DEVELOPMENT OF G-QUADRUPLEX ASSISTED HYBRIDIZATION CHAIN REACTION SYSTEM

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ABSTRACT

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Kanat, Beyza

Master of Science Program in Bioengineering

Advisor: Assoc. Prof. Dr. Osman Doluca

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Hybridization chain reaction (HCR) is a technique used for the diagnosis of nucleic acids and does not require an enzyme. In this technique, the hybridization cascade is initiated with the target molecule DNA or RNA, and this is accomplished by means of toehold mediated strand displacement between hairpin sequences in the environment. Ultimately, the goal is to achieve signal amplification. Although the HCR method is considered as an alternative to the PCR (polymerization chain reaction) method, its still low measurement sensitivity has led to new designs. In this thesis, a study was carried out on a new HCR design. A new HCR mechanism has been developed as a result of the use of fluorescent labeling techniques with the help of alternative DNA structures called G-quadruplex. In this design, it is aimed that the

detection limit is low and the measurement sensitivity is high. The HCR system described in this thesis used G-quadruplex and fluorescent labeling together for the diagnosis of nucleic acid at concentration levels as low as 20 pM. It is concluded that the Amplex Red(™) used in this study provides an increase in fluorescence and reduces the diagnostic limit by 10 times and is explained in this thesis.

Keywords: G-quadruplex, hybridization chain reaction, nucleic acid detection, signal amplification

ÖZET

G-QUADRUPLEKS DESTEKLİ HİBRİDİZASYON
ZİNCİR REAKSİYON SİSTEMİNİN GELİŞTİRİLMESİ

Kanat, Beyza

Biyomühendislik Yüksek Lisans Programı

Tez Danışmanı: Doç. Dr. Osman DOLUCA

Temmuz, 2021

Hibridizasyon zincir reaksiyonu (HCR) nükleik asitlerin teşhisi için kullanılan ve enzim gerektirmeyen bir tekniktir. Bu teknikte hedef molekül olan DNA veya RNA ile hibridizasyon kaskadı başlatılır ve ortamda bulunan hairpin sekansları arasında "toehold temelli iplik yer değiştirme" aracılığı ile gerçekleştirilir. Sonuçta amaç sinyal amplifikasyonunu gerçekleştirmektir. HCR metodu, PCR (polimerizasyon zincir reaksiyonu) metoduna alternatif olarak düşünülse de halen daha ölçüm hassasiyetinin düşük olması yeni tasarımlar yapılmasına yönlendirmiştir. Bu tezde de yeni bir HCR tasarımı ile ilgili çalışma gerçekleştirilmiştir. G-quadrupleks denilen alternatif DNA yapılarının yardımı ile floresan etiketleme tekniklerinin de kullanılması sonucunda

yeni bir HCR mekanizması geliştirilmiştir. Bu tasarımda teşhis limitinin düşük olması ve ölçüm hassasiyetinin yüksek olması amaçlanmıştır. Bu tezde açıklanan HCR sistemi 20 pM kadar düşük konsantrasyon seviyesinde nükleik asit teşhisi için G-quadrupleks ve floresan etiketlemeyi birlikte kullanmıştır. Bu çalışmada kullanılan Amplex Red(™)'in floresan artışını sağladığı ve teşhis limitini 10 kat düşürdüğü sonucuna ulaşılmakta ve bu tezde açıklanmaktadır.

Anahtar Kelimeler: G-quadrupleks, hibridizasyon zincir reaksiyonu, nükleik asit teşhisi, sinyal amplifikasyonu

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CHAPTER 1: INTRODUCTION

DNA is a biological molecule that contains and codes genetic information. It exists in various conformations and has functions. It has a backbone structure where deoxyribose sugar, phosphate groups, and nucleobases are bind together. The most common form is B-DNA and has a normal double-stranded helix structure. Some small molecules bind to the DNA molecule and these can be classified as intercalation and groove binding (Sinden, 1994).

There are also ten different types of alternative DNA molecules that are not called B-DNA. A few of them can be thought of as hairpins, triplexes called H-DNAs, Z-DNAs, G-quadruplexes. In the formation of these alternative structures, DNA sequences should be folded in different conditions and angles compared to B-DNA. As another possibility, the nucleobases must form different and unusual base pairs according to the Watson-Crick definition. It is known that Hoogsteen base pairs are of critical importance in stabilizing the conformation of alternative DNA structures. In addition, the DNA molecule has become important and applied in the fields of nanomaterials, molecular computing and the other critical areas. This is achieved by the well-known double-helix structure and its stiffness and flexibility properties. While well-known DNA structures can be formed with nucleic acid sequences, different structures can also be formed according to various conditions. Over time, it has been understood that the guanine base has a different property compared to other bases. According to the conformation of the guanine bases, it is possible that four guanine bases are bonded together by hydrogen bonding and contain a positive ion such as potassium in the center. These square conformations are called G-tetrad. G-quadruplex structures are formed as a result of folding or stacking of these G-tetrads on top of each other. It is emerging that G-quadruplexes are increasingly important physiologically. Their presence in telomeres has an important role in every stage of central dogma, these can be called transcription, translation, replication (Choi and Majima, 2011).

G-quadruplexes have an enormous role in the realization of cellular processes and mechanisms such as gene expression and mRNA translation in particular (Harkness and Mittermaier, 2017).

Some techniques are used to measure and evaluate the formation capacity of G-quadruplexes. NMR, X-ray crystallography, circular dichroism spectroscopy can be

considered as examples of these techniques that provide structural information. Fluorescent labels are also used for imaging and easy identification. There are algorithms such as Quadparser, Quadruplexes, AllQuads, ImGQfinder. Despite many advantages, computational G-quadruplex prediction approaches also have limitations (Puig Lombardi and Londoño-Vallejo, 2020).

DNA amplification is a critical process that takes place in many analyzes and studies. One of the most common amplification methods is PCR (polymerase chain reaction). In this technique, the selected DNA fragment is specifically replicated. This amplification process using primers is achieved by making the primers anneal to the main chain. Then, these primers that bind with the polymerase enzyme extend and form the new chain. This stage is called extension. As a result, DNA synthesis takes place between the annealed primers and the main chain. In each cycle, the DNA product doubles compared to the previous one and amplification is achieved (Saiki, 1990).

There are three main stages in PCR. The first step is to divide the double chain DNA molecule by denaturing it into two. The second stage is that the primary sequences annealed to the chain, and the last stage is that the primary sequences elongate and complete the new chain. If the molecule to be amplified is not DNA but RNA, cDNA must first be synthesized before PCR and this must be provided with reverse transcriptase enzyme. While PCR cycles take about 3-5 minutes each, generally one process takes place between 20-40 cycles (Schochetman, Ou and Jones, 1988).

Isothermal amplification techniques are produced and used as an alternative to the PCR technique, especially in the field of point-of-care diagnosis. It is a great advantage that it is simple, fast and cost-effective. The isothermal amplification method is applied successfully especially in situ diagnosis of single biomolecules. Common ones can be said as hybridization chain reaction, strand displacement, rolling circle, helicase-dependant, recombinase polymerase amplification. In isothermal amplification methods, a thermal cycler device is not needed here as it is needed in PCR. It has many important advantages such as being easy to use and very good tolerance to inhibitory factors. In these terms, it is preferred over the PCR technique (Kim and Easley, 2011).

The isothermal amplification method based on the nucleic acid sequence called NASBA has an important place. NASBA which is known as Self-Sustained Sequence Replication Techniques (3SR) mimic the stages of some retroviral replication processes in vivo using the RNA template and its purpose is to produce RNA amplicons. The helicase-dependent amplification method, abbreviated as HDA, is a highly competent and high-quality technique for DNA amplification. The helicase enzyme allows the chains to separate from each other in this process. The HDA technique first begins with the helicase enzyme separating the double chain DNA molecule. Two different primers, called forward and reverse, are connected to this chain and the process continues with an elongation process in which the polymerase takes part. The HDA technique takes one or two hours and provides low copy number amplification. RPA refers to the recombinase polymerase amplification method. It provides the reaction taking place at a single temperature in a single tube. This temperature is generally constant between 37°C or 42°C. The RPA technique has been a favorite in point-of-care technology, with its rapid amplification process of 20-40 minutes and sufficient temperature conditions. Loop-mediated isothermal amplification (LAMP), generally works in the range of 60-65°C and performs the process with a minimum of four primer sequences. In this way, a very high specificity detection is achieved. Although the LAMP method is widely used in the diagnosis of pathogens, it has been found much more sufficient than PCR in bacteria, virus and fungal studies. Rolling circle amplification (RCA), has a high-efficiency effect on circular DNA molecules and provides strand displacement with a high level of polymerase activity consumption by using Phi29 bacteriophage DNA polymerase enzyme. Single primary isothermal amplification (SPIA) is a linear amplification technique. Chimeric DNA or RNA sequences are used as primers. It is based on the repeated replication of target sequences. RiboSPIA, on the other hand, provides the replication of original transcript products and provides high yield and quality products. Strand displacement amplification (SDA) is based on multifunctional primer sequences that recognize targets and also contain endonuclease sites. Following chain separation, these versatile primary sequences incorporate and extend restriction target molecules into the amplicon (Craw and Balachandran, 2012).

Hybridization chain reaction (HCR) is a type of isothermal amplification. It generally refers to the hybridization process between two different hairpin sets. It is

also a new technological method that enables fast detection of DNA sequences or other nucleic acids and molecules without using enzymes. Target identification and signal transmission in the use of the HCR in biosensors are realized through different pathways. HCR can be realized with the triggered self-assembly of DNA molecules. HCR is coming up as a by-product for that reason is hairpin forming oligonucleotides (HFOs) can be used as fuel packets. HFOs can be triggered to start the chain reaction. Scientists are realized that this can be used as amplifying signal transducer. Basically, the HCR is storage of potential energy of two hairpins. When adding the trigger which is an 'one strand DNA initiator', this trigger opens the Hairpin 1 (HP1) molecule and then, this opened Hairpin 1 molecule opens the Hairpin 2 (HP2). We can say that HP1 can be identical to original trigger. This HCR process is continued until the HFOs are consumed. The resulting products of HCR, do not need any detection tool. For biosensor applications, DNA and RNA aptamers are important for development of HCR triggers. Scientists are developing HCR systems which are provide quadratic, cubic or exponential growth when it is triggered by initiator (Park et al., 2018).

The main advantages of HCR are less difficulty in process, cheaper than the other methods, sensitivity and convenience. Nucleic acid experiments which are based on HCR should be totally without enzyme. Otherwise, it will repeal the most important advantage of HCR. A very good sensitivity could not be achieved in nucleic acid experiments where we used the HCR method. Various enzymatic amplification schemes were therefore applied to HCR to improve the assay sensitivity. HCR is used at room temperature and there is no need for any thermal cycler, so it is more advantageous than PCR. PCR is provided the exponential amplification and HCR is provided the linear amplification. The vulnerability of enzymes widely limits the applications of HCR nucleic acid assays in complex biological samples. The design of the HFOs should be thermodynamically more quantitative since a relatively small variation may adversely affect the amplification efficiency of HCR and even be a situation like not being able to start the HCR process (Bi, Yue and Zhang, 2017).

Some isothermal amplification techniques, such as RCA, SDA, and LAMP have been used for bio-imaging analysis. These methods need enzymes and often require complicated processes. HCR-based isothermal amplification methods have been reach extend usage to image various targets, such as extracellular matrix, tumor cells, and RNAs, demonstrating its advantages such as simplicity and their enzyme-

free. HCR-based bio-imaging methods include fluorescence in situ hybridization (FISH) imaging and live cell imaging and applications of HCR in biomedicine (Bi, Yue and Zhang, 2017).

It was stated that no enzyme was used in HCR experiments. An activity with an enzyme effect is needed. It is known that hemin/G-quadruplex complexes show peroxidase activity. This activity has the same effect as Horseradish peroxidase. Horseradish peroxidase is an enzyme. It is an enzyme that amplifies the signal by converting chemiluminescent substrates in the detection of target molecules such as protein, nucleic acid. Parameters such as catalytic oxidation rate and inactivation rate depend on the H_2O_2 concentration. Based on this, it can be said that H_2O_2, G-quadruplex forms and hemin are effective and important factors for the reaction (Yang et al., 2011).

Fluorescence Resonance Energy Transfer (FRET) is a mechanism that expresses energy transfer between chromophores. One of the chromophores is the donor and the other is the acceptor. Donor is in excited state. The energy in the donor can transmit non-radiative to the acceptor. The distance between the two chromophores is an important factor that can affect this transfer. FRET efficiency is used to find out if two fluorophores are far enough apart. A fluorophore is a chemical compound. Fluorophores can emit or absorb light in specific spectra. It re-emits light when light is excited. Reducing the fluorescent intensity is called quenching. Quenching is considered the basis for FRET assays. Quenchers absorb energy from a fluorophore. If these are dark quencher, the received energy comes out as heat. If these are fluorescent quenchers, the received energy is reflected as visible light. Examples of dark quenchers are black hole quenchers and there are BHQ1, BHQ2, BHQ3 varieties (Marras, 2008).

The guanine-rich sequences that we use in the HCR technique and in our design, especially the hairpin structures, were designed with the Nupack. A trigger sequence molecule and four hairpin sequences were designed and used in experiments. One of the HFOs, GH2 is also designed with FAM attachment and it is called GH2-F. The designed sequences were delivered to us through a private company. Fluorescence spectroscopy was used for experimental tests. In order to interpret the fluorescent peaks obtained, the bands formed by using the gel electrophoresis method were

observed, and hybridization of the hairpins in different buffers were investigated. Several kind of techniques such as polyacrylamide gel and agarose gel have been applied for gel electrophoresis. It is emphasized that two types of analysis, SDS-PAGE and Native-PAGE, can be performed on polyacrylamide gel. The main difference between them is the use of SDS, which means whether the gel contains sodium dodecyl sulfate.

The manuscript (Ang and Yung, 2016), which we consider as a guide for experiments, was also applied. In the assay containing HP1 and HP2 (100 nM), GT concentrations of 0 nM, 1 nM, 2 nM, 5 nM, 10 nM, 100 nM were compared. The excitation value is set to 520 nm. Peaks were obtained at wavelengths of 560 nm and 660 nm. The intensity values of the peaks at 660 nm are directly proportional to the GT concentrations.

In in silico experiments, it was observed that HFOs did not form complexes in the absence of GT and they were present in high concentration (80 μM) in the medium. However, HFOs are known to form complexes when GT is present. In this case, only GH4 is present in a high concentration. This is because the complexity value is selected as 4. It could be seen that gh4 could polymerize with other HFOs if the complexity had been chosen higher. In the GH1-GH3-GH2-GT complex, four groups of G-G-G base sequences were found. These bases can be folded to form a G-quadruplex. The existence of these regions is one of the goals of our HCR design.

In gel experiments, the effects of magnesium, lithium and potassium on G-quadruplex formation were observed by comparing monovalent ions. As a result of magnesium being more effective, we focused the studies on this ion. This issue was investigated by performing Mg^{++} concentration gradient experiments. Comparisons with higher concentration mg buffer did not yield very distinctive results. We conclude that the working conditions, parameters or the concentration were not increased enough.

G4-HRP activity experiments were performed to examine the operation of the HCR system without FAM dye. In these experiments, GT concentration gradient studies were performed using GH2. Concentrations are 100 fM, 1 pM, 10 pM, 100 pM, 1 nM. DNA stability is increased with the use of FAM dye. In these experiments, DNA stability and the effect of quencher were observed by not using FAM. As a result, 1

nM GT concentration gave the highest peak. It is concluded that the HCR system works and is successful. but insignificant peaks occurred at other concentrations.

The GH system containing GH2-F was tested in fluorescence experiments. The effect of different GT concentrations was observed. GT concentrations of 0.2 nM, 20 nM and 1 μM were studied. The 0.2 nM GT concentration was determined as the lowest possible concentration. It was concluded that in these fluorescence experiments, when AmplexRed was added and incubated for 15 minutes, a linear range was not detected and more work was required to investigate the linear detection limits, however, qualitative detection could be obtained for concentrations as low as 20 pM.

The aim of this thesis is to introduce a new design of the HCR technique and to express that it can be used in many biological and clinical studies and in many fields. We have implemented and demonstrated this new design both bioinformatically and experimentally. We would like to point out that the newly designed HCR assembly is a very new and very efficient technique in its field, with advantages such as isothermal, does not require enzymes, is fast and easy to perform, and does not require a temperature gradient.

CHAPTER 2: LITERATURE REVIEW

2.1 DNA Structures and G-quadruplexes

Double-stranded DNA generally consists of the genetic information in its structure. This common structure is called as right-handed DNA molecule which is a B-form DNA molecule. Hydrogen bonds, where found between bases, provide this B-form structure. The other DNA forms which are called non-B-form DNAs or alternative DNA structures have importance for protein interactions. These proteins can have a role in replication, expression, or recombination processes. On the other hand, alternative DNA structures have another major role in the basis of nucleosomes and in the formation of DNA-containing supramolecular structures (Wood, 2016).

A-form DNA structure and B-form DNA structure formate by random or mixed DNA sequences. These sequence types are responsible only for A-form and B-form DNA structures. For looking at other alternative DNA types, there are need some symmetry elements or exclusive sequence characteristics. If these requirements are provided, the alternative forms which are left-handed DNA form that is called Z-DNA structure, cruciforms, intramolecular triplexes, quadruplex DNA structures, slipped-strand DNA, parallel-stranded DNA forms, also unpaired DNA forms can be obtained (Wood, 2016).

Local alternative DNA forms and differences of DNA supercoiling create opportunities in autoregulation for DNA functions. Canonical B-DNA form is a double helix structure that contains two antiparallel chains, also hydrogen bonds hold these structures together from in base pairs (between A-T and G-C). 10.5 base pairs create one turn for B-DNA structure, also they are perpendicular to the axis of the helical structure. DNA has two grooves which are major and minor and the diameter is 20 Å (Wood, 2016).

Table 1. The table shows that the parameters of B-DNA form using the information in some studies which are about oligonucleotide duplexes in crystals (Source: Wood, 2016).

Features	A DNA	B DNA	Z DNA
Diameter of helix	23 Å	20 Å	18 Å
Rotation	32.7°	34.3°	-30°
Base pair tilt	20	-6	7
Length of the helix	28.2 Å	34 Å	44.4 Å
Axial rise per residue	2.55 Å	3.4 Å	3.7 Å
Residue per helical turn	11	10.5	12
Direction of helix	Right handed	Right handed	Left handed

A-DNA and Z-DNA forms have double-helical structures but their base-pair arrangement is different from the B-DNA forms. 11 base pairs create one turn for A-DNA structure, also grooves of A-DNA form are not too much deep as in B-DNA form (Wood, 2016).

A-DNA forms are created with some physiological conditions and those forms a result of high-energy conformations in double helix structures. A-DNA forms are stabilized with the presence of alcohols in some aqueous liquids or in reducing water activity conditions. Also, it shows that A-DNA structures are a form of double-helical parts of RNA, that's why DNA and RNA hybrid duplexes. On the other hand, there are some proofs about DNA's local transitions as B-form to A-form during transcription. Also, there are proteins that can exchange the DNA to A-form (Dickerson, 1992).

The discovery of the Z-DNA form is a huge event in the science world but it was unnecessary. Because a lot of previous studies show that there are so many alternative DNA forms. There are a lot of X-ray evidence for nucleic acid helical structure and all of them are coming from fiber diffraction studies which are used on natural DNA molecules and simple sequence synthetic polynucleotides. In normal conditions, these evidence are not as certain X-ray crystallography. B-DNA forms can contain any potential sequence, even so, there can be some alternative structures in some conditions which are using some specific sequences and environments. When

looking at all structures, the most different one is the Z-DNA form, because it is left-handed, least twisted, skinniest helix structure. There are some thoughts about the other, new, and different alternative DNA forms in the future. Some evidence and calculations show that the other helixes can be the stable versions which including the left-handed of normal B-DNA forms. High levels of salt or spermine are needed to convert the poly(dG-dC) structure to the Z-DNA form. Pohl and Jovin studied about high salt concentration of poly(dG-dC) with circular dichroism spectrum about ten years ago. Alternative purine and pyrimidine sequences can create Z-DNA forms, so a short stretch of dG-dC can stay as B-DNA form. This process can be realized using recombinant DNA technology as cloning dG-dC sequences in selected plasmids. Also, circular dichroism and NMR technologies contain these sequences (Cantor, 1981).

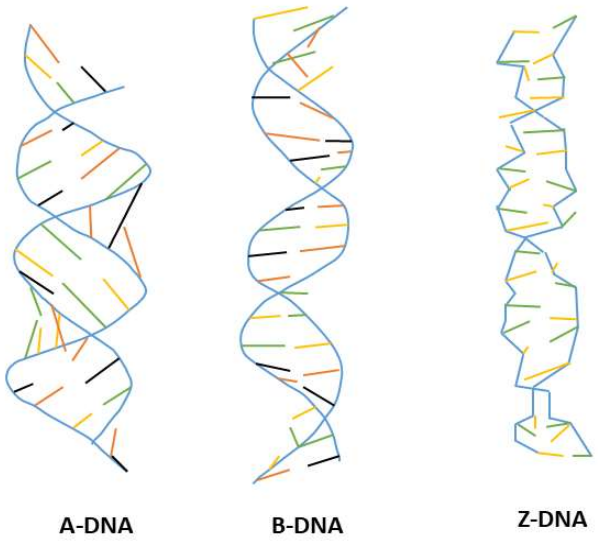


Figure 1. Structures of DNA molecule. From left to the right A-DNA, B-DNA, and Z-DNA respectively.

Some differences in the DNA molecule are dependent on environmental factors and sequence. Also, the dynamic properties and structural features of short oligonucleotides are understandable now with using NMR and X-ray crystallography techniques. Some properties like groove dimensionality which is dependent on sequence are feasible to longer sequences. On the other hand, those properties may have a relationship with DNA in nucleosomes. Even so, these assumptions are not supported with real evidence. There are some features about DNA flexibility, those are not too much understood. The relationship of DNA with macromolecular ligands all the time contains conformational changes in the DNA molecule. Also, there is not too

much information about them. DNA has sequence specific recognition affected by groove binding ligands contains a few factors which are minor groove, hydrogen bonds, displacement of bound water, and also hydrophobic interactions. There are small individual differences in DNA structure which open those minor grooves and increased the interactions by sequence releated induced fit. Additionally, crystal structure of the netropsin dodeca nucleotide complex can shows us those informations (Neidle, Pearl and Skelly, 1987).

2.1.1 Formations of G-quadruplexes and Their Functions

Function of any molecule is depend on its structural properties. According to DNA molecule, the double helix structure is most common known structure type and it provides genetic code with using base pairing. There are so much information about dynamic structure of DNA and its predispositon to adopting secondary structures. G-quadruplex is the most known secondary structure of DNA molecule and it is four stranded (Spiegel, Adhikari and Balasubramanian, 2020).

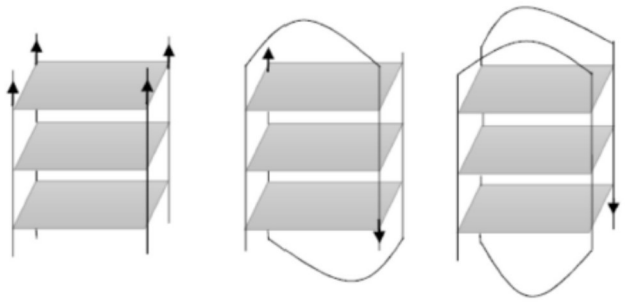


Figure 2. Schematic illustration of non-B-form DNA structures. DNA can assume so many kind alternate conformations depending on sequences. The figure shows that firstly; G4 DNA is formed via a parallel arrangement of four guanine rich DNA strands. Secondly; intermolecular G4 conformation is formed by DNA sequences with G-rich repeats forming hairpins that dimerize to stabilize the bimolecular structure. Finally; intramolecular G4 DNA is formed by a single DNA strand with either four guanine rich repeats or longer guanine tracts that can fold upon themselves to form the G-quadruplex structure.

Guanosine monophosphate and its other types can aggregate as self way. Guanylic acid or guanosine monophosphate formate four strand and right handed helices, also it is stabilised with Hoogsteen hydrogen-bonds for guanines to create co-planar G-quartets and its understood with fibre diffraction. Also, the oligonucleotide

sequences from immunoglobulin switch area and telomeres can increase the stability of G4 structures for in vitro conditions. G quartets are stabilised with cations to O6 of guanine bases and the cation options are respectively K+ > Na+ > Li+. G quadrets can show intermolecular and unimolecular property, also G4s can have different conformations based on strand type, length and direction. X-ray crystallography and NMR techniques and studies show some important informations about DNA G-quadruplex structures and those studies based on some specific human genome promotor regions and human telomeric repeat. There are genomic motifs which are created by algorithms and they defined the G4 structures in genomic DNA. According to the first algorithms, the models show that the loop lengths can not be more than seven and there have to be four continous guanines. There are another factors which are cytosine methylation or guanine oxidation, and those are effective on stability level of G-quadruplexes (Spiegel, Adhikari and Balasubramanian, 2020).

2.1.2 The Other Factors Such as Little Molecules That Bind G-quadruplexes

Telomerase is a molecule which is added new parts to 3'end of DNA molecule, also it is called as reverse transcriptase enzyme that is expressed in cancer. There are need some little molecules which provide inhibity in telomeric ends. Also these molecules are stable and liganded G-quadruplexes. For an approach, daunomycin molecule show up in complex G4 structure which is made up from four d(TGGGGT) strands and it seems with using X-ray structure of that little molecule. Also, there are a lot of little molecule known as complex structure with G4s by NMR and X-ray crystallography. There are some ligands are defined for modulate the expression of genes which can carry a sequence that create G4. On the other hand, there are 1000 small molecules can create complex structure with targeting G4 structures and those are identified in G-quadruplex Ligands Database. These small molecules have aromatic surface and they bind to loops and the other way is grooves of G-quadruplexes. Aromatic structure numbers, positive charges and hydrogen bond donor count can be exceed for reach best suitable conditions for small molecule with very well pharmacokinetic features (Spiegel, Adhikari and Balasubramanian, 2020).

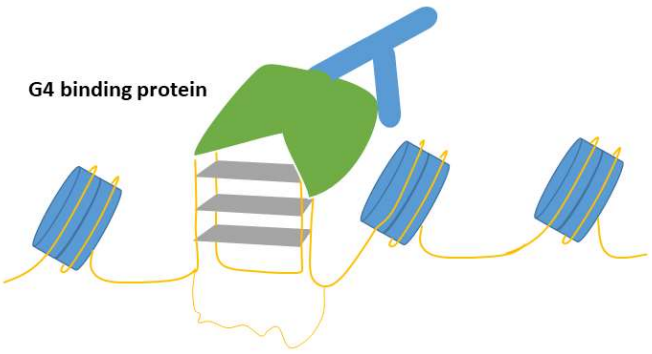


Figure 3. DNA damage can occur as a result of the DNA molecule being in the same environment with the G-quadruplex ligands and interacting with each other. It is seen in the figure where regions connected to G-quadruplexes are identified by ChIP sequencing, which can analyze protein and DNA interaction (Source: Spiegel, Adhikari and Balasubramanian, 2020).

G4 ligands which have no drug-like features are roll up in xenografts of cancer. For another aspect, MM41 is a G4 ligand and its X-ray crystallographic structure shows that structural properties of G4 can use interactions in grooves of that G4 structure. For looking the connection betwenn of backbone and groove regions do not need any straight aromatic structure. For that reason, non-flat compounds or grooves can have a importance for targeting G-quadruplexes. G-quadruplex ligands have structure and activity connection and this relation controls by some specific features such as polarity, lipophilicity, planarity, solubility and permeability (Spiegel, Adhikari and Balasubramanian, 2020).

