Mouse Genome Editing Using the CRISPR/Cas System

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ABSTRACT

The availability of techniques to create desired genetic mutations has enabled the laboratory mouse as an extensively used model organism in biomedical research including human genetics. A new addition to this existing technical repertoire is the CRISPR/Cas system. Specifically, this system allows editing of the mouse genome much more quickly than the previously used techniques, and, more importantly, multiple mutations can be created in a single experiment. Here we provide protocols for preparation of CRISPR/Cas reagents and microinjection into one-cell mouse embryos to create knockout or knock-in mouse models. *Curr. Protoc. Hum. Genet*. 00:15.7.1-15.7.27. © 2014 by John Wiley & Sons, Inc.

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INTRODUCTION

Tools and techniques to introduce foreign genes and to mutate endogenous genes in the mouse were first developed in early 1980s, and those methods remained relatively standard for about three decades. In the past 4 to 5 years however, there has been a storm of newer methods and tools added to gene-editing techniques, which have been elegantly used to create genetic mutations. These include designer nucleases such as Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs), as well as the RNA-guided nuclease system called the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR–associated genes (CRISPR/Cas) system. In this unit, we provide recipes and protocols to create specific mutations in the mouse genome using the CRISPR/Cas system, which has moved to the forefront of gene editing due to its simplicity, efficiency, ease, and robustness (Fujii et al., 2013, 2014; Shen et al., 2013, 2014; Wang et al., 2013; Seruggia and Montoliu, 2014; Yang et al., 2013; Zhou et al., 2014).

The CRISPR/Cas system was originally described as an adaptive immune mechanism against invading viruses in bacteria (Barrangou et al., 2007). The system comprises two RNAs and one protein component: a CRISPR RNA (crRNA), a short RNA that undergoes complementary binding with the foreign DNA; a tracrRNA that hybridizes with the crRNA; and a Cas9 enzyme that interacts with the DNA:RNA complexes and cleaves the DNA at a specific site (Horvath and Barrangou, 2010).

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This system has been redesigned for gene-editing purposes (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013b; Terns and Terns, 2014) by combining crRNA and tracrRNA into a chimeric unit called a single guide RNA (sgRNA), and by codon-optimizing the Cas9 enzyme sequence to suit mammalian expression. Hence, the CRISPR/Cas9 system used for gene editing consists primarily of two components: a guide RNA that detects the specific sequence in the genome and the Cas9 enzyme that binds to the sgRNA and cleaves the DNA at the target site. While Cas9 mRNA is a common component, the sgRNA is the unique component for each specific gene-editing experiment. When DNA is cleaved, it mainly gets repaired through a mechanism called Non-Homologous End Joining (NHEJ), which is a highly error-prone mechanism that causes a few base pair *in*sertions or *del*etions (*indels*) at the cut site. Such an event, in most cases, results in a frame-shift mutation of the coding sequence, eventually leading to gene disruption (a knockout). In cases where a specific mutation is to be introduced at the cut site (called knock-in), a repair template DNA is also needed as a third component of the CRISPR/Cas system, which becomes inserted at the cut site through Homology-Directed Repair (HDR). The repair template DNA can be either a single-stranded oligonucleotide or a double-stranded plasmid/linear DNA.

The CRISPR/Cas9 components (sgRNA, Cas9 mRNA with or without a repair DNA) can be introduced into a one-cell-stage mouse embryo to generate offspring that can potentially contain knockout or knock-in mutations. The process involves four major steps: (1) designing CRISPR targets (Basic Protocol 1); (2) synthesis and purification of RNA and DNA components (Basic Protocol 2); (3) isolation of one-cell-stage mouse embryos, microinjection of CRISPR/Cas components, and transfer of injected embryos into pseudopregnant mice (Basic Protocol 3); and (4) genotyping of offspring to identify mutations (Basic Protocol 4). An overview of CRISPR/Cas-mediated mouse genome–editing steps is presented in Figure 15.7.1.

BASIC PROTOCOL 1

DESIGNING CRISPR TARGETS

The first step in gene editing using the CRISPR/Cas system is to find a specific CRISPR target site (or sites) near the genomic region of interest. The CRISPR target sequence constitutes 20 nucleotides followed by an "NGG" sequence called Protospacer Adjacent Motif (PAM).

It should be noted that due to such a short sequence requirement, there are higher risks of off-target cleavage if sequences closely similar to the target sites exist elsewhere in the genome, as recently reported by several laboratories (Fu et al., 2013; Hsu et al., 2013; Mali et al., 2013a; Pattanayak et al., 2013). In other words, the Cas9 enzyme can tolerate a few base mismatches and can still cleave at such sites: in particular, mismatches in the first 7 nucleotides (of the 20) are more tolerated than those from the 8th position onwards (Cong et al., 2013). Currently, there are several online tools available for designing CRISPR targets (i.e., Cong et al., 2013; Montague et al., 2014; Heigwer et al., 2014; *http://crispr.mit.edu/*, *https://chopchop.rc.fas.harvard.edu/index.php*, and *http://www.e-crisp.org/E-CRISP/index.html*, respectively). As an example, the tool available through *http://crispr.mit.edu/*, from Feng Zhang's Laboratory at MIT/BROAD Institute, is described here. The screenshots from a CRISPR target search exercise are shown in Figure 15.7.2.

1. Visit *http://crispr.mit.edu/*.
2. Enter the "name" for your sequence and an e-mail address.
3. Select the species; e.g., "mouse."

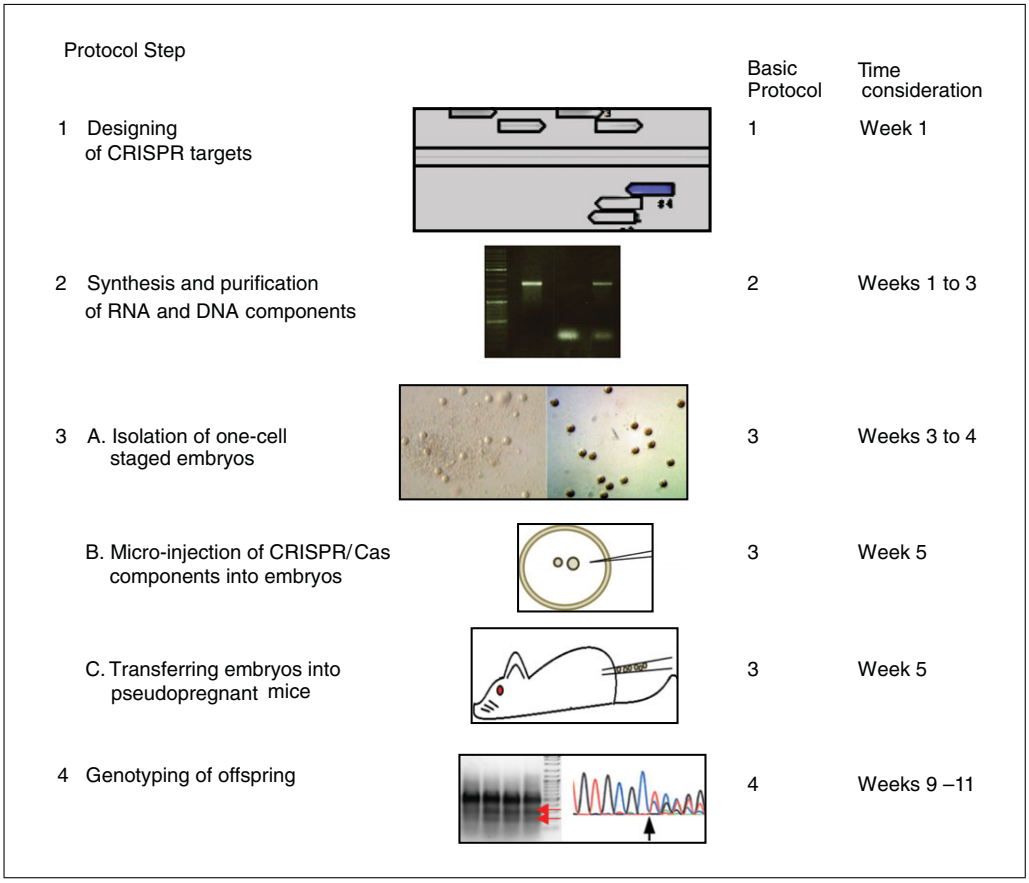

Figure 15.7.1 Overview of CRISPR/Cas-mediated mouse genome-editing steps.

4. Enter the sequence in the box and click "submit." A sequence of up to 250 bases can be entered at once. The job generates two types of clickable output files, (i) guides and off-targets and (ii) nickase analysis:

   i. *Guides and off-targets:* The results present a ranked list of all possible guides in the query sequence ordered by faithfulness of on-target activity computed as 100% minus a weighted sum of off-target hit-scores in the target genome. The higher the guide score, the better the guide sequence.

   ii. Nickase analysis lists pairs of guide sequences that can be used for a double-nicking approach to gene editing (discussed in the Commentary).

5. *Guide Selection:* Guides having an aggregate score of greater than 50% are colored green and should be considered candidate targeting sequences if no high-scoring off-targets fall in marked gene regions (indicated in the table to the right). Guides colored yellow should be considered backups for specific targeting in the case where no suitable green guides are clear of high-scoring, gene off-targets. Guides colored in red have many likely off-target interactions in the target genome and should be avoided.

SYNTHESIS AND PURIFICATION OF RNA AND DNA COMPONENTS

BASIC PROTOCOL 2

The CRISPR/Cas system components include sgRNA and Cas9: the sgRNA guides the Cas9 enzyme to the target site on the genomic DNA and the Cas9 enzyme cleaves the target site, eventually resulting in indel mutations at the cut site. The system also includes a repair DNA as a third component if a knock-in mutation is to be inserted at the cleavage site. The microinjection components used for mouse genome editing using the CRISPR/Cas system are listed in Table 15.7.1. Note that the Cas9 is introduced into the zygotes as mRNA that gets translated into the protein.

