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Andrew Robert Baker
Worcester Polytechnic Institute

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Single-Base Editing of Cellular mRNA by CRISPR/Cas9

*A Major Qualifying Project
submitted to the Faculty of the
Worcester Polytechnic Institute
in partial fulfillment of the requirements for the
Bachelor of Science degree in Biology/Biotechnology
and Biochemistry*

*Andrew Baker
Kimberly Coddling*

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We also owe our gratitude to the 2016 WPI iGEM team, for their previous work in the field. Because of their prior work, we were able to use several protocols, constructs, and reagents already established for the project.

Finally, we thank the entire Biology and Biotechnology department, as well as the Biochemistry department, for giving us the opportunity to complete the project, as well as funding for our necessary materials in the Project Lab.

ABSTRACT

Genetic engineering is currently dominated by CRISPR/Cas9 technology, promising precise multipurpose genome editing. The 2016 WPI iGEM team investigated the potential of adapting this technology to direct single-base editing of mRNA by linking deactivated Cas9 to the C-to-U RNA editor enzyme APOBEC1. We pursued the re-cloning and characterization of the dCas9/APOBEC fusion to identify and eliminate problems related to expression and toxicity. Ultimately, this adapted CRISPR/Cas9 system could provide an advantageous method of editing, particularly for therapeutics.

INTRODUCTION

The natural CRISPR/Cas9 system employs a bacterial adaptive immune response in which clustered regularly-interspaced short palindromic repeats (CRISPRs) are recognized by the nuclease Cas9 and cleaved for the purpose of editing the gene in an effort to ensure organism survival. This system has been specially adapted for the use of targeting virtually any position in the genome by guiding the Cas9 to recognize specific sites of double-stranded DNA (dsDNA), usually via engineered single-guideRNAs (sgRNAs) (Ran et al. 2013). A simple diagram of the mechanism of CRISPR/Cas9 technology is below:

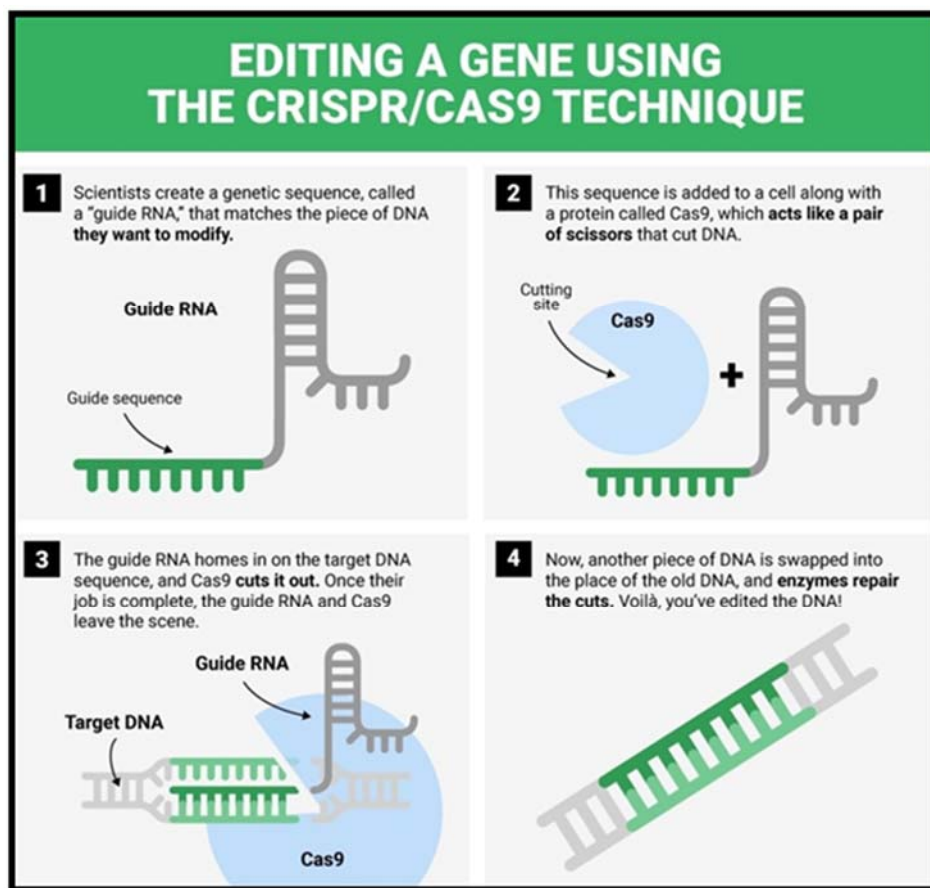


Figure 1: A Diagram of CRISPR/Cas9 Editing Technology (Lewis 2015)

Because of its natural derivation and high efficiency, the CRISPR/Cas9 system has quickly developed into a highly-regarded gene editing technology, through both recombination-based and single-base editing. In recombination-based editing, Cas9 cuts the dsDNA at a target locus, where the DNA is edited in the desired way (i.e. gene edits, knockouts), and the cell undergoes either non-homologous end joining (NHEJ) or homology-directed repair (HDR), although

typically NHEJ (Ran et al. 2013). In single-base editing, the CRISPR/Cas9 system foregoes double-stranded DNA cleavage but retains editing power through an attached DNA-editing deaminase enzyme called APOBEC, which targets specific bases to be changed to a different nucleotide, such as a C→U substitution (Komor et al. 2016).

