YMTHE, Volume *29*

Supplemental Information

Engineered prime editors with PAM flexibility

Jiyeon Kweon, Jung-Ki Yoon, An-Hee Jang, Ha Rim Shin, Ji-Eun See, Gayoung Jang, Jong-Il Kim, and Yongsub Kim

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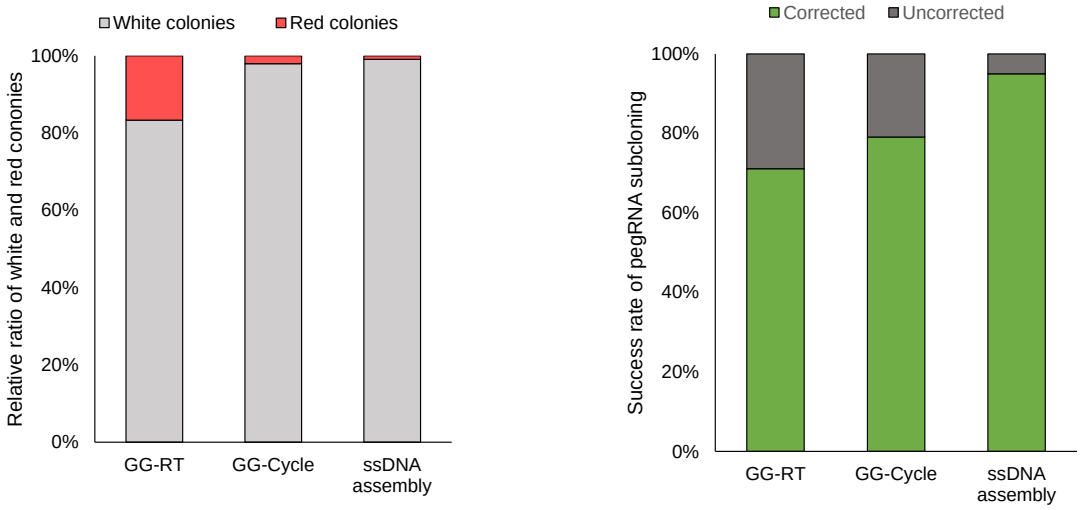
Note S1. Procedure for pegRNA subcloning by ssDNA assembly.

To improve the throughput of pegRNA construction, we used the ssDNA assembly method instead of the Golden Gate (GG) assembly method. In comparison with Golden Gate assembly, ssDNA assembly has no oligonucleotide annealing step in a thermocycler and does not require the phosphorylation of gRNA scaffold oligonucleotides. The ratio of red/white colonies is similar in the ssDNA assembly method (GG-RT: incubation at room temperature for 10 min; GG-Cycle: 8 cycles of 5 min at 16°C and 5 min at 37°C). We also determined the cloning efficiency by the targeted-deep sequencing of pooled transformants. In comparison with conventional methods, ssDNA assembly has a slightly higher success rate in pegRNA cloning. The detailed protocol of pegRNA cloning is described below.



1. Oligonucleotide preparation for pegRNA cloning

    a. Oligonucleotides for the spacer sequence (target-specific component)
    The 18 nt 5' overhang (5'-tggaaaggacgaaacacc-3') and 3' overhang (5'-gttttagagctagaaata-3') are attached to the desired spacer sequence as follows:
    5'- tggaaaggacgaaacaccNNNNNNNNNNNNNNNNNNNgttttagagctagaaata-3',
    where N×20 is the target spacer sequence without PAM, and the spacer sequence must begin with a guanine nucleotide. If the spacer sequence does not begin with a guanine nucleotide, an additional guanine nucleotide should be added for pegRNA transcription by the U6 promoter.

    b. Oligonucleotides for the RT template and PBS sequence (target-specific component)
    The 18 nt 5' overhang (5'-gtggcaccgagtcggtgc-3') and 3' overhang (5'-ttttttaagcttgggcc-3') are attached to the desired spacer sequence as follows:
    5'-gtggcaccgagtcggtgcNNNNNNNNNNNNNNNNNNNttttttaagcttgggcc-3',
    where Ns are the desired RT template and PBS sequence.

    c. Oligonucleotides for the pegRNA scaffold sequence (common component)
    The reverse complementary sequence of gRNA scaffold is as follows:
    5'-gcaccgactcggtgccacttttcaagttgataacggactagccttattttaacttgctatttctagctctaaaac-3'

2. Vector preparation for pegRNA cloning

    The pU6-pegRNA-GG-acceptor (addgene, plasmid #132777) plasmid DNA is digested with

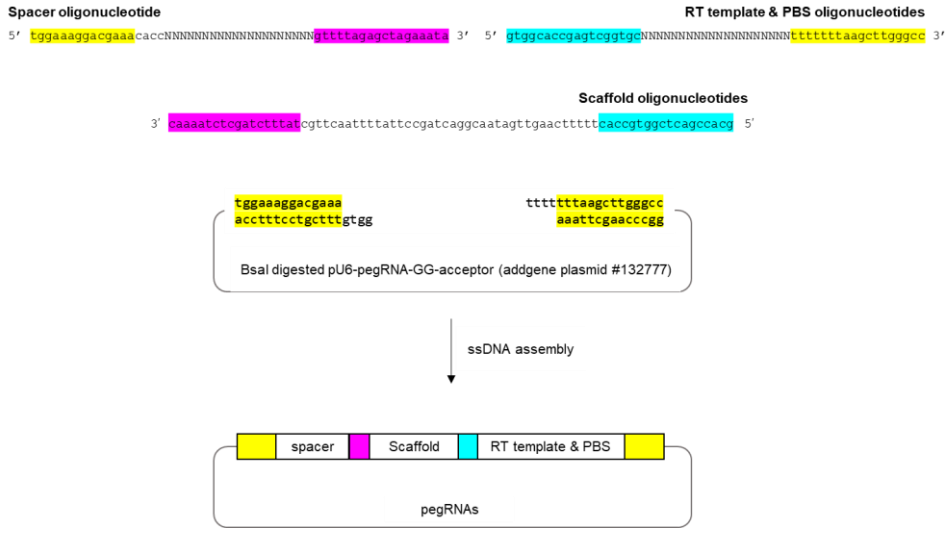
the BsaI-HFv2 (NEB R3733S) restriction enzyme as previously described.