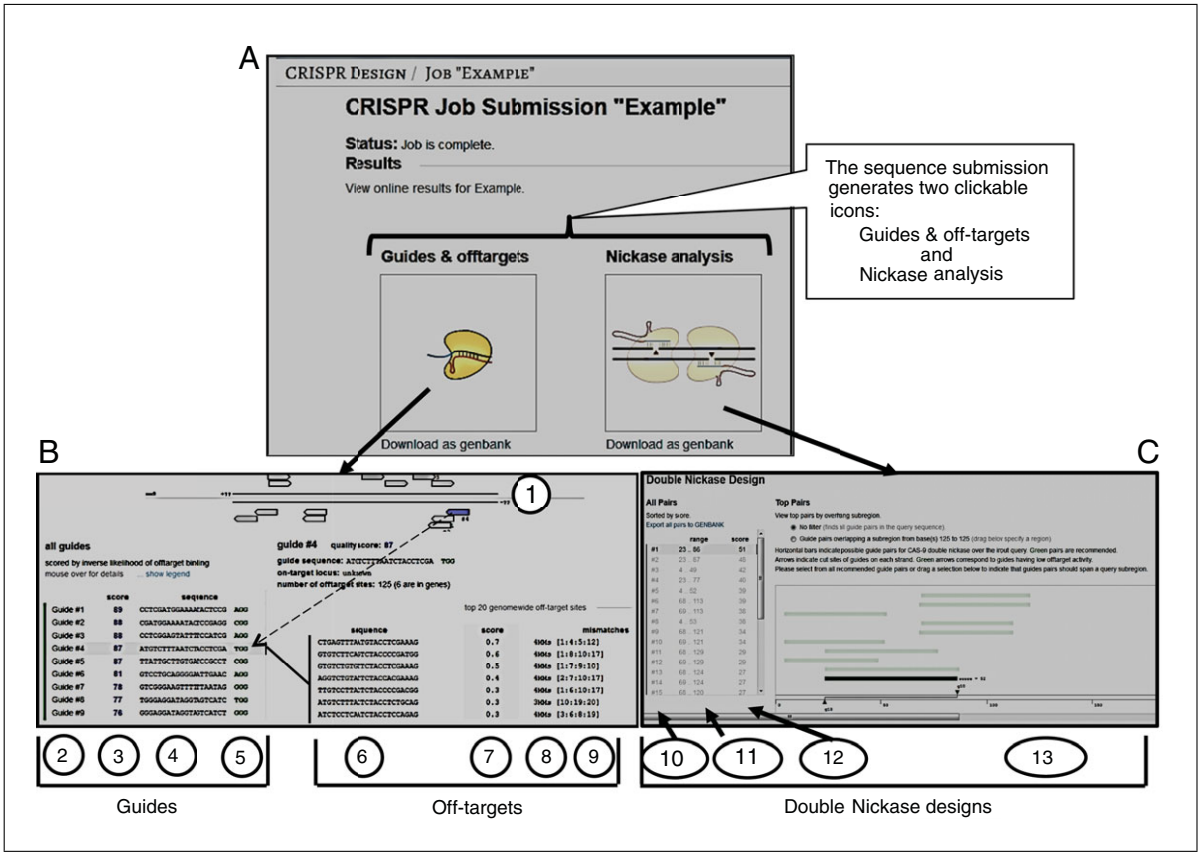

Figure 15.7.2 CRISPR design output files. (**A**) Screenshot of the CRISPR target search results window that shows two clickable icons. (**B**) Screenshot of the "Guides and off-targets window". (1) Graphical display of various targets (the guide with the cursor on it gets highlighted in blue). (2) Serial number of guide sequence. (3) Ranking of the guides displayed from highest to lowest. (4) Guide sequences (20 bases long). (5) PAM sequence. (6) Off-target sequences for a cursor selected guide (guide no. 4 on the left in this example). (7) Ranking of the off-targets displayed from highest to lowest. (8) Number of mismatches. (9) Positions of mismatches. (**C**) Screenshot of double nickase design. (10) Serial number of double nickase pairs. (11) The nucleotide position of pairs with respect to the input sequence. (12) Ranking of double nickase pairs displayed from highest to lowest. (13) Graphical display of double nickase pairs targets (the pair with the cursor on it becomes highlighted in green).

Table 15.7.1 The Microinjection Components Needed for Mouse Genome Editing via CRISPR/Cas System

Component	Molecular nature	Purpose in the CRISPR/Cas system	Method of generation
Cas9 RNA	Capped and polyadenylated mRNA	To produce Cas9 protein	In vitro transcription followed by capping and polyadenylation
sgRNA	Short RNA consisting of CRISPR guide and tracer sequences	Guiding Cas9 to the target sequence	In vitro transcription
Repair DNA	Single-stranded oligonucleotide or double-stranded linear/circular plasmid DNA	Homology-directed repair	Oligonucleotide: commercially synthesized Plasmid: cloning

Materials

sgRNA expression plasmid vectors: e.g., pUC57-sgRNA expression vector (Addgene, plasmid no. 51132; Shen et al., 2014) *or* MLM3636 (Addgene, plasmid no. 43860)

Suitable restriction endonucleasess (REs): e.g., *Bbs*I for pX260 or pX330 and *Bsa*I for digesting pUC57-sgRNA vectors plus REs that cut once downstream of the Cas9 or sgRNA sequences to linearize the plasmids (e.g., pBGK plasmid that

expresses Cas9 can be linearized with *Xba*I and pUC57-sgRNA vector can be linearized with *Dra*I); it is necessary to ensure that the chosen restriction site does not cut the Cas9 or sgRNA sequences.

0.8% TAE or TBE agarose gel (also see *UNIT 2.7*)

Gel-extraction kit (Promega; also available from Qiagen or similar vendors) including elution buffer (EB)

100 μM suitable oligonucleotides containing the target sequence

Quick Ligase and 2× Quick Ligation Buffer (NEB)

T4 polynucleotide kinase (PNK; NEB)

10× T4 ligase buffer (NEB)

Suitable competent bacteria (i.e., DH10B, DH5α)

Dithiothreitol (DTT)

*Esp*3I restriction endonuclease (Thermo Scientific)

TOP 10 electrocompetent cells (Life Technologies)

LB agar plates containing 50 μg/ml ampicillin

Expand Long Template PCR System (Roche)

Plasmid DNAs containing Cas9 sequence, e.g., pBGK-Cas9polyA or hCas9 (Addgene, plasmid no. 41815; Mali et al., 2013b), pX260 (Addgene, plasmid no. 42229) or pX330 (Addgene, plasmid no. 42230; Cong et al., 2013), or any such mammalian codon-optimized Cas9 plasmid that can be used for in vitro transcription

Oligonucleotides:

T7-hCas9-Fw-5′-TAATACGACTCACTATAGGGAGAATGGACAAGAAGTACTCCATTG-3′

hCas9-Rv 5′- CGGTAGGGATCGAACCCTTTCA-3′

T7-sgRNA-Fw 5′-TTAATACGACTCACTATAGGN_{20}-3′ (where N_{20} is the selected CRISPR target, cloned in the sgRNA vector)

sgRNA-Rv 5′- AAAAGCACCGACTCGGTGCC-3′

Expand High Fidelity PCR system (Roche) or similar proof-reading *Taq* polymerase

70% and 100% ethanol

RNase-free H_2O and embryo-tested H_2O (available from most molecular biology suppliers)

3 M sodium acetate, pH 5.2 (*APPENDIX 2D*)

mMESSAGE mMACHINE T7 ULTRA kit (Ambion, cat. no. AM1345)

MegaClear kit (Ambion, cat. no. AM1908)

RNase-free microinjection buffer: 1 mM Tris·Cl, pH 7.5/0.1 mM EDTA prepared with RNase-free H_2O

Thermal cycler

50°, 65°, and 95°C water baths or heat block(s)

Nanodrop spectrophotometer for determination of DNA and RNA concentration

NucAway Spin Columns (Life Technologies; optional)

Ultrafree-MC VV Centrifugal Filter (Millipore, cat. no. UFC30VV25)

Additional reagents and equipment for agarose gel electrophoresis (*UNIT 2.7*), transformation of *E. coli* (Seidman et al., 1997), and spectrophotometric determination of nucleic acids including use of Nanodrop (*APPENDIX 3D*), and loading microinjection mix on injection needles (Basic Protocol 3)

Preparation of Template DNAs for RNA Transcription

A linearized plasmid or a PCR product can be used as a template for synthesis of Cas9 mRNA and sgRNAs. Due to their convenient short size, sgRNAs can also be generated by annealing of commercially synthesized oligonucleotides.

Table 15.7.2 Examples of Plasmid Vectors Available for Synthesis of Cas9 mRNA and for Generating sgRNA Expression Constructs

Plasmid	Promoter	Purpose	Transcription template generation	Reference
pCAG-T3-hCAS-pA	T3	Cas9 mRNA transcription	Linearized with *Sph*I	Fujii et al. (2013)
	T3	sgRNA transcription	Linearized with *Dra*I	Fujii et al. (2013)
pX330	T7 or T3 in primer	Cas9 and sgRNA transcription	Used as a PCR template to amplify Cas9 and sgRNA	Yang et al. (2013)
pBGK	T7	Cas9 mRNA transcription	Linearized with *Xba*I	This unit
pST1374-NLS-flag-linker-Cas9	T7 or T3 in primer	Cas9 mRNA transcription	Used as a PCR template to amplify Cas9 and sgRNA	Shen et al. (2013)
pUC57-sgRNA	T7	sgRNA transcription	Linearized with *Dra*I	Shen et al. (2014)

While certain Cas9 plasmids can be used for RNA synthesis without further alterations, sgRNA target sequences need to be cloned into empty vectors before using them in in vitro transcription reactions. Two alternative sets of steps (steps 1b to 6b and steps 1c to 6c) for cloning sgRNA target sequences into plasmid are given below.

Cloning sgRNA target sequences into a plasmid (template DNA) vector

Currently several CRISPR/Cas system plasmids are available through Addgene.org into which sgRNA targets can be cloned to use as template DNAs. Of these, the plasmids that can be used directly, or after PCR-amplifying the insert, for generating RNA molecules needed for mouse gene-editing experiments are compiled in Table 15.7.2. Some of these vectors allow expression of both Cas9 mRNA and sgRNA in a single plasmid (typically used for gene editing in cultured cells but can also be used for embryo work; Mashiko et al., 2013), whereas the ones that are suitable for mouse embryo injections allow synthesis of Cas9 mRNA and sgRNA separately through in vitro transcription. The sgRNA target sequences can be synthesized as DNA oligonucleotides and are cloned into a plasmid vector following standard protocols.

Cloning sgRNA target sequences into a plasmid (template DNA) vector by standard cloning procedures

1a. Digest 1 μg of sgRNA expression plasmid vector with a suitable restriction endonuclease (e.g., *Bbs*I for pX260 or pX330 and *Bsa*I for pUC57-sgRNA vectors) for 30 min at 37°C.

2a. Gel purify digested vector using Promega Gel Extraction Kit and elute in EB.

3a. *Phosphorylate and anneal each pair of oligonucleotides:* Mix 1 μl each of 100 μM primers, add 1 μl 10× T4 ligase buffer (NEB), 6.5 μl water, and 0.5 μl T4 PNK. Anneal in a thermal cycler using the following parameters: 37°C for 30 min, 95°C for 5 min and then ramp down to 25°C at 5°C/min

The phosphorylation step can be optional. It can be omitted by not adding PNK and skipping the 37°C, 30-min incubation.

4a. Set up ligation reaction and incubate at room temperature for 10 min:

X μl digested vector from step 2a (50 ng)
1 μl 1:250 dilution of phosphorylated and annealed oligonucleotide duplex from step 3a
5 μl 2× Quick Ligation Buffer (NEB)
X μl distilled deionized H_2O
10 μl subtotal

1 μl Quick Ligase (NEB)
11 μl total.

