GE Healthcare

Experimental design considerations for efficient and specific gene knockin using a CRISPR-Cas9 for HDR with synthetic crRNA and tracrRNA

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Abstract

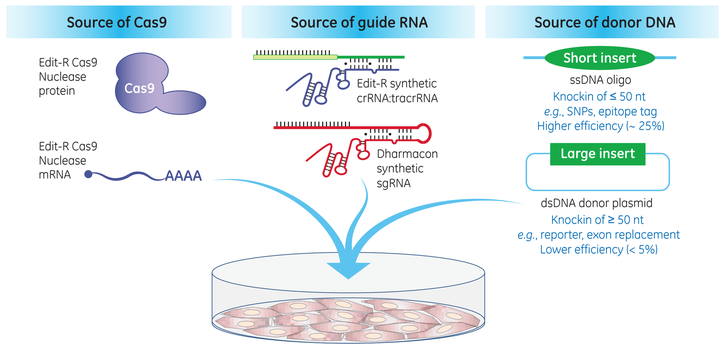
The bacterial CRISPR-Cas9 system has revolutionized the genome engineering world with its efficiency and ease of use. The most common use of the CRISPR-Cas9 technology has been to engender gene knockouts that are generated as a result of imperfect repair of a targeted double-strand DNA break by the non-homologous end joining (NHEJ) pathway.

Precise editing can also be obtained with the CRISPR-Cas9 system by providing a donor template, such as a single-stranded DNA oligonucleotide or double-stranded DNA in the form of a plasmid or linear DNA fragment, to be incorporated into the cell's genome by homology-directed repair (HDR). Efficiency of gene knockin is much lower compared to gene knockout since NHEJ is active throughout the cell cycle, especially G1 phase, while the HDR pathway is only active during the S and G2 phases.

The HDR pathway was initially believed to be error-free, but mutagenic alterations such as substitutions, insertions or deletions, and chromosome rearrangements can still occur during an HDR event. Therefore, it is imperative to optimize the efficiency of the gene editing experiment to increase the likelihood of successful knockin.

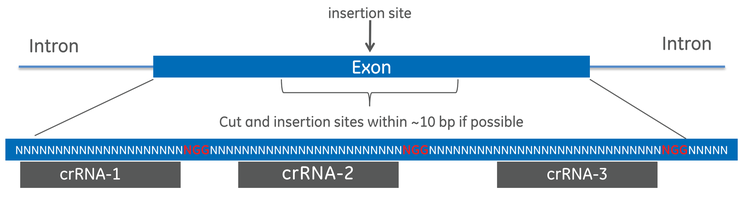
We evaluated the efficiency of single-stranded oligonucleotide and double-stranded DNA plasmid donors for gene knockin. We will demonstrate the characterization of clonal cell lines and additional design and analysis considerations for precise genome engineering with HDR and the CRISPR-Cas9 system.

Genome engineering by HDR with CRISPR-Cas9 and DNA donors



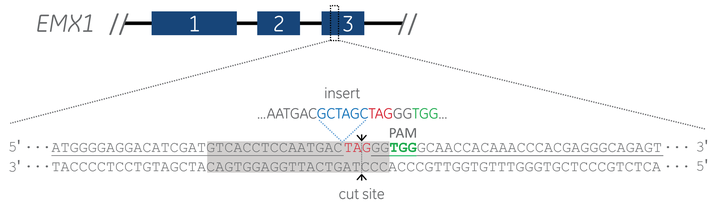
For knockin experiments with CRISPR-Cas9 by the HDR pathway, short single-stranded DNA (ssDNA) donor oligos are integrated into a cell's genome with higher efficiency than long double-stranded DNA (dsDNA) donor plasmids.

Precise genome knockin is more efficient when the insertion site is closer to the DNA double-strand break



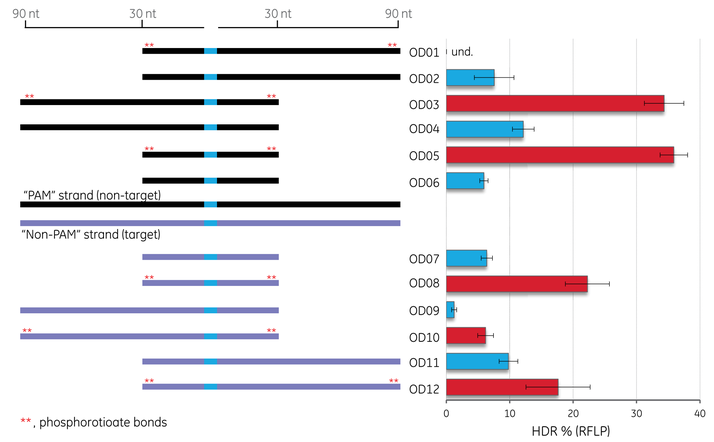
For better efficiency, the site of the specific recombination should be less than 10 bp away from the Cas9 cleaved site [Elliott *et al. Mol. Cell Biol.* **18**, 93-101 (1998)]. Multiple guide RNAs should be tested to choose one with the highest editing efficiency, balanced with highest specificity.

