



Engineering of RNA motifs for nanotechnology applications by utilizing light-up aptamer

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by

Nida Fathima

(Regd. Number: 201801117)

Supervisor: Dr Ashwani Sharma

Assistant Professor

Department of Chemistry and Biology

IISER Tirupati

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Name of student: Nida Fathima

Roll Number: 201801117

Signature of student: 

Date: 05/04/2023

Name of Supervisor: Dr Ashwani Sharma

Signature of Supervisor: 

Date: 05/04/2023

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Name of student: Nida Fathima

Roll Number: 201801117

Signature of student: 

Date: 05/04/2023

Endorsed by

Name of Supervisor: Dr Ashwani Sharma

Signature of Supervisor: 

Date: 05/04/2023

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Abstract

RNA nanotechnology comprises of nanoscale structures of RNA that act as functional and structural elements. RNA nanostructures include two dimensional (2D) structures like triangles, squares, etc., and three dimensional (3D) structures like tetrahedrons.^[1] These nanostructures have been utilized for various applications such as targeted drug delivery, diagnostics, and other biomedical applications.^[2] Different RNA motifs are used to construct RNA nanostructures, most of them are taken from naturally existing RNAs with slight modifications.^[1] For instance, RNA three-way junction (3WJ) taken from pRNA of phi29 bacteriophage can serve as a platform to construct thermodynamically stable RNA nanostructures with tunable size and shape.^[3] Similarly, many other motifs like RNA hairpins, kissing loops, four-way junctions, etc. taken from natural RNAs were also used in the construction of RNA nanostructures.^[4] RNA nanostructure stability depends on the RNA motif embedded in it. But currently, there are no high throughput ways to compare the stability of different RNA motifs. The only way is UV melting which is a difficult and time taking process.^[5] As most of the RNA nanoparticles are constructed from 3WJ motifs, we looked for methods to compare the stabilities of different 3WJ by using a light-up aptamer that lights up in presence of a non-fluorescent dye and makes the dye fluoresce.

In this work, we are trying to compare the stabilities of modified 5S rRNA and phi29 3WJs with its native structure in a high throughput fluorescence-based method. For that, we designed a sensor by merging the engineered 3WJ scaffold with a light-up aptamer called baby spinach. This sensor lights up in the presence of a dye called DFHBI, And the stability of 3WJ correlates with fluorescence intensity. More fluorescence indicates high stability of junction and vice-versa.

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Chapter 1

INTRODUCTION

Biological macromolecules, including DNA, RNA, and proteins, have intrinsic characteristics that make them potential building blocks for the construction of 2D, 3D, and 4D nanostructures.^[6] Both DNA and proteins have been much more extensively studied than RNA in terms of their potential applications in nanotechnology. RNA can be designed and produced with a level of simplicity as DNA. In addition, RNA shows versatility in structure and function similar to that of proteins. The ability of RNA to self-assemble into defined size and structure, its favourable *in vivo* properties, and great therapeutic potential make RNA a suitable candidate for nanotechnological applications.

RNA nanotechnology is an emerging field in which RNA sequences are engineered to self-assemble into nanoscale objects with defined geometries. These nanoscale objects have applications in a variety of fields, including drug delivery, diagnostics, immunology, photonics, nanoelectronics, etc.^[1] The therapeutic applications of RNA nanostructures are of great importance as it has a lot of advantages over existing drugs. Scientists have high hopes for RNA nanostructure-based drugs for targeted drug delivery, especially in the field of cancer therapy. However, the development of RNA nanotechnology has been hampered by a number of obstacles, and one of the main problems was the thermodynamic stability of RNA nanostructures.^[6] The discovery of the thermodynamically stable pRNA-3WJ, which can be assembled from three different RNAs, nearly resolved the thermodynamic instability and *in vivo* dissociation of RNA nanostructures.^[3]

1.1 Nucleic Acids

Nucleic acids are the primary information-carrying molecule of a cell. They are biopolymers composed of monomeric units called nucleotides. Each nucleotide is made up of a pentose sugar connected to a nitrogen-containing aromatic base, which is then bonded to a phosphate group. There are two types of nucleic acids in our body, Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). Each nucleic acid contains

four of five possible nitrogenous bases: adenine (A), guanine (G), cytosine (C), thymine (T), and uracil (U). Among these A and G are classified as purines, and C, T, and U are collectively referred to as pyrimidines. The nucleotides A, C, and G are present in both DNA and RNA, And the fourth base is T in DNA and U in RNA. The absence of a hydroxyl group (OH) on the 2' carbon of the sugar ring distinguishes the pentose sugar in DNA (2'-deoxyribose) from the sugar in RNA (ribose). A nucleoside is a sugar that is joined to one of the bases but does not have a phosphate group. The phosphate group joins the successive sugar residues in a chain by forming a bridge between the 5'-hydroxyl group on one sugar and the 3'-hydroxyl group on the following sugar. In both RNA and DNA, these nucleoside connections are known as phosphodiester bonds.^[7]

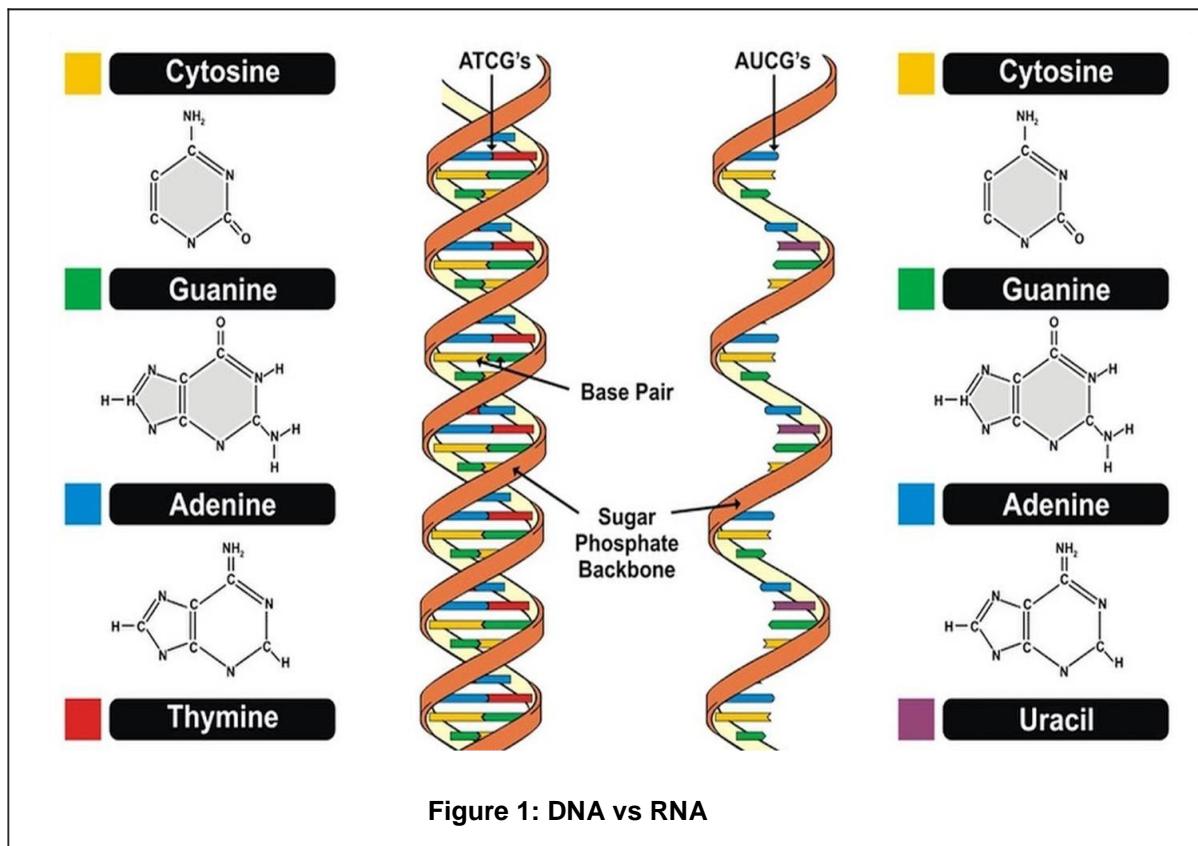
1.1.1 Deoxyribonucleic Nucleic Acid (DNA)

DNA is the hereditary material that store and transmits genetic information for the development and functions of an organism. The nucleotide of DNA contains deoxyribose sugar, phosphate, and four nitrogenous bases Adenine (A), Guanine (G), Cytosine (C), and Thymine (T). In DNA, adenine and guanine are purine bases and thymine and cytosine are pyrimidine bases. Purines form base pairing with pyrimidines. Adenine forms two hydrogen bonds with Thymine, and Guanine forms three hydrogen bonds with Cytosine. This type of base pairing is known as Watson-Crick base pairing. One strand of DNA is held to another by hydrogen bonds between the bases. DNA generally exists in double helical form, and adopts B helix through Watson-Crick base pairing. A phosphodiester bond is formed between the phosphate of one nucleotide and the sugar of the next nucleotide, forming a sugar-phosphate backbone. The combination of this structure and DNA's chemical stability makes it the ideal genetic material. Identical biological information is stored on each of the double-stranded DNA. When the two strands divide, this information is also reproduced. DNA can replicate into multiple copies known as DNA replication and can be transcribed into RNA.^[8]

1.1.2 Ribonucleic acid (RNA)

RNA is a nucleic acid that functions mostly in cellular protein synthesis. Like DNA, RNA is also made up of a long chain of nucleotides. Each nucleotide in RNA consists

of ribose sugar, phosphate, and four nitrogenous bases Adenine, Guanine, Cytosine, and Uracil (U). RNA is mostly single-stranded, shows extensive intramolecular base pairing between complementary sequences, and adopts A-helix which is more compact than B-helix. In addition to canonical Watson and crick base pairing RNA also has non-canonical base pairings like G-A, and G-U, this allows RNA to form a variety of secondary structures including helices, loops, hairpin motifs, pseudoknots, etc. Compared to DNA, RNA is chemically unstable and prone to hydrolysis because of the presence of a highly reactive 2'-hydroxyl group in the ribose sugar.^[1]^[8] Also, RNA is susceptible to attack by RNase which is present almost everywhere and recognizes the 2'-OH.

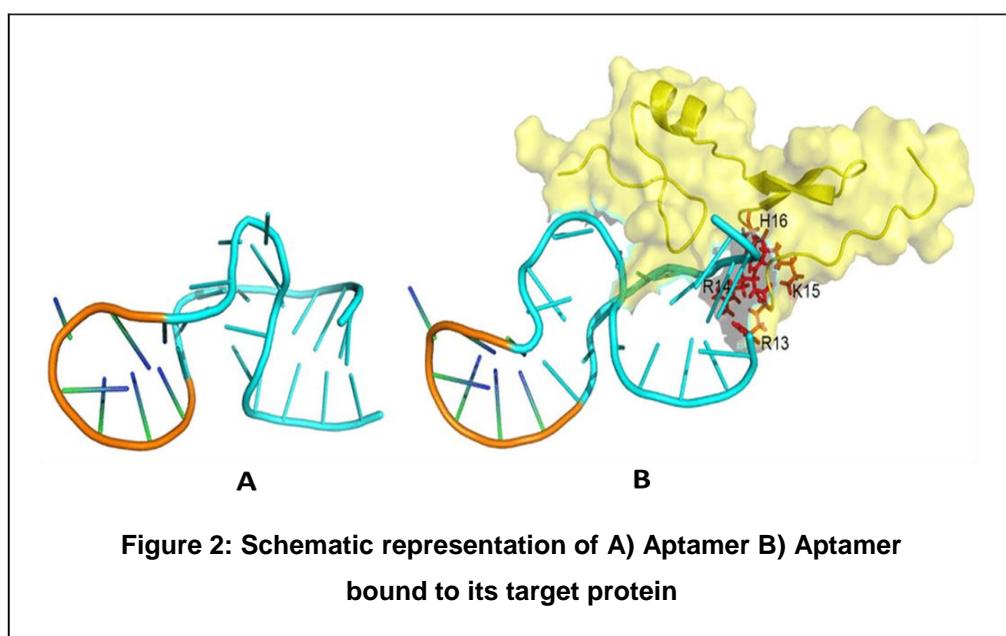


There are different types of RNA exist in our cells. Among them most important types are Messenger RNA (mRNA), which transmits DNA information, transfer RNA (tRNA), which performs a variety of functions during protein synthesis, Ribosomal RNA (rRNA), which serves as the “machinery” in the process of synthesis, etc. The important role of RNA is to convert information from DNA to proteins^[8]. In addition, RNA regulates cellular processes such as cellular catalytic reaction, cell division, differentiation, growth, and death, and also serves as the primary genetic material for

some viruses.^[9] RNA also provides many functional modules such as aptamers, ribozyme, riboswitch, etc. RNA shows versatility in structure as well as functions. This adaptability of RNA makes it a good material for use in nanotechnology.^[6]

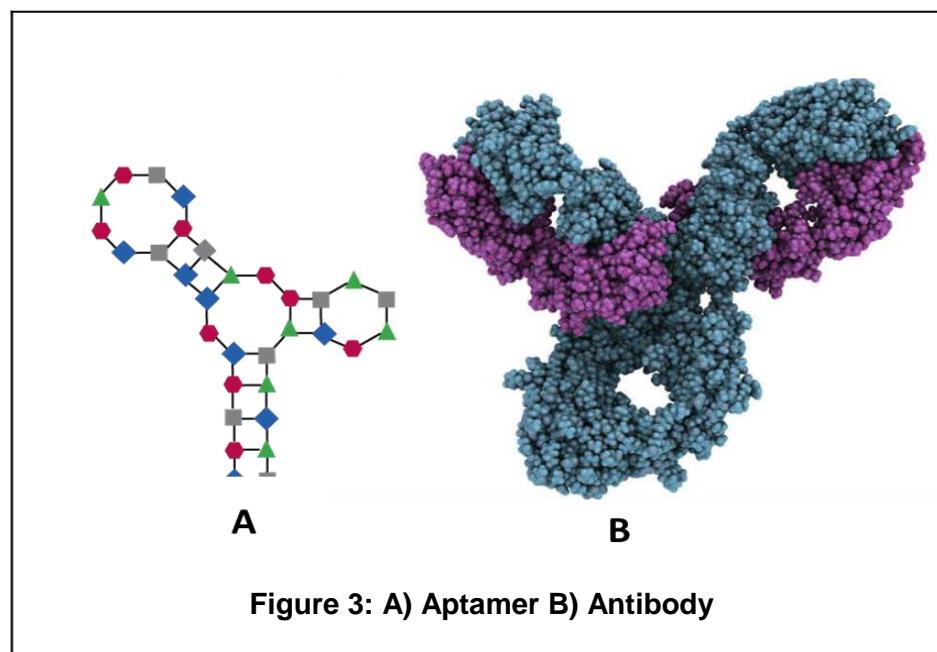
1.2 Nucleic acid-based aptamers

The word aptamer is derived from the Latin word “Aptus” that means ‘fit’ and the Greek word “Meros” that means ‘Part’. Aptamers are short single-stranded DNA/RNA oligonucleotide sequences that bind to a specific target with high specificity and affinity. Similar to monoclonal antibodies, aptamers also bind to their target by folding into a three-dimensional structural conformation.^[10] Most aptamers bind to their target with high binding affinity usually in the range of 10 pM to 10nM, towards a wide range of targets like proteins, metabolites, small organic compounds, carbohydrates, biological cofactors, metal ions, and even whole organisms like yeast, bacteria, mammalian cells, etc .^[11] The first therapeutic aptamer was described 25 years ago. Thus far ‘Pegaptanib’ an aptamer-based drug has been approved for clinical use by FDA, and numerous others are in preclinical or clinical development. Aptamers are analogous to antibodies in the range of target recognition and applications.^[12]



1.2.1 Aptamers vs Antibodies

Although aptamers recognize and bind targets of interest in a manner similar to antibodies, they have a number of advantages over antibodies, including faster target potential generation, lower manufacturing costs, no batch-to-batch variability, higher modifiability, high membrane permeability, better thermal stability, low immunogenicity, and long shelf life.^[13] Raising antibodies against poisons or other non-immunogenic targets is challenging because antibody selection requires a biological mechanism. On the other hand, an aptamer targets any protein because its entire selection is a chemical procedure carried out *in vitro*.^[14]



Aptamers are usually selected by an *in vitro* selection technique called SELEX, but natural aptamers also exist. Aptamer found in riboswitch is an example of a natural aptamer.^[15]

1.2.2 SELEX

Aptamers are typically produced by the SELEX (Systematic Evolution of Ligand by exponential enrichment) technique. This *in vitro* selection technique can separate high-affinity and specific aptamers from DNA or RNA libraries for a variety of target molecules.^[15]

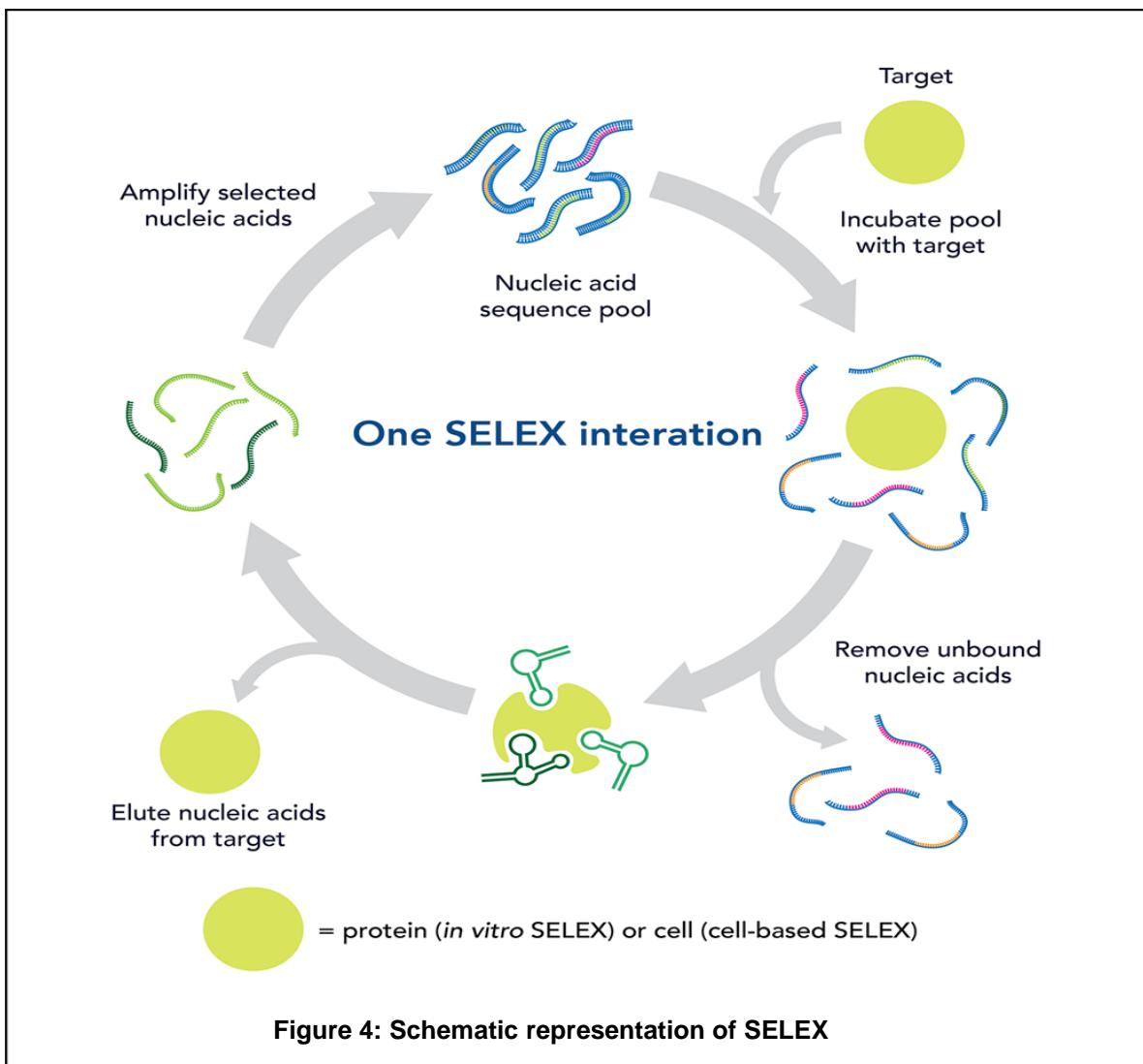


Figure 4: Schematic representation of SELEX

1. The initial step of SELEX is synthesizing a large pool of nucleic acid molecules made from an average of 15-40 bases of the random sequence flanked by primers 5' and 3' end.
2. The pool of DNA is transcribed into an RNA pool, and it is subsequently exposed to the target ligand of interest.
3. Unbound sequences from the mixture are removed and bound sequences are eluted and amplified by RT PCR.
4. Now we have an enriched pool of sequences with which these processes are repeated several times to get the final nucleic acid pool that is cloned and sequenced to get the potential aptamer.

