USING CRISPR TO KNOCKOUT RPA3B IN *ARABIDOPSIS THALIANA*

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Utilizing CRISPR to Knockout RPA3B in *Arabidopsis thaliana*

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Replication Protein A (RPA) is a heterotrimeric single stranded DNA binding (SSB) protein. RPA has functions in DNA metabolism and meiosis (1). Specifically, the subunit RPA3 helps give structure to the heterotrimeric complex, allowing the RPA1 subunit to bind to DNA in order to perform various reactions involved in DNA replication and repair. To further characterize the activity of RPA3B, knockouts will be generated in *Arabidopsis thaliana* using CRISPR, a gene editing technology that has become widely used recently.

CHAPTER I

INTRODUCTION

Maintenance of single stranded DNA (ssDNA) is a cellular function that is critical to successful cellular growth and replication. One protein that is able to unwind double stranded DNA (dsDNA) is Replication Protein A (RPA). RPA is an ssDNA binding protein (SSB) that primarily serves to protect single-stranded DNA from nucleolytic degradation and hairpin formation. RPA and other SSB's play critical roles in DNA replication, recombination, and repair, as well as meiosis (1). Additionally, unlike other SSB's, RPA also has a role in DNA damage signaling (2).

In *Arabidopsis thaliana,* RPA is composed of three subunits: RPA1 (~70 kiloDaltons), RPA2 (~32 kDa), and RPA3B (~14 kDa). Each subunit can be expressed from any of a varying number of paralogous genes. In *A. thaliana*, 5 RPA1 paralogs, 2 RPA2 paralogs, and 2 RPA3 paralogs have been found. Due to some functional redundancy between paralogs, *A. thaliana* mutant for certain RPA paralogs can still survive and develop normally, although this is not always the case (3). While much has been learned about the function and mechanisms of action of RPA1, the role of RPA2 and RPA3 is not as well understood (2). In order to bridge this gap in knowledge, a gene knockout targeting RPA3B will be employed to better study the function of RPA3.

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a naturally occurring bacterial and archaeal immune system that functions by recognizing foreign DNA via base pairing with an RNA protospacer, an oligonucleotide that complimentary to a section of the foreign DNA, and cleaving it via the activity of the Cas9 protein (4). CRISPR was first observed as an array in the genome of certain bacteria without its purpose being known. It was then recognized that many of the spacer sequences between repeats in the array were derived from either plasmid or viral DNA sequences. This lead to the hypothesis that CRISPR could function as an adaptive immune system, which was later shown to be correct (4, 5). Recently, this naturally occurring system has been harnessed to make targeted cuts in the genome of the subject being studied (6). Replacing DNA sequences in the CRISPR array derived from plasmid or viral DNA with a sequence that matches a region of genomic DNA allows CRISPR/Cas9 to make targeted cuts in an organism's genome. By using a binary plasmid system with *Agrobacterium tumefaciens* as a vector, T-DNA can be transferred into plants that, when recombined into the genome, will allow the infected plants to express all components of the CRISPR/Cas9 system necessary for gene knockout.

In this experiment, CRISPR/Cas9 will be used to attempt to knockout *rpa3b*. This will be done in both wild-type *A. thaliana* as well as *A. thaliana* with an *rpa3a* null mutation in order to generate both *rpa3b* single mutant seeds and *rpa3a rpa3b* double mutant seeds. Survival and growth of *rpa3a rpa3b A. thaliana* will indicate that there could be a third RPA3 paralog that has yet to be documented. Following successful knockout, phenotypic traits to be observed in surviving *A. thaliana* may include: leaf shape, date of flowering, number of flowers, root length, sensitivity to ionizing and non-ionizing radiation, sensitivity to depleted dNTP pools, sensitivity to DNA polymerase inhibition, and telomere length (3).

CHAPTER II

MATERIALS AND METHODS

Protospacer Design: The protospacer was designed so as to align with a sequence in the RPA3B gene that is a part of the exon, and as close to the start codon as possible (Figure 1). Since *Streptococcus aureus* Cas9 will be used, the PAM sequence used for designing the protospacer was 5’-NNGAA-3’.

```
ttccaacaaaccggttcggtttaacactattaatttctcgacctcgctcctatcttctcctctcattcgtcaatcca
gagactagggttttcttcccgccgaaacctcaccggaaattctctccggcatctcctaattcgtcaatcgggtactg
ttaaatcacaaggctttagtaatttcttcttatacataggtctaaatctatctgcttgatttgaaatttggattttg
gtctaattagggactgacactaccaatttacttaatgatttgaggataattacttcgattttgacttagatgatatg
aatttgatttagtttcagtgagaaagacgaatgaatgttttgatttttatagagaATGGATACATCAAGTCCTGCTG
CTTTTGTGAATGGAGCTTTGTTGAGAAGGTACATTGGTCAGAAAGTGAGAGCAGTGATTCAAGTTATCAGATCAGAT
GTTGGATCAGTGATTGGTAAATCGACTGATGATCAACAGATTGTTGTTAAAGGTTCTCCTCAACCGCCTTTAACTAC
TTACCTTGAGGTAATTGGAATTGCTGAGACTGACAACACTATTCGTGCTGAAGTTTGGACCAACTTTGGTGATAGTT
TCGgtatgaatttaagcaaaaatcattcctttaataagctatgtcctttatgatttgcaaattcttgtgtcttgatt
tggggtcttggcttgtttgcagATGTGCAAAACTACAATGAGCTATGTAAGCTTGCAAATGGTGAGTTTAGACACTT
GTTCATCTAAatcacagggtattggtgatgaatgtggaaagctttttttcttggagattctttgggttggagctattg
atatgcctggttgatctttagtattgaaattcttctgtttcatgttagtttcacggtttctagtatttatcttatgg
tctttgaaatctgtatgaaaactcttctgtgtcatattagaattaaggtaaactgtgttagtcgatcttactatgaa
tcttatgtttcttgttttgtgttttggttctctttgcttttcttttgattccatcacattggatcctagcttgtaaa
tggtataaaactatataaaatccctaaaaa
```

Figure 1. RPA3B full-length genomic DNA, highlighting the start codon (light green), stop codon (red), 5’-NNGAA-3’ PAM sequence (purple), protospacer sequence (light blue) and exon (yellow font). The protospacer was designed to cut at an early point in the exon in the gene.

