Supplementary Information

Materials and Methods

DNA manipulations. DNA manipulation including DNA preparation, digestion, ligation, amplification, synthesis, purification, agarose gel electrophoresis, *etc.* were conducted according to *Molecular Cloning: A Laboratory Manual* with some modifications. Briefly, targeting chimeric single guide RNA (sgRNA) scaffolds for cell transfection assay were constructed by ligation annealed oligonucleotides (oligos) (Supplementary Table S3) into BsaI-digested pUC19-U6-sgRNA vectors (Supplementary Sequences) or directly *de novo* synthesized (Supplementary Table S4) into pUC19-U6 vector[1].

***De novo* gene synthesis and plasmid construction.** PSI-BLAST program[2] was adopted to identify new CRISPR-Cas12b orthologs. Their coding sequences were humanized[3] and oligos for Cas12b gene and sgRNA synthesis were designed using GeneDesign program[4]. All oligos for gene synthesis were commercially purchased (Taihe Biotechnology Co., LTD). For Cas12b coding genes which are >3 kb were split into 4 "chunks" of ~800 bp (Supplementary Table S1). For gRNAs which are <300 bp were left as is (Supplementary Tables S2 and S4). All oligos for gene synthesis were commercially purchased (Taihe Biotechnology Co., LTD). DNA fragments were synthesized using overlap extension PCR method according to our previous study[1]. Purified products were assembled into expression vectors via homologous recombination in vitro using NEBuilder® HiFi DNA Assembly Master Mix (NEB). The pCAG-2AeGFP and pUC19-U6 vectors[1] were applied for mammalian expression of Cas12b proteins and sgRNAs, respectively. AaCas12b, AkCas12b, AmCas12b, BsCas12b and their cognate sgRNAs have been previously reported[1].

Cell culture and transfection. Human embryonic kidney 293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% Antibiotic-Antimycotic (Gibco) at 37℃ with 5% CO_2 incubation. 293T cells were transfected using Lipofectamine LTX (Invitrogen) following the manufacturer's recommended protocol. For each well of a 48-well plate, a total of 400 ng

plasmid (Cas12b: sgRNA = 2: 1) were used. Then 48 h following transfection, cells were harvested directly for genomic DNA extraction without sorting.

T7 endonuclease I (T7EI) assay and Sanger sequencing. Harvested cells were lysed directly with Buffer L (Bimake) supplemented with Protease K and incubated at 55℃ for 3 h and inactivated at 95℃ for 10 min. Genomic region surrounding the Cas12b target site for each gene was PCR-amplified (Supplementary Table S5). 200 ~ 400 ng PCR products were mixed with ddH_2O to a final volume of 10 μL, and subjected to re-annealing process to enable heteroduplex formation according to previous methods[5]. After re-annealing, products were treated with 1/10 volume of NEBuffer^{TM} 2.1 and 0.2 μL T7EI (NEB) at 37 ℃ for 30 min, and analyzed on 3% agarose gels. Indels were quantitated based on relative band intensities[6]. T7EI assay identified mutated products were subjected to be cloned into TA cloning vector and transformed into competent *E. coli* strain (Transgen Biotech). After overnight culture, colonies were randomly picked out and sequenced.

For each sample, transfection and T7EI assay were repeated twice or more and a representative result was shown. The histogram in this study represented the mean value of two independent repeats.

References

1. Teng, F. et al. Repurposing CRISPR-Cas12b for mammalian genome engineering. *Cell Discov.* **4**, 63 (2018).
2. Altschul, S.F. et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389-3402 (1997).
3. Grote, A. et al. JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. *Nucleic Acids Res.* **33**, W526-531 (2005).
4. Richardson, S.M., Wheelan, S.J., Yarrington, R.M. & Boeke, J.D. GeneDesign: rapid, automated design of multikilobase synthetic genes. *Genome Res.* **16**, 550-556 (2006).
5. Li, W., Teng, F., Li, T. & Zhou, Q. Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems. *Nat. Biotechnol.* **31**, 684-686 (2013).
6. Cong, L. et al. Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819-823 (2013).

