## Title

# Discovery and characterization of sgRNA-independent DNA cleavage from CRISPR/Cas9 in mouse embryo

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## Supplementary-information

**Supplementary TableS1 PCR primers**

| Name | Sequence(5’-3’) |
|---|---|
| Tyr-JDF | AGACTGTTCTTGCTATTATGGGA |
| Tyr-JDR | ATTGGCCGATCCCTGTACTT |
| TYR-lowsg | TTAATACGACTCACTATAGGACCACTATTACGTAATCCGTTTTAGAGCTAGAAATAGC |
| TYR-highsg | TTAATACGACTCACTATATTATGCGATGGAACACCTGAGTTTTAGAGCTAGAAATAGC |
| Scaffold-F | GTTTTAGAGCTAGAAATAGC |
| T7-R1 | AAAAGCACCGACTCGGTGCCA |

**Supplementary TableS2 The cutting efficiency of sgRNAs was verified in Tyr-low group and Tyr-high group**

| Group | Embryo with pronuclear | Number of blastocysts | Mutation mice number |
|---|---|---|---|
| Tyr-low | 15 | 5 | 5 |
| Tyr-high | 20 | 7 | 6 |

**Supplementary TableS3 In vitro development of early mouse embryos in three groups**

| Group | Embryo with pronuclear | 2-cell(%) | Blastocysts(%) |
|---|---|---|---|
| Control | 85 | 72(84.70,72/85)[a] | 43(50.58,43/85)[a] |
| Tyr-low | 111 | 97(87.39,97/111)[a] | 50(45.05,50/111)[a] |
| Tyr-high | 111 | 85(76.57,85/111)[a] | 39(35.14,39/111)[b] |

**Supplementary TableS4 Pregnancy of full-term mice in the two groups**

| Group | 2-cell embryo | Receptor number | New born mice(%) | Mutation mice number(%) |
|---|---|---|---|---|
| Tyr-low | 250 | 5 | 17(6.80, 17/250)[a] | 16(94.12, 16/17)[a] |
| Tyr-high | 176 | 4 | 0(0, 0/176)[b] | 0(0, 0/0) |

**Supplementary Table S5 Genotype statistics of full-term mice in Tyr low group and 13.5d mouse embryos in Tyr high group**

| Tyr-low 组类型 | Tyr-low 组突变类型 | 小鼠只数 |
|---|---|---|
| Type1 | -54bp/-1bp,+1bp | 1 |
| Type2 | -162bp/-1bp | 1 |
| Type3 | +1bp/+1bp | 3 |
| Type4 | -8bp/+1bp | 1 |
| Type5 | -7bp/-116bp,+116bp | 1 |
| Type6 | -58bp/-58bp | 1 |

| | | |
|---|---|---|
| Type7 | -162bp/+1bp | 1 |
| Type8 | -87bp/-170bp | 1 |
| Type9 | -7bp/-7bp | 1 |
| Type10 | -34bp/-1bp | 1 |

| Tyr-high 组类型 | Tyr-high 组突变类型 | 小鼠只数 |
|---|---|---|
| Type1 | -12bp/-40bp | 2 |
| Type2 | -11bp/-2bp,+8bp | 1 |
| Type3 | -12bp/-15bp | 1 |
| Type4 | +1bp/-168bp | 1 |
| Type5 | +1bp/-117bp | 1 |
| Type6 | -12bp/-12bp | 8 |
| Type7 | -12bp/-65bp | 1 |
| Type8 | -16bp/+1bp | 1 |
| Type9 | -8bp/-8bp | 1 |
| Type10 | +1bp/+1bp | 1 |
| Type11 | +1bp/+2bp | 1 |
| Type12 | -12bp/+2bp | 1 |
| Type13 | -11bp/+1bp | 1 |
| Type14 | -12bp/-8bp,+8bp | 1 |

**Supplementary Table S6 Potential off target sequences (POTS) for Tyr-low sgRNA and Tyr-high sgRNA.**

The potential off-target sites of the two sgRNAs were predicted using the online-based tool (http://crispor.tefor.net/). The top three POTS were selected for each sgRNA according to the ranking scores. The blue colored nts are the sgRNA sequences, the red nts are the PAMs, and the red nts with underline is the primers used for PCR and sequence analysis.

| | |
|---|---|
| TYR-low sgRNA potential off target #1 | mm4_intron_Ppp2ca_chr11_51994338<br>aaaataaatcttaaaaaaaaaaaagaatgtcatcttgatcaaaaagtttagcttaacaacgcggt ggtgatgacatatgttcttaatcccagcacttgaaaaatctcttgagtttgaggccagcctggtctac agactgagttctagaacagccagggctacacagagaaaccttgtctcaagaaaacaaaaatataca aacaaaaaaatcagcttatcaaatcaataacaagcagtttctttttttccattctcaagtaacaaatgt ttaagctaaatgacatcataatgtctctatttatttgacattataatgtctgtattagatagagacagg acctctctaatgtaatcctggctgtcctcaaactcaaggatctgcctaactccttcccaagtcctggc tgacccagagctcactatatagattagactgtcacagaagtccatatgcctttgactcttggccatcc |

| | |
|---|---|
| | tcctgcatgagcttccagagtgctgcgattataagcatttataaccacactcaccaagttgatagtat ttacctttttccatctgtggatggaactgtgcctaagatggaactgcatgtgctcacccaaA |
| TYR-low sgRNA potential off target #2 | mm4_exon_Slc7a7_chr14_54646155<br>Cgccatggtcaacagcaccaagtatgaagtggctgctcagcacgaggccgacgatggctctgctc tcggggatggggccagcccagtggcggagcaggtcaaactgaagaaggagatctccctgcttaat ggcgtgtgtctcatagtggggaacatgatcggctccggcatctttgtctcccctaagggtgtgctcat gtacagtgcctcttttggcctctcactggtcatctgggctgtcgggggcattttctccgtctttggggc cctttgttacgctgaactgggtaccaccattaagaaatccgggccagctatgcttatatcctggag gccttcgggggattccttgcgttcatccgcctctggacttctctgctcatcattgaacccaccagcca ggccgtcattgccatcacctttgccaactacatggtgcagcccctcttcccgagctgtggcgctccct atgccgccggccgcctgctggccgctgcctgcatctgtaagtagagatggggcaggattggtgttgt ggaggactgggtagaagtgggggtgtgtgagtccagccaaggaggaggagctgggaaatgattg cctaca |
| TYR-low sgRNA potential off target #3 | mm4_intergenic_Snd1\|Lrrc4_chr6_28799427<br>Ctagcatgcacattttggtcagcctggaagagatgttgagtgctctggacttccttggagtaaaggt aagaatttaaatcccccaaaaggctcttggctgccctgagcttcaggtcagagaatcaggtaataa cctggtatttgacttctagctcttcctgccttagggctggcaatgcctcctgcatgccagaccactgc ccaggaccaggattactaaatccaggagaagcaagccaaaagggatcagtgtttaaagggaaac accaggtagcagacaagaaaaagaagtggtgtgatgaaaaggccaagagagccacttaggaaa atgggactgaacggtggctgcaaatgtcagtagttccgtgatacatatggcagtcgtatatgtacaa ggcaagcgtgggaaccatgtcccctaatatgggaggccatctgaaatgtagagaagcctcacctg ccccgtgcaggcctgcccttacgtagcaatgggatggaaaggcaagcagttttctacttactcaaa gcagagggagagacaggtattggtgccctgcttatccaaagccaggaaaaataagtagtagttctt agtgtctgcatc |