Protospacer Annealing: 2 µL (50 µM) of each protospacer was mixed with 46 µL of double distilled water. Protospacers were annealed at 95°C for 5 minutes, then allowed 20 minutes at room temperature to cool.

Protospacer Ligation: The protospacer was ligated into the entry vector (pen-Sa-Chimera) by mixing 2 µL of the purified digested entry vector, 3 µL of annealed protospacers, 1 µL of T4 Ligase, 1 µL T4 Ligase Buffer, and 3 µL double distilled water. The reaction mixture was then incubated at 16°C for 16 hours.

Preparation of Selective Growth Media: 400 mL MilliQ Water, 20 g LB Agar Medium, and MilliQ Water to bring the total volume to 500 mL were mixed in a 1L bottle, then stirred until the mixture was fully dissolved. The bottle was capped and covered with autoclave tape, then placed in an autoclave bin containing a small amount of water, and autoclaved using an L-20 cycle. The autoclaved flask cooled until it could be touched comfortably. A work station in the flow hood was cleaned by spraying the work station and gloved hands with 70% EtOH, and lighting a Bunson Burner next to your station to help prevent airborne contamination. Add .5 mL of 100 µg/mL carbenicillin solution to the mixture (when later selecting for the destination vector, use the same volume of 100 µg/mL spectinomycin), then pour into 12-15 labeled plates. Allow the plates to cool for a few hours or overnight before plating bacteria. Store plates in a plastic sleeve in the cold room. Test plates by streaking one plate with a known carbenicillin resistant bacteria strain, and another plate with a nonresistant strain to ensure appropriate antibiotic activity. If only resistant bacteria grow, the plates may be used to isolate *E. coli* cells at a later time.

Transformation of *Escherichia coli:* Defrost DH5α *E. coli* cell culture, and add 5 µL of Ligated Entry Vector to the cell culture. Place the cell mixture on ice for 30 minutes, then place the cell mixture in a 42°C water bath for precisely 45 seconds (heat-shocking the cells makes them competent, and allows for the entry of the vector containing the protospacer into the bacteria). Immediately return the tube to ice for 30 minutes, then add 300 µL of Luria Broth to the cellular mixture. Incubate at 37°C in the rotating drum for one hour. After incubation, cells can be plated on Agar supplemented with carbenicillin, or stored at room temperature for up to 3 days. Once plated, incubate overnight at 37°C (Figures 4 and 5 display growth resulting from plating immediately and plating after two days, respectively).

Transformation Confirmation via Colony PCR: Test approximately 5 colonies using the forward oligo and SS-129 as primers when testing for the entry vector. Use SS-42 and SS-43 when testing for the destination vector. For each colony to be tested, prepare a reaction mixture containing: 5 µL 10x Standard *Taq* Reaction Buffer, 1 µL 10 mM dNTP's, 1 µL of 10 µM forward primer (forward protospacer oligo), 1 µL of 10 µM reverse primer (M13 rev), .25 µL of Hot Start *Taq* DNA Polymerase, a small inoculant taken from an *E. coli* colony, and double distilled water to dilute the final reaction volume to 50 µL before use of the thermocycler for DNA amplification (Table 1).

Table 1. PCR conditions used for colony PCR in order to confirm the presence of the protospacer in *E. coli*.

PCR Conditions		
Temperature	Time	
98°C	30 s.	
98°C	10 s.	39 X
56°C	30 s.	
68°C	23 s.	
68°C	5 min	

Load and run the PCR product samples in a 1% agarose gel. Confirm successfully transformed colonies by observing a band at approx. 370 base pairs for the entry vector, or 1 kb for the destination vector.

Miniprep and Plasmid Purification: Select one or two successfully transformed colonies to inoculate a 5 mL liquid growth culture. Miniprep plasmid DNA using Machery-Nagel NucleoSpin Plasmid kit. Centrifuge the liquid culture tubes in order to pellet the *E. coli* cells at the bottom of the tube. Discard supernatant from the culture, and add 250 µL of A1 Buffer to the tube. Resuspend the *E. coli* cells in the buffer by pipetting up and down. Add 250 µL of A2

Buffer to the tube. Mix by gently inverting the tube eight times, then allow five minutes to incubate at room temperature. Add 300 µL of A3 buffer to the tube. Mix by gently inverting the tube until the blue sample turns completely colorless (signaling complete neutralization of the reaction). Centrifuge at 11,000g for 7.5 minutes, or longer if the supernatant is not clear after centrifugation. Load 750 µL of the clear lysate solution into a provided column placed into a collection tube. Centrifuge the column and collection tube it rests in for 1 minute at 11,000g. Discard all flow-through. Wash the silica membrane by adding 600 µL of A4 buffer to the column. Centrifuge the column and collection tube it rests in for 1 minute at 11,000g. Discard all flow-through. Dry the silica membrane by centrifuging the column and collection tube it rests in for an additional 2 minutes at 11,000g. Elute the plasmid DNA from the silica membrane using 50 µL of double distilled water. Nanodrop the elution to determine DNA concentration and salt content. Sequence the purified plasmid using SS42 as a primer to ensure correct integration of the protospacer into the vector.