Figure S1

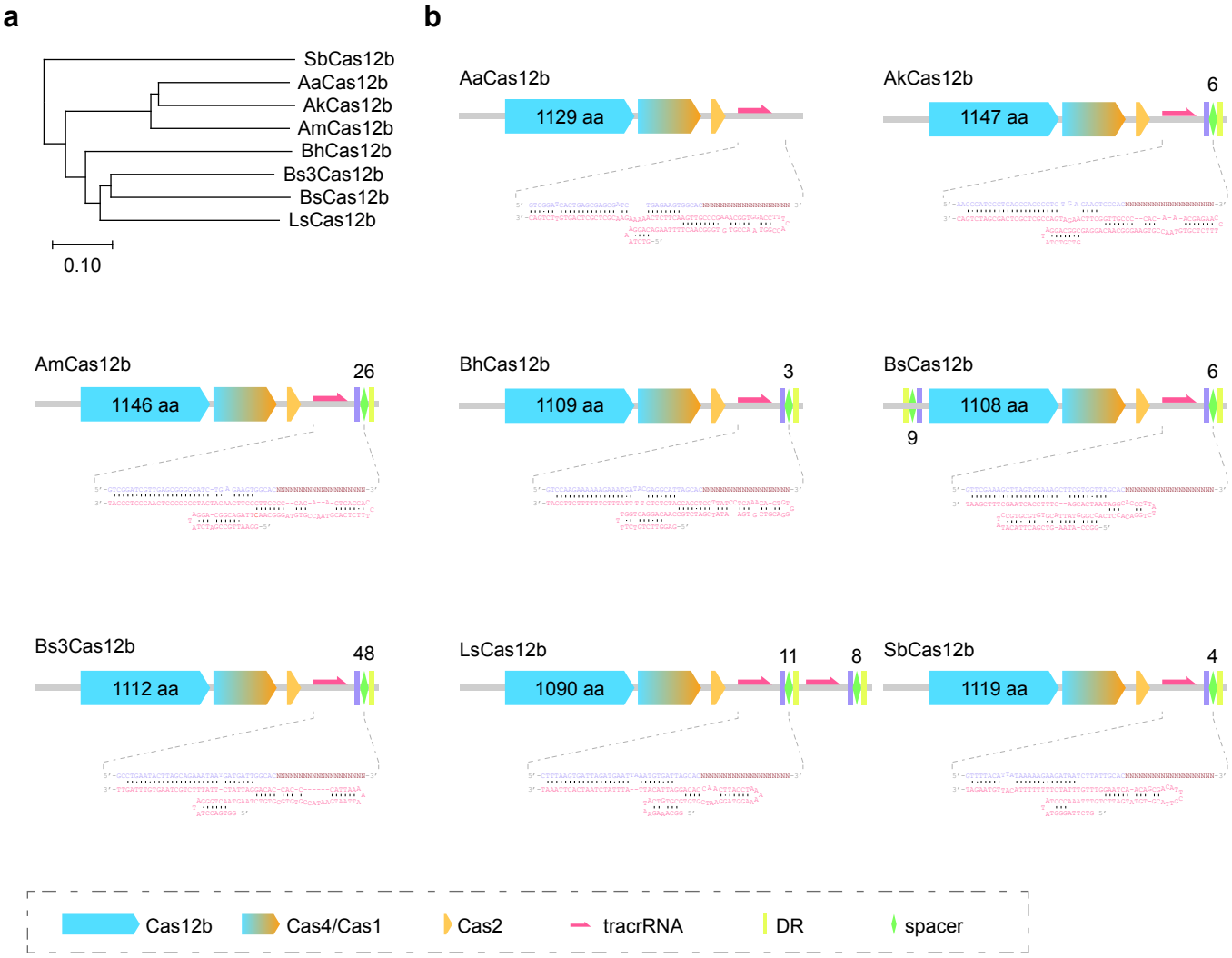

a
SbCas12b
AaCas12b
AkCas12b
AmCas12b
BhCas12b
Bs3Cas12b
BsCas12b
LsCas12b
0.10
b
AaCas12b
1129 aa
AkCas12b
1147 aa
6
AmCas12b
1146 aa
26
BhCas12b
1109 aa
3
BsCas12b
1108 aa
6
9
Bs3Cas12b
1112 aa
48
LsCas12b
1090 aa
11
8
SbCas12b
1119 aa
4
Cas12b
Cas4/Cas1
Cas2
tracrRNA
DR
spacer

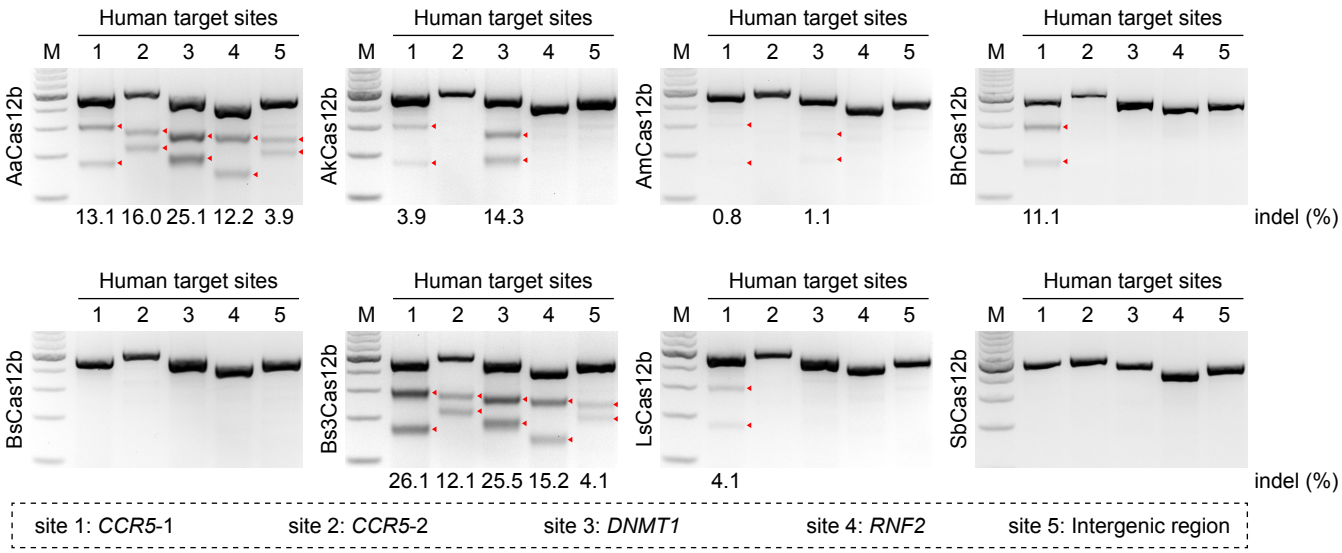
Figure S1 Phylogenetic tree of non-redundant Cas12b orthologs and their loci chosen for genome-editing testing. a Neighbor Joining phylogenetic tree showing the evolutionary relationships of Cas12b orthologs tested in this study. **(b)** Maps of bacterial genomic loci corresponding to the eight Cas12b proteins in Supplementary Fig. S1a. In silico co-folding of the crRNA DR and putative tracrRNA shows stable secondary structure. DR, direct repeat. The number of each bacterial genomic spacers is indicated above their CRISPR array.

Figure S2

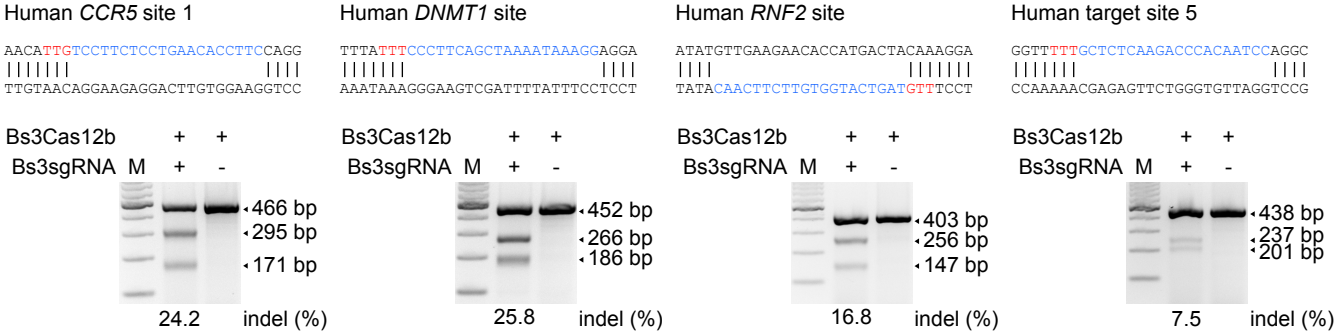
Figure S2 Protein alignment of Cas12b orthologs. Multiple sequence alignment of the amino acid sequences of the eight Cas12b orthologs tested in this study. Residues that are conserved are highlighted with a red background and conserved mutations are highlighted with an outline and red font.

