Engineering Base Changes and Epitope-Tagged Alleles in Mice Using Cas9 RNA-Guided Nuclease

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Mice carrying patient-associated base changes are powerful tools to define the causality of single-nucleotide variants to disease states. Epitope tags enable immuno-based studies of genes for which no antibodies are available. These alleles enable detailed and precise developmental, mechanistic, and translational research. The first step in generating these alleles is to identify within the target sequence—the orthologous sequence for base changes or the N or C terminus for epitope tags—appropriate Cas9 protospacer sequences. Subsequent steps include design and acquisition of a single-stranded oligonucleotide repair template, synthesis of a single guide RNA (sgRNA), collection of zygotes, and microinjection or electroporation of zygotes with Cas9 mRNA or protein, sgRNA, and repair template followed by screening born mice for the presence of the desired sequence change. Quality control of mouse lines includes screening for random or multicopy insertions of the repair template and, depending on sgRNA sequence, off-target sequence variation introduced by Cas9. © 2025 The Author(s). Current Protocols published by Wiley Periodicals LLC.

Basic Protocol 1: Single guide RNA design and synthesis
Alternate Protocol 1: Single guide RNA synthesis by primer extension and in vitro transcription
Basic Protocol 2: Design of oligonucleotide repair template
Basic Protocol 3: Preparation of RNA mixture for microinjection
Support Protocol 1: Preparation of microinjection buffer
Alternate Protocol 2: Preparation of RNP complexes for electroporation
Basic Protocol 4: Collection and preparation of mouse zygotes for microinjection or electroporation
Basic Protocol 5: Electroporation of Cas9 RNP into zygotes using cuvettes
Alternate Protocol 3: Electroporation of Cas9 RNP into zygotes using electrode slides
Basic Protocol 6: Screening and quality control of derived mice
Support Protocol 2: Deconvoluting multiple sequence chromatograms with DECODR

Keywords: Cas9 • electroporation • epitope tags • genome editing • microinjection • mouse • single nucleotide variant

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INTRODUCTION

The application of Cas9 RNA–guided nucleases to mammalian genome editing (Shen et al., 2013; H. Wang et al., 2013; reviewed in Hsu et al., 2014; F. Zhang et al., 2014) has significantly simplified generating precise mouse models of human disease. A mutation is defined as a permanent change in genetic material. However, over time, the term mutation began to be associated with pathogenic changes while the term polymorphism became used for benign changes. It is now standard in the genetics field to refer to base changes as variants, with qualifying terms speaking to pathogenicity and frequency (Richards et al., 2015). Thus, we have updated the title and body of this article to remove the term "point mutation" and substitute base change(s) or variant(s). While a base change is a variant, not all variants are only base changes, e.g., structural or copy number changes are also considered variants.

Timelines to engineer base changes and epitope-tagged alleles in the mouse genome using Cas-type endonucleases are now on the order of 6 months, compared to 1 to 2 years for embryonic stem (ES) cell–based technologies. These timelines are further reduced when strain backgrounds that are not available as ES cells are used as embryo donors avoiding the need to backcross the engineered change onto the target strain background. The mouse can now efficiently be used to evaluate variants of unknown significance and carry out mechanistic and preclinical studies of disease-associated alleles.

This article begins with protocols for design and synthesis of single guide RNAs (sgRNAs) to direct Cas9 endonuclease to the target site (Basic Protocol 1 and Alternate Protocol 1). The main difference between the two sgRNA synthesis protocols are that in Basic Protocol 1, sgRNA template synthesis and in vitro transcription are separate steps, while in the Alternate Protocol 1, the template and in vitro transcription are performed in a single reaction. Thus, while Basic Protocol 1 is more easily scaled if large amounts of sgRNA are needed and adaptable with appropriate primer sequence changes to other Cas enzymes, it is a longer more complex protocol. We routinely use Alternate Protocol 1 to produce sgRNAs as it produces enough sgRNA for several embryo manipulation experiments. The synthesis of Cas9 mRNA has been described elsewhere (H. Wang et al., 2013) and, with a plethora of commercial sources of Cas9 mRNA suitable for embryo microinjection, is not described here. Several commercial sources of Cas9 protein suitable for zygote electroporation with ribonucleoprotein (RNP) also exist. In Basic Protocol 2 we describe the steps for designing the oligonucleotide repair template needed to effect precise genome sequence changes. Next, we describe the preparation of RNA/template mixes for mouse zygote microinjection (Basic Protocol 3) and RNP/template mixes for electroporation (Alternate Protocol 2).

Superovulation protocols and mating mice for embryo production depend on the strain background and animal facility infrastructure. Microinjection is a specialist technique for which practical details are largely dependent on the specific equipment available. Expert surgical techniques are required for embryo transfer into pseudopregnant recipients for gestation and parturition of mice from manipulated embryos. These techniques are

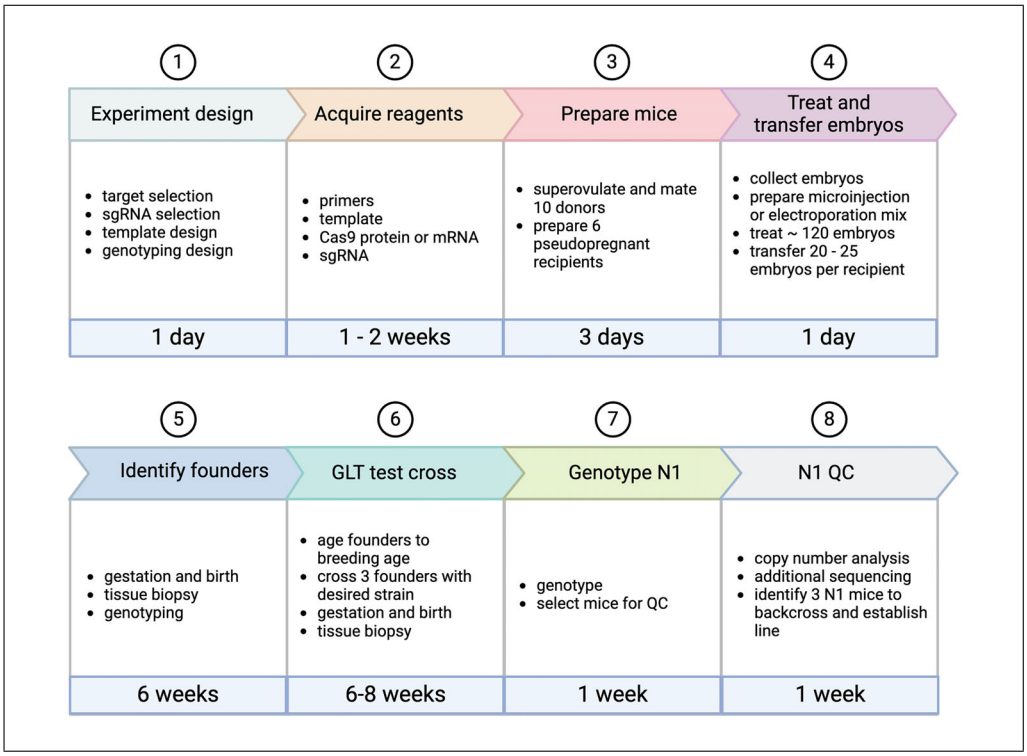

Figure 1 Schematic workflow for generating base-change or epitope-tagged alleles in mice using Cas9 endonuclease and single-strand DNA oligonucleotide repair templates. Step 1. Allele design. Design the desired allele, select the sgRNA protospacer and design the repair template and genotyping PCR assays. Step 2. Acquire reagents. Order the required primers and repair template and synthesize sgRNA. Timing for this step depends on the lead time for ordering reagents. Step 3. Prepare embryo donors and recipients. After reagents are ready, embryo donors are superovulated (start 3 days before embryo treatment) and pseudopregnant recipients are prepared (start 1 day before embryo treatment). Superovulated embryo donors are mated to fertile stud males the day before the experiment. The number of embryos donors required is strain dependent; we typically use 10 embryo donors from C57BL/6J or C57BL6/N mice to obtain 80-120 zygotes for endonuclease treatment and prepare 6 pseudopregnant recipients. The timeline does not account for lead time to acquire embryo donors and prepare stud males. Step 4. Treat and transfer embryos. Prepare the endonuclease mix and keep on ice until embryo treatment. Collect embryos. Electroporate or microinject zygotes according to the appropriate protocol. Transfer ~20-25 viable embryos into each pseudopregnant recipient. Step 5. Screen founders. Isolate DNA from pup tissue biopsies, perform PCR, and sequence amplicons to identify founders with the desired allele. DECODR analysis can assist in identifying founders. Step 6. Germline transmission test breeding. At breeding age, mate each founder to a wild-type mouse from the desired strain background. We typically test cross up to 3 founders and hold additional founders as "backups" until allele germline transmission is confirmed. The timeline in this figure assumes germline transmission in the first litter. We have often obtained germline transmission when only one or two founders were obtained and available for breeding. Step 7. Genotype N1 mice. Genotype mice born from test crosses using the same assays used to identify founders. All N1 mice that carry the allele should be heterozygous (hemizygous in the case of males born from X-linked gene editing experiments). If germline transmission does not occur in the first litter(s), we screen at least 28 pups from each test cross before setting up additional test crosses or repeating the experiment. Step 8. Quality control N1 mice. Use at minimum copy number assessment of the template to QC N1 mice. Subcloning the PCR amplicons from genotyping provides confirmation of the allele sequence. We recommend designing one-step real-time allelic discrimination assays (e.g., Taqman or equivalent assays) or tag-anchored PCR assays for routine genotyping rather than PCR and sequencing. Created using BioRender.

usually performed by academic or commercial service providers rather than at individual laboratories. Furthermore, these techniques are well described elsewhere (Behringer et al., 2014; Wefers et al., 2012) and so are not detailed here.

Basic Protocol 4 describes collecting and preparing zygotes at 0.5 day post coitum (dpc) that are suitable for delivery of Cas9 reagents and repair templates by either microinjection or electroporation. Two electroporation methods are described in Basic Protocol 5 and Alternate Protocol 3. Finally, screening founders and quality control of mice produced by founder breeding to establish high-quality lines are described in Basic Protocol 6.

