

## Supplementary Information for

Behavior of homing endonuclease gene drives targeting genes required for viability or female fertility with multiplexed guide RNAs

Georg Oberhofer, Tobin Ivy, Bruce A. Hay

Corresponding author: Bruce A. Hay

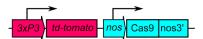
Email: haybruce@caltech.edu

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Figures. S1 to S3



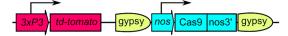
1. White marker excised with Apal and replaced by 3xP3-tomato (primer 17 + 18)



2. Cut with NheI, gypsy insulator inserted (primer 19 +20)



3. Cut with SacII, second gypsy insulator inserted (primer 21 + 22)

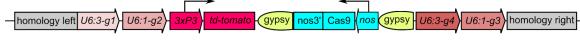


4. Cut with Sapl and Nsil and inversion of insulated nos-Cas9 cassette (primer 23 + 24)

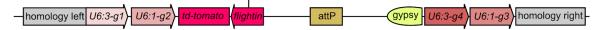
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5. Cut with SnaBI, insertion of left homolgy arm (*yellowG*-HEG: primer 25 + 26, *deformed*-HEG: primer 33 + 34) and two guides (*yellowG*-HEG:primer 27 + 28, *deformed*-HEG: primer 35 + 36)

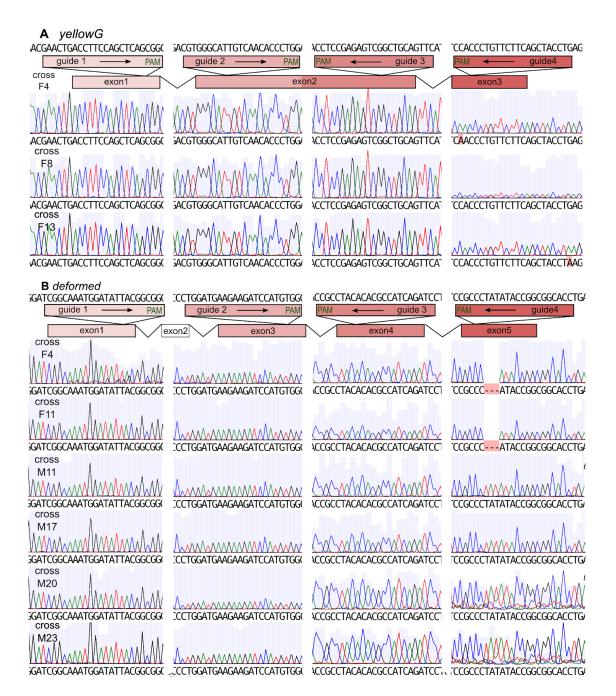
6. Cut with Apal, insertion of right homology arm (*yellowG*-HEG: primer 31 + 32, *deformed*-HEG: primer 39 + 40) and two guides (*yellowG*-HEG: primer 29 + 30, *deformed*-HEG: primer 37 + 38)



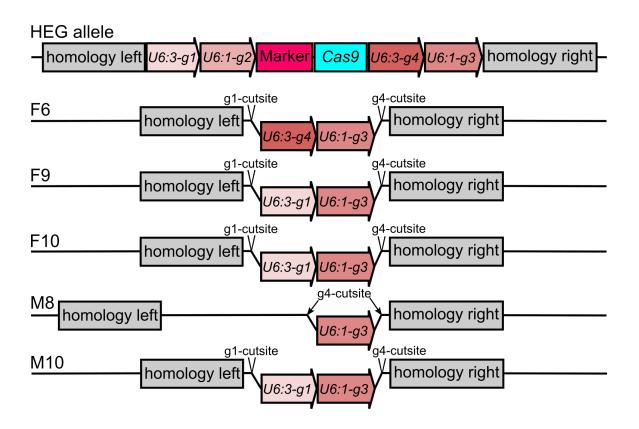
7. Cut with HindIII, insertion of *flightin-td-tomato* and attP landing pad (*td-tomato*: primer 41+42, *flightin* promoter: primer 43+44, attP: primer 45+46)



**Fig. S1. Cloning strategy for HEG and split-HEG constructs.** Step by step assembly guide listing used enzymes and primers. See Material and Methods section for details.



**Fig. S2. gRNA target site sequences of escapers.** Chromatograms from one fly of each cross containing escapers is shown. All flies were of the +/Df genotype **(A) yg-HEG Escapers.** gRNA4 target sequence had a mutated PAM site in progeny of cross F4. Progeny from cross F13 had a mutation in the distal most base of the PAM. All other target sites did not show mutations. **(B)** *Dfd*-HEG Escapers. Progeny coming from cross F4 and F11 had a 3bp deletion in the target sequence. All other target sites were intact.



**Fig. S3: Incomplete homing events.** Shown are details of the incomplete homing events observed in Fig. 3. In all cases a likely recombination event between repetitive sequences within the left and right gRNA cassettes eliminated Cas9 and the marker from the construct.