5a. *Optional, but highly recommended:* Treat ligation reaction with Plasmid-Safe exonuclease to prevent unwanted recombination products (15 μl total volume):

11 μl ligation reaction from step 4a
1.5 μl 10× PlasmidSafe buffer
1.5 μl 10mM ATP
1 μl Plasmid-Safe exonuclease.

Incubate reaction at 37°C for 30 min.

6a. Transform 1 to 2 μl of the final product into competent cells using standard procedures (Seidman et al., 1997), pick colonies, and verify sequence.

Cloning sgRNA sequences into a plasmid vector by Golden Gate Cloning method

The sgRNA target sequences can also be cloned into a plasmid vector by the Golden Gate Cloning method, which bypasses the need to purify linearized vector (Engler et al., 2008; Engler and Marillonnet, 2014). A diagram illustrating the Golden Gate Cloning to build sgRNA expression vectors is shown in Figure 15.7.3.

1b. Design two oligonucleotides carrying the N_{20} nucleotides (identified as indicated in Basic Protocol 1) flanked by *Esp*3I- compatible overhangs as follows: Fw oligonucleotide ACACC-N_{20}-G and Rv oligonucleotide AAAAC-(RC)N_{20}-G.

2b. Prepare 100 μl of a solution of the two oligonucleotides, each at a final concentration of 10 μM, in distilled, deionized water.

3b. Heat the oligonucleotides at 95°C for 5 min, then let cool down 10 min at room temperature.

4b. Set up Golden Gate Cloning reaction:

200 ng circular MLM3636 plasmid
2 μl of the annealed oligonucleotide mix (from step 3b)
2 μl 10× T4 DNA ligase buffer
1 μl 20 mM DTT
1 μl T4 DNA ligase
1 μl *Esp*3I
H_2O up to 20 μl.

5b. Incubate in a thermal cycler with the following cycling program:

5 cycles:	5 min	37°C
	10 min	16°C.

6b. Transform 1 to 2 μl of the final product by electroporation into TOP 10 electrocompetent cells and plate transformants on LB agar plates supplemented with 50 μg/ml ampicillin. Pick colonies and sequence verify.

Generation of PCR-based templates for in vitro transcription of Cas9 and sgRNA

Because of its short size, sgRNA can also be synthesized directly from a double-stranded DNA template obtained by annealing two oligonucleotides that contain T7 promoter sequence on the 5′ end of the sgRNA target sequence, and, therefore, cloning sgRNA sequences into a plasmid vector is not absolutely necessary. Template for Cas9 mRNA

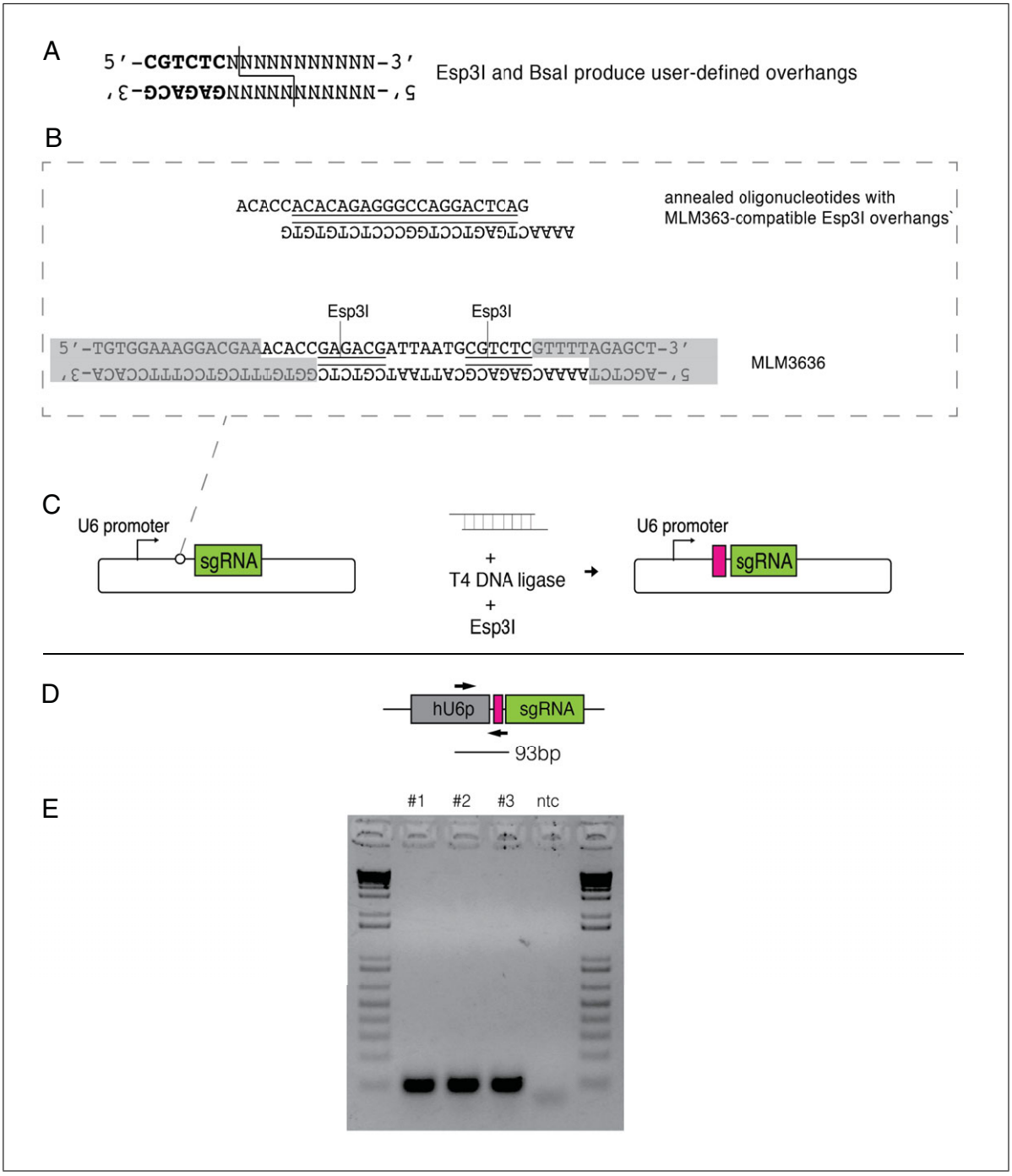

Figure 15.7.3 Diagram illustrating the Golden Gate Cloning to build sgRNA expression vectors. (**A**) Type IIs restriction enzymes are used to produce user-defined overhangs. (**B**) Forward and reverse oligonucleotides corresponding to the CRISPR target site 5′N(20) are designed adding *Esp*3I overhangs compatible with those included in the MLM3636 vector. (**C**) A one-pot digestion-ligation reaction is performed with circular vector and annealed oligonucleotides to obtain the desired sgRNA vector. (**D**) Colony PCR of MLM3636-based sgRNA vectors. A forward primer mapping on the hU6 promoter sequence and the sgRNA reverse oligonucleotide (previously used for the annealing reaction) are used for colony PCR. (**E**) Correctly assembled plasmids yield a 93-bp product: three positive colonies and a negative colony (ntc) are shown.

transcription can also be synthesized by PCR with a T7 promoter-containing primer, and using any codon-optimized Cas9 plasmid (Ran et al., 2013). A schematic of PCR template generation for in vitro transcription of sgRNA and Cas9 mRNA is shown in Figures 15.7.4A and 15.7.4C, and the gel images of transcribed and purified RNA are shown in Figure 15.7.4B-C.

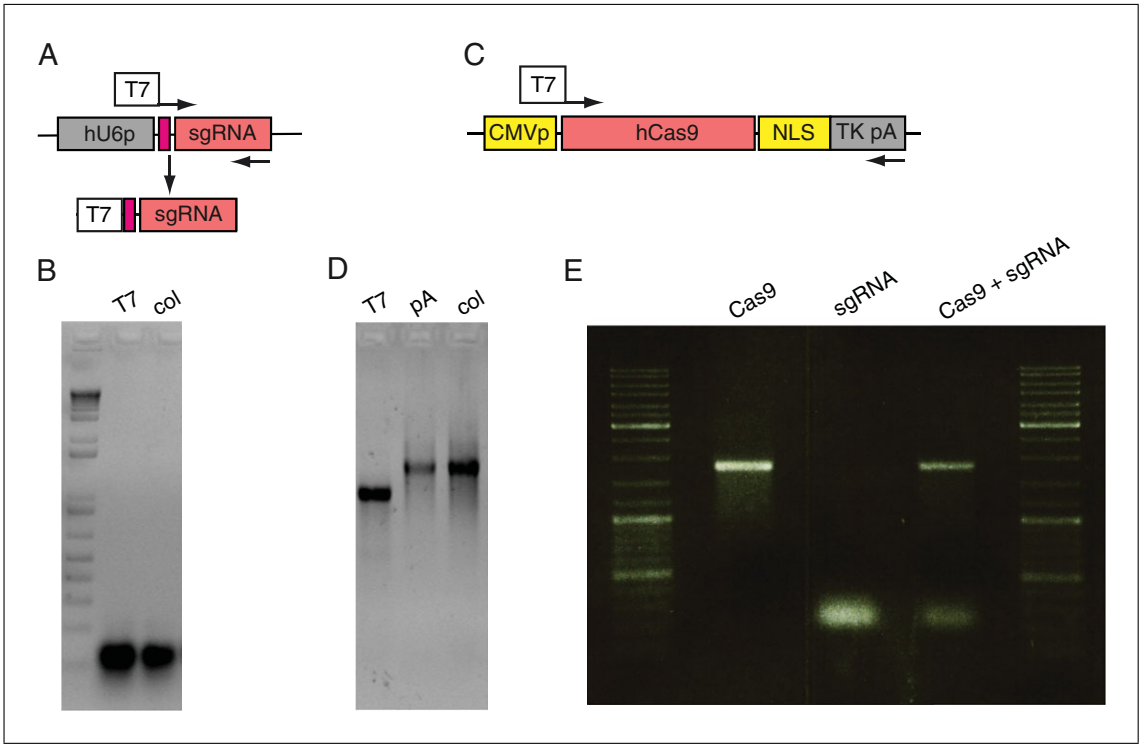

Figure 15.7.4 Representative agarose gel images of CRISPR/Cas RNA components. *(***A**) and (**B**) depict sgRNA in vitro transcription. (A) The sgRNA sequence is PCR-amplified from MLM3636-based vector with primers carrying the T7 RNA polymerase promoter on the 5′ extremity. (B) The resulting PCR is used as a template for T7 RNA polymerase transcription in vitro. A typical gel electrophoresis of an sgRNA after T7 RNA polymerase transcription (T7) and after column purification with NucAway spin columns (Col). (**C**) and (**D**) depict Cas9 in vitro transcription. (C) The Cas9 ORF, including NLS, is PCR-amplified with primers carrying the T7 RNA polymerase promoter on the 5′ extremity. (D) The PCR product is used as a template for in vitro transcription, 5′ capping, and 3′ poly(A) tailing. A representative gel electrophoresis is shown. A supershift is observed after the poly(A) tailing reaction (pA). NucAway spin columns are used for RNA purification. (**E**) A representative gel image of Cas9 mRNA (100 ng), sgRNA (100 ng), and 1:1 microinjection mix of Cas9 and sgRNA (50 ng each) samples.

