Upgrading of specificity Sniper-Cas9 without loss of on-target activity

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Upgrading of specificity Sniper-Cas9 without loss of on-target activity

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TABLE OF CONTENTS

ABSTRACT ····· 1

I. INTRODUCTION ····· 2

II. MATERIALS AND METHODS ····· 4

1. Plasmid construction ····· 4
2. Sniper-Cas9 mutant library construction ····· 5
3. Positive and negative screening for directed evolution ····· 5
4. Site saturation mutagenesis in Sniper-Cas9 ····· 6
5. Oligonucleotide libraries ····· 6
6. Cell culture and transfection ····· 7
7. Production of lentivirus ····· 7
8. Generation of Sniper-Cas9 variant-expressing cell lines and transfection of lentiviral libraries ····· 7
9. Deep sequencing and analysis ····· 8
10. Delivery of Cas9 variants into a cell library using RNPs ····· 9

III. RESULTS ····· 10

1. Directed evolution of Sniper-Cas9 ····· 10
2. High-throughput assessments of the activities and specificities of the Sniper-Cas9 variants ····· 15
3. Sniper2L represents an outlier to the trade-off relationship between general activity and specificity ····· 24
4. High-throughput evaluation of Cas9 variant RNPs compared to WT-Cas9 RNPs ····· 25

IV. DISCUSSION ····· 31

V. CONCLUSION ····· 34

REFERENCES ····· 35

ABSTRACT (IN KOREAN) ····· 38

LIST OF FIGURES

Figure 1. Indel frequencies among clones at two different sites ····· 11

Figure 2. Sniper-screen and sequencing for hits selection ··· 13

Figure 3. Selection of hits by measuring on-target and off-target Indel frequencies ····· 14

Figure 4. Schematics of the subsequent evaluation of Sniper-Cas9 variants at a large number of target sequences ····· 16

Figure 5. Correlations between indel frequencies induced in two technical replicates ····· 17

Figure 6. PAM compatibilities among Sniper-Cas9 variants ·····18

Figure 7. Activities and specificities comparison of Sniper-Cas9 variants ····· 20

Figure 8. Specificity of Sniper-Cas9 variants in various mismatches ····· 22

Figure 9. Activity assessments at target sequences with (G/g)N_{19} or tRNA-N_{20} sgRNAs····· 23

Figure 10. Relationship between the specificity and activity of SpCas9 and Cas9 variants ····· 24

Figure 11. Schematic representation of experimental strategy using RNP delivery ····· 26

Figure 12. Indel frequencies to confirm 6-TG selection ····· 26

Figure 13. PAM compatibilities among high-fidelity Cas9 variants in RNP level ····· 28

Figure 14. High-throughput evaluation of Cas9 activities when delivered as ribonuclear proteins ····· 30

ABSTRACT

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Applications of CRISPR/Cas9 had been limited by off-target effect, which induce cleavage and mutations at untargeted genomic sites. As a strategy to solve these problems, several high-fidelity SpCas9 variants with increased specificity have been reported. Although we also developed a high-fidelity Cas9 called Sniper-Cas9, a trade-off relationship between activity and specificity of Cas9 variants was observed when engineered Cas9 variants, including Sniper-Cas9, were evaluated in a high-throughput platform. It is limitation to apply to the high-fidelity variants when efficient genome editing is required. Here, I developed a next-generation high fidelity variant, Sniper2L, which has improved specificity without loss of on-target activity using directed evolution screening. Sniper2L activity and specificity were evaluated using high-throughput platform that has many target sequences in lentiviral level. A modified high-throughput assessment platform is also developed to measure Cas9 variants activities when they are delivered as ribonucleoprotein (RNP) complexes. It was also confirmed that Sniper2L can induce highly efficient and specific editing on many target sequences when it is delivered as a RNP complex. I envision that Sniper2L will be useful when efficient and specific genome editing is required.

Key words : crispr-cas9, high fidelity cas9 variant, high-throughput screening

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I. INTRODUCTION

The applications of SpCas9-induced genome editing can often be restricted due to insufficient editing or off-target effects. Early assessment of the genome-wide off-target effects of Cas9 resulted in the detection of a large number of off-target sites[1,2], raising safety-related concerns about the development of Cas9-based human therapeutics. The list of side effects caused by Cas9-induced double-strand breaks has grown to include translocations[3], large deletions[3], p53 activation[4], and chromothripsis[5]. Many of these side effects could be minimized if the specificity of Cas9 were improved.

The specificity of Cas9 has been increased by the generation of Cas9 variants containing amino acid substitutions. In one approach, the position and identity of the substitutions were determined based on studies of the Cas9 mechanism. eSpCas9(1.1)[6], Cas9-HF1[7], HypaCas9[8], and Cas9_R63A/Q768A[9] were generated using this rational design approach. Alternatively, Cas9 variant-encoding libraries have been experimentally generated, and variants with increased specificity were selected using various screening systems. evoCas9[10], HiFi Cas9[11], and Sniper-Cas9[12] were developed using this directed evolution approach. However, it was reported that the modifications that decrease off-target cleavage also hamper the on-target cleavage activities of these Cas9 variants, shown a trade-off between the general activity and specificity[13] when tested with

a large number of target sequences. A high-fidelity variant with general activity similar to that of SpCas9 would facilitate the application of Cas9-based genome editing including gene therapy and genetic screening.

In this study, I developed Sniper2L, a next-generation high fidelity variant, using directed evolution. To confirm the specificity and activity of Sniper2L at a large number of target sequences, I evaluated its activity using two different delivery methods; lentiviral expression and delivery as ribonuclear protein (RNP) complex with guide RNAs. The high-throughput evaluations showed that Sniper2L has higher fidelity with retained general activities, overcoming the trade-off between activity and specificity regardless the delivery methods. I believe that Sniper2L will facilitate the applications of genome editing due to its high general activity and low off-target effects.

II. MATERIALS AND METHODS

1. Plasmid construction

Each type of plasmid used in the Sniper-screen contains replication origins and resistance markers that are compatible with each other. The p11a plasmid, which contains the ccdB gene, was double-digested with SphI and XhoI enzymes (Enzynomics, Daejeon, Republic of Korea) and ligated to oligos (Cosmogenetech, Seoul, Republic of Korea) containing the EMX1(1.6) target sequence (gcgccacTggttgatgtgat) with T4 DNA ligase (Enzynomics, Daejeon, Republic of Korea). The pSC101 (sgRNA-expressing vector) and the Sniper-Cas9 library plasmid have been described previously[12]. The EMX(1.6) sgRNA sequence with a mismatch (gcgccacTggttgatgtgat; the mismatched nucleotide at position 13 is capitalized) was cloned into the pSC101 vector after BsaI digestion.

For generating plasmids that express Cas9 variants, the lentiCas9-Blast plasmid (Addgene, Watertown, MA, USA, #52962) was digested with XbaI and BamHI-HF restriction enzymes (NEB, Ipswich, MA, USA) and treated with 1 μl of calf intestinal alkaline phosphatase (NEB, Ipswich, MA, USA) for 30 min at 37 °C. The digested vector was gel purified using a MEGAquick-spin Total Fragment DNA Purification Kit (iNtRON Biotechnology, Seongnam, Republic of Korea) according to the manufacturer's protocol. Mutation sites were introduced into variants by amplifying the lentiCas9-Blast plasmid using primers containing the mutation with Phusion High-fidelity DNA Polymerase (NEB, Ipswich, MA, USA). The mutation sites were chosen according to suggestions from GenScript for inducing high variant expression levels[14,15]. The amplicons were gel-purified (iNtRON Biotechnology, Seongnam, Republic of Korea) and assembled with digested lentiCas9-Blast plasmids using NEBuilder HiFi DNA Assembly Master Mix (NEB, Ipswich, MA, USA) for 1 h at 50 °C.

2. Sniper-Cas9 mutant library construction

Sniper-Cas9 mutant libraries were constructed using three independent protocols for mutagenesis, from XL1-red competent cells (Agilent, Santa Clara, CA, USA), Genemorph II (Agilent, Santa Clara, CA, USA), and Diversify PCR random mutagenesis (Clontech, Mountain View, CA, USA) kits. All reaction conditions have been described previously[12]. The assembled libraries were transformed into Endura™ electrocompetent cells (Lucigen, Middleton, WI, USA) and incubated on LB plates containing chloramphenicol (12.5 μg/mL) at 37 °C overnight. A total of 3×10^6 colonies were obtained for each library, resulting in an overall library complexity of 10^7. Pooled library plasmids were purified using a midi prep kit (NucleoBond Xtra Midi EF, Macherey-Nagel, Allentown, PA, USA).

