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 an 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 genome. 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 knock. Here we demonstrate the use of synthetic single-stranded DNA (ssDNA) oligos in a novel gene editing platform comprised of synthetic crRNA and tracrRNA that program Cas9 nucleases to perform HDR, resulting in precise insertion of short DNA sequences. By carefully optimizing lipid-based transfection conditions, including ssDNA oligo concentrations, we can utilize this platform to create knock-ins. We evaluated several parameters that affect the HDR efficiency, including the length of homology arms needed in the ssDNA 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 30-50 nucleotide sequences with homology arms as short as 10 nucleotides. We compared editing efficiency and HDR efficiencies of crRNA and tracrRNA oligos utilizing a cell line stably expressing Cas9 to cells co-transfected with Cas9-expression plasmid +/- antibiotic selection. Under these conditions, Cas9-integrated HDR edited locus for HDR genome engineering.

Targeted integration via HDR at the VCP locus

**A.** 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 HDR knock-in using a 15 nucleotide homology arm. HDR percentages were calculated using a RFLP assay with HindIII digestion of VCP PCR amplificates. C. PCR-SSCP.

**B.** Donor DNA oligo concentration optimization

**C.** Optimization of homology arm length for maximal HDR knock-in

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

- Cells seeded at a density that gives 75-90% confluence on transfection day
- 200 nglw DharmaFECT Duo™ - Cas9 expression plasmid for use in a stably integrated Cas9-expressing cell line
- Stable cell lines can be created by transfection using DharmaFECT™ and Cas9 nuclease
- DharmaFECT Duo Transfection Reagent (optimized concentration for the cell line)
- 25 nglw plasmid DNA
- Oligo concentration:
  - U2OS (transfection) or Cas9-integrated: 2.5-5.0 nglw donor oligos
  - 3x nucleotide homology arms for small insertions (50 nucleotides)

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**Figure 5.** DharmaFECT Duo Transfection Reagent was used to co-transfect U2OS cells with 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 an EMX1-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 knock-in for each concentration of donor DNA oligo in each transfection. Data presented are from three independent transfections.

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

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**Figure 7.** 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 HDR knock-in using a 30 nucleotide homology arm. HDR percentages were calculated using a RFLP assay with HindIII digestion of EMX1 PCR amplificates. C. PCR-SSCP.