CRISPR-Cas9 Genome Editing in *Drosophila*

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The CRISPR-Cas9 system has transformed genome engineering of model organisms from possible to practical. CRISPR-Cas9 can be readily programmed to generate sequence-specific double-strand breaks that disrupt targeted loci when repaired by error-prone non-homologous end joining (NHEJ) or to catalyze precise genome modification through homology-directed repair (HDR). Here we describe a streamlined approach for rapid and highly efficient engineering of the *Drosophila* genome via CRISPR-Cas9-mediated HDR. In this approach, transgenic flies expressing Cas9 are injected with plasmids to express guide RNAs (gRNAs) and positively marked donor templates. We detail target-site selection; gRNA plasmid generation; donor template design and construction; and the generation, identification, and molecular confirmation of engineered lines. We also present alternative approaches and highlight key considerations for experimental design. The approach outlined here can be used to rapidly and reliably generate a variety of engineered modifications, including genomic deletions and replacements, precise sequence edits, and incorporation of protein tags.

Keywords: CRISPR • Cas9 • homology directed repair • genome engineering • *Drosophila*

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INTRODUCTION

The CRISPR-Cas9 system is significantly advancing the ability of researchers to engineer targeted genome modifications for functional studies of genes and genetic elements. In *Drosophila*, the CRISPR-Cas9 system has been used to disrupt, delete, replace, tag, and edit multiple genes and genetic elements (Bassett et al., 2013; Kondo and Ueda, 2013; Ren et al., 2013; Yu et al., 2013; Gratz et al., 2013a; Gratz et al., 2014; Lee et al., 2014; Port et al., 2014; Sebo et al., 2014; Xue et al., 2014; Yu et al., 2014). The rapid and widespread adoption of CRISPR-Cas9 illustrates the utility of this novel genome engineering platform for generating a wide variety of modifications, and its power for addressing fundamental biological questions, understanding and treating disease, and engineering agriculturally relevant species and their pests.

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CRISPR-Cas9 systems are simple and highly robust genome-engineering tools that are being widely adopted by the research community. The most widely used system, from *Streptococcus pyogenes*, was simplified to two components to facilitate genome engineering: a common endonuclease called Cas9 and a single chimeric RNA referred to as a guide RNA (gRNA) (Jinek et al., 2012). gRNAs interact with Cas9 and guide the nuclease to specific DNA sequences through an easily programmed 20-nt target sequence that directly base pairs with complementary DNA. Upon binding its target, Cas9 utilizes its two nuclease domains to generate a double-strand break (DSB). The only known requirement for a potential cleavage site is the presence of a 3-bp protospacer adjacent motif (PAM) of the form NGG immediately 3′ of the 20-nt target sequence. Thus, *S. pyogenes* CRISPR-Cas9 target sites occur an average of once in every eight basepairs of genomic sequence.

Induction of a DSB in genomic DNA triggers repair by one of two general cellular-repair pathways, both of which can be co-opted for genome engineering. Non-homologous end-joining (NHEJ) is an error-prone process in which broken ends are simply ligated together. This repair pathway can yield small insertions and deletions (indels) that disrupt function at cleavage sites. In contrast, homology-directed repair (HDR) employs homologous DNA sequences as templates for precise repair. By supplying donor templates comprising exogenous sequence flanked by homology-containing stretches (commonly referred to as homology arms), the HDR pathway can be appropriated to make precise modifications including defined deletions, sequence substitutions, or insertions. Beyond the genome engineering applications of the CRISPR-Cas9 system, nuclease-dead Cas9 is being used as a sequence-specific repressor or activator of gene expression, and developed as a tool for probing genome structure and function without causing mutations (Bikard et al., 2013; Chen et al., 2013; Cheng et al., 2013; Fujita and Fujii, 2013; Gilbert et al., 2013; Maeder et al., 2013; Perez-Pinera et al., 2013; Qi et al., 2013; Anton et al., 2014; Kearns et al., 2014).

Here we detail a rapid and efficient CRISPR-Cas9 method for HDR-mediated engineering of the *Drosophila* genome (Gratz et al., 2013a; Gratz et al., 2014). We have used this approach to generate numerous genome modifications, including gene replacements, in-frame protein tag insertions, and conditional alleles. The Basic Protocol covers target site selection; gRNA generation; donor design and construction; and the generation, identification and molecular confirmation of engineered lines. We begin with key considerations for experimental design and discuss alternative approaches (see Strategic Planning).

STRATEGIC PLANNING

Figure 31.2.1 shows a decision tree that can be used as a guide in designing the appropriate strategy for different types of CRISPR-Cas9 genome engineering experiments. Here we discuss the key considerations for each decision point.

What is the goal?

Loss-of-function (lof) allele: If the goal is to generate an lof allele, an approach that relies on either NHEJ or HDR can be employed to achieve the desired aim. The key difference in practice is whether a donor repair template is included. If NHEJ (no donor) is chosen, there are two options: (1) disrupt the locus by targeting a single cleavage event in a critical sequence and recovering disruptive indels; or (2) delete the locus with two flanking gRNAs. It will be necessary to screen candidate mutants using an appropriate molecular approach unless phenotypic screening is possible for the desired mutant. The simplest way to identify relatively small indels is by using high-resolution melt analysis (HRMA), while PCR can be used to detect larger deletions (Bassett et al., 2013; Gratz et al., 2013a). Following sequence verification, the result will be an unmarked lof allele.

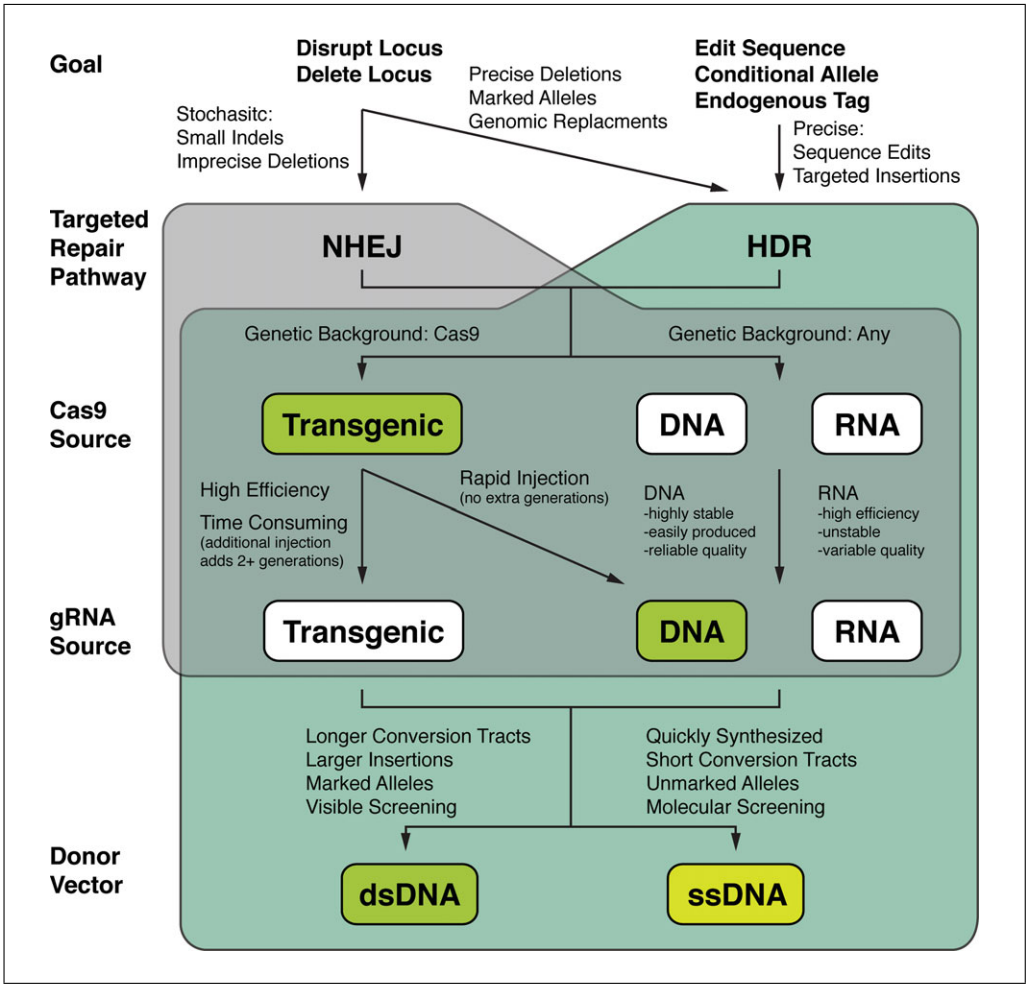

Figure 31.2.1 Strategic planning flowchart. The options outlined in this protocol are indicated by shaded (green) boxes. See text (Strategic Planning) for a detailed discussion of each choice point, including the advantages and disadvantages of each strategy. For the color version of this figure, go to *http://www.currentprotocols.com/protocol/mb3102*.

