

# CRISPR/Cas9-Based Protocol for Precise Genome Editing in Induced Pluripotent Stem Cells

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## Abstract

The advent of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-based genome editing has marked a significant advancement in genetic engineering technology. However, the editing of induced pluripotent stem cells (iPSCs) with CRISPR presents notable challenges in ensuring cell survival and achieving high editing efficiency. These challenges become even more complex when considering the specific target site. P53 activation as a result of traditional CRISPR editing can lead to apoptosis, potentially worsening cell health or even resulting in cell death. Mitigating this apoptotic response can enhance cell survival post-CRISPR editing, which will ultimately increase editing efficiency. In our study, we observed that combining p53 inhibition with pro-survival small molecules yields a homologous recombination rate of over 90% when using CRISPR in human iPSCs. This protocol significantly streamlines the editing process and reduces the time and resources necessary for creating isogenic lines.

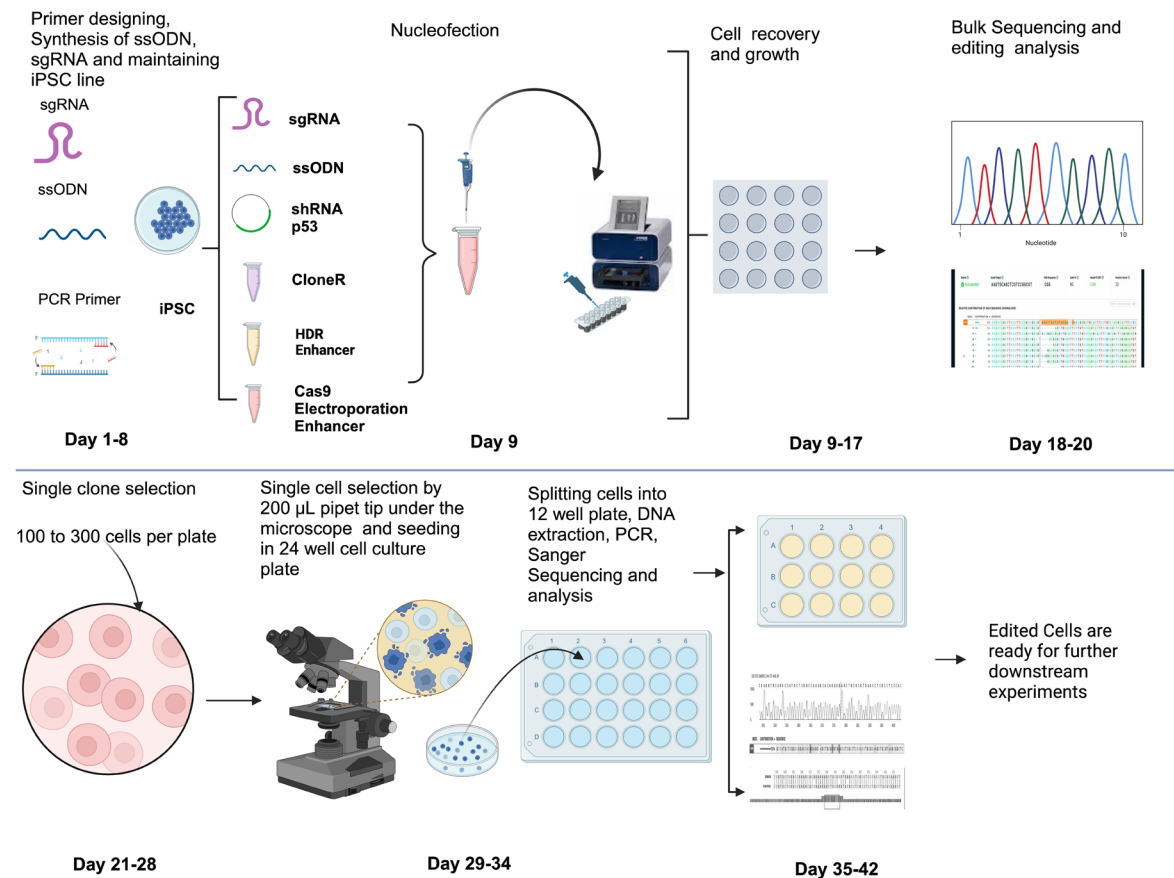
## Key features

- The combination of p53 inhibition and pro-survival small molecules promotes cell survival and increases the efficiency of genome editing.
- Genome editing can be completed in as little as 8 weeks for iPSCs, significantly reducing the total time required.
- Achieves a homologous recombination rate of over 90% in human iPSCs.

