CRISPR/Cas9-Directed Genome Editing of Cultured Cells

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ABSTRACT

Human genome engineering has been transformed by the introduction of the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) system found in most bacteria and archaea. Type II CRISPR/Cas systems have been engineered to induce RNA-guided genome editing in human cells, where small RNAs function together with Cas9 nucleases for sequence-specific cleavage of target sequences. Here we describe the protocol for Cas9-mediated human genome engineering, including construct building and transfection methods necessary for delivering Cas9 and guide RNA (gRNA) into human-induced pluripotent stem cells (hiPSCs) and HEK293 cells. Following genome editing, we also describe methods to assess genome editing efficiency using next-generation sequencing and isolate monoclonal hiPSCs with the desired modifications for downstream applications. *Curr. Protoc. Mol. Biol.* 107:31.1.1-31.1.17.

Keywords: genome engineering • CRISPR • human stem cells

INTRODUCTION

Targeted human genome editing enables functional studies of genetic variation in biology and disease, and holds tremendous potential for clinical applications. To facilitate genome engineering, technologies such as Zinc-Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs) have been developed to enable targeted and programmable modification of endogenous genomic sequences (Miller et al., 2007; Hockemeyer et al., 2011). However, the need to design new complex nucleases for each target site limits the utility of these methods, particularly in multiplexed gene targeting applications.

Recently, the type II bacterial CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) system has been developed as an efficient and versatile technology for genome editing in eukaryotic cells and whole organisms (Jinek et al., 2012, 2013; Cong et al., 2013; DiCarlo et al., 2013; Friedland et al., 2013; Gratz et al., 2013; Hwang et al., 2013; Mali et al., 2013a; Wang et al., 2013). The CRISPR/Cas system was first identified in bacteria and archaea as an RNA-mediated adaptive defense system that safeguards organisms from invading viruses and plasmids (Ishino et al., 1987; Horvath and Barrangou, 2010; Wiedenheft et al., 2012). The hallmark of the CRISPR/Cas system consists of CRISPR arrays composed of spacers interspersed with direct repeats and *cas* genes present in the operons (Bhaya et al., 2011; Terns and Terns, 2011). In CRISPR/Cas-mediated immunity, bacteria and archaea react to viral or plasmid attack in the adaptive phase by first integrating short fragments of foreign nucleic acid (protospacers) into the host chromosome at the proximal end of the CRISPR array. In the expression phase, CRISPR loci are transcribed into precursor CRISPR RNA (pre-crRNA) and further

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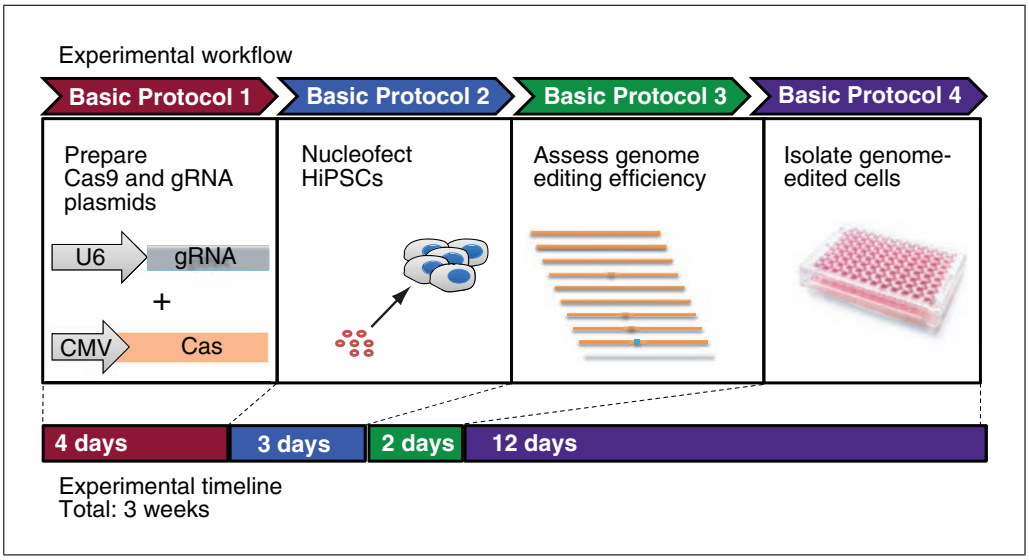

Figure 31.1.1 Workflow and timeline for hiPSC genome engineering. In the complete workflow, the Cas9 and gRNA plasmid constructs are built and subsequently transfected into cells. Genome editing efficiency is assessed using deep sequencing. Finally, monoclonal hiPSC colonies with desired genotype can be isolated using cell sorting. The entire workflow takes 3 weeks to perform.

processed into a library of short CRISPR RNAs (crRNAs) that can recognize and pair with complementary sequences from invading viral or plasmid targets (Carte et al., 2008; Haurwitz et al., 2010; Deltcheva et al., 2011; Gesner et al., 2011; Sashital et al., 2011; Wang et al., 2011). In the final interference phase, crRNAs are packaged with trans-activating crRNA (tracrRNA) and Cas proteins to form ribonucleoprotein complexes that together detect and destroy foreign sequences (Brouns et al., 2008; Hale et al., 2008; Jore et al., 2011; Lintner et al., 2011; Wiedenheft et al., 2011a,b).

