



Cas9-Based Genome Editing in *Drosophila*

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Abstract

Our ability to modify the *Drosophila* genome has recently been revolutionized by the development of the CRISPR system. The simplicity and high efficiency of this system allows its widespread use for many different applications, greatly increasing the range of genome modification experiments that can be performed. Here, we first discuss some general design principles for genome engineering experiments in *Drosophila* and then present detailed protocols for the production of CRISPR reagents and screening strategies to detect successful genome modification events in both tissue culture cells and animals.



1. INTRODUCTION

The development of genome engineering technologies such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases

(TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR) has revolutionized our ability to modify endogenous genomic sequences in *Drosophila* both in cultured cells and *in vivo* (Bassett & Liu, 2014; Bassett, Tibbit, Ponting, & Liu, 2013, 2014; Beumer, Bhattacharyya, Bibikova, Trautman, & Carroll, 2006; Beumer & Carroll, 2014; Bottcher et al., 2014; Gratz et al., 2013; Gratz, Wildonger, Harrison, & O'Connor-Giles, 2013; Gratz et al., 2014; Kondo, 2014; Kondo & Ueda, 2013; Ren et al., 2013; Sebo, Lee, Peng, & Guo, 2014; Yu et al., 2013, 2014). ZFNs, TALENs, and CRISPR all function with similar mechanisms whereby a nonspecific nuclease is combined with a sequence-specific DNA binding element to generate a targeted double-strand break (DSB). The DSB is then repaired using either the non-homologous end joining (NHEJ) pathway or the homologous recombination (HR) pathway (Bibikova, Golic, Golic, & Carroll, 2002; Chapman, Taylor, & Boulton, 2012). The generation of a DSB in a coding region and repair by NHEJ can lead to small insertions or deletions (indel mutations) and therefore generate a knockout of a specific gene. In contrast to NHEJ, HR generally uses the homologous chromosome as a template and repairs the DSB with no sequence alterations. However, this mechanism can be exploited by including a donor construct with “arms” homologous to the target region. At some frequency, the donor will be used by the HR machinery as a template instead of the homologous chromosome, leading to a precise modification of the target site (Bottcher et al., 2014). Depending on the nature of the donor construct, this could be an insertion of exogenous sequence (e.g., GFP), introduction of a mutant allele, etc. Such insertions are generally referred to as knock-ins.

Although all three genome engineering technologies have been used successfully to produce genomic changes, CRISPR appears to function with considerably higher efficiency than ZFNs or TALENs (Beumer, Trautman, Christian, et al., 2013; Bibikova et al., 2002; Yu et al., 2013). Furthermore, generation of the required reagents is considerably simpler, making CRISPR the method of choice in most situations. The CRISPR system requires two components. The first is Cas9, a nonspecific nuclease protein, and the second is a single-guide RNA (sgRNA) molecule, which provides sequence specificity by base pairing with the target genomic sequence (Cong et al., 2013; Mali, Yang, et al., 2013). By altering the sequence of the sgRNA, highly specific DSBs can be generated at defined loci.

In order to take advantage of the CRISPR system in *Drosophila*, several factors must be considered and the approach taken must match the

experimental goals. For example, depending on the desired genomic modification (gene knockout, precise sequence modification, gene tagging, etc.), the sgRNA target site must be positioned differently. Furthermore, off-target effects, mutation efficiency, use of a donor construct, and method of reagent delivery must all be considered to achieve the intended result with high specificity and efficiency.



2. APPLICATIONS AND DESIGN CONSIDERATIONS FOR CRISPR-BASED GENOME EDITING

CRISPR can be used to generate a diverse range of genomic modifications including small random changes, insertions, deletions, and substitutions. In order to achieve the desired outcome, different approaches must be taken and several aspects of sgRNA design should be considered. Here we describe the most common applications and some general approaches to achieve them.

1. *Random mutations at a given target site:* In the absence of a donor construct, DSBs generated with CRISPR will be repaired primarily by NHEJ, leading to small indel mutations at the target site (Chapman et al., 2012). This approach is somewhat limited due to the lack of control over the mutations produced and the small region of sequence affected. Therefore, NHEJ is not the best approach for deletion of large regions of sequence or disruption of poorly characterized elements such as regulatory sequences. NHEJ is however very effective at generating frameshift mutations in coding sequences (Bibikova et al., 2002) and so is the approach of choice for gene disruption.

By targeting a sgRNA to the coding sequence of the gene of interest, frameshifts can be produced with high efficiency (Bassett et al., 2013; Cong et al., 2013; Gratz, Cummings, et al., 2013; Kondo & Ueda, 2013; Mali, Yang, et al., 2013; Ren et al., 2013; Sebo et al., 2014), leading to truncation of the encoded protein. An optimal sgRNA design for this application would target a genomic site close to the 5' end of the coding sequence and in an exon common to all transcripts in order to maximize the chance of ablating protein function.

2. *Insertion of exogenous sequences:* In contrast to knockout of a gene via frameshift-inducing indels, insertion of exogenous sequences, such as for generation of GFP-tagged proteins, requires precise sequence alteration. To achieve this, CRISPR must be used in combination with a donor construct. Donor constructs consist of the sequence to be inserted,

flanked on either side by “arms” with sequences homologous to the target site. Once a DSB has been generated, a subset will be repaired by HR using the donor construct as a template and therefore insert the exogenous sequence into the target site (Auer, Duroure, De Cian, Concordet, & Del Bene, 2014; Bassett et al., 2014; Dickinson, Ward, Reiner, & Goldstein, 2013; Gratz et al., 2014; Xue et al., 2014; Yang et al., 2013). For this application, it is unlikely that an sgRNA target site will be available exactly at the point of insertion so sequences should be selected as close to this as possible to maximize efficiency.

