**Rebuttal letter for JoVE59202 "Minimizing off-target effects of CRISPR-CAS9 without loss of on-target activity"**

**In response to reviewers’ and editor’s comments, we have now revised our manuscript. Our response is underlined.**

**Editorial comments:**  
Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have double checked our spelling and grammar. We also hired an external expert to double check the process.

2. Please print and sign the attached Author License Agreement (ALA) as the currently submitted one has been corrected. Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account.

We have attached ALA.

3. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

The previous publication was made to Nature Communications, which editorial policy allows re-prints. We have uploaded this information in .docx file to our Editorial Manager account.

4. Please provide the institutional affiliation for each author.

All of the authors are affiliated to Toolgen.

5. 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. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Enzynomics, Agilent, Clontech, Zymo research, Thermo, NanoDrop, Macherey-Nagel, Gene pulser II, Bio-Rad, Sigma Aldrich, Jena Bioscience, Turbo Dnase (Ambion), Phusion, Qiagen, etc.

We have removed all of the commercial names which appeared in the paper.

6. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have avoided every personal pronoun we could find.

7. Please revise the protocol to contain only action items that direct the reader to do something (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.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

We have made changes as required.

8. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

We have added more details to the parts suggested by the reviewers.

9. Please ensure that conditions and primers are listed all PCR procedures.

We have added primers and conditions to PCR procedures. However, reviewer #1 felt some of the steps are too specific, limiting the scope of the paper only to the specific type of Cas9 used in the original paper. To address this, we have added these details to the parts that are generally required to all of the Cas9 variants that can be evolved in Sniper-screen.

10. 1.1.4: Please describe how to ligate both fragments using T4 ligase.

We have added relevant details: “1.1.4 Ligate the fragments together using T4 ligase: mix 50 ng of digested pgrg36 and 6 ng of PCR insert in a reaction volume of 20 μL. Incubate at room temperature (RT) overnight.”

11. 1.1.5: Please describe how to confirm the insertion of EMX1 site by standard sequencing or add a relevant reference.

We have added relevant reference. 1.1.5 Transform the ligated plasmid into DH5 alpha competent cells and grow the resulting transformants on Luria broth (LB) agar plates containing ampicillin (100 μg/mL) at 32˚C. Confirm the insertion of the *GOI* fragment by Sanger sequencing13”

12. 3.3.7: Please describe how to Midi-prep the cultured cells.

Midi-prep protocol is available in the purchased kit. Each kit has their own protocol. We do not restrict a specific kind of kit that can be used in this process.

13. 3.3.8: Please indicate the specific screening steps that are being repeated here.

We have indicated the specific screening steps: “3.4.2 Prepare new Sniper-screening cells with a one-mismatch sgRNA plasmid. (See 3.2.) Redo the screening process (3.2-3.3) until survival reaches a plateau. Use 10 ng of the selected Cas9 plasmid for transformation and 10 ng/mL ATC during recovery. Maintain the ATC concentration at 10 ng/mL for the selective condition.”

14. 4.1.1: Please describe how to pick the target sites.

We have described how to pick the target sites as follows: “4.1.1 Pick target sites on <http://www.rgenome.net/cas-offinder/>. Select the PAM type appropriate for the species from which Cas9 was derived and the target genome (human, mouse, zebrafish, *etc*.) Fill in the ‘Query sequences’ tab, choose the ‘Mismatch number’, and click the ‘Submit’ button.

4.1.2 After a few seconds, the on-target (with a mismatch number of ‘0’) and off-target sites will appear. In general, choose the off-target sites with 1-3 mismatches.”

15. 4.2.2: Please provide the composition of PCR mixture.

We have provided the composition of PCR mixture as follows: “4.2.2 Prepare the PCR mixture for amplification of the sgRNA-encoding sequence as follows: combine 10 μL 5X Phusion HF buffer, 0.5 μL crRNA oligo (100 pmol/μl), 0.5 μL tracr RNA oligo (100 pmol/μl), 2.5 μL dNTPs (10 mM each), 0.5 μL Phusion DNA polymerase, and 36 μL NFW.”

