

**Multiplex CRISPR/Cas9-based genome engineering enhanced by
Drosha-mediated sgRNA-shRNA structure**

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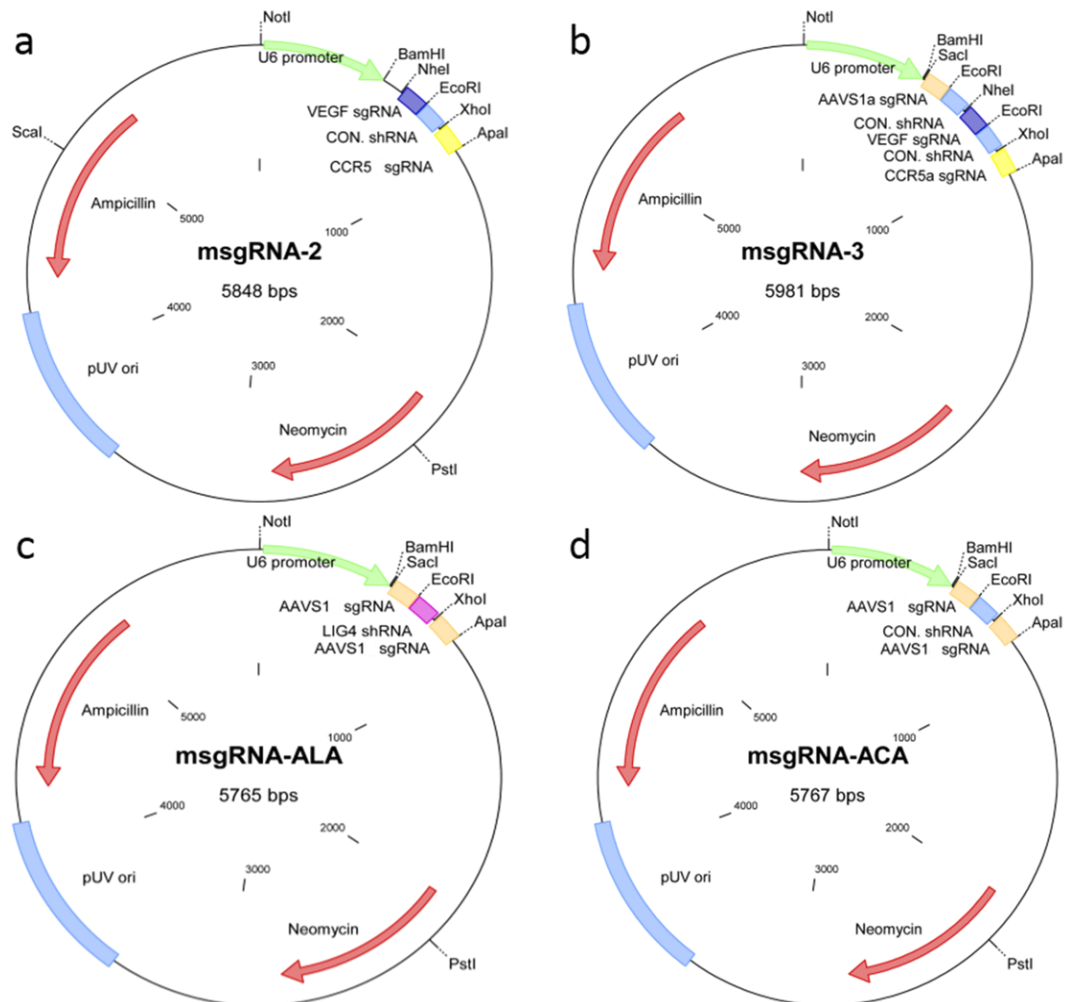
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Supplementary Information



Supplementary Figure 1 Plasmid maps for the constructs: (a) msgRNA-2, (b) msgRNA-3, (c) msgRNA-ALA and (d) msgRNA-ACA

