MAPPING OF STRUCTURAL CHANGES TO THE YKKCD ANTIBIOTIC SENSOR RNA CAUSED BY TETRACYCLINE BINDING

A THESIS

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BY

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Caroline A. Conley
A riboswitch is a noncoding RNA that controls gene expression response to changes in the cellular environment. Most riboswitches regulate genes responsible for transporting, synthesizing or recycling small metabolites in bacteria. These riboswitches, primarily found at the 5’ region of messenger RNA, recognize the metabolic product of the gene being regulated. When a metabolite concentration threshold is reached, expression is turned off. In contrast, the ykkCD putative riboswitch increases production of an efflux pump that ejects toxins from the cell by binding to the antibiotic tetracycline. As tetracycline binds to ykkCD riboswitch, structural changes take place that trigger the production of the efflux pump. We will present terbium fragmentation and other nucleic acid footprinting methods to map the tetracycline site and to visualize the allosteric changes that take place due to tetracycline binding.

References:


2. Howell, L. A. Mapping the structural change caused by tetracycline binding to the ykkCD antibiotic sensor RNA. Ball State University, Muncie, IN, 2013
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Chapter 1: Project Development

1.1 Discovery of Antibiotics

Alexander Fleming discovered penicillin in 1928 at St. Mary’s Hospital, London. The discovery and development of penicillin began a revolution in pharmaceutical chemistry. By the 1940’s the United States required numerous doses of penicillin to combat disease and infection from World War II injuries. The United State of America designed large scale production of the drug for increased availability. Thus, penicillin dawned the age of antibiotics.

Antibiotics are not made synthetically; they are produced from bacteria and fungi which inhibit or kill microbial species. Fleming was studying Staphylococcus aureus when he found a blue-green mold on a petri dish. He noticed that the bacteria were not growing around the mold. The mold (Penicillium notatum) had secreted something that inhibited bacterial growth. Liquid extracted from the mold killed bacteria such as streptococcus, meningococcal and diphtheria bacillus. Fleming’s discovery was published in the British Journal of Experimental Pathology in June 1929. Howard Florey and Ernst Chain of Oxford University focused their work on the purification of penicillin. On February 12, 1942, Albert Alexander was the first human to receive a treatment of penicillin. To increase production, an American company (Pfizer) developed new mold growth methods and fermentation tanks. However, within five years of large scale production, some bacteria became resistant to penicillin.
1.2 Bacterial Resistance

During the 1940’s-1950’s many antibiotics were developed after the booming success of penicillin. Some of the most important developments at this time were Beta-Lactams, Chloramphenicols, Tetracyclines and Macrolides. Overuse of antibiotics has become an increasing problem across the world. The bacteria that antibiotics are designed to kill have adapted to them, making the drugs less effective. Misuse of antibiotics has led to the propagation of mutant bacteria that resist antibiotics. By inappropriate prescription and failure to finish medication; we have increased the number of antibiotic resistant strains. Low levels of antibiotics are introduced to humans in many other ways such as the agricultural use of antibiotics in food production. This is a threat to us, because low levels of antibiotic lead to increased growth of bacteria that is resistant to these antibiotics. Making matters worse, the companies responsible for development of new antibiotics have been steadily decreasing over the last 30 years. This is due to the cost of research and testing that is required to bring a new drug to market. On average this cost is around $5 billion. The rise of cost has forced many antibiotic companies to close their research facilities. Pfizer stopped research in late 2011, Sanofi, Eli Lilly and Bristol-Meyers Squibb all stopped research of antibiotics in the 1990’s. In the last 5 years, only two antibiotics have passed the FDA regulations and made it to market. In efforts to increase antibiotic research, President Obama instated the Generating Antibiotic Incentives Now (GAIN) Act. The GAIN act allows for an extended 5 years on the drug patent, fast track FDA approval and FDA trial guidance. From the GAIN Act, there is a new antibiotic on the market used to treat MRSA called Dalvance. Since February 2014, 17 more antibiotics are
benefiting from the GAIN Act. As bacteria gain resistance, it is crucial to keep researching and studying gene resistant pathways.

Currently, this is what we understand about resistance. Antibiotic resistance is acquired in three different ways, transformation, conjugation and transduction. A mutation changes the structure of a gene which allows for the new form to be passed on to succeeding generations. The mutations are caused by the modification of single base units in DNA or rearrangements of genes and chromosomes. The resistant bacteria will have one or more mutations in its genome to help it survive in the presence of an antibiotic. One bacterium with the mutation that survives the dosage of antibiotic will propagate at alarming speeds using one of the methods described below.

Transformation involves the transfer of the resistant gene from dead bacterium to another live bacterium. A plasmid is a small circular DNA molecule that is not a part of the main circular DNA chromosome of the bacterium. Conjugation will transfer the plasmid carrying the resistant gene to the bacterium. Transduction transfers the resistant gene through a virus. Figure 1 illustrates these processes.

![Diagram of resistant gene transfer by transformation, conjugation and transduction.](image)

**Figure 1:** Resistant gene transfer by transformation, conjugation and transduction.
Once bacteria get the resistant gene, resistance mechanisms are encoded by the plasmid. Resistance mechanisms evolve that chemically modify, inactivate or alter antibiotic target sites. One mechanism involves the production of an enzyme (beta lactamase) to degrade and inactivate the antibiotic. This mechanism is commonly observed in the breakdown of the beta lactam ring in penicillin molecules. The enzyme hydrolyzes the amide bond formed in beta lactam antibiotics. 

![Beta Lactam Ring](image)

**Figure 2:** Hydrolysis of the beta lactam ring present in penicillin. 

Another mechanism allows bacteria to modify the antibiotic target molecule and make the antibiotic ineffective. This can be done through a small structural change in the target molecule that prevents binding. One more mechanism involves transporter proteins, which expel antibiotics from the bacteria. These proteins are called efflux pumps and are located in the membrane of the bacterial cell. Efflux pumps are the most common mechanism of resistance to tetracycline.
Figure 3: Model of antibiotic resistance mechanisms. Antibiotics can be removed from the cell through efflux pumps, degraded by degrading enzymes such as beta lactamase or by structurally altering the target molecule site. When the target is altered, there is no binding.

Tetracycline was designed to fight bacterial infections that cause respiratory tract infections, urinary tract infections, acne, stomach ulcers and Lyme disease. Most recently it has been studied to treat anthrax exposure by respiration. The first synthesis of tetracycline was developed by Robert B. Woodward at Pfizer in 1962. It involved an 18 step linear process to assemble the 4 ring compound. The total synthesis of tetracycline was difficult due to the stereospecific functional groups present. This increased the chemical stability of the compound when in acidic or basic conditions.
1.3 Efflux pumps

An efflux pump is a transporter protein, which expels toxic substances such as antibiotics from inside the cell to outside of the cell. Efflux pumps are found in Gram-positive, Gram-negative and eukaryotic organisms. Pumps differ by the types of substrates they eject. Some efflux pumps are multiple drug resistant (MDR) pumps, meaning multiple drugs can use this pump to exit a cell. They use proton motive force as an energy source to export substrates from the bacterial cell. Efflux pumps are unique in the fact that they have not developed in response to new and changing antibiotics.  

Bacterial genes detect antibiotics in their environment by RNA and protein sensors. The bacterial genes produce efflux pumps. RNA sensors that are capable of changing shape in response to metabolites or other physical or chemical changes trigger gene production. The change in mRNA structure affects transcription or translation of the downstream gene without the use of protein cofactors. Protein sensors called transcription factors are extensively studied in how they detect small molecules and change mRNA structure to alter gene expression. So, how do structural changes in RNA affect gene expression? How does RNA alone detect molecules and alter gene expression? To answer these questions, we need to look at these RNA regulators called riboswitches and understand how they function.
1.4 What is a Riboswitch?

The concept of the riboswitch is a very recent development in the biochemical world, first being introduced in 2002. Prior to 2002, it was believed that proteins were the main source of gene regulation.12

“The term ‘riboswitch’ is used to describe the properties of an mRNA sequence that controls transcription or translation through binding of a metabolite, which typically encodes the production of proteins designed to transport the metabolites.”12

Since 2002, riboswitches are becoming increasingly studied for their gene regulation capabilities at transcription or translation in bacteria, plants, algae and fungi. They take advantage of the ability of RNA to specifically bind a wide range of small molecule ligands.

1.5 Riboswitch Domains and Metabolite Binding

Riboswitches have a conserved secondary structure which allows for specificity in binding of metabolites. Riboswitches are found at the 5’ untranslated region (UTR) of mRNA. 13 The highly conserved region is the riboswitch; this region regulates the downstream coding sequences. The riboswitch is separated into two regions based on their functionality; the aptamer domain and the sensor domain or expression platform. The aptamer domain is the region where ligand binding occurs. The metabolite that is recognized by the riboswitch is closely related to the gene that is regulated. Riboswitches are typically located adjacent to genes that transport or synthesize the metabolite that is recognized. Once the metabolite levels reach their limit, they
bind to the aptamer platform and trigger structural changes in the entire riboswitch. See Figure 3. When the expression platform experiences the structural change, it leads to a change in gene expression through two possible mechanisms. Typically the ligand binding induced structural change turns off gene expression by stopping transcription or inhibiting translation of the gene that is regulated. However, ligand binding may turn gene expression on.\textsuperscript{14} Initiating the anti-termination of transcription or the activation of translation.

\textbf{Figure 5:} (1) shows the riboswitch without metabolite and a terminator stem which stops gene expression. (2) shows the binding of a metabolite (+) and the resulting structural change. This structure has no terminator stem, so gene expression occurs.

There are over 20 subclasses of riboswitches based on nucleotide length and ligands.\textsuperscript{15} Through research, structures of the aptamer domains have been determined for the major classes of riboswitches and the ligands they bind. There are two common structures observed in riboswitches, the pseudoknot and the multihelical junction. The pseudoknot is formed through two stem-loop structures where one stem is intercalated between two halves of another stem.\textsuperscript{16} The multihelical junctions are divided into two subclasses based on the regulatory helix, P1. These groups are Ia and Ib. Group Ia helical junctions can be seen in the TPP riboswitch,
where the conformation is stabilized by tertiary interactions. Group Ib helical junctions are common in riboswitches like SAM-II with small pseduoknots.

Table 1 represents some of these subclasses and their unique variety of size and specific ligands. Table 2 shows the diversity and complexity of structures that recognize each ligand.

Table 1: Riboswitch sub classes

<table>
<thead>
<tr>
<th>Class</th>
<th>Members</th>
<th>Natural Ligand</th>
<th>Size (nucleotides)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enzyme Cofactor</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPP</td>
<td>TPP</td>
<td>TPP, Thiamine Pyrophosphate</td>
<td>100-200</td>
</tr>
<tr>
<td>FMN</td>
<td>FMN</td>
<td>FMN, flavin, mononucleotide</td>
<td>120-140</td>
</tr>
<tr>
<td>AdoCbl</td>
<td>AdoCbl</td>
<td>AdoCbl, adenosylcobalamin</td>
<td>200-220</td>
</tr>
<tr>
<td>SAM-I</td>
<td>SAM</td>
<td>SAM, S-adenosylmethionine</td>
<td>105-125</td>
</tr>
<tr>
<td>SAM-II</td>
<td>SAM</td>
<td>SAM, S-adenosylmethionine</td>
<td>70-90</td>
</tr>
<tr>
<td>SAM-III</td>
<td>SAM</td>
<td>SAM, S-adenosylmethionine</td>
<td>115-135</td>
</tr>
<tr>
<td><strong>Amino Acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>Lysine</td>
<td></td>
<td>165-190</td>
</tr>
<tr>
<td>Glycine I, II</td>
<td>Glycine</td>
<td></td>
<td>110-120</td>
</tr>
<tr>
<td><strong>Nucleotide Bases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guanine</td>
<td>Guanine</td>
<td>Guanine, hypoxanthine</td>
<td>60-80</td>
</tr>
<tr>
<td>Adenine</td>
<td>Adenine</td>
<td></td>
<td>60-80</td>
</tr>
<tr>
<td>PreQ1</td>
<td>Pre-Q1</td>
<td></td>
<td>25-45</td>
</tr>
</tbody>
</table>
Table 2: Examples of Riboswitch diversity and binding ligands.

