

Preparation of a Multi-Part Varkud Satellite Ribozyme Variant for Kinetics Studies

by

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Abstract

The Varkud Satellite (VS) ribozyme is the largest of the “small” nucleolytic ribozymes and is the only one for which there are no high resolution crystal structures available. The VS ribozyme comprises a catalytic domain and a substrate domain. The catalytic domain includes five helices that interact with the stem-loop substrate.

The substrate is docked within a cleft that is formed by helices II and VI. This naturally brings the cleavage site in close proximity to the A730 loop in helix VI. The adenines within the A730 loop are very crucial to the cleavage reaction and any substitution causes a major decrease in the cleavage activity of the ribozyme.

This study is aimed at designing and producing a variant of the Varkud Satellite ribozyme that consists of multiple parts that can be used for detailed studies of ribozyme kinetics and assembly.

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To My Family and Friends

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List of Abbreviations

Å	Ångström
bp	Base pairs
DNA	Deoxyribonucleic Acid
HDV	Hepatitis Delta Virus
LB	Lysogeny broth
μ	Micro
mRNA	Messenger Ribonucleic Acid
ng	nanograms
NMR	Nuclear Magnetic Resonance
nt	Nucleotides
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
tRNA	Transfer Ribonucleic Acid
RNase P	Ribonuclease P
S _N 2	Binuclear Nucleophilic Substitution
SOC	Super Optimal broth with Catabolite repression
VS	Varkud Satellite

Chapter 1: Nucleic Acids and the RNA World

1.1 Introduction

This chapter will provide background information on nucleic acids. It will also cover the structural and functional differences between deoxyribonucleic acids (DNA) and ribonucleic acids (RNA) along with their roles during evolution.

1.2 Nucleic Acids

The discovery of the double helix structures of deoxyribonucleic acids (DNA) by James Watson and Francis Crick in 1953 provided the basis of understanding how the genetic information within a cell is replicated and passed on to future generations [1].

In the following year George Gamow proposed that the structure of proteins were encoded within the DNA sequence, such that every three bases within the DNA sequence makes a codon and each codon sequence represents an amino acid, hence the sequence of bases within the DNA codes for the amino acid structure of proteins [2].

This theory was supported by Francis Crick in his discovery of transfer ribonucleic acids (tRNA) that mediates the translation process by reading the codon sequence of DNA and its involvement in the assembly of amino acids into polypeptides during transcription [3].

In the same year, it was found that some viral particles only contain RNA and proteins but no DNA present which suggests that RNA may also act as a storage system of genetic information.

As more research was performed in this field, the roles of DNA, RNA and proteins became more distinctive. DNA serves as the genetic material storage system, whereas

RNA plays a role as an intermediate aiding the formation of protein molecules and proteins are the machinery that catalyzes most reactions within the cell [4].

1.3 RNA and DNA

DNA and RNA are both nucleic acids sharing similar characteristics. Their monomers both have a phosphate group attached to a pentose sugar and one of four nucleobases. The difference between these two lies on the 2' carbon on the pentose ring, where in DNA there is no hydroxyl group at this position.

Another difference is the type of base that is attached to the pentose sugar. In DNA, adenine, cytosine, guanosine and thymine bind to the pentose sugar where in RNA, thymine is not present and is replaced by uracil. Because of these differences in structure and base attachment, RNA is able to fold into more diverse secondary and tertiary structures allowing them to perform catalytic reactions.

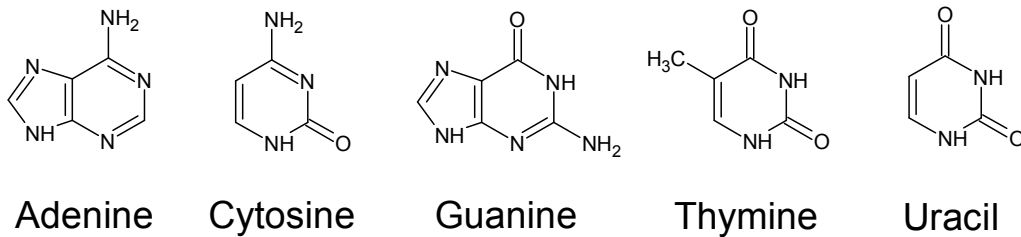


Figure 1. 1 - The five different nucleobases that are attached on the ribose sugar in DNA and RNA

1.4 The RNA World

The simple paradigm of information flow from DNA → RNA → Protein proposed by Watson answered a lot of questions regarding how genetic information was stored and passed on, but at the same time it raised another question: Which species appeared first?

The replication of DNA requires the presence of protein enzymes, but without the sequence encoded in DNA proteins cannot be made. It is highly unlikely that all three species were created simultaneously during evolution.

An answer to this “chicken-and-egg” question was suggested by three scientists: Carl Woese, Watson, Crick and Leslie Orgel all of whom hypothesized that RNA may be the species that existed first, and is responsible for both storage of genetic information and the replication of this information [5-7].

This idea of RNA catalysis was not readily accepted until the discovery of RNA catalysis in the absence of protein molecules by Cech and his co-workers in 1982 [8]. They observed a RNA self-splicing reaction of an *in vitro* transcribed rRNA that is present in *Tetrahymena thermophila* [8]. More evidence of RNA catalysis was demonstrated by Altman and his co-workers in their discovery of catalytic RNA within RNase P [9].

These findings led to a concept called the “RNA World Hypothesis”, which proposes that during early stages of life RNA plays a significant role in genetic information storage, replication and even cell life sustaining catalytic reactions [10].

1.5 RNA in the RNA World Hypothesis

If RNA was the first genetic storage system, how was it made in the first place? How was its building unit, the nucleotide generated? Moreover, was RNA stable enough to store genetic information to be passed on to future generations?

RNA is very susceptible to degradation and hydrolysis reactions in the presence of divalent metals and this limits the lifetime of RNA molecules. One possible explanation is natural selection. The environment in the pre-biotic world may have been very different from the world today. This harsh environment leads to natural selection of more stable RNA structures that allowed replication to occur and through evolution it became stable enough to store genetic information.

1.6 The RNA World Hypothesis

The general idea of the RNA world is that during the development of life on earth, evolution based on RNA replication preceded the appearance of DNA and protein synthesis. It was thought that RNA catalysis played an important role in the early evolution of life.

The discovery of catalytic RNAs initiated discussion of the role of RNA in the origin of life and led to the “RNA World Hypothesis” [10].

There are no strict definitions of the RNA World as many speculations were proposed. But the RNA world hypothesis has three basic assumptions: First, at some point in evolution, the genetic material was based on RNA, and RNA catalyzes its own replication. Second, Watson-Crick base-pairing was the key in RNA recognition and replication that allows RNA to specifically interact with its substrate. Third, genetically encoded protein molecules were not involved as a catalyst in the early stages of life.

There is strong evidence that an RNA World did indeed exist in the early stage of evolution. An example of this is seen in the active site of ribosome [11, 12]. The active site for peptide bond formation within the ribosome lies deep within a central core of RNA where no amino acid side chain comes within 18Å of the active site [13]. The outer part of the ribosome is made of amino acid residues, while the catalytic core is purely made of RNA. This supported the statement made by Crick in 1968 stating that “the primitive ribosome could have been made entirely of RNA”.

There might be other molecules that served as a genetic system before the appearance of RNA molecules, but it is believed that the appearance of DNA and protein molecules was preceded by RNA.

Chapter 2: Catalytic RNA

2.1 Introduction

This chapter will provide some background information on catalytic RNAs and how they perform their catalytic reactions.

2.2 RNA Secondary Structures

RNA is able to fold and form diverse secondary structures. These secondary structures of RNA are generated through various canonical and non-canonical base pairs (Watson-Crick and non-Watson-Crick base pairing) that are stabilized by hydrogen bonding and base stacking interactions. Moreover, RNA is able to form tertiary structures through the interactions of secondary structure interactions; the hammerhead ribozyme and pseudoknots are examples of complex RNA structures. A few examples of various RNA secondary structure motifs are illustrated below.

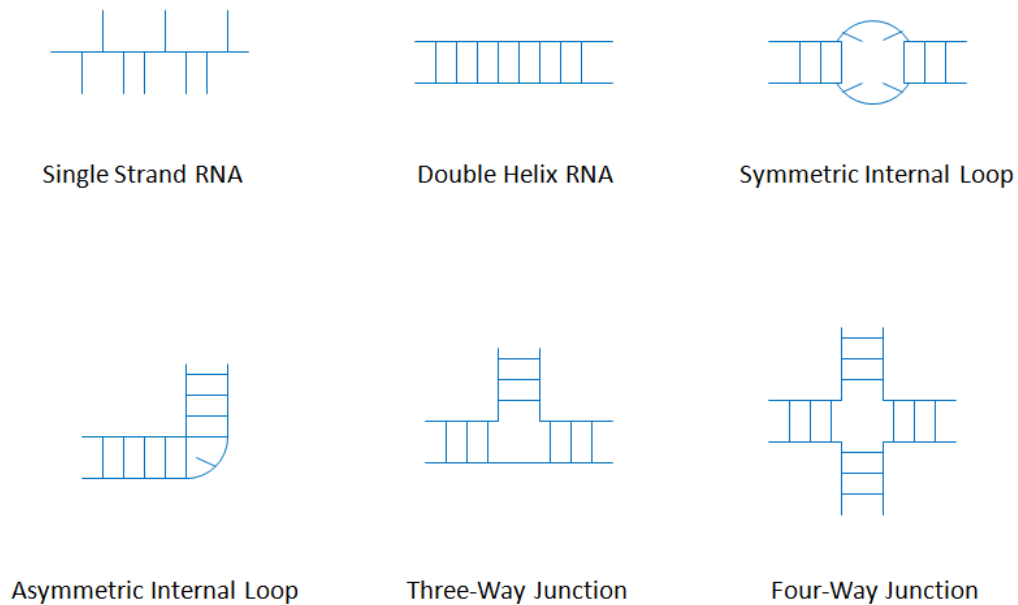


Figure 2. 1 - Various RNA secondary structures

2.3 Ribozymes

Ribozymes are RNA species that have the ability to catalyze reactions similar to protein based enzymes. They have been found to participate in various processes within a cell including RNA processing, protein synthesis, gene expression and viral RNA replication [11, 13, 14].

The naturally occurring ribozymes can be divided into two groups based on their size and reaction mechanisms. The “large” catalytic RNA group includes the group I introns, group II introns and the RNA component of the ribonuclease P (RNase P). These “large” ribozymes are typically hundreds of nucleotide in size and perform cleavage reactions that give products with 3'-hydroxyl and 5'-phosphate termini [15-17].

The “small” nucleolytic ribozymes are generally present in plant viruses or exists as satellite RNA of larger plasmids. They are typically forty to a few hundred nucleotides in size and perform cleavage reactions the give 2'3'-cyclic phosphate and 5'-hydroxyl termini products. These “small” ribozymes include the hammerhead ribozyme, the hairpin ribozyme, hepatitis delta ribozyme (HDV) and the Varkud satellite (VS) ribozyme [15-17].

2.4 General Catalytic Mechanism of Ribozymes

The reaction speed of ribozymes is rather slow compared to protein enzyme catalyzed reactions, typically in the range of 1 min^{-1} [18]. Even though the reaction appears to be slow, it is adequate for the biological functions they perform. Moreover, these ribozymes are only required to work once *in vivo*.

Interactions of ribozyme with their substrate (in most cases, oligonucleotides) are typically via a mixture of Watson-Crick and non-Watson-Crick interactions. In most cases, the presence of metal ions (usually divalent ions such as magnesium ions) is necessary for the ribozymes to perform their reactions.

The reaction mechanisms of the “small” and “large” ribozymes are different. The reaction mechanism of “small” ribozymes is more similar to non-enzymatic hydrolysis of RNA closely following a binuclear nucleophilic substitution (S_N2) reaction.

The general reaction mechanism for these “small” ribozymes begins with the vicinal 2'-hydroxyl group attacking the neighboring phosphate molecule, forming a bipyramidal transition state. This transition state will then be broken down releasing the 5'-oxyanion group completing the cleavage reaction.

Unlike the “small” ribozymes, the reaction catalyzed by the “large” ribozymes is initiated by the attack of an external nucleophile at the 3'5'-phosphodiester bond forming a trigonal bipyramidal transition state. This transition state will rearrange itself releasing the 3'-hydroxyl and 5'-phosphate termini products. The nucleophile that initiates the cleavage reaction is either the 3'-OH of a guanosine, a water molecule or the 2'-OH of an adenosine.

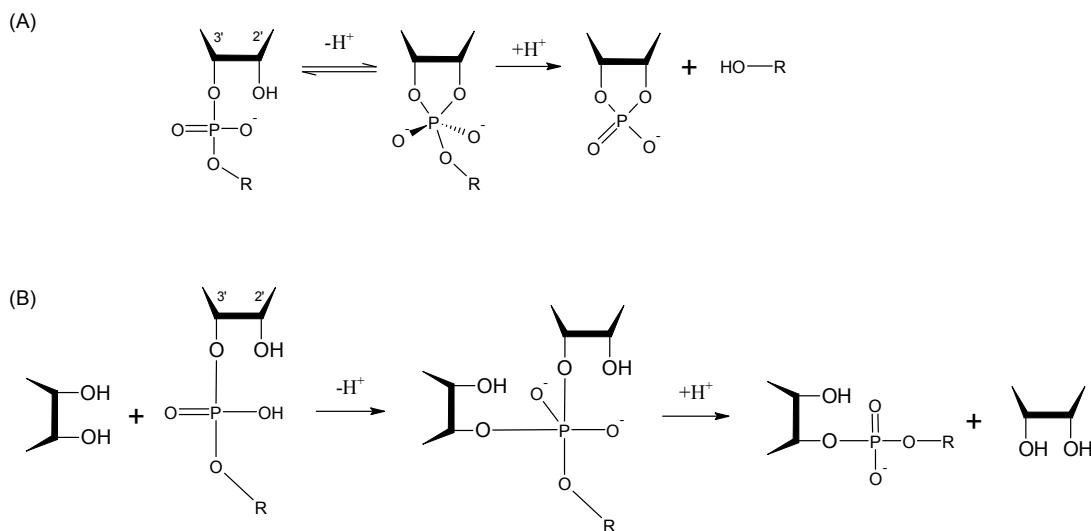


Figure 2.2 - Catalytic mechanism of large and small ribozymes. (A) The transesterification reaction performed by small ribozymes. (B) The transesterification reaction performed by large ribozymes.

