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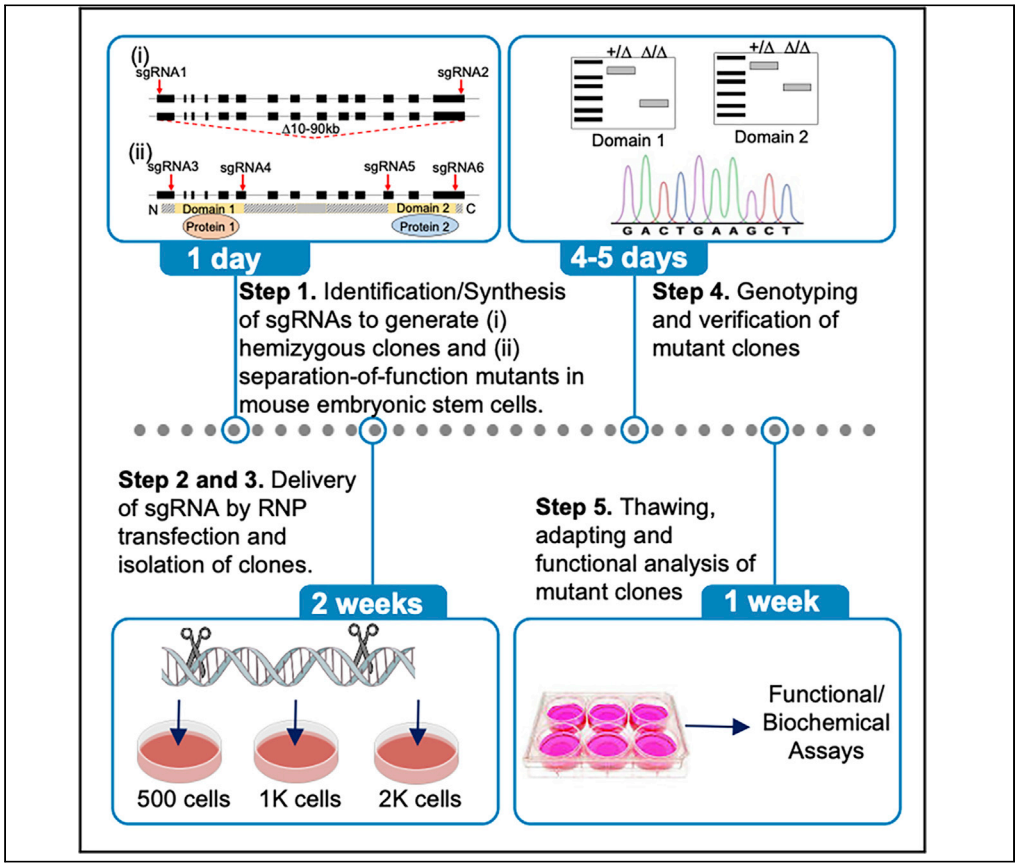
Protocol

Exploiting CRISPR/Cas9 to engineer precise segmental deletions in mouse embryonic stem cells



In this protocol, we use CRISPR/Cas9 to generate large deletions of the entire coding region of a gene of interest, generating a hemizygous cell line. Next, we systematically engineer precise in-frame deletions within the intact wild-type allele, facilitating study of multi-domain proteins. The optimized protocol described here allows us to rapidly screen for effective sgRNA pairs and to engineer either an in-frame deletion or a frameshift mutation in high frequencies in mouse embryonic stem cells.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Generation of large, defined genomic deletions in mouse embryonic stem cells

Synthesis and validation of sgRNA pairs to ensure efficient gene deletion

Generation of precise segmental deletions to study separation-of-function alleles

Functional and biochemical analyses of mutant clones obtained by CRISPR/Cas9

Elango et al., STAR Protocols 3, 101551
September 16, 2022
https://doi.org/10.1016/j.xpro.2022.101551

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Exploiting CRISPR/Cas9 to engineer precise segmental deletions in mouse embryonic stem cells

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https://doi.org/10.1016/j.xpro.2022.101551

SUMMARY

In this protocol, we use CRISPR/Cas9 to generate large deletions of the entire coding region of a gene of interest, generating a hemizygous cell line. Next, we systematically engineer precise in-frame deletions within the intact wild-type allele, facilitating study of multi-domain proteins. The optimized protocol described here allows us to rapidly screen for effective sgRNA pairs and to engineer either an in-frame deletion or a frameshift mutation in high frequencies in mouse embryonic stem cells.