<table>
<thead>
<tr>
<th>Name</th>
<th>Riboswitch</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coenzyme Thiamine pyrophosphate (TPP)</td>
<td><img src="image" alt="Coenzyme Thiamine pyrophosphate" /></td>
<td><img src="image" alt="TPP" /></td>
</tr>
<tr>
<td>Amino Acid Lysine</td>
<td><img src="image" alt="Amino Acid Lysine" /></td>
<td><img src="image" alt="Lysine" /></td>
</tr>
<tr>
<td>Nucleobase pre-queenosine-1 (PreQ1)</td>
<td><img src="image" alt="Nucleobase pre-queenosine-1" /></td>
<td><img src="image" alt="PreQ1" /></td>
</tr>
<tr>
<td>mRNA Glucosamine-6-phosphate</td>
<td><img src="image" alt="mRNA Glucosamine-6-phosphate" /></td>
<td><img src="image" alt="Glucosamine-6-phosphate" /></td>
</tr>
<tr>
<td>SAM-II</td>
<td><img src="image" alt="SAM-II" /></td>
<td><img src="image" alt="SAM-II" /></td>
</tr>
</tbody>
</table>
1.6 The yKKCD Putative Riboswitch

This project involves the study of the ykkCD riboswitch. The ykkCD riboswitch meets certain criteria, which makes it a good candidate for an antibiotic sensor. It is highly conserved in Gram positive bacteria, including some pathogenic organisms. The ykkCD pump is located upstream of the MDR efflux pump gene. 80% of the ykkCD sequence is conserved and appears multiple times in *Bacillus subtilis*.19

This sensor recognizes and binds the antibiotic, tetracycline. Once tetracycline levels reach a threshold limit, the riboswitch binds to tetracycline, triggers production of the efflux pump which in turn facilitates removal of tetracycline from the cell. When bacteria contain the ykkCD toxin sensor and the downstream pump, they show resistance to the tetracycline antibiotic.

The riboswitch is considered “putative” because the information available is currently assumed to exist. In order to show that the ykkCD riboswitch is a toxin sensor, it must specifically bind tetracycline. This binding should trigger the conformational change discussed in Figure 3 leading to an increase in ykkCD efflux pump production. Figure 6 shows the predicted secondary structure of the ykkCD RNA. The unique secondary structure indicates a terminator stem which suggests that regulation will occur during transcription.
Figure 6: Secondary Structure of ykkCD RNA

Previous work completed by Ambar Rana used RT-PCR to determine ykkCD pump levels in the presence of various ligands, including tetracycline that can be transported by the ykkCD pump. These ligands were streptomycin, TPPC, chlotamphenicol and fosfomycin.
Rana’s data concluded that tetracycline is the only antibiotic among ykkCD ligands that increased the production of pump mRNA levels when grown with them in *Bacillus Subtilis* cells.

![Graph showing pump mRNA levels in the presence of various antibiotics](image)

**Figure 7**: ykkCD pump mRNA levels in the presence of various antibiotics.

Binding assays completed by Krystal Roark and Delores James mapped which part of the sensor recognizes tetracycline. The result was important for knowing which specific nucleotides are crucial for tetracycline binding. To do this, mutations were introduced into the ykkCD RNA and their effect on tetracycline binding was studied. The structures were mutant RNA predictions using the online tool, mFold. The mutations would not be expected to eliminate ligand binding unless the nucleotide(s) altered were important for binding. From the binding assays and mutation studies Roark and James completed we can identify a ligand binding site. These important nucleotides are found at the 5’ of the conserved region.
Figure 8: (a.) The mutations seen in this figure did not alter tetracycline binding sites. (b.) Mutants that did alter tetracycline binding.

In order to further study the ykkCD riboswitch, it will be important to understand how the binding of tetracycline effects allosteric conformational change. RNA 2D structure probing tools will be used to visualize conformational changes.

1.7 RNA Footprinting

Robert Holley of Cornell University was the first person to sequence the alanine- tRNA in 1965. In 1968 he received the Nobel Prize for his success and in the years that followed other small RNA molecules were sequenced. This methodology was a great advancement in the field of science and led to the mapping of extremely long DNA molecules.22

RNA footprinting can be summarized by the statement that a cleavage pattern can take RNA molecules that differ in length by as little as one nucleotide and separate them into two distinct
bands using electrophoresis using a thin polyacrylamide gel. The unique patterns of bands that appear on the gel are the “footprint”. RNA footprinting techniques include probing methods that use enzymes or chemicals to resolve the cleavage patterns on thin, denaturing polyacrylamide gels. These gels are referred to as sequencing gels.

Ribonuclease digestion is one way to characterize the RNA secondary structure. Using a variety of RNase enzymes with specific substrate preference, we can study the products of digestion with and without ligand bound.

Comparing the footprint of the ykkCD RNA and the ykkCD RNA-tetracycline complex will reveal the binding site of tetracycline. Where the tetracycline is bound, no bands will be present on the sequencing gel, because this region is protected from ribonuclease digestion. To visualize fragmentation patterns, the nucleic acid is labeled at the 3’ or 5’ end. See Figure 9 to illustrate this process.
Figure 9: The RNA molecule is labeled at the 5’ end. Ligand (tetracycline) is bound. Digestion of the RNA is carried out by various ribonucleases. A denaturing gel maps the footprint of the tetracycline binding site.
1.8 End Labeling:

To visualize the cleavage patterns, the RNA is labeled at either the 5’ or 3’ end. This can be done in many ways with detailed protocols found in the literature. For this project, three end-labeling methods were used. The first method labels the RNA at the 5’ end in a two-step process beginning with a phosphatase treatment followed by a kinase treatment using IR-700-UTP Dye (Figure 7). The phosphatase treatment catalyzes the dephosphorylation of 5’ and 3’ ends of RNA. It also hydrolyses NTP’s and dNTP’s and allows for end labeling with the dye. 3’ end-labeling allows for the addition of homopolymer tails to the 3’ ends of RNA with modified nucleotides (e.g. dUTP). Terminal transferase (TdT) polymerase catalyzes the addition of the deoxynucleotides to the 3’ hydroxyl terminus.

![Figure 10: Structure of IR-700-UTP dye used for 5’ and 3’ end labeling.](image)

The third method uses a radiolabelled nucleotide containing $^{32}$P. These nucleotides are commonly used for nucleic acid labeling. The in vitro synthesized RNA is dephosphorylated and the radiolabeled ATP is incorporated with T4 polynucleotide kinase (PNK). This method is
preferred for increased sensitivity and resolution to visualize the band patterns by autoradiography.

(a)

(b)

**Figure 11:** (a) Illustration of 5’ end radiolabeling using SAP and T4 PNK. (b) [γ-\(^{32}\)P]dNTP labeled nucleotide.
1.9 Probing Techniques

Once RNA is labeled with fluorescent dye or $^{32}\text{P}$, structure probing techniques listed below, are used in identifying single nucleotides, base paired regions and ligand binding sites in the RNA.

1. RNase A, T₁, V₁, and 1 protection assays
2. Hydroxyl radical footprinting
3. In-line probing
4. Tb$^{3+}$ self-cleavage

Protection Assays:

Ribonuclease protection assays (RPAs) are a sensitive method used to detect and identify specific nucleotides in an RNA sequence. There are a wide variety of ribonucleases available for RNA mapping such as RNase A, T₁, V₁, and 1 to name a few. Each ribonuclease cleaves RNA after unique nucleotides and at single or double stranded regions. When the RNA molecule binds with a small molecule ligand, the region where it is bound is protected from cleavage. When used in combination, these methods can characterize secondary structure and RNA conformational changes. Using sequencing gels, the cleavage patterns can be observed.

To interpret protection patterns, 2 ladders will be needed. The first is a hydroxyl (OH) ladder. The OH ladder provides a visual of the hydrolyzed RNA fragments since there is cleavage after every nucleotide. The T₁ ladder cleaves after every G nucleotide in the RNA, because the buffer conditions used for the ladder denature the RNA. Therefore, a ladder is generated of unpaired
G nucleotides. The T₁ ladder should be used in combination with the OH ladder to identify which cleavage site is altered upon ligand binding.

RNase A cleaves single stranded RNA at the U and C nucleotides. Under denaturing conditions, RNase A cleaves after every U and C nucleotide. RNase V₁ will cleave base paired nucleotides. RNase I will degrade all RNA dinucleotide bonds leaving a 5' hydroxyl and a 2', 3' cyclic monophosphate. It has a high specificity for single strand RNA, which works well for protection assays in combination with RNase V₁. Cleavage happens on the 3’ side of pyrimidine bases (C and U). The following is a hypothetical example, not from the ykkCD RNA. Below is a hypothetical experiment of a sequencing gel using RNase T₁, A and V₁. The sequence is identified by the cleavage patterns (Figure 12) and the secondary structure is predicted using mfold analysis (Figure 13)²³
Figure 12: This gel would show the hypothetical cleavage patterns using 5’ radiolabeled RNA sample digested with RNase T₁, A and V₁. Lane 1 shows cleavage in denaturing conditions for all C and U nucleotides by RNase A. Lane 2 shows cleavage in denaturing conditions by RNase T₁ at all G nucleotides. Lane 3 shows cleavage after single-stranded C and U nucleotides using RNase A. Lane 4 shows RNase V₁ to show double stranded regions. Lane 5 cleaves at unique G nucleotides in a stem-loop. On the right is the Secondary Structure of the RNA predicted by mfold analysis.
Hydroxyl Radical Footprinting:

The hydroxyl radical method is designed to cleave the backbone of the RNA structure. Hydroxyl radicals are created from the Fenton reaction. The reaction uses iron (Fe$^{2+}$) and hydrogen peroxide (H$_2$O$_2$) to form the hydroxide radical.

$$[Fe(EDTA)]^{2-} + H_2O_2 \rightarrow [Fe(EDTA)]^{1-} + OH^{-1} + \hat{O}H$$

The hydroxide radicals are very small in size, similar to the size of a water molecule. This allows for easy access to the RNA backbone. This allows for precise mapping of the ligand binding site.

![Diagram of hydroxyl radical footprinting](image)

**Figure 13:** Here the hydroxyl radical is accessing the backbone, removing the 5’ hydrogen and creating a free radical intermediate. The result is the radiolabeled cleavage product.

The hydroxyl radical is unable to access the backbone at the nucleotide(s) where the ligand is bound to the RNA. When analyzed on an electrophoresis gel, there will be gaps in the band pattern where the ligand is bound. Where the backbone is accessible, cleavage will occur.
Figure 14: Hypothetical hydroxyl radical footprinting pattern. The hydroxyl radical cleaves the RNA backbone at all nucleotides not protected by bound ligand.

Due to the small size of the hydroxyl radical, the binding site is mapped with high resolution. However, there are some limitations to the hydroxyl radical method. Samples treated with the radical can only achieve a 2D representation. They do not generate 3D structures such as X-ray crystallography or NMR spectroscopy. The radical reacts very quickly, so optimizing concentration and cleavage patterns can be time consuming.²⁴
**In-Line Probing:**

The In-line probing technique induces RNA hydrolysis through “in-line” nucleophilic attack of the 2’ hydroxyl on the phosphorus center. The 2’ hydroxyl, the phosphorous and 5’ oxygen align which allows the oxygen in the 2’ position to replace the 5’ oxygen and cleave the RNA linkage. Certain nucleotides are fixed, they are not flexible. Those linkages are now stationary, locked in one position making them unlikely to move, which will reduce cleavage. When samples are resolved on a sequencing gel, these areas will be identified by gaps in the cleavage patterns.

*Figure 15:* In-line probing technique to cleave RNA linkage.
**Figure 16**: This is a figure of a hypothetical In-Line probing experiment. When the ligand binds to RNA, changes occur in the secondary structure which make certain linkages less flexible and less likely be in an in-line conformation. At these sites, cleavage is reduced leaving a footprint in the sequence to indicate ligand bound regions.

**Tb\(^{3+}\) self-cleavage:**

Terbium (Tb\(^{3+}\)) is a trivalent lanthanide metal ion used for probing the overall structure of RNA and identifying metal ion binding sites. By deprotonating the 2-hydroxyl group a nucleophilic attack on adjacent phosphodiester bond is more effective for leaving a cyclic phosphate at the 5′-hydroxyl terminus. This technique is similar to inline probing but is more efficient.
Using terbium fragmentation as a footprinting method provides desirable information. This method is unique, because two different concentrations of terbium will result in different cleavage patterns. RNA structure and function is dependent on cations, specifically Mg\(^{2+}\) to stabilize secondary and tertiary structure. When the RNA sample is incubated with low concentrations (micromolar) of Tb\(^{3+}\), we can detect the high-affinity metal-binding sites. Even at low concentrations, Tb\(^{3+}\) will readily displace millimolar concentrations of Mg\(^{2+}\). Tb\(^{3+}\) is similar in size to Mg\(^{2+}\) and easily identifies coordinating positions. These binding sites are crucial to folding the RNA. This is a slow cleavage and footprinting resolution is better after longer incubation periods.

High Tb\(^{3+}\) concentrations (millimolar) will result in backbone cleavage of single stranded regions. These regions are structurally more accessible and flexible for nucleophilic attack. The cleavage patterns reflect the flexible regions in the RNA. This is useful for locating stem loops in the secondary structure.
Figure 18: (Top Left) High-affinity Mg$^{2+}$ binding sites identified by low concentrations of Tb$^{3+}$. (Top Right) cleavage sites of single stranded, flexible regions in the RNA. (Bottom) Hypothetical footprinting pattern observed in sequencing gel.

