Chapter 8
CRISPR/Cas9-Mediated Genome Editing in Human Pluripotent Stem Cells

Jian-Ping Zhang, Amanda Neises, Tao Cheng and Xiao-Bing Zhang

Abstract Induced pluripotent stem cells (iPSCs) hold great promise for gene and cell therapies. Correction of a diseased gene is often required before conducting directed differentiation of iPSCs. In addition, creation of iPSC reporter lines greatly facilitates high-throughput screening and other applications. Recent advances in the CRISPR genome editing technology have made it possible to readily accomplish these goals. Here, we describe a step-by-step procedure to efficiently generate a GFP reporter iPSC line by using a Cas9-sgRNA vector and an optimized donor template plasmid.

Keywords Human pluripotent stem cells · CRISPR · Cas9-sgRNA · Genome editing · Knock-in

8.1 Introduction

The generation of human induced pluripotent stem cells (iPSCs) from adult somatic cells holds great potential for gene and cell therapies. iPSCs are considered an ideal source of autologous cells for cell replacement therapy because iPSCs can be induced to differentiate into more than 200 types of cells. iPSCs can be generated from fibroblasts derived from a skin biopsy or trace amount of renal proximal

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tubular cells present in urine [1]. We and other investigators have generated integration-free iPSCs using mononuclear cells from peripheral blood (PB) [2–6]. PB is the easy to access, minimally invasive, and the most abundant cell source in human body for generating iPSCs. PB-derived iPSCs may be particularly useful in generating blood products, as one can take advantage of the epigenetic memory of these cells [7].

For iPSC-based cell replacement therapy, one needs to correct the diseased gene before differentiating them into cells of clinical interest. On the other hand, to facilitate the efforts in identifying novel approaches for directed differentiation of iPSCs, one may need to generate an iPSC reporter cell line. A recent technological breakthrough has made it possible to edit the genome rapidly and economically. This exciting technology is called CRISPR, or the clustered regularly interspaced short palindromic repeat, which uses a single-guide RNA (sgRNA) in complex with a CRISPR-associated nuclease Cas9 to search and bind with the complementary sequence on the genome, followed by excision of double-stranded DNA at the precise target locus [8, 9].

The CRISPR-Cas9 system has been widely used in genome editing due to the ease of vector construction and high targeting efficiency. A single-guide RNA (sgRNA) targets Cas9 to genomic regions that are complementary to the 20-nucleotide (nt) target region of the sgRNA that contains a 5′-NGG-3′ protospacer-adjacent motif (PAM). The Cas9 nuclease generates a DNA double-strand break (DSB) three base pairs upstream of the PAM [10–12]. The DSB is often repaired by the error-prone non-homologous end-joining (NHEJ) pathway, which induces nucleotide substitution, insertions and deletions (indels) and may lead to frameshift mutations [13, 14]. Alternatively, the DSB may be repaired by high-fidelity homologous recombination (HR) if an exogenous DNA template flanked by homologous sequences to the target site is provided. Targeted editing allows for correction of disease-causing mutations by replacing a mutated gene directly or the insertion of a functional copy of the affected gene into a safe genomic harbor or downstream of its own promoter [15–18].

In an early report, the gene knock-in efficiency using the CRISPR-Cas9 system in human pluripotent stem cells was relatively low, ranging from 1 to 4 % [10]. To enhance its efficiency, investigators have optimized this approach by (a) optimization of transfection; (b) enrichment of the transfected cells; (c) optimizing the design of donor DNA template by flanking it with sgRNA target sites, thereby inducing the cleavage of a donor template plasmid by a Cas9–sgRNA complex; and (d) using NHEJ inhibitors to improve HR efficiency [19–23]. The specificity of CRISPR-Cas9 system was considered a major safety concern for clinical applications [24]; however, whole-genome sequencing studies showed low incidence of off-target mutations induced by Cas9-sgRNA in human iPSCs [25, 26]. Recently, we and other investigators have shown that targeting the specificity can be substantially increased by the use of truncated guide RNAs. When the length of sgRNA is reduced from 20 to 17 bp or 18 bp, the off-target efficiency can be reduced by 1000-fold [12].

