

Protocol

# Design and Generation of *Drosophila* Single Guide RNA Expression Constructs

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The recent advances in CRISPR-based genome engineering have enabled a plethora of new experiments to study a wide range of biological questions. The major attraction of this system over previous methods is its high efficiency and simplicity of use. For example, whereas previous genome engineering technologies required the generation of new proteins to target each new locus, CRISPR requires only the expression of a different single guide RNA (sgRNA). This sgRNA binds to the Cas9 endonuclease protein and directs the generation of a double-strand break to a highly specific genomic site determined by the sgRNA sequence. In addition, the relative simplicity of the *Drosophila* genome is a particular advantage, as possible sgRNA off-target sites can easily be avoided. Here, we provide a step-by-step protocol for designing sgRNA target sites using the *Drosophila* RNAi Screening Center (DRSC) Find CRISPRs tool (version 2). We also describe the generation of sgRNA expression plasmids for the use in cultured *Drosophila* cells or in vivo. Finally, we discuss specific design requirements for various genome engineering applications.



## MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

**RECIPE:** Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

## Reagents

BbsI restriction enzyme (Thermo Scientific)  
Chemically competent *E. coli* and reagents for transformation  
DNA sequencing primer (5'-CAATAGGACACTTTGATTC-3') and reagents  
FastAP enzyme and 10× FastDigest buffer (Thermo Scientific)  
LB liquid medium <R> with ampicillin for selection

*In addition, prepare plates containing LB medium solidified with agar (plus ampicillin).*

PCR purification kit (e.g., QIAGEN)  
Plasmid DNA miniprep kit (e.g., QIAGEN)  
sgRNA cloning vector (p1100 or p118 [Ren et al. 2013; Housden et al. 2015]) (available from authors on request)

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sgRNA oligonucleotides (100  $\mu$ M) (designed according to Steps 8–12)  
T4 polynucleotide kinase (T4 PNK) and 10 $\times$  T4 ligation buffer (New England BioLabs)  
T7 ligase and 2 $\times$  Quick ligase buffer (Enzymatics)

## Equipment

Incubator at 37°C

sgRNA selection tool (DRSC Find CRISPRs [version 2]; [www.flyrnai.org/crispr2](http://www.flyrnai.org/crispr2))

*Because of the abundance of possible target sites in the Drosophila genome, it is often relatively simple to identify sgRNA sequences close to the locus of interest. However, both efficiency and specificity vary greatly between different sgRNAs; therefore, careful target site selection is critical to successful genome modification. Various tools exist for the consideration of variables such as off-target (OT) prediction and mutation efficiency to aid in the selection of the optimal sgRNA for any given application. DRSC Find CRISPRs allows visualization of all possible sgRNA target sites on both strands relevant to any gene or any specific location throughout the Drosophila genome in the context of a genome browser (Ren et al. 2013; Housden et al. 2014). Each sgRNA is annotated with predicted OT sites, a score that predicts mutation efficiency, and restriction enzyme recognition site(s) that might be impacted by DSB. The user has the option to select different thresholds for predicted OT potential as well as apply filters based on the relationship of the OT site to gene annotations. For example, a user can opt to allow sgRNAs with OT predictions if they are not located in the coding DNA sequence (CDS) region of any other gene. Other available tools, each with different criteria for sgRNA target annotation and OT prediction, include CRISPR Optimal Target Finder (<http://tools.flycrispr.molbio.wisc.edu/targetFinder/>) (Gratz et al. 2014), E-CRISP (<http://www.e-crisp.org/E-CRISP/designcrispr.html>) (Heigwer et al. 2014), and CHOPCHOP (<https://chopchop.rc.fas.harvard.edu/index.php>) (Montague et al. 2014).*