1c. Use the Expand Long Template PCR System to amplify the Cas9 coding sequence with the oligonucleotides T7-hCas9-Fw and hCas9-Rv using 10 ng of the hCas9 plasmid as template, with the following cycling program:

1 cycle:	5 min	94°C	(initial denaturation)
30 cycles:	30 sec	94°C	(denaturation)
	30 sec	50°C	(annealing)
	4 min	72°C	(extension)
1 cycle:	7 min	72°C	(hold).

Use the Expand High Fidelity PCR System to amplify the sgRNA sequence with the oligonucleotides T7-sgRNA-Fw and sgRNA-Rv using 10 ng of the sgRNA vector as template with the following cycling program:

1 cycle:	5 min	94°C	(initial denaturation)
30 cycles:	30 sec	94°C	(denaturation)
	30 sec	50°C	(annealing)
	30 sec	72°C	(extension).

2c. Precipitate the PCR product with 300 µl of ice-cold 100% ethanol. Incubate 1 hr at −20°C.

3c. Microcentrifuge 15 min at 16,000 × *g*, 4°C. Remove supernatant.

4c. Wash with 300 μl of ice-cold 70% ethanol. Microcentrifuge 5 min at 16,000 × *g* at 4°C.

5c. Remove the supernatant, air-dry DNA pellet 10 min, and resuspend in 50 μl RNase-free water. Estimate the DNA concentration using a Nanodrop microspectrophotometer (*APPENDIX 3D*).

6c. Use 500 ng of purified PCR product for in vitro transcription as in step 12.

Preparation of RNA Components from Template DNAs

Regardless of the format, the RNA synthesis process starts by generating a linear DNA. If a plasmid DNA (steps 6a and 6b) is used, the DNA is linearized using a restriction enzyme situated immediately downstream of the Cas9 or sgRNA sequence, whereas the PCR products generated in step 6c can be directly used as templates for RNA synthesis (step 12).

Linearize plasmid template DNAs for in vitro transcription of Cas9 and sgRNA

7. Prepare a midiprep or maxiprep of plasmid DNA from transformed bacteria prepared at step 6a or 6b. Linearize 10 μg of plasmid DNA with an appropriate restriction enzyme.

   Optional: Separate the plasmid digest on a 0.8% agarose gel (UNIT 2.7) and gel purify the band of linearized plasmid using a gel extraction kit.

8. Precipitate the eluted DNA by adding 0.1 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol, and incubate for 30 min to 1 hr at room temperature.

9. Pellet the DNA by microcentrifugation for 10 min at 13,000 rpm, room temperature. Wash the pellet with 250 μl of 70% ethanol, microcentrifuge again as before, and air dry the pellet for 5 min.

10. Resuspend the DNA in 10 μl RNase-free water and incubate for 15 min at 37°C.

11. Estimate the DNA concentration by spectrophotometric measurement (*APPENDIX 3D*) and confirm DNA purity and integrity by agarose gel electrophoresis (*UNIT 2.7*).

    A total of ~1 μg DNA is required in the next step, in a volume <6 μl. Therefore, a minimum DNA concentration of 166 ng/μl is required for the in vitro transcription reaction).

In vitro transcription

12. Set up a 20-μl in vitro transcription reaction in a 1.5-ml microcentrifuge tube using the reagents from the mMESSAGE mMACHINE T7 ULTRA kit as follows:

    10 μl T7 2× NTP/ARCA reagent from the kit
    2 μl 10× buffer from the kit
    1 μg linearized template DNA (from step 5)
    Make up to 20 μl with nuclease-free H_2O
    2 μl T7 Enzyme Mix from the kit.

    Mix the reaction and incubate for 2 hr at 37°C.

Polyadenylation reaction

This step is not necessary for sgRNAs and for Cas9 transcribed from plasmids that contain multiple 'A' nucleotides after the coding sequence (e.g, pBGK that has 83 bases of 'A' nucleotides after the Cas9 coding sequence).

13. To each 20 μl-transcription reaction, add the following reagents from the mMESSAGE mMACHINE T7 ULTRA kit as follows:

    36 μl nuclease-free H_2O
    20 μl 5× E-PAP buffer from the kit
    10 μl 25 mM $MnCl_2$ from the kit
    10 mM ATP from the kit.

14. At this step (before adding the E-PAP enzyme), mix and take 2.5 μl as quality-control sample C1.

15. Add 4 μl E-PAP enzyme from the mMESSAGE mMACHINE T7 ULTRA kit to the step 13 reaction mix.

16. Mix and incubate the reaction for 1 hr at 37°C.

Purify RNA

RNA purification using MegaClear kit

Alternatively, follow steps 17b to 21b to use NucAway column purification in place of the MegaClear kit.

17a. To each 100-μl polyadenylation reaction add reagents from the MegaClear kit as follows:

    350 μl binding buffer from kit
    250 μl ethanol (200 proof ACS grade).

    Mix and apply to spin column inserted into a collection tube (also items from MegaClear kit).

18a. Microcentrifuge spin column for 1 min at 16,000 × *g*, room temperature. Discard flowthrough and add 500 μl wash solution from the MegaClear kit.

19a. Repeat step 18a.

20a. Microcentrifuge the column for 30 sec at 16,000 × *g*, room temperature.

21a. Transfer column to the top of a new collection tube, apply 50 μl elution solution (from MegaClear kit), close lid, and incubate 5 min at 65°C.

22a. Microcentrifuge 1 min at 16,000 × *g*, room temperature, apply another 50 μl elution solution, and incubate 5 min at 65°C.

23a. Collect the second eluate by microcentrifugation under the conditions described in step 22a, and pool both eluates.

24a. To 100 μl of the eluate, add 10 μl 5 M ammonium acetate (from kit) and 275 μl 100% ethanol. Incubate 30 min at −20°C.

25a. Microcentrifuge 15 min at 16,000 × *g*, room temperature, discard supernatant, add 500 μl of 70% ethanol (prepared with embryo-tested water).

26a. Microcentrifuge 1 min at 16,000 × *g*, room temperature, repeat the washing step with 70% ethanol, discard supernatant, and air dry the pellet for 5 min.

27a. Resuspend the RNA pellet in 50 μl microinjection buffer (prepared with RNase-free water), then incubate 5 min at 37°C.

28a. Estimate RNA concentration in a Nanodrop spectrophotometer (*APPENDIX 3D*). Distribute RNA into small aliquots (~5 μl) and store at −80°C until required for the preparation of microinjection mix.

Typical yield from one in vitro transcription reaction will be ~30 μg RNA.

RNA purification using NucAway columns

As an alternative to the MegaClear kit, RNA can be purified using column-based gel chromatography. This protocol uses NucAway Spin Columns.

17b. Dissolve the powder contained in the NucAway column in 650 μl of RNase-free microinjection buffer, carefully removing all air bubbles. Cap the tube and hydrate for 5 to 15 min at room temperature.

18b. Remove the cap at the bottom and place the column in a collection tube. Microcentrifuge for 2 min at 750 × *g*, room temperature.

19b. Place the column in a fresh 1.5-ml tube and apply the RNA solution dropwise to the center of the gel bed, without touching the column wall.

20b. Spin the column for 2 sec at 750 × *g*, room temperature, placing the tube in the same orientation as in the previous centrifugation step.

21b. Discard the column and distribute the purified RNA in small aliquots. Take a small aliquot of sample for gel electrophoresis and measure the RNA concentration using a Nanodrop device (*APPENDIX 3D*). Store RNA at −80°C until use.

Typical yield of one reaction is 30 to 50 μg of RNA.

RNA used for mouse embryo injection is best assessed using gel formulations especially suited for RNA electrophoresis, as described in Wefers et al. (2013). However, RNA quality can also be assessed using the TAE gels routinely used for DNA electrophoresis (see, e.g., UNIT 7.4 and APPENDIX 3F).

Representative gel images of RNA components used in a CRISPR/Cas microinjection mix are shown in Figure 15.7.4B-E.

Preparation of repair DNAs

There are two types of repair DNAs; single-stranded DNA with homology arms about 60 bases long for insertion of short sequences (e.g., short immunoaffinity tags, *loxP* sites) or double-stranded plasmid DNA with longer homology arms of over 0.5 kb (e.g., reporter cassettes such as GFP or *lacZ* or minigenes). Short, single-stranded DNAs can be procured as oligonucleotides (primers from any commercial source such as IDT, Operon, etc.). Preparation of larger, double-stranded targeting DNA, on the other hand, involves careful designing strategies and complex construction steps that are described elsewhere (LePage and Conlon, 2006; Hall et al., 2009). The primers used as repair oligonucleotides (commercially synthesized) are preferably reconstituted on the day of the first injection session and frozen as multiple aliquots at −80°C for later injection sessions (if necessary). The plasmid DNAs used as repair DNA are prepared using high-quality plasmid DNA isolation kits (from Promega or Qiagen or any such vendors).

Preparation of microinjection mix

The typical volume of microinjection mix prepared will be 150 to 300 μl.

29. Remove RNA aliquots from −80°C and thaw on ice.

Optionally include the sgRNA, Cas9 RNA, and repair DNAs generated above.

30. Mix desired concentrations of Cas9 RNA, sgRNA, and optional DNAs and prepare for microinjection as described below.

We prefer to make pre-dilutions of each of the components and mix them to obtain the final solution. For example, if only one sgRNA and Cas9 are injected, each at 10 ng/μl concentration, the pre-dilutions of 20 ng/μl sgRNA and 20 ng/μl Cas9 mRNA are made,

and then equal volumes of these solutions are mixed to obtain the microinjection mix. If three components (e.g., two sgRNAs and a Cas9 mRNA or one sgRNA, Cas9 mRNA, and a repair DNA) need to be injected, each of the three components are made as 3× pre-dilutions, and then a third of each of these solutions are mixed to obtain the final solution that will be 1× with respect to each component.

31. Centrifuge 10 min at 16,000 × *g*, 4°C, then pass through Millipore centrifugal filters according to the manufacturer's instructions.

Alternatively, the microinjection mixes can be centrifuged at 30,000 × g for 1 hr, in which case the additional filtration may not be necessary. Spinning and/or filtering are required to eliminate any remaining solid particles, to prevent clogging of the microinjection needle.

The microinjection mix is then loaded on to the needles as described in Basic Protocol 3. The injection needles are preferably kept in a container on ice until they are assembled on to the microinjection setup.