3. Positive and negative screening for directed evolution

BW25141-EMX1(1.6) was co-transformed with p11a (*ccdB* + target sequence) and pSC101 (sgRNA expression) plasmids (from which sgRNA expression can be induced by the addition of anhydrotetracycline (ATC)). The transformed BW25141-EMX1 cells were plated on LB plates containing ampicillin (50 μg/mL) and kanamycin (25 μg/mL), and then incubated overnight at 32 °C. Electrocompetent cells were produced from transformants cultured in liquid S.O.B. medium containing 0.1% glucose, ampicillin, and kanamycin until the OD_{600} reached 0.4. Each Sniper library underwent four round screening. 100 ng of each library plasmid was transformed into 50 μL of electrocompetent BW25141-*EMX1*(1.6) cells using a Gene Pulser (Gene Pulser II, Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. In 1st screening, the transformed cell was incubated without ATC plated on chloramphenicol/kanamycin LB plates (nonselective conditions) and chloramphenicol/kanamycin/arabinose (1.5 mg/mL, Sigma-Aldrich, Burlington, MA, USA) LB plates (selective conditions) without ATC followed by the overnight culture at 32 °C. In the 2nd to 4th screening, the transformed cell was

incubated with 10 ng/ml ATC during recovery plated on nonselective and selective condition LB plates in the absence of ATC. Sniper screening conditions have been described previously[12]. After four rounds of screening, 50 colonies were obtained from selective condition plates, which were incubated in chloramphenicol containing LB medium at 42 °C. Each plasmid was Sanger-sequenced.

4. Site saturation mutagenesis in Sniper-Cas9

For site saturation mutagenesis of the 1007th position in Sniper-Cas9, the pBLC-Sniper-Cas9 plasmid was amplified using primers containing NNK at the appropriate position. The PCR product was digested with DpnI (Enzynomics, Daejeon, Republic of Korea), treated with T4 polynucleotide kinase (Enzynomics, Daejeon, Republic of Korea), and ligated with T4 ligase (Enzynomics, Daejeon, Republic of Korea). The resulting product was transformed in DH5alpha cells. After Sanger-sequencing of plasmids from 100 randomly-selected colonies, variants containing 20 different amino acids at the 1007th position were identified.

5. Oligonucleotide libraries

Sniper-Cas9 mutant libraries were constructed using three independent protocols for mutagenesis

Three oligonucleotide pools, libraries A, B, and C, were described in previous study[13]. Library A was utilized for evaluating PAM sequences and activities at mismatched target sequences. Using library B, indel frequencies induced by variants were measured at a large number of target sequences with (G/g)N_{19} sgRNAs. Library C contained target sequences that were identical with those in library B but used a different sgRNA expression system that resulted in perfectly matched tRNA-N_{20} sgRNAs. All three oligonucleotide libraries were used for examining Sniper-Cas9 variants based on lentiviral delivery, whereas

Library A was applied for comparing high-fidelity variants using the RNP delivery method.

6. Cell culture and transfection

Human embryonic kidney 293T (HEK293T) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum. Cells were transfected using lipofectamine 2000 (Invitrogen, Waltham, MA, USA) at a weight ratio of 1:1 (Sniper-Cas9 variant plasmid: sgRNA expression plasmid) in 48 wells. Genomic DNA was isolated with a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) 72 h after transfection.

7. Production of lentivirus

Lentivirus was produced using a method identical to that utilized in our previous study[13]. In brief, the day before transfection, HEK293T cells were seeded; the following day, the cells were treated with chloroquine diphosphate for up to 5 h, and transfected with lentiviral vector and packaging plasmids. The next day, the lentivirus-containing media was removed and fresh DMEM was added to the transfected HEK293T cells. The supernatant with viral particles was harvested 48 h after transfection; remaining library plasmids were degraded by treatment with Benzonase (Enzynomics, Daejeon, Republic of Korea) [16,17]

8. Generation of Sniper-Cas9 variant-expressing cell lines and transduction of lentiviral libraries

For measuring lentiviral titers, HEK293T cells were transduced with sequentially diluted aliquots of lentivirus-containing supernatant along with 10 μg/ml of polybrene and incubated overnight. The next day, both transduced and untransduced cells were treated with 20 μg/ml of blasticidin S (InvivoGen, San Diego, CA, USA), and the number of surviving cells in the transduced population

was counted when the untransduced cells were no longer viable[16]. Cell lines expressing Sniper-Cas9 variants were continuously maintained in the presence of 20 μg/ml of blasticidin S (InvivoGen, San Diego, CA, USA).

Lentiviral libraries were transduced into Sniper-Cas9 variant-expressing cells using a protocol identical with that previously described[13]. In brief, 2.5 x 10^7 Sniper-Cas9 variant-expressing cells were seeded in each 15-cm dish; two dishes (with a total of 5 x 10^7 cells) were used for libraries A and C and four dishes (with a total of 1.0 x 10^8 cells) were used for library B. Lentiviral plasmid libraries were transduced at an MOI of 0.4 along with 10 μg/ml of polybrene. After 4 days (libraries A, B, and C) and 7 days (library A) of transduction, cells were harvested.

9. Deep sequencing and analysis

To examine the activities of the Sniper-Cas9 variants, samples were prepared and analyzed as previously described[13]. The following formula was used to remove background indel frequencies:

Indel frequencies (%) =

$$\frac{\text{Indel read counts} - (\text{Total read counts x background indel frequency})/100}{\text{Total read counts} - (\text{Total read counts x background indel frequences})/100} \times 100$$

To minimize the errors generated by array synthesis, PCR amplification, or deep sequencing, I excluded target sequences with fewer than 100 total read counts or that exhibited background indel frequencies greater than 8% from the analysis.

10. Delivery of Cas9 variants into a cell library using RNPs

Lentiviral plasmid library A was transduced into HEK293T cells at an MOI of 0.1 to generate a cell library. The cell library was continuously maintained in the presence of 2 μg/ml of puromycin (Invitrogen, Waltham, MA, USA). The *HPRT*-targeting sgRNA templates were generated by annealing two complementary oligonucleotides, which were then incubated with T7 RNA polymerase in reaction buffer (40 mM Tris-HCl, 6 mM $MgCl_2$, 10 mM DTT, 10 mM NaCl, 2 mM spermidine, 3.3 mM NTPs, 1 U/μl RNase inhibitor, at pH 7.9) for 8 h at 37 °C. Transcribed sgRNAs were preincubated with DNase I to remove template DNA and purified using a PCR purification kit (Macrogen, Seoul, Republic of Korea). A total of 3 x 10^7 cells (6 x 10^6 cells per dish x 5 dishes) were transfected with protein variants (WT-Cas9, Sniper1, Sniper2L, HiFi Cas9, and Cas9_R63A/Q768A; 40 μg) premixed with in vitro transcribed *HPRT*-targeting sgRNA (40 μg) and Alt-R Cas9 electroporation enhancer (4 μM, Integrated DNA Technologies, Coralville, IA, USA) using a Neon transfection system (ThermoFisher, Waltham, MA, USA) with the following settings: 1,150 V, 20 ms, and 2 pulses per 2 x 10^6 cells using a 100 μL tip. On day 3 after transfection, a portion of the cell culture was harvested for analysis of indels at the HRPT site. Beginning on day 7 after transfection, cells were maintained with DMEM supplemented with 10 % fetal bovine serum and 30 μM 6TG (Sigma-Aldrich, Burlington, MA, USA). The cells were harvested 14 days after the 6TG selection began. Genomic DNA was isolated with a Blood & Cell Culture DNA Maxi Kit (Qiagen, Hilden, Germany).

III. RESULTS

1. Directed evolution of Sniper-Cas9

Previously, I used "Sniper-screen" for directed evolution of Cas9 in E. coli[12]. In brief, both positive [Cas9-mediated cleavage of a plasmid containing a lethal gene (*ccdB*)] and negative (lack of *E. coli*-killing cleavage at a mismatched off-target genomic site) selection pressure were applied into SpCas9 mutant libraries with random errors of up to 10^7 complexities in the whole Cas9-encoding sequence; a fragment of the human *EMX1* gene was used for the matched and mismatched target sequences. The initial Sniper-screen resulted in the identification of three Cas9 variants named Clone-1, Clone-2, and Clone-3[12]. I selected Clone-1 because it induced high frequencies of on-target indels with many different single-guide RNAs (sgRNAs) compared to Clone-2 and Clone-3, which showed low on-target indel efficiencies with the same sgRNAs. High indel frequencies were observed when these variants were tested with the sgRNA EMX1.3, which was used in the Sniper-screen. To eliminate Cas9 variants with reduced on-target activities such as Clone-2 and Clone-3, I needed to perform the Sniper-screen with a sgRNA that would result in low on-target indel efficiencies with Clone-2 and Clone-3 while retaining WT-level indel efficiencies with Clone-1 (Figure 1). The sgRNA EMX1.6, which targets a different region of the *EMX1* fragment compared to sgRNA EMX1.3, fulfilled this criterion.