3. pegRNA construction by ssDNA assembly
   Each component is mixed for ssDNA assembly as follows:

Vector DNA	50 ng
Oligonucleotides for spacer	1 µl of 100 nM
Oligonucleotides for RT template and PBS	1 µl of 100 nM
Oligonucleotides for gRNA scaffold	1 µl of 100 nM
2× HiFi DNA Assembly Master Mix (NEB #E2621S)	5 µl
H_2O	up to 10 µl

   The reaction mixture is incubated at 50°C for 30 min, and an appropriate amount is transformed using competent cells.

The following scheme summarizes the process of ssDNA assembly in the pegRNA subcloning protocol.



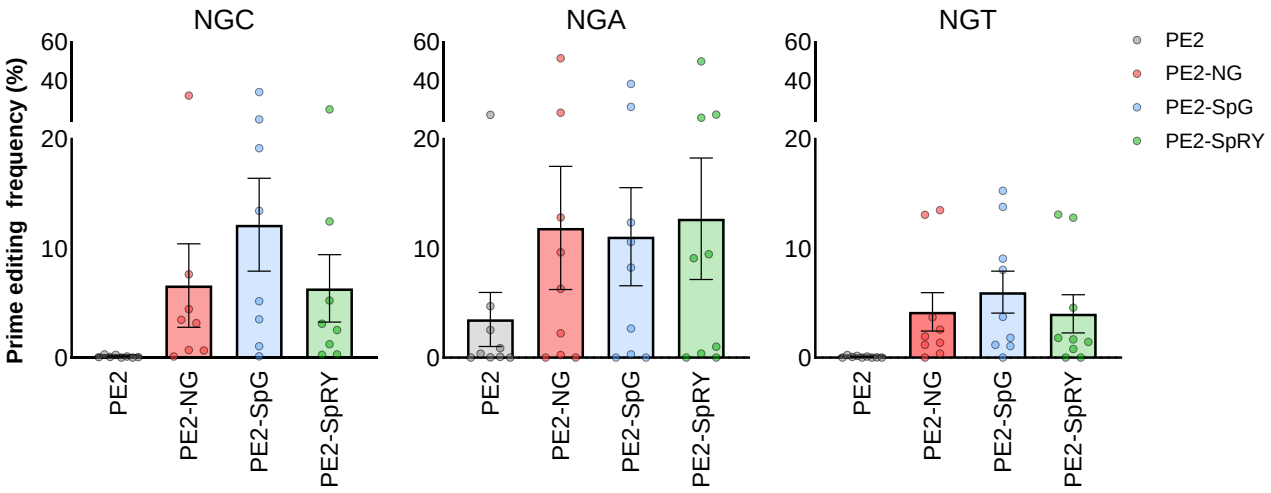


Figure S1. Prime editing activities of the PE2 variants at the NGC, NGA, and NGT PAM sites in HEK293T cells. The PE2-SpG variant showed average prime editing activities of 12.2% and 6.0% at 8 NGC PAM sites and 9 NGT PAM sites, respectively. The wild-type PE2 showed an average prime editing activity of 2.5% at 9 NGA PAM sites. The numerical values of the prime editing activity are listed in Table S1. Mean ± s.e.m. of the average prime editing frequency for each target site.

