CRISPR/Cas9 Mediated Mutagenesis of the ELOVL4 Gene to Generate a Patient Specific Stargardt's Eye Disease Model

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Abstract

The ELOVL4 enzyme is required to generate very long-chain fatty acids with structural and functional importance in the retina.(1) Genetic defects in ELOVL4 are responsible for Stargardt's disease, leading to a loss of central vision. This paper focuses on creating an *in-vitro* model of Stargardt's disease that replicates a key genetic defect observed in patients for the purpose of testing dietary supplements or other treatments that can restore normal long-chain fatty acids in the retina. We are using CRISPR to create a patient-relevant 5 base pair deletion on ELOVL4 in induced pluripotent stem cells (*IPSCs*) that can be grown into retinal organoids in culture. (2) Bioinformatics tools were utilized to generate potential CRISPR guides and repair oligonucleotides that are predicted to effectively cut and repair the gene with the 5bp mutation observed in patients. These guides were tested by transfection into HEK cells, followed by testing for cutting efficiency in the ELOVL4 gene. (3) Future studies will take the successful constructs and generate the desired mutations in *IPSCs* for generation of retinal organoids to study Stargardt's disease.

Introduction

Macular dystrophy as a result of autosomal dominant Stargardt's disease (STGD3) is a rare genetic disorder that is known to cause early onset loss of central vision, "decreased visual acuity, macular atrophy, and extensive fundus flecks". (2) On the inner dorsal position in the eye lies the macula, the central focus point of the eye that allows for greater focus and detailed imaging.

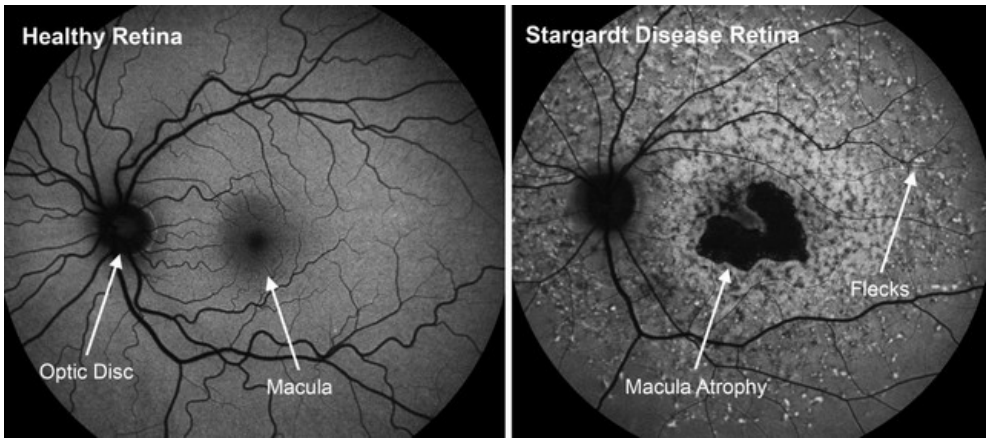


Figure 1: *Images of a normal (left) retina and one from a patient with advanced Stargardt disease (right). The macula is the central area of the retina where high resolution, central vision occurs. This area is greatly affected in Stargardt's disease causing early loss of central vision and greatly affecting the quality of life. While multi-factoral in nature, one key cause of Stargardt's disease is a malfunctioning ELOVL4 gene that results in the inability to generate key, very long chain fatty acids that are required for maintenance of the retinal photoreceptors in the macula.*
Image from Alkaeus Pharmaceuticals; https://www.alkeuspharma.com/stargardt.html

The overall aim of this project is to design a construct specific to STGD3. The cause of STGD3 is a 5 bp mutation on the *Elongation of very long chain fatty acids 4* (ELOVL4) gene that codes for an enzyme that is crucial for the biosynthesis of very long chain polyunsaturated fatty acids (VLC-PUFA). VLC-PUFAs serve important roles in areas of the "brain, retina, skin, Meibomian glands, and testes".(5) Their function in the macula is theorized to be more important in outer retinal tissues, but the lack of VLC-PUFA can cause macular degeneration.(6)

The goal of our particular study is to provide the Lowry Medical Research Institute with the appropriate *in vitro* model of STGD3; to then generate human retinal organoids with relevant VLC-PUFA deficiency. The current *in vivo* models used to replicate age-related macular degeneration (AMD) are insufficient to accurately represent human physiological responses. (7) The knockout of ELOVL4 is known to be neonatally lethal in mice models. However, for most ELOVL4 mutations, the heterozygous genotype is phenotypically normal and Stargardt's is generally considered to be a recessive disorder. However, the specific 5 base-pair deletion mutant that we are studying is a unique example of a dominant disease where only one defective allele is disease causative. It is hypothesized that this particular mutation may result in production of a dominant-negative ELOVL4 protein that may negate the activity of the functional allele. However, more information regarding this particular mutation is required to understand this disease. One strategy for treating Stargardt's disease may be to supplement the retinas with synthetic VLC-PUFAs, but before that treatment modality is used it needs to be demonstrated that those fatty acids can become incorporated into diseased retinae even with the dominant mutation. (6,7) Our study utilizes CRISPR-Cas9 to create an *in vitro* model of VLC-PUFA deficiency by generating the particular 5 base pair deletion observed in these

patients. (8) Through the use of bioinformatic tools, our team designed amplification primers and various CRISPR guides to accurately induce the target mutation in a pX330.puro Cas9 plasmid. This STGD3 mutant plasmid was transformed into *E.coli* to select colonies for plasmid uptake. To preserve successful transformation with mutant plasmid uptake, aliquots are kept at -80° C. The CRISPR Cas9 plasmid and guides were tested in a HEK293 cell line to determine optimal gene amplification primers, sequencing primers, and successful targeting of the gene for CRISPR Cas9 induced cutting and repair. Ultimately, successful CRISPR guides that generate the desired cuts, along with repair oligonucleotides that incorporate our desired mutation into the cuts, will be used to generate the patient-relevant mutation in *IPSCs* that can be grown into retinal organoids for use as a model system for this particular version of Stargardt's disease.