Complex modifications: To engineer defined modifications, such as specific changes to a nucleotide sequence or insertion of a tag or other exogenous DNA (e.g., FRT sites for a conditional allele), it will be necessary to employ the HDR pathway by supplying a donor repair template and screening for the desired repair event. This pathway can also be employed to generate defined deletions and/or to insert screenable marker genes that facilitate recovery of engineered flies. Subsequently, markers that have been flanked by LoxP or FRT sites can be readily removed to minimize alterations to the engineered locus or retained to provide a marked allele.

Irrespective of whether the goal is to employ the NHEJ or HDR pathway, it will be necessary to decide how to introduce Cas9

Transgenic Cas9 source: Unless the experimental goals demand use of a specific genetic strain, we recommend using a transgenic Cas9 source for the highest efficiency and reliability. We have generated transgenic fly lines expressing Cas9 under the control of the germline *vasa* promoter. These and related fly lines are available at the Bloomington *Drosophila* Stock Center (Gratz et al., 2014; Port et al., 2014; Sebo et al., 2014).

Injection of Cas9 as DNA or RNA: If a requirement is to work in a particular genetic background (or non-*melanogaster* species), Cas9 can be introduced as either plasmid DNA or mRNA through injection. A disadvantage in using RNA is the relatively

high cost compared to DNA, which is inexpensive to generate and easy to handle. The primary drawback of injected Cas9 DNA is that, while it appears to work quite reliably, it does so with lower efficiency than Cas9 mRNA (Ren et al., 2013; Gratz et al., 2013a). Optimized promoters are likely to mitigate this difference. In some cases, such as attempts to generate recessive lethal alleles, the lower efficiency observed with DNA constructs may provide an advantage by reducing the levels of biallelic targeting.

All CRISPR experiments require a gRNA to guide Cas9 to the targeted locus. As with Cas9, gRNAs can be supplied transgenically or injected as DNA or RNA

Transgenic gRNA source: gRNAs can be integrated into the genome using phiC31-mediated transgenesis (Kondo and Ueda, 2013; Port et al., 2014; Xue et al., 2014). This approach has the advantage of catalyzing extremely efficient NHEJ, and possibly HDR, though to date only one such experiment has been published (Port et al., 2014). The disadvantage of an integrated gRNA approach is the time and cost of generating a unique gRNA-expressing transgenic fly line for each targeting experiment. Integrating a gRNA more than doubles the timeline for obtaining an engineered fly line and costs several hundred dollars if injections are outsourced, as is commonly the case. However, given the advantage of increased efficacy, this approach is particularly useful if a gene or genomic sequence will be manipulated frequently.

Injection of gRNA as DNA or RNA: Injection of gRNAs, as either DNA or RNA, is the most rapid method for engineering flies, and both DNA and RNA are highly efficient, particularly when combined with transgenic sources of Cas9 (Ren et al., 2013; Gratz et al., 2014; Xue et al., 2014; Yu et al., 2014).

For HDR, but not NHEJ, it will be necessary to supply a donor repair template. Both dsDNA and single-stranded DNA (ssDNA) donors have been used successfully in Drosophila

dsDNA donor: dsDNA donors are highly versatile as they can incorporate large DNA sequences (Gratz et al., 2014; Port et al., 2014; Xue et al., 2014; Yu et al., 2014). We have generated vectors for rapidly cloning locus-specific homology arms into donors with visible markers. The pHD-DsRed-attP and pHD-DsRed vectors, described in detail below, are available from Addgene.

ssDNA donor: ssDNA donor templates can be used to incorporate small modifications (Gratz et al., 2013a; Port et al., 2014; Xue et al., 2014). The primary advantage of using ssDNA donors is that they can be rapidly synthesized, obviating the need for cloning. However, most synthesis companies have a size limit of ~200 nt for single-stranded DNA synthesis, so they cannot be used for larger modifications such as the incorporation of fluorescent tags or for the inclusion of a selection marker for identifying engineered flies. Thus, molecular screening is generally required, increasing the labor necessary to identify and recover the intended allele.

In the Basic Protocol below, we detail our preferred method for efficient generation of engineered flies via HDR: injection of *vasa-Cas9* flies with gRNA plasmids and a positively marked dsDNA donor template. This choice represents a favorable balance of time, cost, efficiency, and reliability. With this approach, we obtain engineered alleles within 1 month at a total reagent cost of approximately $150. Injections generally cost an additional $200 if outsourced. In our experience, an average of 25% (range = 7% to 42%) of fertile injected flies transmit the targeted event to their progeny. In Alternate Protocol 1, we detail HDR with ssDNA donor templates. Alternate Protocol 2 covers HDR in other genetic backgrounds using Cas9 supplied as DNA. In Alternate Protocol 3, we outline our approach for NHEJ using a transgenic source of Cas9 and gRNA supplied as DNA. This approach has also been used successfully by Ren et al. (2013). Together

these protocols offer a versatile toolset amenable for generating a variety of genome modifications in *Drosophila*.

GENOME ENGINEERING OF *DROSOPHILA* VIA HOMOLOGY-DIRECTED REPAIR USING THE CRISPR-Cas9 SYSTEM

BASIC PROTOCOL

Selection of high-quality target sites is essential for the success of any CRISPR-based genome engineering experiment. It is important to identify target sites that will generate DSBs close to the location of the intended modification. In choosing a target site, location must be balanced with target-site specificity and, thus, the potential for off-target DSBs. While originally raised as a significant concern in the editing of transformed cell lines (Fu et al., 2013), with careful target site selection, off-target cleavage does not seem to be a significant problem for genome editing of organisms or human stem cells (Bassett et al., 2013; Chiu et al., 2013; Yang et al., 2013; Gratz et al., 2013a; Duan et al., 2014; Gratz et al., 2014; Kiskinis et al., 2014; Smith et al., 2014; Suzuki et al., 2014; Veres et al., 2014). Nonetheless, our current understanding of Cas9-induced cleavage is far from complete, so it is important to select the most specific sites possible to minimize the potential for off-target mutagenesis. To facilitate the rapid identification of high-quality target sites, we have developed a Web-based tool, CRISPR Optimal Target Finder, that identifies gRNA cleavage sites and evaluates their specificity (Gratz et al., 2014).

It is essential that target sites be identified in sequence obtained from the fly strain that will be edited, not the reference genome. Polymorphisms between a given fly strain and the reference genome are frequent, especially in intergenic regions, and could eliminate or significantly decrease cleavage if they occur within the target sequence. Thus, CRISPR Optimal Target Finder identifies gRNA target sites in user-supplied DNA sequence rather than reference genome sequences. In the Basic Protocol, we use *vasa-Cas9* flies. However, as described in Alternate Protocol 2 below, this approach can be readily adapted to engineer any fly strain.

