Journal of Visualized Experiments

# Generation of Defined Genomic Modifications Using CRISPR-CAS9 in Human Pluripotent Stem Cells

--Manuscript Draft--

| | |
|---|---|
| **Article Type:** | Invited Methods Article - JoVE Produced Video |
| **Manuscript Number:** | JoVE60085R1 |
| **Full Title:** | Generation of Defined Genomic Modifications Using CRISPR-CAS9 in Human Pluripotent Stem Cells |
| **Keywords:** | stem cells; genome editing; CRISPR-CAS9; single strand DNA oligonucleotide; heterozygous mutation, isogenic cell line |
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| **Additional Information:** | |
| **Question** | **Response** |
| Please indicate whether this article will be Standard Access or Open Access. | Standard Access (US$2,400) |
| Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations. | Philadelphia, PA USA |

Cover Letter

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May 10, 2019

Stephanie R. Weldon, PhD
Science Editor
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Dear Dr. Weldon,

We have addressed all of the editorial and reviewer comments in our Response to review. The changes have been incorporated into the text that we are re-submitting for Video production by JoVE.

Thank you very much for your consideration.

Thank you for your consideration.

Best Regards,

Deborah L. French, PhD

**TITLE:**

# Generation of Defined Genomic Modifications Using CRISPR-CAS9 in Human Pluripotent Stem Cells

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**KEYWORDS:**

stem cells, genome editing, CRISPR-CAS9, single strand DNA oligonucleotide, heterozygous mutation, isogenic cell line

**SUMMARY:**

This protocol provides a method to facilitate the generation of defined heterozygous or homozygous nucleotide changes using CRISPR-CAS9 in human pluripotent stem cells.

**ABSTRACT:**

Human pluripotent stem cells offer a powerful system to study gene function and model specific mutations relevant to disease. The generation of precise heterozygous genetic modifications is challenging due to CRISPR-CAS9 mediated indel formation in the second allele. Here, we demonstrate a protocol to help overcome this difficulty by using two repair templates in which only one expresses the desired sequence change, while both templates contain silent mutations to prevent re-cutting and indel formation. This methodology is most advantageous for gene editing coding regions of DNA to generate isogenic control and mutant human stem cell lines for studying human disease and biology. In addition, optimization of transfection and screening methodologies have been performed to reduce labor and cost of a gene editing experiment. Overall, this protocol is widely applicable to many genome editing projects utilizing the human pluripotent stem cell model.

## INTRODUCTION:

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) are valuable tools for modeling human disease due to their capacity for renewal, while maintaining the ability to generate cell types of different lineages[1-4]. These models open the possibility to interrogate gene function, and understand how specific mutations and phenotypes are related to various diseases[5,6]. However, to understand how a specific alteration is linked to a particular phenotype, the use of a paired isogenic control and mutant cell lines is important to control for line to line variability[7-8]. Transcription activator-like effector nucleases (TALENs) and zinc finger nucleases have been used to generate insertion or deletion (indels) mutations in diverse genetic models, including primary cells; but these nucleases can be cumbersome to use and expensive[9-14]. The discovery of the clustered regularly interspaced short palindromic repeat (CRISPR)-CAS9 nuclease has revolutionized the field due to efficiency in indel formation in virtually any region of the genome, simplicity of use, and reduction in cost[15-19].

A challenge in using the CRISPR-CAS9 based genome editing technology has been the generation or correction of specific mutations in one allele without creating an indel mutation in the second allele[20]. The major goal of this protocol is to overcome this challenge by using two single-stranded oligonucleotide (ssODN) repair templates to reduce indel formation in the second allele. Both ssODNs are designed to contain silent mutations to prevent re-cutting by the CAS9 nuclease, but only one contains the alteration of interest. This method increases the efficiency of generating a specific heterozygous genetic modification without inducing indel formation in the second allele. Using this protocol, gene editing experiments in six independent genomic locations demonstrate the precise introduction of the desired genomic change in one allele without indel formation in the second allele and occurs with an overall efficiency of ~10%. The described protocol has been adapted from Maguire et al.[21].

## PROTOCOL:

### 1. Design and construction of guide RNA (gRNA)

NOTE: Each gRNA is made up of two 60 base pair (bp) oligonucleotides that are annealed to generate a 100 bp double stranded (ds) oligonucleotide (**Figure 1A-C**). The timeline for gRNA design, generation, and testing cutting efficiency is approximately 2 weeks (**Figure 2**).

1.1. Select the DNA region of interest to be genome edited and identify 3−4 23 bp sequences that fit the format, 5'-G($N_{19}$)NGG-3'. These sequences should be located within 20 bp of the region of interest.

