Supplementary Figures

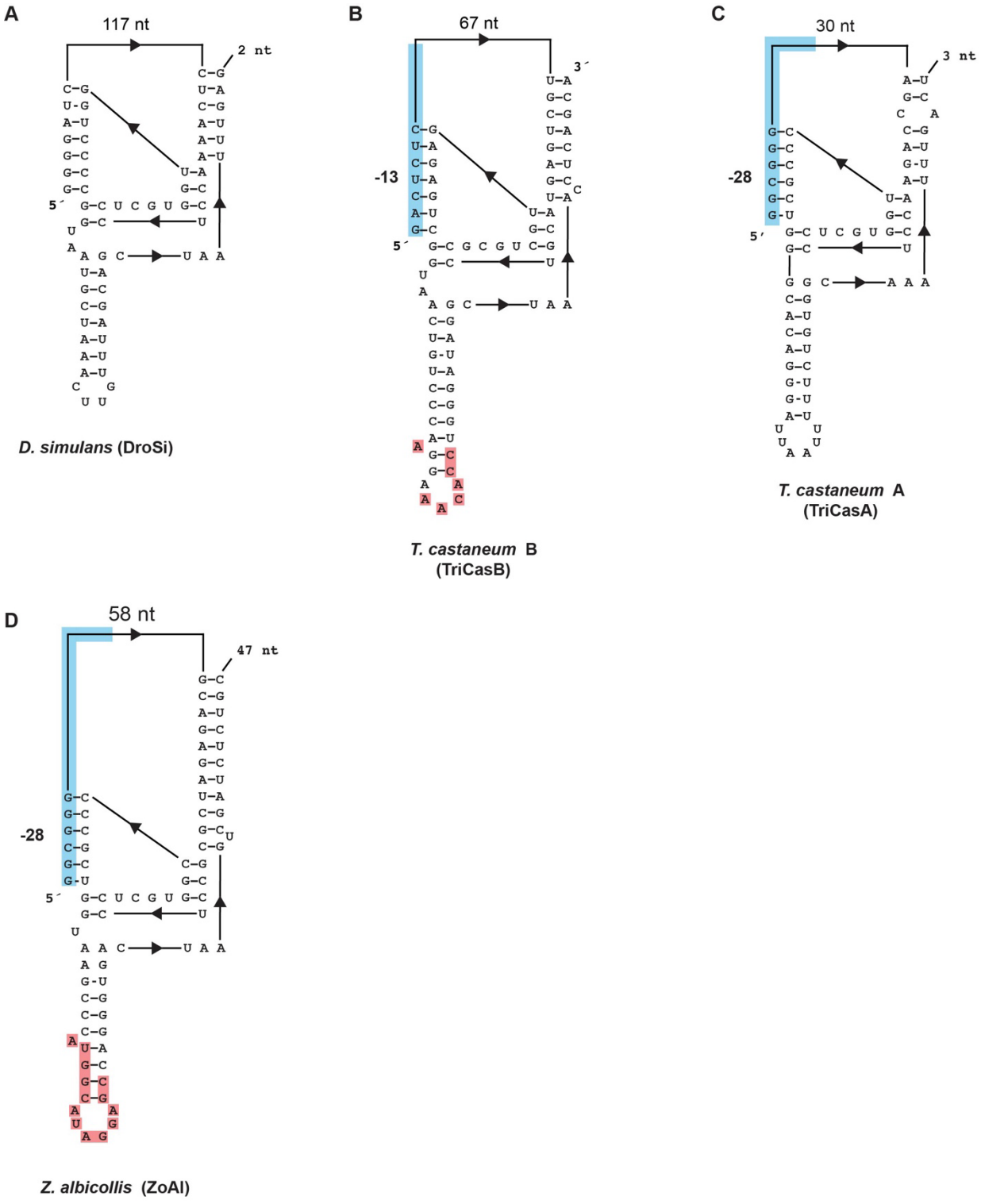


Figure S1. Secondary structures of native-derived R2 Rzs used in this study. Related to Figure 1. Blue indicates regions corresponding to rRNA. Orange indicates non-native sequence.