Longer homology arms have been associated with higher efficiency of HR but only to a certain extent and no further improvement is seen past ~ 1 kb (Beumer, Trautman, Mukherjee, & Carroll, 2013; Bottcher et al., 2014; Urnov et al., 2005). We therefore design all homology arms to be roughly 1 kb in length. In addition, knocking down the *ligase4* gene, a component of the NHEJ repair pathway, biases repair toward HR and can improve efficiency of insertion. This method has been shown to be effective at increasing the rate of HR both *in vivo* and in cultured cells (Beumer et al., 2008; Bottcher et al., 2014; Bozas, Beumer, Trautman, & Carroll, 2009; Gratz et al., 2014).

Note that for some applications, such as generation of a point mutant allele, it may be possible to use a single-stranded DNA oligo as the donor, avoiding the need to generate a longer donor construct. However, this approach is limited to very small insertions (Gratz, Cummings, et al., 2013).

3. *Specific deletions and substitutions*: Similar to insertions, the generation of a deletion or substitution requires precise sequence alteration and so a donor construct should be used in combination with CRISPR. To generate a deletion, homology arms should be designed flanking the sequence to be deleted with no additional sequences cloned in between. In this case, the sgRNA target site can be anywhere within the sequence to be deleted.

Substitutions are produced in a similar manner to insertions with a donor containing the sequence to be inserted flanked by homology arms. The difference in this case is that the homology arms induce recombination at sites that are not directly adjacent but separated by the sequence to be deleted. Again, the sgRNA target should be within the sequence to be replaced.

2.1. Selection of sgRNA target sites

One of the advantages of the CRISPR system over other existing genome editing technologies is the relative lack of sequence limitations in the targeted sites. The only requirement is the presence of a PAM sequence at the 3' end of the target site. For Cas9 derived from *Streptococcus pyogenes* (SpCas9), the optimal PAM sequence is NGG (or NAG although this leads to lower efficiency) (Jiang, Bikard, Cox, Zhang, & Marraffini, 2013), which occurs often throughout the *Drosophila* genome (every 10.4 bp on average). However, in some cases, such as modification of very precise regions, it may be difficult to find an appropriate PAM sequence. In this case, a more distant sgRNA target site can be used with an HR-based approach.

As described above, the target site of sgRNAs should be positioned based on the type of modification desired. Common to all of these approaches, a second consideration in the selection of a suitable sgRNA target site is the possibility of DSB generation at off-target sites. In mammalian systems, several reports suggest that off-target mutations may be a significant issue associated with the use of the CRISPR system (Fu et al., 2013; Hsu et al., 2013; Mali, Aach, et al., 2013; Pattanayak et al., 2013). Likely due in part to the lower complexity of the *Drosophila* genome, off-target events appear to be much less of a concern in this system (Ren et al., 2013). Indeed, no publications have yet reported detection of off-target effects. In addition, the presence of off-target sites may not be a problem for some applications. For example, when performing genome alterations *in vivo*, off-target mutations on nontarget chromosomes can be tolerated because they can be crossed out of the initial stock. In contrast, off-target events anywhere in the genome may be of concern in cultured cells.

Although it is clear from several studies that sgRNAs have widely varying efficiencies, little is currently known about the factors affecting efficiency. It is therefore difficult to predict how well a specific sgRNA will function prior to testing. For this reason, it is often sensible to test the efficiency of several sgRNAs targeting the region of interest in cell culture to determine which are the most likely to generate DSBs at high efficiency before making the investment of time and effort involved with *in vivo* genome engineering. Moreover, in some situations, it may be advantageous to use an sgRNA with lower efficiency, such as when a homozygous mutation is cell lethal. In such cases, sgRNAs with lower efficiency may result in higher recovery of mutant lines due to an increase in heterozygous compared to homozygous mutants.

2.2. Tools facilitating sgRNA design

To aid the design of sgRNAs, we recently developed an online tool allowing the user to browse all possible SpCas9 sgRNA targets in the *Drosophila* genome (Ren et al., 2013) (<http://www.flyrnai.org/crispr2>). sgRNAs can be filtered based on their genomic location (intron, CDS, UTR, intergenic, etc.), predicted off-target annotation with customizable stringency, and PAM sequence type (NGG or NAG). Individual target sites can then be selected from the genome browser interface to access more detailed annotation. This includes details of any potential off-target sites (genomic location, number and position of mismatches, etc.), whether the sgRNA sequence contains features that may prevent activity (such as a U6 terminator sequence), restriction enzyme sites that could be used to screen for mutations, and a score predicting the likely mutation efficiency at the intended target site. Using these predictions, we estimate that 97% of *Drosophila* protein-coding genes can be mutated with no predicted off-target mutations.

Several other tools are also available to facilitate sgRNA design (Mohr, Hu, Kim, Housden, & Perrimon, 2014) (Table 19.1). For example, targetFinder (Gratz et al., 2014) (<http://tools.flycrispr.molbio.wisc.edu/targetFinder/>) can be used to design sgRNAs for many different *Drosophila* species. With e-CRISP (Heigwer et al., 2014) (<http://www.e-crisp.org/E-CRISP>), a specific purpose such as gene knockout or protein tagging can be indicated to aid selection of the most appropriate sgRNA target sites.

