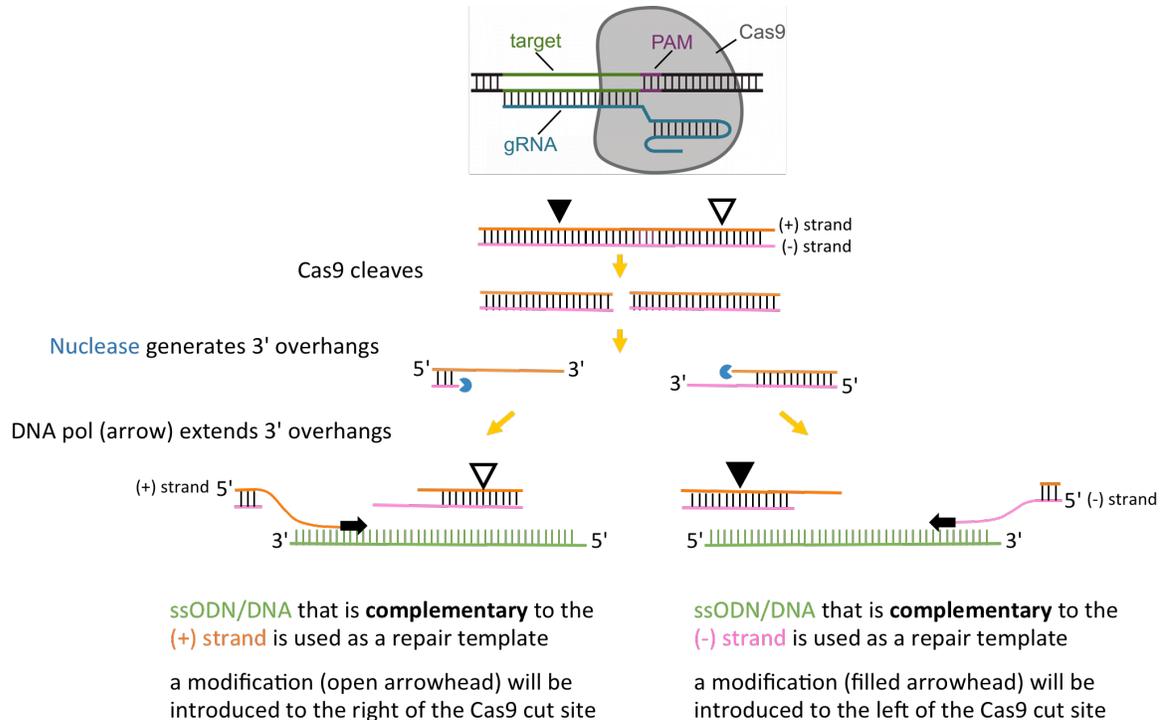


CRISPR-Cas9 experiment using a single-stranded oligo donor (ssODN)

Applications: Introducing a deletion, a short tag (or other sequence), or a point mutation(s)

Repair mechanism:



STEP-BY-STEP DESIGN OF AN ssODN DONOR EXPERIMENT (EXAMPLE: ADDITION OF A SHORT SEQUENCES)

1. Sequence the target region in the strain of the flies that will be CRISPR'ed (we typically sequence ~ 1kb around the site of modification). The reference genome sequence (e.g. what is on FlyBase) can be used as a guideline, but SNPs frequently exist and could affect the outcome of CRISPR-Cas9 cleavage and repair. Therefore, the design of ssODN and guide RNA (gRNA) should use the nucleotide sequence of the fly strain that is to be CRISPR'ed. **Note: only ONE gRNA is necessary when using an ssODN donor!** The ssODN will serve as a template for the repair of only one strand. Only use two gRNAs if you intend to generate a deletion.
2. Obtain the sequence around the target region and incorporate the changes that you intend to make.

