Chapter 2

Simple, Efficient CRISPR-Cas9-Mediated Gene Editing in Mice: Strategies and Methods

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Abstract

Genetic modification of almost any species is now possible using approaches based on targeted nucleases. These novel tools now bypass previous limited species windows, allowing precision nucleotide modification of the genome at high efficiency, rapidly and economically. Here we focus on the modification of the mouse genome; the mouse, with its short generation time and comparatively low maintenance/production costs is the perfect mammal with which to probe the genome to understand its functions and complexities. Further, using targeted nucleases combined with homologous recombination, it is now possible to precisely tailor the genome, creating models of human diseases and conditions directly and efficiently in zygotes derived from any mouse strain. Combined these approaches make it possible to sequentially and progressively refine mouse models to better reflect human disease, test and develop therapeutics. Here, we briefly review the strategies involved in designing targeted nucleases (sgRNAs) providing solutions and outlining in detail the practical processes involved in precision targeting and modification of the mouse genome and the establishing of new precision genetically modified mouse lines.

Key words Cas9, CRISPR, Genome editing, RNA-guided endonucleases, Genetic engineering, Mouse model, Microinjection, transgenic, Humanization, Transgenic, sgRNA

1 Introduction

Since the late 1980s mouse Embryonic Stem Cells (ES) have been the primary tool in engineering genetically modified animals [1, 2]. Although powerful in its time, the use of ES cells to create these animals carried a number of limitations including the poor ES cell strain (and species) availability, the high skill sets needed, and the length of time required to obtain a modified strain. With the advent of targeted nuclease technologies, genetic editing directly in the mouse zygote has become universally efficient, rapid, and economic [3–5]. Direct targeted nuclease modification of zygotes was first demonstrated with Zinc Finger Nucleases (ZFN) [6, 7]. This innovative approach was rapidly followed by the development and use of Transcription Activator-Like Effector Nucleases (TALEN) [8, 9]. Both these
systems are based on custom engineered proteins designed to bind to defined target DNA sequences, leading to a targeted double-stranded DNA cut. As such, their construction is complex, requiring time and a fair degree of skill. In sharp contrast, the CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats, and CRISPR Associated protein 9) [10, 11] system specificity relies on a custom synthetic RNA and its base pairing to the DNA target. These custom RNAs are exceptionally simple to design and to construct. These characteristics, coupled with the observed accuracy and efficiency of genome targeted cutting makes CRISPR-Cas9 the predominant methodology for genetic editing in mouse and other species [4, 5, 12, 13]. Here, we outline the time line (Fig. 1), practical steps and challenges in constructing and using targeted nuclease technologies enabling simple genetic editing directly in mouse zygotes, leading to founder offspring and germline transmission of desired genetic modifications.

1.1 CRISPR-Cas9 and Genome Editing in Zygotes

CRISPR-Cas9 is an RNA-guided endonuclease comprised of two principle parts, a universal DNA endonuclease (Cas9 protein) and a custom-designed synthetic single guide RNA (sgRNA). The sgRNA complexes with and acts as a targeting guide for the Cas9 nuclease. At the time of writing the most commonly used Cas9 nuclease is derived from *Streptococcus pyogenes* (SpCas9). However, novel Cas9 orthologs and modified versions of Cas9 with different properties are being developed, and novel systems discovered [14–17]. The sgRNA sequence is established and
construction is simple, being based on a synthetic RNA scaffold plus a 5’ variable 17–20 nucleotide sequence that defines the genomic target by sequence base homology. Once introduced into a cell, the Cas9-sgRNA complexes, enters the nucleus and scans the genome for the sgRNA defined target sequence. This is first achieved by identifying a triplet sequence called the protospacer adjacent motif (PAM) [18, 19]. The PAM, is defined by the originating species of Cas9 (e.g. “NGG” for SpCas9) and although part of the targeting recognition sequence, it is not represented in the sgRNA guide sequence. Following recognition of a PAM sequence, the sgRNA in association with Cas9 exploits Watson-Crick base pairing of its complementary 5’ targeting region to the putative target genomic sequence. Where the sequences match, the complex causes a blunt-ended double-strand break in the targeted DNA [19] (Fig. 2).

Double-stranded DNA breaks occur naturally often in cells and are rapidly repaired using a Non-Homologous End Joining (NHEJ) repair pathway. Although NHEJ is in general accurate, dsDNA breaks induced by RNA-guided endonucleases often lead to indels; i.e. insertions of one to two base pairs, or more commonly deletions of one to ten’s of base pairs, or occasionally hundreds of base pairs [20, 21]. However, in the presence of homologous (donor) DNA template, the cell’s internal repair systems can make use of an alternative process, the homology-directed repair (HDR) pathway which can lead to incorporation of the exogenous donor sequence albeit at lower frequencies (Fig. 2) [4, 7, 13, 22]. In recent years these approaches have been used to directly edit the genome of many different species, ranging from bacteria, plants, to mice and humans [4, 23–25]. In the final analysis, the power of CRISPR-Cas9 is that it is simple to design, fast to construct and use, and of crucial importance, is highly efficient and accurate in its action.

1.2 Definitions

Definitions for zygote genome editing outlined in this chapter.

(a). Simple Knockout.
Use of one targeting sgRNA aimed at genomic DNA sequence disruption, for example targeting an initiation codon and causing an indel by NHEJ, without donor DNA, and leading to serendipitous frame shift and disruptive mutations (Fig. 2a) [26].

(b). Dropout Knockout.
Use of multiple targeting sgRNAs simultaneously, flanking a contiguous section of genome with the aim of deleting the intervening sequence. This can be designed to disrupt the gene, or with forethought if desired a hypomorph. The targeted regions can be separated by tens to thousands of bases, without donor DNA (Fig. 2b) [27].
(c). **Simple KnockIn.**

Use of one or more targeting sgRNA’s to disrupt a target sequence followed by Homology Directed Repair (HDR) designed to incorporate small genetic modifications (1—~50 bp) using a single-stranded DNA (ssDNA) oligo (100–200 bases) as donor template; for example introducing a SNP, modifying coding sequence to effect amino acids changes, introducing a tag sequence (e.g. V5), or designed to give a small precise deletion (Fig. 2c) [7, 28].

(d). **Large KnockIn.**

Use of one or more targeting sgRNAs to disrupt a target sequence that is repaired by HDR using a donor DNA template (e.g. plasmid) with extensive sequence homology arms.
(2–10 kb) to the target region. This aims at integrating longer, >50 bp, to many kb of novel DNA sequences precisely (e.g. multiple LoxP sites, visualization markers, humanization), or to effect precise larger deletions (Fig. 2c) [29, 30].

It is only since late 2012 that CRISPR-Cas9 has been used to genetically alter mammalian cells hence protocols are in a state of flux and are still being refined. From our own experiences and others it is already apparent that there are a number of common challenges and needs to address when using this system; these include:

CRISPR-Cas9 interacts with its target sequence via an RNA guide sequence, binding by base pairing to its complementary DNA target strand via Watson-Crick-type interactions. Although such base pairing is conceptually simple, it is known that base pair mismatches between the guide sequence (especially at the 5′ end, distal to the PAM) and the target sequence can be tolerated to varying and at times surprising degrees. Such near matches can lead to off-target dsDNA breaks and consequently unintended genetic modification [31–36]. In an attempt to address this issue a number of partial solutions have been developed:


There are numerous web-based software resources where target sequences can be submitted and suggested guide sequences returned, see Table 1. These various web routines are generally simple to use. Crucially many will scan the target species genome and provide a listing of near matching, potential off-target sites. Some of these programs attempt to rank guide sequences however, the rules which govern off target matches are still very much under development and hence any ranking should be regarded as advisory and not absolute [36]; for recent reviews and approaches see [37–39]. As a rule of thumb and after assessment by a web-based program for near matching sites, we further select target guide sequences with GC content of between 40 and 60 %, whilst avoiding runs of four of more nucleotides of the same base, and aim for higher G/C content at the 3′ seed sequence end [19, 32]. In some cases, for example with simple KnockIn (KI)’s the choice of guides sequence may be very limited due to the location of the desired precision modification, which needs to be within the guide recognition sequence (preferably at its 3′, PAM proximal end) in order to prevent recutting post modification. At times, however, due to local sequence constraints the only practical approach is pragmatism coupled with an acceptance that off-target cutting may occur and may require additional screening, and/or back-crossing (relying on allelic segregation) to eliminate any unintended
effects in a new mouse strain. It should also be noted that the reference genome used by many of these programs (e.g. C57BL/6J) may not truly represent the actual strain/genome you are targeting. For example, each inbred mouse line has a multitude of strain-specific polymorphisms. This challenge becomes more significant (and at times useful) when working with outbred animals. Also, if sequentially modifying a strain, previous modifications (i.e. transgenes) may need to be considered when designing the guide sequence.


