THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOCHEMISTRY & MOLECULAR BIOLOGY

IN VITRO EXAMINATION AND DETECTION OF A TRIPLEX STRUCTURE PRODUCED AS A RESULT OF A GAA TRINUCLEOTIDE REPEAT SEQUENCE IN PATIENTS WITH FRIEDREICH'S ATAXIA

ZACHARY PETTORUTO
SPRING 2020

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biochemistry & Molecular Biology
with honors in Biochemistry & Molecular Biology

Reviewed and approved* by the following:

Dr. Maria Krasilnikova
Research Assistant Professor of Biochemistry and Molecular Biology
Thesis Supervisor

Dr. Ming Tien
Professor of Biochemistry
Honors Adviser

* Electronic approvals are on file.

ABSTRACT

Through previous studies performed on the nature of neurodegenerative disorders commonly found within patients worldwide, it was discovered that many of these disorders contain microsatellite repeat sequences within the genome that potentially aid in the development of these disorders through unknown mechanisms. More specifically, Friedreich's ataxia, one of the most common forms of ataxia, contains a GAA trinucleotide repeat, which is hypothesized to form a triplex structure that decreases the stability of the DNA and progresses the disease in patients.

The overall purpose of these experiments was to properly identify the presence of a triplex structure *in vitro* of a DNA sample that contains the GAA trinucleotide repeat. To achieve this goal, many experimental parameters were tested to determine ideal conditions, such as the proper PCR conditions needed to successfully amplify the target sequence, the proper conditions of the biotin label detection protocol to analyze DNA bands on a membrane, and the conditions involved in the generation of a radioactive probe to analyze the potential triplex structure formed *in vitro*.

Once the parameters were tested and idealized, a template sequence containing the trinucleotide repeat was chemically modified with chloroacetaldehyde and potassium permanganate and subsequently digested with ScaI-HF and EcoO109I restriction enzymes. The banding patterns of these samples within a polyacrylamide gel were analyzed alongside generated length controls designed to flank the trinucleotide repeat and aid in the visualization of the triplex structure.

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Maria Krasilnikova for the supervision of the research project throughout the year as well as her continued support in the lab and in the development of my thesis. In addition, I would like to thank my honors adviser, Dr. Ming Tien, for his valuable feedback throughout the thesis writing process. Finally, I would like to thank the other members of my research team: Matthew Pettoruto, Emilie Korchak, and Lauren Duttry. Their contributions to the overall research project were immense and tremendously beneficial throughout my experience on this research project.

INTRODUCTION

Friedreich's ataxia, often referred to as the most common form of ataxia, is a severe neurodegenerative disorder that results from a heritable mutation in the *X25* frataxin gene.[1] With an estimated prevalence of approximately 1 in 50,000 individuals in the United States and approximately 1 in 90 individuals being a carrier for the disorder, Friedreich's ataxia is primarily characterized by ataxia, or weakening, of the limbs and muscles as well as decreased speech and motor functions, dysarthria, and a decrease in the functionality of the proprioceptors, resulting in a decreased awareness of the position of the lower limbs.[2,3] The onset of these symptoms normally occurs within the age range of 5 - 15, but can be undetected until the ages of 20 - 25, and patients with these symptoms have an approximate life expectancy of 45 years.[1,2,4] Though there is no known cure for Friedreich's ataxia, various forms of treatment have undergone clinical trials aimed to delay or halt the progression of the disease.[5] One particular type of antioxidant therapy treatment, Idebenone treatment, has been clinically tested and shown to improve motor and cerebral functions in patients who were early in terms of disease detection and development.[6] Another form of treatment, Deferiprone, targets aconitase in frataxin-depleted cells in order to prevent mitochondrial iron chelation to promote cell proliferation.[7]

In the wild type *X25* gene, the frataxin protein product does not have a conclusive known function, but it is mainly thought to play a role in iron homeostasis and respiratory functions within the mitochondria of yeast and humans.[1,8] As mentioned earlier, Friedreich's ataxia is the result of an autosomal recessive mutation that occurs in the *X25* frataxin gene, which has been mapped on a particular locus located on chromosome 9q13 in humans.[1,9] More specifically,

numerous studies on patients with this neurodegenerative disorder have identified the most common type of mutation in the form of an unstable GAA trinucleotide repeat that occurs within the first intron of *X25*.[1] One particular study conducted by Campuzano *et al.* (1996) analyzed patients with Friedreich's ataxia, and the results of the study lead to the conclusion that though some of the patients had point mutations within *X25* that likely resulted in the disease, the vast majority of the patients they analyzed contained unstable GAA trinucleotide repeats that expanded throughout the genome.[10] From an evolutionary standpoint, trinucleotide repeats have been very commonly found within the human genome and are known to be particularly stable when the number of repeats have been limited. With Friedreich's Ataxia in particular, wild-type alleles of the *X25* gene have been found to contain approximately 7 – 34 GAA trinucleotide repeats, resulting in overall genomic stability and an inability of the disease to occur or progress.[8] Other neurodegenerative disorders, such as Fragile X syndrome and Myotonic dystrophy, with similar pathogenic causes also contain similar ranges of stable trinucleotide repeats that prevent the natural progression of the respective diseases.[8] However, when the *X25* gene becomes mutated, the GAA trinucleotide repeat undergoes expansion from 7 – 34 repeats to more than 100 repeats.[8] As a result of this expansion, the trinucleotide repeats become drastically unstable and contribute to the overall genomic instability in patients with Friedreich's ataxia, which enhances the rapid and relentless progression of the disease.

Though many studies have contributed the progression of Friedreich's ataxia to the unstable expansion of GAA trinucleotide repeats, the mechanism by which this expansion causes the progression of the disease is largely unknown. One theory that could explain the genomic instability that results from the trinucleotide repeat expansion is that a non-canonical DNA structure that deviates from the normal double-helical structure forms at the site of the repeat

expansion.[11] Because Friedreich's ataxia is caused by a polypurine trinucleotide repeat sequence, this theory adopts the idea that a triplex DNA structure forms at the site of the repeat sequence.[11] The non-canonical triplex structure (H-DNA) forms when a single strand of the DNA forms a Hoogsteen hydrogen bond with the polypurine nucleotides found within the major groove of the double helix, resulting in a three-stranded DNA structure that deviates from the standard double-helix structure.[11,12]

The role that the triplex structure would play in the development and progression of the disease is largely unknown, but previous *in vitro* experiments have been performed on H-DNA structure to determine a potential correlation between the length of the polypurine sequence, the formation of the triplex structure, and genomic instability/replication stalling events.[11] For instance, a study was conducted in 2006 in which over 2500 human genes containing long polypurine:polypyrimidine sequences of ≥ 100-250 bp were determined through gene mapping, and expressed/enhanced in various proteins that serve a function in DNA modification and replication events.[13] The results of this study provided a positive correlation between the length of the polypurine□polypyrimidine sequences, the recombination of genetic material during chromosomal translocation events in the cellular life cycle, and the high frequency of mutations found within mammalian cells.[13] In addition, the results also provided validity to the correlation between the increased presence of triplex structures and the destabilization of chromosomal DNA, which contributes to the increased genetic instability in eukaryotic organisms with these long, unstable sequences.[13] In another study conducted by Vasquez and Wang, a polypurine sequence designed to form a triplex structure was ligated into a *c-MYC* promoter, which was transformed into mammalian COS-7 cells.[11] The results of this study concluded that the addition of this particular sequence resulted in DNA damage in the form of double-stranded breaks in the

DNA structure as well as significantly higher mutation frequencies in these transformed COS-7 cells.[11] Overall, these *in vitro* experiments have provided strong evidence of direct correlations between the formation of triplex DNA structures and genomic instability in the form of increased genetic rearrangements and a higher frequency of replication stalling events.

To perform these *in vitro* experiments, a common scientific technique that is often utilized is a two-dimensional agarose gel. This technique, developed in 1983 by Leslie Bell and Breck Byers, was designed to utilize the different electrophoretic mobility of different structures in order to separate various branched DNA intermediates that occur during DNA replication and homologous chromosomal recombination.[14] During two-dimensional agarose gel electrophoresis, the gel is initially performed in the first dimension in the same manner as a standard agarose gel electrophoresis before the gel is rotated and performed in the second dimension in order to effectively isolate branched replication intermediates from linear DNA molecules of the same mass.[15] If performed successfully, the branched intermediates found within the two-dimensional gel will be one of three simple shapes: a Simple Y arc in which the restriction fragment lies between the origin of replication and the terminus, a Bubble-shaped intermediate in which the initiation of replication occurs within the restriction fragment, and a Double Y arc in which the replication terminus occurs within the restriction fragment.[15] That being said, there can be variations of these simple shapes that result from various recombination products and electrophoretic motility, but these variations are established from the foundations established by the different simple shapes.[15] Within the context of this experiment, the results of this particular technique would indicate approximately where within the plasmid that replication stalling will occur, and if this event can be attributed to the presence of the established triplex structure that forms within the DNA sequence.

Another common scientific technique that was used and tested throughout the duration of these experiments was biotin-label detection. The concept behind this technique is that biotin, an essential cofactor and supplement found in consumer products to aid in various metabolic functions within organisms, is covalently linked and fixated to a uracil triphosphate nucleotide.[16,17] Once this label is developed, because biotin is fixated to a nucleotide, it can be incorporated into a target DNA sequence via PCR for further experimentation. The biotin label would then be exposed to a covalently linked streptavidin-alkaline phosphatase complex, where the streptavidin would form a strong non-covalent interaction with the biotinylated nucleotide.[18,19] Finally, the addition of nitroblue tetrazolium-bromo-chloro-indolyl phosphate (NBT/BCIP) serves as a substrate for the alkaline phosphatase, where the reaction results in a visible blue precipitate that can be used to visualize the target sequence.[18] This experimental technique has been utilized extensively, with primary applications that consist of DNA detection and antibody analysis based on the principles of Enzyme Linked Immunosorbent Assay (ELISA).[20,21] For this method of detection, some key advantages are that the label is easy to implement and use in an experiment, the results from biotin label detection can be obtained in a relatively short period of time, and the results obtained from this method of nonradioactive detection have a high degree of precision and accuracy.[22] However, a main disadvantage associated with this method of detection is that the precipitation has a limited sensitivity, thus rendering the results more difficult to interpret compared to other forms of label detection.[22] Within the context of this experiment, biotin label detection was utilized to identify the chemically modified DNA samples and to detect the location of the triplex structure within the pUCNEO plasmid.

During various experiments that involve the use of Southern Blotting, biotinylated detection of target DNA sequences often serves as a suitable non-radioactive labeling detective method.[18] However, another experimental technique that was utilized throughout these experiments with the objective of detecting *in vitro* triplex structures in chemically modified DNA was radioactive label detection. The concept behind this technique is that a probe is developed that contains a radioactive nucleotide with a ^{32}P-label on one of the three phosphate groups on the nucleotide.[23] Once this probe is developed and is hybridized to the target DNA sequence, the radioactive probe is exposed to a film, which is later developed to view the radioactivity of the probe to determine the location of the target DNA sequence.[23] This method of radioactive labeling has widespread applications, with the primary applications consisting of detection/identification of particular DNA sequences and enzyme-labeled antibodies analyzed via Southern Blotting and Western Blotting methods, respectively.[24] This method of radioactive labeling yields many experimental advantages, including high signal sensitivity for easy visualization and reusability of gels and membranes that contain the radioactive probe.[25] However, this method also yields many disadvantages, such as the fact that the probe is more dangerous to work with and the instability of the probe requires rapid utilization to obtain a signal.[25] Within the context of this experiment, radioactive labeling was incorporated into a probe that was developed via PCR and analyzed to determine the presence of a triplex structure *in vitro* with a high degree of sensitivity.

The final experimental technique utilized throughout these experiments was the chemical modification of DNA via chloroacetaldehyde (CAA) and potassium permanganate. When these chemicals are introduced, they alter the DNA structure by creating various DNA adducts in the case of CAA, or by altering the orientation and configuration of the pyrimidine nucleotides in the

case of potassium permanganate.[26,27] Through modification of DNA with these chemicals, the structure of the DNA strand will be altered in a manner that promotes stalling of the replication fork at these modified bases.[28] Chemical modification of nucleic acids has been an increasing area of research in an array of medical and biological applications, such as analysis of gene function, treatment development for various genetic diseases, and analysis of RNA interference, among other critical applications.[29] In the context of this experiment, the aim is to view promote replication stalling at these modified bases to analyze the potential presence of a triplex structure *in vitro*.

The overall purpose of this experiment is to definitively prove that a triplex structure does form at the site of a $(GAA)_{57}$ trinucleotide repeat sequence through *in vitro* chemical modifications of the pUCneo DNA sequence that contains the trinucleotide repeat. To achieve this experimental objective, the portion of the DNA sequence that contains the GAA repeat was exposed to chemical-modifying agents chloroacetaldehyde and potassium permanganate before being amplified through the utilization of Polymerase Chain Reaction (PCR). The amplified sequence was precipitated via ethanol and exposed to a polyacrylamide gel, where the bands were analyzed to determine the presence of a triplex structure occurring *in vitro*.

MATERIALS & METHODS

Biotin Label Condition Testing

Prior to the testing and detection of *in vitro* triplex structures via a biotin label, the components and reagents utilized in the primer extension reactions and Southern Blotting procedures needed to be tested. First, a primer extension reaction was performed on a GAA_{57} DNA template with 10mM solution of dATP, dCTP, and dGTP, a 6.5mM solution of dTTP nucleotides, a Biotin-11-dUTP label, 1X Taq Buffer, and 50 mM magnesium choloride solution. The reaction solution underwent an initial denaturation step at 95°C for 2 minutes before undergoing 30 cycles of denaturation (95°C, 30 sec.), annealing (48°C, 30 sec.), and extension (72°C, 30 sec.).

