Efficient Cas9-based Genome Editing Using CRISPR Analysis Webtools in Severe Early-onset-obesity Patient-derived iPSCs

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The CRISPR system is an adaptive defense mechanism used by bacteria and archaea against viruses and plasmids. The discovery of the CRISPR-associated protein Cas9 and its RNA-guided cleavage mechanism marked the beginning of a new era in genomic engineering by enabling the editing of a target region in the genome. Gene-edited cells or mice can be used as models for understanding human diseases. Given its high impact in functional genomic experiments on different model systems, several CRISPR/Cas9 protocols have been generated in the past years. The technique uses a straightforward "cut and stitch" mechanism, but requires an accurate step-by-step design. One of the key points is the use of an efficient programmable guide RNA to increase the rate of success in obtaining gene-specific edited clones. Here, we describe an efficient editing protocol using a ribonucleotide protein (RNP) complex for homology-directed repair (HDR)–based correction of a point mutation in an induced pluripotent stem cell (iPSC) line generated from a 14-year-old patient with severe early-onset obesity carrying a *de novo* variant of *ARNT2*. The resulting isogenic iPSC line, named CUIMCi003-A-1, has a normal karyotype, expresses stemness markers, and can be differentiated into progenies from all three germ layers. We provide a detailed workflow for designing a single guide RNA and donor DNA, and for isolating clonal human iPSCs edited with the desired modification. This article also focuses on parameters to consider when selecting reagents for CRISPR/Cas9 gene editing after testing their efficiency with *in silico* tools. © 2022 The Authors. Current Protocols published by Wiley Periodicals LLC.

This article was corrected on 23 January 2023. See the end of the full text for details.

Basic Protocol 1: Design of sgRNAs and PCR primers
Basic Protocol 2: Testing the efficiency of sgRNAs
Basic Protocol 3: Design of template or donor DNA
Basic Protocol 4: Targeted gene editing
Basic Protocol 5: Selection of positive clones
Basic Protocol 6: Freezing, thawing, and expansion of cells
Basic Protocol 7: Characterization of edited cell lines

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INTRODUCTION

In 2012, a small team of scientists led by Drs. Jennifer Doudna and Emmanuelle Charpentier showed for the first time the mechanism by which the CRISPR-associated protein Cas9 introduces targeted double-stranded breaks (DSBs) in the genome of bacteria using an RNA-guided sequence (Jinek et al., 2012). This discovery had high impact on the scientific community, and just one year later three different studies demonstrated that the CRISPR/Cas9 system can be used to perform efficiently genome editing in human cells (Cong et al., 2013; Jinek et al., 2013; Mali, Esvelt, & Church, 2013). At the same time, the possibilities offered by human induced pluripotent stem cell (hiPSC) reprogramming from fibroblasts or peripheral blood mononuclear cells (PBMCs) increased the relevance of this technique, which immediately became one of the most important methods to study gene function, drug testing, and gene therapy (Maherali et al., 2007; Park et al., 2008; Takahashi & Yamanaka, 2006; Wernig et al., 2007). Cas9 is a family of endonucleases that use a 20-nt guide RNA (gRNA) to recognize and cut a specific DNA region. Cas9 specificity also relies on recognition of a short sequence (NGG) downstream of the gRNA, known as the protospacer adjacent motif (PAM), which is required for opening the DNA and target cleavage (Nishimasu et al., 2014; Sternberg, Redding, Jinek, Greene, & Doudna, 2014). After introduction of a DSB, two major DNA repair mechanisms can intervene: non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Saleh-Gohari & Helleday, 2004; Urnov, Rebar, Holmes, Zhang, & Gregory, 2010; Zhang, Li, Ou, Huang, & Liang, 2021). The first knocks out the targeted gene by generating nonspecific mutations (insertions/deletions, commonly known as indels) and tends to be error-prone, while the second produces specific modifications determined by an exogenous donor template.

To deliver this extremely powerful tool to cells, two different transfection strategies can be employed: using plasmid-based transient expression of Cas9 and a single gRNA (sgRNA) or using direct delivery of Cas9/sgRNA as a ribonucleoprotein (RNP) complex. Both approaches are well-adapted for genome editing and have different advantages and limitations. To efficiently target the region of interest using the plasmid approach, it is necessary to determine the optimal amount of Cas9 and sgRNA to deliver to the cells. It has been reported, however, that excessive or limited Cas9 or sgRNA can undermine the specificity and efficiency of targeting (Hsu et al., 2013; Pattanayak et al., 2013; Wu et al., 2014). In practical terms, it is time-consuming to estimate and establish the optimal concentration of Cas9 and sgRNA to use for plasmid-based transient expression. In contrast, optimizing the required Cas9 copy number is more feasible when using the RNP complex method. The RNP method results in less off-target effects because nuclease activity in RNPs flats out after 24 hr, whereas plasmids remain active inside the cell for several days, increasing the chance of cutting in regions with sequence similarities to the targeted region. The RNP method is also less toxic, because the commercially available modified gRNAs are chemically modified to decrease cytotoxicity and increase editing efficiency, whereas transfection reagents for plasmid transfection have known cytotoxicity and the

plasmids themselves can be cytotoxic. Finally, the RNP method reduces experimental time, as the gRNA/Cas9 RNP complex is preassembled so there is no need to wait for plasmids to be transcribed; the complex enters the cell nucleus and immediately starts cutting (Kim, Kim, Cho, Kim, & Kim, 2014; Kissling, Monfort, Swarts, Wutz, & Jinek, 2019; Ruan et al., 2019). For all these reasons, the RNP-based approach is becoming the preferred method for CRISPR/Cas9 approaches. The workflow includes several steps, some of which are laborious and impact the rate of success of editing the region of interest. One strategy to increase the likelihood of correct targeting is to test the efficiency of the reagents (sgRNA and donor DNA) in advance to avoid troubleshooting in the later stage of the procedure.

Here we describe a step-by-step RNP-based gene-editing protocol, in which the initial step consists of selecting the best sgRNAs by measuring their efficiency using *in silico* tools, while analyzing in parallel their predicted off-target score. Specifically, we provide the workflow for the correction of a *de novo* heterozygous variant (NM_014862.4,c.388C>G) in exon 4 of *ARNT2* (chr15:80,404,382-80,597,933, GRCh38/hg38) in an iPSC line generated from a 14-year-old girl with severe early-onset obesity (Iannello, Patel, Sirabella, Corneo, & Thaker, 2021). The resulting isogenic control iPSC line (CUIMCi003-A-1) is pluripotent, expresses stemness markers, has normal karyotype, and is mycoplasma negative.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly. All work with cells should be performed in a biosafety cabinet.

DESIGN OF sgRNAs AND PCR PRIMERS

BASIC PROTOCOL 1

Designing the sgRNA is the first critical step for successful gene targeting and is based on important reference steps developed and described in the past several years. An efficient sgRNA should lead to high levels of on-target Cas9 cleavage with minimal off-target effects. Design and optimization of PCR primers for genotyping is the second essential step to avoid downstream problems, and this is also described below.

Materials

Publicly available genome browsers: Ensembl (*https://useast.ensembl.org/index.html*), UCSC Genome Browser (*https://genome.ucsc.edu/*)
DepMap portal (*https://depmap.org/portal/*)
sgRNA design tools: Benchling (*https://www.benchling.com/*), Synthego CRISPR Design Tool (*https://design.synthego.com/#/*), Invitrogen TrueDesign Genome Editor (*https://www.thermofisher.com/us/en/home/life-science/genome-editing/geneart-crispr/invitrogen-truedesign-genome-editor.html*)
Primer designer tools: NCBI Primer-BLAST (*https://www.ncbi.nlm.nih.gov/tools/primer-blast/*), Primer3 Input (*https://primer3.ut.ee/*), or other available software

Additional reagents and equipment for PCR (see Basic Protocol 2) and agarose gel electrophoresis

Identify sgRNA targeting sequence

1. Before starting the design of a target-specific sgRNA, it is necessary to locate the region of interest (Fig. 1A). Use a publicly available genome browser such as Ensembl or UCSC Genome Browser to determine exon/intron regions and search for the target region.

   In this example, we targeted the Homo sapiens (GRCh38.p10) chr15:80,470,411 region in ARNT2 exon 4, where the c.388C>G variant is located.

