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Synthesizing mRNA to Treat Hereditary Cataracts

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ABSTRACT

Hereditary cataracts, the most common cause of blindness, affect hundreds of thousands of children worldwide. The Crystallin Beta B2 (CRYBB2) gene codes for the βB2 protein which maintains the eye's refractive index. When that gene mutates, the eye crystallin proteins aggregate, refracting the light away from the retina, forming a hereditary cataract. Currently, there is no drug to fix cataracts, only surgery which is costly, invasive, and has the risk of developing posterior capsular opacity. This research assesses whether an mRNA eye drop drug can use gene replacement therapy to fix the mutated gene, restoring sight without surgery. Unique from other studies, the test animal was zebrafish due to the similarities between humans and zebrafish such as sharing 70% of genes. Other studies have been conducted solely on mRNA's potential in COVID-specific vaccines, but by 2035, 78% of the mRNA industry will be non-COVID related medicines. Similarly, this research harnessed molecular biology techniques such as double digestion and ligation to ultimately create two DNA templates, one for the Green Fluorescent Protein (msGFP) and one for the **β**B2 protein. Softwares including SnapGene and National Center for Biotechnology Information modeled the ligation of the distinct parts of the template (T7, UTRs, and CDS). Finally, in-vitro transcription (IVT) was performed to change the GFP DNA template into mRNA. Lipid nanoparticles then encapsulated the mRNA for transport into the zebrafish cells, making the fish glow, demonstrating the feasibility of also synthesizing βB2 mRNA to treat hereditary cataracts using the synthesized βB2 DNA template.

Introduction

Cataracts are a leading cause of blindness in the United States. More than 24.4 million Americans over forty years old suffer from age-related cataracts, and more than 200,000 children worldwide suffer from hereditary cataracts. Hereditary cataracts are caused by the inheritance of a faulty gene that results in the formation of not functioning βB2 crystallin proteins in the eye lens that aggregate together to form protein clumps (Shiels and Hejtmancik, 2020). Currently, the only available treatment is surgery, which can cost upwards of $4,000 per eye, and risks developing a condition known as posterior capsular opacity. The eye may have to be treated again, increasing the chances of costs compounding. There is no effective pharmaceutical drug on the market that can prevent or treat hereditary cataracts.

Gene replacement therapy is an effective method that can be used to develop a drug for hereditary cataracts. Gene therapy for the eye has distinct benefits as a target organ for both hereditary and congenital ocular disorders (Bainbridge et al., 2006). Using this method, βB2 mRNA can be synthesized and be replaced with the faulty gene in the test subject, which in turn would stop the formation of not functioning βB2 crystallin protein and start the formation of the normal functioning βB2 crystallin protein in the eye lens. mRNA synthesis, in general, involves a number of steps. First, DNA templates for the βB2 and GFP proteins are created. SnapGene was used to build and visualize the various parts of the DNA templates. These templates are then combined together using recombinant DNA techniques. The combined DNA template is amplified by growing

E. coli cell colonies. Transcription of these DNA templates to mRNA is achieved using in-vitro transcription. In vitro-transcription is a process that uses a strand of DNA to synthesize a strand of complementary mRNA (Beckert and Masquida, 2010). Finally, mRNA for msGFP is microinjected into zebrafish cells to see if the mRNA could be inserted in the same way.

Zebrafish or *Danio rerio* were uniquely chosen as test animals to insert the different samples of synthesized mRNA, as they have βB2 crystallin protein in their eye lens, similar to humans. Zebrafish were chosen due to their fast reproduction time, similar eye structures, and have 70% of the same genes as humans (Burke, 2016). However, the synthesized βB2 mRNA available on the market is not optimized for zebrafish and is very inefficient for translation and gene expression. Using NCBI data, the most efficient mRNA sequence for zebrafish was selected. NCBI identifies the most naturally occurring codons in zebrafish in order to minimize error.

Overall, this research works to evaluate the hypothesis that a gene replacement therapy method, through the creation of the βB2 and/or GFP protein mRNA, can be used to create an effective drug to treat hereditary cataracts. Since mRNA has been used successfully in numerous COVID vaccines, mRNA technology is plausible to treat medical disorders, like hereditary cataracts, outside the COVID world as well, especially because by 2035, 78% of the mRNA industry will be non-COVID related medicines. After its creation (past this research), to deliver the drug, subconjunctival injection and topical submersion (eye drops) methods are possible because they were found to be equally effective in eliminating bacteria from corneal ulcers without causing noteworthy side effects (Baum, 1982). This finding can also be applied to delivering mRNA to treat hereditary cataracts.