SpCas9 can be mutated to function as a nickase, cutting only one of the two strands of the targeted dsDNA. In this inception, two sgRNAs are used in close proximity targeting opposite

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<th>Table 1</th>
<th>Web-based resources. Non-exhaustive listing of web sites to assist and advise on designing targeting sequence, gene analysis databases, and physical reagent resources, see also <a href="http://omictools.com/crispr-cas9-category">http://omictools.com/crispr-cas9-category</a></th>
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DNA strands, resulting in a staggered dsDNA break. Data suggest that this dual sgRNA nickase strategy can considerably lower off-target cutting [31, 40]. A further enhancement of this approach is to use a “dead” Cas9 (i.e. Cas9 with no nuclease activity), fused to an obligate heterodimer, FokI nuclease [41, 42]. Here, only when the heterodimer FokI nucleases are in the correct configuration will a (staggered) dsDNA cut occur. However, the use of paired Cas9’s with their configuration restrictions (e.g. dual co-located PAMs) and possible Cas9-sgRNA differential activities may lower overall efficiency. Modified Cas9’s are readily available from Addgene, see Table 1.

3. *Truncated guides.*

The actual binding interaction of the guide or seed part of sgRNA to target DNA, leading to the cutting of the DNA is not fully understood. However, it has been empirically determined that truncating the target guide sequence from the 20 bases to 18 bases substantially reduces off target cutting [36, 43]. The truncated guide approach is simple to implement and the efficiency of cutting does not seem to be significantly impaired; MVW and BEL’s own observation and [43]. However, when designing these truncated guides it should be noted that most guide design programs listed in Table 1, use a default of 20 bases and will need to be truncated manually at the 5’ end by two bases. Also if the T7 promoter is used to make the sgRNA, a “G” is required at the 5’ end of the sgRNA (see below).

4. *Breeding Founders.*

When creating a genetically modified mouse line, the putative founder mice should be backcrossed at least once. This will help reduce any potential off target effects via genetic segregation, unless they are closely (physically) linked to the desired modification. In special cases where it is known that a guide may modify loci which are not the primary objective (e.g. when targeting a gene family motif, or unintentionally modifying pseudogenes etc.), several cycles of back-crossing coupled with active selection by genotyping against identified off-target alleles may be necessary.

The introduction of CRISPR-Cas9 into the zygote does not necessarily ensure that a NHEJ or HDR event occurs before S-phase or zygote cleavage. In fact, CRISPR-Cas9 events often appear to occur later than the zygote stage, including at 2-, 4-, or even perhaps 8-cell stages (MVW and BEL, own observations). Founder animals that progress from such independent CRISPR-Cas9 blastomere events will develop as genetic mosaics composed of cells where different nuclease-mediated repair events have occurred. Practically this means that when a tail tip, ear notch, or other somatic tissue is collected from founder animals, it is only a sample of the potential...
genetic heterogeneity within the mosaic animal. Further, even where clear results are obtained from such somatic tissue samples; e.g. a perfect biallelic modification, the animal’s gametes (sperm or oocytes) may not totally reflect this (Fig. 3). Such somatic mosaicism also jeopardizes, or minimally complicates using founder animals for direct phenotypic analysis. The take-home message here is that somatic cell biopsies of founder animals cannot be regarded as a complete reflection of the whole animal, including importantly the germ-line. As such, any putative founder animal must be backcrossed to the background strain and the resulting offspring (N1) sequence analyzed to determine precisely their specific mutant allele. At times, such germline mosaicism can be viewed as an opportunity, as a single founder may provide multitude alleles, offering more genome editing events from fewer animals. Once N1 animal(s) that contain the desired events are identified they can then be used to establish the new strain.

**Fig. 3** Sequence chromatographs showing sequence derived from mosaic founder. An alignment of DNA sequence chromatographs obtained from PCRs covering the flanking regions of a CRISPR-Cas9 event using mouse tail derived DNA (inbred mouse line). The sgRNA guide sequence and PAM (inverted and hence CCN) is shown above, with the expected dsDNA break ~3 bp upstream of the PAM. Panel (a) is from a wild-type mouse showing the known homozygous target region. Panel (b) is derived from a founder animal and shows at least four alleles with varying degrees of signal strength for the targeted region, i.e. the tail is genetically mosaic. Panel (c) is derived from an N1 offspring derived from the founder “b” and clearly shows a wild-type and a single-base insertion allele at the expected site, i.e. this N1 offspring is heterozygous with a 1-base insertion allele. Panel (d) shows another, different example of a founder “b” N1 offspring, showing clearly a wild-type and a mutated allele; however, this time the mutation is a 19 base deletion. This demonstrates that although modified founder animals can be identified, it can be difficult to predict what the actual germline contains due to their often mosaic nature. This problem also occurs with KI’s where the correction can be masked by a host of overlaying NHEJ events.
The objective in developing a genetically modified mouse is to generate a resource to either study gene function or develop a disease model - fast. This generally requires germline transmission of the genetic modification, often to homozygosity. In the pursuit of speed it is tempting to pair modified founder animals with the objective of obtaining homozygous modified animals rapidly. The authors strongly caution against this approach. With especially serendipitous indel KOs, this course of action will often result in variable compound heterozygous offspring due to founder animal mosaicism, and will complicate analysis considerably. In the case of founders with precise HDR events, although often still mosaics, these could be intercrossed resulting in rapid development of offspring homozygous for the desired precise event. However, the resulting frequency of homozygous offspring is dependent upon the actual allele frequency in the gametes and not the tail! Further, there is an elevated risk that any off-target events present in founders will become fixed in the nascent homozygous strain, potentially complicating later phenotypic analysis. We suggest that if this latter short cut is practiced, it be done only as a quick but potentially flawed approach to obtain preliminary data. Hence we strongly recommend that founder animals be backcrossed at least once before developing a homozygous strain.

A key advantage in using targeted nucleases is that they can be applied to zygotes derived from any strain or genetic background. It should be remembered and possibly exploited, genetic background will have profound impacts upon mutation phenotype [44]. For the majority of our work we have used inbred backgrounds including C57BL/6J, FVB/J, DBA/2J, Balb/cJ, NOD/ShiLTJ, MRL/MpJ, and CAST/EiJ. Currently, we are increasingly using strains that have undergone previous (often multiple) genetic modification, including KI’s, KO’s, and transgenics, e.g. NSG, NRG, Tg32 FcRn (respectively, The Jackson Laboratory mouse strain reference # 5557, 7799, and 14565). The use of previously modified and characterized strains (models) allows rapid and sequential genomic editing of animals, leading to the development of highly customized mouse models. The use of these perhaps more obscure strains, however, may need to be tempered against availability, embryo yield, and survivability post zygote injection/transfer, each of which can be quite unique to a strain. Recently approaches based on improved superovulation and/or collection and cryopreservation of oocytes have been developed which may alleviate these challenges [45, 46].

A number of publications have used F1 or F2 zygotes derived from crossing different inbred strains for CRISPR-Cas9-mediated modifications [26, 29]. Creation of these zygotes, due to hybrid vigor uses fewer resources and the zygotes often exhibit increased resilience to the traumatic processes involved in microinjection and
embryo transfer. Such embryos have their uses, for example in optimization of methods and training, but caution that their use will severely compromise coherent genetic analysis due to genetic segregation; i.e. offspring will be genetically unique and highly heterogeneous which will cause variability to their phenotype confounding analysis [47, 48].