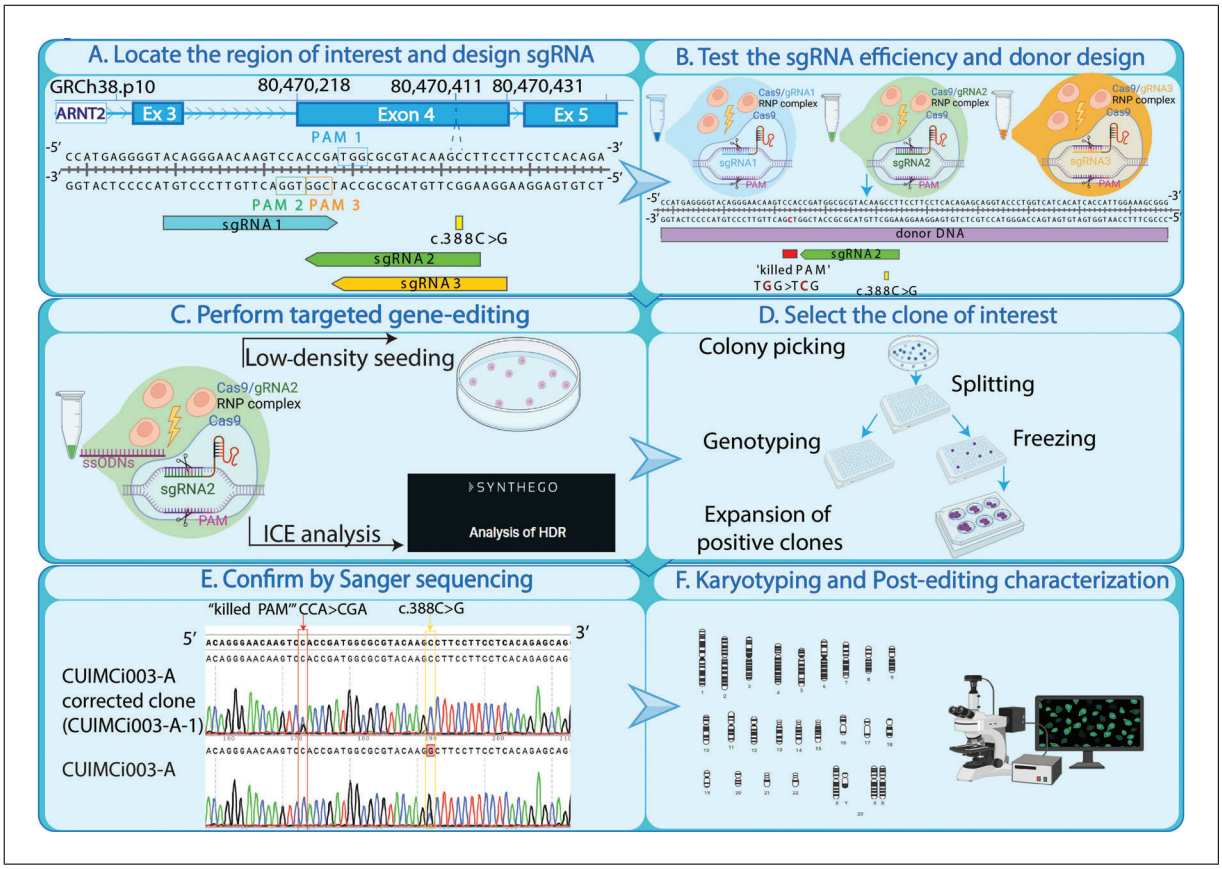

Figure 1 Detailed workflow of protocol for gene editing to correct the c.388C>G variant of the CUIMCi003-A iPSC line. (**A**) Representation of the localization of the region of interest and the designed sgRNAs. The c.388C>G variant occurs in the chr15:80,470,411 region in exon 4 of *ARNT2*. (**B**) Testing of sgRNA efficiency for all guides (three singular electroporation experiments) and donor DNA design after choosing the most efficient sgRNA. The selected sgRNA2 and the respective donor DNA (with killed PAM) are shown. (**C**) Targeted gene-editing is achieved by repeating the electroporation with donor DNA added. Cells are collected for single-cell seeding 48-72 hr after electroporation. In parallel, a portion of electroporated cells is used to assess the efficiency of cleavage and knock-in by ICE analysis. (**D**) Colonies are picked and transferred to a 96-well plate, which is then split to two plates: one for genotyping and one for freezing. After genotyping, the clones in which editing has been confirmed are expanded. (**E**) Sanger sequencing is used to confirm the corrected clone (CUIMCi003-A-1) and killed PAM. (**F**) Post-editing characterization includes karyotyping, assessment of stemness marker expression by immunocytochemistry, verification of potential for differentiation of three lineages, and mycoplasma testing.

2. It is very important to predict how essential the targeted gene is for cell survival, and thus whether and how the editing (knock-out/mutation) could be lethal to the target cells. Search for the gene of interest on DepMap and check the dependency score.

   A highly negative dependency score indicates that cell survival is highly dependent on that gene, and therefore editing could be problematic. If the gene is essential, an inducible system should be considered.

3. Select an sgRNA design web tool such as CRISPR Design Tool, Benchling, or Invitrogen TrueDesign Genome Editor.

   To design an sgRNA for knocking out a specific region, CRISPR Design Tool and Invitrogen TrueDesign Genome Editor are preferred.

4. For CRISPR Design Tool, go to the website, select the species, and enter the name of the gene of interest.

   Online tools such as Benchling and TrueDesign Genome Editor also allow pasting of the sequence of interest in FASTA format.

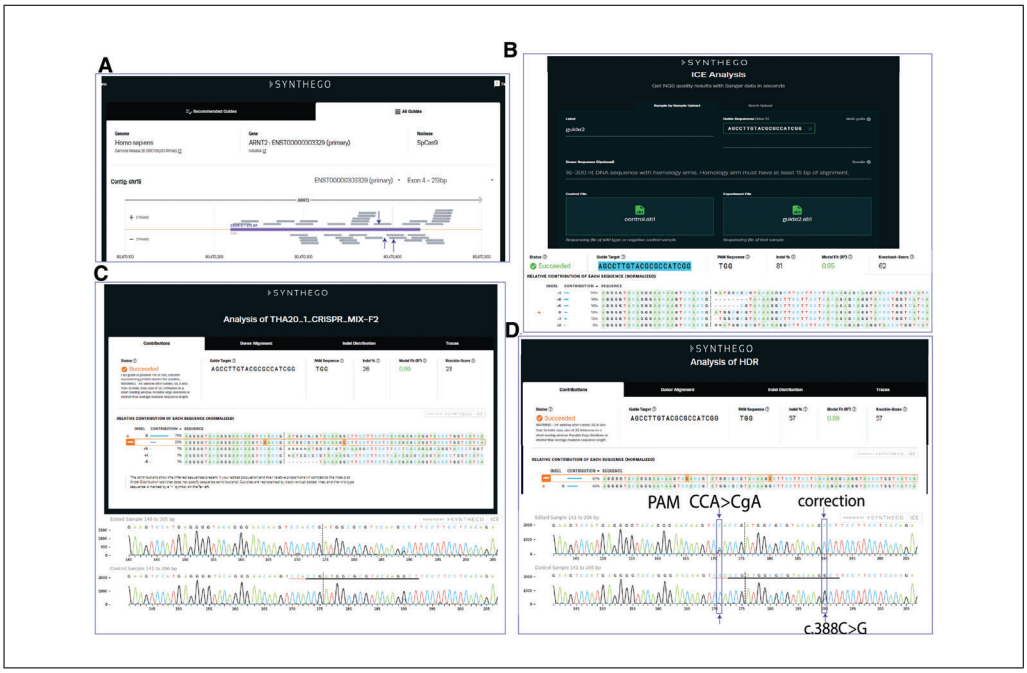

Figure 2 sgRNA design using the Synthego CRISPR Design Tool. (**A**) Screenshot of interface. Arrows indicate three sgRNAs designed for correction of the c.388C>G variant in the chr15:80,470,411 region of exon 4 of *ARNT2*. (**B**) Screenshot of ICE analysis webpage. A brief report showing ICE results for sgRNA2 is shown at the bottom. Vertical dotted line represents the cleavage site. Percentages represent frequency of different indel events present in the mixed population sequenced after electroporation. (**C**) ICE analysis of pooled cells to assess efficiency of HDR. (**D**) HDR efficiency analysis of edited clones.

Table 1 sgRNA Sequences Designed Using the Synthego CRISPR Design Tool for the Project Reported Here

Name	Strand	Sequence (5′ to 3′)	Cut site	Exon	On-target score	Off-target score
ARNT2_g RNA1	+	ACAGGGAACAAGTCCACCGA	80,470,395	4	0.640	0,0,0,4,77
ARNT2_g RNA2	–	AGCCTTGTACGCGCCATCGG	80,470,397	4	0.608	0,0,0,0,8
ARNT2_g RNA3	–	GGAAGCCTTGTACGCGCCAT	80,470,400	4	0.537	0,0,0,2,21

5. Select the exon of interest and analyze the resulting recommended sgRNAs (Fig. 2A).

   a. Choose sgRNAs located within 20 bp of the target site if the target is a single-nucleotide/few-base-pair modification.

   Suitable sgRNAs should be 20 bp in length and located immediately next to the 5′ end of the PAM (Montague, Cruz, Gagnon, Church, & Valen, 2014).

   Since the efficacy of sgRNAs varies considerably, it is recommended to select two or three sgRNAs per site of interest based on low off-target and high on-target activity scores (Table 1). Good and bad on-target (or cutting) scores are relative to the target region. Some target regions yield very efficient sgRNAs with a cutting efficiency of 0.8, which is considered high. Others yield sgRNAs with a cutting efficiency of only 0.5, which is considered low. Choose the highest score available at the target region. The off-target score is another parameter to decide which sgRNA is better: a low off-target score in combination with the highest cutting efficiency score will be the best sgRNA. The output from the CRISPR design tool is a list of potential off-target sites. For example, for ARNT2_g RNA2 the off-target score is 0,0,0,0,8, which indicates 0 identical mismatches, 0 sequences with a single mismatch, 0 sequences with two mismatches, 0 sequences with three mismatches, and 8 sequences with four mismatches. It is generally recommended to select guides that have 0 identical matches, 0 single mismatches, and at least 2 mismatches.