Methods

Protocols

This research required numerous experiments each with its own specific set of protocols. Different protocols were used in order to carry out most of these different experiments. BioLab and Qiagen kits with specific protocols were also used throughout the research process. An overview will be provided; however, specific details on each of the various protocols can be found in the appendices.

DNA Design

The plasmids were designed with a program called SnapGene. NCBI was used to determine the optimized sequence for βB2 Coding Sequence (CDS), βB2 Untranslated Region (UTR), and msGFP. Once the T7 promoter was added to the start of the sequence, SnapGene rendered the desired plasmid design, as shown by Figures 1 and 2. From there, the sequences of the design were ordered to later be assembled.

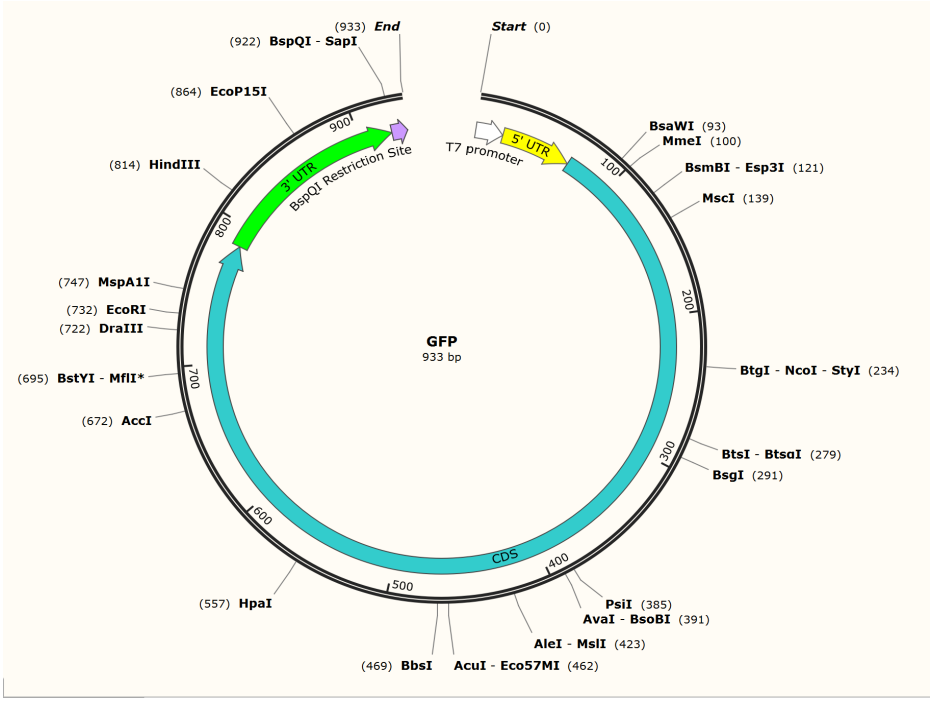

Figure 1. DNA Template for Green Fluorescent Protein (GFP)

SnapGene was the software used to render the model of the GFP DNA template shown in Figure 1. It contains all the parts of the DNA template where restriction enzymes will cut, allowing one to see which specific enzymes to use in double digestion and ligation when putting the different template's parts together.