ISOLATION OF ONE-CELL-STAGE MOUSE EMBRYOS, MICROINJECTION OF CRISPR/Cas COMPONENTS, AND TRANSFER OF INJECTED EMBRYOS INTO PSEUDOPREGNANT MICE

BASIC PROTOCOL 3

In a traditional transgenic experiment, typically about 200 to 300 fertilized oocytes are microinjected with DNA, whereas, due to the relatively high efficiency of the CRISPR/Cas9 system, approximately 50 to 100 fertilized oocytes are sufficient to generate the desired mutations. This number of embryos can be obtained by superovulating 6 to 10 females for each microinjection session. We use FVB inbred or B6/SJL F1 hybrid strains embryos for CRISPR/Cas9 experiments. These animals can be purchased from commercial sources or can be bred in-house. All procedures involving laboratory animals should be performed according to institutional and national guidelines and legislation (see Internet Resources).

It should be noted that the steps to generate mutant mice using the CRISPR/Cas system follow the standard transgenic techniques—comprising multiple steps including embryo production, isolation, and microinjection, as well as transfer of manipulated embryos into recipient females—that have been well established for over three decades. We suggest that the reader refer to the exhaustive resources that are available (Pease and Saunders, 2011; Behringer et al., 2014) describing the critical parameters and troubleshooting involved in generating transgenic mouse models, which are very similar to the steps for generating mutant mice using the CRISPR/Cas system.

Materials

Mice:

Donor females: in our laboratory, we procure 3-week-old B6SJL/J F1 females from The Jackson Laboratory or 4-week-old FVB female from Charles River Laboratories

Stud males: purchased from the respective vendors at 5 to 6 weeks of age

Pseudopregnant recipients: Crl: CD1(ICR) female mice purchased at 5 to 6 weeks of age from Charles River Laboratories, for pseudo-pregnant foster mother (pseudopregnant mice are obtained by mating 5 to 12 week old CD1 females to vasectomized CD-1 males on the day before microinjection; on the morning of the injection day, plug-positive females are used for oviduct transfers; typically 10 to 20 CD-1 females are bred in each session to obtain an average of 4 to 8 plugged females, and about 15 to 25 injected embryos are transferred per female)

Vasectomized males: 5 to 6 week-old CD-1 mice purchased from, Charles River Laboratories; vasectomies performed as described in Behringer et al. (2014)

Hormones: pregnant mares serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG) were obtained from the National Hormone and Peptide Program (Harbor–UCLA Medical Center, Torrance, Calif.)
M2 medium for embryo handling and microinjection (Millipore, cat. no. MR-015-D)
Hyaluronidase for dissociation of cumulus oophorus complex (Millipore, cat. no. MR-051-F)
KSOM + AA for embryo incubation (Millipore, cat. no. MR-106-D)
Microinjection mix (Basic Protocol 2)
Light mineral oil (Millipore, cat. no. ES-005-C)

Individually Ventilated Cages (IVCs; Allentown, Lab Products, or Tecniplast)
Falcon tissue culture dish, 35 × 10 mm (Corning, cat. no. 353001)
Falcon tissue culture dish, 60 × 15 mm (Corning, cat. no. 353002)
Falcon IVF dish (Corning, cat. no. 353653)
Slide warmer
Dissecting instruments including fine forceps
Flexipet oocyte/embryo pipets (Cook Medical, cat. no. K-FPIP-1130-10BS-5)
Model P-97 pipet puller (Sutter Instrument Co.) outfitted with 2.5 mm × 2.5 mm box filament (FB255B)
Glass: (World Precision Instrument Inc., cat. no. TW100F-4) with filament 1.0 mm, 4 in.
MicroFil, 28-G/97 mm long (World Precision Instrument Inc., cat. no. MF28G)
1-cc tuberculin syringes
Nikon Eclipse TE 2000-E with DIC, equipped with Narishige IM 300 microinjector and NT-88-V3 micromanipulators
Condenser lens: LWD 0.52
Objectives:
PLAN 4×/0.10 WD30
PLAN APO 10×/0.45 WD4.0
PLAN FLUOR ELWD 20×/0.45 DIC L/NI
PLAN FLUOR ELWD 40×/0.60 DIC M/NI
Chamber slide (Lab-Tek, cat. no. 177372)
Heated glass insert for chamber slide (Live Cell Instrument, cat. no. HG-T-Z002) with temperature controller (Live Cell Instrument, cat. no. CU-301)
Holding pipets (Humagen, cat. no. MPH-SM-20)

Additional reagents and equipment for transfer of embryos into pseudopregnant mice (Behringer et al., 2014)

Superovulation and collection of fertilized eggs

1. House mice in Individually Ventilated Cages (IVCs) on a 14-10 light cycle (on at 06:00, off at 19:00).
2. Inject donor female mice with 5.0 IU PMSG around noon on Day 1.
3. Approximately 48 hr post-PMSG injection, inject female mice with 5.0 U hCG on Day 3 and breed with stud males overnight.
4. On day 4 morning, prepare the following dishes:
   a. Oviduct collection dish: 60-mm Falcon dish (cat. no. 353002) with 2 ml M2 medium; one dish per up to 10 females.
   b. Hyaluronidase dish: 35-mm Falcon dish (cat. no. 353001) with 1.5 ml Millipore hyaluronidase; one dish per up to 10 females.

c. Wash dish: 35-mm Falcon dish (cat. no. 353001) with 1.5 ml M2 medium; two dishes per session.
d. KSOM rinse dish: 35-mm Falcon dish (cat. no. 353001) with 1.5 ml Millipore KSOM (pre-equilibrated).
e. Incubation dish: Falcon IVF dish (cat. no. 353653) with 1.0 ml Millipore KSOM (pre-equilibrated); two per injection session.
f. ET dish: 35-mm Falcon dish (cat. no. 353001) with 1.5 ml M2 medium.

All KSOM dishes are prepared approximately 30 min prior to use. The dishes are not overlayed with oil, to shorten equilibration time and minimize the chance of oil cross-contamination. KSOM medium pH changes rapidly outside the incubator.

5. Euthanize donor females using an institutionally approved method approximately 20 hr post HCG (about 08:00 on day 4).

6. Dissect out the oviducts and place in a Falcon (cat. no. 353001) culture dish containing M2. Maintain tissue samples at 37°C on heated slide warmer. Make sure all females are processed in less than 10 min post euthanasia.

Alternatively, females can be processed in batches to finish collection in less than 10 min.

7. Once all oviducts have been collected, move to a new clean area and begin dissociating cumulus-oocyte complexes (COC) as in the following steps.

The following steps are performed under a stereomicroscope maintained at 37°C .

8. Place oviducts, one at a time, in the hyaluronidase dish.

9. Dissect out the cumulus complex by disruption of the ampulla with a pair of fine forceps.

10. Continue processing the remaining oviducts, working quickly. If all samples cannot be processed in <10 min, work with smaller sample sets.

Once the last COCs have been expelled from the ampulla, the first set of oocytes should have been dissociated enough to pick and collect individual oocytes from the dish.

11. Using a 130-μm flexipet pipet, transfer the oocytes to the M2 wash dish.

This will inactivate the residual hyaluronidase. Transfer as few hyaluronidase/cumulus cells as possible during this process.

12. *Optional:* Repeat the washing step to remove residual cumulus cells and hyaluronidase.

13. Pool all collected zygotes in a fresh M2 wash dish. Collect zygotes one by one with the flexipet. Count the number of zygotes and unfertilized oocytes. Record this information for fertilization efficiency.

14. Transfer only healthy looking zygotes into the KSOM wash dish; this will dilute out any residual M2 present. Wash all the embryos in this manner and then transfer them into the incubation dish until needed (typically 30 min to 1 hr). Culture the dish at a CO_2 concentration to maintain a pH range of 7.23 to 7.42.

Prepare microinjection needles

15. Prepare injection capillaries freshly on the morning of injection using a Sutter Model P-97 pipet puller outfitted with a 2.5-mm × 2.5 mm box filament (FB255B) using the following program:

Glass: # TW100F-4
Heat: Ramp +5

Pull: 70
Velocity: 120
Pressure: 200
Time: 100 delay.

Sterile technique is important because you will be injecting RNA molecules.

Embryo microinjection

16. Back-fill injection needles with 1 to 2 μl of microinjection mix (from Basic Protocol 2; containing RNA and DNA components) using a 28-G MicroFil connected to a 1-cc tuberculin syringe.

    The MicroFil is prewashed three times with sterile injection buffer.

17. Affix the injection needle in the needle holder.

    Remaining needles should be stored prefilled on ice as an additional precaution to prevent RNA degradation during this step. A needle storage unit is made from a Falcon 351058 culture dish outfitted with a 1-cm rod-shaped piece of plasticine. The injection needles filled with the microinjection solution are stuck to the plasticine and the entire storage unit is placed directly in contact with an ice bath.

18. Program the following parameters into the Narishige IM 300 microinjector:

    Injection pressure: 20 psi
    Balance: 2.2 psi
    Hold: 14 psi
    Clear: 0.20 sec
    Clear Hold: 0.30 sec
    Injection time: 0.08 sec.

19. Prepare the microinjection chamber using a Lab-Tek chamber slide by making two side-by-side 150-μl drops of M2 medium. Flatten these drops (spread out circularly) with a pipet tip to minimize their height and allow for mineral oil overlay. Overlay the flattened drops with ~1 ml of mineral oil.

20. Transfer 20 to 50 zygotes to one of the drops in the microinjection chamber (number depends on skills of injector; inject all zygotes within 10 min). Maintain the injection slide at 37°C with the heated glass insert.

21. Check morphology of zygotes under the microscope (presence of zona pellucida, pronuclei, and both polar bodies). Discard embryos with more than two pronuclei.

22. Prior to injection, make sure the needle is open by placing the injection needle next to an embryo. Press the clear button on the injector. If the embryo rotates freely, the needle is free of any obstruction. If the embryo does not move, gently break the tip of the injection needle off against the holding pipet in a scraping action. Check the needle again for flow rate. Discard if you observe too much embryo movement.

    With experience, the proper i.d. of the needle can be ascertained by the rate of flow and movement of the embryo in this manner.