NOTE: Targeting can be performed on the sense or anti-sense strand.

1.2. Evaluate the gRNA sequences for genomic redundancy and off-target probability using a resource such as CRISPOR (http://crispor.tefor.net).

1.3. Incorporate the 20 bp target sequence (excluding the protospacer adjacent motif or PAM) into two 60-mer oligonucleotides as shown (sequences are 5' to 3' and red and green are reverse complements as shown in **Figure 1C**).

1.4. Order the two 60 bp oligonucleotides from vendor of choice. Once the oligonucleotides are received, resuspend each to a final concentration of 100 µM in $ddH_2O$. Make a working stock of 10 µM.

1.5. Anneal the two oligonucleotides and generate a 100 bp dsDNA fragment using DNA polymerase (**Table of Materials**). Combine 5 µL of 10 µM forward oligonucleotide and 5 µL of 10 µM reverse oligonucleotide in a polymerase chain reaction (PCR) strip tube and incubate at 95 °C for 5 min. Cool the reaction for 10 min at room temperature (RT).

1.6. Set up the PCR as described in **Table 1**. Perform PCR amplification in a thermal cycler using the parameters outlined in **Table 2**.

1.7. Visualize the PCR products on a 1.5% (w/v) ethidium bromide (EtBr) agarose gel electrophoresed at 80−100 V for 40 min. Excise the 100 bp band (**Figure 3A**), visualized on an LED light box, from the gel using a razor blade and purify using a gel extraction kit (**Table of Materials**).

## 2. Design of PCR primers for screening

2.1. Perform screening of the edited DNA using forward and reverse PCR primers that are specifically designed to amplify a 400−500 bp region of the gene of interest (**Table 2**). Use DNA isolated from any control iPSC line to confirm a clean amplicon when performing the screening PCR.

2.2. Visualize PCR products on a 1.5% (w/v) gel following electrophoresis at 80−100 V for 1 h.

NOTE: Sequencing of edited clones is performed using a nested primer that is designed following confirmation of the screening primer set. Samples are sent to a commercial source for sequencing.

## 3. Preparation of gRNA_cloning vector plasmid

3.1. To linearize the cloning vector, take a 1.5 mL centrifuge tube and add 1−5 µg of DNA of the gRNA_cloning vector along with 4 µL of the *Afl*II restriction enzyme buffer and 1.5 µL of *Afl*II restriction enzyme. Bring up the reaction to 24 µL of $ddH_2O$. Mix the reaction by pipetting up and down. Incubate at 37 °C overnight (O/N).

3.2. Electrophorese on a 1% agarose gel at 80−100 V for 1 h and excise bands (expected band size is ~3519 bp).

3.3. Extract and purify as in step 1.6.

**4. Assembly of gRNA vector**

4.1. Set up reactions and assemble DNA fragments using the assembly kit (**Table of Materials**) at 1:5 ratios of *Afl*II digested gRNA cloning vector to 100 bp gel purified insert, as described in **Table 3**.

4.2. Incubate the reaction at 50 °C for 15 min. Dilute the reaction 1:3 in $ddH_2O$ and use 3 µL for bacterial transformation, as per manufacturer's instructions (**Table of Materials**). Plate cells on kanamycin LB/agar plates.

4.3. Pick 3−5 colonies per gRNA. Inoculate each colony in 4 mL of LB and grow O/N at 37 °C in an orbital shaking incubator.

4.4. Purify plasmid DNA using a miniprep plasmid isolation kit, and sequence each gRNA using the following primers to ensure successful cloning: Forward: GTACAAAAAAGCAGGCTTTAAAGG; Reverse: TGCCAACTTTGTACAAGAAAGCT.

**5. Test gRNA cutting efficiency**

5.1. Plate hESCs on irradiated murine embryonic fibroblasts (MEFs) in a 6-well plate, as previously described[21]. When the cells reach 70−80% confluency, prepare the transfection master mix outlined in **Table 4**.

5.2. Mix by pipetting and incubate at RT for 15 min. Add dropwise to the cells and incubate at 37 °C for 48 h (with a media change after 24 h).

5.3. Harvest cells for sorting after 48 h.

5.3.1. Remove MEFs enzymatically (**Table of Materials**) with a 3 min RT incubation.

5.3.2. Rinse cells 1x with hESC medium (**Table 5**) and scrape into hESC medium + 10 µM Y-27632 dihydrochloride (**Table of Materials**).

