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CRISPR/Cas9-Mediated Mutagenesis of Human Pluripotent Stem Cells in Defined Xeno-free E8 Medium

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Abstract

The recent advent of engineered nucleases including the CRISPR/Cas9 system has greatly facilitated genome manipulation in human pluripotent stem cells (hPSCs). In addition to facilitating hPSC-based disease studies, the application of genome engineering in hPSCs has also opened up new avenues for cell replacement therapy. To improve consistency and reproducibility of hPSC-based studies, and to meet the safety and regulatory requirements for clinical translation, it is necessary to use a defined, xeno-free cell culture system. This chapter describes protocols for CRISPR/Cas9 genome editing in an inducible Cas9 hPSC-based system, using cells cultured in chemically defined, xeno-free E8 Medium on a recombinant human vitronectin substrate. We detail procedures for the design and transfection of CRISPR guide RNAs, colony selection, and the expansion and validation of clonal mutant lines, all within this fully defined culture condition. These methods may be applied to a wide range of genome-engineering applications in hPSCs, including those that utilize different types of site-specific nucleases such as zinc finger nucleases (ZFNs) and TALENs, and form a closer step towards clinical utility of these cells.

1. Introduction

Human pluripotent stem cells (hPSCs) possess the capacity to both self-renew and give rise to all derivatives of the three embryonic germ layers, prompting rigorous investigations toward directing and utilizing their differentiation capabilities for regenerative medicine. Interrogating gene function in either self-renewing or differentiating hPSCs also provides a valuable platform towards further understanding human development and disease mechanisms. However to fully realize this potential, we require more efficient and robust methods for genetic manipulation in human cells. While classical gene targeting via homologous recombination has proven to be a potent tool to dissect gene function in mouse embryonic stem cells (mESCs) [1], this process has been hampered by inherent differences in growth characteristics between mouse and human PSCs and by a lack of methods that are permissive for high efficiency genetic manipulation in human cells [2–4].

The recent advent of programmable site-specific nucleases including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated (Cas) systems have

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significantly facilitated genome engineering at the molecular level in a diverse range of cultured cells including hPSCs, *reviewed in* [5]. These chimeric nucleases enable a broad range of genetic modifications by inducing double-stranded breaks at precise locations, triggering the endogenous DNA repair machinery to activate either error-prone non-homologous end joining (NHEJ) or homology-directed repair (HDR). Both mechanisms may be exploited for genetic manipulation by either introducing indels (via NHEJ) to create frameshift mutations that nullify gene alleles, or single nucleotide substitutions (via HDR) to recapitulate human disease mutations.

The rapid progress of genome targeting using engineered nucleases has opened up various avenues for hPSC-based disease studies through strategies such as knocking out a gene or recreating a patient mutation. However, for genome edited hPSCs to be useful for downstream cellular transplantation and disease treatment, we require a completely chemically defined xeno-free culture system for implementation in a clinical setting. Conventional methods of hPSC culture and gene targeting rely on their maintenance and expansion on a feeder layer of fibroblast cells in media containing serum or serum replacement that incorporates animal products such as albumin. Feeders, serum (or serum replacement), and albumin have all demonstrated considerable batch variability, often requiring extensive quality control of new preparations, and uncertainty over their complex undefined components impedes the strict requirements entailed for clinical utilization. For genome manipulation, feeder cells also reduce the transfection efficiency of hPSCs by sequestering transfection reagents, require considerable time and effort in preparation, and can also become depleted when drug selection is employed, necessitating further re-supplementation. Conditions for hPSCs are now beginning to transpire from feeder-dependent cells in animal product containing media to culture on defined extracellular matrix substrates in xeno-free fully defined media [6–9]. Notably, Chen et al. [10] recently reported the development of a simplified hPSC culture medium, E8, which comprises eight completely defined components, for adherent culture of hPSCs on the defined matrix protein, vitronectin. At present, there are few studies that describe genome editing procedures on hPSCs cultured in this fully defined culture condition [11].

We have previously described an efficient platform for genome editing in hPSCs called iCRISPR, using a combination of two gene editing tools – the TALEN and CRISPR/Cas9 system [12]. Using this approach we have successfully created many hPSC lines with biallelic (homozygous or compound heterozygous) or heterozygous loss-of-function mutations in important developmental genes, within the context of culture conditions that depend on feeder cells and contain serum replacement in culture media, *detailed in* [13]. Here we describe an optimized protocol for CRISPR/Cas9-mediated mutagenesis of hPSCs, using our iCRISPR platform, in the defined xeno-free E8 medium on vitronectin substrate. We focus on the generation of gene knockout hPSC lines through the creation of indel frameshift mutations, although our techniques and culture methods are also applicable to the generation of cell lines with precise nucleotide alterations and reporter transgene knock-ins [13], and may be adapted for general applications of TALEN- or CRISPR-mediated gene editing, not limited to the iCRISPR system. This new simplified, efficient protocol for genetic modification serves as a useful platform to study disease mechanisms and facilitates transfer of basic research towards clinical application.