Table 2 Genotyping Primers for *ARNT2*

Primer	Sequence	T_m
ARNT2_F1	5′- GGTGTTAGCCCCTAGTTCCTGG -3′	62.07
ARNT2_R1	3′- TGGCTTCATTCCTTCCTCAACC -5′	60.56
ARNT2_F2	5′- CCCCTCTCCTGATCTCGTGC -3′	61.74
ARNT2_R2	3′- TTGGTGACGCTGGGATTCC -5′	60.00
ARNT2_F3	5′- GCCAGACAAGCTCACCATCC -3′	61.03
ARNT2_R3	3′- CCTTGAGCCCTGGTTCCCC -5′	62.29

b. Check that the sgRNA sequence does not match any other region on the target genome. No mismatch is tolerated in the seed sequence (10-12 bp proximal region from the PAM proximity region). One or two mismatches are tolerated in the non-seed region (10 bp from the 5′ terminus) (Zhang et al., 2020).

c. To improve target specificity and binding, try to avoid regions with single-nucleotide polymorphisms (SNPs). These can be determined by Sanger sequencing of the target region.

The websites ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), dbSNP (https://www.ncbi.nlm.nih.gov/snp/), and Ensembl (https://useast.ensembl.org/index.html) can also be used to identify SNPs.

d. To select the best sgRNA, it is strongly recommended to perform an efficiency test for all selected sgRNAs (see Basic Protocol 2 for ICE analysis).

e. Consider the flexibility of the PAM sequence. To avoid sgRNA re-targeting, it is important to silence (or "kill") the PAM sequence. For this reason, one of the criteria to consider when designing sgRNAs is the possibility of introducing a synonymous variant in the PAM region (Fig. 1B; see Basic Protocol 3).

sgRNA synthesis is commercially available via providers like Synthego and IDT; sgRNA can also be cloned into the appropriate vectors or generated using in vitro transcription. Multiple gRNAs were available for our target sequence, but we selected these three gRNAs (represented in Table 1) because of both their high on-target and low off-target score. These three gRNAs were all tested according to Basic Protocol 2.

Design and optimize PCR primers

6. Design three sets of primers for each target site using the publicly available Primer-BLAST, Primer3 Input, or other available software.

7. Prefer primers that bind at least 100-200 bp away from the PAM site in each direction.

This helps generate clean and readable sequences during genotyping screening. The primers designed for our example are shown in Table 2.

8. As DNA template, use DNA isolated from the cells that will be edited. For PCR conditions, see Basic Protocol 2, steps 27-28.

9. Optimize the PCR reaction by using a combination of these primers and at least three different annealing temperatures.

We tested the three combinations (F1R1, 443 bp; F2R2, 300 bp; F3R3, 203 bp) at three annealing temperatures (Fig. S1A).

10. Determine the best primer set by running the PCR product on a 1.5% to 2% agarose gel.

We chose ARNT2_F1R1 as the best primer set because of the specificity of the amplification (no nonspecific bands were observed) at the annealing temperature of 62°C and

the optimal length of the PCR product obtained (443 bp) for ICE analysis (Fig. S1A). For Sanger sequencing, we chose ARNT2_F2 because it is optimally distanced from the target site and works as an internal primer.

TESTING THE EFFICIENCY OF SGRNAS

BASIC PROTOCOL 2

To assess the efficiency of the sgRNAs selected in Table 1, a simple gene-targeting experiment (Fig. 1B) was undertaken in the CUIMCi003-A iPSC line (Iannello et al., 2021), using the Inference of CRISPR Edits (ICE) online tool from Synthego. As a control, it is important to include the genomic DNA sequence of the non-edited iPSC line to monitor the assay activity.

Materials

hESC or hiPSC cell line with normal karyotype, e.g., CUIMCi003-A (Iannello et al., 2021)
mTeSR Plus medium (Stem Cell Technology, cat. no. 100-0276)
Matrigel GFR Membrane Matrix (Corning, cat. no. 354230) or Cultrex RGF Basement Membrane Extract with Phenol Red (R&D Systems, cat. no. 3433-005-01)
0.5 mM EDTA (see recipe)
Y-27632 (ROCK inhibitor, Tocris, cat. no. 1254)
P3 primary cell 4D-Nucleofector X kit S (Lonza, cat. no. V4XP-3032), including P3 primary cell buffer, Supplement 1, and 16-well electroporation strips
Synthesized sgRNA(s) (Synthego, CRISPRevolution sgRNA EZ Kit, 1.5 nmol, or IDT, Alt-R CRISPR/Cas9 sgRNA, 2 nmol)
Alt-R S.p. HiFi Cas9 Nuclease V3 (Integrated DNA Technologies, cat. no. 1081061)
StemPro Accutase Cell Dissociation Reagent (Life Technologies, cat. no. A11105-01)
1× Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium (Corning, cat. no. MT-21-031-CV)
Trypan blue (Gibco, cat. no. 15250061)
CloneR (Stem Cell Technology, cat. no. 05888)
DNA lysis buffer (see recipe)
Proteinase K (Invitrogen, cat. no. AM2548)
Clontech Labs 3P CloneAmp HiFi PCR Premix (Clontech Labs, cat. no. 3P 639298)
25 nM DNA primers for PCR, standard desalting (Integrated DNA Technologies; see Basic Protocol 1)
DNA template for PCR

6- and 24-well flat-bottom cell culture plates (Corning, cat. nos. 3506 and 3526)
Inverted microscope (Olympus CKX31)
Hemocytometer or other cell counter (e.g., Countess 3 automated cell counter with Countess chamber slides, Thermo Fisher Scientific)
15-ml conical centrifuge tubes (Corning, cat. no. 352097)
Electroporator, e.g., 4D-Nucleofector Core Unit (Lonza, cat. no. AAF-1002B [now discontinued] or AAF-1003B) with X-unit (Lonza, cat. no. AAF-1003X)
37°C incubator at 5% O_2, 5% CO_2 (hypoxic) or 21% O_2, 5% CO_2 (standard) (e.g., Double-Stacked Tri-gas Incubator HeraCell 150i, Thermo Scientific)
1.5-ml microcentrifuge tubes (Fisherbrand, cat. no. 05-408-129)
Nanodrop (Thermo Scientific)
MicroAmp 8-tube strips, 0.2 ml, and 8-cap strips (Applied BioSystem, cat. nos. N801-0580 and N801-0535)
Thermocycler (e.g., Eppendorf MasterCycler Nexus gradient)
ICE Analysis online tool (Synthego, *https://ice.synthego.com/#/*)

Additional reagents and equipment for agarose gel electrophoresis and Sanger sequencing

Culture cells

1. Select a hESC or hiPSC line that has been confirmed to have a normal karyotype. Grow in feeder-free conditions on Matrigel-coated 6-well culture plates using mTeSR Plus medium.

   We previously used mTeSR1 medium, but it must be changed every day, whereas mTeSR Plus is changed every other day. We have also been using Cultrex-coated plates instead of Matrigel. Throughout these protocols, Matrigel can be replaced with Cultrex.

   We try to split cells on Thursday, then change medium on Friday, adding 4 ml mTeSR Plus per well of a 6-well plate. Medium is changed again on Monday and Wednesday at 2 ml/well.

2. Passage cells non-enzymatically using 0.5 mM EDTA before they reach 100% confluency.

   a. Coat one (or more, if needed) 6-well plate with Matrigel. Leave for 1 hr at room temperature.

      Better transfection efficiency is observed when cells are passaged the day before electroporation to reach 50%-60% confluency on the day of electroporation. Prior optimization of the splitting density may be required. Only 2×10^5 cells are required per sgRNA to be tested, so one well of a 6-well plate may be enough.

   b. Warm 2 ml of 0.5 mM EDTA for each well at 37°C.

      Only the required amount should be warmed before the experiment.

   c. Aspirate medium from the cells and rinse cells by adding 1 ml of 0.5 mM prewarmed EDTA.

   d. Aspirate rinse, replace with another 1 ml of 0.5 mM EDTA/well, and incubate at room temperature for 2-3 min and/or check cells under a microscope.

      Colonies start loosening after ∼2 min, and cells start to round up and separate from each other. Some iPSC lines may require more time than others. Cells should not detach from the surface of the plate.

   e. Carefully aspirate EDTA and collect cells by gentle pipetting with 1 ml mTeSR Plus medium with 10 μM Y-27362.

      If the cells have detached from the surface (which happens very rarely), collect them in 0.5 mM EDTA, centrifuge (5 min at 300 × g), aspirate the supernatant, and resuspend the pellet in 1 ml mTeSR Plus with 10 μM Y-27362.

   f. Split cells 1:10 or at the best ratio for your cell line.

   g. Aspirate Matrigel from the coated plate and immediately transfer cells to the coated plate in mTeSR Plus medium with 10 μM Y-27362.

Electroporate cells

3. Prepare a Matrigel-coated 24-well plate and incubate 1 hr at room temperature.

4. Prepare an electroporation reaction mix as follows (21.5 μl per sgRNA to be tested):

   16 μl P3 primary cell buffer
   4 μl Supplement 1
   1 μl (5 μg) sgRNA
   0.5 μl (5 μg) Cas9
   Incubate the mix 20-25 min at room temperature.

   To test all three sgRNAs in Table 1, we prepared enough cells and reagents for three reactions. An additional well of non-electroporated cells is needed for a positive control for PCR and ICE analysis.

5. While the mix is incubating, warm Accutase to room temperature and detach the cells. Aspirate medium from the cells, add 1 ml Accutase per well, and wait 1-2 min and/or check cells under the microscope.
6. Once the cells start detaching from the surface, add 1 ml of 1× DPBS without calcium and magnesium to block the enzyme.
7. Pipette gently to make a single-cell suspension, breaking up any clumps.
8. Count cells by trypan blue exclusion using a hemocytometer or other cell counter.
9. Collect 2×10^5 cells per reaction in a 15-ml tube and bring to 10 ml using 1× DPBS without calcium and magnesium.
10. Centrifuge 5 min at 300 × *g*. Aspirate supernatant, being careful not to disturb the small pellet.
11. Gently resuspend pellet with electroporation mix, being careful to avoid bubbles, and transfer each reaction to a separate well of an electroporation strip.
12. Electroporate using program CA137 or CB150 on a Lonza 4D-Nucleofector Core Unit.

