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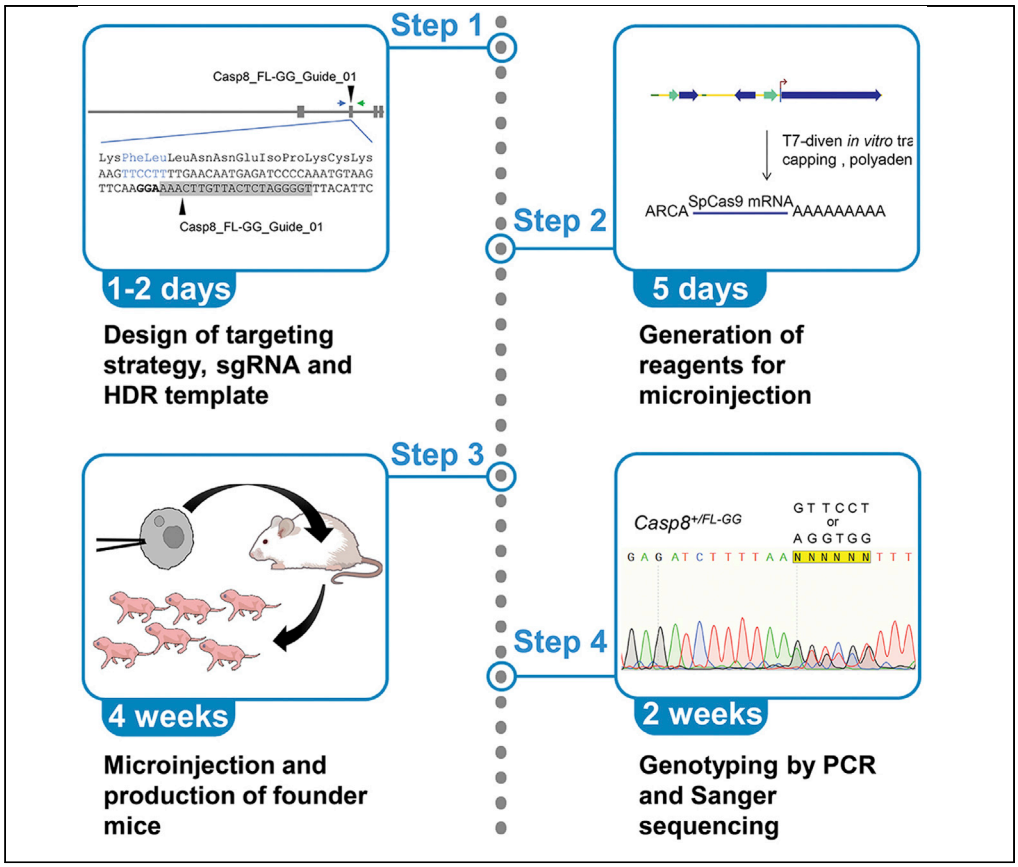
Protocol

Generation of *Casp8*$^{FL122/123GG}$ Mice Using CRISPR-Cas9 Technology



The purpose of this protocol is to describe the generation of missense mutations in mice using CRISPR-Cas9 technology. The current protocol focuses on the generation of a *Casp8*$^{FL122/123GG}$ missense mutation, but it can be adapted to introduce any missense or nonsense mutation.

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HIGHLIGHTS

Protocol describes engineering missense mutations in mice using CRISPR-SpCas9

Steps for design of allele, sgRNA, and homology directed repair template are outlined

sgRNA and Cas9 mRNA for zygote microinjection are generated *in vitro*

Founder mice are characterized by PCR and Sanger sequencing

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Generation of *Casp8*$^{FL122/123GG}$ Mice Using CRISPR-Cas9 Technology

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SUMMARY

The purpose of this protocol is to describe the generation of missense mutations in mice using CRISPR-Cas9 technology. The current protocol focuses on the generation of a *Casp8*$^{FL122/123GG}$ missense mutation, but it can be adapted to introduce any missense or nonsense mutation.

For complete details on the use and execution of this protocol, please refer to Tummers et al. (2020).

BEFORE YOU BEGIN

Amino acid substitutions in mice are generated by insertion of a single DNA double strand break (DSB) using CRISPR-Cas9 and coadministration of a single homology directed repair (HDR) template encoding the amino acid change and additional silent mutations encoding a restriction enzyme recognition sequence. These silent mutations are located within the guide target sequence or the protospacer adjacent motif (PAM) and are designed to prevent further cleavage of the target site by Cas9 upon DSB repair, and to facilitate genotyping. Before preparing reagents for microinjection, one must design the mutant allele, select an appropriate guide sequence, design the HDR template, and design on- and off-target PCR primers that will be used for genotyping (Fig. 1).

Casp8$^{FL122/123GG}$ Allele Design

Timing: 2 h

To assist with the design of alleles, we recommend using a molecular biology software. We use SnapGene (https://www.snapgene.com/). The following describes the steps used for designing alleles in Snap Gene.

1. Download *Casp8* gene information form NCBI - Gene
   a. Go to https://www.ncbi.nlm.nih.gov/gene/ and type Caps8 in the query box
   b. Click on Casp8 from *Mus musculus*, ID: 12370
   c. Scroll down to "Genomic regions, transcripts, and products" and click on: GenBank
   d. Click on "Send to" and select "Complete record," "file," "GenBank" format and "show GI." Click on create file. This will download the Casp8 GenBank file to your browser
   e. Open the file in SnapGene
2. Design Casp8 FLGG allele in silico
   a. Locate the phenylalanine 122 (F122) and the Leucine 123 (L123)

b. Make the following amino acid changes F122G and L123G by changing the nucleotide sequence TTCCTT to GGTGGT
c. Save the allele under a different name than the wild-type (WT) allele

Guide Sequence Selection

Timing: 1 h

The successful manipulation of genomes using CRISPR-Cas9 technology depends, for the most part, on the selection of efficient and selective guide sequences. Guide sequences are selected based on three main criteria:

- The proximity of the cleavage site relative to the editing site
- The selectivity of the guide sequence
- The efficiency of a guide sequence to cleave the on-target site

Whenever possible, select the guide sequence that is closest to the editing site, has the least potential off-target sites. Guide sequences with potential off-target sites having 0–2 mismatches should be avoided. Off-target cleavage sites can be identified using Cas-Offinder (http://www.rgenome.net/cas-offinder/), has a high predicted cleavage efficiency. Cleavage efficiency is affected by nucleotide composition of sgRNAs. In particular, the nature of the nucleotide immediately upstream of the PAM sequence can have a negative or positive effect on Cas9 activity. The presence of a thymine is associated with reduced SpCas9 activity in cultured cells whereas presence of a guanine residue has the opposite effect (Doench et al., 2016). Similarly, the presence of a cytosine in position −3 (relative to the PAM) is preferred by SpCas9 whereas a thymine at position −2, −3, or −4 (Xu et al., 2015) has the opposite effect. Guide sequences with ~50% GC content are ideal. To help with guide selection, we use the Moreno-Mateos Efficiency score than can be found on USCS genome browser CRISPR Targets Track instead of Doench Rule Set 1 score as it considers transcription from the T7 promoter (Doench et al., 2016, Moreno-Mateos et al., 2015). The following table illustrates the effect of nucleotide composition on SpCas9 activity based on Xu et al., 2015. The following table indicates the effect of nucleotide composition at a given position (relative to the PAM sequence) on SpCas9 activity. This table should be used only to compare between 2 or more possible guides. For example, the presence of a thymine at position −1 does not completely abolish Cas9 activity, it reduces it.

