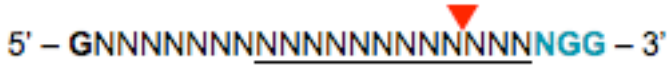
Generating targeting chiRNAs

A. Selecting a target sequence

The genomic target sequence should fulfill the following requirements:

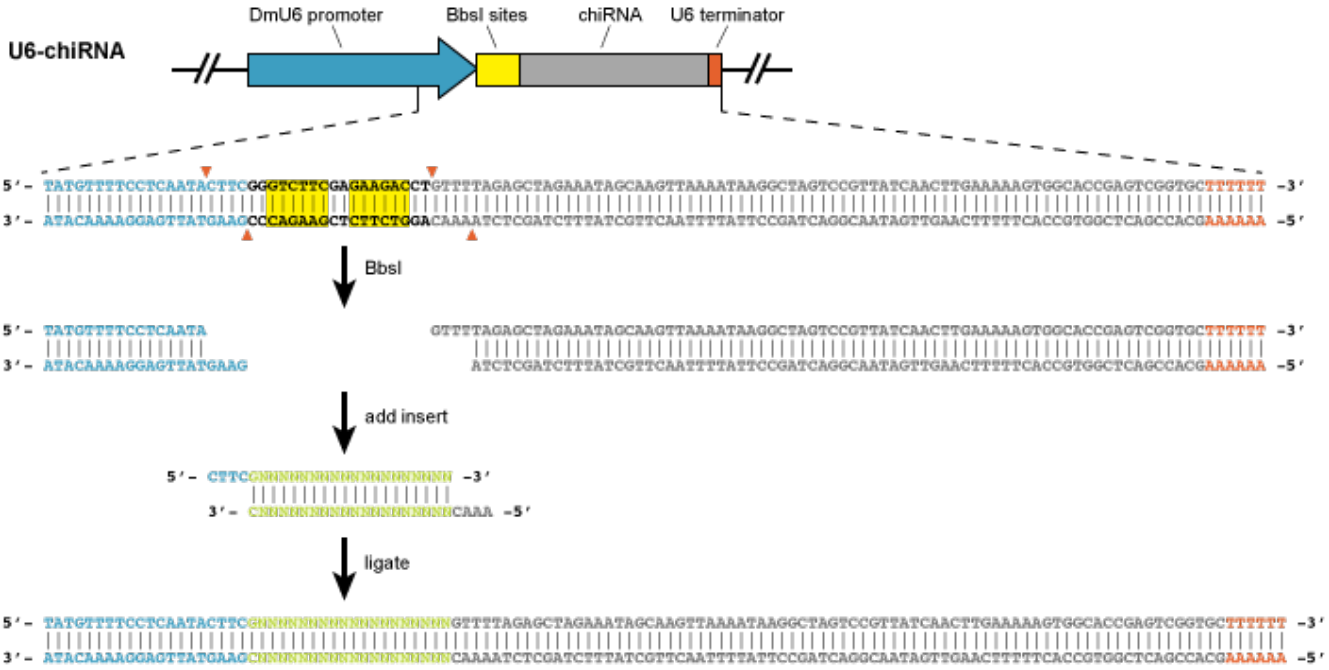
- 20-nt long
- followed by a 3-nt PAM sequence: **NGG**
- begin with a **G** to optimize U6-driven transcription



Cas9 nuclease cuts 3-nt upstream of the PAM site (cleavage site indicated by red arrowhead). The 12 nt upstrearm of the PAM site (underlined) are often referred to as the seed sequence and are the most critical determinants of cleavage specificity.

You can use the flyCRISPR Optimal Target Finder tool to identify highly specific target sites in the Drosophila genome.

B. Cloning

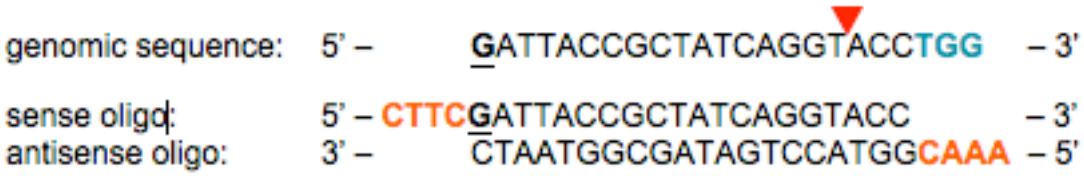


Targeting chiRNAs are easily cloned by annealed oligos into the pU6-BbsI-chiRNA plasmid via the BbsI restriction sites:

1. Design oligos based on the following template:

sense oligo: 5′ – **CTTCG** (19 nt) – 3′
antisense oligo: 3′ – C (19 nt) **CAAA** – 5′

example:



- The overhang sequences 5′ – **CTTC** – 3′ (sense oligo) and 3′ – **CAAA** – 5′ (antisense oligo) are complementary to the overhangs generated by BbsI digestion.
- The **G** in the sense oligo corresponds to the first nt of the chiRNA and is necessary for efficient U6-driven expression. This G is the first nt in the 20-nt targeting sequence.
- The sense oligo19-nt sequence is the EXACT same sequence as the genomic target sequence. Do not include the 3-nt NGG PAM sequence.
- Red arrowhead indicates Cas9 cut site.

