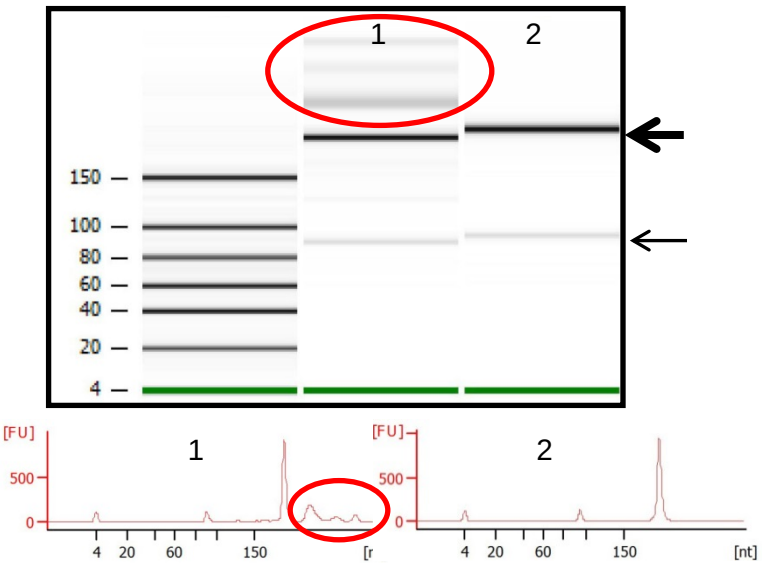
Supplemental Method #1: Preparation and activity testing of sgRNA

1. Define the top scoring 1-3 crRNA sequences that overlap or are as close as possible (ideally < 15bp) to the genomic sequence to be edited with the Design Tool from Feng Zhang's lab (http://crispr.mit.edu). Be sure first nucleotide is a guanine; if it is not, you may substitute the first nucleotide with guanine or simply add a guanine in the first position followed by your 20 nucleotide crRNA sequence.
2. Order Watson (5'*CACC*GN_{19}) and Crick (*AAAC*N_{19}C) complementary crRNAs for cloning into pX330 vector. **Do not include the adjacent PAM in your ordered sequence**.
3. Combine two oligonucleotides (1:1, v/v in nuclease-free H_2O), heat for 5 min at 95°C in a heat block, and slowly cool for 45 min with tubes remaining in the block; the block is removed from heat source and placed on bench for optimal annealing of oligonucleotides.
4. Linearize pX330 with *BbsI* (no need to dephosphorylate vector) and ligate the annealed oligonucleotides (no need to phosphorylate) o/n at 16°C.
5. Transform ligation reaction into suitable bacteria, pick colonies, and prepare DNA for Sanger sequencing to confirm fidelity of cloned crRNA.
6. Meanwhile, PCR clone a ~500bp amplicon around the genomic region to be edited and clone into the *BamHI*/*SalI* sites of the EGxxFP split reporter (addgene, plasmid #50716). This plasmid should then be co-transfected along with each individual pX330 plasmid carrying a sequence-verified sgRNA into HEK293 cells. Be sure to include an empty EGxxFP reporter without a targeted insert as a control. Two days later, assess reconstituted EGFP positivity as a qualitative measure of the DSB cleavage efficiency of each sgRNA (see Supplemental Figure I).
7. Design a forward primer with T7 promoter (5'-TAATACGACTCACTATAGG-3') followed by the crRNA sequence (for a total length of ~39 nucleotides) and combine with the universal reverse primer (corresponding to the 3' end of the tracrRNA in pX330, 5'AAAAGCACCGACTCGGTGCC-3') for PCR amplification of the entire sgRNA in pX330 (use AccuStart II Gel Track PCR Supermix (Cat. #84228)). Perform PCR in all 8 wells of a strip with minimum volume of 20μl/tube to increase PCR product yield.
8. Clean up pooled PCR reactions with appropriate PCR purification kit and aim to achieve at least 500 ng of PCR product (~100 nucleotides) for the in vitro transcription.
9. Perform in vitro transcription on PCR product with the MEGAshortscript T7 Transcription Kit (Thermo Fisher Scientific, Cat. #AM1354) to achieve at least 25-30 μg of in vitro transcribed sgRNA. Reaction time is 4-5 hours, but overnight incubation often yields higher concentrations of sgRNA.
10. Purify in vitro transcribed sgRNA with RNeasy mini kit (part2) or MEGaclear Transcription Clean-up Kit (Thermo Fisher Scientific, Cat. #AM1908) and aim for a concentration of 0.5-1 μg/μl.
11. Assess integrity of sgRNA before injection into mouse zygote with small RNA (6-150 nucleotide) chip of the Agilent 2100 BioAnalyzer. Use ~100ng of sgRNA for this analysis.



BioAnalyzer data of sgRNAs. It is absolutely essential to verify integrity of sgRNA with a BioAnalyzer before submitting to a core for microinjection into a mouse zygote. At left is a typical small RNA gel of two sgRNAs that were processed as above. Bands represent a sgRNA (90-100 nucleotide range, small arrow; note that a sgRNA may often migrate a little faster than its actual size of 100 nucleotides). A dominant higher molecular weight species, likely represents some dimeric structure of the sgRNA (large arrow) and some higher molecular weight species (red circle in sgRNA #1) represent some unknown RNA. The electropherogram traces below illustrate a cleaner sgRNA #2 as compared to the contaminated (red circle) species in sgRNA#1. These samples demonstrate the utility in testing 2 sgRNAs as it is difficult to predict which sgRNA will work best and provide the cleanest final sample preparation for generating genome-edited mice.

