Supplemental Materials for

Improved constructs for bait RNA display in a bacterial three-hybrid assay

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Supplemental Table S1. *E. coli* strains used in this study.

Strain	Genotype	Antibiotic Resistance	Source
NEB5α-F′I^{q}	*E. coli* lacI^{q} host strain for plasmid construction.	TetR	New England Biolabs
KB483	FW102 Δ*hfq::kan* harboring an F′ episome bearing test promoter *plac-O$_L$2-62* fused to *lacZ*.	KanR; TetR; StrR	(Pandey et al. 2020)
MG1655	Strain K-12 F^{-} lambda^{-} *ivlG^{-} rfb*-50 *rph*-1	–	(Blattner et al. 1997)

Supplemental Table S2. Plasmids used in this study.

Plasmid Name	Description	Details	Source or oligos used in this study
p35u4 (Addgene #174786)	pAdapter: pAC-p$_{constit}$-CI-MS2^{CP}	Encodes CI-MS2^{CP} fusion protein under control of a constitutive promoter; p15A origin of replication. Isolated from a pCW17-derived mutagenesis library; confers CmR	(Wang et al. 2021)
pACλCI (Addgene #53730)	pACλCI empty vector	Encodes full-length λCI under the control of the lac*UV5* promoter; confers CmR	(Pandey et al. 2020)
pBr-α (Addgene #53731)	pBR-α-empty vector	Encodes residues 1-248 of the alpha vector fused under control of *lpp* and *lacUV5 promoters*; confers AmpR	(Dove et al. 1997)
pCH1 (Addgene #174663)	pBait-1xMS2^{hp}-empty	pCDF-pBAD-1xMS2^{hp}. Encodes a single MS2^{hp} under the control of an arabinose-inducible promoter, followed by XmaI and HindIII sites. CloDF13 origin of replication; confers SpecR	(Pandey et al. 2020) (schematics in Fig 1B+2A)
pCH6 (Addgene #174662)	pBait-1xMS2^{hp}-ChiX	*E. coli chiX* inserted between XmaI/HindIII sites in pCH1; sRNA encodes its own terminator; confers SpecR	(Pandey et al. 2020)
pCH7	pBait-1xMS2^{hp}-McaS	*E. coli mcaS* inserted between XmaI/HindIII sites in pCH1; sRNA encodes its own terminator; confers SpecR	oKB1192+ oKB1193
pCH8	pBait-1xMS2^{hp}-CyaR	*E. coli cyaR* inserted between XmaI/HindIII sites in pCH1; sRNA encodes its own terminator; confers SpecR	oKB1194+ oKB1195
pCH9	pBait-1xMS2^{hp}-OxyS	*E. coli oxyS* inserted between XmaI/HindIII sites in pCH1; sRNA encodes its own terminator; confers SpecR	(Pandey et al. 2020)

pCH10	pBait-1xMS2^{hp}-RyhB	*E. coli ryhB* inserted between XmaI/HindIII sites in pCH1; sRNA encodes its own terminator; confers SpecR	oKB1198+ oKB1199
pCH12	pBait-1xMS2^{hp}-SgrS	*E. coli sgrS* inserted between XmaI/HindIII sites in pCH1; sRNA encodes its own terminator; confers SpecR	oKB1107+ oKB1108
pCH13	pBait-1xMS2^{hp}-ArcZ	*E. coli arcZ* inserted between XmaI/HindIII sites in pCH1; sRNA encodes its own terminator; confers SpecR	(Pandey et al. 2020)
pHL6	pBait-1xMS2^{hp}-T_{trpA}	Encodes a single MS2^{hp} followed by XmaI and HindIII sites and the intrinsic terminator from *E. coli trpA* gene (T_{trpA}). Hybrid RNA is under the control of an arabinose-inducible promoter. CloDF13 origin of replication; confers SpecR	(Pandey et al. 2020) (schematic: Fig 4A)
pHL28	pBait-1xMS2^{hp}-*rpoS-5′*-T_{trpA}	*E. coli* 5′UTR of *rpoS* (-134 to +3, relative to AUG) inserted between XmaI/HindIII sites in pHL6; confers SpecR	oHL38 + oHL39
pHL29	pBait-1xMS2^{hp}-*eptB*-5′-T_{trpA}	*E. coli* 5′UTR of *eptB* (-106 to +27, relative to AUG) inserted between XmaI/HindIII sites in pHL6; confers SpecR	oHL40 + oHL41
pHL30	pBait-1xMS2^{hp}-*sodB*-5′-T_{trpA}	*E. coli* 5′UTR of *sodB* (-55 to +27, relative to AUG) inserted between XmaI/HindIII sites in pHL6; confers SpecR	oHL42 + oHL43
pHL31	pBait-1xMS2^{hp}-*chiP*-5′-T_{trpA}	*E. coli* 5′UTR of *chiP* (-98 to +12, relative to AUG) inserted between XmaI/HindIII sites in pHL6; confers SpecR	oHL44 + oHL45
pHL32	pBait-1xMS2^{hp}-*mutS*-5′-T_{trpA}	*E. coli* 5′UTR of *mutS* (-74 to +27, relative to AUG) inserted between XmaI/HindIII sites in pHL6; confers SpecR	oHL46 + oHL47
pHL36	pBait-1xMS2^{hp}-*rpoS*-5′-TAA-T_{trpA}	pHL28 with stop codon (TAA) inserted after *5′*UTR: *E. coli* 5′UTR of *mutS* (-134 to +3, relative to AUG), upstream of T_{trpA}; confers SpecR	oHL66 + oHL67
pHL37	pBait-1xMS2^{hp}-*eptB*-5′-TAA-T_{trpA}	pHL29 with stop codon (TAA) inserted after *5′*UTR: *E. coli* 5′UTR of *eptB* (-106 to +27, relative to AUG), upstream of T_{trpA}; confers SpecR	oHL66 + oHL68
pHL38	pBait-1xMS2^{hp}-*sodB*-5′TAA-T_{trpA}	pHL30 with stop codon (TAA) inserted after *5′*UTR: *E. coli* 5′UTR of *sodB* (-55 to +27, relative to AUG), upstream of T_{trpA}; confers SpecR	oHL66 + oHL69

