Generating Mouse Models Using CRISPR-Cas9-Mediated Genome Editing

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The CRISPR-Cas9 system in bacteria and archaea has recently been exploited for genome editing in various model organisms, including mice. The CRISPR-Cas9 reagents can be delivered directly into the mouse zygote to derive a mutant animal carrying targeted genetic modifications. The major components of the system include the guide RNA, which provides target specificity, the Cas9 nuclease that creates the DNA double-strand break, and the donor oligonucleotide or plasmid carrying the intended mutation flanked by sequences homologous to the target site. Here we describe the general considerations and experimental protocols for creating genetically modified mice using the CRISPR-Cas9 system. © 2016 by John Wiley & Sons, Inc.

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INTRODUCTION

Mice are important model organisms for studying human biology, diseases, and therapeutics (Zambrowicz and Sands, 2003). Historically, using well established gene targeting methods, targeted mutagenesis in mice has been achieved, first in mouse embryonic stem cells and subsequently for derivation of mice from the targeted ES clones (Capecchi, 2005). Recently, site-directed DNA endonucleases have been developed into powerful tools for genome editing in various model organisms (Hwang et al., 2013; Li et al., 2013; Hai et al., 2014; Niu et al., 2014), including mice (Wang et al., 2013; Yang et al., 2013). Compared with conventional gene targeting in ES cells, genome editing by site-directed DNA endonucleases is highly efficient and can be performed directly on the zygotes, circumventing the need for a germline-competent embryonic stem cell line.

Site-directed nucleases include homing endonuclease, zinc finger nuclease (ZFN), transcription activator–like effector nuclease (TALEN), and, recently, clustered regularly interspaced palindromic repeat (CRISPR) and CRISPR-associated endonuclease (Cas) (Gaj et al., 2013; Doudna and Charpentier, 2014; Hsu et al., 2014). Owing to its simplicity in design and superior efficiency, the CRISPR-Cas9 system has swiftly become the technology of choice for genome editing in both cell-line and animal-model creation (Doudna and Charpentier, 2014; Hsu et al., 2014). For CRISPR-Cas9, the most widely used system is derived from *Streptococcus pyogenes* (SP), although several orthologous systems from other strains have also been characterized (Doudna and Charpentier, 2014). The protocols in this article are based on the SP CRISPR-Cas9 system.

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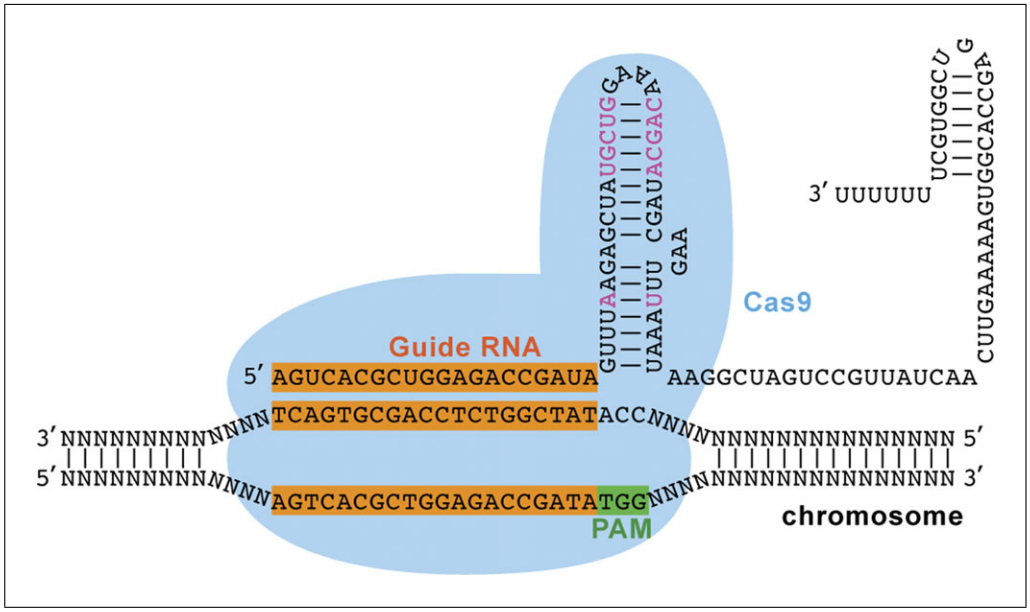

Figure 1 The CRISPR-Cas9 system for genome editing. The CRISPR-Cas9 system has two components—the single guide RNA and the Cas9 nuclease. The guide RNA provides target specificity by Watson-Crick base pairing with the protospacer sequence located in the genomic DNA target site, along with the requirement for "NGG" PAM sequence adjacent to and immediately 3′ from the protospacer sequence of the non-complementary strand. Shown in black font is the sgRNA in its conventional backbone (Jinek et al., 2012; Cong et al., 2013), and colored in pink are the two modifications ("A-U flip" and "stem loop extension") from the improved sgRNA backbone (Chen et al., 2013).

The CRISPR-Cas9 system for genome editing (Fig. 1) involves several components, including the single guide RNA (sgRNA) that specifies target specificity and the Cas9 enzyme that creates the DNA double-strand break (DSB). sgRNA is an artificial fusion of two naturally occurring RNA species, the CRISPR RNA (crRNA) and the trans-activating RNA (tracrRNA) (Jinek et al., 2012; Cong et al., 2013). The 20-nt (nucleotide) RNA sequence located at the 5′ end of the sgRNA specifies the target specificity by sequence complementarity to the protospacer sequence located in the genomic target site. In the SP CRISPR-Cas9 system, target specificity of the Cas9 protein is also dictated by the Protospacer Adjacent Motif (PAM), a trinucleotide sequence of "NGG," directly adjacent to and continuous from the 3′ end of the protospacer sequence of the noncomplementary strand. In an effort to improve targeting efficiency (Chen et al., 2013), two modifications were made to the conventional sgRNA backbone, including "A-U flip" and "stem extension" (Fig. 1). We routinely use this improved backbone in our CRISPR-Cas9 experiments.

Various types of mutations can be created using the CRISPR-Cas9 system, depending on use of the endogenous DNA repair pathways (Doudna and Charpentier, 2014; Hsu et al., 2014; Wiles et al., 2015). When a DNA DSB is created, non-homologous end joining (NHEJ) can come into play and repair the damage by ligating the two ends together, often with nucleotides inserted or deleted in-between the two broken ends. This is the indel (insertion/deletion)–based knockout model. Alternatively, when a donor oligonucleotide or plasmid carrying the intended mutation flanked by sequences homologous to the target site is provided, homology-directed repair (HDR) may be mobilized to incorporate the precise mutation into the target site. This is the HDR-based knock-in model. Depending on configuration of the experiment, the nature of the knock-in mutation can be point mutation incorporation, tag or transgene insertion, or replacement of the murine sequence with its human ortholog. As is evident, if the goal is to disrupt or delete a genomic sequence, only sgRNA(s) and the Cas9 enzyme are needed, while if the goal is to create

a precise genome modification, donor oligonucleotide or plasmid with homology arms flanking the intended mutation will need to be provided.

GENERAL CONSIDERATIONS FOR GENERATING A MOUSE MODEL USING CRISPR-Cas9

The process for generating a mouse model can be arbitrarily divided into five steps, in sequence:

Step 1: Model Design (outcome from this step includes in silico design of the allele, design and validation of the genotyping strategies, and experimental plan)
Step 2: Reagent Synthesis (outcome from this step includes Cas9 mRNA or protein, sgRNA, donor oligonucleotide or donor plasmid)
Step 3: Founder Generation (outcome from this step includes founder mice carrying intended mutation)
Step 4: Germline Transmission (outcome from this step includes F1 mice carrying intended mutation)
Step 5: Study Cohort Generation (outcome from this step includes study cohort carrying intended mutation)

Before starting on benchwork, one should always map out the entire process and derive the final edited allele in silico. Some important considerations are discussed in the following sections.

Region to Target

For a knockout model based on indel mutation mediated by NHEJ, a common practice is to rely on frameshift mutation using one sgRNA or delete a segment of the genomic sequence using two sgRNAs flanking the region to be deleted. These are some of the considerations:

1. Avoid targeting the "ATG" translation start site, if there is a downstream in-frame "ATG" which may be used to produce a truncated protein.
2. Target a region that is relatively 5′ to the gene, downstream from the starting "ATG" within an exon.
3. Target a 5′ exon commonly shared among the transcript variants, if the intent is to target all transcript variants associated with the gene of interest. Alternatively, target an exon used particularly in a transcript variant, if the intent is to delete a specific transcript variant.
4. Target safely within an exon and preserve the splice donor and acceptor sites flanking a particular exon to avoid creation of an aberrant splicing product. Indel mutations created by CRISPR-Cas9 are often small, less than 20 nt in size, and, as such, one should choose an exon that is large enough to accommodate the mutation while leaving the splice donor and acceptor sites unaffected.
5. Alternatively, sequences coding for a functionally important domain could be targeted, such as those mediating ligand binding, catalytic activity, or protein/protein interactions.
6. Another important consideration is sequence complexity in the area of interest. An unusually high GC content could pose a challenge for a polymerase chain reaction (PCR) used in the genotyping assay, or long strings of "A" or "T" could prevent successful sequencing reaction reading through the region. It is essential to have a genotyping strategy designed and validated before finalizing a project plan, including the choice of a region to target, or one may risk not being able to screen for the founder mice.

For a knock-in model, the DSB is ideally placed as close to the mutation-incorporation site as possible; therefore, there will be very limited choices for the target site. For these

projects, one must evaluate the region to be sure there is a valid CRISPR guide choice before embarking on a CRISPR project.

Guide Choices

For a model that relies on NHEJ to create an indel allele, there may be multiple choices for a guide sequence. In this case, the primary consideration may be to minimize off-target damage while maximizing targeting efficiency. There are many sgRNA design programs that identify off-target sequence matches for a given guide sequence (Hsu et al., 2013; Wiles et al., 2015). For designing sgRNAs with high efficiency, in addition to the use of software programs that make an attempt to calculate "efficiency score" (Doench et al., 2014), it is a common practice to transfect the CRISPR guides into a cell line and evaluate their efficacy in vitro, or to inject the guides and analyze targeting efficiency using the blastocysts or fetuses at embryonic day 10.5 or beyond. Also, for indel models, preference may be given to a guide sequence that has a better chance of producing a frameshift mutation based on analysis of microhomology-directed repair (Bae et al., 2014).

Strain-Related Considerations

If the genome sequence of the intended strain is not available, a segment of the genomic DNA sequence from the strain of choice encompassing the target site should be retrieved by PCR and sequence verified before a guide and PAM sequence are chosen, to avoid failure due to sequence divergence between the reference strain and the strain of choice. Also, one must bear in mind that different strains of mice may behave differently with respect to superovulation, microinjection, and surgery (Byers et al., 2006; Yamauchi et al., 2007). As such, protocols may need further refinement when a new strain of mice is used.