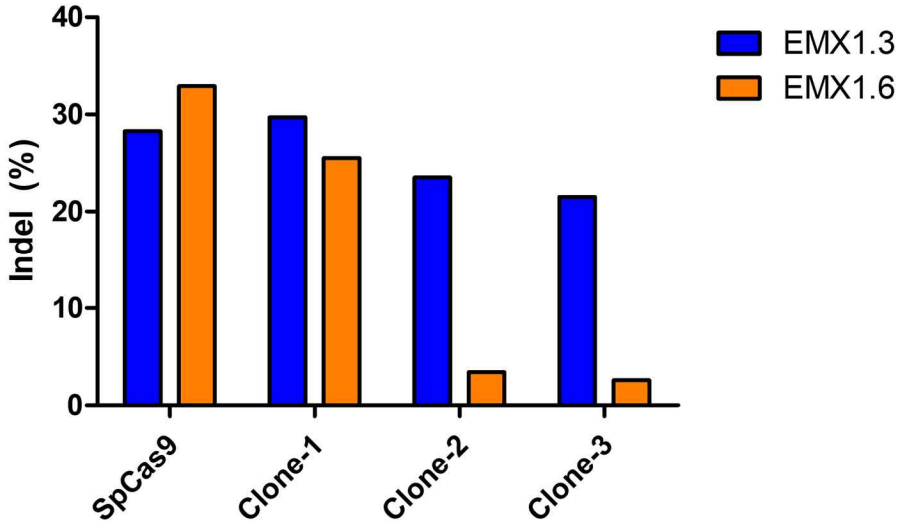
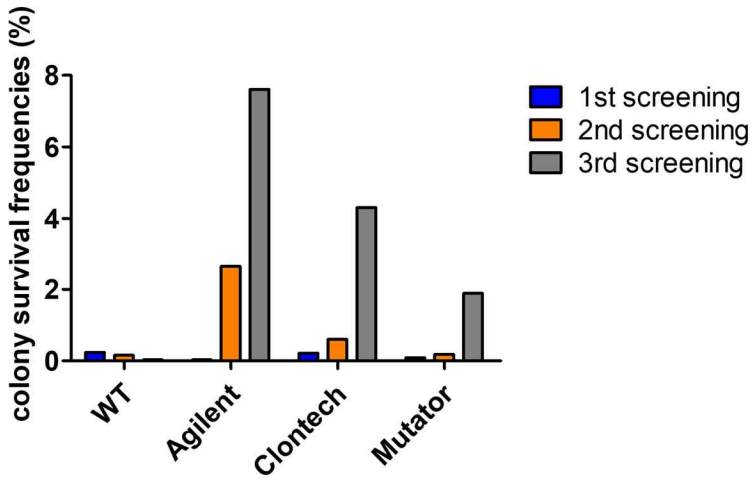


Figure 1. Indel frequencies among clones at two different sites. Indel frequencies induced by Clone-1 (Sniper-Cas9, referred to in this manuscript as Sniper1), Clone-2, and Clone-3 with the EMX1.3 and EMX1.6 sgRNAs, which target two different sites in the human *EMX1* gene.

Libraries encoding mutant versions of Sniper-Cas9 with random errors in the Sniper-Cas9 sequence were constructed using the three different mutagenesis kits that were used in the previous Sniper-screen[12]. The Sniper-screen selection procedure was repeated four times with the EMX1.6 sgRNA (Figure 2a). The final clones were sequenced and a hot spot at the 1007th position of Sniper-Cas9 was identified (Figure 2b). I performed site saturation mutagenesis in the 1007th position and selected the two mutants named Sniper2L and Sniper2P with E1007L and E1007P mutations, respectively, both of which retained high on-target activities with diminished off-target activities when tested in human cells (HEK293 cells) at four different target sequences in comparison with Sniper1 (Figure 3a~e).

a



b

Amino acid, position of WT-Cas9	Ag1	Cl1	Cl2	Cl3	Cl4	Cl5	Mu1	Mu2
Lys, 4	Lys							
Lys, 112	Asn							
His, 137		His						
Ile, 350		Val						
Ile, 492	Phe							
Arg, 671	His							
Gln, 709		Gln						
Lys, 735	Thr							
Ala, 889								Val
Glu, 1007		Val		Val	Gly	Gly		
Tyr, 1021		Cys						
Lys, 1191							Glu	
Lys, 1192						Arg		
Ser, 1277			Gly					
Number of colonies for the clone/ Number of total sequenced colonies	11/12	14/39	8/39	3/39	3/39	2/39	4/48	3/48

Figure 2. Sniper-screen and sequencing for hits selection. a, Colony survival frequencies for cells transformed with three libraries encoding mutant versions of Sniper-Cas9 during three rounds screening. Agilent, Genemorph II error-prone PCR kit from Agilent; Clontech, Diversify PCR random mutagenesis kit from Clontech; and Mutator, XL-1 Red competent cells from Agilent. **b,** Sequencing results of selected hits obtained from the Sniper-screen performed with three classes of libraries encoding mutant versions of Sniper-Cas9. Ag1, Cl1~5, and Mu1~2 respectively indicate selected hits from libraries generated using the Genemorph II error-prone PCR kit, the Diversify PCR random mutagenesis kit, and XL-1 Red competent cells.

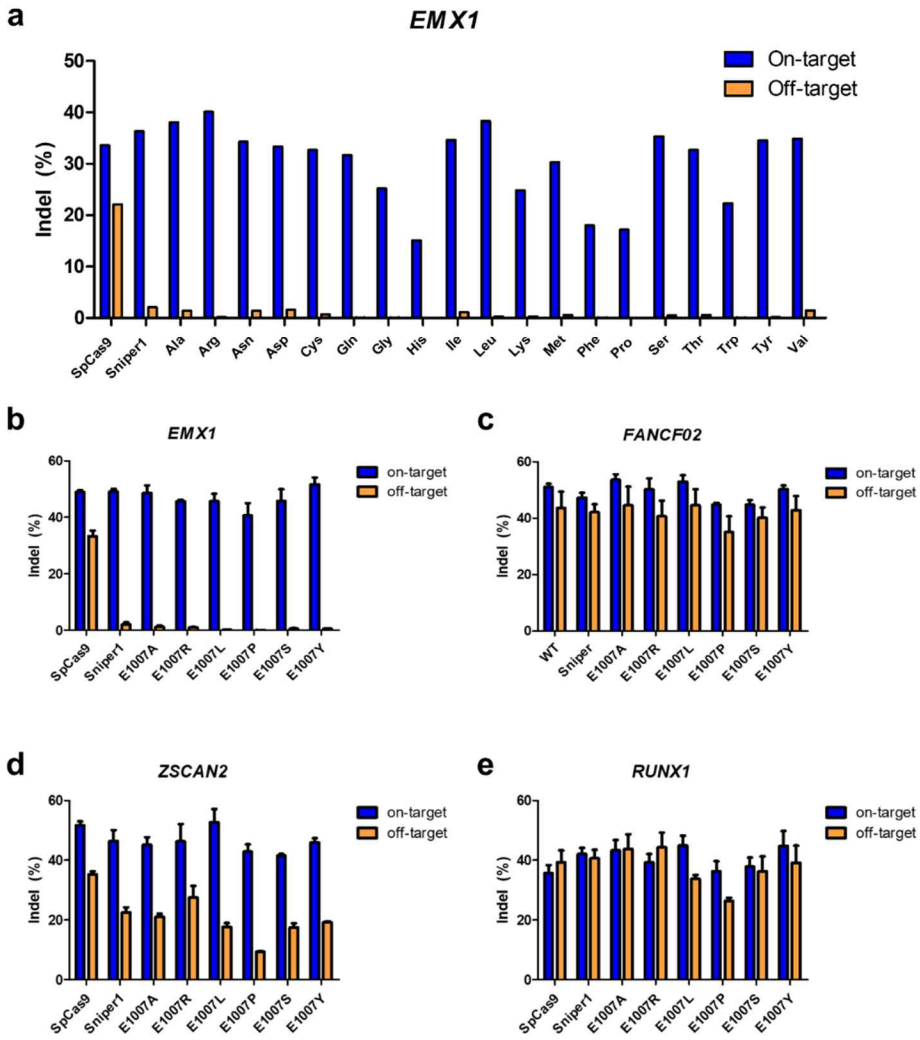


Figure 3. Selection of hits by measuring on-target and off-target Indel frequencies. a, on-target and off-target indel frequencies of SpCas9 and site saturation mutagenesis variants at the 1007th position. (originally a Glu codon) **b~e,** On-target and off-target indel frequencies induced by SpCas9 and Sniper-Cas9 variants, obtained by site saturation mutagenesis at the 1007th position, when targeted to the *EMX1* (**b**), *FANCF02* (**c**), *ZSCAN2* (**d**), and *RUNX1* (**e**) genes in HEK293T cells. Error bars indicate s.e.m. (n = 3)