1.4.2 Target Sequence

When sgRNA guides are designed, it is crucial that the target sequence is fully defined as unintentional mismatches between target and guide may cause a guide to fail, or work very inefficiently (see Table 1 for resources to assist). This is of special concern where the precise sequence of a particular strain is not available. Further, if using F2 or outbred animals, the existence of sequence polymorphisms may raise both on-target and off-target complications (at times this can be used to experimental advantage). Where there is any doubt regarding the genomic target sequence, a simple PCR using the desired strain’s genomic DNA as template, followed by sequence analysis across the region of interest is recommended. This simple step in advance of the microinjection can save significant time and resources, and has the added benefit that the PCR will provide useful data to assist in the screening of putative founders post-microinjection for the CRISPR/Cas9-mediated events.

1.4.3 Delivery of Nuclease: KO/ KI

The choice of cytoplasmic vs. pronuclear microinjection of the sgRNA and Cas9 is determined by the objective: KO vs. KI. For simple KO’s, where no donor DNA template is utilized, delivery of sgRNA and Cas9 by microinjection to the cytoplasm is the most commonly used approach [49]. Most Cas9’s contain one or two nuclear localization signals and the Cas9-sgRNA complex appears to be actively conveyed to the nucleus. A novel approach avoiding microinjection, based on electroporation of zygotes with sgRNA and Cas9 is also a possibility [50]. Where KI’s are desired which use donor DNA (oligo or plasmid), most groups use pronuclear microinjection to deliver the templates to the nucleus, whilst also “lingering” in the cytoplasm to deposit the RNA’s into their appropriate subcellular location. While the skill set needed for pronuclear microinjection is higher, and results in reduced live born compared to cytoplasmic microinjection, this approach has proven to be very efficient for KI’s (MVW and BEL own observations) [26, 29, 51].

1.4.4 KO Targeting

When creating KO animals it is essential to define the goal, i.e., if a null mutation is the absolute need or if potential hypomorphs would be of use. To help ensure that the desired event occurs the gene domain structure needs to be understood, facilitating targeting of critical regions; e.g. the initiation (ATG) codon, a transmembrane domain, or active binding site (see Table 1 for web resources). In this context our experience when using a single targeting sgRNA will often result in an indel of only a few bases around the targeted region.
Although this may be effective, many genes do have complex, often not fully characterized splice variations and may also possess cryptic initiation sites and/or splice acceptors. As such, a single targeting sgRNA may potentially lead to a hypomorph instead of a null mutation, and even the doubt of failing to produce a null can lead to substantial extra work. A more effective approach to gene disruption is to use two sgRNA flanking an essential element within the target, >100 bp—10 kb apart; however, note smaller dropouts occur more efficiently, although 50–100 kb dropouts can be found (Fig. 2b). If carefully designed, this two sgRNA strategy will increase the probability of a null mutation or minimally give two opportunities to disrupt the target region. The use of an oligo or a plasmid containing flanking regions homologous to the intended deleted region is also an option as these can mediate much larger and controlled deletions (≫10 kb) [52, 53].

1.4.5 KI Targeting

For subtle precision KI with changes in the 1–50 bp range, e.g. SNP humanization, tags, integrate sites, and precision deletions, the use of a single Cas9-sgRNA mediated dsDNA cut followed by HDR using a predominantly homologous donor ssDNA oligo (≤200 base) has proven to be highly efficient. A key consideration when designing this type of modification is that once the desired HDR event occurs in the genome that the Cas9-sgRNA will no longer (re)cut the modified targeted region. To achieve this the guide + PAM target site must be sufficiently compromised post-HDR so that the Cas9-sgRNA complex can no longer target the region. We and others have found that this is best done by introducing target changes post modification which have one or more mismatches in the 3′ “seed” end of the guide sequence proximal to the PAM. The PAM of SpCas9 PAM can be used as the mismatch region, although it should be noted that the NGG can functionally substitute NAG and NGA [32, 54]. Synonymous, or silent substitutions to the target genome could also be used, however we suggest that unnecessary changes to the target region, even so-called silent ones be avoided as they may have unintended consequences. For larger KIs (e.g. >100 bp), at the time of writing and in the experience of the authors approaches to incorporate larger donor sequences are still poorly defined, are locus sensitive, and appear to be less efficient in inbred mice vs. F1 or F2 strains (MVW, BEL own observations).

1.4.6 Targeting Multiple Sites by Design

Theoretically many regions could be targeted provided Cas9 does not become limiting. We and others have used multiple sgRNAs targeting three or more genes (with 2xsgRNA’s/gene) simultaneously [29]. This approach decreases the cost/gene, although when multiple modifications occur in a single animal they will, unless linked, segregate independently. In any case, care should be exercised as each guide has a finite probability of off-target cutting. It is also possible to use a
single guide to target multiple copies of a sequence (e.g. gene family motif, putative pseudogenes, or other functional sequence motifs); i.e. all contain the same target sequence (including PAM) (MVW own observations). The efficiencies for the various target sites will vary and screening offspring can be challenging as it requires resolving which of the multiple copies of a target sequence have been modified.

1.4.7 An Abundance of Riches: Too Many Alleles

An unexpected challenge we have seen is having too many founders and offspring with a multitude of various (potentially interesting) mutations. Handling these animals with this abundance of alleles can rapidly overwhelm mouse space, analysis, and financial constraints. The authors have found that selecting two or at most, three (where possible, male) founders with the desired mutation has been sufficient to obtain a desired line rapidly. Once offspring with the desired mutations are identified the other lines are terminated, or where additional lines may need to be preserved as potentially interesting, sperm cryopreservation is used (see also Chapter 3 for details on sperm cryopreservation) [55, 56].

1.5 Future Directions

Nuclease-mediated genetic editing technologies have only recently come onto the scene and their future potential can only be guessed. However, it is obvious that their future use will literally change the world. Key elements that are under active research include:

1. Approaches to increase HDR efficiency vs. NHEJ; e.g. through the use of NHEJ inhibitors [57, 58].

2. Elimination of mosaicism, potentially allowing biallelic modified founder animals to define phenotype [59, 60].

3. Developing systems for larger scale genomic integrations (i.e. hundreds of kb of DNA) using, for example safe harbor sites (e.g. ROSA26 locus), facilitating controlled transgenesis, synthetic biology approaches to gene expression and novel gene control systems [3, 61–65].

4. Redefinition and control of PAM sequence specificity, allowing universal targeting by engineering the Streptococcus pyogenes Cas9 PAM site [15] and/or the use of orthologous Cas9’s [14, 23, 66–68]. For example, Cpf1 is a novel targeting nuclease, with an alternative PAM and perhaps more importantly, it exhibits different physical properties from Cas9, being smaller and causing a staggered dsDNA cut outside the guide sequence [16, 17].

5. The elimination or reduction of off-target concerns providing genetic editing without significant collateral damage; e.g. by the development of proven rules in guide design based on nonbiased off target assessment methods, and a better understanding of RNA-guided endonuclease target interactions [36, 69, 70].
6. Improved control of oocyte production allowing more rapid and sequential modification of modified mouse strains and other species [45, 46].

2 Materials

2.1 Enzymes and Kits

2. High Fidelity PCR; e.g. Phusion® High-Fidelity DNA Polymerase with supplied buffer (New England Biolabs Inc., cat. # M0530).
3. PCR product purification (QIAquick PCR Purification Kit, Qiagen cat. # 28106).
4. PCR product purification kits for sequencing (HighPrep™ PCR MAGBIO, AC-60050).
5. T7 In Vitro Transcription kit for sgRNA (MEGAshortscript™ T7 Transcription Kit, Life Technologies, cat. # AM1354M).
6. SpCas9 mRNA (capped and polyadenylated) home-made or from a commercial source (e.g. Trilink, Cat # L 6125). Store at −80 °C as single-use aliquots (e.g. 5–10 μg aliquots at 1000 ng/μL).

2.2 Reagents

1. RNase-ZAP (Life Technologies, AM9780).
2. Molecular grade water, nuclease-free.
3. Nuclease-free TE pH 8.0, 10 mM Tris pH 8.0, 0.1 mM EDTA (IDT Cat # 11-05-01-09).
5. Isopropanol (ACS Reagent Grade Plus).
6. Tris Borate EDTA (TBE) electrophoresis buffer, used at 0.5x.
7. Electrophoresis gel UV DNA/RNA stain (EnviroSafe® Helixxtec, cat # HDS001).
8. Microinjection TE buffer, nuclease free TE pH 7.5: 10 mM Tris pH 7.5, 0.1 mM EDTA (IDT Cat # 11-05-01-05).

