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Method Article

Two CRISPR/Cas9-mediated methods for targeting complex insertions, deletions, or replacements in mouse

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A B S T R A C T

Genetically modified model organisms are valuable tools for probing gene function, dissecting complex signaling networks, studying human disease, and more. CRISPR/Cas9 technology has significantly democratized and reduced the time and cost of generating genetically modified models to the point that small gene edits are now routinely and efficiently generated in as little as two months. However, generation of larger and more sophisticated gene-modifications continues to be inefficient. Alternative ways to provide the replacement DNA sequence, method of Cas9 delivery, and tethering the template sequence to Cas9 or the guide RNA (gRNA) have all been tested in an effort to maximize homology-directed repair for precise modification of the genome. We present two CRISPR/Cas9 methods that have been used to successfully generate large and complex gene-edits in mouse. In the first method, the Cas9 enzyme is used in conjunction with two sgRNAs and a long single-stranded DNA (lssDNA) template prepared by an alternative protocol. The second method utilizes a tethering approach to couple a biotinylated, double-stranded DNA (dsDNA) template to a Cas9-streptavidin fusion protein.

- Alternative method for generating long, single-stranded DNA templates for CRISPR/Cas9 editing.
- Demonstration that using two sgRNAs with Cas9-streptavidin/biotinylated-dsDNA is feasible for large DNA modifications.

A R T I C L E I N F O
Method name: CRISPR/Cas9-mediated large genetic modifications in mouse
Keywords: CRISPR/Cas9, Gene-editing, Mouse, Zygote, Microinjection, ssDNA, Streptavidin/biotin
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Specifications Table

Subject Area:	Biochemistry, genetics and molecular biology
More specific subject area:	CRISPR/Cas9 gene-editing
Method name:	CRISPR/Cas9-mediated large genetic modifications in mouse
Name and reference of original method:	Method 1: Miura, H., Quadros, R. M., Gurumurthy, C. B., & Ohtsuka, M. Easi-CRISPR for creating knock-in and conditional knockout mouse models using long ssDNA donors. *Nature protocols* (2018) *13*(1), 195. Method 2: Gu, B., Posfai, E., & Rossant, J. Efficient generation of targeted large insertions by microinjection into two-cell-stage mouse embryos. *Nature biotechnology* (2018) *36*(7), 632.
Resource availability:	Availability of all kits, plasmids, and other specialized materials are reported in the method details.

Method details

Animal use

All animal experiments were performed following Portuguese (Portaria 1005/95) and European (Directive 2010/63/EU) legislations regarding housing, husbandry, and welfare. This project received review and approval by the Ethics committee of Instituto Gulbenkian de Ciência and by the Portuguese National Entity, Direcção Geral de Alimentação Veterinária (license reference: 014308).

Common materials for all procedures
- DNase/RNase-free water
- RNase-free Eppendorf tubes
- RNase-free filtered pipette tips
- Thermocycler system
- Heat block or water bath
- Agarose
- 1x TAE buffer
- Gel electrophoresis set up (chamber and power supply)
- Gel loading dye
- DNA ladder (100bp or 1 kb ladder)
- DNA stain (ethidium bromide or alternative)
- Gel imaging system [ultraviolet (UV) imaging]
- Temperature controlled centrifuge
- NanoDrop spectrophotometer or similar equipment

NOTE 1: Common materials and equipment can be used from any reliable company.
NOTE 2: All protocols should be performed under DNase/RNase-free conditions.

A. in vitro *transcription of Cas9 or Cas9-streptavidin mRNA*

Materials.
- Cas9 or Cas9-streptavidin plasmid (this protocol is intended for plasmids containing a T7 promoter)
  - Cas9: pT7-Cas9 (available commercially from OriGene Cat. No. GE100014)
  - Cas9-streptavidin: PCS2+Cas9-mSA (available from Addgene Plasmid No. 103882)
    - NOTE: The SP6 promoter in this plasmid was replaced with a T7 promoter using standard cloning techniques.
  - NOTE: Protocol can be adapted to use a plasmid containing an SP6 promoter.
- Restriction enzyme appropriate to linearize Cas9 plasmid (see note 1c)
- Phenol/Chloroform
- 3 M NaOAc, pH 5.3

- 100% ethanol
- mMessage mMachine T7 Ultra kit (Thermo Fisher Cat. No. AM1345)
  - NOTE: Use SP6 in vitro transcription kit if using a plasmid containing an SP6 promoter.
- RNeasy Mini Kit (Qiagen Cat. No. 74104)
- Dry ice

Protocol

Restriction digest and cleaning of Cas9 or Cas9-streptavidin plasmid.