For complete details on the use and execution of this protocol, please refer to Panday et al. (2021).

BEFORE YOU BEGIN

The entire process of generating single clones containing precise segmental deletions in mouse embryonic stem (mES) cells takes several weeks to complete.

1. For all molecular biology techniques, always use certified RNAse-free water.
2. Wear PPE at all times while preparing and handling RNA: lab coat, gloves, mask.
3. Use filter-tips and autoclaved tubes for all steps.
4. Use DEPC-treated or nuclease-free certified water for all steps.
5. Always keep reagents on ice. Dry ice is used to minimize unwanted thawing of other frozen mES cells on the same plate.
   a. Thawed mES suspension is directly plated onto a tissue culture dish containing a monolayer of irradiated mouse embryonic fibroblasts.

⚠ CRITICAL: sgRNA samples prepared in vitro can be stored at −20°C for at most one month. After this time, the efficiency of gene deletion is reduced dramatically.

sgRNA identification, purification, and quality control

Timing: 1 day

6. Identification and designing sgRNAs to target gene of interest (Figure 1). We order three sgRNA oligos each to the target region at the 5′ and 3′ ends of the gene of interest. In addition, we design and order three forward and three reverse primer pairs for genotyping.

Note: Design three pairs of forward and reverse primers approximately 250 bp away from the predicted sgRNA cut site for genotyping.