Table 19.1 Tools for sgRNA design

Lab	Web site	Reference
DRSC	http://www.flyrnai.org/crispr/	Ren et al. (2013)
O'Connor-Giles	http://tools.flycrispr.molbio.wisc.edu/targetFinder/	Gratz et al. (2014)
DKFZ/Boutros	http://www.e-crisp.org/E-CRISP/designcrispr.html	Heigwer, Kerr, and Boutros (2014)
NIG-FLY/Ueda	www.shigen.nig.ac.jp/fly/nigfly/cas9/index.jsp	Kondo and Ueda (2013)
Center for Bioinformatics, PKU	http://cas9.cbi.pku.edu.cn/	Ma, Ye, Zheng, and Kong (2013)
Zhang	http://crispr.mit.edu/	Hsu et al. (2013)
Joung	http://zifit.partners.org/ZiFiT/	Hwang et al. (2013)



3. DELIVERY OF CRISPR COMPONENTS

To generate a genomic modification using the CRISPR system, it is vital that both Cas9 and one or more sgRNAs are delivered efficiently to the cells of interest (usually the germ line). Various methods have been developed to deliver these components and each is associated with advantages and disadvantages. One option is to generate RNA for both Cas9 and the sgRNA and directly inject these into embryos (Bassett et al., 2013; Yu et al., 2013). This approach is attractive because it does not require any cloning steps. It is also possible to inject purified Cas9 protein (Lee et al., 2014). However, compared to other delivery methods, injection of RNA or protein appears to lead to relatively low mutation rates (Bassett & Liu, 2014; Beumer & Carroll, 2014; Gratz, Wildonger, Harrison, & O'Connor-Giles, 2013; Lee et al., 2014).

An alternative approach is to use *Drosophila* stocks that express Cas9 in the germ line (Table 19.2). Several such stocks have been generated using either *vasa* or *nanos* regulatory sequences to drive SpCas9 expression specifically in the germ cells (Kondo & Ueda, 2013; Ren et al., 2013; Sebo et al., 2014; Xue et al., 2014). This offers the advantages of increased efficiency and that viability effects due to somatic mutations in the injected flies are unlikely, aiding the recovery of deleterious mutations through the germ line.

Using these lines means that Cas9 delivery is no longer a consideration, significantly reducing the effort required to prepare CRISPR components. Delivery of sgRNA into Cas9-expressing flies can be achieved using several different methods. As discussed above, the sgRNA can be generated *in vitro* and injected into Cas9-expressing embryos. Alternatively, the sgRNA can be encoded into an expression vector, usually containing a constitutive promoter such as U6, to drive expression of the RNA. While this requires the greater effort of cloning the sequence into a vector, it generally produces higher mutation efficiency than direct delivery of RNA (Kondo & Ueda, 2013; Ren et al., 2013). A final option is to generate fly lines expressing sgRNA, which can then be crossed to Cas9-expressing flies to generate mutant offspring (Kondo, 2014; Kondo & Ueda, 2013). This approach produces the highest efficiency but is a lengthy process due to the need to establish a new fly stock for every sgRNA.

We have found that the best compromise between mutation efficiency, effort, and time required is achieved by injecting an sgRNA expression plasmid into embryos expressing Cas9 in the germ line.

Table 19.2 CRISPR-related fly lines

Source	Genotype	Description	Reference
BDSC 51323	y1 M{vas-Cas9}ZH-2A w1118/ FM7c	Expresses Cas9 from vasa promoter	Gratz et al. (2014)
BDSC 51324	w1118; PBac{vas-Cas9} VK00027	Expresses Cas9 from vasa promoter	Gratz et al. (2014)
BDSC 55821	y1 M{vas-Cas9.RFP}ZH-2A w1118/FM7a, P{Tb1}FM7-A	Expresses Cas9 from vasa promoter, marked with RFP	Gratz et al. (2014)
BDSC 52669	y1 M{vas-Cas9.S}ZH-2A w1118	Expresses Cas9 from vasa promoter	Sebo et al. (2014)
BDSC 54590	y1 M{Act5C-Cas9.P}ZH-2A w*	Expresses Cas9 from Act5c promoter	CRISPR fly design project
BDSC 54591	y1 M{nos-Cas9.P}ZH-2A w*	Expresses Cas9 from nanos promoter	CRISPR fly design project
BDSC 54592	P{hsFLP}1, y1 w1118; P{UAS-Cas9.P}attP2	Expresses Cas9 from UAS promoter	CRISPR fly design project
BDSC 54593	P{hsFLP}1, y1 w1118; P{UAS-Cas9.P}attP2 P{GAL4::VP16-nos.UTR}CG6325MVD1	Expresses Cas9 from UAS promoter and Gal4 from nanos promoter	CRISPR fly design project
BDSC 54594	P{hsFLP}1, y1 w1118; P{UAS-Cas9.P}attP40	Expresses Cas9 from UAS promoter	CRISPR fly design project
BDSC 54595	w1118; P{UAS-Cas9.C}attP2	Expresses Cas9 from UAS promoter	CRISPR fly design project
BDSC 54596	w1118; P{UAS-Cas9.D10A}attP2	Expresses Cas9 (nickase) from UAS promoter	CRISPR fly design project
NIG-Fly CAS- 0001	y2 <i>cho2 v1</i> ; <i>attP40{nos-Cas9}/CyO</i>	Expresses Cas9 from nanos promoter	Kondo and Ueda (2013)

