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TITLE:

Using Sniper-Cas9 to Minimize Off-target Effects of CRISPR-Cas9 Without the Loss of On-target Activity via Directed Evolution

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KEYWORDS:

Sniper, off-target, on-target, CRISPR, Cas9, RNP, plasmid

SUMMARY:

Here, we present a protocol to optimize CRISPR-Cas9 to achieve a higher specificity without the loss of on-target activity. We use a directed evolution approach called Sniper-screen to find a mutant Cas9 with the desired characteristics. Sniper-Cas9 is compatible with truncated single-guide RNAs and delivery in a ribonucleoprotein format, well-known strategies for achieving higher specificities.

ABSTRACT:

The development of clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) into therapeutic modalities requires the avoidance of its potentially deleterious off-target effects. Several methods have been devised to reduce such effects. Here, we present an *Escherichia coli*-based directed evolution method called Sniper-screen to obtain a Cas9 variant with optimized specificity and retained on-target activity, called Sniper-Cas9. Using Sniper-screen, positive and negative selection can be performed simultaneously. The screen can also be repeated with other single-guide RNA (sgRNA) sequences to enrich for the true positive hits. By using the CMV-PltetO1 dual promoter to express Cas9 variants, the performance of the pooled library can be quickly checked in mammalian cells. Methods to increase the specificity of Sniper-Cas9 are also described. First, the use of truncated sgRNAs has previously been shown to increase Cas9 specificity. Unlike other engineered Cas9s, Sniper-Cas9 retains a wild-type (WT) level of on-target activity when combined with truncated sgRNAs. Second, the delivery of Sniper-Cas9 in a ribonucleoprotein (RNP) format instead of a plasmid format is possible without affecting its on-target activity.

INTRODUCTION:

In this paper, we aim to improve the specificity of Cas9 by combining different strategies. Various methods of avoiding the off-target effects of CRISPR-Cas9 have been developed. For example, truncated sgRNAs can be used to achieve higher specificity¹. Additionally, the method of Cas9 delivery can be changed from a plasmid format to an RNP format to obtain higher specificity². Specific amino acid residues of the *Streptococcus pyogenes* Cas9 (SpCas9) protein have been modified according to the rational design described previously³⁻⁵. Alternatively, amino acid residues have been altered in a random manner and the Cas9 variants with the highest specificity were identified using either a yeast⁶ or an *E. coli*^{7,8} screening system.

However, many groups have reported that Cas9 variants engineered using the design to debilitate the nonspecific interaction between Cas9 and the substrate exhibit low on-target activities⁷⁻¹². We developed an *E. coli*-based directed evolution system, Sniper-screen, to screen randomly mutagenized Cas9 variants. An *E. coli* screening system has advantages over a yeast system because of the faster doubling time and higher transformation efficiencies of *E. coli*.

Both negative and positive selection, based on three different plasmids and a gene of interest (*GOI*) integrated into the *E. coli* genome, are used in Sniper-screen. Cas9 variants are expressed under the CMV-PltetO1 dual-promoter system of a low-copy number plasmid so that candidates identified in *E. coli* can be tested in mammalian cells without the need for subcloning. The *GOI* is introduced into the *E. coli* genome using the Tn7 transposon system. The sgRNA plasmid, which contains a temperature-sensitive origin of replication, expresses an sgRNA targeting the *GOI*; however, the sgRNA and *GOI* sequences are not perfectly matched. A perfectly matched sgRNA target site exists on a third plasmid containing the *ccdB* gene, which encodes a lethal product that poisons gyrase. In this system, cells expressing Cas9 variants with high off-target activities are removed because double-strand breaks (DSBs) are introduced into the mismatched site located in the genomic DNA. On the other hand, cells expressing Cas9 variants with low on-target activities are also removed because of lethal *ccdB* gene expression. The expression level of the Cas9 variants can be changed by altering the concentration of anhydrotetracycline (ATC), which adjusts the selection force.

We reasoned that locating the mismatched sgRNA target site in the genomic DNA rather than on a plasmid would increase the sensitivity of the system. The advantage of this approach is that there is only one genomic site, whereas there would be many plasmids, each containing a target site, within a single *E. coli* cell.

Using this system, we identified a Cas9 variant, Sniper-Cas9, which shows WT-level on-target activities and reduced off-target activities compared to WT Cas9. Sniper-Cas9 can achieve even higher specificity ratios by using truncated sgRNAs or RNP-based delivery rather than plasmid-based delivery.

PROTOCOL:

1. Integration of a human *GOI* into the BW25141 *E. coli* strain

1.1. Cloning of the *GOI*

1.1.1. Polymerase chain reaction (PCR) amplify a 500 bp length of a human *GOI* containing various candidate target sites via standard PCR methods with primers containing NotI and XhoI restriction enzyme sites.

NOTE: In this experiment, the *GOI* was the human *EMX1* gene.

1.1.2. Digest both the PCR product and the pgrg36¹³ vector with NotI and XhoI restriction enzymes.

1.1.3. Gel purify the desired fragments (500 bp and 12 kb).

1.1.4. Ligate the fragments together using T4 ligase. To do this, mix 50 ng of digested pgrg36 and 6 ng of PCR insert in a reaction volume of 20 µL containing 1x ligase buffer and 0.5 U ligase enzyme. Incubate at room temperature (RT) overnight.

1.1.5. Transform the ligated plasmid into DH5-α competent cells. Grow the resulting transformants on Luria broth (LB) agar plates containing ampicillin (100 µg/mL) at 32 °C and incubate overnight. Pick up a colony and grow it in LB media containing ampicillin overnight. Isolate the plasmid DNA from the *E. coli*, using a commercially available miniprep Kit. Confirm the insertion of the *GOI* fragment by Sanger sequencing the plasmid obtained from the mini-plasmid preparation (mini-prep)¹³.