It has been recently demonstrated that the type II CRISPR system from *Streptococcus pyrogenes* can be engineered to induce Cas9-mediated double-stranded breaks (DSBs) in a sequence-specific manner in vitro by providing a synthetic guide RNA (gRNA) composed of crRNA fused to tracrRNA (Jinek et al., 2012). Moreover, the system has been successfully adapted to function in human cells with the use of human codon-optimized Cas9 and customizable 20-nt gRNAs (Cho et al., 2013; Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013a). Once the gRNA identifies its 20-bp target followed by a PAM (protospacer-adjacent motif) sequence–NGG, Cas9 nuclease then cleaves the target sequence, creating a DSB (Jinek et al., 2012). The resulting DSB will either generate nonspecific mutations knocking out a gene through the error-prone NHEJ (non-homologous end joining) pathway, or produce specific modifications dictated by an exogenous repair template through the HDR (homology-directed repair) pathway (Saleh-Gohari and Helleday, 2004; Urnov et al., 2010; Chen et al., 2011). This system greatly enhances the ease of genome engineering through the creation of desired DSBs targeted by RNA sequences that are easy to design, synthesize, and deliver, holding great promise for multiplexed genome editing.

With the advent of human induced pluripotent stem cells (hiPSCs) that can be re-programmed from fibroblasts to a human embryonic stem cell (hESC)–like state with maintained pluripotency, self-renewal, and differentiation capacity, a better understanding of human biology and potential clinical applications is now possible (Takahashi and Yamanaka, 2006; Maherali et al., 2007; Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007; Park et al., 2008). hiPSC technology presents a promising tool for supplying various cell types for transplantation therapy, regenerative medicine, drug testing, and developmental biology experiments. The potential of hiPSCs can be further enhanced

by genome engineering, which may be used to study human gene function, track cells or endogenous proteins with a knock-in reporter, and correct genetic defects for gene therapy.

To harness the full potential of hiPSC technology, this unit provides a streamlined method for conducting genome editing in hiPSCs (Fig. 31.1.1). Basic Protocol 1 describes the construction of Cas9 and gRNA plasmids, including the purification of the Cas9 plasmid from stab cultures obtained from Addgene, bioinformatic analysis to determine an appropriate target sequence, and construction of gRNA plasmid from IDT gBlocks. Basic Protocol 2 describes the transfection of hiPSCs, while the Alternate Protocol outlines the same process for HEK293 cells. Basic Protocol 3 describes the assessment of genome editing efficiency in successfully transfected cells. Finally, Basic Protocol 4 describes a method to isolate monoclonal hiPSC colonies with desired genotype.

PREPARATION OF Cas9 AND gRNA PLASMIDS

BASIC PROTOCOL 1

Plasmids containing Cas9 and the guide RNA are necessary for Cas9-mediated genome editing. This basic protocol outlines the steps necessary to prepare both plasmids for transfection.

Materials

Cas9 plasmid (Addgene, plasmid ID 41815) as bacterial stab in agar
LB agar plate containing 100 μg/ml ampicillin (*UNIT 1.1*)
LB liquid medium containing 100 μg/ml ampicillin (*UNIT 1.1*)
HiSpeed Plasmid Maxi Kit (Qiagen)
PCR-grade sterile deionized water
PCR-Blunt II-Topo kit (Invitrogen, cat. no. K2800-20) including One Shot Top10 Chemically Competent *E. coli* cells (other competent cells for cloning may also be used)
Sterilized glass beads (EMD Millipore, cat. no. 71013-3)
LB agar plate containing 50 μg/ml kanamycin (*UNIT 1.1*)
M13 Forward (5′-GTTTTCCCAGTCACGACG-3′) and M13 Reverse (5′-AACAGCTATGACCATG-3′) universal sequencing primers
LB liquid medium containing 50 μg/ml kanamycin (*UNIT 1.1*)
Qiagen plasmid Mini Kit (Qiagen)

Sterile pipet tips or toothpicks for picking colonies from agar plates
37°C incubator-shaker
Nanodrop microspectrophotometer (*http://www.nanodrop.com*)
Sequence analysis software (e.g., NCBI BLAST, UCSC Genome Browser BLAT, LaserGene)
DNA synthesis facility
42°C incubator for heat-shocking cells
10-ml bacterial culture tubes
Access to Sanger sequencing facility

Additional reagents and equipment for DNA synthesis (*UNIT 2.11*) and Sanger sequencing (*UNIT 7.1*)

Prepare Cas9 plasmid

1. Obtain plasmid from Addgene.
2. Use a sterile pipet tip or toothpick to scrape the bacterial stock from the Addgene bacterial stab, and streak it onto an LB agar plate containing 100 μg/ml ampicillin. Incubate plate at 37°C for 10 hr or overnight.

3. Once colonies are formed, pick a single colony from the plate to inoculate 200 ml of LB liquid medium containing 100 μg/ml ampicillin. Grow overnight at 37°C with shaking at 200 rpm.

4. Isolate plasmid DNA using a plasmid Maxiprep kit. Use Nanodrop microspectrophotometer to measure DNA concentration. Resuspend DNA at ~1 μg/μl in water. Use this product for transfection.

