Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

Curr Protoc Stem Cell Biol. Author manuscript; available in PMC 2019 February 28.

Published in final edited form as:

Curr Protoc Stem Cell Biol. ; 44: 5B.7.1–5B.7.22. doi:10.1002/cpsc.46.

CRISPR-Cas9 Based Genome Editing of Human Induced Pluripotent Stem Cells

Joseph C. Giacalone[1,2,*], **Tasneem P. Sharma**[1,2,*], **Erin R. Burnight**[1,2], **John F. Fingert**[1,2], **Robert F. Mullins**[1,2], **Edwin M. Stone**[1,2], and **Budd A. Tucker**[1,2,†]

[1]Stephen A. Wynn Institute for Vision Research, Carver College of Medicine, University of Iowa, Iowa City, IA

[2]Department of Ophthalmology and Visual Sciences, Carver College of Medicine, University of Iowa, Iowa City, IA

Abstract

Emergence of the CRISPR-Cas9 system has revolutionized the field of genome editing. Specifically, by introducing inexpensive reagents, that are relatively straightforward to design and validate, it is now possible to correct genetic variants or insert desired sequences at any location within the genome. As such, CRISPR based genome editing of patient-specific iPSCs shows great promise for future autologous cell replacement therapies. One caveat however, is that hiPSCs are notoriously difficult to transfect and optimized experimental design considerations are often necessary. In this unit, we describe design strategies and methods for efficient CRISPR-genome editing of patient- specific iPSCs. Additionally, we detail a flexible approach that utilizes positive selection to generate clones with a desired genomic modification, Cre-lox recombination to remove the integrated selection cassette, and negative selection to eliminate residual hiPSCs with intact selection cassettes.

Keywords

hiPSC; genome-editing; CRISPR-Cas9; autologous cell replacement

Introduction

For decades geneticists have used restriction enzymes to manipulate DNA for research and diagnostic purposes. These nucleases arose during evolution as an adaptive immune system in bacteria designed to recognize and cleave specific sequences of foreign DNA (Bikard and Marraffini, 2012). The specificity of typical restriction enzymes is limited by the fact that targeted motifs can occur at multiple locations across the human genome. Unlike traditional restriction enzymes, CRISPR-Cas9, which also has an innate immune function in bacteria, has recently been adapted to genome editing applications in mammalian cells (Mali et al.,

[†]Corresponding author: Budd A. Tucker, Stephen A. Wynn Institute for Vision Research, Department of Ophthalmology and Visual Sciences, Carver College of Medicine, University of Iowa, 375 Newton Road, Iowa City, IA, 52242. Telephone: 319-355-7242; budd-tucker@uiowa.edu.

[*]Both authors contributed equally to this manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

2013; Cong et al., 2013; Jinek et al., 2013; Maeder et al., 2013). The importance of this system derives from the fact that it can be custom engineered by choosing a guide RNA (gRNA) for a specific sequence of interest. Upon recognition of the targeted sequence, the Cas9 nuclease creates a double stranded break (DSB) that is repaired most commonly through non-homologous end joining. This repair pathway results in deletions and insertions, which can be used to modify regulatory or intronic sequences, or for gene deletion. Alternatively, the CRISPR-Cas9 system can be used to repair the genome via homologous recombination (HR), which requires co-delivery of a donor DNA template with homologous sequence flanking the double stranded break. The low-cost and high-efficiency of CRISPR-Cas9-based genome editing makes it a powerful tool for manipulating cells for research and future disease treatment.

With the advent of the human induced pluripotent stem cell (hiPSCs) it is now possible to generate virtually any cell type from any individual regardless of sex or age (Wiley et al., 2016). This makes the iPSC ideal for cell replacement purposes, that is, it has the potential to act as an autologous cell source that is void of the ethical concerns associated with harvest and use of embryonic tissue. For Mendelian disease, the one drawback of using autologous iPSCs for cell replacement is that genetic correction of the patient's disease causing mutation will likely be required prior to differentiation and transplantation.

Although progress in CRISPR-based genome editing has provided scientists with endless opportunities to genetically modify a variety of cell types *in vitro*, hiPSCs are difficult to transfect. Furthermore, selection and expansion of CRISPR corrected iPSC clones is often laborious and time consuming. In this unit, we describe an approach for CRISPR-mediated genome editing of hiPSCs. We then detail two robust methods for delivering CRISPR-Cas9 reagents to patient-specific iPSCs. The first protocol utilizes an electroporation-based system (**Protocol 1**), which requires instrumentation to transfect hiPSCs. As an alternative, the subsequent protocol offers an equally effective reagent-based approach (**Alternate Protocol 1**) that eliminates the need for expensive instrumentation and reduces the complexity. To obtain corrected clones, we include a protocol that details a method for positive selection of corrected clones (**Protocol 2**). Then, we present basic methods for analyzing the experimental outcomes (**Protocol 3**). Finally, we include a protocol for Cre-mediated excision of the reporter construct, and negative selection of the remaining floxed cells (**Protocol 4**).

Experimental Preparation

Design considerations

Cells can respond to DNA damage through multiple repair pathways such as non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ, which occurs most frequently, is typically imperfect, i.e. NHEJ based creation of small insertions and/or deletions is common. Alternatively, HR is a high fidelity DNA repair process, which relies on the use of a homologous DNA template. Not surprisingly, the HR pathway is markedly less efficient then NHEJ, with reported frequencies in hiPSCs between 0.2%–10% (Miyaoka et al., 2016; Mali et al., 2013). In our experience focusing on genes that cause inherited retinal disease (Burnight et al., 2017), most HR efficiencies in patient-specific iPSCs are at

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

the lower end of this range. We hypothesize that this disconnect may be due to a selection bias. Logically early experiments focused on either previously studied or tractable regions of the genome.

Importantly, these two repair pathways allow researchers to modify the genome in two distinct ways, 1) gene disruption or 2) targeted gene modification. If gene disruption is intended, NHEJ is typically the preferred mode of repair. For instance, by targeting early exons NHEJ induces insertions and deletions which can disrupt gene function through non-sense mediated decay and/or induction of a frame shift and creation of an early stop codon. To correct a mutation in the genome or to insert a foreign sequence at a specific location, the homologous recombination pathway is employed. This is accomplished though a repair template containing the desired sequence modifications. Regardless, both approaches require effective use of the CRISPR-Cas9 system.

CRISPR-Cas9 System

The entire CRISPR-Cas9 system can be delivered in a single plasmid. A typical CRISPR-Cas9 plasmid contains two main expression cassettes: an RNA Pol III promoter driving expression of a chimeric, single small guide RNA (sgRNA) and a constitutive RNA Pol II promoter driving the expression of the Cas9 gene. For this protocol, we use pX330-U6-Chimeric_BB-CBh-hSpCas9 (pX330), which is available through Addgene (#42230). This approach requires the researcher to identify and clone a guide sequence of interest to obtain a functional plasmid. The guide sequence, also referred to as a protospacer, is generally 20 base pairs and must be followed by a protospacer adjacent motif (PAM) in the genome.

Many online tools enable researchers to design appropriate guides for a genomic sequence of interest. Some of note are CRISPOR (http://crispor.org) (Haeussler et al., 2016) and benchling.com which streamline the results of multiple published algorithms that assess on-target and off-target predictions. Nuclease activity can vary across a region of interest, and therefore multiple guides should be chosen to find a region amenable to modification. When considering a location in the genome, repetitive regions, which constitute poor candidates for guide sequences due to a drastic increase in off-target sites, should be avoided.

After guide sequences are chosen, oligos can be ordered from companies such as IDT. The px330 contains Bbs1 cloning sites for insertion of the guide sequence. Therefore, the top oligo sequence must be preceded by a 5′CACC sequence and the bottom oligo must be preceded a by 5′AAAC sequence. All final plasmids must be endotoxin free (Macherey-Nagel/Clontech NucleoBond Xtra Maxi EF kit).

Homology Construct Design

Co-delivery of a homologous template during genome editing significantly increases HR efficiencies. There are two types of templates to consider: 1) a double-stranded plasmid DNA or 2) a single-stranded oligodeoxynucleotide (ssODN). For this protocol, we use a plasmid-based homology construct referred to as a homology-directed repair plasmid (pHDR). A typical pHDR design includes 500bp–1000bp homology arms, and the DSB should occur within 200 base pairs of the intended modification (Ratz et al., 2015; Merkle et al., 2015; Byrne et al., 2014). As mentioned previously, the “optimal” distance between DSB

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

and desired modification may not be possible due low complexity regions of the genome. In our experience, there is flexibility in the distance from the DSB to the intended genomic modification as well as the required length of homology arm, which must be determined experimentally. Even after HR occurs, the continued presence of Cas9 nuclease may result in re-cutting events. To avoid unintended NHEJ, homology templates commonly include nucleotide changes to disrupt the PAM sequence or the proximal guide sequence. If the guide sequence falls in an exon, wobble position changes preserve the peptide sequence. Finally, during HR, the invading strand crosses the DSB to repair the genome. Therefore placing the intended genomic modification between the DSB and selection cassette links correction with selection.

For instructive purposes, we will briefly discuss the similarities and differences between the three-pHDR designs presented in this unit. Generally, the plasmid contains ~1–2kb of homologous sequence that spans the targeted region. Also, each expression cassette has an upstream promoter sequence and downstream polyadenylation sequence, which are vital for gene expression. Moreover, for stable integration, the EF1α promoter or PGK promoter is typically chosen because the CMV promoter can be silenced in hiPSCs (Luo et al., 2014). In the first example, we generate a pHDR that contains sequence homology for the gene *MYOC* and a non-integrating mCherry expression cassette to demonstrate the co-delivery of pX330 and pHDR (Figure 1). Additionally, because the reporter is transiently expressed, mCherry positivity only indicates the potential for HR. For the next example, we designed a construct that allows for positive selection (pHDR^{+}) 0f genome-edited hiPSCs (Figure 2). In the second example, the pHDR^{+} contains a floxed puromycin resistance cassette located centrally in the homology template for *MYOC* such that HR incorporates the cassette into an intron (Figure 2). As a result, drug treatment selects for cells corrected via the HR pathway. However, transcription from intronic selection cassettes may disrupt target gene expression (Zou et al., 2011). One can transfect the cells with Cre-recombinase to excise the disruptive sequence, but this creates a heterogeneous population of cells with excised and intact reporters. Therefore, to efficiently remove hiPSCs with intact selection cassettes, we illustrate a dual selection strategy in the final example that utilizes both positive and negative selection (pHDR$^{+/-}$) to correct a mutant *RPGR* allele (Figure 3). To generate the pHDR$^{+/-}$, we replaced the stop codon in the puromycin resistance sequence with a porcine 2A peptide (P2A) coding sequence followed by the Herpes Simplex Virus type 1 thymidine kinase (vTK) gene. Translation of the 2A peptide causes ribosomal skipping resulting in two protein products (Kim et al., 2011). As mentioned previously, flanking the dual selection cassette with loxP sites that are oriented in the same direction enables Cre-mediated excision. If the cassettes persist, the cell continues to express vTK which phosphorylates ganciclovir, a nucleoside analogue, which disrupts DNA synthesis (Chakraborty et al., 2013; Wang et al., 2004). Thus, ganciclovir treatment selects against vTK^{+} cells resulting in a homogenous population of hiPSCs.