2. Materials

2.1 Cell Culture Reagents

1. E8 Medium Kit (Life Technologies, cat. no. A15170-01)
2. Truncated recombinant human vitronectin (VTN-N) (Life Technologies, cat. no. A14700)
3. Dulbecco's Phosphate-Buffered Saline (DPBS) without calcium or magnesium (Life Technologies, cat. no. 14190)
4. Penicillin: Streptomycin Solution (Gemini Bio-products, cat. no. 400-109)
5. EDTA 0.5M, pH 8.0 (KD Medical, cat. no. RGE-3130)
6. Rho-associated protein kinase (ROCK) inhibitor, Y-27632 dihydrochloride (Selleck Chemicals, cat. no. S1049)
7. Dimethyl Sulfoxide (DMSO), cell culture reagent (Santa Cruz Biotechnology, cat. no. sc-358801)

2.2 CRISPR sgRNA Design Reagents

1. Herculase II Fusion DNA Polymerase (Agilent Technologies, cat. no. 600679)
2. MEGAscript T7 Transcription Kit (Ambion, cat. no. AM1354)
3. MEGAclear Transcription Clean-Up Kit (Ambion, cat. no. AM1908)

2.3 sgRNA Transfection Reagents

1. Doxycycline hyclate (Sigma Aldrich, cat. no. D9891)
2. TrypLE Select Enzyme 1X, no phenol red (Gibco, cat. no. 12563)
3. Opti-MEM I Reduced Serum Medium (Gibco, cat. no. 31985)
4. Lipofectamine RNAiMAX Reagent (Invitrogen, cat. no. 13778)

2.4 Genomic PCR and General Molecular Biology Reagents

1. T7 Endonuclease I (New England Biolabs, cat. no. M0302S)
2. DNeasy Blood and Tissue Kit (Qiagen, cat. no. 69506)
3. NEBuffer 2 10X (New England Biolabs, cat. no. B7002S)
4. 1X TAE (40mM Tris acetate, 1mM EDTA)
5. UltraPure Agarose (Invitrogen, cat. no. 16500)
6. Ethidium Bromide, 1% Solution (Fisher Scientific, cat. no. BP1302-10)
7. Orange Gel Loading Dye 6X (New England Biolabs, cat. no. B7022S)
8. 10X Lysis Buffer (0.45% NP-40, 0.45% Tween 20 in 10mM Tris pH8.0), store at 4°C

9. NP-40; IGEPAL CA-630 (Sigma-Aldrich, cat. no. I8896)
10. Tween 20 (Fisher Scientific, cat. no. BP337)
11. Tris Base (Fisher Scientific, cat. no. BP154-1)
12. Proteinase K, recombinant, PCR grade (Roche, cat. no. 3115879001)
13. Adhesive PCR plate seals (ThermoFisher Scientific, cat. no. AB-0558)
14. Zero Blunt TOPO PCR Cloning Kit (Life Technologies, cat. no. 450245)

3. Methods

3.1 Generation of iCas9 hPSCs

3.2 hPSC culture and passaging in E8 conditions

3.2.1 Vitronectin preparation

3.2.2 hPSC maintenance and passaging in E8 with EDTA dissociation

3.3 Design and production of sgRNAs

3.4 SgRNA transfection of hPSCs in E8 to create hPSC mutant lines

3.5 T7 Endonuclease I (T7E1) Assay to determine frequency of indel mutagenesis.

3.6 Replating of transfected cells at clonal density

3.7 Colony picking

3.8 PCR-based screening of mutant colonies and colony passaging

3.9 Colony expansion and validation of mutant lines

3.1 Generation of iCas9 hPSCs

A number of laboratories have now successfully used the CRISPR/Cas9 system for genome editing in hPSCs, *reviewed in* [5]. However, the delivery of Cas9 and single chimeric guide RNA (sgRNA) remains a step that can limit the gene editing efficiency and ease of use. We believe that inducible expression of Cas9 (iCas9) from the *AAVS1* safe harbor locus proffers a robust approach to genetic modification via the CRISPR/Cas9 system. Because this system no longer entails co-transfection of Cas9, the high transfection efficiency of the smaller sgRNA leads to significantly improved rates of genome editing in the target locus. Our laboratory has previously documented generation of iCas9 hPSCs by TALEN-mediated targeting into the *AAVS1* locus [12]. Two donor plasmids were co-electroporated with the *AAVS1*-targeting TALEN constructs. One donor plasmid (Puro-Cas9, Addgene plasmid no. 58409) comprised a doxycycline-inducible Cas9 expression cassette selectable with puromycin, the other (Neo-M2rtTA, Addgene plasmid no. 60843) contained a constitutive reverse tetracycline transactivator selectable with G418 (Geneticin). Once clonal iCas9 lines are established, Cas9 expression is induced by doxycycline treatment (Fig. 1). Because the

iCRISPR platform entails only the transfection of a short sgRNA oligonucleotide to arbitrate gene editing, the system is efficient, cost-effective, convenient and has reduced technical demand, particularly if one desires to model complex human traits caused by multiple gene mutations. One can also envision that an integrated Cas9 line can be easily extended for CRISPR-mediated screens [14], where only the smaller and easier to infect sgRNA library would need to be introduced into the cell. For detailed information on TALEN design and donor vector construction please refer to Gonzalez et al. [12] and Zhu et al. [13]. Alternatively the iCas9 lines can now be procured on three hESC (HUES8, HUES9, MEL1) and one hiPSC (BJ iPSC) backgrounds via the MSKCC Antibody and Bioresource Core Facility (New York, NY, USA).

