APPLICATION NOTE

Homology-directed repair with Dharmacon Edit-R CRISPR-Cas9 reagents and single-stranded DNA oligos.

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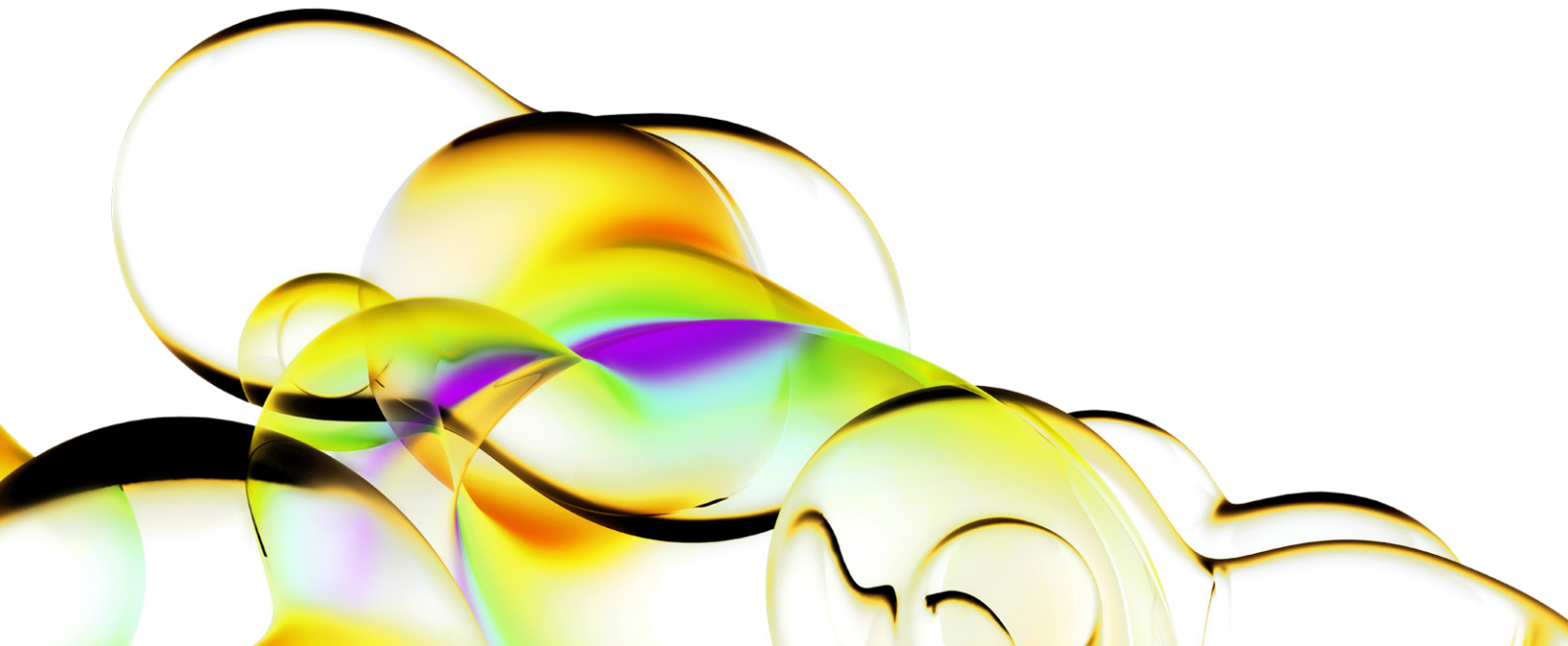
Abstract

CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated protein 9) is a revolutionary tool that utilizes an RNA-guided nuclease for efficient site-directed genome engineering in various eukaryotic systems. The double-strand breaks (DSBs) created by CRISPR-Cas9 are repaired in the cell by two predominant mechanisms: imprecise non-homologous end joining (NHEJ) and precise homology-directed repair (HDR). The Dharmacon™ Edit-R™ CRISPR-Cas9 gene engineering platform consists of both synthetic and expressed guide RNAs. In conjunction with Cas9 expression plasmids and lentiviral particles, it supports multiple experimental workflows. Here we demonstrate an innovative workflow to guide the insertion of 10-12 nucleotides into a gene of interest by HDR with the Edit-R CRISPR-Cas9 system, DharmaFECT™ Duo Transfection Reagent, and a single-stranded donor DNA oligo (ssDNA).

