

Supplementary Information

Experimental Protocols

I. Selection of candidate sgRNAs to test

Candidate single-guide RNAs (sgRNAs) can be identified using flyCRISPR Optimal Target Finder (<http://targetfinder.flycrispr.neuro.brown.edu>, Gratz. et al. 2014). The sgRNA target site should be as close to the intended site of modification as possible. A length of 20 nucleotides works well, and an initial 5' G or GG in the target site sequence is not necessary for in vitro transcription using T7 RNA polymerase. High stringency filtering is sufficient, and only NGG PAM sites should be utilized. Potential off-target sites should be minimized; zero predicted off-target sites is ideal.

The sgRNA target site sequence should be validated in the *Drosophila* strain or species genotype you plan to edit. This should be done by Sanger sequencing the putative site from the strain's genomic DNA. Since sequence polymorphisms are prevalent across the genome of various stocks, the *Drosophila* reference genome sequence should only be taken as a guide, and the stock of interest should be sequence verified.

II. In vitro transcription (IVT) of sgRNA

Make the sgRNA DNA template for IVT:

Perform a 50 µL PCR reaction using a proofreading polymerase (e.g. NEB Phusion HF DNA polymerase, New England Biolabs #M0530S) and the pU6-BbsI-chiRNA plasmid (Addgene #45946) as a template. Design PCR primers as follows to generate a ~120 bp PCR product:

sgRNA_R: AAAAGCACCGACTCGGTGCC (used in all reactions)

sgRNA_F: TTAATACGACTCACTATAGG[sgRNA_sequence_noPAM]GTTTTAGAGCTAGAAATAG
T7 promoter Annealing site

The T7 promoter sequence enables T7 RNA polymerase to initiate transcription. The Annealing site enables the primer to anneal to the plasmid template. Note that the PAM site is not included in the in vitro transcribed sgRNA.

Verify successful PCR amplification using an agarose gel - the product should be ~120 bp in length. Purify the PCR product using standard column purification (e.g. Qiaquick PCR Purification Kit, Qiagen #28106).

IVT:

Use ~300 ng purified PCR DNA in a 20 μ L MEGAscript in vitro transcription reaction (ThermoFisher #AM1333) supplemented with 0.5 μ L Ribolock RNase inhibitor (ThermoFisher #EO0381). Incubate reaction at 37°C overnight in a thermocycler with a heated lid. Purify reaction products using an RNA cleanup column and elute in 20 μ L nuclease-free dH₂O (e.g. New England Biolabs Monarch RNA Cleanup Kit #T2040L). Successful IVT should yield >40 μ g of RNA and should produce a large discrete band on an agarose gel.

III. Assembly of sgRNA-Cas9 RNPs

Mix together at room temperature to a final volume of 5 μ L:

- 1.19 μ L Cas9-NLS protein (IDT #1081058, 10 μ g/ μ L)
- 0.38 μ L 2M KCl
- 2.36 μ g sgRNA
- Nuclease-free dH₂O to 5 μ L

Incubate at room temperature for 10 min. Centrifuge in a microfuge at maximum speed for 10 min at room temperature. Transfer 4 μ L of supernatant into new tube to be loaded into injection needles. Store at room temperature. Prepare RNPs fresh for each day of injections.

IV. Determination of sgRNA Cleavage Efficiency in Embryos

Embryo injections:

Injections are performed in pre-cellularized embryos without dechoriation using Gompel and Schröder's method (<http://gompel.org/wp-content/uploads/2015/12/Drosophila-transformation-with-chorion.pdf>). Injection of 35-40 embryos per batch of sgRNA RNPs should be sufficient. Also perform injections of 35-40 embryos with Cas9-NLS protein only or mock-injections as a control. After injection, rupture any embryos that were skipped during injection due to age or other defects with a needle. Then remove as much of the halocarbon oil as possible from the coverslip. Place coverslip with embryos on an egg-laying plate. Keep plate in a humid chamber at 25°C overnight.

Genomic DNA extraction:

Injected embryos should be harvested ~24 hours post-injection. Use L1 larvae (preferable) or late-stage embryos, indicating survival of the injection process. Use the following single-fly genomic protocol (courtesy of Justin Kumar). Eight or more individuals should be sufficient to screen one sgRNA.