1.2. Preparation of the BW25141-*GOI*

1.2.1. Transform the obtained pgrg36-*GOI* plasmid into the BW25141 *E. coli* strain. It is essential to use a BW25141 strain in order to minimize the number of false positive colonies.

1.2.2. Grow the transformed cells in the LB buffer at 32 °C overnight. *GOI* is inserted into the genomic DNA of the BW25141 strain (BW25141-*GOI*). Remove the pgrg36-*GOI* plasmid from the BW25141-*GOI* strain using the standard pgrg36 protocol¹³. Briefly, dilute a colony (approximately 10⁷-fold) and grow it on an LB plate at 42 °C overnight. Streak the colonies on the LB plate and grow them at 42 °C overnight.

1.2.3. Confirm the correct *GOI* insertion by colony PCR, using the primers suggested in the pgrg36 protocol: 5'-GATGCTGGTGGCGAAGCTGT-3' and 5'-GATGACGGTTTGTCACATGGA-3'. The primer amplifies the genomic DNA insertion site, and the size of the resulting PCR product will be 904 bp plus the size of the insert (500 bp in this case).

1.2.4. Prepare electrocompetent BW25141-*GOI* cells (a detailed protocol is described in steps 3.2.6–3.2.10).

2. Preparation of the Cas9 variant library

2.1. Library preparation

2.1.1. Transform the Cas9 vector⁷ into a commercial *E. coli* mutator strain (**Table of Materials**) and follow the manufacturer's instructions to obtain a variant library (the Mutator library).

2.1.2. Perform error-prone PCR on the whole WT Cas9 sequence in the Cas9 vector, using an error-prone PCR kit (**Table of Materials**).

NOTE: The low-error rate protocol was adopted in the Sniper-Cas9 case to avoid disrupting the original function of the protein.

2.1.3. Digest the Cas9 vector with appropriate restriction enzymes. Gel purify the PCR product (from step 2.1.2) and the digested backbone.

NOTE: The size of the SpCas9 gene is about 4.3 kb. XhoI and KpnI were chosen to digest pBLC-SpCas9 vector which was used in the Sniper-Cas9 case.

2.1.4. Assemble the backbone fragment (from step 2.1.3) and the insert amplified using error-prone PCR (from step 2.1.3) via isothermal *in vitro* recombination.

NOTE: More than 500 ng of backbone is needed to obtain a high concentration of library (error-prone PCR [EP] library). Two different error-prone PCR kits were used to prepare EP libraries in the Sniper-Cas9 case (EP library I and II).

2.1.5. Purify the products from the assembly (from step 2.1.4) using a DNA purification kit that enables low-volume elution (**Table of Materials**). Elute with 6 µL of nuclease-free water (NFW) and measure the concentration of DNA.

2.1.6. Transform more than 500 ng of Cas9 library vector (for each of three libraries) into 50 µL of electrocompetent *E. coli* cells (**Table of Materials**). See the electroporation protocol in steps 3.2.1-3.2.4. For this library preparation, use 1 mL of SOC medium instead of 250 µL per 50 µL of competent cells.

2.1.7. Make 1:100, 1:1,000, and 1:10,000 dilutions of the mixture containing the recovered cells with SOC medium. Plate the diluted cells on 100 mm LB agar plates supplemented with chloramphenicol (12.5 µg/mL). Plate the remaining cells on a 245 mm² plate. Incubate at 37 °C overnight.

2.2. Calculation of library complexity

2.2.1. Photograph the dilution plates via a gel documentation system or an ordinary digital

camera. Run OpenCFU software¹⁴ and upload the photographs of the dilution plates. Set the counting area inside a plate and remove false colonies.

2.2.2. Manually multiply the number of colonies by the dilution factor to obtain the original number of transformants. Convert these numbers into logarithmic form (base 10). Calculate the average to determine the complexity of the library.

2.2.3. When the desired complexity value is obtained, gather all the colonies on the 245 mm square plate (from step 2.1.7) using a spreader and 20 mL of LB supplemented with chloramphenicol. Do not grow the gathered colonies and purify the plasmid library using a commercial midiprep kit.

NOTE: The higher the library complexity, the better. When Sniper-Cas9 was identified, a diversity of 3×10^6 was achieved for each library.

3. Positive and negative screening for evolving Cas9

3.1. Target selection and plasmid construction

3.1.1. Select a target sgRNA spacer sequence in the *GOI*. Substitute one or two residues in the random nucleotide to produce a mismatched sequence.

NOTE: Human *EMX1* target site 3 (GAGTCCGAGCAGAAGAAGAA with GGG PAM) was used in the Sniper-Cas9 case. Followings are the mismatched sequences used: GAGTCCGAGCAGAAgAGAA, GAacCCGAGCAGAAGAAGAA, GAGTCCGAGCAGAgGAAGAA, and GAGcCCGAGCAGAAGAAGAA.

3.1.2. Insert the mismatched sequence (see step 3.1.1) into the sgRNA plasmid using standard oligonucleotide (oligo) cloning procedures⁷.

3.1.3. Insert the mismatched sequence with a PAM at the 3' end into p11-lacY-wtx1 (**Table of Materials**) to construct the *ccdB* plasmid using standard oligo cloning procedures¹⁵.