Figure S3

a



b



c

Human *CCR5* site 1 — indel

```
CATTGTCCTTCTCCTGAACACCT---------TCCAGGAATTCTT
CATTGTCCTTCTCCTGAACACCTgaaggccccaggAGGAATTCTT  Δ3, +12
CATTGTCCTTCTCCTGAACA-------------GGAATTCTT    Δ7
CATTGTCCTTCTCCTGAA---------------GGAATTCTT    Δ9
CATTGTCCTT-----(Δ27 bp)----------              Δ27
```

Human *RNF2* site — indel

```
CCAATTTGTTTGGATATGTTGA---AGAACACCATGACTACAAAG
CCAATTTGTTTGGATATGTTGAtgaAGAACACCATGACTACAAAG  +3
CCAATTTGTTTGGATATGTT------GAACACCATGACTACAAAG  Δ3
CCAATTTGTTTGGAT----------AGAACACCATGACTACAAAG  Δ7
CCAATTTGTTTGGA-----------AGAACACCATGACTACAAAG  Δ8
CCAATTTGTTTGGAT----------CACCATGACTACAAAG      Δ11
CCAATTT-----------------------GACTACAAAG      Δ25
```

Human *DNMT1* site — indel

```
TATTTCCCTTCAGCTAAAATAAAGGAGGAGGAAGCTGCTAAGGAC
TATTTCCCTTCAGCT--------GGAGGAGGAAGCTGCTAAGGAC  Δ8
TATTTCCCTTCAGCTAAAA----------GGAAGCTGCTAAGGAC  Δ10
TATTTCCCTTCAG-----------------GAAGCTGCTAAGGAC  Δ17
TATTTCCCTTCAGCTAAA------------------------GGAC  Δ23
TATTTCCCTTC----------(Δ48 bp)--------------    Δ48
```

Human target site 5 — indel

```
TTTTTGCTCTCAAGACCCACAATCCAGGCCGGAAGAGGCAAGCAT
TTTTTGCTCTCAAGACCCACA-----GGCCGGAAGAGGCAAGCAT  Δ5
TTTTTGCTCTCAAGACCCACAA----------AGAGGCAAGCAT   Δ11
```

Figure S3 Cas12b orthologs mediated genome targeting in human 293T cells. a T7EI assay results indicating the genome targeting activities of the eight Cas12b proteins combined with their cognate sgRNAs in the human genome, respectively. Red triangles indicate the cleaved bands. **b** T7EI assay results indicating the simultaneous multiplex genome targeting mediated by Bs3Cas12b combined with its cognate sgRNAs (Bs3sgRNAs) in human 293T cells. **c** Sanger sequencing showing representative indels induced by Bs3Cas12b combined with Bs3sgRNAs in Supplementary Fig. S3b. PAM and protospacer sequences are colored in red and blue, respectively. Deletions and insertions are symbolled with purple dashes and green lowercases, respectively.

Figure S4

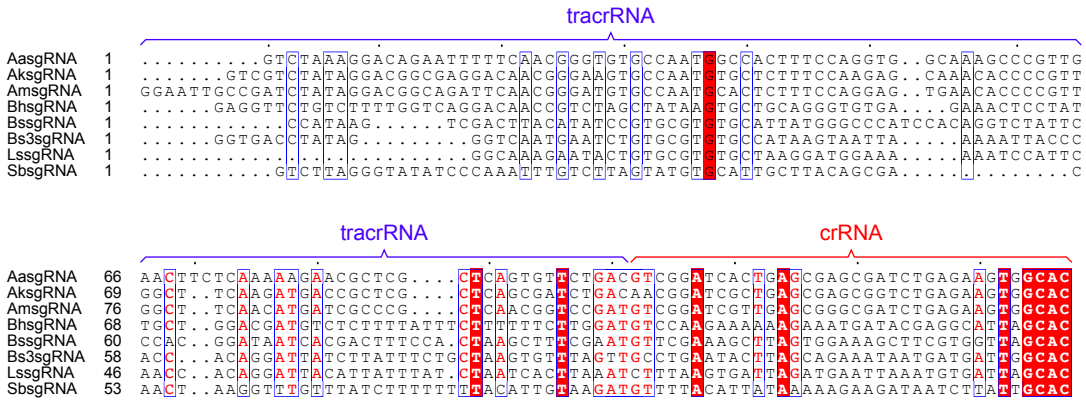
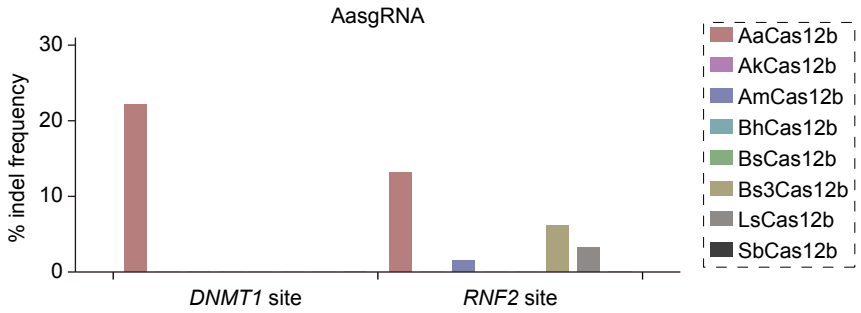

tracrRNA
AasgRNA 1
AksgRNA 1
AmsgRNA 1
BhsgRNA 1
BssgRNA 1
Bs3sgRNA 1
LssgRNA 1
SbsgRNA 1
tracrRNA
crRNA
AasgRNA 66
AksgRNA 69
AmsgRNA 76
BhsgRNA 68
BssgRNA 60
Bs3sgRNA 58
LssgRNA 46
SbsgRNA 53

Figure S4 DNA alignment of sgRNAs of Cas12b. Multiple sequence alignment of the DNA sequences of the 8 sgRNAs derived from the 8 Cas12b loci tested in this study.

