

## DNA NANOTECHNOLOGY: Building and characterizing DNA-constructs for mechano-chemical studies of single DNA-ligand interaction

*Master of Science Thesis*

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THESIS FOR THE DEGREE OF MASTER OF SCIENCE

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Cover picture:

The general scheme of this project, starting from hybridization of two single-stranded oligonucleotides into double-stranded duplexes, enzyme ligation with T4 ligase and finally assembling of concatemers on gold surface by QCM-D

Department of Chemical and Biological Engineering  
Göteborg, Sweden 2012

*To my family ...*

*"Any man who knows all the answers most likely misunderstood the questions"*

*(Nancy Willard)*

# DNA nanotechnology: Building and characterizing DNA-constructs for mechanochemical studies of single DNA-ligand interaction

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## ABSTRACT

Self-assembly of oligonucleotides with designed sequence, is very valuable method for building fairly long linear DNA constructs. Studies of sequence-specific interaction between different ligands and single DNA molecules are becoming increasingly important. An approach toward this end is assembling DNA molecules by oligonucleotides hybridization in solution. In this project we attempted to construct double strand DNA concatemer (>500 base pairs) from two specific sequence- designed 50bp oligonucleotides by hybridization and ligation. Due to complementary base pairing process, two single stranded oligos can spontaneously attach together and form long comcatemeric dsDNA molecules. Various incubation conditions such as oligos concentration, heating and hybridization time, salt and PEG concentration were checked to optimize the building of longer DNA constructs. Also enzyme ligation was performed successfully to join the nicks in the concatemers. Gel electrophoresis was employed to verify size and shape distribution of formed constructs. For obtaining linear constructs and avoiding circular DNA formation, step-wise adding of oligonucleotides on solid surface was done using QCM-D technique. With this method, linear DNA constructs up to hundred base pairs were formed reasonably and can be used as a helpful tool for single-DNA molecule studies.

Keywords:

DNA, Hybridization, Ligation, Gel Electrophoresis, Oligonucleotides, QCM-D

## List of Abbreviations

A	Adenine
APS	Ammonium Persulfate
ATP	Adenosine Tri-Phosphate
BIS	N, N'-methylene-bisacrylamide
bp	base pair(s)
C	Cytosine
DNA	DeoxyriboNucleic Acid
dsDNA	Double-strand DNA
EDTA	Ethylene Diamine Tetraacetic Acid
EEO	Electroendosmosis
EtOH	Ethanol
G	Guanine
GE	Gel Electrophoresis
IEF	Isoelectric Focusing
MBE	Moving Boundry Electrophoresis
mw	Molecular weight
OD	Optical Density
PAAG	PolyAcrylAmide Gel
PAGE	PolyAcrylamide Gel Electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PEG	PolyEthylene Glycol
PFGE	Pulsed-Field Gel Electrophoresis
PNK	PolyNucleotide Kinase
RNA	Ribo-Nucleic Acid
RT	Room temperature
SDS	Sodium Dodecyl Sulfate
ssDNA	Single- strand DNA
T	Thymine
TBE	Tris-borate-EDTA buffer
TEMED	N,N,N',N'-Tetramethylethylenediamine
$T_m$	Melting temperature of DNA
QCM-D	Quartz Crystal Microbalance with Dissipation
ZE	Zone Electrophoresis

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## 1 Introduction

Today nucleic acids play very important role in cellular processes such as cell division (DNA replication) and protein synthesis (transcription and translation). Also as healthy and cancer cells, both are influenced by these processes, nucleic acids are excellent targets for anti-cancer drugs (Palchaudhuri & J Hergenrother, 2007).

DNA nanotechnology involves the design and manufacture of novel constructions with specific geometrical and topological properties (Seeman, 1998).

DNA is a unique structural element for nanotechnology, since it is the building block of living cells with the highest information content and its self-assembly, interactions and biological functions is most predictable compared to other types of biomolecules (Gothelf & H. LaBean, 2005).

The sequence-specific interaction of different ligands with DNA is very interested field because of huge potential applications in biophysics and medical chemistry, the design of medicines that can site-specifically bind to DNA is an important pharmacological task, for example, the therapeutic activity of most of the currently used antitumor drugs depends on the potency and selectivity of their interaction with DNA, for instance DNA-intercalating ligands as anti-cancer drugs has developed significantly (Denny, 1989). Over the past thirty years many small molecules have been discovered that bind to DNA in a sequence selective fashion, several of these are used as therapeutic agents for treating cancer and other diseases.

Recently new techniques have been developed to study the behavior of individual DNA molecule during DNA-ligand binding reaction. Techniques are used for analysis of sequence-specific dsDNA-ligand interaction have developed widely today, for instance by means of fluorescent intercalator probes (Kirschstein, Sip, & Kittler, 2000), but this is the first time that, interaction of DNA-ligand binding at the single DNA level is investigated. The initial purpose is, to construct long linear dsDNA molecules (ca. 500bp in length) by self-assembly of single-stranded oligonucleotides.

## 1. *Introduction*

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Since building such DNA-constructs with desired length is not easy from scratch, our suggestion is to hybridize them by utilizing two 50-mer pre-designed sequenced single strand oligonucleotides, which could be done in a process by which half-slide complementary oligonucleotide pairs spontaneously assemble into some long concatemeric structures. According to Simonova et al report (Simonova, Vladimirova, Zenkova, & Vlassov, 2006) 24 and 25-mer antisense oligonucleotides have been designed for assembling into concatemeric complexes which are used for increasing cellular binding efficiency. As mentioned in this report the ability of such concatemeric structures gets better by increasing length and concentration.

Synthesized oligonucleotides with pre-specific designed sequence are simple and useful materials for self assembly procedure. By varying incubating conditions such as hybridization time, oligonucleotide concentration and salt concentrations, a much optimized method for formation of long DNA concatemers can be achieved.

Agarose gel electrophoresis is the main method to verify the size distribution of constructs and polyacrylamide gel electrophoresis can separate constructs in shape difference. By attaching oligonucleotides onto a solid surface, complimentary oligonucleotides will add to achieve step-wise building of DNA concatemers, QCM-D and SPR can be used for this purpose.

## 2 Theory

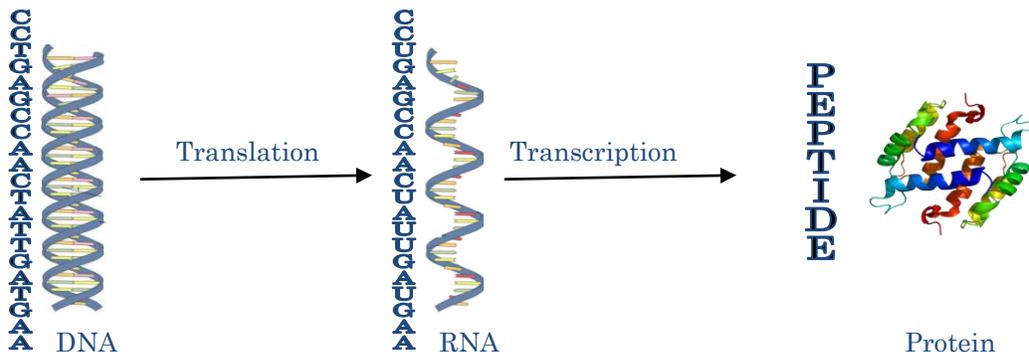
This chapter explains the theoretical background of the various compounds, materials and techniques used in this project.

### 2.1 DNA

"DNA is more than just the secret of life – it is also a versatile component for making nanoscopic structures and devices"

(Nadrian C. Seeman)

DNA has been recognized as the genetic material of living organisms for more than 60 years. As it was proposed by Crick it is central dogma of life and building block of proteins (Crick, 1970) (Figure.1). The human genome is the full complement of DNA from an organism that contains the genetic instructions and carries all the information needed for functioning of living cells.



*Figure. 1 Central dogma of life from DNA to protein*

DNA has macromolecular structure composed of ordinary repeating long polymers formed from nucleotides. According to Watson - Crick Model, DNA molecule consists two polynucleotide strands twisted around each other in a helical structure (Watson & Crick, 1953). The backbone of strands is made from sugar and phosphate groups joined by ester bonds (Figure.2). The sugar in DNA is 2-deoxyribose, which is a pentose sugar. The sugars are joined together by phosphate groups that form phosphodiester bonds between the third and fifth carbon atoms of adjacent sugar rings. The asymmetric ends of DNA strands are labeled the 5' and 3' ends, which the 5' end has a free phosphate group and the 3' end a free hydroxyl group. The double helix DNA (or double strand DNA which refers to dsDNA) has antiparallel structure which means the two polymer strands run in opposite directions, one in the 5' to 3' and the other in the 3' to 5' direction.

## 2. Theory

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The DNA strand consists of two purine bases, namely the Adenine (A) and Guanine (G), and two pyrimidine bases, Thymine (T) and Cytosine (C), which are attached together by hydrogen bonds, adenine to thymine by two hydrogen bonds and cytosine to guanine by three hydrogen bonds (Figure.3). The sequence of bases in one strand is complementary to that in the other strand, which means adenine always pairs with thymine and guanine always pairs with cytosine. The base pairing of two complementary strands of DNA molecule permits a double strand form. The double-helix strands (dsDNA) are held together by two forces, hydrogen bonds between nucleotides and partly base-stacking (hydrophobic) interactions among adjacent, stacked base-pairs.

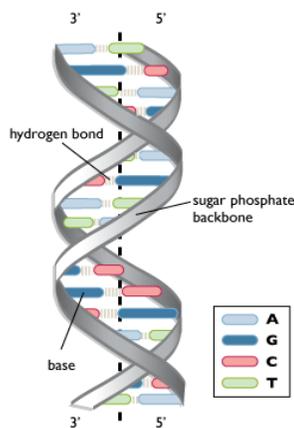


Figure. 2 The DNA double helix structure

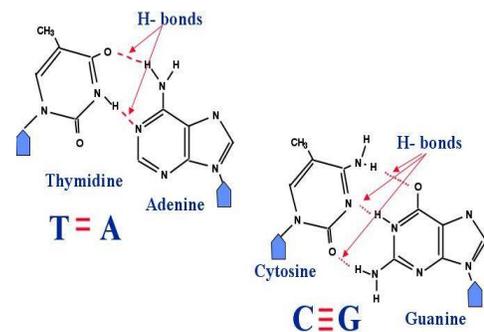


Figure. 3 Base-pairing in DNA molecule

### 2.1.1 DNA Nanotechnology

DNA nanotechnology employs DNA constructs to manipulate the spatial and temporal distribution of matter and can be divided into two fields, structural and dynamic DNA nanotechnology (Zhang & Seelig, 2011).

The specific and versatile properties of DNA, makes it a unique and powerful material for nano engineering applications. DNA has extraordinary properties to construct complex functional nano structures (Gothelf & Brown, 2005). Great number of researches and developments has led to design and characterize of novel constructions of DNA nanostructures and nanodevices within the past few decades.

## 2. Theory

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DNA nanotechnology is now very rapidly growing science and becoming increasingly attractive to different bio engineering fields with interesting large and diverse applications.

Today DNA nanotechnology has advantages, manipulation of DNA constructs is easy because of known DNA local structure, it can also be manipulated using different enzymes, DNA is one of the best nanoscale materials because it can self assembled, self replicate and adopts various states and conformations. So DNA is used as a structural material in nanotechnology to direct the assembly of highly structured material with specific nanoscale features, instead of just the carrier of genetic information (C.Seeman, 2003).

The area of DNA nanotechnology has the potential to greatly impact the future of bioscience, the very specific bonding and binding properties, makes it undoubtedly a widely fascinating structural material.

### 2.1.2 DNA Hybridization

DNA is the molecule of heredity, evolutionary process changes is reflected in changes in the base pairs in DNA molecule. Two species that have evolved from a common ancestor have very similar base pair sequences in their DNA molecules. The degree of relatedness of two species can be assessed by studying how similar their base pair sequences are. DNA hybridization is the method of examining this similarity.

In molecular genetics, for hybridization, single strands of DNA from two different species are permitted to join together to form hybrid double helices. These hybrid fragments of DNA can be applied to determine the evolutionary relatedness of organisms by examining how closely two species in DNA base pair sequences are. The degree of hybridization is proportional to this similarity.

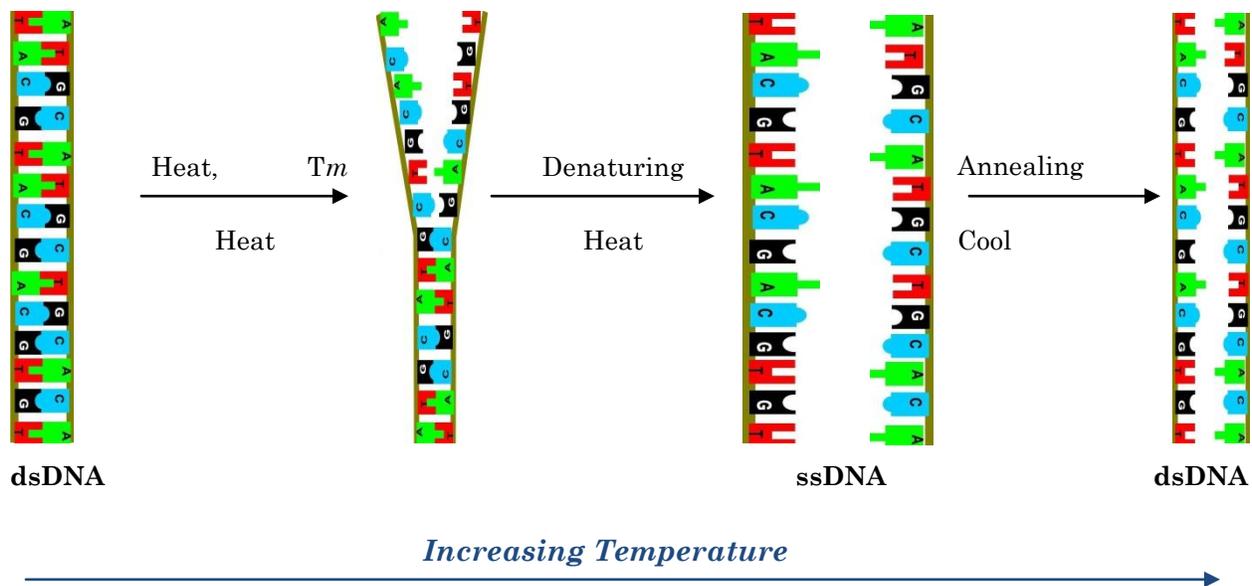
Hybridization of DNA is achieved by denaturing strands of DNA from two different species by heating up to 86° C [186.8° F]. The hydrogen bonds between all complementary base pairs will break and a mixture of single-stranded segments of DNA is incubated, allowing similar strands from both species chemically join together or re-anneal at complementary base pairs by reforming hydrogen bonds to recombine into hybrid DNA (Figure.4).The conversion of dsDNA to ssDNA by heating can be influenced by many factors such as temperature, salt concentration,

## 2. Theory

sequence composition, inorganic solvents and the degree of sequence similarity or mismatch between ssDNA molecules.

As the amount of annealing is directly proportional to the similarity of the DNA strands, it is possible to heating the hybrid DNA and recording the temperature at which the strands separate.

The temperature at which hybrid DNA separation takes place is related to the number of hydrogen bonds formed between complementary base pairs. Consequently, if the two species are strictly related, most base pairs will be complementary and the temperature of separation will be close to 86° C [186.8° F]. But if the two species are not closely related, they will not share many common DNA sequences and fewer complementary base pairs will form. It is because less energy is needed to break hydrogen bonds, therefore the temperature of separation is less than 86° C.