2.3 DNA Oligos and PCR Primers

1. T7-guide sgDNA overlapping PCR primer 5′- TTAATACGACT CACTATA-(GN17-19)- GTTTAAGAGCTATGCTGGAA-3′, where “N” is the sgRNA guide target sequence (see [71]) and below.

2. Common 80mer ssDNA oligo defining the crRNA stem loop region of the sgRNA 5′-AAAAAAGCACCGACTCGGTGCACTTTTTTCAAGTTGATAACGGACTAGCTTAAAC-3′ [71].
3. Various synthetic ssDNA oligos for use as donor templates; e.g. IDT, made up at 1000 ng/μL in 10 mM Tris, 0.1 mM EDTA pH 8.0 aliquoted and stored at −20 °C; e.g. IDT, cat # 11-05-01-09.

2.4 Superovulation and Microinjection Equipment

1. Stereomicroscope (Zeiss Discovery.V8).
2. Inverted microscope (Zeiss AxioObserver.D1).
3. Micromanipulators (Eppendorf NK2).
4. Injectors (Narashige IM-5A, IM-11-2 or Eppendorf Femtojet).
5. Needle puller (Sutter P97, P1000).

2.5 Consumables and Reagents for Superovulation and Microinjection

1. Thin wall capillary tubes with filament (World Precision Instruments (WPI) TW100F-4).
2. Thin wall capillary tubes without filament (WPI TW100-4).
4. Aspirator tube assembly (Sigma A5177; or as an alternate to mouth pipetting, COOK Flexipet).
5. Pregnant Mare Serum Gonadotropin (PMSG) (ProSpec HOR-272).
7. Embryo Culture Media (COOK K-RCVL).
8. Embryo Handling Media, M2 (e.g. Zenith Biotech ZFM2-100).
9. Silicone fluid (Clearco PSF-20cSt), alternatively mineral oil can be used.
10. Hyaluronidase (Sigma H3506-1G).

2.6 Reagents for Founder Screening

1. NaOH tail lysis buffer: 50 mM NaOH, 0.2 mM EDTA.
2. 1 M Tris–HCl pH 8.3.
3. Proteinase K tail buffer: 50 mM KCl, 20 mM Tris pH 8.3, 1 mM beta-mercaptoethanol, 0.5% NP40, 0.5% Tween-20, 1 mM EDTA.
4. Proteinase K.
5. 10 mM Tris pH 8.3.
6. Optional: Qiagen’s DNeasy Blood and Tissue Kit (Qiagen 69506).

3 Methods

3.1 Characterization of Target Region

Where the sequence of the region to be modified is not completely known, approximately 1–2 kb centered on the target should be PCR amplified and sequence verified. If the animals/zygotes are heterozygous for the region, e.g. outbred, F1, F2 etc., this may
result in reduced effective HDR. For larger deletions long range PCR (3–10 kb) or other strategies may need to be developed to cover the region (see Table 1 for resource listing). These data will also be used for subsequent screening of putative founders.

Using the known sequence and established PCR primer standard design programs, primers are designed and ordered. We routinely test and optimize these in advance using ~1–3 ng of pure genomic or 1 μL (~100–300 ng, see below) of crude “tail digest” DNA in a 30 cycle 15 μL PCR, see Table 2. To ascertain an optimal annealing temperature with a good specific signal to noise ratio, we use a temperature annealing gradient PCR to optimize the PCR conditions empirically (Fig. 4). Typically we test annealing temperatures in the range of 48 °C–68 °C. If this fails, for example due to polymorphism or repetitive sequences, other primers may need to be designed and tested.

### 3.2 HDR ssDNA Oligo Donor for Microinjection

For HDR, oligo donor ssDNAs for microinjection can be ordered from various suppliers and are generally supplied lyophilized. When ordering ssDNA as donor DNA, order oligos ~150–200 bp in

<table>
<thead>
<tr>
<th>Reagent</th>
<th>1× μL</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular grade water</td>
<td>11.825</td>
<td>1 95 °C</td>
</tr>
<tr>
<td>10× Standard buffer</td>
<td>1.500</td>
<td>2 95 °C</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>0.300</td>
<td>3 48–68 °C</td>
</tr>
<tr>
<td>Primer Mix each @ [10 μM]</td>
<td>0.300</td>
<td>4 68 °C</td>
</tr>
<tr>
<td>Taq Polymerase (c.e. NEB#M0273)</td>
<td>0.075</td>
<td>Repeat 2–4</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1.000</td>
<td>5 68 °C</td>
</tr>
<tr>
<td>Final volume</td>
<td>15.000</td>
<td>6 4 °C</td>
</tr>
</tbody>
</table>

Fig. 4 Example of optimization PCR. The effect of different PCR annealing conditions. Note that as the annealing temperature increases, specificity and noise to signal ratio improves. In this example, we would select 62 °C as the optimized annealing temperature, as it has a good signal strength combined with high specificity
length and in sufficient quantity (i.e. 100 nM) to allow for PAGE purification. Although we have found that non-PAGE purified ssDNA oligos work well for HDR, PAGE purification will reduce the presence of truncated products that may compete with the full-length donor following CRISPR-induced DSBs. Some publications suggest that the first 2–3 bases 5′ and also the third and second to last 3′ bases are phosphorothioated to improve oligonucleotide stability in vivo [7, 72]. Donor oligos should be dissolved in appropriate volume of microinjection TE buffer (10 mM Tris pH 7.5, 0.1 mM EDTA) made to 1 μg/μL stocks and stored at −20 °C.

3.3 sgDNA Template Preparation

An outline of construction and synthesis of sgRNA is shown in Fig. 5 and is based on [71, 73]. Targeting sgRNAs are synthesized from a dsDNA template containing a T7 promoter and the unique guide sequence (T7-guide) 5′-TTAAATACGACTCACTATA-(G<sub>N17-19</sub>-GTATTAAGAGCTATGCTGGAA-3′, plus a common 80mer defining the crRNA stem loop region of the sgRNA 5′-AAAAAAAGCAC C G A C T C G G T G C C A C T T T T T C A A G T G T G ATAACGGACTAGCCTATTAAAAACTTGCTATGCTGTTTC CAGCATAAGCTCCTAACC [43, 71]. The use of an overlapping PCR synthesis to make an in vitro transcription template avoids time-consuming cloning and quality control sequencing, providing speed and complete flexibility in the process of sgRNA design and production. Using separate guide and tracr RNA's made synthetically should also be considered.

3.4 Design of Targeting Guide for sgRNA

1. A truncated guide for the sgDNA oligo should ideally be 18 bases in length, although 19 and 20 base guides can be used (see Note 1) [43].

2. When designing guides we utilize web software to screen for and minimize potential off-target cutting, selecting those guides with the least near matches (e.g. ZIFIT, see Table 1).

3. Due to the use of the T7 promoter for in vitro transcription, the guide sequence MUST initiate with at least one guanine (“G”), without this it will fail. If necessary, the guide can begin with a non-recognition “G” (i.e. one that does not exist in the genomic target). This will be the first base of the transcribed sgRNA; for a more complete description, see Fu et al 2014 [43]. Once this N17-20 sequence is established it is “inserted” into the T7-guide sequence for oligo DNA synthesis and can be ordered.

3.5 T7-guide PCR Synthesis of sgDNA Template

On ice, assemble reagents for the PCR synthesis of the sgDNA template as outlined in Table 3 (Fig. 5). Once reagents are mixed, gently centrifuge and perform PCR using conditions given in Table 3 (see Notes 2 and 3).
As the next stages involve synthesizing and handling RNA they must be done as clean as possible, avoiding the introduction of RNases that will rapidly degrade sgRNA and Cas9 mRNAs. RNase-ZAP or similar RNase cleaning agents can be used to decontaminate the work area. These stages require to always use clean reagents, dust free disposable plastic supplies, and should be executed whilst wearing fresh gloves. RNA should always be kept on ice or stored at −80 °C to reduce RNase activity.