Methods

Designing of CRISPR Guides

The online software Benchling was utilized to visualize the ELOVL4 gene and elucidate optimum target sequences for CRISPR editing at the region of interest. Following evaluation of on and off target scores, three 20 nucleotide-long DNA segments were chosen to serve as guide sequences based on their relative low off-target scores, high on-target scores, and proximal cut site to the desired mutation within the sequence of interest. All appropriate Cas9 "guides" are required to have a protospacer adjacent motif (PAM) sequence ("NGG") immediately adjacent to each guide as this directs where the Cas9 nuclease cut will occur. On-target and off-target scores are provided by Benchling in references to both Doench, Fusi et al., and Hsu et al. (9) These guides can be seen within the ELOVL4 gene sequence in **Figure 2**. Potential guide sequences were identified, these were synthesized with the sequence CACCG added to the 5' end of the 5' to 3' (forward) strand in which the NGG sequence followed, and the sequence CAAA and C had to be added to the 5' end and the 3' end, respectively, of the reverse. This provided the appropriate restriction enzyme sequences to match with the plasmid subcloning region for insertion of the guides into the pX330.puro plasmid (https://www.addgene.org/110403/). The two oligo sequences for each guide were purchased from Integrated DNA Technologies (IDT). The created guide sequences and corresponding names are depicted in **Table 1.1**. Each guide has specific cut sites, and PAM sequences **Table 1.2**.

Guide Sequence Name	Purchased Sequences (Plasmid Specific Additions Are Bolded & CRISPR Sequence is Underlined)
Guide 1	F 5'**CACCG**AAATATGAAGCTGATTGCAT 3' R 5'**AAAC**ATGCAATCAGCTTCATATTT**C** 3'
Guide 2	F 5'**CACCG**GAGCCTAAGAAACCAAAAGC 3' R 5'**AAAC**GCTTTTGGTTTCTTAGGCTC**C** 3'
Guide 3	F 5'**CACCG**TCTTTCTTAACTTCTACATT 3' R 5'**AAAC**AATGTAGAAGTTAAGAAAGA**C** 3'

Table 1.1 Purchased guide sequences with added sequences for vector addition. Plasmid specific additions to the sequences are bolded.

Guide Sequence Name	Cut Position*	Leading or Lagging Strand	PAM Sequence	On Target:Off-Target Score
Guide 1	30792	Lagging	AGG	55.1:37.9
Guide 2	30859	Leading	TGG	52.1:37.8
Guide 3	30827	Leading	CGG	40.9:31.5

Table 1.2 - Transcribed data from Benchling Software showcasing basic information regarding each guide sequence implemented. *Cut position is in reference to only the ELOVL4 Gene

Designing of Primers

In order to visualize functioning of the CRISPR Cas-9 system in transfected HEK293 cells, creation of proper primer pairs were needed to both amplify the area of interest within the ELOVL4 gene from the human genome of HEK293 cells via PCR amplification, and allow for sequencing of the amplified area via Sanger sequencing by RetroGen Incorporated. These can be visualized in context of the ELOVL4 gene sequence in **Figure 2**. The Primer Design Tool Available on The National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was implemented to determine optimal primer sequences to purchase from IDT. Forward and reverse primers to be utilized for PCR amplification were chosen based on the criteria that they should be less than 400 nucleotides away from the cut site, and forward and reverse primers to be used for sequencing purposes were chosen based on the criteria that they should be no more than 200 nucleotides away from the cut site so that accurate sequencing through the cut site could be obtained by standard Sangar Sequencing methods. Information regarding primers utilized for PCR and sequencing is summarized in **Table 2.** These can also be visualized in the gene context in **Figure 2**.

Primer Function (PCR or Sequencing)	Sequence	Forward or Reverse
PCR	**5' CTAGCCATGGGAGCCAGAAAAC 3'**	Forward
PCR	**5' CCCAAGCTCTCCTTTGCTTCT 3'**	Reverse
Sequencing	**5' GCACACGGCACTGTCTCTTT 3'**	Forward
Sequencing	**5' GCTCACACCATTTGCTGAAA 3'**	Reverse

Table 2 - Purchased Primers. Showcased above are the associated sequences and direction.

SnapGene Viewer was implemented to determine the primer sequence necessary for sequencing the region in the pX330.puro plasmid used as a vector in this study. The forward primer was ordered from IDT to correlate with the U6 promoter associated with the vector reading **5' GACTATCATATGCTTACCGT 3'**. After subcloning the various guides into the pX330 plasmid, sequencing through the subcloning site was performed to confirm successful insertion of the correct guides.

Maintenance of HEK293 Cells

Human Embryonic Kidney (HEK) 293 cells were utilized for the purposes of this lab. Cells were kept in 10 mL Dulbecco's Modified Eagle Medium (DMEM) supplemented with anti-anti, fetal bovine serum, and sodium pyruvate, in T75 cell flasks coated with poly-lysine to aid in attachment, and incubated in standard CO2 incubators at 37° C, with 5% CO2. Stock volumes of media were made mixing 44 mL of DMEM, 5 mL of 10% FBS, 500 µL of anti-anti(antibiotic -antimycotic), 500µL of Sodium Pyruvate and refrigerated at 4° C until utilized.

Cell Splitting of HEK293

Cells were split *ad libitum*. To split cells, excess media was vacuumed from cells into hazardous waste containers. To wash excess residue, 5 mL of Phosphate Buffered Saline (PBS) buffer was added to HEK cells. This was then vacuumed. 2 mL of cell dissociation buffer was then added and was incubated for 10 minutes. Cells were then visualized under a microscope to detect proper detachment from the flask. Following detachment, 4 mL of stock supplemented DMEM solution (described above) were added and mixed via pipetting to create a homogenous mixture. A desired amount of solution was discarded in hazardous waste or kept for DNA isolation and the remaining liquid culture would then be diluted by a preceding volume to create a final solution of 10mL of solution in the flask. All manipulation of the cell culture was carried out in a sterile biosafety cabinet. The cells were then incubated at 37°C with 5% CO2.

Isolation of HEK293 DNA

To test the efficacy of the primers, HEK cell DNA was isolated. 6 mL of HEK cells were pelleted by centrifugation at 750 rpms in a Beckman Coulter Allegra 6KR Centrifuge for 7 min. After pouring off the supernatant of the product, cells were resuspended in 200 µL of PBS. The Qiagen QIAamp DNA Mini Kit (Catalog N. 51304) was then implemented to lyse the cells and isolate the DNA in DNAse free water, according to the manufacturer's protocol, and the concentration of genomic DNA was determined through the use of NanoDrop 2000c Spectrophotometer.