2. High-throughput assessments of the activities and specificities of the Sniper2 variants

Although I compared the activities of Sniper2L and Sniper2P at four target sequences, to compare the general activities of these two variants, much more target sequences should be used[13]. To evaluate the activities of these two variants at a large number of target sequences, I adopted a high-throughput evaluation approach that I previously used to compare activities of various Cas9 variants[13]. For this high-throughput evaluations, I first generated individual cell lines, each containing a single copy of a variant-expressing lentivirus[13], which led to comparable expression levels of Sniper1 and the Sniper2 variants (Figure 4). I then transduced our previously-described lentiviral libraries of pairs of sgRNA-encoding and corresponding target sequences[13,18], into the Sniper-Cas9 variant-expressing cells and indel frequencies at the integrated target sequences were determined by deep sequencing four and seven days after the transduction of lentiviral libraries (Methods). The used libraries were libraries A, B, and C[13], which contained 11,802, 23,679, and 7,567 sgRNA-target pairs, respectively. Because indel frequencies between two technical replicates were well correlated (Figure 5), I combined the read counts from two replicates to draw more accurate conclusions.

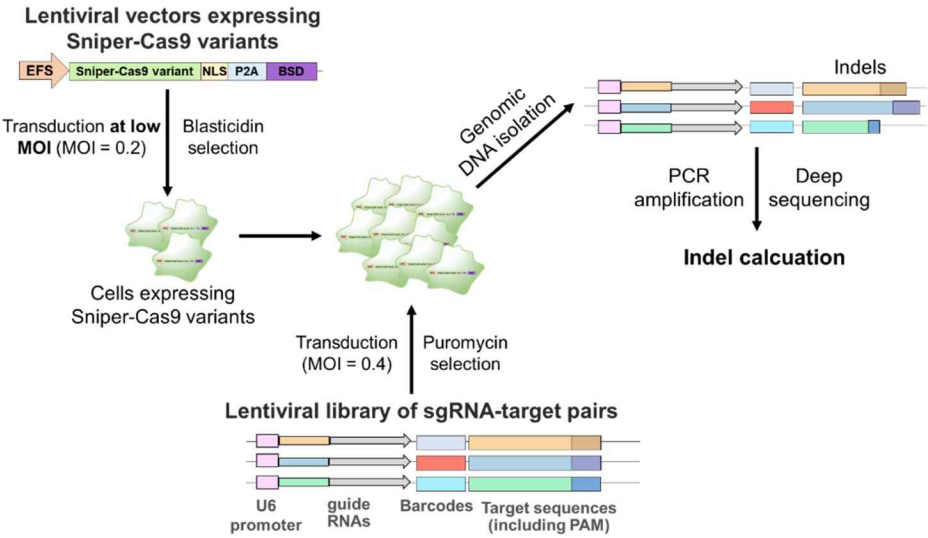


Figure 4. Schematics of the subsequent evaluation of Sniper-Cas9 variants at a large number of target sequences.

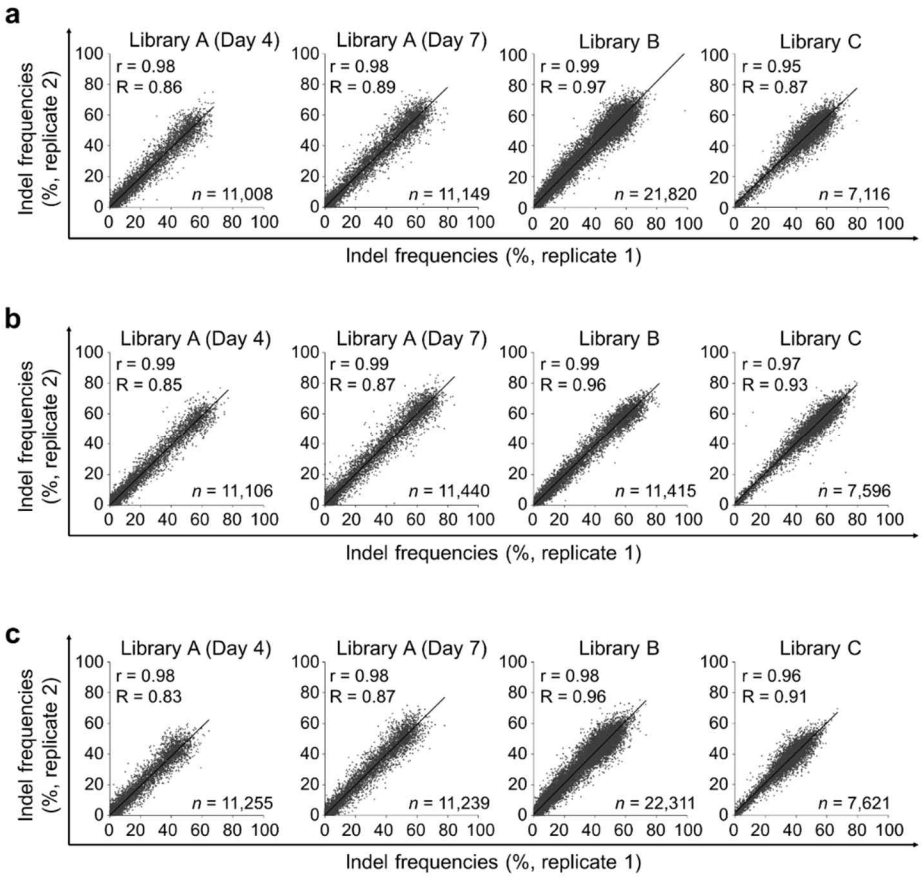


Figure 5. Correlations between indel frequencies induced in two technical replicates. Correlations between indel frequencies induced in two technical replicates by Sniper-Cas9 (**a**), Sniper2L (**b**), and Sniper2P (**c**) in the high-throughput analysis. The Pearson correlation coefficient (r) and the Spearman correlation (R) are shown. In each graph, the number of target sequences (*n*) that I used for analysis is shown.

I first determined the PAM compatibilities recognized by the Sniper2 variants using library A containing target sequences with NNNN PAMs. I found that the PAM compatibilities of Sniper-Cas9 variants were identical and that the highest average activities were observed at target sequences with NGG PAMs (Figure 6). These results are in line with the PAM compatibilities of other high-fidelity variants[13] and would be attributable to the lack of mutations within the PAM-interacting domain of Sniper-Cas9 variants. Based on these results, target sequences with NGG PAMs were chosen for subsequent analysis.

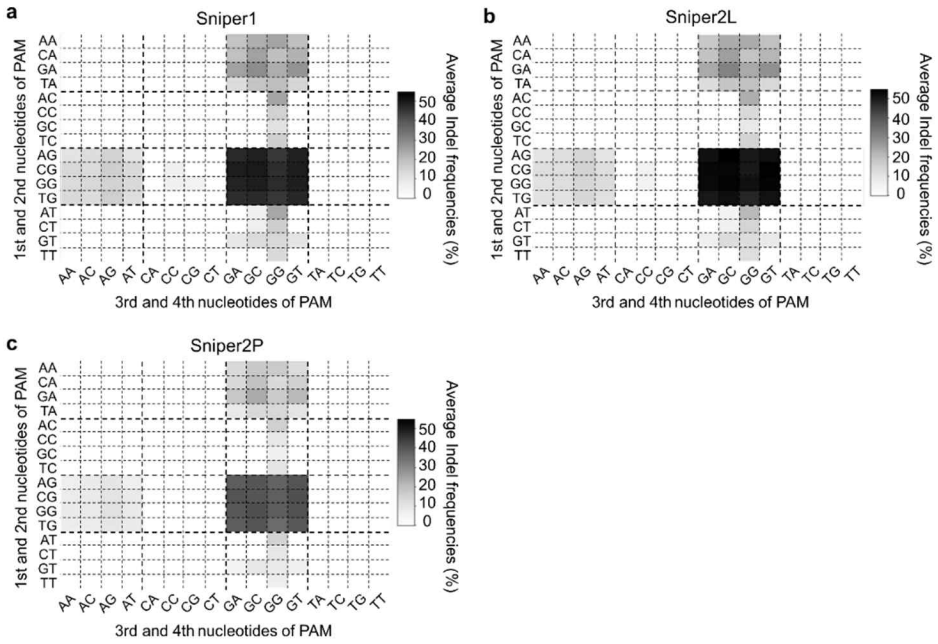


Figure 6. PAM compatibilities among Sniper-Cas9 variants. PAM sequences recognized by Sniper1 (**a**), Sniper2L (**b**), and Sniper2P (**c**). Average indel frequencies four days after the transduction of library A into Sniper-Cas9 variant-expressing cells are shown; Average indel frequencies lower than 5% are indicated as white boxes in the grids. n = 24 - 30 per PAM sequence.