Breeding Considerations

Founder mice from a CRISPR-Cas9 experiment often are mosaic, with each founder mouse carrying more than two alleles. Thus, it is a good idea to breed these founder mice first with the wild-type mice of the chosen strain to transmit and segregate the alleles among the F1 mice. Under certain circumstances, a breeding scheme could be designed to accomplish two purposes—to transmit the allele and to recruit another mutant allele, in one round of breeding. For example, for a conditional knockout model, the founder mice may be bred with a Cre line to transmit the *loxP* allele to F1 mice and to recruit the Cre transgene. For a double-knockout model, the founder mice may be bred with the second knockout model. In addition, in vitro fertilization may be considered to generate a large number of F1 mice, if male founder mice are available. Of particular note is that the alleles that appear among the F1 mice must be carefully examined and the desired mutant allele carried forward for further breeding.

Here we describe the protocols that cover the entire process of generating mouse models using the CRISPR-Cas9 system, including synthesis of sgRNA (Support Protocol 1 and Support Protocol 2), Cas9 mRNA (Support Protocol 3), and donor oligonucleotide (Support Protocol 4), preparing the injection mixture (Basic Protocol 1) for use in microinjection (Basic Protocol 2) or electroporation (Basic Protocol 4) to deliver the CRISPR-Cas9 reagents into the mouse zygotes, and genotyping strategies (Basic Protocol 3) used in a genome-editing experiment.

BASIC PROTOCOL 1

PREPARE MICROINJECTION MIXTURE

Components of the CRISPR-Cas9 system, including the sgRNA, Cas9 mRNA or protein, and donor DNA if it is an HDR knock-in experiment, are brought together into a mixture to achieve the desired final concentrations and the mixture is injected into the mouse zygotes. It is imperative that the preparation be free of particulate matter and, most importantly, RNase contamination.

Materials

500 ng/μl Cas9 mRNA (Support Protocol 3)
500 ng/μl sgRNA (Support Protocols 1 and 2)
500 ng/μl donor oligonucleotide (Support Protocol 4)
500 ng/μl donor plasmid (Support Protocol 5)
TE buffer, pH 7.5: 10 mM Tris·Cl, pH 7.5/0.1 mM EDTA, DNase- and RNase-free

0.2-ml RNase-free PCR tubes (Life Technologies, cat. no. AM12225)
1.5-ml RNase-free microcentrifuge tubes (Life Technologies, cat. no. AM12400)
Galaxy 20R centrifuge (VWR)
BioDrop μLite spectrophotometer (Isogen Life Science)

1. Add CRISPR-Cas9 components, including Cas9 mRNA, sgRNA, and donor oligonucleotide or plasmid, into an 0.2-ml RNase-free PCR tube as follows, and bring to final volume (15 μl) with TE buffer, pH 7.5.

   3 μl of 500 ng/μl Cas9 mRNA (100 ng/μl final)
   1.5 μl of 500 ng/μl sgRNA (50 ng/μl final)
   (3)* μl of 500 ng/μl oligonucleotide (100 ng/μl final)
   (0.6)* μl of 500 ng/μl plasmid (20 ng/μl final)
   TE buffer, pH 7.5, to achieve 15 μl final assay volume.

   **The volumes in parentheses indicate the amount of donor DNA needed, which is only relevant for making HDR knock-in models.*

2. Place the 0.2-ml tube into a 1.5-ml microcentrifuge tube stuffed with a small piece of Kimwipe at the bottom to support the tube.

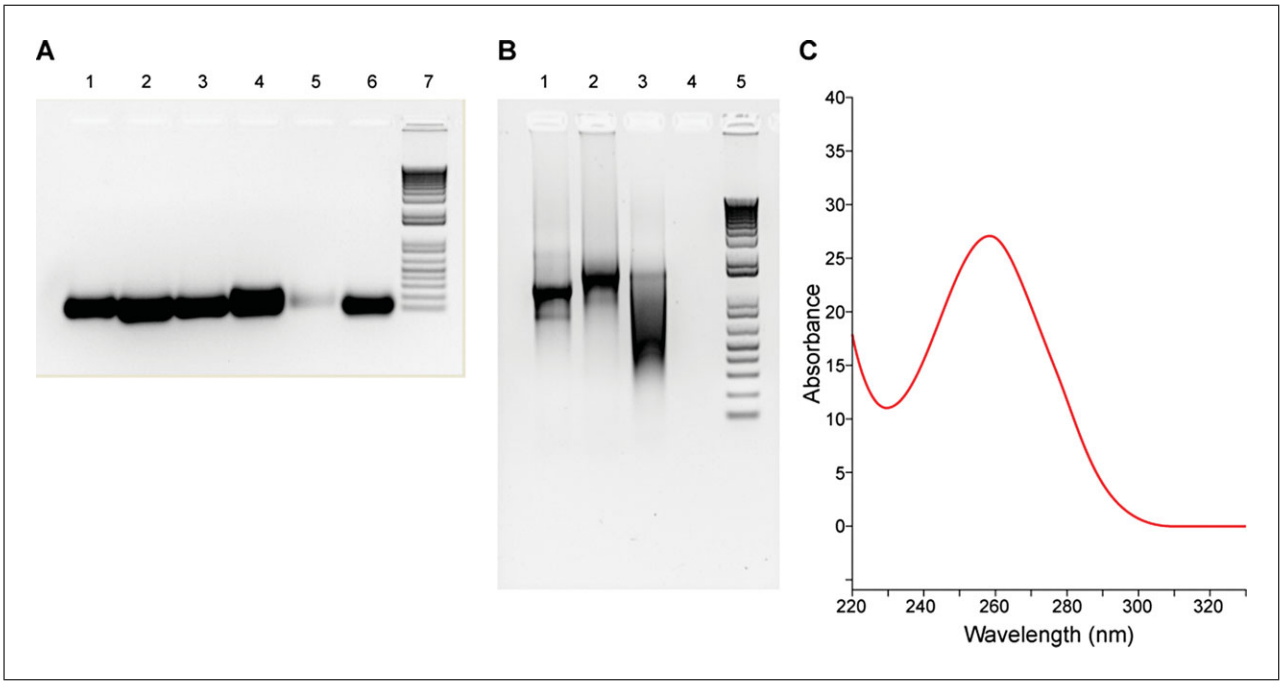

Figure 2 Quality control of the sgRNA and Cas9 mRNA preparations. (**A**) sgRNA seen on a nondenaturing agarose gel, stained with GelRed (Biotium). Lanes are samples 1 to 6, with sample 5 having a lower yield. Lane 7 is 1 Kb plus DNA ladder (Cat. No. 10787-018, Invitrogen). (**B**) Cas9 mRNA seen on a nondenaturing agarose gel, stained with GelRed. Lane 1: 2.5 μl withdrawn from the 96-μl in vitro transcription reaction; lane 2: 2.5 μl withdrawn from the 100 μl after polyadenylation was completed; Lane 3, 2.5 μl withdrawn from the 50-μl elution volume after purification with the MEGAclear kit. Lane 4: blank; Lane 5: 1 Kb plus DNA ladder. (**C**) Cas9 mRNA examined by spectrophotometry. The A_{260}/A_{280} ratio is 2.3 and A_{260}/A_{230} ratio 2.45 for this sample. The concentration is 1.125 ng/μl.

3. Centrifuge for 15 min at 20,000 × *g*, 4°C, to separate supernatant from any possible particulate matter.
4. Transfer the supernatant to a new vial for use in microinjection: Carefully take 10 μl of the supernatant and split at 5 μl/vial into two 0.2-ml RNase-free PCR tubes, pre-labeled with project number.
5. Check for integrity of the preparation by taking absorbance at 230 nm, 260 nm, and 280 nm on a spectrophotometer of the remaining 5 μl of the mixture to be sure that all components are accounted for (indicating that the preparation is free of RNase contamination). Alternatively, perform non-denaturing agarose (Fig. 2) or denaturing polyacrylamide gel electrophoresis (not shown) to be sure of the integrity of the components.

   For example, the concentration of the mixture containing sgRNA and Cas9 mRNA should be 150 ng/μl, if both reagents remain intact. Also, they should appear intact on agarose gel.
6. Place the two vials containing the supernatant portion of the preparation on ice and bring to the microinjection laboratory (see Basic Protocol 2).

   It may be desirable to measure the absorbance of the preparation when microinjection has been completed or perform agarose gel electrophoresis to verify integrity of the preparation throughout the day.

SUPPORT PROTOCOL 1

GUIDE SEQUENCE SELECTION

For each target site, a guide sequence needs to be chosen and evaluated for targeting efficiency as well as for the possibility of off-target damage, based on current understanding of the parameters that influence these outcomes. There are software programs that take these parameters into consideration which can be used to assist with selection of a guide sequence. In addition, a guide can also be evaluated by transfection into a cell line or injection into the mouse zygotes, followed by analysis of targeting efficiency (protocol not provided).

Materials

Computer with Internet access
Sequence management software (e.g., Vector NTI, Geneious, ApE)

1. Go to NCBI homepage (*http://www.ncbi.nlm.nih.gov/*) and, from the drop-down menu, select Gene, then type the name of the gene and press the Search button.
2. From the table listing the gene choices that appears, select the gene from the species of interest (for example, *Mus musculus*).
3. From the NCBI page for the gene that appears, scroll down to NCBI Reference Sequences (RefSeq) and, under Genomic, select NC_0000XX for C57BL/6J or NT_XXXXXX for other strains, such as NOD/ShiLtJ.
4. Click on GenBank.
5. From the GenBank nucleotide page that appears, click on the arrow under Send at the upper right-hand corner to expand and see the drop-down menu, and, under Choose Destination, choose File. Under Format, choose GenBank. Click Create File.
6. From the "Opening sequence.gb" window that appears, choose to open the file with VectorNTI format or other format of choice, if you do not have one set up as default already.

Table 1 Oligonucleotides used in a PCR Reaction to Generate the DNA Templates for In Vitro Transcription of the Cas9 mRNA and sgRNA

Gene	Direction	Sequence (5′ to 3′)	Reference
Cas9	F	TAATACGACTCACTATAGGG AGACCACCATGGACTATAAG GACCACGAC	Cong et al. (2013)
	R	GCGAGCTCTAGGAATTCTTAC	
sgRNA, conventional backbone	F1	gaaattaatacgactcactatag(N20)gttttag agctagaaatagc	Cong et al. (2013)
	R1	aaaagcaccgactcggtgccacttttcaagttg ataacggactagccttattttaacttgctatttctag ctctaaaac	
sgRNA improved backbone	F2	taatacgactcactatag(N20)gtttaagagct atgctggaaac[a]	Chen et al. (2013)
	R2	aaaaaagcaccgactcggtgccacttttcaagt tgataacggactagccttatttaaacttgct atgctgtttccagcatagctcttaaac	

[a]"N20": the 20-nt sequence of the guide unique to each target site.

7. In Vector NTI, under File, choose Save As and save the genomic sequence of the gene of interest in "DNA/RNAs Database."
8. Go to the Ensembl genome browser (*http://www.ensembl.org/index.html*) and under Search, select Mouse. Enter the name of the gene.
9. From the selections that appear, select the gene of interest.
10. In the table for the gene that appears, under the Transcript ID column, select the transcript of choice.
11. In the left-hand column, under Sequence, click on Exons.
12. Highlight the sequence for the exon of interest and copy the sequence.