	Nucleotide Composition						
Positive effect	T	A	A	G	C	G	G
Negative effect	G			T	T	T/C	T
Position relative to PAM (NGG)	−7	−6	−5	−4	−3	−2	−1

For missense mutations, we typically use UCSC genome browser to quickly identify potential guide sequences.

3. Open UCSC genome browser (https://genome.ucsc.edu/).
4. Click on "Blat."
5. Copy the target region from SnapGene and paste into the query box in USCS Genome browser.
6. Select the most recent mouse genome assembly (GRCm38/mm10) and click search.
7. Locate amino acids F122 and L123.
8. Identify possible guide sequences located near the target site. Guide sequences are color coded and will appear under the genomic sequence. For Casp8$^{FL122/123GG}$ mutation, seven

guides can be used to insert the missense mutations. We selected the guide sequence that was the closest to F122 and L123 (TGGGGATCTCATTGTTCAAA chr1:58827267-58827289, minus (-) strand) even though the Moreno-Mateo scores for other guide sequences in this region were, in some cases, higher. To load the CRISPR track onto USCS genome browser.

a. Scroll down to Genes and Gene Predictions
b. Under CRISPR Targets, select: Pack
c. Click on the Refresh button on the right-hand side

9. Once you have selected a guide sequence, make annotation using the "Add feature" function in SnapGene.
   a. Copy the guide sequence from UCSC genome browser and retrieve that sequence in SnapGene using the Ctrl + F function, in WT gene
   b. Click on "Add feature" and provide a name for the guide sequence (e.g., Casp8-FL-GG-Guide 01)
10. Generate a list of potential off-target sites using Cas-Offinder (http://www.rgenome.net/cas-offinder/)
    a. Go to the Cas-Offinder website
    b. Copy and paste your guide sequence in the box under Query Sequence
    c. Select three mismatches (or as many as you want)
    d. Under PAM Type, Select SpCas9 from Streptococcus pyogenes: 5′-NRG-3′ (R = A or G)
    e. Under Target Genome, select vertebrate and then *Mus musculus* (mm10) – Mouse
    f. Click Submit
    g. Once the list has been generated, click on Download results
    h. Keep the list of potential off-target sites for future analysis of potential off-target cleavage
    i. The following was obtained for this guide sequence:

crRNA	DNA	Chromosome	Position	Direction	Mismatches
TGGGGATCTCATTGTTCAAANRG	TGGGGATCTCATTGTTtAAAAAG	chr1	174897358	+	1
TGGGGATCTCATTGTTCAAANRG	TGGGGATCTCAgTGTaCAAAGAG	chr10	123472249	+	2
TGGGGATCTCATTGTTCAAANRG	TGGGGATCTCATTccTaAAACAG	chr8	27020079	-	3
TGGGGATCTCATTGTTCAAANRG	TGGGGATCTCcagGTTCAAAGAG	chr8	29120183	+	3
TGGGGATCTCATTGTTCAAANRG	TaGGGATgTCATTGTTCtAAAGG	chr8	50052954	-	3
TGGGGATCTCATTGTTCAAANRG	TGaGGATCTCATTGTTgtAAAAG	chr8	104840714	-	3
TGGGGATCTCATTGTTCAAANRG	aGGGaATCTCATTGcTCAAAAGG	chr12	67491641	+	3
TGGGGATCTCATTGTTCAAANRG	TGGGGATaTCATTGTTCActGAG	chr3	16667289	-	3
TGGGGATCTCATTGTTCAAANRG	TGGGGATaTCATTcTTtAAAGGG	chr3	38976803	-	3
TGGGGATCTCATTGTTCAAANRG	TGGGGAcCTCAcTGTTCAcAAGG	chr3	81950321	-	3
TGGGGATCTCATTGTTCAAANRG	TGGGGtTCTCtTTcTTCAAAGAG	chr3	132924358	+	3
TGGGGATCTCATTGTTCAAANRG	TaGGcATCTCATTGTcCAAACAG	chr5	40809056	+	3
TGGGGATCTCATTGTTCAAANRG	TGtGGATaTCATTGTTCAgAGAG	chr5	150556979	+	3
TGGGGATCTCATTGTTCAAANRG	TGGGGAgCTCATaGTcCAAAGGG	chr1	54601861	+	3
TGGGGATCTCATTGTTCAAANRG	TGGaGAcCTCtTTGTTCAAAGGG	chr1	94996957	-	3
TGGGGATCTCATTGTTCAAANRG	TGGGtATtTCATTGTTaAAATGG	chr2	109966908	-	3
TGGGGATCTCATTGTTCAAANRG	TGaGGATgTCATaGTTCAAATGG	chr19	49738456	+	3
TGGGGATCTCATTGTTCAAANRG	TGGaaATCTCATTGTTtAAATGG	chr15	54865790	-	3
TGGGGATCTCATTGTTCAAANRG	TGGGGAgCTCtTTGTTCAcAGAG	chr15	71732494	+	3
TGGGGATCTCATTGTTCAAANRG	TGGGaATCTCATgGTTCAgAAAG	chr17	51245545	+	3

(*Continued on next page*)

Continued

crRNA	DNA	Chromosome	Position	Direction	Mismatches
TGGGGATCTCATTGTTCAAANRG	TGtGGATCTCcTTGTTCtAAAAG	chr6	106679097	+	3
TGGGGATCTCATTGTTCAAANRG	TGtGaATCTaATTGTTCAAACAG	chr6	112857527	-	3
TGGGGATCTCATTGTTCAAANRG	TGGGGATgTCAgTtTTCAAAGGG	chr6	136898284	+	3
TGGGGATCTCATTGTTCAAANRG	TGGGGATggCATTtTTCAAAAGG	chr9	72634834	-	3
TGGGGATCTCATTGTTCAAANRG	TGGaGATCTCATctTTCAAATGG	chr9	99656134	+	3
TGGGGATCTCATTGTTCAAANRG	TcGGGtTCTCATTcTTCAAAGGG	chr18	27715630	-	3
TGGGGATCTCATTGTTCAAANRG	TGGGGATCTaATTGTTCAgcAAG	chr18	38911085	-	3
TGGGGATCTCATTGTTCAAANRG	TGGGGtTCTCATTGTaCAgATGG	chr11	60107926	-	3

11. Order the following oligonucleotides which will be used to synthesize the guide RNA (underlined). We typically order oligonucleotides from Integrated DNA technology, IDT (or from any other source):
    a. Casp8_FL-GG_Guide_ 01_F:
    TAATACGACTCACTATAGGTGGGGATCTCATTGTTCAAAGTTTTAGAGCTAGAAATAGCA
        i. Custom oligonucleotide
        ii. 25 nmol scale
        iii. Lyophilized
        iv. Standard desalted
    b. sgRNA-Scaffold-R: AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGAC
    TAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC
        i. Ultramer technology
        ii. 4 nmol scale
        iii. Lyophilized
        iv. Desalted
    c. T7-19: TAATACGACTCACTATAGG
        i. Custom oligonucleotide
        ii. 25 nmol scale
        iii. Lyophilized
        iv. Standard desalted
    d. sgRNA-R: AAAAGCACCGACTCGGTGCC
        i. Custom oligonucleotide
        ii. 25 nmol scale
        iii. Lyophilized
        iv. Standard desalted

Homology Directed Repair Template Design

Timing: 1 h

HDR templates for inserting missense mutations are typically single stranded DNA (ssDNA) molecules of 100–200 nucleotides in length and contain:

- Substitutions encoding the desired amino acid change.
- Silent mutations encoding a restriction site that is absent in the target region (if possible). This restriction site will be used for identifying potentially edited organisms and facilitate PCR-based genotyping of animal models.

