APPLICATION NOTE

Optimized HDR-mediated fluorescent protein knock-in in K-562 cells using Edit-R CRISPR-Cas9 reagents and electroporation.

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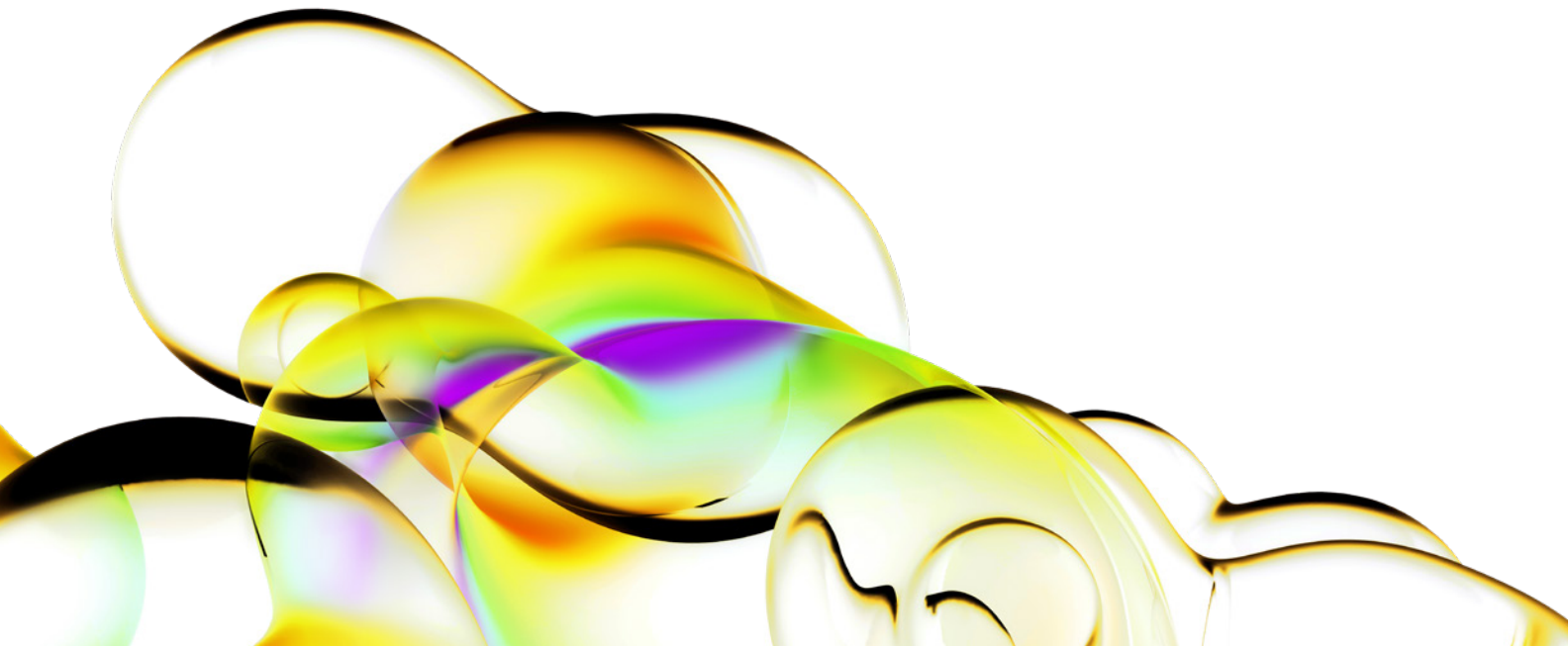
Introduction