Identify appropriate gRNA targeting sequence

5. Using sequence analysis software, identify all 22-bp regions within 50 bp of the intended genomic target in the form of 5′-N19-NGG-3′.

   These 22-bp regions may be located on either strand and should ideally overlap the target sequence.

   The selected target sequence must follow the standard sequence structure of 5′-G-N19-NGG-3′. The 5′ G is necessary for the U6 promoter used on the gRNA plasmid, while the 3' NGG is the protospacer adjacent motif (PAM) that is necessary for Cas9 recognition. It must also be unique to the genomic target site and have minimal alternate targets on the genome.

6. For each candidate sequence, query for alternate binding sites in the reference genome. Because of the higher tolerance of mismatches in the first 7 bp of the target sequence, search the reference genome for the last 13 bp of the target sequence with the NGG protospacer adjacent motif (S_{13}NGG). Use NCBI BLASTN or other online software to choose the one with minimal off-target sites at region of interest. Finalize the design of the customized gRNA expression fragment (455 bp) by including the selected target sequence (N19) in the gRNA expression fragment below.

   This final sequence will contain everything necessary for gRNA expression, including the U6 promoter, customized target sequence, gRNA scaffold, and termination signal, as annotated in Figure 31.1.2.

Create gRNA plasmid construct from IDT gBlock

7. Synthesize the final gRNA expression fragment (455 bp) as a standard gBlock without any 5′ modifications from gene synthesis companies.

Figure 31.1.2 Overview of customized gRNA expression fragment. The chosen selected target sequence is inserted in the red region of the construct above. Of note, G in green indicates the start of the U6-driven transcript. For the color version of this figure, go to *http://www.currentprotocols.com/protocol/mb3101*.

8. Resuspend the gBlock (delivered at 200 ng) in 20 μl of water for a final concentration of 10 ng/μl.

9. Pipet 1 μl gBlock, 1 μl pCRII-Blunt-TOPO vector, and 4 μl salt solution (from PCR-Blunt II-Topo kit) in a 1.5-ml microcentrifuge tube, mixing gently. Incubate at room temperature for at least 5 min.

10. To transform 5 μl of product into Top10 Chemically Competent *E. coli* cells, thaw one aliquot of Top10 cells in ice for 10 min, add 5 μl of the TOPO cloning reaction from the previous step, and incubate on ice for 30 min. Heat-shock the cells at 42°C, then return to ice for 2 min. Add 250 μl of room temperature SOC medium (from PCR-Blunt II-Topo kit) and incubate at 37°C with shaking for 1 hr.

11. Spread 100 μl of the transformation mixture using sterilized glass beads onto a prewarmed LB agar plate containing 50 μg/ml kanamycin by gently swirling the plate or, alternatively, using an inoculating loop for spreading. Incubate overnight at 37°C.

    Expect 10 to 100 colonies, with the majority containing the desired insert.

12. After incubation, pick ~5 colonies for Sanger sequencing (*UNIT 7.7*) using the M13 Forward and M13 Reverse universal sequencing primers.

13. After identifying the colonies with the correct sequence, grow a maxiprep culture of the correct transformant by inoculating 200 ml of LB medium containing 50 μg/ml kanamycin with 100 μl of the original culture (step 11). Grow overnight at 37°C with shaking at 200 rpm.

14. Isolate plasmid DNA using a plasmid maxiprep kit. Resuspend plasmid DNA at ~1 μg/ml in water. Use this product for transfection (see Basic Protocol 2 and Alternate Protocol).

TRANSFECTION OF hiPSCs

BASIC PROTOCOL 2

Genome editing in hiPSCs holds great potential for gene therapy as well as the functional study of genetic variation when hiPSCs differentiate into relevant cell types. While the Cas9 and gRNA plasmids are being prepared, initiate hiPSC culture to prepare for transfection. The proper procedure for genome editing on tissue-cultured hiPSCs is described in this protocol.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and aseptic technique should be used accordingly.

Materials

PGP1 hiPSC cells adapted for growth on Matrigel (see personal genome project Web site: *http://www.personalgenomes.org/*)
Matrigel (hESC-qualified; BD Sciences, cat. no. 354277)
DMEM/F12 medium (Invitrogen)
mTeSR1 medium (StemCell Technologies, cat. no. 05850)
InSolution Rho kinase (ROCK) inhibitor (Calbiochem, cat. no. Y-27632)
P3 Primary Cell 4D-Nucleofector X kit containing P3 and Supplement 1 solutions in addition to 16-well Nucleocuvette Strips (Lonza, cat. no. V4XP-4032)
Cas9 plasmid DNA (see Basic Protocol 1)
gRNAexpression vector (see Basic Protocol 1)

Phosphate-buffered saline (PBS; Life Technologies, cat. no. 20012-050)
TrypLE Express (Invitrogen, cat. no. 12604-013)

6- and 48-well tissue culture–treated plates
15- and 50-ml conical centrifuge tubes (e.g., BD Falcon)
Countess automated cell counter (Invitrogen)
Tabletop centrifuge and plate adapter
Amaxa 4D-Nucleofector System (Lonza, cat. no. CD-MN025)

Additional reagents and equipment for culture of hiPSC in mTeSR medium (see Technical Manual Version 3.0.0 from Stem Cell Technologies; *http://www.stemcell.com/~/media/Technical%20Resources/B/C/A/2/B/29106MAN_3_0_0.pdf*)

Prepare for transfection

1. Culture PGP1 hiPSCs using standard protocol for hiPSC in mTeSR1 medium (in 6-well Matrigel-coated plates, until the cells are 40% confluent.