A portion of the PCR product was extracted from the solution and underwent a precipitation protocol, which consisted of mixing the DNA with polyacryl carrier, 0.3M sodium acetate solution, pure ethanol, and 70% ethanol before dissolving the resulting pellet in a Tris-EDTA (TE) solution. Serial dilutions of the precipitated DNA sample were created up to a dilution factor of 10_4. A positively charged nylon membrane was spotted with each of the serial dilutions, the undiluted precipitated product, the PCR product that was not precipitated, and the Biotin-11-dUTP label. The membrane was cross-linked at a UV-exposure of 400 μjoules × 100. The membrane was washed in 2X saline sodium citrate (SSC) solution.

The reagents utilized in the Biotin Chromogenic Detection Kit (Product #K0662) were prepared prior to Southern Blotting, which consisted of 1X Blocking/Washing Buffer, 1% (w/v)

Blocking Solution, 5000-fold-diluted Streptavadin-alkaline phosphatase (AP) Conjugate, 1X Detection Buffer, and 1X BCIP/NBT Solution. Once the solutions were created from the stock solutions, the Southern Blotting procedure was performed following the steps outlined in the detection protocol for the Biotin Chromogenic Detection Kit #K0662 from Thermo Fisher Scientific, Inc., which consisted of washing the membrane, blocking the membrane to prevent non-specific probe binding, incubating the membrane with the Streptavadin-AP conjugate, washing any unbound conjugates off of the membrane, incubating the membrane in detection buffer and subsequently with the BCIP/NBT solution that acts as the substrate to the alkaline phosphatase. The membrane was left incubated overnight in the dark to preserve the light-sensitive substrate solution.

Following incubation of the membrane, the conjugate and the substrate solution were mixed in detection buffer and left in the dark overnight to determine the viability of the streptavidin-AP conjugate.

For further condition testing, CTT10 pUCNEO plasmid (2 μg) were digested with HindIII restriction enzyme at 37°C before being denatured in boiling water for approximately one minute and immediately placed in ice. Serial dilutions of the digested plasmid were performed up to a dilution factor of 10_5. For the undiluted digestion reaction and dilutions 10_2 – 10_5, each sample was spotted on a small strip of labeled nylon membrane and were all cross-linked at 400 μjoules × 100. For the 10_1 dilution, however, the sample was spotted on three different strips of membranes, which were cross-linked at 400 μjoules × 100, 800 μjoules × 100, and 1200 μjoules × 100, respectively. All of the nylon membranes were hybridized in a hybridization solution consisting of 7% (w/v) sodium dodecyl sulfate (SDS) solution and 0.25M sodium hydrogen phosphate solution, and with denatured salmon sperm DNA to prevent non-

specific binding of the probe to the membrane. Following hybridization, the membranes underwent detection protocol with kit #K0662 overnight.

Testing of Digestion Reaction Conditions

Before actual experimentation on the digestion enzymes was performed, the plasmid map of the pUCNEO plasmid containing the GAA_{57} repeat sequence was analyzed with the resulting fragments that would theoretically result following digestion with XhoI, ScaI, HindIII, and EcoO109I restriction enzymes. Once each enzyme was found suitable for digestion of the plasmid, three digestion conditions were tested on the GAA_{57} DNA template with the following buffers and enzymes: XhoI restriction enzyme in 1X Buffer G (Thermo Fisher Scientific, Inc.) and subsequently in 1X Buffer D (Thermo Fisher Scientific, Inc.), HindIII restriction enzyme in 1X Buffer G, and KpnI restriction enzyme in 1X KpnI buffer (New England Biolabs, Inc.). To analyze the success of the digestion reactions, a 1.5% agarose gel was prepared via the mixture of 0.90g agarose RA in 60 ml Tris base, acetic acid, EDTA (TAE) solution with 0.5 µl ethidium bromide. Each digestion condition was loaded onto the gel and underwent electrophoresis before being analyzed under UV light.

Testing PCR Reagents on GAA_{57} Template

Given the results of the initial biotin-label experiments, the PCR reagents and the conditions of the primer extension reactions were tested throughout multiple experiments. The first PCR reaction was performed on a 1:100 diluted GAA_{57} DNA template with ScaI primer, 5' reverse primer, 10mM dNTP solution, 1X Taq buffer, and 50mM magnesium chloride solution.

Two PCR reactions were set up to test two different Taq polymerases. The reaction mixtures were initially denatured at 98°C for 30 seconds before undergoing 25 cycles of denaturation (98°C, 10 sec.), annealing (46°C, 30 sec.), and extension (72°C, 20 sec.). The PCR products underwent electrophoresis and were analyzed in a 1.5% agarose gel.

Following this particular experiment, the PCR reagents were altered slightly, in which the ScaI forward primer was replaced by a 5' forward primer and the annealing temperature was increased to 55°C due to the new forward primer. Once again, two conditions were tested with two different Taq polymerases, and the resulting products were analyzed on a 1.5% agarose gel.

For the next experiment, two PCR reactions were set up that used two different sets of primers. The first reaction consisted of an R3 forward primer and an R4 reverse primer, and the other reaction consisted of a ScaI forward primer with a 5' reverse primer. All other conditions were held constant with the same Taq DNA polymerase added to both reactions. Given the nature of the primers, the annealing temperature was once again decreased to 46°C, and an additional extension reaction ran for five minutes at 72°C following the 25th cycle. The resulting products were analyzed in a 1.5% agarose gel.

This subsequent conditional experiment was heavily altered, with the 1:100 diluted GAA_{57} DNA template mixed with 2X PCR Master Mix, 5' forward primer, and 5' reverse primer. The annealing temperature was once again raised to 55°C and the additional extension reaction was carried out once again. The product was analyzed in a 1.5% agarose gel.

The results of the previous experiment prompted another PCR condition experiment that utilized a new, fresh 10 mM dNTP solution with the 5' forward primer and 5' reverse primer, keeping the rest of the PCR conditions held constant. The exact temperature and time conditions

of the PCR reaction were also held constant with respect to the previous experiment. The resulting product was analyzed in a 1.5% agarose gel.

Utilizing the fresh solutions of the different nucleotides, a 10mM dATP, dGTP, dCTP solution was prepared and tested in this PCR experiment with a fresh Biotin-11-dUTP label, with all other PCR reagents and conditions remaining constant. The successful incorporation of the label was analyzed in a 1.5% agarose gel.

Biotin Detection Protocol with Tested PCR Conditions

Now that the correct PCR conditions have been determined through multiple rounds of experimentation, the biotin label must be tested with the new PCR product to determine the success of the kit reagents. The PCR product was eluted from the gel, and underwent serial dilutions up to a dilution factor of 10_4. The undiluted and diluted PCR products were spotted on three membranes that were cross-linked at 400 μjoules × 100, 800 μjoules × 100, and 1200 μjoules × 100, respectively. The membranes were not hybridized, but underwent detection protocol with the reagents from kit #K0662 overnight.

Three PCR reactions were labeled “Old”, “No T”, and “T” to identify reactions that contained the old biotin label without the presence of dTTP, a new sample of the biotin label without the presence of dTTP, and the new sample of the biotin label with the presence of dTTP. The reactions were set up with the ScaI forward primer and the 5’ reverse primer, and the annealing temperature was dropped to 46°C, with all other PCR conditions held constant compared to previous experiments. The products were analyzed on an agarose gel before undergoing serial dilutions up to a dilution factor of 10_4, being spotted on three strips of nylon

membrane, and cross-linking at 800 μjoules × 100. The membranes were hybridized and underwent detection protocol with the reagents from kit #K0662.

Analysis of Chemically Modified DNA Samples with Digested Length Controls

Initially, PCR was used to amplify the 1:100 diluted GAA_{57} DNA template via ScaI forward primer and 5' reverse primer with the biotin label and maintaining all other PCR conditions. Three digestion reactions were performed on the amplified GAA_{57} DNA template with XhoI, HindIII, and EcoO109I in the previously tested respective conditions of each enzyme. The digestion reactions were all performed at 37°C for approximately 45 minutes. The digested fragments for each separate digestion reaction served as the template in three primer extended reactions that utilized the ScaI forward primer and the biotin label once again to generate three digested length controls that would be used to help analyze subsequent results. Following the primer extension reaction, the samples were precipitated as done previously via ethanol and dissolved in equal amounts of TE solution and formaldehyde dye.

Pre-made samples of DNA modified with chloroacetaldehyde, potassium permanganate, and a maxi-prep DNA sample were used as templates for the preparation of three primer extension reactions that incorporated the ScaI primer and the biotin label and utilized Klenow Taq in lieu of DNA Taq polymerase. For the thermocycler conditions, however, the additional extension reaction was not performed and PCR ran for 30 cycles rather than 25. These samples were also precipitated via ethanol and dissolved in equal amounts of TE solution and formaldehyde dye.

Prior to analysis of the length controls and chemically modified samples, 6% polyacrylamide gel was prepared with urea, 19:1 acrylamide/bisacrylamide solution, and 1X Tris-Borate-EDTA (TBE) solution. The resulting solution was filtered before 10% ammonium persulfate and tetramethylethylenediamine (TEMED) were added to allow the gel to polymerize. Once loaded into the apparatus, the gel was pre-washed for one hour in 1X TBE solution at 65W before the samples were loaded into the gel. The gel ran for an additional hour in the same electrophoretic conditions following the loading of the samples and the contents were transferred onto a nylon membrane. The membrane was then cross-linked at 800 μjoules × 100 and underwent detection protocol with the appropriate amount of reagents from kit #K0662.

Following this experiment, the fragments produced from an EcoO109I digestion reaction were amplified in two separate PCR reactions via Taq DNA polymerase and Klenow Taq, respectively. Once the samples were generated and precipitated, they were loaded onto a polyacrylamide gel along with the digestion length controls and analyzed on a membrane via biotin detection protocol.

Design and Utilization of Oligo Primers for Trinucleotide Repeat

It was determined that the plasmid map of pUCNEO plasmid should be re-analyzed for the design of new primers that would be effective in isolating the GAA trinucleotide repeat sequence as well as the potential triplex structure that would be formed in proximity to the repeat sequence. In this design, one forward primer was developed that would anneal to the plasmid sequence 100 bp upstream of the repeat sequence, and three reverse primers that would anneal to

the plasmid directly before the repeat sequence (R1 primer), directly after the repeat sequence (R2 primer), and at the EcoO109I cut site (R3 primer).

Once the primers were obtained, to determine the success of these primers, three PCR reactions were performed with the oligo forward primer and the biotin label, and with each reaction utilizing a different reverse primer. All PCR reactions were performed with Taq DNA polymerase. Given the nature of the new primers, the annealing temperature of the PCR reaction was raised to 58°C and the additional extension reaction was performed for five minutes at 72°C following the 30th cycle. The expected PCR products that would form from the R1, R2 and R3 primers were analyzed in a 1.5% agarose gel to determine bands with expected lengths of 100 bp, 299 bp, and 368 bp, respectively. The products were precipitated via ethanol, denatured, and the resulting pellets were dissolved in TE solution and formaldehyde dye.

Once the control lengths (labeled "R1", "R2", and "R3", respectively) were generated, six primer extension reactions were set up with two reactions using the chloroacetaldehyde sample as the DNA template, two reactions using the potassium permanganate sample as the DNA template, and two reactions using the maxi-prep sample as the DNA template. Within those three sets of two reactions, one reaction utilized DNA Taq polymerase and one reaction utilized Klenow Taq, and this was repeated for each type of template used. All reactions ran with the oligo forward primer and the biotin label with the same thermocycler conditions as the control lengths. Following the primer extension reactions, the samples were precipitated with ethanol and dissolved in TE solution and formaldehyde dye. The control lengths and the six samples were all loaded onto a 6% polyacrylamide gel and the resulting bands were transferred onto a nylon membrane. The membrane was cross-linked at 800 μjoules × 100 and underwent detection protocol via the reagents found in kit #K0662.

Given the results of the biotin label, fresh control lengths and the six samples were generated once again using the same procedure and conditions previously used. The fresh samples ran through a 6% polyacrylamide gel and the resulting bands were analyzed on a biotinylated membrane that had just undergone the detection protocol.

Radioactive Detection

As a new means of detecting the triplex structure *in vitro* to potentially yield more conclusive results, it was decided that a radioactive probe should be incorporated onto the membrane via hybridization and developed on an x-ray film to analyze the banding patterns of the chemically modified samples.

Before the probe was generated, however, the chemically modified DNA samples were re-created to generate new, fresh samples. The pUCNEO plasmid containing the trinucleotide repeat was mixed with 1M magnesium chloride solution and 1M Tris, pH 8.0 solution and distributed equally into three separate chemical modification reactions. A diluted solution of chloroacetaldehyde was added to one of the reactions, and the CAA and maxi-prep samples were incubated at 37°C for 8 minutes. For the permanganate sample, 10mM potassium permanganate solution was added to the appropriate reaction and incubated at 37°C for 5 minutes. The permanganate modification was halted through the addition of β-mercaptoethanol. All three modified samples were precipitated via ethanol and dissolved in TE solution. All three samples underwent digestion reactions with ScaI-HF and EcoO109I at 37°C for 60 minutes, and the digested samples were also precipitated via ethanol and dissolved in TE solution. The success of the sample generations and digestion reactions were analyzed in a 1.5% agarose gel. The

chemically modified samples and the control lengths ran on a 6% polyacrylamide gel and the contents were transferred on a nylon membrane and cross-linked at 800 μjoules × 100.

