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TITLE:

CRISPR/Cas9 editing of the *C. elegans rbm-3.2* gene using the *dpy-10* co-CRISPR screening marker and assembled ribonucleoprotein complexes.

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KEYWORDS:

CRISPR/Cas9, *C. elegans,* ribonucleoprotein complexes, genome engineering, *rbm-3.2*, gene editing, microinjection, CRISPR screening

SUMMARY:

The goal of this protocol is to enable seamless CRISPR/Cas9 editing of the *C. elegans* genome using assembled ribonucleoprotein complexes and the *dpy-10* co-CRISPR marker for screening. This protocol can be used to make a variety of genetic modifications in *C. elegans* including insertions, deletions, gene replacements and codon substitutions.

ABSTRACT:

The bacterial Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/*Streptococcus pyogenes* CRISPR-associated protein (Cas) system has been harnessed by researchers to study important biologically relevant problems. The unparalleled power of the CRISPR/Cas genome editing method allows researchers to precisely edit any locus of their choosing, thereby facilitating an increased understanding of gene function. Several methods for editing the *C. elegans* genome by CRISPR/Cas9 have been described previously. Here, we discuss and demonstrate a method which utilizes *in vitro* assembled ribonucleoprotein complexes and the *dpy-10* co-CRISPR marker for screening. Specifically, in this article, we go through the step-by-step process of introducing premature stop codons into the *C. elegans rbm-3.2* gene by homology-directed repair using this method of CRISPR/Cas9 editing. This relatively simple editing method can be used to study the function of any gene of interest and allows for the generation of homozygous-edited *C. elegans* by CRISPR/Cas9 editing in less than two weeks.

INTRODUCTION:

The Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/ *Streptococcus pyogenes* CRISPR-associated protein (Cas) technology enables efficient targeted genome editing in a wide range of organisms[1-4]. The CRISPR system was first discovered as a part of a prokaryotic antiviral immune response[5-7]. The Type II CRISPR system uses an endonuclease such as Cas9, a transactivating RNA (tracrRNA) and a short, target DNA-specific 20-nucleotide long guide CRISPR RNA (crRNA) to recognize an "NGG" Protospacer Adjacent Motif (PAM) and make a double-stranded break in the target DNA[5-12]. This double-stranded break is recognized as a lesion by the cellular DNA repair machinery. Consequently, the generated double-stranded break can be repaired by one of two pathways- i) Non-homologous end joining (NHEJ) or ii) Homology-directed repair (HDR)[13]. NHEJ is often error prone and therefore, when this pathway is used to repair the double-stranded break in the target DNA, it often causes inactivating mutations (insertions, deletions) in the gene of interest. On the other hand, by supplying an exogenous repair template with homology to either side of the double-strand break, the cellular DNA repair machinery can be directed to use HDR to repair the break[13]. The HDR method thus enables precise editing of any locus of interest.

A variety of CRISPR/Cas9 gene editing protocols have been described for *C. elegans*[14-20]. The most commonly employed CRISPR/Cas9 editing methods in *C. elegans* include both cloning-based and cloning-free protocols to generate repair templates for CRISPR/Cas9 editing[14-20]. This protocol discusses in detail a cloning-free CRISPR/Cas9 editing protocol based on using *dpy-10* as a co-CRISPR marker for screening. Until now, the only detailed *C. elegans*-focused CRISPR/Cas9 editing video protocol that exists utilizes a fluorescent marker for screening[21]. However, using a fluorescent marker for screening requires access to a fluorescence microscope, which many laboratories at small Primarily Undergraduate Institutions (PUIs) may find difficult to access. It is encouraging to note that positive correlative results have been obtained in previous studies between worms carrying fluorescent markers and the presence of the edit[21,22]. However, additional studies are necessary to determine the overall efficiency of the fluorescence-based screening method for editing a wide variety of loci with different guide RNAs and repair templates. Finally, since the plasmids encoding for these fluorescent markers form extrachromosomal arrays, variable fluorescence is often produced from these arrays that can make positives difficult to identify[23]. Hence, although the fluorescence-based screening method may be useful to adopt, the above-mentioned issues may limit its applicability.

Using a co-CRISPR marker that produces a visible phenotype greatly reduces the number of progeny that need to be screened to find a positively-edited worm[23-28]. Importantly, the phenotypes that are produced by these markers can be easily detected under a simple dissecting microscope[23-34]. The *dpy-10* co-CRISPR marker is one of the best characterized and widely used co-CRISPR markers for performing *C. elegans* CRISPR/Cas9 genome editing[24,27]. Therefore, this article will discuss the method of performing CRISPR/Cas9 editing in *C. elegans* using the direct delivery of ribonucleoprotein complexes with *dpy-10* as a co-CRISPR marker[27,35]. In this method, the prepared editing injection mix consists of a well-characterized *dpy-10* crRNA and *dpy-10* repair template to mediate the generation of an observable dominant "roller" (Rol) phenotype that is conferred by a known heterozygous *dpy-10(cn64)* mutation within the *dpy-10* gene[24,27,29].

When present in its homozygous state, the *dpy-10(cn64)* mutation causes a dumpy (Dpy) phenotype which produces shorter and stouter worms[24,29]. The Rol phenotype is mediated by the accurate insertion of the co-supplied *dpy-10* repair template into a single copy of the *dpy-10* gene by HDR-mediated CRISPR/Cas9 editing. Therefore, the appearance of Rol *C. elegans* indicates a successful injection as well as a successful HDR-mediated editing event within the injected worm's cells. Since the crRNA and the repair template of the target gene of interest are in the same injection mix with the *dpy-10* crRNA and *dpy-10* repair template, there is a good chance that the identified Rol worms were also simultaneously edited at the target gene of interest. Hence, these Rol worms are then screened for the edit of interest by techniques such as polymerase chain reaction (PCR) (for edits greater than 50 bp) or by PCR followed by restriction digestion (for edits less than 50 bp).

The advantages of using this method for genome editing are: i) CRISPR edits can be generated at a relatively high efficiency (2% to 70%) using this method[27]; ii) the repair templates and guide RNAs that are used in this method do not involve cloning, thereby reducing the time required for their generation; iii) by assembling ribonucleoprotein complexes *in vitro*, the concentrations of the assembled editing complexes can be maintained relatively constant, thereby improving reproducibility; iv) some guide RNAs that fail to generate edits when expressed from plasmids have been shown to work for CRISPR/Cas9 genome editing when supplied as *in vitro* transcribed crRNAs[27]; v) including the *dpy-10* co-CRISPR marker enables easy screening using a dissecting microscope and decreases the number of progeny that must be screened to find positives[24,27]; and vi) DNA sequencing verified homozygous-edited worm lines can be obtained by this method within a couple of weeks[19,27].

Excellent book chapters pertaining to many different *C. elegans* CRISPR methods have been published[14-20,43]. However, the demonstration of the *dpy-10* co-CRISPR method in a video format in a laboratory setting is currently lacking. In this article, we describe and demonstrate the process of using the *dpy-10* co-CRISPR method to edit a representative target gene named *rbm-3.2,* a putative RNA-binding protein (WormBase: https://wormbase.org/species/c_elegans/gene/WBGene00011156#0-9fcb6d-10). Specifically, here we describe in detail the method of introducing three premature stop codons within the *C. elegans rbm-3.2* gene using *in vitro* assembled ribonucleoprotein complexes and an exogenously supplied linear single-stranded repair template. These studies have been successful in generating the first *C. elegans* CRISPR strain with premature stop codons in the *rbm-3.2* gene. Since not much is currently known regarding the function of this gene in *C. elegans,* this strain will serve as a useful tool in dissecting the function of RMB-3.2. This method can also be adopted to make insertions, substitutions, and deletions at any locus within the *C. elegans* genome.

PROTOCOL:

This protocol for CRISPR/Cas9 genome editing was approved by the University of Tulsa Institutional Biosafety Committee following NIH guidelines. Throughout this protocol, sterile technique was practiced. All steps of this protocol were carried out using reagents that were free of nucleases. Special care was taken to prevent RNase contamination such as cleaning gloves as

well as workspaces and equipment (e.g. pipettes, exterior of glassware, etc.) with an RNase decontaminating solution.

1. crRNA design

1.1 Find the PAM that enables Cas9 to cut closest to the edit site.

1.1.1 The PAM site is 5'-NGG-3'.

1.1.2 Remember that the PAM can be on either strand of the DNA.

1.2 Select 20 bp at the 5' end of the PAM as the crRNA sequence.

NOTE: The actual crRNA sequence that is synthesized by commercial companies is longer than 20 bp as it has an additional generic sequence that is automatically added to the target-specific 20 bp crRNA sequence by the synthesizing company. Recent studies have not found off-target effects of Cas9 in *C. elegans*[36-40]. Further, the resultant CRISPR-edited strains are outcrossed. Therefore, we are not very concerned about off-target effects of the designed crRNAs. However, online websites including http://genome.sfu.ca/crispr/ and http://crispor.tefor.net/ can be used to find and select the crRNAs with the least off-target effects[38,41]. crRNAs that end with a G or GG and those with greater than 50% GC-content are predicted to be more efficient[19,23,42].

1.3 Order at least 10 nmol of the crRNA from a company (e.g. IDT, Horizon Discovery, etc.).

1.3.1 Once the lyophilized crRNA arrives, store it at -30°C until the day of the microinjection.

1.3.2 On the day of performing the microinjection, spin the lyophilized crRNA at maximum speed (16,200 x g) for one minute and resuspend it in 5 mM Tris-Cl (pH 7.4) to make a 8 µg/µL stock of the crRNA.

1.3.3 Store the unused crRNA stock frozen at -80 °C.

2. Repair template design

2.1 Download a sequence manipulation software (e.g. CLC sequence viewer, APE, Snapgene, Vector NTI, etc.). We use CLC sequence viewer version 8.0 (Qiagen) as it is user-friendly and can be downloaded for free.

2.2 Paste the sequence of the target DNA of interest into the sequence viewer.

2.3 The orientation of the repair template influences the editing efficiency[28]. For inserting a repair sequence to the 5' end of the PAM, design a single-stranded repair template with DNA sequence from the same DNA strand on which the PAM sequence is located (protospacer

strand)[28]. While for inserting a repair sequence to the 3' end of the PAM, design a single-stranded repair template with DNA sequence from the DNA strand that does not carry the PAM sequence (spacer strand)[28].

2.4 Select 35 base pairs of sequence with uninterrupted homology on both sides (on the 5' and 3' end) of the edit.

2.5 Mutate or delete the PAM to prevent cutting of the repair template or the edited genomic DNA by Cas9.

2.5.1 If mutation of the PAM is not possible, introduce several silent mutations close to the 5' end of the PAM to prevent cutting of the repair template.

2.5.2 Make sure to only introduce silent mutations of the PAM if it is present in an exonic region.

2.5.3 Check the codon usage frequency to ensure that the mutated codon is introduced at a frequency that is comparable to the original non-mutated codon (e.g. https://www.genscript.com/tools/codon-frequency-table). In some cases, it might not be possible to introduce the mutated codon at a similar frequency to the unmutated codon. However, for making mutations of a few amino acids, we do not expect the expression level of the mutant protein to be altered significantly.

2.6 If the edit is small (e.g. mutation of a few bases), introduce a unique restriction site close to the edit by silent mutagenesis. We use http://heimanlab.com/cut2.html to find restriction sites that can be introduced by silent mutagenesis.

2.6.1 Check the codon usage frequency to ensure that the mutated codon is introduced at a frequency that is comparable to the original non-mutated codon. As stated above, this is not always possible. However, for experiments involving mutations of a few amino acids, we do not expect the expression level of the mutant protein to be altered significantly.

2.7 Synthesize and purchase linear single-stranded repair templates for HDR as 4 nmol ultramer oligos.

2.8 Resuspend the lyophilized oligonucleotide repair template in nuclease-free water to make a 1 µg/µL stock of the repair template and store it at -30 °C until further use.

3. Screening primer design

3.1 Using CLC sequence viewer or other similar applications, design 20 to 23 base pair forward and reverse primers on either side of the edit respectively, such that they produce a band of between 400 to 600 base pairs upon PCR amplification.

3.1.1 For larger insertions that are several kilobases in size, design the forward primer to be located just outside the left homology arm of the repair template. Design the reverse primer to be located within the repair template itself. In this case, a PCR product will only be obtained in the case of positively-edited worms.

3.1.2 To identify homozygous-edited worms for longer insertions, amplify the entire region of the insertion by designing forward and reverse primers that are located outside the insertion junctions.

