GE Healthcare

Homology-directed repair using synthetic crRNA and tracrRNA with single-stranded DNA oligos

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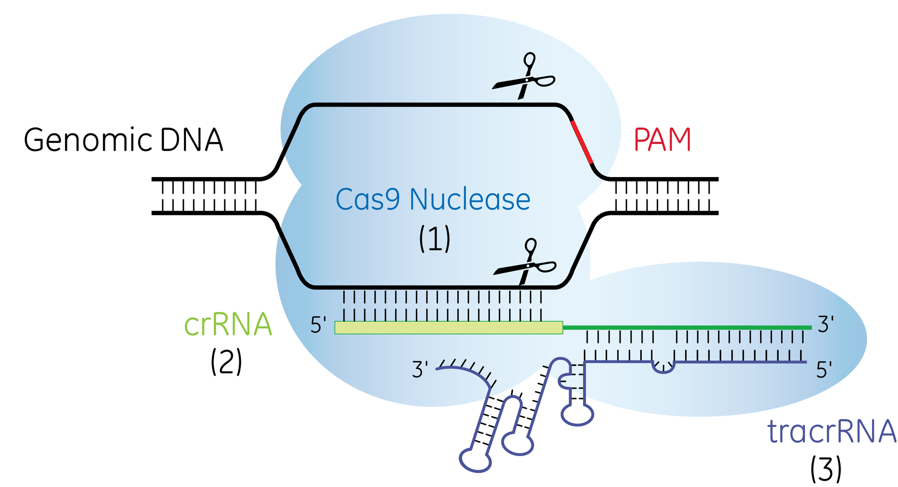
Abstract

The CRISPR-Cas9 system derived from *Streptococcus pyogenes* uses a Cas9 nuclease protein that, when complexed with a crRNA and a targeting tracrRNA containing a 20 nucleotide guide sequence complementary to the genomic target of interest, creates targeted 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 a desired insertion, or knockin.

Here we demonstrate the use of synthetic single-stranded DNA (ssDNA) oligo donors in a novel gene editing platform comprised of synthetic crRNA and tracrRNA that program Cas9 nuclease to perform HDR, resulting in precise insertion of short DNA sequences. By carefully optimizing lipid-based transfection conditions, including ssDNA oligo concentration, we can utilize this platform to create knockins. We evaluated several parameters that affect the HDR efficiency including the length of homology arms needed in the ssDNA oligo donor. We tested homology arm lengths of 10, 20, 30, 40, 50, 60, and 70 nucleotides and found that HDR is able to perform insertion of 10-12 nucleotide sequences with homology arms as short as 20 nucleotides. Interestingly, we also observed that longer ssDNA homology arms become less efficient.

We compared editing and HDR efficiencies of crRNA:tracrRNA and ssDNA oligo utilizing a cell line stably expressing Cas9 to cells co-transfected with Cas9 expression plasmid +/- antibiotic selection. Under these conditions, Cas9-integrated cells resulted in the highest efficiency of gene editing and HDR, ~ 50% and ~ 24%, respectively. 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 Dharmacon™ Edit-R™ Cas9 Nuclease and synthetic crRNA:tracrRNA



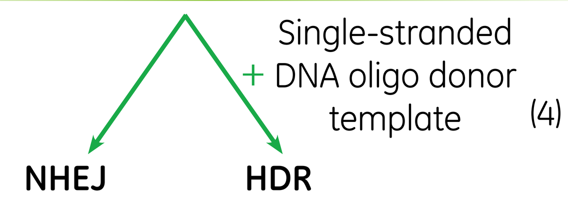


Figure 1. Illustration of Cas9 nuclease (1) bound to the synthetic crRNA (2):tracrRNA (3) complex and targeted to genomic DNA by the guide sequence in the crRNA, adjacent to the PAM (red). Repair of DSB can be achieved by NHEJ in the absence of a donor template, or by HDR when a donor template (4), such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