STRATEGIC PLANNING

Typically, embryo donors must be acclimated to the light cycle of the animal facility before superovulation and mating. Hormone injections begin 3 days prior to the day of embryo harvest. To ensure mice are not ordered and/or superovulated prematurely, synthesis and quality control of sgRNAs should be performed prior to ordering embryo donors. Depending on the synthesis protocol used, this can take up to 2 days (Basic Protocol 1) or several hours (Alternate Protocol 1). On the day of microinjection or electroporation, the Cas9 RNA or RNP mixes should be prepared (Basic Protocol 3 or Alternate Protocol 2) while the embryos are being collected and prepared (see Basic Protocol 4). It is possible to prepare the Cas9 RNA mix ahead of time and store it at –80°C for up to 1 week or prepare the Cas9 RNP mix and store it at 4°C overnight. Figure 1 presents a schematic of this workflow.

NOTE: All protocols involving animals must be reviewed and approved by the appropriate Animal Care and Use Committee and must follow regulations for the care and use of laboratory animals.

BASIC PROTOCOL 1

SINGLE GUIDE RNA DESIGN AND SYNTHESIS

There are several software tools available to assist with sgRNA design and selection. The choice of tool is largely dependent on user preference. Table 1 provides the names, URLs, and published references for some commonly used tools. It is important to choose a tool that provides an accurate assessment of potential off-target sites, with some tools (e.g., Cas-OFFinder) providing more flexibility for off-target searching than others. The selection of the sgRNA will be constrained by the location of the desired sequence change or tag insertion. As a result, it may be necessary to select sgRNAs that are somewhat less specific. Ideally, sgRNAs should have at least three mismatches to all putative off-target sites with at least one of these mismatches in the 11-bp "seed" region adjacent to the protospacer adjacent motif (PAM) (Hsu et al., 2013). Off-target sites adjacent to both

Table 1 Software Tools for sgRNA Design

Tool	URL	Reference
Benchling	*https://www.benchling.com/*	Biology Software
Cas-Database	*https://www.rgenome.net/cas-database/*	Park et al. (2016)
Cas-Designer	*https://www.rgenome.net/cas-designer/*	Park et al. (2015)
Cas-OFFinder	*https://www.rgenome.net/cas-offinder/*	Bae et al. (2014)
CHOPCHOP	*https://chopchop.cbu.uib.no/*	Labun et al. (2016); Montague et al. (2014)
CRISPOR	*https://crispor.tefor.net/*	Haeussler et al. (2016)
Guide Picker	*https://www.deskgen.com/guide-picker/*	Hough et al. (2017a, 2017b)
WTSI Genome Editing	*https://www.sanger.ac.uk/htgt/wge/*	Hodgkins et al. (2015)

–NGG and –NAG PAMs should generally be considered. Whole genome sequence analyses of Cas9-derived mice shows that Cas9-mediated off-target variation (mutagenesis) is rare when guides are appropriately specific such as those with an MIT on-target score >66 (Anderson et al., 2018; Hsu et al., 2013; Peterson et al., 2023). Cutting frequency determination (CFD) score can also predict specificity of *Streptococcus pyogenes* Cas9 guides (Doench et al., 2016) and is based on a broader array of experimental guide–target mismatches than the MIT score. When an apparently less-specific sgRNA sequence must be used due to constraints surrounding the target site, it is important to determine if the predicted off-target sites are linked to the target locus. Off-target sites that are unlinked can be screened and bred away during quality control and production of the mouse line. For both base changes and epitope tags, an sgRNA with a predicted endonuclease cleavage site within ∼20 bp is best, though base changes up to 27 bp from the cleavage site have been successfully introduced.

Two protocols are presented for sgRNA synthesis. One uses a PCR-derived template (Bassett et al., 2013) followed by in vitro transcription using a commercial kit, and the other uses a commercial kit for primer extension and in vitro transcription in a single reaction. Both approaches provide sufficient sgRNAs for multiple rounds of zygote microinjection or electroporation, although injection or electroporation of 80 to 160 embryos in one or two sessions is usually sufficient to obtain the desired base changes or epitope-tagged allele. After synthesis and quantitation, synthesized sgRNA is subjected to quality control by gel electrophoresis (Masek et al., 2005) or capillary electrophoresis (e.g., Agilent Bioanalyzer).

For further details, see Commentary.

Materials

10 µM sgRNA-FWD PCR primer
10 µM sgRNA-REV PCR primer, or 10 µM pX330-REV primer and 1 ng/µl pX330 plasmid, RNase-free (Addgene, cat. no. 42230)
H_2O, nuclease-free (e.g., Life Technologies, cat. no. 10977015)
2× KAPA HiFi HotStart ReadyMix (Kapa Biosystems, cat. no. KK2602)
Monarch PCR & DNA cleanup kit (New England Biolabs, cat. no. T1030), including columns and binding, wash, and elution buffers
Agarose, RNase-free (e.g., Life Technologies, cat. no. 16500500)
10× TAE, RNase-free (e.g., Life Technologies, cat. no. AM9869)
MEGAshortscript T7 kit (Life Technologies, cat. no. AM1354M), including T7 enzyme, 10× reaction buffer, individual NTP solutions, and Turbo DNase
RNA Clean & Concentrator-25 (Zymo Research, cat. no. R1017), including columns and 95% to 100% (v/v) ethanol, nuclease-free water, and Zymo RNA binding, prep, and wash buffers
2× RNA loading dye (e.g., New England Biolabs, cat. no. E2040S)

Nuclease- and pyrogen-free PCR tubes
"Fast" thermocycler
Benchtop microcentrifuge
RNase-free microcentrifuge tubes

Additional reagents and equipment for quantitation of RNA by UV spectrophotometry (Gallagher, 2011) and agarose gel electrophoresis (Masek et al., 2005; Voytas, 2000)

Design sgRNA and primers

1. Select an sgRNA sequence (18 to 20 nt) using any of the many publicly available tools (e.g., Table 1) or by hand.

The sgRNA sequence should match a 20-nt target sequence in the DNA followed by a PAM of NGG. The NGG is absolutely required for cleavage but should not be included in the sgRNA construct.

For T7 promoter transcription, the first two bases should be a GG dinucleotide. Appending GG to the 5' end of the sequence seems to be well tolerated for most sgRNAs and may improve specificity (Cho et al., 2014) when a sequence starting with GG is not available.

2. Order the forward PCR oligonucleotide for synthesizing a T7 promoter–driven sgRNA construct in the form of spacer–T7 promoter–gRNA sequence (N20)–template anchor. Dilute primers to 10 µM in TE buffer or nuclease-free water.

   FWD: TACGTGTAATACGACTCACTATA**GG**(N)18-20gttttagagctagaaatagc
   pX330-REV: AGCACCGACTCGGTGCCACT
   sgRNA-REV:
   AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGAC-
   TAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC

   There are two options for preparing sgRNA templates for in vitro synthesis. One uses pX330 as the PCR template with FWD and pX330-REV primers; the other uses the FWD primer with a reverse primer that encodes the complete 3' sequence of the sgRNA (sgRNA-REV). Both methods work equally well, although the second obviates the need for purified plasmid. The sgRNA-REV primer should be PAGE-purified before use.

   In the FWD primer, Ns represent the sgRNA spacer sequence. One or both of the two G's in bold immediately preceding the gRNA spacer sequence can be removed if the gRNA spacer sequence starts with one or two G's, respectively (see Commentary, Background Information, for additional considerations regarding adding two G's). The nucleotides shown in lowercase in the FWD primer indicate the "anchor" sequence that binds to either pX330 or sgRNA-REV during PCR.

Prepare sgRNA template

3. Prepare template synthesis reactions (40 µl per sgRNA to be synthesized) by combining the following in individual nuclease-free PCR tubes:

   For pX330-REV:

   20 µl of 2× HiFi ReadyMix
   1.2 µl of 10 µM FWD
   1.2 µl of 10 µM pX330-REV
   4 µl of 1 ng/µl pX330
   13.6 µl nuclease-free H_2O.

   For sgRNA-REV:

   20 µl of 2× HiFi ReadyMix
   2.0 µl of 10 µM FWD
   2.0 µl of 10 µM sgRNA-REV
   16 µl nuclease-free H_2O.

4. Cycle in a "fast" thermocycler as follows.

   For pX330-REV:

95°C for 3 min	
5 cycles:	98°C for 20 s
	55°C for 15 s + 1°C/cycle
	72°C for 10 s
20 cycles:	98°C for 20 s
	60°C for 15 s
	72°C for 10 s
72°C for 3 min	
8°C hold	

For sgRNA-REV:

95°C for 3 min	
35 cycles:	98°C for 20 s
	60°C for 15 s
	72°C for 10 s
72°C for 3 min	
8°C hold	

The conditions described here are optimized for thermocyclers with rapid ramp times. Cycling conditions may need to be optimized for other instruments.

Purify PCR amplicon

5. Add 60 µl nuclease-free water and 500 µl DNA binding buffer and mix well.

   This protocol describes purification using the Monarch kit, but any appropriate fragment purification kit can be used.

   If desired, the PCR fragment can be assayed by gel electrophoresis (step 11) before purification.

6. With the column in a collection tube, load sample onto the column. Centrifuge 30 to 60 s at 16,000 × *g*. Discard flowthrough.

   This and all subsequent centrifugations are at room temperature.

7. Apply 200 µl wash buffer to the column and centrifuge 30 to 60 s at 16,000 × *g*. Repeat with another 200 µl wash buffer.

   Ensure that wash buffer is diluted with ethanol per kit instructions.

8. Discard flowthrough and centrifuge column 30 to 60 s at 16,000 × *g*.

9. Transfer column to an RNase-free microcentrifuge tube. Add 20 µl DNA elution buffer and incubate 1 min at room temperature. Centrifuge 1 min at 50 × *g* followed by 1 min at 16,000 × *g*. Discard column and cap the microcentrifuge tube.

10. Quantitate template using a UV spectrophotometer (Gallagher, 2011).

11. Electrophorese ∼200 ng on a 1.5% agarose/1× TAE gel (Voytas, 2000) to ensure a single band of 125 bp.

    If multiple bands are seen, the 125-bp product should be gel purified using a gel purification kit (e.g., Machery-Nagel NucleoSpin gel and PCR purification kit, cat. no. 740609). The purified band should be quantitated per step 10 before continuing with the protocol.

12. Store DNA at –20°C until needed.

    This is a good pause point. The PCR product can be stored at –20°C indefinitely.

Synthesize sgRNA

13. Set up in vitro transcription reactions (20 µl per reaction) at room temperature using the MEGAshortscript T7 kit with:

    Nuclease-free water to 20 µl final volume
    2 µl of 10× T7 reaction buffer
    8 µl NTP premix (2 µl each ATP, CTP, GTP, and UTP)
    200 ng prepared template DNA (∼120 nM final)
    2 µl T7 enzyme mix.