The generation of a radioactive probe was generated through a primer extension reaction, in which the R1 control length served as the DNA template and the oligo R1 primer was used to carry out the reaction. In addition, a 10mM dATP, dGTP, dTTP solution was used in this reaction with a radioactive dCTP nucleotide. The reaction was carried out with Taq DNA polymerase. The thermocycler conditions were carried out with an initial denaturation step at 95°C for 2 minutes, 20 cycles of denaturation (95°C, 30 sec.), annealing (58°C, 30 sec.), and extension (72°C, 5 min.) with an additional denaturation step after the 20_{th} cycle at 98°C for 2 minutes. The probe was hybridized onto the membrane overnight at 65°C and the membrane was washed with a prepared 1L-wash solution containing 30 ml 20X SSC solution and 5 ml 20% (w/v) SDS solution. Once the wash was completed, the membrane was placed in the cassette with the film that would later be developed and analyzed.

To amplify the signal of the radioactive probe, the R1 control length was amplified via PCR with the oligo forward primer and a 10mM dNTP solution. The PCR reaction was carried out for 30 cycles at the same temperatures in the previous reaction, with a five-minute extension reaction following the 30_{th} cycle. Once the template was amplified, a PCR reaction was created with Klenow Buffer, the oligo R1 primer, the R1 amplified template from the previous PCR reaction, 10mM dATP, dGTP, dTTP solution, and the radioactive dCTP nucleotide. The membrane was hybridized with the probe and subsequently washed using hybridization solution and wash solution, respectively. The membrane was then stored at -80°C with the film prior to development. For further analysis, the same probe and contents were analyzed on a different kind of x-ray film.

Following this, the R1 template for the development of the radioactive probe was recreated without the biotin label and with a new Taq DNA polymerase. Once the template was developed, the new Taq DNA polymerase was used to generate the radioactive probe, which was hybridized on the membrane and developed on a new film. Two different films were exposed to the newly generated probe and hybridized membrane at two different exposure times to compare the results.

RESULTS

Biotin Label Detection Conditions Testing

Initially, the reaction conditions of the Biotin Chromogenic Detection Kit (Product #K0662) were tested on a pUCNEO plasmid that contains a GAA_{57} satellite repeat sequence. Throughout these experiments, this pUCNEO plasmid was utilized as a model system for the analysis of chemical modification on the trinucleotide repeat sequence, as the plasmid itself contained 57 repeats of the GAA trinucleotide microsatellite sequence that is flanked by restriction enzyme sites ideal for isolating the sequence for in-depth analysis *in vitro*. In addition, the plasmid also contains various genes and markers, such as the *ampR* gene that encodes for ampicillin resistance and an SV40 origin of replication, that would be key components within the context of *in vivo* experiments.

Once the contents of the biotin labeling reagents were tested and analyzed, a sample of CTT10 pUCNEO plasmid underwent a digestion reaction with HindIII restriction enzyme and denatured before undergoing subsequent dilutions and being spotted on membranes. The cross-linking conditions, dilutions, and amount of digested DNA on the membranes were all tested in this particular experiment. The membranes underwent hybridization protocol with the pre-made PCR probe and subsequent detection with the reagents in the Biotin Chromogenic Detection Kit. Once again, none of the resulting membranes contained any precipitated spots that would normally be visible due to the biotin label.

Testing of Digestion Reaction Conditions

Following analysis of the biotin labeling components, the enzymatic recognition sites of various digestion enzymes within the plasmid were analyzed in the planning and development of digestion reactions that would be performed throughout these experiments. Within the context of this study, the digestion reactions were performed in order to excise the chemically modified samples from the rest of the pUCNEO plasmid so that replication stalling would be analyzed at the modified bases within the repeat sequence and at nearby flanking sequences where the triplex structure was theorized to occur.

The pUC19NEO GAA_{57} cut site map was analyzed with various digestion enzymes, such as XhoI, ScaI, HindIII, and EcoO109I (**Figures 1 & 2**).

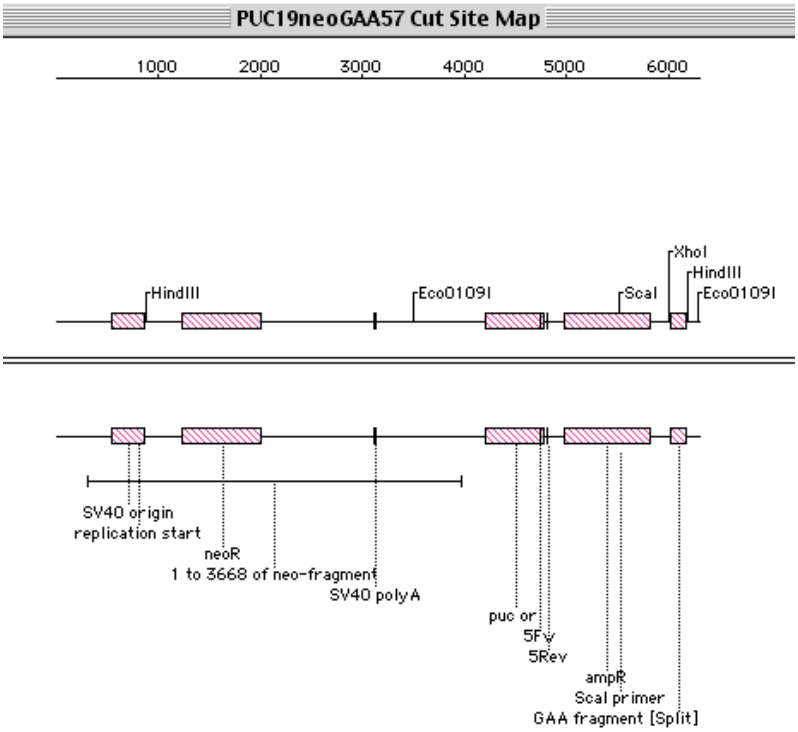

Figure 1. pUCNEO Cut Site Map. The plasmid map above displayed the cut sites of XhoI, HindIII, ScaI, and EcoO109I on the plasmid.

Fragment Size	Left Cut by Overhang	Enzyme	Cut by From : To	Right Cut by Enzyme	Overhang
5826	[+ 4]	XhoI	5997:5525	ScaI	[- 1]
471	[- 1]	ScaI	5526:5996	XhoI	[+ 4]
5627	[+ 4]	HindIII	6196:5525	ScaI	[- 1]
670	[- 1]	ScaI	5526:6195	HindIII	[+ 4]
5535	[+ 2]	EcoO109I	6288:5525	ScaI	[- 1]
762	[- 1]	ScaI	5526:6287	EcoO109I	[+ 2]

Figure 2. Estimated Fragment Lengths Produced from Restriction Enzymes. The predicted fragment lengths were estimate provided from the digestion information found in the cut site map (refer to **Figure 1** above).

In addition, the different resulting fragments and their sizes were analyzed to test and determine which digestion conditions would be ideal to isolate the GAA_{57} trinucleotide repeat and flanking sequences that could contain the triplex structure (**Figure 2**). To test digestion conditions, the GAA_{57} template underwent digestion with XhoI, HindIII, and KpnI in various buffer conditions, including Buffer G, Buffer D, and Cutsmart Buffer. Once the digestions reactions were performed, the resulting fragments were visualized on a 1.5% agarose gel and under UV light (**Figure 3**).

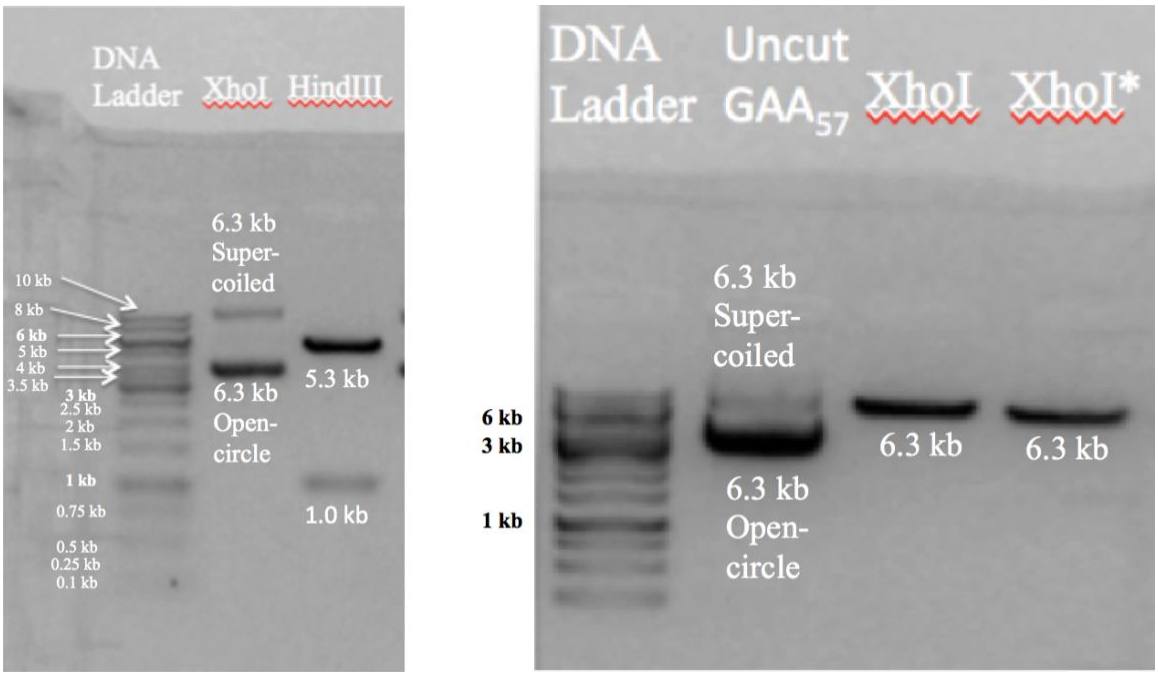

Figure 3. Agarose Gel Analysis of Digestion Conditions on GAA57. a) The gel was used over the course of two digestion experiments to test the conditions of XhoI, HindIII, and KpnI on the GAA57 template. The full DNA ladder is labeled as well as the digestion bands and identification of conformations, if applicable. **b)** The "XhoI" and "XhoI*" nomenclature was used to differentiate the two different XhoI enzymes used during the second experiment. All of the bands were labeled with conformation, if applicable. The same DNA ladder was used for both experiments. Due to the proximity of the bands on the second ladder, only the highly intense bands were labeled, but the bands correspond to the other ladder used in the previous gel.

According to **Figure 3a**, for the first experiment, the results of the gel indicate that HindIII was able to completely digest GAA57 in Buffer G, and XhoI and KpnI were not successful in digesting GAA57 given the identical banding patterns produced by two different restriction enzymes, which correspond to two different conformations of the pUCNEO plasmid. For the second experiment, the linearized single band produced by the digestion of both types of XhoI restriction enzymes indicate a successful digestion within both Buffer D and Cutsmart Buffer (**Figure 3b**). KpnI was found to be ineffective in digesting the pUCNEO plasmid in any reaction conditions (**Figure 3a & 3b**).

Testing PCR Reagents on GAA_{57} Template

Following analysis of the digestion enzymes and their efficiencies, various PCR reagents and conditions were tested on the GAA_{57} template to determine the optimal amplification conditions of the trinucleotide repeat sequence. Following a series of PCR reactions in which various combinations of primers were tested and no PCR products were visualized on the agarose gel, the utilization of a freshly obtained sample of free nucleotides was essential in the visualization of an amplified PCR product. In this particular reaction, two reactions were performed with the fresh dNTP solution, with one reaction containing the ScaI forward primer and the other reaction containing the 5' forward primer. Both reactions were run at an annealing temperature of 46°C and underwent agarose gel electrophoresis. The resulting gel image visually displayed both of the PCR products alongside the DNA ladder, indicating a successful PCR reaction (**Figure 4**).

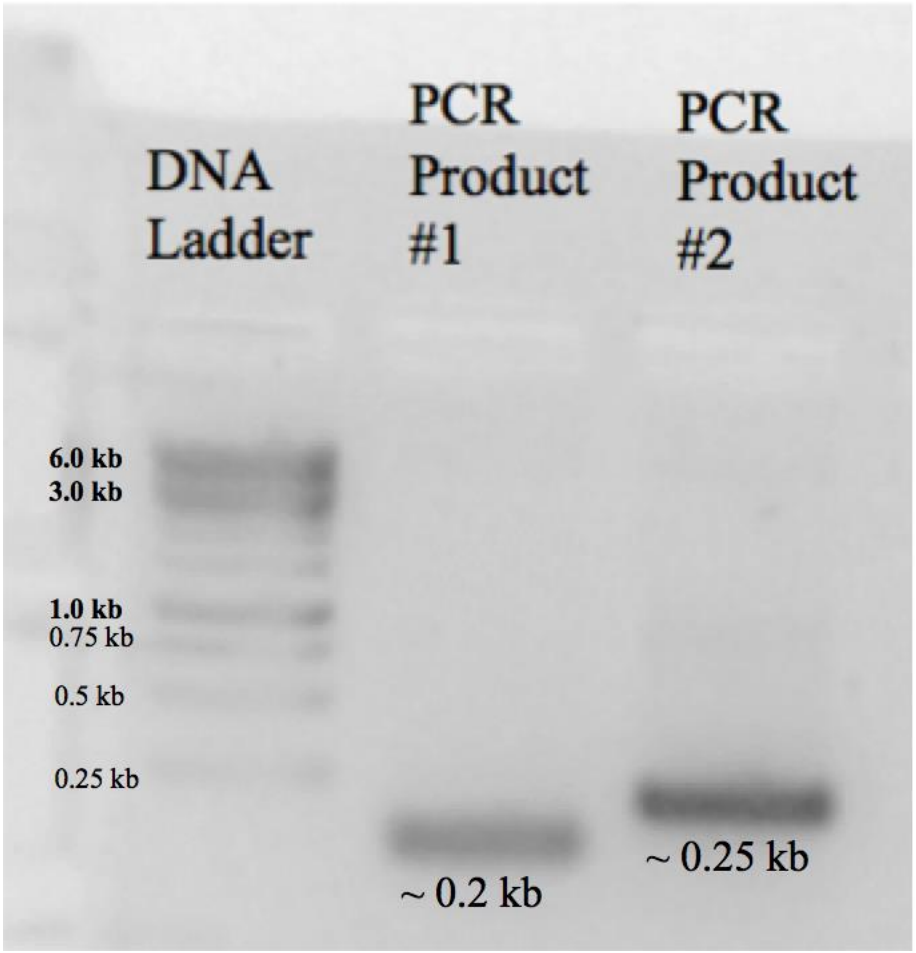

Figure 4. PCR Products from Freshly Obtained dNTP Solution. The gel image above displays the DNA ladder as well as the two PCR reactions that were successful upon addition of the fresh dNTP solution. The smallest three DNA ladder bands as well as the intensity-marker bands were labeled as well as the approximate size of the resulting PCR fragments. "PCR Product #1" was the GAA fragment that was obtained with the utilization of the ScaI forward primer, and "PCR Product #2" was the GAA fragment that was obtained with the utilization of the 5' forward primer.