Materials

vasa-Cas9 fly stocks (Bloomington Drosophila Stock Center)
- y^1 M{vas-Cas9.RFP-}ZH-2A w^{1118}/FM7a, P{Tb^1}FM7-A (stock number 55821)
- w^{1118}; PBac{vas-Cas9}VK00037/CyO, P{Tb^1}Cpr^{CyO-A} (stock number 56552)
- w^{1118}; PBac{vas-Cas9}VK00027 (stock number 51324)

Total DNA purification kit (DNeasy Blood and Tissue Kit; Qiagen)
PCR and sequencing primers (see *UNIT 15.1*; Kramer and Coen, 2001)
Phusion High-Fidelity DNA Polymerase (New England Biolabs)
Gel extraction kit (Wizard SV Gel and PCR Clean-Up Sytem; Promega)
Pair of short complementary oligonucleotides for cloning each target site (see information under step 12)
Nuclease free H_2O
10 U/μl T4 polynucleotide kinase (New England Biolabs)
10× T4 DNA ligase buffer (New England Biolabs)
pU6-BbsI-gRNA (Addgene; plasmid 45946)
*Bbs*I restriction endonuclease
400 U/μl T4 DNA ligase (New England Biolabs)
E. coli DH5α or other suitable cloning strain
LB agar plates containing 100 μg/ml ampicillin (*UNIT 1.1*; Elbing and Brent, 2002)
Plasmid miniprep kit (PureYield Plasmid Miniprep System; Promega)

Sequencing primers (also see *UNIT 7.4A*; Slatko et al., 1999):
T7 (5′-TAATACGACTCACTATAGGG-3′)
T3 (5′- AATTAACCCTCACTAAAGGG-3′)
Endotoxin-Free Plasmid Midi Kit (Macherey-Nagel NucleoBond Xtra Midi EF or similar)
Primers to amplify both homology arms (see information under step 23)
pHD primers:
pHD-BB-1 (5′-ACGAAAGGCTCAGTCGAAAG-3′)
pHD-BB-2 (5′-TGATATCAAAATTATACATGTCAACG-3′)
pHD-HSP70-R (5′-CGGTCGAGGGTTCGAAATCGATAAG-3′)
pHD-SV40-F (5′-GGCCGCGACTCTAGATCATAATC-3′)
10 mM dNTP mix: 10 mM each of dATP, dCTP, dGTP, and dTTP
5× HF Phusion Buffer (New England Biolabs)
pHD-DsRed-attP (Addgene; Plasmid 51019, also called pDSRed-attP)
pHD-DsRed (Addgene; Plasmid 51434)
*Aar*I restriction endonuclease
LB liquid medium containing 100 μg/ml ampicillin (*UNIT 1.1*; Elbing and Brent, 2002)
Adult fly homogenization buffer (see Reagents and Solutions for recipe)
Proteinase K (New England Biolabs)
PCR and sequencing primers for identification and confirmation of CRISPR alleles (also see text under step 41):
pHD-HSP70-R (5′-CGGTCGAGGGTTCGAAATCGATAAG-3′)
pHD-SV40-F (5′-GGCCGCGACTCTAGATCATAATC-3′)
Left-Genomic-F (target locus specific)
Right-Genomic-R (target locus specific)

Thermal cycler
0.2-ml PCR tubes

Additional reagents and equipment for the polymerase chain reaction (PCR; *UNIT 15.1*; Kramer and Coen, 2001), agarose gel electrophoresis (*UNIT 2.5A*; Voytas, 2000), Sanger sequencing (*UNIT 7.4A*; Slatko et al., 1999), determination of nucleic acid concentration by spectrophotometry (*APPENDIX 3D*; Gallagher, 2011), transformation of *E. coli* (*UNIT 1.8*; Seidman et al., 1997), and microinjection of *Drosophila* embryos (Dobson, 2007)

Target site selection

1. Isolate genomic DNA from the Cas9-expressing (or other) fly strain in which the genome modifications will be made. Purify total DNA from about 50 adult flies using a total DNA purification kit.

   This large genomic DNA preparation can be used for many subsequent CRISPR experiments.

2. Design PCR primers (see *UNIT 15.1*; Kramer and Coen, 2001) to amplify a region of about 500 to 1000 bp centered around the target region.

   Only one gRNA is necessary to catalyze HDR. We often design our experiments to include two gRNAs targeting either end of the region to be modified (see Figure 31.2.3). However, the effect of one versus two gRNAs on efficiency or choice of cellular repair pathway is not known.

3. Prepare a 50-μl reaction to amplify the target region (*UNIT 15.1*; Kramer and Coen, 2001) using Phusion High-Fidelity DNA Polymerase.

4. Use agarose gel electrophoresis (*UNIT 2.5A*; Voytas, 2000) to purify the PCR product. Obtain the product using a gel-extraction kit.

5. Sequence the PCR product using Sanger sequencing (*UNIT 7.4A*; Slatko et al., 1999).

6. Identify CRISPR target sites in the sequenced region. Submit the sequence returned from step 5 to the CRISPR Optimal Target Finder (*http://tools.flycrispr.molbio.wisc.edu/targetFinder*).

7. Select the genome release to be searched.

   Target Finder is currently configured to search D. melanogaster release 5 by default. Release 6 is also available, as are genomes for D. simulans, D. yakuba, D. mauritiana, D. sechellia, D. ananassae, D. erecta, D. persimilis, D. pseudoobscura, D. virilis, D. mojavensis, D. willistoni, D. grimshawi, Anopheles gambiae (strains M and S), Aedes aegypti, Apis mellifera, Tribolium castaneum (release 2 and draft release 4), and C. elegans.

8. Select the length of the target sites to be identified.

   Target Finder will identify all target sequences of the selected length in the 500- to 1000-bp sequenced region from step 2. Full-length target sites of 20 nt offer the highest cleavage efficiency and are the most commonly used. Target sequences of a shorter length have been shown to increase the specificity of Cas9 cleavage while decreasing cleavage efficiency (Fu et al., 2014).

9. Target Finder will identify all CRISPR target sequences in the input sequence: to limit identified target sites to those that start with a G (for efficient expression from the U6 promoter when supplying gRNA as plasmid DNA) or those that start with GG (for efficient expression from the T7 promoter using in vitro transcription of gRNAs), select the appropriate option.

   Alternatively, a G can simply be added to the 5′ end of any target sequence when cloning the gRNA plasmid (see below) for efficient U6 transcription.

10. Once CRISPR targets are identified, evaluate their specificity based on user-selected criteria.

    The final 12 nt of the CRISPR target sequence, often referred to as the 'seed' sequence, are more critical for specificity than the distal eight nucleotides. The CRISPR Optimal Target Finder algorithms consider both the number and location of mismatches in the evaluation of potential off-target cleavage sites.

    High-stringency (default setting): defines potential off-target sites as those with (i) perfect matches (zero mismatches) to the seed sequence or (ii) one mismatch in the seed sequence and one or zero mismatches in the distal sequence.

    Maximum stringency: defines potential off-target as sites with (i) perfect matches (zero mismatches) to the seed sequence, (ii) one mismatch in the seed sequence and four or fewer mismatches in the distal sequence, or (iii) two mismatches in the seed sequence and a maximum of one mismatch in the distal sequence.

    PAM: By default, the program will only consider sequences adjacent to a canonical NGG PAM in the evaluation of potential off-target cleavage sites. Putative off-target sites adjacent to a non-canonical PAM sequence of the form NAG can be considered by selecting the 'NGG and NAG' option. In transformed cell lines, target sites adjacent to an NAG PAM were cleaved at one-fifth the efficiency of those adjacent to a canonical NGG PAM sequence (Hsu et al., 2013).

    We recommend use of the default settings for most applications in Drosophila, where little off-target cleavage has been observed to date (Bassett et al., 2013; Gratz et al., 2013a, 2014).

11. Select specific target site(s) for the genome engineering project by balancing proximity to the site to be edited and the potential for off-target cleavage. Target Finder returns all identified target sites in order of specificity. For each target site, the program also provides oligonucleotide sequences designed for gRNA plasmid cloning into a pU6-BbsI-gRNA vector.

gRNA plasmid preparation

To supply gRNAs containing the target-specific sequences from a plasmid DNA source, we have generated vectors for rapid cloning of target-specific sequences using short complementary oligonucleotides and a simple annealing and ligation process. The pU6-BbsI-gRNA vectors utilize the small RNA promoter of a *Drosophila* U6 gene to express the gRNA.

12. Order a pair of short complementary oligonucleotides for each target site.

    The following guidelines can be used to help design the oligonucleotides. The oligonucleotide design incorporates the target sequence and cohesive ends for cloning into the pU6-BbsI-gRNA backbone. The top strand should be designed in the format of 5′-CTTCG$(N)_{19}$-3′, where $G(N)_{19}$ corresponds to the unique target site sequence beginning with a G for efficient transcription from the Drosophila U6 promoter (Fig. 31.2.2). The bottom strand is designed in the format of 5′-AAAC$(N)_{19}$ C-3′, with $(N)_{19}$ C representing the reverse complement of the targeting sequence. Alternatively, to aid in design process, the CRISPR Optimal Target Finder has a feature that will output the oligonucleotide sequences needed for cloning selected target sites.