23. Using the holding pipet, place first zygote in position and fix by applying negative pressure.

24. Perform microinjection:

    For best results, CRISPR/Cas microinjection mixes can be injected into cytoplasm (Horii et al., 2014), if homology-directed repair DNA is not included in the mix. If the mix contains both RNA and the repair DNA, it is best to inject both into cytoplasm and pronucleus.

a. *Cytoplasmic and pronuclear injection:* Align the embryo and the holding needle so that both the opening of the needle and the pronucleus of the embryo are both in focus. If not, continue rotating/aligning the embryo. This will ensure that the pronucleus is not askew from the center of the holding needle. Otherwise, this could cause the embryo to slightly rotate during injection, resulting in missing the pronucleus.
b. Using the injection needle, penetrate the zona pellucida. Move forward into the closest pronucleus. Positive pressure is maintained at all times on the injection needle. Depending on ID of the needle, a slight swelling of the pronucleus (PN) can be seen once the plasma membrane is penetrated. Otherwise, press the injection foot pedal to observe a slight swelling of the PN.
c. Retract the tip of the needle to the cytoplasm and inject another volume of microinjection mix into the cytoplasm. Carefully withdraw the capillary from the zygote. Often, enough positive pressure is present to allow for simultaneous injection of the cytoplasm during needle removal from the pronucleus.
d. *For the injection of Cas9 mRNA and sgRNA without targeting DNA:* Perform the same injection steps, except avoid injection of the mix directly into PN. Penetrate the zona pellucida. Inject directly into the cytoplasm

With DIC and 300× magnification, one can visualize displacement of the granular structures in the cytoplasm of zygotes of certain strains (e.g., C57BL/6 and B6 hybrids).

25. Proceed with remaining zygotes.
26. After injection of all zygotes, use a transfer pipet to collect and transfer them into fresh M2 medium.
27. Sort out lysed zygotes.
28. Incubate surviving zygotes at 37°C in KSOM + AA until embryo transfer.

It is suggested that a certain number of injected zygotes (∼30) be cultured overnight to assess the toxicity of each batch of RNA reagents prepared. A successful injection session should result in 90% to 95% of zygotes progressing into the two-cell stage. If the batch of injection reagents is toxic, one may also notice lysis of a large number of zygotes within ∼1 hr after the injection. Such testing of the RNA when thawed for use in subsequent microinjection sessions is not necessary.

29. Transfer the manipulated embryos into the oviducts of pseudopregnant foster mothers following the surgical procedures described in Behringer et al. (2014).

The optimal number of embryos transferred is 18 per female, bilaterally. It should be noted that transgenic techniques that comprise steps including embryo production, isolation, microinjection, and transfer of manipulated embryos into recipient females have been established over three decades: the collective experience of several labs worldwide has been recently surveyed with respect to standard practices followed in transgenic procedures and the expected outcomes. We recommend reading the published survey results (Fielder et al., 2010; Pease and Saunders, 2011), which give more detailed information related to suggested practices, critical parameters, and troubleshooting of transgenic procedures.

GENOTYPING OF OFFSPRING

Types of mutations that can be achieved by CRISPR/Cas system are: (i) simple deletions and insertions, (ii) replacement mutations using short repair oligonucleotides, and (iii) insertion of larger DNA cassettes using plasmid DNA.

Table 15.7.3 Genotyping Methods to Detect Mutations in CRISPR/Cas-Generated Offspring

Genotyping method	Detects	Intended purpose of CRISPR/Cas system
Surveyor or T7E1 assay	Cas9 induced indels	Cas9 cleavage with or without repair DNA
Flanking PCR and RFLP	Specific small insertions or Cas9 induced deletions	Cas9 cleavage with short repair oligonucleotides
Int+ Ext PCR	Specific insertions	Cas9 cleavage with short repair oligonucleotides or larger plasmid DNA
Sequencing	All types of mutations	All of the above

Genotyping assays

The offspring can be genotyped using several possible assays depending on the purpose. Table 15.7.3 gives a synopsis of these assays. Most of these methods follow standard protocols and are well established over the years, except the T7E1 assay, for which the steps are provided here. A quick overview of each is given below:

T7endonuclease1 (T7E1) and Surveyor assay (this protocol): T7endonuclease 1 (T7E1) cleaves a double-stranded DNA at the site if it has base-pair mismatches. Another enzyme called Cel1 may be used in place of T7E1, which has a similar type of cleavage activity at mismatch sites in heteroduplex DNAs. The assay using the Cel1 enzyme is called the Surveyor assay. This property of mismatch cleaving is perfectly suited for detection of mutations in gene-editing experiments where Cas9 or TALEN or ZFN-mediated DNA breaks invariably lead to *indels*. The assay is performed by PCR amplifying the target sequence using surrounding primers, and the PCR product is denatured and then annealed and incubated with Cel1 or T7E1 enzyme. The denatured and annealed dsDNAs will contain a mixture of homo- and heteroduplexes. The enzyme-digested products, when resolved by agarose gel electrophoresis, will show full-length and expected-size cleavage products if Cas9 cleavage occurred that resulted in indels.

Flanking primer PCR and restriction fragment length polymorphism (RFLP): This method is suitable for detection of short insertions when a repair oligonucleotide is included in the microinjection experiment to achieve targeted knock-in. If the repair oligonucleotide is designed to either contain a new restriction endonuclease site or leads to ablation of an RE site in the genomic locus, the flanking primer PCR product can be subjected to RE digestion and the products can be assessed for the desired replacement mutation. When primers surrounding the target region are used to amplify the target region, such products can show the expected difference in size when subjected to agarose gel electrophoresis.

Internal + external primer PCR: In the insertion experiments, one primer that binds to the repair DNA (internal primer) and a primer that binds outside a corresponding homology arm (external primer) are used to amplify the region to detect the targeted insertion.

Sequencing: It is suggested that all types of mutations be confirmed by sequencing the target site. Mutations can be detected by direct sequencing of the PCR product amplified from the target region. Occasionally, direct sequencing of PCR product may not detect the mutations. In such cases, the PCR product can be cloned using T/A cloning method, and a few random colonies are sequenced to identify the mutation.

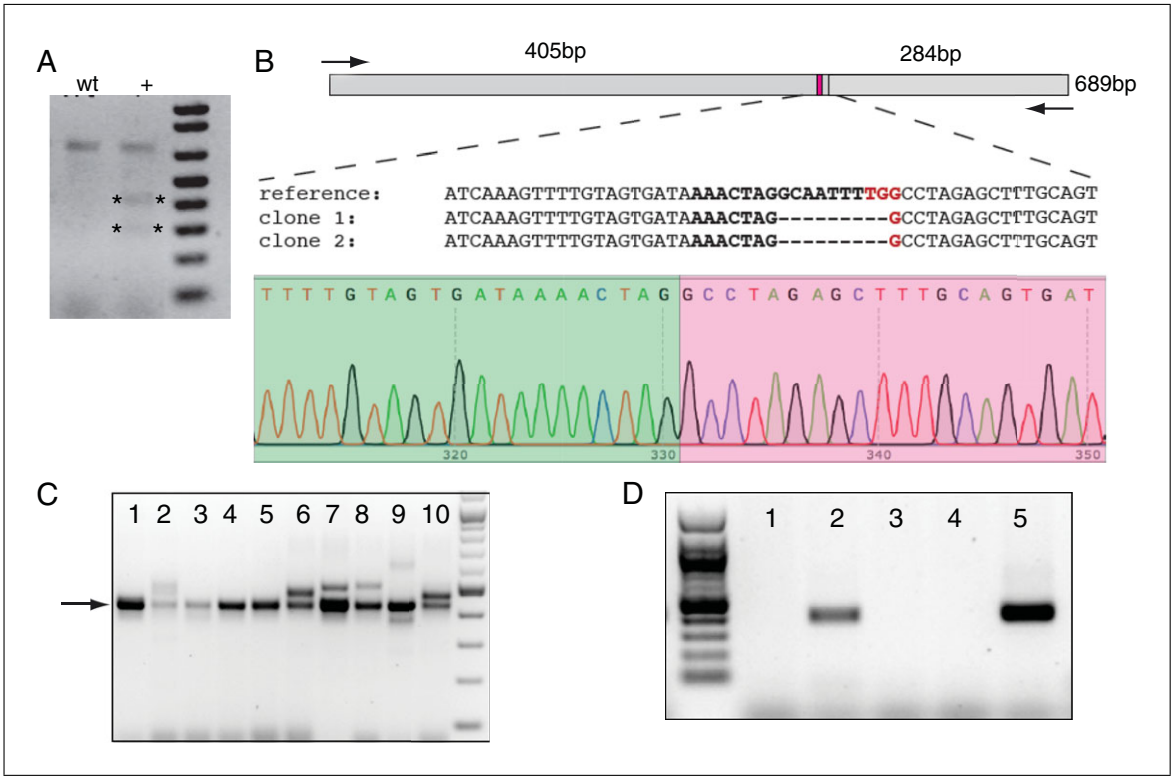

Figure 15.7.5 Examples of genotyping. (**A**) A representative T7E1 assay of a wild type and a mutant sample. The T7E1 cleavage products in mutant sample (+) are marked with asterisks. (**B**) A 689-bp fragment centered on the CRISPR binding site is PCR amplified, cloned, and sequenced. Sequence alignments and chromatogram showing a 9-bp deletion in two independent clones. (**C**) PCR of mutation insertion site in an oligonucleotide-based HDR knock-in experiment showing wild-type band (arrow) along with higher-sized bands resulting from insertion of the oligonucleotides included in the microinjection mix. A smaller-sized band in sample 9 indicates deletion of a few nucleotides. Unequal intensities of the higher/lower-sized bands with that of wild-type band in some samples indicate mosaicism. (**D**) An example of PCR with internal and external primers showing amplification of the expected band only in the positive samples (nos. 2 and 5).

Materials

T7 Endonuclease I (NEB)
NEBuffer 2 (NEB)
2% agarose gel (also see *UNIT 2.7*)
Thermal cycler

Additional reagents and equipment for the polymerase chain reaction (PCR; Kramer and Coen, 2001) and agarose gel electrophoresis (*UNIT 2.7*)

1. Design two oligonucleotide primers to amplify a 400- to 700-bp fragment centered on the targeted sequence.
2. Perform the PCR (Kramer and Coen, 2001) in 25 µl. Make sure that the PCR amplification yields a unique and specific product.
3. Run 5 µl of the PCR product on an agarose gel (*UNIT 2.7*) in TAE buffer to verify the success of the reaction.
4. Melt and anneal slowly the rest of the PCR volume, to allow the formation of heteroduplexes, as follows:

Hold	95°C	10 min
ramp	95°C/85°C −2°C/sec, hold 85°C	1 min
ramp	85°C/75°C −0.3°C/sec, hold 75°C	1 min
ramp	75°C/65°C −0.3°C/sec, hold 65°C	1 min
ramp	65°C/55°C −0.3°C/sec, hold 55°C	1 min
ramp	55°C/45°C −0.3°C/sec, hold 45°C	1 min
ramp	45°C/35°C −0.3°C/sec, hold 35°C	1 min
ramp	35°C/25°C −0.3°C/sec	1 min.

5. Digest 10 μl of the PCR product with 5 U of T7 endonuclease I in 20 μl of 1× NEBuffer 2. Incubate 25 min at 37°C.

6. Run the digestion on a 2% agarose gel (*UNIT 2.7*) in TAE buffer.

Representative genotyping examples of T7E1 assay, flanking primer PCR, internal and external primer PCR, and sequencing of target sites are shown in Figure 15.7.5.

