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Supplementary information for

Efficient precise integration of large DNA sequences with 3′-overhang dsDNA donors using CRISPR/Cas9

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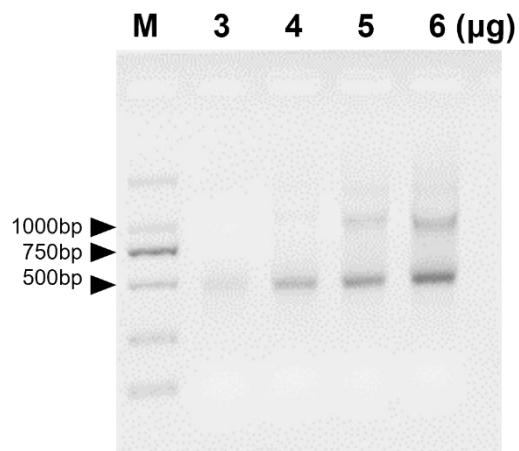
Fig. S1. Testing of the maximum amount of dsDNA subjected to digestion by Lambda exonuclease. The variable amounts of PCR-amplified dsDNA (1,110 bp), as indicated on top of each lane, without any modification, were subjected to digestion by 5 U Lambda exonuclease within 60 min. Noticeably, 3 µg of total 1,110 bp dsDNA were completely digested as evidenced by the disappearance of both ~1,110 bp and ~500 bp dsDNA bands within 60 min.
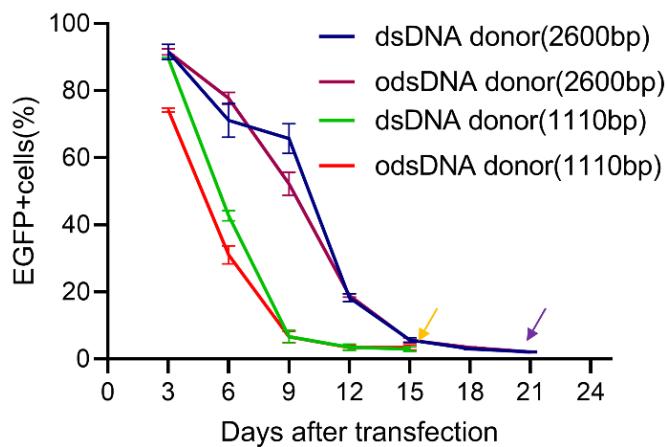
Fig. S2. Determination of EGFP expression background from donor templates. Growing HEK293T cells were transfected with dsDNA or odsDNA donors from PCR-amplified plasmids with varying lengths in the absence of Cas9 RNPs. The percentages of EGFP-positive cells at different days post-transfection as indicated were recorded by flow cytometry. The post-transfection day 15 and 21, when the EGFP background intensities were considered as low as background levels, were selected for subsequent studies for 1,110 bp and 2,600 bp templates, respectively.
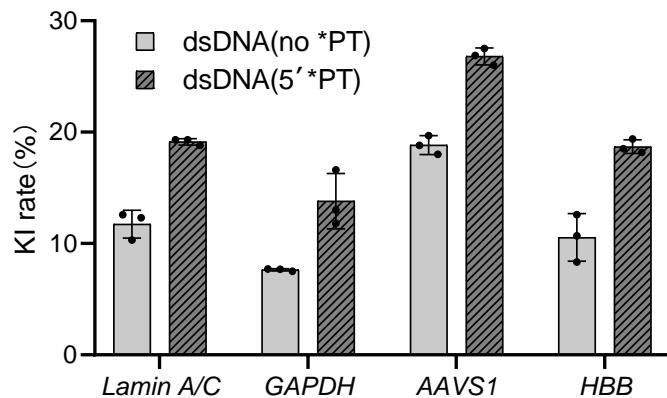
**Fig. S3. Enhanced KI rate with 5′-PT-modified dsDNA donor templates.** A side-by-side comparisons between dsDNA and 5′-PT-modified 1,110bp dsDNA donor templates (with 50bp homology arms on both sides) were performed for measuring KI efficiency across four selective genomic loci (*Lamin A/C, GAPDH, AAVS1*, and *HBB*). 5′-PT-modified donors constantly exhibited higher KI efficiencies than dsDNA donors without modification.
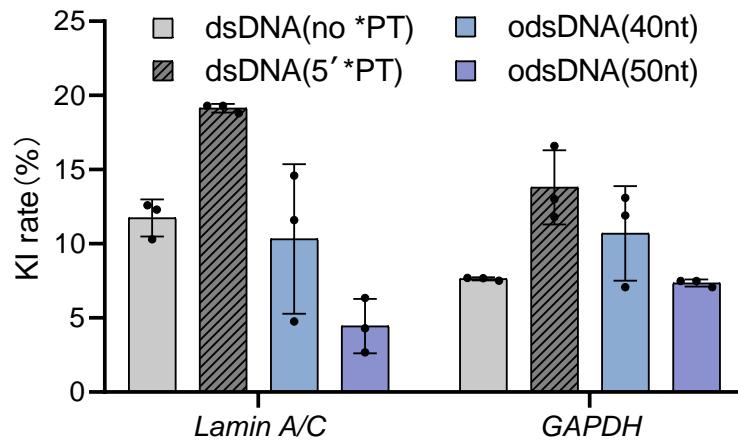
Fig. S4. Comparison of gene-sized (1010 bp) KI rates using odsDNA donors (40- and 50-nt overhangs) at two genomic loci in HEK293T cells. It revealed that odsDNA donors with longer overhangs (40- and 50-nt) exhibited similar, or even lower, KI rates, as compared with 5′ PT-modified dsDNA donors (1).
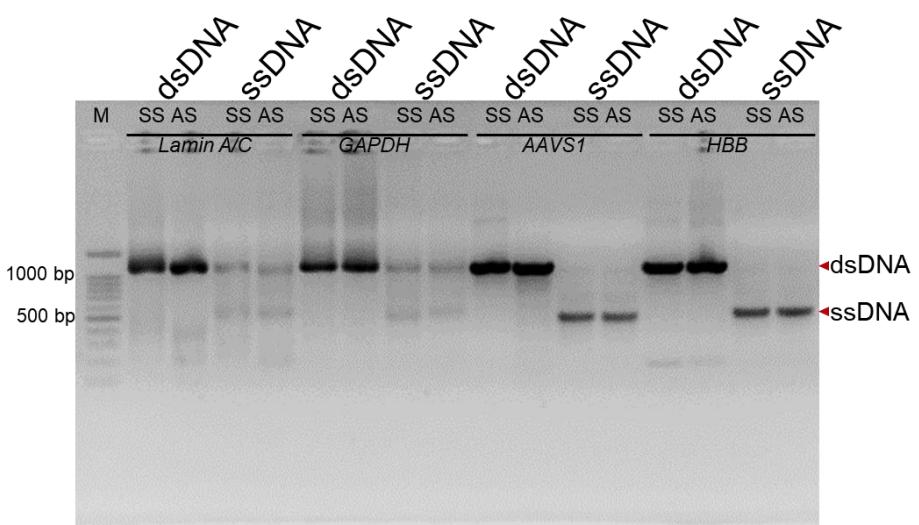
Fig. S5. A representative agarose gel image showing the recovered, newly synthesized ssDNA product in comparison to dsDNA for KI experiments.
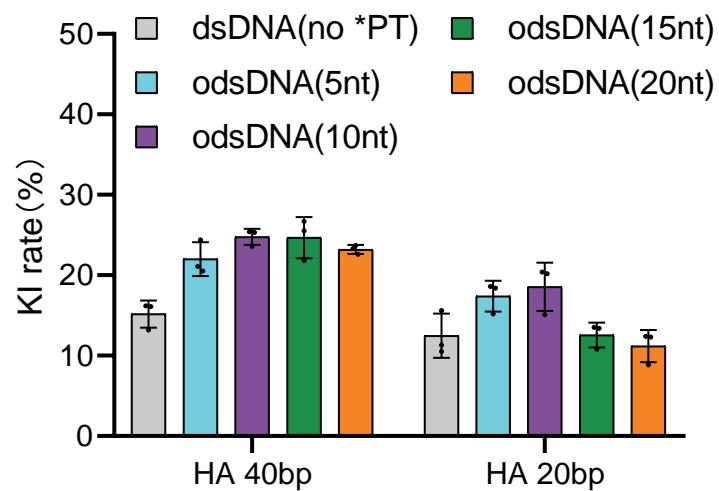
Fig. S6. Comparison of the KI rates for dsDNA donor and odsDNA donors with shorter HA lengths (40 bp and 20 bp).
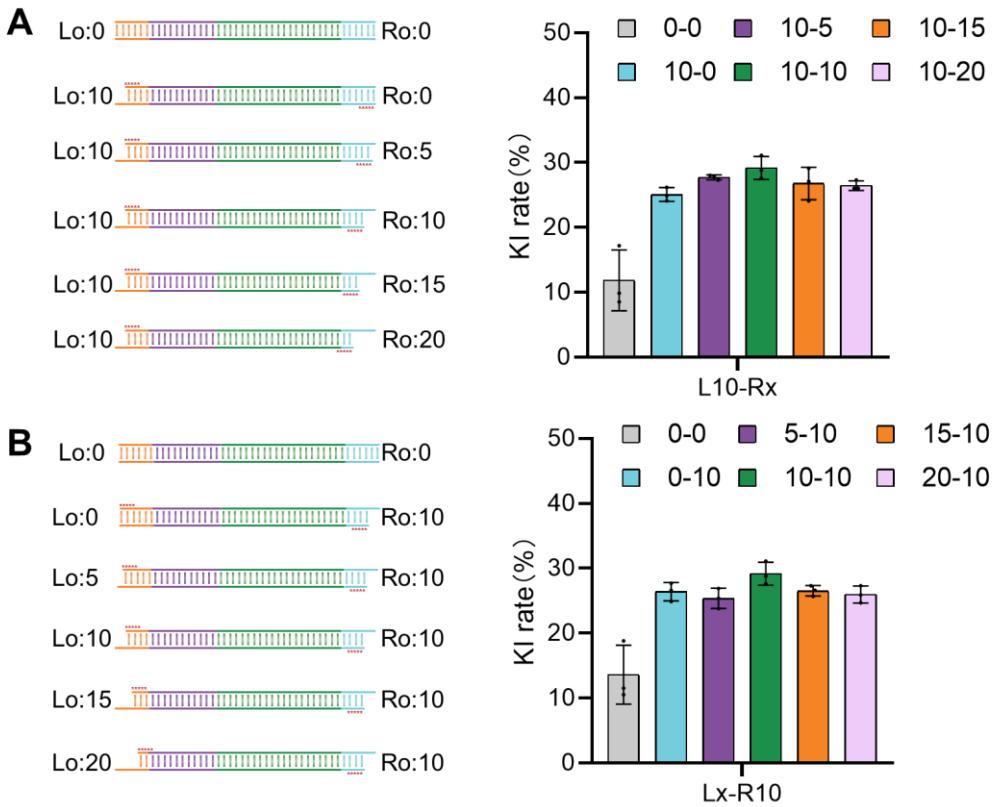
Fig. S7. Comparisons of the KI rates for odsDNA donors with asymmetric overhangs. (**A**) KI rates for odsDNA donors with 10-nt overhang at non-PAM end and the varying overhang lengths (5-, 10-, 15- and 20-nt) at PAM end. (**B**) KI rates for odsDNA donors with the 10-nt overhang at PAM end and the varying overhang lengths (5-, 10-, 15- and 20-nt) at non-PAM end. Lo, Left end of odsDNA; Ro, Right end of odsDNA.
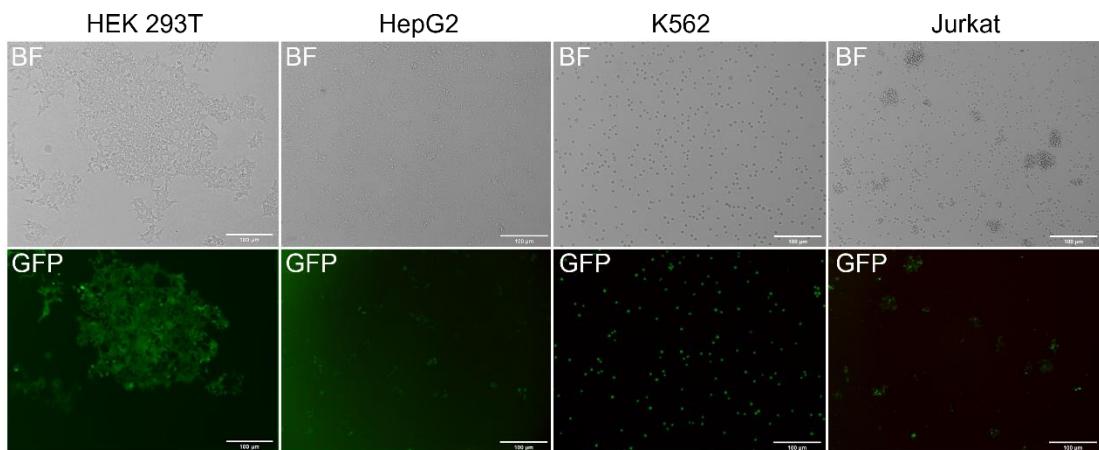
Fig. S8. Representative imaging of 1,110 bp donor KI at the *AAVS1* locus in HEK293T, HepG2, K562, and Jurkat cells by fluorescence microscopy. The EGFP displayed high expression intensities at the *AAVS1* locus. BF, bright field. Image quantifications were performed with ImageJ. Scale bar=100 μm.
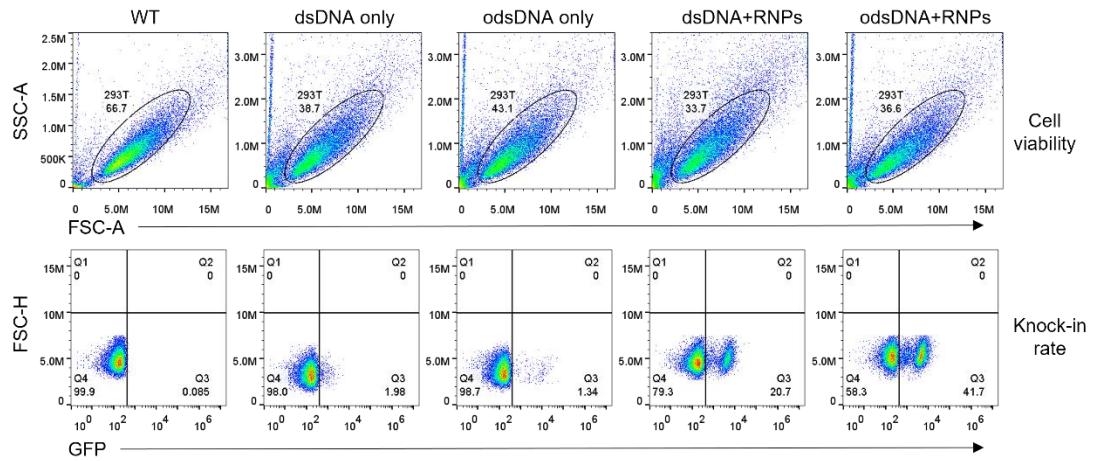
Fig. S9. Comparison of the cell viability and gene KI rates after nucleofection at *AAVS1* locus in HEK293T cells. Cell viability was determined by flow cytometry analysis three days after nucleofection, and the KI rate was examined on basis of the percentage of GFP-positive expression 15 days post-nucleofection. A representative flow cytometry plot was shown for each nucleofection group for donor-only or RNP transfections as indicated on the top of each panel. The RNP nucleofection containing the 10-nt 3′-overhang odsDNA demonstrated the highest KI rate (~42%), as compared to RNP/dsDNA complex (~19%).