LR Reaction: Adjust pEn-Sa-Chimera PCR (entry vector) product concentration to 50 ng/µL. Adjust pDe-Cas9 (destination vector) concentration to 100 ng/µL. Mix 2 µL of purified entry vector PCR product, 3 µL of destination vector, 4 µL TE-buffer, and 1 µL of LR Clonase II (TE-buffer pH 8 contains: 10 mM Tris-HCl, 1 mM EDTA. LR Clonase II catalyzes the recombination of the entry vector into the destination vector using *attL* and *attR* sites). Vortex, then centrifuge the reaction mixture. Allow 2 hours for the reaction to incubate at room temperature. Add 1 µL Proteinase K, then incubate at 37°C for 10 minutes.

Transformation of *Agrobacterium tumefaciens:* Thaw a 50 µL solution of *A. tumefaciens* cells one ice. To the cell solution, at 50 ng of the destination vector. Transfer this solution to an electroporation cuvette that has been chilled at -20°C. Apply the following conditions to the cuvette using the electroporator: 2.00 V, 200 Ω, 25 μF. After electroporation, add 1 mL SOC media to the cuvette, and mix by pipetting up and down. Transfer to a 1 mL microcentrifuge tube, and incubate the transformed cells at 30°C. Incubate for 4 hours. Plate 100 µL of the culture onto an LB plate supplemented with spectinomycin.

A. tumefaciens Mini-Culture: For each colony that is to be minicultured, add 5 mL LB, 5 µL of 50 µg/mL Kanamycin, and 5 µL of 10 µg/mL Gentamycin. Using a pipette tip, collect a small inoculant of *Agrobacterium* cells, and eject the tip into the culture tube. Incubate the cultures at 28°C for 2 days while rotating. The miniculture can then be used to inoculate a 500 mL culture, which is grown to an OD_{600} of about 1 (24-36 hours).

Floral Dip: Suspend transformed *Agrobacterium* to an OD_{600} of 1 in 500 mL of 5% sucrose solution. Add Silwet L-77 to the solution to a concentration of .05%, and Acetosyringone to a concentration of 150 µM. Dip plants to be transformed by *Agrobacteria* in solution for approximately 1 minute, with agitation. Maintain plants in a high humidity environment for 16-24 hours to ensure *Agrobacterium* infection occurs, then continue to grow plants in the growth chamber. Seeds will be ready for planting on MS + Basta media and MS + carbenicillin in approximately 1 week.

Seedling Germination: Seedlings were plated on .5% MS + carbenicillin after sterilization with 70% ethanol.

DNA Isolation: DNA was extracted from plants using a phenol-chloroform extraction method. Tissue samples were collected from each germinated plant, and pulverized after flash freezing. 500 µL 2x CTAB buffer was added to the tissue, and the solutions were then allowed 30 minutes to incubate at 65°C. Following the incubation, 500 µL phenol chloroform was added to each sample. The samples were briefly vortexed, then centrifuged at 15,000 rpm for 20 minutes. After centrifugation, the top layer of the solution was removed and transferred to 500 µL of isopropanol. Samples were refrigerated for approximately 1 hour before centrifuging at 15,000 rpm for 30 minutes. The isopropanol was removed from each solution, leaving only the precipitate. The precipitate was cleaned with 500 µL of ethanol, then dissolved in 100 µL of RNase free water.

CHAPTER III

RESULTS AND DISCUSSION

CRISPR is a very low efficiency procedure, in which several things must happen correctly in order for a large mutation to occur. First and foremost, Cas9 and other proteins that must be used in CRISPR are not naturally occurring in plants, and must be transformed into the cell along with the protospacer. These proteins must then be transcribed and modified with non-native cellular machinery, introducing the possibility of errors. These proteins must then be transported into the nucleus of the cell, along with a guide RNA, which is a very low efficiency procedure. An efficient CRISPR procedure yields transformants from .5% to 5% of the time (7). Additionally, the success rate of floral dip of .4% was much lower than expected (8). With a lower than expected efficiency in transforming plants via the floral dip method, it is not surprising that a transgenic plant was not obtained from the 21 surviving plants.

If plants with a large-scale deletion in RPA3B were obtained via this methodology, we would have assessed whether or not these plants had any reduced ability to replicate or repair DNA. Although it is not known precisely what role the 3rd subunit plays in these functions of RPA, it is suspected that a mutation that would affect the structure of this subunit could affect the activities of the other subunits (1, 3). Had a RPA3B knockout plant been obtained via this procedure, seeds from that plant would have been cultivated via similar procedures as mentioned in this paper. These offspring could have then been assayed via leaf shape, date of flowering, number of flowers, root length, sensitivity to ionizing and non-ionizing radiation, sensitivity to depleted dNTP pools, sensitivity to DNA polymerase inhibition, and telomere length.

Of the 21 plants generated by this study, there were no abnormalities in development. Plants grew over a time frame consistent with wild type plants, and the phenotype of these plants matches the wild type. This is consistent with the similarity in the sequence between all of these plants and the wild type throughout the RPA3B gene. In order to generate mutant plants, this procedure must be repeated with additional wild type plants. Since our floral dipping efficiency was much lower than anticipated, procedural changes, such as shortening the submerged time to be more consistent with published practices, could aid in increasing the probability of obtaining a transgenic seedling.

