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Crispr-mediated Gene Targeting of Human Induced Pluripotent Stem Cells

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Abstract

CRISPR / Cas9 nuclease systems can create double-stranded DNA breaks at specific sequences to efficiently and precisely disrupt, excise, mutate, insert, or replace genes. However, human embryonic stem or induced pluripotent stem cells (iPSCs) are more difficult to transfect and less resilient to DNA damage than immortalized tumor cell lines. Here, we describe an optimized protocol for genome engineering of human iPSCs using a simple transient transfection of plasmids and/or single-stranded oligonucleotides. With this protocol, we achieve transfection efficiencies greater than 60%, with gene disruption efficiencies from 1-25% and gene insertion / replacement efficiencies from 0.5-10% without any further selection or enrichment steps. We also describe how to design and assess optimal sgRNA target sites and donor targeting vectors; cloning individual iPSC by single cell FACS sorting, and genotyping successfully edited cells.

Keywords

gene targeting; CRISPR / Cas9 nuclease; human induced pluripotent stem cells; transfection; genome engineering

Introduction

The development of CRISPR/Cas9 nucleases has enormously expanded our ability to engineer genetic changes in human cells (Mali et al., 2013b). These nucleases can be targeted to specific sequences in the genome to create double-stranded DNA (dsDNA) breaks. When these are repaired using the non-homologous end joining (NHEJ) pathway, small insertion and deletion mutations (indels) are produced and disrupt genes. Two dsDNA breaks can be generated to excise the intervening gene segment. Alternatively, the dsDNA break can be repaired by the homologous recombination pathway – here, specific base pair changes or transgene insertions can be made using a homologous donor targeting vector.

Human induced pluripotent stem cells (hiPSC) can be gene-targeted, cloned, genotyped, and expanded, due to their self-renewing capability. Edited iPSC clones can then be differentiated into a variety of other cell types to analyze the phenotypic effect of the

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induced mutations. The ability to readily genetically modify human iPSC also holds tremendous clinical promise for generating artificial organs and safer gene therapies. However, while immortalized human tumor cell lines have been edited with almost complete efficiency (Fu et al., 2014), lower success rates have been achieved in human iPSC (Mali et al., 2013b; Yang et al., 2013; Byrne et al., 2015).

Here, we describe various strategies for optimal genome editing in human iPSC using a simple transient transfection of plasmid DNA. We describe parameters for selecting sgRNA targeting sites and list detailed protocols for: the transient transfection of DNA into iPSC (Basic Protocol 1); assessing sgRNA activity using next-generation sequencing (Basic Protocol 2); cloning iPSC using single-cell FACS sorting (Basic Protocol 3); and a high-throughput method for purifying genomic DNA for PCR genotyping (Basic Protocol 4). Using the design considerations and protocols listed below, we typically achieve transfection efficiencies greater than 60% along with gene disruption frequencies of 1-25% and homologous gene targeting frequencies of 0.5-10% in human iPSC without any subsequent selection or enrichment steps.

Strategic Planning

For any gene targeting project, the placement of nuclease targeting sites should be carefully chosen according to the experimental goals and structure of the genomic locus. For simple gene disruption, a single cut site can generate indel mutations using the NHEJ repair pathway. When placed within coding exons, such indels can cause frameshifts and disrupt protein expression. Targeting protein reactive domains or coding exons towards the beginning of the gene may be preferable, as mutations here may be less likely to accidentally generate truncated protein artefacts with residual biological activity. Areas possessing relatively unique genome sequences should be chosen, rather than a common domain shared by several homologous members of the same gene family (unless the goal is to target multiple members of the gene family).

Alternatively, one can design two nuclease sites to excise the intervening section of the genome (Cong et al., 2013; Canver et al., 2014; Byrne et al., 2015). These junctions are often re-ligated with perfect precision between the two blunt-ended dsDNA break sites. This strategy allows nuclease sites within introns or outside the gene to be used, which is particularly useful when no satisfactory nuclease sites can be found within a coding region. When the nuclease sites are < 100 bp apart, excisions were the most common outcome (Cong et al., 2013); for a 2.7 Kbp deletion, the ratio of excisions to inversions was roughly 3:1 (Byrne et al., 2015); for sites > 1 Mbp apart, excisions and inversions occurred with similar frequency (Canver et al., 2014). The frequency of excision mutations can often surpass the frequency of indel mutations in hiPSC, especially when the nuclease sites are < 3 Kbp apart (Byrne et al., 2015). Again, the organization of the gene must be carefully considered to avoid alternative exon splicing events or truncated products.

When specific mutations are desired, a donor targeting vector for homologous recombination can be transfected along with the nuclease elements. These donors can be single-stranded DNA oligonucleotides (ssODN) or plasmids for engineering point

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mutations. Here, the nuclease site should be chosen as close to the intended mutation as possible, since targeting efficiencies drop precipitously as the dsDNA break becomes farther from the mutation. For ssODN donors, having the desired mutation in the center of the oligo produced the highest targeting efficiency (Yang et al., 2013). A minimum of 40 bp of ssODN homology on each side of the mutation is necessary for effective gene targeting (Yang et al., 2013; Chen et al., 2011). One report showed that 45 bp of homology worked best, achieving a targeting efficiency of 1.8% in iPSC (Yang et al., 2013). However, longer ssODNs, with homology arm lengths from 60 – 100 bp, are also commonly used (Ran et al., 2013b; Lin et al., 2014a). The highest targeting frequencies occurred when the mutation was within 5-10 bp of the nuclease site; when the mutation was more than 40 bp away, gene targeting was barely detectable (Yang et al., 2013; Chen et al., 2011).

Alternatively, plasmid targeting vectors for homologous recombination can also be used to generate desired point mutations, as well as larger “knock-in” transgene insertions or gene replacements. The plasmid targeting vector can be constructed with homology arms of 1-2 Kbp flanking the desired insertion, although increased homology may still improve targeting of difficult constructs (Hockemeyer et al., 2009; Orlando et al., 2010; Beumer et al., 2013; Hendel et al., 2014; Byrne et al., 2015). The dsDNA break should ideally be positioned within ~200 bp of the mutation, as the presence of homologous sequence between the dsDNA break and the desired mutation can decrease gene targeting efficiency. Finally, gene targeting efficiency declines with larger transgene insertions, although insertions up to 25 Kbp and gene replacements up to 10 Kbp have been reported (Moehle et al., 2007; Urnov et al., 2010; Duportet et al., 2014; Byrne et al., 2015).

Choice of nuclease targeting sites

The *S. pyogenes* Cas9 nuclease targets a 20 bp dsDNA sequence specified by the single guide RNA (sgRNA) next to a 3′ protospacer adjacent motif (PAM) of NGG, although PAM sequences of NAG can be targeted as well (Jinek et al., 2012; Mali et al., 2013b; 2013a). Upon binding to the sgRNA and complementary DNA targeting site, the Cas9 nuclease generates a blunt-ended double-stranded DNA break three base pairs upstream of the PAM. Cas9-sgRNA complexes can potentially tolerate 1-6 bp mismatches between the sgRNA and the target sequence, creating off-target cuts in genomic DNA. Although a “seed” sequence of the 8-13 nucleotides closest to the PAM appears to be more important for Cas9 nuclease specificity, mismatches can sometimes be tolerated here as well (Jinek et al., 2012; Mali et al., 2013a). Off-target Cas9 nuclease activity can also potentially occur when small insertions or deletions are present between the sgRNA and the genomic DNA (sgRNA or DNA “bulges”) (Lin et al., 2014b; Byrne et al., 2015). Several online tools and algorithms are available to identify specific nuclease targeting sites, including: the CRISPR Design Tool (crispr.mit.edu) (Hsu et al., 2013); ZiFiT targeter (zifit.partners.org/ZiFiT) (Fu et al., 2014); CasFinder (arep.med.harvard.edu/CasFinder/) (Aach et al., 2014); and E-Crisp (www.e-crisp.org/E-CRISP/) (Heigwer et al., 2014). In addition, specific Cas9 sgRNA targets for disrupting human exons can be found from published sets of sgRNA screening libraries (Shalem et al., 2014; Wang et al., 2014; Aach et al., 2014). These algorithms are constantly being refined to incorporate further discoveries about Cas9 targeting specificity.

