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Generation of Isogenic Human iPS Cell Line Precisely Corrected by Genome Editing Using the CRISPR/Cas9 System

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Abstract Genome engineering and human iPS cells are two powerful technologies, which can be combined to highlight phenotypic differences and identify pathological mechanisms of complex diseases by providing isogenic cellular material. However, very few data are available regarding precise gene correction in human iPS cells. Here, we describe an optimized stepwise protocol to deliver CRISPR/Cas9 plasmids in human iPS cells. We highlight technical issues especially those associated to human stem cell culture and to the correction of a point mutation to obtain isogenic iPS cell line, without inserting any resistance cassette. Based on a two-steps clonal isolation protocol (mechanical picking followed by enzymatic dissociation), we succeed to select and expand corrected human iPS cell line with a great efficiency (more than 2 % of the sequenced colonies). This protocol can also be used to obtain knock-out cell line from healthy iPS cell line by the NHEJ pathway (with about 15 % efficiency) and reproduce disease phenotype. In addition, we also provide protocols for functional validation tests after every critical step.

Keywords Isogenic human iPS cell · Precise gene correction · CRISPR · Genome editing · Cas9 endonuclease

Introduction

Patient-specific human iPS cells may be the optimal cellular material for disease modeling, drug development and future patient-specific cellular therapies. However, numerous complex diseases such as Bardet-Biedl, Wolfram or Alström Syndrome have not yet been modeled mainly due to the complex correlation between the genetic mutation(s) and the resulting phenotype severity [1, 2]. Indeed, because of the large variability in genetic background between individuals, it is very difficult to detect and study molecular changes resulting from one specific allele. For these pathologies, where no specific or subtle phenotype is observed when comparing mutated cells and corresponding "healthy" control, genome editing is the only way to distinguish disease-relevant changes. Furthermore, a specific phenotype, can be identified between patients and corresponding control, and does not correspond to the disease but is related to the epigenetic status of the patient. Indeed, a number of studies have shown that iPS cells retain epigenetic methylation patterns from the somatic cell of origin, affecting the differentiation process of the cells [3–6]. In this context, genome editing and human iPS cells are two powerful technologies that can be combined to highlight phenotypic differences and permit the identification of pathological mechanisms of complex diseases.

In the past, precise genomic modifications of human stem cells were achieved by laborious and inefficient homologous recombination using mostly BACs (Bacterial Artificial Chromosomes) [7–9]. Adapted to eukaryotes in 2013, the bacterial CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-Associated nuclease 9) became a powerful tool to efficiently and precisely modify eukaryotic genome [10–13]. The engineered Cas9 endonuclease uses a specific 20-nucleotides single guide RNA (sgRNA) to generate a double-stranded break (DSB) at the targeted genomic locus. Following DSB, the cell can activate a repair system through error-prone non-homologous end joining (NHEJ) or

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homology-directed repair (HDR) pathways. Without DNA template, the NHEJ pathway induces uncontrolled deletion/insertion leading to gene invalidation, while the presence of a homologous DNA template favors precise HDR. Although HDR occurs at lower frequencies than NHEJ, the high fidelity repair is, due to the DSB, largely increased as compared to "classical" homologous recombination. Recently, single-stranded oligonucleotides (ssODNs), used as the repair template, have also shown to efficiently generate precise and small genomic modifications [14, 15].

The vast majority of CRISPR/Cas genome editing in human cells - so far - has been performed in human immortalized cell lines such as HEK293T cells or human neonatal fibroblasts, which are easily manipulated and transfected [10–12, 14, 16]. On the contrary, very few data are available regarding gene correction in human iPS cells [17–20]. Moreover, because of the handling difficulties, genome editing in human stem cells requires, most of the time, insertion of a floxed resistance cassette. LoxP sites are introduced by homologous recombination, together with the antibiotic gene. Removal of this cassette leads to residual LoxP sequences that may induce uncontrolled phenotype [18, 21–24].

Here, we describe an optimized stepwise protocol to efficiently generate isogenic disease and control iPS cell lines using CRISPR/Cas genome editing technique without inserting any resistance cassette (Fig. 1). We first describe the detailed protocol used for engineering all the plasmids necessary to perform genome editing by CRISPR/Cas9. Then, we review all the steps necessary to transduce iPS cells, and finally we explain how to select the fraction of colonies that harbor efficient recombining.

Using a transient selection followed by a two-steps clonal isolation, we are able to select and clonally expand corrected human iPS cell with a great efficiency (more than 2 % of the sequenced colonies). This protocol can also be used to obtain knock out cell lines from healthy human iPS cells by the NHEJ pathway (about 15 % of efficiency) and reproduce disease phenotype. In addition, we provide protocols for functional validation after every critical step (Fig. 2).

Materials

Reagents

Design of the Plasmids for CRISPR/Cas9 Genome Editing and Functional Analysis

- Plasmids: pLCas9 (Addgene plasmid #44719), empty sgRNA cloning vector (Addgene plasmid #41824), pJET1.2 blunt cloning vector (CloneJET PCR Cloning Kit, Life technologies, cat. no. K1231) or any other cloning plasmid.
- PCR primers or oligos for sgRNA construction are listed in Table 1 and can be ordered as 25nmole DNA oligos with standard desalting purification at Integrated DNA Technologies (IDT) or any other supplier.
- Phusion® High-Fidelity DNA Polymerase (New England BioLabs, cat. no. M0530S).
- GoTaq® DNA Polymerase (Promega, cat. no. M3001)
- dNTPs Mix 10 mM (Promega, cat. no. U1511)
- QIAquick gel extraction kit (Qiagen, cat. no. 28704)
- QIAprep spin miniprep kit (Qiagen, cat. no. 27106)
- EndoFree® Plasmid purification maxi kit (Qiagen, cat. no. 12362)
- Agarose (Sigma, cat. no. A9539)
- Midori Green Advanced DNA Stain (Nippon genetics Europe GmbH, cat. no. MG 04)
- GeneRuler™ DNA Ladder Mix (Life Technologies, cat. no. SM0331/2/3)
- DNA Gel Loading Dye (6X) (Life Technologies, cat. no. R0611)
- AflII (New England BioLabs, cat. no. R0520S)
- BglII (New England BioLabs, cat. no. R0144S)
- Gibson Assembly® Master Mix (New England BioLabs, cat. no. E2611S)
- DTT, Molecular Grade (Promega, cat. no. P1171)
- One Shot TOP10 chemically competent E. coli (Life Technologies, cat. no. C4040-10)
- Ampicillin, 100 $mg.ml^{-1}$, sterile filtered (Sigma, cat. no. A5354)
- Kanamycin 50 $mg.ml^{-1}$, sterile filtered (Sigma, cat. no. K0254)
- DNeasy® Blood and Tissue Kit (Qiagen, cat. no. 69504)
- QIAquick® PCR Purification Kit (Qiagen, cat. no. 28106)
- T7 Endonuclease I (New England BioLabs, cat. no. M0302S)

Cell Culture

- Gibco® irradiated Mouse Embryonic Fibroblasts (MEF) (Cat. no. S1520-100)
- HEK293T cell line (Life Technologies, cat. no. R70007)
- DMEM, high glucose (Life Technologies, cat. no. 41965-039)
- DMEM/F12 (Life Technologies, cat. no. 31331-028)
- MEM Non-Essential Amino Acids Solution (NEAA), 10 mM (Life Technologies, cat. no. 11140-035)
- Dulbecco's PBS w/o Ca^{2+}, Mg^{2+} (DPBS) (Lonza, cat. no. BE17-512F)

