Embryo microinjection with CRISPR-Cas9 in mice and zebrafish

Mouse

To test the efficiency of CRISPR-Cas9–mediated genome editing in mouse models using either Cas9 recombinant protein, or Cas9 mRNA and *in vitro*–transcribed short guide RNA (sgRNA), we perform pronuclear injections and culture the zygotes to the blastocyst stage. We term this the "blastocyst assay". The blastocyst assay makes itself amenable to multiplexing, and allows the testing of several CRISPR/sgRNAs in a single blastocyst. We have tested 6–8 spatially separated CRISPR/sgRNAs successfully in one experiment. We test the efficiency of nuclease activity of each of the sgRNAs, and the sgRNAs showing good nuclease activity are chosen for injections at the same dosage (as in the blastocyst assay) to generate genetically engineered mice.

The premise of the blastocyst assay is that CRISPR-Cas9 nuclease activity generates double-strand breaks (DSBs), and the cell uses the non-homologous end joining (NHEJ) mechanism to repair the DSB. Formation of indels is a common feature of NHEJ, and the presence of indels allows the formation of heteroduplexes. Heteroduplexes can be resolved on polyacrylamide gels due to their mobility shift, in comparison to homoduplexes.

Genomic DNA obtained from lysed blastocysts is used as a PCR template. Amplified products are subjected to denaturation followed by slow renaturation to facilitate heteroduplex formation and separated on a 6% polyacrylamide gel.

Our experiments with both Cas9 mRNA (Invitrogen™ GeneArt™ CRISPR Nuclease mRNA, Cat. No. A29378) and Cas9 protein (Invitrogen™ GeneArt™ Platinum™ Cas9 Nuclease, Cat. No. B25641) showed positive results. The concentrations used in our experiments are listed below (Table 1). We load 0.5–1 µL of the prepared CRISPR/sgRNA stock volume injection solution in each injection needle prepared by using a micropipette puller and microforge injection (Table 1). Pronuclear injections with 1–3 pL of the injection solution per embryo are performed. Injected embryos are transferred to a pseudo-pregnant recipient mouse (Figure 1). Upon obtaining the founder mice (F_0), we perform genotyping assays using their tail genomic DNA.

Table 1. Concentrations of components in the mouse blastocyst assay.

Component	Stock concentration (ng/µL)	Final concentration (ng/µL)	Stock volume (µL)
GeneArt CRISPR Nuclease mRNA	1,000	100	1.0
sgRNA, gene 1	250	50	2.0
Injection buffer ($T_{10}E_{0.1}$)	NA	NA	7.0
Total volume			**10.0**
GeneArt Platinum Cas9 Nuclease	3,000	300	1.0
sgRNA, gene 1	250	112.5	4.5
sgRNA, gene 2	250	112.5	4.5
Total volume			**10.0**

invitrogen

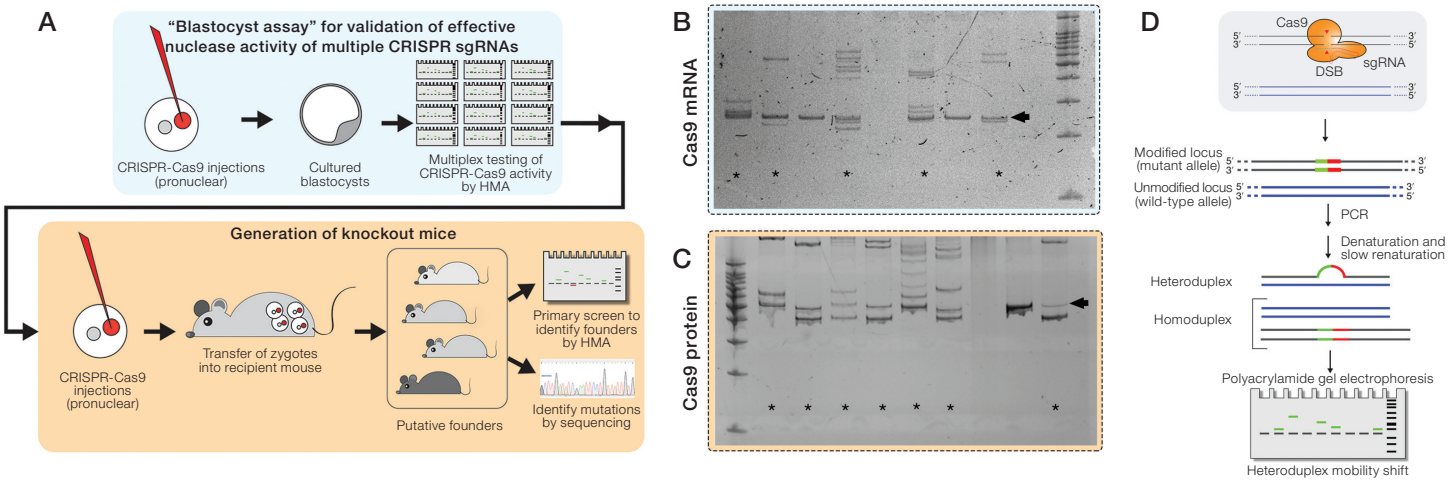

Figure 1. Step-by-step procedure from pronuclear injection to generation of mice. (A) Schematic of the workflow, from injections to blastocyst assay, and then to the generation of mice. **(B)** Heteroduplex mobility assay (HMA) profiles obtained from blastocyst assay (gene 1). **(C)** HMA profiles obtained from potential founder animals (tail DNA, gene 2). **(D)** Schematic illustrating the principle behind HMA. Arrows in B and C indicate wild-type amplicon or band, and asterisks (*) indicate samples with modified alleles (presence of indels).

Zebrafish

To create mutant alleles in zebrafish, we injected the CRISPR/sgRNA stock volume with concentrations given in Table 2. Freshly laid fertilized eggs are collected from breeding tanks and injected with the CRISPR/sgRNA stock volume into the blastomeres using a microinjection setup. A sgRNA targeting the tyrosinase gene involved in melanin biosynthesis resulted in loss of pigmentation in both the body and eyes—showing a range of mosaic phenotypes (Figure 2).

Table 2. Concentrations of components in the zebrafish blastocyst assay.

Component	Stock concentration (ng/µL)	Final concentration (ng/µL)	Stock volume (µL)
Zebrafish tyrosinase sgRNA	1,500	200	0.7
GeneArt Platinum Cas9 Nuclease	3,000	600	1.0
Injection buffer ($T_{10}E_{0.1}$)	NA	NA	3.3
Total			**5.0**

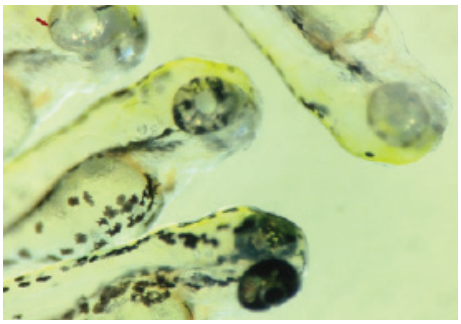

Figure 2. Range of phenotypes observed following injection of CRISPR/sgRNA stock volume in zebrafish blastomeres. Severe (top left), moderate (middle), and mild (bottom) loss of pigmentation in the eyes can be observed in these 2-day–old embryos. Maroon arrows (top left) point to minimal pigmentation in a predominantly unpigmented eye.

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