**Figure. 4 Mechanism of Hybridization.**

$T_m$  refers to the temperature at which 50% of the DNA is hybridized.

Heating the DNA above its melting temperature ( $T_m$ ) will separate two strands. Above the  $T_m$ , DNA is mostly in single-stranded form and below the  $T_m$ , in double-stranded form. Principally the  $T_m$  depends on GC-content so DNA molecules with AT-rich sequences have lower  $T_m$  values, therefore in this way DNA-denaturation (by comparing melting temperatures) can be used as a tool to detect sequence differences.

## 2. Theory

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### 2.1.3 DNA Ligation

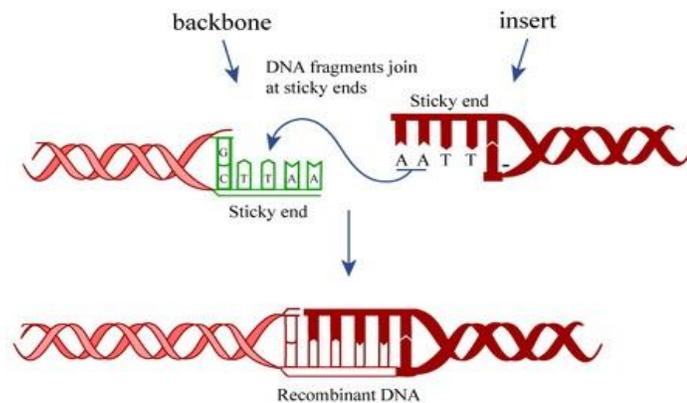
Generally ligation means connecting linear DNA fragments with covalent bonds, but in particular in molecular biology, ligation is generating a phosphodiester bond between 3' hydroxyl and 5' phosphate of two nucleotides.

T4 DNA ligase is used as enzyme for ligating DNA fragments. This enzyme ligates fragments with blunt or cohesive ends. A ligation process needs three components, DNA fragments, T4 DNA ligase and buffer contains ATP.

The amount of DNA ligase needed for ligation depends mainly on the nature of DNA fragments to be ligated, particularly whether they are blunt or cohesive ends. Normally more enzyme has to be used for blunt ends fragments (Gaastra & Hansen, 1985).

**Figure. 5 Simple schematic of DNA ligation reactions.**

(Figure by MIT OpenCourseWare)



## 2.2 Oligonucleotide

An Oligonucleotide (Oligomer) is normally a short nucleic acid polymer with fifty or fewer bases which are formed by covalently binding of the 5'-phosphate group and 3'-hydroxyl groups to form phosphodiester bonds. They are usually synthesized in a sequence-specific manner from single nucleoside phosphoramidites. Synthesis of appropriate length oligonucleotides (50 bases or even longer) is now routine (R.Fox, 1997).

Oligonucleotides with different sequences have specific chemical and biophysical properties and generally are used as primer for DNA synthesis. Some of the techniques that employ oligonucleotides as their prime component are hybridization, sequencing, Southern blotting and polymerase chain reaction (PCR), (Chavali, Mahajan, Tabassum, & Maiti, 2005).

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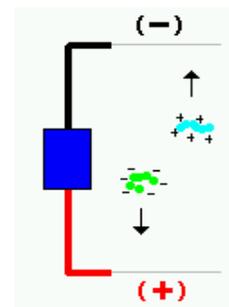
### 2.3 Electrophoretic Techniques

#### 2.3.1 Fundamental Principles

The term electrophoresis was coined from the Greek word *phoresis*, which means 'being carried', in other words being carried by an electrical field, it describes the migration of charged (dispersed) particles under the influence of an electric field. Many important biological molecules, such as amino acids, peptides, nucleic acids, nucleotides and proteins, possess ionisable species either as cations (+) or anions (-). Under the influence of an electric field these charged particles will migrate either to the cathode or to the anode, according to the nature of their net charge.

Electrophoresis is a technique used to separate and sometimes purify macromolecules, especially proteins and nucleic acids - that differ in size, charge or conformation, so it is one of the most widely-used techniques in biochemistry, biophysical chemistry and molecular biology.

*Figure. 6 Migration of charged ions during electrophoresis*



In order to understand better how charged species separate it is necessary to look at some simple equations relating to electrophoresis. When a potential difference (voltage) is applied across the electrodes, it generates a potential gradient,  $E$ , which is the applied voltage,  $V$ , divided by the distance,  $d$ , between the electrodes. When this potential gradient  $E$ , is applied, the force on a molecule bearing a charge of  $q$  coulombs is  $Eq$  newtons. This is the force that drives a charged molecule towards an electrode. But, also there is a frictional resistance that retards the movement of this charged molecule. This force depends on the hydrodynamic size and shape of the molecule, the pore size of the medium in which electrophoresis is running and the viscosity of the buffer.

## 2. Theory

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The velocity,  $v$ , of a charged molecule in an electric field is therefore given by the equation:

$$v = \frac{Eq}{f} \quad \text{where } f \text{ is the frictional coefficient} \quad (2.1)$$

The electrophoretic mobility ( $\mu$ ), defines the ratio of the velocity of the ion to field strength ( $v/E$ ). When a potential difference is applied, therefore, molecules with different overall charges will begin to separate due to different electrophoretic mobilities. Because of different frictional forces, even molecules with similar charges could be separate if they have different molecular sizes (Wilson & Walker, 2010).

During an electrophoresis experiment, a protein or nucleic acid experiences electric force proportional to its effective charge,  $q$ , and also the electric field strength,  $E$ . As the electric force of the molecule is affected by the opposite force from the frictional resistance,  $F_f$ , of the gel matrix, it will very soon move with a constant velocity,  $v$ .

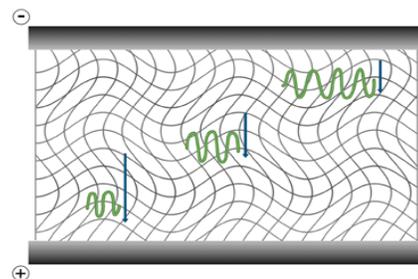
In free solution,  $f$  will follow Stoke's law:

$$f = 6(\pi) r/n = 6\pi\eta R \quad (2.2)$$

Where  $r$  is the radius of the particle moving with velocity  $v$  through a medium of viscosity  $n$ . Mobility in free solutions would then be the same for molecules of same charge to mass ratio. However Stoke's law is not enough to explain the frictional force within a gel matrix, because determination of density and effective pore size of the matrix are important, so the influence of this combination of factors is that, among the molecules with the same charge to mass ratio, smaller molecules move faster and electrophoretic separation occurs by size (Figure.7).

**Figure. 7 The gel electrophoresis of samples having identical charge to mass ratios results in fractionation by size**

larger molecules move more slowly because of their lower electrophoretic mobility



## 2. Theory

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### 2.3.2 Short History of Different Electrophoretic Methods

Historically, the development of electrophoresis began with the pioneering work of the Swedish biochemist, Arne Tiselius, published his first paper on electrophoresis in 1937, it represented a novel technique for studying of physico-chemical properties of different proteins. He was awarded the Noble prize on his work in 1948 (Vesterberg, 1993).

The basic form of electrophoresis performed by A. Tiselius and co-workers using a U-shaped type of glass with an electrode at both end, provided an efficient method for separation of molecules in free solution, this form of moving boundary electrophoresis (MBE) was replaced in the 1950s by zone electrophoresis (ZE), which relied on the separation of molecules on a solid support (e.g. paper or cellulose).

The most commonly method, gel electrophoresis (GE) was also introduced in the 1950s by Oliver Smithies. At first, GE was mainly performed for bioanalytical analyses, but has since evolved as a major preparative technique to partially purify biomolecules before further characterization by other advanced technologies including: immunoblotting, mass spectrometry, and molecular techniques such as polymerase chain reaction (PCR) and DNA sequencing. While starch-block electrophoresis introduced the concept of sieving, other important developments in the 1960s included Svensson's isoelectric focusing (IEF) and Shapiro and colleagues' sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Where IEF allowed separation based on surface charge, the now-popular SDS-PAGE allowed separation of biomolecules on the basis of molecular mass. The 1960s also was the development of capillary electrophoresis (CE) by Hjerten, which has concerned much interest because of high resolution for proteins, nucleic acids, peptides and pharmaceuticals on the analytical scale, and today is very accepted method in the field of clinical chemistry.

***Figure. 8 Arne Wilhelm Kaurin Tiselius (1902 –1971)***

Swedish biochemist who won the Nobel Prize in Chemistry in 1948 for his research on electrophoresis (development of MBE method) and chromatographic adsorption analysis.

Photo by Nobel Foundation



## 2. Theory

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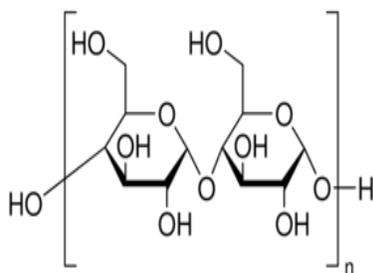
During the last forty years a number of electrophoretic methods have developed quickly to suggest high resolution analytical and preparative separations in biochemistry, bioanalytical chemistry and life sciences for research and various applied purposes. Over the last years electrophoretic separations in gels and numerous applications in nucleic acid (DNA,RNA) field have been much improved , making possible well-organized mapping of the human genome, and many other applications in molecular biology, genetics and medicine (Vesterberg, 1993).

### 2.3.3 Support media

As mentioned before the Nobel Prize in Chemistry in 1948 was awarded to Tiselius for carrying out electrophoresis in free solution, but very soon researchers found out that many problems such as the adverse effects of diffusion and convection currents arise from this approach. Later it was realised that performing electrophoresis on a porous mechanical support could minimise problems. This type of support media reduces convection currents and diffusion. The first used supports were filter paper or cellulose acetate strips, which should wetted in buffer, but the problem is that the separation of macromolecules such as nucleic acids and proteins on such supports is not strong (Wilson & Walker, 2010).

In last decades after introduction of gels as support medium a rapid advancement in methods for analysing of macromolecules happened. Although the earliest gel system was starch gel, but nowadays agarose gels and polyacrylamide gels are the most important used gel systems for electrophoretic techniques.

These gels immerse within an electrophoresis buffer that supply the essential ions for carrying the current and some type of buffer for controlling the pH in a relatively constant range.



**Figure. 9 Chemical structure of starch**

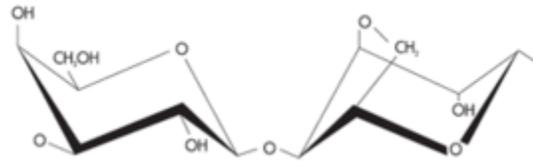
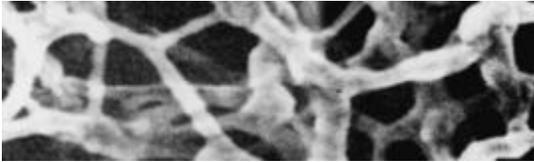
extracted from potato. The first gel-electrophoresis method, have used starch as gel system

## 2. Theory

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### 2.3.4 Agarose gels

Agarose is a linear polysaccharide, extracts from certain seaweed, which is one of the components of agar and consists of agarobiose units with relative molecular mass about 12000 (Wilson & Walker, 2010).



**Figure. 10 Agarobiose**

the repeating unit of agarose, repetition of (1->3)- $\beta$ -D-galactopyranose and of (1->4)-(3,6)-anhydro- $\alpha$ -L-galactopyranose

Agarose is usually used at concentrations of between 0.5% and 2%. The higher concentration makes stiffer gels with smaller pore sizes. Agarose gels are very easy to prepare, just suspending dry powder agarose in aqueous buffer, boiling up to a clear solution, pour it and let be formed in room temperature. It is also non toxic, non hygroscopic, electrically neutral and biologically inert. Based on sulphate concentration agarose has different purity grades, lower sulphate content, the higher agarose purity.

Different agarose concentrations have large range of separation but comparatively low resolving power, fragments of DNA from about 200 to 50,000 bp can be separated.

Agarose electrophoresis is widely used for analyzing the relative molecular weight of DNA, it was shown in previous studies that the mobility of DNA in agarose gel is inversely related to its sedimentation rate in sucrose density gradient (Takahashi, Baba, & Ogino, 1969), and therefore electrophoresis in agarose gel can suitably be used to approximate the relative molecular length of DNA.

## 2. Theory

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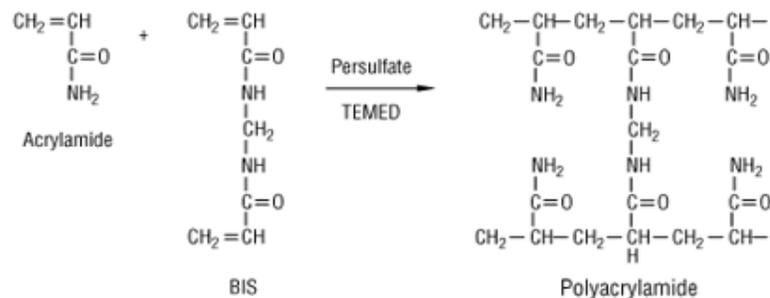
### 2.3.5 Polyacrylamide gels

Polyacrylamide gel electrophoresis (PAGE) is used widely for separation of proteins and nucleic acids because of unparalleled resolution and flexibility of applications.

Cross-linked polyacrylamide gels are formed by copolymerization of acrylamide monomer and N,N'-methylene-bisacrylamide (BIS) in a vinyl addition polymerization reaction which initiated by a free radical-generating system using ammonium persulphate (APS) and the base N,N,N',N'-tetramethylethylenediamine (TEMED) (Fig.2.10). TEMED accelerates the decomposition of persulphate ion and formation of free radicals (Wilson & Walker, 2010) :



For starting polymerization chain reaction, the persulfate free radicals convert acrylamide monomers to free radicals which react with unactivated monomers (Shi & Jackowski, 1998). The elongating polymer chains are crosslinked in a random fashion by bis acrylamide, and a gel with specific porosity characterizations forms, which depends on the polymerization conditions and monomer concentrations (Figure.11).



**Figure. 11** The polymerization of polyacrylamide gel from acrylamide and bis-acrylamide

The effective pore size of polyacrylamide gels is related to acrylamide concentration, when acrylamide concentration increases the gel pore size decreases, so it is an inverse function of "total monomer concentration", (%T), described as the sum of the acrylamide monomer and the crosslinking agent concentrations (Allen, Saravis, & Maurer, 1984).

## 2. Theory

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### 2.3.5.1 Denaturing PAGE

Denaturing polyacrylamide electrophoresis is very helpful technique which is used for many applications such as analysis of proteins and separation and purification of single stranded DNA and RNA. It can also be used for chain length and molecular weight determination of small DNA and RNA molecules (Frank & Köster, 1979).

The electrophoretic analysis of single stranded nucleic acids is complicated by the secondary structures attained by these molecules. Even introduction of thin gel techniques employing higher temperature around 50°C was not exceptionally successful to melt very stable secondary structures (Frank, Müller, & Wollf, 1981), it seems, separation on the basis of molecular weight requires the addition of denaturing agents which unfold the DNA strands and eliminate the influence of shape on their mobility properties.

Fragments between 2 to 500 bases can be separated with this method. Gels with different pore sizes are prepared by varying the amount of acrylamide (Table.3.3), higher concentration gels for separating smaller fragments and lower concentration gels for larger fragments. Hydrogen bonds between bases stabilize nucleic acids structures. Disturbing these hydrogen bonds is needed for denaturing. The most frequently used DNA denaturants are urea and formamide, 7M (mol L<sup>-1</sup>) urea is widely used as denaturant agent resolved in gel structure and formamide usually is added to samples in different concentrations. The denaturing effect of urea is greatly increased by temperature, so electrophoresis usually carry out in a 60 or 70°C incubator, the maximum denaturing effect is reached if the electrophoresis run at this temperature (Southern, 2002). These denaturants effectively lower the melting temperature of DNA concatemers and accomplish the denaturation by forming new hydrogen bonds with the DNA bases, consequently "saturating" H-bond sites and avoiding the formation of inter-base bonds. Other denaturants including NaOH, formaldehyde and DMSO can be used in some cases.