Keywords

Homology-directed repair, homologous recombination, non-homologous end joining, gene editing, genome engineering, Edit-R, Cas9, CRISPR, donor oligo, donor DNA, lipid transfection, homology arms, insertion, repair, DSB, knockin



Introduction

The bacterial adaptive immune system, the CRISPR-Cas9 system, can be repurposed for targeted gene editing of mammalian genomes.[1,2,3] Derived from *Streptococcus pyogenes*, the system uses the Cas9 nuclease protein that complexes with a tracrRNA and a targeting crRNA (which contains a 20 nucleotide guide sequence complementary to the genomic target of interest).[3] The genomic target is amenable to Cas9 targeting if it is upstream of a PAM (protospacer adjacent motif), which for *S. pyogenes* is NGG. The Edit-R CRISPR-Cas9 system mimics the endogenous bacterial system by using a synthetic tracrRNA and custom synthetic crRNA to guide Cas9 to a specific target site in order to create a DSB three nucleotides upstream of the PAM sequence. Once the DSB occurs, the mammalian cell utilizes endogenous mechanisms to repair the broken genomic DNA, the most common methods being NHEJ or HDR. While NHEJ repair of the DSB is often imprecise and error-prone due to the generation of random insertions and deletions (indels), HDR faithfully copies the genetic information from a related DNA sequence, such as a sister chromatid or a foreign donor DNA sequence (plasmid or DNA oligo). By using single-stranded DNA (ssDNA) donors, one can rapidly design and synthesize a donor DNA sequence containing the desired changes to the genomic location of interest.

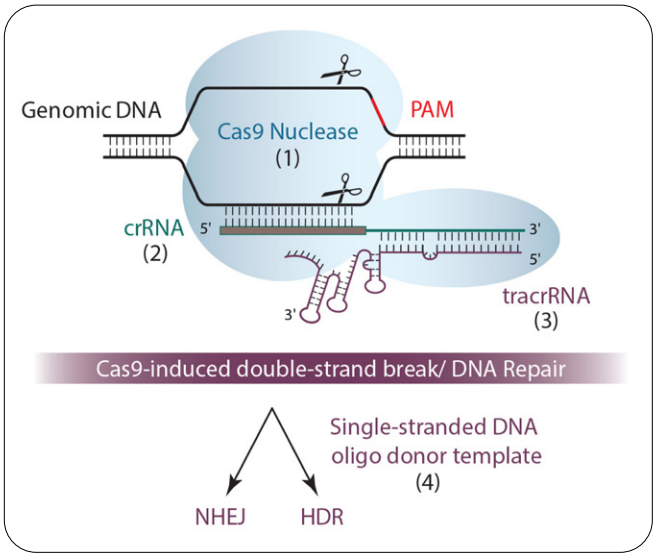


Figure 1: Edit-R CRISPR-Cas9 genome engineering. Illustration of Cas9 nuclease (1) bound to the synthetic crRNA (2):tracrRNA (3) complex and targeted to genomic DNA by the guide sequence in the crRNA, adjacent to the PAM (red). Repair of DSBs can be achieved by NHEJ in the absence of a donor template, or by HDR when a donor template such as a single-stranded DNA oligo (4) is present.