Cre-mediated excision leaves a single loxP site of 34 base pairs in the genome. While the remaining sequence is unlikely to be deleterious (Zhu et al., 2015; Meier et al., 2010), the following strategies avoid disrupting the transcriptome. First, any intronic space proximal to exons should be avoided because there is potential to interrupt sequences important for splicing. Online bioinformatic tools, such as Human Splice Finder (http://www.umd.be/

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

HSF3/HSF.shtml), can predict if a candidate sequence affects splicing (Desmet et al., 2009). Secondly, historical knowledge can be used such as previous experiments targeting the same gene of interest. Lastly, highly conserved, non-coding regions of the genome should be avoided to prevent disrupting features such as enhancers or alternative promoters (Meier et al., 2010). Conservation can be viewed using the UCSC Genome Browser (https://genome.ucsc.edu/).

ssODNs can also be used as homology templates. They are typically designed based on either strand with 90 base pair arms (180 bases total) flanking the intended genomic modification (Ran et al., 2013). Importantly, sgRNAs should be chosen such that the DSBs occur approximately 10 base pairs away from the intended modification for efficient HR (Yang et al., 2013). Because of technical hurdles, earlier ssODN based strategies lacked reporter cassettes (Quadros et al., 2017), and therefore required either high levels of HR to sample a corrected clone or FACS. Cell sorting approaches require MEF feeder layers for hiPSC survival (Byrne and Church, 2015) limiting their use for cell replacement therapies. Experiments using ssODNs often use the term “scarless” since there is no residual motif or sequence such as a loxP. However, this term excludes changes in the genome caused by off-target nuclease activity. Recent studies have successfully used long ssODN containing reporters (Quadros et al., 2017). Both pHDRs and ssODNs can be ordered commercially. To reduce costs, a reusable pHDR$^{+\text{or}+/-}$ can be synthesized commercially with two multiple cloning sites surrounding the selection cassette. Then, the required homology arms can be generated inexpensively by PCR of gDNA and cloned. The pHDR should be endotoxin free (Macherey-Nagel/Clontech NucleoBond Xtra Maxi EF kit).

Methods For CRISPR-Cas9 Genome Editing

We will now describe two different approaches we routinely use to transfect hiPSCs. The first protocol describes use of the NEON/reagent transfection system and the second utilizes a liposome based-reagent for transfection. Only one approach is necessary. Therefore, labs without the NEON transfection system, please begin at **Alternate Protocol 1** (note - if needed, hiPSC lines are publically available at ATCC (https://www.atcc.org/)). If a project requires patient-specific lines, please refer to our method describing the generation of xeno-free, cGMP-compliant patient-specific iPSCs from skin biopsies (Wiley et al., 2017). The following transfection protocols use patient-specific iPSC lines generated as per our xeno-free, cGMP-compliant protocol.

Human Subjects

Stem cells used to demonstrate this protocol were derived from human patients. All patients provided written, informed consent for this study, which was approved by the Institutional Review Board of the University of Iowa (project approval #200202022) and adhered to the tenets set forth in the Declaration of Helsinki.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

BASIC PROTOCOL 1 Electroporation of hiPSCs to deliver CRISPR-Cas9 containing plasmids

The following protocol describes the optimized conditions for using the NEON/reagent transfection system for CRISPR-based genome editing of hiPSCs cultured under xeno-free conditions cultured on recombinant human laminin-521 in E8 media (for detailed iPSC generation and culture protocol please see (Wiley et al., 2017)). These steps can be employed to efficiently deliver the CRISPR-Cas9 plasmid (pX330) alone to induce insertions and deletions through NHEJ or with a homology directed repair plasmid (pHDR) to achieve HR-mediated modifications.

Materials and Equipment

- NEON Transfection system (Cat. No. MPK5000; Thermo Fisher Scientific, Waltham, MA, USA)
- NEON Transfection System 100 ul Kit (Cat. No. MPK10096; Thermo Fisher Scientific, Waltham, MA, USA)
- pX330-U6-Chimeric_BB-CBh-hSpCas9 (Plasmid #42230; Addgene, Cambridge, MA USA)
- Homology-directed Repair Plasmid (pHDR) *optional*
- Essential 8 Medium (Cat. No. A1517001; Thermo Fisher Scientific, Waltham, MA, USA)
- Versene solution (Cat. No. 15040066; Thermo Fisher Scientific, Waltham, MA, USA)
- RevitaCell™ Supplement (100X) (Cat. No. A2644501; Thermo Fisher Scientific, Waltham, MA, USA)
- Primocin™ (Cat. No. ant-pm-1; InvivoGen, San Diego, CA, USA)
- rhFGF2 (Waisman Biomanufacturing, Madison, WI; Cat#: rhFGF)
- Recombinant human laminin-521 (rhLaminin-521) (Corning Life Sciences; Tewksbury, CA; Cat#: 354222)
- Dulbecco's Phosphate Buffered Saline (Cat. No. 15040066; Thermo Fisher Scientific, Waltham, MA, USA)

Preparation of hiPSCs for Neon transfection

1 Expand hiPSCs in Essential 8 (E8) media supplemented with rhFGF2 (10 ng/ml) and primocin (100 μg/ml) on rhLaminin-521 coated six-well tissue culture treated plates.

Note: Each electroporation reaction will need ~7.0×10^5 to 1.0×10^6 cells. Typically, one well will yield around 7×10^5 cells. A single electroporation reaction requires ~7.0×10^5 to 1.0×10^6 cells. We typically include a reaction without plasmid as a control.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

2 Prepare rhLaminin-521 coated, six-well tissue culture treated plates on which to seed the hiPSCs after the electroporation reaction. One well of a six-well tissue culture treated plate is required per transfection.

Note: This step requires advanced planning due to extended coating times. We typically dilute 1 mL of rhLaminin-521 into 19 mL of 1× DPBS. We then coat the necessary number of six-well tissue culture treated plates with 1.5 mL of rhLaminin-521 solution per well. The plates can be sealed and stored overnight at 4°C or incubated for 2 hours at 37°C. To avoid potential sample swaps, we maintain distinct lines on separate plates.

3 Thirty minutes before electroporation, pre-treat hiPSC cultures with E8 media containing 1X RevitaCell™ and rhFGF2 (10 ng/ml) and return them to the incubator.

Note: RevitaCell™ contains a ROCK inhibitor, which may result in a spindle shaped cell morphology. This change is temporary and will not affect the pluripotency of the line. The morphology will reverse after RevitaCell™ is removed.

4 To set up the NEON transfection system, obtain Buffer R, Buffer E2 and 100ul NEON tips.

5 Next, aliquot the appropriate amount of plasmid into a 1.5 mL microfuge tube.

a. For a gene disruption strategy, use 2 μg of pX330 per reaction.

b. For a gene correction strategy, use 1.2 μg pX330 and 800 ng pHDR per reaction. This assumes a 1:1 molar ratio of a ~10kb pX330 and ~6kb pHDR.

Note: The volume of plasmid DNA should not exceed 10% of the electroporation reaction. Additionally, the total amount of plasmid DNA can be increased, but cell viability will decrease.

6 Aspirate medium from the pre-treated cells and incubate with 1.5 mL of Versene solution per well for three to five minutes at 37 °C to loosen cellular attachments.

7 Next, aspirate the Versene. Then, gently lift the cells with 1 mL of 1X DPBS to form a single-cell suspension and transfer the single-cell suspension to a sterile conical tube.

Note: Take care to avoid over-pipetting the cells. In our experience, hiPSCs are viable in 1x DPBS for at least an hour. For experiments spanning multiple hours, consider transfecting the cells in batches.

8 Count the single-cell suspension and transfer the appropriate amount to a new sterile conical tube.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Note: To calculate volume of the single-cell suspension needed, plan on using ~7.0 × 10^5 to 1.0 × 10^6 cells per transfection. Use of any cell counter or hemocytometer is appropriate.

9 Centrifuge the cells at 0.1 RCF for three minutes. During this time, obtain rhLaminin-521 coated tissue culture treated plates from step 3. Carefully remove the residual buffer without scraping the well, and transfer 1.5 mL of E8 media supplemented with rhFGF2 (10 ng/mL) and 1X RevitaCell™ without antibiotics (E8, rhFGF2, 1X RevitaCell™) to each well. This plate will be used after the electroporation protocol.

Electroporation steps

10 Aspirate the supernatant without disturbing the pellet. Then, gently resuspend the pellet in 120 μL of Buffer R per reaction and transfer the suspension to the appropriate microfuge tube(s) from step 7.

Note: we have not observed drastic changes in hiPSC viability for cells stored in 1x Buffer R for at least an hour.

11 Program the NEON transfection system using the following parameters: 1100 Volts, 30 ms, and 1 pulse.

12 To set up the instrument, fill the Neon Tube with 3 mL of Buffer E2 and insert the Neon Tube into the Neon Pipette Station as per manufacturer's instructions.

13 Using the Neon pipette, aspirate the reaction from step 10 with a Neon 100 μL tip.

Note: Avoid incorporating air bubbles in the tip, which will decrease the efficiency of the electroporation. Each tip can be used twice for replicates before discarding.

14 Place the Neon pipette vertically into the Neon Tube located within the Neon Pipette Station and operate the system according to the manufacturer's instructions.

