

Supplemental materials

Materials and Methods

Plasmids construction

All DNA sequences of plasmids used in this study were provided in Supplemental Sequences. Human codon-optimized retron RTs were synthesized and inserted into EcoRI-digested pHG023 backbone or SmaI-digested pHG077 backbone by NEBuilder (New England Biolabs). The cloning of plasmids for expressing 5' rgRNA and 3' rgRNA were based on pHG215 backbone. Donor sequences were cloned and inserted into the replaceable region of retron ncRNA with *EcoRI* digestion and Gibson assembly using NEBuilder. Primers for cloning were listed in Supplemental Table1.

Cell Culture, transfection and flow cytometry sorting

Cell Culture, transfection and flow cytometry were performed as previously described (Xu et al., 2021). Briefly, HEK293T cells cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin were seeded on 24-well poly-D-lysine coated plates (Corning). Transfection was conducted with 4 µl of PEI (Polyscience) following the manufacturer's manual and 1 µg of CR plasmids and 1 µg of rgRNA plasmids. Unless otherwise stated, transfected cells were sorted by MoFlo XDP 72 h after transfection. BFP and mCherry double-positive cells were collected.

msDNA purification and quantification

Four thousand sorted cells were centrifuged down at 7,000 rpm for 5 mins. Cells plate were resuspended in Trizol for isolating rgRNA-msDNA hybrid as the the manufacturer's manual for RNA purification. For quantifying the abundance of *HEK3* msDNA, the rgRNA-msDNA extract was digested by *DdeI* to eliminate the contamination of plasmid DNA and genomic DNA. PCR was conducted using the *DdeI* digested product as template.

Genomic DNA extraction

Four thousand sorted cells were harvested for genomic DNA extraction by addition of 10 µl of lysis buffer (Vazyme) following the manufacturer's manual.

Efficiency analysis of retron editing at human endogenous genomic loci

The genomic DNA extraction was performed as described above. FOR deep sequencing analysis, the genomic region in the vicinity of Cas9 target site was amplified by Phanta Max Super-Fidelity DNA Polymerase (Vazyme) using nested PCR: First round: 98 °C, 3 mins; (98 °C, 30 sec; 58 °C, 30 sec; 72 °C, 1 min) X 20 cycles; 72 °C, 5 min. Second round: 98 °C, 3 mins; (98 °C, 30 sec;

56 °C, 30 sec; 72 °C, 30 sec) X 35 cycles; 72 °C, 5 min; primers with barcode were used. PCR products were purified by Gel extraction kit (Vazyme) and sequenced on an Illumina HiSeq X System (150-bp paired-end reads). Forward reads were aligned to the reference sequences using BWA (v0.7.17-r1188) with parameter of “bwa mem -A2 -O3 -E1”. At each target, editing was calculated as the percentage of total reads containing desired edits without indels within a 10-bp window of the cut site.

Restriction-fragment length polymorphism (RFLP) was performed to determine the CR-mediated precise editing. Purified PCR products were digested with *HindIII* and *BclI* (Thermo) for *EMX1* and *HEK3* amplicon respectively, and incubated for 90 mins at 37 °C. The digested products were analyzed by agarose gel electrophoresis and imaged with gel imaging system (Tanon).

All primers used for genomic DNA amplification are listed in Supplemental Table1.

Activity determination of retron editing at known Cas9 off-target sites

Determination was performed as previous (Anzalone et al., 2019). In brief, the genomic region of interest was amplified using nested PCR: First round: 98 °C, 3 mins; (98 °C, 30 sec; 58 °C, 30 sec; 72 °C, 1 min) X 20 cycles; 72 °C, 5 min. Second round: 98 °C, 3 mins; (98 °C, 30 sec; 56 °C, 30 sec; 72 °C, 30 sec) X 35 cycles; 72 °C, 5 min. Purified PCR products were Sanger sequenced and analyzed as previously described (Brinkman et al., 2018).