3.2 hPSC culture and passaging in E8 conditions

Below we detail routine hPSC culturing and passaging techniques in E8 Medium on vitronectin extracellular matrix substrate. Our protocol is based on experience working with hPSCs on the H1 (WiCell Research Institute), HUES8 (Harvard University) and MEL1 (StemCore, Stem Cells Ltd; University of Queensland) background. Other hPSC lines may have inherent differences in characteristics that may require adjustment or optimization of the protocol.

3.2.1 Vitronectin preparation—We routinely culture our hPSCs in E8 Medium on vitronectin-coated culture vessels, employing a truncated recombinant human form of vitronectin (VTN-N) that has been demonstrated to maintain pluripotency and normal growth characteristics in multiple human PSC lines [10]. A final VTN-N coating concentration of 0.5µg/cm² is recommended, although we find cells can survive in a concentration range similar to this and generally use a working concentration of 5µg/mL in different sized culture vessels (Table 1) (*see Note 1*). We routinely store VTN-N in 60µl aliquots at –80°C.

1. Thaw VTN-N at room temperature and coat surface at 5µg/mL. For example, when coating an entire 6-well plate, thaw 60µl of stock VTN-N and dilute into 6mL Dulbecco's Phosphate-Buffered Saline (DPBS) without calcium or magnesium. Gently mix by pipetting the solution up and down.
2. Add 1mL of diluted VTN-N solution to each well of a 6-well plate.
3. Incubate the coated plates at room temperature for at least 1 h (*see Note 2*). To ensure that VTN-N has not evaporated from wells prior to use, we recommend that coated vessels are not left out at room temperature for more than 4 h.

3.2.2 hPSC maintenance and passaging in E8 with EDTA dissociation—

1. To prepare complete E8 Medium, thaw frozen E8 Supplement at 4°C overnight. Transfer the entire contents of the E8 Supplement (~10mL) into the bottle of E8

¹Although optimal working concentration of vitronectin is cell line dependent, we find that hPSCs tend not to be sensitive to vitronectin concentration and we routinely employ a working concentration of 5µg/mL. The stock concentration of VTN-N (Life Technologies) is 500µg/mL, so we dilute the VTN-N stock 1:100 in DPBS for general tissue culture coating.

²VTN-N coated vessels that are in excess can be sealed with plastic wrap and stored at 4°C for up to a week without drying. Before use, pre-warm the vessel to room temperature for at least 30 min.

Basal Medium (500mL). One can also add 5mL Penicillin: Streptomycin solution (1% v/v final concentration) to the E8 Basal Medium.

2. When hPSC colonies have reached ~80% confluence (Fig. 2), aspirate expended E8 media from the vessel.
3. Rinse once with DPBS without calcium and magnesium. Refer to Table 2 for suggested rinse volumes.
4. To dissociate the hPSCs, add 0.5mM EDTA in DPBS (pre-warmed to room temperature) to the vessel (*see* Note 3). Refer to Table 2 for suggested dissociation volumes. Let cells sit in EDTA at room temperature for 4–5 min, at which point cells should start separating and colony edges will begin to detach. EDTA partially dissociates the cells enabling the hPSCs to adhere as small aggregates. We find that adherence as small colonies is more beneficial to cell survival than individualized hPSCs, as previously reported [9].
5. Before colonies have detached, aspirate the EDTA solution from the cells. With gentle pipetting, use complete E8 Medium to wash and disperse the colonies. Perform quickly as media components rapidly neutralize the EDTA and colonies will start to reattach to the vessel.
6. Collect the desired proportion of cells and plate onto a new pre-coated VTN-N vessel in complete E8 Medium with 10 μ M Y-27632 ROCK inhibitor. For recommended plating volume, refer to Table 2. Gently sway the vessel to ensure even distribution of cells, and incubate at 37°C, in a 5% CO₂ incubator overnight.
7. Change complete E8 Medium daily (without Y-27632 ROCK inhibitor). Depending on the growth characteristics of the particular cell line and their downstream purpose, we customarily passage cells at a ratio of 1:6 to 1:12, every 4–5 days (Fig. 2). We have found that cells can be directly adapted from live feeder-dependent hPSC culture to VTN-N adherent E8 culture. However cells frozen in feeder-dependent conditions may need to be thawed first onto feeders before adaptation to feeder-independent culture, to improve survival rate.

3.3 Design and production of sgRNAs

To generate gene knockout lines using our iCRISPR platform, sgRNAs are transfected into the inducible iCas9 hPSC line. A 120-nt synthetic single-stranded DNA containing the T7 promoter sequence followed by the variable 20-nt sgRNA target sequence and the remaining constant sgRNA sequence is used as the PCR template, and *in vitro* transcription is performed to generate sgRNAs for transfection (Fig. 3) [13,15]. We have found this method more efficient and cost-effective in comparison to the transfection of an sgRNA-expressing plasmid. Cas9 endonuclease induces a double-stranded break, which can trigger DNA repair via NHEJ, leading to random indel mutations. This then allows the creation of heterozygous

³To prevent degradation of E8 Medium components, do not warm the medium at 37°C. Warm the medium at room temperature until it is no longer cool to touch prior to usage.

and biallelic (homozygous or compound heterozygous) gene mutations through codon reading frameshifts. To ensure that gene function is disrupted, we recommend designing an sgRNA such that it targets sequences immediately upstream of or within an essential functional domain of the corresponding protein. If there are multiple splice variants of the gene of interest, target a functional region that is at least relevant to the isoform in question. If a gene is not well annotated, it will be more prudent to design an sgRNA close to the initiation start codon. For the design of the 20-nt variable sgRNA seed sequence, we routinely employ the guidelines set out by the CRISPR Design Tool (<http://crispr.mit.edu>) developed by the Zhang Lab at MIT [16]. The software predicts the most efficient sgRNAs based on high targeting specificity and a minimal number of off-target sites (*see* Note 4). We customarily design three sgRNAs per gene, and go on to generate clonal lines from at least two of these CRISPRs that can effectively induce indel mutations at the target locus, as first determined by a T7E1 Assay.

