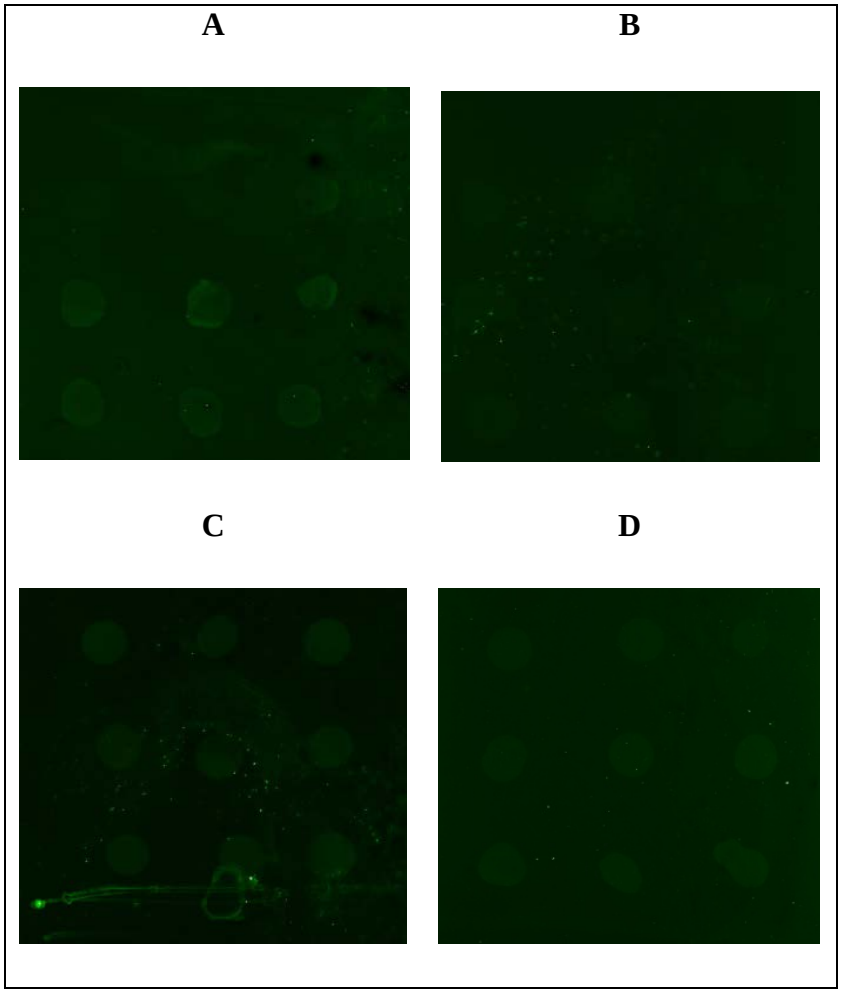

Figure 3.5 Microarray scanner system(Axon Genepix4100A) scanning results of sandwich hybridization assay performed with different blocking and hybridization solutions. **A.** Blocking solution I and Hybridization solution I. **B.** Blocking solution I and Hybridization solution II. **C.** Blocking solution II and Hybridization solution II. **D.** Blocking solution II and Hybridization solution I.

3.1.4 Optimization of Oligonucleotide Type

The selectivity and the stability of formed probe-target hybrid were very important in array platforms. Therefore, locked (LNA-locked nucleic acid) and 2'O-Methyl modified oligonucleotides which had advantages in terms of selectivity and thermal resistance were used to test the performance of the platform besides oligonucleotides

that chemical structures not changed. The other conditions used in study to determine oligonucleotide type shown in Table 3.3.

Table 3.3 The Conditions used in the determination of oligonucleotide type

Probe	Adapter	Mark	Cross Linker	Probe & adapter concent.	Hybridization Temperature	Hybridi. duration
1. DNA 2. LNA 3. 2'O-Methyl	1. miR21 2.miR21-F 3.Ank	FAM	$SM(PEG)_2$	P1 = 20 µM P2 = 20 µM A = 20 µM	30 ^{0}C	18 hours

As will be seen in Figure 3.6, LNA and 2'O-Methyl modified probes gave better results than probes that structures were unsubstituted. According to results, it can be said that oligonucleotide type affected DNA-RNA sandwich system in a pivotal rate; hence, LNA probes were used in the next stage of the study.

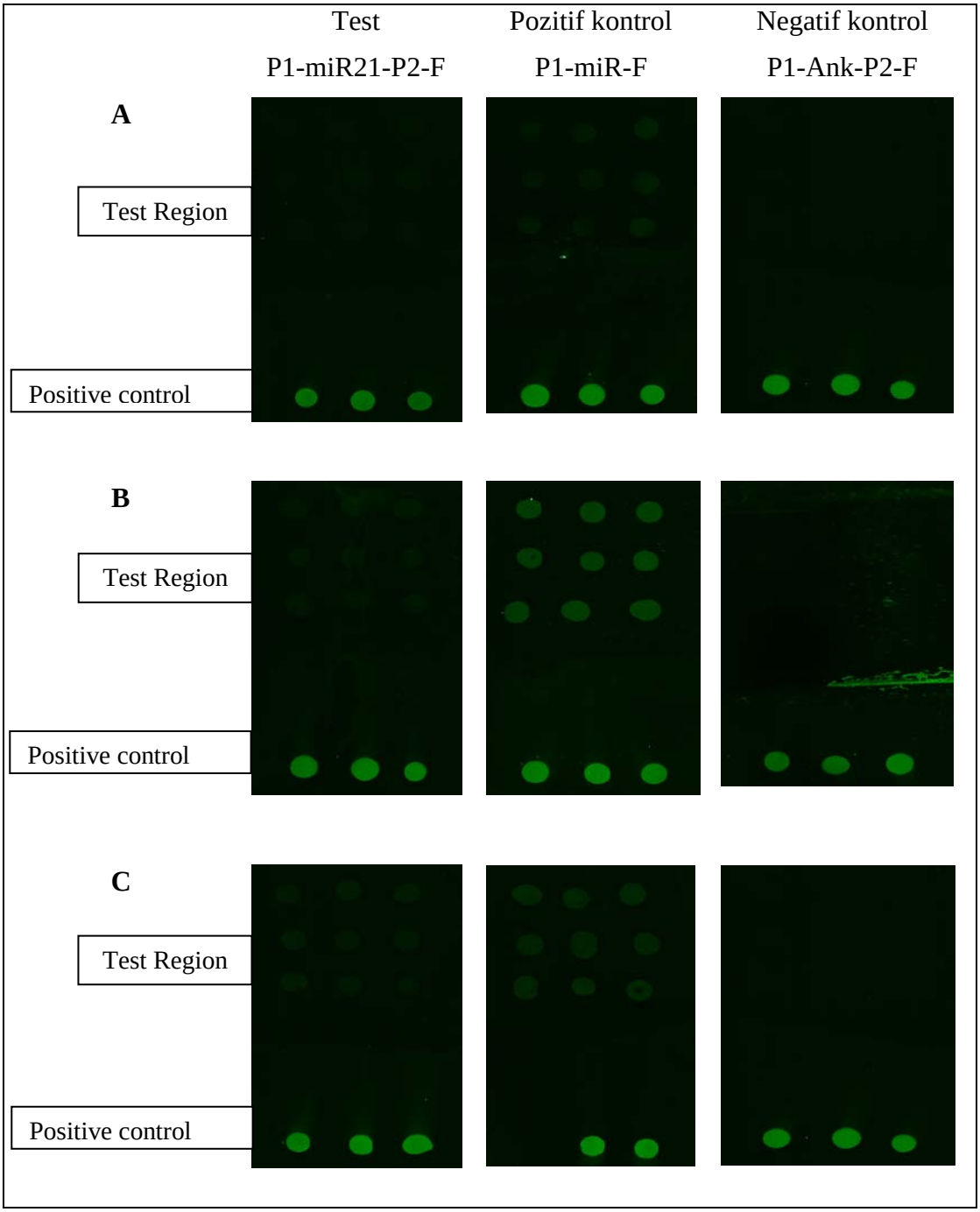

Figure 3.6 Microarray scanner system (Axon Genepix4100A) scanning results of sandwich hybridization assay performed with different oligonucleotide types. **A.** Probes with unsubstitutes chemical structure. **B.** LNA modified probes. **C.** 2'O-Methyl modified probes.

3.1.5 DNA-DNA and DNA-RNA Sandwich System

In order to determine the DNA-DNA and DNA-RNA sandwich systems difference, experiments were done by using DNA sequence of miR21. Sandwich system was repeated by using this adapter and results were compared with results of experiments which were constructed by using miR21adapters. Experiments were carried out using both unsubstituted chemical structure and LNA modified probes. The other conditions used in study to observe DNA-DNA and DNA-RNA sandwich systems shown in Table 3.4.

Table 3.4 The Conditions used in observation of difference between DNA-DNA and DNA-RNA sandwich systems.

Probe	Adapter	Mark	Cross-Linker	Probe& adapter concent.	Hybridi. Tempera.	Hybridi. Duration
1. DNA 2. LNA	1. miR21 2. miR21-DNA 3.miR21-F 4.Ank	FAM	$SM(PEG)_2$	P1 = 20 µM P2 = 20 µM A = 20 µM	30 ^{0}C	18 hours

The optimization conditions of DNA-RNA sandwich system were found to have to be much more sensitive for both oligonucleotide types according to results of experiments which were applied to DNA-DNA and DNA-RNA sandwich systems. In addition to this, it was observed again that LNA modified probes worked much better than the unmodified probes (Figure 3.7).

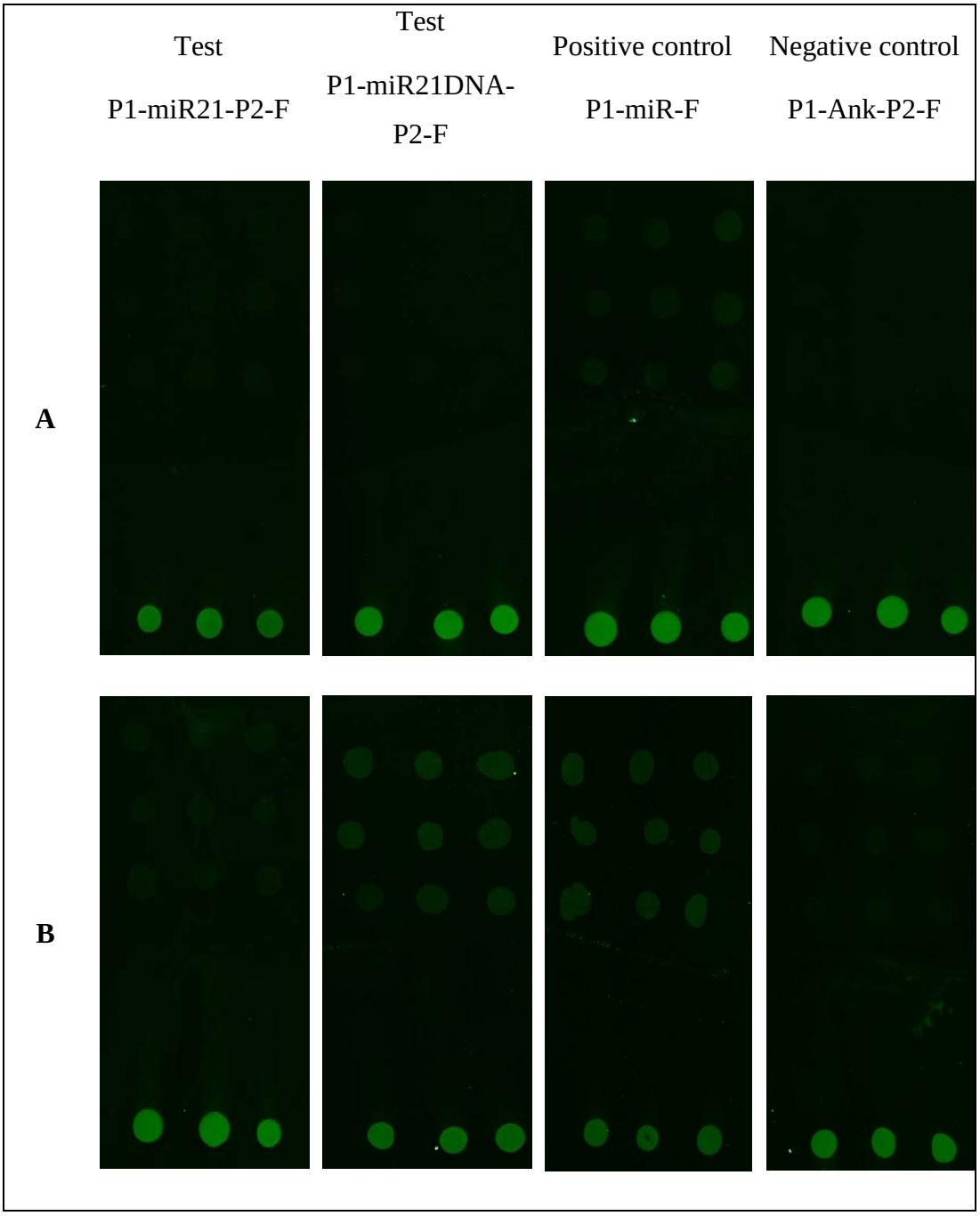

Figure 3.7 Microarray scanner system (Axon Genepix4100A) scanning results of the difference between DNA-RNA and DNA-DNA sandwich hybridization assay performed with different oligonucleotide types. **A.** Probes with unmodified chemical structure. **B.** LNA modified probes.

3.1.6 Optimization of Different Signal Probes

The sandwich system used in this study is a flexible one in terms of signals. In other words, the signal probes can be marked with different markers. In this study, signal probes were marked with two different markers such as FAM and Biotin. The other conditions used in study to test different signal probes shown in Table 3.5.

Table 3.5 The Conditions used in observation of different signal probes.

Probe	Adapter	Mark	Cross-Linker	Probe & adapter concent.	Hybridization Temperature	Hybridization Duration
LNA	1. miR21 2.Ank	1.FAM 2.BIO	$SM(PEG)_2$	P1 = 20 µM P2 = 20 µM A = 20 µM	30 ^{0}C	18 hours

In order to determine the signal probe type, type 1 slide design was used with nine spots. The difference between these two signals which were applied to LNA modified probes, was observed and positive results were found for both signals, seen in Figure 3.8

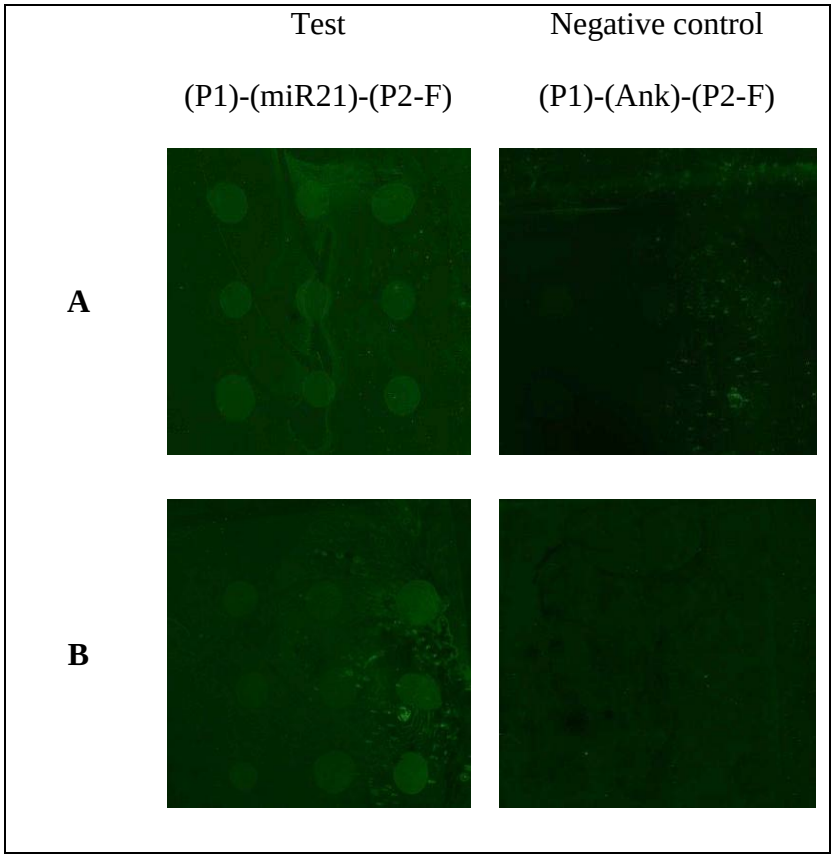

Figure 3.8 Microarray scanner system (Axon Genepix4100A) scanning results of the difference between sandwich hybridization assay performed with different signal probes. **A.** Probes marked with FAM. **B.** Probes marked with Biotin.

It can be said that probes marked with FAM worked much better than probes marked with Biotin and probes marked with FAM were used in the next stages of the study.

3.1.7 Optimization of Capture Probe (Probe 1) Concentration

After the decision on the use of probes which were LNA modified and labelled with FAM, different capture probe (probe 1 (P1)) concentrations were tried primarily for the optimization of sandwich system that is one of the most critical step. The concentration of probe 1 is important factor affecting the hybridization efficiency (Zhou et al., 2004). With an increase in the concentration of probe 1, the captured target on the slide increases. For this purpose, signal probe (probe 2 (P2)) and

adapter concentrations were kept constant as 40 µM and four different values such as 1, 10, 20, and 40 µM were immobilized on to Poly-L lysine coated slides. The other conditions used in study to optimize P1 concentration, shown in Table 3.6.

Table 3.6 The Conditions used in optimization of probe 1 concentration

Probe	Adapter	Mark	Cross-Linker	Probe & adapter concent.	Hybridi. Tempe.	Hybridization Duration
LNA	1. miR21 2.Ank	FAM	$SM(PEG)_2$	P2 = 40 µM A = 40 µM	30 ^{0}C	18 hours

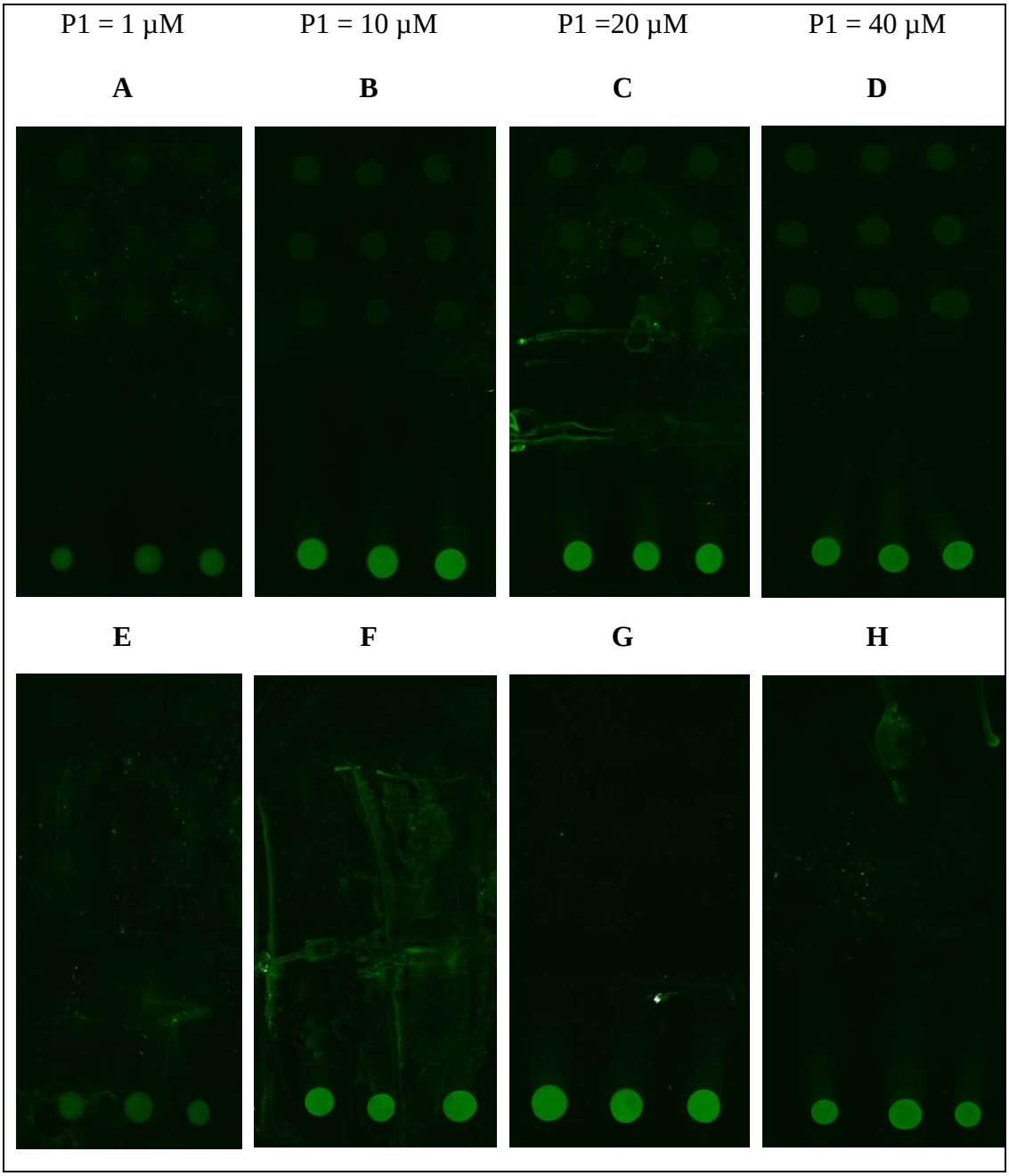

Figure 3.9 Microarray scanner system (Axon Genepix4100A) scanning results of the different probe 1 concentrations. **A.** P1 concentration: 1 µM. **B.** P1 concentration: 10 µM. **C.** P1 concentration: 20 µM. **D.** P1 concentration: 40 µM. **E.** 1 µM negative control. **F.** 10 µM negative control. **G.** 20 µM negative control. **H.** 40 µM negative control.

As can be seen from the Figure 3.9, spots were not very clear at 1 µM concentration which was the lowest concentration tried. Besides that, it can be said that the clearness of spots increased with increasing concentrations. On the other hand, not huge difference was seen between 20 and 40 µM concentrations. Besides that, so increasing the amount of probe 1 density decreases the hybridization efficiency because of steric factors. As a result of this, 20 µM was selected as standard for the concentration of P1 in the continuation of work.