2.5 Various Ribozyme Structures

2.5.1 Hairpin Ribozymes

The hairpin ribozyme is one of the four naturally occurring catalytic RNAs that perform site specific cleavage reactions. Unlike some of the other ribozymes, the hairpin ribozyme does not require metal ions as a cofactor.

This ribozyme is found as a satellite RNA of many plant viruses where it catalyzes the sequence specific cleavage (and ligation) reaction of RNA through transesterification reactions [19].

In the natural form of the RNA, the catalytic domain is responsible for cleaving individual RNA replicates from multimeric transcripts produced by the rolling-circle replication of the RNA. But *in vitro*, the RNA substrate and the catalytic domain can be synthesized as two separate units and can be mixed together to allow the cleavage reaction to take place [20].

The minimum hairpin ribozyme structure consists of two internal loops with a four way helical junction. Folding and interactions of the two stem loops are responsible for creating the active site of the hairpin ribozyme. The catalysis follows a general acid base chemistry where the guanine on position 8 of loop A functions as a base and the adenine on position 38 of loop B functions as an acid. The cleavage site lies on stem loop A, indicated by the arrow in figure 2.3 [20, 21].

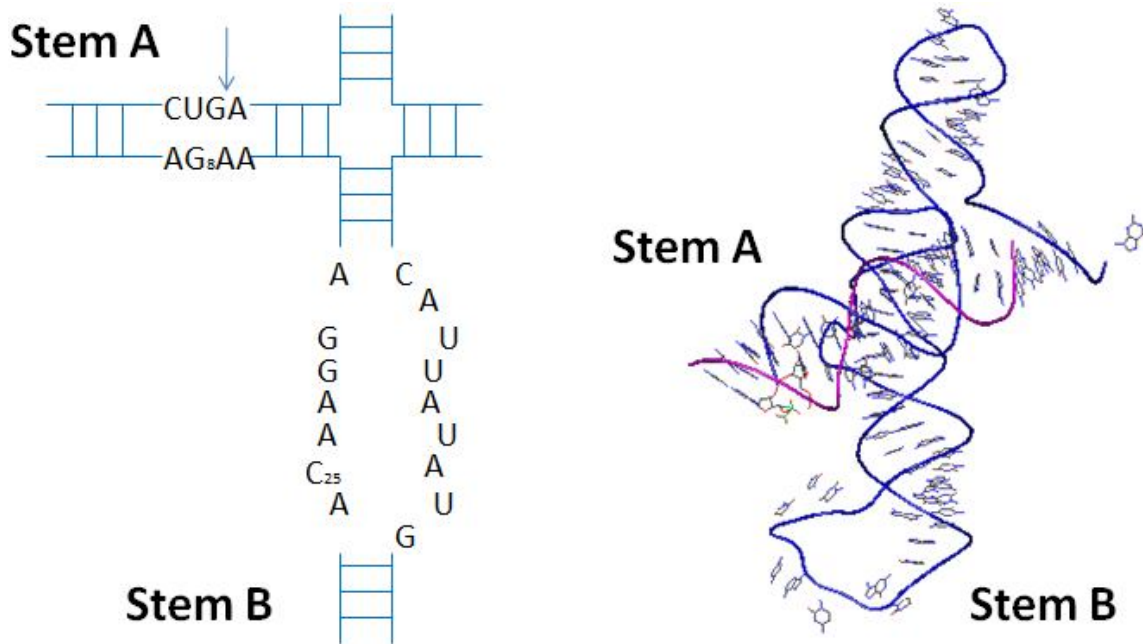


Figure 2.3 - The structure of the hairpin ribozyme. (Left) The secondary structure of the hairpin ribozyme illustrating the two stems loop structure. The cleavage site is indicated by the arrow on stem A. (Right) The crystal structure of the hairpin ribozyme [20, 21].

As with all small ribozymes, the cleavage reaction of the hairpin ribozyme produces 2'3'-cyclic phosphate and 5'-OH termini products. The cleavage reaction mechanism of the hairpin ribozyme is illustrated in figure 2.4 [21].

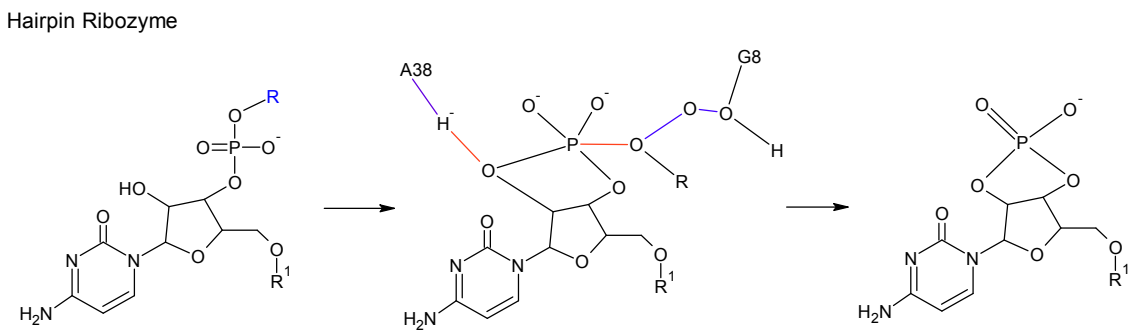


Figure 2.4 - The catalytic mechanism of the hairpin ribozyme [21].

2.5.2 Hammerhead Ribozymes

Hammerhead ribozymes are one of the four types of ribozyme that perform sequence specific hydrolytic cleavage reactions. Similar to the other types of ribozyme, they catalyze cleavage and ligation reactions that produces 5'-OH and 2'3'-cyclic phosphate products [22, 23].

Unlike the hairpin ribozymes, the hammerhead ribozyme requires the presence of divalent metal ions for optimal catalytic activity. Moreover, the substrate RNA sequence must contain an NUX triplet, where N can be any nucleotide; X can be an adenine, uracil or cytosine, but not guanine [24].

The hammerhead ribozyme is found naturally in plant viruses where they are responsible for the cleavage reactions during the replication cycle of its RNA. During RNA replication, multiple units of the RNA are produced in a long strand and the hammerhead RNA will fold into its active catalytic form performing cleavage reactions giving single units of RNA [23].

The minimum RNA sequence of the hammerhead ribozyme required for catalytic activity consists of three secondary structure elements with the absence of tertiary contacts. Functional group studies of the ribozyme have shown that the important elements for catalysis are located far apart from each other, suggesting that the hammer head ribozyme must undergo conformational changes upon substrate catalysis [25].

Furthermore, in the absence of magnesium ions the hammerhead ribozyme exists in an extended form with an unstructured core at the center. However in low concentration of magnesium ions stems II and III rearranges themselves in a coaxial geometry. With further increase of magnesium ion concentration, a second conformation change takes place which generates the active form of the ribozyme [25]. The cleavage reaction of the hammerhead ribozyme follows an acid base catalysis. The guanine on position 8 is believed to be the general acid and the guanine on position 12 is believed to be the general base. Additionally, the guanine on position 5 appears to

interact with the substrate allowing it to be correctly positioned within the active site [24].

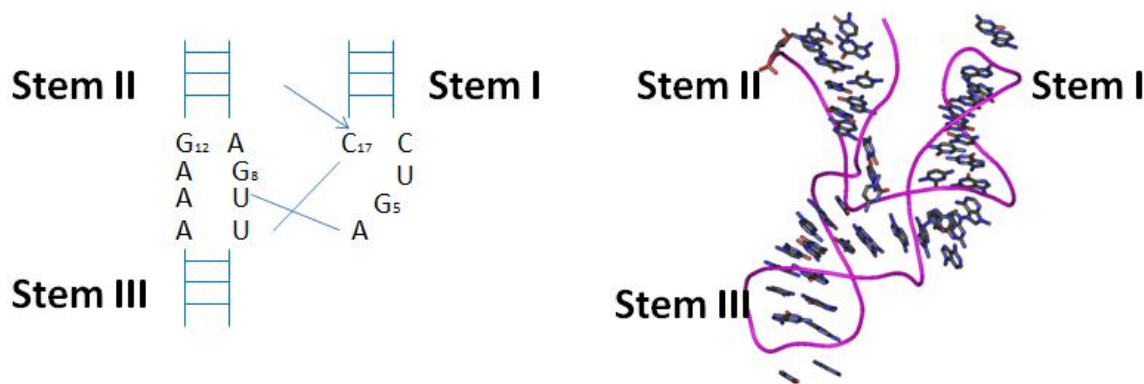


Figure 2. 5 - The structure of the hammerhead ribozyme. (Left) The secondary structure of the minimal hammerhead ribozyme showing how the three stems interact with each other. The cleavage site is indicated by the arrow on stem I. (Right) The crystal structure of the full length hammerhead ribozyme.[24]

The cleavage reaction is initiated by divalent metal ions. Once the substrate is docked within the active site, a metal-hydroxide complex binds to the oxygen of the scissile phosphate bond and deprotonates the 2'-OH group. The 2'-oxy anion performs a nucleophilic attack on the phosphorous atom forming a penta-coordinated transition state, followed by the departure of the 5'-nucleoside giving the products. An illustration of the cleavage reaction performed by the hammerhead ribozyme is shown below [23].

Hammerhead Ribozyme

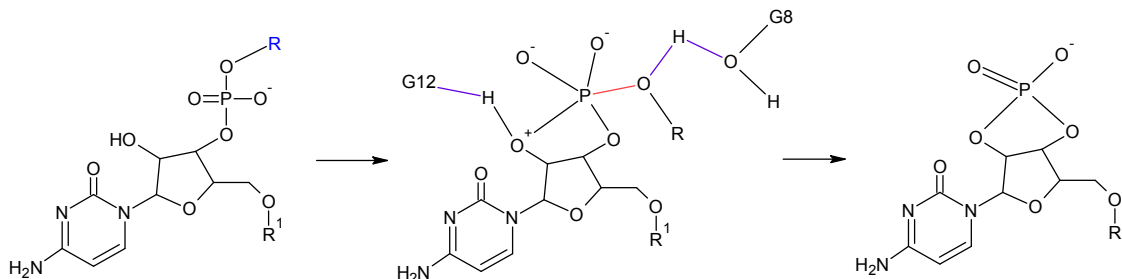


Figure 2. 6 - The catalytic mechanism of the hammerhead ribozyme.[23]

2.5.3 Hepatitis Delta Virus Ribozyme

The hepatitis delta virus (HDV) ribozyme is an RNA satellite virus of the hepatitis B virus. The HDV is a circular single stranded RNA of 1,700 nucleotides and is replicated using a rolling circle replication mechanism. The replication cycle will produce linear multimers of the RNA which will fold and perform cleavage reactions producing unit length RNA [26].

The self cleavage reaction performed by the HDV is similar to other nucleolytic ribozymes. They perform transesterification reaction that produces 2',3'-cyclic phosphate and 5'-hydroxyl termini products. The minimum HDV structure required for catalytic activity consists of 85 nucleotides and surprisingly only one nucleotide after the 5' cleavage site is sufficient for the cleavage activity. The HDV ribozyme is also the fastest known naturally occurring ribozyme with a rate of 52 min^{-1} [27].

The structure of the HDV ribozyme comprises of five helical segments which are connected with two pseudoknot structures. These five helical junctions' forms two parallel stacks where P1, P1.1 and P4 stack nearly co-axially and P2, P3 forms a second co-axial stack as shown in figure 2.7 [27].

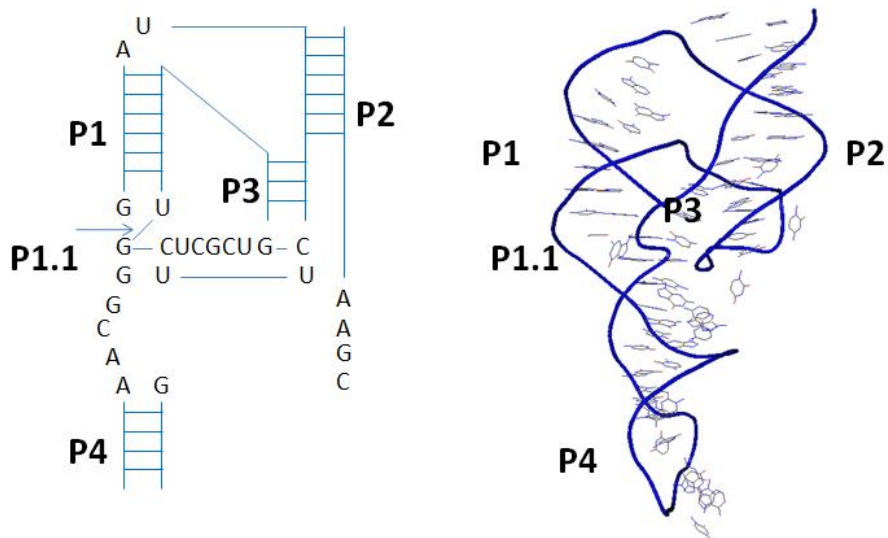


Figure 2. 7 - The structure of the Hepatitis Delta ribozyme. (Left) The minimal secondary structure of the Hepatitis Delta ribozyme. The cleavage site is indicated by the arrow on P1.1. (Right) The crystal structure of the Hepatitis delta ribozyme [27].