While Tb$^{3+}$ footprinting is an easy to follow protocol there are many factors that must be controlled during the cleavage reactions. Concentration, pH, temperature and timing parameters need to be established to optimize cleavage patterns. pH of the reactions should be close to physiological (7.0-7.5) to allow the terbium hydroxide Tb(OH) to accumulate at the cleavage site. Tb$^{3+}$ can also precipitate at pH higher than 7.5. Higher temperatures will result in faster cleavage. Temperatures range from 20°C to 40°C, over a 30 minute to 2 hour period. Using the end labeling and RNase protection assays mentioned above, it is the goal of this thesis to map the structure of the ykkCD riboswitch.
CHAPTER 2: Methods

2.1 ykkCD Riboswitch Construct

To design the ykkCD RNA, the DNA template was amplified from *Bacillus Subtilis* genomic DNA (Bacillus Genetic Shock Center) with EcoR1 site at the 5’ end and BamHI site at the 3’ end. (New England Biolabs) This was done using two primers.

#1 5' -gcgGAATTCtaatacgactcactataggACAGTGTTTTCTAGGGTCCGCAA-3'

and

#2 5' -cgGGATCCACTCCTGGGCTTTTATCCCACCGT-3'

Primer #1 contained the promotor region for T7 RNA polymerase. The highlighted region is the T7 promotor sequence. Two G’s were added to the 5’ side of the native ykkCD sequence to enable efficient transcription with T7 RNA polymerase. The underlined region is the EcoR1 restriction site. The double underlined region is the 5’ of the ykkCD sequence.

Primer #2 contained an overlap with the end of the desired RNA sequence. The dashed line in Primer #2 indicates the BamH1 restriction site. Primer 2 also contains the 3’ compliment to the ykkCD sequence. The two primers were cloned into the pUC19 vector. The sequence was verified by the University of Chicago Sequencing facility.
The Y-RNA construct is used to map the tetracycline binding site. The Y-construct contains the ykkCD riboswitch. The following table represents the ykkCD putative riboswitch construct.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Nucleotide Length</th>
<th>Molecular weight (g/mol)</th>
<th>ε (L/cm*mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>112</td>
<td>36,329.4</td>
<td>1,149,800</td>
</tr>
</tbody>
</table>

The following sequence is the ykkCD aptamer domain from *Bacillus Subtilis* Spizizenii Strain W23.

5’ GUAAAGUUUUCUAGGGGUUCGCCUGUGUAACUGAACAGCCUGGGAGGAGAAAACACA UACGCCUAUUAGAGCGGUGUAGCAGACGGAGGGAGAAAAAGCCCGGGAGAG 3’

### 2.2 Mapping the Tetracycline Binding Site:

The following is a brief summary of the transformation of the ykkCD-pUC19 construct.

DH5α competent cells are transformed with the plasmid containing the DNA sequence ykkCD aptamer domain. Transformation of the DNA into bacterial cells will allow the cells to store and replicate plasmids independently from replicating its own DNA leading to synthesis of large amounts of DNA template. Purification of the plasmid DNA is completed using Qiagen Plasmid Plus Midi Kit. BamH1 restriction enzyme is used for linearization of the plasmid DNA to cut the ykkCD-PUC19 construct after the ykkCD sequence. After linearization, *in vitro* transcription using the T7 promotor synthesizes RNA in high concentrations. To ensure that RNA is pure and free from T7 RNA polymerase (New England BioLabs #M0251S) and unincorporated nucleotides; the RNA clean up kit and gel purification is used. RNA is then ready for end labeling and RNase protections methods. RNA footprinting gels are used to see the cleavage patterns of the RNase protections and map the binding site of tetracycline.
2.3 Protocols:

Transformation Using the Heat Shock Method:

Transformation is used to introduce DNA into a bacterial cell. In order to increase transformation efficiency, competent cells are used. The heat shock method is a common technique used for transformation. Heat shock changes the porosity of the cell membrane so DNA can enter the bacteria at a faster rate.

1. Add 10 μL of Y-DNA to 100 μL DH5α competent cells.
2. Sit on ice for 20 minutes.
3. Heat shock cells in 42°C water bath for 2 minutes.
4. Sit on ice for 2 minutes.
5. Add cells to 900 μL of pre warmed Luria Broth (LB) media.
6. Grow cells in shaker at 130 rpm at 37°C for 1 hour.
7. Transfer the cells to a 1.5 mL centrifuge tube.
8. Spin the cells down for 1 to 2 minutes using a table top centrifuge.
9. Remove the supernatant LB media.
10. Suspend the pellet cells in 100 μL of LB media.
11. Plate cells on a LB 100 μg/ mL Ampicillin plate.
12. Grow overnight at 37°C, do not exceed 20 hours of growth. Only cells that contain the plasmid will be able to form colonies.
13. Wrap the edge of the plates with Parafilm.
14. Label and store plates with Y-DNA colonies in -4°C refrigerator.

LB Recipe:

Mix the following and add to 1 L of deionized water. Autoclave the mixture.
10 g Bacto-tryptone (BD Bioscience Cat. No. 211705), 5 g yeast extract (Fisher BioReagents Cat. No. M-5038) and 10 g Sodium Chloride (OmniPur Cat. No. 7710)

-OR-

Add 25 capsules of LB Medium (MP Biomedicals, LLC Cat. No. 3002-021)to 1 L of purified water.
Plasmid Prep Procedure (2-part inoculation):

1. Begin with fresh LB media from the autoclave, 1000x ampicillin (AMP) antibiotic (Sigma A0166-25G, store at 2-8°C) and Y-DNA transformation colony.
2. Add 1 mL LB and 1 μL AMP (1000x) to a 10 mL Falcon tube and vortex.
3. Using a pipette tip, scrape a Y-DNA colony and add in to the test tube.
4. Place test tubes in shaker for 4-5 hours at 37°C.
5. Add 40 mL of LB and 40 μL 1000x AMP to 50 mL Erlenmeyer flask.
6. Add the 1 mL grow up to the Erlenmeyer flask.
7. Shake overnight at 37°C.
8. Remove inoculation flask from the shaker a place in 4°C fridge for 30 minutes.
9. Pour cells into 50 mL centrifuge tube.
10. Spin down cells for 10 minutes at 3000-5000 rpm. Remove supernatant LB media.
11. Cell pellets can be stores at -80°C for later use.

Qiagen Plasmid Plus Midi Kit (Cat. No. #12943):

Follow instructions from manufacturer for the Quick Start Protocol. The Qiagen Midi Kit is preferred for large scale purification of plasmid DNA. This kit is ideal for its speed, high DNA concentrations and easy removal of bacterial endotoxins. Each anion exchange column is capable of purifying 10 μg of plasmid DNA. The kit takes around 35 minutes to complete. Plasmid DNA concentrations yield an average of 500-700ng/μL with an absorbance reading around 14.0 at 260 nm (Abs260).
**Quantifying DNA Concentration:**

To quantify DNA concentrations, the Cole-Parmer Nanodrop 1000 Spectrophotometer is used. The Nanodrop allows for micro-volume sample sizes (0.5-2.0μL) to be applied directly to the sample pedestal. The instrument can detect low to high concentrations (up to 15,000 ng/μL) in less than 5 seconds. The Nanodrop uses ND-1000 V 3.6.0 software that is user friendly.

1. Clean the sample pedestal with RNase free water
2. Blank with 2 μL of Buffer EB.
3. Measure a blank sample to ensure sample pedestal is cleaned
4. Wipe dry with a Kimwipe
5. Measure 2 μL sample
6. Record the concentration in laboratory notebook.
DNA Linearization:

DNA linearization is required to transform a circular plasmid DNA into a linear molecule. Linearization volumes can vary between 100 μL to 500 μL. Bam H1 is the restriction endonuclease required for linearization. (New England Bio Labs Cat. No. R0136S) T7 RNA Polymerase initiates synthesis at the T7 promoter sequence and produces an RNA transcript of the DNA. The T7 polymerase is too robust to be stopped by the terminator stem in Gram positive bacteria, hence linearization will discontinue transcription at the end of the desired construct. The super coiled DNA will travel faster than linearized DNA when analyzed on an agarose gel. This will confirm that linearization was successful. A 500 μL reaction is prepared as followed. Typical concentration of linearized DNA is around 200-400 ng/μl.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Buffer 3</td>
<td>50</td>
</tr>
<tr>
<td>Bam H1</td>
<td>50</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>350</td>
</tr>
<tr>
<td>RNase free water</td>
<td>50</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>500</strong></td>
</tr>
</tbody>
</table>

Incubate at 37°C overnight.
Agarose Gel Electrophoresis:

To confirm linearization reaction is completed successfully, a 1% agarose gel is prepared as follows.

1. Add 1 g of agarose (Sigma Cat. No. A-9539) to 100 mL of 1x tris-acetate-EDTA (TAE) Buffer (Promega TAE buffer 40X Cat. No. V4281) in an Erlenmeyer flask.
2. Microwave the flask until contents boils (about 1 minute).
3. Allow the flask to cool.
4. Add 10 μL of ethidium bromide (FisherBiotech Cat. No. BP102-5).
5. Pour into the mold and add the combs.
6. Allow the gel to polymerize for 15 minutes.
7. Prepare samples by adding 5 μL of DNA with 1 μL of 6X loading dye.
8. Include DNA ladder as reference.
9. Load Samples into well and run gel for 20 minutes at 100 volts using 1X TAE running buffer.
QIAquick Nucleotide Removal Kit (Cat. No. 28304):

This protocol is designed for cleanup of DNA enzymatic reactions. The kit is designed for the removal of enzymes, salts, and unincorporated nucleotides. Refer to the manufacturer’s handbook for protocol details. This kit is the preferred method after DNA linearization because it recovers about 95% of DNA which can be eluted in small volumes. The kit completes a buffer exchange so DNA is eluted in RNase free water or Buffer EB (10mM Tris, pH 8.5) provided by the manufacturer. By using the kit, a phenol chloroform extraction and DNA precipitation is not required. This saves time since the samples would no longer have to incubate overnight. After the use of the kit, the samples can be immediately analyzed on a gel. Average concentrations of purified linearized DNAs are around 200 ng/μL and Nanodrop absorbance readings (Abs$_{260}$) are around 2-4.
Transcription:

For transcription, T7 RNA polymerase from phage promotes the synthesis for RNA in the 5’ to 3’ direction. T7 allows for high yield, *in vitro* transcription of RNA by the runoff transcription method. The minimum concentration of linearized DNA that contains the T7 phage promoter required for transcription is 0.025 μg/μL. To calculate the volume of linear DNA required for transcription use the $M_1V_1 = M_2V_2$ equation.

For a 500 μL transcription reaction and a linear DNA concentration of 0.366 μg/μL, a sample calculation is shown.

\[
(0.401 \text{μg/μL}) (V_1) = (0.025 \text{ μg/μL}) (500 \text{ μL})
\]

\[
V_1 = 31.2 \text{μL of linear DNA}
\]

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Reagent</th>
<th>Concentration</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigma Aldrich #T87602-1KG</td>
<td>1 M Tris pH 8.0</td>
<td>20 mM</td>
<td>10</td>
</tr>
<tr>
<td>New England BioLabs #N0466S</td>
<td>25 mM GTP</td>
<td>4 mM</td>
<td>20</td>
</tr>
<tr>
<td>New England BioLab #V3151</td>
<td>100 mM DL-Dithiothreitol (DTT)</td>
<td>5 mM</td>
<td>25</td>
</tr>
<tr>
<td>Acros #21568-2500</td>
<td>1% Triton</td>
<td>0.1%</td>
<td>5</td>
</tr>
<tr>
<td>New England BioLabs #M0251S</td>
<td>50,000 U/mL T7 RNAP</td>
<td>1 U/μL</td>
<td>50</td>
</tr>
<tr>
<td>Fisher Chemical #M-13448</td>
<td>100 mM MgCl₂</td>
<td>24 mM</td>
<td>120</td>
</tr>
<tr>
<td>New England BioLabs #M0314S</td>
<td>40 U/μL RNase Inhibitor Murine</td>
<td>1 U/μL</td>
<td>5</td>
</tr>
<tr>
<td>Linear DNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNase Free Water</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td></td>
<td></td>
<td><strong>500</strong></td>
</tr>
</tbody>
</table>

Incubate for 1.5 hours at 37°C
Thermo Scientific Gene JET RNA Cleanup and Concentration Micro Kit (Cat. No. K0841):

Follow information from the manufacturer for the Thermo Scientific Gene JET RNA Cleanup and Concentration Micro Kit. A silica membrane column is used to bind RNA to the column and allow impurities to pass by. Each RNA clean up column is good for 10 μg RNA samples. Two columns were typically used for a 500 μL transcription. Using the kit is ideal, because it eliminates a gel purification step. Gel purification reduces the amount of RNA recovered and involves more time. The kit can be completed in 20 minutes and successfully concentrates RNA while carrying out a buffer exchange. The RNA is eluted in RNase free water. Average absorbance (Abs$_{260}$) using the Nanodrop of recovered RNA is between 20-30 with 1,000-2,000 ng/μL. The RNA can be stored at -20°C for short term and -80°C for long term use.
**5’ RNA End Labeling with T4 Polynucleotide Kinase:**

In order to complete RNA footprinting methods and map the tetracycline binding site, the RNA must be labeled at either the 5’ or 3’ end. This method labels the 5’ end with fluorescent dye for RNA footprinting studies. To increase resolution and sensitivity we use an end-labeled nucleotide to visualize the bands of the footprint. This labeling method is completed in a two-step process beginning with a phosphatase treatment followed by a kinase treatment. Shrimp Alkaline Phosphatase (rSAP) catalyzes the dephosphorylation of 5’ and 3’ ends of RNA. It also hydrolyses NTP’s and dNTP’s and prepares *in vitro* transcribed RNA for end labeling. rSAP is irreversibly inactivated by heating, making removal of rSAP unnecessary.