pHL39	pBait-1xMS2^{hp}-*chiP*-5′-TAA-T_{trpA}	pHL31 with stop codon (TAA) inserted between 5′UTR and HindIII site: *E. coli* 5′UTR of *chiP* (-98 to +12, relative to AUG) upstream of T_{trpA}; confers SpecR	oHL66 + oHL70
pHL40	pBait-1xMS2^{hp}-*mutS*-5′-TAA-T_{trpA}	pHL32 with stop codon (TAA) inserted between 5′UTR and HindIII site: *E. coli* 5′UTR of *mutS* (-74 to +27, relative to AUG) upstream of T_{trpA}; confers SpecR	oHL66 + oHL71
pKB817	pPrey-α-Hfq	Encodes residues 1-248 of the alpha subunit of RNA polymerase fused via three alanine residues to full-length wild-type *E. coli hfq*; confers AmpR	(Berry and Hochschild 2018)
pKB845	pBait-2xMS2^{hp}	pCDF-pBAD-2xMS2^{hp}-XmaI-HindIII; two MS2 RNA hairpins (2xMS2^{hp}) and an XmaI site inserted into pKB822 between BamHI/HindIII sites; confers SpecR	(Berry and Hochschild 2018)
pKB858	pBait-2xMS2^{hp}-Spot42	*E. coli spot42* inserted between XmaI/HindIII sites in pKB845; sRNA encodes its own terminator; confers SpecR	oKB1113 + oKB1114
pKB939	pBait-2xMS2^{hp}-MicF	*E. coli micF* inserted between XmaI/HindIII sites in pKB845; sRNA encodes its own terminator; confers SpecR	oKB1205 + oKB1206
pKB940	pBait-2xMS2^{hp}-GcvB	*E. coli gcvB* inserted between XmaI/HindIII sites in pKB845; sRNA encodes its own terminator; confers SpecR	oKB1207 + oKB1208
pKB941	pBait-2xMS2^{hp}-DsrA	*E. coli dsrA* inserted between XmaI/HindIII sites in pKB845; sRNA encodes its own terminator; confers SpecR	(Wang et al. 2021)
pKB943	pBait-2xMS2^{hp}-GlmZ	*E. coli glmZ* inserted between XmaI/HindIII sites in pKB845; sRNA encodes its own terminator; confers SpecR	oKB1213 + oKB1214
pKB1212	pBait-1xMS2^{hp}-MicA	*E. coli micA* inserted between XmaI/HindIII sites in pKB845; sRNA encodes its own terminator; confers SpecR	oKB1535 + oKB1536
pKB1213	pBait-1xMS2^{hp}-RybB	*E. coli rybB* inserted between XmaI/HindIII sites in pKB845; sRNA encodes its own terminator; confers SpecR	oKB1537 + oKB1538
pLN27	pBait-13GC[1xMS2^{hp}]-ChiX	pCH6 with 13-bp GC clamp flanking an MS2^{hp} inserted between EcoRI/XmaI sites; sRNA encodes its own terminator; confers SpecR	pCH6 + oLN28 + oLN29
pLN28	pBait-5GC[1xMS2^{hp}]-ChiX	pCH6 with 5-bp GC clamp flanking an MS2^{hp} inserted between EcoRI/XmaI sites; sRNA encodes its own terminator; confers SpecR	pCH6 + oLN30 + oLN31

pLN31	pBait-13GC[1xMS2^{hp}]-McaS	*E. coli mcaS* inserted between XmaI/HindIII sites in pSW1; sRNA encodes its own terminator; confers SpecR	oKB1192 + oKB1193
pLN32	pBait-13GC[1xMS2^{hp}]-CyaR	*E. coli cyaR* inserted between XmaI/HindIII sites in pSW1; sRNA encodes its own terminator; confers SpecR	oKB1194 + oKB1195
pLN33	pBait-13GC[1xMS2^{hp}]-OxyS	*E. coli oxyS* inserted between XmaI/HindIII sites in pSW1; sRNA encodes its own terminator; confers SpecR	oKB1196 + oKB1197
pLN34 (Addgene #222403)	pBait-5GC[1xMS2^{hp}]-McaS	*E. coli mcaS* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oKB1192 + oKB1193
pLN35	pBait-5GC[1xMS2^{hp}]-CyaR	*E. coli cyaR* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oKB1194 + oKB1195
pLN36	pBait-5GC[1xMS2^{hp}]-OxyS	*E. coli oxyS* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oKB1196 + oKB1197
pLN37	pBait-1xMS2^{hp}-13GC[*rpoS*-5′]-T$_{trpA}$	*E. coli* 5′UTR of *rpoS* (-134 to +3, relative to AUG; Mikulecky et al. 2004) inserted between XmaI/HindIII sites in pSW2; confers SpecR	oHL38 + oHL39
pLN38	pBait-1xMS2^{hp}-13GC[*eptB*-5′] -T$_{trpA}$	*E. coli* 5′UTR of *eptB* (-106 to +27, relative to AUG) inserted between XmaI/HindIII sites in pSW2; confers SpecR	oHL40 + oHL41
pLN39	pBait-1xMS2^{hp}-13GC[*chiP*-5′]-T$_{trpA}$	*E. coli* 5′UTR of *chiP* (-98 to +12, relative to AUG) inserted between XmaI/HindIII sites in pSW2; confers SpecR	oHL44 + oHL45
pLN40	pBait-1xMS2^{hp}-13GC[*mutS*-5′]-T$_{trpA}$	*E. coli* 5′UTR of *mutS* (-74 to +27, relative to AUG) inserted between XmaI/HindIII sites in pSW2; confers SpecR	oHL46 + oHL47
pLN41	pBait-1xMS2^{hp}-7GC[*rpoS*-5′]-T$_{trpA}$	*E. coli* 5′UTR of *rpoS* (-134 to +3, relative to AUG; Mikulecky et al. 2004) inserted between XmaI/HindIII sites in pSS2; confers SpecR	oHL38 + oHL39
pLN42	pBait-1xMS2^{hp}-7GC[*eptB*-5′]-T$_{trpA}$	*E. coli* 5′UTR of *eptB* (-106 to +27, relative to AUG) inserted between XmaI/HindIII sites in pSS2; confers SpecR	oHL40 + oHL41
pLN43 (Addgene #222404)	pBait-1xMS2^{hp}-7GC[*chiP*-5′]-T$_{trpA}$	*E. coli* 5′UTR of *chiP* (-98 to +12, relative to AUG) inserted between XmaI/HindIII sites in pSS2; confers SpecR	oHL44 + oHL45
pLN44	pBait-1xMS2^{hp}-7GC[*mutS*-5′]-T$_{trpA}$	*E. coli* 5′UTR of *mutS* (-74 to +27, relative to AUG) inserted between XmaI/HindIII sites in pSS2; confers SpecR	oHL46 + oHL47

pLN53	pBait-5GC[1xMS2^{hp}]-GlmZ	*E. coli glmZ* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oKB1213 + oKB1214
pLN54	pBait-5GC[1xMS2^{hp}]-DsrA	*E. coli dsrA* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oKB1209 + oKB1210
pLN80	pBait-5GC[1xMS2^{hp}]-RybB	*E. coli rybB* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oKB1537 + oKB1538
pLN81	pBait-5GC[1xMS2^{hp}]-MicA	*E. coli micA* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oKB1535 + oKB1536
pLN83	pBait-5GC[1xMS2^{hp}]-RyhB	*E. coli ryhB* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oKB1198 + oKB1199
pLN84	pBait-5GC[1xMS2^{hp}]-ArcZ	*E. coli arcZ* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oKB1211 + oKB1212
pLN85	pBait-5GC[1xMS2^{hp}]-MicF	*E. coli micF* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oKB1205 + oKB1206
pLN91	pBait-5GC[1xMS2^{hp}]-SgrS	*E. coli sgrS* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oKB1107 + oKB1108
pLN92	pBait-5GC[1xMS2^{hp}]-Spot42	*E. coli spot42* inserted between XmaI/HindIII sites in pSS1; native sRNA terminator; confers SpecR	oKB1113 + oKB1114
pLN93	pBait-5GC[1xMS2^{hp}]-GcvB	*E. coli gcvB* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oKB1207 + oKB1208
pLN94	pBait-1xMS2^{hp}-OmrA	*E. coli omrA* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oLN66 + oLN67
pLN96	pBait-1xMS2^{hp}-SdhX	*E. coli sdhX* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oLN70 + oLN71
pLN97	pBait-1xMS2^{hp}-MicL	*E. coli micL* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oLN72 + oLN73
pLN98	pBait-1xMS2^{hp}-GadY	*E. coli gadY* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oLN74 + oLN75