Both wild type and *rpa3a* plants were dipped in the *A. tumefaciens* solution, resulting in selective growth of the wild type plants; however, no selection acted upon the *rpa3a* plants. This is likely due to kanamycin resistance having been conferred to these plants during a previous transformation event. Since the results of this selection were invalid, no seedlings could be transferred to soil for further study. It is hypothesized that a *rpa3a x rpa3b* double mutation would result in a lethal phenotype, but this has not been confirmed (3).

An additional experiment was run in parallel to this procedure within The Shippen Lab, where a different protospacer design was used in attempt to accomplish the same goals as set forth here. This was done so as to compare the efficiency of different protospacer adjacent motifs in generating deletion and frameshift mutations in *Arabidopsis*. After sequencing mutant plants, neither protospacer design yielded a large-scale mutation in offspring plants.

CHAPTER IV

CONCLUSION

In this experiment, CRISPR technology was used in an attempt to knockout RPA3B in *Arabidopsis thaliana*. The procedure used was successful in transforming CRISPR genes into *Arabidopsis*; however, there were no knockout mutants generated despite this. Mutations were found in the sequenced RPA3B gene of 2 plants. Both of these mutations were single nucleotide substitutions, and were therefore unlikely to yield mutations affecting the function of RPA3B. For further experimentation, it is recommended to generate additional generations of offspring plants to increase the likelihood of obtaining a plant with a significant CRISPR mutation.

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APPENDIX

Sequences producing significant alignments:	Score (bits)	E Value
AT4G18590.1 | Symbols: | Nucleic acid-binding, OB-fold-lik...	40	4e-04
AT2G18900.1 | Symbols: | Transducin/WD40 repeat-like super...	28	1.5
AT5G05630.1 | Symbols: | Amino acid permease family protei...	26	5.8
AT5G33990.1 | Symbols: | transposable element gene | chr5:...	26	5.8
AT5G45880.1 | Symbols: | Pollen Ole e 1 allergen and exten...	26	5.8
AT5G34965.1 | Symbols: | transposable element gene | chr5:...	26	5.8
AT5G19490.1 | Symbols: | Histone superfamily protein | chr...	26	5.8
AT5G17420.1 | Symbols: IRX3, CESA7, ATCESA7, MUR10 | Cellul...	26	5.8
AT4G30110.1 | Symbols: HMA2, ATHMA2 | heavy metal atpase 2 ...	26	5.8
AT4G05589.1 | Symbols: | transposable element gene | chr4:...	26	5.8
AT3G09930.1 | Symbols: | GDSL-like Lipase/Acylhydrolase su...	26	5.8
AT2G14010.1 | Symbols: | transposable element gene | chr2:...	26	5.8
AT2G43070.1 | Symbols: SPPL3, ATSPPL3 | SIGNAL PEPTIDE PEPT...	26	5.8
AT1G59890.4 | Symbols: SNL5 | SIN3-like 5 | chr1:22043829-2...	26	5.8
AT1G59890.2 | Symbols: SNL5 | SIN3-like 5 | chr1:22043829-2...	26	5.8
AT1G59890.3 | Symbols: SNL5 | SIN3-like 5 | chr1:22043829-2...	26	5.8
AT1G32950.1 | Symbols: | Subtilase family protein | chr1:1...	26	5.8
AT1G32960.1 | Symbols: ATSBT3.3, SBT3.3 | Subtilase family ...	26	5.8
AT1G10450.1 | Symbols: SNL6 | SIN3-like 6 | chr1:3431780-34...	26	5.8
AT1G13540.1 | Symbols: | Protein of unknown function (DUF1...	26	5.8
AT1G21770.1 | Symbols: | Acyl-CoA N-acyltransferases (NAT)...	26	5.8
AT1G59890.1 | Symbols: SNL5 | SIN3-like 5 | chr1:22043830-2...	26	5.8
AT1G42060.1 | Symbols: | transposable element gene | chr1:...	26	5.8

Figure A1. BLAST results returned when searching for the protospacer sequence across the *Arabidopsis thaliana* genome. The sequence at4g18590 is the gene of interest, RPA3B. Only 1 sequence, at location at2g18900, shows alignment at a level where it is expected that up to 1.5 sequences in the *Arabidopsis thaliana* genome would align to that degree or better by random chance. Several sequences show alignment at a level where 5.8 sequences in the genome are expected to align by chance.

```
>AT4G18590.1 | Symbols:  | Nucleic acid-binding, OB-fold-like protein |
           chr4:10236225-10237185 FORWARD LENGTH=961
          Length = 961

 Score = 40.1 bits (20), Expect = 4e-04
 Identities = 20/20 (100%)
 Strand = Plus / Plus


Query: 1   catcaagtcctgctgctttt 20
           ||||||||||||||||||||
Sbjct: 307 catcaagtcctgctgctttt 326
```

```
>AT2G18900.1 | Symbols:  | Transducin/WD40 repeat-like superfamily protein |
           chr2:8188257-8192489 REVERSE LENGTH=4233
          Length = 4233

 Score = 28.2 bits (14), Expect = 1.5
 Identities = 14/14 (100%)
 Strand = Plus / Plus


Query: 1   catcaagtcctgct 14
           ||||||||||||||
Sbjct: 332 catcaagtcctgct 345
```

```
>AT5G05630.1 | Symbols:  | Amino acid permease family protein |
           chr5:1682321-1684191 FORWARD LENGTH=1871
          Length = 1871

 Score = 26.3 bits (13), Expect = 5.8
 Identities = 13/13 (100%)
 Strand = Plus / Minus


Query: 7   gtcctgctgcttt 19
           |||||||||||||
Sbjct: 464 gtcctgctgcttt 452
```