Target site
PAM

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GCTCTGGTTCTGGGTACTTTTATCTGTCCCCTCCACCCCA CAGTGGGGCCACTAGGGAC (WT)
GCTCT-GTTCTGGGTACTTTTATCTGTCCCTCCACCCCA CAGTGGGGCCACTAGGGAC (-2)
-----TCCACagaA t c a g G G G G a t a A C g c a G G A a (-45,+14)
GCTCTGGTTCTGGGTACTTTTATCTGTCCCCTCCACCCCA aCAGTGGGGCCACTAGGGAC (+1) (X3)
G-----A C A t a G G G G C -----C (-49,+2) (X2)
GCTCTGGTTCTGGGTACTTTTATCTGTCCCCTC----- C A G T G G G G C C A C T A G G G A C (-7)
GCTCTGGTTCTGGGTACTTTTATCTGTCCCCTCCACCCCA ----- (-18)
GCTCTGGTTCTGGGTACTTTTATCTGTCCCCTCCACC-----CCACTAGGGAC (-11)
GCTCTGGTTCTGGGTACTTTTATCTGTCCCCT-----CCACTAGGGAC (-16)

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(a) AAVS1 site (11/12, 91.67%)

Target site
PAM

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GGGGGCGCTCGGCCACCACAGGGA A G C T G G GTGA (WT)
-----GTGA (-30)
GGGGGCGCTCGGCCACCACAGGGA t g t g c A G C T G G GTGA (+5)

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(b) VEGF site (2/9, 22.22%)

Target site
PAM

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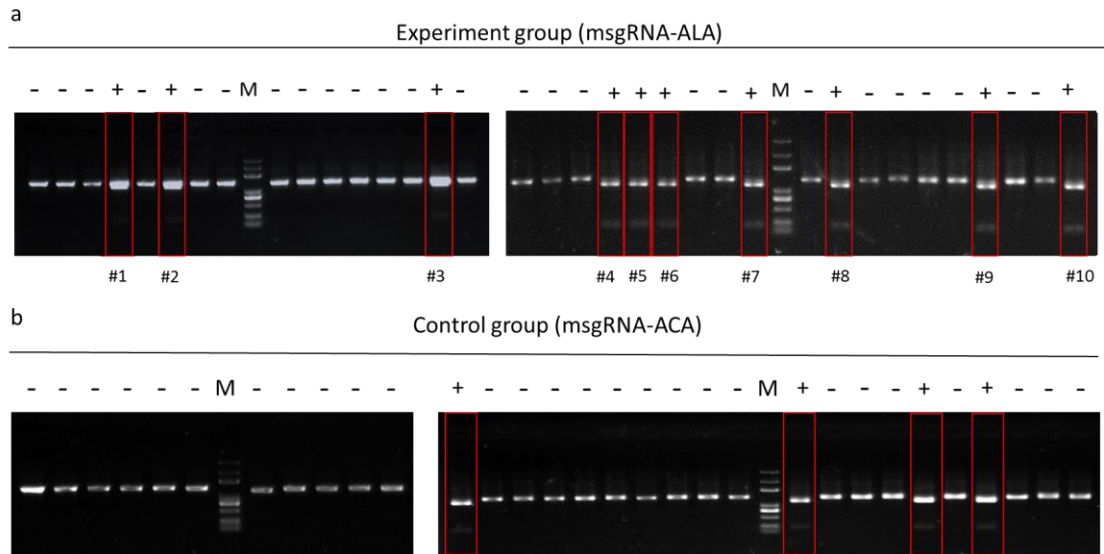
GAT CACTTGTGTCACCACCCCAAAGGTGGACCGTCC (WT)
GAT CACTTGTGTCACCACCCCAA t c t a G G A C C G T C C (-4,+4) (X5)
G t T C A C A C T T G T C A C C A C C C C A A t c t a G G A C C G T C C (-5,+5)