Table 19.2 CRISPR-related fly lines—cont'd

Source	Genotype	Description	Reference
NIG- Fly CAS- 0002	$\gamma 2 cho2 v1 P\{nos-Cas9, \gamma +, v +\}$ $1A/FM7c, KrGAL4 UAS-GFP$	Expresses Cas9 from nanos promoter	Kondo and Ueda (2013)
NIG- Fly CAS- 0003	$\gamma 2 cho2 v1; P\{nos-Cas9, \gamma +, v +\}$ $3A/TM6C, Sb Tb$	Expresses Cas9 from nanos promoter	Kondo and Ueda (2013)
NIG- Fly CAS- 0004	$\gamma 2 cho2 v1; Sp/CyO, P\{nos-Cas9, \gamma +, v +\}2A$	Expresses Cas9 from nanos promoter	Kondo and Ueda (2013)

For delivery of CRISPR components in cell culture, there are fewer options. As for *in vivo*, the components can be delivered either as *in vitro*-generated RNA or in expression plasmids. For example, an expression vector encoding SpCas9 and sgRNA that can be transfected into cultured cells was recently reported (Bassett et al., 2014) and we have developed a similar plasmid (pL018) (Housden et al., unpublished) (Table 19.3).

The major issue associated with genome engineering in cell lines is currently the inability to generate alterations with 100% efficiency. The rate of alterations is limited by both sgRNA efficiency and transfection efficiency, which is generally low in *Drosophila* cell lines (Bassett et al., 2014). A recent report introduced a selection cassette with the CRISPR components, which increased the mutation rate considerably (Bottcher et al., 2014). The persistence of wild-type sequences, however, results in selection against any unfavorable mutations (e.g., that slow growth) and, over time, reversion of the population to wild type. Until methods are developed to overcome these problems, the use of CRISPR in cell culture is limited to the generation of unstable, mixed populations.



4. GENERATION OF CRISPR REAGENTS

As discussed above, there are several methods available to deliver CRISPR components either *in vivo* or in cultured cells. Therefore, the procedures involved in generation of the relevant reagents will depend on the approach taken. Here we will focus on the generation of expression plasmids for delivery of sgRNA into Cas9-expressing flies or for transfection into cultured cells.

Table 19.3 Cas9 and sgRNA expression plasmids and donor vectors

Source	Plasmid name	Plasmid purpose	Reference
Perrimon lab	pL018	Expression of Cas9 (codon optimized) under Act5c promoter and sgRNA under fly U6 promoter	Unpublished
Addgene #49330	pAc-sgRNA-Cas9	Expression of sgRNA and Cas9-Puro in cell culture	Bassett et al. (2014)
Addgene #49408	pCFD1-dU6:1gRNA	Expression of sgRNA under control of the <i>Drosophila</i> U6:1 promoter	CRISPR fly design project
Addgene #49409	pCFD2-dU6:2gRNA	Expression of sgRNA under control of the <i>Drosophila</i> U6:2 promoter	CRISPR fly design project
Addgene #49410	pCFD3-U6:3gRNA	Expression of sgRNA under control of the <i>Drosophila</i> U6:3 promoter	CRISPR fly design project
Addgene #49411	pCFD4-U6:1_U6:3-tandemgRNAs	Expression of two sgRNAs from <i>Drosophila</i> U6:1 and U6:3 promoters	CRISPR fly design project
Addgene #45946	pU6-BbsI-chiRNA	Plasmid for expression of chiRNA under the control of the <i>Drosophila</i> snRNA:U6:96Ab promoter	Gratz, Cummings, et al. (2013)
Addgene #45945	pHsp70-Cas9	Expression of Cas9 (codon optimized) under control of Hsp70 promoter	Gratz, Cummings, et al. (2013)
Addgene #46294	pBS-Hsp70-Cas9	Expression of Cas9 (codon optimized) under control of Hsp70 promoter	Gratz, Cummings, et al. (2013)
NIG-Fly	pBFv-nosP-Cas9	Expression of Cas9 from the nanos promoter	Kondo and Ueda (2013)
NIG-Fly	pBFv-U6.2	sgRNA expression vector with attB	Kondo and Ueda (2013)
Perrimon lab	pBH-donor	Vector for generation of donor constructs	Unpublished

4.1. Cloning of sgRNAs into expression vectors

Few vectors are currently available for expression of sgRNAs. However, those that are available are generally compatible with similar cloning protocols using type II restriction enzymes (Table 19.3). For example, the procedure described below is based on one previously developed for

mammalian CRISPR vectors (Ran et al., 2013) but can be used with pL018 (Housden et al., unpublished), pU6-*BbsI*-chiRNA (Gratz, Cummings, et al., 2013), U6b-sgRNA-short (Ren et al., 2013), pAc-sgRNA-Cas9 (note that this plasmid is compatible with *BspQI* instead of *BbsI*) (Bassett et al., 2014), and pBFv-U6.2 (Kondo & Ueda, 2013) *Drosophila* plasmids. Here we will focus on pL018 but the procedure can be easily modified for other plasmids. When designing sgRNA oligos, be sure to include the relevant 4-bp overhangs to allow ligation into the digested vector and when using a U6 expression plasmid, also include an additional G at the start of the sgRNA sequence to initiate transcription.