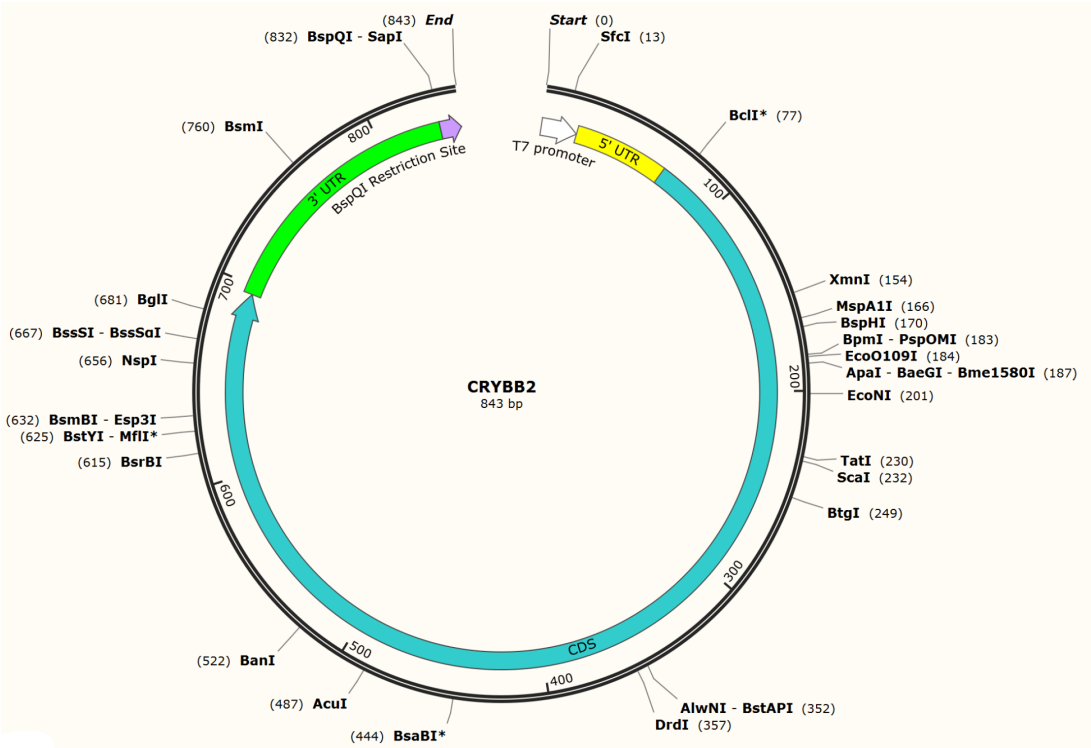

Figure 2. DNA Template for βB2 Protein

Similar to Figure 1, SnapGene rendered this model of the DNA template for the βB2 protein.

DNA Transformation

Next, the DNA templates had to be synthesized, so they could be used to be transcribed into mRNA. First, the Escherichia coli (E. coli) cell lines were transformed with the T7, βB2 CDS, msGFP CDS, and βB2 UTR plasmids by a heat shock. The heat shock causes different E. coli's cell membranes to become more fluid and take up the plasmids. From there, the E. coli was plated on an agar plate (see Appendix A) and placed in the incubator at 37℃ to grow and create multiple colonies with the desired parts of the DNA template from the plasmid: transformation (see Appendix B). One colony from each plate was selected to go into an incubator at 37℃ for miniprep (8mL Luria Bertani (LB) broth) with 8 microliters of kanamycin, thus replicating and giving those copies its exact identical molecular makeup. Then, that mini prepped colony for each plasmid was spun down in the centrifuge at 7000 revolutions per minute for 10 minutes to obtain a cell pellet, as the supernatant was poured out. The pellet was stored overnight at -20℃. The cell pellet was then purified by the Qiagen purification kit to obtain purified plasmids.

Polymerase Chain Reaction (PCR)

The purity of the T7 promoter backbone, βB2 CDS, msGFP, and βB2 UTR plasmids was assessed with a nanodrop machine using 1.5 microliters. Perform PCR under the conditions shown in Figure 3 to amplify the βB2 CDS and msGFP of the DNA template: see Appendix C.

PCR Setup Template (BB2_CDS) :

- T1: 98*C – 30 seconds
- T2: 98*C – 10 seconds
- T3: 72*C – 30 seconds
- T4: 72*C – 37.4 seconds
    - Cycles: 25
- T6: 72*C – 2:00

HOLD TEMPERATE - 4 * C

PCR Setup Template (msGFP):

- T1: 98*C – 30 seconds
- T2: 98*C – 10 seconds
- T3: 72*C – 30 seconds
- T4: 72*C – 42.48 seconds
    - Cycles: 25
- T6: 72*C – 2:00

HOLD TEMPERATE - 4 * C

Figure 3. PCR Conditions for T1-T7 Stages

Digestion

Using the EcoR1 and BamH1 restriction enzymes, the T7 promoter and βB2 UTR were double digested out of the purified plasmids then incubated at 37℃ for 1 hour: see Appendix D. Then, gel electrophoresis was performed in order to see the DNA fragments and to cut the T7 and UTR fragments out of the 1% agarose gel (see Appendix E). The T7 and UTR samples were divided into two wells each, using the 10% rule, per the protocol. The gel ran for 30 minutes at 80 volts. The appropriate fragments were cut out of gel and the extraction kit called New England Biolabs (NEB) #T1020 was used to purify the DNA fragments from the excess gel.

To assess purity, the DNA fragments were nano dropped. A 1% agarose gel was used in a gel diagnostic to determine if the double digestion succeeded in cutting the DNA at the right places (this is another way to check success like nano dropping). Then the gel diagnostic ran for 30 minutes at 80 volts and afterwards the DNA fragments sat in the gel for 30 minutes.