    Program CB150 is suggested by Lonza to electroporate hESCs (H9/WA09). For some lines, we and others have observed that program CA137 gives a better balance of survival and transfection efficiency. We suggest testing both programs when a new iPSC line is used for gene editing. For CUIMCi003-A, we used CA137. If an instrument other than the Lonza 4D is used, or if a transfection reagent is used, check the manufacturer's instructions as well as the literature for the best conditions for transfecting iPSCs.
13. Immediately add 50 µl mTeSR Plus with 10% CloneR to the electroporated cells.

    We have observed increased cell survival, especially of single cells, and better cloning efficiency when CloneR medium is used instead of Y-27362. We use Y-27362 for routine cell maintenance and CloneR for gene editing applications.
14. Aspirate Matrigel from one well of the coated 24-well plate and immediately transfer electroporated cells to that well.
15. Add another 250 µl mTeSR Plus with 10% CloneR to the well.
16. Repeat for each electroporation reaction.
17. Place plate in a 37°C incubator at 5% oxygen, 5% CO_2 (hypoxic) or 21% oxygen, 5% CO_2 (standard). Let cells recover and grow for 48-72 hr.

    Less cell death is observed when cells are kept in hypoxic conditions.
18. On the following day (one day post-electroporation), check cell viability under the microscope, then add another 250 µl mTeSR Plus with 10% CloneR to each well.

Perform PCR amplification and sequencing

19. At 2-3 days post-electroporation, aspirate medium and detach cells using Accutase (steps 5-6) using a 500-µl volume for both Accutase and DPBS.
20. Collect cells and transfer to a 1.5-ml centrifuge tube.
21. Centrifuge 5 min at 300 × *g*. Aspirate the supernatant.

    If DNA extraction will not be performed immediately, the cell pellet can be stored at −20°C.
22. Add 100-500 µl DNA lysis buffer to the cell pellet and mix well by pipetting.

    The volume of lysis buffer may vary based on pellet size or cell number.
23. Add proteinase K to a final concentration of 200 µg/ml and incubate 30 min at 56°C.

24. Inactivate proteinase K by incubating 20 min at 96°C.

25. Allow sample to cool to room temperature and measure the DNA concentration.

26. Prepare serial dilutions of 10, 20, 40, 60, 80, and 100 in dH_2O in 8-tube strips to determine the optimum dilution/concentration for PCR amplification (Fig. S1B).

    We do not determine the exact amount (ng) of DNA for each PCR reaction, but rather use the dilution that gives a more specific and cleaner PCR product. A minimum of 5-10 ng DNA is required.

27. Prepare a PCR master mix as follows (10 µl per reaction):

    5 µl PCR premix
    1 µl (10 ng) DNA template
    1 µl (0.5 µM) Primer F
    1 µl (0.5 µM) Primer R
    2 µl dH_2O

    Use the primers previously identified by optimization (see Basic Protocol 1). We used primer set ARNT2_F1R1 (Fig. S1) with a PCR premix containing a high-fidelity DNA polymerase.

28. Perform amplification using the following program:
    Initial denaturation: 95°C, 5 min
    35 cycles:

    denaturation: 98°C, 20 s
    annealing: 62°C, 20 s
    extension: 72°C, 30 s

    Final extension: 72°C, 1 min

29. Check the amplicon size by running 2 µl on a 1.5% to 2% agarose gel.

30. Use the remaining 8 µl for Sanger sequencing.

    Purified or crude PCR products can be used for Sanger sequencing using an internal (sequencing) primer. The internal primer should be at least 100-200 bp away from the PAM/cut site. The sample consisting of the non-electroporated iPSCs should also be sequenced as a control. In our experiment, we used the internal primer ARNT2_F2 for Sanger sequencing.

Assess editing efficiency by ICE analysis

31. Analyze the electropherograms generated by Sanger sequencing for electroporated and non-electroporated cells using the ICE online tool.

    a. Add the recommended information on the webpage, including the 20-bp sgRNA sequence. Upload the trace files (i.e., electropherograms files generated by Sanger sequencing; Fig. 2B).
    b. Interpret the results.

    The indel percentage is a general indicator of how efficiently a given sgRNA/Cas9 complex cuts under the transfection conditions tested. The same value is also reported in the contribution column (Fig. 2B).

    c. Choose the sgRNA with higher indel and knock-in scores.

    In our example, the three sgRNAs in Table 1 were tested separately in electroporation experiments. ARNT2_gRNA2 was chosen because of its cutting efficiency of 0.6 (knock-out score, Fig. 2B) and better off-target score (Table 1) compared to the other two options (knock-out scores of 0.7 and 0.3 for ARNT2_gRNA1 and ARNT2_gRNA3, respectively). ARNT2_gRNA2 was also chosen because it spans the location of the region we wished to edit (c.388C>G). For a knock-out experiment where more than one sgRNA is used in the same reaction for electroporation, the electroporated cells can be used directly for clonal selection if ICE analysis reveals a knock-out score >10.

DESIGN OF TEMPLATE OR DONOR DNA

BASIC PROTOCOL 3

Homology-directed repair acts in the presence of a single-stranded oligodeoxynucleotide (ssODN) or donor DNA template to specifically modify the target region of the gene of interest (Lin, Staahl, Alla, & Doudna, 2014). Depending on the type of editing (correction/introduction of a point mutation or sequence insertion), the design of a donor template might vary.

Materials

Modified donor ssODN (Integrated DNA Technologies)
Benchling or other available software

1. Design the ssODN as a complementary sequence to the sgRNA target strand (Fig. 1B).
2. Include a sequence of ∼40-50 bp on each side of the PAM sequence (the length of the homology arms can vary).

   An 80- to 100-bp ssODN is recommended when introducing/correcting a single nucleotide mutation or when deleting/inserting a small fragment. To insert a larger fragment in the region of interest, the donor DNA template should include the sequence of interest flanked by homology arms (right and left) of ∼100 bp.

3. The sgRNA could recognize the PAM sequence in the donor DNA template, leading to repetitive cleavage. To avoid this, introduce a single nucleotide change in the PAM sequence, maintaining the same reading frame (i.e., a killed PAM, Fig. 1B). This will avoid sgRNA re-targeting and increase editing efficiency.

   In this example, we chose the sequence

   GGTACCTGCTCTGTGAGGAAGGAAGgCTTGTACGCGCCATCGGTcGACTTGTT CCCTGTACCCCTCATGGACTTCATGTG (Fig. 1B).

   If the synonymous modification in the PAM site is not possible, an alternative is to introduce a synonymous variant at the 3′ end of the sgRNA binding region in the ssODN.

TARGETED GENE EDITING

BASIC PROTOCOL 4

After optimizing the electroporation conditions and choosing the best sgRNA (see Basic Protocol 2), electroporation is repeated, adding donor DNA, if needed (see Basic Protocol 3; Fig. 1C). This electroporation requires more cells (0.5–1 × 10^6 per reaction) and more electroporation mix (100 µl per reaction). Upon genotyping, we use ICE analysis to determine the cleavage efficiency and the knock-in efficiency if a donor is used.

Additional materials *(also see Basic Protocol 2)*

Single-stranded modified donor DNA (ssODN; Integrated DNA Technologies)
12-well flat-bottom cell culture plates (Corning, cat. no. 3513)
P3 primary cell 4D-Nucleofector X kit L (Lonza, cat. no. V4XP-3024), including P3 primary cell buffer, Supplement 1, and 100-µl electroporation cuvettes

Prepare cells and reagents

1. Prepare Matrigel-coated 12-well plates and incubate for 1 hr at room temperature.
2. One hour before electroporation, aspirate medium from the iPSCs to be electroporated and replace with 500 µl mTeSR Plus medium with 10% CloneR.

   Extra cells should be included for a non-electroporated control.

3. Warm Accutase to room temperature.
4. Prepare warm mTeSR Plus medium with 10% CloneR (without antibiotics).

5. Prepare the electroporation reaction mix as follows (100 µl per reaction):

   75 µl P3 primary cell buffer
   18 µl Supplement 1
   3 µl (15 µg) ssODN (for 90-100 bp)
   3 µl (15 µg) sgRNA
   1 µl (10 µg) Cas9

   Incubate 20-25 min at room temperature.

Electroporate cells

6. Prepare iPSCs using Accutase as described (see Basic Protocol 2, steps 5-8).
7. Collect 0.5–1 $\times$ 10^6 cells/reaction in a 15-ml tube and bring to 10 ml with 1$\times$ DPBS without calcium and magnesium.
8. Centrifuge 5 min at 300 $\times$ *g*. Aspirate supernatant, being careful not to disturb the cell pellet.
9. Gently resuspend pellet with electroporation mix, being careful to avoid formation of bubbles, and transfer to an electroporation cuvette for the Nucleofector 4D device.
10. Electroporate cells using the program that works best for your cells.

    We used program CA137. To ensure higher survival after electroporation, do not keep cells in electroporation buffer for long.