Materials

- Complementary oligos carrying sgRNA target sequence (not including PAM)
- Suitable plasmid for the desired application
- *BbsI* restriction enzyme (Thermo Scientific)
- T4 ligase buffer (NEB)
- T4 PNK enzyme (NEB)
- T7 ligase and buffer (Enzymatics)
- FastDigest Buffer (Thermo Scientific)
- FastAP enzyme (Thermo Scientific)

Protocol

1. Set up a restriction digest reaction as shown below and incubate at 37 °C for 30 min:
 - 1 µg pL018 (or other suitable plasmid)
 - 2 µl 10 × FastDigest Buffer
 - 1 µl FastAP
 - 1 µl *BbsI* or other suitable enzyme
 - Water to 20 µl total volume
2. Purify reaction products using a PCR purification kit and normalize concentration to 10 ng/µl.
3. Resuspend sense and antisense sgRNA oligos to 100 µM and anneal using the following reaction mixture:
 - 1 µl 100 µM sense sgRNA oligo
 - 1 µl 100 µM antisense sgRNA oligo
 - 1 µl 10 × T4 ligation buffer
 - 0.5 µl T4 PNK (NEB)
 - 6.5 µl water

Note: In this step, use T4 ligation buffer with the PNK enzyme as this contains ATP required for phosphorylation of the annealed oligos.

Use a thermocycler with the following program to phosphorylate and anneal oligos:

- 37 °C—30 min
 - 95 °C—5 min
 - Ramp from 95 °C to 25 °C at 5 °C/min
4. Dilute the annealed oligos from step 3 by 200-fold using water (to 50 nM). If the concentration is too high, multiple copies may be inserted into the vector.
 5. Ligate annealed oligos into digested vector as follows:
 - 1 µl digested plasmid (from step 2)
 - 1 µl diluted annealed oligos (from step 4)
 - 5 µl 2 × Quick Ligase Buffer
 - 0.5 µl T7 ligase
 - 2.5 µl waterIncubate at room temperature for 5 min.

Note: While 5 min of ligation is generally sufficient, longer incubation periods may be used to increase colony numbers if necessary.

When using new vectors, we recommend performing negative controls in parallel using the same conditions but omitting the annealed oligos from the ligation reaction.

6. Transform 2 µl of ligation product into chemically competent *E. coli* using standard procedures and spread on LB plates containing ampicillin. Incubate the plates at 37 °C overnight.
7. Culture and miniprep single colonies and sequence to confirm successful cloning. In general, we have very high success rates for screening single colonies although more can be tested if necessary.

4.2. Cloning of donor constructs

As described above, single-stranded DNA oligos can be used as donors, making the insertion of small sequences very simple. However, due to the limit on the length of oligos that can be reliably generated, this approach can only be used to make small changes to the genome.

Production of a double-stranded donor construct requires the ligation of three or four components; two homology arms, an insert (for most but not all applications), and a backbone vector. These constructs can be produced using standard restriction digests followed by four-way ligation, although this approach is generally inefficient and time consuming. Instead, more

advanced cloning procedures can be used to generate the construct in a single step. Here, as an example, we describe a detailed protocol to produce a donor construct for insertion of an exogenous sequence using golden gate cloning (Engler, Kandzia, & Marillonnet, 2008; Engler & Marillonnet, 2014). Note that other approaches are also possible to generate these constructs, including Gibson assembly (Gibson et al., 2009).

When designing homology arms for golden gate cloning, it is important to ensure that the restriction enzymes used for construction of the donor plasmid do not cut within these sequences. To facilitate this, the donor vector we use (pBH-donor) is compatible with three type II restriction enzymes: *BsaI*, *BbsI*, and *BsmBI* (Housden et al., unpublished).

Type II restriction enzymes cut outside their recognition sequences. A single enzyme can therefore be used to generate multiple different sticky ends in a single digest reaction. Furthermore, the enzyme recognition sequence is cleaved from the DNA molecule to be cloned during the digest reaction. When the fragments are subsequently ligated together, the restriction enzyme recognition sites will not be present and so the molecule cannot be recut. If the small fragment cleaved from the end of the molecule religates, however, the restriction site will be restored and the molecule can therefore be redigested. Golden gate cloning works on the principle of cycling between digest and ligation conditions in the presence of both the restriction and ligation enzymes. Iterative rounds of digest and ligation therefore drive the accumulation of correctly ligated products even when multiple fragments are present. By including a backbone vector in the reaction, it is possible to transform the products directly without the need for additional cloning steps. Using this easily scalable approach, we generally obtain greater than 80% of constructs from a single reaction by screening only one resulting colony. Screening additional colonies increases the success rate to above 95%.

Materials

- High-fidelity polymerase (e.g., Phusion high-fidelity polymerase from NEB)
- Gel extraction kit (e.g., QIAGEN gel purification kit)
- 10 × BSA
- 10 mM ATP
- NEB buffer 4
- T7 ligase (Enzymatics)
- Type II restriction enzyme (e.g., *BsaI*, *BsmBI*, or *BbsI*)
- Thermal cycler

- Chemically competent bacteria
- Miniprep kit
- Restriction enzymes for test digest
- Oligos for sequencing
- pBH-donor or other suitable vector