I evaluated the activities and specificities of the Sniper2 variants at a large number of matched and mismatched target sequences. For assessing on-target activities, the 8,744 target sequences with NGG PAMs in library B were utilized. I found that Sniper2L exhibited significantly higher efficiencies than Sniper1, whereas Sniper2P induced the lowest indel frequencies (Figure 7a). Next, I compared the specificities of the Sniper-Cas9 variants with that of Sniper1 by comparing activities at mismatched target sequences using library A. Given that a comparison of activities at mismatched target sequences can be biased when the activities at matched target sequences are substantially different between the comparison groups, I used 30 sgRNAs that induced comparable Sniper-cas9 variant-directed indel frequencies either four or seven days after transduction. Each of the 30 sgRNAs was paired with 98 target sequences harboring one-, two-, or three-base mismatches. The activities of Sniper2L at the mismatched target sequences were significantly lower than those of Sniper1 and Sniper2P (Figure 7b). If I define specificity as 1 – (indel frequencies at target sequences that harbor a single-mismatch divided by those at perfectly matched target sequences), the specificity of Sniper2L was significantly higher than those of Sniper2P and Sniper1 (Figure 7c).

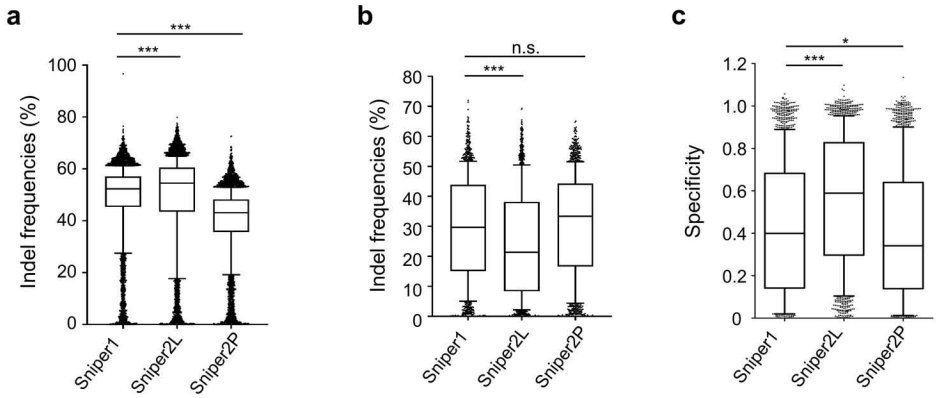


Figure 7. Activities and specificities comparison of Sniper-Cas9 variants. a, Indel frequencies at target sequences containing NGG PAMs. n = 7,702 target sequences. $P = <7.56 \text{ X } 10^{-34}$ for Sniper1 and Sniper2L and Sniper1 and Sniper2P; Kruskal-Wallis test. **b,** Indel frequencies at target sequences with single-base mismatches containing NGG PAMs. n = 1,732. P = 3.16 x 10^{-18} for Sniper1 and Sniper2L and no statistically significant difference between Sniper1 and Sniper2P; Kruskal-Wallis test. **c,** General specificity of variants. Specificity was calculated as 1 – (indel frequencies at target sequences that harbor a single mismatch divided by those at perfectly matched target sequences). n = 1,734, 1,732, and 1,734 for Sniper1, Sniper2L, and Sniper2P, respectively. $P = 2.51 \text{ x } 10^{-32}$ for Sniper1.0 and Sniper2L and 0.015 for Sniper1 and Sniper2P; Mann-Whitney U test.

When I determined the specificity as a function of the mismatch position, I found that all three Sniper Cas9 variants showed higher specificity at the PAM-proximal region as compared to the PAM distal regions (Figure 8a). Similar higher specificities at the PAM-proximal regions were also previously observed in other high-fidelity Cas9 variants[13]. Notably, Sniper2L was less likely to tolerate mismatches in both the PAM-distal and -proximal regions as compared to Sniper1 and Sniper2P; in those regions, local specificity reached high points at positions 5 and 15, respectively. Furthermore, all Sniper variants tolerated single-base wobble mismatches more than single-base transversion mismatches, which is in line with the previous results of SpCas9 variants[1,13] (Figure 8b). The relative indel frequencies at mismatched target sequences containing consecutive two- or three-base transversion mismatches were dramatically reduced (Figure 8c). Based on these results, I selected Sniper2L as a new version of Sniper-Cas9.

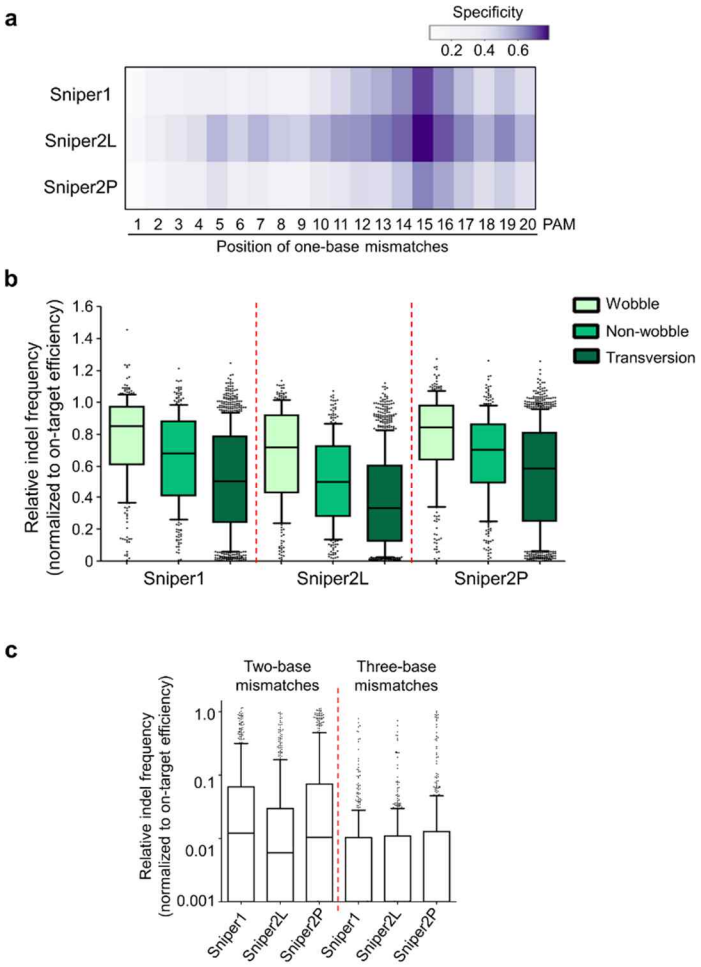


Figure 8. Specificity of Sniper-Cas9 variants in various mismatches. a, Specificity of variants depending on the position of the mismatch **b,** Relative indel frequencies induced by Sniper1, Sniper2L, and Sniper2P at mismatched targets vary depending on the types of single-base mismatches. The boxes represent the 25th, 50th, and 75th percentiles; whiskers show the 10th and 90th percentiles. *n* = 275 (wobble), 304 (non-wobble), and 1,155 (transversion) for Sniper1 and Sniper2P and 275, 304, and 1,153 for Sniper2L. **c,** Relative indel frequencies analyzed at target sequences with consecutive two- or three-base transversion mismatches. *n* = 554 and 531 for two- and three-base mismatches, respectively. The boxes represent the 25th, 50th, and 75th percentiles; whiskers show the 10th and 90th percentiles.