Squish buffer (10 mL): 9.8 mL dH₂O
100 µL 1M Tris pH 8.0
20 µL 0.5M EDTA
50 µL 5M NaCl

Before squishing, add 1 µL proteinase K (20 mg/mL) for every 100 µL of squish buffer.

- 1) Pick a single embryo/L1 larva with a pipet tip and grind it in 20 µL squish mix in a PCR tube. Pipette the mix several times. It is easy to lose the animal, so check under a dissecting scope to ensure that it is still inside the tube and ruptured (easier with larvae).
- 2) Incubate at 37°C for 30 min.
- 3) Incubate at 95°C for 5 min.
- 4) Store genomic preps at 4°C.

T7 endonuclease I assay:

The DNA substrate for the T7EI digestion should be ~700-1200nt in length. This is made by PCR from the genomic DNA of a single embryo. The sgRNA target site should be located close to the center of the amplicon. Verify before running the T7EI assay that you can obtain a single robust PCR product and that T7EI digestion does not produce bands that overlap with predicted cleavage products generated by a sgRNA. Even PCR substrates from uninjected control embryos can produce multiple digestion products after T7EI digestion. T7E1 is a bacteriophage enzyme that naturally recognizes and resolves Holliday junctions. It also cleaves a range of DNA structures including DNA mismatches. However, resolvases also cleave DNA if there are long tracts of A residues, meaning that they will generate background digestion products from DNA substrates if there are A-tracts or other unusual structures present.

1) *Synthesize the T7EI DNA substrate:* Perform a 50 µL PCR reaction for each individual L1/embryo using 3-5 µL genomic DNA as template.

2) *Generate heteroduplexes:* Take 10 µL of PCR reaction in step 1 and perform the following denaturation and re-annealing in a PCR machine:

95°C – 3 min.
94°C – 1 min.
93°C – 1 min.
92°C – 1 min.
... continue downwards in 1 degree increments to...
4°C – 1min.
6°C – 10 sec.
8°C – 10 sec.
10°C – 10 sec.

12°C – hold

3) *Perform T7EI digestion:* To 10 µL re-annealed PCR DNA, add:

2 µL 10X NEBuffer 2

0.2 µL T7 Endonuclease I (New England Biolabs #M0302L)

7.8 µL dH₂O.

Incubate at 37°C for 1 hour.

4) *Analysis of reaction:* Run in a 2% (w/v) agarose gel: 10 µL undigested PCR product side-by-side with 20 µL T7EI reaction products. Ethidium bromide and 0.5X TBE should be used to increase sensitivity to see faint digestion products. Samples with cleavage products at expected sizes from RNP-injected animals that are not present in mock-injected controls are indicative of sgRNA-guided cleavage. Bands may be very faint. It is not unusual for > 50% of individuals to have cleavage products for a good sgRNA.

V. Donor plasmid design

The CRISPR/Cas9 system can be used to introduce various modifications (e.g. protein tags, precise mutations) into the genome of *Drosophila* via homology-directed repair (HDR). To do so, a donor plasmid carrying the intended modification must be introduced into the embryo along with a sgRNA and Cas9-NLS.

The first step is to computationally assemble the donor plasmid using an informatics tool such as Benchling (<https://www.benchling.com>). The donor plasmid typically consists of 5 pieces:

- (1) backbone plasmid with a negative selection marker
- (2) modification of interest
- (3) positive transformation marker
- (4) left homology arm
- (5) right homology arm

(1) Backbone plasmid with negative selection marker

All the pieces necessary for genome editing via HDR need to be inserted via Gibson assembly into a plasmid with a negative selection marker that can be used to screen against integration of the entire plasmid into the genome. We use pBS-GMR-*eya*(shRNA), described in the paper (DGRC #1518). It carries a short hairpin RNAi agent against *eya* mRNA transcripts. Its transcription is driven by the eye-specific GMR enhancer. If it integrates into the *Drosophila* genome, it results in small eyes and can be used in any line with normal eye morphology. The pBS-GMR-*eya*(shRNA) plasmid is 3,845 bp and its annotated sequence is in Supplementary File 1.