3.1.8 Optimization of Signal Probe (Probe 2) Concentration

The density of the signal probe is another important factor for the formation of hybridization and signal. After the determination of P1 concentration, different signal probe (probe 2 (P2)) concentrations were used and optimal P2 concentration was attempted to determine. The concentration of signal probe is very important to specify the strength of acquired signal. For this purpose, different concentrations as 20, 30, and 40 µM were tested. The other conditions, utilized in the study, were shown in Table 3.7

Table 3.7 The Conditions used in optimization of probe 2 concentrations

Probe	Adapter	Mark	Cross-Linker	Probe & adapter concent.	Hybridization Temperature	Hybridization Duration
LNA	1. miR21 2.Ank	FAM	$SM(PEG)_2$	P1 = 20 µM A = 40 µM	30 ^{0}C	18 hours

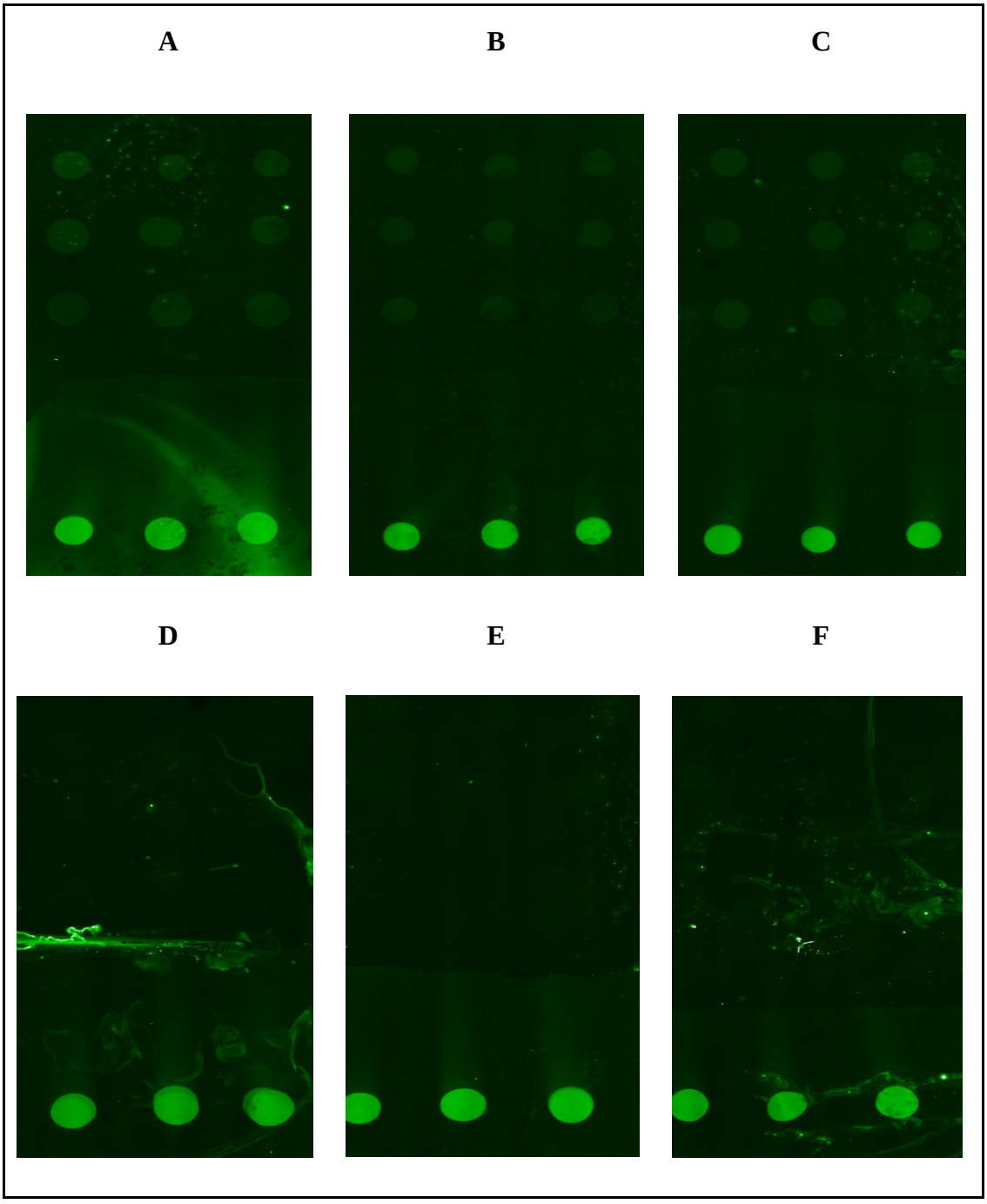

Figure 3.10 Microarray scanner system (Axon Genepix4100A) scanning results of the different probe 2 concentrations. **A.** P2 concentration: 20 µM. **B.** P2 concentration: 30 µM. **C.** P2 concentration: 40 µM. **D.** 20 µM negative control. **E.** 30 µM negative control. **F.** 40 µM negative control.

As can be seen from the Figure 3.10 , the hybridization signals were not very different from each other, in other words, an increase in the P2 concentration do not increase the efficiency of these spots,; therefore, 20 µM was used as P2 concentration in the further steps of the study.

3.1.9 Optimization of Hybridization Duration

The other important parameter is the duration of hybridization. Hybridization duration was also an important parameter in sandwich hybridization system. Hence, different incubation durations were tested: 30 minutes, 1 hour, 1.5 hour, 5 hour, and 18 hour. Other conditions used in testing, shown in Table 3.8.

Table 3.8 The Conditions used in optimization of hybridization time.

Probe	Adapter	Mark	Cross- Linker	Probe & adapter concentration	Hybridization Temperature
LNA	1. miR21 2.Ank	FAM	$SM(PEG)_2$	P1 = 20 µM P2 = 20 µM A = 20 µM	30 ^{0}C

No differences were observed among hybridization times such as 30 minutes, 1 hour, and 1.5 hour. Furthermore, the efficiency of sandwich system decreased and weaker signal was taken when hybridization time was extended to 18 hour, shown in Figure 3.11. The reason for this may become the degradation of fluorescent under extended duration. It was shown that longer hybridization could generate nonspecific background (Zhen Guo, 2002). The extended hybridization duration reduced the signal as seen Figure 3.11 – E.

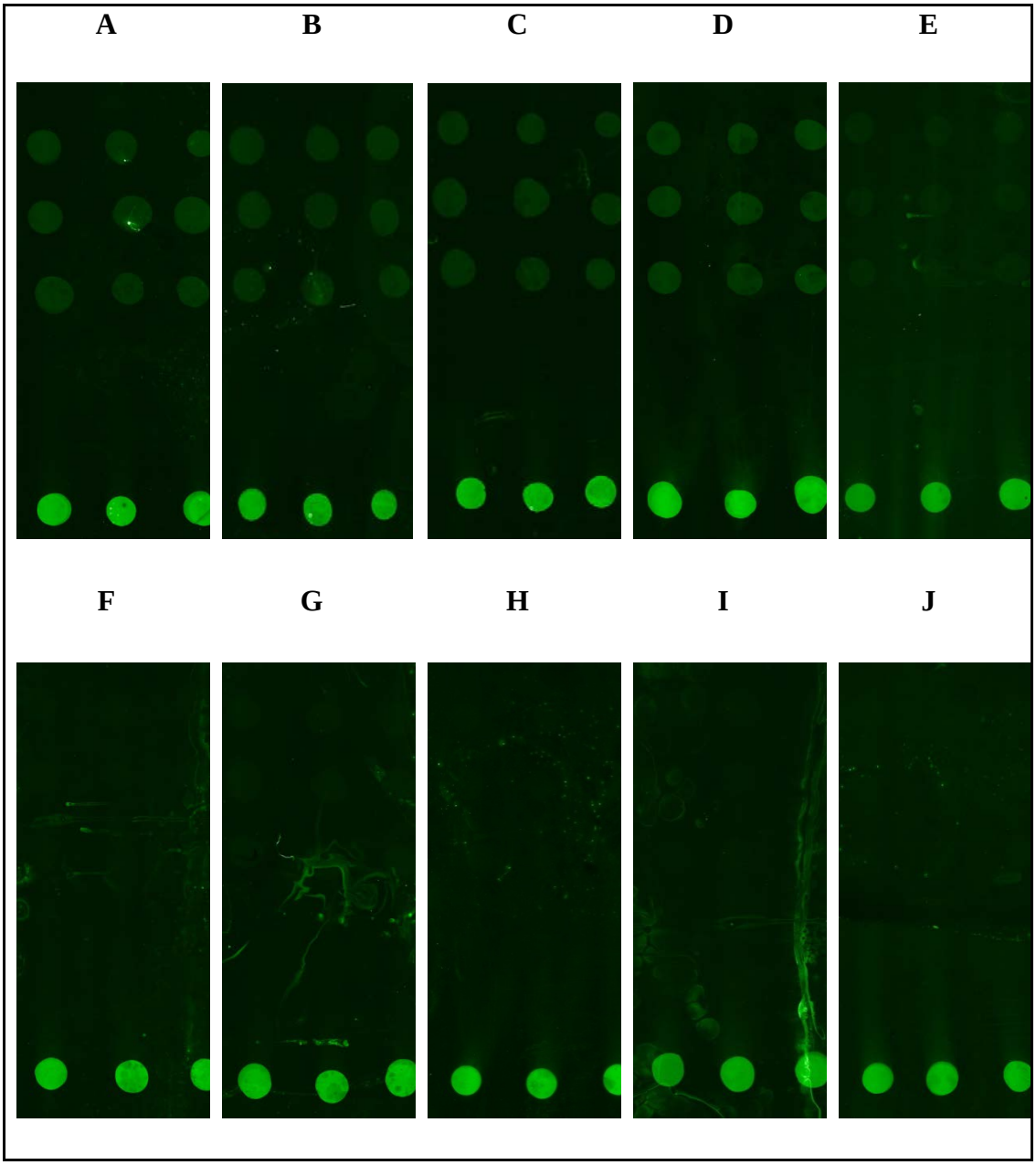

Figure 3.11 Microarray scanner system (Axon Genepix4100A) scanning results of the different hybridization duration. **A.** Hybridization time: 30 minutes. **B.** Hybridization time: 1 hour. **C.** Hybridization time: 1.5 hour. **D.** Hybridization time: 5 hour. **E.** Hybridization time: 18 hour. **F.** 30 minutes, negative control. **G.** 1hour, negative control. **H.** 1.5 hour, negative control. **I.** 5 hour, negative control. **J.** 18 hour, negative control.

In order to increase the yield of the system and shorten the total duration of study, hybridization time was decreased to 30 minutes in the next stages of the study.

3.1.10 Optimization of Hybridization Temperature

Temperature is another parameter that affects hybridization dynamic. Hybridization kinetics of array is temperature-dependent and the specificity and efficiency are based on the hybridization temperature (Tao et al., 2003). The melting temperature of the sequence was determined by the length of the sequence. The target and signal probe were hybridized in tube prior to applying on surface. Hence, it can be said that hybridization temperature was in relation with target and signal probe. The T_m (melt temperature) value of studied environment was 56 ^{0}C. Therefore, different hybridization temperature parameters were carried out to system like, 30 ^{0}C (T_m -25), 35^{0}C (T_m -20), 40^{0}C (T_m -15), and 45^{0}C (T_m -10). Other conditions used in applications, shown in Table 3.9.

Table 3.9 The Conditions used in optimization of hybridization temperature.

Probe	Adapter	Mark	Cross-Linker	Probe & adapter concentration	Hybridization duration
LNA	1. miR21 2.Ank	FAM	$SM(PEG)_2$	P1 = 20 µM P2 = 20 µM A = 20 µM	30 minutes

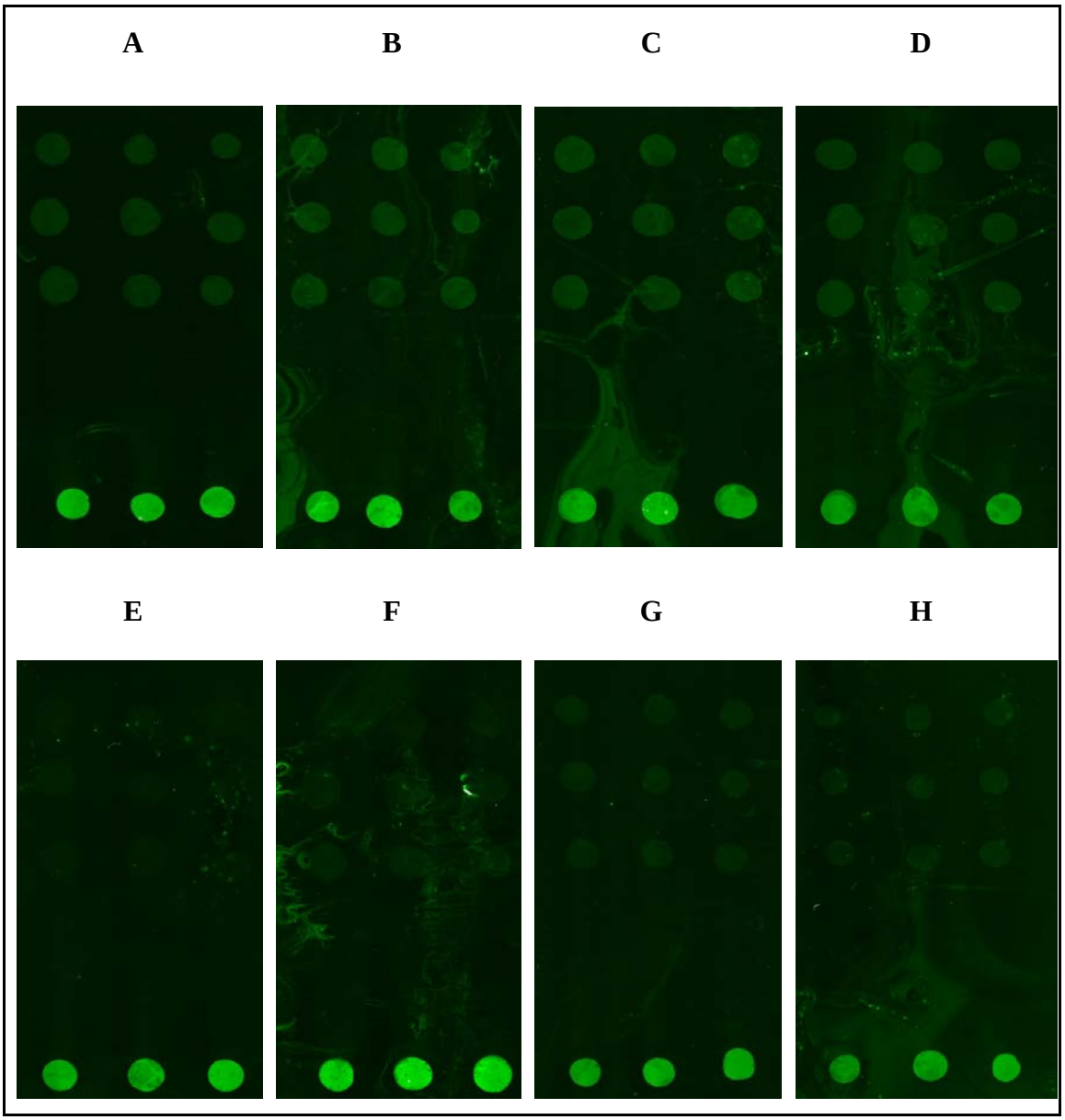

Figure 3.12 Microarray scanner system (Axon Genepix4100A) scanning results of the different hybridization temperatures. **A.** Hybridization temperature: 30^0C, test. **B.** Hybridization temperature: 35^0C, test. **C.** Hybridization temperature: 40^0C, test. **D.** Hybridization temperature: 45^0C, test. **E.** Hybridization temperature: 30^0C, negative control. **F.** Hybridization temperature: 35^0C, negative control. **G.** Hybridization Temperature: 40^0C, negative control. **H.** Hybridization temperature: 45^0C, negative control.

According to results shown in Figure 3.12, it can be said that the binding increased with increasing temperature, in other words, when temperature increased towards Tm value hybridization stringency also increased. However, with an increase in hybridization temperature, the nonspecific bindings increase in negative controls. Hence, 30^0C which had least non-specific bindings was approved as hybridization temperature in next stages of study.

3.1.11 Optimization of Washing Conditions

Washing is another critical step affecting the quality of expression. Insufficient washing results in the unspecific bindings and high background fluorescence and this makes detection of signal impossible (Sipe and Saha, 2007). Therefore, different solution concentration, duration, and agitation speeds were studied and attempted to determine optimum conditions. Washing conditions used in study were listed below.

Washing I:	5XSSC + 0.1% SDS	10 minutes	1 repeat
Washing II:	5XSSC + 0.1% SDS	5 minutes	1 repeat
	1XSSC	5 minutes	1 repeat
	0.1XSSC	5 minutes	1 repeat
	dH_2O	1 minute	3 repeats
Washing III:	1XSSC	5 minutes	1 repeat
	0.1XSSC	5 minutes	1 repeat
	dH_2O	1 minutes	3 repeats

Other conditions used in applications, shown in Table 3.10.

Table 3.10 The Conditions used in optimization of washing conditions.

Probe	Adapter	Mark	Cross-Linker	Probe & adapter concent.	Hybridi. Tempe.	Hybridi. duration
LNA	1. miR21 2.Ank	FAM	$SM(PEG)_2$	P1 = 20 µM P2 = 20 µM A= 20 µM	30 ^{0}C	30 minutes

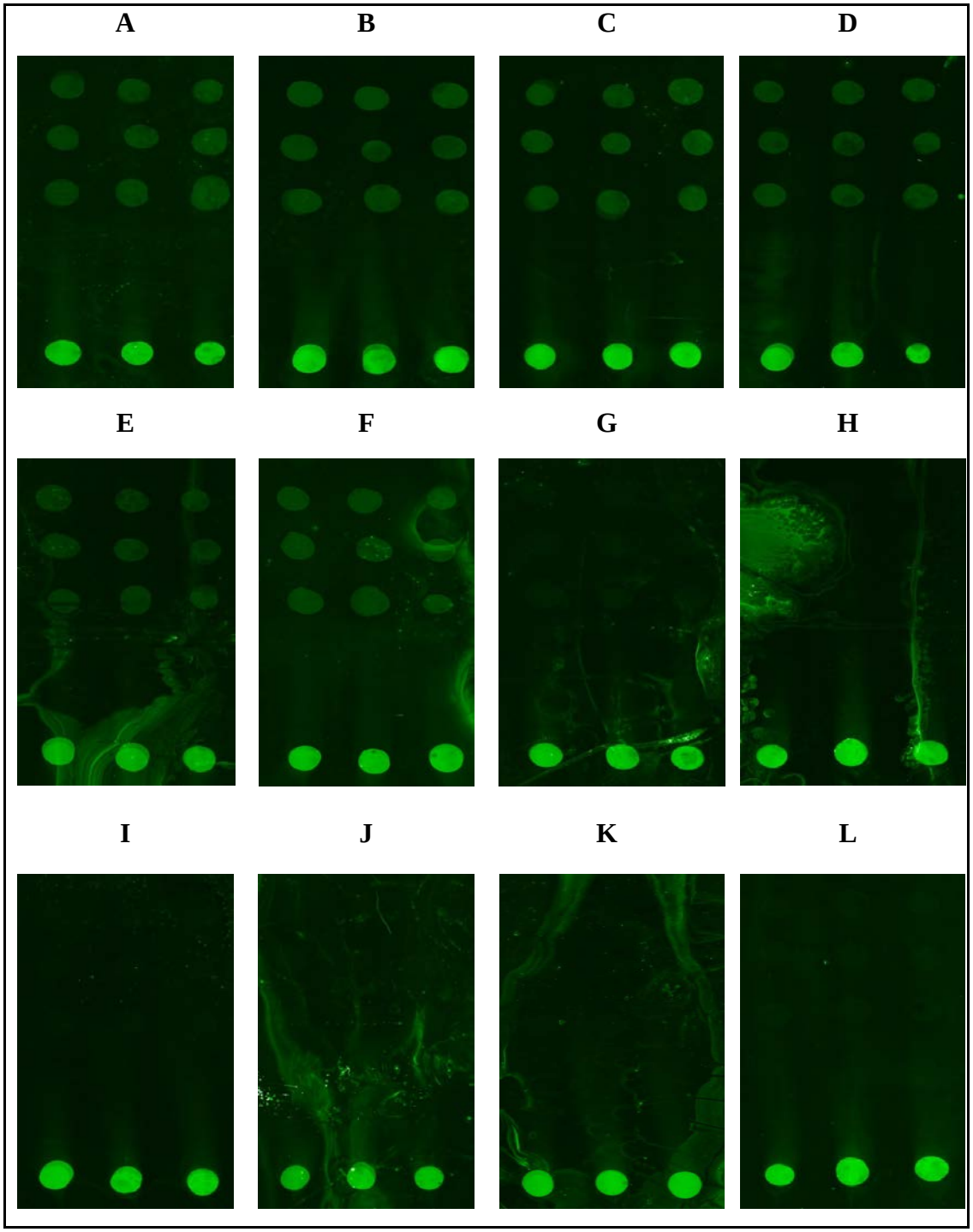

Figure 3.13 Microarray scanner system (Axon Genepix4100A) scanning results of the different washing conditions after hybridization. **A.** Washing I, 100 rpm; test. **B.** Washing II, 100 rpm; test. **C.** Washing III, 100 rpm; test. **D.** Washing I, 130 rpm; test. **E.** Washing II, 130 rpm; test. **F.** Washing III, 130 rpm; test **G.** Washing I, 100

rpm; negative control. **H.** Washing II, 100 rpm; negative control. **I.** Washing III, 100 rpm; negative control. **J.** Washing I, 130 rpm; negative control. **K.** Washing II, 130 rpm; negative control. **L.** Washing III, 130 rpm; negative control.

It is observed that results with low background were taken with washing I at 130 rpm and unspecific binding was also minimized with this washing type (Figure 3.13). Furthermore, this type of washing was preferred in next stages of study because the number of washing steps was diminished with this washing.

3.1.12 Determination of Sensitiveness

After optimization of the platform, the platform sensitivity was searched. Minimum adapter concentration, that is, platform sensitivity, has been able to identify by developed miRNA sequence in studies which were carried out with target miRNA sequence in different concentrations. For this reason, different adapter concentrations such as 0.01, 0.1, 1, 10, and 20 µM were tried. Other conditions used in the determination of sensitivity of platform, shown in Table 3.11 and result in Figure 3.14.

Table 3.11 The Conditions used in determination of sensitivity of platform.

Probe	Adapter	Mark	Cross-Linker	Probe & adapter concent.	Hybridiation Temperature	Hybridi. Duration
LNA	1. miR21 2.Ank	FAM	$SM(PEG)_2$	P1 = 20 µM P2 = 20 µM	30 ^{0}C	30 minutes

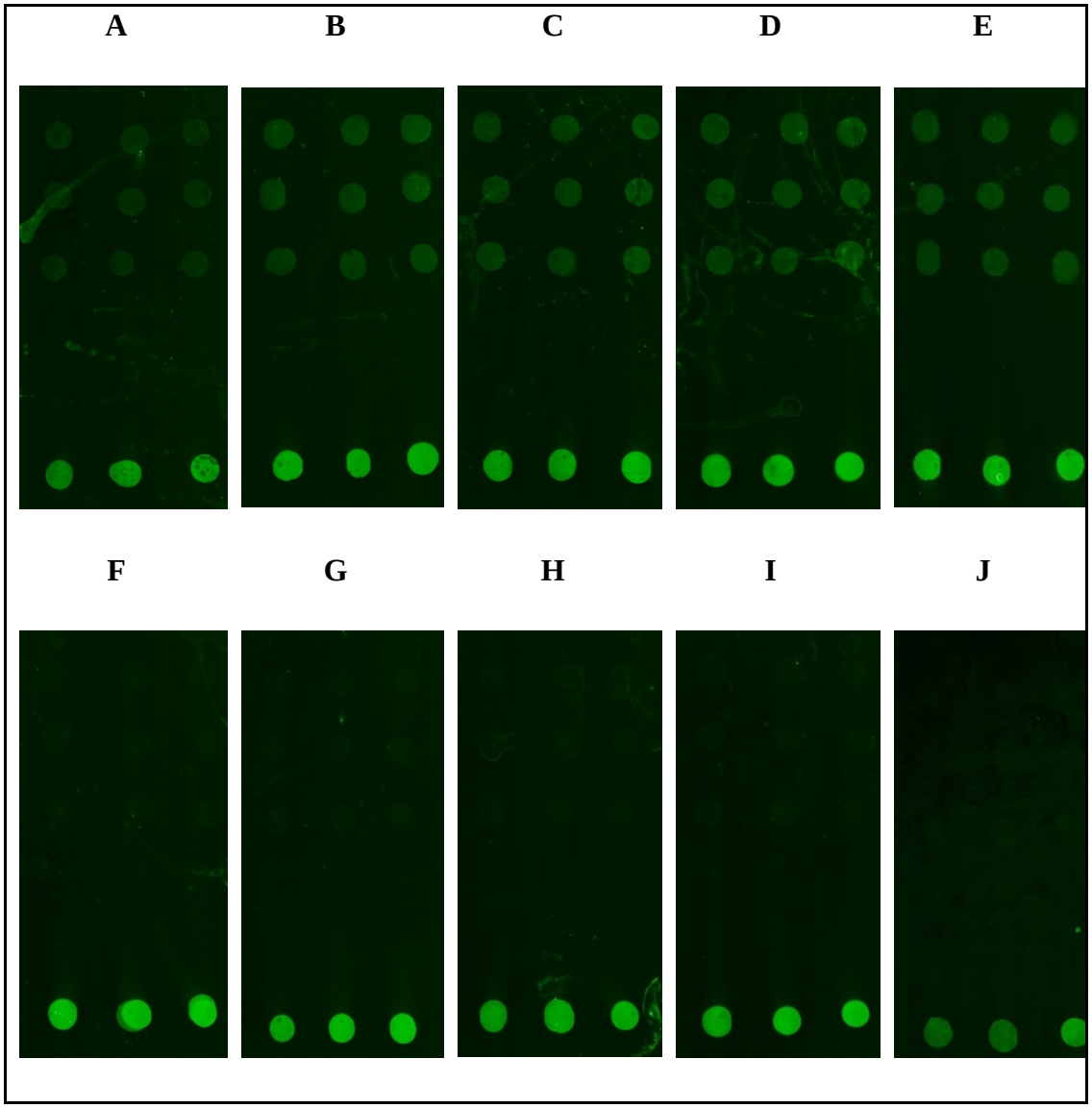

Figure 3.14 Microarray scanner system (Axon Genepix4100A) scanning for platform sensitivity. **A.** 0.01 µM; test. **B.** 0.1 µM; test. **C.** 1 µM; test. **D.** 10 µM; test. **E.** 20 µM; test **F.** 0.01 µM; negative control. **G. 0.**1 µM; negative control. **H.** 1 µM; negative control. **I.** 10 µM; negative control. **J.** 20 µM; negative control.