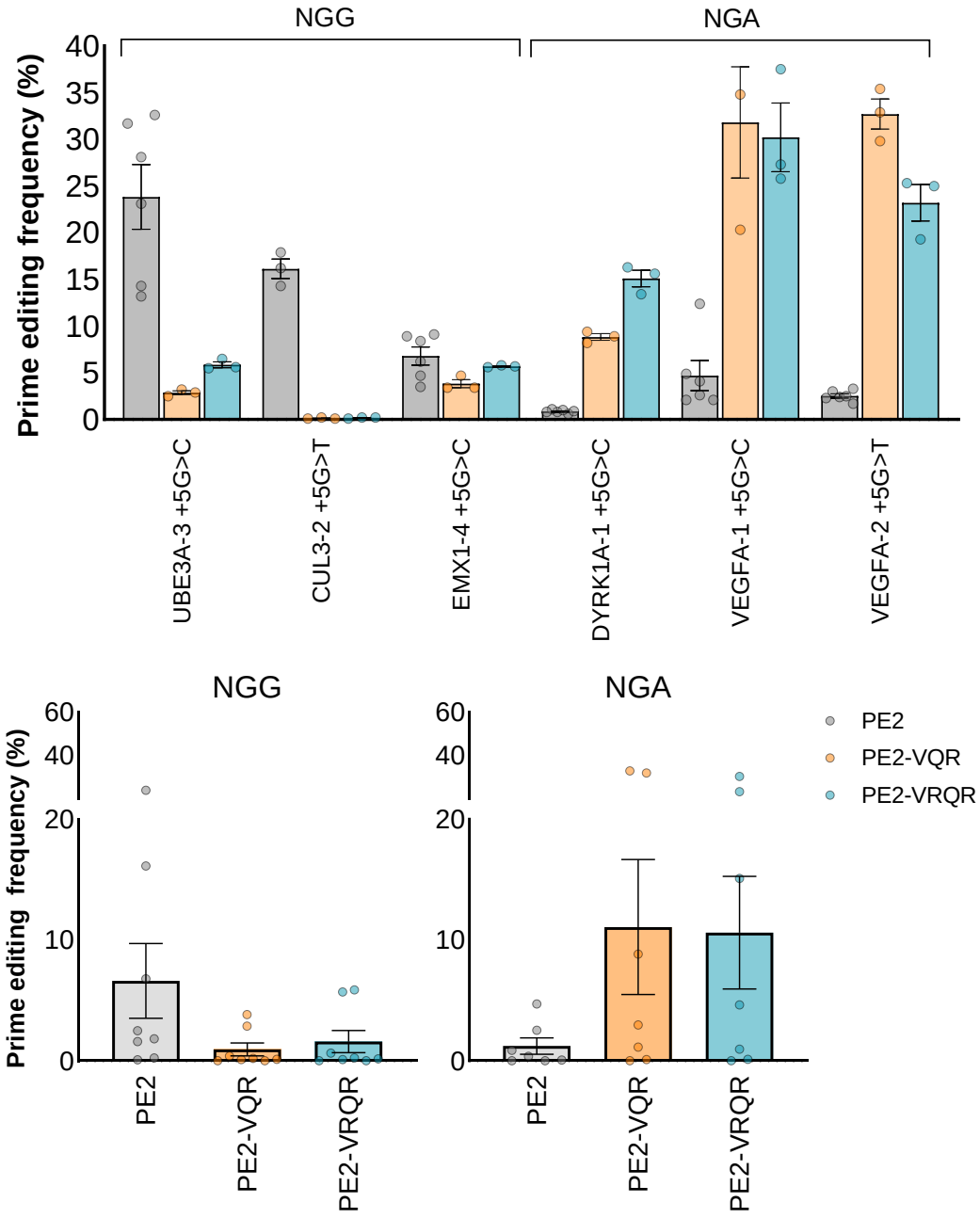


Figure S2. Prime editing activities of the PE2-VQR and PE2-VRQR variants in HEK293T cells. The PE2-VQR and PE2-VRQR variants showed average prime editing activities of 11.1% and 10.7% at 7 NGA PAM sites each. The numerical values of the prime editing activity are listed in Table S1. Mean ± s.e.m. of $n = 3$ or n=6 independent biological replicates in the top panel. Mean ± s.e.m. of the average prime editing frequency for each target site in the bottom panel.

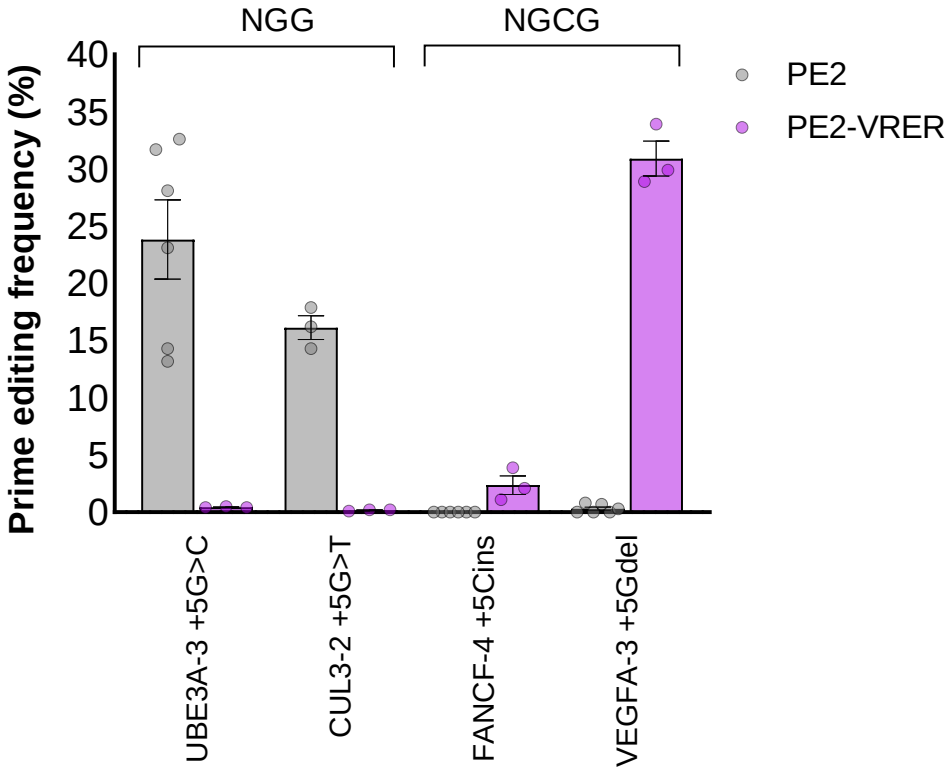


Figure S3. Prime editing activities of the PE2-VRER variant in HEK293T cells. The PE2-VRER variant showed an average prime editing activity of 30.9% at the VEGFA-3+5Gdel site with NGCG PAM. The numerical values of the prime editing activity are listed in Table S1. Mean ± s.e.m. of $n = 3$ or n=6 independent biological replicates.