For selecting aptamer there are different variants of SELEX methods depending on target molecules. Some are briefly described below.

- Cell-SELEX – it utilizes whole live cells as targets
- Nitrocellulose membrane filtration-based SELEX – used for protein targets
- Microfluidic assay-based SELEX -used for the selection of aptamer on a smaller scale.
- Capture-SELEX - aptamers are selected against small molecules.
- Capillary electrophoresis-SELEX which involves the separation of ions based on electrophoretic mobility
- Animal-SELEX - aptamers are selected directly within live animal models, etc.^{[15],[16]}

1.2.3 Applications of Aptamers

Aptamer offers a wide range of possible applications in biotechnology, clinical medicine, and intracellular research.

The following is an explanation and list of some significant applications of aptamers.

Aptamer as a drug: Aptamer provides opportunities for structure-based drug design strategies. Specific and high binding affinity towards its target molecules and low or no immunogenicity and toxicity make aptamer an effective therapeutic reagent.

Aptamer as a drug delivery system: Aptamers that bind to cell surface receptors have been exploited to deliver drugs and a variety of other cargo into cells.

Aptamer uses in bioimaging: Aptamer which is conjugated to a fluorophore can be used for bioimaging. The main advantage of this aptamer-based bioimaging technique is, it is less or non-toxic to the human body. Aptamer's high specificity for its target will increase the certainty of the results obtained during diagnosis or clinical analysis.

Aptamers as a diagnostic tool: Aptamer's properties like high affinity and specificity towards the target, small size, less immunogenicity, stable structures, ease of synthesis, etc make aptamer a good tool for diagnosis. Aptamer-based detection assays are expected to detect the low concentrations of pathogens than conventional antibody detection assays such as ELISA. [17],[18]

1.3 Light up Aptamer

Light-up aptamers are a class of aptamers that can bind to weakly fluorogenic molecules that show negligible fluorescence and enhance their fluorescence on binding with aptamer. Now-a-days light-up aptamers are widely used to detect small molecules and macromolecules both *in vitro* and *in-vivo*.^[19] Light-up aptamers were initially used for RNA imaging in cells, and it shows several advantages over older RNA imaging techniques. When compared to other RNA imaging techniques like MS2 tagging, Green fluorescent protein (GFP), Molecular beacon, Fluorescent in situ hybridization (FISH), etc., the main benefit is that it has a relatively low background fluorescence resulting in a high signal-to-noise ratio.^[20]

Some examples of Light-up aptamers are Spinach aptamer, Malachite green aptamer, Broccoli aptamer, Mango Aptamer, Corn aptamer, Pepper aptamer. The funny vegetable and fruit names were given to these aptamers depending on the wavelength of the color they emit after absorbing light of a particular wavelength. My work is mostly dependent on baby Spinach light-up aptamer that gives green fluorescent which is modified into a sensor to check the stability of three-way junction motifs.

1.3.1 Working principle of Light-up aptamer

RNA aptamer enhances the fluorescence of an otherwise practically nonfluorescent compound. Here the role of the aptamer is to stabilize the planar structure of a small molecule to prevent the dissipation of its energy through nonradiative decay pathways like heat, molecular motions, etc. So, the radiative pathway can dominate, leading to an increase in fluorescence.^[19]

Schematic representation of working principle of light-up aptamer is given in figure 5 below.

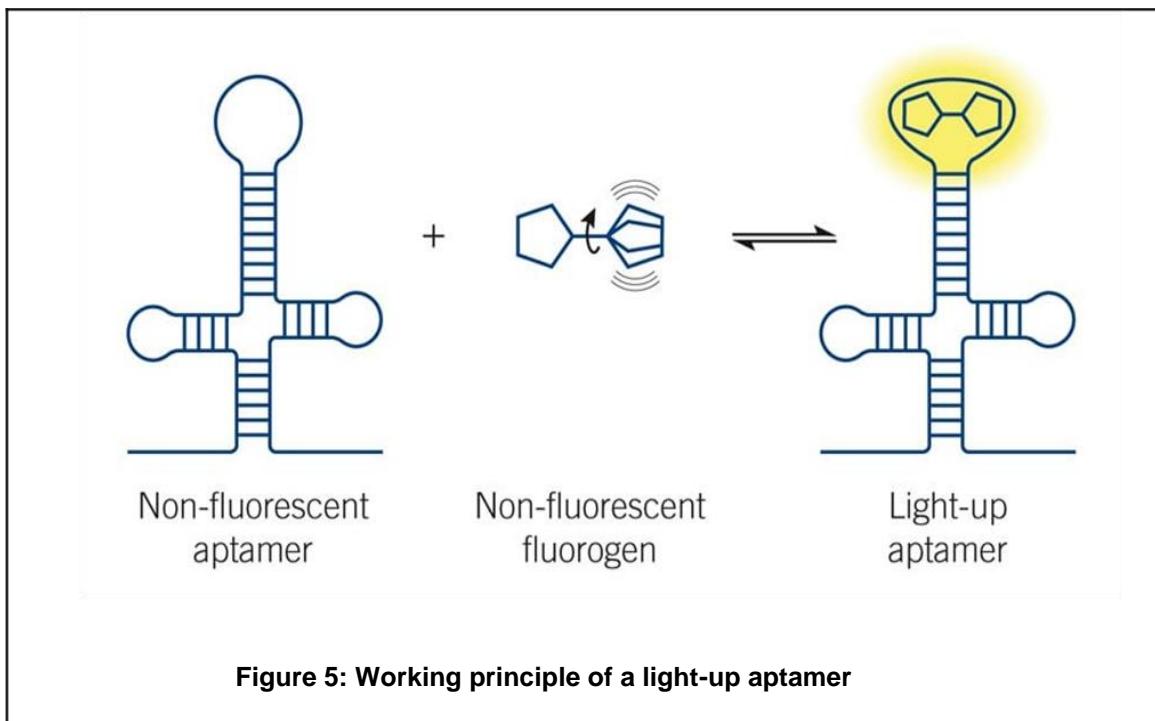


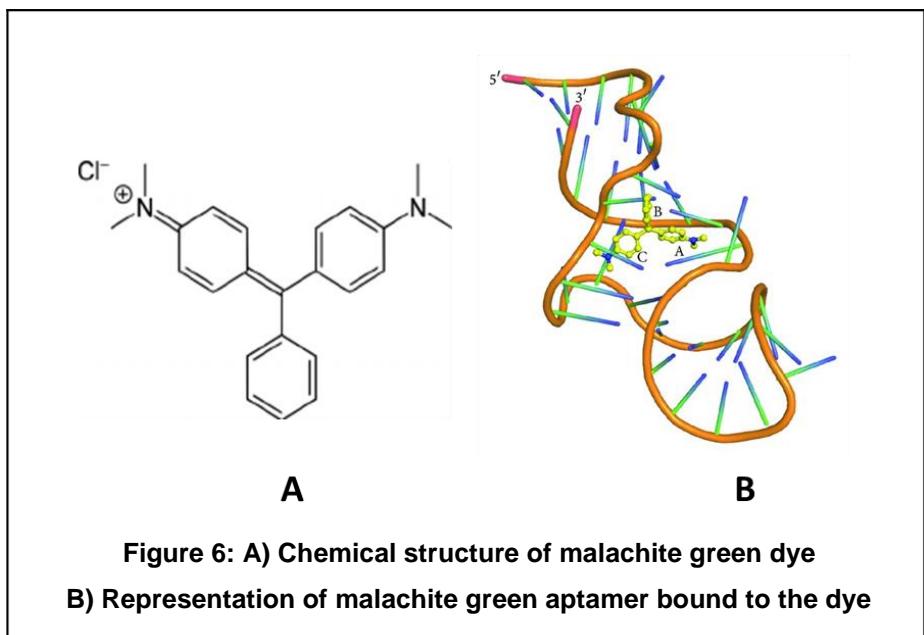
Figure 5: Working principle of a light-up aptamer

1.3.2 Examples of light-up aptamers

Some of the most commonly used Light up aptamers are discussed briefly below.

1.3.2.1 Malachite Green (MG) Aptamer

The malachite green aptamer was the first endogenously produced light-up aptamer. It is a 38-nucleotide-long RNA aptamer that specifically binds to a dye called malachite green. This aptamer was developed by Wilson et al, later in 2003, Tsien et al discovered that it could enhance malachite green's fluorescence by more than 2300-fold. The excitation and emission wavelengths of malachite green are 622 nm and 650 nm respectively. Malachite green was used to construct a light-up RNA aptamer-based sensor for imaging RNAs. Later malachite green dye was discovered to be poisonous to cells and unable to be used for in cell applications.^[21]



1.3.2.2 Spinach aptamer and its derivatives

Spinach aptamer is a 98-nucleotide-long RNA that binds specifically to an otherwise non-fluorescent green fluorophore, DFHBI (3,5-difluoro-4-hydroxybenzylidene imidazolinone), and enhances its fluorescence. The name spinach is based on its green color. Spinach aptamer was developed by Dr. Jaffrey's Group In 2011. DFHBI is a variant of the chromophore of green fluorescent protein called HBI (4-hydroxy benzylidene imidazolinone). As a result, spinach aptamer is recognized as a GFP mimic. DFHBI, like HBI, does not emit fluorescence when in its free form, but after interacting with spinach aptamer, it emits a green fluorescence that is 50% as intense as enhanced GFP (EGFP). DFHBI dye has excitation and emission wavelengths of ~450nm and ~505nm respectively.^[22]

The spinach RNA has a long structure, which consists of two helical domains. These two domains are separated by an internal bulge, which has a G-quadruplex structure as shown in figure 7. This G-quadruplex structure and surrounding nucleotides form a binding site for the fluorophore DFHBI.^[22] Also, because of its small size, DFHBI benefits from cell permeability and exhibits neither cytotoxicity nor phototoxicity. Spinach aptamer was used to tag 5S rRNA and monitor its dynamics in the human embryonic kidney (HEK) 293 T cells.^[23]

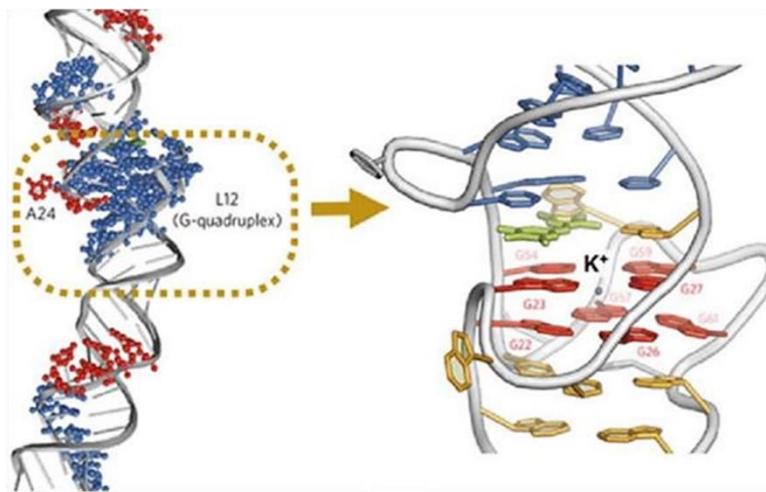
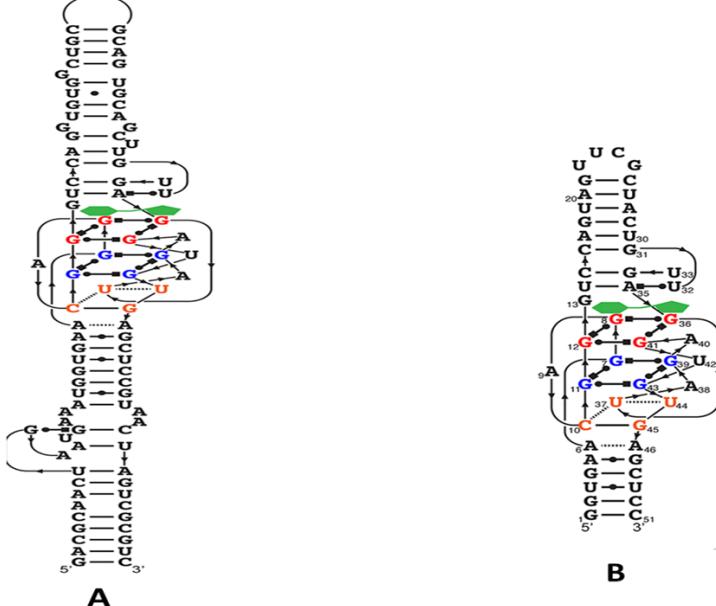


Figure 7: G-quadruplex region in spinach aptamer

To increase thermal stability and folding efficiency Spinach aptamer (98 nucleotide) was modified into its miniature versions named Baby spinach (51 nucleotides), i-spinach (69 nucleotides), etc.



**Figure 8: Secondary structure of A) Spinach aptamer
B) Baby spinach aptamer**

Baby spinach (Bspi) is a truncated version of Spinach aptamer with 51 nucleotides long RNA sequence, and it has a high mechanical stability compared to full length spinach aptamer. Baby spinach aptamer preserves the G-quadruplex in its structure

and it retains fluorescence as much as its parent aptamer spinach when bound to DFHBI.^{[24], [25]} Spinach and baby spinach aptamer are given in figure 8.

1.3.2.3 Broccoli aptamer

Broccoli aptamer is a 49-nucleotide-long RNA aptamer that binds and activates the fluorescence of DFHBI & DFHBI-1T (1,1,1-trifluoromethyl derivative of the DFHBI) dye and exhibits bright green color. It is an improved version of Spinach. Both broccoli and spinach show high in vitro fold efficiency. But broccoli showed lower dependence on magnesium for its stability.^[26] Broccoli is widely used for sensor construction and imaging in bacterial and mammalian cells.^[25]

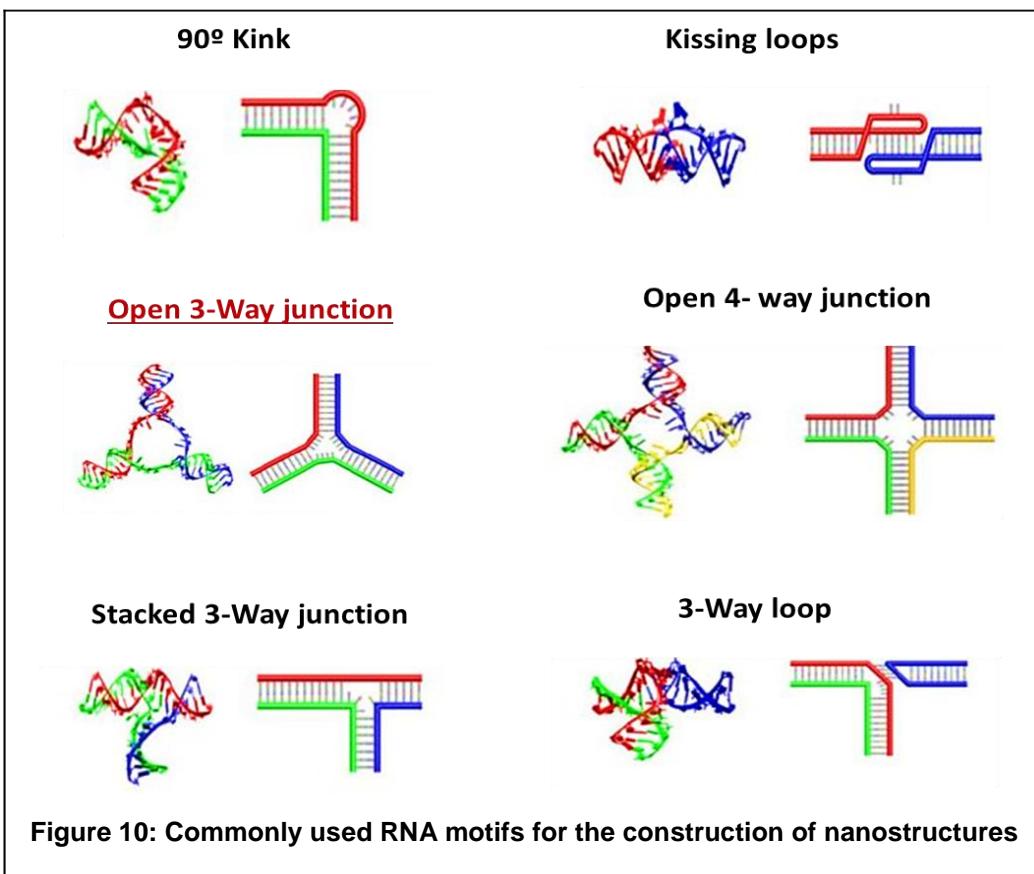
1.4 RNA Nanotechnology

RNA nanotechnology is the branch of nanotechnology that deals with the construction of different RNA nanostructures and their applications. RNA Nanotechnology has seen rapid development in the last two decades because of RNA's ability to construct a variety of nanostructures of defined size and shape. RNA nanostructures can be further decorated with a variety of functional units such as RNA aptamer, ribozyme, siRNA, and miRNA that can be used for drug delivery, siRNA delivery, targeted antigen delivery, etc.^[27]

1.4.1 Building blocks of RNA nanostructures - RNA Motifs

Various RNA motifs have been used as a platform for constructing RNA nanoparticles. These motifs contain multiple RNA-RNA Interactions. Some commonly used Motifs for RNA nanostructure construction are RNA duplex, hairpin, Kink turn, kissing loops, three-way junction, three-way loops, etc.^[27] Few commonly used RNA motifs are given in figure 9.