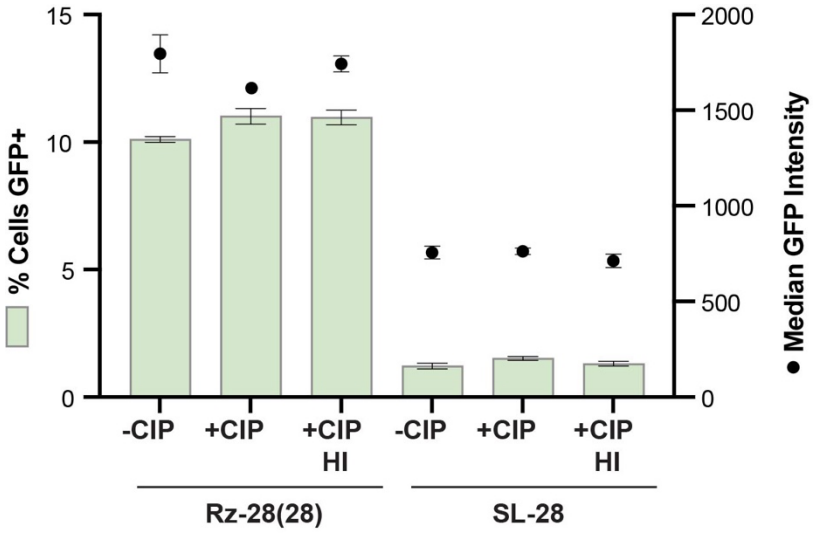
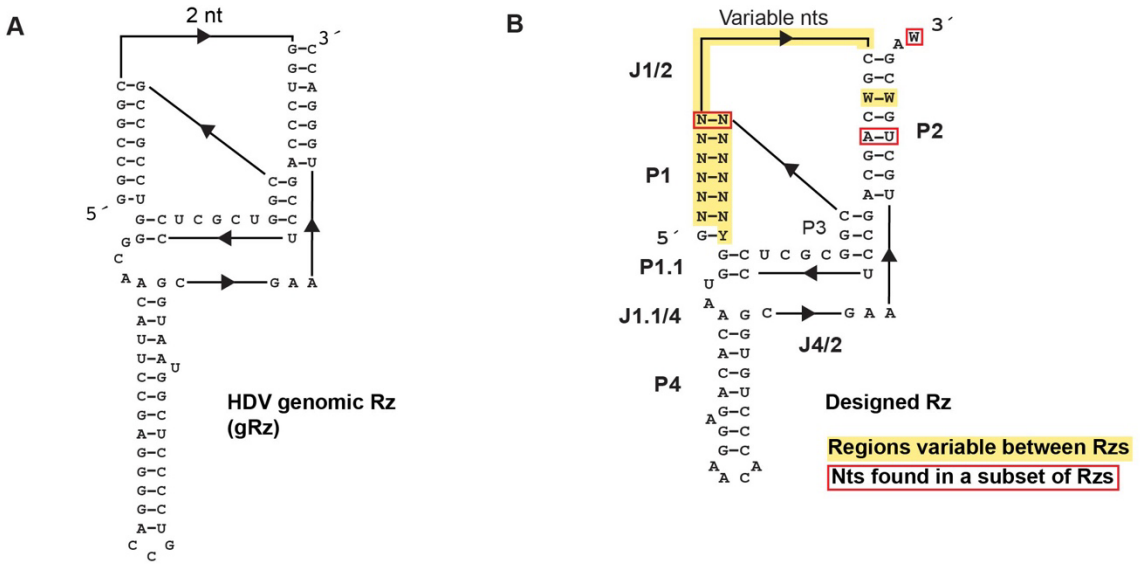


Figure S2. Phosphatase treatment of RNA templates with non-Rz 5′ modules does not impact PRINT efficiency. Related to Figure 3. Quantification of insertion efficiency in PRINT with RNA templates harboring Rz-28(28) and SL-28 5′ modules either not treated or treated with active calf intestinal phosphatase (CIP) or CIP that had been heat-inactivated (HI), as determined by flow cytometry. Values indicate mean ± SD, *n*=3.



