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Chapter 29

Engineering Mutation Clones in Mammalian Cells with CRISPR/Cas9

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Abstract

CRISPR, *C*lustered *R*egularly *I*nterspaced *S*hort *P*alindromic *R*epeat, as a powerful genome engineering system has been widely accepted and employed in gene editing of a vast range of cell types. Comparing to zinc finger nucleases (ZFNs) or transcription-activator-like effector nucleases (TALENs), CRISPR shows less complicated process and higher efficiency. With the development of different CRISPR systems, it can be used not only to knock out a gene, but also to make precise modifications, activate or repress target genes with epigenetic modifications, and even for genome-wide screening. Here we will describe the procedure of generating stable cell lines with a knock-in mutation created by CRISPR. Specifically, this protocol demonstrated how to apply CRISPR to create the point mutation of R249 to S249 on *TP53* exon 7 in human embryonic stem cells (hESC) H9 line, which includes three major steps: (1) design CRISPR system targeting *TP53* genomic region, (2) deliver the system to H9 hESC and clone selection, and (3) examination and selection of positive clones.

Key words CRISPR, Genome editing, Precision gene editing, gRNA design, Donor vector, Stable cell line validation, Southern blot

1 Introduction

It has been a long journey of searching for precision genome editing methods. The emergence of ZFNs for the first time provides a method to produce a double-strand break at any desired DNA locus [1]. Later, the arising of TALENs provides a better tool for cutting and thereby editing specific genomic loci [2–5]. Still, the approach requires considerable amount of work, requiring a new pair of TALEs for each target. In 1993, CRISPR was first discovered as a structure with multiple copies of repeated sequences in microbes [6], later proved to be an adaptive immune system in bacteria [7–9]. A decade later, it is suggested as current name CRISPR, clustered regularly interspaced palindromic repeats [10]. It composes with tandem repeated sequences separated by spacers, and a group of Cas proteins serve as the nuclease. In effect,

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Cas proteins work as programmable restriction enzymes cut DNA at 3 nucleotides upstream of the proto-spacer adjacent motif (PAM) sequences [11]. Soon after Cas9 was proven to cleave DNA in vitro, CRISPR is predicted to be repurposed for genome editing in other organisms [12, 13]. With the discovery of Cas proteins, a small RNA transcribed from a sequence immediately adjacent to the CRISPR locus called trans-activating CRISPR RNA (tracrRNA) and processed CRISPR RNA (crRNAs) have been shown required for specific DNA locus recognition [14]. To reprogram Cas9 with custom-designed spacers to cut any target site [15], people found fusing crRNA and tracrRNA to a single guide RNA (sgRNA) could direct Cas9 function [16]. Especially, Zhang group modified the original sgRNA and found a full-length fusion of sgRNA efficiently cut desired DNA loci in vivo [17]. Briefly, CRISPR systems contain two components: a guide RNA and a CRISPR-associated endonuclease (Cas proteins). Through the design of guide RNAs specific to the DNA locus of interest, CRISPR system can be freely used in any cells.

In this chapter, the steps of the generation of a CRISPR produced point mutation on *TP53* R249 locus in hESC H9 line will be described. First of all, design and generate guide RNA and corresponding donor vector targeting *TP53* genomic locus. Second, deliver these plasmids to H9 cells and following with selection and validation. Clone verification process contains several approaches, including PCR, sequencing, and southern blot. Selected clones need the excision of selection cassette from genome to minimize the impact of exogenous DNA on the insertion locus. This procedure has been used to precisely modify genes in various cell lines, as showed in many publication [18, 19].

2 Materials

2.1 gRNA Cloning

1. pX335-U6-Chimeric_BB-CBh-hSpCas9n (D10A) (Addgene, Plasmid no. 42335).
2. BbsI (BpiI) (New England BioLabs).
3. T4 DNA Ligase (New England BioLabs).
4. LB Agar.
5. 50× TAE.
6. Agarose.
7. DNA ladder mix (Goldbio).
8. QIAquick gel extraction kit (Qiagen).
9. QIAprep spin miniprep kit (Qiagen).
10. PCR machine.

2.2 T7 Endonuclease I Assay

1. OneTaq® Quick-Load 2× Master Mix (New England BioLabs).
2. NEB buffer 2 (New England BioLabs).
3. T7 endonuclease I (New England BioLabs).
4. Human embryonic kidney (HEK) 293T cells (ATCC).
5. Dulbecco's modified Eagle medium (DMEM) with high glucose (Life Technologies).
6. FBS.
7. L-Glutamine solution (GenDEPOT, cat. no. CA009-010).
8. Penicillin/streptomycin solution.
9. Lipofectamine 3000 (Life Technologies).
10. Opti-MEM (Thermo Fisher Scientific).