Figure A2. BLAST results showing sequence alignment between the protospacer and various locations in the *Arabidopsis thaliana* genome. Expected 20 nucleotide alignment occurs between the protospacer and the targeted gene, RPA3B. Fourteen nucleotide alignment occurs between the protospacer and the most similar non-targeted gene (at2g18900; Expect=1.5). Thirteen nucleotide alignment occurs between the protospacer and sequences at the Expect=5.8 level of alignment. Since 15 nucleotides aligning is the minimum level of alignment necessary for CRISPR/Cas9 to cleave DNA, the use of this protospacer will serve to minimize off-target effects.

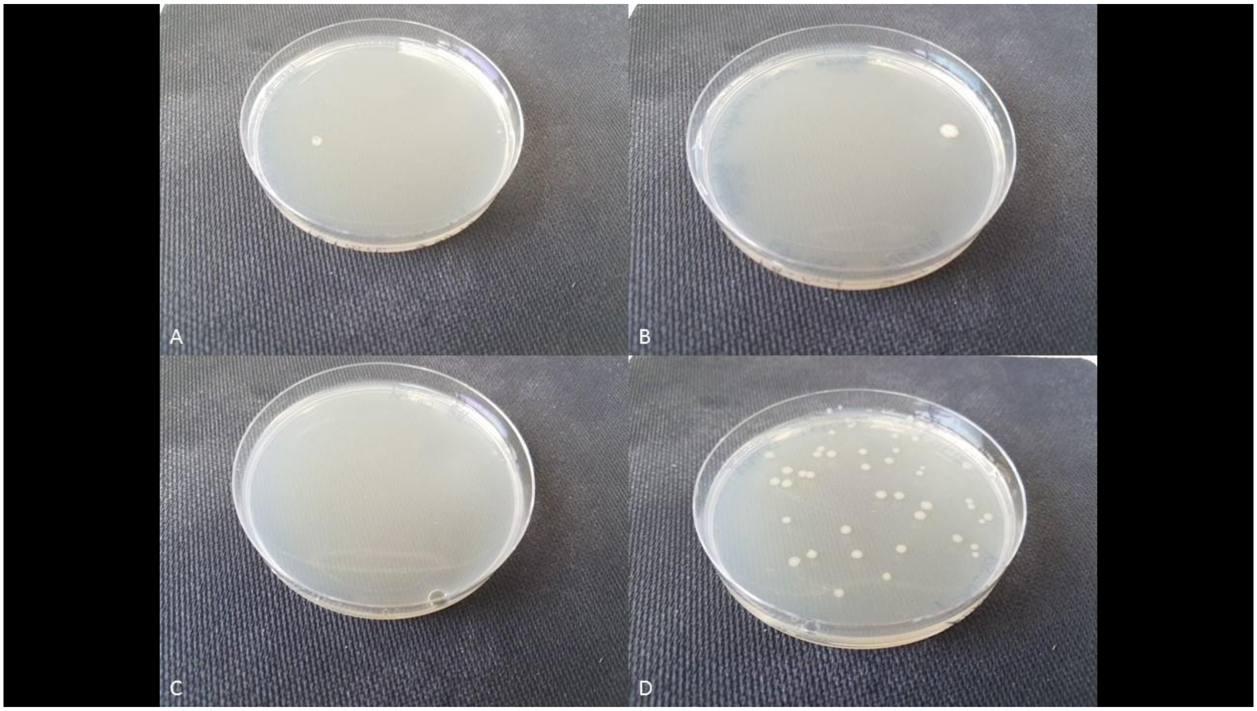

Figure A3. Upon transformation, plates were incubated at 37°C overnight. Displays growth pattern after (A) transformation using Pen-Sa-Chimera ligated with the protospacer by Behailu; (B) transformation using Pen-Sa-Chimera ligated with the protospacer by John; (C) no transformation (negative control); (D) transformation using Pen-Sa-Chimera (positive control). Plates above were used to attempt to isolate the plasmid from *E. coli*.