Since perfectly matched sgRNAs generated by the tRNA-associated processing system could increase the activity of some high-fidelity variants such as eSpCas9(1.1), SpCas9-HF1, and evoCas9, but not HypaCas9 and xCas9[13,19,20], I compared the activities of the Sniper variants at identical targets using library C, based on tRNA-N_{20} sgRNAs, and library B, based on (G/g)N_{19} sgRNAs (hereafter, 20-nt guide sequences with a matched or mismatched 5′ guanosine are described as GN_{19} and gN_{19}, respectively). Such (G/g)N_{19} sgRNAs are expressed from a U6 promoter with a G at the 5' terminus, which is often mismatched with the corresponding nucleotide at the target sequence. I observed that Sniper2L and Sniper2P displayed slightly higher general activities with (G/g)N_{19} sgRNAs than tRNA-N_{20} sgRNAs although tRNA-N_{20} showed slightly higher Sniper2L-induced activities than gN_{19} sgRNAs at target sequences starting with 5'-C or -T (Figure 9).

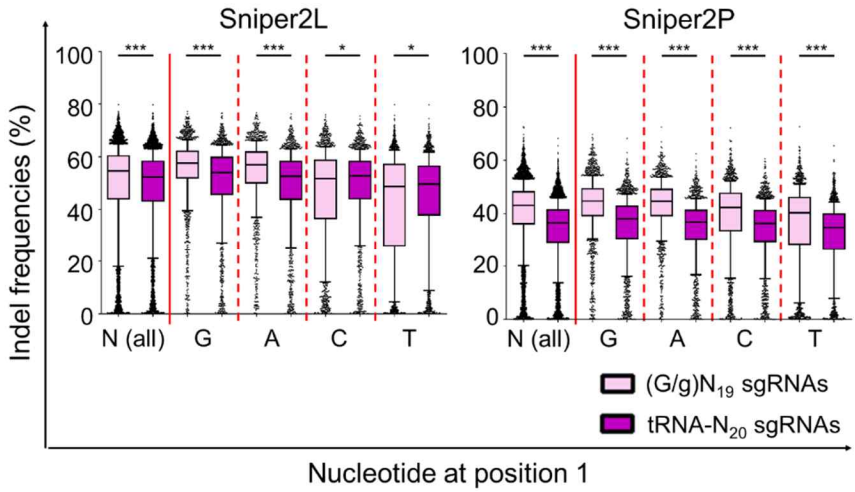


Figure 9. Activity assessments at target sequences with (G/g)N_{19} or tRNA-N_{20} sgRNAs. *n* = 6,321 (N), 1,666, 1,467, 1,626, and 1,562 (T) for Sniper2L, and 6,765 (N), 1,807, 1,587, 1,721, and 1,650 (T) for Sniper2P. *P* = 8.39 x 10^{-20} (N), 5.06 X 10^{-26} (G), 7.56 X 10^{-34} (A), 0.012 (C), and 0.04 (T) for Sniper2L, and 7.56 X 10^{-34} (all) for Sniper2P; Mann-Whitney U test.

3. Sniper2L represents an outlier to the trade-off relationship between general activity and specificity

I previously observed a trade-off between general activity and specificity of SpCas9 variants[13]; when a high-fidelity variant displayed high fidelity or specificity, it also exhibited relatively low general activity. To examine whether the Sniper2 variants followed this trend, I measured their activity and specificity using eight sgRNAs that were previously used in the analysis of the other high-fidelity variants (Figure 10). I observed that Sniper2L displayed both enhanced fidelity and higher on-target activities compared to Sniper1. To my knowledge, Sniper2L is the first and the only high-fidelity variant without sacrificing its general activity, being an outlier to the trade-off relationship between general activity and specificity.

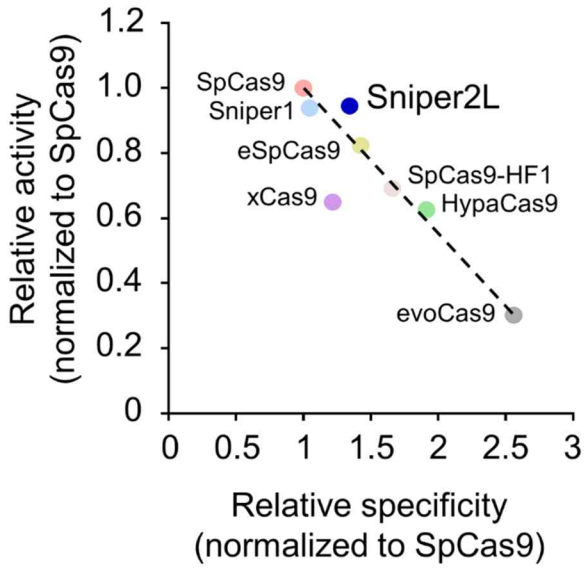


Figure 10. Relationship between the specificity and activity of SpCas9 and Cas9 variants. Sniper2L represents an outlier of the general trade-off. The specificity and activity of the high-fidelity variants were taken from our previous study[13]. The dashed line shows the general trade-off relationship.

4. High-throughput evaluation of Cas9 variant RNPs compared to WT-Cas9 RNPs

Cas9 and sgRNAs can be delivered either in an mRNA or preassembled RNP format when they are developed as therapeutics[21,22]. Hence, I measured the activities of high-fidelity variants, including Sniper2L that had been delivered in RNP format into cells expressing library A (Figure 11). HEK293T cells were transduced with library A lentivirus at an MOI of 0.1. After puromycin selection, I individually transfected SpCas9, Sniper1, Sniper2L, HiFi Cas9[11], and Cas9_R63A/Q768A[9], preassembled with the *HPRT*-targeting sgRNA, into the cell library (Figure 12). HiFi Cas9 and Cas9_R63A/Q768A were selected as the comparison groups because HiFi Cas9 showed low off-target effects when delivered as RNP format[11] and because Cas9_R63A/Q768A is a very recently reported high-fidelity variants of SpCas9[9]. The *HPRT*-targeting sgRNA in the RNP is then expected to be swapped with the sgRNA expressed by the cells[23]. The delivery efficiency of Cas9 protein combined with any sgRNA is expected to be higher than that of apo-Cas9 according to previous research[23]. After the 6TG selection step, genomic DNA was prepared and analyzed in the same manner as that of plasmid-treated samples.

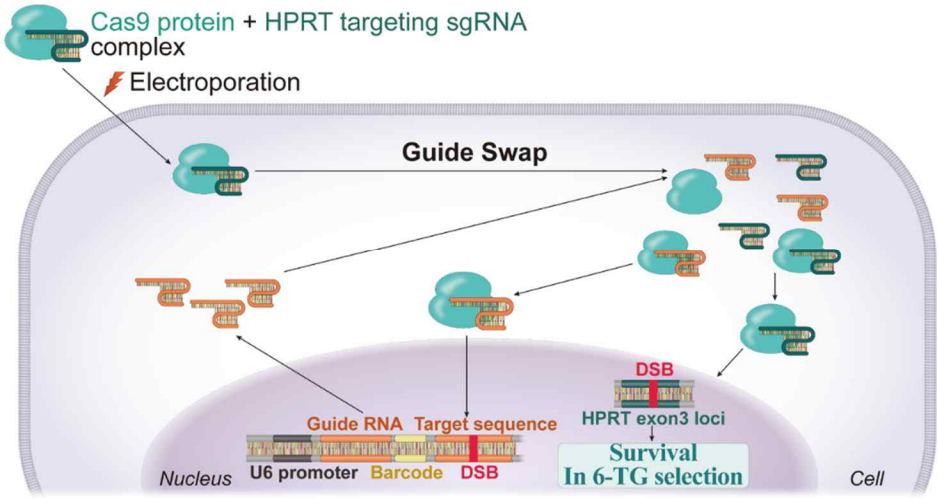


Figure 11. Schematic representation of experimental strategy using RNP delivery.

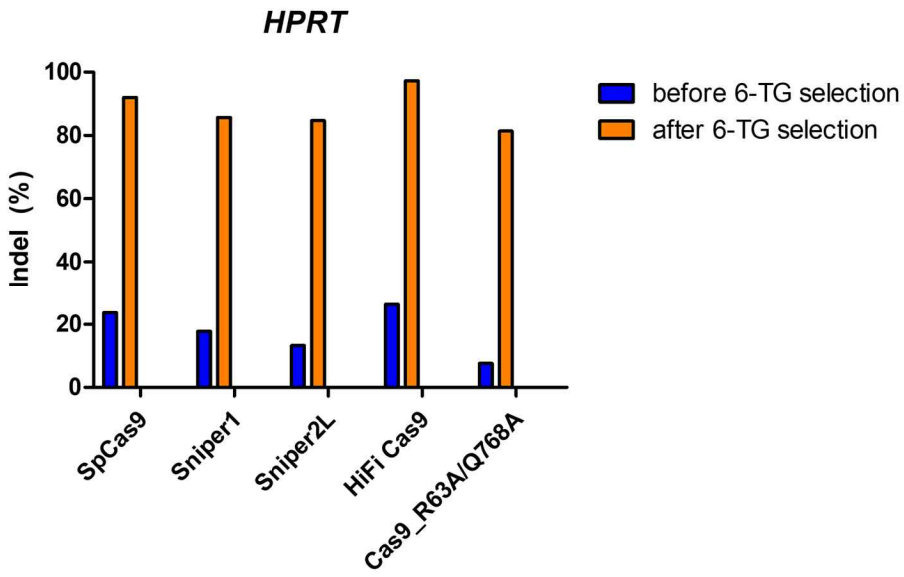


Figure 12. Indel frequencies to confirm 6-TG selection. Indel frequencies induced by Cas9 variants in the human *HPRT* gene in library screening using RNP delivery before and after 6TG selection.