3.2. Preparation of Sniper-screening *E. coli* competent cells

3.2.1. Thaw BW25141-*GOI* electrocompetent cells on ice.

3.2.2. Add 1 ng each of the *ccdB* plasmid and the sgRNA plasmid with double mismatches into 50 μ L of thawed BW25141-*GOI* cells. Gently mix the cells by pipetting and move them into a prechilled 0.1 cm electroporation cuvette.

3.2.3. Transform the *E. coli* with the two plasmids via electroporation. Add 250 μ L of SOC medium immediately after electroporation. Gently pipette the solution to mix the cells and the medium. Transfer the mixture to a 1.5 mL microcentrifuge tube.

NOTE: For maximum efficiency, set the voltage at 1.80 kV, and the runtime should be between 4.8 ms and 5.0 ms.

3.2.4. Recover the transformed cells and incubate them at 32 °C for 1 h with gentle shaking.

3.2.5. Plate 125 µL of the recovered cells on an ampicillin (50 µg/mL)/kanamycin (25 µg/mL) LB agar plate (culture condition). Plate the remaining cells on an ampicillin/kanamycin/arabinose (1.5 mg/mL) LB agar plate (*ccdB*-expressing condition). Incubate at 32 °C overnight.

3.2.6. Check for the absence of surviving colonies on the *ccdB*-expressing condition plate. Gather colonies from the culture condition plate using a spreader and culture them in 250 mL of super optimal broth (SOB) medium supplemented with 50 µg/mL of ampicillin and 25 µg/mL of kanamycin at 32 °C, with gentle shaking.

3.2.7. When the optical density at 600 nm (OD₆₀₀) reaches 0.4, chill the flask on ice. Prepare prechilled deionized water and a prechilled 10% glycerol solution (sterilize before use).

3.2.8. Centrifuge (at 4,000 x *g* for 5 min at 4 °C) the cells and discard the supernatant. Add 200 mL of prechilled deionized water. Resuspend the cells using a 10 mL serological pipette. Repeat this step 3x.

3.2.9. Wash the cells with 50 mL of prechilled 10% glycerol solution. Centrifuge them as before (at 4,000 x *g* for 5 min at 4 °C).

3.2.10. Discard the supernatant and resuspend the pellet in 300 µL of 10% glycerol solution. Make 50 µL aliquots and freeze them in liquid nitrogen. Store the cells (Sniper-screening cells) at -80 °C.

3.3. Sniper-screening

3.3.1. Transform the Sniper-screening cells (from step 3.2.10) with 100 ng of the Cas9 variant plasmids from each library (from step 2.2.3. See steps 2.1.1 and 2.1.4). Follow the electroporation steps described in steps 3.2.1–3.2.3.

3.3.2. Transfer 250 µL of the cells to a fresh 1.5 mL microcentrifuge tube. Add 250 pg of ATC to make a final concentration of 10 ng/mL. Recover both ATC-containing and ATC-free cells (see step 3.2.4 for the recovery step).

3.3.3. Plate 25 µL of recovered ATC-free cells on a chloramphenicol/kanamycin LB agar plate (nonselective condition). Add ATC to the recovered ATC-containing cells to make a final concentration of 100 ng/mL on a 245 mm LB plate. Immediately plate the cells on a chloramphenicol/kanamycin/arabinose LB agar plate (selective condition). Incubate overnight at 32 °C.

NOTE: The size and the number of the LB plate are determined by the size of diversity of the screening covers. In the case of a 100 mm Petri dish with 20 mL of LB, add 2 µg of ATC.

3.3.4. Photograph the plates. Count the number of viable colonies using OpenCFU software¹⁴. (See step 2.2.1) Make sure that the number of colonies on the nonselective plate is at least 10x larger than the diversity of the library to cover all variants.

3.3.5. Calculate the survival frequency as follows.

Survival frequency = the number of colonies on a selective plate / (the number of colonies on a nonselective plate x 10)

3.3.6. Pool the colonies that survived on the selective plates from all three libraries. Incubate the surviving colonies in 250 mL of LB medium supplemented with 12.5 µg/mL chloramphenicol at 42 °C overnight. Isolate the screened Cas9 library DNA using a midiprep kit.

NOTE: This step clears the sgRNA plasmid.

3.3.7. Repeat the screening process from steps 3.3.1–3.3.6 until the survival frequency reaches a plateau. Use 10 ng of the selected Cas9 plasmid for transformation and 10 ng/mL of ATC during recovery. Maintain the ATC concentration at 100 ng/mL for the selective condition.

3.4. Shuffling and the second screening

3.4.1. Shuffle the selected pooled variants using the following DNA-shuffling protocol. PCR amplify the Cas9 insert in the Cas9 plasmid using flanking primers, 150 nucleotides from the insert boundaries. Digest 2 µg of amplified PCR product with DNase I for 1 min at 37 °C.

3.4.2. Purify fragments 70–200 bp in length using 2% agarose gel electrophoresis. PCR amplify the purified fragments. Use the product as a template to PCR amplify the Cas9 insert with appropriate primers flanking Cas9. Use the final PCR product to construct a Cas9 library as described in step 2.1.4.

3.4.3. Prepare new Sniper-screening cells (see section 3.2) with another mismatched sgRNA plasmid (see step 3.1.1). Redo the screening process (sections 3.2–3.3) until the survival rate 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.

3.5. Selection of evolved Cas9 mutant plasmids

3.5.1. After the last screening step, randomly pick one hundred colonies from the selective plate and culture them in chloramphenicol-containing LB medium at 42 °C overnight.

3.5.2. Isolate the plasmids using a miniprep procedure and Sanger-sequence the inserts using

sequencing primers within Cas9.