1. For each CRISPR, design a 120-nt oligo that includes the T7 promoter sequence, the variable 20-nt sgRNA recognition sequence (selected from the CRISPR Design Tool) and the constant chimeric sgRNA sequence (Fig. 3). Dilute the oligo to a 100 μ M stock solution, then prepare a 2.5nM working solution. The 120-nt oligo is used as template for PCR amplification using the T7 forward and Tracr reverse primers (Tables 3–5). Resolve 5 μ L of each PCR reaction by electrophoresis on a 1% ethidium bromide-stained agarose gel to check for appropriate amplification of the template.
2. For the *in vitro* transcription of the 120-nt sgRNA PCR product to yield the final sgRNA product, we employ the MEGAshortscript T7 Transcription Kit, using the reagents indicated in Table 6.

The reaction was incubated at 37°C for 4 h to overnight. 1 μ L of TURBO DNase was added to the reaction to remove the DNA template, and the reaction mix was further incubated at 37°C for 15 min. To purify the RNA transcript, we used the MEGAclean Transcription Clean-Up Kit. We typically eluted the RNA in 100 μ L RNase-free water, yielding ~50–100 μ g total RNA. When possible, the RNA concentration was adjusted to 600ng/ μ L or 300ng/ μ L and stored at –80°C until ready for use.

3.4 SgRNA transfection of hPSCs in E8 to create hPSC mutant lines

hPSCs are typically transfected as cultures in 24-well plates. We suggest using at least two different cell densities (usually 1:4 or 1:2 ratio) to achieve an optimal balance between cell survival and indel mutation rate, which may need to be adjusted as both CRISPR toxicity and cell line can affect the rate of survival following transfection. We generally perform each transfection in duplicate: one well is used to collect genomic DNA for the T7E1 Assay,

⁴Recent studies have identified only a low incidence and minimal effects of off-target mutations from whole genome sequence analysis in individual hPSC mutant clones derived from CRISPR/Cas9 gene editing [19,20]. However, off-target mutations could still lead to difficult to interpret results and impede functional studies. To minimize the effect of confounding phenotypes introduced by mutations at off-target sites, we suggest generating independent mutant lines using at least two different sgRNAs targeting different sequences within the same gene. If the CRISPR mutation is specific, we expect to observe a similar phenotype in cell lines generated by these same loci-targeting sgRNAs.

which provides an estimate of the rate of mutagenesis, and the other well is used for cell replating at clonal density to enable gene knockout lines to be established (Fig. 4).

1. (Day -1) One day prior to transfection, when cells are ~50–60% confluent, treat iCas9 cells with 2 μ g/mL doxycycline and 10 μ M Y-27632 ROCK inhibitor. This will activate transcription of Cas9 endonuclease before sgRNA transfection.
2. (Day 0) Depending on the number of transfections to be performed, coat 24-well tissue culture plates with VTN-N (Table 1) at room temperature for at least 1 h.
3. Dissociate doxycycline- and ROCK inhibitor-treated iCas9 cells with 1X TrypLE Select. It is important that cells are dissociated to individualized cells as opposed to EDTA-treated small aggregates for transfection. This ensures access of cells to the small sgRNA oligo and associated transfection reagents. Aspirate pre-coated VTN-N. Resuspend cells in complete E8 Medium (with 2 μ g/mL doxycycline and 10 μ M Y-27632 ROCK inhibitor) such that they achieve a surface area ratio of 1:4 or 1:2 and perform cell plating in duplicates for each different sgRNA and density. For each dilution, plate an additional well to serve as an untransfected negative control for the ensuing T7E1 Assay.
4. For each pair of duplicate transfections, prepare in a sterile 1.5mL microcentrifuge tube:

Mixture 1: 50 μ L Opti-MEM Medium with 3 μ L Lipofectamine RNAiMAX Reagent

Mixture 2: 50 μ L Opti-MEM Medium with 600ng sgRNA (typically 1–2 μ L)

Combine both mixtures (~100 μ L total) and incubate at room temperature for 5 min.
5. Add 50 μ L of each sgRNA-lipid complex dropwise to newly dissociated cells in duplicate. Each well will receive ~300ng of sgRNA.
6. Incubate transfected cells at 37°C, in a 5% CO₂ incubator overnight.
7. (Day 1) Change complete E8 Medium with 2 μ g/mL doxycycline and 10 μ M Y-27632 ROCK inhibitor.
8. (Days 2–4) Change complete E8 Medium. Depending on transfection survival rates and confluency, proceed to cell harvest for genomic DNA collection or cell deposition at clonal density.