In the gel image shown in **Figure 4**, the PCR product that is visualized in lane #2 signifies the amplified GAA-repeat fragment as well as flanking sequences. Because this amplification reaction utilized the ScaI forward primer, the primer anneals to a portion of the plasmid that corresponds to the *ampR* gene upstream of the repeat sequence (**Figure 1 & 2**). Given the placement of the ScaI primer upstream of the 5' end of the GAA fragment and the

inclusion of the entirety of the GAA fragment, the band corresponds to an estimated length of about 200 bp (**Figure 1-2, 4**). The PCR product that is visualized in lane #3 signifies the amplified GAA-repeat fragment as well as the flanking sequences that were obtained through utilization of the 5' forward primer as compared to the ScaI primer (**Figure 4**). Because the primer annealing site of the 5' forward primer is further upstream of the ScaI primer annealing site (**Figure 4**), the fragment generated from the 5' forward primer is slightly greater in length than the fragment generated from the ScaI primer. Overall, the PCR fragments were able to be visualized in this experiment and not in the previous experiments because the previous solution of free nucleotides were non-functional, whereas this freshly obtained sample contained functional nucleotides that were able to be incorporated into the resulting fragment properly.

Now that PCR was proven to be successful with the freshly obtained dNTP solution, primer extension with the Biotin-11-dUTP label needed to be determined in the same manner. The PCR reaction was run with a fresh solution of dATP, dGTP, & dCTP, the Biotin-11-dUTP label, the 5' forward primer, the 5' reverse primer, and Taq DNA polymerase. The primer extension reaction was performed and visualized on an agarose gel, which indicates that the primer extension product with the biotin label was successful (**Figure 5**).

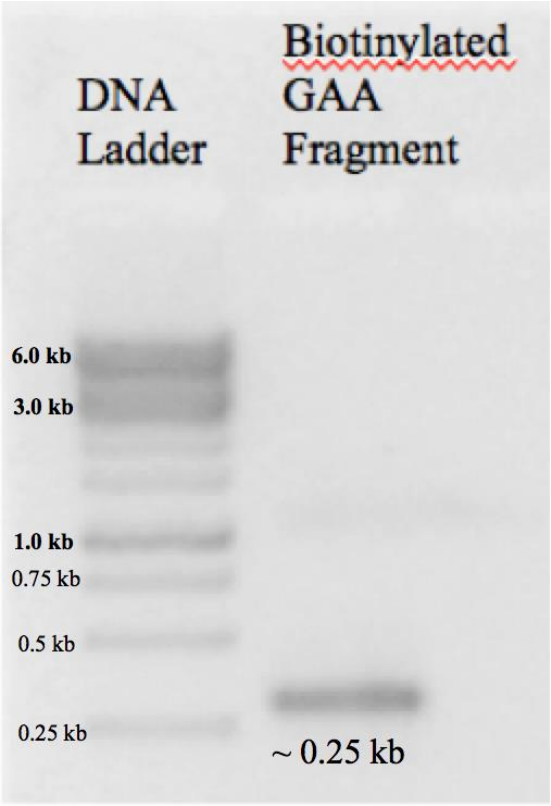

Figure 5. Analysis of Amplification of Biotinylated GAA Fragment. The gel image above displays the DNA Ladder alongside the PCR product that was successfully generated to incorporate the Biotin-11-dUTP label. The smallest three bands and the high-intensity bands of the DNA ladder were labeled in the context of this experiment. The approximate size of the fragment was also identified and labeled.

Biotin Detection Protocol with Tested PCR Conditions

With the proper PCR conditions being evaluated and established, the biotin labeling protocol can be tested with the proper reagents and in tandem with the newly established PCR conditions. The primer extension product was denatured and underwent serial dilutions up to a factor of a 10_4 dilution. The diluted samples were spotted on three membranes labeled "400", "800", and "1200", respectively, to indicate the energy value at which each membrane was cross-linked (μjoules × 100). The purpose of running these experiments and performing the biotin labeling reaction with multiple cross-linking energies was to improve and determine the

optimal cross-linking conditions that would yield in the highest clarity of results. The membranes did not undergo hybridization, but were instead immediately subjected to the Biotin Chromogenic Detection Kit protocol to visualize any precipitate found on the membranes. The results of the label detection are shown in **Figure 6**.

Figure 6. Biotin Chromogenic Detection Kit Utilization on Membranes Cross-Linked at Various Conditions. The image above displays the precipitated DNA produced from the biotinylated PCR product cross-linked at various conditions. From top to bottom, the membranes were labeled "400", "800", and "1200" to indicate the energy at which they were cross-linked. From left to right, the spots were labeled as "1" and "10_1" to indicate the level of dilution of the spotted DNA sample.

As indicated in the image, only the concentrated and 10_1-diluted spots appeared on each of the membranes. However, the membrane cross-linked at 800 μjoules × 100 produced the brightest spots; more specifically, it produced the brightest 10_1-diluted spot.

To test the optimal conditions for the biotin label detection protocol that would yield the most conclusive results, it was determined whether the Biotin-11-dUTP label works best with or without the presence of dTTP in the primer extension reaction. Three primer extension reactions were performed with a previously utilized sample of Biotin-11-dUTP, a freshly obtained sample of the biotin label without dTTP (served as the control), and the freshly obtained sample of the biotin label with the presence of dTTP. The resulting products were serially diluted and spotted on membranes which were cross-linked at 800 μjoules × 100. The membranes then underwent hybridization and Southern Blotting protocols with the detection kit reagents, and the results are shown in **Figure 7**.

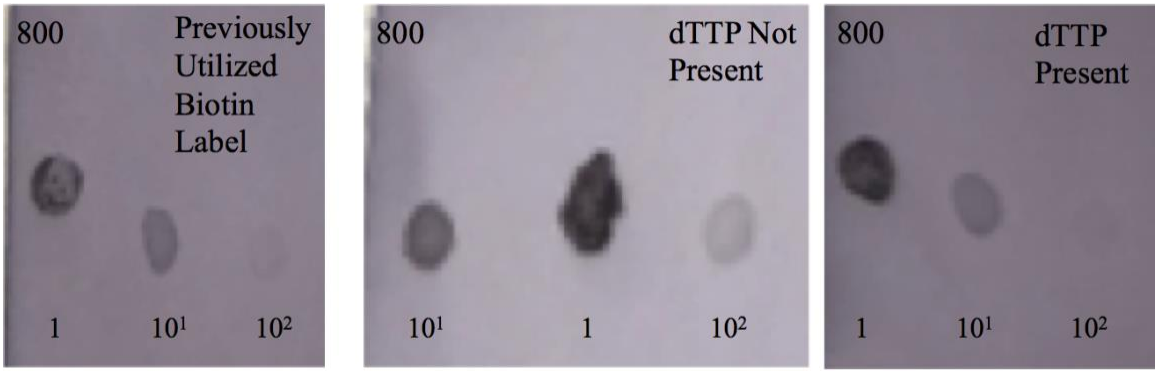

Figure 7. Testing of Biotin Label With and Without the Presence of dTTP Nucleotide. From left to right, the membranes were labeled to indicate the sample labeled with the previously utilized biotin label, "dTTP Not Present" to indicate the freshly obtained biotin label without the presence of dTTP, and "dTTP Present" to indicate the freshly obtained biotin label with the presence of dTTP. The membranes were spotted from left to right with increasing dilutions from 1 to 10_2 (EXCEPTION: On the second membrane, the spotting positions of the undiluted and 10_1 diluted spots were switched).

According to the results of this experiment, on all of the membranes, the undiluted, 10_1 dilutions, and 10_2 dilutions are all visible to varying degrees. However, the freshly obtained biotin label produced brighter precipitation spots than the previously utilized biotin label. On the membrane with the fresh Biotin-11-dUTP label that does not contain dTTP, the 10_3-diluted sample appear very faintly on the membrane, whereas the precipitate of that dilution does not appear on the other two membranes.

Analysis of Chemically Modified DNA Samples with Digested Length Controls

Three digestion reactions were performed on the GAA_{57} template with XhoI, HindIII, and EcoO109I digestion enzymes, respectively. The digested fragments for each of the reactions were amplified via PCR with the Biotin-11-dUTP label and analyzed on a gel to ensure that the reaction was successful, which it was in all cases. Primer extension reactions were then performed on GAA_{57} template DNA that was been amplified with chloroacetaldehyde and

potassium permanganate, respectively, and a maxi-prep sample of the trinucleotide-repeat template. All of the PCR-amplified samples were precipitated with ethanol and loaded onto a 6% polyacrylamide gel, which ran for about an hour before the contents of the gel were transferred onto a nylon membrane. The membrane was then cross-linked and underwent detection protocol (**Figure 8**).

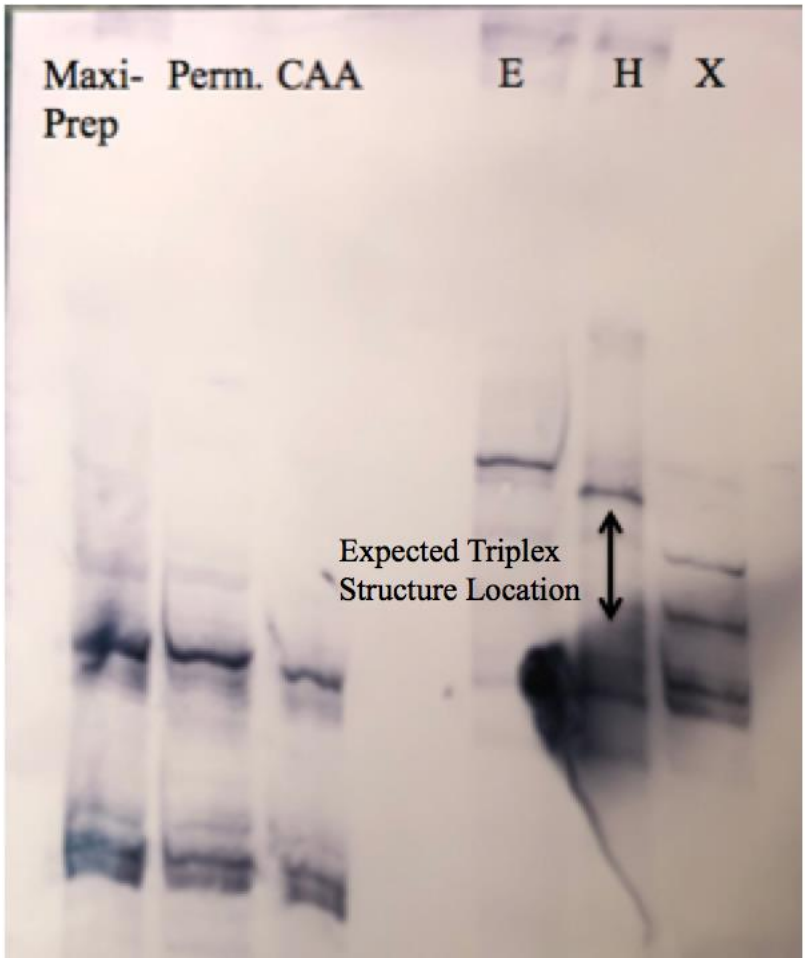

Figure 8. Biotin Label Detection of Chemically Modified Samples Compared to Digestion Controls. The membrane above shows the resulting bands of the chemically modified samples compared to the digestion length controls. From left to right, the lanes were labeled as follows: "Maxi-Prep" to indicate the maxi-prep sample, "Perm." to indicate the potassium permanganate sample, "CAA" to indicate the chloroacetaldehyde sample, "E" to indicate the EcoO109I digestion control, "H" to indicate the HindIII digestion control, and "X" to indicate the XhoI digestion control. The expected location of the triplex structure was also determined based on the location of the digestion controls.

According to the image above, the bands of the chemically modified samples could not be properly analyzed in comparison to the digestion controls, providing inconclusive evidence of triplex structure identification *in vitro*. As identified in the figure, the expected location of the triplex structure was located in between the primary band of the XhoI digestion control and the primary band of the HindIII digestion control because that is the location where replication stalling would have likely occurred. However, based on the migration and banding pattern of the DNA samples within the gel, the triplex structure could not be properly identified.

