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Precise Genome Editing of Drosophila with CRISPR RNA-Guided Cas9

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Abstract

The readily programmable CRISPR/Cas9 system is transforming genome engineering. We and others have adapted the *S. pyogenes* CRISPR/Cas9 system to precisely engineer the Drosophila genome and demonstrated that these modifications are efficiently transmitted through the germline. Here we provide a detailed protocol for engineering small indels, defined deletions, and targeted insertion of exogenous DNA sequences within 1 month using a rapid DNA injection-based approach.

Keywords

CRISPR; Cas9; Genome engineering; Nonhomologous end joining; Homologous recombination; Drosophila

1 Introduction

The CRISPR/Cas9 system is transforming genome engineering with its simplicity. In Drosophila, the CRISPR/Cas9 system has been employed to interrupt, delete, and replace genes [1-7]. These modifications are efficiently transmitted through the germline for the establishment of stable transgenic lines. The rapid adoption of the CRISPR/Cas9 system illustrates its utility and adaptability, while the variety of complex modifications

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successfully generated to date offers only a glimpse into the genome manipulations now within reach.

The endogenous *S. pyogenes* CRIPSR/Cas9 adaptive immune system has been simplified to two components for use in genome engineering: Cas9 and a single chimeric RNA referred to as a chiRNA or guide RNA (gRNA) [8]. The gRNA comprises CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) sequences that direct sequence-specific cleavage of genomic DNA by Cas9 through a simple base-pairing mechanism. gRNAs are easily programmed to recognize a 20-nt target sequence and direct Cas9-dependent cleavage of both DNA strands at a defined site within the target. The only requirement for a CRISPR target site is the presence of a 3-bp protospacer adjacent motif (PAM) of the form NGG immediately 3′ of the 20-nt recognition sequence. Thus, potential CRISPR-Cas9 target sites are common, occurring on average once in every eight base pairs of genomic sequence.

Cas9-induced double-strand breaks (DSBs) trigger DNA repair via two cellular pathways that can be harnessed for genome editing. Nonhomologous end joining (NHEJ) is an error-prone process that can result in small insertions and deletions (indels) that disrupt function at cleavage sites. Homology-directed repair (HDR) employs homologous DNA sequences as templates for precise repair. By supplying an exogenous donor template, this repair pathway can be exploited to precisely edit genomic sequence or insert exogenous DNA.

In this chapter we describe the method for CRISPR/Cas9-mediated gene targeting in Drosophila developed in Gratz et al. [2] (Fig. 1). Specifically, we detail the use of a DNA injection-based approach to generate gene-disrupting indels and defined deletions via NHEJ and to replace genes with exogenous sequences by HDR employing readily synthesized single-stranded DNA (ssDNA) donors. These modifications can be efficiently generated and transmitted through the germline within a month making the promise of rapid genome engineering a reality. Throughout the protocol, we highlight key considerations for initiating a CRISPR genome engineering project. We also note alternatives to and extensions of our protocol. Given the accessibility and adaptability of the system, we expect the range of modifications achieved with CRISPR/Cas9 to expand rapidly as this transformative technology is employed by researchers worldwide.

2 Materials

2.1 Molecular Biology

1. Cloning and expression vectors: pBS-Hsp70-Cas9 for expression of codon-optimized Cas9 and pU6-BbsI-gRNA for cloning and expression of specific gRNAs are available from Addgene.
2. T4 DNA Ligase.
3. T4 Polynucleotide Kinase.
4. *E. coli* DH5α or other suitable strain for general cloning.
5. EndoFree Plasmid Maxi kit (Qiagen).
6. *BbsI* endonuclease.

7. Wizard SV Gel and PCR Clean-up System kit (Promega).
8. Embryo homogenization buffer: 10 mM Tris–HCl (pH 8.2), 25 mM NaCl, 1 mM EDTA, 0.2 % Triton-X100. Immediately before use, add 200 μg/mL proteinase K.
9. Adult fly homogenization buffer: 10 mM Tris–HCl (pH 8.2), 25 mM NaCl. Prior to use, add 200 μg/mL proteinase K.
10. Agarose gel electrophoresis equipment.
11. PCR reagents, including primers designed to amplify a 500–700-bp region flanking your target site.
12. Optional: SURVEYOR Mutation Detection kit (Transgenomic).