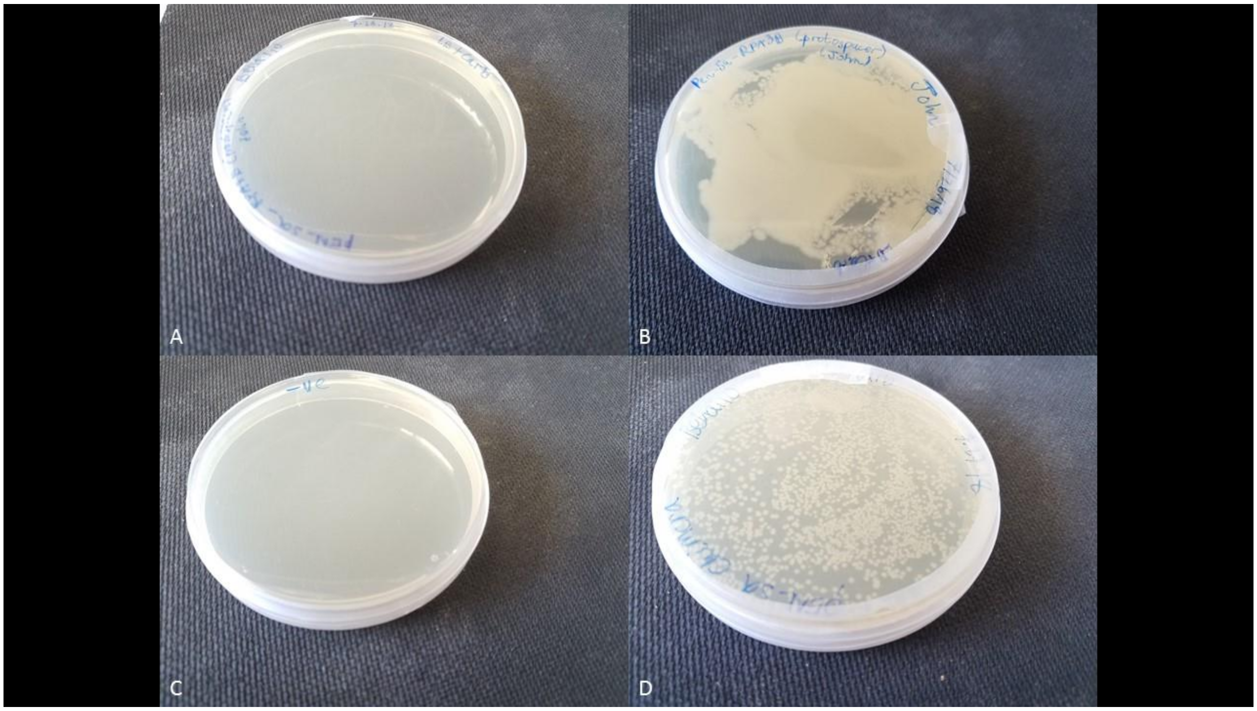

Figure A4. Upon transformation, plates were incubated at 37°C overnight. Displays growth pattern after (A) transformation using Pen-Sa-Chimera ligated with the protospacer by Behailu; (B) transformation using Pen-Sa-Chimera ligated with the protospacer by John; (C) no transformation (negative control); (D) transformation using Pen-Sa-Chimera (positive control). Plasmid purification was not attempted on any of the colonies grown on these plates.

CTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAAC
CAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGGCC
GCTACAGGGCGCTCCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGTTTCGGTGCGGGCCT
CTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGG
TTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAGCGCGACGTAATACGACTCACTATAGGGCG
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AAAACGAAGCTGAGTTTATATACAGCTAGAGTCGAAGTAGTG|ATTGGGGTCTTCGAGAAGACCT|GTT
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CGAGATTTTTACGCGTCCTGAGGACCCAGCTTTCTTGTACAAAGTTGGCATTATAAAAAATAATTGCTC
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CCTTCCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAACATGGTCATAGCT
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AGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTT
TTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTA
CGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGG
ATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACT
TGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCC
ATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGC
TGCAATGATACCGCGAGAACCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAA
GGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAA
GCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTG
TCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCC
CCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGC
AGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTT
TTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTG
CCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAAC
GTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTG
CACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAA
AATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATA
TTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAA
ACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCAC

Figure A5. Shown above is the sequence of the Pen-Sa-Chimera plasmid. Highlighted are the SS-42 primer recognition sequence (light blue), the *BbsI* double recognition site (light green), and the nucleotide sequence removed from the plasmid by *BbsI* (red text).

CTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAAC
CAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGGCC
GCTACAGGGCGCTCCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGTTTCGGTGCGGGCCT
CTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGG
TTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAGCGCGACGTAATACGACTCACTATAGGGCG
AATTGGCGGAAGGCCGTCAAGGCCGCATCAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTT
GCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCTACGCGTCCT
GAGGCTTTTTTTCTTCTTCTTCGTTCATACAGTTTTTTTTTGTTTATCAGCTTACATTTTCTTGAACCGTA
GCTTTCGTTTTCTTCTTTTTAACTTTCCATTCGGAGTTTTTGTATCTTGTTTCATAGTTTGTCCCAGGATT
AGAATGATTAGGCATCGAACCTTCAAGAATTTGATTGAATAAAACATCTTCATTCTTAAGATATGAAG
ATAATCTTCAAAAGGCCCCTGGGAATCTGAAAGAAGAGAAGCAGGCCCATTTATATGGGAAAGAACA
ATAGTATTTCTTATATAGGCCCATTTAAGTTGAAAACAATCTTCAAAAGTCCCACATCGCTTAGATAAG
AAAACGAAGCTGAGTTTATATACAGCTAGAGTCGAAGTAGTG|ATTGCATCAAGTCCTGCTGCTTTT|GT
TTTAGTACTCTGGAAACAGAATCTACTAAAACAAGGCAAAATGCCGTGTTTATCTCGTCAACTTGTTGG
CGAGATTTTTACGCGTCCTGAGGACCCAGCTTTCTTGTACAAAGTTGGCATTATAAAAAATAATTGCTC
ATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTATTTGCTGGGCCTCATGGG
CCTTCCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAACATGGTCATAGCT
GTTTCCTTGCGTATTGGGCGCTCTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGGTAA
AGCCTGGGGTGCCTAATGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTG
GCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCG
AAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCC
GACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTC
ACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGT
TCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATC
GCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTC
TTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCC
AGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTT
TTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTA
CGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGG
ATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACT
TGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCC
ATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGC
TGCAATGATACCGCGAGAACCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAA
GGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAA
GCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTG
TCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCC
CCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGC
AGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTT
TTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTG
CCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAAC
GTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTG
CACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAA
AATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATA
TTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAA
ACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCAC

Figure A6. Shown above is the expected plasmid sequence upon *BbsI* digestion and ligation of the protospacer. Highlighted are the SS-42 primer recognition sequence (light blue) as well as the protospacer (light green).