Ligation

The T7 promoter and the UTRs were ligated with the CDS into one DNA template. Before adding the ligase, the other contents were mixed (see Appendix F), and the mixture sat for 5 minutes on ice. Then, ligase was added, and sat for 10 minutes at room temperature. Finally, the tube was put in a mini heater at 67℃ for 5 minutes. The ligated DNA was transformed overnight.

Liquid cell cultures were used to miniprep a single ligated, transformed cell colony in each tube. 8 microliters of kanamycin were added to each tube, and then one cell colony was swiped from the transformation plate to swirl around and deposit in each tube (one colony per tube). The opening of tubes was wrapped in aluminum and then kept in the incubator at 37℃ for 18 hours. The minipreps were purified (T7 and βB2) using Qiagen kit. The minipreps were nano-dropped. The ligation was then sent off for sequencing by using 10 microliters of DNA and 10 microliters of water for colonies 1, 3, and 4. However, for colony 2, 5 microliters of DNA and 10 microliters of water were used. The T7-UTR ligation's sequencing was successful.

Ligated DNA Linearization and Purification

The DNA was linearized by incubating 7.5 microliters of DNA, 5 microliters of CutSmart buffer, 37.5 microliters of DNase free water, and 1 microliter of BspQI at 37℃ for an hour. After linearization, the DNA was purified in order to prepare for in-vitro transcription (IVT). Note that the following steps were performed with msGFP DNA instead of the linearized and ligated DNA template due to insufficient time. However, the steps are the same, thus showing that the method of preparing DNA for IVT is highly feasible. Starting with 50 microliters of the msGFP DNA, 10 microliters of 3M sodium acetate were added. Then, 60 microliters of phenol/chloroform mixture were added and mixed violently. The mixture was then centrifuged for 7.5 minutes at 17000 relative centrifugal force (rcf) and 22℃. The aqueous phase of the mixture was extracted and moved into a new tube and had 60 microliters of chloroform to that new tube. The tube was then centrifuged for 5 minutes at 17000 rcf at 27℃. 60 microliters of the aqueous phase were transferred to a new tube again, and 120 microliters of 100% ethanol wash was added to that. The new tube was incubated at -80℃ for 60 minutes. After taking the tube out, it was centrifuged for 2 minutes at 16000 rcf at 4℃. The supernatant was removed, and the pellet was rinsed with 500 microliters of 70% ethanol. Then the ethanol was dried out of the DNA at 67℃. The DNA was then resuspended in 10 microliters of DNase free treated water. Finally, a nanodrop was performed to check the purity of the msGFP after performing all the purification steps described before.

In-Vitro Transcription

Moving on to in-vitro transcription, 3 microliters of nuclease free water, 10 microliters of 2x ARCA/NTP mix, 5 microliters of msGFP DNA, and 2 microliters of T7 RNA polymerase were mixed in a tube. The mixture was centrifuged for 30 seconds (all contents must be at the bottom of the tube). The tube incubated at 37℃ for 30 minutes. After taking it out, 2 microliters of DNase I were added then the solution was mixed and incubated at 37℃ for 15 minutes. The mRNA was purified using the NEB #E20655 mRNA kit. Finally, the msGFP mRNA was nano dropped.

Injection

Moving on to injecting the mRNA into the zebrafish without using lipid nanoparticles, the fish were first put to sleep using 10 drops of Tricaine. Three fish were injected with 5 microliters of msGFP mRNA each, while leaving three fish "uninjected" as a control group. Finally, the fish were observed (asleep in a 1% agarose gel).

Results and Figures

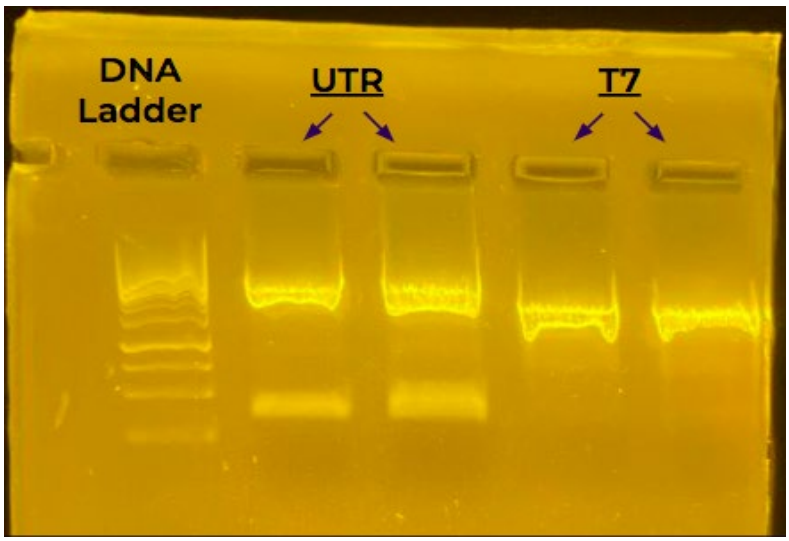

Figure 4. Gel Electrophoresis for T7 Promoter Backbone and βB2 UTR in 1% Agarose Gel