15 When the protocol finishes, remove the Neon pipette and transfer the treated cells to the appropriate well on the plate set up in step 9.

16 Incubate six-well tissue culture treated plate(s) overnight at 37°C, 5% CO_2.

17 On the next day, replace the media with E8 media containing 1X RevitaCell™, (10 ng/ml), primocin (100 μg/mL), and rhFGF2 (10 ng/mL).

Post-electroporation

Because plasmid-based transfections require time for nuclear incorporation and expression, we typically wait ~24–48 hours before proceeding to any of the downstream protocols. To select for corrected clones, proceed to **Protocol 2** after 24 hours. To obtain gDNA and RNA and analyze the nuclease efficiency and genomic correction, proceed to **Protocol 3** after 48–72 hours.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

As an exemplar, we designed an experiment to modify the gene *MYOC* that included a pHDR with a non-intergrating mCherry expression cassette (Figure 1A&B). To monitor delivery of the CRISPR plasmid, we also modified the pX330 plasmid by inserting a GFP expression cassette upstream of the hU6 promoter to gauge the relative efficiency/success of the transfections after 24 hours (Figure 1B). We then transfected the hiPSCs with varying amounts of pX330 and observed reporter expression again after 24 hours (Figure 1C). After 72 hours, we isolated gDNA and analyzed the DSB efficiency using the T7 Endonuclease I assay described in **Protocol 3** (Figure 1D). Finally, co-delivery of pX330 and pHDR was confirmed post-transfection by the co-localization of mCherry and GFP expression in hiPSC after 24 hours (Figure 1F).

ALTERNATE PROTOCOL 1 Reagent-Based Transfection of hiPSCs to Deliver CRISPR-Cas9 Plasmid

This protocol describes an alternative approach that uses Lipofectamine Stem Transfection Reagent for plasmid-based genome editing of hiPSCs cultured under xeno-free conditions cultured on recombinant human laminin-521 in E8 media (for detailed iPSC generation and culture protocol please see (Wiley et al., 2017)). If the Neon Transfection system is being used (**Protocol 1**), then this protocol is not necessary. This technique can efficiently deliver the CRISPR-Cas9 plasmid (pX330) alone to induce insertions and deletions through NHEJ or with a homology directed repair plasmid (pHDR) to achieve HR mediated modifications.

Materials and Equipment

- pX330-U6-Chimeric_BB-CBh-hSpCas9 (Plasmid #42230; Addgene, Cambridge, MA USA)
- Homology-directed Repair Plasmid *optional*
- Opti-MEM® I Reduced Serum Medium (Cat. No. 31985070; Thermo Fisher Scientific, Waltham, MA, USA)
- Essential 8 Medium (Cat. No. A1517001; Thermo Fisher Scientific, Waltham, MA, USA)
- Versene solution (Cat. No. 15040066; Thermo Fisher Scientific, Waltham, MA, USA)
- Lipofectamine Stem Transfection Reagent (Cat. No. STEM00001; Thermo Fisher Scientific, Waltham, MA, USA)
- RevitaCell™ Supplement (100X) (Cat. No. A2644501; Thermo Fisher Scientific, Waltham, MA, USA)
- Primocin™ (Cat. No. ant-pm-1; InvivoGen, San Diego, CA, USA)
- rhFGF2 (Waisman Biomanufacturing, Madison, WI; Cat#: rhFGF)
- Dulbecco’s Phosphate Buffered Saline (Cat. No. 15040066; Thermo Fisher Scientific, Waltham, MA, USA)

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Preparation and Transfection of iPSCs

1. Expand hiPSCs in E8 media supplemented with rhFGF2 (10 ng/mL) and primocin (100 μg/mL) on rhLaminin-521 coated six-well tissue culture treated plates.

   Note: Each transfection reaction will require one well of a six-well tissue culture treated plate of hiPSCs at 40–50% confluency.

2. Thirty minutes before transfection, pre-treat hiPSC cultures with 1.5 mL of E8 media containing 1x Revitacell™, rhFGF2 (10 ng/mL), and primocin (100 μg/mL). Return plate(s) to the incubator.

3. To prepare for the transfection, obtain Opti-MEM®I medium, and Lipofectamine Stem Transfection Reagent and allow the components to warm to room temperature.

   Note: RevitaCell™ contains a ROCK inhibitor, which may result in a spindle shaped cell morphology. This change is temporary and will not affect the pluripotency of the line. The morphology will reverse after RevitaCell™ is removed.

4. Next, aliquot the appropriate amount of plasmid into a 1.5 mL microfuge tube.

   a. For a gene disruption strategy, use 2 μg of pX330 per reaction.

   b. For a gene correction strategy, use 1.2 μg pX330 and 800 ng pHDR per reaction. This assumes a 1:1 molar ratio of a ~10 kb pX330 and ~6 kb pHDR.

      Note: *Please note that the total amount of plasmid DNA can be increased. However this may result in a marked decrease in cell viability.*

5. Add 50 μL of Opti-MEM®I medium per reaction to microfuge tube(s) from step 5.

6. Next, add 12.5 μL of Lipofectamine Stem Transfection Reagent per reaction to the microfuge tube(s) and gently mix.

7. Incubate the Lipofectamine Stem Transfection Reagent complexes for ten minutes at room temperature.

8. Evenly distribute the transfection complex to the appropriate well of the pre-treated plate from step 2.

9. Incubate cells for 24 to 48 hours and observe cells for reporter expression. Removal of complexes by changing the media after transfection is not required.

Post-transfection

Because plasmid-based transfections require time for nuclear incorporation and expression, we typically wait ~24–48 hours before proceeding to any of the downstream protocols. To select for corrected clones, proceed to **Protocol 2** after 24 hours. To obtain gDNA and RNA

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

to analyze the nuclease efficiency and genomic correction, proceed to **Protocol 3** after 48–72 hours.

As indicated above to determine transfection efficiency, our modified pX330-GFP expression plasmid was used (Figure 1E). While both transfection protocols work efficiently, cellular morphology indicates that hiPSCs appear healthier after 24 hours post-transfection when using Lipofectamine Stem Transfection Reagent (Figure 1E) compared to electroporation (Figure 1C).

Basic Protocol 2 Positive Selection of Genomically Modified hiPSCs

The following protocol details a method to select for corrected iPSC clones generated in **Protocol 1** and **Alternate Protocol 1** by co-delivery with a pHDR^{+} or pHDR$^{+/-}$ plasmid. To select for drug resistance clones, we typically start with a low level of puromycin and slowly increase the concentration over two weeks. One six-well plate of untreated cells may be included to monitor for issues with selection (i.e. all untreated hiPSCs should be dead after two weeks of treatment). After seven days, resistant clones should begin to emerge. By day 12 any surviving clones will need to be manually picked and expanded. It is advisable to obtain and analyze gDNA after positive selection. As clones with the desired genomic modifications are identified, generate stocks using the PSC Cryopreservation Kit (Cat. No. A2644601; Thermo Fisher Scientific, Waltham, MA, USA).

Note: For gene disruption strategies delivering pX330 alone skip to **Protocol 3**.

Materials and Equipment

- hiPSCs treated according to either **Protocol 1** or **Alternate Protocol 1**
- Puromycin Dihydrochloride (Cat. No. A1113802; Thermo Fisher Scientific, Waltham, MA, USA)
- StemPro™ EZPassage™ Disposable Stem Cell Passaging Tool (Cat. No. 23181010; Thermo Fisher Scientific, Waltham, MA, USA)
- Essential 8 Medium (Cat. No. A1517001; Thermo Fisher Scientific, Waltham, MA, USA)
- RevitaCell™ Supplement (100X) (Cat. No. A2644501; Thermo Fisher Scientific, Waltham, MA, USA)
- Recombinant human laminin-521 (Cat#: 354222; Corning Life Sciences; Tewksbury, CA)
- Costar 12-well tissue culture-treated sterile plates (Cat#: 3513; Corning Life Sciences, Tewksbury, CA)

Positive Selection (Puromycin) of Corrected iPSCs

1. Begin selection of hiPSCs transfected with pX330 and pHDR^{+} or pHDR $^{+/-}$ in either **Protocol 1** or **Alternate Protocol 1**. For all subsequent positive selection

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

steps, supplement E8 media with primocin (100 μg/mL) and rhFGF2 (10 ng/mL) unless otherwise noted. Coat six-well tissue culture treated plates as necessary.

Note: rhLaminin-521 coated 12-well tissue culture treated plates will be needed to pick clones on day 12. Dilute 1 mL of rhLaminin-521 into 19 mL of 1x DBPS and coat each well with 0.75 mL. rhLaminin-521 coated six-well tissue-culture treated plates will be necessary to expand clones that survive after day 15. Include an untreated control to monitor selection.

2. Aspirate media from six-well tissue culture treated plate(s) of interest.

3. Day 1 – 2: 24 hours after stem cell transfection, treat cells with 2 mL of E8 media containing 0.05ug/mL of puromycin [0.25 μL of the stock (10 mg/uL) into 50 mL of E8].

   Note: Drug selection must begin before cells become overly confluent. For wells between 70–80% confluent, expand the cells before selection.

4. Day 3 – 7: Treat cells with E8 media containing 0.1ug/mL of puromycin (0.5 μL of the stock (10 mg/μL) into 50 mL of E8).

5. Day 8 – ~11: Treat cells with 2 mL of E8 media containing 0.5 μg/mL of puromycin [2.5 μL of the stock (10 mg/μL) into 50 mL of E8].

   Note: If an untreated control is included, these cells should be nearly or completely dead around day 10 or 11. Surviving colonies should be evident at this point, which are large enough to passage for expansion and further analysis.

6. Day 12: Switch all media to E8 medium supplemented with RevitaCell™. Also obtain rhLaminin-521 coated 12-well tissue culture treated plates and replace residual buffer with 0.75 mL of E8 medium supplemented with 1x RevitaCell™. To passage corrected clones, use the StemPro EZPassage Disposable Stem Cell Passaging Tool to loosen colony. The colony can then be gently pipetted and transferred to the appropriate location on the 12-well tissue culture treated plate.