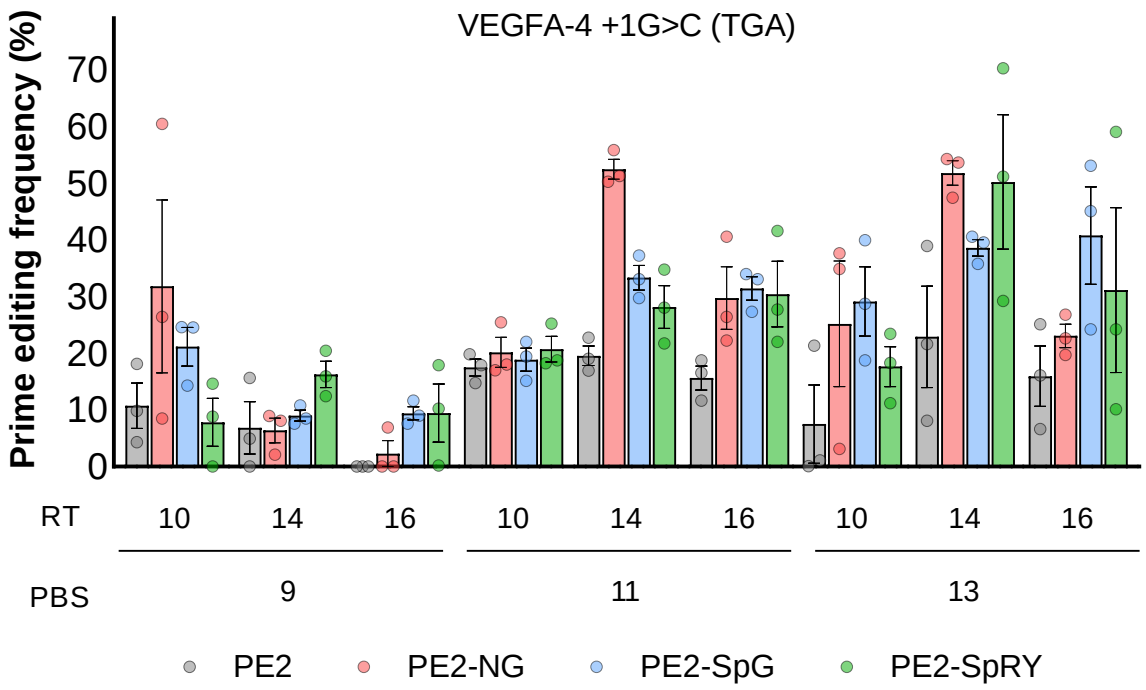


Figure S4. Prime editing activities at the VEGFA-4 +1G>C site with various PBS and RT template lengths in pegRNAs in HEK293T cells. The PAM sequence is shown in the parentheses, and the numerical values of the prime editing activity are listed in Table S1. Mean ± s.e.m. of $n = 3$ independent biological replicates.