PCR Amplification of HEK293 ELOVL4 Gene Near Mutation Site

Amplification of the segment of the ELOVL4 gene that contained the area to occupy the mutation of interest was achieved utilizing the Thermo Fisher Scientific DreamTaq Green PCR Master Mix (2X). Two different ratios of components were utilized to test effective isolation techniques. In one PCR reaction 10µL of Master Mix, 1 µL of the forward PCR primer, 1 µL of the reverse PCR primer 0.79 µL of DNA, and 7.21 µL of DNAse free water were utilized. In another PCR reaction 12.5µL of Master Mix, 0.25 µL of the forward PCR primer, 0.25 µL of the reverse PCR primer 0.79 µL of DNA, and 11.21 µL of DNAse free water were utilized. PCR reactions were placed in MJ Research PTC-100 Programmable Thermal Contoller 95°C for 5 min, and then run through 40 cycles of 95°C for 10 seconds, 60°C for 15 seconds, and 72°C for 30 seconds. The protocol was then set to 72°C for 5 min and held at 4°C.

Gel Extraction Vs. PCR Purification System

Two modalities were tested to elucidate optimum Isolation of the PCR product. One modality entailed utilizing the Promega Wizard PCR Cleanup System with Vacuum Manifold. The other entailed the use of the Qiagen QIAEX II Gel Extraction Kit. Both samples were prepared and sent to RetroGen sequencing to provide results on which procedure would be utilized in the future for analyzing cut and mutated regions of HEK293 Cell DNA based on efficacy of sequence reading utilizing the primers discussed above.

Subcloning of Guides Into pX330.puro Backbone

Insertion of each paired oligo sequences into the pX330.puro vector was conducted via the Junko Shimazu (Gleeson Lab) CRISPR Target Sequence Cloning Protocol implementing the single-step digestion-ligation modality. Pairs of oligos were phosphorylated and annealed by added 1 µL of each oligo (100µM), 1 µL of 10x T4 Ligation Buffer (NEB), 6.5 µL of ddH20, 0.5 µL of T4 PNK (NEB). This was annealed in a thermocycler using a program set to 37°C for 30 min, raised to 95°C for 5 minutes, and then lowered to 25°C at a rate of 5°C/minute. The annealed oligo was then diluted 250 fold. 100 ng of pX330 backbone was then added in a separate PCR reaction tube, and 2 µL of the diluted phosphorylated and annealed oligo duplex was then added, alongside 2 µL of 10x Tango buffer (or FastDigest Buffer), 1 µL of DTT (10mM), 1 µL of ATP (10mM), 1 µL FastDigest Bbsl (Thermo Fisher Fermentas), 0.5 µL T7 DNA ligase. DNAse free water was added to create a final volume of 20 µL. The annealing of

the sequence into the backbone was then achieved by placing the reaction in a thermocycler using a program set to to run for 5 min at 37°C, then 5 minutes at 23°C, running 6 cycles total, (accumulating for a total run time of 1 hour). The reaction was then held at 4°C.

Transformation of Ultra-Competent *E. coli* Cells

Transformation of Ultra-Competent *E. coli* cells with the manipulated pX330.puro vector was performed following the *E. coli* transformation protocol described by Addgene (https://www.addgene.org/protocols/bacterial-transformation/). Bacteria were then plated on agar plates containing 100 µg/mL of antibiotic that were created following Addgene, utilizing an aseptic technique to generate individual colonies (https://www.addgene.org/protocols/pouring-lb-agar-plates/). 100µL of overnight prep and 50µL of overnight prep were individually plated for each guide for growth.

Inoculation of selected for *E. coli* Cells

Three selected colonies for vectors with the guide sequences for guides 1 and guide 3, and 4 selected colonies for vectors with the guide sequences for guide 2 were selected for individual overnight inoculations following a protocol from Addgene (https://www.addgene.org/protocols/inoculate-bacterial-culture/). The Addgene inoculation protocol was followed utilizing 5 mL of SOC media supplemented with Ampicillin (100 µg/mL). Overnight growth in a shaking incubator was allowed.

Glycerol Stock

Aliquots from each overnight culture were then removed from each media to create glycerol stocks from each overnight growth of *E. coli*. This was achieved by following a protocol from Addgene to ensure if proper transformation was successful, stock bacteria was maintained. These were stored in a -80° C Freezer.