14. Mix contents well by gently flicking tube and collect contents by pulse centrifugation. Incubate at least 2 hr and up to 4 hr at 37°C.

15. Add 1 µl Turbo DNase and incubate 15 min at 37°C to remove DNA template.

16. Bring volume to 50 µl by adding 30 µl nuclease-free water.

Purify sgRNA

17. Add 100 µl Zymo RNA binding buffer and mix well by pipetting.
18. Add 150 µl of 95% to 100% ethanol. Mix well by pipetting.
19. Apply mixture to a column in a collection tube and centrifuge 30 s at 16,000 × *g*.
20. Apply 400 µl Zymo RNA prep buffer to the column and centrifuge 30 s at 16,000 × *g*. Discard flowthrough.
21. Apply 700 µl Zymo RNA wash buffer to the column and centrifuge 30 s at 16,000 × *g*. Discard flowthrough. Repeat wash with 400 µl wash buffer and discard flowthrough.
22. Centrifuge 2 min at 16,000 × *g* to dry the column.
23. Transfer column to a nuclease-free microcentrifuge tube and apply 30 µl nuclease-free water (provided in the kit). Centrifuge 1 min at 50 × *g* and then 1 min at 16,000 × *g*. Discard column, cap the tube, and place tube on ice until dilutions for quantitation and quality control are made. Store the sgRNA at –80°C.

    In our laboratory, we use sgRNAs stored at –80°C for up to 6 months after synthesis. We have not tested stability and functionality beyond 6 months.

Quantitate sgRNA and perform quality control

24. Prepare a 1:5 dilution of the sgRNA with 3 µl sgRNA and 12 µl nuclease-free water in a nuclease-free microcentrifuge tube. Vortex briefly and pulse centrifuge to collect tube contents.
25. Check RNA purity and concentration by UV absorbance at 230, 260, and 280 nm (Gallagher, 2011).

    A260/A280 and A260/A230 ratios should be ≥2.0.

    Use the UV absorbance at A260 and the sgRNA extinction coefficient to calculate the sgRNA concentration. Calculate the extinction coefficient using the nearest neighbor method; this is easily done by entering the complete (not just the spacer) sgRNA sequence into the AAT Bioquest RNA concentration calculator, available at https://www.aatbio.com/tools/calculate-RNA-concentration (AAT Bioquest Inc., 2025).

26. Check RNA integrity using one of the following techniques:
    a. *For electrophoresis* (Masek et al., 2005): Denature RNA in 2× RNA loading dye by heating 5 to 10 min at 65° to 70°C, then snap-cool in an ice-water bath. Immediately run on a 1.2% (w/v) agarose/1× TAE gel.
    b. *For bioanalysis*: Run on a Bioanalyzer, ScreenTape, or similar instrument using a service provider (e.g., The Centre for Applied Genomics) or following manufacturer's instructions.

    A single band of ∼100 nt should be observed. Smearing of the band below the expected size indicates RNase contamination. In this case, clean the workspace with RNase Away and repeat template and RNA synthesis. Discrete bands smaller than the expected size suggest premature transcription termination during synthesis and/or incomplete template extension during PCR. A ladder of RNA products can indicate incomplete denaturation prior to electrophoresis. Note that the expected 100-nt product will run slower than a 100-bp DNA band.

ALTERNATE PROTOCOL 1

SINGLE GUIDE RNA SYNTHESIS BY PRIMER EXTENSION AND IN VITRO TRANSCRIPTION

There are several commercially available kits for sgRNA synthesis. Commercial kits can offer a variety of advantages, including the ability to use shorter oligonucleotides, which can reduce both costs and probability of errors during synthesis. They may also be more accessible to the novice user. We use the EnGen sgRNA synthesis kit (New England

Biolabs) with very good results and its one-tube primer extension and in vitro transcription saves significant time.

Additional Materials *(also see Basic Protocol 1)*

1 µM FWD primer in TE buffer or water
EnGen sgRNA synthesis kit (New England Biolabs, cat. no. E3322), including EnGen enzyme, 2× reaction mix, 0.1 M DTT, and DNase I

1. Following the design guidelines outlined in Basic Protocol 1, step 1, order the forward PCR oligonucleotide per kit instructions in the form of spacer–T7 promoter–gRNA spacer sequence–template anchor.

   FWD: TTCTAATACGACTCACTATA**GG**(N)18-20GTTTTAGAGCTAGA

   The Ns represent the sgRNA spacer sequence. One or both of the two G's in bold can be removed if the gRNA spacer sequence starts with one or two G's, respectively (see Commentary, Background Information, for additional considerations regarding adding the two G's). The reverse primer is provided in the kit reaction mix.

2. Thaw the FWD oligonucleotide and 2× EnGen sgRNA reaction mix on ice. Vortex well to mix and pulse centrifuge to collect contents. Leave enzyme mix at –20°C until use.

3. Assemble the reaction at room temperature by adding the following components, in order, to a nuclease-free PCR tube:

   2 µl nuclease-free water
   1 µl of 0.1 M DTT
   10 µl of 2× EnGen sgRNA reaction mix
   5 µl of 1 µM FWD primer
   2 µl EnGen enzyme mix

4. Mix well by gently flicking tube, pulse centrifuge to collect contents, and then incubate 60 min at 37°C.

5. Place tube on ice and add 30 µl nuclease-free water and 2 µl DNase I. Incubate at 37°C for 25 min, then return tube to ice.

   For convenience perform these incubations in a thermocycler set for a 60 min incubation at 37°C, a 10°C hold to add water and DNase I, a 25 min incubation at 37°C that is started by manually advancing from the 10°C hold, and a final hold at 10°C.

6. Clean up RNA, quantitate, and perform quality control as described (see Basic Protocol 1, steps 17 to 26).

DESIGN OF OLIGONUCLEOTIDE REPAIR TEMPLATE

BASIC PROTOCOL 2

To introduce single nucleotide variants, base changes over a stretch of up to ~60 nucleotides, or epitope tags, synthetic single-stranded oligonucleotides are provided as a repair template for the endogenous cellular DNA repair machinery. The oligonucleotide repair template should have the desired change(s) at or near an endonuclease cut site, both flanked by 50- to 70-nt homology arms. The desired change can be either 5' or 3' to the cut site. An important consideration for repair template design is whether the sequence change eliminates the ability of the Cas9 RNP to re-cut the repaired allele with subsequent nonhomologous end joining (NHEJ) repair introducing cis-located insertions or deletions (indels). To obviate the risk of re-cutting, one or more silent changes (i.e., nucleotide changes that preserve the amino acid sequence) can be introduced to change the PAM from –NGG to any sequence except –NAG. If destruction of the PAM with a silent base change is not possible, two or more silent changes in the protospacer sequence "seed" region (11-bp adjacent to the PAM) (Hsu et al., 2013) can be used to reduce the

risk of Cas9 re-cutting the repaired allele. Doench et al. (2016) provide experimentally determined residual cutting efficiencies for mismatches along the gRNA spacer sequence in the article's Supplemental Table ST19. These data can be used to choose silent changes within the sgRNA spacer that will reduce the probability of re-cutting the repaired allele. In general, silent base changes should mimic the codon usage of the original codon as closely as possible. It should be noted that silent mutations may have unintended consequences on transcription, splicing, and/or translation (e.g., Biro, 2008; Doktor et al., 2014; Nielsen et al., 2007). This risk must be balanced against the possibility of Cas9 re-cutting and introducing cis-located indels. It may be possible to obtain the correct allele in the absence of silent mutations depending on the location and number of target changes relative to the protospacer sequence and/or PAM.

Either the target or silent mutations may introduce or destroy a restriction enzyme site that could then be used for genotyping founders by restriction length polymorphism after PCR (see Basic Protocol 6). In the absence of a restriction site polymorphism, derived alleles can be genotyped by real-time or digital-droplet PCR (Mazaika & Homsy, 2014) for the presence of the nucleotide variant.

See Commentary for further details.

Materials

Software for protein alignment

1. Align amino acid sequences of source orthologous protein (usually human) with the mouse ortholog.
2. Identify the target amino acid(s) and nucleotide changes to affect the desired amino acid change(s).

   Epitope tags are usually inserted at the N- or C-terminus of proteins, either immediately following the start codon or immediately before the stop codon, respectively. The location of the tag depends on what is known about the gene's promoter and whether insertion of the epitope tag will disrupt transcription, protein domains, structure, and/or function. Often a flexible linker (e.g., [GGSG]1-3) is used to join the epitope tag to the target protein to reduce the risk of structural interference and maximize the availability of the epitope tag to antibodies.
3. Identify the repair template center, which is the sequence between the endonuclease cut site and the nucleotide changes.
4. Extend the repair template 50 to 70 nt on each side of the center.
5. Order the synthetic oligonucleotide (e.g., Integrated DNA Technologies Ultramers) either lyophilized or normalized at 100 μM in TE buffer, pH 8.

   For microinjection, a template concentration of 100 μM is sufficient; however, a concentration of 200 μM is often needed to prepare an electroporation mix.
6. Before use, dilute the repair oligonucleotide to an appropriate stock concentration in TE buffer or RNase-free microinjection buffer.

   For microinjection, a working stock solution of 10 μM is suitable. For electroporation, no dilution is needed.

BASIC PROTOCOL 3

PREPARATION OF RNA MIXTURE FOR MICROINJECTION

This protocol describes preparation of the mixture of Cas9 mRNA, sgRNA, and oligonucleotide repair template for injection. Microinjection mixes can occasionally perform poorly, as indicated by difficulty of injection, clogged microinjection needles, or high embryo lysis rates. Filtering the mixes through an RNase-free filter column can improve injection performance. Quality control of the microinjection mixtures can also assist

with troubleshooting. Pre- and post-injection fractions of the microinjection mixture are checked for integrity by agarose gel electrophoresis.

Materials

1 mg/ml Cas9 mRNA (e.g., Life Technologies, cat. no. A29378; TriLink, cat. no. L-6112)
≥300 ng/µl sgRNA (see Basic Protocol 1 or Alternate Protocol 1)
10 µM oligonucleotide repair template in TE or microinjection buffer (see Basic Protocol 2)
RNase-free microinjection buffer (see Support Protocol 1)

Nuclease- and pyrogen-free microcentrifuge tubes
Vortexer
Benchtop microcentrifuge, 4°C and room temperature
Spin-X RNase-free centrifuge tube filters, 0.22-µm CA membrane (Corning, cat. no. 8160)

Prepare microinjection mix

1. Calculate the amount of Cas9 mRNA, sgRNA, and oligonucleotide repair template needed for 75 µl of the desired final concentration, as follows.