1. Following sgDNA template production, reserve 1–5 μL of PCR product for quality control DNA gel electrophoresis.
2. Purify remaining PCR product using Qiaquick PCR Purification Kit, eluting in 30 μL nuclease-free molecular grade water.
3. Quantify by spectrophotometry. A typical concentration expected is ~75–150 ng/μL; i.e. a total yield of ~2.4–4 μg from a 100 μL PCR reaction.
4. Gel electrophorese using a 2% agarose made and run in 0.5× TBE, loading 1 μL of PCR (pre and post purification) with appropriate markers to visually verify the sgDNA is present and is a single band at ~120 bp (see Fig. 6).
5. Store Purified sgDNA at −20 °C, or proceed immediately to the in vitro transcription using the MEGAscript T7 Transcription kit.

### sgDNA Template Quality Control

#### Table 3

<table>
<thead>
<tr>
<th>Reagent</th>
<th>1× μL</th>
<th>PCR Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular grade water</td>
<td>76.00</td>
<td>1 98 °C 30 s</td>
</tr>
<tr>
<td>5× HF buffer</td>
<td>20.00</td>
<td>2 98 °C 10 s</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>2.00</td>
<td>3 60 °C 30 s</td>
</tr>
<tr>
<td>Common stem loop 80 bp oligo [100 μM]</td>
<td>0.50</td>
<td>4 72 °C 15 s</td>
</tr>
<tr>
<td>Phusion polymerase</td>
<td>1.00</td>
<td>Repeat 2–4 35×</td>
</tr>
<tr>
<td><strong>Master mix volume</strong></td>
<td><strong>99.50</strong></td>
<td></td>
</tr>
<tr>
<td>T7-guide (target specific) Primer [100 μM]</td>
<td>0.50</td>
<td>5 72 °C 10 min</td>
</tr>
<tr>
<td><strong>Final volume</strong></td>
<td><strong>100.00</strong></td>
<td>6 4 °C Hold</td>
</tr>
</tbody>
</table>

### sgRNA In Vitro Transcription

gRNA synthesis using, e.g. MegaShortscript T7 Kit and the T7 promoter-containing sgDNA template created above (see Note 4).

1. Follow manufacturers protocol combining reagents at room temperature and in order as outlined in Table 4 (for convenience,
the nucleotides can be combined and 8 μL of the premix used per reaction).

2. Mix, quick spin, and then incubate at 37 °C for 2–4 h (longer will increased RNA yields slightly).

3. Add 1 μL of Turbo DNase (provided in the kit) and incubate at 37 °C for another 15 min in order to remove the sgDNA template, leaving the sgRNA intact.

Fig. 5 Outline dsDNA T7 template to sgRNA synthesis. Outline of the process to construct a dsDNA template for sgRNA synthesis using PCR, followed by T7 promoter-driven IVT synthesis of the sgRNA. Section (a) is the generalized sequence for the T7-guide PCR primer, which is the main variable and crucial to specificity in sgRNA design. When using truncated guides the G+N16–17 defines the guide protospacer, i.e. the target sequence for the sgRNA. Note, the T7 polymerase promoter has an obligate “G” as its final 3′ base, in order for the T7 to be active it is crucial that the first base of the guide sequence is a “G”. However, we have found where this cannot be arranged that G+18 bases, where the “G” is silent and not part of the recognition sequence will function well. For guides of 20 bases, G+20 where the “G” does not match the target also appear to be functional. Section (b), shows the common 80 base oligo defining stem loop structure of the sgRNA which also contains 20 bases (3′) which are complementary (overlapping) to the T7-guide PCR primer. Section (c), the T7-guide primer and the common 80 base oligo are combined in an overlapping PCR to produce the sgDNA template. Section (d), after PCR and purification, the sgDNA template is used in a T7 driven IVT, synthesizing large amounts of sgRNA. The sgRNA construction and synthesis is fast, and the only variable is the T7-guide, which can be synthesized and delivered within 24–72 h. The subsequent processes (PCR, PCR purification, IVT, and sgRNA purification) can be completed within a single day.
4. Transfer 20 μL of the in vitro transcription reaction to an Eppendorf tube for phenol chloroform extraction.

5. Reserve the remaining ~1 μL for quality control gel electrophoresis, keep on ice −4 °C, or better −20 to −80 °C.

6. Add 115 μL molecular grade water to the reaction and 15 μL ammonium acetate (provided in the MegaShortscript T7 kit) giving a total volume of 150 μL (20 + 115 + 15 μL).


8. Vortex for 30 s, then spin at 20,000 × g for 5 min at RT in a bench top Eppendorf centrifuge.

9. Remove ~100 μL of the upper layer containing the sgRNA to a fresh tube (see Note 5).

**Fig. 6** TBE agarose gel showing sgRNA samples before and after extraction cleanup. Five independent sgDNA’s PCR synthesis, before and after clean-up (0.5× TBE, 1.5 % agarose). The expected band is ~120 bp and approximately 100 ng product was loaded in each lane.

**Table 4**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>1× μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified sgDNA PCR Product [75–150 ng/μL]</td>
<td>8.00</td>
</tr>
<tr>
<td>10× T7 Reaction Buffer</td>
<td>2.00</td>
</tr>
<tr>
<td>75 mM T7 ATP Solution</td>
<td>2.00</td>
</tr>
<tr>
<td>75 mM T7 CTP Solution</td>
<td>2.00</td>
</tr>
<tr>
<td>75 mM T7 GTP Solution</td>
<td>2.00</td>
</tr>
<tr>
<td>75 mM T7 UTP Solution</td>
<td>2.00</td>
</tr>
<tr>
<td>T7 Enzyme Mix</td>
<td>2.00</td>
</tr>
<tr>
<td>Final volume</td>
<td>20.00</td>
</tr>
</tbody>
</table>
10. To precipitate the sgRNA add an equal volume (100 μL) isopropanol (propan-2-ol), vortex, and quick spin down.

11. Place the tubes into −20 °C, −80 °C, or dry ice and chill for ≥15 min (this can be left overnight if desired).

12. Thaw if needed, and pellet the sgRNA by centrifugation in a refrigerated microcentrifuge; e.g. bench top Eppendorf centrifuge at 20,000×g for 15 min at 4 °C.

13. Remove supernatant with care, leaving a small white pellet of sgRNA at the bottom of the tube.

14. Wash twice in 500 μL 70% ethanol, spinning at 20,000×g for 5 min at 4 °C each time, and taking care not to disturb the pellet.

15. Use a P10 tip to remove as much of the ethanol as possible whilst avoiding the pellet.

16. Allow the sgRNA pellet to dry for 5–10 min (see Note 6).

17. Resuspend the sgRNA pellet in 30 μL in 10 mM Tris, 0.1 mM EDTA, pH 7.5 and keep on ice for immediate use, otherwise, store at −80 °C.

### 3.8 sgRNA Quality Control

1. Dilute sgRNA sample (e.g. ~1:5, 1–4 μL IDTE) and use to measure the concentration of the sgRNA (keep sgRNA at 4 °C or if for longer periods, −80 °C).

2. Quantify by spectrophotometry (e.g. using a Eppendorf Biospektrometer, Nanodrop), typical concentration is ~300 ng/μL; i.e. ~50 μg yield from 100 μL of the in vitro transcription reaction.

3. Back-calculate to determine the actual sgRNA stock concentration; in this case 5× the measured concentration of the diluted sgRNA.

4. To visualize the sgRNA quality, use some/all of the remaining dilution (~3 μL; aim for ~100–400 ng total sgRNA per lane in order to prevent overloading the gel) and gel-electrophoresis on a standard 2.0% agarose electrophoresis 0.5× TBE gel with EnviroSafe DNA Stain to visualize RNA and DNA ladder); see Note 7. If desired, also run the reserved (unpurified sgRNA from the in vitro transcription step). Figure 7 shows example sgRNA gel images before and after purification.

5. Once sgRNA material is confirmed it should be stored at −80 °C until needed. We have not found it useful to conduct in vitro assay to determine sgRNA activity (see Note 8). If multiple microinjections are planned using a single sgRNA batch, freezing down multiple aliquots of the sgRNA is recommended to reduce freeze-thaw cycles.
Table 5 outlines starting point concentrations of the various CRISPR-Cas9 and donor DNA for zygote microinjection. Once microinjection reagents are mixed they must be kept at 4 °C. It is also strongly suggested as a quality control measure, that samples pre and post microinjection be kept and gel electrophoresed to ascertain if the preps have been compromised (Fig. 8). The secondary structures (dimers) seen here in panel (b) with purified sgRNA are often seen at this stage.