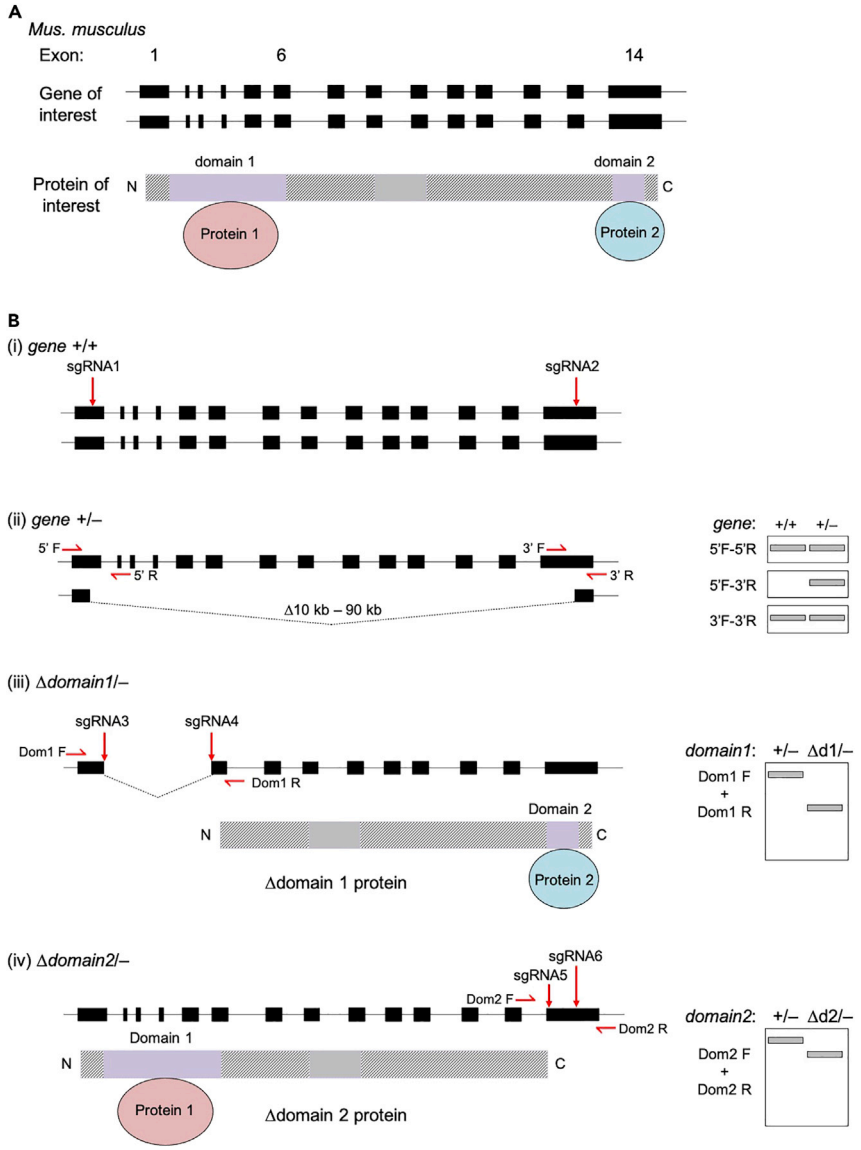

Figure 1. Schematic showing CRISPR/Cas9 design in mES cells

(A) Representative mouse gene (gene of interest) containing 14 exons (black rectangles) and representative mouse protein (protein of interest). Exons 1–6 code for domain 1 that binds protein 1 (pink oval). Exon 14 codes for domain 2 that binds protein 2 (blue oval).

(B) Schematic showing stepwise domain deletions and corresponding predicted protein structures. (1) Schematic showing both alleles of a mouse gene of interest (*gene +/+*), with positions of sgRNAs (red arrows) to engineer a hemizygote. (2) Generation of a hemizygote (gene +/–) using sgRNAs. Primer pairs (red half arrows) used to validate the large deletion, and the intact left and right junctions are detected by PCR as shown. Representative gel (right) showing expected PCR products in clones. (3) Deletion of domain 1 (*Δdomain1/–*) using sgRNA pairs 3+4 (red arrows) to generate an in-frame mutation (top) and a gel image showing the expected PCR outcome using primers shown (red half arrows). The predicted protein structure (Protein of Interest Δdomain 1) is also shown (bottom). (4) Deletion of domain 2 (*Δdomain2/–*) using sgRNA pairs 5+6 (red arrows) to generate an in-frame mutation (top) and gel image showing the desired deletion outcome using primers shown (red half arrows). The predicted protein structure (Protein of Interest Δdomain 2) is also shown (bottom).

Note: This oligo is commercially synthesized and is shipped in a lyophilized form. It is compatible with commercial in vitro transcription kits used to generate sgRNAs.

Note: Each of these lyophilized oligos used for sgRNA synthesis are diluted to 1 μM with nuclease-free water.

Preparation of mouse embryonic stem cells for transfection

Timing: 7–10 days

7. Thaw parental mES cell line containing a single copy of the Tus/*Ter* (Willis et al., 2014, 2017) on an approximately 80% confluent monolayer of irradiated mouse embryonic fibroblasts (MEF). Thaw mES cells in ES cell medium containing DMEM with 15% serum, 20 mM HEPES pH 7.6, 0.1 mM beta mercaptoethanol, 500 U/mL LIF, 1× MEM NEAA, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.001 M sodium pyruvate and MycoZap prophylactic (Willis and Scully, 2021).
8. Once the thawed mES cells reach approximately a medium to large size (with refractile edges), they are ready for adaptation.
9. For adaptation, add 1 mL of sterile 0.2% Gelatin (w/v) made in 1×PBS to a fresh 6-well plate. Depending on size and number of colonies, trypsinize cells with 250 μL 0.25% trysin and quench cells with 750 μL mES cell media and passage cells 1:5 or 1:3 onto the freshly gelatinized plate devoid of any MEF feeder layer.
10. After approximately 3 passages, the mES cells are fully adapted to growth on gelatin and are ready for transfection.

Preparation of MEF coated wells for picking clones

Timing: 30–45 min

11. We routinely pick approximately 96 clones for analysis by PCR and sequencing. Before picking single clones of mES cells following CRISPR/Cas9 treatment, we plate four 24-well plates with a monolayer of irradiated MEFs and incubate at 37°C for 4–5 days until the MEFs are approximately 80% confluent.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
sgRNA oligos	Thermo Fisher Scientific	N/A
Nuclease-free water	New England Biolabs	cat# B1500L
Engen sgRNA Synthesis Kit, *S. Pyrogenes*	New England Biolabs	cat# E3322
RNA Clean and Concentrate kit	Zymo Research	cat# R1017
10% TBE-Urea Novex Gel	Life Technologies	cat# EC6875BOX
Low Range ssRNA ladder	New England Biolabs	cat# N03645
5× TBE Running Buffer	Life Technologies	cat# LC6675
4%–20% TBE-Acrylamide Gels, 1.0 mm, 15 well	Life Technologies	cat#EC62255BOX
Lipofectamine 2000	Life Technologies	cat#11668019
Gibco Opti-MEM (serum-free media)	Life Technologies	cat#31985070
Genomic DNA isolation: Cell lysis solution	QIAGEN	cat#158908
Genomic DNA isolation: Protein precipitation solution	QIAGEN	cat#158912
Genomic DNA isolation: DNA hydration solution	QIAGEN	cat#158914