## 2. Theory

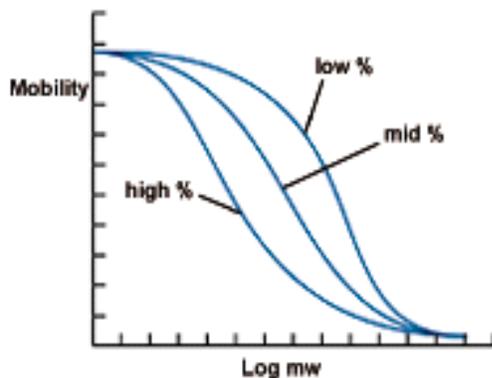
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### 2.3.5.2 Native PAGE

As seen from the nomenclature, native PAGE separations run in non-denaturing conditions, therefore here a denaturing agent is not needed. Double stranded DNA maintains its double helical structure, so with rodlike shape migrates through the gel. But single stranded DNA can assume various conformations under native conditions, it depends to solution, temperature and environment.

As DNA molecules have a uniform negative charge density provided by phosphate groups in its backbone, in free solution it has mobility which is independent of molecular size. But in a sieving medium such as polyacrylamide gel, the ability of molecule to find a way through the gel pores determines the relative mobility properties of molecule.

It was shown that there is a linear relation between the effective radius of the molecule and the log of the molecular weight. As a result, on a gel of specific concentration and porosity properties, the distance that a given fragment could migrate is proportional to the log of its molecular weight (log mw) (Figure.12).



*Figure. 12 Electrophoretic mobility versus molecular weight*

Photo by [www.nationaldiagnostics.com](http://www.nationaldiagnostics.com)

This proportionality of log mw with mobility is valid just for certain conditions. For small size molecules, as the gel could not sieve the molecule effectively, these small molecules and others with smaller size, migrate at the same rate. Also for extremely large DNA molecules are unable to find a way through the gel and the migration distance is almost zero.

Sequence of bases in dsDNA in some cases affects the mobility of the molecule, certain sequences cause bending of helical structure, which can decrease the speed of migration.

## 2. Theory

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### 2.4 QCM-D

#### 2.4.1 The QCM-D principle

The Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D) is a particular type of QCM technique based on the ring-down rather than impedance analysis which commercialized by Chalmers group (Rodahl & Kasemo, 1996). It is a useful technology in surface analysis. In brief, it used for measuring structure, viscoelastic properties and mass changes in real-time with nano-sensitivity.

#### 2.4.2 History of QCM

For more than 50 years, QCM (Quartz Crystal Microbalance) is a traditional, accepted method for analyzing mass changes on rigid surfaces, most successfully as a sensor in, e.g. film deposition and film growth in air and vacuum medium. So it is used commonly as the frequency determining element of an electronic oscillator and recording the oscillation frequency changes (Rodahl & Kasemo, 1996). QCM was not performed normally in liquids because the liquid overdamp the oscillation (Johannsmann, 2008).

The basic principle of QCM is that a voltage is applied to a quartz crystal causing it to oscillate at a specific frequency. According to Sauerbrey equation (Eq.2.5) changes in mass on quartz surface are directly related to changes in frequency of the oscillating crystal:

$$\Delta m = -C \cdot \Delta f \quad (\text{Simple form of Sauerbrey equation}) \quad (2.5)$$

Where  $\Delta m$  is mass change (g) and  $\Delta f$  is frequency change (Hz).

The Sauerbrey equation is valid just for rigid, suitably thin adsorbed layers and applies for oscillation in air only, so for soft, viscoelastic films or measurements should be performed in liquid, Sauerbrey relationship undervalue the mass and therefore another technique is needed to fully analyze such material.

There are some disadvantages of using an oscillator that only permits measurements of resonant frequency: such as (1) admixture of true frequency with frequency from the energy supplying elements due to for instance a mass increase,

## 2. Theory

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(2) unknown phase shift of electronic oscillator is used for QCM resonant frequency, (3) missing of dissipation factor which in many cases is important (Rodahl & Kasemo, 1996).

### 2.4.3 Dissipation

QCM-D measures frequency and dissipation of quartz crystal together. When the driving voltage is turned off, dissipation happens, where the energy from the oscillating crystal dissipates from the system. D describes dissipation and is defined as follows:

$$D = E_{\text{lost}} / 2\pi E_{\text{stored}} \quad (2.6)$$

Where  $E_{\text{lost}}$  is the energy lost during one oscillation cycle and  $E_{\text{stored}}$  is the total energy stored in the oscillator.

Dissipation analysis allows qualitative measurements of the structural properties of adsorbed layers. By combining frequency and dissipation measurements, the QCM-D technology enables quantitative analysis of the thickness, viscosity, morphology and shear elastic modules of the adsorbed layers where these types of analyses are beyond the Sauerbrey approved limitations. Also with this technique it is possible to make certain which materials can be studied in the Sauerbrey regime or not.

Consequently QCM-D makes it possible measuring both mass and structural properties in real-time. By measuring dissipation parameter, analysis of soft films and other materials that do not follow the linear relation between mass and frequency changes, could be done.

### 2.4.4 Applications in Bio- science

In recent years biology-based studies using QCM-D technique is rapidly growing. In many biomolecular studies the dissipation parameter and the consequently extracted viscoelastic parameters are essential for many applications. For example in cellular adsorption applications, the QCM frequency and Sauerbrey relationship can be used for underestimating of adsorbed mass of cell (Dixon, 2008).

## *2. Theory*

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DNA as an important biological material has been used in a wide range of researches using QCM-D technique. Some applications are DNA-protein interactions, gene delivery, DNA hybridization, DNA-ligand binding and adsorption of linear and supercoiled DNA on surface (Dixon, 2008).

QCM-D also is used widely for studying of proteins, lipids and cells as very strong tool for different applications.

### 3. Material and Methods

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## 3 Materials and Methods

### 3.1 PBS

PBS (phosphate buffered saline) is a water-based salt solution used as a buffer to maintain a constant pH for biological usage. A 200 ml stock of 200 mM PBS solution with desired pH (around 7.5), was prepared by adding 0.768 g of  $K_2HPO_4 \cdot H_2O$  (Potassium phosphate monobasic) and 5.986 g of  $Na_2HPO_4 \cdot 2H_2O$  (Sodium phosphate dibasic) and diluted with MilliQ-water, according to table 3.1.

Potassium Phosphate Monobasic Anhydrous g/L	Sodium Phosphate Dibasic Heptahydrate g/L	23 °C pH	Potassium Phosphate Monobasic Anhydrous g/L	Sodium Phosphate Dibasic Heptahydrate g/L	23 °C pH
22.4	3.49	5.7	10.80	29.51	6.9
22.08	4.29	5.8	9.36	32.73	7.0
21.60	5.37	5.9	7.92	35.95	7.1
21.05	6.60	6.0	6.72	38.63	7.2
20.40	8.05	6.1	5.52	41.31	7.3
19.56	9.93	6.2	4.56	43.46	7.4
18.60	12.07	6.3	3.84	45.07	7.5
17.64	14.22	6.4	3.12	46.68	7.6
16.44	16.90	6.5	2.52	48.55	7.7
15.00	20.12	6.6	2.04	49.09	7.8
13.56	23.34	6.7	1.68	49.89	7.9
12.24	26.29	6.8	1.27	50.81	8.0

*Table 3.1. Phosphate buffer table- for preparation of 200 mM buffer solution*

Other desired concentrations like 5mM PBS can be prepared from this stock solution. In most experiments 5 mM phosphate buffer was used, but in some cases 200 mM solution was employed also.

### 3. Material and Methods

#### 3.2 Oligonucleotides

The synthesized oligonucleotides used in this project were purchased from ATDBio Ltd, Southampton, with following sequence.

Oligo ID	Sequence (50-mer)	Mt	T molar conc.	OD 260nm	$\epsilon$
A9661	TCTCG GACTA ACCCT GAGGT CAGCG CCAGT GAGAG TGCTG CAGGC ACGGT	15480.6	273.49	143.8	525.8
A9662	CGCTG ACCTC AGGGT TAGTC CGAGA ACCGT GAATC CAGCA CTCTC ACTGG	15409.6	171.85	90.1	524.3

Table 3.2. Sequences of oligonucleotides

All oligonucleotide samples were prepared by dissolving in 5 mM PBS buffer with a pH of 7.2 to make 20 or 25  $\mu$ M concentration from stock solutions. To verify the accurate sample concentration, the sample absorption was measured at 260 nm with a Varian Cary 5000 spectrophotometer. The concentrations were calculated using extinction coefficient values reported by supplier.

In this report AB represents A9961 oligo and A'B' will present A9662 oligo.

##### 3.2.1 Hybridization of the oligonucleotides

As mentioned before, our strategy in this project for construction of DNA concatemers was assembling them by hybridization of single-stranded oligonucleotides (Figure.13).

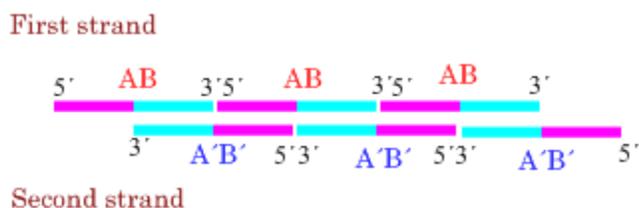


Figure. 13 Scheme of DNA-duplex formation

Double-stranded duplexes was formed by mixing two oligonucleotides (AB and A'B') and hybridize them by heating to 90°C for dissociating hydrogen bonds, and then cooling samples for different times (one hour up to overnight) to let complementary base pairs start to hybridize.

### 3. Material and Methods

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#### 3.3 Enzymes

All enzymes used in this project were obtained from New England Biolabs.

##### 3.3.1 T4 DNA Ligase

T4 DNA Ligase catalyzes the formation of two covalent phosphodiester bonds between the 5'-phosphate and the 3'-hydroxyl groups of adjacent nucleotides of two DNA strands. It used for blunt or cohesive ligation of DNA fragments and has no activity on single-stranded nucleic acids. For inactivation it should be heated at 65°C for 10 minutes (Helfman, Fiddes, & Hanahan, 1987).

T4 DNA ligase plays important roles in living cells and involves in such vital processes like DNA replication, DNA recombination and DNA repair (Gaastra & Hansen, 1985).

##### 3.3.2 T4 Polynucleotide Kinase

T4 Polynucleotide Kinase (T4 PNK) catalyzes exchange reaction between  $\gamma$ -phosphate from ATP and 5'-terminus of polynucleotides (dsDNA or ssDNA) or mononucleotides bearing a 3'-phosphate group (Berkner & William, 1979).

It is usually used for end-labeling of DNA and DNA sequencing, subsequent ligation of oligonucleotides by adding of 5'-phosphates and subtraction of 3'-phosphoryl groups. For inactivation it should be heated at 65°C for 20 minutes

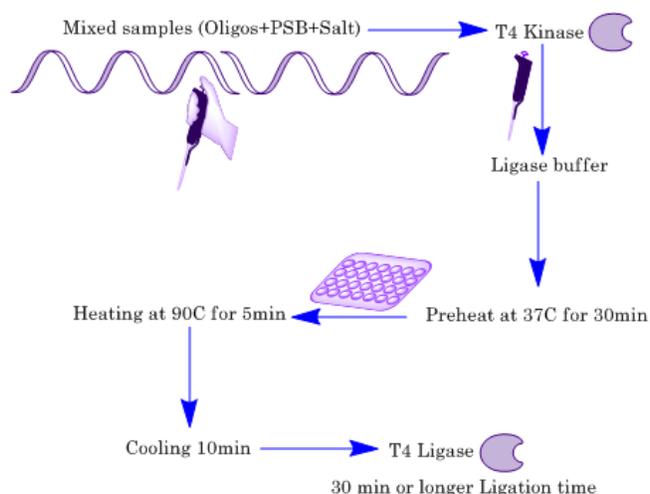
##### 3.3.3 Ligation of the concatemers

The protocol we used for hybridization, consists stepwise increasing of concatemer length in a mode of 25 base pairs addition of oligonucleotides, it means after every 25 pairs, the double-stranded duplex contains a notch. Therefore for sticking the both ends of concatemer a ligation step is needed.

The protocol was employed in this project work for ligation was according to scheme in Figure.14.

### 3. Material and Methods

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**Figure. 14** Scheme of ligation process by T4 ligase

#### 3.4 Buffer system

The buffer system in electrophoresis is used to control the pH of the gel, avoiding damage to sample molecules and in some cases, controlling the ionization state of the molecules. Also carrying the current flowing through the electrophoresis gel is done by buffer ions.

In denaturing PAGE electrophoresis of DNA which is a homogeneous system, the type and concentration of buffers in the tank and gel are the same, here the buffer prevents large swings in pH and also controls the conductivity of the gel. For the native electrophoresis of proteins, the buffer pH has the extra function of controlling the state of ionization of the samples. In this type of electrophoresis, even minor changes in pH can result in great effects on the relative mobility of sample components. But in a multiphasic system, such as SDS-PAGE electrophoresis of proteins, as buffers in the tank and gel are not the same, the considerations of buffer properties can take on an even larger level of complexity.

It is very important to keep the ionic strength of buffer in the gel in a satisfactory level to maintain the sample in solution and provide sufficient buffering capacity. To get sharper bands higher concentrations of gel buffer must be used because it will slow the diffusion of sample, but it should be considered that higher buffer concentrations will increase the electrical conductivity and the current will be greater and more heat will be generated. With higher concentration, at a given voltage, the current will be greater and more heat will be generated. For decreasing of excessive heating problems it is better to employ high buffer concentrations at a low voltage gradient.

### 3. Material and Methods

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#### 3.4.1 TBE buffer

In all experiments Tris-borate-EDTA (TBE) was used as electrophoresis buffer. For making TBE buffer Boric acid, Tris base and EDTA is needed. A stock solution of 2000ml 10×TBE buffer was prepared by adding 61.83 g Boric acid, 121.14 g Trizma base and 9.31 g EDTA ( dehydrate salt of EDTA was used ) and diluting up to a final volume of 2 L with MilliQ-H<sub>2</sub>O.

The solution can be stored at room temperature. However because a precipitate will form after long time in older solutions, storing in a refrigerator may increase life-time of solution and prevents precipitation.

From this stock solution 1×TBE buffer was prepared by diluting with MilliQ-water by 10×.

#### 3.5 Salts

##### 3.5.1 NaCl

A stock solution of 10 ml of 4M NaCl solution was prepared by dissolving 2.337 g of sodium chloride in MilliQ-water up to 10 ml final volume. Other NaCl concentrations (50, 100, 200, 300 and 400 mM) were prepared from this stock solution.

##### 3.5.2 PEG

100 ml solution of 40% w/w PEG-8000 solution was prepared by adding 40 g PEG-8000 powder to 60 ml MillQ-H<sub>2</sub>O, the solution stirred for 2 hours, the solution was allowed to stir for overnight to obtain a homogenous solution. Other concentrations (20, 10 and 5%) were prepared from this stock solution.

### 3. Material and Methods

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#### 3.6 Instrumentation

##### 3.6.1 Agarose gel electrophoresis

Agarose gel electrophoresis experiments were carried out in 2.5% agarose gel (gels are prepared as percentage w/v solutions, it means the weight of agarose in gram per 100 ml buffer solution, thus a 2.5% gel is 2.5 g agarose in 100 ml buffer ) .