Thermocycler

## METHOD

### Designing sgRNAs Using DRSC Find CRISPRs

1. Load the home page at <http://www.flyrnai.org/crispr2> (Fig. 1A).
2. Enter the FlyBase gene ID, CG number, gene symbol, or chromosome location of the desired editing event (Box 1).
3. Select the OT requirements and features of the targeted sequence (e.g., CDS, untranslated region [UTR], intron, intergenic) (Box 2).
4. Select PAM sequences to include for use with Cas9 derived from *Streptococcus pyogenes* (Box 3).  
*Note that NGG results in considerably higher efficiency than NAG.*
5. Select OT stringency (Box 4).  
*The least stringent option considers sites with up to three mismatches as OTs. The most stringent option considers sites with up to five mismatches as OTs.*
6. Click “Submit” to view all sgRNA target sites in the context of a genome browser. Identify the region of interest.  
*Note that the first CDS exon can be either to the left or to the right, depending on the strand.*  
*See Troubleshooting.*
7. Click each sgRNA site to view detailed information such as OT annotation and efficiency score (Fig. 1B). Select the optimal sgRNA designs according to the guidelines provided in Figure 1C.  
*See Discussion.*

### Cloning sgRNAs

#### Designing Oligonucleotides

8. Copy the selected sgRNA target sequence from the DRSC Find CRISPRs tool.
9. Remove the NGG PAM from the 3' end to obtain the sense sgRNA oligo sequence.
10. To obtain the antisense oligo sequence, find the reverse complement of the sense oligo.



11. Add the following cloning sequences to the sense and antisense oligos.

Cloning vector	Sequences to add
For pl100	sense: "CTTCG-20 bp" antisense: "AAAC-20 bp-C"
For pl18	sense: "GTTCG-20 bp" antisense: "AAAC-20 bp-C"

*Both pl100 and pl18 express the sgRNA from a U6 promoter. This requires that the expressed sequence start with G. We therefore add G to the start of every sgRNA, although this is not necessary when the first position of the sgRNA target is already G.*

12. Synthesize the sense and antisense oligos and purify by standard desalting. Resuspend the oligos in H<sub>2</sub>O to a final concentration of 100 μM.

### Annealing sgRNA Oligos

13. Assemble the following reaction mixture.

Reagent	Amount to add
Sense sgRNA oligo (100 μM)	1 μL
Antisense sgRNA oligo (100 μM)	1 μL
10× T4 ligation buffer	1 μL
T4 PNK	0.5 μL
H <sub>2</sub> O	6.5 μL

14. Transfer the mixture to a thermocycler and run the following program.

Temperature	Time
37°C	30 min
95°C	5 min
Ramp from 95°C to 25°C	5°C/min

15. Dilute the annealed oligos 200-fold using H<sub>2</sub>O.

*The final oligo concentration is 50 nM.*

### Linearizing the Cloning Vector

16. Assemble the following digestion reaction to linearize the selected cloning vector.

Reagent	Amount to add
Vector DNA (pl100 or pl18)	1 μg
10× FastDigest buffer	2 μL
FastAP enzyme	1 μL
BbsI	1 μL
H <sub>2</sub> O	to 20 μL

17. Incubate the reaction mixture for 30 min at 37°C.

18. Purify the reaction products using a PCR purification kit following the manufacturer's instructions.

19. Dilute the linearized plasmid to 10 ng/μL using H<sub>2</sub>O.

*A large preparation of linearized plasmid can be produced and stored at -20°C for later use.*

### Ligating the sgRNA Fragment into the Linearized Vector

*When performing this protocol for the first time, we recommend including a negative control in which the annealed oligos are omitted. This sample should not produce colonies if the vector is correctly linearized.*

20. Assemble the following ligation reaction mixture.

Reagent	Amount to add
Linearized plasmid (10 ng) from Step 19	1 $\mu$ L
Annealed oligos (50 nM) from Step 15	1 $\mu$ L
2 $\times$ Quick ligase buffer	5 $\mu$ L
T7 ligase	0.5 $\mu$ L
H <sub>2</sub> O	2.5 $\mu$ L

21. Incubate the ligation mixture for 5 min at room temperature.
22. Transform 2  $\mu$ L of ligation mixture into chemically competent *E. coli* using standard methods. Spread the cells onto plates containing solid LB agar with ampicillin. Incubate overnight at 37°C.
23. Select two individual colonies for culture in LB liquid medium with ampicillin. Prepare plasmid DNA minipreps from each clone using standard methods.
24. Sequence the miniprep DNA using the sequencing primer (5'-CAATAGGACACTTTGATTC-3') to check for successful cloning of a single sgRNA sequence.

See *Troubleshooting*.