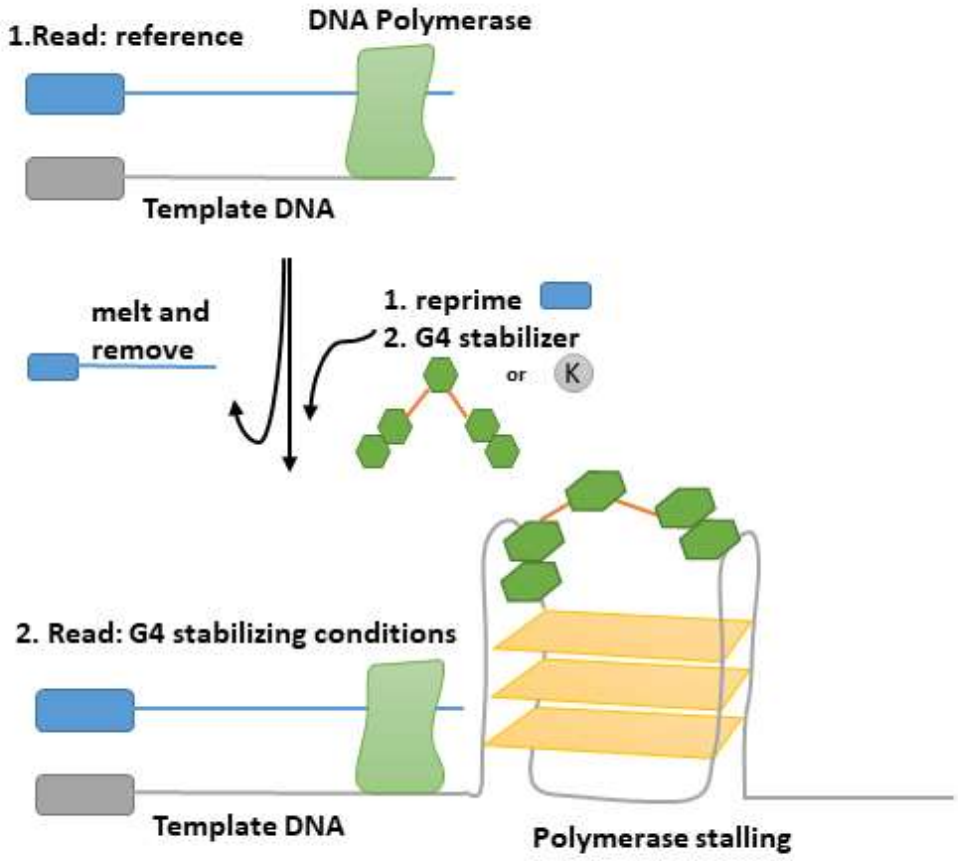


Figure 4. The sequencing of G-quadruplex structures found in the human genomic DNA is shown in the figure. Under conditions where G-quadruplexes can be stabilized, two sequential sequences are run and diagnosis of polymerase stalling attached to G-quadruplexes is performed.

There are algorithms that can predict the G-quadruplex structures and their motifs based on the sequence in the human genome. G-quadruplex structures are identified firstly in ciliate telomeres using an antibody that is specific to the G-quadruplex structure. This telomeric G-quadruplex creation is depend on protein interactions and they controlled those interactions dynamically. On the other hand, there is a discourse that G-quadruplexes are found in human cells or cancer tissues. Therefore, the antibodies can use for illustration those G-quadruplex movements and behavior. The telomeric G-quadruplex antibody can colocalize with human telomerase. G4 ligand molecules which are radiolabelled can show the place of telomeres. There is a say, G-quadruplexes can identify and detected with the application of alkyne functionalized G4 ligand molecules. In that process, cell fixation and azide-alkyne cycloaddition steps are followed. Fluorescent molecules have

different emission and excitation maxima values, and their decay lifetime when bind to G-quadruplexes is developed to illustrate living cells. Also, removing these molecules from nucleus and changing their place by G4 ligand pyridostatin observed in living cells by fluorescence microscopy. On the other hand, the specificity of G-quadruplexes increased by colocalization of G-quadruplex and G4 antibody complex in fixed cells (Spiegel, Adhikari and Balasubramanian, 2020).

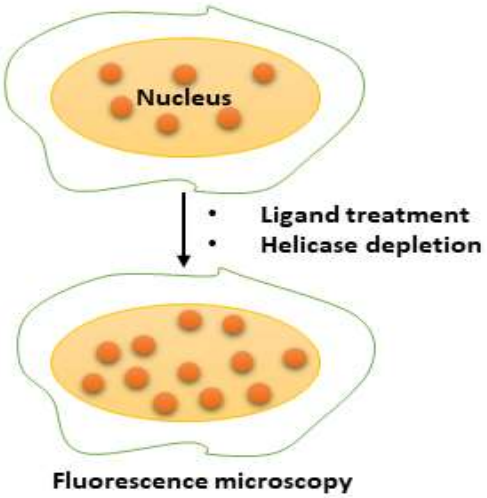


Figure 5. Some structure-specific antibodies or fluorescent G4 ligands are used to display G-quadruplex structures in cells. The number of G4s diagnosed on imaging can be increased with helicase depletion or ligand treatment. This is expressed in the figure.

2.1.3 Examination of G-quadruplex Structures for Their Biological Aspect

The place of G-quadruplexes which have been at area that regulate of genome function is included to G-quadruplexes which have role in biological processes. In mouse, RTEL1 which is mean regulator of telomere elongation Helicase-1 can seperate the G-quadruplex structures and then it protect the telomere integrity. In fact, G-quadruplex structures are expected to spoil the telomerase function. Also, healing to function can be also critical and important. Genetic and epigenetic instability is an important factor for G-quadruplex structures as in some conditions, especially when G-quadruplexes are not well regulated. For an example, PIF1 Helicase in yeast is effective in counterworking of DNA breaks and genomic instability which is based on G-quadruplexes. On the other hand, PIF1 Helicase in human can increase the healing of DNA breaks for encouraging the homologous recombination at sequences which have a chance to formate G-quadruplexes. Also, G-quadruplexes can be a sensor or

trapping sites of DNA damage when reactive oxygen species be caused that problem. G-quadruplexes have another role in replication process, for an example the G4s can restrain the replication fork process when helicases are broken. There is a thought about G-quadruplexes can trigger the initation of DNA replication. This thesis come up from genome mapping of four different human cell lines with using deep sequencing are caused of G4 enrichment. If there is a G4 structure formation, complemantary DNA chain can nat use Watson-Crick base pairing. Also, there can be a opposite chain which is C-rich and it can be single chain, then that chain can formate a complex structure with single stranded binding protein molecules. The mapping processes of endogenous G4s like ChIP sequencing, footprinting or immunofluorescence have been compatible with G4s which are marking the genes that doing transcription process. For an inference, G-quadruplex structures are related to the working and control mechanisms in biology, cancer cell growth, telomere biology, replication or genome instability. Also, G4 structures are over represent in genes which are cancer-promoting. If there are too much G4 structures in cancer states, G4 molecules can be select as molecular target for cancer. On the other hand, pyridostatin, RHPS4 and the other G4 ligands can be create DNA breaks in cancer cells and also those ligands can effect to DNA repair pathways as a good way. It can said, G4s can be promising targets for future therapeutics (Spiegel, Adhikari and Balasubramanian, 2020).

2.1.4 The Use of G-quadruplexes as Tools in The Biological Field

G-quadruplexes are defined as a tool in a lot of situation as binding to targets. There are aptamers which contain G-quadruplex are used for diseases as a therapeutic and diagnostic agents. Also, these aptamers should be stable as chemically and thermodynamically and they have to be low immunogenicity, also good cellular uptake. Aptamers which have contained G4s are responsible to recognize enzymes, small molecules or protein structures and the best example of that is thrombin-binding aptamer which is called TBA. That is defined by SELEX which is mean systematic evolution of ligands by exponential enrichment. There is a result with crystal structure of TBA, that is DNA G4s are in antiparallel conformation with two quartet planes. On the other hand, that can said thrombin stimulate the TBA for create G4s conformation to binding. Studies about TBA and the other similar molecules show that they can used for biosensing processes as well as with nanomolar affinity. According to biosensing

application examples, hemin binding aptamer PS2.M which is containing G4 shows catalytic features for binding hemin. There have been some controls of sequence and also structure obligations for those catalytic features to verify the critical task of G4s in that peroxidase mimicking system (Kwok and Merrick, 2017).

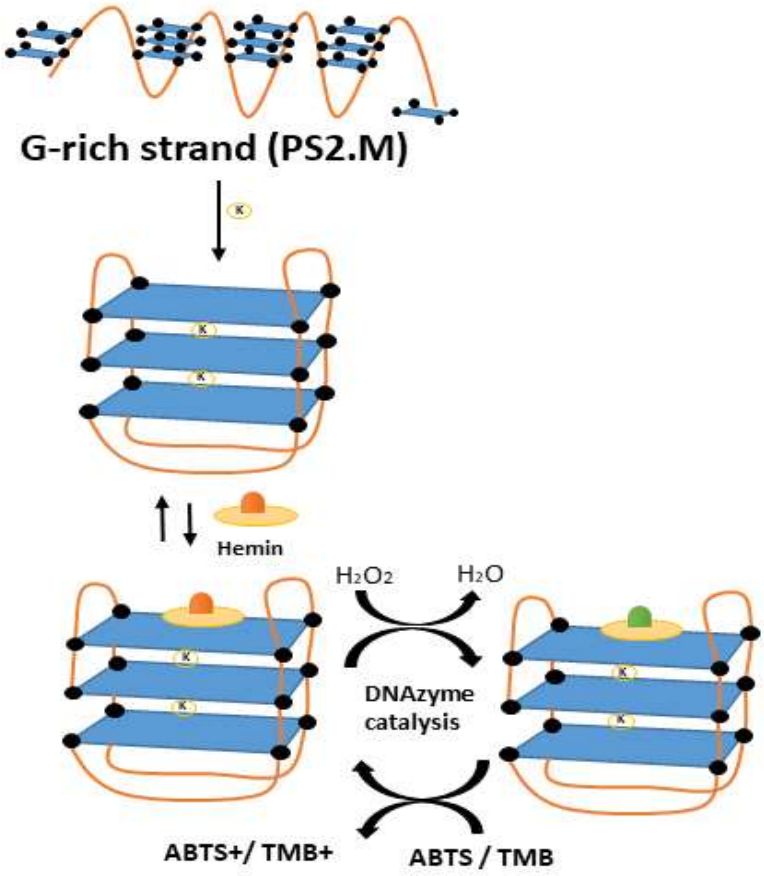


Figure 6. In the figure, it is seen that heme behaves like DNAzyme. The region named PS2.M is a sequence rich in the presence of guanine base, and this sequence folds into G-quadruplex in the presence of potassium ion. It then connects to hemin. This hemine and G-quadruplex structure shows peroxidase properties. Substrates such as ABTS or TMB can be used for colorimetric imaging (Source: Kwok and Merrick, 2017).

The other critical and important G4s application is QPA which is mean quadruplex priming amplification. It contains self dissociation of DNA duplex structures and then production G4 structures with primer extension reaction. After that, fluorescence signal is used to identify G4s (Kwok and Merrick, 2017).

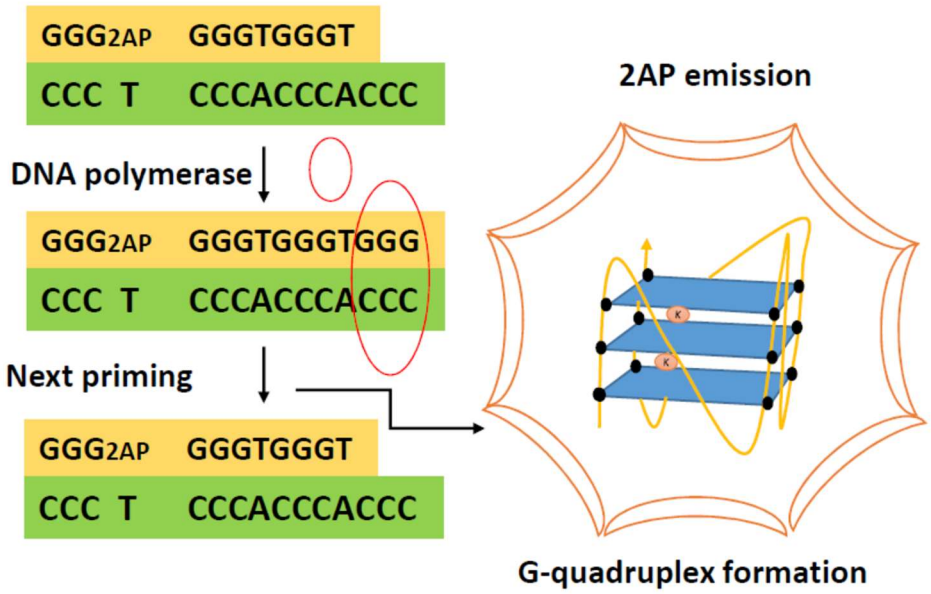


Figure 7. The figure shows the stage of preparing the quadruplex structure for amplification. The region called 2AP is the region abundant in guanine and lacks a guanine to form a G-quadruplex. DNA polymerase compensates for this deficiency. It does this by supporting the formation of G-quadruplex. G-quadruplex formation releases 2AP in the loop structure and thus a fluorescent signal is generated (Source: Kwok and Merrick, 2017) .

2.1.5 Understanding The G-quadruplex Structures with Computational Methods

In intramolecular structures, guanine repeats are found in same DNA strand. There are some intermolecular G-quadruplex structures found in sense and antisense strands. There are many algorithms are published for intramolecular quadruplexes to understand and know the potential G4s production from DNA sequence and these algorithms can be Quad-Parser, QGRS Mapper, G4P Calculator, QuadBase and also G4 Hunter. Understanding the intermolecular G-quadruplex structures are more complex, because both DNA strands have importance. At the same time, algorithms are create for RNA. Used computational methods are divided to biophysical and biochemical methods. For looking as an example, conformation of G-quadruplex structures can be monitored by circular dichroism signals. G4s structures with parallel topology have negative and positive circular dichroism signal levels at 240-262 nm respectively. But in anti parallel topology these signals are 262 and 295 nm levels respectively. On the other hand, thermostability of G-quadruplexes identified with observing UV signals at 295 nm value (Kwok and Merrick, 2017).

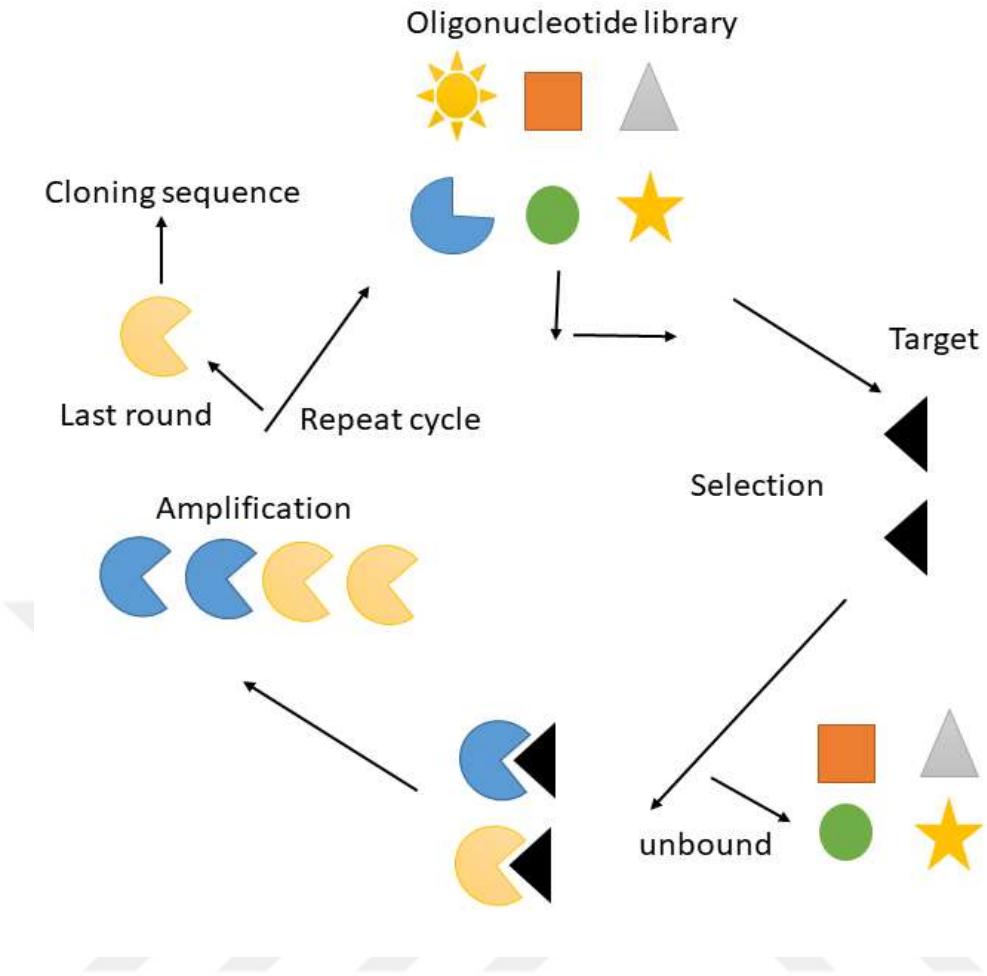


Figure 8. Stages of the SELEX process. A single stranded DNA/RNA molecule is synthesized and treated with the target molecule. Unbound sequences are avoided during the washing step. The linked sequences are amplified by PCR or RT-PCR (Source: Kwok and Merrick, 2017).

Cell imaging methods are used for illustration of G-quadruplexes. There are probes in living cells are investigate in time and it will be important which are called as 'light-up' probes. For another situation, there is Sty49 that is first quadruplex-specific antibody and it used for illustration of G4 structures in macronuclei of ciliate. Also, those contain huge amount telomeric DNA which are include a lot of G-quadruplexes. There is a problem with the detection of single G-quadruplex motifs and the advent of NGS provides designing G-quadruplex specific NGS methods at the genome and transcriptome level. They classified as antibody originated pull-down approach and polymerase stalling approach. For the first approach, the ChIP of G-quadruplexes is studied in a short time. Suitable antibodies can not be identified easily, because native chromatin content masks the majority of G-quadruplex epitopes.

Because putative G-quadruplex sequences folded in G-quadruplexes only at some specific situations. In all of these conditions, NGS used in especially the presence or absence of conditions (potassium ions and G-quadruplex stabilizing ligand pyridostatin) that favor quadruplex folding (Kwok and Merrick, 2017).

There are some ways that explain how we can detect G-quadruplexes using NGS. G4s can block polymerases and this process similar to the polymerase stop assays which is used for query individual structures. Potassium can stabilize the G4s, nevertheless, sodium and lithium ions have similar strength with potassium but their effect is the opposite. Also, pyridostatin stabilized G4s more than the others. Firstly, genomic DNA is sequenced in unfavoring conditions by Illumina to identify every fragment. Synthesized strand is peeled and again it is sequenced in G-quadruplex favoring conditions. Bases are added randomly by polymerase when it reaches to the stable G-quadruplex. It causes discontinuities in sequence detection and base quality. For example, when we look to primary human B lymphocytes, G-quadruplex sequence sites can compatible with computationally predicted G-quadruplexes (Vogt, 2015).

Non-canonical G-quadruplexes' detection is hard and these are enriched in gene bodies. Some new G-quadruplexes can detect with biophysical methods. There is not an option for know to all G-quadruplex sequence detects' fraction and which structures are generated in the cellular environment. When we used pyridostatin to find sites, it provides structural specificity and it does not overlap with those detected when using potassium. Some assays provide the detection of G-quadruplex motifs that are stable in physiological situations that disturb the biochemical mechanism, similar to polymerase delay at replication forks. There are some ideas that are about these structures that are irrelevant. As a result, there is so much evidence about genome instability and quadruplexes. For example, there is a study that shows G-quadruplexes can be found in BRCA1, BRCA2, and MAP3K8 genes which are related to the cancer disease. There is an assumption that G-quadruplexes are caused to generate genetic dysfunction and then create cancer because resolvase-type enzymes which are removed G-quadruplexes are consumed in tumors. There are studies which working in pin-down mechanism and these studies try to put G-quadruplex in a cellular content with immunoprecipitation-based enrichment, for instance (Vogt, 2015).

The other way is using the pull-down protocol with the HF2 antibody. There is a TBE urea gel and the pull-down of G-quadruplex oligonucleotides' analysis is realized on these gels with using HF2. Not single-stranded DNA is captured by HF2. G-quadruplex oligonucleotides are conquered with using HF2 in the asset of excess sonicated salmon sperm DNA. GeneRuler Ultra Low Range DNA Ladder and KIT-2 quadruplex are used in this process (Lam et al., 2013).

The other way is RefSeq genomic sequences that were used for the analysis of GQ/imGQ abundance and distribution in the human genome. Data are obtained from UCSC. Non-overlapping motifs' frequencies in the genome are calculated by ImGQfinder. Fragments that contain 4-7 guanine bases are called one putative GQ/ImGQ, 8-10 guanine bases are called two putative GQ/ImGQ. Non-G4 fragments are chosen randomly from ten million 33-nt random chromosome fragments. It repeated ten times. Non-G4 G-rich fragment distribution depends on Transcription Start Site (TSS)/ Transcription Termination Site (TTS) sites and exon/intron boundaries. Another situation is about PDS (pyridostatin) as a ligand that stabilizes G-quadruplexes and is used as a platform for advancing matrices for elective G-quadruplex isolation. It can be caused double-strand DNA breaks and it provides G-quadruplex detection and mapping in genomic DNA (Varizhuk et al., 2017).

2.1.6 Signaling Pathways and Detection of Nucleic Acids Such As DNA, RNA and Related Other Molecules

DNA, RNA, and the other nucleic acids also various type conformations on nucleic acids like G-quadruplex structures can be considered as markers in specific diseases. These detection ways can be realized with HCR methods which are seperated to two different groups and they are based on solid support or solution. DNA or RNA are used as trigger molecule in hybridization chain reaction of two hairpins which are kinetically trapped. Also that process can be ended with creation of DNA polymeric nanowires.

HCR products are included a lot of repeated parts and it provides an amplification platform for detection by working with a few labels to create signals. These processes contain fluorescent and calorimetric ways. Fluorescent biosensors which are based on HCR process, binding affect of labels are critical factors for signal transduction. There are some systems which are detection systems that have pyrene-

labelled hairpins. In that system which is inovated by Tan's group, pyrene units are adjusted on hairpin close to the pyrene molecule on contiguous hairpin. Transition from pyrene to pyrene excimer, emission shift in 375 nm- 398 nm range to 485 nm can be observed. That process provides sensitive detection of DNA molecule at low concentrations as fM and also it is feasible as quantitation of nucleic acid molecules in fluids. The other example in this way, is related to fluorescence quenching effects for graphene oxide. That can be realized between hairpins which are fluorophore labelled and HCR products.

2.2 Amplification and Techniques About DNA Synthesis

DNA amplification methods have critical role in bioanalysis and biomedical study areas as their powerful, easy and cheap properties. There are so many signal amplification or DNA amplification methods in literature, also in applications. Those methods can be PCR that is means polymerase chain reaction, RCA (rolling circle amplification), SDA which is called as strand displacement amplification, and HCR which is the main topic of our study area and it is called as hybridization chain reaction. For looking normal type HCR, there is an analyte and it triggers the opening process of DNA hairpin structures. HCR is efficient amplification method in terms of detection in high sensitivity for large aspect of analytes. Those can be nucleotides, proteins or small molecules. Scientists are designed some monomers for create non-linear HCR which can be branched HCR, also dendritic (Bi, Yue and Zhang, 2017).

2.2.1 Molecular Methods and PCR for Handling DNA Copies

There are a lot of molecular approaches and hybridization methods which we can named as Southern and Northern blot hybridization, dot and slot blot hybridization, FISH (fluorescent in situ hybridization), microarrays, quantitative PCR. Also those methods are used for identify microbial communities, quantification of some species as microorganism in specific environmental samples. The other area is determine the activity of some specific organisms. For use these methods there is need some material that is nucleic acid which can be DNA and RNA. Those are handled from samples without using cultivation of organisms. These sample kinds and nucleic acid types are effect the choosing the best extraction method. Extraction methods are depend on blow up cells and purified the final material that is nucleic acid from all other parts of cells. This blowing up or distruption process can be realized using chemical, physical ways

or enzymes. Critical step of this process that is purification need phenol-chloroform extraction step and ethanol precipitation. If there is need to only DNA, may use RNA free DNases. But if there is need to only RNA, use DNA free RNases. There are some kits which are used for handled nucleic acid easily. There is no need use phenol-chloroform extraction step and ethanol precipitation with this kits (Kaksonen, 2007).

2.2.2 PCR Which is a Technique Used in DNA Amplification and Synthesis

PCR is the most known method and it is a hybridization method. When there is a need to synthesis of DNA as nucleic acid, PCR can be useful in this respect. PCR is a in vitro method that provide a lot of DNA sample with amplified the DNA fragments. For this application the inital DNA should be denaturated by heat. After that, oligonucleotide primers are goes and stick to seperated chains of DNA and this step is called annealing. There are producing copy parts of selected areas of DNA strand with using polymerase enzyme and that process called as extension. All of these three steps and its repeating procedure is an exponential amplification of target (Kaksonen, 2007).

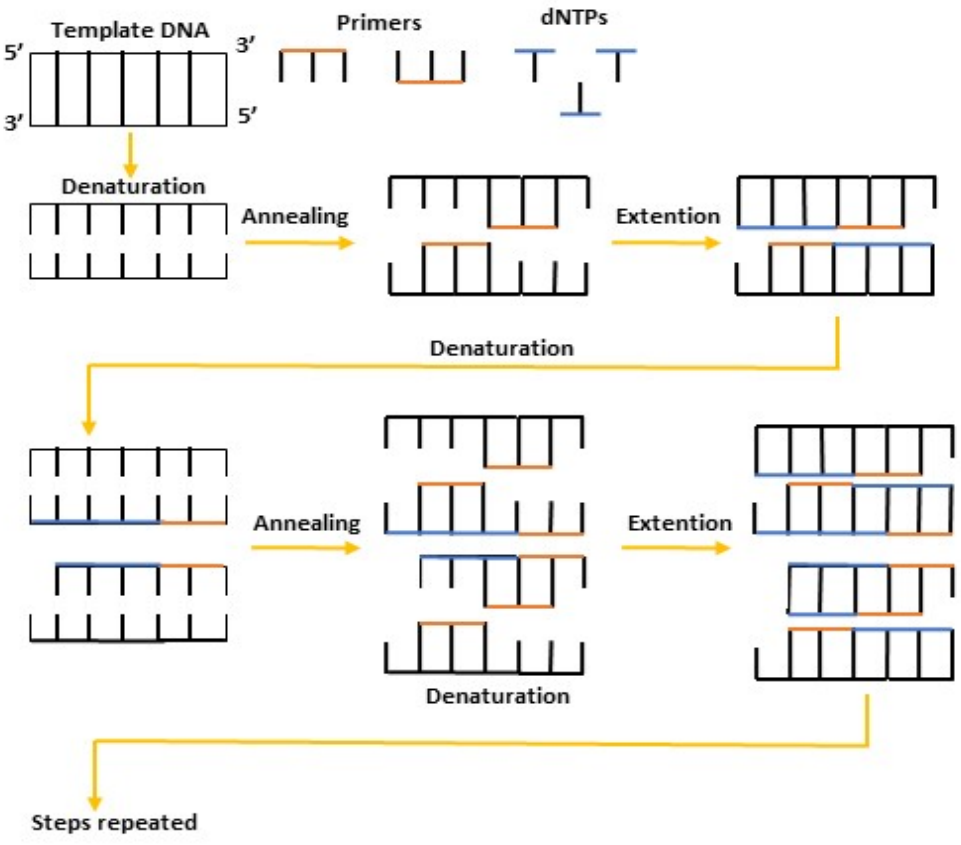


Figure 9. Steps of the PCR method (Source: Kaksonen, 2007).

Realizing this PCR method can be happened by thermal cycling machine. Cooling and heating steps are programmed automatically. PCR process needs a mixture which contains DNA polymerase enzyme, dNTPs, primers, buffer and template DNA strand. Primers are present the short parts of complementary strand. Also, these are the starting point of DNA amplification process (Kaksonen, 2007).

PCR has some disadvantageous as there is no detectable final product and obtain low yield product because of inhibitors. These inhibitors can be phenolic compounds or metals. Also, there are another problems like preferential amplification, mispair-extension of primers, creation primer-dimer and their competition for generate the final product. If increase the specifity and yield of final product, reaction conditions should be change as optimal situation by avoiding contamination risks, changing concentrations or temperature or cycle number. On the other hand, the primer and polymerase quality are effective factors for result (Kaksonen, 2007).