1 Perform a restriction digest of the Cas9 or Cas9-streptavidin plasmid. Incubate reaction overnight at appropriate temperature for restriction enzyme.
   a Basic reaction conditions:Plasmid10 μg10x Enzyme Buffer5 μlRestriction Enzyme2 μlWaterto 50 μl
   b NOTE: Obtain a large amount of plasmid through standard midi-prep or maxi-prep procedures.
   c NOTE: Restriction enzyme should linearize the plasmid immediately after the Cas9 or Cas9-streptavidin sequence within the plasmid. This allows for "run off" transcription. It is possible to use an enzyme that cuts multiple times within the plasmid backbone as long as it generates a linear piece of plasmid containing the T7 priming site and the entire Cas9 or Cas9-streptavidin sequence.
2 Run a small amount (~200 ng) of the cleaned restriction digest on a 0.8% agarose gel to confirm plasmid was properly linearized.
3 Add 50 μl of DNase/RNase-free water to bring the reaction to 100 μl.
4 Add 100 μl of phenol/chloroform and shake vigorously until reaction becomes opaque.
5 Spin solution at maximum speed for 5 min at room temperature.
6 Carefully remove the upper, aqueous layer of the reaction and move to a new tube.
   d NOTE: It is very important to avoid contamination with the lower layer.
7 Add 1/10th volume (10 μl) of 3 M NaOAc, pH 5.3, and mix well.
8 Add 2.5 volumes (250 μl) of ice cold 100% ethanol and mix well.
9 Chill on dry ice for >30 min or at -20 °C overnight to precipitate DNA.
10 Spin reaction at maximum speed for 30 min at 4 °C.
11 Carefully remove supernatant taking care not to disturb the DNA pellet.
12 Wash DNA by adding 500 μl of ice-cold 70% ethanol.
13 Spin at maximum speed for 10 min at 4 °C.
14 Carefully remove all of the supernatant taking care not to disturb the DNA pellet.
15 Air dry DNA pellet at room temperature for 5–10 minutes or until all traces of liquid have evaporated.
16 Resuspend DNA in a small volume (~15 μl) of DNase/RNase-free water and measure concentration on a NanoDrop or similar.
   e NOTE: The desired DNA concentration is between 1–2 μg/μl to have enough DNA template for the subsequent transcription reaction.

Cas9 or Cas9-streptavidin mRNA synthesis: mMESSAGE mMACHINE T7 Ultra Kit. Follow the manufacturer's protocol with the following notes:

1 Incubate the transcription reaction for 2 h at 37 °C.
2 Increase to 2 μl TURBO DNase, mix well, and incubate for 15 min at 37 °C.
   a Proper mixing will ensure that all traces of DNA template are removed.
3 Save 2 μl of the solution (Sample 1) before adding the E-PAP reagent.
4 Incubate with the Poly(A) tailing reagents for 30 min at 37 °C.
5 Save 2 μl of the solution (Sample 2) after completing the Poly(A) tailing procedure.
   a Dilute both reserved samples with 9 μl of water and add RNase-free gel loading dye.
   b Run samples on a 0.8% agarose gel to determine if Poly(A) tailing was successful (Fig. 1).

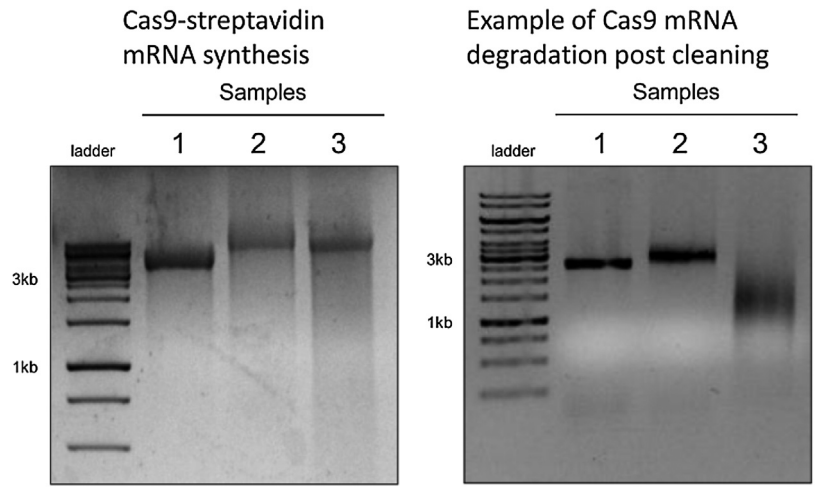

Fig. 1. Synthesis of Cas9 or Cas9-streptavidin mRNA. Images of Gel Safe stained 0.8% agarose gels of samples 1–3 of preparation of Cas9-streptavidin mRNA (section A). Left gel shows expected size increase pre- and post-Poly(A) tailing (samples 1 and 2) and a single non-degraded Cas9-streptavidin mRNA product after cleaning (sample 3). Right gel shows example of appropriate Poly (A) tailing (samples 1 and 2) but degradation of the Cas9 mRNA product following cleaning (sample 3). Ladder: 1 kb ladder, 1 kb and 3 kb bands marked.