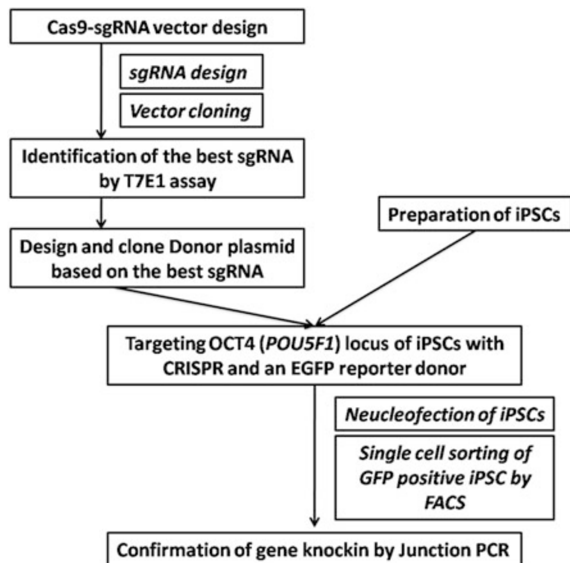

Fig. 8.1 An outline of procedures to generate a GFP reporter human iPSC line at OCT4 locus

In this chapter, to exemplify genome editing in iPSC cells, we describe a step-by-step procedure to generate a GFP reporter line at the OCT4 (POU5F1) locus (Fig. 8.1). We recommend designing three sgRNAs to target the DNA surrounding the stop codon of OCT4, followed by identifying the best sgRNA with the highest cleavage efficiency. The cleavage efficiency will be determined by a T7E1 mutation detection assay, an enzyme mismatch cleavage method, after transfecting 293T cells with individual sgRNA together with Cas9. To target the C terminus of OCT4, the stop codon of OCT4 is deleted and the last OCT4 codon is fused in frame with a 2A-GFP-Wpre sequence, where 2A is a self-cleaving peptide. To enhance the knock-in efficiency, the sgRNA target sequence is also included to flank the donor template. After the GFP knock-in, GFP is under the control of the endogenous OCT4 promoter; thus, the GFP intensity reflects the OCT4 expression in cells. As follows, we describe in detail the optimized design of sgRNA targeting system to improve efficiency and specificity, culture of iPSCs, and nucleofection of iPSCs with sgOCT4 and a GFP reporter donor and conclude with the selection of the GFP-positive clones by flow cytometry.

8.2 Materials

8.2.1 Cell Culture

1. 293T culture medium. DMEM (Dulbecco’s modified Eagle medium; HyClone, Cat. No. SH30243.01B) was supplemented with 10 % fetal bovine serum (FBS;

GIBCO, Cat. No. 16000-044) and 1 % P/S (penicillin/streptomycin; ABM, Cat. No. G255). Store the medium at 4 °C for up to 4 weeks.
2. iPSC culture medium. PB-derived iPSCs were generated in our laboratory. iPSCs were maintained in E8 medium (Essential 8 Medium; GIBCO, Cat. No. A15169-01).
3. 0.25 % trypsin/EDTA (GIBCO, Cat. No. 25200-056).
4. Accutase (GIBCO, Cat. No. A11105-01).
5. 0.5 mm EDTA (cell disassociation buffer). Dilute 500 μl of 0.5M EDTA (GIBCO, Cat. No. 15575) in 500 ml PBS.
6. ROCK inhibitor (Y-27632; ABM, Cat. No. G604).
7. Matrigel-coated plates. Dissolve 1 ml Matrigel (BD, Cat. No. 354230) in 50 ml cold Knockout DMEM/F12 medium (GIBCO, Cat. No. 12660-012). Add 1 ml diluted Matrigel to each well of 6-well plates. Incubate at 37 °C for 1 h or at 4 ° C for overnight. Aspirate out the medium before cell culture.
8. T7E1 (Peking polymath technology, Cat. No. E001L).

8.2.2 Transfection Reagents and Nucleofection Kits

1. Lipofectamine 2000 (Invitrogen, Cat. No. 11668-019).
2. Human Stem Cell Nucleofector® Kit (Lonza, Cat. No. VPH5022).

8.2.3 DNA Extraction, PCR, and Cloning

1. TaKaRa MiniBEST Universal Genomic DNA Extraction Kit (Takara, Cat. No. 9765).
2. QIAquick PCR purification kit (Qiagen, Cat. No. 28104).
3. 2× FastPfu PCR SuperMix (TransGen, Cat. No. as221-1).
4. KAPA HiFi polymerase (Kapa Biosystems, Cat. No. KK2102).
5. Phusion® High-Fidelity PCR Master Mix with HF Buffer (NEB, Cat. No. M0531).
6. CloneJET PCR Cloning Kit (Thermo, Cat. No. K1232).
7. Gibson Assembly® Cloning Kit (NEB, Cat. No. E5510S).