**Keywords:** iPSCs, CRISPR Cas9, Nucleofection, Editing efficiency, Homologous recombination rate

**This protocol is used in:** Sci Rep (2024), DOI: 10.1038/s41598-024-60766-4

## Graphical overview



## Outline of Protocol

## Background

Genome editing has revolutionized biotechnology by allowing scientists to make precise changes to an organism's DNA. The ability to modify an organism's genome allows experts to study difficult evolutionary and medical problems in more complex systems [1]. The application of gene editing in induced pluripotent stem cells (iPSCs) to create genetically modified isogenic lines can enhance our understanding of how genetic mutations cause disease. Gene editing ranges from conceptually straightforward gene knockouts to more intricate modifications such as point mutations or whole gene insertions. However, currently, gene editing is still confronted with challenges [2–4]. In plant and animal models, clustered interspaced short palindromic repeats (CRISPR) have emerged as the tool of choice for genome editing [5]. Nowadays, the nuclease versions Cas9 and Cas12a are commonly used [1]. The CRISPR-Cas9 and Cas12a systems work with a guide RNA (gRNA) that is divided into two parts: a trans-activating CRISPR RNA (tracrRNA) that binds directly to the Cas nuclease to create ribonucleoprotein (RNP) and a CRISPR RNA (crRNA) that indicates the genomic target location [6]. When compared with previous editing techniques, the CRISPR-Cas innovation offers more precise targeting and editing proficiency as well as being easier and less expensive to configure, allowing it to be used more extensively [7–9]. However, the CRISPR-Cas editing systems still require improvement, particularly for point mutations. In certain cases, there may be several risk variants associated with a target gene, necessitating the creation of multiple lines with distinct single nucleotide polymorphisms (SNPs) or the sequential insertion of genetic modifications. Therefore, to enable more laboratories

to produce the needed genetically modified lines, it is essential to develop a gene editing technique that is both highly efficient and easily adaptable. Currently, our attention is on overcoming the barriers to introducing point mutation in cell lines and improving the method to be as effective as possible. Editing efficiency may be increased by preventing cell death with the use of a Rho-related protein kinase (ROCK) inhibitor and blocking the p53 pathway [7]. In this protocol, we used pro-survival small molecules in conjunction with p53 inhibition to attain a homologous recombination rate exceeding 90%.

## Materials and reagents

### Biological materials

1. iPSC line (human NDC1) [10]

### Reagents

1. mTeSR Plus basal (Stem Cell Technologies, catalog number: 100-0274)
2. mTeSR Plus 5× supplement (Stem Cell Technologies, catalog number: 100-0275)
3. CloneR (Stem Cell Technologies, catalog number: 05888 10 mL)
4. Revitacell supplement (Gibco, catalog number: A2644501 100×)
5. 1× D-PBS (Gibco, catalog number: 10010049 500 mL)
6. DMEM/F12 (Gibco, catalog number: A4192001 500 mL)
7. Matrigel (Corning, catalog number: 47743-706 5 mL)
8. Accutase (VWR, catalog number: AT104 100 mL)
9. ReLeSR (Stem Cell Technologies, catalog number: 100-0484)
10. Alt-R Cas9 HDR enhancer (stock solution 3 mM) (IDT, catalog number: 1081062)
11. pmaxGFP (LONZA, catalog number: V4XP3032 50 µL at 1 µg/µL)
12. P3 primary cell nucleofector solution (LONZA, catalog number: V4XP3032 675 µL)
13. P3 primary cell nucleofector supplement (LONZA, catalog number: V4XP3032 150 µL)
14. Single-guide RNA (sgRNA) (IDT custom-made, 100 µM)
15. Alt-R S.p. HiFi Cas9 Nuclease V3 (IDT, catalog number: 10810559 500 µg at 10 µg/µL)
16. Single-strand oligo donor (ssODN) (IDT, 10 nmol)
17. shp53-f2 plasmid (Addgene)
18. Alt-R Cas9 electroporation enhancer (IDT, catalog number: 1075915 100 µm)
19. Molecular-grade H<sub>2</sub>O (Cytiva, catalog number: 82007-334 500 mL)
20. Zymo quick DNA™ MicroPrep kit (Zymo Research, catalog number: D3021)
21. Agarose (VWR Life Sciences, catalog number: N605-500G)
22. Zymo Clean™ Gel DNA Recovery kit (Zymo Research, catalog number: D4002)

