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Jack Wang April 27, 2022

Development of a DNAzyme-based, Self-catalyzing Reaction and Self-assembly Gold Nanoparticle Reaction for the Detection of Dengue Virus

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An abstract of
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Abstract

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Dengue virus is one of the most dangerous and rapidly spreading mosquito-borne viral diseases in the world, with localized outbreaks and epidemics being observed especially in the developing countries of Latin America, South America, Africa, and Southeast Asia. Despite its prevalence and the significant amount of research already put into studying it, it is likely that activity of the virus is still being underestimated due to lack of clinical suspicion and diagnostic tests. Therefore, it is important that rapid and sensitive tests that can be used in non-clinical settings in rural regions be developed and deployed to combat Dengue. This project focuses on developing a test for heavily conserved Dengue RNA components. The system utilizes a DNAzyme-based, self-catalyzing reaction coupled with a gold nanoparticle self-assembly reaction to produce a colorimetric readout in the presence of viral DNA/RNA targets. The system can also be easily converted to test for RNA and DNA components of various other viruses.

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Development of a DNAzyme-based, Self-catalyzing Reaction and Self-assembly Gold Nanoparticle Reaction for the Detection of Dengue Virus

Introduction

Over the last few decades, Dengue virus (DENV 1-4) has spread rapidly to many countries in different regions all over the world, causing more and more outbreaks and localized epidemics. DENV is a flavivirus that fuses with host cells and releases its RNA into the cytoplasm[1]. Dengue outbreaks have been recently observed in many countries in central Africa as well as the majority of Latin American countries, the Indian Subcontinent, and Southeast Asian countries. Today, Dengue has overtaken Malaria to be the most dangerous and rapidly spreading mosquito-borne viral disease in the world. Despite the already extensive research on the prevalence and spread of the virus throughout the world, it is possible that activity of the virus is still being underestimated due to lack of clinical suspicion and diagnostic tests[2]. Therefore, it is vitally important that more rapid and sensitive tests be developed that can be used in non-laboratory settings like the rural regions of the countries affected. One such possibility for a novel test to detect Dengue genomic components in a sample is with the use of DNAzyme-based self catalyzing reactions combined with gold nanoparticle self-assembly reactions. This research project will focus on developing a DNAzyme system that will self-catalyze in the presence of specific, conserved sequences of the Dengue genome and a DNAzyme- directed assembly of gold nanoparticles for colorimetric readout.

A DNAzyme is an innovative use for DNA, which is traditionally known for its use in storing information or more recently in DNA nanostructures. DNAzymes are a class of catalytic nucleic acids which can cleave specific nucleic acid substrates in the presence of cofactors acting as catalysts. The DNAzyme is usually formed from an "enzyme" strand and a "substrate" strand. The enzyme strand is a specific strand of ssDNA that can form a functional secondary structure in the presence of metallic ion cofactors like Pb^{2+} or Mg^{2+}. Surrounding the enzyme site will be two sense arms to attach substrate strands[3]. The DNAzyme is very sensitive towards the presence of cofactors, and therefore DNAzymes have been used in DNA biosensors for the presence of lead ions[4]. Although other metallic ion cofactors have been demonstrated as possible catalysts for the DNAzyme reaction, lead has proven to be the most effective catalyst by far[5]. The substrate strand contains a single RNA nucleotide that acts as a cleavage site, which is flanked by two arms of antisense DNA to the enzyme strand. Not only are these DNAzymes able to cleave substrates with efficiency, they are also extremely specific, as only one nucleotide mismatch in the antisense arms will significantly decrease cleaving activity[6] (Figure 1). Cascade reactions similar to biological signal transduction reactions using DNAzymes have been demonstrated in previous work; however, amplification of the signal has proven to be more difficult to accomplish[7].

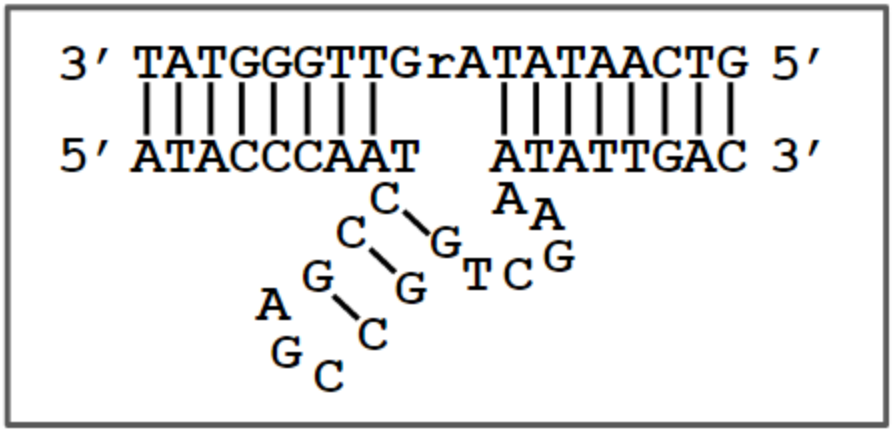

Figure 1: Basic DNAzyme and substrate design with conserved DNA and RNA sequences.

The bottom strand represents the DNAzyme ssDNA strand. It includes a conserved sequence segment that will hybridize into a specific conformation as well as flanking recognition sites. The top strand is the substrate. It includes an RNA base where the cutting will occur as well as flanking recognition sites to bind with the DNAzyme.

Gold nanoparticle sensors are utilized to provide a colorimetric readout after the DNAzyme reaction is complete. Colorimetric sensors are simple and can minimalize costs associated with instrumentation. Gold nanoparticles being used for this type of sensing in previous work has shown that different aggregation states of metal nanoparticles results in distinctively different colorimetric readouts (red versus blue readouts)[8] (Figure 2). Gold nanoparticles can be covalently modified with DNA strands, allowing us to tailor the system to fit our needs. The sensitivity and well defined differences in readout coupled with the relatively simple readout technique make this system an ideal way to detect the action of the DNAzyme self-catalyzing reaction[9].

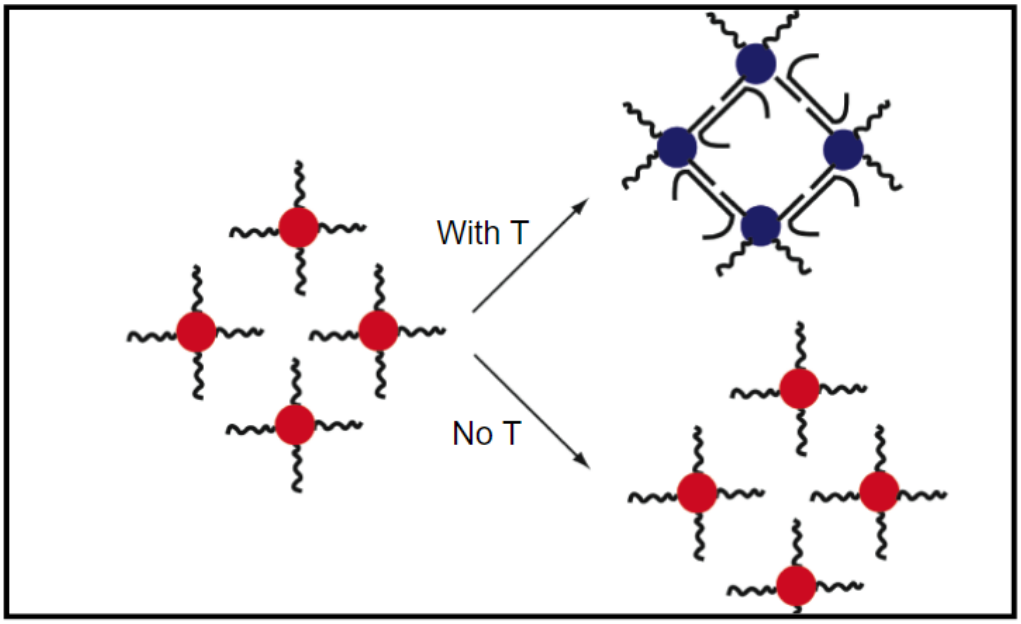

Figure 2: Design schematic of the gold nanoparticle aggregation reaction.

Without a Dengue Trigger (T), the DNAzymes do not function, and there is no readout. In the presence of a viral RNA trigger, the DNAzymes have cleaved their substrates, and the short strand products from the cleaving begin to aggregate the gold using their covalently bound complementary sequences.