While CRISPR technology has become a rather valuable tool in modern molecular biology, the current system is not without its issues, both technically and ethically. Biologically, Cas9's ability to cut so efficiently at specific sequences on the genomic level is what makes it such a useful tool; however, sequences similar to the target sequence are often also cut, resulting in unpredictable and irreversible off-target effects (Harrison et al. 2014). After the initial cut by Cas9, cells routinely use the NHEJ repair pathway to repair the genomic DNA. This repair mechanism is often inaccurate and leads to frequent insertions, deletions, and changes of nucleotides. These indel and single-point mutations are unpredictable and irreversible, with the potential to yield drastic, undesirable results (Harrison et al. 2014). Lastly, the extent of complex post-transcriptional modifications and splicing variation allows for a myriad of uncontrollable outcomes, due to the nature of editing primary genetic material. Our project was motivated partially due to these technical concerns, and partially due to the share of ethical and safety concerns that naturally come from genetic engineering.

In response to these concerns, one adaptation to the current CRISPR/Cas9 gene-editing system has been to target RNA instead of DNA. It has been shown that CRISPR/Cas9 can in fact be programmed to recognize single-stranded RNA (ssRNA) while avoiding the corresponding DNA sequences, and further cleave the targeted RNA in this manner (O'Connell et al. 2014). Furthermore, the CRISPR/Cas9 system has been demonstrated to be useful for live RNA tracking in vivo (Nelles et al. 2016). Finally, very recent progress by the Huang group has addressed the need to develop an mRNA-based CRISPR tool; their work validated the possibility of efficient targeting of cellular mRNA by an enzymatically dead Cas9, without affecting its corresponding DNA segments (2016).

Therefore, understanding the technical and ethical issues presented by the common system of DNA-targeting CRISPR/Cas9, having studied the precedent of RNA targeting with CRISPR/Cas9, and inspired by the foundational work of the 2016 Worcester Polytechnic Institute (WPI) iGEM team in this field, we developed an adapted CRISPR/Cas9 system that also targets mRNA (WPI_Worcester, 2016). Another significant change from the current technology is that it utilizes the DNA/RNA editing enzyme APOBEC to cause specific single-base edits.

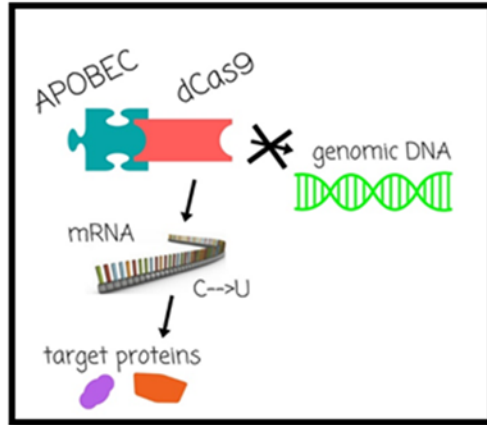


Figure 2: A Diagram of Targeted mRNA Editing by dCas9/APOBEC

In this way, we can make very precise adjustments to the mRNA of a desired organism, regulating its related protein levels, without editing a single nucleotide of DNA. Additionally, the application of this tool would be a temporary therapy; in the case of any unforeseen error or mutation, the same regulation could be easily reversed by stopping the editing of mRNA. We propose that this mRNA/CRISPR/Cas9-APOBEC system can be used for the same research and therapeutic purposes as the original CRISPR gene-editing technology, but with significantly reduced scientific and ethical risk, as well as increased accuracy.

Our project continued the foundational work done by the 2016 WPI iGEM team in its efforts to develop this promising adapted CRISPR/Cas9 therapy. Herein, we describe our attempt to re-clone the dCas9/APOBEC construct. We also describe our conclusion of the project with transfection trials of several constructs into mammalian cells.

MATERIALS & METHODS

Preparation of pcDNA3.1+

We transformed 2 uL pcDNA3.1+ template vector DNA (provided by the WPI 2016 iGEM team) into 50 uL chilled, thawed competent DH5a *e. coli* cells. They were then incubated on ice for 30 minutes, heat shocked at 42°C for 1 minute, and incubated back on ice for 5 minutes. 200 uL of SOC media was added to each 52 uL cell/DNA mix for a brief outgrowth period. The SOC media contained the following ingredients and then was sterilized and filtered: 0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, and 20 mM Glucose. The cells in the media were put into a shaking incubator at 37°C for 2 hours, then plated (in various quantities) on LB plates (made with 1 mL Ampicillin at a concentration of 100 mg/mL). These sat overnight in a 37°C incubator. Single colonies were picked for overnight cultures in 3 mL LB (w/Amp) each. These were mini-prepped using the Macherey-Nagel NucleoSpi Plasmid Mini Kit (Bethleme, PA) per the manufacturer's instructions. The mini-prep products were run through a 1% agarose gel at room-temperature and 100 Volts, visualized by SYBR (from Qiagen), then cut out and gel-purified by Macherey-Nagel Gel and PCR Clean-Up Kit per the manufacturer's instructions (Registry of Standard, n.d.). Verification gel(s) can be found in Figure 1 of the Appendix.