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
dNTP kit	Fisher	cat#10297018
Taq DNA Polymerase (250 U)	QIAGEN	cat#201203
Experimental models: Cell lines		
Mouse embryonic stem cells Genotype: Conditional Brca1 $^{flox/hygro}$–*Ter* reporter cell line. Sex: Male	(Willis et al., 2014, 2017)	N/A
Oligonucleotides		
Primer: Fancm 5′ exon2 breaksite sgRNA transcription 5′-GGTTCTTTTCCTGACACCGCAGG-3′	Thermo Fisher Scientific	N/A
Primer: FancmΔ85/Δ 3′ exon2 breaksite sgRNA transcription 5′-GATGAAGCTCATAAGGCACTTGG-3′	Thermo Fisher Scientific	N/A
Primer: Fancm exon23 breaksite sgRNA transcription 5′-GTTACCAAATGATCTTAACCAGG-3′	Thermo Fisher Scientific	N/A
Primer: Fancm exon2 sense PCR and sequencing 5′-CTACCTCAAGCTCCAGAGTCCTGG-3′	Thermo Fisher Scientific	N/A
Primer: Fancm exon2 antisense PCR and sequencing 5′-AGTTCCCATCACTGAGACTTATTCC-3′	Thermo Fisher Scientific	N/A
Primer: Fancm exon23 sense PCR and sequencing 5′-CTACCTCAAGCTCCAGAGTCCTGG-3′	Thermo Fisher Scientific	N/A
Primer: Fancm exon23 antisense PCR and sequencing 5′-AGGGATGACCTGAGGTTGTC-3′	Thermo Fisher Scientific	N/A
Primer: Fancm MM1 5′ breaksite sgRNA transcription 5′-ATGCTGACACTGTTAAACAAAGG-3′	Thermo Fisher Scientific	N/A
Primer: Fancm MM1 3′ breaksite sgRNA transcription 5′-GTGAACAGCTCTTCTTCCAATGG-3′	Thermo Fisher Scientific	N/A
Primer: Fancm MM2 5′ breaksite sgRNA transcription 5′-CAAGAAGAGCTGAGGACTGACGG-3′	Thermo Fisher Scientific	N/A
Primer: Fancm MM2 3′ breaksite sgRNA transcription 5′-TCTGATAGGACTCGCACCCTGGG-3′	Thermo Fisher Scientific	N/A
Primer: Fancm DEAH 5′ breaksite sgRNA transcription 5′-TGGTAAATGACCTTACTAGAGGG-3′	Thermo Fisher Scientific	N/A
Primer: Fancm DEAH 3′ breaksite sgRNA transcription 5′-ATGAAGCTCATAAGGCACTTGGG-3′	Thermo Fisher Scientific	N/A
Primer: Fancm MM1 sense PCR and sequencing 5′-CTTGTTTGGTAGGGTGAATGCA-3′	Thermo Fisher Scientific	N/A
Primer: Fancm MM1 antisense PCR and sequencing 5′-GGGAGAACGGGATAAAAATCTCT-3′	Thermo Fisher Scientific	N/A
Primer: Fancm MM2 sense PCR and sequencing 5′-TAGATGATGATTCTGAACCTGAAGAC-3′	Thermo Fisher Scientific	N/A
Primer: Fancm MM2 antisense PCR and sequencing 5′-TGTGCTCCTGACTCTCTGCT-3′	Thermo Fisher Scientific	N/A
Primer: Fancm DEAH sense PCR and sequencing 5′-CTACCTCAAGCTCCAGAGTCCTGG-3′	Thermo Fisher Scientific	N/A
Primer: Fancm DEAH antisense PCR and sequencing 5′-AGTTCCCATCACTGAGACTTATTCC-3′	Thermo Fisher Scientific	N/A
Software and algorithms		
Flojo 10		
Microsoft XL		
Prism 7.0		
Others		
Beckman Coulter Cytoflex LX fitted with yellow (561 nm) and blue (488 nm) lasers		

STEP-BY-STEP METHOD DETAILS

sgRNA identification, purification, and quality control

Timing: 1 day

This section provides step-by-step details for the identification, synthesis, and purification of sgRNA pairs used to generate precise genomic deletions in mES cells. This section also provides various methods to assure good quality of sgRNA prior to tranfection.