    T4 polynucleotide kinase (PNK) can be used to add the 5′ phosphates to standard oligonucleotides, as described below, or 5′ phosphorylated oligonucleotides can be ordered.

    As noted above, target sites without an endogenous 5′ G can be used by simply adding a G to the 5′ end of a 20-nt target site in the format of $G(N)_{20}$ to achieve efficient transcription. In this case, the top strand should be designed in the format of 5′-CTTCG$(N)_{20}$-3′ and the bottom strand designed in the format of 5′-AAAC$(N)_{20}$ C-3′.

13. Resuspend oligonucleotides at a concentration of 100 μM in nuclease-free water.

14. Combine 1 μl of the top-strand oligonucleotide (100 μM stock), 1 μl of the bottom-strand oligonucleotide (100 μM stock), 1 μl of T4 DNA ligase buffer (10×), 6 μl of nuclease-free water, and 1 μl of T4 polynucleotide kinase (10 U/μl). In a thermal cycler, incubate at 37°C for 30 min, then at 95°C for 5 min, then ramp down to 25°C at a rate of −5°C/min.

15. Digest 1 μg of pU6-BbsI-gRNA with 10 U *Bbs*I for 2 hr at 37°C.

    The pU6-BbsI-gRNA plasmid expresses the gRNA under the control of the U6-2 promoter.

16. Use agarose gel electrophoresis (*UNIT 2.5A*; Voytas, 2000) to purify the digested vector. Obtain the product using a gel-extraction kit. Determine the DNA concentration using a spectrophotometer (*APPENDIX 3D*; Gallagher, 2011).

17. Ligate the annealed insert into pU6-BbsI-gRNA as follows. Combine 1 μl of annealed insert (from step 14), 50 ng of *Bbs*I-digested pU6-BbsI-gRNA (from step 15), 1 μl of T4 DNA ligase buffer (10×), 1 μl of T4 DNA ligase (400 U/μl), and enough nuclease-free water to bring the reaction to 10 μl. Incubate at 25°C for 1 hr.

18. Transform the ligation reaction into DH5α cells (*UNIT 1.8*; Seidman et al., 1997) and select colonies on LB plates containing 100 μg/ml ampicillin.

    A control transformation of digested pU6-BbsI-gRNA vector alone can be performed to ensure that no contaminating undigested plasmid was collected in step 16.

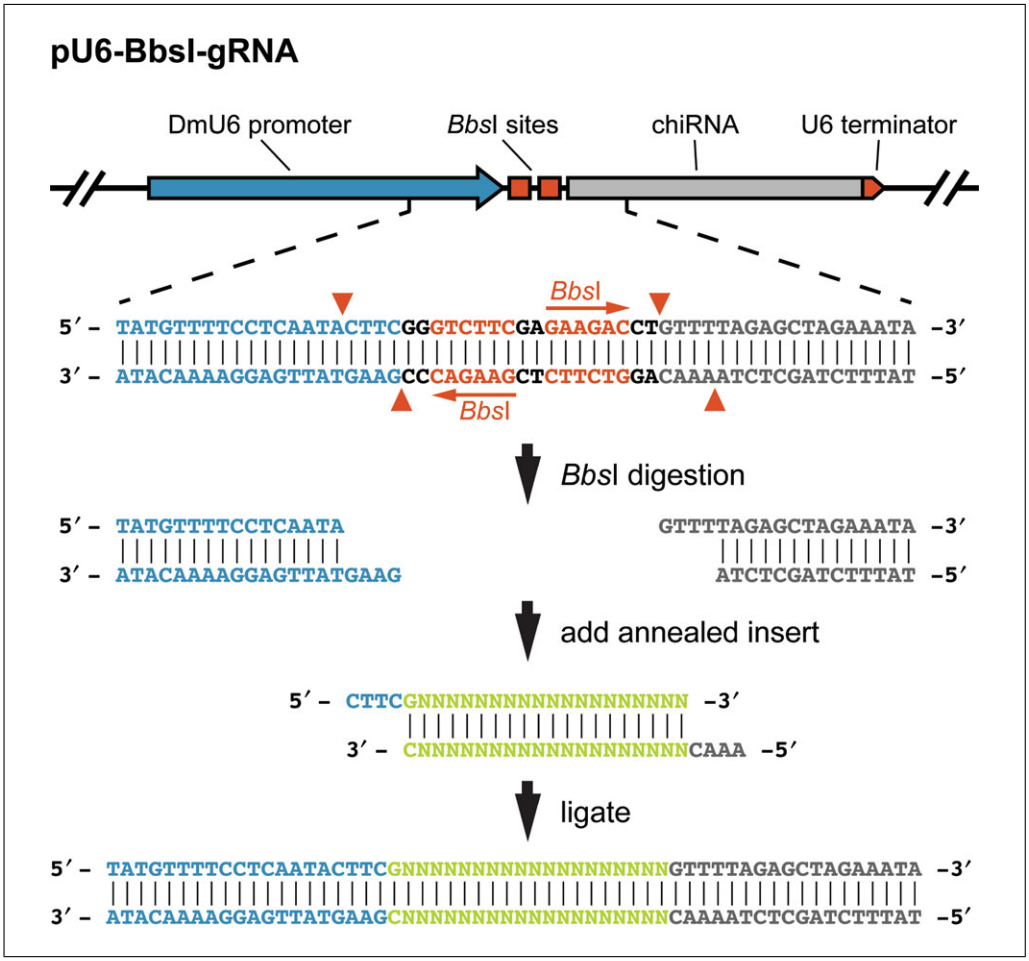

Figure 31.2.2 gRNA plasmid cloning. The pU6-BbsI-gRNA vector contains two *Bbs*I cut sites between the *Drosophila* U6-2 (snRNA:U6:96Ab) promoter and the common portion of the gRNA. Specific target site sequences are synthesized as complementary oligonucleotides designed to generate appropriate cohesive 5′ overhangs once annealed. The annealed oligonucleotides, once phosphorylated, are then ligated into the *Bbs*I-digested pU6-BbsI-gRNA vector. U6-2 sequence (blue), *Bbs*I recognition sequences (red), target site-specific sequence (green), and the common portion of the gRNA (gray) are indicated. Red arrowheads denote the breakpoints generated by *Bbs*I cleavage. For the color version of this figure, go to *http://www.currentprotocols.com/protocol/mb3102*.

19. Isolate plasmids from two to four individual colonies using a plasmid miniprep kit. Screen for plasmids with incorporated oligonucleotides by Sanger sequencing (*UNIT 7.4A*; Slatko et al., 1999) using T7 and/or T3 primers.

    Alternatively, positive colonies can be identified via colony PCR using a target-site oligonucleotide in combination with a T3 or T7 primer. Use a pipet tip to scratch a visible amount of an individual colony into a PCR tube and use the tip to inoculate 4 ml of LB containing 100 μg/ml ampicillin. Add 10 μl of PCR master mix to the PCR tube and use the appropriate cycling parameters to amplify the diagnostic product.

20. Prepare DNA for injection from a positive clone using an Endotoxin-Free Plasmid Midi Kit. Elute in nuclease-free water.

    As with all Drosophila injection-based techniques, preparing high-quality DNA is important for successful CRISPR-mediated genome engineering.