2.3 Donor Vector Cloning

1. OneTaq® Quick-Load 2× Master Mix (New England BioLabs).
2. pGEM-T Easy (Promega).
3. DpnI (New England BioLabs).
4. EcoRI (New England BioLabs).
5. BamHI (New England BioLabs).
6. NotI (New England BioLabs).
7. pFrt-PGK-EM7-NeoR-bpA-Frt (Addgene, Plasmid no. 22687).

2.4 H9 Human Embryonic Stem Cell (H9 hESC) Culture

1. hESC line H9 (WiCell Institute).
2. Matrigel.
3. DMEM/F12.
4. StemMACS™ iPS-Brew XF (Miltenyi Biotec).
5. Accutase Cell Detachment Solution (Corning).
6. hESC medium: DMEM/F12 with 20% KnockOut Serum replacement (Life Technologies), 1% Gibco GlutaMax (Life Technologies), 1% NEAA (Corning), 0.0007% β-mercaptoethanol (Sigma), and 10 ng/mL FGF2 (EMD Millipore).
7. Type A Gelatin from porcine skin (Sigma-Aldrich).
8. 0.1% Gelatin solution (wt/vol): To prepare 1 L of 0.1% (wt/vol) gelatin solution, dissolve 1 g of gelatin powder in 1000 mL distilled H_2O. Autoclave the solution, then filter with the 0.22 μm filter to eliminate any impurity.
9. Filter unit (500 mL, 0.22 μm).

2.5 Cell Electroporation

1. Embryo Max Electroporation Buffer (Millipore).
2. Electroporation Cuvettes (Bio-Rad).

3. Bio-Rad Gene PulserXcell System (Bio-Rad).
4. NEO-resistant MEFs, CF6Neo Irradiated, Neomycin Resistant (MTi GlobalStem).
5. G418 Sulfate Solution.
6. ROCK inhibitor Thiazovivin (Millipore Sigma).

2.6 PCR and Southern Blot Validation

1. Easy DNA gDNA purification kit (Life Technologies).
2. PCR DIG Probe Synthesis Kit (Roche).
3. Depurination solution: 250 mM HCl. Mix 20.80 mL of (37%) HCl solution with deionized water and make final volume to 1 L.
4. Denaturation solution: 0.5 M NaOH, 1.5 M NaCl. Dissolve 20 g NaOH and 87.75 g NaCl in a 1 L glass beaker containing 700 mL of deionized water. Mix well and make final volume to 1 L with deionized water.
5. Neutralization solution: 0.5 M Tris–HCl (pH 7.5), 1.5 M NaCl. Add 87.75 g NaCl to 500 mL 1.0 M Tris–HCl (pH 7.5) to a 1 L glass beaker. Mix well and add deionized water to a total volume of 1 L.
6. 20× SSC.
7. DIG wash and block buffer set (Roche).
8. Low stringency buffer: 0.1% SDS, 2× SSC. Add 1 g SDS and 100 mL 20× SSC buffer to deionized water, mix well, and adjust volume to 1 L.
9. High stringency buffer: 0.1% SDS, 0.5× SSC. Add 1 g SDS and 25 mL 20× SSC buffer to deionized water, mix well, and adjust final volume to 1 L.
10. Anti-Digoxigenin-AP, Fab fragments (Roche).
11. CDPstar chemiluminescent substrate (Roche).
12. Nylon membranes.
13. Hybridization bags.

2.7 FNF Cassette Removal

1. pCAGGS-flpE-puro (Addgene, # 20733).
2. Puromycin (RPI).

3 Methods

3.1 Design and Generate the CRISPR Plasmids to Target Your Gene of Interest

To make the precise modification of the gene of interest, homology-directed repair (HDR) is a good method to choose. In order to employ HDR in gene editing, a DNA repair template with the desired alteration, the gRNA and Cas9 nickase have to be introduced to your cell of interest at the same time. The DNA repair