**Phosphatase Treatment:**

Mix the following in a 1.5 mL centrifuge tube:

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Reagents</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>New England BioLabs #B7204S</td>
<td>PhosphataseFastAP Buffer</td>
<td>10</td>
</tr>
<tr>
<td>New England BioLabs #M0317S</td>
<td>PhosphataseFastAP</td>
<td>5</td>
</tr>
<tr>
<td>New England BioLabs #M0314S</td>
<td>RNase inhibitor Murine</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>RNase free water</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Clean RNA</td>
<td>75</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

1. Mix by pipetting.
2. Incubate for 10 minutes at 37°C.
3. Heat inactivate the phosphatase for 5 minutes at 65°C.
**Kinase Treatment:**

Once the terminal phosphate is removed, a kinase treatment with T4 polynucleotide kinase (PNK) is performed to catalyze the exchange of $P_i$ from the gamma position of ATP to the 5′ hydroxyl terminus of single stranded RNA. The label is light sensitive so the experiment should be carried out in low light. Use 5 times the label to RNA ratio by the following sample calculation.

$$\text{Abs}_{260} \text{ clean RNA} = 4.252$$
$$4.252 = (1,149,800)(c)(1 \text{ cm})$$
$$c = 3.698 \times 10^{-6} \text{M}$$

$$(3.698 \times 10^{-6} \text{ M})(110 \mu\text{L}) = (1.0 \times 10^{-3} \text{ M})(X)$$
$$X = 0.4 - 0.5 \mu\text{L} \text{ of Label}$$

Mix the following to the previous Phosphatase treatment tube:

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Reagents</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jena BioScience #NU-821-680</td>
<td>Labeled NTP (IR_700_UTP)</td>
<td>0.5</td>
</tr>
<tr>
<td>New England BioLabs #M0201S</td>
<td>T4 Kinase</td>
<td>5</td>
</tr>
<tr>
<td>New England BioLabs #M0314S</td>
<td>RNase inhibitor Murine</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td></td>
<td><strong>106.5</strong></td>
</tr>
</tbody>
</table>

1. Mix by pipetting.
2. Incubate for 30 minutes at 37°C.
3’ RNA End Labeling with Terminal Transferase:

Another labeling method that can be used on RNA for footprinting involves labeling the RNA at the 3’ end. This labeling method allows for the addition of homopolymer tails to the 3’ ends of RNA with modified nucleotides (e.g. dUTP). Terminal transferase (TdT) is a polymerase that catalyzes the addition of the deoxynucleotides to the 3’ hydroxyl terminus of nucleic acids. The addition of Co^{2+} increases tailing efficiency. Aminoallyl-dUTP-dye is recommended for fluorescent labeling. Keep the dUTP away from light sources and conduct experimental procedures in low light is desired. Follow labeling protocol from manufacturer.

1. Set up reaction as followed:

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>New England BioLabs #B0315S</td>
<td>RNA (200pmol)</td>
<td>X</td>
</tr>
<tr>
<td>Jena BioScience #NU-803-680-S</td>
<td>10X Terminal Transferase Reaction buffer</td>
<td>7.5</td>
</tr>
<tr>
<td>New England BioLabs #B0252S</td>
<td>100 μM dUTP</td>
<td>15</td>
</tr>
<tr>
<td>New England BioLabs #M0315S</td>
<td>CoCl_{2}</td>
<td>7.5</td>
</tr>
<tr>
<td>New England BioLabs #M0315S</td>
<td>Terminal Transferase (20,000 U/mL)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>H_{2}O</td>
<td>X</td>
</tr>
</tbody>
</table>

2. Total reaction volume: 75 μL.

3. Incubate the reaction at 37°C for 1.5 hours.
P-32 Radioactive End-Labeling:

The most common and traditional labelling method used for RNA sequencing is 5’ labelling with γ-32P-ATP. This method is preferred for increased sensitivity and resolution to visualize the band patterns by autoradiography. 32P was chosen over other similar radioactive atoms such as 35S because the β particles emitted by 32P are ten times stronger. This reduces the exposure time required to visualize band patterns after gel electrophoresis. 32P labelling protocols have been optimized and protocols are easily reproduced. Some disadvantages to 32P end labelling results in unstable samples which means gel electrophoresis must be run the same day as the sequencing reactions and there is increased safety risk with radioactive samples. Radiation safety training is required to work with 32P and radiation exposure levels should be monitored by wearing a radiation badge. 32P has a half-life of 14.29 days, thus we need to plan experiments to optimize the usage of the label before decay affects results.

[γ-32P]dNTP labeled nucleotide
Labeling in vitro Transcribed RNA:

SAP Treatment: Mix the following reagents in a 1.5 mL micro centrifuge tube and incubate at 37°C for 30 minutes. Inactivate the SAP at 65°C for 20 minutes.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 pmoles RNA (1μM = 1 pmole/μL)</td>
<td>X</td>
</tr>
<tr>
<td>Kinase Buffer</td>
<td>2</td>
</tr>
<tr>
<td>Shrimp alkaline phosphatase (SAP)</td>
<td>1</td>
</tr>
<tr>
<td>RNase Inhibitor Murine</td>
<td>1</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Fill to 20 μL</td>
</tr>
</tbody>
</table>

Kinase Treatment: Mix the following reagents with the SAP treatment. Incubate at 37°C for 30 minutes.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAP treated RNA</td>
<td>20</td>
</tr>
<tr>
<td>10X Kinase buffer</td>
<td>1</td>
</tr>
<tr>
<td>γ-P32-ATP (25μM Perkin Elmer)</td>
<td>1</td>
</tr>
<tr>
<td>Polynucleotide kinase T4 (PNK)</td>
<td>1</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Fill to 30 μL</td>
</tr>
</tbody>
</table>
Gel Purification of labeled RNAs:

To successfully complete nucleic acid footprinting the labeled RNA must be very pure, therefore gel purification methods are used to remove any degraded products or unincorporated label. Gel purification must be completed after the labeling protocols. Gel purification reduces RNA yields by 30%-50%. This method involves a series of steps to prepare reagents and the gel mixture. Prepare the following.

1. 40 % acrylamide
2. Gel mix
3. 10% APS
4. 10X TBE and 0.5X TBE buffer
5. Urea Dye

For gel purification, prepare 1.0 mm gel casting plates. Make sure plates are clean and will not leak when gel mix is added. 15 mL of gel mix will make 2, 1.0mm gels. Add 100 μL of APS and 10 μL of tetramethylethylenediamine (TEMED) (Pierce #17919) to the gel mix. TEMED will catalyze the polymerization of acrylamide. Pour the solution in between the glass plates and insert the single, 1.0mm large well comb. Allow 15-20 minutes for the gel to polymerize. Once gel is completely polymerized, remove the comb and wash the well with water using a pipette to remove any excess gel obstructing the well. Fill the apparatus chamber with 0.5X TBE Buffer. Pre-run the gel for 30 minutes at 1000 volts, 150 mA and 15 watts.
40% (19:1) Acrylamide:

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigma #A-8887</td>
<td>Acrylamide</td>
<td>380 g</td>
</tr>
<tr>
<td>Sigma #M7279</td>
<td>N, N’-Methyl bis-acrylamide</td>
<td>20 g</td>
</tr>
<tr>
<td></td>
<td>H₂O</td>
<td>Fill to 1L</td>
</tr>
</tbody>
</table>

*Use low heat to help dissolve the acrylamide.

10% Gel Mix:

To a 50 mL centrifuge tube, prepare the gel mix:

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acros #197460050</td>
<td>Urea</td>
<td>4.09g</td>
</tr>
<tr>
<td></td>
<td>40% acrylamide</td>
<td>3.98 mL</td>
</tr>
<tr>
<td></td>
<td>5X TBE</td>
<td>1.59 mL</td>
</tr>
<tr>
<td></td>
<td>RNase free H₂O</td>
<td>Fill to 10 mL</td>
</tr>
</tbody>
</table>

10% Ammonium Persulfate (APS):

In a 1.5 mL micro centrifuge tube, prepare a 10% solution of APS (Acros #40116-1000).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>0.1g</td>
</tr>
<tr>
<td>H₂O</td>
<td>Fill to 1 mL</td>
</tr>
</tbody>
</table>

*Mix by vortex.

10X TBE:

Prepare gel running buffer.

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluka #93352</td>
<td>Tris Base</td>
<td>108.0 g</td>
</tr>
<tr>
<td>OmniPur #2710</td>
<td>Boric Acid</td>
<td>55.0 g</td>
</tr>
<tr>
<td>Fisher #E478-500</td>
<td>0.5 M EDTA (pH 8.0)</td>
<td>40 mL</td>
</tr>
<tr>
<td></td>
<td>H₂O</td>
<td>Fill to 1 L</td>
</tr>
</tbody>
</table>

*Dilute 10X TBE to 0.5X TBE. Mix 50 mL of 10X TBE and 950 mL distilled water.
6M Urea Dye:

In a 1.5 mL centrifuge tube, prepare 6M urea dye.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>0.36g</td>
</tr>
<tr>
<td>10X TBE</td>
<td>50 μL</td>
</tr>
<tr>
<td>Bromophenol Blue (BPB)</td>
<td>10 μL</td>
</tr>
<tr>
<td>Xylene Cylenol dye</td>
<td>5 μL</td>
</tr>
<tr>
<td>H₂O</td>
<td>Fill to 1 mL</td>
</tr>
</tbody>
</table>

Prepare the sample for Gel Purification:

Once the gel had polymerized, prepare the end-labeled RNA sample to be loaded.

1. Add 1:1 volume of 6M urea dye to the RNA.
2. Boil the samples at 100°C for 10 minutes. Vortex and spin down.
3. Load the sample.
4. Run the gel at the same settings as the Pre-run.
5. Run the gel until the xylene cylenol dye front runs to the bottom of the gel. On average, the dye front runs out in 30 minutes.
Band Visualization of IR-700 labeled RNAs:

For IR-700 labeled RNAs, the Odyssey Gel Scanner is used to visualize the RNA. This scanner allows for higher resolution and easy viewing of the end-labeled RNA. A bright band can be viewed for band excision. The scanner has user friendly software which can be used to adjust brightness and saturation of the sample. It is also useful for printing an actual size image of the gel for band excision after purification. Follow the instrumentation manual from LI-COR and complete proper training before using the instrument.

1. The labeled RNA is still light sensitive. Protect the gel from light while transporting the gel to the scanner.
2. Open the lid of the scanner and place the gel with the thick side of the glass on the scanner, in the bottom left corner.
3. Select “New Analysis”, leave setting on “custom”.
4. Check the 700 intensity.
5. Click the large ‘f’ icon to set the focus. For a 0.75mm gel, set the focus to 3.15mm. For a 1.0mm gel set the focus to 3.17mm.
6. Adjust the scan area to ensure the entire gel is scanned.
7. Click the start button to begin the scan.
8. The scan takes approximately 12 minutes to complete.
9. Adjust the view, saturation and color when scan is complete.
10. Print the image the same size as the gel.
11. Save the scan.

Band Excision:

1. Cover the printed gel scan with a layer of clear plastic wrap.
2. Remove the gel from the glass plates and align the gel with the printed image.
3. Excise the RNA band using a sterile razor blade and forceps.
4. Prepare dialysis tubing if needed.
5. Clamp one end of the dialysis tubing.
6. Add 3 mL of 0.25X TBE Buffer.
7. Place the excised band into the tubing and clamp the top. Try to avoid bubbles.
Dialysis Tubing Preparation:

1. Remove a large piece of Spectrapor Membrane tubing. (Molecular weight cut off 6,000-8,000. Thickness: 001 (Scientific Products)).
2. Boil the tubing in a 600 mL beaker in solution #1 for 10 minutes.

Solution #1:

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fisher #E478-500</td>
<td>0.5M EDTA (pH 8.0)</td>
<td>1 mM</td>
<td>800 μL</td>
</tr>
<tr>
<td>Fisher #BP328-500</td>
<td>Sodium Bicarbonate</td>
<td>2%</td>
<td>8 g</td>
</tr>
<tr>
<td></td>
<td>H₂O</td>
<td></td>
<td>Fill to 400 mL</td>
</tr>
</tbody>
</table>

3. Rinse the tubing in RNase free water.
4. Boil the tubing for 10 minutes in solution #2.

Solution #2:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5M EDTA (pH 8.0)</td>
<td>1 mM</td>
<td>800 μL</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>Fill to 400 mL</td>
</tr>
</tbody>
</table>

5. Store tubing at 4°C in RNase free water.
**Gel Extraction:**

Two methods were commonly used to extract the purified RNA band from the gel. The first method uses Electroelution and an over-night buffer exchange. The second method is commonly referred to as the “Crush and Soak” method. Here the gel containing the RNA is crushed and soaked in an elution buffer over-night. Precipitation of the RNA is completed the following morning. After experimenting with the two methods, the crush and soak method proved to have higher yields of RNA after precipitation and decreased degradation of the RNA.