Donor vector design

dsDNA donor vectors can be made in many configurations to facilitate the generation of an endless variety of genome modifications (Fig. 31.2.3). This Basic Protocol focuses on the design of donor constructs using the pHD-DsRed-attP or pHD-DsRed vectors

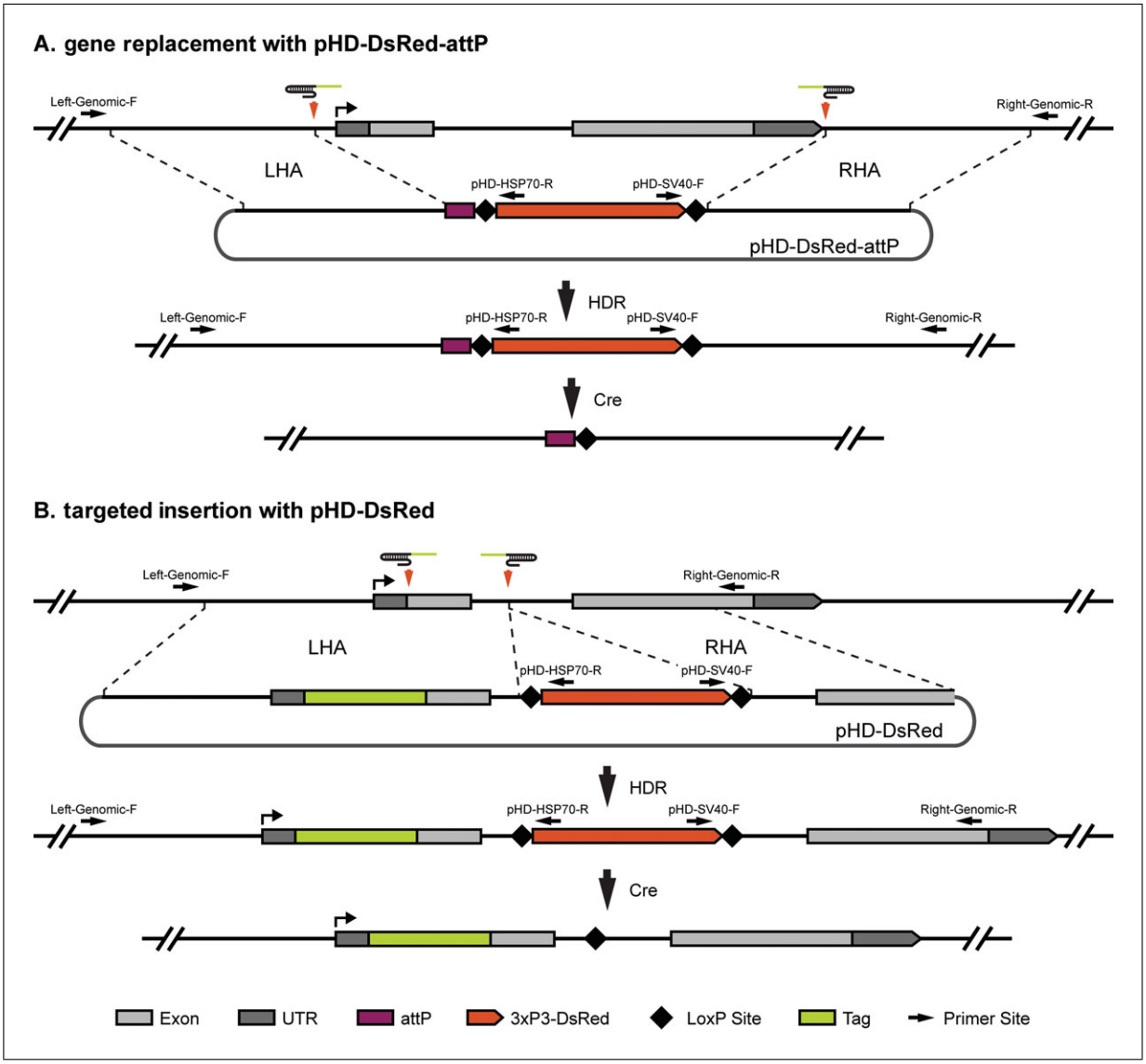

Figure 31.2.3 Donor construct design. The (**A**) pHD-DsRed-attP vector and (**B**) pHD-DsRed donor vectors and their typical uses are depicted. Both vectors contain a removable 3×P3-dsRed marker flanked by LoxP sites and two multiple-cloning sites for insertion of the left (LHA) and right (RHA) homology arms. pHD-DsRed-attP also contains the recombination-based docking site attP. (A) In the case of replacing a locus using the pHD-DsRed-attP vector, two target sites flanking the region to be replaced are chosen. Homologous sequences immediately flanking the cleavage sites should be cloned into the MCSs. Upon Cas9-mediated cleavage and HDR, the region between the two gRNA cut sites is replaced with the attP site and removable DsRed marker. Using Cre recombinase, the DsRed marker can be removed leaving only the attP docking site and a single LoxP site. (B) In the case of tagging a gene using the pHD-DsRed vector, select a target site close to the tag insertion site and another target site in a nearby intron where the DsRed marker will be placed. Homology arms will include sequences immediately flanking the cleavage sites. In addition, one of the homology arms will contain the in-frame tag and sequences between the tag and the DsRed marker. Upon Cas9 cleavage and HDR, the untagged region is replaced with a tagged region and a visible 3×P3-DsRed marker. Using Cre recombinase, the DsRed marker can be removed, leaving only the tagged coding sequence and a single LoxP site. Black arrows indicate the primer binding sites used for molecular characterization for candidate alleles. Note that the two locus-specific primers are in the genomic region outside of the homology regions used in the donor vector. For the color version of this figure, go to *http://www.currentprotocols.com/protocol/mb3102*.

available through Addgene. The design of ssDNA donors is described in Alternate Protocol 1, below. The pHD-DsRed-attP vector is used for generating marked knock-out alleles that harbor an attP phage recombination site for serial manipulations of the target locus catalyzed by phiC31. pHD-DsRed is used for generating positively marked targeted insertions or sequence edits. Both vectors include a removable DsRed marker expressed

strongly in the eye for visual identification of lines with targeted events, and contain multiple cloning sites for inserting locus-specific homology arms. The following steps describe the use of the type IIS restriction enzymes *Aar*I and *Sap*I for seamless integration of homology arms into pHD-DsRed-attP or pHD-DsRed. These vectors have a number of additional restriction sites that can be used for homology arm cloning. Gibson assembly and related techniques offer an attractive and rapid alternative approach, but are not described here. Finally, DNA synthesis is becoming an increasingly affordable option for generating donors.

21. Identify the two ~1-Kb sequences that flank the cleavage site and the genomic region that will be modified.

    Extensive analysis of zinc-finger nuclease–catalyzed HDR has demonstrated that homology arms of 1 Kb mediate efficient HDR (Beumer et al., 2013), and we and others have found this to be the case with CRISPR-based HDR (Gratz et al., 2014; Port et al., 2014; Xue et al., 2014; Yu et al., 2014).

22. Ensure that neither *Aar*I nor *Sap*I restriction sites occur within the homology arms, as these restriction enzymes are used for cloning the donor vector.

    If the homology arms contain AarI or SapI sites, alter the design and/or experimental protocol accordingly. For example, if the 5′ homology arm contains an AarI cut site, it may be necessary to 'invert' the locations of the 5′ and 3′ homology arms in the donor such that the donor cassette is placed on the antisense strand. If the 3′ arm contains an AarI cut site, it will simply be necessary to clone the 5′ homology arm first. Alternatively, the multiple cloning sites can be used instead of AarI or SapI.

23. Design and order primers to amplify both homology arms.

    Homology arms should contain sequence immediately adjacent to the cleavage sites (Cas9-mediated DSBs are generated 3-bp upstream of the PAM) for the most efficient HDR. When deleting or replacing a locus, the homology arms will simply comprise the sequence flanking the deleted region (Fig. 31.2.3A). For inserting exogenous sequences or editing genomic sequences, design overlapping extension PCR oligonucleotides to construct a fragment that contains (i) a 1-Kb homology region, (ii) the desired insertion or edit, and (iii) the genomic sequence between the edit and the marker insertion site, usually in an adjacent intron (Fig. 31.2.3B). For detailed guidance on the design of overlapping extension PCR primers, see UNIT 3.23 (Miklos et al., 2012).

    Left homology arm (AarI): The forward primer should follow the format 5′-NNNN*CACCTGC*NNNNTCGC$(N)_{20}$-3′ where the *Aar*I site is italicized, the cohesive end generated by *Aar*I digestion in underlined, Ns represent spacer sequences required for efficient cleavage and accurate cohesive end generation, and $(N)_{20}$ represents the hybridization sequence. The reverse primers vary slightly for the two vectors because only one includes the attP sequence in the AarI overhang. For the pHD-DsRed-attP vector, the reverse primer should follow the format 5′-NNNN*CACCTGC*NNNNCTAC$(N)_{20}$-3′ (Fig. 31.2.4A and B). For pHD-DsRed cloning, the reverse primer should follow the format 5′-NNNN*CACCTGC*NNNNTTAT$(N)_{20}$-3′ (Fig. 31.2.4A and C).