Plasmid Purification and Analysis of Transformed Vectors

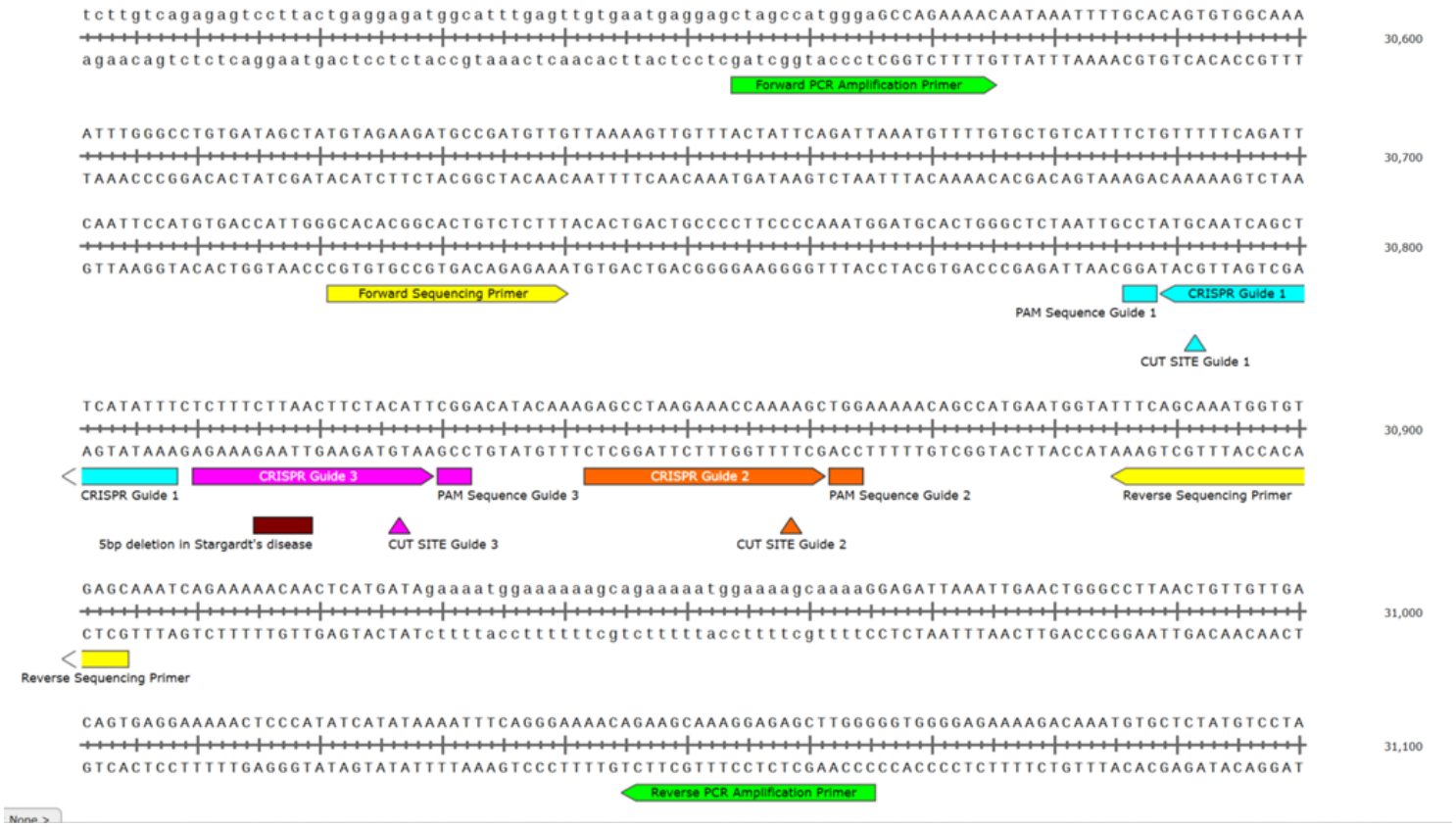
Plasmid Purification from overnight cultures was performed utilizing the QIAprep Spin Miniprep Kit kit from Qiagen. Successful elution of vectors was ascertained via gel electrophoresis. Successful insertion of oligo sequences was ascertained via RetroGen Sequencing.

RESULTS

Map of Annotated ELOVL4 Gene

Showcased is the annotated ELOVL4 gene near the area of interest. PCR amplification primers and sequencing primers are highlighted in green and yellow, respectively. Each CRISPR guide attachment site, associated cut site, and associated PAM sequence are also showcased. CRISPR Guides 2 and 3, running from the 3' to 5' end of the bottom strand in the figure, denote attachment to the top strand utilizing the subsequent PAM sequence found in the top strand. Alternatively, Guide 1, running from the 5' end to the 3' end of the bottom strand in the figure, denotes attachment to the bottom strand utilizing the subsequent PAM sequence in the bottom strand. The Directionality of Primers are noted via the arrows that point at the ends of each label. Cut sites are designated by triangle arrows, and PAM sequences by boxes. The figure was created using SnapGene Viewer.

Figure 2: *Annotated Form of ELOVL4 Gene Sequence showcasing Primer pairs (amplification and sequencing), and the CRISPR guide sequences associated with the desired mutation. The amplification primers are shown in green and the sequencing primers in yellow. The three guide constructs and the adjacent PAM sequences are also shown, designed to be near the 5bp deletion shown in dark red.*



Testing Primer Functionality

One of the primary goals of the study was to effectively extract and amplify the ELOVL4 mutation site seen in **Figure 2** from the human HEK293 cells in order to edit and repair the site as needed. The amplification primers were designed in order to extract this portion of the DNA while the sequencing primers were nested within to amplify a more precise sequence closer to the mutation site.

To test the accuracy of these amplification primers, the HEK DNA was extracted, amplified, and run on a native gel. The goal was to identify a band at ~500 bp as that was the length of our target region. **Figure 3A** depicts the gel that was loaded with two amplified samples that tested two different concentrations of primers. The result is a band at around 500 bp as predicted. We determined the 0.25µL concentration to be more appropriate going forward since it resulted in the same band as the 1µL while using a quarter of each of the primers. **Figure 2B** depicts the replicated gel loaded with the forward and reverse PCR amplification primer samples prior to extracting and sending them to sequencing.

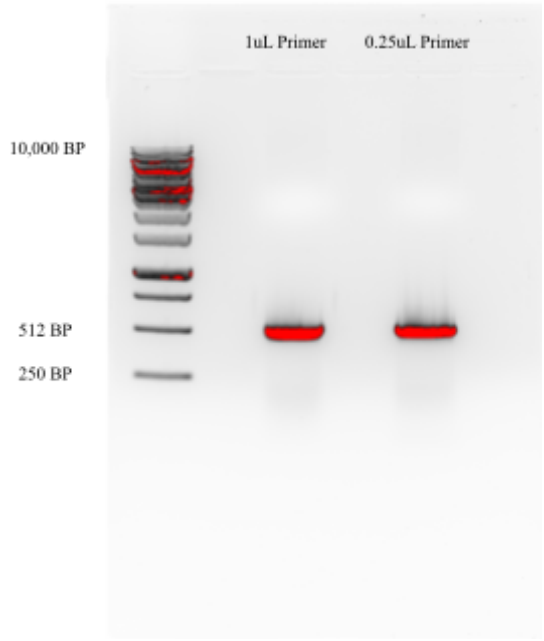


Figure 3A: *Amplified HEK DNA region testing two different primer concentrations*

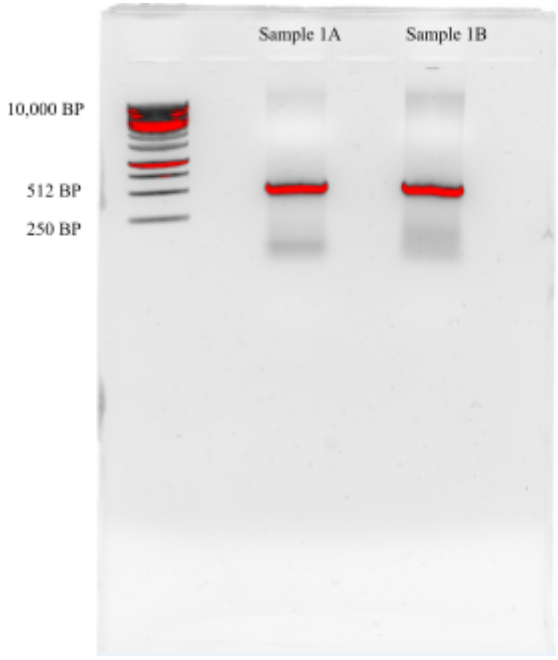


Figure 3B: *Isolated HEK DNA prior to gel extraction and sequencing*

In order to test the accuracy of the sequencing primers as well as to confirm the accuracy of the amplification primers, the samples from **Figure 3B** were sent for sequencing. **Figure 4A** represents the initial sequencing attempt that was made. The “N’s” reflect a lack of good

sequencing and represent unclear nucleotides. This first sequence also produced an inconclusive BLAST result suggesting an unsatisfactory sequence.