From crystal structure studies, it was revealed that the cytosine at position 75 participates in the cleavage reaction as a base where it deprotonates the 2'OH group. The proposed reaction mechanism of the hepatitis delta virus ribozyme is illustrated in figure 2.8.

HDV Ribozyme

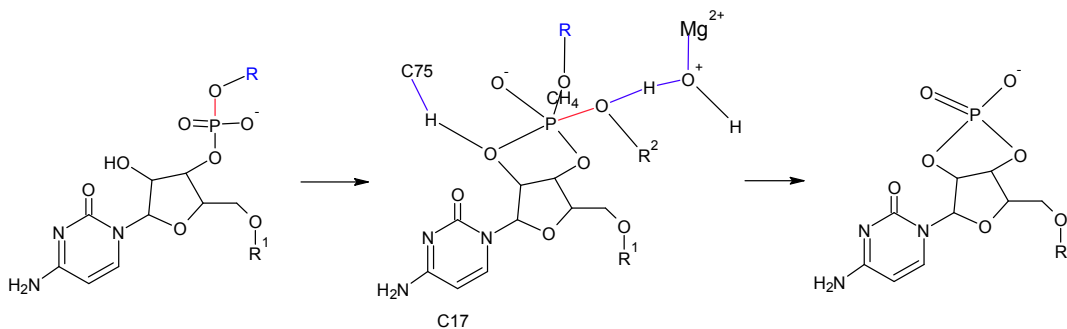


Figure 2. 8 - The catalytic mechanism of the Hepatitis Delta ribozyme [27].

2.5.4 The Varkud Satellite Ribozyme

The Varkud Satellite (VS) ribozyme is the largest of the small nucleolytic ribozyme. Its substrate recognition and cleavage reaction differs from the other small ribozymes. More about this ribozyme will be discussed in the next chapter.

Chapter 3: The Varkud Satellite Ribozyme

3.1 Introduction

The Varkud Satellite (VS) Ribozyme is the largest of the “small” nucleolytic ribozymes. It is approximately 150 nucleotides (nt) in size and currently there are no three dimensional high resolution structures available for this ribozyme. The VS ribozyme is unique in size, structure and substrate interaction compared to other small nucleolytic ribozymes. It is similar to the small ribozymes and catalyzes site specific transesterification reactions that give 2'3'-cyclic phosphate and 5'-hydroxyl products [28].

The VS RNA is a single-stranded plasmid (~881 nucleotides in size) found in the mitochondria of certain isolates of the species *Neurospora crassa* [29]. The VS ribozyme is formed by the VS RNA during the replication cycle of the VS RNA. During the replication process, the VS RNA is reversely transcribed to produce the VS DNA. This VS DNA serves as a template for the RNA polymerase encoded within the Varkud plasmid to create long strands of multimeric concatamers. Each concatamer will fold up forming multiple units of the VS ribozyme. Through self-cleavage and ligation reactions, the multimeric concatamers will be cleaved into circular monomeric VS RNA [29].

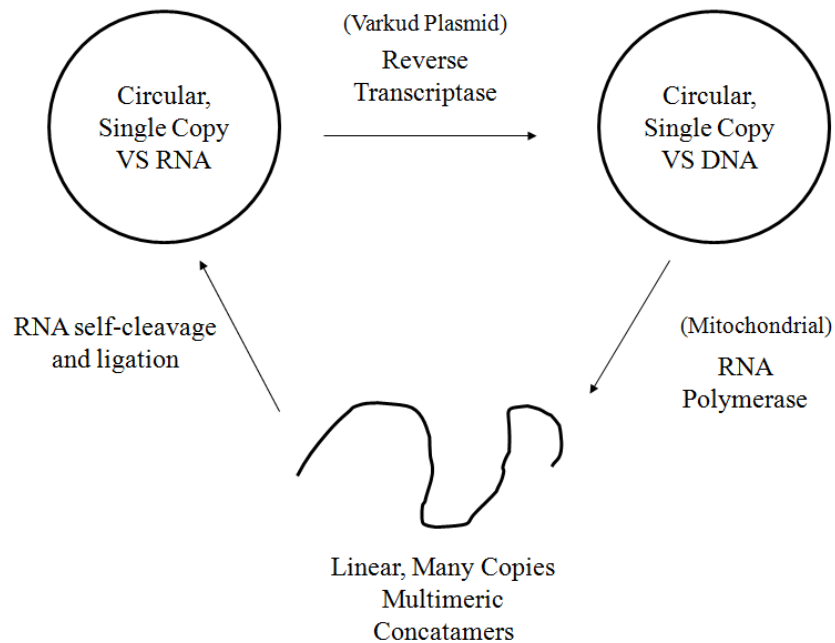


Figure 3. 1 - The life cycle of the Varkud Satellite RNA [29].

The entire VS RNA contains 881 nucleotides, but only a portion of the RNA is required for catalytic activity.[30] For the cleavage reaction to occur, one nucleotide upstream and 153 nucleotides downstream of the cleavage site are required [31]. The VS ribozyme is capable of performing the reverse reaction (ligation) as well as the cleavage reaction [32].

The secondary structure of the VS ribozyme was determined through biochemical and mutational analysis [32, 33]. The general structure of the VS RNA consists of six different helices. Helix I is a 5' stem-loop structure with an internal loop where the cleavage site is located (indicated by the arrow on figure 3.2). Helix I is attached to an H-shaped structure formed by helices II to VI as illustrated in figure 3.2 [32].

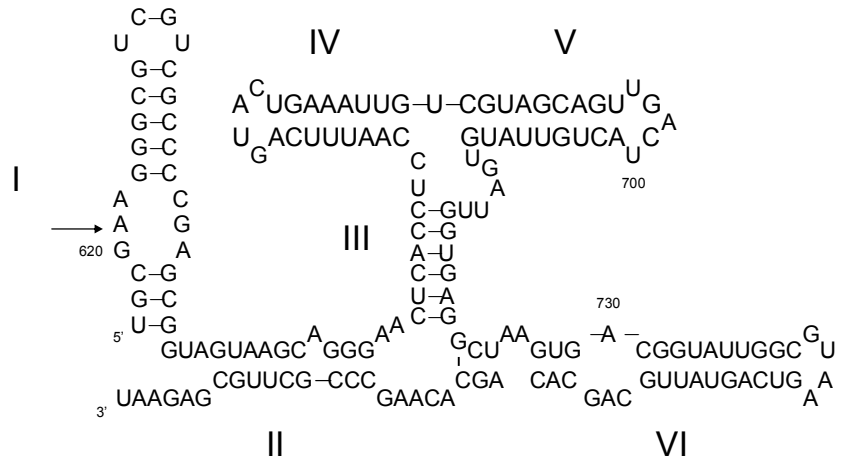


Figure 3. 2 - The secondary structure of the Varkud Satellite RNA [32].

Similar to other small ribozymes, the substrate strand (helix I) can be physically detached from the rest of the RNA and the VS ribozyme can perform the cleavage reaction *in trans* [34]. When the ribozyme acts *in trans* on its substrate, it can perform multiple turnover reactions. Even when the VS ribozyme performs its cleavage reaction *in trans*, it is able to achieve cleavage reaction rates just like the natural *cis*-acting ribozyme [34].

3.2 Structure of the VS Ribozyme

Although there are no crystal structures for the VS ribozyme, the global structure of the ribozyme was determined using fluorescence resonance energy transfer (FRET), nuclear magnetic resonance (NMR) and gel electrophoresis methods [32, 33, 35-38]. It was found that helix I is the substrate domain and helices II to VI forms the catalytic domain. This catalytic domain adopts an H-shaped structure formed by two three-way junctions. The first junction is formed between helices II, III and VI, the second junction is formed between III, IV, and V.

The VS RNA has been extensively studied in the past and many important elements have been discovered through mutagenesis studies [38]. First, the secondary structure must be preserved for catalytic activity. But base substitution in many regions of the RNA sequence do not appear to affect the activity of the ribozyme as long as the secondary structure is conserved and provided that bases within the active site remain unchanged [38].

The entire VS RNA consists of 881 nucleotides in length but a large portion of sequence can be removed and still retain catalytic activity [39]. The majority of helix IV and the end of helix VI can be removed and the ribozyme remains functional, but the lengths of helices II and V are important for the catalytic functions of the ribozyme [39]. Furthermore, the nucleotide sequence within the terminal loop of helix V is required for activity and any changes at this position will result in a great loss of catalytic activity. This suggests that the bulges within helices II and VI may participate in the catalytic activity of the ribozyme [40].

Helix I, the substrate strand has been extensively studied using NMR techniques. The first study was based on the internal loop of the helix, which contains the cleavage site [41]. It was found that there were several non-canonical base pairs within this area where the cleavage site is located. The cut site is located between a G-A base pair and an A⁺-C mismatch pair as indicated by figure 3.3 [42].

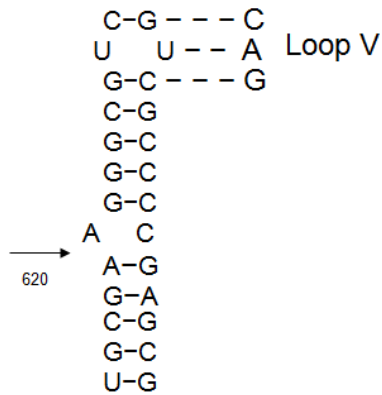


Figure 3.3 - The substrate strand of the Varkud Satellite Ribozyme. The cleavage site is indicated by the arrow at the G-C mismatch. Loop V interacts with the terminal loop of the substrate strand as indicated by the dotted line [41].

3.3 Helical Junctions of the VS Ribozyme

The activity of the VS ribozyme is highly dependent on the structure and sequence of the three-way junctions. These helical junctions are responsible for forming the correct active structure that allows catalytic activity. The two three-way junctions of the VS ribozyme are important in forming the active structure of the ribozyme [35]. The importance of proper folding of RNA has been clearly demonstrated in the hairpin and hammerhead ribozymes. Without the formation of helical junctions that allow the folding of RNA into its three dimensional structures, these ribozymes were unable to perform any catalytic activity [20, 43].

From *in vitro* selection techniques, it was found that the VS ribozyme has two conformations: the “active” structure, and the “inactive” structure [44]. This was deduced from the presence and absence of a ribozyme domain upon binding of magnesium ions. In the presence of magnesium ions, helices III and VI change conformation and become coaxially stacked on each other forming an acute angle ($\sim 65^\circ$) between helices II, IV and a large angle between helices III, VI ($\sim 160^\circ$). This change in conformation creates a cleft where the substrate is believed to bind [35].

The lengths of helices II and VI are extremely important for the function of the VS ribozyme. These two helices are responsible for proper interaction with the substrate allowing it to be correctly positioned so that it is in close proximity to the active site. Helix III is responsible for the formation of the dihedral angle between helices II and VI, creating a cleft that allows the substrate to dock within the active site of the ribozyme [39].

3.4 Catalysis of the VS Ribozyme

The naturally occurring VS ribozyme functions in *cis*, where the substrate unit and the catalytic unit are in one long strand of RNA. However it is possible to split the VS ribozyme into two separate domains, the catalytic domain (helices II to VI) and the substrate domain (helix I). In this case the ribozyme is able to perform multiple turnover reactions as long as the substrate is provided [39]. Using this technique, the cleavage reaction can be monitored as a function of time to determine the kinetics of the ribozyme. From this type of study it was found that the ribozyme has an overall reaction rate of about 2 min^{-1} with an apparent K_M of approximately $1 \mu\text{M}^{-1}$ [40].

3.5 Interaction of the substrate with the VS ribozyme

Most ribozymes interact with their substrates through Watson-Crick and non-Watson Crick base pairing, but this was not observed in the VS ribozyme. From mutagenesis studies it was found that the VS ribozyme interacts with its substrate primarily through tertiary interactions. The exact mechanism is still unclear with the present biochemical data available but in the mean time some models have been proposed [34].

In the proposed tertiary structure of the VS ribozyme, it was shown that there are two contact points between the substrate and the catalytic unit. In the natural *cis*-acting form of the ribozyme, the substrate interacts with the 5'-end of helix II and the terminal loop of helix V. Under these constraints, the only position that the substrate can be placed is within the cleft formed between helices II and VI. This brings the cleavage site of the substrate in close proximity of the A730 loop of helix VI, which is believed to be the active site of the ribozyme [34].

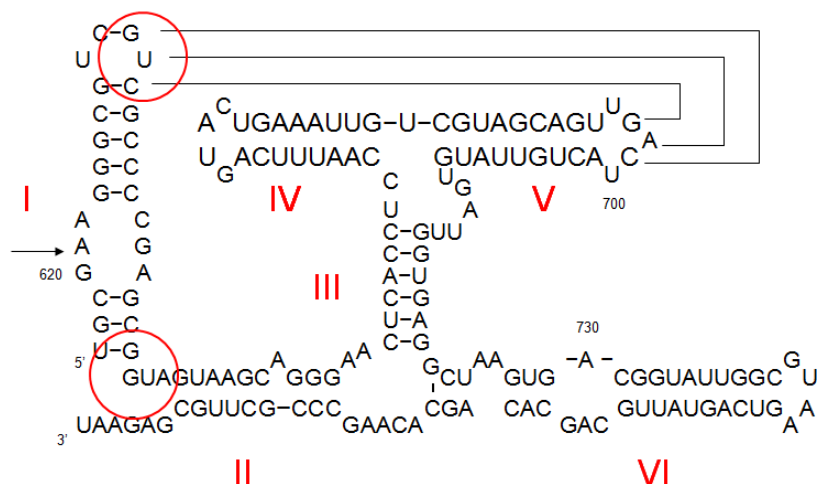


Figure 3. 4 - The secondary structure of the VS ribozyme. The cleavage site is marked by the arrow on helix I. The contact area between the substrate and the ribozyme is marked with the lines and circle.