3.2 Test the primers and optimize PCR conditions with wild-type genomic DNA prior to using the primers for genotyping.

3.2.1 Ensure that a single band of expected size is produced upon PCR amplification of the genomic DNA.

4. Preparing young adult worms for injection

4.1 Pick L2-L3 stage *C. elegans* onto a fresh bacterial lawn on an MYOB plate and incubate at 20 °C overnight.

NOTE: The protocol for making MYOB plates can be found here: http://www.wormbook.org/wli/wbg13.2p12a/

4.2 On the day of microinjection, pick young adult worms with fewer than 10 embryos in the uterus to inject.

5. Preparing the injection mix

5.1 Prepare the injection mix in the same order as shown in **Table 1** in sterile nuclease-free tubes.

NOTE: The components of the injection mix can be scaled down to make 5 µL injection mixes (instead of 20 µL) if future injections with this mix are not anticipated.

5.2 Mix the injection mix by pipetting.

5.3 Incubate the injection mix at 37 °C for 15 minutes to assemble ribonucleoprotein complexes.

5.4 Spin the injection mix at 4 °C at 16,200 x g for 5 minutes.

6. Microinjection into the *C. elegans* gonad

6.1 Perform microinjection of the CRISPR injection mix into the *C. elegans* gonad, as

described in Iyer et al. 2019[43].

6.1.1 Inject about 30 worms with CRISPR injection mix. The unused injection mix can be re-used by storing at 4°C for a period of about 6 months without loss of efficiency[49].

6.1.2 Inject both gonad arms if possible. Injected worms are considered the P_0 generation.

7. Injected worm recovery and transfer

7.1 After microinjection, move the microinjected P_0 worms using a worm-pick to a 60 mm MYOB agar plate seeded with OP50 *E. coli* and let them recover at room temperature for about one hour.

7.1.1 Note that the recovery temperature will depend upon the genotype of the injected worms. For example, for temperature-sensitive strains, worm recovery at a different temperature may be necessary.

7.2 Using a platinum wire worm pick, transfer each injected worm onto a single seeded 35 mm MYOB agar petri plate and allow them to lay eggs at 20 °C until the next day.

7.2.1 Note that some worms will die as a result of injury from the microinjection procedure. Only pick those worms that are alive and exhibit movement.

7.3 After 24 hours, transfer the injected worms to new individual plates (1 worm per plate).

8. Picking *C. elegans* for screening

8.1 3 to 4 days at 20°C after the injection was performed, monitor all the plates that contain the progeny of the injected worms using a dissecting microscope.

8.2 Identify plates that have F_1 progeny exhibiting roller (Rol) and dumpy (Dpy) phenotypes. A Dpy phenotype is where worms appear shorter and stouter than control worms at the same developmental stage (WormBase: https://wormbase.org/species/all/phenotype/WBPhenotype:0000583#0--10). Plates with Rol and Dpy worms represent plates where the F_1 progeny have been successfully edited with the *dpy-10(cn64)* mutation to be present in its heterozygous or homozygous state, respectively.

8.3 Pick the plates with the most roller and dumpy worms for screening.

8.4 Single out 50 to 100 F_1 Rol worms from these plates to their own individual plates (1 worm per plate) and allow them to lay eggs.

8.4.1 Allow L4-staged F_1 Rol worms to lay eggs and produce progeny (F_2) for 1 to 2 days.

9. Single worm lysis and PCR

9.1 After producing F_2 for 1 to 2 days, transfer each singled F_1 Rol mother into 2.5 µL of lysis buffer **(Table 2)** with a 1:100 dilution of 20 mg/mL Proteinase K.

9.2 Freeze the tubes at -30 °C for 20 minutes. The worms can be stored at this stage for an extended period until further analysis.

9.3 Perform single worm lysis on a PCR thermocycler with the following conditions: 60 °C for 1 hour, 95 °C for 15 min and hold at 4 °C[44].

9.4 Add the following reagents directly to 2.5 µL of the lysed worm containing PCR tube: 12.5 µL of 2x PCR mix containing dNTPs, DNA polymerase, $MgCl_2$ and loading dye, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, and sterile water to 22.5 µL. The total volume of each PCR reaction is 25 µL.

NOTE: In case of screening multiple F_1 worms, it is faster and more convenient to make a PCR master-mix for multiple reactions and add 22.5 µL of the master-mix to each lysis tube.

9.5 Set up PCR reactions on a thermocycler with the following conditions: 95 °C for 1 minute, 35 cycles of 95 °C for 15 s, 55 °C for 15 s (optimize for each primer set), 72 °C for 1 minute (optimize for each target DNA). Hold reactions at 4 °C.

10. Restriction digestion and agarose gel electrophoresis

NOTE: A restriction digestion is only necessary while screening for small edits (less than 50 bp).

10.1 Transfer 10 µL of the PCR DNA from step 9 to a new tube.

10.2 Add between 2 to 4 units of restriction enzyme and 1x restriction enzyme buffer (1.5 µL of 10x reaction buffer) per 15 µL reaction.

10.3 Incubate at 37 °C (or at other enzyme-specific temperature) for 2 hours (shorter incubation times may be possible with fast acting enzymes).

10.4 Heat inactivate the restriction digest by heating the PCR tubes at 65 °C (or other enzyme-specific temperature) for 10 to 15 minutes.

10.5 Load the entire reaction from each PCR tube into a single well of a 1%-2% agarose gel and run gel at 110 mA until proper band separation is achieved.

NOTE: We use a PCR mix that already has a loading dye included for visualization while running on an agarose gel. This mix does not interfere with the restriction digestion reaction and is therefore convenient to use for screening many worms at one time.

11. Identify positively-edited worms

11.1 Visualize agarose gels under UV light (for ethidium bromide gels) to detect DNA band sizes.

NOTE: Positively-edited worms will display an extra band of DNA at the expected size due to editing using the repair template carrying the restriction site at the desired locus within the worm genome. F_1 roller worms are expected to be heterozygous for the edit and are therefore expected to exhibit three bands (one wild-type uncut PCR product and two smaller fragments from the edited cut PCR product). Although rare, it must be noted that homozygotes do arise occasionally.

11.2 Save all the original positively-edited plates until the presence of the edit is verified by Sanger sequencing.

12. Homozygose edit of interest

12.1 Pick between 8 and 12 wild-type looking non-rolling F_2 worms from the respective positively-edited F_1 Rol worm plates and allow them to lay eggs and produce progeny for 1 to 2 days.

NOTE: Since *C. elegans* are self-fertilizing hermaphrodites, if the edited allele does not affect viability or development, the proportion of expected homozygous mutants should be approximately 25%. Non-rolling F_2 worms must be wild-type for the *dpy-10* locus. Picking non-rolling worms enables the generation of edited worms that have lost the *dpy-10(cn64)* mutation and only have the mutation at your gene of interest.

12.1.1 Note that the *dpy-10* gene is present on chromosome II. If your gene of interest is linked to *dpy-10*, you may not be able to easily segregate the *dpy-10* mutation away from your gene of interest.

12.2 Perform steps 9 thru 11 to identify homozygous worm lines.

13. Confirm edit by sequencing

13.1 Once homozygous worms are identified, perform lysis and PCR as described in step 9.

13.1.1 Set up at least 3 PCR reactions for each homozygous line to be sequenced.

13.2 Purify the PCR reactions using a PCR purification kit.

13.3 Measure DNA concentration using a nanodrop spectrophotometer.

13.4 Send sample with the respective forward primer for Sanger sequencing. Ensure that the forward primer is designed to be at least 50 bases away from the edit to be sequenced.

13.5 Analyze the sequencing results using sequence analysis software to confirm the presence of the edit.

REPRESENTATIVE RESULTS:

rbm-3.2 is a putative RNA-binding protein that has homology to human cleavage stimulation factor subunit 2 tau variant (WormBase: https://wormbase.org/species/c_elegans/gene/WBGene00011156#0-9fcb6d-10). The RBM-3.2 protein was identified as a binding partner of Protein Phosphatase 1 (GSP-1) and its regulators Inhibitor-2 (I-2^{SZY-2}) and SDS-22 in a previous study that identified these proteins as novel regulators of *C. elegans* centriole duplication (data not shown)[45]. Presently, very little is known regarding the function of the *rbm-3.2* gene in *C. elegans.* Hence, to further investigate the biological role of the *C. elegans rbm-3.2* gene, the *rbm-3.2*-null allele *rbm-3.2(ok688)* was obtained from the Caenorhabditis Genetics Center (CGC).

Unfortunately, in addition to possessing a deletion of the entire *rbm-3.2* gene, the *rbm-3.2(ok688)* allele also results in a partial deletion of an overlapping gene, *rbm-3.1,* thereby complicating genetic analysis. Therefore, in order to accurately investigate the role of the *rbm-3.2* gene in *C. elegans,* we used CRISPR/Cas9 editing to introduce three premature stop codons very close to the start of the *C. elegans rbm-3.2* coding region, leaving the overlapping *rbm-3.1* gene intact.

To introduce these premature stop codons into the *C. elegans rbm-3.2* gene, we designed a crRNA with a PAM motif that was located on the opposite strand (template strand) of DNA **(Figure 1A)**. The Cas9 cut site was located 6 bases away from the *rbm-3.2* start codon ATG. To introduce a premature stop codon into the *rbm-3.2* gene, we designed a repair template with the following five major characteristics: 1) 35 bases of uninterrupted homology to *rbm-3.2* upstream of the *rbm-3.2* start codon (left homology arm) 2) A short stretch of bases containing the PAM motif was deleted 3) An EcoRI restriction site was introduced immediately after the start codon for screening 4) The second and the third codons of *rbm-3.2* were deleted and three stop codons were introduced after the fifth RBM-3.2 codon to stop translation of the *rbm-3.2* mRNA 5) 35 bases of uninterrupted homology to the first intron of *rbm-3.2* were included downstream of the edit (right homology arm) **(Figure 1B)**.

The injection mix for this CRISPR experiment was prepared as indicated in **Table I**. Note that 1 µL of 1 M KCl was used in the injection mix as compared with 0.5 µL of 1 M KCl recommended previously[27]. We find that using a higher concentration of KCl in the injection mix improved the editing efficiency of commercially purchased Cas9. The injection mix was incubated at 37 °C for 15 minutes to assemble ribonucleoprotein complexes. The mix was then centrifuged and loaded into the pulled microinjection needle. Microinjection into the *C. elegans* gonad was performed as described in Iyer et al. 2019[43].

Figure 2 depicts the experimental timeline for generating edited *C. elegans* using this protocol. Although we typically inject 30 worms for each CRISPR experiment, some laboratories inject fewer worms (between 10 to 20) for each CRISPR experiment. Since injecting more worms increases the probability of finding positive edits, we prefer to inject a larger number of worms. Many laboratories pick "jackpot" broods (plates with greater than 50% Rol and Dpy progeny) for screening. In our experience after performing several CRISPR experiments, although jackpot broods do arise occasionally, a majority of times, the Rol worms that are picked for screening often come from many different P_0 plates, each consisting of a few Rol progeny. No jackpot broods were obtained in this experiment.

In total, we screened the genomic DNA of 73 F_1 Rol worms that were obtained from 7 injected P_0 worms for the presence of the edit. The sequences of the screening primers and their locations with respect to the start codon of the *rbm-3.2* gene are represented in **Figure 3A**. Through the analysis, 7 out of 73 F_1 worms were found to be positive for the edit (9.5%) **(Figure 3B)**. Unedited worms displayed a single DNA fragment of 445 bp upon EcoRI digestion. Whereas, worms carrying a premature stop codon in the *rbm-3.2* gene exhibited two fragments of 265 bp and 166 bp respectively upon EcoRI digestion. Heterozygous-edited worms displayed three fragments upon EcoRI digestion: one wild-type unedited DNA fragment of 445 bp and two DNA fragments of 265 bp and 166 bp respectively from the edited copy of the *rbm-3.2* gene.

To identify worms carrying homozygous edits, we transferred 12 F_2 worms from the identified positive F_1 heterozygotes onto new individual plates and allowed them to produce progeny (F_3). The F_2 worms were then screened for homozygosity as described earlier. 6 out of 12 (50%) screened worms were found to be homozygous for our edit of interest **(Figure 4A).** The genomic DNA of an identified homozygous-edited worm line was used to set up a DNA sequencing reaction. DNA sequencing analysis confirmed the presence of the edit in its homozygous state **(Figure 4B)**. In general, it is beneficial to sequence multiple homozygous worm lines to ensure that all homozygous-edited worm lines exhibit the same phenotype (if the null-mutation produces a specific phenotype). Further, it might also be necessary to validate gene knockouts using an expression-based assay such as western blotting or RT-PCR or via phenotype analysis. This is because, it is possible that in-spite of introducing premature stop codons at the beginning of the gene, an alternative ATG might be present downstream of the premature stop codons. This ATG could be used to initiate protein expression, resulting in a partially-functional truncated protein.

