Protocol

Design and Generation of Donor Constructs for Genome Engineering in *Drosophila*

Benjamin E. Housden[1,3] and Norbert Perrimon[1,2,3]

[1]*Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115;* [2]*Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts 02115*

The generation of precise alterations to the genome using CRISPR requires the combination of CRISPR and a donor construct containing homology to the target site. A double-strand break is first generated at the target locus using CRISPR. It is then repaired using the endogenous homologous recombination (HR) pathway. When a donor construct is provided, it can be used as a template for HR repair and can therefore be exploited to introduce alterations in the genomic sequence with single base-pair precision. Here we describe a protocol for the generation of donor constructs using Golden Gate assembly and discuss some key considerations for donor construct design for use in *Drosophila*.

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

Agarose
Chemically competent *E. coli* and reagents for transformation
DNA sequencing primers and reagents
    BHF: 5′-GGGAAACGCCTGGTATCTTT-3′
    BHR: 5′-GCATTACGCTGACTTGAC-3′
dNTPs (10 mM) (e.g., New England BioLabs)
Gel purification kit (e.g., QIAGEN)
Genomic DNA template (from the genetic background to be engineered)
Golden Gate assembly reagents
    Adenosine triuphosphate (ATP) (10 mM) (e.g., New England BioLabs)
    Bovine serum albumin (BSA) (10×) (e.g., New England BioLabs)
    NEBuffer 4 (New England BioLabs)
        NEB CutSmart buffer can be used in place of NEBuffer 4 and BSA.
    pBH-donor vector DNA (available from authors on request)

[3]Correspondence: bhousden@genetics.med.harvard.edu; perrimon@receptor.med.harvard.edu

Cite this protocol as *Cold Spring Harb Protoc;* doi:10.1101/pdb.prot090787

T7 ligase (Enzymatics)
Type IIS restriction enzyme (BbsI, BsaI-HF, or BsmBI) (New England BioLabs)

High-fidelity polymerase enzyme and buffer (e.g., Phusion with 5× HF buffer; New England BioLabs)
LB liquid medium <R> with kanamycin for selection

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

Oligonucleotide primers to amplify homology arms (10 μM) (designed according to Steps 1–5)
Plasmid DNA miniprep kit (e.g., QIAGEN)
XbaI and XhoI, with CutSmart buffer (New England BioLabs)

Equipment

Incubator at 37°C
NanoDrop or Qubit for DNA quantification
Primer3 (http://bioinfo.ut.ee/primer3) or similar program for primer design
Thermocycler
UV gel imager

METHOD

Designing Homology Arms

The design of a donor construct is highly dependent on the relevant application, although in all cases two homology arms are required. If the intended application is the insertion of sequence into the genome or replacement of endogenous sequence with a transgene, then an insert component is also required in the donor construct. The most important consideration for donor design is the placement of homology arms, which is also dependent on the desired application. For insertions or single base-pair alterations, the two homology arms should be immediately adjacent to one another, whereas for deletions or replacement of endogenous sequence, the homology arms should flank the sequence to be removed. In all cases, the design of homology arms should be paired with the single guide RNA (sgRNA) design, described in Protocol: ***Design and Generation of* Drosophila *Single Guide RNA Expression Constructs*** *(Housden et al. 2016). Moreover, in cases where the homology arms are close together, the sgRNA should be as close to the join as possible. Wherever possible, the sgRNA target site should be disrupted by a successful editing event to avoid further modification. In addition, the sgRNA should not target either homology arm. When homology arms are separated (e.g., for deletions), the sgRNA can be placed anywhere within the intervening sequence.*

1. Search for sgRNAs as described in Protocol: **Design and Generation of *Drosophila* Single Guide RNA Expression Constructs** (Housden et al. 2016). Select an sgRNA that targets as close to the intended change in genomic sequence as possible.

   In cases where a large region is to be deleted or replaced, the sgRNA can target anywhere between the two homology arms. In addition, multiple sgRNAs can be expressed simultaneously within this region to increase the editing rate.