- Additional silent mutations disrupting the protospacer element (the sequence recognized by the guide RNA) or the protospacer adjacent motif (PAM). These changes will prevent subsequent cleavage of the site by Cas9. A minimum of three substitutions should be introduced to prevent further cleavage by Cas9.
- Homology arms of 25–70 nucleotides flanking the target site.
- Although previous studies in cultured cells have reported differences in targeting efficiencies between sense and antisense HDR oligos (with respect to guide target sequence), we have not found any meaningful differences in targeting when using sense or antisense HDR oligos (with respect to the guide target sequence) for the generation of mouse models (unpublished data from > 200 mouse models engineered using CRISPR-SpCas9 technology). Consequently, and for simplicity, we always select the sense strand (with respect to the gene) for the design of HDR templates.

For the generation of Casp8$^{FL122/123GG}$ allele, the following HDR template was used:

GTTTCCTGCCACAGGGTCATGCTCTTTAAGCTCTCAGAAGAAGTGAGCGAGTTGGAATTGAGA
TCTTTTAAAGGTGGAATGTAAGCTGGAAGATGACTTGGTA
AGACCTAATCTCCTGAAGATGGGTCACCTCTGG

This HDR template contains:

- substitutions encoding the desired amino acid changes (TTCCTT to GGTGGT), in yellow. In this case the substitutions also eliminated the PAM sequence which is sufficient to prevent further cleavage by SpCas9 after repair;
- a silent mutation (in blue) encoding DraI site (TTTAAA), underlined; and
- homology arms of 71 and 79 nucleotides on each side of the nucleotide substitutions.

Note: the region corresponding to guide target site is highlighted in gray and is, in this case, antisense.

12. Once the HDR template is designed, make nucleotide substitutions in silico as described above under allele design.
13. Order HDR templates from IDT (or from any other source). For ssDNA fragments, shorter that 200 nucleotides, select:
    a. Ultramer technology
    b. 4 nmol scale
    c. Lyophilized
    d. Desalted

On- and Off-Target Genotyping Primer Selection

Timing: 30 min

For each on- or off-target locus, use UCSC genome browser BLAT function to retrieve the target sequence location in the genome and download the DNA sequence overlapping the target site (~1,000 bp, with ~500 bp on each side of the target site). Design PCR primers to amplify a ~500–700 bp amplicons. For on-target PCR genotyping, primer locations should be outside the HDR template homology arms. For off-target sites, PCR primers should be at least 200 nucleotides away from the potential cleavage site. Primers should have an annealing temperature (Ta) of ~60°C. The following procedure describes the steps to design PCR primers using NCBI Primer blast.

14. Visit NCBI Primer blast website: http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome.

15. Paste your target region, in fasta format, in the query box. And set the following parameters as follows:
    a. Range: Set forward primer (beginning of sequence to 300) and reverse primer (from 723 to end of sequence). Since the target site is from 500 to 523, this will only return primer pairs that are at least 200 bp away from the target site on either side. You may need to adjust these parameters if you have repeat elements and/or low complexity sequences. Avoid amplifying repeat elements as this will affect sequencing performance
    b. PCR product size: set to min 400, max 700. Those are sizes that are convenient for amplification and that can be sequenced completely on both strands by Sanger sequencing
    c. Number of primer pairs to return: Use default setting: 10. You can choose more primers if no suitable primers were identified
    d. Primer melting temperatures: use default setting

    Note: if no primer pairs can be found because of unusual %GC content, increase the primer melting temperature or decrease/increase length of primers.
    e. Search mode: No user guidance
    f. Database: Select Genome (reference assembly from selected organisms)
    g. Organism: Type the species (e.g., *Mus musculus*) and select from the drop-down menu
    h. Misprimed product size deviation: select 1,000
    i. Click on "Show results in a new window" and use new graphic view

16. Click on "Get Primers." When you obtain the results, select appropriate primer pair, and do the following:
    a. Add on-target primers to SnapGene files (wild-type and modified alleles)
    b. Write down PCR genotyping banding pattern obtained from both WT and modified alleles. Include banding pattern result if restriction digest is involved in the PCR genotyping strategy
    c. The following primer pair was selected for the amplification of the on-target site:
        i. Casp8_FL-GG_F01: TTCCCCCAAATCCTCGCATC
        ii. Casp8_FL-GG_R01: TTAGCAGGGCTCTCTGGTCT
17. Order primers from IDT
    a. Standard synthesis
    b. 25 nmol scale
    c. Lyophilized
    d. Desalted
18. Repeat the procedure for each possible off-target site. Site with three or more mismatches are typically not cleaved by SpCas9 when generating mice via zygote injection of SpCas9 mRNA transcript. Also, off-target sites with one or more mismatches and sub-optimal 5′-NAG-3′ PAM sequence are usually not cleaved when generating mice via zygote injection of SpCas9 mRNA transcript. For alternatives to using Cas9 mRNA, please refer to the "generation of sgRNA" section of the Step-By-Step Method Details.

On-Target Genotyping Banding Pattern

Timing: 30 min

19. Using primers Casp8_FL-GG_F01 and Casp8_FL-GG_R01, the following banding pattern is obtained:

Allele	Casp8_FL-GG_F01 + Casp8_FL-GG_R01	Casp8_FL-GG_F01 + Casp8_FL-GG_R01 + DraI digest
Casp8	430 bp	430 bp
$Casp8^{FL122/123G}$	430 bp	313 and 117 bp