After double digestion was performed, the DNA was loaded for gel electrophoresis. From Figure 4's completed gel, it can be observed if the DNA pieces were cut and separated correctly for both the βB2 insert and the T7 backbone. The desired DNA fragments were also cut out of the gel.

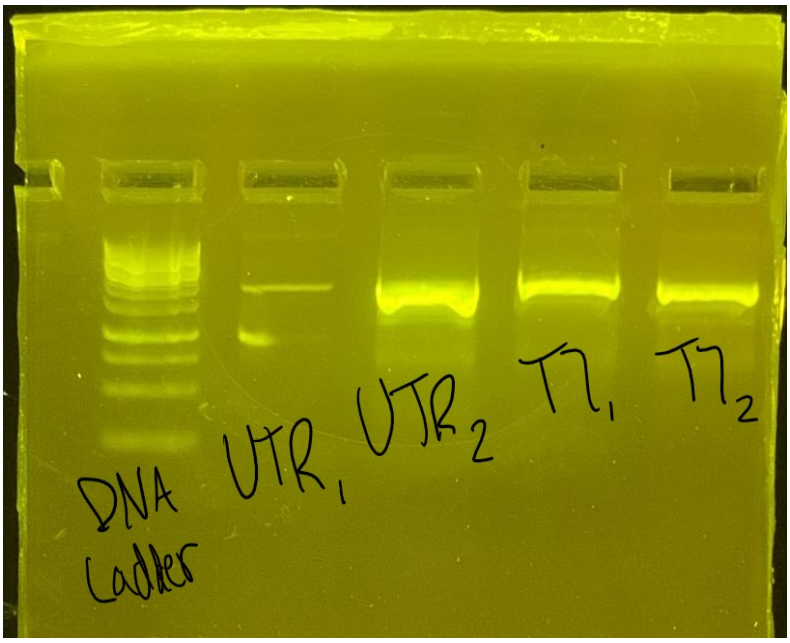

Figure 5. Gel Diagnostic for T7 Promoter Backbone and βB2 UTR in 1% Agarose Gel

The gel diagnostic in Figure 5 was used to double check that the double digestion cut the T7 and UTR out of their plasmids properly, and that the DNA fragments were cut out of the 1% agarose gel correctly.

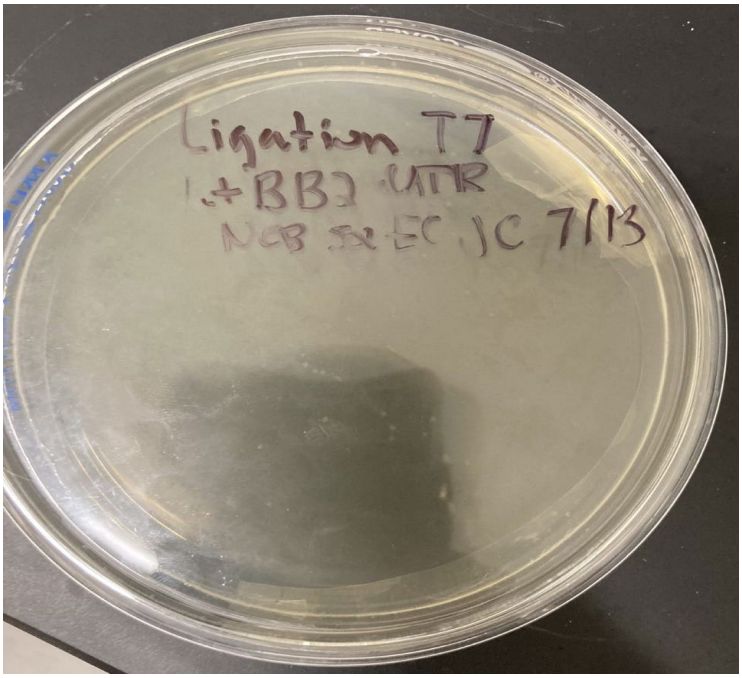

Figure 6. Transformed Cell Colonies of T7 Promoter and βB2 UTR Ligation

Cell colonies, as shown in Figure 6, were transformed to have the ligated T7 promoter and βB2 UTR. The transformed bacterial colonies can be seen on an agar plate.