5.3.3. Pellet cells at 300 x *g* for 3 min and resuspend in 0.5 mL of hESC medium + 10 µM Y-27632 dihydrochloride.

5.3.4. Filter into a 5 mL tube through a 35 µm cell-strainer cap.

5.4. Using fluorescence activated cell sorting (FACS), gate on live cells and sort the green fluorescent protein (GFP) positive cells.

5.5. Transfer a maximum of 1.5 x $10^4$ sorted cells directly into a 10 $cm^2$ dish coated with 1:3 basement membrane matrix (**Table of Materials**) and irradiated MEFs in hESC medium (**Table 5**) containing Y-27632 dihydrochloride.

5.6. Change medium daily using the hESC medium (**Table 5**) without Y-27632 dihydrochloride and manually pick clones after 10−15 days, when colonies are ~1 mm in diameter.

5.6.1. Using a P200 pipette and microscope, carefully scrape a single clone and draw cells into the pipette.

5.6.2. Disperse the cells by gently pipetting 3−4x in a 96 well plate in the medium drawn up with the colony.

5.6.3. Dispense into PCR strip tubes for screening and pellet the cells by centrifugation at 10,000 x *g* for 5 min. Pick 20 colonies per gRNA.

**6. Clone screening**

6.1. Isolate DNA by incubating cell pellets in 20 µL of proteinase K buffer (**Table 5**) (1 h at 55 °C and 10 min at 95 °C) and vortex vigorously. Centrifuge at 10,000 x *g* for 5 min and collect the supernatant.

6.2. Perform screening PCR (section 2) in a total volume of 20 µL using a master mix including primers designed to amplify the region of interest and 5 µL of the proteinase K digest. Use genomic DNA isolated from the cell line that was gene edited as a control.

6.3. Evaluate size changes of PCR products following 1 h electrophoresis at 70−90 V on a 2.5% (w/v) agarose gel (**Figure 3B,C**). Any size difference is indicative of cleavage.

**7. Precise genome editing in pluripotent stem cells using single strand oligo DNA (ssODNs)**

7.1. Design 100 bp ssODNs centered around the most efficient gRNA sequence determined to have the best cutting efficiency.

7.2. Prevent re-cleavage of the recombined ODN by introducing silent mutations in the gRNA sequence. A single silent mutation in the PAM sequence is sufficient, but if not possible, 3−4 silent mutations will work.

NOTE: To facilitate screening of targeted clones, the introduction of a restriction site within ~20 bp of the gRNA sequence is ideal.

7.3. Design one ODN with the desired base change(s) to create the mutation of interest and one ODN without the base change(s).

NOTE: These changes should be no more than ~20 bp from the predicted CRISPR/CAS9 cut site, as recombination drops off considerably at greater distances.

7.4. Order the two ssODNs from vendor of choice and resuspend in water to make 1 µg/µL stocks. Store stocks at -20 °C.

**8. Transfection setup**

8.1. To transfect the ssODN and the CRISPR-CAS9 plasmids, plate the target cell line in a 6-well dish on irradiated MEFs to reach 70–80% confluency after an O/N incubation.

8.2. Set up the transfection reaction as described in **Table 6**. Mix the reaction by pipetting and incubate for 15 min at RT. Add the transfection reaction mixture dropwise to the cells.

8.3. After 48 h, prepare the cells for cell sorting as described in section 5.3.

8.4. Pick colonies ~10 days after plating single cells using a 200 µL pipette. Transfer 100 µL of cells to one well of a 24 or 48 well plate previously coated with gelatin and irradiated MEFs in hESC medium with Y-27632 dihydrochloride. Use the remaining 100 µL for DNA isolation as described in section 6.

**9. Checking for mutations in single colonies**

9.1. To check for successful integration of the ssODN, take 5 µL of DNA isolated from each colony to perform PCR using the screening primers designed in section 2. Purify the PCR products (**Table of Materials**) and prepare the restriction enzyme digestion using the unique enzyme site created in the ssODN.

NOTE: This digestion also includes the restriction enzyme buffer and the manufacturer's recommended concentration of restriction enzyme in a volume of 40 µL.

9.2. Mix the reaction by pipetting and incubate at the manufacturer's recommended temperature for 1–3 h.

9.3. Visualize the digested PCR products on a 1.5% (w/v) EtBr agarose gel electrophoresed at 80–100 V for 40 min. If successful integration of the ssODN has occurred, sequence specific mutations using a nested primer.