   To coat plates with Matrigel, do the following:
   a. Thaw a vial of 300 µl Matrigel on ice.
   b. Transfer 24 ml cold DMEM/F12 into a 50-ml conical polypropylene tube.
   c. Transfer 300 µl Matrigel into the tube. Invert to mix.
   d. Add 1 ml of this mixture per well of a 6-well plate, then leave the plate at room temperature for 1 hr.
   e. Aspirate Matrigel and replace with 2 ml cells/medium.

2. At a time point 2 hr before electroporation, replace the medium of the hiPSCs with 2 ml prewarmed mTeSR1 medium containing 2 µl/ml ROCK inhibitor.

3. At a time point 1 hr before electroporation, prepare destination wells for transfected cells:
   a. Thaw a vial of 300 µl Matrigel on ice
   b. Transfer 24 ml cold DMEM/F12 into a 50-ml conical polypropylene tube.
   c. Transfer 300 µl Matrigel into the tube. Invert to mix.
   d. Add 500 µl of this mixture per well of 48-well plate (one well will be needed per transfection), then leave the plate at room temperature for 1 hr.
   e. Aspirate Matrigel and replace with prewarmed 500 µl mTeSR1 medium with 2 µl/ml ROCK inhibitor.

   The small surface area of the wells of 48-well plates promotes high cell density and healthy growth after transfection.

4. Prepare a transfection master mix (scale appropriately):
   16.4 µl P3 and 3.6 µl Supplement 1 from Nucleofactor X kit
   1 µl 1 µg/µl Cas9 plasmid
   1 µl 1 µg/µl gRNA plasmid
   22 µl per reaction, total.

Transfect hiPSCs

5. Aspirate the ROCK inhibitor–containing medium from the wells containing hiPSCs and wash each well with 2 ml room temperature PBS.

6. Aspirate PBS, add 1 ml TrypLE Express, and incubate the plate at 37°C for 5 min.

7. Resuspend cells with 3 ml mTeSR1 medium and gently pipet up and down several times to generate a single-cell suspension. Transfer disassociated cells into a 15-ml centrifuge tube containing 10 ml mTeSR1 medium.

8. Count cells with cell counter and calculate total volume required for 1×10^6 cells/transfection, scaling as needed.

   Given the toxicity of transfection, the minimum number of cells per transfection required to isolate transfectants is 200,000. However, higher cell counts decrease the efficiency of transfection by increasing the number of targets. A titration of cell counts ranging from 200,000 to 1×10^6 may help find the optimal balance.

9. Place desired quantity of cells (in this case 1×10^6) in 15-ml centrifuge tube, centrifuge at $200 \times g$ for 5 min at room temperature, and aspirate supernatant.
10. Resuspend each unit of 1×10^6 cells in 22 µl of the transfection master mix prepared in step 4.
11. Quickly transfer cells into the central chamber of one well of a Nucleocuvette strip. Place the strip into 4-D Nucleofector device.
12. Nucleofect cells using program CB150.
13. Quickly add 80 µl of prewarmed mTESR1 medium containing 2 µl/ml ROCK inhibitor to each well of electroporated cells. Pipet up and down once or twice to mix.
14. Transfer cells from the strip to wells of the Matrigel-coated plate containing mTeSR1 medium with 2 µl/ml ROCK inhibitor prepared in step 3.
15. Centrifuge the plate 3 min at $70 \times g$, room temperature. Place cells into 37°C incubator.
16. After 24 hr, change to fresh mTeSR1 medium without ROCK inhibitor.
17. Harvest cells 3 days after electroporation. Follow protocol for assessing targeting efficiency in Basic Protocol 3.

TRANSFECTION OF HUMAN HEK293 CELLS

ALTERNATE PROTOCOL

Genome editing in HEK293 cells is efficient and convenient, thus serving as an ideal system to test and optimize reagent before moving to hiPSCs. Here, we describe the transfection procedure on HEK293 cells with the Cas9/gRNA plasmids.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and aseptic technique should be used accordingly.

Additional Materials *(also see Basic Protocol 2)*

HEK 293 cells (Invitrogen)
Complete DMEM medium (see recipe)
Lipofectamine 20000 (Invitrogen, cat. no. 11668027)
Opti-MEM medium (Invitrogen, cat. no. 31985062)
Cas9 plasmid DNA (see Basic Protocol 1)
gRNAexpression vector (see Basic Protocol 1)
12-well tissue culture treated plates

Plate 293 cells for transfection

1. Culture HEK 293 cells in complete DMEM medium in 6-well plates until the cells are ~70% confluent.
2. Aspirate medium and wash cells with 2 ml room temperature PBS.

3. Aspirate PBS, add 1 ml TrypLE Express, and incubate at 37°C for 2 min.
4. Resuspend cells with 5 ml prewarmed complete DMEM medium
5. Count cells using an automated cell counter and calculate volume required for 200,000 cells per transfection.
6. Place desired volume of cells into 15-ml centrifuge tube. Centrifuge 5 min at 200 × *g*, room temperature, and aspirate supernatant.
7. Resuspend cell pellet in 1 ml complete DMEM medium.
8. Plate cells in a 12-well tissue culture plate and return to incubator.

