APPLICATION NOTE

Knock-in a SNP alteration in iPSCs using Edit-R reagents.

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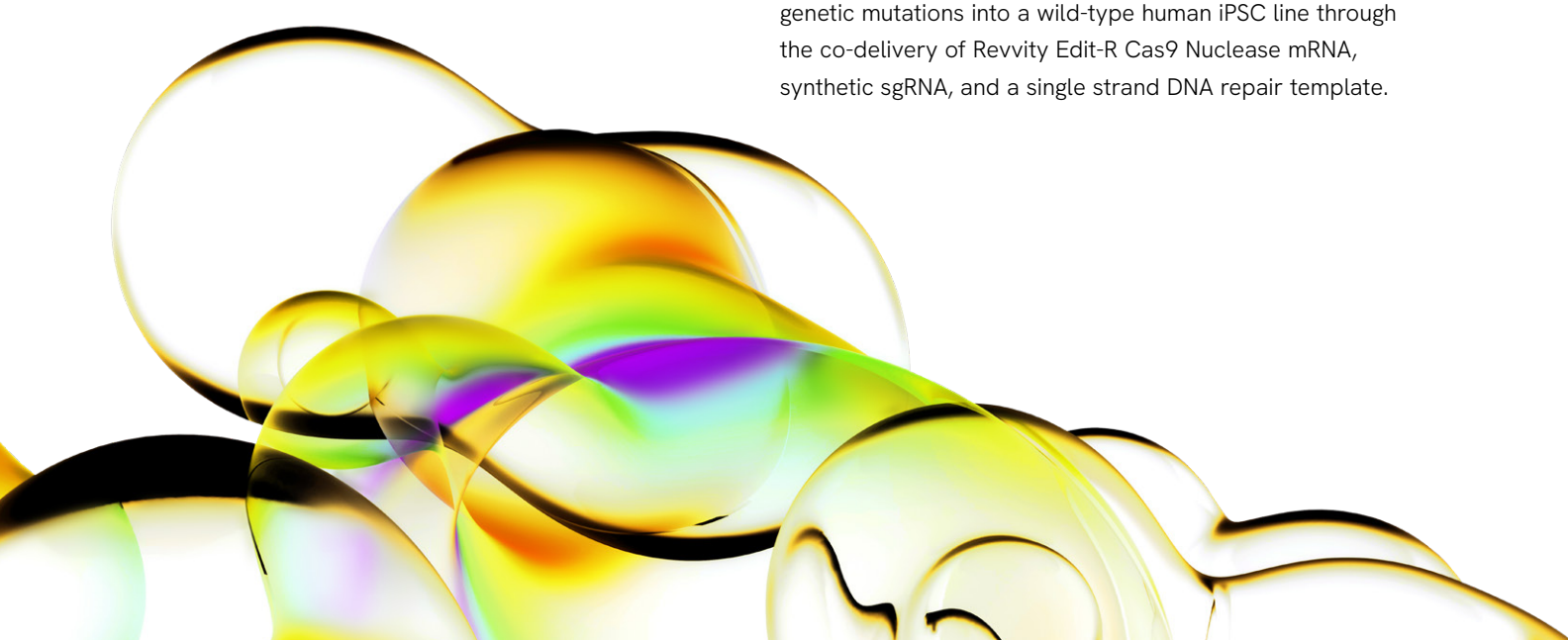
Introduction

An increasing number of human diseases have been found to have a genetic component. To study the phenotypic effects of these mutations, genome editing technologies such as the CRISPR-Cas9 gene editing system can be used to manipulate a cell's genomic DNA (Jinek 2012, Mali 2013, Cong 2013). By co-delivery of a Cas9 nuclease and a DNA-targeting guide RNA, a targeted double-strand DNA break is made, prompting the cellular machinery to repair the lesion. The co-introduction of a synthetic repair template can be used by the cell during repair to incorporate precise genomic changes desired by the researcher through the homology-directed repair (HDR) pathway (Waddington 2016, Jehuda 2018). In this way, disease-associated mutations can be precisely introduced into a wild-type cell background, or patient-derived disease models can be corrected to the wild-type sequence. This generates an otherwise isogenic pair of disease and control cell lines for study in the laboratory.