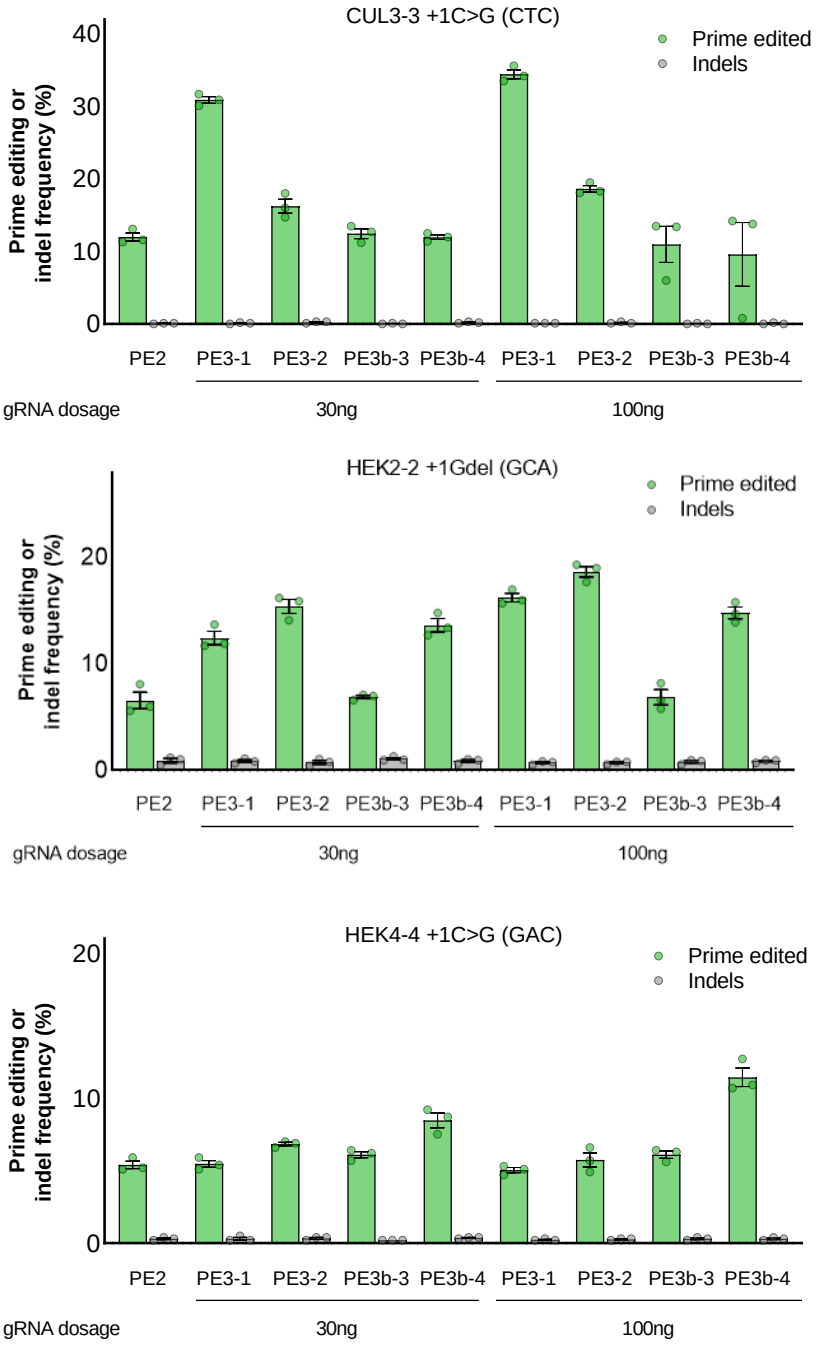


Figure S5. Prime editing using the PE3 and PE3b systems at three target sites in HEK293 cells. Two different amounts (30 ng or 100 ng) of gRNAs were used for PE3 or PE3b as described in the Materials and Methods section, and the frequencies of prime editing activity with 30 ng of gRNA were plotted as shown in Figures 3A-C. There was no significant increase in prime editing activity or indels with an increased amount of gRNA at three target sites. The PAM sequences are shown in the parentheses, and the numerical values of the prime editing activity are listed in Table S1. Mean ± s.e.m. of $n = 3$ independent biological replicates.

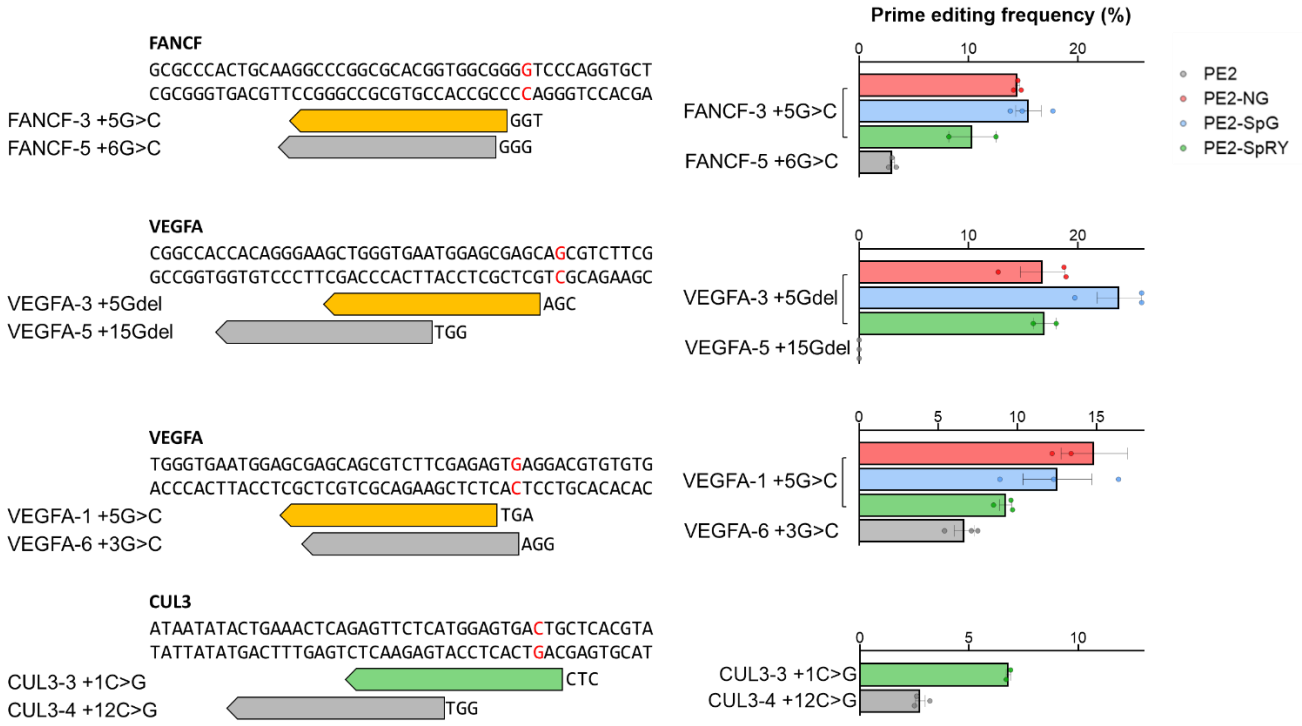


Figure S6. Comparison of prime editing activities between wild-type PE2 and PE2 variants in HEK293T cells. The pegRNAs for wild-type PE2 were designed to introduce the same mutations introduced by PE2 variants. The positions of the intended mutations are highlighted in red, and the PAM and spacer sequences of each pegRNA are shown below the target sequences. The numerical values of the prime editing activity are listed in Table S1. Mean ± s.e.m. of n = 2 or 3 independent biological replicates.