Given that such RNP-based library screening had not been conducted previously, I first examined the PAM sequences that were recognized by the high-fidelity variants to verify a new strategy. Among target sequences containing all possible 4-nt PAM sequences (NNNN), variants displayed the highest indel frequencies at targets with NGG PAMs. In contrast to previous results obtained from cell lines that stably express Cas9 variants, I barely observed activities higher than 5% at target sequences containing non-canonical PAM sequences such as NGA or NAG (Figure 13). These results suggest that the shorter time of exposure to Cas9 (delivered in an RNP format) affected the efficiencies of the high-fidelity variants, such that they preferentially cleaved targets containing the most active PAM sequences.

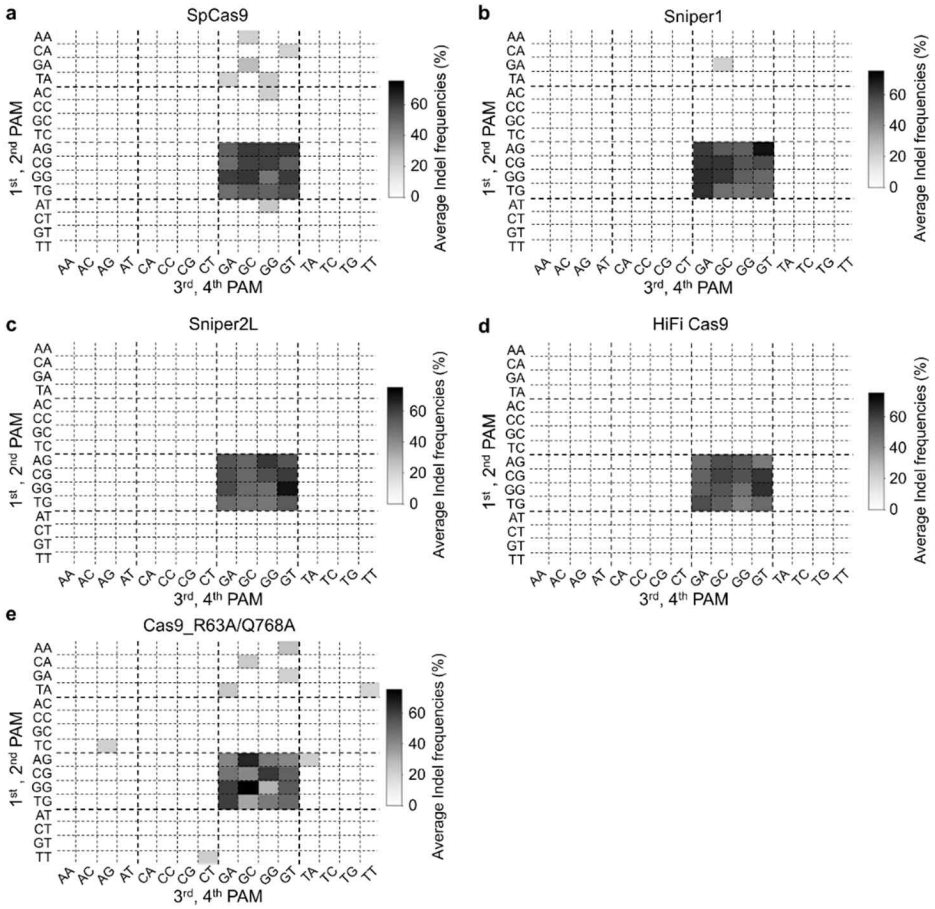


Figure 13. **PAM compatibilities among high-fidelity Cas9 variants in RNP level.** Average indel frequencies associated with all possible 4-nt PAM sequences. I excluded PAM sequences associated with indel frequencies less than 5%; such PAMs are indicated as white boxes in the grid. n = 14 – 28 (**a**), 15 – 29 (**b**), 12 – 28 (**c**), 13 – 29 (**d**), and 7 – 26 (**e**) target sequences per 4-nt PAM.

I next assessed nuclease activities at 30 perfectly matched target sequences, each with 5 different barcodes, in library A and found that the activities of the high-fidelity variants were similar except that Cas9_R63A/Q768A showed a tendency of relatively lower activities, which is in line with the previous report[9], although this difference was not statistically significant (Figure 14a). I also measured indel frequencies at mismatched target sequences and found that Sniper2L was highly inactive at the mismatched targets as compared to the other variants (Figure 14b). Wobble single-base mismatches were more tolerated as compared to transversion mismatches for all variants (Figure 14c). Taken together, the results indicate that Sniper2L exhibits high on-target activities along with relatively low off-target activities compared to previously reported high-fidelity variants when delivered in either lentiviral or RNP format.

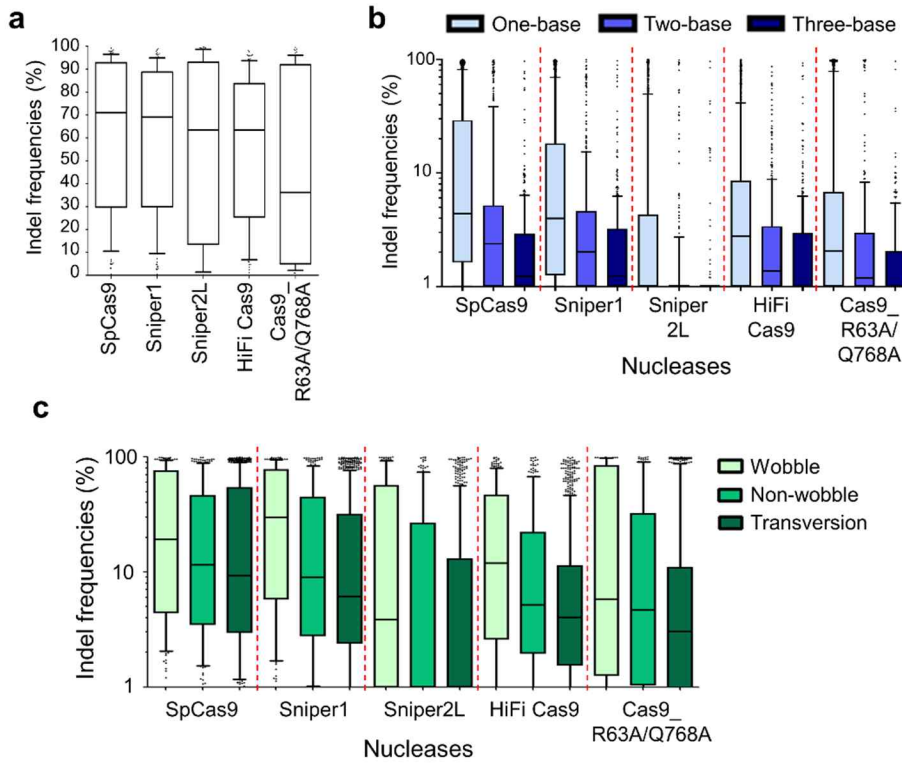


Figure 14. High-throughput evaluation of Cas9 activities when delivered as ribonuclear proteins. a, Indel frequencies at perfectly matched target sequences with NGG PAMs. *n* = 105, 113, 81, 113, and 69 for SpCas9, Sniper1, Sniper2L, HiFi Cas9, and Cas9_R63A/Q768A, respectively. No statistically significant difference; Kruskal-Wallis test. **b,** Effects of the number of mismatches between the sgRNA and target sequence. *n* = 2,236 (one-base), 448 (two-base), and 414 (three-base) for SpCas9, 2,352, 446, and 296 for Sniper1, 1,641, 322, and 296 for Sniper2L, 2,248, 441, and 414 for HiFi Cas9, and 1,398, 278, and 245 for Cas9_R63A/Q768A. **c,** Activities of variants at target sequences with single-base mismatches as a function of the type of mismatch. *n* = 214 (wobble), 237 (non-wobble), and 923 (transversion) for SpCas9, 223, 246, and 982 for Sniper1, 169, 159, and 695 for Sniper2L, 223, 236, and 934 for HiFi Cas9, and 127, 144, and 604 for Cas9_R63A/Q768A. The boxes represent the 25th, 50th, and 75th percentiles; whiskers show the 10th and 90th percentiles.