2.2 Embryo Injections

1. Grape juice agar plates and yeast paste for collecting Drosophila embryos.
2. Population cages for embryo collection.
3. Inverted microscope equipped with a micromanipulator, micropipette holder, and a microinjector.
4. Glass capillary for injection needles.
5. Micropipette puller.
6. Microscope slides and cover slips.
7. Halocarbon oil 700 and 27 (Halocarbon Products Corporation).

2.3 Fly Stocks

1. Because CRISPR RNA/Cas9 components are introduced through injection into embryos, any fly stock can be engineered. Lines containing a phenotypically marked element in the targeted locus will allow for negative screening for the genome modifications. In appropriate genetic backgrounds, donor templates carrying visible markers facilitate positive screening. It may also be desirable to carry out modifications in a particular fly strain to control for genetic background, for example, in behavioral, quantitative trait loci, and evolutionary studies. Finally, genome engineering can be carried out in a fly line that transgenically expresses Cas9 in the germline such that only the gRNA vectors and HDR donor vector must be supplied through injection [3-6].

3 Methods

3.1 Select CRISPR Target Sites

1. Once you have determined the genome modification you wish to make and designed a general strategy (*see* Note 1), the first step is to identify best site or sites

[1]A key consideration in designing your strategy is determining how you will identify flies in which the desired modification was induced, and it is essential that you consider this critical step early in your planning process. We discuss several screening options in Subheading 3.7.

for targeting Cas9-induced DSBs. For mutagenesis via indel formation, you will need to identify a single CRISPR target site in a region where a small insertion or deletion has the potential to be disruptive—for example, by inducing a frameshift in the coding sequence of a targeted gene. To generate a defined deletion of, for example, an entire gene, you will need to identify two CRISPR target sites flanking the region to be excised. If your goal is to incorporate a tag, such as FLAG, into the endogenous coding sequences of a gene, you will want to identify a single target site as close as possible to the desired modification.

2. Sequence the target regions in the specific fly line you will be editing. Polymorphisms between fly lines and the reference genome are frequent, especially in intergenic regions, and could significantly decrease cleavage frequency if they occur within your gRNA sequence.

3. For each target region, identify a 20-nt CRISPR target site flanked on the 3′ end by the PAM sequence (NGG) such that the target site is 5′-$(N)_{20}$(NGG). The PAM sequence is not part of the gRNA but is required at the genomic target site for DNA cleavage by Cas9.

4. CRISPR target sites should be selected to minimize potential off-target cleavage. Several studies have shown that the 12-nt "seed" region of the target site immediately adjacent to the PAM is the most critical region for efficient cleavage and should be unique, if possible [9-17]. Furthermore, PAMs of the form NAG permit low-efficiency cleavage in cell lines [12]. Based on current understanding in the field, highly specific gRNAs can be generated by selecting CRISPR target sites with the fewest potential off-target sites in the genome, defined as:

   (a) PAM-adjacent sites with ≥11/12 matches to the target seed sequence.

   (b) PAM-adjacent sites with ≥18/20 matches to the full target sequence.

   (c) Sites fitting above criteria adjacent to a PAM in the form of NAG as well as NGG.

To facilitate high-quality gRNA design, we have created a web application, CRISPR Optimal Target Finder, that identifies specific target sites in Drosophila and other invertebrate species [3]. The program can be accessed at http://tools.flycrispr.molbio.wisc.edu/targetFinder/.

3.2 Generate gRNA Expression Plasmids

1. CRISPR targeting sequences are synthesized as oligonucleotides, annealed and incorporated into the gRNA expression vector, pU6-BbsI-gRNA (Fig. 2). Cohesive ends for seamless cloning of targeting sequences into the gRNA backbone are included in the oligonucleotide design. The top strand should be designed in the format of 5′-CTTCG$(N)_{19}$-3′, where G$(N)_{19}$ corresponds to your unique target site sequence beginning with a G for efficient transcription from the Drosophila U6 promoter (*see* Note 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. You can either order 5′ phosphorylated oligonucleotides or, as described

below, use T4 Polynucleotide Kinase (PNK) to add the 5′-phosphates to standard oligonucleotides. Oligonucleotides should be resuspended at a concentration of 100 μM in nuclease-free H_2O.

