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A Synthetic CRISPR-Cas9 System for Homology-directed Repair

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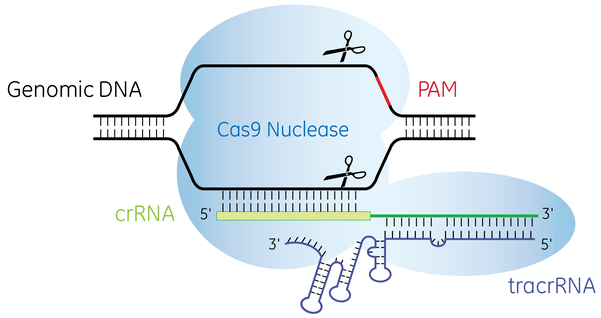
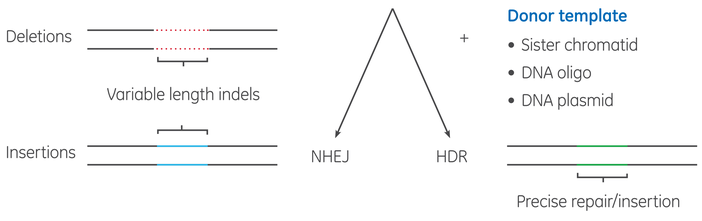
Abstract

CRISPR-Cas9 is increasingly becoming the most widely used genome engineering tool due to its ease of use and ability to cause double strand breaks (DSBs) at almost any locus of interest. The cellular homology-directed repair (HDR) pathway can be used to introduce exogenous genetic content, although this application of CRISPR-Cas9 is not as straightforward as exploiting the endogenous non-homologous end joining (NHEJ) pathway to create gene knockouts. We use a synthetic dual-RNA approach based on the natural bacterial CRISPR-Cas9 system for genome editing in mammalian cells that allows for rapid analysis of multiple chemically synthesized guide RNAs. Here we demonstrate the utility of this system to HDR genomic engineering applications and provide guidelines for improving CRISPR Cas9-assisted HDR. We present examples and design recommendations for the use of short, single-stranded DNAs as donor templates for small insertions. We show that homology arm length of the single-stranded donor DNA affects the efficiency of HDR. We also demonstrate the use of plasmid DNA donor templates for large insertions, using fluorescent protein fusions as a model. Lastly, we outline techniques and methods for characterization of HDR-generated cell lines for precise genomic engineering.

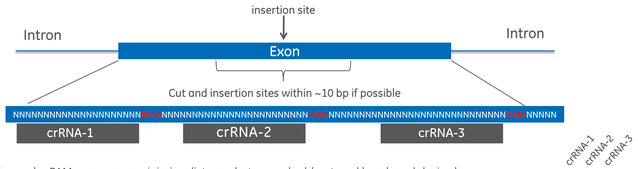
Cas9 programmed with dual synthetic RNAs



Cas9-induced double-strand break and DNA Repair

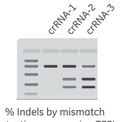


crRNAs for HDR applications



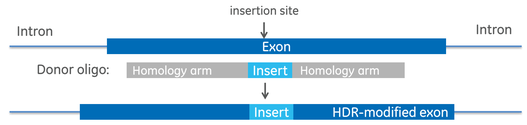
- Identify nearby PAM sequences; minimize distance between double-strand break and desired insertion site
- Assess efficiency of the corresponding crRNAs (ability to create indels)
- Analyze crRNA for potential off-targets (may be a trade-off with efficiency in some cases) :

dharmacon.gelifesciences.com/resources/tools-and-calculators/crispr-specificity-tool



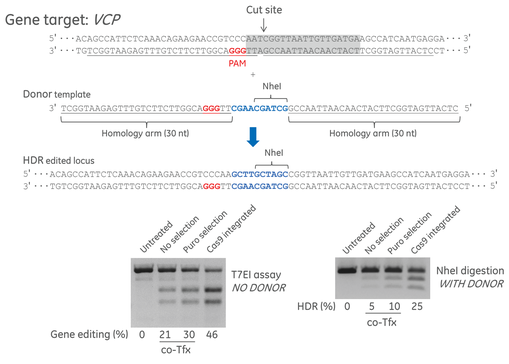
% Indels by mismatch detection assay using T7EI