The plasmid is linearized via restriction enzyme digestion before Gibson assembly. pBS-GMR-*eya*(shRNA) can be linearized with *EcoRV* (recognition sequence: GATATC), which is located in the multi-cloning site of pBluescript. Since *EcoRV* digestion generates blunt ended fragments, no nucleotides will be removed by the 5'→3' exonuclease activity of the Gibson assembly, and thus the assembled insert should be placed right at the cut site.

(2) Modification of interest

The modification of interest (MOI) should be placed as close to the sgRNA target site as possible to minimize the possibility of homologous recombination occurring between the sgRNA target site and the MOI. We have had success with the MOI located less than 30 bp from the sgRNA target site. Placement within the sgRNA site is ideal, as it will also inactivate the sgRNA site in the donor plasmid.

(3) Positive transformation marker

The 3xP3-DsRed marker gene is used to screen for positive integration of the intended modification via HDR. Note that 3xP3-DsRed fluorescence is only visible in a small number of ommatidia in a wildtype eye color background, making the fluorescence difficult though not impossible to observe.

The 3xP3-DsRed cassette from the pScarlessHD-DsRed plasmid (Addgene #64703) is flanked by piggyBac transposition sites (TTAA) that can be used to cleanly excise the entire marker gene after successful integration of the modification via HDR. After excision, the remaining genome sequence will be reduced to a single TTAA site. Thus, the scarless cassette should be placed either in a native TTAA site near the sgRNA site (ideally less than 30 bp) or within a TTAA site in the intended modification. Placement within a TTAA inside the sgRNA site is ideal, as it will also inactivate the sgRNA site in the donor plasmid.

(4) Left and right homology arms

For successful HDR, homology arms of native genomic sequence must be present on either side of the MOI and sgRNA target site. Lengths of ~1000 bp are standard. Lengths can be slightly increased or reduced to provide ideal sequences for Gibson assembly (e.g. moderate GC content and nonrepetitive sequence).

Important: If you cannot inactivate the sgRNA site in the donor plasmid either by inserting the MOI or scarless-DsRed cassette into the sgRNA core, then you need to mutate at least a single basepair in the PAM site or the sgRNA core of the donor plasmid.

Design of primers for Gibson assembly

Once an ideal donor plasmid is computationally designed, Gibson assembly can be used to assemble the necessary DNA fragments into the donor plasmid. DNA fragments can be generated in 3 ways:

- (1) PCR of genomic or plasmid template
- (2) Restriction digest of plasmid
- (3) Commercial de novo synthesis (e.g. IDT GBlocks)

In most cases, the backbone plasmid is generated via *EcoRV* digest, and the homology arms are PCR amplified from genomic DNA from the same *Drosophila* strain or species to be used for injections. The positive transformation marker is typically generated via PCR from pScarlessHD-DsRed plasmid DNA. The modification of interest can be generated either via PCR or de novo synthesis.

To design ideal primers to generate DNA fragments for Gibson assembly, use the NEBuilder tool <http://nebuilder.neb.com> with the following build settings:

Product Kit: NEBuilder HiFi DNA Assembly Master Mix

Minimum Overlap: 30 nt

Circularize: Yes

PCR Polymerase/Kit: Phusion High-Fidelity DNA Polymerase (HF Buffer)

PCR Primer Conc.: 500 nM

Min. Primer Length: 18

Max. Primer Length: 60 (not a build setting, but necessary for standard IDT order)

Try to alleviate flagged issues if possible, though not all issues can be resolved. For example, you cannot change the ends of the cut backbone plasmid, even if they are not ideal for Gibson assembly. Ends of homology arms can be slightly altered to improve Gibson overlap regions, and a synthesized gBlocks fragment can be altered to do the same.

Avoid placing repetitive regions like the very ends of the piggyBac transposition sites into Gibson overlap regions. Overlap regions can be slightly altered via junction properties in NEBuilder. For the scarless 3xP3-DsRed cassette, the 17 bp at both ends of the cassette are identical (5'-TTAACCCTAGAAAGATA-3') and thus should not be used in Gibson overlap regions. If synthesizing the MOI via gBlocks, one potential workaround is to extend the gBlocks fragment through the adjacent transposon end of the 3xP3-DsRed cassette to place the Gibson overlap region deeper into a nonrepetitive region of the cassette. We have verified that the following sequences within the 3xP3-DsRed cassette can be used as Gibson overlap regions:

piggyBac left (5') region: 5'-GTCGTTATAGTTCAAAATCAGTGACACTTA-3'

piggyBac right (3') region: 5'-AGATAATCATGCGTAAATTGACGCATGTG-3'

Once all primers are designed, verify that they all will bind in your computationally assembled donor plasmid.