**3.9 Assembly of Reagents for Microinjection**

![TBE agarose gel showing 11 sgRNA samples, before (a) and after (b) clean up via Phenol:Chloroform Extraction. The result of 11 examples of sgRNA IVT using the overlapping PCR to construct the sgDNAs (0.5× TBE, 2.0 % agarose). The sgRNA is ~120 bases and runs at about this equivalent size as the dsDNA marker. Note: sample #2 sgRNA had a reduced yield, and the Pre-cleanup gel (a) demonstrates that the IVT was poor for this sample, and not sample loss during clean-up. Further, if this were due to RNA degradation it would run as a faint smear (Fig. 8). The secondary structures (dimers) seen here in panel (b) with purified sgRNA are often seen at this stage.](image)

Fig. 7 TBE agarose gel showing 11 sgRNA samples, before (a) and after (b) clean up via Phenol:Chloroform Extraction. The result of 11 examples of sgRNA IVT using the overlapping PCR to construct the sgDNAs (0.5× TBE, 2.0 % agarose). The sgRNA is ~120 bases and runs at about this equivalent size as the dsDNA marker. Note: sample #2 sgRNA had a reduced yield, and the Pre-cleanup gel (a) demonstrates that the IVT was poor for this sample, and not sample loss during clean-up. Further, if this were due to RNA degradation it would run as a faint smear (Fig. 8). The secondary structures (dimers) seen here in panel (b) with purified sgRNA are often seen at this stage.

1. Combine RNA reagents: Microinjection TE buffer, sgRNA’s, and Cas9 mRNA on ice in a PCR tube. Use a thermocycler to denature RNAs, using a program that steps down from 90 to 4 °C, holding at 90, 80, 70, 60, 50, 40, 30, 20, and 10 °C each for 1 min, and maintaining samples at 4 °C until ready to proceed.

2. Add where required other components (i.e. donor DNA) and RNAsin (see Note 9).

3. Mix, and transfer the entire preparation to a 1.5 mL microcentrifuge tube and spin at 20,000 × g at 4 °C for 10 min. This helps sediment any microparticulates that will block the injection needle (see Note 10).
### Table 5
Suggested starting point for zygote microinjection reagent concentrations

<table>
<thead>
<tr>
<th></th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Simple KO: Cytoplasmic injection + 1 sgRNA</strong></td>
<td></td>
</tr>
<tr>
<td>Microinjection TE buffer 10 mM Tris pH7.5, 0.1 mM EDTA</td>
<td>Make to 25 μL</td>
</tr>
<tr>
<td>Cas9 mRNA</td>
<td>100 ng/μL</td>
</tr>
<tr>
<td>sgRNA</td>
<td>50 ng/μL</td>
</tr>
<tr>
<td>RNAsin</td>
<td>0.2 U/μL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Dropout KO: Cytoplasmic injection + 2 sgRNA’s</strong></th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microinjection TE buffer 10 mM Tris pH7.5, 0.1 mM EDTA</td>
<td>Make to 25 μL</td>
</tr>
<tr>
<td>Cas9 mRNA</td>
<td>100 ng/μL</td>
</tr>
<tr>
<td>sgRNA #1</td>
<td>25 ng/μL</td>
</tr>
<tr>
<td>sgRNA #2</td>
<td>25 ng/μL</td>
</tr>
<tr>
<td>RNAsin</td>
<td>0.2 U/μL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Simple KI: Pronuclear injection + oligo</strong></th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microinjection TE buffer 10 mM Tris pH7.5, 0.1 mM EDTA</td>
<td>Make to 25 μL</td>
</tr>
<tr>
<td>Cas9 mRNA</td>
<td>60 ng/μL</td>
</tr>
<tr>
<td>sgRNA</td>
<td>30 ng/μL</td>
</tr>
<tr>
<td>Donor Oligo (ssDNA, ~100–200mer)</td>
<td>1–10 ng/μL</td>
</tr>
<tr>
<td>RNAsin</td>
<td>0.2 U/μL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Large KI: Pronuclear injection + plasmid</strong></th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microinjection TE buffer 10 mM Tris pH7.5, 0.1 mM EDTA</td>
<td>Make to 25 μL</td>
</tr>
<tr>
<td>Cas9 mRNA</td>
<td>60 ng/μL</td>
</tr>
<tr>
<td>sgRNA</td>
<td>30 ng/μL</td>
</tr>
<tr>
<td>Donor Plasmid (dsDNA) Supercoiled</td>
<td>1–20 ng/μL</td>
</tr>
<tr>
<td>RNAsin</td>
<td>0.2 U/μL</td>
</tr>
</tbody>
</table>

4. With care, transfer 20 μL of the supernatant into a new 0.2 mL PCR tube, leaving the remaining ~5 μL plus debris in the 1.5 mL tube. Keep both tubes on ice; use the 20 μL supernatant for microinjection. The remaining 5 μL is reserved to confirm that the RNAs were intact prior to microinjection by gel electrophoresis.

5. After microinjection, gel electrophorese the remaining microinjection sample through a 1.5% agarose 0.5× TBE gel, to check for degradation during the microinjection process (Figs. 8 and 9).
**Fig. 8** RNAs pre and post Microinjection. An example of RNA preparations before and after microinjection, gel is 1.5% agarose in 0.5× TBE (non-denaturing), shown with two size DNA markers, Molecular Weight Ladders (MWL), 100 bp MWL (Promega) and a 1 KB MWL (NEB). Note, the microinjection preparation samples (a–c) here have been denatured as outlined in the text and so the sgRNA’s move as single band size ~120 bp. In contrast, the individual RNA components of the sample (Cas9, sgRNA #1, and #2) did not receive this treatment and so the secondary structures (e.g. dimers) are evident in those lanes. This gel confirms that the microinjection sample was intact and present before microinjection (“a”), and that no significant RNA degradation occurred during the microinjection process (“b” and “c”, two aliquots of the same preparation sample)

**Fig. 9** Visualization of degraded RNA (RNase Activity). Examples of degraded Cas9 and sgRNA due to RNase activity. This gel (1.5% agarose in 0.5× TBE) shows the same samples as used in Fig. 8 but subjected to RNases for a few seconds at room temperature and directly loaded into a gel. The result shows RNase degradation leading to a low molecular weight smear of all the samples, which is especially prominent with the Cas9 mRNA. Such degraded RNA would probably fail to yield modified pups post microinjection.
1. The production of large numbers of synchronized zygotes via superovulation greatly increases microinjection throughput, while minimizing the vivarium footprint. Here we will briefly outline their production and isolation from C57BL/6J mice. It must be noted however, that there is no universal protocol for inducing superovulation as genetic background profoundly impacts the procedure and zygote yield. When optimizing superovulation protocols for a specific strain it is important to also consider age and weight, in relation to hormone dose \[74\], as well as the time of hormone injection in conjunction with the room light cycle \[75\].

2. Calculate the number of donor females necessary to complete the requested experiment. For example with C57BL/6 J, 10 plugged donor females, 24–28 days old are expected to yield 200–250 good quality zygotes.

3. Three days before microinjection, at ~10.00 AM (mouse room lights 12 h on, 12 h off, 6 AM–6 PM) inject intraperitoneal (IP) 5 international units (IU) of PMSG in C57BL/6J at 24–28 days of age.

4. Forty seven hours post PMSG administration, inject IP 5 IU of hCG and immediately setup donor females at 1:1 with proven C57BL/6J stud males (see Note 11).

5. The following day at ~7.00 AM check for copulation plug. Those females displaying a plug are segregated for zygote collection (see Note 12).

6. For zygote collection females are euthanized via cervical dislocation, the oviduct excised and placed in 35 mm Petri dish with 3 mL of M2 media at RT. The ampullae lysed and the oocyte clutches removed. Hyaluronidase is added to the media at ~3 mg/mL to digest the cumulus cells and release zygotes, which are then transferred immediately to fresh M2 media at RT. Zygotes should be washed several times in M2 to remove cumulus cells, debris, and hyaluronidase \[75\] (see Note 13).