FIGURE AND TABLE LEGENDS:

Table 1: Components of the injection mix for CRISPR/Cas9 editing using ribonucleoprotein complexes and *dpy-10* as a co-CRISPR marker. Use sterile techniques and RNase-free reagents while making the injection mix. Please note that sterile, non-DEPC treated nuclease-free water was used to make the injection mix.

Table 2: Worm lysis buffer recipe. The worm lysis buffer can be made in bulk, autoclaved, filtered and aliquoted for long term storage (NOTE: the lysis buffer can be stored for over a year at room temperature). Add a 1:100 dilution of 20 mg/mL proteinase K to the worm lysis buffer just before

each use.

Figure 1: Schematic showing crRNA and repair template design for *rbm-3.2* premature stop CRISPR. **A**. Schematic displaying the coding and template strands of the *rbm-3.2* gene with the crRNA sequence and the PAM motif sequence located on the template strand. **B**. Schematic representing the different characteristics of the repair template that was synthesized to introduce three premature stop codons into the *rbm-3.2* gene.

Figure 2: Experimental timeline for generating homozygous-edited *C. elegans* by CRISPR/Cas9 editing using preassembled ribonucleoprotein complexes and *dpy-10* as a co-CRISPR marker. A day-by-day breakdown of the steps that need to be performed to generate homozygous-edited *C. elegans* using this method of CRISPR/Cas9 editing. Briefly, 30 worms were injected with the CRISPR editing mix using microinjection and segregated onto individual MYOB plates seeded with OP50 *E. coli*. After 24 hours, the injected P_0 worms were transferred onto fresh new MYOB plates and allowed to continue laying eggs. On Days 3 and 4 after microinjection, the plates were examined for the presence of Rol F_1 worms. The plates with the maximum number of Rol and Dpy F_1 worms were selected and 73 F_1 Rol worms (we usually pick between 50 to 100 F_1 Rol worms per CRISPR experiment) were singled onto new individual MYOB plates (1 worm per plate) and allowed to lay eggs for about 2 days. On Day 6 after microinjection, worm lysates were prepared from the F_1 worms that had produced progeny (F_2) and were screened for the presence of the edit by PCR followed by restriction digestion with EcoRI and agarose gel electrophoresis. On Day 7, 12 non-Rol, non-Dpy F_2 worms were transferred from the positive plates onto new individual plates and allowed to produce progeny (F_3). On Day 9, the F_2 worms were screened for homozygosity of the edit as described previously. On Day 10, worm lysis, PCR and PCR cleanup were performed for the homozygous F_3 worms and the DNA concentration was measured using a NanoDrop spectrophotometer. On Day 11, Sanger sequencing reactions were set up for positive samples and the reactions were sent for DNA sequencing. On Day 12, the sequencing results were analyzed, and the presence of the edit was verified using a sequence analysis software (e.g. CLC sequence viewer).

Figure 3: Screening for *C. elegans* that are heterozygous for the *rbm-3.2* premature stop codons. Agarose gel electrophoresis images of *C. elegans* genomic DNA digested with EcoRI. 73 individual F_1 worms were genotyped and screened for the presence of the *rbm-3.2* edit. Red numbers and asterisks indicate positively-edited worms. All the 7 identified positively-edited *C. elegans* were heterozygous for the edit as they exhibited one wild-type unedited DNA fragment of 445 bp and two DNA fragments of 265 bp and 166 bp respectively from the edited copy of the *rbm-3.2* gene upon EcoRI digestion.

Figure 4: Identifying and verifying homozygous-edited *C. elegans* carrying the *rbm-3.2* premature stop codons. A. Screening for *C. elegans* that are homozygous for the *rbm-3.2* premature stop codons**.** Agarose gel electrophoresis images of *C. elegans* genomic DNA digested with EcoRI. 12 individual F_2 worms from positive plates were genotyped and screened for homozygosity of the *rbm-3.2* edit. Red: homozygous-editedhomozygous-edited worms. 6 out of the 12 screened F_2 worms (50%) were homozygous for the *rbm-3.2* premature stop codons. No

PCR product was present for worm 7. **B.** Confirming the insertion of the premature stop codons in *rbm-3.2* by DNA sequencing. Schematic showing the comparison of DNA and protein sequences of unedited and edited homozygotes. Analysis of DNA sequencing results of genomic DNA from homozygous-edited worms confirmed the presence of the three premature stop codons in the *rbm-3.2* gene upon CRISPR/Cas9 editing. All the resultant amino acid changes after CRISPR/Cas9 editing are indicated in either red letters or red asterisks.

DISCUSSION:

We have used the above protocol to edit several genes besides *rbm-3.2.* The editing efficiencies for different loci, guide RNAs and repair templates (single-stranded and double-stranded) have varied between 2% and 58% (data not shown). The observed editing efficiencies are comparable to the previously reported editing efficiencies of 2% to 70% for this protocol[27]. We have also been successful in using this technique in making gene deletions. Using two crRNAs we replaced a gene that is close to 6 kb in length with the coding sequence for green fluorescent protein (GFP) (data not shown). For this experiment, about 14% of the Rol F_1 worms that were analyzed were found to be positive for the gene deletion and replacement with GFP (data not shown). However, additional experiments are required to determine the maximum length of gene deletions and replacements that can be performed using this technique.

This technique can be used to make insertions that are about 1.6 kb in length[19]. A recent study has shown that for making insertions that are over 1.6 kb in length using this protocol, generating two double-strand breaks and using repair templates with longer homology arms can enable the insertion of much larger fragments of DNA (~10 Kb)[28]. Alternatively, multiple rounds of gene editing with this protocol may be performed to generate larger edits. Other plasmid-based *C. elegans* CRISPR/Cas9 gene editing protocols may also be adopted for CRISPR experiments involving the insertion of DNA fragments larger than 1.6 kb[46-48].

Prior to using this protocol for gene editing, it is necessary to ensure that the gene of interest is not linked to the *dpy-10* locus on chromosome II. In the case of a target gene being linked to *dpy-10*, it may be problematic to segregate the *dpy-10* mutation away from your edit of interest. Hence, in the event that the gene of interest is linked to the *dpy-10* locus, other co-CRISPR markers such as *unc-58* (X-chromosome)*, unc-22* or *zen-4* (chromosome IV)*,* and *ben-1* or *pha-1* (chromosome III) that are located on different chromosomes may be used[24-26,28]. Data from the Meyer lab demonstrate that *ben-1* and *zen-4* mutations can be used as successful co-CRISPR markers for screening with this method[28]. However, it is important to note that using *zen-4* and *pha-1* as co-CRISPR markers necessitates performing the CRISPR experiment in non-wild-type *zen-4(cle10*ts) or *pha-1(e2123*ts*)* backgrounds respectively[26,28]. Further, CRISPR experiments involving some co-CRISPR markers such as *ben-1* may require the preparation of special plates (e.g. plates containing benzimidazole)[28]. We have successfully used the *unc-58* co-CRISPR marker to screen for positive edits for a gene that is linked to the *dpy-10* using this method (data not shown). The *unc-58(e665)* mutation confers a visible phenotype (paralysis) that can be effectively used to screen for positively-edited worms[24]. Alternatively, if access to a fluorescent microscope is available, a fluorescently-tagged *gtbp-1* gene can also be used as a co-CRISPR marker for this protocol[19].

For a high editing efficiency while using this method of genome editing, the edit site must be within 10 to 30 bases from the Cas9 cutting site. If the edit site is over 30 bases away from the Cas9 cutting site, the editing efficiency drops drastically[19,35,37]. However, a recent study from the Meyer lab has demonstrated that creating two double-strand breaks at a distance from one another can enable the insertion of edits far away from the Cas9 cut site using this protocol[28].

In the current protocol, the repair template was designed so that all the three inserted stop codons appear in the same reading frame. However, an alternative strategy to create null-mutants would be to use a universal 43-bases long knock-in STOP-IN cassette that has been described previously[50]. Importantly, this cassette has stop codons in all the three possible reading frames and causes frameshift mutations. This is an especially useful strategy for generating null-mutants when improper or incomplete repair occurs at the edit site.

In conclusion, due to its short duration and the recent advances in this method, this is an excellent method for routine laboratory experiments involving the addition of short immunogenic epitope tags, fluorescent tags, making gene deletions, gene replacements and codon substitutions.

ACKNOWLEDGMENTS:

This work was supported by University of Tulsa (TU) start-up funds and the TU Faculty Development Summer Fellowship awarded to Jyoti Iyer. We would like to thank the Chemistry Summer Undergraduate Research Program (CSURP) and the Tulsa Undergraduate Research Challenge (TURC) for awarding stipends to the students involved in this study. We would like to acknowledge Ms. Caroline Dunn for her technical assistance. Finally, we would like to thank Drs. Jordan Ward, Aimee Jaramillo-Lambert, Anna Allen, Robert Sheaff, William Potter and Saili Moghe for their critical comments on this manuscript.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Sander, J.D., Joung, J.K. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nature Biotechnology*. **32** (4), 347–355 (2014).
2. Carroll, D. Genome engineering with targetable nucleases. *Annual Review of Biochemistry*. **83**, 409-439 (2014).
3. Chandrasegaran, S., Carroll, D. Origins of Programmable Nucleases for Genome Engineering. *Journal of Molecular Biology*. **428** (5), 963–989 (2016).
4. Knott, G.J., Doudna, J.A. CRISPR-Cas guides the future of genetic engineering. *Science*. **361** (6405), 866-869 (2018).
5. Barrangou, R. et al. CRISPR provides acquired resistance against viruses in prokaryotes. *Science*. **315** (5819), 1709-1712 (2007).
6. Marraffini, L.A., Sontheimer, E.J. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science*. 322 (5909), 1843-1845 (2008).

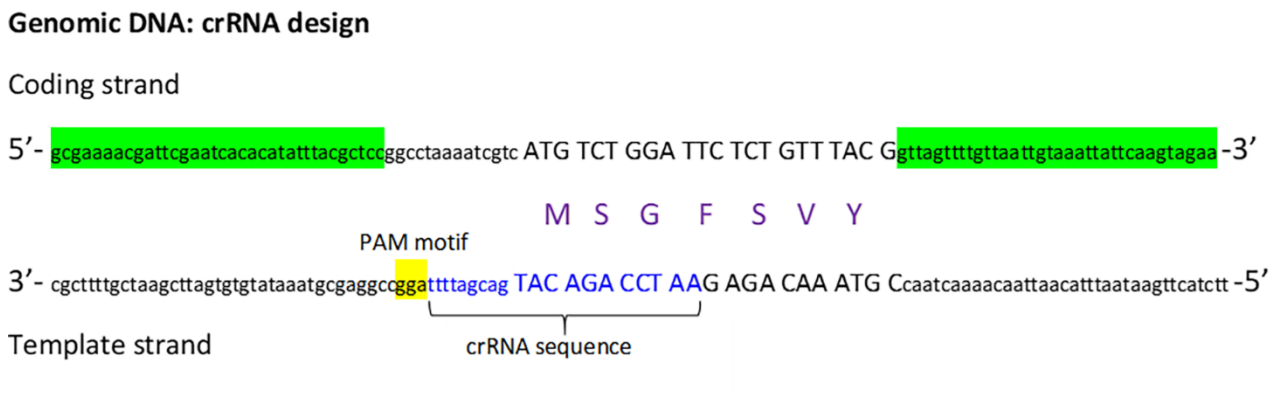
7. Brouns, S.J. et al. Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science*. **321** (5891), 960-964 (2008).
8. Mojica, F.J., Díez-Villaseñor, C., García-Martínez, J., Almendros, C. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology*. **155** (3), 733-740 (2009).
9. Garneau, J.E. et al. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature*. **468** (7320), 67-71 (2010).
10. Gasiunas, G., Barrangou, R., Horvath, P., Siksnys, V. Cas9–crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proceedings of the National Academy of Sciences*. **109** (39), E2579-E2586 (2012).
11. Jinek, M. et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*. **337** (6096), 816-821 (2012).
12. Makarova, K.S., Koonin, E.V. Annotation and classification of CRISPR-Cas systems. In *CRISPR, Methods in Molecular Biology*. **1311**, 7-75 (2015). Humana Press, New York, NY.
13. Pawelczak, K.S., Gavande, N.S., VanderVere-Carozza, P.S., Turchi, J.J. Modulating DNA Repair Pathways to Improve Precision Genome Engineering. *ACS Chemical Biology*. **13** (2), 389–396 (2018).
14. Frøkjær-Jensen, C. Exciting prospects for precise engineering of Caenorhabditis elegans genomes with CRISPR/Cas9. *Genetics*. **195** (3), 635-642 (2013).
15. Waaijers, S., Boxem, M. Engineering the Caenorhabditis elegans genome with CRISPR/Cas9. *Methods*. **68** (3), 381-388 (2014).
16. Xu, S. The application of CRISPR-Cas9 genome editing in Caenorhabditis elegans. *Journal of Genetics and Genomics*. **42** (8), 413-421 (2015).
17. Dickinson, D.J., Goldstein, B. CRISPR-based methods for Caenorhabditis elegans genome engineering. *Genetics*. **202** (3), 885-901 (2016).
18. Nance, J., Frøkjær-Jensen, C. The Caenorhabditis elegans transgenic toolbox. *Genetics*. 212 (4), 959-990 (2019).
19. Paix, A., Folkmann, A., Seydoux, G. Precision genome editing using CRISPR-Cas9 and linear repair templates in C. elegans. *Methods*. **121**, 86-93 (2017).
20. Kim, H.M., Colaiácovo, M.P. CRISPR-Cas9-Guided Genome Engineering in Caenorhabditis elegans. *Current Protocols in Molecular Biology*. **129** (1), e106 (2019).
21. Prior, H., MacConnachie, L., Martinez, J. L., Nicholl, G. C., Beg, A. A. A Rapid and Facile Pipeline for Generating Genomic Point Mutants in C. elegans Using CRISPR/Cas9 Ribonucleoproteins. *Journal of Visualized Experiments*. **134**, e57518 (2018).
22. Prior, H., Jawad, A.K., MacConnachie, L., Beg, A.A. Highly efficient, rapid and Co-CRISPR-independent genome editing in Caenorhabditis elegans. *G3: Genes, Genomes, Genetics*. **7** (11), 3693-3698 (2017).
23. Farboud, B., Meyer, B.J. Dramatic enhancement of genome editing by CRISPR/Cas9 through improved guide RNA design. *Genetics*. **199** (4), 959-971 (2015).
24. Arribere, J.A., Bell, R.T., Fu, B.X., Artiles, K.L., Hartman, P.S., Fire, A.Z. Efficient marker-free recovery of custom genetic modifications with CRISPR/Cas9 in Caenorhabditis elegans. *Genetics*. **198** (3), 837-846 (2014).
25. Kim, H. et al. A co-CRISPR strategy for efficient genome editing in Caenorhabditis elegans. *Genetics*. **197** (4), 1069–1080 (2014).