VI. Construction of the donor plasmid via Gibson assembly

(1) Backbone plasmid with negative selection marker

Digest 5-10 µg of pBS-GMR-*eya*(shRNA) with EcoRV-HF (New England Biolabs #R3195S) at 37°C for 15 min. Digested product should be run on a 1% agarose gel, using multiple lanes to accommodate the large volume of digest. Bands of linearized plasmid should be quickly and carefully excised from gel, minimizing exposure to UV light, and purified using Monarch DNA Gel Extraction Kit (New England Biolabs #T1020S) to avoid contamination of Gibson assembly reactions with trace uncut plasmid.

(2) Scarless DsRed cassette

Perform a 50 µL PCR reaction using a proofreading polymerase (e.g. New England Biolabs Phusion HF) and 30-50 ng of pScarlessHD-DsRed plasmid as template (Addgene #64703). Touchdown PCR is recommended to reduce non-specific bands. The entire PCR product should be run on a 1% agarose gel, and the desired product should be gel extracted as above to avoid contamination of the Gibson assembly reaction with template plasmid. Similarly, gel extraction should be performed on any other PCR reaction that uses plasmid as template.

(3) Left and right homology arms

Perform a 50 µL touchdown PCR reaction for each homology arm using a proofreading polymerase (e.g. New England Biolabs Phusion HF) and 50 ng of genomic DNA from the same *Drosophila* strain or species that will be used for injections. If multiple bands are present, then purify via gel extraction. Otherwise, standard column purification is sufficient (e.g. Qiaquick PCR cleanup, Qiagen #28106).

(4) Synthesized DNA fragments

Any synthesized DNA fragment (e.g. IDT GBlocks) should be briefly centrifuged and resuspended in molecular grade dH₂O to a final concentration of 10 ng/µL. Incubate at 50°C for 15 minutes to facilitate better resuspension.

Assembly:

Empirically determine concentration of all fragments using a fluorometer (e.g. Qubit) or spectrophotometer (e.g. Nanodrop). For a 5 piece Gibson assembly reaction, fragments should be added in equimolar amounts, with total DNA content of the reaction not exceeding 0.5 pmol. The combined volume of DNA fragments should be 10 µL or less. Adjust volume using dH₂O. 0.08 - 0.1 pmol per fragment

works well. The accompanying Gibson assembly calculator can be used to determine appropriate volumes.

To perform the Gibson assembly reaction:

1. Mix all DNA fragments together. Combined volume should be less than 10 μ L. Add dH₂O to 10 μ L.
2. Mix 10 μ L of combined DNA fragments with 10 μ L NEBuilder HiFi DNA Master Mix (New England Biolabs #E2621). Mix well.
3. Incubate at 50°C for 1 hour in a thermocycler with heated lid.
4. Transform into competent *E. coli*. As even successful Gibson assembly reactions produce a small number of colonies, it is important to use *E. coli* with as high transformation efficiency as possible. Electrocompetent *E. coli* typically have higher efficiency than chemically competent *E. coli*.

A successful reaction will produce one to several hundred colonies. Performing a negative control reaction in parallel is useful to distinguish a successful low-yield reaction from non-specific colonies. Negative control reactions typically contain NEBuilder HiFi DNA Master Mix and only the backbone plasmid and scarless DsRed cassette fragments, as these are most likely to introduce contaminants. Individual colonies can be picked and screened via PCR for successful assembly across 1-2 junctions. Confirm correct assembly of the entire inserted region via Sanger sequencing. Polymorphisms in noncoding regions of homology arms are not uncommon, but ensure that there are no disabling mutations in the scarless DsRed cassette or coding regions of the homology arms.