Clean Cas9 or Cas9-streptavidin mRNA: RNeasy Mini Kit. Follow the manufacturer's protocol with the following notes:

1 Elute in 40 μl of RNase-free water instead of provided elution buffer.
2 Measure the concentration of the eluted RNA on a NanoDrop of similar.
3 Run 200–500 ng of mRNA on a 0.8% agarose gel (Sample 3) to confirm that it has not degraded during the cleaning procedure (Fig. 1).
4 Store mRNA at -80 °C.
   a NOTE: Aliquot into smaller volumes and avoid freeze-thaw cycles which might degrade the product. Run a small amount of mRNA on a gel prior to every experiment to insure product has not degraded.

B. in vitro *transcription of sgRNA (skip this procedure if purchasing commercially synthesized sgRNA)*
Refer to method validation for further information on choosing sgRNA(s)

Materials
- sgRNA plasmid (protocol is intended for plasmids containing a T7 promoter due to restrictions of MEGAshortscript T7 kit).
  - sgRNA plasmids can be made in-house (e.g. Using pT7-sgRNA commercially available from OriGene Cat. No. GE100025) or purchased from numerous vendors.
  - Linear T7-sgRNA template can also be generated via PCR. Proceed immediately with in vitro transcription if using this method.
- Restriction enzyme appropriate to linearize sgRNA plasmid (see note 1c).
- Phenol/Chloroform.
- 3 M NaOAc pH5.3.
- 100% ethanol

- MEGAshortscript T7 Kit (Thermo Fisher Cat. No. AM1354)
- MEGAclear transcription clean-up kit (Thermo Fisher Cat. No. AM1908)
- Dry ice

Protocol

Restriction digest and cleaning of sgRNA plasmid.

1 Perform a standard restriction digest of the sgRNA plasmid. Incubate reaction overnight at appropriate temperature for the restriction enzyme.
   a Basic reaction conditions:Plasmid25 μg10x Enzyme Buffer5 μlRestriction Enzyme2-3μlWaterto 50 μl
   b NOTE: Obtain a large amount of plasmid through standard midi-prep or maxi-prep procedures.
   c NOTE: Restriction enzyme should linearize the plasmid immediately after the sgRNA scaffold within the plasmid. This allows for "run off" transcription. It is possible to use an enzyme that cuts multiple times within the plasmid backbone as long as it generates a linear piece of plasmid containing the T7 priming site and the entire sgRNA scaffold.
2 Run a small amount (~200 ng) of the cleaned restriction digest on a 0.8% agarose gel to confirm plasmid was properly linearized.
3 Add 50 μl of DNase/RNase-free water to bring the reaction to 100 μl.
4 Add 100 μl of phenol/chloroform and shake vigorously until reaction becomes opaque.
5 Spin solution at maximum speed for 5 min at room temperature.
6 Carefully remove the upper, aqueous layer of the reaction and move to a new tube.
   a NOTE: it is very important to avoid contamination with the lower layer.
7 Add 1/10th volume (10 μl) of 3 M NaOAc pH 5.3 and mix well.
8 Add 2.5 volumes (250 μl) of ice cold 100% ethanol and mix well.
9 Chill on dry ice for >30 min or at -20 °C overnight to precipitate DNA.
10 Spin reaction at maximum speed for 30 min at 4 °C.
11 Carefully remove supernatant taking care not to disturb the DNA pellet.
12 Wash DNA by adding 500 μl of ice-cold 70% ethanol.
13 Spin at maximum speed for 10 min at 4 °C.
14 Carefully remove all of the supernatant taking care not to disturb the DNA pellet.
15 Air dry DNA pellet at room temperature for 5–10 minutes or until all traces of liquid have evaporated.
16 Resuspend DNA in a small volume (~15 μl) of DNase/RNase-free water and measure the concentration on a NanoDrop or similar.
   a NOTE: The desired DNA concentration is between 1–2 μg/μl to have enough template DNA for the subsequent transcription reaction.

In vitro transcription of sgRNA: MEGAshortscript T7 kit. Follow the manufacturer's protocol with the following notes

1 Incubate the transcription reaction at 37 °C for 4 h.
   a If drops accumulate in the tube's lid, collect the reaction contents with a short spin at room temperature (advisable, every 1.5 h).
2 Increase to 2 μl TURBO DNase, mix well, and incubate for 30 min at 37 °C.
   a Proper mixing will ensure that all traces of DNA template are removed.