Human-induced pluripotent stem cells (iPSCs) are an excellent model system for the study of genetic diseases because they are a renewable, karyotypically normal cell type that can be manipulated in the pluripotent state and then differentiated into many clinically relevant isogenic cell types. Additionally, there are a variety of methods for generating iPSCs from patient samples with minimally invasive procedures (Malik 2013). Generating iPSC models remains challenging in part due to differences in HDR-mediated editing efficiencies between cell lines and locus-to-locus variability.

Here, we demonstrate the introduction of disease-associated genetic mutations into a wild-type human iPSC line through the co-delivery of Revvity Edit-R Cas9 Nuclease mRNA, synthetic sgRNA, and a single strand DNA repair template.



These DNA-free reagents prevent potentially confounding random genomic integration, preserving the integrity of the iPSC models' genetic composition. Additionally, synthetic sgRNA and Cas9 Nuclease mRNA are active in the cell for less time than plasmid-based reagents, which reduces the risk for off-target editing activity. For technical ease of use, Cas9 Nuclease mRNA also precludes the need for using a reagent with a cell-line specific optimized promoter.

Results

To mimic two known disease-associated genetic mutations in a healthy iPSC background, we chose two well-characterized genotypes. The R225X mutation in the LMNA gene introduces a premature stop codon resulting in various laminopathies (Capell et al. 2006; Rankin et al. 2006). The MYH7 D239N variant replaces an aspartic acid with an asparagine and is known to be associated with early-onset hypertrophic cardiomyopathy (Adhikari et al., 2016). Both are dominant traits where a phenotype can be observed in patients with mono-allelic single-nucleotide polymorphisms (SNPs) inherited from a single parent. Therefore, to properly model the genetic disease, editing must only be made to one allele while maintaining a wild type allele. This type of gene editing can be particularly challenging in iPSCs where HDR is not the preferred dsDNA break repair method.

To trigger HDR in iPSCs that would result in the introduction of a SNP, we relied on electroporation conditions previously optimized to achieve high percentages of indel formation with the hypothesis that the more efficient the delivery of gene editing reagents, the greater likelihood of observing rare HDR events (see Methods). Edit-R Cas9 Nuclease mRNA was co-delivered with a specific sgRNA designed with the Revvity CRISPR Design Tool to target the genome within 15 bp of the desired editing location. A single strand DNA oligo was also designed for co-delivery using the Revvity HDR Donor Designer that was complementary to the editing region and included the disease-associated point mutation, as well as necessary silent mutations to prevent further CRISPR-Cas9 recognition and cleavage after HDR repair (Figure 1).

