



**Figure S3** Strategy used to recover and maintain the recombinant balancer chromosome. On two occasions, a balanced male was recovered that appeared to have a double dose of the wild type red eye color (*w+*) reporter that identified the triple knock-out (3KO) chromosome. In both cases, the male was virtually sterile, but when crossed to a stock bearing the reciprocal translocation *apterous<sup>Xa</sup>*, it was possible to recover the *CyO* balancer bearing the triple knock out and maintain it in a stock. PCR was used to confirm the balancer had lost the three *Drosophila STE24* paralogs. Note that in this reciprocal translocation (the *apterous<sup>Xa</sup>* chromosome), the region containing the three *STE24* paralogs now resides over on the third chromosome rather than the second. The original parental reciprocal translocation chromosome *apterous<sup>Xa</sup>* was obtained from the Bloomington Indiana Stock center, stock #3234, where the balancer for the second is *In(2L)Cy, In(2R)Cy, Cy[1]*. The instability for the *CG9000* region was unanticipated, and thus when discovered, the original Bloomington stock was rebuilt over *CyO; TM6*.