Protocol

1. Design oligos for PCR amplification of two homology arms and insert fragment. These oligos should add type IIs restriction enzyme cut sites to the PCR products required for later cloning steps.
2. PCR amplify each of the homology arms using a high-fidelity polymerase.
3. Run PCR products on a gel to check the sizes of the bands. We recommend using 1 kb for all homology arms.
4. Gel purify homology arms from the gel using standard kits according to manufacturer's instructions.
5. PCR amplify insert sequence and gel purify if necessary (if using a short sequence, this can also be produced as complementary oligos annealed together).
6. Set up a golden gate reaction using the gel-purified homology arms, insert fragment and backbone vector:
 - 10 ng each homology arm
 - 10 ng donor vector
 - 10 ng insert fragment
 - 1 μ l 10 \times BSA
 - 1 μ l 10 mM ATP
 - 1 μ l NEB buffer 4
 - 0.5 μ l T7 ligase
 - 0.5 μ l type IIs restriction enzyme
 - Water to 10 μ l
7. Place samples in a thermal cycler and run the following program:
 1. 37 °C—2 min
 2. 20 °C—3 min
 3. Repeat steps 1 and 2 a further nine times
 4. 37 °C—2 min
 5. 95 °C—5 min
8. Transform 5 μ l of reaction product into chemically competent *E. coli* using standard procedures and spread onto a kanamycin plate. Incubate overnight at 37 °C.
9. Culture two of the resulting colonies overnight in selective media.

10. Miniprep samples using a standard kit according to manufacturer's instructions.
11. Send samples for sequencing using suitable primers for the plasmid used. For pBH-donor, sequence with the primer 5'-GAATCGCAGACCGATACCAG-3'.

Using this cloning approach, we generally find that a high proportion of clones carry the desired components, correctly assembled, and so screening one or two clones is sufficient.

As an alternative approach, it is not necessary to include the backbone vector in the golden gate reaction. The homology arms and insert can be assembled by golden gate cloning and then reamplified by PCR using a high-fidelity polymerase before cloning into a vector of choice using standard procedures. This can be a useful alternative approach if none of the restriction enzymes compatible with the donor vector are appropriate for the homology arms being generated.

4.3. Isolation of *in vivo* genome modifications

Once the relevant reagents have been generated and injected into fly embryos, the next stage is the identification and recovery of the desired genome modification events. As described below, there are various methods that can be used to detect modifications but the injected G0 flies must first be crossed to obtain nonmosaic animals before screening can be performed (Fig. 19.1). In order to do this, we generally cross G0 flies to a line with balancers on the chromosome of interest. The resulting F1 flies can then be collected and screened with one of the methods described below before re-crossing to the same balancer line to isolate the stock.



5. DETECTION OF MUTATIONS

Several methods are available to detect genome alterations induced using CRISPR. For most cases involving insertions, deletions or substitutions, customized methods must be used to detect the change. One option for insertions or substitutions is to include a visible marker such as miniwhite or $3 \times P3$ -dsRed in the inserted sequence. This then allows simple selection of flies carrying the desired modification (Gratz et al., 2014). However, in some cases it may be undesirable to insert the additional sequences associated with these markers, in which case PCR-based screening approaches may be more appropriate.

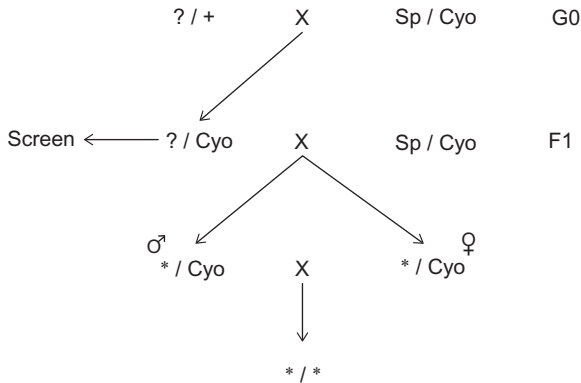


Figure 19.1 Crossing scheme to generate modified *Drosophila* lines. Following injection of CRISPR components into embryos, the modified chromosome must then be detected and isolated. This can be done by first crossing the injected G0 flies to a suitable balancer stock (second chromosome balancers are used as an example: e.g., *Sp/Cyo*) and isolating individual F1 flies for screening by restriction profiling, surveyor, HRMA, or other suitable approach. Screening can be performed using nonlethal methods and so flies carrying the desired modification (*) can then be recrossed to balancers to isolate the chromosome in a stable stock.

Detection of indels caused by NHEJ is more difficult due to the general lack of visible phenotypes and unreliable effects on PCR-based assays. Many mutations caused by NHEJ are very small (1-bp insertions or deletions are relatively common) (Cong et al., 2013; Mali, Yang, et al., 2013; Ren et al., 2013) and so will not necessarily affect amplification with an overlapping PCR primer. Therefore, alternative screening methods must be used.

Several methods are available, including restriction profiling, endonuclease assays and high-resolution melt assays (HRMAs) (Bassett et al., 2013; Cong et al., 2013; Wang et al., 2013). Each of these methods has advantages and disadvantages and should be chosen based on the number of samples to screen and the availability of suitable reagents. Note that following all of these screening methods, it is recommended to sequence the target site to confirm sequence alteration and determine the nature of the mutation.

5.1. Preparation of genomic DNA from fly wings

In order to screen flies prior to establishing stocks, it is possible to extract genomic DNA from a single wing for screening without killing the flies

(Carvalho, Ja, & Benzer, 2009). This allows selection of the correct F1 adults prior to crossing and therefore significantly reduces the workload compared to whole fly screening, which requires all crosses to be established first. All three of the screening methods detailed below require the preparation of genomic DNA. Note that for preparation of genomic DNA from cells, a similar protocol can be used in which the cells are resuspended in squishing buffer and lysed in a thermocycler as described.