A *Lamin A/C*, 1010bp insertion

$y=-0.01474*x^2+0.8998*x+13.25$
$KI_{max}, T_m=30.52$

KI rate (%)
OBS T_m (℃)

B *GAPDH*, 1010bp insertion

$y=-0.009444*x^2+0.6625*x+7.965$
$KI_{max}, T_m=35.07$

KI rate (%)
OBS T_m (℃)

C *AAVS1*, 1010bp insertion

$y=-0.01044*x^2+0.636*x+12.98$
$KI_{max}, T_m=30.46$

KI rate (%)
OBS T_m (℃)

D *HBB*, 1010bp insertion

$y=-0.01652*x^2+1.117*x+12.36$
$KI_{max}, T_m=33.81$
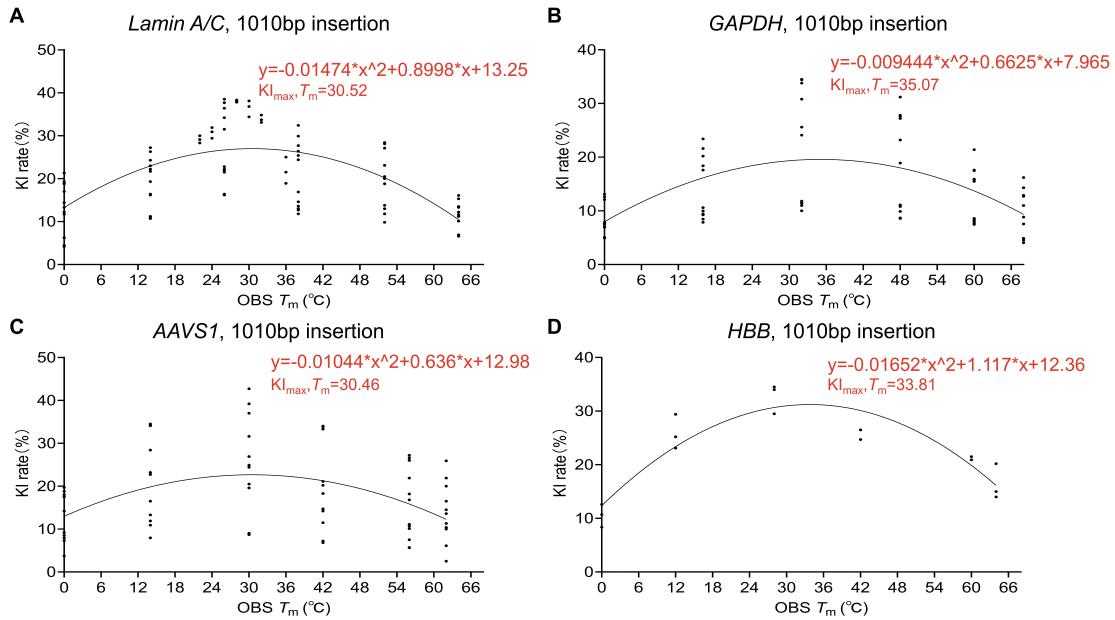
KI rate (%)
OBS T_m (℃)

Fig. S10. The nonlinear quadratic fitting curves showing the varied KI rates across different genomic loci with variable T_m of 3′-overhang of odsDNA. The T_m was calculated based on the perfect base-pairing between 3′-overhang of the DSB and OBS in the odsDNA donor. All the odsDNA donors with 1,010 bp in length harbored 50 bp HAs on both ends and were designed against four genomic loci, including *Lamin A/C* locus (**A**), *GAPDH* locus (**B**), *AAVS1* locus (**C**) and *HBB* locus (**D**).

A

Schematic diagram of genotyping PCR	Gene KI site	Donor template	Size of expected 5' junction PCR	Size of expected 3' junction PCR	Size of expected full length PCR	
					No insertion	Knock-in
	Lamin A/C	EF-1α core promoter-EGFP	419bp	474bp	553bp	1563bp
	GAPDH	EF-1α core promoter-EGFP	438bp	376bp	474bp	1484bp
	AAVS1	EF-1α core promoter-EGFP	428bp	498bp	486bp	1496bp
	HBB	EF-1α core promoter-EGFP	400bp	467bp	411bp	1421bp
	Lamin A/C	EF-1α promoter-EGFP- bGH poly(A) signal	478bp	477bp	553bp	3053bp

B

M: Marker(5000bp)
1: WT
2: dsDNA(no *PT)
3: dsDNA(5' *PT)
4: odsDNA(5nt)
5: odsDNA(10nt)
6: odsDNA(15nt)
7: odsDNA(20nt)
8: odsDNA(30nt)