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The nuclease activity among different sgRNAs can vary widely. Cas9 nuclease activity is positively correlated with areas of open chromatin (Yang et al., 2013; Kuscu et al., 2014); however, substantial variations in activity can still be found among neighboring sgRNAs in the same locus. More active sgRNAs for *S. pyogenes* were associated with a G at the 20th base pair position (adjacent to the PAM), while less active sgRNAs were associated with a C (Doench et al., 2014). Other characteristics associated with higher levels of *S. pyogenes* sgRNA activity are: targeting sequences with between 20-80% GC content, sgRNAs targeting the non-transcribed strand, and purines in the last four bases of the spacer sequence (Wang et al., 2014). While these criteria were statistically significant, they still did not account for all of the observed variation in sgRNA activity.

In addition to the originally described Cas9 nuclease from *S. pyogenes*, other Cas9 nucleases from other bacterial species have also been identified and characterized. As these different Cas9 nucleases bind to different sgRNA backbone sequences and possess different PAM sequence requirements, they may be used orthogonal to one another (Esvelt et al., 2013). The *S. aureus* Cas9 is especially notable for its smaller size, which makes it amenable for packaging into viral vectors (Ran et al., 2015). While the *S. pyogenes* Cas9 is the best well characterized, analyses and online tools are being developed to include these other Cas9 nucleases.

Due to the ease of cloning sgRNAs, and the ongoing questions regarding sgRNA specificity and activity, we recommend that users select a few sgRNA target sites and test them empirically. While it is important to try to select sgRNAs that are as specific as possible, a perfectly unique sequence may not exist suitably close to your desired mutation.

An increasingly wide selection of plasmids for CRISPR/Cas9 genome editing, with instructions for cloning, are available from the Addgene plasmid repository (www.addgene.org/CRISPR/). The protocols listed below were specifically developed with the plasmids to express human-codon optimized Cas9 and sgRNAs from (Mali et al., 2013b). While the CMV promoter has been widely used for constitutive gene expression in mammalian cells, several reports have shown that it is silenced in human iPSC. Indeed, when Cas9 is expressed by alternative constitutive promoters EF1α or CAGGS, we have found a several-fold increase in gene disruption activity in iPSC compared to CMV. Thus, an EF1α promoter was used for Cas9 expression in the following protocols. When expressing sgRNA from a U6 promoter, an extra G must be placed before the sgRNA construct (if one is not naturally present) to initiate transcription.

Plasmid donor vectors containing homology arms can be easily cloned using isothermal assembly (Gibson et al., 2009) or synthesized as gene fragments (Integrated DNA Technologies). Homology arm sequences should ideally be cloned from the cell line being targeted to obtain identical (isogenic) sequences, since any polymorphic differences between the targeting vector and the genomic locus can decrease gene targeting frequencies (Deyle et al., 2013). Any high-fidelity hot-start DNA polymerase (Qiagen, New England Biolabs, KAPA) can be used for cloning together the targeting vector, although some optimization of PCR buffer and annealing temperature conditions may be necessary. We recommend that the targeting vectors be transfected as circular plasmids, even though linearized targeting

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vectors have been widely used in the past. Linear targeting vectors showed a reduced gene targeting efficiency (Byrne et al., 2015), in part due to reduced nucleofection efficiency, and have a higher propensity for inserting randomly into the genome. The use of Cas9 nucleases usually increases the frequency of correctly gene-targeted events to far surpass the frequency of random integrations; nevertheless, the possibility of random (or partial) integration remains, and should be considered, especially when using selection markers.

All plasmids for nucleofection into iPSC should be endotoxin-free (Qiagen Endo-free Plasmid Maxi Kit) and dissolved in TE buffer at a concentration greater than 2 mg/ml, so as not to dilute the nucleofection buffer. Oligo donors (ssODN) should be HPLC-purified and resuspended in sterile distilled water.

Basic protocol 1. Transfection of Plasmids into Human Induced Pluripotent Stem Cells

With this protocol, we can consistently introduce plasmid DNA into human iPSC with 60-70% transfection efficiency. Once the plasmids and cells are ready, the nucleofection process takes a few hours. Once the iPSC are dissociated, it is important to work as quickly as possible, as remaining in the nucleofection buffer for too long will decrease cell viability. The transient transfection will subside after 3 days of culture and protein expression will turnover after 5-10 days of culture. While this protocol focuses on human iPSC, it can be adapted for use in other cell types, using culture conditions and nucleofection protocols suitable for that cell type (although the amounts of plasmid / ssODN and promoters for Cas9 expression may need adjustment).

This protocol uses the Amaxa 4D-Nucleofector X Unit (Lonza), but we have also gotten good transfection efficiencies in human iPSC from the Neon Transfection system (Life Technologies). High transfection efficiencies can also be achieved using lipid- or polymer-based methods such as Lipofectamine (Life Technologies) or Stemfect (Stemgent), according to manufacturer's protocols for human iPSC, although the Cas9 plasmid amounts and ratios may need to be adjusted accordingly. Traditional electroporation methods in PBS will produce much lower transfection efficiencies. The amounts listed below are for the 20 μl Nucleocuvette strips; if using the 100 μl single Nucleocuvettes, increase all quantities five-fold. When using any transfection method for the first time, a control reaction transfecting a fluorescent protein-expressing plasmid is recommended to verify transfection efficiency.

A number of different human iPSC lines are available from cell line resources such as Coriell (coriell.org), ATCC (atcc.org), and the Harvard Stem Cell Institute (hsci.harvard.edu), among others. Furthermore, numerous academic and commercial facilities offer iPSC derivation services from primary human tissues. Detailed protocols for culturing human ES and iPSC are available in Current Protocols in Stem Cell Biology Unit 1C.2 “Defined, Feeder-Independent Medium for Human Embryonic Stem Cell Culture” as well as online (wicell.org, stembook.org). Here, we have used iPSC derived from open-consented participants in the Personal Genome Project (Lee et al., 2009), but this protocol is widely applicable to any human ES or iPS cell line. These cells have been adapted to culture

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under feeder-free conditions, in the defined mTeSR1 medium on Matrigel-coated tissue culture plates. We have found lower transfection efficiencies (40-60%) when transfecting iPSC growing on irradiated mouse embryonic fibroblasts (MEF) feeder layers, due to incomplete separation of the iPSC from the MEFs immediately before nucleofection.