Following this experiment, the digestion length controls were compared with samples identified by both Taq DNA polymerase and Klenow Taq to determine which enzyme works better in the context of this experiment (**Figure 9**).

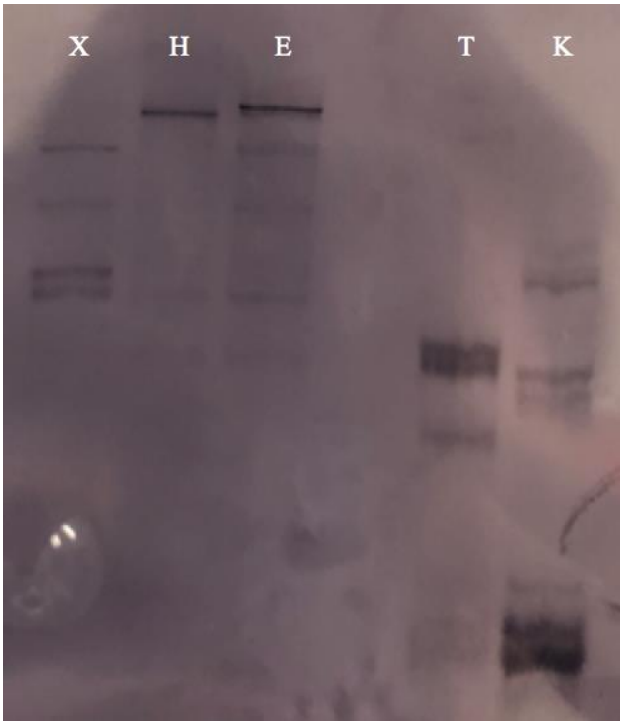

Figure 9. Membrane Analysis of Taq DNA Polymerase and Klenow Taq Effectiveness in Amplification of Sequence with Potential Triplex Structure. The membrane image above was labeled as follows from left to right: "X" to indicate the XhoI digestion control, "H" to indicate the HindIII digestion control, "E" to indicate the EcoO109I digestion control, "T" to indicate the sample amplified by Taq DNA polymerase, and "K" to indicate the sample amplified by Klenow Taq.

According to the membrane image above, the presence of many shorter bands in the “K” lane indicate that the Klenow Taq had difficulty in amplifying the PCR product through the triplex structure. The Taq DNA polymerase, however, is able to amplify the product through the triplex structure.

Design and Utilization of Oligo Primers for Trinucleotide Repeat

The plasmid map for the pUC19neo plasmid containing the trinucleotide repeat was analyzed once again, and this map was used to create an oligo forward primer and three reverse primers designed to end right before the trinucleotide repeat sequence, right after the trinucleotide repeat sequence, and at the EcoO109I cut site. The primer placements on the sequence can be visualized in **Figure 10**.

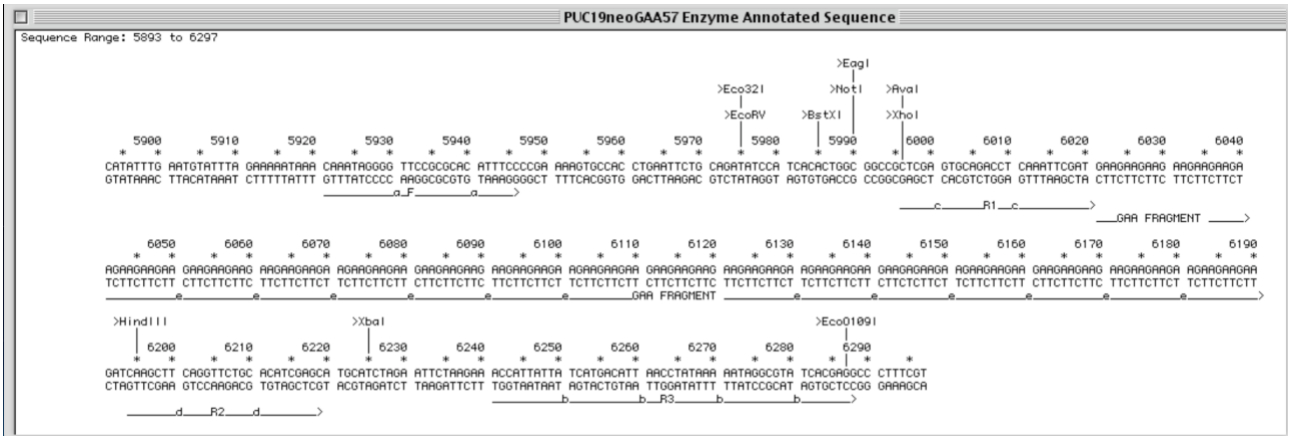

Figure 10. Primer Annealing Map of Oligo Primers to Annotated pUCNEO Plasmid Sequence. The image above displays the annotated flanking and repeat sequences along with the placement of the forward primer (F) and the three reverse primers (R1, R2, and R3, respectively) from left to right in the sequence.

The primers were designed based on the complementary sequences of the template DNA sequence. Once the primers were obtained and prepared, they were all performed on the GAA57

template strand to generate fragment control lengths to be subsequently utilized on a polyacrylamide gel. The control lengths were analyzed on an agarose gel, and the results of the gel indicated that three fragments were generated at lengths of approximately 100 bp, 299 bp, and 368 bp, respectively, which align with the expected lengths of the control products (**Figure 11**).

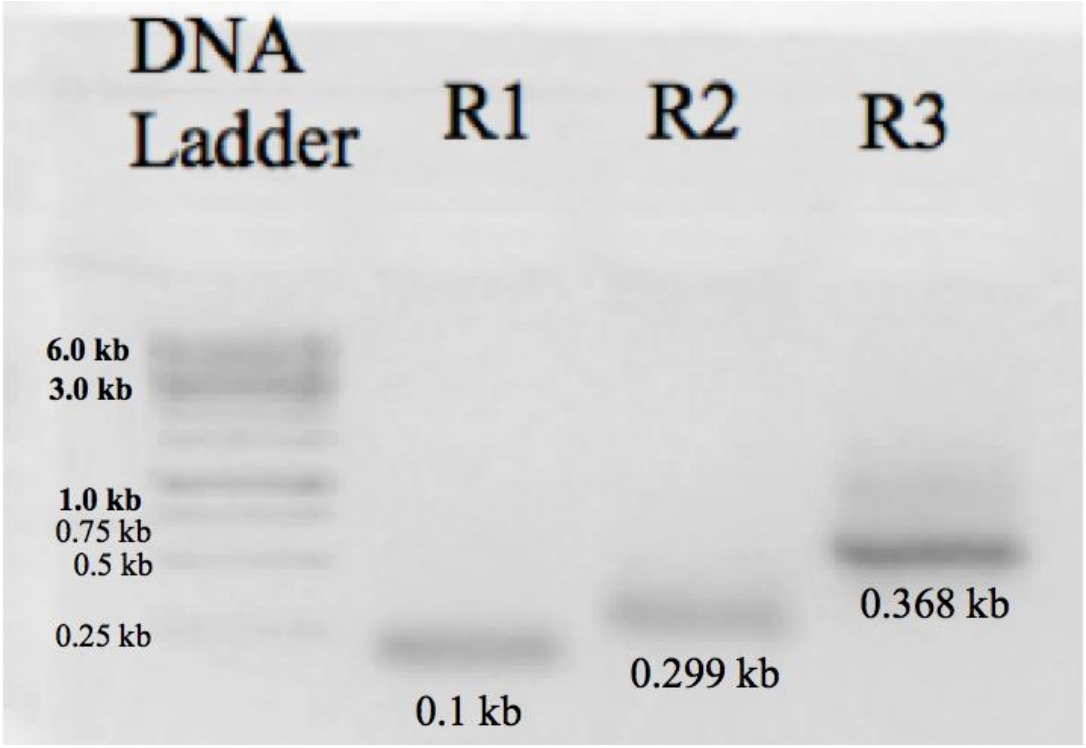

Figure 11. Agarose Gel Analysis of Length Control Amplifications. The gel above displays the DNA ladder as well as the labeled lanes that contained the respective length control fragments obtained from PCR with the oligo primers. The high intensity bands and the smallest three DNA ladder fragments were labeled for the context of this experiment, as well as the approximate sizes of the control lengths.

Once the control lengths were generated, six samples were created via PCR utilizing both Klenow Taq and DNA Taq polymerase on the maxi-prep DNA sample as well as the samples modified with chloroacetaldehyde and potassium permanganate. All of the samples were precipitated with ethanol and ran on polyacrylamide gel. The contents of the gel were transferred onto a nylon membrane and underwent Biotin Chromogenic Detection Kit protocol.

The results of the membrane could not be properly analyzed due to the smearing of the length controls on the membrane (**Figure 12**).

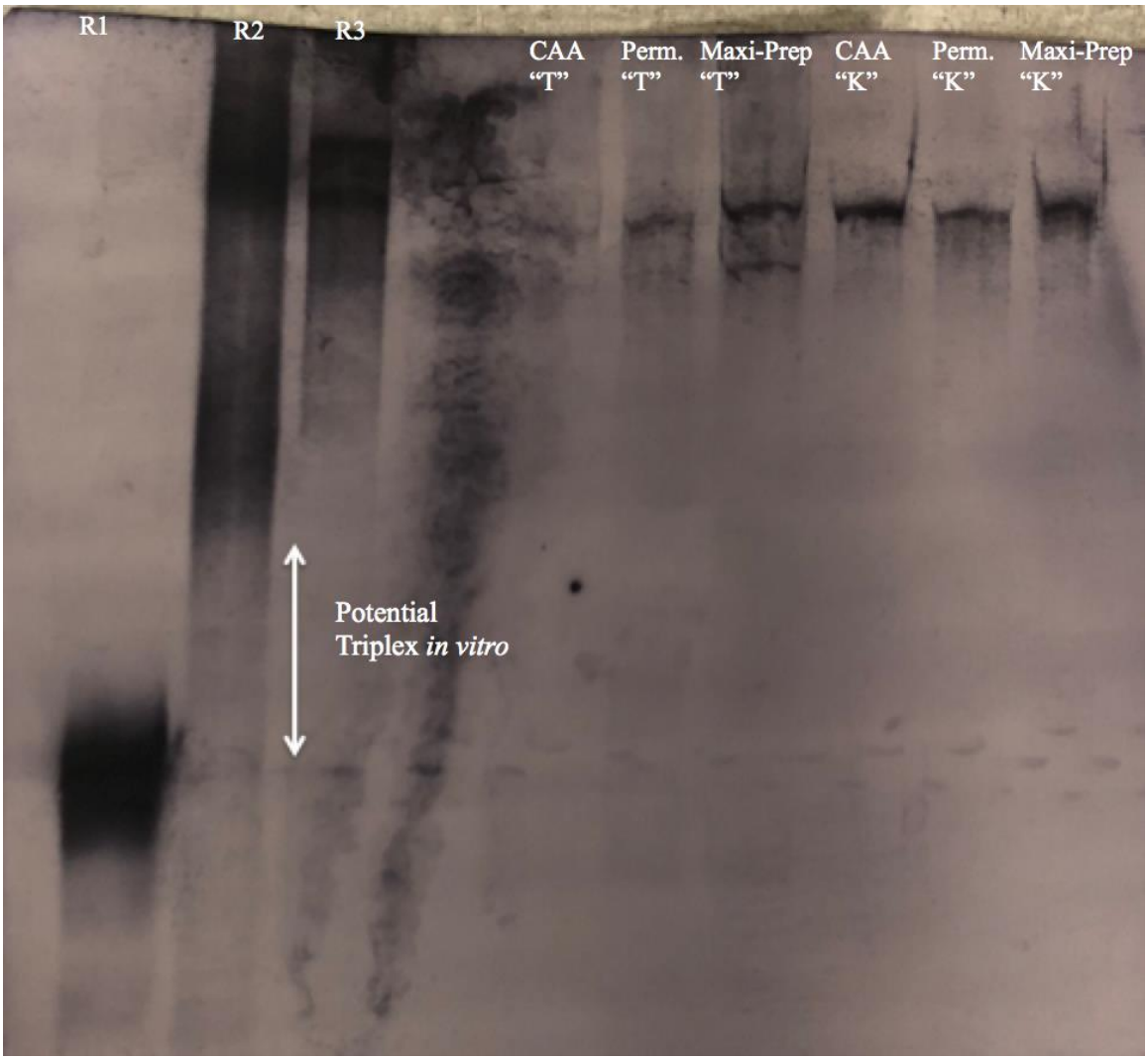

Figure 12. Analysis of Chemically Modified DNA Samples Amplified in Comparison with Oligo Length Controls. The membrane above indicates the length controls generated from the three different reverse primers and the chemically modified DNA samples amplified with both Taq DNA polymerase (as indicated by "T") and Klenow Taq (as indicated by "K"), respectively. The approximate location of the predicted triplex structure was identified in relation to the R1 and R2 length control.

According to the membrane image above, the triplex structure identification could not be properly identified due to the intense smearing of the length controls (**Figure 12**).

This protocol was repeated with newly generated length controls and chemically modified DNA samples, and they ran on another polyacrylamide gel to undergo biotin detection once again. The results of the detection on the membrane are visualized in **Figure 13**.