### Solutions

1. Ribonucleoprotein (RNP) complex (see Recipes)
2. CloneR media (see Recipes)
3. Nucleofection reaction solution (see Recipes)
4. Nucleofection media (see Recipes)
5. P3 nucleofection solution mix (see Recipes)
6. mTeSR complete media (see Recipes)
7. Matrigel (see Recipes)

## Recipes

### 1. RNP complex

This should be freshly prepared.

Reagent	Final concentration	Volume
Cas9 nuclease V3	64 mM	0.8 $\mu$ L
sgRNA	100 $\mu$ M	1.2 $\mu$ L
D-PBS (1 $\times$ )		3.0 $\mu$ L
Total		5.0 $\mu$ L

### 2. CloneR media

This should be freshly prepared. Can be stored at 4 °C for one week.

Media component	Volume
mTeSR complete	8.9 mL
Clone R	1 mL
Revitacell	100 $\mu$ L
Total	10 mL

### 3. Nucleofection reaction solution

This should be freshly prepared.

Component	Test reaction	GFP control	No pulse control
P3 primary cell nucleofector solution	1 $\mu$ L	7 $\mu$ L	1 $\mu$ L
shp53-f2 plasmid	1 $\mu$ L	1 $\mu$ L	1 $\mu$ L
Alt-R electroporation enhancer (100 $\mu$ M)	1 $\mu$ L	1 $\mu$ L	1 $\mu$ L
ssODN (single-stranded oligodeoxynucleotide) (100 $\mu$ M)	1 $\mu$ L	0 $\mu$ L	1 $\mu$ L
RNP complex	5 $\mu$ L	0 $\mu$ L	5 $\mu$ L
GFP (0.5 $\mu$ g/ $\mu$ L)	0 $\mu$ L	1 $\mu$ L	0 $\mu$ L
Cells (1 million/reaction) ( $\mu$ L can be altered)	11 $\mu$ L	11 $\mu$ L	11 $\mu$ L
Total	20 $\mu$ L	20 $\mu$ L	20 $\mu$ L

### 4. Nucleofection media

This should be freshly prepared.

Component	Volume
CloneR media	1 mL
Alt-R Cas9 HDR enhancer	10 $\mu$ L

### 5. P3 nucleofection solution mix

This should be freshly prepared.

Component	Volume
P3 primary cell nucleofector solution	16.4 $\mu$ L
P3 primary cell nucleofector supplement	3.6 $\mu$ L
Total	20 $\mu$ L

### 6. mTeSR Plus complete media

This should be freshly prepared and can be stored at 4 °C for two weeks.

Component	Volume
mTeSR Plus basal	40 mL
mTeSR Plus 5 $\times$ supplement	10 mL
Total	50 mL

## 7. Matrigel

Dilute Matrigel right before the experiment with DMEM/F12. The diluted Matrigel can be stored at 4 °C for 2 weeks.