Amplification of dCas9 and APOBEC

dCas9: We amplified dCas9 DNA template (provided by the WPI 2016 iGEM team) via PCR reactions with Q5 master mix (New England BioLabs, Beverly, MA), using the following protocol:

1x	[95 for 02:00]
30x	[95 for 00:30]
	[59 for 00:45]
	[72 for 04:30]
1x	[72 for 10:00]
	Hold at 10 degrees for infinity

The PCR product was run in a 1% agarose gel, visualized by SYBR (from Cambrex in Rockland, Maine), cut out and gel-purified by Qiagen purification kit. Verification gel(s) can be found in the Appendix.

The temperature for annealing in the dCas9 amplification protocol was established by performing a gradient-temperature PCR reaction. We ran 12 identical reactions, each of 15 uL, spanning from 45 to 62 degrees Celsius. The brightest bands when visualized in an agarose gel by SYBR allowed us to calculate the optimal annealing temperature to be 59 degrees. For the gel results and calculation, please refer to Figure 2 of the Appendix section.

APOBEC: We amplified APOBEC with 1Xten linker, 2Xten linker, and 3Xten linker (provided by the WPI 2016 iGEM team) via PCR reactions with Q5 master mix, using the following protocol:

```
1x    [95 for 02:00]
30x   [95 for 00:30]
      [55 for 00:45]
      [72 for 01:00]
1x    [72 for 10:00]
Hold at 10 degrees for infinity
```

The PCR products were treated as described above.

Cloning of dCas9 into pcDNA

We used Gibson cloning method to attempt to clone dCas9 into our pcDNA3.1+ vector. We mixed 100 ng of gel-purified pcDNA3.1+ vector DNA with 200 ng of gel-purified dCas9 insert DNA, along 10 uL of NEBuilder HiFi DNA Assembly Master Mix and the remaining amount needed of deionized water to create a 20 uL total reaction. The reactions were incubated in a PCR machine for 30 minutes at 50°C. The reaction product was then transformed by mixing 2 uL of the product into 50 uL of thawed competent DH5α *e. coli* cells (obtained by Professor Farny's competent cell stock in the -80 degrees Celsius freezer), incubated on ice for 30 minutes, heat shocked at 42°C for 30 seconds, and put back on ice for 2 minutes. 950 uL of room temperature SOC media was added, the mix was then incubated at 37°C with vigorous shaking for 1 hour, then plated on prewarmed LB (w/Amp) plates, and incubated overnight at 37°C (Gibson, 2009 and New England BioLabs, n.d.). We further attempted to repeat this with un-gel-purified dCas9 insert DNA, all the rest remaining the same. Our control for these reactions was to plate the plasmid without insert DNA, and cells without any transformation plasmid or insert DNA (Registry of Standard, n.d.).

Transfection of dCas9/APOBEC Constructs

For transfection, we followed the protocol found with TransFectin (from Bio-Rad), a highly efficient, lipid-based transfection reagent. We plated 600,000 cells into the wells of a 6-well plate and allowed 24 hours for adherence. We then added 2 µg of DNA with 10 µL TransFectin and counted this as time = 0 hours for imaging by microscopy. We performed two transfections using this basic process.

The first transfection was conducted with H1299 cells, which is a human non-small cell lung carcinoma cell line, and was done as described above. The DNA inserted into the cells were the following constructs: APOBEC 1X, APOBEC 2X, APOBEC 3X, BE2, dCas9, and pRETRO. The control was cells with no DNA. The cells were incubated at a stable 37 degrees Celsius. After 48 hours, the cells were imaged (please see Results section).

The second transfection was conducted with MCF7 cells, a breast cancer cell line, and was done as described above, with the following exception: the cells were incubated for 4 hours before changing media, completely removing the transfection reagent. The media used was standard DMEM, 10% FBS and 1% Pen-Strep (Gibco). The following three variations were the conditions used: transfection reagent and DNA, DNA only, and transfection reagent only. The DNA added for this transfection was the BE2 construct (Addgene Plasmid #73020). The control well contained only cells. All cells were imaged at 24 hours (please see Results section).

RESULTS

The first part of our project consisted of the re-cloning of dCas9 into the pcDNA3.1+ vector, to be followed by APOBEC with the 1X, 2X, and 3X size linkers. We first amplified our dCas9 DNA through multiple PCR reactions, but rarely were able to produce enough DNA to begin molecular cloning. Because of this issue, we optimized the PCR protocol by performing a gradient-temperature PCR reaction in which the annealing temperature of the reaction was increased incrementally. Below, Figure 1 illustrates the results collected.