2. Design primers to amplify homology arms on either side of the target locus.

   After the optimal sgRNA design is selected and the genomic sequences upstream of as well as downstream from the sgRNA cutting site are retrieved, we recommend using Primer3 (http://bioinfo.ut.ee/primer3) or a similar program for primer design. Polymerase chain reaction (PCR) products should be 500–1200 bp in size. The sequences of both PCR products should be scanned for BbsI, BsaI, and BsmBI restriction enzyme recognition sites to determine the appropriate enzyme for Step 3. If the recognition sites of all the three enzymes are found within the PCR products, the PCR primers must be redesigned.

3. Select a suitable Type IIS restriction enzyme for the Golden Gate reaction: BbsI, BsaI, or BsmBI.

   Ensure that the selected restriction enzyme does not cut within any of the sequences to be assembled. Any suitable backbone vector can be used, but pBH donor was designed to be compatible with multiple different restriction enzymes (BbsI, BsaI, and BsmBI) and contains a ccdB *gene to facilitate the selection of correctly assembled clones. Note that optimal activity of BsmBI occurs at a higher temperature than BsaI or BbsI. This enzyme therefore often results in reduced efficiency using the Golden Gate reaction conditions described below, and BsaI or BbsI should be used preferentially whenever possible.*

4. Add the restriction enzyme binding sequences and overhangs for cloning to the primers designed in Step 2.

The target sequences for BbsI, BsaI, and BsmBI are GAAGACnnXXXX, GGTCTCnXXXX, and CGTCTCnXXXX, respectively. X indicates the four base pairs that will be used for cloning and must be complementary to those on the respective ligation partner. For example, when assembling two homology arms into pBH-donor using BsaI, the sequences added to each primer should be:

Left homology arm forward primer: GGTCTCnGACC-target-specific sequence
Left homology arm reverse primer: GGTCTCnGAAC-target-specific sequence
Right homology arm forward primer: GGTCTCnGTTC-target-specific sequence
Right homology arm reverse primer: GGTCTCnTATA-target-specific sequence

When assembling two homology arms plus an insert into pBH donor, the sequences should be:

Left homology arm forward primer: GGTCTCnGACC-target-specific sequence
Left homology arm reverse primer: GGTCTCnGAAC-target-specific sequence
Insert forward primer: GGTCTCnGTTC-target-specific sequence
Insert reverse primer: GGTCTCnGCCC-target-specific sequence
Right homology arm forward primer: GGTCTCnGGGC-target-specific sequence
Right homology arm reverse primer GGTCTCnTATA-target-specific sequence

5. Synthesize each set of primers and purify by standard desalting.

Generating Donor Constructs

6. Perform PCR to amplify each of the homology fragments (left arm and right arm) and insert, if applicable, using the primer sets from Step 5 as follows.

Reagent	Amount to add
Genomic DNA template	20 ng
5× Phusion HF buffer	10 µL
10 mM dNTPs	1 µL
Forward primer (according to fragment) (10 µM)	1 µL
Reverse primer (according to fragment) (10 µM)	1 µL
Phusion polymerase	0.5 µL

These are standard reaction conditions. Optimization for each fragment may be required.

7. Transfer the samples to a thermocycler and run the following program.

	Temperature	Time
Start	98°C	3 min
29 cycles	98°C	30 sec
	57°C	30 sec
	72°C	30 sec
Hold	10°C	

These are standard cycling conditions. Optimization for each fragment may be required.

8. Purify each fragment product using standard methods for agarose gel purification, and elute in H_2O.
9. Quantify the gel-purified products, and dilute to 20 ng/µL using H_2O.
10. Assemble the Golden Gate reaction as follows.

Reagent	Amount to add
pBH-donor vector	15 ng
Left homology arm	15 ng
Right homology arm	15 ng
Insert fragment (optional)	15 ng
10 mM ATP	1 µL
10× BSA	1 µL
NEB Buffer 4	1 µL
T7 ligase	0.5 µL
Type IIS restriction enzyme	0.5 µL
H_2O	to 10 µL

11. Transfer the samples to a thermocycler and run the following program.

Temperature	Time
37°C	2 min
20°C	3 min
Repeat cycle	14 times
37°C	30 min

12. Transform 5 µL of the reaction products into chemically competent *Escherichia coli* using standard methods. Spread the cells onto plates containing solid LB agar with kanamycin. Incubate overnight at 37°C.