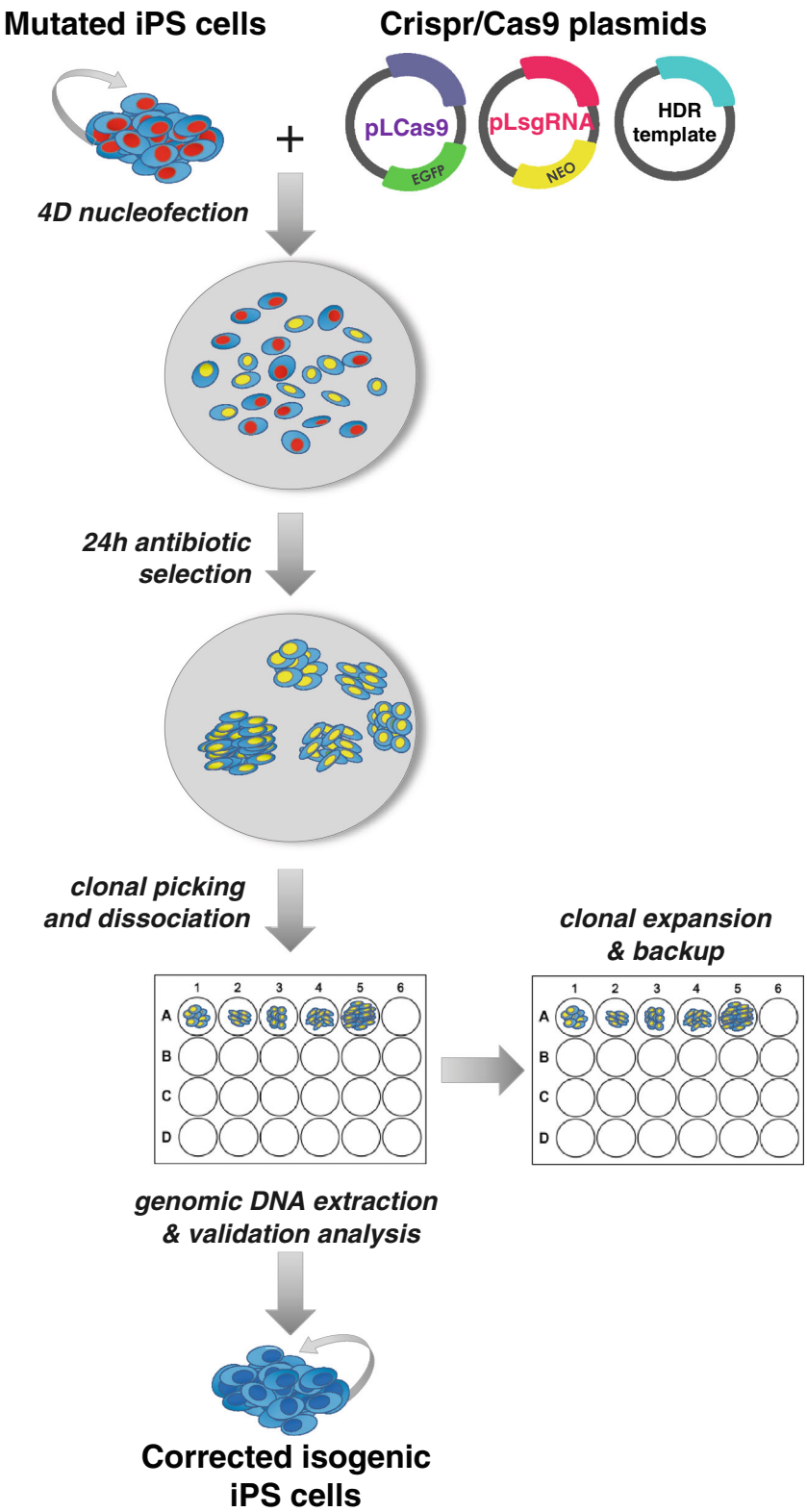

Fig. 1 Overview of the strategy used for genome editing of human iPS cells using CRISPR/Cas9. Human iPS cells are nucleofected with genome editing plasmids and HDR template and selected 2 days later during 24 h with G418. Resistant colonies appear within 2 weeks. They are mechanically and clonally picked prior to be dissociated and transferred into separated well (two-steps clonal isolation). After clonal expansion and constitution of a backup plate, genomic DNA can be extracted from each clone and analyzed for gene correction by sequencing

- Fetal Bovine Serum (FBS) (Life Technologies, cat. no. 10270-106)
- Knock-out Serum Replacement (KO-SR) (Life Technologies, cat. no. 10828-028)
- Opti-MEM I reduced-serum medium (Life Technologies, cat. no. 11058-021)
- Penicillin/streptomycin (Pen/strep), 100× (Life Technologies, cat. no. 15140-122)