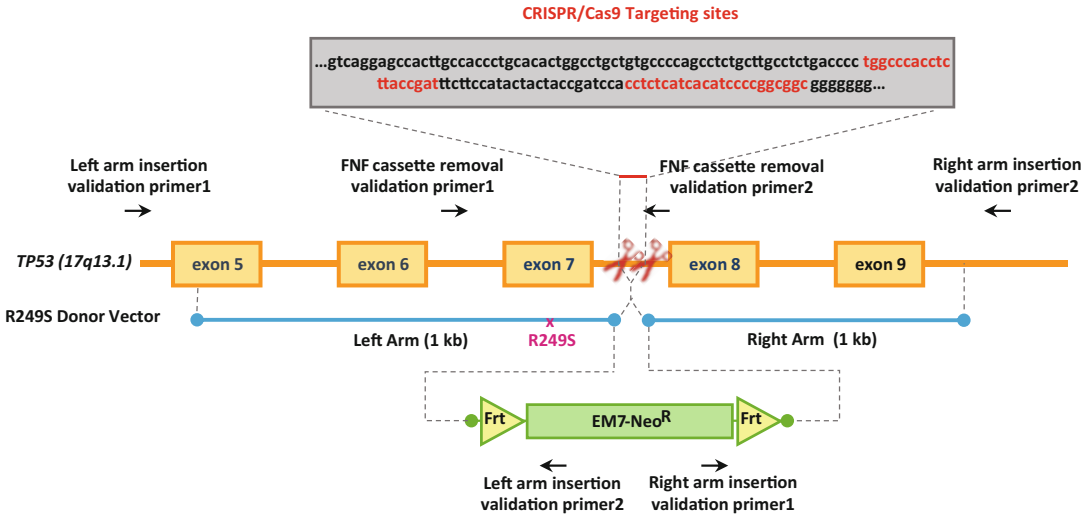

Fig. 1 Schematic diagram of CRISPR/Cas9 design for creating R249S mutation on *TP53*. The gRNA targeting loci are depicted and sequences are in red in the top box. The donor vector consists of the left homology arm (with p53 R249S mutation), right homology arm, and a FNF cassette. Gene-specific primers are designed for validation of the left arm and right arm insertion and FNF cassette removal

template is also named donor vector, which contains left and right homology arms and selection marker for picking up positive cells. The desired DNA alteration needs to be located within the homology arms, along with its upstream and downstream sequences. Figure 1 illustrates the design of donor vector and gRNAs to generate the point mutation that convert Arginine 249 to Serine 249 on *TP53* exon 7.

3.1.1 CRISPR Target Sites Selection, Guide RNA (gRNA) Design and Making

1. Searching CRISPR target sites for your gene of interest using the website: https://benchling.com/crispr. Import your target regions by type in the official gene symbol or transcript ID, or entry your own sequence. After define parameters, such as "single guide" or "pair guide," the website will analyze the gene or sequence you entered, then produce a list of guide oligoes each with its on-target and off-target scores. For instance, the official gene symbol of human p53 is *TP53* and its reference transcript ID is NM_000546.5. Click "Design CRISPR Guides" and select "Paired guides" to design gRNA sequence binding to sense and antisense strand. Choose the gRNA sequence with both higher on-target and off-target scores (*see* **Notes 1** and **2**). Two pairs of gRNA were selected for targeting *TP53*R249 locus (Fig. 1), and are shown in Table 1. Add BbsI enzyme sequence (5′-caccg-gRNA sense-3′; 5′-aaac-gRNA antisense-c-3′) to the gRNA oligoes for subsequent cloning convenience.

Table 1
gRNA oligoes for targeting p53/R249 locus

Oligo name	Sequence (5′–3′)
gRNA1 sense	*caccg*tggcccacctcttaccgat
gRNA1 antisense	*aaac*atcggtaagaggtgggccac
gRNA2 sense	*caccg*cctctcatcacatccccggcggc
gRNA2 antisense	*aaac*gccgccggggatgtgatgagaggc

2. Generate gRNA-expressing pX335 construct.
   (a) After received oligoes from commercial vendors, resuspend them at a concentration of 20 μM in sterile DNase/RNase-free ddH_2O.
   (b) Prepare 50 μL of the oligo duplex mixture by mixing 5 μL of 10× NEB buffer 2, 2 μM of forward and reverse oligo each in sterile DNase/RNase-free ddH_2O.
   (c) Boil 1 L water in a glass beaker on the heating block. Incubate the tube of the oligo duplex mixture in the boiling water for 5–8 min. Turn off the heater and let the temperature of the gRNA duplex mixture gradually down to room temperature, which usually takes 4–6 h. The annealed gRNA duplex can be kept at 4 °C for later use (*see* **Note 3**).
   (d) Digest 6 μg of pX335 vector with BbsI, then run the digested vector on a 0.8% (wt/vol) agarose gel. Cut and extract the digested vector from the agarose gel with a gel extraction kit. Ligate the purified vector with annealed gRNA duplex in a mixture of 1 μL annealed gRNA oligo, 0.5 μL digested vector, 1 μL 10× NEB T4 DNA ligase buffer, 1 μL NEB T4 DNA ligase, and 6.5 μL ddH_2O. Incubate the ligation mixture at room temperate for 1 h or 16 °C overnight. Following with transformation of gRNA-vector ligation product into DH5α competent cells.
3. Validate the pX335-gRNA construct.
   Pick up antibiotic-resistant clones, culture them, isolate plasmids by using the miniprep kit, and check gRNA insertion with Sanger sequencing. The sequencing primer for this particular construct is: 5′-TGCATATACGATACAAGGCTGTTAG-3′.