26. Ward, J.D. Rapid and precise engineering of the Caenorhabditis elegans genome with lethal mutation co-conversion and inactivation of NHEJ repair. *Genetics*. **199** (2), 363-377 (2015).
27. Paix, A., Folkmann, A., Rasoloson, D., Seydoux, G. High efficiency, homology-directed genome editing in Caenorhabditis elegans using CRISPR-Cas9 ribonucleoprotein complexes. *Genetics*. **201** (1), 47-54 (2015).
28. Farboud, B., Severson, A.F., Meyer, B.J. Strategies for efficient genome editing using CRISPR-Cas9. *Genetics*. **211** (2), 431-457 (2019).
29. Levy, A.D., Yang, J., Kramer, J.M. Molecular and genetic analyses of the Caenorhabditis elegans dpy-2 and dpy-10 collagen genes: a variety of molecular alterations affect organismal morphology. *Molecular Biology of the Cell*. **4** (8), 803-817 (1993).
30. Kramer, J.M., Johnson, J.J., Edgar, R.S., Basch, C., Roberts, S. The sqt-1 gene of C. elegans encodes a collagen critical for organismal morphogenesis. *Cell*. **55** (4), 555-565 (1988).
31. Schnabel, H., Schnabel, R. An organ-specific differentiation gene, pha-1, from Caenorhabditis elegans. *Science*. **250** (4981), 686-688 (1990).
32. Granato, M., Schnabel, H., Schnabel, R. pha-1, a selectable marker for gene transfer in C. elegans. *Nucleic Acids Research*. **22** (9), 1762-1763 (1994).
33. Chalfie, M., Thomson, J.N. Structural and functional diversity in the neuronal microtubules of Caenorhabditis elegans. *Journal of Cell Biology*. **93** (1), 15-23 (1982).
34. Severson, A.F., Hamill, D.R., Carter, J.C., Schumacher, J., Bowerman, B. The aurora-related kinase AIR-2 recruits ZEN-4/CeMKLP1 to the mitotic spindle at metaphase and is required for cytokinesis. *Current Biology*. **10** (19), 1162-1171 (2000).
35. Paix, A., Schmidt, H., Seydoux, G. Cas9-assisted recombineering in C. elegans: genome editing using in vivo assembly of linear DNAs. *Nucleic Acids Research*. **44** (15), e128-e128 (2016).
36. Chiu, H., Schwartz, H.T., Antoshechkin, I., Sternberg, P.W. Transgene-free genome editing in Caenorhabditis elegans using CRISPR-Cas. *Genetics*. **195** (3), 1167-1171 (2013).
37. Paix, A. et al. Scalable and versatile genome editing using linear DNAs with microhomology to Cas9 Sites in Caenorhabditis elegans. *Genetics*, **198** (4), 1347-1356 (2014).
38. Au, V. et al. CRISPR/Cas9 methodology for the generation of knockout deletions in Caenorhabditis elegans. *G3: Genes, Genomes, Genetics*. **9** (1), 135-144 (2019).
39. Dickinson, D.J., Ward, J.D., Reiner, D.J., Goldstein, B. Engineering the Caenorhabditis elegans genome using Cas9-triggered homologous recombination. *Nature Methods*. **10** (10), 1028-1034 (2013).
40. Friedland, A.E., Tzur, Y.B., Esvelt, K.M., Colaiácovo, M.P., Church, G.M., Calarco, J. A. Heritable genome editing in C. elegans via a CRISPR-Cas9 system. *Nature Methods*. **10** (8), 741-743 (2013).
41. Haeussler, M. et al. Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. *Genome Biology*. **17** (1), 148 (2016).
42. Gagnon, J.A. et al. Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs. *PloS One*. **9** (5), e98186 (2014).
43. Iyer, J., DeVaul, N., Hansen, T., Nebenfuehr, B. Using microinjection to generate genetically modified Caenorhabditis elegans by CRISPR/Cas9 editing. *Microinjection, Methods in Molecular Biology*. **1874***,* 431-457 (2019).

704 44. JoVE Science Education Database. Basic Methods in Cellular and Molecular Biology. PCR:
705 The Polymerase Chain Reaction. *Journal of Visualized Experiments* (2017).
706 45. Peel, N., Iyer, J., Naik, A., Dougherty, M. P., Decker, M., O'Connell, K.F. Protein
707 phosphatase 1 down regulates ZYG-1 levels to limit centriole duplication. *PLoS Genetics*. **13** (1),
708 e1006543 (2017).
709 46. Dickinson, D.J., Pani, A.M., Heppert, J.K., Higgins, C.D., Goldstein, B. Streamlined
710 genome engineering with a self-excising drug selection cassette. *Genetics*. **200** (4), 1035-1049
711 (2015).
712 47. Norris, A.D., Kim, H.M., Colaiácovo, M.P., Calarco, J.A. Efficient genome editing in
713 Caenorhabditis elegans with a toolkit of dual-marker selection cassettes. *Genetics*. **201** (2), 449-
714 458 (2015).
715 48. Schwartz, M.L., Jorgensen, E.M. SapTrap, a toolkit for high-throughput CRISPR/Cas9
716 gene modification in Caenorhabditis elegans. *Genetics*. **202** (4), 1277-1288 (2016).
717 49. Ghanta, K.S., Mello, C.C. Melting dsDNA Donor Molecules Greatly Improves Precision
718 Genome Editing in Caenorhabditis elegans. *Genetics*. **216** (2) (2020).
719 50. Wang, H., Park, H., Liu, J., Sternberg, P. W. An efficient genome editing strategy to
720 generate putative null mutants in Caenorhabditis elegans using CRISPR/Cas9. *G3: Genes,*
721 *Genomes, Genetics*. **8** (11), 3607-3616 (2018).

Figure 1

A



B

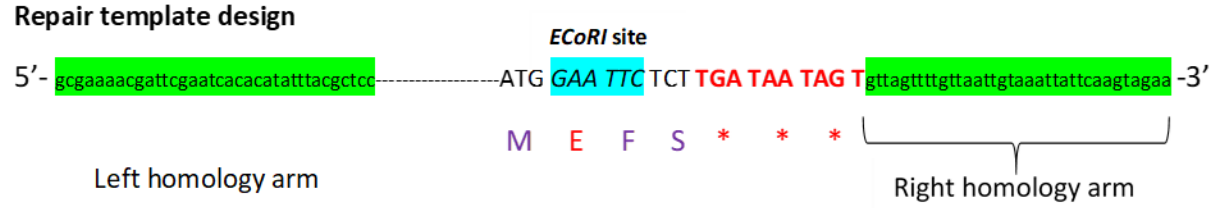


Figure 2

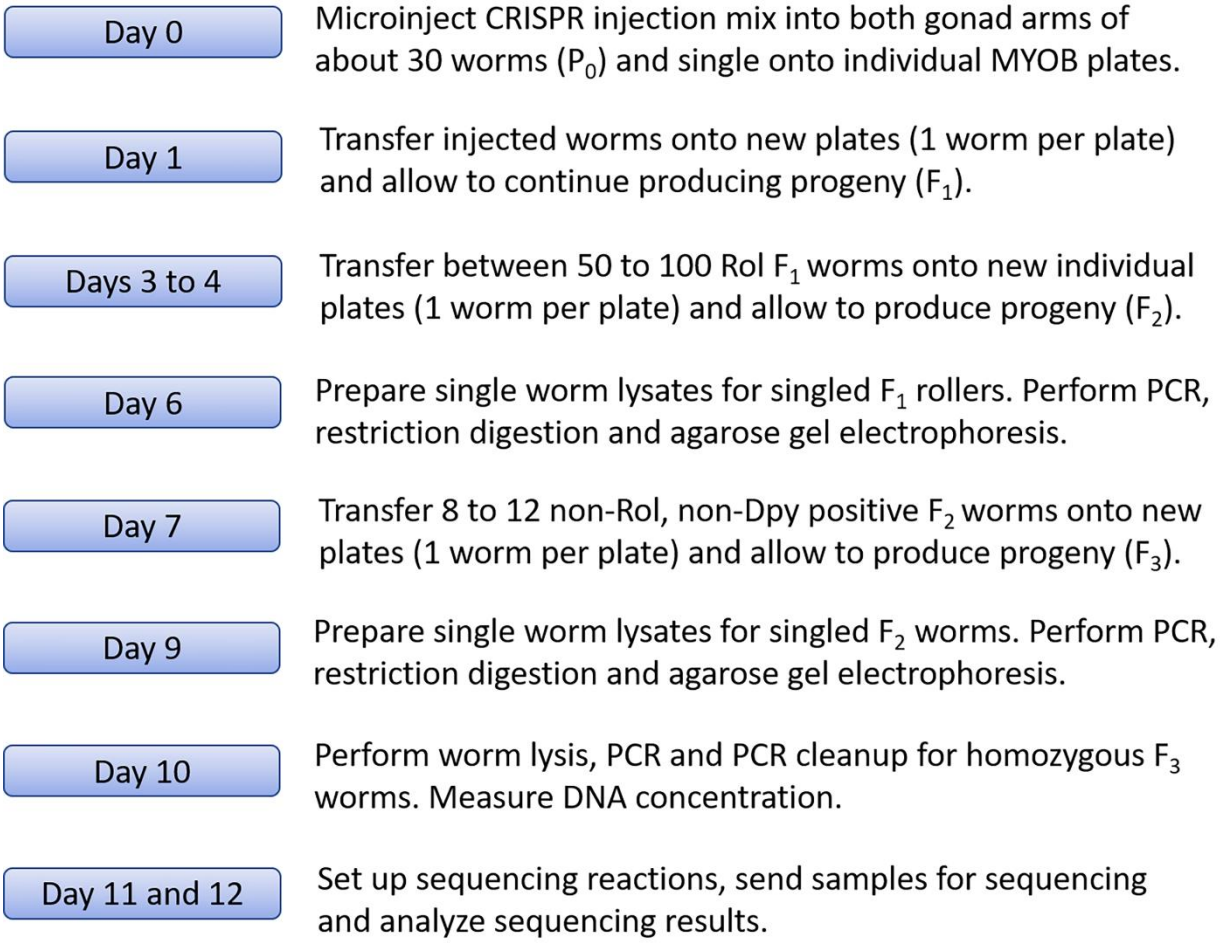


Figure 3

A

***rbm-3.2* premature-stop codon CRISPR screening primers**

Forward primer: 5'- AGAATGAGGAATAAAAGACGCAG - 3'
(Located 176 bp before the start codon for the *rbm-3.2* gene)