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse Strains		
Mouse zygotes (C57BL/6J)	The Jackson Laboratory	Stock # 000664
Pseudopregnant females (CD-1 IGS Mouse)	Charles River	Strain code: 022
Chemicals, Peptides, and Recombinant Proteins		
Proteinase K	Invitrogen	Cat # 25530031
dNTP Mix, PCR Grade (800 µL)	QIAGEN	Cat # 201901
DraI	New England Biolab	Cat # R0129S
PmeI	New England Biolab	Cat # R0560S
Critical Commercial Assays		
Taq DNA Polymerase (1,000 U)	QIAGEN	Cat # 201205
QIAquick gel extraction kit	QIAGEN	Cat # 28704
MEGAshortscript T7 Transcription Kit	Invitrogen	Cat # AM1354
MEGAclear Transcription Clean-Up Kit	Invitrogen	Cat # AM1908
mMESSAGE mMACHINE T7 Ultra Kit	Invitrogen	Cat # AM1345
ExoSAP-IT PCR Product Cleanup Reagent	Invitrogen	Cat # 78201
Phusion High-Fidelity DNA Polymerase	New England Biolabs	Cat # M0530L
Oligonucleotides		
Casp8_ FL-GG_Guide 01_F TAATACGACTCACTATAGGTGGG GATCTCATTGTTCAAAGTTTTAGAGCT AGAAATAGCA	Integrated DNA Technologies	n/a
sgRNA-Scaffold-R AAAAAAGCACCGACTCGGTGCCAC TTTTTCAAGTTGATAACGGACTAGCCTTA TTTTAACTTGCTATTTCTAGCTCTAAAAC	Integrated DNA Technologies	n/a
T7-19 TAATACGACTCACTATAGG	Integrated DNA Technologies	n/a
sgRNA-R AAAAGCACCGACTCGGTGCC	Integrated DNA Technologies	n/a
Casp8_FL-GG_F01 TTCCCCCAAATCCTCGCATC	Integrated DNA Technologies	n/a
Casp8_FL-GG_R01 TTAGCAGGGCTCTCTGGTCT	Integrated DNA Technologies	n/a
Casp8_FL-GG_HDR1 GTTTCCTGCCACAGGGTCATGCTC TTTAAGCTCTCAGAAGAAGTGAGCGAG TTGGAATTGAGATCTTTTAAAGGTGG TTTGAACAATGAGATCCCCAAATG TAAGCTGGAAGATGACTTGGTAAGACC TAATCTCCTGAAGATGGGTCACCTCTGG	Integrated DNA Technologies	n/a
Recombinant DNA		
pCDNA3.3TOPO-T7-hCas9	Pelletier et al., 2015	Addgene Cat #161876
Software and Algorithms		
UCSC genome browser	UCSC	https://genome.ucsc.edu/
SnapGene	GraphPad Software	https://www.snapgene.com/
NCBI Gene page	NIH	https://www.ncbi.nlm.nih.gov/gene/
Primer Blast	NIH	=http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome
Cas-Offinder	Bae et al., 2014	(http://www.rgenome.net/cas-offinder/)

MATERIALS AND EQUIPMENT

Tail lysis buffer. Prepare tail lysis buffer as described below.

Reagent	Final Concentration	Amount
KCl (2 M)	500 mM	12.5 mL
Tris pH 8.3 (1 M)	100 mM	5.0 mL
Gelatin	0.1 mg/mL	5.0 mg
NP40 (100%)	1% (v/v)	0.5 mL
Tween20 (100%)	1% (v/v)	0.5 mL
Proteinase K (50 mg/mL)	500 μg/mL	500 μL
ddH_2O	n/a	31 mL
Total	n/a	50 mL

Note: Gelatin takes several hours to dissolve. Before adding Proteinase K, the solution can be autoclaved to help dissolve gelatin.

STEP-BY-STEP METHOD DETAILS

Generation of Cas9 mRNA Transcript

Timing: 1 day

This section describes the steps used for the generation of SpCas9 mRNA transcript. This transcript is produced from a modified version of the codon optimized SpCas9 plasmid (Mali et al., 2013) (Plasmid 41815: hCas9, Addgene) in which the T7 promoter was inserted upstream of the SpCas9 cDNA (Pelletier et al., 2015). The transcript is synthesized in vitro using the mMESSAGE mMACHINE T7 Ultra Kit (Invitrogen, AM1345). The insertion of the T7 promoter allows for the rapid preparation of large quantities of Cas9 transcript directly from the linearized plasmid. RNA transcripts are subsequently enriched using the MEGAclear Kit from Invitrogen (AM1908M) and analyzed using the 2100 Bioanalyzer from Agilent Technologies (Fig. 2).

1. Generation of SpCas9 DNA template
   a. Linearize pCDNA3.3TOPO-T7-hCas9 by setting up the following restriction digest:
      i. pcDNA3.3TOPO-T7-hCas9 plasmid: 10 μg
      ii. PmeI (R0560S 10,000 units/mL): 2 μL
      iii. NEBuffer Cutsmart: 5 μL
      iv. Add ddH_2O up to 50 μL
      v. Incubate at 37°C for 2–4 h
   b. Run a few microliters of the digested and undigested plasmid on 1% agarose gel
   c. Image gel and save the image for your records
   d. Purify the remaining of the reaction using QIAquick gel extraction kit (QIAGEN, 28704)
2. Setup the RNA transcription assay using mMESSAGE mMACHINE T7 Ultra Kit as described by the manufacturer (Invitrogen, AM1345). Cap and polyadenylate the transcript.
3. Purify RNA using MEGAclear Kit according to manufacturer's instruction.
4. Measure RNA concentration with Nanodrop Spectrophotometer (Thermo Fisher Scientific, 13-400-519).
5. Aliquot 8 μg of RNA transcript in 1.5 mL microfuge tubes. Label and store −80°C.
6. Save 2–3 μL to run on 2100 Bioanalyzer (1 μL is enough per run).
7. Assess the quality of RNA on bioanalyzer according to manufacturer's recommendations.

Generation of the sgRNA Transcript

Timing: 1 day

sgRNA transcripts for zygote injection are produced in vitro from double stranded DNA templates. DNA templates are generated by annealing two ssDNA oligonucleotides, fill-in of protruding ends, and the amplification of the full-length template by PCR. The following describes the step-by-step procedure for the generation of the dsDNA template and the single guide RNA transcript (Fig. 3).

8. Generation of the sgRNA DNA template
   a. Re-hydrate all oligonucleotides to a concentration of 100 μM using ddH_2O
   b. Setup PCR reactions on ice as follows:

	PCR Reactions			
	1	2	3	4
Reagents	Volume (μL)			
5× HF Phusion buffer	10	10	10	10
dNTPs (10 mM each)	1	1	1	1
sgRNA specific primer (100 μM)	1	1	0	1
sgRNA common primer (100 μM)	1	1	1	0
ddH_2O	36	37	38	38
Phusion (2,000 units/mL)	1	0	0	0

Note: Reactions 2, 3, and 4 are control reactions.

   c. Use the following cycling conditions:

Step	Number of Cycles	Temperature	Time
1	1	98°C	2 min
2	5	98°C	10 s
		50°C	15 s
		72°C	15 s
3	1	72°C	10 min
4	1	4°C	Hold

   d. Load 5 μL of annealing reaction on a 2% agarose gel/in 1× TAE buffer and run at 120 V for 30–60 min
   e. Image gel and keep for your records
   f. Dilute PCR reaction 1:200 in ddH_2O
   g. Setup PCR reactions on ice as follows:

Reagents	Volume (μL)
5× HF Phusion buffer	10
dNTPs, 10 mM each	1
T7-19 primer (100 μM)	0.25
sgRNA-R primer (100 μM)	0.25

(*Continued on next page*)

Continued

Reagents	Volume (μL)
ddH_2O	37
Phusion (2,000 units/mL)	0.5
DNA (diluted dsDNA from step previous step)	1
Total (μL)	50

h. Use the following cycling conditions:

Step	Number of Cycles	Temperature	Time
1	1	98°C	2 min
2	25	98°C	10 s
		55°C	15 s
		72°C	15 s
3		72°C	10 min
4	1	4°C	Hold

i. Load 5 μL of PCR products on 2% agarose gel in 1× TAE buffer and run at 120 V for 30–60 min
j. Image gel and keep for your reference
k. Clean DNA fragment, 45 μL of PCR product, from steps a-h, using QIAquick PCR purification kit, using the centrifugation method
l. Measure DNA concentration and store at −20°C

9. Synthesize the sgRNA using MEGAshortscript T7 kit as described by the manufacturer (Invitrogen, AM1354).
10. Quantify DNA using a nanodrop spectrophotometer.
11. Save 2–3 μL to run on 2100 Bioanalyzer (1 μL is enough per run).
12. Assess the quality of RNA on bioanalyzer according to manufacturer's recommendations.
13. Store the remaining sgRNA transcript at −80°C.