Transfect 293 cells

9. After a day of incubation, replace medium on cells with 1 ml fresh prewarmed complete DMEM medium. Return to incubator and allow to incubate while preparing DNA mix.
10. Add 5 μl Lipofectamine 2000 to 50 μl Opti-MEM in a 1.5-ml microcentrifuge tube. Invert several times to mix. Incubate the mixture at room temperature for 5 min.
11. Add 1 μg Cas9 plasmid and 1 μg gRNA to 50 μlOpti-MEM in a 1.5-ml microcentrifuge tube.
12. Add diluted DNA from step 11 to diluted Lipofectamine mixture from step 10, flicking the tube several times to mix.
13. Incubate the mixture 15 min at room temperature.
14. Add 100 μl of the mixture dropwise to the cells.
15. Replace medium after 24 hr with fresh prewarmed complete DMEM medium.

    High concentrations of Lipofectamine can be toxic. Monitor cell conditions. If high cell toxicity is observed, change to fresh DMEM medium after 8 hr.

16. Harvest cells 3 days after transfection.

BASIC PROTOCOL 3

GENOTYPING TRANSFECTED CELLS USING NEXT-GENERATION SEQUENCING

After transfection, the targeting efficiency needs to be assessed to determine whether isolation of genome-targeted cells from a heterogeneous population is feasible. Normally, targeting efficiency on the order of 1% is expected for hiPSCs using Cas9-gRNA system without selection. This basic protocol describes the assessment of the targeting efficiency using next-generation sequencing techniques that can yield high read depths on the targeted site from a population of nucleofected cells.

Materials

Illumina forward sequence (ACACTCTTTCCCTACACGACGCTCTTCCGATCT)
Illumina reverse sequence
(GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT)
Transfected hiPSCs (Basic Protocol 1 or Alternate Protocol) growing in culture
Phosphate-buffered saline (PBS; Life Technologies, cat. no. 20012-050)
mTeSR1 medium (StemCell Technologies, cat. no. 05850)
prepGEM gold buffer (ZyGEM)
prepGEM tissue protease enzyme (ZyGEM)
KAPA Hifi Hotstart Readymix (KAPA Biosystems)
Illumina amplification primers (see step 3)

Illumina index primers (ScriptSeq Index PCR Primers)
Illumina PCR primer (AATGATACGGCGACCACCGAGATCTACACTCTTTCC-CTACACGACGCTCTTCCGATCT)
2-log DNA ladder (New England Biolabs)
QIAquick PCR purification kit (Qiagen)

Computer running Primer3 software (*http://primer3.sourceforge.net/*) for primer identification
15-ml conical tubes (BD Falcon)
Tabletop centrifuge
Thermal cycler
Access to MiSeq sequencer

Additional reagents and equipment for agarose gel electrophoresis (*UNIT 20.5A*) and measuring DNA concentration (*APPENDIX 3D*)

Design Illumina amplification primers for the targeting region

1. Select a ~500-bp region around the targeting site.
2. Use Primer3 to identify optimal targeting primer sets that amplify 200 to 300 bp around the targeting site.
3. Finalize the design of and order the customized Illumina amplification primers:

   a. Append the Illumina forward sequence (ACACTCTTTCCCTACAC-GACGCTCTTCCGATCT) to the 5′ end of the forward primer from step 2.
   b. Append the Illumina reverse sequence (GTGACTGGAGTTCAGACGTGT-GCTCTTCCGATCT) to the 5′ end of the reverse primer from step 2.

   The Illumina amplification scheme is summarized in Figure 31.1.3.

Harvest cells and create sequencing library

4. Aspirate the mTeSR1 medium from the cultured, transfected hiPSCs and wash the cells gently with PBS.
5. Aspirate PBS, add 1 ml TrypLE Express, and incubate the plate at 37°C for 5 min.
6. Transfer disassociated cells into a 15-ml conical tube containing 10 ml mTeSR1 medium and centrifuge 5 min at 200 × *g*, room temperature.
7. Aspirate supernatant and resuspend the cell pellet with the residual medium left in the conical tube.
8. Prepare a 10-μl cell lysis reaction with the following reagents in a PCR strip:
   8.9 μl cell pellet suspension
   1 μl prepGEM gold buffer (ZyGEM)
   0.1 μl of prepGEM tissue protease enzyme (ZyGEM)
9. Incubate the reaction in a thermal cycler:
   75°C for 15 min
   95°C for 5 min.
10. Prepare a 20-μl PCR reaction to obtain the amplicon of the targeting region.
    1 μl of the reaction from step 9
    10 μl 2 × KAPA Hifi Hotstart Readymix
    0.2 μl 100 mM each Illumina amplification primer (see step 3)
    Water to 20 μl.
11. Perform PCR with the following parameters:

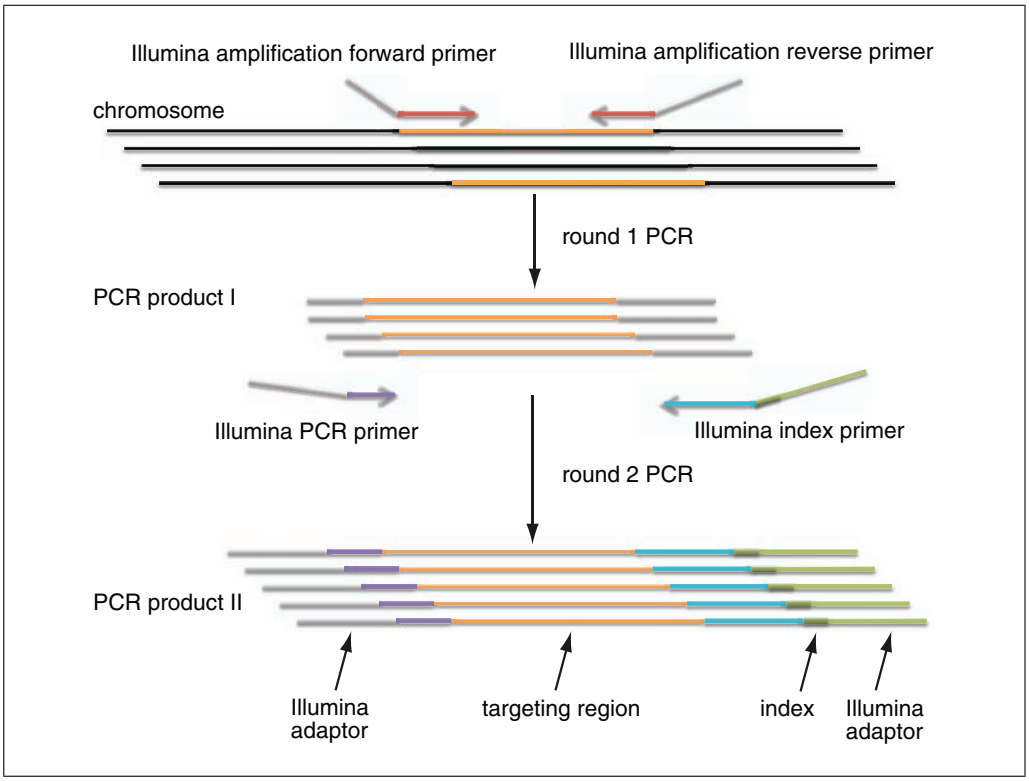

Figure 31.1.3 Schematic of Illumina sequencing library preparation. The first round of PCR amplifies the targeting region with the universal forward and reverse sequences necessary to anneal to the Illumina proprietary primers. The second round of PCR adds an index primer necessary for deconvoluting separate sequencing pools, as well as the adaptor necessary for attachment to the sequencing flow cell.

1 cycle:	5 min	95°C	(initial denaturation)
15 to 25 cycles:	20 sec	98°C	(denaturation)
	20 sec	65°C	(annealing)
	20 sec	72°C	(extension).

If you do not get clear product bands, try more cycles to amplify desired product.

12. Prepare the second round of PCR reaction to add the Illumina sequence adaptor.
    5 µl of the reaction from step 11
    10 µl KAPA Hifi Hotstart Readymix
    1 µl Illumina index primer
    0.1 µl of 100mM Illumina PCR primer
    Water to 20 µl.

    There are 48 orthogonal Illumina index primers from the ScriptSeq Index PCR Primers kit. Choose independent index primers for different reactions.

13. Perform the second round of PCR with the following parameters:

1 cycle:	5 min	95°C	(initial denaturation)
15 to 25 cycles:	20 sec	98°C	(denaturation)
	20 sec	65°C	(annealing)
	20 sec	72°C	(extension)
1 cycle:	4 min	72°C	(final extension).

If you do not get clear product bands, try more cycles to amplify desired product.

14. Run PCR product on a 2% agarose gel (*UNIT 20.5A*) against a 2-log DNA ladder and verify the correct amplicon length.

The Illumina sequencing adapter adds 160 bp to the genomic amplicon.

15. PCR purify the product with QIAquick PCR purification kit. Measure concentration of each sample (*APPENDIX 3D*) and pool each sample at the same concentration to ensure equal sequencing coverage. Submit for sequencing with MiSeq Personal Sequencer.

16. After the sequencing results arrive, analyze the results using the bioinformatics platform of choice.

    The authors recommend the CRISPR genome analyzer: http://54.80.152.219/.

    The incorporation frequency is defined by the percentage of sequences that have mutated away from the wild-type sequence from the original sample. An incorporation frequency of 1% or more is ideal for the downstream protocols.

SINGLE-CELL ISOLATION OF GENOME-TARGETED MONOCLONAL hiPSCs

BASIC PROTOCOL 4

After a successful round of Cas9-mediated genome engineering, the next step is to isolate monoclonal hiPSC colonies with the desired genotype. This can be accomplished by fluorescence-activated cell sorting (FACS) and genotyping of single cell–derived colonies. Once genome-edited monoclonal hiPSC colonies have been isolated, they can be utilized for downstream applications—e.g., differentiating into relevant tissue types to interrogate functionality in biology and disease.

