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

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Abstract

The CRISPR-Cas9 system derived from Streptococcus pyogenes uses the Cas9 nuclease protein that complexes with a tracrRNA and a targeting crRNA containing a 20 nucleotide guide sequence complementary to the genomic target of interest, to create double-strand DNA breaks. Once the double-strand break occurs, the mammalian cell utilizes endogenous mechanisms to repair the broken genomic DNA. In the presence of a donor sequence, the double-strand break can be repaired precisely using homology-directed repair (HDR) resulting in the desired insertion or knockin.

Here we demonstrate the use of synthetic single-stranded DNA oligo donors in a novel gene editing (Dharmacon™ Edit-R™) platform comprised of synthetic tracrRNA and crRNAs which program Cas9 nuclease to perform HDR, resulting in precise insertion of short DNA sequences. By carefully optimizing lipid-based transfection conditions, we can utilize this platform to create knockins with efficiencies as high as 25%. We evaluate several parameters that affect the HDR efficiency including the length of homology arms needed in the single-stranded DNA oligo donor. Our data show that HDR is able to perform insertion of 10-12 nucleotide sequences with as little as 20 nucleotide homology arms. We additionally provide experimental workflows to perform simple and effective lipid-based HDR transfections in a 96-well plate format. The methods presented within 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.

Genome engineering with Edit-R Cas9 and synthetic crRNA:tracrRNA

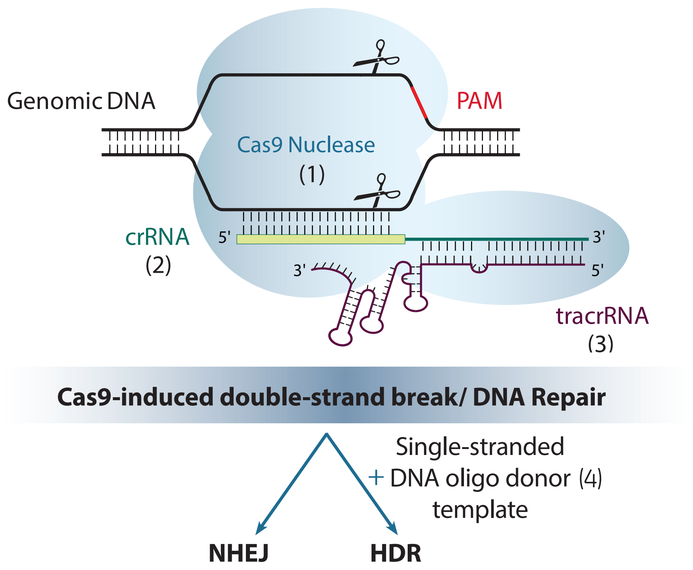

Figure 1. Illustration of Cas9 nuclease (1) bound to the synthetic crRNA (2):tracrRNA (3) complex and targeted to genomic DNA by the guide sequence in the crRNA, adjacent to the PAM (red). Repair of DSB can be achieved by NHEJ in the absence of a donor template, or by HDR when a donor template (4) such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

HDR lipid transfection workflow

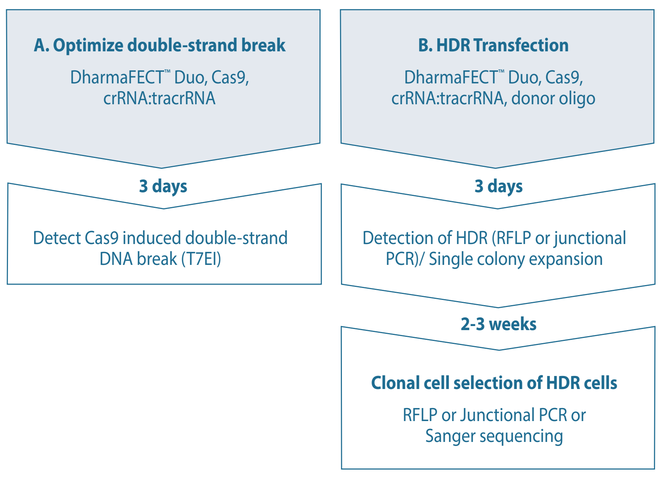

Figure 2. A Cas9 expression plasmid co-transfection workflow with DharmaFECT Duo lipid transfection reagent. Optimization of maximal double-strand breaks **(A)** is necessary to increase the amount of double-strand breaks to be repaired by HDR when co-transfected with a donor DNA oligo **(B)**.