According to Figure 3.14, developed platform gave a signal even at 0.01 µM concentration. It can be said that the sensitivity of platform was very effective.

3.1.13 Optimized Conditions for miR21

DNA-RNA sandwich system was developed for miR21 adapter and optimization studies were performed in the first stage of the study. The conditions acquired from the first stage of the study, shown in Table 3.12.

Table 3.12 The Optimized conditions for miR 21.

Probe	Mark	Cross-Linker	Probe & adapter concent.	Blocking Solution	Hybridi. Solutio	Hybridi. Temp.	Hyb. Dur.
LNA	FAM	$SM(PEG)_2$	P1;20 µM P2;20 µM A;0.1 µM	2%BSA	0.6X SSC, 0.5% SDS, 2% BSA	30 ^{0}C	30 min.

3.2 Application of Different miRNA Samples to Developed Array Platform

In the second stage of study, the different miRNAs were worked to detect by using array platform. The sensitivity and selectivity of miRNA based platform were studied by selecting miRNAs in different lengths that expressions change when met cancer and miRNAs that become a members of same family and have similar sequences. For this reason, the miRNAs were determined that expressions increased and decreased with breast cancer and the probes were used that were consistent with miRNAs to obtain specific signals when these were applied to system. The hybridization duration, temperature, washing conditions after hybridization were studied to arrive optimized conditions.

3.2.1 miRNA Probe Sets on The Developed Platform

Probe sets, planned to study, were applied to system by using optimized conditions for miR 21, firstly. Conditions used in the study, shown in Table 3.13.

Table 3.13 The Conditions for trying miRNA probe sets.

Probe & adapter concent.	Blocking Solution	Hybridi. Solution	Hybridi. Temperature	Hybridi. Duration	Washing Conditions
P;10 µM P2;10 µM A;10 µM	2%BSA	0.6X SSC, 0.5% SDS, 2% BSA	30 ^{0}C	30 min.	1X / 10 min / 5X SSC + 0.1% SDS

Each probe sets were studied on different slides. Besides this, negative control slide was prepared by constructing sandwich hybridization with P1, P2, and Ank. The slide design used in these studies shown in Figure 2.5 and results were in Figure 3.15.

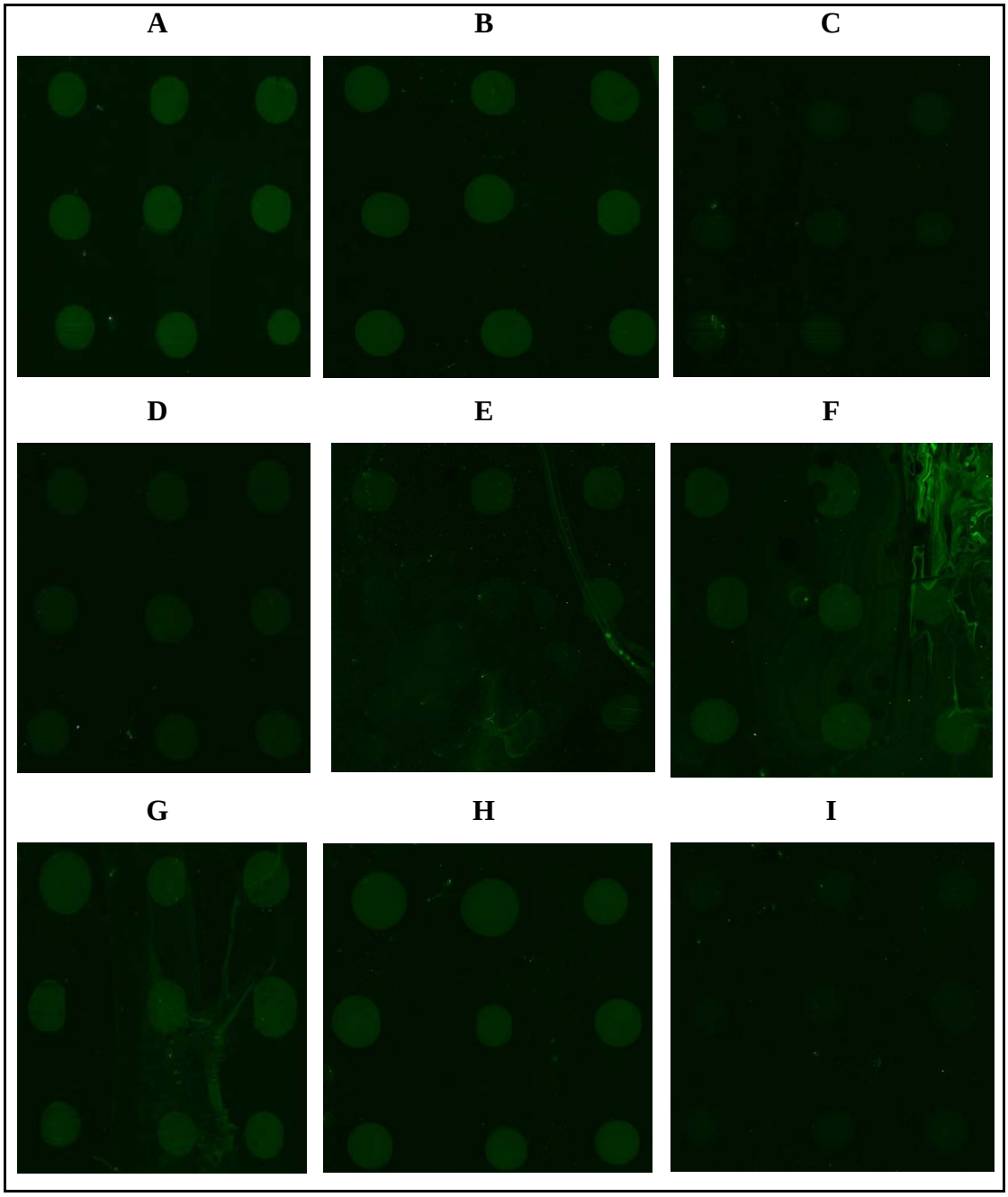

Figure 3.15 Microarray scanner system (Axon Genepix4100A) scanning for sandwich hybridization system done with different miRNA probe sets, by using optimized conditions for miR 21. **A.** miR 21. **B.** miR 10b. **C.** miR 145. **D.** miR 125. **E.** miR 200a. **F.** miR 200b. **G.** miR 200c. **H.** U6. **I.** Negative control.

According to results in Figure 3.15, it can be said that optimized conditions for miR 21 were appropriate for miR 21, miR 10b, miR 200b, and miR 200c but, conditions must be optimized for miR 145, miR 125, and miR 200a. Hence, P1 and P2 concentrations were increased for these probe sets at low efficiency in the rest part of the study.

3.2.2 miRNA Probe Sets on Single Slide

After the optimization of probe sets on slides one by one, experiment was repeated that all probes were applied on a single slide. The slide design was shown in Figure for this purpose. This slide design was used for the next stages of study. Conditions used in the study, shown in Table 3.14 and results in Figure 3.16.

Table 3.14 The Conditions for trying miRNA probe sets on single slide

Probe & adapter concent.	Blocking Solution	Hybridi. Solution	Hybridi. Temperature	Hybridi. Duration	Washing Conditions
P1;10 µM P2;10 µM A;10 µM	2%BSA	0.6X SSC, 0.5% SDS, 2% BSA	30 ^{0}C	30 minutes	1X / 10 min / 5X SSC + 0.1% SDS

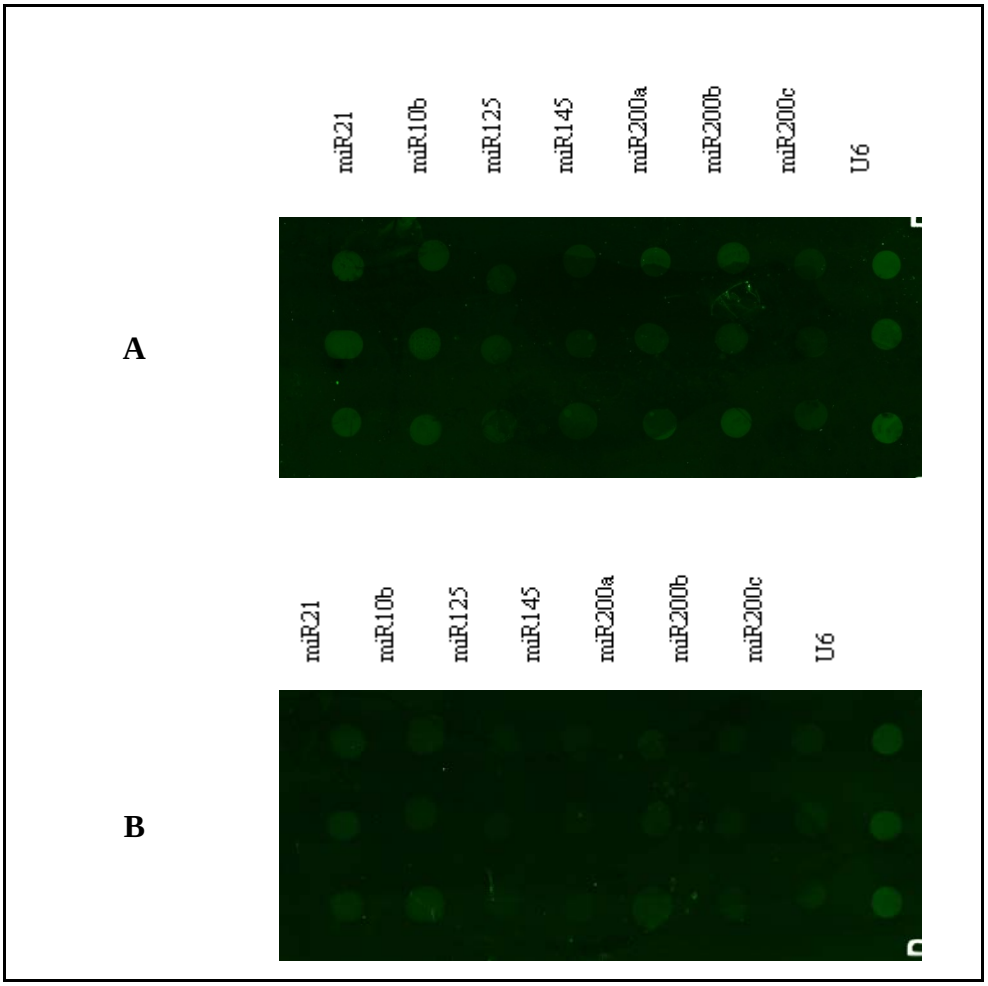

Figure 3.16 Microarray scanner system (Axon Genepix4100A) scanning for sandwich hybridization system done with different miRNA probe sets on single slide, by using optimized conditions for miR 21. **A.** miRNA platform involving 8 probe sets. **B.** Negative control.

Unwanted bindings were observed on slides formed with all probe sets as seen during the study that was done with probe sets one by one.

3.2.3 Optimization of washing conditions

As indicated before, the washing conditions are very important in order to prevent unwanted bindings and decrease background. For this reason, hybridization system was worked to optimize by increase in washing conditions. In this optimization studies, tried washing conditions were given below and other conditions in Table 3.15.

Washing I:	5XSSC + 0.1% SDS	15 minutes	1 repeat
	dH2O	1 minute	3 repeats
Washing 2:	5XSSC + 0.1% SDS	5 minutes	1 repeat
	1XSSC	5 minutes	1 repeat
	0.1XSSC	5 minutes	1 repeat
	dH2O	1 minute	3 repeats

Table 3.15 The Conditions for trying new probe sets

Probe& adapter concent.	Blocking Solution	Hybridization Solution	Hybridization Temperature	Hybridization Duration
P1 = 10 µM P2 = 10 µM A = 10µM	2%BSA	0.6X SSC, 0.5% SDS, 2% BSA	30 ^{0}C	30 minutes

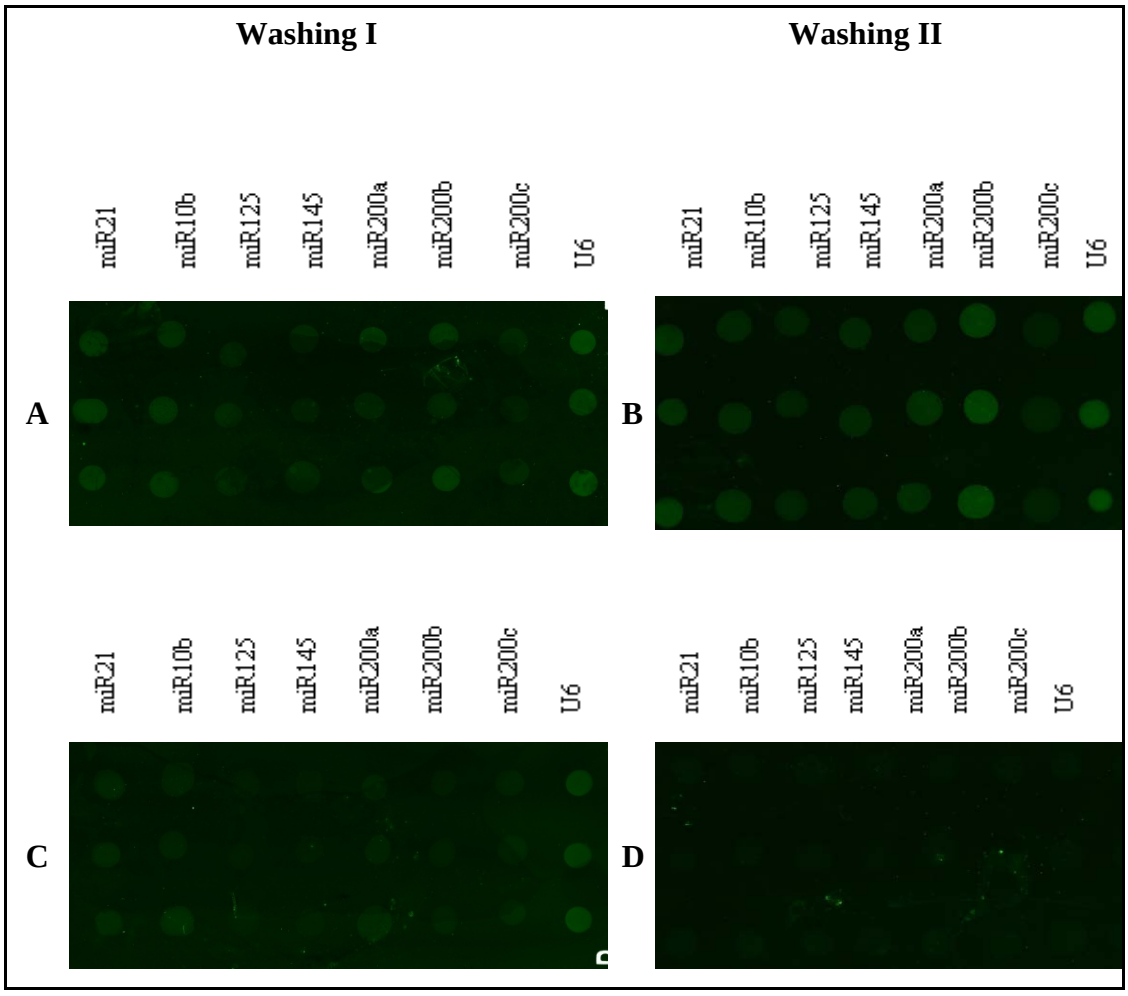

Figure 3.17 Microarray scanner system (Axon Genepix4100A) scanning for sandwich hybridization system done with different washing conditions **A.** miRNA platform with washing I, positive control. **B.** miRNA platform with washing I, negative control. **C.** miRNA platform with washing II, positive control. **D.** miRNA platform with washing II, negative control.

It was observed that when the washing steps increase, the unspecified bindings decrease (Figure 3.17). Consequently, washing II conditions were utilized in the next stages of the study.

3.2.4 The Test of P1-P2 Binding

All probes as P1 and P2 used in study were analyzed with several soft wares and it was determined that the unwanted hybridization between P1 and P2 probes were not exist. On the other hand, the negative control experiment was prepared to test the existence of unwanted hybridization between different probe sets. The capture probes were spotted for one type miRNA for each line in hybridization region as shown in Figure 2.5 and only P2 probes were incubated without the addition of adapters.

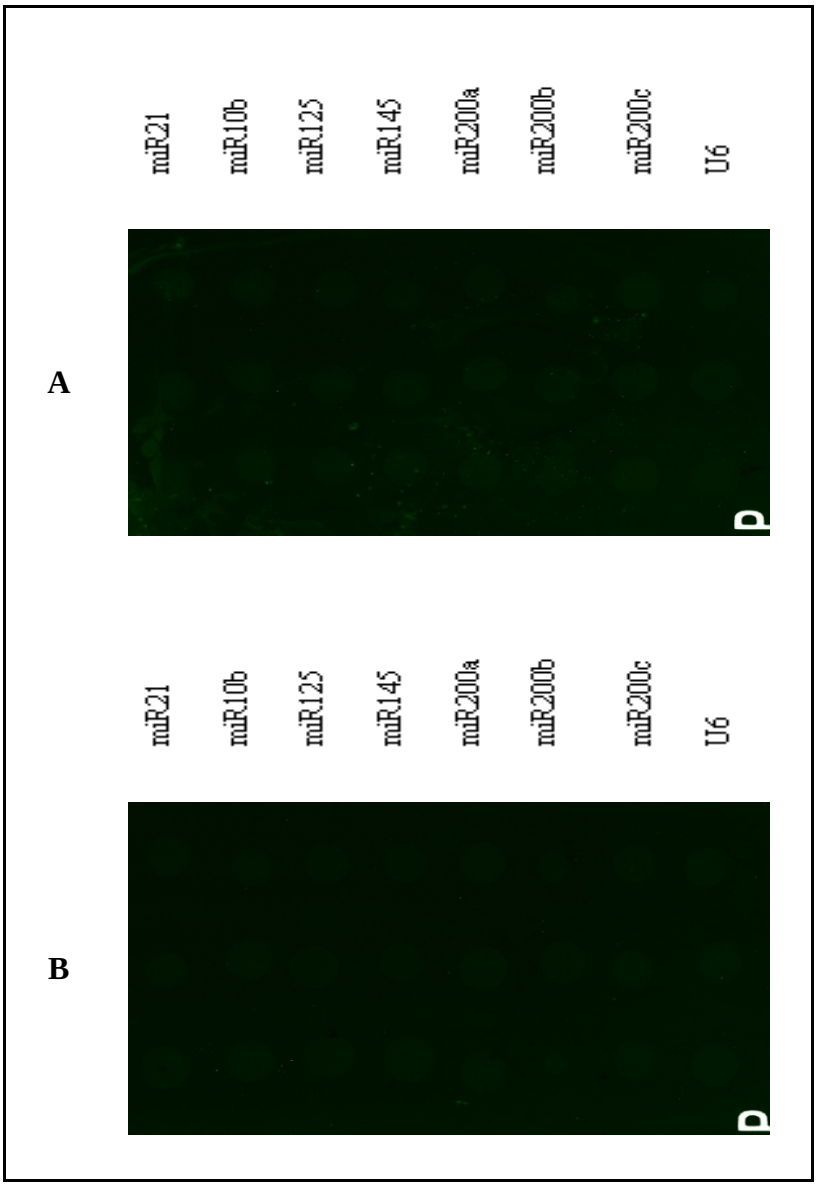

Figure 3.18 The possibility of P1 and P2 probes bindings A) P1 + P2 B) P1 + Ank + P2

According to results in Figure 3.18, for both same probe sets and different probe sets, it can be said that undesirable hybridization was not observed.

3.2.5 The test of platform with FAM labeled adapter

Labeled miR-21 was used as positive control in studies which was applied for the optimization of miR-21 probe set. FAM labeled miR-21 was used to test performance of prepared platform with different miRNA samples. P1 was spotted for one type miRNA for each line located in hybridization region as in Figure 2.5. In order to test platform with FAM labled adapter, instead of adapter and P2, only FAM labeled miR-21 was incubated. The expectation was that miR-21-FAM sequence was bond only P1 capture probe in the form of P1-miR21-L and any luminescence was not seen on other captıre probes.

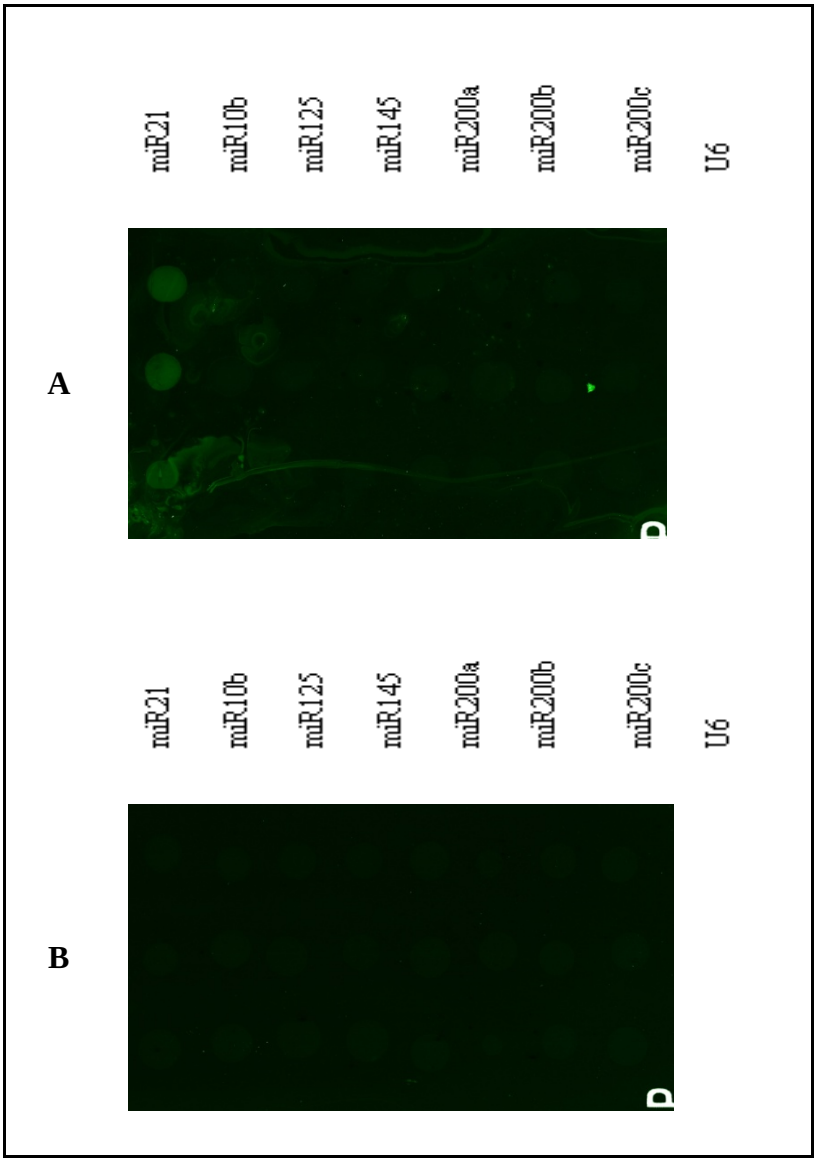

Figure 3.19 The performance of platform with FAM labeled P1 A) P1 + P2 B) P1 + Ank + P2

The negative control slide was prepared by using Ank adapter in studies done with FAM labeled miR-21, also. It was observed that labeled miR-21 probe bond especially own capture probe, but in negative control slides, binding was seen although very little (Figure 3.19). So, this shows that study needs to continue to work.