Cleaning sgRNA transcript: MEGAclear transcription clean-up kit. Follow the manufacturer's protocol with the following notes

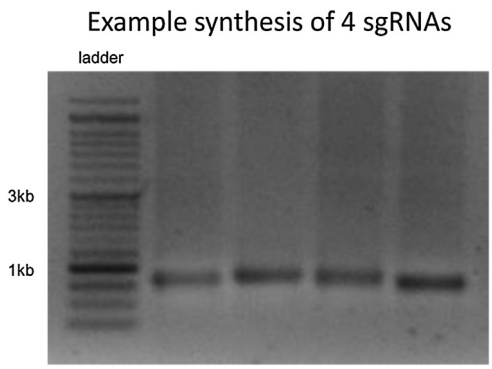

Fig. 2. Synthesis of sgRNA. Image of Gel Safe stained 2% agarose gel of final sgRNA product (section B). Single band is observed showing no signs of degradation following procedure. Ladder: 1 kb ladder, 1 kb and 3 kb bands marked.

1 Make sure all centrifugation steps do not exceed an RCF of 15,000xg. This will prevent damage to the spin columns.
2 Use RNA elution option 1. Elute in 50 μl of DNase/RNase-free water and not the provided elution buffer. Incubate at 70 °C in a heat block for 10 min.
3 Do not repeat the elution procedure as suggested by the manufacturer. In our hands this does not increase the RNA yields significantly and reduces final sgRNA concentration.
4 Following cleaning, measure the RNA concentration on NanoDrop or similar.
5 Run ~500 ng on a 2% agarose gel to confirm production of a single sgRNA product (Fig. 2).
   a NOTE: In some cases the RNA might give two apparent products, one with the expected size and another a bit bigger. This pattern results from the RNA acquiring different conformations that run differently in the gel.
6 Store sgRNA at -80 °C
   a NOTE: Aliquot into smaller volumes and avoid freeze-thaw cycles which might degrade the product. Run a small amount of sgRNA on a gel prior to every experiment to insure product has not degraded.

C. Synthesis of single stranded DNA (ssDNA) template

Materials.
- Targeting plasmid template
  - Refer to method validation for further information on design of targeting plasmid template.
- Primer set for template generation (forward and reverse)
  - One primer must contain a biotin modification on the 5′ end
- High Fidelity Taq Polymerase kit with proof reading activity (e.g. HotStar HiFidelity Polymerase kit, Qiagen Cat. No. 202602)
- PCR purification system (e.g. QIAquick PCR purification kit, Qiagen Cat. No. 28104)
- Dynabeads MyOne Streptavidin C1 beads (Thermo Fisher Cat. No. 65001)
- Magnetic tube rack
- 3 M NaOAc, pH 5.3
- 1 M Tris HCl, pH 7.5
- 100% ethanol
- Buffer A
  - 10mM Tris HCl pH 7.5
  - 30mM NaCl
  - 1mM EDTA
- Buffer B

- 0.15
- 1mM EDTA
- Dry ice
- Tris-EDTA (TE) buffer
  - 10mM Tris HCl
  - 1mM EDTA

Protocol

1 PCR primers should be designed to amplify desired gene-edited sequence and contain 100-200bp of flanking homology upstream and downstream to the site of editing within the genome.
   a NOTE: Only one PCR primer should contain a biotin modification on the 5′ end.
   b NOTE: It has not been empirically tested whether the choice of primer containing the biotin modification (forward or reverse) affects the efficiency of gene-editing.
2 Set up 5–10 PCR reactions to amplify template sequence from the targeting plasmid template.
   a NOTE: Use of a proof-reading enzyme is essential to reduce the probability of introducing mutations.
   b NOTE: Minimize the primer concentration as much as possible to allow for more efficient oligo removal during step 3.
   c NOTE: Check PCR reaction by running a small amount (2–5 μl of the reaction) on an agarose gel to confirm reaction generates single PCR product. If reaction cannot be optimized to eliminate un-desired bands, proceed with a gel PCR purification method to obtain desired band.
3 Pool PCR reactions and clean using a column-based PCR purification system to remove the unused biotinylated primer that will interfere with the reaction in step 4. The typical elution volume is 50 μl. Elute in DNase/RNase-free water instead of provided elution buffer.
   a Reserve 2 μl of cleaned product to serve as a loading control (Sample 1).
   b Calculate the total yield of purified DNA. This will be necessary to calculate the volume of beads necessary in step 4.
4 Wash Dynabeads MyOne Streptavidin C1 beads twice with 500 μl of Buffer A. Use 1.5 mL Eppendorff tubes with a magnetic rack to precipitate beads.
   a Follow manufacturer's recommendations to determine volume of beads required to bind biotinylated DNA.
5 Dilute purified PCR product in 300 μl of Buffer A and incubate with streptavidin beads at room temperature for 1 h with rotation.
   a NOTE: For PCR products greater than 1 kb, increase the incubation time to several hours or to overnight to increase efficiency of biotin/streptavidin binding.
6 Collect beads using a magnetic rack and save the supernatant (Sample 2). This will be used to estimate the efficiency of biotin/streptavidin binding.
7 Wash beads 2 x 500 μl with Buffer A at room temperature.
8 Denature the DNA to liberate the un-biotinylated DNA strand with 300 μl of Buffer B at room temperature for 25 min with rotation.
   a NOTE: For PCR products greater than 1 kb, increase elution time to several hours for higher efficiency.
9 Collect the beads using a magnetic rack. Carefully recover the supernatant ensuring no beads are carried over (Sample 3).
   a NOTE: Do NOT use non-sticky Eppendorf tubes as this will result in losing the ssDNA in the next steps.
   b Neutralize reaction with 30 μl of 1 M Tris HCl, pH 7.5.
10 Precipitate DNA from Sample 2 and Sample 3.
   a Add 30 μl of 3 M NaOAc, pH 5.3 and 900 μl of 100% ethanol and mix thoroughly.
   b Place tubes on dry ice for at least 30 min.
11 Recover precipitated DNAs by centrifugation at maximum speed for 30 min at 4 °C.
   a Remove supernatant and allow DNA pellet to air-dry for 5–10 min or until any remaining liquid has evaporated.

