CRISPR-Cas-Mediated Targeted Genome Editing in Human Cells

Luhan Yang, Prashant Mali, Caroline Kim-Kiselak, and George Church

Abstract

The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) systems have evolved as an adaptive surveillance and defense mechanism in bacteria and archaea that uses short RNAs to direct degradation of foreign genetic elements. Here, we present our protocol for utilizing the *S. pyogenes* type II bacterial CRISPR system to achieve sequence-specific genome alterations in human cells. In principle, any genomic sequence of the form N₁₉NGG can be targeted with the generation of custom guide RNA (gRNA) which functions to direct the Cas9 protein to genomic targets and induce DNA cleavage. Here, we describe our methods for designing and generating gRNA expression constructs either singly or in a multiplexed manner, as well as optimized protocols for the delivery of Cas9-gRNA components into human cells. Genomic alterations at the target site are then introduced either through nonhomologous end joining (NHEJ) or through homologous recombination (HR) in the presence of an appropriate donor sequence. This RNA-guided editing tool offers greater ease of customization and synthesis in comparison to existing sequence-specific endonucleases and promises to become a highly versatile and multiplexable human genome engineering platform.

Key words CRISPR, Cas9, Human genome engineering, hiPSCs

1 Introduction

The CRISPR-Cas system, found in most bacteria and archaea, provides immunity against invading genetic elements [1–3]. In the immunization phase, upon exposure to viral DNA or foreign plasmids, short fragments of exogenous DNA are integrated into the CRISPR locus in the prokaryote genome as spacers between repeat sequences [4]. The locus consists of CRISPR-associated (Cas) genes in operons in addition to the spacer-repeat array. In the immunity phase, the spacer-repeat array is expressed as a pre-CRISPR RNA (pre-crRNA), and the transcript is processed into individual spacer-repeat units as small guiding crRNAs. These crRNAs then form a complex with Cas proteins to induce cleavage of foreign nucleic acids complementary to the spacer sequence [5].
The CRISPR-Cas systems described thus far fall into three major classes, each with distinct mechanisms of action and composed of different Cas gene family [6]. While the type I and III systems utilize multiple distinct effector proteins to direct endonuclease activity, the type II system relies primarily on a single protein Cas9 to both generate crRNA and cleave corresponding target DNA and thus represents the most convenient format for transferring this machinery into eukaryotic cells. Specifically, in type II system in *S. pyogenes*, transactivating crRNA (tracrRNA) first hybridizes to the repeat regions of pre-crRNA. This duplex is then recognized and cleaved by Cas9 and RNase III, and the resulting mature crRNA remains in complex with tracrRNA and Cas9 to form the functional unit that ultimately targets and cleaves foreign DNA homologous to the crRNA spacer sequence.

Jinek et al. [7] recently demonstrated that a single tracrRNA-crRNA chimera in complex with the Cas9 protein was capable of introducing sequence-specific double-stranded DNA cleavage in vitro. We engineered this two-component system for function in eukaryotic cells [8] by (1) inducing direct expression of an optimized tracrRNA-crRNA chimera construct, which we have termed guide RNA (gRNA), and (2) expressing the human codon-optimized Cas9 (hCas9) protein (Fig. 1a).

Cas9 detects genomic targets by unwinding the DNA duplex and scanning for complementarity between the genomic DNA and the spacer sequence in the gRNA. However, Cas9 will only cleave DNA if the correct protospacer-adjacent motif (PAM) is detected [7, 9]. While each type II system has individual PAM requirements, the *S. pyogenes* system described in this protocol requires an NGG sequence immediately downstream of the protospacer, with N being any nucleotide (Fig. 1b).

The Cas9 protein contains two nuclease domains, an HNH domain and a RuvC-like domain, which together generate a double-stranded break at the 3′ end of the protospacer following target recognition. If one of the two nuclease domains is inactivated, Cas9 will function as a nickase in vitro [7] as well as in human cells [8]. Cas9-induced double-stranded breaks or single-stranded nicks are subsequently detected by host DNA repair mechanisms, most often through the nonhomologous end-joining (NHEJ) pathway in mammalian cells, an error-prone process which can result mutations at targeted genomic sites.