**Electroelution:**

This method is used to extract the labeled RNA from the gel. An electrophoresis chamber is set up and a negative current is applied. The current allows the RNA to migrate out of the gel. Running a positive current for 1-2 minutes will prevent RNA from sticking to the dialysis tubing.

1. Place the tubing containing the gel band into the Agarose gel electrophoresis apparatus.
2. Fill the chamber with 0.25X TBE buffer.
3. Place the tubing in the chamber.
4. Cover the apparatus to keep light from damaging the sample.
5. Run for 30 minutes at 90V.
6. Switch the positive and negative electrodes and run for 1 minute to prevent RNA from sticking to dialysis tubing.
Buffer Exchange:

1. Place the tubing in a 500 mL beaker with a stir bar.
2. Add 500 mL of RNase free water.
3. Change the water after 2 hours of dialysis and allow the buffer exchange to continue overnight.
4. The next day, transfer the RNA equally between 2 micro centrifuge tubes.
5. Cover the tops of the tubes with Parafilm and poke small holes in the top.
6. Freeze the samples before vacuuming to reduce degradation of RNA.
7. Vacuum the samples until liquid is gone.
8. Suspend the pellet in 100 μL of RNase free water.
9. Aliquot the labeled RNA into smaller samples for further use.
10. Store the labeled RNA in -80°C.

Crush and Soak:

To the excised band, add 1-2 mL of elution buffer (1X TBE and 0.3M NaOAc). Place in 4°C refrigerator on an agitator overnight. The next morning, complete the following protocol.

1. Centrifuge the tube for 10 Minutes at max speed (13,500 rpm)
2. Remove the supernatant to a clean 1 mL centrifuge tube.
3. Add 2.5x vol. of 100% cold ethanol
4. Incubate at -20°C for 1 hour.
5. Centrifuge at max speed for 20 minutes. Pour off supernatant, keep the pellet.
6. Wash the pellet with 75% cold ethanol.
7. Centrifuge for 10 minutes.
8. Speed vacuum the sample for 10 minutes to remove any excess ethanol.
9. Suspend the pellet in 50-100 μL of dH₂O.
10. Store the labeled RNA at -20°C.
Gel Purification/ Band Excision for $\gamma$$^{32}$P-ATP labeled RNAs:

Complete gel purification with a 10% acrylamide gel as before and run at constant voltage. Once the dye front has moved three quarters down the gel, stop the electrophoresis to avoid radioactive materials from entering the buffer chambers and contaminating the apparatus. Remove the gel from between the glass plates and carefully transfer the gel to a piece of plastic wrap and cover on both sides. Expose the gel to the phosphor screen for 5 minutes and scan using the Storm Scanner. Print the gel image to actual size. Use a sterile razor blade to excise the RNA band of interest. This band will migrate into the upper region of the gel and appear as the darkest band. Place the excised band into a 2 mL micro centrifuge tube.

Crush and Soak:

To the excised band, add 1-2 mL of elution buffer (1XTBE and 0.3M NaOAc). Place in 4°C refrigerator on an agitator overnight. The next morning, complete the following protocol.

1. Centrifuge the tube for 10 Minutes at max speed (13,500 rpm)
2. Remove the supernatant to a clean 1 mL centrifuge tube.
3. Add 2.5x vol. of 100% cold ethanol
4. Incubate at -20°C for 1 hour.
5. Centrifuge at max speed for 20 minutes. Pour off supernatant, keep the pellet.
6. Wash the pellet with 75% cold ethanol.
7. Centrifuge for 10 minutes.
8. Speed vacuum the sample for 10 minutes to remove any excess ethanol.
9. Suspend the pellet in 60 $\mu$L of dH$_2$O. This should be enough volume for 3 sequencing gel experiments.
10. Store the $^{32}$P-labelled RNA in a radioactive marked container at -20°C.

RNA protection methods: Complete RNA protection methods (section 2.4) using radioactive safety techniques.
2.4 RNase Protection Assays:

Knowledge of RNA secondary structure is necessary to understand the functionality of complex RNA molecules. Structure probing techniques such as RNase A, T1, and V1 protection assays, hydroxyl radical, in-line probing and Tb$^{3+}$ self-cleavage assist in discovering RNA secondary and tertiary structure. When used in combination, these methods can detect ligand binding sites and RNA conformational changes.
OH and T₁ Ladder:

The OH ladder is required to interpret footprinting patterns. The purpose of the ladder is to identify the nucleotides where the RNA is cleaved. The OH ladder cleaves after every nucleotide. Each cleavage band will be matched to a specific nucleotide. A 1x alkaline hydrolysis buffer and end-labeled RNA is required for this reaction. In this procedure, 3 hydrolysis times are used to generate optimal cleavage patterns. Select the hydrolysis time of the ladder that provides the best visualization of the cleavage. The best resolution was obtained with a 15 minute cleavage for IR end labeled RNA and 5 minutes with radiolabeled RNA.

The RNase T₁ ladder cleaves single stranded RNA from the 3’ end to G nucleotides in denaturing conditions. Therefore, a ladder of G nucleotides is generated. This reaction uses a 1X RNA sequencing buffer and RNase T₁ (ThermoScientific 1,000U/μL Cat. No. EN0541) Use dilutions of RNase T₁ to determine what concentration will provide the best digestion pattern.
OH Ladder:

1. Prepare a 1X alkaline Hydrolysis Buffer:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Na₂CO₃(pH 9.2)</td>
<td>200</td>
</tr>
<tr>
<td>0.5 M EDTA</td>
<td>20</td>
</tr>
<tr>
<td>H₂O</td>
<td>Fill to 10 mL</td>
</tr>
</tbody>
</table>

2. Prepare the OH Ladder Master Mix:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X Alkaline Hydrolysis Buffer</td>
<td>X</td>
</tr>
<tr>
<td>0.1-0.3μg end labeled RNA</td>
<td>X</td>
</tr>
</tbody>
</table>

*Add sufficient volume of 1X alkaline Hydrolysis buffer to bring total volume to 15 μL. Do not exceed 5 μL of end labeled RNA.

3. Aliquot the master mix into 3 centrifuge tubes, 5μL each.
4. Heat each sample at 95°C.
5. Remove one tube after 2 minutes. Remove the second tube after 5 minutes and remove the third tube after 15 minutes. Place sample on ice after heating.
6. Add equal volumes of Urea dye to each tube to stop the reaction.
T1 Ladder:

1. Prepare the 1X RNA Sequencing Buffer:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>7M Urea</td>
<td>4.20g</td>
</tr>
<tr>
<td>20 mM Sodium Citrate (pH 5)</td>
<td>500 μL</td>
</tr>
<tr>
<td>0.5 M EDTA</td>
<td>20 μL</td>
</tr>
<tr>
<td><strong>Total Volume:</strong></td>
<td>Fill to 10 mL</td>
</tr>
</tbody>
</table>

2. Prepare the T1 Master Mix:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X RNA Sequencing Buffer</td>
<td>X</td>
</tr>
<tr>
<td>0.15-0.3μg end label RNA</td>
<td>X</td>
</tr>
</tbody>
</table>

*Add sufficient 1X RNA Sequencing buffer to bring reaction to a total volume of 36μL.

Use no more than 10 μL of end labeled RNA.

3. Aliquot 9μL each into 4 micro centrifuge tubes.
4. Heat to 50°C for 5 minutes. Cool to room temperature.
5. Add 1 μL RNase T1 into tube #2 and mix by pipetting.
6. Transfer 1 μL from tube #2 into tube #3 and mix by pipetting.
7. Incubate all tubes at room temperature for 15 minutes.
8. Stop the reaction by adding equal volumes of LI-COR Stop Solution.
**RNase A Protection Assay:**

RNase A (Cat. No. 2274) will cleave at the 3’ end of single stranded RNA at the U and C bases. This protection method is used in combination with RNase T1 for mapping single stranded nucleotides in RNA. This protocol requires 10X RNA structure buffer (100mM Tris, pH7, 1M KCl and 100mM MgCl2) found in Ambion’s ribonuclease set (Cat. No.AM2275). Use dilutions of RNase A to determine what concentration will provide the best cleavage pattern.

1. Prepare the RNase A Master Mix:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X RNA Structure Buffer</td>
<td>4.0</td>
</tr>
<tr>
<td>0.2-0.4 μg end label RNA</td>
<td>X</td>
</tr>
</tbody>
</table>

*Add nuclease-free water to bring reaction to a total volume of 36 μL.

2. Aliquot 9 μL of the master mix into 4 microcentrifuge tubes.
3. To tube #2, add 1 μL of RNase A and mix by pipetting.
4. Transfer 1 μL from Tube #3 to tube #4 and mix by pipetting.
5. Incubate all tubes at room temperature for 15 minutes.
6. Stop the reaction by adding equal volumes of Urea dye.
RNase V₁ Protection Assay: base paired nucleotides

RNase V₁ will cleave base paired nucleotides. This method is useful for mapping structured regions of RNA.

1. Prepare the RNase V₁ Master Mix:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X RNA Structure Buffer</td>
<td>4.0</td>
</tr>
<tr>
<td>0.2-0.4 μg end label RNA</td>
<td>X</td>
</tr>
</tbody>
</table>

   *Add nuclease-free water to bring reaction to a total volume of 36 μL.

2. Aliquot 9 μL of the master mix into 4 microcentrifuge tubes.
3. To tube #2, add 1 μL of RNase V₁ (Cat. No. #2275) and mix by pipetting.
4. Transfer 1 μL from Tube #3 to tube #4 and mix by pipetting.
5. Incubate all tubes at room temperature for 15 minutes.
6. Stop the reaction by adding equal volumes of urea dye.
RNase I Protection Assay (Ambion #AM2294/ #AM2295):

RNase I will degrade all RNA dinucleotide bonds leaving a 5’ hydroxyl and a 2’, 3’ cyclic monophosphate. It has a high specificity for single strand RNA degradation which works well for protection assays. Cleavage happens on the 3’ side of pyrimidine bases (C,T and U).

1. Prepare the RNase I Master Mix:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X RNA Structure Buffer</td>
<td>X</td>
</tr>
<tr>
<td>10 μg end label RNA</td>
<td>X</td>
</tr>
</tbody>
</table>

*Add sufficient 10X RNA Structure buffer to bring reaction to a total volume of 27μL. Use no more than 10 μL of end labeled RNA.

3. Aliquot 9μL into 3 micro centrifuge tubes.
5. Add 1 μL RNase I(100U/μL) into tube #2 and mix by pipetting.
6. Transfer 1 μL from tube #2 into tube #3 and mix by pipetting.
7. Incubate all samples at 37°C for 30 minutes.
8. Stop the reaction by adding equal volumes of urea dye.
Fragmentation with Tb$^{3+}$:

Fragmentation with Terbium (Tb$^{3+}$) is used for probing the overall secondary structure of RNA at nucleotide resolution where the RNA is flexible enough for cleavage. Tb$^{3+}$ has a low pKa which increases formation of Tb(OH)$^{2+}$ to hydrolyze the RNA backbone. By deprotonating the 2-hydroxyl of the ribose, nucleophilic attack of the resulting oxyanion on the 3,5-phosphodiester forms 2’,3’-cyclic phosphate and releases the adjacent 5’ hydroxyl terminus. At low concentrations, this self-cleavage protocol allows Tb$^{3+}$ to bind in the same location as Mg$^{2+}$, thus revealing the Mg$^{2+}$ binding sites in the RNA. The best fragmentation has been observed with 100mM Tb$^{3+}$ and a 90 minute incubation period.

1. Refold the end labeled RNA.
   a. Heat for 2 minutes at 100°C. Quick Spin. 10 minutes on ice.
2. Prepare 3 concentrations of TbCl$_3$ from a 100 mM stock solution. (0.3 mM, 0.5 mM and 100mM). Dissolve 3.73g TbCl$_3$ in 100 mL of 5 mM sodium cacodylate buffer.
3. Sodium Cacodylate buffer, 5 mM (pH 5.5): to make a 100 mM stock concentration add 15.9g/L. Add NaOH to adjust the pH to 7.0. Dilute 100mM stock to 5 mM. Store at room temperature.
4. Prepare the following mix for each concentration of TbCl$_3$.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>2</td>
</tr>
<tr>
<td>5X Reaction Buffer with HEPES (pH 7.0-7.5)</td>
<td>2</td>
</tr>
<tr>
<td>TbCl$_3$ (Alfa Aesar #44472)</td>
<td>2</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>10</strong></td>
</tr>
</tbody>
</table>

*5X Reaction buffer: 100mM HEPES pH 7.0, 500mM KCl, 5mM MgCl$_2$.