2.2.3 Isothermal Amplification and Related Methods

Amplification is a method which provides an opportunity to multiplying copies of chromosomal regions within a chromosome arm in molecular biology. For this area, polymerase chain reaction is a good selection to creating multiple copies of small parts of DNA molecule. Isothermal amplification of nucleic acids is actually quite simple and can accumulate nucleic acids quickly without the need for changing temperature gradients (Zhao et al., 2015).

Amplification technologies are also valid for recombinant DNA technology besides molecular biology. With these techniques, small amounts of nucleic acids can be diagnosed and analyzed. As is known when it comes to DNA amplification, PCR is the most commonly used and common technique. We witness the widespread use of PCR especially in the identification and threat of infectious diseases, genetic disorders and other situations. Unfortunately, in order to use PCR technology, it is necessary to have a thermocycling machine. With this machine, the DNA molecule is separated into two chains and then amplified. The fact that PCR requires a machine causes both expensive and long processes. In addition, the necessity of using enzymes is among the disadvantages. As today's technology develops, a light has been shed on this machine requirement problem. Instead, options have been developed to allow DNA amplification in vivo, without the need for a thermocycling machine under isothermal

conditions (Gill and Ghaemi, 2008).

A wide variety of isothermal amplification methods have been developed in the early 1990s and these have been an alternative to PCR. Isothermal amplification methods perform the identification of nucleic acid target sequences aerodynamically, exponentially, while independent of thermal cycling. From the moment the reaction starts, it is expected that the polymerase enzyme will perform the chain separation for the relevant sequence (Zhao et al., 2015).

For DNA replication, the DNA polymerase enzyme has to work with a number of accessory proteins. By identifying these proteins, it was possible to imitate in vivo mechanisms and to develop new isothermal amplification methods in vitro. There are many amplification methods, and there are studies on isothermal nucleic acid amplification methods such as transcription-based amplification, nucleic acid sequence-based amplification, signal-based amplification of RNA technology, strand displacement amplification, rolling circle amplification, loop-mediated isothermal amplification of DNA, isothermal multiple displacement amplification, helicase-dependent amplification, single primer isothermal amplification, and circular helicase (Gill and Ghaemi, 2008).

It was known that isothermal amplification methods take place at a certain temperature (generally 60-65 °C), under simple conditions and are much less complicated compared to PCR. Isothermal amplification methods are also distinguished among themselves according to various conditions and targets. Factors such as enzyme type, primary use status, sensitivity and specificity affect this situation. Apart from the methods mentioned above, Recombinase Polymerase Amplification and Exponential Amplification Reaction are also available (Üvey and Ünal, 2018).

Considering the isothermal amplification methods in general, they can be examined under 3 different main headings and a grouping can be made as follows:

1-Exponential Amplification Methods

2-Linear Amplification Methods

3-Cascade Amplification Methods

The varieties examined under the name of exponential amplification are as follows:

- Nucleic acid Sequence-Based Amplification
- Exponential Strand Displacement Amplification
- Loop-Mediated Isothermal Amplification
- Exponential Rolling Circle Amplification
- Helicase-Dependent Amplification
- Recombinase Polymerase Amplification
- Whole Genome Amplification
- Emerging Exponential Isothermal Amplification

The type examined under the name of linear amplification is as follows:

- Linear SDA
- Linear RCA
- Transcription based Amplification
- Signal Amplification Strategies

The classification examined under the name of Cascade amplification is as follows:

- SDA-Combined Cascade Amplification
- RCA-Combined Cascade Amplification
- Additional Cascade Amplification (Zhao et al., 2015)

If we look to nucleic acid sequence based amplification (NASBA) and transcription mediated amplification method (TMA), we can see that both are very similar. The basis of these techniques lies in using the function of RNA polymerase to obtain RNA by a promoter designed from the primer site. Reverse transcriptase enzyme is also known to assist in the process of obtaining DNA from RNA template. With the activity of a third enzyme, RNAse H, this RNA amplification technology is highly evolving and advancing. This enzyme ensures the removal of RNA from the cDNA, and the heat-denaturation step is not performed in this process. For this reason, the use of the thermocycling machine is eliminated and thus the isothermal amplification method called 3SR called self sustained sequence replication is produced. The determination of the final products obtained with the NASBA technique can be performed with gel electrophoresis and fluorescent probes. The method in which fluorescent probes are used is called real time NASBA, while the method in

which colorimetric assays are performed is called NASBAELISA. Based on this, the FDA, the Food and Drug Administration office of United States of America, has developed a technique for molecular detection of some microorganisms such as HIV1 (human immunodeficiency viruses) or HCV (hepatitis C virus) and named it NASBAECL (Gill and Ghaemi, 2008).

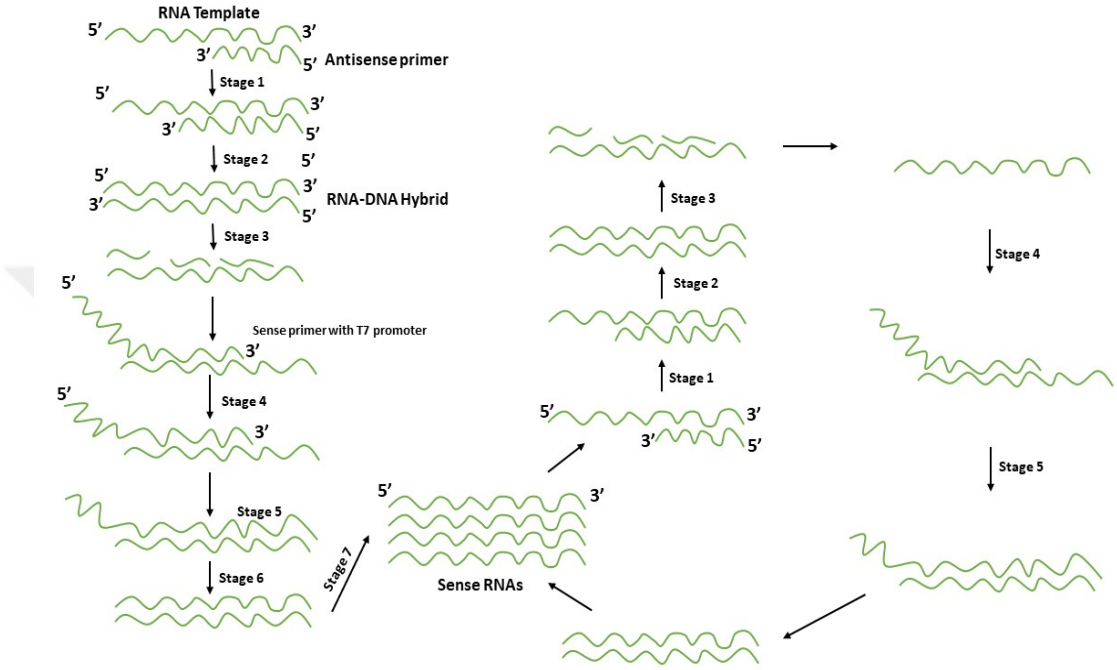


Figure 10. The phased representation of the NASBA method. The antisense primer in the environment attaches to the RNA template chain. Primer extends throughout the template. The complementary DNA chain is extended by reverse transcriptase. The combined state of DNA and RNA chain interacts with RNase H. The T7 promoter region is annealed to the newly synthesized single stranded DNA molecule. Complement DNA chain is extended by enzyme. A double stranded DNA molecule containing the T7 promoter region is used as a template. Sense target RNA is synthesized with T7 RNA polymerase enzyme. The RNAs formed as a result of this synthesis rejoin the process and cause RNA amplicon to form and accumulate (Source:Gill and Ghaemi, 2008).

Signal-based amplification method (SMART) is another isothermal amplification technique in RNA technology. With this technique, the essence of the work is the formation of a 3WJ structure called three way junction. This method is based on signal amplification and does not require a thermal cycling machine. Also,

copying target sequences is not among the principles of this method. In this method, a highly target dependent signal is produced and is well suited for identification of DNA or RNA targets. There are two single-stranded oligonucleotide probes in SMART technology and these probes are considered as extension and template. Each probe has a region that can hybridize to the target at adjacent positions, and the other shorter region will hybridize to the other probe. In the presence of a specific target, these two probes are annealed to each other. This formation is called the 3WJ structure. After this formation, Bst DNA polymerase enables the short oligonucleotide to be elongated, facing the template probe. This is called the extension stage. In this way, the double-stranded T7 RNA polymerase promoter sequence is produced. With this promoter, T7 RNA polymerase is able to produce multiple copies of RNA amplicons. Thus, the 3WJ structure is allowed to be produced only when a specific target molecule exists. Each RNA amplicon can be self-amplified by linking to a second template probe sequence and extended by the DNA polymerase enzyme. In this way, a double chain promoter region is created. As a result, the increase of RNA amplicons with ELOSA (an enzyme linked oligosorbent assay) is followed. This process is a signal enhancement technique in which the target sequence is consequently not self-amplifying (Gill and Ghaemi, 2008).

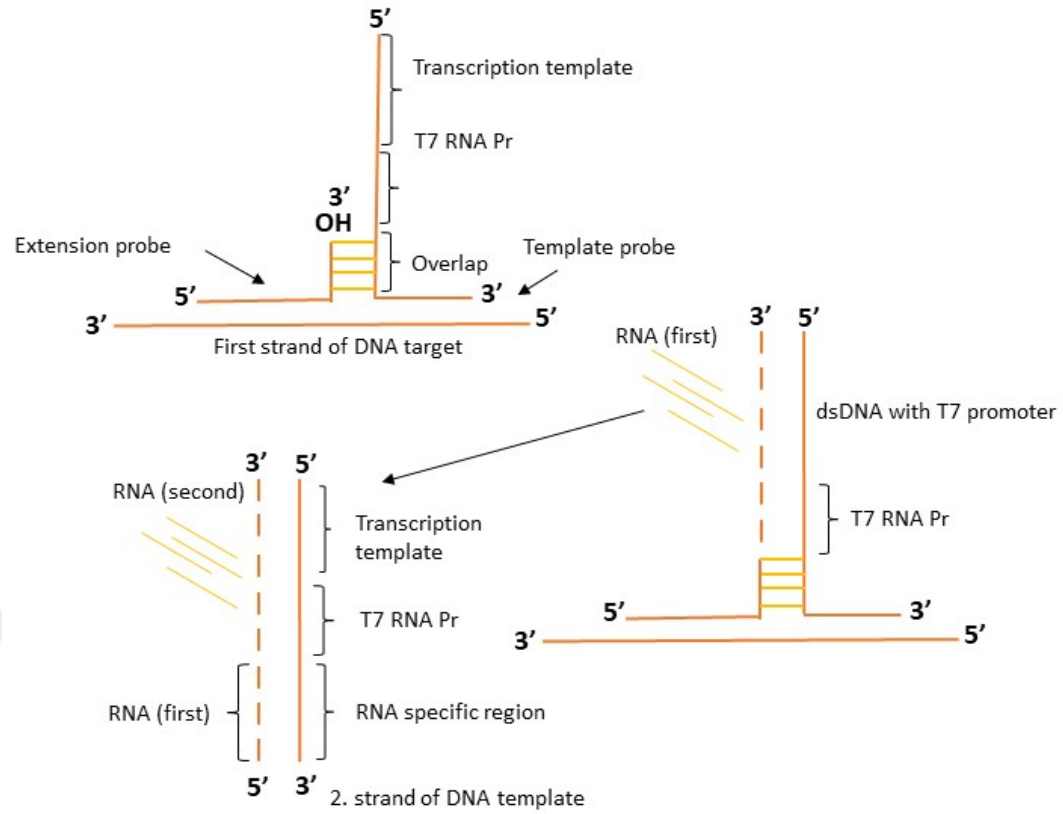


Figure 11. Representation of the stages of the SMART process. The first part describes the 3WJ formation. Probes are shown to be anneal to the target. The template probe contains the T7 promoter region. This is called Pr for short. Required for diagnosis of RNA signal. The 3' end of this probe sequence was inhibited by phosphorylation. In the next step, the RNA signal is created by transcription. A region (called the Bst DNA polymerase region) contained in the extension probe forms the Pr. In this way, many copies of RNA signals are transcribed with T7 RNA polymerase (Source: Gill and Ghaemi, 2008).

Another isothermal amplification method, strand displacement amplification, which is called SDA, allows fold amplification of the target DNA sequence in as little as 15 minutes. Due to the isothermal nature and nature of SDA, some advantages such as ease of use and cost-effectiveness can be achieved. In addition, it is possible to use the same fluorescent detector probes in many different analyzes. This advantage has a very important potential in genetic analysis and we can deduce this from the determination of factors such as susceptibility to diseases and therapeutic effect with multiple nucleic acid markers. The most common use of SDA technique is the diagnosis of infectious diseases. If we look at the foundation of the SDA technique,

we can say that it was introduced by Walker and his colleagues in 1992. This technique is based on the coordinated activity of two enzymes, the restriction endonuclease enzyme and the strand displacing DNA polymerase enzyme. These cause primary-directed amplification of the target sequence under isothermal conditions. The SDA method is realized by the gradual occurrence of some molecular events. These molecular events begin with the addition of target bearing, heat-denatured single-strand genomic DNA, primers and 4 deoxynucleotide triphosphate. This SDA procedure begins with some primer extension and displacement activities. Displacement activities are known to produce the dsDNA fragment. This fragment contains the copy of the target sequence via the recognition region of restriction endonucleases derived from the 5' end of SDA primers (Hellyer and Nadeau, 2004).

BD ProbeTec ET System has a great place and importance in the widespread use of SDA technology in the clinical field. This system is actually a temperature controlled kinetic fluorescence reader device. This device requires a 96-well microtiter plate. FRET probes are needed for homogeneous amplification and identification of target analytes. As a result, the advantages of this system are ease of use, minimum laboratory requirement and high-throughput potential. There is another advantage in this system compared to other complex systems. It is the ability to perform tests without the need for unidirectional workflow or dedicated workspaces. As in every system and method, false-positive results can occur in this. The reasons for false positive results may be cross contamination that may occur while preparing the sample or improper use of the amplified material. There are also some minor technical disadvantages to SDA. These include the design and maintenance of the device. Another type of SDA method is the reverse transcriptase SDA method. This is briefly referred to as RT-SDA. In addition, this technique is used to measure in vitro transcripts of viruses such as HIV. The RT-SDA method has some requirements before the amplification process, and this can be said as the conversion of RNA to cDNA. For this, SDA primer can be used or one or more bumper primers connected with SDA primers need to perform the reverse transcription reaction. It is stated that RT-SDA can have a sensitivity of less than 100 target molecules per reaction. It is known that the SDA method is very well suited to in situ applications such as tissue morphology in many respects (Hellyer and Nadeau, 2004).

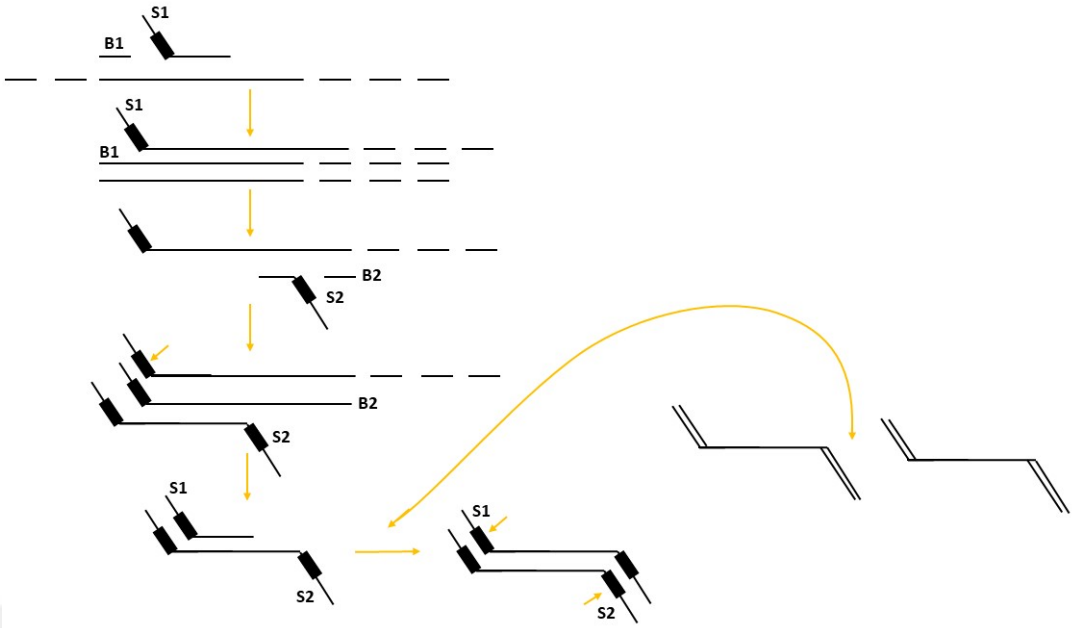


Figure 12. Stages of SDA process and production of target amplicon. There are 4 primers B1, S1, B2, S2. B1 hybridizes to S1. Polymerase enzyme provides elongation from the 3 'end of B1. The same process applies to primers B2 and S2 (Source: Hellyer and Nadeau, 2004).

Rolling circle amplification is another isothermal amplification technique, abbreviated as RCA. RCA can create many copies of a sequence for the DNA amplification process. Although RCA is an isothermal and enzymatic process, it amplifies short DNA or RNA primers into longer single-stranded versions. While doing this, it uses the circular DNA template and some special DNA or RNA polymerase enzymes take part in this process. The product formed as a result of the RCA process is actually a concatemer structure consisting of dozens or maybe hundreds of tandem repeats. The RCA method is popular in the fields of biomedical and nanobiotechnology in terms of its versatile possibilities and ease. The RCA method has been used from the very beginning for sensitive diagnostic methods of target molecules. These target molecules are known to include DNA, RNA, proteins, some cells and small molecules. The importance of the RCA technique in the field of nanobiotechnology is that it assembles the nano-samples periodically and uses it as a template, and as a result, it uses the template consisting of these repetitions to produce long and single-chain nucleic acids. Modifications can also be created in RCA products by making some design changes in the Circular template. Through this method, it is possible to produce complex DNA nanostructures and DNA origami,

nanoribbons and nanotubes can be considered as examples of these structures. Another benefit of RCA in nanotechnology is that it has a very important use in biodetection, bioseperation and also drug delivery. The most important and common DNA and RNA polymerase enzymes used in RCA technique are known as Phi29, Bst and T7. Phi29 and Bst polymerases have uses for the DNA molecule, while T7 polymerase has uses for the RNA molecule. The widespread use of Phi29 polymerase is due to its exceptional processability and strand displacement ability (Ali et al., 2014).

As in PCR, there is no need for thermal cycler device in RCA. Because the RCA technique works at a constant temperature and this is usually 37 °C, which is mean room temperature. There is also a need for a solid support material or a complex biological environment. This biological environment may be the cell surface or the interior of the cell. The production of some multifunctional materials with biorecognition, sensing and maybe imaging can be achieved by hybridizing RCA products to oligonucleotides. These oligonucleotides are complementary parts linked by structures such as fluorophores, biotin and antibodies. RCA is used effectively in many areas other than diagnostics. These areas are cell-free sequencing and cloning, whole genome sequence, genotyping. The basic components of a classical RCA process are DNA polymerase, a suitable buffer, a short DNA or RNA primer, dNTPs and circular DNA template. Rolling circle amplification method and its products can be viewed and examined with many signal reading techniques. The most common is gel electrophoresis. Some imaging techniques such as fluorescence spectroscopy and flow cytometry can be implemented by hybridization or working together of fluorescent materials with RCA products. These imaging and examination processes can be performed using materials that are common in nanotechnology such as gold nanoparticles, magnetic beads, and also quantum dots. This diagnosis can be explained as colorimetric and spectroscopic (Ali et al., 2014).

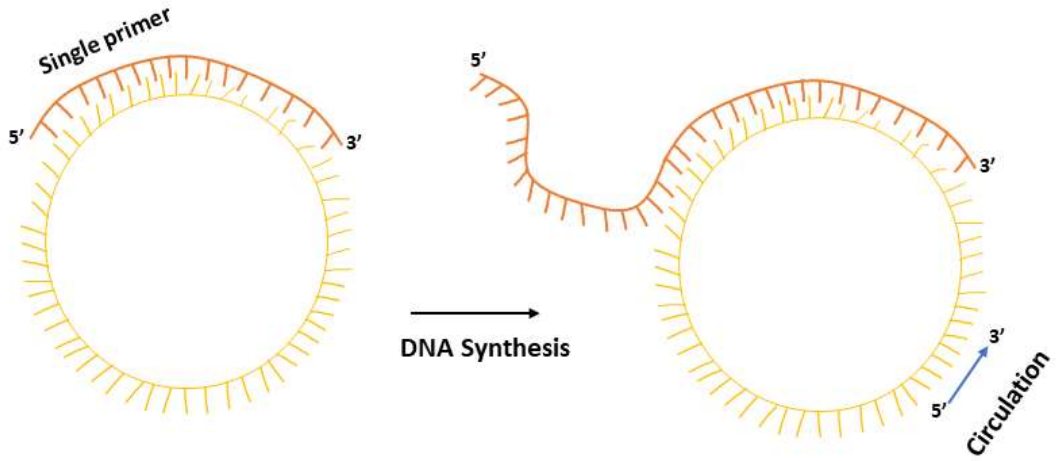


Figure 13. It is a demonstration of the RCA method. In this method, using a single primer, the DNA is carried over the structure called the minicircle. The reaction starts with the hybridization of the linear DNA molecule and the DNA minicircle. The products appear as a large smear structure in gel electrophoresis (Source: Gill and Ghaemi, 2008).

Loop mediated isothermal amplification method is a newly discovered technique that can be considered as a gene amplification method. With this technique, speed, high precision and simplicity come to the fore. From the isolation of nucleic acids to the diagnosis of amplification, simplicity and convenience are based on all processes. The entire reaction develops at a constant temperature and two types of elongation processes are repeated and at the same time this process takes place in the loop regions. This process, which is of two types, consists of the self-elongation phase of the template starting from the stem loop region, followed by the attachment process, the phase where the new primers elongation for the loop region (Notomi et al., 2015).

The loop mediated isothermal amplification method, which is abbreviated as LAMP, has a wide range of uses. It is widely used in point of care tests, rapid performance of food and environmental based tests, and genetic tests. We use the LAMP method, which we know that the primary use area is genetic tests, also in the diagnosis of infectious diseases. Hepatitis or tuberculosis are examples of these infectious diseases. The use of LAMP has emerged as a need, especially for the diagnosis of these infectious diseases by PCR and due to the disadvantages of PCR. In genetic tests, it is seen that the nucleic acid extraction from the samples with the PCR method, the amplification of the genes and finally the identification part is performed.

However, it is stated that due to disadvantages such as equipment requirement, cost and difficult usage conditions, the LAMP method has been switched quickly and clinical applications are easier with this method. It is known that a constant temperature is sufficient for the reaction in the LAMP technique and the fact that only one type of enzyme is required makes it different from other amplification techniques. It targets 6 gene regions in the amplification process. In addition, it provides a good level of specificity due to the basic features of the inner primer. The sensitivity level of this method, which takes place in about an hour, is similar to the nested PCR method. It is stated that very small amounts of genes can be amplified in a short time. It is also possible to use it for amplification of the target RNA sequence. If the reverse transcriptase enzyme is included in this process, one step amplification can be performed and it is known that this enzyme exhibits strand displacement activity. Among the diagnostic methods, it is reasonable to use fluorescence or turbidity factors because of the high specificity and the large amount of amplification products obtained. Both of these factors provide real time detection and imaging control to the LAMP reaction. Therefore, the use of normal DNA probes is also possible for the diagnostic process. Amplified products consist of many repeating units and these repeats are linked in opposite directions to each other. In the LAMP method, no special reagent is required and it is possible to do this in a very basic biology laboratory (Notomi et al., 2015).

Many studies have been done for the LAMP method and the change of metal ions while amplification is taking place in some have been investigated. It is seen that pyrophosphate is produced in large amounts in DNA amplification. This material is known to bind with metal ions to form insoluble salts. As a result, the concentration of metal ions in the environment is drastically reduced. Generally, manganese is used as metal ion and calcein as fluorescent molecule in these studies. It is observed that with the interaction of these materials, the reaction is quenched, resulting in an orange image. It is seen that the pyrophosphate ion affects manganese and that the combination of manganase-calsein does not occur, so calsein interacts with magnesium and as a result, a higher fluorescent signal is obtained. The fact that these processes are based on the LAMP method highlights the genetic tests, which are the common use of LAMP, more. In addition, these genetic tests should be accessible not only in the field of medicine, but also in agriculture and food tests, which are thought

to be unrelated, as well as environmental measurements and tests. This will be supported by the LAMP method and its innovations (Tomita et al., 2008).

The Isothermal Multiple Displacement Amplification method is based on the use of many primers and strand displacement replication of nucleic acid sequences with them. In this multiple displacement amplification application, it is stated that two sets of primers are used and one of them is the complement of a chain of nucleic acid molecule and the other primer set is the complement of the opposite chain. It is known that the 5 end for both primer sets is far from the nucleic acid sequence. Because primers hybridize to each other for amplification. One of the main features of this method, which we can abbreviate as IMDA, is that the primers intervening during the replication process are replaced by the polymerase enzyme. Amplification is maintained by the replication process, in which the polymerase enzyme initiates each primer and spontaneous termination can continue. In this way, many overlapped copies of the entire genome can be synthesized in a very short time (Asiello and Baeumner, 2011).

By means of the isothermal multiple displacement amplification method, which is made by taking the whole human genome, a large number of reproduction of clinical samples that may be very limited and therefore very high value is performed. However, many factors such as how much the obtained copy DNA loses its originality when compared with the original DNA sample or whether these copies are of high quality, and whether they will allow for future diagnostic tests should be tested (Luthra and Medeiros, 2004).

The IMDA method provides a good overall quality review of the genome. With some studies, it is known that the amplification deviation values of the IMDA method between 8 chromosomal loci are 3 times less than the method called Whole Genome Amplification method, in which the whole genome is amplified and abbreviated as WGA. This shows that the efficiency of PCR-based WGA method is less than IMDA. Biological samples such as blood or tissue culture cells can be taken directly for the amplification process. It is stated that DNA amplified by IMDA method is useful for many methods. These include methods such as genotyping, single nucleotide polymorphisms or chromosome painting, subcloning (Dean et al., 2002).

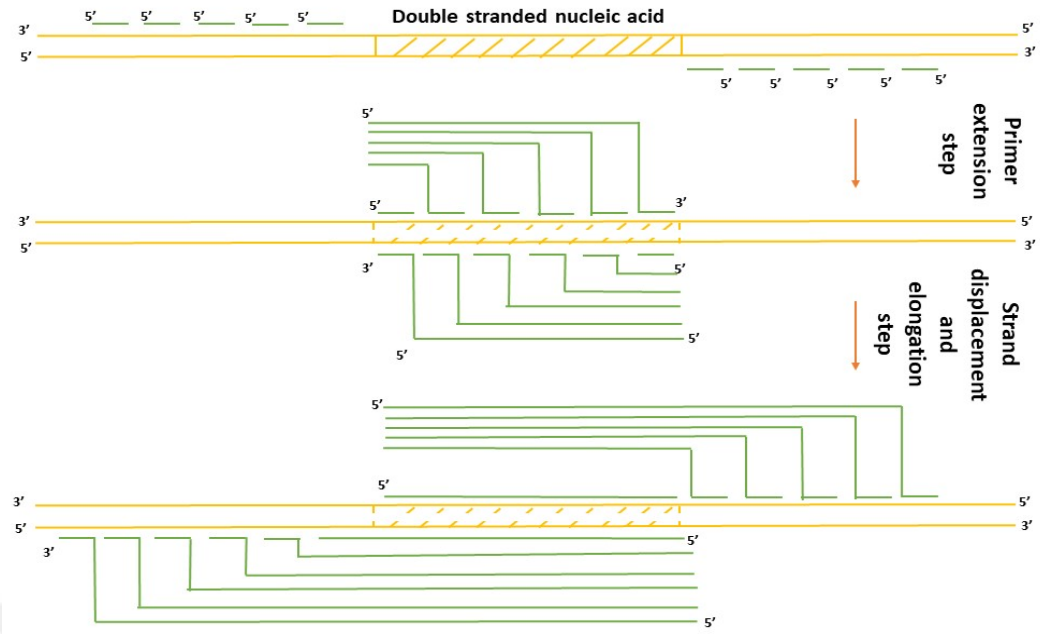


Figure 14. IMDA progressive explanation. Double stranded nucleic acid containing hatched regions is shown. Primers cling to the chain on the right and left sides and elongation occurs. The polymerase enzyme replaces the chains extended by the primers with the extended chain. Below the figure refers to the multiple chains of the more elongated nucleic acid (Source: Asiello and Baeumner, 2011).