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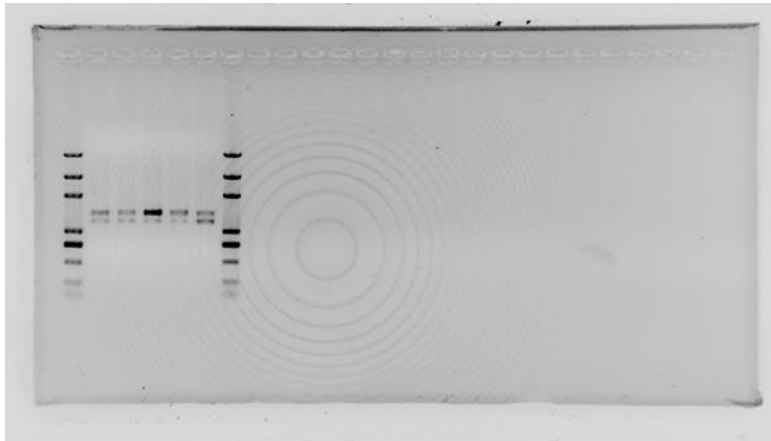
(c) CCR5 site (6/7, 85.71%)

Supplementary Figure 2 The indels within the detected “T-A clones” from the cell pool

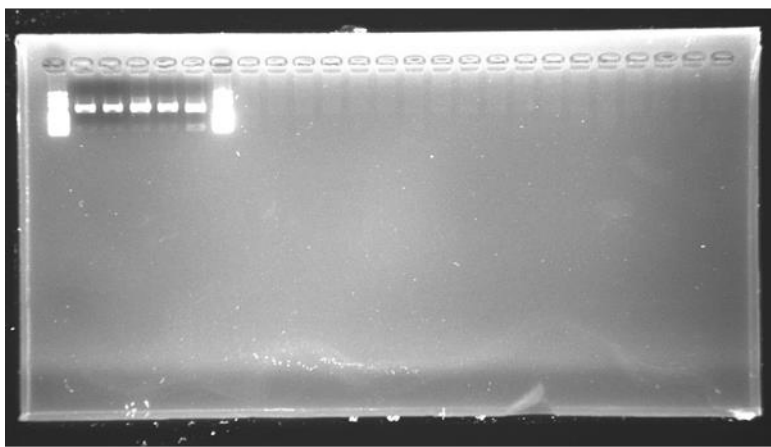
targeted by msgRNA-3. (a) AAVS1 site, (b) VEGF site, (c) CCR5 site



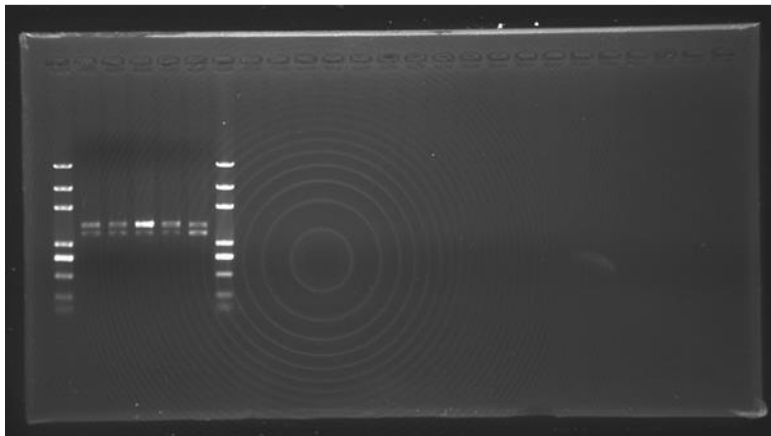
Supplementary Figure 3 The results for the detection of genome edited positive clones by *EcoR I* -digesting assay. The PCR product was supposed to be 1397 bp in length and could be digested into two fragments (1194 bp and 203 bp) if the genome DNA had been successfully edited by HDR-based repair. The PCR product would be partially digested if the clone was heterozygotes with only one allele edited. Percentage of digestion positive clones within detected clones was calculated to evaluate the precise genome editing efficiency. The results demonstrated 10 positive clones out of 35 cell clones (10/35) for the msgRNA-ALA group, while 4 out of 31 clones (4/31) for the msgRNA-ACA control. However, the pictures and the bands were not clear for confirming whether one or both alleles were edited. Further repeat detection of these positive clones demonstrated that they were all heterozygotes with one allele edited (refer to Fig.4e in the manuscript).



a



b



c

Supplementary Figure 4 Full-length gel images of representative results for the detection of positive clones by EcoR I digestion. (a) Full-length gel of Fig. 4e in the manuscript. (b)

Overexposure after a shorter electrophoresis period for a clear vision of the 203bp band. (c)
 Normal-length exposure after a longer electrophoresis period for a clear vision of the 1397 bp
 and 1194 bp bands.