pLN100	pBait-5GC[1xMS2^{hp}]-OmrA	*E. coli omrA* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oLN66 + oLN67
pLN102	pBait-5GC[1xMS2^{hp}]-SdhX	*E. coli sdhX* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oLN70 + oLN71
pLN103	pBait-5GC[1xMS2^{hp}]-MicL	*E. coli micL* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oLN72 + oLN73
pLN104	pBait-5GC[1xMS2^{hp}]-GadY	*E. coli gadY* inserted between XmaI/HindIII sites in pSS1; sRNA encodes its own terminator; confers SpecR	oLN74 + oLN75
pSS1 (Addgene #222405)	pBait-5GC[1xMS2^{hp}]	pCDF-pBAD-5GC[1xMS2^{hp}]. Encodes a single MS2^{hp} flanked by 5-bp GC clamp, followed by XmaI and HindIII sites; hybrid RNA is under the control of an arabinose-inducible promoter, CloDF13 origin of replication; confers SpecR	pCH1 + oLN30 + oLN31 (schematic in Fig 2D)
pSS2 (Addgene #222406)	pBait-1xMS2^{hp}-7GC - T_{trpA}	pCDF-pBAD-1xMS2^{hp}-7GC[XmaI-HindIII]-TAA-$T_{trpA.}$ Encodes a single MS2^{hp} followed by XmaI and HindIII sites flanked by a 7-bp GC clamp, a stop codon (TAA) and the intrinsic terminator from *E. coli trpA* gene (T_{trpA}). Hybrid RNA is under the control of an arabinose-inducible promoter. CloDF13 origin of replication; confers SpecR	pSS2x + oSS3 + oSS4 (schematic in Fig 4D)
pSS2x	n/a	Intermediate construct used to make pSS2 from pHL6	pHL6 + oSS1 + oSS2
pSS3	pBait-1xMS2^{hp}-7GC[*sodB*-5′]-T_{trpA}	*E. coli* 5′UTR of *sodB* (-55 to +27, relative to AUG) between XmaI/HindIII sites in pSS2 (cloned by inserting 7GC clamp into pHL38); confers SpecR	pSS3x + oSS3 + oSS7
pSS3x	n/a	Intermediate construct used to make pSS23 from pHL38	pHL30 + oSS5 + oSS6
pSW1	pBait-1xMS2^{hp}-13GC[1xMS2^{hp}]	pCDF-pBAD-13GC[1xMS2^{hp}]. Encodes a single MS2^{hp} flanked by 13-bp GC clamp, followed by XmaI and HindIII sites; hybrid RNA is under the control of an arabinose-inducible promoter, CloDF13 origin of replication; confers SpecR	oLN28 + oLN29 (schematic in Fig 2C)
pSW2	pBait-1xMS2^{hp}-13GC-T_{trpA}	pCDF-pBAD-1xMS2^{hp}-13GC[XmaI-HindIII]-TAA-T_{trpA} Encodes a single MS2^{hp} followed by XmaI and HindIII sites flanked by a 13-bp GC clamp, a stop codon (TAA) and the	pSW2x + oSW3 + oSW4 (schematic in Fig 4C)

		intrinsic terminator from *E. coli trpA* gene (T_{trpA}). Hybrid RNA is under the control of an arabinose-inducible promoter. CloDF13 origin of replication; confers SpecR	
pSW2x	n/a	Intermediate construct used to make pSW2 from pHL6	pHL6 + oSW1+ oSW2
pSW3	pBait-1xMS2^{hp}-13GC[*sodB*-5′]-T_{trpA}	pHL38 with 13-bp GC clamp flanking *E. coli* 5′UTR of *sodB* and stop codon (TAA) inserted between HindIII site and the second GC flank; confers SpecR	pSW3x + oSW3 + oSW7
pSW3x	n/a	Intermediate construct used to make pSW3 from pHL38	pHL30 + oSW5 + oSW6

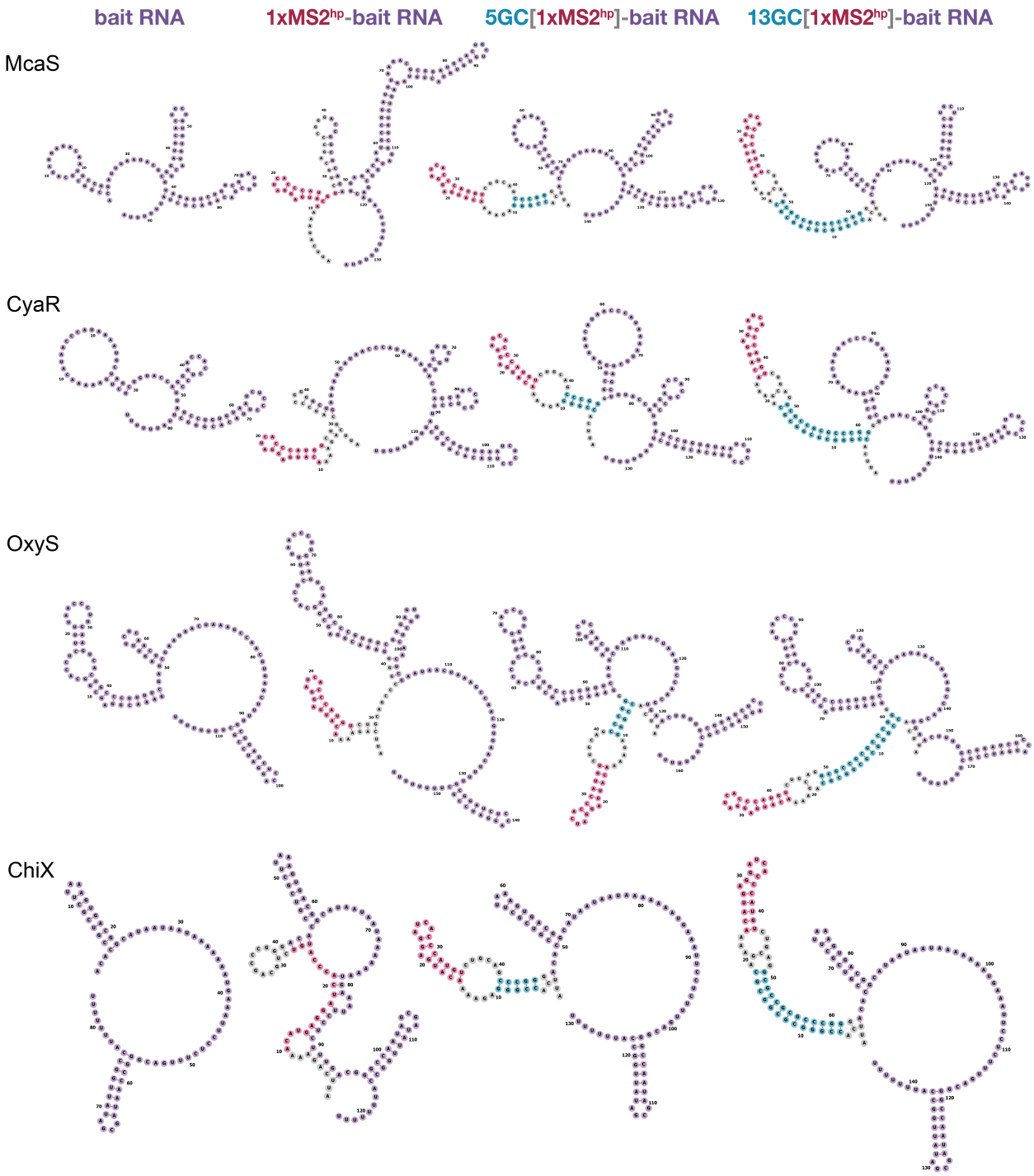
Supplemental Table S3. Oligonucleotides used in this study.