3.5.3. Select the top three most frequent variants to test them in human cell lines.

4. Delivery of Cas9 as an RNP with truncated sgRNA

4.1. Target gene selection using Cas-OFFinder

4.1.1. Pick target sites with Cas-OFFinder (<http://www.rgenome.net/cas-offinder/>). Select the PAM type appropriate for the specific type of Cas9 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 one to three mismatches.

4.2. Preparation of the sgRNA template

4.2.1. Order crRNA and tracrRNA oligos with the following template sequences, namely crRNA sequence: 5'-TAATACGACTCACTATAGGNNNNNNNNNNNNNNNNNNNGTTTGTAGAGCTAGAA-3'; tracrRNA sequence: 5'-AGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTATTTTAACTTGCTATTTCTAGCTCTAAAAC-3'. Fill N20 in the crRNA sequence with the target sequence obtained in step 4.1.2. To design truncated sgRNAs, remove bases from the 5' end to obtain N19, N18, or N17 sgRNA sequences.

4.2.2. Prepare the PCR mixture for amplification of the sgRNA-encoding sequence as follows: combine 10 µL of 5x buffer, 0.5 µL of crRNA oligo (100 pmol/µL), 0.5 µL of tracrRNA oligo (100 pmol/µL), 2.5 µL of dNTPs (10 mM each), 0.5 µL of DNA polymerase, and 36 µL of NFW (**Table of Materials**).

4.2.3. Amplify the template using the following conditions, namely, for the initial denaturation: 1 min at 98 °C; for the denaturation, annealing, and extension: 10 s at 98 °C, 15 s at 54 °C, 20 s at 72 °C, for 25 cycles; for the final extension: 5 min at 72 °C.

4.2.4. Analyze 2 µL of the amplified template DNA (from step 4.2.3) on a 2% agarose gel. Purify the template using a PCR purification kit.

NOTE: The size of the template DNA is 125 bp.

4.3. Synthesis of sgRNA

4.3.1. Prepare the reaction mixture for sgRNA synthesis as follows: combine 8.5 µL of template DNA (from step 4.2.3), 1 µL of UTP (25 mM), 1 µL of CTP (25 mM), 1 µL of GTP (25 mM), 1 µL of

ATP (25 mM), 4.2 µL of MgCl₂ (100 mM), 4.5 µL of T7 RNA polymerase (50 U/µL), 3 µL of 10x T7 RNA polymerase buffer, 1.2 µL of pyrophosphatase (0.5 U/µL), 0.75 µL of RNase inhibitor (40 U/µL), and 4.2 µL of NFW.

4.3.2. Incubate the reaction mixture at 37 °C overnight (at least for 10 h). Add 0.5 µL of DNase (2 U/µL) to the reaction mixture and incubate at 37 °C for 15–30 min. Purify the sgRNA using an RNA purification kit.

4.3.3. To reduce the toxicity caused by an innate immune response triggered by the 5'-triphosphate on the sgRNA¹⁶, remove the 5'-triphosphate from the guide RNAs with calf intestinal alkaline phosphatase (CIP) as follows: treat 10 µg of *in vitro*-transcribed RNA with 250 U of CIP for 3 h at 37 °C in the presence of 100 U of RNase inhibitor. Purify the CIP-treated sgRNA using an RNA purification kit.

5. WT- and Sniper-Cas9 protein expression and purification

5.1. Protein expression in *E. coli*

5.1.1. Transform pET plasmids¹⁸ encoding His-tagged WT- and Sniper-Cas9 into the BL21 (DE3) *E. coli* strain.

5.1.2. Inoculate 50 mL of LB medium containing 50 µg/mL kanamycin with a fresh colony harboring the pET-Cas9 expression plasmid and shake it overnight (200 rpm) at 37 °C (preculture).

5.1.3. Transfer 10 mL of overnight culture to 500 mL of fresh LB medium containing 50 µg/mL kanamycin. Incubate the culture with shaking (200 rpm) at 37 °C for 2 h.

5.1.4. Monitor the OD₆₀₀ until the culture reaches the mid-log phase of growth (OD₆₀₀ ≈ 0.6–0.7).

5.1.5. Induce the expression of the WT- or Sniper-Cas9 protein with isopropyl β-D-1-thiogalactopyranoside (IPTG, at a final concentration of 0.25 mM). Incubate the culture at 18 °C overnight.

5.2. Protein purification

5.2.1. Harvest the cells by centrifugation at 5,000 x *g* for 10 min at 4 °C.

5.2.2. Resuspend the pellet in the lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 4 mM dithiothreitol [DTT], 5 mM benzamidine, 100 mM phenylmethylsulfonyl fluoride [PMSF], pH 8) at 20 mL per gram wet weight.

5.2.3. Add PMSF, DTT, and lysozyme to a final concentration of 1 mg/mL each and incubate the mixture on ice for 30 min.

5.2.4. Sonicate the cells on ice. Pulse repeatedly for 10 s at 200–300 W with a 10 s cooling period between each pulse, for a total time of 20 min.

5.2.5. Centrifuge the lysate at 6,000 x *g* for 30 min at 4 °C.

5.2.6. Remove the supernatant to a fresh tube. Add 1 mL of His-Bind agarose resin to 5 mL of cleared lysate and shake gently for 1 h at 4 °C.

5.2.7. Load the lysate/His-Bind Agarose resin mixture onto a column with a capped bottom outlet.

5.2.8. Remove the bottom cap and collect the column flow-through.

5.2.9. Wash the column 2x with wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8); collect the wash fraction for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

5.2.10. Elute the protein 10x with 1 mL of elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8), collecting samples for SDS-PAGE.