13. Select four individual colonies for culture in LB liquid medium with kanamycin. Prepare plasmid DNA minipreps from each clone using standard methods.

    See Troubleshooting.

14. Assemble the following digestion reaction for each clone.

Reagent	Amount to add
Plasmid miniprep DNA	200 ng
XbaI	0.2 µL
XhoI	0.2 µL
Cutsmart buffer	1 µL
H_2O	to 10 µL

15. Incubate the reactions for 1 h at 37°C.

16. Visualize the digested products on an agarose gel. Calculate the expected band sizes based on the desired product and analyze the gel image to determine whether the correct construct has been produced.

    XbaI and XhoI cut the pBH-donor vector close to the joins with the homology arms. The digested products should therefore include a band at 1624 bp, indicating the presence of the pBH-donor vector, plus additional bands depending on the size and presence of relevant restriction sites in the homology arms or insert sequences.

    See Troubleshooting.

17. Sequence the clones that produced the correct banding patterns in Step 16 using the BHF and BHR sequencing primers.

    For long homology arms, or when an insert sequence is cloned between homology arms, additional sequencing primers will be needed to cover the whole construct.

18. Perform a local sequence alignment to a reference sequence or the experimentally determined sequence of the starting constructs.

TROUBLESHOOTING

Problem (Steps 13 or 16): No colonies are produced from the transformation, or a component is missing from Golden Gate product.

Solution: Golden Gate assembly performs optimally when all components are present in equimolar ratios. For simplicity, we generally calculate the amounts of each component using DNA mass (Step 10). However, depending on the molecular mass of each component, efficiency might be increased by calculating these amounts based on molar ratios. Repeat the assembly process including equimolar amounts of each component.

DISCUSSION

Repair of a CRISPR-induced double-strand break (DSB) using HR is an extremely powerful approach to genome engineering and allows almost any modification to the genomic sequence with single base-

pair precision. To achieve this, a donor construct must be provided that contains the modified sequence flanked by regions homologous to the target site, although the specific design will vary depending on the application. In addition to generating a desired modification to the genomic sequence, the use of a donor also allows the inclusion of a selection cassette, greatly easing the identification of successful editing events.

There are two main approaches to donor-based genome engineering that have been used successfully in *Drosophila* (Gratz et al. 2013, 2014; Bassett et al. 2014). A simple option is to generate a single-stranded oligo with short regions of homology (15–50 bp) flanking the DSB site. This option is attractive due to the ease with which these oligos can be generated, but it severely limits the length of sequence that can be inserted into the genome. It is therefore not possible to include a selection cassette using this approach. A second option is to produce a construct containing long homology regions (generally 500–1000 bp) on either side of the DSB site. This allows a much greater length of sequence to be included, but cloning these constructs requires considerably more work than producing a short oligo.

Including a selection cassette or visible marker by using long donor constructs can provide a significant advantage. Small indel mutations, such as those used to generate gene knockouts, generally require indirect molecular screening methods to identify correctly modified animals (Housden et al. 2014). By including a donor construct carrying a visible marker such as *mini-white* or 3XP3-RFP, successfully modified animals can be easily identified in the F_1 generation following injection (Gratz et al. 2014), considerably reducing the effort involved in generating a stable stock. Antibiotic resistance genes could be used in a similar manner in cell culture.

This protocol describes the use of Golden Gate assembly for the production of donor constructs; however, other methods, such as Gibson assembly, are also available (Gibson et al. 2009; Gibson 2011). These approaches are comparable in ease of use and robustness. We generally favor Golden Gate assembly because components can be provided either as linear fragments or intact plasmids. This greatly simplifies the cloning procedure when generating many donors in parallel involving some of the same components. For example, when generating donors for inserting green fluorescent protein (GFP) tags on multiple genes, the GFP can be provided as a plasmid without the need for processing before assembly.

RECIPE

LB Liquid Medium

In 1 L H_2O, 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

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.

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