Materials

Human induced pluripotent stem cells (e.g. PGP1 iPSC from Coriell #GM 23338)

mTeSR1 defined feeder-free medium for human iPSC culture (StemCell Technologies #05850)

Knockout DMEM/F12 medium (Life Technologies #12660-012)

Matrigel human ES cell-qualified matrix (Corning #354277), aliquotted according to manufacturer's instructions

Y-27632 Rho kinase inhibitor (10 mM stock solution, see recipe)

P3 Primary Cell 4D-Nucleofection kit (Lonza, V4XP-3032)

endotoxin-free plasmid DNA expressing the Cas9 nuclease

endotoxin-free plasmid DNA expressing single guide RNA

endotoxin-free plasmid DNA (or HPLC-purified ssODN) donor construct

Accutase (EMD Millipore #SF006; StemCell Technologies #07920; or other supplier)

Trypan Blue solution (0.4%) (Life Technologies #15250)

37°C humidified incubator with 5% CO_2

Sterile tissue culture-treated plates and dishes

4D-Nucleofector system with X Unit (Lonza #AAF-1002B and 1002X)

Tabletop centrifuge

Cell counter (either an automated system like the Countess Cell counter (Life Technologies) or a hemacytometer chamber slide)

Protocol steps

1. Expand hiPSC cells under feeder-free conditions in mTeSR1 medium on tissue culture plates coated with ES-qualified Matrigel according to standard protocols.

   Each nucleofection reaction will need 0.5×10^6 cells, although a range of $0.2 – 2 \times 10^6$ iPSC per reaction can be used. Generally, one 10 cm dish will yield approximately $5\text{-}10 \times 10^6$ iPSC when ready to passage.

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2. Prepare Matrigel-coated 24-well tissue culture plates, one well per nucleofection reaction. Coat each well with 0.3 ml of Matrigel diluted in DMEM/F12 according to manufacturer's instructions. Incubate at room temperature for at least one hour.

   The precise dilution of Matrigel varies by lot and specific aliquot volumes will be noted in the included product information. Frozen aliquots will be around 270-350 μl and stored at -20°C. For use, each aliquot should be quickly dissolved in 25 ml cold DMEM/F12 and can be kept at 4°C for a few weeks. As Matrigel is temperature-sensitive, and can begin to gel above 10°C, It is important to follow the manufacturer's instructions for preparing and diluting aliquots.

3. Change hiPSC culture medium to fresh mTeSR1 containing 10 μM ROCK inhibitor (1000× of 10 mM stock solution) for at least 30 minutes before nucleofection. Use 5 ml to treat a 10 cm dish or 1 ml per well in a 6-well plate. Cells treated with ROCK inhibitor should display the characteristic change in morphology of colonies with jagged edges.

4. Combine Nucleofector solution P3 with the provided supplement solution according to manufacturer's instructions (Lonza). For each nucleofection reaction, dilute and combine the DNA mixtures in Nucleofector solution P3 (with supplement) to a final volume of 10 μl. Each nucleofection should contain 0.5 μg of Cas9-expressing plasmid and 1-1.5 μg of sgRNA-expressing plasmids. (When multiple sgRNA-expressing plasmids are used, mix them in equal amounts for a total of 1-1.5 μg plasmid.) If a plasmid targeting vector is being used, include 2 μg per nucleofection reaction. If an ssODN donor is being used, include up to 200 pmol per nucleofection reaction.

   DNA plasmids must be concentrated enough such that the total volume of DNA does not exceed 10% of the nucleofection reaction (2 μl for a 20 μl Nucleocuvette). DNA amounts exceeding 4 μg per nucleofection may have an adverse effect on iPSC viability.

5. Aspirate the mTeSR1 with Rock inhibitor media from the cells and incubate with Accutase dissociating enzyme for 5-10 min at 37°C. Once iPSC have detached, add an equal volume of mTeSR1 with Rock inhibitor, and pipet gently to achieve a single cell suspension. Transfer cells into a sterile conical tube and centrifuge the cells at 110 × g for 3 min. at room temperature.

6. Resuspend the cell pellet in 5 ml mTeSR1 with Rock inhibitor. Remove a sample of cells for counting. Combine cells with an equal volume of trypan blue solution and pipet onto the counting slide.

7. Transfer the required number of iPSC into a new conical tube and centrifuge at 110 × g for 3 min. Aspirate off most of the media. Use a pipette to remove the final layer of media without disturbing the cell pellet.

8. Resuspend cell pellet in 10 μl Nuclofector solution P3 (with supplement) for each reaction.

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9. For each reaction, promptly combine 10 μl of DNA mixture with 10 μl of resuspended cells and transfer the whole 20 μl into a Nucleocuvette. Ensure that the sample is at the bottom of the cuvette.

10. Place Nucleocuvette into the Nucleofector device and run program CB-150.

11. Add 80 μl mTeSR1 with Rock inhibitor medium into each nucleocuvette well and pipet once or twice to resuspend cells. Transfer each reaction into one well of a Matrigel-coated 24-well plate containing 0.5 ml warm mTeSR1 with Rock inhibitor medium. A high plating density post nucleofection is important for cell survival.

12. 24 hours post nucleofection, iPSC transfected with a fluorescent protein-expressing plasmid may be examined to assess the transfection efficiency. Change the media to mTeSR1 without Rock inhibitor. Since the iPSC were plated at a high density, they may appear confluent. As most of the Cas9-induced cell death occurs between 1 and 2 days post nucleofection, we advise waiting until 2 days post nucleofection to passage the iPSC. Transfected iPSC can then be propagated using regular iPSC culture protocols. After 4 or 5 days post nucleofection, the transient transfection will have subsided, and the cell population can be assayed for gene editing efficiency.

Basic protocol 2. Assessment of Cutting Efficiency

In order to assess and compare the gene disruption activity of various sgRNAs, a mismatch-specific endonuclease assay – either T7 endonuclease I (New England Biolabs), or Cel-1 Surveyor nuclease (Transgenomic) – is commonly used (Qiu et al., 2004; Kim et al., 2009). These assays involve PCR amplifying a short region (roughly 500 bp) around the intended sgRNA targeting site from the genomic DNA of the population of potentially edited cells. These PCR products are melted, re-annealed, cleaved by the mismatch-specific endonuclease, and quantitated by gel electrophoresis. While the endonuclease assays offer a rapid and cheap measure of gene disruption activity, we find that repeated measurements of the same sample can vary by more than 10%, and the limit of detection is around 3% of sequences.

We prefer a next-generation sequencing-based assay that has a much lower limit of detection (< 0.1%) and provides additional sequence information about the edited sgRNA site (Yang et al., 2013). Here, a 100-200 bp region around the edited sgRNA targeting site is PCR amplified and sequenced on a MiSeq system (Illumina). The initial set of genome-specific PCR primers are designed with the requisite MiSeq adaptor sequences appended to the 5′ end. Then, a second round of nested PCR with standard index primers incorporates the barcodes (ScriptSeq from Epicentre or Nextera from Illumina (Yang et al., 2014b). The resulting next-generation sequencing data can be analyzed by the online CRISPR Genome Analyzer platform, which accepts the sequencing reads, the genomic sequence being targeted, and a donor sequence for homologous recombination (if applicable), and calculates the rate of indels and successful homologous recombination (crispr-ga.net) (Guell et al., 2014).

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A preliminary experiment testing a few sgRNAs designed against the region of interest (along with a previously-published positive control sgRNA) will help verify the hiPSC transfection conditions and Cas9 plasmid constructs, particularly for investigators new to Cas9 genome editing. The sgRNA with the highest activity can then be chosen. Determining the overall gene editing efficiency among the whole post-transfection hiPSC population will help estimate the number of cells that should be sorted and screened to obtain a successfully-edited clone. Potential off-target sites in the genome may also be analyzed in this manner. While each MiSeq run (150 bp, paired-end) can be expensive, smaller-scale Micro and Nano kits are now available from Illumina, and multiple samples can be barcoded, pooled, and sequenced in parallel (Yang et al., 2013). Compared to the cost and labor of cloning, expanding, and screening hundreds of hiPSC clones, it is prudent to first ensure that some successfully-edited hiPSC exist in the population. The PCR and data analysis can be performed fairly quickly; the rate-limiting step may be access to the MiSeq sequencer machine. The transfected hiPSC cultures may be frozen down during this process, or hiPSC may be transfected fresh once the optimal sgRNAs and conditions have been determined.