Oligo Name	**Sequence (5′ to 3′)**	**Details (F: Forward primer, R: Reverse primer)**
oHL38	GGCCGGCCCGGGACACGCTTGCATTTTGAAATTCG	F XmaI *rpoS*-5′ (pHL28, pLN37, pLN41)
oHL39	GGCCGGAAGCTTCATAAGGTGGCTCCTACCCGTGATC	R HindIII *rpoS*-5′ (pHL28, pLN37, pLN41)
oHL40	GGCCGGCCCGGGGCGCGTGTAGATTTTACTTATCTGAC	F XmaI *eptB*-5′ (pHL29, pLN38, pLN42)
oHL41	GGCCGGAAGCTTCTGTGTAATCGATTTGATGTATCTCATG	R HindIII *eptB*-5′ (pHL29, pLN38, pLN42)
oHL42	GGCCGGCCCGGGATACGCACAATAAGGCTATTGTACG	F XmaI *sodB*-5′ (pHL30)
oHL43	GGCCGGAAGCTTTGGTAGTGCAGGTAATTCGAATGAC	R HindIII *sodB*-5′ (pHL30)
oHL44	GGCCGGCCCGGGGTAGTCAGCGAGACTTTTCTCAACGC	F XmaI *chiP*-5′ (pHL31, pLN39, pLN43)
oHL45	GGCCGGAAGCTTAAACGTACGCATGGGTTAATCCTCTTTG	R HindIII *chiP*-5′ (pHL31, pLN39, pLN43)
oHL46	GGCCGGCCCGGGTGCGCCTTATGTGATTACAACGAAA ATA	F XmaI *mutS*-5′ (pHL32, pLN40, pLN44)
oHL47	GGCCGGAAGCTTGGCGTCGAAATTTTCTATTGCACTC	R HindIII *mutS*-5′ (pHL32, pLN40, pLN44)
oHL66	TAAAAGCTTAGCCCGCCTAAT	F Q5 TAA (pHL36, 37, 38, 39, 40)

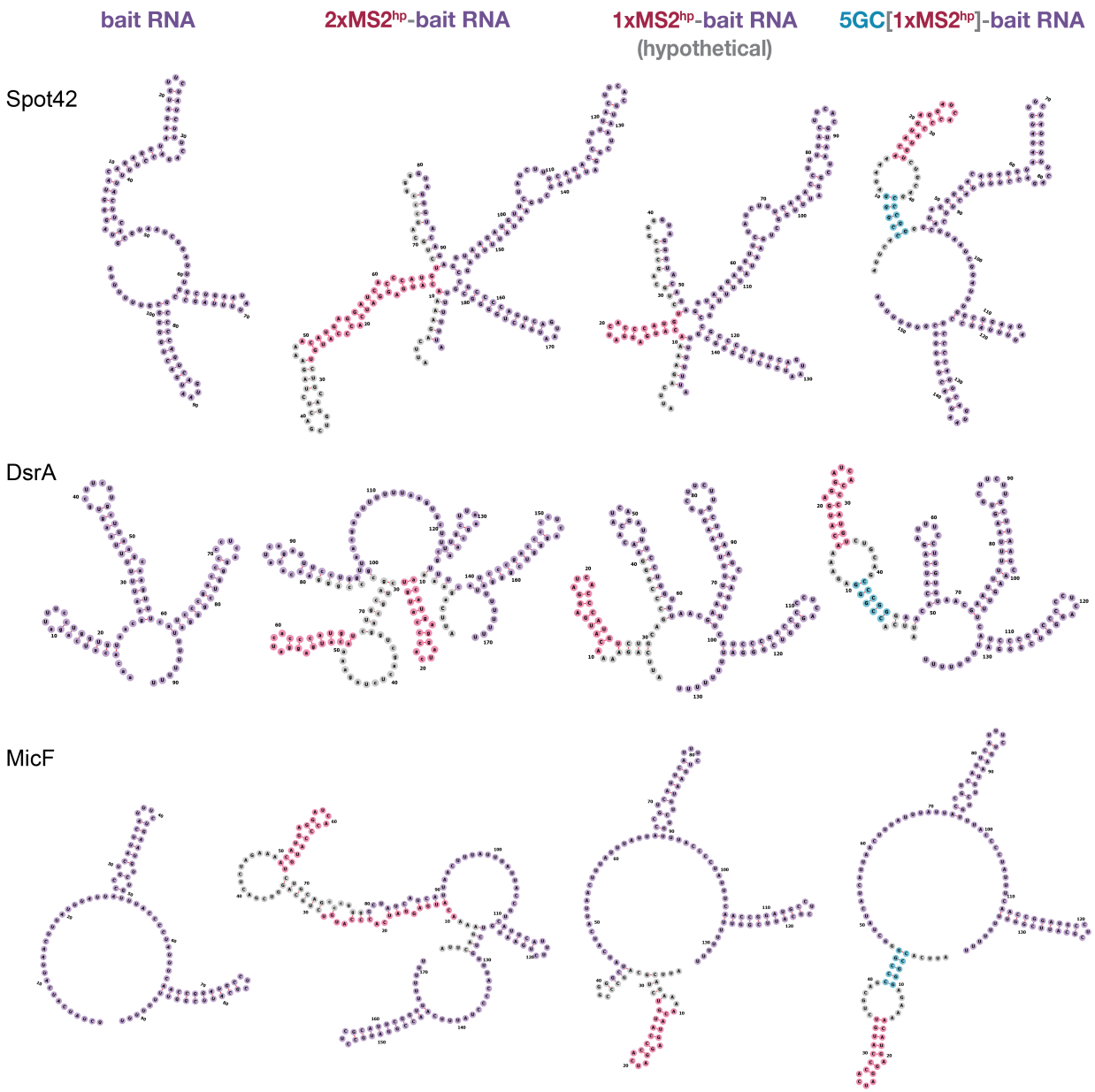
oHL67	CATAAGGTGGCTCCTACC	R Q5 *rpoS-5′* TAA (pHL36)
oHL68	CTGTGTAATCGATTTGATGTATCTC	R Q5 *eptB-5′* TAA (pHL37)
oHL69	TGGTAGTGCAGGTAATTCG	R Q5 *sodB-5′* TAA (pHL38)
oHL70	AAACGTACGCATGGGTTAATC	R Q5 *chiP-5′* TAA (pHL39)
oHL71	GGCGTCGAAATTTTCTATTGC	R Q5 *mutS-5′* TAA (pHL40)
oKB1107	TCCCCCCGGGGATGAAGCAAGGGGGTGCC	F XmaI SgrS (pCH12, pLN91)
oKB1108	CCGGCCAAGCTTAAAAAAAACCAGCAGGTATAATCTGCTGG	R HindIII SgrS (pCH12, pLN91)
oKB1192	TCCCCCCGGGACCGGCGCAGAGGAG	F XmaI McaS (pCH7, pLN31, pLN34)
oKB1193	CCGGCCAAGCTTAAAAAATAGAGTCTGTCGACATCCGCC	R HindIII McaS (pCH7, pLN31, pLN34)
oKB1194	TCCCCCCGGGGCTGAAAAACATAACCCATAAAATGCTAGC	F XmaI CyaR (pCH8, pLN32, pLN35)
oKB1195	CCGGCCAAGCTTAAAAAATAAGCCCGTGTAAGGGAGATTAC	R HindIII CyaR (pCH8, pLN32, pLN35)
oKB1196	TCCCCCCGGGGAAACGGAGCGGCACCTC	F XmaI OxyS (pCH9, pLN33, pLN36)
oKB1197	CCGGCCAAGCTTAAAAAAAAGCGGATCCTGGAGATCC	R HindIII OxyS (pCH9, pLN33, pLN36)
oKB1198	TCCCCCCGGGGCGATCAGGAAGACCCTCG	F XmaI RyhB (pCH10, pLN83)
oKB1199	CCGGCCAAGCTTAAAAAAAAGCCAGCACCCGG	R HindIII RyhB (pCH10, pLN83)
oKB1205	TCCCCCCGGGGCTATCATCATTAACTTTATTTATTACCGTC	F XmaI MicF (pKB939, pLN85)
oKB1206	CCGGCCAAGCTTAAAAAAAACCGAATGCGAGGCATC	R HindIII MicF (pKB939, pLN85)
oKB1207	TCCCCCCGGGACTTCCTGAGCCGGAACG	F XmaI GcvB (pKB940, pLN93)
oKB1208	CCGGCCAAGCTTAAAAAAAGCACCGCAATTAGGCG	R HindIII GcvB (pKB940, pLN93)