Further studies showed that helix I is unhindered at the 5' end of the RNA and can be extended in length without affecting the catalytic activity of the ribozyme. In addition, helix I can be detached from the VS RNA and act *in trans* with the VS ribozyme without significant loss of catalytic activity [34]. Moreover, helix I can be attached to the catalytic unit in various ways (attaching with the 5'-end or the 3'-end) and it does not affect the cleavage activity of the ribozyme.

The VS ribozyme can also catalyze the ligation reaction (reverse) as opposed to the cleavage reaction. The ligation reaction of the VS ribozyme has not been extensively studied, but it was found that extension of the 5' and 3' termini leads to better ligation of the RNA. It was shown in other studies that the ligation reaction can be promoted by extending the length of helix I by more than 10 nucleotides. This extension appears to prevent the dissociation of the substrate RNA before ligation reaction occurs [45].

3.6 The Active Site of the VS Ribozyme

There are several important nucleotides that are required for catalytic activity of the VS ribozyme. One of the most important nucleotide is the adenine at position 730 within the A730 loop in helix VI.[40] Any substitution or deletion of this nucleotide will result in a significant loss of cleavage activity in both the *cis* and *trans* form of the ribozyme even if the VS RNA was able to fold into its secondary structure. Studies of the secondary structure of the VS ribozyme explain this observation. When the substrate is docked within the cleft formed by helices II, III and IV, it naturally brings the cleavage site on the substrate in close proximity to the adenine at position 730, suggesting that the A730 loop may be the active site of the ribozyme [38].

Within the A730 loop, the A756 also appears to play an important role in the cleavage reaction. Any substitution of this adenine with any other nucleotide causes a 300-fold reduction in cleavage and ligation activity even though the substrate is able to bind to the ribozyme [40].

Functional group modification studies on the A756 showed that any removal, replacement or addition of hydroxyl groups or translocation on the purine would lead to a major decrease in activity [40]. The studies also showed that the adenine appears to be involved in the cleavage reaction using the Watson-Crick edge of the base. It is still unclear exactly how the A756 participates in the reaction. It is also speculated that A756 may have a structural role in the reaction; yet, further studies are required to confirm these speculations.

3.7 Catalytic Mechanism of the VS Ribozyme

What can be concluded is that the active site of the ribozyme is located within the A730 loop but the exact catalytic mechanism is still unknown. The interaction between the ribozyme and the substrate differs from other small nucleolytic ribozyme.

From the similarities observed in the VS ribozyme and the small nucleolytic ribozyme, it was predicted that the reaction mechanism of the VS ribozyme should also follow the reaction scheme for the small nucleolytic ribozyme [37].

The reaction begins with a nucleophilic attack of the 2'-oxygen on the 3'-phosphorus in a S_N2 reaction mechanism forming an intermediate. It then proceeds with the 5'-oxygen producing the final cyclic 2'3'-phosphate product. In the reverse reaction, the 5'-oxygen attacks the 2'3'-phosphorus of the cyclic phosphate to form the internucleotide phosphodiester linkage.

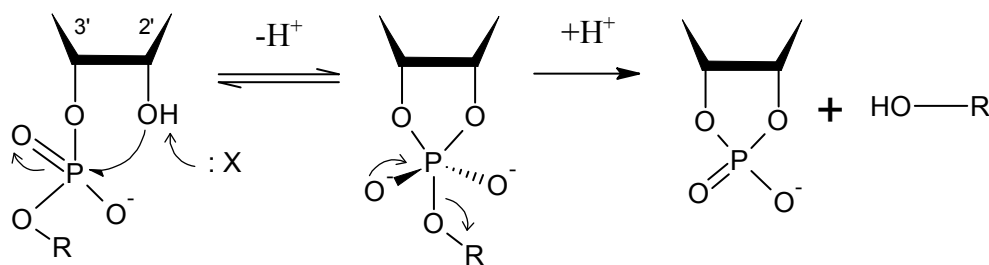


Figure 3. 5 - The proposed catalytic mechanism of the Varkud Satellite RNA [35].

3.8 Research Objectives

The primary goal of the research was to synthesize a catalytically active version of the Varkud Satellite ribozyme that is made up of multiple parts that can be used in detail studies of ribozyme kinetics and its dependence on ribozyme assembly. To achieve this goal three separate approaches were tested and are summarized in the following chapters.

Chapter 4: Results and Discussion

4.1 Introduction

This chapter covers all the experimental details in the synthesis of the VS ribozyme. The primer design and experimental protocols will be discussed for each study along with all the problems encountered in the production of the VS ribozyme.

4.2 Study 1: Three Part System with Hepatitis Delta Ribozyme Sequence

In order to characterize the changes in conformation within the helices of the VS ribozyme, a “divide and conquer” approach was used. This method involves preparing the VS ribozyme as a three-part system.

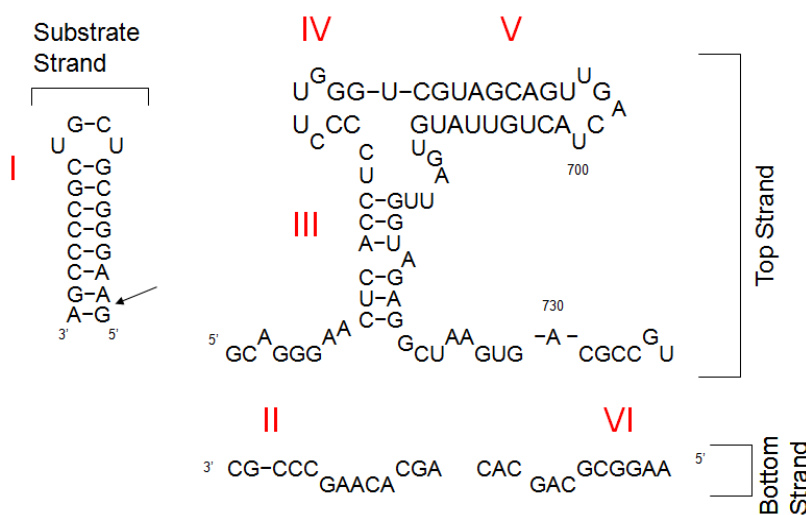


Figure 4. 1 - The secondary structure of the VS ribozyme as a three-part system

The first part is the substrate strand, consisting of helix I. This RNA is commercially synthesized with a fluorescence tag at the 5'-end of the RNA. During the cleavage reaction of the ribozyme the substrate would be cleaved and resulting a smaller RNA fragment containing the fluorescence tag. This change in size can be

detected using polyacrylamide gel electrophoresis allowing kinetics studies to be performed.

The second part of the VS ribozyme is the “bottom” strand consisting of helices II and VI. This RNA would be synthesized from a DNA template with the following sequence.

5'- GGGCGGGCTTGTGCTGTGCTGCCGTCCTATAGTGAGTCGTATTA -3'

The sequence marked in red is the T7 promotor region and the region in blue is the DNA template for the VS “bottom” strand sequence.

This template was ordered from Sigma Aldrich, and upon arrival it was dissolved in nuclease free water. Transcription reactions were performed using the DNA template to create the VS “bottom” strand and was analyzed using 12% polyacrylamide gel electrophoresis. The band of containing the RNA was cut out using a scalpel and “crush-and-soak” was performed to extract the RNA from the gel fragments. Ethanol precipitation was performed to precipitate out the RNA. After precipitation, the ethanol was removed and the RNA pellet was dissolved in nuclease free water. A successful transcription of the “bottom” strand RNA is shown in figure 4.2.

The lane of the left is the RNA transcription result and the lane on the right is a RNA loading dye. The RNA loading dye contains xylene cyanol FF and bromophenol blue and on a 12% polyacrylamide gel they migrate approximately 70 bp and 20 bp respectively. The DNA template used for the transcription is 44 bp which correlates to the top band as it migrated slightly below the xylene cyanol FF. The expected size of the RNA is 26 bp which migrated slightly above the bromophenol blue, hence it was concluded that the RNA bottom strand was successfully synthesized.

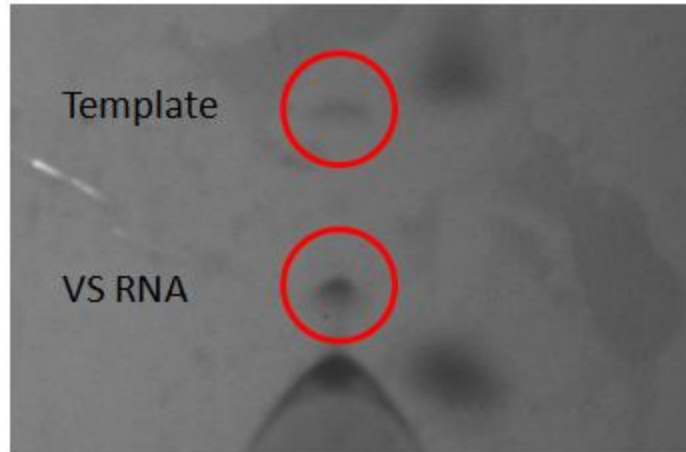


Figure 4. 2 - Transcription result for the VS "bottom" strand. The lane on the left is the transcription result and the lane on the right is the RNA ladder. The band with the largest size is the DNA template, the middle band is the VS "bottom" RNA the bottom strand are the unused nucleotides.

The top part of the catalytic unit is synthesized from a DNA template produced by a series of primers from a PCR reaction. A T7 promotor sequence is incorporated before the VS ribozyme sequence with a hepatitis delta ribozyme sequence at the end. The T7 promotor sequence is placed before the VS ribozyme sequence so that the RNA can be synthesized using the T7 bacteriophage RNA polymerase. A hepatitis delta virus RNA sequence was placed after the VS ribozyme sequence because T7 RNA polymerase mediates non-templated addition of nucleotides during transcription such that one or two extra nucleotides are added at the 3'-end of the transcript. The purpose of the hepatitis delta ribozyme RNA sequence is to perform self-cleavage reaction after the RNA has been transcribed thereby removing unwanted nucleotides to create the desired 3' end.

The template that was used to create the VS "top" RNA is shown below.

5'-

GGTAATACGACTCACTATAGGGCAGGGAACCTCACCTCCCCTTGGGTCGTAGCAGTTGACTACTGTTATGT
 GATTGGTAGAGGCTAAGTGACCGTCCGGGTCGGCTGCAGACCCAGCCTCCTCGCGGCCCGACCTGGGCA
 TCTTCGGATGGCGAATGGGTACCTCGAGTCC -3'

The T7 promotor is highlighted in red, the VS RNA template is highlighted in blue and the hepatitis delta virus ribozyme template is highlighted in green.

Using the primer oligonucleotides ordered from Sigma Aldrich, PCR reactions were performed to generate the DNA template, but several problems were encountered in this process. Originally a temperature of 59°C was used for the renaturation in the first round of PCR, but this was also the melting temperature for the first forward primer. This gave a product that was approximately 125 bp in length which was far too small to be the expected template size indicating alternate products were formed. Various different settings were tested for the renaturation step and it was found that three PCR reactions were required to generate the correct size product.

Instead of the protocol listed in the experimental section, a variation of the original protocol was used. The primers are dissolved in nuclease-free water to make a final concentration of 100 nmol/μL. 5 μL of each of the forward and reverse set of primers were mixed together to create the primer solution. 3 μL of this primer mix was added to 39.75 μL of nuclease-free water, 5.0 μL of 10x *Pfu* buffer, 1.25 μL of 10mM dNTP mix solution and 1.0 μL of *Pfu* DNA polymerase. This solution was made in a 0.2 mL microcentrifuge tube and placed in the PCR thermal cycler. The sequence was amplified using the following cycle: 94°C for 1 minute, 70°C for 30 seconds, 72°C for 90 seconds and 72°C for 10 minutes. This cycle was repeated 30 times. The template formed from the first PCR reaction is then used as a template for the second round of PCR.

The second round of PCR was performed using 1.0 μL of the template from PCR cycle 1, 39.75 μL of nuclease-free water, 1.0 μL of *Pfu* DNA polymerase, 5.0 μL of 10X *Pfu* buffer, 1.25 μL of 10mM dNTP mix solution, 1.0 μL of the forward primer and 1.0 μL of the reverse primer. This second PCR reaction mixture was subjected to 94°C for 3 minutes, 59°C for 30 seconds, 72°C for 10 minutes, 72°C for 90 seconds. This cycle was repeated 30 times creating template #2.

1.0 μL of template #2 was then used for a third round of PCR with 39.75 μL of nuclease-free water, 5.0 μL of 10x Pfu buffer, 1.0 μL Pfu DNA polymerase, 1.25 μL of 10mM dNTP, 1.0 μL of the forward primer and 1.0 μL of the reverse primer. The third PCR cycle goes through a 94°C for 2 minutes and 20 seconds, 57°C for 20 seconds, 72°C for 15 seconds and is repeated 30 times.