5.2.11. Concentrate the eluted WT- or Sniper-Cas9 protein using a 100 kDa column filter. Store the samples in a solution of 10 mM Tris-HCl, 150 mM NaCl, and 50% glycerol at -80 °C.

6. RNP delivery

6.1. Transfection and preparation of cells for RNP delivery

6.1.1. Maintain HEK293T cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics at 37 °C with 5% CO₂.

6.1.2. Mix WT- or Sniper-Cas9 protein (2 µg) with sgRNA (2 µg) and incubate for 10 min at RT to make RNP complexes.

6.1.3. Trypsinize and count the cells. Prepare 2 x 10⁴ cells per one reaction. Wash the cells with phosphate-buffered saline (PBS) and centrifuge. Aspirate the supernatant and resuspend the pellet with electroporation buffer.

6.1.4. Electroporate RNP complexes into the cells using the following settings, namely 1,300 V, 30 ms, and one pulse. Plate the cells on a 48-well plate filled with 500 µL of DMEM supplemented with FBS and antibiotics (as described in step 6.1.1) right after the electroporation. Incubate at 37 °C with 5% CO₂.

6.1.5. Isolate the genomic DNA with a gDNA preparation kit, 48 h after the transfection.

7. Transfection of plasmids encoding Sniper-Cas9 and sgRNA

7.1. Construction of an sgRNA plasmid

7.1.1. Order forward and reverse oligos with the following template sequences, namely forward: 5'-CACCGNNNNNNNNNNNNNNNNNNNNNN-3'; reverse: 5'-AAACNNNNNNNNNNNNNNNNNNNNNNNC-3'. Replace N20 with the target sequence obtained in step 4.1.2. Shorten the target sequence to a length of N19, N18, or N17 to synthesize truncated sgRNA.

7.1.2. Anneal both oligos in 1x T4 DNA ligase buffer.

7.1.3. Digest the pRG2 vector with BsaI restriction enzyme.

7.1.4. Gel purify the digested vector (3,300 bp) using a 0.8% agarose gel.

7.1.5. Ligate the annealed oligo and the purified fragment using T4 ligase at 37 °C: mix 50 ng of digested pRG2 and 1 ng of annealed oligo in a reaction volume of 20 µL. Incubate at RT for 15 min.

7.1.6. Transform the ligation mixture into the DH5 alpha strain and grow transformants on LB agar plates containing ampicillin (100 µg/mL) at 37 °C. Confirm the insertion of the oligo in the vector by standard sequencing.

7.2. Transfection of plasmids encoding Sniper-Cas9 and sgRNA

7.2.1. Maintain HEK293T cells in DMEM supplemented with 10% FBS and 1% antibiotics at 37 °C with 5% CO₂. The day before transfection, trypsinize and count the cells. When working at a 48-well scale, plate 1 x 10⁵ cells per well in 250 µL of complete growth medium. The cells should be 50%–80 % confluent on the day of transfection.

7.2.2. At the 48-well scale, prepare 250 ng of p3s-Cas9 plasmid and 250 ng of sgRNA plasmid for transfection, using a lipid-based transfection reagent. Mix the plasmids in 25 µL of serum free-MEM.

7.2.3. Dilute 1 µL of transfection reagent with 25 µL of serum free-MEM. Incubate the mixture at RT for 5 min. Combine the two mixtures and incubate the resulting solution at RT for 20 min to form plasmid-lipofectamine complexes.

7.2.4. After 20 min of incubation, add 50 µL of the solution containing the plasmid-transfection reagent complexes directly to each well containing cells, and mix gently by rocking the plate back and forth. Incubate the cells at 37 °C in a CO₂ incubator for 48–72 h post transfection before assaying for transgene expression.

8. Calculation of indel frequencies to determine on-target and off-target activities

8.1. Targeted deep sequencing for the analysis of on-target and potential off-target sites

8.1.1. Isolate genomic DNA from step 6.1.5 or 7.2.4 with a gDNA preparation kit. Generate deep sequencing libraries by PCR amplification of the gDNA with primers targeting on-target and off-target.

8.1.2. Use index primers to label each sample. Subject pooled libraries to paired-end sequencing using a next-generation sequencing machine.

8.2. Deep sequencing analysis using Cas-Analyzer

8.2.1. Analyze deep sequencing data using the Cas-Analyzer assessment tool¹⁷.

8.2.2. Choose the Fastq files under the **Read 1** and **Read 2** tabs (Read 1 = XX_SXX_L001_R1_001.fastq, Read 2 = XX_SXX_L001_R2_001.fastq).

8.2.3. Fill out the **Basic Information** tab and the **Analysis Parameters** tab. Click the **Submit** button.

REPRESENTATIVE RESULTS:

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, sgRNAs with varying lengths (from 17- to 20-mers) must be tested and both on-target and off-target activities must be measured to optimize specificity.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative Sniper-screens performed with different libraries of random Cas9 variants. Mutator library, EP library I, and EP library II indicate libraries made using different commercial kits. First, second, and final indicate the number of times the enrichment screen was performed⁷. This figure has been modified from Lee et al.⁷.

Figure 2: On-target and off-target activities of WT-Cas9 or Sniper-Cas9 paired with a 20-mer guide sequence delivered via plasmid or RNP. Specificity ratios were determined by dividing the on-target activity by the off-target activity. Error bars indicate SEM ($n = 3$)⁷. This figure has been

modified from Lee et al.⁷.