	Final concentration for pronuclear injection	Final concentration for cytoplasmic injection
Cas9 mRNA	30 ng/µl	100 ng/µl
sgRNA	10 ng/µl	10 ng/µl
Repair template	10 ng/µl (~0.25 µM)	100 ng/µl (~2.5 µM)
Microinjection buffer	to 75 µl	to 75 µl

2. Thaw sgRNA and repair template on ice, then combine components in RNase- and pyrogen-free microinjection buffer in a nuclease- and pyrogen-free microcentrifuge tube to a final volume of 75 µl. Mix well by vortexing.

3. Centrifuge 5 min at 20,000 × *g*, 4°C, to pellet any particulates that may clog the injection needle.

Filter mix (optional)

4. Apply 50 µl RNase-free microinjection buffer to a Spin-X filter in a collection tube. Centrifuge 30 s at 10,000 × *g*, room temperature.

5. Discard flowthrough and collection tube. Place filter in a clean RNase- and pyrogen-free collection tube.

6. Apply microinjection mixture to the filter and centrifuge 15 s at 10,000 × *g*, room temperature.

7. Remove filter, then centrifuge eluent 5 min at 20,000 × *g*, 4°C.

   This step is necessary to remove fibers that originate from the filter. Without this step, needles may get clogged with particulate matter, likely from the filter.

Store injection, pre-injection, and post-injection fractions

8. Carefully remove the top 50 µl of mixture to a clean RNase- and pyrogen-free microcentrifuge tube and store on ice until injection. Store the remaining 25 µl at –80°C as the pre-injection fraction for later quality control.

   The microinjection mix may be stored at –80°C for up to 1 week prior to injection.

9. After microinjection, store remaining microinjection mix at –80°C as the post-injection fraction.

Perform quality control of mix

10. Test RNA integrity in the pre- and post-injection fractions by agarose gel electrophoresis (see Basic Protocol 1, step 29).

    Bioanalyzer, ScreenTape, or equivalent analysis are acceptable alternatives.

    There should be two bands, one at ~4.1 kb (Cas9 RNA) and one at ~100 nt (sgRNA) and 140 to 200 nt (oligonucleotide). Intensities should be equivalent between the two samples with no obvious degradation. The sgRNA and oligonucleotide often overlap in agarose gels. In this case, integrity of the Cas9 mRNA can be used as a reporter of sgRNA integrity.

SUPPORT PROTOCOL 1

PREPARATION OF MICROINJECTION BUFFER

The quality of microinjection buffer is critical and should be tested prior to use for injection experiments. Preparation and quality control are described below.

Materials

1 M Tris, pH 7.6 (Sigma, cat. no. T2444)
0.5 M EDTA (Sigma, cat. no. E7889)
Embryo-tested water (Sigma, cat. no. W1503)
RNA (e.g., ThermoFisher Century-Plus RNA Markers, cat. no. AM7145)

Sterile PETG bottle
0.22-µm surfactant-free cellulose acetate (SFCA) vacuum filter units (e.g., Nalgene, cat. no. 157-0020)
20-ml Luer-lock syringe (BD Biosciences, cat. no. 309611), γ-radiation-sterilized
Whatman Anotop Plus filter, 0.02-µm (VWR, cat. no. 28138-011)
Sterile PETG serum vials and sterile closures (e.g., Nalgene, cat. nos. 342032-0010 and 342158-0021)
Incubator or heat block, 37°C

Additional reagents and equipment for agarose gel electrophoresis (see Basic Protocol 1 and Masek et al., 2005)

NOTE: Prepare in a biosafety cabinet to maintain stock solution sterility.

1. Combine the following in a sterile PETG bottle and mix well.

   2 ml of 1 M Tris, pH 7.6 (10 mM final)
   100 µl of 0.5 M EDTA (0.25 mM final)
   198 ml embryo-tested water

2. Set up a disposable 0.22-µm SFCA vacuum filter unit in the biosafety cabinet. Rinse filter with 100 ml microinjection buffer by vacuum filtration.

3. Transfer rinsed filter to a new collection bottle, discarding the first bottle with rinse buffer.

4. Filter the remaining 100 ml buffer through the rinsed filter. Store in the sterile collection bottle for the next step.

5. Aspirate 20 ml buffer into a γ-radiation-sterilized 20-ml Luer-lock syringe. Attach a 0.02-µm Whatman Anotop Plus filter to the filled syringe.

6. Filter and discard 5 ml buffer to rinse the filter, then filter the remaining buffer into two aliquots in sterile PETG serum vials. Close with a sterile closure and store at room temperature.

7. To confirm that buffer is RNase-free, add 2 µg RNA to 18 µl filtered microinjection buffer and incubate at 37°C for ≥30 min. Assess integrity of the RNA by agarose gel electrophoresis or bioanalysis compared to freshly prepared RNA.

   No RNA degradation should be observed.

8. Confirm that buffer is suitable for microinjection by injecting 40 to 60 embryos (0.5 dpc, see Basic Protocol 4) with 0.02-µm filtered buffer. Transfer to pseudopregnant recipients in parallel with 40 to 60 embryos injected with previously QC'd buffer. Dissect mid-gestation and score number of implantation sites and number of live embryos.

   Implantation and live embryo rates for test buffer should be within 10% of rates for previously QC'd buffer.

PREPARATION OF RNP COMPLEXES FOR ELECTROPORATION

ALTERNATE PROTOCOL 2

Zygote microinjection is a specialist technique with practical limitations on throughput. Many types of genetic modifications can be produced using the less specialized technique of electroporation to deliver Cas9 RNP to mouse zygotes (S. Chen et al., 2016; Hashimoto et al., 2016; W. Wang et al., 2016). Cas9 protein is pre-complexed with sgRNAs before adding the oligonucleotide repair template. Electroporation is higher throughput and more efficient than microinjection and requires less technical expertise. Nonetheless, it is still critical to have expertise in embryo handling and embryo transfer surgery.

Materials

≥400 ng/µl sgRNA (see Basic Protocol 1 or Alternate Protocol 1)
200 µM oligonucleotide repair template in TE buffer (see Basic Protocol 2)
H_2O, nuclease-free
5× Cas9 RNP buffer (see recipe)
Cas9 protein (e.g., PNABio cat. no. CP01, Integrated DNA Technologies, cat. no. 1074182)

Benchtop centrifuge
Nuclease- and pyrogen-free 0.5-ml microcentrifuge tubes
Incubator or heat block, 37°C
Ice

1. Thaw sgRNA and oligonucleotide repair template on ice. Mix well and pulse centrifuge to collect contents.

2. To prepare 2× RNPs in 1× Cas9 RNP buffer, combine the following in a 0.5-ml nuclease- and pyrogen-free microcentrifuge tube:

   Nuclease-free water to a total of 5 or 11 µl (including oligonucleotide repair template)
   1 × Cas9 buffer
   12 µM sgRNA
   8 µM Cas9 protein.

   Each electroporation requires 10 µl RNP mix when performing electroporation in a cuvette, 2.5 µl when using a slide. Thus, 11 or 5 µl should be prepared per electroporation.

   sgRNAs are used in 50% molar excess to Cas9 protein (e.g., 12 µM sgRNA is ~420 ng/µl).

3. Mix well by flicking the tube and pulse centrifuge to collect any droplets.

4. Incubate at 37°C for 10 min. Pulse centrifuge to collect droplets and transfer to ice.

5. Add oligonucleotide repair template to a final concentration of 20 µM.

6. Mix well by flicking the tube and pulse centrifuge to collect droplets. Place on ice until use (no more than 18 hr).

BASIC PROTOCOL 4

COLLECTION AND PREPARATION OF MOUSE ZYGOTES FOR MICROINJECTION OR ELECTROPORATION

One advantage of Cas9 mutagenesis in zygotes is the ability to produce variants in specific inbred backgrounds rather than relying on a few specific background strains available as ES cells. Zygotes from many inbred strains can be collected with some variation for age and hormone doses for superovulation (Byers et al., 2006; Luo et al., 2011). The steps below describe the collection and preparation of embryos.

Because zygote microinjection requires specialized equipment and skill sets and is typically performed by service cores located in accredited animal facilities rather than at individual labs, the specifics of superovulation and mating, microinjection, and embryo transfer are not detailed here. These protocols are fully described elsewhere (Behringer et al., 2014; Wefers et al., 2012) and should be routine for any transgenic or mouse model service core facility. Two electroporation methods, using cuvettes or slides, are described below (see Basic Protocol 5 and Alternate Protocol 3, respectively).

Materials

KSOM^{AA} medium, e.g., EmbryoMax KSOM (Sigma, cat. no. MR-121-D) or Global medium (Cooper Surgical, cat. no. LGGG-050) with 1 mg/ml Global protein supplement (Cooper Surgical, cat. no. LGPS-020)
Paraffin oil, embryo-tested (Cooper Surgical, cat. no. LGPO-500)
Superovulated, mated, and plugged 0.5 dpc–female mice as embryo donors
70% (v/v) ethanol
M2 medium (Sigma, cat. no. MR-015D) with 4 mg/ml embryo-tested BSA (Sigma, cat. no. A3311)
10× hyaluronidase (see recipe)

35- and 100-mm plastic Petri dishes (e.g., Falcon, cat. nos. 351008 and 353003)
Incubator at 37°C, 6% CO_2
Absorbent bench pads (e.g., VWR, cat. no. 82020-845)
Instruments for oviduct dissection, including scissors, forceps, watchmaker's forceps, fine scissors
Stereomicroscope with transmitted light, 16×, 25×, and 40× magnification
26-G needles and 1-cc syringes (optional)
Pipette with 20-µl tips
Embryo-handling pipet with freshly pulled glass capillaries

Additional reagents and equipment for humane euthanasia and for embryo culture in microdrops under paraffin oil (Behringer et al., 2014)

Dissect oviducts

1. Set up a 35-mm Petri dish with KSOM^{AA} microdrops under paraffin oil for embryo culture (Behringer et al., 2014). Pre-equilibrate overnight at 37°C/6% CO_2.

2. On the morning of injection, humanely euthanize plugged 0.5-dpc females by cervical dislocation following approved animal use protocols.

   Typically, 8 to 15 embryo donors are used per electroporation or microinjection session. More mice are usually needed for microinjection since not all embryos will have visible pronuclei suitable for injection. The age and number of mice needed to produce sufficient embryos varies by strain.