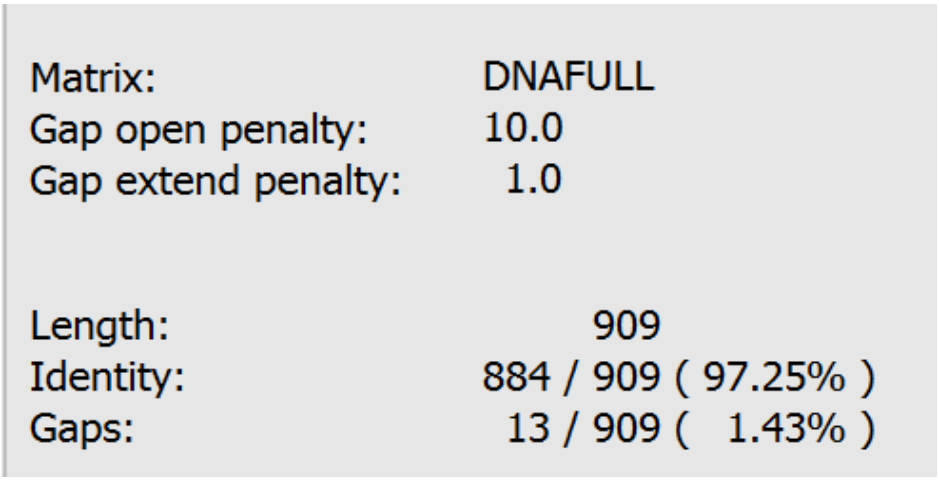

Figure 7. Colony 1's Sequencing Results

The sequencing results for the first mini-prepped colony of the T7-UTR ligation are shown in Figure 7. It is the measure of the template ligation's accuracy with regards to the SnapGene model as the goal.

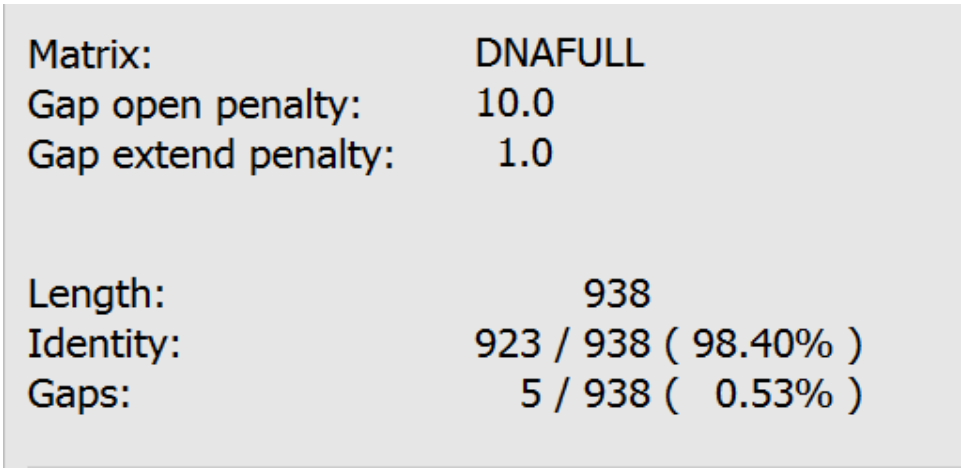

Figure 8. Colony 2's Sequencing Results

Similar to Figure 7, the sequencing results for the second mini-prepped colony of the T7-UTR ligation are shown in Figure 8.

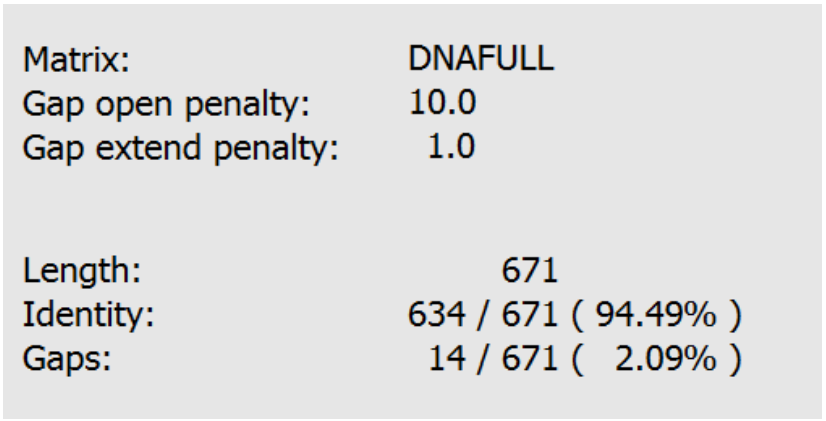

Figure 9. Colony 3's Sequencing Results

Similar to Figure 7, the sequencing results for the third mini-prepped colony of the T7-UTR ligation are shown in Figure 9.