Supplementary Table 1 Transfection groups set for the DsRed-eGFP surrogate reporter assay

	Cas9 vector	msgRNA	Reporter	Group
Molar ratio	1	1.5	0.8	
VEGF target groups	pll3.7-U6-CMV-hStCas9	msgRNA-2	pRG.VEGF	Experiment group
	pll3.7-U6-VEGF-CMV-hStCas9	pcDNA3.1(+)	pRG.VEGF	Positive control
	pll3.7-U6-CMV-hStCas9	pcDNA3.1(+)	pRG.VEGF	Negative control
CCR5 target groups	pll3.7-U6-CMV-hStCas9	msgRNA-2	pRG.CCR5	Experiment group
	pll3.7-U6-CCR5-CMV-hStCas9	pcDNA3.1(+)	pRG.CCR5	Positive control
	pll3.7-U6-CMV-hStCas9	pcDNA3.1(+)	pRG.CCR5	Negative control

Supplementary Table 2 Transfection groups set for the multiplex genome targeting assay

	Cas9 vector	msgRNA	Reporter	Group
Molar ratio	1	1.5	0.8	
AAVS1 target groups	pll3.7-U6-CMV-hStCas9	msgRNA-3	pRPG.AAVS1	Experiment 1
	pll3.7-U6-AAVS1-CMV-hStCas9	pcDNA3.1(+)	pRPG.AAVS1	Positive control 1
VEGF target groups	pll3.7-U6-CMV-hStCas9	msgRNA-3	pRPG.VEGF	Experiment 2
	pll3.7-U6-VEGF-CMV-hStCas9	pcDNA3.1(+)	pRPG.VEGF	Positive control 2

CCR5 target	pll3.7-U6-CMV-hStCas9	msgRNA-3	pRPG.CCR5	Experiment 3
groups	pll3.7-U6-CCR5-CMV-hStCas9	pcDNA3.1(+)	pRPG.CCR5	Positive control 3

Supplementary Table 3 Diplex and triplex targeted positive clones detected in the multiplex genome targeting assay

Groups	Number of diplex and triplex targeted positive clones/Detected cell clones			
	AAVS1 & VEGF	VEGF & CCR5	AAVS1 & CCR5	AAVS1 & VEGF & CCR5
Experiment 1	1/12	0/12	2/12	1/12
Experiment 2	1/10	1/10	1/10	1/10
Experiment 3	0/10	0/10	1/10	1/10
General frequency	6% (2/32)	3% (1/32)	13% (4/32)	9% (3/32)

Supplementary Table 4 The overall mutated clones for each targeted locus in the multiplex genome targeting assay

Groups	Mutated clones/Detected clones			Remarks
	AAVS1	VEGF	CCR5	
Experiment 1	11/12	2/12	4/12	pRPG.AAVS1 as reporter
Experiment 2	6/10	3/10	5/10	pRPG.VEGF as reporter
Experiment 3	9/10	1/10	2/10	pRPG.CCR5 as reporter
General frequency	81% (26/32)	19% (6/32)	34% (11/32)	
Positive control 1	100% (6/6)	-	-	pRPG.AAVS1 as reporter
Positive control 2	-	100% (8/8)	-	pRPG.VEGF as reporter

Supplementary Table 5 Primers used in Quantitative RT-PCR assay

Primer name	Sequence	Remarks
qP-LIG4.F	GCCCCGAGGCCAGTTAAACGAGAAG	For <i>LIG4</i> gene in the
qP-LIG4.R	GTGGTTCTTATGAAGAGCATCATG	qRT-PCR assay
ACTB forward	ATTGCCGACAGGATGCAGA	For β -actin gene in the
ACTB reverse	GAGTACTTGCGCTCAGGAGGA	qRT-PCR assay

Supplementary Sequence 1 The DNA sequence designed for the msgRNA-2 construct

(between *NheI* and *ApaI* cutting site).