Among these junctions, Three-way junction (3WJ) is a highly stable and flexible RNA motif that can be used as a building blocks for the construction highly stable RNA nanostructures. So, we chose 3WJ for our study



1.4.2 Three-way junction RNA motif

RNA nanostructures have various therapeutic applications including pathogen detection, drug/gene delivery, etc. But *in-vivo* instability of nanostructures has become a hindrance to its development in therapeutics.^[5] Researchers have found that packaging RNA (pRNA) unit of bacteriophage phi29 DNA packaging motor contains a highly stable 3WJ RNA motif. This 3WJ RNA motif is resistant to 8 M urea denaturation, has a high melting temperature, and is highly stable even in low concentrations.^[5] The 3WJ motifs are one of the primary motifs used for the construction of RNA nanostructures of defined size and shape. In addition to phi29 pRNA 3WJ, numerous other stable 3WJ are also discovered. For example, both prokaryotes' and eukaryotes' 5S-ribosomal RNA contains a stable three-way junction known as 5S rRNA 3WJ.^[28]

3WJ remains functional *in-vitro* and *in-vivo*, suggesting that the three-way junction core can be used as a building block for constructing a variety of multifunctional nanoparticles.^[29]

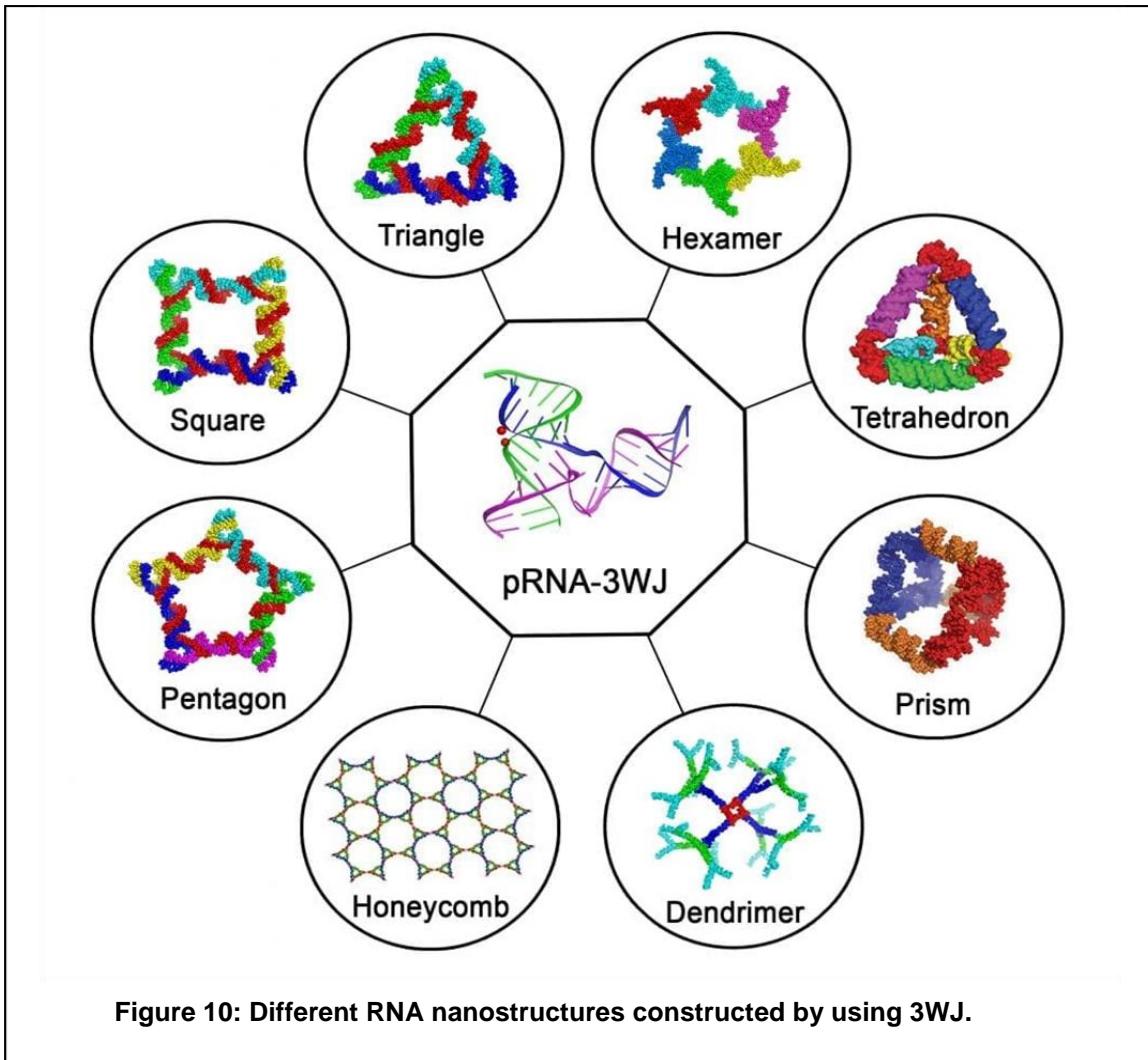


Figure 10: Different RNA nanostructures constructed by using 3WJ.

1.5 Objective and scope of the work

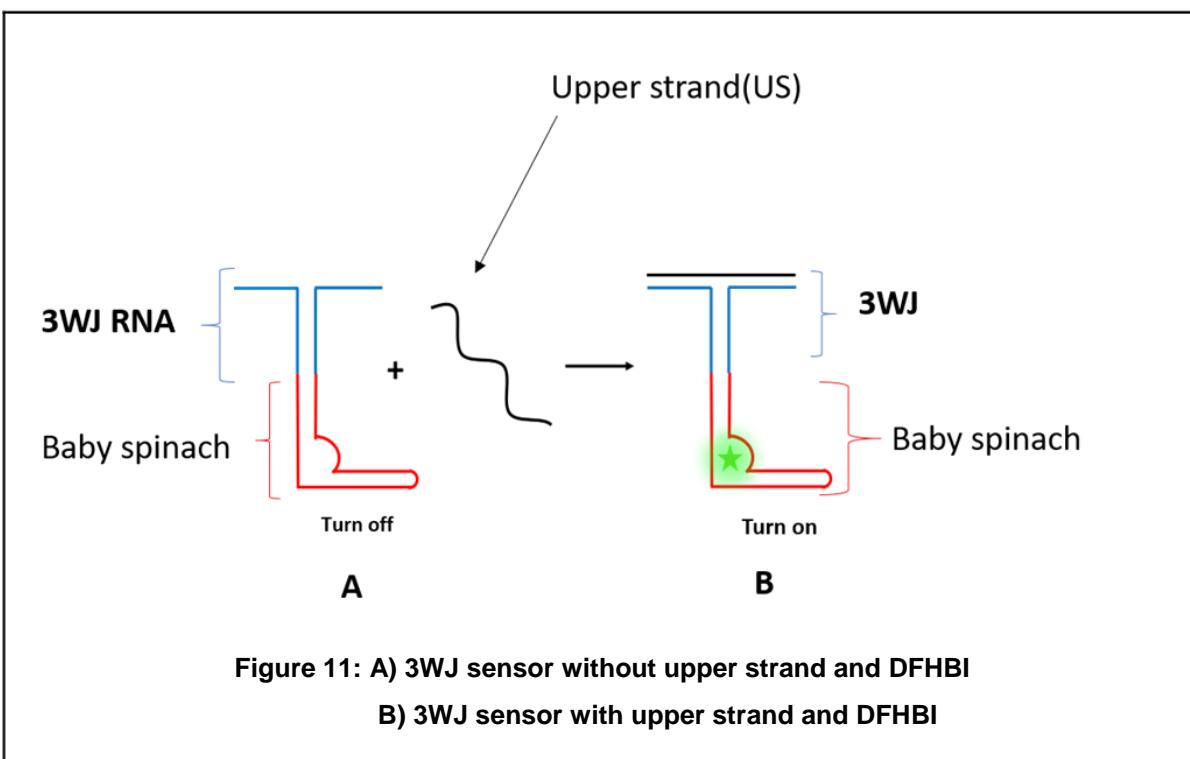
To Engineer a highly stable 3WJ for RNA nanoparticle construction

3WJ RNA motifs are one of the primary motifs for constructing RNA nanoparticles. 3WJ had been used to construct multifunctional nanoparticles of defined size and shape. RNA nano-constructs include triangles, squares (2D structures), tetrahedrons (3D designs) etc.

phi29 RNA 3WJ and 5S rRNA 3WJ are two of the stable 3WJ known. phi29 3WJ is found in packaging RNA (pRNA) of bacteriophage phi29, it shows exceptional stability and can self-assemble from three different fragments of RNA with high efficiency.^[3] And 5S rRNA 3WJ is found in 5S rRNA of both prokaryotic and eukaryotic ribosomes.^[28]

Here, we aimed to do few base modifications in these two 3WJ scaffolds (phi29 3WJ & 5S rRNA 3WJ) and to check how the stability changes according to these modifications in order to get a highly stable 3WJ. The modification includes deletion, addition, or mutation of one or more nucleotides in the core region of the 3WJ.

Earlier researchers checked the stability of different 3WJ, by comparing its thermodynamic parameters either through thermal gels or through UV optical melting that requires more time and is not high throughput. Here we are developing a high throughput method to compare stabilities of different RNA 3WJs using fluorescence method. The design strategy has been provided below (Figure 11). High thermal stability of 3WJ will lead to high fluorescence output.



**Figure 11: A) 3WJ sensor without upper strand and DFHBI
B) 3WJ sensor with upper strand and DFHBI**

In order to carry out our hypothesis we designed a sensor based on RNA light-up aptamer, baby spinach. For that, we have merged different modified 3WJs (phi29, 5s

rRNA) with few base truncated baby spinach (to minimize the background signal of the sensor). The design of the sensor is in such a way that in the presence of three strands of 3WJ it will light up and give fluorescence. In the absence of one of the strands, there will be no fluorescence or minimal fluorescence emission. Fluorescence emitted by the sensor will be directly proportional to the stability of the three-way junction, more fluorescence indicated high stability and vice-versa. This is because, in the presence of all three strands of 3WJ the secondary structure of baby spinach aptamer will restore to its original state, whereas in the absence of one of the strands the secondary structure will be disrupted, hence the change in fluorescence. So, by this method, we can easily compare the stability of different 3WJ.

Chapter 2

MATERIALS AND METHODS

All the DNA sequences used in this project were either ordered as single stranded DNA from IDT (Integrated DNA technologies) or synthesized in house by using K&A H6/H8 DNA/RNA synthesizer. Double stranded DNA templates for *in-vitro* transcription were prepared by PCR amplification using C1000 Touch™ Thermal cycler (Bio-Rad). The PCR products were purified by using a MinElute PCR purification kit (Qiagen). Nucleic acid concentrations were measured using a Nano-Drop One^c (Thermo Scientific). RNAs were in vitro transcribed using an AmpliScribe™ T7 high yield Transcription kit (Lucigen), and were purified by 8M Urea PAGE. Fluorescence measurements were recorded using microplate reader (BioTek Synergy H1). DFHBI (SML1627-5MG) and all other chemicals were purchased from Sigma and SRL.

2.1 PCR amplification

PCR (Polymerase chain reaction) is a technique used in molecular biology to make multiple copies of a segment of DNA of interest, generating a large number of copies from a small initial sample. By PCR, even a single DNA segment can be produced in millions of copies.

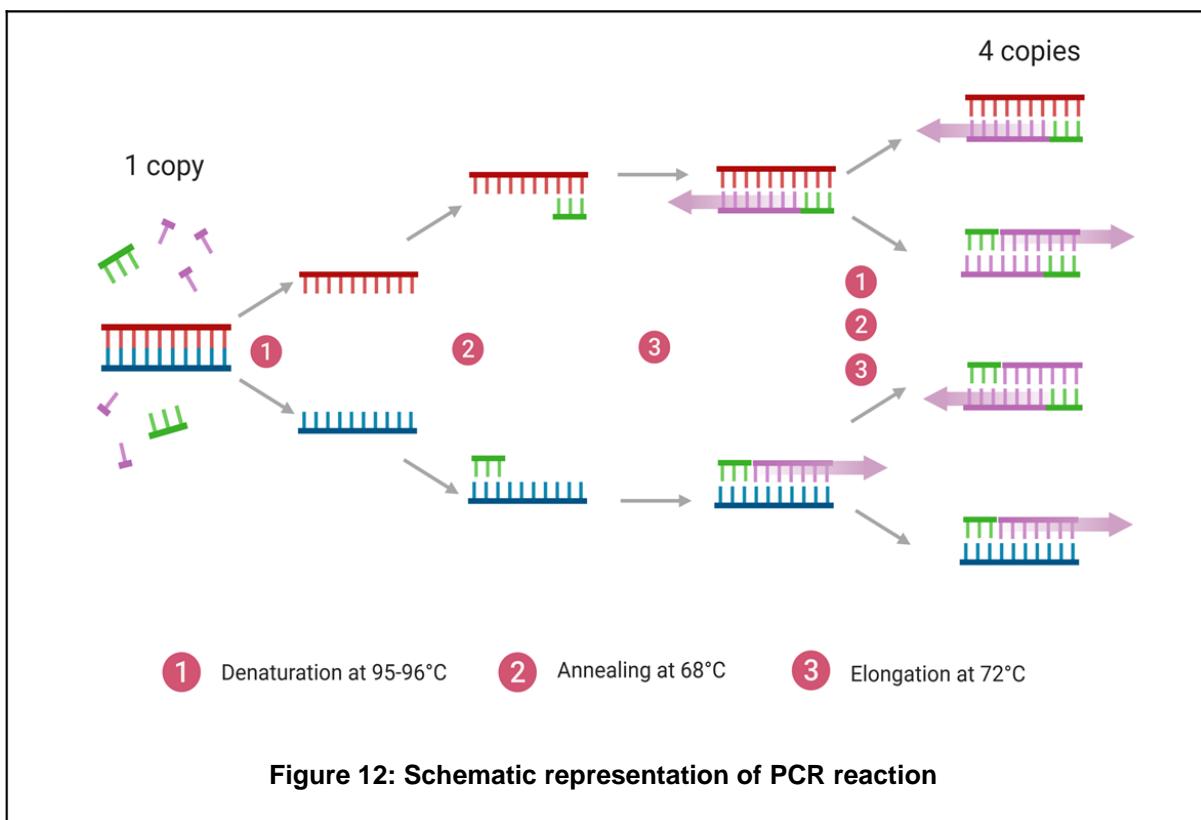
The basis for the PCR method is the enzymatic replication of DNA. Primer-mediated enzymes are used to amplify a short DNA segment or template DNA. DNA Polymerase enzyme synthesizes new strands of DNA that are complementary to the template DNA. The DNA polymerase can add a nucleotide to the 3'-OH group of an already existing nucleotide only. Hence a primer is needed for DNA polymerase to work. PCR reaction involves 3 major steps, which are briefly explained below.^[30]

Denaturation: The reaction mixture is heated to 94°C or higher temperature for 15 seconds to 2 minutes to denature it. The two intertwined strands of DNA will split from one another during the denaturation process, generating the single-stranded DNA template required for replication by the thermostable DNA polymerase.

Annealing: During this step, the temperature is lowered to 50-65°C for 20-40 seconds. At this temperature, the oligonucleotide primers (Forward primer, Reverse primer) can anneal to their complementary sequences on the denatured template DNA.

Extension: The annealing of primers provides a free 3'-OH group for the synthesis of double stranded (ds) DNA by DNA polymerase using single stranded (ss) DNA as a template. The DNA polymerase synthesises the complementary strand of the DNA template by adding nucleotides in the 5'-3' direction. Extension is usually done at 72°C.

Following the first round, the procedure is repeated by going back to the initial reaction temperature and doing the denaturation, annealing, and extension steps again. These three-step temperature cycles will get repeated. [30]



In PCR, the template can be ssDNA or dsDNA. In the case of double-stranded DNA, both forward primer and reverse primer can anneal after the separation of strands. But for ssDNA, in the first cycle of PCR, the reverse primer that is complementary to the template gets annealed at the 5' end of the template DNA and extends the template to form dsDNA. This dsDNA separates in the following cycle, enabling both primers to anneal the template and amplify.

Firstly, we performed a test PCR for all the required DNA in order to optimize the number of amplification cycles and template concentration to eliminate undesired duplexes. So, we conducted PCR of the same template DNA at five different concentrations 0, 2.5, 5, 7.5, and 10 ng/ μ l with 7 cycles and 10 cycles PCR reactions. Test PCR was analyzed by running a 1.5 % agarose gel. Among these reactions, The PCR reaction that produces the minimum undesirable bands was chosen for the scale-up PCR reaction. The reaction mixture for one PCR reaction is given below in table 1.

Reagents	Amount of volume
10X PCR Buffer	2.5 μ l
25Mm MgCl ₂	2.5 μ l
2.5mM dNTPs	1.5 μ l
10uM Forward primer	1.25 μ l
10uM Reverse primer	1.25 μ l
Taq polymerase	0.25 μ l
Template	X μ l (required volume)
DEPC Water	Y μ l (volume to make up to 25 μ l)

Table 1: PCR reaction mixture for 1 reaction (25 μ l)

2.2 Ethanol precipitation of DNA

The PCR product was ethanol precipitated by adding 10 % of the total volume of 3M sodium acetate and 2.5 times 100% ethanol. The sample was stored at -80°C for at least 4 hours for the DNA to precipitate. After incubation, the samples were centrifuged for 30 minutes at 13,000 rpm at 10 °C. The supernatant was discarded and the pellet was washed with 200 μ l of 70 % ethanol by centrifuging again for 15 minutes at 13000 rpm. Again, discarded the supernatant and kept the pellet for airdry. After complete drying, the DNA was dissolved in 100 μ l of DEPC H₂O.

