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Using CRISPR-Cas9 as a Restriction Enzyme

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USING CRISPR-CAS9 AS A RESTRICTION ENZYME

By

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ABSTRACT

Restriction digests are a commonly utilized process for cleaving DNA at specific, but relatively common sites. Restriction enzymes have widespread use in DNA manipulation. CRISPR/Cas9 is a recently identified endonuclease which utilizes a customizable guide sequence to recognize and cut specific ~20 bp sites located in a DNA sequence. This preliminary research aimed to exploit the potential benefit of DNA restriction using the CRISPR/Cas9 procedure through alterations of different components involved in that system. We sought to refine existing CRISPR/Cas9 protocols and make a budget friendly, user-selectable CRISPR/Cas9 restriction digest protocol. The motivation for this research was to simplify and adapt known CRISPR protocols in hopes of using CRISPR as a targeted restriction enzyme. This project yielded negative results, however, important insights into the dilution of target sequences was achieved.

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CHAPTER 1

INTRODUCTION

1.1 A Brief History of Genetic Improvement after Mendel's Research

Over 150 years ago Gregor Mendel identified segregation patterns in pea plants (Mendel 1866), leading to a revolution in our understanding of inheritance (van Dijk et al., 2018). Mendel's work was focused on determining the mathematical concepts of how plants pass on traits to progeny (Mendel 1866). Despite his incredible achievement, Gregor Mendel's work would not be rediscovered until the early 1900's (Moore, 2001). The contribution of Mendel's work would see immediate success in helping plant breeders efficiently use cross-breeding to introduce desired traits in plant varieties (Larkin, 1986). The plant variants generated from crossbreeding have increased yields in various crop types across the world over the span of the last 100 years. In North America alone, corn yield has increased from approximately 2 tonnes per hectare in 1940 to over 10 tonnes per hectare in 2014 (Ritchie and Roser, 2019) and worldwide cereal production has nearly tripled over the course of 1961 to 2014. Though past (and present) biological research has drawn extensively from the findings of Mendel (Figure 1.1), there are limitations to the cross-breeding of plants. While cross-breeding is faster than trait selection in plants, the long time and high expense it may take to get "pure lines" with desired traits is still a major concern (Larkin, 1986). The biggest drawback to using cross-breeding is that the

number of traits that can be introduced in new lines of plants is limited by the amount of genetic variation available in the plant species as a whole (Vlaams Institute voor Biotechnologie, 2018).

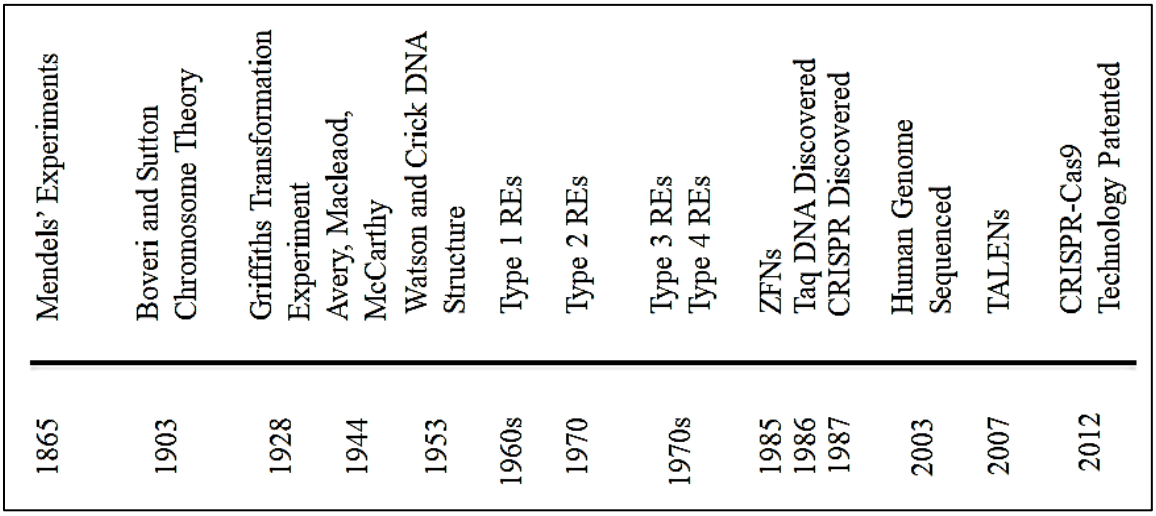

Figure 1.1. Timeline of restriction enzymes' discovery with general historical landmarks.

Through the years 1889 to 1907 Thomas Boveri carried out a series of experiments investigating sea urchins and their chromosomes (Boveri, 1889, 1902, 1905, 1907). The result of Boveri's research was a better understanding of how important chromosomes are in embryonic development (Laubichler and Davidson, 2008). In 1902 Walter Sutton published a paper about his work with grasshoppers and their chromosomes where he submitted his revelations regarding chromosomes (Sutton 1902). In 1903 Sutton published another paper which divulged more about the significance of chromosomes and helped further establish a connection between Mendel's thoughts on heredity and chromosomes (Sutton 1903). As a result of their contributions the Boveri-Sutton chromosome theory took shape (O'Connor and Miko, 2008). In 1910, Thomas Hunt Morgan carried out a series of experiments investigating fruit fly traits (Morgan

1910). In a short amount of time his experiments yielded the evidence needed to establish the link between chromosomes and heredity (Morgan, 1910). With Morgan's contribution confirming what Boveri and Sutton had postulated years prior, the theory of chromosome inheritance was developed (Gleason, 2017).

In 1928 Frederick Griffith carried out a series of experiments with bacteria of the pneumococcal species; these experiments showed that bacteria could acquire new genetic information through a "transformation factor" (Griffith, 1928). Griffith's experiment served as a basis for an experiment conducted by Oswald Avery, Colin Macleaod, and MacLynn McCarty in 1943; this experiment pointed to DNA as the genetic material (Avery et al., 1943). In 1953 James Watson and Francis Crick discovered the structure of DNA by analyzing pre-existing x-ray diffraction results and building models of DNA (Watson and Crick 1953).

In the late 1950's and into the early 1960's Francis Crick proposed a theory as to how the relationship between DNA to RNA and RNA to proteins could function. This relationship was referred to as the central dogma of biology (Crick, 1958). His theory was shown to be true and this relationship amongst genetic material is a key factor in understanding bio-molecular relationships (Figure 1.2).