3.2.6 The determination of binding rates between adapters and other probe sets

It is very important that each of the adapter to connect to own probe sets in terms of the specificity of hybridization in platform which was formed for 8 adapter set. Hence, many trials were planned to determine the specificity of probe sets and confirm the positive signals.

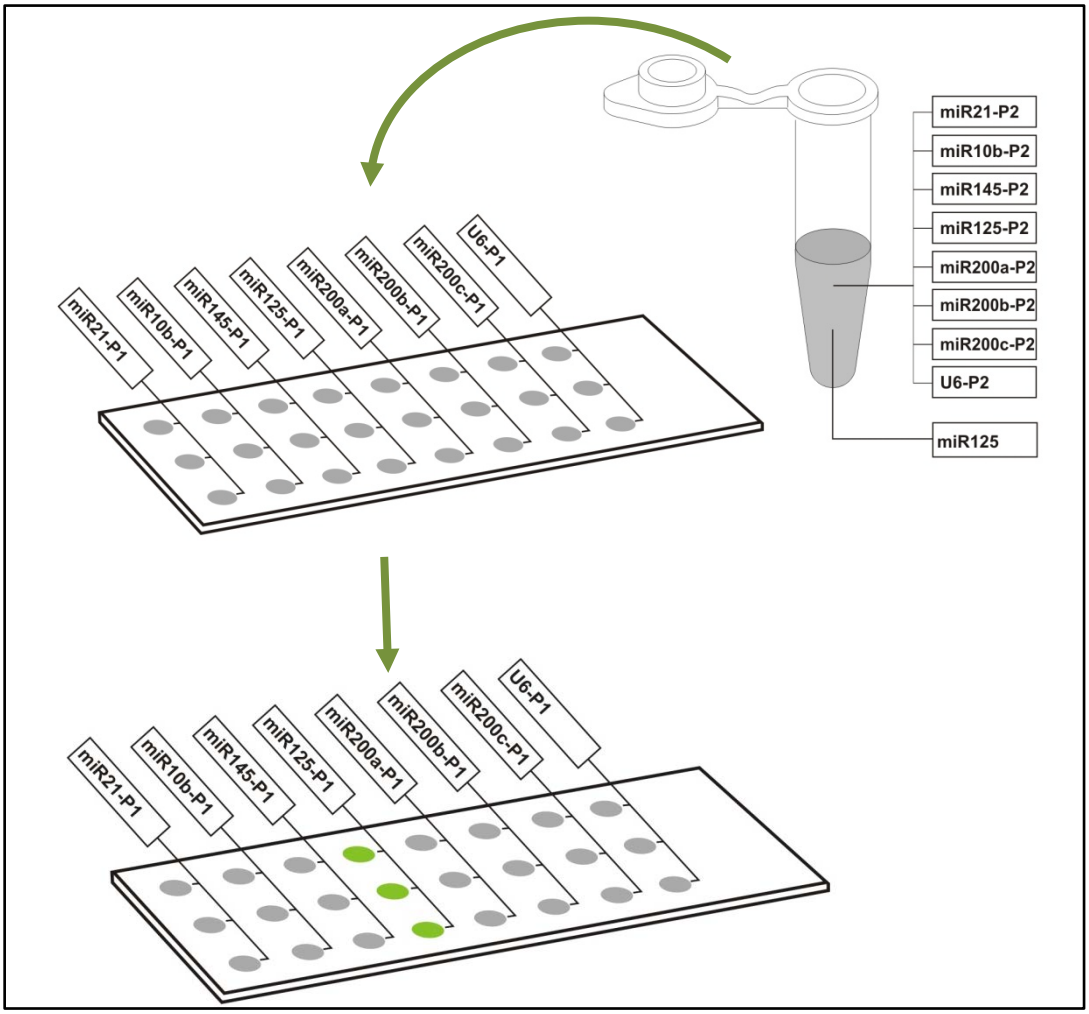

Figure 3.20 The slide design for the determination of specificity of adapters

In this study, one slide was prepared specifically for each adapter type and P1 was spotted for one type miRNA for each line in hybridization regions of these slides. After that, all P2 for miRNAs such as miR-21, miR10b, miR125c, miR145, miR200a, miR200b, miR200c, U6 were incubated with only one miRNA adapter and this solution was applied on the capture probes. The occurence of formation of signal only in the region that solution (all P2 and one type miRNA) involving which miRNA was anticipated .(Figure 3.20). As a positive control, all P2 and all adapters were incubated and was loaded onto a slide; consequently, the sandwich system was formed as P1-A-P2. Beisde positive control, a negative control slide was prepared. The conditions used for the determination of specificity shown in Table 3.16.

Table 3.16 The Conditions for testing the specificity of platform

Cross-Linker	Probe & adapter concent.	Blocking Solution	Hybridization Solution	Hybridization Duration	Washing Conditions after Hybridization
$SM(PEG)_2$	P1 = 5 µM P2 = 5 µM A = 5 µM	2%BSA	0.6X SSC, 0.5% SDS, 2% BSA	30 minutes	1X/ 10 min/ 5X SSC + 0.1% SDS 1X / 5 min / 1X SSC 3X / 1 min / dH2O

As seen in Figure 3.21, different binding properties were observed when adapters whether individually incubated with all P2 and applied to the platform. For example, while miR-21 and miR-10b bond to only own probes, miR-200a, miR-200b, and miR-200c belonging to the same miRNA family bond to probes not only own probes but also other probes that were belong to other family members. It was seen in studies which was carried out with FAM labeled miR-21 that miR-21 adapter bond especially to its own probe. In the same way, in order to test bindings of adapters to their own signal probes, 9 slides were prepared. In these slides, P1 was spotted for one type miRNA for each line in hybridization region as in Figure 2.5.

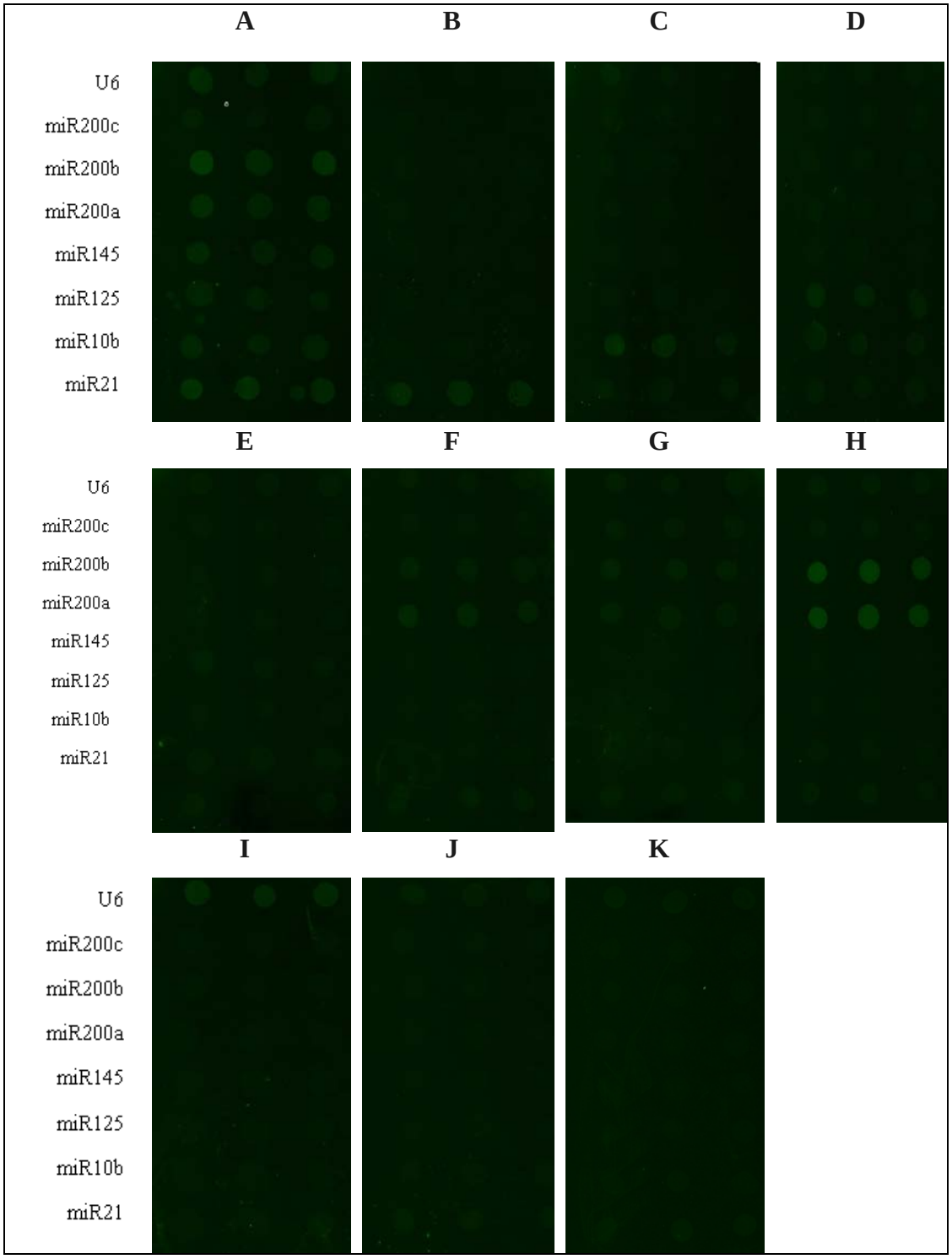

Figure 3.21 The specificity of platform A) Positive control B) miR21 C) miR10b D) miR125 E) miR145 F) miR200a G) miR200b H)miR200c I) U6 J) Ank (Negative control) K) P1-P2 (negative control)

All P2 (miR21-P2-L, miR10b-P2-L, miR145-P2-L, miR145-P2-L, miR200a-P2-L, miR200b-P2-L, miR200c-P2-L, U6-P2-L) and only one type adapter were incubated and loaded onto slides. The formation of signal just as in the line involving miRNA' capture probe was excepted.For instance, in Figure 3.21, miR-21 adapter was incubated with all P2 (miR21-P2-L, miR10b-P2-L, miR145-P2-L, miR145-P2-L, miR200a-P2-L, miR200b-P2-L, miR200c-P2-L, U6-P2-L) and this was loaded onto slide. The expectation was that the formation of signal was only in line with miR-21 capture probe.

According to results in Figure 3.21, miR-21, miR-10b, and U6' adapters were bond to their own signal probes but in other probe sets, undesirable hybridizations were observed.

3.2.7 The optimization of Hybridization temperature for miRNA probe sets

After the optimization of washing conditions, for improving the hybridization of all miRNAs many tests were done. For this purpose, many hybridization temperatures such as 30˚C, 35˚C, 40˚C, and 45˚C were tried and 3 slides were prepared for every temperature. P1 was pointed for one type miRNA to each line in hybridization region as shown in Figure 2.5. All P2 as miR21-P2, miR10b-P2, miR145-P2, miR145-P2, miR200a-P2, miR200b-P2, miR200c-P2, and U6-P2 and all addapters like miR21, miR10b, miR145, miR125, miR200a, miR200b, miR200c, and U6 were incubated in tube for first slide. After incubation, this solution was loaded onto the slide; thus, P1-A-P2 sandwich system was formed. For second slide, all P2 and only miR-21 were incubated and this solution was loaded onto slide that consists of all P1. The hybridization specificity was tested with this slide. miR-21 adapter must be connected with only its own probe in specific hybridization temperature and the hybridization should occur only in region that miR-21 and its P1 connected. For final slide, negative control was formed by using Ank instead of adapter. The conditions used in the optimization of hybridizationn temperature were shown in Table 3.17.

Table 3.17 The Conditions for optimization of hybridization temperature

Cross-Linker	Probe & adapter concent.	Blocking Solution	Hybridization Solution	Hybridization Duration	Washing Conditions after hybridization
$SM(PEG)_2$	P1;5 µM P2 ;5 µM A ;5 µM	2%BSA	0.6X SSC, 0.5% SDS, 2% BSA	30 minutes	1X / 10 min / 5X SSC + 0.1% SDS 1X / 5 min / 1X SSC 3X / 1 min / dH2O

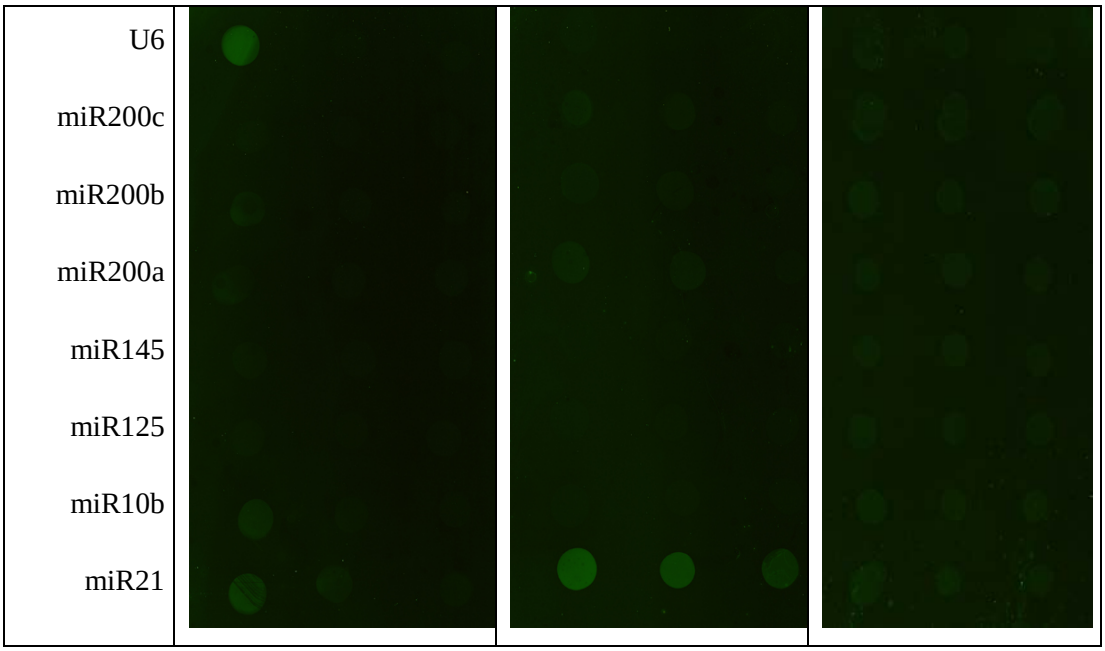

Figure 3.22 Results obtained for platform in hybridization temperature as 30˚C A) miRNA platform involving 8 probe sets B) Platform with only miR21 Adapter C) Negative control

P1-A-P2 connecion was not been good enough in 30˚C as seen from Figure 3.22. Furthermore, when looked at second and thirrd slide, although, it was seen that miR-

21 bond to only its probe set, spots were observed in negative control slide, that is, the formation of unspecific bindings. For this resullt, it can be sait that conditions in Table 3.17, were not suitable for hybridization temperature as 30˚C.

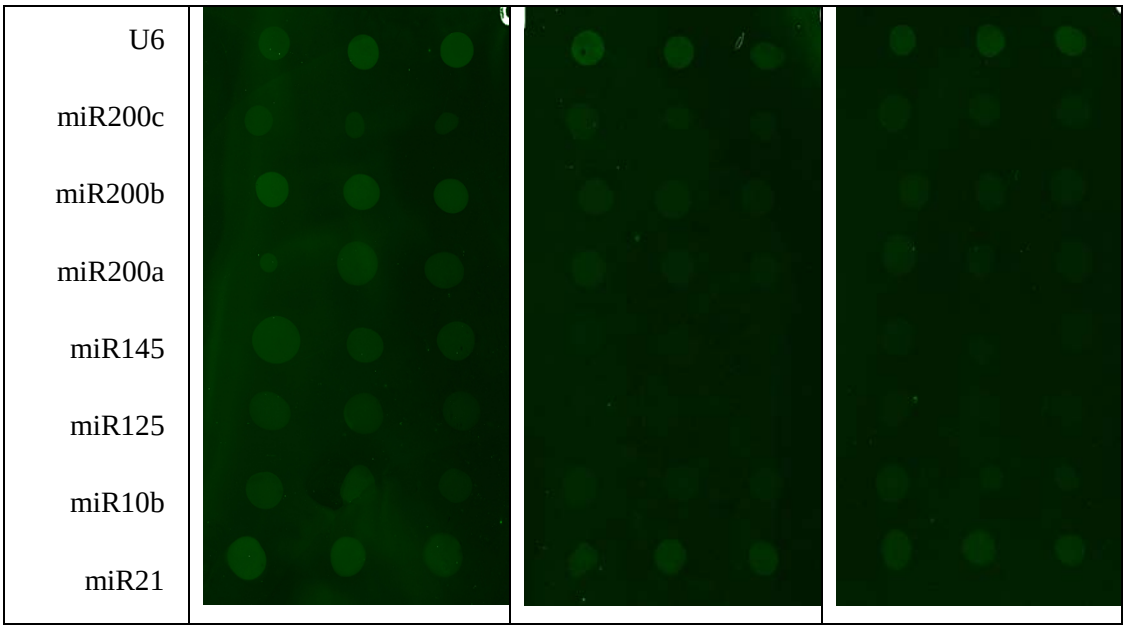

Figure 3.23 Results obtained for platform in hybridization temperature as 35˚C A) miRNA platform involving 8 probe sets B) Platform with only miR21 Adapter C) Negative control

By increasing the temperature of hybridization as 35˚C, P1-P2-A binding became stronger but in negative control slide, unwanted bindings were continued to observe. Also, miR-21 bond besides its probe set connected to other probe sets (Figure 3.23). Hence, it was determined that 35˚C hybridization temperature was not appropriate for platform.

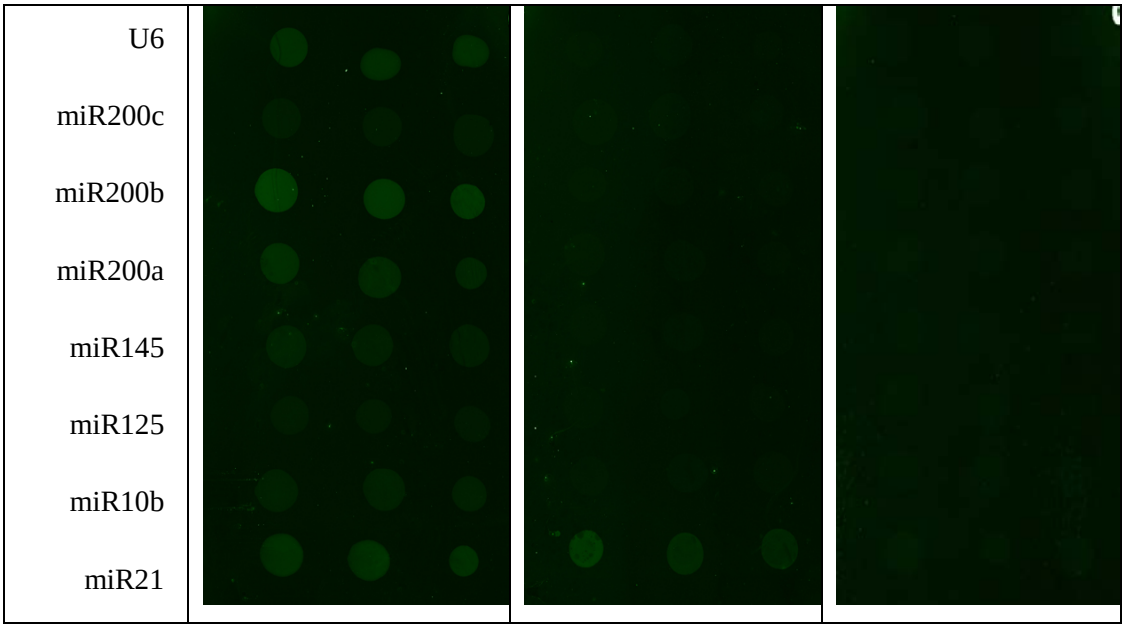

Figure 3.24 Results obtained for platform in hybridization temperature as 40˚C A) miRNA platform involving 8 probe sets B) Platform with only miR21 Adapter C) Negative control

According to results in Figure 3.24, it can be said that unspecific bindings decreased in negative control slide when hybridization temperature was incresed. Also, hybridization rate was increased rather in hybridization temperature as 40˚C when compared to results at temperatures as 30˚C and 35˚C. In study, as hybridization temperature, 45˚C was tried, finally. When temperature increased to 45˚C, P1-A-P2 connection and the binding specificity of adapters to their probes increased and also, the undesirable bindings were minimized in negative control (Figure 3.25).

According to results, 45˚C was determined as hybridization temperature in the next stages of the study.

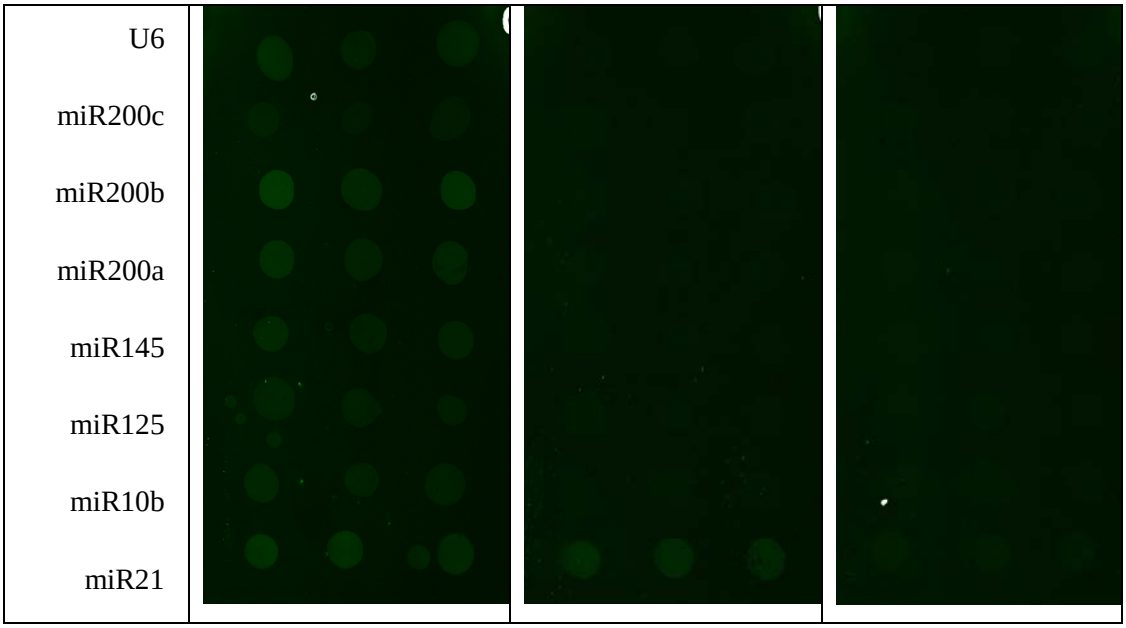

Figure 3.25 Results obtained for platform in hybridization temperature as 45˚C A) miRNA platform involving 8 probe sets B) Platform with only miR21 Adapter C) Negative control

3.2.8 The test of specificity in miR200 family

miR-200a, miR-200b, and miR-200c which comes from same family, can be bond to each other probes as seen in Figure 3.23. For this result, hybridization temperature was increased to 50˚C for these 3 adapter sets by using conditions given in Table 3.17.