Component	Volume
DMEM F12	11 mL
Matrigel (1 mg/mL)	1 mL
Total	12 mL

## Laboratory supplies

1. 6-well culture plate (Thermo Scientific, catalog number: 12556004)
2. 24-well plate (Thermo Scientific, catalog number: 12556006)
3. 12-well plate (Thermo Scientific, catalog number: 12556005)
4. 1.7 mL microtube (Genesee Scientific, catalog number: 24-282C)
5. 1,250 µL tips (GeneMate, catalog number: P-1237-1250)
6. 200 µL tips (Neptune, catalog number: 89140-902)
7. 20 µL tips (GeneMate, catalog number: P-1237-20)
8. 10 µL tips (GeneMate, catalog number: P-1234-10)
9. 15 mL Falcon tubes (Sarstedt, catalog number: 62.554.205)
10. 16-well Nucleocuvette Strip (Lonza, catalog number: V4XP3032)

## Equipment

1. Nucleofector (Lonza, model: 4D)
2. Centrifuge (Sorvall, model: ST16R)
3. Gel imager (Bio-Rad, model: ChemiDoc™ MP Imaging System)
4. Gel electrophoresis machine (Labnet, model: Enduro GelXL)
5. NanoDrop (Thermo Scientific, model: Nanodrop One)
6. PCR thermocycler (Bio-Rad, model: T100™ thermal cycler)
7. Microscope (EVOS, Advance Microscopy group)
8. Incubator (SANYO CO<sub>2</sub> incubator)
9. Biosafety cabinet (Nuaire, model: LabGard, Class II, Type A2, Biological Safety Cabinet)
10. Hemocytometer (Hausser Scientific Horsham, model: Bright-Line)

## Software and datasets

1. Synthego.com (<https://design.synthego.com/#/>)
2. IDT ([https://www.idtdna.com/site/order/designtool/index/CRISPR\\_CUSTOM](https://www.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM))
3. ImageJ (<https://ij.imjoy.io/>)

## Procedure

### A. In silico design sgRNA and ssODN

1. Design sgRNA and ssODN by using IDT software:  
<https://www.idtdna.com/site/order/designtool/index/HDRDESIGN>.
2. The exact amount to resuspend sgRNA (Table 1) and ssODN (Table 2) to a 100 µM concentration can be

determined using IDT Resuspension Calculator: <https://www.idtdna.com/calc/resuspension/>.

**Table 1. Information of guide RNA used in this protocol**

dsSNP ID	Sequences	PAM Site
rs121918393	GAGGCGCACCCGCAGCTCCT	CGG

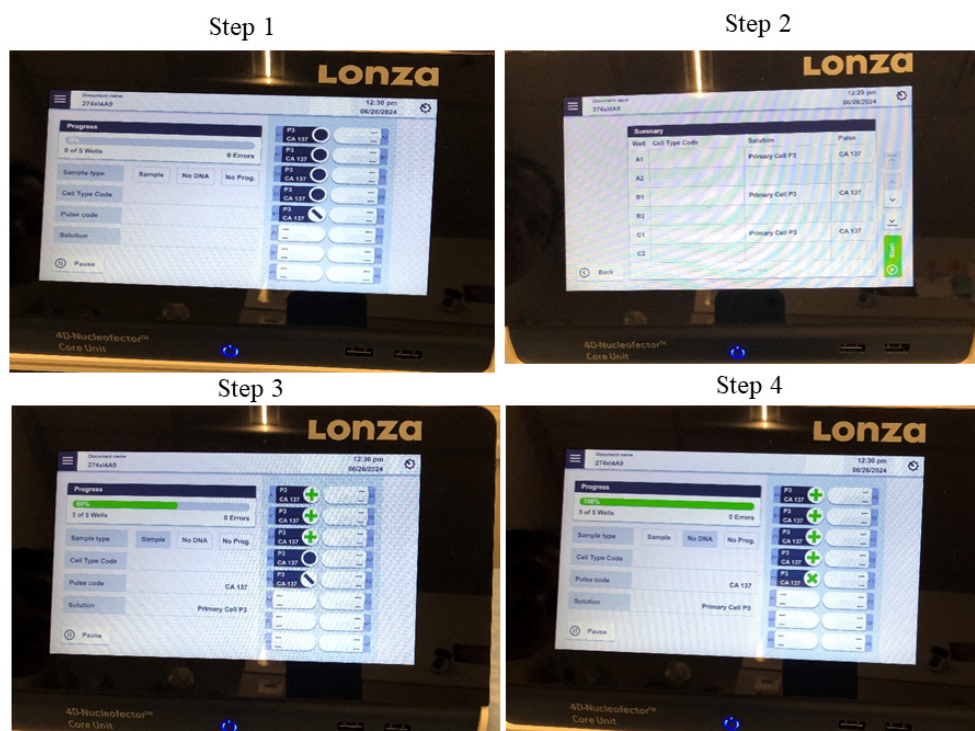
**Table 2. Information on HDR templates used in this study**