Figure S5

a



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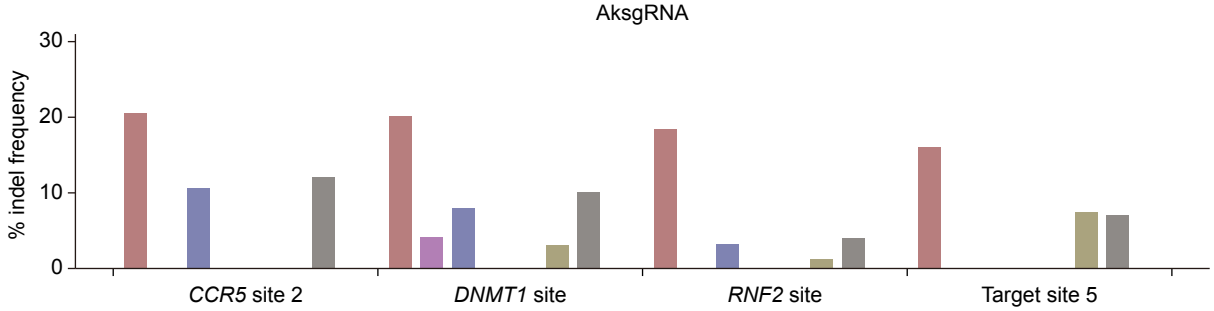

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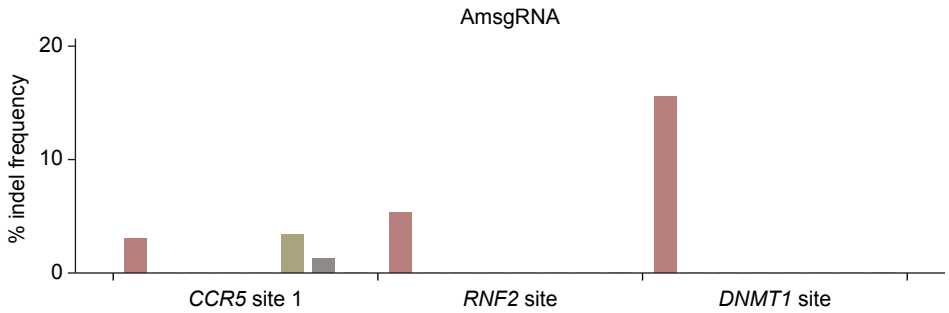

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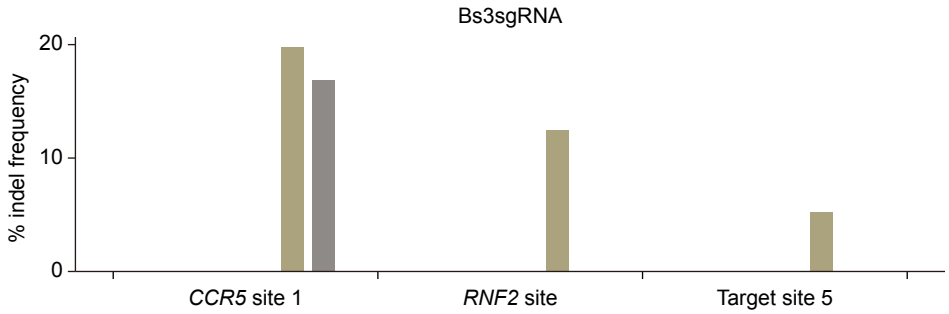

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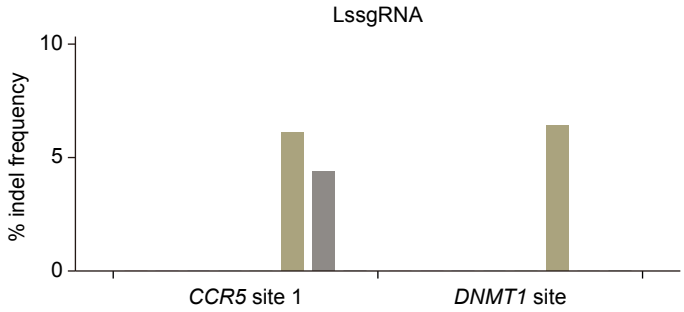


Figure S5 Interchangeability between Cas12b orthologs and their sgRNAs. T7EI assay results indicating the average genome targeting activities of the eight Cas12b orthologs directed by AasgRNA **(a)**, AksgRNA **(b)**, AmsgRNA **(c)**, Bs3sgRNA **(d)** and LssgRNA **(e)** in human 293T cells, respectively. Red triangles indicate the cleaved bands. n = 2.