Dharmacon™ Edit-R™ CRISPR-Cas9™ genome engineering is a fast and relatively simple way to knockout gene function, or precisely knock-in a DNA sequence for gene correction or gene tagging. Targeted gene knockout is achieved through generation of a double-strand break (DSB) in the DNA using Cas9 nuclease and guide RNA (gRNA). The DSB is then repaired, often imperfectly, by random insertions or deletions (indels), through the endogenous non-homologous end joining (NHEJ) repair pathway. For knock-in experiments, in addition to the Cas9 nuclease and gRNA, a DNA donor template is required and the DSB is precisely repaired with the donor template through the homology-directed repair (HDR) pathway. Different formats of Cas9 nuclease may be used: vector expressed, mRNA or recombinant protein. gRNA can also be expressed from a vector as a chimeric single guide RNA (sgRNA) or chemically synthesized as sgRNA or as dual components, CRISPR RNA (crRNA) and tracrRNA.

Knock-in using a donor template, either a single-stranded DNA (ssDNA) donor oligo or donor plasmid, has a relatively low efficiency, often in the 1-20% range. Therefore, successful HDR-mediated knock-in experiments require important design considerations and experimental optimization. First, the NHEJ-mediated editing levels need to be optimized by identifying the gRNA (crRNA:tracrRNA or sgRNA) with the highest cutting efficiency when combined with a Cas9 nuclease.



The editing efficiency must also be balanced with the proximity of the cut site to the desired insertion site; the closer these are to each other, the higher frequency of knock-in. The Dharmacon CRISPR RNA Design Tool is an online gRNA design tool that incorporates predicted functionality, specificity, and proximity to the genomic alteration site to recommend guide RNAs for HDR. Next, a DNA donor template needs to be designed with proper homology arms (HA) to incorporate precise genomic alteration(s). The online Dharmacon Edit-R HDR Donor Designer aids in designing and ordering the appropriate donor template for the specific HDR-mediated applications. A ssDNA donor is appropriate for alterations of ~50 nt or less, such as SNP correction or epitope tag addition. A donor plasmid allows for larger insertions, such as a fluorescent reporter or exon replacement. Once the donor template is generated, reagent amounts and analysis timepoint need to be experimentally determined. Finally, the precise knock-in must be confirmed by clonal cell isolation, expansion and Sanger sequencing. This Application Note will focus on optimized delivery of the HDR reagents in K-652 cells using electroporation as a reagent delivery method, since these cells are resistant to transfection with conventional transfection reagents.

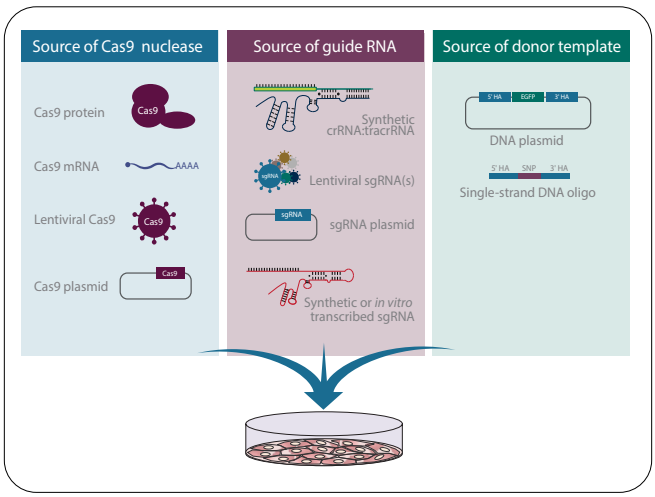


Figure 1: The sources of Cas9, guide RNA, and donor template that can be utilized in HDR-mediated CRISPR-Cas9 gene editing experiments (one of each is required). Four options are available for Cas9 nuclease: Cas9 protein, Cas9 mRNA, lentiviral Cas9 and Cas9 plasmid. Sources of guide RNA include synthetic crRNA:tracrRNA, lentiviral sgRNA, sgRNA plasmid and synthetic or *in vitro* transcribed sgRNA. Finally, there are two options for donor repair template: a single-stranded DNA donor oligo for short insertions or alterations of ~ 50 nt or less, or a donor plasmid that allows for large insertions.