5. Incubate samples at 37°C for 15 minutes.
6. After 15 minutes remove 5 μL from each tube and add it to a new tube with 1 μL 0.5M EDTA.
7. Allow the remaining 5 μL to incubate at 37°C for an addition 1.5 hours.
8. Add 1 μL of EDTA to the samples after 1.5 hours.
9. Add equal volumes of urea dye to each sample.
In-Line Probing:

The in-line probing method is one of the oldest cleavage methods around and is very simple to complete. This method takes advantage of RNAs natural instability. A nucleophilic attack cleaves the RNA phosphodiester linkage by aligning the 2’ oxygen with the 5’ oxygen leaving group to cleave the RNA linkage. Cleaving the RNA will result in fragmentation. However, this method uses very restrictive conditions that tend to differ from in vivo environments. A 5X Tris-based reaction buffer is required for this reaction. Best cleavage patterns were observed after 72 hours. Do not let cleavage proceed longer than 72 hours.

1. Mix the following in a 1.5 mL micro centrifuge tube:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X Tris Buffer</td>
<td>2</td>
</tr>
<tr>
<td>End-labeled RNA</td>
<td>2</td>
</tr>
<tr>
<td>H2O</td>
<td>6</td>
</tr>
</tbody>
</table>

*5X Tris Buffer: 100mM Tris pH 8.0, 500mM KCl, 20mM MgCl₂

2. Allow sample to incubate for 24 hours at room temperature. Keep sample out of light.
3. After 24 hours remove 2 μL and mix with 2 μL of urea dye. Freeze sample at -20°C.
4. After an additional 24 hours, remove 2 μL and mix with 2 μL of urea dye. Freeze sample at -20°C.
5. After an additional 24 hours, remove 2 μL and mix with 2 μL of urea dye. Freeze sample at -20°C.
6. Label samples as 24 hr., 48 hr., and 72 hr.
7. Add equal volumes of urea dye to each sample.
8. Heat samples at 100°C for 2 minutes prior to loading on a gel.
Hydroxyl Radical:

This is an additional cleavage technique, however due to time constraints this protocol has not been optimized with the ykkCD RNA. This method involves the generation of a hydroxyl radical by the reduction of hydrogen peroxide ($\text{H}_2\text{O}_2$) by iron and ascorbic acid. The hydroxyl radical reacts with the RNA by breaking down the phosphate backbone. However, the hydroxyl radical method is not sensitive to the secondary structure and only detects accessibility of the backbone; not individual bases. When the OH radical cleavage pattern of RNA and RNA-ligand complexes are compared, the hydroxyl radical allows us to see where the RNA is protected from cleavage.

1. Prepare the following solutions:
   - 100 mM EDTA pH 8.0
   - 250mM ascorbic acid
   - 2.5% hydrogen peroxide
   - 1M Thiourea
   - 50mM Fe(NH$_4$)$_2$(SO$_4$)$_2$ *6H$_2$O

2. Prepare the hydroxyl radical buffer:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 mM HEPES Buffer (pH 7.8)</td>
<td>384</td>
</tr>
<tr>
<td>100 mM MgCl$_2$</td>
<td>240</td>
</tr>
<tr>
<td>500 mM NH$_4$Cl</td>
<td>480</td>
</tr>
<tr>
<td>100 mM DTT</td>
<td>120</td>
</tr>
<tr>
<td>Add dH$_2$O to total volume</td>
<td>2000</td>
</tr>
</tbody>
</table>

3. Add 2 μL of RNA to 19 μL of hydroxyl radical buffer.
4. In a separate microcentrifuge tube, mix the following:
   - 4 μL 50mM Fe(NH$_4$)$_2$(SO$_4$)$_2$ *6H$_2$O
   - 4 μL 100 mM EDTA pH 8.0
   - 4 μL 250mM ascorbic acid
5. Mix 4 μL of the solution from step 4 with the RNA-buffer mixture from step 3.
6. Incubate the sample for 3 minutes at room temperature.
7. After 3 minutes, remove 8 μL and add to a tube containing 8 μL of 1M thiourea.
8. Incubate sample for an additional 2 minutes.
9. After 2 minutes, remove 8 μL and add to a tube containing 8 μL of 1M thiourea.
10. Incubate the sample for an additional 5 minutes.
11. After 5 minutes, add 8 μL of 1M thiourea to the tube.
12. Add equal volumes of LI-COR Stop Solution to each sample.
2.5 RNA Sequencing Gel:

The Model 4300 LI-COR DNA Analyzer is used for RNA sequencing of IR-700 labeled RNAs.

The instrument uses fluorescence to visualize the chain terminated fragments of DNA/RNA by separation on an acrylamide gel. The fragments are excited by a diode laser as they pass by the detection window. The image data is displayed on the computer monitor using e-Seq software. Refer to the manufacturer's application manual for more detailed instruction.

Choosing a Gel:

For IR-700 labeled RNA footprinting, a 41 cm gel with 0.35 mm spacers is used. The well comb chosen for these experiments contained 20 wells, 0.64 cm wide.

Reagent Preparation:

The following reagents need to be prepared prior to assembling the gel. All reagents need to be at room temperature.

1. Gel Slick Solution (Lonza Cat. No. 50640) -OR- mix 50 μL of bind silane to 10 mL of 100% ethanol.
2. 5.5% Acrylamide Gel Mix

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% Acrylamide</td>
<td>5.5 mL</td>
</tr>
<tr>
<td>10X TBE</td>
<td>4.0 mL</td>
</tr>
<tr>
<td>Urea</td>
<td>19.2 g</td>
</tr>
<tr>
<td>Fill to 40 mL with DI water</td>
<td>Total Volume: 40 mL</td>
</tr>
</tbody>
</table>

3. 230 μL of 10% APS (0.1g APS to 1 mL deionized water)
4. 23 μL of TEMED
5. 1L of 0.8X TBE running buffer
6. 1% Agarose sealing gel (1 g agarose in 100 mL deionized water, microwave for 1 minute or until boiling)
Plate Assembly:

Use the following to assemble the electrophoresis apparatus.

1. Gloves
2. Kim Wipes
3. Front plate (41 cm)
4. Back plate (41 cm)
5. 0.35 mm spacers
6. 0.35 mm comb
7. 1 set of rail assemblies
8. 50 mL centrifuge tube
9. Bind Silane
10. 1000 μL pipette and tips

Begin by cleaning the front and back plate with dish detergent and a bristle brush. Rinse well with water and dry. Follow assembly steps below.

1. Using a Kimwipe, apply a small amount of bind silane to the inner surface of the front and back plates. Allow the solution to completely dry for 3 minutes.
2. Place the spacers along the edge, on the inside of the back plate.
3. Place the front plate on top of the back plate. Realign the spacers if necessary.
4. Attach the left and right rail assemblies over the plate edges. Make sure the glass plates are flat against the bench top and the rails fit tightly against the edges of the plates. The rail assembly should have the groove for the upper buffer tank pointing up.
5. Tighten the rail assembly just finger tight to avoid damaging the glass plates.
6. Microwave the 1% agarose sealing gel for 1 minute or until boiling.
7. Using a pasture pipet, seal the bottom and sides of the glass plates with the sealing gel. Allow 5 minutes for gel to polymerize.
8. Stand the gel up right (90°) on the bench top and use excess sealing gel to ensure the bottom of the glass plates is sealed before pouring the gel.
9. Mix 40 mL of the 5.5% acrylamide gel mix with 23 μL of TEMED and 230 μL of 10% APS.
10. Use a 1000 μL pipette to add the gel mix between the glass plates until full.
11. Use a bubble hook to remove any bubbles from the gel.
12. Insert the well comb.
13. Allow an hour and a half for the gel to polymerize.
Pre-Running the Gel:

1. Insert the bottom, black buffer chamber in the LI-COR 4300 Analyzer.
2. Set the bottom of the gel in the bottom buffer chamber and hook the support arms on the rail assemblies into the notches on the analyzer.
3. Unscrew the top, upper buffer tank knob on the rail assemblies.
4. Place the upper buffer tank into the notch on the rail assembly and tighten the knobs finger tight.
5. Fill the bottom buffer tank and upper buffer tank to the fill line with 0.8X TBE running buffer.
6. Let the comb be submerged in the running buffer for 10 minutes prior to removing the comb.
7. After 10 minutes, remove the comb and rinse the wells using a syringe and a razor blade to remove any excess gel from around the wells.
8. Attach the bottom and upper buffer tank lids ensuring the wire electrodes are submerged in the running buffer.
9. Attach one end of the double-ended jumper to the high voltage connector port on the upper buffer chamber and the other end to the port on the analyzer.

e-Seq Software:

1. Select New run from the file menu.
2. Specify folder to save the run data.
3. Enter name and password (user/user).
4. Enter the electrophoresis conditions (4300 25 cm .25 mm)
5. Choose labeling channels (700/800)
6. Select Begin Pre Run(pre run lasts 20-25 minutes)
7. When pre-run finishes, open the door to the analyzer, remove the upper buffer tank lid. Heat samples at 100°C for 2 minutes prior to loading. Load 1-2 μL samples using a 0.5-10 μL pipette with 0.4 mm flat multi-flex tips (FisherScientific Cat. No. 05-408-150).
8. Once all samples are loaded, put the lid back on the upper buffer chamber, close the door to the sequencer and select Begin Run on the monitor.
9. Run will take 2-5 hours to complete. Data will automatically be saved to the computer. Gel runs are safe to let run over night if needed.
ImageJ Software:

ImageJ is a Java-based image processing program available for free through the National Institute of Health. ImageJ is able to edit the .TIF image files saved by the 4300 Analyzer.

1. Open the ImageJ toolbar.
2. Select **File** then **Open** and choose the .TIF image from your foot printing gel.
3. Select **Edit** and then **Invert** to reverse the black and white color settings. This makes the bands easier to analyze.
4. Click and drag anywhere on the image to select the region containing the band patterns.
5. Select **Image** then **Crop**
6. Select **Image** then **Adjust** then **Brightness/Contrast** to best visualize the bands.
7. Select **File** and **Save As** to save your image as a .jpeg
8. The image can now be opened in other programs such as Word or Adobe and printed.
RNA Sequencing Gel:

Choosing a Gel:

For radiolabeled RNA footprinting, a 45x20 cm gel with 0.35 mm spacers is used. The well comb chosen for these experiments contained 20 wells, 0.64 cm wide.

Reagent Preparation:

The following reagents need to be prepared prior to assembling the gel. All reagents need to be at room temperature.

1. Gel Slick Solution (Lonza Cat. No. 50640) -OR- mix 50 μL of bind silane to 10 mL of 100% ethanol.
2. 15% Acrylamide Gel Mix

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% Acrylamide</td>
<td>94 mL</td>
</tr>
<tr>
<td>10X TBE</td>
<td>25 mL</td>
</tr>
<tr>
<td>Urea</td>
<td>120 g</td>
</tr>
<tr>
<td>Fill to 40 mL with DI water</td>
<td>Total Volume: 250 mL</td>
</tr>
</tbody>
</table>

3. 400 μL of 10% APS (0.1g APS to 1 mL deionized water)
4. 40 μL of TEMED
5. 1L of 1X TBE running buffer
Plate Assembly:

Use the following to assemble the electrophoresis apparatus.

1. Gloves
2. Kimwipes
3. Front plate
4. Back plate
5. 0.35 mm spacers
6. 0.35 mm comb
7. Black binder clips (8 large size)
8. Bind Silane
9. 250 mL beaker
10. Scotch Packing Tape

Begin by cleaning the front and back plate with dish detergent and a bristle brush. Rinse well with water and dry. Follow assembly steps below.