Materials

PCR kit with a high-fidelity DNA polymerase (*e.g.* KAPA HiFi HotStart #KK2501)

genomic DNA purification kit for mammalian cell culture (Sigma #G1N70, Qiagen #13323, or other supplier

SYBR Green I Nucleic Acid Stain (Life Technologies #S-7563)

Nextera dual index primers (Illumina #FC-121-1011 or FC-121-1012)

Qiaquick gel extraction kit (Qiagen #28704)

quantitative PCR machine capable of detecting SYBR Green

PCR plates or tubes with optically clear films or lids

electrophoresis apparatus for running 2% agarose gels

Nanodrop spectrophotometer or other system for measuring DNA concentration

access to MiSeq sequencing facility

Protocol steps

Design PCR primers for MiSeq analysis

1. Design a pair of specifically PCR primers to amplify a 150-200 bp region around the sgRNA targeting site of interest (either on-target or off-target) using Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) or a similar program
2. Append the forward (5′ CTTTCCCTACACGACGCTCTTCCGATCT 3′) and reverse (5′ GGAGTTCAGACGTGTGCTCTTCCGATCT 3′) Illumina adapter sequences to the 5′ end of each sequence-specific primer.

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For example, for the positive control sgRNA targeting the human THY1 exon 2: GGGTC AGGCT GAACT CGTAC TGG

The forward and reverse primers amplifying this 195 bp gene region are thus:

forward: 5′ CTTTCCCTACACGACGCTCTTCCGATCT GTATTTGCTGGTGAAGTTGGTTCGG 3′

reverse: 5′ GGAGTTCAGACGTGTGCTCTTCCGATCT CAGAAGGTGACCAGCCTAACGG 3′

With the Illumina adapter sequences underlined and followed by the THY1-specific primer sequences

3. Verify that the PCR primers can specifically amplify the correct-size product from ~100 ng of purified human genomic DNA using a high-fidelity DNA polymerase.

   The PCR reaction conditions for each set of primers may need to be optimized for buffer and annealing temperature. Ideally, the purified genomic DNA should be from the same cell line being targeted, in case any SNPs may be present in the primer annealing sites.

First round of PCR amplification

4. Harvest a population of CRISPR gene-targeted human iPSC and purify genomic DNA according to manufacturer's kit instructions.

5. PCR amplify desired gene segment from the purified genomic DNA using the validated primers and primer cycling conditions. Include SYBR Green to follow the PCR amplification curves on a quantitative real-time PCR machine. Dilute the SYBR Green I solution 1:500 in dH2O to make a working stock.

To continue the THY1 example:

5 µl 5× KAPA HiFi Fidelity buffer

0.75 µl 10 mM KAPA dNTP mix (0.3 mM final concentration)

0.75 µl 10 µM forward PCR primer (0.3 µM final concentration)

0.75 µl 10 µM reverse PCR primer (0.3 µM final concentration)

50-100 ng of purified genomic DNA

0.25 µl SYBR Green working stock (

0.5 µl KAPA HiFi HotStart DNA polymerase

ddH20 to 25 µl

PCR cycling conditions:

1. 95°C for 5 min
2. 98°C for 30 sec
3. 66°C for 30 sec

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4. 72°C for 30 sec
5. Repeat steps 2-4 for 35 cycles
6. Stop amplification reaction once the SYBR Green amplification curve begins to plateau (typically after around 25 cycles)

Overamplification of the PCR products can lead to a skewed assessment of the gene targeting efficiency.

6. Run PCR reaction on a 2% agarose gel.

7. Excise the predicted-size PCR product from the gel.

8. Gel purify PCR reaction using a gel extraction kit according to manufacturer's instructions (*e.g.* Qiagen QIAquick Gel extraction kit)

Careful gel purification of the PCR reaction (as opposed to a PCR purification kit) is recommended, as off-target PCR products may be present when amplifying from genomic DNA. A PCR purification kit may also not fully remove the longer PCR primers, which will interfere with subsequent reactions.

9. Measure the DNA concentration of the purified PCR product using a Nanodrop spectrophotometer or other method.

Second round of PCR amplification

10. Perform a second round of PCR amplification to add MiSeq index primers. Using index primers containing different barcodes, multiple samples can be combined together and analyzed in a single MiSeq run. The resulting .fastq data files returned by the sequencer will already be organized by sample barcode.

To continue the example:

20 μl 5× KAPA HiFi Fidelity buffer

3 μl 10 mM KAPA dNTP mix (0.3 mM final concentration)

5 μl 10 μM index 1 primer (N7xx) (0.3 μM final concentration)

5 μl 10 μM index 2 primer (N5xx) (0.3 μM final concentration)

100 ng of purified PCR product from step 8

1 μl SYBR Green working stock

2 μl KAPA HiFi HotStart DNA polymerase

ddH20 to 100 μl

Divide 25 μl each into four wells of a quantitative PCR plate

PCR cycling conditions:

1. 95°C for 5 min
2. 98°C for 30 sec

3. 66°C for 30 sec
4. 72°C for 30 sec
5. Repeat steps 2-4 for 25 cycles
6. Stop amplification reaction once the SYBR Green amplification curve begins to plateau (typically after around 5-10 cycles)

11. Gel extract DNA and quantitate PCR reaction repeating steps 6-9.

12. Pool PCR reactions together in equal amounts and submit for MiSeq sequencing analysis (150 bp, paired-end, supplemented with 20% PhiX).

13. Analyze MiSeq results using the CRISPR Genome analyzer: (crispr-ga.net) (Guell et al., 2014)

Basic protocol 3. Cloning IPSC by Single Cell Facs Sorting

Several days post nucleofection, after the transiently transfected plasmids have been lost and the Cas9 nuclease activity has subsided, targeted iPSC may be individually cloned to generate a culture of successfully targeted cells. If a positive selection marker for antibiotic resistance has been integrated into the genome (such as those for neomycin, hygromycin, or puromycin), that antibiotic may be added to the culture to remove unrecombined antibiotic-sensitive cells. Emerging antibiotic-resistant stem cell clones can then be individually picked by hand and cultured.

Alternatively, human iPSC may be cloned by FACS sorting individual cells into separate wells of a 96-well plate. We find that this is less labor-intensive than picking colonies manually and ensures that each clone is derived from a single cell. To preserve the viability of the dissociated single iPSC, a cocktail of small molecule inhibitors (termed SMC4, from Biovision) is added to the culture (Valamehr et al., 2012). (Recent reports state that inhibiting TGF-β may be detrimental for the long-term maintenance of human iPSC and induce differentiation, particularly in transgene-free iPSC, and instead now advocate omitting the TGF-β inhibitor SB431542 and including LIF (Valamehr et al., 2014). However, we have not yet tested this.)

We find that the viability of isolated iPSC is further enhanced by sorting the cells (previously cultured in feeder-free mTeSR1 media) onto a feeder layer of irradiated MEFs in human ES cell medium. Eight days after FACS sorting, colony formation should be apparent from the individually sorted iPSC. When the colonies have grown to a passagable size, the SMC4 inhibitors can be removed from the ES cell medium. We usually achieve 20-60% iPSC survival and colony formation post sort. The gene targeting efficiency in the iPSC population (measured as described in the previous section) can be used to estimate the number of wells needed for sorting to obtain a successfully targeted viable clone. The iPSC colonies may then be cultured and expanded using standard protocols on a MEF feeder layer for a few passages before being transitioned to feeder-free iPSC conditions. A portion of each potentially targeted iPSC clone may be taken for genomic DNA extraction and genotyping.