C

	Length	P1	J1/2	P2	P3		P1.1		P3´	P1´	P1.1´	J1.1/4	P4	J4/2	P2´	
Rz-28(28)	110 nt	GGCGGG	39 nt	CGUCAGCA	GCC	U	CC	UCGC	GGC	CCCGGC	GG	UA	21 nt	CGAA	UGCUGACG	A
Rz-28(14)	92 nt	GGCGGG	21 nt	CGUCAGCA	GCC	U	CC	UCGC	GGC	CCCGGC	GG	UA	21 nt	CGAA	UGCUGACG	A
Rz-28(18)	96 nt	GGCGGG	25 nt	CGUCAGCA	GCC	U	CC	UCGC	GGC	CCCGGC	GG	UA	21 nt	CGAA	UGCUGACG	A
Rz-28(22)	106 nt	GGCGGG	35 nt	CGUCAGCA	GCC	U	CC	UCGC	GGC	CCCGGC	GG	UA	21 nt	CGAA	UGCUGACG	A
Rz-28(23)	107 nt	GGCGGG	35 nt	CGUCAGCA	GCC	U	CC	UCGC	GGC	CCCGGC	GG	UA	21 nt	CGAA	UGCUGACG	AU
Rz-28(24)	109 nt	GGCGGG	37 nt	CGUCAGCA	GCC	U	CC	UCGC	GGC	CCCGGC	GG	UA	21 nt	CGAA	UGCUGACG	AU
Rz-28(25)	109 nt	GGCGGG	37 nt	CGUCAGCA	GCC	U	CC	UCGC	GGC	CCCGGC	GG	UA	21 nt	CGAA	UGCUGACG	AU
Rz-28(26)	108 nt	GGCGGG	37 nt	CGUCAGCA	GCC	U	CC	UCGC	GGC	CCCGGC	GG	UA	21 nt	CGAA	UGCUGACG	A
Rz-28(27)	110 nt	GGCGGG	38 nt	CGUCAGCA	GCC	U	CC	UCGC	GGC	CCCGGC	GG	UA	21 nt	CGAA	UGCUGACG	AU
Rz-28(30)	111 nt	GGCGGG	39 nt	CGUCAGCA	GCC	U	CC	UCGC	GGC	CCCGGC	GG	UA	21 nt	CGAA	UGCUGACG	AU
Rz-28(32)	114 nt	GGCGGG	42 nt	CGUCAGCA	GCC	U	CC	UCGC	GGC	CCCGGC	GG	UA	21 nt	CGAA	UGCUGACG	AU
Rz-13(11)	77 nt	GACUCUC	5 nt	CGACGCA	GCC	U	CC	UCGC	GGC	GAGAGUU	GG	UA	21 nt	CGAA	UGCGUCG	AA
Rz-13(13)	79 nt	GACUCUC	7 nt	CGACGCA	GCC	U	CC	UCGC	GGC	GAGAGUU	GG	UA	21 nt	CGAA	UGCGUCG	AA
Rz-21(21)	99 nt	GUAACUA	27 nt	CGUCAGCA	GCC	U	CC	UCGC	GGC	UAGUUAC	GG	UA	21 nt	CGAA	UGCUGACG	AU
Rz-24(24)	105 nt	GGAGUAA	31 nt	CGUCAGCA	GCC	U	CC	UCGC	GGC	UUACUCC	GG	UA	21 nt	CGAA	UGCUGACG	AU
Rz-36(36)	129 nt	GGGUAAA	55 nt	CGUCAGCA	GCC	U	CC	UCGC	GGC	UUUACCC	GG	UA	21 nt	CGAA	UGCUGACG	AU

Figure S3. Secondary structures of HDV genomic Rz and designed HDV-like Rzs. Related to Figures 3-4. **A.** Secondary structure of HDV genomic Rz. **B.** Predicted secondary structure of designed Rzs used in this study. Yellow indicates regions which vary between designed Rzs. Red-boxed nts are found only in a subset of Rzs. **C.** Table summary of sequences of designed Rzs used in this study.