In contrast to other recent technologies such as zinc finger nucleases and TALENs [10, 11], the CRISPR system relies upon easily engineered RNA molecules for sequence specificity versus DNA-binding proteins that must be reconfigured for each targeted region. Here, we describe a robust platform for gRNA synthesis and expression which can be used to generate single gRNAs as well as large pooled libraries synthesized from custom DNA arrays. Towards this, as a ready reference, a genome-wide resource of ~200,000 unique gRNAs targeting ~40% of human exons featuring
the NGG PAM sequence, at a multiplicity of ~2.4 sites per targeted exonic region as described in Mali and Yang et al. [8], is available for reference at http://arep.med.harvard.edu/human_crispr/. Finally, we describe our protocols for targeted genome editing in human cells using this engineered system. With its simplicity of design and construction, the Cas9-gRNA system represents an appealing and facile approach for human genome engineering with broad applications in basic and applied medical research.

2 Materials

2.1 Determination of gRNA Target Sequence

1. Sequence analysis software.
2. Sequence alignment search software such as NCBI BLAST or UCSC Genome Browser BLAT.

2.2 gRNA Synthesis

2.2.1 Option A

1. gBlock (IDT) containing desired gRNA target sequence.
2. PCR-grade sterile deionized water.
3. Option 3a:
(a) PCR-Blunt II-TOPO kit (Invitrogen, K2800-20) including One Shot Top10 Chemically Competent *E. coli* cells.

(b) Incubator, 42 °C.

(c) SOC medium, provided with One Shot Top10 cells (Invitrogen).

(d) Incubator shaker, 37 °C.

(e) Sterilized glass beads.

(f) LB agar: 1 % (w/v) Bactotryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) sodium chloride, 1.5 % (w/v) agar.

(g) LB broth: 1 % (w/v) Bactotryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) sodium chloride.

(h) Kanamycin stock at 50 mg/mL in water.

(i) 10 mL bacterial culture tubes.

(j) 1.5 mL microcentrifuge tubes.

(k) Qiagen plasmid miniprep kit (Qiagen).


(m) M13 Forward (5′-GTTTTCCCAGTCACGACG-3′) and M13 Reverse (5′-AACAGCTATGACCATG-3′) universal sequencing primers.

(n) Access to Sanger sequencing facility.

(o) HiSpeed Plasmid Maxi Kit (Qiagen).

Option 3b:

(a) PCR primers gRNA_F (5′-TGTACAAAAAAAGCAGGC TTTAAAG-3′) and gRNA_R (5′-TAATGCCAACTTTG TACAAGAAAG-3′).

(b) 2× KAPA HiFi HotStart ReadyMix (Kapa, KK2601).

(c) PCR tubes.

(d) Thermocycler.

(e) E-Gel EX_Gel 2 % (Invitrogen).

(f) E-Gel agarose gel electrophoresis system (Invitrogen).

(g) 2-log DNA ladder (NEB).

(h) PCR purification kit (Qiagen).

(i) 1.5 mL microcentrifuge tubes.

(j) NanoDrop 2000 (NanoDrop).

(k) Speedvac.

**2.2.2 Option B**

1. PCR-grade sterile deionized water.

2. 60mer oligonucleotides Insert_F and Insert_R, sequence in Subheading 3.2.2, step 1.

3. 1.5 mL microcentrifuge tubes.
2.3 **gRNA Preparation from Microarrays for Multiplexible Targeting**

CRISPR-Cas-Mediated Targeted Genome Editing in Human Cells

1. 200 bp oligonucleotide pool (Custom Array Inc.).
2. PCR-grade sterile deionized water.
3. 2× KAPA HiFi HotStart ReadyMix (Kapa, KK2601).
4. PCR primers.
   (a) gRNA pool F1: TATGAGGACGAATCTCCCGCTTATA
   (b) gRNA pool R1: GGTCCTTGACAAACGTGTGCTTGTAC
   (c) Target-specific barcode forward primer (25 bp).
   (d) Target-specific barcode reverse primer (25 bp).
   (e) gRNA pool F2: tttcttggctttatatatcttgtggaaaggac
(f) gRNA pool R2: GACTAGCCTTATTTTAACTTG-CT ATTTCTAGCT

5. Thermocycler.
6. QIAquick PCR purification kit (Qiagen).
7. 1.5 mL microcentrifuge tubes.
8. AflII restriction enzyme (NEB, R0520).
9. E-Gel EX_Gel 2 % (Invitrogen).
10. E-Gel agarose gel electrophoresis system (Invitrogen).
11. 2-log DNA ladder (NEB).
12. QIAquick Gel Extraction Kit (Qiagen).
14. One Shot Top10 Chemically Competent E. coli cells (Invitrogen, C4040-03).
15. SOC medium, provided with One Shot Top10 cells (Invitrogen).
16. LB agar.
17. Kanamycin stock at 50 mg/mL in water.
18. Incubator.
19. LB broth.
20. Incubator shaker (37 °C).
21. 10 mL bacterial culture tubes.
22. Qiagen plasmid miniprep kit (Qiagen).
25. HiSpeed Plasmid Maxi Kit (Qiagen).