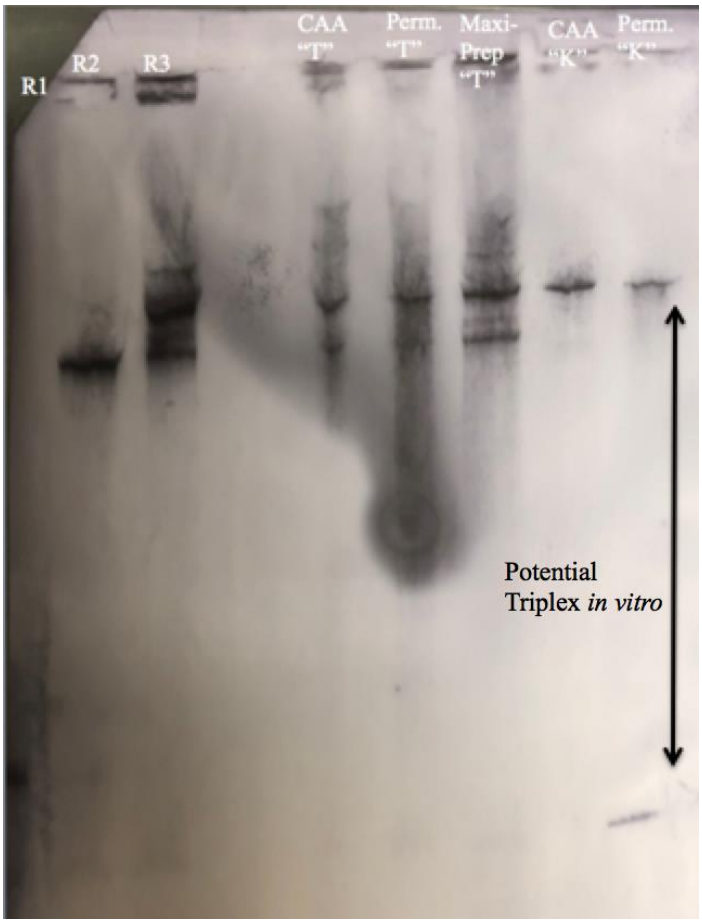

Figure 13. Analysis of Chemically Modified DNA Samples Replication Experiment. The membrane image above shows the results of the chemically modified samples in relation to the length controls with the same labeling nomenclature as the previous experiment (refer to **Figure 12**). The potential triplex *in vitro* structure is identified and labeleld in the image.

According to the image above, the triplex structure *in vitro* was potentially identified due to the lack of smearing and the clarity of the DNA bands, but more tests were performed for certainty due to the lack of sensitivity of the biotin label (**Figure 13**).

Radioactive Detection

To increase the assay sensitivity, it was decided that a radioactive probe would be used in lieu of the previously used Biotin label. However, before this could begin, the chemically modified DNA samples were reproduced to obtain fresh and potentially unexpired samples for further experimental use. Once the samples were created, digestion reactions were performed on the CAA, permanganate, and maxi-prep samples with EcoO109I and ScaI-HF restriction enzymes (**Figures 14 & 15**).

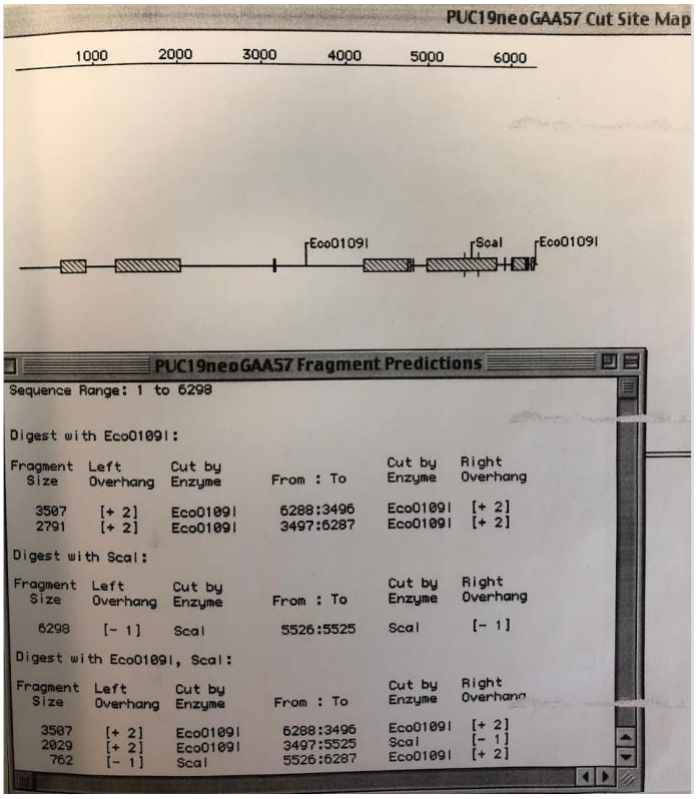

Figure 14. Cut Sites and Estimated Fragment Sizes of DNA Template Digested with ScaI-HF and EcoO109I. The gel image above indicates the cut site map of the pUCNEO plasmid as well as the length and number of fragments that would result from digestion with both ScaI-HF and EcoO109I digestion enzymes.

According to the cut site map, because EcoO109I has two cut sites and ScaI-HF only has one cut site, the digestion would likely produce three fragments with lengths of 3507 bp, 2029 bp, and 762 bp, respectively.

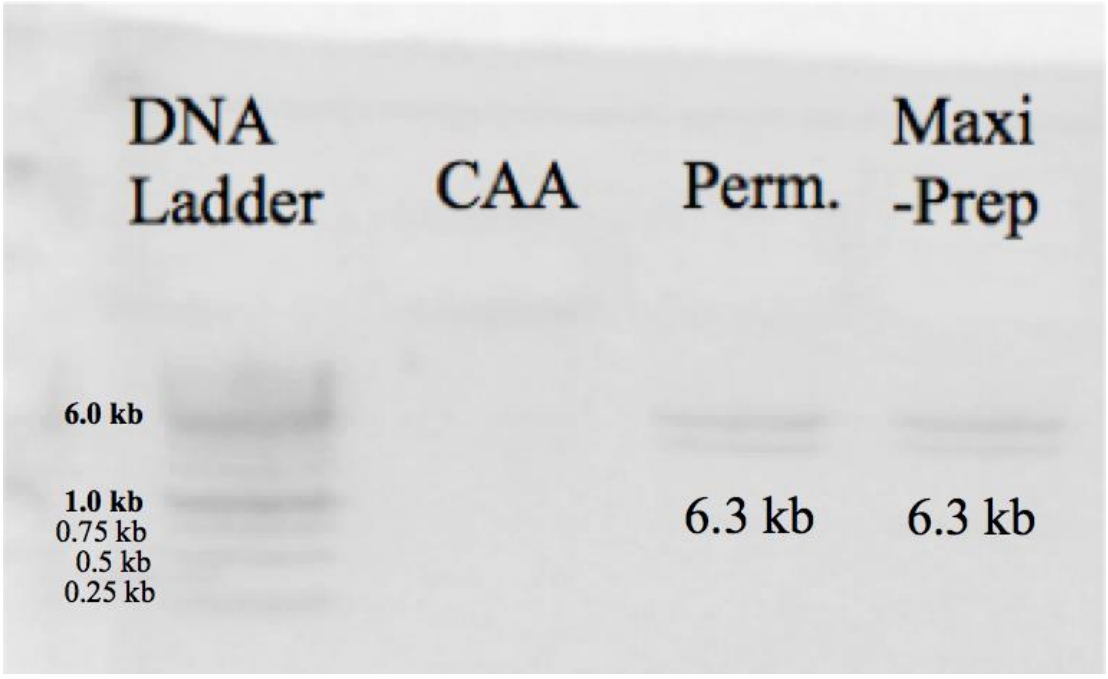

Figure 15. Agarose Gel Analysis of Chemically Modified Samples Digested with ScaI-HF and EcoO109I. The gel image above displays the DNA ladder and the lanes that were loaded with the chloracetaldehyde (CAA), potassium permanganate (Perm.), and Maxi-Prep DNA samples, respectively. Given the minimal spreading of the DNA ladder bands, only the bottom three bands and the 1.0-kb & 6.0-kb high-intensity bands were labeled for the context of this experiment. The approximate sizes of the "Perm." and "Maxi-Prep" samples were also labeled to correspond to the size of the plasmid.

According to the gel image above, the chloacetaldehyde sample did not produce any fragmented/digested bands within the gel, while the permanganate and maxi-prep samples both produced doublet bands identical to one another. The presence of the doublet band and a faint, shorter singlet band indicated a successful digestion of the latter two samples based on the predicted fragments identified in the cut site map (**Figure 14**). Based on these results, the chloroacetaldehyde sample needed to be recreated and digested in a subsequent experiment. The digestion of the chloroacetaldehyde sample produce a doublet band followed by a faint singlet

band further down in the gel lane. Because these results aligned with the digestion of the previous two samples and correspond to the predicted fragments shown in the cut site map, digestion of the sample was found to be successful.

The digested samples were then amplified via PCR, precipitated, and loaded onto a polyacrylamide gel along with the three length controls generated from the oligo primers. Once the contents of the gel were transferred to a nylon membrane, a radioactive probe was generated via PCR utilizing the R1 length control fragment as a template for radioactive ^{32}P-dCTP. The membrane was then hybridized with the probe and developed onto a film.

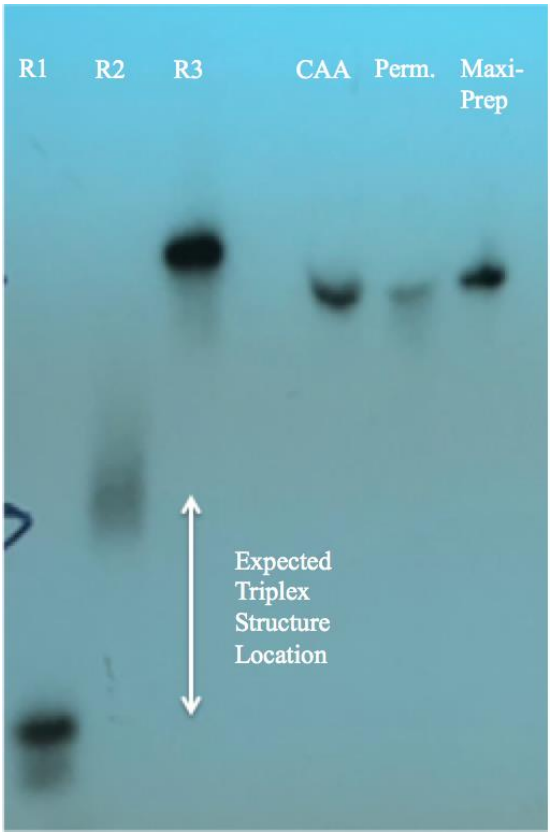

Figure 16. X-Ray Analysis of the Radioactive Probe on the Length Controls and Chemically Modified Samples. The film above displays the approximate location of the length controls and the chemically modified and digested samples, which are labeled on the film above. In addition, the expected location of the triplex structure was also identified based on the banding pattern of the R1 and R2 length controls.

According to the image above, it was determined that the concentration of dCTP needed to be increased to develop a more desirable visualization of the membrane given that the triplex structure could not be identified for the chemically modified samples in relation to the location of the length controls (**Figure 16**). In addition, based on the results of the membrane above, the ideal conditions were established that would allow the DNA samples to migrate to the optimal position within the gel and on the membrane. According to these results, when running the samples on a 6% polyacrylamide gel, the contents should run in the membrane until the xylene cyanol band runs 8 cm from the ladder. The experimental implications of determining the optimal conditions of the gel electrophoresis is that those conditions could be applied to future experiments with varying degrees of sensitivity to yield the highest quality results.

To combat this, the template for the radioactive probe was amplified via PCR to produce a higher concentration of templates that would allow for more radioactive probes to be generated through an elongated extension reaction. The PCR probe was amplified to produce a stronger signal, and the results were displayed on another film.

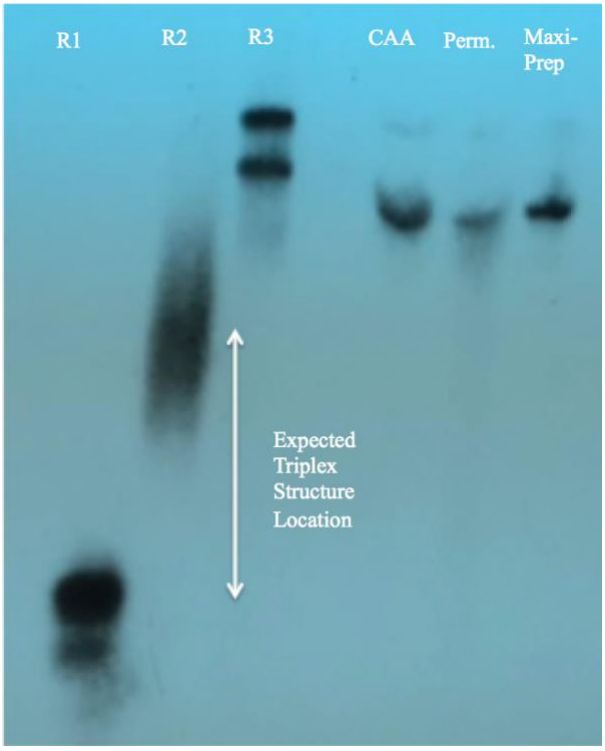

Figure 17. X-Ray Analysis of an Increased Amount of Radioactive Probe on Length Controls and Chemically Modified Samples. The film above displays the length controls and the chemically modified samples at increased sensitivity in relation to the previous experiment based on the higher concentration of radioactive probe.

According to the image above, the control lengths were smeared on the film, inhibiting the proper identification of the triplex structure. The same probe and contents were analyzed on a different type of film, and once again, the bands were too smeared to provide an accurate result (**Figure 18**).