11. Immediately add ~100 µl mTeSR Plus medium 10% CloneR to the cuvette.
12. Aspirate Matrigel from the coated 12-well plate and add 500 µl/well mTeSR Plus medium with 10% CloneR.
13. Transfer electroporated cells to one well of the 12-well plate.
14. Place plate in the incubator (preferably hypoxic; see Basic Protocol 2, step 17) and grow for 24 hr.
15. On the following day, check cell viability under the microscope, add another 500 µl mTeSR Plus medium with 10% CloneR, and allow cells to grow for another 24-48 hr.

Proceed to ICE analysis and single-cell seeding

16. Detach cells using 0.5 mM EDTA as described (see Basic Protocol 2, step 2), but use a volume of 500 µl for EDTA solution and mTeSR Plus with 10% CloneR for this plate format.
17. Count cells. Keep ~5 $\times$ 10^3 cells for single-cell seeding (see Basic Protocol 5) and use the rest for DNA extraction and ICE analysis (see Basic Protocol 2, steps 19-31) (Fig. 1C).

    ICE analysis is required at this point in our case, as we performed a knock-in experiment (adding a donor DNA to correct the c.388C>G variant). For a knock-out experiment, if donor DNA is being used, ICE analysis can be repeated at this point or one can proceed directly to single-cell seeding.

    ICE analysis will help predict the percentage of knock-in present in the electroporated cells. The number of colonies to pick for genotyping will be determined by ICE analysis results. The knock-in score represents the percentage of sequences in the sample that contains the desired alteration. The same value is also reported on the contribution column (Fig. 2C). The higher the knock-out or knock-in score, the fewer colonies need to be picked. In our case, ICE analysis showed a knock-in score of 23 (Fig. 2C).

18. Proceed with low-density (single-cell) seeding and colony picking for genotyping if the knock-in score is >10.

    It is possible to get an edited clone even when the knock-in score is below 10, but it may require screening of many more colonies.

SELECTION OF POSITIVE CLONES

BASIC PROTOCOL 5

Cells are ready for low-density seeding and selection (Fig. 1C) 48-72 hr after electroporation. Colonies are picked and expanded, and then DNA is isolated from each colony and analyzed by genotyping. Positive colonies are further expanded for deeper analysis (cloning of alleles and sequencing).

Additional materials *(also see Basic Protocol 2)*

10-cm cell culture dishes (Thermo Scientific BioLite, cat. no. 130182, *optional*)
Cell strainer (*optional*)
96-well flat-bottom cell culture plates (Corning, cat. no. 3596)
Multichannel aspirator with 8-channel adaptor (Corning, cat. nos. 4930 and 4931)
Multichannel pipettor (Gilson)
Sterile disposable reagent reservoirs (Corning, cat. no. 4871)
96-well PCR plate (Thermo Scientific, cat. no. AB0900W)
Microplate sealing film (Axygen, cat. no. UC500)

Seed and grow single cells

1. Prepare a Matrigel-coated 6-well plate or 10-cm culture dish.
2. Detach cells using 0.5 mM EDTA as described (see Basic Protocol 2, step 2).
3. Count cells using trypan blue exclusion and a hemocytometer or other cell counter.
4. Prepare a single-cell suspension. If any cell clumps are observed, use a cell strainer to remove them.

   Cell clumps must be strictly avoided to isolate single clones.

5. Seed cells in Matrigel-coated dishes using mTeSR Plus medium with 10% CloneR. Seed $\sim 5 \times 10^3$ cells in 10 ml medium for a 10-cm dish or $\sim 1 \times 10^3$ cells in 2 ml medium per well of a 6-well plate.

   We usually seed two 10-cm dishes, although one is generally enough to obtain enough colonies to seed a full 96-well plate.

6. Place plate(s) in a 37°C incubator at 5% O_2, 5% CO_2 (hypoxic) or 21% O_2, 5% CO_2 (standard) and grow overnight.
7. The next day, add 5 ml mTeSR Plus medium with 10% CloneR to a 10-cm plate or 1 ml medium per well of a 6-well plate.
8. Allow cells to grow for ~1 week or until there are ~50-100 cells per colony. Change medium every other day.

Pick colonies

9. Coat a 96-well plate with 50 µl Matrigel/well and leave for 1 hr.

   The number of plates will depend on the ICE results and on how many colonies are picked. ICE analysis is a good reference tool to predict the potential number of clones with the alteration of interest, but, as a bioinformatic tool, it has some limitations. As a standard practice (and in this particular experiment), we pick a full 96-well plate for genotyping. In a knock-out editing experiment, for a score of 50, we suggest picking 20-30 colonies.

10. At the same time, change the medium on the cells with fresh medium and leave for at least 1 hr.

11. When ready to pick colonies, aspirate Matrigel from the 96-well plate and replace with 50 µl/well mTeSR Plus medium with 10% CloneR.
12. Manually pick colonies using a microscope inside a biosafety cabinet or picking hood. Position the plate under the microscope to select a colony, then pick manually using a pipette and transfer to one well of the 96-well coated plate (Fig. 1D).

    We usually use a P200 pipette with filtered tips. A fresh tip must be used for each colony.
13. When all colonies have been picked, place the plate in the incubator and grow cells overnight.
14. The next day, add another 100 µl mTeSR Plus medium with 10% CloneR to each well.
15. Grow colonies for ∼1 week, changing the medium every other day.

Perform genotyping and allele sequencing

16. Check confluency under the microscope. When the colonies are ∼70% confluent (∼1 week), detach cells using 0.5 mM EDTA and split to two fresh Matrigel-coated 96-well culture plates (Fig. 1D).

    One plate will be used for genotyping (below) and the other for freezing as a backup of all clones picked (see Basic Protocol 6).
17. Allow cells to grow again to 70% confluency.

    Once the cells reach 70% confluency, proceed to freezing the plate to be used as a backup (see Basic Protocol 6).
18. For the plate to be genotyped, remove medium from each well using a multichannel aspirator or multichannel pipette, being sure to change tips after each row.
19. Wash cells with 100 µl/well of 1× DPBS without calcium and magnesium. Aspirate DPBS.

    Once washed, cells can be stored in the 96-well plate up to 24 hrs at −20°C, although it is strongly recommended to proceed immediately to DNA lysis.
20. Add 100 µl DNA lysis buffer with 200 µg/ml proteinase K to each well and allow cells to lyse for 20 min at room temperature.
21. Transfer lysates to a 96-well PCR plate using a multichannel pipettor and seal the plate with sealing film.

    Care must be taken to avoid any cross-well DNA contamination when transferring lysates, which are viscous and sticky.
22. Incubate 1 hr at 56°C to lyse cells, and then 20 min at 95°C to inactivate the proteinase K.
23. Measure DNA concentration from three to four wells using a spectrophotometer or Nanodrop and calculate the mean concentration from these wells.
24. Use the multichannel pipettor to dilute the entire 96-well plate at the optimal dilution determined previously (see Basic Protocol 2, steps 26-30). Change tips with each row and be careful to avoid cross-contamination.
25. Perform PCR as described (see Basic Protocol 2, steps 27-28).
26. Check amplification and size of the PCR products by running 23 µl on a 1.5%-2% agarose gel. Use the remaining PCR product for genotyping/screening by Sanger sequencing or target-specific restriction enzyme digestion.

    Electropherograms from Sanger sequencing can be analyzed manually or by ICE analysis. We sequenced 48 of the 96 colonies we picked and found four that showed the desired correction, indicating successful editing.

FREEZING, THAWING, AND EXPANSION OF CELLS

This protocol first describes how to freeze and thaw the backup 96-well plate containing all clones picked. When the cells in the 96-well plate reach 70% confluency (see Basic Protocol 5), they are ready for freezing. Once genotyping is complete, the plate can be thawed and used to expand and validate only those clone(s) carrying the desired editing in the region of interest (Fig. 1D).

NOTE: It is important to freeze and thaw plates quickly to minimize cell loss. All reagents and supplies should be prepared and arranged ahead of time in a biosafety cabinet to make the process fast. All steps should be performed using a multichannel pipettor. Efficient handling of a multichannel pipettor is a must.

Additional materials *(also see Basic Protocol 2)*

Freezing medium (see recipe)
70% (v/v) reagent (denature) alcohol (Ricca Chemical, cat. no. 2546705)

Multichannel pipettor (Gilson)
Sterile disposable reagent reservoirs (Corning, cat. no. 4871)
Parafilm
Styrofoam box or other cell freezing plate container
–80°C freezer
37°C water bath
Centrifuge for 96-well plates
6-, 12-, and 24-well flat-bottom cell culture plates (Corning, cat. nos. 3506, 3513, and 3526)

Freeze 96-well plate for backup

1. Warm 0.5 mM EDTA at 37°C.
2. Aspirate medium from the wells of the first row and wash with 100 µl/well of 1× DPBS without calcium and magnesium.

   Two rows can be processed at a time, if needed.

3. Add 100 µl prewarmed 0.5 mM EDTA per well and incubate for 2 min, checking the cells under a microscope. When colonies are loose, gently remove the EDTA.
4. Add 100 µl freezing medium per well and mix cells by pipetting gently four to six times.
5. Repeat for all rows of the plate.

   To avoid long exposure to the DMSO in the freezing medium, cells should be frozen in batches using two 96-well plates. Simply transfer the cells from half of the plate to a second Matrigel-coated plate and immediately transfer it to the –80°C freezer, then proceed with the remaining wells in a separate batch.

6. Wrap the plate with Parafilm and cover with multiple layers of tissue paper.
7. Place the plate in a Styrofoam box and transfer that to a –80°C freezer.

   Placing the plate in a Styrofoam box applies gradual freezing, which prevents cold shock to the cells. We do not recommend storing 96-well plates in liquid nitrogen, as it can leak into the wells and damage the cells.