   Note: Exclude drug selection during passaging. Gently loosen the colony using StemPro EZPassage Disposable Stem Cell Passaging Tool. Applying too much pressure will dislodge the colony. If this occurs, change media to avoid contaminating subsequent colonies.

7. Day 13 – 15: Treat isolated colonies with E8 medium containing 0.5 μg/mL puromycin.

   Note: Not all of the isolated clones survive at this point which is normal.

At this point, we expand the surviving clones on six-well tissue culture treated plates. Maintain at least three wells of a six-well tissue culture treated plate per clone so that gDNA and RNA can be collected and analyzed using **Protocol 3**. As a proof of concept, we

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

generated patient-specific stem cells from an individual with a mutation in the *MYOC* gene and designed a pHDR$^+$ capable of correcting the targeted mutation (Figure 2A&B).

Assessment of Genomic Modification (PROTOCOL 3)

This protocol describes basic methods to obtain gDNA and RNA as well as methods to analyze the experimental results. For amplifying genomic DNA, design primers using bioinformatics tools such as Primer3 or IDT's PrimerQuest tool. To assay for Cas9 nuclease activity, PCR amplify across the targeted genomic region. Then, proceed to the heteroduplex formation step, which is followed by the T7 endonuclease I assay. This sequence of events denatures the PCR products and slowly anneals them to allow for mismatched products to rejoin. These mismatches result from NHEJ repair of Cas9-mediated DSBs. T7 endonuclease I will cleave any PCR products containing mismatches. Alternatively, to screen for the correct placement of the selection cassette in the genome, design one primer to target a unique region of the selection cassette and the other primer to exclude sequences found in the homology construct. This design strategy prevents amplification of residual plasmid DNA. Finally for rt-PCR, Primer-BLAST can be used for primer design.

Materials and Equipment

- NucleoSpin RNA (cat. no. 740955; Macherey-Nagel/Clontech, Mountain View, CA, USA)
- NucleoSpin Tissue (cat. no. 740952; Macherey-Nagel/Clontech, Mountain View, CA, USA)
- AccuPrime Taq DNA Polymerase, high fidelity (Cat. No. 12346086; Thermo Fisher Scientific, Waltham, MA, USA)
- T7 Endonuclease I (cat. no. M0302; New England BioLabs, Ipswich, MA, USA)
- NanoDrop One Microvolume UV-Vis Spectrophotometer (Cat. No. ND-ONE-W; Thermo Fisher Scientific, Waltham, MA, USA)
- T100 Thermal Cycler (Cat. No. 1861096; Biorad, Hercules, California, USA)
- MinElute PCR Purification Kit (Cat. No. 28004; Qiagen, Germantown, MD, USA)
- E-Gel Agarose Gels Starter Pak, 2% (Cat. No. G600002; Thermo Fisher Scientific, Waltham, MA, USA)
- SuperScript VILO cDNA Synthesis Kit (Cat. No. 11754–050; Thermo Fisher Scientific, Waltham, MA, USA)

Isolation of RNA and cDNA synthesis

1 Remove culture medium from the wells containing cells of interest.

Note: When appropriate, the subsequent steps can be performed under non-sterile conditions.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

2. Perform RNA isolation as per manufacturer's instructions with one modification. For the first step, 350 μL Buffer RA1 and 3.5 μL β-mercaptoethanol directly to each well of interest.

   Note: hiPSCs can be collected under sterile conditions by using ***Protocol 1*** *steps 7–8.*

3. Determine concentration and quality of each RNA sample using the Nanodrop. Other forms of DNA quantification are also sufficient.

4. Generate cDNA using SuperScript VILO cDNA Synthesis Kit per the manufacturer's instructions.

Isolation of gDNA

5. Remove culture medium from the wells containing cells of interest.

   Note: When appropriate, the subsequent steps can be performed under non-sterile conditions.

6. Perform gDNA isolation as per manufacturer's instructions with one modification. For the first step, add 200 μL Buffer T1, 25 μL Proteinase K solution, and 200 μL Buffer B3 directly to each well of interest.

   Note: hiPSCs can be collected under sterile conditions by using ***Protocol 1*** *steps 7–8.*

7. Determine concentration of each gDNA samples using the Nanodrop. Other forms of DNA quantification are also sufficient.

PCR Set Up

8. Set up PCR reaction(s) as follows:

   a. PCR components

      i. AccuPrime Reaction Buffer II 5 μl

      ii. gDNA 100 ng

      iii. target oligo F (20nM) 2 μl

      iv. target oligo R (20nM) 2 μl

         note: One primer must target sequence not contained in the homology directed repair plasmid to avoid amplification of residual plasmid DNA. Repeat this process for the upstream junction. For rt-PCR it is important to include amplifications of housekeeping genes such as POL2RA as a control.

      v. AccuPrime Taq 0.25 μl

      vi. ddH_2O to 50 μL

   b. Cycling Parameters

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

i. 94°C for 3 minutes

ii. 94°C for 15 seconds

iii. 58°C for 20 seconds

iv. 72°C for 1 minute

v. Repeat steps ii-iv 34 times

Note: For amplification of cDNA repeat steps ii–iv 40 times.

vi. 72°C for 5 minutes

vii. 4°C for ∞

9 9. Check amplification by running 5ul of each PCR reaction on a 2% E-gel.

Note: Any method of DNA electrophoresis can be substituted for the E-gel system. It is important that the PCR results in a single product of the correct size.

10 10. Column purify the remainder of each reaction using the Qiagen MinElute PCR purification kit per the kit's instructions

Note: We typically elute the purified PCR product with 10 μL of ddH2O.

11 Determine concentrations of the purified PCR products using the Nanodrop.

Note: To continue with the next section, at least 200 ng of the PCR product are required. For inefficient PCRs, perform replicates and purify them together.

Heteroduplex Formation

Heteroduplex formation is only necessary to perform the T7 Endonuclease I assay. This protocol denatures the PCR products and slowly anneals them to allow for mismatching products to form. These mismatches are due to Cas9-medaited DSBs inducing NHEJ.

12 Set up the heteroduplex reaction as follows:

a. Reaction components

i. 10X NEB Buffer 2 μL (*Note this is supplied with the T7 Endonuclease I)*

ii. Purified PCR product 200 ng

iii. ddH_2O to 20 μL

b. Heteroduplex parameters

i. 95°C for 10 minutes

ii. 95–85°C, −2°C/s

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

iii. 85°C 1 minute

iv. 85–75°C, −2°C/s

v. 75°C 1 minute

vi. 75–65°C, −2°C/s

vii. 65°C 1 minute

viii. 65–55°C, −2°C/s

ix. 55°C 1 minute

x. 55–45°C, −2°C/s

xi. 45°C 1 minute

xii. 45–35°C, −2°C/s

xiii. 35°C 1 minute

xiv. 35–25°C, −2°C/s

xv. 25°C 1 minute

xvi. 25–4°C, −2°C/s

xvii. 4°C hold

T7 Endonuclease I Assay

This assay detects imperfectly annealed PCR products resulting in a cleavage event. Therefore, it is ideal to detect Cas9 nuclease activity at a particular locus. Figure 2D demonstrates the results of the T7 Endonuclease I assay.

13 Add 1 μL of T7 Endonuclease I to each heteroduplexed reaction and incubate at 37 °C for 30 minutes.

14 Run entire reaction on a 2% E-gel.

Note: SNPs and sequences with secondary structures may result in false positives. Therefore, an untreated control is needed to validate the results.

As mentioned previously, we generated patient-specific stem cells from an individual with a mutation in the *MYOC* gene and designed a pHDR$^+$ capable of correcting the targeted mutation. To validate the location of the *MYOC* selection cassette, we amplified the gDNA using a forward primer in the SV40 polyadenylation sequence and a reverse primer sequence located outside of the pHDR (Figure 2C). We typically use Sanger sequencing to validate the sequence at a single nucleotide level.

We screened for clonal purity by amplifying across the location of the guide sequence and performed the T7 Endonuclease I assay (**Protocol 3**) (Figure 2D). In short, we denatured and re-annealed the PCR products allowing for mismatched templates to anneal. T7 Endonuclease I can detect imperfectly annealed PCR products resulting in a cleavage event.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

For example, lane C contains the PCR product of gDNA collected before CRISPR-based genome editing, which results in one distinct band (Figure 2D). The resulting clones can be compared to the control to identify a homogenous population, such as that shown in lane P6. If the genomic modification is correctly located and sequenced, proceed to Cre-Lox recombination to remove the selection cassette (**Protocol 4**).

Removal of Selection Cassette From Genomically Modified hiPSCs and Negative Selection (Protocol 4)

The following protocol details a method to remove the selection cassette after positive selection (**Protocol 2**). To begin, obtain one well of a six-well plate containing the cells of interest at 40%–50% confluency. These cells will be transfected with a Cre-recombinase plasmid to facilitate removal of the selection cassette. After the cells recover, we manually pick colonies expanded from single cells to obtain a homogenous population of cells. Alternatively, for dual selection cassettes, we will use negative selection to remove cells that continue to express vTK. As clones with the desired genomic modifications are identified, it is important generate stocks using the PSC Cryopreservation Kit (Cat. No. A2644601; Thermo Fisher Scientific, Waltham, MA, USA).

Note: This protocol is only necessary for gene editing strategies utilizing a selection cassette (pHDR^{+} or $^{+/-}$). Therefore, skip this step if no selection cassette was used.

Materials and Equipment

- hiPSCs treated according to **Protocol 2**
- Essential 8 Medium (Cat. No. A1517001; Thermo Fisher Scientific, Waltham, MA, USA)
- RevitaCell™ Supplement (100X) (Cat. No. A2644501; Thermo Fisher Scientific, Waltham, MA, USA)
- Recombinant human laminin-521 (Cat#: 354222; Corning Life Sciences; Tewksbury, CA)
- pCSCre2 (Plasmid #:31308; Addgene, Cambridge, MA USA)
- Ganciclovir (Cat#:sud-gcv; Invivogen, San Diego, CA, USA)
- Lipofectamine Stem Transfection Reagent (Cat. No. STEM00001; Thermo Fisher Scientific, Waltham, MA, USA)
- Opti-MEM® I Reduced Serum Medium (Cat. No. 31985070; Thermo Fisher Scientific, Waltham, MA, USA)

Cre-Lox recombination

1 Expand hiPSCs generated in **Protocol 2** in E8 media supplemented with rhFGF2 (10 ng/mL) and primocin (100 μg/mL) on rhLaminin-521 coated six-well tissue culture treated plates.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Note: Each transfection reaction will require one well of a six-well tissue culture treated plate of hiPSCs at 40–50% confluency

2 Maintain rhLaminin-521 coated-6-well tissue culture treated plates to passage hiPSCs after Cre recombinase (pCSCre2) treatment. When passaging from six well plates, we typically distribute ¼ of one well to an entire six-well tissue culture treated plate (~ 100,000 to 300,000 cells per well).