Optimization of Cas9 protein:crRNA:tracrRNA with HDR DNA donor plasmid in K-562 cells

For HDR-mediated knock-in it is important to optimize the delivery (transfection or electroporation), in two steps:

1. maximize the NHEJ-mediated editing, and **2.** titrate in the donor template (donor oligo or donor plasmid). Here we demonstrate the optimization of HDR-mediated fluorescent protein knock-in in frame with *LMNA* in K-562 cells. Electroporation is a robust delivery method typically used when working with difficult-to-transfect or suspension cell lines and has been shown to work well in CRISPR-Cas9 gene editing experiments with varied Cas9 and gRNA sources[1-5] (Figure 1). This electroporation optimization was performed in a 96-well format with synthetic crRNA:tracrRNA, Cas9 protein, and an HDR DNA donor plasmid containing EGFP and ~ 1000 bp 5′ and 3′ homology arms corresponding to the *LMNA* gene.

Optimized electroporation conditions for NHEJ-mediated gene editing

Initial conditions for NHEJ-mediated editing originated from guidelines provided in the Lonza™ 96-well Shuttle™ System protocol for K-562 cells. K-562 cells (200 000) were electroporated with Edit-R synthetic crRNA targeting human *LMNA* (custom designed with the Dharmacon CRISPR Design tool), Edit-R synthetic tracrRNA (Cat #U-002000-20) and Edit-R Cas9 Nuclease protein NLS (Cat #CAS11730) as a ribonucleoprotein (RNP) complex with increasing molar ratios of crRNA:tracrRNA to Cas9 protein from 1:1 to 4:1 (see Cas9 protein nucleofection protocol for more details). Data from a DNA mismatch detection assay using T7 Endonuclease I (T7EI) to estimate indel formation resulted in the highest editing efficiency for a 2:1 ratio of crRNA:tracrRNA (60 pmols) to Cas9 protein (30 pmols; data not shown).

Optimized electroporation conditions for HDR-mediated knock-in

Electroporations were then repeated using optimized conditions for NHEJ-mediated gene editing (200 000 cells, 2:1 ratio of crRNA:tracrRNA to Cas9 protein) plus the addition of a donor plasmid designed using the HDR Donor Designer for an in-frame N-terminal *LMNA* knock-in of EGFP. A negative control of donor plasmid alone was paired with each HDR-mediated knock-in sample to normalize for EGFP expression resulting from the plasmid alone.

After electroporation, cells were maintained until there was minimal EGFP expression detected using fluorescent microscopy from the donor plasmid alone control samples. At this point (day seven, after two cell passages), FACS analysis was performed to capture the percent of the cell population with knock-in of EGFP at the N-terminus of *LMNA*

