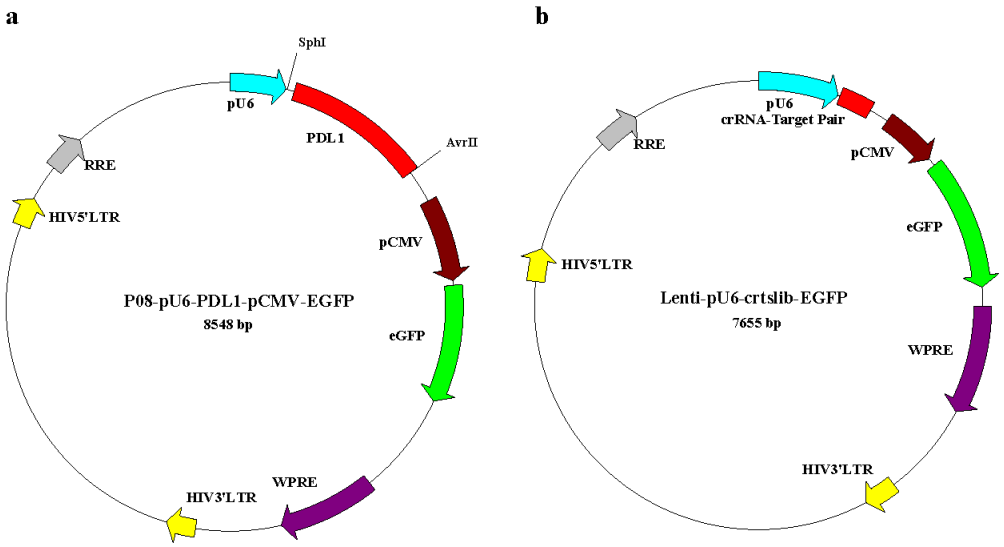
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Plasmid maps and sequences

(1) Lenti-pU6-crtslib-EGFP (b): Backbone plasmid for the construction of paired guide–target libraries, modified from the following plasmid: P08-pU6-PDL1-pCMV-EGFP (a).



- U6 promoter
- SphI restriction site
- AvrII restriction site
- EcoRI restriction site
- XbaI restriction site

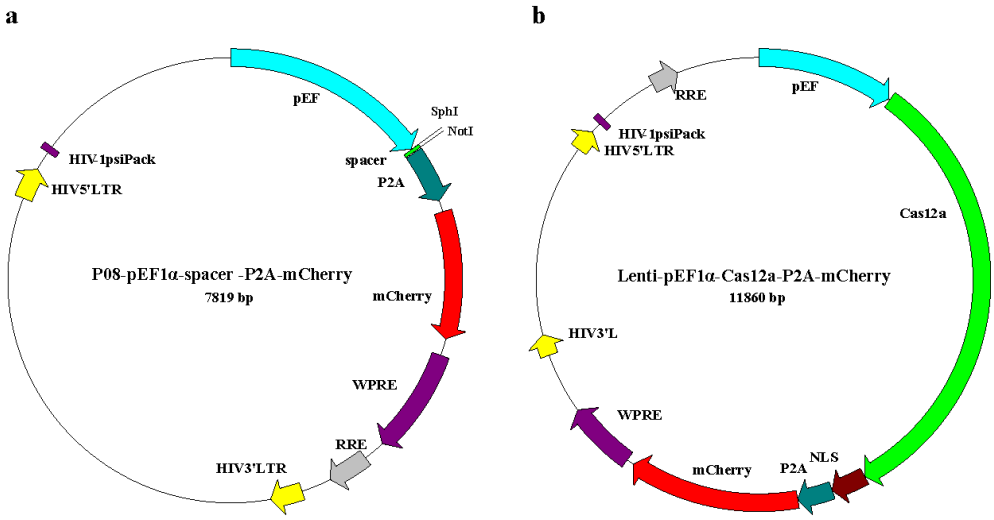
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(2) Lenti-pEF1α-Cas12a-P2A-mCherry: Backbone plasmid for the construction of Cas12a-expressing lentivirus vectors, modified from the following plasmid: P08-pEF1α-spacer -P2A-mCherry.