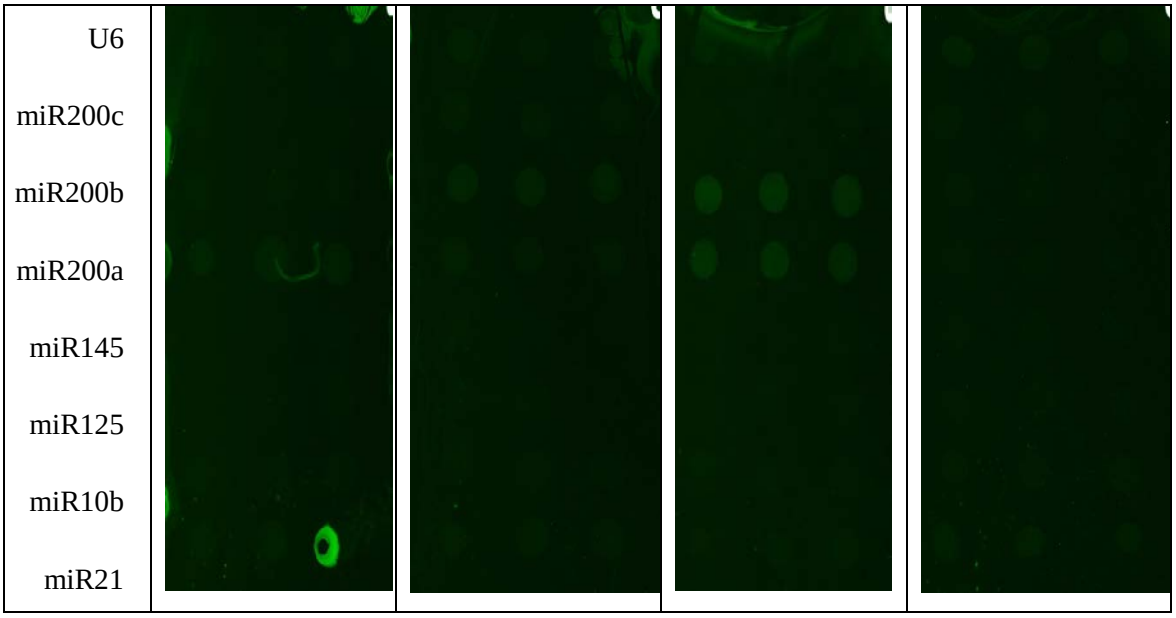

Figure 3.26 The specificity of miR200 family members in hybridization temperature as 50˚C A) miR200a B) miR200b C) miR200c

As seen in Figure 3.26, increasing the hybridization temperature did not increase the selectivity of the adapter. Thus, the studies were continued to perform with 45˚C in the next stages of the study.

3.2.9 The optimization of miR200c binding conditions

Since the beginning of the study, miR200c gave a very faint spots in all trials. In other words, sandwich hybridization platform could not be obtained efficiently for miR200c with these conditions. Therefore, by increasing the concentration of probes, experiments were carried out in different temperatures. The applied conditions were shown in Table 3.18.

Table 3.18 The Conditions for the improvement of miR200c binding

Condition	Cross Linker	Probe & adapter concent.	Blocking Solution	Hybridi. Solution	Hybridi. Tempe.	Hybridiza. Duration	Washing Conditions after hybri.
1	$SM(PEG)_2$	P1 = 10 µM P2 = 10 µM A = 10 µM	2%BSA	0.6X SSC, 0.5% SDS, 2% BSA	30˚C	30 min.	1X/ 10 min/ 5X SSC + 0.1% SDS 1X / 5 min / 1X SSC 3X / 1 min / dH2O
2	$SM(PEG)_2$	P1 = 10 µM P2 = 10 µM A = 10 µM	2%BSA	0.6X SSC, 0.5% SDS, 2% BSA	45˚C	30 min.	1X/ 10 min/ 5X SSC + 0.1% SDS 1X / 5 min / 1X SSC 3X / 1 min / dH2O
3	$SM(PEG)_2$	P1 = 20 µM P2 = 20 µM A = 20 µM	2%BSA	0.6X SSC, 0.5% SDS, 2% BSA	45˚C	30 min.	1X/ 10 min/ 5X SSC + 0.1% SDS 1X / 5 min / 1X SSC 3X / 1 min / dH2O

During the trials, only miR200c probe and adapter set was used. When preparing slides, 9 spots were formed and one negative control slide was prepared for each trial. According to results in Figure 3.27, a change in hybridization temperature did not increase the binding rate of adapter while an increase in the concentration affected the results positively. But still, miR200c probe-adapter set that Tm values was not different from other probe adapter set gave much more faint spots than other sets although different conditions were applied. The reason for this may become from a deformation in miR200c adapter or probe set. Hence, new probe sets were

ordered and experiments for miR200c were repeated but still, the desired spots were not achieved for miR200c.

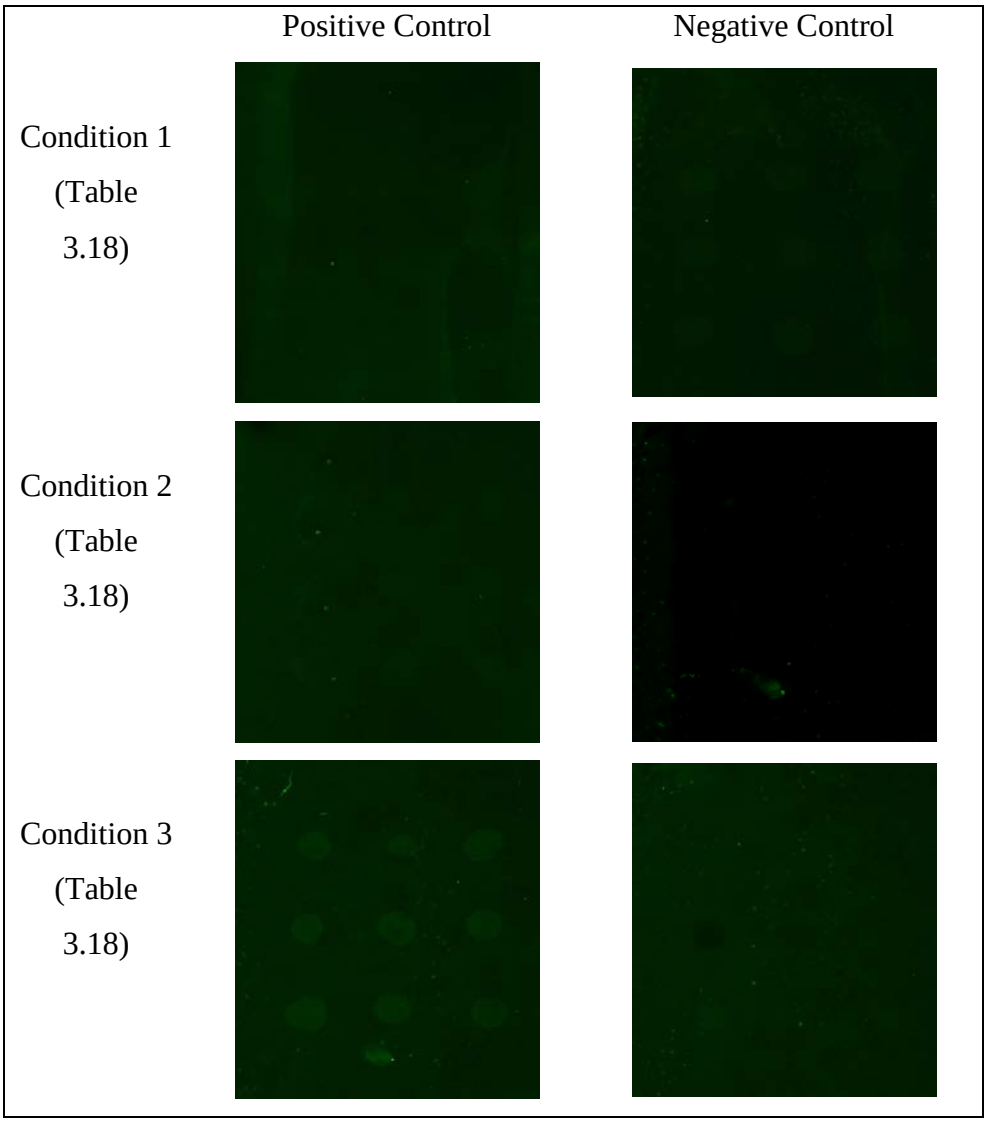

Figure 3.27 The test of miR200c binding conditions. **A.** Condition 1 **B.** Condition 2 **C.** Condition 3

3.2.10 The determination of platform sensitivity

The identification of platform sensitivity and becoming platform in high sensitivity have great importance. For this purpose, it was worked to determine minimum

adapter concentration identified by developed miRNA array platform in experiments which performed with different miRNA concentrations. In other words, the sensitivity of platform was worked to identify. Many concentrations such as 0.0001 µM, 0.001 µM, 0.01 µM, 0.1 µM, 1 µM, 5 µM ve 10 µM and all probes and adapter sets were utilized to determine sensitivity of platform.

Table 3.19 The Conditions for determination of the sensitivity of platform

Cross linker	Probe concentra.	Block. Solu.	Hyb. Solu.	Hybridi. Duration	Washing conditions after hybridization
$SM(PEG)_2$	miR21;5µM miR10b;5µM miR125;10µM miR145;10µM miR200a;5µM miR200b;5µM miR200c;20µM U6; 5µM	2%BSA	0.6X SSC, 0.5% SDS, 2% BSA	30 minutes	1X/ 10 min/ 5X SSC + 0.1% SDS 1X / 5 min / 1X SSC 3X / 1 min / dH2O

The studies for the identification of platform sensitivity were started with 5 µM probe concentration for all adapters except miR200c. However, it was seen that the spots were very pallid for miR125 and miR145 when probe and adapter concentrations were decreased to 5 µM. For this reason, the conditions given in Table 3.19 were carried out for the determination of platform sensitivity.

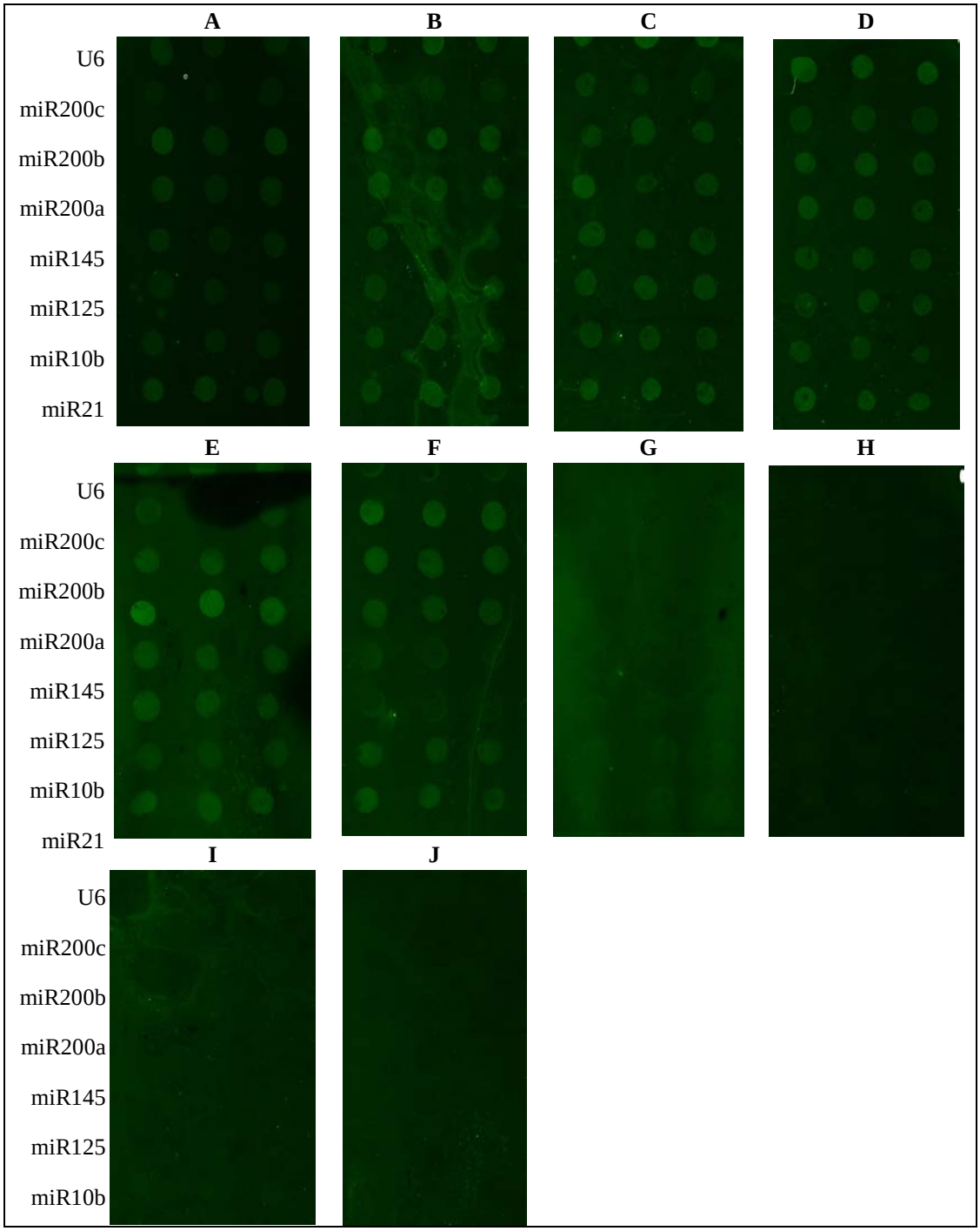

Figure 3.28 The sensitivity of platform A) 10µM B) 5µM C) 1µM D) 0.5µM E) 0.1µM F) 0.01µM G) 0.001µM H) 0.0001µM I) Ank (negative control J) P1-P2 (negative control)

According to results in Figure 3.28, it can be said that the developed platform can detect 0.01 µM adapter concentration clearly. The spots became very faint under this concentration.

3.3 Application of Different miRNA Samples to Developed Array Platform

The nucleic acid based array platform which was developed and optimized in the first two stages of the study was performed with real total RNA samples and the performance of platform was investigated in the third stage of the study (Figure 3.29). For this purpose, immortal and non-tumorigenic breast cell lines such as MCF10A ve MCF12A, metastasis negative cell line as MCF7, and metastasis positive cell line as MDA-MB-231 cell line and commercially available total RNA samples obtained from tumorigenic and normal tissues.

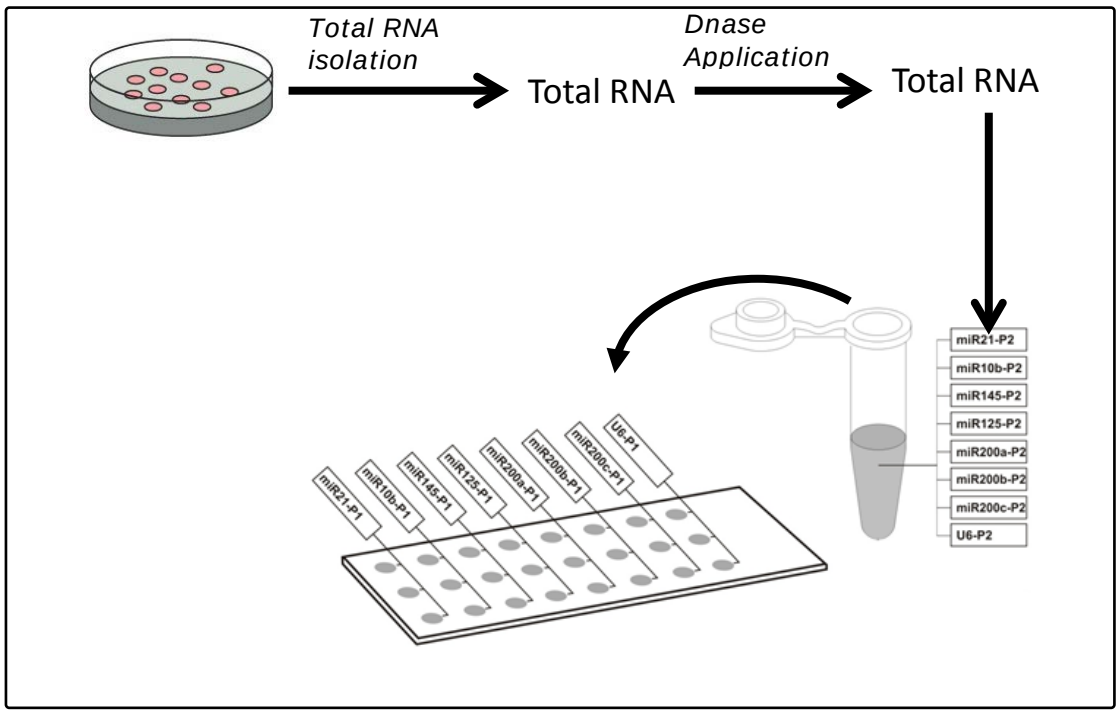

Figure 3.29 The steps in third stage of the study

Moreover, by using total RNA samples isolated from cell lines, RT-PCR was performed to compare results obtained from the platform.

3.3.1 Total RNA isolation

In this study, in order to make RNA isolation, firstly, MCF10A, MCF12A, MCF7, and MDA-MB-231 cell lines were grown in mediums shown in Table 2.2. After isolation, total RNA amounts were measured with NANODROP and obtained results were given at Table 3.20.

Table 3.20 Total RNA concentration

Cell Lines	Total RNA concentration
MCF 10A	425.9 ng/µl
MCF 12A	1064.4 ng/µl
MCF 7	230.9 ng/µl
MDA-MB-231	74 ng/µl

3.3.2 DNase Application

It is very important that the purity of RNA which is used in the array platform studies. Hence, DNase application was carried out to the isolated RNA before applying RNA samples to platform to prevent any DNA existence.

After DNase application, RNA samples were measured with NANODROP again, the obtained results were given in Table 3.21.

Table 3.21 Total RNA concentration after DNase Treatment

Cell Lines	Total RNA concentration
MCF 10A	108.1 ng/µl
MCF 12A	73.5 ng/µl
MCF 7	87.9 ng/µl
MDA-MB-231	54.7 ng/µl

After this process, PCR was carried out for samples by using GAPDH primers. The conditions used in PCR were shown in Table 2.3 and 2.4.

The products obtained from PCR were loaded onto gel and scanned. It was expected to see no bands on the gel because DNase treatment should prevent any DNA existence. It was decided that DNase treatment was applied again because very slight bands were observed on gels. After second DNase process, samples were measured again with NANODROP (Table 3.22) and PCR was applied again for samples (Figure 3.30).

Table 3.22 RNA concentration for cell lines

Cell lines	**RNA concentration**
MCF 7	61.3 ng/µl
MDA 231	29.7 ng/µl
MCF 12A	49.1 ng/µl
MCF10A	73.1 ng/µl

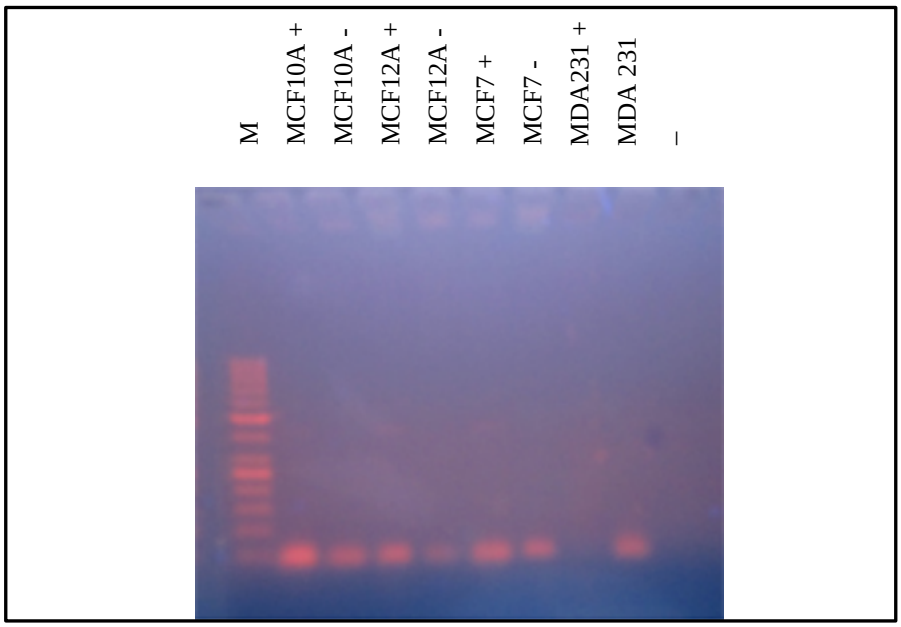

Figure 3.30 The samples applied DNase process

According to Figure 3.30, it can be accepted that the obtained RNA samples purity was appropriate to apply platform.

3.3.3 cDNA Analysis

cDNA samples were prepared via reverse transcription method by using obtained total RNA samples. In order to realize cDNA analysis, the conditions were used in Table 2.5.

3.3.4 RT-PCR

The existence of miRNA and their expressions in different cell lines was confirmed with RT-PCR analysis and Taqman probes were used in these studies. cDNAs were obtained from total RNA samples via reverse transcription method and these cDNAs were qualified by using Taqman primers with RT-PCR method (Figure 3.31). RT-PCR made the detection for the expression of all miRNAs in study, results were shown in Appendix C. The total RNA samples which were commercially available

and obtained from normal tissues were utilized for calibration. Three replicates were prepared for each type cell line, U6 was used as reference material and the conditions used in Table 2.6.