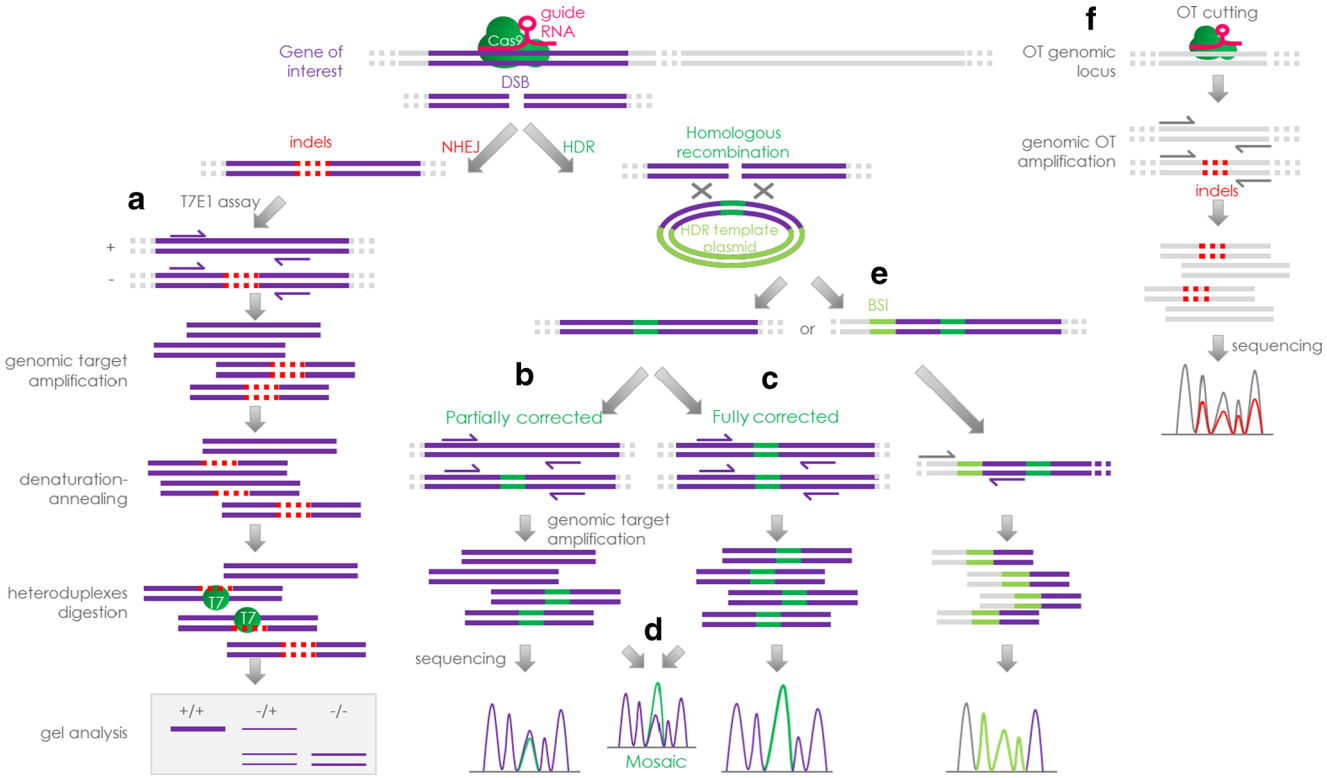

Fig. 2 Protocols for the validation of isogenic iPS cell line. Using a specific 20-nucleotides sgRNA, Cas9 endonuclease generates a DSB at the targeted genomic locus. This DSB can be repaired by the cell through the NHEJ or HDR pathways. Without any DNA template, the NHEJ pathway induces uncontrolled indels leading to gene knock out while the presence of a homologous DNA template favors precise HDR. **a** T7E1 assay is used to detect indels. First, genomic targeted locus is amplified. Homoduplex amplicons are then denatured and re-annealed to form heteroduplexes, which are finally digested by the T7E1. Heterozygous (+/−) cell line detection is sufficient as successful functional validation in iPS cell line but can be reused to generate KO iPS cell line via an additional CRISPR/Cas9 experiment. (**B-C-D-E**) HDR events can be detected by sequencing after corresponding genomic locus amplification. Use healthy or mutated chromatogram sequence as control. **b** In the presence of a partially corrected iPS cell line, two equal peaks instead of one can be detected for the targeted HDR locus. **c** In presence of a fully corrected iPS cell line, the corrected nucleotide can be detected clearly without peak superposition at the targeted HDR locus. **d** Mosaic colonies are a mix of modified and unmodified cells resulting from nucleofection of clustered cells. Two distinct peaks not equal in size can be observed. **e** Backbone sequence can be detected at both side of the HDR site. **f** Unspecific OT cutting can be predicted by homology based on sgRNA sequence. OT genomic locus can be amplified to detect indels via T7E1 assay or sequencing. Indels appear on the chromatogram as sequence frameshifts and can be confirmed by sequence alignment with the unmodified sequence

- G418 disulfate salt (Sigma, cat. no. G1279)
- β-mercaptoethanol (pure liquid) (Sigma, cat. no. M7522)
- Trypsin-EDTA 0.05 % (1X) (Life Technologies, cat. no. 25300054)
- Lipofectamine 2000 transfection reagent (Life Technologies, cat. no. 13778-075)
- Geltrex LDEV-free reduced growth factor basement membrane matrix (Life Technologies, cat. no. A1413202)
- Accutase™ cell detachment solution (100 ml) (Stemcell Technologies, cat. no. 07920)
- Rho-associated protein kinase inhibitor (ROCK-I) (Y-27632) (Millipore, cat. no. 688000-1)
- Amaxa P3 primary cell 4D-Nucleofector X kit S, 32 RCT (Lonza, cat. no. V4XP-3032)
- Basic Fibroblast Growth Factor (bFGF) (Peprotech, cat. no. 100-18B)

Equipment

- Steritop-GP, 0.22 μm, polyethersulfone (PES) Express PLUS, 250 mL 45 mm, radio-sterilized (Millipore, cat. no. SCGPT02RE)
- Standard microcentrifuge tubes, 1.5 ml (Eppendorf, cat. no. 0030 120.086)
- Falcon 15 ml Conical Centrifuge Tube (Fisher Scientific, cat. no. 14-959-49B)
- Tissue culture dish, 60×15 mm (Corning, cat. no. 430-166)
- Tissue culture plate, 24 wells (Corning, cat. no. 3526)
- Nunc Tissue culture dish, 100 mm (Thermo Scientific, cat. no. 150-350)
- T75 Flasks (Corning, cat. no. 430-641)
- T175 Flasks (Corning, cat. no. 431-080)

Table 1 Primers sequence

Section(Step)	Primer	Sequence	Tm (°C)	Purpose
3.1 (3)	pLCas9-5' Forward	GGCTCTAGTGCCTCTGCTAACC	59	pLCas9 sequencing
3.1 (3)	M13 Reverse	AGCGGATAACAATTTCACACAGG	57	pLCas9 sequencing
3.2 (3)	Oligo Forward: CACCGNNNNNNNNNNNNNNNNNNN	TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAA	Ramp	sgRNA design
3.2 (3)	Oligo Reverse: AAACNNNNNNNNNNNNNNNNNNNC	GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTA	Ramp	sgRNA design
3.2 (12)	Sp6 Forward	ATTTAGGTGACACTATAG	43	pLsgRNA sequencing
3.2 (12)	T7 Forward	TAATACGACTCACTATAGGG	48	pLsgRNA sequencing
3.6 (8c)	pJET1.2 Forward	CGACTCACTATAGGGAGAGCGGC	70	pJet1.2 sequencing primer
3.6 (8c)	pJET1.2 Reverse	AAGAACATCGATTTTCCATGGCAG	70	pJet1.2 sequencing primer

N, in green: nucleotides corresponding to the guide sequence, homologue to the target locus. N, in red: complementary nucleotides

- Scepter™ handheld automated cells counter 2.0 (Millipore, cat. no. PHCC20060)
- Scepter™ cell counter sensors, 60 μm (Millipore, cat. no. PHCC60050)
- NanoDrop 2000 device, UV spectrophotometer (Thermo Scientific)
- 4D-Nucleofector™ System (Lonza, cat. no. AAF-1002B and AAF-1002X)

Softwares and Online Tools

- CRISPR/Cas plasmids and resources on Addgene at https://www.addgene.org/CRISPR/
- BLAST, human genome online tool: http://blast.ncbi.nlm.nih.gov/Blast.cgi
- Zhang's Lab genome editing online tool: www.genome-engineering.org/
- ImageJ, quantification software available at http://rsbweb.nih.gov/ij/
- Serial Cloner 2.6, molecular biology software available at http://serialbasics.free.fr/Serial_Cloner.html

Culture Medium

- **HEK293T cell culture medium** (500 ml): 440 ml DMEM, 50 ml FBS, 5 ml NEAA, 5 ml Pen/Strep, 5 μl β-mercaptoethanol. Store at 4 °C.
- **KO-SR medium** for human iPS cell culture (500 ml): 395 ml DMEM/F12, 100 ml KO-SR, 5 ml NEAA, 5 μl β-mercaptoethanol. Store at 4 °C.
- **KO-SR conditioned medium** for human iPS cell culture in feeder-free condition: thaw irradiated MEFs in HEK293T cell medium on gelatin coated T175 flasks. The day after, replace the HEK293T cell medium by 50 ml of KO-SR medium per T175 flask. Incubate 24 h at 37 °C, 5 % CO_2. Collect the conditioned KO-SR medium daily during max 2 months and store it at −20 °C. The medium has to be filtered through Steritop-GP before use. Store at 4 °C for maximum 1 week.

Methods

Design of the Plasmids for CRISPR/Cas9 Genome Editing and Functional Analysis

Our method is based on plasmids available at Addgene. Basically, CRISPR/Cas9 components consist in two plasmids: one containing the sgRNA, a second encoding for the Cas9 endonuclease. These two plasmids can be used together to induce

DSB in healthy iPS cells, invalidating a target gene and generating knock-out iPS cell line. To correct a point mutation in a mutated iPS cell line, the HDR DNA template is transfected with the sgRNA and the Cas9. This HDR DNA template contains the corrected sequence and will recombine by homology at the DSB site. Here we provide the stepwise protocol to correct a point mutation in human iPS cells.