1) 5 end adaptor of the Cas12a coding region

- Kozak sequence
- N-terminal SV40 NLS

ACCGGTGCCACCATGGCCCCAAAGAAGAAGCGGAAGGTC

2) 3 end adaptor of the Cas12a coding region (without stop codon)

- C-terminal NLS: Nucleoplasmin NLS (bipartite nuclear localization signal from nucleoplasmin)
- 3×HA tag

AAACGTCCGGCGGCCACGAAAAAAGCCGGCCAGGCAAAAAAGAAAAAAGGCTCCTACCCGTACGATGTTCCGGATT
ACGCGTATCCGTACGACGTGCCGGATTATGCATACCCGTATGATGTCCCGGACTATGCC

The vector backbone sequences between restriction sites EcoRI and XbaI are the same using P08-pU6-PDL1-pCMV-EGFP. Here, we show the different parts used to express Cas12a.

- EF1α promoter
- SphI restriction site
- 20bp spacer: between two adjacent restriction sites to avoid steric hindrance impairing digestion efficiency in a double digestion reaction
- NotI restriction site
- P2A linker
- mCherry

GCCCGTCAGTGGGCAGAGCGCACATCGCCCACAGTCCCCGAGAAGTTGGGGGGAGGGGTCGGCAATTGAACCGGT
GCCTAGAGAAGGTGGCGCGGGGTAAACTGGGAAAGTGATGTCGTGTACTGGCTCCGCCTTTTTCCCGAGGGTGGG
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AGTGCCGTGTGTGGTTCCCGCGGGCCTGGCCTCTTTACGGGTTATGGCCCTTGCGTGCCTTGAATTACTTCCACC
TGGCTGCAGTACGTGATTCTTGATCCCGAGCTTCGGGTTGGAAGTGGGTGGGAGAGTTCGAGGCCTTGCGCTTAA
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GGGTAGTCTCAAGCTGGCCGGCCTGCTCTGGTGCCTGGCCTCGCGCCGCCGTGTATCGCCCCGCCCTGGGCGGCA
AGGCTGGCCCGGTCGGCACCAGTTGCGTGAGCGGAAAGATGGCCGCTTCCCGGCCCTGCTGCAGGGAGCTCAAAA
TGGAGGACGCGGCGCTCGGGAGAGCGGGCGGGTGAGTCACCCACACAAAGGAAAAGGGCCTTTCCGTCCTCAGCC
GTCGCTTCATGTGACTCCACGGAGTACCGGGCGCCGTCCAGGCACCTCGATTAGTTCTCGAGCTTTTGGAGTACG
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AGGGCGAGGAGGATAACTCCGCCATCATCAAGGAGTTCCTGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAACG
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CCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGA
AGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACT
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TGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGC
GGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACG
ACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTACAACGTCAACATCAAGT
TGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCG
GCGGCATGGACGAGCTGTACAAGTGGTAA

Humanized Coding Sequences of 20 Cas12a Effectors

The coding region sequences of 16 Cas12a orthologues and 4 Cas12a variants used in this study, corresponding to P1–P20 in Supplementary Table S2, are presented here.

The full coding regions of 16 Cas12a orthologues were synthesized by BGI Geneland Scientific (China). When synthesizing gene fragments, universal adaptors were added on either side of the coding region for amplification and to provide homologous arms for cloning into the expressing vector. The N-terminal adaptor contains a partial EF1α promoter, a kozak sequence, and SV40 NLS, while the C-terminal adaptor includes a C-terminal NLS and the 3×HA sequence.

N-terminal adaptor:

CAAAGTTTTTTTCTTCCATTTCAGGTGTCGTGAGCATGCACCGGTGCCACCATGGCCCCAAAGAAGAAGCGGAAG
GTC

C-terminal adaptor:

AAACGTCCGGCGGCCACGAAAAAAGCCGGCCAGGCAAAAAAGAAAAAAGGCTCCTACCCGTACGATGTTCCGGAT
TACGCGTATCCGTACGACGTGCCGGATTATGCATACCCGTATGATGTCCCGGACTATGCC

The coding region sequences of 16 Cas12a orthologues without stop codons are listed as follows, with their corresponding GenBank accession numbers:

Cas12a orthologue	GenBank accession number
AsCas12a	OK557997
LbCas12a	OK557998
FnCas12a	OK557999
MbCas12a	OK558000
BsCas12a	OK558001
HkCas12a	OK558002
ArCas12a	OK558003
PrCas12a	OK558004
PxCas12a	OK558005
Mb3Cas12a	OK557996
PdCas12a	OK558006
PiCas12a	OK558007
Lb2Cas12a	OK558008
ErCas12a	OK558009
EeCas12a	OK558010
CeCas12a	OK558011