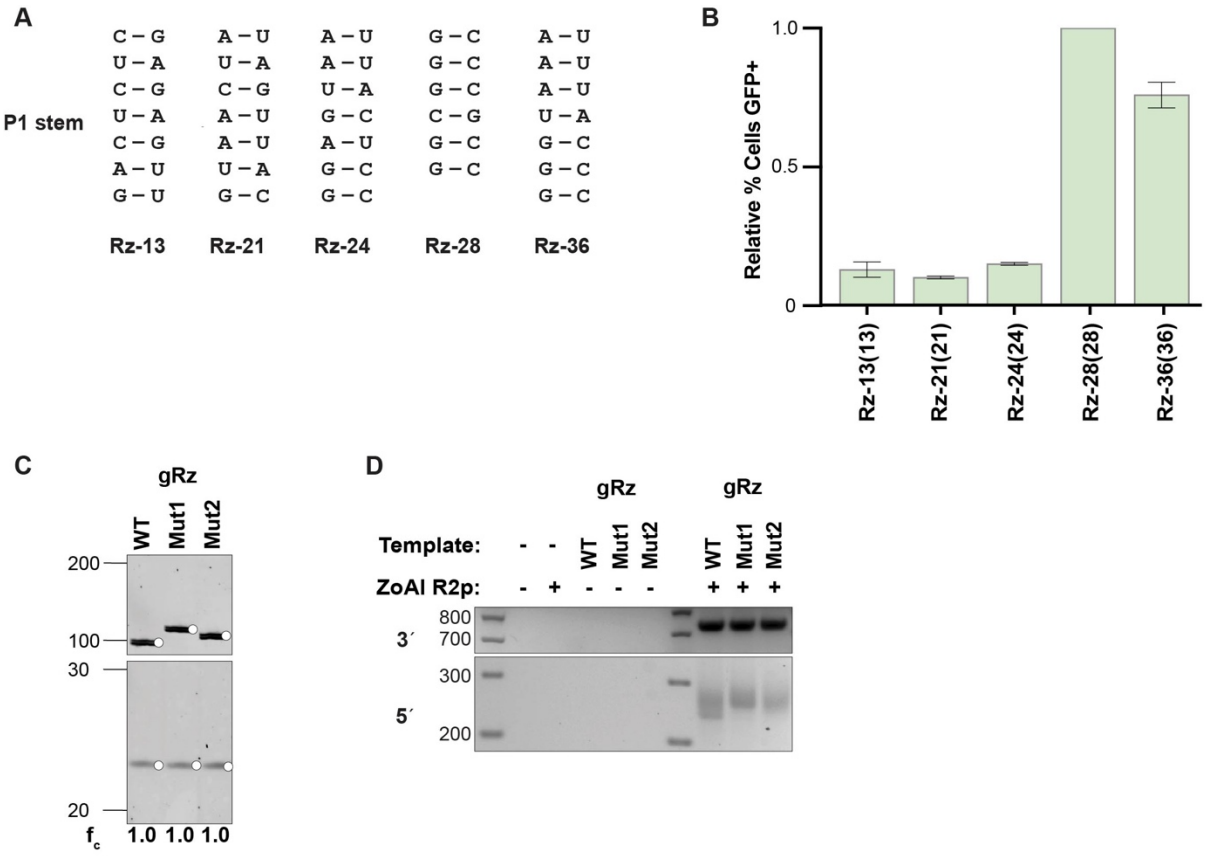


Figure S4. GC content of HDV Rz P1 stems influences 5´ module impact on PRINT efficiency. Related to Figure 4. **A.** Predicted P1 stems of designed HDV-like Rzs used in this study. **B.** Quantification of relative insertion efficiency of designed HDV-like Rz 5´ module RNA templates used in this study, as measured by flow cytometry. Rz-28(28) is set equal to 1. **C.** Analysis of gRz and gRz mutant cleavage from a PP7 hp leader. Reaction products were separated by 12% Urea-PAGE. Apparent size differences between Rzs are due to folding and not differences in molecular weight (see Methods). Cleaved reaction products are indicated by white circles and fraction cleaved (f_c) is quantified below each lane. **D.** PCR amplification of 3´ (top) and 5´ (bottom) rDNA-transgene junctions from gDNA in a representative replicate of the experiment in Figure 4H.