Casting and running the agarose gel was performed according to following steps:

1. For preparation of 2.5% agarose gel, 2.5 g agarose dissolved into 100 ml 1×TBE buffer and swirled, melting of agarose was done by heating up to 200°C (it can be done also in microwave) until boiling. Then allowed the agarose to cool for 5 min to 60°-70°C and poured the gel into the tray, insert the comb and let it for gelation at RT for at least 30 minutes.
2. After the gel hardens, put it into electrophoresis tank and filled with 1×TBE buffer, load the samples and ladders and run the electrophoresis at constant 4V/cm voltage for 2-3 hours.
3. Scanning the gel using a Typhon™ 9410 Variable Mode Imager gel scanner.



*Figure. 15 Components of Agarose gel electrophoresis*

### 3. Material and Methods

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#### 3.6.2 Pulsed-Field gel electrophoresis (PFGE)

The agarose gel electrophoresis enables to separate DNA fractions of 60 kb or less. The development of Pulsed-Field gel electrophoresis (PFGE) made it possible to separate larger DNA fragments up to  $2 \times 10^3$  kb, which has expanded the range of resolution for larger DNA molecules (Schwartz & Cantor, 1984).

This method is based on applying two electric fields alternately at different angles for definite period of time (e.g. 60s), introduction of first electric field will stretch coiled molecules in the horizontal plane and push them to move through the gel, activation of second electric field interrupts the first field and move the molecule in the new direction. Since the smaller molecules realigns faster (because of length-dependent relaxation behaviour) and larger molecules take longer to realign, molecules according to their size will separate with periodic reversing of the field (Wilson & Walker, 2010).

#### 3.6.3 Polyacrylamide gel electrophoresis

Native and denaturing polyacrilamide electrophoresis experiments were carried out. In following sections they have explained separately. All polyacrilamide gels were hand casted.

#### 3.6.4 Denaturing PAGE

For denaturing urea polyacrilamide gel electrophoresis, 7M urea was used. Different PAAG concentrations were prepared according to following table:

Gel %	Acrylamide 40% (ml)	10 × TBE (ml)	Urea (g)	MilliQ-H <sub>2</sub> O(ml)	APS (μl)	TEMED (μl)
5	1.875	1.5	6.31	11.515	105	5.3
10	3.75	1.5	6.31	9.64	105	5.3
20	5.625	1.5	6.31	7.765	105	5.3
<b>Total Volume : 15 ml</b>						

Table 3.3. Composition of different 7M urea PAAG concentrations

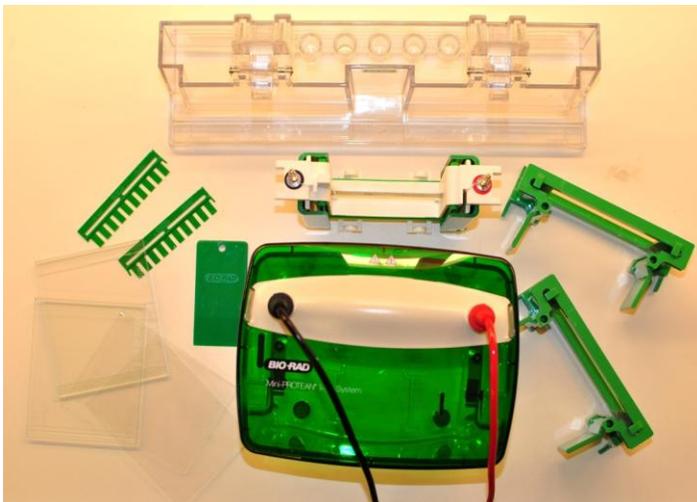
All electrophoresis experiments were done using BIO-RAD mini-PROTEIN® Tetra system (Figure.16).

### 3. Material and Methods

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Setting up, casting and running of urea PAGE was done according to the following protocol:

1. Cleaning glass plates with water rinse and wipe to dry by EtOH and air.
2. Assembling the glass plates into casting frame and casting stand, sealing glass plates with parafilm in order to prevent any leakage.
3. Preparation of desired gel concentration according to above table.
4. Degassing with N<sub>2</sub> and pouring the gel using a serological pipette, be careful not introducing air bubbles, let the gel polymerize for 60 minutes.
5. Place the gel in apparatus, fill the tank with 1×TBE buffer and blow out wells with pipette.
6. Prerun the gel for 30 min at constant 8V/cm voltage and 70°C.
7. Blow out wells again and loading the samples (approx. 5-6 µl) into each well and also loading DNA ladders (50bp and 500bp ladders).
8. Allow the gel to run for 30min to 1.5h according to the expected size of DNA.
9. After finishing the electrophoresis, soak the gel in staining solution (5 µl syber gold in 50 ml H<sub>2</sub>O).
10. Scanning the gel using a Typhon™ 9410 Variable Mode Imager gel scanner.



*Figure. 16 Components of Polyacrilamide gel electrophoresis*

### 3. Material and Methods

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#### 3.6.5 Native PAGE

Native polyacrylamide electrophoresis experiments were done, using the same protocol as denaturing gel, just without using urea and water bath, all other steps were the same.

#### 3.6.6 QCM-D

The QCM-D measurement was performed using a Q-Sense E4 system by Q-Sense AB.

At first the quartz crystals were cleaned according to the following procedure:

1. Rinsing the crystals with MilliQ-H<sub>2</sub>O and dry the surface using N<sub>2</sub> gas.
2. Espousing the crystals to UV-ozone for 15 min, in order to taking any biomolecules or organic particles away.
3. Cleaning the crystals in a 5:1:1 solution of MilliQ: NH<sub>3</sub>: H<sub>2</sub>O<sub>2</sub> at 80°C for 10 min, boiling until bubbles appears on the surface.
4. Rinsing the crystals using MilliQ-H<sub>2</sub>O, dry with N<sub>2</sub> and put them into Biotin solution and keeping in dark for 12 h.

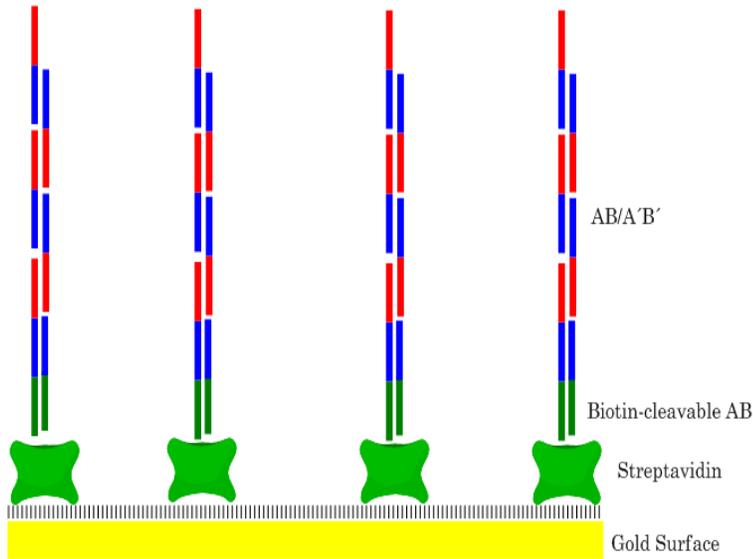
Before starting the measurement the crystals were put into ethanol and sonicated for 5 min. The crystal was mounted in the temperature controlled chamber and the chamber was rinsed with buffer (5 mM phosphate buffer + 200 mM NaCl). The temperature was set to 22°C and the experiment was started. Streptavidin was introduced into chamber first, after frequency stabilization, biotin-cleavage AB was added and then Cleavable A' followed by adding A'B' and AB respectively (Figure.3.3). After any addition and before adding the next solution, the chamber should be rinsed with buffer first, it takes 3-4 min to get a stable frequency and starting the next measurement after rinsing with buffer.

Biotin is broadly used as 5'- end-labelling in oligonucleotide studies, because affinity of biotin-streptavidin interaction is enormously high (Olejnik, Krzymańska-Olejnik, & Rothschild, 1996).

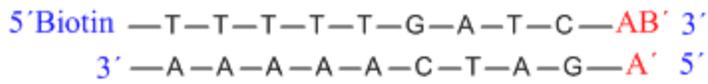
### 3. Material and Methods

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The experiment was continued until assembling a 400bp strand, it took almost 7 h and 30 min to finish. The results were analyzed using QTools software.



*Figure.16 Schematic view of assembling DNA strand on quartz surface*



## 4. Results & Discussion

This chapter contains the results obtained from gel electrophoresis (Agarose & PAAG) and QCM-D experiment. The electrophoresis part is divided to two parts, in the first part the raw results are presented and some important comments have been mentioned. The second part includes some detailed analysis information for better interpretation of results. There is also a supplementary part at the end of the report (index chapter) where, more experiment results have been collected.

### 4.1 Agarose gel electrophoresis

All agarose gel experiments were performed in 1×TBE buffer (50 mM Tris, 50 mM borate, 1.25 mM EDTA, pH 8.2) at RT at 4V/cm for 3 hours. Agarose gels (2.5%) were prepared using D1 Low EEO agarose from CONDA. O'RangeRuler™ 50 bp and 500 bp DNA ladders from Fermentas were used as size standards.

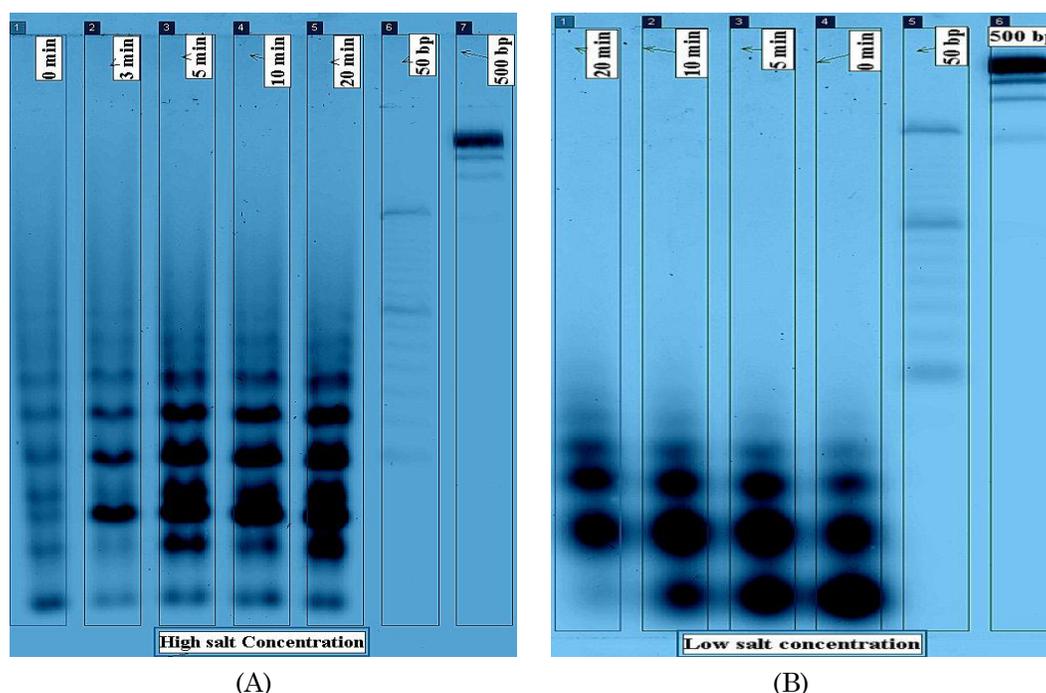
All samples were stained by 0.2 µl YO-PRO®-1 dye from invitrogen before running. A Typhon 9410 scanner with laser excitation wavelength at 488 nm and a 520 BP 40 emission filter was used for visualization of results.

#### 4.1.1 Different heating times

As mentioned earlier, before starting hybridization process, samples were heated for some minutes at 90°C to certify of dissociation of hydrogen bonds. In most cases, samples were heated up to 90°C for 5 minutes, in this experiment we have tested different heating times (0 min to 20 min) to verify if longer heating times had any influence or not.

Figure.18 shows the results, in two different conditions (High & Low salt concentration) after one hour hybridization time. The results show that increasing heating time from 5 to 20 min doesn't have a big affect on length of formed concatemers, but comparing between high and low salt concentration results, confirms significantly the role of salt addition for making longer strands.

## 4. Results & Discussion



**Figure. 17. 2.5% Agarose electrophoresis, Different heating times**

(A) High salt concentration (B) Low salt concentration, heating at 90°C, 2.5  $\mu$ M oligo. One hour hybridization time, gel run at 4V/cm for 3h.

### 4.1.2 Different hybridization times

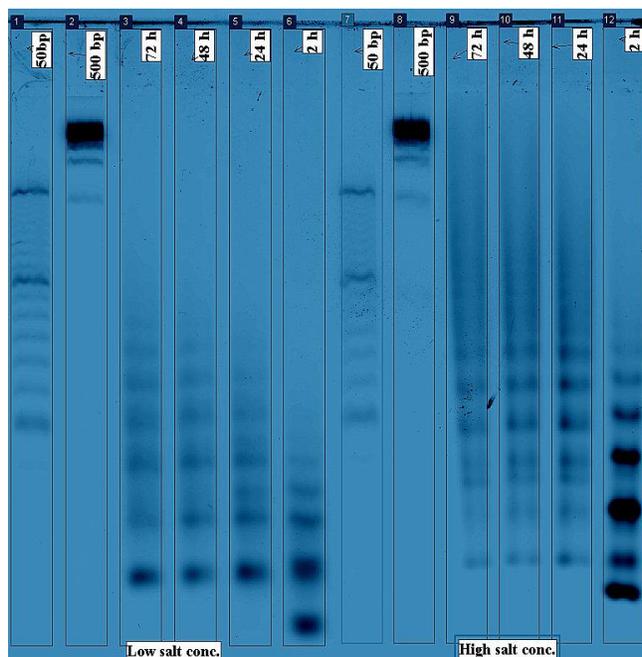
Figure.19 shows how the duration of hybridization affects the concatemer distribution. In this experiment different hybridization times, starting from 2 hours up to 72 hours were checked to verify which hybridization time is more reasonable for building longer strands.

The experiment was done using 2.5  $\mu$ M oligo concentration and hybridization was performed at room temperature. Again two parallel experiments, the first in presence and the second in absence of salt were done.

The results show longer hybridization times beyond 2 h have very slight effect on the size distribution, that is almost can be disregarded, but again the positive influence of salt concentration can be seen clearly.

## 4. Results & Discussion

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**Figure. 18. 2.5% Agarose electrophoresis, Different hybridization times.**

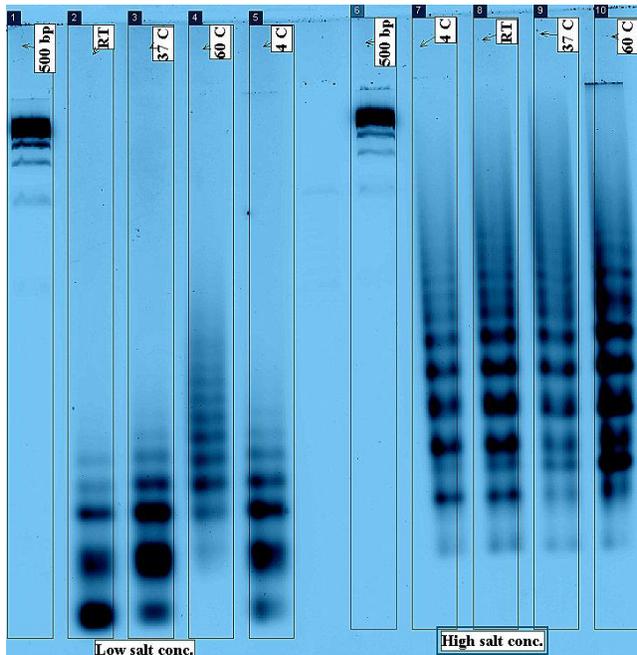
2.5  $\mu$ M oligo, 5mM phosphate buffer, 1h hybridization time at RT, gel run at 4V/cm for 3h.