dsSNP ID	HDR Template Sequences
rs121918393	GGAGCCGCTTACGCAGCTTGCAGGTGGGAGGCGAGGCTCACGCGCAGC TCCTCTGTGCTCTGCCCAGCATGGCTGCACCTCGCCGCGTACTGCAC

## B. Cell culture and gene editing

- Maintain the iPSC line in mTeSR Plus complete media (see Recipe 6).  
*Note: Maintain the iPSC line in mTeSR Plus complete media at 37 °C in a humidified 5% CO<sub>2</sub> incubator. Change media every other day. Passage cells when 75%–80% confluence is achieved.*
- Change cell culture media to mTeSR Plus complete media with additional 1% Revitacell supplement 1 h before the start of nucleofection.
- Prepare a Matrigel-coated 24-well plate (see Recipe 7) for the nucleofected cells >1 h prior.
- After aspirating Matrigel, add 500 µL of nucleofection media (see Recipe 4). Use half these amounts if using a 48-well plate.
- Aspirate media from the iPSC cell culture.
- Add 2 mL of 1× D-PBS to clean/rinse the cell monolayer and aspirate.
- Add 0.5 mL of Accutase, incubate at 37 °C for <10 min (8 min is optimal), and check that cells have been released using a microscope.
- Add 2 mL of DMEM/F12 to neutralize the Accutase.
- Gently wash cells off the plate using a p1000 pipette and transfer to a 15 mL conical tube.
- Add additional DMEM/F12 to make up to 10 mL.
- Take 10 µL of cell suspension and count using a hemocytometer or other cell counting methods. A cell concentration of 1 million cells per reaction is recommended (see cell counting protocol in File S1).  
*Note: We recommend the user to optimize the cell density when a new line is used.*
- Take the total number of cells (per milliliter of cell suspension) needed for the experiment depending on how many separate reactions are being done.  
*Note: For example, three reactions would need 3 million cells in total. If the cell concentration was 0.5 million/mL, then 6 mL would need to be taken from the counted suspension to have 3 million cells in total.*
- Centrifuge the cell suspension at 200× g for 7 min at 4 °C with acceleration at 9 and deceleration at 5.
- Aspirate and resuspend cells in 11 µL per reaction of P3 primary cell nucleofector solution mix (see Recipe 5).
- In each Nucleofection reaction mixture, add the cell suspension and 5 µL of the pre-prepared RNP complex and pmaxGFP, ssODN, shp53-f2 plasmid, and Alt-R Cas9 electroporation enhancer and add additional P3 primary cell nucleofector solution if required (see Recipe 3).
- Pipette suspension up and down 2–3 times to mix and transfer 20 µL of the nucleofection reaction mixture to the corresponding 16-well nucleocuvette strip wells.
- Gently tap the 16-well nucleocuvette strip on the counter inside the biosafety cabinet to ensure that there are no air bubbles.
- Turn on the Lonza 4D nucleofector, select *X*, and select the 16-strip Nucleocuvette with the *P3 program CA137*.  
*Note: We recommend optimizing by testing 4–8 programs initially when using a new cell line.*
- Place the nucleocuvette strip into the Lonza 4D system and nucleofect.
- Check corresponding wells to program. Ensure that the no-pulse control does not have an assigned program, then select *START*. The nucleofection will take only ~30 s (Figure 1). Any errors will show on the screen.