2.3. In-vitro transcription (IVT)

In vitro transcription is a process that enables the template-directed synthesis of RNA molecules of any sequence, from small oligonucleotides to those of several kilobases. It is based on designing a DNA template with a bacteriophage promoter sequence upstream of it, then transcribing using the corresponding RNA polymerase. [31]

We used the Lucigen Ampliscribe kit for in-vitro transcription. The reaction mixture for transcription (Given in table 2) was prepared and incubated at 37°C for 4-5 hours. After incubation, 1 µl RNase-free DNase was added and again set for 30 minutes at 37°C. The sample was then kept for ethanol precipitation overnight at -80°C. After precipitation. The next day, the sample was taken from -80°C and centrifuged at 13,000 rpm speed for 30 minutes at 10°C. The pellet was washed with 200 µl of 70% ethanol. The excess ethanol was removed by using speed vac for 5 minutes. The dried RNA was then dissolved in 20 µl of nuclease-free water. RNA was analyzed by using denaturing UREA PAGE.

Reagents	Amounts of volume
10X reaction buffer	2 µl
ATP	1.5 µl
GTP	1.5 µl
CTP	1.5 µl
UTP	1.5 µl
DTT	2 µl
Template	Around 700 ng
Ribonuclease RNA inhibitor	0.5 µl
T7 polymerase enzyme	2 µl
DEPC water	Make volume up to 20µl

Table 2: Reaction mixture of in-vitro transcription (For 20 µl reaction)

2.4. Gel electrophoresis

Electrophoresis is a technique used to separate and sometimes purify macromolecules, especially proteins and nucleic acids that differ in size, charge, or conformation. Gel electrophoresis is a technique that separates molecules based on how quickly they move through a gel under the influence of an electrical field. There are two main types of gel electrophoresis, Agarose gel electrophoresis (AGE) and Polyacrylamide gel electrophoresis (PAGE). AGE is a horizontal electrophoresis in which agarose is used as the gel matrix to separate molecules of different sizes. And PAGE is a vertical electrophoresis in which polyacrylamide is used as the gel matrix to separate molecules. Two types of PAGE generally denatured (Urea) PAGE and

native PAGE are routinely used in our lab. Urea PAGE is a denaturing gel which uses urea as a denaturing agent but in native PAGE, dsDNA or RNA is not denatured but are separate under native conditions.

In gel electrophoresis different gel percentage are used for the separation of different sized molecules. i.e. percentage of gel will depend upon the size of our sample. For smaller sized molecule we need to use higher percentage gel because it migrates faster in gel.

2.4.1 Agarose gel electrophoresis (AGE)

Agarose gel electrophoresis is routinely used to determine the presence, purity, and size of PCR products. it is a convenient analytical method for separating DNA fragments with sizes ranging from 70 bp to 25 kb. For smaller-size DNA native PAGE is preferred over AGE.

For DNA having a size around 100 bp 1.5 % agarose can be used. 1.5 % Agarose gel is prepared by adding 1.5 g of agarose in 100ml 1X TAE (Tris-Acetic acid -EDTA) buffer. Heat the mixture of agarose and TAE buffer until the agarose is completely dissolved. Cool it a little, add 1 μ l of EtBr, carefully pour it into the gel caster, and then wait for the gel to solidify.

A 1.5% agarose gel was run at 80 V for 60 minutes to analyse the PCR DNA both before and after purification, along with a template-less negative control. The samples were prepared and loaded in the gel with a DNA ladder and length control to confirm the size and purity.

2.4.1.1 Sample Preparation and gel imaging for AGE

For making samples for agarose gel, we took 5 μ l of PCR sample, and 1 μ l of 6X loading dye was added to it. In the case of column purified DNA, we took around 100ng of DNA, made the volume up to 5 μ l with DEPC (Diethyl pyrocarbonate) water, and then added 1 μ l of 6X loading dye. Similarly, prepared the sample for size standard also. Then loaded the samples in the gel along with the size standard. Applied the necessary voltage and time to the gel. Scanned the gel using a Gel-doc or Amersham Typhoon gel scanner after the run is complete.

2.4.2 Native PAGE

Sometimes we used native PAGE to check the purity of PCR products. Native PAGE is also used to confirm the annealing of two or more DNA/RNA sequences.

An 8 % native PAGE gel is made as follows. Take 2 ml of 40% acrylamide solution in a beaker, then 1 ml 10X TBE (Tris-Borate-EDTA) buffer and 6.9 ml Milli-Q water were added along with 90 μ l of 10% APS (ammonium persulfate) and 10 μ l of TEMED (N, N, N', N'-tetramethyl ethylene diamine). Mix the solution well and cast the gel. Here APS and TEMED helps to catalyze the PAGE's polymerization reaction and harden the gel.

2.4.2.1 Sample Preparation and gel imaging of Native PAGE

Sample preparation for native PAGE is the same as agarose gel (Explained in section 2.4.1.1). Before loading the samples clean the wells of the gel by using a syringe with 1X TBE buffer (Running buffer). Load the samples in the wells along with the size standard. Run the gel at the required voltage and time. Once the running is over gently take the gel out from the caster and put it in staining (EtBr in1X TBE) solution for 10-15 minutes to stain the DNA. After that, scan the gel by using the Amersham Typhoon gel scanner.

2.4.3 Urea PAGE

Urea PAGE, also known as denaturing urea polyacrylamide gel electrophoresis, uses 6-8 M urea to denature secondary DNA or RNA structures and separates them depending on their molecular weight in a polyacrylamide gel matrix. We usually use 8M Urea PAGE to determine the size and purity of the transcription product. The percentage of gel will depend upon the size of our transcription product. For RNA having a size of more than 50 bases we use 8% urea, and for smaller RNA we usually use 16 % gel.

The following steps are used to prepare the 8% urea page. 10 ml of the 8% Urea PAGE solution, which contains 8M Urea, is added into a beaker. Then, 90 μ l of the 10% APS and 10 μ l of the TEMED were added to the beaker. Mixed the solution well and cast the gel. Similar way we can prepare 16% Urea gel.

2.4.3.1 Sample Preparation and gel staining for Urea PAGE

For making samples for analysis gel, 1 μ l of transcription product is taken and 9 μ l of nuclease-free water is added to make 1: 10 dilution. 1 μ l and 3 μ l of the 1:10 dilution is taken in two vials, made volume up to 5 μ l with nuclease-free water, and 5 μ l of 2X Denaturing dye is added to it. Then the samples were heated at 85°C for 5 minutes and cooled it down by keeping the samples on the ice for the next 5 minutes. Similar way prepared samples for size standard also. Before loading the samples, the wells were cleaned by using a syringe with 1X TBE buffer (Running buffer) to ensure that the urea concentration in each well was the same. Loaded the samples in the wells along with the size standard. Run the gel at the required voltage and time. The gel was gently taken from the caster when the running was over, and it was kept in staining (Etbr in 1X TBE) solution for 10-15 minutes to stain it. After that, the Amersham Typhoon gel scanner was used to scan the gel. We also used DFHBI staining if the RNA samples are light-up aptamers.

2.5 Purification of DNA

For PCR DNA purification, a MinElute PCR Purification Kit from Qiagen was used. The protocol for DNA purification is explained below.

First, add five times (500 μ l) of buffer PB (binding buffer) to the 100 μ l PCR sample. Then transfer it to the MinElute column. Centrifuge the column for one 1 Min 30 Sec and discard the flow through. Then, add 750 μ l of buffer PE (wash buffer) and Centrifuge for 1 min, discard flow through, do an empty spin for 1 min, and discard flow through again. Transfer the column to a fresh 1.5 ml collecting tube. Add 10 μ l DEPC water to the centre of the MinElute column and elute the DNA by centrifuging it for 1 min. Elute once more with 10 μ l of DEPC water. After purification, we can check the concentration and yield of DNA by using a Nanodrop spectrophotometer. By running an agarose gel or native PAGE, we can determine whether the PCR product is pure.

2.6 Purification of RNA

Two different techniques—the crush-soak method and ZYMO kit purification were used to purify RNA. The crush and soak method is typically used to purify RNA that

shows multiple bands in addition to the target band in the analysis gel. But if RNA is showing only one band in the analysis gel then the purification was done using ZYMO purification kit.

2.6.1 ZYMO column purification of RNA

The ZYMO purification kit is typically used to purify RNA that shows only one band in the analysis gel. The following protocol is used for ZYMO column purification.

In the first step, add 40 µl of RNA Binding Buffer to 20 µl of the in vitro transcription reaction (ethanol precipitated). Then add 60 µl of 100% ethanol (three times the volume of transcription product) to it. Mix it well by vortexing. Then transfer the entire volume to the spin column that was included with the ZYMO kit. Centrifuge the column for 1 minute at 13,000 rpm and discard the flow through. Then add 400 µl of RNA Prep Buffer and Centrifuge for 1 minute at 13,000 rpm, and discard the flow through. After that add 700 µl of RNA Wash Buffer and Centrifuge for 1 minute at 13,000 rpm, and discard the flow through. Again, add 400 µl of RNA Wash Buffer and Centrifuge for 1 minute at 13,000 rpm, and discard the flow through. Then give an empty spin for 2 minutes and remove all the wash buffer left. Now take a fresh 1.5 ml tube and transfer the column to it. Add 20 µl DEPC water to the centre of the column and Centrifuge for 1 minute at 13,000 rpm. Again, elute the RNA with 20 µl more DEPC water and Centrifuge for 1 minute at 13,000 rpm. Now we can check the concentration of purified RNA by using a nanodrop spectrophotometer.

2.6.2 Crush and Soak method for RNA Purification

If RNA is having multiple bands, then the crush and soak method is used to purify it. In this method, First, we will run the entire RNA which is needed to purify in a Urea PAGE gel. For that first cast a Urea PAGE purification gel by using a 1.5 mm spacer comb as it gives more thickness which was helpful while cutting tiny pieces from the gel. Mix the entire volume of RNA with the same volume of 2X Denaturing Dye. Heat it at 85°C for 5 minutes and snap cool for another 5 minutes on the ice. And load the RNA samples in Urea PAGE gel and run it for enough voltage and time so that our required RNA band will come around the middle of the gel. If the gel is running for more time keep the entire gel system on an ice tray to reduce the effect of heat generated during electrophoresis. Gently take the gel out once the running is

completed, and place it on a TLC plate. Use a UV handheld device for visualization of RNA in the gel. From the gel carefully cut the band of interest (Our required RNA band). Crush this gel fraction well and take it in a 1.5 ml tube. Add 1 ml of elution buffer (0.5 M Ammonium acetate, 0.1 mM EDTA, and 0.1% SDS) to it and keep it at 37 °C for overnight incubation. After completion of incubation time, take elution buffer out avoiding gel pieces, and then do the ethanol precipitation. After that centrifuge the sample for 30 minutes at 13,000 rpm at 10 °C. Discard the supernatant and keep the pellet for airdry. After complete drying dissolve the pellet in 40 µl of nuclease-free water. Now the RNA will be pure and we can confirm it by running one more Urea PAGE.

2.7 NUPACK analysis

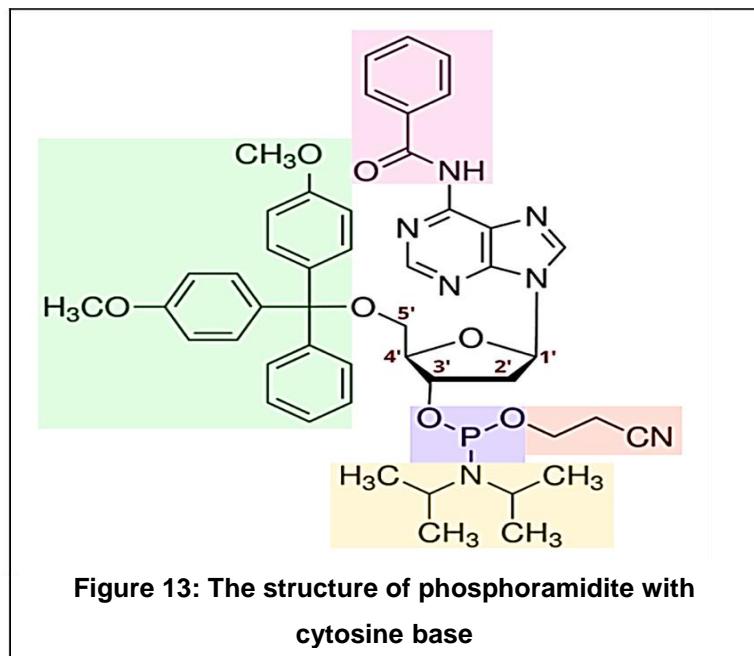
The Nucleic Acid package (NUPACK) software program is used to analyze and design and structure of nucleic acid systems.^[33]

2.8 Oligonucleotide DNA/RNA Synthesis

DNA/RNA sequences required for all the experiments were chemically synthesized in our lab by using H6/H8 DNA-RNA Synthesizer. Here the Oligonucleotides are synthesized using the solid phase synthesis method. As the name implies, solid phase synthesis is carried out on a solid support, from which the synthesized oligonucleotide has to be cleaved. In this method, the 3' hydroxyl group of sugar molecule is covalently attached to the solid support material known as resins which will allow all reagents to freely pass-through columns. Controlled pore glass (CPG) and polystyrene, which are insoluble particles, are the two most commonly used solid support materials.^[32]

Commercially available Phosphoramidites are used in solid-phase synthesis as the building blocks for oligonucleotides. A phosphoramidite ((RO)₂PNR₂) is a modified nucleoside containing a phosphite ester group. Except for thymine, each base possesses an exocyclic NH₂ group that functions as a nucleophile and that is protected by using a benzoyl or other group. Diisopropylamine and 2-cyano ethane are attached to the phosphorus through N- and O-linkage that is further connected to 3' C of a sugar molecule where one nucleotide binds to another nucleotide via a phosphodiester link (Figure 13). The Dimethoxytrityl (DMT) group is used as a protecting group of the

hydroxyl group at the 5' position of the sugar molecule. The nucleotide with all these protecting groups is called a nucleoside phosphoramidite molecule. In contrast to DNA biosynthesis, which is carried out from 5'-3' oligonucleotide biosynthesis is carried out from the 3'-5' direction.



A cycle of oligonucleotide synthesis involves the following steps as shown in Figure 14.

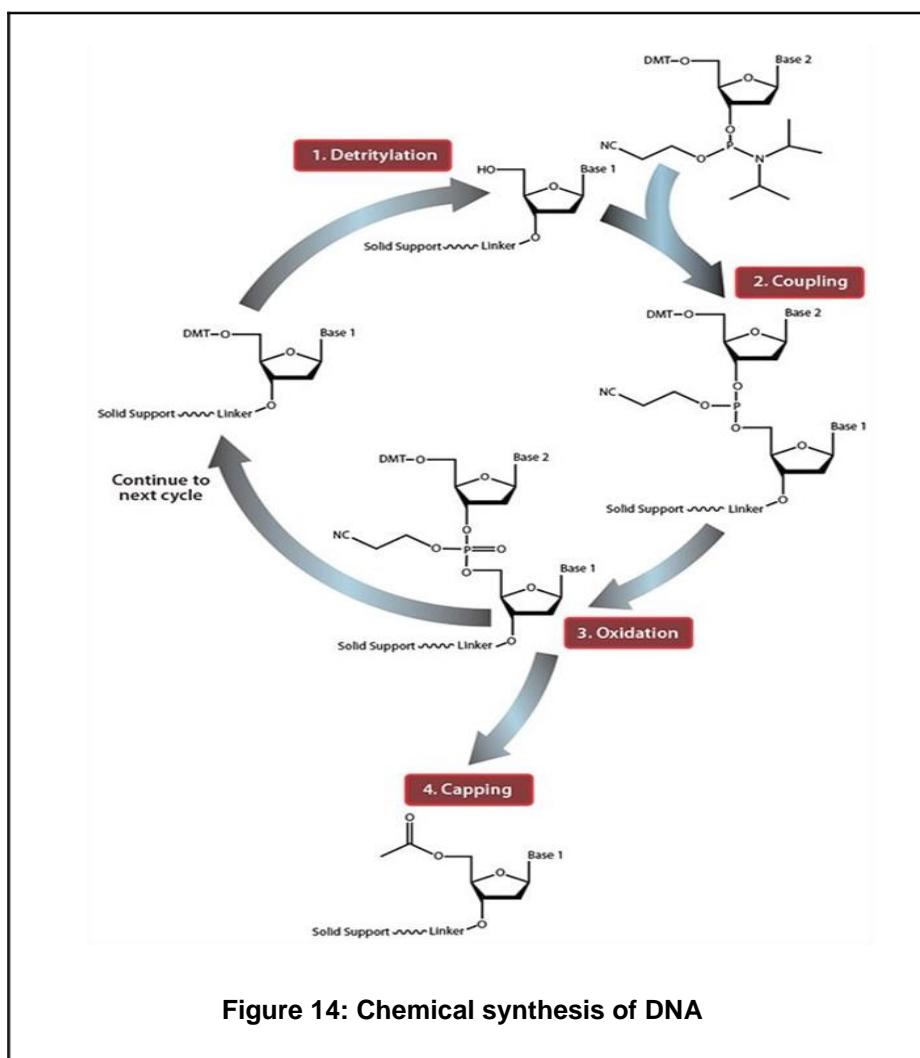
Detritylation: The DNA/RNA synthesis cycle is initiated by the removal of the 5'-DMT protecting group by TCA (trichloroacetic acid) from the first solid-support-linked nucleoside. During the functionalization of the solid support resin, the 5'-DMT prevents the nucleoside from polymerizing.

Coupling: when the DMT is removed, free 5'-OH of the solid-support-linked nucleoside can react with the next nucleoside, which is added as a phosphoramidite monomer. This process is called a coupling reaction. A coupling reaction results in the formation of a dinucleoside with a phosphite triester linkage and a free diisopropylamine group.

Oxidation: Prior to the beginning of the following cycle, the unnatural and unstable phosphite triester that results from the coupling reaction must be changed into a more

stable phosphorus species. The phosphite triester is transformed into the stable phosphate triester by oxidation.