3.1.2 Test gRNA Targeting Efficiency Using T7 Endonuclease 1 Assay

1. Plate HEK293T cells in 6 cm cell culture dishes using DMEM + 10% FBS + 1% L-Glutamine + 1% P/S medium.
2. Start the transfection until cells reach 50% confluence. Prepare the transfection mixture with 3 μg pX335-gRNA plasmid in 300 μL of Opti-MEM I reduced-serum medium. Mix the DNA cocktail with 10 μL Lipofectamine 3000 as describe by manufacturer protocol.

3. Harvest cells at 48 h after transfection and isolate genomic DNA using genomic DNA extraction kit.
4. Amplify fragments bracketing gRNA targeted region using primers (5′-TGTAAAACGACGGCCAG TGCCTCCCCTG CTTGCCACAG-3′ and 5′-CAGGAAACAGCTATGACCGGG AGCAGTAAGGAGATTCC-3′). Run 10 μL PCR product on 2% agarose gel to make sure one single fragment as the predicted size (*see* **Note 4**).
5. Reannealing 13.6 μL PCR product with 1.6 μL NEB buffer 2 using the cycle in Table 2 to generate heteroduplex DNA.
6. Add 0.4 μL NEB buffer 2 and 0.2 μL T7 endonuclease I to the heteroduplex DNA mixture and digest at 37 °C for 30 min. Run the digested product on a 2% agarose gel. Multiple cleavage bands should be present below the original PCR product if gRNA can target the designed region (Fig. 2). Choose the gRNA pair with the highest cleavage efficiency for future step.

Table 2
Reannealing program

Temperature (°C)	Time
95	5 min
95–85	−2 °C/s
85–25	−0.1 °C/s
25	10 min

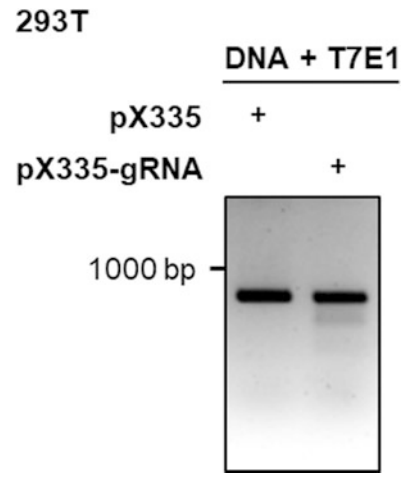

Fig. 2 Representative result of T7E1 assay. The right lane shows T7E1 assay performed after transfection of the gRNA vectors, while the left lane shows negative control after transfection of empty vectors

3.1.3 Donor Vector Design and Synthesis

1. As shown in Fig. 1, the left and right homology arms are at up- and downstream of the gRNA targeting site, respectively. Both arms have been designed about 1 kb long, flanking several *TP53* exons and introns (*see* **Note 5**). Using the H9 hESC genomic DNA as template and the specific primers amplify homology arms (Fig. 1). The left arm primers are 5′-GAA TTCCGCGTCCGCGCCATGGCCATCTACAAGCAGT CAC AG-3′ and 5′-GAATTCAGGCCAGTGTGCAGGGTGGCAAG TGGCTCCTGACCT-3′. The right arm primers are 5′-GGA TCCGCTGTGCCCCAGCCTCTGCTTGCCTCTGACCCCT GG-3′ and 5′-GCGGCCGCCAGGCTAGGCTAAGCT ATGAT GTTCCTTAGATTAGG-3′. After confirming the PCR product on a 0.8% agarose gel, cut and extract the band from the agarose gel with a gel extraction kit. Ligate it with a pGEM-T Easy vector according to manufacturer's protocol. Incubate the ligation mixture at room temperate for 1 h or 16 °C overnight. Following with transformation of pGEM-T vector ligation product into DH5α competent cells.
2. Generate the R249S mutation in the left arm/pGEM-T construct by PCR using the primers: 5′-TGCATGGGCGGCATG AACCGGAGTCCCATCCTCACCATC-3′ and 5′-GATGGT GAGGATGGGACTCCGGTTCATG CCGCCCATGCA-3′. Digest the PCR product using DpnI at 37 °C for 2 h and then transform digestion product into DH5α cells.
3. Pick up antibiotic-resistant clones, culture them, isolate plasmids by using the miniprep kit, and send for Sanger sequencing to validate the point mutation.
4. Digest the left homology arm/pGEM-T construct with EcoRI and right homology arm/pGEM-T construct with BamHI and NotI to get the left and right arm fragments. At the first time digest the pFrt-PGK-EM7-NeoR-bpA-Frt (pFNF) vector with EcoRI, then ligate the left arm fragment with this digested pFNF vectors using T4 ligase. Following with transformation of ligation product into DH5α competent cells. Isolate plasmids and confirm the successful insertion of left arm in the pFNF vector. Next, digest this left arm containing pFNF vector with BamHI and NotI, then ligate with right arm fragment, flowing with transformation and confirmation. The end pFNF plasmid should contain both left arm and right arm with desired DNA modification.