Reverse primer: 5'- GGCTGAAGTAGTTGCCAAGG -3'
(Located 266 bp after the start codon for the *rbm-3.2* gene)

B

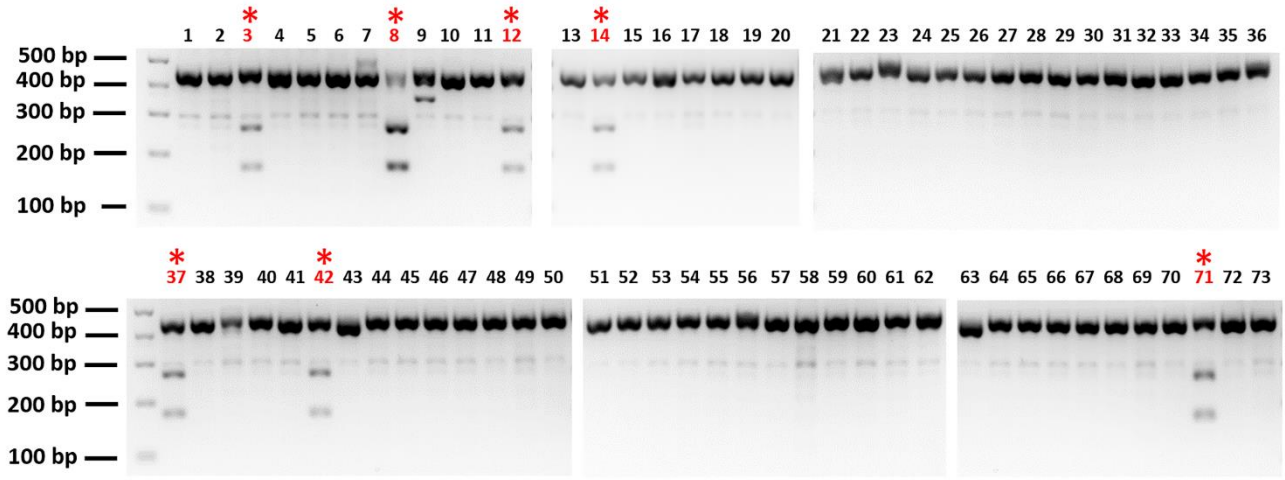
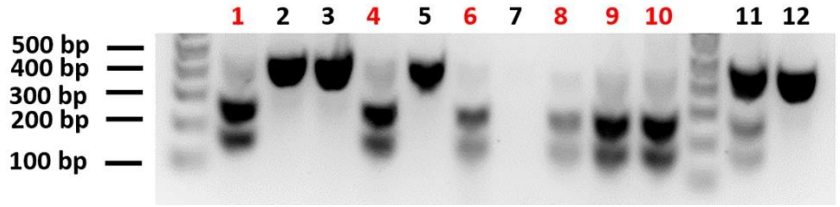


Figure 4

A



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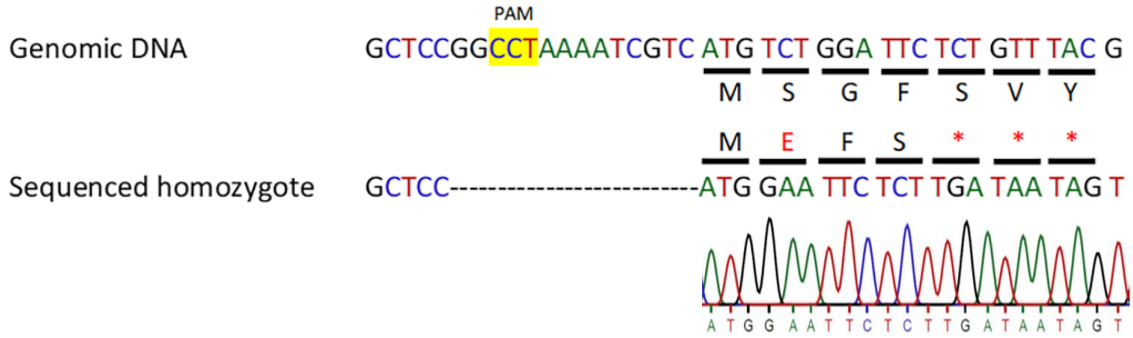

Table 1

Reagent	**Concentration**	**Volume to Add**
Cas9 protein in nuclease-free water with 20% glycerol	2 µg/µL	5 µL
KCl in sterile nuclease-free water	1 M	1 µL
tracrRNA in 5 mM Tris-Cl pH 7.5	4 µg/µL	5 µL
dpy-10 crRNA in 5 mM Tris-Cl pH 7.5	8 µg/µL	0.4 µL
rmb-3.2 crRNA in 5 mM Tris-Cl pH 7.5	8 µg/µL	1 µL
dpy-10 repair template in sterile nuclease-free water	500 ng/µL	0.55 µL
rmb-3.2 repair template in sterile nuclease-free water	1 µg/µL	2.2 µL
sterile nuclease-free water	-	4.85 µL

Table 2

Reagent	Concentration	Volume to add
KCl	1 M	5 mL
Tris-HCl pH 8.3	1 M	1 mL
MgCl2	1 M	250 µL
NP-40 (or IGEPAL CA-630)	100%	450 µL
Tween-20	100%	450 µL
Gelatin	2%	500 µL
Water	-	92.35 mL

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Barcoded DNA sequencing tubes	Eurofins Genomics	SimpleSeq Kit Premixed	N/A
Cas9 protein	PNA Bio	CP01	Lyophilized Cas9 protein was resuspe
crRNAs	Horizon Discovery/ GE Dharmacon	N/A	Lyophilized crRNAs were resuspended
ECoRI	New England Biolabs	R3101S	Stored at -30 °C
Minelute PCR purification kit	Qiagen	28004	Spin columns stored at 4 °C
MyTaq Redmix	Meridian Bioscience	BIO-25043	Stored at -30 °C
Primers	IDT	N/A	Standard 20 nmol oligos were synthes
Proteinase K	Gold Bio	P-480-SL4	Stored at -30 °C
Repair template oligos	IDT	N/A	4 nmol Ultramer oligos were synthesi
tracrRNA	Horizon Discovery/ GE Dharmacon	U-002005-50	Lyophilized tracrRNA was resuspende

ıded in 20%glycerol containing nuclease-free water, aliquoted and stored at -80 °C

l in 5 mM Tris-Cl pH 7.5, aliquoted and stored at -80 °C

ɜized, resuspended in sterile nuclease-free water and stored at -30 °C

ƶed, resuspended in sterile nuclease-free water and stored at -30 °C

d in 5 mM Tris-Cl pH 7.5, aliquoted and stored at -80 °C

November 2, 2020

Ref: Revisions to JoVE62001

Dear Dr. Nguyen,

We thank the reviewers and the editors for their thorough review of our manuscript titled "CRISPR/Cas9 editing of the *C. elegans rbm-3.2* gene using the *dpy-10* co-CRISPR screening marker and assembled ribonucleoprotein complexes." The critical comments that have been obtained upon the editorial and peer-review of our manuscript have resulted in greatly improving the quality of this manuscript. As you will note from our revised manuscript, we have spent a considerable amount of time and effort in addressing every editorial and reviewer comment as thoroughly as possible. Our responses to the editorial and reviewer comments have been included below in red and blue. We have included two pdfs of our revised manuscript- one showing the changes tracked and another showing the original markup without the changes tracked. The line numbers that have been referenced in our responses to the reviewer and editorial comments correspond to the manuscript which shows the changes tracked. With these revisions, we hope that you will now find our revised and much-improved manuscript suitable for publication in JoVE. We thank you very much for facilitating the peer-review of our manuscript. We are also greatly appreciative of the reviewers for taking the time to provide thoughtful and insightful comments to improve the quality of our manuscript.

Best Regards,

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Phone: 918-631-2070

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We have reviewed the manuscript and made the appropriate spelling and grammar changes.

2. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
We have implemented these changes in the manuscript.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all

commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: RNaseZAP, lines 154-155 (Our laboratory uses CLC sequence viewer…versions); please rephrase lines 166-167 to "Please find restriction sites…mutagenesis; we use http://heimanlab.com/…"; IDT (line 171); MyTaq Redmix (Bioline)
We have removed commercial language in addition to fixing the specific changes outlined in the two examples provided. [Lines 136, 155, 163-166, 195, 203, 301-303, 327-330].

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.
We have adjusted the numbering system of the Protocol to reflect these recommendations.

5. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.
We have formatted the spacing of the protocol steps per this request, and we have highlighted the portions of the protocol section to be included in the video.

6. Please express all centrifugation parameters in this format: 13,000 × g
We have changed this to be denoted as 16, 200 x g as this is the equivalent speed to 13,000 rpm for our centrifuge. [Lines 158, 246]

7. Line 179 (PCR conditions): For filming, please provide specific details or cite a reference.
We have cited a new reference #44 for additional details pertaining to the PCR procedure. [Line 299]

8. Please move the description of Figure 2 (lines 315-332) into its legend appearing after Representative Results. Please discuss the figure—modifications to the protocol, points to note etc in the Representative Results.
We have moved the description of Figure 2 to appear after the Representative Results [Line 494-511] and have included a discussion of the figure in the representative results section (Lines 420-427).

9. As we are a methods journal, please add limitations of the technique to the Discussion section.-
We believe that the limitations of this technique have already been included in the discussion section, examples of which are referenced in lines 537-539 which state that "additional experiments are required to determine the maximum length of gene deletions and replacements that can be performed using this technique" in addition to lines 567-571 which state that "For a high editing efficiency while using this method of genome editing, the edit site must be within 10 to 30 bases from the Cas9 cutting site. If the edit site is over 30 bases away from the Cas9 cutting site, the editing efficiency drops drastically[19,35,37]. However, a recent study from the Meyer lab has demonstrated that creating two double-strand breaks at a distance from one another can enable the insertion of edits far away from the Cas9 cut site using this protocol[28]."

10. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please note spaces between Volume and issue. Also, please do not abbreviate any journal names, issue should not be bold, and capitalize the first letters of the words in the journal name.
We have edited our references to reflect the requested changes.

11. Please sort the Materials Table alphabetically by the name of the material.
We have sorted the Materials Table alphabetically as requested.

_____

Reviewers' comments:
Reviewer #1:
This manuscript (and accompanying video) provide details of a popular approach for introducing genomic changes in the nematode C. elegans. The authors describe in detail how to use dpy-10 as a co-CRISPR marker that produces an easily visible phenotype for screening. Although this method can be applied to introduce fluorescent tags or other sequences and make deletions or substitutions, the paper does not provide detailed modification of the protocol, if any, for how to do so.

Specific comments:
- The title of the paper can be simplified: CRISPR/Cas9 editing of the C. elegans rbm-3.2 gene using the dpy-10 co-CRISPR screening marker and assembled ribonucleoprotein complexes- Thank you very much for this suggestion. The title of the paper has been amended accordingly. [Lines 2-3]

- In the introduction highlights dpy-10 as a widely used co-CRISPR marker. Some addition of thoughts on the limitations of using this approach would be useful (e.g., off-target effects). The limitations to using the *dpy-10* co-CRISPR marker have been discussed in the Discussion section. Specifically, we have mentioned that if a gene being edited by CRISPR/Cas9 is located on chromosome 2 (the same chromosome as *dpy-10*), alternative co-CRISPR markers such as *unc-58, pha-1, zen-4,* etc. can be used. Regarding off-target effects, we have mentioned in the protocol section that current studies in *C. elegans* have not revealed any off-target effects of CRISPR/Cas9 editing in *C. elegans.*
[Lines 555-571, 147-151, respectively]

- Line 120: rbm-2.3 should say rbm-3.2- We thank the reviewer for pointing out this error. We have made this change. [line 125]

1. Protocol
- It may be more reader friendly to use a,b,c… or 1,2,3 for the main steps and a different numbering/lettering for the substeps, rather than the numbering used for steps and substeps in the current version. Sections A,B,C,D etc., should be consistently upper or lower case.- Thank you for this suggestion. However, the journal requires us to follow specific guidelines for formatting the steps of the protocol. In fact, the JoVE editorial team has recommended us to name the steps as 1.1 and sub-steps as 1.1.1. To comply with publishing requirements, we followed the journal's formatting requirements.