Materials

- Squishing buffer (10 mM Tris-HCl pH 8.2, 1 mM EDTA, 25 mM NaCl, 400 µg/ml Proteinase K (add fresh from 50× stock stored at -20 °C))
- Blender or homogenizer pestles
- Thermocycler

Protocol

1. Remove one wing from fly (tear the wing close to the hinge but avoiding damage to the thorax) using forceps and place in a 1.5-ml microcentrifuge tube. The exact position of the tear can vary as long as the thorax is not damaged.
2. Add 20 µl of squishing buffer and homogenize well in the tube using a pestle or blender.
3. Transfer to a PCR tube and run the following program in a thermocycler:
 - 50 °C—1 h
 - 98 °C—10 min
 - 10 °C—hold

5.1.1 Restriction profiling

This screening approach relies on the disruption of a genomic restriction enzyme recognition sequence by an NHEJ-induced mutation. Particularly when generating gene knockouts, there are often several possible sgRNA targets that can be used, allowing selection of one that overlaps with such a restriction site. Note that these restriction sites are annotated in the DRSC sgRNA design tool described above. In order to detect mutations, a fragment surrounding the target site must first be amplified by PCR from the genomic DNA prepared as described above from F1 generation flies. Next, the PCR product is digested with the relevant restriction enzyme and visualized on a gel. Any alteration of wild-type sequence that disrupts the restriction site will change the band pattern produced, therefore indicating the presence of a mutation.

5.1.2 Surveyor assay to detect indels

It will often be the case that no suitable restriction sites are present at the sgRNA target locus for screening. One alternative possibility is to use endonuclease assays. These work by first amplifying a fragment from genomic DNA containing the sgRNA target site and then melting and reannealing to form homoduplexes and heteroduplexes between wild-type and mutant sequences. An endonuclease enzyme is then used that specifically cuts mismatches in the heteroduplexed molecules, resulting in a change in the band pattern when the samples are visualized on a gel (Fig. 19.2). We generally use SURVEYOR Mutation Detection Kit (Transgenomics) to detect indels generated by NHEJ, although other enzymes can also be used (e.g., T7 nuclease).

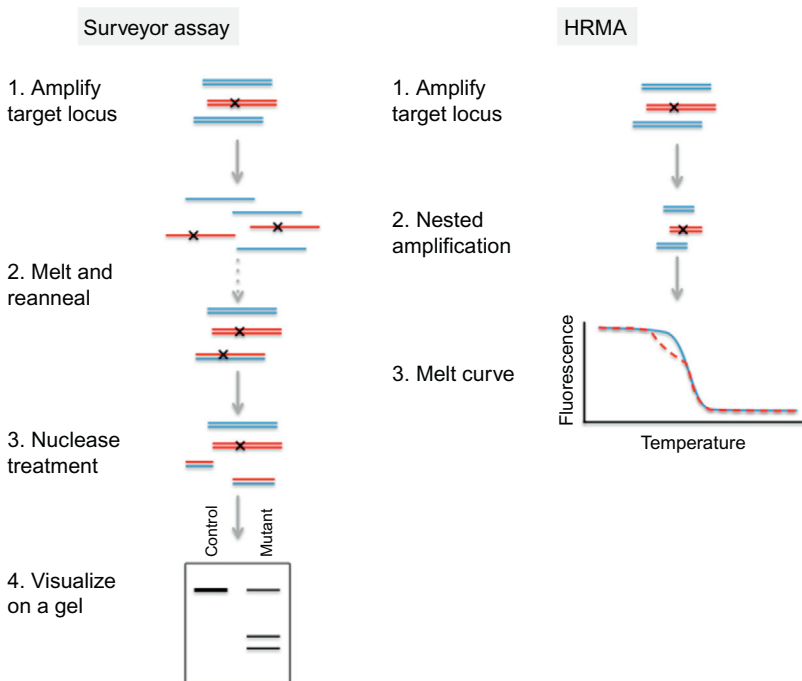


Figure 19.2 Surveyor and HRMA screening protocols. Surveyor assays rely on the ability of the surveyor endonuclease to cleave mismatched DNA strands. Melting and reannealing fragments amplified from a mixture of wild-type and mutant alleles lead to the formation of heteroduplexes, which are then cleaved by the surveyor enzyme to alter the band pattern when the products are visualized on a gel. HRMA measures differences in the melt curves between amplified fragments. These differences may be small depending on the sequence change and so specialized software may be required to detect mutated samples.

Materials

- Suitable primers for PCR amplification
- Standard PCR reagents including a high-fidelity polymerase enzyme
- PCR purification kit (e.g., QIAquick PCR purification kit from QIAGEN)
- Thermocycler
- Taq PCR buffer
- Surveyor mutation detection kit (Transgenomic)

Protocol

1. Extract genomic DNA as described above, either from whole flies or individual wings.
2. Design and optimize surveyor primers such that a single, strong band is produced by PCR and amplify fragments from genomic DNA using optimized PCR conditions and a high-fidelity polymerase. The optimal fragment length is around 500 bp as this allows reliable amplification and easy visualization of changes in band sizes following nuclease treatment.

It is important to use a high-fidelity polymerase for this step to prevent the introduction of sequence differences due to PCR errors. These would be detected by the surveyor nuclease and generate false-positive results.

3. Purify PCR products using a PCR purification kit and normalize to 20 ng/μl with water.

Note that primer optimization is a key factor and generating a specific PCR product is very important for surveyor success. Including a negative control consisting of unmutated genomic DNA is also recommended.