Thaw 96-well plate

8. Remove the 96-well plate from the −80°C freezer. Remove the tissue paper and transfer plate (still sealed with Parafilm) to a 37°C water bath. Monitor carefully to ensure that the medium is thawing gradually and that water does not enter the wells.

9. When more than half of the medium has thawed, move the plate to the biosafety cabinet.
10. Clean the surface of the plate with 70% alcohol and then remove the Parafilm.
11. Add 100 µl mTeSR Plus with 10% CloneR to each well.
12. Centrifuge plate 5 min at 300 × *g*.

    Cells should be deposited at the bottom of the wells.
13. Carefully remove 150 µl medium from each well and add 100 µl/well fresh mTeSR Plus with 10% CloneR.
14. Place plate in a 37°C incubator at 5% O_2, 5% CO_2 (hypoxic) or 21% O_2, 5% CO_2 (standard) and leave overnight.
15. The next day, add another 50 µl/well mTeSR Plus with 10% CloneR.
16. For further culture maintenance, change medium every other day.

    In our case, two of the four positive clones did not survive freezing and thawing, and we proceeded with expansion of the remaining two clones.

Expand desired clones

17. After thawing, allow cells to grow for 2 days in hypoxic or standard incubator conditions. Check the wells with positive clones under a microscope every day to confirm viability.
18. When the cells reach ∼70% confluency (2-3 days), detach cells for each positive clone using 0.5 mM EDTA (see Basic Protocol 2, step 2) and split to one well of a Matrigel-coated 24-well plate using 300 µl/well mTeSR Plus medium with 10% CloneR.
19. When cells reach confluency (2-3 days), split cells again to one well of a Matrigel-coated 12-well plate using 500 µl/well medium.
20. When cells reach confluency (2-3 days), split once more to one well of a Matrigel-coated 6-well plate using 2 ml/well medium.

    Alternatively, expansion can be done directly from the 96-well plate to a 12-well plate and then a 6-well plate.
21. Make frozen stocks of positive clones and keep some cells growing for confirmation by genotyping.
22. Reconfirm the editing in these cells by genotyping (Fig. 1E).

    Once the genotyping is confirmed, cells are ready for further expansion and characterization for expression of stemness markers, three lineage differentiation potential, karyotyping, and absence of mycoplasma contamination (Fig. 1F). In our example, from the two clones that were expanded, one clone (CUIMCi003-A-1) was chosen for complete characterization.

BASIC PROTOCOL 7

CHARACTERIZATION OF EDITED CELL LINES

Upon generating and expanding an iPSC cell line with the desired editing, it is necessary to confirm that the cell line shows a normal karyotype, is pluripotent, and expresses stemness markers (Fig. 1F). Pluripotency is assessed by differentiation using a commercial kit and immunostaining for the germ-layer-specific markers OTX2 (ectoderm), Brachyury (mesoderm), and SOX17 (endoderm). The list of antibodies used for immunostaining of stemness and differentiation markers is shown in Table 3. Additional characterization includes sequence analysis, testing for mycoplasma contamination, and off-target analysis. Short tandem repeat (STR) testing can also be used to confirm that the edited line is

Table 3 Antibodies for Immunocytochemistry

	Antibody	Dilution	Supplier (cat. no., RRID)
Stemness markers	Rabbit anti-Oct4 (Alexa Fluor 488)	1:50	Cell Signaling Technology (5177S, AB_10693303)
	Rabbit anti-NANOG	1:400	Cell Signaling Technology (4903P, AB_10559205)
	Mouse anti-SOX2 (Alexa Fluor 488)	1:50	Santa Cruz Biotechnology (sc-365823, AB_10842165)
Differentiation markers	Goat anti-OTX2	1:20	R&D Systems (AF1979, AB 2157172)
	Goat anti-Brachyury	1:20	R&D Systems (AF2085, AB_2200235)
	Goat anti-SOX17 (NL557)	1:10	R&D Systems (NL1924R, AB_2195645)
Secondary antibodies	Goat anti-rabbit IgG (Alexa Fluor 488)	1:1000	Thermo Fisher Scientific (A11008, AB_143165)
	Rabbit anti-goat IgG (Alexa Fluor 488)	1:1000	Thermo Fisher Scientific (A27012, AB_2536077)

derived from the parental mutated iPSC line. This can be carried out by providers such as Cell Line Genetics or WiCell.

In our case, characterization of the edited line CUIMCi003-A-1, which is the isogenic control for the patient-derived CUIMCi003-A iPSC line, is summarized in Figure 3. It demonstrated typical pluripotent stem cell morphology (Fig. 3A, 200 µm). Assessment of stemness marker expression by immunostaining, three-lineage differentiation potential, and mycoplasma testing were performed following protocols in Iannello et al. (2021) (Fig. 3, Fig. S2). In the steps below, we have indicated the passage number at which each test was performed, but there is no ideal passage number for characterization of the edited line.

Additional materials *(also see Basic Protocol 2)*

Human Pluripotent Stem Cell Functional Identification kit (R&D Systems, cat. no. SC027)
Primary and secondary antibodies for stemness and differentiation markers (Table 3)
4% paraformaldehyde (PFA) in PBS (Santa Cruz, cat. no. sc-281692)
0.1% (v/v) Triton X-100 (Thermo Scientific, cat. no. 85111) in DPBS
Protein Block (Agilent DAKO, cat. no. X0909)
7.5% bovine serum albumin (BSA, Gibco, cat. no. 15260037)
DAPI nuclear counterstain
e-Myco Plus Mycoplasma PCR Detection kit (Intron, cat. no. 25234)
DNA lysis buffer (see recipe)

T25 vented cell culture flask (Corning, cat. no. 430639)
Fluorescence microscope (Olympus IX73 inverted microscope connected to a XM10 monochrome camera)

Perform sequence analysis (Fig. 3A)

1. Isolate and purify genomic DNA from edited and unedited iPSC lines and perform Sanger sequencing using the primer set identified in Basic Protocol 1.

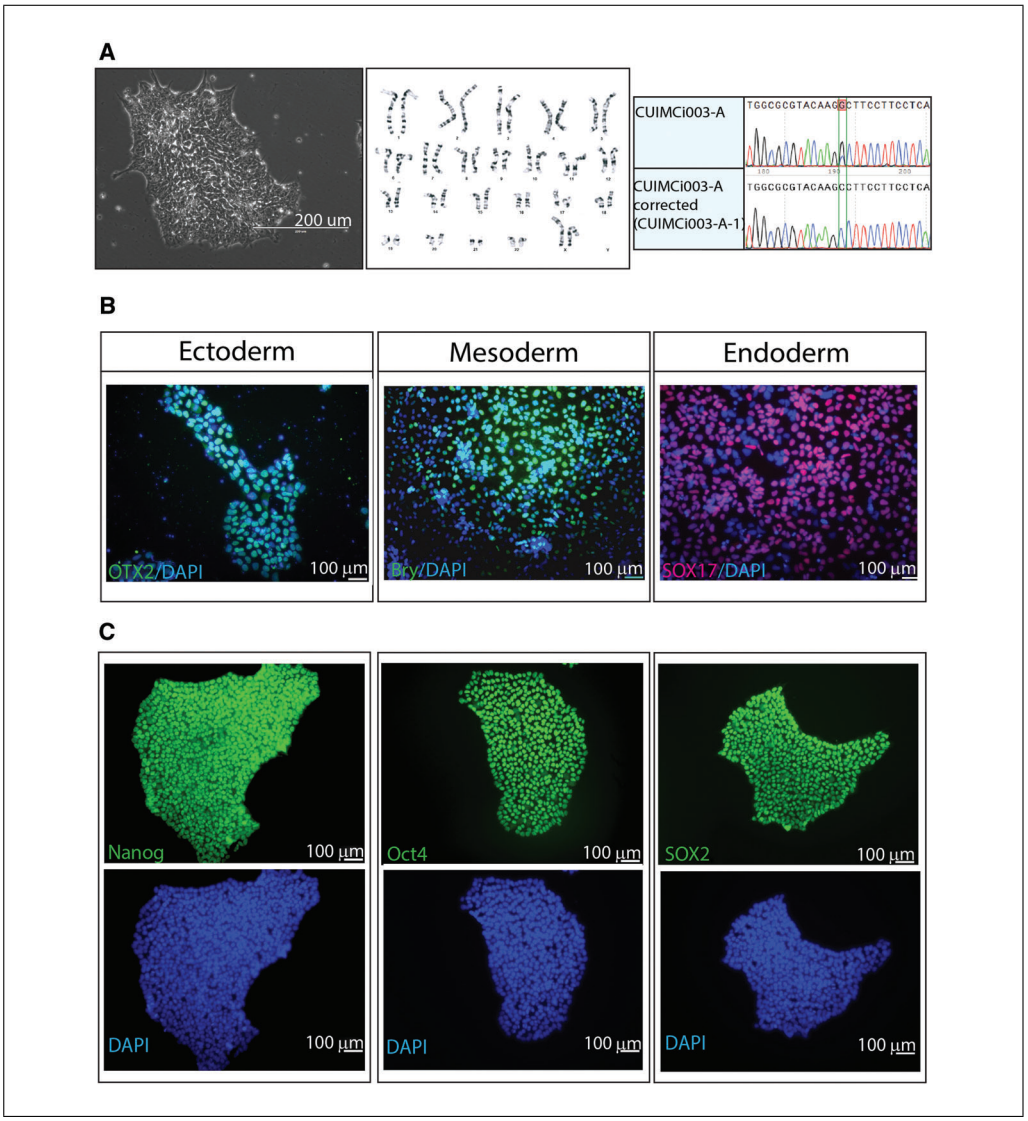

Figure 3 (**A**) CUIMCi003-A-1, the isogenic control for CUIMCi003-A, shows normal morphology and karyotype after editing. Sanger sequencing confirms correction of the c.388C>G mutation in CUIMCi003-A-1. (**B**) The pluripotency of CUIMCi003-A-1 is confirmed by expression of markers from all three germ layers (OTX2 for ectoderm, Brachyury for mesoderm, SOX17 for endoderm) upon differentiation. (**C**) Immunostaining shows expression of stemness markers Oct4, Nanog, and SOX2 in undifferentiated cells.