16. 4.2.5, 8.1.2, etc.: Please add more specific details about how this is done.

We have combined 4.2.5 with 4.2.4. “8.1.1 Isolate genomic DNA from 7.2.6. with a gDNA preparation kit. Generate deep sequencing libraries by PCR amplification of the gDNA with primers targeting on-target and off-targets.”

17. Please note that steps 3.2.1-3.2.4 should be highlighted for filming because they are repeated in highlighted steps (3.3.1 and 3.3.2).

3.2.1 -3.2.4 are not repeated in highlighted steps. Only 3.3.1-3.3.6 are repeated as indicated.

18. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

We have combined some protocol steps: 3.3.6+ 3.3.7, 4.1.1 +4.1.2 +4.1.3 4.2.4 +4.2.5, 4.2.2+4.2.3+4.2.4, 4.3.5+ 4.3.6.

19. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted most important part of the protocol.

20. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

We have done as required.

21. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We have highlighted relevant details as suggested.

22. Please discuss all figures in the Representative Results.

We have indicated figure number in representative result as follows: “After Sniper-screen is performed, the percentage of survival colonies can be calculated by dividing the number of colonies on the LB plate containing chloramphenicol, kanamycin, arabinose, and ATC (CKAA) by the number of colonies in the LB plate containing chloramphenicol and kanamycin only (CK). This percentage was usually very low when Sniper-screen was performed with the libraries of SpCas9. True-positive hits can be enriched by repeating the screen with the surviving pool. In this representative Sniper-screen, for example, a 100% survival rate was obtained after the third screen (Figure 1). Transfections using RNPs or plasmid-encoded Sniper-Cas9 can be done for various targets and the resulting on-target and off-target activities measured by targeted amplicon sequencing (Figure 2). At most targets, Sniper-Cas9 shows the same level of on-target activities and higher specificity ratios compared to the WT. Truncated sgRNAs can also be used to further improve specificity (Figure 3). However, their use is limited to only a few targets because they result in low on-target activities compared to full-length sgRNAs in most cases. Therefore, one must test sgRNAs with varying lengths (from 17- to 20-mers) and measure both on-target and off-target activities to optimize specificity.”

23. Discussion: Please discuss any future applications of the technique.

We have added future applications of the technique as follows: “The specificities of other DNA endonucleases that induce DSBs, such as SaCas9 or Cpf1s, could also be improved by using Sniper-screen. Unfortunately, Sniper-screen cannot be used to increase the specificity of base editors directly, because base editors do not induce DSBs in the genomic DNA of *E. coli*. Because base editors use the nickase or dead version of Cas9 in the core of their system, the specificities of base editors could be increased by using the hits obtained from Sniper-screen.”

24. Please remove the embedded figure(s) and Table of Materials from the manuscript.

We have removed embedded figures and Table of Materials from the manuscript.

25. Figure 1: Please remove commercial language (Agilent, Clontech).

We have removed the commercial language.

26. References: Please do not abbreviate journal titles.

We have changed the abbreviated journal titles back to normal.

27. Table of Equipment and Materials: Please upload it in the form of an .xls or .xlsx file. Please remove trademark (™) and registered (®) symbols and sort the items in alphabetical order according to the Name of Material/ Equipment.  
We have removed trademark and registered symbols and sorted the items in alphabetical order.

**Reviewers' comments:**  
  
**Reviewer #1:**  
Manuscript Summary:  
The manuscript by Lee and colleagues details a method utilized to generate a high-fidelity Cas9 enzyme. Specifically, the method, known as "Sniper screen," utilizes both a simultaneous positive and negative selection to identify variants with low off-target activity without compromising on-target activity. The method is amenable to evolving Cas9 enzymes for any genomic loci, and can yield variants that can be coupled with other high-fidelity genome engineering technologies, such as truncated sgRNAs and RNP delivery.  
  