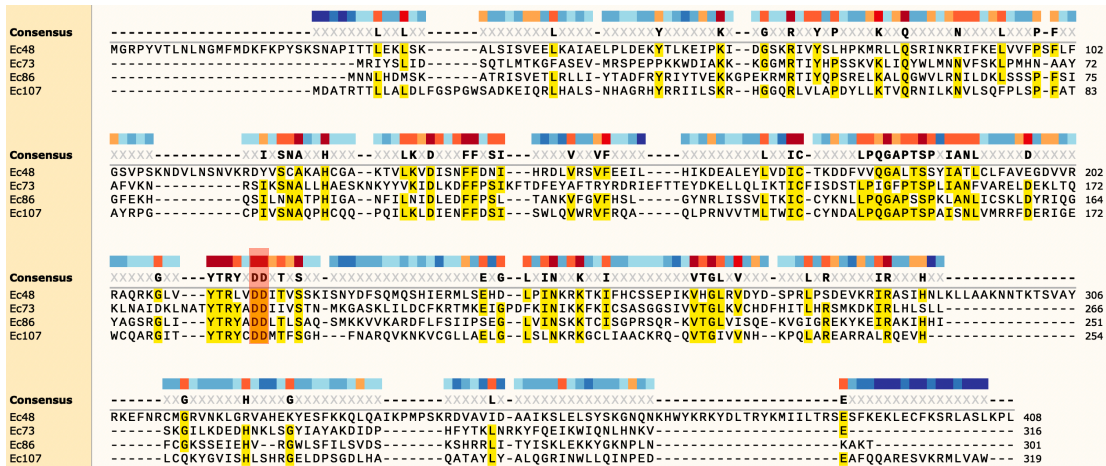
Supplemental References

Anzalone, A.V., Randolph, P.B., Davis, J.R., Sousa, A.A., Koblin, L.W., Levy, J.M., Chen, P.J., Wilson, C., Newby, G.A., Raguram, A., *et al.* (2019). Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* 576, 149-+.

Brinkman, E.K., Kousholt, A.N., Harmsen, T., Leemans, C., Chen, T., Jonkers, J., and van Steensel, B. (2018). Easy quantification of template-directed CRISPR/Cas9 editing. *Nucleic Acids Res* 46, e58.