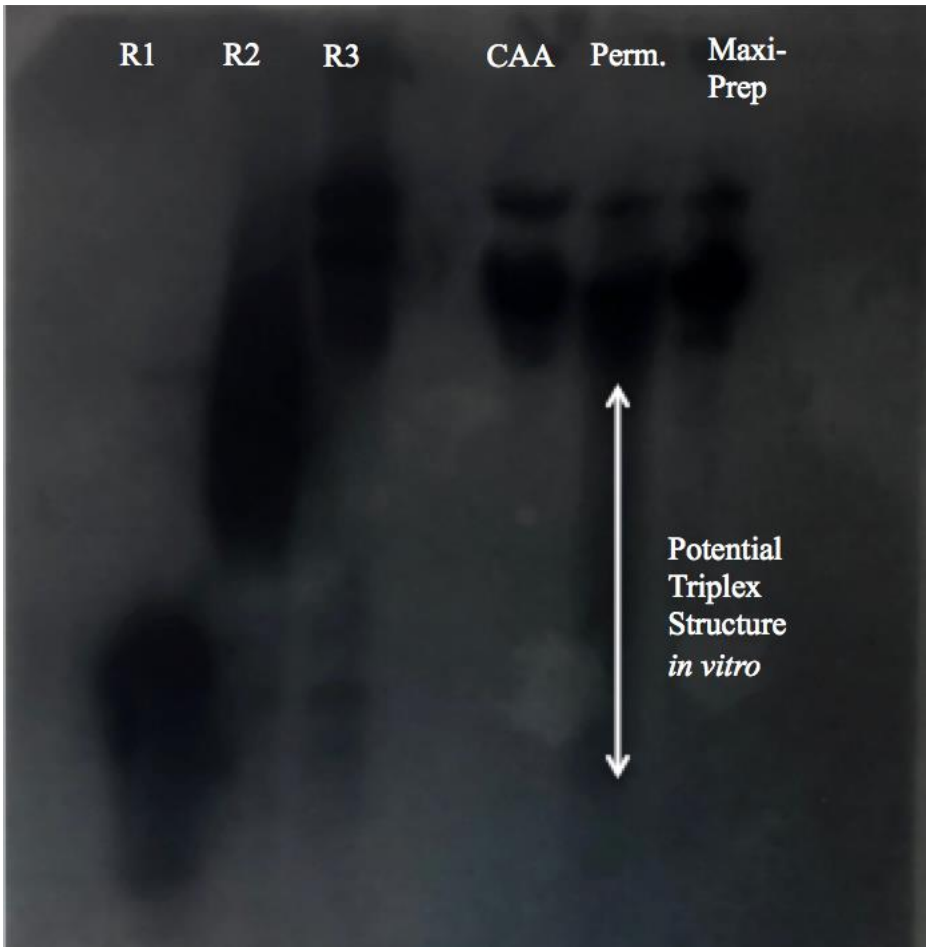

Figure 18. Radioactive Probe Analysis of Length Controls and Chemically Modified Samples on Alternate X-Ray Film. The new film was developed with the same contents as the previous two experiments, with the length controls and chemically modified samples being analyzed based on the radioactive probe.

Based on the film image above, the smear on the gel pertaining to the sample modified with potassium permanganate could likely correspond to the area of the plasmid that contains the non-canonical triplex structure. However, more experimentation was required with a lower degree of sensitivity and a higher degree of clarity to avoid background smearing that would potentially alter the results. As a result of this, the R1 template was regenerated without the biotin label and with a new, highly functional Taq polymerase. The same membrane was

hybridized with the newly generated probe and the results of the hybridization are shown below (**Figure 19**).

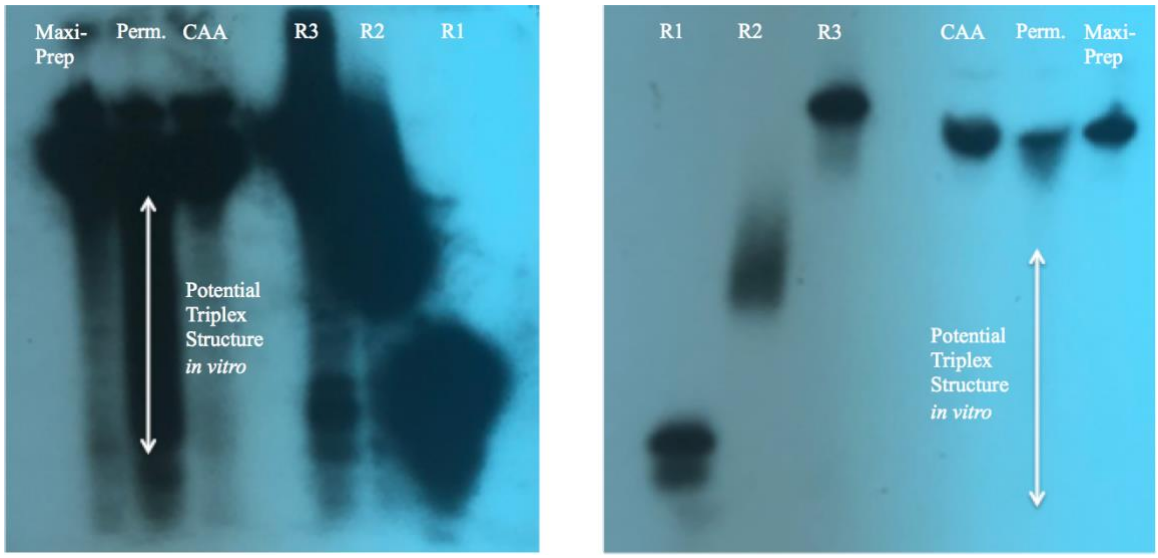

Figure 19. Analysis of Radioactive Probe Generation with Fresh Taq Polymerase and Hybridization on Membrane at Different Exposure Durations. a) The labeled film above describes the lanes that correspond to the modified DNA samples and the control length fragments. The potential triplex structure was identified in the smeared lane containing the "Perm." sample. **b)** The membrane shows the same banding pattern as **Figure 19a**, but the film had less exposure time to the membrane to prevent intense smearing.

According to the films above, the increased generation of the radioactive probe combined with the long exposure time of approximately 48 hours resulted in the intense smearing of the DNA samples that could not provide conclusive evidence of a triplex structure as contrasted to the expected results of individual bands that should appear for the chemically modified samples. However, the intense smearing of the lane containing the sample modified with potassium permanganate has the potential of corresponding to a triplex structure formed *in vitro*. However, better resolution was required to be able to observe individual bands within the lanes rather than smearing as a result of the fusion of the bands. Because of the intense smearing of the film, the exposure time was decreased to approximately two hours with the same membrane to improve the clarity of the film. The decreased exposure time produced the following results shown below

(**Figure 19b**). The film shows much less smearing with these conditions, but the individual bands that would correspond to the triplex structure could not be visualized within the context of this experiment.

DISCUSSION

Biotin Label Condition Testing

The initial condition testing for these experiments consisted of analysis of the functionality of the reagents utilized in the biotin labeling precipitation reaction. Analysis of the process and of these reagents leads to the conclusion that the reagents were likely to be functional, which led to analysis of the plasmid containing the GAA_{57} trinucleotide repeat sequence. As stated previously, the plasmid contains not only the trinucleotide repeat sequences, but also flanking sequences that contain the *ampR* gene, an SV40 origin of replication, and other genes and biological markers. Another study conducted by Potaman *et al.* in 2004 analyzed the presence of intramolecular triplex structures within recombinant plasmids with varying microsatellite repeat lengths.[30] However, in that experiment, the plasmids contained flanking AT-repeat sequences that flanked the trinucleotide repeat sequences, whereas the pUCNEO plasmid used throughout this experiment did not contain such sequences.[30] The purpose of utilizing the A-T flanking sequences in those plasmids to promote formation and stability of the triplex structure that would likely develop in those regions of the plasmids.[30]

To test the latter conclusion once again, digestion reactions were performed on the CTT10 pUCNEO plasmid, diluted, and spotted on several strips of nylon membrane to be cross-linked to varying degrees. As indicated previously, none of the membranes had any precipitation on them. Because neither the PCR-amplified GAA_{57} sequence nor the digested CTT10

pUCNEO plasmid had generated any precipitated spots, it was concluded that the biotin label was no longer functional and that a new label needed to be purchased.

Testing of Digestion Reaction Conditions

While the new biotin label was in the process of being acquired, the conditions of various digestion reactions were tested that would later be used when trinucleotide repeat sequence would be successfully amplified and ready to be chemically modified and examined. Various buffer conditions were tested for XhoI, HindIII, and KpnI restriction enzymes over the course of two separate experiments in which the results were combined to produce clear, concise conclusions. According to **Figure 3a**, during the first experiment in which XhoI and HindIII were mixed in Buffer G (Thermo Fisher Scientific, Inc.) and KpnI was mixed in 1X KpnI Buffer (New England Biolabs, Inc.), only the HindIII digestion enzyme was successful in digesting the GAA_{57} template, as it produced expected fragment lengths of 670 bp and 93 bp based on the digestion of the GAA_{57} repeat sequence alone (**Figures 1-3b**). On the flip side, however, the XhoI and KpnI were not functional in digesting the plasmid as both enzymes yield the exact same banding pattern, which correspond to the open-circle and supercoiled conformations of the plasmid, but not to an actual digestion of the plasmid (**Figure 3**).

For the next experiment, two different XhoI enzymes were placed in Buffer D (Thermo Fisher Scientific, Inc.) and Cutsmart Buffer (New England Biolabs, Inc.). Because XhoI has only one cut site in the GAA_{57} repeat sequence, the presence of one band found in both conditions lead to the conclusion that XhoI is functional in both Buffer D and Cutsmart Buffer. The KpnI enzyme was going to be tested again in FastDigest Buffer (Thermo Fisher Scientific,

Inc.), but the reaction was halted before it was analyzed in the agarose gel because further analysis of the GAA_{57} cut site map indicated that KpnI does not have a cut site in the sequence and therefore would not be functional in any given condition or context in this particular experiment.

Testing PCR Reagents on GAA_{57} Template

As stated previously, the initial biotin-labeling experiment yielded two conclusions: There was an error in the PCR reagents, or there was an error in the detection protocol. Because it was found that the kit reagents are functional, the PCR reagents were tested on the GAA_{57} template. The initial condition testing involved the utilization of the ScaI primer and the 5' reverse primer in the amplification of the GAA_{57} template via two different Taq polymerases and the analysis of the two PCR products in an agarose gel. Because this experimental set up failed to obtain a visible PCR product within the agarose gel, many experimental conditions were altered and manipulated, primarly the combinations of primers, until a freshly obtained solution of free nucleotides was implemented into the experiment, which resulted in the amplification of the GAA fragment and flanking sequences that can be observed in **Figure 4**. In that particular reaction, both the ScaI and 5' forward primers were used in combination with the 5' reverse primer, respectively, in order to aid in the amplification of the target sequence. This result leads to the conclusion that regardless of the previous primer combinations, the PCR reaction continued to fail because the 10mM dNTP solution used in each of the previous reactions had expired and therefore were no longer functional. This conclusion also provided valuable hindsight into the initial biotin-label detection experiment. The lack of

precipitation found on the initial membrane was due to the fact that the non-functional dNTP solution prevented the PCR products from being produced in the first place, meaning there was no viable DNA on the membrane to begin with.

In the study conducted by Potaman *et al.*, the target genomic DNA sequences were amplified via PCR with primers that contained the recognition sequences for the EcoRI and BamHI restriction enzymes.[30] Within the context of that experiment, the purpose of utilizing those primers was to implement those recognition sequences in the resulting plasmids that would properly digest the entire trinucleotide repeat sequence and any desired flanking sequences for in-depth analysis.[30] Within the context of this experiment, the ScaI forward primer likely contained the ScaI recognition sequence that would have allowed the ScaI restriction enzyme to digest the GAA_{57} sequence and flanking sequences from the pUCNEO plasmid.

Now that the primary cause of the PCR failure was determined, the incorporation of the biotin label must be tested to determine if the label had any interference with the success of the PCR reaction. To test this condition, a fresh 10mM solution of dATP, dGTP, and dCTP from the new reagents were created and incorporated into the PCR reaction with the new Biotin-11-dUTP label, and the amplified product was analyzed in an agarose gel. According to **Figure 5**, the PCR product was clearly visualized in the agarose gel, indicating that the biotin label was successfully incorporated into the PCR product and it had no negative effects on the progression of the PCR reaction.

Biotin Detection Protocol with Tested PCR Conditions

Now that the PCR conditions have been properly identified and it has been determined that the PCR amplification is successful with the biotin label, the reagents of the detection kit #K0662 needed to be re-examined and analyzed to determine the optimal detection conditions that would lead to the most sensitive and definitive conclusions for future experiments.

Because the entire PCR product was analyzed on the agarose gel, the product was eluted from the agarose gel and underwent serial dilutions up to a dilution factor of 10_4. The diluted and undiluted PCR products were spotted on three membranes that were cross-linked at varying conditions to determine the optimal cross-linking condition that would yield the most conclusive results. According to **Figure 6**, all three membranes had spots that were produced on only the undiluted and the 10_1 diluted samples, with the remaining subsequent dilutions not producing spots that could be detected by the naked eye. Though precipitated DNA samples were analyzed on the membrane, it was previously expected that the more diluted samples would produce visible spots, but the lack of spots was attributed to the likelihood that the biotin label was not fully functional, but functional enough to carry out subsequent experiments. In addition, because the 10_1 diluted spot on the membrane cross-linked at 800 μjoules × 100 was the brightest relative to the other two membranes, it was concluded that the optimal cross-linking condition was 800 μjoules × 100 and all subsequent membranes would be cross-linked at that energy level throughout the duration of the study.