    You may want to use a program (http://reverse-complement.com/cleanup.html) to clean up the sequence (get rid of spacing, etc.).

13. Copy the sequence and find this sequence in the gene in the VectorNTI file. Pay special attention to the region intended to be targeted.
14. Copy about 100 nt including the region to be targeted and paste into the window at CRISPR Design (*http://crispr.mit.edu/*).
15. Select the guide overlapping with the site intended for targeting and with the least sequence match elsewhere in the genome.
16. Alternatively, copy and paste sequence into the Benchling program (*https://benchling.com/*) and select the guide or guides that have the best "efficiency score" and "specificity score."
17. For indel models, select guide(s) with best possibilities of creating a frameshift mutation based on microhomology-directed repair analysis (*http://www.rgenome.net/mich-calculator/*).

You may want to create a composite sequence in VectorNTI format that is the DNA template for in vitro transcription, including the T7 promoter sequence, the guide sequence, and the rest of the sgRNA sequence. You can then copy and paste the guide sequence into this file any time you want to synthesize a new guide. This file will provide you with the sequence for the forward primer, including T7 promoter, guide, and a part of the crRNA sequence that overlaps with sequence of the reverse primer, as listed in Table 1.

SUPPORT PROTOCOL 2

SINGLE GUIDE RNA SYNTHESIS

sgRNA is an artificial fusion of two naturally occurring RNA species—the CRISPR RNA (crRNA) and the trans-activating crRNA (tracrRNA) (Jinek et al., 2012)—about 124 to 130 nt in size, depending on the sgRNA backbone used (Fig. 1). We routinely use the improved sgRNA backbone that has been shown to improve targeting efficiency in cell lines (Chen et al., 2013). An sgRNA can be transcribed from a linearized plasmid or a linear DNA template generated from a PCR reaction. In this protocol, we describe synthesis of the sgRNA from a DNA template generated from a PCR reaction and incorporating the T7 promoter from the forward primer sequence (Table 1).

Materials

sgRNA forward primer (Table 1, ordered as 4 nmol ultramer from IDT)
sgRNA reverse primer (Table 1, ordered as 4 nmol ultramer from IDT)
Nuclease-free H_2O
dNTP mixture: 2.5 mM each dNTP
PrimeSTAR GXL DNA Polymerase (Clontech, cat. no. R050B) and 5× PrimeSTAR GXL buffer
QIAquick PCR purification kit (Qiagen, cat. no. 28106)
MEGAShortScriptT7 Kit (Life Technologies, cat. no. AM1354)
MEGAclear Kit (Life Technologies, cat. no. AM1908)
GelRed stain (Biotium, cat. no. 41003)

Thermal Cycler (BioRad, cat. no.1851148)
BioDrop μLite spectrophotometer (Isogen Life Science)

Additional reagents and equipment for PCR (Kramer and Coen, 2000) and agarose gel electrophoresis (Voytas, 2000)

1. Reconstitute the oligonucleotides to 10 μM (10 pmol/μl) with nuclease-free water
2. Set up the 50-μl PCR reaction as indicated below.

   In this case, the sgRNA forward and sgRNA reverse primers are both the template and the primers.

   30.0 μl nuclease-free H_2O
   10 μl 5× PrimeSTAR GXL buffer (1× final)
   4 μl dNTP mixture (2.5 mM each dNTP) (200 μM final)
   2.5 μl 10 μM sgRNA forward primer (0.5 μM final)
   2.5 μl 10 μM sgRNA reverse primer (0.5 μM final)
   1.0 μl PrimeSTAR GXL DNA Polymerase (1.25 U/50 μl reaction)
3. Perform PCR (Kramer and Coen, 2000) using the following cycling conditions:

1 cycle:	30 sec	98°C	(initial denaturation)
35 cycles:	10 sec	98°C	(denaturation)
	15 sec	55°C	(annealing)
	30 sec	68°C	(extension)
1 cycle:	5 min	68°C	(final extension)
1 cycle:	indefinitely	4°C	(hold).

4. Separate an aliquot of the PCR product on a 2% (weight/volume) agarose gel in TAE buffer (Voytas, 2000) to assess its yield and to verify that the product is unique and of the expected size.

   The size of the PCR product is 124 to 130 nt, depending on the choice of the sgRNA backbone sequence.

5. Purify the T7 promoter/sgRNA PCR product using the QIAQuick PCR purification kit according to manufacturer's instructions. Reconstitute to 50 ng/μl or higher concentration using elution buffer provided with the kit.

6. Perform in vitro transcription to produce sgRNA using the MEGAShortScript T7 kit according to manufacturer's instructions, adding 400 to 1000 ng of DNA template in an 8-μl volume (20-μl reaction volume, total).

7. Purify the sgRNA using the MEGAclear kit and elute with elution buffer according to the kit protocol. If the yield is found to be low, use an alternative protocol and purify with phenol:chloroform extraction/alcohol precipitation, or ammonium acetate precipitation as described in the kit manual.

8. Mix an aliquot of the sgRNA with the Gel Loading Buffer II provided in the MEGAShortScript T7 kit and separate on 2% nondenaturing agarose gel in 1× TAE solution (Voytas, 2000). Stain the gel with GelRed according to the manufacturer's instructions to assess yield and quality (Fig. 2A).

9. More conveniently, examine yield and quality by taking absorbance at 230 nm, 260 nm, and 280 nm, similar to the example provided for a Cas9 mRNA preparation (Fig. 2C), paying special attention to 260/280 ratio (a ratio of 2.0 or above is indicative of a clean RNA preparation, free of protein, phenol, or other contaminants that absorb at or near 280 nm) and 260/230 ratio (expected 260/230 ratios are commonly in the range of 2.0 to 2.2, indicative of absence of contaminants that absorb at 230 nm, including EDTA and phenol).

10. Dilute the sgRNA to 500 ng/μl, aliquot at 5 μl per vial, and store at −80°C until use.

Cas9 mRNA SYNTHESIS

SUPPORT PROTOCOL 3

Cas9 mRNA from SP and used in this protocol is 4.3 kb in size and can be synthesized from an in vitro transcription reaction. The T7 promoter is added to the forward primer (Table 1) and the DNA template produced from the pX330 plasmid in a PCR reaction using the forward and reverse primers specified in Table 1. The PCR template will carry the T7 promoter at the 5′ end followed by coding sequence for the SpCas9 gene that has been codon-optimized to that from human. All reagents for mRNA synthesis and polyadenylation are included in the kit. Alternatively, Cas9 mRNA and protein can be purchased from various vendors.

Materials

Cas9 forward primer (Table 1, ordered from IDT)
Cas9 reverse primer (Table 1, ordered from IDT)
pX330 plasmid (plasmid #42230, Addgene)
Nuclease-free H_2O
Phusion High-Fidelity DNA Polymerase (New England Biolabs, cat. no. M0530L) and 5× Phusion HF buffer
dNTP mixture (2.5 mM each dNTP)

QIAquick PCR purification kit (Qiagen, cat. no. 28106)
mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Cat. No. AM1345, Life Technologies)
MEGAclear Kit (Life Technologies, cat. no. AM1908)
RNase AWAY Decontamination Reagent (Life Technologies, cat. no. 10328-011)

Thermal Cycler (BioRad, cat. no. 1851148)
BioDrop μLite spectrophotometer (Isogen Life Science)

Additional reagents and equipment for PCR (Kramer and Coen, 2000) and agarose gel electrophoresis (Voytas, 2000)

1. Reconstitute the oligonucleotides to 10 μM (10 pmol/μl) with nuclease-free water.
2. Set up a 50-μl PCR reaction as indicated below:

   33 μl nuclease-free H_2O
   10 μl 5× Phusion HF buffer (1× final)
   1 μl dNTP mixture (2.5 mM each dNTP) (200 μM final)
   2.5 μl 10 μM Cas9 forward primer (0.5 μM final)
   2.5 μl 10 μM Cas9 reverse primer (0.5 μM final)
   0.5 μl 50 ng/μl pX330 (<250 ng final)
   0.5 μl Phusion DNA Polymerase (1.0 U/50 μl final).
3. Perform PCR (Kramer and Coen, 2000) using the following cycling conditions:

1 cycle:	30 sec	98°C	(initial denaturation)
35 cycles:	15 sec	98°C	(denaturation)
	30 sec	55°C	(annealing)
	4 min	72°C	(extension)
1 cycle:	5 min	72°C	(final extension)
1 cycle:	indefinitely	4°C	(hold).

4. Separate an aliquot of the PCR product (2 μl) on a 1% (weight/volume) agarose gel in TAE buffer (Voytas, 2000) to assess yield and to verify that the product is unique and of the expected size (4.3 kb).
5. Purify the T7 promoter/Cas9 PCR product using the QIAQuick PCR purification kit according to manufacturer's instructions. Reconstitute to 100 ng/μl or higher concentration using elution buffer provided with the kit.

   Alternatively, the PCR product can also be cloned into a plasmid and the plasmid linearized and used as the template for in vitro transcription.
6. Perform in vitro transcription to produce the Cas9 mRNA using the mMESSAGE mMACHINE T7 ULTRA Transcription Kit according to manufacturer's instructions, adding 500 to 1000 ng of DNA template in a 6-μl volume (20-μl reaction volume, total). Save 2.5 μl out of the 96 μl total as the "before pA addition" preparation for analysis by agarose gel electrophoresis later on.
7. Add poly(A) tail following manufacturer's instructions and save 2.5 μl as the "after pA addition" preparation for analysis by agarose gel electrophoresis later on.
8. Purify the Cas9 mRNA using the MEGAclear kit according to manufacturer's instructions and elute with 50 μl elution buffer according to the kit protocol. Alternatively, purify with phenol:chloroform extraction/isopropanol precipitation, or lithium chloride precipitation as described in the kit manual. Save an aliquot as the "after column" preparation for analysis by agarose gel electrophoresis.

9. Perform nondenaturing agarose gel electrophoresis (Voytas, 2000) of the three preparations (before pA, after pA, and after column) on 1% agarose (weight/volume) gel in TAE buffer (Fig. 2B). Make sure all setups have been treated with RNase Away and are RNase free.

   Alternatively, denaturing polyacrylamide gel electrophoresis may be used.

10. Examine the yield and quality of the Cas9 mRNA preparation by taking absorbance at wavelengths of 230 nm, 260 nm, and 280 nm (Fig. 2C). As described earlier for the sgRNA preparation, pay special attention to 260/280 and 260/230 ratios.

11. Dilute the Cas9 mRNA to 500 ng/μl in elution buffer.

12. Aliquot at 5 μl per vial and store at –80°C until use.

DONOR OLIGONUCLEOTIDE SYNTHESIS

SUPPORT PROTOCOL 4

An oligonucleotide up to 200 nt may be synthesized using a commercial vendor. A donor oligonucleotide is used when the intended mutation is relatively small and, in addition to the intended mutation, the left and right homology arms of minimally 30 to 50 nt each could all be accommodated into the oligonucleotide. These include point mutation incorporation and tag insertion (3× FLAG, His, V5, *loxP*, etc.). The oligonucleotide is designed such that the mutation is centered with homology arms flanking each side.