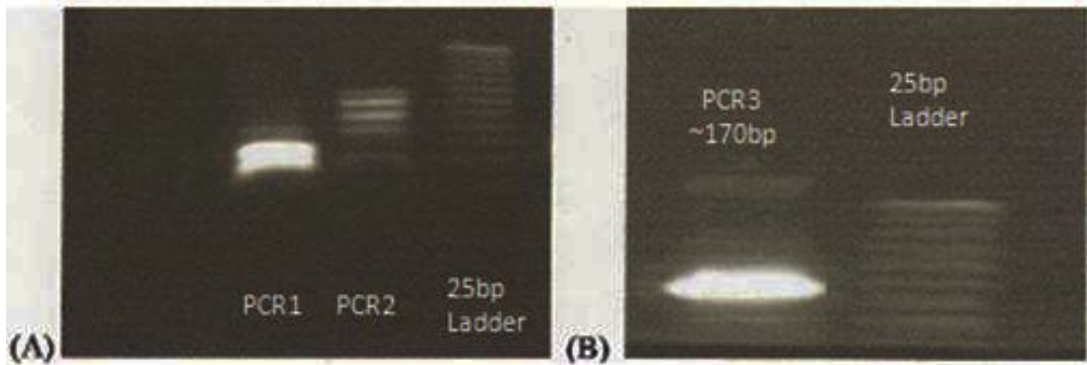


Figure 4.3 – PCR product for study 1. (A) The first (lane 1) and second (lane 2) rounds of the PCR reaction along with a 25bp DNA ladder (lane 3). (B) Results of the third round of PCR reaction (left lane) and the 25bp DNA ladder (right lane).

Results of the PCR reactions are shown in figure 4.3. In figure 4.3, (A) the first round of the PCR produced a fragment that was far too small to be the expected sequence, but used as a template for the second round of PCR. The second PCR had its renaturation temperature lowered from 70°C to 59°C to allow the binding of the first forward primer and the last reverse primer on the template.

Lane 3 in figure 4.3 (A) shows the product of the second round of PCR reaction, as seen from this figure, the lane shows various different band fragments. This product is not the desired product but is used as a template for the third PCR reaction. The results of the third round of PCR are shown in figure 4.3 (B). In this figure, there is a bright band present at approximately 171 bp, which is the correct size of the desired sequence indicating that PCR had successfully created the correct size template.

The 171 bp fragment was extracted from the agarose gel using a scalpel and was purified then cloned into the pSC-B cloning vector. This vector was chosen because the PCR product was blunt ended, and the vector allows ligation of blunt ended DNA sequences. After cloning and purification the plasmid was sent to the Mobix Laboratory in the University of McMaster for sequencing.

The results from the Mobix laboratory showed that the DNA sequence is indeed correct and is in the forward direction. But this plasmid sequence cannot be used as a template for transcription because the pSC-B vector has a T7 promotor sequence itself while the DNA template design also has a T7 promotor sequence. This would cause inefficient transcription because of the presence of two promoter binding areas. Therefore the template sequence was transferred from the pSC-B vector to a high copy pUC-18 vector. The template and vector were both double digested using *Bam*HI and *Hind*III restriction enzymes and ligation reaction was performed after.

The ratio 1:1 of insert: vector was found to produce the greatest amount of white colonies when grown on agar plates with ampicillin. A ratio of 1:3 had also produced white colonies but in smaller amount where the 3:1 ratio did not produce any growth of colonies. Using the 1:1 ratio, the insert and vector were transformed into SURE competent cells. The results of this transformation are shown in figure 4.4.

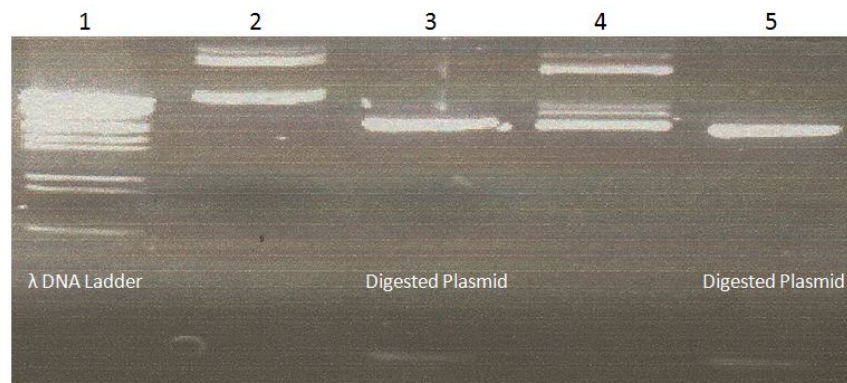


Figure 4. 4 - Transformation result of the VS DNA template into the pSC-B plasmid. Lane 1 is the λ-DNA marker and lanes 2 to 5 are the transformation sample.

Lane 1 is the λ -DNA marker and lanes 2 to 5 are the transformation samples. Samples in lane 3 and 5 had the desired sequence successfully cloned into the pUC-18 vector, but lanes 2 and 4 shows incomplete digestion. The product from lane 5 was purified and extracted using phenol: chloroform followed by ethanol precipitation. Once the DNA is precipitated it was transcribed using T7 RNA polymerase enzyme and the result is verified using 6% polyacrylamide gel electrophoresis.

Different magnesium concentrations with various heating and cooling cycles have been tested for the cleavage reaction of the hepatitis virus delta virus so that the VS ribozyme sequence “top” strand can be extracted but it was unsuccessful.

Folding analysis of the hepatitis delta virus ribozyme sequence and the VS ribozyme sequence was performed using the M-Fold program [46]. This program revealed that several extremely stable alternative secondary structures can be formed. Formation of these structures likely has prohibited the hepatitis delta virus ribozyme from folding into its correct structure and hence the VS “top” strand RNA was not properly cleaved.

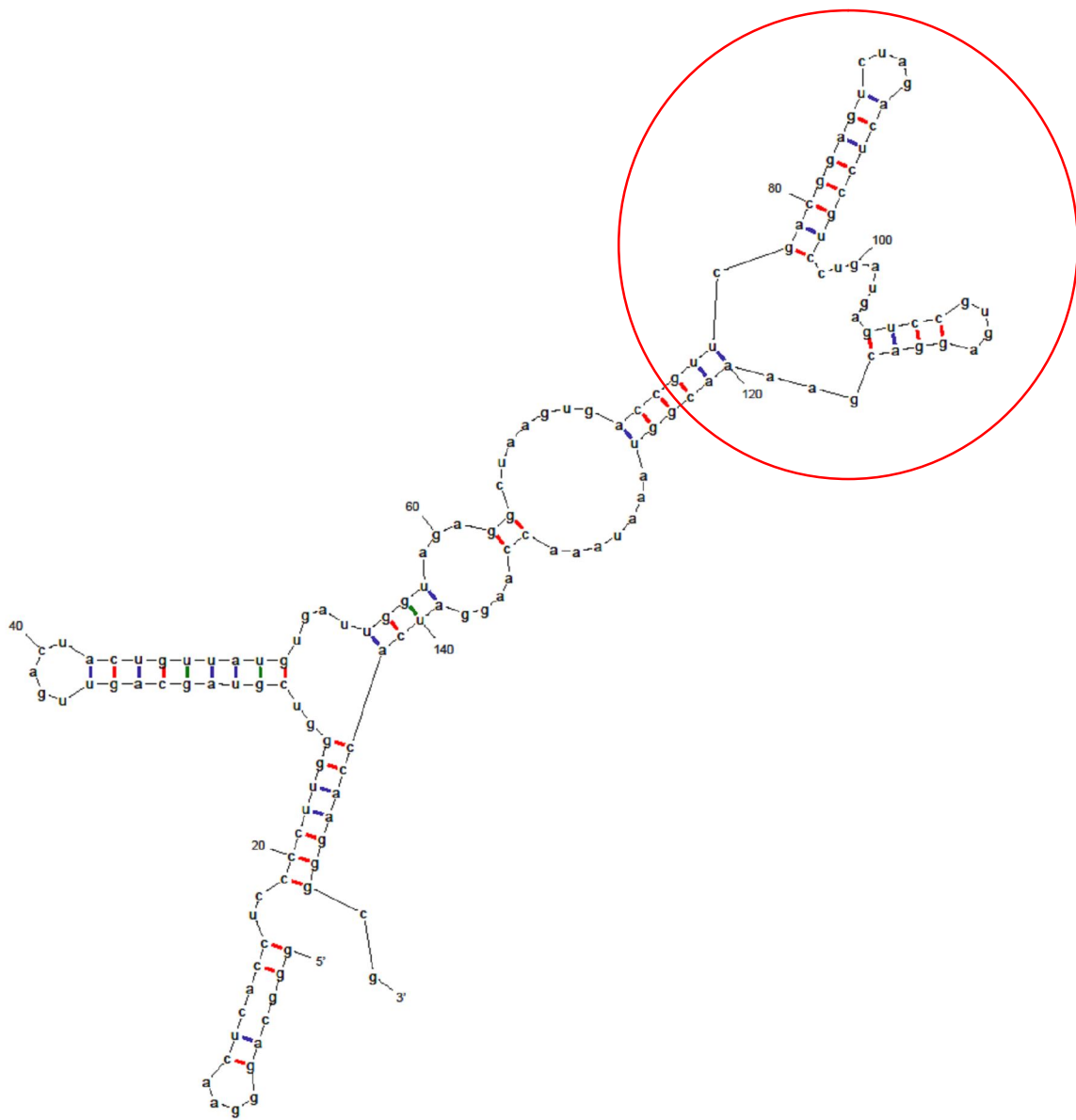
4.3 Study 2: Three Part System with the Hammerhead Sequence

Sequence analysis and RNA folding studies revealed that the hepatitis delta ribozyme was not formed properly to allow the cleavage reaction to take place. Therefore a new sequence was designed to generate the VS “top” RNA strand. From the study of other ribozyme structures, it was concluded that the hammerhead ribozyme sequence combined with the VS “top” RNA sequence was able to generate a proper RNA structure that should allow the cleavage reaction to occur. The following sequence was designed:

5’-

GGTAATACGACTCACTATAGGGCAGGGAACTCACCTCCCCTTGGGTCGTAGCAGTTGACTACTGTTATGT
GATTGGTAGAGGCTAAGTGACCGTTCGACGGAGTCTAGACTCCGTCCTGATGAGTCCGTGAGGACGAAA
ACGGTAAATAACCAAGGATCACC -3’

The sequence marked in red is the T7 promotor sequence, the sequence marked in blue is the VS “top” sequence and the sequence marked in green is the hammerhead RNA sequence. The T7 promotor is placed before the desired RNA sequence creating a position for the T7 polymerase to bind allowing transcription to begin. Similar to the hepatitis delta virus RNA, the hammerhead RNA and the VS “top” RNA will be transcribed as a single stranded RNA. What is expected to happen is that the RNA would fold into its secondary structure forming the VS “top” RNA and the hammerhead ribozyme will perform self cleavage reaction releasing the VS “top” RNA.



$dG = -54.63$ [initially -58.20] 09Jul27-15-55-18

Figure 4. 5 - M-Fold prediction of the RNA product that will be transcribed using the template for study 2. The hammerhead ribozyme structure is circled in red [46].

In principle, only two rounds of PCR should be sufficient to produce the full length product, but even after three rounds of PCR, the correct size template was not observed. The PCR protocol for this part of the study is in the appendix. The expected size product was obtained after the fourth round of PCR as shown in figure 4.7.

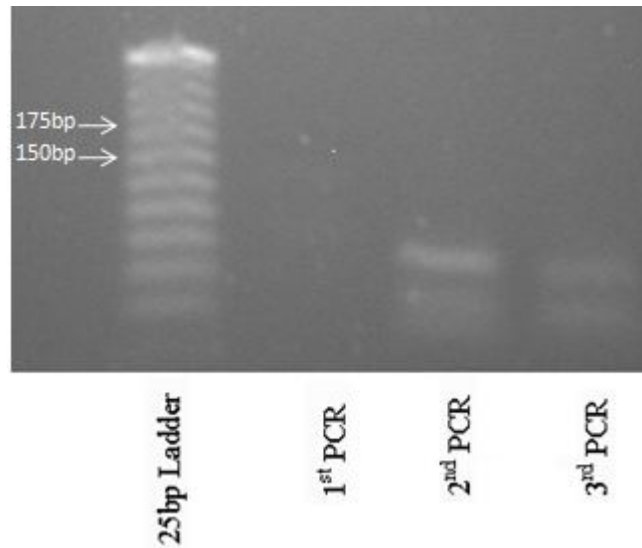


Figure 4. 6 - The first three rounds of PCR for study 2.

PCR reactions 1-3 did not form the entire sequence of length 163 bp as shown in figure 4.6. After the fourth round of PCR, a band of approximately 163 bp was shown in figure 4.7, which is the size the expected template.

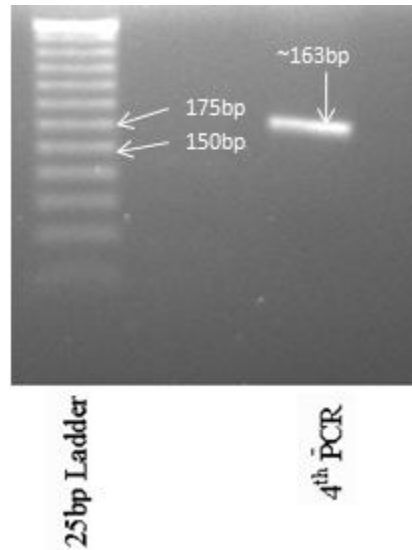


Figure 4. 7 - The fourth round of PCR for study 2. The fourth round of PCR generated the full length DNA template.

The template was extracted, purified and ligated into pSC-B plasmid and then transformed into SURE competent cells using heat shock transformation. Cells that were white are grown in LB-amp broth and the plasmid is extracted, digested and was analyzed on 1% agarose gels. A sample of the digested plasmid was sent to the Sequencing Facility in the Biology Department of the University of Waterloo.