Forward:

NNNNNNNNNNNNNNNNNNNNNNNCCNNNNTGNNNGGNCNNGNTCCTTATNNGAGTCACTGGACTATCCTCTGCCCG
GGTTACTGACAATTCTGAGGTCAAACATTGNCAACCACANCAAGGTGNNNGAAANCANCTNCGCGTAGANTTACACG
ATGAAAGTGGGGAAATTCNNGCANCTGGCCTNNATGACCAANTGGANTGTTTCANGAAATCTTTCANGAGTGTNCTG
CTCNTANNTANCCCGTGATCTTGTGCCNATTGCNCAAAAACAATAGNANANTCTNNAAAACTAATANGANCNGTTGT
CNNAANNNGATACCCCGGNTAGAAAGCTGATCTATCCATCCCCCGTCCGCNTNGCTAATTNNANTTATNTATNGCCA
CNCTATNTCACTANNTNCCAAANATTCTNTTTTTNNTTTNNTTGGTGNACC

Reverse:

NNNNNNNNNNNNANNNNNNNNNNTGCGNANTNNTCGGTCTCTCNCAATATNNGAATAAATGGATATTTNTGCNNGNN
TTNNTNAAAAATCTGAGGCAAGCATTGNCTAATCAACNAGGTGAAGGAAAGCTTTTTATCGTAAATTTACTCGATGA
AAGTGGNNNATTCCNGCAACNGCTNANNGACCNGATTGATGCCTTTTNCNTATCTTACANGAATGTTCGGCTNTNTN
TTCTCGTTGTCCTGTGAATGTTN

Figure 4A: *First nucleotide sequencing of amplified ELOVL4 site*

This sequencing result led us to attempt a different purification method: the Promega Wizard® PCR Preps DNA purification system. This vacuum manifold technique saved time when compared to the gel electrophoresis, and resulted in a cleaner sequencing. Both methods were directly compared after being sent for sequencing. **Figure 4B** depicts the second attempt at gel purification and the corresponding sequence. The gel extraction was once again unable to produce a conclusive BLAST due to the number of unclear nucleotides in contrast to the sequencing results following the Qiagen PCR purification method (**Figure 4C**).

Gel purification (Forward):

NNNNNNNNNNNNNNNTGNNGCACTGGGCTCTATTGCCTATGCANNCGGNTNNNNATTTCACTTTAAGNGANTNGNNG
NGNNTTNCAGNNCNGCNAGTCNNCCCGGCGAGAAAAAACNNTCNGGGNAAGAGAGCTNTGCCGCGTGACGGNNNGAG
AATTANTGGGGATATAAACGGGAGAGTAGNGNAAAAGGGCATGTCATCAGAAAAGATANACAAATTGGGTNNGTCNG
CATCTTTCACCGNNGTATTNTCNCCCNGTTTNGGACTCCGGGGANAATATAGTGNTGTCNGNCTCCGGTTNGTGATT
NAAGTTTTGNGATTTCNTGATGNN

Gel purification (Reverse:

NNNNNNNNNNNNNNNNNNNNNANNNNNNCNTNTCATCCTNTACAGCNGGCCGCNTNNNGTTTCATTTTCCAGCNNCA
NNNNNGNNNNANGCGTCACCACTCAGNCTNNGNGNCNNGGCCACCCNTNTCTGATAATGAGAGAGTGNCGACTGNTG
GNAGNACNNAANGAGNNACNNAATTGCTATGTAGCGNNCNNANATNTCTTTANACNAGATNGANNACNNGGNNNCTN
TGTTATTGACANNGCGATNCNCTCTCNNCTNNTCA

Figure 4B: *Second sequencing attempt with gel purification*

PCR purification (Sys) forward:

NNNNNNNNNNCAATGGATGCACTGGGCTCTAATTGCCTATGCAATCAGCTTCATATTTCTCTTTCTTAACTTCTACA
TTCGGACATACAAAGAGCCTAAGAAACCAAAAGCTGGAAAAACAGCCATGAATGGTATTTCAGCAAATGGTGTGAGC
AAATCAGAAAAACAACTCGTGATAGAAAATGGAAAAAAGCAGAAAAATGGAAAAGCAAAAGGAGATTAAATTGAACT
GGGCCTTAACTGTTGTTGACAGTGAGGAAAAACTCCCATATCATATAAAATTTCAGGGAAAACAGAAGCAAANAGAG
CTTGNNCNCTNGATTTTGGGTTNTNTNNTCTCGGATAGAGNNATGGAGTNAANNNATATTTTAGAAANAAAACGGAA
ANNA

PCR purification (Sys) Reverse:

NNNNNNNNNNNCNGCTTTTGGTTTCTTAGGCTCTTTGTATGTCCGAATGTAGAAGTTAAGAAAGAGAAATATGAAGC
TGATTGCATAGGCAATTAGAGCCCAGTGCATCCATTTGGGGAAGGGGCAGTCAGTGTAAAGAGACAGTGCCGTGTGC
CCAATGGTCACATGGAATTGAATCTGAAAAACAGAAATGACAGCACAAAACATTTAATCTGAATAGTAAACAACTTT
TAACAACATCGGCATCTTCTACATAGCTATCACAGGCCCAAATTTTGCCACACTGTGCAAAATTTATTGTTTTCTGG
CTCCCATGGCTAGA

Figure 4C: *Wizard PCR Preps PCR purification system sequencing results*

Figure 4C shows the PCR purification sequencing result that was able to produce a conclusive BLAST result that can be seen in **Figure 5**. This led us to conclude that the PCR purification would be the technique to use moving forward.