In our case, genomic DNA was isolated from the iPSC line with variant c.388C>G and the corrected line CUIMCi003-A-1. Sanger sequencing was performed by Genewiz (now Azenta) using the primer ARNT2_F2. Correction of the mutation was confirmed in the edited line.

Perform karyotyping (Fig. 3A)

2. Coat a T25 flask with Matrigel and leave for 1 hr at room temperature.
3. Split cells and seed onto the coated flask (one T25 is equivalent to three wells of a 6-well plate).
4. Let cells grow to 30%-40% confluency.
5. Use a reputable service provider for karyotyping and request standard G-banding on at least 20 metaphases at 425-band resolution.

We use Columbia University Medical Center-Clinical Laboratory Services (New York City) or Cell Line Genetics (Madison, Wisconsin). When shipping live cells to an outside service provider, use a trustworthy delivery service like FedEx. Fill the flask with medium, wrap the lid with Parafilm, wrap the flask with bubble wrap, and ship at room temperature using priority overnight. It may be better to use feeder cells instead of Matrigel to guarantee cells remain attached during transportation. Avoid shipping when severe weather is forecast to avoid cold temperatures and delivery delays.

Cytogenetic analysis by Giemsa-banding, performed on CUIMCi003-A-1 at passage 18, confirmed a normal female karyotype (46, XX).

Assess in vitro differentiation potential (Fig. 3B)

6. Assess germ layer differentiation potential using the Human Pluripotent Stem Cell Functional Identification kit according to manufacturer's instructions.

Line CUIMCi003-A-1 at passage 29 was successfully differentiated in vitro into all three germ layers. The pluripotency of this line was confirmed by expression of ectoderm (OTX2), mesoderm (Brachyury), and endoderm (SOX17) markers from all three germ layers (Table 3).

Assess stemness marker expression by immunostaining (Fig. 3C)

7. Fix the edited line with 4% PFA for 10 min at room temperature.

We performed immunostaining on CUIMCi003-A-1 at passage 29.

8. Wash cells twice with 1× DPBS.
9. Permeabilize cells with 0.1% Triton X-100 for 30 min at room temperature.
10. Block cells in Protein Block for 30 min at room temperature.
11. Stain with primary antibodies (Table 3) diluted in 1× DPBS containing 1% BSA overnight at 4°C.
12. Wash twice with 1× PBS containing 1% BSA.
13. If needed, incubate with secondary antibody (Table 3) in 1× DPBS containing 1% BSA for 1 hr at room temperature.
14. Wash three times with 1× DPBS containing 1% BSA.
15. Stain nuclei with DAPI and capture images using a fluorescence microscope.

Immunostaining showed expression of stemness markers Oct 4, Nanog, and SOX2 in undifferentiated cells.

Test for mycoplasma contamination (Fig. S2)

16. Test iPSCs for mycoplasma using a PCR-based kit according to manufacturer's instructions.

Our mycoplasma test was performed at passage 30 and was negative.

Perform off-target analysis

17. Confirm integrity of off-target sequence(s) by amplification and Sanger sequencing of the top three predicted off-target candidates.

The gRNAs design software tools mentioned above automatically recommend guides with the highest chances of gene knock-out/knock-in and the lowest off-target effects. sgRNA length is also optimized; truncated sgRNAs with <20 base homology display less off-target activity (Fu, Sander, Reyon, Cascio, & Joung, 2014). Because RNPs persist only briefly inside the cells, the chance of off-target editing is reduced (Kim et al., 2014; Liang et al., 2015). The top three to five predicted off-target candidates could also be predicted using the COSMID46 tool (https://crispr.bme.gatech.edu) or the MIT CRISPR design

tool (http://crispr.mit.edu). The integrity of the off-target sequence(s) can be confirmed by amplification and Sanger sequencing of the top three predicted off-target candidates. We selected three sgRNAs for further testing based on the off-target scores in Table 1, obtained using the Synthego CRISPR Design Tool. We did not perform specific off-target analysis in the positive clone.

REAGENTS AND SOLUTIONS

DNA lysis buffer

2.22 g Trizma HCl
1.325 g Trizma base (final 50 mM Tris, pH 8.0)
1.864 g 50 mM KCl
0.46525 g 2.5 mM EDTA
2.25 ml 0.45% (v/v) NP-40 (or equivalent, called "Igepal")
2.25 ml 0.45% (v/v) Tween-20
dH_2O to 500 ml
Filter using a 0.2-µm 500-ml sterile disposable filter unit (Thermo Scientific, cat. no. 5660020)
Store in 50-ml aliquots at 4°C

Buffer can be stored indefinitely if no precipitates or contaminants are observed.

EDTA, 0.5 mM

0.5 M EDTA (Life Technologies, cat. no. 15575-020)
1× DPBS without calcium and magnesium (Corning, cat. no. MT-21-031-CV)
Filter using a 0.2-µm 500-ml sterile disposable filter unit (Thermo Scientific, cat. no. 5660020)
Store at room temperature

EDTA must be diluted in DPBS without calcium and magnesium, as it does not work in the presence of these ions. It can be stored indefinitely if no precipitates or contaminants are observed.

Freezing medium

5 ml mTeSR Plus medium (Stem Cell Technology, cat. no. 100-0276)
4 ml knock-out serum replacement (Life Technologies, cat. no. 10828-028) *or* heat-inactivated fetal bovine serum (FBS, Life Technologies, cat. no. 10082147)
1 ml dimethyl sulfoxide (DMSO, Hybri-Max, Sigma, cat. no. D2650)
10 µl Y-27632 (ROCK inhibitor, Tocris, cat. no. 1254)
Store up to 1 week at 4°C

COMMENTARY

Background Information

The advent of CRISPR/Ca9 technology efficiently improved how scientists approach disease-modeling by using patient-derived iPSCs and correcting/introducing a mutation to generate isogenic controls. To date, this is the closest model to monitor human diseases that allows understanding of if and how a genetic variant can disrupt gene function, leading to recapitulation *in vitro* of the phenotype observed *in vivo*. For this reason, it is important to efficiently generate genome-edited iPSCs to be used for functional studies. Since the technique was first discovered, many protocols have been generated that employ plasmids or RNP complexes to transfect cells. As for all approaches, each has its advantages and disadvantages. The use of plasmids is more time-consuming (optimizing the plasmid concentration), has more cell toxicity (the RNP complex is degraded in shorter time), and results in more off-target effects compared to RNP complexes (Kim et al., 2014; Kissling et al., 2019; Ruan et al., 2019). The latter is a very delicate point, since the goal is to generate an iPSC line that differs from the pre-edited cell line exclusively for the specific targeted region without any other random genomic alteration that could be generated during transfection.

In this context, our goal is to use the most specific method for transfecting cells and creating an optimized protocol to efficiently generate the desired gene-edited iPSC line. The protocol described here focuses on choosing the optimal sgRNA before proceeding with cell electroporation in order to increase the rate of success of the experiment. A gene-editing experiment takes a lot of time and effort, and by testing the sgRNAs before performing the "real" electroporation experiment we reduce troubleshooting of subsequent steps. The efficiency testing of the gRNAs is done by ICE analysis, a user-friendly bioinformatics tool provided by Synthego, where the calculated score gives information about the cutting efficiency of each sgRNA submitted. During this process, the off-target score for each gRNA is also evaluated, allowing one to choose the sgRNA that is less likely to affect untargeted regions. It is important to remember that *in silico* tools have limitations, and these must be considered when analyzing the output from the ICE analysis tool. In our specific case, after testing three sgRNAs using ICE analysis, we selected the one with the highest cutting efficiency score (0.6) and better off-target score (Table 1). With this sgRNA (ARNT2_g RNA2) and the correspondent donor DNA, we were able to efficiently generate the isogenic control line CUIMCi003-A-1 from the patient-derived iPSC line by correcting the c.388C>G variant.

Critical Parameters and Troubleshooting

Successful genome editing relies on many critical steps and reagents as well as timing. Here, we focus on culture conditions, electroporation conditions and reagents, genotyping, and freezing/thawing of cells. Reported here are a few factors to consider throughout the protocol.

A clean, specific PCR amplification depends on many factors. Choosing the primer set that gives the best specificity (fewest nonspecific bands) and the optimal length of PCR amplicons is an extremely critical part of the genotyping and ICE analysis. Make sure to test more than one set of primers for genotyping, test the PCR conditions, and then select the best primer set. For successful genotyping, it is also important to determine the optimal concentration of genomic DNA to amplify. Test multiple dilutions of the DNA of interest in your PCR conditions and choose the best one. Storage of DNA lysates may also influence the quality of amplification, and we suggest storing DNA lysates at –20°C. We have observed poor PCR results when DNA samples were stored at room temperature, possibly because DNA isolated with the lysis buffer used here is not "clean" enough.