Homology-directed repair lipid transfection workflow

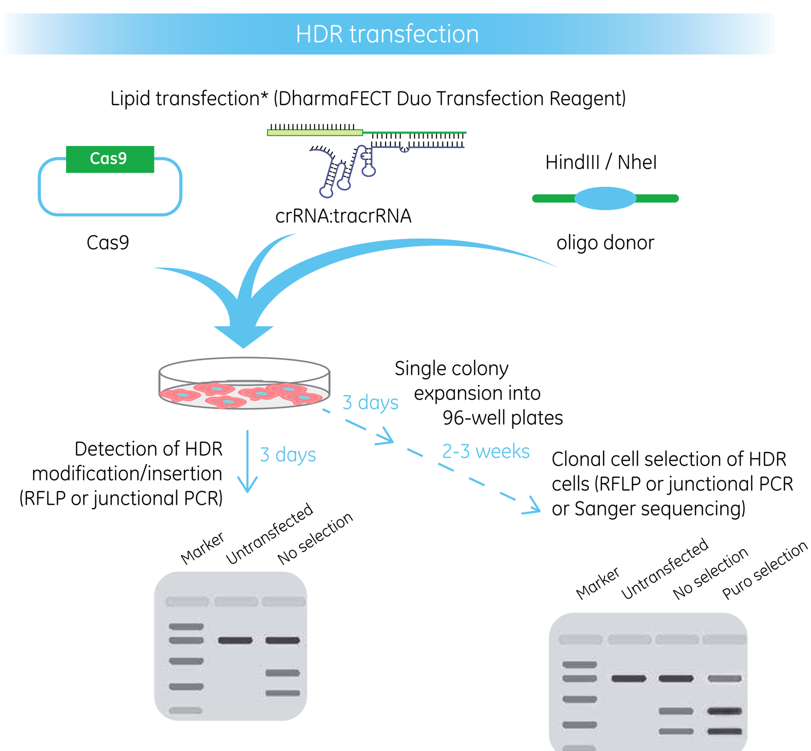


Figure 2. A Cas9 expression plasmid co-transfection workflow with Dharmacon™ DharmaFECT™ Duo lipid transfection reagent. Detection and optimization of double-strand breaks prior to performing the HDR transfection ensures maximal double-strand breaks for HDR.