GGGGGGGGGGGGGGGGGGCGCTAGATTTGATTGATAAACATCTTCATTCTTAAGATATGAAGATAATC
TTCAAAAGGCCCCTGGGAATCTGAAAGAAGAGAAGCAGGCCCATTTATATGGGAAAGAACAATAGTA
TTTCTTATATAGGCCCATTTAAGTTGAAAACAATCTTCAAAAGTCCCACATCGCTTAGATAAGAAAAC
GAAGCTGAGTTTATATACAGCTAGAGTCGAAGTAGTGATTGCATCAAGTCCTGCTGCTTTTGTTTTAGT
ACTCTGGAAACAGAATCTACTAAAACAAGGCAAAATGCCGTGTTTATCTCGTCAACTTGTTGGCGAGA
TTTTTACGCGTCCTGAGGACCCAGCTTTCTTGTACAAAGTTGGCATTATAAAAAATAATTGCTCATCAA
TTTGTTGCAACGAACAGGTCACTATCAGTCAAGATAAAATCATTATTTGCTGGGCCTCATGGGCCTTCC
GCTCACTGCCCGCTTTCCAGTCGGTAAACCTGTCTTACCAACTGCATTAACATGGTCATACCTGTTTCC
TTGCGTATGGGGCGCTCTCTCCTTCCTCGCTCGCTCAGTGACTGGATCGGTCGTTCGGATAAACCAGGG
ATGGGTGATGAGCAAAGAGGCAG

Figure A7. Shown above is the result of sequencing the plasmid extracted from colonies plated by Behailu. Highlighted in light green is the protospacer sequence inserted into the plasmid at the *BbsI* restriction site.

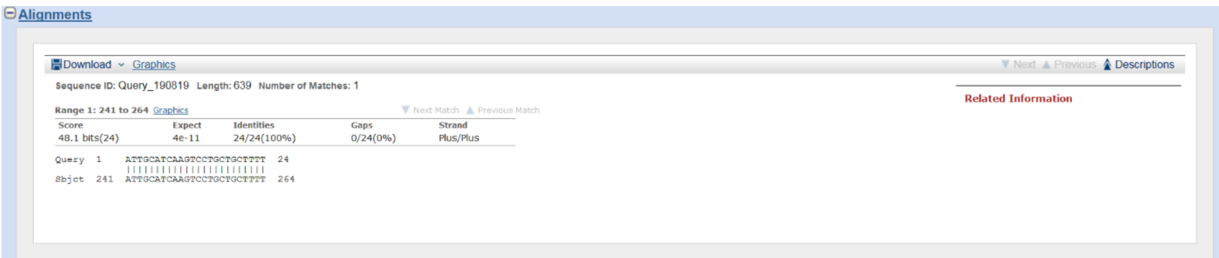

Figure A8. BLAST results showing a 24 nucleotide alignment between the forward protospacer sequence and the sequencing results.

CRISPR RPA3B Sample 17

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Query  5    TTCTTCTTATA-ATAGGTCT-AATCTATCTGCTTGATTTGAAATTTGGATTTTGGTCTAA  62
            ||||||||||| |||||||| |||||||||||||||||||||||||||||||||||||||
Sbjct  178  TTCTTCTTATACATAGGTCTAAATCTATCTGCTTGATTTGAAATTTGGATTTTGGTCTAA  237

Query  63   TTAGGGACTGACACTACCAATTTACTTAATGATTTGAGGATAATTACTTCGATTTTGACT  122
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  238  TTAGGGACTGACACTACCAATTTACTTAATGATTTGAGGATAATTACTTCGATTTTGACT  297

Query  123  TAGATGATATGAATTTGATTTAGTTTCAGTGAGAAAGACGAATGAATGTTTTGATTTTTA  182
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  298  TAGATGATATGAATTTGATTTAGTTTCAGTGAGAAAGACGAATGAATGTTTTGATTTTTA  357

Query  183  TAGAGAATGGATACATCAAGTCCTGCTGCTTTTGTGAATGGAGCTTTGTTGAGAAGGTAC  242
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  358  TAGAGAATGGATACATCAAGTCCTGCTGCTTTTGTGAATGGAGCTTTGTTGAGAAGGTAC  417

Query  243  ATTGGTCAGAAAGTGAGAGCAGTGATTCAAGTTATCAGATCAGATGTTGGATCAGTGATT  302
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  418  ATTGGTCAGAAAGTGAGAGCAGTGATTCAAGTTATCAGATCAGATGTTGGATCAGTGATT  477

Query  303  GGTAAATCGACTGATGATCAACAGATTGTTGTTAAAGGTTCTCCTCAACCGCCTTTAACT  362
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  478  GGTAAATCGACTGATGATCAACAGATTGTTGTTAAAGGTTCTCCTCAACCGCCTTTAACT  537

Query  363  ACTTACCTTGAGGTAATTGGAATTGCTGAGACTGACAACACTATTCGTGCTGAAGTTTGG  422
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  538  ACTTACCTTGAGGTAATTGGAATTGCTGAGACTGACAACACTATTCGTGCTGAAGTTTGG  597

Query  423  ACCAACTTTGGTGATAGTTTCGGTATGAATTTAAGCAAAAATCATTCCTTTAATAAGCTA  482
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  598  ACCAACTTTGGTGATAGTTTCGGTATGAATTTAAGCAAAAATCATTCCTTTAATAAGCTA  657