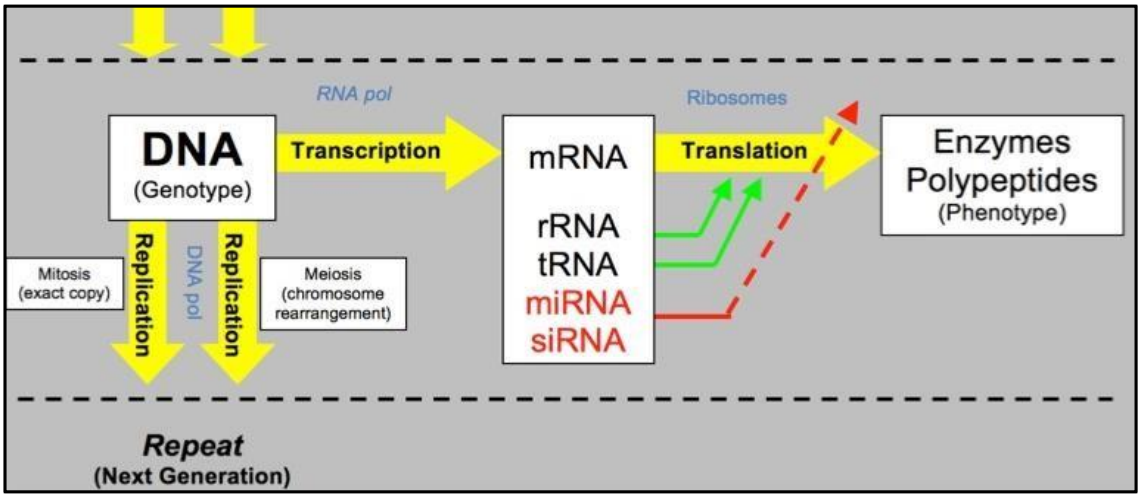

Figure 1.2. A diagram of the Central Dogma. *Provided by Dr. Shultz.*

The onset of genetic engineering began with research into recombinant DNA in the late 1960's (Jackson et al., 1972). The first experiment utilizing recombinant DNA was reported by David Jackson in 1971. This experiment involved the early use of restriction enzymes.

By the 1980's, genetic engineering had opened a new facet of research (Berg and Mertz, 2010) and led to the development of several tools utilized to edit gene sequences as shown in Figure 1.3. These tools include restriction enzymes, zinc finger nucleases (ZFNs), transcription activator-like endonucleases (TALENs), and clustered regularly interspaced short palindromic repeats/ CRISPR associated protein systems (Amitai et al., 2016).

Figure 1.3. The different types of restriction enzymes; ZFN and TALEN sequences specificity relates to specific triplet or limited specific bp sequences respectively. Pink highlights represent binding sites for indicated restricition enzyme or endonuclease. Bold lines indicate cleavage sites. "M" indicates DNA methylation.

1.2 Restriction Enzymes

Restriction enzymes are defensive mechanisms that help protect bacterial DNA from being altered by viral DNA. This is accomplished by compromising the viral DNA through cleavage (Loenen et al., 2014). Restriction enzymes were initially discovered in the 1950's (Roberts, 2005) but were not truly understood until the 1960's (Loenen et al., 2014). Studies investigated interactions between the phage Lambda and bacterial DNA, revealing that bacteria exposed to bacteriophages could use restriction enzymes to defend against a viral attack. Initial studies observed that bacteriophages had varying degrees of success when attacking different strains of the same type of bacteria (Roberts, 2005). The varying success of the bacteriophage was predicted to be the result of enzymatic reactions that modify DNA (Roberts, 2005). The next significant study saw the purification of restriction enzymes from *E. coli* and the subsequent testing of how these enzymes select cleaving sites (Loenen et al., 2014).

There are currently 4 types of restriction enzymes (Table 1.1, Figure 1.3). Type 1 restriction enzymes were the first to be discovered and were determined to be a defense mechanism against DNA entry utilized by *E. coli* and other enteric bacteria (Loenen et al., 2014). Type 2 restriction enzymes are the broadest category and feature enzymes that recognize specific patterns in DNA and cleave at or near these sites (Pingoud et al, 2014). Type 3 enzymes have an intermediate function between type 1 and type 2 restriction enzymes (Loenen et al., 2014). Type 4 is the rarest type and only works with modified DNA sequences (Loenen et al., 2014).

Table 1.1. Types of Restriction Enzymes (Loenen et al., 2014)

Enzymes	Features
Type 1	Cleave variably, often far from the recognition site
Type 2	Cleave within or at fixed positions close to recognition site
Type 3	Cleave at fixed position outside of recognition site
Type 4	Cleave m6A, m5C, hm5C, and/or other modified DNA

1.3 Zinc Finger Nucleases and Transcription Activator-Like Endonucleases

Zinc Finger Nucleases (ZFNs) were the first chimeric endonucleases to be created and had some success when used for gene editing (Amitai and Sorek, 2016). ZFNs have a modular design featuring domains which recognize nucleotide triplets. These nucleases have a critical disadvantage that would be overcome by future nucleases, namely the specificity that must be adhered to when designing these nucleases. They are specific, but so specific that their design does not lend well to being flexible for research. Transcription Activator-Like Endonucleases (TALENs) incorporate similar technology to ZFNs (Amitai and Sorek, 2016). TALENs offer a unique improvement when compared to ZFNs because their domains match with individual nucleotides. Their inherent flexibility offers a wider array of applications and an easier system to utilize; their flexibility led to ZFNs as a research tool.

1.4 CRISPR Associated Protein/ CRISPR-Cas9

Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR associated protein (CRISPR-Cas) is a DNA editing tool that was discovered as a result of research into the ‘adaptive’ immunity of bacteria (Richter et al., 2012). This DNA-editing technology utilizes a unique guide-RNA which targets a selected DNA site through complementary base-pair alignment (Loureiro and Da Silva, 2019). Once the CRISPR-

Cas9/gRNA complex binds to the target site, a double strand break (DSB) is created in the DNA sequence (Loureiro and Da Silva, 2019). This break can be further utilized by either allowing DNA repair mechanisms to create a new sequence in this spot or by inserting a custom sequence (Loureiro and Da Silva, 2019).

The guide sequence that Cas9 utilizes is composed of CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) (Amitai and Sorek, 2016). Both crRNA and tracrRNA are necessary for CRISPR-Cas9 to successfully target a DNA sequence (Figure 1.4). These two components must work in conjunction with a protospacer adjacent motif (PAM) site located downstream from the target DNA sequence. (Amitai and Sorek, 2016). The combination of crRNA and tracrRNA can be used in a pre-created guide sequence known as single guide RNA (sgRNA). CRISPR/Cas9 has not made TALEN technology obsolete, however it has offered a viable alternative to TALENs that can be utilized in unique ways at a lower cost with simpler materials.