The Edit-R CRISPR-Cas9 synthetic guide RNA experimental workflow (Figure 1) consists of (1) a plasmid expressing a mammalian codon-optimized gene sequence encoding Cas9, (2) a synthetic crRNA designed to target a genomic site of interest, and (3) a synthetic tracrRNA. The use of chemically synthesized RNA components allows facile generation of indels through NHEJ which can result in gene disruption (knockout). However, this same workflow can also be combined with (4) an ssDNA donor oligo for rapid genome engineering using HDR. Here, we first demonstrate the application of Edit-R crRNA:tracrRNA in an experimental workflow (Figure 2) including ssDNA oligos to knock in short DNA sequences into two different genomic regions of interest. Second, we establish lipid-based transfection as an effective technique to accomplish HDR experiments with knockin efficiencies as high as 25%. Last, we optimize ssDNA oligo homology arm length to provide DNA donor oligo design recommendations for use with the Edit-R genome engineering platform. The methods presented within this application note can be applied to HDR-based insertion of epitope tags such as a FLAG™ tag, SNPs, precise stop codons, and amino acid changes in the active site of enzymes.

Results

Genome engineering using CRISPR-Cas9 requires expression of the Cas9 nuclease with the crRNA and tracrRNA. This can be achieved by co-transfection of a plasmid expressing Cas9 and crRNA:tracrRNA (Figure 2A), or by creation of a cell line in which the Cas9 cassette is delivered using lentiviral particles and stably integrated and expressed prior to transfection with crRNA:tracrRNA. For HDR and creation of knockins, it is important to optimize experimental conditions to obtain the maximal levels of DSBs. Therefore, experiments to optimize DSBs without a donor template should be performed (Figure 2A) to assess and maximize levels of gene editing. These experiments should be performed prior to experiments using the donor template (Figure 2B) and be used as a preliminary assessment for overall levels of DSB generation.

Genome engineering at the VCP locus: A crRNA was designed to target exon 2 of the human *VCP* gene and insert a DNA sequence containing two restriction sites for detection of the knockin (Figure 3A). First, a U2OS cell line was co-transfected with a hCMV-PuroR-Cas9 nuclease expression plasmid and the synthetic crRNA:tracrRNA complex using DharmaFECT Duo Transfection Reagent according to transfection conditions optimized for maximal indel formation (Figure 2A). Transfected cell populations were either not selected or selected with puromycin to enrich for Cas9 expression. Additionally, U2OS cells stably expressing Cas9 were transfected with the *VCP* crRNA:tracrRNA. All cell populations were assessed 72 hours post-transfection with a DNA mismatch detection assay using T7 endonuclease I (T7EI) to estimate relative indel formation (Figure 3B).

The above transfections were also performed in the presence of a ssDNA donor oligo containing homology arms and a 12 nucleotide sequence for insertion that contains two adjacent restriction enzyme sequences (Figure 3A). Transfected cells were assayed for knockin of the inserted sequence 72 hours post-transfection by a Restriction Fragment Length Polymorphism (RFLP) assay (Figure 3C). The highest amounts of HDR knockin were observed in conditions corresponding to the highest amount of gene editing as measured by indels using the mismatch detection assay (Figure 3B and C). This result highlights the importance of optimizing experimental conditions to ensure maximal creation of CRISPR-Cas9-mediated DSBs.