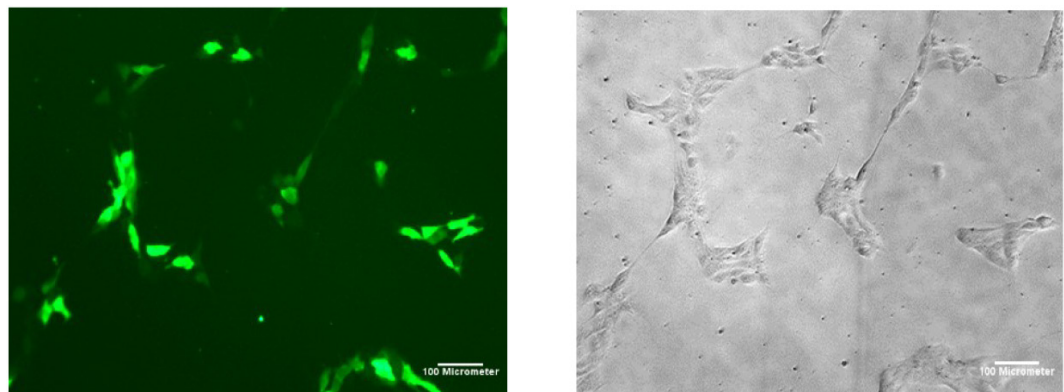


**Figure 1. Pictorial presentation of setting Lonza nucleofector for gene editing.** Step 1: Set the Lonza nucleofector; select pulse code, solution, and cell type. Step 2: Select the program. Step 3: Run the program after selecting all required wells. Step 4: Completion of nucleofection.

21. Remove the Nucleocuvette from the nucleofector and incubate at 37 °C for 10–20 min.  
*Notes:*
  - a. Do not add anything, move, or disturb the cuvette strip during this time.
  - b. This allows the lipid bilayer of the cells to reposition/organize.
22. After 10–20 min incubation, add 20 µL of the prewarmed nucleofection media (see Recipe 4) from the pre-prepared 24-well plate to each well in the nucleocuvette.
23. Using a p20 pipette, add the 40 µL nucleoporated cell suspension into the 500 µL of nucleofection media already in the Matrigel-coated 24-well plate.
24. Incubate cells at 37 °C; then, check and change media at 24 h with CloneR media (see Recipe 2).
25. Forty-eight hours after nucleofection, replace the CloneR media with fresh CloneR media. A complete change is not required. This is to compensate for the evaporation/degradation of previous CloneR.