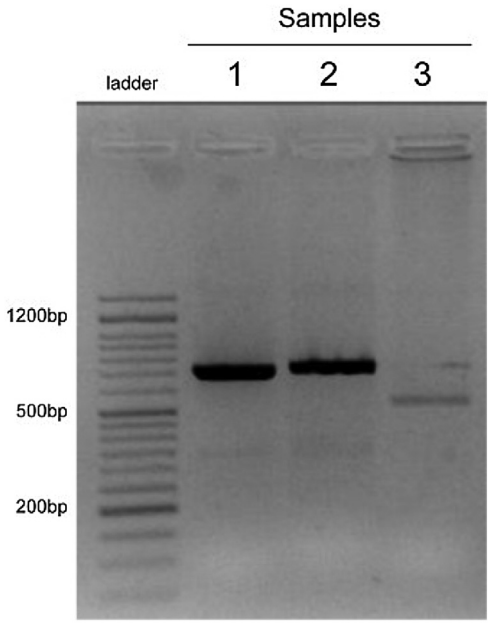

Fig. 3. Generation of ssDNA product. Image of Gel Safe stained 1% agarose gel of samples 1–3 of generating a 738bp ssDNA product (section C). 15.5 μg of biotinylated PCR product was bound to streptavidin-conjugated beads (sample 1). 1.8 μg of unbound DNA was recovered in the supernatant following binding (sample 2). 2 μg of ssDNA product was recovered in the final steps (sample 3). The weak ssDNA band and smear within the lane is what is typically observed. Approximately 350 ng of DNA (dsDNA or ssDNA) was loaded per lane. Ladder: 100bp ladder, 200bp, 500bp, and 1200bp bands marked.

b Resuspend Sample 2 in 5 μl of TE buffer or water.
c Resuspend Sample 3 (ssDNA) in a small volume (11–15 μl) of RNase-free water.
d NOTE: Precipitated ssDNA frequently adheres to the tube wall along the entire vertical length instead of forming a pellet. To resuspend, use the pipette-tip to move the droplet of water along the tube. Liquid will form droplets along the tube. Recover the liquid with a short centrifugation and repeat the process until droplets no longer adhere to the tube wall, which means that the DNA is dissolved. Recover the whole volume with a short spin.

12 Measure the concentration of Sample 2 and Sample 3 on a NanoDrop or similar.
13 Run Samples 1–3 on a 1% agarose gel (Fig. 3).
a Run 1–2 μl of Sample 3 (ssDNA).
b Try to use comparable amounts of all samples to improve comparison.
c NOTE: ssDNA runs different from PCR products and signal can be difficult to detect using most gel visualization methods.
14 ssDNA can be stored at -20 °C or -80 °C.

D. Synthesis of biotinylated double stranded DNA (dsDNA)

Materials.

- Targeting plasmid template
  - Refer to supplemental information for further notes on design of targeting plasmid template
- Primer set for template generation (forward and reverse)
  - Both primers must contain a biotin modification on the 5′ end.
- High Fidelity Taq Polymerase kit with proof-reading activity (e.g. HotStar HiFidelity Polymerase kit, Qiagen Cat. No. 202602)

- PCR purification system (e.g. QIAquick PCR purification kit, Qiagen Cat. No. 28104)

Protocol

1 PCR primers should be designed to amplify desired gene-edited sequence and contain 100-200bp of flanking homology upstream and downstream to the site of editing within the genome.
2 Set up 5–10 PCR reactions to amplify template sequence from the targeting plasmid template.
   a NOTE: Use of a proof-reading enzyme is essential to reduce the probability of introducing mutations.
   b NOTE: Minimize the primer concentration as much as possible to allow for more efficient oligo removal during step 3.
   c NOTE: Check PCR reaction by running a small amount (2–5 μl of the reaction) on an agarose gel to confirm reaction generates single PCR product. If reaction cannot be optimized to eliminate undesired bands, proceed with a gel PCR purification method to obtain desired band.
3 Pool PCR reactions and clean using a PCR purification system. This step is essential to remove the unused primers that will interfere with the binding of the DNA to the Cas9-mSA molecule upon injection (section E). The typical elution volume is 50 μl. Elute using RNase-free water and not supplied elution buffer.
   a Measure concentration of cleaned PCR product on NanoDrop or similar.
4 Run a small amount (200–500 ng) of cleaned PCR product on a gel to confirm size and purity of product.
5 Biotinylated dsDNA can be stored at -20 °C or -80 °C.