Different iPSC lines may respond differently to the electroporation conditions, with some lines surviving better than others. When starting with an iPSC line, different electroporation programs should be tested. We always test programs CB150 and CA137 in the Nucleofector 4D. If the cells do not survive or are not electroporated successfully, consider using a device from a different manufacturer (e.g., Life Technologies Neon). Excess Cas9/sgRNA can trigger nonspecific targeting and sometimes targeting of the donor DNA (Wu et al., 2014). It is important to use the optimal concentration of each electroporation reagent (Cas9/sgRNA and ssODN). Also make sure to use a healthy cell culture that is not overgrown. Split the cells the day before electroporation so they are ∼50% confluent on the day of electroporation. Before starting, check the karyotype of your cell line and make sure it shows no abnormality. If you are correcting a mutation, confirm the presence of the mutation on the iPSC line before starting.

To achieve maximum cell recovery after freezing and thawing a 96-well plate requires a lot of attention and efficient time management. Every step should be planned in advance, and all material should be cold. Be efficient and avoid exposing cells to room temperature for long. A 96-well plate can be processed in two or three batches, and cells may also be transferred to other properly labeled plates or freezing vials.

Troubleshooting guidelines are outlined in Table 4.

Understanding Results

We previously generated the iPSC line CUIMCi003-A, derived from a patient with severe early-onset obesity (Iannello et al., 2021). This line carries a c.388C>G *de novo* variant in exon4 of *ARNT2* (Fig. 1A). Using the present CRISPR/Cas9-based protocol, we generated an isogenic control line, CUIMCi003-A-1, in which we corrected the c.388C>G mutation to develop an iPSC-based disease model.

To correct the c.388C>G mutation at chr15:80,470,411 in exon 4 of *ARNT2*, three sgRNAs were designed at the vicinity of the target correction site (Table 1, arrows in Fig. 2A) using the Synthego CRISPR Design Tool. These sgRNAs were used for the

Table 4 Troubleshooting Gene Editing With the CRISPR/Cas9 System

Problem	Possible cause	Solution
Failed ICE analysis (donor/sgRNAs)	Length of donor/sgRNA sequences	Use the combination of primers with higher specificity that amplifies a longer fragment. For ICE analysis, have the total sequence between 400-800 bp to properly align the guide in the control file and identify where the CRISPR edit was performed.
	Poor quality sequencing traces	Use an internal primer for Sanger sequencing. Use a primer set with better specificity. Check DNA quality and determine the optimal DNA concentration.
Poor PCR/sequencing signal	DNA concentration	Check DNA quality and concentration. Determine optimal DNA concentration for PCR.
	DNA storage	Store DNA and any dilution at –20°C. Storage at 4°C or RT may affect DNA integrity.
	PCR profile to optimize	Change PCR conditions (annealing temperature, extension time) according to primers used.
Low hiPSC viability after electroporation	Cell handling	Use gentle pipetting when handling cells.
	Cell clumping	When using Accutase, collect cells once they start detaching from the surface.
	Electroporation program	Test more than one program and choose according to your experiment.
	Long exposure to electroporation reagents	Do not keep cells in the reaction mix for too long.
Low gene-targeting efficiency	Medium present in cells to be electroporated	Aspirate all medium when collecting cells for electroporation. Medium in the reaction mix may impact electroporation efficiency.
	Cells are not in dividing stage	Better efficiency is observed when cells are passaged the day before electroporation so they are 50%-60% confluent on the day of electroporation.
	Knock-in score is less than 10%	Multiple screening by genotyping. Design a more efficient sgRNA and donor. Tips for design are found in the text.
Cell death after thawing	Long exposure of cells to DMSO in freezing medium	Freeze 96-well plates quickly to minimize cell loss. Keep all reagents/pipettes handy to make process fast. Freeze plates in batches to minimize DMSO exposure.

next steps (Fig. 2A), in which CUIMCi003-A cells were electroporated with the three different sgRNAs plus Cas9 and subjected to target-specific PCR amplification followed by Sanger sequencing. Trace files from the unedited and electroporated iPSCs were uploaded to the ICE analysis tool to determine the editing efficiency of each of the three sgRNAs. Based on the scores determined by ICE analysis, ARNT2_g RNA2 produced the best editing efficiency, generating an 81% indel score (Fig. 2B). Therefore, we chose ARNT2_g RNA2 for the subsequent steps. We then designed a ssODN compatible with ARNT2_g RNA2 and performed electroporation of CUIMCi003-A using ARNT2_g RNA2 with Cas9 and the ssODN. Electroporated cells were subjected to low-density seeding and allowed to grow as colonies. Genotyping was performed in the resulting colonies and ICE analysis was performed to assess the efficiency of HDR in the positive clones. Considering that the c.388C>G mutation is heterozygous, we expected 50% HDR efficiency in the corrected clones and observed a 57% HDR score in the edited clones. We believe this discrepancy may be due to a limitation of the ICE tool. To further confirm our results, we analyzed chromatogram data generated during ICE analysis, and determined that the

Table 5 Time Considerations

Procedure	Steps	Time for individual step	Total time	Stopping point
Basic Protocol 1: sgRNA design	Identification of sgRNA targeting sequence	2-4 hr	~3-4 days[a]	Yes
	Design of PCR primers	2 hr		
	Optimization of PCR primers	5 hr (including DNA extraction, PCR, agarose gel electrophoresis)		
Basic Protocol 2: Testing sgRNA efficiency	hiPSC culture	3 hr	~15 days[b]	No
	Electroporation	1-2 hr		No
	PCR	5 hr (including DNA extraction, PCR, agarose gel electrophoresis)		Yes
	Sequencing	1 day		Yes
	ICE analysis	2-3 hr		Yes
Basic Protocol 3: DNA template/donor design	DNA template or donor design	2-4 hr	2-4 hr	Yes
Basic Protocol 4: Targeted gene editing	Preparation	2 hr	4-5 days[c]	No
	Electroporation	1-2 hr		
	ICE analysis	2-3 hr		
Basic Protocol 5: Selection of positive clones	Single-cell seeding	2 hr	15-20 days	No
	Culture	1-2 weeks		
	Colony picking	3-4 hr (depending on number of plates)		
	Sequencing and ICE analysis	1 day		
	Genotyping and allele sequencing	5-7 days (depending on cell growth)		
Basic Protocol 6: Freezing, thawing, expansion	Freezing	3 hr	At least 3-5 days between freezing and thawing	Yes
	Thawing	3 hr		No
	Expansion	2-3 weeks	1-2 weeks	Yes
Basic Protocol 7: Characterization	Assessment of stemness marker expression	2-3 weeks	15-21 days	Yes
	Karyotyping	14 days		

[a] Including time to receive primers.
[b] Including time to receive sgRNAs/donor, hiPSC cell culture, 48-72 hr incubation for gene editing, and time to receive sequencing results.
[c] Including 48-72 hr cell growth after electroporation and time to receive sequencing results.

chromatograms from edited clones were clean and that the 7% variation is noise from sequencing that the software can pick up (Fig. 2D). Sanger sequencing shows that the mutation was corrected in the positive (edited) iPSC clones (Fig. 1E). iPSCs from two confirmed positive clones were expanded. One of these two clones (CUIMCi003-A-1) was chosen and characterized for pluripotency potential, chromosomal

integrity, and absence of mycoplasma (Fig. 3, Fig. S2).

The edited line CUIMCi003-A-1 showed an iPSC-like morphology and a normal G-banded female karyotype (46, XX) (Fig. 3A). The chromatograph obtained from Sanger sequencing confirmed the correction of the target mutation (Fig. 3A). Next, cells were tested for potential to differentiate into the three germ lineages by immunostaining of differentiated cells for marker expression specific to ectoderm (OTX2), mesoderm (Brachyury), and endoderm (SOX17). The presence of all three markers confirms the potential of the edited line to differentiate into all three germ layers (Fig. 3B). Expression of the stemness markers Oct 4, Nanog, and SOX2 is a hallmark of iPSC characterization. Immunostaining of undifferentiated edited iPSCs showed that all three markers were expressed. In addition, we showed that the edited iPSC line is mycoplasma free (Fig. S2).

Both iPSC lines—the mutant and the isogenic control generated with this protocol—are available upon request.

Time Considerations

The total protocol can give rise to the desired gene editing in 12-16 weeks. For a breakdown of time needed for different steps, see Table 5.

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Author Contributions

Achchhe Patel: Conceptualization, data curation, formal analysis, investigation, methodology, project administration, resources, software, validation, visualization, writing (original draft, review, and editing); **Grazia Iannello**: Conceptualization, data curation, formal analysis, investigation, methodology, project administration, resources, software, validation, visualization, writing (original draft, review, and editing); **Alejandro Garcia Diaz**: Conceptualization, investigation, methodology, resources, validation; **Dario Sirabella**: Methodology, writing (review and editing); **Vidhu Thaker**: Conceptualization, funding acquisition, project administration, resources, supervision, writing (review and editing); **Barbara Corneo**: Conceptualization, data curation, funding acquisition, investigation, methodology, project administration, resources, supervision, writing (review and editing).

Conflict of Interest

The authors declare no conflict of interest.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon request.

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CORRECTIONS

In this publication, the following corrections have been made:

Page 8, step 4, “0.5µl (10µg) Cas9” has been replaced with the correct amount.

Page 12, step 5, “1µl (20µg) Cas9” has been replaced with the correct amount.

The current version online now includes these corrections and may be considered the authoritative version of record.