Figure 3: On-target and off-target activities of Sniper-Cas9 compared to WT-Cas9 obtained using sgRNAs with variable lengths targeting the FANCF01 and AAVS sites. Specificity ratios were determined by dividing indel frequencies at on-target sites by those at the respective off-target sites. sgRNAs with a matched guanine at the 5' terminus (GX18 or GX19) and those with a mismatched guanine (gX17, gX18, gX19, or gX20) are indicated. Specificity ratios were not calculated when the normalized on-target activities were <70%⁷. This figure has been modified from Lee et al.⁷.

DISCUSSION:

For those who want to avoid cumbersome screening procedures to obtain Sniper-Cas9, the Sniper-Cas9 protein and the encoding plasmid are available. Using these materials, the optimum length of the sgRNA, that providing the highest specificity ratio, should be determined. In addition, delivery of Sniper-Cas9 and sgRNA in an RNP format is recommended because it usually results in a higher specificity ratio than delivery in a plasmid format. Unlike Sniper-Cas9, other engineered Cas9 variants are not compatible with truncated sgRNAs^{6,7} or delivery in RNP form⁸ (with the exception of HiFi-Cas9).

For Sniper-screen, the selection of the mismatched sequence is the most important step. Selection of a mismatched sgRNA that is associated with low cleavage activity toward the *GOI* sequence should be avoided. If not, Cas9 variants with a WT level of specificity will not cleave the *E. coli* genomic DNA with the mismatched target site. This effect will result in a large number of background colonies, jeopardizing the whole screening procedure.

Because *E. coli* has a fast doubling time and high transformation efficiency compared to yeast, Sniper-screen is advantageous compared to yeast-based screening methods. Additionally, Sniper-screen should be more sensitive than other *E. coli*-based systems in which the mismatched site is carried on a plasmid: there is one copy of the genomic DNA and thus only one copy of the mismatched site in our system, but a large number of plasmids within a single *E. coli* cell.

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*. As 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.

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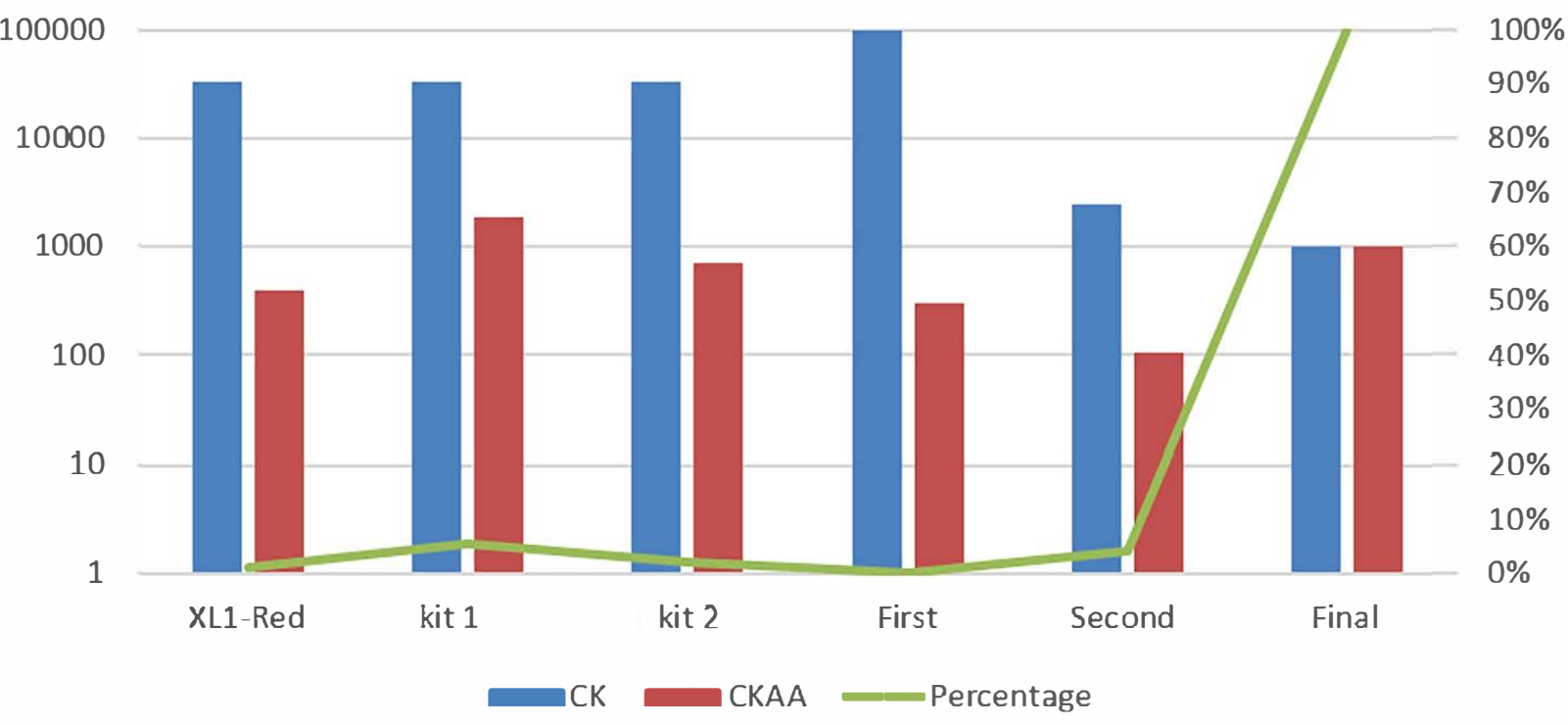
DISCLOSURES:

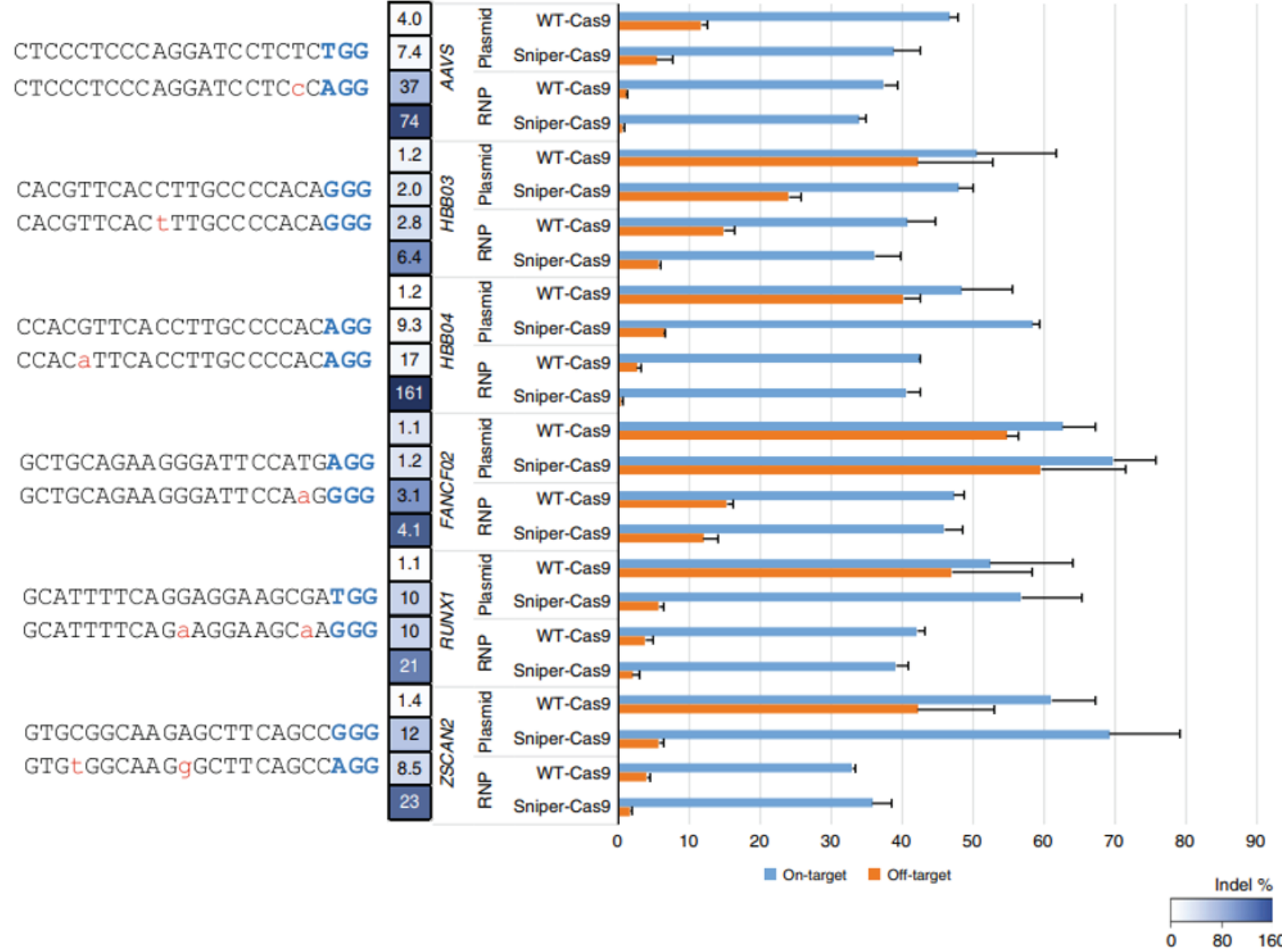
ToolGen has filed a patent application (PCT/KR2017/006212) covering Sniper-screen (status: pending, inventor: Jungjoon K. Lee). Jungjoon K. Lee, Joonsun Lee, Minhee Jung, and Euihwan Jeong are employees of ToolGen, Inc.