Figure S6

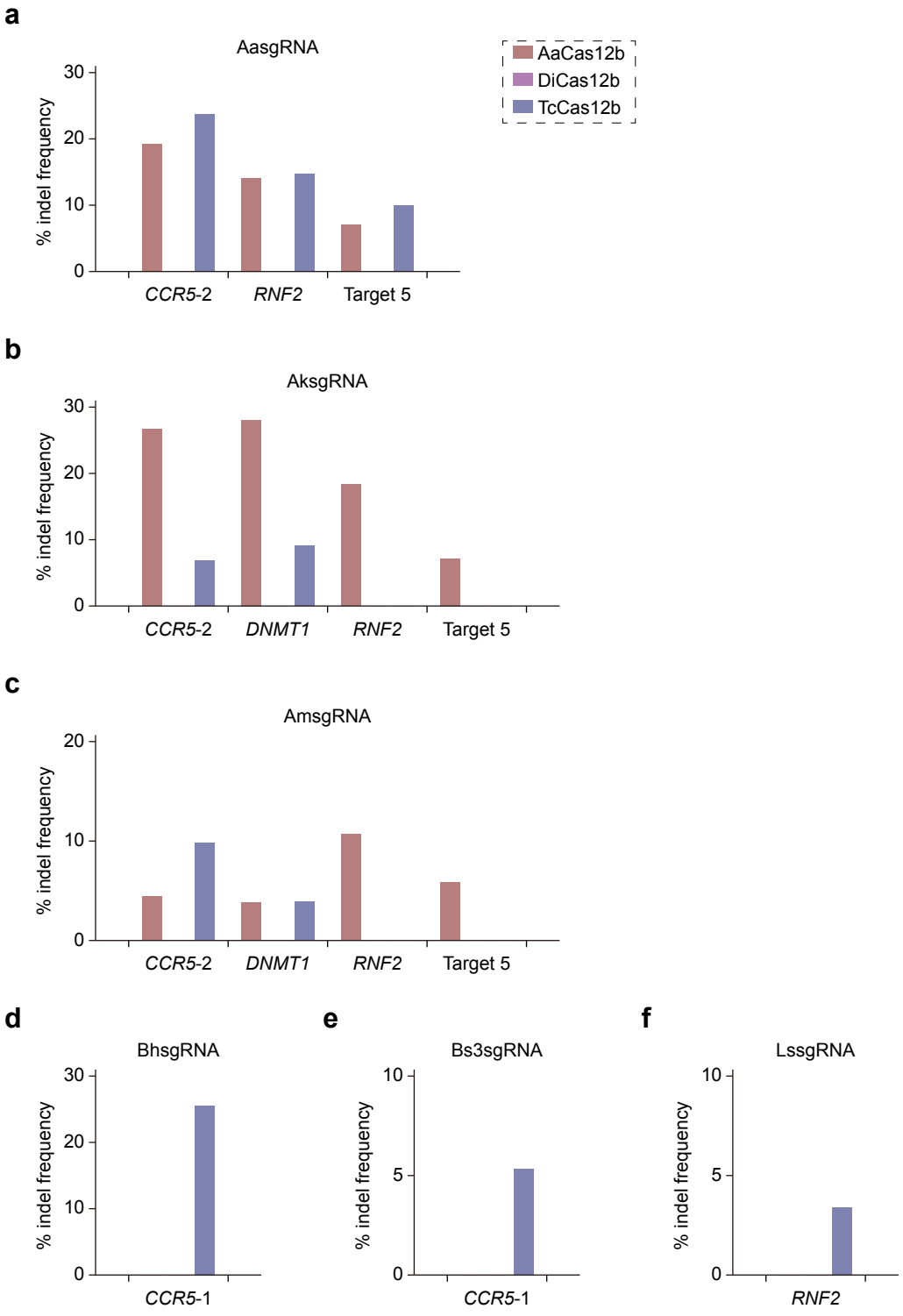
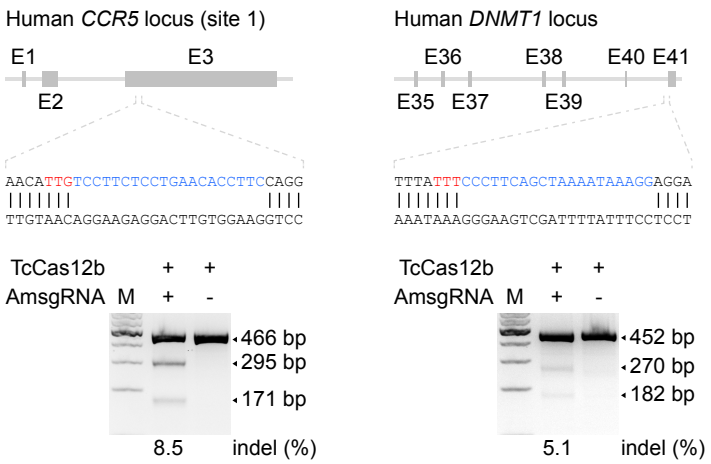

a
AasgRNA
AaCas12b
DiCas12b
TcCas12b
% indel frequency
30
20
10
0
CCR5-2
RNF2
Target 5
b
AksgRNA
% indel frequency
30
20
10
0
CCR5-2
DNMT1
RNF2
Target 5
c
AmsgRNA
% indel frequency
20
10
0
CCR5-2
DNMT1
RNF2
Target 5
d
BhsgRNA
% indel frequency
30
20
10
0
CCR5-1
e
Bs3sgRNA
% indel frequency
10
5
0
CCR5-1
f
LssgRNA
% indel frequency
10
5
0
RNF2

Figure S6 Orthologous sgRNAs directed Cas12b for genome editing. T7EI assay results indicating the average genome targeting activities of AaCas12b, DiCas12b and TcCas12b directed by AasgRNA **(a)**, AksgRNA **(b)**, AmsgRNA **(c)**, BhsgRNA **(d),** Bs3sgRNA **(e)** and LssgRNA **(f)** in the human 293T cells, respectively. Red triangles indicate the cleaved bands. n = 2.

Figure S7

a



b

AksgRNA-*CCR5*-1	indel
TACAACATTGTCCTTCTCCTGAACACCTTCCAGGAATTCTTTGGCCTGAATAATTGCAGT	
TACAACATTGTCCTTCTCCTGAACACCT------AATTCTTTGGCCTGAATAATTGCAGT	Δ6
TACAACATTGTCCTTCTCCTGAACA----------TCTTTGGCCTGAATAATTGCAGT	Δ12
TACAACATTGTCCTTCTCCTGAA--------------------------TAATTGCAGT	Δ27
AksgRNA-*DNMT1*	**indel**
CCTTTTATTTCCCTTCAGCTAAAATAAAGGAGGAGGAAGCTGCTAAGGACTAGTTCTGCC	
CCTTTTATTTCCCTTCAGCTAAA-----GGAGGAGGAAGCTGCTAAGGACTAGTTCTGCC	Δ5
CCTTTTATTTCCCTTCAGCTAAAAa--------AGGAAGCTGCTAAGGACTAGTTCTGCC	Δ9, +1
CCTTTTATTTCCCTTCAGCTAAA----------TGCTAAGGACTAGTTCTGCC	Δ17