Plasmid that contains the correct sequence was double digested with BamHI and *HindIII* restriction enzymes and the insert was transferred into pUC18 plasmid. This is because the pSC-B plasmid has a T7 promotor within the plasmid sequence and the VS “top” RNA sequence designed for this study also had a T7 promotor sequence within it. Having two different T7 promotor sequence will cause inefficient binding of the T7 RNA polymerase and will result in impaired transcription.

The pUC-18 with the VS “top” RNA was then transformed into StrataClone™ cells from Stratagene using the heat shock protocol. Cells were selected using x-gal and the colonies that appear white was chosen and grown in LB-amp media. Plasmid

preparation was performed to determine if the sequence was cloned into the vector properly and a sample of the plasmid was sent for sequencing using the M13 primer.

At this point there were errors in the sequencing results and all the samples sent were returned as failed. For some unknown reason the reverse M13 primer within the pUC-18 plasmid was dysfunctional and is unable to be used as the primer for sequencing. As all samples sent for sequencing requested the use of this primer, all sequences came back as failed. Therefore, more cells were grown, purified and digested and sent for sequencing with the hopes of getting a positive sequencing result. However, none of the cells were able to provide a functional M13 reverse primer and hence it was unable to determine if the VSHH sequence was cloned into the vector successfully. In troubleshooting what might have gone wrong, another approach was attempted to determine if the chosen colony contained the plasmid with the VSHH insert.

After reviewing the restriction enzyme sites on the plasmid and the insert, the *HindIII* restriction enzyme was chosen for the digestion process. After linearization of the plasmid, samples were prepared and sent for sequencing again but using the M13 forward primer. This would produce a complimentary sequence of the VSHH, an inverse correlation to the forward sequence. By analysis with the BLAST program online and comparing the sequence design, it was confirmed that VSHH sequence was present in the plasmid.

Cells that contained the correct template sequence were grown in LB-amp media to produce large amount of the template sequence to allow transcription reaction to be performed. The plasmid from these cells was extracted and purified using Qiagen Maxi Preparation Kit and then linearized using *EcoRI* restriction enzymes. The linearized plasmid was purified and dissolved in nuclease free water.

Multiple transcription tests were performed with the sequence and the Promega Transcription Kit gave the best result. As the RNA sequence produced contains a hammerhead ribozyme sequence, folding and cleavage of RNA often occurs during the transcription process. When the transcription reaction was complete, it was analyzed using electrophoresis using 8% polyacrylamide gel and a total of four bands can be perceived. The first and largest band was assigned to be the plasmid, the second largest band to be the uncleaved VS RNA and hammerhead ribozyme transcript (size of approximately 163 bp), the third fragment is the cleaved VS “top” RNA sequence (size of approximately 97 bp) and smallest fragment is the cleaved hammerhead RNA with a size of 66bp.

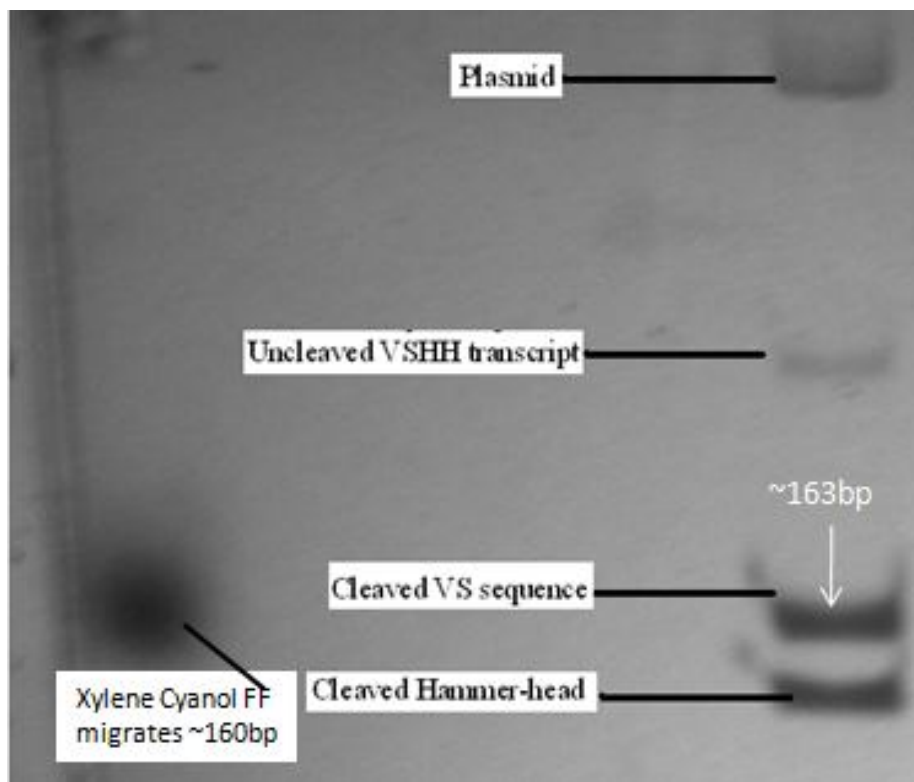


Figure 4. 8 - Transcription result from of the DNA template from study 2

Unfortunately, when the transcription reaction was replicated using the same protocols and solution, no RNA was made. Many different conditions were tried hoping to get the transcription to work but failed. It was believed that the hammerhead ribozyme formed was undergoing non-specific cleavage reaction and therefore whatever that was transcribed was cleaved as well.

4.4 Study 3: Two Part System

Due to transcription problems of the previous approach, a new RNA construct was designed. The sequence used for this study does not include an extra ribozyme but instead a restriction enzyme site at the end so that transcription will stop when it reach the end of the template. The sequence design for this study includes most of the VS ribozyme catalytic unit with the removal of six nucleotides at the 3'-end. This structure was chosen so that binding studies between the substrate and the VS catalytic unit can be performed using the Axela™ DotLab System.

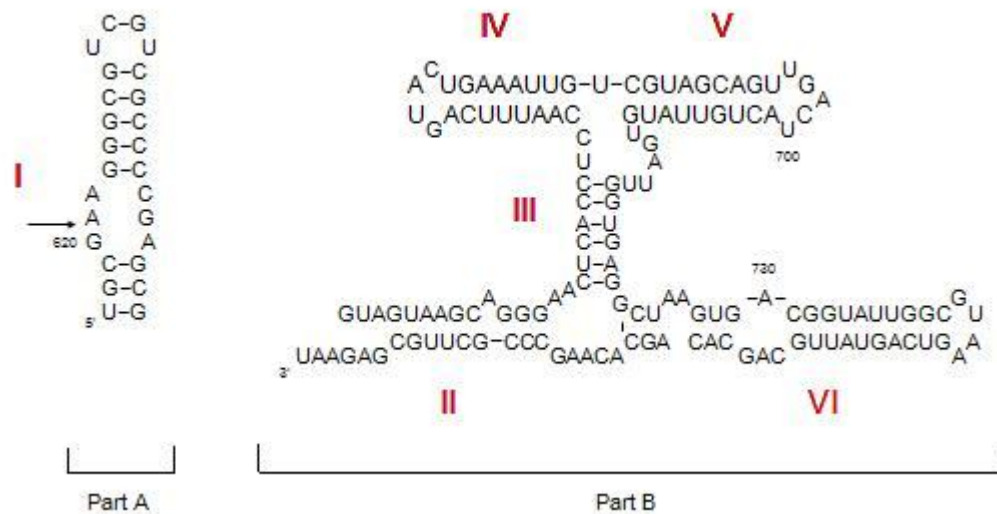


Figure 4. 9 - Structure of the Varkud Satellite ribozyme as a two part system

The sequence that was designed to generate the VS catalytic unit is shown below

5'-

GUAAGCAGGGAACUCACCUCCAUUUCAGUACUGAAAUUGUCGUAGCAGUUGACUACUGUUAUGU
 GAUUGGUAGAGGCCUAAGUGACGGUAUUGGCGUAAGUCAGUAUUGCAGCACAGCACAAGCCCGCUU
 ACGAGAAU -3'

The DNA template that was designed to create this template is shown below.

5' –

AGTGGAAATTCGCAAGCGGGCTTGTGCTGTGCTGCAATACTGACTTACGCCAATACCGTCACTTAGCCTCT
ACCAATCACATAACAGTAGTCAACTGCTACGACAATTCAGTACTGAAATTGGAGGTGAGTTCCCGCTTA
CTATAGTGAGTCGTATTAAAGCTTGGG -3'

The sequence marked in red is the T7 promoter sequence, the sequence marked in blue is the VS RNA template sequence and the sequence marked in green are the two restriction enzyme sites (the first one is the *EcoRI* site and the second one is the *HindIII* site)

Six primers were designed using the PCR Oligo Maker[47]: Four assembly primers and two flanking primers. The PCR reactions were successful and the second PCR reaction gave a single product of approximately 170bp in size.

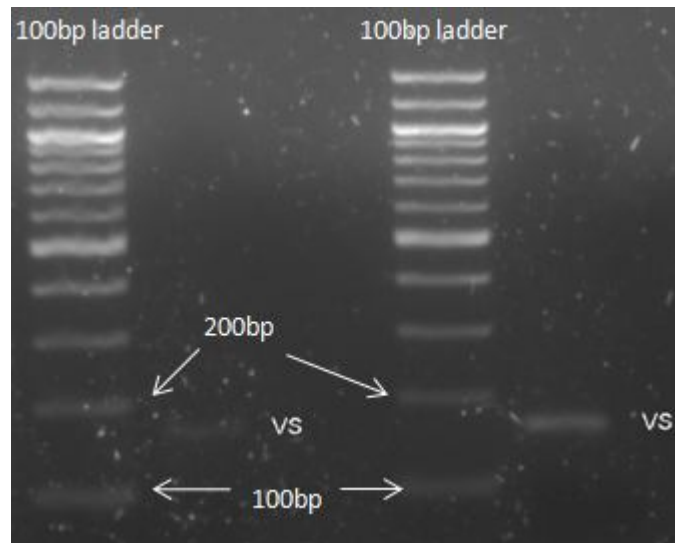


Figure 4. 10 - PCR result of the DNA template VS design #1

The PCR product was purified, digested and ligated with the pUC-18 vector. The plasmid was transformed into competent cells and was grown on LB-ampicillin selection

plate containing x-gal. Cells that appear white on the selection plates were re-grown in LB media and plasmid extraction was performed. A sample of the plasmid was sent for sequencing at the Biology department of the University of Waterloo.

Sequencing results indicated that the correct sequence is present within the plasmid. Therefore large scale production of the plasmid template was performed.

Multiple transcriptions were performed with the plasmid template but there was no detectable production of RNA. It was first thought that transcription solution was not optimized and therefore many conditions were tested without success.

Further study of the DNA template indicated that a uracil was placed at the second position of the transcript causing impaired transcription; hence no transcription product was obtained. Therefore the RNA sequence was modified and the uracil was changed to a guanine and base paired with a cytosine. This change should not affect the catalytic activity of the ribozyme because this base pair is not involved in the catalytic reaction and the secondary structure of the VS ribozyme was not altered. The new RNA sequence is shown below.

5'-
GGAAGCAGGGAACUCACCUCCAUUUCAGUACUGAAUUGUCGUAGCAGUUGACUACUGUUAUGU
GAUUGGUAGAGGCCUAAGUGACGGUAUUGGCGUAAGUCAGUAUUGCAGCACAGCACAAGCCCGCUU
CCGAGAAU -3'

The DNA sequence that is used to generate the RNA sequence is shown below.

5'-
ATTCTCGGAAGCGGGCTTGTGCTGTGCTGCAATACTGACTTACGCCAATACCGTCACTTAGCCTCTACCAA
TCACATAACAGTAGTCAACTGCTACGACAATTCAGTACTGAAATTGGAGGTGAGTTCCTGCTTCCCTAT
AGTGAGTCGTCTTA -3'

The sequence in red is the T7 promotor and the sequence in blue is the VS RNA template sequence. To generate this DNA template, six primers were designed using the PCR Oligo Maker program for the PCR reaction[47]: Four assembly primers for the first PCR and two flanking primers for the second PCR.

The PCR reactions were very successful. In the first round of PCR a clear band of approximately 170 bp was shown. Using the crude PCR1 reaction mixture, amplification of this full length template was performed in PCR2 using the two flanking primers. The results of the PCR reactions are shown in figure 4.11.

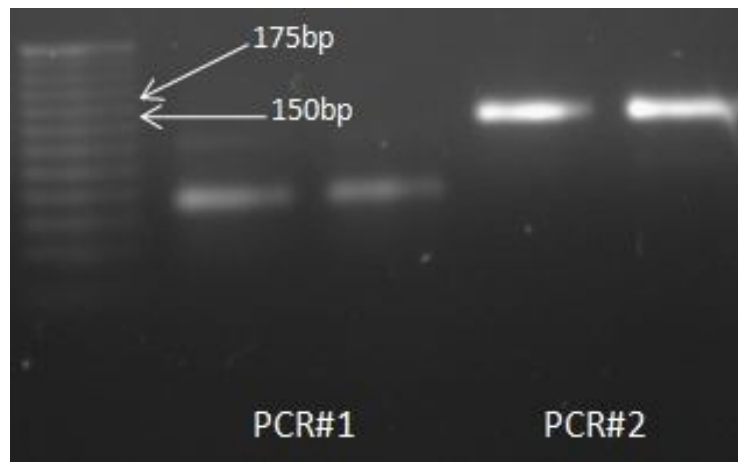


Figure 4. 11 - PCR of the DNA template VS design #2

Lane 1 is the 25bp DNA step ladder, lanes 2 and 3 are the products of the first PCR reaction and lanes 4 and 5 are the products of the second PCR reaction. During the first PCR cycle, DNA of various sizes were obtained but after amplification in the second PCR reaction, a clear band was seen.