Introducing short inserts with HDR and a synthetic DNA donor oligonucleotide



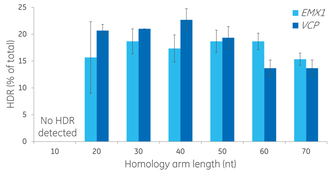
1. Design synthetic DNA donor oligo
   Identify homology arms surrounding target region
2. Optimize transfection conditions and reagents
   Cas9 + crRNA:tracrRNA + donor oligo
3. Verify HDR modification

Using HDR to generate a 10 nt insertion



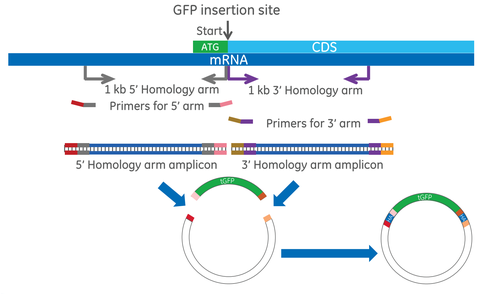
RFLP analysis with NheI was used to detect the presence of the insertion. The HDR efficiency achieved was 5-25%, depending on whether Cas9 was co-transfected as a plasmid or stably expressed in the cells.

Homology arm length for maximal HDR knockin



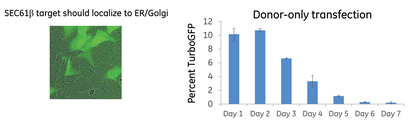
Different lengths of homology arms on the DNA donor oligo were assessed for efficiency of knockin with two different gene targets. The optimal HDR was found to occur when homology arms were 30-40 nt in length. ND = none detected.

Inserting an N-terminal GFP tag into SEC61β with HDR and a donor plasmid



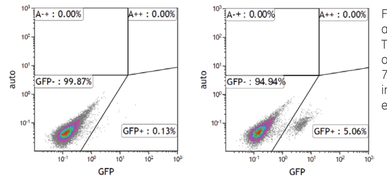
1. Construct donor plasmid
   Design and amplify homology arms from target region
   Assemble donor plasmid
   Optimize transfection conditions
2. Cas9 + crRNA:tracrRNA + donor plasmid
3. Verify HDR modification

Minimizing background TurboGFP expression from the donor plasmid



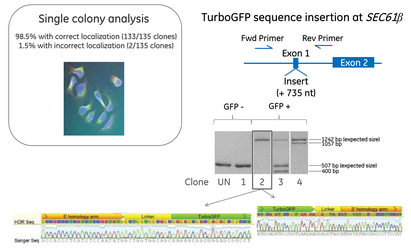
Since the donor plasmid homology arm includes endogenous promoter sequence, the donor plasmid alone was transfected to assess its ability for GFP expression. While it did self-express GFP, by 7 days post-transfection this non-specific background was virtually undetectable; this was the time point selected to assess efficiency of HDR knockin.

Identifying the TurboGFP-tagged positive cell population by FACS



FACS sorting was used to identify, separate, and quantify the percentage of GFP+ cells. The left plot measures residual fluorescence of the donor-alone transfected cells after 7 days, while the right plot illustrates the increase in GFP+ cells due to tagging of the endogenous locus by HDR.

Further characterization of the tagged target



Clone 2 shows correct localization of TurboGFP-SEC61B, as well as an increased PCR amplicon size where TurboGFP was integrated at all alleles present. This was confirmed using Sanger sequencing. Clones 3 and 4 contained additional editing events, underscoring the need to carefully characterize the resulting cell line after performing editing. Sanger sequencing (data not shown) confirmed that in addition to the desired TurboGFP integration, at least one of the alleles in clone 3 contained a 107 bp deletion (the band about 100 bp lower than the wild type band at 507 bp), while clone 4 contained an allele with an incomplete addition of TurboGFP (the additional band running lower than the expected size (1242 bp) with the TurboGFP insertion).

HDR with CRISPR-Cas9 and synthetic crRNA:tracrRNA

When selecting a crRNA target site, balance location, functionality, and specificity

- Test 3-5 (or more) crRNAs as close as possible to the desired insertion site

Optimize transfections to improve DSB efficiency

- More double-strand breaks yield more DNA ends to repair with HDR

Disrupt the CRISPR site in the donor DNA to prevent ongoing Cas9 cleavage of the HDR-modified locus

- Change PAM site
- Disrupt crRNA target site

Always sequence the resulting HDR cell line to verify proper insertion

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