IV. DISCUSSION

Unlike the high-fidelity variants like Cas9-HF1, HypaCas9 et al developed by rational design based on Cas9 crystal structure[24], Sniper-Cas9 was obtained by directed evolution screening leading to random error. It was expected that additional screening would allow finding other mutations that can improve specificity. Previously, when developing the Sniper1, The original Sniper-screen was performed using four different sgRNAs targeted to the EMX1.3 region, with mismatches in the (5^{th} and 6^{th}), 7^{th}, 17^{th}, or (17^{th} and 18^{th}) positions. To select Cas9 variants with mismatch intolerance in a wide range of positions in the sgRNA, EMX1.6 sgRNA which contains a mismatch in the 13^{th} position (gcgccacTggttgatgtgat; the mismatched nucleotide at position 13 is capitalized) was used in the new Sniper-screen for Sniper2L. Thus, Sniper2L which was obtained through the addition of a further point mutation to Sniper-Cas9 was developed by directed evolution screen based on Sniper-Cas9 sequences and site saturation mutagenesis.

Sniper2L was then characterized using a high-throughput assessment platform that was previously used to compare Cas9 variants with high specificities[13].In brief, library A included 8,130 and 3,672 pairs to evaluate protospacer adjacent motif (PAM) compatibility and mismatch tolerance, respectively. Library B, which contained 8,744, 12,093, and 2,660 pairs with NGG, NGH, and non-NG PAMs, respectively, was used for validating variants at a large number of target sequences. In contrast to libraries A and B, Library C utilized perfectly matched N_{20} sgRNAs generated by transfer RNA (tRNA)-associated processing , with the majority of target sequences taken from library B. Sniper2L showed higher specificity without significant loss of on-target activity compared to Sniper1 in high-throughput evaluation of lentiviral level.

Cas9 and sgRNAs can be delivered either in an mRNA or preassembled RNP format when they are developed as therapeutics[21,22]. The results drawn from high-throughput analysis following lentiviral delivery might not be directly

relevant to RNP- or mRNA-based approaches, given that Cas9 RNPs show higher specificities than plasmid-expressed Cas9[25]. Therefore, Sniper2L was compared and evaluated with previous reported high-fidelity variants including spCas9 using high-throughput platform in RNP level. The original high-throughput assessment protocol has an antibiotic selection step to remove the cells that do not express Cas9. When the Cas9 delivery platform is changed from plasmid to RNP, this step is no longer available. To overcome this limitation, I delivered Cas9 protein together with an *HPRT*-targeting sgRNA. Because *HPRT* knockout provides resistance to 6-thioguanine (6TG), the cells in which Cas9 delivery has occurred can be selected via 6TG selection, similar to the antibiotic selection step. Accordingly, a modified high-throughput assessment platform for measuring indel frequencies induced by Cas9 variants delivered in an RNP complex was also developed. Although on-target activities of the high-fidelity variants were similar, Sniper2L exhibited decreased activity in mismatched target than other variants in high-throughput evaluation of RNP level. Taken together, Sniper2L exhibited high on-target activities along with low off-target activities relatively compared with variants when delivered in either lentiviral or RNP complex.

While some previously reported variants have substitution in the REC3 domain, the 1007th mutation of Sniper2L is located in the RuvC domain considering the difference between Sniper2L and other variants mechanistically. The REC3 domain in Cas9 protein senses PAM-distal mismatches and controls the transition of the HNH domain into an active conformation, which serves as a mechanism to block the cleavage in the presence of mismatches. To decrease off-target activity, variants such as Cas9-HF1[7] and HypaCas9[8] contain mutations into the REC3 domain increasing the energetic barrier for transition of the HNH domain. These variants showed not only an increased specificity but also a decreased on-target activity simultaneously. Unlike these variants, I developed improved version of Sniper-Cas9, Sniper2L, which contain E1007L in RuvC domain. In a recent study, a new high-fidelity variant named Superfi-Cas9 was

developed, in which seven residues in the RuvC domain responsible for mismatch stabilization were mutated to aspartic acid[26]. They observed that substrates containing PAM-distal mismatches are stabilized by reorganization of a loop in the RuvC domain. In comparison with WT-Cas9, Superfi-Cas9 with mutagenesis of mismatch-stabilizing residues in RuvC domain displayed a similar on-target cleavage rate and a 500-fold slower off-target cleavage rate. Because the 1007th position in the Sniper2L sequence is close to the locations of the 7-D mutations in the SuperFi-Cas9 RuvC domain (Y1010, Y1013, Y1016, V1018, R1019, Q1027, and K1031), I speculate that the mechanism for the improved specificity and retained activity of Sniper 2L may be similar to that of SuperFi-Cas9.

Sniper2L is developed defying the trade-off between activity and specificity. It is considered to prevent Sniper2L from the activity-specificity trade off relationship using directed-evolution screening system, improving specificity without loss of on-target activity.

Furthermore, high-throughput assessment platform has been modified successfully in the way that RNP delivery shows improved modality. The Cas9 protein was preassembled with *HPRT*-targeting sgRNA and the genome-edited library cells were enriched by 6-TG selection. The enriched cells were assessed and the activities and specificities of Cas9-sgRNA RNPs were successfully measured. This modified protocol widens the applicability of the high-throughput assessment platform such that Cas9 delivered in any type of modality could be tested.

V. CONCLUSION

Sniper2L which was obtained through the addition of a further point mutation to Sniper-Cas9 was developed by directed evolution screen based on Sniper-Cas9 sequences and site saturation mutagenesis. Sniper2L were evaluated using high-throughput platform that involves libraries of pairs of RNA-encoding and corresponding target sequences in lentiviral and RNP level. A modified high-throughput assessment platform for measuring indel frequencies induced by Cas9 variants delivered in an RNP complex was developed. Sniper2L showed higher specificity without significant loss of on-target activity compared to Sniper-Cas9 in two high-throughput evaluation, by lentiviral and RNP delivery. Notably, this improvement is an outlier to the previously found trade-off between activity and specificity.

In conclusion, it is confirmed that a new high-fidelity variant shows great improved trade-off relationship between general activity and specificity compared with other Cas9 variants. It is expected that Sniper2L will be used when high-efficiency and low-off-target effects are required.

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ABSTRACT (IN KOREAN)

표적 활성도의 손실 없이 특이성을 증가시킨 Sniper-Cas9 개발

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김영훈

CRISPR/Cas9 유전자가위를 사용함에 있어서 비표적 유전자 지역의 원하지 않는 절단이나 변이는 CRISPR/Cas9 유전자가위의 한계로 지적되고 있다. 최근 이러한 문제를 해결하기 위해 특이성이 증가된 높은 정확도를 가진 다양한 Cas9 유전자가위 변형체들이 소개되고 있다. 그러나 이러한 Cas9 유전자가위 변형체들은 특이성은 증가하지만 활성도가 떨어지는 트레이드 오프 관계를 보인다는 것을 대용량 처리 플랫폼을 이용하여 평가되었고 이는 효율적인 유전자 편집을 요구하는 상황에서 Cas9 유전자가위 변형체들의 단점으로 작용될 수 있다.
본 연구에서는 유도진화선별을 이용하여 표적 활성도의 손실 없이 향상된 특이성을 가지는 차세대 Cas9 유전자가위 변형체인 Sniper2L을 개발하였다. Sniper2L의 활성도와 특이성은 렌티바이러스 수준에서 대용량 처리 플랫폼을 이용하여 평가되었다. 그리고 리보핵산단백질의 형태로 유전자가위를 전달하여 활성도와 특이성을 계산할 수 있는 변형된 대용량 처리 플랫폼 기술도 개발하였다. 이를 이용하여 대용량 처리 플랫폼에서 렌티바이러스와 리보핵산단백질로 전달되었을 때 Sniper2L은 높은 활성도와 특이성을 가진다고 평가되었다.
결론적으로 다른 Cas9 유전자가위 변형체들과 비교했을 때 본 연구에서 개발된 Sniper2L은 활성과 특이성 사이의 향상된 트레이드 오프 관계를 보이는 것을 확인하였고 Sniper2L은 높은 효율과 정확성을 요구하는 유전자 편집 분야에서 유용하게 사용될 수 있을 것으로 판단된다.

핵심되는 말 : 크리스퍼, 유전자 편집, cas9 변이체, 대량스크리닝