2. Phosphorylate and anneal the target sequence oligonucleotide pairs in one step: 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, 6 μL of H_2O, and 1 μL of T4 Polynucleotide Kinase. Incubate at 37°C for 30 min, and 95 °C for 5 min, and then ramp to 25 °C at a rate of −5 °C/min.
3. Prepare pU6-BbsI-gRNA for cloning by transforming DH5α cells and selecting colonies on plates containing 100 μg/mL ampicillin. Purify plasmid DNA and resuspend in nuclease-free H_2O.
4. Determine the DNA concentration using a spectrophotometer. Digest 1 μg of pU6-BbsI-gRNA with *BbsI*.
5. Gel purify cut vector to remove any uncut vector. We use the Promega Wizard SV Gel and PCR Clean-up System kit. Determine the DNA concentration using a spectrophotometer.
6. Ligate the annealed insert and purified *BbsI*-digested pU6-BbsI-gRNA. Combine 1 μL of annealed insert, 50 ng of *BbsI-digested* pU6-BbsI-gRNA, 1 μL of T4 DNA Ligase buffer, 1 μL of T4 DNA Ligase, and enough H_2O to bring the reaction to 10 μL. Incubate at 25 °C for 1 h.
7. Transform the ligation reaction into DH5α cells and select colonies on plates containing 100 μg/mL ampicillin.
8. Isolate plasmids from 2 to 4 individual colonies using a mini-prep kit. Screen for plasmids with incorporated oligonucleotides by Sanger sequencing.
9. Prepare DNA for injection from a positive clone using the Qiagen EndoFree Plasmid Maxi kit.
10. Determine the DNA concentration using a spectro photometer.
11. Aliquot and store at −20 °C.

3.3 Prepare Cas9 Expression Plasmid (Unless Injecting into a Cas9-Expressing Line)

1. Transform DH5α cells with pBS-Hsp70-Cas9 and select on ampicillin-containing plates. Purify plasmid DNA using the Qiagen EndoFree Plasmid Maxi kit and resuspend in nuclease-free H_2O.
2. Determine the DNA concentration using a spectrophotometer.
3. Aliquot and store at −20 °C (*see* Note 3).

[2]A study in zebrafish and our experiences indicate that G nucleotides can be added as an additional base pair on the 5′ end of the 20-nt target sequence without significantly affecting efficiency [20]. This can be useful when a suitable target beginning with G is not available.

[3]Injection of Cas9 and a targeting gRNA as mRNA and RNA, respectively, has also been shown to efficiently generate indels in Drosophila [1, 7].

3.4 Design and Prepare Donor Template for HDR

1. To incorporate exogenous sequences, you will need to generate a donor repair template for HDR. Here we outline the method we used to replace the *yellow* locus with a 50-bp attP ΦC31 phage recombination site using an ssDNA donor template. An analogous strategy can be used to insert other short exogenous sequences or to introduce polymorphisms. The primary advantage of using ssDNA donor templates is that they can be rapidly synthesized, obviating the need for cloning. However, the size of ssDNAs is generally limited to ~200 nt. Larger double-stranded DNA (dsDNA) donor templates can be employed to efficiently incorporate larger exogenous sequences or modify sequences over a large region [3].

   Design an ssDNA donor with homology arms corresponding to sequences immediately adjacent to the targeted cleavage sites flanking the attP sequence (Fig. 3). We use the 50-nt attP sequence: CTACGCCCCCAACTGAGAGAACTCAAAGGTTACCCCAGTTGGGGCACT AC. Homology arms of ~40–60 nt have been shown to mediate efficient HDR with ssDNA donors in Drosophila [2, 18, 19]. Cas9-mediated DSBs are generated 3 bp upstream of the PAM, so homology regions in the repair template should be designed to include the portion of the targeting sequence that remains following cleavage. The repair template should also be designed to avoid recutting of the genomic locus following successful HDR. This can be achieved by truncating or mutating the CRISPR target sequence within the donor template. The orientation of the single-stranded donor is critical to the success of the experiment. During DNA repair, free 3′ ends created by rescission at the DSB search for and invade homologous DNA. It is therefore essential for incorporation of donor sequences that the ssDNA bears complementarity to the free 3′ end.

