APPLICATION NOTE

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Knock-in a fluorescent tag into iPSCs using Edit-R™ reagents

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Introduction

The CRISPR-Cas9 toolbox has been expanded by the inclusion of repair templates, which can be used in the homology-directed repair (HDR) pathway for precise genome modifications. The introduction of large tags through HDR-mediated gene editing enables applications in the laboratory, such as imaging fluorescently tagged proteins of interest and generating reporter cell lines.

Induced pluripotent stem cells (iPSCs) are an effective model for the study of normal and disease-associated cell biology because they are a renewable, karyotypically normal cell type that can be manipulated in the pluripotent state and differentiated into a variety of isogenic cell types. 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 present the ability to endogenously tag a protein of interest with a fluorescent marker in a human iPSC line with Horizon Discovery Edit-R CRISPR reagents. 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 demonstrate the use of Edit-R gene editing reagents to introduce a fluorescent tag that would be endogenously expressed to illuminate a protein of interest, we chose to design reagents for tagging Fibrillarin (FBL), a protein known to localize to the fibrillar component of the nucleolus. Additionally, fibrillarin has previously been shown to tolerate an endogenous green fluorescent protein (GFP) tag in stem cells (Roberts *et al.*, 2017).

Edit-R synthetic sgRNA was designed using the Horizon CRISPR Design Tool to target the C-terminus of Fibrillarin within 15 bp of the desired GPF insertion site. We recommend that two or three sgRNAs are tested in parallel as best practice because different targeting sequences may result in different editing efficiencies. A repair template plasmid design was generated using the Edit-R HDR Plasmid Donor Designer, and the full plasmid was then generated with an Edit-R HDR Plasmid Donor Kit.

These reagents were co-delivered with Edit-R Cas9 Nuclease mRNA using electroporation methods previously optimized (see Methods) for high indel formation. We hypothesized that the more efficient the delivery of gene editing reagents, the greater likelihood of observing rare HDR events in this cell type, which does not favor HDR repair.

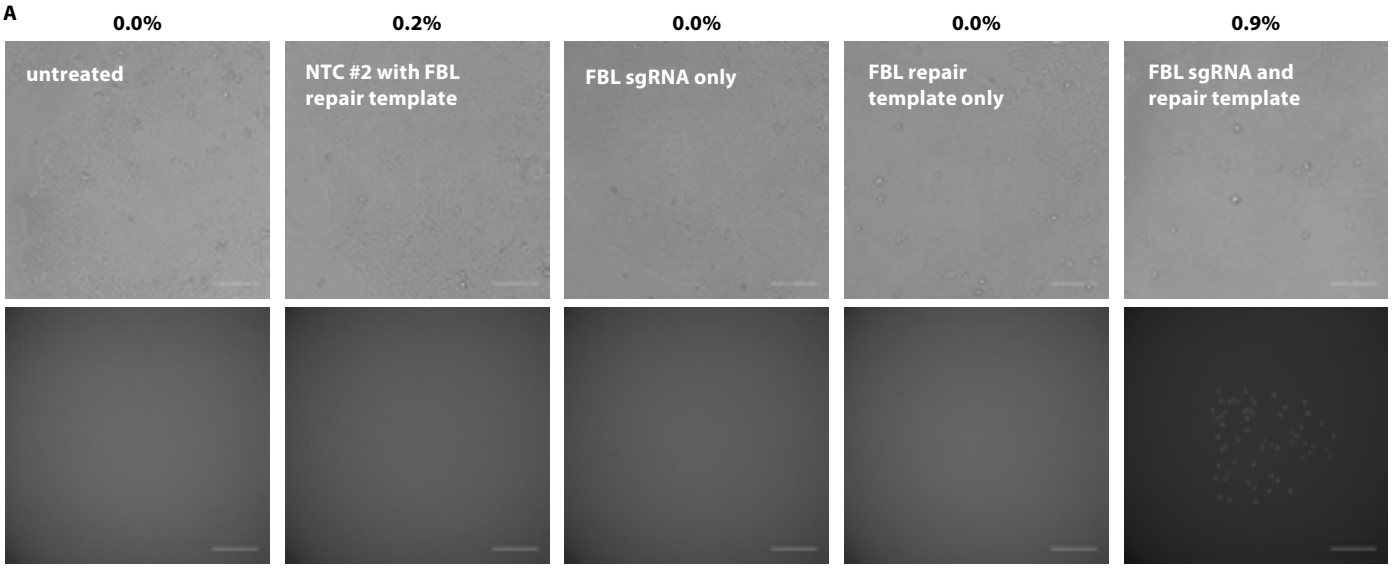
Five days after electroporation, cells were imaged (Figure 1A) and then passaged into single-cell suspension for live analysis by flow cytometry to determine the percentage of cells that expressed GFP. Untreated samples and those treated with only the sgRNA or only the repair template had no background GFP signal. Samples treated with the repair template and a non-targeting control sgRNA did have a small amount (0.2%) of background GFP signal of low intensity, compared to the 0.9% of cells that were GFP positive and relatively bright in the sample treated with the FBL-targeting sgRNA and the repair template plasmid (Figure 1B). Importantly, only cells from the sample treated with the FBL-targeting sgRNA and repair template plasmid exhibited GFP fluorescence localized to the nucleolus. Although background fluorescence was detected in the non-targeting control sample, no GFP fluorescence could be detected by microscopy, either as correctly localizing FBL-GFP or as a diffuse cytoplasmic signal.