Fig. S11. Genomic PCR productions analysis of cells after gene KI. (**A**) Summary of the primer design for the examination of the on-target indels by PCR amplification across a panel of four genomic loci with variable length of dsDNA and odsDNA donors in HEK293T, HepG2, K562 and Jurkat cells. For each genomic locus, three pairs of primers were designed, including a pair of full-length primers, as well as left and right junction primers as indicated by the arrows. (**B**) Agarose gel visualization of the full-length genomic PCR amplicons containing no or predicted insertion size as indicated by the arrows. The markers and the donors used were labelled on the top for each panel.
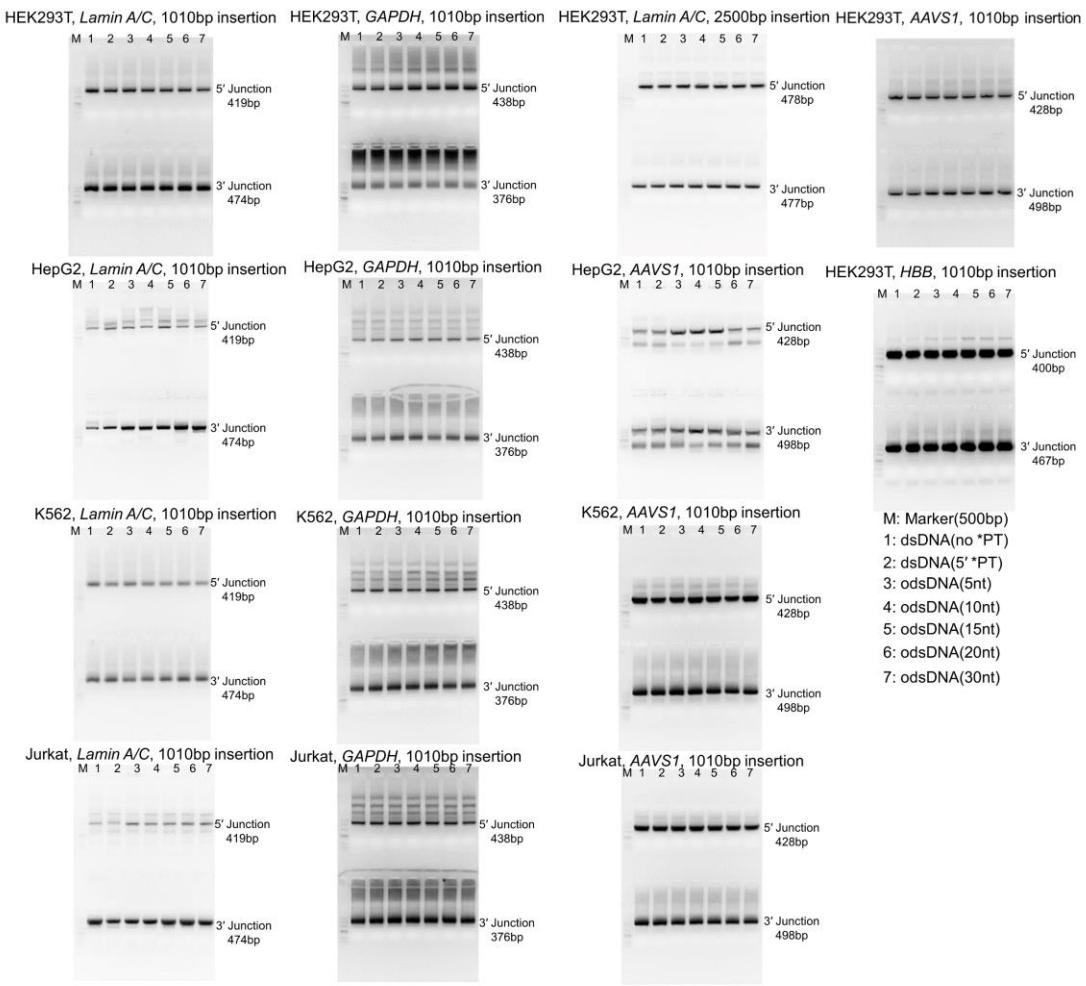
Fig. S12. Correct gene-sized target integration for junctions as visualized by agarose gel. The 1010-bp and 2500-bp donors (including dsDNA, 5′-end modified dsDNA, odsDNA with 5-nt, 10-nt, 15-nt, 20-nt and 30-nt overhangs, respectively) designed to target across four genomic loci (*Lamin A/C*, *GAPDH*, *AAVS1* and *HBB* loci) were co-delivered along with Cas9/sgRNA to the cell lines (HEK293T, HepG2, K562 and Jurkat cells). The junction PCR followed by 2% agarose gel electrophoresis was carried out to verify the correct target insertion.
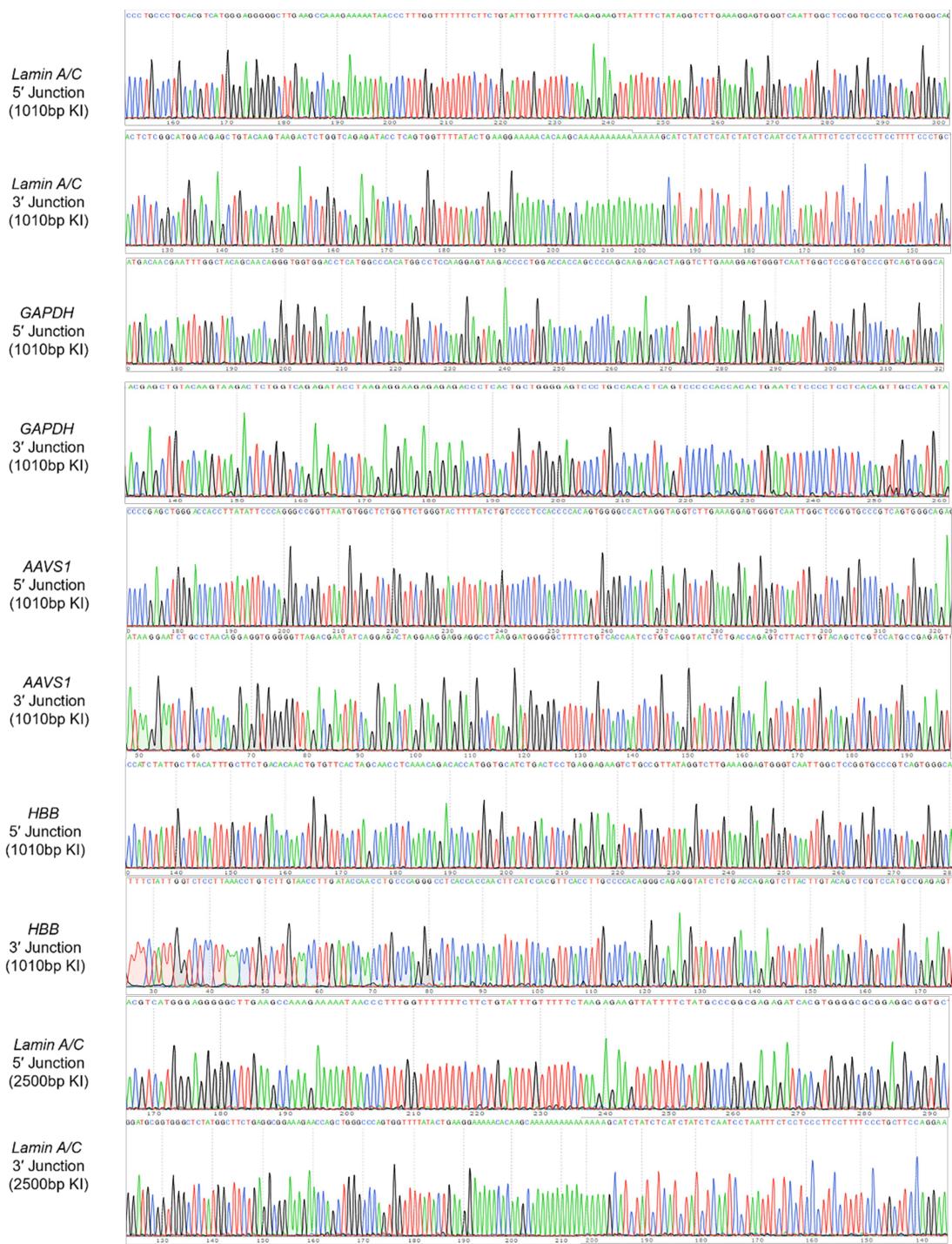
Fig. S13. Sanger sequencing validation of the Junction PCR products at each locus after targeted gene KI in HEK293T cells. Two gene-sized large-fragment (1010 and 2500 bp) donors were employed to conduct KI across the four genomic *Lamin A/C*, *GAPDH*, *AAVS1* and *HBB* loci as indicated.
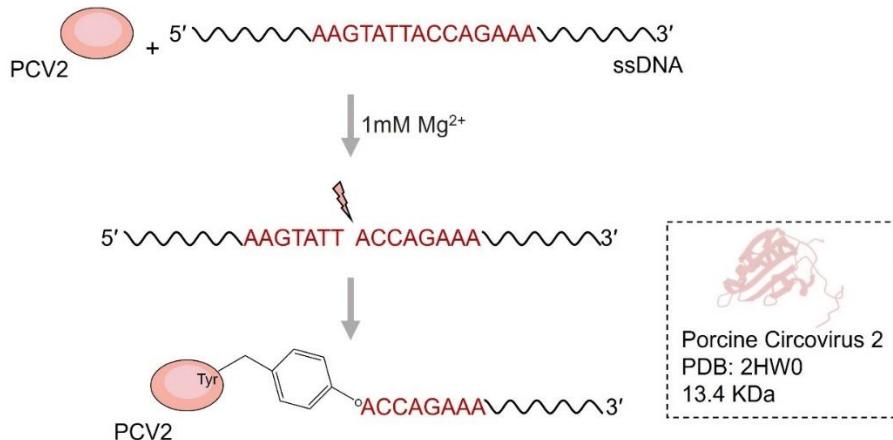
Fig. S14. Schematic diagram showing the reaction of Porcine Circovirus 2 (PCV2) with ssDNA. PCV2 recognizes and cleaves ssDNA containing motif sequence (AAGTATTACCAGAAA) in the presence of 1 mM Mg^{2+}, and covalently attaches to the ssDNA (2).
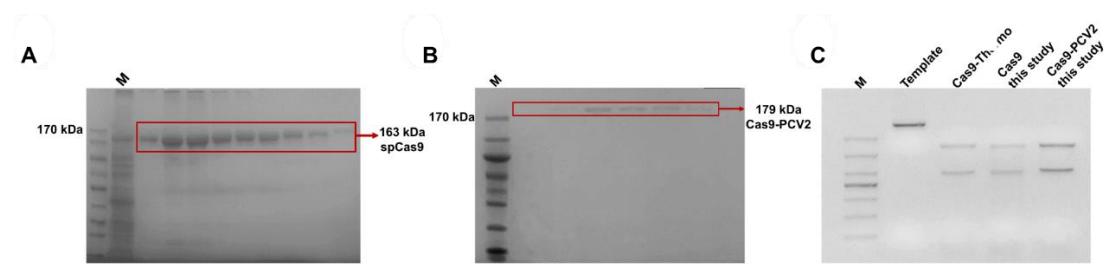
Fig. S15. Purification and activity detection *in vitro* of Cas9 and Cas9-PCV2 fusion proteins. (**A**) Verification of purified spCas9 protein by SDS-PAGE. (**B**) Verification of purified Cas9-PCV2 fusion protein by SDS-PAGE. (**C**) The DNA cleavage assay for the Cas9-PCV2 activity *in vitro* with a common DNA substrate.
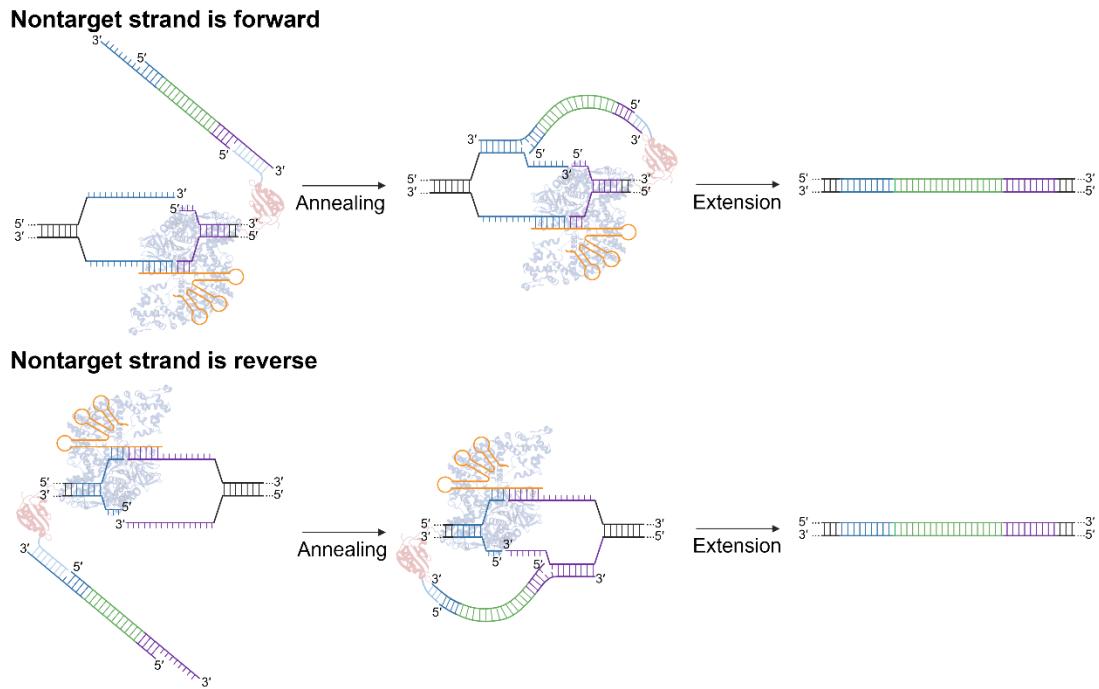
Fig. S16. Design of the Cas-PCV2/linker-directed tethering of odsDNA donors under two different circumstances. When the nontarget strand is at forward strand, Cas9-PCV2 attaches to the 3′-overhang (purple) on the right side of the odsDNA via PCV2-linker, and the nontarget strand without PAM (blue) anneals to the 3′-overhang (blue) on the left end of the odsDNA (upper panel). When the nontarget strand is at reverse strand, Cas9-PCV2 attaches to the 3′-overhang (blue) on the left side of the odsDNA via PCV2-linker, and the nontarget strand without PAM (purple) annealed to the 3′-overhang (purple) on the right end of the odsDNA (lower panel). The structure of Cas9 (no. 4CMP) and PCV2 (no. 2HW0) is adapted from PDB database.