c

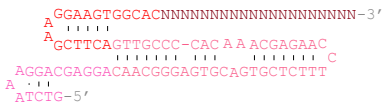
AmsgRNA-*CCR5*-1	indel
TACAACATTGTCCTTCTCCTGAACACCTTCCAGGAATTCTTTGGCCTGAATAATTGCAGT	
TACAACATTGTCCTTCTCCTGAACACCTga-AGGAATTCTTTGGCCTGAATAATTGCAGT	Δ3, +2
TACAACATTGTCCTTCTCCTGAACA---TCCAGGAATTCTTTGGCCTGAATAATTGCAGT	Δ3
TACAACATTGTCCTTCTCCTGAACACCga--AGGAATTCTTTGGCCTGAATAATTGCAGT	Δ4, +2
TACAACATTGTCCTTCTCCTGAACACCT----GGAATTCTTTGGCCTGAATAATTGCAGT	Δ4
TACAACATTGTCCTTCTCCTGAACACC-------AATTCTTTGGCCTGAATAATTGCAGT	Δ7
TACAACATTGTCCTTCTCCTGAA--------------------------TAATTGCAGT	Δ27
TACAACATTGTCCTTCTCC--------------------------------AATTGCAGT	Δ32
AmsgRNA-*DNMT1*	**indel**
CCTTTTATTTCCCTTCAGCTAAAATAAAGGAGGAGGAAGCTGCTAAGGACTAGTTCTGCC	
CCTTTTATTTCCCTTCAGCTAAAtata--GAGGAGGAAGCTGCTAAGGACTAGTTCTGCC	Δ6, +4
CCTTTTATTTCCCTTCAGCTAAAAT------GGAGGAAGCTGCTAAGGACTAGTTCTGCC	Δ6
CCTTTTATTTCCCTTCAGCTAAAg----------GGAAGCTGCTAAGGACTAGTTCTGCC	Δ11, +1
CCTTTTATTTCCCTTCAGCTAAA----------GGAAGCTGCTAAGGACTAGTTCTGCC	Δ11
CCTTTTATTTCCCTTCAGCT---------------GAAGCTGCTAAGGACTAGTTCTGCC	Δ15

Figure S7 TcCas12b-mediated multiplex genome editing. a T7EI assay results indicating the simultaneous multiplex genome targeting mediated by TcCas12b combined with AmsgRNAs in human 293T cells. **b-c** Sanger sequencing showing representative indels induced by TcCas12b combined with AksgRNAs in Fig. 1h **(b)** and AmsgRNAs in Supplementary Fig. S7a **(c)**. PAM and protospacer sequences are colored in red and blue, respectively. Deletions and insertions are symbolled with purple dashes and green lowercases, respectively.

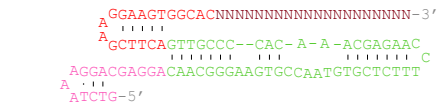
Figure S8

a

artsgRNA1



artsgRNA2



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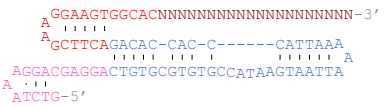
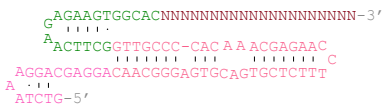


artsgRNA4



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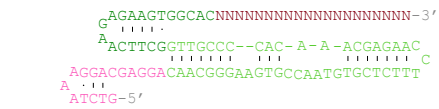

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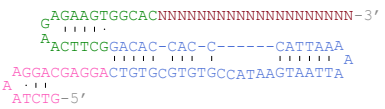

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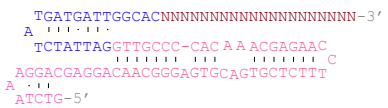

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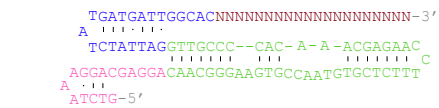

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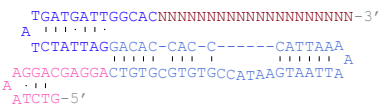

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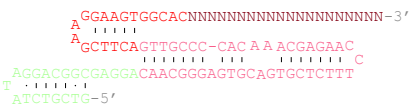

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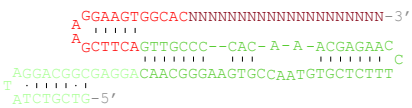

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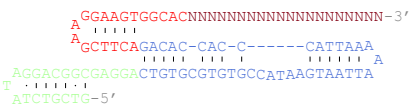

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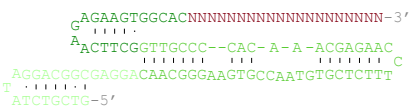

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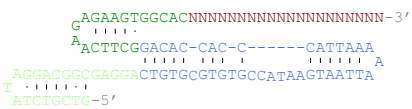

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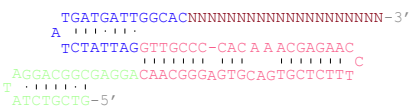

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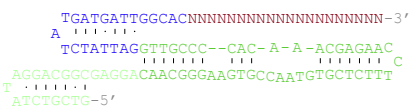

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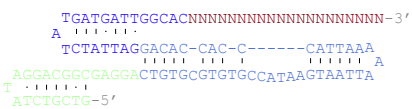

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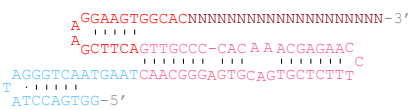

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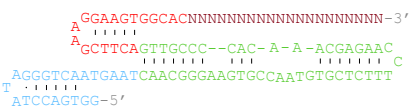
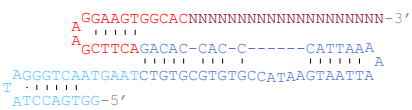


artsgRNA21



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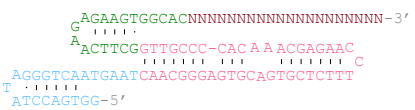

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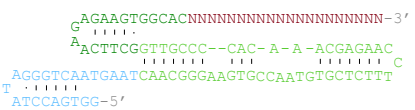

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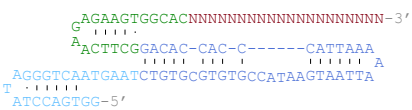

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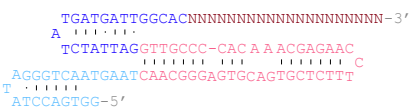

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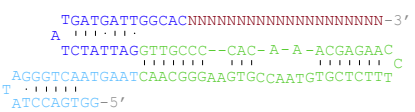

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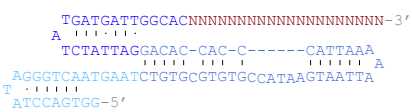

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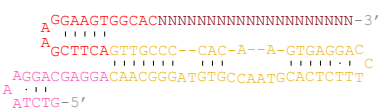