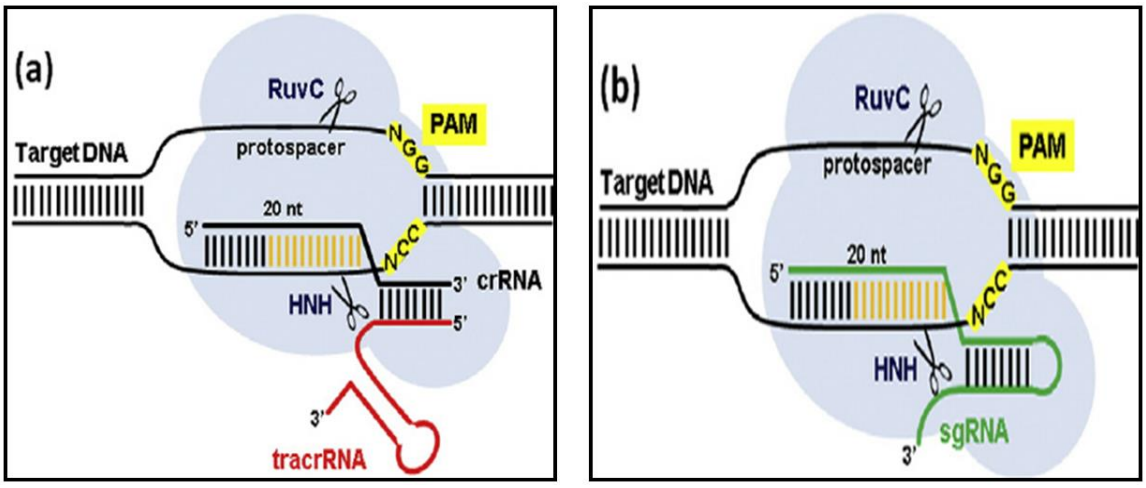

Figure 1.4. Functional components of the CRISPR-Cas9 system (Bortesi, L. and Fischer, R., 2014). Panel (a) shows the individual RNA components necessary for proper Cas9 function. Panel (b) shows the RNA components joined together into an sgRNA sequence.

1.5 Project Goals

The motivation for this research was to simplify and adapt known CRISPR protocols in hopes of using CRISPR as a targeted restriction enzyme. The developed protocol would be utilized as a molecular biology tool. CRISPR can take the basic application of restriction enzymes and improve upon that function by supplying a vast array of specific target sites that restriction enzymes do not have the flexibility to recognize. Our objective was to combine the function of a restriction enzyme with the specificity of ZFN/TALEN procedures by using CRISPR/Cas9 to knock out a specific gene. This was done in the hopes of making Cas9 more accessible and simpler to use. The simplest way of carrying out CRISPR/Cas9 reactions is by manipulating/changing sgRNA sequences. The major drawback of using sgRNA sequences is the cost associated with these sequences. The smallest sample size offered by IDT on their website is a 2 nmol sample of sgRNA at 195.00$ a sequence (IDT, 2019).

A total of three different hypotheses were tested within this study.

H1, A unique, user-selectable restriction digest protocol can be created for CRISPR-Cas9 by adapting and altering current CRISPR-Cas9 protocols.

H2, The designed protocol will be effective at incorporating unconventional guide sequences to be used in conjunction with Cas9.

H3, The designed CRISPR-Cas9 protocol will have increased cleavage specificity relative to standard restriction enzymes.

CHAPTER 2

MATERIALS AND METHODS

2.1 Source of DNA and Primer Design

Promega Lambda DNA (Cat. # D1501) was used in this project. Stock DNA was diluted to a 1:40 working ratio for all PCR reactions. This particular source of Lambda DNA has proven reliable through multiple iterations of Genetics and PCR Methods laboratory courses (Shultz, 2019).

A primer pair (Forward 5'- TAAATTCGCACAGCAGCAAC -3'; Reverse 5'- ACGTTTTCAGGTTGGCATTC -3') was created to amplify a 1,000bp region of the Lambda phage genome (GenBank ID: J02459.1). Lyophilized IDT primer tubes were spun down in a microcentrifuge, then TE buffer (Promega, cat. # PAV6231) was added to each primer (10 x nmol value). The primer tubes were then briefly centrifuged and placed in a 60^{o}C heat block for 60 minutes, then spun down. Next, 400uL of molecular biology grade water was pipetted into a labeled dilution tube, followed by 50uL of the left and 50uL of the right primers.

PCR reactions were prepared to test primer functionality. A master mix of 100uL GoTaq, 30uL molecular biology water, and 30uL Lambda (1:40) DNA template was created. Eighteen microliters of the master mix was aliquoted into 12 PCR tubes. Two microliters of primers were added to each PCR tube. The PCR tubes were spun down and placed into a BioRad T100 Thermal Cycler using an initial denaturing step of 5 minutes

at 95° C, 35 cycles of denaturing for 60 seconds at 95° C, annealing for 75 seconds at 60° C, and extending for 45 seconds at 72° C, and a final extending period for 5 minutes at 72° C. All samples were held at 10^{o} C until removed from the thermal cycler.

After the PCR program was run, the products were loaded in an agarose gel. The gel was run for an hour at 160V to confirm that they produced a 1000bp band. A picture of the gel was taken using UVP documentation system (BioDoc-It Imaging System, M-26). The primers were then used to create identical PCR reactions for use as stock PCR product for subsequent experiments.

2.2 CRISPOR Program

The CRISPOR program (Haeussler, M., and Concordet, J., 2019) was used to identify potential target sequences for Cas9 within the 1,000 bp PCR product and five guide sequences were selected. These five sequences were ordered as "crDNA," which is to say that they mimic traditional crRNA sequences, but have the structure of DNA (Table 2.1). These "crDNAs" were also ordered with uracil replacing the thymine (Table 2.1).