- Sequence manipulation software should be mentioned earlier because they are needed in the "crRNA design" section.- We understand the reviewer's concern. However, locating the PAM motif and designing the crRNA can be easily performed without using a sequence manipulation software. Therefore, we decided to introduce the sequence manipulation software in a later section. [Line 163-166]
- Providing more generality of the primer design for different kinds of edits would be useful, rather than only emphasizing the 400-600 bp size criterion. For example what if one wanted to integrate a gene that is 6 kb? Primers have to be designed to amplify junctions of integration. Once the junctions are confirmed, the entire 6kb region has to be amplified and sequenced to ensure single-copy intact

integration.- We thank the reviewer for this suggestion. We have now included additional directions on how to design primers for longer insertions in the screening primer design section. [Lines 213-219]

- For "preparing the injection mix", does incubation at 37C for 15 min to 'activate' Cas9 with tracrRNA and crRNA need the homology repair template?- We thank the reviewer for pointing out that the word "activate" can be misleading. To prevent confusion, we have amended this sentence to "Incubate the injection mix at 37°C for 15 minutes to assemble ribonucleoprotein complexes" [Line 243-244, 414-415]

- Line 221: it should say "allow L4-staged F1 Rol worms to lay eggs (F2) for 1-2 days" and not "allow L4 stage F1 Rol worms to produce lay eggs and produce progeny (F2) for 1-2 days". We thank the reviewer for pointing out this error. We have made this change. [Line 288]

- In the section "Homozygous edit of interest", step 3 states to repeat steps 9.3 to 12, which is confusing because of differences in detail. It would be better to explicitly spell out what needs to be done at this stage.- We understand the reviewer's concern. We have amended the protocol and changed this sentence to read "Perform steps 9 thru 11 to identify homozygous worm lines." However, we respectfully disagree with the reviewer's evaluation regarding this point. As evidenced by the other reviewers' comments, most of the other reviewers did not have any concern with understanding the protocol directions in this section. We believe that we re-stating what we had already previously stated in steps 9 thru 11 will be redundant and will not add further value to the protocol. Additionally, since this part of the protocol will also be filmed in a video format, we hope that this will serve to eliminate any potential confusion regarding this section. [Line 362]

- Throughout the protocol, there is storage at -30C vs at -80C. Is this incidental or is there a specific reason for the two temperatures. Explaining the rationale for temperature choice would be helpful.- We thank the reviewer for making this observation. We have modified this sentence to read "Freeze the tubes at -30°C for 20 minutes." to avoid any confusion regarding the freezing temperature. [Line 295]

- Line 257: "integration of the" should be "editing using" because the homology repair template is not integrated into the worm but rather is used to change the genomic sequence of the worm.- We thank the reviewer for this observation. We have made the change that the reviewer has suggested. [Line 336]

2. Figures & legends
- Figure 1: Panel A can have the words "genomic DNA: crRNA design" written above the figure so that the readers know this is the target DNA being used to design the crRNA sequence. The repeated "Genomic DNA" label can be removed and just 'coding strand' and 'template strand' can be indicated. Panel B can similarly say "Repair template design". Boxes around each figure can be removed. We thank the reviewer for this observation. We have made the suggested changes to Figure 1.

- Figure 3 & 4: Inverted gel images may be easier on the reader's eyes to see faint bands. We thank the reviewer for this observation. We have inverted the gel images for Figures 3 and 4 so that the bands are easier to visualize.

- Figure 4: Simplify labeling and indication of lanes of interest to be similar to figure 3 and refer to lanes of interest consistently (e.g., why is lane 4 not labeled in red?). We thank the reviewer for this suggestion. We have modified Figure 4 to appear similar in formatting to Figure 3. We have also labeled lane 4 in red. The figure legend for Figure 4 has also been modified accordingly.

- The reagents table with company, catalog # etc., should be labeled and referred to in the manuscript as necessary. Although this is a good suggestion, JoVE policies prohibit authors from mentioning specific company names and catalog numbers within the manuscript.

Reviewer #2:
Review of Smith et al. JoVE manuscript Sept. 2020

This manuscript describes a very widely used CRISPR strategy to edit genes in C. elegans. It would be very beneficial to have a video example for those new to CRISPR to watch prior to tackling this technical advance alone. The comments below are meant to improve the presentation and the explanation of the technique, assuming many naïve readers will be the audience.

Major concerns:
1. The authors should explain why Dpys and Rollers would come from these dpy-10 CRISPR mixes. They should explain why one would see both.- We thank the reviewer for their suggestion. We have now included two sentences, one in the Introduction section and another in the protocol section 8.2.2 explaining the significance of the Dpy phenotype and why both Dpys and Rollers come from the *dpy-10* CRISPR mixes. [Lines 88-93, 280-282]

2. The authors should discuss how orientation of the repair oligo influences the efficiency of repair.- We thank the reviewer for this suggestion. We have now included an additional step 2.3 in the protocol section for "Repair template design" which provides directions about which orientation of the repair template should be used for making edits to the 5' end versus the 3' end of the PAM. [Lines 170-175]

3. Table 1 has few concerns. Paix et al, 2017 Methods stated that one should avoid DEPC-treated buffers or water. When the authors state RNase-free, do they mean DEPC treatment? – We apologize for being unclear regarding this point. By RNase-free, we do not mean DEPC treatment. We use nuclease-free, non-DEPC-treated water for making our injection mixes. This point has been clarified in the amended legend for Table 1. [Lines 477-478]

And isn't 20ul a rather large injection mix considering the expense of all the reagents. Since injecting 30 animals doesn't even use a single microliter, perhaps the authors should suggest 5 ul mixes to save on reagents.- The reviewers bring up a good point. However, the reason that we make a 20 ul injection mix is because our microinjection needle often gets clogged while performing *C. elegans* microinjections. This necessitates pulling and loading new microinjection needles. We usually load 1.5 ul of the injection mix into a single microinjection needle. Preparing 5 ul of the microinjection mix will not be sufficient to load more than 3 new needles. Moreover, often, we also use the same microinjection mix in the future to inject *C. elegans* strains with different genetic backgrounds as needed. According to a recent study from the Mello lab (Ghanta, K.S., & Mello, C.C. Melting dsDNA Donor Molecules Greatly Improves Precision Genome Editing in Caenorhabditis elegans. *Genetics,* 216 (2), (2020). https://doi.org/10.1534/genetics.120.303564), prepared injection mixes can be stored at 4°C for a period of up to 6 months without loss of efficiency. Therefore in conclusion, the reasons that we prefer making 20 ul injection mixes are: i) to ensure that we have enough injection mix to load the needle in the case that the needle gets clogged ii) to ensure that we do not need to re-make the same injection mix for injecting *C. elegans* strains with different genetic backgrounds. However, we have added an additional note in the "Preparing the injection mix" section 5.1.1 mentioning "Note: The components of the injection mix can be scaled down to make 5 µl injection mixes (instead of 20 µl) if future injections

with this mix are not anticipated." Further, we have also included an additional note in the "Microinjection into the *C. elegans* gonad" section 6.1.1.1 indicating that "The unused injection mix can be reused by storing at 4°C for a period of about 6 months without loss of efficiency[50]"
[Lines 238-239, 252-253]

4. Figure 1: what is the logic in not using PAM sites in codon 3 or codon 8 of coding strand? Isn't the coding strand easier to work with? Especially since changing sequences 3' of the PAM site is optimal. We thank the reviewer for their critical analysis. The reason that we used the PAM that we used in this study was because this guide sequence had the least predicted off-target editing effects. Moreover, we had successfully used the crRNA corresponding to this PAM sequence in a different prior experiment for making a different type of edit to the N-terminus of the *rbm-3.2* gene. In our previous experiment, we found that the crRNA corresponding to this PAM worked well and yielded several positive edits. Since we knew that this crRNA worked well from our previous experiment, we just wanted to maintain the same conditions for introducing the premature stop codons at the N-terminus of the *rbm-3.2* gene.

5. The example shown in Fig 1 is confusing because it looks like the repair deletes ~15 nucleotides of the 5' UTR and then changes the first 5 amino acids to 4 before adding stop codons. The Sternberg published a strategy in which they put stop codons in all 3 reading frames in case the CRISPR repair is not perfect and there is a tiny indel. In this example, the 3 stop codons are in the same frame. Perhaps the authors should at least mention this as an alternative strategy for making early stops.- We thank the reviewer for this suggestion. We have now included a paragraph in the discussion section which discusses the strategy from the Sternberg lab as an alternative strategy for making null-mutants. [Lines 579-584]

6. The authors should emphasize that a few lines should be isolated and confirmed by sequencing. This will strengthen any conclusions made about all the lines that should have the same phenotype- We thank the reviewers for this suggestion. We have now added a sentence to the representative results section to reflect this addition. [Lines 462-464]

Minor concerns:
Lines 97-98 are confusing. Do the authors mean the size of the edit being < or > 50 bp?- We apologize for the confusion. We have now replaced the symbol by the words "less than" or "greater than".
[Lines 101-102]

Lines 136-144: the authors should make it clear that the crRNA sequence is longer than 20 nucleotides, but the synthesis company adds the rest since it does not depend on the gene sequence.- We thank the reviewer for this suggestion. We have included this information in Section 1.2.1 of crRNA design.
[Lines 144-146]

Line 144: how much crRNA should one order. The smallest amount from Dharmacon may not be enough for many injections.- We thank the reviewer for this important suggestion. In Section 1.3 of "crRNA design" we have now included the sentence "Order at least 10 nmol of the crRNA from a company"
[Line 155]

Line 157: "both", not either.- We thank the reviewer for pointing this out. We have made this change.
[Line 177]

Line 160: what should one do if they cannot mutate the PAM site without disrupting the coding

sequence? The authors should suggest mutating a few codons as long as they are silent changes 5' of the PAM site. If mutation of the PAM is not possible, introduce several silent mutations close to the 5' end of the PAM to prevent cutting of the repair template.- We thank the reviewer for their suggestion. We have now included an additional sentence in the "Repair template design" section 2.5.1 mentioning "If mutation of the PAM is not possible, introduce several silent mutations close to the 5' end of the PAM to prevent cutting of the repair template." [Lines 182-183]

Line 175 section: if inserting a restriction site, the authors should state the one should check there are not ones nearby; it should be a unique site in the region.- We thank the reviewer for their suggestion. We have amended section 2.6 of "Repair template design" to "If the edit is small (e.g. mutation of a few bases), introduce a unique restriction site close to the edit by silent mutagenesis." [Lines 193-194]

Line 192 and 311: does 37°C really activate Cas9 or is this step just meant to allow the RNP to assemble?- We thank the reviewer for this observation. We have now changed the words "activate Cas9 enzyme" to "assemble ribonucleoprotein complexes" in both these places. [Line 243-244, 414-415]

Line 210: what is the difference between the first and second plate? Should investigator keep and monitor both?- The P_0 worms are transferred to new individual plates on Day 2 to prevent the plate from Day 1 from starving out. As we have not stated at any point in our protocol to discard any of the plates, we assume that it is clear to the readers to keep and monitor both plates. [Lines 343-344]

Lines 218-220: Aren't these called "Jackpot" broods? "pick from the plates with the most….". How many jackpot broods should one pick from? What is considered a jackpot? 5? 10? 20 rollers?- We thank the reviewer for their question. We have clarified this point further in representative results section. [Line 423]

Line 221: "produce lay eggs" needs fixing.- We thank the reviewer for this observation. We have fixed this mistake. [Line 288]

Line 225: "After producing F2 for 1-2 days…..".- We thank the reviewer for this observation. We have made this change. [Line 292]

Will the video show how to pick single worms into 2.5 ul of lysis buffer? This can be a challenging skill to perform.- Yes. The video will show how to pick single worms into 2.5 ul of lysis buffer.