1. Using a Kimwipe, apply a small amount of bind silane to the inner surface of the front and back plates. Allow the solution to completely dry for 3 minutes.
2. Place the spacers along the edge, on the inside of the back plate.
3. Place the front plate on top of the back plate. Realign the spacers if necessary.
4. Using the Scotch Package tape, tape the bottom portion of the gel and the entire left and right side of the gel plates. Be sure to remove any air bubbles in the tape. Use the black binder clips to help secure the edges.
5. Lean the gel at a slight angle (30°) on the bench top.
6. Mix 100 mL of the 15% acrylamide gel mix with 40 μL of TEMED and 400 μL of 10% APS.
7. Slowly, pour the gel mix between the glass plates until full.
8. Use a bubble hook to remove any bubbles from the gel.
9. Insert the well comb.
10. Allow an hour for the gel to polymerize.
11. Clean the outside of the glass plates with dH₂O and ethanol if necessary.
Pre-Running the Gel:

1. Insert the bottom of the glass plates into the lower buffer chamber of the Owl Electrophoresis apparatus.
2. Finger tighten the side rails to hold the glass plate to the apparatus.
3. Fill the bottom buffer tank and upper buffer tank to the fill line with 1X TBE running buffer.
4. Let the comb be submerged in the running buffer for 10 minutes prior to removing the comb.
5. After 10 minutes, remove the comb and rinse the wells using a syringe and a razor blade to remove any excess gel from around the wells.
6. Attach the bottom and upper buffer tank lids ensuring the wire electrodes are submerged in the running buffer.
7. Pre Run the gel at constant voltage for 10 minutes.
8. Load samples remembering that they are radioactive. Use caution.
9. Run the gel for 2-3 hours.
Drying and Exposing the Radioactive Gel:

1. Remove the buffer from the upper chamber of the apparatus.
2. Loosen the side knobs and remove the glass plates from the apparatus.
3. Carefully remove the top glass panel.
4. Rinse the gel with a wash bottle filled with 10% glacial acetic acid and 10% methanol. Start rinsing the gel from the top of the wells to the bottom of the plate. This will remove excess urea from the gel and speed up the drying process. The gel will start to wrinkle as it lets go of the bottom glass panel.
5. Finish rinsing the gel with dH₂O from the top of the gel to the bottom of the gel.
6. Cut 3-5 sheets of gel drying paper to the correct size of the gel. Lay 1 sheet of the paper on top of the gel and slowly turn the glass plate upside down to help transfer the gel to the paper.
7. Lay the gel into a tray with enough distilled water to cover the gel. Use this try to help remove any wrinkles in the gel while it was transferred to the paper.
8. Set the paper and gel on top of the remaining sheets of drying paper that was cut. This will help absorb excess water.
9. Cover the gel with plastic wrap and place on the vacuum gel dryer. Set the temperature of the gel dry to 80°C for 1-2 hours. Set the vacuum timer for 2-3 hours. Allow the gel to cool and dry before removing it from the vacuum set up to avoid shattering and cracking the gel.
10. Cut the gel to fit the area of the phosphor screen and expose the gel overnight.
11. In the morning, scan the gel using the Storm scanner. ImageJ software can be used to improve the image quality.
12. Clear the phosphor screen by exposing it to white light.
13. Dispose of the gel in the proper radioactive waste container. Check that no areas of the lab were contaminated by the radioactive samples.
Chapter 3: Results

The following data has been collected throughout the duration of this thesis. Techniques were optimized to improve efficiency, increase resolution of sequencing gels and provide valuable information for future students working on this project. All optimizations to procedures are reflected in Chapter 2, Protocols.

**ykkCD Construct RNA Purification:**

DH5α competent cells are transformed with the plasmid coding for the ykkCD riboswitch and incubated in selective medium to only grow bacteria that harbors the ykkCD construct. Transformation efficiency is increased by using competent cells. Heat shock method allows DNA to enter the bacteria at a faster rate. Cells were successfully cultivated after overnight incubation. Cells were harvested after 2-part inoculation for a 24 hour growth period.

![Figure 20](image)

*Figure 20:* (a.) ykkCD Bacterial cells grown in *Bacillus Subtilis* containing the ykkCD construct, plated on an ampicillin treated agar plate. (b.) Bacterial cell growth after 2-part inoculation.
Once cells were collected, purification of plasmid DNA is completed using the Qiagen Plasmid Plus Midi Kit. The DNA is referred to as “uncut DNA” or “super coiled” DNA, because it has not been linearized at this point. Average yields of uncut DNA were around 500 ng/μL in 200 μL volume. Linearization of the DNA transforms the circular plasmid DNA into a linear molecule using BamHI reaction enzyme. This DNA is referred to as “Cut” DNA. To be sure that linearization takes place, both samples are run on a 1% agarose gel with a 1kb (0.1kb-10kb) DNA ladder. In a successful linearization, the uncut DNA will travel faster and further than the cut DNA.

![Image of agarose gel](image)

**Figure 21:** 1% agarose gel used to confirm the success of DNA linearization with BamH1 restriction enzyme. The left lane contains the MW ladder, the middle lane shows the uncut, supercoiled DNA and the third lane shows a successfully linearized DNA.

Phenol chloroform (PC) extraction and DNA precipitation was used to remove the BamHI enzyme and perform a buffer exchange after linearization. The DNA pellet was suspended in RNase free water. By completing the buffer exchange, a more accurate DNA concentration can
be identified by the Nanodrop spectrophotometer. After DNA PC extraction and precipitation, average yields of linearized DNA were 200-500 ng/μL in 100 μL.

As mentioned earlier, transcription requires the use of T7 RNA polymerase to transcribe the DNA into RNA. To confirm the success of transcription, a 10% denaturing PAGE was used. Figure 22 shows what happens when T7 RNA polymerase is omitted from the transcription mix. Transcription does not take place and RNA is not produced. When T7 is included, a dark band appears containing the RNA. The gel is stained with ethidium bromide and visualized with UV light. RNA PC extraction and RNA precipitation will remove any unwanted side products of the reaction, complete buffer exchange and reduce the transcription volume prior to gel purification. This is a good protocol to complete after transcription and before gel purification.

**Figure 22:** 10% denaturing PAGE of mock transcription reaction and transcription with T7 RNA polymerase. Lane 1, is the mock transcription, sample contains no T7, the thin band present represents the linear DNA template. Lane 2 is blank, it contains no sample. Lane 3 contains the transcription reaction with T7.
Gel purification is required to ensure the RNA is free from degradation products and unincorporated nucleotides before end labeling. Figure 23 shows a 10% denaturing PAGE used for gel purification. The top, dark band is the RNA. It migrates towards the top region of the gel due to its molecular weight. The darker band that appears at the bottom of the gel is the urea-xylene cyanol dye front. The band containing the RNA is excised using sterile technique, followed by the Crush and Soak procedure found in Chapter 2. Figure 24 shows a 1% agarose gel with a successful progression of uncut DNA, cut DNA and purified RNA after the crush and soak protocol. The RNA is smaller in size than the DNA and should travel further in the gel as seen in Figure 24. The RNA is ready for end labeling. It is clear that the purified RNA is not contaminated with template DNA.

**Figure 23**: RNA gel purification visualized by UV shadowing. The top band contains the RNA. The bottom band is the urea dye front.
Figure 24: 1% agarose gel showing successful uncut DNA, cut DNA and purified RNA used for end labeling.

ykkCD RNA 5’ End Labeling:

As described in the previous chapters, three methods of end labeling were attempted during this project. The following data, will define which labeling method is most efficient.

Method 1: 5’ End Labeling with IR-700 dye

Optimization of the 5’ end labeling protocol began on 02-02-2014. Two different phosphatase enzymes were used. The first enzyme, FastAP from Fermentas required an inactivation temperature of 75°C. The protocol was followed using this phosphatase many times, but it was believed that the higher inactivation temperature was damaging the RNA. There also could have been contamination during the phosphatase treatment that was degrading the RNA. The second phosphatase was shrimp alkaline phosphatase (rSAP) from New England Biolabs. The inactivation temperature for rSAP is 65°C and it costs less than the FastAP. In both cases the phosphatase treatment was followed by IR dye labeling the RNA with T4 polynucleotide kinase. Using the rSAP showed a strong band that migrates the same distance as the unlabeled RNA. This band was excised and extracted by the crush and soak method.
RNase protection assays and Tb$^{3+}$ fragmentation was completed on the 5’ labeled RNA. Small 10% denaturing PAGE were run before attempting to sequence the pattern on the larger sequencing gel. After many months of running these trials it became apparent that there was something unacceptable with the 5’ end labeling of the RNA. The untreated RNA showed a distinct pattern of bands, when it should have been a single, clean, non-degraded sample. The protection assays and Tb$^{3+}$ fragmentations showed the same pattern of bands that the untreated sample did. To determine if this was caused by degradation, all solutions were made again under RNase free conditions and new enzymes were purchased. The band patterns were better seen during Tb$^{3+}$ fragmentation, which indicated that Tb$^{3+}$ fragmentation would be a good cleavage method to pursue. The protection assays were completed again, using T$_1$, V$_1$ and T$_1$ and OH ladders were generated. The gels showed no signs of cleavage with the ribonucleases. The lack of cleavage pattern observed could be contributed to the IR-700 dye blocking the ribonuclease from cleaving the RNA. Since interpretation of Tb$^{3+}$ fragmentation
patterns requires comparison to RNase T₁ ladder without the ability to perform RNase T₁ fragmentation. Tb³⁺ fragmentation of IR-dye labeled samples alone are not useful. The figures below show what these patterns looked like.

Figure 26: 10% denature PAGE, run at constant voltage for 30 minutes. (a.) Tb³⁺ fragmentation (b.) V₁ Ribonuclease protection (c.) RNaseI and T₁ ribonuclease protection

ykkCD RNA 3’ End labeling:
3’ end labeling was another available option to visualize the cleavage patterns. This method uses terminal transferase and the same IR-700 dye as 5’ end labeling. The protocol for the 3’ end labeling takes less time to complete than the 5’ end labeling protocol; this can decrease RNase contamination and degradation by reducing incubation time and using lower temperature. The first experiment completed was a comparison of 3’ end labeled RNA versus 5’ end labeled RNA. See Figure 27. With the new labeling method, the RNA traveled into the gel.
Terminal transferase is known to add more than one nucleotide to the end of the RNA. This might account for the change in migration between the 5’ and 3’ labeled RNA samples.

**Figure 27**: 10% denature gel used to visualize 3’ and 5’ end labeling prior to gel purification.

In order to determine which band in the 3’ labeled RNA contained the RNA; an experiment was performed using a sample that has the 3’ end labeled RNA and a sample without the RNA. Since the mock reaction contained everything except the RNA, the RNA band could be identified. The samples were resolved on a 10% denaturing gel. See Figure 28. There are some bands that are similar in each sample; but the top band is only present in the sample containing the RNA. This is why the top band was identified as the labeled RNA.
Figure 28: 10% denature PAGE with 3’ end labeled RNA and control sample of IR-700 dye and terminal transferase.

Next, a larger volume of 3’ end labeled RNA was purified. Figure 29 shows what this purification looked like. The first dark band from the top was extracted to collect the labeled RNA. The third bottom band was the urea dye front and it was not collected. Since the top two bands are in close proximity, purification gels had to be run longer to increase separation. After gel purification, it was decided to attempt the first sequencing gel.
Figure 29: Gel purification of 3’ end labeled RNA. The gel shows three bands from the top to the bottom. The top band contains the labeled RNA. The middle band is degraded product and unincorporated dye. The bottom band is the urea dye front.

Optimization of the LI-COR DNA analyzer was required. In the past, the instrument had a history of a leaking buffer chamber, which produced streaking and a “smiling” effect when samples are run. Rubber gaskets were replaced around the buffer chambers and the leaking stopped. Sequencing gels were pre-run, loaded and were run at constant voltage for 6 hours. The gel was extremely overloaded with labeled sample, but there was an increase in cleavage bands present that could not be seen with the small, 10% denature PAGE.
Figure 30: First attempted sequencing gel of 3’ end labeled RNA.

There is an area that appears to have dead pixels or damage to the scanning device inside the instrument. For future sequencing gels, avoid loading the three, furthest left lanes. The LI-COR analyzer is a very sensitive instrument. It will identify any cleavage bands present in the sample and adjust the background contrast based on intensity of the sample. For this reason, it was important to optimize, specifically how much sample needed to be loaded onto the gel to visualize the best cleavage patterns. Each RNA sample contained 3 picomoles of RNA. Serial dilutions were used to reduce the RNA concentration as seen in Figure 31.
**Figure 31**: Dilutions of 3’ end labeled RNA to determine which dilution provides the best cleavage pattern. The (1/1000) dilution provided a clear cleavage pattern.
Figure 32 shows a sequencing gel with 3’ end labeled RNA, optimized for the amount of labeled RNA sample loaded, acrylamide gel percentage and run time. This gel showed distinct cleavage bands, however the control RNA sample was not clean. The OH ladder cleavage pattern looks the same as the control sample. The T₁ ladder showed no cleavage, this may mean that higher concentration of T₁ would be required to see the cleavage. While this sequencing gel looked promising, the samples were not clean enough to interpret a sequencing pattern.

**Figure 32**: Sequencing gel of 3’ end labeled RNA.
The 3’ end labeling protocols and protection assays were completed many times with similar results. Figure 33 is an image of the sequencing gel that had the least contamination present in the control RNA sample. However, just like the 5’ labeling with the IR-700 dye; the protection assays and fragmentation methods were showing no signs of cleavage.

**Figure 33:** 3’ end labeled sequence gel containing Tb^{3+}, T₁ and RNase A protection samples.
**ykkCD RNA 5’ End labeling with γ-^{32}P-ATP:**

On April 16, 2015, the lab had completed the requirements for radiation safety. An area of the lab was designated to working with radioactive samples and lab members completed course work for radiation safety training. Radioactive labeling has classically been the best way to visualize cleavage patterns in sequencing gels. With the help of Dr. Emil Khisamutdinov, the lab purchased a Storm Scanner, Storm Scanner computer software and a phosphor imager screen to use with radioactive end labeling. γ-^{32}P-ATP was ordered from Perkin Elmer. To ^{32}P label the *in vitro* transcribed RNA follow the protocol found in chapter 2. Figure 34 shows the first attempts of labeling and gel purification of radioactive labeled RNA at the 5’ end with γ-^{32}P-ATP. The top, darkest bands were excised and eluted from the gel using the crush and soak method. Two bands are present, because the volume of radiolabeled RNA exceeded the volume capacity of one sample well in the gel.