1. Identify your target genetic locus of interest (e.g., *Fancm*) in the mouse genome.
   a. We design sgRNAs by identifying the protospacer adjacent motif (PAM) sequence 'NGG' (*Streptococcus pyogenes* Cas9), flanking the regions of *Fancm* to be deleted. We designed several mutants of *Fancm* including: *Fancm* Δ85/Δ frameshift mutants, *Fancm* MM1Δ/Δ, *Fancm* MM2Δ/Δ and *Fancm* DEAHΔ/Δ in-frame mutants (Panday et al., 2021).
   b. We use online tools to design oligos compatible for *in vitro* transcription. https://cctop.cos.uni-heidelberg.de:8043/

   ⚠ CRITICAL: Several other validated online portals could be used to design sgRNAs for CRISPR/Cas9 mediated gene deletion. For examples, https://portals.broadinstitute.org/gppx/crispick/public.

   c. Before the oligos are ordered, we generate an oligo sequence using the tool mentioned below. This tool generates a sequence that contains the target gene sequence and also includes the T7 promoter sequence at the 5′ end and a 14-nt overlap sequence at the 3′ end. If there is no G at the 5′ end of the target gene sequence, the tool will add a G to the generated oligo. https://sgrna.neb.com/#!/sgrna.

   ⚠ CRITICAL: To generate frame-shift mutations in the target gene, ensure that the two sgRNAs are designed to cut in different reading frames. Similarly, to engineer an in-frame mutation, it is critical to design sgRNAs that cut in the same reading frame (Panday et al., 2021).

2. Use commercial *in vitro* transcription kit (NEB cat# E3322) to synthesize sgRNAs as per manufacturer's instructions. https://www.neb.com/protocols/2016/05/11/engen-sqrna-synthesis-kit-s-pyogenes-protocol-e3322.

   ⚠ CRITICAL: Addition of DTT as per manufacturer's instructions is essential and yields may be dramatically improved if samples incubated for 1–3 h vs the 30 min recommended time.

3. Following sgRNA synthesis, degrade DNA by addition of DNAase for 15 min at 37°C.
4. Purify newly synthesized sgRNA using commercially available RNA purification kit (Zymo Research cat# R1017). Elute sgRNA using 30 μL nuclease-free water.
5. Estimate the concentration and purity of sgRNA using Thermo Scientific Nanodrop One and ensure the A260:280 ratio shows 2.0.
6. To further ensure the quality of the freshly synthesized sgRNA, we denature sgRNA using RNA sample buffer and heat at 70°C for 10 min.
7. Run denatured sgRNA on a 10% TBE-Urea in 1× TBE running buffer. Use denatured RNA molecular weight ladder in parallel. Run the gel at 180 V for 60 min.
8. Remove gel and stain in 2 μg/mL ethidium bromide in water for 20 min at 25°C with gentle shaking.
9. Image gel. Typically, you will see one prominent band. Detection of multiple bands or light smearing indicates degradation of sgRNA.

   ⚠ CRITICAL: Always keep sgRNAs on ice. Store at −20°C for ∼ 1 week. Store at −80°C for long term storage.

Delivery of sgRNA-Ribonucleoprotein (RNP) by transfection

Timing: 5 days

Using sgRNA-Ribonucleoprotein (RNP) transfection method, we can rapidly validate sgRNA pairs that cut the desired genomic loci most efficiently. This validation of sgRNAs narrows down the downstream efforts required to generate a mutant mES cell clone and provides a cost-effective method for parallel generation of clones of various mutant genotypes.