### 4.1.3 Different hybridization temperatures

Figure.20 shows the affect of hybridization temperature as an important parameter on size distribution. Here different hybridization temperatures (4°, RT, 37° and 60°C) were examined applying on 2.5  $\mu$ M oligo samples. Again two different salt concentrations were assessed.

The result show considerable effect of hybridization temperature for assembling longer constructs, as it is observable that longer concatemers has been formed in higher hybridization temperatures. Again the optimistic effect of high salt concentration is clear.

## 4. Results & Discussion



**Figure. 19. 2.5% Agarose electrophoresis, Different hybridization temperatures**

2.5  $\mu$ M oligo, 5mM phosphate buffer, 1h hybridization time at RT, gel run at 4V/cm for 3h.

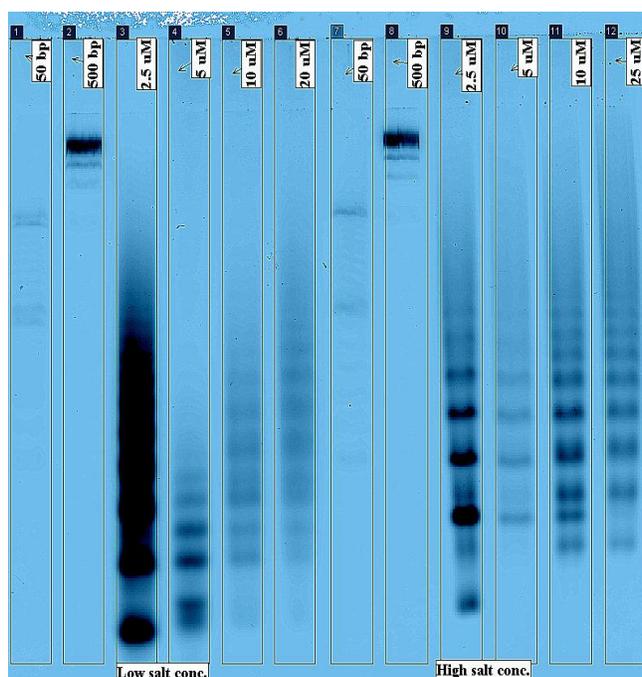
### 4.1.4 Different oligo concentrations

Figure.21 shows results of testing different oligonucleotide concentrations (2.5, 5, 10 and 20  $\mu$ M). It is apparent that increasing of oligo concentration helps to form longer concatemers, these results are in agreement with the previously published reports (Simonova, Vladimirova, Zenkova, & Vlassov, 2006).

It is clear from the gel in Figure.21 that high salt concentration facilitates the formation of longer concatemers with better efficiency even in low oligonucleotide concentrations.

## 4. Results & Discussion

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**Figure. 20. 2.5% Agarose electrophoresis, Different oligo concentrations**

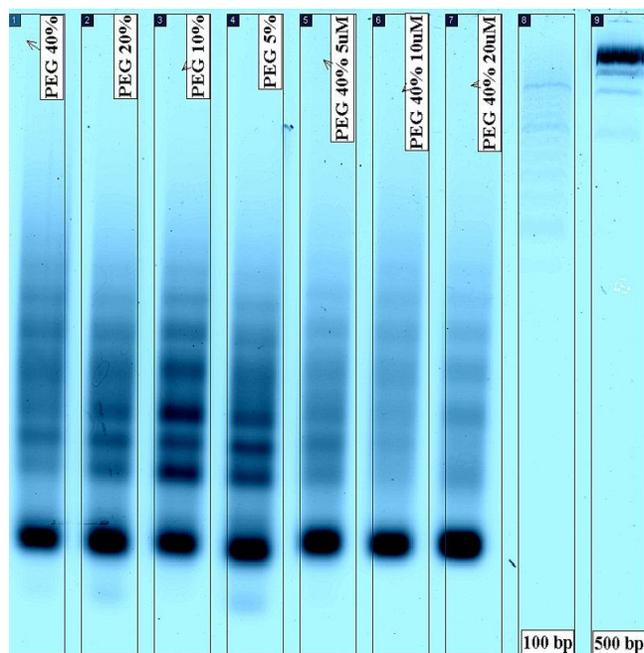
5mM phosphate buffer, 1h hybridization time at RT, gel runs at 4V/cm for 3h.

### 4.1.5 Different PEG concentrations

Figure.22 shows the results of checking different PEG (PolyEthylene Glycol) concentrations (5, 10, 20 and 40% w/w). Also different oligo concentrations vs. PEG 40% w/w was checked to clarify different PEG conc. effect on the concatemers distribution.

As can be seen from the result, higher PEG concentration didn't have a significant effect for building longer constructs, it seems 5 or 10% PEG concentration is efficiently enough for forming long concatemers.

## 4. Results & Discussion



**Figure. 21 . 2.5% Agarose electrophoresis, Different PEG concentrations**

5mM phosphate buffer, 1h hybridization time at RT, gel runs at 4V/cm for 3h.

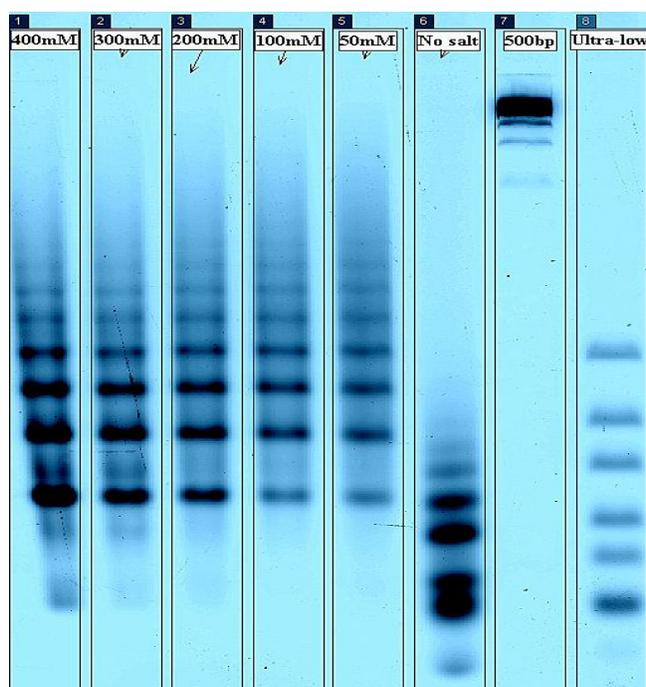
### 4.1.6 Different salt concentrations

In all previous experiments a 400 mM solution of NaCl was used as salt additive and confirmed that, the presence of salt has a positive effect for constructing double-stranded duplexes, via reduction of repulsion forces between the single-stranded structures. In this experiment different concentrations of NaCl (50, 100, 200, 300 and 400 mM) were used to check the role of salt concentration itself.

For this experiment 2  $\mu$ M oligonucleotide was used and the samples were hybridized for 2 h before running in the gel.

As expected, salt addition was increased the length of DNA concatemer (see figure.23), but looking more carefully into the results, illustrates that maximum effect of salt concentration is until 200 mM , beyond this value, addition of higher salt concentration has less effect on size distribution of formed concatemers.

## 4. Results & Discussion



**Figure. 22. 2.5% Agarose electrophoresis, Different NaCl concentrations**

5mM phosphate buffer, 2 h hybridization times at RT, gel runs at 4V/cm for 3h.

### 4.2 Polyacrylamide electrophoresis

All PAGE experiments (5% & 10% native and denaturing) were performed in 1×TBE buffer (50 mM Tris, 50 mM borate, 1.25 mM EDTA, pH 8.2). Native PAGE was performed at 8V/cm for 30 min and urea-denaturing PAGE was performed at 70°C at 8V/cm for 30 min. O'RangeRuler™ 50 bp and 500 bp DNA ladders from Fermentas were used as size standards.

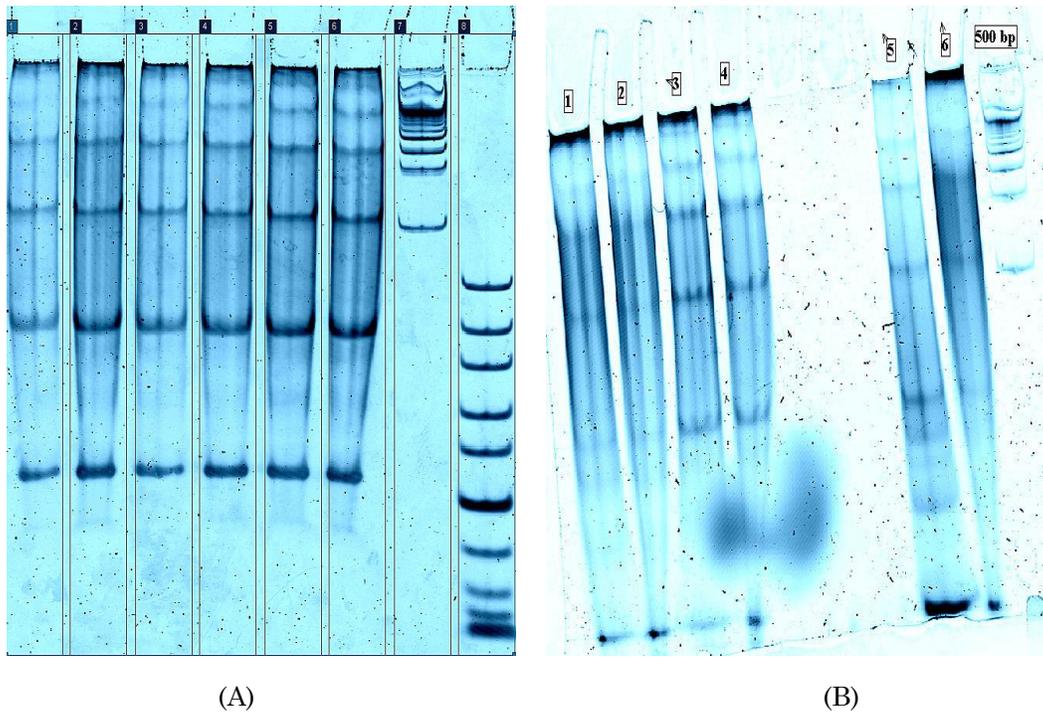
All samples were post-stained by Syber gold solution (5 µl in 50 ml MilliQ-water). For scanning the gels, a Typhon 9410 scanner with laser excitation wavelength at 488 nm and a 520 BP 40 emission filter was used.

#### 4.2.1 Native PAGE

Figure.24 (A) shows 5% native PAGE for ligated samples. Different ligation times (30min, 2h, 4h and overnight) were examined for 20 µM oligonucleotide samples (lanes 1,2,3 and 4 respectively). In lane 5, 10 min heating time and overnight ligation time and in lane 6, 20 min heating time and overnight ligation time was checked. Lane 7 is 500 bp ladder standard and lane 8, GeneRuler™ Ultra Low Range DNA ladder.

#### 4. Results & Discussion

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**Figure. 23. 5% Native Polyacrylamide electrophoresis**

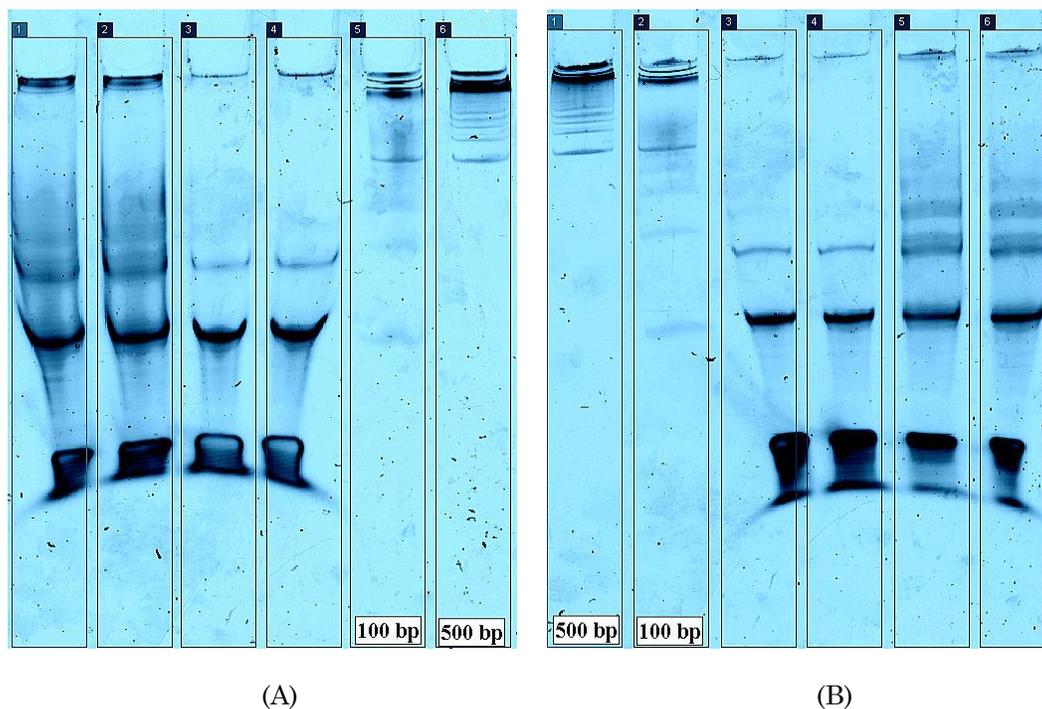
(A) Ligation with T4 Ligase, 25  $\mu$ M oligo, gel run at 8V/cm for 30 min. (B) Heated vs. Non-heated samples, 30 min hybridization, gel run at 8V/cm for 35 min.

Figure 24(A), clearly indicates that increasing temperature (from 5 to 20 min) doesn't affect the ligation efficiency remarkably, this assumption proves in Figure 25(B) where lanes 3 and 4 are samples without heating.

Figure.24 (A) clearly confirms the formation of long linear concatemers after ligation.

#### 4. Results & Discussion

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**Figure. 24. 5% Urea-denaturing Polyacrylamide electrophoresis**

Ligation with T4 Ligase, 25  $\mu$ M oligo, different ligation times, gel run at 8V/cm for 30 min. (A) without formamide addition, (B) formamide addition

Figure. 25 show 5% urea PAGE experiment for different ligation times (30 min, 2, 4 and 24 h). 25  $\mu$ M oligo was used in two different experiments, in the first experiment (Figure.25A) formamide was not added to the samples, but in second experiment (Figure.25B) formamide was added to all samples before running.

## 4. Results & Discussion

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### 4.3 Size distribution and intensity analysis

This section involves a detailed analysis for some of the selected results which enable us to have a better understanding about size distribution and intensity of formed double-stranded DNA concatemers.

As discussed in previous section about the role of salt addition and its positive effect on size length, it was decided to do the analysis only for high salt concentration results.

Two parallel assessments have been done, first quantification of the distributed bands (each band corresponds to specific size length) and second the intensity analysis of these bands which signify the amount of different-sized fragments. Four results among various experiments have picked for detailed analysis, including the results for different oligo concentrations, different hybridization times, different hybridization temperatures and different salt concentrations.