(Figure courtesy of Ke Lab)

Methods

Design of the DNAzyme Components

The Dengue trigger sequence was pulled from a highly conserved sequence of the DENV 2 and 4 genome. All DNAzyme components were designed and tested via computer simulations like Nupack and IDT. Self annealing, Group annealing, melting temperatures, and mismatches were found through Nupack and IDT simulations (Figure 3)..

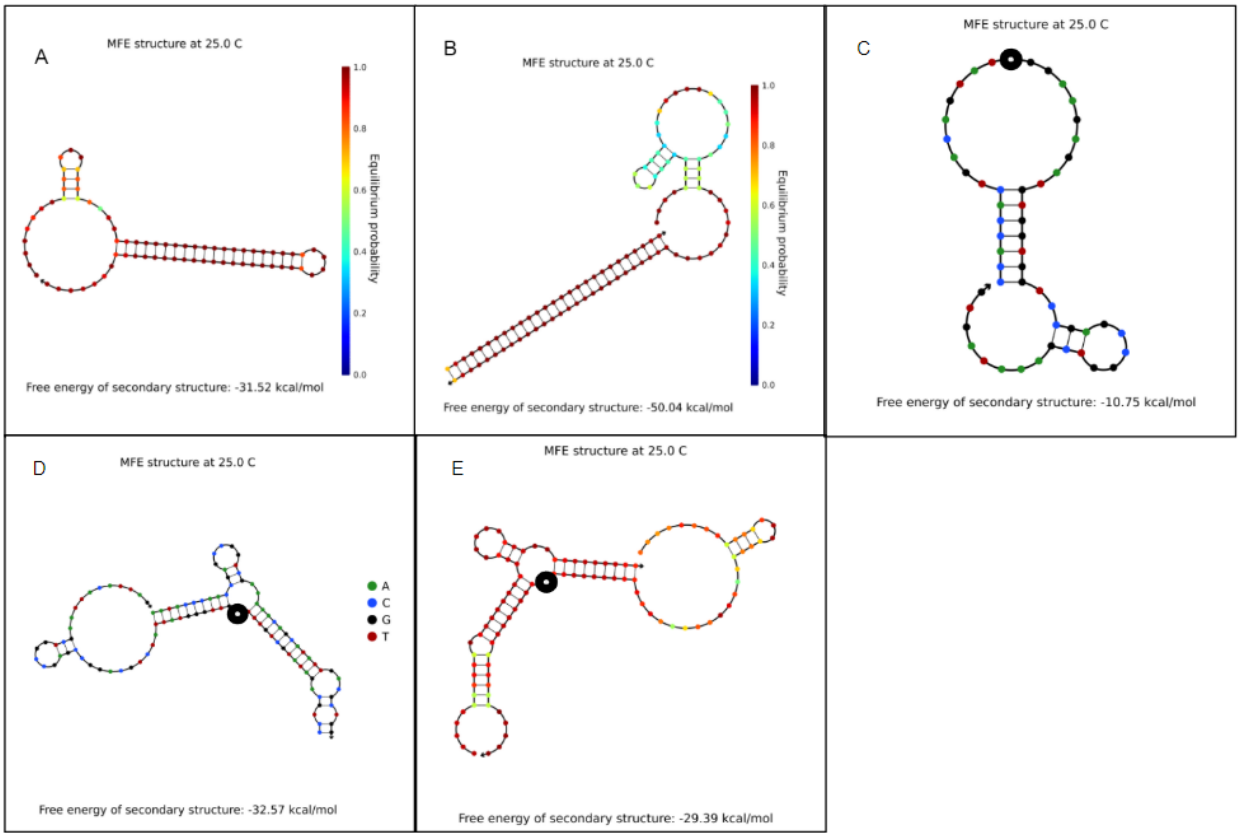

Figure 3: Nupack simulations demonstrating hybridization of DNAzyme components

A. Structure and free energy depiction of Component 0: containing the C0/C2 DNAzyme and the inhibitor sequence complementary to the Dengue trigger. B. The binding of the Dengue Trigger to C0. The inhibitor sequence has completely hybridized to the trigger and the DNAzyme is released. C. Basic structure of Component 1 and 2. Both have a similar conformation. The black circle represents the location of the RNA nucleotide. D. Binding between a lone C0/C2 DNAzyme and Component 1. The DNAzyme conformation can be visibly seen on top of the RNA base. E. Binding between a cut and active Component 1 and an inactive Component 2. Again, the DNAzyme complex can be visibly seen.

All DNA was ordered through IDT. Lead Acetate was procured through Thermo Fisher. Once arrived, the DNA was purified using large denaturing PAGE gels and gel elution kits. DNAzyme reactions are run with 50 mM tris-HCl (150 mM NaCl, pH 7.4). 2 µM lead acetate

was used for each reaction. Sample concentrations varied but a 1 μM baseline concentration was used for most samples. Reaction time and temperature varied. Prior to running reactions, individual components were heated to 95ºC for 10 min, chilled on ice for 10 min, incubated at room temperature for 30 minutes, and subsequently stored at 4ºC. Each reaction sample was 10 μL large (Figure 4).

The DNAzyme reaction has four components. The first component is a trigger consisting of a specific DNA sequence from the Dengue genome that is highly conserved among many variants. The second component is an initiator sequence, called Component 0 (C0). C0 contains the C0/C2 DNAzyme and its two flanking arms. One of the flanking arms is blocked by an inhibitor sequence, which is the antisense to the Dengue trigger. The inhibitor is attached to C0 using a small hairpin and contains a toehold for strand displacement (Figure 5A). After the addition of the Dengue trigger, a strand displacement reaction occurs, causing the trigger and inhibitor sequence to hybridize. This leaves the arm of the C0/C2 DNAzyme open and able to bind to a substrate (Figure 5B). The substrate that the C0/C2 DNAzyme is able to bind to and cleave is part of a hairpin on the third component, Component 1 (C1). C1 contains the C1 DNAzyme with one arm hybridized to a complementary sequence, which is bound to C1 with the hairpin containing a cleavage site. Once an activated C0 binds to C1, the C0/C2 DNAzyme will Cleave C1, removing the blocker to the C1 DNAzyme arm and activating the enzyme. The active C1 DNAzyme is then able to bind to and cleave the substrate site on the last component, Component 2 (C2). C2 has a similar structure as C1 and also contains the C0/C2 DNAzyme.. Once C2 has been cleaved by C1, the C0/C2 DNAzyme arm is revealed and the enzyme is

activated. Once active, C2 can cleave other unreacted C1, thereby feeding into a positive feedback loop (Figure 6).

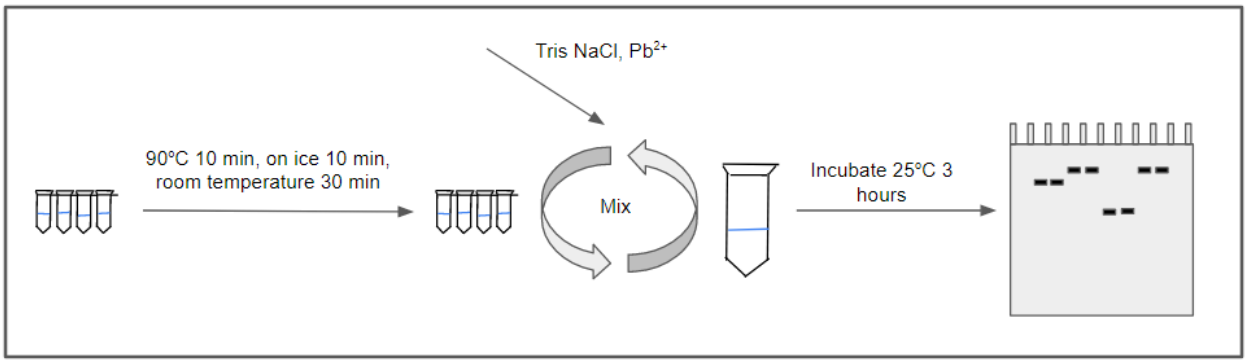

Figure 4: Basic experimental diagram for testing DNAzyme components

Variables changed as needed.