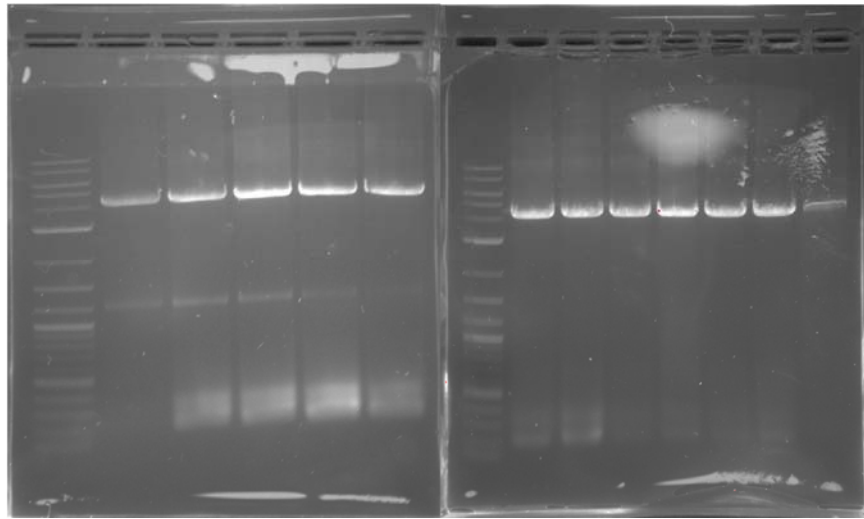


Figure 3: Gradient-Temperature PCR Gel Results

The lanes in the two gels sequentially represent reactions 1-12 of the temperature gradient, ranging from 45 to 62 degrees. The results shown in Figure 1 suggest an optimal annealing temperature between 57.78 (reaction 9) and 59.2 (reaction 10) degrees. Based on these results, the annealing temperature used in future PCRs was set to 59 degrees (full calculation can be found in Figure 2 of the Appendix). Using this modified PCR protocol, we continued amplifying the dCas9 DNA and began attempting to clone the DNA into pcDNA 3.1+ via Gibson assembly.

Inserting the dCas9 DNA into the pcDNA 3.1+ vector proved difficult for unknown reasons. We believe that because of dCas9's size (approximately 4.2 kb), the DNA was simply too big to successfully integrate into the pcDNA 3.1+ plasmid in one step. We suggest that in the future, the dCas9 gene could be split into two or three parts, amplified separately via PCR and inserted individually into the plasmid. By splitting the gene into parts, the size of the insert is dramatically reduced - possible making the cloning more successful.

Because we had little time left in our MQP to complete the cloning process and transfect with our own constructs, we had the fortune to use iGEM's previously made/purchased constructs for

investigating transfection/expression of dCas9 and APOBEC in mammalian cells. We began by first taking each individual component of the pRETRO iGEM-constructs, as well as the AddGene plasmid BE2, and transfecting H1299 mammalian cells. The table below clarifies each of the DNA constructs we added to our transfection(s):

Figure 4: Table of DNA Constructs Used in Transfection(s)

DNA Construct	Plasmid Backbone	Contents	Source
“pRETRO”	pRETRO	none	AddGene
“dCas9”	pRETRO	dCas9	iGEM
“APOBEC 1X”	pRETRO	APOBEC 1X	iGEM
“APOBEC 2X”	pRETRO	APOBEC 2X	iGEM
“APOBEC 3X”	pRETRO	APOBEC 3X	iGEM
“BE2”	pCMV	dCas9/APOBEC	AddGene

The following figure shows the images taken from the first transfection, which was done with H1299 cells:

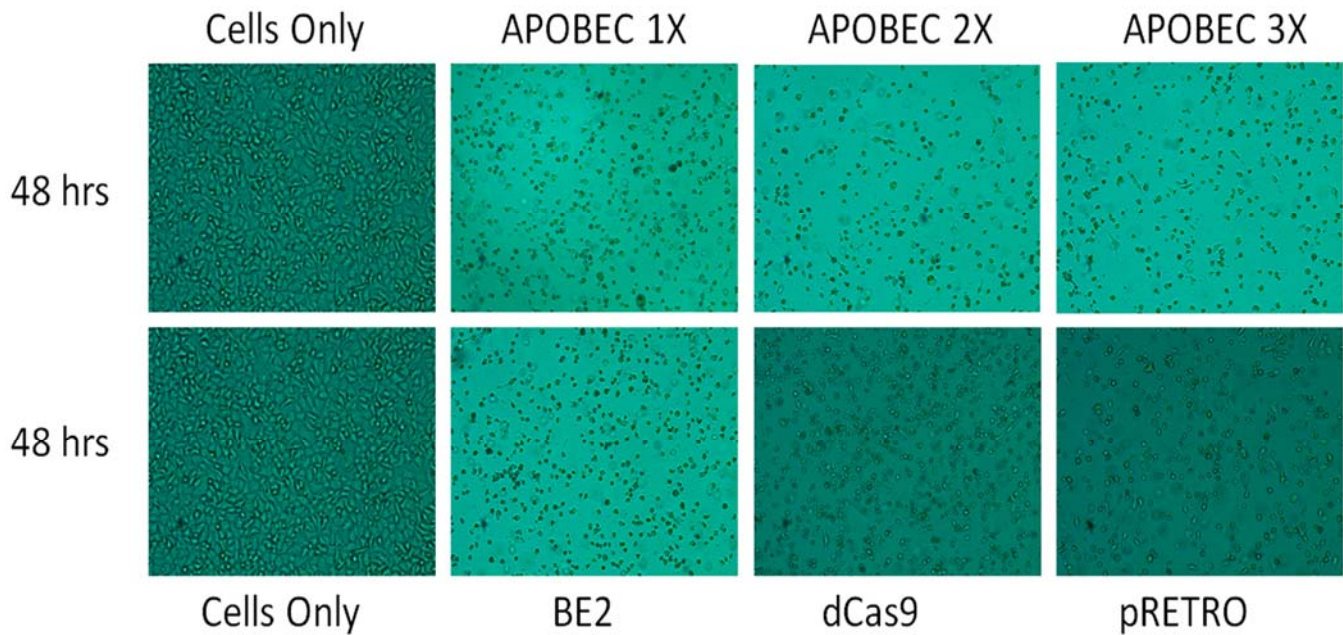


Figure 5: Cells 48 Hours Post-Transfection with Various Constructs Compared to Control

From this transfection, we realized the incidence of massive cell death in every well except for the one that contained only cells (no transfection reagent and no DNA) - specifically, after 48 hours, the cells with DNA added became suspended in the media, over 80% dead as compared to the anchored, 90% alive “cells only” well. This was an unexpected result, especially because a simple vector such as pRETRO should not theoretically cause cell death. Therefore, we wanted to determine which of the two added transfection ingredients (reagent or DNA) was the one responsible for killing the cells. To do this, we conducted a second experiment including proper controls to make a more thorough and substantiated conclusion. This second transfection involved transfecting MCF7 mammalian cells with only BE2. We decided to switch to the new cell type, MCF7, to determine if the cell type played any role in the results, and to prevent cell death if caused easily to the previous cell types tried (293T’s and H1299’s).

The following figure shows the images taken from the second transfection, which was done with MCF7 cells:

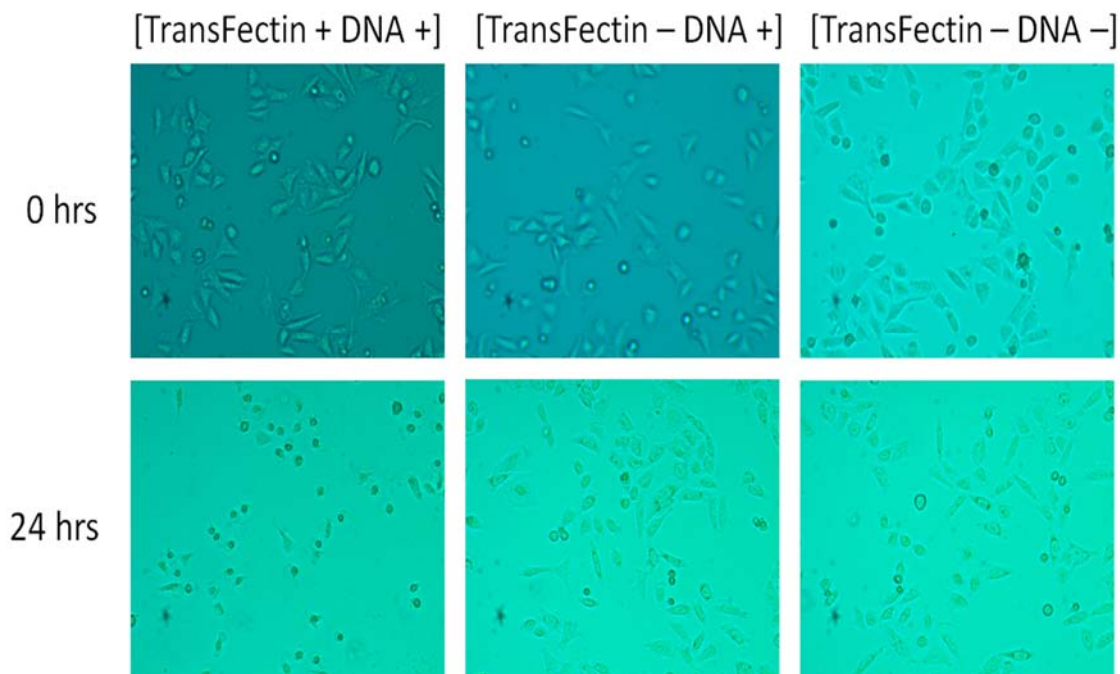


Figure 6: Cells 0 and 24 Hours Post-Transfection, Testing for Toxicity Cause

The images collected above indicate that the transfection reagent used (TransFectin) is responsible for the massive cell death observed in both Figures 2 and 3. This result could be due to a variety of reasons including: the age of the TransFectin (4+ years), or the plasmid itself. While the second column in Figure 3 indicates that the DNA is not killing the cells, the DNA is