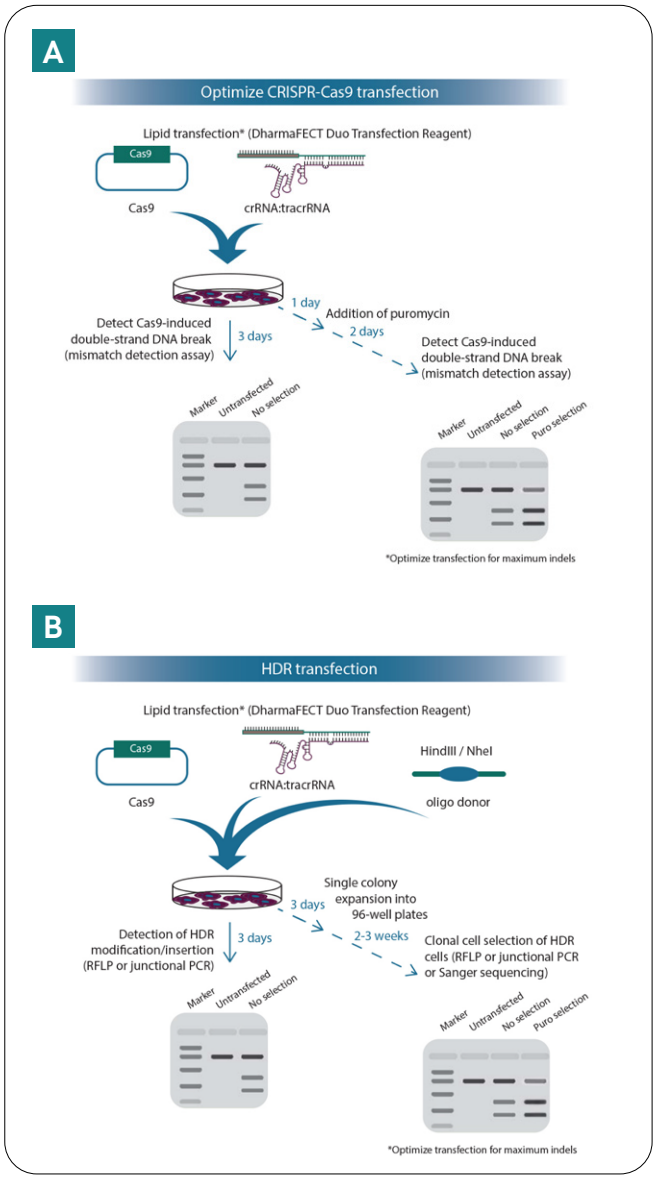


Figure 2: HDR-based experimental workflow for co-transfection of synthetic RNAs, Cas9 expression plasmid and synthetic DNA oligo donor. A Cas9 expression plasmid co-transfection workflow with DharmaFECT Duo Transfection Reagent. Detection of double-strand breaks (A) prior to performing the HDR transfection (B) ensures maximal double-strand breaks for HDR repair. Optimization of maximal DSBs (A and dashed arrows) is necessary to increase the amount of DSBs to be repaired by HDR.

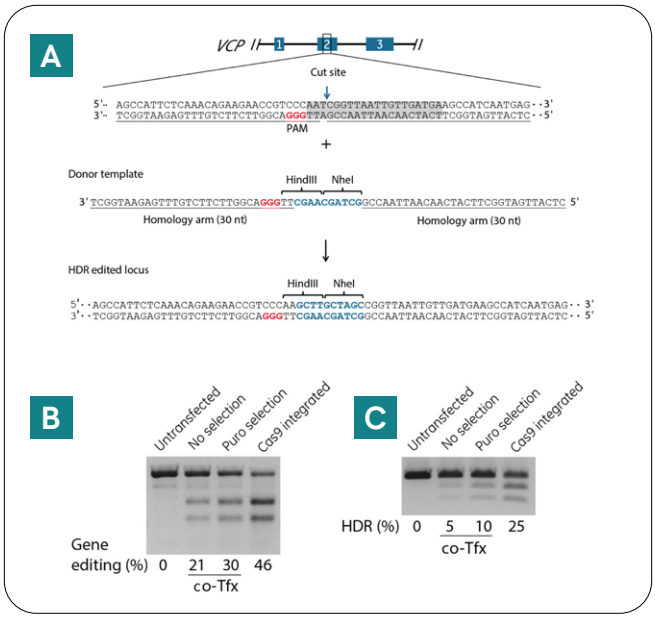


Figure 3: Gene editing at the *VCP* locus. Expressed Cas9 and synthetic crRNA:tracrRNA were used to target the VCP locus for HDR genome engineering in U2OS cells. A. Schematic of the VCP locus. Exons are indicated by the blue boxes. The guide sequence in the synthetic crRNA is indicated by the gray highlighted region, the PAM is indicated in red, and the cut site is indicated by the blue arrow. The inserted sequence is indicated in blue. B. Analysis of the efficiency of indel mutations using mismatch detection analysis of untransfected cells and transfected cells without (no selection) and with (puro selection) puromycin selection or using a Cas9-expressing cell line (Cas9 integrated). The percent editing is indicated below the gels. C. Analysis of the efficiency of HDR-based insertion of a HindIII and NheI restriction enzyme sites introduced using a 30 nucleotide homology arm DNA oligo. Percent HDR editing was calculated using an RFLP assay with NheI digestion of VCP PCR amplicons. co-Tfx = co-transfection.