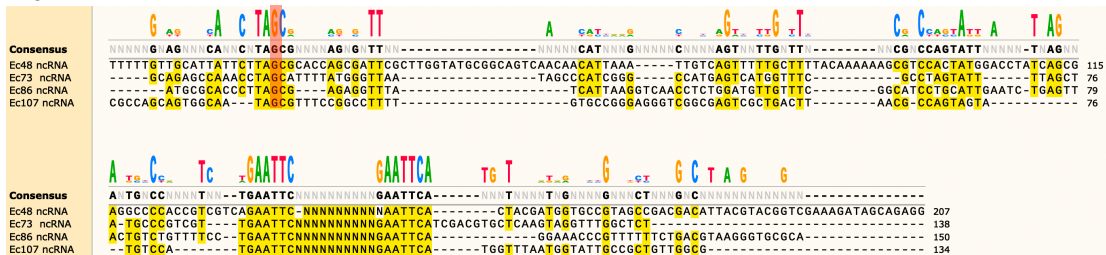
A

Alignment results of retron RT protein sequences

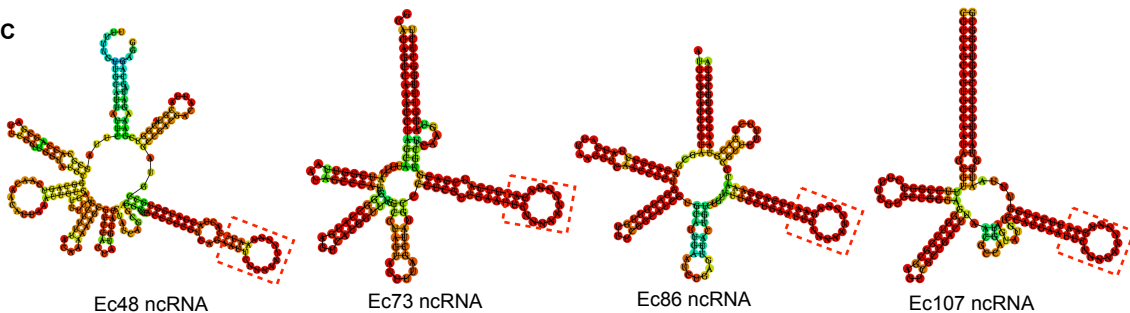


B

Alignment results of retron ncRNA

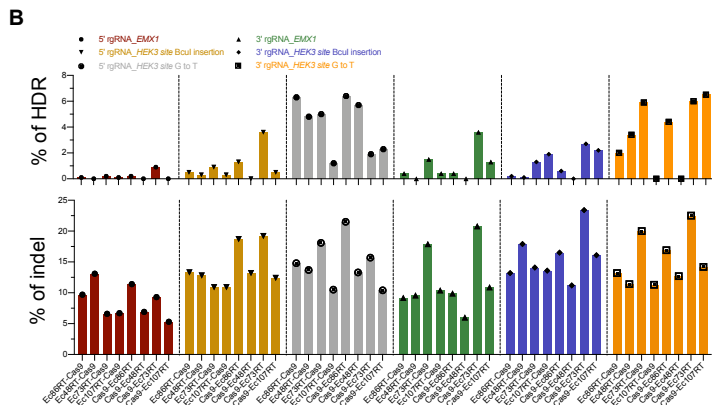
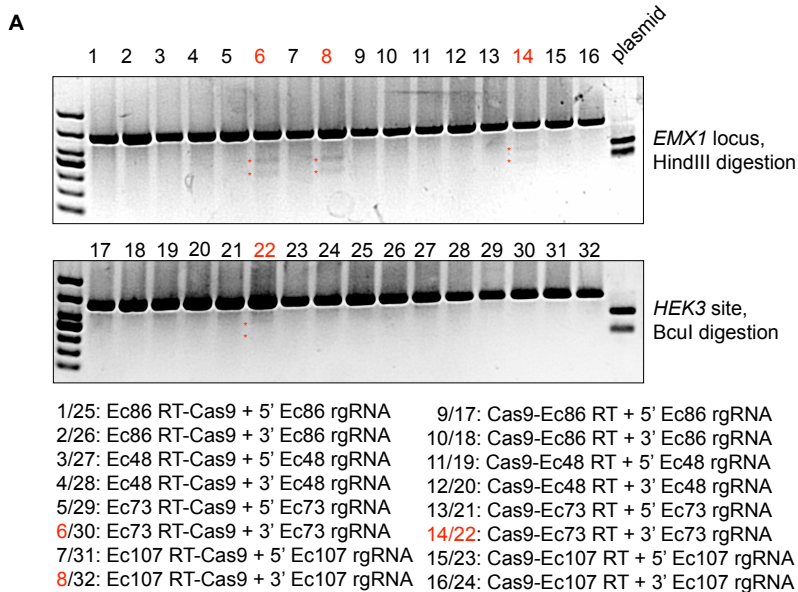