Since the discovery of PCR, the importance and use of amplification methods have increased greatly. One of the few methods used for DNA amplification is the helicase dependent amplification method and is abbreviated as HDA. It has been determined that HDA is very useful in reproducing DNA in isothermal conditions, as DNA helicase can dissolve the double-stranded DNA molecule without the need for heat. Especially in living systems, DNA or RNA amplification is a very important process. At the same time, a leading copy is needed. Aside from its feasibility and role, it also has an important capacity in the field of conventional molecular biology and especially in biotechnology. There are many manipulation methods for DNA amplification and we know that the most known of these is PCR. Using very small amounts of nucleic acid as a source and amplifying it by means of enzymes is an old and inconvenient and slow process. With these old techniques, a lot of benefits were gained, especially in the field of clinical diagnosis. This has been replaced by sequence-dependent and much faster amplification methods. Thanks to these methods, which are more sensitive and easy to use in diagnosis, progress has been made in the clinical field. As one of these new methods, HDA has made it possible to overcome

many problems. HDA has advantages in preserving chromosome morphology and many other limitations. New techniques such as isothermal amplification are used to overcome many limitations by mimicking the in vivo DNA replication process. We can achieve this through the mechanisms in nucleic acid replication in living systems and their development. As the name of HDA suggests, the helicase enzyme plays the leading role in this technique, and it was inspired by the replication fork. In the presence of ATP, the double stranded DNA molecule is opened by the helicase enzyme. These opening chains are wrapped in single-stranded binding proteins called SSB. For this DNA molecule that is now separated as two single chains, primers are placed at the 3 'end of the single chains. Chain completion and new chain formation of primers is performed by means of DNA polymerase enzymes that do not contain Exonuclease. The result is two double-stranded DNA molecules, which will be substrates of the helicase for the next round. In this way, we see that exponential amplification of the target DNA sequence can be performed by simultaneous chain reaction. There are some proteins fundamental to the HDA mechanism. These play an important role in mimicking the replication fork. These, especially DNA polymerase and helicase, are based on chemical energy while allowing DNA synthesis to take place. Helicase also has various other roles. It is effective in nucleic acid metabolism, genome replication as well as performing repair-reperation processes and especially in performing these in living systems. Many viruses and eukaryotic systems are able to code for the helicase enzyme, proving them to be highly suitable targets for developing antiviral systems (Jeong, Park and Kim, 2009).

Helicase enzymes are molecular motor proteins that can migrate along the nucleic acid molecule and split the double chain into two through the energy obtained by NTP hydrolysis. These helicase enzymes are divided into various families and superfamilies and subjected to some sort of classification. While doing this, aspects such as 5 'and 3' ends are looked at or evaluated according to the substrate specificity. Oligomerization is another classification factor and according to this factor it is known that hexameric helicases do not perform NTP hydrolysis and cannot open the chain of nucleic acid molecule to two. Nucleic acid polymerase enzyme catalyzes the reaction that will support the addition of nucleotides to the 3 'end of the nucleic acid molecule. There are many polymerase enzymes and their types, and they perform the activity they have during the reaction depending on some factors. These factors are template,

synthetic products, presence or absence of primer. The polymerase of *E.coli* bacteria was discovered at the time, and studies were conducted to shed light on the mechanism and logic of many polymerase enzymes. The most commonly used polymerases are listed as Taq, Pfu, reverse transcriptase. Depending on the studies, purified DNA or RNA polymerase enzymes are also produced. From this point of view, the most common RNA polymerases are T7, T3, SP6. Among other proteins, the single stranded binding protein is abbreviated as SSB protein, especially it binds to single stranded parts in the DNA molecule. Although it is a sequence-independent protein, it is present in many organisms, including viruses. Reannealing of unwound single-stranded DNA molecules produced by the helicase activity is prevented by SSB proteins and their structure is protected by preventing their degradation. SSBs trigger the breakdown of the secondary structure of DNA and, in turn, cause an increase in the activity of polymerase enzymes. Therefore, SSBs are needed for the transcription of template structures of single-stranded unwound DNA molecules. The basic requirements commonly used and needed in HDA method are DNA helicase and SSB. As stated in one of the studies, UvrD DNA helicase was used for DNA helicase and T4 gene 32 and perhaps RB 49 gene 32 proteins were used for SSB. It is stated as a requirement for them to perform DNA amplification in vitro. Already, the first use of the HDA system for isothermal amplification was performed with *E.coli* UvrD helicase enzyme and helicase-loader MutL protein (Jeong, Park and Kim, 2009).

In conclusion, to summarize the mechanism of the HDA method, this mechanism is dependent on the unwinding activity of the DNA helicase enzyme. The use of heat is unnecessary to separate the two chains, DNA helicase does this function. Next, primers specific to the sequence attach to chains that are split into two and form the complementary chain by means of DNA polymerase. This process will be repeated until an exponential amplification and increase is observed at a single temperature level. Repeating this process many times at a constant temperature completely eliminates the need for a thermo-cycling device. HDA method amplicons can be diagnosed by gel electrophoresis or ELISA tests (Gill and Ghaemi, 2008).

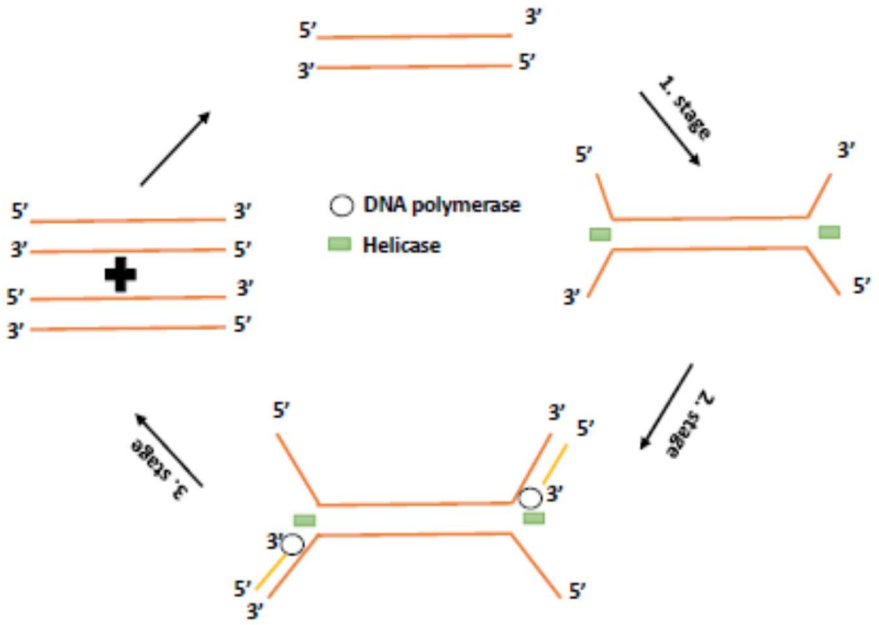


Figure 15. Representation of the HDA method. The helicase enzyme splits the DNA double strand into two, primer molecules attach to the single strands of this DNA molecule. The DNA polymerase enzyme enables the primers to elongate and thus to create two duplexes from 1 duplex. This cycle is repeated and HDA process is performed as many times as required (Source: Gill and Ghaemi, 2008).

The amplification method in which a few copies of double stranded DNA and one primer are sufficient is known as single primer-triggered isothermal amplification (SAMP). The abbreviation of this method is also found in some sources as SPIA. However, it is also known that the abbreviation SPIA is passed as single primary isothermal amplification method. In this method, nicking endonuclease enzyme is used without using any chemical or temperature changes. In this way, it is possible to use a constant temperature in each step from the very beginning to the end of the reaction. In addition, the basic factors required by the SAMP method are very reasonable and these are the only primer, nicking endonuclease enzyme, DNA polymerase enzyme. As a result, the need for complicated processes in primer design has disappeared. Although this process is very important, it can be performed easily. The most common use of the SAMP method is in the discovery, design, production and application of portable diagnostic devices in point of care tests. The primary design is of great importance in amplification efficiency. The primary molecule cannot self-hybridize in the absence of the target molecule. On the contrary, we see that the primer hybridizes with a single stranded DNA molecule when the target molecule is present. Then we

see that this primer is extended by the polymerase enzyme to form the complementary chain. At the end of this process, the extended primary separates from the single-stranded DNA molecule. This allows the primer to self-hybridize (Serhan et al., 2019).

In general, a single, chimeric primer is involved in this SPIA technique. It is seen that this primer is placed at the 3 'end in the DNA molecule and the 5' end in the RNA molecule. DNA-based processing is normally called SPIA, while RNA-based processing is called Ribo-SPIA. In addition to the primer, RNase H and DNA polymerase enzyme also effectively show strand displacement activity. The DNA amplification process is expected to begin with the hybridization of the chimeric primer to the target DNA molecule. Extension of this primer occurs by the polymerase enzyme and the 5 RNA portions of this elongated primer are split by RNase H, allowing for a new chimeric primer to be attached. It tries to bind to the complementary DNA molecule by competing with the newly attached chimeric primer. This process and competition are stabilized as the DNA polymerase enzyme binds. The stages before repetition take place. That is, there is primer elongation, 5 RNA portions of this elongating primer are split in two by RNase H, the primary binding site is released. SPIA is especially used for special genomic sequences and amplification of synthetically created oligo DNA molecules. The Ribo-SPIA method is suitable for the amplification of global as well as target-specific RNA molecules. Among other types of RNA molecules, it functions as a linear method, especially in isothermal amplification of mRNA. The replication process takes place approximately 10,000 times as part of the process. In other words, when looking at the result, we can perform the amplification of very large nucleic acid samples with the SPIA method, despite the limited biological samples. If a circular DNA molecule is considered as a template and the amplification process is to be performed, the circular helicase dependent amplification, in short, the cHDA method, is widely used. A basic system is required for the DNA polymerase enzyme and helicase. In this method, because the system is based on the T7 replication process, there are single-chain binding proteins called T7 helicase, T7 DNA polymerase and SSB. By means of T7 helicase, the template chain is divided into two, specially designed primers adhere to the template and the primers start to elongate from the 3 'end, and these are achieved by the rolling circle mechanism. All steps of the method take place at a constant temperature of 25°C (Gill and Ghaemi, 2008).

The recombinase polymerase amplification method, called RPA, is an amplification technique operating at low temperatures. Recombinase, DNA polymerase, binding proteins are used to enable recombination and repair processes. The complex version of the recombinase and primer supports the binding of the primers to the DNA strand at 37°C. SSB proteins provide stabilization of the displaced strand, and the DNA polymerase enzyme required for the displaced strand also supports primer elongation. The result is that two double stranded DNA molecules replicate themselves and as a result, an exponential amplification occurs naturally. The RPA technique can be applied to the target region in DNAs with a copy number of less than 10 and around copies. The diagnosis of these amplified target regions is performed by some methods. These can be real time analysis using nucleic acid dye, specially designed and used fluorophore probes and lateral flow technique (Asiello and Baeumner, 2011).

In order not to use the heat denaturation step in the PCR technique, there are two key proteins in the RPA technique. As is known, one of them is SSB proteins and the other is E. coli RecA recombinase. In general, if we need to look at the sequence and method of RPA, first of all there is the DNA polymerase enzyme responsible for replication and having strand displacement activity. It has this activity to make primers elongate. Proteins that are also used commercially are sold in some kits. The most common of these is the kits in which SauDNA polymerase enzyme is used and it is known that this enzyme is obtained from Staphylococcus aureus. In this process, there are some accessory proteins and supportive cofactors. As an example, consider the T4 UvsY protein. This supports RecA during the formation of nucleoprotein filaments. An example of a contributing factor is creatine kinase. This supports the production of ATP for the energy required in the system using phosphocreatine. In this method, the recombinase enzyme forms nucleoprotein filaments together with the single-chain oligonucleotide primer and probes, and the recombinase enzyme is also supported by the loading factor. These filaments search for double-stranded DNA target molecules for homologous sequences, and when they find that double-stranded target DNA molecule, the D-loop structure is formed, and local separation occurs as a chain stabilized by SSB proteins and the chain to which the primers are attached. The RecA protein hydrolyzes the ATP molecule and allows extension of the primers by the strand displacement DNA polymerase enzyme, with the removal of recombinases from the

protein-induced nucleoprotein filament structures. The use of the RPA method with other methods such as ELISA is also quite common. This collaboration, which is necessary for food safety tests, targets various allergens, GMO, potentially harmful bacteria and fungi. This collaboration works as well as the collaboration of PCR and ELISA. The reproducibility and sensitivity of the test gives similar results in these two different collaborations. In addition, the RPA technique can achieve its speed, flexibility and isothermal properties through a composition that we can call "protein soup", so that the steps of in vivo recombination can be performed imitatively. According to the obtained data, although the tests based on the RPA method are not approved in clinical researches, this is expected to occur in the near future due to many advantageous features such as cost effective and isothermal properties (Daher et al., 2016).

Tools and techniques used in the diagnostic and clinical fields are expected to have certain properties for biomolecular diagnostics and are generally characterized as sensitivity, selectivity and high applicability, low cost. Amplification of nucleic acids lies at the most fundamental stage of the techniques used to identify the DNA molecule. In this way, it provides the identification of limited amount of target molecules and supports the improvement of the sensitivity of the technique and method used. Especially the combination of such techniques with microfluidic devices and their co-existing work requires less amount of samples used and keeps analysis time short. Besides, by using a single device, various possibilities are provided in automation and integration. For nucleic acid analysis, temperature gradient tests, which are the basis of PCR, are performed in many systems, and analysis and various tests of these nucleic acid molecules are performed. However, as mentioned many times, in isothermal amplification methods, analyzes are performed at normal temperature and a temperature gradient is not needed. Thus, many studies today can include micro-fluid devices and their simple properties to assist these isothermal analysis. As a result of the use of microfluidic devices together with isothermal amplification techniques, it is ensured that the amplification product is produced with a higher efficiency and percentage, and that error deviations are reduced. In this way, single cell genome amplification is also possible (Zanoli and Spoto, 2013).

2.2.4 New and Common Method Hybridization Chain Reaction

In general, HCR has isothermal amplification principles and so easy technique, also it is recommended by Dirks and Pierce. HCR appeared at 2004 so, it kind of new technique. In normal HCR system, there are 2 types of Hairpin and one and so importantly trigger seqeunce. There have been cascade of hybridization process to Hairpins by trigger sequences. That process provides to creation of nicked double helix structure and that process will be continou until all DNA hairpins are consumed away.

HCR is also good and versatile tool for biological study areas which are biosensing, bioimaging and may be medical areas. These will be happen because of important spesifications of HCR those are, its ultrahigh sensitivity, its flexible structure, and its no need enzyme feature.

Biosensing have a function with using moities like fluorophores, some electrochemical reagents, and also they can be nanoparticles. HCR is used for detection of nucleic acids, enzyme activities, also proteins, ions and the other small molecules as sensitively. On the other hand, recoognition molecules can work together with HCR system for improve new techniques or methods such as in situ and intracellular imaging ways. These help us to understand and know the biological processes and their working principles. Therefor, some methods can apply to disease diagnosis and cure pathways. In a manner, HCR products and molecules are used in the drug delivery systems and this situation is found an application area in biomedicine. HCR products have a large capacity in carriers in drug delivery. Nanomedicine and nanotechnology are represent to working area with using HCR products.

2.2.5 Properties of HCR System

Trigger molecules are initiate the DNA hairpins in their specific sites which is called cross opening process. Also, this process ends with create DNA polymeric nanowires. When there is no initiator, Hairpin 1 (HP1) and Hairpin 2 (HP2) will be stable by their relationship with kinetic trap. If initiator molecule is added, the HP1 structure is opened and its sticky end of HP1. Part of HP1 is act as another trigger sequence. Therefor, it can be connect to sticky end of HP2. It is stated that the sticky end of the HP2 molecule has the same or rather high similarity to the molecule initiating the reaction. Dirks and Pierce who are discovered of this system, said that molecular weight of products in HCR system is more than initiator molecule.

If the comparison is necessary, HCR is a technique which does not require any enzyme or enzymatic reaction and it is isothermal amplification method compared to polymerase chain reaction (PCR) can be said. Also, hybridization chain reaction represents the how can probe-amplification technique be. On the other hand, PCR shows that target amplification technique as known. False-positive outcomes are reduced noticeably and decreased the risk of cross-contamination caused by amplicons. HCR system should contains two hairpin structures and they include toehold, stem, loop parts. There is a short loop structure on hairpin and it is protected by stem part to store energy. Toehold sequence is effect to kinetic energy with changing its length and binding strength. So that, length and sequence design of HCR system has carries importance. For an example, when the toehold length was so short, the reaction can not start. There are some ways to activate the HCR system and those are photoactivation, and pH changing.

2.2.6 Application Areas of HCR

Bioimaging and biomedicine contain huge part of application area of HCR. FISH imaging, cell imaging which might be live cell imaging and imaging for cell surface properties are example of these areas. On the other hand, HCR products which are nanometer size molecules are used like drug carriers, although transferring of these molecules into cell is hard process in drug delivery. There are other difficulties such as size and charge of used nucleic acid molecule. Also, size and weight of HCR products can be hazardous for biomedical applications because there can be non specific bindings and therefore can happened false signals, then normal cells can be dead. Considering the many advantages of HCR as well as its disadvantages, this technology is the bright field of the future.

2.2.7 Types of HCR System

HCR systems are divided into many types and grouped in terms of various features. The first of these is the HCR system. Nanostructures can be produced by means of a HCR system, and these net-shaped structures can have different mesh shape properties such as quadrilateral or hexagonal. In this way, quite a lot of success in programming and high efficiency in amplification are obtained. The design of two types of probes named HF and HT for this system by Qin and his colleagues and their realization of the cross linking HCR mechanism shed light on this system. In this study,

it is seen that the target molecule named as IFN-γ activates the system and creates nanostructures of the designed probes. In this study, it is ensured that FRET signals are used and thus HCR products with netlike feature are specified and monitored. As a result of the values reached with the limit of detection calculation, it is observed that the sensitivity is at picomolar level (Duan et al., 2020).

In this important study by Qin and his colleagues, it is seen that the HCR system is used for high-precision diagnosis of cytokine molecules. FAM fluorescence donor probe named HF and HT probe as TAMRA fluorescence acceptor. It is stated that the hairpin aptamer probe does not work and remains stable when the initiator molecule is not in the environment. Therefore, the HCR system formed by two hairpin molecules in the environment is inhibited. It is expected that the FRET signal will logically drop from FAM dye to TAMRA. However, as stated by Qin et al., when there is an initiator molecule in the environment, the aptamer probe called HA will be activated. In this way, a conformational change occurs and the trigger region is freed (Paquin et al., 2015).

The in-situ HCR method is called fluorescence in situ hybridization technique because it works with fluorescence and is abbreviated as FISH. With the FISH technique, DNA probes are labeled and sent inside the cells to bind with the target molecule. In this way, the imaging and monitoring of DNA mapping and mRNA expression is realized. In this method, the introduction of small DNA probes into living tissues or cells and following an enzyme-independent pathway result in signal generation. In this signal generation, specificity is expected to be at a high level. The FISH method includes the penetration of very high levels of molecules and the amplification of signals as well as their localization. The most common use of this method is to perform multiple nucleic acid identification of environmental organisms. In one of the studies for this method, a zebrafish was used and RNA probes were used. In a study of Choi et al. DNA probes were designed and amplification of the double HCR method was provided with two HCR amplification molecules. All these processes are carried out on a single in situ trigger molecule (Duan et al., 2020).

In one of the studies on in-situ HCR method, split probe and normal probes were compared. This comparison has been tried in chicken embryos, challenging factors such as sample thickness and autofluorescence that are actually major

problems. The normal probes used in the study were enlarged in size and this was achieved by adding some previously untested probes to the medium. In the test using split probes, it is stated that increasing the size of the probes does not cause any difference in general. In this study, the automatic background supression concept was tested as a result, and without the need for any probe size adjustment, an increase in the background signal is achieved by simply increasing the probe size (Choi et al., 2018).

Localized hybridization chain reaction is another type of HCR method. With this method, it is possible to increase the amount and concentration of nucleic acid products and reactants in the local area. This is achieved by fixing probes on nano-scale molecules. In this way, there is a remarkable increase in the sensitivity of the diagnosis as well as the increase in the reaction rate. As a result, it can be said that in this method, hairpins are limited directly in relation to the size of the scaffold (Duan et al., 2020).

Localized HCR system is based on the principle that all semi-stable hairpin molecules are connected to the long DNA track without any problem. All these hairpins need to be tied in this way, but not hybridized. As in all HCR mechanisms, it is known that the reaction starts when the initiator molecule enters the environment. The initiator molecule attaches to the area of hairpin 1 called external toehold and hybridizes there. As a result, hairpin 1 is replaced with the stem part. The loop structure opens structurally and forms the stem part of the hairpin 2. This process continues up to 6 hairpin molecules in the environment. Continuous previous hairpin molecule replaces the stem part of the next hairpin molecule and the process proceeds in this way. This localized HCR method is only possible if there is a long DNA track, and a noticeable detail is that the kinetic ratio is higher than other systems when there is a DNA track (Bui et al., 2017).

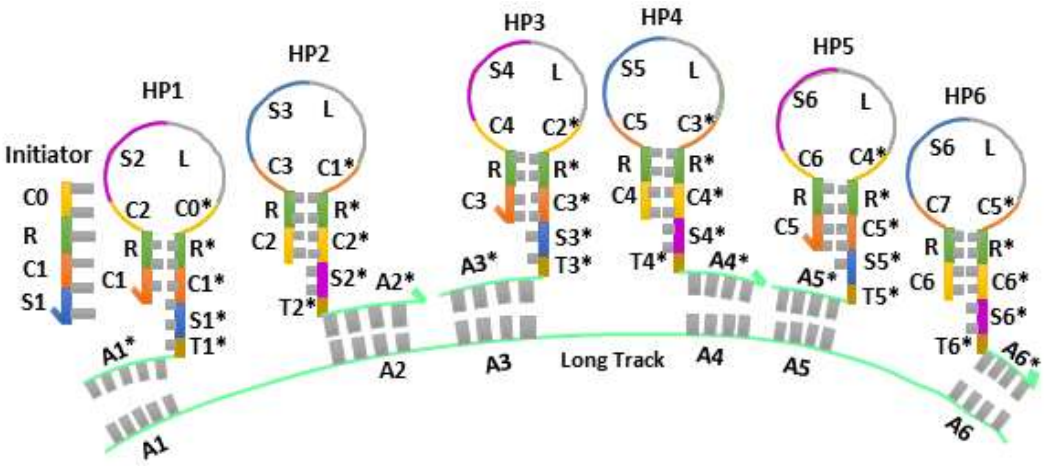


Figure 16. Localized HCR method. It has been shown that 6 hairpins attach to the long DNA chain. When the trigger molecule is added, the hybridization of the metastable hairpins begins (Source: Bui et al., 2017).

There is another type of HCR called Migration HCR. It was designed and unearthed by Pierce as recently as 2007. The basis of this method is the polymerization of the two chains to each other. There is a target structure called “A chain” in the environment and there is a secondary structure called “R chain” that acts as an auxiliary. As a result of their combination and polymerization, a duplex structure in the form of “A * R” emerges. In this technique, called four way branch migration hybridization chain reaction, the sequence design process can be very easy and minimized. In this method, there are many mechanisms and methods required for the identification of the target molecule, and the most common of these is fluorescence technology. Fluorescent sensors based on FRET are in a very advantageous position in terms of high sensitivity. Studies have shown that the use of FRET technology together with other isothermal methods results in a high level of identification of target molecules. In one of the studies, Huang et al. took an important step in this regard, combining FRET technology with the HCR technique, an isothermal amplification method, and succeeded in imaging tumor-associated mRNA molecules in single cells and some tissues, and achieved this with high precision. In this method, there are four hairpin molecules. Some of these are known to be in large loops, leading to difficult and complex designs. In this study, the target molecule is identified and this is achieved thanks to the nucleic acid molecule called complementary or helper. The parts of the “A * R” structure called sticky ends will be able to recognize the HP1 molecule and the two separate ends of this molecule in a much more comfortable and good level.

Copies of the target molecule formed as a result of amplification can create long nicked double stranded DNA and these molecules consist of many repeated FRET parts. In this way, the diagnostic level of the signal will be very high (Ren et al., 2019).

The ability to measure cancer cells at a very high sensitivity level is very important in terms of stopping the progression of cancer, intervening and applying the necessary treatment. Specialized cancer cells, ie "circulating tumor cells" in the bloodstream, are very few and difficult to diagnose cells. For this reason, the analysis of "circulating tumor cells", abbreviated as CTC, can provide very critical information. The diagnosis of these cancer cells in the peripheral blood circulation is a big problem due to the low concentration. As a result, analysis methods are important for both the cancer field and the other clinical diagnostic area. These analysis methods, which are of great importance, are based on the detection of some biomarkers. We can achieve this by using the lysis process of cancer cells followed by the PCR technique. This whole process is very time consuming, wasting money and at the same time not suitable for “point of care”. For this reason, a technique called multibranched hybridization chain reaction has been developed (Zhou et al., 2014).

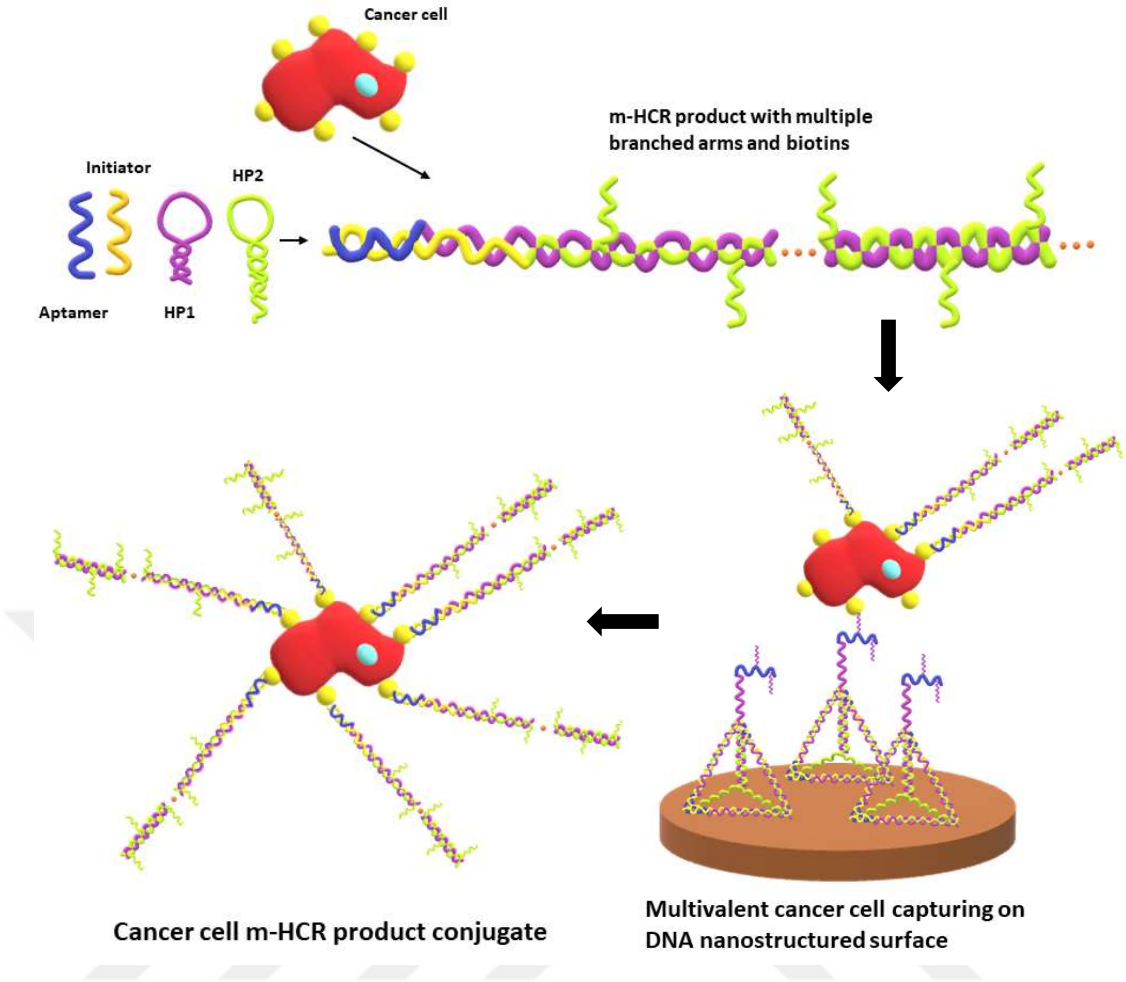


Figure 17. The multibranched HCR technique is demonstrated. It is the technique in which long products are produced by means of long biotin molecules and long arms. When cancer cells and mHCR products are combined, cancer cells attach to a surface that has a DNA nanostructure and accomplish this by polyvalent linking. Ultimately electrochemical signal is produced (Source: Zhou et al., 2014).