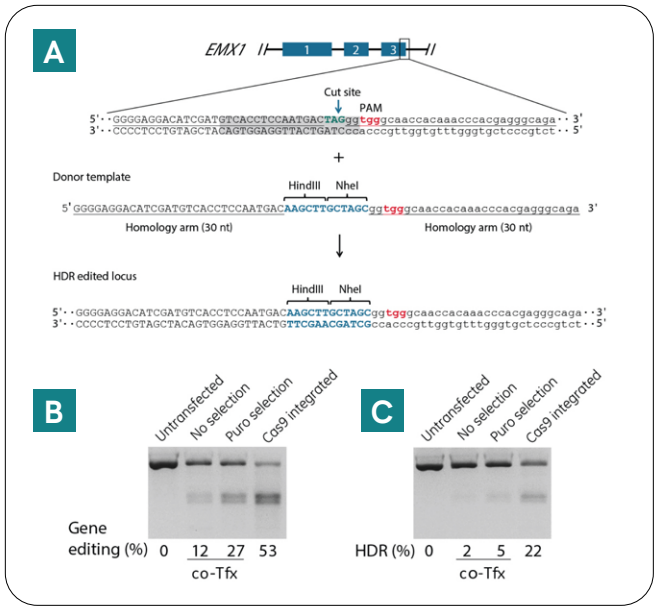


Figure 4: Gene editing at the *EMX1* locus. Expressed Cas9 and synthetic crRNA:tracrRNA were used to target the *EMX*1 locus for HDR genome engineering in U2OS cells. A. Schematic of the *EMX*1 locus. Exons are indicated by the blue boxes and uppercase lettering; introns are indicated by the lowercase lettering. The guide sequence in the crRNA is indicated by the gray highlighted region, the PAM is indicated in red, and the cut site is indicated by the blue arrow. The inserted sequence is indicated in blue, and the endogenous stop codon is green. B. Analysis of the efficiency of indel mutations using mismatch detection analysis of untransfected cells and transfected cells without (no selection) and with (puro selection) puromycin selection or using a Cas9-expressing cell line (Cas9 integrated). The percent editing is indicated below the gels. C. Analysis of the efficiency of HDR insertion of a HindIII and NheI restriction enzyme sites introduced using a 30 nucleotide homology arm DNA oligo. Percent HDR editing was calculated using an RFLP assay with NheI digestion of *EMX1* PCR amplicons. co-Tfx = co-transfection.

Genome engineering at the *EMX*1 locus: DharmaFECT Duo Transfection Reagent was used to co-transfect U2OS cells with a hCMV-Puro^R-Cas9 nuclease expression plasmid and synthetic crRNA:tracrRNA complex targeting exon 3 of the human *EMX*1 gene, and to transfect synthetic crRNA:tracrRNA into a Cas9-integrated U2OS cell line (Figure 4A). Transfections were performed as described above, including a ssDNA donor oligo. Total gene editing (indel %) and HDR knockin in the nonselected, puromycin-selected, and Cas9-integrated U2OS cell populations were analyzed 72 hours post-transfection (Figure 4B and C). Consistent with the results from targeting VCP, the highest amounts of HDR knockin at the EMX1 locus were observed in conditions corresponding to the highest amount of gene editing as measured by indel formation using the mismatch detection assay.