C

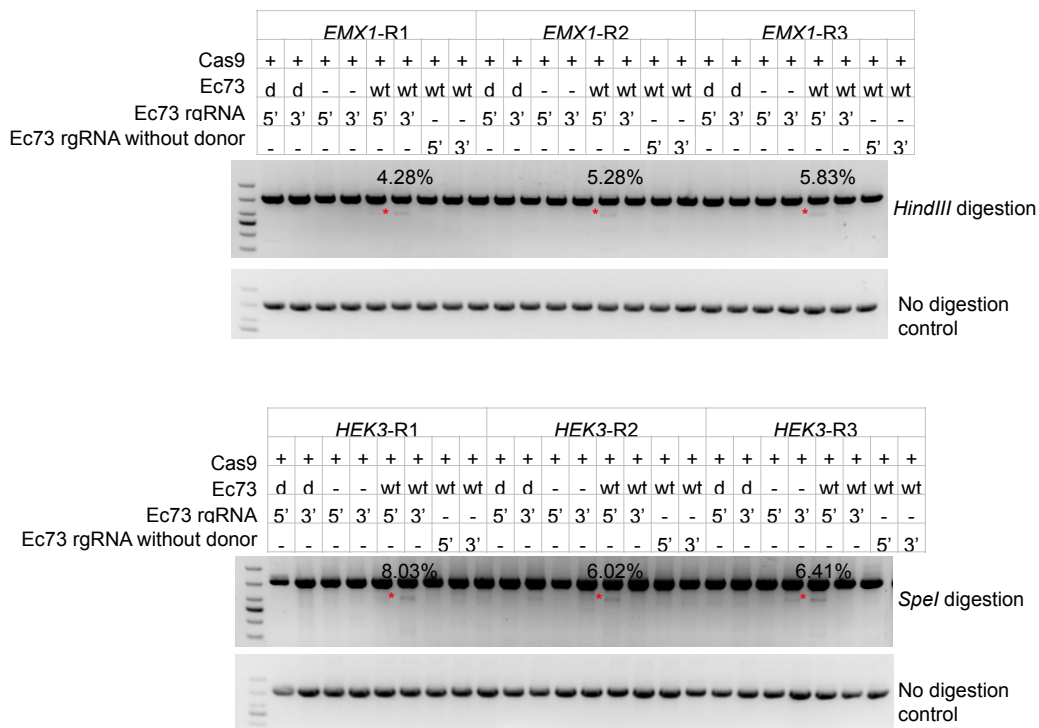


Supplemental Figure 1. Comparison of four retrons.

(A) Protein sequences alignment of Ec48-RT, Ec73-RT, Ec86-RT and Ec107-RT. Conserved double aspartic acid (DD) were highlighted in red. (B) Alignment of Ec48-ncRNA, Ec73-ncRNA, Ec86-ncRNA and Ec107-ncRNA. Conserved priming G was highlighted in red. (C) Predicted secondary structure of Ec48-ncRNA, Ec73-ncRNA, Ec86-ncRNA and Ec107-ncRNA using RNAfold web server. The replaceable region of retron ncRNA was marked with red dotted line.

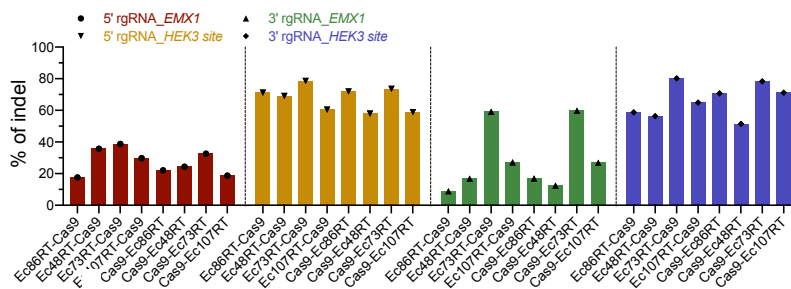
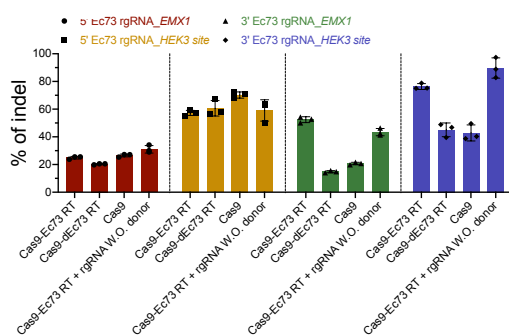


Supplemental Figure 2. Retron editing system-stimulated precise editing and indels. (A) Determination of retron editing system-mediated precise genome editing by restriction-fragment length polymorphism (RFLP) analysis. Digested products were marked by asterisks. (B) Efficiency determination of retron editing with 90nt donor by TIDER analysis. Transfected cells were directly collected without FACS sorting for genomic DNA extraction and TIDER analysis.