Targeted integration via HDR at the VCP locus

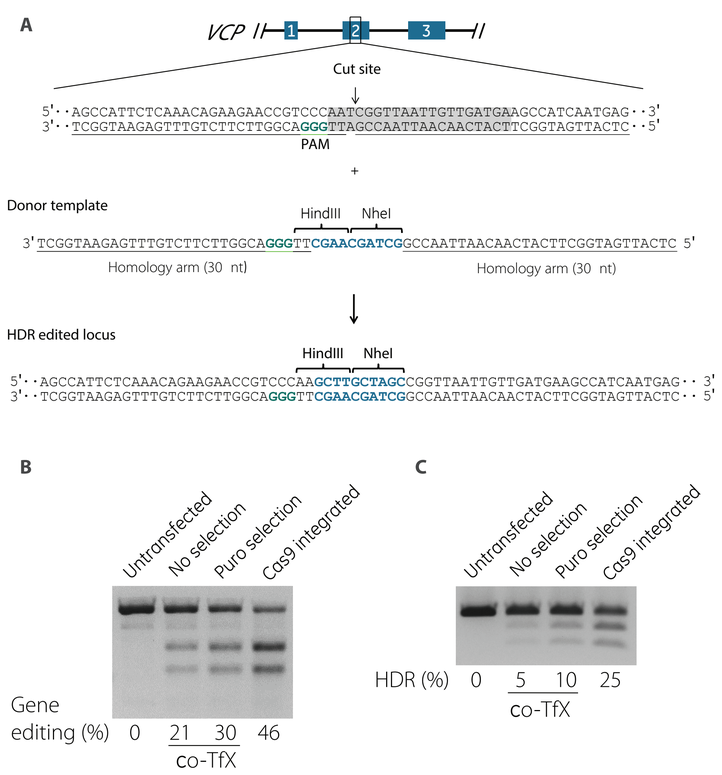

Figure 3. U2OS cells were used to target the VCP locus for HDR genome engineering. **(A)** Schematic of the VCP locus. Exons are indicated by the blue boxes. The guide sequence in the crRNA is indicated by the gray highlighted region, the PAM is indicated in green, and the cut site is indicated by the arrow. The additional inserted sequence is indicated in blue along with the restriction enzyme sites. **(B)** Analysis of the efficiency of indel mutations using T7EI analysis of untransfected and transfected cells (with and without Cas9 plasmid selection or using a Cas9-integrated cell line). The level of gene editing is indicated below the gels. **(C)** Analysis of the efficiency of HDR insertion of a HindIII/NheI restriction enzyme site (blue) introduced via a 30 nucleotide homology arm DNA oligo. HDR percentage was calculated using an RFLP assay with NheI digestion of VCP PCR amplicons.