1. Multiple CRISPR plasmids and cloning protocols from distinct research teams are available at Addgene (http://www.addgene.org/crispr). Maps and features of those plasmids are also available online (see **Note 1**).
2. We are currently using plasmids from K. Musunuru and G. Church Labs. Musunuru's plasmid encodes a Cas9 protein, under the CAG promoter, bearing a C terminus SV40 nuclear localization signal and EGFP (pLCas9, #44719). Church's plasmid is an empty sgRNA expression vector. After cloning, the specific sgRNA is controlled by the human U6 polymerase III promoter. This plasmid contains a Neomycin/G418 resistance gene (empty sgRNA cloning vector, #41824).
3. Plasmids should be sequenced and kept as endotoxin-free stock solutions at −20 °C. Avoid freeze and thaw cycles. See Table 1 and Addgene for sequencing primers.

Design of the sgRNA and Cloning into the Empty sgRNA Cloning Vector (#41824)

1. Design of the sgRNA and validation steps should be adapted to the plasmid of your choice. Use the online design tool (http://tools.genome-engineering.org) from Zhang Lab to design your sgRNA sequences and predict off-targets. Choose at least three distinct high rated guide sequences close to the mutation with the lowest off-targets (see off-targets analysis steps). Note that the Cas9 cleaves DNA approximatively 3 bp upstream the Protospacer Adjacent Motif (PAM) sequence.
2. CRISPR cloning protocols are already published and available on Addgene. We follow the sgRNA synthesis protocol of Church's team (option B) (http://www.addgene.org/crispr/church/) starting from targeted genomic sites where NGG is the PAM sequence and the 19**N** represents the predicted guide sequence: 5′-**GNNNNNNNNNNNNNNNNNN**NGG -3′. The sequence needs a 5'G base to allow efficient translation by the RNA polymerase III from the U6 promoter [25] (see **Note 2**).
3. Guide sequences need to be flanked by additional bases to allow their cloning into the delivery plasmid. Order the two following 60-mer DNA oligos at IDT (www.idtdna.com) or any other supplier. Standard desalting purification is sufficient.

   Forward Oligo: TTTCTTGGCTTTATATATCTTGTG GAAAGGACGAAACACC**GNNNNNNNNN NNNNNNNNNN**
   Reverse Oligo: GACTAGCCTTATTTTAACTTGCTA TTTCTAGCTCTAAAAC**NNNNNNNNNNNNNNNNNNNC**

4. Dissolve both oligos at 100 μM with nuclease-free water.
5. Anneal the two oligos and extend them to one 100 bp DNA fragment using the mix described in Table 2 and the following cycling conditions: denaturation 30s at 98 °C, ramp from 98 to 85° at −2 °C/s and from 85 to 25 °C at −0.1 °C/s, final extension 5 min at 72 °C and hold at 4 °C.
6. Linearize the sgRNA cloning vector using AflII at 37 °C for 60 min as described in Table 3.
7. Purify the linearized vector on a 1 % agarose gel using QIAquick Gel Extraction Kit following manufacturer instructions.
8. Determine DNA concentration of both 100 bp DNA fragment and linearized vector using a NanoDrop device.
9. Incorporate the 100 bp DNA fragment (from **step 5**) into the linearized vector (from **step 7**) using Gibson Assembly™ Master Mix (Table 4) and incubate at 50 °C for 60 min. The product can be stored at −20 °C or on ice before the next step.
10. Transform this product into competent cells as described elsewhere [26]. Briefly, thaw one aliquot of Top10® chemically competent cells on ice and add 2 μl of ligation product (from **step 9**). Keep on ice during 30 min. Heat shock at 42 °C for 30s and immediately transfer the tube back on ice during 2 min. Add 950 μl of LB medium and let the cells recover at 37 °C during 60 min with agitation. Centrifuge, resuspend the pelleted cells with 200 μl of LB medium and spread 100 μl on a LB agar plate containing kanamycin 50 μg/ml (sgRNA cloning vector contains kanamycin resistance gene). Incubate overnight at 37 °C.

Table 2 sgRNA oligos annealing mix

Component	Amount (μl)	Final concentration
5x Phusion HF buffer	4	1x
10 mM dNTPs	0.4	200 μM
100 μM forward oligo	1	5 μM
100 μm reverse oligo	1	5 μM
Phusion DNA polymerase	0.2	0.5unit/20 μl
Nuclease-free water	13.4	

Table 3 Linearization of empty sgRNA cloning vector with AflII

Component	Amount (μl)
10x NEBuffer	5
Empty pLsgRNA cloning vector (1 μg/μl)	1
AflII (20 000units/ml)	1
Nuclease-free water	43

11. Next day, check for colony growth and analyze 5–10 colonies by sequencing (see **Note 3**). Keep the remaining plate at 4 °C if picking more colonies is necessary.
12. Sequence pLsgRNA from each colony by Sanger sequencing using the T7 or the Sp6 forward primer (see Table 1). Reference the sequencing results against the empty sgRNA cloning vector sequence to check that the 20-nucleotides guide sequence is inserted next to the U6 promoter.
13. Amplify, extract plasmid DNA (containing target guide DNA sequence) using QIAGEN EndoFree Plasmid Maxi kit following manufacturer instructions and make frozen stocks of pLsgRNA (see **Note 4**). Avoid freeze and thaw cycles.

Functional Validation of the sgRNA Specificity

Upon cell transfection, the Cas9 and sgRNA will be simultaneously expressed. Then, they need to assemble together into a ribonucleoprotein (RNP) complex to induced DNA cutting and subsequent NHEJ events. This step of the genome editing process is validated in HEK293T cells by the detection of these NHEJ events after transfection with both plasmids (pLCas9 and pLsgRNA) or each plasmid alone as negative controls (see **Note 5**).

1. HEK293T cells are maintained in culture in T75 flask in HEK293T cell medium (see reagent setup) at 37 °C with 5 % CO_2.
2. Remove the medium. Rinse the HEK293T cells once with DPBS and harvest them with trypsin-EDTA during 20 min at 37 °C. Resuspend the cells in fresh HEK293T cell medium. Centrifuge 5 min at 200 g. Remove the medium and resuspend the pelleted cells with HEK293T cell medium. Plate $0.5–1\times10^5$ cells per well in a 6-well tissue culture plate and incubate at 37 °C (see **Note 6**).
3. Transfect the HEK293T cells when they are 60 % confluent with Lipofectamine® 2000 or any other transfection reagent using manufacturer instructions. For one transfection reaction, dilute 500 ng pLCas9 DNA and 2 μg pLsgRNA DNA in 250 μl of OPTI-MEM I medium. In another tube, dilute 5 μl of Lipofectamine® 2000 in 250 μl OPTI-MEM I medium. Incubate 5 min at RT. Combine the diluted DNA and the diluted Lipofectamine® and incubate 20 min at RT. Add the 500 μl of complexes to one well containing the HEK293T with 2 ml of remaining culture medium. Mix gently the plate and incubate 6 h at 37 °C, 5 % CO_2. After 6 h, change for fresh medium. After 24–48 h, check for transfection efficiency based on the EGFP expressed by pLCas9 (see **Note 7**).
4. After 48 h, harvest cells using trypsin-EDTA and pellet them 5 min at 200 g. Cells can be store at −20 °C for subsequent genomic DNA isolation, amplification and T7E1 assay (see **next steps**).