2.4 Prepare hCas9 Plasmid

1. hCas9 bacterial glycerol stock (Addgene, plasmid ID 41824).
2. LB agar.
3. Ampicillin stock at 50 mg/mL in water.
4. Incubator(37 °C).
5. LB broth.
6. Incubator shaker.
7. HiSpeed Plasmid Maxi Kit (Qiagen).
8. PCR-grade sterile deionized water.

2.5 Transfection of 293 HEK Cells

2.5.1 Day 0: Plate 293 Cells for Transfection

1. 293 human embryonic kidney cells (Invitrogen).
2. 6 well tissue culture-treated plates.
3. Humidified incubator, with cells maintained at 37 °C and 5 % CO₂.
4. High-glucose DMEM media (Invitrogen).
2.5.2 Day 1: Transfection

1. Complete DMEM media (10 % FBS, 1× P/S, and 1× NEAA).
2. Lipofectamine 20000 (Invitrogen, 11668027).
3. Opti-MEM Medium (Invitrogen, 31985062).
4. 1.5 mL microcentrifuge tube.
5. hCas9 plasmid DNA generated in Subheading 3.3.
6. gRNA expression vector generated in Subheading 3.2.
7. 1.5 mL microcentrifuge tubes.
8. Humidified incubator, with cells maintained at 37 °C and 5 % CO₂.

2.6 Transfection of PGP1 iPS Cells

2.6.1 Matrigel Preparation

1. PGP1 iPS cells adapted for growth on Matrigel.
2. Humidified incubator, with cells maintained at 37 °C and 5 % CO₂.
4. DMEM/F12 media (Invitrogen).
5. 48 well tissue culture-treated plates (BD Biosciences).
6. mTeSR1 medium (Stemcell Technologies, 05850).
8. PBS (Invitrogen).
10. Countess automated cell counter (Invitrogen).
11. 15 mL centrifuge tubes.
12. Tabletop centrifuge.
13. P3 Primary Cell 4D-Nucleofector X kit containing P3 and Supplement 1 solutions in addition to 16-well Nucleocuvette strips (Lonza, V4XP-4032).
15. hCas9 plasmid DNA generated in Subheading 3.3.
16. gRNA expression vector generated in Subheading 3.2.
17. 15 mL centrifuge tubes.

3 Methods

The following is a general protocol (Fig. 2) for performing CRISPR-mediated genome editing through the identification of gRNA target sites (Fig. 3), generation of gRNA expression vectors either singly or in multiplexed fashion (Figs. 4 and 5), and the transfection of CRISPR elements into 293 HEK and PGP1 iPS cell lines (Fig. 6).

In this system, we have utilized the human U6 promoter to directly drive gRNA transcription, due to its well-defined transcriptional start and end points [12, 13]. However, this approach requires that gRNA transcription initiate with G, which is then followed by the remaining 19 nt sequence target in genomic sequence preceding the NGG PAM sequence (Fig. 1). The inclusion of this initial G in the final gRNA, which may not be present in the genomic sequence, does not affect sequence specificity due to its presence at the 5′ end of the spacer region, where mismatches are tolerated [7, 9].

This protocol also specifically describes our method for generating a large library of gRNA sequences targeting the human exome, designed in a 200 bp format compatible with multiplex synthesis on custom DNA arrays and efficient cloning into a common expression vector [8]. This permits the targeted retrieval of individual gRNA sequences as each sequence is associated with a
**Step 1:** Find all 23bp genomic sites of the form 5’-NGG-3’ near your intended target site (ideally ±50bp). These may reside on the + or -strand. Representative example:

<table>
<thead>
<tr>
<th>Target sequence</th>
<th>PAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’-TAATACTTTATCTGTCCCCTCCACCCCACAGTGGGGCCAC-3’</td>
<td>N19 NGG</td>
</tr>
<tr>
<td>3’-ATTATGAAAATAGACAGGGGAGGTGGGGTGTCACCCCGGTATCCCTGTCTAAACCAGCTGTCTTTCGGG-5’</td>
<td></td>
</tr>
</tbody>
</table>

**Step 2:** Using NCBI blast, select sequences for which none or very few sequences of the form 5’-NNNN NNSSSS SSSSS SSSSS NGG-3’ exist at any other location in the human genome (here the S’s represent the actual sequence bases at the target genomic location). Representative example:

```
5’-TAATACTTTATCTGTCCCCTCCACCCCACAGTGGGGCCAC-3’
3’-ATTATGAAAATAGACAGGGGAGGTGGGGTGTCACCCCGGTATCCCTGTCTAAACCAGCTGTCTTTCGGG-5’
```