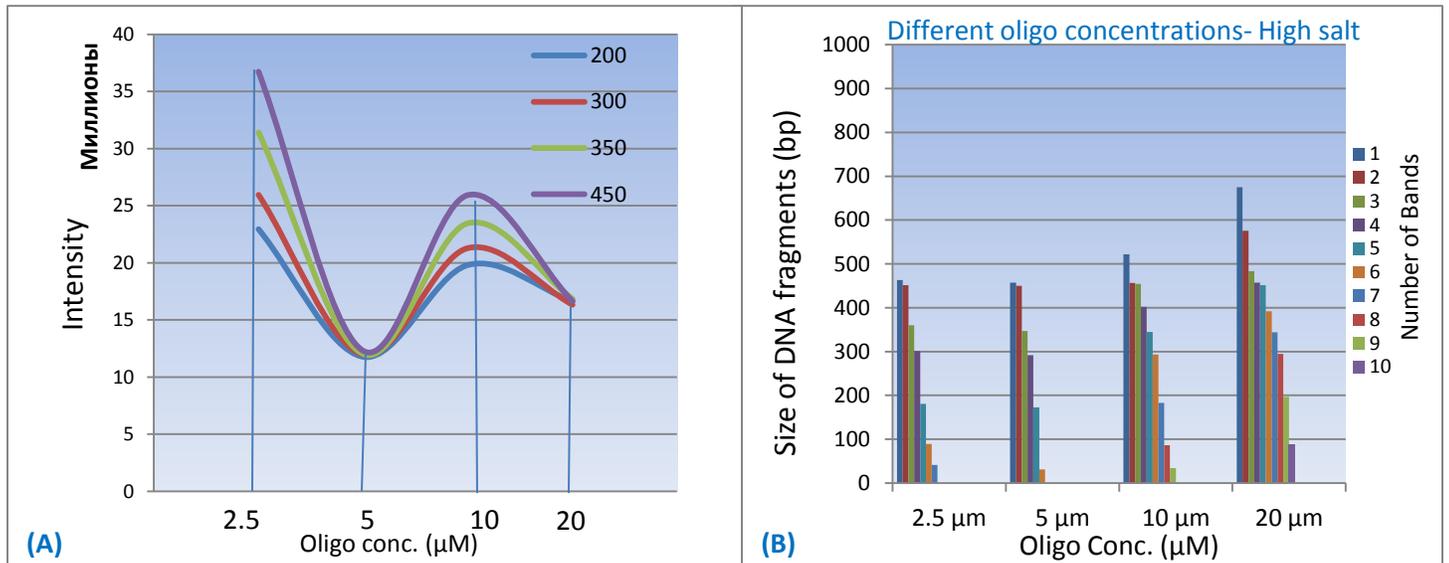
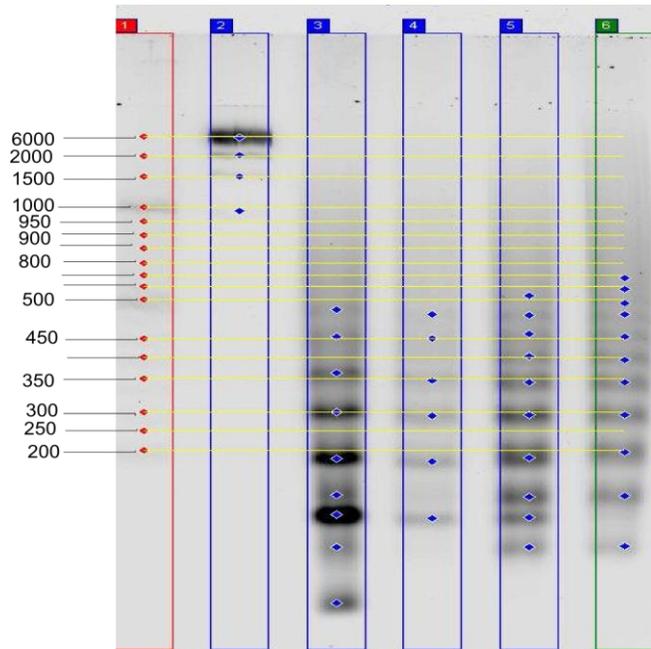
Comments and discussions were mentioned in previous sections, mostly are based on following analysis, so in this part we just try to underline some important remarks which are more noticeable from the derivative charts.

## 4. Results & Discussion

**Figure. 26. Size distribution of different oligo concentrations**

Lanes 2-6 are 2.5, 5, 10 and 20  $\mu\text{M}$  oligos respectively

Figure. 27 show higher intensity for 2.5  $\mu\text{M}$  oligo but almost longer length sizes for higher oligo concentrations. 5 $\mu\text{M}$  oligo has lowest intensity and fewer bands.



**Figure. 27. Size distribution and intensity analysis of Fig.26**

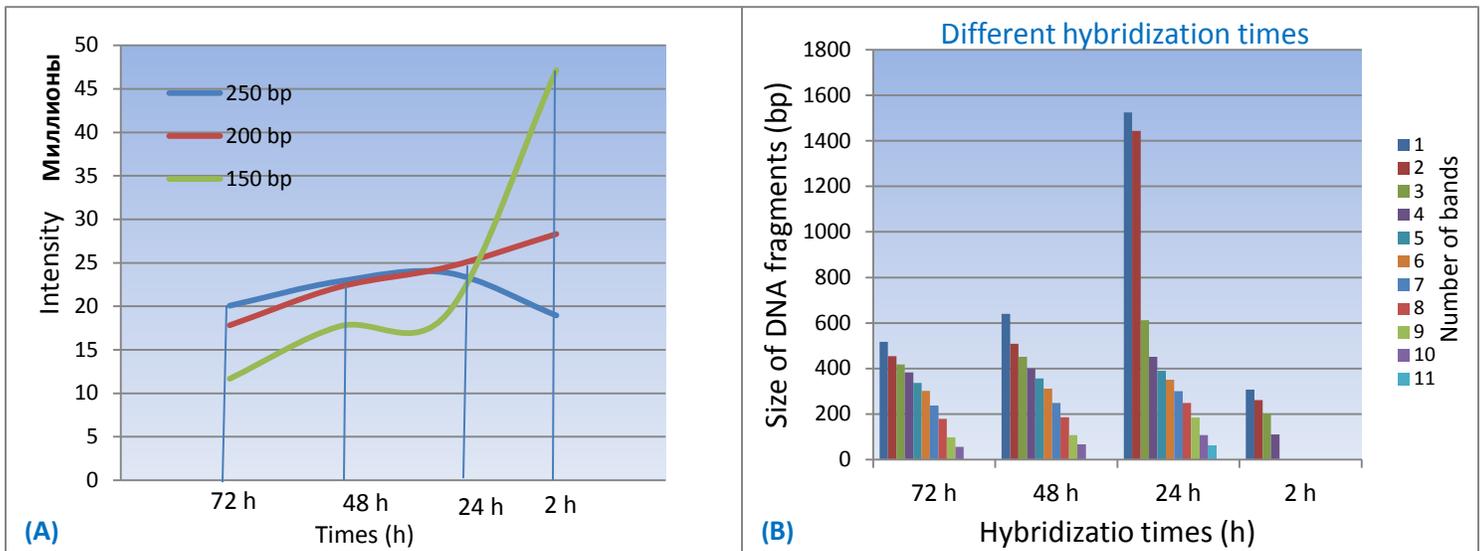
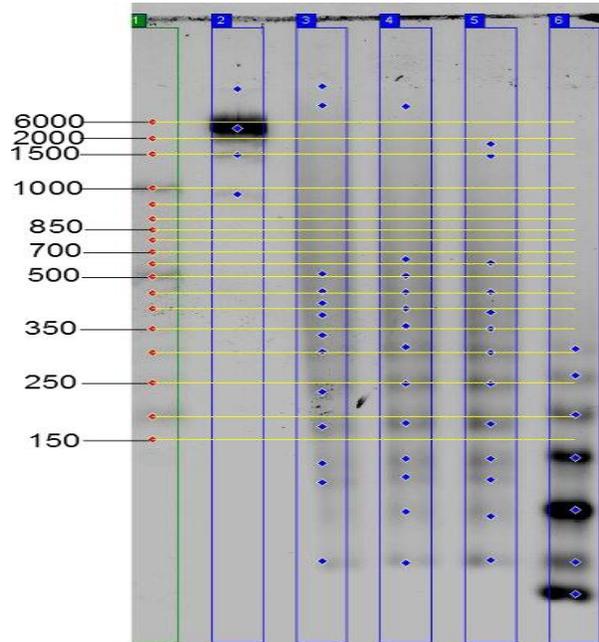
(A) Intensity analysis for four different DNA fragments (200,300,350 and 450bp) (B) Size distribution analysis, 1-10 is the number of distributed bands for different oligo concentrations

#### 4. Results & Discussion

**Figure. 28. Size distribution of different hybridization times**

Lanes 2-6 represent 72, 48, 24 and 2h respectively

Higher intensity belongs to 2h hybridization time, it seems after 24 h hybridization, length size and number of fragments doesn't change significantly.



**Figure. 29. Size distribution and intensity analysis of Fig.28**

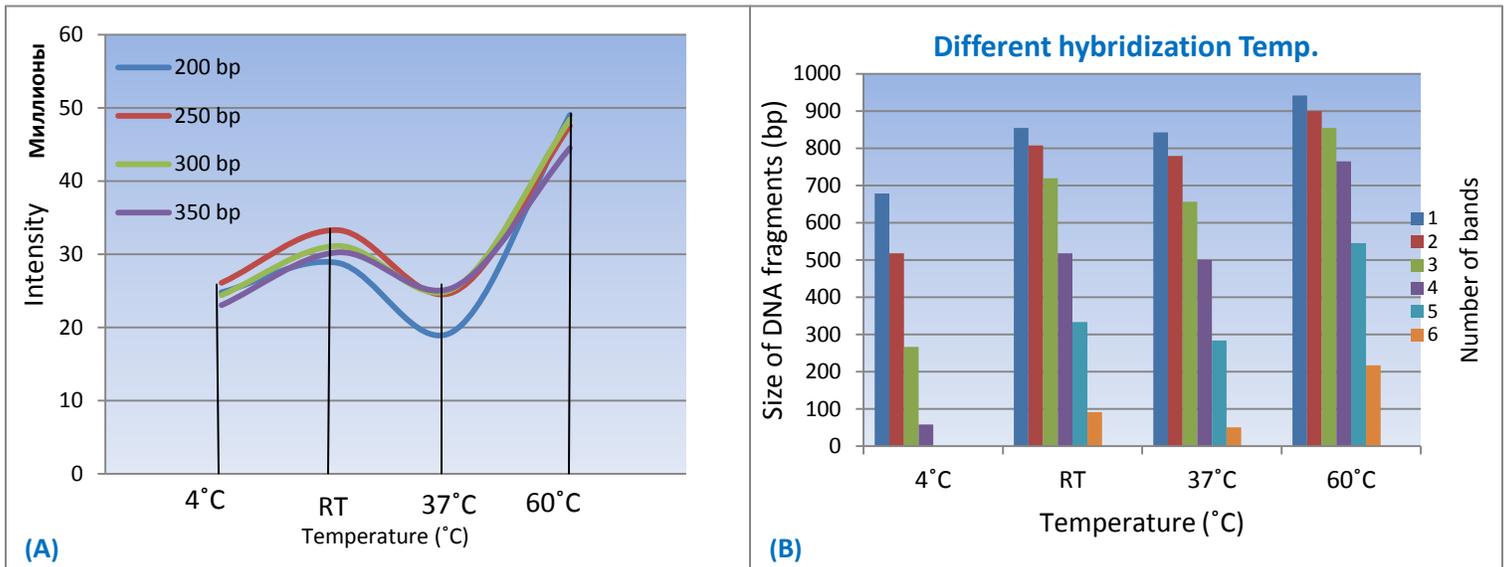
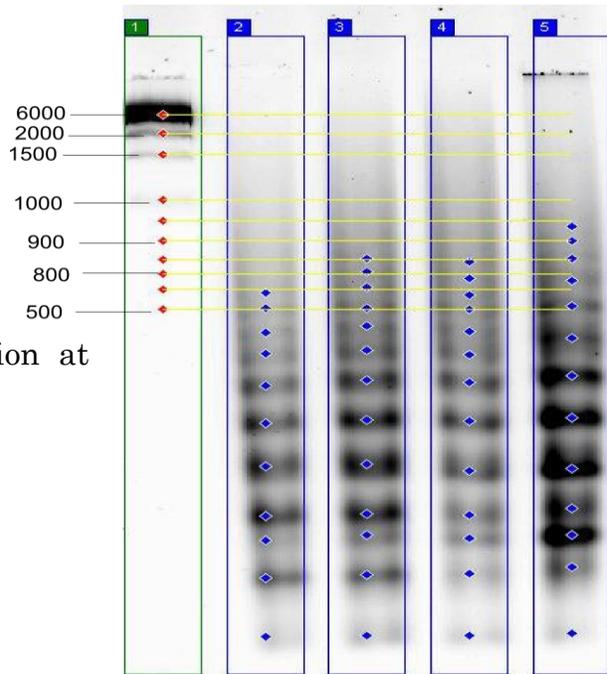
(A) Intensity analysis for three different DNA fragments (150,200 and 250bp) (B) Size distribution analysis, 1-11 is the number of distributed bands for different hybridization times

#### 4. Results & Discussion

**Figure. 30. Size distribution of different hybridization temperatures**

Lanes 2-5 are 4°, RT, 37° and 60°C respectively

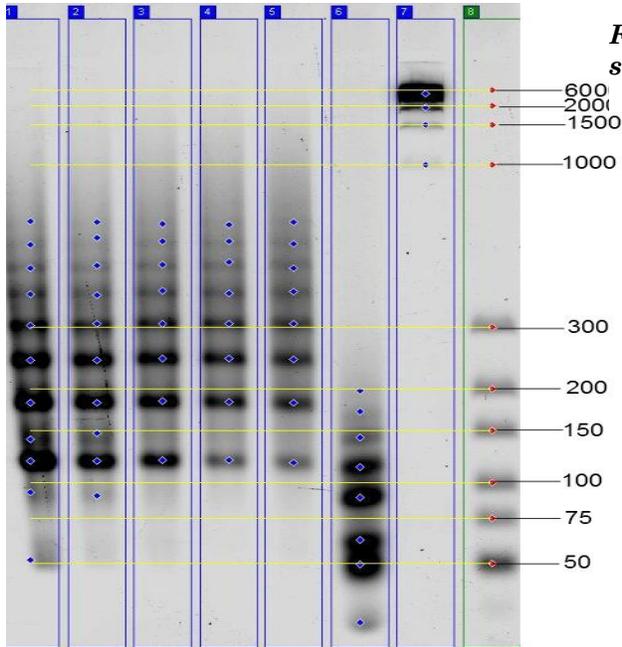
It appears that higher temperature helps to form longer DNA fragments, also more intensity for constructs were formed at 60°C, but there is slight difference between room temperature and incubation at 37°C.