Example:

```
tgaggacgccattaacgctgcggtgttatcaacactggaCTTCTTAAAATTACAAAGTACGTCCCCGC
TGTGGGATTTTTTAAAAATTTGTTATAATTTCGACGATCTTTTAGTTGTGTTTTATTTATTTTCGACTAA
TTCGACCTTAAACGCGAAACTTCACTTGACGATCTGGCCGGAACAACACACCACAATGCGTGACCACA
TGGTCCTTCATGAGTATGTAAATGCTGCCGGTATCACCGGTGGCGGCGGAAGTGGAGGTGGAGGCTCGGG
TGGAGGCAGTTCGCCAAGTGAAATAATTACTTTGCAGCTTGGGCAGTGCGGCAATCAAAGtaggtttctg
aaatcaggacgagtaccatctgcattgctaaaatcgtttaaacgcagTTGGCTTTGAGTTCTGGAAAAGA
TTGTGCCTGGAGCATGGCATCTCCCCTAGCGGCGTGCTGGAGGACTTTGCCAATGATGGCTTGGACCGC
```

Nucleotides to be inserted into the genome

- Use a guide RNA finder program (we use TargetFinder, available through the flyCRISPR website: <http://tools.flycrispr.molbio.wisc.edu/targetFinder/>) to find a guide RNA targeting sequence and corresponding PAM site that is nearby the intended modification (preferably within 10-20 nt from the site of editing due to the limited size of ssODN that can be synthesized).

Example:

tgaggacgccattaaacgctgcggtggttatcaacactggaCTTCTTAAAATTACAAAGTACGTCCCCGC
 TGTGGGATTTTTTAAAAATTTTGTATAATTCGACGATCTTTTAGTTGTGTTTTATTTTATTTTCTGACTAA
 TTCGACCTTAAACGC**GAAACTTCACCTGACGATTC****TGG**CCGGAAACAACACACCACAATG**CGTGACCACA**
TGGTCCTTCATGAGTATGTAATGCTGCCGGTATCACCGGTGGCGGCGGAAGTGGAGGTGGAGGCTCGGG
 TGGAGGCAGTTTCGCCAAGTGAAATAATTACTTTGCAGCTTGGGCAGTGCGGCAATCAAAGtaggtttctg
 aaatcaggacgagtaccatctgcattgctaaaatcgtttaaacgcagTTGGCTTTGAGTTCTGGAAAAGA
 TTGTGCCTGGAGCATGGCATCTCCCCTAGCGGCGTGCTGGAGGACTTTGCCAATGATGGCTTGGACCGC

guide RNA sequence

PAM sequence

Nucleotides to be inserted into the genome

- Design the ssODN.

The ssODN is comprised of three parts: two homology arms sandwiching the region to be edited.

- The 5' homology arm (HA):** this homology arm will encompass the ~50 nt upstream of the Cas9 cleavage site. The Cas9 cleavage site is 3 nt upstream of the PAM site (see diagram below).
- The edited region:** the genome sequence that will be modified by an addition to and/or an alternation of the endogenous sequence. This also includes sequence between the targeted modification and the Cas9 cleavage site.
- The 3' homology arm (HA):** this homology arm, also ~50 nt, will match the genomic sequence directly downstream of the edited sequence.

Example:

tgaggacgccattaaacgctgcggtggttatcaacactggaCTTCTTAAAATTACAAAGTACGTCCCCGC
 TGTGGGATTTTTTAAAAATTTTGTATAATTCGACGATCTTTTAGTTGTGTTT**TATTTTTATTTCTGACTAA**
TTCGACCTTAAACGCGAAACTTCACCTGACGA **/** **TTC****TGG**CCGGAAACAACACACCACAATG**CGTGACCAC**
ATGGTCCTTCATGAGTATGTAATGCTGCCGGTATCACCGGTGGCGGCGGAAGTGGAGGTGGAGGCTCGG
GTGGAGGCAGTTTCGCCAAGTGAAATAATTACTTTGCAGCTTGGGCAGTGCGGCAATCAAAGtaggtttct
 gaaatcaggacgagtaccatctgcattgctaaaatcgtttaaacgcagTTGGCTTTGAGTTCTGGAAAAG
 ATTGTGCCTGGAGCATGGCATCTCCCCTAGCGGCGTGCTGGAGGACTTTGCCAATGATGGCTTGGACCGC