Concentration of synthetic single-stranded donor DNA oligo: In order to determine the optimal concentration of the donor template for HDR, we performed a dose curve of the ssDNA oligo targeting the *EMX1* locus. Non-selected and puromycin-selected U2OS cells were transfected with fixed amounts of the hCMV-Puro^R-Cas9 nuclease expression plasmid (200 ng/ well), synthetic crRNA:tracrRNA complex (25 nM final/well), and increasing amounts of ssDNA donor oligo (0.5, 1.0, 2.5, 5.0, 10.0, 20.0, 40.0, 60.0, and 100.0 nM final/well) in a 96-well plate. Cas9-integrated U2OS cells were transfected with fixed amounts of the synthetic crRNA:tracrRNA complex (25 nM final/well), and increasing amounts of ssDNA donor oligo (0.5, 1.0, 2.5, 5.0, 10.0, 20.0, 40.0, 60.0, and 100.0 nM final/well) in a 96-well plate. Cells were harvested 72 hours post-transfection and HDR knockin was detected using the RFLP assay. Both non-selected and puromycin-selected U2OS cell populations display the highest levels of HDR knockin using a final concentration range of 2.5 nM to 10.0 nM ssDNA donor oligo (Figure 5). Similarly, the Cas9-integrated U2OS cell line exhibited the highest levels of HDR knockin using a final concentration range of 2.5 nM to 10.0 nM ssDNA donor oligo (Figure 5).

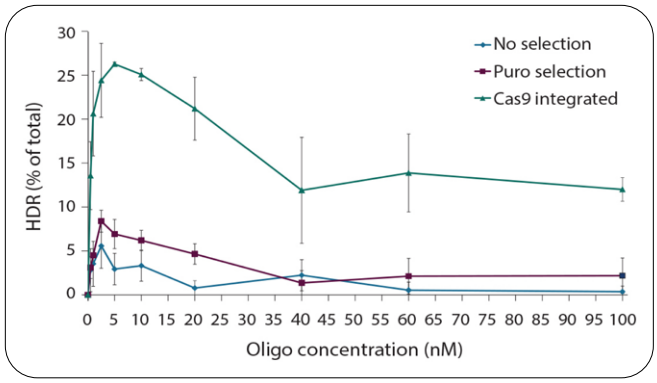


Figure 5: Donor DNA oligo concentration optimization. DharmaFECT Duo Transfection Reagent was used to co-transfect U2OS cells with a Cas9 expression plasmid, *EMX1* crRNA:tracrRNA, and increasing concentrations of a donor DNA oligo with 30 nucleotide homology arms. Alternatively, a Cas9-integrated U2OS cell line was transfected with crRNA:tracrRNA to target *EMX1*, and increasing concentrations of a donor DNA oligo with 30 nucleotide homology arms. The RFLP assay was used to determine the amount of HDR knockin for each concentration of donor DNA oligo in each transfection. Data presented is from three independent transfections.

Effect of homology arm length on HDR efficiency: When targeting *VCP* or *EMX*1 (Figures 3 and 4), the ssDNA oligo donor sequences contained 30 nucleotides of homology on each arm. In order to determine how homology arm length can affect HDR efficiency, Cas9-integrated U2OS cells were transfected with complexed crRNA:tracrRNA and ssDNA donor oligos with increasing homology arm lengths. Donor DNA oligo homology arms were designed to have 10 nucleotides of homology per arm, increasing in 10 nucleotide increments up to 70 nucleotides total per homology arm. Cell populations were assayed at 72 hours post-transfection and the percent HDR knockin was determined by RFLP (Figure 6). HDR was detected with homology arms as short as 20 nucleotides at a knockin percentage comparable to the longer homology arms that were tested (30-70 nucleotides).

Discussion

The Edit-R CRISPR-Cas9 genome engineering platform employing chemically synthesized RNAs is a quick and easy method to test multiple guide sequences for optimizing % indel formation through NHEJ, and achieving functional gene knockouts. Here, we have demonstrated the ability to utilize the same experimental workflow to rapidly create knockins through the HDR pathway simply by including a single-stranded DNA oligo donor template. We have further demonstrated the use of lipid-based transfection using DharmaFECT Duo Transfection Reagent to achieve precise sequence insertion through the HDR pathway, whereas, in the literature, this is commonly achieved through expensive electroporation methods and use of costly Cas9 nuclease reagents. Recommended experimental conditions for performing similar successful sequence insertion experiments using these Edit-R CRISPR-Cas9 reagents and workflows are shown in Table 1.