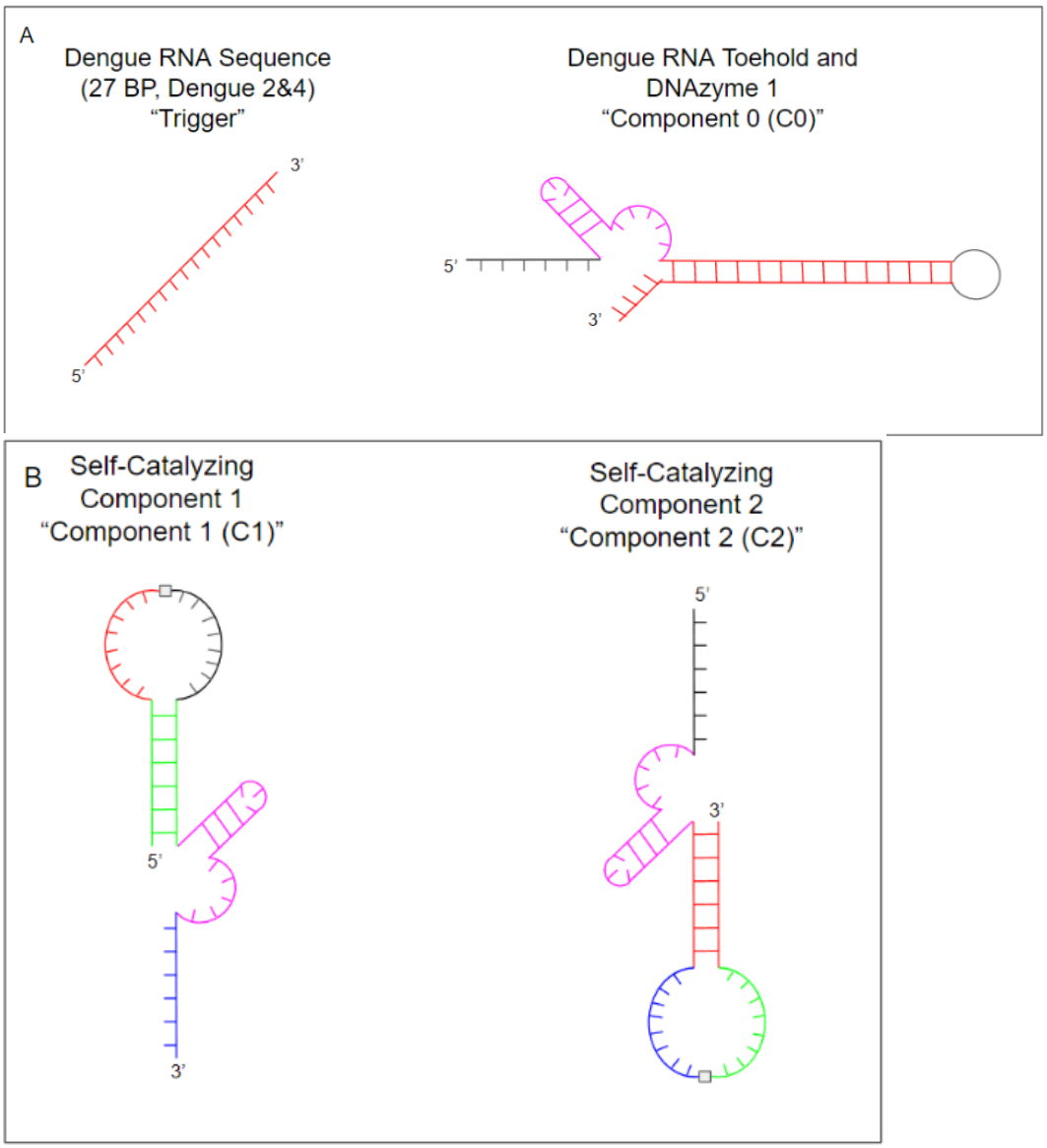

Figure 5: Design models of components

A. Trigger strand and Component 0. B. Component 1 and Component 2

Reactions were run to verify the functionality of the DNAzymes, of each component, and of the self-catalytic reaction as a whole. All results were analyzed using denaturing and native PAGE gels. After verification of the reaction's functionality and specificity, optimization tests were run to determine the most effective concentrations to use to produce the most specific and sensitive reaction.

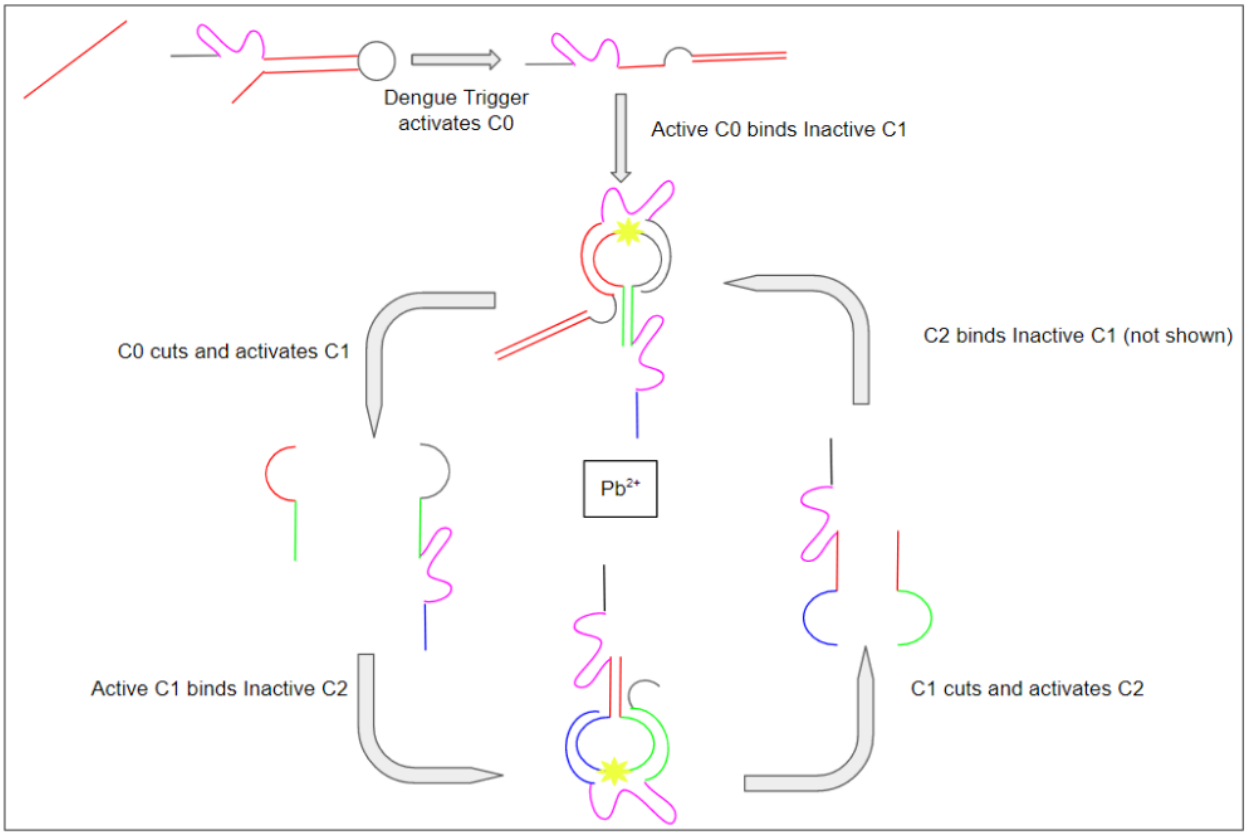

Figure 6: Full DNAzyme self-catalyzing reaction schematic.

Design of the Gold Nanoparticles

Following the DNAzyme tests, four sets of gold nanoparticles will be ordered. Each set of nanoparticles will have DNA sequences antisense to one of the DNAzyme arms of activated C1 and C2 conjugated to the surface. Reactions will be run to determine if the nanoparticles, when in the presence of cleaved C1 and C2, are able to give a colorimetric readout. This readout will be analyzed using UV-vis extinction spectra.