Materials

0.1% (w/v) gelatin (StemCell Technologies, cat. no. 07903)
Irradiated CF-1 mouse embryonic fibroblasts (Michalska, 2007)
hES cell medium (see recipe)
Recombinant fibroblast growth factor (Millipore)
SMC4 (BD Biosciences; for 1× SMC4, supplement 500 ml of medium with one vial of SMC4 purchased from BD)
Fibronectin (StemCell Technologies)
Heterogenous pool of edited hiPSC (Basic Protocol 2)
mTeSR1 medium (StemCell Technologies, cat. no. 05850) supplemented with SMC4 (BD Biosciences) at final concentration of 1× (one vial per 500 ml medium)
mTeSR1 medium (StemCell Technologies, cat. no. 05850), unsupplemented
Phosphate-buffered saline (PBS; Invitrogen, cat. no. 20012-050)
Accutase (Millipore)
ToPro-3 viability dye (Invitrogen)
Matrigel (hESC-qualified; BD Sciences, cat. no. 354277)

96-well plates
BD FACSAria II SORP UV (BD Biosciences) with 100-mm nozzle
Centrifuge for 96-well plates
Access to Sanger sequencing facility

Additional reagents and equipment for obtaining amplicons of the targeting region (see Basic Protocol 3, steps 4 to 11)

1. One day before the experiment, prepare 96-well plates with CF-1 mouse embryonic fibroblast (MEF) as follows:
   a. Coat the plate by incubating 15 min with 50 μl/well of 0.1% (w/v) gelatin at room temperature, and wash with PBS.

b. Thaw and plate MEF in the gelatin-coated 96-well at a concentration of 1×10^6 cells/well in complete DMEM medium.
c. Incubate the MEF plate in the 37°C incubator overnight.
d. Following the overnight incubation, change the medium to hES cell medium supplemented with 100 ng/ml fibroblast growth factor, 1× SMC4 (one vial per 500 ml medium), and 5 mg/ml fibronectin.

2. Replace the medium on the hiPSCs in a 48-well plate from Basic Protocol 2 with mTeSR1 medium supplemented with 1× SMC4 (one vial per 500 ml medium) for at least 2 hr before FACS analysis is to be performed.

3. Aspirate the medium from the cultured hiPSCs, then wash the cells gently with PBS.

4. Aspirate PBS, add 200 μl/well Accutase (or enough to cover the well), and incubate at 37° for 5 to 10 min.

5. Generate the single-cell suspension by adding 1 ml mTeSR1 (unsupplemented) to each well and pipetting up and down gently several times.

6. Place cell suspension in a 15-ml conical tube, then centrifuge 5 min at 200 × *g*, room temperature. Aspirate supernatant.

7. Resuspend the cells with 1 ml mTeSR1 and add 0.5 μl of the viability dye ToPro-3.

8. Using a BD FACSAria II SORP UV with 100-mm nozzle under sterile conditions, sort single cells into individual wells of the 96-well plates prepared in step 1.

   The 100-mm nozzle is critical for the FACS experiment, to minimize the stress on hiPSCs.

9. After collection, centrifuge plates 3 min at 70 × *g*, room temperature. and place the plate into the tissue culture incubator

10. Four days after sorting, colony formation should be apparent; at this point replace the culture medium with hES cell medium supplemented with 1× SMC4.

11. Eight days after sorting, replace medium with hES medium (unsupplemented).

    SMC4 is beneficial for cell viability post sorting. However, long-duration exposure of cells to SMC4 may lead to cell differentiation. We recommend removing SMC4 from the culture medium once the colony formation is stable.

12. Passage the monoclonal hiPSC cells into Matrigel-coated 96-well plate and save half of the cells for genotyping.

    To coat wells with Matrigel, do the following:
    a. Thaw a vial of 300 μl Matrigel on ice.
    b. Transfer 24 ml cold DMEM/F12 into a 50-ml conical polypropylene tube.
    c. Transfer 300 μl Matrigel into the tube. Invert to mix.
    d. Add 100 μl of this mixture per well of a 96-well plate, then leave the plate at room temperature for 1 hr.
    e. Aspirate Matrigel and replace with 200 μl cells/medium.

13. Perform steps 4 to 11 in Basic Protocol 3 to obtain amplicons of the targeting region.

    The amplicon produced in the first round of Illumina PCR is sufficient for Sanger sequencing. Either the forward or the reverse primer can be used as the sequencing primer.

14. Perform Sanger sequencing to check the genotype of the targeting region.

15. Choose a colony containing the correct mutation for downstream differentiation or processing.

Table 31.1.1 Troubleshooting Common Problems with the CRISPR/Cas9 System

Problem	Possible cause	Solution
Low hiPSC viability after electroporation	DNA plasmid purity is low	Use maxiprep kit to generate high-quality DNA
	Too much DNA	Reduce the amount of DNA
	Cell density is too high before electroporation	Transfect cells under exponential growth phase
	Cell number is not sufficient	Use a minimum of 300,000 cells per transfection
	Delay of cell recovery after electroporation	Speed the recovery after electroporation by preparing all the necessary plates and pipets in advance and recovering the cells as soon as the electroporation is complete
	Cell is sensitive to trypsin treatment	Use nonenzymatic method to generate single-cell suspension, such as EDTA treatment
	gRNA off-target effect is prevalent and toxic to the cell	Try alternative gRNA targeting site
Low genome targeting efficiency	DNA transfection efficiency is low, or cell viability is low after transfection	Use Cas9-GFP construct followed by FACS to enrich transfected cell
	The targeting site is not accessible/targetable	There is no systematic knowledge yet regarding the impact of the targeting sequence on the targeting efficiency. We recommend that users design/generate/test multiple gRNAs near the region of interest.
Unable to obtain amplicon of the targeting region	Primer design is not optimal	Use Primer3 or other primer design software to optimize the design of primer on the targeting region
	Insufficient cell number	Start with at least >1000 cells
	Lysis reaction is not sufficient	Elongate the prepGEM digestion time
	Too much lysis reaction in the PCR reaction	Use no more than 1/10 volume of lysis reaction in the final PCR reaction