With the multibranched HCR technique, long structures with multiple arms, but also containing biotin molecules, can be produced. These multi-branched and biorine long structures support the increase in the value of the signal resulting from the amplification to be obtained with HCR. They do this by enabling and mediating many avidin HRP molecules to bind to the target. In addition, multi-strand structures can achieve a multivalent bonding on top of it using an electrode with a gold-containing nanomaterial structure. There is a structure called epithelial cell adhesion molecule and the aptamer of this molecule is partially linked to the trigger molecule. The mHCR method is used in some studies to increase multivalent binding and signal values, which are effective in detecting cancer cells (Zhou et al., 2014).

In one of the studies conducted by Feifei Lan et al., the mhcr method was used to obtain a product designed as a long molecule. For this, arms with many branches were used, and thus the process of adhering to nanoparticles became easier. With this adhesion, the oxidation of the hydroquin molecule is catalyzed. For this, hydrogen peroxide anoxide has been studied. The aim of this study is to capture and capture carcinoembryonic antigens. With mHCR, it has opened the door to clinical immunoassay areas and new techniques of other biological structures and molecules (Lan et al., 2016).

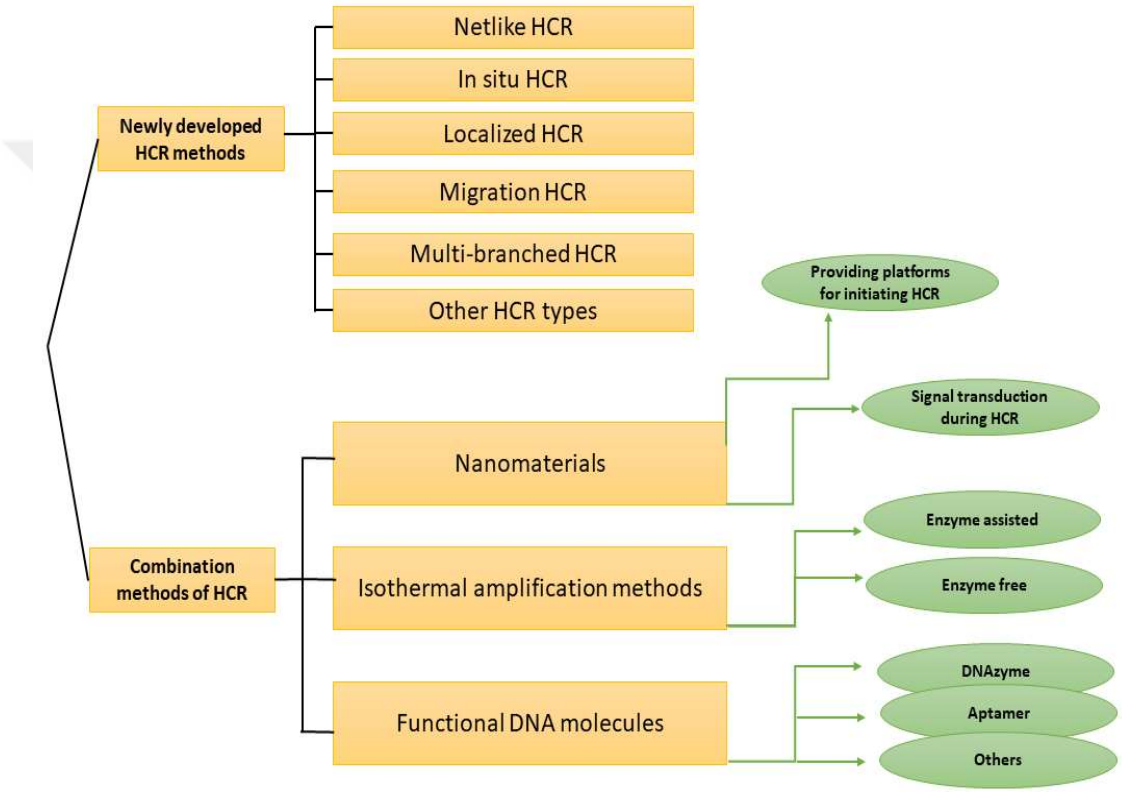


Figure 18. HCR methods and their use / classification in the field of biosensors (Source: Duan et al., 2020).

So many scientists and researchers are studied about hybridization chain reaction and other a lot of amplification methods in many ways. In this way Nannan Liu et. al. group is one of them with their work which has a concept as DNA hybridization chain reaction and supersandwich self assembly methods for detection of molecules and markers. Their goal was handle sensitive sensors to detect of biomarkers like nucleic acids, proteins etc. there are two important methods to reach this goal which are called DNA hybridization chain reaction and DNA supersandwich self-assembly methods. Also these methods have major importance as enzyme-free

way and it provides to increase the sensitivity of sensors. This group are used one dimensional DNA nanostructure for these two methods (Liu et al., 2017).

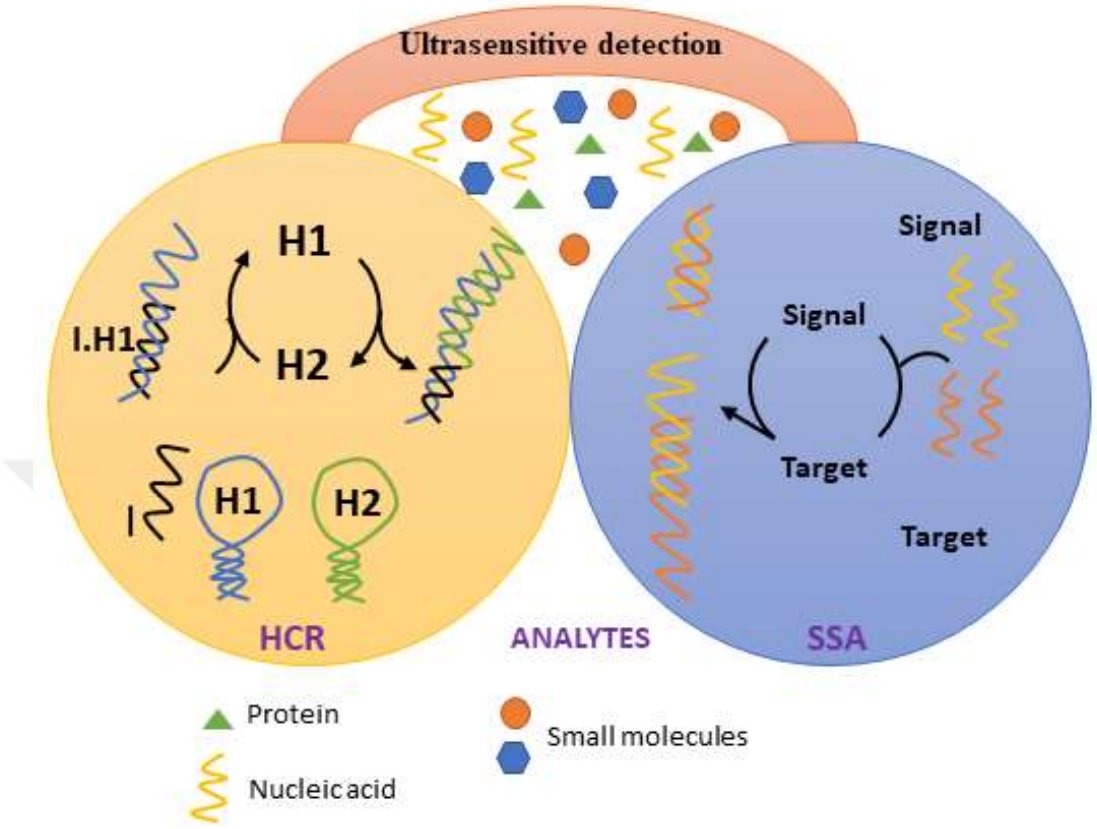


Figure 19. HCR and DNA super sandwich self-assembly methods are demonstrated to create one-size nanostructures for high precision detection (Source: Liu et al., 2017).

Nannan Liu and others examine these strategies as three parts and the first one is nucleic acid detection. In this part, pyrene excimers and their formation and electrochemical biosensors are determined by circular strand displacement polymerase reaction and HCR. Also, in this part of the experiment, colorimetric detection was made using gold nanoparticles. Protein detection forms the second part of the study. For this, DNAzyme supersandwich nanostructure was used as molecular tags and with this structure based on hemin / G-quadruplex, electrochemical immunosensor has been explained. The last part of the study consists of the detection of small molecules and ions by the DNA supersandwich method (Liu et al., 2017).

HCR has many varieties and some of them are as follows: multi-branched HCR, migration HCR, localized HCR, in-situ HCR, netlike HCR are examined in terms of biosensing ability by Chuyan Zhang et. al. in an article which determines

supporting together with nanomaterials and functional DNA molecules (Duan et al., 2020).

Jin Huang et. al. are studied nucleic acid detection in some biological fluids with using HCR method, but this method based on pyrene-excimer probes. Comparison of PCR, LCR (ligase chain reaction) and HCR is found in this study. Also, discussed quantitation of nucleic acid in biological fluids in terms of biomedical area. They are pointed to the importance of signals in probes and biological fluids respect. On the other hand, pyrene is used as sensitive fluorescent dye and that is so crucial because of the long life time and its association with biological materials. This assosiaciton can be represent as strong cellular background signal (Huang et al., 2011).

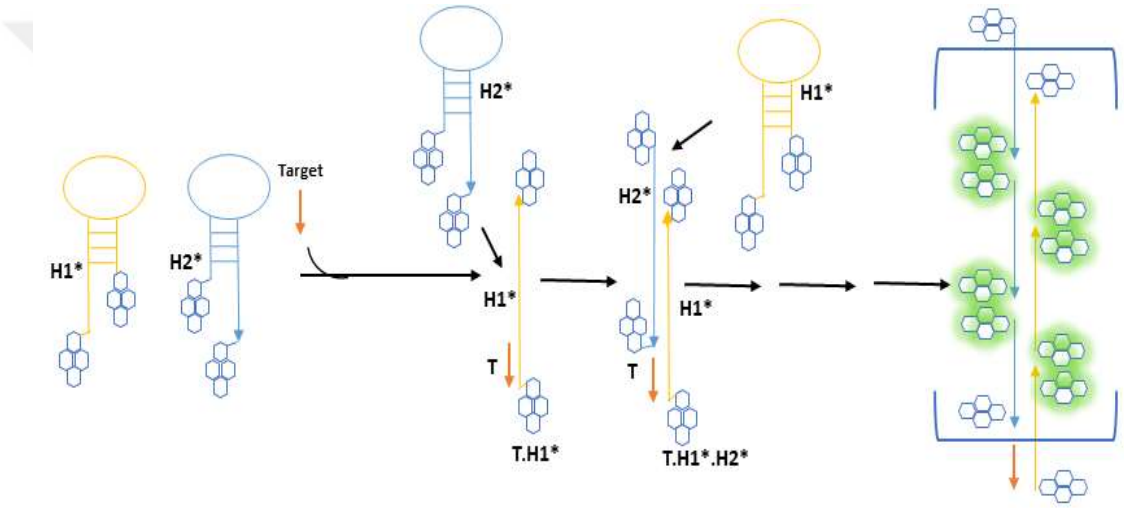


Figure 20. DNA diagnosis process by HCR amplification and formation of pyrene excimers are shown (Source: Huang et al., 2011).

Hongqian Chu and the others are focused on a HCR study with aim control the signal amplification process in living cells at any time by HCR system and help of near-infrared light. They said that engineering of HCR system especially in biological systems has a challange in two ways temporally and spatially. Optical controls provide some regulations as an advantage in this respect but of course they have some disadvantagous such as photodamage to cells and shallow depth of tissue penetration. For that reason lanthanide-ion-doped upconversion nanoparticles (UCNPs) are used to obtain NIR light from longer wavelength to shorter wavelenght and those nanoparticles can used as NIR to UV transducers (Chu et al., 2019).

Nowadays the clinical applications carry a huge importance and the multiplex detection of microRNA is a part of it. For that reason DaganZhang et. al. worked at a study which is about realitionship between multiplex detection of microRNA and HCR. Multiplex detection can be performed by HCR and polydopamine (PDA) encapsulated photonic crystal (PhC) barcodes are necessary for HCR. Those barcodes are immobilized and then they provide a subsequent reaction with amino-modified hairpin probes. MicroRNA quantification process can be realized with changing colors of those barcodes. Dagan Zhang and the others have so low detection limit as low as 8.0 fM (Zhang et al., 2019).

Some FISH processes can have problems in terms of detection because of low concentration of rRNA in target organisms especially in environmental microbiology area. So that, Yamaguchi T. et. al. put forward that in situ DNA-HCR method can prevent this problem. They studied bacteria from seawater and archea from sludge sample by (catalyzed reporter deposition) CARD-FISH or normal, regular FISH. For looking to the results Yamaguchi T. et. al. said that DNA-HCR method is so simple for detection of single microbial cells (Yamaguchi et al., 2015).

Circulating miRNAs and their quantitation indicates disease type. Andrea Miti and the others supports this idea with work and they used biosensors which based on HCR. Andrea Miti and the others used gold nanoparticles inn detection of miR-17. Using of hairpin surface-tethered probes provide high sensitivity on detection and HCR method increased and amplified that signal detection. All that process take 1 hour and obtained 1 pM detection limit. That limit is known good enough to used in diagnostic applications (Miti et al., 2020).

Yanting Nie et. al. are described that gold nanoparticles increase the potential of HCR. This may enhance the sensitivity of important electrochemical immunologic tests such as carbohydrate antigen 125 which is a biomarker in overian cancer. When looking to previous HCR based tests, the DNA immobilized onto gold nanoparticles, so that dendrimeric chains are obtained on electrod after HCR. When amount of DNA molecules is increased onto the electrode, phosphate groups are increased, then electrical signal too. The limit of detection in their work is 50 $\mu U.mL^{-1}$ which means there is huge need of signal amplification in clinical area (Nie, Yang and Ding, 2018).

Meijuan Liang and the others work on microRNAs and used cascade HCR as enzyme free isothermal amplified method. Result of their work is discussed by gel electrophoresis and also, atomic force microscope helps to examine of branched DNA nanostructure and its morphology. So that, cascade HCR is more helpful and utilize than regular HCR method because of its higher amplification efficiency. Performans and quality of sensors which used in this study are determined by voltammetric and chronocoulometric analysis. Lowest limit of detection is 11 pM (Liang et al., 2018).

2.3. Horseradish Peroxidase (HRP) Activities

Enzymes are molecules that catalyze and speed up reactions. Horseradish peroxidase (HRP) is also an enzyme. Enzyme tags are used by catalyzing enzymes such as HRP to produce signal amplification in biosensors. In some cases, the disadvantages of natural-based enzymes were observed in the reactions. Since nucleic acids are more advantageous than protein enzymes, their use has become widespread. DNAzymes, on the other hand, are DNA-based catalysts and they have high chemical stability and programmability. In addition, they can be easily synthesized and modified. Considering all these features, hemin/G-quadruplex DNAzyme is widely used. This field of use is indicated for electrochemical and colorimetric diagnostics (Alizadeh, Salimi and Hallaj, 2020).

It has been stated that hemin/G-quadruplex molecules have peroxidase activity especially recently. This advantageous feature is widely used in the colorimetric identification of target molecules. In order to understand the substrate specificity, inactivation mechanism and kinetics of this complex, DNAzyme, it is necessary to compare it with HRP. Hemin/G-quadruplex complex has higher inactivation rate and substrate specificity than HRP. This is because the center of the catalytic hemin is exposed. Inactivation of the hemin/G-quadruplex complex occurs by degradation of hemin by H_2O_2 rather than elimination of G-quadruplexes. For this reason, factors such as the catalytic oxidation rate and inactivation rate of the hemin/G-quadruplex complex are related to the H_2O_2 concentration. Therefore, the intermediates formed by the hemin/G-quadruplex complex and H_2O_2 represent the branch point of the catalysis and inactivation process. When the substrates in the reaction are reduced, they react with intermediates, affecting the inactivation of the hemin/G-quadruplex complex. The higher substrate specificity of hemin/G-quadruplex complexes than HRP may be due

to hemin accumulation in the terminal regions of G-quadruplexes. In this case, it is known that hemin, H_2O_2 and G-quadruplex structures are effective factors for the reaction (Yang et al., 2011).

2.4. Fluorescence Dyes/Quenchers and FRET

The emergence of cheap and easy methods to label nucleic acids has facilitated access to nucleic acid hybridization probes. Since non-radioactive labels are used, it is possible to see these probes especially in clinical studies and biosensors. Observation of the nucleic acid amplification process can be realized through the use of fluorescent hybridization probes that only produce a fluorescent signal when bound to the target molecule. These probes come in a wide variety of colors. Each different colored probe can be used to detect different nucleic acid sequences. This can be seen from the fact that they are labeled with different colored fluorophores. Multiplex diagnostics can also be performed. This requires multiple hybridization probes. Selecting probe tags depends on the type of hybridization probe and the number of target molecules (Marras, 2008).

Many fluorescent hybridization probes have been produced, introduced and used in studies especially in recent years. Although fluorescent hybridization probes differ from each other, they are all labeled by a fluorophore. This fluorophore absorbs energy from the light source, transfers it, and emits it at a wavelength suitable for its type. When a photon is absorbed from the light source, the fluorophore goes from the ground state to the excited singlet state. It happens in as little as 1 fs (femto second). The excited singlet state can be called stable. When the emission of light occurs, it returns to the ground state. The emission of light into the singlet state is called fluorescence. In the meantime, some energy loss occurs. Emitted fluorescent light is less than absorbed. Therefore, emission takes place at a longer wavelength than absorption (Marras, 2008).

There may be decreases in fluorescent intensity and this is called quenching. Fluorescent diagnostic methods can be considered in two ways. It occurs through energy transfer between fluorophores and from a fluorophore to a non-fluorescent molecule, during which fluorescence is quenched (Marras, 2008).

Fluorescent Resonance Energy Transfer (FRET) is a quenching mechanism and energy transfer between two molecules. It is a non-radiative process. The photon

from the excised fluorophore is called a donor. An electron with a higher vibrational level than the Excited state is called an acceptor. The donor raises the energy level of the acceptor. As a result, the energy of the donor fluorophore returns to the ground state. At this time, it cannot emit fluorescence. This is related to the distance between acceptor and donor molecules. Also, an important point is that the fluorescent emission spectrum range of the donor and the absorption spectrum of the acceptor should overlap. The acceptor may or may not be a fluorophore. If it is a fluorophore, the transferred energy will be emitted as fluorescence. If the acceptor is not fluorescent, this energy will be lost as heat. Quenching of a fluorophore can be achieved as a result of the formation of complex non-fluorescent molecules. This is called contact quenching. Here, the donor and acceptor interaction takes place by proton-coupled electron transfer (Marras, 2008).

Single chain hybridization probes are used in the molecular beacon probe process. These consist of stem and loop structure. The loop portion of the oligonucleotide is the probe sequence and is complementary to the target molecule. This probe sequence portion is located between the two arm sequences. These arm sequences are complementary to each other. But it is never linked to the probe sequence or the target molecule. These arm sequences fold into each other to form a double helical structure (stem hybrid) and transform the probe sequence into a hairpin structure. The fluorophore is attached to one end of this oligonucleotide sequence. The other end is connected to the non-fluorescent quencher moiety. Stem hybrid structure brings fluorophore and quencher closer. In this way, contact quenching takes place from the fluorophore to the quencher. There are quencher labels for fluorescent hybridization probes. The most common of these are Black Hole Quenchers (BHQs). The absorption maxima of BHQ-1 is 534 nm. BHQ-2 is 580 nm. BHQ-3's is 670 nm. BHQ dyes absorb excitation energy from a fluorophore and lose this energy as heat. If this is a Fluorescent quencher, it will emit most of the energy as light (Marras, 2008).

2.5. Design of Oligonucleotide Sequences and Tools

Organizing oligonucleotide sequences in computer environment is of great importance for in vitro studies. Some parameters are required for these designs to be specific and highly sensitive studies. Choosing the most suitable tool for the study and target molecules comes first among these parameters (Hendling and Barišić, 2019).

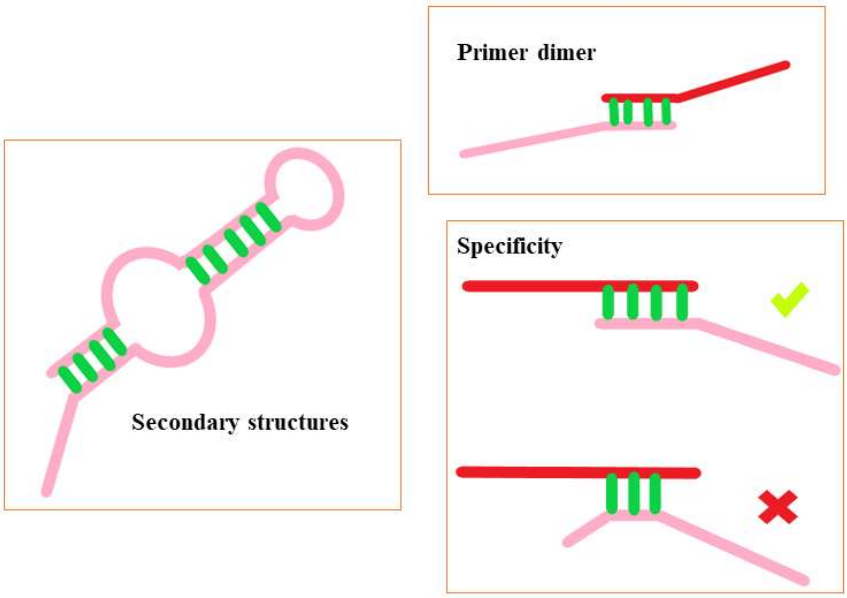


Figure 21. Schematic representation of the pathway used in the design of oligo. Factors such as formation of secondary structures, appropriate selection of oligos, formation of primer-dimer structures are the most important parts of these stages (Source: Hendling and Barišić, 2019).

2.5.1. Parameters in Oligonucleotide Design

Oligonucleotide length is one of these parameters. Because length is directly linked to four important factors. These factors are specificity, cost, efficiency and stability. It makes sense that the longer an oligonucleotide sequence, the less base similarity to other target sequences. This will increase the theoretical specificity. Likewise, as the sequence length increases, the tolerance of mismatch increases. That is, long sequence oligonucleotides tend to bind to sequences of high heterogeneous level. It is known that the longer the sequence, the higher the probability of forming a hairpin structure or primer-dimer. The other parameter is the melting temperature. This temperature is the temperature at which, as a percentage, half of the sequence is paired with the bases in the complementary chain. As it is known, the reaction temperature must be low in order for the oligo to attach and match to the target DNA chain. However, if the temperature is too low, the efficiency of secondary structure formation or non-specific binding will decrease. Therefore, attention is paid to factors such as Tm, reaction temperature. The sequence content of the oligos used to adjust this temperature, i.e. GC amount and salt concentration, and their interactions with their closest neighbor (NN) are of great importance. While oligo design software normally

takes into account GC content, recently salt concentration has also become an influencing factor. As a result, Tm estimates and oligo designs based on NN interactions give the best results. The third parameter is specificity adn with this parameter, it is aimed that oligos cannot bind to non-target DNA molecules. For this reason, target sequences should be planned correctly during design. In this way, it should be able to match with the real target DNAs in the reaction. There is a sequence alignment technology that checks if oligo binds to unwanted target sequences. However, this alone is not enough because DNA will not cover duplex thermodynamics. For this reason, it is not recommended to use only the BLAST tool. Mismatches sometimes contribute to the stability of duplex structures, but nevertheless, attention should be paid to mismatches, especially those at the 3 'end, as they can cause problems with the amplification of target sequences. Another important parameter is secondary structure formation. Since oligoes are single chain, they can form secondary structures by folding and matching. They may also limit the hybridization of oligos to target sequences. It is stated that NN-based models should be developed with factors such as salt concentration, loops, terminal dangling ends, mismatches. Salt concentration factor greatly affects the choice of buffer to be used in studies. It should also be known that the mismatch factor can have both stabilizing and destabilizing effects on duplex structures. We can compare this as terminal and internal mismatch (Hendling and Barišić, 2019).

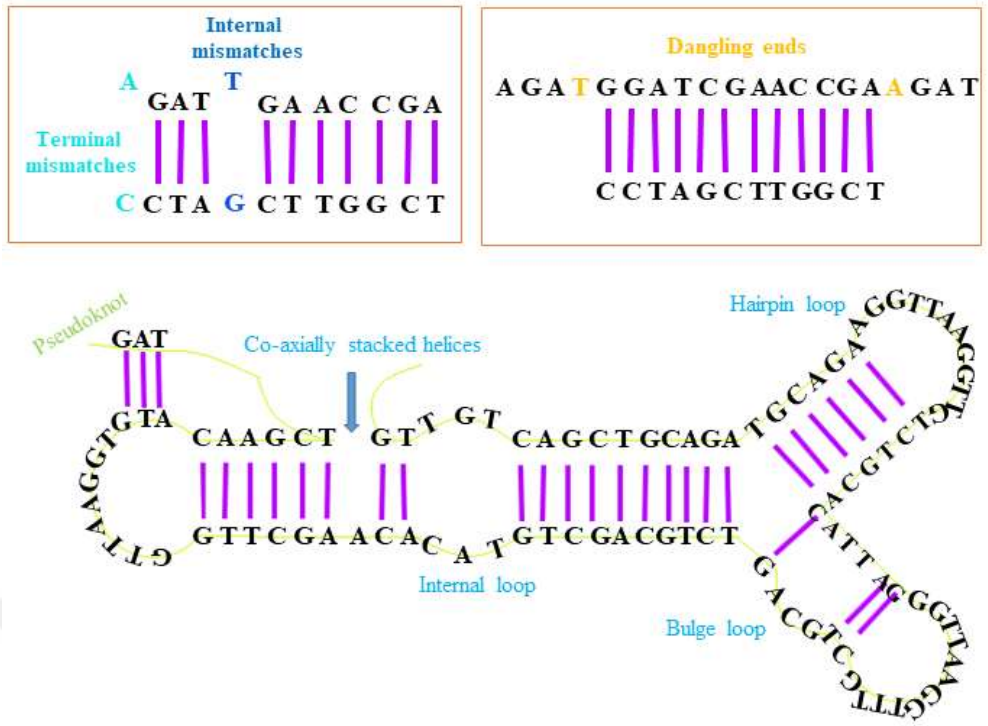


Figure 22. Schematic representation of secondary structures expected to be formed by considering some loop patterns. Vertical black lines indicate matching of bases. Color illustrations show the conformation of secondary structures (Source: Hendling and Barišić, 2019).