E. Conditions for microinjection of CRISPR/Cas9 reagents

Materials.

- Cas9 protein (option for Method 1 only)
- Cas9 or Cas9-streptavidin mRNA (see procedure A)
- sgRNA(s) (see procedure B)
- ssDNA template (see procedure C, for Method 1 only)
- Biotinylated-dsDNA template (see procedure D, for Method 2 only)
- Microinjection buffer
  - 8mM Tris HCl, pH 7.5
  - 0.2

Method 1: Gene-editing using Cas9, gRNA(s), and ssDNA template

Cas9 protein	100 ng/μl	–
Cas9 mRNA	–	37.5 ng/μl
sgRNA(s)	20 ng/μl (each if using multiple)	
ssDNA	20 ng/μl	

Method 2: Gene-editing using Cas9-streptavidin fusion protein mRNA, gRNA(s), and Biotinylated-dsDNA template

Cas9-streptavidin mRNA	35-40 ng/μl
sgRNA(s)	20 ng/μl (each if using multiple)
Biotinylated-dsDNA	20 ng/μl

CRISPR/Cas9 reagents were injected into the pronuclei of fertilized oocytes using standard procedures [1]. Gene-editing can be assessed by PCR analysis and/or Southern Blotting as appropriate for the desired modification.

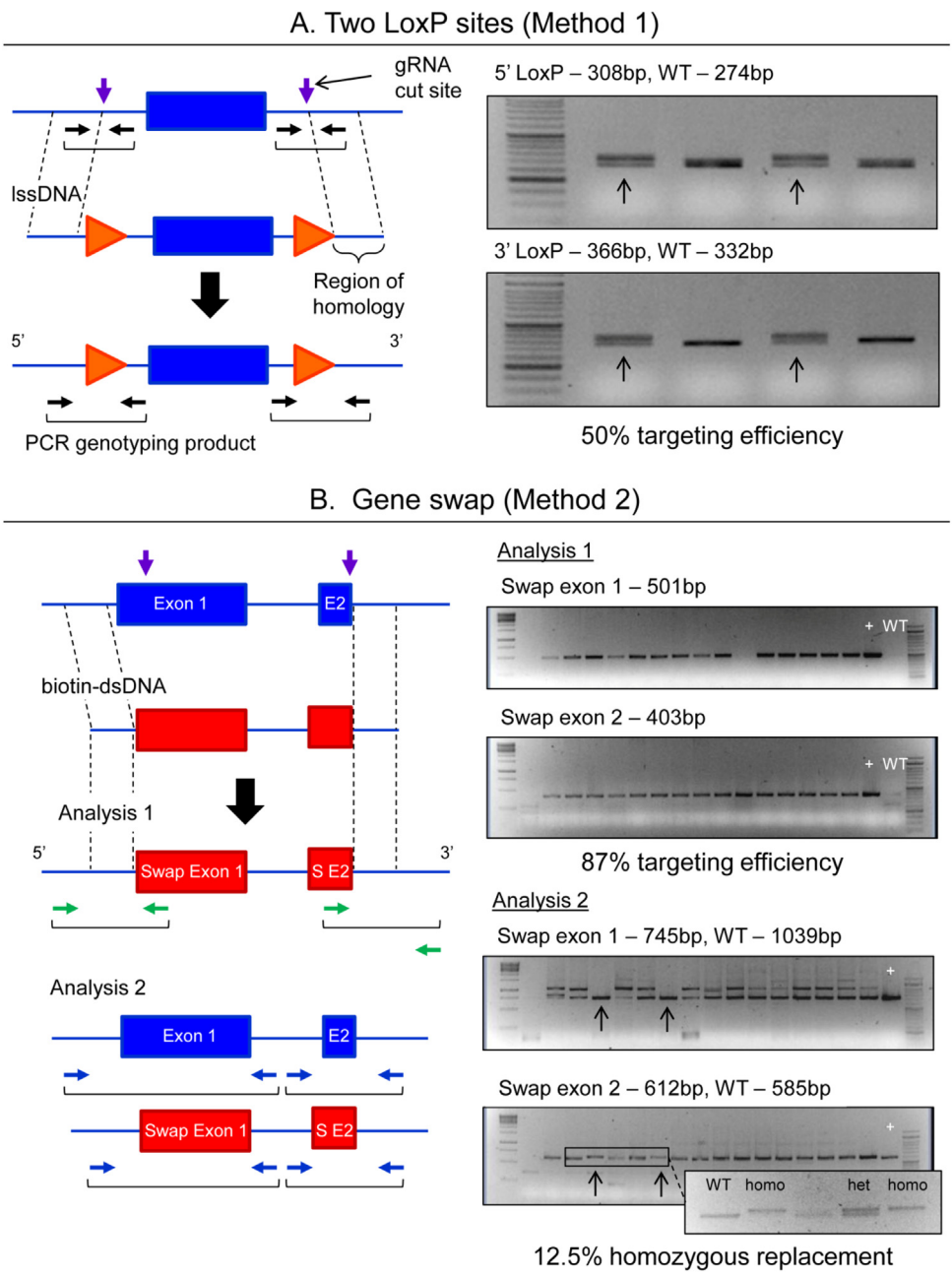

Fig. 4. Generation of large genetic modifications in mouse. (A) Insertion of two LoxP sites 700bp apart using Method 1 (section E). Diagram represents targeting strategy using two sgRNAs (purple arrows) and a lssDNA template to insert two LoxP sites (orange triangle) flanking an exon (blue box). Homology regions are noted by black dashed lines. Representative locations of PCR primers (black arrows) within the endogenous and edited sequence are marked and expected band sizes are listed above gel images. Gels (right) show PCR analysis for the 5′ and 3′ LoxP sites of 4 founder (F0) animals, two heterozygote animals marked by black arrows. (B) Gene swap of coding exons using Method 2 (section E). Diagram represents targeting strategy using two sgRNAs (purple arrows) and a biotinylated dsDNA template to replace two exons (blue boxes – endogenous exons, red boxes – swap exons) while preserving flanking and intronic sequence (blue line). Homology regions are noted by black dashed lines.

NOTE: It is of critical importance to prepare all stock reagents (Cas9/Cas9-streptavidin mRNA, sgRNAs, and replacement DNA) as concentrated as possible. Thus, when reagents are diluted for microinjection, potential contaminants are diluted beyond detection and will not obstruct the injection needle.

Method validation

We have used both Method 1 and Method 2 to generate a diverse array of gene-edited mouse models including;

- Conditional allele via simultaneous incorporation of two LoxP sites
- Gene swaps through removing and replacing the coding regions of Gene A with Gene B

