

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,

Jyoti Iyer, Ph.D
Assistant Professor
Department of Chemistry and Biochemistry
University of Tulsa
800 S. Tucker Dr.
Tulsa, OK-74133
Email: jgi2708@utulsa.edu
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 protocol²⁸.”

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 37°C 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 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 -30°C vs at -80°C. 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 efficiency⁵⁰”
[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 P₀ 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 should be 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 P₀ 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 DNA⁵⁻¹²" [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 (“Homozygote 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 F₂ worms carrying the homozygous edit. On Day 10, the lysis is performed for F₃ worms from homozygous edited parent F₂ worm plates. In theory, we could use the PCR products from the identified homozygous F₂ 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 F₂ 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 F₃ worms and use this as a template to set up the PCRs for DNA sequencing reactions. Further, since we will be picking the F₃ worms to propagate and maintain the edited line, we prefer verifying the F₃ 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 or 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 worm²³⁻²⁸. Importantly, the phenotypes that are produced by these markers can be easily detected under a simple dissecting microscope²³⁻³⁴." 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 cite 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, "...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). 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 Rol 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 F₁ worms were picked from 7 different injected P₀ 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 response⁵⁻⁷.” [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 20°C 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 mutation 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.