In addition, intramolecular folding has an important role. Because this intramolecular folding includes the loop structures formed in the folded DNA molecule. As a result, these can prevent the hybridization of oligos to the target DNA. Therefore, factors related to loop structures are important in oligo designs. These can be called hairpin loops, bulge loops, internal loops, co-axially stacked helices, and multi-branched loops. The last parameter is primary-dimer structures. It is called hybridization of two primers to each other. Primary dimers can show different effects in different experiments, suppressing hybridization. Therefore, attention should be paid to this detail during design (Hendling and Barišić, 2019).

2.5.2. Tools Used in Oligonucleotide Design

There are many paid and free software and webtools for oligo design on the market. Some of the parameters mentioned in free ones may not be taken into account. This may be caused by the logical ratio between the analysis and design time and the consideration of all parameters. Single-stranded oligos can fold against unwanted

targets or on themselves. Such situations prolong the analysis and design time. Some simulations are required to accurately predict the interactions between oligo. These are called molecular dynamics simulations. To implement this simulation, the webtool called oxDNA.org should be used. In this way, the thermodynamic and structural properties of double or single-stranded DNA molecules can be examined and analyzed. Common tools in in silico oligo design and multiple sequencing are shown in the Table 2 (Hendling and Barišić, 2019).

Table 2. Commonly used tools for multiple alignment design and analysis of nucleic acids (Source: Hendling and Barišić, 2019).

Name of Tool	Used Method
Clustal Omega	Progressive alignment, guide tree
MUSCLE	Progressive alignment, iterations
Kalign	Progressive alignment, Wu-Manber string-matching algorithm
T-coffee	Progressive alignment
DIALIGN	Greedy and progressive alignment
MAFFT	Progressive alignment, iterations

OligoAnalyzer ™ Tool, OligoArchitect ™ Online, oli2go, OligoMiner, Nupack tools can be discussed for the most well-known software used in oligonucleotide design. Oli2go is an automatic multiplex oligo design tool that is highly efficient and easy to use for the user. While designing primers and hybridization probes, it also provides controls for specificity and cross-dimers within the same run. The most important feature of this tool is that it performs many steps in a single step. For example, oli2go does not use a single species for specificity control, it uses the genome sequence of all organisms and species at once. In this way, very high specificity oligos are designed in multiplex studies (Hendling et al., 2018).

OligoMiner provides individual control over the parameters affecting each designed probe. In particular, it provides fast and accurate analysis and design opportunity for the genome-scale design of probes to be used in the FISH method. With this tool, it demonstrates the design and discovery of genome-scale probes and their performance with diffraction-limited and single-molecule superresolution

imaging of chromosomal and RNA targets (Beliveau et al., 2018).

2.5.2.1. Nupack Used in Design and Analysis

Matching the bases of RNA or DNA molecules and analyzing and designing them has become widespread for use in many researches and also in the engineering of biological systems. Nupack tool is used especially in analyzing the secondary structures of nucleic acids and in the design part, which has a very important function. Calculation of partition functions and minimum free energies for DNA and RNA chains interacting in a large and complex way is basically performed. It functions in the calculation of equilibrium concentration. Partition function and concentration are known to be used to calculate the base-pairing balance, especially in dilute solutions (Allouche, 2012).

In the analysis part of the Nupack tool, the thermodynamic properties of nucleic acid chains that do not contain pseudoknot are analyzed. There are 3 stages of analysis as input, computation and result. Input; It offers the user the ability to determine the content and conditions of the solution, although it varies depending on whether the material used is DNA or RNA. It also offers the right to determine the temperature, the number of chains and the number of complexes. It contains sections where the sequences will be written. The concentration amount should be specified below each sequence information. There is also a pane under the name "Advanced options". We can customize the settings here on request. With this option, details such as selecting and applying energy models in accordance with the sample, specifying the salt concentration, and allowing pseudoknots can be accessed. While entering information for all this used sample into the system, the calculation time is also specified specifically. In the computation part of the analysis, base matching properties and their balance, partition function, energy models are calculated. In cases where the maximum complex size is more than 1, it uses information about partition function calculations, strand concentration. In cases where the data related to the strand concentration needs to change, these changes can be made in the result part of the analysis. The equilibrium properties of the sample solution used are described here. Melt profile plot, ensemble pair fractions plot and equilibrium concentration histogram data and results are available here. At the same time, minimum free energy (MFE) structure plot and pair probability plot results can be reached graphically. In the design part, the user is

allowed to design one or more stranded sequences. The input part of the design is for the selection and determination of some features. In the design part, the type of molecule (RNA or DNA), temperature, number of independent sequence designs parameters are determined. Target secondary structure is also determined and dot-parens-plus notation is used while doing this. While "dot" refers to unpaired bases here, each base pair is symbolized with a "parenthesis", and every nick between chains is symbolized with a "plus" sign. In the computation part, optimizations are made to reduce ensemble defects. The process of calculating the number of mismatched bases is also performed. In the result section, the designability summary, sequence design table showing GC content, target structure plot, pair probabilities plot are shown (Allouche, 2012).

2.6. Techniques Used For Analysis and Detection of G-quadruplexes

Nucleic acid molecules contain large amounts of guanine bases in some regions. G-quadruplex structures are formed by the succession of guanine bases in these regions and forming a certain conformation. The discovery of G-quadruplexes, especially in living organisms, determination of their location and examination of their functions have a very important role. The reason why these procedures have not been performed at a very high level of success is the lack of suitable G-quadruplex probes. Artificial G-quadruplex probes can produced. These probes must be of high affinity and specificity so that they can be properly and correctly connected to G-quadruplexes. These artificial probes are especially used in some specific studies to identify G-quadruplexes in human cells or other experimental animals, mice and chickens, using methods such as the Chip-Seq technique. At the same time, small ligand molecules and proteins that can interact with G-quadruplexes, especially in the last 20 years, have taken place in studies for the diagnosis of G-quadruplexes. There are many alternatives to these ligands for identification, and these detect G-quadruplex structures with the fluorescence generated. Some studies use native G4 interacting proteins, but it is known that their specificity is low because they are also linked to non-G4 structures. In addition to these diagnostic methods, diagnosis is possible with immunostaining or immunoprecipitation methods. However, both of these methods require G4 antibodies and are not efficient as antibodies have trouble penetrating living cells. It is common to use fluorescent signals and some devices for this to diagnose G-quadruplexes. Fluorescence spectroscopy and circular dichroism spectrometer devices are also

widely used in signal diagnosis experiments (Zheng et al., 2020).

2.6.1. Fluorescence Spectrometer

Fluorescent is a 3-step process. The fluorescent probe molecule is a fluorophore specially designed to settle at a specific location in a biological material or to react to various stimuli. These 3 stages are named as excitation, excited stated lifetime and emission. The following can be said for the excitation phase; A photon is emitted from a laser and this photon is absorbed by a fluorophore. Excited electronic singlet state (S1 ') is created. Fluorescence is separated from chemiluminescence with this step. The second phase, which is the excited state, is available for finite time. It does not occur for a long time, there are approximately 1–10 x 10-9 seconds. It is seen that the fluorophore experiences some conformational changes in this limited time period and it experiences a wide variety and different interactions in the molecular environment in which it is located. Two different results are reached here. First, the energy of (S1') would be partially spent. The second result is that even if all molecules are excited by absorption at the beginning of the process, they will not be able to return to ground state (S0) by emission. The measurement of the excited state lifetime stage is explained by fluorescent quantum yield. The concept of fluorescent quantum yield is expressed as the ratio of the number of fluorescence emitted to the number of fluorescence absorbed. In the 3rd stage, fluorescence emission; An energy photon is emitted to cause the fluorophore to return to the (S0) stage. Since the energy is emitted due to the 2nd stage (excited state lifetime), the energy of the photon becomes low. Therefore, it has a longer wavelength than excited state lifetime. The difference between this energy and wavelength is called "Stokes shift". Stokes shift is very important for the concept of sensitivity in methods using fluorescence. Because emission photons are separated from excitation photons, and provide diagnosis on low background (Wiederschain, 2011).

Fluorescent processes are cyclical. The same fluorophore can be repeatedly excited and diagnosed. Discrete electronic transitions are being replaced by a wide spectrum of energy. These broad spectra are specified as fluorescence excitation spectrum and fluorescence emission spectrum. The bandwith factor becomes important during the simultaneous detection of many fluorophores (Wiederschain, 2011).

Fluorescence spectroscopy is referred to by a few more names and these are referred to as fluorimetry and spectrofluorometry. This device is an electromagnetic spectroscopy instrument where fluorescence analysis of the tested samples is performed. The electrons of the samples tested by using beam of light are excited, thus enabling the light to spread. While ultraviolet light is generally used, visible light is also used rarely. Intensity fluctuations caused by the emission of light are measured by means of single or double fluorophores. There are many energy levels of the materials, hence the samples used. Fluorescence is related to the electronic and vibrational stages of these levels. Often materials have a ground energy level as well as a high level of excited electronics. First of all, the tested sample is excited by a photon from ground electronic level to excited level. By means of fluorescence spectroscopy, different frequencies and intensity states of the light can be tested together and the vibrational states can be determined by interpreting them. While measuring in a fluorescent emission device, the excitation wavelength is kept constant and the detection wavelength is changed (Mehta, 2013).

At the same time, the samples used are in solution form and optically diluted by adjusting according to the excitation wavelength. The device where the solution is placed are square shaped quartz tubs. The reason why this shape is special is that the light can come at right angles and be observed (Eisinger and Flores, 1979).

Especially biochemical applications of fluorescence are for the use of intrinsic protein fluorescence. When we consider all biopolymers, proteins are prominent in showing intrinsic fluorescence properties. Lipid, saccharide and membrane structures do not fluoresce. Almost no intrinsic fluorescence feature of the DNA molecule has a negative effect on usage. Tryptophan is one of the amino acid structures that show fluorescence. In addition, phenylalanine and tyrosine are two other amino acid members with fluorescent properties. It is also ironic that these are very small amounts in protein complexes. Tryptophan is the intrinsic fluorescence with the extensive characteristic, but the percentage of it found in proteins is very low as 1 mol%. If all 20 amino acid molecules were fluorescent instead of these 3 amino acids, protein emission would be very complex. One of the notable properties of intrinsic proteins is the high sensitivity of tryptophan to its environment. Some changes in the emission spectrum of tryptophan may occur in response to conformational changes, binding of substrates or denaturation. Tryptophan and tyrosine have high anisotropy properties.

This means that the sensitivity to the conformation of the proteins is high. Tryptophan is known to have complex spectral properties. The reason for this is that there are two very close isoenergetic excited states. The emission of proteins is suppressed by tryptophan. Its absorbance occurs at long wavelengths and its extinction coefficient value is quite large (Lakowicz, 2013).

2.6.2. Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy is an effective method to understand and analyze the conformation of proteins and many molecules. Newly developed bioinformatics methods are effective for CD spectra analysis to have a more effective level. With CD spectra, not only proteins and structural properties of proteins, but also polypeptides and peptide structures can be examined. Such methods are more efficient for molecules with helical structure. Because the helical structures tend to be regular and they form very close spectra with well-defined ϕ, Ψ angles. These spectra of the helical structures form highly intense and highly efficient CD signals (Whitmore and Wallace, 2008).

The basis of the working principle of the CD spectroscopy device is that there is an absorption difference between left and right-handed polarized light passing through an active molecule, which is called a chiral molecule. The difference between these two forms of polarized light is measured in the CD spectrum of the sample under test and this is performed as a function of wavelength. A variant of this, UV-CD spectroscopy is used to examine the natural layers and folding of membrane proteins, and to monitor and observe the secondary structures they form. The wavelength between 190-250 nm is called the far-UV region and when looking at the CD spectrum of any protein molecule in this region, the main chromophore is the peptide bond to be observed. CD spectroscopy has many good advantages. It is known to be used together with other biophysical methods and support each other thanks to its fast and efficient nature. This can be seen especially in the characterization of proteins and when studying their relationships with other molecules. By examining and analyzing spin paramagnetic resonance, nuclear magnetic resonance, and surface plasmon resonance, the relationship of proteins with other molecules and structures can be seen (Kumagai, Araujo and Lopes, 2017).

CD is a very important and efficient method for observing and examining the structures of proteins in the analyzed solution. It consists of two circularly polarized parts, left and right, and CD indicates the different absorptions of these two components. If the absorption of these left and right components does not occur after the polarized light passes through the analyzed sample, the polarized radiation will be regenerated. If the left and right have different absorption, the radiation appears as elliptical polarization. If the chromophore is not optically passive, that is, it is a chiral molecule, it will be possible to observe the CD signal. The CD device measures the difference between the absorption of left and right components called L and R (Kelly, Jess and Price, 2005).

In principle, the difference between the absorbance of left and right handed polarized light of a molecule measuring CD is measured, and we can express this with Beer's law for diluted samples. This law is said to be the difference between molar extinction coefficients. It is formalized as " A=ε.d.c ". "A" is mean absorption, while "ε" stands for exticnction coefficient, "d" for thickness, "c" for concentration (Petralli-Mallow et al., 1993).

2.6.3. Gel Electrophoresis

Gel electrophoresis is the sorting of large molecules such as protein, nucleic acid or smaller parts on a gel according to their size and charge. When electric current is applied to this gel, the analyzed samples pass through the pores of the gel. Although this analysis is mostly applied after PCR, it can be used beforehand as a preparation stage for techniques such as RFLP, cloning, Southern blotting. DNA electrophoresis, as used in PCR, is considered essential for several other analytical techniques, and these can be considered as restriction enzyme mapping, applications related to plasmid inserts, sequence analysis, polymorphism comparison. After all, many molecular biology methods are dependent on electrophoresis technique. When it comes to electrophoresis, three applications should come to mind and these should be slab gel, capillary electrophoresis and 3rd microfabricated tools. The most common type for the DNA electrophoresis technique is slab gel electrophoresis. For this, a polymer material, conductive medium and voltage application are required. In capillary electrophoresis technique, there are thin channels. It is important for the process of separating DNA fragments. Microfabricated devices, are separated from DNA

fragments according to a single feature determined and considered a fairly new method. One of the important factors in electrophoresis and especially in slab gel electrophoresis is the conductive medium. The purpose of this is to provide the correct and regular distribution of the applied electric field. The voltage gradient is determined along the length of a gel. By providing the electric field, the negative charge of the DNA fragments allows them to move on the gel. Generally, the principles set for double-stranded DNA molecules also apply to denatured and single-stranded DNA molecules and single-stranded RNA molecules. However, it should be noted that in these single-chain molecules, heat and denaturants are used to prevent unwanted hybridization. Also, attention should be paid to the alkaline level of the environment in an analysis to be made for the RNA molecule because these conditions hydrolyze the RNA. Historically, electrophoresis of DNA dates back to 1942. Coleman and his colleague Miller stated for the first time in those years that the neutral hexoses progressed to the anode in borax solution. But the most important breakthroughs and changes in DNA electrophoresis were experienced in 1971 (Brody and Kern, 2004).

The gel matrix has a sieve-like effect that allows the analyzed samples to be separated according to their size. The type of this gel matrix is chosen depending on several factors and is used in studies. These factors are; electrical charge can be thought of as molecular weight. The most commonly used types are polyacrylamide gels and agarose gels. Starch gels are also used, although rarely. For the starch gel, the analyzed substance is proteins, and the method of analysis can be expressed as pouring into slab or tubes. Molecules analyzed in agarose gel are known to be very large protein molecules or molecules such as nucleic acids. Substances analyzed in acrylamide gel are proteins and nucleic acid molecules. In addition, cross linking feature in agarose and acrylamide gel is remarkable. To examine agarose, it is known to be a repetition of disaccharide units, including galactose and 3,6-anhydrogalactose. The degree of porosity obtained in agarose is very high and this is known to be particularly higher than starch gel. Adjustment of porosity can be changed with the initial concentration. The porosity to be obtained in the low concentration selection will be large. It is common to use this gel for separating DNA and its fragments. There is a special reason for this, the uniform electrical charge distribution in nucleic acid molecules and knowing how to determine the mobility of these molecules through the agarose gel matrix. However, attention should be paid to the limited mechanical stability, which

is a negative factor. If we need to examine acrylamide gel, it is known that both proteins and nucleic acid molecules are separated. This gel formation is formed by the polymerization of acrylamide. It is mechanically very strong and inert, and at the same time a high level of controllability in porosity is ensured. This is achieved by methylene bonds and cross linking. In general, the chemical ratio of acrylamide and methylene bisacrylamide is preferred as 40/1. While this ratio is often preferred in protein separation, the ratio 19/1 is used for the separation of DNA molecules. The most important detail in the gel factor of the electrophoresis process is the staining stage. First, when the analyzed samples are loaded into the gel and voltage is applied, it should be monitored that they move electrically within the gel. This can be observed by staining the sample molecules with a suitable chemical. If the DNA molecules loaded on the gel are stained with ethidium bromide, they can be observed with UV light. Coloring can be done in two ways. First, DNA samples can be loaded by staining with ethidium bromide without loading the gel, and the second can be performed by washing the gel with an ethidium bromide solution and shaking it at the end of all electrophoretic processes (Magdeldin, 2012).

2.6.3.1. Polyacrylamide Gel Electrophoresis (SDS PAGE)

SDS-PAGE is described as a “sodium dodecyl sulfate polyacrylamide gel”. It is an important tool and method for identifying and determining the molecular weights of unknown protein molecules. To accomplish this, molecular weight standards and protein samples are distributed on the same gel. The graph is then created with a link between the molecular weight and the travel distance in the gel. Using this graph, the unknown protein is found by the intercalation method. In this technique, a supportive medium is prepared by PAGE and protein denaturation and linearization are provided by SDS. This method increases the ability to predict the relative molecular mass of proteins, as well as the amount of protein in the analyzed sample and the distribution of protein fractions. SDS is known to be a detergent and negatively charged. This also plays a role in dissolving secondary and tertiary molecules by dissolving hydrophobic structures. Polyacrylamide has a gel matrix structure that acts as a sieve and consists of monomers called acrylamide. Through this sieve function, we understand that large molecules slow down the speed of movement on the gel and at the same time accelerate the passage of small molecules through the pores in the gel. The orientation of the passage through the pores takes place with the electric field created. The mobility of

the analyzed samples in the gel depends not only on the size of the gel pores, but also on the electrical charge and shape of the samples (protein, DNA, etc.). At the end of the electrophoresis process, The gel should be stained with Coomassie Brilliant Blue or silver dye to see the samples moved in the gel and separated according to different sizes and loads. To make this distinction more accurate, a molecule called a DNA marker (ladder) is added to a separate well. By using the DNA marker, the ladder, bands of known sizes are created. By looking at these bands, information about the sample can be obtained (Manns, 2011).

2.6.3.2. Agarose Gel Electrophoresis

DNA separation in the range of 0.5-25 kb is generally performed in agarose gels. It performs identification and purification as well as separation. There are three stages of agarose gel electrophoresis, the first of which is to prepare an agarose gel that will form a pore in the appropriate size for DNA. The second step is to load the DNA samples into the wells and to ensure the mobility of the samples in the gel with an appropriate amount of voltage. In the last step, if ethidium bromide has not been added to the gel before, the staining process is performed and the bands are displayed under UV light with the imaging device (Johansson, 1972).

Agarose gel electrophoresis is performed horizontally in a special container. The gel is placed horizontally so that it remains in the buffer. Vertical versions are also used, although very rarely. One of the limitations in the agarose gel electrophoresis process is the voltage. If the voltage is increased too much, the gel begins to melt and problems occur in the wells and therefore in the mobility of the samples into the gel. On the contrary, if the operation is continued at very low voltage, the bands will expand for small DNA molecules. DNA cannot be seen in visible light. Special dyes are needed and can be selected according to the size of the DNA molecule. While Xylene cyanol is used for large DNA molecules, Bromophenol blue is used for small DNA molecules (Johansson, 1972).

CHAPTER 3: MATERIALS AND METHODS

3.1. Reference HCR Design

Hairpin 1 (HP1) , Hairpin 2 (HP2) and trigger sequences are included in the design (Table 3), which was prepared as the first experiment based on the manuscript (Ang and Yung, 2016). HP1 and HP2 molecules were adjusted to 100 nM. Cy3 is paired with HP1 and Cy5 is paired with HP2. 5X SSCT hybridization buffer (750 mM NaCl and 75 mM trisodium citrate with 0.1% Tween-20, pH 7.0) was used as the buffer. GT concentration values were determined as follows: 0 nM, 1 nM, 2 nM, 5 nM, 10 nM, 100 nM. The samples were held at 90 °C for 5 minutes. It was then kept at room temperature for 30 minutes. Measurements were made in a fluorescence spectrometer. The excitation value is selected as 520 nm and the start and end wavelength values are determined as 530 nm-800 nm, respectively.

3.2. Oligonucleotide Sequences Used in This Thesis

In this study, 5 oligonucleotide sequences forming hairpin molecules were used. These are called GH1, GH2, GH2-F, GH3 and GH4. In addition, in order for the HCR mechanism to work, the presence of a molecule that initiates the reaction is required. This is called a trigger molecule or initiator. Here, it is reffered as GT. The sequences used in the study are shown in the Table 3.

Table 3. The DNA sequences used in this study. The bold nucleotides indicate guanines that are expected to participate in formation of G-quadruplexes.

Component Name	Sequence (5'-3')
GH1	5'-AAGATGGAAGGAGGCCCAGTTTCATGGGCCTCCTTC**GGG**A -3'
GH2-FAM	5'-/FAM/TGGGCCTCCTTCCGTTTTGAAGGAGGCCCATGAAAC/BHQ-1/-3'
GH2	5'- TGGGCCTCCTTCCGTTTTGAAGGAGGCCCATGAAAC -3'
GH3	5'-A**GGG**TA**GGG**C**GGG**AAAACGGAAGGAGGCCCACCATTATGGGCCTCCTTC-3'
GH4	5'-TGGGCCTCCTTCCATCTTGAAGGAGGCCCATAATGG -3'
GT	5'-TGGGCCTCCTTCCATCTT -3'
HP1-Cy3	/5Cy3/ GGA ATT GGG AGT AAG GGC TGT GAT GCC CTT ACT CCC
HP2-Cy5	GCC CTT ACT CCC AAT TCC GGG AGT AAG GGC ATC ACA /3Cy5/

3.3. Using The Nupack Tool

GH1, GH2, GH2-F, GH3, GH4 and GT sequences were designed bioinformatically using the Nupack webtool. It is possible to see the interactions, folding, thermodynamic behavior and conformation of the designed sequences with Nupack analysis. Parameters suitable for the experiment are set. DNA is chosen as the nucleic acid type, the reaction temperature is 30 °C as it is the temperature of the laboratory environment. Complexity level is set to four. Number of strand species is set to five because there are five hairpin sequences. These are GH1, GH2, GH3, GH4 and GT. The concentrations of the HFOs are made 100 µM and the concentration of the Na^+ salt is set at 0.1 M. Two analyzes are performed in the presence and absence of GT. By setting the GT concentration to zero, it is observed how the hairpin arrays behave.

3.4. Our Hybridization Chain Reaction Design

HFOs were designed to have a main stock of 100 µM. GH mixture is prepared by taking 5 µl from each hairpin. 5x SSCT is used as a buffer. This buffer contains 0.1% (v / v) Tween20, 75 mM sodium citrate, 750 mM sodium chloride and 150 mM potassium chloride and its pH value is 7.0. The GH mixture is heated at 85 °C for 10 minutes and quickly put on ice for 10 minutes. The GH mixture is incubated overnight with GTs of different concentrations. The reaction mixture is then added to the GH-GT mixture. The reaction mixture content is as follows: 5x SSCT buffer, 50 nM hemin, 200 µM H_2O_2 and 20 µM AmplexRed(™). All fluorescence measurements were performed with excitation at 490 nm and with bandwidths of 5 nm-5 nm.

3.5. Fluorescent Spectroscopy Used For Signal Diasgnosis in HCR Experiments

The GH system, which also contains HFOs and trigger molecules, is prepared in the appropriate condition as stated in the section 3.3. The fluorescent signals of the samples are measured in wavelength in a fluorescent spectrometer as peak. The quartz crystal cuvette used exclusively for this device should not contain any material or even dust particles. For this reason, the inside of the tubs are filled and emptied with distilled water to be completely washed. This process is optimally done three times.

With dust-free napkins, all water drops on the inside and outside of the tubs are wiped away. If necessary, this can be done with the pump that dries the inside of the tub. After the quartz crystal tubs are washed and dried completely, it is important not to touch the sides, because the light sent by the fluorescence spectrometer enters from the side surfaces of the quartz crystal tub during the measurement. Any stain or dust on the surface of the quartz crystal tubs reduces the accuracy and precision of this measurement.

700 µl of samples are taken, placed in quartz crystal tubes and measured. Since the inside of the tub is narrow, the pipette tip cannot literally go inside. For this reason, the solution should be transferred from the edge of the mouth of the tub. This should be done very slowly, because any bubbles or foams that form on the solution will cause the measurement to be inaccurate.

The program named SpectraManager is opened on the computer and the program is run by clicking on the fluorescence measurement option. The device is kept

on for about 30 minutes before taking a measurement. In this way, it is expected that the lamps in the device are warmed up and optimized. It is extremely important in terms of measurement accuracy that no external light source can penetrate inside. Measurement options also vary depending on the work done in the fluorescence spectrometer program. “Spectra Measurement” for normal measurement and “Time Course Measurement” options if measurement will be made in a certain order according to time.

After Spectra Measurement is selected, a screen appears. Parameters related to the sample can be changed here. After the parameters are set, the name of the sample to be measured is written by pressing the Sample-Start mark and the measurement is started.

3.5.1. Steps of G4-HRP Activity Experiments

GH1, GH2, GH3, GH4 and GT were used. We set the final concentration of the GH system to be 100 pM. The final concentrations of hemin, H_2O_2 and AmplexRed$^{(TM)}$ to enter the system were determined as 10 pM, 1 mM, and 10 µM, respectively. 1X SSCT was put into the system as a buffer. GTs were prepared first. 1 µM intermediate stock was prepared by taking 5 µl of 100 µM master stock GT and adding 495 µl of TE (Tris EDTA buffer pH 8.0) to it. 50 µl of 1 µM intermediate stock was taken and 450 µl of TE buffer was added, thus obtaining a 100 nM intermediate stock. A 10 nM intermediate stock was obtained by taking 50 µl of 100 nM intermediate stock and adding 450 µl of TE buffer to it. Finally, 50 µl of 10 nM intermediate stock is taken and 450 µl of TE buffer is added on top of it and 100 µM intermediate stock is prepared. Then, for GH mix, 5 µl of GH1, GH2, GH3, GH4 is taken and 580 µl of 1X SSCT buffer is added. The GH mix is distributed in five separate tubes as 100 µl each. To keep the volume equal to the other tubes, 8 µl of TE buffer is added to the 0 GT tube. Additions are made to these tubes from the intermediate stocks of GTs. Thus, the final concentrations of GTs are 100 fM, 1 pM, 10 pM, 100 pM, 1 nM. The 'Time Course Measurment' option is selected from the Fluorescence Spectrometer. Excitation wavelength is set as 560 nm and emisson wavelength is set as 580 nm.

3.6. Gel Electrophoresis Method Used to Get Results

Gel electrophoresis experiments were carried out. With this technique, we can qualitatively see that the HFOs are bind to each other and the HCR system is working. The binding of HFOs to each other according to their molecular weight and complexity can be examined. There are also varieties of gel electrophoresis technique. These are called SDS-PAGE, polyacrylamide gel, agarose gel. It depends on the type and content of the gel used.

3.6.1. Steps of Polyacrylamide Gel Electrophoresis (SDS-PAGE)

We perform SDS-PAGE technique in order to obtain a distinction according to the molecular weights of proteins, nucleic acids or HFOs. SDS is an anionic detergent and is referred to as sodium dodecyl sulphate. This anionic detergent disrupts the natural structure and conformation of proteins, making them linear and negatively charged. PAGE stands for polyacrylamide gel electrophoresis. The important reasons for using polyacrylamide gel are that it is inert, neutral, hydrophilic and transparent. This gel dissolves in Tris-HCl buffer and polymerizes in the presence of APS and TEMED. In our experiments, we want oligos or proteins to move simultaneously in the gel. Some proteins are known to reach the end of the gel at different times, which creates some problems. Therefore, it is aimed to load all of the proteins analyzed for simultaneous progression with the same load. This is achieved using SDS. In the SDS-PAGE study, resolving gel containing 10% acrylamide was primarily prepared. The amount of chemicals used for this is determined as in the Table 4.