After purification of the PCR product, the DNA was subjected to double digestion using both *EcoRI* and *HindIII* restriction enzyme and was digested for 3 hours. Result of this digestion is shown in the figure 4.12.



Figure 4. 12 - Double digestion of the DNA template design #2

Analysis using 2% agarose gel showed that the DNA template was not digested at all. Further studies of the DNA template using BLAST analysis indicated that there are no restriction enzyme sites within the designed template.

A solution to this problem was to add the restriction enzyme sites with an additional PCR step. The idea is to use two addition primers that will partially overlap the 5' and the 3' ends of the template generated from the second PCR step. Along with the overlap regions a restriction enzyme binding sequence will be added at each end. An illustration of this addition is shown in the figure below.

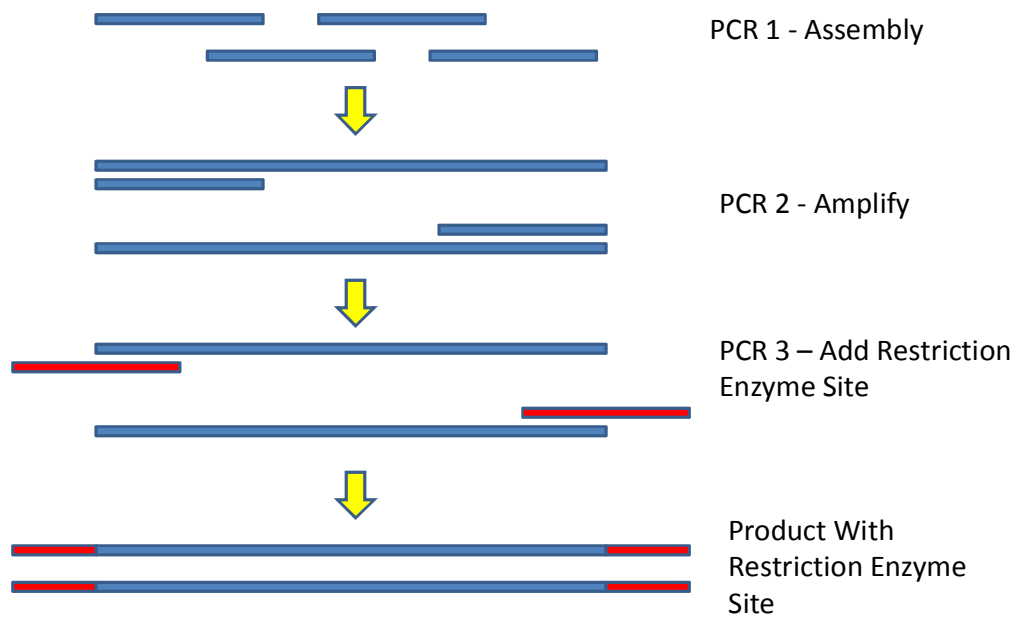


Figure 4. 13 - Illustration diagram for addition of restriction enzyme sites on a DNA template

After purifying this sequence, double digestion of the template was performed using *EcoRI* and *HindIII* restriction enzyme. Analysis of this digestion using 2% agarose gel gave surprising results. Various fragments of different lengths were observed on the gel with all the PCR samples that were performed. It suggested that various primers were binding at the incorrect places forming different DNA templates and hence giving this result.

The fix to the previous problem was unable to produce the desired result; therefore a new DNA template was designed that included restriction enzyme sites and the T7 RNA polymerase promoter sequence.

5'-

AGTGAAGCTTAATACGACTCACTATAGGAAGCAGGGAACTCACCTCCAATTCAGTACTGAAATTGTCG
TAGCAGTTGACTACTGTTATGTGATTGGTAGAGGCTAAGTGACGGTATTGGCGTAAGTCAGTATTGCAGC
ACAGCACAAGCCCGCTCCGAGAATTCGGG -3'

The sequence marked in red is the T7 promoter sequence, the sequence marked in blue is the VS RNA template sequence and the sequence marked in green are the two restriction enzyme sites (the first one is the *Hind*III site and the second one is the *Eco*RI site)

Using the PCR Oligo Maker and the designed DNA template, six primers were created for the PCR reaction [47]: four assembly primers and two flanking primers.

Results of the PCR reaction is shown in figure 4.14. Lane 2 and 3 are the result from the first PCR reaction where various size fragments were observed, but there is a band of approximately 170 bp was also present. After amplification of the template from the second PCR reaction, this band was more intense as seen in lanes 4 and 5, indicating that the DNA template was successfully created.

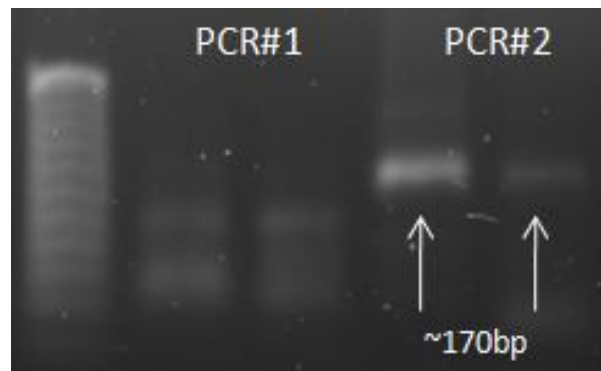


Figure 4. 14 - PCR of the DNA template VS design #3

Double digestion of the DNA template was performed using both *Eco*RI and *Hind*III restriction enzyme so that the template can be inserted into the pUC-18 vector.

From the 2% agarose gel analysis, it showed that digestion was successful; a slightly smaller band was observed when comparing the digestion mixture sample with the original DNA template.

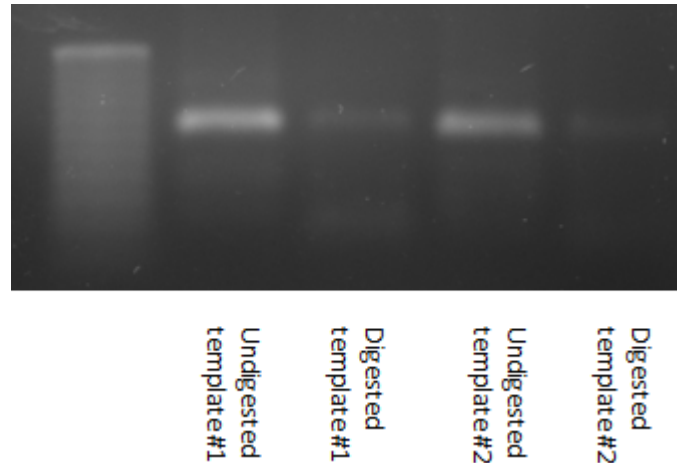


Figure 4. 15 - Double digestion of the DNA template VS design #3

Similarly the pUC-18 vector was also double digested with *EcoRI* and *HindIII* restriction enzyme to allow the ligation of insert to plasmid. Both insert and vector were purified and dissolved in nuclease-free water. Ligation of the insert and vector was then performed overnight followed by transformation of the plasmid into competent cells. Colonies that appeared white were re-grown in LB media, followed by plasmid extraction and sequencing at the Mobix Laboratory in the University of McMaster. Sequencing results are shown below. The M13 reverse primer was chosen for sequencing hoping that it will be able to sequence most of the insert within the pUC-18 plasmid.

NNNNNNNNNNNTNNNNCGANNTCNCGNNNNGGGCTGTGTGCTGTGCAATACTGACTTACGCCNATAACCGTCACTT
 AGCCTCTACCNATCNCATAACAGTAGTCAACTGCTACGACAATTTTCAGTACTGAAAATTGGAGGTGAGTTCCCTGCTTCC
TATAGTGAGTCGTATTAAGCTTGGCACTGGCCGTCGTTTACAACGTCGTGACTGGGAAAACCTGGCGTTACCCAA
 CTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAAC
 AGTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTACACCCGCAT
 ATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCCGTGACGCG
 CCCTGACGGGCTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTGAGAGGT
 TTTCACCGTCATCACCGAAACGCGCGAGACGAAAGGGCTCGTGATACGCCTATTTTATAGGTTAATGTCATGATAAT
 AATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGAAAATGTGCGCGGAACCCCTATTTGTTTATTTTCTAAATACATT
 CAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTC
 AACATTTCCGTGTCGCCCTTATCCCTTTTTTGGCGCATTTTGCCTTCTGTTTTGCTCACCCAGAAACGCTGGTGAAAG
 TAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTACATCGAACTGGNTCTAACAGCGTAAGATCCTTGANN
 GTTTTGCCCCGAANAACGTTTTCCAATGATGAGCACTTTTAAANTTCTGCTATGTNGCGCGGTATTATCCCNTATTGAC
 GCCNNGNNNNNNNCAACTCGGTCGCCGCATACACNANTNCTNANANNNNNNNNNNNNANTACTNNNCANNNNCANA
 AAAANCNTCNNNNNNNNNNNNNNGNNNNNNNNNNNNANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
 NNNNNNANNN

From the sequencing results obtained, the T7 promoter sequence is present (sequence in bold) but most of the insert sequence were not able to be sequenced. This is because of the binding of the M13 primer that was used for sequencing was in close proximity to the VS sequence, therefore some parts of the VS sequence was not able to be sequenced. Even so, with the presence of the T7 promoter within the plasmid it was concluded that the VS template was successfully cloned into the pUC-18 vector. Large scale production of the plasmid was performed to obtain sufficient template for transcription reactions to produce the VS RNA catalytic unit. Plasmid extraction was performed and the plasmid was linearized using *EcoRI* restriction enzyme. The result of the restriction enzyme digestion is shown in figure 4.16. Lane 1 is the 100bp DNA ladder, lane 2 is the undigested plasmid and lanes 3 to 6 are the digested product. As seen in figure 4.16, the linearization was successful where only one band was seen in lanes 3 to 6.

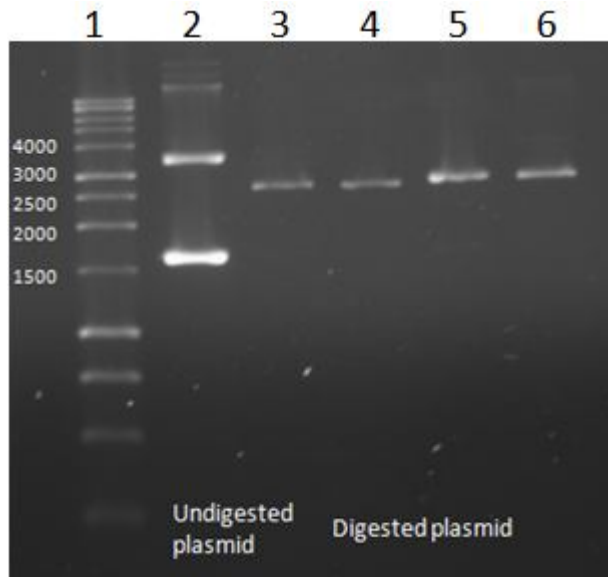


Figure 4. 16 - Double digestion of VS DNA in pUC18 plasmid. Lane 1 is the 100bp DNA ladder, lane 2 is the undigested plasmid and lanes 3 to 6 are the digested product. As seen in figure 4.16, the linearization was successful where only one band was seen in lanes 3 to 6.

The linearized template was purified and dissolved in nuclease free water to a final concentration of 100 ng/ μ L. Transcription reactions were performed using this template and then analyzed using 8% polyacrylamide gel electrophoresis.

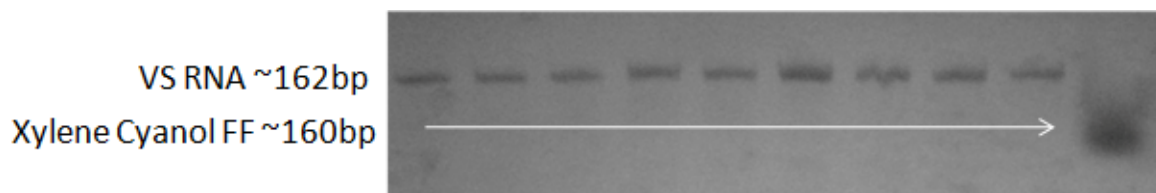


Figure 4. 17 - Transcription result of the DNA template design #3

The expected size of the RNA fragment is 162 bp, and from gel analysis there is a band right above the xylene cyanol FF, this dye migrates as \sim 160bp on an 8% polyacrylamide gel. From this it was deduced that the transcription was successful and the VS RNA catalytic unit was made.

The VS RNA catalytic unit was extracted from the polyacrylamide gel by crush-and-soak in TEN buffer followed by ethanol precipitation. The purified RNA was dissolved in nuclease-free water and was used for kinetic studies.

For the cleavage reaction, 5nM of the substrate was mixed with $\sim 1\mu\text{M}$ of the VS catalytic unit and the reaction was initiated by an addition of 11mM Mg^{2+} . Aliquots of the reaction mixture were taken out and the reaction was terminated by an addition of a solution containing formaldehyde and EDTA.