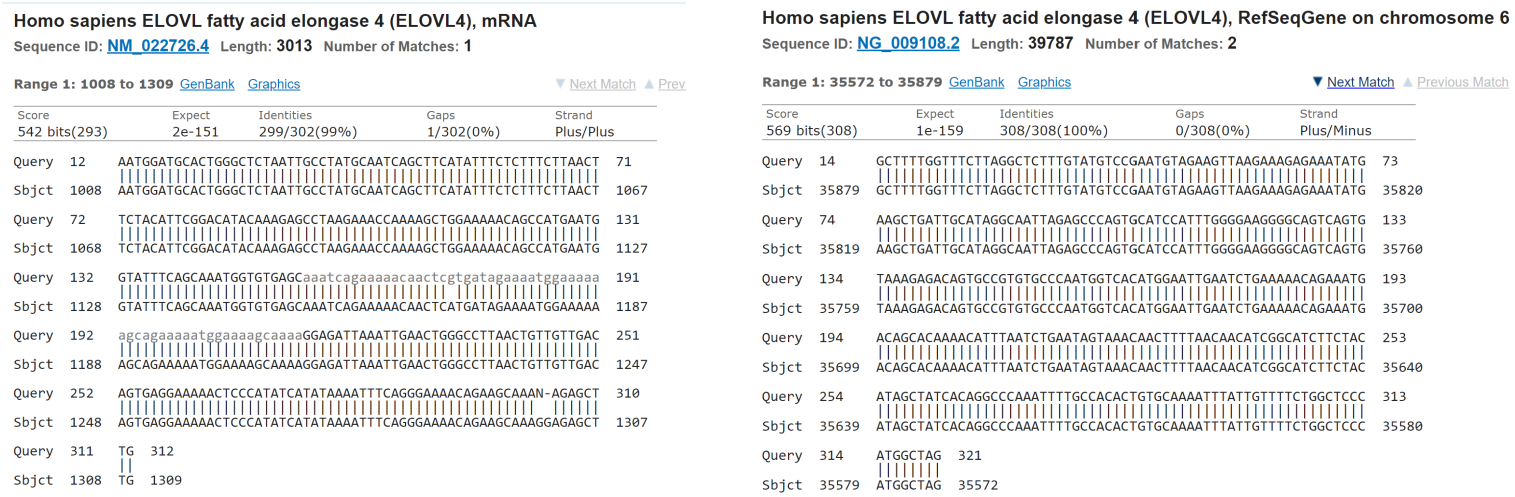


Figure 5: *PCR purification forward and reverse BLAST results demonstrating successful amplification and sequencing of the human ELOVL4 gene from HEK cells.*

Guide Insertion

Another goal of the study was to successfully introduce CRISPR guides into a plasmid, and transform that plasmid into a bacterial line. **Figure 6A** illustrates the first failed attempt at the guide insertion. Although there are several reasons as to why this could have occurred, the protocol was edited and attempted again with a different PCR machine. **Figure 6B** demonstrates

that guides 1.1, 1.2, 1.3, 2.1, 2.2, 2.3 and 3.2 were successfully uptaken by plasmid, having DNA present at ~8,000 bp.

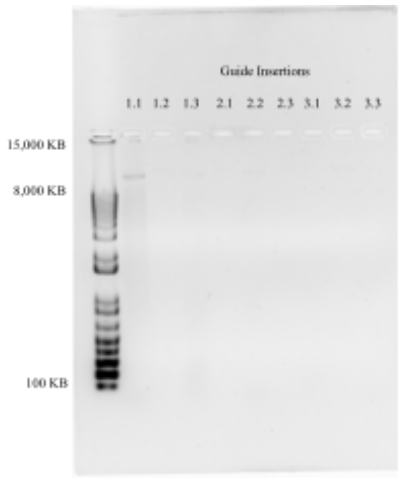


Figure 6A: *Failed plasmid native gel with no reading of any guides*

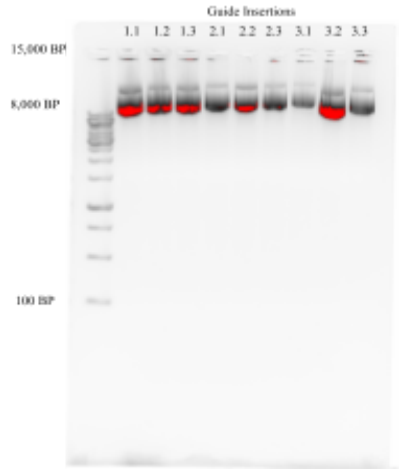


Figure 6B: *Plasmid present ~8000 BP*

To better support these findings and to verify the target sequence; each plasmid was sent for sequencing. **Figures 7A-C** function to display the plasmid sequencing, as well as BLAST results of each of the three guides. **Figures 7A** and **7B** correlate to the appropriate insertion of guides 1 and 2, as represented by the green guide sequence in each of the sequencings. Additionally, both sequences were BLASTed which produced an accurate identification of the ELOVL4 mutation site. Guides 1.2, 1.3, 2.2, 2.3, and 2.4 also replicated a successful insertion after sequencing which highlights the strength of both guides.

Guide 1-1: **AAATATGAAGCTGATTGCAT**

NNNNNNNNNNNNNNNGNNNNNNNNNNTNNNTGGCTTTTNTATCTTGTGGAAAGGACGAAACACCG**AAATATGAAGCTGAT**
TGCATGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTG
CTTTTTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTTTTAGCGCGTGCGCCAATTCTGCAGACAA
ATGGCTCTAGAGGTACCCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGAC
GTCAATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTAC
ATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTGTGCCCAGTAC
ATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTCGAGGTGAGCCCCAC
GTTCTGCTTCACTCTCCCCATCTCCCCCCCCTCCCCACCCCCAATTTTGTATTTATTTATTTTTTAATTATTTTGTGCAG
CGATGGGGGCGGGGGGGGGGGGGGGCGCGCGCCAGGCGGGGCGGGGCGGGGCNAGGGGCGGGGCGGGGCNAGGCGNANA
GGTGCGGCGGCAGCCNATCAAANCGGCGCGCTCCNAAAGTTTCCTTTTNNNGNGAGN

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Sequence ID: **Query_44559** Length: **9905** Number of Matches: **3**