Table 2.1. crDNA Sequences

Sequence ID	Cut Site (bp)	Sequence
SeqT1	151/fw	5′-AGAGCAGAAAAAGACCTGGG-3′
SeqT2	210/fw	5′-TGAAGTCCGGCTGGAGTGAG-3′
SeqT3	404/fw	5′-GTGGGGATTGTCGGGAGTAT-3′
SeqT4	635/fw	5′-ATGCGCGGCTATGCCACCGG-3′
SeqT5	766/fw	5′-AGGTCCGGCTGCTCTGAAGG-3′
DNA Sequences with Uracil		
SeqU1	151/fw	5′-AGAGCAGAAAAAGACCUGGG-3′
SeqU2	210/fw	5′-UGAAGUCCGGCUGGAGUGAG-3′
SeqU3	404/fw	5′-GUGGGGAUUGUCGGGAGUAU-3′
SeqU4	635/fw	5′-AUGCGCGGCUAUGCCACCGG-3′
SeqU5	766/fw	5′-AGGUCCGGCUGCUCUGAAGG-3′

2.3 Origene Functionality Test

Experiment 1 was conducted by adhering closely to the steps and reagents recommended in the Origene protocol (Origene, 2019). Duplex reactions were made to combine the crDNA and tracrDNA sequences by mixing 1uL of 100uM crRNA, 1uL of 100uM tracrRNA (IDT, cat. # 1072533), and 8uL of nuclease-free duplex buffer (IDT, cat. # 11010301); this was done individually for all guide sequences used. The guide sequences used for this protocol included the following: 210, 635, 210U, and 635U (Table 2.1). The duplex reactions were heated at 95 degrees Celsius for 5 minutes and then cooled to room temperature.

Four solution types were made by diluting one of each of the following in nuclease free water: stock crDNA, stock crDNA containing Uracil, crDNA with tracrRNA, and crDNA containing Uracil with tracrRNA. Three different concentrations of the stock DNA were made using nuclease free water. The Cas9 reagent (NEB, cat. # M0386T) was diluted immediately before the experiment took place by using a mixture containing reaction buffer (NEB, cat. # B0386) diluted in nuclease free water. Each guide sequence sample was diluted from an initial concentration of 100uM to a final concentration of 1uM in nuclease free water.

The two tubes that contained both guide sequences and tracrRNA were then diluted from 10uM to 1uM by putting 1uL of the initial solution into 9uL of nuclease free water. Three DNA template tubes were prepared by diluting the original concentration of 1.264uM to the following concentrations: 100uL total solution 1uL DNA, 1.5uL DNA and 6uL DNA. There were diluted with 99uL, 98.5uL, and 94uL nuclease-free water, respectively. One buffer working solution tube was prepared by diluting the 10x buffer to

1x by using 2uL of 10x buffer and 17uL of nuclease-free water. One Cas9 working solution tube was prepared. The Cas9 (NEB, cat. # M0386T) was diluted from 20uM to 1uM in 1X buffer and 1uL of nuclease-free water. Overall, 1uL of Cas9 (NEB, cat. # M0386T), 2uL of 1x buffer, and 17uL nuclease-free water were combined and then used immediately.

In total 14 reactions tubes were made. The first 12 reactions contained either crDNA or crDNA-U and no tracrRNA was added. Four different crDNAs were used which were comprised of 2 crDNA and 2 crDNA-U sequences. Three different concentrations of DNA template were used $^{2}/_{3}$, 1, and 4 times the calculated value. The other 2 reactions were standard, but had crDNA and crDNA-U with tracrRNA added.

The reactions were prepared by mixing the following: 21uL of nuclease-free water, 2.9uL of 10X Cas9 nuclease reaction buffer, 1uL of 1uM sgRNA , 1uL of 1uM Cas9 Nuclease (NEB, cat. # M0386T), and 1uL of template DNA with one of the the three concentrations prepared. The contents were mixed and spun down in a microcentrifuge. The reactions were incubated at 37 ^{o}C for 1 hour, then heated at 65 ^{o}C for 10 minutes to deactivate the Cas9 nuclease (NEB, cat. # M0386T). Sample sizes of 12uL were taken from each reaction tube and electrophoresed in order to determine band size.

2.4 Adapted Origene Protocol

The second experiment featured the crDNA and crDNA with uracil segments used in conjunction with wtCas9 (Table 2.1, Table 2.2). The second experiment also followed the Origene protocol but featured increased concentrations of template DNA and Cas9 (NEB, cat. # M0386T). Two PCR tubes were prepared by combining 1uL of

100uM crDNA and 1uL of 100uM tracrRNA (IDT, cat. # 1072533) with 8uL of nuclease free duplex buffer (IDT, cat. # 11010301). Each tube was prepared with a unique crDNA; either 635 or 635U crDNA sequences. The duplex tubes were heated at 95 ^{o}C for 5 minutes and were cooled to room temperature.

Four guide DNA tubes were prepared next and served as dilution tubes for the sequences that were being tested (635, 635U, 210, and 210U). The guides were diluted from an initial molarity of 100uM to 1uM by pipetting 1uL of stock guide DNA into 99uL of nuclease-free water.

Two duplex working solution tubes were prepared by diluting 1uL of 10uM stock duplex solution into 9uL of nuclease-free water for a final dilution of 1uM. A Cas9 dilution tube was made by combining 2uL of 10x Cas9 reaction buffer (NEB, cat. # B0386) and 17uL of nuclease-free water. One microliter of 20uM Cas9 (NEB, cat. # M0386T) was added to the solution immediately before the reaction tubes were assembled.

Twelve reaction tubes were made. Six reactions contained 15uL of nuclease free water, 2.9uL of 10X Cas9 nuclease reaction buffer, 1uL of 1uM guide DNA, 1uL of 1uM Cas9 (NEB, cat. # M0386T), and 10uL of undiluted PCR product. These 6 reactions contained the following guides: 635, 635U, 210, 210U, 635 with tracrRNA, and 635U with tracrRNA. The second set (3X Cas9 set) was comprised of 6 reactions that contained the following: 13uL of nuclease-free water, 2.9uL of 10X Cas9 nuclease reaction buffer, 1uL of 1uM sgRNA, 3uL of 1uM Cas9 nuclease (NEB, cat. # M0386T), 10uL of undiluted PCR product. The contents were mixed and spun down in a microcentrifuge. The reactions were incubated at 37 ^{o}C for 1 hour, then heated at 65 ^{o}C for 10 minutes to

deactivate the Cas9 nuclease (NEB, cat. # M0386T). A total of 12uL were taken from each reaction tube and electrophoresed in order to determine band size.

2.5 IDT Protocol Functionality Test

Experiment 3 incorporated the Alt-R Cas9 protocol from IDT (IDT, 2019). Four duplex tubes were made by mixing crDNA, tracrRNA (IDT, cat.# 1072533) and nuclease-free duplex buffer (IDT, cat. # 11010301). The final concentration was 10uM, which was done by mixing 1uL of 100uM crDNA with 1uL of 100uM tracrRNA in 8uL of nuclease-free duplex buffer. The duplex tubes were heated at 95^{o} Celsius for 5 minutes. The duplex tubes were then cooled to room temperature. A dilution of the DNA template was then prepared using 2uL of DNA template diluted into 48uL of nuclease-free water.