3. Lay the mice on absorbent paper and wet the abdomen with 70% ethanol. Pinch the skin and make an incision at the midline with scissors. Holding the skin firmly above and below the incision, pull the skin apart and toward the head and tail until the abdomen is completely exposed and the fur is out of the way.
4. Using watchmaker's forceps and fine scissors, cut the body wall (peritoneum) and push the coils of the gut out of the way. Locate the two horns of the uterus, the oviducts, and the ovaries.
5. Grasp the upper end of one of the uterine horns with fine forceps and gently pull the uterus, oviduct, ovary, and fat pad away from the body cavity. Use the closed tips of fine forceps or scissors to separate the membrane (the mesometrium) that connects the reproductive tract to the body wall and carries a prominent blood vessel near the oviduct.
6. Pull one oviduct, ovary, and fat pad with fine forceps and cut between the oviduct and ovary with fine scissors. Reposition the forceps and cut the uterus near the oviduct. Transfer the oviduct and attached segment of uterus to a 100-mm Petri dish containing M2 medium at room temperature.
7. Repeat with the second side and remaining animals until all oviducts are removed by dissection.

Isolate embryos

8. Transfer one oviduct at a time to a new Petri dish with M2 medium and view under a stereomicroscope at 16× or 25× magnification to locate the ampulla where the zygotes are located.
9. Use watchmaker's forceps to grasp the oviduct next to the infundibulum and hold it firmly on the bottom of the dish. Use another watchmaker's forceps or a 26-G needle attached to a 1-cc syringe to tear the oviduct near ampulla, releasing the clutch of cumulus cells. Repeat steps 8 and 9 with all dissected oviducts.
10. Use a pipette with a 20-µl tip to collect and transfer all cumulus masses into a single 100-µl drop of freshly diluted 0.3 mg/ml hyaluronidase in M2.
11. Incubate at room temperature until the cumulus cells separate from the zygotes.

    Pipet the cumulus mass up and down with an embryo-handling pipet, if necessary. Do not leave the embryos in hyaluronidase for ≥3 min, as it is harmful for the embryos.

12. Transfer embryos through at least two fresh M2 drops to rinse off the hyaluronidase, cumulus cells, and debris.
13. Using a stereomicroscope at 40× magnification (or higher), screen embryos to select only fertilized zygotes by checking for the presence of the polar body (rotating the embryos with the pipet to visualize it) and pronuclei.
14. Transfer fertilized embryos in groups of 20 to 40 into $KSOM^{AA}$ microdrops. Keep at 37°C/6% CO_2 until injection.

    Pronuclear injection, cytoplasmic injection, or electroporation are usually performed immediately after collection and within 4 hr. One or two groups of embryos can be removed from the incubator at a time, with the remainder kept in the incubator. Microinjection times may vary depending on the visibility of the pronucleus. Embryos will continue to develop in the incubator. Underdeveloped embryos can be cultured until the pronuclei are clearly visible; however, in embryos that are a bit more developed, pronuclear envelopes may start to dissolve and/or zygotes may begin to divide if they are maintained in the incubator too long. Treated embryos should be transferred into 0.5 dpc–pseudopregnant females on the same day.

ELECTROPORATION OF Cas9 RNP INTO ZYGOTES USING CUVETTES

Electroporation of zygotes can be done in 0.1 mm–gap cuvettes or on an electrode slide. The former enables the use of standard equipment and consumables available in most laboratories. Its drawback is that the relatively large volumes of buffer needed to recover the embryos for transfer necessitates additional time and effort for embryo recovery. With either method, the zona pellucida of the embryo is weakened with acid Tyrode's prior to electroporation. After electroporation, embryos are transferred to pseudopregnant females for gestation and birth.

This protocol employs two different media. $KSOM^{AA}$ medium is not buffered for use outside of the incubator, while M2 medium is. All manipulations of embryos outside the incubator are carried out in M2, which is buffered appropriately for handling embryos "on the bench." Embryos should be cultured or temporarily held in the incubator in pre-equilibrated $KSOM^{AA}$. It is important to rinse away any M2 through sequential rinses in $KSOM^{AA}$ prior to placing the embryos in microdrops for culture (or temporary storage) in the incubator.

Materials

$KSOM^{AA}$ medium, e.g., EmbryoMax KSOM (Sigma, cat. no. MR-121-D) or Global medium (Cooper Surgical, cat. no. LGGG-050) with 1 mg/ml Global protein supplement (Cooper Surgical, cat. no. LGPS-020) equilibrated overnight at 37°C/6% CO_2
Paraffin oil (Cooper Surgical, cat. no. LGPO-500)
Acid Tyrode's solution (Sigma, cat. no. T1788)
M2 medium (Sigma, cat. no. MR-015D) with 4 mg/ml embryo-tested BSA (Sigma, cat. no. A3311)
0.5-dpc zygotes in $KSOM^{AA}$ culture (see Basic Protocol 4)
Opti-MEM (ThermoFisher Scientific, cat. no. 31985062)
Cas9 RNP mix (see Alternate Protocol 2)

35- and 100-mm plastic Petri dishes (e.g., Falcon, cat. nos. 351008 and 353003)
Incubator at 37°C, 6% CO_2
Stereomicroscope with transmitted light, 16× or 25× magnification
Embryo-handling pipet with freshly pulled glass capillaries
Electroporator (e.g., BioRad Gene Pulser Xcell with ShockPod)
Pipette with 20-µl tips
1-mm-gap cuvettes (e.g., Cell Projects, cat. no. EP-201, BioRad, cat. no. 165-2089)

Additional reagents and equipment for embryo culture in microdrops under paraffin oil (Behringer et al., 2014)

Weaken zona pellucida

1. Prepare two 35-mm Petri dish with $KSOM^{AA}$ microdrops as described in Behringer et al. (2014) and pre-equilibrate the plates overnight at 37°C/6% CO_2.

   The rinse plate needs three to four drops per group of embryos; the culture plate needs one drop per group.

2. Place one 100-µl drop of acid Tyrode's solution and two to four drops of M2 medium in a 100-mm Petri dish.

   One drop of M2 is needed for each group of embryos.

3. Using 16× or 25× magnification and an embryo-handling pipet, transfer one group of 20 to 40 embryos in a minimal amount of medium into the acid Tyrode's drop and quickly spread them around.

Embryos may be treated with acid Tyrode's immediately after collection in Basic Protocol 4, step 13, by transferring them in groups of 20 to 40 directly from M2. Although it is possible to treat larger groups in acid Tyrode's, this extends the time that embryos are exposed to acid, because it takes longer to collect the embryos back into the pipet. Longer incubation can lead to loss of the zona pellucida and compromise embryo survival.

4. Immediately retrieve embryos in a minimal volume of acid and place them in a drop of M2.

   Acid exposure should be no more than 7 to 10 s.

5. Repeat for remaining groups of embryos, placing each group in a separate drop of M2.

   If embryos are to be electroporated immediately, they can be transferred to the 50-µl drop of Opti-MEM in step 10 directly from the M2 rinse.

6. Rinse groups of embryos by transferring from M2 through three to four drops of CO_2-equilibrated $KSOM^{AA}$ medium in the prepared wash plate.

7. Place embryos in fresh pre-equilibrated $KSOM^{AA}$ microdrops in the embryo culture plate and return to incubator until electroporation.

   Embryos can be used immediately for electroporation. Only one or two groups of embryos should be removed from the incubator at any given time. While there is in theory no maximum amount of time that embryos can be left in the incubator, they will continue to develop. Zygotes that are a bit more developed may begin to divide if they are maintained in the incubator too long.

Perform electroporation

8. Set up the electroporator to deliver 12 pulses (30 V, square wave, 1 ms duration) with 100-msec intervals between pulses.

   For the BioRad Xcell, use a program that delivers six 30 V, 1-ms pulses with 100-ms intervals and execute it twice in succession on each set of embryos.

9. Set up a 100-mm Petri dish with a ~50-µl drop of Opti-MEM in the center and a 10-µl drop of Opti-MEM more peripherally.

10. Collect the embryos from M2 (step 5) or $KSOM^{AA}$ (step 7) using an embryo-handling pipet and rinse them in the 50-µl drop of Opti-MEM.

    Electroporation of up to 80 embryos can be performed in the same cuvette.

11. Transfer embryos to the 10-µl drop of Opti-MEM, being careful to minimize medium carry-over.

12. Immediately add 10 µl Cas9 RNP mix.

13. Using a pipette with a 20-µl tip, transfer the embryos in the 20-µl mix to the bottom of a 1-mm-gap cuvette.

    Use a pipette and tip that will reach the bottom of the cuvette, both for placing embryos into the cuvette and for retrieving the embryos after electroporation. The Rainin L20 LTS with L20 tips is a suitable pipette for this purpose.

14. Place cuvette into the cuvette chamber and execute the program.

15. Remove cuvette from the chamber and carefully add 50 to 100 µl (depending on cuvette capacity) of CO_2-equilibrated $KSOM^{AA}$. With the pipette set to 20 µl, gently but vigorously pipet up and down so that embryos float in the medium.

16. Remove ~18 µl embryo suspension at a time to a Petri dish, avoiding air bubbles in the resulting drops.

Embryos are removed from the cuvette in small aliquots (<20 µl) because of the size limitations of the pipette and cuvette. Only the L20 pipet and tip has proven small enough to fit into the bottom of the cuvette for maximal embryo recovery.

17. Retrieve embryos from the $KSOM^{AA}$ drops in the Petri dish using an embryo-handling pipet and collect them into one $KSOM^{AA}$ drop. Count embryos to ensure that the majority were recovered. Rinse cuvette with more $KSOM^{AA}$, if necessary.

    It is important to use enough medium to recover the embryos. Adding <50 µl medium to the cuvette prior to removing embryos (step 15) results in the loss of as many as 20% to 50% of embryos. Adding a larger volume of medium (50 to 100 µl) and mixing with the pipet tip at or near the bottom of the cuvette then rinsing at least twice with an additional 50 to 100 µl, allows recovery of 95% to 100% of the embryos.

18. Transfer the embryos to a $KSOM^{AA}$ microdrop culture dish and place in the 37°C/6% CO_2 incubator until ready for embryo transfer surgery.

    Embryo transfer should be performed on the same day.

ALTERNATE PROTOCOL 3

ELECTROPORATION OF Cas9 RNP INTO ZYGOTES USING ELECTRODE SLIDES

Using a slide for electroporation simplifies embryo manipulations and decreases the amount of Cas9 reagents used. However, the slides are not disposable and must be thoroughly rinsed with nuclease-free water and dried between uses to minimize the chance of reagent carryover between embryo groups.