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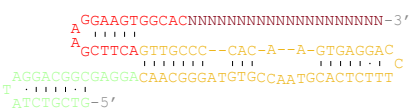

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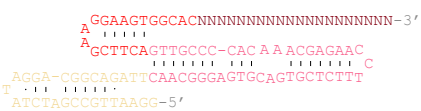

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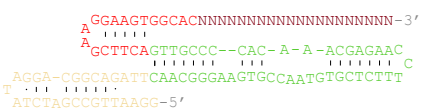

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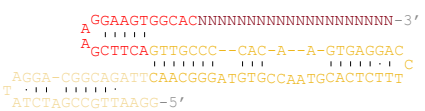
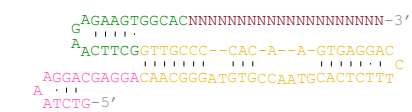


artsgRNA33



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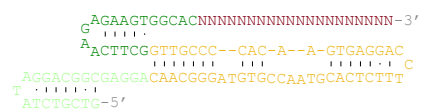

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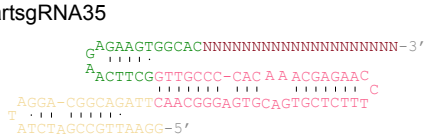

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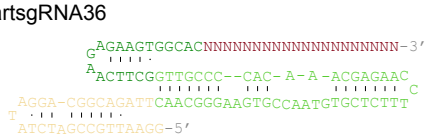

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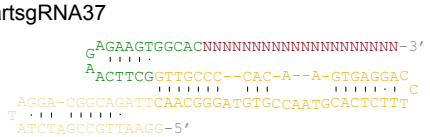

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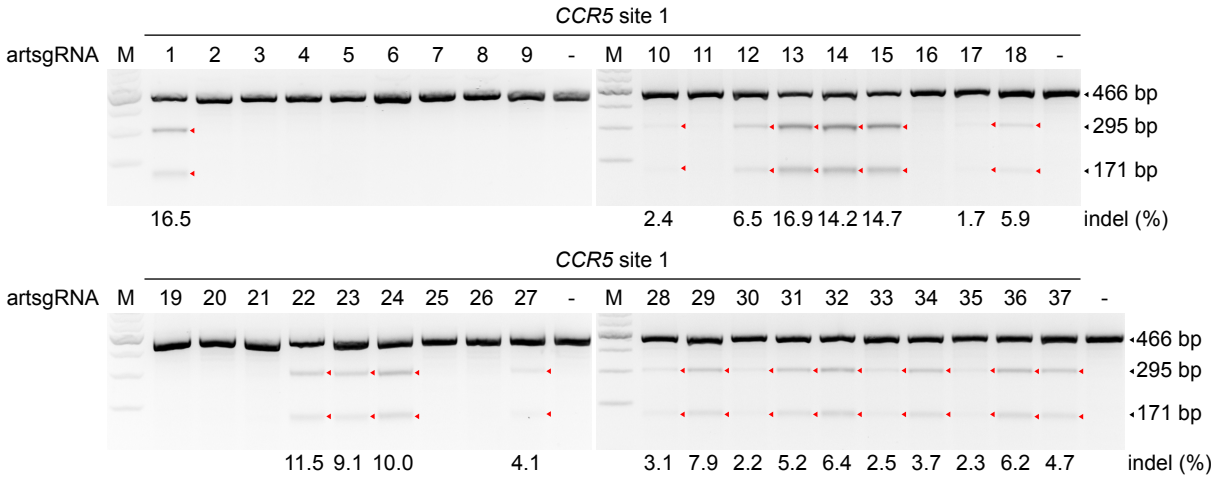
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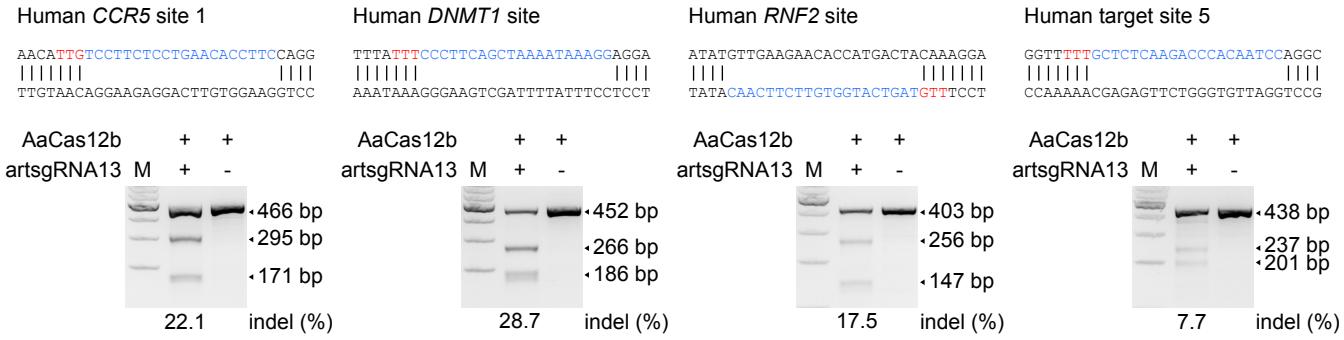
artsgRNA M 1 2 3 4 5 6 7 8 9 - M 10 11 12 13 14 15 16 17 18 -
466 bp
295 bp
171 bp
16.5 2.4 6.5 16.9 14.2 14.7 1.7 5.9 indel (%)
CCR5 site 1
artsgRNA M 19 20 21 22 23 24 25 26 27 - M 28 29 30 31 32 33 34 35 36 37 -
466 bp
295 bp
171 bp
11.5 9.1 10.0 4.1 3.1 7.9 2.2 5.2 6.4 2.5 3.7 2.3 6.2 4.7 indel (%)

c


Human CCR5 site 1
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TTGTAACAGGAAGAGGACTTGTGGAAGGTCC
AaCas12b + +
artsgRNA13 M + -
466 bp
295 bp
171 bp
22.1 indel (%)
Human DNMT1 site
TTTATTTCCCTTCAGCTAAAATAAAGGAGGA
AAATAAAGGGAAGTCGATTTTATTTCCTCCT
AaCas12b + +
artsgRNA13 M + -
452 bp
266 bp
186 bp
28.7 indel (%)
Human RNF2 site
ATATGTTGAAGAACACCATGACTACAAAGGA
TATACAACTTCTTGTGGTACTGATGTTTCCT
AaCas12b + +
artsgRNA13 M + -
403 bp
256 bp
147 bp
17.5 indel (%)
Human target site 5
GGTTTTTGCTCTCAAGACCCACAATCCAGGC
CCAAAAACGAGAGTTCTGGGTGTTAGGTCCG
AaCas12b + +
artsgRNA13 M + -
438 bp
237 bp
201 bp
7.7 indel (%)