    Right homology arm (SapI): The right homology arm primer design is the same for both pHD-DsRed-attP and pHD-DsRed. The forward primer should follow the format 5′-NNNN*GCTCTTC*NTAT$(N)_{20}$-3′ where the *Sap*I site is italicized, the cohesive end generated by *Sap*I digestion is underlined, Ns represent spacer sequences required for efficient cleavage and accurate cohesive end generation, and $(N)_{20}$ represents the hybridization sequence (Fig. 31.2.4D). The reverse primer should follow the format 5′-NNNN*GCTCTTC*NGAC$(N)_{20}$-3′ (Fig. 31.2.4D).

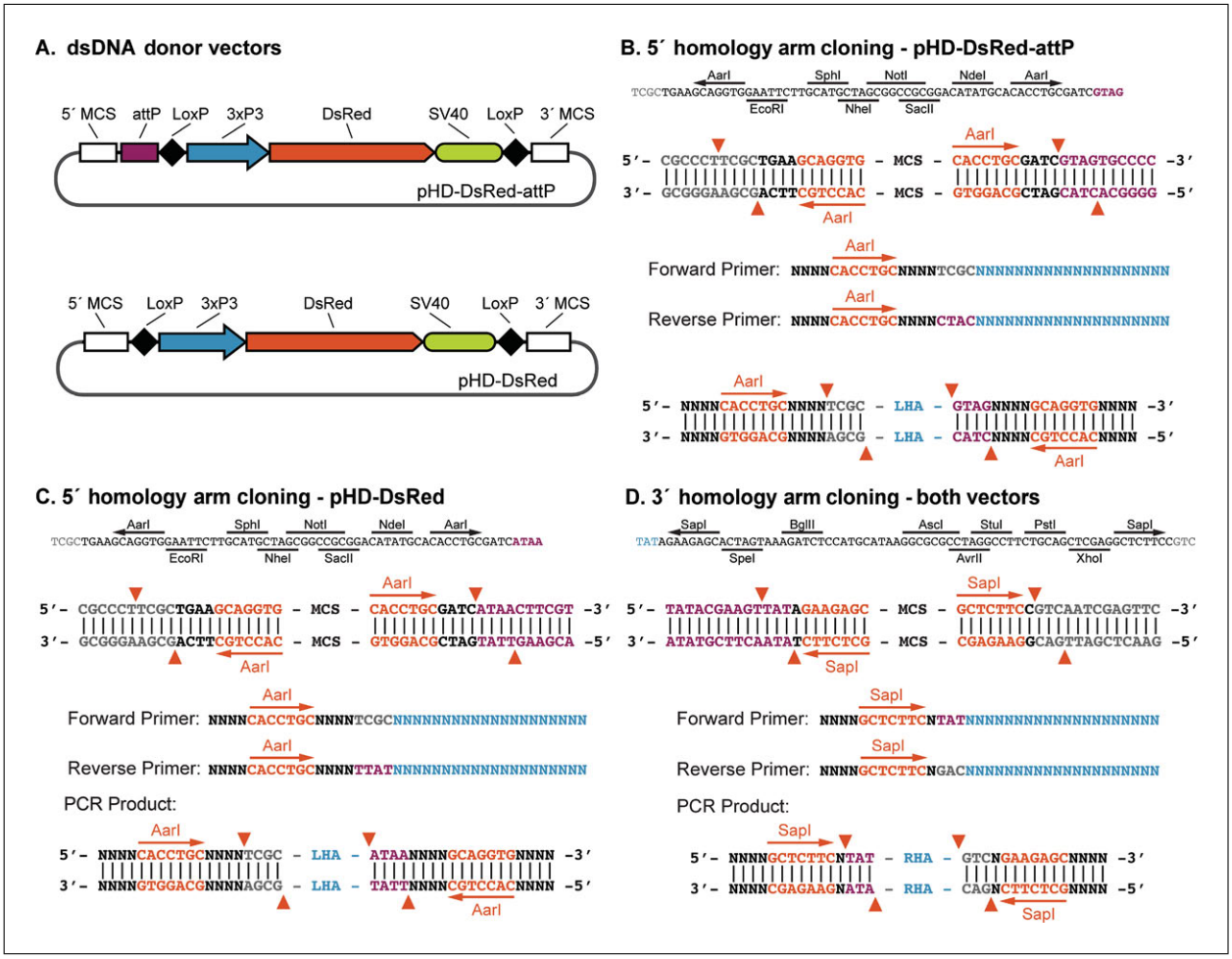

Figure 31.2.4 Donor plasmid cloning. (**A**) Schematic of the pHD-DsRed-attP and pHD-DsRed donor vector including the MCSs. (**B**-**D**) Multiple cloning site sequences and primer design for type IIS restriction site (*Aar*I or *Sap*I)–based cloning of homology arms for the LHA of pHD-DsRed-attP (**B**), the LHA of pHD-DsRed (**C**), and the RHA of both pHD-DsRed-attP and pHD-DsRed (**D**). Note that the LHA of pHD-DsRed-attP and pHD-DsRed require slightly different primers due to the presence or absence of the attP site. Vector backbone sequences (gray), attP/LoxP sequence (purple), *Aar*I/*Sap*I recognition sites (red), and locus-specific hybridization sequences (blue) are indicated. Red arrows indicate the breakpoints generated by *Aar*I or *Sap*I digestion. For the color version of this figure, go to *http://www.currentprotocols.com/protocol/mb3102*.

24. If the target sequences remain intact in the homology arms, incorporate silent mutations to disrupt the PAM or two seed sequence nucleotides into the design.

    This will ensure that neither the donor construct nor the successfully engineered locus are targets for cleavage.

Donor vector construction

25. Prepare a 50-µl reaction to amplify each homology arm (also see *UNIT 15.1*; Kramer and Coen, 2001).

    Homology arms should be amplified from the targeted fly line as maximal homology increases the efficiency of HDR (Deng and Capecchi, 1992; Nassif and Engels, 1993).

    0.5 µl of template DNA
    2.5 µl of 10 mM forward primer (from step 23)
    2.5 µl of 10 mM reverse primer (from step 23)
    1.0 µl of 10 mM dNTP mix
    10 µl of 5× HF Phusion Buffer

0.2 μl of Phusion High-Fidelity DNA Polymerase
H_2O to 50 μl.

26. Perform PCR (also see *UNIT 15.1*; Kramer and Coen, 2001) with the following parameters:

1 cycle:	2 min	94°C	(initial denaturation)
30 cycles:	10 sec	98°C	(denaturation)
	30 sec	45°-72°C	(annealing)
	30 sec/Kb	72°C	(extension)
1 cycle:	10 min	72°C	(final extension).

27. Purify homology arms via agarose gel electrophoresis (*UNIT 2.5A*; Voytas, 2000) and a gel-extraction kit. Determine the DNA concentration using a spectrophotometer (*APPENDIX 3D*; Gallagher, 2011).

28. Simultaneously digest 1 μg of the pHD-DsRed-attP or pHD-DsRed vector and 1 μg of the left homology arm PCR product with 4 U of *Aar*I restriction endonuclease for 4 hr at 37°C.

29. Use agarose gel electrophoresis to purify both the *Aar*I-digested pHD-DsRed-attP or pHD-DsRed vector and left homology arm PCR product. Obtain the product using a gel-extraction kit. Determine the DNA concentration using a spectrophotometer (*APPENDIX 3D*; Gallagher, 2011).

30. Ligate the left homology arm into pHD-DsRed-attP or pHD-DsRed vector as follows. Combine 50 ng of *Aar*I-digested pHD-DsRed-attP, 3:1 molar ratio of digested left homology arm PCR product, 1 μl of T4 DNA ligase buffer (10×), 1 μl of T4 DNA ligase (400 U/μl), and enough nuclease-free water to bring the reaction to 10 μl. Incubate at 25°C for 1 hr.

31. Transform the ligation reaction into DH5α cells (*UNIT 1.8*; Seidman et al., 1997) and select colonies on LB plates containing 100 μg/ml ampicillin.