Additional Materials *(also see Basic Protocol 5)*

H_2O, nuclease-free

1-mm-gap electrode slide (e.g., BEX, cat. no. CUY21 EDIT II, via Protech International, cat. no. LF501PT1-10) and wires to attach to electroporator

Kimwipes

1. Prepare embryos by weakening the zona pellucida as described (see Basic Protocol 5, steps 1 to 7).
2. Set up the electroporator as described (see Basic Protocol 5, step 8).
3. Set up two stereomicroscopes next to each other: one with the electrode slide placed inside a 100-mm Petri dish and connected to the electroporator, the other with a 100-mm Petri dish for preparation of embryos and RNP mixtures.

   It is convenient to perform this procedure with two adjacent microscopes, but a single microscope can be used.

4. Place one ~50-µl drop of Opti-MEM in the middle of the embryo prep dish.
5. Near the periphery of the dish, place one ~50-µl drop of M2 medium for each group of embryos to be electroporated and three ~50-µl drops of CO_2-equilibriated $KSOM^{AA}$ per group for rinses.

   It is convenient to divide the dish into two halves to process two groups of embryos in quick succession. Up to 80 embryos may be processed for electroporation in a single slide.

6. Transfer one group of embryos into an M2 drop.
7. Prepare the RNP mix for the first group of embryos by placing 2.5 µl Opti-MEM next to the drop containing embryos and then adding 2.5 µl Cas9 RNP mix.
8. Transfer the 5-µl Opti-MEM/RNP mix to the gap between the electrodes on the slide.

9. Rinse the group of embryos in the 50-µl Opti-MEM drop, taking care to transfer a minimal volume of M2.
10. Transfer embryos (with a minimal volume of Opti-MEM) to the Opti-MEM/RNP drop between the electrodes and line them up parallel to the electrodes. Work quickly to minimize evaporation of the mix in the slide.
11. Execute the electroporation program.
12. Remove embryos from the slide and return them to the original M2 drop in the embryo prep dish.

    All embryos can be recovered using an electroporation slide and embryo handling pipet, so there is no need for rinses (as with a cuvette) and the expected recovery is 100%.
13. Rinse the slide with copious amounts of nuclease-free water and wipe dry with a clean laboratory wipe (e.g., Kimwipe) before repeating steps 6 to 12 with the next group of embryos.
14. After all embryos have been treated and returned to M2, rinse each group of embryos through at least three CO_2-equilibriated $KSOM^{AA}$ drops and return them to the $KSOM^{AA}$ microdrop culture dish. Incubate at 37°C/6% CO_2 until embryo transfer surgery.

    Embryo transfer should be performed on the same day.

SCREENING AND QUALITY CONTROL OF DERIVED MICE

BASIC PROTOCOL 6

Mice born from microinjected or electroporated embryos must be screened to identify founders. Founders are then backcrossed to the original strain (when using inbred lines) or to the desired background strain (when using F1 or other embryo donors). Quality control of the N1 mice ensures that the allele is the correct sequence and that no random or extra integrations of the repair template have occurred. N1 mice may also be screened for the presence of Cas9-induced off-target variants. Once N1 mice have passed quality control requirements, they can be used to establish the line.

The following steps outline the methods we use for screening founder and N1 mice. Detailed procedures can be found in references cited below and an overview of the principles of mouse line quality control can be found in Lintott and Nutter (2023).

See Commentary for additional details.

Materials

Mice born from microinjected or electroporated embryos
PCR primers >150-bp distal to and flanking the target change
Amplification primers and hydrolysis probe for real-time PCR detection of oligonucleotide repair template sequence that does not differentiate between wild-type and engineered sequences

Additional reagents and equipment for:
Screening founder mice:
Tissue biopsies, tissue lysates, and genomic DNA preparation (Behringer et al., 2014)
PCR of genomic DNA (Kramer & Coen, 2001)
Gel electrophoresis (Voytas, 2000) and sequencing (Dorit et al., 2001) of PCR amplicons
Screening N1 mice:
Tissue biopsies

DNA purification (Liu & Harada, 2013)
Subcloning PCR amplicons (e.g., TOPO TA cloning kit, ThermoFisher cat. no. K457501, or Finney et al., 2001)
Sequencing plasmids (Slatko, Albright et al., 2001; Slatko, Heinrich et al., 2001)
Quantitative real-time PCR (qrtPCR) (L. Ma & Chung, 2014) or digital PCR (dPCR) (Mazaika & Homsy, 2014)

Screen and backcross founders

1. Identify (e.g., by ear punch) and obtain tissue biopsies from mice born from microinjected or electroporated embryos.
2. Prepare genomic DNA and perform PCR to amplify the target sequence with >150 bp on each side flanking the target site.

   Crude DNA preparations are sufficient for most end-point PCR applications. If the target region is difficult to amplify, DNA can be purified using commercial kits or organic extraction prior to PCR.

3. If a restriction site polymorphism is predicted by the sequence change, digest the PCR products with the appropriate restriction enzyme and analyze by gel electrophoresis.

   Founders are likely to be mosaic and thus both wild-type and variant alleles (from either heterozygous or mosaic animals) may be present. If destruction of a restriction site is being used for genotyping, be aware that indels introduced by NHEJ repair of the DNA double-stranded break, rather than homology-directed repair (HDR), could also destroy the restriction site.

4. Analyze amplicons by gel electrophoresis and sequence them to confirm the presence of the desired base change(s) or epitope tag.

   Founders may contain alleles with the templated change at the target site and indels. If using Sanger sequencing capillary-based fluorescent sequencing, chromatograms from animals with only the wild-type and base change allele will show two peaks at the target site(s): one with base(s) for the wild-type allele and one with base(s) for the variant allele. For epitope tag insertions, two overlapping chromatograms will be observed beginning at the target site. Overlapping chromatograms can be deconvoluted in silico using Poly-Peak Parser (Hill et al., 2014) or DECODR (see Support Protocol 2). If using Sanger sequencing with autoradiography, two bands will appear on the gel. If chromatograms cannot be deconvoluted, subcloning and sequencing may be required to identify founders.

5. Breed founders with the desired sequence change with the desired background strain.

Screen and QC N1 mice

6. Identify (e.g., by ear punch) and obtain tissue biopsies from born N1 mice.
7. Purify genomic DNA and perform PCR to amplify the target sequence.

   For quantitative PCR, inhibitors and variations in DNA amounts found in crude tissue lysates can compromise results. Using purified DNA (from commercial kits or organic extraction) is recommended for these applications. Using such DNA for the initial screen obviates the need to resample mice.

8. Verify the integrity of the flanking genomic sequence and the presence of the targeted changes by sequencing the PCR amplicon and by restriction site polymorphism, if applicable.
9. For epitope tag insertions, subclone the PCR products and sequence the resultant plasmid inserts to confirm that the epitope tag has the correct sequence.

Subcloning and sequencing are usually not necessary for substitutions of only one or a few nucleotides, as these can be readily confirmed by direct PCR amplicon sequencing.

10. Design and acquire PCR primers and hydrolysis probes for qrtPCR or dPCR.

    Most companies that supply qrtPCR or digital PCR reagents also offer free or low-cost design services. Design principles for primers and probes for dPCR are outlined elsewhere (Mazaika & Homsy, 2014). Depending on primer and probe compatibility with the target gene, Tfrc or Tert can be used as a reference gene for both qrtPCR and dPCR of genomic DNA.

11. Determine the copy number of the target sequence (template) using qrtPCR or dPCR.

    By designing the amplification primers and hydrolysis probe to recognize sequences within the template that are common to the wild-type and variant sequences, wild-type DNA can be used as two-copy (for autosomal genes) or one-copy (for sex-linked genes in males) controls.

12. Using the appropriate software for the qrtPCR or dPCR platform, determine the copy number of the target sequence.

    For QC purposes, we consider target copy numbers ranging from 1.8 to 2.2 for autosomal genes and 0.8 to 1.2 for sex-linked genes in males as wild type (pass). Copy numbers outside of these ranges are provisional pass (1.6 to 2.4 for autosomal and 0.6 to 1.4 for sex-linked) or as failing (>2.4 for autosomal or >1.4 for sex-linked).

DECONVOLUTING MULTIPLE SEQUENCE CHROMATOGRAMS WITH DECODR

SUPPORT PROTOCOL 2

Founders generated with Cas9 endonuclease are often mosaic, sometimes with more than two alleles if editing occurred after DNA replication. An estimate of the proportion of founders' alleles with the desired sequence changes helps when choosing founders for GLT breeding. When the multiple sequence chromatograms in mosaic founders are not easily distinguished, we estimate the proportion of the desired allele in each founder using the sequence deconvolution tool, DECODR (*https://decodr.org/*; Bloh et al., 2021). This tool estimates both the indel sizes and locations, and if given a template sequence, can estimate the proportion of templated insertions or single-nucleotide changes. Generally, we choose founders with the highest proportion of the desired modification for GLT breeding.

Materials

DECODR, v3.0 (*https://decodr.org/*)
Founder ab1 files
Reference (wild-type) sequence in a text, fasta, fastq or ab1 file
Endonuclease spacer sequence(s) (DECODR can analyze Cas9 or Cas12a cut sites)
HDR template sequence (plain text)

DECODR analysis

1. To use DECODR, create a free login.
2. In the DECODR tool's six entry fields, enter the following:
   a. Sample Title. Name for your sample; DECODR will remember your recent analyses so make this meaningful.
   b. Sample Type. Choose "Bulk."
   c. Guide Sequence. Choose "Cas9" or "Cas12," or for another endonuclease choose "none." Analysis for "none" is limited, see Bloh et al. (2021). Enter the guide spacer sequence, do not include the PAM. Press return to add the guide. If needed, up to two guide sequences can be added.

Figure 2 DECODR analysis can assist with identifying correctly tagged alleles in mosaic founders. (**A**) DECODR output for four founders' ab1 sequence files. Boxed bases indicate nucleotides different from the wild-type sequence. In this example we expected a 48-bp insertion. DECODR identified a 48-bp insertion in founder 1 and only partial insertions in the other three founders. (**B**) More detailed DECODR output (obtained by clicking on the file name shown in (**A**)) indicates that in founder 1, 45.7% of the chromatograms match the HDR (template) sequence and 54.3% of the chromatograms have a single base insertion at the endonuclease cut site. (**C**) The sequence alignment (SnapGene software) of founder 1's ab1 file to the desired tagged allele sequence. In the DECODR output, the green bar indicates the gRNA spacer sequence; the red bar indicates the PAM; and the pipe (vertical line) in the sequence indicates the predicted endonuclease cut site. Created in BioRender.

d. Donor Sequence. Paste in the template sequence if one was used. Leave blank if no template was used.
e. Wild-type/Control File. Either select "Text Input" and paste in the wild-type sequence as text or use "File Input" and drag in the relevant control sequence file as an ab1, fasta, fastq or txt file. We routinely use the text input.
f. Comparison/Experiment File(s). Drag in the ab1 files from the founder mice. We routinely input up to 30 files with no issues.