oKB1209	TCCCCCCGGGAACACATCAGATTTCCTGGTGTAAC	F XmaI DsrA (pKB941, pLN54)
oKB1210	CCGGCCAAGCTTAAAAAAAATCCCGACCCTGAGGG	R HindIII DsrA (pKB941, pLN54)
oKB1211	TCCCCCCGGGGTGCGGCCTGAAAAACAGTGC	F XmaI ArcZ (pCH13, pLN84)
oKB1212	CCGGCCAAGCTTAAAAAATGACCCCGGCTAGACC	R HindIII ArcZ (pCH13, pLN84)
oKB1213	TCCCCCCGGGGTAGATGCTCATTCCATCTCTTATGTTCG	F XmaI GlmZ (pKB943, pLN53)
oKB1214	CCGGCCAAGCTTAAAAAAAACAGGTCTGTATGACAACAAGTGG	R HindIII GlmZ (pKB943, pLN53)
oKB1535	GGCCGGCCCGGGGAAAGACGCGCATTTGTTATCATCATCC	F XmaI MicA (pKB1212, pLN81)
oKB1536	CCGGCCAAGCTTAGAAAAGAAAAAGGCCACTCGTGAGTG	R HindIII MicA (pKB1212, pLN81)
oKB1537	GGCCGGCCCGGGGCCACTGCTTTTCTTTGATGTCCCC	F XmaI RybB (pKB1213, pLN80)
oKB1538	CCGGCCAAGCTTAACAAAAAACCCATCAACCTTGAACCG	R HindIII RybB (pKB1213, pLN80)
oLN28	AATTCACCGGGCGCGGCGCAGAAAACATGAGGATCACCCATGTCTGCAGGCGCCGCGC	F EcoRI 13GC (pLN27, pSW1)
oLN29	CCGGGCGCGGCGCCTGCAGACATGGGTGATCCTCATGTTTTCTGCGCCGCGCCCGGTG	R XmaI 13GC (pLN27, pSW1)
oLN30	AATTCACCGGGAGAAAACATGAGGATCACCCATGTCTGCAGC	F EcoRI 5GC (pLN28, pSS1)
oLN31	CCGGGCTGCAGACATGGGTGATCCTCATGTTTTCTCCCGGTG	R XmaI 5GC (pLN28, pSS1)
oLN66	AAGCTTACAGAATTTTAAGTGCTTC	F XmaI OmrA (pLN94, pLN100)
oLN67	ATTAGGTGACATCACGAAGG	R HindIII OmrA (pLN94, pLN100)
oLN70	TCCCCCCGGGATATCTGTAATAAGAAATAGCCCTCGCC	F XmaI SdhX (pLN96, pLN102)
oLN71	CCGGCCAAGCTTACAAAAAAGGCCATCATACGATGG	R HindIII SdhX (pLN96, pLN102)