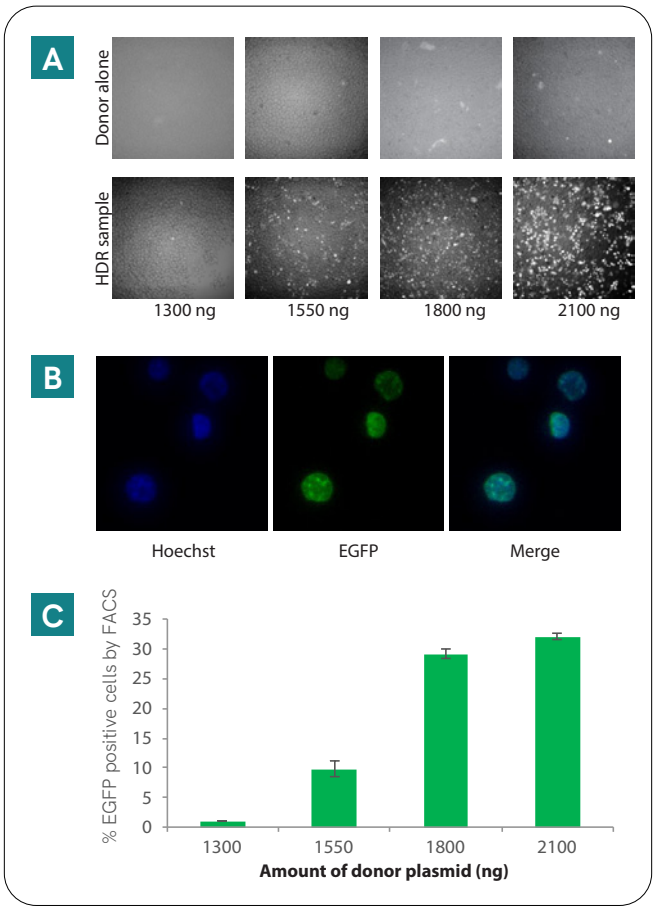


Figure 2: Optimization of HDR-mediated reporter knock-in with electroporation in K-562 cells. A. Electroporation of *LMNA*-EGFP donor plasmid alone at increasing concentration shows low amounts of nonspecific EGFP expression. Electroporation of Cas9 protein:crRNA:tracrRNA with increasing concentration of *LMNA*-EGFP donor plasmid shows correlating increase in EGFP expression. B. Upon increased magnification the localization of the EGFP expression in the HDR-mediated knock-in samples appears as expected in the nucleus for *LMNA* and co-localizes with Hoechst staining. C. Flow cytometry analysis of the HDR-mediated knock-in cell population gives increasing EGFP expression up to 32% with increased amount of DNA donor plasmid. The corresponding analysis of the DNA donor plasmid alone controls showed less than 0.5% EGFP expression (data not shown) for all doses.

Results

Fluorescent microscopy images of the HDR-mediated knock-in samples at day seven show that EGFP expression intensity increases with increased amount of DNA donor plasmid included in the electroporation, with the greatest fluorescent intensity observed between 1800-2100 ng donor plasmid (Figure 2A). The corresponding DNA donor plasmid alone shows low amounts of EGFP expression from the plasmid in mostly dead-appearing K-562 cells and debris, likely due to initial electroporation toxicity that is not fully diluted from the suspension population (Figure 2A). Additionally, with increased magnification, the localization of the EGFP expression in the HDR-mediated knock-in samples appears as expected in the nucleus for LNMA and co-localizes with Hoechst staining (Figure 2B).

Flow cytometry analysis of the HDR-mediated knock-in cell population shows increasing EGFP expression up to 32% with increased amount of DNA donor plasmid (Figure 2C), whereas the corresponding analysis of the DNA donor plasmid alone controls showed less than 0.5% EGFP expression (data not shown) for all doses. For the highest two amounts of DNA donor plasmid (1800 ng and 2100 ng) the percent HDR knock-in efficiency of EGFP was 29% and 32%, respectively. For the lower doses the increase between doses was much greater (~ 9 fold for 1300 ng to 1550 ng and then ~ 3 fold from 1550 ng to 1800 ng) indicating that a plateau is approached around 2100 ng of DNA donor plasmid per electroporation sample.

Conclusion

We have provided electroporation guidelines for precise knock-in of a fluorescent reporter to a desired genomic location using plasmid donor template, Cas9 protein, and synthetic crRNA:tracrRNA. We demonstrated that 1800-2100 ng of donor plasmid is the optimal amount for an HDR knock-in efficiency of 32% in K-562 cells. It is important to fully optimize design of experiments, amounts of the CRISPR-Cas9 reagents and donor template, as well as the delivery conditions to obtain the highest efficiency of HDR-meditated knock-in. Future work would include creation of clonal cell lines and confirmation of the precise EGFP insertion by Sanger sequencing. By following these guidelines to obtain the highest efficiency, fewer clonal cell lines will need to be analyzed to obtain the precisely edited clonal cell line for future studies of any specific protein, pathway or disease model.

References

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