Oligos should be ordered 5′ phosphorylated or phosphorylated using T4 PNK.

2. Anneal oligos

Using oligos phosophorylated by the manufacturer.
Dilute oligos at 100 µM in H_2O and set up the following reaction:

1µL oligo 1
1µL oligo 2
1µL 10x T4 ligation buffer
7µL water
Run the following thermocycler program:
95°C for 5 min, then ramp to 25°C at a rate of -0.1°C/sec.

When using PNK to phosphorylate the oligos.
Dilute oligos at 100 µM in H_2O and set up the following reaction:

1µL oligo 1
1µL oligo 2
1µL 10x T4 ligation buffer
1µL T4 Polynucleotide Kinase (NEB)
6µL water
Run the following thermocycler program:
37°C for 30 min, 95°C for 5 min, then ramp to 25°C at a rate of -0.1°C/sec.

3. Cut the pU6-BbsI-chiRNA plasmid with BbsI enzyme and de-phosphorylate.

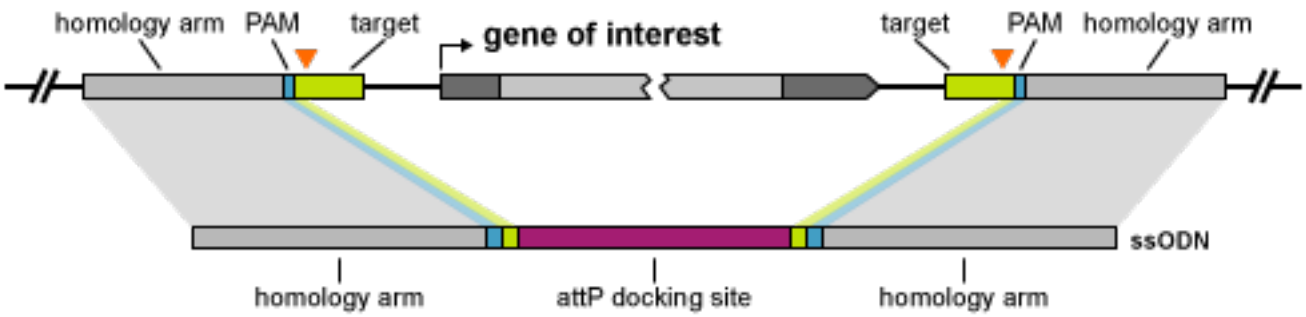
Digest 1µg of pU6-BbsI-chiRNA the BbsI (NEB) following the manufacturer's protocol. Half way through the digestion add 1µL of Calf Intestinal Alkaline Phosphatase (NEB).

Gel purify the digested product to aid in removal of undigested vector.

4. Ligate the annealed oligos with cut pU6-BbsI-chiRNA and transform E. coli.

XµL BbsI digested pU6-BbsI-chiRNA (50ng)
1µL annealed oligo insert
1µL 10x T4 ligation buffer
1µL T4 DNA ligase (NEB)
H_2O to 10µL
Incubate at 25°C for one hour then transform. Confirm inserts by sequencing with either T7 or T3 oligos.

Designing an ssODN template for HR-mediated repair



5’— (~60-nt 5’ homology) **CCNNNN** (**attP sequence**) **NNNNGG** (~60-nt 3' homology) – 3’

- **PAM**
- **NNN** corresponds to the 3-nt adjacent to the **PAM** site that remain following Cas9 cleavage. In this example, the 5’ PAM site is on the antisense strand and the 3’ PAM site is on the sense strand.
- **attP sequence (50-nt):**
  GTAGTGCCCCAACTGGGGTAACCTTTGAGTTCTCTCAGTTGGGGGCGTAG

Single-stranded oligonucleotides (ssODNs) of up to 200-nt can be ordered from Integrated DNA Technologies and other companies. Do not have the oligonucleotide PAGE-purified, as this can introduce trace chemical contaminates that reduce embryo viability.