3.5 T7 Endonuclease I (T7E1) Assay to determine frequency of indel mutagenesis

Cas9 cleavage triggers endogenous mechanisms of DNA repair that can eventually lead to mutations through NHEJ. These mutations include deletions, insertions, as well as deletions combined with insertions, and may occur in one allele or both. Because genome editing with engineered Cas9 nucleases will not target all intended loci with similar efficiencies, the mutation hit rate at a particular locus should be assessed prior to the selection and sequencing of isolated clones. The genomic DNA from targeted cells is first amplified by PCR. The PCR products are denatured and hybridized to allow heteroduplex formation

between wildtype and CRISPR/Cas9-mutated DNA. We use the enzyme T7E1 to recognize and cleave mismatched DNA. The resulting cleaved PCR products are visualized by gel electrophoresis and band intensity quantified to provide an evaluation of mutation rate (Fig 5). Because T7E1 does not detect single base indels effectively, the T7E1 mutation rate is generally an underestimate of the CRISPR mutation rate at a specific locus [17] (*see Note 5*).

1. Three to four days after sgRNA transfection, cells should be 50–80% confluent depending on cell density and CRISPR toxicity. From one well of each duplicate, harvest cells with TrypLE Select and proceed to genomic DNA isolation using the DNeasy Blood and Tissue Kit. Also extract genomic DNA from the untransfected well, which will serve as a control in the T7E1 Assay.
2. Perform PCR using primer pairs designed to amplify a genomic region of about 500 bp to 1,000 bp flanking the CRISPR target site. We recommend using high-fidelity Herculanase II Fusion DNA Polymerase, with suggested reaction mixtures and cycling conditions detailed in Tables 7 and 8.
3. To perform the T7E1 Assay, PCR-amplified DNA is first denatured and hybridized (Tables 9 and 10), then digested with T7E1 enzyme (Table 11). DNA denaturation and hybridization is performed in a thermocycler.

Incubate reaction at 37°C for 30 min, then proceed to T7E1 mutagenesis quantification. The remaining 10µL of denatured and hybridized PCR product can be used as an undigested control during quantification.

4. To quantify the T7E1 Assay, resolve T7E1-digested hybridized samples alongside T7E1-undigested samples and the untransfected PCR-amplified sample by gel electrophoresis on a 2.5% ethidium bromide-stained 1X TAE agarose gel (Fig. 5). We recommend using Orange G gel loading dye as opposed to Bromophenol blue to prevent obstruction of the resolved bands by the gel tracking dye. Illuminate the resolved gel under UV fluorescence and image with a Gel Doc XR+ System (Bio-Rad), or equivalent gel imager. Image Lab Software (Bio-Rad) or ImageJ Software (National Institutes of Health, Bethesda, MD) is used to determine the relative band intensities of cut and uncut DNA to calculate the frequency of indel mutagenesis, using the formula: Indel Percentage = $(1 - ((1 - (b + c)) / (a + b + c))) \times 100$, where *a* is the intensity of the undigested PCR product, and *b* and *c* are the intensities of the T7E1-cleaved product (Fig. 5). The expected size of each cleavage product can be estimated by the position of the forward and reverse primers and the intended position of Cas9 cleavage. Cas9 (from *S. pyogenes*) is known to make a blunt cut between the 17th and 18th bases

⁵Although the percentage cut determined by the T7E1 Assay may be an underestimate of the real sgRNA mutation rate, we find excellent correlation between the T7E1 Assay and the number of mutant lines identified by sequencing. This emphasizes the importance of performing the T7E1 Assay in parallel with clonal deposition of transfected cells and prior to mechanical picking of isolated colonies. Alternatively, the indel mutation rate can be determined through a Restriction Fragment Length Polymorphism (RFLP) Assay if a CRISPR is designed such that the Cas9 cleavage site (3 bp upstream of the PAM recognition motif) is in close proximity to a restriction enzyme cutting site (~5 bp). Since the assay directly measures the loss of a restriction site, it may be more sensitive at detecting the indel mutagenesis rate compared to the T7E1 Assay.

in the 20-nt crRNA target sequence, three bases 5' of the NGG PAM recognition motif [18].

3.6 Replating of transfected cells at clonal density

After determining the rate of indel mutagenesis by the T7E1 Assay, identify the best transfection condition for each CRISPR. Typically, three to four days after sgRNA transfection, cells are harvested from the corresponding duplicate well for plating at clonal density to allow isolation of individually mutated clones. To accomplish this, cells are harvested by dissociation into single cells with TrypLE Select and plated at 2,000 cells per 100mm tissue culture dish for colony growth.

1. For each CRISPR, coat two 100mm tissue culture dishes with VTN-N (Table 1) at room temperature for at least 1 h.
2. Three to four days after sgRNA transfection, cells should be 50–80% confluent depending on cell density and CRISPR toxicity. From the remaining well (which was not harvested for the T7E1 Assay) of each duplicate, rinse once with 500µL DPBS without calcium and magnesium.
3. To dissociate the hPSCs, add 250µL TrypLE Select to each well. Incubate cells at 37°C for 4–5 min, at which point cells should start to detach. Gently pipette the colony up and down and collect in a 15mL Falcon tube.
4. Rinse each well with 2mL DPBS and collect with cells in the 15mL Falcon tube.
5. Centrifuge at $200 \times g$ for 4 min at room temperature and aspirate the supernatant.
6. Resuspend in an appropriate volume of complete E8 Medium to perform a cell count. We recommend resuspending cells in 5mL E8 Medium if using the Beckman Coulter Vi-CELL XR or an equivalent automated cell viability analyzer. If the cell count is to be performed manually via a hemocytometer, resuspend cells in a smaller volume of E8 Medium. Determine the volume required for 2,000 cells.
7. Aspirate VTN-N coating from 100mm dishes, and add 10mL E8 Medium with 10µM Y-27632 ROCK inhibitor.
8. Add 2,000 cells per dish for each different CRISPR. Perform in duplicate. Gently swirl the dish and incubate cells at 37°C, in a 5% CO₂ incubator overnight.
9. To allow cells to settle, do not change media the following day. Two days following single cell deposition, change with E8 Media without ROCK inhibitor. Thereafter, change media daily until colonies are ready for picking.