Table 4. Resolving gel content with 10% acrylamide.

dH_2O	4.1 ml
Acrylamide/bis (30% 37.5:1)	3.3 ml
1 M Tris-HCl buffer (pH: 8.8)	2.5 ml
SDS solution, 10%	100 µl
Ammonium persulfate (APS), 10%	32 µl
N,N,N′,N′-tetramethylethylene-diamine (TEMED)	10 µl

Two solutions are prepared for the SDS-PAGE experiment. These are resolving gel solution and stacking gel solution. These solutions are transferred to between two

thin glasses. Resolving gel solution and stacking gel solution freeze and turn into a gel form. It is important to do these steps quickly because after APS and TEMED are added, the gel reacts very quickly and polymerizes. It is necessary to avoid any bubble formation. At the end, 1 ml of isopropanol is added because the top of the gel should be flat and smooth. It is important to create smooth looking wells in the gel. Comb is used for this. After about 30 minutes the gel should have frozen. It is added to the tank from 1X Runnig buffer. 1X Running buffer solution contains 28.8 grams of glycine, 6.04 grams of Tris base, 20 ml of 10% SDS. It is completed with 2 liters of dH_2O in total. Its pH is adjusted to 8.3.

Table 5. Stacking gel content.

dH_2O	6.1 ml
Acrylamide/bis (30%, 37.5:1)	1.3 ml
Tris–HCl (1 M, pH 6.8)	2.5 ml
SDS, 10%	100 µl
TEMED	10 µl
Ammonium persulfate (APS), 10%	100 µl

The comb is slowly and beveled out of the gel. Care is taken not to damage and tear the wells. Before loading the samples into the wells, a voltage is applied at 60 V for 15 minutes as a preliminary. Then, samples are loaded into the wells and the system is run in this way, first at 60 V for 90 minutes, then at 120 V for 90 minutes. The formation of small bubbles is observed to ensure that the system is working. 4 µl of 5X loading buffer is added separately to samples. The DNA marker (ladder) is placed in the last well. In our own studies, a 100 bp DNA marker was added as 6 ul. The samples move to the last part of the gel. When they reach the appropriate place, the electric current is cut off. The gel is removed between two thin glasses and placed in an empty container. The gel is washed with distilled water and imaged on the imaging device.

3.6.1.2. Native Gel Electrophoresis

This is a variant of the SDS-PAGE method that does not contain SDS. First of all, a solution called "Resolving gel" is prepared. This must be prepared quickly because it freezes immediately and can cause problems with forming wells. The

solution contains dH_2O (distilled water), Tris-base, APS, TEMED, 40% acrylamide solution. To prepare APS, 0.1 grams of APS in powder form is taken, dissolved in 1 ml of distilled water and stored at -20°C. Samples to be loaded into the wells must be bound with a dye (loading buffer) to be visualized on the gel. The loading buffer contains bromophenol blue, glycerol, Tris-HCl, distilled water. Except that it does not contain SDS, all other operations are performed as in the SDS-PAGE method.

3.6.2. Steps of Agarose Gel Electrophoresis

The percentage of agarose gel is adjusted according to the desired pore size. In our studies, this is usually 2.5 %. For this reason, 2.5 grams of powder agarose is weighed and 100 ml of 1X TAE buffer is added on it. This mixture is placed in a flask and heated in the microwave until it dissolves and forms a transparent image. It is provided to cool down to 50°C or 60°C. Add 5 µl ethidium bromide or its alternative GelRed. It is shaken slightly to be homogeneous. The prepared gel is poured into the gel tank. It is necessary to be slow in order not to form bubbles. Then, appropriate well combs are placed. The gel is allowed to freeze for about 30 minutes. After the gel has frozen, 1X TAE Buffer is added to the tank. Combs are removed gently and without damaging the wells. Samples are mixed with loading dye and transferred to the wells. The appropriate DNA marker is loaded into the last or first well. In our study, 6 µl of a 100 bp DNA marker is added. The complex containing GH1, GH2, GH3, GH4 was called GH1-4. Likewise, The complex containing GH1, GH2, GH3 was called GH1-3.

To prepare the 2.5 M MgCl buffer, 4.41 g sodium citrate is weighed and 600 µl of 2.5 M 20X MSC buffer is added. Ultra pure water is added so that the final volume is 50 ml. The final concentration is aimed to be 0.3 M. The pH is adjusted to 7.0. To prepare the 5X MSCT buffer, 12.5 ml of 20X MSC buffer is used. 50 ul Tween20 is added. Distilled water is added to the last 50 ml. For preparing the 5X PSCT buffer, 5.51 g of trisodium citrate is weighed. 14.0 g of KCl are added. It is completed with 220 ml of distilled water. The pH value is adjusted to 7.0 using HCl. It is completed with distilled water to its final volume of 250 ml. For preparing the 5X LSCT buffer, it is aimed to be 0.001 mole in 10 ml, so weigh 0.0424 g of LiCl. Make up to 10 ml with 100 mM Tris + 10 mM EDTA buffer. The voltage of the tank is set to 100 V for 40-60 minutes.

CHAPTER 4: RESULTS AND DISCUSSION

In the experiments of Hairpin 1 (HP1) and Hairpin 2 (HP2) based on the manuscript (Ang and Yung, 2016), it was stated that the fluorescence intensity would increase at 660 nm and decreased at 550 nm as the GT concentration increased. The results of our experiment are expected to be in this direction. The results are shown in Figure 23. Based on this, we made our HCR design.

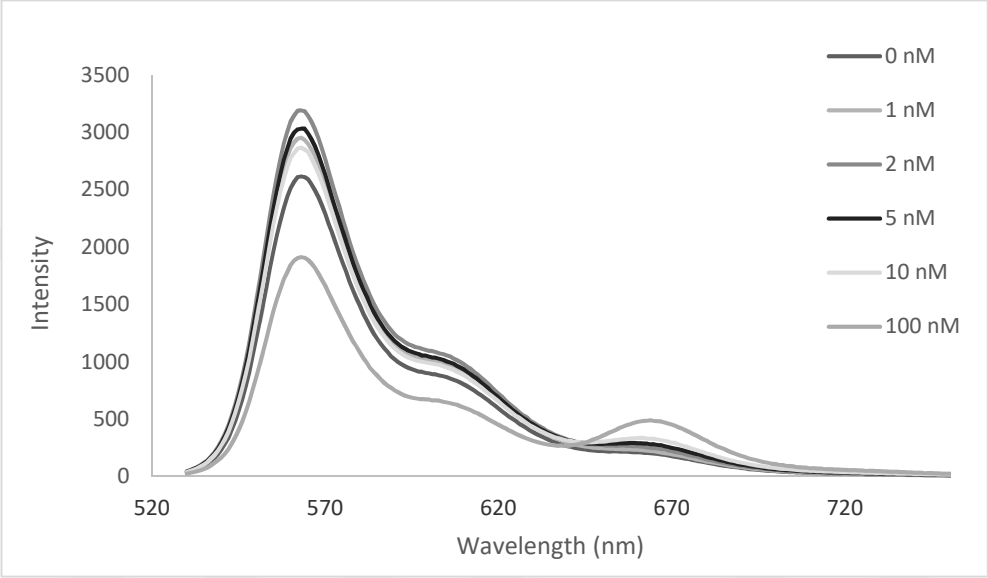


Figure 23. The peak formation from top to bottom at 570 nm is 2 nM, 5 nM, 1 nM, 10 nM, 0 nM, and 100 nM, respectively. The peak formation as GT concentration from top to bottom at 660 nm is 100 nM, 10 nM, 5 nM, 2 nM, 1 nM, and 0 nM, respectively.

Next we have demonstrated the new HCR system. This system is composed of four hairpin-forming oligonucleotides (HFO). To design we used computer assisted softwares and demonstrated the effect of a trigger sequence (GT) on the assembly of the oligonucleotides into a complex. These designs were than tested in vitro using gel electrophoresis and fluorescence spectrometer. To detect the presence of GT more sensitively we had incorporated a G-quadruplex forming sequence. The sequences were designed so that G-quadruplexes would form only if the HFOs are assembled. We rely on the fact that G-quadruplexes may have horseradish peroxidase (HRP) activity and convert AmplexRed(™) into fluorescently active resofurin molecule. We aimed to detect GT concentration through detection of resofurin through fluorescence. Furthermore we have tested modified HFOs with fluorescent reporter and quenchers GH2-F was designed to provide an alternative to HRP activity.

GH1 is expected to interact with the GT, bond and then dissolve its hairpin structure. The other part of GH1 is expected to bind to the GH2-F molecule. Since GH2-F no longer has a hairpin structure, the FAM moves away from the BHQ. In this way, it can show fluorescence even at 520 nm wavelength levels. By binding half of the GH2-F to GH1, the other half allows binding with GH3. Likewise, the process continues and binding is performed for GH4. The sequence at the 5' end of the GH4 is equivalent to the GT sequence. In this way, it supports interaction with another GH1 in the environment and allows the process to continue. This process only lasts until the equilibrium point is reached. The determination of the equilibrium point depends on the GT concentration.

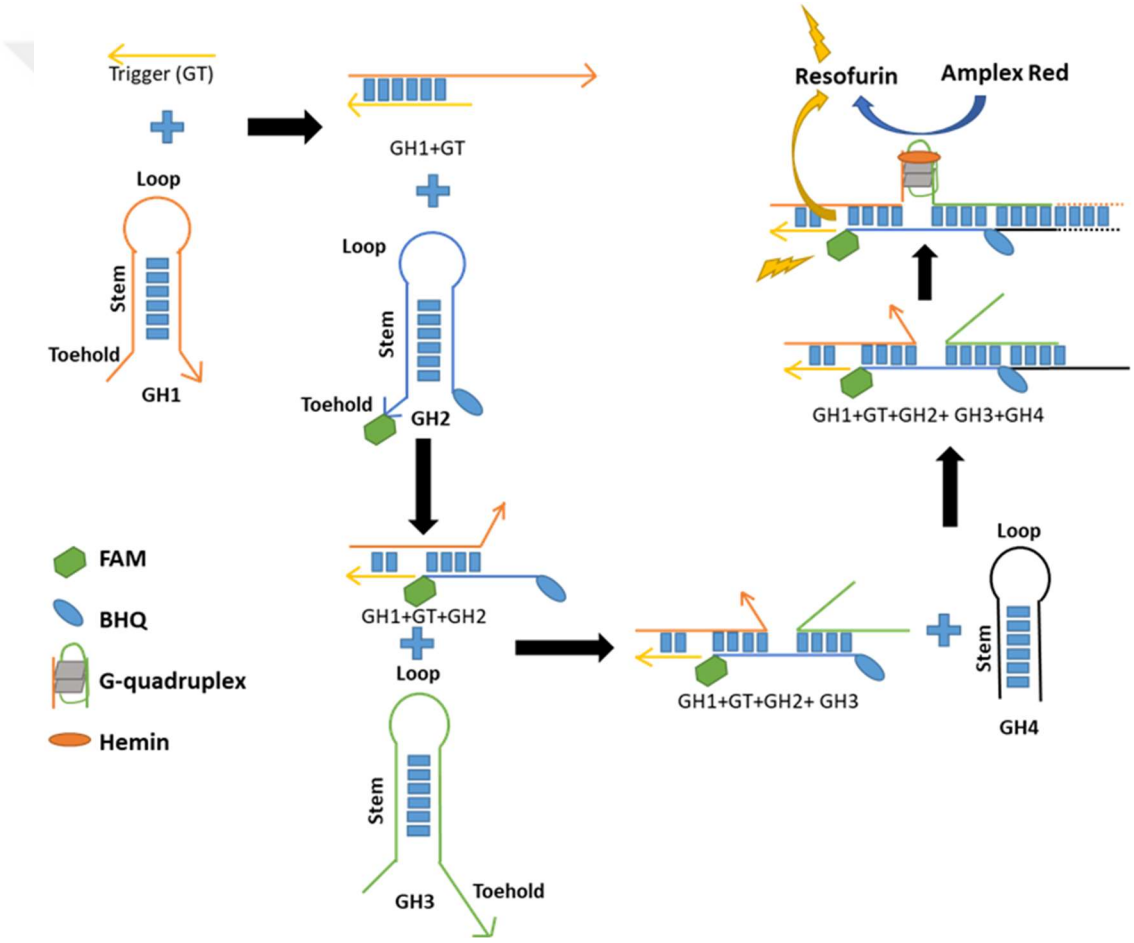


Figure 24. Illustration of the design of our G-quadruplex supported HCR mechanism.

4.1. In silico Experiments

A webtool called Nupack was used to design and simulate HFO assembly. In the experiment performed according to the newly designed HCR system, four HFO sequences were designed and their assembly was simulated using the Nupack webtool.

Additionally a short oligonucleotide called GT sequence, the sequence that will start the HCR reaction, was also used as our trigger. The assembly that all the HFOs and the trigger sequence can form with each other has been analyzed and shown through the Nupack tool.

In Figure 27, it is seen that the strands that do not bind to each other in the absence of GT are folded on themselves. However, in Figure 45 when GT is added, it is seen that different strands form complexes together. It is seen that GT is an important factor for polymerization. Longer complexes could also occur, but the complexity level was set to 4 in Nupack analyses. For this reason, we do not see longer complexes. With Nupack analysis settings, the level of complexity can be further increased but the analysis time is were too long and difficult to obtain the data.

4.1.1. In silico Assembly with HFOs

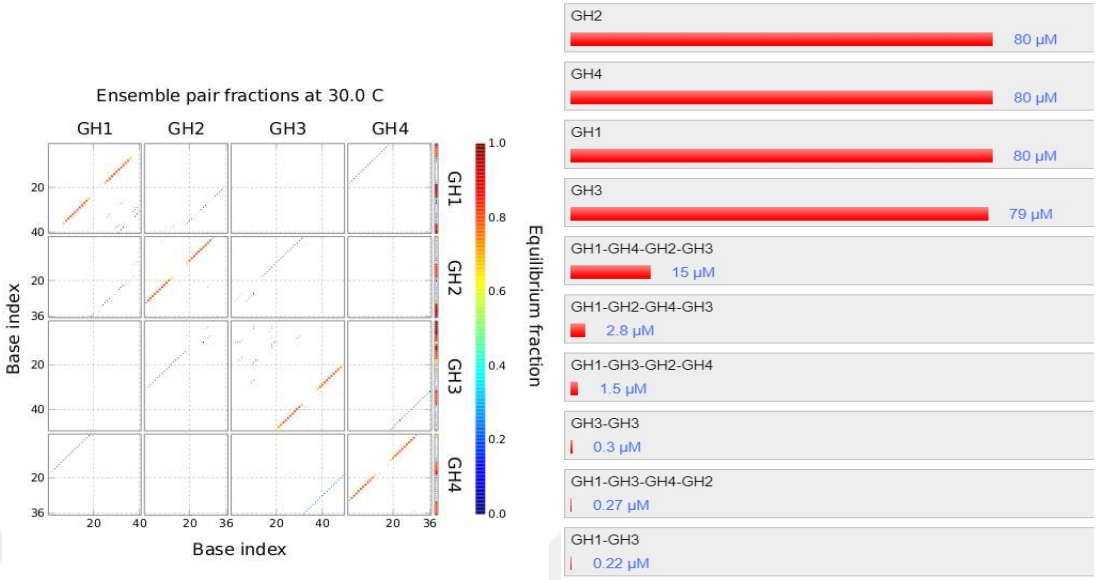


Figure 25. Ensemble pair fraction graph and distribution and interactions of HFOs are shown in the absence of GT.

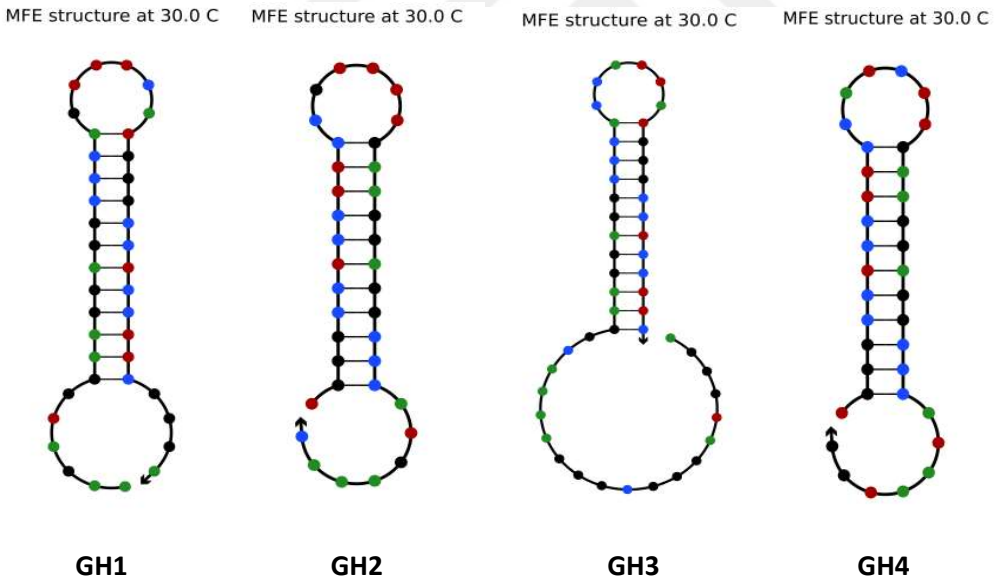
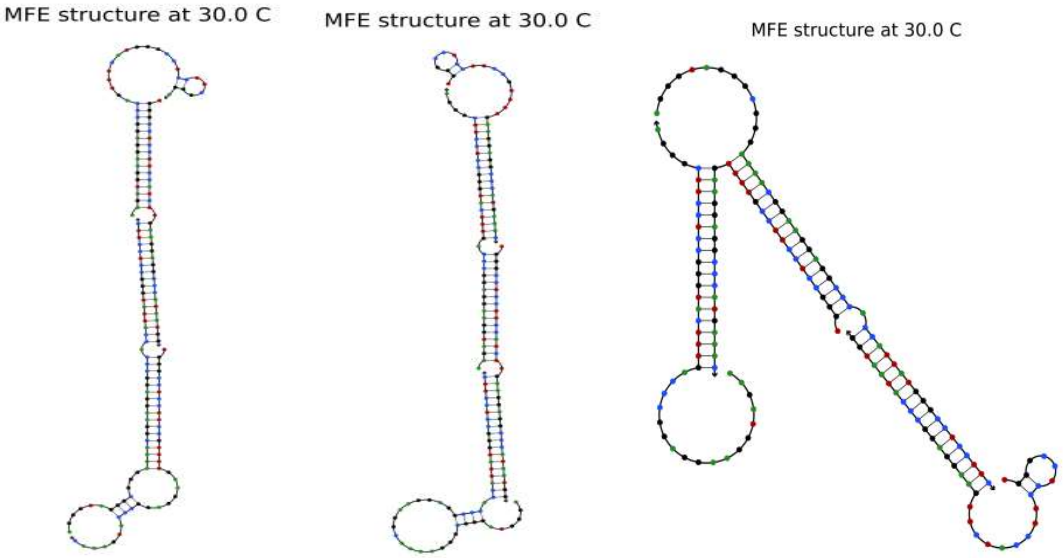
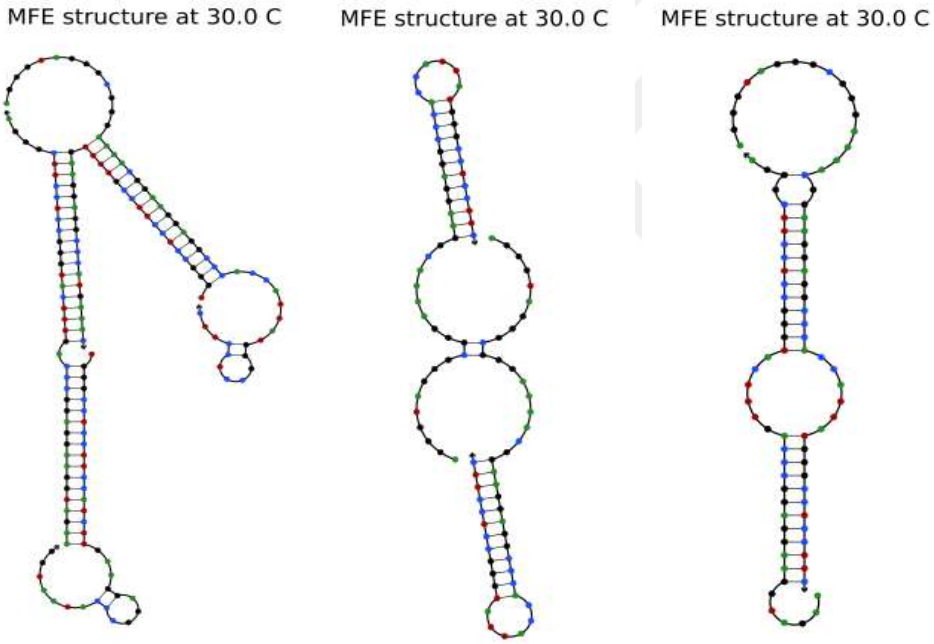


Figure 26. Nupack image of HFOs. (The colors of bases are represent as Green: A, Blue: C, Black: G, Red: T)



GH1-GH4-GH2-GH3 **GH1-GH2-GH4-GH3** **GH1-GH3-GH4-GH2**



GH1-GH3-GH2-G4 **GH3-GH3** **GH1-GH3**

Figure 27. Interactions between HFOs in the absence of GT are shown. The conditions of the assembly are 100 µM HFOs and 0.1 M Na^+ concentration. (The colors of bases are represent as Green: A, Blue: C, Black: G, Red: T)

4.1.2. In silico Assembly with HFOs and GT

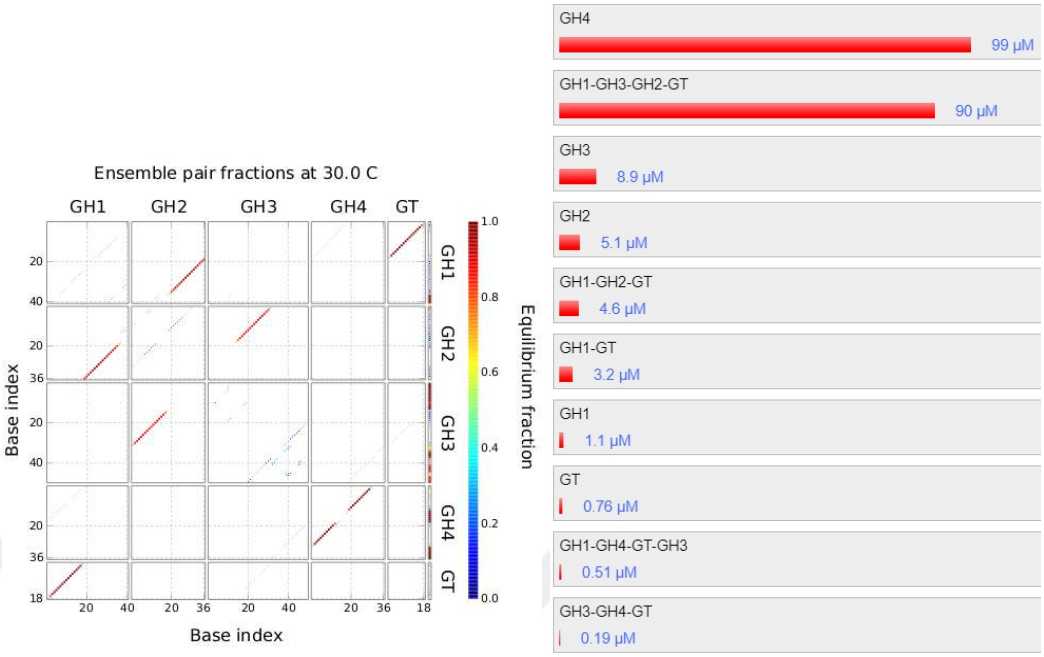
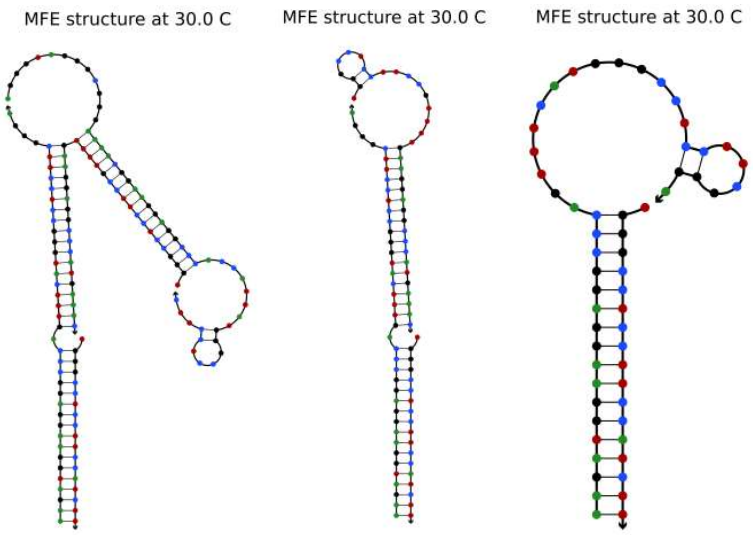
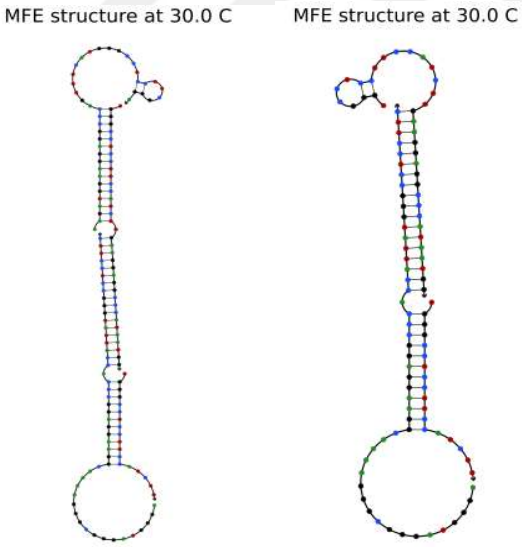


Figure 28. Ensemble pair fraction graph and distribution and interactions of HFOs are shown while GT is 100 µM.

Na^+ salt concentration was also reduced to 0.1 M in in silico assembly performed in the presence of GT. The temperature is 30 °C. In these conditions, the concentrations of GH4 and G1-GH3-GH2-GT complex are very close to each other and are almost 1 µM. Other HFOs appear to remain in low concentrations, with almost all of them being spent to form complexes but this cannot be said for GH4. The concentration of GH4 in the environment is too high. This is because the complexity level is four. If complexity is increased, GH4 would polymerize and GH4 concentration will decrease. We would not increase complexity any further since it became too complex for computation.



GH1-GH3-GH2-GT **GH1-GH2-GT** **GH1-GT**



GH1-GH4-GT-GH3 **GH3-GH4-GT**

Figure 29. Nupack image of HFOs is given in the presence of GT. (The colors of bases are represent as Green: A, Blue: C, Black: G, Red: T) The appearance of GH1, GH2, GH3 and GH4 HFOs is the same here as in Figure 26. So it is not shown again.

It is seen that GT addition causes G-repeats to come close. This can also result in G-quadruplex formation. In the presence of GT, it is seen in the GH1-GH3-GH2-GT complex that guanine repeats come together as groups of four. These four guanine groups fold to form a G-quadruplex. A zoomed-in view of the G-quadruplex forming region in the GH1-GH3-GH2-GT complex is shown in Figure 30.

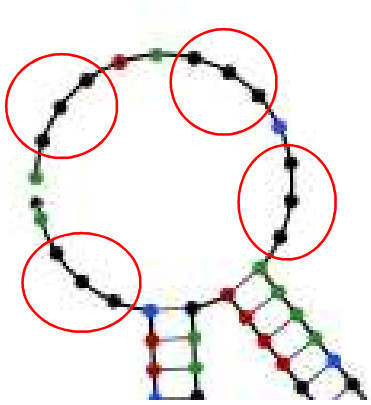


Figure 30. The GH1-GH3-GH2-GT complex shown in Figure 29 is zoomed in and shown. Here, there are repeats of Guanine in the regions marked with the red circle. When the quaternary guanine repeat groups come together, folding is expected to occur and a G-quadruplex structure is formed.