**Step 3 (option A):** Incorporate 19bp of the selected target sequence as highlighted here: 5’-NNNN NNSSSS SSSSS SSSSS NGG-3’ into the DNA fragment as indicated below:

```
TGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGGTAC
CAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATA
TACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACA
AAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTT
TGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTT
GAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACC
GGCCAC
```

This 455bp fragment bears all components necessary for gRNA expression, namely: **U6 promoter + target sequence + guide RNA scaffold + termination signal.**

**Fig. 3** Design of Cas9-gRNA targeting site (the sequence and the BLAST results are hypothetical for the purpose of demonstration)

**Fig. 4** Schematic outline of gRNA expression constructs design and synthesis approaches A (Subheading 3.2.1)
unique barcode. We anticipate that users will develop many alternative approaches to this basic design, such as using barcodes associated with several gRNA sequences for pooled retrieval or generating cheaper 100 bp arrays as a more cost-effective approach for certain applications.

While we have had success with these methods thus far as presented, several aspects of this protocol may require additional optimization, especially when applied to other cell lines. We discuss this topic as well as other potential limitations of this system in Subheading 4 of the manuscript.

3.1 Determination of Target Sequence

1. Using sequence analysis software, identify all 22 bp regions within 50 bp of your intended genomic target in the form of 5'-N_{19}-NGG-3'. These 22 bp regions may be located on either strand and should ideally overlap the target sequence (Fig. 3).

2. Use an alignment search tool to check whether the selected target sequences are unique in the genome to prevent any undesired off-target effects. Exact correspondence in the 13 bp
region immediately proximal to the NGG PAM sequence (Fig. 3) is required for Cas9-gRNA specific recognition, while mismatches in the 5′ region are better tolerated. Thus, to determine whether Cas9-gRNA cleavage sequence is unique to the genome, one needs to check the existence of other sites in the genome in the form of $S_{13}$NGG ($S, N = A, T, C, G$) (Fig. 3). If multiple hits besides the desired genomic target are observed, other alternative sites need to be chosen and checked. The specificity of gRNA target sequence design is discussed below (see Note 1).

### 3.2 gRNA Synthesis

1. Design gRNA expression constructs according to the target sequence 5′-N$_{19}$-NGG-3′. Incorporate the customized N$_{19}$ sequence into the gRNA expression fragment design. This final fragment contains all of the components necessary for gRNA expression, including the U6 promoter, customized target sequence, the gRNA scaffold, and termination signal (Fig. 4).

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Fig. 6 Schematic representation of introducing Cas9/gRNA into HEK293 cells (Subheading 3.5) and hiPSCs (Subheading 3.6) for specific genomic targeting
2. Synthesize the gRNA expression construct. The gRNA expression fragment can be ordered directly through IDT (Subheading 3.2.1) in the form of a gBlock or synthesized with an in-house protocol (Subheading 3.2.2). We discussed the application of different methods in Subheading 4 (see Note 2).

3.2.1 Option A

1. Synthesize the final gRNA expression fragment (455 bp) as a standard gBlock without any 5′ modifications from IDT (https://www.idtdna.com/pages/products/genes/gblocks-gene-fragments).

2. The synthesized gBlock can be amplified in two ways (Fig. 4):
   (a) Clone the synthesized gBlock into the PCR-Blunt II-TOPO vector from Invitrogen.
   • Resuspend the gBlock (delivered at 200 ng) in 20 μL of water for a final concentration of 10 ng/μL.
   • Pipet 1 μL gBlock, 1 μL PCR-BluntII-TOPO vector, and 4 μL salt solution in a 1.5 mL microcentrifuge tube, and mix gently. Incubate at room temperature for at least 5 min.
   • Transform 5 μL of product into Top10 Chemically Competent E. coli cells. Thaw one aliquot of Top10 cells in ice for 10 min, then 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 S.O.C. medium and incubate in a 37 °C shaker for 1 h.
   • Spread 100 μL of the transformation using sterilized glass beads onto a prewarmed LB agar-kanamycin (50 μg/mL) plate and incubate overnight at 37 °C. Expect 10–100 colonies, with the majority containing the desired insert.
   • Pick 1–5 colonies the next day. Culture each colony in 5 mL LB medium containing 50 μg/mL kanamycin overnight at 37 °C with shaking at 200 rpm.
   • Use a plasmid miniprep kit to isolate DNA from the majority of each 5 mL culture. Elute DNA from each column with 30 μL water. Save the remainder of each culture by storing at 4 °C.
   • Determine DNA concentration for each sample with a NanoDrop, and submit samples for Sanger sequencing using M13 Forward and M13 Reverse universal sequencing primers to determine correct transformants.
   • 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
miniprep culture. Grow overnight at 37 °C with shaking at 200 rpm.