>AaCas12a

ATGACCCAGTTCGAGGGCTTTACCAACCTGTATCAGGTGAGCAAAACCCTGCGTTTTGAGCTGATCCCGCAGGGC
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AAACCGATCATCGATCGTATCTACAAAACCTATGCCGACCAGTGCCTGCAGCTGGTGCAGCTGGATTGGGAGAAC
CTGAGCGCCGCCATCGACTCCTATCGCAAAGAGAAAACCGAGGAGACCCGTAACGCCCTGATCGAGGAGCAGGCC
ACCTATCGCAATGCCATCCATGACTACTTCATCGGCCGTACCGACAACCTGACCGATGCCATCAATAAACGCCAT
GCCGAGATCTACAAAGGCCTGTTCAAAGCCGAGCTGTTTAATGGCAAAGTGCTGAAACAGCTGGGCACCGTGACC
ACCACCGAGCATGAGAACGCCCTGCTGCGTAGCTTCGACAAATTTACCACCTACTTCTCCGGCTTTTATGAGAAC
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ACCCAGACCCAGATCGACCTGTATAACCAGCTGCTGGGCGGCATCTCTCGTGAGGCAGGCACCGAGAAAATCAAA
GGCCTGAACGAGGTGCTGAATCTGGCCATCCAGAAAAATGATGAGACCGCCCATATCATCGCCTCCCTGCCGCAT
CGCTTCATCCCGCTGTTTAAACAGATCCTGTCCGATCGTAACACCCTGTCTTTCATCCTGGAGGAGTTTAAAAGC
GACGAGGAAGTGATCCAGTCCTTCTGCAAATACAAAACCCTGCTGCGCAACGAGAACGTGCTGGAGACCGCCGAG
GCCCTGTTTAACGAGCTGAACAGCATCGACCTGACCCATATCTTCATCAGCCATAAAAAACTGGAGACCATCAGC
AGCGCCCTGTGCGACCATTGGGATACCCTGCGTAATGCCCTGTATGAGCGTCGCATCTCCGAGCTGACCGGCAAA
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GCCGCAGGCAAAGAGCTGAGCGAGGCCTTCAAACAGAAAACCAGCGAGATCCTGTCCCATGCACATGCCGCCCTG
GATCAGCCGCTGCCGACCACCCTGAAAAAACAGGAGGAGAAAGAGATCCTGAAATCTCAGCTGGACAGCCTGCTG
GGCCTGTACCATCTGCTGGACTGGTTTGCCGTGGATGAGTCCAACGAGGTGGACCCGGAGTTCTCTGCCCGTCTG

ACCGGCATCAAACTGGAGATGGAGCCGTCTCTGAGCTTCTACAACAAAGCCCGCAATTATGCCACCAAAAAACCG
TACTCCGTGGAGAAATTCAAACTGAACTTTCAGATGCCGACCCTGGCCTCTGGCTGGGACGTGAATAAAGAGAAA
AACAATGGCGCCATCCTGTTTGTGAAAAACGGCCTGTACTATCTGGGCATCATGCCGAAACAGAAAGGCCGTTAT
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>CeCas12a

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TACAACTGGTTCGACTTCATCCAGAACCGCCGCTTC

For the four Cas12a variants, R substitutions were performed by replacing the codons at the corresponding amino acid positions of the wild-type protein with the codon for arginine (AGA), while the A substitution was performed by replacing the codon at the corresponding position with the codon for alanine (GCC).

>enAsCas12a-HF(E174R/N282A/S542R/K548R)

We deposited the three novel engineered 3R versions of the Cas12a variants in Addgene under the ID numbers shown below:

>PrCas12a-3Rv(E162R/N519R/K525R) Addgene#179708
>Mb3Cas12a-3Rv(D180R/N581R/K587R) Addgene#179709
>BsCas12a-3Rv(K155R/N512R/K518R) Addgene#179710

Detailed Legend for Figures 1-5

Here, we provide supplementary details for the legends of Figures 1-5, including the targets involved, the statistical analysis used, detailed *p* values, and other descriptions.

Related to Figure 1

(**d**) Synt.R1 and R2 show the target sites from synthetic biological replicates. 'D12' represents a 12 bp deletion repair outcome generated by Cas12a editing after alignment with the original target site

sequence. The repair outcome containing bases shown in bold red text denoting the microhomology unit (MH unit) indicates that the repair outcome was generated via MMEJ-based pathway.

Related to Figure 2

(**b**) Note that the PrCas12a bacterial loci contain no DR array. Here, we applied an optimized crRNA scaffold sequence with a loop region variant as UAUG for the editing activity assay of PrCas12a. Box-whisker plots: median line, quartiles for box edges, 1-99% whiskers. Differences in the editing activity between Cas12a orthologues were determined by non-parametric Kruskal–Wallis test (H test), followed by Dunn's multiple comparison post hoc test. ****$p < 0.0001$. For p values less significant than **** p <0.0001, (**c**) HEK293T: Pr v.s. Bs, ** p =0.0062; Lb v.s. Lb2, *** p =0.0002. (**d**) RKO: Mb3 v.s. Lb, ** p =0.0027.