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Materials

0.1% gelatin solution (Millipore #ES-006B)

DMEM-10 (see recipe)

Irradiated CF-1 mouse embryonic fibroblasts (MEF) (Global Stem #6001G)

hES cell medium (see recipe)

Recombinant basic fibroblast growth factor (100 μg/ml stock solution; see recipe)

mTeSR1 and hES cell medium supplemented with SMC4 inhibitors (see recipe)

Fibronectin solution (1 mg/ml, Sigma #F1141) (do not vortex or agitate stock)

Gene targeted iPSC (Basic Protocol 1)

mTeSR1 defined feeder-free medium for human iPSC culture (StemCell Technologies #05850)

Accutase (EMD Millipore #SF006; StemCell Technologies #07920; or other supplier)

Fluorescently-conjugated antibody (optional) (for example, mouse anti-human Thy1, clone 5E10) (BD Biosciences, eBioscience)

Cell viability dye for FACS analysis (e.g. propidium iodide, DAPI, YoPro from Life Technologies, BD Biosciences, eBioscience, or other supplier)

96-well flat-bottom plates, sterile, tissue culture treated

Cell strainer (Corning #352350) or 70 μm nylon mesh, cut into squares and autoclaved (Spectrum labs #146490)

FACS sorter capable of sterile sorting into 96-well plates (such as the Beckman Coulter MoFlo Astrios or BD Biosciences FACS Aria)

Tabletop centrifuge with a swinging-bucket rotor for 96-well plates

37°C humidified incubator with 5% CO_2

Protocol steps

Prepare 96-well plates with MEF feeder layer

1. The day before FACS sorting, prepare the MEF feeder layer on gelatin-coated 96-well flat-bottom plates. Add 60 μl of 0.1% gelatin per well and incubate at room temperature for at least 20 min.
2. Thaw a frozen vial of irradiated MEFs in DMEM-10 media. Approximately 10^6 cells will be needed for each 96-well plate. Quickly thaw each frozen vial in a 37°C water bath and gently pipet the contents into 10 ml of DMEM-10 in a conical tube.

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Gently invert the tube to mix the cells and centrifuge at 120 × g for 3 min at room temperature.

3. Resuspend the MEF cell pellet in DMEM-10 using 10 ml for 10^6 cells.

4. Remove the gelatin solution from the plates by aspirating. If many plates are being prepared, the solution washes can also be done by inverting and shaking the plate over paper towels inside the tissue culture hood. Be sure to keep the plates sterile and not in contact with the paper towels.

5. Add 0.1 ml of resuspended MEFs (approximately 10^4 cells) to each well. Culture plates overnight at 37°C.

   The MEF feeder layer is best prepared the day before FACS sorting, although plates can be used from 8 hours to 2 days after plating. After plating, the MEFs should form a confluent layer over each well.

Pretreat human iPSC with SMC4 inhibitors

6. Change media on the iPSC plates to fresh mTeSR1 supplemented with SMC4 inhibitors. Incubate for at least one hour.

This step can also be done the night before FACS sorting. iPSC treated with the SMC4 inhibitors will develop a flattened morphology.

Prepare MEF feeder cultures with hES medium with SMC4 inhibitors.

7. For each 96-well plate, prepare 10 ml of hES medium with SMC4 inhibitors (see recipe) and add fibronectin to a final concentration of 5 μg / ml.

8. Remove DMEM-10 media from the 96-well MEF plate by aspirating or shaking over paper towels. Add 0.1 ml / well of the hES medium with SMC4 inhibitors and fibronectin. Return plates to the incubator until ready for FACS sorting.

The addition of the fibronectin solution is optional, but it may be included to improve the initial seeding of iPSC. It does not need to be included in subsequent media changes.

Dissociate iPSC for FACS sorting

9. Remove mTeSR1 with SMC4 media from iPSC cultures by aspirating. Add Accutase solution to cells (for a 6-well plate, use 1 ml / well; for a 10 cm dish, use 5 ml). Incubate at 37°C for 10-20 min until the cells detach.

The presence of the SMC4 inhibitors causes the cells to adhere more strongly to the plate; therefore an extended Accutase incubation time may be needed. As thiazovivin is also a Rho kinase inhbitor, Y-27632 is not required.

10. Stop the Accutase reaction with an equal volume of mTeSR1 with SMC4 inhibitors. Transfer cells into a conical tube and centrifuge for 120 × g for 3 min at room temperature. Aspirate off supernatant.

11. Resuspend the iPSC pellet in mTeSR1 with SMC4 inhibitors.

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Extracellular antibody stain for iPSC surface antigens (optional)

12. Centrifuge cells for 120 × g for 3 min at room temperature.

13. Resuspend cells in antibody stain. Dilute fluorescently-conjugated antibody according to manufacturer's recommendations in mTeSR1 with SMC4 inhibitors supplemented with 10% ES cell-qualified FCS. Use approximately 0.1 ml of antibody stain for 10^6 cells. Incubate cells at 4°C for 30 min in the dark.

14. Wash cells twice with mTeSR1 with SMC4 media, centrifuging at 120 × g for 3 min at room temperature.

Prepare final cell sample for FACS sorting

15. Centrifuge cells at 120 × g for 3 min at room temperature.

16. Resuspend cell pellet in at least 0.4 ml mTeSR1 with SMC4 media. Filter through nylon mesh or cell strainers into the FACS tube. Keep cells in the dark on ice.

17. Right before sorting, add 0.2 ml mTeSR1 with SMC 4 with viability dye into each FACS tube.

Any viability dye that can be used for live / dead cell discrimination for flow cytometry can be used. Dilute in mTeSR1 with SMC4 media according to the manufacturer's recommendations. Be sure that the viability dye is compatible with any fluorescent proteins or antibody stains that may have also been used.

FACS sorting single cells into 96-well plates

18. Using a FACS sorter under sterile conditions, sort single viable iPSC into each well of the prepared 96-well MEF plates.

If available, using a 100 um nozzle may improve cell survival after FACS sorting. As a control, sort 10-20 cells into one well of the 96-well plate. Cells should be first be gated on for size (FSC vs. SSC), then gated for singlets (either SSC-H vs. SSC-W, FSC-H vs. FSC-W, or FSC-A vs. FSC-H), and finally gated for exclusion of the viability dye. If fluorescent markers or antibody stains were used, single viable cells can be sorted based on these parameters as well. Since only ~100 cells will be sorted per plate, the gating criteria may be made particularly selective, to choose only the most viable cells.

19. After sorting, centrifuge the plates at 70 × g for 3 min at room temperature to help the iPSC attach to the MEF feeder layer. Return plates to the 37°C tissue culture incubator.

Expansion of sorted iPSC clones

20. Four days after sorting, change the media on the plates to 0.1 ml / well of fresh hES media with SMC4 inhibitors (no fibronectin).

Media changes should only be necessary at day 4 and day 8 post-sort, with more frequent media changes thereafter.

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21. Eight days after sorting, colonies should be visible. Change the media on the plates to 0.1 ml / well of fresh hES media.

Once the iPSC colonies have grown large enough to be self-sustaining, supplementation with the SMC4 inhibitors is no longer required. If colonies still appear small after 8 days, cells can be maintained in hES media with SMC4 inhibitors for up to 12 days.