8.2.4 Plastics and Equipment

1. TC-treated 6-well plates (BD Falcon; Cat. No. 353046).
2. 5- or 15- or 50-ml polystyrene tubes (BD Falcon).

3. Pipettes and pipettors.
4. Nucleofector (Lonza; Amaxa II).
5. CO_2 incubator.
6. Centrifuge.
7. PCR thermal cycler.
8. FACSAria II flow cytometer.
9. Inverted microscope.

8.3 Methods

8.3.1 sgRNA Design and Vector Cloning

8.3.1.1 sgRNA Design

1. Retrieve the sequence of ~100 bp surrounding OCT4 stop codon by using Genome Browser (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start).
2. Design regular 20-bp sgRNAs at CHOPCHOP Web site (https://chopchop.rc.fas.harvard.edu/) by pasting the input sequence. Truncate the length of the sgRNA to 17 or 18 bp by deleting 2–3 bases at the 5′ end (see note 1).
3. Preferentially pick sgRNAs with a G or an A at the 3′ end. The targeting sequences are (N16)GNGG, (N16)ANGG, (N16)CNCC, or (N16)TNCC (see note 2).
4. Preferentially pick sgRNAs with a G at the 5′ end. If the first nucleotide of sgRNA is an A, C, or T, add a G in front of it, as U6 promoter-mediated transcription starts at a G.
5. Search off-target sites at TagScan (http://ccg.vital-it.ch/tagger/tagscan.html). For 17-bp sgRNAs, abandon the ones that have a perfect match at other loci of the human genome. For 18-bp sgRNAs, abandon the ones that have less than 1 mismatch at other loci of the human genome.

8.3.1.2 Cas9-sgRNA Vector Cloning

6. Use plasmid pU6-sgBbsI-EF1-Cas9-E2A-Puro-wpre to provide the sgRNA vector backbone.
7. Digest pU6-sgBbsI-EF1-Cas9-E2A-Puro-wpre vector with BbsI enzyme at 37 °C overnight (see note 3).

Prepare the following mixture:

Plasmid	5 μg
BbsI	3 μl
10× buffer	5 μl
H_2O	Bring total volume to 50 μl

Run the PCR product on a 1 % agarose gel at 70 V for 2 h. Cut out the correct band (9.5 kb) and purify the PCR products with the QIAquick PCR purification kit according to the manufacturer's instructions.

8. Synthesize sgRNA template: TATATATCTTGTGGAAAGGACGAAACACCG NNNNNNNNNNNNNNNNN GTTTTAGAGCTAGAAATAGCAAGTTAAAAT. PCR primers are listed as follows: sgRNA-F: TATATATCTTGTGGAAAGGACGAA and sgRNA-R: ATTTTAACTTGCTATTTCTAGCTCTAA. Use the KAPA HiFi polymerase to amplify the sgRNA product, using the following cycling conditions: 98 °C for 2 min, 1 cycle; 98 °C for 5 s, 60 °C for 20 s, 20 cycles. Purify the PCR products with the QIAquick PCR purification kit according to the manufacturer's instructions.
9. Assemble 100 ng of the sgRNA backbone and 10 ng of the sgRNA PCR product using Gibson Assembly® Cloning Kit in a total volume of 20 μl. Incubate in a thermocycler at 50 °C for 15 min. After incubation, use 1 μl for transformation. After overnight culture at 37 °C, pick 3 clones into 15-ml tubes with 1–2 ml LB medium and shake at 37 °C for 8–16 h.
10. Isolate the plasmid DNA from cultures by using a QIAprep Spin Miniprep Kit according to the manufacturer's instructions. Verify the sequence of each clone by Sanger sequencing. The sequencing primer is U6-F: GGGCAGGAAGAGGGCCTAT.
11. Pick the clone with correct insert. Culture 100 μl *Escherichia coli* in 250 ml LB medium for 16–18 h, followed by extracting plasmids with Qiagen Plasmid Plus Maxi Kit.

8.3.2 Identification of the Best sgRNA by T7E1 Assay

8.3.2.1 Preparation of 293T Cells for Transfection

1. 293T cells are maintained in 6-well plates. Passage cells when reaching 80–90 % confluency.
2. To passage, remove the medium and add 2 ml PBS to rinse the cells.
3. Remove the PBS, add 0.4 ml of 0.25 % trypsin/EDTA to each well, and incubate at 37 °C.