Overall, the protocol presented is detailed and thorough, and accomplishes the major goals for consideration, but can be made suitable for publication by addressing the following simple concerns:  
  
Major Concerns:  
1. Overall, the protocol varies from being too detailed in certain experimental sections, to not having enough detail in other sections. For example, plasmid choice (pBLC-SpCas9) and thus associated cloning strategies (such as restriction enzyme choice and PAM sequence) is very specific, and does not allow for the generalization of the method to other Cas9 enzymes. Especially with the advent of novel Cas9 enzymes/orthologs recognizing different PAM sequences, for this work to be useful to the genome editing community, the methodologies described should generalize to any Cas9 construct. On the other hand, certain steps are not augmented properly. For example, DNA shuffling seems to be a critical step in achieving library diversity to obtain high-fidelity variants. However, the authors simply point to a general manufacturer's instructions. The same can be said for the section of targeted deep sequencing. It would be advised that the authors expand these sections and provide details of the methodology (i.e. how to clone shuffled fragments, etc.).

Thank you for the suggestions. We have removed unnecessary details that are specific to spCas9. We have added more details to other general steps including DNA shuffling and targeted deep sequencing.

2. As this work is submitted as a methods paper, it can be benefited from at least one figure that graphically summarizes the workflow of the screening procedure. The original manuscript (Lee, et al. Nat. Comm 2018) does contain a graphic of the procedure, but an adapted version must be included to provide experimentalists a general idea of the protocol.

Thank you for your suggestion. However, Representative result is for the actual data that we could not fit in schematics in this section.

Minor Concerns:  
3. The authors need to carefully review the grammar of the manuscript to ensure ease of reading by readers. Many sentences, especially in the introduction and discussion, omit modifiers words and articles ("a", "an", "the", etc.) that make it difficult to read properly.  
We have double checked the grammar and spellings with external expert.  
  
**Reviewer #2:**  
Manuscript Summary:  
This article titled " Minimizing off-target effect of CRISPR-CAS9 without loss of on-target effect" presents a nice method to screen for better version of Cas9 in terms of specificity and efficiency. Because of the great potentials of Cas9 in broad applications, generation of Cas9 variants that are specific and effective to the selected target is essential especially for those to be used clinically. One of the pathway leading to the desirable Cas9 variants is to perform Cas9 engineering either through rational design based on its gRNA binding surface or random evolution such as described in the article. Therefore the topic of the article should be of great interest to the genome editing community using Cas9 as a tool.  
  
Major Concerns:  
1. Fig. 2. 1) more mismatch sequences per target site should be tested, ideally every position as Cas9 would have different activities for variations at different positions relevant to the spacer; 2) a comparison of the ratios of the off-target vs on-target activities should be performed in order to know if Sniper-Cas9 is better than WT-Cas9 as it seems for some targets there are no big differences between them. The same concern is true for Fig. 3.  
Thank you for your suggestion. However, this is method paper that highlights the protocol that is used in the original paper. We will take the suggestion into account for future research.

Minor Concerns:  
As the article listed the protocols in details step by step, it would be better to briefly state the purposes of some steps to facilitate the understanding such as why some vectors are used and if they can be replace by alternative ones. The second concern is the obscure logic of some steps. For example, in the 2.1.1 it stated that it is to get a variant library (XL1-red) by transfecting the pBLC-SpCas9 into XL1-red cells, is this step for making more plasmid. Another one is the evaluation of library complexity, what is the standard to judge if the library complexity is desirable.

We have erased detailed names for the some of the plasmids like pBLC-Cas9 since they can be replaced by alternative ones as suggested. For the rest of the suggestions, we have included more details to state the purpose. “2.1.1 Transform the Cas9 vector7 into a commercial *E. coli* mutator strain (see the table of materials) and follow the manufacturer’s instructions to obtain a variant library (the Mutator library).” and “2.2.3 When the desired complexity value is obtained, gather all of the colonies on the 245 mm square plate using a spreader. Purify the plasmid library using a Midi-prep kit. (Note: the higher the library complexity, the better. When Sniper-Cas9 was identified, a diversity of 3 X 106 was achieved for each library.)”