**REPRESENTATIVE RESULTS:**

**Generation of gRNAs and screening for indels**

Each gRNA will be cloned into a plasmid vector and expressed using the U6 promoter. The AflI restriction enzyme is used to linearize the plasmid (addgene #41824) and is located after the U6 promoter. The 100 bp band generated after annealing the two 60 bp oligos is cloned into the gRNA expression vector using the DNA assembly. Once the gRNA plasmids are generated, they are transfected into hESCs or iPSCs along with a CRISPS-CAS9 GFP plasmid (addgene #44719). The GFP+ cells are sorted after 2 days to enrich for transfected cells and plated (see section 5). After 10−14 days, single cell derived colonies are picked and used to isolate DNA to screen for indel formation generated for each gRNA. A PCR amplification with primers spanning the gRNA site is used to visualize indel formation using a 2.5% (w/v) agarose gel with EtBr, electrophoresed at 70−90 V for 1 h.

**Generation of 100 bp ssODN to introduce specific mutations**

Two ssODN oligos are designed around the gRNA with the most efficient cutting. Each gRNA is 100 bp and contains silent mutations, preferably at the PAM sequence, to avoid re-cutting (**Figure 4A**). A silent mutation in the PAM sequence can generate a unique restriction site, which helps to screen for successful integration into one or two alleles (**Figure 4B**).

**ssODN recombination outcomes**

Using this protocol, the frequency of targeting events for six different genes using the two ssODN approach is shown in **Figure 5**. By examining genomic modification at the gRNA site in both alleles, different outcomes were expected including recombination of the wildtype (WT) ssODN, the mutant ssODN and indel formation. The clones in which only one allele had undergone recombination, the most common outcome was indel formation in the second allele (10−42%). The clones that had integration of the ssODNs in both alleles led to three different outcomes: 1) integration of the WT ssODN in both alleles (0−8%), 2) integration of the mutant ssODN in both alleles (0−25%), and 3) integration of both the WT and mutant ssODN WT (8−21%).

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Overview of gRNA generation.** (**A**) In the region of interest, design four gRNAs ending in GG that are not more than 20 bp apart. (**B**) Each gRNA of 23 bp has a PAM sequence of NGG at the 3' end. (**C**) The 60 bp primers are comprised of a 40 bp sequence complementary to the gRNA backbone plasmid and the 20 bp gRNA sequence without the PAM sequence.

**Figure 2: Protocol timeline for gRNA generation and clone screening.** The timeline for gRNA generation and screening clones is shown. Designing and cloning gRNA expression plasmids takes approximately one week. Approximately 48 hours after transfection into human PSCs, GFP+ cells are sorted and plated at limiting dilution. Colonies should be visible between 7−10 days and at approximately day 20, clones can be picked for screening, expansion, and sequence confirmation.

**Figure 3: gRNA test for cutting efficiency.** (**A**) For gRNA construction, a 100 bp band is excised from a 1.5% agarose gel. (**B**) Following cell transfection, validation of gRNA cutting is visualized using a 2.5% agarose gel. A 180 bp PCR product is used to check for indel formation in which an uncut control and different clones are analyzed for band shifts indicative of indel formation. (**C**) Different gRNAs may have different cutting efficiencies.

**Figure 4: Generation and screening of mutations using two ssODNs.** (**A**) To avoid CRISPR-CAS9 re-cutting of the edited alleles, the PAM sequence can be modified using a G to A silent mutation. This modification adds a unique EcoRI restriction site that can be used for screening. In addition to this silent mutation, the mutant ssODN contains the desired base change. (**B**) Screening for oligonucleotide recombination using EcoRI restriction enzyme digestion can result in three possible outcomes: no cutting represented by a WT band at ~650 bp, recombination in one allele represented by an uncut band of 650 bp and two smaller bands or insertion into both alleles represented by only smaller bands.

**Figure 5: ssODN integration efficiency and outcomes.** Integration efficiency and outcomes of the two ssODN approach were determined by analyzing six different genes. If only one ssODN integrated, indel formation was usually detected in the other allele. If two ssODNs integrated, three possible outcomes were detected, as shown.

**Table 1: gRNA cloning PCR conditions.**

**Table 2: PCR cycling parameters.**

**Table 3: gRNA assembly reaction conditions.**

**Table 4: Cell transfection master mixture.**

**Table 5: Cell culture medium and proteinase K digestion buffer.**

**Table 6: ssODN cell transfection master mixture.**

**DISCUSSION:**

In this protocol, the use of CRISPR-CAS9 along with two ssODN repair templates to generate specific heterozygous or homozygous genome changes is demonstrated in human pluripotent stem cells. This method resulted in the successful generation of isogenic cell lines expressing heterozygous genomic changes with an efficiency close to 10%. This protocol has been optimized for both human ESCs and iPSCs grown on irradiated MEFs which support cell growth and survival after culturing cells at low density after cell sorting. Cell death can be minimized by maintaining cells with 10 ng/mL bFGF and Y-27632 dihydrochloride. It is possible that this protocol may be adapted to feeder free culture systems, but further optimization may be necessary.