7. For microinjection, high quality zygotes are selected and placed into 30 μL microdrops of embryo culture media (e.g. COOK K-RCVL) under silicone fluid (see Note 14).

---

**3.11 Microinjection of CRISPR Reagents**

It is important when preparing glasswear, microinjection needles etc. for Cas9-sgRNA zygote injection that extra effort is made to minimize RNase contamination, including wearing gloves. Any reagent degradation will considerably reduce CRISPR efficiency.

1. The holding side pipettes and handling pipettes can be prepared in advance but the microinjection needles should be prepared the day of the experiment (or purchased from a reputable vendor). Table 6 illustrates an effective injection needle pulling
program for a Sutter P97 using WPI TW100F-4 thin wall capillary tubes (see Note 15).

2. At microinjection and immediately before use, CRISPR reagents should be prepared and stored on ice. After loading the needle into the injector, back pressure must be established immediately to prevent dilution of the reagents by the injection medium.

3. After the needle is loaded and mounted, collected zygotes are placed onto a slide containing 150–200 μL of M2 media. The number of zygotes placed on a slide should not exceed the number that can be injected within 20–30 min.

### 3.11.1 Cytoplasmic injection

1. Move the needle through the zona and into the cytoplasm of the zygote. The needle must pierce the oolemma. Once pierced it is possible to visualize the reagents leaving the needle as a disturbance of the cytoplasm. Exit from the zygote should be swift to minimize zygote lysis. It is estimated that 2–10 pL can be deposited in the cytoplasm without cell lysis (see Note 16).

2. After zygotes have been injected, remove and wash through three 30 μL drops of equilibrated K-RCVL and place back into culture in a K-MINC-1000 benchtop incubator at 37 °C, 5% CO₂/5% O₂/90% N₂.

### 3.11.2 Pronuclear Plus Cytoplasmic Injection

1. Zygotes are loaded onto a slide as outlined above. The needle is prepared also as outlined.

2. The zygote is orientated in order to ensure proper focal plane alignment between the needle tip and the pronucleus. Entry into the pronucleus is confirmed visually by observing swelling of the pronucleus. Upon exit from the pronucleus the injector should consciously linger a few seconds to deposit material in the cytoplasm. After completing injections, remove the zygotes and wash through three 30 μL drops of equilibrated K-RCVL and place into culture in a K-MINC-1000 benchtop incubator at 37 °C, 5% CO₂/5% O₂/90% N₂.

### 3.12 Transfer Embryos Into Pseudopregnant Recipients

It is essential that high quality pseudos’ are available to receive the injected zygotes. We prefer to transfer injected zygotes on the same day (i.e. at one cell stage) as this reduces the time zygotes are in an artificial environment however, transfer at the two cell stage is also

<table>
<thead>
<tr>
<th>Filament size</th>
<th>Pressure</th>
<th>Heat</th>
<th>Pull</th>
<th>Velocity</th>
<th>Delay</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 × 4.5 (FB245B)</td>
<td>500</td>
<td>Ramp minus 13–15</td>
<td>75</td>
<td>70</td>
<td>80</td>
</tr>
</tbody>
</table>
commonly used [75] (see Note 17). Pseudopregnant recipient mice can be readily provided by a good mouse unit [75], or can also be purchased from reputable dealers.

3.13  Screening of Putative Founder Animals for CRISPR Meditated Events

PCR screening for CRISPR mediated events: For crude DNA tail lysate preparation ear punches (~1 mm circle) or tail tips (≤1 mm) are collected at 2–3 weeks of age from putative founder mice and subjected to either NaOH or Proteinase K digestion to make crude DNA available for PCR analysis (see Note 18).

3.14  Crude NaOH DNA Tail Lysate

NaOH tail lysis buffer is 50 mM NaOH, 0.2 mM EDTA and can be made up in advance and stored at RT.

1. One ear punch or ~1 mm of tail tip tissue is digested in 100 μL NaOH DNA tail lysate at 95 °C for 1 h; e.g. using 200 μL 8-strip tubes and PCR machine.
2. Neutralize the NaOH by the addition of 10 μL 1 M Tris–HCl pH 8.3.
3. Mix samples, then centrifuge at max speed (~6000 × g) in a bench top mini-centrifuge at RT to sediment hair, cartilage, and other tissue remnants.
4. For most PCRs 0.1–1 μL (30–300 ng DNA) of this crude supernatant is sufficient in a 15 μL PCR reaction (more may suppress the PCR).
5. NaOH derived DNA tail lysate can be stored at 4 °C if used that day, or at −20 °C for long-term storage.

3.15  Crude Proteinase K DNA Tail Lysate

Proteinase K tail buffer is 50 mM KCl, 20 mM Tris pH 8.3, 1 mM beta-mercaptoethanol, 0.5% NP40, 0.5% Tween-20, 1 mM EDTA and can be made up in advance (i.e. without proteinase K) and stored at RT.

1. Immediately before use, add proteinase K (stored at −20 °C) to the buffer to a final concentration of 0.25 mg/mL.
2. One ear punch or ~2 mm of tail tip tissue is digested in 100 μL proteinase K DNA tail lysate with proteinase K using 200 μL 8-strip PCR tubes at 55–60 °C for at 5–18 h.
3. After digestion, the crude proteinase K DNA tail lysate samples are mixed and centrifuged at max speed (~6000 × g) in a bench top mini-centrifuge at RT to sediment hair, cartilage, and other debris.
4. Before using Crude Proteinase K DNA tail lysate in the PCR, samples are diluted 1:10 in 10 mM Tris pH 8.3 (e.g. 5 μL/45 μL) and must be heated to 94 °C for ~3 min. This critical step both denatures the sample DNA and destroys proteinase K activity.
5. For most screening PCRs, 1 μL (~30 ng DNA) of the 1/10 diluted and denatured crude supernatant is sufficient for use in a 15 μL PCR reaction.

6. Crude Proteinase K DNA tail lysate can be stored at 4 °C if used that day or at −20 °C for long-term storage.

3.16 Crude DNA Tail Lysate Purification

Purification of crude DNA lysate is only rarely required and should be avoided except for particularly finicky PCR reactions. Where required, we suggest Qiagen’s DNeasy Blood and Tissue Kit be used, following the manufacturer’s protocol.

3.17 PCR Screening for Founder Animals

Depending upon the exact strategy, potential founder animals are screened for KO and/or KI by PCR. Where large regions have been potentially deleted or a specific “in-out” PCR strategy has been applied, a positive result PCR is usually quite obvious/predictable. However, we suggest that these PCR products be sequenced to begin defining the exact nature of the modification and help prioritize which animals to breed from.

In Fig. 10 we show a typical gel obtained from a simple KI founder screen. Obvious serendipitous deletions are also apparent. In this example, while only 3/24 PCR bands show an obvious CRISPR-mediated effect (insertion or deletion), subsequent sequence analysis identified 15 additional mutated founders (although mostly mosaic), three of which appeared to contain the desired HDR KI.

3.18 Selection of Optimal Founders to breed: KO and KI, What to Look for

1. The selection criteria for which identified founder animals/s to breed is project specific and often not clear cut due to mosaicism. As a general rule, we recommend selecting ~3 putative founder animals where the desired event can be seen, ideally males due to their ability to sire more offspring rapidly.

2. For KO’s, the putative founder(s) animals with the largest deletions are typically the most likely to result in a functional null. However, an understanding of the gene structure will allow appropriate ranking of founders.