Discussion

Here we have demonstrated the use of Edit-R Cas9 Nuclease mRNA, synthetic sgRNA, and a dsDNA repair template to introduce a fluorescent tag to an endogenously regulated protein of interest in iPSCs. Edited cells could be further enriched by fluorescence-activated cells sorting (FACS) to study a genetically diverse population of tagged cells as a population, or clonal cell lines could be derived from this enriched population and genotyped for precise editing as previously described (Roberts 2017).

The methods presented in the creation of this endogenously tagged iPSC line could be adapted to create other useful reporters or tools. For example, an iPSC line engineered with a reporter specifically expressed in differentiated cell types, or an iPSC line expressing a functional protein from a safe harbor site.

This application note demonstrates the utility of the Edit-R Cas9 Nuclease mRNA, synthetic sgRNA, and dsDNA HDR repair templates as a convenient source of CRISPR reagents for effective genome editing in human iPSCs.



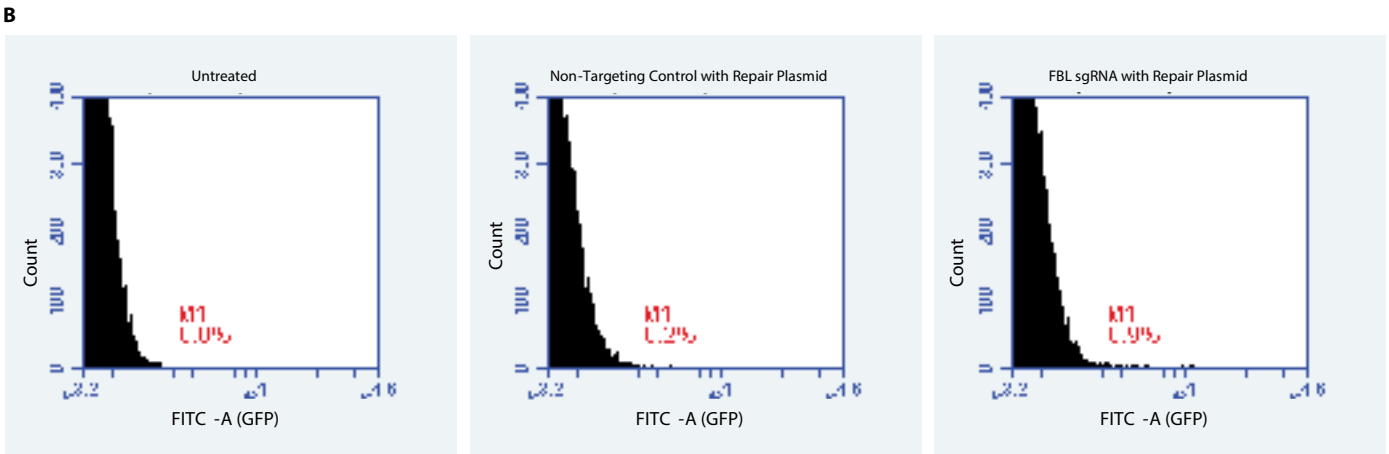


Figure 1. Edit-R Cas9 Nuclease mRNA, synthetic sgRNA, and a dsDNA repair template introduce a fluorescent tag to the Fibrillarin protein in human iPSCs. A) Percent GFP positive cells detected by flow cytometry for each experimental sample. Fluorescent images (2nd row) show the Fibrillarin-GFP protein correctly localized to the nucleolus of iPSCs only when the targeting sgRNA was used with the appropriate repair template. Images were taken 5 days after nucleofection (including one passage). Scale bars are 100 µm. B) Example flow cytometry gating with GFP fluorescence on the X-axis.

Materials and methods

Cell culture

A Human Episomal iPSC Line (Cat # A18945, Thermo Fisher Scientific) 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 (Cat # A14700, Thermo Fisher Scientific) 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 (Cat #15040066, Thermo Fisher Scientific) 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 (Cat # A1110501 Thermo Fisher Scientific) 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 x 10^5 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 Edit-R synthetic sgRNA, and 100 ng dsDNA repair template in 20 µL reactions.

Custom guide RNA targeting sequence: AACTGAAGTTCAGCGCTGTC

The donor template was constructed such that there were 870 base pairs of homology 5' of the terminal FBL stop codon and 693 base pairs of homology 3' of the terminal FBL stop codon (including the stop codon) with a AGGS-(EGFP)-GGS linker and fluorescent protein sequence inserted between the two homology arms.