Transfection efficiency can be variable from cell line to cell line, but the use of 3 µg of DNA and 3 µL of a lipid transfection reagent generally gave the best results of between 0.5–2% transfection efficiency. However, if transfection efficiency is lower, optimization of the amount of DNA and lipid reagent can be performed. Other transfection reagents can also be tested by the investigator.

The efficiency of indel generation will vary with different gRNAs and can be influenced by genomic location. Within the vast majority of cases, if four gRNAs are designed, at least one and many times 2 to 3, will work efficiently. Additionally, prior to testing gRNAs, optimization of the PCR strategy to visualize indels with a clean single band DNA product is important. For screening ssODN based genome editing, it is best to add a restriction enzyme site when introducing a silent mutation(s), but if not possible, deletion of a restriction enzyme site can be used as well. In addition, homology directed repair requires actively cycling cells so the cell density of cultures prior to transfection is critical to enhance the frequency of clones repaired using the introduced ssODN template.

Some of the limitations of this protocol are related to the position in the genome that will be edited. When coding regions are modified, ssODN carrying silent mutations prevent the Cas9 from re-cutting the edited site and the silent mutations do not alter the protein product. However, editing in regulatory or non-coding regions using this methodology becomes more difficult as silent mutations are not possible. If the base to be edited is part of a PAM sequence, this can be done successfully but only homozygous changes can be generated efficiently.

The protocol described here is useful to generate or correct heterozygous coding mutations without the addition of unintended indels in the second allele. This protocol will facilitate the use of human PSCs to study a wide range of topics from developmental biology to modeling of genetic diseases. The use of isogenic lines is critical to define functions of a given coding mutation without confounding effects due to differing genetic backgrounds.

**ACKNOWLEDGMENTS:**
This research was supported by funding from the National Heart, Lung, and Blood Institute (NHLBI), National Institutes of Health through grants U01HL099656 (P.G. and D.L.F.) and U01HL134696 (P.G. and D.L.F.).

**DISCLOSURES:**
The authors have nothing to disclose.

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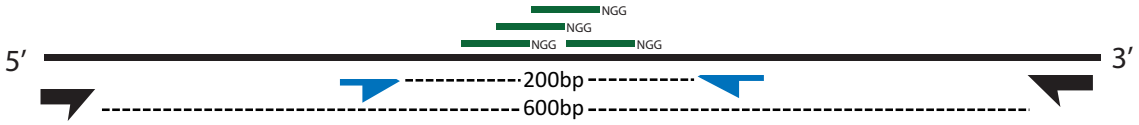
437 *Biology*. **48** (1), e64 (2019).
438

A. Region of interest




B. gRNA
5′-NNNN NNNNN NNNNN NNNNN NGG-3′

C. Oligo F: TTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACC**G**NNNNNNNNNNNNNNNNNNN
Oligo R: GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAANNNNNNNNNNNNNNNNNNN**C**

Figure 2



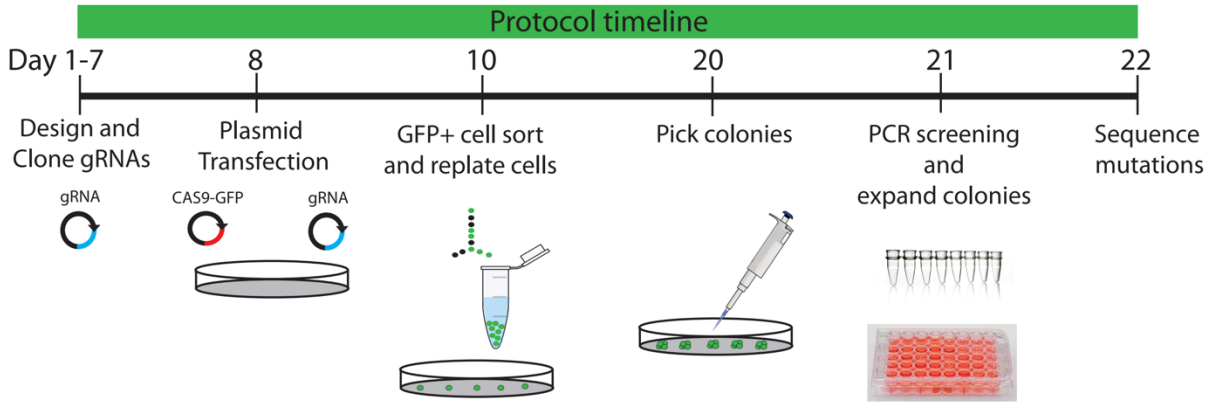
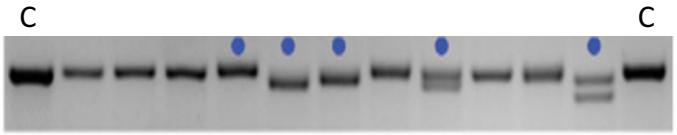