- Isolate plasmid DNA using a plasmid maxiprep kit. Resuspend plasmid DNA at ~1 μg/mL in water. Use this product for transfection.

(b) Alternatively, directly PCR amplify the fragment for transfection and expression.

- Resuspend the gBlock (delivered at 200 ng) in 20 μL of water for a final concentration of 10 ng/μL; resuspend gRNA F and gRNA R primers at 100 mM in water.

- Prepare 100 μL PCR reaction as following and aliquot 25 μL reaction into each PCR tube.
  - 4 μL gBlock.
  - 4 μL gRNA F primer (10 μM).
  - 4 μL gRNA R primer (10 μM).
  - 50 μL 2× KAPA HiFi HotStart ReadyMix.
  - 38 μL PCR-grade water.
  - 100 μL total volume.

- Place tubes in thermocycler, and run the following program:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
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<td>20 s</td>
<td>30</td>
</tr>
<tr>
<td>65 °C</td>
<td>15 s</td>
<td></td>
</tr>
<tr>
<td>72 °C</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>72 °C</td>
<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>4–10 °C</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>

- Dilute 2 μL PCR product into 18 μL water and run alongside 2-log ladder on 2 % EX_Gel to confirm whether a specific band of 455 bp is obtained.

- Purify PCR product using a PCR cleanup kit, and elute the DNA from the column in 30 μL water.

- Measure the purified PCR product concentration with NanoDrop, then speedvac and resuspend the DNA at 1 μg/μL in water. Use this product for transfection.

3.2.2 Option B

1. Incorporate 19 bp of the selected target sequence (i.e., 5′-N₁₉ of 5′-N₁₉-NGG-3′) into two 60mer oligonucleotides which have 20 nt complementary region with each other at 3′ ends (bold).
Underlined region in Insert_R is the reverse complement of 19 bp (N_{19}) (Fig. 5).

(a) Insert_F: TTTCTTGCTTTATATATCTTG TGGA-AAGGACGAAACACCGN_{19}

(b) Insert_R: GACTAGCCTTTATTTTAAC TTGCTATTCTAGCTCTAAAAACN_{19}C

2. Order the two 60mer oligonucleotides (25 nmol) from a commercial source such as IDT.

3. Suspend each 60mer oligonucleotide at 100 mM in water.

4. Anneal the two oligos to create a DNA fragment containing 20 bp double-stranded regions flanked by 40 nt 5’ overhangs at each end.

(a) Mix 10 μL Insert_F (100 μM) and 10 μL Insert_R (100 μM) in a PCR tube.

(b) Place the tube in a thermocycler with the following protocol:

<table>
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<th>Temperature</th>
<th>Time</th>
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<tbody>
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<td>Ramp to 4 °C</td>
<td>0.1 °C/s</td>
</tr>
<tr>
<td>4–10 °C</td>
<td>∞</td>
</tr>
</tbody>
</table>

(c) The resulting product can be stored at 4 °C.

5. Extend the annealed DNA fragment using the Phusion polymerase to create a 100 bp fully double-stranded DNA product.

(a) Create the following reaction mixture:

• 2 μL annealed DNA product (50 μM).
• 4 μL 5× Phusion HF buffer.
• 1 μL 10 mM dNTPs.
• 1 μL Phusion DNA polymerase.
• 12 μL nuclease-free H_{2}O.
• 20 μL total volume.

(b) Place tubes in thermocycler, and run the following program:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 °C</td>
<td>15 min</td>
</tr>
<tr>
<td>4–10 °C</td>
<td>∞</td>
</tr>
</tbody>
</table>
(c) Purify the reaction with PCR cleanup kit and elute the product in 30 μL water.

(d) The final product is a 100 bp dsDNA fragment. Measure the concentration of purified product with a NanoDrop.

6. Linearize the gRNA cloning vector using AflII.

(a) Obtain the gRNA cloning vector from Addgene (plasmid ID 41824) as a bacterial stab in agar.

(b) Use the tip of a sterile pipet tip or toothpick to scrape the frozen bacterial stock, then streak onto an LB agar-kanamycin plate (50 μg/mL). Incubate plate at 37 °C overnight.

(c) Pick a single colony from the plate the next day to inoculate 200 mL of LB-kanamycin (50 μg/mL), and grow overnight at 37 °C with shaking at 200 rpm.