3. For simple KI’s, when examining founder animal derived sequence it is not uncommon for the desired sequence modification to be obscured by other mutant (NHEJ) alleles often due to mosaicism. As such, this process can take some patience, and good sequence analysis software is invaluable (e.g. CodonCode Aligner). Aligning the sequence chromatograms to the reference sequence will identify where the mutations begin (and end, if sequencing from both sides of the target). This will eliminate some candidates based on the likelihood that the desired location is modified. We find when assessing founder carrier animals that a simple although subjective rating helps; e.g. \( A = \) clear trace evidence of HDR, \( B = \) strong trace evidence of HDR, \( C = \) some trace evidence of HDR and \( D = \) faintest trace evidence HR.
4. For Large KI’s, the challenge is to distinguish a successfully targeted KI event from a random transgenic (i.e. an illegitimate, nontargeted genomic integration). We screen founders using two approaches sequentially. First, using a PCR specific to the exogenous sequence we identify any/all animals that contain the donor sequences, regardless of integration position. Typically, this reduces the candidate pool considerably. Next, we screen this smaller candidate pool using a long range “In/Out” PCR strategy, using an appropriate PCR kit (e.g. LongAmp™ Taq PCR Kit, NEB cat # E5200S). This PCR uses a primer designed within the donor DNA region, plus a second primer that lies in the genome outside of the donor HDR region; i.e. beyond the homology arms. Using this In/Out PCR design a correct sized product is produced only when HDR has occurred at the intended locus. For better verification, this PCR should be used...

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**Fig. 10** Examples PCR and electrophoresis gel for screening putative Founders. Electrophoresis gel (1.2% agarose, in 0.5× TBE) of a genotyping PCR assay of potential Founder animals using tail derived DNA from a project designed to introduce a small modification (simple KI). A total of 24 mice were born and tissue biopsies were subjected to PCR/Sequencing analysis. Clear evidence of a serendipitous deletion is shown in two samples #4 and #13, while an insertion is also obvious in sample #21. However, the majority of the mutants, including the correctly targeted KI Founders, were not identified until after sequence analysis of the PCR product. Sequence analysis identified an additional 15 mutants, 3 with the desired HDR event, and only 6/24 mice were shown to be WT by sequence analysis (indicated in the gel image with *). Gel shows: Neg PCR, i.e. negative control; Pos1, i.e. PCR positive control using crude DNA lysate isolated from wild-type; Pos2, i.e. PCR positive control using purified DNA (the slight differences in running is probably due to salt concentration differences in the PCR due to DNA used and isolation method).
performed across both junction points. The region should also be sequenced to verify the completeness and accuracy of the HDR event in the founder, and in N1 offspring.

Upon selecting the most promising founders animals, these mice should be set up for breeding typically at 6–7 weeks of age, to wild-type (same) background animals. To maximize offspring numbers, male founders are preferred and used in trio, or harem matings, or by rotating females after they become pregnant. In this manner, more N1 offspring can be produced to rapidly assess the germline transmission of the modified allele. With putative female founders a single male is sufficient, although if the females are not pregnant within 4 weeks, this male should be replaced. It is essential that defined criteria be established to select those N1 animals which will be used to establish the strain as soon as possible to contain costs. At this time we recommend sperm cryopreservation be considered. This would use 1–2 male carriers and will safeguard against catastrophic loss [55, 56].

Lastly, if not already established, it is essential that a simple and robust PCR genotyping assay be developed for the established line; i.e. not based on sequencing. Often this cannot be fully optimized until after the mutant allele has been characterized from sequences of an N1 mouse. Once developed, this PCR assay should allow simple rapid genotyping of the new line without the time and cost of sequencing.

Now it is only necessary to phenotype the new mouse lines.

4 Notes

1. The PAM sequence; e.g. NGG is intrinsic to the CAS9 and must not be part of the targeting sgDNA primer sequence.

2. Lyophilized oligos (T7-guide oligo and the common step loop 80 bp oligo) should be reconstituted at 100 \( \mu \)M in 10 mM Tris, 0.1 mM EDTA pH 8.0. To prevent freeze/thaw degradation, it is recommended that these oligo stocks be aliquotted and stored at −20 °C until use. Also it is recommend to use Phusion® or other high-fidelity DNA polymerase for sgDNA synthesis to ensure fidelity.

3. The common stem loop 80 bp oligo can be replaced with a shorter primer: 5′-AAAAAAAGCACCGACTCGGTGC-3′ and a plasmid at very low concentration (<1 ng/\( \mu \)L) which contains the 80 bp stem loop sequence (e.g. Addgene 51024).

4. RNA is very sensitive to RNases, which are ubiquitous, keeping the sample cold or preferably frozen will reduce any contaminating activity and prevent your sample from “disappearing”.

5. To avoid bringing over contaminating material from the interface we suggest leaving behind a considerable amount of the aqueous phase, e.g. in this case, collecting only ~100 \( \mu \)L of the
~150 μL aqueous phase. We have found that the amount of sgRNA isolated is still >50 μg with this approach, which is more than adequate for tens of standard microinjections.

6. It is crucial that there is no remaining wash liquid left in the tube.

7. The 0.5× TBE gels are non-denaturing gel, however they provide a rapid and good indication as to sgRNA quality (i.e. degraded or not). For RNA work we recommend that the gel box and “teeth” are clean, and that only fresh running buffer be used.

8. The CRISPR-Cas9 approach exhibits a very high routine functionality in zygotes without prior testing. Also in the case of mouse, germline testing would require a high level of resources/time; i.e. it is faster and directly relevant to use the sgRNA and assess its activity directly in vivo rather than in cell lines.

9. RNAsin is a protein and would be destroyed by the denaturation protocol if added before this step. Also, dsDNA (plasmid) should not be subjected to the denaturation protocol as resulting nicks/breaks could result in a more difficult sample to microinject.

10. It is unclear where such microparticulate debris can accrue from, however we have found this centrifugation step prevents frustration during microinjection due to blocked microinjection needles.

11. Stud male colonies used for zygote production must be maintained with detailed records on the mating performance (i.e. success of females with copulation plug) and age. This will allow purging of nonproductive males to maintain optimal fecundity.

12. Often checking for the presence of a copulation plug is considered optional. We have found that for certain strains, including C57BL/6J, C57BL/6NJ, females without a copulation plug can yield zygotes. As such, harvesting all paired females is a viable option. For other strains including NOD/ShiLtJ, NSG, NRG (respectively, JAX mouse strain reference # 1976, 5557, and 7799), paired females without visible copulation plugs rarely contain zygotes. When plug rates fall below 50% it is not worth the effort to harvest these females. For expensive or difficult to obtain mouse strains, females (depending on the starting age) with no copulation plug may be reused after 2–4 weeks (if not pregnant), however with variable limited success.

13. At this stage processed zygotes can be graded for fertility and morphology. Careful grading will allow more efficient use of injection systems. High quality optics are required to identify any zygotes worth injecting which should show polar bodies and the presence of two distinct pronuclei.

14. Culture dishes containing microdrops under silicone fluid need to have been prepared ~24 h in advance to equilibrate to 37 °C, in 5% CO₂/5% O₂/90% N₂; e.g. in a K-MINC-1000 benchtop incubator (or equivalent).
15. CRISPR-Cas9 modification often requires the microinjection of high concentrations of nucleic acids, this may require developing a needle pulling protocol for pronuclear/cytoplasmic injections using a gradual elongated taper from the shoulder to the point. In addition, the temper of the glass should be such to allow for multiple chips while retaining a sharp point.

16. If possible the reagents should be deposited deep within the cytoplasm and the needle should swiftly exit the cell. If deposited close to the oolemma the chances of the cell lysing increase greatly. If a plume of cellular material is seen external to the oolemma (under the zona) after exiting, the likelihood of zygote survival is small.

17. Survival of zygotes to live born is dependent upon zygote quality, mouse background, microinjectionist skill, cleanliness, and reagent concentrations of microinjected material. Survival also depends upon the nature of the zygote injection, for example with C57BL/6 J zygotes post cytoplasmic injections, we see 30–40% survival to live born, whilst with pronuclear injection, routinely we observe 20–30% survival. Lastly, putative genetically edited founder animals are a precious resource. To help ensure success surgically implanted females should be monitored closely on and around their due date. It is also strongly advised that dedicated foster litters be produced in conjunction with all surgically generated litters. This allows in the case of dystocia or other birthing difficulties, that viable pups can be rescued by performing a Caesarean section and using arranged foster mothers.

18. It can be tempting to believe that using more tissue is better, however this is not the case. These methods are easily overloaded, and the use of too much tissue will inhibit the subsequent PCR reaction. For most robust PCR reactions, 0.1–1 μL of crude NaOH lysate (estimated at ~30–300 ng) will work well, however especially for longer range PCRs (>2 kb) the proteinase K digestion preps may be preferable.

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