22. Continue changing the media on the plates every two days until the iPSC colonies are large enough to passage.

23. The day before passaging, prepare another 96-well plate with gelatin and irradiated MEFs as in steps 1 to 5.

24. The day of passaging, remove DMEM-10 medium from new MEF plates and add 60 μl fresh hES medium supplemented with 10 μM Y-27632.

25. Change the media on the iPSC plates to 100 μl fresh hES medium with 10 μM Y-27632.

26. Mechanically passage the iPSC clones using a 200 μl pipet tip to scrape the bottom of the well. Pipet up and down to break apart the colony. Transfer the entire contents of the well (medium, iPSC, and MEFs) into the new MEF-coated 96-well.

27. Cells can continue to be propagated on MEF feeder culture in hES medium according to established protocols. Once a robust culture is established, portions of the culture can be split for cryopreservation or genomic DNA extraction.

Basic protocol 4. Purification of genomic DNA in 96-well tissue culture plates

Once potentially targeted iPSC clones have been expanded, they must then be genotyped to identify successful gene targeting. Typically, genomic DNA is purified from a portion of each expanded clone (while freezing or continuing to expand the remaining culture) and analyzed using a series of PCR reactions. While many kits for purifying genomic DNA from mammalian cell culture are commercially available (Sigma #G1N70; Qiagen #13323), they can be laborious and expensive when processing hundreds of samples. Alternatively, a cell lysate (e.g. prepared using Prepgem Tissue by Zygem #PTI0050) may be used as a PCR template, but this unpurified genomic DNA may not work for some PCR reactions, especially when amplifying a large PCR product or a tricky gene locus. For a convenient and cheap method of isolating genomic DNA, we use a protocol that lyses the cells and precipitates the genomic DNA directly in the original 96-well polystyrene tissue culture plate (Ramírez-Solis et al., 1992).

Materials

96-well tissue culture plate containing iPSC colonies

Cell lysis buffer (see recipe)

NaCl-saturated Ethanol (see recipe)

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70% Ethanol (molecular biology grade)

TE buffer (0.5×) (see recipe)

humidified 55°C incubator

multichannel pipet

paper towels

Protocol steps

1. Since the entire 96-well tissue culture plate will be used for genomic DNA extraction, be sure to set up separate plate to continue culturing the potentially gene-targeted iPSC clones.

   A somewhat overgrown plate yields higher amounts of DNA, since these iPSC are being harvested it does not matter if they begin to differentiate. Plates coated with Matrigel or a MEF feeder layer can both be used, provided that the PCR genotyping primers are specific for human DNA.

2. Aspirate media and add 50 μl of cell lysis buffer to each well.

3. Incubate plates in a 55°C humidified incubator for 3 hours.

   If the incubator is not humidified, the buffer in the plates will evaporate during the incubation. Placing a pan of water inside the incubator will suffice to prevent this.

4. Remove plates from incubator and allow to return to room temperature.

   Cells should be completely lysed and no longer visible under the microscope.

5. Add 100 μl per well of ice-cold NaCl-saturated ethanol.

   The NaCl will form a suspension in the saturated ethanol and not dissolve completely. Keep the slurry mixed in suspension with frequent stirring and pipetting.

6. Allow plates to sit undisturbed at room temperature for at least one hour.

   After this incubation, a light web of genomic DNA should be visible clinging to the bottom of the well. The subsequent wash steps must be done gently, as too forceful decanting and pipetting may detach the genomic DNA from the polystyrene tissue culture plate.

7. Gently invert the plate over paper towels to remove the supernatant, being careful not to detach the genomic DNA or cross-contaminate the wells.

8. Gently wash the plate with 200 μl per well of 70% Ethanol, and invert onto paper towels. Repeat two more times.

9. After the last 70% ethanol wash, allow the plate to air dry at room temperature without the lid until all of the residual ethanol has evaporated (typically 15-30 min).

10. Add 50 μl per well of TE buffer (0.5×) to dissolve the DNA and store at 4°C.

    For long-term storage, plates may be wrapped in plastic film to prevent evaporation.

Reagents and Solutions

Cell lysis buffer for genomic DNA purification

10 mM Tris HCl pH 7.6

10 mM EDTA

100 mM NaCl

0.5% SDS

This lysis buffer may be prepared ahead of time and stored indefinitely at room temperature. Right before use, add:

0.1 mg/ml Proteinase K (Roche #03115879001)

DMEM-10 for mouse embryonic fibroblast culture

Add 50 ml of heat-inactivated ES cell-qualified fetal calf serum (Life Technologies #10439 or other supplier) to 450 ml high-glucose DMEM supplemented with glutamine and sodium pyruvate (Life Technologies #10569). Filter sterilize and store for a few weeks at 4°C.

Human embryonic stem cell medium (for feeder culture)

200 ml Knockout DMEM/F12 media (Life Technologies #12660-012)

50 ml Knockout Serum Replacement (Life Technologies #10828-028)

2.5 ml L-Glutamine (Life Technologies #25030)

2.5 ml penicillin / streptomycin (Hyclone #SV30010)

2.5 ml MEM-non essential amino acids (Life Technologies #11140; Hyclone #SH30238.01)

2.5 ml HEPES (Hyclone #SH30237.01)

1.75 μl 2-Mercaptoethanol (Sigma #M3148)

250 μl recombinant human bFGF stock solution (see recipe) to a final concentration of 100 ng / ml

Filter sterilize and store for a few weeks at 4°C.

NaCl-saturated Ethanol (75 mM NaCl in ethanol)

For each 100 ml of 100% Ethanol (molecular biology grade), add

1.5 ml of 5M NaCl

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Recombinant basic fibroblast growth factor (bFGF) (100 μg/ml stock)

Reconstitute recombinant human basic fibroblast growth factor, certified for embryonic stem cell culture (Millipore #GF003 or eBioscience #34-8986) to 100 μg / ml in a sterile-filtered solution of 0.5% BSA, 1 mM DTT, and 10% glycerol in PBS. Aliquot and store at -20°C.

SMC4 supplemented mTeSR-1 or hES medium

For each 10 ml of mTeSR-1 or hES medium, add:

5 μM thiazovivin (10 μl of 5 mM stock solution; Biovision #1993-1)

1 μM CHIR99021 (1 μl of 10 mM stock solution; Biovision #1991-1)

0.4 μM PD0325901 (0.8 μl of 5 mM stock solution; Biovision #1990-1)

2 μM SB431542 (4 μl of 5 mM stock solution; Biovision #1992-1)

Calculate the amount needed for that day and prepare fresh. The inhibitors are supplied as a sterile solution in DMSO; when first thawed, make aliquots of the stock solution and freeze at -20°C

TE buffer (0.5×) for resuspending genomic DNA

5 mM Tris HCl pH 8.0

0.5 mM EDTA pH 8.0

Y-27632 Rock inhibitor

Y-27632 Rock inhibitor (EMD Millipore #688000; R&D Systems #1254/10; Abcam #ab120129; or other supplier)

Dissolve to 10 mM in ddH2O and filter-sterilize. Aliquot and store at -20.

Commentary

Background Information

Targeted gene replacements have been essential tools for exploring gene function; however, the low frequency of homologous recombination (10^{-3}-10^{-7}) limits this technique even when using antibiotic selection markers and homology arms of up to 14 Kbp (Kass and Jasin, 2010; Bollag et al., 1989; Moehle et al., 2007; Deng and Capecchi, 1992; Hockemeyer et al., 2009). The development of sequence-specific nucleases such as zinc finger nucleases, (ZFN), transcription activator-like effector nucleases (TALEN), or CRISPR/Cas9 nucleases have enormously expanded our ability to engineer genetic changes in human cells (Urnov et al., 2010; Joung and Sander, 2012; Mali et al., 2013b), particularly human iPSC which were previously resistant to gene editing. The CRISPR/Cas9 nuclease system has been favored due to its easy construction and multiplexability as well as its lower toxicity (Ding et al., 2013; Mali et al., 2013b).