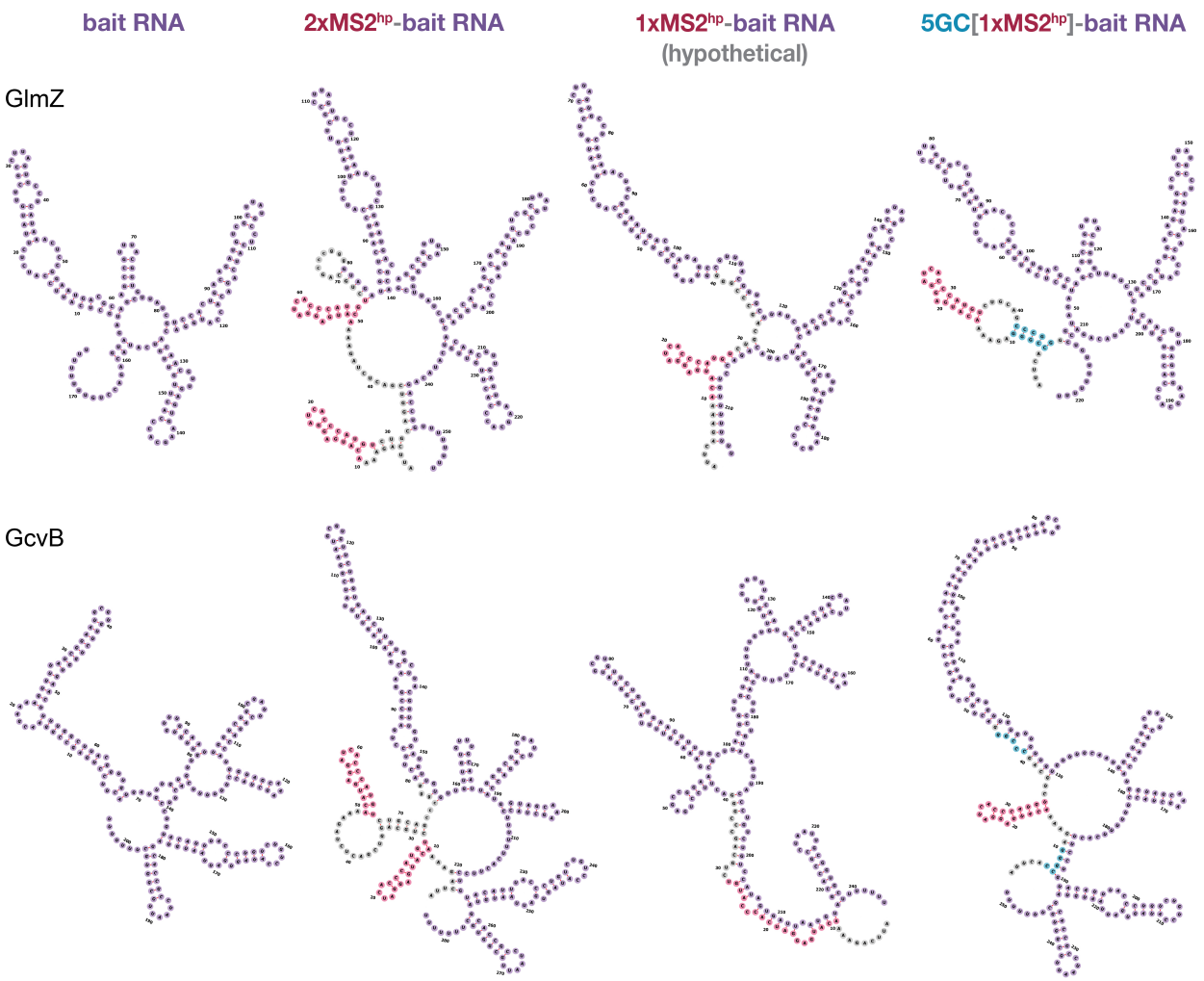
oLN72	TCCCCCCGGGATTTTTACCGTTGCATCATGTCGC	F XmaI MicL (pLN97, pLN103)
oLN73	CCGGCCAAGCTTAAAAAAAGGCCCCTGTTGAAATTGC	R HindIII MicL (pLN97, pLN103)
oLN74	TCCCCCCGGGACTGAGAGCACAAAGTTTCCCG	F XmaI GadY (pLN98, pLN104)
oLN75	CCGGCCAAGCTTAAAAAAAACCCGGCATAGGGGAC	R HindIII GadY (pLN98, pLN104)
oSS1	GGCCCGGGACCTGCAGGCAT	F Q5 7GC (flank 1) (pSS2x)
oSS2	CTGCAGACATGGGTGATCCTCATG	R Q5 7GC (flank 1) (pSS2x)
oSS3	GGGCCAGCCCGCCTAATGAGCGG	F Q5 7GC (flank 2)-TAA (pSS2, pSS3)
oSS4	GGTTAAAGCTTGCATGCCTGCAGG	R Q5 7GC (flank 2)-TAA (pSS2)
oSS5	GGCCCGGGATACGCACAATA	F Q5 7GC (flank 1) (pSS3x)
oSS6	CTGCAGACATGGGTGATC	R Q5 7GC (flank 1) (pSS3x)
oSS7	GGTTAAAGCTTTGGTAGTGCAGGTAATTCG	R Q5 7GC (flank 2)-TAA (pSS3)
oSW1	GCCCCCCGGGACCTGCAGGCAT	F Q5 7GC (flank 1) (pSW2x)
oSW2	GGCCCTGCAGACATGGGTGATCCTCATG	R Q5 7GC (flank 1) (pSW2x)
oSW3	GGGCGGCCAGCCCGCCTAATGAGCGG	F Q5 7GC (flank 2)-TAA (pSW2, pSW3)
oSW4	CCCGGTTAAAGCTTGCATGCCTGCAGG	R Q5 7GC (flank 2)-TAA (pSW2)
oSW5	GCCCCCCGGGATACGCACAATA	F Q5 7GC (flank 1) pSW3x
oSW6	GGCCCTGCAGACATGGGTGATC	R Q5 7GC (flank 1) (pSW3x)
oSW7	CCCGGTTAAAGCTTTGGTAGTGCAGGTAATTCG	R Q5 7GC (flank 2)-TAA (pSW3)



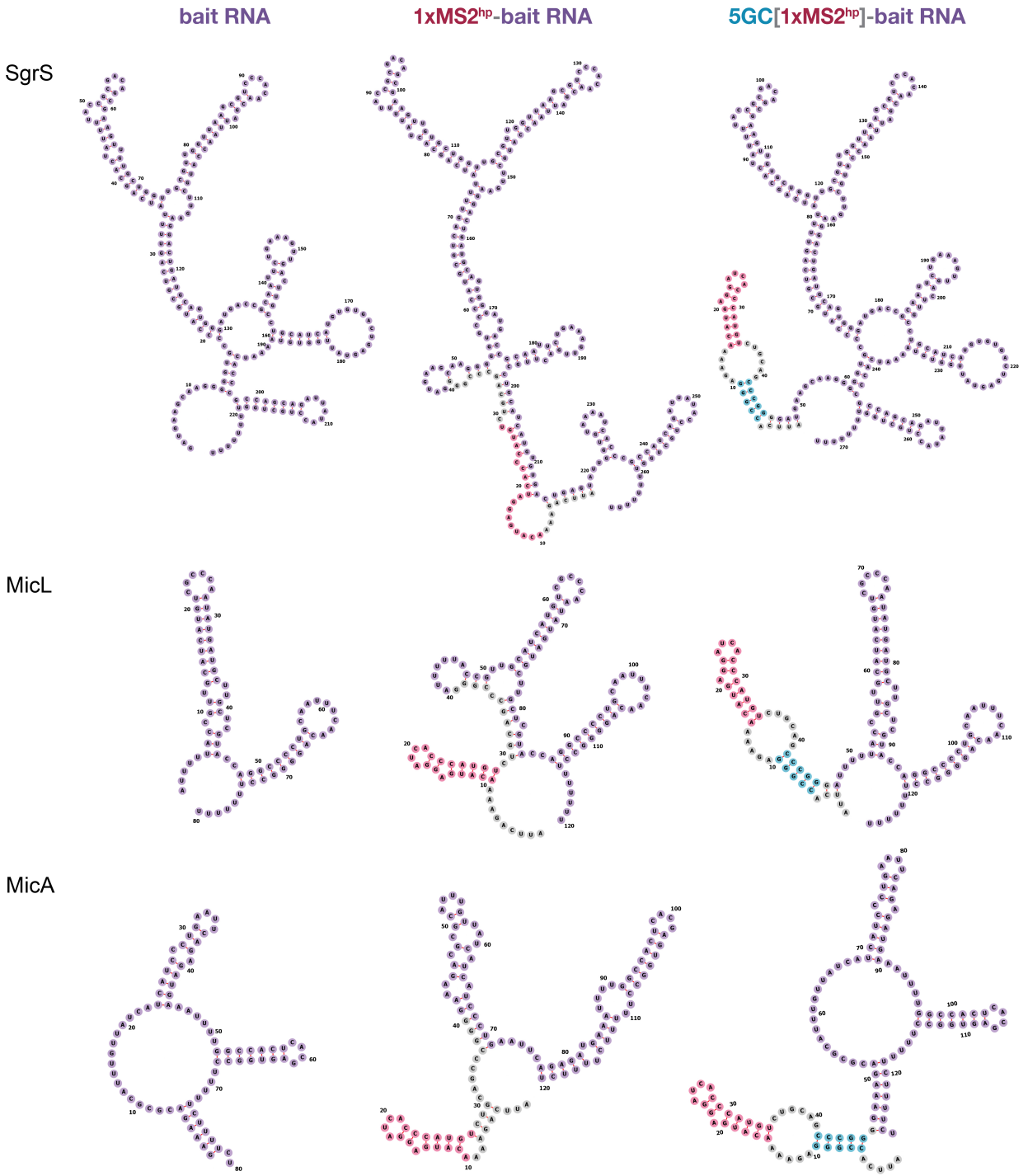
Supplemental Figure S1. Predicted secondary structures for sRNA pBait constructs. Secondary structure predictions of *E. coli* sRNAs used in this study (McaS, CyaR, OxyS and ChiX) as isolated sRNA sequences (column 1) and the corresponding hybrid RNAs when each sRNA is inserted into the 1xMS2^{hp} pBait plasmid (column 2) or the short or long GC-clamp constructs (5GC[1xMS2^{hp}] and 13GC[1xMS2^{hp}]; column 3 and 4, respectively). This panel of sRNA pBait constructs corresponds to data in Fig. 2. RNA structure predictions and visualizations here and throughout the paper were generated using forna (Kerpedjiev et al. 2015).