10. Expand cell line 3 days before transfection to obtain approximately 80% confluency on the day of transfection.
11. One day prior to transfection, reseed mES cell culture(s) on a plate conditioned by previous ES cell culture, to minimize transfection stress.
12. On the day of transfection, harvest cells by trypsinizing and re-suspend cells to a final concentration of 0.8 M cells/mL. Seed 0.2 mL (160,000 cells) per well of a gelatinized 24-well plate.
13. Prepare two transfection mixes in separate tubes in the concentrations denoted below.
    a. In tube 1, dilute commercially available Engen NLS-Cas9 (cat# E3322) to 3 μM (20 μM stock) in Opti-MEM at RT.
    b. In the same tube, dilute each individual sgRNA to 3 μM in Opti-MEM at 25°C.
    c. Assemble RNP in concentrations depicted below.

1× RNP reaction mix	Final concentration	Volume
3 μM sgRNA 1 in Opti-MEM	0.045 μM	0.5 μL
3 μM sgRNA 2 in Opti-MEM	0.045 μM	0.5 μL
3 μM NEB EnGen Cas9 NLS in Opti-MEM	0.045 μM	0.5 μL
Opti-MEM		33 μL

    d. Incubate the RNP mix at RT for 10 min.
    e. In tube 2, mix lipofectamine 2000 in Opti-MEM and incubate at RT for 10 min as depicted below.

1× Reaction mix for lipofectamine	Volume
Lipofectamine 2000	1.2 μL
Opti-MEM	33 μL

14. After appropriate incubation, mix lipofectamine reaction mix with RNP reaction mix and incubate at RT for 10 min.
15. Pipette 66 μL into each well (step 12) and mix plate by shaking to ensure equal distribution of the transfection mixture into each well.
16. Incubate these cells in a 37°C humidified incubator at 6% CO_2 for 6 h.
17. At 6 h, feed cells with 1 mL of prewarmed ES cell medium and incubate cells for 18 h at 37°C in a humidified incubator at 6% CO_2.
18. After 18 h, aspirate transfection medium and refeed cells with fresh ES cell medium to minimize lipofectamine induced toxicity.

Genomic DNA isolation and plating for retrieval of mutant clones

Timing: 6–7 days

This section focuses on the technique used to isolate genomic DNA from mES cells. After DNA extraction, pure DNA is used to amplify the specific region of the genome targeted by

CRISPR/Cas9. This allows us to identify the optimal sgRNA pairs before picking and isolating mutant clones.

19. Harvest the cells 72 h after transfection for genomic DNA isolation to verify the sgRNA pairs that worked most efficiently in the pool.
20. Use PCR to validate successful transfection and to detect the deleted mutant copy.
    a. For small deletions (<150 bp), a stronger 'dropout' band amplified from the deleted product suggests a more efficient gene deletion.
    b. For screening for hemizygotes in the pool, use primers outside of the deleted regions, allowing for amplification of the just the deleted segment (the wild-type allele cannot be amplified because of its large size). Run PCR product on a 1% TAE agarose gel.
21. Once sgRNA pairs have been successfully validated, repeat transfection using the above-mentioned protocol. 72 h after transfection, harvest cells and plate them in three 60 mm dishes containing a confluent layer of irradiated MEFs.
    a. To ensure sufficient separation of individual mutant clones and to account for plating efficiency, plate 500, 1000 and 2000 cells/60 mm dish.

PCR reactions to detect deleted DNA region following CRISPR/Cas9 treatment:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	30 s	35 cycles
Annealing	60°C	30 s	
Extension	72°C	1 min 30 s	
Final extension	72°C	5 min	1
Hold	12°C	forever	

Note: If the genomic deletion is <150 bp in size, run the PCR product in a 2% gel agarose gel for better resolution. If multiple sgRNA pairs are being validated, it is advised to transfect cells with varying combinations of sgRNA and use all the cells for genomic DNA isolation, PCR and validation of the deletion product. Then, repeat the transfection using a single validated sgRNA pair to plate for single cells and clone picking.

Picking mES clones and validation of individual genotypes

Timing: 20–25 days

After RNP transfection, the mutant clones are ready to be retrieved. This section focuses on the methods involved in picking individual clones, isolation of genomic DNA, and the validation of the genotype of these clones.