**Figure. 31. Size distribution and intensity analysis of Fig.30**

(A) Intensity analysis for four different DNA fragments (200,250,300 and 350bp) (B) Size distribution analysis, 1-6 is the number of distributed bands for different hybridization temperatures

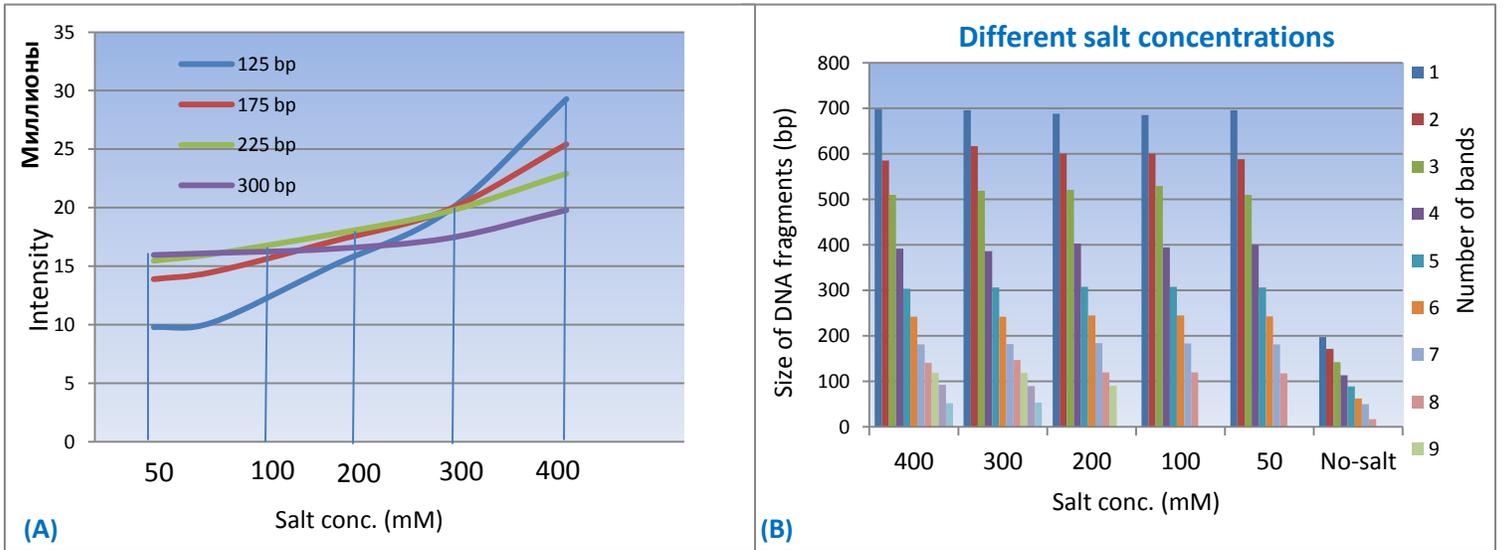
#### 4. Results & Discussion



**Figure. 32. Size distribution of different salt concentrations**

Lanes 1-5 are 400, 300, 200, 100, 50 mM salt respectively, lane 6 is no-salt condition

As discussed before, it seems that 100-200 mM salt concentration has sufficient effect for assembling long fragments. The only influence of higher salt concentration is higher intensity of formed bands.



**Figure. 33. Size distribution and intensity analysis of Fig.32**

(A) Intensity analysis for four different DNA fragments (125,175,225 and 300bp) (B) Size distribution analysis, 1-9 is the number of distributed bands for different salt concentrations

## 4. Results & Discussion

### 4.4 QCM-D

QCM-D experiment was performed for assembling a 400 bp cocatemer. As reviled before in section 3, first a layer of streptavidin was covered the surface, then a biotin-cleavable of AB (cleavable duplex) sequence was bounded to the streptavidin and then cleavable A' added and after this addition, the stepwise addition of A'B' and AB was done until a 400 bp strand was formed. Between any additions a rinsing step was run.

Figure.34 shows the result of QCM-D experiment. The blue line indicates the changes in resonance frequency (decreasing) due to added mass and formation of strands and the red line is dissipation changes (increasing). Different curves indicate frequency and dissipation data from different harmonics.

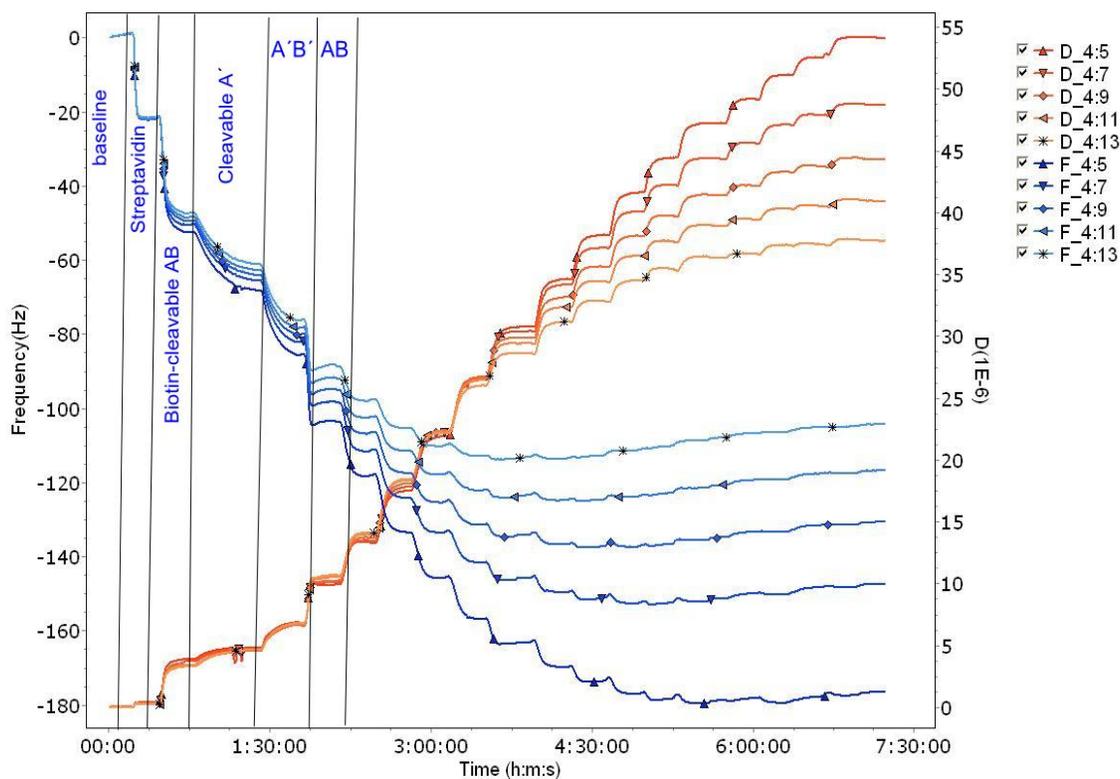


Figure. 34. QCM-D data

#### *4. Results & Discussion*

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The result shows that the linear concatomers were formed on the surface productively. For checking of size distribution of formed structures, the strand could be cleaved off and investigated by gel electrophoresis.

## 5. Conclusion

In this project formation of the concatemeric duplexes at different hybridization conditions have been studied. Two 50-mer synthesized oligonucleotides employed as bottom-up material for assembling long linear double-stranded DNA concatemers in solution and long linear concatemers with different lengths were effectively constructed.

For increasing the length, different conditions were assessed to achieve an optimized protocol, such as oligo concentration, hybridization time and salt concentration, among them, it seems salt concentration enhances the efficiency of duplex formation sensibly. Comparing length of formed concatemers in presence and in the absence of salt, confirmed that addition of enough concentration of salt, forms similarly long concatemers either in shorter hybridization time and lower oligo concentration, therefore in absence of salt or low salt concentration, higher oligo concentration and higher hybridization time is needed for assembling long concatemers. Ligation also was employed as a tool for joining the nicks between formed concatemers.

QCM-D was used for assembling of concatemers on planar surface (a 400 bp strand was formed by this technique as an example for studying). Maybe one of the advantages of QCM-D is that more control is possible during assembling process, unlike hybridization in bulk solution, that a variety of concatemers form with different length distribution. Washing steps also help to form just a limited range of concatemers on surface. But a major drawback of this method is the long time of assembling route, it takes hours to build a hundred base-pairs strand (in our case it took 7 h and 30 min to build a 400 bp stand).

This work was a bottom-up approach for designing specific-sequence DNA constructs for studying binding thermodynamics and kinetics on a single molecule level.

## 6. Perspectives

As mentioned before sequence-specific interaction of different ligands with DNA molecule has very important role in different branches of bioscience. The primary building block for investigating this interaction is to construct longer DNA sequences for further ligand binding studies.

During my thesis work, I took great advantages of gel electrophoresis as a tool for in-depth characterization of formed constructs by varying incubation conditions to get the optimized conditions for hybridization in bulk. Concatemers were formed by varying many different conditions for hybridization and ligation. Our intention was to accomplish the experiments in an optimized condition (for instance in shorter time or lower concentration by employing various strategies), but such an optimization is greatly dependent to a variety of parameters, some of them are not controllable in parallel or are out of control.

Actually the most part of my work involved nucleic acid thermodynamics (DNA hybridization, denaturation and annealing) which significantly is related to the melting temperature ( $T_m$ ) of DNA.  $T_m$  itself depends on the length and sequence of DNA molecule. Maybe more researches related to  $T_m$  and parameters influence melting temperature is required, for instance our focus in this project was set on AT-rich sequences, but higher temperature is needed for GC-content sequences, also  $T_m$  depends on the solvent conditions, for instance higher salt concentrations (e.g. NaCl) increases the  $T_m$ , because the positive sodium ions, shield the negative charges of the phosphate groups on the DNA backbone and repelling of bonds will decrease, but some organic solvents lower the  $T_m$  by increasing the repelling actions.

Another critical aspect of my thesis work was QCM-D technique for attaching the formed concatemers on surface. The long time of assembling procedure and the yield of the final structure are important factors that should be considered. A suggestion for increasing the synthesis yield (by increasing surface area) and a faster assembly is performing the experiment on gold particles instead of planar surface.

## 6. Perspectives

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Finally, the last question is changing the ligation strategy to “Click Nucleic Acid Ligation” by the copper-catalyzed azide-alkyne (CuAAC) reaction. As recently a published report from (El-Sagheer & Brown, 2012) has mentioned the procedure of a biocompatible click DNA linkage. It seems this method is more efficient and well-suited with large scale synthesis.

### **Future directions**

This thesis work was part of a major project, which will focus on improving our understandings about DNA-ligand interaction on single molecule level. In fact this work was primary steps for manipulating DNA constructs with desired properties, so a lot of works still has to be done. The next step, is studying elastic behaviour of formed duplexes by mechanical stretching using optical tweezers. Also, the other main part of whole project is investigation of sequence-dependent binding of these constructs to different ligands, such as functional proteins, therapeutic agents or specific drugs. Among them, binuclear ruthenium complexes (which exhibit a remarkable sequence specific binding properties to DNA) are very interesting target compounds, but at the first stage, Actinomycin D (Act D) as an important antineoplastic antibiotic that inhibits cell proliferation, specifically will be examined. Hopefully this project will be a step forward, in the field of DNA-ligand interaction, an area with great potential and a wide range of future applications.

## 7. Acknowledgments

First of all I would like to thank Professor Björn Åkerman for allowing me to do my thesis project in his lab. It was a great opportunity for me to work at biophysical chemistry department, a place I learned a lot, met different people and had a chance to discover matters I had never experienced in my life!

My gratitude goes to my supervisor Lu sun, for all the help and guidance during my project, thanks to answer even my simple questions with patience and your eternal smile!

I would also like to thank everyone at the division of Physical Chemistry for helping me and made a warm and friendly working environment, and also all diploma workers in that dark room! Although that room had no windows but with you it was always brightened.

During my time at Chalmers, I had really everlasting memories, good friends, great teachers and fantastic atmosphere, a university I really recommend for studying.

Lastly, but more importantly, I would like to thank my family, those whom I respect and admire them always, who have supported, encouraged and motivated me during my studies. My deepest appreciation belongs to my dear parents, especially my beloved mother, thank you for your everlasting attention and kindness although I was always far from you but you are in my heart forever. This thesis work is dedicated to you...

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## 9. Appendix

### 9. Appendix

#### 9.1 Reagents & Chemicals



The list of whole reagents and chemicals were used in this project.

Name	Formula/Cat. No	Company
Acrylamide/Bis solution*	#161-0146	BIO-RAD
Agarose D1 Low EEO	8008	CONDA
Ammonia Solution 25%	NH <sub>3</sub>	Scharlau
APS	(NH <sub>4</sub> ) <sub>2</sub> S <sub>2</sub> O <sub>8</sub>	BIO-RAD
Boric acid	H <sub>3</sub> BO <sub>3</sub>	SIGMA
500bp DNA Ladder	#SM0643	Fermentas
50bp DNA Ladder	#SM0613	Fermentas
Ultra Low Range DNA Ladder	#SM1213	Fermentas
EDTA	C <sub>10</sub> H <sub>14</sub> N <sub>2</sub> Na <sub>2</sub> O <sub>8</sub> .2H <sub>2</sub> O	Fluka
Formamide	HCONH <sub>2</sub>	-
Hydrogen Peroxide 30%	H <sub>2</sub> O <sub>2</sub>	Scharlau
Sodium chloride	NaCl	SIGMA
Oligonucleotide 50-mer	A9661	atdbio
Oligonucleotide 50-mer	A9662	atdbio
PEG-8000	#57H0144	SIGMA
Photassium phosphate monobasic	K <sub>2</sub> HPO <sub>4</sub> .H <sub>2</sub> O	Sigma/Aldrich
Sodium phosphate dibasic	Na <sub>2</sub> HPO <sub>4</sub> .2H <sub>2</sub> O	Sigma/Aldrich
SYBER® gold	#S11494	invitrogen
T4 DNA Ligase	M0202L	BioLabs
T4 Polynucleotide Kinase	M0201S	BioLabs
TEMED	#161-0800	BIO-RAD
Trizma base	C <sub>4</sub> H <sub>11</sub> NO <sub>3</sub>	SIGMA
Urea	CH <sub>4</sub> N <sub>2</sub> O	SIGMA
SYBER® gold	#S11494	Invitrogen
YO-PRO®-1	-	Invitrogen

\*Acrylamide (CH<sub>2</sub>CHCONH<sub>2</sub>) and Bisacrylamide (N,N-methylenebisacrylamide) C<sub>7</sub>H<sub>10</sub>N<sub>2</sub>O<sub>2</sub>