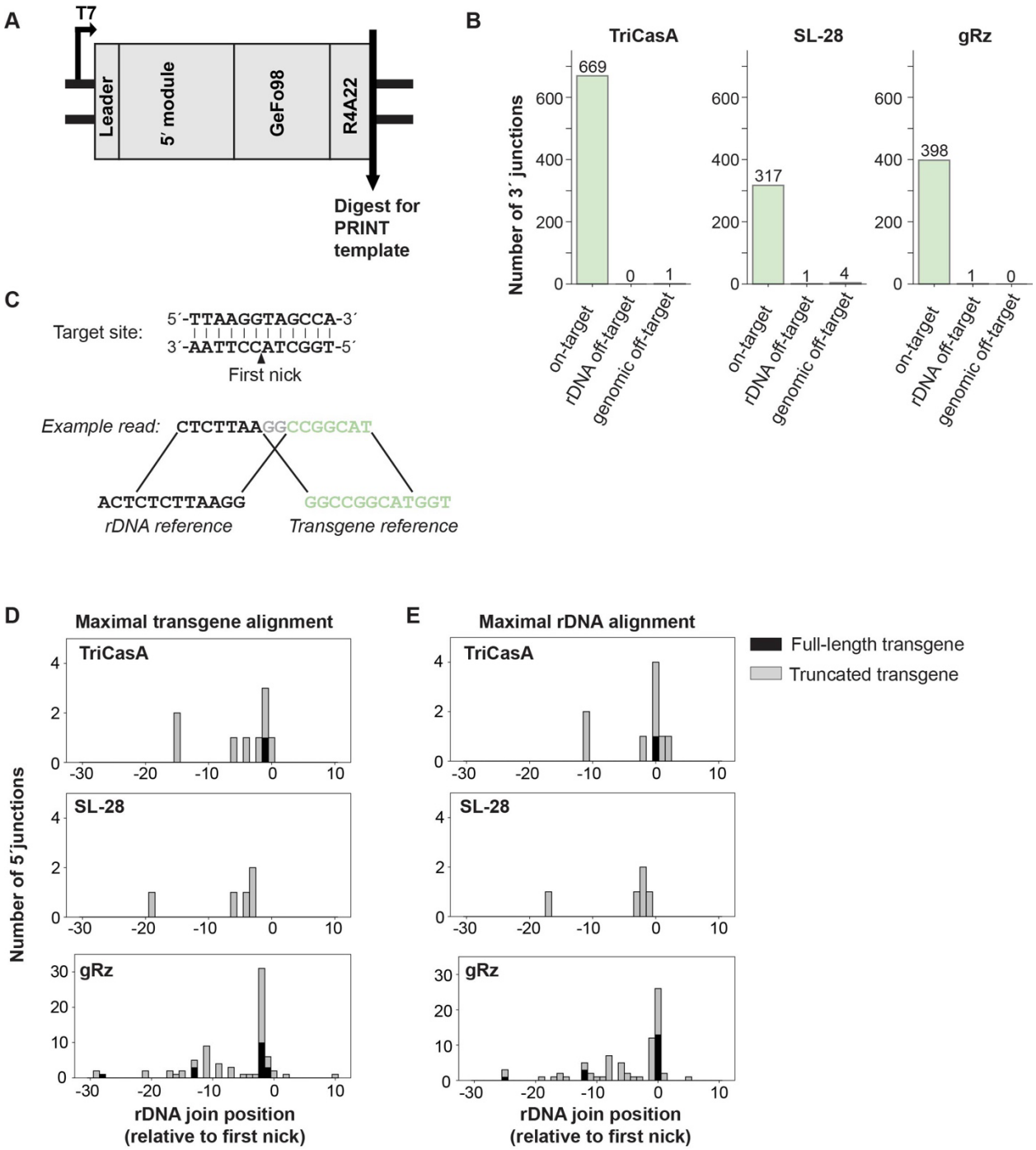


Figure S5. WGS analysis of transgene insertions from different 5′ module RNA templates. Related to Figure 6. **A.** Schematic of minimized RNA template co-delivered with ZoAI R2p mRNA to cells for WGS analysis. GeFo98 is a 98-nt truncated version of GeFo 3′ UTR that retains specificity and affinity for ZoAI R2p. **B.** Analysis of off-target insertions generated from RNA templates with different 5′ modules. Off-target insertions are assessed as either within rDNA or elsewhere in the genome. **C.** Schematic depicting analysis of 5′ junction join positions. Top, target-site sequence. Join positions are inferred relative to the depicted location of first-

strand nicking. Bottom, join positions may be inferred either by maximal rDNA alignment by assigning nts of microhomology to the rDNA reference (left and **E**), or by maximal transgene alignment by assigning nts of microhomology to the transgene reference (right and **D**). **D.** Join positions determined for transgenes with microhomology at 5′ junctions using maximal transgene alignment. Transgenes are classified either as full-length (FL, black) or truncated (Trunc., gray). **E.** Join positions determined for transgenes with microhomology at 5′ junctions using maximal rDNA alignment.

Supplementary Table Legends

Table S1. Additional sequences identified at rDNA-transgene 5´ junctions by WGS.

Catalog of sequencing reads containing extra nts inserted between rDNA-aligned and transgene-aligned sequence from gRz WGS data. For transgene reference coordinates, negative numbers indicate inclusion of uncleaved L8 leader sequence (GGGTAAAC).

Table S2. Plasmids used in this study. Identifier, figure used, and sequence are specified. Each PRINT template was cloned into an *E. coli* pUC57-Mini vector.