Related to Figure 3

(**a**) The numbers of the target sites with diverse PAM sequences involved in each group are as follows. **AsCas12a**: TTTV, n=1,240; VTTV, n=457; TTCV, n=833; and TRTV, n=3,512. **BsCas12a**: TTTV, n=1,640; VTTV, n=348; TTCV, n=501; and TRTV, n=1,875. **PrCas12a**: TTTV, n=2,635; VTTV, n=300; TTCV, n=482; and TRTV, n=2,770. **Mb3Cas12a**: TTTV, n=2,844; VTTV, n=314; TTCV, n=386; and TRTV, n=3,169. Box-whisker plots: median line, quartiles for box edges, 1-99% whiskers. V = A/C/G; R = A/G. (**b**) The targeted DNA contains the target site with a TTTA PAM; the 4 nt PAM parts of the non-target strand and target strand DNA are highlighted in red and yellow, respectively, marked with the corresponding bases and positions in PAM, such as NT(-1)DA, representing an adenine nucleotide at position −1 in the 4 nt PAM on the non-target strand. Ten amino acid residues proximal to PAM are highlighted in blue. Substitutions of these residues were expected to alter the PAM recognition pattern of Cas12a. (**c**) Based on an amino acid alignment with AsCas12a, we introduced the three arginine substitutions mentioned above into the corresponding residue positions of three Cas12a orthologues showing potential in our editing activity assessment (BsCAs12a, PrCas12a, and Mb3Cas12a). Three Cas12a-3R variants were generated: BsCas12a-3Rv (K155R/N512R/K518R), PrCas12a-3Rv (E162R/N519R/K525R), and Mb3Cas12a-3Rv (D180R/N581R/K587R).

Related to Figure 4

The same set of target sites were chosen to evaluate the editing efficiency between WT Cas12a and engineered Cas12a mutants. The number of target sites with diverse PAM sequences involved in each group is as follows. (**a**) PrCas12a: TTTV, n=2,635; VTTV, n=300; TTCV, n=482; and TRTV, n=2,770. (**b**) Mb3Cas12a: TTTV, n=2,844; VTTV, n=314; TTCV, n=386; and TRTV, n=3,169. (**c**) BsCas12a: TTTV, n=1,640; VTTV, n=348; TTCV, n=501; and TRTV, n=1,875. (**d**) AsCas12a: TTTV, n=1,240; VTTV, n=457; TTCV, n=833; and TRTV, n=3,512. Box-whisker plots: median line, quartiles for box edges, 1-99% whiskers. **** $p < 0.0001$ (Mann–Whitney,

two-tailed). V=A/C/G; R=A/G.

Related to Figure 5

Target sites involved in Figure 5 had at least 300 read counts per crRNA–target pair in one sample. The number of target sites involved in each group is as follows. (b) PrCas12a-3Rv: TGTV, *n*=1,249; TATV, *n*=715. Mb3Cas12a-3Rv: TGTV, *n*=840; TATV: *n*=1,250. BsCas12a-3Rv: TGTV, *n*=995; TATV, *n*=605. enAsCas12a-HF: TGTV, *n*=1,500; TATV, *n*=1,437. (c) PrCas12a-3Rv: TTCV, *n*=1,019; ATCV, *n*=436; CTCV, *n*=541; and GTCV, *n*=482. Mb3Cas12a-3Rv: TTCV, *n*=1,095; ATCV, *n*=252; CTCV, *n*=500; and GTCV, *n*=464. Box-whisker plots: median line, quartiles for box edges, 1-99% whiskers. Differences in the editing activity between groups were determined by non-parametric Kruskal–Wallis test (H test), followed by Dunn's multiple comparison post hoc test. **** p <0.0001. For p values less significant than **** p <0.0001, (b) Mb3Cas12a-3Rv: TGTV vs. TATV, (ns) p =0.0750; enAsCas12a-HF: TGTV vs. TATV, (ns) p =0.0684. (c) PrCas12a-3Rv: ATCV vs. CTCV, (ns) p =0.2305; ATCV vs. GTCV, (ns) p >0.9999. Mb3Cas12a-3Rv: TTCV vs. ATCV, (ns) p >0.9999; CTCV vs. GTCV, (ns) p =0.6449.