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_NheI_
gctagcctcg gccaccacag ggaagcgttt tagagctaga aatagcaagt taaaataagg ctagtccggt atcaactga aaaagtggca ccgagtcggt gctttgaatt
cgatcggagc cgggtggtgc ccttcgcaaa atctcgatct ttatcgttca atttttatcc gatcaggcaa tagttgaact ttttcacggt ggctcagcca cgaacttaa
VEGF sgRNA scaffold
_XhoI_
ccgaggcagt aggcacacac tcaaccttta tatttactac atctgtggtc tcactagtaa atataaaggT tgagtgttcg ctcactgtca acagcatata cttctcgag
ggctccgtoa tccgtgtgtg agttggaaat ataaatgatg tagacaccga agtgatcatt tatatttcca actcacaagc gagtgcagct tgctgtatat ggaagagctc
drosha cutting site CON. shRNA drosha cutting site
_ApaI_
cacacttgtc accaccccaa gttttagagc tagaaatagc aagttaaaat aaggctagtc cgttatcaac ttgaaaaagt ggcaccgagt cgggtcgttt ttgggcccgt
gtgtgaacag tgggtgggtt caaaatctcg atctttatcg ttcaatttta ttccgatcag gcaatagtty aactttttca ccgtggctca gccacgaaaa aaccgggcca
CCR5 sgRNA scaffold

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Supplementary Sequence 2 The DNA sequence designed for the msgRNA-3 construct

(between *SacI* and *ApaI* cut sites).

SacI
 gagctcctgt cccctccacc ccacaggttt tagagctaga aatagcaagt taaataagg ctagtccgtt atcaactga aaaagtggca ccgagtcggt gctttgact ccgaggcagt aggcacacac tcaacttta
 ctcgaggaca ggggaggtgg ggtgtccaaa atctcgatct ttatcgttca atttttatcc gacagggcaa tagtgaact ttttccact ggctcagcca cgaacttaa ggctccgtca tccgtgtgtg agttggaat
 AAVS1 sgRNA scaffold Drosha cutting site

NheI
 tattactac atctgtggt tcaactagta atataaagt tgagtgttcg ctcactgtca acagcatata ccttgctagc ctgggccacc acagggaaag gttttagagc tagaaatagc aagttaaaat aaggctagtc
 ataaaatgat tagacacaga agtgatcatt tatatttcca actcaaaagc gaggacagt tgcgttatat ggaacgacag gggccggctg tgcctcttcg caaaactcag atctttatcg tcaatttta ttcgacacg
 CON.shRNA Drosha cutting site VEGF sgRNA scaffold

EcoRI
 cgttatcaac ttgaaaaagt ggcaccgagt cgtgtcttg aattccgagg cagtaggcac acactcaacc tttatatta ctacatctgt ggcttcaact gtaaatata aggttgagt ttcgtcact gccaacagca
 gcaatagttg aactttttoa cgttgctca gccacgaac ttaaggctcc gtcctcgtg tgtgagttgg aatataat gatgtagaca ccgaagtgat catttatatt tccaactcac aagcagtgca cagttgtcgt
 Drosha cutting site CON.shRNA Drosha cutting site

XbaI
 tataccttct cgggcacact tgcaccacc ccaagtttta gagctagaaa tagcaagtta aaataaggt agtcggtat caactgaaa aagtggacc gactcgtgc tttttgggc ccgtttaaac ccgctgatca
 atatggaaag gctcgtgtga acagtggtg ggtcaaat ctogatcttt atcgttcaat tttattcca tcaggaata gttgaactt ttcacgttg ctagccacg aaaaacccc ggcgaatttg ggcgactagt
 CCR5 sgRNA scaffold