Targeted integration via HDR at the *VCP* locus

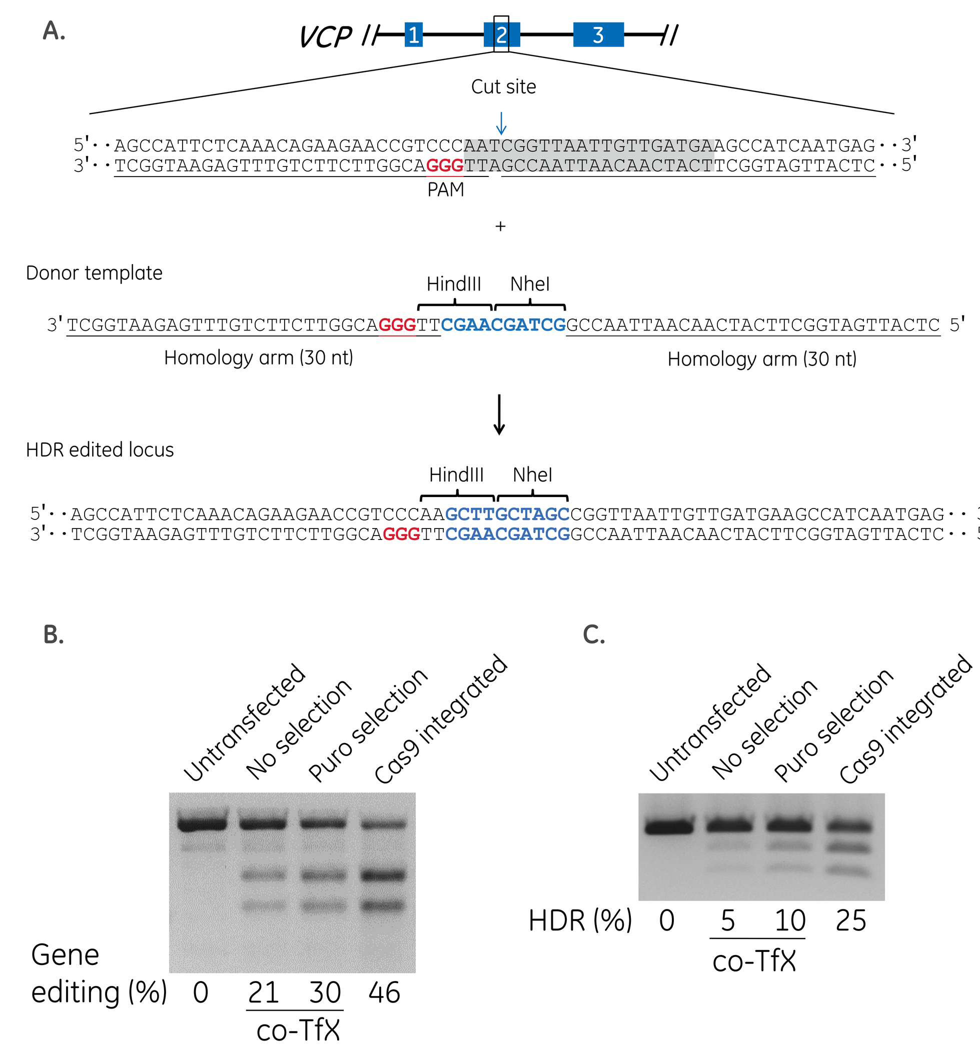


Figure 3. U2OS cells were used to target the *VCP* locus for HDR genome engineering. **A.** Schematic of the *VCP* locus. Exons are indicated by the blue boxes. The guide sequence in the crRNA is indicated by the gray highlighted region, the PAM is indicated in red, and the cut site is indicated by the blue arrow. The additional inserted sequence is indicated in blue along with the restriction enzyme sites. **B.** Analysis of the efficiency of indel mutations using a mismatch detection assay with T7EI 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 using a 30 nucleotide homology arm DNA oligo. HDR percentage was calculated using a RFLP assay with NheI digestion of *VCP* PCR amplicons. Co-TfX = co-transfection.

Targeted integration via HDR at the *EMX1* locus

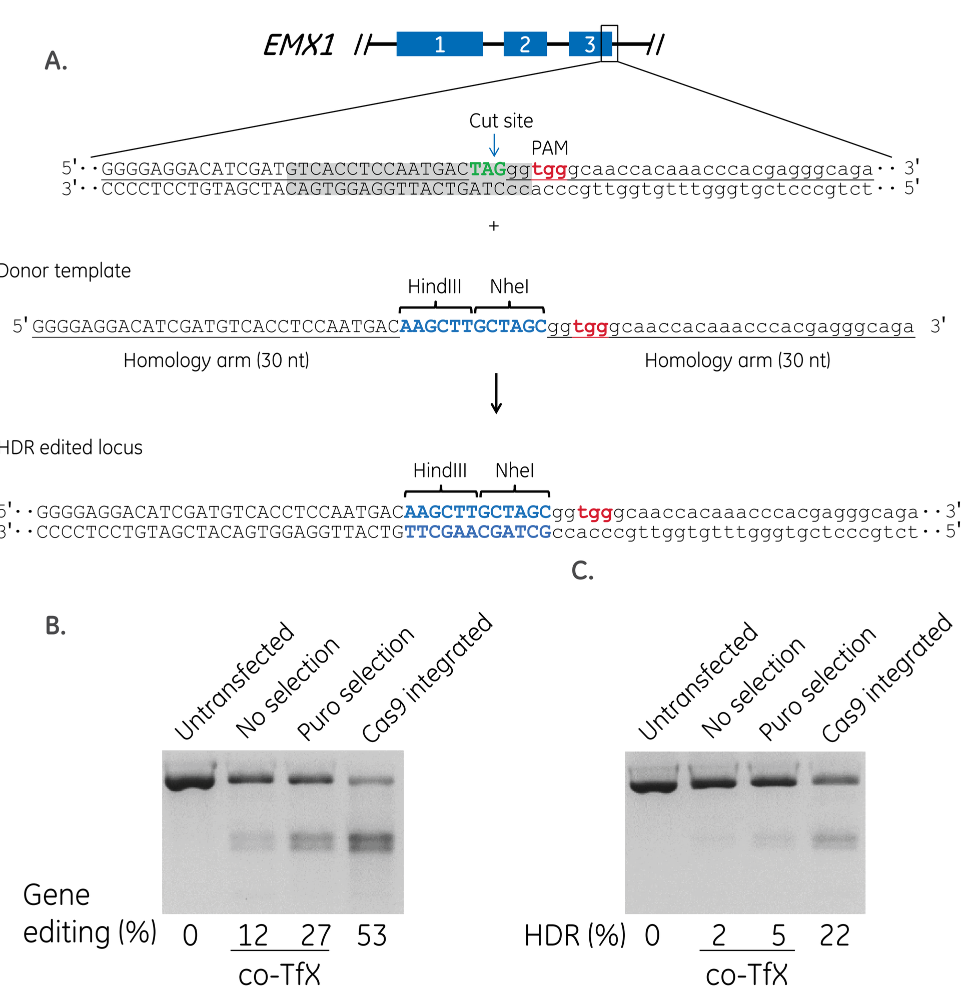


Figure 4. U2OS cells were used to target the *EMX1* locus for HDR genome engineering. **A.** Schematic of the *EMX1* locus. Exons are indicated by the blue boxes. The guide sequence in the crRNA is indicated by the gray highlighted region, the PAM is indicated in red, and the cut site is indicated by the blue arrow. The additional inserted sequence is indicated in blue along with the restriction enzyme sites. **B.** Analysis of the efficiency of indel mutations using a mismatch detection assay with T7EI 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 using a 30 nucleotide homology arm DNA oligo. HDR percentage was calculated using a RFLP assay with NheI digestion of *EMX1* PCR amplicons. Co-TfX = co-transfection.