(d) Use a plasmid maxiprep kit to purify the gRNA cloning vectors per manufacturer’s instruction.

(e) Measure the concentration with a NanoDrop.

(f) Digest the gRNA cloning vector:
   - 1 μg gRNA cloning vector.
   - 1 μL AflII restriction enzyme.
   - 2 μL 10× NEBuffer 4.
   - 2 μL BSA (1×).
   - H2O to 20 μL.
   - 20 μL total volume.

(g) Incubate digest reaction at 37 °C for 1 h.

(h) Dilute 20 μL digestion reaction with 40 μL water, load 20 μL diluted reaction on 1 % EX Gel alongside a 2-log DNA ladder. Excise the 3519 bp band and purify the linearized vector using a gel extraction kit. Elute the DNA from the column in 30 μL water. Measure the concentration with a NanoDrop.

7. Incorporate the 100 bp DNA fragment using isothermal assembly to create the final gRNA expression vector.

(a) In a PCR tube, combine the following:
   - 100 ng double-stranded DNA product from step 3.
   - 100 ng digested gRNA cloning vector from step 4.
   - 10 μL Gibson Assembly Master Mix (2×).
   - H2O to 20 μL.
   - 20 μL total volume.
(b) Place tubes in thermocycler, and run the following program:

<table>
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<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 °C</td>
<td>60 min</td>
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<tr>
<td>4–10 °C</td>
<td>∞</td>
</tr>
</tbody>
</table>

(c) Transform 5 μL of product into Top10 Chemically Competent *E. coli* cells. Thaw one aliquot of Top10 cells in ice for 10 min, and then add 5 μL of the isothermal-assembly 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 S.O.C. medium and incubate in a 37 °C shaker for 1 h.

(d) Spread 100 μL of the transformation using sterilized glass beads onto a prewarmed LB agar-kanamycin (50 μg/mL) plate and incubate overnight at 37 °C.

(e) Pick ~10 colonies the next day. Culture each colony in 5 mL LB medium containing 50 μg/mL kanamycin overnight at 37 °C with shaking at 200 rpm.

(f) Use a plasmid miniprep kit to isolate DNA from the majority of each 5 mL culture. Elute DNA from each column with 30 μL water. Save the remainder of each culture by storing at 4 °C.

(g) Determine DNA concentration for each sample, and submit samples for Sanger sequencing using M13 Forward and M13 Reverse universal sequencing primers to determine transformants that have successfully incorporated the 100 bp DNA containing the target sequence.

(h) 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 miniprep culture. Grow overnight at 37 °C with shaking at 200 rpm.

(i) Isolate plasmid DNA using a plasmid maxi prep kit, resuspend DNA at ~1 μg/μL in water. Use this product for transfection.

3.3 gRNA Preparation from a Microarray for Multiplexible Genome Engineering

1. Design oligonucleotide library of a 200 nt oligonucleotide pool from Custom Array Inc. (Design provided in Supplementary Table 2: http://arep.med.harvard.edu/human_crispr/.)

2. Perform the first round of PCR to amplify the full oligonucleotide library.
3. Resuspend gRNA pool F1 and gRNA pool R1 primers at 100 mM in water.

4. In a PCR tube, add the following:
   (a) 10 ng pooled oligonucleotide library template.
   (b) 2.5 μL gRNA pool F1 primer (10 μM).
   (c) 2.5 μL gRNA pool R1 primer (10 μM).
   (d) 1.5 μL DMSO.
   (e) 25 μL 2x Phusion GC Buffer Master Mix.
   (f) Add H₂O to 50 μL.
   (g) 50 μL total volume.

5. Place tubes in thermocycler, and run the following program:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
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<tr>
<td>98 °C</td>
<td>10 s</td>
<td>25</td>
</tr>
<tr>
<td>70 °C</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>72 °C</td>
<td>10 s</td>
<td></td>
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<tr>
<td>72 °C</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>4 °C</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>

6. Purify the product using a PCR cleanup kit. Elute the DNA from each column with 30 μL water. Measure the concentration with a NanoDrop.