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% CO2. 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 and passaged one time before harvest.

TIDE 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 (Cat #F-518L, Thermo Fisher Scientific) with 5 µL each of Proteinase K (Cat# E00492, Thermo Fisher Scientific) and RNase A (Cat #EN0531, Thermo Fisher Scientific), 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 (Cat #F-518, Thermo Fisher Scientific), using 0.5 µL Phusion Hot Start II DNA Polymerase (Cat #F-549S, Thermo Fisher Scientific), 200 µM each dNTP (Cat #F-549S, Thermo Fisher Scientific), 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 verified to be single amplicons by running 5 µL of the reaction on 1.2% agarose gels before proceeding with Sanger sequencing. The Resulting AB1 files were analyzed by an in-house program that utilizes an adapted version of the TIDER algorithm described in Brinkman et al. (2018) to estimate the likely indel frequencies present within a Sanger sequence trace. Samples are first assessed and filtered on the basis of sequence trace quality, and the analysis parameters determined for each individual input. Treated samples are then aligned to and compared against untreated controls to calculate the sample editing efficiency, the frequency of indels, the rate of indel occurrence, and corresponding p-values. Each possible indel within the specified window of detection was individually tested to determine the likelihood of such an indel being explained by the observed Sanger trace data, and a confidence estimate was then assigned to this indel.

Flow cytometry analysis

On the day of harvest, replicate wells of a 96-well plate were washed once in DPBS (Cat #SH30028, Fisher Scientific) and passaged into a single cell suspension using StemPro Accutase Cell Dissociation Reagent (Cat # A1110501 Thermo Fisher Scientific) and resuspended in mTeSR™ Plus media supplemented with 10 µM ROCK inhibitor molecule Y-27632. Replicate wells were combined in 0.2 mL total volume and analyzed on a BD Accuri(TM) C6 Plus Flow Cytometer equipped with a 405 nm laser to detect GFP with a 530/30-nm filter. Forward scatter height and area (FSC-H, FSC-A), and side scatter height and area (SSC-H, SSC-A) were used to exclude debris and doublets. Single cells were visualized on a GFP (FITC-A) histogram.

References

1. Roberts, *et al.* Systematic gene tagging using CRISPR/Cas9 in human stem cells to illuminate cell organization. *Molecular Biology of the Cell*. **28**(1), (2017).

2. E. Brinkman, *et al.* Easy Quantification of template-directed CRISPR/Cas9 editing. *Nucleic Acids Research*. **46**(10): e58, (2018).

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