Note that oligonucleotide synthesis can incorporate mutations, and at 99.5% coupling efficiency, a preparation of a 200-nt oligonucleotide carries only 36.7% and a 124-nt oligonucleotide carries only 53.7% full length, wild-type product. As such, it may be desirable to use oligonucleotides of shorter lengths whenever possible and without compromising targeting efficiency, or one may want to use PAGE or HPLC to enrich for the full-length product.

Design the donor oligonucleotide following these guiding principles:

1. Total length is less than 200 nt, ideally around 125 nt.

2. The two homology arms flank the intended mutation, with minimally 40 to 50 nt allocated to each arm.

3. The intended mutation is positioned such that it disrupts (for tag or loxP insertion models) or replaces (for point mutation incorporation models) the PAM or the guide sequence, particularly the guide sequence proximal to the PAM sequence.

4. If such arrangement of the intended mutation is not possible, consider creating synonymous mutations such that the newly created HDR allele will not continue to be a substrate for CRISPR-Cas9 cleavage. Choose a synonymous mutation that has a comparable codon usage level as compared to that of the wild-type codon, or the synonymous mutation may work out as a knockdown or a knockout mutation and complicate interpretation of the phenotype associated with the intended mutation (Kimchi-Sarfaty et al., 2007). In addition, make sure that the silent mutation will not create a cryptic splice donor or acceptor sites that lead to production of aberrant splicing product.

5. However, it is possible to create mutant alleles free of synonymous mutations, if a larger number of mice can be generated to compensate for the possible lower efficiency of recovering the mutant allele, due to CRISPR-Cas9 cleaving the HDR allele.

6. After finalizing the design, order the oligonucleotide with a vendor considering using PAGE or HPLC to enrich for the full-length, wild-type oligonucleotide. Store at 4°C until use.

SUPPORT PROTOCOL 5

DONOR PLASMID ASSEMBLY

When the intended mutation is transgene insertion or replacement of murine genome sequence with its human ortholog, a donor plasmid can be assembled by molecular cloning techniques carrying the exogenous gene flanked by homology arms of a few thousand nt. Different from a targeting vector intended for a conventional gene-targeting experiment, the donor vector created for an HDR experiment mediated by CRISPR-Cas9 does not require positive/negative selection cassettes. Also, the donor plasmid is used as a circular, supercoiled preparation to minimize random integration into the genome.

Materials

NucleoBond Xtra Midi EF kit (Clontech, cat. no. 740420.50)

Additional reagents and equipment for standard molecular cloning techniques (Ausubel et al., 2016)

1. Design the donor plasmid with these guiding principles:

   a. Perform "repeat masker" analysis (*http://www.repeatmasker.org/*) to understand the sequence around the region to be mutated.
   b. Position the homology arms around the region of interest to maximize inclusion of unique sequences and such that total combined length of the homology arms is between 5 to 15 Kb.
   c. Take into consideration the need for long-range PCR, restriction fragment length polymorphism analysis, and the 5′ and 3′ Southern blot strategies (Fig. 3), and incorporate restriction sites, if necessary.

2. Assemble donor plasmid using molecular cloning techniques, including PCR, gene synthesis, restriction digestion/ligation, Gibson assembly, or recombineering (see Ausubel et al., 2016), and clone all components, including the exogenous gene, the left homology arm and the right homology arm into a plasmid (pUC57, etc.).

3. Perform restriction fragment length polymorphism analysis to identify the correct clones and sequence the selected clones to be sure of the sequences, particularly around the junctions of the fragments.

4. Produce a midiprep of DNA, free of RNase contamination, using the Clontech NucleoBond Xtra Midi EF kit, according to manufacturer's instructions.

5. Elute and adjust to 500 ng/μl.

BASIC PROTOCOL 2

MICROINJECTION TO DELIVER THE CRISPR-Cas9 REAGENTS INTO MOUSE ZYGOTES

For an indel model mediated by NHEJ, the microinjection mixture, containing sgRNA(s) and Cas9 mRNA, is injected into the cytoplasm of the zygote. For a knock-in model mediated by HDR, we inject into both the pronuclei and the cytoplasm. The embryos are transferred the same day into pseudopregnant female mice and carried to term. A latent period of a few hours is expected during which the Cas9 mRNA will be translated into the protein nuclease. Also, it is possible that the CRISPR-Cas9 reagents may continue to be available while the embryos divide and the chromosomes duplicate. This may explain the observation that founder mice from a CRISPR-Cas9 experiment are often mosaic, carrying a variety of alleles in each founder. See Figures 4 and 5, and Video 1.

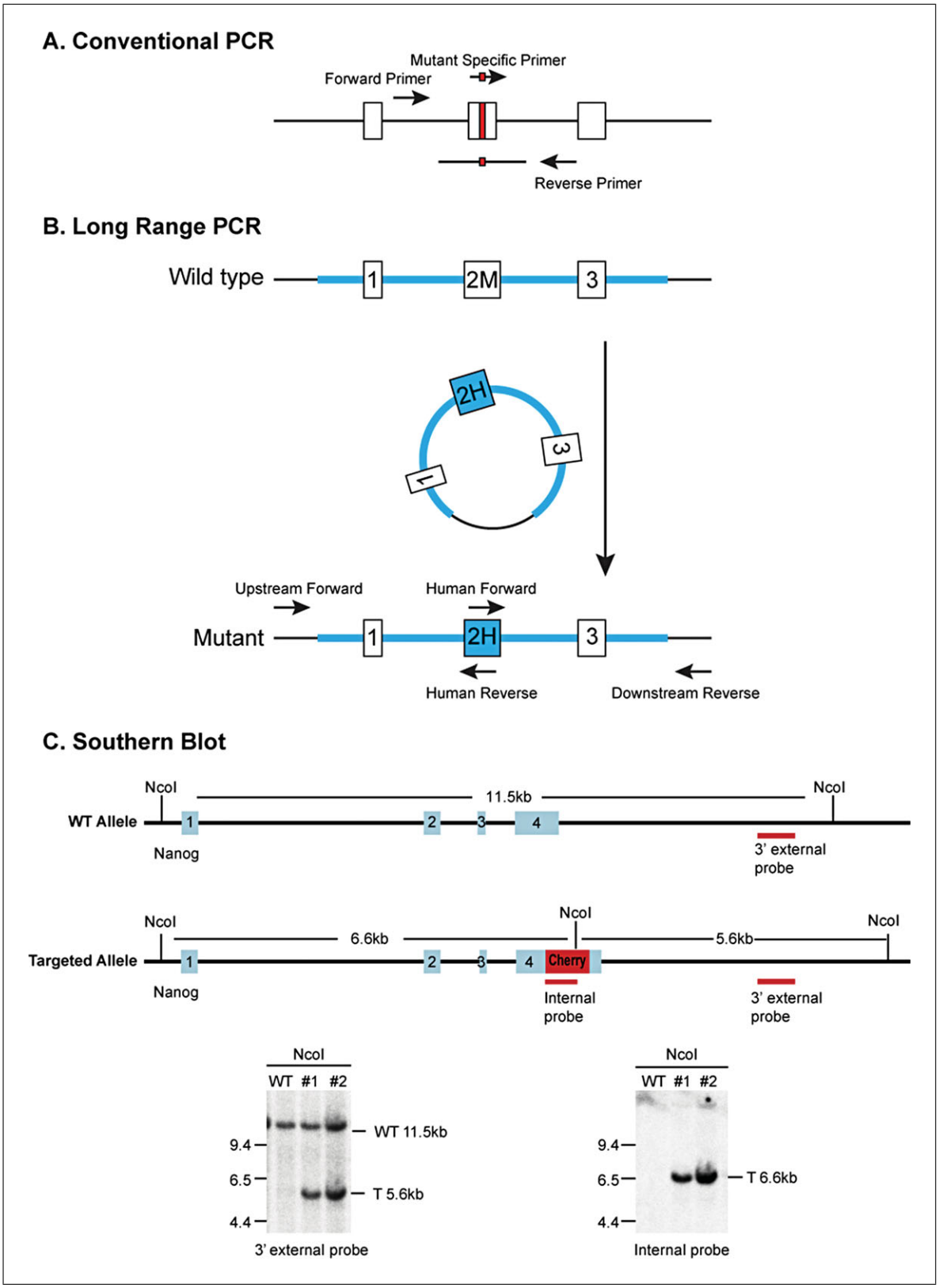

Figure 3 *(legend appears on next page)*

Materials

Donor female mice (various strains; The Jackson Laboratory)
Pregnant mare serum gonadotrophin (PMSG; Prospec, cat. no. HOR-272)
Human chorionic gonadotrophin (hCG; Prospec, cat. no. HOR-250)
Stud male mice (various strains; The Jackson Laboratory)
M2 medium (Sigma-Aldrich, cat. no. M7167-100ML)
Hyaluronidase (Sigma, cat. no. H3506)
K-RVCL-50 medium (Cook Medical)
Mineral oil (Sigma-Aldrich, cat. no. M8410)
Pseudopregnant female mice (CByB6F1/J; The Jackson Laboratory, stock no. 100009; 9 to 11 weeks)

Microdissecting forceps
MINC Benchtop Incubator (Cook Medical)
Microscope slides
Zeiss AxioObserver.D1 Microscope (Zeiss)
TransferMan NK2 micromanipulator (Eppendorf)
Narashige IM-5A Pneumatic Injector (Tritech Research, Inc.)
Thin-wall glass capillaries (World Precison Instruments, cat. no. TW100F-4)
P-97 micropipet puller (Sutter Instrument Company)
1.8-ml cryogenic tube with round bottom (ThermoFisher Scientific, cat. no. 363401)

Additional reagents and equipment for injection (Donovan and Brown, 2006a) and euthanasia (Donovan and Brown, 2006b) of rodents, and microinjection (Hogan et al., 1986)

1. To each donor female mouse (age determined by strain), administer 5 IU intraperitoneally (i.p.) of PMSG followed 47 hr later by 5 IU of hCG (also i.p.).

   Protocols for injection of rodents are found in Donovan and Brown (2006a).

2. Immediately post administration of hCG, mate each female mouse with a stud male mouse.

3. Twenty-two hours later, examine female mice for the presence of a copulation plug in the vaginal tract.

4. Euthanize (Donovan and Brown, 2006b) female mice displaying a copulation plug, excise the oviducts, and place them into M2 medium.

5. Transfer the oviducts into M2 medium containing hyaluronidase (0.3 mg/ml).

Figure 3 *(image appears on previous page)* Genotyping strategies. (**A**) Genotyping by conventional PCR. For indel models, a pair of PCR primers encompassing the target region is used. For HDR models mediated by a donor oligonucleotide, the pair of primers should be positioned 5′ and 3′ to the region overlapping with the donor oligonucleotide. In addition, mutant-specific primers can be designed to amplify only the mutant allele. (**B**) Genotyping by long-range PCR. If a model is created mediated by a donor plasmid, the mice can be genotyped by a pair of primers with the upstream or downstream primer positioned outside of the homology arm and the second primer unique to the transgene sequence. (**C**) Genotyping by Southern blot. Southern blot can be used to assess successful integration of the transgene into the target genomic locus. In this example, *Nco*I digestion releases an 11.5 Kb genomic DNA fragment from the Nanog locus from the wild-type mice. When the mCherry transgene has been successfully inserted into the Nanog locus, it brings along an additional *Nco*I site, generating a 5.6 Kb genome fragment in mutant mice that could be detected by the 3′ probe located outside of the 3′ homology arm. Southern blot with a probe from the mCherry gene confirms there is only one integration event per genome.