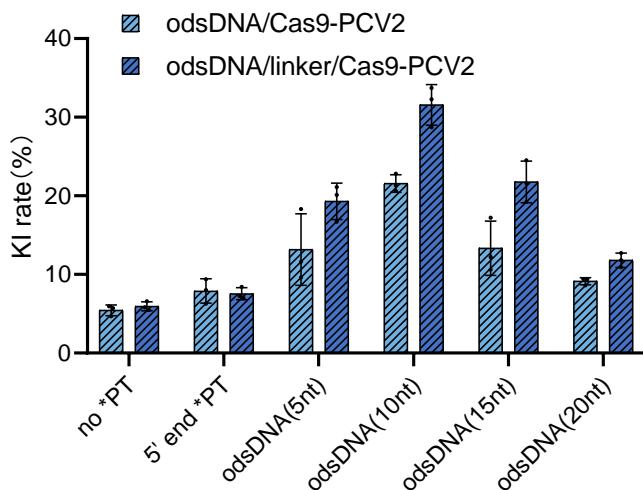
Fig. S17. Enhanced KI efficiency with Cas9-PCV2 fusion protein-tethered 2,600 bp odsDNA donor with 10-nt overhangs. Compared with non-tethered odsDNA donor, the Cas9-PCV2 tethered odsDNA donor, harboring 10-nt base-paring between 3′-overhang of odsDNA and PCV2 linker, exhibited the highest KI efficiency. Of note, the KI rate varies with the variable length of overhangs.
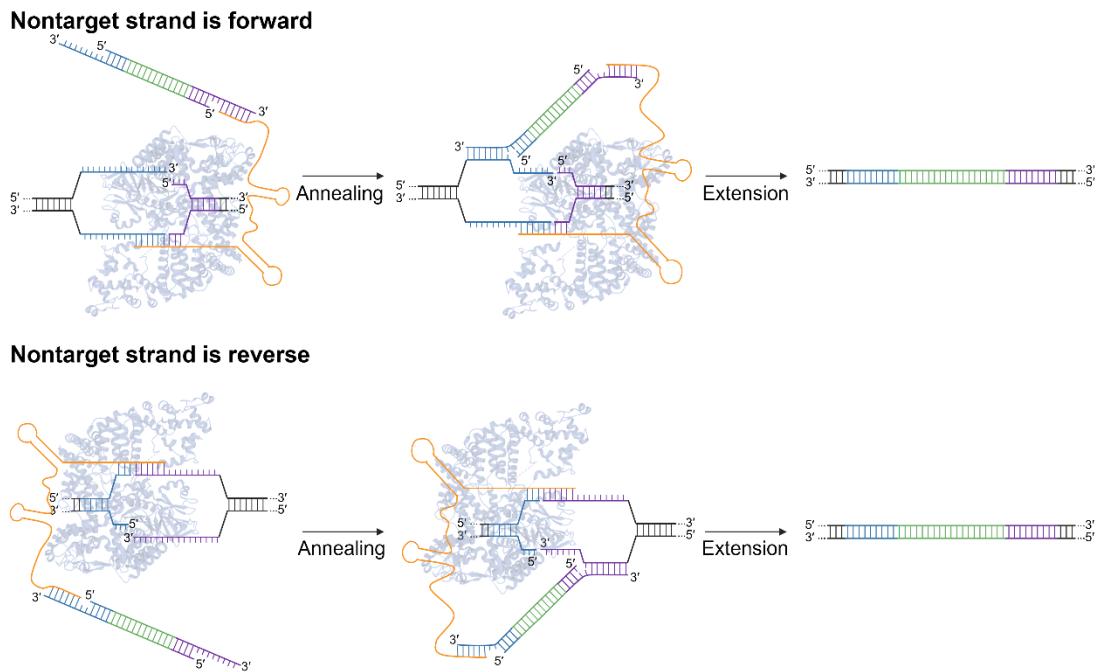
Fig. S18. Design of the 3′ esgRNA for tethering the odsDNA donor to the DSB sites for improved target KI in two different scenarios. When the nontarget strand is at forward strand, esgRNA annealed to the 3′-overhang (purple) on the right side of the odsDNA, and the nontarget strand without PAM (blue) annealed to the 3′-overhang (blue) on the left end of the odsDNA. When the nontarget strand is at reverse strand, esgRNA annealed to the 3′-overhang (blue) on the left side of the odsDNA, and the nontarget strand without PAM (purple) annealed to the 3′-overhang (purple) on the right end of the odsDNA. The structure of Cas9 (no. 4CMP) from PDB database.
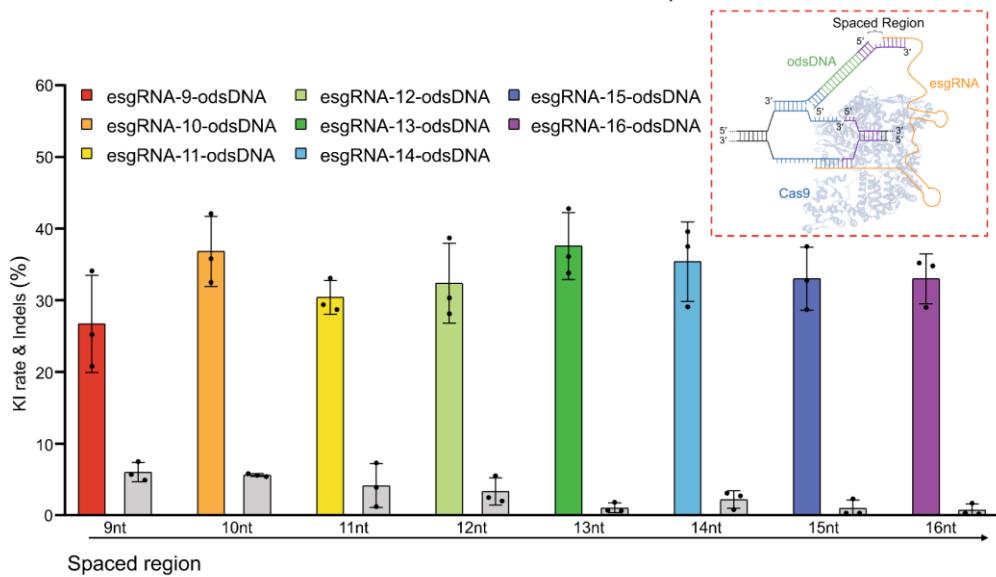
Fig. S19. Testing of KI efficiency and indels with esgRNA harboring variable lengths of OBS. For practical application, we designed a generic esgRNA with a fixed extended sequence consisting of OBS. The variable lengths of spaced region ranging from 23-nt to 16-nt in the 3′-overhang of odsDNA without base-pairing were selected for optimizing the precise KI insertion. The KI efficiencies were determined by the EGFP signal encoded by the 2,500 bp odsDNA donors. The indels frequencies were examined by TIDE pipeline analysis as described before. Gray bar are indels rates.
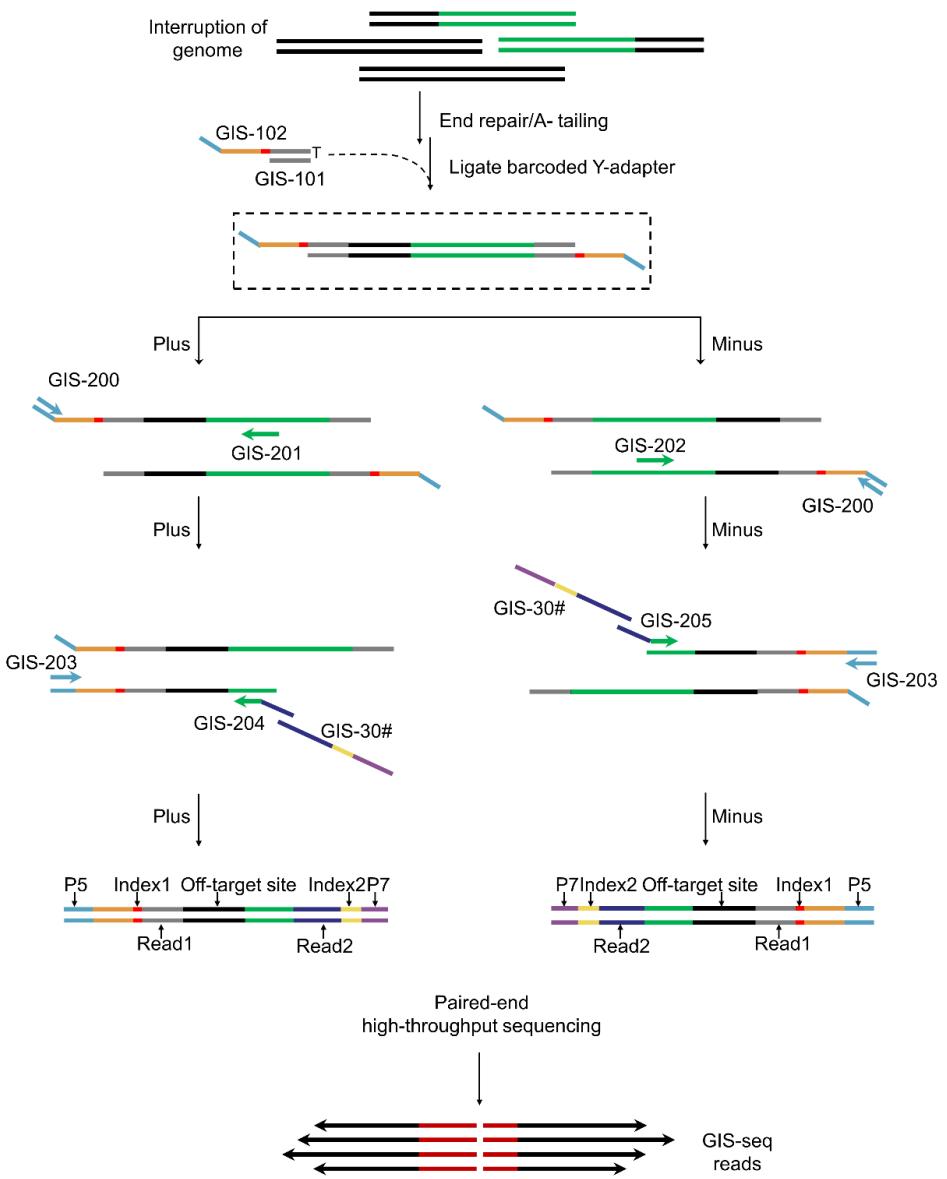
Fig. S20. A general summary of steps for preparing GIS-seq library for paired-end analysis as modified from a previous protocol (3). The gene-sized donors (dsDNA or odsDNA) were integrated into the host genomes by CRISPR RNP nucleofection. The genomic DNA was extracted and fragmented to an average of ~350 bp. After end-repair and A-tailing, the Y-shaped adapters were ligated to the genomic inserts. The target integration junctions were obtained by two rounds of nested PCR amplification, respectively, and the off-target sites were examined by NGS sequencing.