## 9. Appendix

### 9.2 Instruments

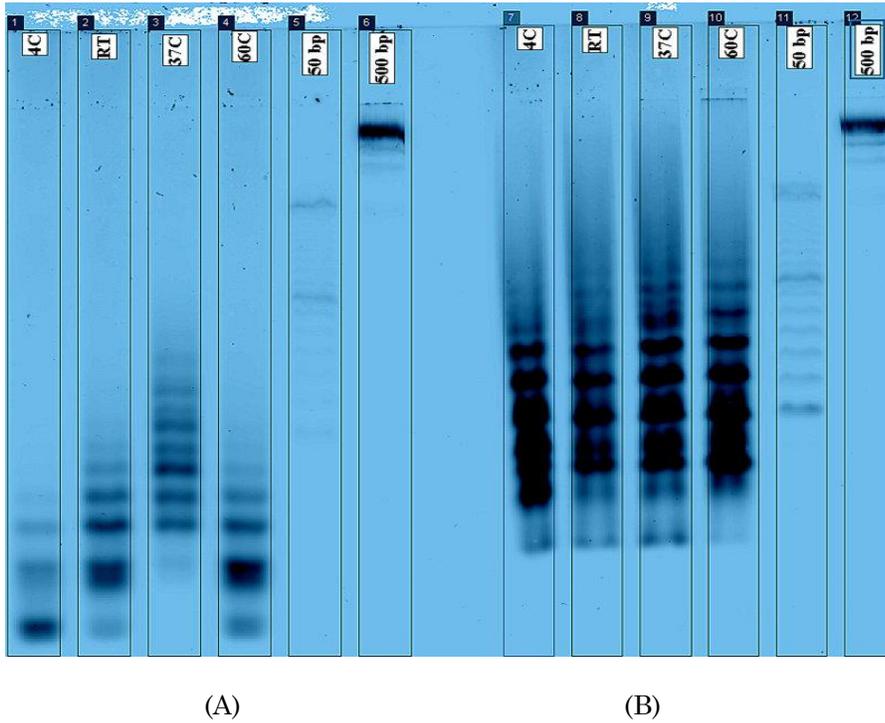
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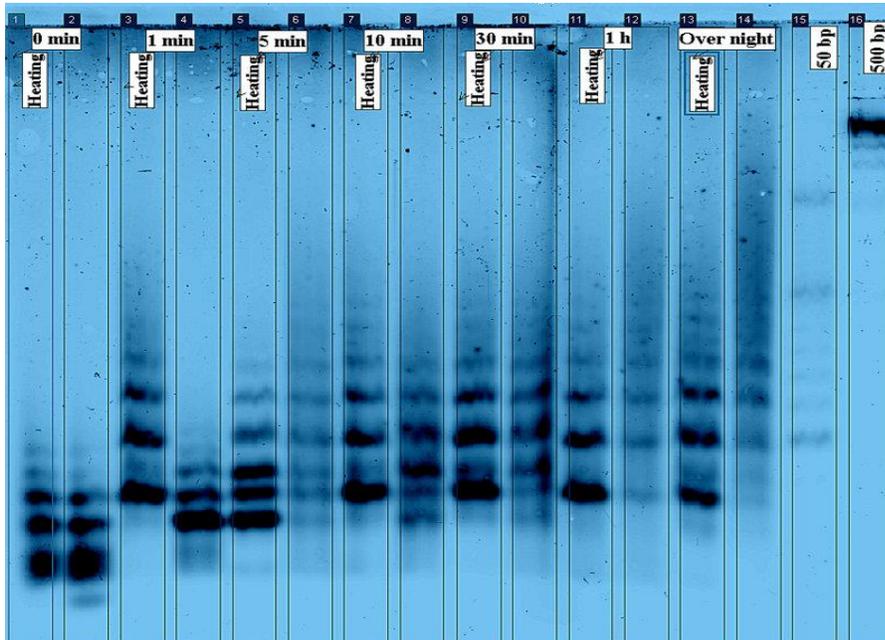
Name	Model/Company
Balance	PL 300/ Mettler
Electrophoresis Power supply	EPS 601- GE Healthcare
Electrophoresis System	BIO-RAD Mini-PROTEIN® Tetra System
Gel Scanner	Typhon 9410/ GE Healthcare
Heating block	QBD2- Grant Instruments Ltd
QCM-D	Q-Sense E4- Biolin Scientific / Q-Sense
Spectrophotometer	Cary 5000/ Varian
Water Bath	-

## 9. Appendix

### 9.3 Agarose gel electrophoresis results

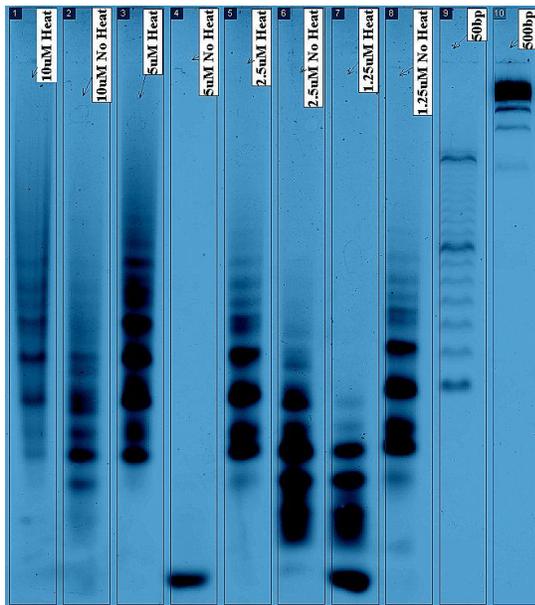


**Figure.9.3.1** 2.5% Agarose electrophoresis, 2.5 μM oligos, different hybridization temperatures, (A) 5 mM PSB, (B) 200 mM PSB, gel run at 4V/cm for 3h.

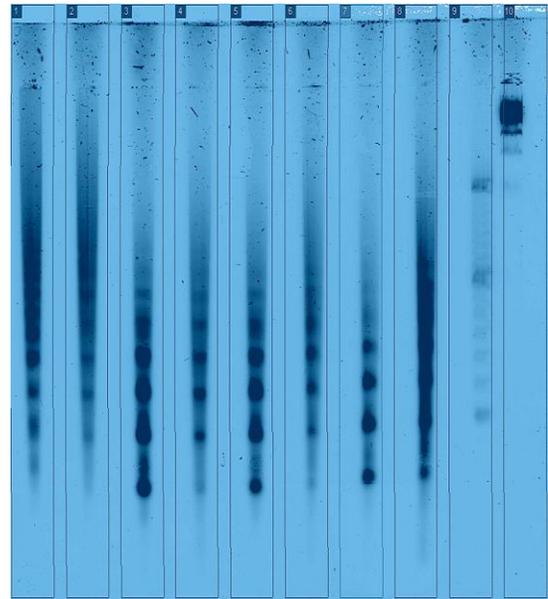


**Figure.9.3.2** 2.5% Agarose electrophoresis, 1.25 μM oligos, different hybridization times, gel run at 4V/cm for 3h.

## 9. Appendix

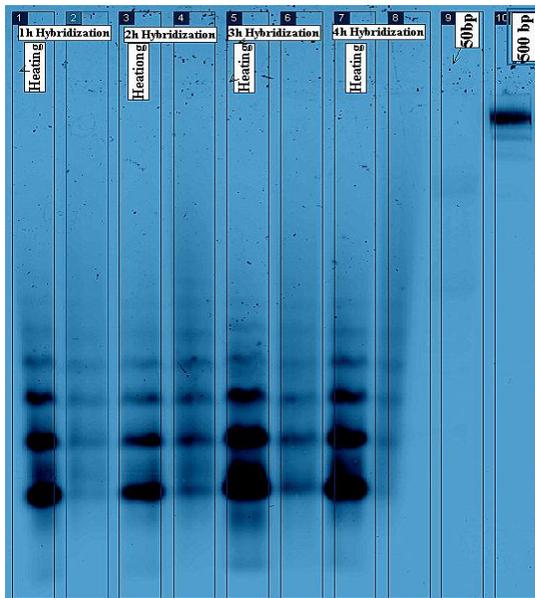


(A)

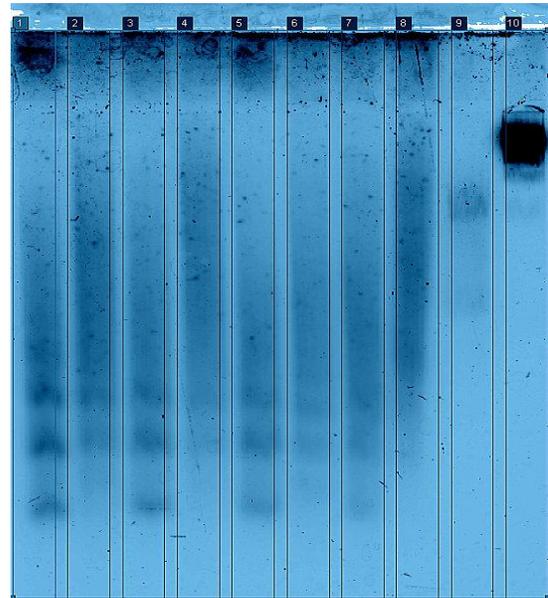


(B)

**Figure.9.3.3** 2.5% Agarose electrophoresis, heating vs. non-heating for different oligo concentrations, (A) 5 min heating, (B) 10 min heating, 200 mM phosphate buffer, 1h hybridization time at RT, gel run at 4V/cm for 3h.



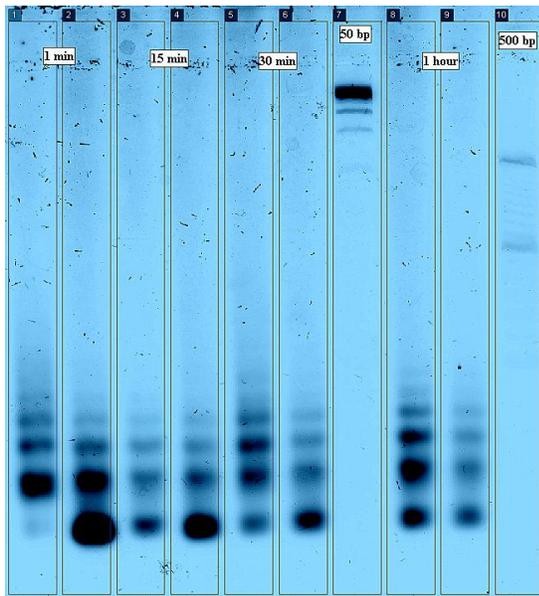
(A)



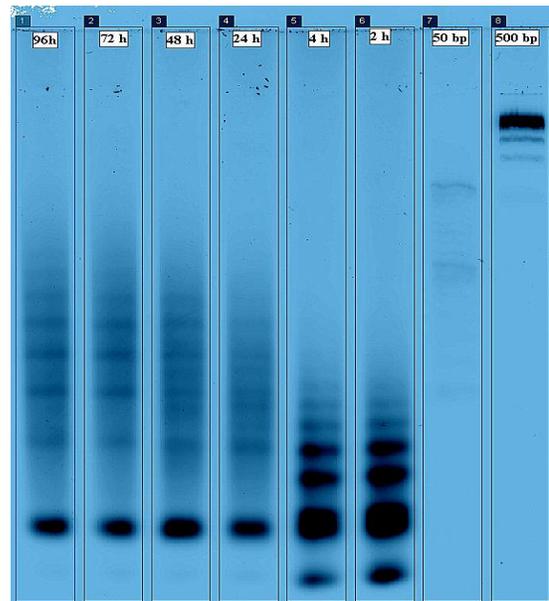
(B)

**Figure.9.3.4** 2.5% Agarose electrophoresis, 1.25  $\mu$ M oligos, two different protocols for different hybridization times (1, 2, 3 and 4h) (A) same starting point (B) same ending point, gel run at 4V/cm for 3h.

## 9. Appendix

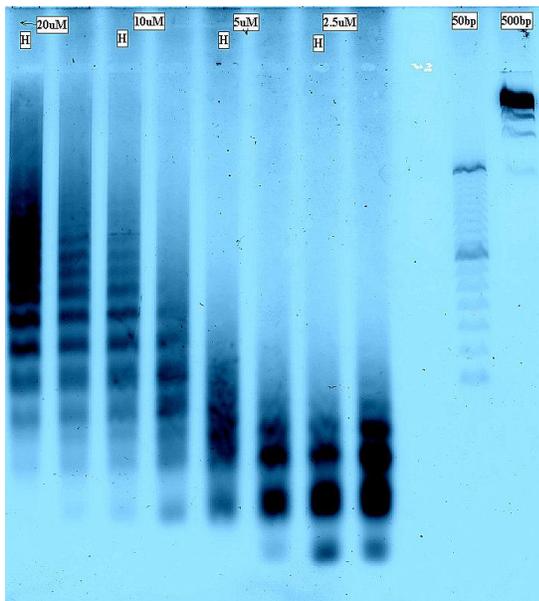


(A)

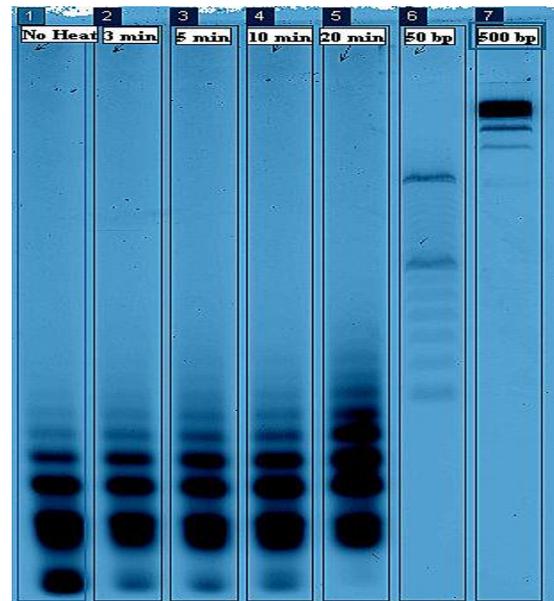


(B)

**Figure.9.3.5** 2.5% Agarose electrophoresis, 2.5  $\mu$ M oligos, different hybridization times (A) (1,15,30 min and 1h) 5 min heating (B) (96,72,48,24,4 and 2h) no heating, gel run at 4V/cm for 2h and 45 min.



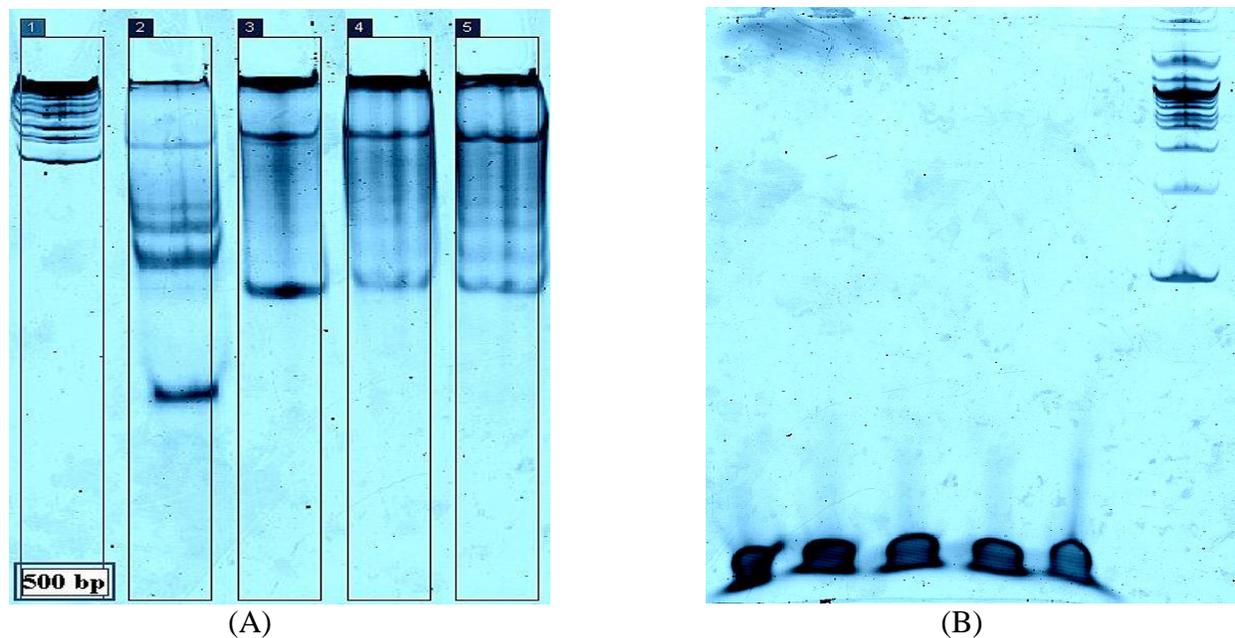
(A)



(B)

**Figure.9.3.6** 2.5% Agarose electrophoresis, (A) different oligo concentrations (20,10,5 and 2.5  $\mu$ M) (B) different heating times, 2.5  $\mu$ M oligo, 1 h hybridization in RT, low salt concentration, gel run at 4V/cm for 3h.

#### 9.4 Polyacrilamide gel electrophoresis results



**Figure.9.4.1** (A) 5% Native PAGE electrophoresis, Ligation of 20  $\mu$ M oligo (B) 5% urea PAGE electrophoresis, Ligation of 20  $\mu$ M oligo, gel run at 8V/cm for 30 min.