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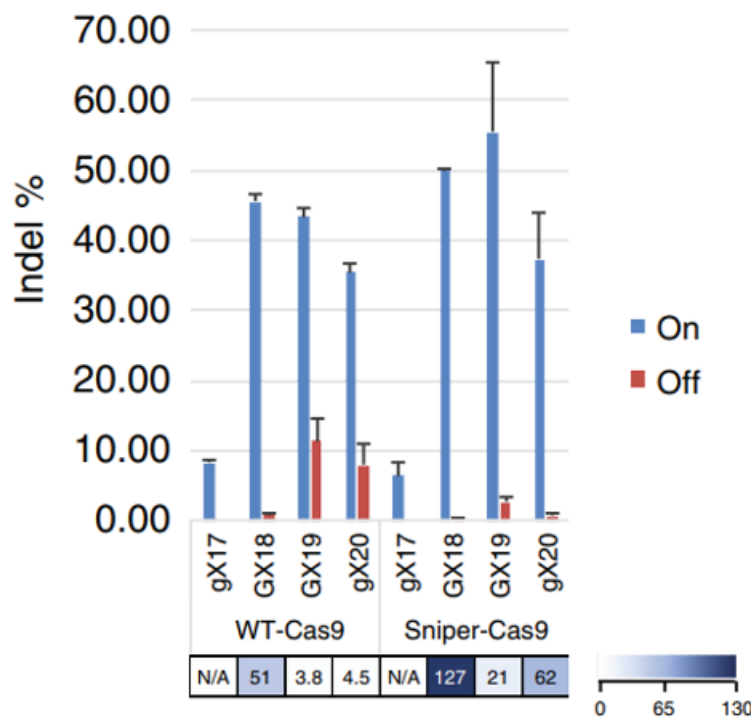
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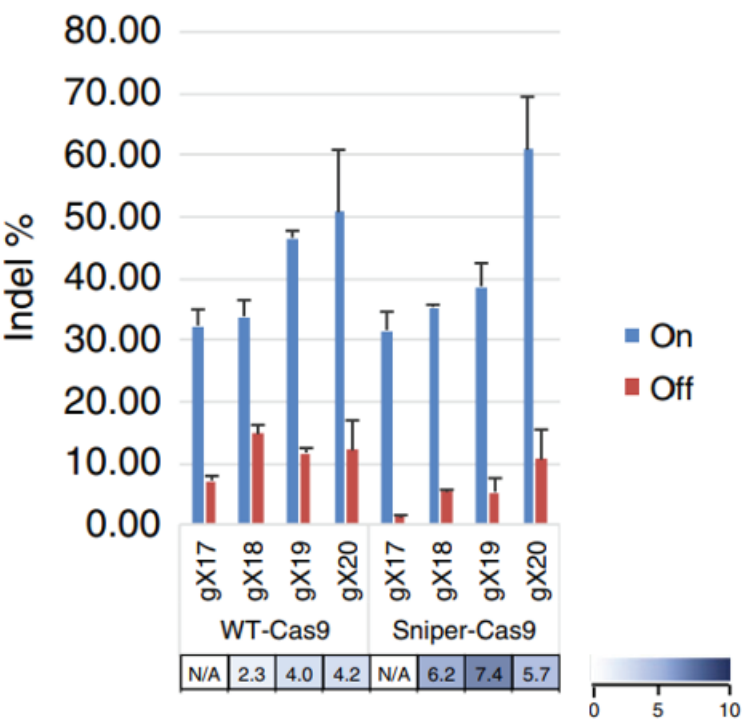


FANCF01



GGAATCCCTTCTGCAGCACCTGG
GGAACCCCgTCTGCAGCACCAAG

AAVS



CTCCCTCCCAGGATCCTCTCTGG
CTCCCTCCCAGGATCCTCcCAGG

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Alkaline Phosphatase, Calf Intestinal (CIP)	NEB	M0290L	
Ampicillin Sodium Salt	Fisher Chemical	BP1760-25	
Anhydrotetracycline hydrochloride	Sigma Aldrich	37919 or 94664	
Antibiotic Antimycotic solution	Welgene	LS203-01	Media component
BamHI	Enzynomics	R003L	
Chloramphenicol	Sigma Aldrich	R4408	
Diversify PCR Random Mutagenesis	Clontech	630703	Error-prone PCR kit
DNA-Shuffling Kit	Jena Bioscience	PP-103	
Dulbecco's Modified Eagle's Medium (DMEM)	Welgene	LM001-17	Culture media
Endura Electrocompetent Cells (DUO)	Lucigen	60242-2	<i>E. coli</i> electrocompetent cells for library preparation
Fetal Bovine Serum (FBS)	Welgene	S101-01	Media component
Gene Pulser II	Bio-Rad		<i>E. coli</i> electroporation equipment
GeneMorph II Random Mutagenesis Kit	Agilent	200550	Error-prone PCR kit
genomic DNA prep kit	GenAll	106-152	gDNA-prep kit
Glycerol	BioShop	GLY001.1	
GP/MP Cuvette, 0.1cm	Bio-Rad	BR165-2089	<i>E. coli</i> electroporation equipment
HEK293T cell	ATCC	CRL-11268	Human cell line
Kanamycin sulfate	Acros Organics	450810500	
L-(+)-Arabinose	Sigma Aldrich	A3256-25G	

LaboPass PCR Purification Kit	Cosmogene tech	CMR0112	
LaboPass Plasmid Mini	Cosmogene tech	CMP0112	Mini-prep kit
LB Agar Miller	Formedium	LMM0202	
LB Broth Miller	Formedium	LMM0102	
Lipofectamine	Invitrogen	11668019	Transfection reagent
NEBuilder HiFi DNA Assembly Master Mix	NEB	E2621L	Gibson assembly (isothermal <i>in vitro</i> recombination) mixture
Neon Transfection System	Thermo	MPK5000	RNP Electroporation equipment
Neon Transfection System 10 µL Kit	Thermo	MPK1096	RNP Electroporation equipment
NucleoBond Xtra Midi EF	Macherey- Nagel	740420.50	Midi-prep kit
Oligo Clean & Concentrator	Zymo Research	D4061	DNA purification kit that enables low-volume elution
Opti-MEM	Gibco	LS31985070	Transfection media
p11-lacY-wtx1	Addgene	#123945	
pgrg36	Addgene	#16666	<i>E. coli</i> mutator strain
Phusion High-Fidelity DNA Polymerase	NEB	M0530L	
Pyrophosphatase	NEB	M2403L	
Ribonucleotide Solution Set	NEB	N0450L	
RNase inhibitor	NEB	M0314L	
T7 RNA polymerase	NEB	M0251L	
Turbo Dnase	Ambion	AM2238	
XbaI	Enzynomics	R013L	
XL1-Red Competent Cells	Agilent	200129	



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Author(s):

Joonsun Lee, Min-hee Jung, Euihwan Jang, Jongjaan K Lee

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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.

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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-prepare the cultured cells.

Midi-prepare 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.

Commented [HM1]: use “a” here rather than “an” because the “o” in “one” is pronounced with a “w” sound

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 replaced 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 3×10^6 was achieved for each library.)"

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