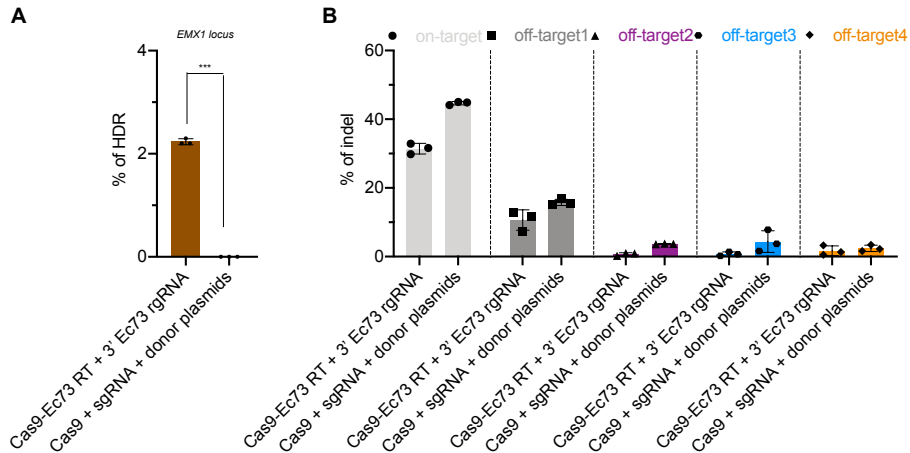


Supplemental Figure 3. RFLP analysis of retron editing system mediated precise editing.

Digested products were marked by asterisks. d represents predicted catalytically dead Ec73 RT, wt indicates wild-type Ec73 RT.

A**B**

Supplemental Figure 4. Deep sequencing analysis of retron editing system induced indels. (A) Related to Fig. 2C and (B) related to Fig. 2D



Supplemental Figure 5. Off target of retron editing at known Cas9 off target sites of *EMX1* locus.

(A) Retron editing-mediated HDR at *EMX1* locus. *** $p < 0.001$, determined by Student's t-test, $n=3$. **(B)** Retron editing-mediated indel at on-target site and four known Cas9 off target sites of *EMX1* locus, $n=3$. Transfected cells were directly collected after 3 days of transfection for genomic DNA extraction and TIDER analysis, with no FACS sorting.