22. 7–10 days after plating transfected cells on 60 mm dishes, the clones will be ready for picking.
23. To pick clones, place the inverted tissue culture microscope in a tissue culture hood, so that the eyepiece is outside and the cell platform inside.
    a. Sterilize the microscope platform with 70% ethanol wipe down.
24. Prepare a sterile 96-well plate with 100 µL 0.25% trypsin per well (use trough and 8-multichannel pipette to dispense the trypsin).
25. Replace ES medium with 4 mL of 1× PBS in the 60 mm dish containing single clones.
26. With the plate under the microscope, use a P200 pipette with a barrier filter tip to suction an individual mES cell colony into the filter tip.
    a. Setup pipette to 50 µL, expel air from the tip and submerge tip into PBS.
    b. Find the end of the pipette tip in the field of view.

c. To loosen mES cell colony, nudge on the edge of the colony using the end of the pipette tip. Withdraw 50 μL of PBS/mES cell colony mix rapidly into the pipette tip.
d. Expel the colony into a well of the 96 well-plate containing the trypsin, pipette 2–4 times to disaggregate the colony into single cells.

27. Transfer disaggregated cells plus trypsin to a 24-well plate coated with a monolayer of irradiated MEFs and incubate cells for 18 h in a 37°C humidified incubator at 6% CO_2.
28. Refeed cells with ES cell medium after 18 h to remove the excess trypsin in the medium.
29. After 6–7 days, trypsinize cells and reseed the entire well to itself to obtain increased cell numbers for expansion and freeze downs.
30. The day after reseeding, the cells will be ready for freezing and expanding.
    a. Prepare a 24 well plate containing freezing mix that contains 250 μL of 20% DMSO in mES grade FBS. Label lid of each well with unique clone number (e.g., 1–24, 25–48 etc).
    b. In parallel, prepare four 6 well plates gelatinized for 5 min. Label lids of each well of each 6-well plate to match the clone numbers of the 24-well freezing mix plate.
    c. After gelatinization is complete, replace gelatin with 2 mL mES prewarmed ES cell medium.
    d. Harvest clones from the previously reseeded 24 well plate by incubation with 2 drops of 0.25% trypsin in PBS for 1 min at 37°C, followed by quenching with 500 μL FBS.
    e. Disaggregate cells by trituration using a P1000 pipet with a filter barrier tip.
    f. Transfer 250 μL of trypsinized mES cell suspension to the prelabeled 24-well plate containing 250 μL of freezing mix (step 30a), mix well and store this aliquot of cells immediately at −80°C for future use.
    g. Transfer remaining 250 μL of trypsinized mES cell suspension to the previously gelatinized 6 well plate, taking care to match clone numbers between frozen aliquot and cultured aliquot. Incubate these cells in a 37°C humidified incubator at 6% CO_2.
31. After approximately 7 days, the 6-well plates will be ready for lysis and genomic (g) DNA preparation.
32. Use this gDNA to genotype individual clones using the same primer pairs and PCR conditions used in step 21. Confirm genotypes of both wild-type and mutant clones by DNA sequencing of PCR products.

Note: We also confirm loss of domain expression by using RTqPCR with primers flanking the deleted domain and validate the protein abundance (or absence of a protein in a mutant clone) using western blotting similar to (Panday et al., 2021).

Thawing and adaptation of single mES mutant clones

Timing: 14 days

In this section, we describe in detail the various steps involved in thawing mES cells on a monolayer of irradiated mouse embryonic fibroblast cells. Following thawing, we describe the protocol to adapt these freshly thawed clones onto gelatin. These processes allow us to acclimatize mES cells to gelatin and prepare these cells for downstream functional and biochemical analyses.

33. Place frozen plate on dry ice to prepare cells for thawing.
    a. The clones that are validated by PCR and sequencing are thawed by addition of 1 mL of pre-warmed ES cell media, one well at a time. As the frozen mix thaws, transfer it to fresh labeled 15 mL tubes. Keep adding 1 mL medium until all the frozen mix has thawed and been transferred.
34. Centrifuge these tubes containing thawed clones at 1,500 × *g* for 5 min at 4°C. Carefully remove supernatant and transfer pellet to a 6-well plate containing a monolayer of irradiated MEFs.

Note: The cell pellet will be very small and sometimes is invisible. Be careful while discarding the supernatant to ensure the pellet is not dislodged.