Table 4 Ligation of the sgRNA into the linearized cloning vector using Gibson Assembly™ master mix

Component	Amount (μl)
100 bp DNA fragment (50 ng/μl)	1
Linearized empty pLsgRNA (50 ng/μl)	2
2x Gibson assembly master mix	10
Nuclease-free water	7

Genomic DNA Isolation, Amplification and Purification

1. Genomic DNA can be extracted from pelleted cells. We use the DNeasy® Blood and Tissue kit or any other DNA extraction kit following manufacturer instructions. Extracted DNA is eluted with 200 μl of elution buffer (EB) from the kit and can be store at −20 °C several months.
2. Amplify 200 ng of genomic DNA using the Phusion® High-Fidelity PCR kitwith the following master mix (see Table 5 and **Note 8**).Perform a PCR using the following cycling conditions: initial denaturation 30s at 98 °C, followed by 35 cycles of denaturation 15 s at 98 °C, annealing 60s at 60 °C and extension 30s at 72 °C.Finally, perform an extension step for 10 min at 72 °C. Keep the final product at 4 °C.

Table 5 Genomic PCR mix

Component	Amount (μl)	Final concentration
5x Phusion GC buffer	10	1x
10 mM dNTPs	1	200 μM
10 μM forward primer	2.5	0.5 μM
10 μM reverse primer	2.5	0.5 μM
Genomic DNA (200 ng/μl)	1	<250 ng
DMSO	1.5	3 %
Phusion DNA polymerase	0.5	1unit/50 μl
Nuclease-free water	31	

3. Run 5 μl of PCR product on a 2 % agarose gel to check for specific single-band and purify the remaining PCR product (45 μl) using QIAquick® PCR Purification Kit or any other PCR purification kit following the manufacturer instructions. Elute the purified product with 50 μl EB. Purified PCR products can be store at −20 °C for several months.

T7 Endonuclease 1 (T7E1) Assay

1. NHEJ events lead to indels (insertion or deletion). T7E1 allows the detection of these mismatches after one step of denaturation-annealing. Mismatches can also be detected using Surveyor nuclease but this enzyme has been shown to be less sensitive than T7E1 [27] (see Fig. 2a).
2. Use 10 μl of purified PCR products (0.5–1 μg) to form heteroduplexes according these cycling conditions (see **Note 9**): denaturation 30s at 98 °C, ramp from 98 to 85° at −2 °C/s and from 85 to 25 °C at −0.1 °C/s and then hold at 4 °C.
3. Heteroduplexes can be stored at −20 °C or directly digested with the T7E1 for 20 min at 37 °C using the following mix: Table 6.
4. Stop the reaction with 2 μl of gel loading buffer and analyze the products directly on a 2 % agarose gel. Use 10 μl of undigested purified PCR products (from **step 2**) as negative control.
5. Visualize T7E1 assay and quantify the cleavage efficiency as describes in Ran et al., 2013. In summary, the sum of cleaved bands (predicted with the sgRNA design) should be equal to the PCR fragment. The negative controls should have only one band corresponding to the size of the PCR product. Occasionally, nonspecific cleavage bands of other sizes may be present but will not interfere with analysis if they are distinct in size from the target cleaved bands. Using ImageJ or any other gel quantification software, measure the integrated intensity of the PCR amplicon and cleaved bands. For each lane, calculate the fraction of the cleaved PCR product (*fcut*) using the following formula: $f_{cut} = (b + c)/(a + b + c)$, where a is the integrated intensity of the undigested PCR product and b and c are the integrated intensities of each cleaved product. NHEJ events can be estimated with the following formula (based on the binomial probability distribution of duplex formation): $indels\ (\%) = 100 \times [(1 - (\sqrt{1 - f\,cut}))]$.

Table 6 T7E1 digestion mix

Component	Amount (μl)
10x NEB buffer 2	2
Heteroduplexes (from step 25)	10
T7E1 (10,000 units/ml)	0.25
Nuclease-free water	7.75

DNA Template Design

The induced DSB can be repair via HDR pathway either by a single-stranded oligonucleotides [15] (ssODNs) (**step 1)** or by a double-stranded DNA template (dsDNA) (**step 2**) [28]. It is important to note that the PAM sequence (NGG) present in the repair template, will also be cut by Cas9, leading to reduced efficiency of the process. We recommend changing one of the two G of the NGG when possible (see **Note 10**). Targeting efficiency can also widely vary depending on iPS cell line, targeted locus and location of the mutation relative to the DSB site. Including an artificial silent restriction site in the repair template is recommended to facilitate clonal screening after editing.

1. ssODNs DNA template, sense or antisense, can be ordered directly from IDT or any other supplier as 4nmole ultramer® DNA. Order oligos with homology arms of at least 60 nucleotides (min 120 bases oligos) [14]. PAGE-purification is not required. Resuspend in sterile nuclease-free water at 10 μM ready-to-use. Although ssODNs DNA templates have been shown to be more efficient than dsDNA templates for correcting point mutation [14, 15, 21], however, those ssODNs DNA did not work in our hands.
2. dsDNA template can be built up from the WT genomic sequence of any "healthy" (non-mutated) human iPS cell line. More than 1kbp of homology arms are recommended [29]. Note that when using this type of repair template, backbone sequence insertion at both sides of the homologous region needs to be checked (see **Validation of the corrected human isogenic iPS cell lines steps**). We obtain the best results with dsDNA HDR strategy using a 3kbp homologous fragment design as described below (see **Note 11**).
3. Extract genomic DNA from one healthy human iPS cell line and amplify the 3kbp HDR fragment with high-fidelity polymerase (**§3.4)** (see **Note 12**).
4. Run 50 μl of the PCR products on 1 % agarose gel and purify on gel the 3kbp HDR fragment and sequence it.
5. Using BLAST (www.ncbi.nlm.nih.gov/blast/), align the sequenced fragment with the human genome and find out mutations (see **Note 13**).
6. Insert the HDR fragment into the pJET1.2/blunt using CloneJET PCR Cloning Kit© following the blunt-end cloning protocol. Prepare the mix as described in Table 7 and incubate 30 min at RT.

Table 7 Ligation of the HDR DNA fragment into HDR donor plasmid

Component	Amount (μl)
2X Reaction Buffer	20
3kbp Purified HDR fragment (10 ng/μl)	15
pJET1.2/blunt cloning vector (50 ng/μl)	1
T4 DNA ligase	1
Nuclease-free water	40

7. Store at −20 °C or transform directly 10 μl into competent cells (section 3.2. **step 10**).
8. Next day, check for colony growth and analyze 5–10 recombinant colonies by PCR (**step 8a**) or enzyme restriction analysis (**step 8b**) before sending them to sequencing (**step 8c**).

   a. Prepare (n+1) PCR master mix for n colonies to analyze as described in Table 8.

– Pick individual colony and resuspend each in 20 μl of the PCR master mix. Keep tips in LB medium for subsequent amplification (see next **step**).
– Perform PCR using following cycling conditions: initial denaturation 3 min at 95 °C, followed by 35 cycles of denaturation 30s at 94 °C, annealing 30s at 60 °C and extension 3 min at 72 °C. Finally, perform an extension step for 10 min at 72 °C. Analyze for the presence of the 3kbp amplicon on a 1 % agarose gel.

   b. In order to analyze the recombinant colonies for the presence of the DNA insert using enzyme restriction digestion, inoculate each of the colonies into 3 ml of LB medium with 100 μg/ml ampicillin and incubate overnight at 37 °C under agitation.

– The next day, extract plasmid DNA from the cells using QIAprep spin Miniprep kit using manufacturer instructions.
– Digest 1 μg of plasmid DNA from recombinant clones with BglII enzyme (or any other restriction enzyme cutting the pJET1.2/blunt at the multiple cloning site) 1 h at 37 °C. Analyze on a 1 % agarose gel for the presence of the 3kbp HDR fragment.

   c. Extracted DNA can also be sent for sequencing using the Forward pJET1.2 sequencing primer (see Table 1).