The final test that needed to be used to determine the optimal biotin-labeling condition was whether or not the label is more prominent with or without the presence of dTTP in the reaction solution. To test this, three PCR reactions were created with one utilizing the old biotin label, one utilizing the new label without dTTP, and one utilizing the new label without dTTP.

According to the results found in **Figure 7**, all three membranes had spots that were visible for the undiluted, 1:10 diluted, and 1:100 diluted PCR products. However, the old biotin label produced precipitated DNA spots that were not as intense as the ones produced by the new label, indicating that the newer biotin label was more effective and easier to visualize overall. In addition, all three membranes had no visible spots presented for the 10_4 or 10_5 diluted samples. Finally, the labeling condition that utilized the new biotin label without the presence of dTTP was the only condition that yielded a faint, yet visible spot for the 10_3-diluted sample. Therefore, it was concluded from this experiment the biotin detection protocol works best when the label is not competing with the dTTP nucleotide for incorporation in the resulting PCR product.

Analysis of Chemically Modified DNA Samples on with Digested Length Controls

With all of the conditions for PCR and the biotin detection protocol tested and properly determined, proper identification of the triplex structure could be tested with the chemically modified samples. To aid in the proper identification of the triplex structure *in vitro*, digested length controls were created with XhoI, HindIII, and EcoO109I, respectively. According to the GAA_{57} cut site map, the restriction enzymes would digest the plasmid in such a manner that would keep the trinucleotide repeat sequence intact, and the triplex structure would theoretically occur between the XhoI cut site and the HindIII cut site (**Figure 1**).

Once the digestion length controls were generated, primer extension reactions were performed on GAA_{57} DNA samples that had already been chemically modified with chloroacetaldehyde and potassium permanganate, and the maxi-prep DNA sample that served as the negative control throughout this study. The chemically modified samples and the digested

length control ran through a polyacrylamide gel, were transferred onto a nylon membrane, and were subjected to the detection protocol (**Figure 8**). According to the membrane image, because the digested length controls were amplified with Taq DNA polymerase and the chemically modified samples were amplified with Klenow Taq, the triplex structure could not be identified on the membrane. This led to further experimentation to determine the nature of Taq DNA polymerase vs. Klenow Taq on the chemically modified samples (**Figure 9**). These experiments lead to the conclusion that Klenow Taq was ineffective at replicating the PCR target product through the chemically modified bases and the modified biotin label of the DNA samples. All experimentation with these digested length controls and the previously made chemically modified DNA samples were inconclusive and ineffective in properly identifying the triplex structure *in vitro*.

According to the previous study conducted by Potaman *et al.*, the results of that study led to the conclusion that once polymerase encounters a stall in the replication fork, the enzyme can unbind and bind back to that particular DNA structure in order to continue replication and diminish the incidence of replication stalling.[30] Based on these results and the banding pattern that resulted in **Figure 9**, one possible conclusion for the banding pattern is that once Taq polymerase encountered the potential triplex structure at the modified bases, the enzyme could have continued to replicate the sequence by unbinding and rebinding to the trinucleotide repeat sequence, or by simply replicating through the modified bases. Theoretically, based on the conclusions drawn in the previous study, this act of unbinding and rebinding could lead to the expansion of the GAA trinucleotide repeat because the replicated DNA sequence would increase in the number of base pairs as a result of the rebinding of the polymerase to that sequence.[30] Therefore, within the context of this experiment, an important experimental implication is that

Klenow Taq would not be a suitable enzyme because the lower activity of the polymerase compared to DNA Taq polymerase would prevent the enzyme from rebinding and thus expanding the trinucleotide repeat sequence.

Design and Utilization of Oligo Primers for Trinucleotide Repeat

Because the experiments involving the digested length controls and the chemically modified samples were not yielding conclusive results, the plasmid map of the GAA_{57} microsatellite repeat and the flanking sequences were analyzed in the development of new oligo primers that would be more effective at amplifying length controls that would provide more conclusive evidence of a triplex structure forming proximally to the GAA_{57} repeat sequence. According to the annotated sequence of the trinucleotide repeat and the flanking sequences, the oligo forward primer was designed to bind to the negative-sense strand of the plasmid approximately 100 bp upstream of the GAA_{57} repeat sequence. The first reverse primer (referred to as the "R1" primer) was designed to bind to the positive-sense strand directly upstream of the trinucleotide repeat sequence. The R2 primer was designed to bind to the positive-sense strand approximately 3-30 bp downstream of the repeat sequence, and the R3 primer was designed to bind to the positive-sense strand approximately 50-98 bp downstream of the trinucleotide repeat sequence at the EcoO109I cut site of the sequence. Together, these primers would be used to generate three length control fragments (R1 fragment, R2 fragment, and R3 fragment, respectively) that would be used to flank the triplex structure and potentially provide more definitive evidence of a triplex structure formed within the chemically modified samples.

To determine the success in the development of the control fragments, each fragment was analyzed in a 1.5% agarose gel (**Figure 11**). The lengths of the fragments are 100 bp, 299 bp, and 368 bp, respectively, which was identified on the agarose gel in relation to the DNA ladder, indicating that the fragments were successfully created with the biotin label incorporated within the fragments.

Once the fragments were successfully generated, they were run on a polyacrylamide gel as controls alongside the chemically modified samples and the maxi-prep samples that were prepared with Taq DNA polymerase and Klenow Taq, respectively. The contents were then transferred to a membrane and underwent the detection protocol (**Figure 13**). According to the membrane image, a potential triplex structure was properly identified in the chemically modified samples that were generated using the Taq polymerase. However, the biotin label in this experimental scenario was not sensitive enough, which lead to the use of another detection method that would generate bands at a higher sensitivity for easier visualization of the triplex structure.

Radioactive Detection

For easier visualization of the triplex structure, instead of using the biotin label, a more sensitive radioactive probe would be generated and developed on an x-ray film. This technique was also utilized in the study conducted by Potaman *et al.*, as the chemical probe analysis was performed with radiolabeled primers that served as the signal to analyze the banding pattern of the plasmids.[30] Beforehand, however, new chemically modified samples were generated as it was hypothesized that the creation of these new samples would provide more prominent bands on the

membrane to also aid in detection of the triplex structure. The samples were developed and the digestion of the chemically modified samples with ScaI-HF and EcoO109I was analyzed on a 1.5% agarose gel (**Figures 15**). The digestions were analyzed based on the predicted fragment sizes and numbers generated from the cut site map of the pUCNEO plasmid with ScaI-HF and EcoO109I (**Figure 14**). According to the initial digestion of the three modified samples, the presence of three bands for the permanganate and maxi-prep DNA samples indicate a successful and complete digestion. However, the chloroacetaldehyde sample needed to be re-generated and digested due to the high concentration of chloroacetaldehyde that initially degraded the DNA sample. Regeneration and digestion of the sample was later proved to be successful.

Once the radioactive probe was generated using the R1 fragment as the template, the membrane that contained the control fragments and the fresh chemically modified samples were hybridized with the probe and the contents were analyzed on x-ray film (**Figure 16**). According to the film, the detection of the triplex structure could not be heavily analyzed, which was attributed to the fact that the radioactive nucleotide had too little concentration in the reaction mixture and therefore interrupted with the development of the probe. This assumption was made based on the lack of individual nucleotides present within the banding patterns of the chemically modified samples (**Figure 16**).

To combat this problem, the R1 fragment was amplified via PCR to serve as a more viable template to produce more probes that would be able to generate brighter bands on the film for easier detection of the triplex structure. The newly generated probe was hybridized with the same membrane and developed on another film (**Figure 17**). Once again, the triplex structure could not be properly identified due to the smearing of the length controls and the lack of individual nucleotides present within the chemically modified sample lanes (**Figure 17**). Based

on the results present in the study conducted by Potaman *et al.*, within the lanes that contain the chemically modified samples, the evidence of a triplex structure would be properly identified by the visualization of individual nucleotide banding pattern in those lanes due to replication stalling at various chemically modified bases throughout the entire target sequence.[30] However, within the context of this experiment, the lack of those individual bands proves difficult for the observation of a triplex structure *in vitro*.

The results of this film prompted the development of the same membrane on a different type of x-ray film as a means of trying to increase the sensitivity and clarity of the bands bound to the radioactive probe. The contents of the membrane on the new film was developed (**Figure 18**) in which the smearing of the length controls increased and once again made it difficult to properly identify the triplex structure *in vitro*. However, though a definitive triplex structure could not be identified, the intense smearing of the samples, particularly the fusion of many bands within the "Perm." lane suggests that upon optimal resolution, a triplex structure could be identified within that region. There were two conclusions that were drawn from these consistent results: The biotin label in the R1 fragment were interfering with the development of the probe, and/or the Taq DNA polymerase was not fully functional and had difficulty in developing the probe.

To troubleshoot both of these issues simultaneously, a new R1 length control was developed without the presence of the biotin label and with a fresh sample of Taq DNA polymerase, which was also used to generate the radioactive probe. The fresh Taq was highly successful in the generation of a radioactive probe as shown in **Figure 19a**, where the excess probe and high exposure time resulted in intense smearing of the DNA samples developed onto the film. The identification of a triplex structure *in vitro* would require the presence of

individual bands found within the chemically modified samples that would prevent replication events from proceeding further, but the smearing of the samples prevented the identification.

To potentially bypass this issue, it was hypothesized that decreasing the exposure of the film to the membrane would decrease the smearing and allow for more clear observations of the samples. According to the resulting film (**Figure 19b**), the smearing was significantly decreased to allow for increased analysis of the contents of the film. However, the expected bands that would correspond to the triplex structure were not present in the chemically modified samples. Based on this result, it was theorized that before the transfer occurred between the gel and the membrane occurred, the bands diffused in the gel, which would cause the bands to become smeared and difficult to visualize due to the smaller size of the observed fragments.

The results above highly suggest that upon chemical modification of the DNA sequence with potassium permanganate, a potential triplex structure *in vitro* could be identified with the optimal sensitivity conditions of the radioactive or non-radioactive detection method of analyzing the contents of the polyacrylamide gel. Further study on this particular subject area would require conclusive evidence of a triplex structure present *in vitro* located in the pUCNEO plasmid proximal to the GAA trinucleotide repeat sequence. Upon proper identification of the triplex structure, the next step would be to analyze the formation of the same triplex structure *in vivo* via transformation of the pUCNEO plasmid into mammalian fibroblast cells.

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ACADEMIC VITA

Zachary T. Pettoruto

ztp5031@psu.edu

EDUCATION

THE PENNSYLVANIA STATE UNIVERSITY — **University Park, PA**
Schreyer Honors College — *Class of 2020*
Eberly College of Science | Pre-Major

AVON GROVE HIGH SCHOOL — **West Grove, PA**
Graduated: June 2016

EXPERIENCE

GLOBAL YOUTH SUMMIT ON THE FUTURE OF MEDICINE — **Brandeis University, Boston, MA**
Participant/Delegate — *June 2015*

- Visited Koch Institute and Broad Institute and gained hands-on experience learning about medical research
- Attended medical symposia given by health leaders in their respected fields on diverse medical topics
- Networked with health professionals and professors on staff at Harvard Medical School
- Collaborated with other delegates on the possibility for developing a future technological innovation dealing with the development of medicine

DENTAL SHADOWING EXPERIENCE — **State College, PA**
Observer — *May 2019 – August 2019*

- Observed many dental procedures on a consistent basis, including root canals, extractions, crown restorations, and sedations, among others
- Learned about the relationships and interactions between the health professional and the patients he/she cares for

INDEPENDENT RESEARCH LAB — **State College, PA**
Researcher — *May 2019 – May 2020*

- Performed many laboratory techniques, such as pipetting microscopic amounts of reagents, performed Polymerase Chain Reactions, developed agarose and polyacrylamide gels, and other techniques to achieve the experimental goal
- Worked with my research professor extensively to troubleshoot potential issues and draw conclusions based on experimental results to determine the presence of a triplex structure *in vitro* caused by a trinucleotide repeat sequence

LEADERSHIP AND ACTIVITIES

SCHREYER HONORS COLLEGE ORIENTATION — **University Park, PA**
Team Leader – Community Builders & Finale Collaboration Committee — *January 2019 – August 2019*

- Coordinated the development of many of the activities and logistical events that occurred during the Schreyer Honors College Orientation, such as the SHO Time Finale and various community-builder activities designed to encourage engagement among the incoming scholars
- Conveyed my previous experience as a SHO Time Mentor as well as important SHO Time information and updates to best prepare my committee of mentors for the three-day orientation with their various orientation groups

PHI ETA SIGMA HONOR SOCIETY — **University Park, PA**
Primary THON Chair — *March 2017 – March 2018*

- Worked with three other THON chairs about fundraising efforts and member engagement for the THON 2018 season
- Served as the primary source of communication between the Communications/Donor & Alumni Relations liaisons and the members of Phi Eta Sigma

THON 2019 COMMUNICATIONS CAPTAIN — **University Park, PA**
Student Outreach Chair — *April 2018 – April 2019*

- Communicated with various Mini-THON programs at several high schools in order to ensure the overall success of those events and therefore, the success of THON as a whole
- Co-lead a committee of 33 dedicated THON volunteers through weekly meetings in which important information regarding THON was conveyed to my committee

SKILLS, AWARDS, & INTERESTS/ACTIVITIES

- Intermediate ability in Italian (written and oral)
- Penn State President's Freshman Award
- Avon Grove General Citizenship Award
- Avon Grove High School Italian Language Award
- Avon Grove High School Top Ten Award
- SHC Academic Excellence Scholarship