7. Perform the second round of PCR to specifically amplify the desired subpopulation of the oligonucleotide library.
   (a) The PCR primers for this step will be specific to the desired target(s) and consist of the 25 bp barcodes flanking the target sequence(s) (barcodes available in Supplementary Table 2 at http://arep.med.harvard.edu/human_crispr/). Resuspend primers at 100 mM in water before use.
   (b) In a PCR tube, add the following:
      • 10 ng PCR product from step 2.
      • 1 μL target-specific barcode forward primer (10 μM).
      • 1 μL target-specific barcode reverse primer (10 μM).
      • 12.5 μL 2x KAPA HiFi HotStart ReadyMix.
      • Add H₂O to 25 μL.
      • 25 μL total volume.
(c) Place tubes in thermocycler, and run the following program:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95 °C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>98 °C</td>
<td>20 s</td>
<td>30</td>
</tr>
<tr>
<td>65 °C</td>
<td>15 s</td>
<td></td>
</tr>
<tr>
<td>72 °C</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>72 °C</td>
<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>4–10 °C</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>

(d) Purify the product using a PCR cleanup kit. Elute the DNA from each column with 30 μL water. Measure the concentration with a NanoDrop.

8. Perform the final round of PCR to subtract the identifying barcode from each gRNA target sequence.

(a) Resuspend gRNA pool F2 and gRNA pool R2 primers at 100 mM in water.

(b) In a PCR tube, add the following:
- 10 ng pooled oligonucleotide library template.
- 2.5 μL gRNA pool F1 primer (10 μM).
- 2.5 μL gRNA pool R1 primer (10 μM).
- 1.5 μL DMSO.
- 25 μL 2× Phusion GC Buffer Master Mix.
- Add H2O to 50 μL.
- 50 μL total volume.

(c) Place tubes in thermocycler, and run the following program:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>98 °C</td>
<td>30 s</td>
<td>1</td>
</tr>
<tr>
<td>98 °C</td>
<td>10 s</td>
<td>25</td>
</tr>
<tr>
<td>68 °C</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>72 °C</td>
<td>10 s</td>
<td></td>
</tr>
<tr>
<td>72 °C</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>4 °C</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>

Purify the product using a PCR purification kit. Elute the DNA from each column with 30 μL water.

(d) Linearize the gRNA cloning vector using AflIII as described in Subheading 3.2.2, step 4.

(e) Incorporate the target-containing fragment from step 4 into the linearized gRNA cloning vector via isothermal
assembly and perform transformation as described in Subheading 3.2.2, step 5. Use this product for transfection.

### 3.4 Prepare hCas9 Plasmid

1. Obtain the hCas9 plasmid from Addgene (plasmid ID 41815) as a bacterial stab in agar.
2. Use the tip of a sterile pipet tip or toothpick to scrape the bacterial stock, streak onto an LB agar-ampicillin plate (100 μg/mL), and then incubate plate at 37 °C overnight.
3. Pick a single colony from the plate the next day to inoculate 200 mL of LB-ampicillin (100 μg/mL), then grow overnight in at 37 °C with shaking at 200 rpm.
4. Isolate plasmid DNA using a plasmid maxiprep kit, resuspend DNA at ~1 μg/μL in water. Use this product for transfection.

### 3.5 Transfection of 293 HEK Cells

#### 3.5.1 Day 0: Plating 293 Cells for Transfection

1. Culture cells in 6 well plates until cells are healthy and at ~70 % confluence.
2. Gently 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 3 mL prewarmed DMEM containing 10 % FBS, 1× P/S, and 1× NEAA (hereafter referred to as “complete DMEM media”).
5. Count cells and calculate volume required for 0.1×10^6 cells (single transfection, scale as needed).
6. Place desired volume of cells into 15 mL centrifuge tube.
7. Spin at 200 × g for 5 min at room temperature and aspirate supernatant.
8. Resuspend in 1 mL media.
9. Plate cells in a 12-well tissue culture plate, return to incubator.

#### 3.5.2 Day 1: Transfection

1. Change media on cells to 1 mL fresh prewarmed complete DMEM media, return to incubator while preparing DNA mix.
2. Add 5 μL Lipofectamine 2000 to 50 μL Opti-MEM in a 1.5 mL microcentrifuge tube, flip several times to mix. Incubate the mixture in the room temperature for 5 min.
3. Add 1 μg Cas9 plasmid and 1 μg gRNA to 50 μL Opti-MEM in a 1.5 mL microcentrifuge tube.
4. Add diluted DNA to diluted Lipofectamine mixture from step 2, flick several times to mix.
5. Incubate 15 min at room temperature.
6. Add 100 μL mixture dropwise to cells.
7. Change media after 24 h to fresh prewarmed complete DMEM media.

8. Harvest cells 3 days after transfection, use desired method to assess targeting efficiency.

3.6 Transfection of PGP1 iPSC Cells

3.6.1 Matrigel Preparation

1. Store frozen Matrigel at −80 until use.

2. Thaw Matrigel in the cold room over night before using.

3. Quickly aliquot 300 μL barely thawed Matrigel into cold 1.5 mL microcentrifuge tubes on ice. Of note, Matrigel will solidify if allowed to come to room temperature and will no longer dissolve in media.