Purification of the donor plasmid:

Purify the donor plasmid DNA for injection using the HiSpeed Plasmid Midi Kit (Qiagen #12643) with additional removal of endotoxins using two reagents from the EndoFree Plasmid Mega Kit (Buffer ER and Buffer QN, Qiagen #12381) to reduce toxicity in injected embryos. The Midi Kit is used as directed by the manufacturer with several modifications, as indicated in red below:

- (1) Pellet 50 mL of an overnight LB culture at 6000 x g for 15 min at 4°C.
- (2) Decant supernatant and resuspend pellet in 6 mL Buffer P1 with added RNase A by vortexing.
- (3) Add 6 mL Buffer P2 and mix well by inverting 4-6 times. Incubate at RT for 5 min.
- (4) During incubation, screw the cap onto the outlet nozzle of the QIAfilter Cartridge. Place the cartridge into a rack or fresh 50 mL conical tube.
- (5) Add 6 mL prechilled Buffer P3 to lysate and mix well by inverting 4-6 times.
- (6) Pour lysate into the QIAfilter Cartridge and incubate at RT for 10 min.
- (7) Remove the cap, insert the plunger, and filter the solution through the syringe filter into a fresh 50 mL conical tube.
- (8) Add 1 mL (EndoFree Mega) Buffer ER to the filtered solution and incubate on ice for 30 min.
- (9) During incubation, equilibrate a HiSpeed Tip with 4 mL Buffer QBT.

- (10) Apply the incubated solution from step 8 to the QBT-equilibrated HiSpeed Tip and allow to flow through.
- (11) Wash the HiSpeed Tip 2 x 10 mL with Buffer QC.
- (12) Place the HiSpeed Tip over a fresh 50 mL conical tube and elute by applying 5 mL (EndoFree Mega) Buffer QN.
- (13) Add 3.5 mL isopropanol to the eluted solution. Mix by inverting and incubate at RT for 5 min.
- (14) During incubation, remove the plunger from a 20 mL syringe and attach the QIAprecipitator Module onto the outlet nozzle.
- (15) Place the QIAprecipitator over a spare 50 mL conical tube. Transfer the eluate mixture into the syringe and insert the plunger. Filter the mixture through using constant pressure.
- (16) Remove the QIAprecipitator from the syringe and pull out the plunger. Re-attach the QIAprecipitator and add 2 mL 70% EtOH to the syringe. Insert the plunger and push the 70% EtOH through.
- (17) Remove the QIAprecipitator from the syringe and pull out the plunger. Attach the QIAprecipitator again and insert the plunger. Dry the membrane by pressing air through the QIAprecipitator. Repeat this step several times.
- (18) Dry the outlet nozzle of the QIAprecipitator with a Kimwipe.
- (19) Remove the plunger from a new 5 mL syringe, attach the QIAprecipitator and hold the outlet over a 1.5 mL collection tube. Add 1 mL Buffer TE to the syringe. Insert the plunger and elute the DNA into the collection tube using constant pressure.
- (20) Remove the QIAprecipitator from the 5 mL syringe and pull out the plunger. Re-attach the QIAprecipitator to the syringe.
- (21) Transfer the eluate from step 19 to the 5 mL syringe and elute for a second time into the same 1.5 mL tube.

This final elution should be performed using TE buffer to maximize recovery of the plasmid DNA. However, TE buffer is not appropriate for injections, and the donor plasmid needs to be concentrated before injection. Perform an ethanol precipitation as follows:

- (1) Estimate volume of DNA solution and add 1/10 volume of 3M sodium acetate pH 5.2. Mix well.
- (2) Add 3 volumes of 100% molecular-grade ethanol.
- (3) Incubate at -80°C for 30 minutes.
- (4) Spin at max speed for 15 minutes at 4°C. Split into multiple 1.5 mL tubes if necessary.
- (5) Remove supernatant and wash pellet twice in 800 µL of 70% ethanol.
- (6) After final wash, remove supernatant and allow to air-dry at RT 5-10 minutes.
- (7) Resuspend in 40 µL nuclease-free dH₂O.
- (8) Measure concentration using NanoDrop or Qubit. Final concentration should be ~240 nM or higher.

VII. Injection and screening of transformants

Injections are performed in pre-cellularized embryos without dechoriation using Gompel and Schröder's method (<http://gompel.org/wp-content/uploads/2015/12/Drosophila-transformation-with-chorion.pdf>). Injections should be performed in the same *Drosophila* strain or species used for sgRNA prescreening.