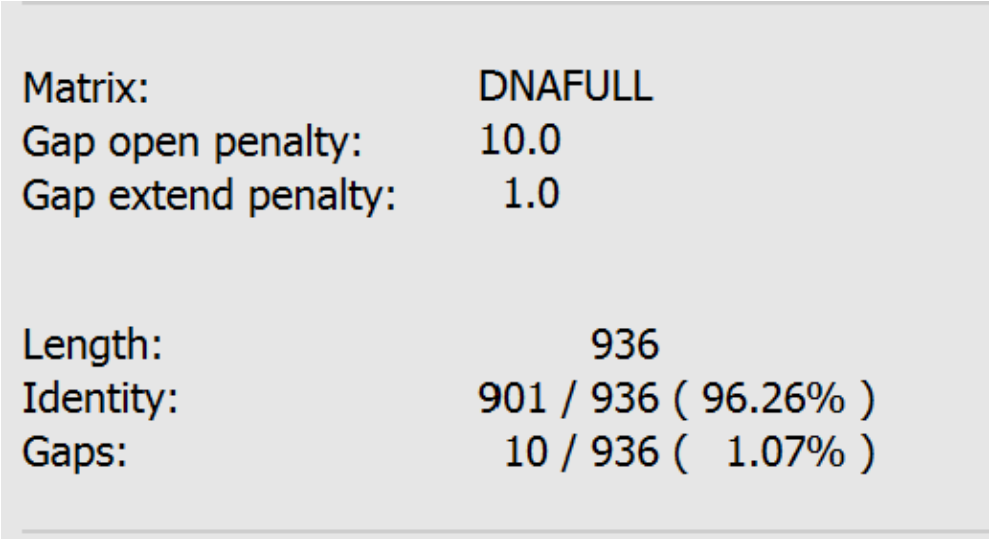

Figure 10. Colony 4's Sequencing Results

Similar to Figure 7, the sequencing results for the fourth mini-prepped colony of the T7-UTR ligation are shown in Figure 10.

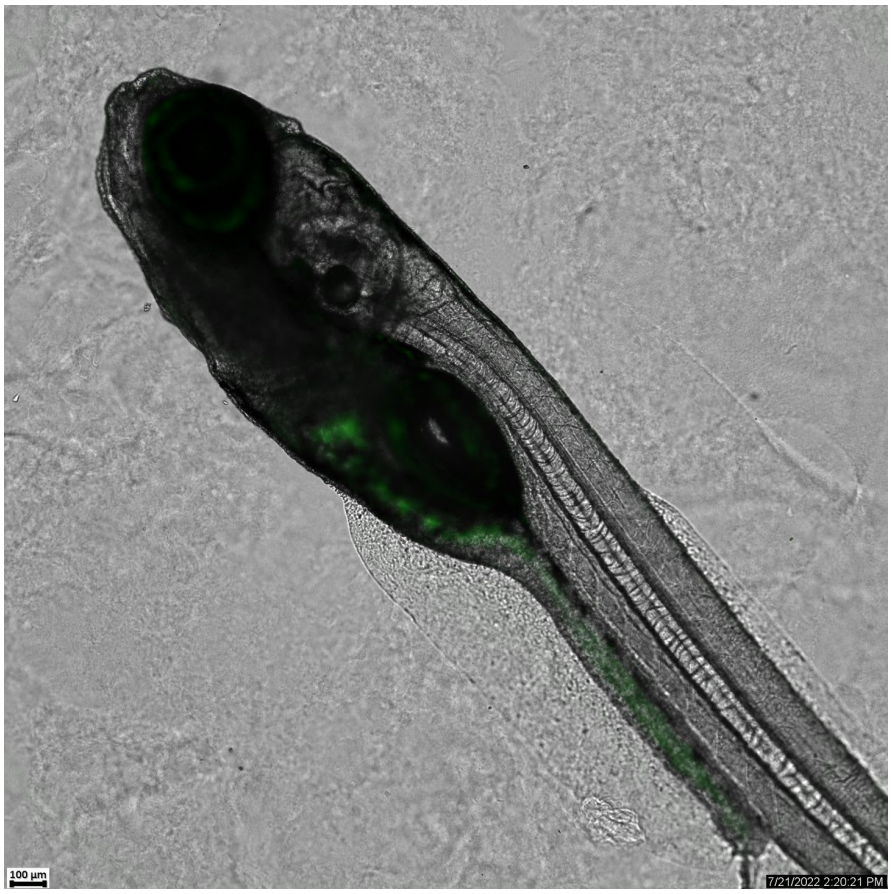

Figure 11. Fish without Injected msGFP mRNA

Pictures were taken of zebrafish without the injected msGFP mRNA to function as the control group. As shown in Figure 11, there is no unnatural fluorescence.