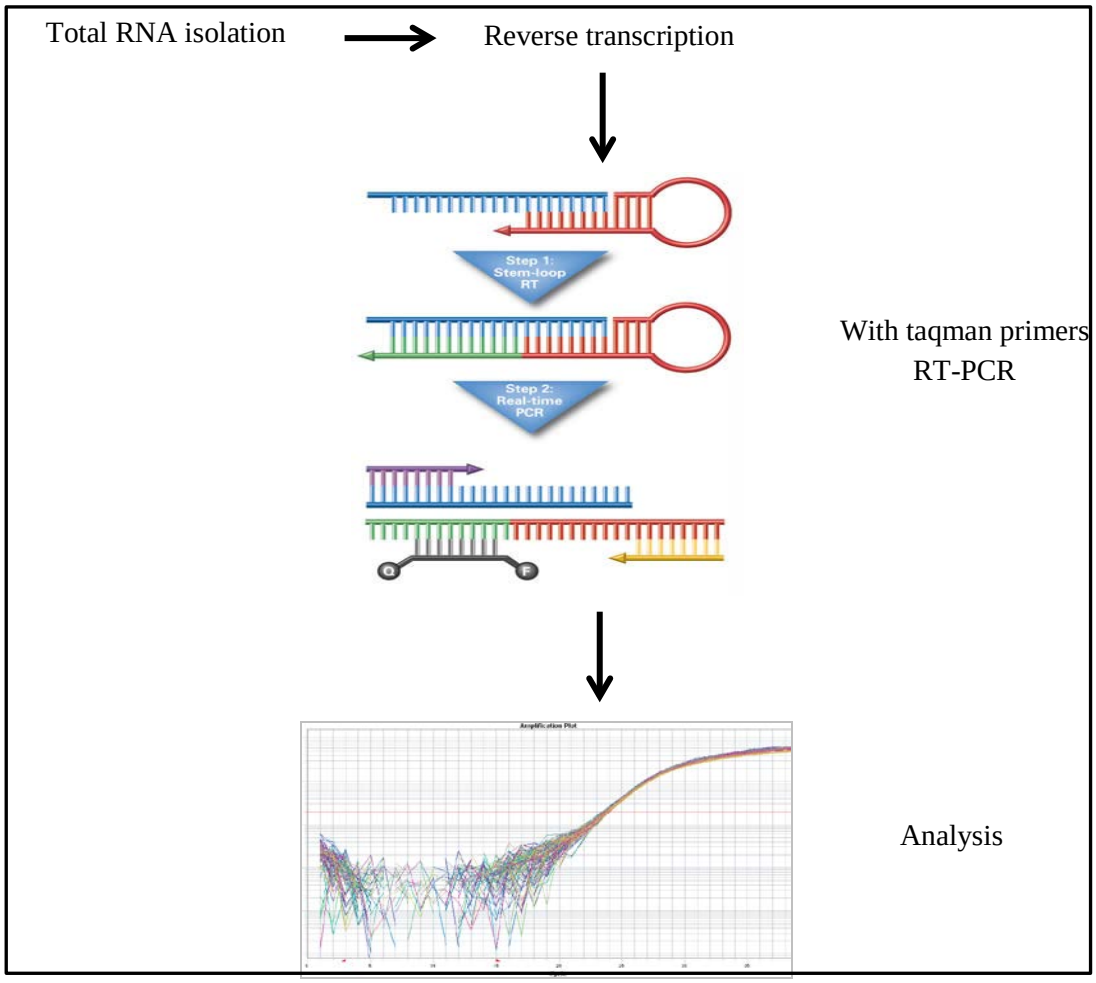

Figure 3.31 The steps in RT-PCR

As indicated in literature, the results obtained from RT-PCR supported that miRNA concentrations show differences between tumor and normal cell lines. For example, in literature, miR-21 expressions increase in breast cancer [Romero-Cordoba et al., 2012; Frankel et al., 2008; Zhu et al., 2007]. According to RT-PCR results in our study, when compared tumorigenic metastasis negative as MCF7 and metastasis positive as MDA-MB-231 cell lines with commercially available cell lines such as

total RNA samples from normal breast tissues, it was observed that miR21 and miR10b increased in both cell types. On the other hand, when the comparison was made with total RNA samples from commercially available normal breast tissue and non-tumorigenic cell lines, these increases did not seen. For instance, when the amount of miR21 was compared in terms of total RNA obtained from commercially available and non-tumorigenic cells, while only 0.22 fold increases were observed in non-tumorigenic cell line as MCF10A 6.7 fold increases were observed in tumorigenic cell line like MCF7. When looked at literature, it is known that miR125 and miR145 expressions decrease in tumorigenic cell lines [Romero-Cordoba et al., 2012]. However, this reduction was not observed in studies.

3.3.5 The application of total RNA samples to platform

The DNase applied total RNA samples were tried to platform which was optimized with synthetic miRNA adapters. At this stage, isolated RNA amounts were measured with NANODROP and the measurements were given in Table 3.24. The measured total RNA samples with NANODROP were carried out the platform by proportioning with respect to concentrations. The conditions used in this study were shown in Table 3.23.

Table 3.23 The conditions used in the test of platform with total RNA samples from breast cancer cell lines

Cross-linker	Probe & adapter concent.	Blocking Solution	Hybridi. Solution	Hybrdi. Duration	Hybridi. Temp.	Washing conditions Aftee r Hybridi.
$SM(PEG)_2$	P1; 5 µM P2; 5 µM MCF7;2.39 µl MDA231;4.92 µl MCF12A;3 µl MCF10A;2 µl	2% BSA	0.6X SSC, 0.5% SDS, 2% BSA	30 min.	45 ^{0}C	1X/ 10 min/ 5X SSC + 0.1% SDS 1X / 5 min / 1X SSC 3X / 1 min / dH2O

The results obtained when total RNA samples were applied to the platform were given in Figure 3.32.

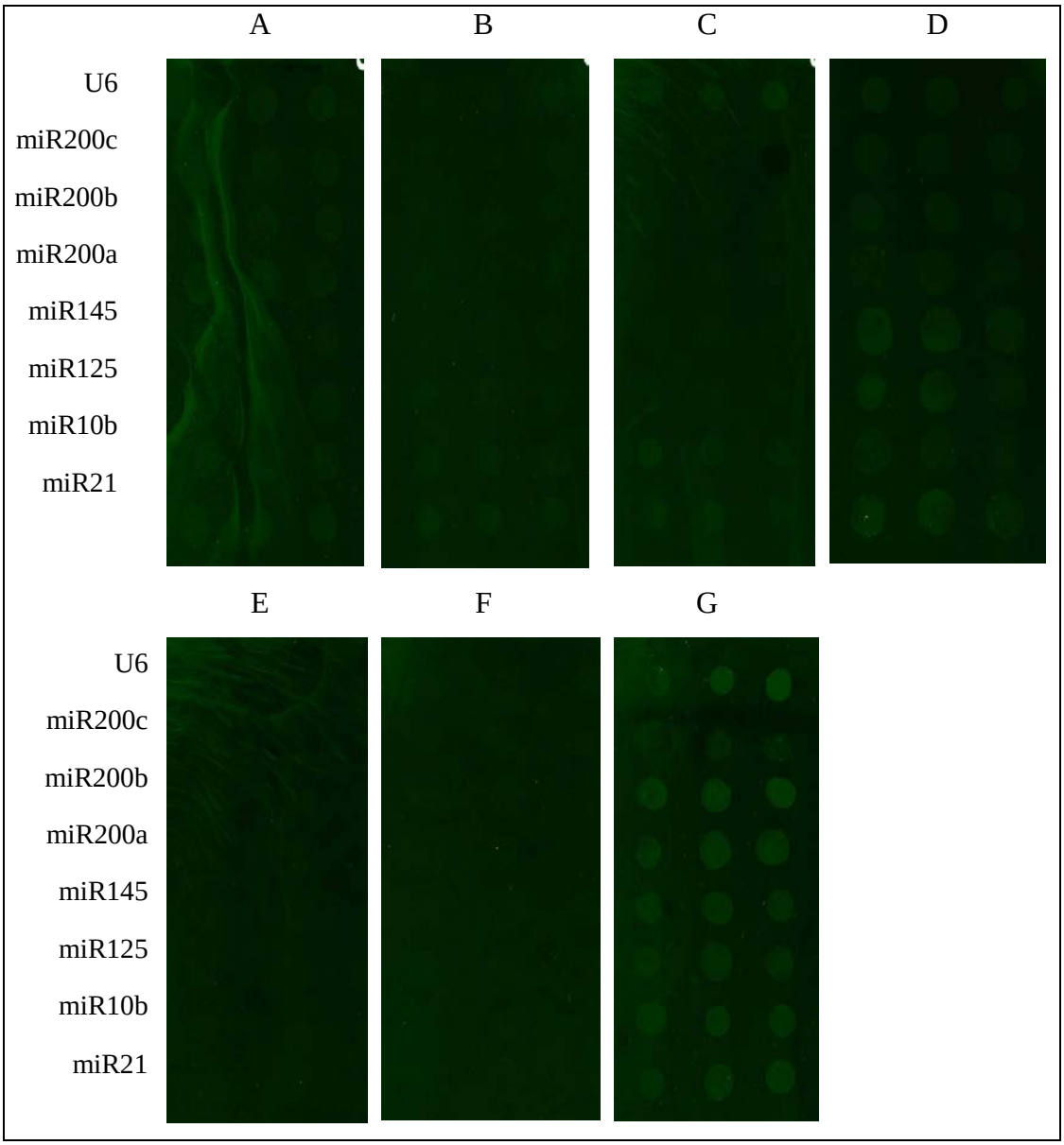

Figure 3.32 Cell lines **A.** MCF7 **B.** MDA231 **C.** MCF 12A **D.** MCF10A **E.** P1+Ank +P2 **F.** P1 +P2 **G.** Positive control

As seen in Figure 3.32, when total RNA samples were applied to the platform, more signals were taken in cell lines with cancer cell lines as A and B in Figure 3.32

according to normal cell lines C and D in Figure 3.32, that is, further binding was observed. However, when looked at all tryings, obtained spots were weak. These images were good, but they were need to turn into quantifiable data. For this purpose, ImageJ is useful for getting information from images, including pixel intensity. We used ImageJ to get intensity information from images and this formula to calculate the corrected total cell fluorescence (CTCF).

CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings)

According to CTCF values, we drew a graph and compared results of figures in terms of cell lines.

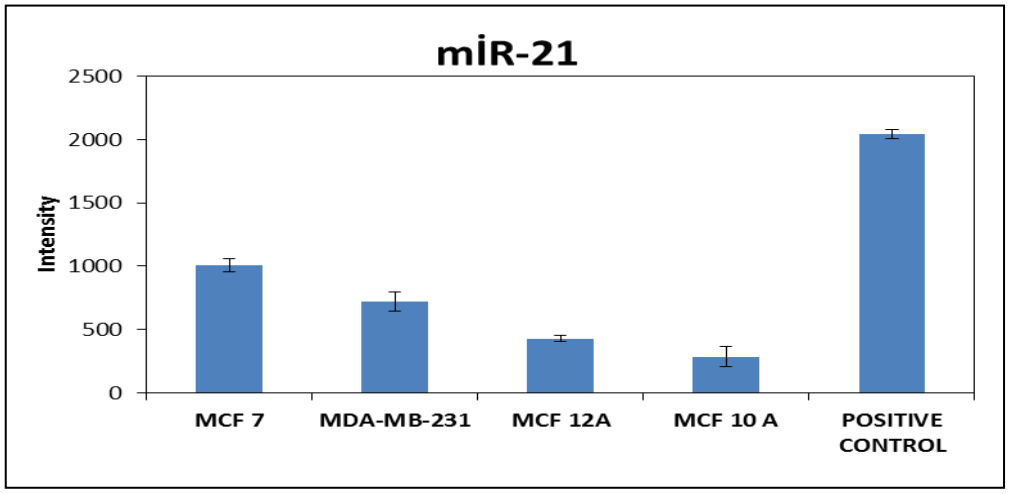

Figure 3.33 Changes of intensities in miR-21 in different cell lines

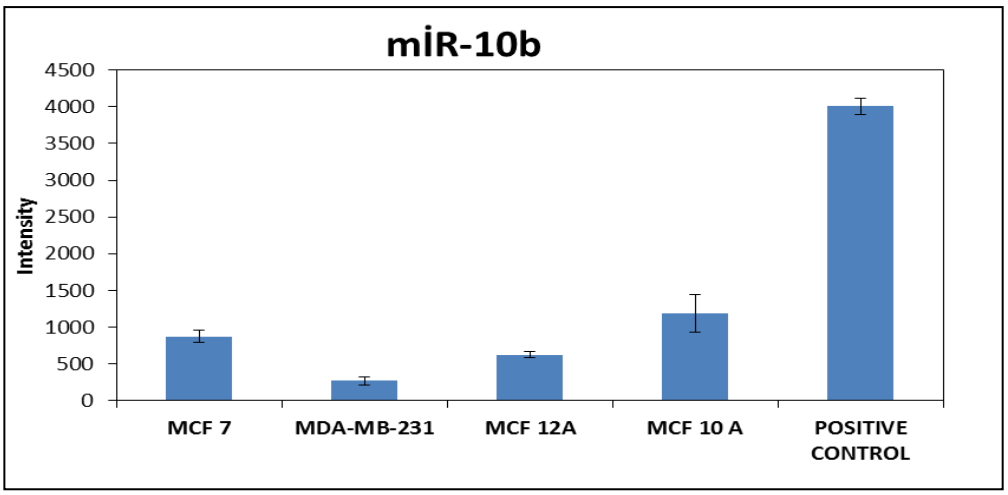

Figure 3.34 Changes of intensities in miR-10b in different cell lines

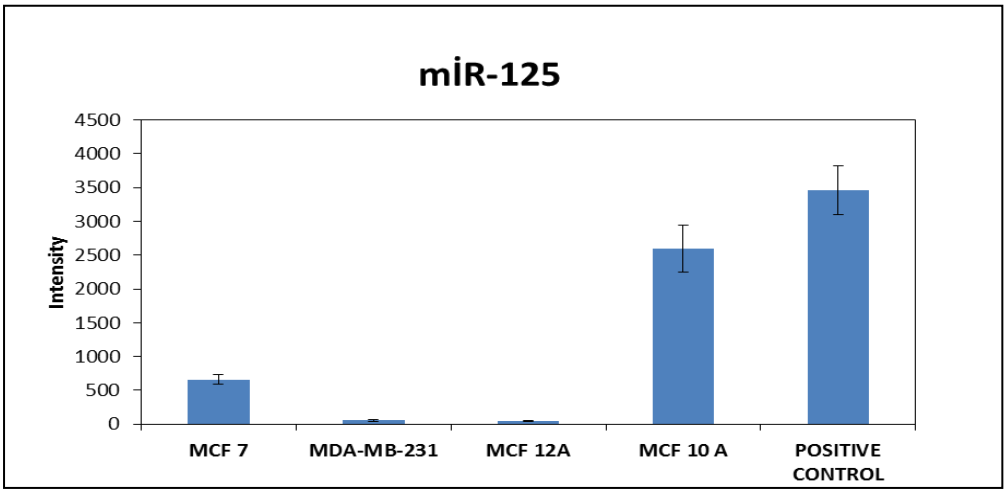

Figure 3.35 Changes of intensities in miR-125 in different cell lines

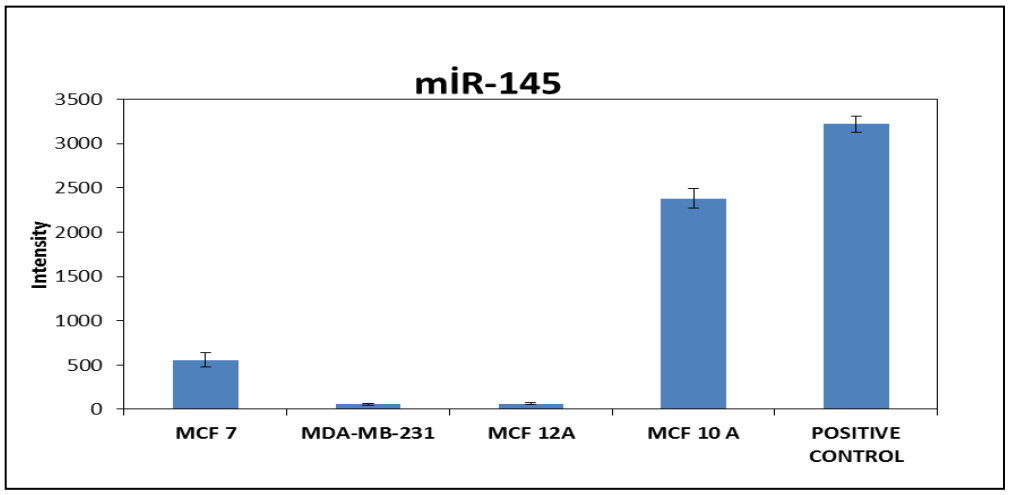

Figure 3.36 Changes of intensities in miR-125 in different cell lines

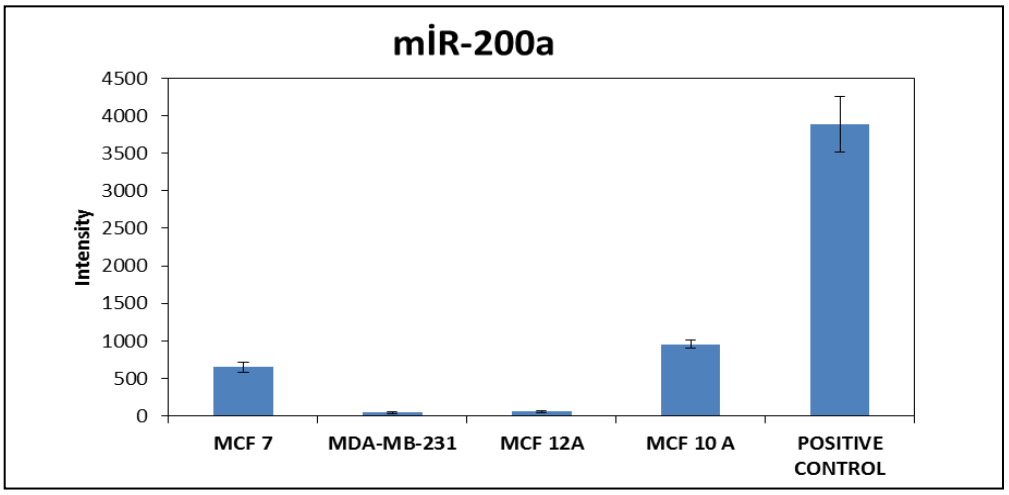

Figure 3.37 Changes of intensities in miR-200a in different cell lines

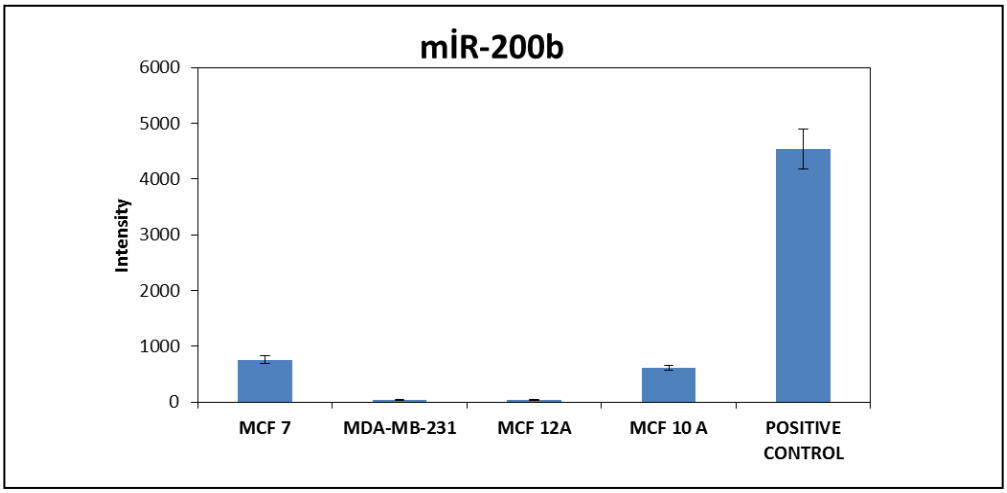

Figure 3.38 Changes of intensities in miR-200b in different cell lines

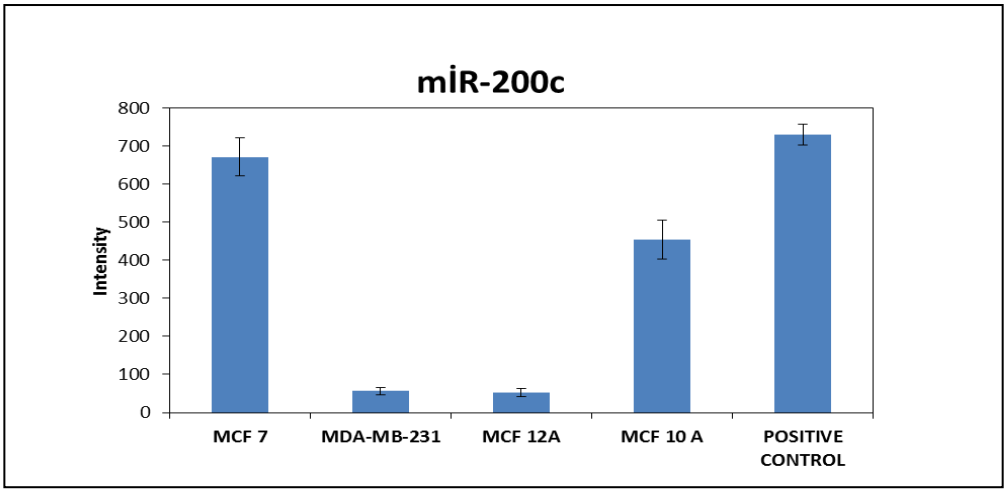

Figure 3.39 Changes of intensities in miR-200c in different cell lines

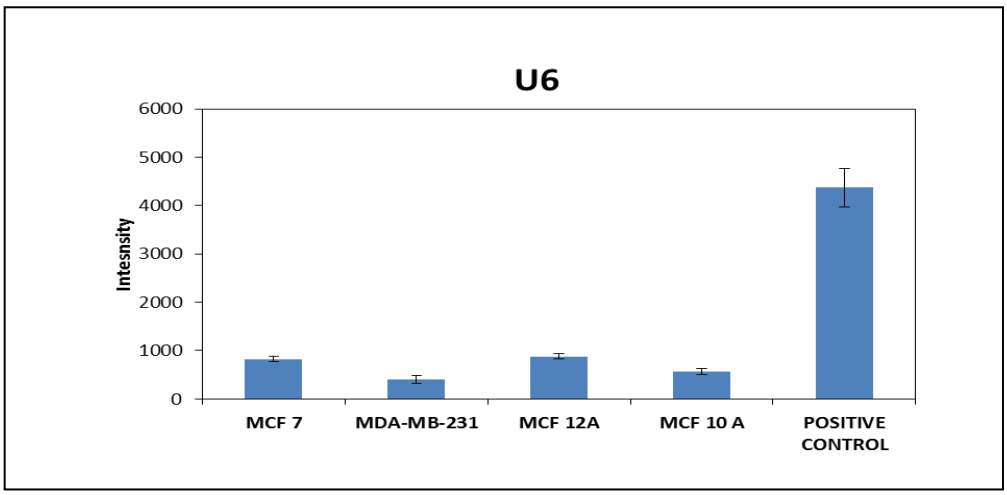

Figure 3.40 Changes of intensities in U6 in different cell lines

As can be seen from figures which shown the color intensities of spots suited to miRNAs, these gave a good result only for miR-21. Positive control result was very high as should be and miR-21 amount was higher in breast cancer cell lines such as MDA-MB-231 and MCF7 than in normal breast cells like MCF10A and MCF12A as can be seen from Figure 3.33. On the other hand, it was not possible to do this review for other miRNAs' results. These results not gave a reliable and compatible results when compared to literature research.

The obtained signals, color intensities were weak. For this reason, by holding other conditions constant, experiments were repeated with high concentration RNA to obtain much clearer spots. The conditions utilized in tryings were given in Table 3.24 and results in Figure 3.41.