| | |
|---|---|
| TYR-high sgRNA potential off target #1 | mm4_intergenic_Gm6594\|Pkdcc_chr17_82876378<br>Aattagactagcgagtgccagccacaataataataaatgcctagcatggtaggttaagttcactttc atctaactgccagaaagctgaatgatactgatgtgcaatttcaacatctttctcttgtctcagaagtaa ctcatttaacataaattgtttttcacagatcatttgtagcagatcagaagagtataatagccatcaggt ttatgcgatggaacacctgagggaccactattacgtaatcctggaaaccatgacaaagccaaaac ccccaggctcccatcttcagcagatgtggaattttgtctgagtttgacccagtatgaatctggatcaat ggatagaactgccaatttcagctttagaaacacactggaaggtaatatccttgtgttcattaattttga ttttttttaatttataatttcttttcagaaattctgggctcagagatgtttataatttaaaattttgtgagtt ttgaaatgttatgcttcaattatacccatggtgatagtaaacttagctagcatagagtatctcactata ctccagaaatttagtgatataaaagggaaaggaagcgaaggaaaggaaagaaaaggaaaggg aagggaagggaaggg |
| TYR-high sgRNA potential off target #2 | mm4_intron_Spats2_chr15_99193920<br>Ttgatttttcaagacaggatttctttgtgtagctctggctgccctttagaccacgctggtctcagactca cagagatctactgcctctgcctcctgagtgctgggcatgagccactctgcctggctgttttaagttttg agaaaataaaatcaagtaaaaatattacctgggaatattactgcatggtttagagtattatctggtta tgtaattggttttgagatcaccacagttgtatgtaaaatactgtttttctaatgcagtaaacataaaca |

| | |
|---|---|
| | agtaataattattttgataccaaggaacacctgaaggcgaggctagaatcaaaatgactagtaac gtgttaggtacttcagtcaaaggccttgtgtgaagtcacaggcacagaatgtatctcagagacgcta gtggtgatgctgcagtaacagctgctctgaccaataaattgcacaaaagctttaaaataagttcagc aatcacagtgcttagataagaaggcctggctggaggggggggtgagatagagaccagtcagcta tcagtgttttccctaatgatagtggccaggtaagcaaagcatattacttgattaaagaaaagat |
| TYR-high sgRNA potential off target #3 | mm4_intergenic_Gm13198\|Ccdc3/Gm26776_chr2_5215861 Aattctccaagataaacagtcttgttgtgtctggctctggatatggccacgtggacaaagtcactat tctgtacgaagcaaatacaagcaggccctcagctgctaagttcccattgcattccccagcagggtgg gcggaaggagctcttggagtttccagccagccatgggatgccaggcaaatgcaggagtcacaagg aaaaaaatcagactcgactgcctctcgaagtggaagagctagagactgaatgctaactatcagtgt caggtcaaaggtgcgtcagatcatttgcaccatgtgatggaacacctgtgggatggagcaccagt gagagggagcatcggtaagagggagcacccatgggatggagcacccatgagagggagtaccagt ggaatggagcacttgtggaatcgagcaccagtgagatggagcaggaacagagaggttctgctcta ggattcctcttctcagtggagctggtgccacagacacttggtcttcagtccagcctgacactccatatc cttgtgagatggatgtgttatagatgtttcctcta |

**Supplementary Table S7 preliminary comparison of Cut&Tag sequencing data**

| | Control | Edit |
|---|---|---|
| Reads Counts | 12,883,306 | 36,660,240 |
| Alignment rate | 86.32% | 80.51% |

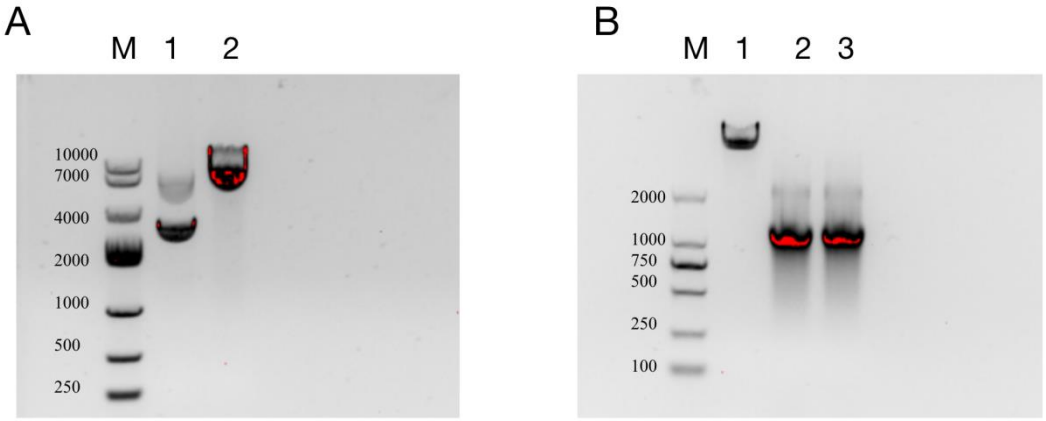
**Supplementary Table S8 On target and off targets information of Tyr-low sgRNA**

| MARK | guideSeq | offtargetSeq | mismatchPos | mismatchCount | mitOfftargetScore | cfdOfftargetScore | chrom | start | end | strand | locusDesc |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A | GGACCACTATTACGT AATCCTGG | TGACCACTAATAAGTCATC CAGG | | 4 | 0.097067 | 0 | chr1 | 40737262 | 40737284 | + | intron:Slc9 |
| B | GGACCACTATTACGT AATCCTGG | AGACCAAAATTACTTAATC CAGG | | 4 | 0.152973 | 0.144 | chr4 | 78431802 | 78431824 | - | intergenic:C 260-Gm11 |
| C | GGACCACTATTACGT AATCCTGG | GGCCCACTCTTACGTACT CAGGG | | 4 | 0.15314 | 0.021609 | chr5 | 71104752 | 71104774 | - | intergenic:C 2-Cox7b2 |
| D | GGACCACTATTACGT AATCCTGG | GGACCACTCTCCAGGTAAC CCGG | | 4 | 0.036801 | 0.022263 | chr5 | 149329336 | 149329358 | - | intergenic:A p-BC02847 |
| E | GGACCACTATTACGT AATCCTGG | GGAATACTATTAAGTTATC CTGG | | 4 | 0.100057 | 0 | chr6 | 106175424 | 106175446 | - | intron:Cntn |
| F | GGACCACTATTACGT AATCCTGG | GGACCAGGATTACTAAAT CCAGG | | 4 | 0.037227 | 0.053278 | chr6 | 28799429 | 28799451 | - | intergenic:S Lrrc4 |
| G | GGACCACTATTACGT AATCCTGG | GGACCACTATTACGTAAT CCTGG | | | | | chr7 | 87483642 | 87484272 | | Ontar |
| H | GGACCACTATTACGT AATCCTGG | GGACCATTATTCTGTAACC CAGG | | 4 | 0.036468 | 0.064145 | chr9 | 20961883 | 20961905 | - | intergenic:I -S1pr2 |
| I | GGACCACTATTACGT AATCCTGG | GGACCACTACTACGCGCT CCAGG | | 4 | 0.02231 | 0.004936 | chr9 | 105387117 | 105387139 | + | intergenic:C 619/Nek11 Nek11 |
| J | GGACCACTATTACGT AATCCTGG | GGGCCACAATGACATAAT CCAGG | | 4 | 0.116657 | 0.146667 | chr10 | 44363996 | 44364018 | - | exon:Atg5 |
| K | GGACCACTATTACGT AATCCTGG | AGACCACTTTTACCTAAGC CTGG | | 4 | 0.028252 | 0.077143 | chr10 | 48517224 | 48517246 | + | intergenic:C 584-Gm24 |
| L | GGACCACTATTACGT AATCCTGG | GGACCACTATGAAGTCAT ACTGG | | 4 | 0.015884 | 0 | chr10 | 110284174 | 110284196 | - | intergenic:I E2f7 |
| M | GGACCACTATTA CGTAATCCTGG | GGACCTCTCTAATGTAATC CTGG | | 4 | 0.108371 | 0.214286 | chr11 | 52103512 | 52103534 | - | intergenic:I a/Gm2415 ca |
| N | GGACCACTATTACGT AATCCTGG | GGACCACAAATCCCTAAT CCTGG | | 4 | 0.092156 | 0.077336 | chr11 | 67423855 | 67423877 | - | intergenic:I -Gas7 |
| O | GGACCACTATTACGT AATCCTGG | GTACCACCATTAAGAAATC CGGG | | 4 | 0.155902 | 0.1417 | chr14 | 54408699 | 54408721 | - | exon:Slc7a |
| P | GGACCACTATTACGT AATCCTGG | GGCCCGCAATTACGTAAT CCGGA | | 3 | 0.289504 | 0.017007 | chr15 | 76307382 | 76307404 | - | exon:Oplah |
| Q | GGACCACTATTACGT AATCCTGG | GGGCCACTAATACTTGAT CCAGG | | 4 | 0.034983 | 0.026862 | chr16 | 44530212 | 44530234 | - | intergenic:I Mir3081 |
| R | GGACCACTATTACGT AATCCTGG | GGACCACTATTATGAACT CAGGG | | 4 | 0.022728 | 0.035759 | chrX | 40328836 | 40328858 | + | intergenic:C 86-Gm146 |
| S | GGACCACTATTACGT AATCCTGG | GGACAACTATTTGGGAAT CCAGG | | 4 | 0.073007 | 0.001299 | chrX | 168802166 | 168802188 | + | intron:Arhg |

**Supplementary Table S9 Analysis of on target and off targets site reads**

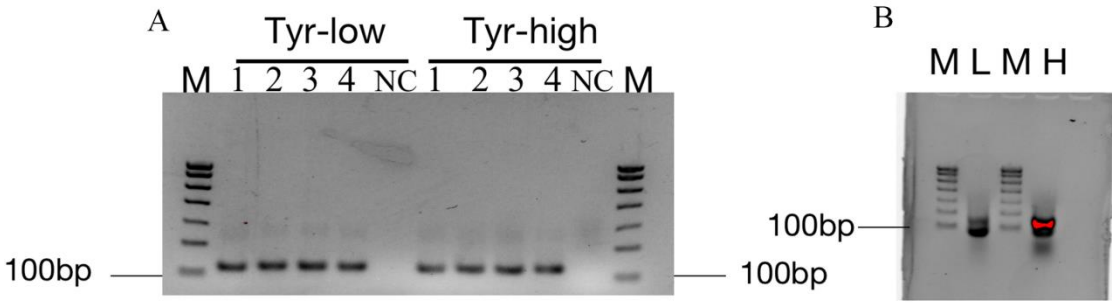
| Chr | Star | End | Reads | Sign |
|---|---|---|---|---|
| chr1 | 40730000 | 40740000 | 296 | A |
| chr5 | 149322206 | 149339358 | 319 | D |

| | | | | |
|---|---|---|---|---|
| chr7 | 87475000 | 87480000 | 2 | G |
| chr9 | 20951883 | 20960000 | 783 | H |
| chr9 | 105382206 | 105387206 | 1 | I |
| chr10 | 48520000 | 48525000 | 3 | K |
| chr10 | 110274174 | 110282206 | 18 | L |
| chr10 | 110282206 | 110294196 | 810 | L |
| chr11 | 52093512 | 52095000 | 557 | M |
| chr11 | 52095000 | 52110000 | 702 | M |
| chr11 | 67413855 | 67415000 | 364 | N |
| chr14 | 54398699 | 54415000 | 409 | O |
| chr15 | 76297382 | 76300000 | 2548 | P |
| chr15 | 76300000 | 76315000 | 336 | P |
| chrX | 40318836 | 40330000 | 875 | R |



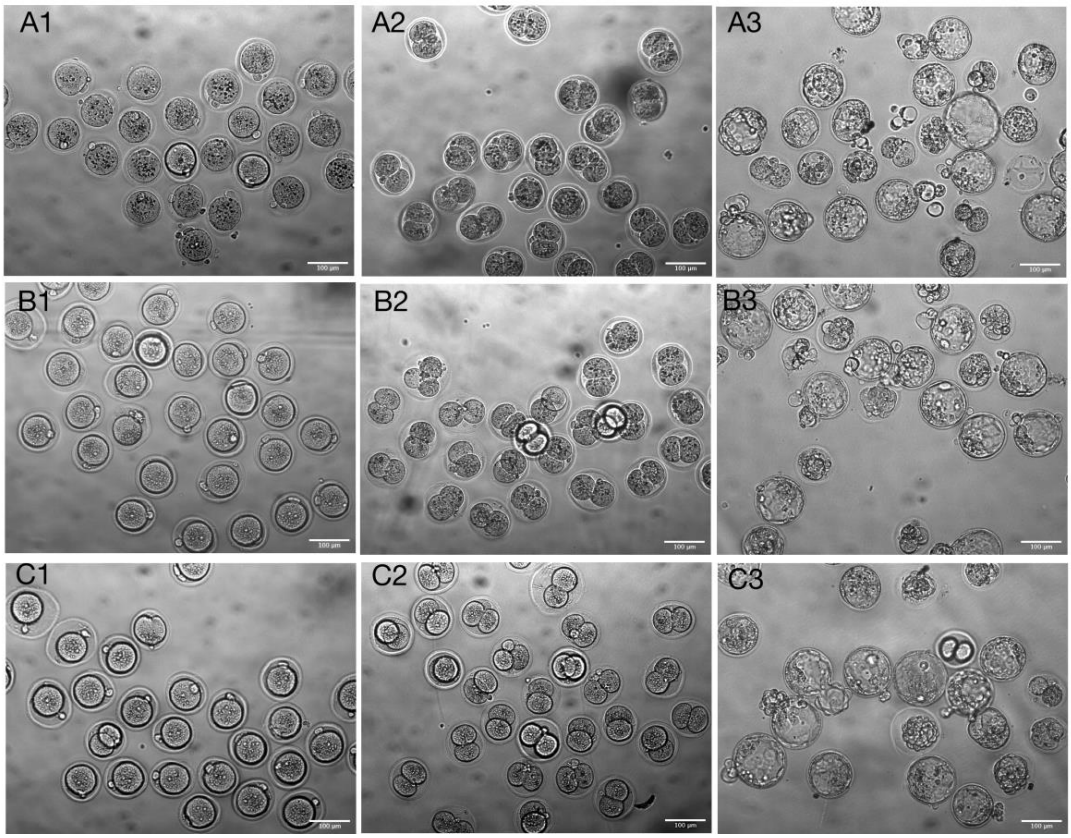

**Supplementary Figure S1. Synthesis of spCas9 mRNA.**

(A) Agarose gel for the linear pT7-Cas9 vector after enzyme digestion. M, DL10000 DNA marker. 1, pT7-Cas9 vector. 2, linear pT7-Cas9 vector after enzyme digestion. (B) Agarose gel map of spCas9 mRNA (2&3). M, DL 2000 DNA marker. 1, linear pT7-Cas9 vector. 2&3, spCas9 mRNA



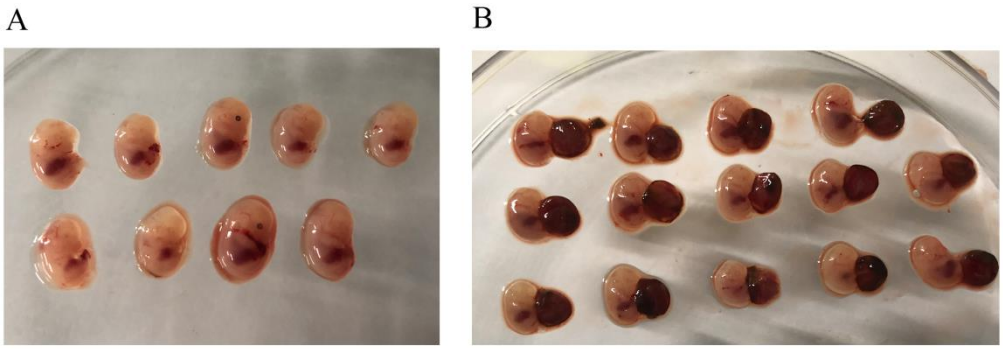

**Supplementary Figure S2. Design and synthesis of sgRNA.**

(A) sgRNA in vitro transcription template agarose gel map for Tyr-low group and Tyr-high group. M, 250 bp DNA Ladder. NC, Negative Control. (B) Synthesis of Tyr-low sgRNA(L) and Tyr-high sgRNA(H) agarose gel map. M, 250 bp DNA Ladder.



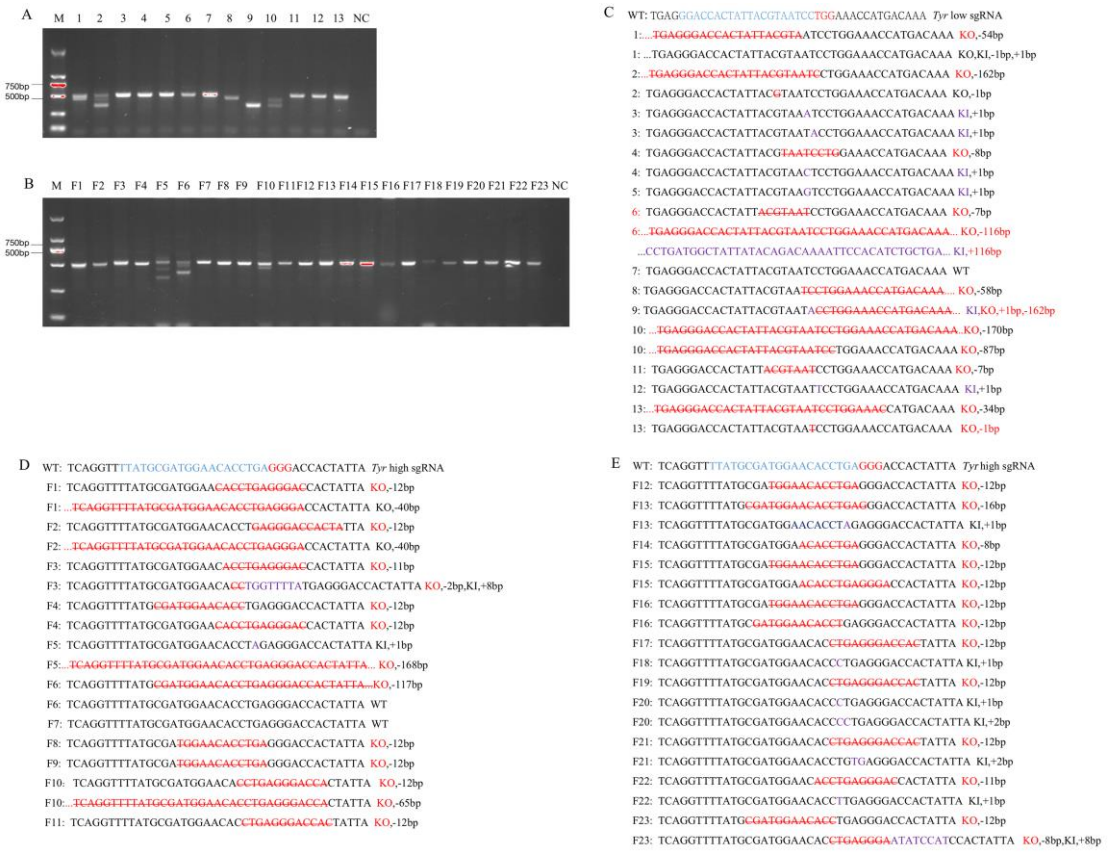

**Supplementary Figure S3. Example of embryo in vitro development of control, Tyr-low sgRNA injected group and Tyr-high sgRNA injected group.**

(A1~A3), In vitro development of uninjected embryos. A1, pronuclear stage. A2, 2-cell. A3, blastocyst. (B1~B3), In vitro development of embryos injected with Tyr low sgRNA. B1, prokaryotic period. B2, 2-cell. B3, blastocyst. (C1~C3), In vitro development of embryos injected with Tyr high sgRNA . C1, pronuclear stage. C2, 2-cell; C3, blastocyst



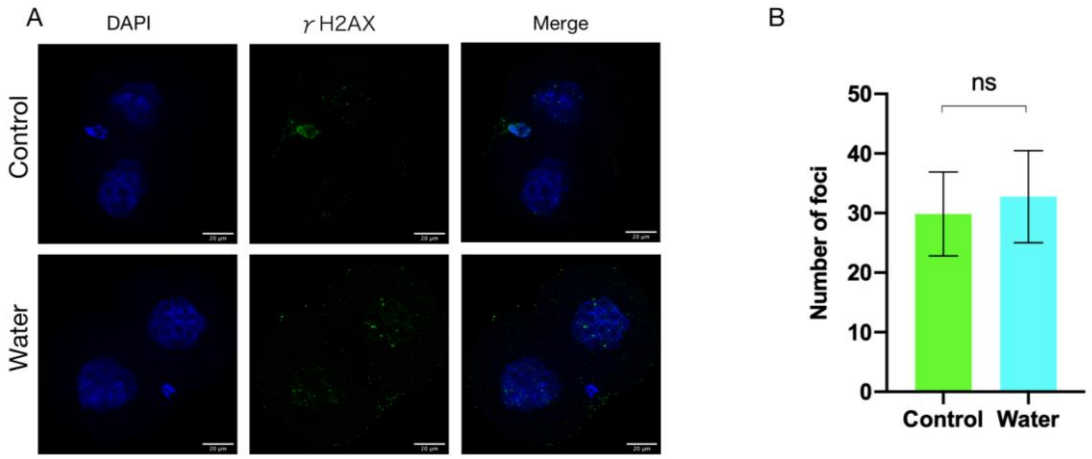

**Supplementary Figure S4.. Embryo in vitro development of three groups and the full term development of the transplanted embryos**

(A&B)13.5d fetus from embryos co-injected Spcas9 mRNA and *Tyr*-high sgRNA.




**Supplementary Figure S5 Agarose gel map and genotype of Tyr-low1-13# and Tyr-high1-23#**

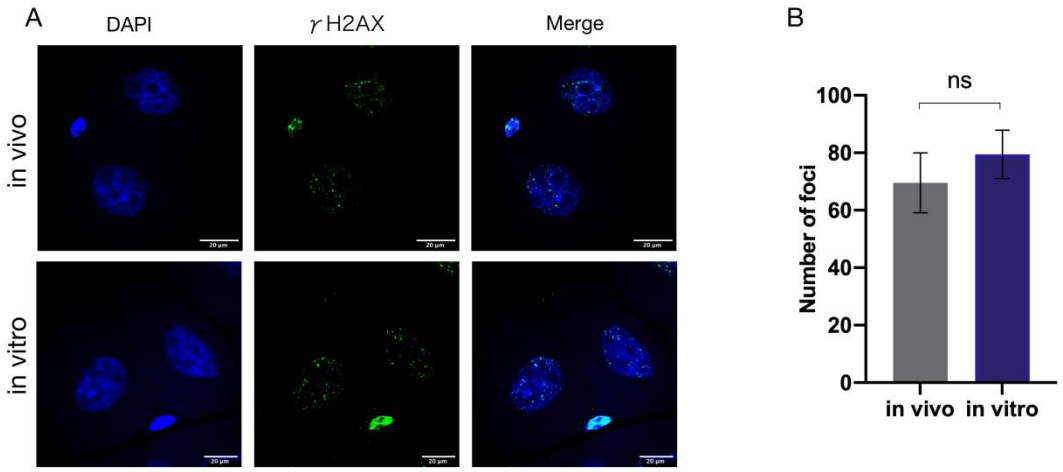
(A)Agarose gel map of full-term birth knockout mice in Tyr-low group. (B) Agarose gel map of 13.5d anatomical knockout mice in Tyr-high group. (C) Genotype analysis of 13 birth mice in Tyr low group. (D&E) Genotype analysis of 13.5d anatomical knockout fetus in Tyr high group.




**Supplementary Figure S6 Comparison of the number of foci of cytoplasmic injected water and unmanipulated mouse embryos at 18 h**

(A) γH2AX immunofluorescence staining of cytoplasmic injected water and unmanipulated (Control) mouse embryos cultured for 18 hours (bar=20μm, means ± sem). (B) γH2AX focus statistical histogram of cytoplasmic injected water and unmanipulated mouse embryos cultured for 18 hours.

(B)




**Supplementary Figure S7 Comparison of the number of foci of embryos in vivo and in vitro at 15 h**

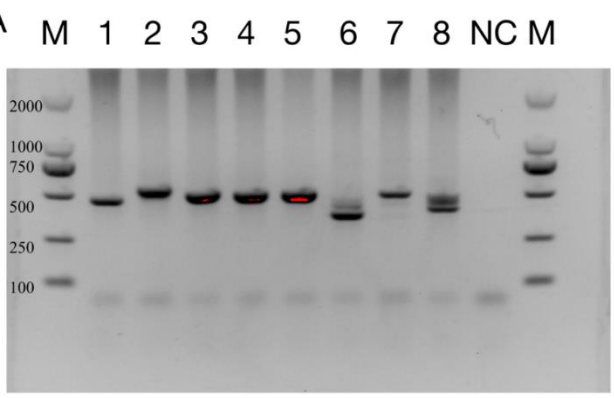
(A) γH2AX immunofluorescence staining of mouse early embryos cultured in vivo and in vitro for 15 hours (bar=20μm, means ± sem). (B) γH2AX focus statistical histogram of mouse early embryos cultured in vivo and in vitro for 15 hours.

| **Edit:** | **Control:** |
|---|---|
| **36660240 reads**; of these: | **12883306 reads**; of these: |
| 36660240 (100.00%) were paired; of these: | 12883306 (100.00%) were paired; of these: |
| 8669516 (23.65%) aligned concordantly 0 times | 2267614 (17.60%) aligned concordantly 0 times |
| 24070402 (65.66%) aligned concordantly exactly 1 time | 8738255 (67.83%) aligned concordantly exactly 1 time |
| 3920322 (10.69%) aligned concordantly >1 times | 1877437 (14.57%) aligned concordantly >1 times |
| ---- | ---- |
| 8669516 pairs aligned concordantly 0 times; of these: | 2267614 pairs aligned concordantly 0 times; of these: |
| 145369 (1.68%) aligned discordantly 1 time | 75309 (3.32%) aligned discordantly 1 time |
| ---- | ---- |
| 8524147 pairs aligned 0 times concordantly or discordantly; of these: | 2192305 pairs aligned 0 times concordantly or discordar of these: |
| 17048294 mates make up the pairs; of these: | 4384610 mates make up the pairs; of these: |
| 14290370 (83.82%) aligned 0 times | 3524661 (80.39%) aligned 0 times |
| 2382010 (13.97%) aligned exactly 1 time | 718799 (16.39%) aligned exactly 1 time |
| 375914 (2.20%) aligned >1 times | 141150 (3.22%) aligned >1 times |
| **80.51% overall alignment rate** | **86.32% overall alignment rate** |

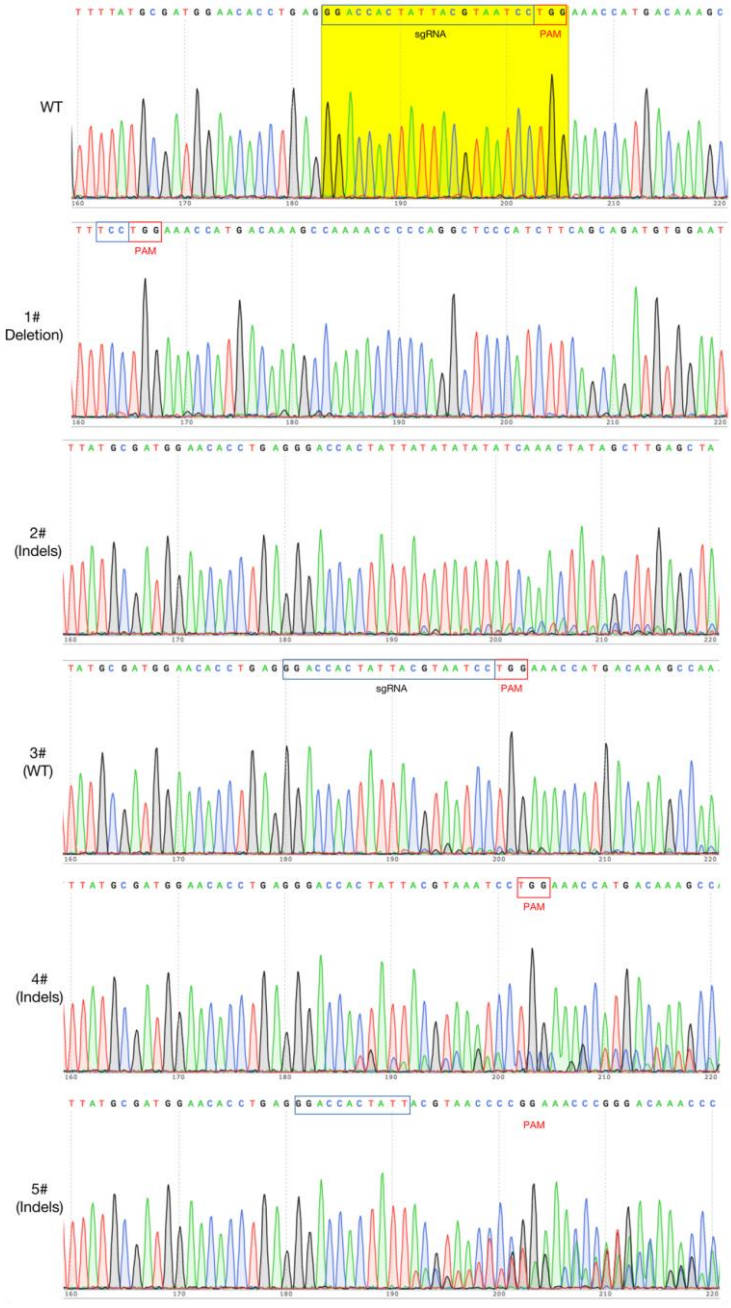
```
intronic    Adgrb3  chr1    25470000    25480000    0   0   comments:Control    0.0 comments:Edit   12.0
intronic    Prim2   chr1    33570000    33580000    0   0   comments:Control    0.0 comments:Edit   21.0
exonic  D630023F18Rik   chr1    65110000    65120000    0   0   comments:Control    0.0 comments:Edit   30.0
exonic  Mreg    chr1    72160000    72170000    0   0   comments:Control    0.0 comments:Edit   44.0
exonic  Tns1    chr1    73990000    74000000    0   0   comments:Control    0.0 comments:Edit   13.0
exonic  Iqca    chr1    90100000    90110000    0   0   comments:Control    0.0 comments:Edit   22.0
exonic  Gal3st2b    chr1    93940000    93950000    0   0   comments:Control    0.0 comments:Edit   33.0
exonic  Gli2    chr1    118850000   118860000   0   0   comments:Control    0.0 comments:Edit   13.0
intronic    Tmem163 chr1    127520000   127530000   0   0   comments:Control    0.0 comments:Edit   20.0
exonic  Dars    chr1    128360000   128370000   0   0   comments:Control    0.0 comments:Edit   16.0
exonic  Fcmr    chr1    130870000   130880000   0   0   comments:Control    0.0 comments:Edit   18.0
exonic  Rab7b   chr1    131700000   131710000   0   0   comments:Control    0.0 comments:Edit   21.0
exonic  Edem3   chr1    151800000   151810000   0   0   comments:Control    0.0 comments:Edit   16.0
exonic  Colgalt2    chr1    152500000   152510000   0   0   comments:Control    0.0 comments:Edit   12.0
exonic  Qsox1   chr1    155800000   155810000   0   0   comments:Control    0.0 comments:Edit   12.0
exonic  Kifap3  chr1    163910000   163920000   0   0   comments:Control    0.0 comments:Edit   16.0
```

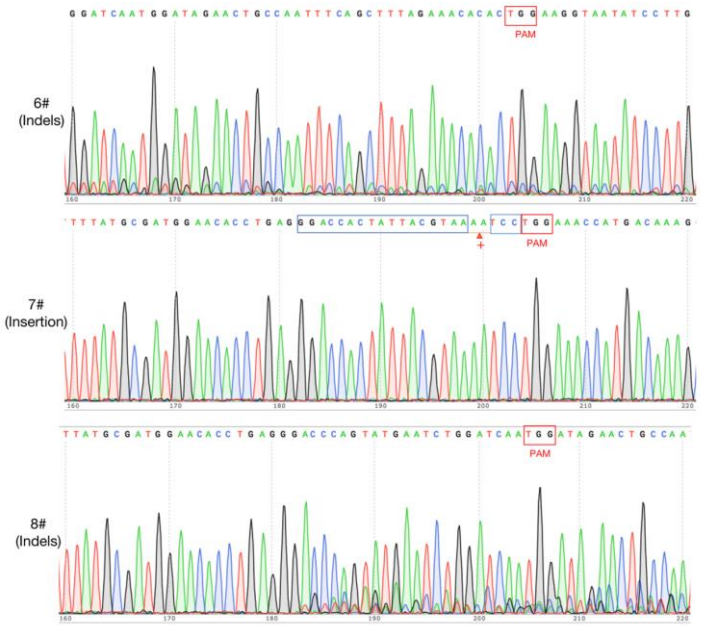
**Supplementary Figure S8 Annotation of sgRNA independent Random off target sites**(Exon，Intro, UTR, Control=0，Edit>10)

A


M 1 2 3 4 5 6 7 8 NC M
2000
1000
750
500
250
100

B

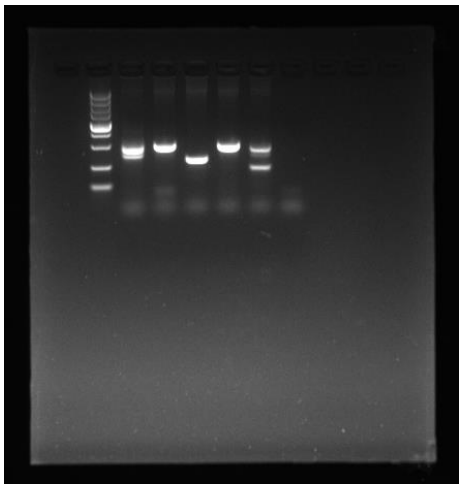

WT
sgRNA
PAM
1#
Deletion)
2#
(Indels)
3#
(WT)
4#
(Indels)
5#
(Indels)



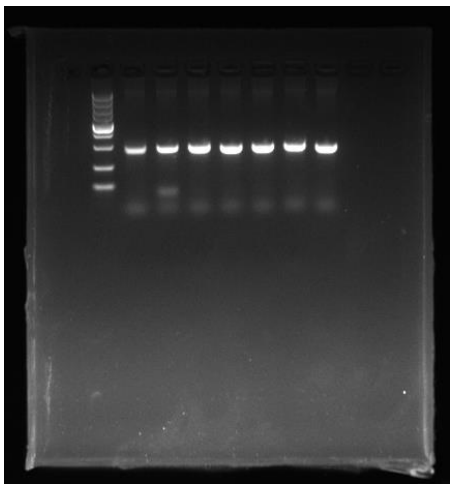

**Supplementary Figure S9 Agarose gel map and the DNA sequence of fragments of *Tyr*-low group in 15h after injection**

(A) Agarose gel map of Tyr-low group at 15h after CPI. M, DL2000 DNA Marker. NC, Negative Control.1-8#, *Tyr*-low group 2-cell embyro in 15h after injection. (B) Chromatograms of Tyr-low group at 15h after CPI. WT, Wild Type. 1-8#, *Tyr*-low group 2-cell embyro in 15h after injection. The PAM was boxed in red, the sgRNA squence was boxed in blue,