Supplemental Method #2: Quick Summary of CRISPR Path to Engineering Genetic Mouse Model

1. Study the genomic region of interest on the UCSC Genome Browser (https://genome.ucsc.edu) and select optimal region(s) to be edited based on ENCODE and other experimental data.
2. Select crRNA(s) either manually or with assistance of any number of search algorithms (see text).
    a. Follow procedures in Supplemental Method 1 for cloning crRNA into plasmid.
    b. Purchase Cas9 mRNA (TriLink BioTechnologies) or Cas9 protein (PNA Bio or New England Biolabs) and, if doing 3c CRISPR, HDR template (single-strand oligonucleotide as PAGE purified Ultramer from Integrated DNA Technologies).
    c. Do not resuspend SSO until just before microinjections.
3. Test activity of sgRNA by transfecting cells with pX330 plasmid carrying Cas9 and sgRNA in HEK-293 cells with an EGxxFP split reporter containing PCR amplified ~500 bp target genomic DNA.
4. Purify sgRNA per Supplemental Method 1 and submit with Cas9 mRNA or protein and, if necessary, HDR template to an academic Transgenic Core for microinjections.
5. Cores vary slightly, but we typically have components mixed just before microinjection at these concentrations: Cas9 mRNA (100 ng/μl), sgRNA(s) (25-50 ng/μl), and single-strand oligonucleotide repair template (100 ng/μl; dissolved and mixed just before injecting). Components are mixed in 10 mM Tris-HCl, pH 7.5 + 0.1 mM EDTA.
6. Components are injected into cytoplasm of C57BL/6J zygotes. Note, pup yields may be higher with hybrid zygotes such as FVB/C57BL/6J, but these mice will need to be back-crossed.
7. Pups may be genotyped as early as day 7 by toe clip or day 20 by ear punch with pre-optimized PCR genotyping assays (see Supplemental Method 3).
8. Putative positive founder DNA is further analyzed by gel purification of band of interest (*e.g.*, shifted band with 3xFLAG or *loxP*, deletion, or precision PCR of 3c CRISPR with discriminating primers, see Supplemental Figures IV, VI-VII); cloned in suitable vector (TOPO), and Sanger sequenced.
9. Same genomic DNA from positive founder should be interrogated for unintended edits based on computer-predicted off-target sites. DNA is amplified with primers flanking putative off-target site, TOPO cloned, and Sanger sequenced. Several individual colonies from TOPO cloning here and in Step 8 should be sequenced since multiple alleles may be present in founders (see Supplemental Figure V and text for more details on mosaicism).
10. Sequence-confirmed founders should be bred for germ line transmission of intended edits. Once transmission of allele occurs in the F_1 generation, founders may be studied if biallelic targeting occurs (*e.g.*, tissue expression of target gene associated with a deleted enhancer).
11. If using genome editing in a hybrid strain, back-cross to the appropriate strain to generate homozygous mice.
12. If off targeting events were noted in the founder mouse, repeat analysis in the F_1 or later generation to determine whether the off target edit was lost during breeding.
13. Test two independent founder-derived F_1 lines to validate phenotypes.

Supplemental Method #3: Pre-Optimization of Multiplex PCR Assay for Genotyping 3c CRISPR Mice

1. Define the subtle edit and test activity of sgRNA according to Supplemental Method #1.
2. Download ~1 kb of genomic sequence with the subtle edit placed in center of sequence.
3. Design primers (with suitable restriction sites) that flank the edited region by ~200-300 nt on each side. Use the PrimerQuest tool under the Integrated DNA Technologies Tool bar; http://www.idtdna.com/site). To save time, we typically order two pairs of primers to test each in case there is PCR failure.
4. PCR clone the genomic fragment into a host plasmid and sequence validate.
5. Perform site-directed mutagenesis (QuikChange kit) on the validated plasmid with oligos that contain the same mutations that will be created in the single-strand oligonucleotide HDR repair template. The goal is to simulate the genomic edit that will exist in the mouse. Sequence validate the mutant plasmid.
6. Design two forward primers that terminate at the edited nucleotides with one (wild type) containing a ~30-35 nt 5' extension of irrelevant sequence to the mouse genome and the other (mutant) with the same core sequence but without the 5' extension and ending with the SNP or multi-base mutations Design reverse primer ~150 bp downstream of the sgRNA (see Supplemental Figure VIIC and below).
7. Create a word document (below) summarizing overall strategy with sgRNAs, PCR primers etc.
8. Spike mouse genomic DNA with about one genomic equivalent of either the wild type or mutant plasmid generated in steps 4-5 and optimize PCR conditions (varying the annealing step first and then cycle number) that will result in the wild type and mutant primers only amplifying their respective wild type and mutant test plasmids. Then try to multiplex with both primers and the common reverse primer in a reaction with equal parts of wild type and mutant test plasmid.
    a. Use AccuStart II Gel Track PCR Supermix (Cat. #84228) for all PCR reactions.
9. Run PCR reactions on at least a 2% agarose gel until the dye front has moved at least ¾ length of a 14 cm gel. We sometimes find it necessary to increase the agarose concentration to 2.5%.
10. Because the forward primers are 30-35 nt different in size, a mutant and wild type allele should be resolved (see image at right and Supplemental Figure VIIC).
11. This approach has been published (reference 127 in the text) and we since have developed similar assays for several new projects, including those with single base pair mutations.