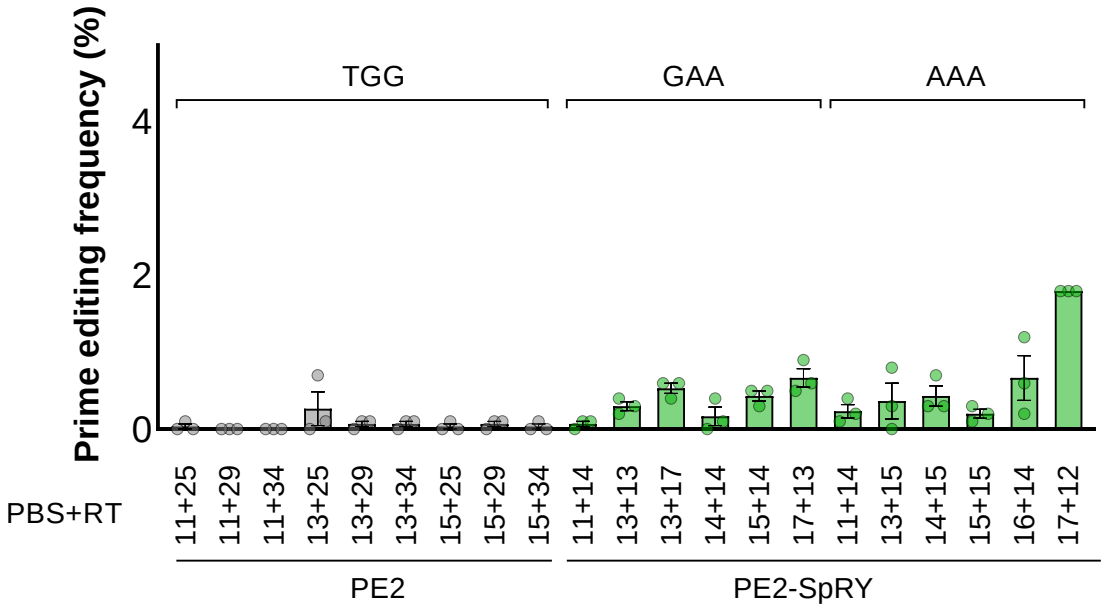


Figure S7. Prime editing activities at the BRAF V600E site with various PBS and RT template lengths in pegRNAs. The pegRNA with 13 nt PBS and 25 nt RT template showed the highest prime editing activity (average of 0.3%) for wild-type PE2, and the pegRNA with 17 nt PBS and 12 nt RT template showed 1.8% prime editing activity for PE2-SpRY. The numerical values of the prime editing activity are listed in Table S1. Prime editing was conducted using HEK293T cells. Mean ± s.e.m. of n = 3 independent biological replicates.

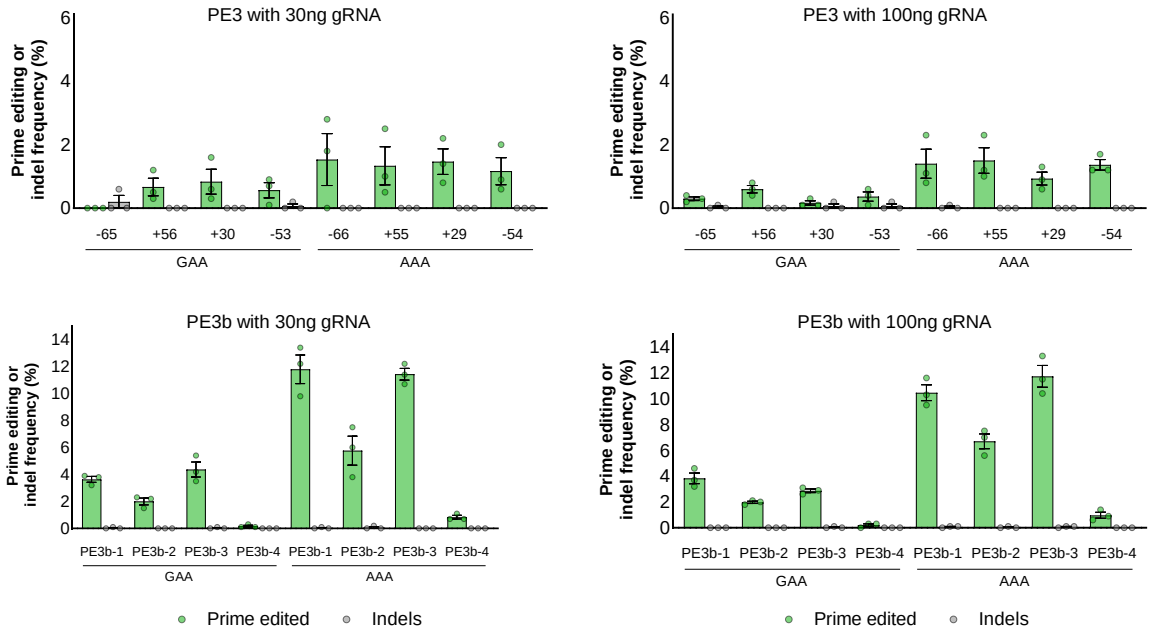


Figure S8. Prime editing using the PE3 and PE3b systems at the BRAF V600E site. BRAF-GAA and BRAF-AAA pegRNAs were evaluated with four gRNAs in the PE3 system. Each gRNA could induce nicks to the non-edited strand; the nicked positions are indicated with the locations of pegRNA-induced nicks. The numerical values of the prime editing activity are listed in Supplementary Table 1. Prime editing was conducted using HEK293T cells. Mean ± s.e.m. of n = 3 independent biological replicates.

