Figure S4 Orientation of PAMs at two nearby Cas9 cleavage sites influences HDR efficiency
Cas9 was directed to two locations in close proximity (224 bp, Target A and Target B), to make two DSBs and thereby stimulate insertion of an exogenously supplied homologous repair template. To examine the effect of PAM orientation on HDR efficiency, two strains were made that had identical sequences at the insertion site except for the orientation of Target B. One strain had PAMs (red) in an IN/IN orientation, placing the PAMs in the excised genomic DNA, while the other strain had PAMs in an IN/OUT orientation, placing one PAM in the excised genomic DNA and the other at a free end of the cleaved chromosome. The mNeonGreen, double-stranded DNA reporter repair template had 500 bp homology arms corresponding to genomic sequences adjacent to the Cas9 DSBs. Repair templates

with either the IN/IN or IN/OUT orientation were identical except for sequences corresponding to Target B sites. The IN/IN repair template included cleavage remnants of Target B that lacked a PAM, while the IN/OUT repair template included cleavage remnants of Target B that had the PAM (see diagram; reporter insert shown in pink). PCR was used to screen 180 Dpy or Rol F1s for each set of PAM orientations. A primer (left brown arrow) that annealed to genomic sequence located outside the bounds of the repair template and a second primer (right brown arrow) that annealed to the *dpy-30* promoter within the repair template only amplified sequences from worms having the desired genomic insertions at Target A. A PCR reaction using the primer (left brown arrow) outside the repair template and a third primer (pink arrow) within the right homology arm produced a 320 bp product from wild-type unedited genomic DNA. Percent (%) of Dpy or Rol F1s with precise insertions for each set of PAMs is shown. Homozygous insert-containing offspring for two positive F1s from each PAM orientation were subjected to Sanger sequencing. In all four cases, the edited genomes had precisely inserted repair templates. The percent of repair from the IN/IN orientation was statistically greater than that for the IN/OUT orientation ($P = 0.001$, chi-square)