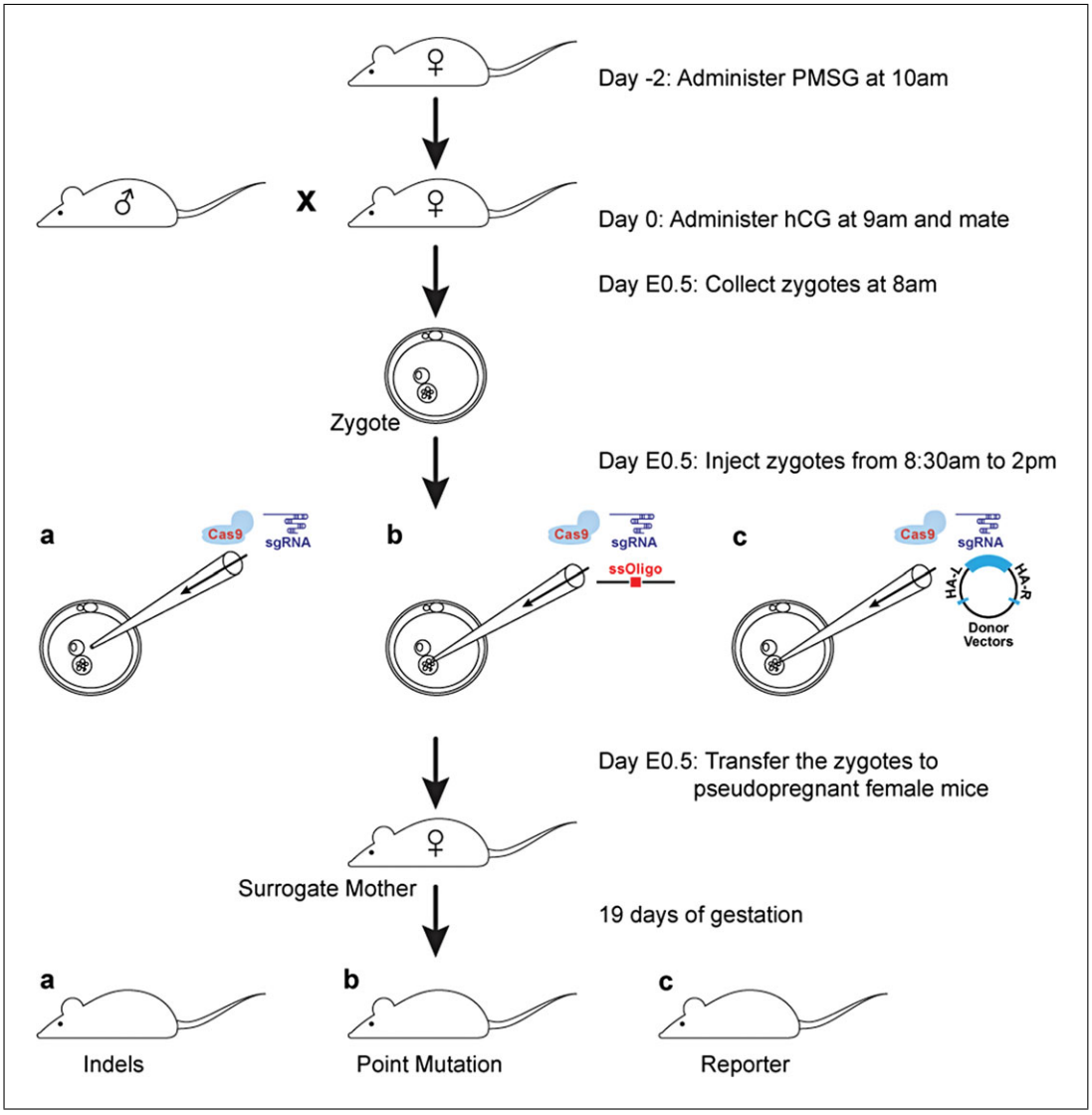

Figure 4 Flow scheme of CRISPR-Cas9-mediated mouse model generation. This is the flow scheme for the C57BJ/6 J strain. Female donor mice are administered PMSG 2 days prior (5 IU), followed 47 hr later by hCG (5 IU), and mated with a stud male mouse immediately. Embryos are collected in the morning, injected with the CRISPR-Cas9 reagents, and transferred the same day. Founder mice can be genotyped when they are 10 to 14 days old.

6. Release the oocyte clutch by puncturing the ampulla with a pair of microdissecting forceps and allow them to incubate in the M2 medium containing hyaluronidase until the cumulus cells fall off.
7. Transfer zygotes to a dish containing fresh M2 medium and grade for fertilization and viability by examining for presence of the two pronuclei and integrity of the membranes enclosing the embryo.
8. Pass the graded zygotes through two washes of fresh M2 medium and then place in microdrops of K-RVCL-50 medium that have been equilibrated under mineral oil for 24 hr in a Cook MINC benchtop incubator (37°C, 5% CO_2/5% O_2/nitrogen).
9. Remove the zygotes from culture and place onto a slide containing 150 μl of fresh M2 medium.
10. Perform microinjection on a Zeiss AxioObserver.D1 using Eppendorf NK2 micromanipulators in conjunction with Narashige IM-5 A injectors. Follow standard

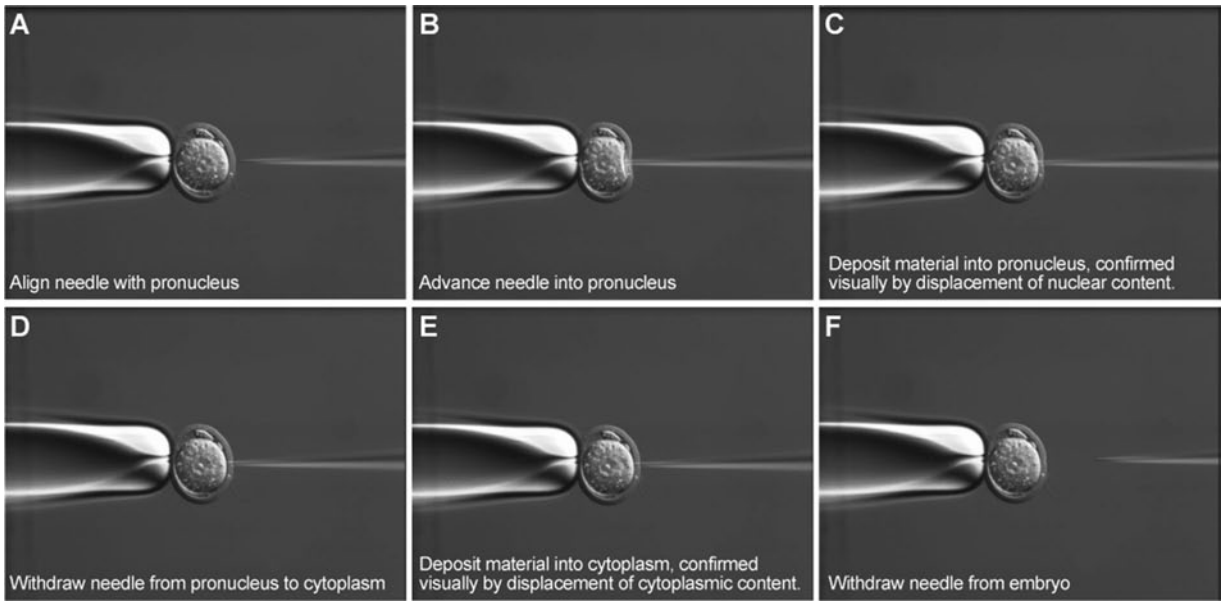

Figure 5 Microinjection of CRISPR-Cas9 reagents into the mouse zygote. Panels (**A**) to (**F**) show how to deposit the CRISPR-Cas9 reagents into both the pronucleus and the cytoplasm.

zygote microinjection procedure (Hogan et al., 1986), with special care taken to deposit the CRISPR-Cas9 reagents into both the pronucleus and the cytoplasm of the zygote (Fig. 4).

Needles for microinjection are pulled fresh daily using World Precison Instruments TW100F-4 thin wall glass capillaries.

11. Fill the microinjection needle with the CRISPR-Cas9 reagents (microinjection mixture from Basic Protocol 1) by placing the back end of the needle into the vial containing the reagents and allow it to fill via capillary action to the tip. When there is visual confirmation that the tip of the needle has filled, remove the needle from the vial and load into the injector (Narashige IM-5A).

12. To prevent the medium on the slide from diluting the reagents inside the needle through the opening at the tip, establish back pressure in the needle before moving it to the slide. On the slide, open the needle further (chip it) by gently flicking it against the holding pipet.

    Once the needle is opened, pressure is applied to establish flow of the reagents out of the tip of the needle. It is critical that positive pressure be maintained at all times to prevent influx of the medium and dilution of the reagents.

13. To generate NHEJ-mediated knockout models, orient the zygote so that the needle can enter without piercing the pronucleus. Move the needle along the *x* axis to penetrate the zona and the oolemma layers of the zygote, and deposit reagents into cytoplasm. Visually confirm that reagents are being deposited by observing the displacement of the cytoplasm at the tip of the needle.

    If a bleb forms, the oolemma has not been pierced and the injection was not successful.

14. To generate HDR-mediated knock-in models, orient the zygote to allow clear access to the pronucleus to be injected (Fig. 4A). Move the needle along the *x* axis to penetrate through the zona and cytoplasm of the zygote, enter, and deposit reagents into the pronucleus (Fig. 4B, C, D).

The pronucleus will swell as reagents are deposited, and once that has been confirmed, retract the needle into the cytoplasm and pause briefly to also deposit reagents there (Fig. 4E, F).

Displacement of the cytoplasm indicates a successful injection (Video 1).

15. Remove the injected zygotes from the slide, rinse through three 30-μl drops of equilibrated K-RVCL-50, and then place into a separate 30-μl microdrop of equilibrated K-RVCL-50.

16. Remove the zygotes from culture and place into a 1.8-ml screw-cap cryogenic tube preloaded with 900 μl of pre-warmed M2 medium for transport to the surgical suite.

17. Remove the zygotes from the tube and place into culture (K-RVCL-50 under oil, Cook MINC benchtop incubator, 37°C, 5% CO_2/5% O_2/nitrogen).

18. At the time of transfer, remove the zygotes from culture, place into pre-warmed M2 medium, and transfer via the oviduct into day-0.5 pseudopregnant CBYB6F1/J females (9 to 11 weeks) following standard methodologies (Hogan et al., 1986).

GENOTYPING BY CONVENTIONAL PCR AND SEQUENCING OF PCR PRODUCT

BASIC PROTOCOL 3

To genotype NHEJ or HDR models mediated by CRISPR-Cas9 using a donor oligonucleotide, conventional PCR and sequencing of the PCR product may suffice. The PCR primers should be positioned to encompass the area to be targeted and should produce a product of 500 to 600 nt to allow good-quality sequencing data for genotype analysis (Fig. 3A). If possible, for HDR models, mutant-specific PCR primers should be designed to enable identification of founder mice carrying the intended mutation, among other mutant alleles (Fig. 3A). This could be very helpful, considering that founder mice generated with the CRISPR-Cas9 nuclease are often mosaic and the mutant allele may only be one of those existing in a particular founder mouse.