COMMENTARY

Background Information

Historic perspectives on mouse genome engineering

Genetic engineering techniques to manipulate the mouse genome have been established over three decades. There are three major types of engineered mutations in mice: *transgenic* mice, in which an exogenous DNA is inserted into the genome; *knockout* mice, in which the endogenous gene is disrupted; and *knock-in* mice, in which the endogenous gene segment is replaced with either a modified version of the gene or an exogenous DNA. The first step in creating any of these types of mutations involves building of a DNA construct using standard molecular biology protocols. While transgenic constructs contain elements needed for expression of a coding sequence, and do not need to contain specific DNA homology arms, targeting vectors used for knockout and knock-in, on the other hand, are required to contain homology arms, and also involve careful design strategies. Steps for generating transgenic and knockout vectors are described in detail elsewhere (*UNIT 15.2*; LePage and Conlon, 2006; Hall et al., 2009; Haruyama et al., 2009).

Creating a transgenic mouse involves direct injection of a DNA of interest into one-cell-stage mouse embryos. On the other hand, generation of knockout or knock-in mice involves first targeting the mutation in mouse embryonic stem (ES) cells through homologous recombination, and then injection of the manipulated ES cells into blastocyst-stage embryos. In the early days of the procedure, the knockout mutations created primarily involved complete deletion, where one or more coding exons were replaced by a positive selection marker such as neomycin. In past two decades, more sophisticated designs have become possible with the introduction of site-specific recombinases such as those used in the Cre-Lox and Flp-Frt systems. Nevertheless, the requirement for an ES cell step to create knockout or knock-in mutations could not be bypassed.

Recent technical advances that expedite the mouse genome engineering process

A key tool that has favored the use of mouse as a model genetic organism compared to other species has been the ability to exploit embryonic stem cells. Using embryonic stem cells and the well established procedures to introduce mutations into them through homologous recombination, several thousand mutant alleles have been created in the mouse genome. The recent technical advances that can bypass the use of ES cells, however, have made it possible to manipulate the genome of any organism. These methods include ZFNs, TALENs, and the CRISPR/Cas system. Among these new technologies, ZFNs and TALENs have been around for about for 4 to 5 years at the time of this writing (Geurts et al., 2009; Rémy et al., 2010; Panda et al., 2013; Sung et al., 2013), and there are some articles that describe using these methodologies to create mutations in the mouse genome (Wefers et al., 2013; Hermann et al., 2014). Of these, CRISPR/Cas is the newest technique, and because of its simplicity and robustness, it shows great promise for creating knockout or knock-in mutations at an unprecedented speed. CRISPR/Cas-mediated knockout or knock-in mouse models can be generated in less than 3 months. More importantly, multiple mutants

can be generated using the CRISPR/Cas system in a single experiment, which is almost impractical using traditional approaches.

Critical Parameters and Troubleshooting

Because the CRISPR/Cas system is a relatively new technique, there are not enough reports yet to make definitive comments about the critical parameters, even though the system has shown a remarkable success rate in creating genetic mutations in many organisms including the mouse. Discussed below are certain critical parameters based on the available reports and our experience in using the system.

Basic Protocol 1: Design

i. *Input sequence:* Since the CRISPR target sequences are 23 nucleotides long (including PAM sequence), the system does not accept sequence less than 23 bases long. Also, the system may not find potential target sites even if the sequence entered is >23 bases.

ii. In a standard knock-in experiment, up to ~80 extra nucleotides can be easily inserted using a single-stranded repair oligonucleotide that will have ~60-nucleotide homology arms on either side. Such designs force CRISPR targets to be identified very near the desired insertion site (preferably within ± 40 bases). This is because the currently available synthesis limit of oligonucleotides is about 200 bases (ultramers from Integrated DNA Technologies). If only point mutations are desired that do not warrant addition of extra nucleotides, the target selection area can be extended up to ±80 bases from the desired site. As reported in cell-based experiments that used a double-nicking approach (see below), the targeted knock-in mutation through HDR is higher when the chosen sgRNA targets are close (about ± 30 bases) to the insertion site (Ran et al., 2013). There are currently no reports available about such limitations for direct mouse zygote experiments. If the design tool does not yield target sequences in the near vicinity (within about 40 nucleotides; the closer, the better) of the desired site for creating a knock-in mutation using a oligonucleotide-mediated HDR, the search can be extended further, in which case plasmid-based HDR can be an option.

iii. Targeting pre-existing restriction sites (or creating new ones after the genetic alteration) may be useful to facilitate the genotyping process.

iv. The appropriate genomic DNA sequences exactly corresponding to the mouse strain that will be used in vivo should be carefully reviewed. Always take into account that the mouse genomic DNA reference is C57BL/6J, but genomic sequences of many additional inbred mouse strains are available (i.e., Ensembl-EBI, Wellcome–Trust Sanger Institute, Mouse Genomic Informatics-MGI-JAX, Phenome Database).

Basic Protocol 2: Synthesis and purification of RNA and DNA components

i. The quality of the microinjection mix components (RNA, DNA and even the microinjection buffer) have to be of highest quality for mouse embryo injections. The utmost care should be taken in preparing the reagents in high-quality nuclease-free water, and final microinjection mixes are prepared using microinjection buffer. Since Cas9 mRNA is a common component in all CRISPR injections, it would be ideal to prepare Cas9 mRNA in larger batches (e.g., starting reaction of 100 µl instead of 20 µl MegaClear RNA synthesis) and store the polyadenylated and purified Cas9 mRNA in single-use aliquots of ~5 µg/vial in −80°C. After obtaining satisfactory results in the first injection session, single-use aliquots of such batches can be used for future experiments (we have used Cas9 mRNA stored up to 5 months). Once an aliquot is thawed, re-freezing of unused mRNA is not recommended.

ii. *HDR DNA:* For simple knock-in mutations such as site-directed mutagenesis or insertion of short sequences, a template DNA 200-bp long is sufficient for HDR. If larger cassettes (e.g., reporters or minigenes) need to be inserted, a targeting vector with longer homology arms, similar to those used in ES-cell-based targeting, needs to be designed. There are not many reports available yet regarding the critical length of homology arms needed in such vectors in the CRISPR/Cas system. We anticipate that length of homology arms largely depends on the locus in question. Such vectors need to contain longer homology arms (typically 1 kb or more on each side), but need not contain selection (positive and negative) markers. An additional important requirement in this case is that the repair plasmids should not contain the CRISPR target sequences. It is also reported that circular plasmid can be co-injected with RNA components for direct embryo-targeting experiments (Mashiko et al., 2013).

Table 15.7.4 Concentrations of RNA and DNA Components in Published CRISPR/Cas Mouse Genome Editing Reports[a]

Cas9 mRNA (ng/μl)	sgRNA(ng/μl)	Donor oligonucleotide (ng/μl)	Reference
20 - 200	20 to 50	Not Done	Wang et al. (2013)
100	50	100	
100(Cyto)	50(Cyto)	Plasmid 500(Cyto)	Yang et al. (2013)
100(Cyto)	50(Cyto)	Plasmid 200(Cyto)	
100(Cyto)	50(Cyto)	Plasmid 50(Cyto)	
100(Cyto)	50(Cyto)	Plasmid 10(Cyto)	
5(Nuc)	2.5(Nuc)	Plasmid 10(Cyto)	
100(Cyto)	50(Cyto)	50(Nuc)	
100(Cyto)	50(Cyto)	10(Nuc)	
100(Cyto)	50(Cyto)	2(Nuc)	
20	20	Not done	Shen et al. (2013)
20	2.5 to 5 ng each of 5 or 10 sgRNAs	Not done	Zhou et al., (2014)
100 μg/ml (Cyto)	10 μg/ml (Cyto)	Not done	Fujii et al. (2013)

[a]Abbreviations: Cyto, site of injection is cytoplasmic; Nuc, site of injection is nuclear.

Basic Protocol 3: isolation of one-cell staged mouse embryos, microinjection of CRISPR/Cas components, and transfer of injected embryos into pseudopregnant mice

The majority of mutant mouse generation steps in the CRISPR/Cas system follow protocols via pronuclear injection used in traditional transgenic mouse production technology, with a few exceptions. Mouse transgenic technology has been established over years of painstaking work by hundreds of skilled technicians and researchers, and is still constantly being perfected. There are multiple and exhaustive resources available that describe critical parameters and troubleshooting of this technology (International Society for Transgenic Technologies, 2011; Behringer et al., 2014). A few parameters specifically related to CRISPR/Cas are discussed below.

i. *Concentration of Cas9 mRNA, sgRNAs:* A wide range of concentrations of Cas9 mRNA and sgRNAs has been reported to work in mouse embryos (listed in Table 15.7.4). This clearly demonstrates the flexibility and robustness of the CRISPR/Cas system. There are some indications that higher concentrations of Cas9 mRNA can yield higher number of mutants in which both alleles are targeted (Zhou et al., 2014). It is cautioned, however, that higher Cas9 concentration can also lead to higher off-target cleavages, at least in cell-based experiments (Hsu et al., 2013). There are no such systematic reports available yet in mice.

ii. *Strain of mouse embryos:* The strains in which the CRISPR/Cas system has been reported to work are C57BL6J, B6D2F1, B6SJLF1, and B6/CBAF1. Many labs are currently trying the CRISPR/Cas system under various strain backgrounds, and as the data become available, it may likely indicate that most strains are amenable to this system. While there are varying strain efficiencies, as noted in standard transgenesis experiments (Auerbach et al., 2003), the efficiencies of CRISPR/Cas-induced mutations may also vary among different strains. We presume that such strain variations, if they occur, would result from technical challenges associated with embryo production, isolation, microinjection, and transfer steps, rather than in vivo mechanistic efficiency of the CRISPR/Cas system in different strains. The fact that CRISPR/Cas-mediated gene editing has been proven to efficiently work in other species supports this argument. It may not be an overstatement if we say "CRISPR/Cas can be successfully used to create mutants under any mouse strain background" provided that the technical challenges pertaining to embryology for that strain are streamlined.

iii. *Site of microinjection:* Unlike in a typical transgenic production where the DNA is microinjected into the pronucleus (preferably

the bigger of the two or both), Cas9, TALENs, and ZFN systems are introduced as mRNAs, which can be injected into the cytoplasm. A recent study compared the efficiency of CRISPR/Cas-mediated DNA cleavage when the Cas9 mRNA and sgRNAs were injected into (i) only cytoplasm, (ii) only nucleus, and (iii) both cytoplasm and nucleus (Horii et al., 2014). The results show that cytoplasmic injections had the highest efficiency. Nonetheless, the other two methods also yielded sufficiently high numbers of mutant genomes. The study, however, did not evaluate knock-in efficiency using oligonucleotide or plasmid-based repair DNA in their injection mixes. The available reports so far that used repair DNA in their CRISPR/Cas injections have not involved cytoplasmic-only injections, and it would be hard to conclude at this point whether cytoplasmic-only injections yield desired results for mixes that comprise repair DNA.