4. After 3–5 min of incubation, add 1 ml of warm DMEM/10 % FBS to inactivate the trypsin, dissociate the cells by pipetting them up and down gently, and then transfer the cells to a 15-ml tube.
5. Count the cells using a hemacytometer.
6. Seed the cells into 24-well plates at a density of $1–2 \times 10^5$ per well in a total volume of 500 μl (see note 4).

8.3.2.2 Transfection of 293T Cells Using Lipofectamine 2000

7. One day later, transfect 0.5 μg of the Cas9-sgOCT4 plasmid and include a GFP plasmid control to 293T cells, following Lipofectamine 2000 manufacturer's instructions.
8. Add lipofectamine and DNA mix to the cells gently.
9. Check the transfection efficiency after 24 h by using a fluorescence microscope (if the GFP control plasmid was cotransfected).
10. Incubate the cells for a total of 72 h after transfection.

8.3.2.3 Harvesting Cells for DNA Extraction

11. 72 h after transfection, dissociate the transfected cells and harvest them by centrifugation at 1000 rpm for 5 min at room temperature.
12. Aspirate out the medium completely, leaving the cell pellet at the bottom of the tube.
13. Isolate the genomic DNA using Genomic DNA Extraction Kit (Qiagen) according to the manufacturer's instructions.

8.3.2.4 Amplifying Targeted Locus by PCR

14. Dilute the extracted DNA to a final concentration of 100–200 ng/μl with ddH2O.
15. Set up a 20 μl PCR reaction using the 2× FastPfu PCR SuperMix (see note 5) as follows:

2× FastPfu PCR SuperMix	10 μl
Forward primer	1 μl
Reverse primer	1 μl
DNA template	1 μl
H_2O	7 μl

16. Perform a PCR with the following cycling conditions (see note 6):

    1 cycle: 98 °C for 2 min (initial denaturation)
    35 cycles: 98 °C for 10 s (denaturation)
    62 °C for 20 s (annealing)
    72 °C for 1 min (extension)
    1 cycle: 72 °C for 2 min (final extension)

17. Run 2–5 μl of the PCR product on a 1 % agarose gel to verify the amplification.
18. Purify the PCR product with the QIAquick PCR purification kit according to the manufacturer's instructions (see note 7).

8.3.2.5 Digestion of the DNA Heteroduplex with T7E1

19. Mix 10 μl of the PCR product, 1 μl T7E1, 2 μl 10× T7E1 buffer, and 7 μl H_2O to a total volume of 20 μl.
20. Incubate for 30 min at 37 °C.
21. Load 10 μl of the sample with loading buffer on a 2 % agarose gel.
22. Run the gel until the loading buffer (blue dye) has migrated to the bottom of the gel.
23. Capture the gel image with a UV imaging station.

8.3.2.6 Calculation of the Cleavage Efficiency

24. Save the gel image in the TIFF format that can be opened in ImageJ.
25. Measure the integrated intensity of the PCR amplicon and cleaved bands by using ImageJ.
26. Estimate cutting efficiency using the following equation:

$$\text{indel}\% = 100 \times (1 - (1 - \text{f}_{\text{cut}})1/2), \quad \text{f}_{\text{cut}} = (b + c)/(a + b + c)$$

    where a is the integrated intensity of the undigested PCR product and b and c are the integrated intensities of each cleavage product.

8.3.3 Design and Clone Donor Plasmid Based on the Best sgRNA

1. Retrieve the sequence of 1 kb surrounding OCT4 stop codon by using Genome Browser (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start).
2. Conduct primary PCR to obtain the homology arm sequence.

Design primers by Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3-plus.cgi). Set the product size ranges as 1.0–1.5 kp. Make sure that there is at least 400 bp at both left and right homology arms.

