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Protocol for generating *D. mojavensis* knockout flies as in:

Mate discrimination among subspecies through a conserved olfactory pathway

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Generation of *Drosophila mojavensis* loss-of-function alleles using CRISPR/Cas9

The protocol listed below was used to generate loss-of-function alleles of the *Or65a* gene in *Drosophila mojavenis wrigleyi* with a set of two *Or65a* specific sgRNAs. To pre-select individuals with potential genome editing events, two additional sgRNAs targeting the *white* gene were injected. All four sgRNAs were delivered as RNA oligonucleotides in combination with Cas9 protein (both Synthego**,** sgRNAs carried a 2'-O-methyl 3' phosphorothioate modification in the first and last three nucleotides for increased *in vivo* stability).

Drosophila mojavensis **microinjections**

Transgenesis was performed following a protocol by the Gompel lab (http://gompel.org/methods) with a few *D. mojavensis wrigleyi* specific modifications:

1. Amplify *D. mojavensis wrigleyi* flies on standard fly food.

2. Prepare egg laying agar plates (1% agar, 20% fruit juice – either grape or apple). Plates can be stored at 4°C for up to 1 month and should be transferred to room temperature prior to injections.

3. Set up a laying pot with roughly 200, 10 day-old *D. mojavensis wrigleyi* flies (both sexes, sexual maturation in *D. mojavensis* takes ~6 days) 1-2 days prior to injections.

4. Pull micropipettes from glass capillaries (1.0 mm OD borosilicate, with filaments, Sutter instruments #BF100-50-10) using a Sutter P-97 micropipette puller (example parameters which have to be adapted: Heat = 510; Pull = 20; Velocity = 55; Time= 250).

5. On the day of injections, soak a few grams of Formula 4-24® Instant *Drosophila* Medium, Blue (Carolina Biological Supply Company) in water, add a spatula on top of fresh egg laying plates, add yeast powder and place this plate on the laying pot. Renew plates 30 min prior to injections.

6. Mix the individual sgRNAs with Cas9 protein (1.5:1) in a 0.2 ml tube and incubate the mixes for 10 min at room temperature. Combine all four sgRNA/Cas9 mixes for injections (final concentrations: 3 µM each sgRNA, 2 µM Cas9) and keep the injection mix on ice. All mixes (final and individual sgRNAs/Cas9) can be stored for up to 1 week at 4°C.

7. Fill the micropipettes with the injection mix via capillary motion.

8. Replace the egg laying agar plate, collect embryos with a paintbrush and wash them briefly in water. Avoid leaving embryos in water for too long as, in our hands, this affects the survival of *D. mojavensis*.

9. Align embryos on a cover slip as described (http://gompel.org/methods), discard embryos of later developmental stages (stage 3 and older), and let the chorion dry for attachment to the cover slip.

10. Cover the embryos with olive oil and perform injections. *D. mojavensis* are smaller than *D. melanogaster* embryos and the relative injection volume has to be adjusted accordingly.

11. Wash off olive oil with 70% ethanol and water after injections and place the cover slip into a new food vial. Injected *D. mojavensis wrigleyi* embryos usually exhibit a developmental delay and will take longer to hatch compared to non-injected wildtype embryos.

12. After hatching, cross individual injected flies with wildtype flies and visually screen progeny (F1) for the lack of eye pigmentation. Note that the *white* gene is located on the X chromosome – only male progeny of injected female flies will display a visible phenotype. The progeny of injected male flies can be in-crossed for another generation prior to visual screening.

Note: To identify a loss-of-function allele at the *Or65a* locus, we injected roughly 200 embryos resulting in 40 viable flies. Of these, 18 flies produced sufficient progeny for visual screening and we identified white eyed individuals in 3 crosses in the F1

generation. Via PCR screening (below) we could identify 2 genome editing events at the *Or65a* locus and recover 2 loss-of-function alleles. We focused on one where the *Or65a*$^{-/-}$ animals carry a 2 bp deletion (see figure below).

PCR based screening for loss-of-function mutations

1. If *white* mutant males are recovered within G0 progeny (F1), cross individual males to wildtype virgins (a mutant male is preferred to set up the breeding as it mates with multiple females and hence produces more offspring).

2. As soon as virgins are mated and eggs and larvae are observed, extract genomic DNA from a single wing of the male F1 fly (see non-lethal genotyping section for more details) using the MyTaq™ Extract-PCR Kit (Bioline Cat No: BIO-21126).

3. PCR amplify the sgRNA-target site of choice and confirm genome editing events via sequencing. The sequencing result of this heterozygous individual typically displays several double-peaks in the sequencing chromatogram 3-4 nucleotides upstream of the protospacer adjacent motif (PAM) sequence***** (see figure below). If a desired loss-of-function allele is detected in the male F1 fly, 50% of its progeny (F2) should be heterozygous mutants.