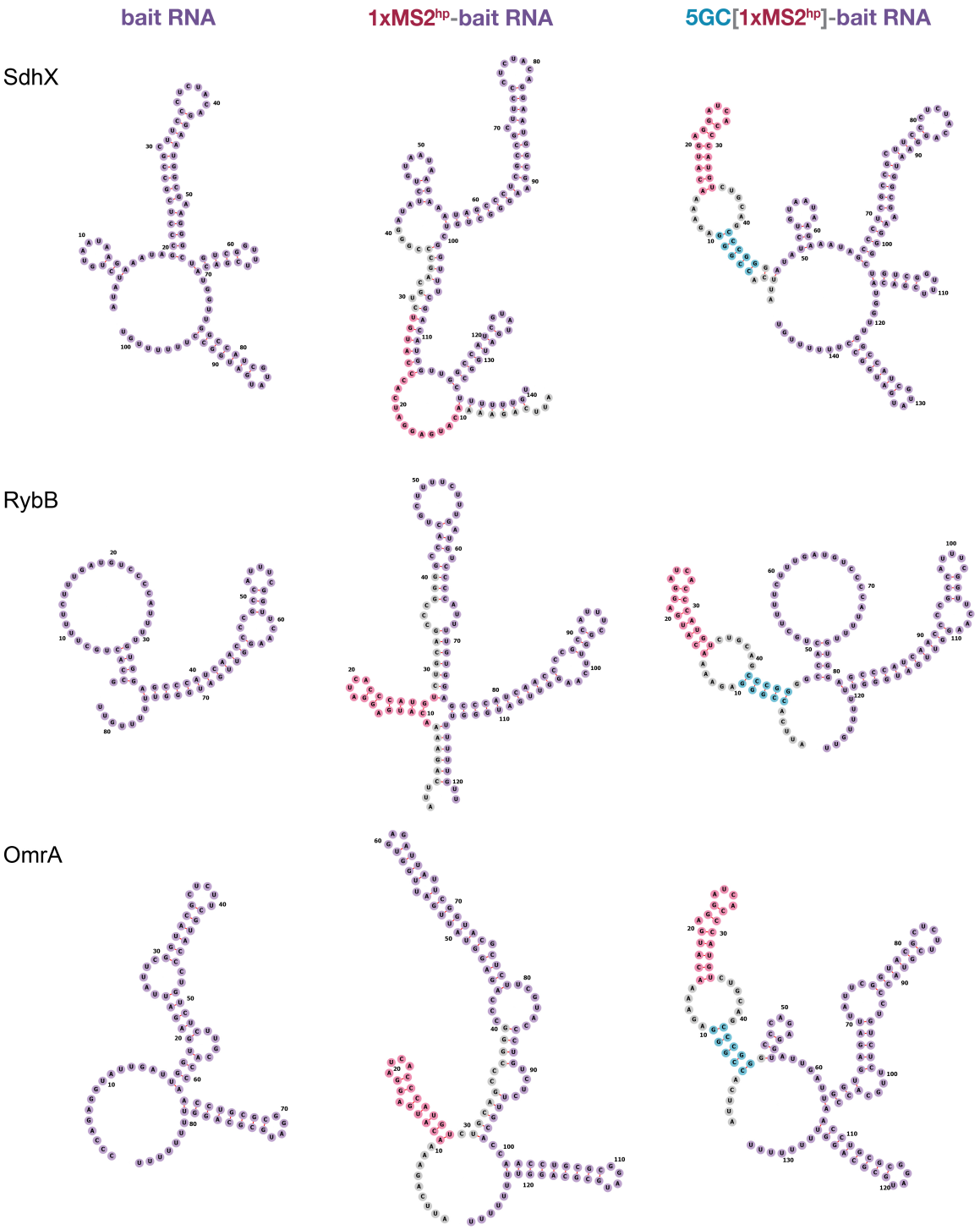
Supplemental Figure S2. Predicted structures of sRNAs originally cloned into 2xMS2^{hp} pBait constructs (group 1 of 2). Secondary structure predictions of *E. coli* Spot42, DsrA and MicF sRNAs on their own (column 1) and the corresponding hybrid RNAs when each bait RNA was inserted into the original 2xMS2^{hp} plasmid (column 2) and the short GC clamp construct (5GC[1xMS2^{hp}]; column 4). These pBait constructs correspond to data shown in Fig. 3. For comparison, column 3 shows predictions for the structures of hypothetical hybrid RNAs that would have been made with the 1xMS2^{hp} pBait plasmid, even though, for this set of sRNAs, these constructs have not been cloned or tested in the B3H assay; additional constructs in this set are shown in Supplemental Fig. S3.