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Complete medium for HEK 293 cells

High-glucose DMEM medium (Invitrogen) supplemented with:
10% fetal bovine serum (FBS)

continued

Table 31.1.2 Time Considerations for CRISPR/Cas9 Protocols

Procedure	Substep	Hands-on time	Total experiment time	Stopping point
Basic Protocol 1—Preparation of hCas9 and gRNA plasmids		5 hr	~ 4 days	Yes
	Prepare hCas9 plasmid	2 hr	2 hr	Yes
	Identify appropriate gRNA targeting sequence	1 hr	~3 days	Yes
	Plasmid construction of gRNA construct from IDT gBlock	2 hr	1 day	Yes
Basic Protocol 2—Transfection of Human iPS cells		3 hr	~3 days (recovery after transfection)	No
Basic Protocol 3—Genotyping transfected cells using next generation sequencing		4 hr	~2 days	Yes
	Harvest cells and create sequencing library	3 hr	3 hr + 1 day (sequencing)	Yes
Basic Protocol 4—Isolate genome-edited hiPSCs			~12 days	
	FACS sorting	3 hr	1 day (prepare MEF plate) +1 day (FACS) + 8 days (colony growth)	Yes
	Harvest cells and create sequencing amplicon	3 hr	3 hr + 1 day (Sanger sequencing)	Yes

1× nonessential amino acids (NEAA)
1× penicillin/streptomycin solution (pen/strep)
Store up to 3 to 6 months at 4ºC

hES cell medium

DMEM/F12 medium (e.g., Invitrogen) containing:
20% (v/v) knockout serum replacement (KOSR)
5 to 10 ng/ml bFGF
1 mM L-glutamine
100 μM nonessential amino acids
100 μM 2-mercaptoethanol
1× penicillin/streptomycin solution (pen/strep)
Store up to 3 to 6 months at 4ºC

COMMENTARY

Background Information

Cas9 is a tool for easily editing the genome of human cells. Compared with other genome editing methods, such as ZFNs and TALENs, the RNA-guided Cas9 system has certain advantages.

First, the simplicity of its design and construction make the tool more accessible. Second, the mere requirement of small RNAs for each new target allows for multiplexible genome targeting. Third, independent studies indicate that the Cas9 system is more efficient than other tools targeting the same region (Hwang et al., 2013; Mali et al., 2013a). However, the specificity of Cas9-mediated genome targeting is still under investigation. Judicious selection of the targeting site is necessary to minimize off-target effects.

Critical Parameters

As with any transfection, the quality of the DNA plasmid is paramount. We recommend plasmid maxiprep kits providing transfection-level DNA, especially for hiPSCs. The amount of DNA used in the transfection is also an important parameter, since higher transfection efficiency and dosage usually yield higher genome-targeting efficiency. When a new cell line is used for the first time, the amount of DNA needed for optimal transfection efficiency should be determined by titration. Finally, the concentration of DNA used in transfection is another important parameter, since the DNA volume used for hiPSC electroporation should be less than 1/10 of the total reaction volume to achieve effective transfection without incurring significant cell death. If the concentration is too low to satisfy this requirement, use a Speedvac evaporator to evaporate some of the water in the DNA solution, thereby increasing concentration.

The hiPSC density before transfection is important, as we have observed that genome editing on cells at exponential growth phase yields higher efficiency. We recommend that users conduct transfection on cells that have reached 30% to 40% confluence.

Finally, when assessing the efficiency of genome editing, the number of cells used in genotyping is critical for successful genotyping following this protocol. We tested the sensitivity of genotyping and found that a minimum of four cells is required to enable the amplification reaction. However, empirically, robust target region amplification occurs with >1000 cells.

Troubleshooting

Table 31.1.1 describes some problems commonly encountered with the protocols described in this unit, along with accompanying solutions.

Anticipated Results

We can achieve $\sim$2% genome targeting efficiency in hiPSCs and $\sim$30% genome targeting efficiency in HEK293 cells using the methods described above. The efficiency in hiPSCs varies with the targeting sites and locations. We detected 0.2% to 15% targeting efficiency in hiPSCs and 1% to $>$50% targeting efficiency in HEK 293 cells without any transfection enrichment and selection. We recommend that a transfection-enrichment strategy be used to maximize the efficiency.

Double-nickases represent an alternative approach for genome editing with mitigated off-targeted effects. It has been shown that the efficiency achieved by double-nickases is comparable to that of nuclease in HEK293 (Mali et al., 2013b; Ran et al., 2013). However, it is still under investigation whether the double-nickase strategy would work in hiPSCs.

Time Considerations

See Table 31.1.2 for a description of the time required for the protocols described in this unit.

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