Figure 3



A.


B.




C.

| | # Clones picked | Cleavage | Efficiency (%) |
|---|---|---|---|
| gRNA1 | 14 | 7 | 45 |
| gRNA2 | 5 | 2 | 40 |
| gRNA3 | 3 | 0 | 0 |
| gRNA4 | 8 | 3 | 38 |
| gRNA5 | 16 | 1 | 6 |

.

Figure 4



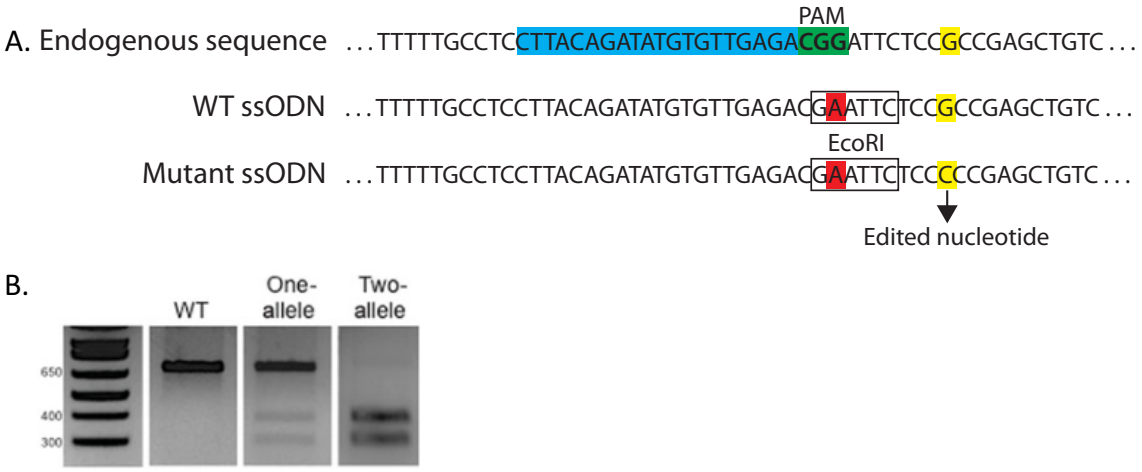


Figure 5



| Gene | # Clones | One-allele integration | | Two-allele integration | | |
|---|---|---|---|---|---|---|
| | | Indels | without indels | WT/WT | MUT/MUT | WT/MUT |
| Gene 1 | 24 | 5 (21%) | 0 | 0 | 1(4%) | 5 (21%) |
| Gene 2 | 48 | 5 (10%) | 0 | 0 | 0 | 6 (13%) |
| Gene 3 | 22 | 4 (18%) | 0 | 1 (5%) | 0 | 2 (9%) |
| Gene 4 | 12 | 5 (42%) | 0 | 1 (5%) | 3(25%) | 1 (8%) |
| Gene 5 | 24 | 7 (29%) | 0 | 0 | 0 | 2 (8%) |
| Gene 6 | 24 | 3 (13%) | 0 | 0 | 0 | 2 (8%) |

| Reagent | Volume (µL) |
|---|---|
| dNTPs (10 mM) | 0.4 |
| Taq polymerase | 0.2 |
| PCR buffer | 4.0 |
| Annealed oligos (10 µM) | 10.0 |
| $ddH_2O$ | 5.4 |

| PCR program | |
|---|---|
| Step 1 | 98 °C for 30 s |
| Step 2 | 98 °C for 10 s |
| Step 3 | 55 °C for 20 s |
| Step 4 | 72 °C for 30 s |
| Step 5 | Repeat steps 2−4 for 30 cycles |
| Step 6 | 72 °C for 5 min |
| Step 7 | Hold at 4 °C |