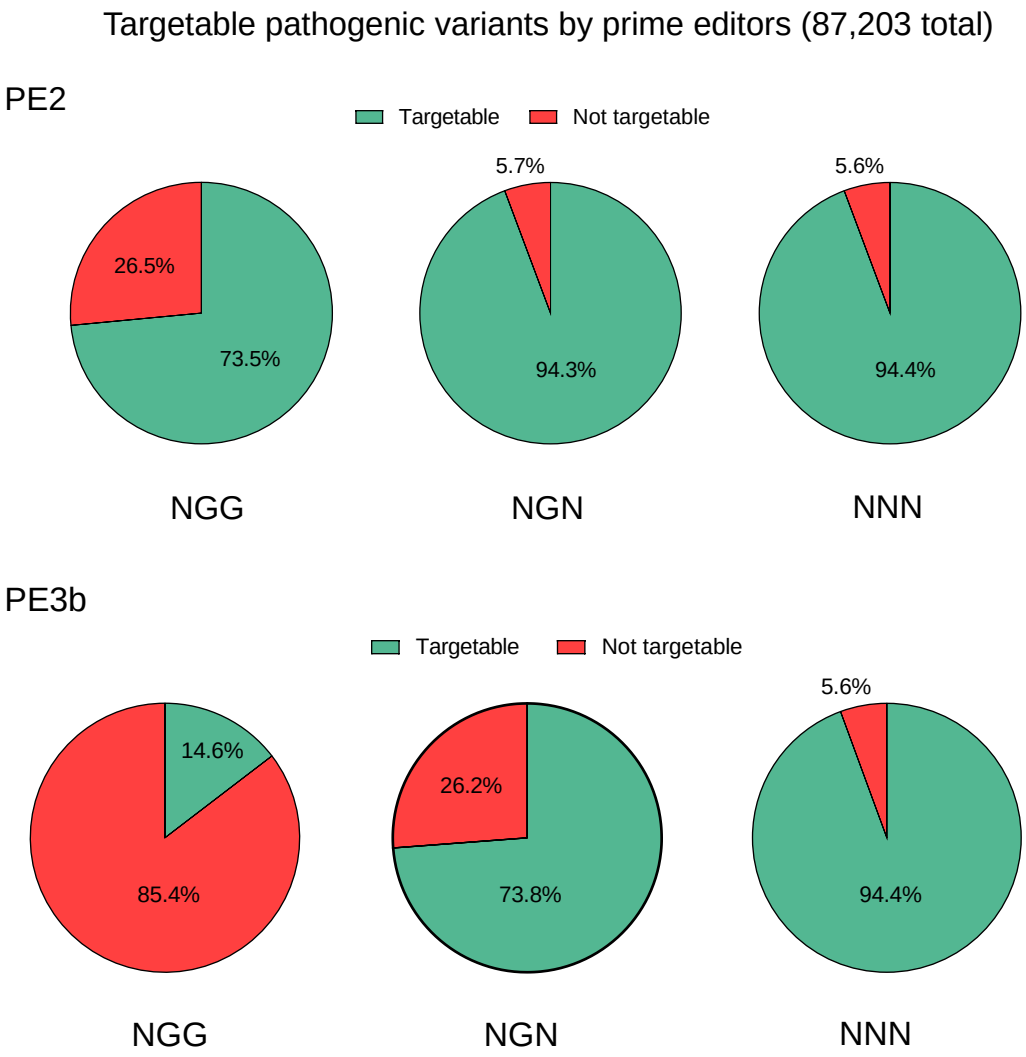


Figure S9. Fraction of human pathogenic variants that could be targeted with the PE2 or PE3b system. Overall, 73.5% of pathogenic variants were targetable for wild-type PE2 (with NGG PAM), and 94.4% of pathogenic variants were targetable for PE2-SpRY (with NNN PAM). In the case of the PE3b system, only 14.6% of variants were targetable for wild-type PE2; in contrast, targetable pathogenic variants were greatly increased up to 94.4% for PE2-SpRY. The remaining 5.6% of pathogenic variants that cannot be targeted were large deletions, insertions, or complex mutations. Total counts are listed in Supplementary Table 4.

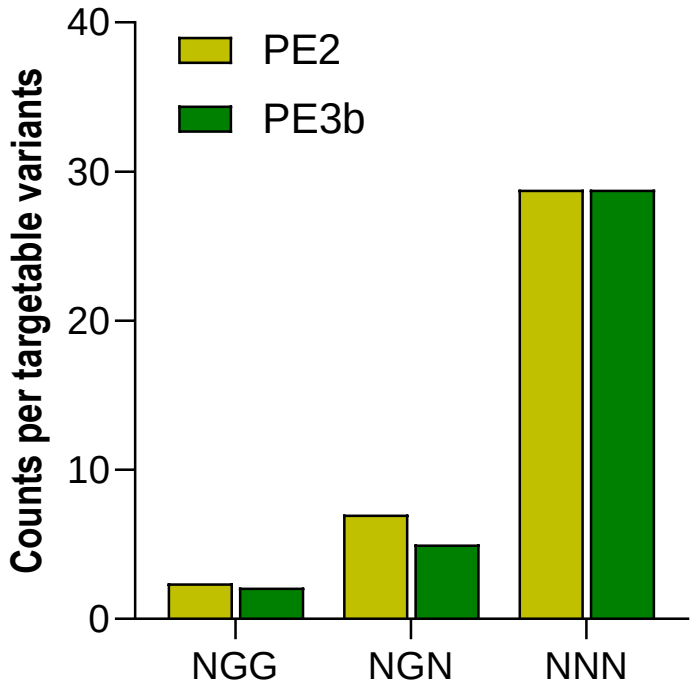


Figure S10. Number of target sites per targetable variant in the PE2 and PE3b systems. The PE2-SpRY variant increased the number of designable pegRNAs per pathogenic variant (from 2.4 to 28.8 pegRNAs in the PE2 system and from 1.6 to 28.8 pegRNAs in the PE3b system). Considering the diverse activity of prime editing, the more pegRNAs that can be designed, the higher the success rate of prime editing. Total counts are listed in Supplementary Table 4.