Results and Discussion

DNAzyme Design 1

Dengue Trigger	5′ AAACAGCATATTGACGCTGGGAAAGAC 3′
Component 0	5′AATACCCAACCGAGCCGGTCGAAATATTGACGCTGGGAAAGACAAA AGTCTTTCCCAGCGTCAATATGCTGTTT3′
Component 1	5′CCTGCAGATCGTCAATAT/rA/TTGGGTATTATCTGCAGGCCGAGC CGGTCGAATTACATTAG 3′
Component 2	5′AATACCCAACCGAGCCGGTCGAAATATTGACGCTAATGTAA/rA/C CTGCAGTAATGCTGTTT3′

Table 1: Design set 1

With the first set of DNA ordered, I ran the first set of reaction tests (Table 1). The first test was a “shot in the dark” attempt to try to generate proof of concept data for the function of the DNAzyme system (Figure 7). From this native gel stained with ethidium bromide, we can see that the trigger appears to be annealing successfully with C0. We can also see a noticeable difference between the “inactive” sample (the last column) and the two reaction columns. Besides this, it is difficult to parse what is actually happening in the reaction itself. From this trial, we determined that we have to reanalyze the samples using a denaturing PAGE gel to access cutting. Also, Sybr Gold should be used to stain the ssDNA samples. Finally, the samples should be checked more closely for purity and concentration differences.

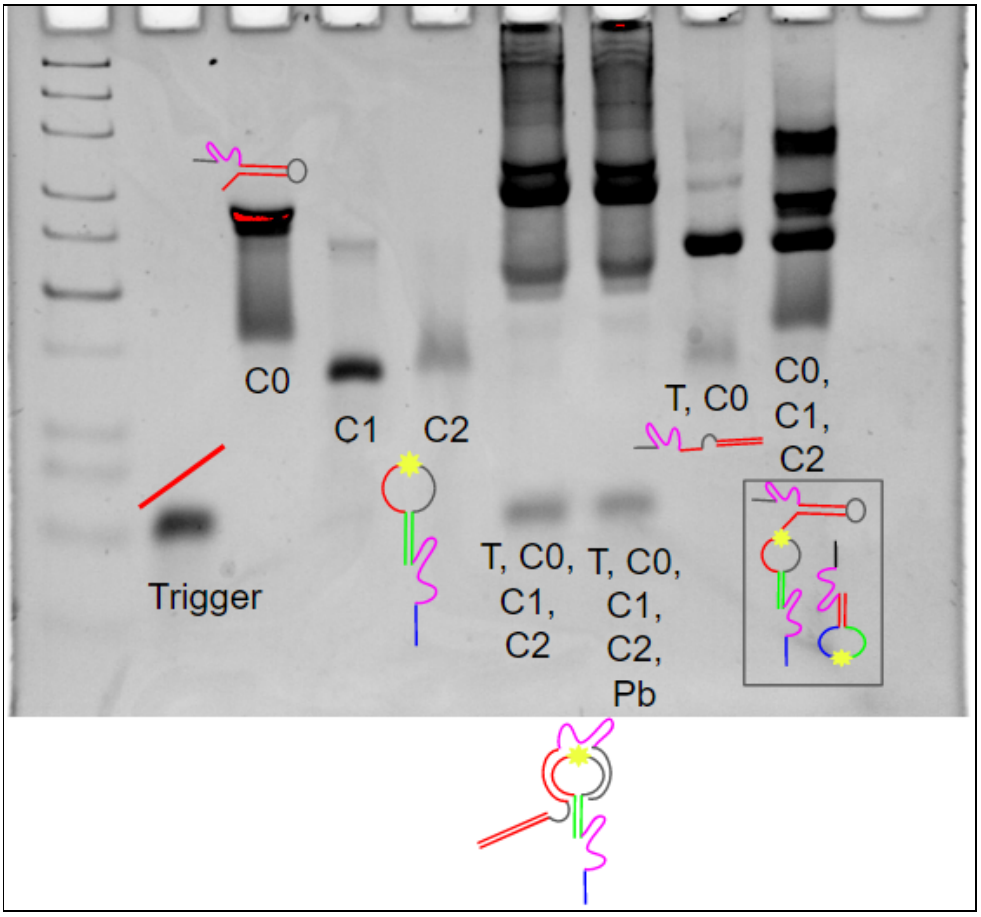

Figure 8: Proof of concept reaction experiment shows promising results but is difficult to fully analyze

From left to right: Ultra Low Range DNA Ladder, Trigger (T), C0, C1, C2, T+C0+C1, T+C0+C1+Pb^{2+}, C1+C2, C0+C1+C2. The sample shows annealing between T and C1 as well as visible differences between the inactive C0+C1+C2 sample and two T+C0+C1 samples.

Due to the difficulty in understanding the results of the previous test, separate "bare" DNAzymes and substrates were ordered to test the functionality of the DNAzymes by themselves (Table 2). The second reaction test utilized the bare DNAzymes and sites, and the samples were analyzed using a denaturing gel (Figure 9) From this test, we cannot see any

visible cutting products (extremely short strands). Indicating that the DNAzyme reaction did not happen. One of the main concerns was that the reaction was not given enough incubation time or incubation temperature.

C0/C2 DNAzyme	5′ AATACCCAACCGAGCCGGTCGAAATATTGACG 3′
C1 DNAzyme	5′ATCTGCAGGCCGAGCCGGTCGAATTACATTAG 3′
C1 Site	5′CCTGCAGATCGTCAATAT/rA/TTGGGTATTATCTGCAGG 3′
C2 Site	5′ATATTGACGCTAATGTAA/rA/CCTGCAGATCGTCAATAT 3′

Table 2: Design set 1 "bare" short DNAzyme and site strands

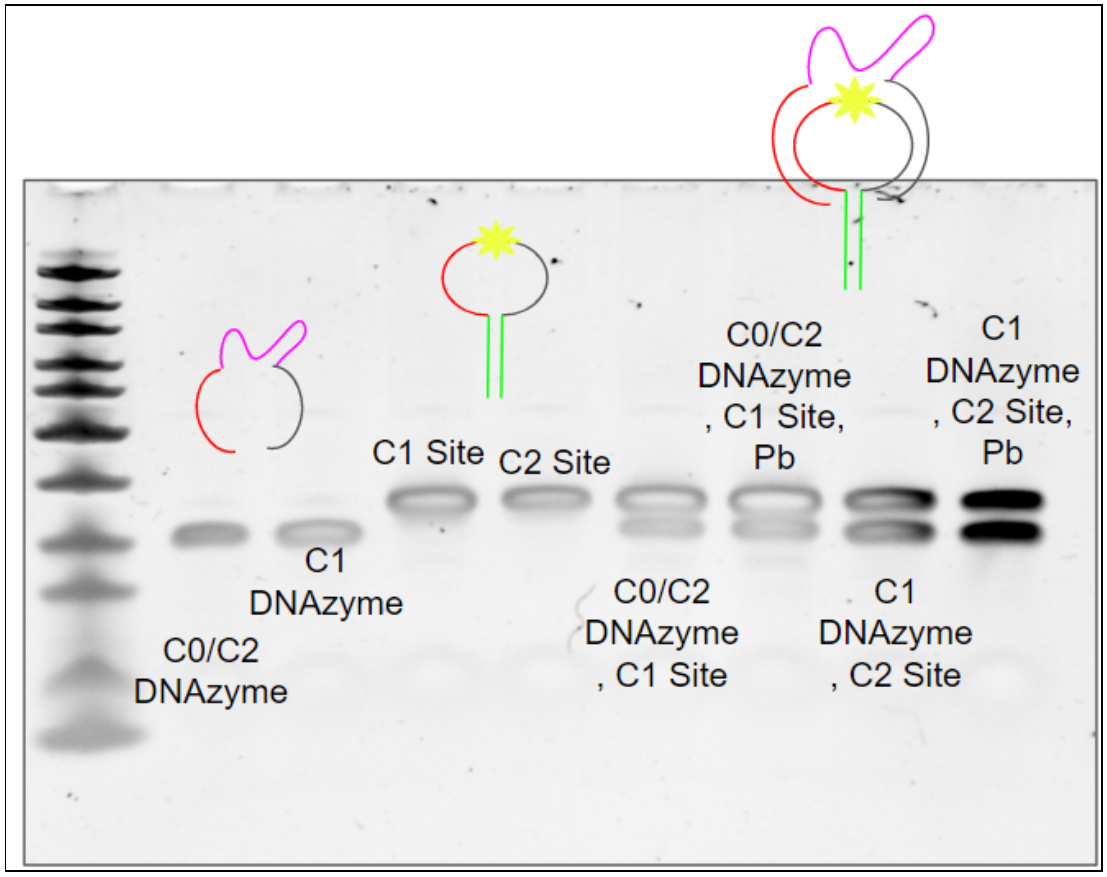

Figure 9: Bare strands test using denaturing PAGE gel analysis indicates no cutting reaction.