For our gene editing strategies, potential sgRNAs are manually identified to generate a double strand break at the 5′ and 3′ boundaries of the region of DNA to be edited. It is important that the Cas9 generated cut site is as close as possible to the desired editing site to maximize the efficiency of homology directed repair. Thus, the relative number of potential sgRNAs to be considered will vary depending on the nature of the desired edit. Publically available online tools can be used to evaluate the potential efficiency of sgRNAs; however, Cas9-mediated cutting efficiency should be empirically determined for each sgRNA. After the sgRNA(s) have been validated, the targeting plasmid vector can be designed and generated using conventional cloning techniques or custom synthesized commercially. We use 100-200bp of flanking homology for both the ssDNA and biotinylated-dsDNA targeting methods. The size of the homology arm is based on evidence provided by other published CRISPR methodologies and experience in our laboratory [2–5]. A targeting plasmid containing 300bp or more of flanking homology allows enough flexibility to pick optimal primer pairs for generating DNA templates.

The choice of using Method 1 or Method 2 must be determined empirically and will differ depending on the nature of the desired edit. We have successfully generated “floxed” alleles using Method 1; however, no evidence of editing was observed when we attempted to generate a gene swap (Fig. 4A). Alternatively, we achieved very high gene editing efficiency when creating a complex gene swap using Method 2 (Fig. 4B). It is important to note that the efficiency of gene editing will vary depending on the target locus and the nature of the edit being generated. Efficiency of editing also depends on the quality of the CRISPR reagents used for microinjection and it is of critical importance to insure that RNA based components show no signs of degradation prior to every round of microinjection.

Additional information

The commercial availability of CRISPR/Cas9 reagents and numerous advancements in efficiency of CRISPR-based gene editing have allowed this technology to be routinely used in laboratories throughout the life sciences and medicine disciplines. The generation of novel gene-edited mouse models is now substantially easier and faster than using conventional stem cell-based gene-editing technologies. CRISPR/Cas9 is now routinely used for the generation of point mutations, “indels” to create loss-of-function alleles, and insertion of DNA sequences including protein tags like FLAG or HA and fluorescent reporters like GFP [6,7]. However, efficient editing and/or insertion of large sequences of DNA, even in the case of fluorescent reporters, remain a big challenge in the field.

Representative locations of PCR primers to generate a unique band if exon swap occurs (Analysis 1, green arrows and brackets) are shown in upper diagram, PCR primers to screen for complete exon swap (Analysis 2, blue arrows and brackets) are shown in lower diagram, expected band sizes are listed above gel images. Gels (right) show PCR analysis for exon swaps of 16 F0 animals. Black arrows on Analysis 2 gels show two animals targeted to homozygous efficiency. Boxed region, shown in higher magnification in inset, demonstrates banding patterns for wild type (WT), heterozygous (het), and homozygous (homo) targeted Exon 2 swap. Targeting plasmid (+) and wild type genomic DNA (WT) were used as controls.