Figure S8 Artificial sgRNAs directed TcCas12b for genome editing. a Schematic illustrating the secondary structures of the 36 artificial sgRNA (artsgRNA) scaffolds (scaffold: 1 - 12 and 14 - 37). **b** T7EI assay results indicating the genome targeting activity of TcCas12b directed by artsgRNAs in the human 293T cells. Red triangles indicate the cleaved bands. **c** T7EI assay results indicating the simultaneous multiplex genome targeting mediated by AaCas12b combined with artsgRNA13s in human 293T cells.

Supplementary Table 1 Oligonucleotides (oligos) for Cas12b gene synthesis. (see attached XLSX file)

Supplementary Table 2 Oligos for sgRNA scaffold synthesis. (see attached XLSX file)

Supplementary Table 3 Protospacer sequences of human genomic targets. Protospacer targets designed based on type V-B CRISPR-Cas12b locus and type V-A CRISPR-Cas12a locus with their requisite PAMs against different genes in the human genome.

CRISPR-Cas	Gene	Protospacer ID	Protospacer sequences (5′ - 3′)	5′PAM	Strand
Cas12b	*CCR5*	*CCR5*-1	TCCTTCTCCTGAACACCTTC	TTG	+
		CCR5-2	TTTGGCCTGAATAATTGCAG	TTC	+
	DNMT1	*DNMT1*	CCCTTCAGCTAAAATAAAGG	TTT	+
	RNF2	*RNF2*	TAGTCATGGTGTTCTTCAAC	TTG	-
	Intergenic region	Target 5	GCTCTCAAGACCCACAATCC	TTT	+
Cas12a	*CCR5*	*CCR5*-1	TCCTTCTCCTGAACACCTTCCAG	TTG	+
		CCR5-2	GCCTGAATAATTGCAGTAGCTCT	TTTG	+
	DNMT1	*DNMT1*	CCTTCAGCTAAAATAAAGGAGGA	TTTC	+
	RNF2	*RNF2*	GATATGTTGAAGAACACCATGAC	TTTG	+
	Intergenic region	Target 5	CTCTCAAGACCCACAATCCAGGC	TTTG	+

Supplementary Table 4 Oligos for artificial sgRNA (artsgRNA) synthesis. (see attached XLSX file)

Supplementary Table 5 Primers used for T7EI assay in this study.

Primer ID	Primer sequences (5′ – 3′)	Product length (bp)	Cleaved bands (bp)
CCR5-1F CCR5-1R	GTCCTGCCGCTGCTTGTCAT CCCACTTGAGTCCGTGTCACA	466	295 + 171
CCR5-2F CCR5-2R	GCAGCTCTCATTTTCCATACAGT GATCGGGTGTAAACTGAGCTTG	493	277 + 216
DNMT1-F DNMT1-R	CTCCTGCTCGGTGAATTTGG TAGTTGATAAGCGAACCTCACAC	452	270 + 182
RNF2-F RNF2-R	GGAGCTGTAGGCGATTATAGTTGAA TTCTCAAACCCTGGAAAGCACTTT	403	256 + 147
Target5-F Target5-R	ACTTCCACCCTCTGTCTTATCTC CCAGCTTCCTCAAATCTTATGCA	438	237 + 201

Supplementary Sequences

Humanized BhCas12b coding sequence from *Bacillus hisashii* strain C4 (GeneBank ID: NZ_NJGA01000060.1)

ATGGCCACCCGCAGCTTCATCCTGAAGATCGAGCCCAACGAGGAGGTGAAGAAGGGCCTGTG
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GCCAGGAGGCCATCTACGAGCACCACGAGCAGGACCCCAAGAACCCCAAGAAGGTGAGCAAG
GCCGAGATCCAGGCCGAGCTGTGGGACTTCGTGCTGAAGATGCAGAAGTGCAACAGCTTCAC
CCACGAGGTGGACAAGGACGAGGTGTTCAACATCCTGCGCGAGCTGTACGAGGAGCTGGTGC
CCAGCAGCGTGGAGAAGAAGGGCGAGGCCAACCAGCTGAGCAACAAGTTCCTGTACCCCCTG
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GAAGGAGCGCCAGGAGCAGCTGCTGCGCGACACCCTGAACACCAACGAGTACCGCCTGAGCA
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Humanized Bs3Cas12b coding sequence from *Bacillus sp.* V3-13 contig_40 (GeneBank ID: NZ_PGUZ01000040.1)

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Humanized DiCas12b coding sequence from *Desulfovibrio inopinatus* DSM 10711 (GeneBank: NZ_KE386879.1)

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Humanized LsCas12b coding sequence from *Laceyella sediminis* strain RHA1 (GeneBank ID: NZ_PVTZ01000002.1)

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Humanized SbCas12b coding sequence from *Spirochaetes bacterium* GWB1_27_13 (GeneBank ID: MIAN01000063.1)

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Humanized TcCas12b coding sequence from *Tuberibacillus calidus* DSM 17572 (GeneBank ID: NZ_KE387196.1)

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pUC19-U6-BhsgRNA partial sequence

(U6-BhsgRNA1_scaffold-*BasI*-*BasI*-terminator**)**

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pUC19-U6-Bs3sgRNA partial sequence

(U6-BssgRNA1_scaffold-*BasI*-*BasI*-terminator**)**

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TGAATACTTAGCAGAAATAATGATGATTGGCACA*GAGACC*GAGAGAG*GGTCTC*Atttttttt
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pUC19-U6-LssgRNA partial sequence

(U6-BssgRNA1_scaffold-*BasI*-*BasI*-terminator**)**

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pUC19-U6-SbsgRNA partial sequence

(U6-BssgRNA1_scaffold-*BasI*-*BasI*-terminator**)**

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