From left to right: Ultralow ladder, C0/C2 DNAzyme, C1 DNAzyme, C1 Site, C2 Site, C0/C2D+C1S, C0/C2D+C1S+Pb^{2+}, C1D+C2S, C1D+C2S+Pb^{2+}. The similarities of the last four reaction column to the first four control columns and the lack of short strand products indicate that the DNAzyme reaction did not occur

In the third test, one set of samples was incubated at 25°C overnight, while the second set was incubated at 37°C for three hours the following day. The samples were analyzed using a denaturing gel (Figure 10). From this test, we saw that the reaction still did not occur even with varying incubation times and temperatures. From this, we concluded that there might be an issue with the lead concentration, or there is potentially a design error in the DNAzyme.

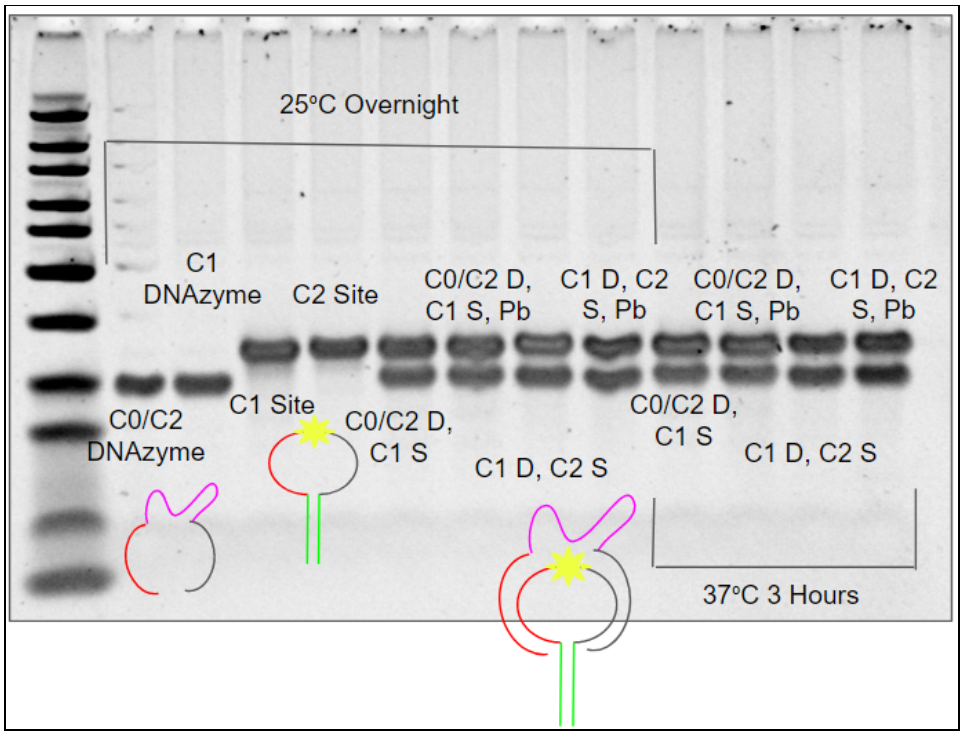

Figure 10: Lack of short strand products indicate that DNAzyme reaction did not occur. Potential issues may be lead concentration of DNAzyme design flaw.

From left to right: Ultralow ladder, C0/C2 DNAzyme, C1 DNAzyme, C1 Site, C2 Site, C0/C2D+C1S, C0/C2D+C1S+Pb^{2+}, C1D+C2S, C1D+C2S+Pb^{2+}, C0/C2D+C1S, C0/C2D+C1S+Pb^{2+}, C1D+C2S, C1D+C2S+Pb^{2+}

After the realization that there may be a design error, we wanted to check to confirm that at the DNAzyme was able to anneal with the site at all. A lack of annealing may be caused by the error and may be causing the lack of cutting. The reaction test was repeated and analyzed using a native PAGE gel (Figure 11). The results told us that the DNAzyme was interacting with the sites, meaning that any design error must be on the DNAzyme region itself.

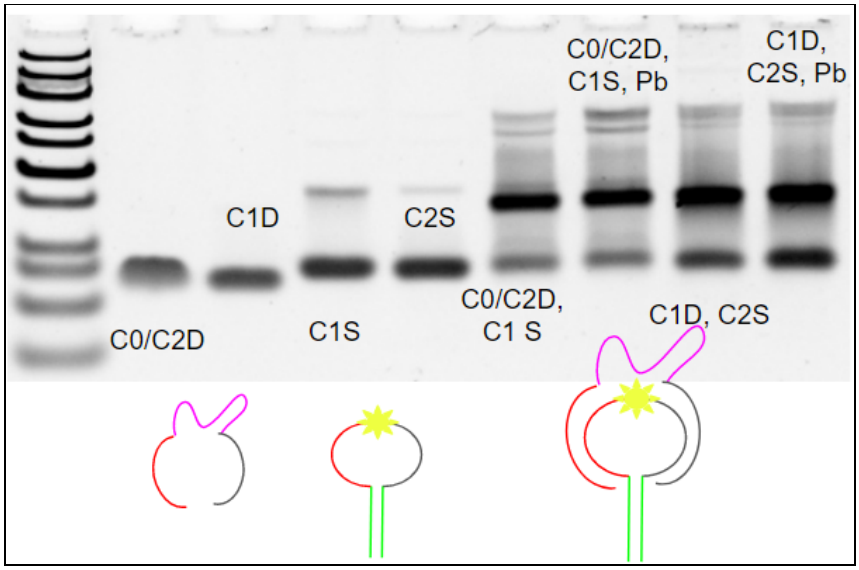

Figure 11: Native PAGE gel analysis demonstrates that DNAzymes are successfully annealing with sites, indicating that design issue lies within the DNAzyme region itself.

From left to right: Ultralow ladder, C0/C2 DNAzyme, C1 DNAzyme, C1 Site, C2 Site, C0/C2D+C1S, C0/C2D+C1S+Pb^{2+}, C1D+C2S, C1D+C2S+Pb^{2+}

DNAzyme Design 2

Upon reviewing the designs, it was realized that an important conserved sequence of the DNAzyme region had not been added. Many papers cited that these non-matching nucleotides are required for the function of the DNAzyme[3-5](Figure 12). It was likely that these nucleotides were removed during design accidentally due to IDT's RNA oligo order size limit (Maximum 60 bp). These bases were subsequently added onto the design again. Bases were removed from the non-hairpin sides of the DNAzymes to keep the oligo under 60 bp (Table 3).

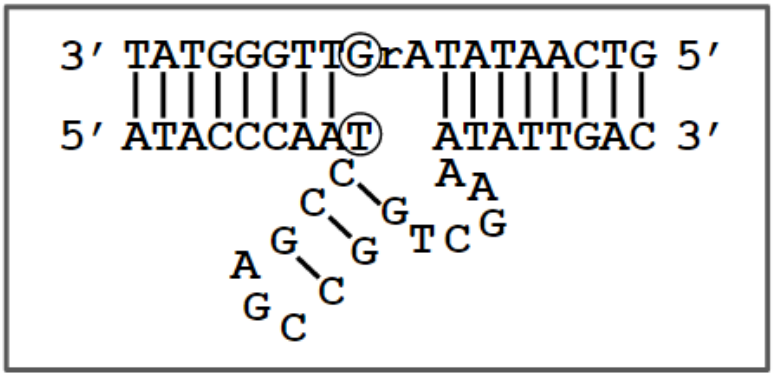

Figure 12: Basic DNAzyme design highlighting conserved nucleotide mismatch.

Construct 0'	5′_ATACCCAA**T**CCGAGCCGGTCGAAATATTGACGCTGGGAAAGACAAA AGTCTTTCCCAGCGTCAATATGCTGTTT3′
Construct 1'	5′CCTGCAGATCGTCAATAT/rA/**G**TTGGGTAT_ATCTGCAGG**T**CCGAG CCGGTCGAA_TACATTAG 3′
Construct 2'	5′_ATACCCAA**T**CCGAGCCGGTCGAAATATTGACGCTAATGTA_/rA/**G** CCTGCAGATCGTCAATAT3′
C0/C2 DNAzyme'	5′_ATACCCAA**T**CCGAGCCGGTCGAAATATTGACG 3′
C1 DNAzyme'	5′ATCTGCAGG**T**CCGAGCCGGTCGAA_TACATTAG 3′
C1 Site'	5′CCTGCAGATCGTCAATAT/rA/**G**TTGGGTAT_ATCTGCAGG 3′
C2 Site'	5′ATATTGACGCTAATGTA_/rA/**G**CCTGCAGATCGTCAATAT 3′

Table 3: DNAzyme design 2 with the addition of the conserved nucleotides

Bold represents added nucleotides. Blank represents removed nucleotides.