Short single-stranded oligodeoxynucleotides (ssODN) are routinely used as the template DNA sequence for introducing small modifications to the genome [8–10]. The main drawback to this method is commercial oligo synthesis has a maximum size of around 200bp from most vendors, which is insufficient for the generation of larger gene edits. Longer single-stranded DNA (lssDNA) fragments can now be synthesized up to 2 kb from some vendors, but these fragments can be costly to purchase and still may not be sufficient in size for all desired genome modifications. Miura et al previously described a two-step method for synthesizing lssDNA, coined *iv*TRT [4]. This method first involves the *in vitro* transcription of the targeting plasmid followed by reverse transcription of the RNA to generate the lssDNA. Here we provide an alternative method for generating lssDNA templates that does not require reverse transcribing DNA from an RNA template. With this methodology we have successfully generated lssDNA templates up to 3.5 kb in size. Importantly, we could insert simultaneously two LoxP sites in a given allele without the need of retargeting schemes to introduce the second LoxP site.

Several groups have explored methods to increase the efficiency of homology directed repair (HDR) though the active recruitment or tethering of the template DNA sequence to either Cas9 or the sgRNA [11–14]. The Cas9 fusion protein to monomeric streptavidin used in Method 2 described here was initially developed to improve the efficiency of generating fluorescent reporter alleles [15]. They report targeting efficiencies up to 97% when using a single sgRNA, Cas9-streptavidin, and a biotinylated-dsDNA template to knock in a particular DNA sequence into a specific position in the genome. The main difference between this report and our method is the demonstration of effective targeting using two sgRNAs to replace a particular genomic sequence for another one (e.g. a gene or enhancer swap). In our hands, the use of two guides does not appear to interfere with homologous recombination or the orientation of the edited sequence, as might be predicted. This new method might prove particularly useful for generating alleles requiring gene-editing of large sequences of DNA.

Declaration of Competing Interest

The authors declare no conflicts of interest

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References

[1] B. Hogan, F. Costantini, E. Lacy, Manipulating the Mouse Embryo: a Laboratory Manual, Vol. 34, Cold spring harbor laboratory, Cold Spring Harbor, NY, 1986.
[2] G.F. Codner, et al., Application of long single-stranded DNA donors in genome editing: generation and validation of mouse mutants, BMC Biol. 16 (1) (2018) 70.
[3] D.G. Lanza, et al., Comparative analysis of single-stranded DNA donors to generate conditional null mouse alleles, BMC Biol. 16 (1) (2018) 69.
[4] H. Miura, et al., CRISPR/Cas9-based generation of knockdown mice by intronic insertion of artificial microRNA using longer single-stranded DNA, Sci. Rep. 5 (2015) 12799.
[5] R.M. Quadros, et al., Easi-CRISPR: a robust method for one-step generation of mice carrying conditional and insertion alleles using long ssDNA donors and CRISPR ribonucleoproteins, Genome Biol. 18 (1) (2017) 92.
[6] H. Wang, et al., One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering, Cell 153 (4) (2013) 910–918.
[7] H. Yang, et al., One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering, Cell 154 (6) (2013) 1370–1379.
[8] P. Singh, J.C. Schimenti, E. Bolcun-Filas, A mouse geneticist's practical guide to CRISPR applications, Genetics 199 (1) (2015) 1–15.
[9] H. Yang, H. Wang, R. Jaenisch, Generating genetically modified mice using CRISPR/Cas-mediated genome engineering, Nat. Protocols 9 (8) (2014) 1956–1968.
[10] F.A. Ran, et al., Genome engineering using the CRISPR-Cas9 system, Nat. Protoc. 8 (11) (2013) 2281–2308.
[11] E.J. Aird, et al., Increasing Cas9-mediated homology-directed repair efficiency through covalent tethering of DNA repair template, Commun. Biol. 1 (2018) 54.

[12] J. Carlson-Stevermer, et al., Assembly of CRISPR ribonucleoproteins with biotinylated oligonucleotides via an RNA aptamer for precise gene editing, Nat. Commun. 8 (1) (2017) 1711.
[13] K. Lee, et al., Synthetically modified guide RNA and donor DNA are a versatile platform for CRISPR-Cas9 engineering, Elife (2017) 6.
[14] M. Ma, et al., Efficient generation of mice carrying homozygous double-floxp alleles using the Cas9-Avidin/Biotin-donor DNA system, Cell Res. 27 (4) (2017) 578–581.
[15] B. Gu, E. Posfai, J. Rossant, Efficient generation of targeted large insertions by microinjection into two-cell-stage mouse embryos, Nat. Biotechnol. 36 (7) (2018) 632–637.