not able to enter the cells because of the absence of TransFectin, thus it would be useful to recapitulate these results with a Transfectin-only culture. This is important to note because once TransFectin is added, the DNA forms complexes with the TransFectin and enters the cells. BE2 contains pRETRO as its plasmid backbone - a DNA sequence that originates from viruses. We suggest that cell death may be occurring in Figures 2 and 3 because of the use of pRETRO. As the DNA enters the cell, it may be integrating into the host genome and inducing apoptosis through either integrating in a non-optimal location (disrupting essential genes), or leading to interferon production in the cell which then leads to cellular apoptosis. We also recommend attempting the same transfections as above in media that does not contain antibiotic. While bacterial antibiotic, like Penn Strep, should not usually cause mammalian cell death, the increase in cell permeability via lipid-based transfection may be contributing to the observed cell death. Similarly, it is recommended that alternative transfection protocols be attempted, such as calcium-phosphate.

DISCUSSION

The foundational work completed by the iGEM team led to an exciting prospect for our MQP. It is indisputable that CRISPR technology offers boundless potential for applications ranging from medical to environmental. However, its technical concerns simultaneously present ethical concerns for the scientific community and the society at large. Firstly, accidental release of any genetically modified organism could lead to severe ecological disruptions. Any experimental organisms released into an ecosystem could quickly proliferate, spreading possibly hazardous DNA sequences throughout the ecosystem (Rodriguez 2016). A second, more economical concern, is the recent practice of patenting DNA sequences. CRISPR technology is frequently used to create and insert laboratory-produced DNA sequences that are then often used in applications for patents. This creates the ethical concern of whether it is morally right to patent genes or other DNA sequences (Rodriguez 2016). A final and most severe ethical concern associated with CRISPR is the practice of editing human genomes. Already, UNESCO has issued the Universal Declaration on the Human Genome and Human Rights which recommends a moratorium on human germline genome editing. Despite similar recommendations to avoid using tools like CRISPR on the human genome, some have argued the potential to treat a wide range of diseases, such as cystic fibrosis or sickle cell disease, through the tool (Rodriguez 2016). It is these concerns that have placed CRISPR into a negative light in terms of public opinion and have founded the need for a safer, less controversial alternative to CRISPR.

While we encountered obstacles in the lab that prevented us from progressing further in the development of this adapted CRISPR/Cas9 technology, we enjoyed the experience immensely and learned boundless skills in troubleshooting and critical thinking. We stress the importance of further developing this adapted CRISPR technology and highly encourage a future MQP team to

pick up where we left off. To this end, we have compiled a list of major routes that we suggest such a team to proceed on. We look forward to the future development of this project.

Our first major suggestion is the continued cloning of dCas9/APOBEC into the pcDNA3.1+ vector. Future persistence in these cloning aims should include APOBEC inserts of various size linkers into pcDNA3.1+, as well as into other standard and atypical vectors. Given the trouble we faced with cloning in dCas9, we propose splitting the dCas9 DNA into multiple parts and cloning them into the vector in pieces. Reducing the substantial size of the insert when cloning the DNA may facilitate uptake of the gene into a plasmid.

Our second major suggestion is determining successful alternate transfection protocols to reduce toxicity to the cells. We suggest attempting the same transfection as done above, but in media that does not contain any antibiotic (our attempt at this yielded large contamination, making the cells difficult to image- we suggest trying several concentrations/quantities of antibiotic). We also suggest transfecting the cells using plasmids that do not originate from viruses in order to reduce possible interferon production post-transfection. Finally, we also suggest finding alternate methods of transfecting the DNA into the cells, in order to accurately determine the effect of the dCas9 construct(s), such as a calcium phosphate method.

Once the dCas9/APOBEC construct(s) have successfully been cloned into an appropriate plasmid vector and the construct(s) have been transfected into mammalian cells, the next major step in the project can be continued. Firstly, one would need to verify the expression of the construct *in vivo*. Following that is to proceed with introducing guide RNA to target the CRISPR/Cas9 system for editing. Finally, conduction of RNA collection and sequencing, as well as protein analysis, would both be done to test for successful mRNA editing.

REFERENCES

- AddGene: the non-profit plasmid repository. Accessed September 2016.
<https://www.addgene.org/>
- Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A., & Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature methods*, 6(5), 343-345.
- Harrison, M. M., Jenkins, B. V., O'Connor-Giles, K. M., & Wildonger, J. (2014). A CRISPR view of development. *Genes & development*, 28(17), 1859-1872.
- Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A., & Liu, D. R. (2016). Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature*.
- Lewis, Tanya (2015). Scientists may soon be able to 'cut and paste' DNA to cure deadly disease and design perfect babies. *Business Insider: Science section*.
- Liu, Y., Chen, Z., He, A., Zhan, Y., Li, J., Liu, L., ... & Huang, W. (2016). Targeting cellular mRNAs translation by CRISPR-Cas9. *Scientific Reports*, 6.
- Nelles, D. A., Fang, M. Y., O'Connell, M. R., Xu, J. L., Markmiller, S. J., Doudna, J. A., & Yeo, G. W. (2016). Programmable RNA tracking in live cells with CRISPR/Cas9. *Cell*, 165(2), 488-496.
- New England BioLabs, Inc. Instruction Manual: NEBuilder HiFi DNA Assembly Master Mix/NEBuilder HiFi DNA Assembly Cloning Kit. *DNA Modifying Enzymes*. (Accessed October 2016)
- O'Connell, M. R., Oakes, B. L., Sternberg, S. H., East-Seletsky, A., Kaplan, M., & Doudna, J. A. (2014). Programmable RNA recognition and cleavage by CRISPR/Cas9. *Nature*, 516(7530), 263-266.
- Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature protocols*, 8(11), 2281-2308.