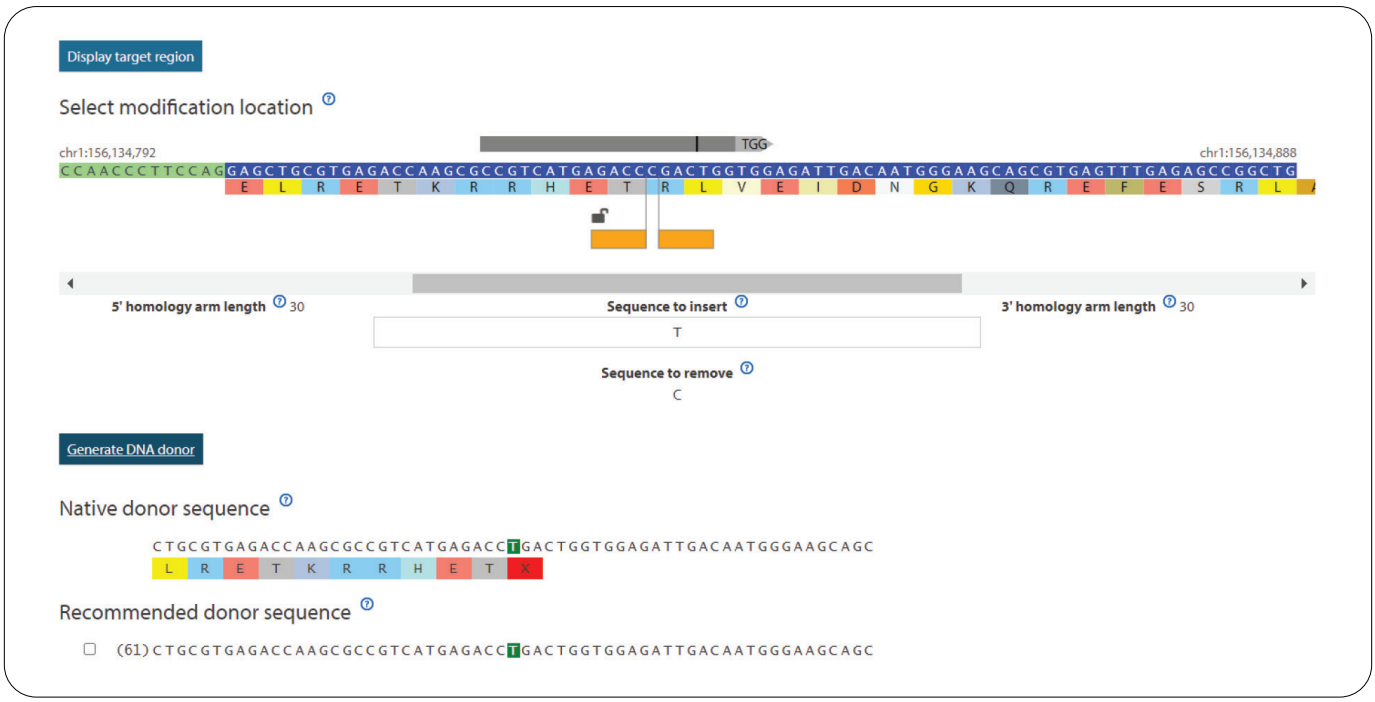


Figure 1: Example of the Revvity HDR Donor Designer. This tool automatically generates a recommended donor sequence designed from user input of the modification location and sequence change desired. In the LMNA gene shown here, arginine (R) at position 225 is converted to a stop codon by the insertion of a thymine (T) and removal of cytosine (C).

Seventy-two hours post-electroporation, cells were imaged to show that the iPSCs maintained healthy morphology after manipulation (Figure 2A), and the edited and control populations were lysed for PCR amplification of the genomic region of interest. Amplicons were gel-purified, and next-generation amplicon sequencing was performed. Analysis of NGS data revealed that indels were the prominent editing outcome from this experiment, and a small fraction of alleles (3-8%) contained the disease-associated point mutation, as expected for this cell type (Figure 2B-C).