The RNP complex tubes were prepared for both the NEB and IDT Cas9 tubes. A total of 8 tubes were made; 4 with NEB Cas9 (cat. # M0386T) and 4 with IDT Alt-R Cas9 (cat. # 1081058). These mixtures were prepared by mixing the crDNAs with Cas9 in PBS. The NEB Cas9 RNPs were prepared by mixing 2uL of 10uM guide DNA with 1uL of 20uM NEB Cas9 (cat. # M0386T) in 17uL of PBS. The Alt-R Cas9 tubes were made by combining 5uL of 10uM guide DNA with .8uL of 62uM Alt-R Cas9 in 44.2 uL of PBS. Both sets of tubes were incubated at room temperature for approximately 10 minutes.

A total of twelve reactions were prepared. Four of these reactions were NEB Cas9 (cat. # M0386T) and another 4 were Alt-R Cas9. The last 4 reactions varied the amount of DNA in the reaction tubes. These reactions included the following reagents: 10X reaction buffer (NEB, cat. # B0386), Cas9 RNP, DNA substrate, and nuclease-free water.

The four reactions made with NEB Cas9 (NEB, cat. # M0386T) were made with the following amounts: 1uL of 10X Cas9 reaction buffer, 1uL of 1uM NEB Cas9 RNP 1uL, 1uL of 50nM DNA substrate, and 7uL of nuclease-free water. The four reactions made with Alt-R Cas9 were made with the following amounts: 1uL of 10X Cas9 reaction buffer, 1uL of 1uM Alt-R Cas9 RNP 1uL, 1uL of 50nM DNA substrate, and 7uL of nuclease-free water. The 4 extra reactions were made with 3 times the amount of template DNA. These reactions were then incubated at 37^{o} C for 60 minutes. Each reaction had 1uL of proteinase K added before a final incubation step of 10 minutes at 56^{o}C. A total of 12uL were taken from each reaction tube and electrophoresed in order to determine band size.

2.6 Adapted IDT and Origene Protocol

The final series of reaction sets featured new guide sequences based off of the crDNA sequences used in the prior reactions (Table 2.2). These new sequences were dubbed sgDNA sequences because they included both the original crDNA sequence as well as a series of base pairs that were designed to mimic a standard tracrRNA sequence. Because these sequences contained the mimicked tracrRNA sequence, no tracrRNA was added to the final reaction sets.

Table 2.2. sgDNA Sequences

Sequence ID	Cut Site (bp)	Sequence
Seq1	210/fw	5′-UGAAGUCCGGCUGGAGUGAGGUUUUAGAGCUAGAA AUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUG AAAAAGUGGCACCGAGUCGGUGCUUU-3′
Seq2	635/fw	5′-AUGCGCGGCUAUGCCACCGGGUUUUAGAGCUAGAA AUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUG AAAAAGUGGCACCGAGUCGGUGCUUUU-3′

The first reaction sets were prepared using the IDT protocol. Tubes were prepared by putting 1uL of sgDNA 210 and sgDNA 635 into tubes labelled d210 and d635 respectively. The D210 and D635 tubes were diluted by putting 9uL of nuclease-free water into both tubes. Tubes were labelled as gCas210 and gCas635 and had 1uL of Alt-R Cas9 (IDT, cat. # 1081058) , 44uL of PBS, and 5uL of d210 and d635 respectively. All gCas tubes were incubated at room temperature for approximately 10 minutes. Tubes were prepared as follows: 210-1 with 1uL of gCas210, 210-2 with 2uL of gCas210, 210-4 with 4uL of gCas210, 210-8 with 8uL of gCas210, 635-1 with 1uL of gCas623, 635-2 with 2uL of gCas635, 635-4 with 4uL of gCas635, and 635-8 with 8uL of gCas635. Next, 2uL of nuclease-free reaction buffer (NEB, cat. # B0386) was added to each reaction tube. After this, 6uL of template DNA was added to each tube. Nuclease-free water was added to each tube in the following amounts: 11uL to 210-1 and 635-1, 10uL to 210-2 and 635-2, 8uL to 210-4 and 635-4, and 4uL to 210-8 and 635-8. These reactions were then incubated at 37^{o} C for 60 minutes. Each reaction had 1uL of proteinase K added before a final incubation step of 10 minutes at 56^{o}C.

Individual reactions were prepared by putting 1uL of sgDNA 210 and sgDNA 635 into tubes labelled d210 and d635 respectively. The D210 and D635 tubes were diluted

by adding 9uL of nuclease-free water. Tubes were labelled as gCas210 and gCas635 and had 1uL of NEB Cas9 (cat. # M0386T), 44uL of PBS, and 5uL of d210 and d635 respectively. All gCas tubes were incubated at room temperature for approximately 10 minutes. Reactions were prepared as follows: 210-1 with 1uL of gCas210, 210-2 with 2uL of gCas210, 210-4 with 4uL of gCas210, 210-8 with 8uL of gCas210, 635-1 with 1uL of gCas623, 635-2 with 2uL of gCas635, 635-4 with 4uL of gCas635, and 635-8 with 8uL of gCas635. Next, 2uL of nuclease-free reaction buffer (NEB, cat. # B0386) was added to each reaction, followed by 6uL of template DNA. Nuclease-free water was added to each tube in the following amounts: 11uL to 210-1 and 635-1, 10uL to 210-2 and 635-2, 8uL to 210-4 and 635-4, and 4uL to 210-8 and 635-8.. These reactions were then incubated at 37^{o} C for 60 minutes. Each reaction had 1uL of proteinase K added before a final incubation step of 10 minutes at 56^{o}C.

The last reaction set was prepared using the Origene protocol. Dilution tubes were labelled as d210 and d635; they had 1uL of sgDNA added to each tube. Next, 99uL of water was added to each tube. A Cas9 dilution was prepared by putting 4uL of nuclease-free reaction buffer (NEB, cat. # B0386), 34uL of water, and 2uL of Cas9 (NEB, cat. # M0386T). 1uL of sgDNA into tubes labelled: 210-1, 210-2, 210-4, 210-8, 210-12, 635-1, 635-2, 635-4, 635-8, and 635-12. A total of 8uL template DNA was added to each reaction tube, followed by 2.9uL reaction buffer. Water was added to each reaction tube in the following amounts: 1uL to 210-1 and 635-1, 2uL to 210-2 and 635-2, 4ul to 210-4 and 635-4, 8uL to 210-8 and 635-8, and 12uL to 210-12 and 635-12. The contents were mixed and spun down in a microcentrifuge. The reactions were incubated at 37 ^{o}C for 1 hour, then heated at 65 ^{o}C for 10 minutes to deactivate the Cas9 nuclease (NEB, cat. #

M0386T). Finally, a total of 12uL were taken from each reaction tube and electrophoresed in order to determine band size.