9. Correct clone(s) can be amplified and purified with Endotoxin-Free Plasmid Purification Maxi Kit following manufacturer instructions and store at −20 °C.

Table 8 Colony PCR mix

Component	Amount (μl)
10x GoTaq® buffer	2
dNTPs mix (2 mM each)	2
10 μM Forward pJET1.2 sequencing primer	0.4
10 μM Reverse pJET1.2 sequencing primer	0.4
25 mM $MgCl_2$	1.2
GoTaq® DNA Polymerase (5units/μl)	0.1
Nuclease-free water	13.9

Genome Editing in Human iPS Cells

Human iPS Cell Culture

1. Human iPS cells are routinely cultured in 60 mm tissue culture plates in feeder-free condition on Geltrex with KO-SR conditioned medium supplemented with 4 ng/ml bFGF. Typically, one 60 mm culture dish of human iPS cells at 60–70 % of confluence is sufficient to perform two genome editing experiments as described below.
2. Dilute Geltrex stock at 1:100 in fresh and cold DMEM/F12. Diluted Geltrex can be kept at 4 °C for maximum 1 week.
3. Two hours before the nucleofection, coat one 100 mm tissue culture plate with 7 ml of diluted Geltrex and incubate at 37 °C (see **Note 14**).
4. At the same time, 2 h before the nucleofection, change the medium of the human iPS cells with 3 ml of fresh KO-SR conditioned medium supplemented with 4 ng/ml bFGF and 10 μM Y-27632.

Delivery of the Plasmids for CRISPR/Cas9 Genome Editing in iPS Cells

1. Genome editing plasmids are delivered into the human iPS cells using the P3 primary cell 4D nucleofector kit. We typically nucleofect between 5 and 8 μg of total DNA for 200 000 cells (see **Note 15**).
2. Prepare in advance:

   – The DNA mix: We typically use 2 μg of each plasmid (pLCas9, pLsgRNA and HDR plasmid) filled up to 20 μl with S1-supplemented P3 nucleofection solution at RT.
   – A 10 ml aliquot of fresh KO-SR conditioned medium with 4 ng/ml bFGF and 10 μM Y-27632.
   – A 1.5 ml microcentrifuge tube filled with 90 μl DPBS.

3. Remove the medium and incubate human iPS cells with 1 ml Accutase during 30 min at 37 °C for dissociation (see **Note 16**).

4. Add 3 ml of KO-SR (out of the aliquot) onto the dissociated cells to detach them and transfer them into a 15 ml Falcon tube. Count the number of cells using the Scepter automated cell counter by pipetting 10 μl and diluting them into 90 μl of DPBS.
5. Transfer 200,000 dissociated cells (Lonza's recommendations for nucleofection) from the 15 ml Falcon tube to another 15 ml Falcon tube and centrifuge them 5 min at 120 g.
6. Switch on the 4D nucleofector device and load the CB150 program. Remove the medium from the pelleted cells, add the DNA mix and gently resuspend the cells by tapping the tube.
7. Transfer the resuspended cells into one electroporation cuvette. Nucleofect using the program CB150 (see **Note 15**).
8. Rapidly but gently reseed the nucleofected cells in the Geltrex-coated 100 mm culture plate with 7 ml of KO-SR (out of the aliquot).
9. Change the medium 12 h after nucleofection (the next day) with 7 ml KO-SR conditioned medium containing 4 ng/ml bFGF and 10 μM Y-27632.
10. 48 h after nucleofection, add G418 antibiotic (100 μg/ml) to the medium during 24 h (see **Note 17**).
11. After selection, replace the medium and refeed daily the cells with fresh KO-SR conditioned medium with 4 ng/ml bFGF.
12. Let the human iPS cell colonies grow (see **Note 18**). Human iPS cell colonies should appear between 7 and 15 days.

Isolation of Clonal iPS Cell Lines by Picking Followed by Enzymatic Dissociation (Two-Steps Clonal Isolation)

The isolation of clonal human iPS cell lines is a critical point. FACS sorting or clonal dilution are in theory the most efficient strategies to obtain clonal iPS cells but induce a high rate of cell death that greatly reduces the efficiency of the experiment. To increase cell survival and efficient regrowth of clonal iPS cells, we therefore isolate the clones using a two-steps dissociation procedure: mechanical picking followed by enzymatic dissociation. First, each colony is mechanically picked using a 100 μl-pipette tip and drop off in a 24-well plate. Secondly, the isolated colony is incubated with Accutase for enzymatic dissociation.

1. According the number of iPS cell colonies:
   - Incubate the appropriate number of wells of a 24-well plate with 500 μl of Geltrex (1:100) at 37 °C during 2 h.
   - Fill an equivalent number of wells with 150 μl of Accutase.
2. Remove the medium from the 100 mm culture plate and rinse the iPS cells twice with KO-SR.
3. Add 10 ml of KO-SR to the cells.
4. Under the microscope, pick up each colony with a 100 μl tips (see **Note 19**) and dip it into one well filled with Accutase. Incubate for 20 min at 37 °C.
5. Add 500 μl of KO-SR conditioned medium supplemented with 4 ng/ml bFGF and 10 μM Y-27632 into each well.
6. Transfer the well content into 1,5 ml sterile microcentrifuge tube (one tube for each clone) and centrifuge 5 min at 120 g.
7. Aspirate the medium and replate the cells onto Geltrex-coated wells with KO-SR conditioned medium supplemented with 4 ng/ml bFGF and 10 μM Y-27632 (one well for each clone).
8. The remaining cells in the 100 mm culture plate can further be dissociated with Accutase, collected and pooled together for DNA isolation, amplification and T7E1 assay (see **Note 20**).
9. Refeed daily the cells with fresh KO-SR conditioned medium with 4 ng/ml bFGF.

Expansion of Clonal Human iPS Lines

1. We recommend performing validation of each clone (see **next step**) as fast as possible but it can sometimes take more than 1 week between the DNA isolation and the sequencing results. Expansion of each clone is necessary to have enough amounts of cells for both validation procedure and keeping them in culture. Do not forget to use distinct tips, tubes and wells for each clone.
2. When the clones reach confluence, prepare a 24-well plate with 500 μl of Geltrex per well during 2 h at 37 °C.
3. Remove the Geltrex from the 24-well plate and add 500 μl per well of KO-SR conditioned medium with 4 ng/ml bFGF and 10 μM Y-27632.
4. Remove the medium from the clones and dissociate the iPS cell colonies by adding 150 μl Accutase per well during 30 min at 37 °C.
5. Add 500 μl of KO-SR into each well containing the Accutase-treated cells.
6. Well by well, pipette 50 μl of the dissociated cells and transfer them - clone by clone - into the newly coated 24-well plate filled up medium. Refeed daily the cells with fresh KO-SR conditioned medium with 4 ng/ml bFGF and repeat clonal expansion steps every 5–7 days (see **Note 21**).
7. Transfer the remaining cells, well by well, into 1.5 ml microcentrifuge tube and centrifuge those 5 min at 120 g.

8. Remove the medium by inverting each tube. Pellets can be store at −20 °C for DNA isolation and genotyping.

Validation of the Corrected Human Isogenic iPS Cell Lines

Sanger Sequencing

1. Purified PCR products (after **Genomic DNA isolation, amplification and purification** step) can be sequenced to detect both NHEJ (make sure the deleted sequence does not contain the primer sequence) and HDR events by alignment with uncorrected genomic sequence (see Fig. 2b-c-d).
2. Analyze each clone by sequencing using a primer close to the corrected locus (ideally no further than 150–200 bases) (**Note 22**).