    A control transformation of digested plasmid alone can be included to ensure that the sample is not contaminated with undigested plasmid.

32. Select two to four individual colonies and culture in 4 ml of LB liquid medium containing 100 μg/ml ampicillin overnight. Isolate plasmid DNA using a plasmid miniprep kit. Screen for plasmids with incorporated homology arms by restriction enzyme digestion or by PCR and Sanger sequencing (*UNIT 7.4A*; Slatko et al., 1999) using pHD-BB-1 and pHD-HSP70-R sequencing primers.

    Alternatively, positive colonies can be identified via colony PCR as described above.

33. Simultaneously digest 1 μg of positive constructs from step 32 and 1 μg of the right homology arm PCR product with 5 U of *Sap*I restriction endonuclease for 2 to 4 hr at 37°C.

34. Use agarose gel electrophoresis (*UNIT 2.5A*; Voytas, 2000) to purify both the *Sap*I digested construct and right homology arm PCR product. Obtain the product using a gel-extraction kit. Determine the DNA concentration using a spectrophotometer (*APPENDIX 3D*; Gallagher, 2011).

35. Ligate the right homology arm into pHD-DsRed-attP as follows. Combine 50 ng of *Sap*I-digested pHD-DsRed-attP, 3:1 molar ratio of digested left homology arm PCR product, 1 μl of T4 DNA ligase buffer (10×), 1 μl of T4 DNA ligase (400 U/μl), and enough nuclease-free water to bring the reaction to 10 μl. Incubate at 25°C for 1 hr.

36. Transform the ligation reaction into DH5α cells (*UNIT 1.8*; Seidman et al., 1997) and select colonies on LB plates containing 100 μg/ml ampicillin.

A control transformation of digested plasmid alone can be included to ensure that the sample is not contaminated with undigested plasmid.

37. Isolate plasmids from two to four individual colonies using a plasmid miniprep kit. Screen for plasmids with incorporated oligonucleotides by restriction enzyme digestion or by PCR and Sanger sequencing (*UNIT 7.4A*; Slatko et al., 1999) using pHD-BB-2 and pHD-SV40-F.

    Alternatively, positive colonies can be identified via colony PCR as described above.

38. Prepare high-quality DNA for injection from a positive clone using an Endotoxin-Free Plasmid Midi Kit. Elute in nuclease-free water.

Injection of CRISPR components

CRISPR components are injected using standard *Drosophila* injection techniques. Here we provide the injection mixture for HDR in *vasa-Cas9* flies.

39. Prepare the injection mixture with 500 ng/μl plasmid donor vector (from step 38) and 100 ng/μl of each gRNA vector (from step 20) in sterile PCR-grade water.

    This parameter can be altered based on your experience. We have tested a range of concentrations and find that gRNA plasmid concentrations between 50 and 250 ng/μl and dsDNA donor concentrations between 250 and 500 ng/μl yield successful HDR.

40. Inject 150 to 300 embryos using standard *Drosophila* techniques (e.g., Dobson, 2007).

Identification and molecular confirmation of CRISPR alleles

Following injection and an appropriate outcross of injected flies, candidate CRISPR alleles are easily identified by screening for F1 progeny with red fluorescent eyes in F1 progeny. Once these candidates have been crossed to a balancer line, they can be sacrificed for molecular characterization to verify recovery of the intended genome modification. Below we describe our strategy of performing three PCRs that, in combination with Sanger sequencing, confirm targeted and precise editing (see Fig. 31.2.3).

41. Design and order primers (Fig. 31.2.3).

    First, perform two flanking PCRs to amplify regions extending from within the DsRed marker cassette to the right and left flanking genomic regions. To confirm precise incorporation at the targeted locus, it is critical that the genomic primer-binding sites be outside the homology arms of the donor vector, as depicted in Figure 31.2.3. Candidate alleles are then confirmed via a spanning PCR using the two genomic primers. This reaction will amplify the entire modified region and rule out undesirable cross-over ("ends-in") repair events that result in the incorporation of the entire donor vector including backbone (Yu et al., 2014).

Isolate genomic DNA from the F1 candidate flies identified by DsRed expression in the eyes

42. Anesthetize a single fly and place it in a 0.2-ml PCR tube. Using a P-200 pipet tip draw up 50 μl of freshly prepared adult fly homogenization buffer. Keeping the buffer in the pipet, use the tip to disrupt and homogenize the fly. Once the fly is homogenized, dispense the remaining buffer.

43. Incubate at 37°C for 30 to 45 min followed by a 5-min incubation at 95°C.

Perform PCR reaction

44. Use 1 μl of genomic DNA (isolated in steps 42 and 43) per 50 μl PCR reaction mixture.

45. Prepare three separate 50-μl reactions for each candidate.

    Primer sets:

    1. Left-Genomic-F to pHD-HSP70-R
    2. pHD-SV40-F to Right-Genomic-R
    3. Left-Genomic-F to Right-Genomic-R

    Reaction mixture:

    1 μl of the genomic DNA from step 43
    2.5 μl of 10 mM forward primer
    2.5 μl of 10 mM reverse primer
    1.0 μl of 10 mM each dNTPs
    10 μl of 5× HF Phusion Buffer
    0.2 μl of Phusion High-Fidelity DNA polymerase
    H_2O to 50 μl.

46. Use agarose gel electrophoresis (*UNIT 2.5A*; Voytas, 2000) to purify positive PCR products. Obtain the product using a gel-extraction kit.

47. Sequence the PCR product using Sanger sequencing (*UNIT 7.4A*; Slatko et al., 1999) to confirm expected sequence.

HDR WITH SINGLE-STRANDED DNA DONORS

ALTERNATE PROTOCOL 1

When engineering small modifications, it may be desirable to use ssDNA donors, which can be rapidly synthesized. However, ssDNA donors are generally limited to 200 nt, and thus cannot be used for engineering large modifications (such as the integration of a fluorescent tag). They also cannot be designed to include a visible marker for screening, so molecular screening is required, which increases the time and labor required to recover engineered flies.

***Additional Materials** (also see Basic Protocol)*

ssDNA donor with homology arms (see text under step 1)

Perform the steps of the Basic Protocol with the following modifications:

1. Design an ssDNA donor with homology arms corresponding to sequences immediately adjacent to the targeted cleavage sites and the intended modification or insertion.

   Homology arms of ~40 to 60 nt have been shown to mediate efficient ssDNA-based HDR in Drosophila (Banga and Boyd, 1992; Beumer et al., 2013; Gratz et al., 2013a; Port et al., 2014; Xue et al., 2014). The orientation of the ssDNA is critical to the success of the experiment. During DNA repair, free 3′ ends created by rescission at the DSB invade homologous DNA. Therefore, to serve as a template for repair, it is essential for the ssDNA be complementary to the free 3′ end. Note that the PAM is not required for cleavage of single-stranded DNA, so it is important that the ssDNA does not include an intact target site or it may be a target of cleavage (Jinek et al., 2012).

2. The injection mixture should contain 100 ng/μl of ssDNA.

3. Engineered flies will need to be identified by phenotype, modification-specific PCR amplification, or a modification-spanning PCR followed by sequencing. DNA can be obtained from F1 flies after outcrossing.

   Alternatively, a non-lethal method, such as isolating wing or leg DNA, can be used prior to outcrossing of F1 candidates.

ALTERNATE PROTOCOL 2

HDR IN ANY GENETIC BACKGROUND

For many applications, it is necessary or desirable to engineer a specific fly strain. This is easily accomplished using an injectable source of Cas9 such as pBS-Hsp70-Cas9. Targeting efficiency is lower than with a transgenic Cas9 source, so it is advisable to inject a larger number of embryos.

***Additional Materials** (also see Basic Protocol)*

pBS-Hsp70-Cas9 plasmid (Addgene; plasmid 46294)

Perform the steps of the Basic Protocol with the following modifications:

1. Prepare the injection mixture with 250 to 500 ng/μl of pBS-Hsp70-Cas9, 500 ng/μl plasmid donor vector, and 100 ng/μl of each gRNA vector in sterile PCR-grade water.
2. Inject 250 to 500 embryos using standard *Drosophila* techniques.