Query  483  TGTCCTTTATGATTTGCAAATTCTTGTGTCTTGATTTGGGGTCTTGGCTTGTTTGCAGAT  542
            ||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  658  TGTCCTTTATGATTTGCAAATTCTTGTGTCTTGATTTGGGGTCTT-GCTTGTTTGCAGAT  716

Query  543  GTGCAAAACTACAATGAGCTATGTAAGCCTGCAAATGGTGAGTTTAGACACTTGTTCATC  602
            |||||||||||||||||||||||||||| |||||||||||||||||||||||||||||||
Sbjct  717  GTGCAAAACTACAATGAGCTATGTAAGCTTGCAAATGGTGAGTTTAGACACTTGTTCATC  776

Query  603  TAAATCACAGGGTATTGGTGATGAATGTGGAAAGCTTTTTTCTTGGAGATTCTTTGGGTT  662
            ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  777  TAAATCACA-GGTATTGGTGATGAATGTGGAAAGCTTTTTTCTTGGAGATTCTTTGGGTT  835

Query  663  GGAGCTATTGATATGCCTGGTTGACCTTTA-TATTGAAAATCATCTGTT  710
            |||||||||||||||||||||||| ||||| |||||||| || ||||||
Sbjct  836  GGAGCTATTGATATGCCTGGTTGATCTTTAGTATTGAAATTCTTCTGTT  884
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Figure A9. Sanger sequencing results of the 1st of 2 samples resulted in one example of single nucleotide mutations in the RPA3B gene that were not localized to the protospacer sequence. Highlighted in blue is the RPA3B exon, in yellow the RPA3B intron, in green the protospacer sequence, and in red the area where the RPA3B gene of the sequenced mutant did not align with the consensus RPA3B gene sequenced.

CRISPR RPA3B Sample 19

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Query  6    TTCATCTTATA-ATAGGTCTAAATCTATCTGCTTGATTTGAAATTTGGATTTTGGTCTAA  64
            |||  ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  178  TTCTTCTTATACATAGGTCTAAATCTATCTGCTTGATTTGAAATTTGGATTTTGGTCTAA  237

Query  65   TTAGGGACTGACACTACCAATTTACTTAATGATTTGAGGATAATTACTTCGATTTTGACT  124
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  238  TTAGGGACTGACACTACCAATTTACTTAATGATTTGAGGATAATTACTTCGATTTTGACT  297

Query  125  TAGATGATATGAATTTGATTTAGTTTCAGTGAGAAAGACGAATGAATGTTTTGATTTTTA  184
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  298  TAGATGATATGAATTTGATTTAGTTTCAGTGAGAAAGACGAATGAATGTTTTGATTTTTA  357

Query  185  TAGAGAATGGATACATCAAGTCCTGCTGCTTTTGTGAATGGAGCTTTGTTGAGAAGGTAC  244
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  358  TAGAGAATGGATACATCAAGTCCTGCTGCTTTTGTGAATGGAGCTTTGTTGAGAAGGTAC  417

Query  245  ATTGGTCAGAAAGTGAGAGCAGTGATTCAAGTTATCAGATCAGATGTTGGATCAGTGATT  304
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  418  ATTGGTCAGAAAGTGAGAGCAGTGATTCAAGTTATCAGATCAGATGTTGGATCAGTGATT  477

Query  305  GGTAAATCGACTGATGATCAACAGATTGTTGTTAAAGGTTCTCCTCAACCGCCTTTAACT  364
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  478  GGTAAATCGACTGATGATCAACAGATTGTTGTTAAAGGTTCTCCTCAACCGCCTTTAACT  537

Query  365  ACTTACCTTGAGGTAATTGGAATTGCTGAGACTGACAACACTATTCGTGCTGAAGTTTGG  424
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  538  ACTTACCTTGAGGTAATTGGAATTGCTGAGACTGACAACACTATTCGTGCTGAAGTTTGG  597

Query  425  ACCAACTTTGGTGATAGTTTCGGTATGAATTTAAGCAAAAATCATTCCTTTAATAAGCTA  484
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  598  ACCAACTTTGGTGATAGTTTCGGTATGAATTTAAGCAAAAATCATTCCTTTAATAAGCTA  657

Query  485  TGTCCTTTATGATTTGCAAATTCTTGTGTCTTGATTTGGGGTCTTGGCTTGTTTGCAGAT  544
            |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  658  TGTCCTTTATGATTTGCAAATTCTTGTGTCTTGATTTGGGGTCTT-GCTTGTTTGCAGAT  716

Query  545  GTGCAAAACTACAATGCAGCTATGTAAGCTTGCAAATGGTGAGTTTAGACACTTGTTCAT  604
            |||||||||||||||| |||||||||||||||||||||||||||||||||||||||||||
Sbjct  717  GTGCAAAACTACAATG-AGCTATGTAAGCTTGCAAATGGTGAGTTTAGACACTTGTTCAT  775

Query  605  CTAAATCACAGGGTATTGGTGATGAATGTGGAAAGCTTTTTTCTTGGAGATTCTTTGGGT  664
            |||||||||| |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  776  CTAAATCACA-GGTATTGGTGATGAATGTGGAAAGCTTTTTTCTTGGAGATTCTTTGGGT  834
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Figure A10. Sanger sequencing results of the 1st of 2 samples resulted in one example of single nucleotide mutations in the RPA3B gene that were not localized to the protospacer sequence. Highlighted in blue is the RPA3B exon, in yellow the RPA3B intron, in green the protospacer sequence, and in red the area where the RPA3B gene of the sequenced mutant did not align with the consensus RPA3B gene sequenced.