3. Click "Analyze data".

   This takes a few seconds to minutes to run, depending on how many experiment files are uploaded

4. The initial output window shows a list of all successfully analyzed experimental files (Fig. 2A).

   For each founder's sequence file there will be one line for each unique chromatogram identified. Boxed bases indicate differences from the wild-type sequence. The indel sizes and their relative proportions of deconvoluted chromatograms are indicated to the left of each unique chromatogram. In the example shown in Figure 2, three chromatograms were identified for founder 4; however, we often see more than three.

5. To see a more detailed analysis, click on the filename.

   This is especially helpful if a template (donor) sequence was included. In the new window are additional details, including the proportion (%) of chromatograms that matched the

template, labeled as HDR in this window (Fig. 2B). For comparison, Figure 2C shows the aligned chromatograms for founder 1 in SnapGene software (www.snapgene.com).

6. Evaluate each founder and select the founder(s) with the highest proportion of the desired allele.

   If the sequence change is expected to affect mouse viability or fertility, founders with ≤50% of the desired sequence change may have a higher probability of transmitting the desired allele to their offspring as heterozygosity rather than homozygosity is more likely in these founders and may be more permissive for germline transmission (Elrick et al., 2024).

REAGENTS AND SOLUTIONS

Use nuclease-free water in all recipes and protocol steps.

Cas9 RNP buffer, 5×

500 µl of 1 M KCl (Sigma, cat. no. 60142)
100 µl of 1 M HEPES buffer, pH 7.2 to 7.4 (Life Technologies, cat. no. 15630)
400 µl embryo-tested water (Sigma, cat. no. W1503)
Prepare in a nuclease- and pyrogen-free 1.5-ml microcentrifuge tube
Mix well
Store up to 1 year at room temperature
Before opening tube, centrifuge briefly to clear lid of any droplets

Hyaluronidase, 10×

Dissolve hyaluronidase (Sigma, cat. no. H4272) at 3 mg/ml in M2 medium (Sigma, cat. no. MR-015D) containing 4 mg/ml embryo-tested BSA (Sigma, cat. no. A3311). Store in aliquots up to 1 year at –20°C. Before use, thaw at 4°C and dilute in M2 medium to a working concentration of 0.3 mg/ml. Store thawed undiluted hyaluronidase up to 4 weeks at 4°C.

COMMENTARY

Background Information

The mouse has been the preeminent mammalian model system due to the ease of manipulating its genome. Beginning with germline-competent transgenic mice (Brinster et al., 1981; Costantini & Lacy, 1981; Gordon & Ruddle, 1981) and gaining sophistication with the isolation of pluripotent embryonic stem (ES) cells (Evans & Kaufman, 1981; Martin, 1981) and subsequent ability to engineer mutations by homologous recombination (Thomas & Capecchi, 1986), genetically modified mice have been used to dissect gene function in both normal and pathological development. The application of zinc-finger nucleases and TAL effector nucleases to modify the genome of mouse zygotes (Wefers et al., 2012) was a turning point in mouse genome engineering, opening the door to direct genome modification in any strain background, not just those for which high-quality ES cells were available. The failure of these technologies to completely replace ES cell-based genome engineering was primarily due to the complexity of design for zinc-finger nucleases and the necessity to engineer new proteins for each target (reviewed in Gaj et al., 2013; H. Kim & Kim, 2014). In 2013, the repurposing of the CRISPR-Cas9 bacterial adaptive immune system to a programmable genome engineering tool (H. Wang et al., 2013; Yang et al., 2013) transformed mouse model production.

The Cas9 proteins are a family of RNA-guided nucleases (Ran et al., 2015) whose target specificity is dictated by a short spacer sequence (18 to 20 nt) within a longer guide RNA (gRNA) (Cong et al., 2013; Jinek et al., 2012) in the assembled ribonucleoprotein. Genomic target sites must be adjacent to a 3' PAM (Ran et al., 2015). The most used Cas9 for mammalian genome editing is a "humanized" version of *Streptococcus pyogenes* Cas9 (Spy Cas9) (Cong et al., 2013) that uses an –NGG PAM, although lower cleavage efficiency is observed adjacent to the non-canonical PAMs –NAG and –NGA (Hsu et al., 2013; Y. Zhang et al., 2014). This, along with some tolerance for base mismatches between the spacer sequence and the genomic target, contributes to the Cas9-induced off-target variation that has been observed for Spy Cas9 (Hsu et al., 2013; Lin et al., 2014). However,

several studies using whole genome sequencing to assess off-target variation (mutation) after Spy Cas9 genome engineering showed that Cas9-induced off-target variation is rare (Anderson et al., 2018; Iyer et al., 2015; Mianne et al., 2016; Nakajima et al., 2016; Peterson et al., 2023; Shen et al., 2014). Importantly, Peterson et al. (2023) demonstrated that the naturally occurring variation among inbred mice from well-maintained colonies was far greater than any reported Cas9-mediated off-target variation when appropriately specific guides were used. They further showed that among putative off-target sites with more than four mismatches, mice derived from untreated embryos were as likely to show variation at these sites as Cas9-treated mice.

Approaches developed to mitigate the risk of off-target mutagenesis include using Cas9 nickase (Shen et al., 2014), using truncated gRNAs (17 to 18 nt) (Fu et al., 2014), using gRNAs with two G residues added to the 5' end of gRNA spacer sequence (5'-GG) (Cho et al., 2014), and using Cas9 RNP instead of plasmid expressed Cas9 (S. Kim et al., 2014). Cas9 nickase is consistently less efficient at introducing sequence changes than Cas9 endonuclease, and the changes in efficiency using truncated or 5'-GG gRNAs varies by gRNA.

In the absence of a homologous repair template, double-stranded breaks (DSBs) are most often repaired by NHEJ, which can introduce indels (Guirouilh-Barbat et al., 2004). However, homology-directed repair (HDR) does occur at DNA DSBs when an appropriate homologous template is present, though at lower rates than NHEJ-mediated repair (Inui et al., 2014; Yang et al., 2013). The efficiency of HDR using a synthetic oligonucleotide repair template in mouse zygotes is sufficient to recover single nucleotide changes and/or insertion of short sequence tags (Inui et al., 2014; Yang et al., 2013). As a result, direct genome editing in zygotes using Cas9 and single-stranded oligonucleotide repair templates has become the primary method for producing alleles with short (<50 bp) stretches of nucleotide changes and short sequence tags.

Critical Parameters

Target selection

As mentioned above, insertion of epitope tags is generally done at the 5' or 3' end of the open reading frame, immediately after the start codon or before the stop codon, respectively. The choice between these locations depends on what is known, if anything, about the functional effect of tagging the protein and/or the location of important functional domains (e.g., transmembrane domains or signal peptides) that may be cleaved from the target protein. For N-terminal (5') insertions one must also consider possible effects on promoter function and gene expression levels. The inclusion of a flexible linker sequence (e.g., [GGSG]1-3; Klein et al., 2014) may mitigate changes to protein function and make the epitope tag more available to antibodies.

When engineering base changes, particularly those intended to mimic patient-associated single nucleotide variants, there are several considerations. The first is whether the mouse contains an ortholog to the protein in question. The Mouse Genome Informatics (MGI) databases (*http://www.informatics.jax.org/*) have orthology information for many vertebrates, including humans. Searching MGI with the human gene symbol will often provide the annotated mouse ortholog. If this fails, searching organism-specific databases (e.g., HGNC at *https://www.genenames.org/* for humans) can provide orthologs in mice. The next step is to obtain and align the two (or more, in the case of alternatively spliced genes) protein-coding sequences to ensure that the target amino acid(s) are conserved between species. Finally, one should assess whether there is gene duplication in one species but not the other. If there is, it may be necessary to either mutate multiple targets (if duplication occurs in the mouse but not the original species) or be aware that phenotypes may not be fully penetrant due to possible compensation for gene dysfunction by the duplicated gene. If the gene is duplicated in the original species and not the mouse, phenotypes in the mouse may be more severe, possibly impacting viability or fertility, which in turn could compromise the ability to obtain the desired variant (Elrick et al., 2024).

sgRNA design

A critical aspect of sgRNA design is to select a spacer sequence that is specific, but specificity must sometimes be compromised to introduce a DSB near the target site. In general, templated repair is more efficient closer to the DSB than further away (unpublished data; F. Chen et al., 2011; Liang et al., 2017; Paquet et al., 2016). As noted above, there are methods reported to improve Cas9 specificity. Our efficiencies to date (75% to 85%, depending on allele type; unpublished data) are largely obtained using 5'-GG sgRNAs. 5'-GG

sgRNAs also facilitate synthesis by in vitro transcription and remove the requirement for the selected sgRNA spacer sequence to begin with one or more G's for T7-based in vitro transcription. In addition, using Cas9 mRNA for microinjection or Cas9 RNP for electroporation limits the exposure of the genome to Cas9 and is likely to reduce the risk of Cas9-induced off-target variation (S. Kim et al., 2014).

A second consideration is that the efficiency with which DSBs are introduced by Cas9 is likely positively correlated with stimulation of HDR (Liang et al., 2017). There are several algorithms to predict Cas9 RNP activity with specific sgRNA (e.g., Doench et al., 2014; H. K. Kim et al., 2019; Moreno-Mateos et al., 2015). Note that these algorithms have not been extensively tested in mouse embryos under conditions that directly detect DSBs or decouple the effects of Cas9-mediated changes from embryo development. Nonetheless, when more than one sgRNA choice is available, predicted activity can assist in selecting between sgRNAs with equivalent specificity. Another consideration with respect to activity, is that higher efficiency cutting can result in a high frequency of bi-allelic editing. In cases where the target gene is homozygous lethal, this may result in lower birth and founder rates (Elrick et al., 2024). See Troubleshooting for more information.