Injection Mix

Dilute all plasmids and/or single-stranded donors in dH20 to the following concentrations and inject following standard embryo injection protocols. To increase survival we try to keep the total concentration of the injection mixture 1,000 ng/µL or less.

Cas9
Cas9 can either be introduced through coinjection of hsp70-Cas9 or it can provided through germline expression by injecting into vasa-Cas9 flies. While both methods work reliably, vasa-Cas9 expression leads to the higher targeting efficiency. We inject hsp70-Cas9 at concentrations of 250 ng/µL to 500 ng/µL. When injecting into vasa-Cas9, you do not need to add any hsp70-Cas9 to the injection mix.

gRNAs
Depending on the intended modification, one or two guideRNAs (gRNAs) may be used. For generating mutations via imperfectly repaired DSBs a single gRNA is used. Larger deletions can generated by using two gRNAs that define the limits of the intended deleiton. For homology-directed repair we also used two gRNAs injected with a homology containing donor. We inject gRNAs at concentrations of 100 ng/µL to 500 ng/µL depending on the application.

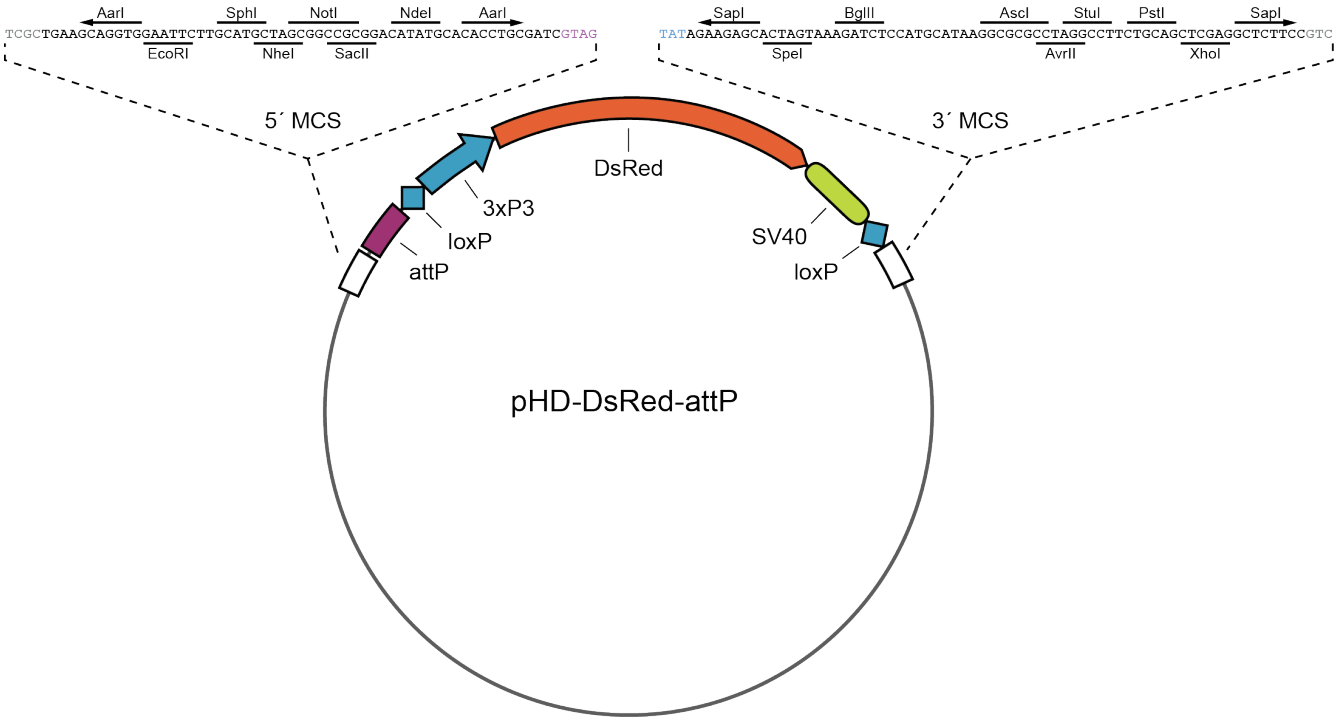
Donors
For homology-directed repair we have used both small single-stranded donors (ssODNs) and larger dsDNA donors. We inject ssODNs at a concentration of 100 ng/µL. For dsDNA donor vectors, like pHD-DsRed-attP, we typically inject at a concentration of 500 ng/µL.