Table S1. The list of sequences for CRISPR/Cas9 target sites tested in this study. Four target sites were selected by CHOPCHOP software (https://chopchop.cbu.uib.no/). PAM sequences are underlined.

Target sites	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2	MM3	Efficiency
Lamin A/C locus	AGAGAAGTTATTT TCTACAG<u>TGG</u>	Chr1: 156139255	+	30	0	0	0	1	16	-
GAPDH locus	AGCCCCAGCAAG AGCACAAG<u>AGG</u>	Chr12: 6539186	+	60	1	0	6	9	26	49.17
PPP1R12C (*AAVS1*) locus	ACAGTGGGGCCA CTAGGGAC<u>AGG</u>	Chr19: 55115754	+	65	2	0	0	1	5	-
HBB locus	CTTGCCCCACAG GGCAGTAA<u>CGG</u>	Chr11: 5226968	-	60	2	0	0	0	15	48.52

Table S2. Sequences of the target insert in donor plasmids used as PCR templates in this study. <mark>Homology arm</mark> (HA) sequences are highlighted in yellow.

Lamin A/C locus donor sequence (EF-1α core promoter-EGFP 1110bp)
<mark>ctttggtttttttcttctgtatttgtttttctaagagaagttattttcta</mark>taggtcttgaaaggagtgggtcaattggctccggtgcccgtcagtgggcagag cgcacatcgcccacagtccccgagaagttggggggagggggtcggcaattgatccggtgcctagagaaggtggcgcggggtaaactgggaaa gtgatgtcgtgtactggctccgccttttccccgagggtgggggagaaccgtatataagtgcagtagtcgccgtgaacgttcttttttcgcaacgggttt gccgccagaacacaggaagcttgccaccatggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggc gacgtaaacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccgg caagctgcccgtgccctggcccacectcgtgaccaccctgacctacggcgtgcagtgcttcagccgctaccccgaccacatgaagcagcacg acttcttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcgccgaggtga agttcgagggcgacacectggtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagta caactacaacagccacaacgtctatatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacg gcagcgtgcagctcgccgaccactaccagcagaacacccccatcggcgacggcccccgtgctgctgcccgacaaccactacctgagcaccca gtccgccctgagcaaagaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctcggcatggacg agctgtacaagtaagactctggtcagagatacct<mark>cagtggttttatactgaaggaaaaacacaagcaaaaaaaaaaaaaaagca</mark>
GAPDH locus donor sequence (EF-1α core promoter-EGFP 1110bp)
<mark>atggcctccaaggagtaagacccctggaccaccagccccagcaagagcac</mark>taggtcttgaaaggagtgggtcaattggctccggtgcccgtca gtgggcagagcgcacatcgcccacagtccccgagaagttggggggagggggtcggcaattgatccggtgcctagagaaggtggcgcggggt aaactgggaaagtgatgtcgtgtactggctccgccttttccccgagggtgggggagaaccgtatataagtgcagtagtcgccgtgaacgttcttttt cgcaacgggtttgccgccagaacacaggaagcttgccaccatggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcg agctggacggcgacgtaaacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcat ctgcaccaccggcaagctgcccgtgccctggcccaccctcgtgaccaccctgacctacggcgtgcagtgcttcagccgctaccccgaccacat gaagcagcacgacttcttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttcttcaaggacgacggcaactacaagacccg cgccgaggtgaagttcgagggcgacacectggtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcac aagctggagtacaactacaacagccacaacgtctatatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaac atcgaggacggcagcgtgcagctcgccgaccactaccagcagaacaccccatcggcgacggcccccgtgctgctgcccgacaaccactacc tgagcacccagtccgccctgagcaaagaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctc ggcatggacgagctgtacaagtaagactctggtcagagatacct<mark>aagaggaagagagagaccctcactgctggggagtccctgccacactca</mark>

<mark>gt</mark>

AAVS1 locus donor sequence (EF-1α core promoter-EGFP 1110bp)
<mark>gttctgggtacttttatctgtcccctccaccccacagtggggccactagg</mark>taggtcttgaaaggagtgggtcaattggctccggtgcccgtcagtg ggcagagcgcacatcgcccacagtccccgagaagttggggggagggggtcggcaattgatccggtgcctagagaaggtggcgcgggtaaa ctgggaaagtgatgtcgtgtactggctccgcctttttcccgagggtggggggagaaccgtatataagtgcagtagtcgccgtgaacgttcttttttcgc aacgggtttgccgccagaacacaggaagcttgccaccatggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagc tggacggcgacgtaaacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctgc accaccggcaagctgcccgtgccctggcccaccctcgtgaccaccctgacctacggcgtgcagtgcttcagccgctaccccgaccacatgaa gcagcacgacttcttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcgc cgaggtgaagttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaag ctggagtacaactacaacagccacaacgtctatatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatc gaggacggcagcgtgcagctcgccgaccactaccagcagaacacccccatcggcgacggccccgtgctgctgcccgacaaccactacctga gcacccagtccgccctgagcaaagaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctcggc atggacgagctgtacaagtaagactctggtcagagatacct<mark>gacaggattggtgacagaaaagcccccatccttaggcctcctccttccta</mark>

HBB locus donor sequence (EF-1α core promoter-EGFP 1110bp)
<mark>tcaaacagacaccatggtgcatctgactcctgaggagaagtctgccgtta</mark>taggtcttgaaaggagtgggtcaattggctccggtgcccgtcagt gggcagagcgcacatcgcccacagtccccgagaagttggggggagggggtcggcaattgatccggtgcctagagaaggtggcgcgggtaa actgggaaagtgatgtcgtgtactggctccgccttttttcccgagggtggggggagaaccgtatataagtgcagtagtcgccgtgaacgttcttttttcg caacgggtttgccgccagaacacaggaagcttgccaccatggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgag ctggacggcgacgtaaacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctg caccaccggcaagctgcccgtgccctggcccaccctcgtgaccaccctgacctacggcgtgcagtgcttcagccgctaccccgaccacatgaa gcagcacgacttcttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcgc cgaggtgaagttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaag ctggagtacaactacaacagccacaacgtctatatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatc gaggacggcagcgtgcagctcgccgaccactaccagcagaacacccccatcggcgacggccccgtgctgctgcccgacaaccactacctga gcacccagtccgccctgagcaaagaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctcggc atggacgagctgtacaagtaagactctggtcagagatacct<mark>ctgccctgtggggcaaggtgaacgtggatgaagttggtggtgaggccctg</mark>