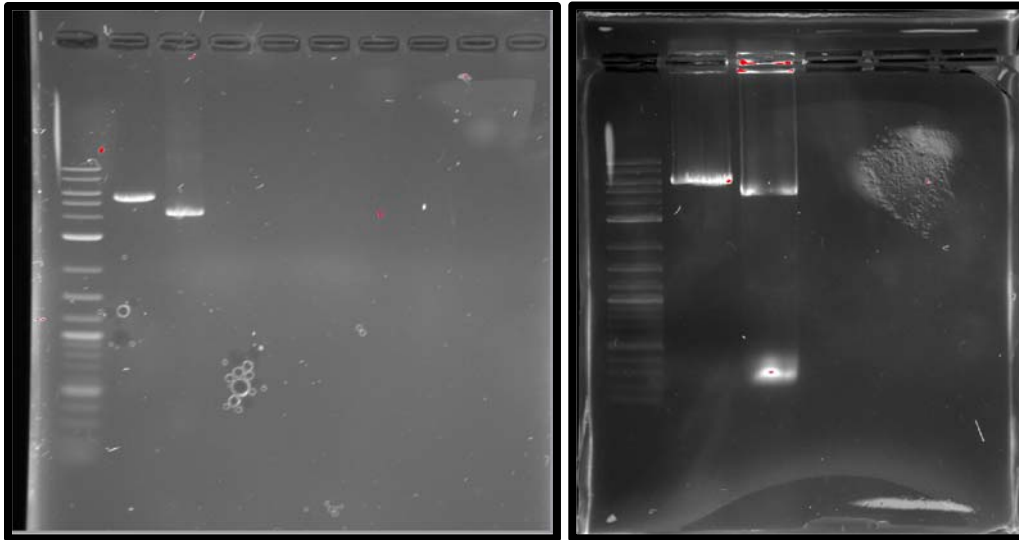
Registry of Standard Biological Parts. (n.d.) Help:Protocols. *iGEM*. Accessed September 2016.
<http://parts.igem.org/wiki/index.php?title=Help:Contents&redirect=no>.

Rodriguez, E. (2016). Ethical Issues in Genome Editing using Crispr/Cas9 System. *Journal of Clinical Research & Bioethics*, 2016.

WPI_Worcester Team (2016). RICE CRISPRs: RNA Inosine/Uracil Conversion Editing Using CRISPR Technology. *iGEM 2016*.

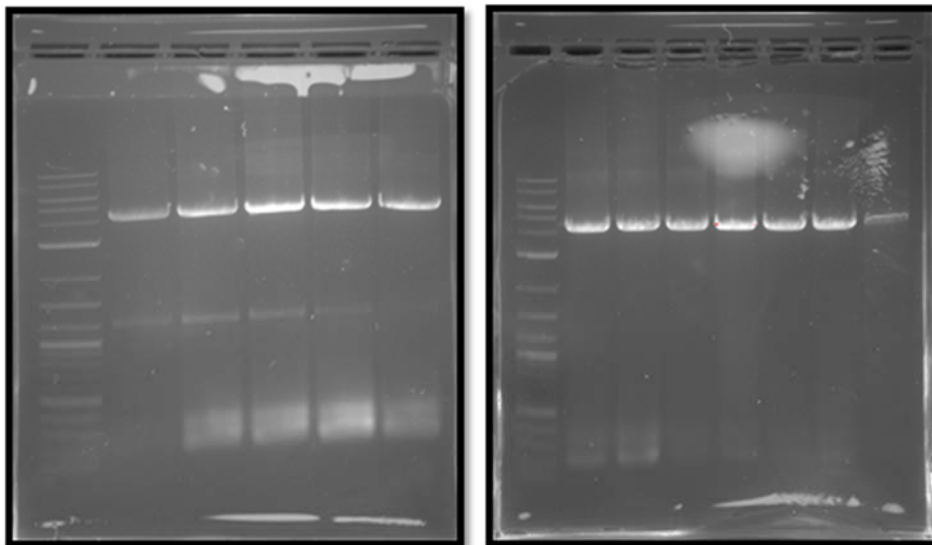
APPENDIX

Figure 1: Verification gels of pcDNA3.1+ and dCas9



Lane 1 in both gels contains pcDNA3.1+ and Lane 2 in both gels contains amplified dCas9.

Figure 2: Visualization Gels for Gradient-temperature Reactions in dCas9 PCR Protocol Optimization



The temperature range over the 12 reactions was 45 to 62, spanning 17 degrees, each reaction representing a difference of $12/17=1.42$ degrees.

