***Gateya* 4th chromosome balancer**

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**Description:**

*Gateya* (also known as *Gat[eya]*) is recessive lethal mutation in the *Gat* gene (CG1732) that is marked by the *GMR-eya(shRNA)* construct that causes a dominant, 100% penetrant, small eye phenotype (Nyberg et al., 2020) similar to the *Drop* (*Dr*) mutation. Notably, enough eye tissue remains in the *Gateya*/+ flies that *w*+ or fluorescent eye markers can be scored in heterozygotes.

The *Gateya* chromosome serves as a 4th chromosome balancer because there is essentially no recombination on the fourth chromosome, the *Gateya* mutation causes a dominant scorable phenotype and is a recessive lethal mutaton (1,435/1,435 adults in a stock of *Gateya*/*CrkdsRed* chromosome were dsRed, small eyed *Gateya*/*CrkdsRed* heterozygotes).

The *Gateya* mutation was created by targeting the *Gat* locus with CRISPR using the two sgRNAs detailed below and an HDR template constructed by Gibson assembly of the Scarless 3xP3 dsRed marker (Addgene #64703, <https://flycrispr.org/scarless-gene-editing/>, (Bier et al., 2018)) flanked by *Gat* 5’ and 3’ homology arms (see below) in the pBS*-GMR-eya*(shRNA) backbone (Addgene #78356, (Nyberg et al., 2020)). Homologous repair from this template should result in deletion of all but the first 10 amino acids of the *Gat* open reading frame. The *GMR-eya(shRNA)* served as a marker for screening for aberrant repair events that resulted in integration of the vector instead of homologous repair from the HDR template. Of three dsRed positive lines established from embryos (BDSC stock #55821 y[1] M{GFP[E.3xP3]=vas-Cas9.RFP-}ZH-2A w[1118]) injected with the *Gat*/Scarless dsRed HDR template and two *Gat* sgRNAs, two were repair events (dsRed positive, WT eyes) and one was an integration event (dsRed, small eye). The line resulting from the integration event was crossed to a Piggyback transposase line (BDSC stock #32070 w[1118]; Herm{3xP3-ECFP, alphatub-piggyBacK10}M6; MKRS/TM6B, Tb[1]) to excise the dsRed transformation marker. A small-eyed non-dsRed line was established from a single male which founded the *Gateya* chromosome. Neither the nature of the original insertional event nor the molecular nature of the *Gateya* mutation produced by the dsRed excision has been investigated. The *vas-Cas9*, piggyBac transposase, other markers and potential off-target mutations were removed from the background by backcrossing the *Gateya* allele against *w1118* for five generations.

**Methods for construction of *Gat* HDR Repair template and sgRNA plasmids**

*Gat* HDR template

Assembled order of the circular *Gat*/Scarless dsRed HDR template construct:

*pBS eya(shRNA)* backbone…*Gat* 5’ homology…Scarless dsRed marker…*Gat* 3’ homology…*pBS* *eya(shRNA)* backbone

The sequences of the primers used to create that *Gat* targeting construct were (*Gat* sequences underlined):

Gat 5’ homology arm forward: 5’ – CCGGGCTGCAGGAATTCGATCAGGATCAATAGCCAAGTCGATCT – 3’

Gat 5’ homology arm reverse: 5’ – CTTTAACGTACGTCACAATATGATTATCTTTCTAGGGTTAAGTCACCATCGCTTGCGGA – 3’

Gat 3’ homology arm forward:   
5’ – GAGCAATATTTCAAGAATGCATGCGTCAATTTTACGCAGACTATCTTTCTAGGGTTAAAGTGGTATGCCAGAAATATCTAG – 3’

Gat 3’ homology arm reverse: 5’ – CGACGGTATCGATAAGCTTGATCATATTCACTCTTGTGAATAGACAC – 3’

Primers for amplifying the Scarless dsRed marker:

Scarless dsRed forward: 5’ – ATATTGTGACGTACGTTAAAGAT – 3’

Scarless dsRed reverse: 5’ – GCATTCTTGAAATATTGCTCTCT – 3’

*Gat* sgRNA plasmids

The following oligonucleotides were annealed and ligated into pU6-BbsI-chiRNA (Addgene #45946; RRID:Addgene\_45946) as describe in <https://flycrispr.org/scarless-gene-editing> to create two plasmids that produce sgRNAs targeting the 5’ and 3’ regions of the *Gat* gene:

*Gat* sgRNA1 forward: 5'- CTTCGCCGCAAGCGATGGTGACGG-3'

*Gat* sgRNA1 reverse: 5'- AAACCCGTCACCATCGCTTGCGGC -3'

*Gat* sgRNA2 forward 5'- CTTCGTTGTCGTACTTACTTAAAG-3'

*Gat* sgRNA2 Reverse 5'- AAACCTTTAAGTAAGTACGACAAC-3'

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**References:**

Bier, E., Harrison, M.M., O'Connor-Giles, K.M., Wildonger, J., 2018. Advances in Engineering the Fly Genome with the CRISPR-Cas System. Genetics 208, 1-18.

Nyberg, K., Nguyen, J., Kwon, Y., Blythe, S., Beitel, G.J., Carthew, R.W., 2020. Adaptable and Efficient Genome Editing by sgRNA-Cas9 Protein Co-injection into Drosophila. BioRxiv 2020.05.07.080762.