After receiving and purifying the new DNAzyme and site strands, a reaction test was set up to analyze the DNAzyme's ability to cut the site. The samples were analyzed using a denaturing PAGE gel (Figure 13). This test showed that the DNAzymes were able to cut some of the sites. There were visible short band products in the reactions samples. However, the cutting efficiency seemed to be low. Also, the C0/C2 DNAzyme no lead sample had short strand products. Through this data, it was believed that that cutting efficiency may be due to insufficient incubation time or temperature. The product in the no lead sample may have been caused by protocol error (accidentally adding lead).

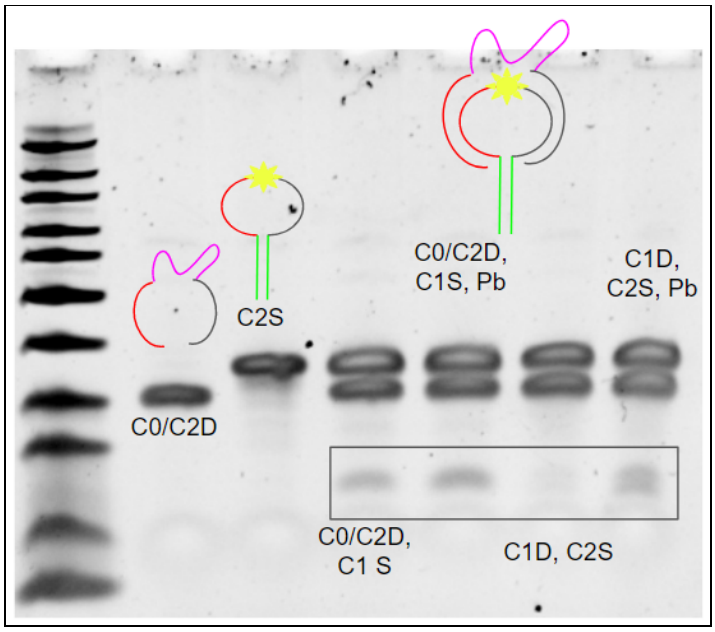

Figure 13: Denaturing PAGE gel showing successful cutting reaction between DNAzymes and site.

From left to right: Ultralow ladder, C0/C2 DNAzyme, C2 Site, C0/C2D+C1S, C0/C2D+C1S+Pb^{2+}, C1D+C2S, C1D+C2S+Pb^{2+}. Cutting efficiency appeared to be low, and C0/C2D+C1S sample had cutting product despite not having lead.

Following the previous test, it was determined to test a higher incubation temperature to see if it would improve cutting efficiency. The samples were run at 25ºC and 35ºC at the same time for three hours. The samples were analyzed using a native PAGE gel (Figure 14). The results showed that it was still difficult to identify all of the conformations present in the reaction samples. A proposed theory was that the slower band was DNAzyme and site that had cut but not dissociated from each other. The lack of visible short strand products indicated that the DNAzyme and cut sites were not dissociating. Also, the concentration of DNAzyme appeared to be lower than site concentration, giving an explanation for why not all of the site strands were annealed. From this, it was decided that running the same reaction but pulling samples out at varying incubation times might help us understand what the differering bands were.

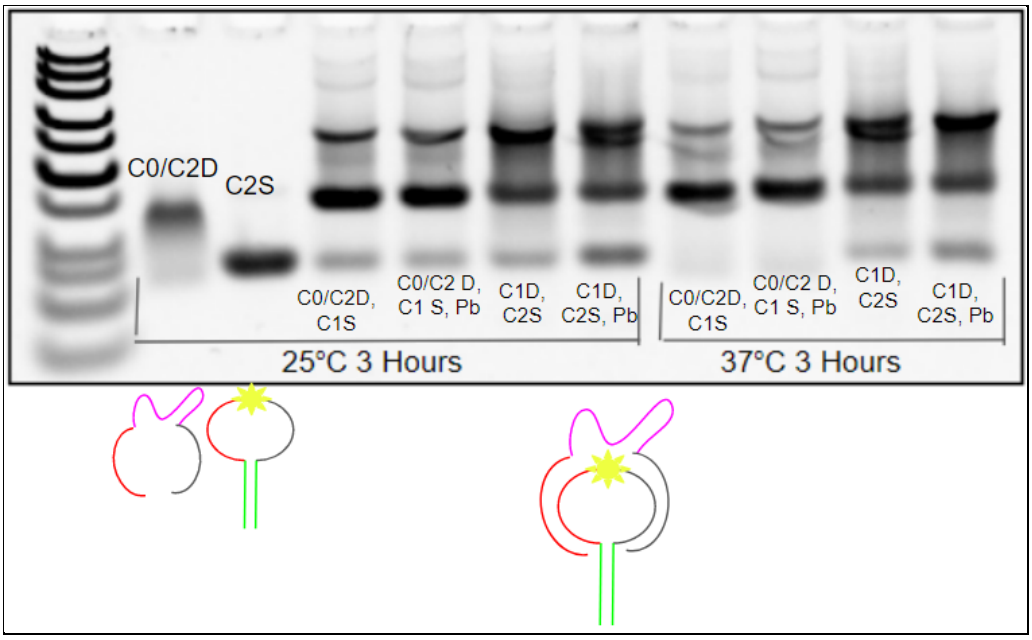

Figure 14: Varying binding conformations between the DNAzyme and site prove difficult to analyze.

From left to right: Ultralow ladder, C0/C2 DNAzyme, C1 DNAzyme, C1 Site, C2 Site, C0/C2D+C1S, C0/C2D+C1S+Pb^{2+}, C1D+C2S, C1D+C2S+Pb^{2+}, C0/C2D+C1S, C0/C2D+C1S+Pb^{2+}, C1D+C2S, C1D+C2S+Pb^{2+}

Next, the reaction was run, but samples were taken out of incubation at time 0, time 1:30, and time 3:00 and put on ice. The samples were analyzed using both a native and denaturing PAGE gel (Figure 15). The native gel saw similarities between all of the time stamp reactions, indicating that ice may not have stopped the reaction from occurring. All of these look similar to the results of the previous test, meaning all of these are post-incubation samples. The denaturing gel saw better cutting efficiency, likely due to the addition of a more even amount of DNAzyme to site. The C0/C2 DNAzyme no lead samples still saw cutting, indicating that they may not require lead to cut (although the cutting efficiency is lower).