Reactions 8-11 are the most effective, evidenced by the brightest, cleanest, thickest bands. Therefore, Reaction 9 is $45+(9*1.42)=57.78$ degrees. Reaction 10 is $45+(10*1.42)=59.2$ degrees. Averaging the temperatures of reactions 9 and 10, we can conclude that the optimal temperature for the annealing step in our PCR protocol for dCas9 should be 58.49 or 58.5 degrees.

The temperature we were using before performing this optimization was 55 degrees.

Figure 3: Verification gel of dCas9 Amplification

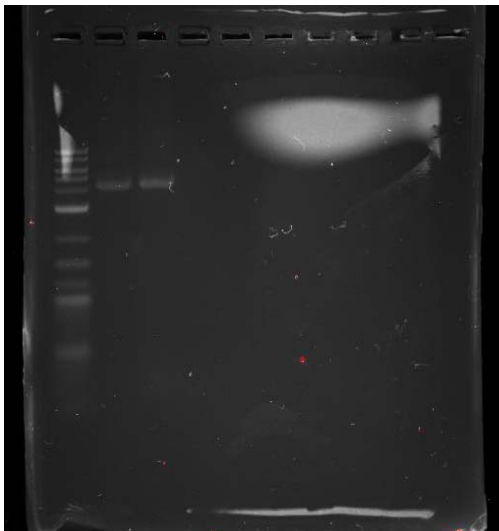


Figure 4: Vector Map of pcDNA3.1+ Plasmid (Addgene)

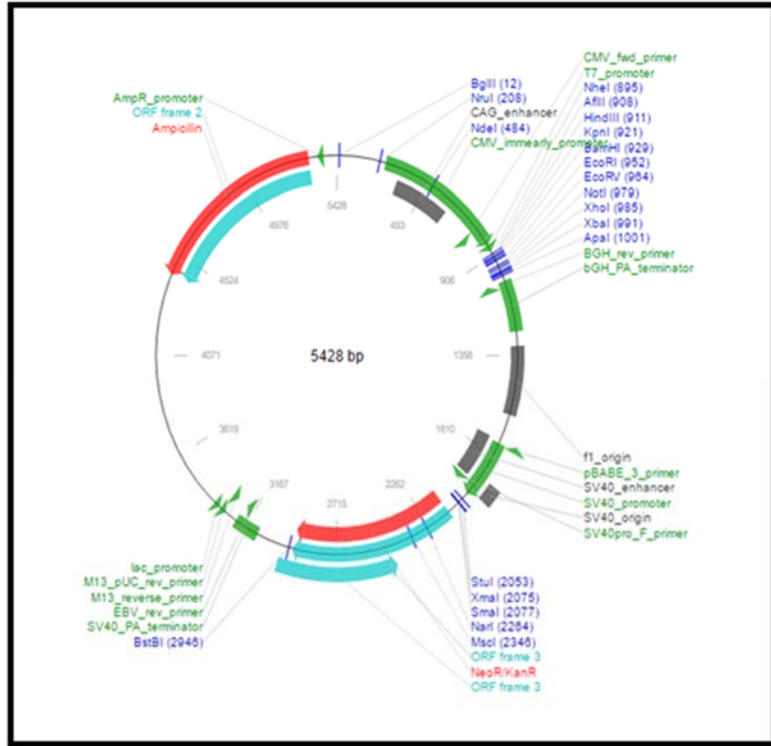


Figure 5: Vector Map of BE2 Plasmid Construct (Addgene)

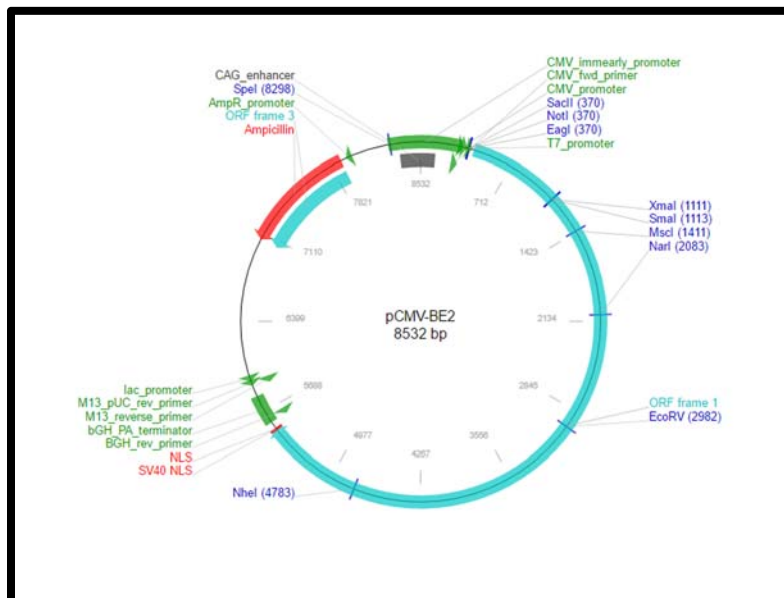


Figure 6: Vector Map of pRETRO Plasmid (Addgene)