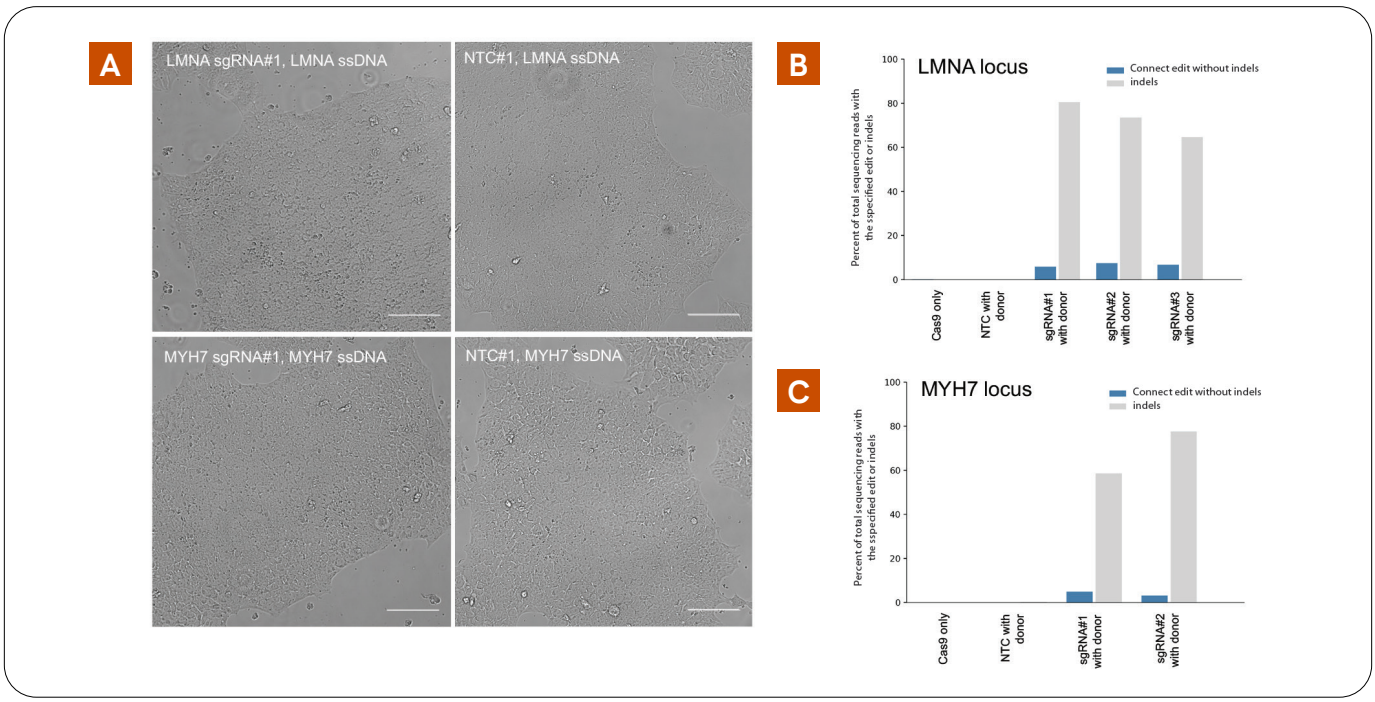


Figure 2: Gene Editing in iPSCs with Cas9-mRNA Nuclease, synthetic sgRNAs, and ssDNA oligo repair template delivered by nucleofection is non-perturbing to iPSC morphology and can be used to introduce small changes to the genome. A) Phase images taken 48 hours post-nucleofection. Scale bars are 100 µm. B-C) Analysis of the percent of total NGS sequencing reads with the specified desired edit (blue) or undesired INDELs (grey) for the LMNA locus (B) and MYH7 locus (C).

Discussion

Here we have demonstrated the ability to introduce specific disease-associated genetic variants with Edit-R CRISPR Cas9 gene editing reagents in human iPSCs. Because iPSCs demonstrate low levels of HDR-mediated repair, the overall incidence of alleles containing the disease-associated mutation is low, and the generation of clonal cell lines is recommended to recover those with the desired genotype.

When continuing this workflow, the researcher must consider the prevalence of the desired mutation and the unedited allele when determining the number of clones necessary to screen in order to recover the desired genotype. In this example, where 8% of alleles have the desired mutation and 20% of alleles are wild type, an expected 1.6% of the population is predicted to have the mono-allelically mutated genotype (8% x 20% = 1.6%). Clonal iPSC lines can be generated through a number of techniques, including low-density seeding of single-cell suspension iPSCs and manual clonal isolation, which does not require any special equipment (Haupt 2018). Individual clones can then be assessed for genotype through PCR amplification of the targeted loci and Sanger sequencing, high-throughput droplet digital PCR techniques (Miyaoka 2018), or in some cases enzymatic evaluation through restriction fragment length polymorphism (RFLP) analysis. This application note demonstrates the utility of the Edit-R Cas9 Nuclease mRNA, synthetic sgRNA, and ssDNA HDR repair templates as a convenient source of CRISPR reagents for effective genome editing in human iPSCs.

Materials and methods

Cell culture. A Human Episomal iPSC Line found to be karyotypically normal was cultured on non-tissue culture treated plastics (recommended for use with Vitronectin coating) coated with truncated Vitronectin (VTN-N) Recombinant Human Protein according to manufacturer's recommendations. Cells were cultured in antibiotic-free mTeSR™ Plus media (Cat # 05825 StemCell Technologies). Cultures were routinely maintained by clump passaging two times a week with Gibco™ Versene Solution per manufacturer's instructions. Cells were incubated at 37 °C and 5% CO_2.