Capping: 100% coupling efficiency is not possible. Some solid-support-linked nucleosides may have unreacted 5'-OH. These hydroxyl groups will react during the following cycle if they are not inhibited or blocked, which will result in a missing base. During repeated rounds, the accumulation of these deletion mutations would result in a complicated mixture of "shortmers" that are challenging to purify, which may make the oligonucleotide unusable. In order to avoid shorter accumulation, capping of the unreacted 5'-OH is necessary. Capping results in the formation of the solid-support-linked nucleoside with an acetylated 5'-OH.^[32]



The second cycle starts with step 1, detritylation, followed by the next three cycles coupling, oxidation, and capping. The number of cycles repeated equals the required

number of bases. In our lab, we synthesize oligonucleotides from 15 to 100 bases. Schematic representation of chemical synthesis of DNA is given in figure 14.

2.8.1 Resin cleavage

Once the synthesis process is completed, we need to separate the solid support/linker from the oligonucleotide and also cleave cyanoethyl linkage. Cleavage is necessary to free up the 3'-OH group since they may take part in biochemical reactions, such as DNA polymerase extension during PCR, which uses the oligonucleotide as a primer. Both cleavage and removal of cyanoethane is done using ammonium hydroxide.

After synthesis is complete, remove the columns and separate the synthesized sequences that are sandwiched between solid support. Add 1.5 ml of the AMA (aqueous Ammonium hydroxide + Methylamine) solution in a 1:1 ratio. Incubate the samples at 65°C for 1 hour 30 minutes and then keep them on ice for 30 minutes. Centrifuge the samples to settle down the resin. Collect the AMA solution in a fresh 2 ml tube avoiding resin. Wash resin with 2 x 100 µl of DEPC water and add in AMA solution avoiding resin. Evaporate the AMA solution completely using a speed vac. If the sample is DNA after completely evaporating the AMA solution, dissolve the DNA in 1 ml nuclease-free water.

2.8.2 TBDMS deprotection in RNA synthesis

RNA 2'-OH is generally protected with TBDMS groups and they need to be removed after cleavage from resin. After evaporating the AMA solution completely, add 250 µl of DMSO (Dimethyl sulfoxide) to the dried RNA above. Heat at 65 °C for 5 minutes and vortex to dissolve the pellet completely. Add 250 µl of TEA.3HF (Triethylamine trihydrofluoride) and incubate for 2.5 hrs at 37°C. Then neutralize the sample by adding 1.5 M Ammonium carbonate drop by drop around 1.5 ml. Later ethanol precipitate the sample.

2.8.3. DNA/RNA Purification by using the Glen-Pak column

The procedure for the Glen-pack column purification is given below.

First, remove the top cap of the Glen-pack column first and then the bottom cap, otherwise, the bed will disturb. Wash the column with 25 ml of water. Wait till the flow

stops. Then Load exactly 1 ml of DNA/RNA sample, do not collect the fraction. Add 1.5 ml of water, do not collect this fraction. Then Elute with 500 μ l water and collect this fraction (Fraction 1). Next elute with 2 ml water and collect this fraction in a 2 ml tube (Fraction 2). Again elute with 2 x 500 μ l water and collect the fractions (Fractions 3 & 4). Check the nanodrop of Fractions 1-4. Discard the fraction with a nanodrop reading of less than 20 ng/ μ l. If the nanodrop of fraction 4 is more than 100 ng/ μ l, do one more 500 μ l elution with water. Mix all fractions. Nanodrop it. Run a gel to check the purity of the DNA/RNA sample and store it in the refrigerator. Finally, wash the column with 10 ml water.

2.9 Fluorescence Studies by using Microplate spectrophotometer

A Microplate spectrophotometer also called a microplate reader is an instrument used to detect chemical, biological, or physical reactions by measuring emitted light. A microplate reader can measure the light signal of the sample in relative fluorescence units. We used 96 well plate fluorescence microplate reader (Synergy H1 microplate reader) for checking the fluorescence measurements of aptamer-based sensors.

An example of sample preparation for a fluorescence experiment is given below. Add 20 μ l 5X sensor buffer (50mM Tris-HCl, 500mM KCl, 50mM MgCl₂) to a PCR tube along with 2.5 μ l of 10 μ M sensor RNA (0.25 μ M) and 2.5 μ M DFHBI which is 10 times more concentrated than the sensor RNA. Add nuclease-free water to it to make the total volume 100 μ l. Transfer the prepared samples to a 96-well plate clear bottom with a black side and incubated for 30 minutes. After incubation, measure the fluorescence intensity using an excitation wavelength of 460 nm and an emission wavelength of 505 nm. By directly dividing the Sample's fluorescence with the control samples, the fold change in fluorescence intensities was calculated. All the fluorescence experiments were triplicated, and the data from three independent experiments were averaged and plotted using MS Excel or origin.

Chapter 3

RESULTS AND DISCUSSION

Nanotechnology has great importance in all areas of our life, especially in the field of medicine and therapeutics. Many different 2D and 3D structures can be made out of RNA using bottom-up RNA nanotechnology techniques. RNA three-way junction (3WJ) is an important structural motif for the stability of RNA nanostructures. A 3WJ derived from Phi29 bacteriophage named Phi29 3WJ had been used a lot in literature for the construction of highly stable RNA nanoparticles. In addition, 5S-rRNA 3WJ (5S-3WJ) from 5S-ribosomal RNA is also an important junction that can be used in the construction of RNA nanoparticles. More stable new junctions are always needed for the stability of the RNA nanostructures. however, no strategy currently exists that can be used to compare the stability of different 3WJs in a high-throughput way.

To compare the stabilities of different 3WJs, we used the strategy by designing a fluorescent sensor in which a baby spinach aptamer is attached to the core of the 3WJ, and its arms are extended with a stable duplex (see section 1.5). The baby spinach aptamer binds to a nearly non-fluorescent molecule DFHBI and enhances its fluorescence by several folds. The stability of the 3WJ core correlates with the fluorescence output of the baby spinach aptamer. i.e., higher stability of 3WJ will be indicated by more fluorescence, and vice versa.

3.1 Objective 1

Our first objective was to compare the stability of a 5S-3WJ (Figure 15 A) by using the above-mentioned strategy and comparing it with commonly used Phi29-3WJ (Figure 15 B)

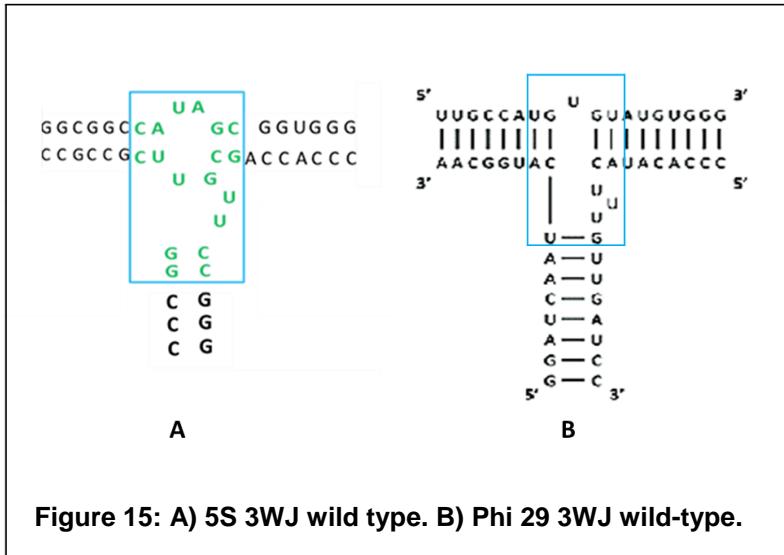
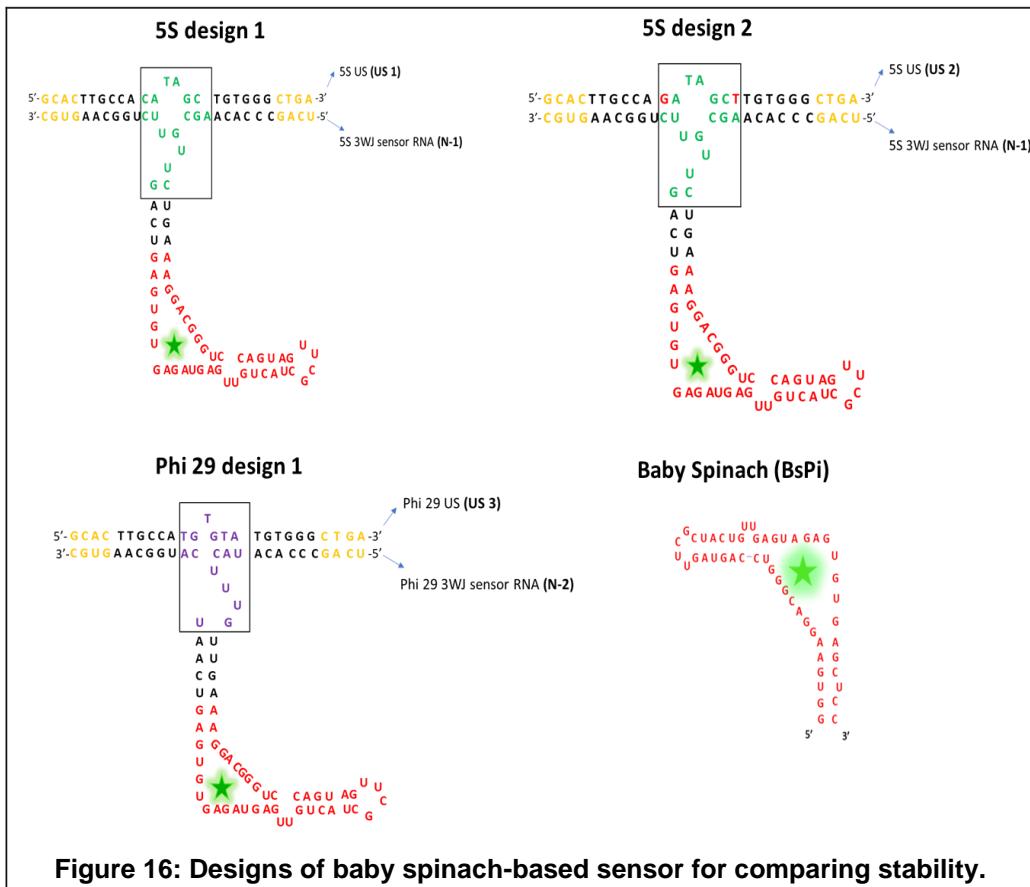


Figure 15: A) 5S 3WJ wild type. B) Phi 29 3WJ wild-type.

For this, the core of the wild type 5S-3WJ and Phi29-3WJ (shown in the cyan box in figure 15) was inserted into a common RNA scaffold having a baby spinach aptamer at one end (shown in red in different designs of Fig 16). The upper strand of 5S-3WJ was also modified to include base pairing with the lower two strands which was thought to increase 5s-3WJ's stability (shown in red in US-2, 5s-design 2, Fig 16)



Here we used DNA as the upper strand instead of RNA because the stability of the DNA-RNA hybrid is assumed to be similar to the RNA-RNA hybrid. The upper strands (US-1, US-2, and US-3) were synthesized by using a DNA synthesizer, while the longer lower strands (N-1 and N-2) were transcribed using PCR and in vitro transcription. The baby Spinach (BsPi) RNA was used as positive control for fluorescence assays.

3.1.1 Synthesis of baby spinach RNA

3.1.1.1 Sequence design

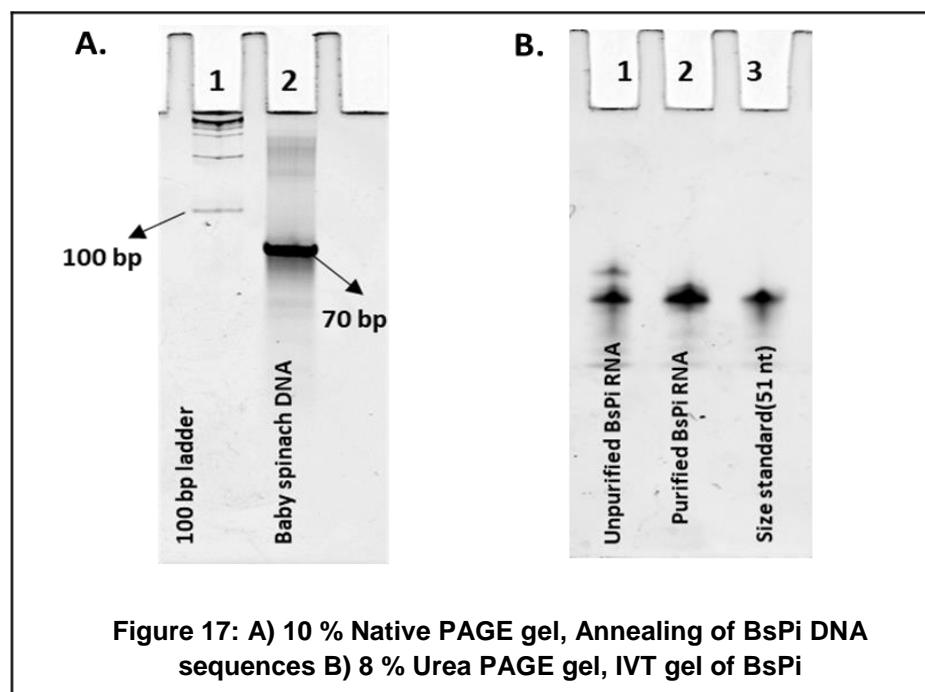
Name of DNA	Sequence (5'-3')	Length (nt)
BsPi DNA +T7 promotor	TAA TAC GAC TCA CTA TAG GTG AAG GAC GGG TCC AGT AGT TCG CTA CTG TTG AGT AGA GTG TGA GCT CC	70
(BsPi DNA +T7 promotor) comp	GGA GCT CAC ACT CTA CTC AAC AGT AGC GAA CTA CTG GAC CCG TCC TTC ACC TAT AGT GAG TCG TAT TA	70

Table 4: sequence details of baby spinach + T7 promotor DNA and its complimentary sequence.

To make baby spinach RNA, we designed the baby spinach DNA sequence which was extended with the T7 promotor sequence (marked in bold) and its complementary sequence. (Sequence details are shown in table 4). Here, the T7 promotor region is required for the recruitment of the T7 polymerase enzyme during transcription. Both sequences of baby spinach were taken in an equimolar ratio in TMS (40 mM Tris-HCl, 10 mM MgCl₂, and 100 mM NaCl) buffer. And they were annealed by heating at 95°C for 5 minutes and then slowly cooled down at the rate of 5 °C per minute by using a thermal cycler. After annealing, the product was analyzed with 10 % Native PAGE run at 100 V for 40 minutes to confirm annealing. The analysis gel is given in figure 17-A that shows the dsDNA after annealing to be of right length (70 bp).

3.1.1.2 Synthesis and purification of baby spinach RNA

The annealed product was used to carry out in-vitro transcription. A 40 μ L reaction was conducted by using the Lucigen Ampliscribe transcription kit. The crude transcription product was subjected to ethanol precipitation. Then analyzed with an 8% Urea Page. It was observed that the transcript was having two bands, so the desired band which matched with size reference was purified using the crush and soak method. The purified RNA was then analyzed by 8% Urea Page, given in Fig 17 B.



The concentration of purified baby spinach was determined by using a nanodrop spectrophotometer and the yield from a 40 μ l reaction was 14.4 μ g. Baby spinach RNA was pure and can be used for further experiments.

3.1.2 Synthesis of N-1 and N-2 RNA sequences

3.1.2.1 Test PCR and scale-up PCR of N-1 and N-2

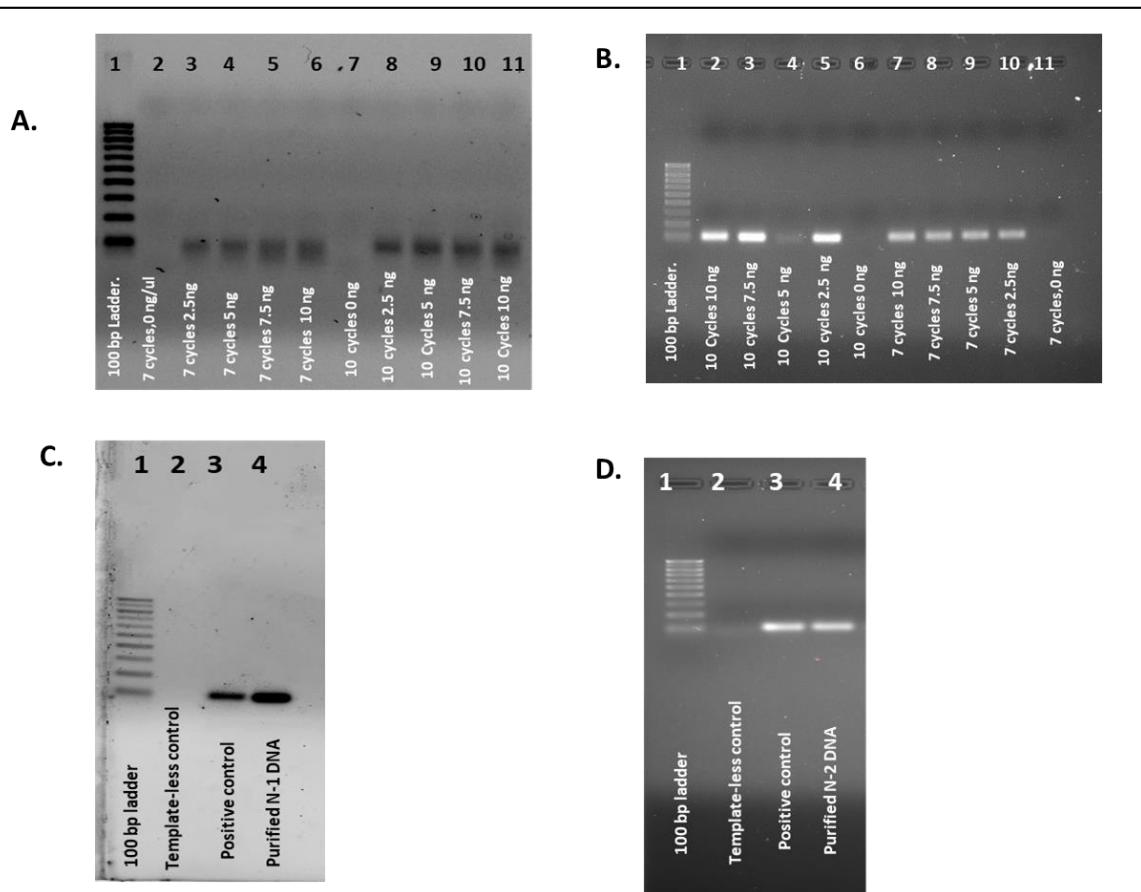
To make the N-1 and N-2 sequences, DNA templates, forward primer, and reverse primer needed for a PCR process were designed. Sequence details are given in table 5 below.