Donor DNA oligo concentration optimization

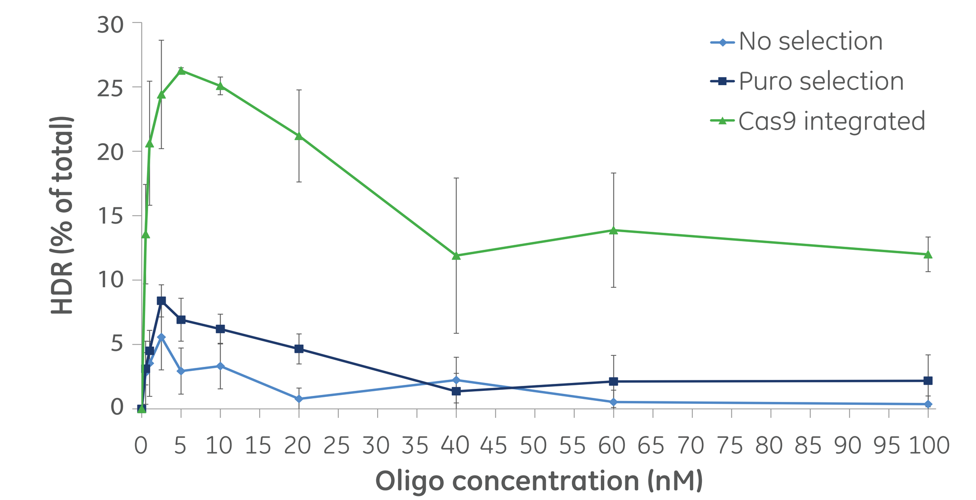


Figure 5. DharmaFECT Duo Transfection Reagent was used to co-transfect U2OS cells with a Cas9 expression plasmid, EMX1-crRNA:tracrRNA, and increasing concentrations of a donor DNA oligo with 30 nucleotide homology arms. Alternatively, DharmaFECT Duo Transfection Reagent was used to transfect a Cas9-integrated U2OS cell line with EMX1-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 are from three independent transfections.

Optimizing homology arm length for maximal HDR knockin

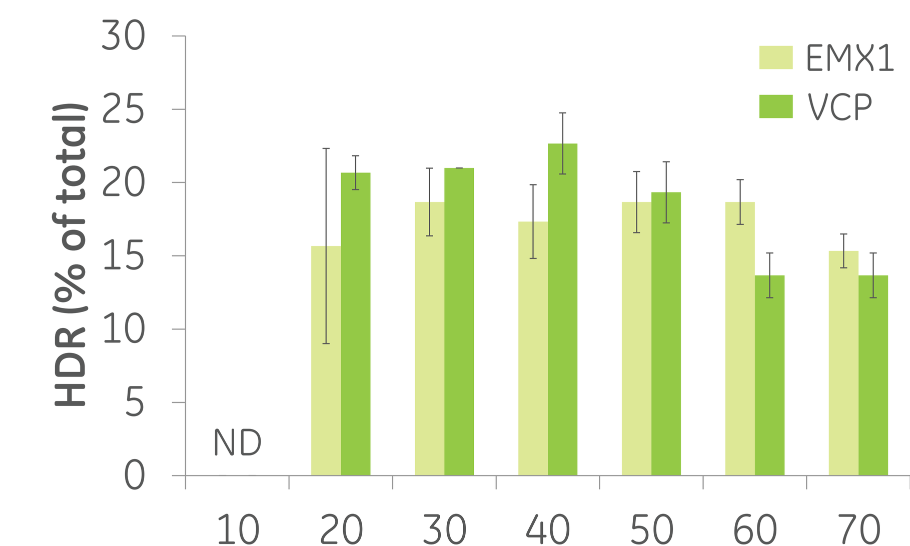


Figure 6. DharmaFECT Duo Transfection Reagent was used to transfect a Cas9 integrated U2OS cell line with either EMX1-crRNA:tracrRNA or VCP-crRNA:tracrRNA and a donor DNA oligo. Different donor DNA oligos were used, each with increasing homology arm length. A RFLP assay was performed on three independent 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 Dharmacon™ 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 Nuclease
- DharmaFECT Duo Transfection Reagent (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 nucleotides)

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