## TROUBLESHOOTING

**Problem (Step 6):** There are no suitable sgRNA target sites.

**Solution:** Although sgRNA target sites are common throughout the genome, there are regions in which suitable sequences are sparse. For example, PAM sequences may be less common in AT-rich regions such as introns. To increase the number of target sites displayed by the DRSC Find CRISPRs tool, reduce the OT stringency threshold, select more tracks with different OT features, or select NAG instead of NGG for the PAM sequence (Fig. 1A).

**Problem (Step 24):** No oligo inserts are detected, or there are multiple oligo inserts present in the cloning vector.

**Solution:** The method described is generally extremely robust and only one or two clones need to be sequenced to find a correctly cloned construct. If no insert is detected, it is likely that the vector linearization was incomplete. This can be checked by performing a negative control ligation omitting the annealed oligos. A completely linearized vector should produce zero colonies under these conditions. If multiple inserts are detected, increase the ratio of oligo dilution to 1:500 in Step 15.

## DISCUSSION

The above protocol provides a general idea of how to use DRSC Find CRISPRs (version 2) to generate sgRNA expression plasmids. However, the specific positioning and nature of the sgRNA will vary depending on the desired application. For example, to generate a frameshift gene knockout, a single sgRNA targeting downstream and close to the start of the coding sequence should be designed. In contrast, to delete a defined locus, two sgRNAs flanking that region or one or two sgRNAs in combination with a donor construct should be used. The specific requirements for some common applications are illustrated in Figure 1C.

One major concern associated with the use of CRISPR in mammalian systems has been the occurrence of OT effects. Indeed, a recent report indicated that OT mutations occur at high efficiency and at multiple loci (Tsai et al. 2015). In addition, many of the OT sites identified in the study were not predicted by current algorithms, making the specificity of any given sgRNA unknown.

In contrast, OT effects appear to be much less of a concern in *Drosophila*, and although several groups have reported high-efficiency on-target mutation, OTs have not yet been observed. However, unbiased OT detection methods such as GUIDE-seq (Tsai et al. 2015) have not yet been used in

*Drosophila*, so it is possible that OT mutations remain undetected. From current prediction algorithms, it appears that 97% of genes can be targeted without OT effects (Ren et al. 2013), suggesting that with careful sgRNA design, OTs are not a major concern in *Drosophila*.

Several modifications to the CRISPR system have been developed to reduce the occurrence of OT mutations. For example, Cas9 can be converted to a nickase to generate single-strand breaks by mutating one of the two endonuclease domains. Single-strand breaks are considerably less likely to lead to indel mutations than double-strand breaks (DSBs) but can still be repaired by homologous recombination (HR) (Davis and Maizels 2014; Rong et al. 2014). This version of the system can therefore be used to make targeted changes with reduced OT mutation frequency. However, Cas9 nickase appears to be considerably less efficient than wild-type Cas9 at inducing HR events, and OT indels may still occur at a low level (Ren et al. 2014). Despite these disadvantages, this is a useful approach where high specificity is important.

A modification of the nickase approach is to use two nickase Cas9 constructs targeting sites in close proximity (Mali et al. 2013; Ran et al. 2013, 2014; Cho et al. 2014). This leads to a DSB only at loci bound by both sgRNAs, whereas OT sites will be cleaved only on a single DNA strand. This likely biases the generation of indel mutations toward the on-target site, but similar to the single nickase approach, efficiency is reduced compared to wild-type Cas9 (Ran et al. 2013). In addition, although the use of two sgRNAs may decrease OT mutation at any given site, it may increase the number of OT sites at which single-strand breaks are generated.

A common application of CRISPR is the induction of frameshift mutations to generate gene knockouts. For this to be successful, DSBs must be induced efficiently but the resulting mutation must also generate a frameshift. Although the repair process often appears to be random, microhomology in the surrounding sequence leads to bias in the resulting repaired sequence (Bae et al. 2014). These effects can be predicted by considering the extent of microhomology and distance from the DSB site. A tool was recently developed to predict the likelihood of generating a frameshift mutation (Bae et al. 2014). One future application for which this may be particularly useful is tissue-specific mutagenesis. It was recently shown that CRISPR can be implemented in defined *Drosophila* tissues to generate inducible mutations (Xue et al. 2014). Note that the DRSC Find CRISPRs tool described above includes predicted frameshift scores for all sgRNAs in the *Drosophila* genome as well as a stand-alone page for users to calculate frameshift scores for their own designs (<http://www.flyrnai.org/evaluateCrispr/>).