CHAPTER 3

RESULTS

3.1 Primer Design

The primer pair designed to amplify a 1,000bp region in the lambda genome amplified the desired product size. The PCR product was tested and verified via gel electrophoresis as shown in Figure 3.1. Both wells containing PCR product showed bands at the 1,000bp region.

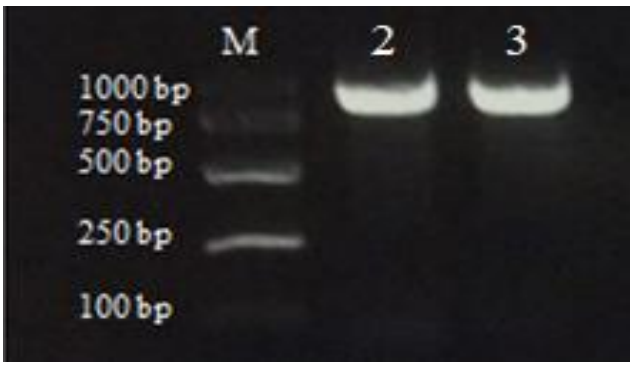

Figure 3.1. Gel electrophoresis of Lambda DNA with designed primers (target size 1000bp). Well 1 shows a size standard (indicated with "M") and wells 2 and 3 show successful PCR.

3.2 Origene Protocol Functionality Test

The first experiment using NEB Cas9 nuclease in conjunction with the Origene protocol yielded no observable results. As evident in Figure 3.2, all reactions failed to

produce a visible Cas9 restriction product. The reactions that had four times the DNA amount were visible on the gel but were very faint and still localized to the 1000bp region. The concentrated PCR product used as a size standard was very bright in every well it was present. Wells 2-4, 6-8, 10-12, and 14-16 show failed CRISPR-Cas9 reactions which utilized crDNA sequences only. Wells 18 and 19 show failed CRISPR-Cas9 reactions which utilized crDNA sequences combined with tracrRNA.

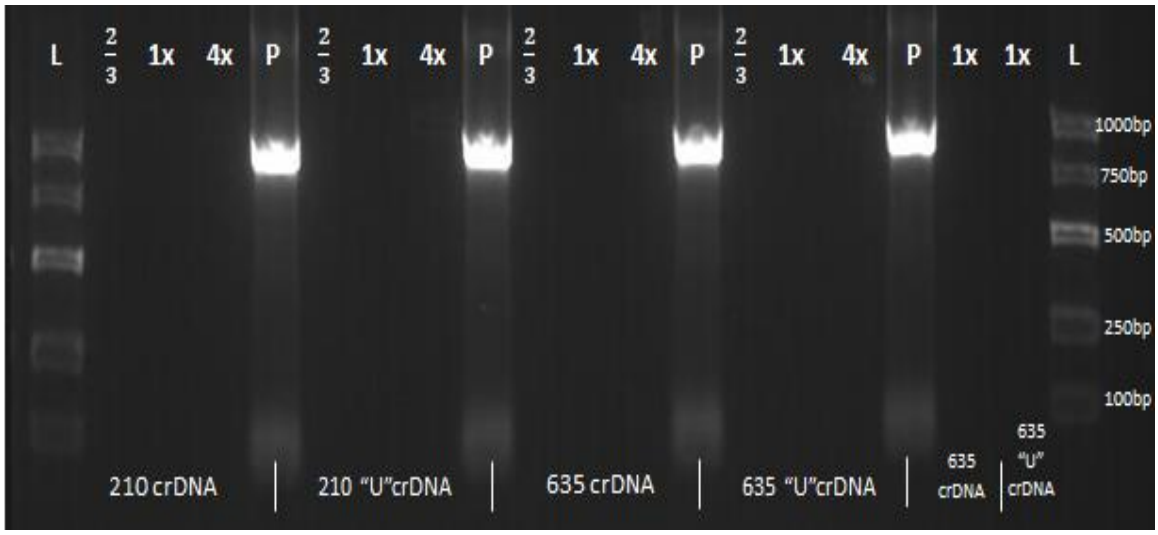

Figure 3.2. Gel electrophoresis of the Origene-based CRISPR-Cas9 protocol. Wells containing ladders are labeled with "L". Wells containing uncut, stock PCR product are labeled "P". The labels $^{2}/_{3}$,1X and 4X indicate the concentration of DNA used in the reaction. The standard concentration was 1X. Wells 2-4, 6-8, 10-12, 14-16, 18, and 19 show CRISPR/Cas9 reaction products.

3.3 Adapted Origene Protocol

The apparent dimness of the resulting DNA was further explored in the second experiment with an increased amount of DNA template (Figure 3.3). The amount of Cas9 was increased for some of these reactions in order to further test that variable. The resulting bands were as bright as the size standard controls in wells 2 and 16. Wells 3-8 and 10-15 had no discernable difference in band size or brightness. Wells 3-6 show failed

CRISPR-Cas9 reactions which utilized crDNA sequences only, but with pure, non-diluted template DNA; all other reagent concentrations remained unaltered form the original protocol. Wells 7 and 8 show failed CRISPR-Cas9 reactions which utilized crDNA sequences combined with tracrRNA; all other reagent concentrations remained unaltered form the original protocol. Wells 10-13 show failed CRISPR-Cas9 reactions which utilized crDNA sequences only, but with pure, non-diluted template DNA and 3x the amount of Cas9 reagent; all other reagent concentrations remained unaltered form the original protocol. Wells 14 and 15 show failed CRISPR-Cas9 reactions which utilized crDNA sequences combined with tracrRNA, but with pure, non-diluted template DNA and 3x the amount of Cas9 reagent; all other reagent concentrations remained unaltered from the original protocol.