Off-Targets (OT) Analysis

1. The 20-nucleotides sgRNA is designed to direct the Cas9 to one specific target gene. However, the interaction between the sgRNA and genomic DNA can tolerate some mismatches leading to upecific cuttings (with decreased efficiencies) elsewhere in the genome [30]. Here, we provide a simple procedure based on sequence homology to detect OT events (see Fig. 2f).
2. Use the online design tool (http://tools.genome-engineering.org) from Zhang Lab to predict off-targets from a given sgRNA.
3. BLAST predicted OT sequences to find out genomic locus containing each of those.
4. Design primers to amplify around 200 nucleotides each side of the OT cutting site. The Cas9 is supposed to cleave DNA around 3 bp upstream the PAM sequence.
5. Using the mix from Table 9 with specific OT primers, perform PCR following cycling conditions: initial denaturation 30s at 98 °C followed by 35 cycles of denaturation 15 s at 98 °C, annealing 60s at 60 °C and extension 30s at 72 °C. Finally, perform an extension step for 10 min at 72 °C and then hold at 4 °C. Purify PCR products, sequence them and analyze for indels (**Note 23**).
6. To detect indels, align each sequenced OT to the full length OT locus.

Table 9 OT PCR mix

Component	Amount (μl)	Final concentration
5x Phusion GC buffer	10	1x
10 mM dNTPs	1	200 μM
10 μM OT forward primer	2.5	0.5 μM
10 μM OT reverse Primer	2.5	0.5 μM
Genomic DNA (200 ng/μl)	1	<250 ng
DMSO	1.5	3 %
Phusion DNA polymerase	0.5	1unit/50 μl
Nuclease-free water	31	

Backbone Sequence Insertion (BSI) Analysis

1. Backbone insertion can occur when HDR is induced by plasmid DNA template. This analysis is not necessary if you use ssODN as repair template (see Fig. 2e).
2. Design primers to amplify genomic sequence of around 200 nucleotides at both ends of the homologous insertion site (upstream and downstream) (see Table 1).
3. Perform PCR using these BSI specific primers, purify, sequence and analyze for BSI by sequence alignment.

Basic Human iPS Cell Characterization and Protein Rescue

1. As any newly generated iPS cell line, the corrected human iPS cell line needs to be characterized regarding stem cell and pluripotency properties. Ensure that any unwanted cutting was made in pluripotent genes performing immunolabelings and qRT-PCR regarding pluripotency genes such as NANOG, OCT4 and SOX2 as described elsewhere [24]. The corrected iPS cell line should also be able to differentiate spontaneously into cells from the three germ layers in vitro without additional morphogenes and in vivo in nude mice [31]. These validation steps constitute a prerequisite to any further phenotype identification by comparing mutant and corrected cells. The rescue of the protein of interest should be checked by immunolabeling and/or western blot if a specific antibody is available.

Notes

1. Order only plasmids bearing human promoters. Moreover, we recommend plasmids with eukaryotic resistance gene and enhanced-green fluorescent protein (EGFP). Take a look at the different cloning protocols before ordering. In this method, we use separate plasmids encoding for the Cas9 and the sgRNA. Cas9 and sgRNA can also be expressed within one vector (see PX plasmids family from Zhang's Lab on Addgene). Moreover, Zhang and colleagues develop a strategy based on the D10A mutant nickase version of Cas9 (Cas9n) [11,

13, 32, 33] to improve the specificity of Cas9-mediated genome editing. Cas9n induces single-stranded break in the DNA and can be used with two distinct sgRNAs to induce a DSB, thereby increasing the targeting specificity and decreasing OT [33]. We recommend you to switch to Cas9n if OTs are detected in the transfected culture. Cas9n plasmids are also available on Addgene.

2. To design the sgRNA: the first nucleotide of the sgRNA should be a G to allow efficient translation by the RNA polymerase III from the U6 promoter [25]. An extra G is appended at the 5′ of the sgRNA where the 20-nucleotides guide sequence does not begin with G.
3. After Gibson Assembly, the inserted sequence is 20 bp length. Analysis of recombinant colonies by PCR followed by gel migration should be avoided. We recommend you to sequence 5–10 recombinant colonies.
4. Purification and stock build up for all CRISPR plasmids should be in endotoxin-free condition.
5. Following this protocol, you can check different sgRNA/Cas9 complex. We basically recommend you to test three distinct sgRNA. We do not recommend transfecting an HDR template in HEK293T and spending a lot of time to find out HDR events.
6. Plate HEK293T cells in a sufficient number of wells for subsequent transfection (one well per transfection condition). Do not forget to take into account the negative controls: each plasmid alone and a condition without DNA.
7. If the transfection efficiency is less than 70 %, redo the transfection and adapt plasmid concentrations. Antibiotic selection is not necessary.
8. High fidelity polymerases are used since accurate, robust and clean genomic PCR amplification is crucial. Amplify approximately 250 bp at both sides of the predicted edited site. Master mix PCR (type of buffer, DMSO and $MgCl_2$ concentration) needs to be optimized depending on the genomic locus. PCR annealing step must be adapted to the primers (For T_m used see Table 1).
9. It is recommended to use a negative control, such as unmodified or WT DNA sequence.
10. If possible, inducing silent mutations in the HDR template replacing one G in the PAM sequence will increase the recombination efficiency. For example: triplet CGG encode for arginine and can be mutated into CGA, CGC or CGU (coding also for arginine). TGG is the only triplet encoding for tryptophan and cannot be changed. These modifications of the HDR template can be done by PCR mutagenesis as described elsewhere.
11. As already mentioned, efficiency of HDR widely vary depending on the genomic locus and the repair template length and type [14]. Don't hesitate to try both types of templates.
12. The HDR template should be more than 1kbp length and have the corrected base approximately in the middle of the sequence [33]. Use high-fidelity DNA polymerase to avoid insertion of any mutation in the template. Accurate and robust genomic PCR amplification is crucial.
13. Single-nucleotide polymorphism (SNP) can be observed. If polymorphisms lead to amino acid change, redo the PCR, use another high-fidelity polymerase or start from another WT iPS cell line.
14. The 100 mm plates will be kept in culture during a long period of time (usually more than 2 weeks) without antibiotic. To avoid loss of material due to infections and to ensure at least one full experiment, we usually perform two experiments at a time.
15. The critical point is to determine the correct balance between the amount of DNA to transfect and the antibiotic concentration necessary to get 10–20 recombinant human iPS cell colonies per 200,000 transfected cells. The total amount of transfected DNA can widely vary between iPS lines. Too much DNA is lethal, while too little DNA decreases the probabilities to obtain corrected colonies. The nucleofection program may also be adapted to the iPS line. This aspect needs to be optimized before, using control pMAX-GFP plasmid. As plasmids are different in size, use molar quantities to compare. 24–48 h after nucleofection, you should have more than 60 % efficiency. If not, adapt program and DNA concentrations.
16. Avoid mechanical dissociation of the cells. Check by the microscope and favor a longer incubation to obtain single cells.
17. To determine the minimal lethal antibiotic concentration that will be used for selection after nucleofection, an antibiotic dose-response curve for each iPS cell line needs to be performed. Incubate iPS cells in presence of several dilutions of your antibiotic in KO-SR conditioned medium with bFGF. After 24 h remove the medium and add fresh culture medium without antibiotic daily during at least 2 weeks.
18. Let the colonies grow as much as possible in order to have enough cells to maximize the chance of recovery after the two-steps clonal isolation. Do not let the colony spontaneously differentiate or overlap.
19. Ensure to be clonal. Avoid moving the plate during the picking. Change the tip after each colony. Pick only well separated and distinct round colonies. Do not pick colonies exhibiting weird shapes, which may come from an undissociated cluster of cells.
20. After the clonal picking, remaining cells from the 100 mm culture dish can be collected and analyzed by T7E1 assay. Low global cleavage efficiency decreases the chance of HDR event and can be use as indication to determine the pursuit of the experiment.