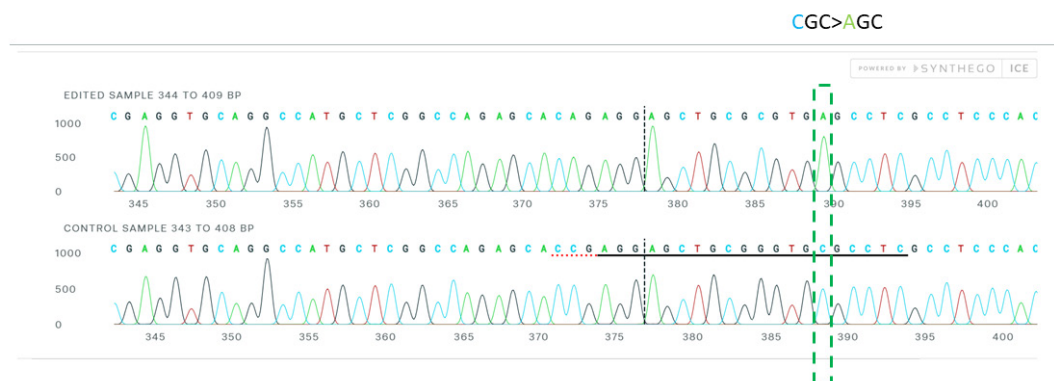
#### Post-nucleofection

1. Twenty-four hours after nucleofection, count the percentage of GFP-expressing cells at 10× magnification. Take two images (Figure 2) with the brightfield filter and two images of the same field of view with the GFP filter per reaction (well). Repeat in 48 h. Save these images onto a USB to count using ImageJ software. After 48 h, the ability to see GFP under the microscope filter slowly dissipates.
2. Once cells reach 70%–80% confluency, extract genomic DNA using the Zymo quick DNA™ MicroPrep kit.
3. Quantify genomic DNA levels using a Nanodrop and perform PCR for the desired gene.  
*Note: For achieving high-quality results in genomic DNA applications like PCR, it is crucial to use good-quality DNA and ensure the following practices: quantification of genomic DNA, optimization of PCR conditions, and running a gel for verification.*
4. Run 20 µL of the PCR product on 1% agarose gel to check the quality of the PCR product.
5. Cut the PCR product and purify by Zymo Clean™ Gel DNA Recovery kit.



**Figure 2. Induced pluripotent stem cell (iPSC) colonies with pmaxGFP 24 h post-nucleofection GFP vs. brightfield at 10× magnification**

6. Quantify the eluate using Nanodrop to determine the optimal amount for Sanger sequencing.
7. Results from Sanger sequencing can be analyzed using Synthego's ICE online software and/or gene viewer programs, e.g., SnapGene.
8. To analyze using ICE, download the ab.1 file for the desired sample to analyze and the corresponding control file.
9. Go to <https://ice.synthego.com> and drag and drop the ab.1 files into the corresponding *control* and *experiment* sample boxes.
10. Use the guide and repair ssODN sequences.
11. Select *add samples to analysis* and *analyze* (Figure 3).



**Figure 3. Sanger sequence view showing edited and control sequences in the region around the guide sequence.** This shows sequence base calls from both the control and the edited sample .ab1 files. The horizontal black underlined region represents the guide sequence. The horizontal red underline is the PAM site. The vertical black dotted line represents the actual cut site.

12. After confirmation of gene editing, cells can be dissociated and isolated into single cells.

#### Single clone selection

1. Coat a 10 cm plate with Matrigel for <1 h.
2. Aspirate media from bulk cell plate, wash with 2 mL of 1× D-PBS, and aspirate.
3. Add 0.5 mL (for a 6-well plate) and 0.125 mL (for a 24-well plate) of Accutase for <10 min at 37 °C.
4. Add 1 mL of DMEM/F12 to the well to wash and separate.
5. Transfer cell suspension to a 15 mL conical tube and top up to 10 mL with DMEM/F12.



*Note: This further dilutes the dissociation reagent to ensure cell health.*

6. Centrifuge at 100× g for 5 min.
7. Aspirate supernatant and flick tube to loosen cell pellet.
8. Add 1 mL of cloning media to the cell pellet and pipette to mix.
9. Count the cells by hemocytometer (File S1).
10. Seed two 10 cm cell culture dishes with 100 and 300 cells in 10 mL of media.
11. Keep the plate in the incubator at 37 °C for 48–72 h.
12. Examine the cells under the microscope after 48 h to see if they are single cells or not.
13. Cells should be ready for picking after 72–96 h of seeding (when cells form single colonies) (Figure S1).
14. After 72–96 h, when small colonies originating from single cells appear, prepare the microscope in the biosafety cabinet.
15. Prepare a 24-well plate with Matrigel media <1 h prior to picking.
16. With the appropriate plate under the microscope (Figure 4) at 4× magnification, use a p200 pipette on the 100 µL setting (to pick a 24-well), and pick colonies that are clearly isolated with a good size (up to 5–10 µm) and shape (Video S1).