![Figure 34](image.jpg)

**Figure 34:** 5’ end labeling of RNA with γ-^{32}P-ATP using 10% denature PAGE. Top, circled bands were purified.
RNA cleavage experiments were completed on the radiolabeled RNA. Each sample contained 1 picomoles of radiolabeled RNA with 2-4 μg tRNA. Samples were loaded with 8M urea dye onto a 10% denaturing PAGE. A higher concentration of urea dye was used to help load the samples into the well; the higher concentration of urea allows the sample to sink into the wells and reduces risk of overflowing the well and contaminating other samples. The sequencing gel was exposed over-night against a phosphor imager screen and read out on the Storm scanner. Figure 35 shows the result of the RNase protection using radiolabeled RNA. Unfortunately, the RNA showed signs of degradation. To resolve radiolabeled samples on a sequencing gel, an apparatus that is different from the LI-COR sequencer had to be used. The usage of this equipment had to be mastered before samples can be run. It’s plausible that the RNA has degraded during this time.
Recall that the goal of this thesis is to identify where tetracycline binds to the ykkCD RNA. This goal is achieved by comparing ykkCD RNA fragmentation patterns without tetracycline to the fragmentation pattern of the RNA with bound tetracycline.
Figure 36 contains the first footprinting gel with the ykkCD riboswitch RNA-tetracycline complex. Tetracycline tends to stabilize the RNA and help reduce degradation of the control sample. However, this gel still shows degradation. The cleavage bands are not defined enough for interpretation, but two conclusions can still be drawn from this data. The first is that 5 minute incubation of the RNA with the hydroxyl ladder mix appears to provide an interpretable OH ladder. The second is, the last dilution of RNase A appears to be suitable to see RNase A cleavage patterns.

Figure 36: ykkCD Riboswitch RNA + Tetracycline complex, sequencing gel to visualize footprinting patterns to map the binding site of tetracycline.
After seeing the success of these radioactive sequencing gels, it was decided to abandon the IR-700 labeling methods and pursue the $^{32}$P methods and protocols. Once the techniques were optimized, it was decided the samples need to be labeled with a higher concentration radioisotope to better distinguish the cleavage patterns. Due to cost of label and possible degradation in the samples seen in figures 35 and 36, all reaction buffers were made again under RNase free conditions. Samples were prepared for protection assays, the sequencing gel was run, and the gel was exposed overnight.

The next morning (05-18-2015) the gel needed to be read by the Storm scanner. The laptop connected to the scanner suffered hardware damage and the scanner software could not be revived. At this point the radioactive sample was decaying, the gel was slowly losing its radioactivity and it was uncertain if the computer would be repaired soon enough to interpret the gel. On (06-04-15) a new laptop arrived. Although this was 18 days after the samples were run on the gel, we read the gel using the new laptop and scanner. Figure 37 is the scan of this gel.
Figure 37: $^{32}$P-labeled RNA sequencing gel.
The control RNA sample in this gel is fairly clean compared to previous samples. Looking at this gel we can conclude that the OH ladder shows the best cleavage after 5 minutes of incubation. The RNase master mix did not degrade the RNA, which shows the buffers used for digestion are not contaminated. The RNase A digestions still appear to be too potent to visualize the cleavage. The RNase T<sub>1</sub> showed prominent bands that were consistent across the decreasing concentration of T<sub>1</sub> ribonuclease. The 1/100 serial dilution showed the best resolution of the cleavage pattern. However, the nucleotides at the 5’ end are hard to visualize on this gel. It is too difficult to identify the individual nucleotides. Ways this could be optimized is by adjusting the amount of radio labeled RNA in each sample and using a lower percentage gel to resolve the samples. The in line probing method without tetracycline showed little to no cleavage after 72 hour incubation at room temperature. The in line probing of the RNA-tetracycline complex showed a unique cleavage pattern with 6 distinct bands. It is possible that the tetracycline sample was contaminated with ribonuclease hence future studies with the tetracycline stock will be remade. If the distinct difference between the in line probing patterns of the ykkCD RNA and the ykkCD RNA-tetracycline complex is real and not due to degradation, it would imply that tetracycline binding causes a significant change in RNA structure. More tests need to be done to examine this possibility.

Crucial time was lost while the laptop was being replaced. The half-life of the radioactive material is 14 days. Due to time constrains of graduation deadlines and submission dates, it is uncertain if more radioactive sequencing gels can be completed. Chapter 4 of this thesis will discuss the data collect thus far.
Chapter 4: Discussion

The following discussion states the most recent knowledge of the mapping of the structure of the ykkCD riboswitch.

By transcribing the RNA template in vitro, we are using artificial conditions which are different from what would be occurring under normal biological conditions. An advantage to this approach is that it allows us to study the effect of tetracycline on the ykkCD RNA structure individually and without the effect of a complex biological system. In vitro processes are easy to manipulate, optimize and duplicate in high yield while in low volume. A drawback is that it can be difficult to relate the in vitro data to biological conditions.

Once the ykkCD RNA was isolated by gel purification, it was determined that the best way to extract the RNA from the gel was through the crush and soak method discussed in the protocols. Electroelution was attempted many times with low yield and it was very difficult to not contaminate the RNA in the process.

Once the RNA was purified, three labeling methods were attempted; 5’ end labeling with IR-700 dye, 3’ end tailing with TdT and 5’ γ-32P-ATP labeling. Of the three labeling methods, each had its advantages and disadvantages.

5’ end labeling with the IR-700 dye is beneficial since the IR dye has no half-life like the radioactive 32P. This means that experiments can be run at a slower pace, which is good for students who are not trained to use radioactivity or are not full time workers in the lab. Some disadvantages to this labeling method come from the structural change it causes to the RNA.
The IR dye molecule is about the size of a nucleobase and it appears to be blocking the ribonuclease from cleaving the RNA where it should during sequencing. This labeling experiment also involved the most time to complete.

3’ end tailing with TdT was a different approach to label the RNA with the same IR-700 dye. This method seemed more promising than the 5’ end labeling, because we could better identify the labeled RNA during gel purification. This reduced the contamination to the sample and this method was also less time consuming. Again, since the IR dye has no half-life the samples can be stored and don’t have to be used immediately. With this method, we noticed that labeled RNA was migrating a different distance than the RNA labeled with at the 5’. This is another indication that the IR dye was altering the structure of the RNA. At one point, it was decided to try footprinting studies. From the data previously presented, these gels could not be interpreted. The cleavage patterns could not be determined, because they showed the same cleavage patterns as the control RNA. This raised the question whether the IR dye is blocking the nuclease from cleaving the RNA.

It is possible the IR dye is blocking the ribonuclease from cleaving near the end of the sequence because RNase I, V₁, T₁ and A are all endonucleases. Since endonuclease cleave the phosphodiester bond in the internal linkages, this would explain why we only see some cleavage bands near the center of the sequencing gels. Using exonucleases may result in more cleavage bands near the ends of the RNA because exonucleases break down the RNA by removing nucleotides from the end of the chain. Exonucleases also have the ability to inhibit cleavage.
In order to use the IR dye, the best approach would be to use reverse transcription (RT) with an IR active primer. This approach is being optimized by another graduate student, Beau Champ. He is using RT and SHAPE chemistry. This method is difficult to develop because the primer site needs to be optimized to ensure that it is accessible in the RNA. The RT process can be time consuming and involves expensive reagents.

The $^{32}$P labeling method is classically the method of choice for RNA footprinting. Since $^{32}$P is another isotope of phosphorous it does not alter the structure of the RNA like the IR dye. The RNA bands were prominent during gel purification and migrated in correlation with the number of base pairs the RNA contains. This method is time sensitive due to the 14 day half-life of $^{32}$P. Once the RNA is labeled, it is important to run the protection assays and the sequencing gel within a narrow time frame. RNA labeling with $^{32}$P can be time consuming since extra safety precautions need to be taken. In this stage of the process, the labeled RNA is very radioactive. Speed and rushing cannot be a factor here, be sure to manage your time during this experiment to not contaminate yourself or the lab with radioactive sample. The equipment and instrumentation required for radiolabeling is expensive and must be treated with care.

The $^{32}$P labeled RNA showed an increased resolution of the cleavage band patterns over the IR dye, which makes them easier to interpret. After completing some sequencing gels using radiolabeled RNA, cleavage patterns became visible. Many weeks were spent to optimize the reaction conditions. For these reasons, the radiolabeling technique will be continued in the future.
To map the structure of the tetracycline binding site, many footprinting protocols were discussed. Each method will be important to consider as this project progresses in the future.

During this project I have had much success with the nuclease protection. Nuclease protections have been used for many years and their protocols are easy to follow and well established. When using the nuclease with IR end labeled RNA, we saw that no cleavage was taking place; this could be caused by the size of the nuclease. Most of the nucleases are large, so they do not gain access to the RNA backbone. When used with radiolabeled RNA, cleavage became detectable. The reactions can be completed under a variety of conditions to optimize the best visualization of the cleavage patterns. These nucleases are sensitive to changes in the secondary structure and will provide detailed results of where the tetracycline is binding to the ykkCD RNA. Three nuclease protocols were optimized for RNase T₁, V₁ and A. Refer to Figure 35, 36 and 37. In these figures, it is still difficult to see the individual nucleotides. It will be near impossible to interpret these gels until the OH ladder is clear enough to see each nucleotide. The OH ladder is a required standard for footprinting procedures. From the data collected, it appears RNase T₁ and V₁ have better resolution of their fragmentation products. I would pursue these nuclease protections over RNase A since RNase A is a very potent nuclease. It takes many dilutions before a cleavage pattern becomes noticeable. RNase T₁ and V₁ require fewer dilutions which saves time during the experiment. Comparing figures 36 and 37 is also a challenge. The sequencing gel in Figure 36 was completed with the RNA-tetracycline complex. Figure 37 contained no tetracycline in the nuclease protections, only one of the in line probing samples contained tetracycline. It is believed that the RNA-tetracycline complex is more stable. This could explain why there are less cleavage patterns visible under the nuclease protections in
figure 36. This sequencing gel also shows that the RNA-tetracycline complex produces the best OH ladder after 5 minutes of incubation.

Self-cleavage of the RNA using Tb\(^{3+}\) was previously explored by Laura Howell using IR-700, 5’ end labeled RNA. She tried to optimize the protocol to visualize cleavage patterns, but due to the restrictions of the IR-700 dye she was unsuccessful. Tb\(^{3+}\) samples were prepared for sequencing prior to the radioactive Storm scanners malfunction, using the concentrations of Tb\(^{3+}\) and incubations periods optimized by Howell. These samples will be used in the future if time permits.

In Figure 37, the two left most lanes contain samples of radiolabeled, in line probing self-cleavage experiments. This technique is very easy to complete. It involves mixing the RNA with a total 1 μM concentration of tetracycline and incubating at room temperature. Unfortunately, there is not much room for optimization of this protocol. The only parameters that can be adjusted are incubation time and tetracycline concentration. Timed samples were removed from incubation every 24 hours for 3 days to see which incubation period had the best cleavage. This protocol was also tried by Howell. The 72 hour incubation consistently showed better cleavage, so this is how long the in line probing samples were incubated prior to sequencing in Figure 37. The in line probing of the RNA-tetracycline complex showed a unique cleavage pattern with 6 distinct bands. Since the control samples are difficult to interpret it is tough to identify what these 6 bands represent.

In 2006, Ron Breaker and Jeffrey Barrick published an article with an in-line probing structure of the ykkC/ykkD RNA. In the future we will be able to compare our in-line data as reference.
Figure 38: In-line probing of the ykkC/ykkD element. Lane 1 shows untreated RNA (NR), Lane 2 has the T₁ Ladder, Lane 3 is the OH Ladder and Lane 4 (-) contains the in-line probing sample. This sample was incubated for 48 hours at 25°C in a buffer around pH 8.3.

Where the bands appear in lane 4, we can identify flexible regions in the RNA. Areas where there are blank spaces indicate well-structured or base paired regions.²⁸

Regrettably, due to software malfunction with the radioactive Storm scanner, crucial time was lost and no further sequencing gels could be run before the submission of this thesis. In conclusion, the work completed during the time of this thesis has provided advancing information towards mapping the structural changes in the ykkCD riboswitch with and without
binding tetracycline. RNA purification methods have been perfected. 5’ radioactive end labeling has been chosen as the best end labeling method. The LI-COR DNA analyzer is fixed and working. The radioactive equipment and instrumentation are now in working order and the lab is equipped for radioactive materials. Ribonuclease and self-cleavage methods have been optimized and the sequencing gels are getting closer to being interpretable. I believe it is possible to successfully sequence this RNA and identify the tetracycline binding site using the protocols and methods discussed in this thesis.
References:


