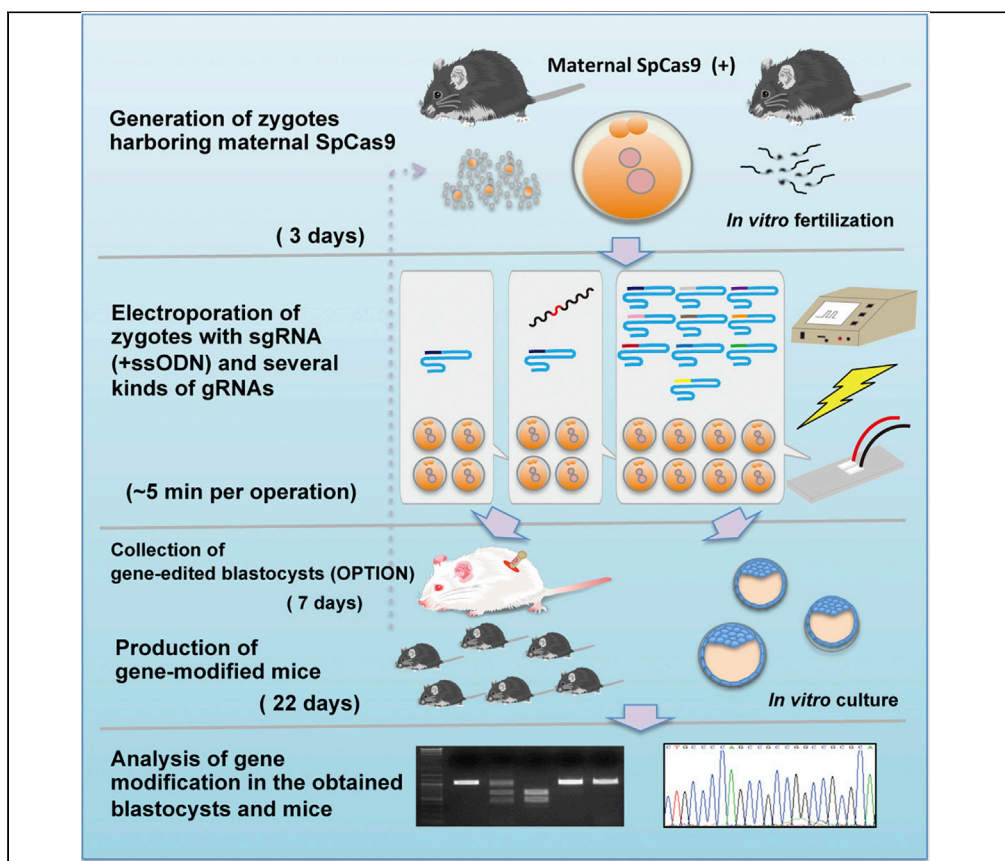


Protocol

Production of single- and multiple-gene-modified mice via maternal SpCas9-based gene editing



Maternally and transiently accumulated SpCas9 (maternal SpCas9) in a zygote derived from a systemically SpCas9-expressing transgenic mouse strain was used to generate single- and multiple-gene-modified mice. Maternal SpCas9-based gene editing allows for high indel and knockin mutation efficiency, low mosaicism, increased pup delivery rate, and simultaneous induction of mutations at multiple loci in contrast to conventional CRISPR/SpCas9-based gene editing.

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Highlights

Single- and multiple-gene-modified mice can be generated using maternal SpCas9

Higher indel and knockin mutation efficiency

Lower mosaicism and increased pup delivery

Mutations can be simultaneously induced in at least nine loci

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Protocol

Production of single- and multiple-gene-modified mice via maternal SpCas9-based gene editing

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SUMMARY

Maternally and transiently accumulated SpCas9 (maternal SpCas9) in a zygote derived from a systemically SpCas9-expressing transgenic mouse strain was used to generate single- and multiple-gene-modified mice. Maternal SpCas9-based gene editing allows for high indel and knockin mutation efficiency, low mosaicism, increased pup delivery rate, and simultaneous induction of mutations at multiple loci in contrast to conventional CRISPR/SpCas9-based gene editing. For complete details on the use and execution of this protocol, please refer to Sakurai et al. (2020).

BEFORE YOU BEGIN

This protocol describes the steps for the production of single-gene-modified and multiple-gene-modified mice using maternal SpCas9-containing zygotes from SpCas9 transgenic mice.

For single-gene-modified mice, one should use all the steps except step 16 of the [before you begin](#) and steps 1–8 of the [step-by-step method details](#).

For multiple-gene-modified mice, one should use all the steps in the [before you begin](#) and step 9 in the [step-by-step method details](#).

Mice

⌚ **Timing:** several months for sCAT

⌚ **Timing:** several weeks for ICR

All mouse experiments were conducted in accordance with institutional guidelines and were approved by the Animal care and Use committee of Shinshu University, Japan.

1. sCAT (Figure 1A)

We have established SpCas9 transgenic (Tg) mice (B6; B6D2-Tg (CAG-hCas9) 2Saku) that systemically express humanized (h) SpCas9 in almost all tissues, termed systemically Cas9-expressing Tg mice (sCAT) (Sakurai et al., 2016, 2020). In this strain, nine copies of the hSpCas9 transgene (CAG-hSpCas9) are inserted into exon 9 of the GTP-binding protein 10 (*Gtpbp10*) gene on chromosome 5. Since its establishment in 2013, sCAT has not exhibited any abnormalities in morphology



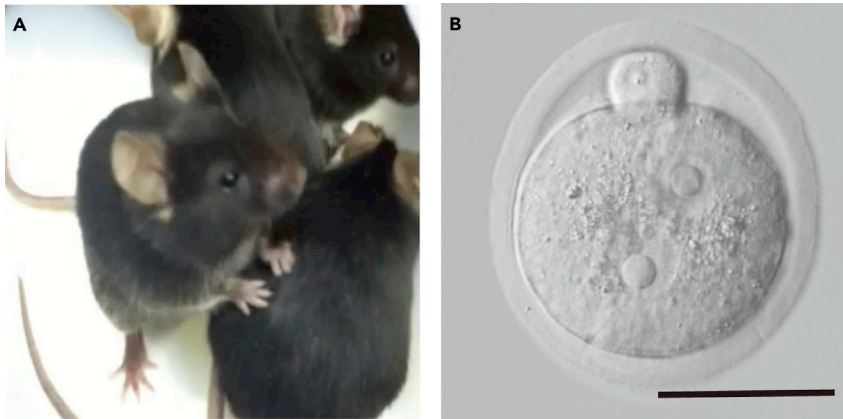


Figure 1. Systemically Cas9-expressing transgenic (sCAT) mouse strain

(A) Adult mice.

(B) Zygote at 6–7 h after fertilization. Bar = 50 μm .

or fertility. Furthermore, sCAT can be bred in either a Tg/+ or Tg/Tg form.

Single- and multiple-gene-modified mice can be generated by electroporating only sgRNAs (-+ssODN), without exogenous SpCas9 mRNA or protein, into zygotes containing maternal SpCas9. The zygotes are generated through conventional *in vitro* fertilization (IVF) methods generally using sCAT females (Tg/+ or Tg/Tg) and males (+/+) because eggs derived from sCAT females (Tg/+ or Tg/Tg) accumulate maternal SpCas9 during oogenesis. The amount of maternal SpCas9 then decreases from zygotes to 2-cell-stage embryos. Therefore, gene editing at the zygote developmental stage affects the efficiency of maternal SpCas9 gene editing. Zygotes are used at 6–7 h after fertilization (before the DNA synthesis phase) to achieve a high efficiency of maternal SpCas9 gene editing.

- a. Available from the RIKEN BioResource Center (<https://mus.brc.riken.jp/en/>, Cat# RBRC10984).
- b. Breed sCAT mice under a 12-h light/dark (7:00–19:00/19:00–7:00) cycle in an SPF environment.
- c. Prepare 3–4 week-old Tg/+ (or Tg/Tg) female and 8–15-week-old +/+ male animals to generate zygotes containing maternal SpCas9 (Figure 1B). 8–12 week-old Tg/+ (or Tg/Tg) female mice can also be used.

Alternatives: There are two additional reports on the generation of modified mice using maternal SpCas9 comprising both the mRNA and protein (Zhang, et al. 2016; Cebrian-Serrano et al., 2017). These, together with our previous reports (Sakurai et al., 2016, 2020), suggest that a mouse strain expressing SpCas9 during oogenesis can be used to generate zygotes containing maternal SpCas9. However, as the amount of maternal SpCas9 contained in the zygote differs between strains, nuclease activity would also be different.

2. ICR

Mice of the outbred ICR strain were used as foster and recipient.

- a. Purchase from a commercial animal breeder (e.g., CLEA Japan, Inc.) and breed until use.
- b. Breed ICR mice under a 12-h light/dark (7:00–19:00/19:00–7:00) cycle in an SPF environment.
- c. Prepare 8–16-week-old female and 8–20-week-old male animals to generate foster female mice.
- d. Prepare 8–16-week-old female and 8–20-week-old vasectomized male mice to generate recipient female mice. Details regarding the production of vasectomized mice can be found in various laboratory manuals/reference books (for example, Behringer et al., 2014).

sgRNA design and preparation

Ⓞ Timing: 4–6 days

sgRNA is composed of CRISPR RNA (crRNA) and tracrRNA. Numerous protocols have been reported for sgRNA preparation, and any of these can be used (for example, Pelletier et al. (2020)). Our procedure is based on *in vitro* sgRNA synthesis using T7 RNA polymerase and an sgRNA DNA template (Figure 2).

Herein, the design and preparation of sgRNA against the *klf5* gene is shown as an example (Sakurai et al., 2016, 2020).

⚠ CRITICAL: For sgRNA preparation, it is also possible to synthesize crRNA and tracrRNA separately and then anneal them. However, we observed that sgRNAs synthesized integrally (Figure 2) are more efficient for gene editing than those derived from annealing crRNA and tracrRNA using maternal SpCas9 (unpublished data).

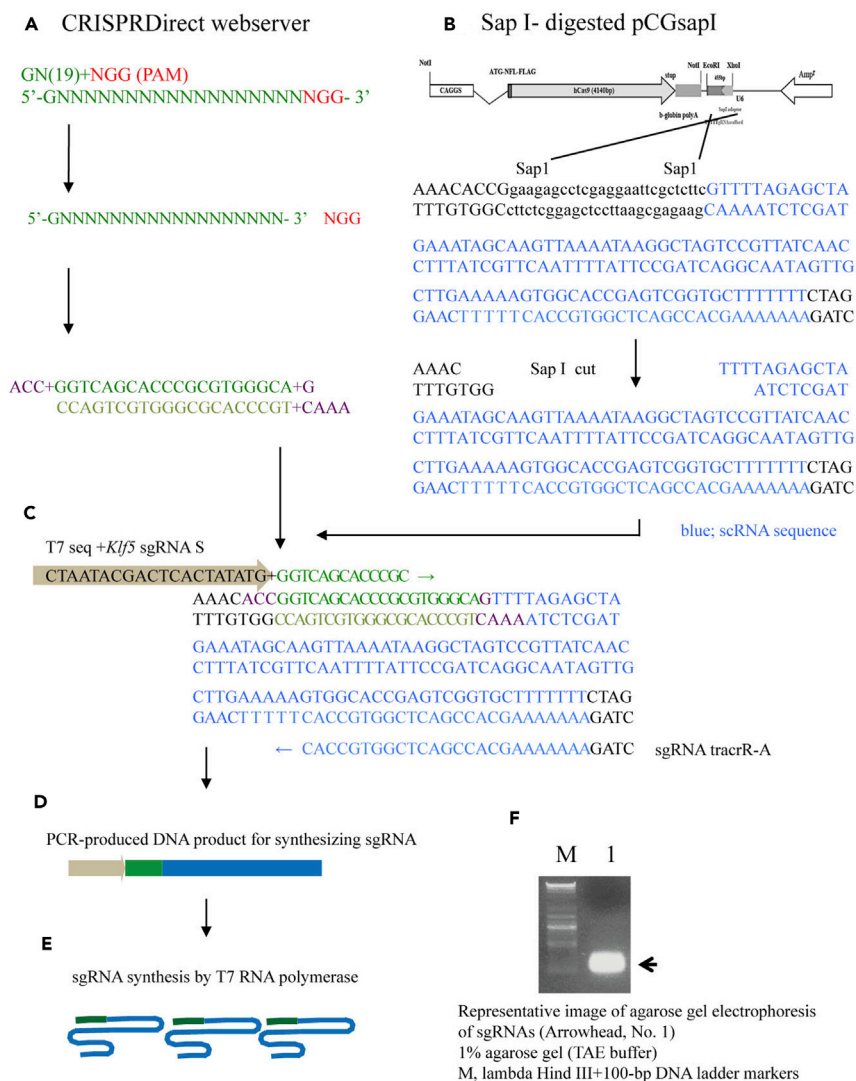


Figure 2. Schematic outline of representative sgRNA preparation for editing the target region of the *klf5* gene

3. Download the DNA sequence of the *klf5* gene from NCBI (<https://www.ncbi.nlm.nih.gov/gene/>).
 - a. Enter the name *klf5* in the query box.
 - b. Choose the house mouse *klf5* (ID12224).
 - c. Copy and save genomic data (GenBank) from the NCBI Reference Sequences (RefSeq) section.
4. Design an sgRNA oligo for the *klf5* gene via the CRISPRDirect webserver (Naito Y, et al., 2015; <https://crispr.dbcls.jp/>).
 - a. Open the CRISPRDirect webserver.
 - b. Enter the genomic DNA sequence obtained in the query box.
 - c. Select "NGG" in the PAM sequence requirement section.
 - d. Select "Mouse (Mus musculus) genome" in the Specificity check section.
 - e. Click on "design".
 - f. Choose 1–3 "target sequence" candidates (GN(19)NGG) of the gene region where sgRNA targeting is desired (GC%; more than 50).
 - g. Copy and save the target sequence, 5'-CCATGCCACGCGGGTGCTGACC-3', which is located around the translation start ATG codon this time (Figure 2A).
5. Order the following oligonucleotides based on the chosen target sequence (Figure 2A). ACC and G bases are added to the 5' and 3' ends of the guide sequence forward strand, respectively; CAAA bases are added to the 5' end of the guide sequence reverse strand (Figure 2A). We typically use Integrated DNA technology (IDT) for that.
 - a. *klf5* sgRNA 1: ACCGGTCAGCACCCGCGTGGGCAG
klf5 sgRNA 2: AAACTGCCACGCGGGTGCTGACC
 T7 seq+ *klf5* sgRNA S: TCTAATACGACTCACTATAG+GGTCAGCACCCG
 sgRNA tracrR-A: CTAGAAAAAAGCACCGACTCGGTGCCAC
 - b. Open IDT the webpage and order.
 - i. Custom oligonucleotide
 - ii. 25 nmol scale
 - iii. 100 μM in TE buffer (pH 8.0)
 - iv. Standard-desalted
6. Anneal and clone the *klf5* sgRNA oligos (Figure 2A).
 - a. Mix the following reagents:

Reagent	Amount
100 μM <i>klf5</i> sgRNA 1	2.5 μL
100 μM <i>klf5</i> sgRNA 2	2.5 μL
1M NaCl /0.5 M TrisCl (pH 7.5) buffer	2.5 μL
ddH ₂ O (double-distilled water)	Up to 25 μL

- b. Heat the mixture for 5 min at 95°C and then keep it at 21°C–25°C for 30 min.
- c. Store on ice until use.

7. Linearize the pCGSapI vector by SapI digestion (NEB). Our vector, pCGSapI (SapI-digested), contains the crRNA sequences (Sakurai et al., 2016; 2020, Figure 2B).
 - a. Mix the following reagents:

Reagent	Amount
pCGSap I vector	10 μg
NEB CutSmart buffer	4 μL
Sap I (10 unit/μL)	2–3 μL
ddH ₂ O (double-distilled water)	Up to 40 μL

- b. Incubate for 2 – 15 h at 37°C.
- c. Electrophorese the reaction solution in a 1% agarose gel (TAE buffer).
- d. Gel-purify the linearized vector using the FastGene Gel/PCR Extraction Kit (Nippon Genetics, FG-91302).
- e. Quantify DNA using a spectrophotometer (GeneQuant 100, GE).
- f. Store on ice until use.

▮▮ **Pause point:** The linearized vector can be stored at –20°C for up to a year.

8. Ligate the annealed oligos and the linearized vector with a DNA Ligation Kit Ver.2.1 (TAKARA #6022) (Figures 2A and 2C).
 - a. Mix the following reagents:

Reagent	Amount
Annealed oligos	1 μL
Linearized pCGsapI	50 ng
Ligation solution I	3 μL
ddH ₂ O (double-distilled water)	Up to 6 μL

- b. Incubate for 2 h at 16°C.

9. Transform competent bacteria (JM109, Dh5a) with 3–6 μL of the ligation solution and plate the bacteria on an LB-Ampicillin (50–100 μg/mL) agar plate.
10. Incubate the plate for 12 – 15 h at 37°C.
11. Perform a colony PCR of 3–5 bacterial colonies to select those harboring the *kif5* sgRNA oligos using Tks Gflex DNA polymerase (TAKARA, #R060A).
 - a. Prepare the following PCR solution (per bacterial colony):

Reagent	Amount
25 μM T7 seq+ <i>kif5</i> sgRNA S	0.2 μL
25 μM sgRNA tracrR-A	0.2 μL
Tks Gflex buffer (x2)	10 μL
Tks Gflex (1.25 unit/μL)	0.5 μL
ddH ₂ O (double-distilled water)	9.1 μL

- b. Introduce a portion of a bacterial colony in the PCR solution of each tube. Number the bacterial colonies added.
- c. Run the colony PCR as follows:

PCR step	Temperature	Time	Cycles
Initial denaturation	95	2 min	1
Denaturation	98	10 s	35
Annealing	55	15 s	
Extension	68	30 s	
Final Extension	-	-	-

- d. Electrophorese the PCR solution (5–10 μL) in a 2% agarose gel (TAE buffer). Observe the PCR product from positive colonies (expected size: 243 bp).

- e. Inoculate 2–3 positive bacterial colonies separately in 2 mL of LB-Ampicillin (50–100 µg/mL) medium and incubate for 8 – 12 h at 37°C.
12. Isolate and purify the *klf5* sgRNA oligo-ligated pCGsapI using a QIAprep Spin Miniprep Kit (- QIAGEN #27106) in accordance with the manufacturer’s instructions.
 - a. Use 1–2 mL bacterial culture media for each tube.
 - b. Elute the plasmids by adding 50 µL TE buffer to the center of each QIAprep 2.0 spin column.
 - c. Quantify the DNA concentration using a spectrophotometer (GeneQuant 100)
 - d. The purified plasmids can be used for DNA sequencing.
 - e. Electrophorese 2 µL of the purified DNA solution in a 1% agarose gel (TAE buffer) to assess DNA quality.
 13. Confirm the *klf5* sgRNA sequence using Sanger sequencing.
Perform sequencing using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, #4337455) with a T7 primer-5' AATACGACTCACTATAG 3', in accordance with the manufacturer’s instructions.
 - a. Submit to the support center of our university or commercially available contractors for sequencing.
 - b. Analyze the sequencing data using the free multiple sequence alignment web program CLUSTALW (<https://www.genome.jp/tools-bin/clustalw>) in accordance with the manufacturer’s instructions.
 14. Prepare the DNA template with the T7 sequence added to support sgRNA synthesis (Figures 2C and 2D).
 - a. Mix the following reagents:

Reagent	Amount
Sequencing-confirmed vector	10–20 ng
25 µM T7 seq+ <i>klf5</i> sgRNA S	0.4 µL
25 µM sgRNA tracrR-A	0.4 µL
Tks Gflex buffer (x2)	50 µL
Tks Gflex (1.25 unit/µL)	1 µL
ddH ₂ O (double-distilled water)	Up to 100 µL

- b. Run the two-step PCR as follows:

PCR step	Temperature	Time	Cycles
Initial denaturation	95	2 min	1
Denaturation	98	10 s	40
Annealing/Extension	68	20 s	
Final Extension	68	20 s	1

- c. Electrophorese the reaction solution in a 2% agarose gel (TAE buffer).
- d. Gel-purify the PCR products (expected size: 243 bp) using the FastGene Gel/PCR Extraction Kit (Nippon Genetics) and elute them by adding 50 µL TE buffer to the center of the kit’s spin column.
- e. Quantify DNA using a spectrophotometer (GeneQuant 100)
- f. Electrophorese 1–3 µL of the purified DNA solution in a 2% agarose gel (TAE buffer) to assess DNA quality.
- g. Adjust the DNA concentration to approximately 50 ng/µL in TE buffer and store on ice until use.

Pause point: The prepared DNA template with the T7 sequence can be stored at –20°C for up to a year.

15. Synthesize and purify the *klf5* sgRNA using the MEGA Shortscript T7 Transcription Kit (Invitrogen) and the MEGAclear Kit (Invitrogen), both in accordance with the manufacturer's instructions (Figure 2E).
 - a. Mix the following reagents:

Reagents (75% DPBS) 10 mL	Amount
DPBS (-)	7.5 mL
Water (Merck, Cat# W1503-500ML)	2.5 mL

Reagents (MEGA shortscript T7 transcription kit)	Amount
T7 seq-added DNA template solution	4–5 μ L
10 \times T7 Reaction buffer	4 μ L
10 \times ATP	4 μ L
10 \times CTP	4 μ L
10 \times GTP	4 μ L
10 \times UTP	4 μ L
T7 Enzyme MIX	4 μ L
RNase-free water (included in the kit)	Up to 40 μ L

- b. Incubate for 4 h at 37°C
- c. Add 2 μ L of TURBO DNase (included in the kit) and incubate for 15 min at 37°C
- d. Purify the *klf5* sgRNAs using the MEGAclear Kit (Invitrogen)
- e. Elute the *klf5* sgRNAs by adding 50 μ L of 75% DPBS (85°C–95°C) to the center of the MEGAclear kit's spin column
- f. Repeat step d again. One hundred microliters of *klf5* sgRNA solution in 75% DPBS would be obtained.
- g. Electrophorese 1–2 μ L of the purified *klf5* sgRNA solution in a 1% agarose gel (TAE buffer) to assess RNA quality (Figure 2F)
- h. Quantify the sgRNA concentration using a spectrophotometer (GeneQuant 100, GE). Store at –80°C until use. The synthesized sgRNA should be adjusted to 1–2 μ g/ μ L in 75% DPBS.

Pause point: The prepared sgRNAs can be stored at –80°C for up to a year.

16. Repeat steps 3–15 of [before you begin](#) for each sgRNA of the other genes. In [Sakurai et al. \(2020\)](#), we synthesized 12 kinds of sgRNA for 12 mouse genes, including *klf5*.

100–200-nt ssODN design and preparation

⌚ Timing: 4–6 days

Single-stranded oligodeoxynucleotides (ssODNs) were synthesized commercially (e.g., via IDT). To generate 100–200-nt ssODNs, 50–100-nt regions of DNA sequences flanking the third nucleotide on the guiding side of the selected sgRNA's PAM sequence were annealed with a Tag sequence (for example, FLAG or loxP sequences with a restriction nuclease site, such as *Cla*I or *Eco*RI) desired to be knocked-in (KI) into the target DNA region. Either forward or reverse ssODN sequences were used.

Herein, the design and preparation of ssODN for KI of a 3 \times FLAG sequence into the *klf5* gene is shown as an example ([Sakurai et al., 2020](#)).

17. Use the genomic DNA data (step 3 of [before you begin](#)) and the selected sgRNA sequence (step 4 of [before you begin](#))
 - a. Search 50–100-nt regions of DNA sequences flanking the third nucleotide on the guide sequence side of the sgRNA PAM sequence
 - b. Add the 3× FLAG sequence and *Clal* site

As a result, the following ssODN sequence (*klf5*+ 3× FLAG, 169 nt) was set:

5'-GGATCGCGATCGCTCCGTGTC~~CCGCTCCCGTAATCCCCAGACCGTCCATGGACTACAAAGA~~
~~CCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAGGGACCCAC~~
 GCGGGTGTGACCATGAGCGCCCGCTGGGACCACTG CCCCAGCC -3'

Bold: *Clal* site, **Underlined:** 3× FLAG

18. Order the ssODN. We typically use IDT.
 - a. Open the IDT webserver
 - b. Order the ssODN
 - i. Ultramer technology
 - ii. 4 nmol scale
 - iii. Lyophilized
 - iv. Desalted
 - c. Adjust to 2–3 µg/µL in 75% DPBS and store at –80°C until use.

PCR primer design and condition for on-target genotyping

⌚ **Timing: 4–6 days**

Numerous webserver are available for specific primer design for the sgRNA-targeted genomic region (e.g., Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>), Primer 3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>)). The PCR primers are designed against 200–600-bp regions flanking the target sequence.

19. Open Primer-BLAST webserver
 - a. Use the genomic DNA data (step 3 of [before you begin](#)) and the selected sgRNA sequence (step 4 of [before you begin](#))
 - b. Enter the genomic DNA sequence in the query box
 - i. PCR product size: 400–1200 bp
 - ii. T_m , min: 60 to max: 65
 - iii. Database: RefseqRNA or Genome
 - iv. Organism: *Mus musculus*
 - v. Others: default
 - c. Click on “Get Primers” and select candidate primer pairs based on the PCR product size and the specificity of the sgRNA-targeted genomic region. If the appropriate primers cannot be set, change the parameters, and try again. The following primer pair was selected:
 - i. CR(*klf5*)-1S: GGTACGCGCTCTTCTTAGTGT
 - ii. CR(*klf5*)-1A: GCGTGTTTCAGATCGTCTCC
 - d. Open the IDT webpage and order:
 - i. Custom oligonucleotide
 - ii. 25 nmol scale
 - iii. 100 µM in TE buffer (pH 8.0)
 - iv. Standard-desalted

20. Perform PCR for on-target genotyping

- a. Use the Tks Gflex DNA polymerase kit (TAKARA) in accordance with the manufacturer's instructions. Prepare the following PCR reagents per sample:

Reagent	Amount
Mouse genomic DNA*	100 ng
25 μ M CR(<i>klf5</i>)-1S	0.2 μ L
25 μ M CR(<i>klf5</i>)-1A	0.2 μ L
Tks Gflex buffer (x2)	0.2 μ L
Tks Gflex (1.25 unit/ μ L)	0.5 μ L
ddH ₂ O (double-distilled water)	Up to 25 μ L

* Genomic DNA was extracted from the ear or tail tip of the sCAT mouse.

- b. Run the PCR as follows:

PCR step	Temperature	Time	Cycles
Initial denaturation	95	2 min	1
Denaturation	98	10 s	30–35
Annealing/Extension	68	40 s	
Final Extension	68	40 s	1

- c. Electrophorese 2.5 μ L of the PCR solution in a 1% agarose gel (TAE buffer).
 d. Observe the PCR products (expected size, 1113 bp)
 e. Purify the remaining PCR products using FastGene Gel/PCR Extraction kit (Nippon Genetics) and elute the PCR products by adding 25 μ L TE buffer to the center of the kit's spin column. The purified PCR products can be used for analyzing DNA double-strand break (DSB) activity of the *klf5* sgRNA as a DNA template.

PCR primer design and condition for off-target genotyping (optional)

⌚ Timing: 4–6 days

- Numerous methods are available for predicting off-target DNA regions (e.g., Cas-Offinder, <http://www.rgenome.net/cas-offinder/>). We generally use CRISPRDirect (Naito, et al., 2015; <https://crispr.dbcls.jp/>) to predict candidate off-target sequences. The PCR primers are designed against 200–600 bp regions flanking the candidate sequence.
- Repeat steps 3 and 4 of [before you begin](#).
- Click on "number of target site" of the candidate *klf5* target sequence, 5'-CCATGCC-CACGCGGGTGCTGACC-3', and analyze "both sides"
- Check the "off-target sequence" and check the candidates. Copy and save 3–5 "off-target sequence" candidates. The following ones were selected:
 - klf5*Offt1: Chr4:46452023-46452037, CCTTGCCCACGCGGGCTCCCCGG
 - klf5*Offt2: Chr11:102467168-10246718, CCCTGCCCACGCGGGGCTCTTGG
 - klf5*Offt3: Chr3:96197318-96197328, CCCTGCCCACGCACCCAGACAA
- Click on "Chr location (ex. Chr4:46452023-46452037)" to obtain the genomic DNA sequence information around the off-target sequence.
- Copy and save the flanking 600-bp (total size: 1200 bp) regions of genomic DNA around the off-target sequence
- Design PCR primers for off-target genotyping using the Primer-BLAST webserver
 - Perform the method described in step 19 of [before you begin](#)
 - The following primers were selected:
 - klf5*Offt1-1: GGTGTTCGGGTCCCTGAG,

- klf5*Offt1-2: CTCACCCGGCGGAGGAAG, expected size: 559 bp
- ii. *klf5*Offt2-1: GCCCAGGCTTGCCTTGTATGT
- klf5*Offt2-2: GGAAAAGCGCGATGAGGCAG, expected size: 508 bp
- iii. *klf5*Offt3-1: GCGAAAATGCGTCTCGCTGC
- klf5*Offt3-2: TCGGAGAGACGATAGGGCTCTG, expected size: 456 bp
28. Order the PCR primers (as in step 19 of [before you begin](#))
 29. Perform PCR for off-target genotyping
 - a. Repeat as described in step 20 of [before you begin](#)
 - b. Electrophorese 2.5 μ L of the PCR solution in a 1% agarose gel to assess the quality of PCR products
 - c. Purify the remaining PCR products using the FastGene Gel/PCR Extraction kit (Nippon Genetics) and elute the PCR products by adding 25 μ L TE buffer to the center of the kit's spin column. The purified PCR products can be used for Sanger sequencing.

Analyses of *in vitro* DSB activity of the sgRNA against the on-target sequence

⌚ Timing: 2 h

We utilize the *in vitro* assay using Cas9RNP to estimate the DNA double-strand break (DSB) activity of the synthesized sgRNA. The selection of sgRNAs with high DSB activity is an important factor for the successful production of genetically modified mice.

30. Use PCR products including the sgRNA target region from the wild-type genomic DNA sample (step 20e of [before you begin](#)) and adjust their concentration to 20–30 ng/ μ L in ddH₂O.
31. Prepare the following Cas9RNP solution per sample:

Reagent	Amount
<i>klf5</i> sgRNA (0.1–0.2 μ g/ μ L 75% DPBS)	2 μ L
SpCas9 protein (0.5 μ g/ μ L 75% DPBS)	1 μ L
10 \times NEB3 buffer	1.5 μ L
ddH ₂ O (double-distilled water)	8.5 μ L

32. Store the Cas9RNP solution at 21–25°C for 20 min or more.
33. Add 2 μ L of PCR solution (step 30 of [before you begin](#)) to 13 μ L of the Cas9RNP solution per sample.
34. Incubate the mixture at 37°C for 1 h.
35. Add 1 μ L of Proteinase K (1 μ g/ μ L ddH₂O) to the mixture and incubate it at 37°C for 15 min.
36. Electrophorese the Cas9RNP-treated PCR solution in a 1%–2% agarose gel (TAE buffer) ([Figure 3](#)).
37. Estimate (Select) an sgRNA with high DSB activity for subsequent gene editing in mouse zygotes.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
sCAT mouse	Sakurai et al., (2016, 2020)	RIKEN Resource Center (RBRC10984)
ICR mouse	CLEA Japan, Inc.	ICR (SPF)

(Continued on next page)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Continued		
Chemicals, peptides, and recombinant protein		
Albumin from bovine serum (BSA)	Merck	Cat# A-3311-10G
Alt-R spCas9 V3	IDT (Integrated DNA Technologies)	Cat# 1081058
Calcium chloride dihydrate (CaCl ₂ · 2H ₂ O)	Merck	Cat# C7902
Cl ₂ I	New England Biolabs	Cat# R0197
D(+)-Glucose	Merck	Cat# G7021-100G
DPBS	Thermo Fisher	Cat# 14190144
EDTA · 2Na	FUJIFILM Wako	Cat# 343-01861
Gelatin	Merck	Cat# G2500-100G
HEPES (mouse embryo tested)	Merck	Cat# H6147-100G
Human chorionic gonadotropin (hCH)	ASKA Animal Health Co., Japan	Cat# GONATROPIN
Kanamycin	Merck	Cat# K1377-5G
L-Glutamine	Merck	Cat# G8540-25G
Magnesium sulfate heptahydrate (MgSO ₄ · 7H ₂ O)	FUJIFILM Wako	Cat# 131-00405
Opti-MEM	Thermo Fisher	Cat# 31985062
Paraffin Liquid (Specially Prepared Reagent)	Nacalai Tesque Inc.	Cat# 26137-85
Phenol red	Merck	Cat# P0290-100ML
Potassium chloride (KCl)	FUJIFILM Wako	Cat# 163-03545
Potassium dihydrogen phosphate (KH ₂ PO ₄)	FUJIFILM Wako	Cat# 169-04245
Pregnant mare serum gonadotropin (PMSG)	ASKA Animal Health Co., Japan	Cat# SEROTROPIN
Proteinase K	Merck	Cat# 3115836001
Sap I	New England Biolabs	Cat# R0569
Sodium bicarbonate (NaHCO ₃)	Merck	Cat# S5761-500G
Sodium chloride (NaCl)	FUJIFILM Wako	Cat# 191-01665
Sodium DL-lactate	Merck	Cat# L4263-100ML
Sodium hydroxide (NaOH)	FUJIFILM Wako	Cat# 198-13765
Sodium pyruvate	Merck	Cat# P4562-25G
Tks Gflex DNA Polymerase	Takara, Inc.	Cat# R060A
Trizma base	Merck	Cat# T1503
Trizma hydrochloride	Merck	Cat# T5941
Tween-20	Tokyo Chemical Industry Co., Ltd.	Cat# 9500-64-5
T7 Endonuclease I	New England Biolabs	Cat# M0302S
Water	Merck	Cat# W1503-500ML
Yeast tRNA	Thermo Fisher	Cat# AM7119
Critical commercial assays		
BigDye™ Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems	Cat# 4337455
FastGene Gel/PCR Extraction kit	Nippon Genetics Co., Ltd.	Cat# FG-91302
Mighty TA-cloning Reagent Set Kit	Takara, Inc.	Cat# 6019
Milllex GP Filter Unit (0.22 mm)	Merck	Cat# SLGP033RS
MEGashortscript T7 Transcription Kit	Invitrogen	Cat# AM1334
MEGAclear Kit	Invitrogen	Cat# AM1908
QIAprep Spin Miniprep Kit	QIAGEN	Cat# 27106
DNA Ligation kit Ver.2.1	Takara, Inc.	Cat# 6022
Oligonucleotides		
klf5Offt1-1: GGTGTTCCGGGTCCTGAG	Synthesized by IDT	N/A
klf5Offt1-2: CTCACCCGGCGGAGGAAG	Synthesized by IDT	N/A
klf5Offt2-1: GCCCAGGCTTGCTTGATGT	Synthesized by IDT	N/A
klf5Offt2-2: GGAAAAGCGCGATGAGGCAG	Synthesized by IDT	N/A

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>klf5</i> Offt3-1: GCGAAAATGCGTCTCGCTGC	Synthesized by IDT	N/A
<i>klf5</i> Offt3-2: TCGGAGAGACGATAGGGCTCTG	Synthesized by IDT	N/A
<i>klf5</i> sgRNA 1: ACCGGTCAGCACCCG CGTGGGCAG	Synthesized by IDT	N/A
<i>klf5</i> sgRNA 2: AAAGTCCCACGCGG GTGCTGACC	Synthesized by IDT	N/A
M13 Rev: CAGGAAACAGCTATGAC	Synthesized by IDT	N/A
M13 M4: GTTTTCCCAGTCACGA	Synthesized by IDT	N/A
T7 seq+ <i>klf5</i> sgRNA S: TCTAATACGAC TCACTATAGGGTCAGCACCCG	Synthesized by IDT	N/A
sgRNA tracrR-A: CTAGAAAAAAGCACCGACTCGGTGCCAC	Synthesized by IDT	N/A
T7: AATACGACTCACTATAG	Synthesized by IDT	N/A
CR(<i>klf5</i>)-1S: GGTACGCGCTCTTCTTAGGTT	Synthesized by IDT	N/A
CR(<i>klf5</i>)-1A: GCGTGTTTCAGATCGTCTCC	Synthesized by IDT	N/A
<i>klf5</i> + 3x FLAG: GGATCGCGATCGCT CCGTGTCGGCTCCGTAATCCCCA GACCGTCCATGGACTACAAAGACCA TGACGGTGATTATAAAGATCATGACA TCGATTACAAGGATGACGATGACAA GGGACCCACGCGGTGCTGACCAT GAGCGCCCGCTGGGACCACTG CCCCAGCC	Synthesized by IDT	N/A
Recombinant DNA		
pCGsap I	Sakurai et al., (2016, 2020)	N/A
Software and algorithms		
CRISPRdirect	Naito et al., (2015)	https://crispr.dbcls.jp/
ClustalW	Kyoto University Bioinformatics Center	https://www.genome.jp/ tools-bin/clustalw
ICE	Synthego Corporation	https://www.synthego.com/
NCBI	NIH	https://www.ncbi.nlm.nih. gov/gene/
Primer-BLAST	NIH	https://www.ncbi.nlm.nih. gov/tools/primer-blast/
Others		
Cover glass	Matsunami Glass Ind., Ltd.	Cat# C218181
Electroporator	BEX Co., Tokyo, Japan	Cat# CUY21EDIT II
Platinum electrodes	BEX Co., Tokyo, Japan	Cat# LF501PT1-10
Stereoscopic microscope	Olympus, Tokyo, Japan	Cat# SZX16

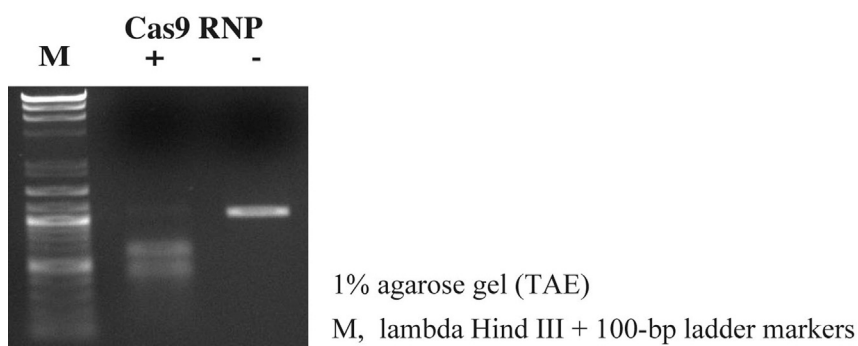


Figure 3. Representative analysis of the *in vitro* DNA double-strand break (DSB) activity of synthesized *klf5* sgRNAs
The size of the PCR product is 1113 bp. The 462 bp and 651bp cleavage PCR products are expected from the DSB activity of *klf5* sgRNAs. Lane Cas9RNP(+) shows a PCR product with DSB, and lane Cas9RNP(-) shows a PCR product without DSB (negative control).

MATERIALS AND EQUIPMENT

Culture media

Stock solution

Reagent	Conc.	Stock solution*	Up to 10 mL by water	Expiration time
NaCl	1.0 M	58.44	0.584 g	3 months
KCl	0.1 M	74.55	0.075 g	3 months
KH ₂ PO ₄	0.1 M	136.09	0.136 g	3 months
MgSO ₄ · 7H ₂ O	0.1 M	246.48	0.246 g	3 months
Lactate-Na	1.117 M (60% sol. 10 dil.)	112.1	1 mL	3 months
Glucose	0.1 M	180.16	0.198 g	1 week
Pyruvate-Na	0.1 M	110	0.11 g	1 week
NaHCO ₃	1.0 M	84.01	0.84 g	1 week
CaCl ₂ · 2H ₂ O	0.171 M	147.01	0.25 g	2 weeks
Glutamine	0.2 M	146	0.292 g	1 week
EDTA	10 mM	372.24	0.037 g	
HEPES	1 M	238.31	2.383 g	
NaOH	0.5 M	40	0.2 g	
Phenol red	0.5%			
Kanamycin	4 mg/mL		0.04 g	(Store at -20°C)
	* Store at 4°C			

Media

mHTF+BSA (expiration time: 2 weeks)KSOM+BSA, (exp. time 2 weeks)KSOM+HEPES+BSA, (exp. time 2 weeks)

mHTF+BSA (10 mL)

Stock sol.	Dosage	Final conc.
1.0 M NaCl	1.017 mL	101.7 mM
0.1 M KCl	469 µL	4.69 mM
0.1 M KH ₂ PO ₄	40 µL	0.4 mM
0.1 M MgSO ₄ · 7H ₂ O	20 µL	0.2 mM
0.117 M Lactate-Na	160 µL	183 mM
0.1 M Glucose	278 µL	2.78 mM
0.1 M Pyruvate-Na	34 µL	0.34 mM
1.0 M NaHCO ₃	250 µL	25 mM
0.171 M CaCl ₂ · 2H ₂ O	300 µL	5.14 mM
0.5% Phenol red	10 µL	
4 mg/mL Kanamycin	100 µL	40 µg/mL
	Up to 10 mL by water	
	Add BSA 40 mg	
	Filtrate 0.22 µm	

KSOM+BSA (10 mL)

Stock sol.	Dosage	Final conc.
1.0 M NaCl	950 µL	95 mM
0.1 M KCl	250 µL	2.5 mM
0.1 M KH ₂ PO ₄	35 µL	0.35 mM
0.1 M MgSO ₄ · 7H ₂ O	20 µL	0.2 mM
0.117 M Lactate-Na	85 µL	10 mM
0.1 M Glucose	20 µL	20 mM

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Continued

KSOM+BSA (10 mL)

0.1 M Pyruvate-Na	20 μ L	20 mM
1.0 M NaHCO ₃	250 μ L	25 mM
0.171 M CaCl ₂ · 2H ₂ O	100 μ L	1.71 mM
0.2 M Glutamine	50 μ L	1 mM
10 mM EDTA	10 μ L	0.01 mM
0.5% Phenol red	10 μ L	
4 mg/mL Kanamycin	100 μ L	40 μ g/mL
	Up to 10 mL with water	
	Add 10 mg BSA	
	Filter through 0.22 μ m	

KSOM+HEPES+BSA (10 mL)

Stock sol.	Dosage	Final conc.
1.0 M NaCl	950 μ L	95 mM
0.1 M KCl	250 μ L	2.5 mM
0.1 M KH ₂ PO ₄	35 μ L	0.35 mM
0.1 M MgSO ₄ · 7H ₂ O	20 μ L	0.2 mM
0.117 M Lactate-Na	85 μ L	10 mM
0.1 M Glucose	20 μ L	20 mM
0.1 M Pyruvate-Na	20 μ L	20 mM
1.0 M NaHCO ₃	25 μ L	2.5 mM
0.171 M CaCl ₂ · 2H ₂ O	100 μ L	1.71 mM
0.2 M Glutamine	50 μ L	1 mM
10 mM EDTA	10 μ L	0.01 mM
1 M HEPES	200 μ L	20 mM
0.5 M NaOH	160 μ L	8 mM
0.5% Phenol red	10 μ L	
4 mg/mL Kanamycin	100 μ L	40 μ g/mL
	Up to 10 mL with water	
	Add 10 mg BSA	
	Filter through 0.22 μ m	

Alternatives: Solutions similar to KSOM+BSA are commercially available (e.g., ARK Resource Co., Ltd., Kumamoto, Japan).

STEP-BY-STEP METHOD DETAILS

- ⌚ **Timing:** 3 days: Preparation and gene editing of maternal SpCas9-containing zygotes; zygote transfer into pseudopregnant mice
- ⌚ **Timing:** 7 days: Collection of gene-edited blastocysts
- ⌚ **Timing:** 22 days: Gestation of gene-modified mice

Details regarding IVF, zygote transfer, and cesarean sections can be found in various laboratory manuals/previous reports (e.g., [Behringer et al., 2014](#)). In this protocol, these procedures are described only with regard to the key points. ([Table 1](#))

Generating mice with single-gene modifications

- ⌚ **Timing:** 3 days for step 1

Table 1. Timetable for the generation of mice with single/multiple gene modifications

Step no.	Days	Time	Action
1	-3	18:30	PMSG injections (superovulation treatment of sCAT female)
2	-2	Evening	Mating of ICR female with male (foster female)
2	-1	Morning	Confirmation of mating (foster female)
3		Evening	Mating of ICR female with vasectomized male (recipient female)
1		18:00–18:20	hCG injections (sCAT female)
3	0	Morning	Confirmation of mating (recipient female)
1		7:30–9:30	IVF (superovulation-induced sCAT female)
1		13:30	IVF (selection of zygotes)
4, 9		15:30–16:30	Electroporation (single sgRNA or several sgRNAs, etc.)
5		~17:30	Oviduct transfer of electroporated sCAT zygotes
6	4	Optional	Blastocyst analysis
7	18	Morning	Delivery of foster mother ICR
7	19	Afternoon	Delivery of recipient mother ICR. If mice do not give birth by the afternoon, cesarean section should be carried out. The pups can be raised in foster ICR.
8	40–49		Offspring genotyping: analysis of gene modifications in offspring mice

⌚ Timing: 10 min for step 1d

⌚ Timing: 30 min for step 1e

⌚ Timing: 10 min for step 1f

⌚ Timing: ~40 min for step 1g

⌚ Timing: 2 days for step 2

⌚ Timing: 2 days for step 3

⌚ Timing: ~20 min for step 4a-d

⌚ Timing: ~10 min for step 4e-f

⌚ Timing: ~5 min for step 4g-i

⌚ Timing: ~5 min for step 4j-n

⌚ Timing: 3–5 min per operation (1 h after zygote collection; around 15:30–16:30) for step 4o-r

⌚ Timing: ~5 min per operation for step 4s-u

⌚ Timing: 1–3 h for step 5

⌚ Timing: 3–4 h for step 6

⌚ Timing: 1–3 h for step 7

⌚ Timing: 2 h (T7EI), 2–3 days (Seq) for step 8c

⌚ Timing: 2 h (Cas9RNP), 4–5 days (TA-cloning and sequencing), 2 days (sequencing and ICE) for step 8f

⌚ Timing: 2 h (RE), 4–5 days (TA-cloning and sequencing) for step 8m

1. Generation of Zygotes Harboring Maternal SpCas9

- a. Day –3 Evening (Superovulation; 18:30); inject 5–7.5 IU pregnant mare serum gonadotropin (PMSG) intraperitoneally into 3–5 sCAT female mice (3–4-week-old (Tg/+ or Tg/Tg, depending on the purpose) or 10 sCAT female mice (8–12-week-old (Tg/+ or Tg/Tg depending on the purpose) (See [troubleshooting 1](#)).

Note: For 3–4 week-old sCAT females, approximately 30–50 eggs were obtained per mouse. For 8–12 week-old sCAT females, approximately 10–30 eggs were obtained per mouse.

Note: When the IVF pair of sCAT female (Tg/+) × male (+/+) is used, pups with and without the SpCas9 transgene can be obtained.

- b. Day –1 evening (superovulation; 18:00–18:20); inject 5–7.5 IU human chorionic gonadotropin (hCG) intraperitoneally into the sCAT females primed with PMSG on day –3.
- c. Day –1 evening; for day 0 IVF implementation:
 - i. Set up culture dishes. Place several 100- μ L droplets (for sperm culture) and several 200- μ L droplets (for egg culture) of mHTF+BSA in a 3.5-cm Petri dish and layer droplets with paraffin oil to prevent evaporation.
 - ii. Place the dish in a CO₂ incubator (5% CO₂, 37°C) for 12–13 h.
- d. Day 0 (07:30–08:00; sperm collection):
 - i. Remove a dish for sperm incubation from the CO₂ incubator.
 - ii. Collect the sperm mass from the cauda epididymis of a sCAT male (+/+), place it in 100- μ L mHTF+BSA drops on the sperm incubation dish and return the dish to the CO₂ incubator.
- e. Day 0 (08:00–09:20; egg collection):
 - i. During sperm incubation (1.5–2 h), collect the egg mass from the ampulla of the oviduct tube of superovulating sCAT females.
 - ii. Place them in the 200- μ L mHTF+BSA drops on the egg culture dish and return the dish to the CO₂ incubator.
- f. Day 0 (09:30); insemination:
 - i. Remove dishes for sperm and egg incubation from the CO₂ incubator.
 - ii. Using a 20- μ L pipettor, add sperm solution containing approximately 8000–20,000 sperms to 200- μ L mHTF+BSA drops containing eggs in the egg culture dish. We used a hemocytometer to estimate sperm counts.
 - iii. Return the dish to the CO₂ incubator until use (next part g-ii).
 - iv. After insemination, prepare the required number of dishes to be used for culturing zygotes. Place 9 × 30- μ L drops of KSOM+BSA media per 3.5-cm dish and layer with paraffin oil to prevent evaporation (zygote culture dish).
 - v. Maintain the dishes in a CO₂ incubator (5% CO₂, 37°C) for 4 h until use (next part g-v).
- g. Day 0 (4 h after insemination around 13:30; zygote collection):
 - i. Place several 100–200- μ L drops of KSOM+HEPES+BSA in the dish (zygote-washing dish). Use this dish for washing zygotes.
 - ii. Remove the egg culture dishes from the CO₂ incubator.
 - iii. Collect the eggs released from the second polar body from the drops in the dishes, using a self-made embryo handling pipette from a Pasteur glass tube (tip diameter, 150–200 μ m), the tip of which should be tapered using a gas burner.
 - iv. Wash polar body-released eggs in the drops in the zygote-washing dish.
 - v. Subsequently, remove the zygote culture dish prepared in the morning from the CO₂ incubator and collect the eggs extruded from the second polar body in the drops in the dishes.

- vi. Return the dishes to the CO₂ incubator and maintain the dishes at 37°C in a 5% CO₂ environment for 1 h until the electroporation step for gene editing (step 4j).

Note: In our results, the ratio of second polar body extruded eggs per the total number of eggs was approximately 50%–90%.

2. Prepare the foster parent ICR females

We performed ICR mating on day –2 to obtain females one day after delivery at day +18 as a foster. The mating performance achieved in our laboratory was approximately 50%.

- a. Day –2 evening; mate 4–8 ICR females (8–16-week-old) in estrus and ICR males (8–20-week-old) to obtain the 2–4 foster parent ICR females.
- b. Day –1 morning (at approximately 09:00); check the plug of ICR females mated with males. Females with plugs should be separated from males and kept in one breeding cage.

3. Prepare the recipient parent ICR females

We performed ICR mating at day –1 to obtain females for zygote transfer at day +19 as a recipient. The mating performance achieved in our laboratory was approximately 50%.

- a. Day –1 evening; mate 6–12 ICR females (8–16-week-old) in estrus and vasectomized ICR males (8–20-week-old) to obtain the 3–6 zygote-recipient parent ICR females.
- b. Day 0 morning (from approximately 07:00–09:00); check the plug of ICR females mated with vasectomized males. Females with plugs are separated from males and kept in one breeding cage.

4. Electroporation

Day 0, preparation of the electroporation solution for delivering sgRNA or sgRNA+ssODN into the zygote

- a. Prepare 20 μL of *klf5* sgRNA solution (50–400 ng/μL in 75% DPBS) or 20 μL of *klf5* sgRNA + ssODN solution (50–400 ng/μL + 800 ng/μL in 75% DPBS) in a 0.2 mL PCR tube.
20 μL of (50–400 ng/μL) *klf5* sgRNA solution

Reagent	Amount
<i>klf5</i> sgRNA (2 μg/μL 75% DPBS) (before you begin 15h)	0.5–4 μL
75% DPBS	19.5–16 μL

or 20 μL of sgRNA + ssODN solution (50–400 ng/μL + 800 ng/μL

Reagent	Amount
<i>klf5</i> sgRNA (2 μg/μL 75% DPBS) (before you begin 15h)	0.5–4 μL
75% DPBS	17–13.5 μL
<i>klf5</i> + 3× FLAG ssODN (2 μg/μL) (before you begin 18c)	2.5 μL

- b. Heat the tube at 75°C for 5 min and then keep it at 21°C–25°C for 5–10 min.
- c. Thereafter, add 20 μL of Opti-MEM (final sgRNA concentration, 25–200 ng/μL or sgRNA + ssODN of 25–200 ng/μL + 400 ng/μL in a 1:1 mixture of 75% DPBS and Opti-MEM).
- d. Keep on ice until use.
Day 0, electroporator setting (Figure 4)
- e. Set up the electroporation system for gene editing (Figures 4A–4F).
 - i. A stereoscopic microscope (Olympus SZX16, Tokyo, Japan)
 - ii. An electroporator (CUY21EDIT II electroporator; BEX Co., Tokyo, Japan) (Figures 4B–4C)
 - iii. A pair of platinum electrodes set in a glass plate (#LF501PT1-10; 1-mm gap, 10-mm length, 3-mm width, and 0.5-mm height; BEX Co.) (Figure 4D)
 - iv. A self-made embryo-handling pipette (Figure 4E), as described in the zygote collection section (step 1g.-iii of [step-by-step method details](#))

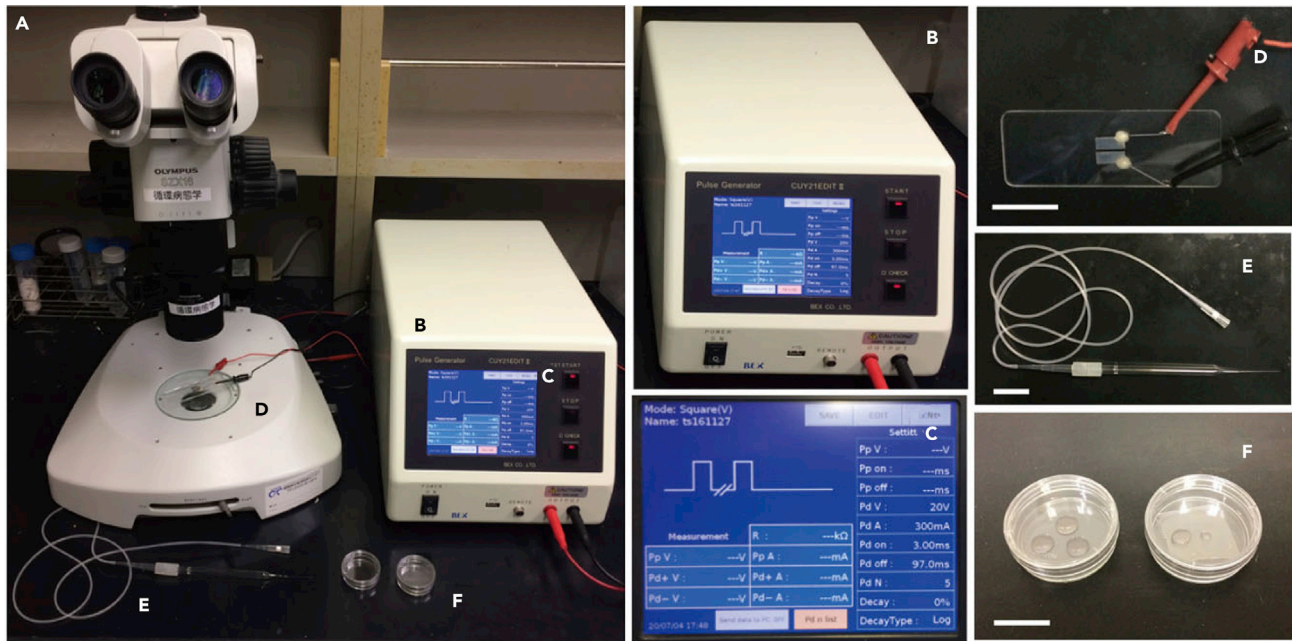


Figure 4. Setup of the electroporation system for the delivery of sgRNAs or sgRNA+ssODN into sCAT zygotes

- (A) Electroporation system used in this study.
 (B) CUY21EDIT II electroporator (BEX Co., Tokyo, Japan).
 (C) Display of the CUY21EDIT II.
 (D) Electroporation chamber with platinum electrodes (#LF501PT1-10; BEX Co.).
 (E) A self-made embryo handling pipette.
 (F) Two dishes for washing zygotes before and after electroporation. Bar = 2 cm.

- f. Turn on the power of the CUY21EDIT II and upload the program.
 - i. Electroporation conditions (Figure 4C)
 - ii. Constant 20 V
 - iii. Switching between on and off for 3 ms and 97 ms, respectively
 - iv. 5 cycles

Alternatives: Electroporators from other manufacturers can be used (e.g., Nepa Gene Co., Ltd., Chiba, Japan). However, the electroporation conditions need to be optimized by investigators.

Day 0, preparation of the two dishes for washing zygotes before and after electroporation (Figures 4F and 5)

- g. For zygote washing dish I, place several 200- μ L droplets of KSOM+HEPES+BSA in the 3.5-cm dish (Figure 5A).
- h. For zygote washing dish II (Figure 5B)
 - i. First, place a glass cover slip (Matsunami Glass Ind., Ltd., Osaka, Japan) in the 3.5-cm dish.
 - ii. Thereafter, place a 100- μ L drop of 1:1 medium (75% DPBS:Opti-MEM) (arrow, Figure 5B) on the cover slip.
 - iii. Then place a 5–10 μ L drop of sgRNA or sgRNA+ssODN solution (arrowhead, Figure 5B) on the cover slip.
- i. Cover the dish with the lid to prevent evaporation of the medium when not in use.
 Day 0, washing sCAT zygotes immediately before electroporation

△ CRITICAL: These operations should be performed in the shortest possible time to reduce stress on the zygotes.

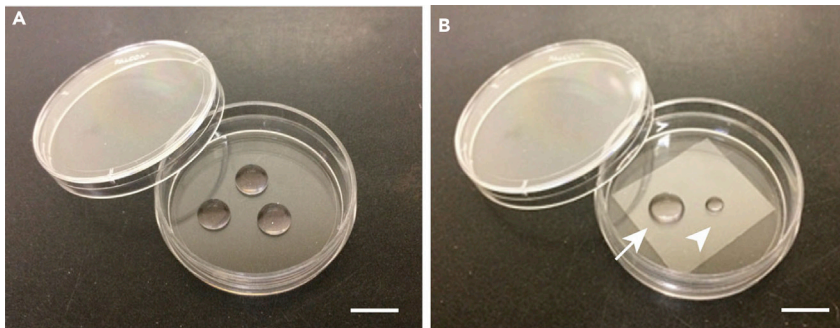


Figure 5. The two 3.5-cm dishes for washing zygotes before and after electroporation

(A) Zygote washing dish I.

(B) Zygote washing dish II. Arrow, 100- μ L drop of 1:1 medium of 75% DPBS and Opti-MEM. Arrowhead, 5–10 μ L drop of sgRNA or sgRNA+ssODN solution. Bar = 1 cm.

- j. Remove the zygote culture dish from the CO₂ incubator.
 - k. Transfer 10–40 zygotes to the drop in washing dish I and wash them using a self-made embryo-handling pipette from a Pasteur glass tube (tip diameter, 150–200 μ m; [Figure 4E](#)).
 - l. Select zygotes containing two pronuclei and a second polar body ([Figure 1B](#)).
 - m. Thereafter, transfer the selected zygotes to the 1:1 medium drop in washing dish II and transfer them to the drop containing sgRNA or sgRNA+ssODN in washing dish II.
 - n. Hold them in this drop until electroporation.
- Day 0, implementation of electroporation for sCAT zygotes ([Figure 6](#))

△ CRITICAL: These operations should be performed rapidly to reduce stress on the zygotes.

CAUTION: To avoid an electric shock, never touch the platinum electrodes when you press the electroporator program switch.

- o. Fill the gap between the electrodes ([Figures 4D](#) and [6A](#)) with 5 μ L of sgRNA or sgRNA+ssODN solution.
- p. Thereafter, transfer the zygotes (10–40) in the drop in washing dish II into the gap between the electrodes ([Figure 6B](#)).
- q. Arrange them in a row as shown in [Figures 6B](#) and [6C](#).
- r. Press the program switch and let the current flow once (See [troubleshooting 2](#)).

Note: In our results, the EDIT II electroporator records approximately 100–200 mA.

Day 0, washing sCAT zygotes immediately after electroporation

△ CRITICAL: Transfer the electroporated zygotes to the drop in washing dish I ([Figure 5A](#)) in the shortest possible time, as the Opti-MEM is harmful to the zygotes.

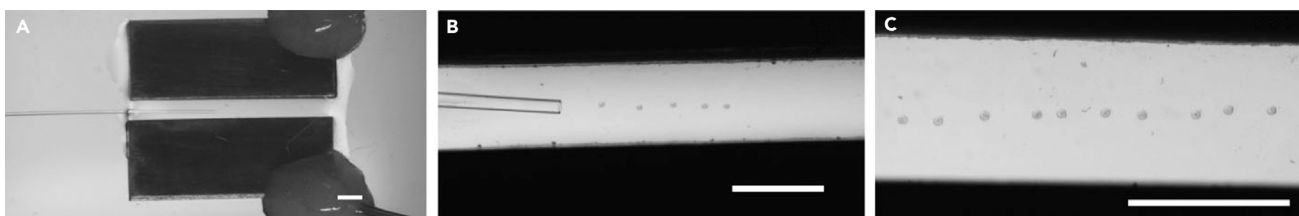


Figure 6. Electroporation chamber with platinum electrodes, and placement of the sCAT zygotes between electrodes

(A) A platinum electrodes set in a glass plate (#LF501PT1-10; BEX Co.).

(B and C) sCAT zygotes are arranged in a straight line. Bar = 1 mm.

- s. Transfer the electroporated zygotes to the drop in washing dish I using a self-made embryo-handling pipette.
 - t. Keep them at 21°C–25°C for 5 min and then return them to the drop in the zygote culture dish.
 - u. Return the dish to the CO₂ incubator.
5. sCAT zygote transfer (1 h after electroporation [Day 0; around 17:30])
The electroporation-treated zygotes are transplanted into the oviducts of recipient ICR females via the conventional method of mouse embryo oviduct transfer (for example, [Behringer et al., 2014](#)), approximately 1 h after the electroporation treatment. Zygote transfer methods can be found in various laboratory manuals/previous reports (e.g., [Behringer et al., 2014](#)). In this protocol, this procedure has been described only with regard to the key points.
- a. Transfer 10–20 zygotes in both oviducts (5–10 zygotes per oviduct) of the embryo-recipient ICR females (step 3 of [step-by-step method details](#)).

Note: Embryo-recipient ICR females lacking swollen ampulla of the uterine tube were not used for zygote transplantation.

Alternatives: For analysis at the blastocyst stage, zygote culturing continued for 4 days without zygote transplantation (step 6 of [step-by-step method details](#)).

6. (OPTIONAL) a blastocyst assay for time-saving indel or KI genotyping ([Sakurai et al., 2014](#))
 - a. Use the blastocysts derived from the electroporation-treated zygotes cultured *in vitro* for 4 days without zygote transplantation (See [troubleshooting 3](#))
 - b. Transfer a blastocyst into each 0.2-mL PCR tube using a self-made embryo handling pipette.
 - c. Add 10–12 µL of the blastocyst lysis solution to each tube.
Lysis solution 100 mL (Autoclaved and stored at –20°C)

Reagent	Amount
1M Tris HCl (pH 8.3)	10 mL
1M KCl	10 mL
Tween 20	0.45 mL
Gelatin	0.02 mg
ddH ₂ O (double-distilled water)	Up to 100 mL

Blastocyst lysis solution 1 mL (Store on ice until use)

Reagent	Amount
Lysis solution	1 mL
tRNA (10 mg/mL, Ambion)	6 µL
Proteinase K (10 µg/µL ddH ₂ O)	12.5 µL

- d. Heat at 56°C for 10 min and then at 95°C for 10 min.
- e. Store on ice until use.

▮▮ **Pause point:** The heated solution can be stored at –20°C for several months.

- f. Use the Tks Gflex DNA Polymerase Kit (TAKARA) in accordance with the manufacturer's instructions. Prepare the following PCR reaction reagents per sample:

Reagent	Amount
Blastocyst lysis solution	2.5 μ L
25 μ M CR(<i>kIf5</i>)-1S	0.2 μ L
25 μ M CR(<i>kIf5</i>)-1A	0.2 μ L
Tks Gflex buffer (x2)	12.5 μ L
Tks Gflex (1.25 unit/ μ L)	0.5 μ L
ddH ₂ O (double-distilled water)	Up to 25 μ L

- g. Run the two-step PCR as follows:

PCR step	Temperature	Time	Cycles
Initial denaturation	95	2 min	1
Denaturation	98	10 s	40–45
Annealing/Extension	68	40 s	
Final Extension	68	40 s	1

- h. Electrophorese 2.5 μ L of the PCR solution in a 1% agarose gel (TAE buffer). Observe the PCR products (expected size, 1113 bp)

Note: The different size or several bands of PCR product observed are attributed to indel or KI mutations.

- i. Purify the remaining PCR products using the FastGene Gel/PCR Extraction Kit (Nippon Genetics) and elute the PCR products by adding 25 μ L ddH₂O to the center of the kit's spin column. The purified PCR products can be used for a T7 Endonuclease I (T7EI) assay or Sanger DNA template sequences.
- j. For the analysis of indel mutations, prepare the following mixtures per sample:

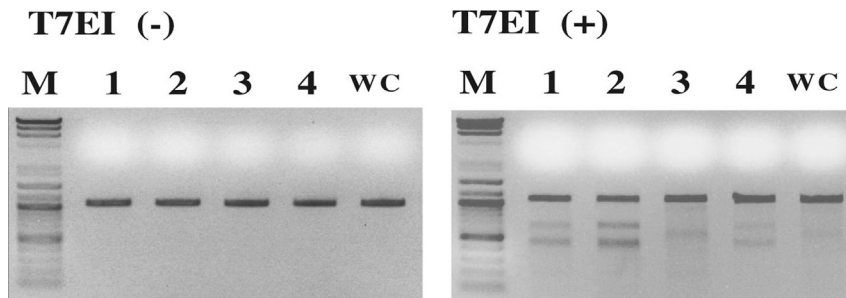
Reagent	Amount
PCR solution (derived from <i>kIf5</i> sgRNA-treated blastocyst)*	150–300 ng
10 x NEB2 buffer (included in the T7EI reagents, NEB)	1.3 μ L
ddH ₂ O (double-distilled water)	Up to 13 μ L

* The PCR products derived from non-*kIf5* sgRNA-treated samples are used as a wild-type control.

- i. Heat at 95°C for 5 min and then store at 21°C–25°C for 30 min~
- ii. Add 2 μ L of T7EI (0.5–1 U/ μ L) to the heated mixtures and incubate them at 37°C for 1–2 h.
- iii. Electrophorese 15 μ L of the solution in a 2%–3% agarose gel (TAE buffer).
- iv. Determine *kIf5* indel mutation (Figure 7).
- k. For the analysis of KI mutations, prepare the following mixtures per sample:

Reagent	Amount
PCR solution (step 6i of step-by-step method details)	100–150 ng
10 x M buffer (included with the Cla I reagent, TAKARA)	1 μ L
Cla I (10 U/ μ L)	1 μ L
ddH ₂ O (double-distilled water)	Up to 10 μ L

- i. Incubate at 37°C for 1 h



1% agarose gel (TAE buffer)
M, lambda Hind III + 100-bp ladder markers

Figure 7. Representative T7 endonuclease I (T7EI) analyses of the *klf5*-sgRNA target DNA region
Lanes 1, 2, and 4 show indel mutations. Lane 3 shows a non-indel mutation. WC: wild-type control.

- ii. Electrophorese 10 μ L of the *Cla*I-treated mixture in a 1% agarose gel (TAE buffer).
 - iii. Determine *klf5* KI mutation (Figure 8A).
 - l. Perform Sanger sequencing with the PCR primers; CR(*klf5*)-1S or CR(*klf5*)-1A in the same way as described in step 13 of [before you begin](#) if you need to confirm the indel or KI mutation sequence (Figure 8B).
7. Delivery of foster and recipient mother ICR
- The foster ICR mice are used when zygote-transferred ICR females were subjected to a cesarean section on Day +19. The recipient mice usually give birth 19 days after zygote transfer. If the pups were not delivered by the afternoon on the 19th day, they were harvested through a cesarean section (for example, [Behringer et al., 2014](#)). In this case, the foster ICR female(s) nursed them, and female cages were not replaced for about 7–10 days after birth of the offspring.
- Day +18, delivery of foster mother ICR
- a. Check the delivery of foster mother ICR (step 2 of [step-by-step method details](#)) by the afternoon.
 - b. Adopt the mother ICR calmy holding pups (observe milk line, white around the stomach) for the foster if needed on the next day (next part).
- Day +19, delivery of recipient mother ICR
- c. Check the delivery of the zygote-transferred mother ICR (step 3 of [step-by-step method details](#)) by the afternoon.
 - d. Continue to breed females that have been confirmed to give birth until Day 40–49.
 - e. Carry out cesarean section if the mice do not give birth by the afternoon. The pups can be raised in foster ICR (step 7b of [step-by-step method details](#)) (See [troubleshooting 4](#)).

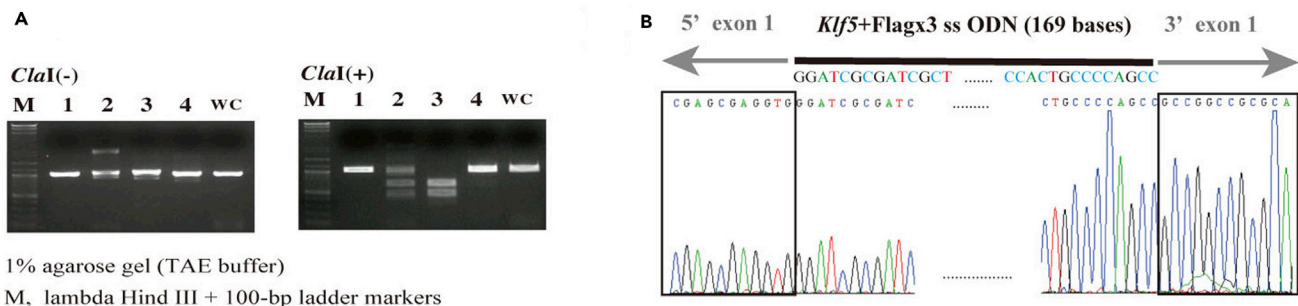


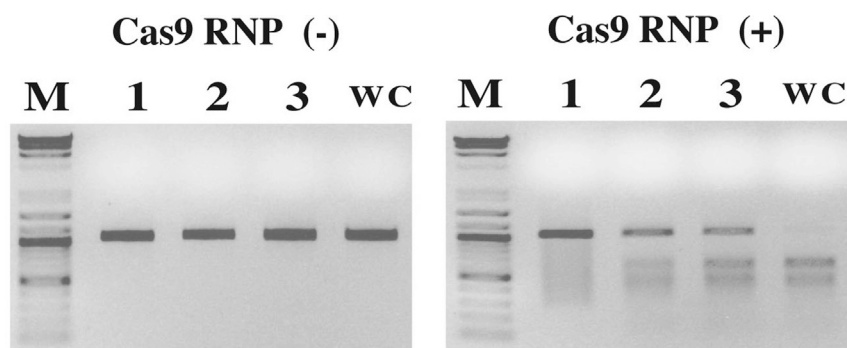
Figure 8. Our representative determination of a knockin (KI) mutation of the *klf5* gene via restriction enzyme digestion and Sanger sequencing
(A) Determination of KI of the 3 \times FLAG tag with a *Cla*I site into the *klf5* gene. Lane 2 shows a mono-allelic KI, lane 3 shows a bi-allelic KI, while lanes 1 and 4 show non-KI products.
(B) Sequence of the 5' and 3' junction site in a *klf5*+ 3 \times Flag KI pup.



Figure 9. On- and off-target genotyping of *klf5* gene in the fetuses developed from *klf5* sgRNA-electroporated sCAT zygotes

(A) A representative result of on-target genotyping via the ICE software.
 (B) A representative result of on-target genotyping via CLUSTALW software.
 (C) A representative result of PCR genotyping of 3 candidate off-target regions of the *klf5* gene (Sakurai et al., 2020).

Note: A tentative evaluation of gene editing rates in the offspring is possible using genomic DNA extracted from the placenta recovered during the cesarean section (step 8 of [step-by-step method details](#))



1% agarose gel (TAE buffer)
 M, lambda Hind III + 100-bp ladder markers

Figure 10. Representative determination of zygosity for indel mutations of the *klf5* gene using the Cas9 RNP cut assay
 Lane 1 shows a biallelic indel. Lanes 2 and 3 show monoallelic indels, while lane WC shows a wild-type control.

8. Offspring genotyping

Around days +40 to 49, the pups are weaned from the ICR foster females. The gene editing efficiency for the pups is to be evaluated using the methods described hereafter.

Subsequent phenotypic analysis is performed for the obtained gene-edited pups. Furthermore, mating and breeding will be carried out to obtain offspring for further phenotypic analysis, confirming the establishment of a mouse strain (See [troubleshooting 5](#)).

- a. Extract genomic DNA from an ear fragment or tail biopsy of the offspring using the DNeasy Blood & Tissue Kit (QIAGEN #69504) in accordance with the manufacturer's instructions.
- b. Perform PCR with primers CR(*klf5*)-1S or CR(*klf5*)-1A and 50–100 ng of the genomic DNA extracted as a template, purified in the same way as described in step 6f–l of [step-by-step method details](#). For indel mutations, use T7EI analysis and Sanger sequencing
- c. Perform T7EI analysis with the purified PCR products described in step 6j (see [Figure 7](#) as similar analysis).
- d. Perform Sanger sequencing with PCR primers CR(*klf5*)-1S or CR(*klf5*)-1A as described in step 13 of [before you begin](#) and step 6–l of [step-by-step method details](#).
- e. Analyze the sequence using free WEB programs (for example, the Inference of CRISPR Edits (ICE) software; Synthego Corporation, Silicon Valley, CA, USA) in accordance with program instructions ([Figure 9A](#)).
For the zygosity of indel mutations, use a Cas9RNP cutting assay or sequencing analysis via TA cloning of the purified PCR product or via a free WEB program (ICE software)
- f. Perform the Cas9RNP cutting assay with purified PCR products as described in steps 30–37 of [before you begin](#) ([Figure 3](#)). See [Figure 10](#) as a representative result.
- g. Carry out TA cloning of the purified PCR product using the Mighty TA-cloning Reagent Set Kit (TAKARA #6019).
 - i. Add A-overhang to the PCR product as follows, per sample:

Reagent (Mighty TA-cloning Reagent Set Kit)	Amount
Purified PCR	~100 ng
10 × Buffer	1 μL
dATP	0.5 μL
A-overhang enzyme	0.5 μL
ddH ₂ O (double-distilled water)	Up to 10 μL

- ii. Incubate at 65°C for 5 min.
- iii. Mix the following reagents:

Reagent (Mighty TA-cloning Reagent Set Kit)	Amount
A-overhang PCR solution	1 μL
pMD20-TA	1 μL
ddH ₂ O (double-distilled water)	Up to 5 μL

- iv. Add 5 μL of Ligation Mighty Mix (total 10 μL) and incubate for 0.5–1 h.
- v. Transform 25 μL of competent bacteria (JM109, Dh5a) with 2.5 μL of the ligation solution and plate the bacteria on an LB-Ampicillin (50–100 μg/mL) agar plate.
- vi. Incubate the plate for 12–15 h at 37°C.
- vii. Perform a colony PCR of 3–5 bacterial colonies with specific PCR primers M13 Rev and M13 M4 as described in [before you begin](#) step 11. Electrophorese the PCR solution (5–10 μL) in a 1% agarose gel (TAE buffer).
M13 Rev: CAGGAAACAGCTATGAC
M13 M4: GTTTTCCCAGTCACGA
- h. Inoculate 2–3 positive bacterial colonies separately in 2 mL of LB-Ampicillin (50–100 μg/mL) medium and incubate for 8 h–15 h at 37°C.
- i. Isolate and purify the PCR product–ligated PMD20-TA (step 20 of [before you begin](#)).