35. After 5–6 days, colonies will be visible on the 6-well plate and cells must be reseeded onto the same well to provide further conditioning for the freshly thawed cells.
36. 2 days after reseeding, the cells are passaged (1:5) onto freshly gelatinized 6 well plates. This process will facilitate adaptation of the mES cells onto gelatin.
37. The remaining cells are centrifuged at 1,500 × *g* for 5 min at 4°C. Discard the supernatant, resuspend the pellet in 10%DMSO (in FBS) and freeze in −80°C for 24 h and then in liquid N_2 indefinitely.
38. Once the cells are adapted to gelatin and there are no MEFs visible under the microscope, the mES cells are ready for transfection and further assays.

Note: Typically, we thaw equal numbers of mutant clones and isogenic wild type clones that retained wild-type genotype following CRISPR/Cas9 treatment.

EXPECTED OUTCOMES

Typically, we pick 96 independent clones for DNA prep, PCR, and subsequent sequencing. The frequency of mutant clones varies depending on the size of genomic deletion, the quality and efficiency of sgRNA cutting and possibly due to the positioning of nucleosomes with respect to the sgRNA cut sites (Schep et al., 2021). We were able to experimentally retrieve 15/96 *Fancm*$^{\Delta 85/\Delta}$ frameshift clones, 2/168 hemizygous *Fancm* clones, 12/96 in-frame mutant clones for MM1 domain, 8/96 in-frame mutant clones for MM2 domain and 3/96 in-frame mutant clones for the DEAH domain. All these clones (and their wild-type isogenic controls) were confirmed by PCR and sequencing.

LIMITATIONS

Although we routinely generate mutant clones that are biallelically deleted for the gene of interest, the frequency of such events is relatively rare. To enhance the probability of generating increased number of mutant clones, we first generate a clone that contains a complete deletion of one allele of the gene of interest, while retaining a wild type second allele. It is important to determine whether this hemizygous cell displays haploinsufficiency. Although this maneuver greatly enhances the number of mutant clones, it does expose the cells to increased CRISPR/Cas9 treatment thereby increasing the possibility of deleterious off-target effects. We address these concerns by rescuing the phenotypic defects by transient or stable expression of our cDNA of interest.

TROUBLESHOOTING

Problem 1

Degradation of newly synthesized sgRNA (step 9).

Potential solution

The appearance of multiple bands in a TBE-Urea gel suggests degradation of sgRNA. This degradation can be significantly reduced by wearing gloves and facial mask to avoid any aerosol contamination. In addition, keeping the sgRNA on ice until the final storage at −20°C will reduce degradation.

Problem 2

Low frequency of mutant clones following CRISPR/Cas9 treatment (step 9).

Potential solution

We observe a dramatic reduction in the frequency of single mutant clones after the sgRNA has been stored for more than one month. It is advisable to make synthesize fresh sgRNA before each CRISPR/Cas9 maneuver.

Problem 3

Low number of cells for genomic DNA isolation and freeze down (step 31).

Potential solution

This phenotype is routinely seen when the targeted gene is essential or is involved in multiple metabolic processes. In such a case, we allow the cells to grow in a hypoxic incubator to minimize oxidative stress. In addition, we reseed the individual cells into the same well several times over several weeks to increase the cell numbers.

Problem 4

Generation of a large unintended deletion in one of the alleles.

Potential solution

While deleting several independent genes, we observed a predicted deletion in one allele but a large, unintended deletion in the other allele resulting from a CRISPR/Cas9 off-target effect (Kosicki et al., 2018; Panday et al., 2021). We confirmed this deletion by whole-genome sequencing (Panday et al., 2021). Generation of a hemizygote allows us to make precise deletions without having to work with unmapped and unintended deletions.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Ralph Scully (rscully@bidmc.harvard.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze [datasets/code].

ACKNOWLEDGMENTS

This work was supported by AACR fellowship 19-40-12-PAND (to A.P.), and NIH grants R01CA095175 and R01CA217991 (to R.S.).

AUTHOR CONTRIBUTIONS

R.E., N.A.W., and R.S. developed the methodology. R.E. and N.A.W. optimized and established CRISPR/Cas9 mediated gene deletions in mES cells. A.P. performed *Fancm* related gene deletions. R.E. and R.S. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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