Lamin A/C locus donor sequence (EF-1α promoter-EGFP 2600bp)
<mark>ctttggtttttttcttctgtatttgtttttctaagagaagttattttcta</mark>tgcccggcgagagatcacgtggggcgcggaggcggtgctgctgggggcacg gccgtccagcctcggcggccatattttttgaggggctgttcatctcgttcacacgctctgtccgccatgtttgtgagtggaagcgccattacccctttca agcgactgaaggctgcagggcctctggtggcccgcatggggagaccagacccgccaggccccgcctttccgcactcagtccgggcttactttat tttgtgagacaggggtctcgcctagaggctccggtgcccgtcagtgggcagagcgcacatcgcccacagtccccgagaagttggggggaggg gtcggcaattgaaccggtgcctagagaaggtggcgcgggtaaactgggaaagtgatgtcgtgtactggctccgcctttttcccgagggtgggg gagaaccgtatataagtgcagtagtcgccgtgaacgttcttttttcgcaacgggtttgccgccagaacacaggtaagtgccgtgtgtggttcccgcg ggcctggcctctttacggggttatggcccttgcgtgccttgaattacttccacgcccctggctgcagtacgtgattcttgatcccgagcttcgggttgg aagtgggtgggagagttcgaggccttgcgcttaaggagcccctcgcctcgtgcttgagttgaggcttggcctgggcgctggggccgccgcgt gcgaatctggtggcaccttcgcgcctgtctcgctgctttcgataagtctctagccatttaaaattttttgatgacctgctgcgacgcttttttttctggcaag atagtcttgtaaatgcggggccaagatctgcacactggtatttcggttttttggggccgcgggcggcgacggggcccgtgcgtcccagcgcacatg ttcggcgaggcggggcctgcgagcgcggccaccgagaatcggacggggggtagtctcaagctggccggcctgctctggtgcctggcctcgcg ccgccgtgtatcgccccgccctgggcggcaaggctggcccggtcggcaccagttgcgtgagcggaaagatggccgcttcccggccctgctg cagggagctcaaaatggaggacgcggcgctcgggagagcgggcgggtgagtcacccacacaaaggaaaagggccttttccgtcctcagccg tcgcttcatgtgactccacggagtaccgggcgccgtccaggcacctcgattagttctcgagctttggagtacgtcgtctttaggttggggggagg ggttttatgcgatggagtttcccatactgagtgggtggagactgaagttaggccagcttggcacttgatgtaattctccttggaatttgcccttttttga gtttggatcttggttcattctcaagcctcagacagtggttcaaagtttttttcttccatttaaggtgtcgtgaaaactaccccaagctggcctctgaggc

caccatggctgtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaacggccacaagttca

gcgtgtccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagctgcccgtgccctggcc

caccctcgtgaccaccctgacctacggcgtgcagtgcttcagccgctaccccgaccacatgaagcagcacgacttcttcaagtccgccatgccc

gaaggctacgtccaggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcgccgaggtgaagttcgagggcgacaccctggt

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atatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgccgacca

ctaccagcagaacacccccatcggcgacggccccgtgctgctgcccgacaaccactacctgagcacccagtccgccctgagcaaagacccc

aacgagaagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctcggcatggacgagctgtacaagtaaaagcttggg

gatcaattctctagagctcgctgatcagcctcgactgtgccttctagttgccagccatctgttgtttgcccctcccccgtgccttccttgaccctggaa

ggtgccactcccactgtcctttcctaataaaatgaggaaattgcatcgcattgtctgagtaggtgtcattctattctgggggggtggggtggggcagg

acagcaaggggggggattgggaagacaatagcaggcatgctggggatgcggtgggctctatggcttctgaggcggaaagaaccagctgggc

c<mark>cagtggttttatactgaaggaaaaacacaagcaaaaaaaaaaaaaaagca</mark>

Table S3. A list of primer sequences (5′→3′) for preparing ssDNA, dsDNA and odsDNA donors through PCR amplification in this study. "*" represents phosphorothioate (PT) modification, "P" represents phosphorylation modification.

Lamin A/C locus	EF-1α core promoter-EGFP (1110bp) primers or EF-1α promoter-EGFP-ploy A signal (2600bp) primers
L50-0-F	ctttggtttttttcttctgtatttgttttctaagagaagttattttcta
L50-0-P-R	P-tgctttttttttttttttgcttgtgtttttccttcagtataaaac
L50-0-P-F	P-ctttggtttttttcttctgtatttgttttctaagagaagttattttcta
L50-0-R	tgctttttttttttttttgcttgtgtttttccttcagtataaaac
L-50-F	ctttggtttttttcttctgtatttgttttctaagagaagttattttctaTAGGTCTTGAAAGGAGTGGG
L-50-R	tgctttttttttttttttgcttgtgtttttccttcagtataaaaccactgAGGTATCTCTGACCAGAGTC
L-50S-F	ctttggtttttttcttctgtatttgttttctaagagaagttattttctaT*A*G*G*T*CTTGAAAGGAGTGGG
L-50S-R	tgctttttttttttttttgcttgtgtttttccttcagtataaaaccactgA*G*G*T*A*TCTCTGACCAGAGTC
L-40S-F	ctttggtttttttcttctgtatttgttttctaagagaagt*t*a*t*t*ttctaTAGGTCTTGA
L-40S-R	tgctttttttttttttttgcttgtgtttttccttcagtata*a*a*a*c*cactgAGGTATCTCT
L-30S-F	ctttggtttttttcttctgtatttgttttc*t*a*a*g*agaagttattttcta
L-30S-R	tgctttttttttttttttgcttgtgtttttc*c*t*t*c*agtataaaaccactg
L-20S-F	ctttggtttttttcttctgta*t*t*t*g*ttttctaagagaag
L-20S-R	tgctttttttttttttttgct*t*g*t*g*tttttccttcagtat
L-15S-F	ctttggtttttttctt*c*t*g*t*atttgttttctaag
L-15S-R	tgcttttttttttttt*t*t*g*c*ttgtgtttttccttc
L-14S-F	ctttggtttttttct*t*c*t*g*t

L-14S-R	tgcttttttttttttt*t*t*t*g*c
L-13S-F	ctttggtttttttc*t*t*c*t*gt
L-13S-R	tgctttttttttttt*t*t*t*t*gc
L-12S-F	ctttggttttttt*c*t*t*c*tgt
L-12S-R	tgctttttttttttt*t*t*t*t*tgc
L-11S-F	ctttggtttttt*t*c*t*t*ctgt
L-11S-R	tgcttttttttttt*t*t*t*t*ttgc
L-10S-F	ctttggttttt*t*t*c*t*tctgtatttgtttttt
L-10S-R	tgctttttttt*t*t*t*t*tttgcttgtgtttttt
L-9S-F	ctttggtttt*t*t*t*c*ttctgt
L-9S-R	tgctttttttt*t*t*t*t*ttttgc
L-8S-F	ctttggttt*t*t*t*t*cttctgt
L-8S-R	tgcttttttt*t*t*t*t*tttttgc
L-5S-F	ctttgg*t*t*t*t*tttcttctgtatttg
L-5S-R	tgcttt*t*t*t*t*tttttttttgcttgtg
L40-0-F	tttcttctgtatttgtttttctaagagaagttattttcta
L40-0-R	ttttttttgcttgtgtttttccttcagtataaaaccactg
L40-5-F	tttctt*c*t*g*t*atttgtttttctaagagaagttattttcta
L40-5-R	tttttt*t*t*g*c*ttgtgtttttccttcagtataaaaccactg
L40-10-F	tttcttctgta*t*t*t*g*tttttctaagagaagttattttcta
L40-10-R	tttttttttgct*t*g*t*g*tttttccttcagtataaaaccactg
L40-15-F	tttcttctgtatttgt*t*t*t*t*ctaagagaagttattttcta
L40-15-R	ttttttttgcttgtgt*t*t*t*t*ccttcagtataaaaccactg
L40-20-F	tttcttctgtatttgtttttc*t*a*a*g*agaagttattttcta
L40-20-R	ttttttttgcttgtgtttttc*c*t*t*c*agtataaaaccactg
L20-0-F	ctaagagaagttattttctaTAGGTC
L20-0-R	ccttcagtataaaaccactgAGGTAT
L20-5-F	ctaaga*g*a*a*g*ttattttctaTAGGTC
L20-5-R	ccttca*g*t*a*t*aaaaccactgAGGTAT
L20-10-F	ctaagagaagt*t*a*t*t*ttctaTAGGTC
L20-10-R	ccttcagtata*a*a*a*c*cactgAGGTAT
L20-15-F	ctaagagaagttattt*tctaTAGGTC
L20-15-R	ccttcagtataaaacc*a*c*t*g*AGGTAT
L20-20-F	ctaagagaagttattttctaT*A*G*G*T*C
L20-20-R	ccttcagtataaaaccactgA*G*G*T*A*T
L50-10-F	ctttggttttt*t*t*c*t*tctgtatttgtttttctaagagaagttattttcta
L50-0-R	t*g*c*t*t*tttttttttttttgcttgtgtttttccttcagtataaaac
L50-10-F	ctttggttttt*t*t*c*t*tctgtatttgtttttctaagagaagttattttcta
L50-5-R	tgcttt*t*t*t*t*t*ttttttttgcttgtgtttttccttcagtataaaac
L50-10-F	ctttggttttt*t*t*c*t*tctgtatttgtttttctaagagaagttattttcta
L50-10-R	tgctttttttt*t*t*t*t*tttgcttgtgtttttccttcagtataaaac

L50-10-F	ctttggttttt*t*t*c*t*tctgtatttgttttttctaagagaagttattttcta
L50-15-R	tgctttttttttttt*t*t*g*c*ttgtgttttttccttcagtataaaac
L50-10-F	ctttggtttt*t*t*c*t*tctgtatttgttttttctaagagaagttattttcta
L50-20-R	tgctttttttttttttttgct*t*g*t*g*ttttttccttcagtataaaac
L50-0-F	c*t*t*t*g*gttttttttcttctgtatttgttttttctaagagaagttattttcta
L50-10-R	tgctttttttt*t*t*t*t*tttgcttgtgttttttccttcagtataaaac
L50-5-F	ctttgg*t*t*t*t*t*tttcttctgtatttgttttttctaagagaagttattttcta
L50-10-R	tgctttttttt*t*t*t*t*tttgcttgtgttttttccttcagtataaaac
L50-15-F	ctttggtttttttctt*c*t*g*t*atttgttttttctaagagaagttattttcta
L50-10-R	tgctttttttt*t*t*t*t*tttgcttgtgttttttccttcagtataaaac
L50-20-F	ctttggtttttttcttctgta*t*t*t*g*ttttttctaagagaagttattttcta
L50-10-R	tgctttttttt*t*t*t*t*tttgcttgtgttttttccttcagtataaaac
GAPDH locus	EF-1α core promoter-EGFP primers
G50-0-F	atggcctccaaggagtaagac
G50-0-P-R	P-actgagtgtggcagggac
G50-0-P-F	P-atggcctccaaggagtaagac
G50-0-R	actgagtgtggcagggac
G-50-F	atggcctccaaggagtaagacccctggaccaccagccccagcaagagcacTAGGTCTTGAAAGGAGTGGG
G-50-R	actgagtgtggcagggactccccagcagtgagggtctctctcttcctcttAGGTATCTCTGACCAGAGTC
G-50S-F	atggcctccaaggagtaagacccctggaccaccagccccagcaagagcacT*A*G*G*T*CTTGAAAGGAGTGGG
G-50S-R	actgagtgtggcagggactccccagcagtgagggtctctctcttcctcttA*G*G*T*A*TCTCTGACCAGAGTC
G-40S-F	atggcctccaaggagtaagacccctggaccaccagccccag*c*a*a*g*agcacTAGGTCTTGA
G-40S-R	actgagtgtggcagggactccccagcagtgagggtctctct*c*t*t*c*ctcttAGGTATCTCT
G-30S-F	atggcctccaaggagtaagacccctggacca*c*c*a*g*ccccagcaagagcac
G-30S-R	actgagtgtggcagggactccccagcagtga*g*g*g*t*ctctctcttcctctt
G-20S-F	atggcctccaaggagtaagac*c*c*c*t*ggaccaccagcccca
G-20S-R	actgagtgtggcagggactcc*c*c*a*g*cagtgagggtctctc
G-15S-F	atggcctccaaggagt*a*a*g*a*cccctggaccaccag
G-15S-R	actgagtgtggcaggg*a*c*t*c*cccagcagtgagggt
G-10S-F	atggcctccaa*g*g*a*g*taagacccctggacc
G-10S-R	actgagtgtgg*c*a*g*g*gactccccagcagtg
G-5S-F	atggcc*t*c*c*a*aggagtaagacccct
G-5S-R	actgag*t*g*t*g*gcagggactccccag
AAVS1 locus	EF-1α core promoter-EGFP primers
A50-0-F	gttctgggtactttatctgtcccc
A50-0-P-R	P-taggaaggaggaggcctaagg