Electroporation. Prior to electroporation, cells were treated with 10 µM ROCK inhibitor molecule Y-27632 (Cat #72302, StemCell Technologies) for 1-4 hours to promote viability post-electroporation. Cells were passaged into a single cell suspension using StemPro Accutase Cell Dissociation Reagent and resuspended in mTeSR™ Plus media supplemented with 10 µM ROCK inhibitor molecule Y-27632. Cell line-specific (see Cell Culture section for specific lineage) electroporation conditions, previously optimized with pMax-GFP expression plasmid, were as follows: Lonza™ P3 Primary Cell 4D-Nucleofector™Kit (Cat # V4SP-3096) was used to transfect 3.4 × 105 cells per reaction in an Amaxa™ 96-well Shuttle™ Device (Cat # AAM-1001S) using setting CM-113. Each reaction contained 1 µg Edit-R EGFP Cas9 Nuclease mRNA (Cat #CAS11860) and 2 µM custom Edit-R synthetic sgRNA, and 0.3 µM ssDNA repair template in 20 µL reactions.

The Custom guide RNA targeting sequences:

LMNA locus

- Targeting sequence 1: GCGCCGTCATGAGACCCGAC
- Targeting sequence 2: CCGTCATGAGACCCGACTGG
- Targeting sequence 3: ATTGTCAATCTCCACCAGTC

MYH7 locus

- Targeting sequence 1: TTGGCAATGCCAAGACCGTC
- Targeting sequence 2: GTTGTCGTTCCGGACGGTCT

The Custom ssDNA repair template sequences:

LMNA locus

- Donor: AAACTCACGCTGCTTCCCATTGTCAAT CTCGACGAGTCATGTCTCATGACGGC GCTTGGTCTCACGCAG

MYH7 locus

- Donor: AGGGACCACTCACGAAGCGGGAGGA GTTGTTGTTCCGGACTGTCTTTGCATT GCCAAAGGCCTCCAGAGCAGGGTTG

After transfection was performed, 80 µL mTeSR™1 Without Phenol Red media (Cat # 05876 StemCell Technologies) supplemented with 10 µM ROCK inhibitor molecule Y-27632 was added to each reaction well, and 25 µL of each reaction was plated into a prepared Vitronectin-coated 96-well plate containing 0.2 mL pre-equilibrated mTeSR™1 Without Phenol Red media supplemented with 10 µM ROCK inhibitor molecule Y-27632. Cells were incubated at 37 °C and 5% CO_2. After 6-20 hours, cells were imaged for GFP fluorescence from the Cas9-GFP mRNA as an indication of transfection efficiency. Twenty-four hours post-transfection, media was changed to mTeSR™ Plus, and cells were fed daily until the time of harvest.

NGS analysis. On the day of harvest, replicate wells were washed 1X in DPBS (Cat #SH30028, Fisher Scientific) and lysed in 0.1 mL/well Thermo Scientific 1× Phusion HF Buffer with 5 µL each of Proteinase K and RNase A, and incubated for 1 h at 56 °C then heat-inactivated at 98 °C for 10 min. Fifty microliter PCR reactions were carried out in 1× Phusion HF buffer, using 0.5 µL Phusion Hot Start II DNA Polymerase, 200 µM each dNTP, 0.5 µM forward and reverse primers, and 5 µL direct cell lysis template. Touchdown PCR cycling was run for each sample: Denature/enzyme activation at 98 °C for 3 min followed by 10 cycles of 98 °C for 10 s, 72 °C for 15 s –1 °C/cycle, and 72 °C for 30 s, then 25 cycles of 98 °C for 3 min followed by 10 cycles of 98 °C for 10 s, 62 °C for 15 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min. Each PCR product was gel purified on a 2% agarose gel and extracted with GeneJet Gel Extraction Kit per the manufacturer's protocol. Extracts were eluted in cell culture grade water and normalized to 20 ng/µL after quantification by Qubit™ Fluorometric Quantification and Qubit™ dsDNA HS Assay Kit and submitted to Genewiz for Amplicon EZ Sequencing. Using a custom Python script, paired-end reads were merged.

Reads containing mismatches in the overlapping region were filtered out, while the higher Phred score for each overlapping base was kept for matches. The non-overlapping portions of each sequence were trimmed off, and any read containing a base with a Phred score < 30 were also filtered out. The remaining reads were aligned using Bowtie2 (Langmead et al., 2012), and a pileup of reads was generated using SAMtools (Li et al., 2009). Custom Python scripts were used to summarize the editing percentages and to generate the corresponding plots.

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For more information:

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