Figure 12. Fish with Injected msGFP mRNA

Pictures were taken of zebrafish with the injected msGFP mRNA to function as the experimental group. As shown in Figure 12, there is much more fluorescence in addition to the natural.

Table 1. First Nanodrop of the T7 Promoter and βB2 UTR

Type of Colony	DNA Concentration (ng/µl)	260/280	260/230
T7 C1	17.4	1.79	2.23
T7 C2	123.9	1.84	2.14
βB2 UTR C1	50.1	1.81	2.29
βB2 UTR C2	89.7	1.83	2.28

Table 2. Nanodrop of msGFP and βB2 CDS

Type of Colony	DNA Concentration (ng/µl)	260/280	260/230
GFP C1	59.3	1.79	2.04
GFP C2	64.5	1.72	1.73
βB2 CDS C1	62.2	1.78	1.96
βB2 CDS C2	55.1	1.8	2.07

Table 3. Nanodrop of βB2 UTR and T7 promoter

Part of DNA Template	DNA Concentration (ng/µl)	260/280	260/230
βB2 UTR	11.0	1.62	0.34
T7 promoter	31.8	1.85	1.00

Table 4. Nanodrop of 4 Colonies of Ligated βB2 UTR and T7 promoter

Colony Number	DNA Concentration (ng/µl)	260/280	260/230
1	120	1.86	2.09
2	254.5	1.88	2.33
3	162.6	1.88	2.32
4	189.8	1.82	1.8

Table 5. Nanodrop of Linearized msGFP

DNA Concentration (ng/µl)	260/280	260/230
178.7	1.75	1.89

Table 6. Nanodrop of msGFP mRNA

mRNA Concentration (ng/µl)	260/280	260/230
105.4	2.27	2.87

Discussion & Conclusions

The preliminary results using GFP show that the mRNA synthesis is feasible to treat hereditary cataracts, but further research using βB2 is needed to prove that gene replacement therapy will be successful. DNA templates for GFP and βB2 were individually digested and ligated to include all of the correct parts. Gel electrophoresis and a gel diagnostic were both performed to check whether the digestion gave the proper results, and the results show that the cuts were accurate. Nanodrops were performed throughout the process to test how pure the DNA or mRNA was. All the nanodrops continually showed that the nucleic acids were pure, so the process could be continued. Some nanodrops portrayed that certain colonies had a higher concentration of DNA than others, so those colonies were what was continued with. GFP mRNA was synthesized correctly, but due to time constraints, msGFP was instead injected into zebrafish. The zebrafish experimental group glowed under a UV light when injected with msGFP mRNA, which means that the mRNA was successfully inserted. Unfortunately, the msGFP mRNA was injected into the swim bladder, not the eye, but we did not have sufficient time to fix the missed injection. However, the fish did still glow green, meaning that the mRNA was properly synthesized.

Due to time constraints, the BB2 mRNA was not synthesized and tested in zebrafish. However, the promising results of the successful msGFP injection laid the groundwork and showed that mRNA can be coded for βB2 protein in the zebrafish. Continued research would include completing Gibson Assembly and receiving positive sequencing results for the βB2 mRNA. This treatment could be encapsulated through lipid nanoparticles and delivered into the eye through submersion. Then, if, due to the drug, the zebrafish regains sight after having their CRYBB2 gene knocked out by CRISPR-Cas9, more work can be done to move the treatment from zebrafish to humans. There is much potential because of the groundwork that this study has done and will continue to do.

This novel method will not just prevent the need for cataract surgery but assist in advancing the mRNA therapeutics industry and providing more accessible treatments. The future of mRNA technology is promising due to its new approved applications and potential to target virtually any disease. mRNA pharmaceuticals allow for personalized treatments and are cheaper than alternative treatments (Weng et al., 2020). One study shows that mRNA vaccines can be used to treat cancer (Miao et al., 2021). This revolutionary method could be a huge gateway into the future of cancer research (cancer immunotherapy) and many other diseases.

Acknowledgments

I would like to thank my advisor for the valuable insight provided to me on this topic.

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