3.2 Deliver gRNA and Donor Vector into the Target Cells

1. Maintain H9 hESCs in StemMACS™ iPS-Brew XF medium on Matrigel-coated plates for 2 weeks to reach its optimal condition for electroporation.

   Making Matrigel coating plates: Thaw Matrigel aliquots for at least 3–4 h on ice or at 4 °C overnight. Dilute Matrigel at a 1:50 ratio in DMEM/F12 medium. Add 4 mL diluted Matrigel into a 10 cm plate and gently shake the plate until the plate

is evenly covered. Leave the Matrigel-coated plate at room temperature for 30 min and then remove any remaining Matrigel by aspiration before plating cells. Otherwise, keep Matrigel inside plates and place at 4 °C overnight. The plate is ready to use the next day.

2. On day 1, prepare MEF feeder cells coated culture dishes.
   (a) Making gelatin-coated culture dishes for MEF feeder cells. Use 5 mL 0.1% gelatin solution to cover a 10-cm plate, put into a 37 °C incubator for at least 15 min. Before use, aspirate off the gelatin and seed cells immediately. For a long time storage, more solution should be used. Gelatin-coated plates can remain in a 37 °C incubator for up to 7 days before use.
   (b) Prepare MEF feeder cells plated culture dishes. The maintenance of clonal H9 ESCs requires supporting from MEF feeder cells. Thaw a cryo vial of NEO-resistant MEFs and plate them onto four to six gelatin-coated 10-cm plates. The dilution of MEFs seeding on culture dishes should be determined by the frozen cell concentration or according to the provided protocol. Incubate overnight at 37 °C and allow cells to attach.
3. On day 2, perform the electroporation transfection of H9 hESCs.
   (a) H9 hESCs are grown in StemMACS™ iPS-Brew XF medium on one 10 cm Matrigel-coated dish at 80–90% confluency. Remove the medium and wash with 5 mL 1× DPBS. Replace the DPBS with 1 mL Accutase and incubate at 37 °C for 3–5 min. Resuspend cells in 10 mL DMEM/F12 medium and centrifuge the cells at 1000 rpm, 4 °C for 5 min.
   (b) Aspirate and discard the supernatant. Resuspend cells in 10 mL Opti-MEM medium to remove remaining FBS, Accutase, etc. in the medium. Count cells and centrifuge 1×10^7 cells for each electroporation reaction at 1000 rpm, 4 °C for 5 min. Remove the supernatant and resuspend cells (1×10^7) in 600 μL Embryo Max electroporation buffer (EEB).
   (c) Mix 50 μg pFNF donor vector plasmid, 5 μg pX335-gRNA1 and 5 μg pX335-gRNA2 in a total volume of 50 μL. Then add this DNA mixture to cells/EEB suspension drop wise and mix well.
   (d) Transfer the cells/DNA/EEB mixture into electroporation cuvettes, and immediately perform electroporation at 300 V/500 μF.
   (e) Transfer transfection mixture into 12 mL hESC medium containing conical tube right after the electroporation.

Seed cells to six 10 cm MEF-coated dishes with different cell solution volumes (1, 1, 2, 2, 3, and 3 mL). Add hESC medium with 2 μM ROCK inhibitor to a final volume of 10 mL.

4. On day 3, check cells and replace with 10 mL fresh hESC medium with 50 μg/mL G418.
5. Change with fresh hESC medium plus 50 μg/mL G418 every 2–3 days for clone growth.
6. When clones are visible, remove the medium and rinse the cells twice with 5 mL 1× DPBS. Prepare Matrigel-coated 48-well plate, and add 500 μL hESC medium with 2 μM ROCK inhibitor to each well. Pick up every single clone using 200 μL pipet tips and plant to each well.