*Notes:*

- a. *Cell colony may need to be lightly scraped to lift it off the plate before pipetting up.*
- b. *After several colonies have been picked, the media in the plate wells may need to be topped up in order to continue picking.*



**Figure 4. Image of microscope setup inside the biosafety cabinet for colony pickup**

17. Allow colonies in each well to form large, good sized colonies (this can take 3–5 days post-picking).
  18. Once the colonies are big enough to be split, prepare/prewarm a new Matrigel-coated 24-well plate with 500 µL of cloning media in each well.
  19. Split corresponding cells into a 12-well plate from a 24-well plate by using ReLeSR as a digestive agent and extract genomic DNA from half of the cells.
  20. After genomic DNA extraction, perform a standard PCR, send the sample for sequencing, and verify with a sequencing analysis program or perform ICE analysis (the same as steps 7 to 11).
- Note: We use commercially available genomic DNA extraction kits for better DNA quality.*
21. After confirmation, perform downstream experiments from the edited cell lines.

## Validation of protocol

This protocol or parts of it has been used and validated in the following research article:

- Singh et al. [11]. A high efficiency precision genome editing method with CRISPR in iPSCs. *Scientific Reports*.

## General notes and troubleshooting

### General notes

1. A passage number below p25 will result in better editing efficiency.
2. On the day of nucleofection, the media should be changed to mTeSR plus with (1%) Revitacell supplement.
3. Try to use the IDT resuspension calculator to resuspend the sgRNA and ssODN.
4. Before use, these reagents should be centrifuged for a brief amount of time.
5. The Cas9 enzyme should always be taken out from the -20 °C freezer directly before use such as when making the RNP complex.
6. If using Accutase to detach, you should use double the volume of DMEM/F12 media to dilute the Accutase.
7. After nucleofection, immediately keep the vessel in the incubator at 37 °C for 10–20 min.
8. For single-cell colony picking, seed 100 and 300 cells in two different 10 cm plates. For **single-cell colony picking**, seeding cells at the correct density is crucial to ensure that individual cells are spaced far enough apart to grow into isolated colonies. Our approach to seed **100 and 300 cells in two different 10 cm plates** is a strategy to balance **cell density** and the ease of picking single colonies.
9. It is highly recommended to cut the desired PCR product from the gel, purify it, and send it for sequencing.

### Troubleshooting

Problem 1: Low cell survival rate after nucleofection.

Possible cause: i) The cell number is low.

Solution: i) Use the appropriate cell number with a minimum of 1 million cells per reaction.

Possible cause: ii) Issue with Cas9 concentration.

Solution: ii) We recommend optimizing the Cas9 concentration by using different concentrations of Cas9 in a titration experiment.

Possible cause: iii) Harsh pipetting and centrifugation.

Solution: iii) Pipette very gently during the cell handling and perform centrifugation with centrifugation breaking methods.

Problem 2: Low editing efficiency.

Possible cause: The guide RNA or ssODN may not be working. Cas9 concentration may be incorrect.

Solution: i) Always try 2–3 different guideRNAs and ssODN.

Solution: ii) Optimize Cas9 concentration.

Problem 3: Poor sequencing results from Sanger sequencing.

Possible cause: Problems with PCR primers or the selection of the correct PCR product.

Solution: Redesign the PCR primer and always run an agarose gel, cut the expected size fragment, elute with the gel recovery kit, quantify, and send for sequencing.

Problem 4: Cells are not attaching in the 10 cm dish after plating the single cell suspension.

Possible cause: Lack of cell survival molecules.

Solution: Use CloneR in the growth media.

## Acknowledgments

We acknowledge NIH grant 1R03AG070415, Veterans Administration Merit Research Award 1 I01 BX006391, University of Minnesota Faculty Research Development Grant, funds from the University of Minnesota, Institute of Translational Neuroscience (ITN) to SHY. SHY is an ITN Scholar. Figures were created with BioRender.com. We also acknowledge our previous work published in *Scientific Reports* (2024), DOI: <https://doi.org/10.1038/s41598-024-60766-4> on which the current protocol is based [11].

## Competing interests

There are no conflicts of interest or competing interests.

Received: August 20, 2024; Accepted: October 14, 2024; Available online: November 05, 2024; Published: December 20, 2024

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## Supplementary information

The following supporting information can be downloaded [here](#):

1. File S1. Protocol for cell counting by using hemocytometer slide
2. Figure S1. Image of single cell
3. Video S1. Video of colony piking under the microscope