Targeted integration via HDR at the *EMX*1 locus

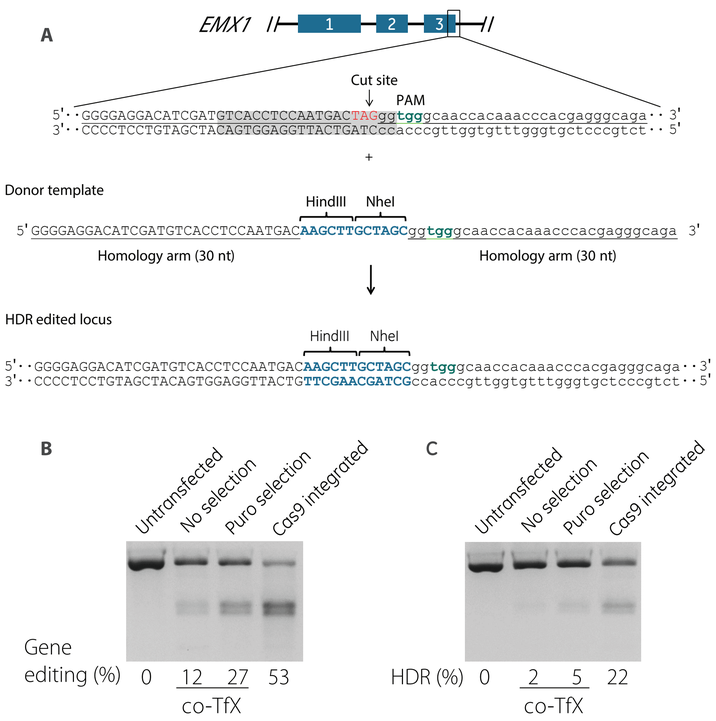

Figure 4. U2OS cells were used to target the EMX1 locus (A) for HDR genome engineering. **(A)** Schematic of the EMX1 locus. Exons are indicated by the blue boxes. The guide sequence in the crRNA is indicated by the gray highlighted region, the PAM is indicated in green, and the cut site is indicated by the arrow. The additional inserted sequence is indicated in blue along with the restriction enzyme sites. **(B)** Analysis of the efficiency of indel mutations using T7EI analysis of untransfected and transfected cells (with and without Cas9 plasmid selection or using a Cas9-integrated cell line). The level of gene editing is indicated below the gels. **(C)** Analysis of the efficiency of HDR insertion of a HindIII/NheI restriction enzyme site (blue) introduced via a 30 nucleotide homology arm DNA oligo. HDR percentage was calculated using an RFLP assay with NheI digestion of *EMX*1 PCR amplicons.

Donor DNA oligo concentration optimization

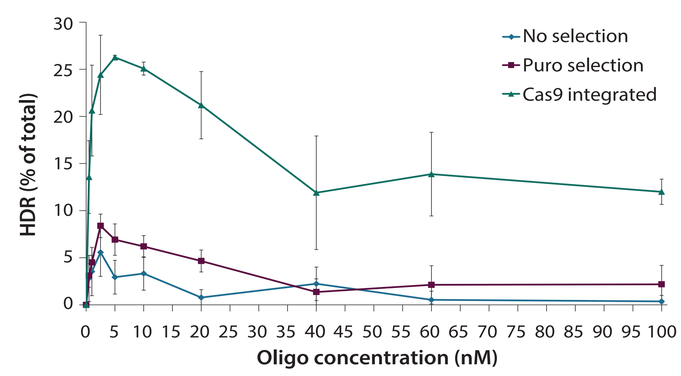

Figure 5. DharmaFECT Duo was used to co-transfect U2OS cells with a Cas9 expression plasmid, EMX1-crRNA:tracrRNA, and increasing concentrations of a donor DNA oligo with 30 nucleotide homology arms. Alternatively, DharmaFECT Duo was used to transfect a Cas9 integrated U2OS cell line with *EMX*1-crRNA:tracrRNA, 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.

Optimizing homology arm length for maximal HDR knockin

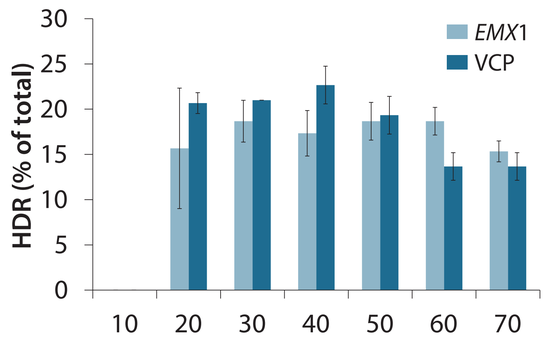

Figure 6. DharmaFECT Duo was used to transfect a Cas9 integrated U2OS cell line with either EMX1-crRNA:tracrRNA or VCP-crRNA:tracrRNA and a donor DNA oligo. Different donor DNA oligos were used, each with increasing homology arm length. A RFLP assay was performed on three independent transfections to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

Guidelines for HDR lipid transfections in 96-well cell culture dishes

- Cells seeded at a density that gives 70-90% confluency on transfection day
- 200 ng/well Edit-R Cas9 expression plasmid (or use a stably integrated Cas9-expressing cell line)
  - » Stable cell lines can be created by transduction using Edit-R Lentiviral Cas9
- DharmaFECT Duo (optimized concentration for the cell line)
- 25 nM/well crRNA:tracrRNA
- Oligo concentration:
  - » U2OS (co-transfection or Cas9 integrated): 2.5 – 10 nM/well donor oligo
- 30 nucleotide homology arms for small insertions (<50 nt)

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