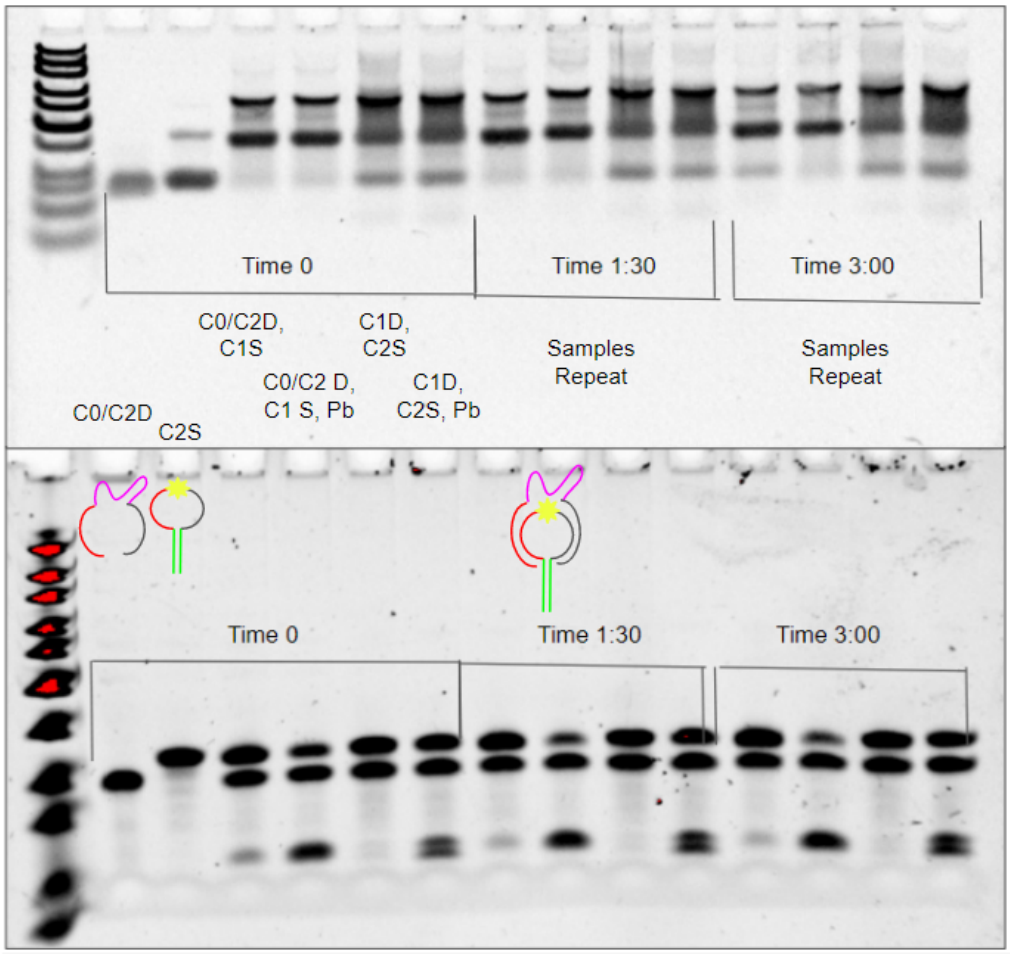

Figure 15: Native and denaturing PAGE analysis of timestamp reaction demonstrates no change among samples, indicating storing on ice did not stop the DNAzyme reaction.

For both gels from left to right: Ultralow ladder, C0/C2 DNAzyme, C2 Site, C0/C2D+C1S, C0/C2D+C1S+Pb^{2+}, C1D+C2S, C1D+C2S+Pb^{2+}, C0/C2D+C1S, C0/C2D+C1S+Pb^{2+}, C1D+C2S, C1D+C2S+Pb^{2+}, C0/C2D+C1S, C0/C2D+C1S+Pb^{2+}, C1D+C2S, C1D+C2S+Pb^{2+}.

Next we tested the annealing conformations of the samples at time 0 only to try to determine which conformations appear immediately and which ones appear after incubation. Samples were mixed, incubated at room temperature for 8 minutes to anneal, then immediately

run on gels. These reactions were analyzed using a native and denaturing PAGE gel (Figure 16). These results indicated that the DNAzyme reaction in the presence of lead occurs almost immediately following mixing. Various conformations were also formed immediately following mixing. DNAzyme levels still appeared to be less than site levels. These results indicate that the reaction is extremely sensitive to lead, but there is still no dissociation between DNAzymes and cut sites.

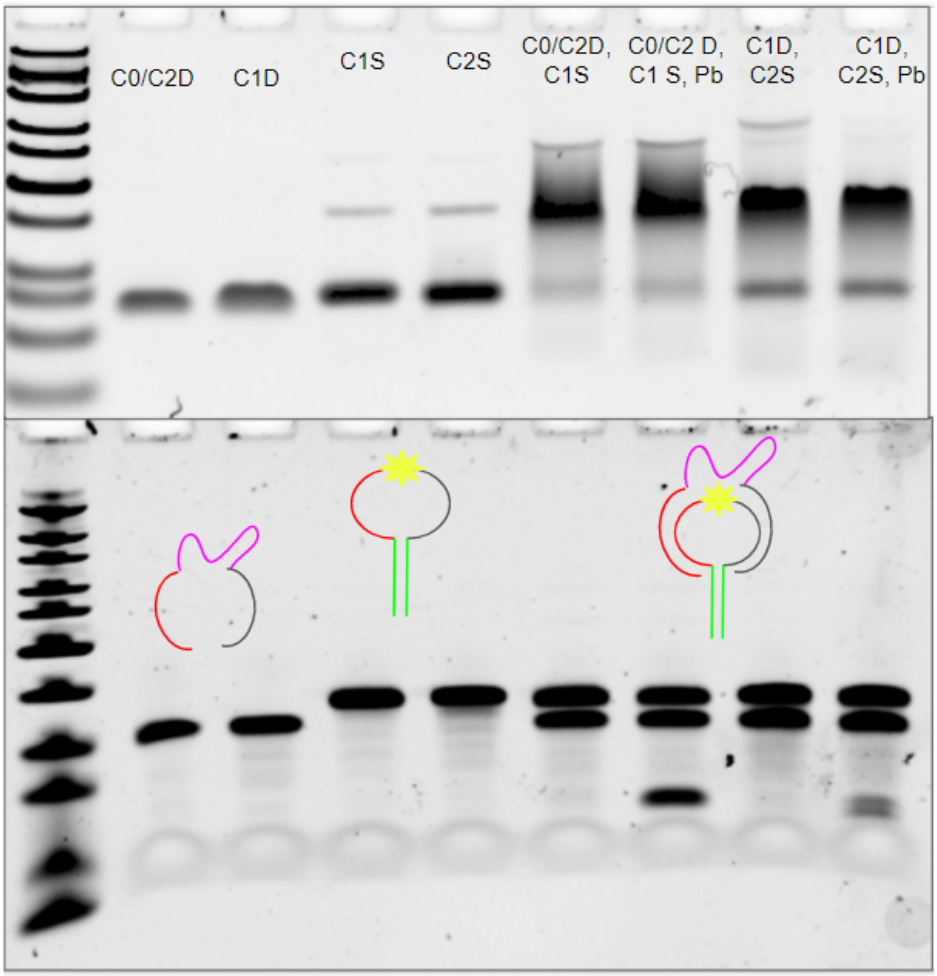

Figure 16: 0 Time reactions indicate that reaction is sensitive to lead and occurs rapidly, but is unable to dissociate following reaction.

For both gels from left to right: Ultralow ladder, C0/C2 DNAzyme, C1 DNAzyme, C1 Site, C2 Site, C0/C2D+C1S, C0/C2D+C1S+Pb^{2+}, C1D+C2S, C1D+C2S+Pb^{2+}.

The next test was to determine whether the addition of a flood of DNAzyme will allow some cut sites to dissociate in a 0 time reaction. The DNAzyme was added in a 5:1 ratio compared to the sites. The samples were mixed, incubated at room temperature, and analyzed immediately using a native gel (Figure 17). The analysis showed that many of the same conformations are still being generated by the 5x reaction sample. However, there may be other bands within the dark bands that are not visible due to insufficient gel running time.