Alternative Strategies

Recombinant Cas9 Protein and Synthetic sgRNAs. As an alternative to in vitro transcription for the generation of sgRNAs and Cas9 mRNA, synthetic sgRNAs can be used in combination with SpCas9 protein for microinjection. These can be purchased from various commercial sources including New England Biolabs, IDT, Invitrogen, Sigma-Aldrich, and others.

sgRNA Quality Control. In addition to, or as an alternative strategy, to assess the quality of in vitro transcribed sgRNAs, a functional in vitro Cas9 assay can be performed by combining recombinant Cas9 protein, the in vitro transcribed sgRNA and their substrate, a double stranded DNA fragment containing the sgRNA target sequence. This DNA fragment can be obtained by PCR amplification of the target site as described under "PCR optimization." The following steps describes this procedure.

14. For each sgRNA, setup set up the following in vitro Cas9 assay reactions. The first reaction contains all components of the reactions whereas the other three reactions serve as negative controls where either recombinant Cas9 protein, the sgRNA or the on-target PCR product (see PCR optimization for the generation of on-target PCR fragments) is omitted.

	Reaction 1	Reaction 2	Reaction 3	Reaction 4
Components	Volume (μL)			
ddH_2O (nuclease-free)	Adjust to 30 μL final	Adjust to 30 μL final	Adjust to 30 μL final	Adjust to 30 μL final
NEB3.1 buffer (NEB, B7203S)	3	3	3	3
Cas9 Nuclease (NEB, M0386S) (1 μM)	1	-	1	1
sgRNA (400 ng/μL)	1	1	-	1
On-target DNA (on-target PCR product), 30 nM	3	3	3	-

15. Incubate reactions at 37°C for 30 min.
16. Add 1 μL of Proteinase K (500 μg/mL) to each sample and incubate at 55°C for 10 min, to inactivate Cas9.
17. Load 10 μL of each reaction on a 1%–2% agarose gel supplemented with GelRed 1× at 120 V for approximately 40 min.
18. Image gel.
19. DNA fragments of 310 and 120 bp, corresponding to the cleaved PCR product should be observed in the reaction 1. Full-length PCR products (430 bp) should be observed in reaction 2 and reaction 3, and no DNA should be found in reaction 4.

Preparation of Microinjection Mixes and Injections

Timing: 15 min

This step-by-step procedure describes the preparation of Cas9 mRNA, sgRNA, and HDR template mix used for mouse zygote microinjections.

20. Prepare the Cas9 mRNA transcript, sgRNA and HDR template as follows:
    a. Cas9 mRNA transcript, 100 ng/μL. Use 1 aliquot (8 μg) previously frozen
    b. sgRNA, 50 ng/μL (Total of 4 μg)
    c. HDR oligonucleotides, 1.2 pmol/μL (~1 μL of a 100 μM)
    d. RNase free ddH_2O, up to 80 μM
21. Make 20 μL aliquots and keep frozen until ready for microinjection.

Microinjection of Pronuclear Stage Zygotes

Timing: several weeks

Microinjection of pronuclear stage zygotes is typically done by transgenic core facilities. For detailed procedures, we recommend the following manual: Manipulating the Mouse Embryo; A Laboratory Manual, Fourth Edition (Behringer, 2014).

PCR Optimization

Timing: 1 day

This step describes the procedure to optimize PCR reactions for the characterization of mice obtained from zygote injections using QIAGEN Taq Polymerase (QIAGEN, 201205). One approach to assay optimization is to determine the optimal annealing temperature of the primers by testing identical reactions containing fixed primer concentrations, across a range of annealing temperatures. This procedure also tests for the optimal concentration of $MgCl_2$ to be used for amplification.

This procedure should be performed prior to obtaining mice from zygote injection so that when mice are obtained from the transgenic facility, genotyping can be performed quickly.

22. Prepare PCR mix with low $MgCl_2$ concentration as indicated below:

Reagents	Volume (μL) for 1 Reaction	No. of Reactions	Volume (μL) for All Reactions
10× CoralLoad PCR Buffer	5	10	50
5× Q-Solution	10	10	100
$MgCl_2$	0	10	0
dNTP (10 mM each)	1	10	10
Forward primer (100 μM)	0.1	10	1
Reverse primer (100 μM)	0.1	10	1
Taq DNA Polymerase	0.5	10	5
ddH_2O	32.8	10	328
Wild-type genomic DNA	0.5	10	5

23. Prepare PCR mix with high $MgCl_2$ concentration as indicated below:

Reagents	Volume (μL) for 1 Reaction	No. of reactions	Volume (μL) for All Reactions
10× CoralLoad PCR Buffer	5	10	50
5× Q-Solution	10	10	100
$MgCl_2$	2	10	20
dNTP (10 mM each)	1	10	10
Forward primer (100 μM)	0.1	10	1
Reverse primer (100 μM)	0.1	10	1
Taq DNA Polymerase	0.5	10	5
ddH_2O	30.8	10	308
Wild-type genomic DNA	0.5	10	5

24. Distribute master mix in 8 wells of a 96 well plate so that each well has a different annealing temperature.
25. Use the following cycling conditions:

Step	Number of Cycles	Temperature	Time
1	1	94°C	4 min
2	35	94°C	30 s
		50°C–70°C	30 s
		72°C	60 s
3	1	72°C	10 min
4	1	4°C	Hold