3.3 Validation of the Positively Modified Clones by PCR, Sanger Sequencing, and Southern Blot

3.3.1 Confirmation of Donor Vector Insertion at Correct Locus by PCR

1. When a single clone expands to 90% confluency in one well of 48-well plate, passage all cells to one well of 6-well plate. When the cells in one well of 6-well plate are 90% confluent, keep 10% of the cells for continuing culture, and extract genomic DNA from 90% of the cells using Easy-DNA gDNA purification kit following the manufacturer's protocol.
2. PCR amplify the inserted region with primers (5′-TGTAAAA CGACGGCCAGTCT AGCTCGCTAGTGGGTTGC-3′ and 5′-TCCAGACTG CCTTGGGAAA-3′ for the left arm, and 5′-GGGGAGGATTGGGAAGACAA-3′ and 5′-CAGGAAA CAGCTATGACCGCCCA GGAGGGTATAATGAGCTA-3′ for the right arm).
3. Run the PCR product on a 0.8% agarose gel using 1× TAE buffer until good separation is achieved. The single band at correct size (Fig. 3) indicates the precise insertion into the correct locus.

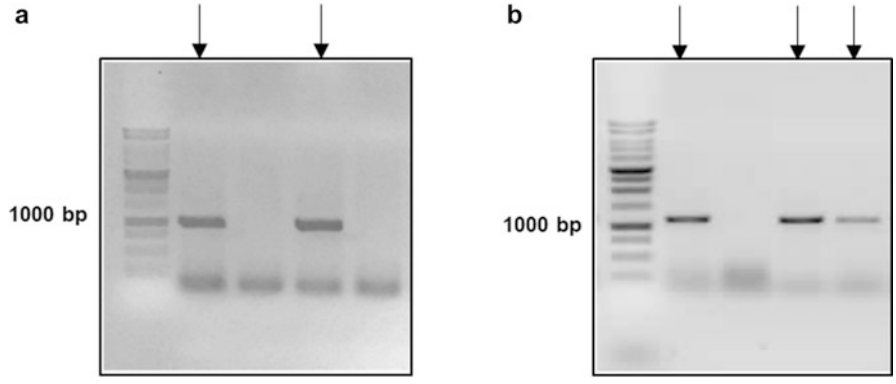

Fig. 3 Representative results of PCR validation of left homology arm and right homology arm insertion. Arrows indicate positive left arm insertion clones with a band around 1000 bp in panel (**a**), and positive right arm insertion with a band around 1200 bp in panel (**b**)

3.3.2 Sanger Sequencing

Pick positive clones according to above PCR results. Perform gel purification of the PCR products and send them for Sanger sequencing to confirm the knock-in point mutation in the selected clones. The primer for sequencing is 5′-TCCAGACTGCCTTGGG AAA-3′.

3.3.3 Southern Blotting

PCR and Sanger sequencing prove the presence of desired modification at the correct location; however, these assays are not able to exclude the possibility of multiple insertion of the donor vector. Southern blotting is the only method to identify single insertion clones.

1. Synthesize a probe that targets the neomycin region using primers 5′-ATGGGATCGGCCATTGAACAAGAT-3′ and 5′-TCAGAAGAACTCGTCAAGAAGGCG-3′ according to the instruction of PCR DIG Probe Synthesis Kit.
2. Digest 10 μg of genomic DNA from positive clones overnight at 37 °C water bath with *BamHI*. Load the digested products on a 0.7% agarose gel 100–150 V electrophoresis for 60 min to achieve a good separation.
3. Prepare the gel for transfer.
   (a) Wash the gel once for 10 min in the depurination solution with gentle shaking, then rinse the gel once with ddH_2O.
   (b) Wash the gel twice for 15 min each time in the denaturation solution with gentle shaking, then rinse the gel once with ddH_2O.
   (c) Wash the gel twice for 15 min each time in the neutralization solution with gentle shaking.
   (d) Rinse the gel once for 10 min in the 20× SSC buffer.
4. Set up the blot transfer "sandwich," and transfer DNA to the nylon membrane overnight in 2× SSC buffer. Disassemble the transfer "sandwich" the next day and mark the wells of the gel on the nylon membrane with a pencil.
5. Expose the wet membrane to UV light at 120 mJ energy to cross-link the membrane. Rinse the membrane briefly with ddH_2O.
6. Preheat the hybridization oven and pre-warm DIG Easy Hyb buffer at 42 °C. Place the membrane into a hybridization bag, fill with DIG Easy Hyb buffer, seal the bag around the membrane, and eliminate any air bubbles. Put the bag on the rotator in the hybridization oven, incubate for 30–60 min.
7. Add 4 μL of probe to a 1.5 mL Eppendorf tube containing 50 μL ddH_2O. Place the tube to boiling water bath for 5 min to denature the probe. Chill the denatured probe immediately on

ice for 2 min. Add the denatured probe to 13 mL pre-warmed DIG Easy Hyb buffer to make the hybridization buffer. Replace the DIG Easy Hyb buffer in the hybridization bag with probe-containing hybridization buffer and avoid making air bubbles. Incubate the membrane in the hybridization buffer at 42 °C in the oven with rotation overnight.