Range 1: 215 to 826 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1038 bits(1150)	0.0	600/615(98%)	4/615(0%)	Plus/Plus

```
Query  1    TGGCTTT-TNTATCTTGTGGAAAGGACGAAACACCGAAATATGAAGCTGATTGCATGTTT  59
Sbjct  215  TGGCTTTATATATCTTGTGGAAAGGACGAAACACCGGGTCTTCGAGAAGA---CCTGTTT  271

Query  60   TAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCA  119
Sbjct  272  TAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCA  331
```

Figure 7A: *Sequencing and BLAST results for Guide 1. The results of the sequenced plasmid was compared with the standard pX330 plasmid. The highlighted portion that does not line up is where the guide sequence was successfully inserted into the plasmid.*

Guide 2-1: **CACCGGAGCCTAAGAAACCAAAAGC**

NNNNNNNNNNNTNTTGGCTTTATATATCTTGTGGAAGGACGAAA**CACCGGAGCCTAAGAAACCAAAAGC**GTTTTAGAGCT
AGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTGTTTTAGAG
CTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTTTTAGCGCGTGCGCCAATTCTGCAGACAAATGGCTCTAGAGGTAC
CCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAGTAACGCCA
ATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATAT
GCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTGTGCCCAGTACATGACCTTATGGGACT
TTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTCGAGGTGAGCCCCACGTTCTGCTTCACTCTC
CCCATCTCCCCCCCCTCCCCACCCCCAATTTTGTATTTATTTATTTTTTAATTATTTTGTGCAGCGATGGGGGCGGGGGG
GGGGGGGGGGCGCGCGCCAGGCGGGGCGGGGCGGGGCGAGGGGCGGGGCGGGGCGAGGCGAANAGGTGCGGCGGCAGCCA
ATCAGAGCGGCGCGCTCCGAAAGTTTCCTTTTATGGCGAGGNGN

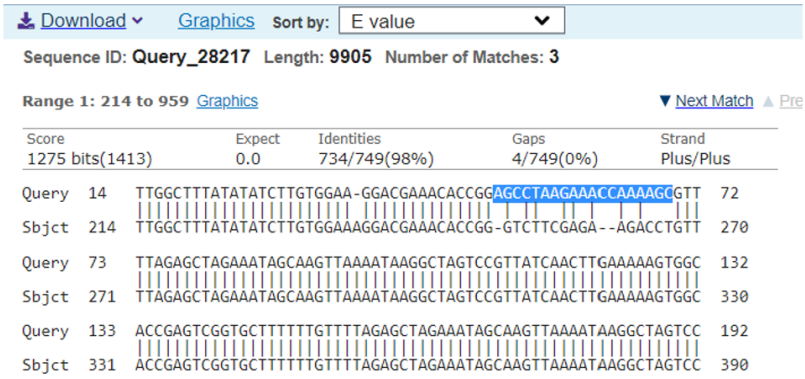

Figure 7B: *Sequencing and BLAST results for Guide 2. The results of the sequenced plasmid was compared with the standard pX330 plasmid. The highlighted portion that does not line up is where the guide sequence was successfully inserted into the plasmid.*

Figure 7C demonstrates the failure of guide 3 to be inserted into the plasmid sequence, as addressed by the red sequence. This was the case for guides 3.1, 3.2, and 3.3.

Guide 3-1 X: **TCTTTCTTAACTTCTACATT**

NNNNNNNNNNNNNNNNTGGCTTTATATATCTTGTGGAAGGACGAAACACCGGGTCTTCGAGAAGACCTGTTTTAGAGCTA
GAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTGTTTTAGAGC
TAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTTTTAGCGCGTGCGCCAATTCTGCAGACAAATGGCTCTAGAGGTACC
CGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAGTAACGCCAA
TAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATG
CCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTGTGCCCAGTACATGACCTTATGGGACTT
TCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTCGAGGTGAGCCCCACGTTCTGCTTCACTCTCC
CCATCTCCCCCCCCTCCCCACCCCCAATTTTGTATTTATTTATTTTTTAATTATTTTGTGCAGCGATGGGGGCGGGGGGG
GGGGGGGGGCGCGCGCCAGGCGGGGGCNGGGCGGGNCGAGGGGCGGGGCGGGGCNAGNCNGANAGGTGCGGCGGCAGCCA
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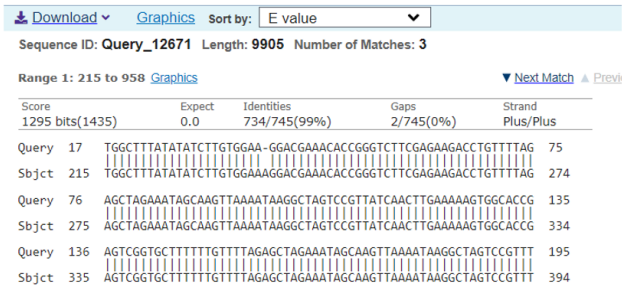

Figure 6C: *Sequencing and BLAST results for Guide 3. There is no insertion in this plasmid sequence as indicated by the lack of non-aligned regions showing perfectly aligned sequences with no guide insertion.*

DISCUSSION

PCR and SEQ Primers for ELOVL4 Mutation Site

Proper amplification and sequencing primers were designed and applied to the HEK293 genome in order to analyze the desired ELOVL4 mutation region. This was necessary for the foundation of the study in order to identify the desired region and certify that sequencing of that region is possible. **Figure 3B** ensures that our amplification primers were successful in isolating the HEK DNA due to the bands at ~500 bp. This amplification success allows us to isolate the mutation region and carry on with the study. **Figure 4A** and **4B** conclude that our gel purification method was unsuccessful due to the accumulation of N's that represent unclear nucleotides and a bad sequence. **Figure 4C** depicts the success of the Promega Wizard® PCR Preps DNA purification system and **Figure 5** confirms this success according to the accurate BLAST. The sequencing success not only confirms that the amplification primers selected for the correct DNA region, but also ensures that sequencing of this region will be possible going forward once the later steps of the study have concluded.