26. Load 10 μL of PCR product on a 1%–2% agarose gel supplemented with GelRed 1× at 120 V for approximately 40 min.

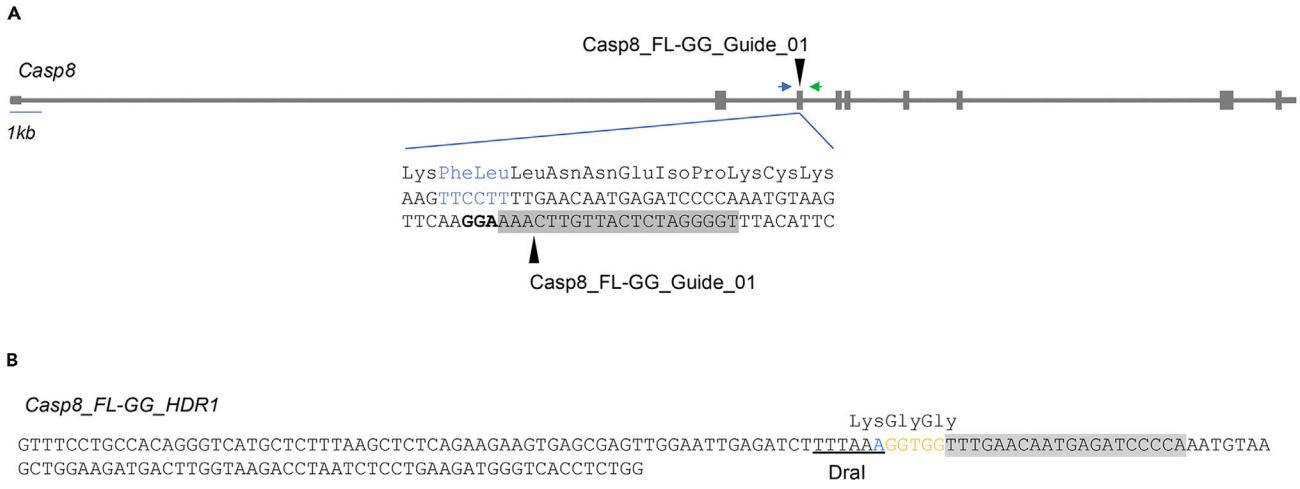

Figure 1. *Casp8*$^{FL122/123GG}$ Targeting Strategy

(A) Schematic representation of the mouse Casp8 gene, the sgRNA, and HDR template used to introduce the F122G and L123G mutations. Black arrowheads indicate the location of Cas9 cleavage site in exon 3 of the Casp8 gene, immediately downstream of amino acids 122 (Phe) and 123 (Leu) using Casp8-FL-GG_Guide 01. Gray boxes indicate exons, gray lines indicate introns. Blue and green arrows represent the genotyping primers. Gray shaded area indicates the guide target sequence which is antisense of the gene. The PAM sequence is indicated in bold.

(B) HDR template. The HDR template contains the Phe to Gly and Leu to Gly mutations (yellow) and a silent mutation (blue) generating a DraI restriction site that can be used to distinguish the WT and the mutant alleles as shown in Figure 4.

27. Image gel and save picture for your records.
28. Select the best PCR condition.

On-Target PCR Genotyping

Timing: 1 week

This step-by-step procedure describes the characterization at the genomic level of mice obtained from zygote injection. To analyze on-target gene editing, the guide target region is PCR amplified and each amplicon is analyzed by restriction enzyme digestion, gel electrophoresis, and Sanger sequencing (Fig. 4).

29. After receiving toe biopsies from your transgenic facility, extract genomic DNA by incubating each tail biopsy in tail lysis buffer (KCl, 500 mM; Tris pH8.3, 100 mM; Gelatin, 0.1 mg/mL; NP40, 1% (v/v); Tween 20, 1% (v/v); Proteinase K, 500 μg/mL), 16 h at 55°C.
30. The next day, heat inactivate the Proteinase K by incubating digested biopsies at 95°C for 10 min.
31. For each DNA sample, setup PCR reactions on ice using previously established PCR conditions. Also include:
    a. Negative Control (no genomic DNA)
    b. Positive Control (Wild-type DNA used for optimizing PCR conditions)
32. Following amplification, save:
    a. 10 μL to run on agarose gel (1%–2%)
    b. 10 μL to digest with appropriate restriction enzyme and run on agarose gel
    c. 25 μL for ExoSAP-IT (Invitrogen, 78201) and sequencing reactions
        i. 10 μL of each "exoSAP"ed sample for sequencing with target-specific forward primer
        ii. 10 μL of each "exoSAP"ed for sequencing with target-specific reverse primer

33. Set up restriction digest as follows:

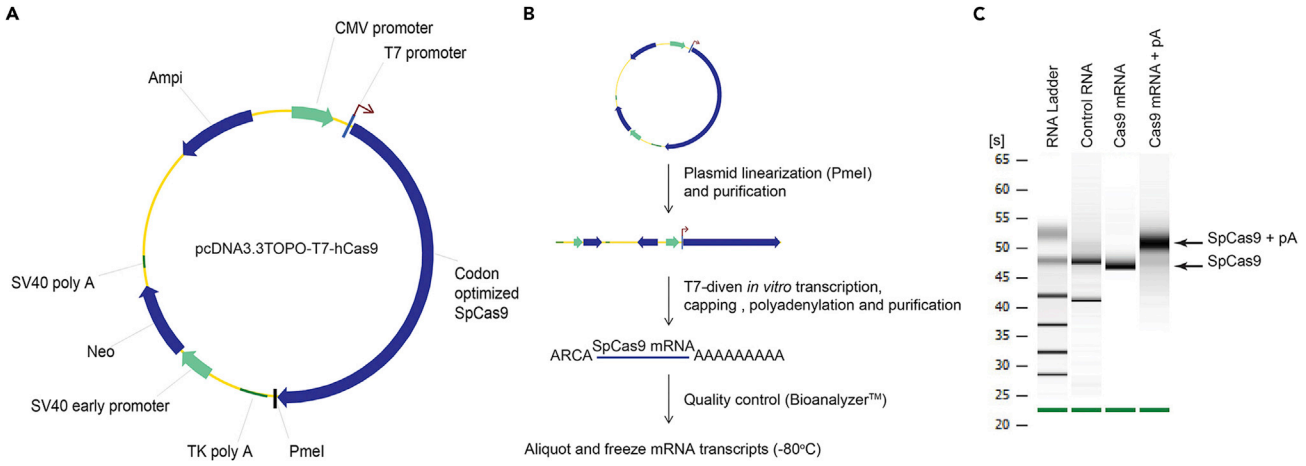

Figure 2. SpCas9 mRNA Transcript Synthesis

(A) Schematic representation of pcDNA3.3TOPO-T7-hCas9 used for the generation of the human codon optimized Cas9 (hCas9) mRNA transcript. pcDNA3.3TOPO-hCas9 (Plasmid 41815: hCas9, Addgene) was modified to insert the T7 promoter upstream of the hCas9 cDNA.

(B) Generation of ARCA-capped polyadenylated (pA) hCas9 transcripts. pcDNA3.3TOPO-T7-hCas9 is linearized using PmeI restriction enzyme and purified using the QIAquick PCR purification kit. The linearized DNA is then used as template for the *in vitro* transcription assay using the mMESSAGE mMACHINE T7 Ultra Kit and purified using the MEGAclear Kit from Invitrogen. mRNA transcripts are then analyzed using a bioanalyzer (Agilent Technologies, Inc.).

(C) Migration of hCas9 mRNA transcripts using the 2100 Bioanalyzer from Agilent Technologies. The retention time of polyadenylated Cas9 mRNA transcript (Cas9 + pA) is typically between 50 and 55 s (lane 3) whereas the retention time of non-polyadenylated Cas9 mRNA transcript is between 40 and 50 s (lane 2). RNA ladder sizes from top to bottom are 6,000, 4,000, 2,000, 1,000, 500, 200, and 25 nucleotides. The control RNA was obtained from an adult mouse spleen.