Oligonucleotide repair template design

Best practices for oligonucleotide repair template design continue to evolve as experimental evidence accumulates. Most suggestions for improving recovery of alleles repaired by HDR come from systematic studies of oligonucleotide design that are generally performed in human cell lines. Due to the high costs of systematic studies using mouse zygotes, the following HDR design strategies are less robustly tested in mouse zygotes. Asymmetric oligonucleotides that anneal to the first DNA strand released from the Cas9 RNP-DNA complex (i.e., complementary to the PAM-containing strand), were more efficient at directing the desired repair than oligonucleotides complementary to the DNA strand that remains complexed with the RNP (Richardson et al., 2016). Adding 5' modifications to the oligonucleotide or using short double–stranded oligonucleotides were also reported to improve repair efficiencies (Ghanta et al., 2021). Finally, changing the polarity of the single-stranded oligonucleotide depending on the sequence change location relative to the DSB may improve efficiency (Liang et al., 2017; Schubert et al., 2021).

In our hands, using symmetrical oligonucleotides that are complementary to the PAM containing strand with 50- to 70-nt homology arms has worked well for introducing single base changes and epitope tags.

Cas9 mRNA and protein

The choice to use Cas9 mRNA vs RNP largely depends on the method of delivery. Both Cas9 mRNA and Cas9 RNP can be used successfully for microinjection to introduce base changes and short sequence tags (e.g., Mianne et al., 2016; Paix et al., 2017; Yang et al., 2013). However, in our hands, Cas9 RNP is significantly more efficient than mRNA when electroporation is used.

Modifications to the *S. pyogenes* Cas9 amino acid sequence may also improve specificity. Several groups have developed higher-fidelity Cas9 (e.g., Kleinstiver et al., 2016; Slaymaker et al., 2016) tested in cell lines, and a proprietary high-fidelity Cas9 is commercially available (Integrated DNA Technologies, cat. no. 1078727). Although these modified Cas9 enzymes may improve specificity of DNA cleavage, they may not improve the integration of sequence-specific changes.

M. Ma et al. (2017) reported improvements in precise repair using an avidin-Cas9 fusion protein with biotinylated repair templates in mice. This approach was used to improve mouse reporter allele generation (Gu et al., 2018). This technology may improve efficiencies for generating base changes and epitope-tagged alleles, but to our knowledge it has not been extensively tested.

Reagent delivery

As iterated above, zygote microinjection requires specialized equipment and skillsets and is typically performed by service core facilities rather than in individual labs, and thus the specifics of microinjection are not described here. Several excellent protocols are available (Behringer et al., 2014; Wefers et al., 2012). For simple alleles (exon deletions, NHEJ-mediated indels, single base changes, and epitope tags), electroporation is a straightforward, efficient, and scalable method for mouse line production. In our experience, electroporation is more efficient (requires fewer embryos per founder obtained) than microinjection. This may be due to the increased survival (live born rate) of embryos manipulated by electroporation compared to microinjection.

Cas9 dose

Changes in Cas9 amounts (concentration delivered to zygotes) can affect efficiency of allele generation. Attention should be paid to the desired outcome of the experiment prior to either increasing or decreasing the amount of Cas9 delivered (whether as mRNA or RNP). For some sequence changes and/or gene targets, particularly those affecting viability and/or fertility (Elrick et al., 2024), increasing the amount of Cas9 may decrease recovery rates, while for other changes and/or targets, it may improve recovery rates. See Troubleshooting for more details.

Zona pellucida treatment

Acid Tyrode's activity can vary between lots and is hard to standardize due to the variation in sizes of embryo-handling pipets and amounts of medium transferred with each group. Weakening the zona pellucida provides more reproducible results in our hands using the electroporation parameters outlined in this protocol. Other groups have eliminated this step from their protocols (Hashimoto & Takemoto, 2015a, 2015b; Horii et al., 2017).

Off-target mutations

Two types of Cas9 off-target mutations may arise when engineering base change or epitope-tagged alleles in mice: random integration of the repair template, or indels at off-target sites. Routine quality control (QC) to detect random integration of repair templates is described in Basic Protocol 6. Off-target indels introduced by Cas9 can be detected by (a) PCR and sequencing of predicted off-target sites, or (b) whole-genome sequencing. The prediction algorithms for Cas9 off-target activity are evolving (e.g., Abadi et al., 2017), and unbiased approaches such as whole-genome sequencing are becoming more affordable. Since off-target mutagenesis rates are very low (Anderson et al., 2018; Iyer et al., 2015; Mianne et al., 2016; Nakajima et al., 2016; Peterson et al., 2023; Shen et al., 2014), additional QC may not be necessary except when sgRNAs with low sequence specificity are used. When an sgRNA with lower than optimal specificity is used, sequencing linked, high-probability predicted off-target sites should be done as part of N1 QC. With decreasing costs of whole genome sequencing, genome wide assessment could also be done, but natural variation (Peterson et al., 2023) may confound the interpretation of apparent Cas9-mediated off-target variation. To control for both natural and Cas9-mediated variation, control and experimental cohorts should be generated from the same breeding colony, e.g., to obtain both wild-type and experimental (homozygous) mice from the same set of heterozygote intercrosses (Lintott & Nutter, 2023).

Mouse line maintenance

To minimize genetic drift from commercial wild-type strains, lines should be maintained by back-crossing heterozygous mutants to the wildtype strain. Homozygous experimental cohorts should be produced by intercrossing heterozygous mice to generate wild-type controls and homozygotes from the same generation of intercrosses. Since genetic drift can result in tens of new single-nucleotide variants and new indel mutations each generation (Uchimura et al., 2015), and commercial colonies provide mice from a range of generations (e.g., Charles River, 2016; Taft, et al., 2006), it is not appropriate to maintain control and experimental lines separately or to order commercial mice as controls.

Troubleshooting

Efficiencies vary from locus to locus but can range from 1% to 40% of live born pups carrying the desired templated sequence change and about double that number of indel alleles. The most common causes of failure are: (a) poor reagent quality as indicated by RNA degradation upon post-injection QC, (b) a low activity Cas9-sgRNA combination, or (c) effects of the sequence change(s) on viability and/or fertility (Elrick et al., 2024). If reagents pass post-injection QC, yet the desired sequence change(s) is not recovered after one round of microinjection or electroporation, assess (a) the number of pups born, and (b) the level of Cas9 activity, as indicated by the recovery of indel alleles at the target site. If good birth rates (>20% of embryos delivered after microinjection or >40% for electroporation) and indel rates (>25% of alleles mutated) are observed, but there is no templated repair, this indicates the Cas9 RNP activity is good. Increasing the amount of oligonucleotide in the microinjection or electroporation mix for repeated attempts may be effective. If good birth rates occur but few or no indel alleles are identified, as a first effort, increase the amount of Cas9 or try a different sgRNA, if available. Removing the non-templated 5'-GG may also help if the spacer sequence begins with one or more G's. RNP cleavage activity can be tested in vitro if low activity is suspected. This is done by amplifying genomic DNA with

the target site and using Cas9 RNP to digest the fragment (New England Biolabs, 2024). While no or little cleavage in vitro definitively marks an inactive or low activity sgRNA, respectively, good in vitro activity does not always correlate with high in vivo activity. If few or no pups are born, try reducing the amount of Cas9 to reduce possible toxic effects of the gene modification by decreasing the likelihood of compound heterozygotes or homozygotes and/or increasing mosaicism. Another method to increase mosaicism is to inject one blastomere of 2-cell embryos rather than zygotes (for a description of 2-cell microinjection, see Gu et al., 2020).

It is possible, even in mosaic animals, that bi-allelic editing will occur in most edited cells, generating cells with two variant alleles, e.g., one with the desired change and one with an indel introducing a frameshift that generates a null allele. In the case of cell or developmental essential genes (Cacheiro et al., 2020), a null allele may result in only wild-type cells contributing to the developed mouse and/or germline, preventing germline transmission of the edited allele to the next generation. In addition, experiments producing compound heterozygotes with the variant and a loss-of-function indel or two loss-of-function indels, may have poor founder rates due to lethality (Elrick et al., 2024). If the desired sequence change is lethal, the FLEx approach can be used to generate a conditional allele (Lindner et al., 2021; Schnutgen & Ghyselinck, 2007). If it appears that inadvertent null alleles are preventing recovery of the desired allele use an alternate approach that reduces the risk of NHEJ. For example instead of using a single sgRNA that directs cutting within an exon, replace the wild-type exon with a variant exon using intronic sgRNAs and deliver the larger DNA template using adeno-associated virus (S. Chen et al., 2019) or perform targeting in ES cells (Gertsenstein et al., 2020) to generate the desired allele through homologous recombination followed by chimera generation and germline transmission.

Anticipated Results

Among mice born, 1% to 40% should have the desired sequence change, with ∼2 to ∼3 fold the number of indel alleles identified. In general, indel alleles are directly correlated with Cas9 activity, whereas templated sequence changes are dependent on the activity of the endogenous cellular DNA repair machinery. The variability is a result of several factors, including accessibility of the locus to Cas9 activity, Cas9-sgRNA efficiency, and the activity of the cellular DNA repair machinery. The Troubleshooting section provides possible explanations for negative results and ways to attempt to rescue failed projects.

Time Considerations

Once reagents arrive (e.g., synthetic oligonucleotides), sgRNA synthesis and QC take 1 to 2 days. Timing of mouse orders for embryo donors, superovulation, mating, and embryo harvest may vary slightly between animal facilities. Preparation of microinjection or electroporation mixes take <1 hr and can reasonably be completed during the time it takes to collect and prepare embryos for reagent delivery when being done by another person. The complete microinjection procedure, from embryo collection to embryo transfer, takes ∼8 hr for 80 to 120 embryos. Electroporation of the same number of embryos can save ∼2 hr of processing time. Once embryo transfer is complete, the remaining experimental timeline is largely dependent on the reproductive biology of the mouse: ∼3 weeks until parturition, ∼2 weeks until born mice can be sampled for genotyping, ∼6 weeks after that until founders can be bred for germline transmission testing. Genotyping and quality control can generally be completed in 2 to 4 days, depending on the number of mice being assayed, once reagents are in hand. Figure 1 presents a schematic of the timeline for this workflow.

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Author Contributions

Marina Gertsenstein: Investigation; methodology; writing—original draft; writing—review and editing. **Lauri Lintott:** Investigation; methodology; visualization; writing—review and editing. **Lauryl Nutter:** Conceptualization; investigation; methodology; supervision; writing—original draft; writing—review and editing.

Conflict of Interest

The authors declare no financial conflicts of interest with respect to reagents or products listed or used in this protocol. The Centre for Phenogenomics offers services to generate genetically engineered mice on a fee-for-service basis.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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