A tail snip or ear punch sample may be taken when the mice are 10 to 14 days old and processed by the HotSHOT method (Truett et al., 2000) to screen for potential founder mice. As discussed earlier, founder mice from a CRISPR-Cas9 experiment are often mosaic, with the founder mouse carrying more than two alleles. Allele composition in a particular founder mouse could be analyzed using the TIDE software program, which decomposes the alleles (Brinkman et al., 2014; Fig. 6A) or PCR products encompassing the target site can be cloned and individual clones sequenced to assess the nature and frequencies of appearance of the alleles.

Materials

2-mm tail snips or ear punches from mice to be genotyped
Alkaline lysis solution: 25 mM NaOH/0.2 mM EDTA, pH 12
Neutralization solution: 40 mM Tris·Cl, pH 5.0
PrimeSTAR GXL DNA Polymerase (Clontech, cat. no. R050B) and 5× PrimeSTAR GXL buffer
dNTP mixture (2.5 mM each dNTP)
Forward and reverse PCR primers (designed based on the principles in Fig. 3; ordered from IDT and reconstituted to 10 pmol/μl with DNase-free water)
6× DNA gel loading dye (Life Technologies, cat. no. R0611)
HighPrep PCR reagent (MAGBIO, cat. no. AC-60050)
70% ethanol
Elution buffer: H_2O, Tris·Cl, pH 8.0, or TE buffer
Sequencing primer (ordered from IDT and reconstituted to 10 pmol/μl with DNase-free water)

96-well thermal cycling plate (VWR, cat. no. 89049-178)
MicroAmp Clear Adhesive Film (Life Technologies, cat. no. 4306311)
Galaxy 20R centrifuge (VWR)
12.5 μl, 12-channel VIAFLO II electronic pipet (Integra Biosciences, Part No. 4621)
125 μl, 12-channel VIAFLO II electronic pipet (Integra Biosciences, Part No. 4622)
1250 μl, 12-channel VIAFLO II electronic pipet (Integra Biosciences, Part No. 4624)
Thermal Cycler with 96-Well Fast Reaction Module (BioRad, cat. no. 1851196)
96R Magnet Plate (Alpaqua, cat. no. A001219)

Additional reagents and equipment for PCR (Kramer and Coen, 2000) and agarose gel electrophoresis (Voytas, 2000)

1. Place 2-mm tail tip (or ear punch) sample into a well of a 96-well thermal cycling plate, accommodating all samples from one project and multiple projects on the same plate. When finished collecting the samples, seal the plate with a plate cover.

   Biopsy samples can then be transported from the animal facility to a molecular biology laboratory.

2. Centrifuge briefly to collect the samples to the bottom of the wells and peel off the cover.
3. Add 50 μl of the alkaline lysis solution to each well using a multichannel pipet.
4. Cover the plate with MicroAmp Clear Adhesive Film.
5. Centrifuge briefly to make sure samples are submerged in lysis solution and there are no air bubbles trapped in the solution.
6. Boil in a PCR machine with heated lid at 98°C for 60 min, and cool to room temperature at the end of the incubation period.
7. Centrifuge briefly to collect all contents at the bottoms of the wells, and then peel off the cover.
8. Add 50 μl of neutralization solution and mix well by pipetting up and down a few times. Apply a new cover.
9. Centrifuge briefly to collect all contents at the bottoms of the wells.
10. Use 1 μl of the supernatant in a 25-μl PCR reaction (see step 12). Avoid touching the bottom of the well and picking up hair and the tissue chunks that may not have been dissolved.
11. Store the remaining tissue lysate at 4°C for a few months or, for longer-term storage, at –20°C.
12. Set up 25-μl PCR reaction in a new 96-well plate as recommended by the manufacturer:

    5.0 μl 5× PrimeSTAR GXL Buffer (1× final)
    2.0 μl dNTP mixture (2.5 mM each dNTP; 200 μM each, final)
    0.5 μl 10 pmol/μl forward primer (0.2 pmol/μl final)
    0.5 μl 10 pmol/μl reverse primer (0.2 pmol/μl final)
    1.0 μl template (crude lysate)
    0.5 μl PrimeSTAR GXL DNA polymerase (0.625 U/50 μl final)
    15.5 μl sterile distilled H_2O.

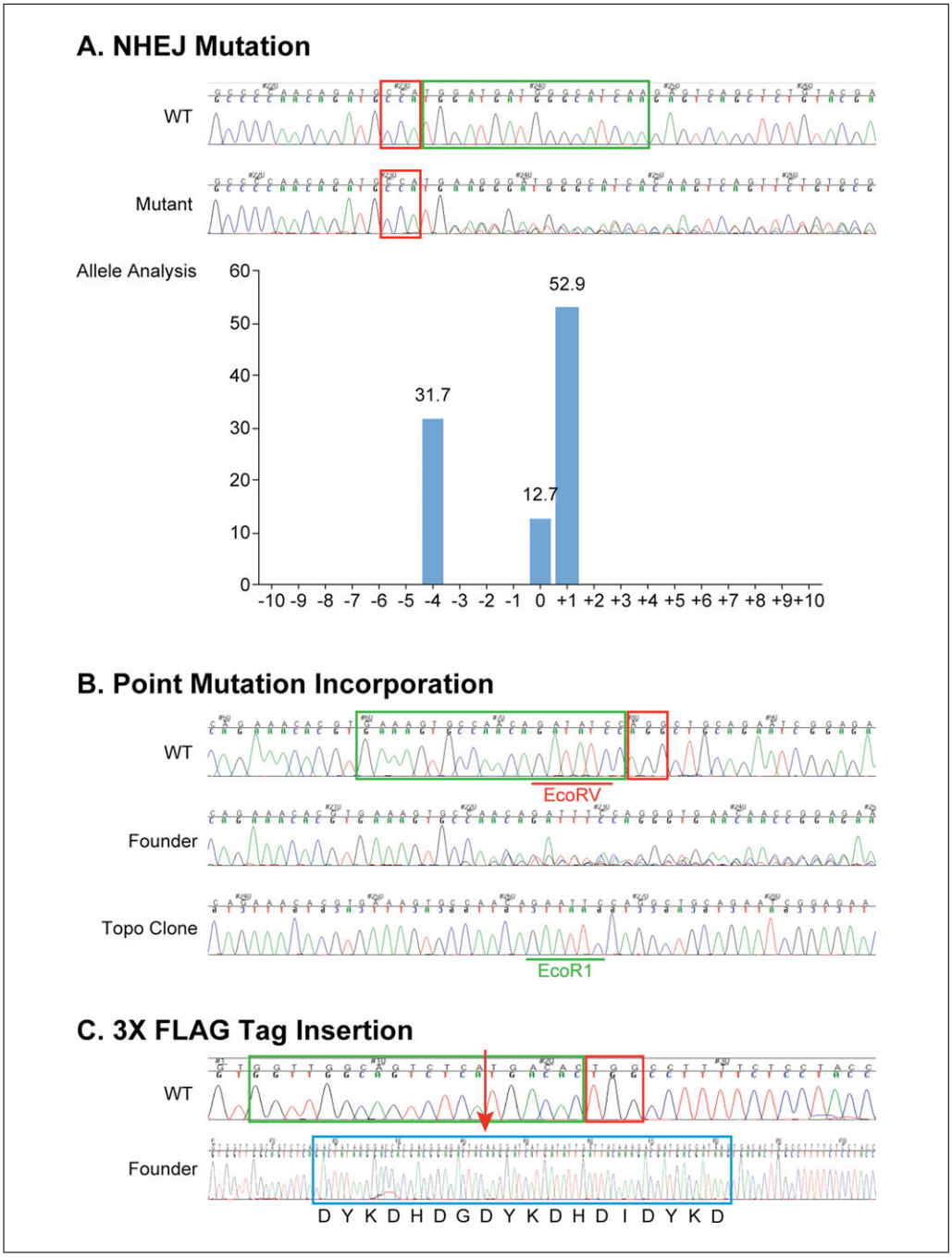

Figure 6 Genotyping CRISPR-Cas9 mice by sequencing of PCR product. (**A**) NHEJ mutation: Sequencing data from a founder mouse is provided, along with sequencing data from a wild-type littermate. The PAM sequence is boxed in red and guide sequence in green. The wild-type mouse exhibits clean wild-type reference sequence. The founder mouse shows overlapping of three sequencing traces starting from the third nt counting from the PAM sequence. Sequence analysis using the TIDE program reveals the existence and relative abundance of three alleles in the founder mouse, including alleles with 1 nt inserted (52.9%), 4 nt deleted (31.7%), and no change in length (12.7%), when compared to the wild-type sequence. (**B**) Point mutation incorporation. The founder mouse carries several alleles, and the correctly targeted allele carrying the intended *Eco*RV to *Eco*RI conversion is confirmed by cloning of the PCR product and sequencing of individual clones. Boxed in red is the PAM sequence and in green is the guide sequence. (**C**) 3× FLAG tag insertion. Successful insertion of the 3× FLAG tag was confirmed by sequencing of the cloned PCR product. Boxed in red is the PAM, in green is the guide, and in blue is the 3× FLAG tag sequences. The 3× FLAG insertion site is indicated by a red arrow.

by pipetting 24 μl of the PCR reaction mixture into each well on the plate and then transferring 1 μl of the crude lysate prepared from the mouse biopsy sample into each well.

Primers are designed based on the principles described in Figure 3.

13. Perform PCR (Kramer and Coen, 2000) using the following cycling conditions:

30 cycles:	10 sec	98°C	(denaturation)
	15 sec	55°C	(annealing)
	1 min/Kb	68°C	(extension).

14. Add 5 μl of the 6× DNA loading dye to the 25-μl PCR reaction, mix well by pipetting up and down several times, microcentrifuge briefly to collect contents, and separate 5 μl on 1% (weight/volume) agarose gel (Voytas, 2000) to assess yield and uniqueness of the PCR product.

    When validating the PCR strategy using wild-type genomic DNA as template, if multiple PCR products are observed on the gel, it may be necessary to increase the annealing temperature or optimize other parameters until a clean and robust PCR product is produced. In addition, PCR product amplified from a founder mouse could appear as multiple bands. This may be the first evidence that a founder mouse has just been identified and tthat it carries deletions in the region that are large enough to be resolved on an agarose gel. Occasionally, a larger band may appear, indicative of larger deletions or insertions in the region encompassed by the PCR product. All could be confirmed definitively by cloning and sequencing of the PCR products.

15. Mix the HighPrep PCR reagent (magnetic beads) well and add 45 μl of the reagent to the 25-μl PCR reaction in the 96-well sample plate.

16. Mix well by pipetting up and down six to eight times, centrifuge briefly, and incubate at room temperature for 5 min (PCR product should now bind to the magnetic beads).

17. Place the sample plate on the Magnet Plate for 2 to 3 min until the solution clears (beads will pull to the side of the well).