3. Double-check PCR product by using in silico PCR tool at Human Genome Browser Gateway. (http://genome.ucsc.edu/cgi-bin/hgPcr?hgsid=425024239_nHNy7CS8MQDKSKIRALRKaq4r39ns). Make sure there is only product at OCT4 locus.
4. Conduct PCR using KAPA HiFi polymerase, using the following cycling conditions: 98 °C for 2 min, 1 cycle; 98°C for 20 s, 64 °C for 15 s, 72 °C for 30 s, 30 cycles. Purify the PCR products with the QIAquick PCR purification kit and confirm the product by Sanger sequencing.
5. Conduct secondary PCR to generate 3 elements of knock-in donor template with ~25-bp overlapping sequence: (1) left HR arm, (2) E2A-GFP, and (3) right HR arm. In the primer design, add the sgOCT4 recognition sequence, including the NGG PAM at the 5′ end of the left HR arm and at the 3′ end of the right HR arm. If the sgRNA targets the OCT4 ORF sequence, introduce 2 sense mutations at the sgRNA target site (see note 8).
6. Conduct PCR using KAPA HiFi polymerase, using the following cycling conditions: 98 °C for 2 min, 1 cycle; 98 °C for 20 s, 64 °C for 15 s, 72 °C for 15 s, 4 cycles; 98 °C for 20 s, 68 °C for 15 s, 72 °C for 15 s, 20 cycles. Purify the PCR products with the QIAquick PCR purification kit.
7. Conduct tertiary PCR to assemble the 3 pieces together. Use the KAPA HiFi polymerase and the following cycling conditions: 98 °C for 2 min, 1 cycle; 98 °C for 20 s, 64 °C for 15 s, 72 °C for 30 s, 4 cycles; 98 °C for 20 s, 68 °C for 15 s, 72 °C for 30 s, 20 cycles. Purify the PCR products with the QIAquick PCR purification kit.
8. Clone the PCR product into pJET1.2/blunt cloning vector using CloneJET PCR Cloning Kit. Set up the following ligation reaction on ice:

2× reaction buffer	10 µl
Non-purified PCR product from step 4	1 µl
pJET1.2/blunt cloning vector	1 µl
Water, nuclease-free up to	19 µl
T4 DNA ligase	1 µl

Vortex briefly and centrifuge for 3–5 s. Incubate the ligation mixture at room temperature for 5–30 min. Use the ligation mixture directly for transformation. Incubate the *E. coli* overnight at 37 °C. Pick 3 clones into 1–2 ml LB medium and shake it vigorously at 37 °C for overnight. The next day, isolate the plasmid DNA by using a QIAprep Spin Miniprep Kit according to the manufacturer’s instructions. Verify the sequence of each clone by using Sanger sequencing. Isolate the correct clone using Qiagen Plasmid Plus Maxi Kit (see note 9).

8.3.4 Creating the OCT4-GFP Reporter iPSC Line

8.3.4.1 Preparation of iPSCs

1. Prepare a 50 ml aliquot of E8 medium supplemented with 10-μM ROCK inhibitor.
2. Coat the 6-well tissue culture plate with 1 ml diluted Matrigel 1:50 in cold DMEM/F12 medium and place the plate at 4 °C overnight or at least 30 min at 37 °C before use.
3. Thaw a vial of cells by gently agitating them around in a 37 °C water bath, transfer the cells to a 15-ml tube, add 5 ml of E8 medium, and centrifuge at 400 g for 5 min at room temperature.
4. Aspirate the supernatant, and seed the cells in a Matrigel-coated 6-well plate. Culture the cells with 2 ml prewarmed E8 medium containing 10-μM ROCK inhibitor.
5. Replace with E8 medium without ROCK inhibitor the next day and refeed cells with fresh E8 medium daily.
6. Passage the cells two days before transfection using Accutase at an appropriate density to achieve roughly 70–80 % confluency in 48 h.

8.3.4.2 Nucleofection of iPSCs

7. iPSCs were cultured in medium supplemented with 10-μM ROCK inhibitor 2–4 h before electroporation.
8. Wash the cells in the 6-well plate with 1–2 ml prewarmed PBS.
9. Add 0.5 ml/well prewarmed Accutase to the digest the iPSCs for 3–5 min at 37 °C.
10. Add 2× volume of prewarmed PBS and pipet gently to dissociate the iPSCs into single cells.
11. Count the number of cells using hemacytometer.
12. Centrifuge the cells at 200 g for 5 min at room temperature.
13. Remove the supernatant and resuspend 8×10^5 iPSCs in 100 μl Human ES Cell Nucleofector Solution with 4 μg of sgOCT4-Cas9 plasmid and 4 μg of GFP donor plasmid.
14. Transfer cells to an electroporation cuvette. Nucleofect the cells with the B-016 program.
15. Add prewarmed 0.5 ml E8 medium supplemented with 10-μM ROCK inhibitor into the cuvette immediately after nucleofection, gently mix the nucleofected cells using the plastic pipet provided with the Nucleofector Kit (see note 10).
16. Transfer the nucleofected cells to one well of the Matrigel-coated 6-well plate. Culture the cells with 2 ml E8 medium containing 10-μM ROCK inhibitor.