4. Melt and reanneal PCR products using the following conditions:

Reaction mixture:

2.5 μl 10 × Taq PCR buffer

22.5 μl 20 ng/μl purified PCR product

Place samples in a thermocycler and run the following program:

95 °C—10 min

Ramp from 95 °C to 85 °C (−2.0 °C/s)

85 °C—1 min

Ramp from 85 °C to 75 °C (−0.3 °C/s)

75 °C—1 min

Ramp from 75 °C to 65 °C (−0.3 °C/s)

65 °C—1 min

Ramp from 65 °C to 55 °C (−0.3 °C/s)

55 °C—1 min
 Ramp from 55 °C to 45 °C (−0.3 °C/s)
 45 °C—1 min
 Ramp from 45 °C to 35 °C (−0.3 °C/s)
 35 °C—1 min
 Ramp from 35 °C to 25 °C (−0.3 °C/s)
 25 °C—1 min
 4 °C—hold

5. Set up surveyor digest reactions as shown below and incubate for 30 min at 42 °C:
 - 25 µl annealed product from step 4
 - 3 µl 0.15 M MgCl₂
 - 1 µl SURVEYOR nuclease S
 - 1 µl SURVEYOR enhancer S

6. Add 2 µl Stop Solution from the kit and visualize on an agarose gel to detect mutations.

When screening cell-based samples, it can be useful to estimate the proportion of mutated alleles in the population. This can be done as follows:

1. Measure the integrated intensity of the uncleaved band A and cleaved bands B and C using ImageJ or other gel quantification software.
2. Calculate f_{cut} as: $f_{\text{cut}} = (B + C) / (A + B + C)$.
3. Estimate the indel occurrence by: $\text{indel}(\%) = 100 \times (1 - \sqrt{1 - f_{\text{cut}}})$

5.1.3 Detection of mutations using HRMA

A cost-effective and scalable approach to mutation detection is to use HRMAs (Bassett et al., 2013). Many companies sell RT-PCR machines with built-in modules for HRMA analysis but the reactions can be performed on almost any RT-PCR machine as long as a melt curve can be performed with fluorescence reads at intervals of 0.1 °C.

The principle of HRMA is that a small fragment is first amplified from the genomic locus potentially containing a mutation. This is then slowly melted and the amount of double-stranded DNA measured throughout the melting process. This produces a melt curve similar to those produced as quality controls in standard RT-PCR assays but with higher resolution. Changes in the sequence of the DNA fragments will alter the shape of this curve thereby allowing detection of samples containing mutations by comparison with curves generated from wild-type samples (Fig. 19.2). Such changes are often very small, especially when using samples containing many

different mutations, as is often the case in cell culture, so specialized software is required to detect them.

This assay is also more sensitive than the other methods, meaning that flies can be screened at the G0 generation and therefore reducing the amount of work required to isolate mutant lines. To generate genomic DNA from the G0 generation, it is recommended that crosses are set up to produce the F1 generation for all G0s and whole flies are used for DNA preps once it is clear that the crosses will produce progeny. Note that wing DNA preps cannot be used in this case because mutations are likely to be in the germ line.

Materials

- Standard PCR reagents including a high-fidelity polymerase enzyme
- Suitable primers for nested amplification
- Precision melt supermix (Bio-Rad) or other similar reaction mix
- RT-PCR machine with high-resolution melt ability

Protocol

1. Prepare genomic DNA as described above for wings or using 50–100 μl squishing buffer for whole flies.
2. PCR amplify a fragment (300–600 bp) around the sgRNA target site using the following reaction mixture and PCR program:

Reaction mixture:

- 2 μl DNA
- 10 μl buffer
- 1 μl dNTPs (25 mM each)
- 1 μl primers (10 μM each)
- 0.5 μl Phusion polymerase
- 1.25 μl MgCl_2 (100 mM)
- 34.25 μl water

PCR program:

- 98 °C—3 min
- 98 °C—30 s
- 50 °C—30 s
- 72 °C—30 s
- Goto step 2—34 times
- 10° C hold

3. Run 5 μl of reaction products on a gel to determine whether the correct size fragment has been produced. It is not necessary to obtain a specific product because nonspecific bands will not be reamplified in the following steps.

4. Dilute PCR product 1:10,000 using water.
5. Set up a nested PCR and melt assay in an RT-PCR machine as follows:

Reaction mixture:

- 1 μl DNA template
- 5 μl precision melt supermix
- 0.3 μl left primer (10 μM)
- 0.3 μl right primer (10 μM)
- 3.4 μl water

HRMA program:

- 95 °C—3 min
- 95 °C—18 s
- 50 °C—30 s

Fluorescence read

Repeat 50 times

- 95 °C—2 min
- 25 °C—2 min
- 4 °C—2 min

Melt curve from 55 °C to 95 °C with fluorescence reads every 0.1 °C

Even small amounts of nonspecific product can affect the results of the HRMA. The method described above uses nested PCR in order to avoid the need to optimize the original PCR. However, it is possible to skip the first PCR amplification step when highly specific primers can be designed.

5.2. Analysis of HRMA data

Many RT-PCR machines come with commercial HRMA analysis software, which can be used to identify samples carrying mutations following the manufacturer's instructions. However, if such software is not available, an online tool can be used. For example, we recently developed HRMAAnalyzer (<http://www.flyrnai.org/HRMA>) (Housden, Flockhart, & Perrimon, unpublished), which can be used to identify samples carrying mutations either using a clustering-based approach or via statistical comparison with control samples.

ACKNOWLEDGMENTS

We would like to thank David Doupé and Stephanie Mohr for helpful discussion in the preparation of this chapter. Work in the Perrimon lab is supported by the NIH and HHMI.

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