8. Stop the hybridization and prepare the membrane for signal detection.
   (a) Transfer the membrane onto a plastic tray the next day. Wash the membrane twice for 5 min each time with 100 mL low stringency buffer.
   (b) Preheat the high stringency buffer to 68 °C. Wash the membrane three times for 10 min each time in pre-warmed 100 mL high stringency buffer with gentle shaking.
   (c) Block the membrane at room temperature by incubation with 100 mL blocking solution for 30 min.
   (d) Incubate the membrane with 50 mL diluted Anti-Digoxigenin-AP solution (1:5000 diluted in blocking solution) for 30–60 min with gentle shaking.
   (e) Wash the membrane twice with 100 mL washing buffer.
9. Incubate the membrane with 20 mL detection buffer for 3 min. Pour off the detection buffer and add 1–2 mL CDPstar chemiluminescent substrate to cover the membrane. Use pipette to make the substrate distribute evenly on the membrane. Incubate the membrane at room temperature for 2 min. Remove excess chemiluminescent substrate and place the membrane into a film cassette. Expose the film.
10. Single band with the predicted size indicates there is no off-target insertion. Choose clones with single insertion for the following experiments (Fig. 4).

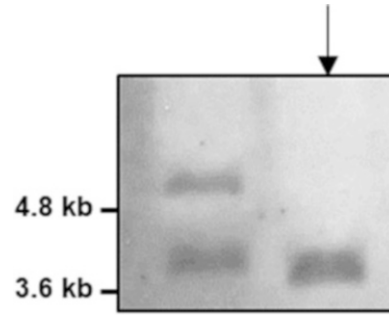

Fig. 4 Representative result of Southern blot. The right lane demonstrates a positive clone without off-target insertion

3.4 The FNF Cassette Removal

After examined by PCR, sequencing, and Southern blot, the correct clone will be picked for FNF cassette removal. In order to eliminate the risk that the FNF cassette affects normal gene function around the insertion locus, it is necessary to remove the cassette from the correct clone genome.

3.4.1 Express Flp Recombinase into Correct Clone Cells

1. Day 1, seed the picked H9 hESC clones into a Matrigel-coated 6-well plate, grow cells at 37 °C and 5% CO_2 overnight to reach 70% confluence at transfection.
2. Day 2, transfect the cells with 2 μg pCAGGS-flpE-puro plasmid each well of the 6-well plate using Lipofectamine 3000 reagent according to the manufacturer's protocol. The pCAGGS-flpE-puro plasmid will express Flp recombinase to excise neomycin-resistant cassette from the genome, at the same time expressing puromycin resistance to provide the selection opportunity.
3. Day 3, change cells with fresh hESC medium, and it is better to do this in the morning.
4. Day 4, 48 h after transfection, begin puromycin treatment at the dosage of 0.5 μg/mL (*see* **Note 6**). Only treat cells with puromycin for 2 days. At the end of puromycin treatment, cells without successful pCAGGS-flpE-puro plasmid transfection will be killed. Gently wash cells with medium once, replace with fresh hESC medium and continue culturing cells for 1–2 more days.
5. Day 6, prepare MEF feeder cells plated culture dishes. Thaw a cryo vial of MEFs and plate them onto four to six gelatin-coated 10-cm plates. The dilution of MEFs seeding on culture dishes should be determined by the frozen cell concentration or according to the provided protocol. Incubate overnight at 37 °C allow cells to attach.
6. Day 7, wash transfected and puromycin treated H9 hESC cells with 2 mL 1× DPBS, add 0.5 mL Accutase into each well, and incubate at 37 °C for 5 min. Neutralize cells with 2 mL fresh medium, and centrifuge cells at 1000 rpm for 5 min. Resuspend cells with 10 mL medium. Count cells and seed 500 cells to each MEF-coated 10 cm plate.
7. Change medium every 2–3 days until clones are visible. Pick up single clones using 200 μL tip to each well of Matrigel-coated 48-well plate.