A50-0-P-F	P-gttctgggtactttatctgtcccc
A50-0-R	taggaaggaggaggcctaagg
A-50-F	gttctgggtactttatctgtcccctccaccccacagtggggccactaggTAGGTCT TGAAAGGAGTGGG
A-50-R	taggaaggaggaggcctaaggatggggggcttttctgtcaccaatcctgtcAGGTAT CTCTGACCAGAGTC
A-30S-F	gttctgggtactttatctgtcccctccacc*c*c*a*c*a
A-30S-R	taggaaggaggaggcctaaggatggggggctt*t*t*c*t*g
A-20S-F	gttctgggtactttatctgt*c*c*c*c*t
A-20S-R	taggaaggaggaggcctaagg*a*t*g*g*g
A-15S-F	gttctgggtacttta*t*c*t*g*t
A-15S-R	taggaaggaggaggcc*t*a*a*g*g
A-10S-F	gttctgggtac*t*t*t*t*atctg
A-10S-R	taggaaggagg*a*g*g*c*ctaag
A-5S-F	gttctg*g*g*t*a*cttttatctg
A-5S-R	taggaa*g*g*a*g*gaggcctaag
HBB locus	EF-1α core promoter-EGFP primers
H50-0-F	tcaaacagacaccatggtgcatc
H50-0-P-R	P-cagggcctcaccaccaactt
H50-0-P-F	P-tcaaacagacaccatggtgcatc
H50-0-R	cagggcctcaccaccaactt
H-50-F	tcaaacagacaccatggtgcatc
H-50-R	cagggcctcaccaccaactt
H-30S-F	tcaaacagacaccatggtgcatctgactcct*g*a*g*g*a
H-30S-R	cagggcctcaccaccaacttcatccacgttc*a*c*c*t*t
H-20S-F	tcaaacagacaccatggtgca*t*c*t*g*act
H-20S-R	cagggcctcaccaccaacttc*a*t*c*c*a
H-15S-F	tcaaacagacaccatg*g*t*g*c*atc
H-15S-R	cagggcctcaccacca*a*c*t*t*c
H-10S-F	tcaaacagaca*c*c*a*t*ggtgcatc
H-10S-R	cagggcctcac*c*a*c*c*aactt
H-5S-F	tcaaac*a*g*a*c*accatggtgcatc
H-5S-R	cagggc*c*t*c*a*ccaccaactt
Lamin A/C loucs	EF-1α promoter-EGFP-ploy A signal (2600bp) primers for esgRNA
L-12S-F	ctttggttttttt*c*t*t*c*tgtatttgtttttctaagag
L-9S-R	attgagatagatgagatagatgctttttttt*t*t*t*t*tttt
L-10S-R	attgagatagatgagatagatgcttttttttt*t*t*t*t*ttt
L-11S-R	attgagatagatgagatagatgctttttttttt*t*t*t*t*tt
L-12S-R	attgagatagatgagatagatgcttttttttttt*t*t*t*t*t
L-13S-R	attgagatagatgagatagatgctttttttttttt*t*t*t*t*g
L-14S-R	attgagatagatgagatagatgcttttttttttttt*t*t*t*g*c

L-15S-R	attgagatagatgagatagatgctttttttttttttt*t*t*g*c*t
L-16S-R	attgagatagatgagatagatgcttttttttttttttt*t*g*c*t*t

Table S4. A list of primer sequence (5′→3′) for sgRNA or esgRNA synthesis in this study.

Lamin A/C	sgRNA primers
L-gRNA-F	TAATACGACTCACTATAGagagaagttattttctacag
L-gRNA-R	TTCTAGCTCTAAAACCTGctgtagaaaataacttctct
GAPDH	sgRNA primers
G-gRNA-F	TAATACGACTCACTATAGagccccagcaagagcacaag
G-gRNA-R	TTCTAGCTCTAAAACCTGcttgtgctcttgctgggggct
AAVS1	sgRNA primers
A-gRNA-F	TAATACGACTCACTATAGacagtggggccactagggac
A-gRNA-R	TTCTAGCTCTAAAACCTGgtccctagtggccccactgt
HBB	sgRNA primers
H-gRNA-F	TAATACGACTCACTATAGcttgcccacagggcagtaa
H-gRNA-R	TTCTAGCTCTAAAACCTGttactgccctgtggggcaag
Lamin A/C	esgRNA primers
L-esgRNA-F	TAATACGACTCACTATAGAGAG
L-esgRNA-R	TCTATCTCATCTATCTCAATCC

Table S5. The template sequence of esgRNA (5′→3′) was used in this study (4) (Different colors to distinguish T7 promoter, target sequence, stem-loop region of sgRNA, annealing sequence)

Template sequence of esgRNA
TAATACGACTCACTATAGAGAGAAGTTATTTTCTACAGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAGAAATTAGGATTGAGATAGATGAGATAGA

Table S6. A list of primer sequence (5′→3′) for genomic DNA PCR (full length and junctions) in this study. All primers were designed in the Primer-BLAST section of NCBI website (Primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi).

Lamin A/C	Primers used in genotyping PCR
Lamin A/C 5′ FP	tgctacctcccttctaggggc
Lamin A/C 3′ FP	cgaccactaccagcagaacac
Lamin A/C 5′ RP	cagtttaccccgcgccac
Lamin A/C 3′ RP	gctggcggagaagcctctat
Lamin A/C 2500KI	Primers used in genotyping PCR
Lamin A/C 5′ FP	tgctacctcccttctaggggc
Lamin A/C 3′ FP	tccttgaccctggaaggtgcca
Lamin A/C 5′ RP	gtctggtctccccatgcggg
Lamin A/C 3′ RP	gctggcggagaagcctctat
GAPDH	Primers used in genotyping PCR
GAPDH 5′ FP	ctcctctgacttcaacagcgac
GAPDH 3′ FP	cgaccactaccagcagaacac
GAPDH 5′ RP	cagtttaccccgcgccac
GAPDH 3′ RP	agtaactggttgagcacagggt
AAVS1	Primers used in genotyping PCR
AAVS1 5′ FP	ttctcctgtggattcgggtc
AAVS1 3′ FP	ctggagtacaactacaacagcc
AAVS1 5′ RP	accttctctaggcaccggat
AAVS1 3′ RP	ctctctggctccatcgtaag
HBB	Primers used in genotyping PCR
HBB 5′ FP	tttgaagtccaactcctaagcca
HBB 3′ FP	ctggagtacaactacaacagcc
HBB 5′ RP	cagtttaccccgcgccac
HBB 3′ RP	gtcagtgcctatcagaaacccaa

Table S7. A list of sequences (5′→3′) for testing of Cas9-PCV2 ssDNA linkers. Upper-case letters are target sequences recognized by PCV2 and lower-case letters are sequences annealed to the 3′-overhang of odsDNA.

Lamin A/C	Cas9-PCV2 ssDNA linker sequences
PCV2-5	AAGTATTACCAGAAAtgctt
PCV2-10	AAGTATTACCAGAAAtgcttttttt
PCV2-15	AAGTATTACCAGAAAtgctttttttttttt
PCV2-20	AAGTATTACCAGAAAtgctttttttttttttttgc
PCV2-30	AAGTATTACCAGAAAtgctttttttttttttttgcttgtgttttt
PCV2-40	AAGTATTACCAGAAAtgctttttttttttttttgcttgtgttttccttcagtat

PCV2-50	AAGTATTACCAGAAAtgcttttttttttttttttgcttgtgtttttccttcagtataaaaccactg

Table S8. The sequences (5′→3′) of fluorescent ssDNA strands for labeling.

Name	sequences
PCV2-linker-QF	5`BHQ1-TAAGTATTACCAGAAA/i6FAMdT/cctcttgtcccacagatatccagaaccctgaccctgccgtgtaccagct
esgRNA-RC	5`Cy5-AAAAAAAGCATCTATCTCATCTATCTCAAT

Table S9. The plasmid sequence for Cas9-PCV2 fusion protein expression and purification. Cas9-PCV2 protein-coding sequences are underlined.