Table 3



| Reagent | Volume (µL) |
|---|---|
| linearized gRNA vector using *Afl* II (i.e., 50 ng/µL) | 1.0 |
| 100 bp DNA (i.e., 250 ng/µL) | 1.0 |
| Master mix 2x | 10.0 |
| $ddH_2O$ | q.s. to 20 |

Table 4



| Reagent | Amount |
|---|---|
| DMEM/F12 | 50.0 µL |
| pCas9_GFP vector (addgene plasmid 44719) | 0.5 µg |
| gRNA plasmid | 0.5 µg |
| lipid transfection reagent | 3.0 µL |

**hESC medium**

| Reagent | Final concentration |
|---|---|
| DMEM/F12 | |
| Knockout serum replacement (KDR) | 15% (v/v) |
| Nonessential amino acids | 100 µM |
| Sodium pyruvate | 1 mM |
| Glutamine | 2 mM |
| β-mercaptoethanol | 0.1 mM |
| bFGF human (basic fibroblast growth factor) | 10 ng/mL |

**10x Proteinase K buffer**

| | |
|---|---|
| Tris.HCl pH:7.4 | 50 mM |
| Ammonium sulfate pH: 9.3 | 15 mM |
| $MgCl_2$ | 2.5 mM |
| Tween 20 | 0.1% (v/v) |
| Proteinase K | 100 µg/mL |

| Reagent | Amount |
|---|---|
| DMEM/F12 | 50.0 µL |
| pCas9_GFP vector (addgene plasmid 44719) | 0.5 µg |
| gRNA plasmid | 0.5 µg |
| ssODN (0.5 µg of each ssODN) | 1.0 µg |
| lipid transfection reagent | 3.0 µL |

| Name of Material/ Equipment | Company | Catalog Number |
|---|---|---|
| 5-ml polystyrene round-bottom tube with cell-strainer cap | Corning | 352235 |
| 6-well polystyrene tissue culture dishes | Corning | 353046 |
| *Afl* II restriction endonuclease | New England Biolabs | R0520 |
| Agarose | VWR | N605 |
| DMEM/F12 medium | ThermoFisher | 11320033 |
| dNTPs | Roche | 11969064001 |
| Fluorescence-activated cell sorter (FACS) apparatus | | |
| Gel extraction kit | Macherey-Nagel | 740609 |
| Gibson Assembly Kit | New England Biolabs | E2611 |
| gRNA_Cloning Vector | Addgene | 41824 |
| LB agar plates containing 50 µg/ml kanamycin | | |
| Lipofectamine Stem Reagent | ThermoFisher | (STEM00001) |
| Matrigel Growth Factor Reduced (GFR) | Corning | 354230 |
| Murine embryonic fibroblasts (MEFs) | | |
| Nucleospin Gel Extraction and PCR Clean-up Kit | Macherey-Nagel | 740609 |
| Orbital shaking incubator | | |
| pCas9_GFP vector | Addgene | 44719 |
| PCR strip tubes | USA Scientific | 1402-2900 |
| Phusion High Fidelity DNA Polymerase and 5× Phusion buffer | New England Biolabs | M0530 |
| PurelinkTM Quick Plasmid Miniprep Kit | Invitrogen | K210011 |
| Proteinase K | Qiagen | Qiagen 19133 |
| StellarTM electrocompetent *Escherichia coli* cells | Takara | 636763 |
| SOC medium | New England Biolabs | B9020S |
| TrypLE Express Enzyme | ThermoFisher | 12605036 |
| Y-27632 dihydrochloride**/**ROCK inhibitor (ROCKi) | Tocris | 1254 |

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| | |
|---|---|
| Title of Article: | Generation of defined genomic modifications using CRISPR-CAS9 in human pluripotent stem cells |
| Author(s): | Fabian L. Cardenas-Diaz, Jean Ann Maguire, Deborah L. French and Paul Gadue |

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**CORRESPONDING AUTHOR**

| | |
|---|---|
| Name: | Deborah L French |
| Department: | Pathology and Laboratory Medicine |
| Institution: | The Children's Hospital of Philadelphia |
| Title: | Associate Professor |
| Signature: | Deborah L French |
| Date: | 4/2/19 |

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Dear Dr. Cao,

Sincerest thanks for the positive response and comments. We have modified the paper in response to editorial and reviewer comments. Please refer to the point-by-point responses.

**Editorial comments:**
Changes to be made by the author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

- We have proofread the manuscript to correct spelling and grammar issues

2. Please revise lines 95-98, 154-159, 168-170, 171-177 to avoid previously published text.

- We revised the designated text

3. Keywords: Please provide at least 6 keywords or phrases.

- Added on page 1 of manuscript

4. Please add a Summary section before the Abstract section to clearly describe the protocol and its applications in complete sentences between 10–50 words: “Here, we present a protocol to …”

- Done

5. Abstract: Please expand to provide an overview of the method and a summary of its advantages, limitations, and applications.