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Critical Parameters

iPSC pluripotency—Even though gene targeting in human iPSC is more difficult than in immortalized tumor cell lines, human iPSC are often chosen for gene targeting projects due to their self-renewing and pluripotent properties. Human iPSC provide a ready and controllable source of cells that can be gene targeted, cloned, genotyped, expanded, and subsequently differentiated into other tissue types, particularly cell types with limited growth potential. Thus, maintaining the pluripotency of iPSC during the genome editing process is paramount. Cells used for gene targeting should be of a low passage number and free of karyotypic abnormalities. Cells should exhibit normal iPSC morphology and express pluripotency markers such as Tra-1/60 and SSEA4. Care should be taken throughout the culturing process to maintain iPSC at an appropriate density, such that they do not die or differentiate. Any cultures that display a differentiated morphology or exhibit large amounts of cell death should not be continued. Once successfully-targeted clones have been genotyped and verified, cells should be examined again for a normal karyotype, expression of pluripotency markers, and ability to form all three germ layers in teratoma assays. For these reasons, we sought to develop gene targeting protocols using a single transient transfection of plasmid DNA, with no further enrichment or selection steps.

Positive controls—As the isolation and genotyping of edited iPSC clones can be time consuming, laborious, and expensive, positive controls should be included during the process to verify successful transfection, cutting, and gene targeting, and provide guidance for troubleshooting. Examining a portion of the targeted iPSC population will assess the frequency of gene editing and estimate how many clones should be genotyped.

The simplest way to verify iPSC transfection efficiency is by transfecting a plasmid that constitutively expresses a fluorescent reporter, and examining the percent of fluorescent cells the following day (either by flow cytometry or microscopy). Most transfection kits for iPSC come with a positive control plasmid (such as pMaxGFP included in the Lonza nucleofection kit), which can also be used to compare the toxicity of homemade plasmid preparations versus commercial ones. While transfection conditions may need to be optimized for a particular iPSC line (number of cells, amount of DNA, plating density, electroporation settings) the manufacturer's protocol should provide a general range of expected transfection efficiencies for iPSC for that reagent.

To verify the cutting activity of the Cas9 and sgRNA plasmids, a positive control reaction with a published sgRNA (such as the THY1 sgRNA mentioned here) can be included. This will be able to distinguish between a general problem with the nuclease versus sgRNAs that simply happen to have an inherently low activity. The activity of various sgRNAs can be compared using the next-generation sequencing protocol (Basic Protocol 2), or, if the gene being disrupted or inserted is expressed by human iPSC, by flow cytometry or microscopy.

For engineering small mutations using ssODN, a positive control can be the sgRNA and ssODN donor oligos targeting the AAVS1 safe harbor locus published in (Yang et al., 2013). If a gene segment is being inserted into the genome, an EGFP transgene can be knocked into the AAVS1 locus (Hockemeyer et al., 2009). Another positive control targeting vector for the same sgRNA targeting site can be constructed using the same

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homology arms as the insertion targeting vector, except that a constitutively-expressed fluorescent protein cassette is being inserted into the genome. This will provide a quick estimate of "knock-in" insertion frequencies at that locus using the same sgRNA and homology arms.

For gene excisions using multiple sgRNAs, once the activity of the individual sgRNAs has been verified, a dilution PCR around the excised segment can be done on genomic DNA from the edited cell population. This may also be done for gene insertions, although care must be taken to ensure that the PCR reaction does not simply amplify residual amounts of the transfected donor fragment itself (De Semir and Aran, 2003). PCR primers designed to anneal to genomic DNA sequences outside of the targeted homology region may be used to ensure that only integrated segments are detected. For insertions less than 100 bp, next-generation sequencing may also be used to estimate gene insertion frequency.

Genotyping successfully-edited clones by PCR—For checking simple gene disruptions or small bp changes, PCR amplification and Sanger sequencing around the targeted locus will suffice. Heterozygous base pair changes will appear as a double peak on the Sanger sequencing trace. Heterozygous indels can similarly be identified by deconvoluting a biallelic Sanger sequencing trace (Dmitriev and Rakitov, 2008). Alternatively, the biallelic PCR product can be subcloned into a plasmid vector (*e.g.* TOPO from Life Technologies) for each allele to be sequenced in a separate reaction. Any potential off-target nuclease sites may also be genotyped by PCR and sequencing to check for unintended mutations.

To genotype larger gene deletions, a PCR reaction with primers that span the two nuclease sites can be sequenced. A second PCR reaction with primers located within the two nuclease sites can identify any un-excised alleles, and determine whether the gene deletion is homozygous or heterozygous.

For targeted "knock-in" gene insertions, one must not only ensure that the entire transgene has been incorporated into the genome, but also that both homology arms have been recombined to the correct site, without recombination into other areas or duplication of the homology arms. A series of PCR reactions can confirm complete and correct integration of the "knock-in" construct into the targeted locus. One set of PCR primers that spans the inserted gene can confirm complete insertion, while two other sets of primers that span each of the homology arms (with one primer annealing outside of the homology arm region) can confirm proper recombination on each end.

Off-target Cas9 nuclease activity—When selecting sgRNA sites, one should check if any close off-target sites are present in the genome. Several online tools are available for this (http://crispr.mit.edu and http://www.e-crisp.org/E-CRISP/designcrispr.html) although the algorithms for predicting sgRNA activity are continually being refined. Ideally, a perfectly specific sgRNA site will possess a unique 13 bp "seed" sequence not found alongside any other active PAM site. Even then, Cas9 nuclease activity may still occur at off-target sites containing 1-2 mismatches in the seed sequence.

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While initial studies examining a population of immortalized tumor cell lines found substantial rates of off-target Cas9 nuclease activity (Fu et al., 2013; Hsu et al., 2013; Pattanayak et al., 2013; Kuscu et al., 2014), this may be less of a concern for gene editing individual stem cell clones (Wu et al., 2014; Yang et al., 2014a; Byrne et al., 2015). For cells with a lower rate of on-target nuclease activity, like transiently transfected iPSC, the off-target sites may be less of an issue. With the above protocol transiently transfecting Cas9 nuclease and sgRNA into human iPSC, we typically find low off-target gene disruption frequencies around 0.1-0.2%, even at sites with identical seed sequences. However, since neither the on-target nor off-target Cas9 nuclease activities can currently be completely predicted through computational analysis, we recommend that any close off-target sgRNA sites also be checked when assessing gene disruption frequency in the cell population and when genotyping successfully targeted clones. Off-target gene disruption frequencies can be lowered by titrating down Cas9 nuclease activity (by transfecting a smaller quantity of plasmid, or expressing the Cas9 nuclease under a weaker promoter), although this will also decrease the on-target nuclease activity.