21. Accutase removal by centrifugation is not necessary because of the high dilution ratio.
22. Detection of mosaic colony. Mosaic colonies can be present following nucleofection, when cells are not perfectly dissociated into single cell. These colonies are a mix of modified and unmodified cells resulting from nucleofection of clustered cells. A first clue is the shape of the mosaic colony, which is not well circular from the center. In addition, by analyzing the sequencing chromatograph, for the targeted nucleotide, two peaks with different dimensions (not equal in size) can be observed (see Fig. 2d). In the presence of those colonies, dissociate the cells with Accutase into single cell and transfer those into 100 mm culture plate. Repeat clonal picking and expansion steps.
23. Mismatches at OT sites can also be detected by T7E1 assay. Purified products from OT analysis steps can be denatured-annealed to form heteroduplexes prior to be digest with T7E1.

Conflict of Interest The authors indicate no potential conflicts of interest.

References

1. Sebat, J., Levy, D. L., & McCarthy, S. E. (2009). Rare structural variants in schizophrenia: one disorder, multiple mutations; one mutation, multiple disorders. *Trends in Genetics, 25*, 528–535.
2. Dipple, K. M., & McCabe, E. R. (2000). Phenotypes of patients with "simple" Mendelian disorders are complex traits: thresholds, modifiers, and systems dynamics. *American Journal of Human Genetics, 66*, 1729–1735.
3. Musunuru, K. (2013). Genome editing of human pluripotent stem cells to generate human cellular disease models. *Disease Models & Mechanisms, 6*, 896–904.
4. Kim, H., Jang, M. J., Kang, M. J., & Han, Y. M. (2011). Epigenetic signatures and temporal expression of lineage-specific genes in hESCs during differentiation to hepatocytes in vitro. *Human Molecular Genetics, 20*, 401–412.
5. Lister, R., Pelizzola, M., Kida, Y. S., et al. (2011). Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. *Nature, 471*, 68–73.
6. Ruiz, S., Diep, D., Gore, A., et al. (2012). Identification of a specific reprogramming-associated epigenetic signature in human induced pluripotent stem cells. *Proceedings of the National Academy of Sciences of the United States of America, 109*, 16196–16201.
7. Zwaka, T. P., & Thomson, J. A. (2003). Homologous recombination in human embryonic stem cells. *Nature Biotechnology, 21*, 319–321.
8. Placantonakis, D. G., Tomishima, M. J., Lafaille, F., et al. (2009). BAC transgenesis in human embryonic stem cells as a novel tool to define the human neural lineage. *Stem Cells, 27*, 521–532.
9. Song, H., Chung, S. K., & Xu, Y. (2010). Modeling disease in human ESCs using an efficient BAC-based homologous recombination system. *Cell Stem Cell, 6*, 80–89.
10. Jinek, M., East, A., Cheng, A., Lin, S., Ma, E., & Doudna, J. (2013). RNA-programmed genome editing in human cells. *eLife, 2*, e00471.
11. Cong, L., Ran, F. A., Cox, D., et al. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science (New York, NY), 339*, 819–823.
12. Mali, P., Yang, L., Esvelt, K. M., et al. (2013). RNA-guided human genome engineering via Cas9. *Science (New York, NY), 339*, 823–826.
13. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science (New York, NY), 337*, 816–821.
14. Lin, S., Staahl, B. T., Alla, R. K., & Doudna, J. A. (2014). Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. *eLife, 3*, e04766.
15. Chen, F., Pruett-Miller, S. M., Huang, Y., et al. (2011). High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases. *Nature Methods, 8*, 753–755.
16. Doench, J. G., Hartenian, E., Graham, D. B., et al. (2014). Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. *Nature Biotechnology, 32*, 1262–1267.
17. Huang, X., Wang, Y., Yan, W., et al. (2015). Production of gene-corrected adult beta globin protein in human erythrocytes differentiated from patient iPSCs after genome editing of the sickle point mutation. *Stem Cells, 33*, 1470–1479.
18. Li, H. L., Fujimoto, N., Sasakawa, N., et al. (2015). Precise correction of the dystrophin gene in duchenne muscular dystrophy patient induced pluripotent stem cells by TALEN and CRISPR-Cas9. *Stem Cell Reports, 4*, 143–154.
19. Miyaoka, Y., Chan, A. H., Judge, L. M., et al. (2014). Isolation of single-base genome-edited human iPS cells without antibiotic selection. *Nature Methods, 11*, 291–293.
20. Reinhardt, P., Schmid, B., Burbulla, L. F., et al. (2013). Genetic correction of a LRRK2 mutation in human iPSCs links parkinsonian neurodegeneration to ERK-dependent changes in gene expression. *Cell Stem Cell, 12*, 354–367.
21. Soldner, F., Laganiere, J., Cheng, A. W., et al. (2011). Generation of isogenic pluripotent stem cells differing exclusively at two early onset Parkinson point mutations. *Cell, 146*, 318–331.
22. Ye, L., Wang, J., Beyer, A. I., et al. (2014). Seamless modification of wild-type induced pluripotent stem cells to the natural CCR5Delta32 mutation confers resistance to HIV infection. *Proceedings of the National Academy of Sciences of the United States of America, 111*, 9591–9596.
23. Yusa, K. (2013). Seamless genome editing in human pluripotent stem cells using custom endonuclease-based gene targeting and the piggyBac transposon. *Nature Protocols, 8*, 2061–2078.
24. Wen, Z., Nguyen, H. N., Guo, Z., et al. (2014). Synaptic dysregulation in a human iPS cell model of mental disorders. *Nature, 515*, 414–418.
25. Guschin, D. Y., Waite, A. J., Katibah, G. E., Miller, J. C., Holmes, M. C., & Rebar, E. J. (2010). A rapid and general assay for monitoring endogenous gene modification. *Methods in Molecular Biology, 649*, 247–256.
26. Froger, A., Hall, J. E. (2007). Transformation of plasmid DNA into E. coli using the heat shock method. *Journal of Visualized Experiments: JoVE*, 253.
27. Vouillot, L., Thelie, A., & Pollet, N. (2015). Comparison of T7E1 and surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases. *G3, 5*, 407–415.

28. Smithies, O., Gregg, R. G., Boggs, S. S., Koralewski, M. A., & Kucherlapati, R. S. (1985). Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. *Nature, 317*, 230–234.
29. Hasty, P., Rivera-Perez, J., & Bradley, A. (1991). The length of homology required for gene targeting in embryonic stem cells. *Molecular and Cellular Biology, 11*, 5586–5591.
30. Lin, Y., Cradick, T. J., Brown, M. T., et al. (2014). CRISPR/Cas9 systems have off-target activity with insertions or deletions between target DNA and guide RNA sequences. *Nucleic Acids Research, 42*, 7473–7485.
31. Takahashi, K., Tanabe, K., Ohnuki, M., et al. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell, 131*, 861–872.
32. Gasiunas, G., Barrangou, R., Horvath, P., & Siksnys, V. (2012). Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proceedings of the National Academy of Sciences of the United States of America, 109*, E2579–E2586.
33. Ran, F. A., Hsu, P. D., Lin, C. Y., et al. (2013). Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell, 154*, 1380–1389.