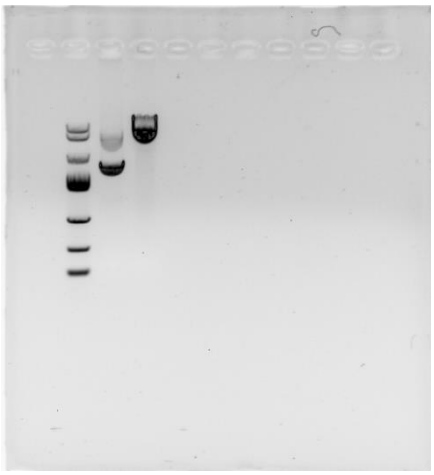
**Full -length gel in the article**



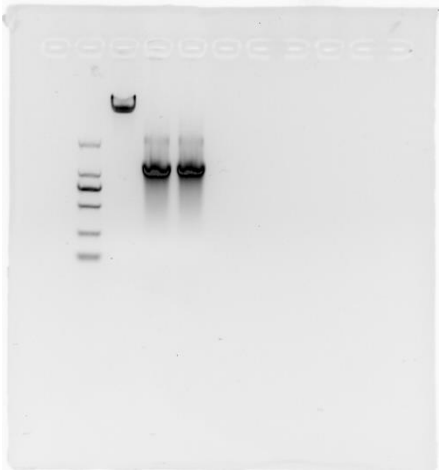
Full-length gel of Figure 1A up



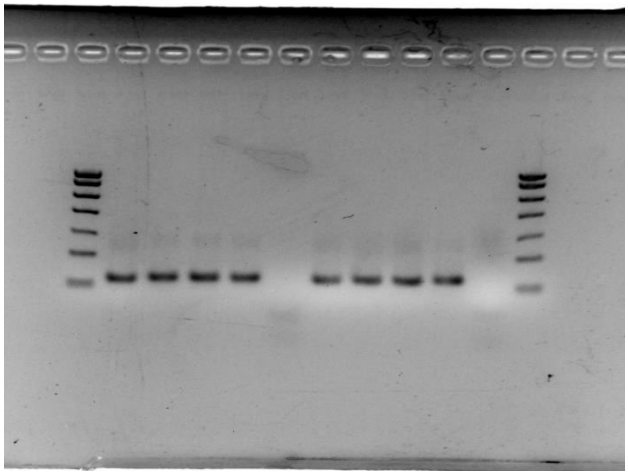
Full-length gel of Figure 1A down



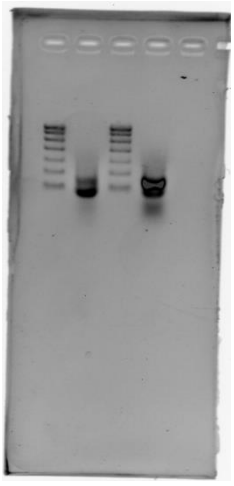
Full-length gel of Figure S1A



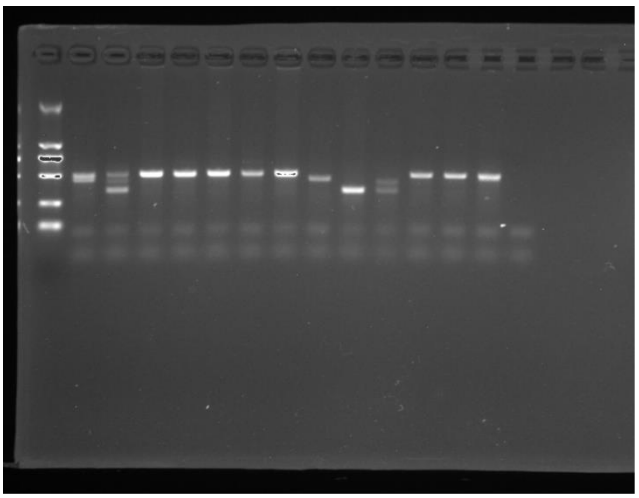
Full-length gel of Figure S1B



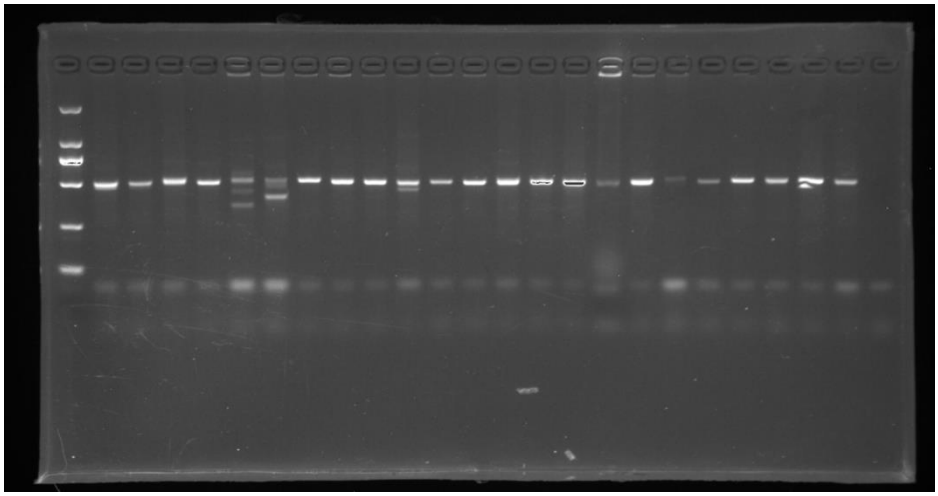
Full-length gel of Figure S2B



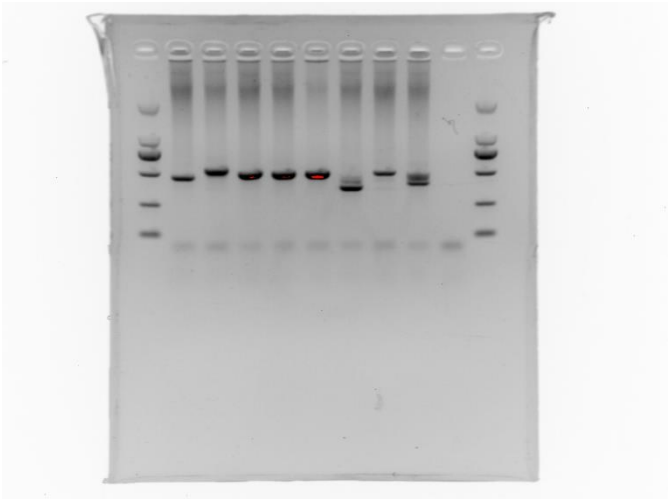
Full-length gel of Figure S2C



Full -length gel of Figure S4A



Full-length gel of Figure S4B



Full-length gel of Figure S9A