2. Resuspend the ssDNA in nuclease-free H_2O to a final concentration of 1 μg/μL.

3.5 Embryo Injections

1. Prepare an injection mixture appropriate for your desired modification. If you are using a single gRNA to generate indels via NHEJ, make an injection mixture containing 500 ng/μL of pBS-Hsp70-Cas9 and 250 ng/μL of the U6-gRNA vector. To generate defined deletions by NHEJ, make an injection mixture of 500 ng/μL of pBS-Hsp70-Cas9 and 250 ng/μL of each U6-gRNA. For gene replacement by HDR, prepare an injection mixture containing 500 ng/μL of pBS-Hsp70-Cas9, 250 ng/μL of each U6-gRNA, and 100 ng/μL of the ssDNA donor template (*see* Note 4). Prepare injection mixtures in nuclease-free H_2O.

2. Establish a small population cage of the fly line to be injected. Provide flies fresh yeast paste on grape-juice plates.

[4]The concentrations of the CRISPR/Cas9 components can be titrated to balance cleavage efficiency and the potential for off-target cleavage [12, 14]. As high concentrations induce more off-target cleavage and may be toxic, it might be necessary to try multiple concentrations if viability or fertility is low. Based on our molecular analysis of targeting in embryos, concentrations as low as 50 ng/μL of pBS-Hsp70-Cas9 and 25 ng/μL of pBS-U6-gRNA can catalyze cleavage in somatic cells.

3. Allow flies to lay embryos for 30 min, collect the embryos, and place them in a drop of water on a cover slip. Align the embryos side by side along the edge of a cover slip with the posterior end of the embryos oriented toward the edge for injection through the cuticle (*see* Note 5). Allow the embryos to dry until they adhere to the cover slip (1–5 min), and then cover the embryos with a 7:1 mixture of halocarbon oil 700 and halocarbon oil 27. Place the cover slip on a slide for injection. Injections must be completed prior to cellularization of pole cells approximately 1 h after laying at 25 °C. We perform injections at 18 °C to slow development and expand this time frame.
4. Inject embryos with a microinjector and a micromanipulator on an inverted microscope using glass capillary needles pulled on a micropipette puller.
5. After injection, allow the halocarbon oil to drain off the embryos. Transfer the cover slip with attached embryos to a plate of Drosophila agar-based sugar food containing a dab of yeast paste. Upon hatching, larvae will move to the yeast paste. After 3–4 days, transfer yeast paste and larvae to a food vial and rear to adulthood.

3.6 Assess Efficiency of Targeting in Injected Embryos (Optional)

1. There is significant variability in targeting efficiency at different genomic loci and, within a single locus, at different target sites [1, 2, 7]. To rapidly estimate the targeting efficiency of our gRNAs before embarking on a full experiment, we collect 10–15 embryos 24 h after injection for molecular analysis (*see* Note 6).
2. To isolate embryonic genomic DNA for molecular analysis, place each embryo in a 0.2 mL PCR tube. Using either a P20 or P200 pipette tip, draw up 20 μL of freshly prepared embryo homogenization buffer. Keeping the buffer in the pipette, use the tip to homogenize the embryo against the wall of the tube. Dispense the homogenization buffer.
3. Incubate at 37 °C for 30–60 min followed by a 5-min incubation at 95 °C.
4. Use 2 μL of embryonic genomic DNA in a 50 μL PCR to amplify the region spanning the targeted cleavage site(s). The PCR primers should be designed to generate a product from the mutant locus that can be readily distinguished from the wild-type locus. We generally design our reactions to generate ~500–700-bp products.
5. Defined deletions and gene replacements that result in size differences greater than 25 bp can be readily recognized by the size of the PCR product (*see* Note 7), whereas small indels and single base-pair mutations can be efficiently detected using the SURVEYOR Mutation Detection kit (*see* Note 8). Briefly, PCR products

[5]Dechorionated embryos can also be injected using standard protocols.
[6]The factors that determine either CRISPR target-specific or locus-specific differences in efficiency are not understood. Thus, a rapid method for assessing whether a particular targeting experiment is efficiently generating the intended modification is desirable. Molecular characterization of injected embryos can be used to assess somatic transformation rates. However, these numbers may not correlate directly with germline transmission rates.
[7]In gene replacement experiments, some cells will repair the Cas9-induced DSB by HDR and incorporate the donor sequence, while others will employ NHEJ and generate a defined deletion lacking the exogenous sequence. In our gene replacement experiments, we generally observe doublets that reflect repair by both pathways in somatic cells of injected embryos.

spanning the targeted site are denatured and reannealed. Any heteroduplexes formed by the annealing of wild-type DNA to indel-containing DNA will be cleaved by the SURVEYOR nuclease.