18. Remove and discard the supernatant by pipetting (be careful not to disturb the beads).

19. Wash at least twice, each time by adding 100 μl of 70% ethanol to each well, incubating for 30 sec at room temperature, and removing and discarding the supernatant by pipetting.

20. Air dry the samples for about 5 min until all traces of ethanol have evaporated.

21. Remove the sample plate from the Magnet Plate and add 40 μl of elution buffer (water, Tris·Cl, pH 8.0, or TE buffer).

22. Mix thoroughly by pipetting up and down a few times, incubate for about 2 min, and place back on the Magnet Plate to clear the contents.

23. Use 5 μl of PCR product and 1 μl of the sequencing primer (5 pmol/μl) for a sequencing reaction.

    The sequencing primer could be the same as the primers used for the PCR reaction or nested to the PCR primers.

24. Download sequencing data and use any sequence analysis software (e.g., Sequencher, Vector NTI; Geneious) for analysis, comparing to sequencing data from a wild-type mouse (Fig. 6A).

    As discussed earlier, founder mice from a CRISPR-Cas9 experiment are often mosaic. The number and nature of the alleles each founder carries can be determined by the

TIDE software program (http://tide.nki.nl; Fig. 6A) or more definitively by cloning of the PCR products and sequencing of individual clones.

Mice carrying successful point mutation incorporation and tag insertion can also be genotyped by conventional PCR and sequencing of the PCR product (Fig. 6B,C).

LONG-RANGE PCR ANALYSIS

SUPPORT PROTOCOL 6

When a donor plasmid is used in a CRISPR-Cas9 experiment, it is necessary to differentiate the HDR allele from random integration of the donor plasmid into the genome. This can be accomplished by long-range PCR or Southern blot analysis. A long-range PCR experiment could be designed such that one primer is located in the genomic region beyond the region overlapping with the homology arms of the donor plasmid and the second primer unique to the intended mutation (Fig. 3B). In this arrangement, a successful PCR product will only be produced when the intended mutation has been incorporated by HDR, as compared to having been integrated randomly into the genome.

Materials

Gentra Puregene Mouse Tail Kit (Qiagen, cat. no. 158267)
QIAquick PCR Purification Kit (Cat. No. 28106, Qiagen)
70% ethanol
10 mM Tris·Cl, pH 8.0

Additional reagents and equipment for conventional PCR (Basic Protocol 3)

1. Extract genomic DNA from mouse biopsy samples using the Gentra Puregene Mouse Tail Kit according to manufacturer's instructions.
2. Perform long-range PCR as in Basic Protocol 3, with extension time adjusted to allow amplification of longer PCR product (with each additional 1 Kb of sequence, add 1 min to the extension time).
3. Purify PCR product using the QIAquick PCR purification kit, following manufacturer's instructions (adequate for recovery of PCR products 100 nt to 10,000 nt in size).
4. Depending on the genotyping strategies designed for the model, perform restriction fragment length polymorphism (RFLP) analysis or sequence the PCR product to determine whether intended mutation has been incorporated.

Founder mice from a CRISPR-Cas9 experiment are often mosaic. It may be necessary to clone the product products and sequence individual clones to determine whether the intended mutation has been incorporated.

SOUTHERN BLOT ANALYSIS

SUPPORT PROTOCOL 7

Southern blot analysis (Southern, 2006; also see Brown, 1999) is a well-established protocol in molecular biology. The major advantage over long-range PCR analysis is that it provides unequivocal data regarding integrity of the targeted allele. If the targeting event has occurred as predicted, a well thought-out Southern blot design will produce a banding pattern that supports the notion that the targeted allele is correct and does not involve aberrant recombinant product at the intended site. Southern blot analysis should always be performed for HDR allele mediated by CRISPR-Cas9 with a donor plasmid.

Here are some basic considerations (see Fig. 3C for an example):

1. Construct DNA sequences for the wild-type and the targeted alleles in silico.

2. Perform restriction analysis to identify a restriction site that would satisfy these criteria:

   a. It is not a substrate for CpG methylation, or if it is, the cognate restriction enzyme is not sensitive to CpG methylation. Some examples are *Bam*HI (GGATCC), *Bgl*II (AGATCT), *Hin*dIII (AAGCTT), *Kpn*I (GGTACC), *Mfe*I (CAATTG), *Nco*I (CCATGG), *Spe*I (ACTAGT), and *Xba*I (TCTAGA).
   b. In the wild-type allele, a pair of restriction sites should exist, with one restriction site positioned outside of the homology arm, extending into the genomic locus. The second restriction site is best positioned also outside of the other homology arm. Use two different restriction sites, if a pair of the same restriction sites is not available.
   c. For the targeted allele, a convenient design is for the exogenous gene to bring in this same restriction site that, combining with the restriction site outside of the homology arm, produces a restriction fragment that differs in size from that of the wild-type allele. Alternatively, if the inserted transgene is large enough, the use of the same two naturally occurring restriction sites outside of the homology arms will produce a mutant fragment that is larger than the wild-type sequence, and the two bands can be resolved on agarose gel and subsequently, on the blot.
   d. The fragment encompassed by the two restriction sites should be less than 20 Kb in size to allow efficient transfer of the DNA fragment from the agarose gel to the membrane during the blotting process.
   e. There should be enough sequence in between the end of the homology arm and the restriction site outside of the homology arm to allow its use as the probe for Southern blotting. A typical probe for a Southern blot is from 500 nt to 800 nt in size and unique in sequence. Uniqueness of this sequence could be verified using a software program such as RepeatMasker analysis (*http://www.repeatmasker.org/).*
   f. The DNA fragment released by this restriction enzyme between the wild-type allele and mutant allele would differ in size such that they will be separated during agarose gel electrophoresis and subsequently, differentiated on the blot. If the two bands are close in size, they may be separated by prolonged electrophoresis.

3. Follow standard procedures for Southern blot (e.g., Brown, 1999).

   Southern blot analysis performed for founder mice will identify the targeted allele if this allele is a major allele in the founder mice. However, when the targeted allele exists as a minor allele, Southern blot may not be adequate to discern this allele on the blot. In this case, long-range PCR product could be produced, cloned, and individual clones sequenced to identity this allele. In addition, a mutant-specific PCR strategy could be designed to enable identification of the mutant allele among other alleles.

BASIC PROTOCOL 4

CRISPR-Cas9 DELIVERY BY ELECTROPORATION

Although CRISPR-Cas9 reagents have traditionally been injected directly into zygotes as described in Basic Protocol 2, several groups, including ours, have shown that efficient genome editing can be achieved by using electroporation to deliver the reagents (Kaneko et al., 2014; Hashimoto and Takemoto, 2015; Qin et al., 2015; Video 2).

Materials

Cas9 mRNA (Support Protocol 3)
sgRNA (Support Protocols 1 and 2)
Donor oligonucleotide (Support Protocol 4)
TE buffer, pH 7.5: 10 mM Tris·Cl, pH 7.5/0.1 mM EDTA, DNase- and RNase-free
M2 medium (Sigma-Aldrich, cat. no. M7167-100ML)
EmbryoMax M2 Medium (EMD Millipore, cat. no. MR-015-D)

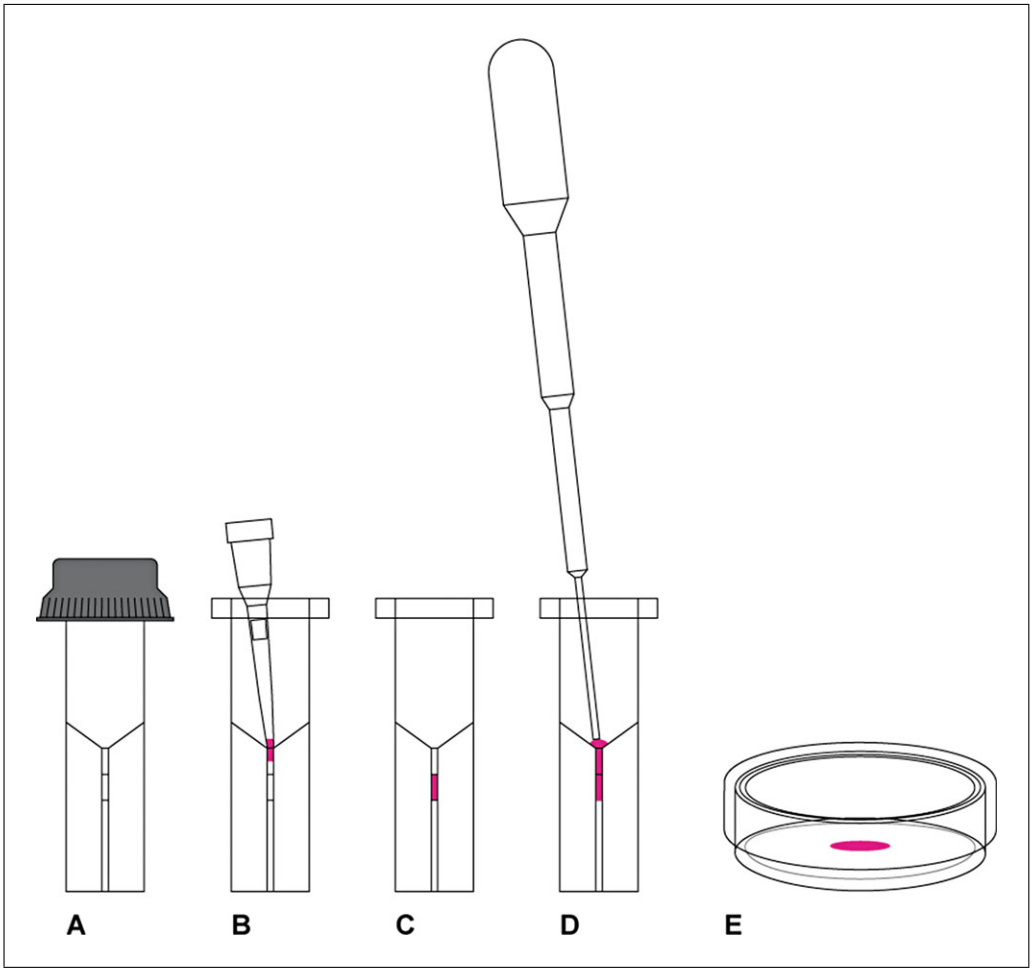

Figure 7 CRISPR-Cas9 delivery by electroporation. (**A**) We use the ECM 830 Square Wave Electroporation System from Harvard Apparatus and the electroporation cuvette from the same vendor with 1-mm gap between the electrodes. The minimum volume is 20 μl and maximum 90 μl. (**B**) The 20 μl of embryos suspended in the CRISPR-Cas9 reagents is loaded into a 20-μl pipet tip and ready to be released into the chamber of the cuvette. (**C**) The 20 μl of embryos suspended in the CRISPR-Cas9 reagents is released into the chamber and spread evenly at the bottom of the chamber by gentle tapping. The cuvette is then transferred to the electroporator and pulses delivered. (**D**) Use the sterile plastic transfer pipet that comes with the cuvette to pick up 100 μl of prewarmed and pre-equilibrated KSOMaa Evolve medium with 1 mg/ml BSA and release the contents into the bottom of the cuvette. (**E**) Pipet up and down a few times to release the embryos that may have adhered to the wall of the chamber, transfer to a 35-mm Petri dish, and prepare the embryos for transfer into pseudopregnant female mice.