pET28b-3×NLS-Cas9-NLS-PCV2-6×His
agcgcctgatgcggtattttctccttacgcatctgtgcggtatttcacaccgcaatggtgcactctcagtacaatctgctctgatgccgcatagttaagccagtatacactccgctatcgctacgtgactgggtcatggctgcgccccgacacccgccaacacccgctgacgcgccctgacgggcttgtctgctcccggcatccgcttacagacaagctgtgaccgtctccgggagctgcatgtgtcagaggttttcaccgtcatcaccgaaacgcgcgaggcagctgcggtaaagctcatcagcgtggtcgtgaagcgattcacagatgtctgcctgttcatccgcgtccagctcgttgagtttctccagaagcgttaatgtctggcttctgataaagcgggccatgttaagggcggttttttcctgtttggtcactgatgcctccgtgtaaggggatttctgttcatgggggtaatgataccgatgaaacgagagaggatgctcacgatacgggttactgatgatgaacatgcccggttactggaacgttgtgagggtaaacaactggcggtatggatgcggcgggaccagagaaaaatcactcagggtcaatgccagcgcttcgttaatacagatgtaggtgttccacagggtagccagcagcatcctgcgatgcagatccggaacataatggtgcagggcgctgacttccgcgtttccagactttacgaaacacggaaaccgaagaccattcatgttgttgctcaggtcgcagacgttttgcagcagcagtcgcttcacgttcgctcgcgtatcggtgattcattctgctaaccagtaaggcaaccccgccagcctagccgggtcctcaacgacaggagcacgatcatgcgcacccgtggggccgccatgccggcgataatggcctgcttctcgccgaaacgtttggtggcgggaccagtgacgaaggcttgagcgagggcgtgcaagattccgaataccgcaagcgacaggccgatcatcgtcgcgctccagcgaaagcggtcctcgccgaaaatgacccagagcgctgccggcacctgtcctacgagttgcatgataaagaagacagtcataagtgcggcgacgatagtcatgccccgcgcccaccggaaggagctgactgggttgaaggctctcaagggcatcggtcgagatcccggtgcctaatgagtgagctaacttacattaattgcgttgcgctcactgcccgctttccagtcgggaaacctgtcgtgccagctgcattaatgaatcggccaacgcgcggggagaggcggtttgcgtattgggcgccagggtggtttttcttttcaccagtgagacgggcaacagctgattgcccttcaccgcctggccctgagagagttgcagcaagcggtccacgctggtttgccccagcaggcgaaaatcctgtttgatggtggttaacggcgggatataacatgagctgtcttcggtatcgtcgtatcccactaccgagatatccgcaccaacgcgcagcccggactcggtaatggcgcgcattgcgcccagcgccatctgatcgttggcaaccagcatcgcagtgggaacgatgccctcattcagcatttgcatggtttgttgaaaaccggacatggcactccagtcgccttcccgttccgctatcggctgaatttgattgcgagtgagatatttatgccagccagccagacgcagacgcgccgagacagaacttaatgggcccgctaacagcgcgatttgctggtgacccaatgcgaccagatgctccacgcccagtcgcgtaccgtcttcatgggagaaaataatactgttgatgggtgtctggtcagagacatcaagaaataacgccggaacattagtgcaggcagcttccacagcaatggcatcctggtcatccagcggatagtaatgatcagcccactgacgcgttgcgcgagaagattgtgcaccgccgcttacaggcttcgacgccgcttcgttctaccatcgacaccaccacgctggcacccagttgatcggcgcgagatttaatcgccgcgacaatttgcgacggcgcgtgcagggccagactggaggtggcaacgccaatcagcaacgactgtttgcccgccagttgttgtgccacgcggttgggaatgtaattcagctccgccatcgccgcttccactttttcccgcgttttcgcagaaacgtggctggcctggttcaccacgcgggaaacggtctgataagagacaccggcatactctgcgacatcgtataacgttactggtttcacattcaccaccctgaattgactctcttccgggcgctatcatgccataccgcgaaaggttttgcgccattcgatggtgtccgggatctcgacgctctcccttatgcgactcctgcattaggaagcagcccagtagtaggttgaggccgttgagcaccgccgccgcaaggaatggtgcatgcaaggagatggcgcccaacagtcccccggccacggggcctgccaccatacccacgccgaaacaagcgctcatgagcccgaagtggcgagcccgatcttccccatcggtgatgtcggcgatataggcgccagcaaccgcacctgtggcgccggtgatgccggccacgatgcgtccggcgtagaggatcgagatctcgatcccgcgaaattaatacgactcactatagggggaattgtgagcggataacaattcccctctagaaataattttgtttaactttaagaaggagatata<u>atgccaaagaaaaaaagaaaggtttctcgtgcggatcctaaaaagaaacggaaagtgtctcgcgcagatccgaaaaaaaaacgcaaggttgcggccgcactcgagatggacaagaagtactccattgggctcgatatcggcacaaacagcgtcggctgggccgtcattacggacgagtacaaggtgccgagcaaaaaattcaaagttctgggcaataccgatcgccacagcataaagaagaacctcattggcgcccctcgttcgactccggggagacggccgaagccacgcggctcaaaagaacagcacggcgcagatatacccgcagaagaatcggatctgctacctgcaggaga</u>

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tggataaccgtattaccgcctttgagtgagctgataccgctcgccgcagccgaacgaccgagcgcagcgagtcagtgagcgaggaagcggaag

Table S10. The odsDNA overhang sequences (5′→3′) tested for various loci and the corresponding T_m values.

Locus	Overhang length (nt)	DNA sequence	T_m (°C)
Lamin A/C	5	ctttg	14
	8	ctttggtt	22
	9	ctttggttt	24
	10	ctttggtttt	26
	11	ctttggttttt	28
	12	ctttggtttttt	30
	13	ctttggttttttt	32
	14	ctttggtttttttc	36
	15	ctttggtttttttct	38
	16	ctttggtttttttctt	40
	17	ctttggtttttttcttc	44
	18	ctttggtttttttcttct	46
	19	ctttggtttttttcttctg	50
	20	ctttggtttttttcttctgt	52
	30	ctttggtttttttcttctgtatttgttttt	64
GAPDH	5	atggc	16
	10	atggcctcca	32
	15	atggcctccaaggag	48

	20	atggcctccaaggagtaaga	60
	30	atggcctccaaggagtaagacccctggacc	68
AAVS1	5	gttct	14
	10	gttctgggta	30
	15	gttctgggtactttt	42
	20	gttctgggtacttttatctg	56
	30	gttctgggtacttttatctgtcccctccac	62
HBB	5	tcaaa	12
	10	tcaaacagac	28
	15	tcaaacagacaccat	42
	20	tcaaacagacaccatggtgc	60
	30	tcaaacagacaccatggtgcatctgactcc	64

Table S11. The synthesized ssDNA sequences (5′→3′) used for strand-annealing to form short dsDNA and odsDNA donors (* indicates phosphorothioate modification and the insertion sequences are highlighted in red).

Lamin A/C dsDNA HDR indel-F	CTTTGGTTTTTTTCTTCTGTATTTTTTTTTCTAAGAGA AGTTATTTTCTAgaattcCAGTGGTTTTATACTGAAGGAA AAACACAAGCAAAAAAAAAAAAAAGCAT
Lamin A/C dsDNA HDR indel-R	ATGCTTTTTTTTTTTTTGCTTGTGTTTTTCCTTCAGT ATAAAACCACTGgaattcTAGAAAATAACTTCTCTTAGA AAAAAAAATACAGAAGAAAAAAACCAAAG
Lamin A/C odsDNA HDR indel-F	T*T*T*C*T*TCTGTATTTTTTTTTCTAAGAGAAGTTAT TTTCTAgaattcCAGTGGTTTTATACTGAAGGAAAAACA CAAGCAAAAAAAAAAAAAAGCAT
Lamin A/C odsDNA HDR indel-R	T*T*T*T*T*TTTGCTTGTGTTTTTCCTTCAGTATAAAA CCACTGgaattcTAGAAAATAACTTCTCTTAGAAAAAAA AATACAGAAGAAAAAAACCAAAG
GAPDH dsDNA HDR indel-F	ATGGCCTCCAAGGAGTAAGACCCCTGGACCACCAGC CCCAGCAAGAGCACgaattcAAGAGGAAGAGAGAGAC CCTCACTGCTGGGGAGTCCCTGCCACACTCAGT
GAPDH dsDNA HDR indel-R	ACTGAGTGTGGCAGGGACTCCCCAGCAGTGAGGGT CTCTCTCTTCCTCTTgaattcGTGCTCTTGCTGGGGCTG GTGGTCCAGGGGTCTTACTCCTTGGAGGCCAT
GAPDH odsDNA HDR indel-F	A*G*G*A*G*TAAGACCCCTGGACCACCAGCCCCAGC AAGAGCACgaattcAAGAGGAAGAGAGAGACCCTCAC TGCTGGGGAGTCCCTGCCACACTCAGT
GAPDH odsDNA HDR indel-R	G*C*A*G*G*GACTCCCCAGCAGTGAGGGTCTCTCTC TTCCTCTTgaattcGTGCTCTTGCTGGGGCTGGTGGTCC AGGGGTCTTACTCCTTGGAGGCCAT

Table S12. Primer sequences (5′→3′) for indels detection by amplicon-seq analysis. (Red is the index sequence.)

Lamin A/C Indel F	CCCTACACGACGCTCTTCCGATCTGAAGCCAAAGAAAAATAACCCTT
Lamin A/C Indel R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCGGTTTTAAGGCAGATGTGGA
GAPDH indel F	CCCTACACGACGCTCTTCCGATCTCCCTGACAACTCTTTTCATCTTC
GAPDH indel R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCAAGGGGTCTACATGGCAA
I5comm	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC
SIP01	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACG
SIP02	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACG
SIP03	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACG
SIP04	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACG
SIP05	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACG
SIP06	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACG
SIP07	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACG
SIP08	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACG
SIP09	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACG
SIP10	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACG

Table S13. Primer sequences (5′→3′) for off-target detection by GIS-seq. (Red is the index sequence.)

GIS-101	5Phos-GATCGGAAGAGC*C*A
GIS-102-L	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGACACTCTTTCCCTACACGACGCTCTTCCGATC*T
GIS-102-G	AATGATACGGCGACCACCGAGATCTACACTAGATCGCACACTCTTTCCCTACACGACGCTCTTCCGATC*T

GIS-200	AATGATACGGCGACCACCGAGATCTAC
GIS-201-L	CTCTAGGCACCGGATCAATTGCCGAC
GIS-202-L	CCCCAACGAGAAGCGCGATCACA
GIS-203(GIS-200)	AATGATACGGCGACCACCGAGATCTAC
GIS-204-L	CCTCTCTATGGGCAGTCGGTGACCAACTTCTCGGGGACTGT
GIS-205-L	CCTCTCTATGGGCAGTCGGTGAGTCCTGCTGGAGTTCGTGA
GIS-201-G	GCACCGGATCAATTGCCGACCCCT
GIS-202-G	GTCCGCCCTGAGCAAAGACCCCAA
GIS-204-G	CCTCTCTATGGGCAGTCGGTGAAACTTCTCGGGGACTGTG
GIS-205-G	CCTCTCTATGGGCAGTCGGTGAATCACATGGTCCTGCTGG
GIS-301	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTGACTGGAGTCCTCTCTATGGGCAGTCGGTGA
GIS-302	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTGACTGGAGTCCTCTCTATGGGCAGTCGGTGA
GIS-303	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTGACTGGAGTCCTCTCTATGGGCAGTCGGTGA
GIS-304	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTGACTGGAGTCCTCTCTATGGGCAGTCGGTGA
GIS-305	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTGACTGGAGTCCTCTCTATGGGCAGTCGGTGA
GIS-306	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTGACTGGAGTCCTCTCTATGGGCAGTCGGTGA
GIS-307	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTGACTGGAGTCCTCTCTATGGGCAGTCGGTGA
GIS-308	CAAGCAGAAGACGGCATACGAGATCCTCTCTGGTGACTGGAGTCCTCTCTATGGGCAGTCGGTGA

SI References

1. Y. Yu *et al.*, An efficient gene knock-in strategy using 5'-modified double-stranded DNA donors with short homology arms. *Nat. Chem. Biol.* **16**, 387-390 (2020).

2. K. N. Lovendahl, A. N. Hayward, W. R. Gordon, Sequence-Directed Covalent Protein-DNA Linkages in a Single Step Using HUH-Tags. *J. Am. Chem. Soc.* **139**, 7030-7035 (2017).

3. N. L. Malinin *et al.*, Defining genome-wide CRISPR-Cas genome-editing nuclease activity with GUIDE-seq. *Nat. Protoc.* **16**, 5592-5615 (2021).

4. A. V. Anzalone *et al.*, Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* **576**, 149-157 (2019).