3.7 Screen F1 Progeny for Germline Transmission of Genome Modifications

1. If the phenotype is known and can be used to screen for the desired modification, cross injected flies to an appropriate fly line and screen F1 progeny for the expected phenotype. Chromosomal deletions that uncover the target locus can be used to simplify subsequent molecular characterization of candidates.
2. If you cannot identify your modification by phenotype, you can design your engineering strategy to utilize one of the alternatives outlined below.

Negative selection—Targeting strategies can be designed to remove a marked element in the targeted locus upon successful editing to enable screening of F1 progeny for loss of the visible marker. For making defined deletions, one consequence of this strategy is an increase in the size of the deletion that must be generated. We have used this strategy to identify defined deletions of approximately 15 kb [3].

Positive selection—Large dsDNA donors can be designed to include a visible marker, such as mini-*white*, along with the desired modification for positive screening of F1 progeny. The visible marker can be flanked by FRT or LoxP sites for subsequent removal. While not covered in this protocol, we have successfully used CRISPR/Cas9 and dsDNA donors to replace genes that are large as 25 kb with an attP docking site and 3×P3-DsRed marker, to generate conditional alleles and insert in-frame protein tags [3] (O'Connor-Giles, Harrison and Wildonger labs, unpublished data).

Molecular identification—After outcrossing, individual F1 progeny can be sacrificed for genomic DNA and subjected to PCR-based analysis for identification of targeted events. This approach was successfully applied by Yu et al. [7], who identified Cas9-induced indels in four genes through a combination of direct sequencing and restriction enzyme-based analysis of PCR products (*see* Note 9).

3.8 Molecularly Confirm Genome Modifications

1. If you have not already done so during your screening process, confirm your modification through PCR and sequencing analysis. Always confirm the entire modification and surrounding DNA because cellular DNA repair processes can result in unexpected modifications or rearrangements, such as repair events that are correct on one end but not the other.

3.9 Evaluate Potential Sites of Off-Target Cleavage

1. Once fly lines with the correct targeted modification have been recovered, an assessment of off-target mutations can be conducted. Using the criteria outlined in

[8]High-resolution melting analysis offers a highly efficient alternative for the detection of indels [1].
[9]The percentage of analyzed progeny with induced mutations ranged from 2 to 99 % [7].

Subheading 3.1 or CRISPR Optimal Target Finder, identify potential off-target cleavage sites for evaluation by PCR and sequence analysis (*see* Note 10).

2. To isolate genomic DNA for molecular analysis, anesthetize a single fly and place it in a 0.2 mL PCR tube. Using a P200 pipette tip draw up 50 μL of freshly prepared adult fly homogenization buffer. Keeping the buffer in the pipette, use the tip to homogenize the fly. Once the fly is homogenized, dispense the remaining buffer.
3. Incubate at 37 °C for 30–60 min followed by a 5-min incubation at 95 °C.
4. Use 1 μL of adult genomic DNA in a 50 μL PCR to amplify 500–700-bp regions spanning any potential off-target cleavage sites.
5. Sequence the PCR products to identify any small indels induced by Cas9 at potential off-target cleavage sites.
6. If you identify any off-target mutations, standard recombination methods can be used to separate them from the intended genome modification. *See* Note 11 for additional suggestions for reducing off-target cleavage.

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Abbreviations

CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
DSB	Double-strand break
dsDNA	Double-stranded DNA
gRNA	Guide RNA
HDR	Homology-directed repair
Indel	Insertion-deletion

[10]Whole-genome analyses of the off-target effects of Cas9-induced DSBs have not yet been conducted in Drosophila. Because off-target cleavage is expected to be sequence based, individual inspection of the most likely off-target cleavage sites should be effective. However, this approach may not detect larger rearrangements that can occur during DSB repair.