## RECIPE

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### *LB Liquid Medium*

In 1 L H<sub>2</sub>O, dissolve 10 g of Bacto Tryptone, 5 g of yeast extract, and 10 g of NaCl. Adjust the pH to 7.5 with NaOH. Sterilize by autoclaving.

## ACKNOWLEDGMENTS

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We thank David Doupé and Stephanie Mohr for helpful discussions during the preparation of this manuscript. Work in the Perrimon Laboratory is supported by grants from the National Institutes of Health and the Howard Hughes Medical Institute.

## REFERENCES

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- Bae S, Kweon J, Kim HS, Kim JS. 2014. Microhomology-based choice of Cas9 nuclease target sites. *Nat Methods* 11: 705–706.
- Cho SW, Kim S, Kim Y, Kweon J, Kim HS, Bae S, Kim JS. 2014. Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res* 24: 132–141.
- Davis L, Maizels N. 2014. Homology-directed repair of DNA nicks via pathways distinct from canonical double-strand break repair. *Proc Natl Acad Sci* 111: E924–E932.
- Gratz SJ, Ukken FP, Rubinstein CD, Thiede G, Donohue LK, Cummings AM, O'Connor-Giles KM. 2014. Highly specific and efficient CRISPR/

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- Cas9-catalyzed homology-directed repair in *Drosophila*. *Genetics* **196**: 961–971.
- Heigwer F, Kerr G, Boutros M. 2014. E-CRISP: Fast CRISPR target site identification. *Nat Methods* **11**: 122–123.
- Housden BE, Lin S, Perrimon N. 2014. Cas9-based genome editing in *Drosophila*. *Methods Enzymol* **546**: 415–439.
- Housden BE, Valvezan AJ, Kelley C, Sopko R, Hu Y, Roesel C, Lin S, Buckner M, Tao R, Yilmazel B, et al. 2015. Identification of potential drug targets for tuberous sclerosis complex by synthetic screens combining CRISPR-based knockouts with RNAi. *Sci Signal* **8**: RS9.
- Mali P, Aach J, Stranges PB, Esvelt KM, Moosburner M, Kosuri S, Yang L, Church GM. 2013. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat Biotechnol* **31**: 833–838.
- Montague TG, Cruz JM, Gagnon JA, Church GM, Valen E. 2014. CHOP-CHOP: A CRISPR/Cas9 and TALEN web tool for genome editing. *Nucleic Acids Res* **42**: W401–W407.
- Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y, et al. 2013. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* **154**: 1380–1389.
- Ren X, Sun J, Housden BE, Hu Y, Roesel C, Lin S, Liu LP, Yang Z, Mao D, Sun L, et al. 2013. Optimized gene editing technology for *Drosophila melanogaster* using germ line-specific Cas9. *Proc Natl Acad Sci* **110**: 19012–19017.
- Ren X, Yang Z, Mao D, Chang Z, Qiao HH, Wang X, Sun J, Hu Q, Cui Y, Liu LP, et al. 2014. Performance of the Cas9 nickase system in *Drosophila melanogaster*. *G3 (Bethesda)* **4**: 1955–1962.
- Rong Z, Zhu S, Xu Y, Fu X. 2014. Homologous recombination in human embryonic stem cells using CRISPR/Cas9 nickase and a long DNA donor template. *Protein Cell* **5**: 258–260.
- Tsai SQ, Zheng Z, Nguyen NT, Liebers M, Topkar VV, Thapar V, Wyvekens N, Khayter C, Iafrate AJ, Le LP, et al. 2015. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR–Cas nucleases. *Nat Biotechnol* **33**: 187–197.
- Xue Z, Wu M, Wen K, Ren M, Long L, Zhang X, Gao G. 2014. CRISPR/Cas9 mediates efficient conditional mutagenesis in *Drosophila*. *G3 (Bethesda)* **4**: 2167–2173.



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*Cold Spring Harb Protoc*; doi: 10.1101/pdb.prot090779

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