This figure was adapted from Pelletier et al. (2015).

a. Setup restriction enzyme digests for each PCR reactions as follows:

Reagent	Volume (μL)
10× CutSmart Buffer	3
ddH_2O	16.5
DraI (20,000 units/mL)	0.5
PCR product obtained from step 31	10

b. Incubate 2–18 h at 37°C

34. Run both digested and undigested PCR products for each sample on 1%–2% agarose gel. Load samples as follows:
    a. DNA Ladder
    b. Sample 1
    c. Sample 2
    d. Sample 3
    e. S…
    f. Positive control
    g. Negative control
    h. Empty Space
    i. DNA Ladder
    j. Sample 1 digested
    k. Sample 2 digested
    l. Sample 3 digested …
    m. Positive control digested

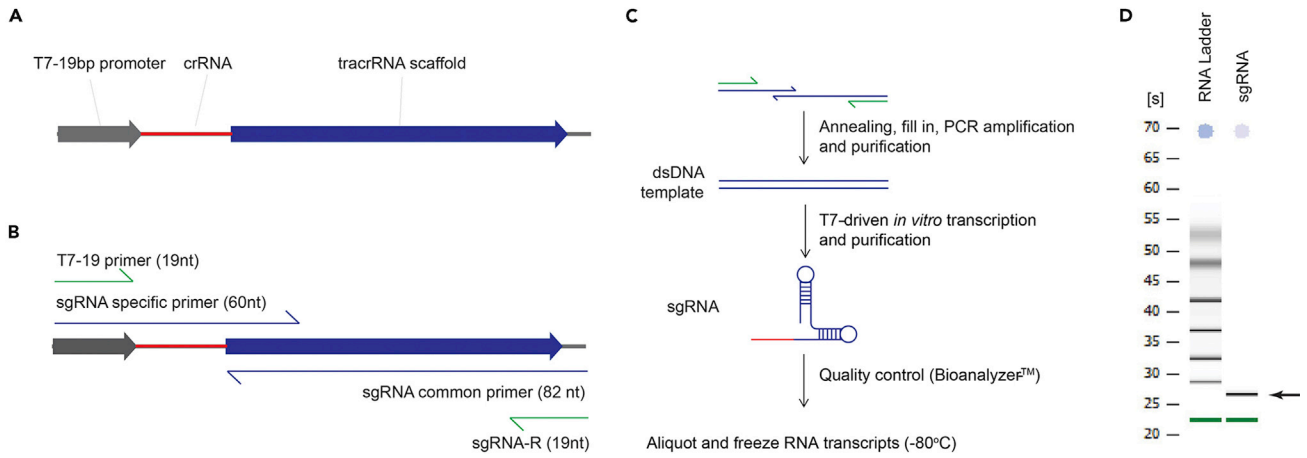

Figure 3. sgRNA transcript Synthesis

(A) Schematic representation of the dsDNA template used for sgRNA generation. Gray arrow, T7 promoter; red line; crRNA which is specific to the genomic target sequence; blue arrow, sgRNA scaffold.

(B) Primers used for the generation of target-specific sgRNAs. T7-19, sgRNA-R, sgRNA common primer, sgRNA specific primer. sgRNA common primer and sgRNA specific primers have overlapping complementary sequences pairing the two oligonucleotides together.

(C) Flowchart detailing the steps of sgRNA generation. The first step in the generation of sgRNAs consists of generating a dsDNA template (A) that will subsequently be used in an *in vitro* transcription assay for short RNA transcripts. The dsDNA template is generated by annealing a target-specific oligonucleotide called sgRNA specific primer together with the sgRNA common primer. Following the annealing reaction, a fill-in reaction produces the dsDNA that will subsequently be amplified by PCR using the T7-19 and sgRNA-R primers. The PCR product is then purified using the QIAquick PCR purification kit (QIAGENQIAGEN) and used in *in vitro* transcription assays using the MEGAshortscript T7 Kit from Invitrogen. The transcript is then enriched using the MEGAclear Kit from Invitrogen and analyzed using the 2100 Bioanalyzer from Agilent Technologies, Inc.

(D) Migration of sgRNAs using the 2100 Bioanalyzer from Agilent Technologies. Retention times of sgRNAs transcripts are typically between 25 and 30 s (black arrow). RNA ladder sizes from top to bottom are 6,000, 4,000, 2,000, 1,000, 500, 200, and 25 nucleotides.

This figure is adapted from Pelletier et al. (2015).

n. Negative control digested

35. Image gel and keep for your records.
36. Set up ExoSAP-IT reactions as follows:
    a. Mix 25 μL of a post-PCR reaction product with 1 μL of ExoSAP-IT reagent (Affymetrix, 78200) in a well of a 96-well plate or 200 μL microfuge tube
37. Incubate at 37°C for 30 min to degrade remaining primers and nucleotides.
38. Incubate at 80°C for 15 min to inactivate ExoSAP-IT reagent. The PCR product is now ready for use in DNA sequencing.
39. Setup sequencing reactions as follows:
    a. 11 μL "exoSAPped" PCR product + 0.5 μL of 10 μM forward primer
    b. 11 μL "exoSAPped" PCR product + 0.5 μL of 10 μM forward primer
    c. Submit for sequencing
40. Analyze sequencing data using SnapGene as follows:
    a. Open Ab1 files using SnapGene
    b. Analyze the chromatogram and look for insertion or deletion of genetic materials, and insertion of the desired mutations

Off-Target PCR Genotyping

Timing: 1 week

Although off-target cleavage is infrequent when generating mice via microinjection of SpCas9 mRNA transcript, off-target cleavage may occur. To identify off-target cleavage in mice harboring the desired mutation, off-target loci are PCR amplified and each amplicon sequenced. For off-

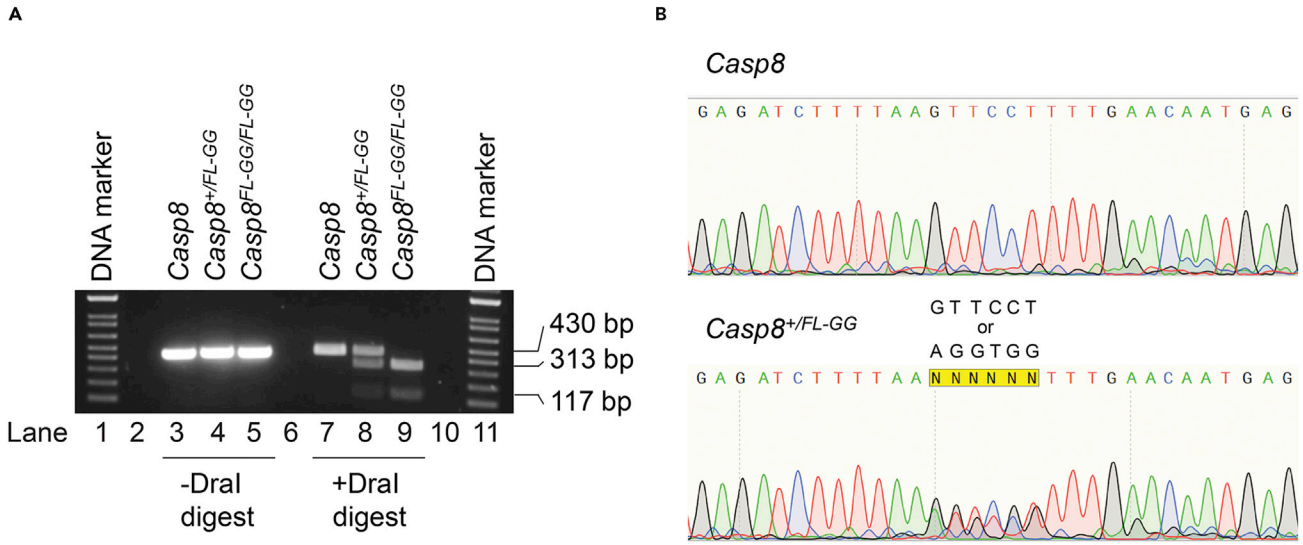

Figure 4. Representative PCR Genotyping Results

(A) Mouse genotypes were routinely determined by PCR on tail genomic DNA extracts. By using the Casp8_FL-GG_F01 and Casp8_LG-GG_R02 primers, a single amplicon of 430 bp is generated for both alleles (lanes 3, 4, and 5). Only PCR amplicons generated from *Casp8*$^{FL122/123GG}$ allele (*Casp8*$^{FL-GG}$) are sensitive to DraI digest (lanes 8 and 9). Note the complete digestion of the PCR amplicon generated from *Casp8*$^{FL-GG/FL-GG}$ mice by DraI (lane 9).