E. Coli line with Guide Inserts

Due to a clear BLAST product from both amplifying primers and sequencing primers, the designed CRISPR guides were able to be inserted into the pX330 plasmid. Guide insertion was not initially successful, as **Figure 6A** illustrates, which led us to proceed with a different apparatus. This new protocol led to the successful insertion of two of the three guides that were tested: guides 1 and 2. **Figure 6B** depicts the gel that was loaded with the different guides and shows the successful uptake of plasmids in various replicates. These were taken to sequencing in order to confirm if the guides themselves were actually inserted into the DNA, which can be seen in **Figures 7A-C**. Guides 1 and 2 were identified within the plasmid sequence, which can be seen in **Figures 7A** and **7B**. These guides also were inserted in each of the other replicates: 1.2, 1.3, 2.2, and 2.3 which suggests complete insertion success.This was a significant result of the study because we now have two different lines of E coli. that have desired CRISPR guides within their plasmid that would target the ELOVL4 mutation site. **Figure 7C** shows the failed uptake of guide 3 which was bound to happen with at least one of the guides. Glycerol stocks for each guide replicate were also stored so new colony batches can be created if more DNA is required.

Future Directions

The immediate next step in this project is to test the newly generated CRISPR guide plasmids to see if there is successful cutting in the HEK cells. The pX330 CRISPR Cas9 plasmid with the inserted guides will be transfected into HEK cells. After 1 - 2 weeks of growth, the HEK cells will be lysed and the genomic DNA extracted using the Qiagen kit. Using our primers and optimized protocols, the ELOVL4 gene sequence will then be amplified, and sent for sequencing using the sequencing primers. If the CRISPR guides cause the desired cuts within the genome, the cells will attempt to repair the double-stranded DNA. However, without a repair guide, these repairs are random and result in InDels (Insertions and deletions) in the sequence as they repair. This is generally how CRISPR is used to generate functional knockouts. For our analyses, the sequencing will tell us if the guides are effective in generating cuts in the ELOVL4 sequence. Each cell whose genome is cut will repair it differently due to random formation of the InDels. Thus, sequencing whereby we see accurate sequence generation up to the cut site, followed by a lack of consensus sequencing from the bulk genomic DNA will indicate differences in sequence at the site of the cut. The same will be true for the reverse sequencing guide, which should also generate consensus sequence information right up to the cut site, with a lack of consensus sequencing afterwards. If the sequencing yields consensus sequencing all the way through the cut sites and beyond, then these guides were not effective in guiding the Cas9 enzyme to the correct genomic sequence for cutting.

In future laboratory studies, potential repair sequences should be devised to insert the mutated gene of interest where indels were created in preliminary steps. Perhaps a fourth alternative guide sequence could be utilized as well to supplement the failed annealing of guide 2 in the pX330.puro plasmid. Off-target effects would then need to be deduced and visualized as well, likely through Benchling, or a similar software, to ensure necessary genes were not undesirably altered. Once the successful guides and repair combination has been created and confirmed in HEK cells, transfection, and subsequent mutation of induced pluripotent stem cells (IPSCs) could be attempted. Monoclonal cultures then utilizing IPSCs could be grown to be utilized as candidates for human organoid creation for future studies involving potential modalities to treat and improve the lives of individuals with Stargardt's Disease.

REFERENCES

1. Liu A, Lin Y, Terry R, Nelson K, Bernstein PS. Role of long-chain and very-long-chain polyunsaturated fatty acids in macular degenerations and dystrophies. Clin Lipidol. 2011;6(5):593-613. doi: 10.2217/clp.11.41. PMID: 25324899; PMCID: PMC4196247.

2. Zhang K, Kniazeva M, Han M, Li W, Yu Z, Yang Z, Li Y, Metzker ML, Allikmets R, Zack DJ, Kakuk LE, Lagali PS, Wong PW, MacDonald IM, Sieving PA, Figueroa DJ, Austin CP, Gould RJ, Ayyagari R, Petrukhin K. A 5-bp deletion in ELOVL4 is associated with two related forms of autosomal dominant macular dystrophy. Nat Genet. 2001 Jan;27(1):89-93. doi: 10.1038/83817. PMID: 11138005.
3. Thomas P, Smart TG. HEK293 cell line: a vehicle for the expression of recombinant proteins. J Pharmacol Toxicol Methods. 2005 May-Jun;51(3):187-200. doi: 10.1016/j.vascn.2004.08.014. PMID: 15862464.
4. Cheng V, Rallabandi R, Gorusupudi A, Lucas S, Rognon G, Bernstein PS, Rainier JD, Conboy JC. Influence of very-long-chain polyunsaturated fatty acids on membrane structure and dynamics. Biophys J. 2022 Jul 19;121(14):2730-2741. doi: 10.1016/j.bpj.2022.06.015. Epub 2022 Jun 16. PMID: 35711144; PMCID: PMC9382336.
5. Hopiavuori BR, Anderson RE, Agbaga MP. ELOVL4: Very long-chain fatty acids serve an eclectic role in mammalian health and function. Prog Retin Eye Res. 2019 Mar;69:137-158. doi: 10.1016/j.preteyeres.2018.10.004. Epub 2018 Oct 25. PMID: 30982505; PMCID: PMC6688602.
6. Agbaga, M.-P., Brush, R. S., Mandal, M. N., Henry, K., Elliott, M. H., & Anderson, R. E. (2008). Role of stargardt-3 macular dystrophy protein (ELOVL4) in the biosynthesis of very long chain fatty acids. *Proceedings of the National Academy of Sciences*, *105*(35), 12843–12848. https://doi.org/10.1073/pnas.0802607105
7. Shida Chen, Nicholas A Popp & Chi-Chao Chan (2014) Animal models of age-related macular degeneration and their translatability into the clinic, Expert Review of Ophthalmology, 9:4, 285-295, DOI: 10.1586/17469899.2014.939171
8. Hryhorowicz M, Lipiński D, Zeyland J, Słomski R. CRISPR/Cas9 Immune System as a Tool for Genome Engineering. Arch Immunol Ther Exp (Warsz). 2017 Jun;65(3):233-240. doi: 10.1007/s00005-016-0427-5. Epub 2016 Oct 3. PMID: 27699445; PMCID: PMC5434172.
9. Doench, J. G., Fusi, N., Sullender, M., Hegde, M., Vaimberg, E. W., Donovan, K. F., Smith, I., Tothova, Z., Wilen, C., Orchard, R., Virgin, H. W., Listgarten, J., & Root, D. E. (2016). Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nature biotechnology*, *34*(2), 184–191. https://doi.org/10.1038/nbt.3437