3.7 Colony picking

We usually allow single cell colonies to grow for 10–12 days, at which point they should reach ~2mm in diameter, but should not have merged with neighboring colonies (Fig. 6). Each clone is picked by mechanical disaggregation using a handheld pipette with filter tip and deposited into single wells of a 96-well plate. When cells have reached confluence, they are passaged onto a new 96-well plate and the remaining cells spun down for genomic DNA

extraction and sequence analysis. Alternatively, colonies could be picked and deposited into duplicate 96-well plates, with one plate for maintenance and expansion, and the other for DNA preparation. The number of colonies picked per sgRNA transfected will depend on the rate of indel mutagenesis. We routinely pick 48 clones for indel efficiencies >20%, and about 96 clones for indel efficiencies between 5–20%. It takes ~60–90 minutes to pick 96 clones (*see* Note 6).

1. For the method involving direct picking and deposition of a colony into a single well, coat the relevant number of 96-well tissue culture plates with VTN-N (Table 1) at room temperature for at least 1 h.
2. Aspirate VTN-N coating from 96-well plates, and add 100 μ L/well E8 Medium with 10 μ M Y-27632 ROCK inhibitor.
3. Change each 100mm dish with fresh E8 Medium before colony picking.
4. Using a manual p200 pipette set at 25 μ L with a filter tip, mechanically disaggregate a well separated colony into a crude grid structure. Dislodge colony pieces and transfer small digested clumps into one well of a new 96-well plate. To avoid cross contamination, ensure all dislodged pieces are removed before dissection of the next clone with a new filter tip. The smaller the colony pieces disaggregated, the better the survival rate (*see* Note 7).
5. Incubate 96-well colony plates at 37°C, in a 5% CO₂ incubator overnight.
6. The following day, change 96-well plates with E8 Media without ROCK inhibitor (100 μ L/well) (*see* Note 8). Thereafter, change media daily until colonies are ready for DNA extraction.

3.8 PCR-based screening of mutant colonies and colony passaging

When cells have reached confluence, colony plates are prepared for DNA extraction and concurrently passaged onto new 96-well plates. We routinely screen for mutant clones through a simplified process of crude genomic DNA extraction without phenol/chloroform purification and precipitation, followed by direct PCR amplification of the region of interest and Sanger sequencing of the unpurified PCR product. This method permits a large number of clones to be screened rapidly with the aid of multichannel pipettes, and provides an accurate and efficient method to assay for mutant clonal lines. PCR amplification is performed using the same forward and reverse primers and PCR cycling parameters as for the T7E1 Assay. Sanger sequencing is performed using a forward primer internal to the one utilized in the T7E1 Assay to increase amplification specificity.

⁶It is feasible for a trained individual to mechanically pick 384 colonies (4 $\times$ 96-well plates) in one sitting, which should take ~4–5 h under a dissecting microscope.

⁷Ensure that only well-separated colonies are picked for cell line establishment. While the majority of mutant lines that we have generated are homogeneous with regard to the mutated allele/s, it is possible that a given mutant line may have arisen from more than one cell, and this is the stage at which the cross-contamination is most likely to occur. After a clonal line is established, we highly recommend confirming the presence of the mutated allele/s through TOPO TA cloning. Where contamination is suspected, cell lines should be sub-cloned.

⁸We do not recommend treating cells with ROCK inhibitor for an extended period of time as the effects of prolonged usage has not been thoroughly tested, and we have found instances where it affects general cell morphology when treatment is applied continually. Where possible, replenish media without ROCK inhibitor the next day following cell passage.

1. Coat an equivalent number of 96-well tissue culture plates for passaging of colonies with VTN-N (Table 1) at room temperature for at least 1 h.
2. Aspirate VTN-N coating from 96-well plates, and add 100 μ L/well E8 Medium with 10 μ M Y-27632 ROCK inhibitor.
3. On 96-well plates with colonies, rinse each well once with 100 μ L DPBS without calcium and magnesium.
4. To dissociate the colonies, add 50 μ L 0.5mM EDTA in DPBS (pre-warmed to room temperature) to each well. Let cells sit in EDTA at room temperature for 4–5 min, at which point cells should start separating and colony edges will begin to detach. It is important at this step to use EDTA instead of TrypLE Select as the passaging of colonies is sensitive to single cell disaggregation, and colonies survive better when passaged in small aggregates.
5. Before colonies have detached, aspirate the EDTA solution from the cells.
6. Add 100 μ L/well E8 Medium with 10 μ M Y-27632 ROCK inhibitor and gently pipette up and down to disperse the colonies. Passage cells onto new VTN-N coated plates. For the first passage in which genomic DNA is concurrently extracted, we customarily perform a split ratio of 1:4. For subsequent passages we routinely passage colonies 1:6.
7. Gently sway the newly passaged 96-well plate to ensure even distribution of cells, and incubate at 37°C, in a 5% CO₂ incubator overnight. The following day, change 96-well plates with E8 Media without ROCK inhibitor (100 μ L/well) (*see* Note 8). Thereafter, change media daily until colonies are ready for passaging.
8. To the plate with the remaining cells, centrifuge at 1,000 $\times$ *g* for 4 min at room temperature and aspirate the supernatant in preparation for cell lysis.
9. Add 35 μ L 1X Lysis Buffer/well. 1X Lysis Buffer is prepared from a 10X Lysis Buffer stock solution in 10mM Tris pH8.0 with 100 μ g/mL Proteinase K.
10. Seal each plate with adhesive qPCR film or equivalent to prevent evaporation of Lysis Buffer and incubate plates at 55°C for 2–4 h.
11. To inactivate the Proteinase K, incubate plates at 85°C for 20 min.
12. Store genomic DNA plates at 4°C until ready for PCR amplification.
13. Perform 25 μ L PCR reactions with the same primer set as per the T7E1 Assay, using 1 μ L of cell lysate for each reaction.
14. Resolve a sampling of reactions by electrophoresis on a 1% agarose gel to ensure that PCR amplification is specific to proceed with sequencing.
15. We customarily submit samples for Sanger sequencing using 1 μ L of unpurified PCR product, with an internal forward primer designed to minimize problems caused by sequencing of non-specific PCR bands.