PAM sequence

/ Cas9 cleavage site (the Cas9 cleavage site falls within the gRNA target sequence)

For simplicity, the gRNA sequence is not shown in this diagram (see diagram above)

Nucleotides to be inserted into the genome

Prevent Cas9 from cleaving the target site after the desired modification is introduced:

To prevent unwanted Cas9 cleavage after the desired modification is introduced, introduce a point mutation within the PAM or in the 12 nt upstream of the PAM (these 12 nt are called the seed, or PAM proximal, sequence). This Cas9-blocking mutation and your desired modification should both be either 3' or 5' of the cleavage site (e.g., they should be on the same side relative to the cut site). For example, if

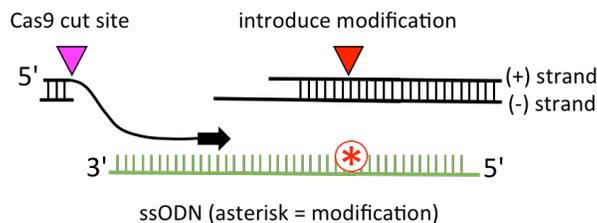
your intended modification is 3' of the cut site (as it is in this example), the mutation to prevent subsequent Cas9 cleavage should also be 3' of the cut site. If the intended modification is 5' of the cleavage site, the mutations to prevent subsequent Cas9 cleavage should also be 5' of the cut site. Also, if the targeted region contains coding sequence or a splice site junction, make sure the mutation does not alter splicing or the protein sequence.

```

tgaggacgccattaaacgctgcggtggttatcaacactggaCTTCTTAAAATTACAAAGTACGTCCCCGC
TGTGGGATTTTTTAAAAATTTTGTATAATTCGACGATCTTTTAGTTGTGTTTATTTTTATTTGACTAA
TTCGACCTTAAACGCGAAACTTCACTTGACGA/TTCTcGCCGGAAACAACACACCACAATCGTGACCAC
ATGGTCCTTCATGAGTATGTAATGCTGCCGGTATCACCGGTGGCGCGGAAGTGGAGGTGGAGGCTCGG
GTGGAGGCAGTTCGCCAAGTGAAATAATTACTTTGCAGCTTGGGCAGTGGCAATCAAAGtaggtttct
gaaatcaggacgagtaccatctgcattgctaaaatcgtttaaacgcagTTGGCTTTGAGTTCTGGAAAAG
ATTGTGCCTGGAGCATGGCATCTCCCTAGCGGCGTGTGGAGGACTTTGCCAATGATGGCTTGACCGC
  
```

5. Consider the repair process, and determine the proper ssODN orientation. The repair process is directional, and it is critical that the ssODN be complementary to the strand that initiates repair in the direction that will incorporate your desired modification (see cartoons below and cartoon on the first page). The position of the desired modification relative to the Cas9 cleavage site determines the orientation of the ssODN. The ssODN is complementary to the strand that initiates repair in the direction of your desired modification.

- a. Example 1: the Cas9 cleavage site is upstream of where the modification will be introduced (this corresponds to the mock CRISPR experiment in this document):



The ssODN corresponds to the "-" strand. The ssODN will be the reverse complement, e.g. order:

```

5' - CTTGGCGAACTGCCTCCACCCGAGCCTCCACCTCCACTTCCGCGCCACCGGTGATACCGGCAGCA
TTTACATACTCATGAAGGACCATGTGGTCACGCATTTGTGGTGTGTTGTTTCCGGCgAGAA/TCGTCAAGT
GAAGTTTCGCGTTTAAGGTCGAATTAGTCGAAATAAAATAA -3'
  
```

In this example the 50-nt HAs + insertion = 176 nt; we typically extend the HAs up to the 200-nt limit of oligo synthesis. So, in this example we would extend the HAs by 12 nt on each side.

- b. Example 2: the Cas9 cleavage site is downstream of the targeted modification. The ssODN corresponds to the "+" strand.