Line 243: Restriction enzyme buffers are usually 10X. The authors should state the final volume of the total digest to determine how much 10X buffer to add.- We thank the reviewer for their recommendation. We have amended section 10.2 of the "Restriction digestion and electrophoresis" section to read "Add between 2 to 4 units of restriction enzyme and 1X restriction enzyme buffer (1.5 µl of 10X reaction buffer) per 15 µl reaction." [Lines 316-317]

Line 245: Not all restriction enzymes work at 37°C. -We thank the reviewer for this observation. We have now changed section 10.3 of the "Restriction digestion and electrophoresis" section to read "Incubate at 37°C (or other enzyme-specific temperature) for 2 hours (shorter incubation times may be possible with fast acting enzymes)." [Lines 319-320]

Line 247: not all restriction enzymes can be inactivated at this temp. -We thank the reviewer for this observation. We have now changed section 10.4 of the "Restriction digestion and electrophoresis"

section to read “Heat inactivate the restriction digest by heating the PCR tubes at 65°C (or other enzyme-specific temperature) for 10 to 15 minutes.” [Lines 322-323]

Line 248: 2% gels may not always be necessary.- We agree with the reviewer. We have now changed section 10.5 of the “Restriction digestion and electrophoresis” section to read “Load the entire reaction from each PCR tube into a single well of a 1%-2% agarose gel and run gel at 110 mA until proper band separation is achieved.” [Lines 325-326]

Line 256: The authors should explain that the F1 roller is likely a het and has the WT uncut band and the cut fragments. They should also explain that rare F1 homozygotes sometimes arise.-We thank the reviewer for their suggestion. Sections 11.2.1 and 11.2.2 have been added to clarify this issue. [Lines 338-341]

Line 264: The authors say Rol but earlier they said it was okay to also pick Dpy animals? Why is that? Please explain.- We apologize for the confusion. However, to our knowledge, we have not recommended picking Dpy animals in our manuscript. Our instructions specify to pick Rol worms. [Lines 286-287]

Line 271: the authors should explain that homozygotes will now lack the WT uncut band.- We understand that the reviewer’s concern. However, we feel that including this level of detail is excessive. We have already discussed how a heterozygote should look like upon agarose gel electrophoresis. Using this information, the readers should be easily able to figure out how homozygotes would look. [Lines 335-340]

Line 320: how many P0s did the 73 Rollers come from? It would be important to share this data so naïve readers will know what a jackpot brood looks like. If all 73 were picked from 3 P0s. it shouldbe stated.- We thank the reviewer for their suggestion. We have indicated in the Representative Results section “In total, we screened the genomic DNA of 73 F1 Rol worms that were obtained from 7 injected P_0 worms for the presence of the edit.” [Lines 446-447]

Lines 337-338: why do restriction enzyme digested fragments not add up to 445 like WT?- The reason that the digested fragments do not add to 445 bp as wildtype is because as shown in Figure 1B, additional sequences were added as well as deleted in the repair template. This resulted in the edited band being 391 bp in size as opposed to the 445 bp wild-type fragment.

Table 2: the authors should state how long lysis buffer can be stored at 4°C and how long lysis buffer with Prot. K can be stored at 4°C.- We thank the reviewer for their suggestion. We have included an additional note in the figure legend for Table 2 mentioning “Note: the lysis buffer can be stored for over a year at room temperature) [Lines 481-482]

Line 374: "with with"- We thank the reviewer for pointing this mistake. The legend for Figure 3 has been updated accordingly. [Line 514]

Line 425: State on which chromosome unc-58 resides.- We apologize for the confusion. However, we have clearly stated in the discussion section of our manuscript that the *unc-58* gene is located on the X-chromosome. [Line 559]

Line 436 what is meant by "short duration"?- We apologize for the confusion. Short duration means short experimental timeline.

Line 437: "involving the addition of….."- We thank the reviewer for their suggestion. We have re-written this sentence to read “In conclusion, due to its short duration and the recent advances in this method, this is an excellent method for routine laboratory experiments involving the addition of short immunogenic epitope tags, fluorescent tags, making gene deletions, gene replacements and codon substitutions.” [Line 587]

Last sentence is awkward and should be broken into two sentences.- We thank the reviewer for their suggestion. However, we respectfully disagree with the reviewer’s evaluation. Since none of the other reviewers have expressed any issues with interpreting or understanding this sentence, we prefer to leave it as it has been currently written. [Lines 586-589]

Reviewer #3:

Manuscript Summary:

CRISPR/Cas9 techniques are well developed in C. elegans, notably for the cloning free approach. Although several protocol papers had been already published, the addition of a visual, step by step protocol will be a great tool for the worm's community.

The protocol is well written, and I only have minor comments

Major Concerns:

None

Minor Concerns:

-line 51: maybe say that the most common length of the spacer sequence (crRNA) is 20nt- We thank the reviewer for their suggestion. We have amended the sentence to read “The Type II CRISPR system uses an endonuclease such as Cas9, a transactivating RNA (tracrRNA) and a short, target DNA-specific 20-nucleotide long guide CRISPR RNA (crRNA) to recognize an “NGG” Protospacer Adjacent Motif (PAM) and make a double-stranded break in the target DNA[5-12]” [Line 53]

-line 75 and 417: fluorescent co-CRISPR method can be used without the need of a plasmid based approach: see Paix et al, Methods, 2017 using gtbp1 locus tagging with a fluorescent protein. – We thank the reviewer for their comment. We have now included an additional sentence in the discussion section to convey that fluorescently-tagged *gtbp-1* can also be used as a co-CRISPR marker for this protocol. [Lines 569-571]

Also Arribere et al, Genetics 2014, use sqt-1 as an alternative to dpy-10: even if both are on Chr2, they have similar phenotype and are in different genomic positions, therefore in some cases sqt-1 can be used as an alternative to dpy10.-We thank the reviewer for their suggestion and agree with their evaluation. However, we felt that since *sqt-1* is also on chromosome 2, to make genetic crossing more convenient, it might be better to use co-CRISPR markers that are located on different chromosomes.

-line 107: there is a "v" typo after the ref 27- We apologize for the confusion. However, the “v” is not a typo and refers to point v) in the sentence. [Line 111]

-line 117: premature STOP insertion / frameshift approach to KO a gene had been used in Paix et al,

Genetics, 2014. It might be useful to say that since STOP codon can be sometime skipped (I believe), it is useful to put several STOP codons and a frameshift (as done by the authors). Also it could be useful to say that it might be necessary to validate the KO using expression assay (WB) or genetically (phenotype) since in theory it is not impossible that a alternative ATG, downstream the premature STOPs, could be used.- We thank the reviewer for their insightful comment. Reviewer 2 also had a similar suggestion. We have accordingly amended a paragraph in the discussion section which discusses the strategy from the Sternberg lab as an alternative strategy for making null-mutants. Additionally, we have also included additional detail in our "Representative Results" section clarifying that additional expression-based or phenotype-based assays must be performed to verify that the strain is a true null. [Lines 464-469, 579-584,]

-line 160: sometimes it is not possible to mutate the PAM with a silent mutation (due to codon usage) or to delete it. In this case it will be useful to say that silent mutations / deletion can be done in the spacer sequence (crRNA).- We thank the reviewer for pointing this out. We have now included an additional sentence in the "Repair template design" section 2.5.1 mentioning "If mutation of the PAM is not possible, introduce several silent mutations close to the 5' end of the PAM to prevent cutting of the repair template." [Lines 182-183]

-line 162/168: in many occasions, it is impossible to make a silent mutation with a similar codon usage, maybe it will be useful to say that for few codons, it should not matter so much- We thank the reviewer for pointing this out. We have now included additional details in sections 2.5.3 and 2.6.2 of the "Repair template design" section to clarify this issue. [Lines 186-191, Lines 198-201]

-line 203: how the P0 are moved to the agar plate after injection?- We thank the reviewer for bringing this to our attention. We have now amended section 7.1 of the "Injected worm recovery and transfer" section to read "After microinjection, move the microinjected P0 worms using a worm-pick to a 60 mm MYOB agar plate seeded with OP50 E. coli and let them recover at room temperature for about one hour." [Line 258]

-line 221: it seems that the sentence should not be here (copy / paste error?)- We apologize for the confusion. However, this sentence is a side-note that is meant to clarify the conditions for section 8.4. [Lines 288-289]

-line 236: maybe it will be useful to add the primer sequences and where they are on the locus (fig 1?)- We thank the reviewer for their suggestion. We have now created a new figure (Figure 3A) indicating the primer sequences and their positions in the genome with respect to the start codon for the *rbm-3.2* gene. We have also added a new sentence in the "Representative Results" section mentioning "The sequences of the screening primers and their locations with respect to the start codon of the rbm-3.2 gene are represented in **Figure 3A**." [Lines 447-448]

-line 243: the final volume of the restriction digest reaction should be written- We thank the reviewer for their suggestion. We have now mentioned the final volume of the restriction digest reaction. [Line 317]

-line 263: maybe say that if the edited allele does not affect viability / development, the proportion of homozygous mutants should be 25%. Also, it should be clear for non C.elegans readers that this model is hermaphrodite / self fertile.- We thank the reviewer for their suggestion. We have added an additional note to section 12.1.1 ("Homozygose edit of interest" section) mentioning "Since *C. elegans* are self-

fertilizing hermaphrodites, if the edited allele does not affect viability or development, the proportion of expected homozygous mutants should be approximately 25%." [Lines 352-354]

-line 298: maybe say that it is not necessary that the crRNA sequence is on the + or – strands- We thank the reviewer for their suggestion. We have mentioned in the crRNA design section 1.1.2 of the protocol that the PAM can be on either strand of the DNA. We hope that this is sufficient to clarify that the crRNA sequence can be on either strand of DNA. [Line 141]

-line 337 and fig 1/4: it is not clear what the dash lanes represent…it is a deletion of the corresponding nucleotides on the locus or they just represent the same sequence than the locus? It is not clear based on the size of the restriction digest fragments obtained. - We apologize for the confusion. The reviewer correctly guesses that the dashes represent a deletion. Upon carefully observing the sequence of the repair template in Figure 1B, it can be determined that a 14 base sequence carrying the PAM located prior to the start codon was deleted in the repair template. This results in the edited PCR product being shorter than the wild-type PCR product.

-fig 4: any idea why the proportion of homozygous edited is so high? The nb 4 should also be in red?
-references: 37 and 48 are the same- We thank the reviewer for their observation and comments. We do not know why the proportion of homozygous edited is so high. However, we do notice that the proportion of homozygotes is higher than expected in some CRISPR/Cas9 editing experiments. We have fixed Figure 4 to ensure that the homozygous edited lane is labeled red. Many thanks to the reviewer for pointing out that references 37 and 48 are the same. This completely skipped our attention. We have fixed this issue.

-fig 1: according to Paix et al, Methods, 2017 , the homology arms (in green in the figure) should be counted starting from the Cas9 cut and/or the last inserted mutation.- We understand what the reviewer is trying to convey. However, the Paix et al, Methods 2017 paper conveys that the homology arms should have 35 bases of uninterrupted homology. Since there is a 14 base deletion between the start codon and the last base of the left homology arm, we did not include the start codon in the homology arms.

-fig 1/4: sequences and sequencing results should be aligned for clarity- We thank the reviewer for their suggestion. We have aligned the sequences and the sequencing results in Figure 4B.

-fig 2: would be useful to say clearly that worms should be single cloned in plates. Also why in day 9 and 10 the worms lysis and PCR have to be repeated?- We thank the reviewer for their suggestion. We have now modified Figure 2 and specified to transfer "1 worm per plate" to indicate that the worms should be single cloned in plates. The single worm lysis on Day 9 is performed to screen F_2 worms carrying the homozygous edit. On Day 10, the lysis is performed for F_3 worms from homozygous edited parent F_2 worm plates. In theory, we could use the PCR products from the identified homozygous F_2 worms as a template to set up additional PCR reactions to send the sample for DNA sequencing. However, in our experience, sometimes using the PCR product from F_2 worms as a template for additional PCRs results in the generation of non-specific PCR products in subsequent PCRs. Therefore, we prefer to perform worm lysis of the F_3 worms and use this as a template to set up the PCRs for DNA sequencing reactions. Further, since we will be picking the F_3 worms to propagate and maintain the edited line, we prefer verifying the F_3 worms directly.

-table 2: NP40, Tween and gelatin concentration could be written in the concentration column (100%

and 100% I believe, and 2%)- We thank the reviewer for their suggestion. We have amended Table 2 accordingly.

Reviewer #4:

Manuscript Summary:

This manuscript describes a protocol for making edits in the C. elegans genome using a co-CRISPR marker leading to a visible phenotype that can be observed directly in a dissecting microscope. The previously published video protocol for C. elegans editing used a fluorescent marker that requires a more expensive microscope for screening edited candidate worms. The protocol here is described and demonstrated using a candidate gene, rbm-3.2, that is expected to behave similarly to any other locus that would be of interest to the reader for editing.

Overall review: The manuscript provides a comprehensive discussion of the protocol that will be useful in a video format for investigators. It is well written and in need of minor edits.