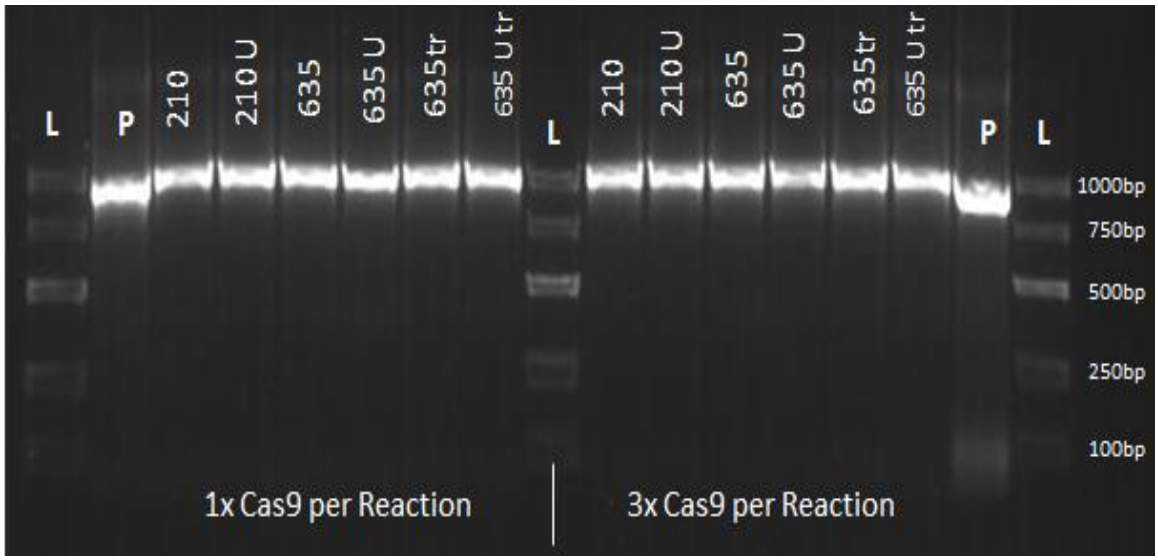

Figure 3.3. Gel electrophoresis of an adapted version of the Origene-based CRISPR-Cas9 protocol with increased template DNA concentrations and increased Cas9 reagent concentrations. Wells containing ladders are labeled with "L". Wells containing uncut, stock PCR product are labeled "P". Wells 3-6, 7, 8,10-13, 14 and 15 contain CRISPR/Cas9 reaction product. All reactions contained 10uL of template DNA.

3.4 IDT Protocol Functionality Test

In the third experiment, the bands of uncut DNA still present in the reaction gel lanes were more apparent than they had been on the gel picture for experiment 1 (Figure 3.4). The few lanes that contained reactions with 3X the amount of DNA (Lanes 11-14 Figure 3.4) showed a faint 1,000bp band. Wells 3-6 show failed CRISPR-Cas9 reactions which utilized crDNA sequences combined with trcrRNA; the Cas9 reagent used in wells 3-6 was from NEB (cat. # M0386T). Wells 7-10 show failed CRISPR-Cas9 reactions which utilized crDNA sequences combined with tracrRNA; the Alt-R Cas9 reagent used in wells 7-10 was from IDT (cat. # 1081058). Wells 11-14 show failed CRISPR-Cas9 reactions which utilized crDNA sequences combined with tracrRNA, but with 3x the amount of template DNA; all other reagent concentrations remained unaltered from the original protocol.

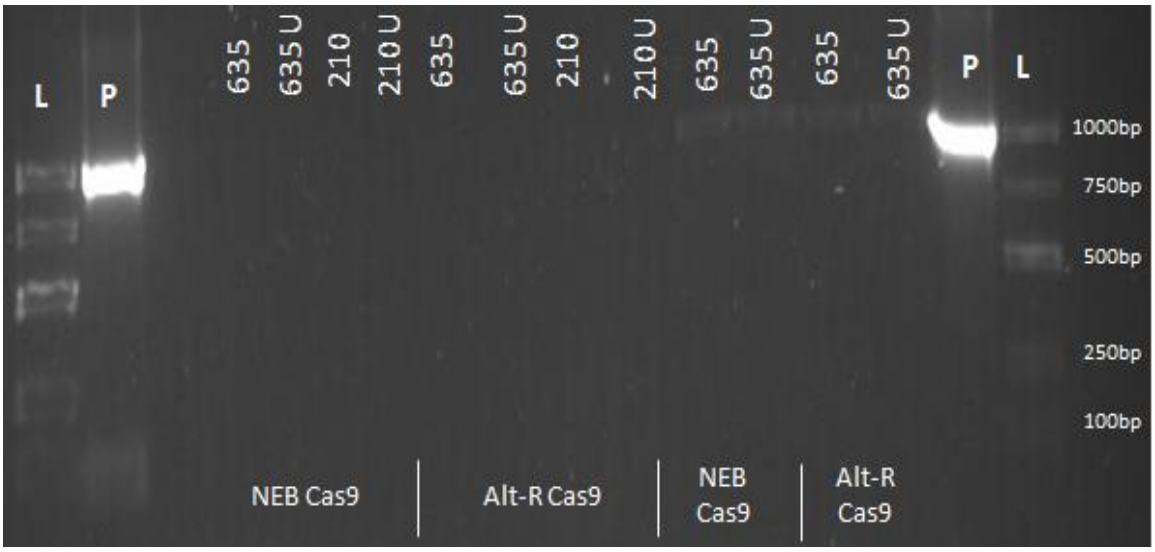

Figure 3.4. Gel electrophoresis of an adapted version of the IDT-based CRISPR-Cas9 protocol. Wells containing ladders are labeled with "L". Wells containing uncut, stock PCR product are labeled "P". Well 2 does not contain any product. Wells 3-6, 7-10, and 11-14 contain CRISPR/Cas9 reaction product. All reactions contained tracrRNA. Wells 11-14 contain 3x the amount (uL) of template DNA.

3.5 Adapted Origene and IDT Protocols

The fourth experiment reactions showed that reactions prepared with sgDNA resulted in no discernable difference from the other sets (Figure 3.5). This experiment also tested a wider variety of Cas9 amounts in each reaction; with no discernable difference in reaction results. Wells 3-10 show failed CRISPR-Cas9 reactions which utilized the IDT protocol; the Alt-R Cas9 reagent used in wells 3-10 was from IDT (cat. # 1081058). Wells 13-20 show failed CRISPR-Cas9 reactions which utilized the IDT protocol; the Cas9 reagent used in wells 13-20 was from NEB (cat. # M0386T). Wells 23-32 show failed CRISPR-Cas9 reactions which utilized the Origene protocol; the Cas9 reagent used in wells 23-32 was from NEB (cat. # M0386T).