Typical Hsp70-Cas9 Injection Mixtures
- **Indels**: phsp70-Cas9 (500 ng/µL), pU6-BbsI-chiRNA (500 ng/µL)
- **Deletions**: phsp70-Cas9 (500 ng/µL), pU6-BbsI-chiRNA (250 ng/µL each)
- **HDR with ssODNs**: phsp70-Cas9 (500 ng/µL), pU6-BbsI-chiRNA (250 ng/µL each), ssODN (100 ng/µL)
- **HDR with dsDNA**: phsp70-Cas9 (250 ng/µL), pU6-BbsI-chiRNA (100 ng/µL each), pHD-DsRed-attP donor (500 ng/µL)

Typical vasa-Cas9 Injection Mixtures
- **Indels**: pU6-BbsI-chiRNA (250 ng/µL)
- **HDR with dsDNA**: pU6-BbsI-chiRNA (100 ng/µL each), pHD-DsRed-attP donor (500 ng/µL).

Rapid dsDNA donor cloning with the pHD-DsRed-attP vector

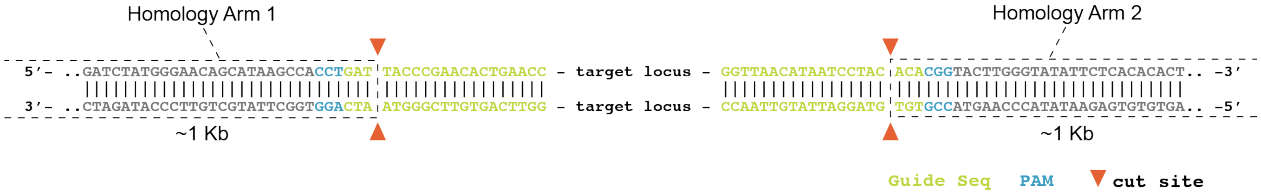
pHD-DsRed-attP is a vector for making dsDNA donor templates for homology-directed repair (HDR). This vector is designed for replacing a targeted locus with a 50-bp attP phage recombination site and is positively marked with a removable (floxed) 3XP3-dsRed construct for screening. It has 2 multiple cloning sites (MCS) for inserting homology arms that immediately flank the targeted locus. Work from Dana Carroll's lab indicates that homology arms of ~1Kb mediate efficient HR (Beumer et al., 2013).



Step 1. Homology arms

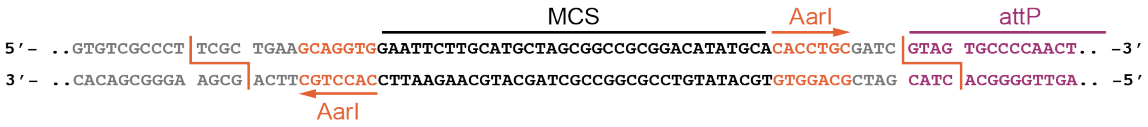
Homology arms should contain sequence **immediately** adjacent to your CRISPR/Cas9 cleavage sites for efficient HDR. In the example below two flanking CRISPR gRNAs are used to generate a defined deletion of a target locus. The intervening locus can be replaced with attP by incorporating homology arms immediately flanking both cleavage sites into pHD-DsRed-attP. Note that the cleavage sites are within the CRISPR target sites and the remaining portion of the target sequence should be included in each homology arm.

example:



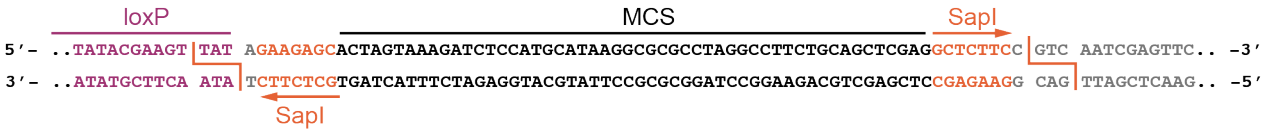
Step 2. Primer design for homology arm 1 (AarI)

In addition to traditional restriction enzyme sites, the pHD-DsRed-attP MCSs contain type IIs restriction sites (AarI for homology arm 1 and SapI for homology arm 2) for seamless and convenient cloning. Use of the type IIs sites requires precise primer design defined below.



AarI (site: CACCTGC) is a 4/8 cutter meaning it creates a 4-bp 5′ overhang 4-bp downstream of the cut site (illustrated as red lines). Primers should contain extra 5′ sequence (Ns) and 4-bp of "filler" sequence (Ns) followed by the appropriate overhang.

Step 3. Primer design for homology arm 2 (SapI)



SapI (site: GCTCTTC) is a 1/4 cutter meaning it creates a 3-bp 5′ overhang 1-bp downstream of the cut site (illustrated as red lines). Primers should contain extra 5′ sequence (Ns) and 1-bp of "filler" sequence (Ns) followed by the appropriate overhang.