We think that G-quadruplex will be formed when guanine bases repeats are closely aligned. This is one of the goals of our HCR design. But we cannot see this with Nupack, because the Nupack algorithm is not designed for this. Only Watson-Crick modeling can be performed with this algorithm.

4.2. In-gel Assembly Experiments

4.2.1. Monovalent Ion Comparison

Gel electrophoresis method was used to observe the results of the experiments of the newly designed HCR system. With this method, it is aimed to examine the reaction relationship of HFOs and GT sequence in different buffers. At this stage, four HFOs, namely GH1, GH2, GH3 and GH4 oligonucleotide sequences were used (Table 3). These oligo sequences were tested with different buffers and it was investigated whether the HCR system was realized. This system containing with GH1, GH2, GH3 and GH4 is called GH1-4 system. The molecular sizes of the complexes were evaluated by comparing them with the DNA ladder in terms of molecularity.

Agarose gel electrophoresis system was prepared in order to see the effect of Mg^{++}, K^{+} or Li^{+} versus different GT concentrations in GH1-4 system. The GH1-4 system was prepared by mixing the HFOs at 5 µM concentration. The mixture was added hemin and DMSO. We have prepared this system in three different buffers containg, Mg^{++}, K^{+} and Li^{+}. and added different concentrations of GT so that GT concentration vs GH1-4 system would be at 1:100, 1:10 and 1:1 ratios. Additionally,

in order to show the assembly of the HFOs, we have prepared same system without GH4, which is refered as GH1-3 system. It was intended that absence of GH4 should inhibit formation of high-order structures and that the assebmly is completed including binding of the last HFO, GH4. After incubation, the mixtures were separated through gel electrophoresis. Complete assembly between HFOs and the trigger sequence are observed in the lane 4 with 1:1 GT concentration in all experiments according to Figure 31. At this level all of HFOs were incorporated into a high-order complexes. These complexes were observed in form of a smear or band above 100 bp according to DNA ladder. In addition, it is observed that there are important binding in the second and third lanes in presence of Mg^{++} (Figure 31-C). For Li^{+} and K^{+}, lane 2 did not show any significant difference in comparison to the negative controls which did not contain any GT. Only a smear was observed in lane 3 where GT concentration was at 1:10 ratio.

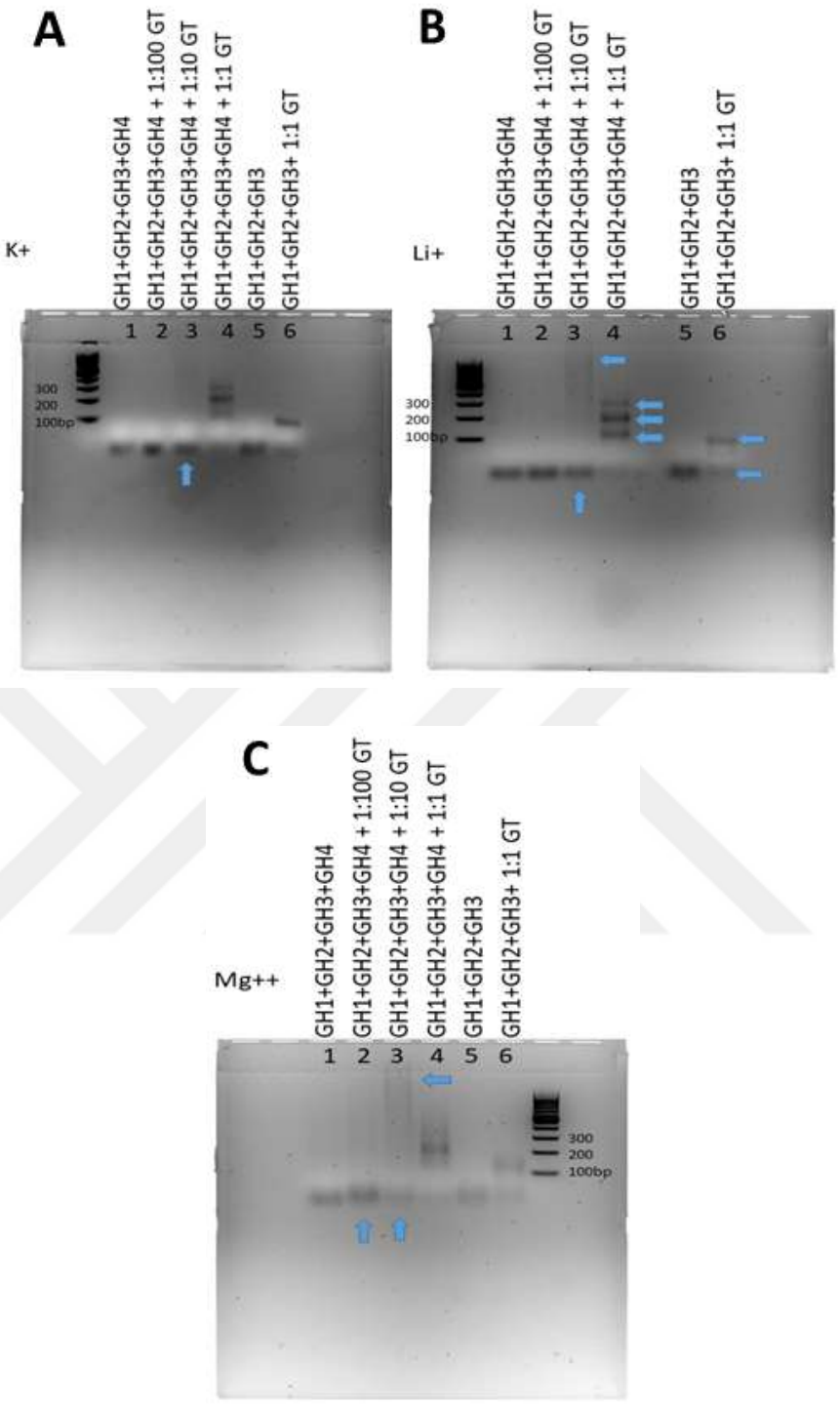


Figure 31. In gel electrophoresis, GT concentrations of 1:100, 1:10 and 1:1 were tested with HFOs. HFOs differ as GH1-4 and GH1-3 systems. A) The effects of HFOs and GTs at different concentrations are seen in the buffer containing K^+. B) The effect of HFOs and GTs at different concentrations are seen in the buffer containing Li^+. C) The effects of HFOs and GTs at different concentrations are seen in the buffer containing Mg^{++}.

The GH1-3 system without GH4 from the HFOs specified in the GH1-4 system was also tested. The system with GH1, GH2, GH3 consists of a mixture of HFOs at a concentration of 5 μM. As with GH1-4, hemin and DMSO were added to this system. For the system tested in buffers containing Mg^{++}, K^{+} and Li^{+}, 1:1 GT and 0 concentration GT were examined.

The gel experiments were in general showed that HCR system works. However, much lower GT concentration was expected to be detected, yet the assembly was inhibited siginificantly even at 1:10 concentration ratio.

There are some conditions of the hybridization process for the HCR reaction that is critical to the efficiency. In the light of these findings, it was concluded that HCR showed better assembly with GT in the presence of magnesium and decided to continue the experiments under these conditions.

4.2.2. Mg^{++} Concentration Gradient Experiment

When Mg^{++} is used in the buffer, a clear band can be observed, it was sufficient for the HFOs to hybridize and provide evidence as a band in the hybridization reaction, but it is seen that the yield is low compared to other buffers. Since more significant banding is observed in analyzes containing magnesium buffer, this parameter was focused on. In order to examine the effects of Mg^{++} more clearly, the interaction of HFOs and GT molecule was investigated in the system containing 2.5 M $MgCl_2$ vs 5X MSCT buffer. In Figure 32, an experiment was made with the GT concentration gradient to understand whether the difference in Mg^{++} concentration has an effect on hybridization.

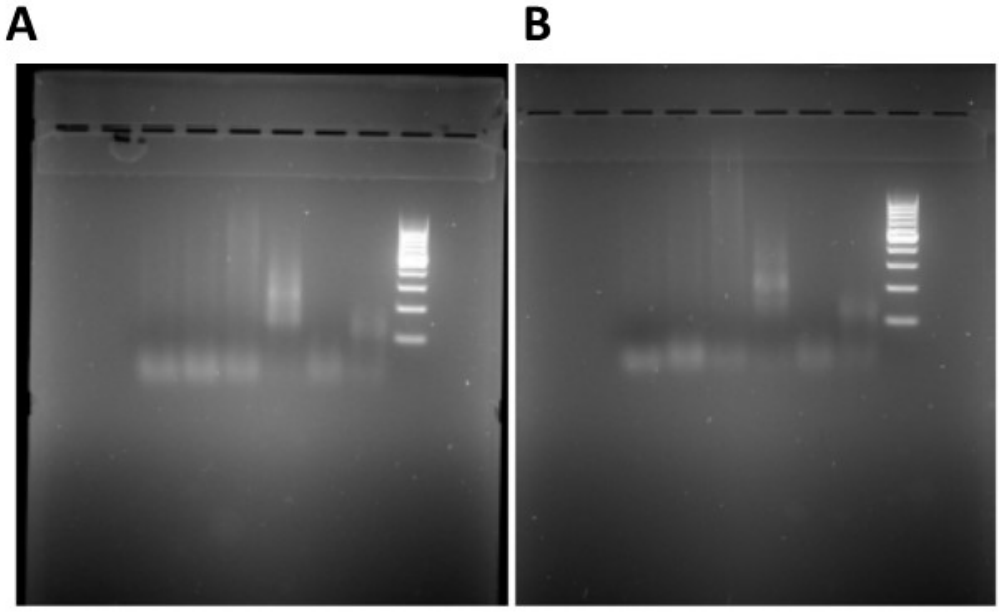


Figure 32. The figure shows the behavior of the GH1-4 system in 2.5M Mg^{++} and 5X MSCT buffers. A) Gel electrophoresis result of GH1-4 system in 2.5 M Mg^{++} buffer. B) Gel electrophoresis result of the GH1-4 system in 5x MSCT buffer.

While the effect of different buffers can be observed in the gel electrophoresis method containing the GH1-4 system, the two buffers containing different concentrations of Mg^{++} did not show significant difference.

We are trying to make the Mg^{++} concentration more than 5x MSCT. For this, we increase it to 2.5 M. But the 2.5 M Mg^{++} concentration may not be high enough. A higher concentration can be tried. As another reason, the concentration level that Mg^{++} can affect is only as much as the concentration of 5x MSCT. Even if the concentration is increased more, Mg^{++} cannot catalyze any reaction as it reaches the saturation point.

4.3. Fluorescence Enhancement Experiments

While the gel experiments indicate that our HCR system is working, an alternative mode of detection may provide quantitative and reproducible result. In order to test an alternative mode, we have decided to use fluorescence.

We modified GH2 with FAM and BHQ1 (GH2-F) in order to change fluorescence between hairpin form and complex form. FAM is the most common fluorescent dye material that is used as an attachment to oligonucleotides. FAM and BHQ1 pair is often used in TaqMan probes (Marras, Kramer and Tyagi, 2002).

The HFOs of GH1, GH2-F, GH3, and GH4 were prepared to be 5 µM and it was called GHmix. GHmix in the absence of GT is expected to quench any excitation at 490nm. When GT is introduced and a high-order molecular complex is formed, it is expected that GH2-F is no longer in hairpin form and FAM and BHQ1 are distant to each other. This inceased distance result in increased fluorescence by FAM.

Addition of FAM dye/quencher groups to the designed hairpin probes increases DNA stability. In dual-labeled oligonucleotide probe (ODN), if the sequence form is hairpin, fluorophores and quenchers added to opposite ends can interact directly. Therefore, thermodynamic aspects of dye and quencher interactions should be considered when designing HFOs.

GT concentrations between 0nM, 0.2 nM, 1 nM, 2 nM, 10 nM, 20 nM, 200 nM and 1 µM levels were tested. By scanning emission between the wavelength range of 500-700 nm, it was aimed to see the fluoresence peak due to FAM fluorescence. Fluorescence observed at 0.2 nM and 1 nM did not provide a very distinctive difference. There is a significant increase starting at 20 nM, and continues to increase until 200 nM with the highest intensity value at 1 µM.

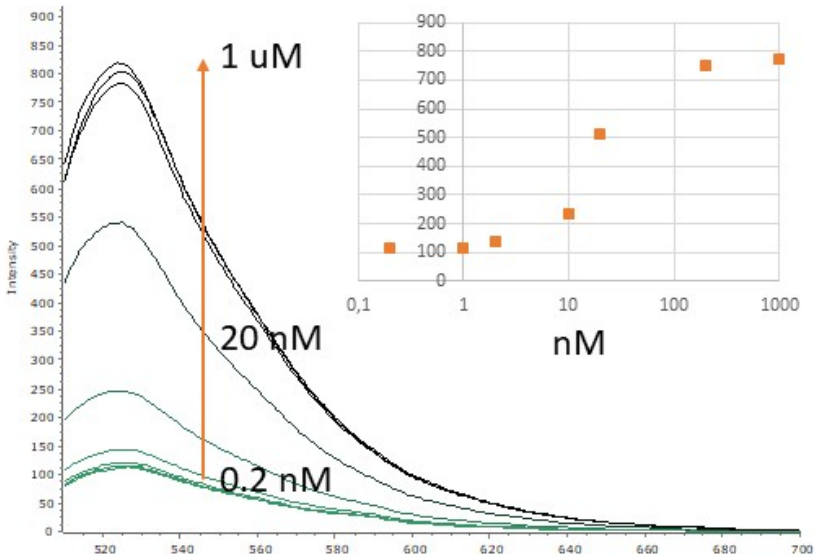


Figure 33. Evaluating the GT concentrations as 0.2 nM, 20 nM and 1 µM by fluorescent spectrometry.

The absence of large differences between 0.2 nM-10 nM GT concentration levels negatively affects the accuracy of the experiment at this level. In order to make

a clearer distinction, a higher concentration of GT should be tested. However, while this is the case, the result that the HCR system cannot operate efficiently at low GT concentration calls into question the success of the entire system. Based on this, a large increase in fluorescence at concentrations higher than 10 nM and the formation of more distinctive peaks indicate that the HCR system is successful in general, but its sensitivity is low.

In the HCR system, in line with these results, the formation of distinctive peaks only at high GT concentrations leads to the conclusion that some factors in the experiment should be changed. It raises the question of whether, by changing the parameters or improving the ambient conditions, lower GT concentrations can be achieved and a high-intensity peak can be obtained and a distinction that will make a difference in fluorescence. For this reason, the idea of adding H_2O_2 and AmplexRed(™) to the experimental setup and detecting HRP activity has emerged.

4.4. G4-HRP Activity Experiments

We performed the G4-HRP experiments to see how the HCR system would work without the FAM dye. To express the experimental setup, a system consisting of GH1, GH2, GH3 and GH4 hairpins was arranged so that the GH system was 100 pM. GT concentrations are 0 fM, 10 fM, 100 fM, 1 pM and 10 pM. 10 µM AmplexRed(™), 10 pM hemin and 1 mM H_2O_2 were added to the system and waited 20 minutes. It is aimed to obtain more accurate results by using the time-dependent self-measurement option of the fluorescent spectrometer device.

The excitation wavelength was determined as 560 nm. The measuring range was set between 570 nm and 720 nm wavelengths. The peak value of all samples is seen at 584 nm. The maximum intensity is seen at 1 nM GT concentration. Then the peaks of 100 pM, 100 fM, 1 pM and 0 GT are seen very close to each other. The lowest peak value is observed at 10 pM. The fact that 0 GT is higher than 10 pM led to inconsistency in the experiment. However, the highest intensity of 1 nM indicates that the HCR system is working correctly.

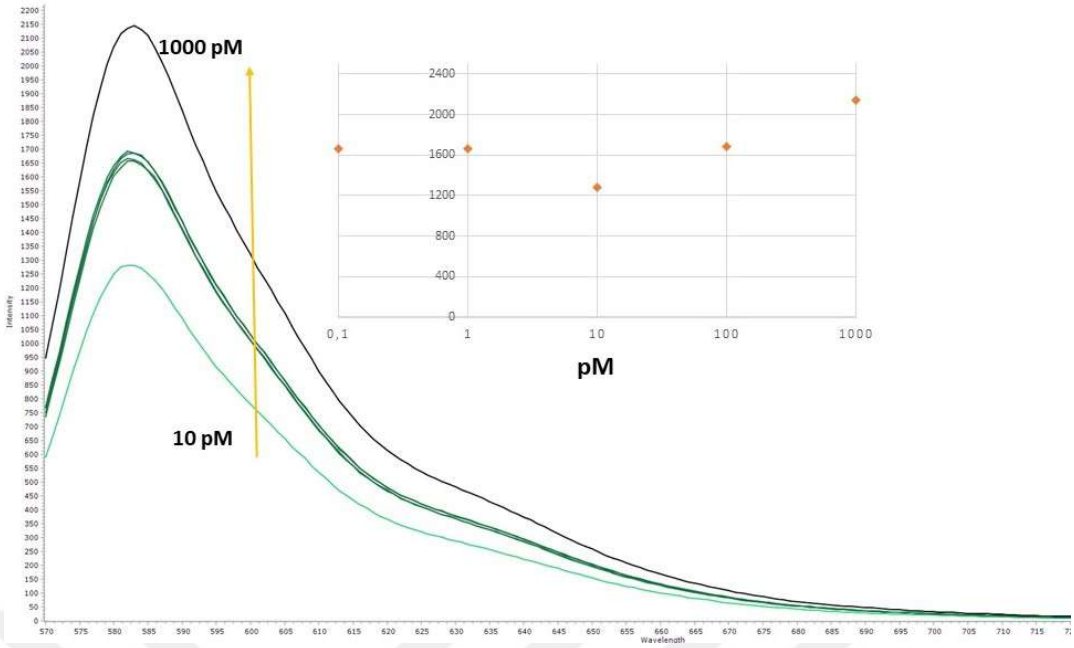


Figure 34. 10 pM and 1000 pM (1 nM) values are shown in the G4-HRP experiment and used 1X SSCT buffer.

The results are not exactly as expected. Except for 1 nM, the peak values given by the other GT concentrations are confusing. The peaks of 100 pM, 100 fM, 1 pM and 0 GT are very close to each other and the lowest peak value is observed at 10 pM. However 1 nm showed highest peak as expected. The discrimination power of the our HCR may have remained low.

4.5. FRET Assisted G4-HRP Activity Detection Experiments

According to our HCR design, when GT is present, HFOs form complexes and G-quadruplexes are formed. G-quadruplexes are known to have HRP activity. However, this activity takes place in the presence of hemin, H_2O_2 and ABTS. The peroxidase activity of the G4/hemin complex allows for colorimetric diagnosis for many target molecules. AmplexRed$^{(TM)}$ can also be used instead of ABTS, thereby revealing the fluorescent product. It is known that HRP activity can only be seen with complex formation, since G-quadruplex will not occur in the HCR system that takes place in the hairpin form.

In this thesis, after reaching the equilibrium in the HCR experiment, hemin is added to bind to the newly formed G-quadruplex structures in the environment. The complexes formed by the binding of G-quadruplex and hemins exhibit an enzymatic activity similar to horseradish peroxidase. If there is H_2O_2 in the solution, G-

quadruplex and hemin complex will react with AmplexRed(™). As a result, it will produce resofrin. Resofrin is a fluorescent molecule and absorbs light around 580 nm wavelength. In the design made in this thesis, it is aimed to determine the concentration of the trigger molecule, namely the GT molecule, with this emission. In order to increase the sensitivity, it was aimed to detect the signal by stimulating the FAM at 490 nm wavelength by choosing to use the förnster resonance energy transfer between FAM and resofrin. This enabled the excitement of resofrin only by FAM in close proximity if the FAM-linked one of the GH2 sequence is unwound.

The same GT concentrations were used in the experimental setup, 0.2 nM, 20 nM and 1 µM GT were included in the experiment. GH1, GH2-F, GH3 and GH4 are prepared to be HFOs 5 µM. AmplexRed(™) and H_2O_2 were added to the experiment and scanning was performed in the range of 500 nm -700 nm after an hour incubation time. As a result, peaks with high separation were expected to be seen.

A new peak formation is observed around the wavelength of 580 nm. The intensity values of this new peak are in the range of 100-240 and are lower than the intensity values of the peak occurring around 520 nm wavelength.

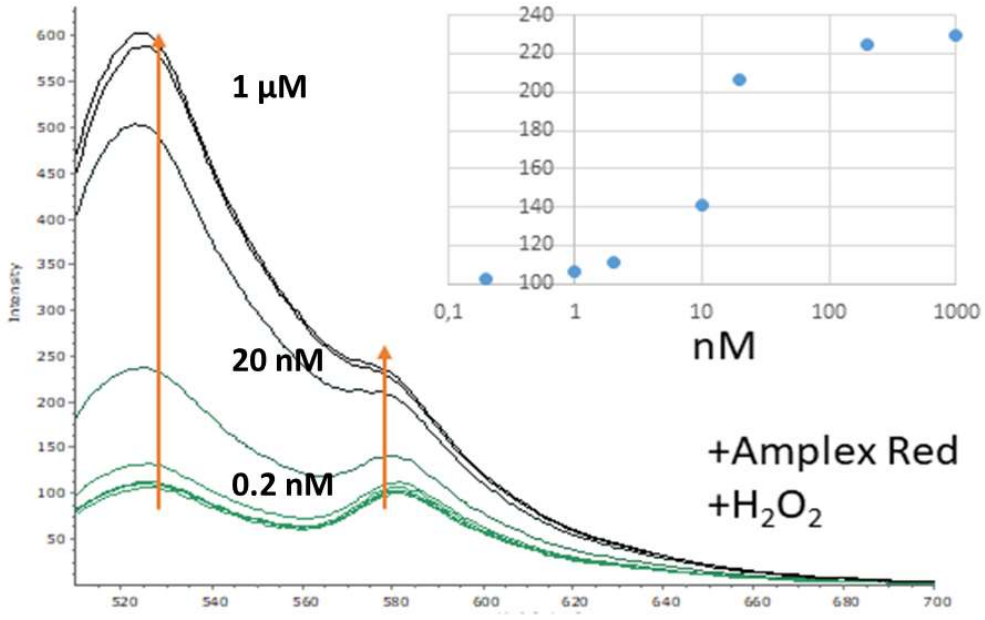


Figure 35. Amplex Red (™) was added to the reaction mix and incubated and also, evaluating the GT concentrations as 0.2 nM, 20 nM and 1 µM.

Under the condition that AmplexRed(™) was added, after an incubation period of an hour and a new resorufin peak was formed. It can be said that the detectability of this resorufin formed was realized through FRET, and this can be achieved because the FAM is excited at the wavelength of 490 nm, the energy is transferred to resofurin and resofurin emitted at the 580 nm. The 0.2 nM and 20 nM concentrations are still quite poor for detectability and discrimination, but for higher concentration levels than these values, increased fluorescence can be mentioned. The ability and competence to reach FRET means that FAM can actually provide its closeness to resorufin, and the reason for this can be shown as the intercalation of resorufin to DNA due to its aromatic structure. As a result of these studies with different concentrations, a linear range could not be detected specifically. However qualitative measurement is possible. The lowest GT concentration used in this experimental setup was determined as 0.2 nM, and although the distinction at high GT concentration levels could not be observed, the peak formation, providing fluorescence, shows that the 0.2 nM GT concentration was the lowest we could reach for our studies.

It was concluded that in these fluorescence experiments, when AmplexRed(™) was added and incubated for 15 minutes, a linear range was not detected and more work was required to investigate the linear detection limits, however, qualitative detection could be obtained for concentrations as low as 20 pM. For this, a design change in the G-quadruplex forming GH1 and GH3 sequence can increase the interaction and stability of the HFOs. As another option, the sequences can be aimed to be linear by increasing the HFOs to a high temperature such as 90 °C for incubation and separating all HFOs (even those that are tied together at room temperature). Then, by placing them in a 0 °C environment, the conformational change of the HFOs and their bonding can be realized by heat shock. Thus, lower GT concentration level can be achieved.

CHAPTER 5. CONCLUSION

The aim of this thesis is to introduce a new design of the HCR technique and to express that it can be used in many biological and clinical studies and in many fields. We have implemented and demonstrated this new design both bioinformatically and experimentally. We would like to point out that the newly designed HCR assembly is a new and efficient technique in its field, with advantages such as isothermal, does not require enzymes, is fast and easy to perform, and does not require a temperature gradient. HCR has many advantages. It is also an important technique that can be used in point of care studies.

The hairpin sequences used in the HCR experiments were designed with the Nupack webtool. While designing the hairpins, the complexity level was chosen as four. This level could have been higher. In this way, the probability of seeing four guanine regions lined up side by side can be increased. The regions where the four guanine bases line up side by side are prone to G-quadruplex formation. In this way, it is possible to foresee all possible G-quadruplexes and make changes in the experimental conditions if necessary. Nupack does not give any information about the formation of G-quadruplex. In addition, assuming that the system operates at 30°C, design and analysis were made. Although the temperature of the laboratory where the experiment is carried out and the tubes containing the samples is set to 30°C, it is always within a margin of error.

Another important factor is metal ions. The buffers used contain different metal ions (lithium, magnesium, and potassium), allowing a comparison. In this way, changes in hairpin folding or hybridization chain reactions that may occur can be seen. After comparing the diversity of metal ions, their concentration differences are also examined. Since the HCR system works more successfully in the magnesium environment, another experiment was conducted by increasing the magnesium concentration to see the effect of the ion in more detail. A separate trial can be designed by increasing the concentrations of lithium and potassium. So that more accurate and fair comparisons can be made. Considering the time constraints and the data obtained, only the magnesium ion was preferred for the concentration comparison experiments. In addition, there was no significant difference as a result of the comparison with high

and low concentration of magnesium. Despite all this, the HCR system worked in buffers containing all the different ions.

GT gradient was tested in G4-HRP experiments. Here, at the highest concentration (1 nM), the peak formation is highest. The correlation between GT concentration and fluorescence was not lineer. Experimental errors may have been made in these experiments. GT concentrations other than 1 nM can be too close to each other. Therefore the results may be uncorrelated to GT concentrations. Mixture of GH system and GTs was incubated overnight. This time may have been too long. During the overnight period, the reaction may have occurred and the hairpins in the environment may have been exhausted and the reaction may have ended. For these reasons the range of GT concentrations could be kept wider. Instead of incubating the GH and GT mixture for a long time, a 2-hour incubation can be done. The amount of reaction mixture (hemin, AmplexRed$^{(TM)}$, H_2O_2 and 1X-SSCT buffer) added to the GH and GT mixture during measurement can be changed. Another experiment can be made by lowering the concentration of H_2O_2 with a final concentration of 1 mM and increasing the concentration of hemin with a final concentration of 10 pM.

We can compare the results of the experiment that we use as a reference in fluorescence experiments with the results of our own fluorescence experiments. They did a study with a LOD value of 100 nM in the manuscript (Ang and Yung, 2016). In the fluorescence experiments we conducted, we achieved very low LOD results. The LOD value reached in Fluorescence Enhancement Experiments is 2 nM. According to the manuscript result (Ang and Yung, 2016), it is a very good LOD concentration. AmplexRed$^{(TM)}$ was used in the G4-HRP Activity Experiment and the LOD concentration was 1 nM. The LOD value in FRET-Assisted G4-HRP Activity Detection Experiments is 1 nM. Going from 100 nM to 2 nM and 1 nM levels according to the referenced study are great results. However, we can understand that the last two fluorescence experiments did not make much difference, since the results were the same as 1 nM.

Spreading the use of the HCR strategy will shed light on many scientific areas, and also, with the flexible detection design of the HCR, the ability to detect target molecules with various signals can be considered in this advantageous situation. There are various signal sources such as metal ions, fluorescent dyes, electron transfer

materials and DNAzymes in HCR technique and studies. As with the HCR technique presented in this thesis, more sensitive signals can be detected by increasing the signal of the target molecule by means of HCR. We understand that the HCR technique will be useful in many areas such as disease diagnosis, food and environmental analysis, in line with its characteristics. In this way, it can be understood that many platforms using the HCR technique can be developed and produced and can be used as biosensors in the future.

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