3.4.2 Validation of Successful Removal of the FNF Cassette

1. When cells are confluent in 6-well plate, maintain 10% of the cells for continuing culture and extract genomic DNA from 90% of the cells using Easy-DNA gDNA purification kit.
2. Use primers flanking the CRISPR editing locus (Fig. 1) (5′-TGTAAAACGACGGCCAGTGCCTCCCCTGCTTG CCACAG-3′ and 5′-CAGGAAACAGCTAT GACCGGGAG-CAGTAAGGAGATTCC-3′) to perform PCR examination.

Fig. 5 Representative result of FNF cassette removal by PCR validation. The right lane shows a positive clone without FNF cassette. The upper band indicates the mutant allele with a Frt scar and the lower band indicates the WT allele without any genome editing

Run the PCR product on a 0.8% agarose gel until the separation is ideal. A 329 bp PCR fragment indicates a WT *TP53* allele without any genomic editing. A 363 bp product (329 + 34 bp Frt scar) indicates a mutant *TP53* allele with Frt insertion, which the majority of FNF cassette has been excised (Fig. 5). At this time the clone is ready to expand for future applications.

4 Notes

1. Several criteria need to be met when designing the gRNA. It is better to choose gRNA located in the intron region and avoid any splicing sequence to reduce the risk of unwanted change on the target DNA. Make sure not to include PAM sequence in the oligoes.
2. When using "https://benchling.com/crispr" for gRNA design, it provides multiple candidates to choose from. Oligoes with higher on- and off-target scores usually work better, but has exceptions. Start with several pairs of gRNA with the highest scores, and test their efficiency by T7 E1 assay before delivery into cells.
3. Cooling down the gRNA duplex mixture slowly is essential for annealing gRNA oligoes perfectly. After turning off the heater, the beaker can be covered to prevent fast temperature dropping.
4. The PCR product size could be variable depending on the genomic DNA region, the length around 500 bp is an optimal size for following steps.
5. The length of left and right homologous arms depends on the genomic region of your target gene; usually, the longer the gene, the longer the homology arms. Also, it tends to design longer homology arm for increasing homologous recombination efficiency. Typically, one side of homology arm is 500–1000 bp.
6. It is necessary to test the tolerance dosage of puromycin for your cell of interest. Generally, the dose is ranging between 0.5 and 2 μg/mL for mammalian cells.

References

1. Bibikova M et al (2001) Stimulation of homologous recombination through targeted cleavage by chimeric nucleases. Mol Cell Biol 21 (1):289–297
2. Zhang F et al (2011) Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. Nat Biotechnol 29(2):149–153
3. Miller JC et al (2011) A TALE nuclease architecture for efficient genome editing. Nat Biotechnol 29(2):143–148
4. Xu A et al (2018) Establishment of a human embryonic stem cell line with homozygous TP53 R248W mutant by TALEN mediated gene editing. Stem Cell Res 29:215–219
5. Zhou R et al (2018) A homozygous p53 R282W mutant human embryonic stem cell line generated using TALEN-mediated precise gene editing. Stem Cell Res 27:131–135
6. Mojica FJ, Juez G, Rodriguez-Valera F (1993) Transcription at different salinities of Haloferax mediterranei sequences adjacent to partially modified PstI sites. Mol Microbiol 9 (3):613–621
7. Mojica FJ et al (2005) Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. J Mol Evol 60 (2):174–182
8. Pourcel C, Salvignol G, Vergnaud G (2005) CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. Microbiology 151 (Pt 3):653–663
9. Bolotin A et al (2005) Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. Microbiology 151(Pt 8):2551–2561
10. Jansen R et al (2002) Identification of genes that are associated with DNA repeats in prokaryotes. Mol Microbiol 43(6):1565–1575
11. Marraffini LA, Sontheimer EJ (2008) CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. Science 322 (5909):1843–1845
12. Deveau H et al (2008) Phage response to CRISPR-encoded resistance in Streptococcus thermophilus. J Bacteriol 190(4):1390–1400
13. Horvath P et al (2008) Diversity, activity, and evolution of CRISPR loci in Streptococcus thermophilus. J Bacteriol 190(4):1401–1412
14. Deltcheva E et al (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature 471(7340):602–607
15. Gasiunas G et al (2012) Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. Proc Natl Acad Sci U S A 109(39): E2579–E2586
16. Jinek M et al (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337 (6096):816–821
17. Cong L et al (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339(6121):819–823
18. Tu J et al (2018) Generation of human embryonic stem cell line with heterozygous RB1 deletion by CRIPSR/Cas9 nickase. Stem Cell Res 28:29–32
19. Huo Z et al (2019) Generation of a heterozygous p53 R249S mutant human embryonic stem cell line by TALEN-mediated genome editing. Stem Cell Res 34:101360

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