Designing ssDNA donor oligo for HDR knockin with CRISPR-Cas9



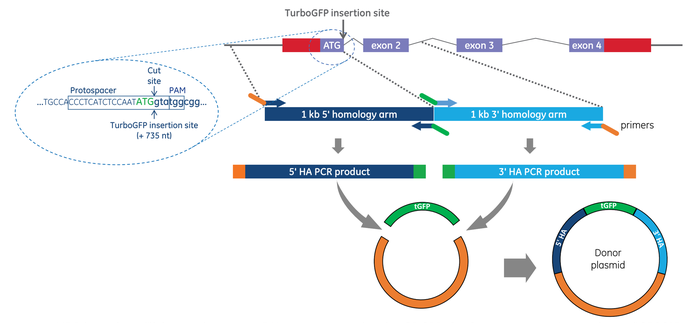
To check for the effect of donor oligo length, symmetry and oligo stabilization by chemical modifications, we inserted 6 nt (blue) upstream of the *EMX1* termination codon (red) in exon 3 using CRISPR-Cas9 by selecting a crRNA target site (grey box; the PAM site in shown in green) very close to the insertion site. The additional sequence introduces an NheI restriction site to the EMX1 amplicon that was used to determine the frequency of ssDNA integration by a restriction fragment length polymorphism (RFLP) analysis. Donor oligonucleotides were designed surrounding the insertion site having various homology arm length, symmetry, and modifications (see next panel).

Knockin is more efficient when donor oligos are stabilized by phosphorothioate bonds at their 5' and 3' ends



ssDNA donor oligos were chemically synthesized containing two homology arms, with each homology arm ranging from 30 to 90 nt in length, and either no modifications or two phosphorothioate bonds at both the 5' and 3' ends of the oligo. The donor oligos were co-transfected with synthetic crRNA:tracrRNA into U2OS cells with an integrated Cas9 nuclease gene. We did not observe a clear effect on strand preference (black or purple oligo size bars) or oligo symmetry. However, in our experimental conditions, donor oligos stabilized by two phosphorothioate bonds at their 5' and 3' ends (red bars) showed the highest frequency of donor integration independent of homology arm asymmetry.

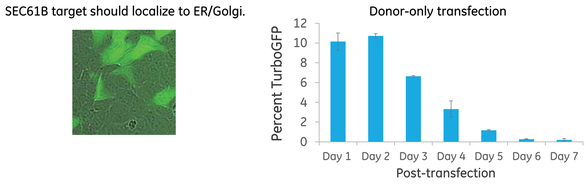
Using HDR and CRISPR-Cas9 to tag SEC61B with TurboGFP™ donor plasmid



To evaluate HDR insertion efficiency of a long dsDNA donor plasmid with CRISPR-Cas9 reagents, the N-terminus of SEC61B protein was tagged with TurboGFP (Evrogen, Moscow, Russia). Approximately 1 kb of homology arms flanking the TurboGFP insertion site were amplified from U2OS genomic DNA with primers containing extended sequences homologous to the TurboGFP cloning vector. Using a ligation independent cloning protocol, the 5' and 3' homology arms were assembled into a final plasmid.

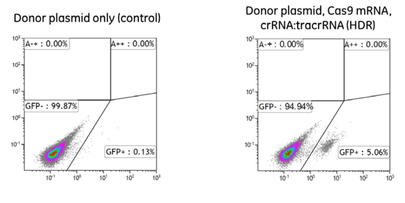
U2OS cells were then co-transfected with Dharmacon™ Edit-R™ Cas9 Nuclease mRNA (Cat #CAS11195), synthetic SEC61B-crRNA (custom synthesis) and synthetic tracrRNA (Cat #U-002000), and TurboGFP-SEC61B donor plasmid using DharmaFECT Duo transfection reagent (Cat #T-2010-01).