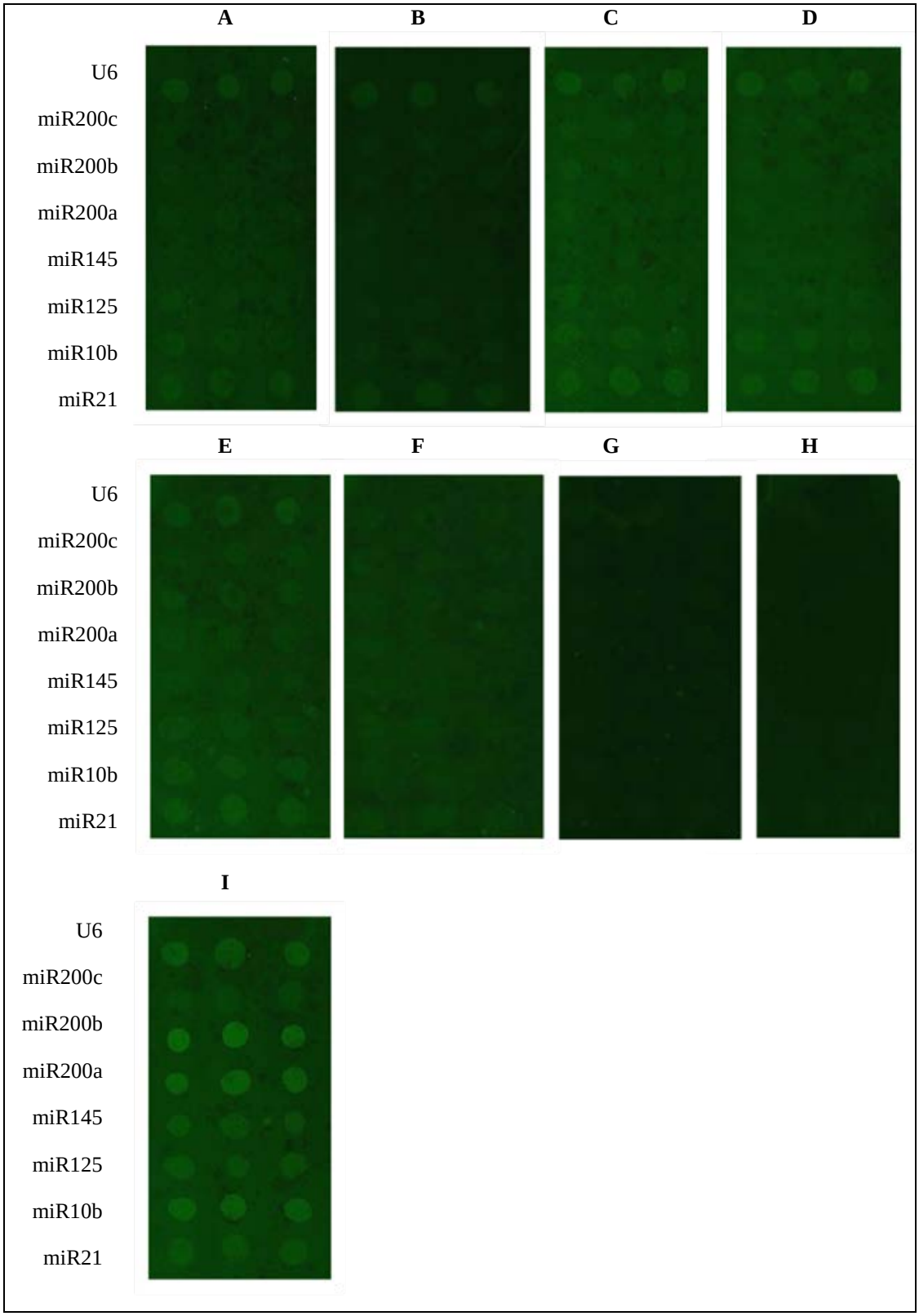
A
B
C
D
U6
miR200c
miR200b
miR200a
miR145
miR125
miR10b
miR21
E
F
G
H
U6
miR200c
miR200b
miR200a
miR145
miR125
miR10b
miR21
I
U6
miR200c
miR200b
miR200a
miR145
miR125
miR10b
miR21

Figure 3.41 Cell lines A) MCF12A B) MCF10A C) MCF 7 D) MDA-MB-231 E) Tumor total RNA F) Breast total RNA G) Negative control (P1+Ank+P2) H) Negative control (P1 + P2) I) Positive control

Table 3.24 The conditions used in the test of platform with total RNA samples from breast cancer cell lines

Cross-linker	Probe & adapter concentrrat.	Blocking Solution	Hybridi. Solution	Hybrdi. Duration	Hybridi. Temperature	Washing conditions afteer Hybridization
$SM(PEG)_2$	P1;10µM P2;10µM MCF7;4.7µl MDA231;9.84 µl MCF12A;6µl MCF10A;4µl	2% BSA	0.6X SSC, 0.5% SDS, 2% BSA	30 min.	45 ^{0}C	1X/ 10 min/ 5X SSC + 0.1% SDS 1X / 5 min / 1X SSC 3X / 1 min / dH2O

As seen in Figure 3.41, an increase in total RNA concentration increaes the performance of miRNA array platform. When the results were analysed it can be said that U6 which was used as positive control was observed in all slides studied with normal cell lines and cell lines with cancer. By becoming parallel to the RT-PCR results, the spots in slides studied with total RNA from cell lines with cancer for miR21 and miR10b were much clearer than with total RNA from normal cell lines. However, it was not observed any decreases in the spots for mir10b, miR125 and miR145 which was specified to be lower in cancer cells in literature studies.

These images were good, but they were need to turn into quantifiable data. For this purpose, ImageJ is useful for getting information from images, including pixel

intensity. We used ImageJ to get intensity information from images and this formula to calculate the corrected total cell fluorescence (CTCF).

CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings)

By using, ImageJ, we obtained CTCF values and according to CTCF values, we drew a graph and compared results of figures in terms of cell lines.

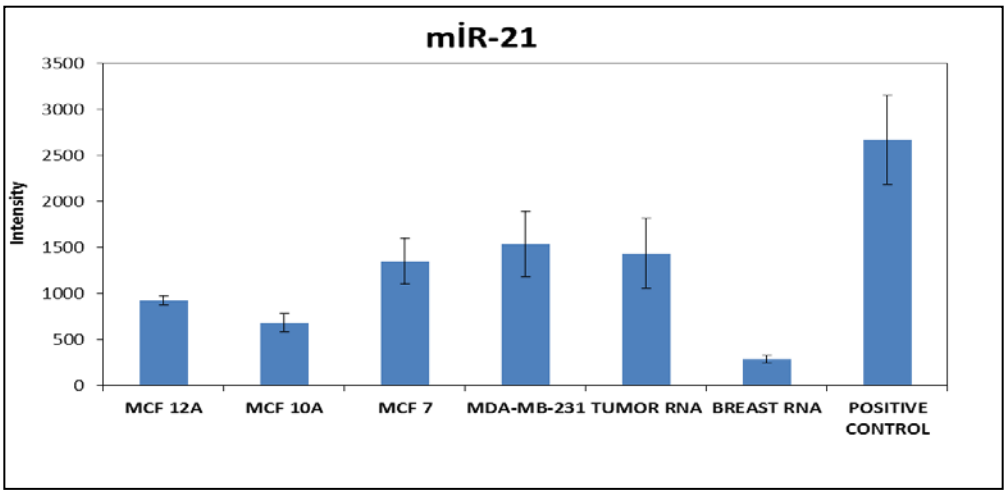

Figure 3.42 Changes of intensities in miR-21 in different cell lines

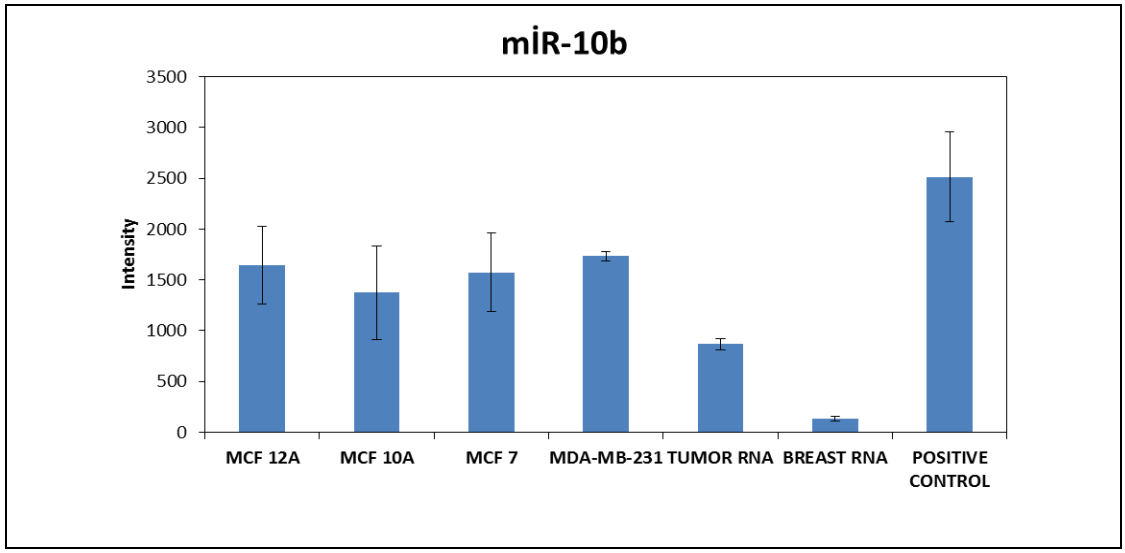

Figure 3.43 Changes of intensities in miR-10b in different cell lines

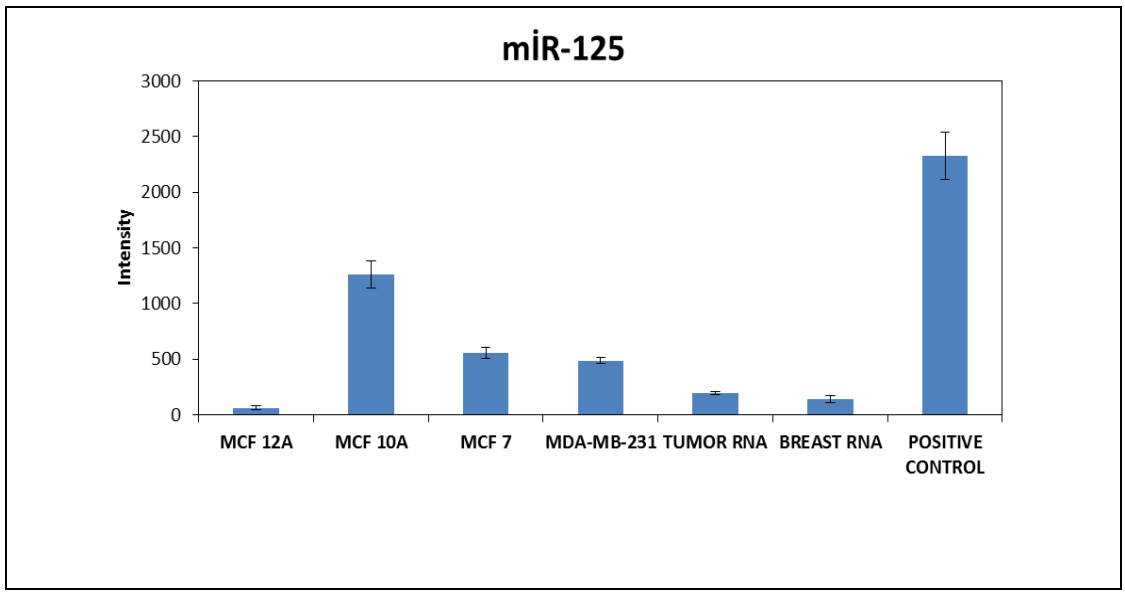

Figure 3.44 Changes of intensities in miR-125 in different cell lines

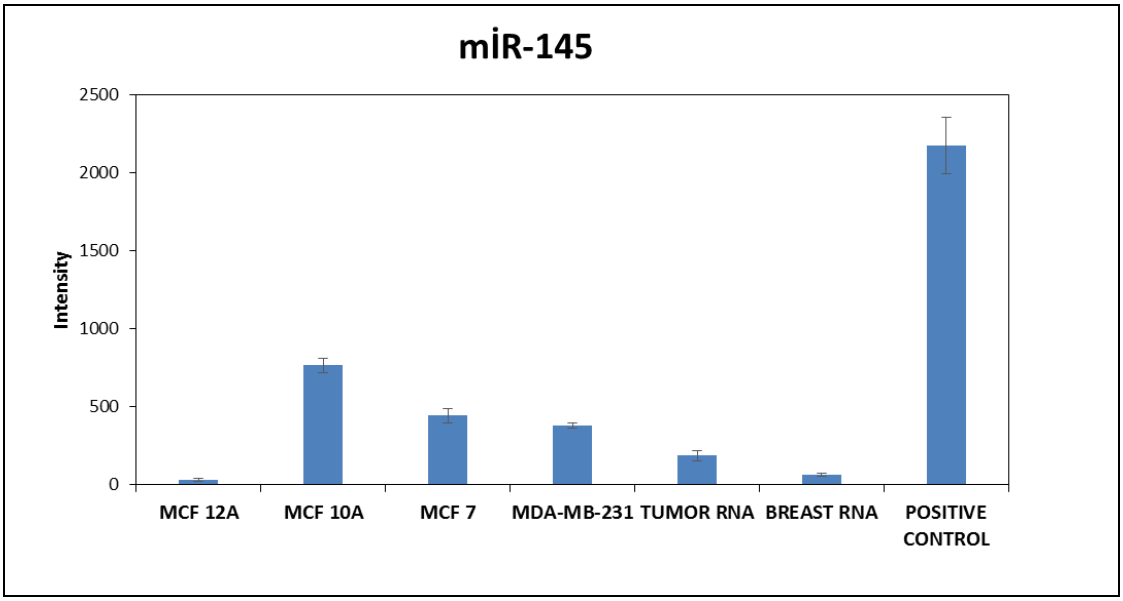

Figure 3.45 Changes of intensities in miR-145 in different cell lines

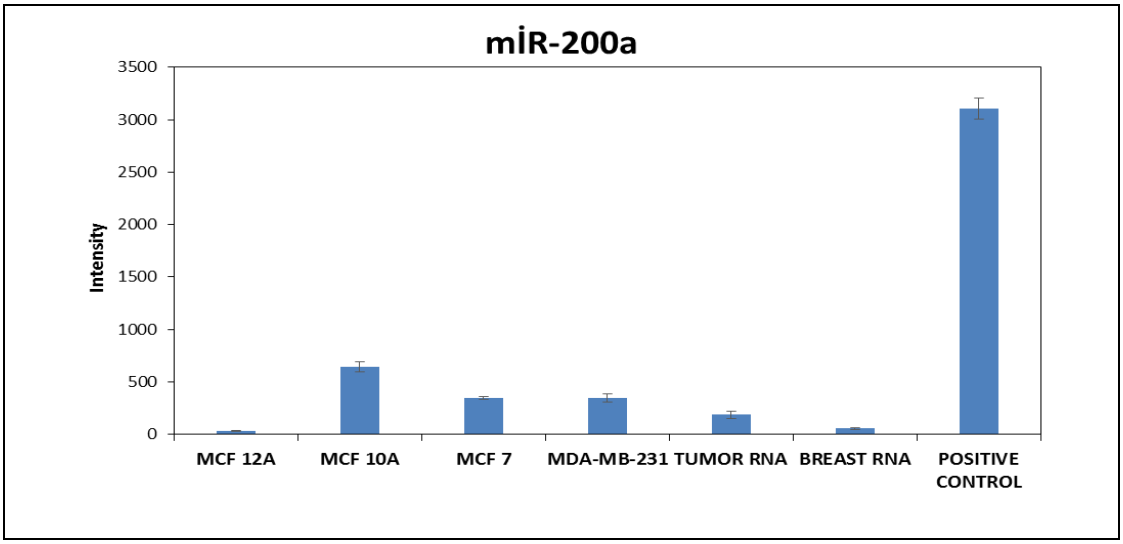

Figure 3.46 Changes of intensities in miR-200a in different cell lines

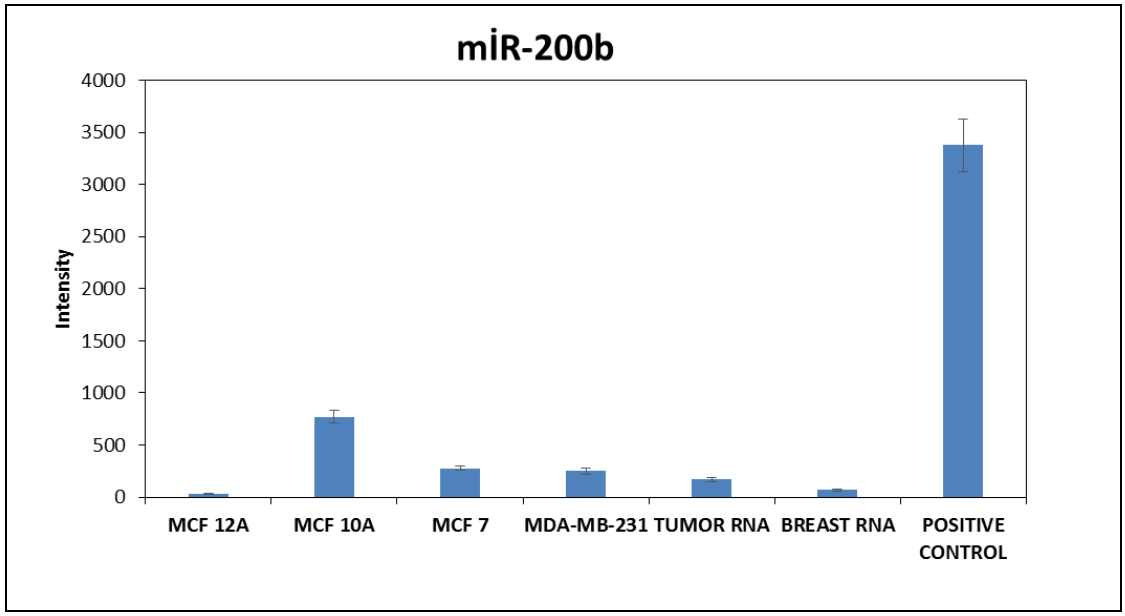

Figure 3.47 Changes of intensities in miR-200b in different cell lines

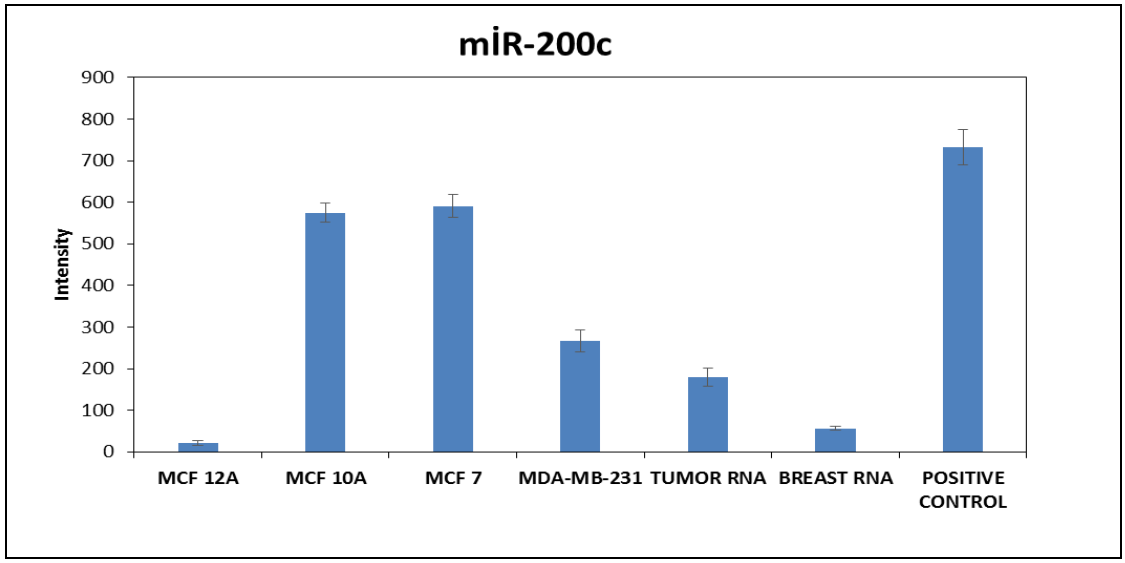

Figure 3.48 Changes of intensities in miR-200c in different cell lines

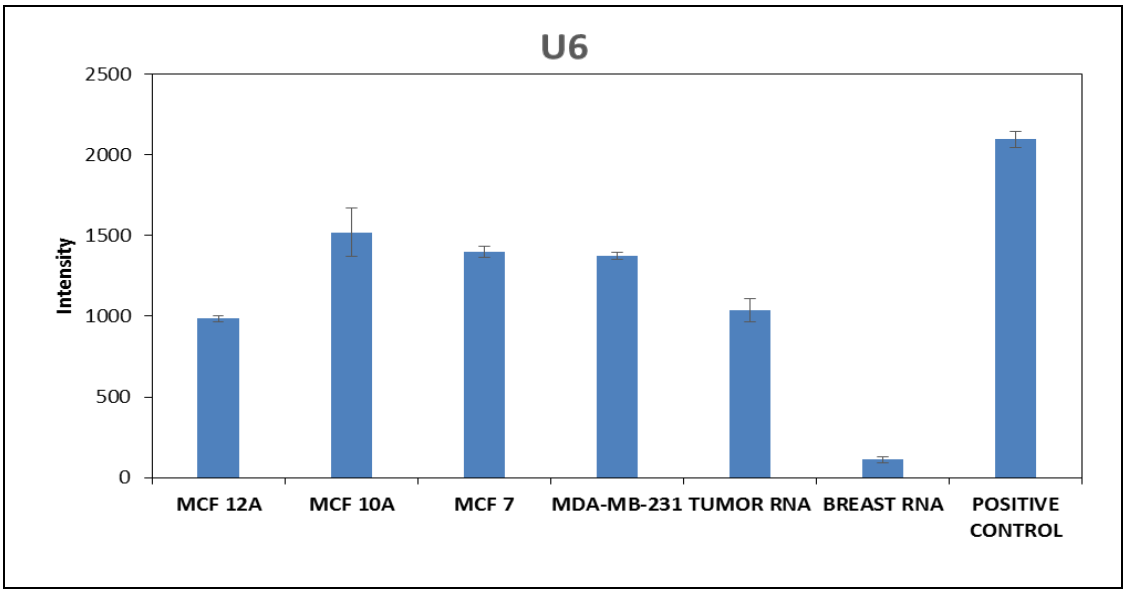

Figure 3.49 Changes of intensities in U6 in different cell lines

As can be seen from figures, only miR-21 gave a good result. Positive control result was very high as we expected and miR-21 amount was higher in breast cancer cell lines such as MDA-MB-231 and MCF7 than in normal breast cells like MCF10A and MCF12A as can be seen from Figure 3.42. Moreover, the intensity of miR-21 was higher in total breast RNA in tumorous cells than total breast RNA in normal cells. However, for other micro RNAs meaningful results cannot be taken. In short, this platform gave good results when only miR-21 applied to the system. However, when much microRNAs were applied to the system, results became very complicated.

CHAPTER 4

CONCLUSION

Sandwich hybridization method is one of the various techniques that have been developed for faster, more sensitive, and reliable nucleis acid detection. It is a flexible system and the target can be a single or double stranded nucleic acid. Sandwich hybridization does not require a labelling and/or cDNA synthesis steps and have minimal equipmetn requirements. Therefore, non-labelled double stranded PCR products, genomic DNA fragments and RNA sequences can be directly applied to the system. Sandwich hybridization platform can also be integrated into microfluidic systems for lab-on-a-chip type applications.

In this work, proof-of-concept for the detection of miRNAs in breast cancer developing a platform depends on the sandwich hybridization system was realised.

Firstly, the determination of cross linker was investigated and SM(PEG)2 gave the best signal which amount was related to immobilized target density. After the determination of cross-linker, different capture and signal probes concentrations were evaluated for platform. In order to optimize blocking, hybridization, washing conditions, oligonucleotide and signal probe types, hybridization temperature, and hybridization duration.

At the end of all experiments, the optimized conditions for sandwich hybridization for all microRNAs were obtained cross-linker as SM(PEG)2, 5 μM capture probe concentration, 5 μM signal probe concentration, 1 hour blocking time, 30 minutes

hybridization time at 45^0C. Under these conditions, it was possible to generate a sandwich hybridization platform at nanomolar range.