Table S3. List of off-target sites in this study.

pegRNA names		Chr.	Spacer sequences	PAM	PBS	RT template	Reference for off-target sites
HEK4 +2G>T	ON	Chr.20	GGCACTGCGGCTGGAGGTGG	GGG	GCGGCTGGAGG	TTGGGGTTAA	Anzalone *et al*.
	OFF	Chr.10	GGCACgaCGGCTGGAGGTGG	GGG	aCGGCTGGAGG	TTGGGGgTtg	
FANCF-4 +5Cins	ON	Chr.11	GCAGAAGGGATTCCATGAGG	TGC	AAGGGATTCCATG	AGGTCGCGCGAAGGC	Walton *et al*.
	OFF	Chr.11	GaAGAAGGGtTTCCATGAGG	AGA	AAGGGtTTCCATG	AGGaCGatacctgag	
EMX1-4 +5G>C	ON	Chr.2	GTCACCTCCAATGACTAGGG	TGG	CCTCCAATGACTA	GGGTCGGCAACCA	
	OFF	Chr.17	GTCACCTgtAATGACTAGGG	AGA	CCTgtAATGACTA	GGGaCaGtAAtgg	
MECP2-3 +1G>C	ON	Chr.X	GGGTGGTTCCATAATCTGTG	TAT	GGTTCCATAATCT	CTGTATACCTAAG	
	OFF	Chr.4	GcGTGGTTaCATAATCTGTG	GAG	GGTTaCATAATCT	CTGgAggggTgca	
CUL3-3 +1C>G	ON	Chr.2	GAGTTCTCATGGAGTGACTG	CTC	TCTCATGGAGTGA	GTGCTCACGTAAC	In this study
	OFF1	Chr.14	aAGTTtTCATGGAGTGACTG	TCA	TtTCATGGAGTGA	GTGtcacaGagta	
	OFF2	Chr.2	GtGTTaTCATGGAGTGACTG	AGA	TaTCATGGAGTGA	GTGagaAtGacAC	
	OFF3	Chr.8	GAGTTCTgATGGAGTGACTG	CTG	TCTgATGGAGTGA	GTGCTggtGccca	
HEK2-4 +1Cins	ON	Chr.5	GGGCGGGCCAGCCTGAATAG	CTG	GGGCCAGCCTGAA	CTAGCTGCAAACAA	
	OFF1	Chr.13	GGGCtGGCCAGCCTGAtTAG	AAT	tGGCCAGCCTGAt	CTAGaataggctAA	
	OFF2	Chr.22	GGGCGaGCCAGCCTG(g)AATAG	GTA	GaGCCAGCCTG(g)AA	CTAGgTaCtggCct	
	OFF3	Chr.5	aGGCtGGCCAGCCTGAATAG	CTT	tGGCCAGCCTGAA	CTAGCTtCAgcagg	

Mismatches are highlighted in blue with lowercase letters. At the HEK2-3 +1Cins OFF-2 site, () indicates a bulge position compared with the on-target site. The intended mutations of pegRNAs are shown in red.

Table S4. Specificity ratio of on- and off-target prime editing activities.

pegRNA name		Specificity ratio					
		Nucleases			Prime editors		
		wildtype	SpG	SpRY	PE2	PE2-SpG	PE2-SpRY
HEK4 +2G>T		1.88	1.55	5.17	6.18	6.19	15.18
FANCF-4 +5Cins		N/A	2.98	20.67	N/A	> 35	> 35
EMX1-4 +5G>C		164.5	3.37	5.67	> 150	> 124	> 30
MECP2-3 +1G>C		N/A	N/A	54.85	N/A	N/A	> 93
CUL3-3 +1C>G	OFF1	N/A	N/A	50	N/A	N/A	57.6
	OFF2	N/A	N/A	225	N/A	N/A	> 192
	OFF3	N/A	N/A	112.5	N/A	N/A	> 192
HEK2-4 +1Cins	OFF1	N/A	N/A	40.33	N/A	N/A	113
	OFF2	N/A	N/A	121	N/A	N/A	> 150
	OFF3	N/A	N/A	60.5	N/A	N/A	> 150

Table S5. Analysis of targetable pathogenic variants using PE variants.

Types		Number of targetable variants		number of pegRNAs per target variants	
Prime editors	PAM	Counts	Percentage (%)	Total counts	pegRNAs per targetabel variants
PE2	NGG	64,121	44.8%	156,862	2.4
	NGN	82,239	79.2%	572,093	7.0
	NNN	82,283	89.6%	2,372,052	28.8
PE3b	NGG	12,769	7.9%	26,589	2.1
	NGN	64,315	51.3%	320,763	5.0
	NNN	82,283	83.8%	2,372,052	28.8

*A total of 87,203 variants from the ClinVar database were analyzed.