Minimizing background TurboGFP expression from the donor plasmid to avoid false positive results



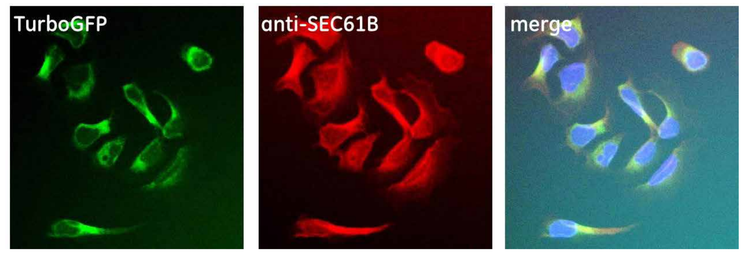
Spurious TurboGFP expression can occur from endogenous promoter sequences potentially present in the 5' homology arm when insertion is on the N-terminus of the protein. Non-specific background expression is drastically reduced by multiple cell divisions/passages. For this experiment, we performed analysis of HDR efficiency at seven days post-transfection when fluorescence of donor plasmid was minimal.

Enrichment of TurboGFP-tagged positive cell population by FACS for clonal cell isolation



Seven days post-transfection, a significant number of U2OS cells (5%) showed TurboGFP expression due to the donor plasmid recombination after generation of double-strand DNA breaks with Cas9 nuclease and synthetic crRNA:tracrRNA targeting exon 1 of *SEC61B* when compared to the control cells with donor plasmid only.

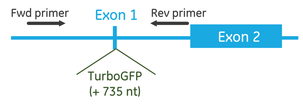
TurboGFP co-localizes with SEC61B in clonal cell lines



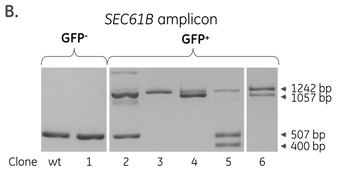
TurboGFP and immunofluorescence with anti-SEC61B-specific antibody showed co-localization of endogenous SEC61B with TurboGFP-tagged SEC61B in GFP-positive cell clones indicating correct insertion of the GFP reporter in frame with SEC61B.

Characterization of TurboGFP-SEC61B clonal cells showed a combination of HDR and NHEJ gene editing

A.

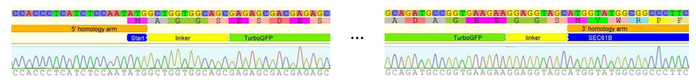


B.

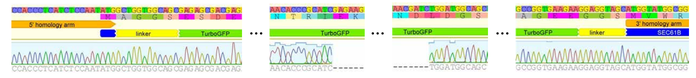


C.

Clone 3, 1242 bp fragment



Clone 6, 1057 bp fragment



D.

Clone	Phenotype	Genotype
1	GFP-	SEC61B^{wt}, homozygous
2	GFP+	tGFP-SEC61B/SEC61B^{wt}, heterozygous
3	GFP+	tGFP-SEC61B homozygous
4	GFP+	tGFP-SEC61B/SEC61B$^{ins(616\ nt)}$
5	GFP+	tGFP-SEC61B/SEC61Bwt/SEC61B$^{del(107\ nt)}$
6	GFP+	tGFP-SEC61B/tGFP$^{del(184\ nt)}$-SEC61B

Analysis of GFP-positive clonal cell lines for correct fusion-protein localization by fluorescence microscopy showed that 98% of cells exhibited the expected intracellular localization. However, PCR-based detection of TurboGFP integration in the SEC61B locus **(A)** showed unexpected bands from gDNA amplification of target region **(B)** for some clonal cell lines (clones 4, 5 and 6). Further analysis by Sanger sequencing of GFP-positive cells [two examples in **(C)**] revealed that all GFP-positive clonal cells (clones 2-6) had at least one allele with the correct tGFP-SEC61B fusion, but three of them (clones 4, 5 and 6) also had incomplete/imprecise recombination or indels **(D)**. Such indels observed in a reporter-tagging experiment can result in an allele with a truncated, untagged gene or in fusion proteins that may affect the cell phenotype. It is essential to not only observe the phenotype, but also to perform careful characterization of clonal lines.

Summary and recommendations for HDR knockin

- When planning a CRISPR-Cas9 HDR experiment, consider the size of the exogenous DNA to be inserted. Short inserts (< 50 nt) with single-stranded oligonucleotides can be up to three times or more efficient than large inserts with donor plasmids.
- Use of two phosphorothioate modifications at the 5' and 3' ends of ssDNA donor oligos improve integration efficiency.
- The Cas9 cut site and/or PAM in the donor DNA should be disrupted to prevent ongoing Cas9 cleavage of the HDR-modified locus (see Application Note at dharmacon.gelifesciences.com).
- When working with donor plasmids, allow several cell passages to eliminate non-integrated donor plasmids before isolating clonal cells.
- Analyze and fully characterize multiple clonal cell lines for the expected phenotype and always sequence the resulting HDR cell line to verify the correct insertion.

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