ALTERNATE PROTOCOL 3

GENERATION OF DISRUPTIVE INDELS AND DELETIONS VIA NHEJ

If your goal is to generate a disruptive allele, you can target the NHEJ repair pathway by introducing Cas9 and one or two gRNAs in the absence of a donor repair template. Using one gRNA to target a single cleavage event in critical sequence, disruptive indels can be recovered. With two gRNAs, the intervening sequence can be deleted.

***Additional Materials** (also see Basic Protocol)*

gRNAs for introducing a disruptive indel (see text under step 1)

Perform the steps of the Basic Protocol with the following modifications:

1. Design and generate gRNAs.

   For introducing a disruptive indel, design one gRNA in a location where the insertion or deletion of a small number of nucleotides is expected to interfere with function. Indels are often less than 10 bp, but can be much longer (Koike-Yusa et al., 2014). For deleting a genomic region, design two gRNAs flanking the region to be deleted. We have used this approach to delete regions as large as 14 Kb in Drosophila (Gratz et al., 2014). Note that the deletion will not be precise and a small number of nucleotides will likely be lost or gained at the repaired junction.

   The generation of deletions by NHEJ with two gRNAs can be conducted in lines with a marked element in the targeted locus (Gratz et al., 2014). This allows for the detection of deletions by loss of the marker in the element.

2. The injection mixture should contain 100 to 250 ng/μl of each gRNA.

   When using a single gRNA, we have increased the concentration to 500 ng/μl.

3. Engineered flies will need to be identified molecularly or by phenotype. Indels can be identified by HRMA, while PCR can be used to detect larger deletions.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 4.

Adult fly homogenization buffer

10 mM Tris·Cl, pH 8.2 (*APPENDIX 2A*)
25 mM NaCl
1 mM EDTA
Store at room temperature for up to 6 months
Add 1 μl of 20 mg/ml proteinase K to 99 μl of homogenization buffer just prior to use

COMMENTARY

Background Information

The CRISPR-Cas9 system is a highly accessible and effective tool for genome engineering in *Drosophila* (Gratz et al., 2013b; Bassett and Liu, 2014; Harrison et al., 2014; Kondo, 2014). The Basic Protocol in this unit details an optimized CRISPR-Cas9 approach that has several advantages. We use a transgenic source of Cas9, expressed in the germline under the control of the *vasa* promoter, to achieve highly efficient and reliable genome engineering. The introduction of gRNA using rapidly constructed plasmids is quick and inexpensive. Our donor vectors facilitate streamlined cloning of locus-specific donor templates, and the incorporation of a removable DsRed marker makes identification of candidate alleles markedly easier than identification through molecular characterization.

While not covered in this protocol, other groups have successfully applied the CRISPR-Cas9 system in *Drosophila* using a variety of methods for introducing gRNAs and Cas9. NHEJ has been successfully accomplished using transgenic Cas9 + gRNA supplied as RNA (Xue et al., 2014), transgenic Cas9 + transgenic gRNA (Kondo and Ueda, 2013; Port et al., 2014; Xue et al., 2014), Cas9 DNA + gRNA plasmid (Ren et al., 2013; Gratz et al., 2013a), and Cas9 mRNA + gRNA supplied as RNA (Bassett et al., 2013; Yu et al., 2013). Successful HDR has been reported using Cas9 DNA, gRNA plasmid, and either a dsDNA or ssDNA donor (Gratz et al., 2013a, 2014). However, efficiency is higher with a transgenic Cas9 source, and all other HDR experiments reported in *Drosophila* to date have been conducted in Cas9-expressing flies using either gRNA plasmid (Gratz et al., 2014), gRNA supplied as RNA (Xue et al., 2014; Yu et al., 2014), or a transgenic gRNA source (Port et al., 2014).

Critical Parameters

Sequencing of target sites: Due to the prevalence of polymorphisms between distinct genetic backgrounds in *Drosophila*, it is critical to sequence the intended target locus in the genetic background in which the genome engineering experiment will be performed. Even a single-basepair change in a target site can be detrimental to the success of the experiment.

Donor template construction: To protect both the donor template and the modified locus from unintended cleavage, it is critical that the donor template not contain an intact gRNA target site.

Molecular confirmation of engineered lines: Because unexpected events can always occur during DNA repair, it is important to thoroughly confirm all candidate alleles. To do this, we suggest three PCRs (Fig. 31.2.3B), which together will confirm that engineered DNA has been incorporated at the target locus and that the locus is free of additional modifications, including the integration of donor vector backbone sequences (Yu et al., 2014).

Finally, it is important to note that, while CRISPR-Cas9 works quite well in *Drosophila*, the system is not yet fully understood. For example, locus- and sequence-specific effects on cleavage efficiency are poorly understood. The Perrimon group (Harvard Medical School) has developed a tool that uses data from high-throughput experiments in S2 cells to predict cleavage efficiency based on gRNA target sequence (*http://www.flyrnai.org/evaluateCrispr/*). An understanding of how donor composition and other experimental design features may influence the efficiency of HDR or the DNA repair pathway utilized by the cell awaits further study.

Troubleshooting

Poor viability: Reduce the concentration of gRNAs and donor vector in the injection mixture. While this may reduce efficiency, we have found that reducing the overall concentration of the injection mixture can increase viability significantly. If the poor viability is due to highly efficient generation of a lethal allele, use an injected DNA source of Cas9 (pBS-hsp70-Cas9) instead of *vasa-Cas9*. This will decrease cleavage efficiency, and thus the occurrence of biallelic events, facilitating the recovery of recessive lethal lesions.

Poor efficiency: In the event that a given targeting experiment fails to yield engineered alleles, the gRNAs should be tested for cleavage efficiency. For genome engineering strategies using a pair of gRNAs, a simple PCR spanning the two gRNA target sites can be performed on embryos 24 hr after injection of both gRNAs into *vasa-Cas9*. Amplicons indicating a deletion between the two targeted cleavage sites demonstrate that both gRNAs are capable of generating DSBs. For strategies using one gRNA, cleavage efficiency can be tested using HRMA or a mismatch-specific nuclease assay on embryos 24 hr post injection. This approach can also be used to assess gRNA efficiency prior to embarking on a CRISPR-based experiment.

Anticipated Results

Using the approach described above to replace genes with attP docking sites in multiple experiments, an average of 24% of injected flies produced correctly engineered progeny. The deleted genes ranged in size from 2 to 27 Kb. Interestingly, we have not observed a strong correlation between deletion size and efficiency, suggesting that locus- or gRNA-specific effects may play a larger role in determining differences in efficiency between targeting experiments. We have also used this approach to insert in-frame tags in a number of loci at a similar average efficiency of 26%.

The highest probability off-target cleavage sites can be identified by sequence similarity to the targeted site. In a subset of our experiments, we have assayed these sites and found no evidence of off-target cleavage (Gratz et al., 2014). Based on this and similar findings by others, we expect that with careful target site selection, engineered fly lines can be generated with few or no off-target mutations in most cases (Bassett et al., 2013; Gratz et al., 2013a; Gratz et al., 2014).

Time Considerations

Target site selection, preparation of gRNA plasmids, and construction of donor templates can be accomplished in 1 week with 1 to 4 hr hands-on time per day. Embryo injection can be accomplished in 1 day with 4 hr hands-on time. After 10 days, injected flies can be outcrossed in approximately 1 hr. After another 10 days, F1 progeny are screened and outcrossed (2 hr) before molecular confirmation, which can be completed in 2 days (5 hr hands-on time).

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Internet Resources

General Web sites

http://flycrispr.molbio.wisc.edu

flyCRISPR.

http://www.crisprflydesign.org

CRISPR fly design.

http://www.shigen.nig.ac.jp/fly/nigfly/cas9/

FlyCas9.

http://oxfcrispr.org

OXfCRISPR.

Reagents

http://flystocks.bio.indiana.edu/Browse/misc-browse/CRISPR.htm

Bloomington Drosophila Stock Center CRISPR stocks.

http://www.addgene.org/CRISPR/drosophila/

Addgene CRISPR plasmids.

Target finder tools

http://tools.flycrispr.molbio.wisc.edu/targetFinder/

CRISPR Optimal Target Finder.

http://www.flyrnai.org/crispr2/

DRSC Find CRISPRs.

http://crispr.mit.edu

CRISPR Design.

http://www.e-crisp.org/E-CRISP/

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