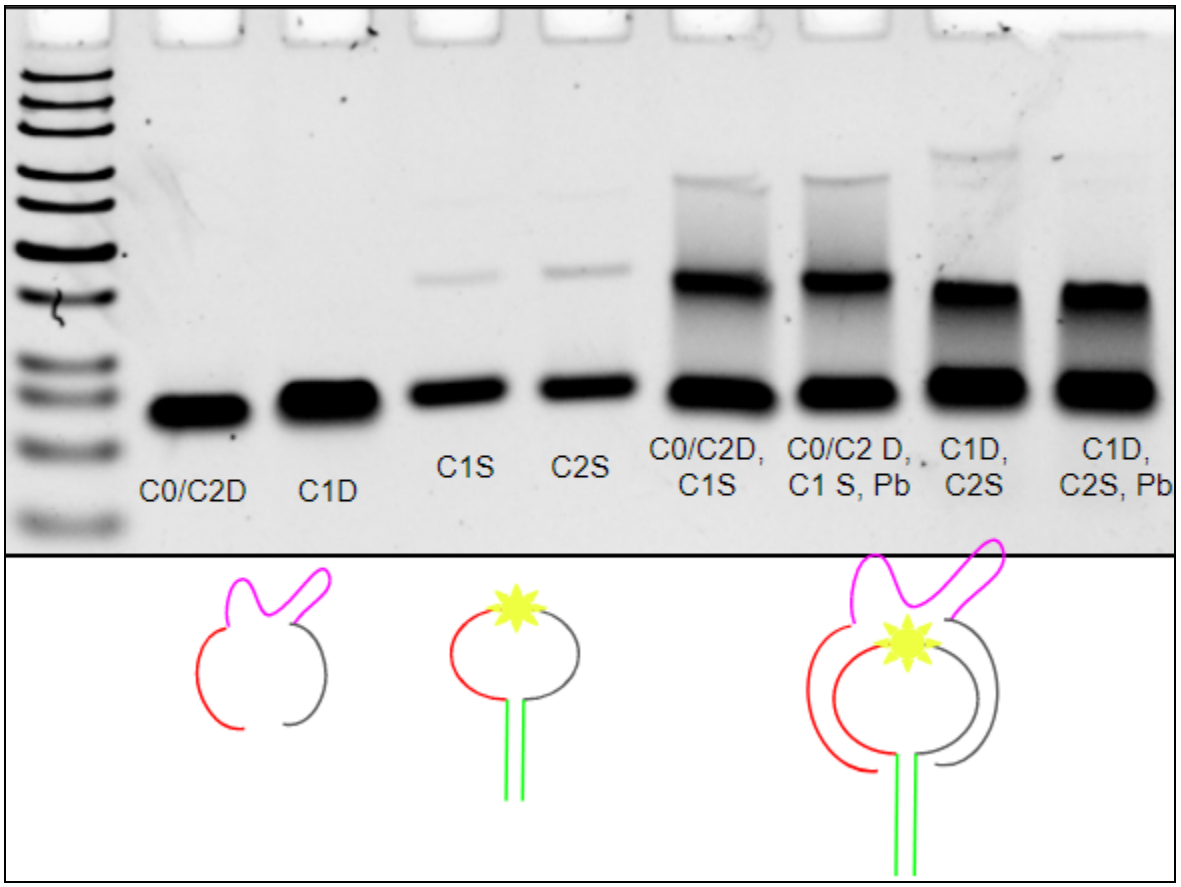

Figure 17: 5:1 DNAzyme concentration at 0 time reaction shows similar bands to regular DNAzyme concentration samples.

From left to right: Ultralow ladder, C0/C2 DNAzyme, C1 DNAzyme, C1 Site, C2 Site, C0/C2D+C1S, C0/C2D+C1S+Pb^{2+}, C1D+C2S, C1D+C2S+Pb^{2+}.

In the next test, a 5x DNAzyme reaction was set up, but the analytical native gel was run for a long period of time, allowing the bands to separate more (Figure 18). The results show multiple new bands, indicating that the large increase in DNAzyme concentration may be forming new, unwanted secondary structures with each other and with the sites. It was

determined that more moderate concentrations of DNAzymes like 1.5x or 2x should be used in future reactions.

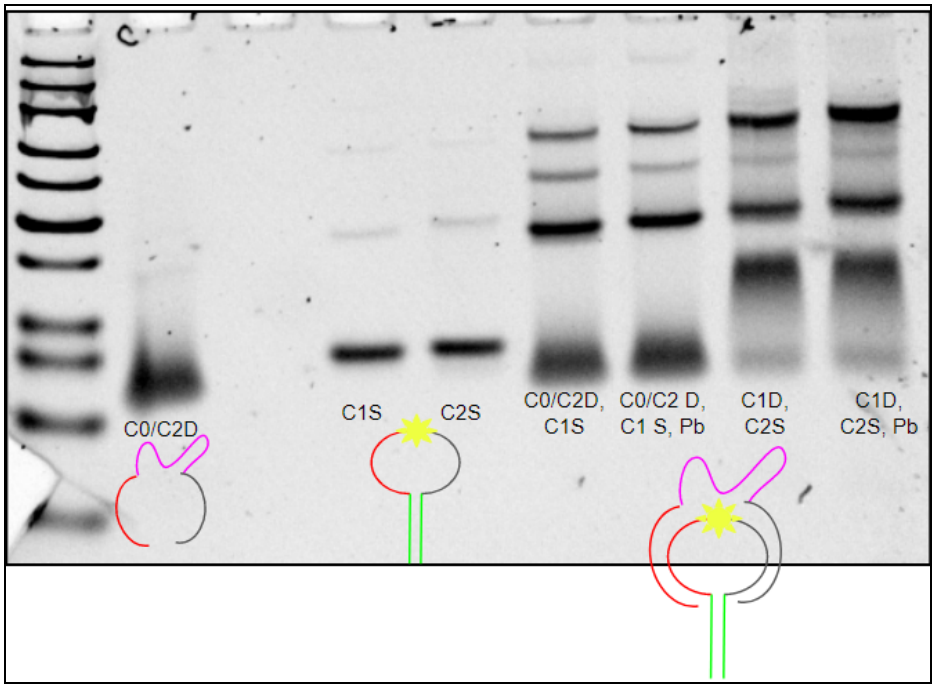

Figure 18: Long gel run time reveals new secondary structures, indicating that high concentrations of DNAzyme may not be usable.

From left to right: Ultralow ladder, C0/C2 DNAzyme, blank, C1 Site, C2 Site, C0/C2D+C1S, C0/C2D+C1S+Pb^{2+}, C1D+C2S, C1D+C2S+Pb^{2+}.

Gold Nanoparticle Tests

Through previous work done in the Ke lab, we were able to determine the functionality of the gold nanoparticle aggregation reaction. Gold nanoparticles covalently modified with DNA strands were tested with and without the presence of an aggregating trigger. An aggregation target concentration gradient was used to test the resulting colorimetric readout of the gold nanoparticles (Figure 19). From the result, we can see a rapid and dramatic shift from red to blue

emission following the addition of any target, indicating that the system is sensitive towards the presence of target strands.

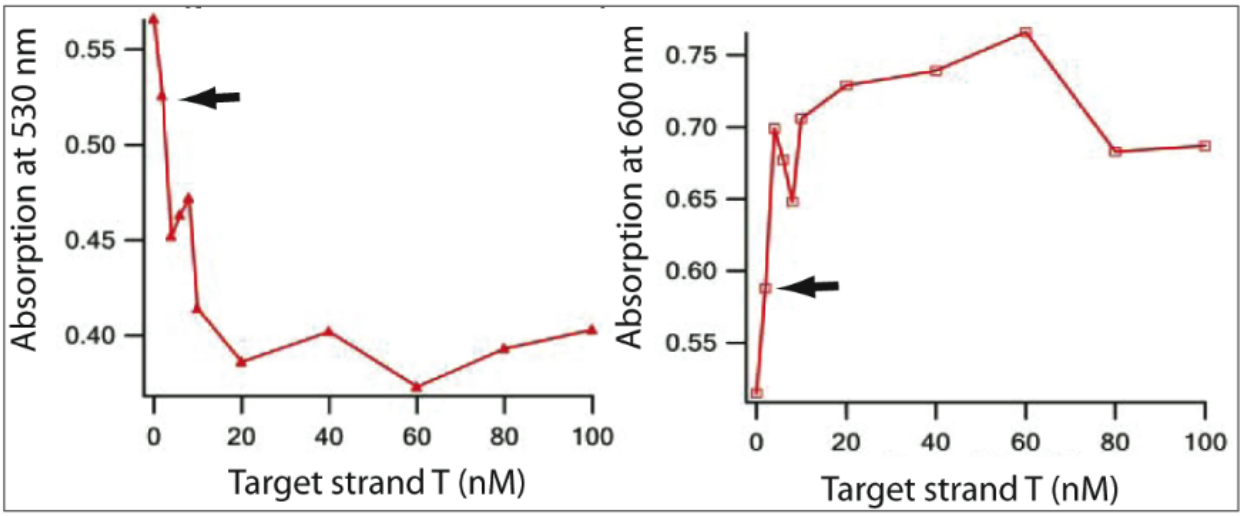

Figure 19: Colorimetric readouts of gold nanoparticle aggregation reactions indicate rapid and dramatic absorption shifts following the addition of target.

Arrows indicate the first data point where the target strand was added.

Overall Discussion

Although it is unfortunate that we were unable to reach a satisfying conclusion to our research, the results still indicate that the DNAzyme self-catalyzing reaction and gold nanoparticle aggregation reactions show much promise. Future steps will be to continue testing the DNAzyme system to ensure its specificity and sensitivity. After optimizing the bare DNAzymes and sites, tests can be run using the full constructs to determine their functionality. Finally, the self-catalyzing reaction can be joined with the gold nanoparticle-self assembly reaction to create a reliable test for Dengue virus that only requires a couple hours of incubation

at room temperature to function. If pulled off, this technology can be revolutionary in the field of low-cost virus tests as it can be tailored to test for any DNA/RNA sequence.

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