- j. Perform Sanger sequencing with PCR primers CR(*klf5*)-1S or CR(*klf5*)-1A and the purified plasmid (200–300 ng) as described in step 13 of [before you begin](#).
- k. Analyze the sequence using one of several free Multiple Sequence Alignment WEB programs (for example, CLUSTALW, <https://www.genome.jp/tools-bin/clustalw>) in accordance with the manufacturer's instructions ([Figure 9B](#)).

Alternatives: Without using TA-cloning, directly perform Sanger sequencing with PCR primers CR(*klf5*)-1S or CR(*klf5*)-1A and the purified PCR products (20–30 ng, step 8b) as described in step 13 of [before you begin](#).

- l. Analyze the sequence using a free WEB program (for example, ICE software), in accordance with the program instructions (see [Figure 9A](#) for a similar analysis).

Note: Bindel-PCR, non-sequencing-based method ([Sakurai et al., 2019](#)), can be used for the determination of zygosity.

For KI mutation, use restriction enzyme (RE) analysis of PCR products and sequencing analysis by TA cloning of the purified PCR product.

- m. Perform RE analysis with the PCR products in the same way as described in step 6k of [step-by-step method details](#) (see [Figure 8A](#) for a similar analysis).
- n. Perform sequencing analysis by TA cloning of the purified PCR product in the same way (step 8g-k of [step-by-step method details](#), see [Figure 8B](#) for a similar analysis).
Use sequencing analysis for off-target mutations (OPTIONAL)
- o. Perform PCR with the PCR primer sets *klf5*Offt1-1/2, *klf5*Offt2-1/2, and *klf5*Offt3-1/2, as well as 50–100 ng of the genomic DNA extracted as a template (8a), purified as described in step 30 of [before you begin](#).
- p. Perform direct Sanger sequencing with these PCR primers and the purified PCR products (20–30 ng) as described in step 13 of [before you begin](#).
- q. Analyze the sequence with free WEB programs CLUSTALW or ICE in accordance with program instructions ([Figure 9C](#)).

Generation of mice with multiple gene modifications

⌚ Timing: ~20 min

For generating mice with multiple mutations, the same procedure was performed except for the preparation of electroporation solution for delivering sgRNA or sgRNA+ssODN into the zygote (steps 4a–d of [step-by-step method details](#)). We performed 10 simultaneous genetic modifications in [Sakurai et al., 2020](#). However, if necessary, a number of sgRNAs in the range of 2–9 can be selected (See [troubleshooting 5](#)).

Herein, we only describe the preparation of an electroporation solution for the delivery of 10 sgRNAs with various target sites ([Sakurai et al., 2020](#)).

9. Day 0, preparation of the electroporation solution for delivering 10 sgRNAs into the zygote
 - a. Prepare 30 μ L of mixed sgRNAs (50 ng/ μ L per sgRNA, total 500 ng/ μ L in 75% DPBS) in a 0.2-mL PCR tube.

Reagent (before you begin 15h)	Amount
1 sgRNA (1–2 μ g/ μ L 75% DPBS)	50 ng
2 sgRNA (1–2 μ g/ μ L 75% DPBS)	50 ng
3 sgRNA (1–2 μ g/ μ L 75% DPBS)	50 ng
4 sgRNA (1–2 μ g/ μ L 75% DPBS)	50 ng
5 sgRNA (1–2 μ g/ μ L 75% DPBS)	50 ng

(Continued on next page)

Continued

Reagent (before you begin 15h)	Amount
6 sgRNA (1–2 μg/μL 75% DPBS)	50 ng
7 sgRNA (1–2 μg/μL 75% DPBS)	50 ng
8 sgRNA (1–2 μg/μL 75% DPBS)	50 ng
9 sgRNA (1–2 μg/μL 75% DPBS)	50 ng
10 sgRNA (1–2 μg/μL 75% DPBS)	50 ng
75% DPBS	Up to 30 μL

- b. Heat the tube at 75°C for 5 min and then keep it at 21°C–25°C for 5–10 min.
- c. Thereafter, add 30 μL of Opti-MEM (final sgRNA concentration, 25 ng/μL per sgRNA, total 250 ng/μL in a 1:1 mixture of 75% DPBS and Opti-MEM).
- d. Keep on ice until use (60 μL in the 1:1 medium).

Note: In our analysis (Sakurai et al., 2020), the upper limit of sgRNA concentration of the mixture solution was set to 250 ng/μL. However, up to approximately 400 ng/μL would be considered possible (unpublished data). If necessary, each sgRNA concentration can be increased to >25 ng/μL to increase the biallelic mutation rate. However, when targeting a gene whose functional deficiency causes lethality, the sgRNA concentration is to be preferably maintained at approximately 25 ng/μL (Sakurai et al., 2020).

EXPECTED OUTCOMES

According to the protocol described herein, approximately 66% of the mice obtained from *klf5* sgRNA-electroporated sCAT zygotes were *klf5* indel mutants, and approximately 48 % of the mice obtained from *klf5* sgRNA+ssODNs-electroporated sCAT zygotes were *klf5*+3× FLAG KI mutants. For the generation of multiple-gene-modified mice, 10 different sgRNAs were simultaneously introduced into sCAT zygotes by electroporation (Sakurai et al., 2020). As a result, the mice harboring mutations at 1 locus, 2–3 loci, 4–5 loci, 6–7 loci, 8–9 loci, were obtained as 3%, 6%, 48%, 26%, 14% of the 35 mice generated in total, respectively.

The maternal SpCas9-based gene-edited mice can be generated by introducing sgRNA (+ssODN) alone into maternal SpCas9-containing zygotes without the need to co-introduce exogenous SpCas9 mRNA or SpCas9 protein. The presented maternal SpCas9-based gene editing system exhibits a high degree of indel mutation efficiency and is superior to the conventional CRISPR/Cas9-based strategy owing to lower mosaicism, higher KI mutation efficiency, a higher pup delivery rate, and the potential to simultaneously induce mutations at 9 loci (Sakurai et al., 2020).

If pups harboring the SpCas9 transgene are selected among gene-modified mice derived from sCAT zygotes, they systemically express SpCas9 in a manner similar to that of the parent sCAT mice. Therefore, additional *in vivo* gene editing would be feasible in these mice, and maternal SpCas9-based gene editing can be repeatedly performed using zygotes derived thereafter (Sakurai et al., 2016).

LIMITATIONS

There is a possibility for off-target cleavage while using maternal SpCas9 in sCAT zygotes. However, the off-target cleavage activity by SpCas9 seemed to be low. We performed off-target analysis for 7 types of sgRNAs using several candidate genes to explore the possibility of SpCas9-induced off-target mutations. We observed no such off-target mutations (Sakurai et al., 2016, 2020). In addition, we performed off-target analysis for *Ar*-sgRNA, which is known to exhibit off-target activity, and observed that its mutation rate did not increase because of SpCas9 (Sakurai et al., 2020).

The genetic background of sCAT mice is the (BDF1 × C57BL/6J) × C57BL/6J backcross (Sakurai et al., 2016, 2020). Therefore, gene-modified mice with a different genetic background cannot be generated. If required, it is necessary to generate an sCAT strain with the indicated genetic background or to obtain a congenic strain through repeated backcrosses with a pure strain such as C57BL/6.

Defining the potential number of simultaneous gene modifications is a challenging question. In our results (Sakurai et al., 2020), simultaneous multiple-gene modifications in sCAT zygotes were limited to nine sites, probably owing to the limited amount of maternal SpCas9 that can be contained in sCAT zygotes. The generation of another sCAT line with higher SpCas9 expression than that in the current line may overcome this limitation.

TROUBLESHOOTING

Problem 1

The number of eggs recovered from superovulating sCAT female mice is less.

Potential solution

We used 3–4 week-old or 8–12-week-old sCAT female mice to obtain eggs following superovulation treatment. sCAT female mice that are 5–7 week-old and 13 week or older are not suitable for egg collection.

Problem 2

Many zygotes die after electroporation.

Potential solution

Under our electroporation system, zygote mortality after electroporation is around 1% or less.

If you are using the same system as us, reconsider the treatment of zygotes during electroporation. If you are using another electroporation device, reconsider the electroporation conditions.

Problem 3

The incidence of blastocysts is poor (less than 50%).

Potential solution

The probable causes are as follows:

Inadequate zygote treatment after electroporation

Poor quality of sgRNA or sgRNA+ssODN purification. The sgRNA or sgRNA+ssODN should be re-purified through ethanol precipitation; a highly pure solution is required.

The gene targeted by the sgRNA is important for embryogenesis.

Problem 4

The pup delivery rate of the electroporated zygotes is poor.

Potential solution

If you are skilled in embryo culture and embryo transfer techniques, the possible causes could be:

Inadequate zygote treatment following electroporation. It is necessary to practice zygote electroporation via a blastocyst assay (step 6 of [step-by-step method details](#)).

Poor quality of gRNA or gRNA+ssODN purification. The sgRNA or sgRNA+ssODN should be re-purified through ethanol precipitation; a highly pure solution is required

The gene targeted by the sgRNA is important for embryogenesis. Approximately 25 ng/μL sgRNA is adopted for gene editing. When targeting a gene whose functional deficiency is lethal, the sgRNA concentration was set to 25 ng/μL. In our experiments, most of the mice obtained were monoallelically gene edited (Sakurai et al., 2020).

Problem 5

The production rate of single- and multiple-gene-modified mice is poor.

Potential solution

The possible causes could be:

Low nuclease activity of sgRNA used in the zygote. It is necessary to prepare sgRNA with high activity. To examine its activity, *in vitro* analysis of the DNA DSB activity of synthesized sgRNAs (step 30–37 of [before you begin](#)) and/or a blastocyst assay (step 6 of [step-by-step method details](#)) can be performed.

The time to perform zygote electroporation. The amount of maternal SpCas9 decreases from zygotes to 2-cell-stage embryos. Therefore, as the developmental stage of the zygote progresses, the efficiency of maternal SpCas9 gene editing steadily decreases. The time of the electroporation procedure should be fixed at 15:30–16:30 on day 0 (steps 4e–u of [step-by-step method details](#)).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Takayuki Sakurai (tsakurai@shinshu-u.ac.jp)

Materials availability

The sCAT strain has been deposited to the RIKEN BioResource Center (Cat# RBRC10984).

Data and code availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

T. Sakurai wrote the manuscript, and T. Shindo discussed the content of the manuscript.

DECLARATION OF INTERESTS

T. Sakurai and T. Shindo have filed a patent application relating to the work described in this manuscript. The Japan Science and Technology Agency (JST) applied for this patent with an international application, “Genetic modification of non-human organisms, egg cells, fertilized eggs, and a method for modifying target genes” (number PCT/JP2016/085391 filed on November 29, 2016).

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