Note: As mentioned previously, this step requires advanced planning due to long rhLamin-521 coating times. We typically dilute 1 mL of rhLaminin-521 into 19 mL of 1x DPBS. We then coat the necessary number of six-well tissue culture treated plates with 1.5 mL of laminin solution per well. The plates can be sealed and stored overnight at 4°C or incubated for 2 hours at 37°C. Coated plates are stable at 4°C for 1 week. To reduce the scale and cost of reagents, we routinely coat less than six wells of a plate. To avoid unintentionally swapping samples, maintain each unique line on separate plates.

3 Thirty minutes before transfection, pre-treat hiPSC cultures with 1.5 mL of media containing 1x RevitaCell™, rhFGF2 (10 ng/mL), and primocin (100 μg/mL). Return plate(s) to the incubator.

4 To prepare for the transfection, obtain Opti-MEM®I medium, and Lipofectamine Stem Transfection Reagent and allow the components to warm to room temperature.

5 Next, aliquot 4 μg of pCSCre2 plasmid per reaction into a 1.5 mL microfuge tube.

6 Add 50 μL of Opti-MEM®I medium per reaction to microfuge tube(s) from step 5.

7 Next, add 12.5 μL of Lipofectamine Stem Transfection Reagent per reaction to the microfuge tube(s) and gently mix.

8 Incubate the Lipofectamine Stem Transfection Reagent complexes for 10 minutes at room temperature.

9 Evenly distribute the transfection complex onto cells cultured in 2 mL of E8 medium containing 1X RevitaCell™, rhFGF2 (10 ng/mL) and primocin (100 μg/mL).

10 For the next 3 days, feed treated cells with 2 mL of E8 medium containing 1X RevitaCell™, rhFGF2 (10 ng/mL) and primocin (100 μg/mL).

Note: RevitaCell™ contains a ROCK inhibitor, which may result in spindle shaped cell morphology. This change is temporary and will not affect the pluripotency of the line. The morphology will reverse after RevitaCell™ is removed.

11 Allow the treated cells to recover until the cells are 80%–85% confluent over the next four to seven days.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Note: Feed cells daily to improve viability. If the cells recover in a shorter period of time, proceed to the next steps. The iPSC cultures must be maintained at a subconfluent state to avoid spontaneous differentiation.

12 Aspirate medium from the pre-treated cells and incubate with 1.5 mL of Versene solution per well for three to five minutes at 37 °C to loosen cellular attachments.

Note: During this time, obtain rhLaminin-521 coated six-well tissue culture treated plates from step 1. Carefully remove the residual buffer without scrapping the bottom of the well. Transfer 1.5 mL of E8 medium supplemented with rhFGF2 (10 ng/ml), 1X RevitaCell™, and primocin (100 μg/mL) to each well.

13 Aspirate the Versene. Gently lift ¼ of the well using E8 medium supplemented with rhFGF2 (10 ng/ml) and RevitaCell™ and distribute 100,000–300,000 cells per well in a six-well tissue culture treated plate to achieve 10%–20% confluency. Continue to step 20 on the following day.

Note: This step does not require a single cell suspension and therefore minimal pipetting is necessary. Overly confluent wells will hinder efficient selection due to the activated drug passing through gap junctions of cells. The cells can be passaged over multiple wells to allow for gDNA and RNA isolation.

Negative Selection (Ganciclovir) of Corrected iPSCs

14 Maintain rhLaminin-521 coated six-well tissue-culture treated plates for passaging. For all subsequent negative selection steps, supplement E8 media with primocin (100 μg/mL) and rhFGF2 (10 ng/mL) unless otherwise noted.

Note: This protocol differs from puromycin selection because the concentration of ganciclovir will be increased over multiple expansions.

15 To begin negative selection, replace media with complete E8 media containing 4 nM ganciclovir.

Note: The concentration of the ganciclovir stock solution is 39.2 mM. To obtain a working stock solution of 4 μM ganciclovir, add 5.1 uL to 50mL of complete E8 media. This solution can then be diluted as required. Because negative selection results in cell death, we typically include one well of our positively selected cells that have not been treated with Cre recombinase as a control. In order to achieve meaningful negative selection, these cells must die during ganciclovir treatment.

16 Feed the cells daily with complete E8 media supplemented with 4 nM ganciclovir (add 50uL of the *4 μM ganciclovir* working stock to 49.95 mL of E8

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

media) until the well is approximately 80%–85% confluent. Then, passage the well according to steps 18–19.

Note: Do not treat with ganciclovir again for 24 hours.

17 On the following day, replace media with complete E8 media containing 40 nM ganciclovir (add 500uL of the *4 μM ganciclovir* working stock to 49.5 mL of E8 media).

18 Feed the cells daily with complete E8 media containing a 40 nM ganciclovir until the well is approximately 80%–85% confluent. Then, passage the well according to steps 18–19.

Note: Do not treat with ganciclovir again for 24 hours.

19 On the following day, replace the media with complete E8 containing 4 uM ganciclovir.

20 Finally, feed the cells daily with complete E8 media containing 4 uM ganciclvoir until the well is approximately 80%–85% confluent.

21 At this point, a homogenous population of corrected cells has been achieved.

22 Expand the cells to maintain them while collecting both gDNA and RNA

Note: If no cell survival is observed, repeat ***Cre-Lox recombination*** *steps to increase the efficiency.*

To illustrate the dual selection, we designed a strategy to correct a mutation in the gene *RPGR* (Figure 3A). To confirm the proper location of the selection cassette, we amplified gDNA using a forward primer unique to the selection cassette (SV40 polyadenylation sequence) and a reverse primer only found in the genome (Reverse 1) (Figure 3B). After Cre-mediated recombination and negative selection, no amplification occured using the gDNA from surviving cells as template (Figure 3B). Importantly, using the same gDNA template, we were able to amplify across the break point region after Cre-mediated recombination (Figure 3C). We hypothesized that incorporation of an intronic expression cassette may disrupt gene expression of *RPGR*. To test this, we generated RNA and cDNA based on **Protocol 3**. Using the rt-PCR modification of the PCR method in **Protocol 3**, we amplified multiple *RPGR* transcripts with a forward primer in exon 11 and a reverse primer in a region of exon 15 shared by both $RPGR^{ORF15}$ and $RPGR^{1–19}$ to exclude gDNA amplification. By comparing the rt-PCR results of the untreated cells with the corrected cells and the corrected cells with the Cre treated cells, we demonstrated loss of the *RPGR* transcript after correction, and restoration by Cre-mediated excision of the selection cassette (Figure 3E). We also amplified *POL2RA* transcripts to exclude the possibility of poor amplification due to degraded cDNA. Robust amplification was observed for each sample supporting our conclusion. (Figure 3D).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

COMMENTARY

Background Information

hiPSCs are capable of self-renewal making them ideal for genetic modification, clonal selection, and subsequent expansion. Earlier studies employed transcription activator-like effector nucleases (TALENs) and Zinc finger nucleases (ZFNs) to achieve gene editing in hiPSCs (Zou et al., 2009; Ma et al., 2013; Hockemeyer et al., 2009), which derive their sequence specificity from protein-DNA interactions (Gaj et al., 2013). Both of these technologies use a chimeric nuclease, which consists of an engineered DNA binding domain linked to a non-specific nuclease. Zinc Finger domains are commonly occurring DNA binding motifs in eukaryotes, and ZFNs derive their specificity from linking of multiple domains (Gaj et al., 2013). While ZFNs were one of the first sequence-specific nucleases used in genome editing, their widespread adoption has been limited by significant design complexities (Mandell and Barbas, 2006). As an alternative, researchers have achieved genomic modification using TALENs, which consist of transcription activator-like effector (TALE) proteins. TALE proteins are prokaryotic in origin and utilize repeats consisting of 33–35 amino acids to recognize one base pair via two hyper-variable residues (Gaj et al., 2013). Importantly, researchers can reliably predict the sequence specificity of TALENs resulting in a reduced design complexity compared to ZFNs (Boch et al., 2009; Moscou and Bogdanove, 2009).

The most recently developed genome editing technology is the clustered regularly interspaced short palindromic repeats CRISPR-Cas9 system (Cong et al., 2013; Mali et al., 2013; Jinek et al., 2012; Hsu et al., 2013; Jinek et al., 2013). The system utilizes RNA-DNA interactions to allow for targeting of specific genomic locations. Moreover, compared to ZFNs and TALENs, a single chimeric guide can be used to cleave at a specific location, and the independent expression of nuclease and guide RNA allows for more than one location to be targeted simultaneously (Cong et al., 2013). The CRISPR-Cas9 system is highly efficient in achieving genome editing in hiPSCs (Ding et al., 2013). Given the relative design facility and low cost, the emergence of the CRISPR-Cas9 system has revolutionized genome editing by improving the efficiency and reducing its complexity in hiPSCs.

Continual innovation in experimental techniques renders genetic manipulation of stem cells achievable. Previous methods include nucleofection (Byrne and Church, 2015), which requires expensive instrumentation with kits that vary by cell type. While the methods described in this protocol focus on genomic correction of a disease variant, the technique can be extended to a wide range of other applications such as the development of reporter systems and the evaluation of genomic regulatory regions.

Critical Parameters and Troubleshooting

For common pitfalls and troubleshooting, please refer to Table 1.

Anticipated Results

For simplicity, genome editing in this protocol either disrupts a target via NHEJ (pX330 transfections) or modifies a target through HR (pX330 and pHDR transfections). For

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

example, a researcher studying a dominant disease with only one mutant allele may be able to restore the normal protein function by specifically targeting the disease allele. In contrast, recessive diseases must have two disease alleles present, and the underlying defect is due to a loss of function. Therefore, HR mediated repair of only one allele is necessary.