[11]As noted earlier, careful CRISPR target selection and low concentrations of CRISPR components can reduce off-target cleavage. Alternatively, a version of Cas9 that has been modified into a nickase by disrupting one of its nuclease domains can be utilized in HDR experiments [8]. Because DNA nicks are less prone to NHEJ but still catalyze HDR, use of the nickase version of Cas9 may substantially reduce off-target effects. Fly lines expressing the nickase version of Cas9 were generated by Kate Koles and Avi Rodal's lab and are available at http://www.crisprflydesign.org/flies. Finally, gRNA length has been correlated with specificity, with longer gRNAs showing less specificity but higher activity [14].

NHEJ	Nonhomologous end joining
PAM	Protospacer adjacent motif
ssDNA	Single-stranded DNA
tracrRNA	Trans-activating CRISPR RNA

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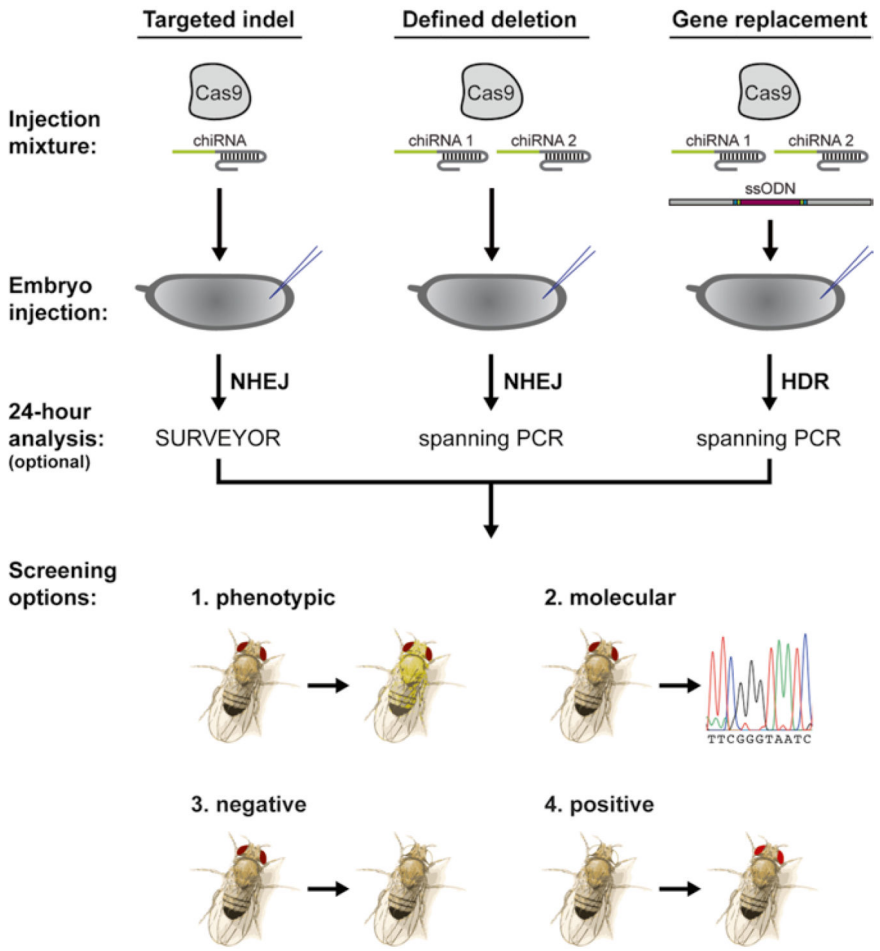

Fig. 1.

Experimental overview. Injection mixtures containing pHsp70-Cas9, the appropriate pBS-U6-gRNA(s), and, for gene replacement, an ssDNA donor are injected into pre-blastoderm embryos. Twenty-four hours after injection, a subset of embryos can be assayed molecularly for the presence of targeted modifications to assess efficiency. Rear remaining embryos to adulthood and outcross to recover heritable modifications. Methods for identifying flies with the targeted modification include (1) phenotypic screening if the phenotype of the targeted modifications is known and readily observable, (2) molecular screening by PCR-based analysis, (3) negative screening for the removal of a marked transposable elements in the targeted locus, and (4) positive screening using a dsDNA donor with a visible marker

