Figures and figure supplements

Efficient single-copy HDR by 5’ modified long dsDNA donors

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Figure 1. Modification of 5’ ends of long dsDNA fragments prevents in vivo multimerization. (A) Schematic representation of long dsDNA donor cassette PCR amplification with universal primers (black arrows) complementary to the cloning vector backbone outside of the assembled donor cassette (e.g. gfp with homology flanks). Bulky moieties like Biotin at the 5’ ends of both modified primers (red octagon) prevent multimerization/NHEJ of dsDNA, providing optimal conditions for HDR-mediated single-copy integration following CRISPR/Cas9-introduced DSB at the target locus (grey scissors). Representation of locus (Lf/Lr) and internal gfp (Gf/Gr) primers for PCR genotyping of putative HDR-mediated gfp integration events. (B) Southern blot analysis reveals the monomeric state of injected dsDNA fragments in vivo for 5’ modification with Biotin or Spacer C3. Long dsDNAs generated with control unmodified primers or Amino-dT attached primers multimerize as indicated by a high molecular weight ladder apparent already within two hours post-injection (hpi). Note: 5’ moieties did not enhance the stability of injected DNA.

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**Figure 1—figure supplement 1.** Schematic representation of the donor plasmids. (A–D) Schematic to-scale representation of respective target locus (A, *rx2*; B, *rx1*; C, *actb*; D, *dnmt1*) with UTR (white boxes with red outlines). **Figure 1—figure supplement 1 continued on next page**
Figure 1—figure supplement 1 continued

and exons (red boxes) highlighted. Homology flanks (HF, grey), sgRNA target sites (white scissors) and respective locus primers (black arrows, Lf, Lr, see Supplementary file 2) are indicated. Respective assembled donor plasmid (A, B, C Golden GATEway (Kirschmaier et al., 2013) or D, conventional cloning) that served as a template for PCR amplification of the unmodified/modified long dsDNA gfp donor cassette is depicted below (FL, flexible linker; backbone sequence in blue). Entry vectors (EV) or restriction enzyme sites for cloning are indicated. The position of primers flanking the donor cassette is indicated, modification highlighted by a red octagon.

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Figure 2. Modification of 5' ends of long dsDNA fragments promotes HDR-mediated single-copy integration. (A) GFP expression in the respective expression domain after HDR-mediated integration of modified dsDNA gfp donor cassettes into rx2, rx1, actb and dnmt1 ORFs in the injected generation. (B) Individual embryo PCR genotyping highlights efficient HDR-mediated single-copy integration of 5'Biotin modified long dsDNA donors, but not unmodified donor cassettes. Locus PCR reveals band size indicative of single-copy gfp integration (asterisk) besides alleles without gfp integration (open arrowhead). Amplification of gfp donor (white arrow) for control.

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Figure 2—figure supplement 1. Quantification of survival and GFP expression of injected embryos. Embryos injected with unmodified or 5’ Biotin, Amino-dT or Spacer C3 modified long dsDNA gfp-rx2 donor cassettes were scored for survival at one dpi, and for GFP expression at two dpi. n, total number of injected embryos.

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**Figure 2—figure supplement 2.** 5’Biotin modification of long dsDNA donors strongly enhances HDR-mediated integration. (A) nx2 locus PCR genotyping of individual GFP-Rx2 positive embryos injected with unmodified or 5’Biotin or Spacer C3 modified long dsDNA gfp donor cassettes (green asterisk, single-copy HDR-mediated integration of gfp, 2547 bp, open arrowhead, nx2 allele without gfp integration, 1719 bp;). Horizontal bar, individual embryo; L, nx2 locus PCR with nx2 Lf/nx2 Lr; G, gfp internal PCR for control with Gf/Gr. (B) Qualitative summary of band spectrum (single-copy HDR-mediated gfp integration, nx2 allele without gfp integration, other) resulting from PCR genotyping in (A). n, number of genotyped GFP-Rx2 expressing embryos.

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Figure 2—figure supplement 3. Stable germline transmission of the single-copy HDR-mediated precise gfp integration. (A) Schematic to-scale representation of the gfp-rx2 locus with UTR (white boxes with red outlines), exons (red boxes) and homology flanks (HF, grey) highlighted. (B, C) Individual GFP-Rx2 expressing embryos (F0, B; F1, C) were genotyped using primers rx2 Lf/rx2 Lr (black arrows) and sequenced with gfpf and gfpr primers (green arrows).

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Figure 3. Single-copy integration of long dsDNA donor establishes stably transmitted gfp-rx2 fusion gene. (A) F2 homozygous embryos exhibit GFP-Rx2 fusion protein expression in the pattern of the endogenous gene in the retina. (B) Southern Blot analysis of F2 gfp-rx2 embryos reveals a single band for a digestion scheme cutting outside the donor cassette (BglII/HindIII) or within the 5′ donor cassette and in intron 2 (ScaI/HindIII) indicating precise single-copy donor integration. (B′) Schematic representation of the modified locus indicating the restriction sites and the domain complementary to the probe used in (B). (C) RT-PCR analysis on mRNA isolated from individual homozygous F3 embryos indicates the transcription of a single gfp-rx2 fusion mRNA in comparison to the shorter wild-type rx2 mRNA as schematically represented in (C′).

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**Figure 3—figure supplement 1.** Stably transmitted single-copy integration of the gfp-rx1 donor cassette. (A) Southern Blot analysis of F2 gfp-rx1 embryos reveals a single band for a digestion scheme cutting outside the donor cassette and within the 3’ donor cassette (HindIII/XmaI) or within the 5’ donor cassette and in intron 1 (NcoI/EcoRI) indicating precise single-copy donor integration. (A’) Schematic representation of the modified locus indicating the restriction sites and the domain complementary to the probe used in (A).

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