4. Perform individual sibling matings of the F2 progeny.

5. Once eggs and larvae are observed, PCR amplify the target site of F2 parental flies. If both parents are genotyped as heterozygote carriers of the loss-of-function allele, 25% of their progeny (F3) should be homozygous mutants.

6. Perform individual brother-sister matings of the F3 progeny followed by genotyping and establish a homozygote loss-of-function stock.

***Note:** Sequencing results could be compared to the reference sequence using TIDE (https://tide.nki.nl/) to deconvolute sequencing traces.

Non-lethal genomic DNA extraction on fly wings

1. Add 3.5 µl double-distilled water (ddH2O) into a 0.2 ml PCR tube.

2. Anesthetize a fly and clip one wing near its base with a razor blade.

3. Submerge the wing in the ddH2O in the PCR tube and ensure that the wing stays in the bottom of the PCR tube.

4. Transfer the PCR tube to -80°C for 10 min (this helps in homogenizing the tissue afterwards and hence increases DNA yield).

5. Add 1 µl Buffer A and 0.5 µl Buffer B of the MyTaq™ Extract-PCR Kit (Cat. No.: BIO-21126, bioline) to the PCR tube.

6. Incubate the sample at 75°C for 5 min before inactivating the reaction by heating to 95°C for 10 min.

7. Spin down the debris and take the supernatant for PCR amplification.

8. Design a set of target specific primers (in our case Dmoj_65F and Dmoj_65R, amplicon size: 175bp) and amplify the sgRNA-target site using the following reaction mix and a standard PCR protocol:

10 µl	5X Q5 Reaction Buffer
10 µl	5X Q5 High GC Enhancer
1 µl	10 mM dNTPs
2 µl	Genomic DNA extraction
2 µl	Fwd primer
2 µl	Rev primer
22.5 µl	Nuclease-Free Water
0.5 µl	Q5 DNA polymerase
50 µl	

9. Determine amplicon sequence via Sanger sequencing.

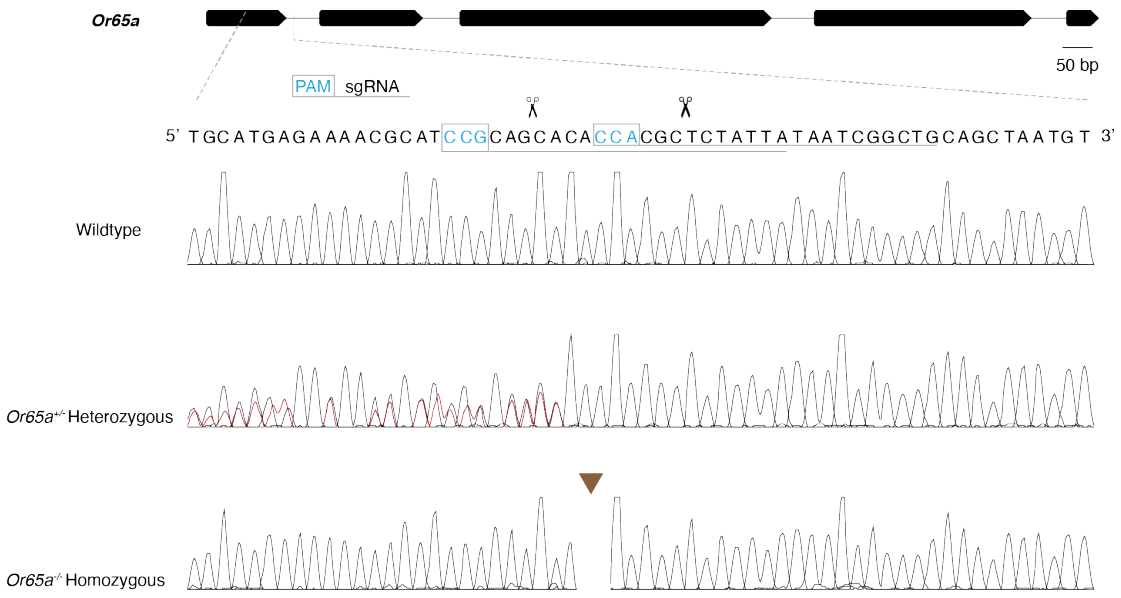

Figure legend: Schematic drawing of the structure of the *Or65a* gene illustrating the strategy for generating knockouts using CRISPR-Cas9. The two guide RNA and PAM sequences (in blue) are shown; scissors denote the cutting positions. Heterozygous flies typically display several double-peaks in the sequencing chromatogram 3-4 nucleotides upstream of the PAM sequence. The *Or65a*$^{-/-}$ mutants shown here carry a 2 bp deletion, resulting in a frame-shift and loss-of-function.