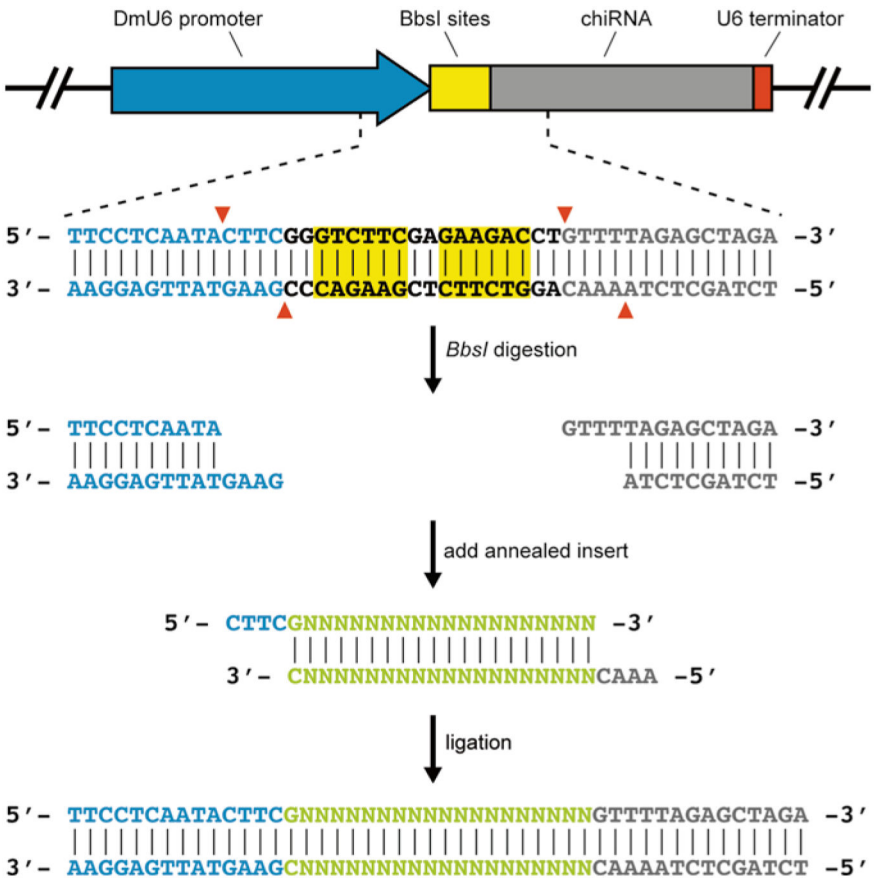

Fig. 2.

gRNA cloning. The pBS-U6-gRNA vector contains two recognitions sites for the type IIs restriction enzyme *BbsI*. Following *BbsI* digestions, unique 4-nt overhangs mediate seamless cloning targeting sequences into the gRNA backbone. Targeting sequence inserts are generated as two 5′ phosphorylated oligonucleotides that are annealed to create complementary overhangs (5′-CTTC, top strand; 5′-AAAC, bottom strand) for ligation into linearized pBS-U6-gRNA

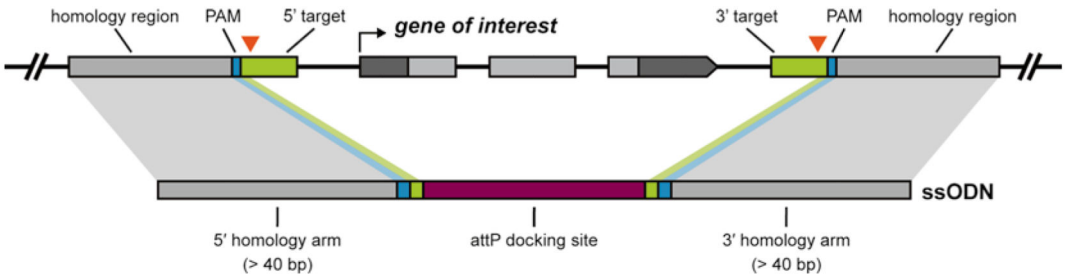

Fig. 3.

ssDNA design. Single-stranded oligonucleotide donors are designed to contain approximately 60 nt of homology to the target locus sequences immediately adjacent to each predicted Cas9-mediated DSB. The homology regions flank a 50-nt attP sequence that provides subsequent access to the targeted locus. *Red arrowheads* mark the predicted Cas9 cleavage sites. Target site sequences (*green*) and PAMs (*blue*) are indicated