The pHDR^{+} or $^{+/-}$ approach is a power tool because of the inherent flexibility. For unpublished targeted regions, the bioinformatics tools fail to fully explain the variation in predictions and observations. This is why multiple guides should be chosen to find a region with Cas9 nuclease activity. To further complicate the process, researchers targeting tissue specific genes not expressed in hiPSC, may struggle to achieve high efficiencies because chromatin states determine the accessibility of the genome. Integrating the selection cassette drastically reduces the uncertainty for HR because selection allows the sampling of rare events. Therefore, in most cases, the pHDR^{+} or $^{+/-}$ approach has a high chance of success regardless of the efficiency of the repair event.

Illustrative Examples

The transfection protocols presented have high efficiencies and depending on the targeted sequence, result in robust double stranded breaks (Figure 1). As an exemplar, we designed a genome editing-based strategy targeting the *MYOC* gene (Figure 1A & B). The sgRNA effectively targeted the *MYOC* gene over a range of plasmid concentrations, which was examined using the T7 Endonuclease I assay (Figure 1C & D). We found that for this target between 1800 ng to 2000 ng was the optimal amount of total plasmid DNA for either **Protocol 1** or **Alternative Protocol 1**. While both methods are effective, there is significantly less cell death at 24 hours post-transfection when the reagent-based method was used (Figure 1E). To demonstrate an approach for genomic correction without the integration of a selection cassette, we co-delivered the CRISPR plasmid expressing a non-integrating GFP cassette with a homology directed repair plasmid containing a non-integrating mCherry expression cassette (Figure 1F). These cells can be flow sorted, clonally expanded and subsequently sequenced for genomic correction (Byrne and Church, 2015).

Protocol 2 provides a strategy to select for HR-mediated genomic correction. To illustrate this strategy, the second pHDR contains a homology template for *MYOC* that flanks a floxed puromycin resistance cassette (pHDR^{+}) (Figure 2A). Based on the location of the selection cassette within the homology arms, the HR repair process inserts the puromycin resistance cassette in intron 2 of *MYOC* (Figure 2A). We generated an induced pluripotent stem cell line from a patient with a G367R mutation in *MYOC* (Figure 2B). Following CRISPR-correction and drug selection we screened eight drug-resistant colonies for the intended genomic modification (Figure 2C & D). To screen across a breakpoint, we designed a primer to target a feature inside of the drug-resistance cassette and a primer targeting a region only located in the genome (Figure 2C). To screen for clonal purity, we screened the colonies using the T7 Endonuclease I assay (Figure 2D). Mismatches resulted in the digested DNA present in lines P2, P4, P5 and P7; only P1, P3, P6 and P8 are suitable for downstream use.

The final pHDR example contains a floxed dual selection cassette (pHDR$^{+/-}$) which efficiently remove cells still expressing vTK after Cre-lox recombination (Figure 3A). This

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

pHDR$^{+/-}$ corrects a mutation in exon 13 by inserting the dual selection cassette in intron 12 (Figure 3A). We demonstrated the correct genomic placement and removal via dual selection by amplifying across the break points (Figure 3B&C). Finally we demonstrated restoration of *RPGR* by Cre-Lox recombination and negative selection (Figure 3D &E).

Time Considerations

The total amount of time and work is largely dependent on whether the experiment utilizes the NHEJ or HR repair pathway. NHEJ events are modulated solely by the Cas9 nuclease activity. Therefore, the user needs only to clone a guide into the pX330 plasmid and transfect the cells. After 48 hours the guide efficiency can be assessed. Multiple guides should be considered at the same time to find a region with sufficient levels of Cas9 nuclease activity. The HR experiments employ plasmids containing homology templates. Generating the desired pHDR will take more or less time depending on the sequences being cloned and whether it is being commercially synthesized. Both selection steps necessitate two weeks time but mostly require minimal effort such as media changes or passaging. Additionally, there is an extra week necessary for excising the floxed selection casette. Table 2 contains an estimation of the total number of days for each task to be completed by a researcher unfamiliar with the techniques.

Acknowledgments

We would like to acknowledge our funding sources: National Eye Institute (RO1EY026008), Wynn Institute for Vision Research, Carver Non-Profit Genetic Testing Laboratory, The Stephen A. Wynn Foundation.

Literature Cited

Bikard D, Marraffini LA. Innate and adaptive immunity in bacteria: mechanisms of programmed genetic variation to fight bacteriophages. Current opinion in immunology. 2012; 24:15–20. [PubMed: 22079134]

Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S, Lahaye T, Nickstadt A, Bonas U. Breaking the code of DNA binding specificity of TAL-type III effectors. Science. 2009; 326:1509–1512. [PubMed: 19933107]

Burnight ER, Gupta M, Wiley LA, Anfinson KR, Tran A, Triboulet R, Hoffmann JM, Klaahsen DL, Andorf JL, Jiao C, et al. Using CRISPR-Cas9 to Generate Gene-Corrected Autologous iPSCs for the Treatment of Inherited Retinal Degeneration. Molecular therapy : the journal of the American Society of Gene Therapy. 2017; 25:1999–2013. [PubMed: 28619647]

Byrne SM, Church GM. Crispr-mediated Gene Targeting of Human Induced Pluripotent Stem Cells. Current protocols in stem cell biology. 2015; 35:5A.8.1–22. [PubMed: 26544538]

Byrne SM, Mali P, Church GM. Genome editing in human stem cells. Methods in enzymology. 2014; 546:119–138. [PubMed: 25398338]

Chakraborty S, Christoforou N, Fattahi A, Herzog RW, Leong KW. A robust strategy for negative selection of Cre-loxP recombination-based excision of transgenes in induced pluripotent stem cells. PLoS ONE. 2013; 8:e64342. [PubMed: 23717601]

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, et al. Multiplex genome engineering using CRISPR/Cas systems. Science. 2013; 339:819–823. [PubMed: 23287718]

Desmet FO, Hamroun D, Lalande M, Collod-Béroud G, Claustres M, Béroud C. Human Splicing Finder: an online bioinformatics tool to predict splicing signals. Nucleic acids research. 2009; 37:e67–e67. [PubMed: 19339519]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Ding Q, Regan SN, Xia Y, Oostrom LA, Cowan CA, Musunuru K. Enhanced efficiency of human pluripotent stem cell genome editing through replacing TALENs with CRISPRs. Cell stem cell. 2013; 12:393–394. [PubMed: 23561441]

Gaj T, Gersbach CA, Barbas CF. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends in biotechnology. 2013; 31:397–405. [PubMed: 23664777]

Haeussler M, Schönig K, Eckert H, Eschstruth A, Mianné J, Renaud JB, Schneider-Maunoury S, Shkumatava A, Teboul L, Kent J, et al. Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. Genome biology. 2016; 17:148. [PubMed: 27380939]

Hockemeyer D, Soldner F, Beard C, Gao Q, Mitalipova M, DeKelver RC, Katibah GE, Amora R, Boydston EA, Zeitler B, et al. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. Nature Biotechnology. 2009; 27:851–857.

Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, et al. DNA targeting specificity of RNA-guided Cas9 nucleases. Nature Biotechnology. 2013; 31:827–832.

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012; 337:816–821. [PubMed: 22745249]

Jinek M, East A, Cheng A, Lin S, Ma E, Doudna J. RNA-programmed genome editing in human cells. eLife. 2013; 2:e00471. [PubMed: 23386978]

Kim JH, Lee SR, Li LH, Park HJ, Park JH, Lee KY, Kim MK, Shin BA, Choi SY. High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. PLoS ONE. 2011; 6:e18556. [PubMed: 21602908]

Luo Y, Liu C, Cerbini T, San H, Lin Y, Chen G, Rao MS, Zou J. Stable enhanced green fluorescent protein expression after differentiation and transplantation of reporter human induced pluripotent stem cells generated by AAVS1 transcription activator-like effector nucleases. Stem cells translational medicine. 2014; 3:821–835. [PubMed: 24833591]

Ma N, Liao B, Zhang H, Wang L, Shan Y, Xue Y, Huang K, Chen S, Zhou X, Chen Y, et al. Transcription activator-like effector nuclease (TALEN)-mediated gene correction in integration-free β-thalassemia induced pluripotent stem cells. The Journal of biological chemistry. 2013; 288:34671–34679. [PubMed: 24155235]

Maeder ML, Linder SJ, Cascio VM, Fu Y, Ho QH, Joung JK. CRISPR RNA-guided activation of endogenous human genes. Nature methods. 2013; 10:977–979. [PubMed: 23892898]

Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM. RNA-guided human genome engineering via Cas9. Science. 2013; 339:823–826. [PubMed: 23287722]

Mandell JG, Barbas CF. Zinc Finger Tools: custom DNA-binding domains for transcription factors and nucleases. Nucleic acids research. 2006; 34:W516–23. [PubMed: 16845061]

Meier ID, Bernreuther C, Tilling T, Neidhardt J, Wong YW, Schulze C, Streichert T, Schachner M. Short DNA sequences inserted for gene targeting can accidentally interfere with off-target gene expression. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2010; 24:1714–1724. [PubMed: 20110269]

Merkle FT, Neuhausser WM, Santos D, Valen E, Gagnon JA, Maas K, Sandoe J, Schier AF, Eggan K. Efficient CRISPR-Cas9-mediated generation of knockin human pluripotent stem cells lacking undesired mutations at the targeted locus. Cell reports. 2015; 11:875–883. [PubMed: 25937281]

Miyaoka Y, Berman JR, Cooper SB, Mayerl SJ, Chan AH, Zhang B, Karlin-Neumann GA, Conklin BR. Systematic quantification of HDR and NHEJ reveals effects of locus, nuclease, and cell type on genome-editing. Scientific reports. 2016; 6:23549. [PubMed: 27030102]

Moscou MJ, Bogdanove AJ. A simple cipher governs DNA recognition by TAL effectors. Science. 2009; 326:1501–1501. [PubMed: 19933106]

Quadros RM, Miura H, Harms DW, Akatsuka H, Sato T, Aida T, Redder R, Richardson GP, Inagaki Y, Sakai D, et al. Easi-CRISPR: a robust method for one-step generation of mice carrying conditional and insertion alleles using long ssDNA donors and CRISPR ribonucleoproteins. Genome biology. 2017; 18:92. [PubMed: 28511701]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. Nature Protocols. 2013; 8:2281–2308. [PubMed: 24157548]

Ratz M, Testa I, Hell SW, Jakobs S. CRISPR/Cas9-mediated endogenous protein tagging for RESOLFT super-resolution microscopy of living human cells. Scientific reports. 2015; 5:9592. [PubMed: 25892259]

Vouillot L, Thélie A, Pollet N. Comparison of T7E1 and surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases. G3 (Bethesda, Md). 2015; 5:407–415.