- Done

6. Introduction: Please rephrase to include a clear statement of the overall goal of this method.

- Done

7. Please define all abbreviations (MEF, RT, etc.) before use.

- Done

8. Please abbreviate liters to L (L, mL, µL) to avoid confusion. Please use the micro symbol µ instead of u.

- Done

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- Removed all commercial language from manuscript

10. Please revise the Protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

- Done

11. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the

"how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

- Added detail to supplement actions and answered 12-28 below

12. 1.5.1: What container is used here?
13. 1.6: Please describe how to excise and purify the 100 bp band. Alternatively, please provide a relevant reference.
14. 2.2: Please describe how to optimize the PCR reaction using control genomic DNA and specify the parameters used for agarose gel electrophoresis. Alternatively, please provide a relevant reference.
15. 3.1: Please describe how this is actually done. What container is used? Is the mixture mixed by pipetting or vortexing?
16. 3.2: Please spell out O/N.
17. 3.3: Please specify the parameters used for agarose gel electrophoresis.
18. 3.4: Please describe how.
19. 4.2: Please provide a reference for the bacterial transformation protocol.
20. 5.1: Please specify the culturing conditions.
21. Line 133: Please specify the incubation temperature here.
22. 5.3.2: Please provide the composition of ROCKi. If it is a commercial term, please replace it with a generic term.
23. 5.3.4: Please specify the mesh size of the strainer.
24. 5.4: Please specify the gating strategies.
25. 6.2: What happen after centrifugation? Please specify.
26. 7.2: Please describe how this step is actually done.
27. 7.6: What are resuspended? The two ssODNs?
28. 9.1: Please specify PCR primers and conditions.

29. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

- Combined shorter protocol steps

30. Please apply single line spacing throughout the manuscript, and include single-line spaces between all paragraphs, headings, steps, etc.

- Done

31. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

- Done

32. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia.

- Done

33. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

34. Tables: Please use the micro symbol µ instead of u and abbreviate liters to L (L, mL, µL) to avoid confusion. Please include a space between all numerical values and their corresponding units: 15 mL, 5 g, 7 cm, 37 °C, 60 s, 24 h, etc. Please use subscripts in chemical formulae to indicate the number of atoms, e.g., $H_2O$, $MgCl_2$, etc. Please replace commercial language (Lipofectamine) with a generic term.

- Changed symbols, including appropriate spacing, added subscript, replaced commercial with generic

35. Table of Materials: Please make the sheet containing “Reagents and solutions” a separate table and reference it in the manuscript.

- Now separate Table 5

36. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

- Done

37. Please remove the titles and figure legends from the uploaded figures. Please include all the figure Legends together at the end of the Representative Results in the manuscript text.

- Removed from figures and put into appropriate location in text

38. Please use superscript arabic numerals to cite references in text. The superscript number is inserted immediately next to the word/group of words it applies to but before any punctuation.

- Done

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Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

- Done

**Reviewers' comments:**
We appreciate both reviewer’s comments. We made all minor corrections suggested by Reviewer 1:

Reviewer #1:
Page 2 Line 89: Says 1% agarose gel, but Figure 3A says 1.5% agarose gel. Please confirm if it's one or the other
Page 2, line 100
The authors have a table of materials with name of material/equipment in one table (Page 21) and company/cat# in another page (page 22). Not sure if this is a journal requirement or a decision of the authors, but I think it will be hard for somebody that has never done this kind of experiments to understand for example which company/cat# is the gRNA cloning vector.
Please keep consistent in naming the same medium, it won't be easy otherwise to follow for a unexperienced reader:
Page 3, line 136
Please correct HESC medium with hESC medium
Page 3, line 143
Please change "growth medium" to hESC medium
Page 4, line 192
Please change "stem cell media" to hESC medium

We clarified the sentence in comment 1, softened the PAM sequence modification, and added the cited reference for comment 3.

Reviewer #2:

1. "Within 20 bps of this region, identify three to four 23 bp sequences that fit the format" this sentence is difficult to understand.
2. In Figure 4A, authors suggested to replace G with A in the PAM sequence. Actually, Cas9 can also Cut NGA or NAG PAMs, although the efficiency is low (PMID: 24956376).
3. PMID: 22967807, this paper is the first genome editing paper in the cardiovascular society, and should be cited.