Optimizing gene editing experimental conditions using a mismatch detection assay is essential before performing HDR applications because of the generally lower baseline rate of HDR when compared to NHEJ for repair of double-strand DNA breaks. One method for increasing the percent editing in a population of transfected cells is to subject the cells to the selective pressure of antibiotic resistance, *e.g.*, puromycin selection. We have observed that transfected cell populations selected for Cas9 expression by application of puromycin correlated with higher levels of knockin through HDR. Furthermore, we have observed higher levels of knockins when using a cell line that stably expresses Cas9 nuclease. We observed that when comparing the genomic engineering of the two targets presented in this study, the mismatch detection assay and the RFLP assay results did not correlate, which is consistent with the overall lower efficiencies of HDR compared to the NHEJ. Yet, for each gene target tested, the highest amount of gene editing, as estimated by mismatch detection, is associated with the highest amount of HDR knockin. It is important to note that careful transfection optimization is a critical process to maximize HDR and decrease the overall effort of screening individual clonal lines to find the correct HDR-induced modification. For example, one would need to screen ten times more individual clonal lines when HDR knockin levels are observed at 1% compared to 10%. Additionally, expression of HDR machinery in cell lines and cell types can vary, which can affect HDR efficiency.

To this end, we were able to identify the optimal amounts of ssDNA donor required (ranging from 2.5 nM to 10.0 nM) for maximal levels of HDR in U2OS, for both co-transfection of Cas9 plasmid plus synthetic crRNA:tracrRNA complex, and transfection of synthetic crRNA:tracrRNA complex into a Cas9-integrated cell line (Figure 5). Our data show that HDR is possible with donor homology arms as short as 20 nucleotides for each arm. However, the variability of the knockin is potentially greater at this arm length; therefore, we recommend each individual homology arm length to be at least 30 nucleotides flanking the desired insert.

When designing donor oligos for HDR, one must consider where the Cas9 DSB occurs with respect to the homology arms of the oligo donor. If the CRISPR protospacer and PAM sequence occurs in one of the homology arms of the oligo donor, silent mutations must be made at one of the two guanine bases of the NGG PAM and/or SNPs in the protospacer sequence proximal to the PAM to prevent Cas9 from cutting the donor oligo-repaired HDR site. Lastly, it has been found that SNP correction rates are as much as four-fold lower when the DSB occurs 100 bp away from the SNP;[4] thus, try to design your crRNA to create a Cas9-mediated DSB as close to the insertion site as possible for maximal HDR efficiency.[5]

Conclusion

The Edit-R CRISPR-Cas9 genome engineering platform simplifies the experimental workflow by removing tedious cloning steps prior to testing gene knockout, gene deletion and HDR. With careful optimization of editing efficiency in each desired cell line, precise insertion of short sequences using synthetic DNA oligo donors can be achieved utilizing the HDR cellular process.

Materials and methods

Lipid transfection: U2OS and Ubi-GFP U2OS-Cas9 cells (Thermo Scientific™ BioImage™ Proteasome Redistribution Assay Cat #R0402102) were seeded in a 96-well plate at 10,000 cells per well. Edit-R synthetic crRNAs and tracrRNA were individually resuspended in 10 mM Tris-HCl (pH7.5), to 100 µM. crRNA and tracrRNA were combined and the RNA was further diluted to 2.5 µM using 10 mM Tris-HCl (pH7.5). A final concentration of

25 nM crRNA:tracrRNA complex (25 nM of each crRNA and tracrRNA) was used for transfection. Cells were transfected with 0.4 µL/well DharmaFECT Duo Transfection Reagent (Dharmacon Cat #T-2010-03) using 200 ng of Cas9 plasmid (for co-transfections) and 25 nM crRNA:tracrRNA complex. A final concentration range of 0.5 to 100 nM ssDNA donor oligo was used for transfections.