Wang J, Lu XX, Chen DZ, Li SF, Zhang LS. Herpes simplex virus thymidine kinase and ganciclovir suicide gene therapy for human pancreatic cancer. World journal of gastroenterology. 2004; 10:400–403. [PubMed: 14760766]

Wiley LA, Anfinson KR, Cranston CM, Kaalberg EE, Collins MM, Mullins RF, Stone EM, Tucker BA. Generation of Xeno-Free, cGMP-Compliant Patient-Specific iPSCs from Skin Biopsy. Current protocols in stem cell biology. 2017; 42:4A.12.1–4A.12.14.

Wiley LA, Burnight ER, DeLuca AP, Anfinson KR, Cranston CM, Kaalberg EE, Penticoff JA, Affatigato LM, Mullins RF, Stone EM, et al. cGMP production of patient-specific iPSCs and photoreceptor precursor cells to treat retinal degenerative blindness. Scientific reports. 2016; 6:30742. [PubMed: 27471043]

Yang L, Guell M, Byrne S, Yang JL, De Los Angeles A, Mali P, Aach J, Kim-Kiselak C, Briggs AW, Rios X, et al. Optimization of scarless human stem cell genome editing. Nucleic acids research. 2013; 41:9049–9061. [PubMed: 23907390]

Zhu Z, Verma N, González F, Shi ZD, Huangfu D. A CRISPR/Cas-Mediated Selection-free Knockin Strategy in Human Embryonic Stem Cells. Stem cell reports. 2015; 4:1103–1111. [PubMed: 26028531]

Zou J, Maeder ML, Mali P, Pruett-Miller SM, Thibodeau-Beganny S, Chou BK, Chen G, Ye Z, Park IH, Daley GQ, et al. Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. Cell stem cell. 2009; 5:97–110. [PubMed: 19540188]

Zou J, Mali P, Huang X, Dowey SN, Cheng L. Site-specific gene correction of a point mutation in human iPS cells derived from an adult patient with sickle cell disease. Blood. 2011; 118:4599–4608. [PubMed: 21881051]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Significance Statement

Human induced pluripotent stem cells (hiPSCs) are the ideal cell source for autologous cell replacement. However, for patients with Mendelian disease it is likely that genetic correction of the patient's original disease causing mutation will be required prior to cellular differentiation and transplantation. In this unit we describe a series of protocols for delivery of CRISPR-Cas9 reagents to hiPSCs and clonal expansion of genetically modified cell lines suitable for autologous cell replacement.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

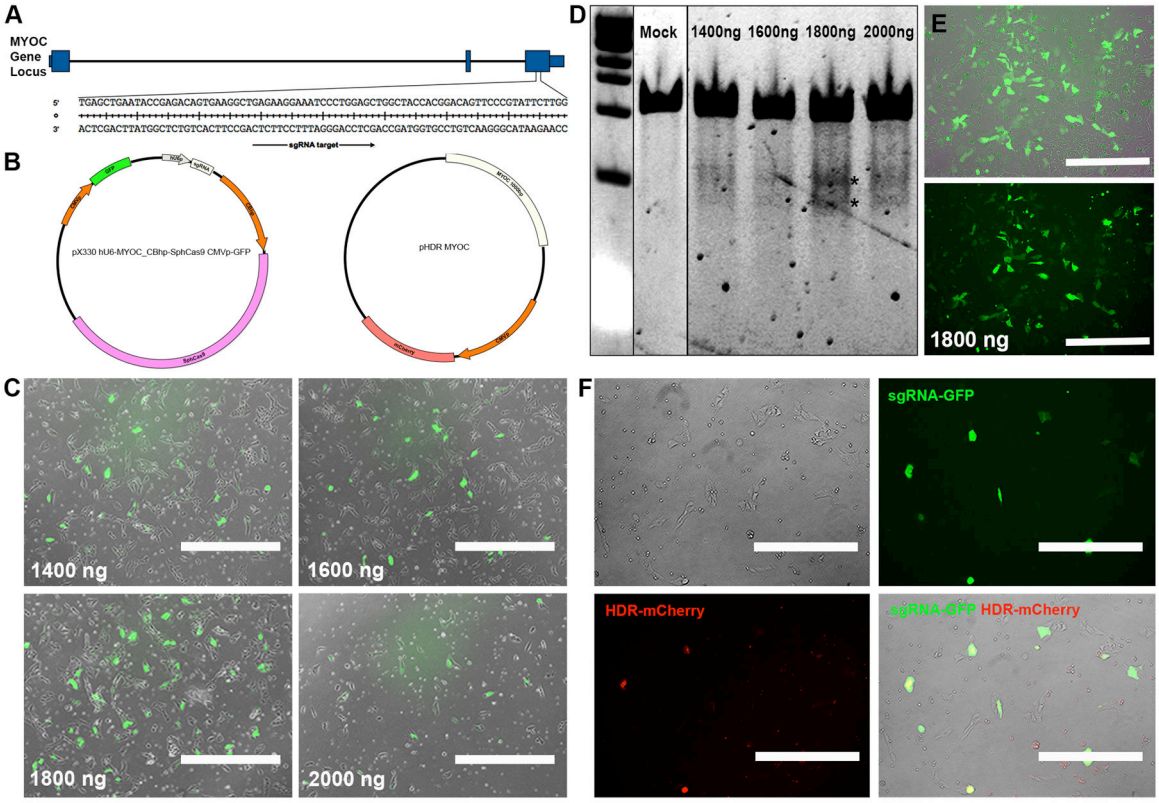

Figure 1. CRISPR-Cas9 Genome Editing of *MYOC*

A) Gene diagram of *MYOC* showing specificity of gRNA. **B)** Plasmid maps are shown for the CRISPR-Cas9 system expressing GFP and a *MYOC* homology directed repair (pHDR) construct containing a non-integrating mCherry cassette. **C)** GFP expression at 24 hours is shown after transfecting hiPSCs with 1400, 1600, 1800, and 2000 ng of pX330-U6-Chimeric_BB-CBh-hSpCas9_CMV-GFP (px330-GFP). **D)** T7 Endonuclease I assays of CRISPR-Cas9 mediated double stranded breaks. Asterisk notes the location of the cleaved PCR products. This assay varies over replicates so caution is needed when comparing the intensity of the cleaved products. The limit of detection is ~5% (Vouillot et al., 2015) **E)** Using 1800 ng of plasmid the Lipofectamine Stem Transfection Reagent effectively delivers the pX330-U6-Chimeric_BB-CBh-hSpCas9_CMV-GFP to hiPSC. **F)** Co-delivery of CRISPR plasmid and *MYOC* HDR plasmid.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

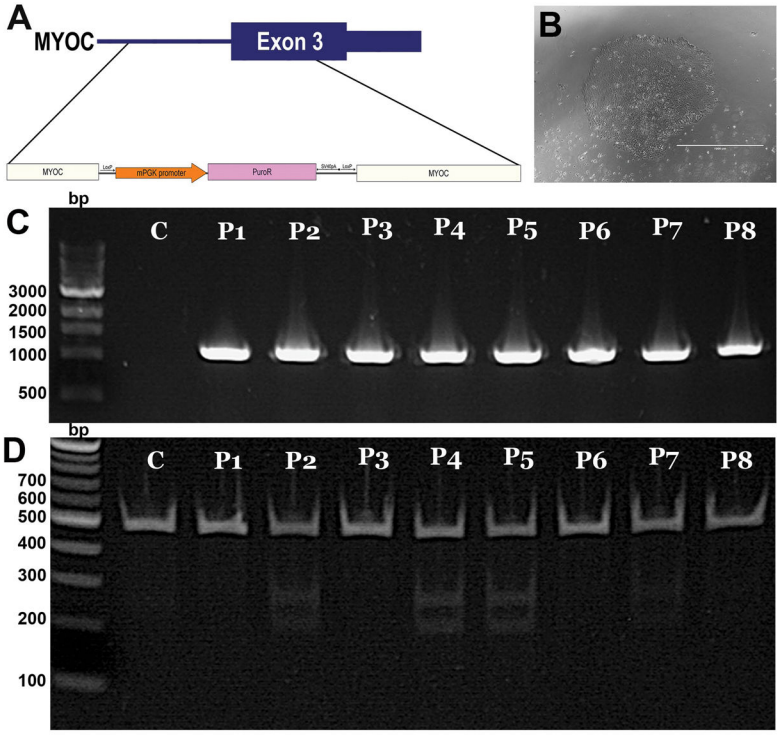

Figure 2. CRISPR-Cas9 Mediated Homologous Recombination of *MYOC*

A) Gene diagram of homology directed repair construct with an integrating drug selection cassette pHDR$^+$ was synthesized for *MYOC*. **B)** Human induced pluripotent stem cell line generated from a patient with a mutation in *MYOC*. **C)** PCR analysis of CRISPR-corrected clones (P1–P8) and an untreated sample (C) using a forward primer targeting SV40pA and a reverse primer unique to the genome. A positive PCR result excludes random genomic incorporation of pHDR$^+$. **D)** T7 Endonuclease I assay screening for clonal purity by indirectly detecting differences in the genome of cells at the guide sequence. Clones P1, P3, P6 and P8 contain at least 95% homogeneity.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