Name of the DNA	Sequence (5'-3')	Length (nt)
N-1 Template	TCA GCC CAC AAG CGT TCC TGA AAG GAC GGG TCC AGT AGT TCG CTA CTG TTG AGT AGA GTG TGA GTC AGG TTC TGG CAA GTG C	81
N-1 FP	TAA TAC GAC TCA CTA TAG GTG A TCA GCC CAC AAG CGT TCT TGA	40
N-1 RP	GCA CTT GCC AGA ACT TGA CTC	21
N-2 Template	TCA GCC CAC ATA CTT TGT TGA AAG GAC GGG TCC AGT AGT TCG CTA CTG TTG AGT AGA GTG TGA GTC AAT CAT GGC CAA GTG C	82
N-2 FP	TAA TAC GAC TCA CTA TAG GTG A TCA GCC CAC ATA CTT TGT TGA	40
N-2 RP	GCA CTT GGC CAT GAT TGA CTC	21

Table 5: DNA sequences required for making N1 and N2

In order to amplify sequences N-1 and N-2, we had to optimize the number of amplification cycles and template concentration to eliminate undesired duplexes. For that test PCR of both sequences was conducted at five different concentrations of DNA templates (0, 2.5, 5, 7.5, 10 ng) with both 7 and 10 cycles. Products were analyzed by running a 1.5 % agarose gel in 100 v for 40 min. Here 2.5 ng, the 7-cycle reaction showed the lowest intensity of the undesired duplexes, compared to the band of interest (Figure 18 A & B). So, proceeded to scale up PCR with the same.

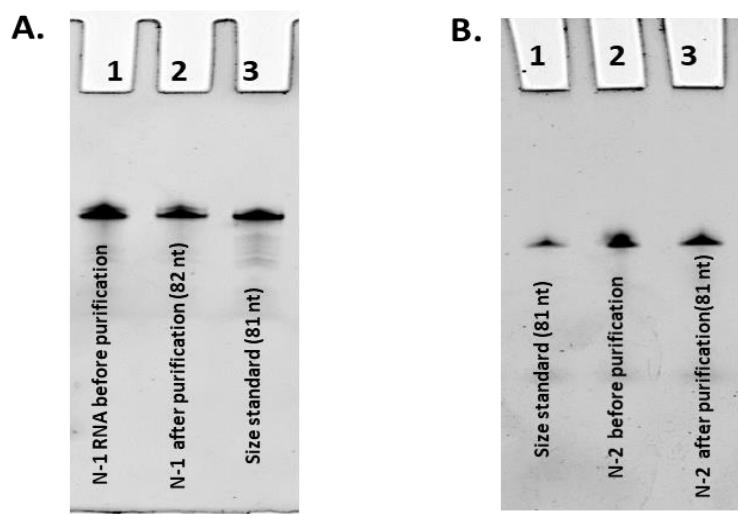
After test PCR, scale-up PCR (400 μ l PCR reaction) of N-1 and N-2 was carried out with 7 cycle reaction and template concentration of 2.5 ng. The crude amplified products of scale-up PCR were ethanol precipitated and purified by using a Qiagen Min Elute PCR purification column. Purified DNAs were eluted in 20 μ l DEPC water and analyzed with 1.5 % agarose gel (Figure 18 C & D). The concentration of DNA sequences was determined by using a nanodrop spectrophotometer and a yield of 5.78 μ g was obtained for N-1 and 4.8 μ g was obtained for N-2.



**Figure 18: A & B - 1.5% Agarose gel -Test PCR gel of N-1 and N2 respectively
C & D - 1.5 % agarose gel- scale up PCR gel of N-1 and N2 respectively**

3.1.2.2 RNA Synthesis of N-1 and N-2 by IVT

Scale-up PCR products were used to carry out in-vitro transcription. The Lucigen Ampliscribe transcription kit was used to carry out a 40 μ L transcription reaction. Transcription products were subjected to ethanol precipitation and then analyzed with 8% Urea PAGE. From the gel, we analyzed that the transcripts were having a single band which also matched the size standard. So, we purified both RNA sequences by using ZYMO column purification. The purified products were then analyzed with another 8% Urea Page. Analysis gel and purification gels are given in the figure 19.



**Figure 19: A – 8% Urea PAGE, transcription gel of N-1
B – 8 % Urea PAGE, Transcription gel of N-2**

Both N-1 and N-2 RNA sequences were pure. The yield of the RNA was calculated and it was 24.6 µg for N-1 and 26 µg for N-2. The sequences were stored at -20⁰ C and later used for fluorescence experiments.

3.1.3 Synthesis of Upper strands (US-1, US-2, and US-3)

5S 3WJ upper strand (US-1) and 5S 3WJ upper strand modified (US-2) were synthesized by using DNA-RNA synthesizer. The synthesized sequences were then analyzed using an 8% urea PAGE. The gel is given in figure 20.

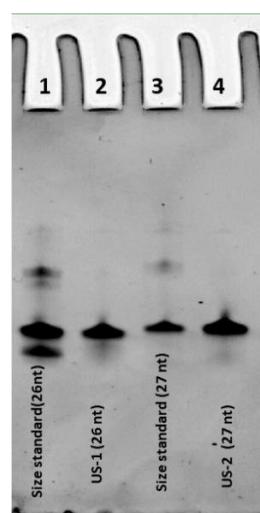


Figure 20: 8% urea PAGE, analysis gel for synthesized US-1 & US-2

Both of the synthesized sequences showed single, pure bands in the gel (lanes 2 and 4) exactly matching the size standards.

Phi 29 3WJ US DNA (US-3) was already available in the lab. That was directly used for the fluorescence experiment.

3.1.4 Fluorescence experiment to compare the stability of 3WJs

Now we have all the required sequences for the fluorescence experiment. The experiment conditions were set up for all the controls i.e., with and without the upper strand of each design to compare the fluorescence emission of both. The fluorescence analysis of 5S design 1, 5S design 2, Phi29 design 1, and baby spinach RNA were performed and their stabilities were compared based on their fluorescence output.

Fluorescence results are shown in figure 21.

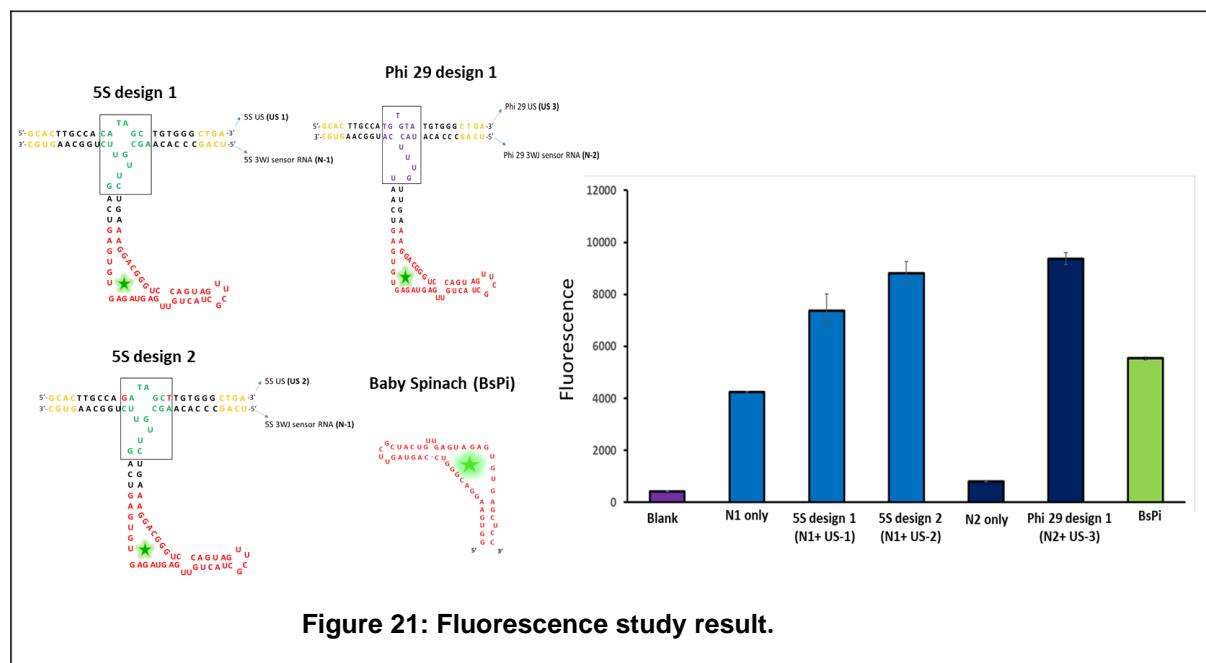


Figure 21: Fluorescence study result.

In Figure 21, 5S design 2 showed better fluorescence than 5S design 1. It indicates that the modifications made to 5S 3WJ made 3WJ more stable than the native 5S 3WJ. The graph also shows that the stability of the modified 3WJ is equivalent to that of phi29 3WJ (Phi 29 design 1). This strategy can be used to screen many modifications with high throughput. We extended this experiment by introducing more modifications to the 3WJs. This will be discussed in objectives 2 and 3.

3.2 Objective 2

Our second goal was to further increase the stability and fluorescence of both Phi 29 and 5S 3WJ using a similar strategy. For that, we further modified the core region of both Phi 29 and 5S 3WJ. In the case of 5S 3WJ, we modified 5S 3WJ with a modified upper strand (5S design 2 in objective 1) because it was having more stability than wild-type 5S 3WJ.

To achieve this, we designed five modified Phi 29 3WJ (Phi 29 M1 to Phi 29 M5) and four modified 5S 3WJ (5S M1- 5S M4). Modifications in Phi 29 3WJ designs are shown in figure 22 and modifications in the 5S 3WJ are given in figure 23 below.

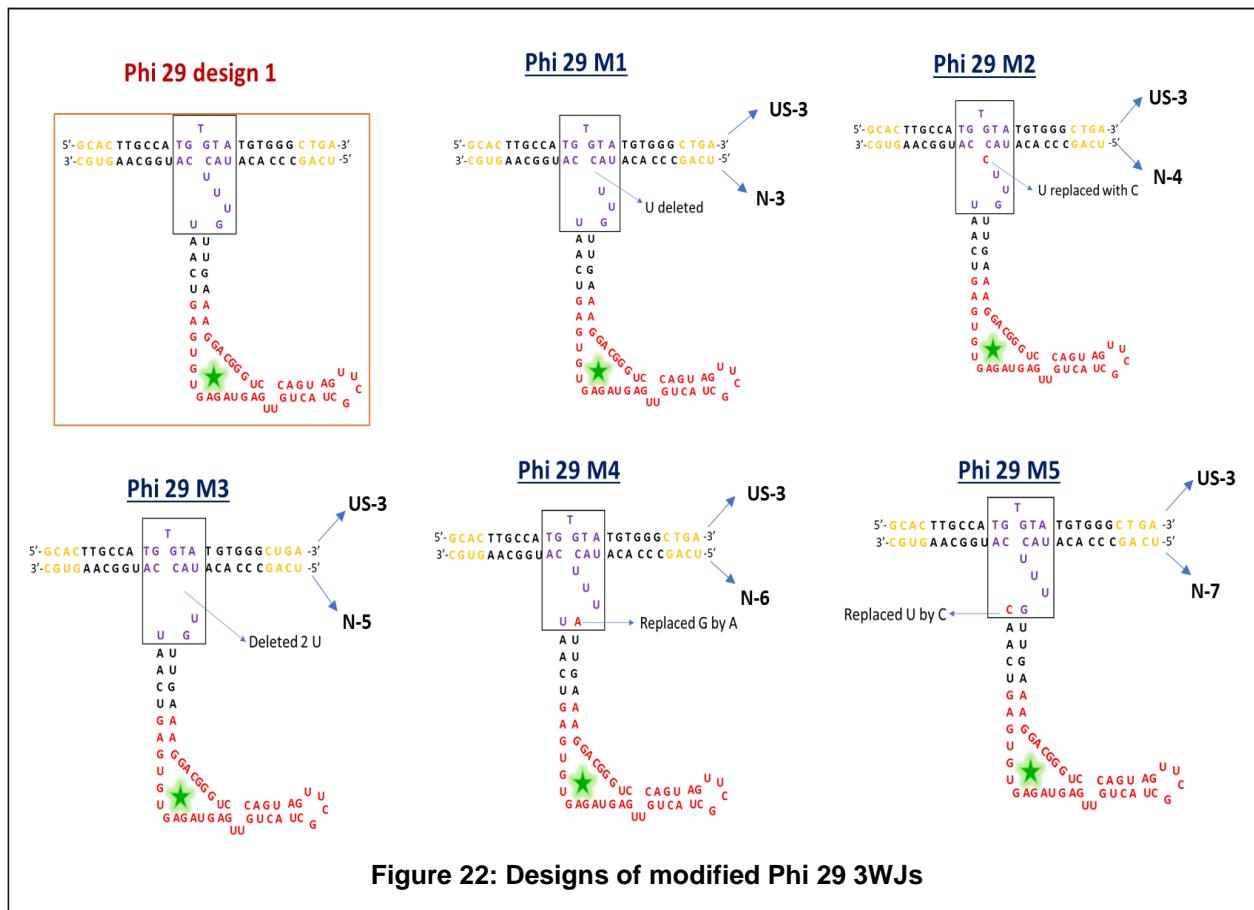


Figure 22: Designs of modified Phi 29 3WJs

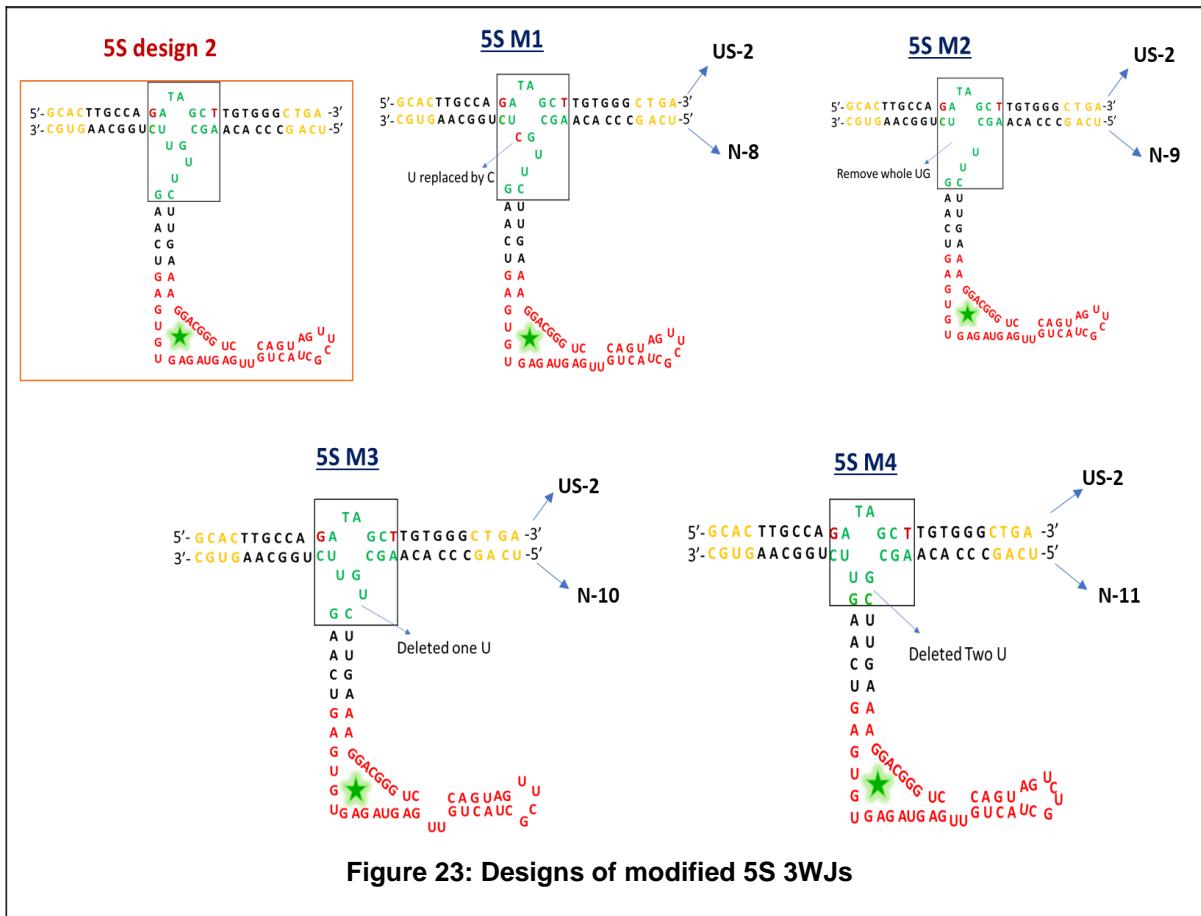


Figure 23: Designs of modified 5S 3WJs

RNA sequences N-3 to N-7 need to be synthesized in order to make designs Phi29 M1 to Phi29 M5. Similarly, RNA sequences N-8 to N-11 are needed in order to make designs 5SM1 to 5SM4.

3.2.1 Synthesis of N-3 to N-11

3.2.1.1 Designing and synthesis of Template, FP, and RP

To synthesize N-3 to N-11 RNA sequences, for each required RNA we designed a DNA template, forward primer, and reverse primer. DNA sequences required for making N-3 to N-7 are given in Table 6 and N-8 to N-11 are given in Table 7. All the sequences were directly synthesized from our lab by using a DNA-RNA synthesizer.

S.No.	Name of DNA/RNA Sequence	Sequence (in 5' → 3')	Length (nt)
1	Phi-29 M1 Temp:	TGA AAG GAC GGG TCC AGT AGT TCG CTA CTG TTG AGT AGA GTG TGA GTC AAT CAT GGC AAG TGC	64
2	Phi-29 M1 F.P	TAA TAC GAC TCA CTA TAG GTC AGC CCA CAT ACT TGT TGA AAG GAC GGG TCC AGT	54
3	Phi-29 M2 F.P	TAA TAC GAC TCA CTA TAG GTC AGC CCA CAT ACC TTG TTG AAA GGA CGG GTC CAG T	55
4	Phi-29 M3 F.P	TAA TAC GAC TCA CTA TAG GTC AGC CCA CAT ACT GTT GAA AGG ACG GGT CCA GT	53
5	Phi-29 M4 F.P	TAA TAC GAC TCA CTA TAG GTC AGC CCA CAT ACT TTA TTG AAA GGA CGG GTC CAG T	55
6	Phi-29 M5 Temp	TCA GCC CAC ATA CTT TGT TGA AAG GAC GGG TCC AGT AGT TCG CTA CTG TTG AGT AGA GTG TGA GTC AAC CAT GGC AAG TGC	81
7	Phi-29 M5 RP	GCA CTT GCC ATG GTT GAC T	19

Table 6: sequences required for making N3-N7 sequences.