Mix together at room temperature:

- 1.19 μ L Cas9-NLS protein (IDT #1081058, 10 μ g/ μ L)
- 0.38 μ L 2M KCl
- 2.36 μ g IVT sgRNA
- 0.60 pmoles donor plasmid DNA
- Nuclease-free dH₂O to 5 μ L final volume

Incubate at room temperature for 10 min. Centrifuge in a microfuge at maximum speed for 10 min at room temperature. Transfer 4 μ L of supernatant into new tube to be loaded into injection needles. Store at room temperature. Prepare RNPs fresh for each day of injections.

Injection of 300-350 embryos is typically sufficient to obtain at least 1 germline transformant. After injection, remove as much oil as possible and place coverslip with injected G0 embryos in a standard food vial. Keep the vial in a humid chamber at 25°C overnight.

Once G0 adults eclose, they should be individually crossed to healthy virgins or males from a wild-type or appropriate balancer line. We typically use *w¹¹¹⁸* flies for injections and the initial cross in order to maintain a consistent genetic background. G1 adults are screened for the expression of 3xP3-DsRed and the absence of *eya(shRNA)* phenotypes. Positive G1 adults typically contain the desired edit and should be individually crossed to an appropriate balancer. Note that you might obtain multiple positive G1 adults from the same G0 parent. These may or may not be independent genome modifications. However, you can be confident that G1 adults taken from different G0 parents will have independent edits.

Once lines are established and stable, verification of the anticipated editing/modification needs to be done by PCR analysis and Sanger sequencing. Errors do occur.

VIII. Removal of the DsRed marker with piggyBac transposase

To precisely excise the 3xP3-DsRed marker cassette and achieve scarless genome editing, set up the following crosses:

- (P) Cross DsRed+ lines to flies expressing the piggyBac transposase. Bloomington stock 32070 contains a piggyBac transposase transgene under control of the α -*tubulin* promoter and tightly linked to a 3XP3-CFP transgenic marker. This is

located on chromosome 2. The stock also contains 3rd chromosome balancers (MKRS/TM6B,Tb), facilitating tracking of the 3rd chromosomes independent of the piggyBac transposase. If DsRed is on the X chromosome, cross virgin DsRed⁺ females to males of the piggyBac transposase line.

- (F₁) If your 3xP3-DsRed gene is on the X chromosome, select several DsRed⁺/CFP⁺ males. If your 3xP3-DsRed gene is on an autosome, select several DsRed⁺/CFP⁺ males. Cross males to 10-20 virgin females with an appropriate balancer. The piggyBac transposase is only weakly efficient, so DsRed⁺ should still be visible albeit mosaic in F₁ flies.
- (F₂) If the DsRed was on an autosome, select single flies that have the appropriate balancer chromosome and are both DsRed⁻ and CFP⁻ and cross again to an appropriate balancer to make a balanced stock. If the DsRed was on the X chromosome, select single female flies that are both DsRed⁻ and CFP⁻, and cross to males from an appropriate balancer line to make a balanced stock. Removal of DsRed typically occurs 10% of the time or less, so make sure crosses are large enough to produce hundreds of F₂ progeny to screen through.

If the genome editing has been performed on a species other than *D. melanogaster*, it will be necessary to inject the 3xP3-DsRed lines with a plasmid vector containing the piggyBac transposase gene under alpha-tubulin promoter control. This plasmid is commercially available (Drosophila Genome Resources Center #1155). Injections can be performed in pre-cellularized embryos without dechoriation using Gompel and Schröder's method (<http://gompel.org/wp-content/uploads/2015/12/Drosophila-transformation-with-chorion.pdf>). Injections should be performed using a concentration of 0.6 mg/mL plasmid DNA dissolved in 0.1 mM Sodium Phosphate pH 7.8 + 5 mM KCl. Cross individual G₀ adults to an appropriate strain and screen G₁ adult offspring for the absence of DsRed eye fluorescence. Since the pBac transposase plasmid vector requires active P element transposase to integrate into an injected embryo's genome, there should be no retention of the transposase gene in G₁ adults.

To ensure that the genome edit is still present after scarless excision, verify via PCR analysis and Sanger sequencing.

References

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