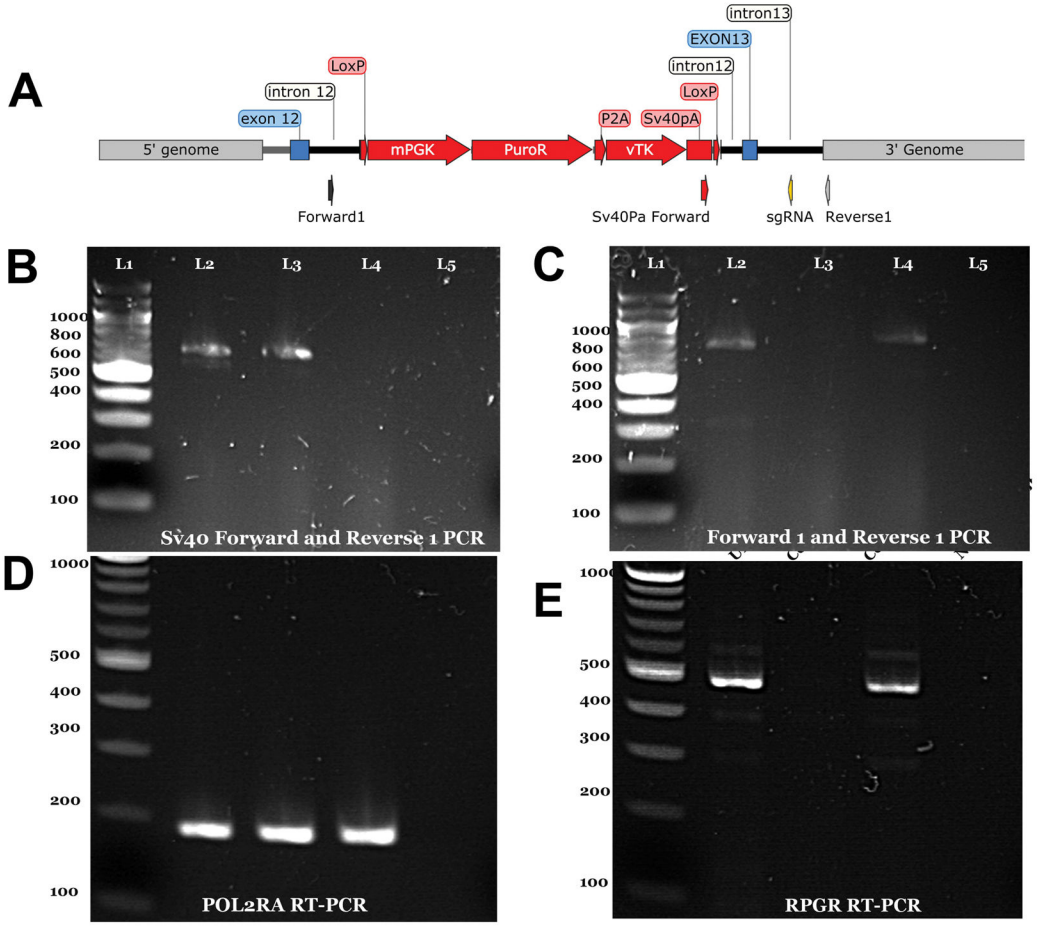

Figure 3. CRISPR-Cas9 Mediated Homologous Recombination of *RPGR*

A) Gene diagram of a homology directed repair construct (pHDR$^{+/-}$) for CRISPR correction of *RPGR*. **B–C)** gDNA analysis; *lane1: Ladder lane 2: CRISPR-mediated HR without positive selection, lane 3: CRISPR-mediated HR with positive selection, Lane 4: CRISPR-mediated HR with dual selection, Lane 5: ddH20 control.* **B**) PCR amplification using a forward primer targeting the SV40 polyadenylation sequence and Reverse Primer 1, which is located only in the genome, shows elimination of the selection cassette via negative selection. **C)** PCR break point analysis using Forward 1 and Reverse 1 demonstrates Cre-mediated excision of the selection cassette. The amplification in lane 2 is due to the presence of heterogeneous outcomes before selection. **(D–E)** cDNA analysis; *lane1: Ladder, lane2: Untreated, lane3: CRISPR-mediated HR with positive selection, lane4: CRISPR-mediated HR with dual selection.* **D)** rt-PCR analysis of a housekeeping gene, *POL2RA,* shows robust amplification which excludes the possibility of degraded cDNA. **E)** rt-PCR analysis of *RPGR* transcripts during the dual selection process demonstrates *RPGR* expression after dual selection.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 1

Troubleshooting

Technique	Common Pitfalls	Possible Causes	Solutions
Electroporation of hiPSCs to deliver CRISPR-Cas9 containing plasmids (PROTOCOL 1)	Poor cell survival	Air bubbles were trapped in the tip.	Air bubbles cause a dramatic spark during electroporation. Always check the Neon Pipette Tip for bubbles before running the protocol.
		Poor hiPSC culture technique.	Feed hiPSCs daily and passage the cells before they reach confluency.
		Over handling of the hiPSCs.	Perform every step up to but not including the electroporation. If poor cell survival is observed, use gentler technique.
		dsDNA induced cellular toxicity.	Large quantities of plasmid such as >5 μg drastically decrease cell survival. If no nuclease activity is apparent with 2 μg of pX330 consider testing additional guides.
		Endotoxin contamination.	Use a kit rated to remove endotoxins.
		Laminin-521 coating issues.	If the colonies grow in “hot dog” shaped patterns, the coating is insufficient. Refer to manufacturer instructions.
		Media not properly maintained.	Refer to manufacturer’s protocol.
	Poor gene disruption efficiency	SNPs	Always use a tool such as the UCSC Genome Browser during guide sequence design. The iPSC line can also be directly sequenced.
		Air in the tip will cause a spark.	Refer to comments above.
		Genomic region refractory to genome-editing	Generate more guides upstream and downstream of the region to find a new target. Try different Cas9 orthologues.
		Transfection optimization issues	Use plasmids with reporters such as pSpCas9(BB)-2A-GFP (pX458) available from Addgene.
		Cas9 orthologue has a low activity	Try a different Cas9 nuclease.
	No observable HR	Rare HR events.	Increase the length of the homology arms. Avoid placing the selection cassette in a low complexity region. For rare events, the number of replicates must be increased.
Reagent Based Transfection of hiPSCs to Deliver CRISPR-Cas9 Containing Plasmid (ALTERNATE PROTOCOL 1).	Poor cell survival	dsDNA toxicity, Endotoxin contamination.	*see above*
		Improper hiPSC culture technique.	*see above*
	Poor gene disruption	*see above*	*see above*
Positive Selection of Genomically Modified hiPSCs (Protocol 2)	No cell survival	Increasing the drug concentration too quickly.	If proper location of selection cassette is observed by PCR, extend the length of each by a day or two. If not, perform selection for a week. Then take a heterogeneous gDNA sample and retest the PCR. This will enrich for the targeted genome-editing event. If distinct colonies are not visible, consider redesigning the pHDR. We commonly find that clones taking more then a week to emerge are due to random plasmid incorporation.
		Varying resistance from line to line.	Change the dosage escalation accordingly. Note that 0.05 μg/mL of puromycin should not cause rapid cell death even in a sensitive line. One approach is to change the media

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Technique	Common Pitfalls	Possible Causes	Solutions
			early, and observe the cells by the end of the day. For sensitive lines, 6–10 hours is sufficient to affect the cell viability. Remove the media and rinse the well. Treat the cells at a lower dosage for an additional 1–2 days.
	No cell death	Too many cells.	Overly confluent cells will render the drug dosage ineffective. However, if the selection began at an appropriate confluency, then the drug dosage needs to be escalated faster.
		Varying resistance from line to line.	For most lines 0.5 μg/mL of puromycin is sufficient for selection. We have observed that 1 μg/mL is necessary in limited cases. Include untreated cells as a control.
	Cells die after clonal section	"Herd resistance".	Some clones seem to survive in the presence of corrected clones and do not contain the genomic modification of interest.
		User technique.	Larger colonies survive the process more often than smaller colonies.
		"Hidden" cells.	Check the margins of the well for hiPSCs. It is easier to miss these cells and assume the well is negative.
	The selection cassette is properly located but disease allele is not corrected	A design or sampling issue.	While respecting the other design considerations, the arm spanning the DSB should be oriented in this order: *(most distal to center) double stranded break---> sequence modification --> selection cassette (most proximal to center)*. Center refers to the middle of homology sequence. Swapping the order of the DSB and modification will include HR repair that includes selection but excludes the desired modification.
	No amplification of the selection cassette at the correct location in the genome.	Random plasmid integration.	If clones emerge after 7 days, they typically do not carry the desired genomic modification. Use multiple sets of primers to rule out a false negative. Additionally, try different high fidelity polymerases.
Removal of Selection Cassette From Genomically Modified hiPSCs and Negative Selection (Protocol 4)	No cell survival	Gap junction-mediated bystander effect.	A high density of cells will prevent cell survival. Transient plasmid transfections of Cre recombinase will at best result in excision of 40%–60% of the selection cassettes. Therefore low ganciclovir concentrations and low plating densities limit the extent of the bystander effect. Alternatively, hiPSCs can be transfected with Cre recombinase an additional time before starting negative selection. Always collect a gDNA sample after Cre recombinase treatment to rule out issues with the LoxP sequences.
	No cell death	This can happen due to mutations in the viral thymidine kinase sequence.	To avoid this, take at least 3–5 clones through the entire dual selection process.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 2

Cumulative Experimental Time

STRATEGY	STEP	RATIONALE	DAYS
Gene disruption Strategies (NHEJ)	pX330 Guide Sequence Cloning	For most people with limited experience, designing and cloning a single 20 bp insert is relatively straightforward and can be accomplished in a week.	7
	Transfection (Neon or Lipofectamine Stem)	While the Neon protocol is more involved, both protocols can be performed in one day. However, the treated cells cannot be analyzed for at least 48 hours.	2
	Downstream analysis	Most of the techniques in **Protocol 3** take a day to complete.	1
			Projected number of days: 10
Gene Correction Strategies (HR)	pX330 Guide Sequence Cloning	See above	7
	pHDR design	This step takes 1–2 months, which depends on how the plasmid is generated and the target sequence. For advanced users, this can be significantly shorter.	45
	Transfection (Neon or Lipofectamine Stem)	see above	1
	Positive Selection	This step takes multiple days because rapid selection is toxic to even the drug resistant cells. This requires minimal hands on time.	14
	Cre-Lox Recombination	This step takes multiple days because the cells need to recovery. This requires minimal hands on time.	7
	Negative Selection	see positive selection	14
	Downstream Analysis	Most of the techniques in **Protocol 3** take a day to complete.	1
			Projected number of days: 89