17. Grow the cells overnight at 37 °C.
18. Refeed the cells with fresh E8 medium without ROCK inhibitor on the next day after nucleofection.

8.3.4.3 Single-Cell Sorting of GFP-Positive iPSCs by FACS

19. Three to four days after nucleofection, when the cells have reached 70–80 % confluency, dissociate the cells with 0.5 ml of 0.5 mM EDTA buffer at 37 °C for 5 min.
20. Remove the EDTA bufferand dissociate the cells by adding 600 µl of E8 medium, and dissociate the cells by gentle pipetting.
21. Transfer the resuspended cells into a 5-ml tube and centrifuge the cells at 200 g for 5 min at room temperature.
22. Aspirate out the medium and resuspend the cells in 200–400 µl of FACS medium.
23. Filter the cells into a 5-ml FACS tube and put the cells on ice until sorting.
24. Coat the 96-well plate with 50 µl Matrigel per well at 4 °C overnight and add 100 µl E8 medium containing 10-µM ROCK inhibitor before sorting (see note 11).
25. GFP-positive iPSCs were sorted into 96-well plates at 1 cell per well on the BD FACSAria.
26. Grow the cells at 37 °C by changing the E8 medium every 2–3 days until reaching 10–20 % confluency.
27. Expand the cells in the 96-well plates into 2 sets of 24-well plates until reaching 80 % confluency.

8.3.4.4 Confirmation of Gene Knock-in by Junction PCR

28. Harvest cells in one set of 24-well plates for DNA extraction.
29. Dissociate the cells using 0.5 mM EDTA buffer and centrifuge the cells at 200 g for 5 min at room temperature.
30. Remove the medium and use the Genomic DNA Extraction Kit (Qiagen) to extract genomic DNA.
31. Dilute the genomic DNA to a final concentration of 100–200 ng/µl with H_2O.
32. Design a pair of PCR primers to amplify the junction between OCT4 genome and inserted GFP donor. Calculate the length of the PCR product.
33. Set up a 20-µl PCR reaction using the 2× FastPfu PCR SuperMix as follows:

2× FastPfu PCR SuperMix	10 µl
Forward primer	1 µl
Reverse primer	1 µl
DNA template	1 µl
H_2O	7 µl

34. Perform a PCR with the following cycling conditions:

    1 cycle: 98 °C for 2 min (initial denaturation)
    35 cycles: 98 °C for 10 s (denaturation)
    62 °C for 20 s (annealing)
    72 °C for 1 min (extension)
    1 cycle: 72 °C for 2 min (final extension)

35. Run the PCR products on a 1 % agarose gel to verify the amplification.
36. Pick the clones with the corrected knock-in for further culture.

8.4 Notes

1. The specificity of the Cas9 nuclease is determined by the 20-nt guide sequence within the sgRNA. Our unpublished data and other investigators show that 17- or 18-nt truncated sgRNA improves the target specificity without affecting the targeting efficiency.
2. A previous study shows that the nucleotide just in front of NGG significantly affects the targeting efficiency of the sgRNA. If the nucleotide is G, the targeting efficiency is highest, followed by A, C, and T.
3. Occasionally, BbsI does not cut the plasmid efficiently. In this case, before the enzyme digestion, treat the DNA at 60 °C for 5 min followed by cooling down on ice for 1 min.
4. To achieve high transfection efficiency, the cells should be evenly distributed. Two approaches may help prevent the clustering of cells in the center of the 24 wells: (1) Rock the plate front to back twice, right before placing it back into the incubator; (2) decrease the volume of culture medium from 500 to ~300 µl.
5. To minimize error in the amplified PCR product, it is important to use a high-fidelity polymerase. Other high-fidelity enzymes, such as KAPA HiFi (Kapa Biosystems) or Phusion High-Fidelity DNA Polymerases (NEB) may be used.
6. Generally, a step of 4 °C for ∞ is unnecessary for the PCR reaction.
7. If there are no primer dimers or extra bands, it is unnecessary to purify the PCR product.
8. Introducing the sgRNA targeting sequences flanking the knock-in donor DNA increases the knock-in efficiency by fivefold or more.

9. For pJET cloning, the volume of reaction can be reduced to a total volume of 3 μl.
10. Treating iPSCs with ROCK inhibitor before and after nucleofection is critical to minimize cell death.
11. Sorting iPSCs into well plates pre-seeded with MEF feeder cells may further increase survival of iPSCs.

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