Supplemental Table1. Primers and oligos used in this study

Primers/Oligos for cloning		
Primer Name	Primers Sequences	
Cas9-Ec86RT F	gcagcagcgggggtcaAGCAAAGCGCCGAGTACCTGAATAC	Retron RTs fused to the carboxy terminus of Cas9
Cas9-Ec86RT R	GGTTCTTTTTGAGCCGCCAGAGGTCTTAGCTTTGTTTCAGAGGATTG	
Cas9-Ec48RT F	ggcagcagcgggggtcaAGCGGCAGACCTTACGTGACCCTG	
Cas9-Ec48RT R	GGTTCTTTTTGAGCCGCCAGACAGGGGCTTGAGGCTGGCCAG	
Cas9-Ec73RT F	ggcagcagcgggggtcaAGCCGGATCTACAGCCTGATTGATAG	
Cas9-Ec73RT R	GGTTCTTTTTGAGCCGCCAGATTCCACTTTGTTGTGCAGGTTG	
Cas9-Ec107RT F	ggcagcagcgggggtcaAGCGACGCCACAAGAACCACCCCTG	
Cas9-Ec107RT R	GGTTCTTTTTGAGCCGCCAGACCAGGCCACCAGCATCCGTTTC	
Ec86RT-Cas9 F	ATCCACGGAGTCCCAGCAGCCcccgggAAAAGCGCCGAGTACCTGAATAC	Retron RTs fused to the amino terminus of Cas9
Ec86RT-Cas9 R	cctccgctagatcctccgacccgggGGTCTTAGCTTTGTTTCAGAGGATTG	
Ec48RT-Cas9 F	ATCCACGGAGTCCCAGCAGCCcccgggGGCAGACCTTACGTGACCCTGAAC	
Ec48RT-Cas9 R	gagcctccgctagatcctccgacccgggCAGGGGCTTGAGGCTGGCCAG	
Ec73RT-Cas9 F	TATCCACGGAGTCCCAGCAGCCcccgggCGGATCTACAGCCTGATTGATAG	
Ec73RT-Cas9 R	agcctccgctagatcctccgacccgggTTCACACTTTGTTGTGCAGGTTG	
Ec107RT-Cas9 F	TATCCACGGAGTCCCAGCAGCCcccgggGACGCCACAAGAACCACCCCTGCTG	
Ec107RT-Cas9 R	agcctccgctagatcctccgacccgggCCAGGCCACCAGCATCCGTTTC	
oligo template for <i>EMX1</i> donor	AAACGGCAGAAGCTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGCTTAAGGGCTCCCATCACATCAACCGGTGGCGCATTGCCACGAAGCAG	PCR template
oligo template for <i>HEK3</i> donor with BcuI insertion	cttctccagccctggcctgggtcaatccttggggcccagactgagcactaGtgatggcagaggaaaggaagccctgcttctccagagggcgctcgag	
oligo template for <i>HEK3</i> donor with G to T transversion	cttctccagccctggcctgggtcaatccttggggcccagactgagcacttgatggcagaggaaaggaagccctgcttctccagagggcgctcgag	
Ec86- <i>EMX1</i> donor F	atctgagttactgtctgtttccctACTAGTACAAACGGCAGAAGCTGGAGGA	Expressing 5' rgRNA and 3' rgRNA containing <i>EMX1</i> donor sequences
Ec86- <i>EMX1</i> donor R	CGCACCCCTTACGTACAGAAgAAACGGGTtCCTCtctaggTGACATCGATGTCTCCCCATTGGCCTGCTTCGTGGCAATGC	
Ec48- <i>EMX1</i> donor F	CAGCGAGGCCCCACCGTCGTCAACTAGTACAAACGGCAGAAGCTGGAGGA	
Ec48- <i>EMX1</i> donor R	GGTACGGCACCATCGTAGTcttaggTGACATCGATGTCTCCCCATTGGCCTGCTTCGTGGCAATGC	
Ec73- <i>EMX1</i> donor F	TATTTTAGCTATGCCCGTCGTACTAGTACAAACGGCAGAAGCTGGAGGA	
Ec73- <i>EMX1</i> donor R	AACCTACTTGTAGCAGTCGATccttaggTGACATCGATGTCTCCCCATTGGCCTGCTTCGTGGCAATGC	
Ec107- <i>EMX1</i> donor F	TTAACGCCAGTAGTATGTCCATACTAGTACAAACGGCAGAAGCTGGAGGA	
Ec107- <i>EMX1</i> donor R	AGCGGCAATACCATTAAACCATccttaggTGACATCGATGTCTCCCCATTGGCCTGCTTCGTGGCAATGC	
Ec86- <i>HEK3</i> donor F	aatctgagttactgtctgtttccctACTAGTgcttccagccctggcctgggt	Expressing 5' rgRNA and 3' rgRNA containing <i>HEK3</i> donor sequences
Ec86- <i>HEK3</i> donor R	GCGCACCCCTTACGTACAGAAgAAACGGGTtCCTCtctaggccctgtctagaaaagctgtcctgcgacgcccctctggag	
Ec48- <i>HEK3</i> donor F	AGCGAGGCCCCACCGTCGTCAACTAGTgcttccagccctggcctg	
Ec48- <i>HEK3</i> donor R	GTCGGCTACGGCACCATCGTAGTcttagggcccctgtctagaaaagctgtcctgcgacgcccctctggag	
Ec73- <i>HEK3</i> donor F	TAGTATTTTAGCTATGCCCGTCGTACTAGTgcttccagccctggcctg	
Ec73- <i>HEK3</i> donor R	AAACCTACTTGTAGCAGTCGATccttagggcccctgtctagaaaagctgtcctgcgacgcccctctggag	
Ec107- <i>HEK3</i> donor F	ACTTAACGCCAGTAGTATGTCCATACTAGTgcttccagccctggcctg	
Ec107- <i>HEK3</i> donor R	CAGCGCAATACCATTAAACCATccttagggcccctgtctagaaaagctgtcctgcgacgcccctctggag	