4. Immediately store at −20 °C until use (good for 2 months).

3.6.2 Transfection

1. Culture PGP1 iPSC cells in 6 well plates until healthy and at ~80 % confluency.

2. On day of transfection, prepare wells for transfected cells by coating with Matrigel.
   (a) Thaw a vial of Matrigel on ice.
   (b) Add 300 μL of cold Matrigel to 24 mL cold DMEM/F12 on ice.
   (c) Invert to mix.
   (d) Add 500 μL per well of 48-well plate (1 well per transfection), then leave the plate at room temperature for 1 h.
   (e) Aspirate Matrigel and replace with prewarmed 500 μL mTESR media with ROCK inhibitor (2 μL/mL) before plating transfected cells.

3. Change media to 2 mL prewarmed mTeSR media with ROCK inhibitor (2 μL/mL), 2 h before nucleofection.

4. Aspirate the media, wash cells with 2 mL room temperature PBS.

5. Aspirate PBS, add 1 mL TrypLE Express, incubate at 37 °C for 5 min.

6. Resuspend cells by gently pipetting up and down five times, place into 15 mL centrifuge tube, and add 10 mL mTeSR-1 followed by several rounds of gentle pipetting.

7. Count cells with cell counter and calculate volume required for 1 × 10^6 cells (single transfection, scale as needed).

8. Place desired quantity of cells in 15 mL centrifuge tube, spin at 200 × g for 5 min at room temperature and aspirate supernatant.

9. Resuspend 1 × 10^6 cells in master mix of 21 μL, including the following:
(a) 16.4 μL P3.
(b) 3.6 μL Supplement 1.
(c) 1 μL Cas9 plasmid (1 μg/μL).
(d) ~21 μL total.
10. Add 1 μL of gRNA (1 μg/μL) to cell suspension mixture.
11. Quickly transfer cells into central chamber of one well of a Nucleocuvette strip, and place strip into the 4D-Nucleofector device.
12. Nucleofect cells using program CB150.
13. Quickly add 80 μL of prewarmed mTESR media containing ROCK inhibitor to each well of electroporated cells, pipet up and down 1–2× to mix.
14. Transfer cells from the strip to Matrigel-coated plate in mTeSR1 media with ROCK inhibitor (prepared in Subheading 3.6.2, step 2).
15. Centrifuge plate at 70 × g for 3 min at room temperature.
16. Place cells into incubator.
17. After 24 h, change to fresh mTeSR1 media without ROCK inhibitor.
18. Harvest cells 3 days after nucleofection and use desired method to assess targeting efficiency (see Note 3).

4 Notes

1. gRNA Design and Specificity.
Here, we describe a method for gRNA design for conducting human genomic targeting against sites with the pattern of 5′-N_{19}-NGG-3′. Despite the simplicity of the gRNA design and its broad applications, we emphasize the following caveats when one attempts to use this system to conduct genome engineering. The works of Jinek et al. [7] and Cong et al. [14] have indicated that the 13 nt immediately upstream of the PAM in the targeted sequence (N_{13} of 5′-N_{13}-NGG-3′) is primarily responsible for the specificity of Cas9-gRNA. Thus, the requirement of an NGG PAM motif adjacent to the targeting site and the limited numbers of nucleotides (13 + 2 = 15 bp) in gRNA which define specificity represent the primary constraints to choosing unique Cas9-gRNA targeting sites in the human genome. Additionally, a deeper understanding of the specificity of Cas9-gRNA is under current investigation [15]. We expect these rules to evolve as we further evaluate the specificity of Cas9-gRNA. It is important to note that because the nature of off-target events with this technology remains an open question, we strongly recommend that users screen for...
potential off-target sites in the genome after obtaining edited monoclonal cells with the desired targeting event.

2. Different Assembly Methods.
We present three different gRNA assembly methods. We suggest ordering synthesized gRNA expression constructs from a commercialized source (https://www.idtdna.com/pages/products/genes/gblocks-gene-fragments) as a convenient method, while Gibson Assembly starting with either 60mer ssODNs or 200-mer oligonucleotide pools is amenable to construction of multiple gRNA expression constructs.

3. Expected Targeting Efficiency and Toxicity in hiPSCs.
The expected cell confluence of PGP1 hiPSCs is approximately 30% on the second day after transfection. Additionally, the rate of mutation at the targeted site via NHEJ is approximately 1% using this protocol [8]. However, the toxicity of transfection and targeting efficiency may vary from cell line to cell line. Thus, optimization of the amount of transfected DNA is recommended when applying this protocol to other human iPS cell lines.

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References

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