Furthermore, this platform was used to diagnose cancer and miRNA which are more tissue specific than mRNA and expression profilling of them is unique and not affected from RNAase activity due to their small sizes were utilized as biomarker. After the deveopment of platform, the platform was studied with synthetic miRNA sequences, tested with total RNA samples from different cell lines and miRNAs that intensities change in breast cancer. The studies show that all miRNA sequences were detected with developed platform based on sandwich hybridization by using FAM labeled probes. A low yield was obtained for signal in some microrna. The reason for this may be miRNA concentration in total RNA is very low. The investigation of different visualization techniques will be useful to increase signal amount. Moreover, when we made quantification by using ImageJ, we can say that this platform gave good and compatible results for miR-21 when we applied all microRNAs with cell lines such as normal and cancer.

In this regard, all of purposes were reached and the concept validation studies were concluded with success, we can say that because miR-21 was generally used as marker for the diagnosis of many diseases. Our system can be developed for the detection of miR-21 in blood and urine and can be used as an alternative in the detection of many diseases.

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APPENDIX A

PREPARATION OF SOLUTIONS AND BUFFERS

100mM (10X) Phosphate Buffered Saline

(1370 mM NaCl, 100 mM Phosphate, 27 mM KCl)

80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 were dissolved in 1L of DEPC-treated distilled H_2O and pH was adjusted to 7.2. After adjustment, solution was autoclaved and the buffer was stored at room temperature.

5X Saline-Sodium Citrate Buffer

(75 mM sodium citrate, 750 mM sodium chloride)

22.06 g Sodium citrate and 43.8 g Sodium chloride were dissolved in 1L of DEPC-treated distilled H_2O and pH was adjusted to 7.0. After adjustment, solution was autoclaved and the buffer was stored at room temperature.

10% SDS

10 g SDS was dissolved in 100 Ml of DEPC-treated distilled H_2O. Sterilization of solution was made by filter sterilization and the solution was stored at room temperature.

2% BSA in 10mM PBS (1X)

2 g BSA was dissolved in 100 Ml of 10mM PBS Buffer and this solution was prepared fresh at every time.

1mM TCEP

0.00286 g of TCEP was dissolved in 100 Ml of DEPC-treated distilled H_2O. This solution was separated into aliquots and stored at -20^{0}C for maximum 2 months.

33.3mM SM(PEG)$_n$ (CL18)

1.418 mg SM(PEG)$_n$ was dissolved in 100 µL DMSO and separated into aliquots and stored at -20^{0}C for maximum 2 months. DMSO was stored at room temperature but it must be wrapped with aluminium foil to prevent it from light degradation.
This solution is very moisture sensitive chemical for this reason prepare stock solutions in DMSO. Take out SM(PEG)$_n$ bottle from refrigerator before 15 min to use preventing condensation.

APPENDIX B

THE SEQUENCES OF PROBES, TARGETS IN TABLES

B.1 The probe 1 sequences used in the first stage of study

Probe	P1 Sequences
▪ P1	5’-/5ThioMC6-D AAAAAAAAATCAACATCAGT-3’
▪ P1 –L	5’-/5ThioMC6-D AAAAAAAAATCAACATCAGT-3’
▪ P1 –M	5’-/5ThioMC6-D AAAAAAAAAUCAACAUCAGU-3’

**Locked and 2'O-methyl modified nucleotides are shown as underlined..*

B.2 The probe 2 sequences used in the first stage of study

Probe	P2 Sequences
▪ P2-F	5’-TGATAAGCTAAAAAAAAAA/6-FAM/-3’
▪ P2-L-F	5’-TGATAAGCTAAAAAAAAAA/6-FAM/-3’
▪ P2-M-F	5’-UGAUAAGCUAAAAAAAAAA/6-FAM/-3’
▪ P2-B	5’-TGATAAGCTAAAAAAAAAA/3Bio/ -3'
▪ P2-L-B	5’-TGATAAGCTAAAAAAAAAA/3Bio/ -3'
▪ P2-M-B	5’-UGAUAAGCUAAAAAAAAAA/3Bio/ -3'

** Locked and 2'O-methyl modified nucleotides are shown as underlined..*

B.3 The adapter sequences used in the first stage of study

Adapter	Adapter Sequences
▪ miR21	5’-UAGCUUAUCAGACUGAUGUUGA-3’
▪ Eşlenik Olmayan Adaptör (Ank)	5’-CACGUUCGGAUACAUCGAUCGAC-3’

B.4 The positive control sequences used in the first stage of study

Positive control	Positive control Sequences
▪ P1-FAM	5’-/5ThioMC6-D/AAAAAAAAATCAACATCAGT/36-FAM/-3’
▪ miR21 FAM	5’-UAGCUUAUCAGACUGAUGUUGA/36-FAM/-3’
▪ miR21-DNA	5’-TAGCTTATCAGACTGATGTTGA-3’

B.5 The probe 1 sequences used in the second stage of study

Probe	Probe 1 Sequences
▪ miR21-P1-L	5’-/5ThioMC6-D/AAAAAAAAATCAACATCAGT-3’
▪ miR10b-P1-L	5’-/5ThioMC6-D/AAAAAAAAACACAATTCGG-3’
▪ miR145-P1-L	5’-/5ThioMC6-D/AAAAAAAAAAGGGATTCCTG-3’
▪ miR125-P1-L	5’-/5ThioMC6-D/AAAAAAAAATCACAAGTTAG-3’
▪ miR200a-P1-L	5’-/5ThioMC6-D/ AAAAAAAAAACATCGTTACC -3’
▪ miR200b-P1-L	5’-/5ThioMC6-D/ AAAAAAAAATCATCATTACC -3’
▪ miR200c-P1-L	5’-/5ThioMC6-D/ AAAAAAAAATCCATCATTAC -3’
▪ U6-P1-L	5’-/5ThioMC6-D/AAAAAAAAACGATACAGAGAAGAT-3’

** Locked and 2'O-methyl modified nucleotides are shown as underlined..*

B.6 The probe 2 sequences used in the second stage of study

Probe	Probe 2 Sequences
▪ miR21-P2-L-F	5’- /TGATAAGCTAAAAAAAAAA/36-FAM/ -3’
▪ miR10b-P2-L-F	5’- /TCTACAGGGTAAAAAAAAAA/36-FAM/ -3’
▪ miR145-P2-L-F	5’- /GAAAACTGGACAAAAAAAAA/36-FAM/ -3’
▪ miR125-P2-L-F	5’- / GTCTCAGGGAAAAAAAAAA /36-FAM/ -3’
▪ miR200a-P2-L-F	5’- / GACAGTGTTAAAAAAAAAA /36-FAM/ -3’
▪ miR200b-P2-L-F	5’- / GGCAGTATTAAAAAAAAAA /36-FAM/ -3’
▪ miR200c-P2-L-F	5’- / CGGCAGTATTAAAAAAAAAA /36-FAM/ -3’
▪ U6-P2-L-F	5’- / AGCATGGCCCCTGCAAAAAAAAA /36-FAM/ -3’

** Locked modified nucleotides are shown as underlined..*

B.7 The adapter (miRNA) sequences used in the second stage of study

Adapter	Adapter Sequences
▪ miR21	5’- UAGCUUAUCAGACUGAUGUUGA-3’
▪ miR10b	5’- UACCCUGUAGAACCGAAUUUGUG -3’
▪ miR145	5’- GUCCAGUUUUCCCAGGAAUCCCU -3’
▪ miR125	5’- UCCCUGAGACCCUAACUUGUGA -3’
▪ miR200a	5’- UAACACUGUCUGGUAACGAUGU -3’
▪ miR200b	5’- UAAUACUGCCUGGUAAUGAUGA-3’
▪ miR200c	5’- UAAUACUGCCGGGUAAUGAUGGA-3’
▪ U6	5’- GCAGGGGCCAUGCUAAUCUUCUCUGUAUCG-3’

B.8 The sequences of probes and adapters to test sandwich system

P/A	Sequences
P1	5’-/5ThioMC6-D/AAA AAA AAA GCA TCT TCA ACG ATG GCC TTT CCT TT-3’
P2	5’-TCG CAA TGA TGG CAT TTG TAG GAG CAA AAA AAA A/6-FAM-3’
Ank	ACC CTG TAA ACG ATC ATC CCC ATT TTT TAC GGC CAATTG GAG GCCTCC CAAT-3’
A	5’- GCT CCT ACA AAT GCC ATC ATT GCG ATA AAG GAA AGG CCA TCG TTG AAG ATG C-3’

APPENDIX C

QUANTIFICATION OF MICRORNAS BY USING RT-PCR

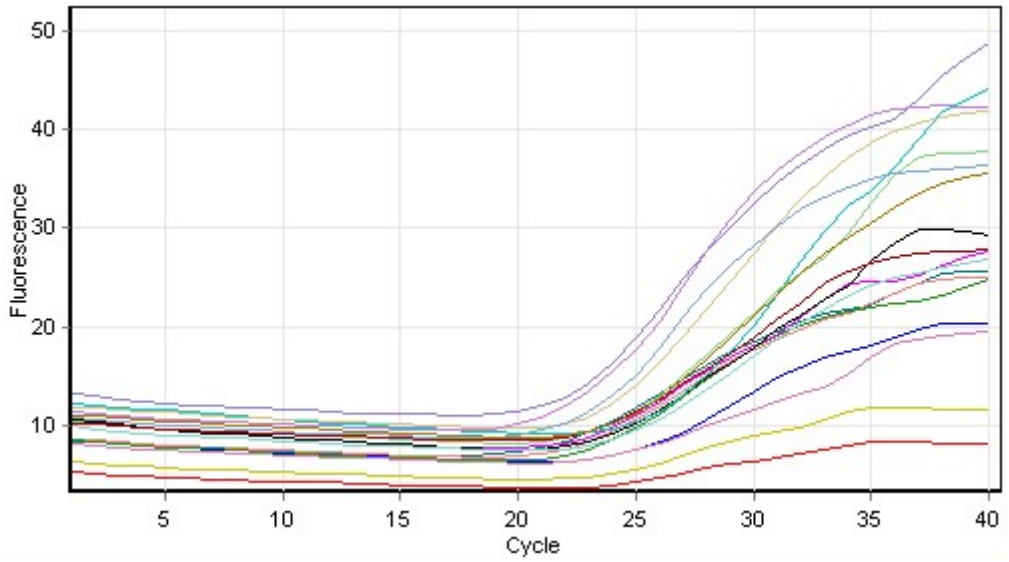

Figure C.1 Concentration of miR-21

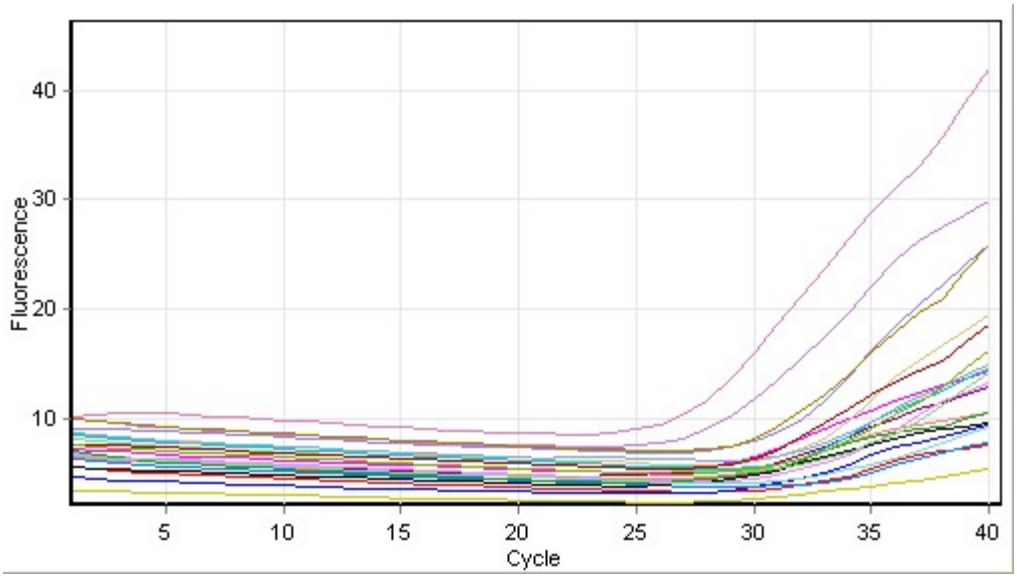

Figure C.2 Concentration of miR-10b

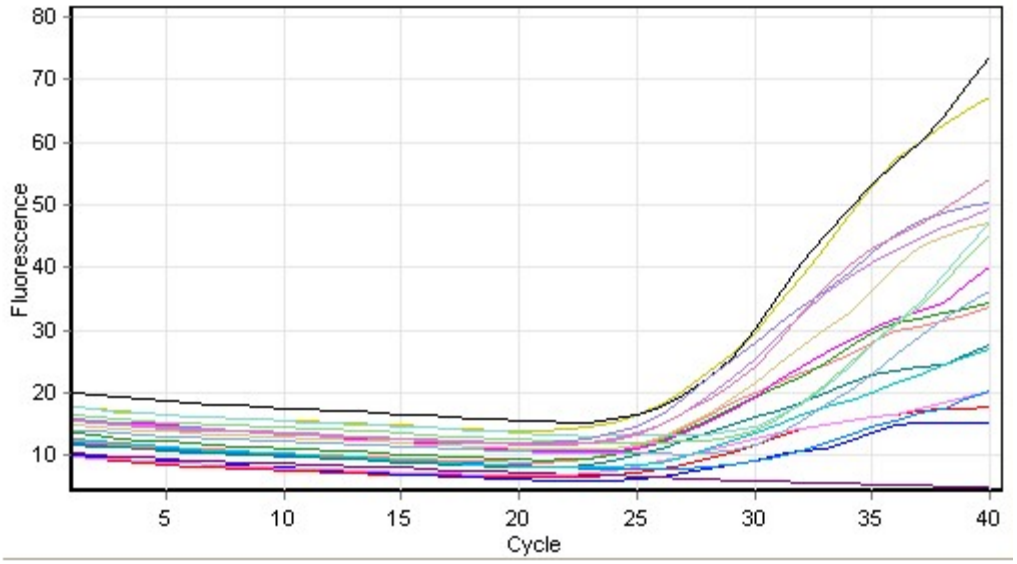

Figure C.3 Concentration of miR-125b

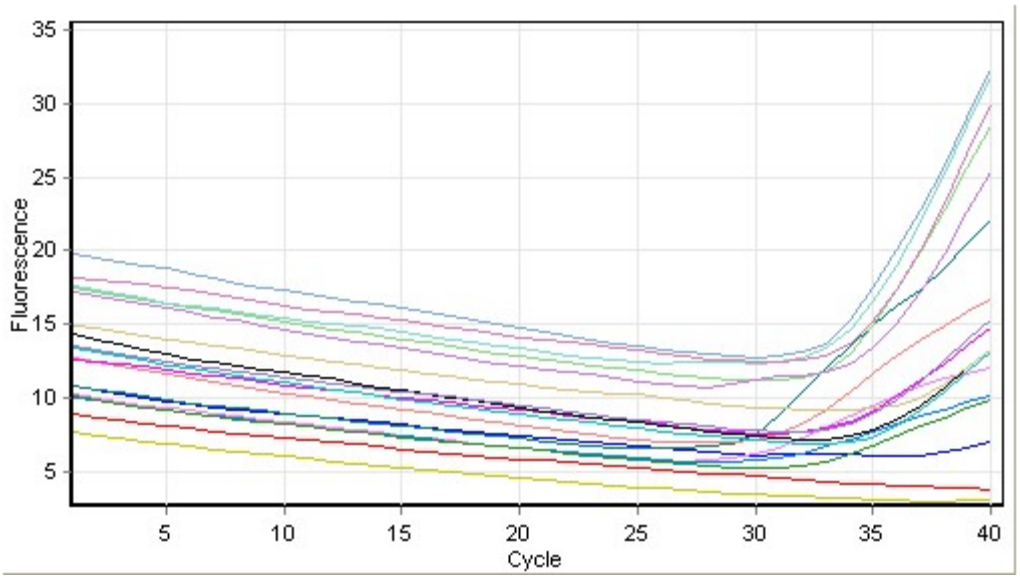

Figure C.4 Concentration of miR-145

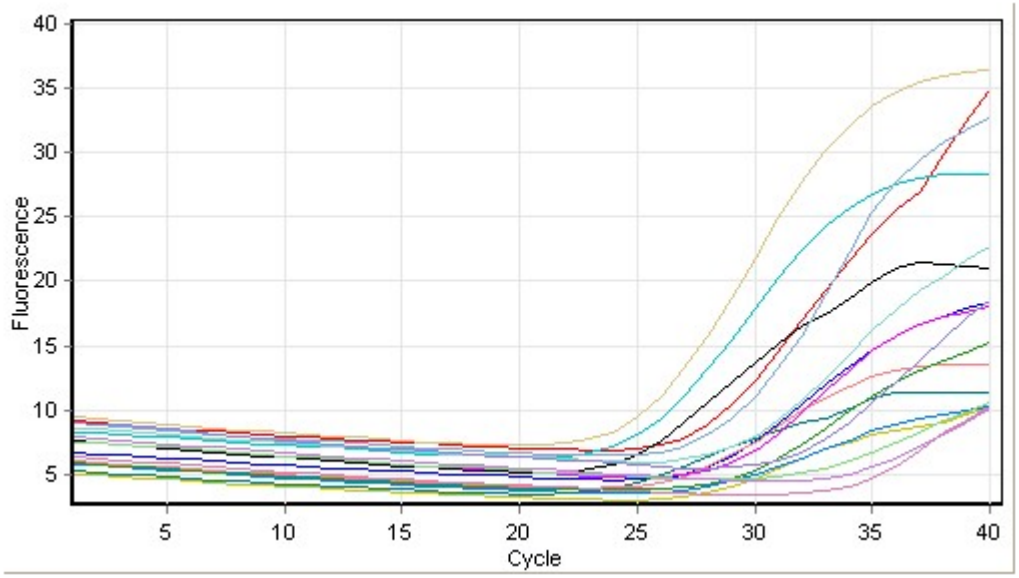

Figure C.5 Concentration of miR-200a

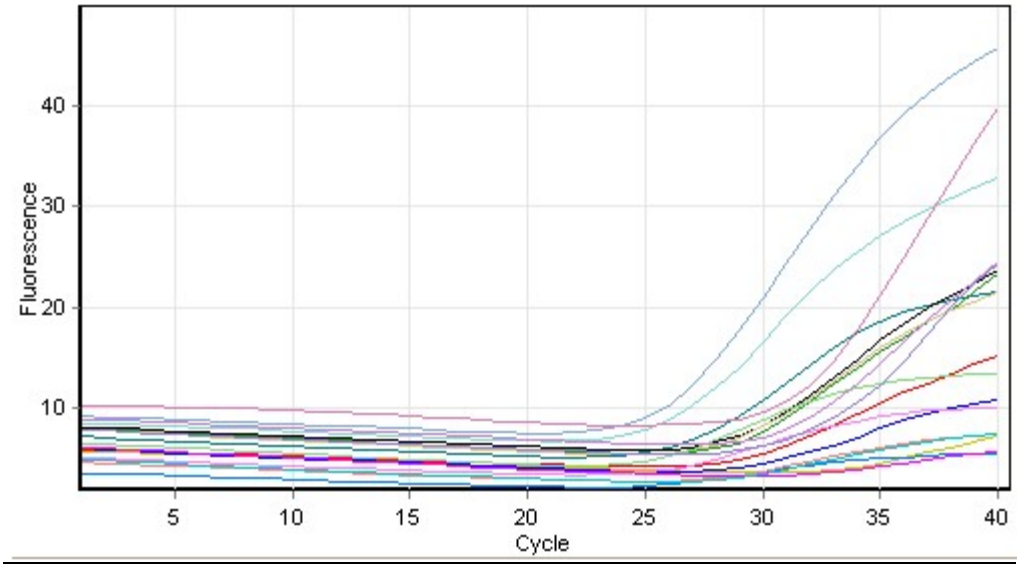

Figure C.6 Concentration of miR-200b

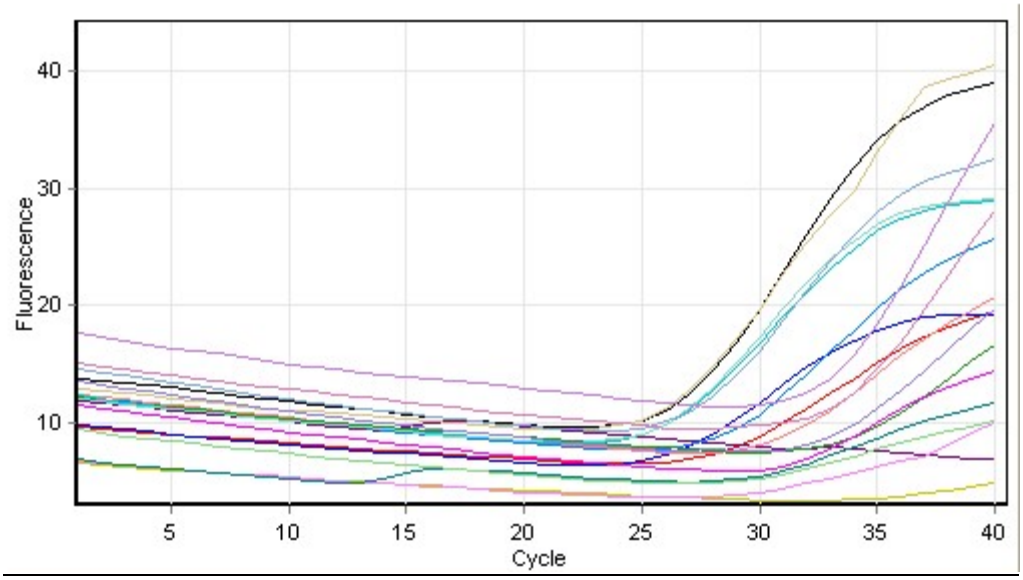

Figure C.7 Concentration of miR-200c

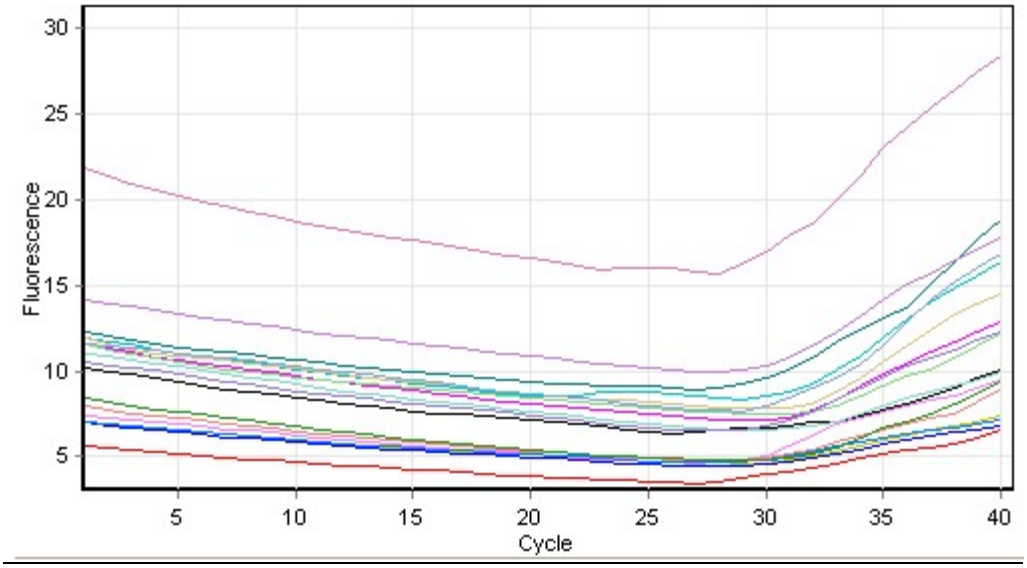

Figure C.8 Concentration of U6