Supplemental Table1. Primers and oligos used in this study

Primers for genomic DNA amplification		
<i>EMX1</i> F1	AAAACACCCTTCTCTCTGGC	For RFLP
<i>EMX1</i> R1	GGAGATTGGAGACACGGAGAG	
<i>EMX1</i> F2	CCATCCCCTTCTGTGAATGT	
<i>EMX1</i> R2	GGAGATTGGAGACACGGAGA	
<i>HEK3</i> F1	tggcaaatgaggctggagagg	
<i>HEK3</i> R1	cagagctgtcctctggtctg	
<i>HEK3</i> F2	ggcatgagaaacctggaga	
<i>HEK3</i> R2	cagagctgtcctctggtct	
<i>EMX1</i> DSeq F1	CCATCCCCTTCTGTGAATGT	For deep sequencing
<i>EMX1</i> DSeq R1	TaGCCTGTTTCTCCTGCTGT	
<i>EMX1</i> DSeq F2	gaattcNNNNNNNnggatccCAGGTGAAGGTGTGGTTCCAG	
<i>EMX1</i> DSeq R2	gaattcNNNNNNNnggatccCCCTCGTGGGTTTGTGGTTG	
<i>HEK3</i> DSeq F1	tggcaaatgaggctggagagg	
<i>HEK3</i> DSeq R1	cttagctggcatggaatgtctc	
<i>HEK3</i> DSeq F2	gaattcNNNNNNNnggatccCTGCTGCAAGTAAGCATGCA	
<i>HEK3</i> DSeq R2	gaattcNNNNNNNnggatccGCCAGCCAAACTTGTCAAC	
<i>EMX1</i> OT1 TIDE F1	GACAGCTTCTTATCCCTGTCTTCCAG	For off-target analysis by TIDER assay
<i>EMX1</i> OT1 TIDE R1	TTCCATTCTCTGCATCACCATTCTG	
<i>EMX1</i> OT1 TIDE F2	ATTCTGAAGACCTGTAATCTGACTC	
<i>EMX1</i> OT1 TIDE R2	TTCTCTGCATCACCATTCTGGTC	
<i>EMX1</i> OT2 TIDE F1	GATGAATCCTCCTCCTGTTCTCAG	
<i>EMX1</i> OT2 TIDE R1	GTAAGAGATTACATACTCTTGCATGTTTG	
<i>EMX1</i> OT2 TIDE F2	CATCCTCCTCATCTGAGGACTCCA	
<i>EMX1</i> OT2 TIDE R2	TTGCATGTTTGATAACACATAAGGTAAGG	
<i>EMX1</i> OT3 TIDE F1	CAAAGGAGACCTGGAGACGACTG	
<i>EMX1</i> OT3 TIDE R1	GACCCAGCTCACCTCACTC	
<i>EMX1</i> OT3 TIDE F2	AGGCACTTGC GGTTTGC ACTG	
<i>EMX1</i> OT3 TIDE R2	CCCTCCGGTTGCCCTTAC	
<i>EMX1</i> OT4 TIDE F1	aagaagccataccgagcggttg	
<i>EMX1</i> OT4 TIDE R1	tgtgtacagagttctcatgtgacctctg	
<i>EMX1</i> OT4 TIDE F2	gaagccataccgagcggttgG	
<i>EMX1</i> OT4 TIDE R2	tacagagttctcatgtgacctctgac	
Primers for testing expression of msDNA		
<i>HEK3</i> FP	gcttccagccctggcctg	
<i>HEK3</i> RP	gccctgtctaggaaagctgtc	