3.9 Colony expansion and validation of mutant lines

Following sequence analyses of colonies, we select for clones with biallelic frameshift indel mutations (either homozygous or compound heterozygous) for knockout studies. Depending on the genetic phenotype to be determined, we also identify colonies with heterozygous mutations, with several wildtype clones generated from the same transfection experiment selected for downstream application as control lines. Mutant and wildtype clones are expanded and cryopreserved until ready for experimental analysis.

96-well colony plates are routinely passaged until completion of sequence analysis. For cell line expansion, desired clones are dissociated with 0.5mM EDTA and passaged onto VTN-N coated 24-well plates. When cells have reached confluence, they are passaged onto VTN-N coated 6-well plates for cryopreservation. E8 Freezing Medium is prepared using 90% complete E8 Medium and 10% DMSO. We typically freeze three vials per one well of a 6-well plate.

We recommend resequencing of desired clones following colony expansion to ensure that no cross-contamination of clonal lines has occurred. In the case of heterozygous and compound heterozygous mutant lines, uneven overlapping peaks on chromatogram files may make it difficult to interpret sequence traces with complete accuracy. To determine the sequence of each allele in these clones, we recommend performing PCR amplification, followed by TOPO TA cloning and sequencing of individual alleles. We employ the same primer set used for the T7E1 Assay for PCR amplification and clone the PCR product using the Zero Blunt TOPO PCR Cloning Kit, as per the manufacturer's instructions. We typically select 8–10 individual transformed bacterial colonies for determination of monoallelic sequence reads. Where an appropriate antibody and assay exists for the protein in question, mutant lines may also be validated by Western Blot or Flow Cytometric analysis.

4. Conclusions

The entire process to establish a clonal mutant line from CRISPR design to mutant sequencing and colony expansion takes a trained individual ~2 months, although this will inevitably vary accordingly with how many gene loci are to be targeted. We have found higher CRISPR targeting efficiencies when sgRNA transfection and clonal deposition are performed in feeder-free VTN-N/E8 conditions compared to irradiated MEF (iMEF) feeder-dependent culture conditions. We believe that this is due to increased cell survival following transfection and single cell seeding, and the absence of iMEFs that may sequester transfection reagents. Although we discuss here only the generation of loss-of-function mutations through indel formation in our feeder-free conditions, these methods can easily be translated to other forms of genome targeting such as the generation of precise nucleotide alterations with a homology directed repair template, or methods that do not utilize the iCRISPR system.

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Fig. 1.

Generation of iCas9 hPSCs through TALEN-mediated gene targeting at the *AAVS1* locus. To establish the iCRISPR system, Puro-Cas9 and Neo-M2rtTA donor vectors were co-electroporated into hPSCs, and clonal lines established through drug selection. Expression of Cas9 is induced following treatment of cells with doxycycline. SA, splice acceptor; 2A, self-cleaving 2A peptide; Neo^R, neomycin resistance gene; CAG, constitutive synthetic promoter; M2rtTA, reverse tetracycline-controlled transactivator sequence; Puro^R, puromycin resistance gene; TRE, tetracycline response element.

Fig. 2.

Expected hPSC morphology following passage in E8 Medium on vitronectincoated culture vessels using EDTA dissociation. **(a)** hPSCs in culture 24 h after a 1:6 ratio passage. **(b)** hPSCs after 48 h begin to form tightly packed colonies. **(c)** hPSCs that are ready for passage are routinely around 80% confluency.

Single-stranded DNA sgRNA template (120-nt)

T7 promoter seq: 5' TAATACGACTCACTATAGGG 3'

sgRNA target seq: selected from CRISPR Design Tool

sgRNA backbone seq: 5' GTTTTAGAGCTAGAAATAGCAAGTTAAATAAGGCTAGTCCGTTATCAACTT
GAAAAAGTGGCACCGAGTCGGTGCTTTT 3'

Fig. 3.

Sequence structure for the 120-nt single-stranded sgRNA template used for sgRNA production. The template includes a 20-nt T7 promoter sequence, the 20-nt variable sgRNA guide sequence that specifies target recognition, and the 80-nt invariant sgRNA backbone sequence. The oligo is PCR-amplified, RNA is transcribed *in vitro* and purified in preparation for transfection.