Major Concerns:

none

Minor Concerns:

Minor content revisions needed:

1. The introduction needs to be rearranged. The logic of a co-CRISPR marker should be explained first in the second paragraph—why and how such a marker is used. Then the use of a specific, named, dpy-10 mutation should be described—particularly how a heterozygous mutation leads to a Rol phenotype (which should be defined) and a homozygous mutation leads to a Dpy phenotype (defined as well). The section about the only editing video protocol that exists uses a fluorescent marker for screening should be moved to a paragraph that precedes the last paragraph. The way the introduction is written now, placing that point front and center is distracting especially with no context for co-CRISPR markers.- We thank the reviewer for their suggestion. We have now included how the different phenotypes (Rol vs Dpy) are caused due to heterozygous of homozygous *dpy-10(cn64)* mutations. Specifically, we have now included two sentences, one in the Introduction section and another in the protocol section 8.2.2 explaining the significance of the Dpy phenotype and why both Dpys and Rollers come from the *dpy-10* CRISPR mixes. Regarding the flow of the introduction section, we respectfully disagree with the reviewer's evaluation. In our opinion, we feel that the introduction section has been written in a logical manner. We have, in fact, provided a context for the co-CRISPR markers in the introduction section by mentioning "Using a co-CRISPR marker that produces a visible phenotype greatly reduces the number of progeny that need to be screened to find a positively-edited worm[23-28,]. Importantly, the phenotypes that are produced by these markers can be easily detected under a simple dissecting microscope[23-34]." Since none of the other reviewers have expressed a concern with the flow of the introduction section, we prefer to keep it as it is. We hope that as authors of this manuscript, we are provided the leeway to present our thoughts in a manner which, in our opinion, makes the manuscript easy to follow and understand for a majority of the readers. Edits: [Lines 88-93, 280-282] Referenced lines: [82-85]

2. There are several places in the manuscript where (WormBase) is used as a reference. The specific web address for the information should be included instead. For example, if there are no articles to site regarding rbm-3.2 then at least the specific webpage that describes the gene should be listed.- We thank the reviewer for their suggestion. We have now included the complete web address for each of the contexts in which WormBase was used as a reference. [Lines 120-121, 278-279, 383-384]

3. It should be noted with the crRNA design that off-target effects are not the only issues when considering the design of the crRNA, but the best efficiency can be predicted using online tools as well. For example, the IDT website allows you to upload a genomic region and then provides you with a list of options to choose from taking efficiency into account as noted on lines 142-143.- We thank the reviewer for their suggestion. However, as we have never used the IDT website to predict crRNA efficiency, we are not sure how accurate their predictions are. Further, in our experience, the most critical factors for ensuring a high editing efficiency is the proximity of the PAM site to the edit site and the orientation of the repair template. We have now included additional details regarding which orientation of the repair template will yield the best editing efficiency in the repair template design section. [Lines 170-175]

4. A link to a codon usage frequency table (line 162) should be provided.- We thank the reviewer for their suggestion. We have now provided a link to the codon usage frequency table in Section 2.5.3 of the "Repair template design" section. [Lines 186-188]

5. It would be useful to note that when choosing a restriction site to add to the repair template and designing the location of the PCR fragment that will be amplified for screening, having the same restriction site that is already present in the genome will provide an internal control for the restriction digest and for optimizing the experiment prior to doing the injections. And, on line 179 it should state "optimize the PCR conditions with wild-type genomic DNA." It should be noted that a large deletion may not require a new restriction site to be introduced because screening can be done with PCR directly to note any bands that are the appropriate smaller size.- We thank the reviewer for their suggestions. According to the reviewer's suggestion, we have now included in the "Screening primer design" section 3.2 a sentence mentioning "Test the primers and optimize PCR conditions with wild-type genomic DNA prior to using the primers for genotyping." The reviewers make an excellent point about having the same restriction site that is already present in the genome as an internal control for restriction digestions. However, in our experience, we are often severely restricted in the type of restriction site that can be introduced by silent mutagenesis. Further, using a restriction site that is already present in the genome as an internal control will necessitate the synthesis of new primers flanking the restriction site. If no positives are identified upon restriction digestion in our CRISPR experiment, in our opinion, a better alternative would be to use other confirmed CRISPR-generated worm lines that carry the same restriction site to check that the restriction enzyme is working properly. Therefore, we have not mentioned this as an option in our protocol. Regarding the reviewer's point to note that a larger deletion may not require a restriction site to be introduced, we have mentioned in the Introduction section that, "..RoI worms are then screened for the edit of interest by techniques such as polymerase chain reaction (PCR) (for edits greater than 50 bp) or by PCR followed by restriction digestion (for edits less than 50 bp). We have also mentioned in the "Restriction digestion and agarose gel electrophoresis" section that "(Note: a restriction digestion is only necessary while screening for small edits (less than 50 bp))". We hope that this addresses the reviewer's concerns. [Lines 221, 100-102, 312-313]

6. After allowing the injected animals to recover, consideration should be given for those that are clearly dead or not moving. It will save the investigator time to not have to screen through plates that have no chance of having progeny that will be edited.- The reviewer makes a good point. We have now added an extra note in the "Injected worm recovery and transfer" section 7.2.1 of the protocol mentioning "Note that some worms will die as a result of injury from the microinjection procedure. Only pick those worms that are alive and exhibit movement." [Lines 267-268]

7. The company Bioline where the MyTaq was purchased no longer exists. I believe it is now Meridian

Bioscience, but the authors should verify that.- We thank the reviewer for pointing this out. We have now made this correction in the Materials Table.

8. When describing the PCR for single worm lysis, it should be stated more clearly that the total reaction is 25 µl. The way it is written in step 4, a reader could think the total reaction is 22.5 µl.- We thank the reviewer for their suggestion. To clarify that the total reaction volume is 25 µl, we have added an extra sentence to section 9.4 of the "Single worm lysis and PCR" mentioning that "The total volume of each PCR reaction is 25 µl." [Line 304]

9. It should be noted that a desired edit on chromosome II that could be linked to dpy-10 can still be identified by screening a higher number of worms since most edits are heterozygous and rolling worms only have one dpy-10 locus edited so the desired edit could be on the other strand and could be segregated away from the dpy-10 mutation. Or non-rolling siblings from plates that have a lot of rollers could be screened for the desired mutation.- We thank the reviewer for their suggestion. Although the strategy that the reviewer suggests is definitely a valid strategy, we are afraid that it could be expensive to adopt this strategy. For example, screening hundreds of additional worms would necessitate the purchase of additional PCR reagents and will also require a significantly greater time investment to pick and process the additional worm samples. Therefore, we feel that in the case that a gene is linked to *dpy10,* it might be preferable to use a co-CRISPR marker that is located on a different chromosome rather than picking additional worms.

10. State that the primer used for Sanger sequencing should be at least 50 bp away from the edit so that the region of interest can be clearly seen in the sequencing results.- We thank the reviewer for their suggestion. We have now included a note in section 13.4.1 of the "Confirm edit by sequencing" section mentioning that "Ensure that the forward primer is designed to be at least 50 bases away from the edit to be sequenced." [Lines 375-376]

11. Remove "data not shown" from the results section (lines 286-287) when a reference is stated where the data should be.- We apologize for not being clear. The reference that was cited in this sentence was included because it identified the proteins GSP-1, I-2^{szy-2} and SDS-22 as novel regulators of centrosome duplication. Although RBM-3.2 was identified as a binding partner of these proteins in this study, these data were not included in the published manuscript. Therefore, in addition to citing the relevant reference, we feel it is important to indicate "(data not shown)". [Lines referenced: 384-387]

12. There is some evidence that the location of the repair either to the 3' or 5' side of the DSB affects the efficiency of the edit. That should be noted if there is an option for which PAM to choose.- We thank the reviewer for their suggestion. We have now included an additional step 2.3 in the protocol section for "Repair template design" which provides directions about which orientation of the repair template should be used for making edits to the 5' end versus the 3' end of the PAM. [Lines 170-175]

13. Indicate in the legend to Table 1 where the reagents were obtained (e.g., the Cas9). For the injection mix, the dpy-10 crRNA and repair templates as well as the specific tracrRNA to be used should be specified.- We thank the reviewer for their suggestion. The journal requires that such information be included in the Table of Materials. Accordingly, we have included the relevant information such as the company name, catalog number, etc. for the reagents used in the Table of Materials.

14. In Figure 2, indicate that the RoI worms are best taken from jackpot plates since those have the best chance of being edited.- We thank the reviewer for this suggestion. However, we did not obtain jackpot

plates (plates with >50% Rol and Dpy progeny) in this particular CRISPR experiment. Hence, the Rol F_1 worms were picked from 7 different injected P_0 worms in this experiment. This information has been included in the Representative Results section for Figure 2. [Lines 423-427, 446-447]

Minor editing revisions needed:
1. In the majority of CRISPR-Cas9 editing papers, Homology-directed repair is abbreviated HDR and not HR. Use the more common abbreviation.- We have made this change. [Lines 58, 62, 95, 96, 203]

2. Lines 49-50 in the introduction contains a sentence fragment and not a sentence. Add a verb.
We thank the reviewer for pointing this out. The word "which" was deleted so that the sentences reads as "The CRISPR system was first discovered as a part of a prokaryotic antiviral immune response[5-7]." [Lines 51-52]

3. Line 59 say "to either side" not "to either sides"- We thank the reviewer for pointing this out. We have made this change. [Line 61]

4. Line 135 remind the reader what to look for with a PAM site.- We thank the reviewer for their suggestion. We have now included an additional note in the "crRNA design" section 1.1.1 reminding the reader that the PAM site is 5'-NGG-3'. [Line 140]

5. Line 184 say "Pick L2-L3 stage C. elegans onto a fresh bacterial lawn on an MYOB plate" and a link should be provided for a protocol to make those plates.- We thank the reviewer for their comment. We have made these changes. [Lines 227-230]

6. Line 271 has a number formatting issue.- We have modified the formatting for this line. [Line 362]

7. "positively-edited worms" needs a dash- We have made this change. [Lines 216, 332, 335, 343, 348, 516, 517, 569]

Reviewer #5:
Manuscript Summary:
This protocol is very clearly written and provide step by step detail. Many research labs interested in the CRISPR-Cas9 of C. elegans will refer o=to their protocol. THe setting they describes are accessible to any C. elegans lab. This protocol will attract many readers.

Major Concerns:
No major concern.

Minor Concerns:
1. Line #91, I suggest 'roll' to Rol phenotype.- We have made this change. [Line 95]

2. In the protocol section 2.4 (line #159), modifying the PAM sequence is not only to prevent the CRISPR Cas9 RNP complex to the cut the DNA repair template during delivery, but to prevent the RNP complex to recut the edited genome. For the mutated PAM sequence, we suggest introducing into the sgRNA target site to prevent Cas9 recut the genome after HDR in case mutating the PAM sequence is impossible.- The reviewer brings up a good point. We have now edited this sentence to read "Mutate or

delete the PAM to prevent cutting of the repair template or the edited genomic DNA by Cas9." [Lines 180-181]

3. In the protocol section 5 (line #183), I think prepare L2-L3 stage worms would be too young. L4 worms at 20oC overnight is a good stage for microinjection.- We thank the reviewer for their comment. We have tried incubating L4 stage worms overnight 20°C and find that they are often too old (have too many embryos) by the time we are ready to inject (we prepare the injection mix in the morning and perform injections in the afternoon). Therefore, we recommend incubating L2-L3 stage worms at 20°C overnight. [Lines referenced 227-228]

4. In the protocol section 6 (line #188), to make the microinjection mix, are the denature and annealing steps required to make crRNA + tracrRNA = gRNA complex? I know crRNA from some other companies require this step to form gRNA.- We do not perform denaturation and annealing to make the crRNA+tracrRNA complex.

5. Line #220, 221: Can you handle 50 to 100 F1 rollers practically? I suggest picking less F1 than 50 to 100.- We agree with the reviewer that picking 50 to 100 F1 rollers is on the higher side. However, we have performed several CRISPR experiments in our lab now and our editing efficiency for this method has ranged from 2% to 58%. Since there is no way to predict what the editing efficiency for a particular locus is going to be, we prefer picking more worms so that we do not have to repeat the injections.

6. The homozygote screening strategy with restriction enzyme digestion in this protocol; Is there any risk to misinterpret the missing band (or no digestion) caused from the homozygote edits causing embryonic lethality? For example, if we can only see wild-type band and heterozygote patterns in Figure 4A but there is no homozygote pattern among the twelve F2 samples, how do we know whether it is embryonic lethal mutantion or caused by incomplete digestion?- We understand the reviewer's concern. Usually, if there is an incomplete digestion of a homozygote, the intensity of the cut band of a homozygote is much stronger than that of a heterozygote. This can be clearly seen in Figure 4. For example, the intensities of the cut bands of the homozygotes in Lanes 1, 4, 6, 8, 9 and 10 are much stronger than that of the heterozygote in Lane 11. So, we conclude that the weak uncut wild-type bands of the homozygotes are caused due to an incomplete digestion of the PCR product. Further, we have also sequenced one of these homozygotes and confirmed homozygosity of the edit by DNA sequencing.