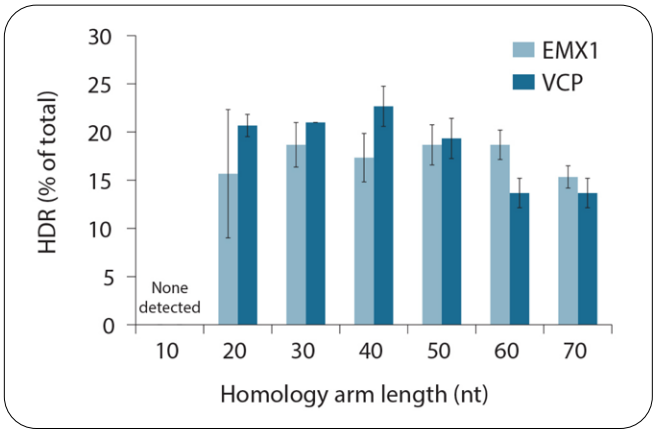


Figure 6: Optimizing homology arm length for maximal HDR knockin. DharmaFECT Duo Transfection Reagent was used to transfect a Cas9-integrated U2OS cell line with crRNA:tracrRNA targeting *EMX1* or *VCP* and a donor DNA oligo. Different donor DNA oligos were used, each with increasing homology arm length. A RFLP assay was performed on three independent transfections to determine HDR-based knockin for each donor DNA oligo with different homology arm lengths.

Table 1: Recommendations for HDR-based gene editing using Edit-R expressed Cas9 and synthetic crRNA:tracrRNA in 96-well cell culture dishes.

Cells seeded at a density that gives 70-90% confluency on transfection day ▪ Stable cell lines can be created by transduction using Edit-R Cas9 Lentiviral Particles
DharmaFECT Duo Transfection Reagent (optimized concentration for each cell line)
25 nM/well Edit-R synthetic crRNA:tracrRNA
Oligo concentration: ▪ U2OS cells (co-transfection): 2.5-10 nM/well donor DNA oligo ▪ U2OS-Cas9 cells (integrated): 2.5-10 nM/well donor DNA oligo
30 nucleotide homology arms for small insertions (< 50 nucleotides)

Note: Conditions were optimized in U2OS cell lines. Conditions may need optimization in your cells of interest.

Puromycin selection: Twenty-four hours post-transfection, cells were exposed to 1.5 µg/mL of puromycin (Invivogen Cat #ant-pr-5). Cells surviving selection were harvested, analyzed, and are referred to as Puro selection.

ssDNA donor oligos: DNA oligos were synthesized in-house using standard solid-phase DNA synthesis and desalting procedures. Each DNA oligo used contained 2 phosphorothioate linkages on the first two and last two DNA bases. Oligos were ethanol precipitated and resuspended (100 µM) in 10 mM Tris-HCl (pH7.5). Oligos were further diluted to 1 µM prior to transfection.

Genomic DNA isolation and DNA mismatch detection assay: Genomic DNA was isolated 72 hours post-transfection by direct lysis of the cells in Phusion™ HF buffer (Thermo Scientific Cat #F-518L) proteinase K (Thermo Scientific Cat #EO0491) and RNase A (Thermo Scientific Cat #EN0531) for 1 hour at 56 °C followed by heat inactivation at 96 °C for 5 minutes. PCR was performed with primers flanking the cleavage sites. PCR products (500 ng) were treated with T7 endonuclease I (T7EI; NEB Cat #M0302L) for 25 minutes at 37 °C and the samples were separated on a 2% agarose gel. Percent editing in each sample was calculated using ImageJ software (NIH, imagej. nih.gov/ij, 1997-2014).

Restriction Fragment Length Polymorphism assay: Genomic isolation was performed as described above 72 hours post-transfection. PCR was performed with primers flanking the restriction enzyme knockin site and outside of the homology arms used for each donor DNA oligo. PCR products were further purified and eluted in water. PCR products (500 ng) were digested in FastDigest Buffer and 1 U of FastDigest NheI enzyme (Thermo Scientific Cat FD0974) for 30 minutes at 37 °C and the samples were separated on a 2% agarose gel. HDR knockin for each sample was calculated using ImageJ software (NIH, imagej nih.gov/ij, 1997-2014).

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For more information:

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