S.No.	Name of DNA/RNA Sequence	Sequence (in 5' → 3')	Length of sequence (in nt)
1	5S M1 Temp	TCA GCC CAC AAG CGT TCT TGA AAG GAC GGG TCC AGT AGT TCG CTA CTG TTG AGT AGA GTG TGA GTC AAG CTC TGG CAA GTG C	82
2	5S M1 RP	GCA CTT GCC AGA GCT TGA CTC 3'	21
3	5S M2 Temp	TCA GCC CAC AAG CTT CTT GAA AGG ACG GGT CCA GTA GTT CGC TAC TGT TGA GTA GAG TGT GAG TCA AGT CTG GCA AGT GC	80
4	5S M2 FP	TAA TAC GAC TCA CTA TAG GTC AGC CCA CAA GCT TCT TGA	39
5	5S M2 RP.	GCA CTT GCC AGA CTT GAC TC	20
6	5S M3 Temp	CT TGA AAG GAC GGG TCC AGT AGT TCG CTA CTG TTG AGT AGA GTG TGA GTC AAG TTC TGG CAA GTG C 3'	66
7	5S M3 FP	TAA TAC GAC TCA CTA TAG GTC AGC CCA CAA GCG TCT TGA AAG GAC GGG TCC A	52
8	5S M4 FP	TAA TAC GAC TCA CTA TAG GTC AGC CCA CAA GCG CTT GAA AGG ACG GGT CCA G	52

Table 7: DNA sequences required for making N8-N11.

3.2.1.2 Purification of synthesized DNA sequences

After resin cleaving and desalting all the synthesized DNA sequences were purified by using Glen-pack columns. Then the DNA sequences were analyzed by using 8 % urea PAGE given in figure 24. The sizes of synthesized sequences are confirmed using appropriate size standards.

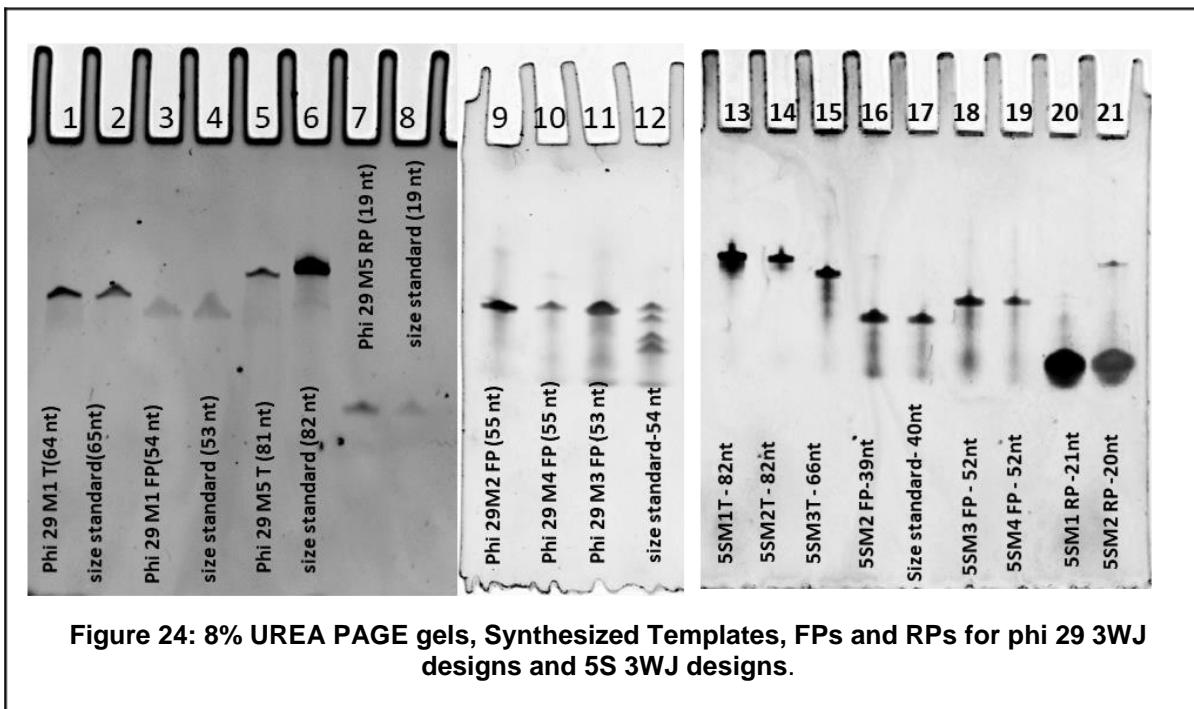


Figure 24 makes it clear that all the synthesized DNA sequences were pure and of the right size.

3.2.1.3 Scale up PCR of N-3 to N-11

Scale-up PCR of N-3 to N-11 were carried out by using synthesized templates and primers. PCR products were purified by using Qiagen MinElute PCR purification columns. 1.5 % Agarose gel was run to confirm the PCR and check the purity of the DNA sequences. Gels are given in figure 25.

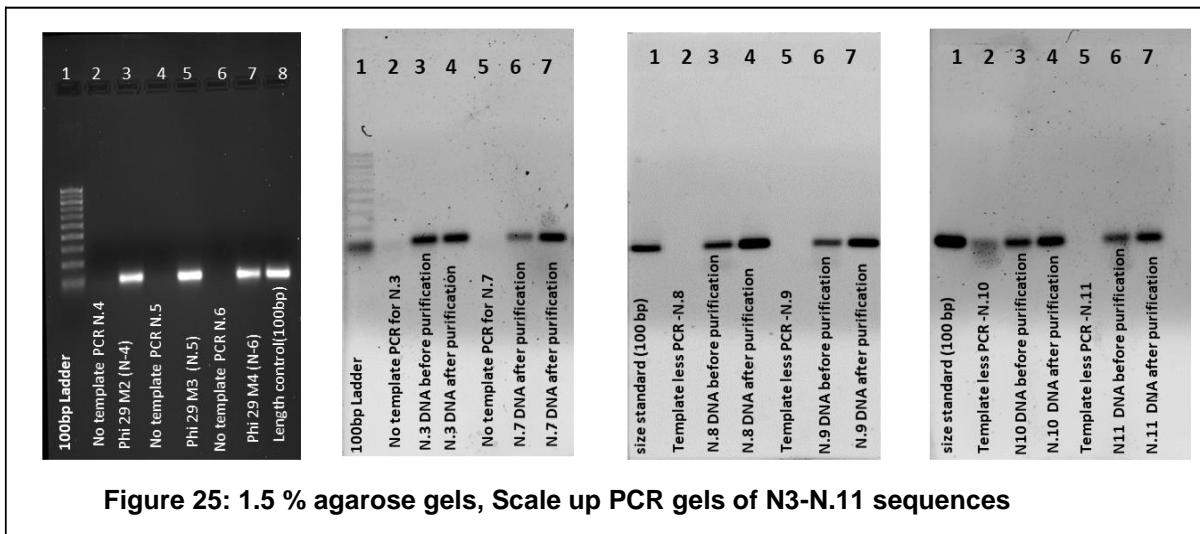


Figure 25: 1.5 % agarose gels, Scale up PCR gels of N3-N.11 sequences

From the gel, we analyzed that all the PCR products were pure. We checked the nanodrop reading to make sure that we are having enough yield to go for transcription.

3.2.1.4 Synthesis of N-3 to N-11 RNA sequences by IVT

Scale-up PCR products were used to perform transcription of N-3 to N-11 in order to produce RNA, and after transcription, RNA sequences were analyzed by running 8 % urea PAGE, given in figure 26.

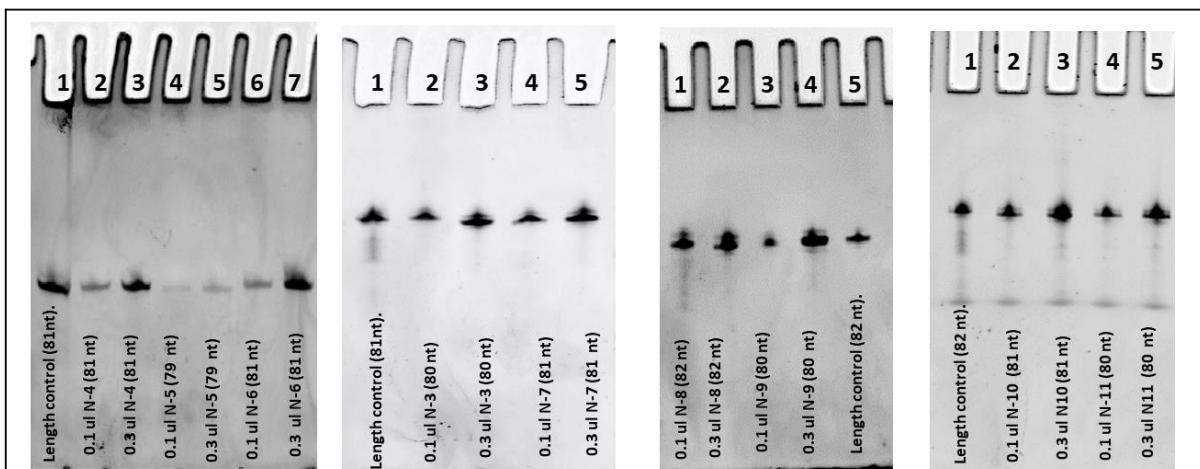
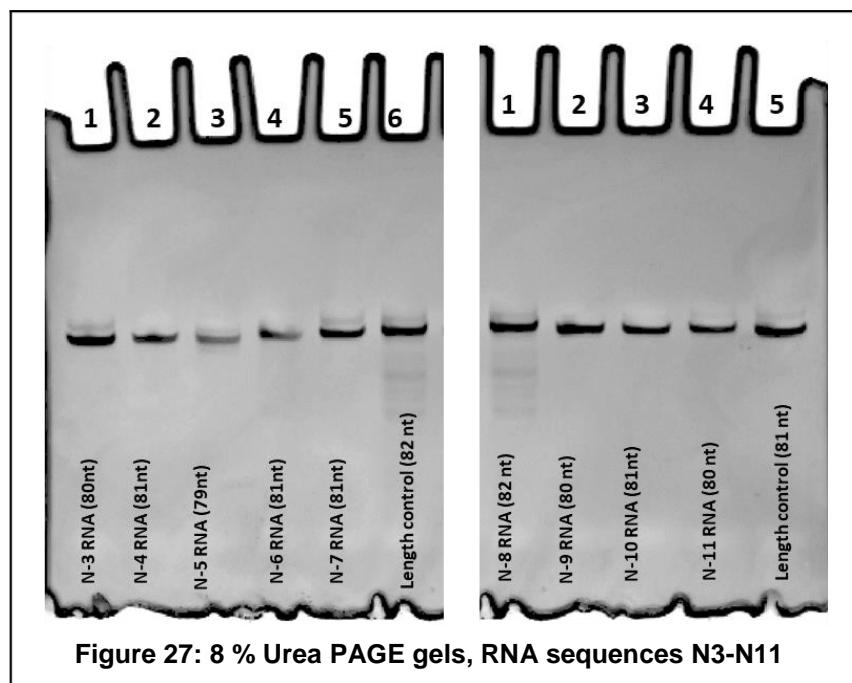


Figure 26: 8 % Urea PAGE gels, Transcription products N3-N11 before purification.

The analysis gel, shown in figure 26, showed a single band for each transcription product, which was also matched with the size standards used. So, we used ZYMO

columns to purify the RNA sequences. Again 8% urea PAGE was performed after purification to verify the RNA's purity. Purification gels are given in figure 27.



All the RNA sequences were pure. All nine RNA (five Phi 29 RNA and four 5S RNA) required for the fluorescence experiment are now available. We used the same upper strands used in the last objective because here we didn't modify the upper strands. In order to compare the stability of 3WJ designs, we continued with the fluorescence experiment.

3.2.2 Fluorescence experiment

The fluorescence experiment was carried out for all nine designs, with and without the upper strand, to compare the fluorescence emission of both. Fluorescence results are shown in figure 28.

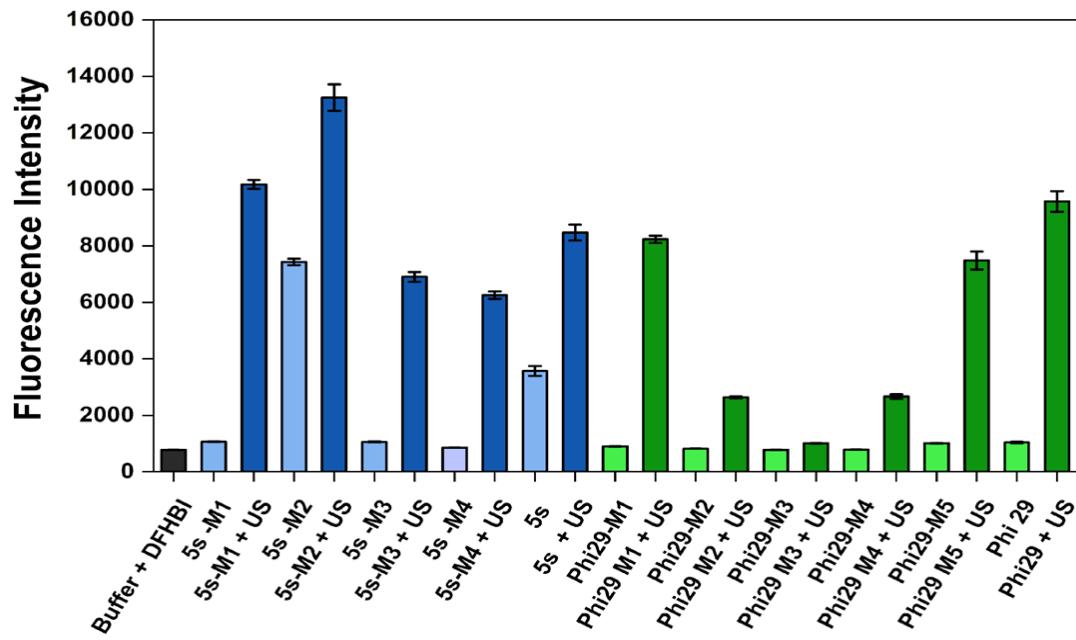


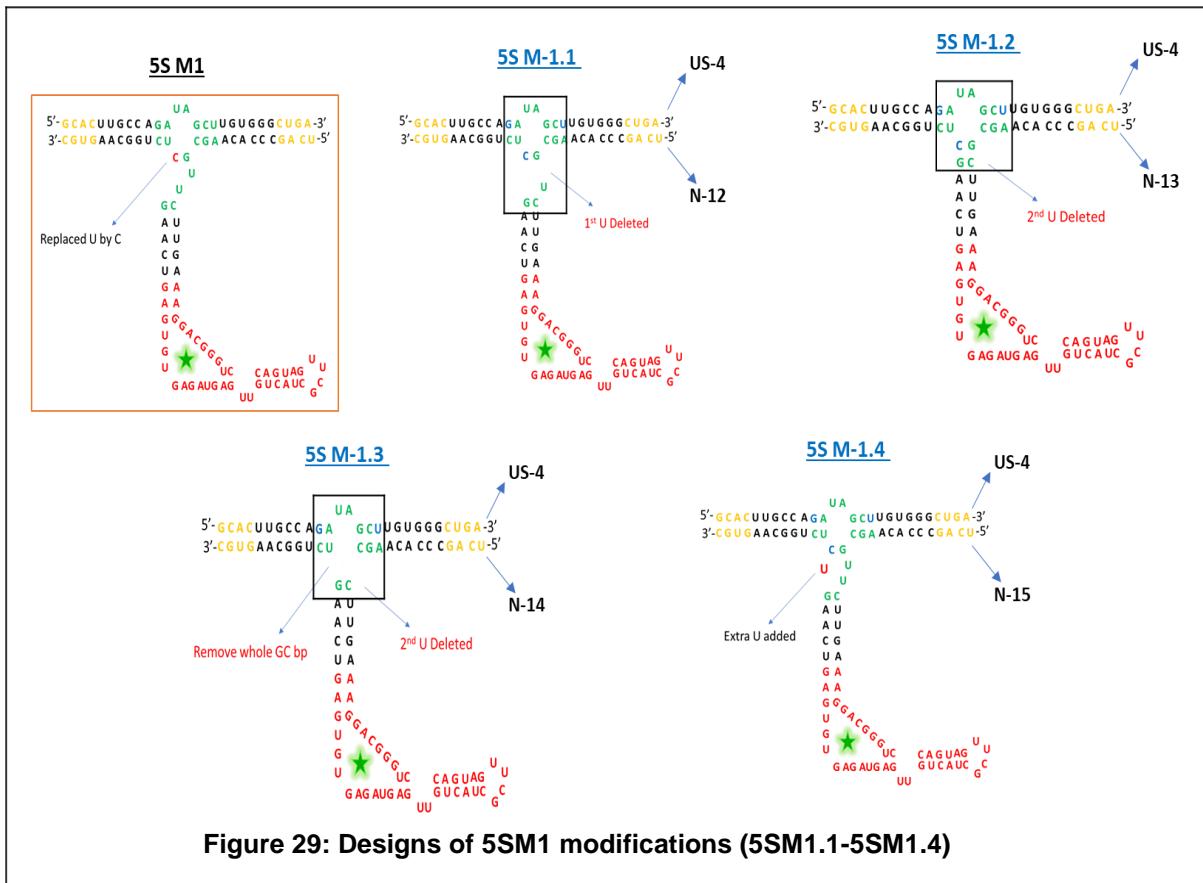
Figure 28: Fluorescence experiment of 5S 3WJ and Phi29 3WJ modifications.

Among all the 5S 3WJ designs 5SM1 and 5SM2 were showing high fluorescence. That means 5SM1 and 5SM2 were more stable than other 5S 3WJs. But 5S M2 was having high background signal and 5SM1 was showing a very less background signal. Among all the 5 Phi 29 designs, none of the 3WJ was showing better results than wild type phi 29 3WJ. That means all the modifications we did reduce the stability of phi 29 3WJ.

Here 5SM1 is the most stable 3WJ. So, we selected 5SM1 for further modification. This will be explained in the next objective.

3.3 Objective 3

From the last objective, we got 5SM1 as one of the most stable 3WJ that showed high fluorescence value than wild-type 3WJ and all other modified 3WJs. Our next objective was to check can we further increase the stability of 5SM1 3WJ by using the same fluorescence method. For that, we introduced four modifications in 5S M1 3WJ to get designs 5S M1.1, 5S M1.2, 5S M1.3, and 5S M1.4. Modifications are given in figure 29.



For this experiment, RNA upper strand (US-4) was used instead of DNA which was synthesized from our lab. In order to make designs 5SM1.1, 5SM1.2, 5S M1.3, and 5S M1.4 we need to make lower RNA sequences N-12, N-13, N-14, and N-15.