If no specific sgRNA targeting sites can be located, (e.g., a gene locus that shares sequence homology with other conserved gene family members), a Cas9 nickase mutant (D10A) may be used to generate the dsDNA break instead of a Cas9 nuclease (Jinek et al., 2012). For gene disruption using paired Cas9 nickases, the highest rate of indel formation was achieved using two offset sgRNAs where the double nicks resulted in a 5′ DNA overhang. Indel formation was greatest with a 20-50 bp 5′ overhang, although detectable up to 130 bp (Mali et al., 2013a; Ran et al., 2013a; Cho et al., 2014). Recently, further specificity has been achieved using catalytically-inactive nuclease-null Cas9 proteins fused to a FokI homodimer nuclease domain – a pair of sgRNAs can bring the attached FokI domains together at the target site to generate a dsDNA break (Guilinger et al., 2014; Tsai et al., 2014). However, while the paired nickase strategy possesses greater gene editing specificity, we find that the overall frequency of gene editing in iPSC is much lower, and recommend that users begin with the full Cas9 nuclease to assess the initial levels of sgRNA activity at their desired locus.

Troubleshooting

Poor transfection efficiency—If iPSC transfection efficiencies are low, even with a commercially-prepared fluorescent control plasmid, then the electroporation conditions may need to be optimized for a particular cell line. Both Lonza and Life Technologies can provide a series of electroporation settings to test their respective nucleofector machines. If a particular cell line appears to be especially resistant to nucleofection (or no such machine is available in the lab) several lipid- or polymer-based transfection methods (Lipofectamine, Stemgent) have also been shown to work well for human iPSC.

Poor cell survival after transfection—Some cell death should always be expected following the transfection process; however, most of the iPSC should remain viable and re-attached to the tissue culture plate the following day. Plating the cells at a high density post nucleofection is critical for their survival, as is supplementing the iPSC media with the Y-27632 Rock inhibitor before, during, and after passaging. A negative control reaction

transfecting cells with no DNA (buffer alone) will help distinguish whether the toxicity is due to the plasmid DNA or the cell handling. If large amounts of cell death occur even with no DNA, further care must be taken to passage and transfect the cells as quickly and gently as possible, or optimize the electroporation program, or use a different transfection reagent.

If there is good cell survival with no DNA, but poor cell survival when transfecting a commercially-prepared fluorescent control plasmid, then the quantity of DNA in the transfection reaction may be titrated down. If there is good survival with the control fluorescent plasmid, but poor survival with the prepared Cas9 and sgRNA plasmids, ensure that the maxiprep plasmid preparations are endotoxin-free and > 2 mg / ml. (While miniprep plasmid DNA may sometimes be suitable for transfecting other cell types, we find that it is often not suitable for transfecting iPSC.) Also ensure that the sgRNA does not target a gene essential for cell survival – control reactions using published sgRNAs that target the AAVS1 safe harbor locus or the THY1 surface marker may be used for comparison.

Poor gene disruption efficiency—If iPSC transfection efficiencies are > 50% and most of the cells remain viable afterward, but no gene disruption is seen, even with published positive control sgRNAs, there may be a problem with the Cas9 and sgRNA plasmids. Ensure that the plasmid sequences are correct, the Cas9 nuclease is codon-optimized for mammalian cell expression and contains a nuclear localization signal, and the promoter is suitable for iPSC. Intracellular staining with a monoclonal anti-Cas9 antibody (Diagenode) may be used to verify Cas9 protein expression. One may test an alternate set of published Cas9 expression plasmids (available from Addgene), or a plasmid that expresses both Cas9 and the sgRNA. Different iPSC lines can exhibit different levels of gene disruption efficiency, even using the same plasmids and protocols, although some gene disruption should still be evident (Byrne et al., 2015).

An alternative approach is to use a plasmid that expresses Cas9 along with GFP (Ding et al., 2013). Thus, the hiPSC population can be enriched for successfully-transfected GFP^+ cells by FACS sorting 24-48 hours after transfection. This measure may be particularly beneficial in cases of low hiPSC transfection efficiency. However, when FACS sorting hiPSC so soon after transfection, we recommend to collect and culture them as a population (not single cell cloning), as the cells will still be undergoing a significant amount of gene editing and genotoxic stress. Once the transient transfection and gene editing has subsided and the hiPSC culture has recovered, the enriched hiPSC may then be cloned and genotyped. Detailed protocols for this process are described in Current Protocols in Stem Cell Biology Unit 5B.3: "TALEN- and CRISPR/Cas9-Mediated Gene Editing in Human Pluripotent Stem Cells Using Lipid-Based Transfection".

If gene disruption frequencies with positive control sgRNAs are in line with what has been published for iPSC, but very low gene disruption is seen at new sgRNA targeting sites, then the new sgRNA may simply have low cutting activity. Alternate sgRNAs may be designed, keeping in mind the parameters for designing more active guides outlined in the Strategic Planning section.

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Poor gene targeting efficiency—If the desired sgRNA exhibits a fair amount of cutting activity (as indicated by gene disruption frequency), but very little gene correction or gene targeting, the amounts of donor ssODN or plasmid may need to be optimized. For donor ssODN, check that the oligo is centered over the intended mutation and that the mutation is within 5-10 bp of the dsDNA break. For donor plasmids, ensure that the homology arm sequences are at least 2 Kbp long and the sequence is identical to that of the cell line. Ensure that no sequence homology exists between the dsDNA break and the gene insertion or within the gene insertion. Check that the Cas9 / sgRNA does not cut the donor construct (through sequence analysis or an in vitro Cas9 nuclease assay). An antibiotic selection marker may be included with the transgene to enrich for targeted events.

Poor cell survival after single-cell FACS sorting—Prior to cell sorting, the population of potentially gene-targeted cells should have recovered from the transfection procedure and possess growth kinetics and survival comparable to before the transfection. Cells should be dissociated and stained right before sorting, and kept on ice while in suspension. The live and dead cell staining should be clearly distinguishable on the FACS plot. Since only ~100 iPSC are required per 96-well plate, the FACS sorting gates can be drawn tightly to choose only those cells that are clearly healthy and express the right markers. The FACS sorter should be carefully calibrated and aligned to accurately deposit one correct cell in the center of each well.

Ensure that the hES media is freshly made, that the SMC4 inhibitors and growth factors have been prepared and stored correctly, and that the MEF feeder cell layer is confluent prior to sorting. iPSC may also be cloned using other methods, such as manual colony picking with a pipet tip, or limiting dilution.

Anticipated Results

Using the described nucleofection methods, we consistently achieve iPSC transfection efficiencies >60%, as measured by flow cytometry analysis one day after transfection. Our gene disruption efficiencies using a single guide RNA with the Cas9 nuclease range from 1-25% of the total cell population, as assayed by next-generation sequencing analysis five days after transfection. Using two sgRNAs with the Cas9 nuclease, we can achieve homozygous deletions of up to 86 Kbp with up to 8% frequency, although this is highly sgRNA-dependent. Our gene insertion or replacement efficiencies (without any subsequent selection or enrichment) range from 0.5-10% of the total cell population depending on the location and size of the insertion and the particular iPSC line (Byrne et al., 2015).

Time Considerations

Once the sgRNA sites have been designed, it should take about a week for them to be synthesized or cloned into the sgRNA backbone plasmid. However, assembling a donor targeting vector with long homology arms may take longer to clone.

Once plasmids and iPSC cultures are ready, the nucleofection process will take a few hours, depending on the number of samples. One day after transfection, cells should express the Cas9 enzyme or fluorescent protein for assessing transfection efficiency. Two or three days

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after transfection, iPSC cultures may need to be passaged into a larger well. Three to five days after transfection, the transient transfection will have subsided, along with the gene editing process, and a sample of cells may be taken to measure gene disruption efficiency. Five to ten days after transfection (depending on the rate of protein turnover), individual iPSC may be cloned by single-cell FACS sorting.

Eight days after sorting, iPSC colonies should be visible, although it may take another week of growth before the colonies are large enough to passage. From there, cultures should be passaged every 3-4 days until they have expanded enough for freezing and genotyping.

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