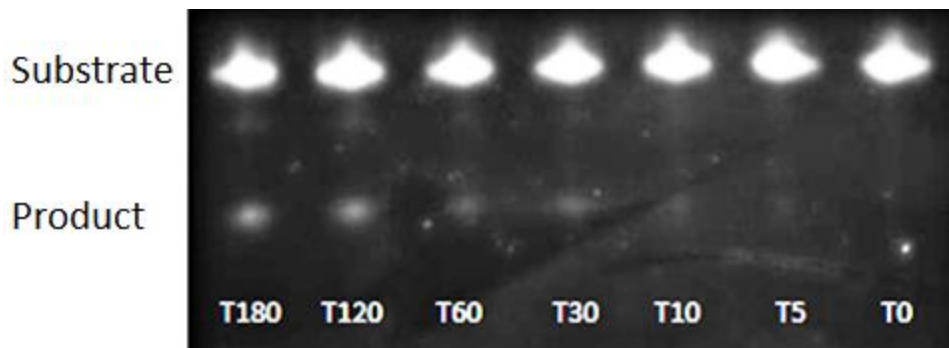


Figure 4. 18 - Cleavage reaction of the VS catalytic unit. The cleavage reaction was performed by mixing the VS catalytic unit with its substrate. Aliquots of the reaction were taken out at 5, 10, 30, 60, 120 and 180 minutes.

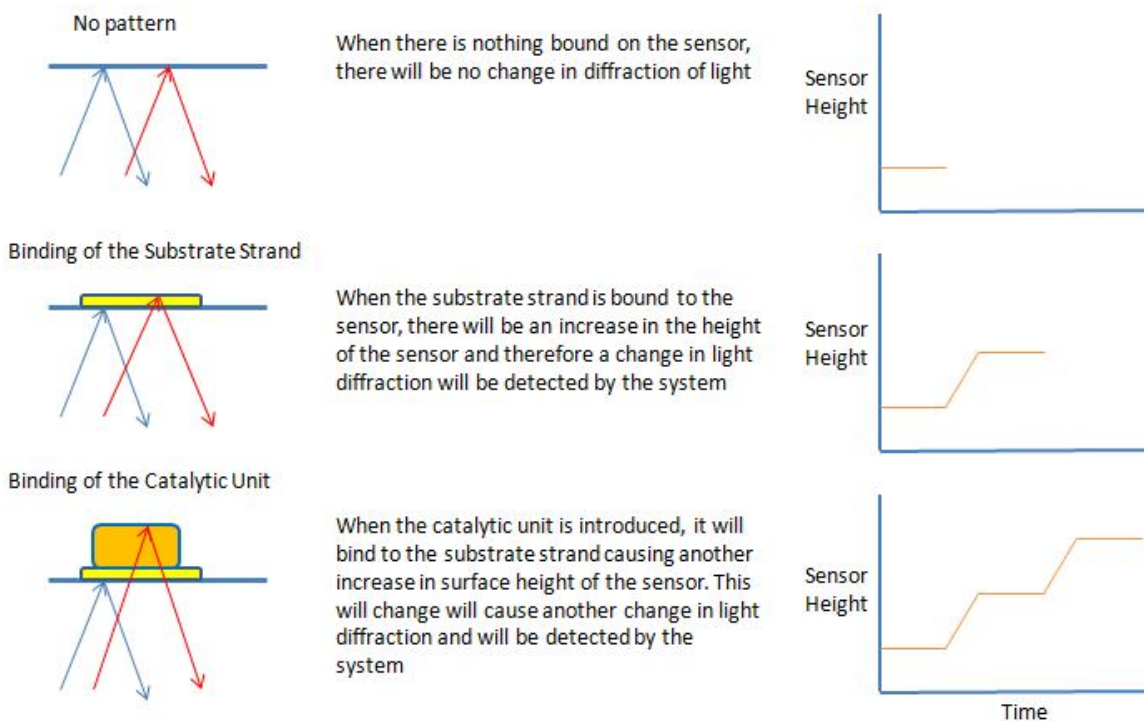
The cleavage reaction was analyzed using gel electrophoresis on a 12% polyacrylamide gel. As shown in figure 4.18, there was a gradual increase in product from the 10 minute mark. A clear band of product was seen after 30 minutes of the cleavage reaction.

The reaction rate of the two part VS ribozyme is very slow. Further optimization of the reaction conditions must be performed to increase the cleavage rate. However this figure clearly demonstrates that a catalytically active version of the VS ribozyme was successfully synthesized.

4.5 Conclusion and Future Studies

The VS RNA catalytic unit was successfully synthesized as shown by the cleavage assay. Extraction and purification of the RNA followed by kinetics studies can be performed with the substrate strand. Optimization of cleavage activity will need to be performed before detailed binding studies can be undertaken.

Binding studies of the substrate and ribozyme can be performed using the Axela DotLab System. The Axela DotLab system measures binding activity based on change in light refraction. It detects changes that occur on the surface of the sensor chip. Initially the substrate strand would be immobilized on the surface of the sensor followed by the introduction of the VS ribozyme catalytic unit. This would cause an increase in surface height of the sensor which can be detected by the Axela DotLab System. This study offers an additional source of information on the binding and release of the VS ribozyme on its substrate. The Axela DotLab System allows real-time kinetic studies by measuring k_{binding} directly.



Chapter 5: Experimental Procedures

5.1 Introduction

This chapter covers the materials and methods used for the synthesis and analysis of the VS RNA. The basic protocol for each step of the reaction is outlined and any modifications will be discussed.

The VS RNA used in this study was prepared enzymatically from a synthetic DNA template using T7 RNA polymerase and unlabelled dNTPs. The DNA template is generated using polymerase chain reaction (PCR) and cloned into high copy plasmids. The plasmid is then transformed into competent cells so that these bacterial cells can be grown to produce large amounts of DNA template to be used for transcription reactions.

5.2 PCR Reactions

The DNA template design is based on the RNA sequence of the VS ribozyme structure. Using the RNA sequence, the DNA template is generated using the PCR Oligo Maker by Johnson's Laboratory group in York University [47]. Restriction enzyme sites need to be manually designed after the template has been generated so that the template can be cloned into suitable vectors.

Once the DNA template is designed, the template is fed into the program to produce suitable primers for PCR reactions. For most of the PCR products, two PCR reactions are required to generate the DNA template. The first PCR reaction is to generate the full length product while the second PCR reaction amplifies the sequence.

All of the primers that are used for PCR are ordered from Sigma Aldrich. Upon arrival, the oligodeoxynucleotides that are used for the first PCR reaction were diluted to 0.125 $\mu\text{g}/\mu\text{L}$ with nuclease free water, while the oligodeoxynucleotides used for the second PCR cycle are diluted to 0.25 $\mu\text{g}/\mu\text{L}$ with nuclease free water.

The reaction protocol for the first PCR reaction is shown in the table below.

Table 1 - Reaction protocol for the first PCR reaction.

Each Assembly Primers (0.125 µg/µL)	4 µL
5mM dNTP mix (5mM of each dATP, dCTP, dGTP, dTTP)	4 µL
10x Thermopol Buffer (NEB)	10 µL
Vent DNA Polymerase (2000 units/mL)	1.5 µL
Nuclease free Water	68.5 µL
Cycles of PCR	8
Denaturation Temperature	94°C for 90 seconds
Annealing Temperature	54°C for 120 seconds
Elongation Temperature	72°C for 180 seconds
Special Steps	<ol style="list-style-type: none">1. During the first cycle the 94°C was performed for 7 minutes2. After the last cycle is complete an additional 5 minutes incubation at 72°C

The reaction protocol for the second PCR reaction is shown in the table below

Table 2 - Reaction protocol for the second round PCR reaction.

Crude Reaction Mixture from the First PCR reaction	1 μ L
The Forward and Reverse flanking Primers (0.250 μ g/ μ L)	4 μ L
5mM dNTP mix (5mM of each dATP, dCTP, dGTP, dTTP)	4 μ L
10x Thermopol Buffer (NEB)	10 μ L
Vent DNA Polymerase (2000 units/mL)	1.5 μ L
Nuclease free Water	75.5 μ L
Cycles of PCR	25
Denaturation Temperature	94°C for 30 seconds
Annealing Temperature	54°C for 120 seconds
Elongation Temperature	72°C for 90 seconds
Special Steps	<ol style="list-style-type: none"> 1. During the first cycle the 94°C was performed for 5 minutes 2. After the last cycle is complete an additional 5 minutes incubation at 72°C

The PCR reactions were analyzed using agarose gel electrophoresis to determine if the correct template was made. The expected size for all the template that was used were approximately 170 nucleotides in length. Therefore for all the PCR reactions, a 2% agarose gel concentration was chosen because it is able to separate DNA fragment sizes 50 to 2000 base pair DNA. A 25bp DNA step ladder was also loaded along with the sample to determine the size of the template formed. As a comparison band, a 6X loading dye was also loaded along with the sample. The 6X loading dye contains 0.04%

Orange G, 0.03% Bromophenol Blue and 0.03% Xylene cyanol FF where they migrate approximately 50bp, 300bp and 4000bp respectively on a 2% agarose gel.

5.3 Restriction Enzyme Digestion and Ligation Protocol

Restriction enzyme digestion was performed in various steps in generating the DNA template. Restriction enzyme digestion was performed on the PCR product it can be cloned into the vector of interest. Digestion was also performed on the plasmid containing the DNA template to linearize the plasmid to allow transcription reaction to happen.

The general restriction enzyme digestion protocol is shown below

Table 3 - Restriction enzyme digestion protocol.

Total Volume	30 μ L
Purified DNA Template	10 μ L (~0.1 μ g)
10x <i>Eco</i> RI Buffer	3 μ L
Restriction Enzymes	0.3 μ L of each Restriction Enzyme
Nuclease Free Water	Up to 30 μ L, to make up to the final volume
Incubation Temperature	37°C
Incubation Time	3 hours

Double digestion of the DNA template was analyzed using agarose gel electrophoresis to determine if the digestion was complete. A DNA ladder and the undigested template will also be loaded on the same gel to determine the size of the digested sample and allow comparison of the undigested template verses the digested template.

Ligation of the digested DNA template and the digested vector was performed so that it forms a plasmid that can be transformed into competent cells to allow large scale production of DNA templates.

The general ligation protocol is shown below

Table 4 - Ligation protocol for cloning DNA template into plasmid.

Total Volume	10 μ L
Purified and digested DNA insert	3 μ L (~300ng)
Purified and digested vector	1 μ L (~100ng)
10x Ligase Buffer	1 μ L
T4 DNA Ligase Enzyme	2 μ L of each Restriction Enzyme
Nuclease Free Water	Up to 10 μ L, to make up to the final volume
Incubation Temperature	4°C
Incubation Time	8-12 hours (overnight)

The ligation reaction is analyzed using agarose gel electrophoresis. A DNA ladder and the undigested vector will also be loaded on the same gel to determine the size of the ligated product and allow comparison of the plasmid versus the vector.

5.5 Purification of Samples

Purification of the DNA template is required for each step in generating the plasmid template. Purification of samples removes enzymes, undigested DNA templates, unused nucleotides and nonsense DNA fragments so that it does not interfere with future steps.

Purification of PCR products was performed using the illustra™ MicroSpin G-25 Columns to remove the primers, nucleotide and enzymes from the desired template. The purified PCR product was dissolved in nuclease free water.

Purification of restriction enzyme digestion products was performed using the QIAquick PCR Purification Kit from Qiagen to remove undigested plasmid and enzymes from the digested plasmid. The purified product is dissolved in nuclease free water.

Concentration determination of DNA after purification steps was performed using the Thermo Scientific NanoDrop machine. It is a UV spectrometer that determines DNA, RNA and protein concentration by measuring the sample under various light wavelengths and calculates the concentration of the sample.

5.6 Transformation of Cells

To allow large scale production of plasmid in the most cost efficient manner, the plasmid was introduced into bacterial cells so that the bacterial cells can generate multiple copies of the plasmid. Transformation of the plasmid containing the insert into competent cells was performed using the heat shock protocol. The plasmid were transformed into Stratagene StrataClone competent cells and grown on lysogeny broth (LB) agar plate that contained ampicillin and 40 µL of 2% X-gal.

The heat shock protocol used for experiments performed is outlined below.

One tube of the StrataClone SoloPack competent cell was used for each ligation reaction. 2 µL of ligated product was added to the competent cells and the reaction mixture was incubated on ice for 20 minutes. Heat-shock of the transformation mixture

was performed at 42°C for 45 seconds and incubated on ice for 2 minutes. 250 µL of Pre-warmed SOC was added to the transformation mixture and the cells were allowed to recover for one hour at 37°C. The cells were then grown on lysogeny broth (LB) agar plate that contained ampicillin and 40 µL of 2% X-gal.

Cells that appear white would be re-plated and grown in 5 mL LB broth at 37°C for 8 hours. Plasmid extraction and purification was performed Promega™ Wizard Plus SV Minipreps DNA Purification System. The purified plasmid was dissolved in nuclease free water to a final concentration of 100 ng/µL.

5.7 Transcription of RNA

Transcription reactions of the purified and linearized plasmid were performed using T7 RNA polymerase. The protocol that gave best transcription results is shown below.

Table 5 - T7 RNA polymerase transcription protocol.

Chemical Solution	Final Concentration	Volume for 1000 μ L Reaction
Tris pH 8.0 1M	80 mM	80 μ L
DTT 1M	15 mM	15 μ L
MgCl ₂ 2M	33 mM	16.6 μ L
dNTP 10mM (mixture containing 10mM of dATP, dCTP, dGTP, dUTP)	3.75 mM of each	752 μ L
Spermidine 100mM	1 mM	10 μ L
Linearized Template 100 ng/ μ L	~0.1 μ g	8 μ L
T7 RNA Polymerase		100 μ L
Nuclease Free Water		Up to 1000 μ L, to make up to the final volume
Incubation Temperature		37°C
Incubation Time		6 hours

Transcription reactions are analyzed on 8% polyacrylamide gel electrophoresis under UV light. An RNA loading dye containing xylene cyanol FF and bromophenol blue was used to estimate the size of the transcribed product.

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