Acidic Tyrode's solution (Sigma-Aldrich, cat. no. T1788)
Opti-MEM medium (Life Technologies, cat. no. 31985)
KSOMaa Evolve medium (Zenith Biotech, cat. no. ZEKS-050)
Bovine serum albumin (Sigma-Aldrich, cat. no. A2153)

Electroporation Cuvette Plus, 1 mm gap (Harvard Apparatus, cat. no. 45-0124)
ECM830 Square Wave Electroporation System (UX-02894-18, Harvard Apparatus; Fig. 7A)
35 × 10 mm Petri dish (Corning, cat. no. 351008)
Pseudopregnant female mice (CByB6F1/J; The Jackson Laboratory, stock no. 100009, 9 to 11 weeks)

Additional reagents and equipment for harvesting embryos (Basic Protocol 2) and embryo transfer (Hogan et al., 1986)

1. Prepare the CRISPR-Cas9 reagents at 2× concentration in a volume of 10 μl in TE buffer, pH 7.5, in a DNase- and RNase-free vial, prepared fresh on the day of experiment as follows:

   1200 ng/μl Cas9 mRNA,
   600 ng/μl sgRNA
   2000 ng/μl donor oligonucleotide.

2. Harvest the embryos as described in Basic Protocol 2.
3. Remove the embryos from the K-RVCL medium and wash in pre-warmed EmbryoMax M2 medium.
4. In groups of 50 to 150, depending on the skill level of the personnel handling the embryos, place the embryos in the acidic Tyrode's solution for 10 sec.
5. Remove the embryos and wash through three 100 μl drops of pre-warmed EmbryoMax M2 medium.
6. Pick up the embryos with a pipet and, immediately before electroporation, place into a 10 μl drop of Opti-MEM medium that has been pre-warmed and equilibrated.
7. Add the 10 μl of CRISPR-Cas9 reagents, consisting of the Cas9 mRNA, sgRNA, and donor oligonucleotide (if relevant) reconstituted in TE buffer (pH 7.5), to the embryos in Opti-MEM medium and pipet gently up and down a few times to disperse the embryos into the CRISPR-Cas9 reagents.
8. Pick up the 20 μl of embryos suspended in CRISPR-Cas9 reagents with a 20-μl pipet tip and deposit the contents into the chamber of a 1-mm electroporation cuvette, making sure that the pipet tip is securely positioned between electrodes of the cuvette (Fig. 7B).
9. Cap the cuvette and tap gently to collect all content to the bottom of the chamber, eliminating any bubbles and spreading the content evenly on the bottom of the chamber (Fig. 7C).
10. Electroporate using the ECM830 Square Wave Electroporation System with the conditions of 30 V, 1 msec pulse duration and two pulses separated by 100 msec pulse interval.
11. Following delivery of the pulses, with the sterile plastic transfer pipet that comes with the cuvette, pick up 100 μl of prewarmed and pre-equilibrated KSOMaa Evolve medium with 1 mg/ml BSA and release into the bottom of the cuvette (Fig. 7D).
12. Remove the embryos from the cuvette and place into a 35 × 10 mm Petri dish (Fig. 7E).
13. Rinse the cuvette with additional 100 μl of KOSMaa Evolve containing 1 mg/ml BSA and add to the embryos in 35 × 10 mm Petri dish.
14. Transfer the embryos into CByB6F1/J pseudopregnant female mice following standard embryo transfer protocol (Hogan et al., 1986).

COMMENTARY

Background Information

Although mouse models carrying targeted mutations have been created in the last 30 years using gene-targeting technology (Capecchi, 2005), they are limited to those strains that have available germline-competent ES cell lines and are prone to technical challenges inherent in the technology. The nuclease technologies, including ZFN, TALEN, and CRISPR-Cas9, have recently been invented and found to be highly efficient such that they can be injected directly into mouse zygotes and

Table 2 Troubleshooting Guide to Generating Mouse Models Using CRISPR-Cas9-Mediated Genome Editing

Problem	Possible cause	Solution
Cas9 mRNA degraded	RNase might have been introduced into the system	One must be mindful that RNase is ubiquitous in a laboratory environment and exercise vigilance by decontaminating the counter top and all glassware and plasticware used to support Cas9 mRNA synthesis; use RNase-free tips and vials. Also avoid talking while working with RNA samples.
No or low recovery of the sgRNA from column purification	The MEGAclear Kit is a glass filter-based system for purification of single-stranded RNA transcript and suitable for RNA transcripts greater than 100 nt. The sgRNA in a CRISPR/Cas9 experiment is often 120 nt in size and may not be adequately retained in the column.	Consider using phenol:chloroform extraction and alcohol precipitation to purify the sgRNA preparation instead of MEGAclear kit, or use alcohol precipitation directly.
Microinjection mixture clogging the needle	This may be caused by particulate matter carryover from column purification or a high concentration of the donor DNA	Centrifuge the microinjection mixture at 20,000 × g for 15 min and use the supernatant for microinjection
No or low live birth for manipulated embryos	Embryos compromised in the process	Whenever available, transfer unmanipulated embryos as a control to determine whether media, reagents, or environmental conditions (excessive noise, construction, etc.) may be the problem.
No targeting	The guide may not have worked	Choose another guide or carry more than one guide in parallel if possible to maximize the chance of successful delivery of the model at the end of the process. Also, it may help to evaluate the guides in vitro in a cell line or injecting into mouse embryos and screen the guides before choosing a guide for a full-scale experiment.
Poor recovery of the embryos from the electroporation cuvette	The embryos may have adhered to the sides of the cuvette	Be sure to use the plastic transfer pipet that comes with the cuvette to transfer embryos. Also, the embryos can be recovered by an additional round of rinsing of the cuvette.

mutant mice derived among a limited number of mice born. We first reported the successful generation of mutant mice using a piezo drill–based injection method to deliver the CRISPR-Cas9 reagents into the mouse zygotes (Wang et al., 2013; Yang et al., 2013). In this protocol, we focus on pronuclear microinjection, which is a more widely used method, and present electroporation as an alternative effective method to deliver the CRISPR-Cas9 system.

Critical Parameters

Quality of the RNA preparations

Central to the CRISPR-Cas9 protocols is the use of Cas9 mRNA and sgRNA, which, although straightforward to synthesize, are prone to degradation by RNases which are ubiquitous in a laboratory environment. Special care must be exercised to avoid introducing RNase into the process while handling the samples during synthesis and injection. All countertops, glassware, and plasticware must be cleansed with solution formulated to eliminate RNase activity (for example, RNase Away from Life Technologies), and tips and vials must be certified to be RNase-free. RNA samples should be analyzed by spectrophotometry or agarose gel electrophoresis to assess integrity of the samples (Fig. 2).

Table 3 NHEJ Founder Efficiency Among the Major Inbred Strains of Mice

Strain	Projects	Guides worked	Total guides	Founder mice	Total mice	Founder efficiency (%)
C57BL/6J	6	9	10	94	214	44
C57BL/6NJ	2	3	3	8	74	11
NOD/ShiLtJ	22	38	44	200	643	31
NSG	10	15	18	91	291	31
NRG	1	2	2	16	29	55
DBA/2J	5	3	5	3	22	14
BXSB/MpJ	1	1	1	17	18	94
Total	47	71	83	429	1291	33

Guide efficiency

Although parameters impacting efficiency of the CRISPR-Cas9 system are starting to be understood (Doench et al., 2014), it remains a challenge to predict in vivo efficacy of a given guide. It is imperative to use more than one guide per experiment, if possible, to maximize the chance for a successful experiment. In addition, guides could be screened by transfecting into a cell line or injecting into mouse embryos to assess targeting efficiency. For a knock-in experiment, depending on the site to be mutated, there may be a limited number of guide choices for the spCas9. In this case, one may want to look into Cas9 with altered PAM specificities (Kleinstiver et al., 2015), which may provide additional genome coverage and flexibility.

Inbred strains of mice

Inbred strains of mice may respond differently to superovulation. To work with a particular inbred strain of mice, the protocol for superovulation may need to be adjusted accordingly, For example, in response to the same hormone regimen (5.0 IU PMSG and 5.0 IU of hCG), while the 129S1/SvlmJ mice produced 39.5 normal oocytes per female donor, the C57BL/6J mice produced 25 oocytes and the A/J produced only 5.4 (Byers et al., 2006). In addition, zygotes from inbred strains of mice may respond differently to the trauma of microinjection (Yamauchi et al., 2007) and electroporation. When dealing with unconventional or difficult inbred strains of mice, it may be useful to incorporate control experiments to monitor the entire process and to identify the cause of any problem that may be encountered.

Genotyping

Although some founders may be homozygous or heterozygous for the intended mutation and can be readily identified, others may carry the intended mutation, along with other mutant alleles. As such, it may require a special screening strategy to uncover the mutant allele. This could be accomplished by mutant-specific PCR strategies, cloning of the PCR products, and sequencing of the individual clones.

Troubleshooting

Table 2 lists various problems that can arise with the protocols in this article, along with their possible causes and solutions.

Anticipated Results

NHEJ knockout models

We have been successful delivering founder mice carrying NHEJ mutations for the more than 60 models that we have screened, if we use two guides per model. The average number of mice screened per guide is 15 and average founder efficiency 33% among 1,291 mice that we have genotyped (Table 3). It is understood that this efficiency rate may reflect the biology of the CRISPR-Cas9 system and, in addition, our learning curve working with the CRISP-Cas9 system, as these are among the first of our CRISPR-Cas9 experiments.

HDR knock-in models mediated by a donor oligonucleotide or a donor plasmid

We successfully delivered founder mice for 37 models out of the 42 models that we have genotyped. Founder efficiency differs widely among the models and strains but could be over 50%. Also, mice carrying homozygous

HDR alleles have been identified among the founder mice from certain projects.

Time Considerations

NHEJ knockout and HDR knock-in models mediated by a donor oligonucleotide

It is possible to derive founder mice within 2 months from concept to delivery of the founder mice, including 1 to 2 weeks to prepare reagents (donor oligonucleotide and sgRNA), 3 weeks for mice to be born, and 3 weeks for mice to age and be genotyped.

HDR knock-in models mediated by a donor plasmid

The timeline depends on successful synthesis or assembly of a donor plasmid. It is possible to derive founder mice within 2 months, when all reagents (Cas9 mRNA, sgRNA and a donor plasmid) are available.

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Internet Resources

http://www.ncbi.nlm.nih.gov/

Download genomic sequence for a gene.

http://www.ensembl.org/index.html

Exon/intron structure and sequence analysis.

http://www.uniprot.org/

Post-translational processing and protein functional domains.

http://crispr.mit.edu/

Off-target sequence match analysis.

https://benchling.com/

On-target and off-target efficiency scores.

http://www.rgenome.net/mich-calculator/

Micro-homology-mediated repair.

http://www.repeatmasker.org/

Repeat masking.

https://www.sanger.ac.uk/sanger/Mouse_SnpViewer/rel-1303

Sequence divergence among mouse strains.