Fig. 4.

Procedure for generating gene knockout mutant lines in iCas9 hPSCs in a feeder-free system. sgRNAs targeting specific loci are transfected into iCas9 hPSCs in duplicate and Cas9 expression is induced with doxycycline (DOX). Cells in one well are used to measure the rate of indel mutagenesis by the T7E1 Assay. Cells in the other well are used for replating at clonal density. Once colonies have grown, they are picked for sequence determination and expansion to establish clonal mutant lines.

Fig. 5.

T7E1 Assay to measure the rate of indel mutagenesis. CRISPR-transfected cells are lysed and genomic DNA extracted and PCR-amplified around the target locus. PCR products are denatured and hybridized to enable heteroduplex formation between wildtype DNA and mutated DNA. T7E1 recognizes indels generated by non-homologous end joining (NHEJ) induced by a CRISPR/Cas9-mediated double-stranded break, and cuts the reannealed mismatched DNA. Cleaved products can be visualized by gel electrophoresis and band intensity quantified for determination of percentage indel mutagenesis.

Fig. 6. Expected morphology of colonies cultured for 10–12 days following clonal density deposition in E8 Medium on vitronectin-coated culture vessels. The colonies may vary slightly in size, but should not have merged with neighboring colonies.

Table 1

Suggested VTN-N coating volumes for different sized culture vessels

Culture vessel	Volume of diluted VTN-N (5µg/mL) solution
100mm dish	5mL
6-well plate	1mL per well
12-well plate	500µL per well
24-well plate	250µL per well
96-well plate	50µL per well

Table 2

Suggested reagent volumes for culturing in E8 conditions in different sized culture vessels

Culture vessel	DPBS for rinse	0.5mM EDTA in DPBS	Complete E8 Medium
100mm dish	10mL	5mL	10mL
6-well plate	2mL per well	1mL per well	2mL per well
12-well plate	1mL per well	500µL per well	1mL per well
24-well plate	500µL per well	250µL per well	500µL per well
96-well plate	100µL per well	50µL per well	100µL per well

Table 3

Primers for PCR amplification of the 120-nt sgRNA oligo

Primer	Sequence (5' to 3')
T7 forward	TAATACGACTCACTATAGGG
Tracr reverse	AAAAGCACCGACTCGGTGCC

Table 4PCR reaction mix to amplify the 120-nt sgRNA oligo (50 μ L)

Component	Quantity (in μ L)
Distilled water (dH ₂ O)	35.5
5X Herculase II reaction buffer	10
Herculase II fusion DNA polymerase	0.5
dNTP mix (25mM of each dNTP)	0.5
T7 forward (10 μ M)	1.25
Tracr reverse (10 μ M)	1.25
120-nt sgRNA oligo template (2.5nM)	1

Table 5

PCR cycling parameters to amplify the 120-nt sgRNA oligo

Cycle number	Denature	Anneal	Extend
1	95°C, 2 min		
2–31	95°C, 20 s	60°C, 20 s	72°C, 1 min
32			72°C, 3 min

Table 6*In vitro* transcription reaction mix (20μL)

Component	Quantity (in μL)
T7 10X reaction buffer	2
T7 ATP solution	2
T7 CTP solution	2
T7 GTP solution	2
T7 UTP solution	2
T7 enzyme mix	2
PCR-amplified sgRNA DNA template	8

Table 7PCR reaction mix to amplify genomic region for T7E1 Assay (25 μ L)

Component	Quantity (in μ L)
Distilled water (dH ₂ O)	to 25
5X Herculase II reaction buffer	5
Herculase II fusion DNA polymerase	0.25
dNTP mix (25mM of each dNTP)	0.25
Forward primer (10 μ M)	0.625
Reverse primer (10 μ M)	0.625
sgRNA-transfected genomic DNA	100–200ng

Table 8

PCR cycling parameters to amplify genomic region for T7E1 Assay

Cycle number	Denature	Anneal	Extend
1	95°C, 2 min		
2–36	95°C, 20 s	55–65 °C, 20 s	72°C, 1 min
37			72°C, 3 min

* If possible, perform a gradient PCR for annealing temperature, to find the optimal temperature for each new primer pair that will enable efficient and specific amplification of targets. We routinely find an ideal range between 55°C and 65°C.

Table 9Reaction mix for DNA denaturation and hybridization (20 μ L)

Component	Quantity (μ L)
Unpurified PCR Product	8
NEBuffer 2 10X	2
Distilled water (dH ₂ O)	10

Table 10

Thermocycling parameters for gradual denaturation and hybridization

Temperature	Duration	Thermocycler conditions
95°C	10 min	
85°C	1 min	Ramp to 85°C at 2°C/s
75°C	1 min	Ramp to 75°C at 0.3°C/s
65°C	1 min	Ramp to 65°C at 0.3°C/s
55°C	1 min	Ramp to 55°C at 0.3°C/s
45°C	1 min	Ramp to 45°C at 0.3°C/s
35°C	1 min	Ramp to 35°C at 0.3°C/s
25°C	1 min	Ramp to 25°C at 0.3°C/s
4°C	Hold	

Table 11

Reaction mix for T7E1 digestion

Component	Quantity (μL)
Denatured and hybridized PCR product	10
T7E1 (10U/μL)	0.1 (1U)