(B) Representative Sanger sequencing chromatogram obtained for the PCR amplicons generated from Casp8 and *Casp8*$^{+/FL-GG}$ mice. Note the double peaks where the nucleotide substitutions occurred. GTTCCT to AGGTGG.

targets, PCR optimization steps are not required unless no amplification is observed. For sequencing reactions, only forward sequencing data are needed unless no information can be obtained. Only the top five potential off-targets should be screened. If no off-target activity is detected, we consider the mouse line acceptable.

41. Setup PCR reactions using off-target primer pairs as described below. Analyze genomic DNA from each potential founder. Also include:
    a. Negative Control (water only)
    b. Positive Control (DNA from same species)

Reagents	Volume (μL) for 1 Reaction	No. of Reactions (no. of Founders)	Volume (μL) for All Reactions
10× CoralLoad PCR Buffer	5	3	15
5× Q-Solution	10	3	30
$MgCl_2$	2	3	6
dNTP (10 mM each)	1	3	3
Forward primer (100 μM)	0.1	3	0.3
Reverse primer (100 μM)	0.1	3	0.3
Taq DNA Polymerase	0.5	3	1.5
ddH_2O	30.8	3	92.4
Wild-type genomic DNA	0.5	3	1.5

42. Use the following cycling conditions:

Step	Number of Cycles	Temperature	Time
1	1	94°C	4 min
2	35	94°C	30 s
		58°C	30 s
		72°C	60 s
3	1	72°C	10 min
4	1	4°C	Hold

43. Use 10 μL to run on agarose gel.
44. Image gel and keep for your records.
45. Set up ExoSAP-IT reactions as follows:
    a. Mix 25 μL of a post-PCR reaction product with 1 μL of ExoSAP-IT reagent (Affymetrix, 78200) in a well of a 96-well plate or 200 μL microfuge tube.
46. Incubate at 37°C for 30 min to degrade remaining primers and nucleotides.
47. Incubate at 80°C for 15 min to inactivate ExoSAP-IT reagent. The PCR product is now ready for use in DNA sequencing.
48. Setup sequencing reactions as follows:
    a. 11 μL "exoSAPped" PCR product + 0.5 μL of 10 μM forward primer
    b. 11 μL "exoSAPped" PCR product + 0.5 μL of 10 μM forward primer
    c. Submit for sequencing
49. Analyze sequencing data using SnapGene as follows:
    a. Open Ab1 files using SnapGene
    b. Analyze the chromatogram and look for insertion or deletion of genetic materials, and insertion of the desired mutations

EXPECTED OUTCOMES

Assuming that the missense mutation engineered is not deleterious to mouse development, 10–50% of animals will harbor the desired mutation. For the generation of Casp8$^{FL122/123GG}$ mouse line, we obtained 28 mice from zygote injection. From these 28 mice, 4 harbored the Casp8$^{FL122/123GG}$ mutation (14%).

LIMITATIONS

There are two important limitations to the use of this protocol for mouse genome engineering. The first is the possibility of introducing undesired mutations within the genome of the mouse. The second is the possibility of being unable to identify guide target sequence in near the area where the missense mutation is to be inserted. The first issue can be resolved by selecting highly specific guide sequences as detailed here but also, by outbreeding founder mice with wild-type animals to eliminate potential undesired mutations. The second issue can be solved by using Cas9 protein with distinct PAM requirements. Several Cas9 and related endonucleases have been identified and can be used as an alternative to SpCas9 (Pelletier et al., 2015).

TROUBLESHOOTING

Problem 1

No PCR products are obtained when optimizing PCR reactions

Potential Solution

If no PCR product is obtained, consider designing a new pair of primers.

Problem 2

Issues with the quality or yield of in vitro sgRNA and Cas9 mRNA synthesis

Potential Solution

The strategy presented here for the synthesis of sgRNA and Cas9 mRNA has consistently yielded enough high-quality material for the generation of mouse models. It has been used for the generation of more than 300 sgRNAs and the production of several hundreds of mouse models. We recognize however that this method may not be convenient to all. As an alternative to in vitro transcription for the generation of sgRNAs and Cas9 mRNA, synthetic sgRNAs can be used in combination with recombinant SpCas9 protein for microinjection. These can be purchased from various companies including New England Biolabs, IDT, Invitrogen, Sigma-Aldrich, and others.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Douglas R. Green (doug.green@stjude.org).

Materials Availability

Casp8FL122/123GG mouse line generated in this study can be obtained from Dr. Green's laboratory at St. Jude Children's Research Hospital, Memphis, TN, 38105. pCDNA3.3TOPO-T7-hCas9 can be obtained from Dr. Pelletier's Laboratory at Indiana University School of Medicine, Indianapolis, 46202 or thru Addgene (161876).

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AUTHOR CONTRIBUTIONS

S.P., B.T., and D.R.G. wrote the article, S.P. designed the allele, and B.T. provided data.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Bae, S., Park, J., and Kim, J.-S. (2014). Cas-OFFinder: A fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics *30*, 1473–1475.

Behringer, R. (2014). Manipulating the Mouse Embryo: a Laboratory Manual (Cold Spring Harbor Laboratory Press).

Doench, J.G., Fusi, N., Sullender, M., Hegde, M., Vaimberg, E.W., Donovan, K.F., Smith, I., Tothova, Z., Wilen, C., Orchard, R., et al. (2016). Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. Nat. Biotechnol. *34*, 184–191.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., Dicarlo, J.E., Norville, J.E., and Church, G.M. (2013). RNA-guided human genome engineering via Cas9. Science *339*, 823–826.

Moreno-Mateos, M.A., Vejnar, C.E., Beaudoin, J.D., Fernandez, J.P., Mis, E.K., Khokha, M.K., and Giraldez, A.J. (2015). CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9 targeting in vivo. Nat. Methods *12*, 982–988.

Pelletier, S., Gingras, S., and Green, D.R. (2015). Mouse genome engineering via CRISPR-Cas9 for study of immune function. Immunity *42*, 18–27.

Tummers, B., Mari, L., Guy, C.S., Heckmann, B.L., Rodriguez, D.A., Ruhl, S., Moretti, J., Crawford, J.C., Fitzgerald, P., Kanneganti, T.D., et al. (2020). Caspase-8-Dependent Inflammatory Responses Are Controlled by Its Adaptor, FADD, and Necroptosis. Immunity *52*, 994–1006.e8.

Xu, H., Xiao, T., Chen, C.H., Li, W., Meyer, C.A., Wu, Q., Wu, D., Cong, L., Zhang, F., Liu, J.S., et al. (2015). Sequence determinants of improved CRISPR sgRNA design. Genome Res *25*, 1147–1157.