Basic Protocol 4: Genotyping

Surveyor or T7E1 assay: Genotyping using the Surveyor assay or T7E1 assay can be challenging in certain situations: (i) mosaicism (resulting in more than two types of alleles in some samples), (ii) large indels (occasional preference of PCR for shorter or larger alleles in the samples or inefficient hetero-duplex formation of strands during the assay), (iii) X-linked genes (single-copy genes: any mutation in such genes cannot be detected unless it is mosaic). Efficiency of this assay is also of concern in certain cases such as (i) presence of nonspecific bands in the PCR, (ii) incomplete removal of primer dimers before the PCR product is subjected to the assay, (iii) poor efficiency of Cel1 or T7E1 enzyme activity, (iv) inefficient formation of heteroduplexes, and (v) inability to detect very short fragments if the mutation is close to one end of the PCR product. Suggested remedies in these cases would be to (a) adjust PCR conditions to obtain a clean PCR product, (b) purify the PCR product before the assay, (c) use the suggested amount (less than 200 ng) of PCR product in the assay, (d) redesign PCR primers to place the expected indel mutation at about one-third or two-thirds of length of the PCR product, (e) consider alternate genotyping options such as RFLP and/or sequencing.

An additional point to keep in mind is the presence of Single Nucleotide Polymorphisms (SNPs), microsatellite variations, or Simple Sequence Length Polymorphisms (SSLPs) in the genomic region used for PCR genotyping, as they may lead to false positives by the T7E1 assay or Surveyor assay due to the associated mis-pairing of the base pairs. Therefore, we suggest including genomic DNA of the mouse strain used in these genotyping assays, to anticipate any unexpected false positives.

An example of a false negative Surveyor assay result that was detected by direct sequencing of a target region PCR product is shown in Figure 15.7.6.

Off-target effects

As discussed in the design section (Basic Protocol 1), the CRISPR/Cas system is likely to have high off-target effects simply because of the very short sequence requirement in sgRNAs for target recognition. Such off-target effects can be minimized significantly by:

i. *Careful selection of target sequences:* Currently, there are two online tools available for target selection for the mouse genome see Basic Protocol 1). It is expected that such tools will be improved further as additional experimental data are accumulated and additional tools become available. We suggest that the same sequence be queried using more than one search tool and the common best sequence(s) selected that result from multiple search tools that may decrease the likelihood of off-target effects.

ii. *Using double-nicking (also called offset nicking) approach:* Certain mutations in the Cas9 protein cause DNA nicking instead of double-stranded breaks. This feature is elegantly used, in a similar fashion to using two molecules of ZFNs and TALENs, to cause nicking on opposite strands. A requirement for the orchestrated functioning of two independent sgRNA sequences significantly decreases the likelihood of off-target effects. An example of a Cas9 mutant that is shown to be useful in double-nicking strategy is D10A (termed Cas9n: Cas9 nickase; Mali et al., 2013a; Ran et al., 2013; Fujii et al., 2014; Shen et al., 2014).

iii. *Screening of off-target effects:* Even though off-target mutations (if present) can be eliminated by breeding of the mutants in subsequent generations, it is prudent to screen the mutant(s) chosen for further experiments to detect the presence of off-target mutations. The screening can be done if the selected sgRNA has only a few potential off-target sites, using the Surveyor or T7E1 assay of PCR products amplified from the off-target sites. Screening of the potential off-target sequences with up to four mismatches (particularly in the PAM distal nucleotides of the target sequence)

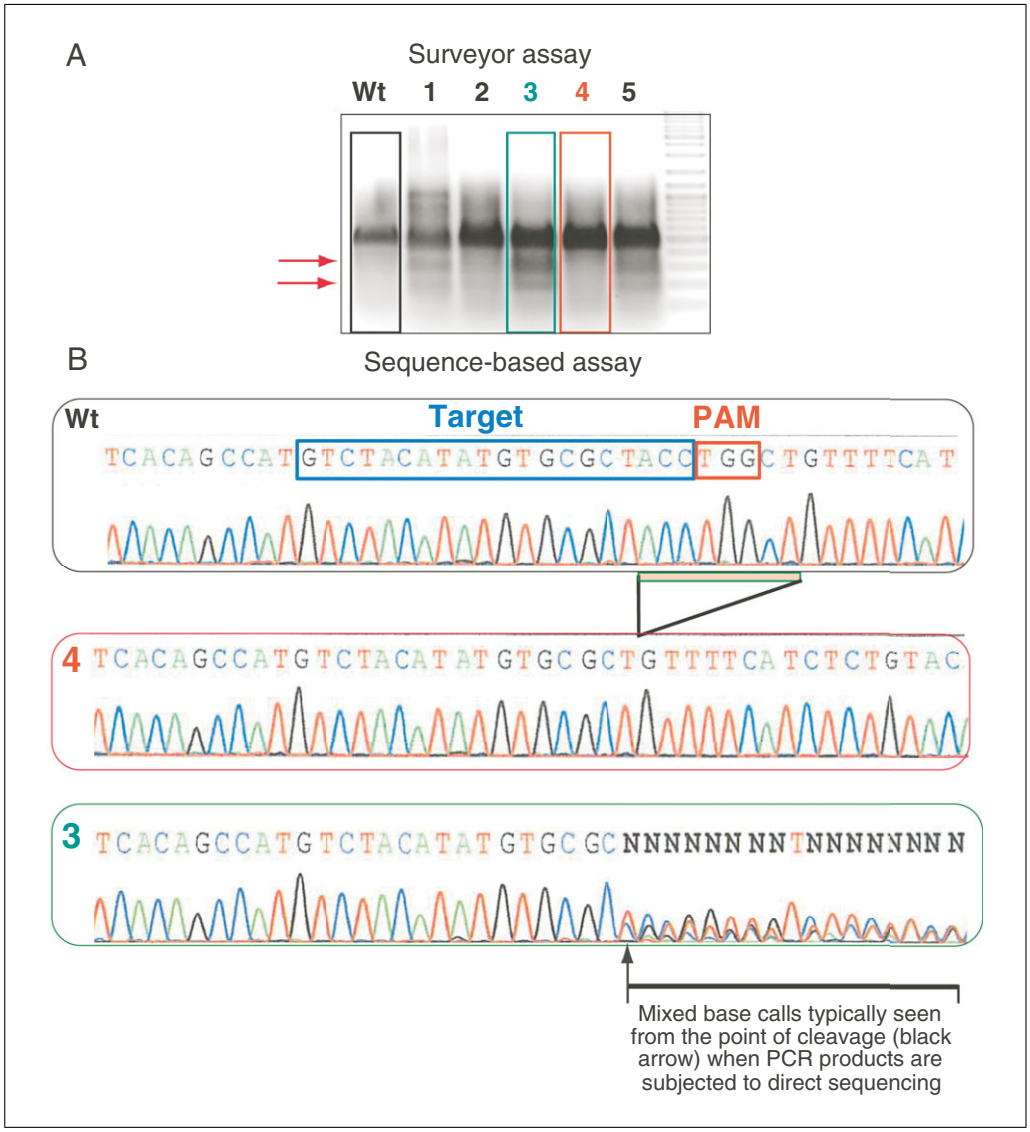

Figure 15.7.6 An example of Surveyor assay combined with sequencing to detect CRISPR/Cas mutations on the X chromosome. (**A**) Surveyor assay of DNA samples isolated from CRISPR/Cas injected pronuclei cultured up to the blastocyst stage showing negative (sample 4) and positive (remaining) samples. Red arrows indicate cleaved bands of 261- and 184-bp from the 445-bp PCR product. Notably, all samples were positive by sequencing assay, including those that were Surveyor negative. Note that occasionally the Surveyor assay results in very weak cleavage products (e.g., sample 2). (**B**) Direct sequencing of PCR products from wild type, a Surveyor -positive (sample 3), and a Surveyor-negative (sample 4) sample. Sample number 4 had deletion of 8 nucleotides even though the Surveyor assay was negative. This could be due to the sample being male and having only one X-chromosome. Sample 3 showed typical overlapping peaks after the cut site (arrow), indicative of two (or more if mosaic) templates: this sample could be a female embryo with only one allele mutated (or more if mosaic) or a male with mosaic activity of CRISPR/Cas.

is strongly suggested. Alternatively, samples can be subjected to expensive methods such as whole-genome sequencing to critically rule out all any off-target mutations.

Anticipated Results

As observed in published reports so far, CRISPR/Cas-induced mutations occur at a higher efficiency compared to standard transgenic mouse production. However, even though both of these techniques use one-cell mouse embryo injections, they cannot be directly compared with each other (except for the number of mutants generated), because the intended end results are quite different. Typical transgenic rates (number of transgenic mutants obtained/100 embryos injected) through pronuclear DNA injection range from 0.6 ±

0.8 % to 2.8± 3.1%, depending on various parameters including strain and type of DNA (Fielder et al., 2010). All the reports that have used the CRISPR/Cas system so far, either to create indels or HDR knock-in mutations using short oligonucleotides, have observed a several-fold higher success rate than that what is achieved using traditional transgenesis. Because of such high efficiency, about a third or a quarter of starting number of embryos injected will lead to mutant pups in the CRISPR/Cas system. This may be compared to traditional transgenesis, where typically about 200 to 300 zygotes are injected to obtain three to four independent founder mice. It is anticipated that the CRISPR/Cas system may become the most widely used and preferred method for mouse genome editing in the near future.

Time Considerations

Generation of knockout or knock-in mice using standard ES-cell based approaches involves at least two to three major steps that take about a year to produce chimeras. The CRISPR/Cas system, on the other hand, can generate mutant mice in one major step and can be completed in <3 months. Furthermore, multiple mutants (more than one gene or locus) can also be generated in a single experiment using the CRISPR/Cas system. A typical time frame for CRISPR/Cas experimental procedures is outlined below and depicted in Figure 15.7.1:

Week 1: Searching CRISPR target sequences and designing of constructs.

Weeks 1 to 2: Procuring of primers for amplifying Cas9 and/or sgRNA templates and primers for genotyping assays.

Weeks 2 to 3: Construction of vectors (as needed) and generating and purifying RNA and DNA components.

Weeks 2 to 3: Procuring of animals and testing genotyping primers on wild-type genomic DNA.

Week 4: Making sure that all the injection components are ready. Initiation of superovulation.

Week 5: Microinjection and embryo transfer.

Weeks 9 to 11: Genotyping of offspring.

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Internet Resources

http://crispr.mit.edu/

This site searches SpCas9 target sites within the sequence of interest and allows users to enter a 23 to 250 base DNA sequence. The site is hosted and maintained by Dr. Feng Zhang's group at Massachusetts Institute of Technology. The algorithm used by this program is based on the specificity analysis performed in Hsu et al. (2013). Details related to this Web site and how to use the tool are given in Basic Protocol 1 and outlined in Figure 15.7.2.

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