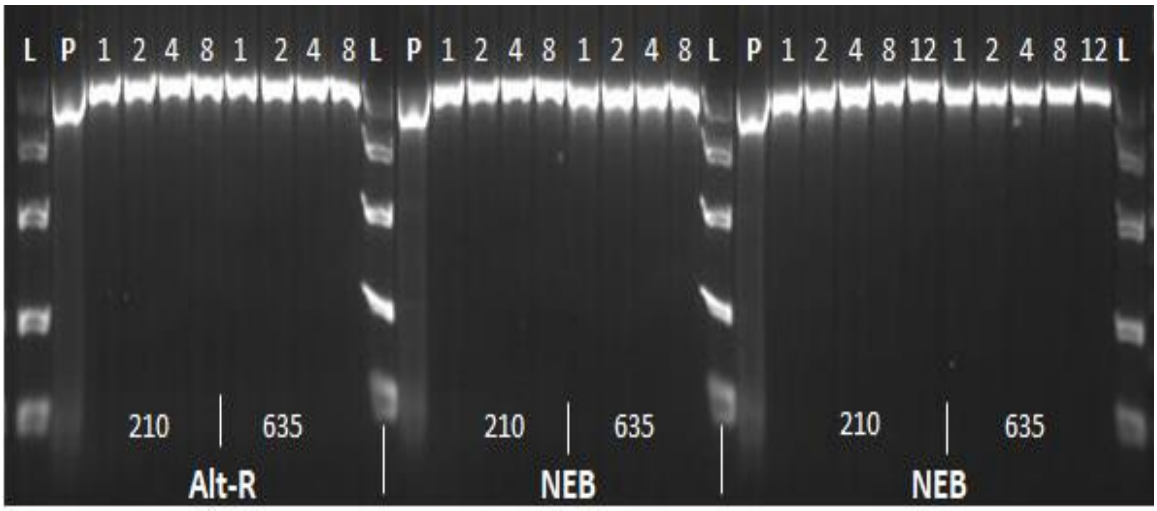

Figure 3.5. Gel electrophoresis of CRISPR/Cas9 reaction products that were made using sgDNA sequences. Wells containing ladders are labeled with "L". Wells containing uncut, stock PCR product are labeled "P". Wells 3-10, 13-20, 23-32 contained CRISPR/Cas9 reaction product. The amount of cas9 (uL) added to each reaction is listed at the top. The first two sets of reactions utilized the IDT protocol; the last set of reactions utilized the Origene protocol.

CHAPTER 4

DISCUSSION

4.1 Mendelian Genetics

CRISPR represents a unique opportunity in comparison to Mendelian methods of organism improvement. The initial goal of this research was to investigate whether CRISPR-Cas9 can knock-out dominant traits and remove the need for traditional genetic selection. Theoretically, CRISPR can knock-out any gene that has a PAM sequence in the correct location and a unique ~20bp sequence to match. Our objective was to combine the function of a restriction enzyme with the specificity of ZFN/TALEN procedures by using CRISPR/Cas9 to knock out a specific gene. Integrating these proved to be too optimistic for a single project. The first step to this process would be to devise a targetable (and distinctive) restriction digest procedure.

4.2 Restriction Enzymes

Restriction enzymes have proven to be a crucial tool in gene editing and have helped to improve the understanding of gene properties as a whole. CRISPR can take the basic application of restriction enzymes and improve upon that function by supplying a vast array of specific target sites that restriction enzymes do not have the flexibility to recognize. There were two major hurdles that this project sought to overcome – the high

cost of RNA-based sgDNA and the lack of a simple method for in-vivo restriction. This project was unable to achieve either of these difficult tasks.

4.3 Zinc Finger Nucleases and Transcription Activator-Like Endonucleases

ZFNs and TALENS have proven to be invaluable stepping stones to the development of new and improved gene altering technologies, namely CRISPR-Cas9. This research was to provide evidence to the fact that CRISPR-Cas9 has improved flexibility when compared to ZFNs or TALENS. Although all three types are usable for gene editing, the specificity of CRISPR is extremely attractive because it has the potential to be far more accessible to both experts and beginners alike.

4.4 CRISPR Associated Protein/ CRISPR-Cas9

CRISPR-Cas9 is becoming the cutting edge of genetic research. Utilization of CRISPR-Cas9 can prove challenging due to its unique method of operation; however the overall process of using it lends itself well to easy reproducibility. These initial experiments sought to bring the functionality of the CRISPR system into widespread use as a restriction enzyme. It was at this point that we made the most fundamental mistake. We had attempted to utilize commercial protocols (IDT,Origene) for the application of CRISPR enzymes. Only to realize that these protocols were designed to introduce a gene into a sequence, that use selection protocols to identify successful integration.

The problem with our solution was dilution. These protocols required the dilution of our target DNA to undetectable (on agarose) levels. Even if our experiments had worked, it would have been on such a small scale that we could never have known.

4.5 Project Outcomes

We sought to investigate three hypotheses. The first was that a user selectable restriction digest protocol could be created. The project resulted in failure to produce a simplified protocol, to successfully utilize CRISPR/Cas9 and to also make DNA sequences with Uracil replacing the Thymine bases to work with the Cas9 nuclease. For every experiment the choice was made to use crDNA sequences that were designed to produce fragments with disproportionately different sizes to enable easy visualization of a gel. If any of the digests had successfully been performed, the sizes would be roughly at 2/8 ratio or a 6/4.

The majority of the reactions from the first and third experiments had heavily diluted reagents that resulted in the decreased possibility of visualizing a successful restriction. Not only was there a decreased possibility of seeing a successful restriction, but the resulting bands of un-successfully restricted DNA were also difficult (if not impossible) to visualize. This was most likely caused by the very small amounts of template DNA included in most of the reactions.

Ideally a control would have been used to confirm the functionality of the reagents that were used. This step was initially skipped due to cost and time concerns. Ultimately, the testing of the reagents was not completed. The sheer cost associated with the traditional method of determining the functionality of reagents and then following through with subsequent troubleshooting would make this research impractical. If the expected approach was used then multiple variables would need to be tested. Comparing control Cas9 reaction with the experimental reactions would require standardization and could result in more money and time being spent than was available.

Although CRISPR/Cas9 is less specific in relation to ZFNs and TALENs, CRISPR/Cas9 has superior specificity to restriction enzymes. This research sought to compare the restriction potential of CRISPR/Cas9 to standard restriction enzymes. This project failed to show the superior specificity of CRISPR/Cas9.

CHAPTER 5

CONCLUSION

By testing the cleaving ability of CRISPR/Cas9 we hoped that a more user-friendly and cost effective method of utilizing CRISPR/Cas9 could be devised to restrict specific DNA targets. We hoped that a more accessible and easy to understand protocol could be developed and that using DNA instead of RNA as a guide sequence would dramatacially reduce cost. If it is discovered that new combinations of genetic material and Cas9 can achieve a new function, this could potentially lead to specific, user selectable restriction digest capabilities. Although the experiments did not succeed, they have led to important insights that can be utilized in the next round of research. In future experiments, the concentration of end product will be increased or the capacity to detect this product will be improved. In addition, the manipulation of provided controls will be the focus, which will provide positive functional controls.

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