3.3.1 Synthesis of N-12 to N-15 RNA sequences

3.3.1.1 Designing and synthesis of Template, FP, and RP

To synthesize N-12 to N-15 sequences, we designed DNA template, Forward primer, and reverse primer for each required RNA. Sequence details are given in table 8. All the sequences were directly synthesized from our lab.

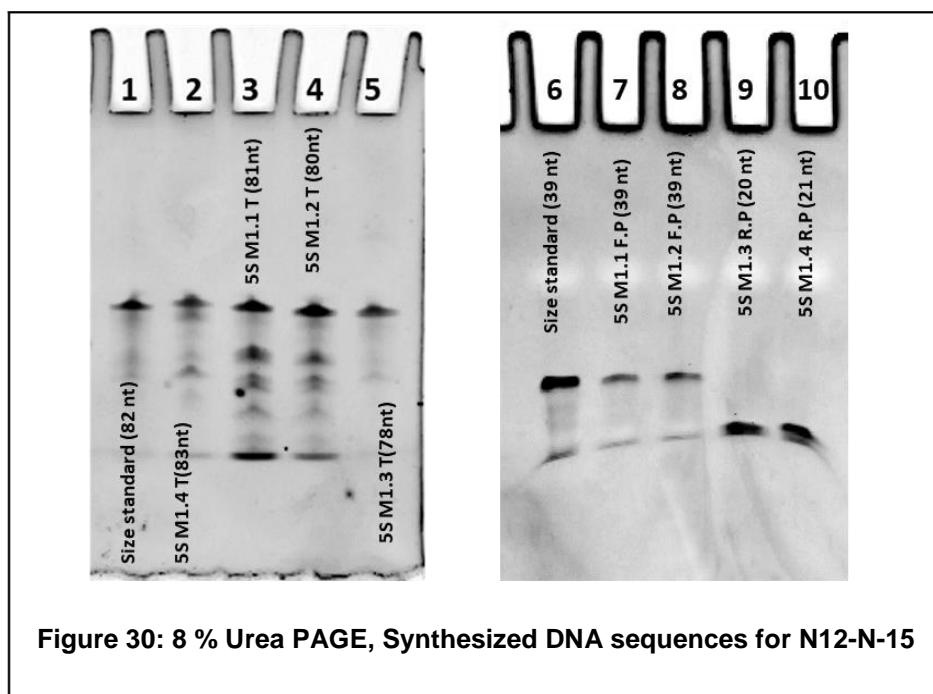
S/N	Name of DNA Sequence	Sequence (in 5' → 3')	Nt.
1	5s M-1.1 Temp	TCA GCC CAC AAG CGT CTT GAA AGG ACG GGT CCA GTA GTT CGC TAC TGT TGA GTA GAG TGT GAG TCA AGC TCT GGC AAG TGC	81
2	5s M-1.1 F.P.	TAA TAC GAC TCA CTA TAG GTC AGC CCA CAA GCG TCT TGA	39

3	5s M-1.2 Temp	TCA GCC CAC AAG CGC TTG AAA GGA CGG GTC CAG TAG TTC GCT ACT GTT GAG TAG AGT GTG AGT CAA GCT CTG GCA AGT GC	80
4	5s M-1.2 F.P.	TAA TAC GAC TCA CTA TAG GTC AGC CCA CAA GCG CTT GAA	39
5	5s M-1.3 Temp	TCA GCC CAC AAG CCT TGA AAG GAC GGG TCC AGT AGT TCG CTA CTG TTG AGT AGA GTG TGA GTC AAG TCT GGC AAG TGC	78
6	5s M-1.3 R.P.	GCA CTT GCC AGA CTT GAC TC	20
7	5s M-1.3 F.P.	TAA TAC GAC TCA CTA TAG GTC AGC CCA CAA GCC TTG AA	38
8	5s M-1.4 Temp	TCA GCC CAC AAG CGT TCT TGA AAG GAC GGG TCC AGT AGT TCG CTA CTG TTG AGT AGA GTG TGA GTC AAG TCT CTG GCA AGT GC	83
9	5s M-1.4 R.P.	GCA CTT GCC AGA GAC TTG ACT	21

Table 8: DNA sequences required for making N12 to N15.

3.3.1.2 Purification of synthesized DNA sequences

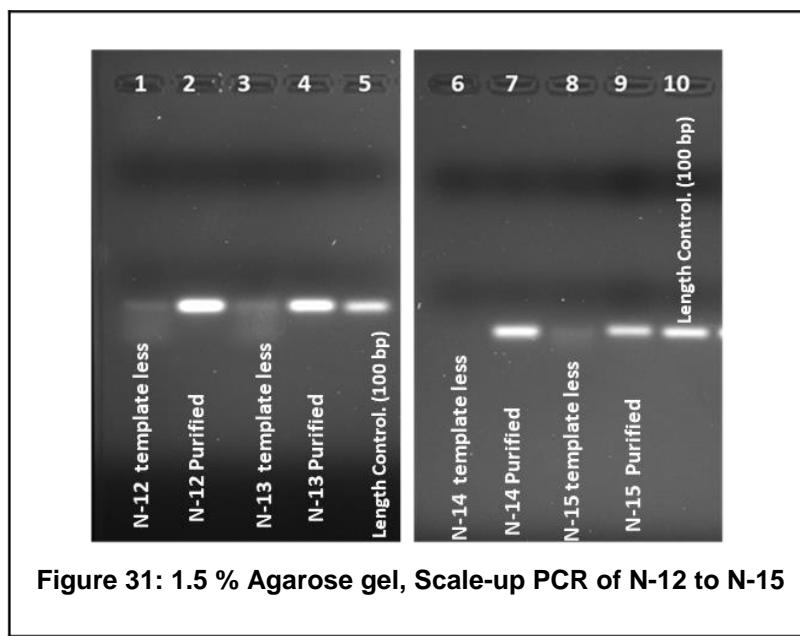
After resin cleaving and desalting all the synthesized DNA sequences were purified by using Glen-pack columns. The DNA sequences were analyzed by using 8 % urea PAGE given in figure 30. The sizes of synthesized sequences are checked by using appropriate size standards.



All the synthesized DNA sequences except 5SM 1.T and 5SM.2 T were pure and matched with size standards. 5S M1.T and 5SM2.T were purified by crush and soak method.

3.3.1.3 Scale up PCR of N-12 to N-15

Scale-up PCR of N-12 to N-15 were carried out by using synthesized templates and primers. PCR products were purified by using a PCR purification column. 1.5 % Agarose gel was run to confirm the PCR and check the purity of DNA sequences.



From the gel (Figure 31) we analyzed that all the DNA sequences were pure. We checked the nanodrop reading to make sure that we are having enough yield to go for transcription.

3.3.1.4 Synthesis of N-12 to N-15 RNA sequences by IVT

Scale-up PCR products were used to perform transcription to get RNA N-12 to N-15. After transcription, all the RNA sequences were analyzed by running 8 % urea PAGE. Also, we purified all the RNA by using ZYMO columns. One more 8% urea PAGE was performed after purification to verify the RNA's purity. Both analysis gel and purification gel are given in Figure 32 below.

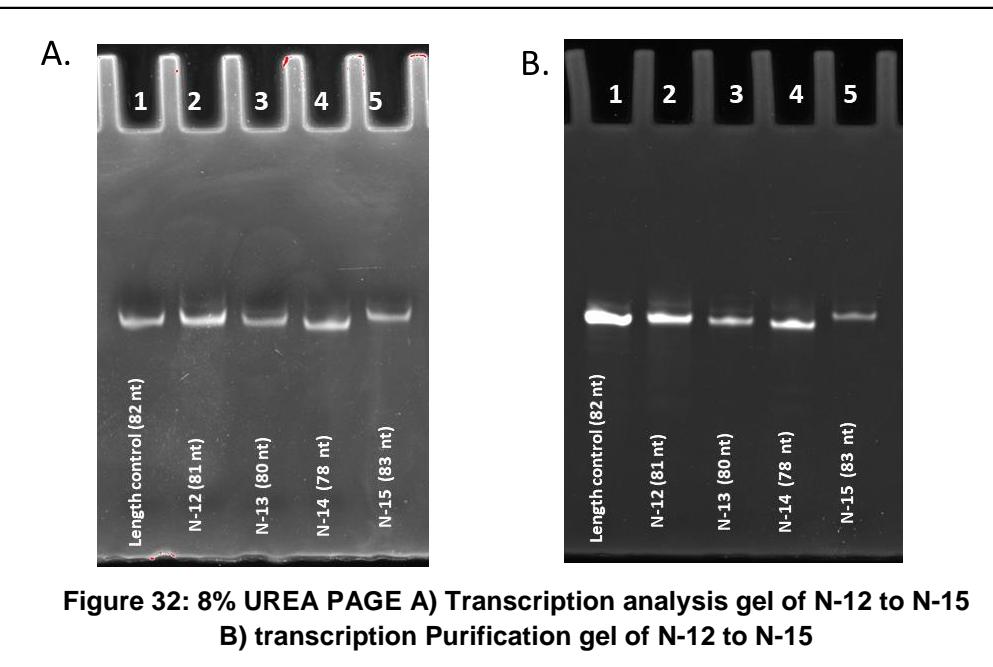


Figure 32: 8% UREA PAGE
A) Transcription analysis gel of N-12 to N-15
B) transcription Purification gel of N-12 to N-15

All 4 of the RNA sensors and upper strands needed for designs 5SM1.1 to 5SM1.4 are now ready. We continued the fluorescence experiment to compare the stability of 3WJ designs.

3.3.2 Fluorescence experiment of 5S-M1.1 to 5SM1.M4

The fluorescence experiment was carried out for all four 5SM1 designs. The experimental conditions were set up, with and without the upper strand for each 3WJ to compare the fluorescence emission of both. We also compared the stability of 5SM1 modified 3WJs with wild-type 5S 3WJ, 5SM1, also with wild-type Phi 29 3WJ. Fluorescence results are shown in figure 33.

Fluorescence data make it obvious that 5SM1 continues to outperform all other modifications, however 5SM1.1 also looks comparable to 5S-M1. Moreover, it has a higher fluorescence value than wild-type Phi 29 3WJ. This suggests that Phi 29 3WJ and all other 5S designs we have been less stable than 5SM1 3WJ.

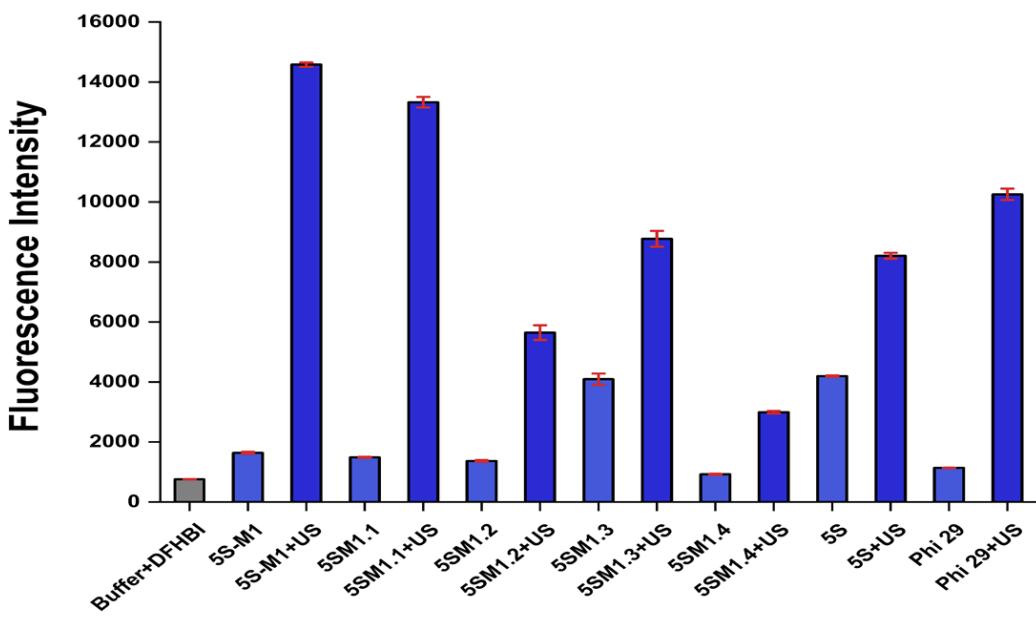


Figure 33: Fluorescence experiment results of 5S-M1.1 to 5S-M1.4

Future plan

My future plan is to choose a 3WJ that is more stable than wild-type 3WJ, and construct RNA nanoparticles like triangles, squares, tetrahedrons, etc. by putting the chosen 3WJ in the corners of those structures. After getting stable nano structure, we will attach several RNA modules to it, such as aptamers and siRNAs, etc. and check how well they work for targeted drug delivery.

Also, we will check the thermal stability of few selected stable 3WJs by UV melting experiment and compare the results with our fluorescence data.

Designs of RNA nanosquare and nanotriangle by using 5SM1 3WJ in the corners are given below in figure 34.

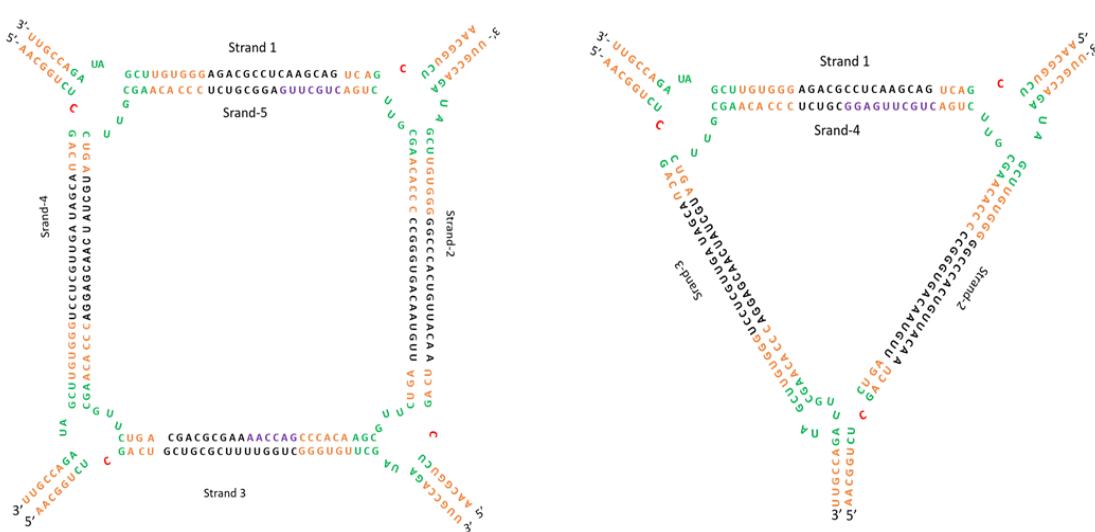
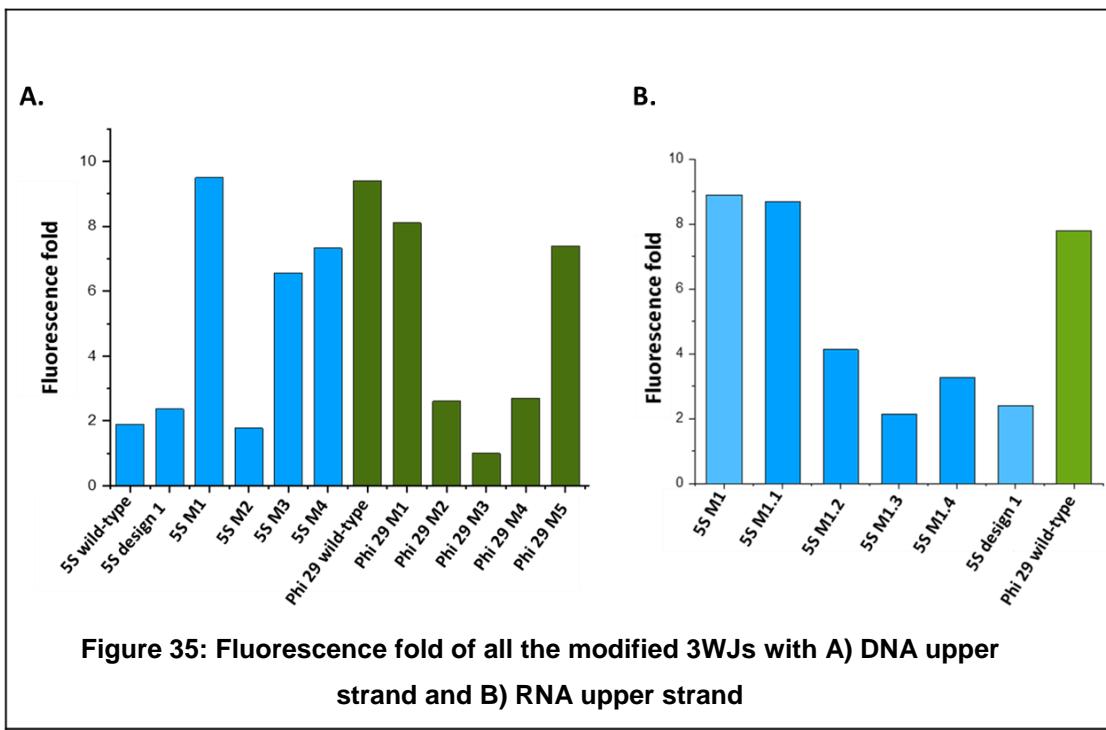


Figure 34: Design of RNA nanostructures by using 5SM1 3WJ

Chapter 4

CONCLUSION

RNA three-way junctions (3WJ) are important motifs that are used in the construction of RNA nanoparticles. In this project, we synthesized different variants of wild-type 5S rRNA 3WJ and phi29 3WJ by mutating few nucleotides in their core region, and compared their stabilities using a high throughput fluorescence-based method. To achieve this, we designed a 3WJ based fluorescent sensor by utilizing baby spinach light-up aptamer attached to one of its end. The stability of the 3WJ core correlates with the fluorescence output of the baby spinach aptamer. i.e., higher stability of 3WJ will be indicated by more fluorescence. The fluorescence fold of all the modified 3WJs has been provided below (Figure 35).



Based on fluorescence results, it is concluded that 5SM1 and 5SM1.1 are the most stable 3WJs among all other 3WJs showing a fluorescence fold change of 9.2 and 8.8 respectively, and are much better than 5S wild-type that shows a fluorescence fold

change of 1.9 only. However, for Phi29 3WJs, none of the modified junction could show better fold change than the wild-type Phi29 3WJ. Based on this data, we can say that 5SM1 and 5SM1.1 can be further used for constructing highly stable RNA nanostructures of different size and shapes.

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