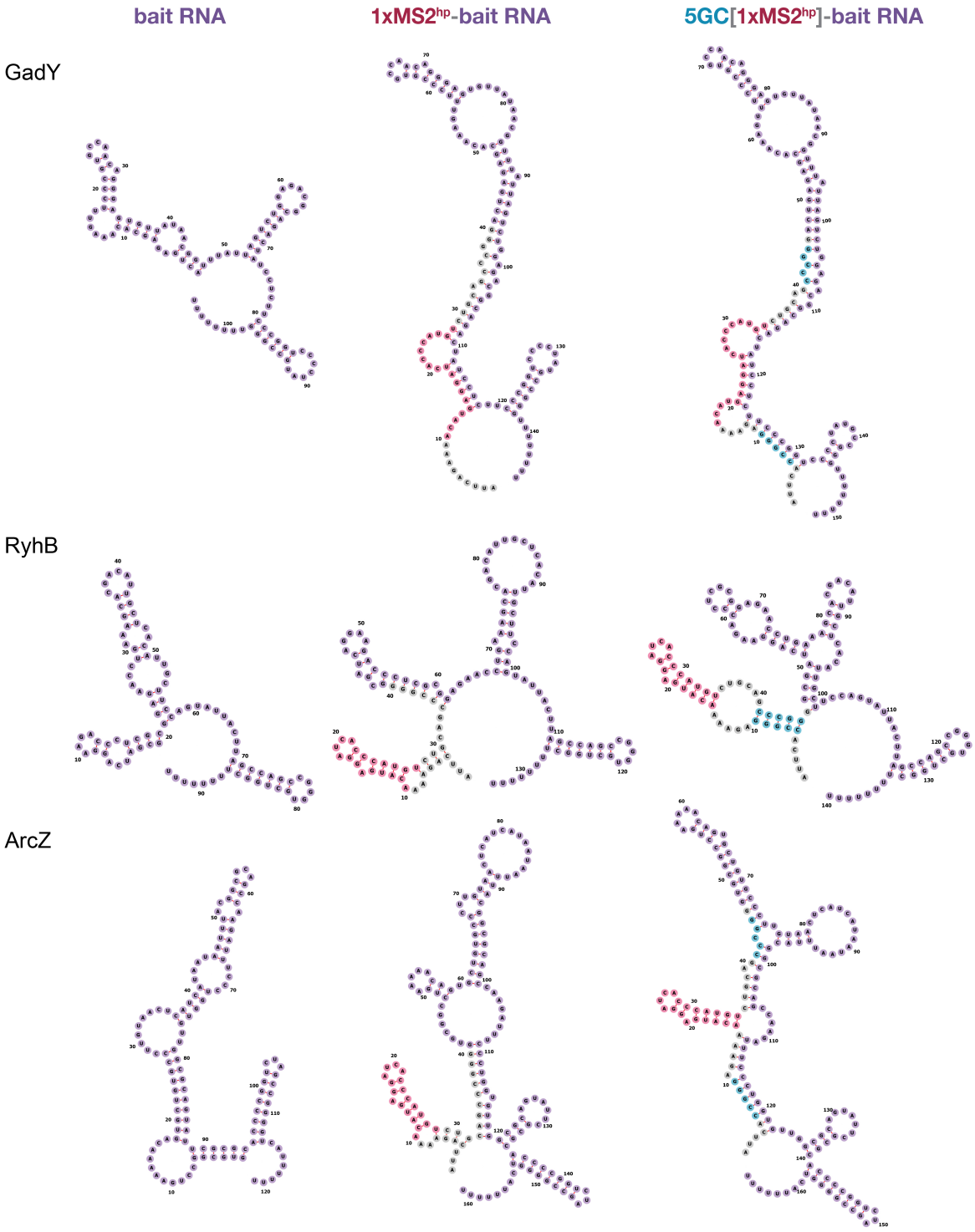
Supplemental Figure S3. Predicted structures of sRNAs originally cloned into 2xMS2^{hp} pBait constructs (group 2 of 2). Secondary structure predictions of *E. coli* GcvB and GlmZ sRNAs on their own (column 1) and the corresponding hybrid RNAs when each sRNA was inserted into the original 2xMS2^{hp} plasmid (column 2) and the short GC clamp construct (5GC[1xMS2^{hp}]; column 4). These sRNA pBait plasmids correspond to data shown in Fig. 3. For comparison, column 3 shows predictions for the structures of hypothetical hybrid RNAs that would have been made with the 1xMS2^{hp} pBait plasmid, even though these 1xMS2^{hp} constructs have not been cloned or tested in the B3H assay for this set of sRNAs; additional constructs in this set are shown in Supplemental Fig. S2.



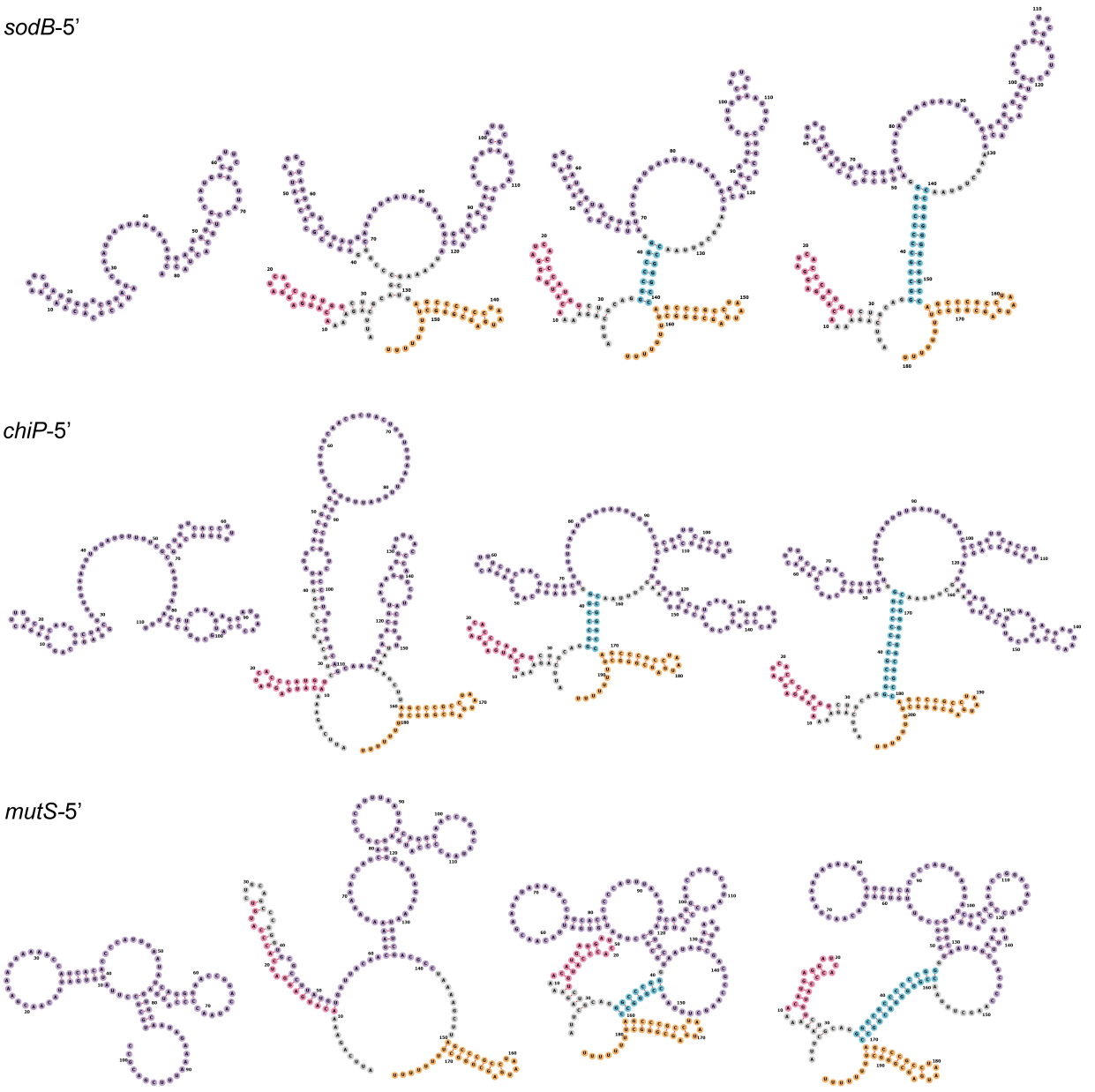
Supplemental Figure S4. Predicted secondary structures for sRNAs originally cloned into 1xMS2^{hp} pBait constructs and predicted to fold properly in the short GC-clamp constructs (group 1 of 2). Secondary structure predictions of *E. coli* SgrS, MicL and MicA sRNAs on their own (column 1) and the corresponding hybrid RNAs when each sRNA was inserted into the 1xMS2^{hp} pBait plasmid (column 2) or the short GC-clamp construct (5GC[1xMS2^{hp}]; column 3). These constructs represent the set in Fig. 3 that were originally cloned into 1xMS2^{hp} pBait constructs and for which forna predicts that both the sRNA and MS2^{hp} moiety fold properly in the presence, but not the absence, of the 5GC clamp; additional constructs in this set are shown in Supplemental Fig. S5.



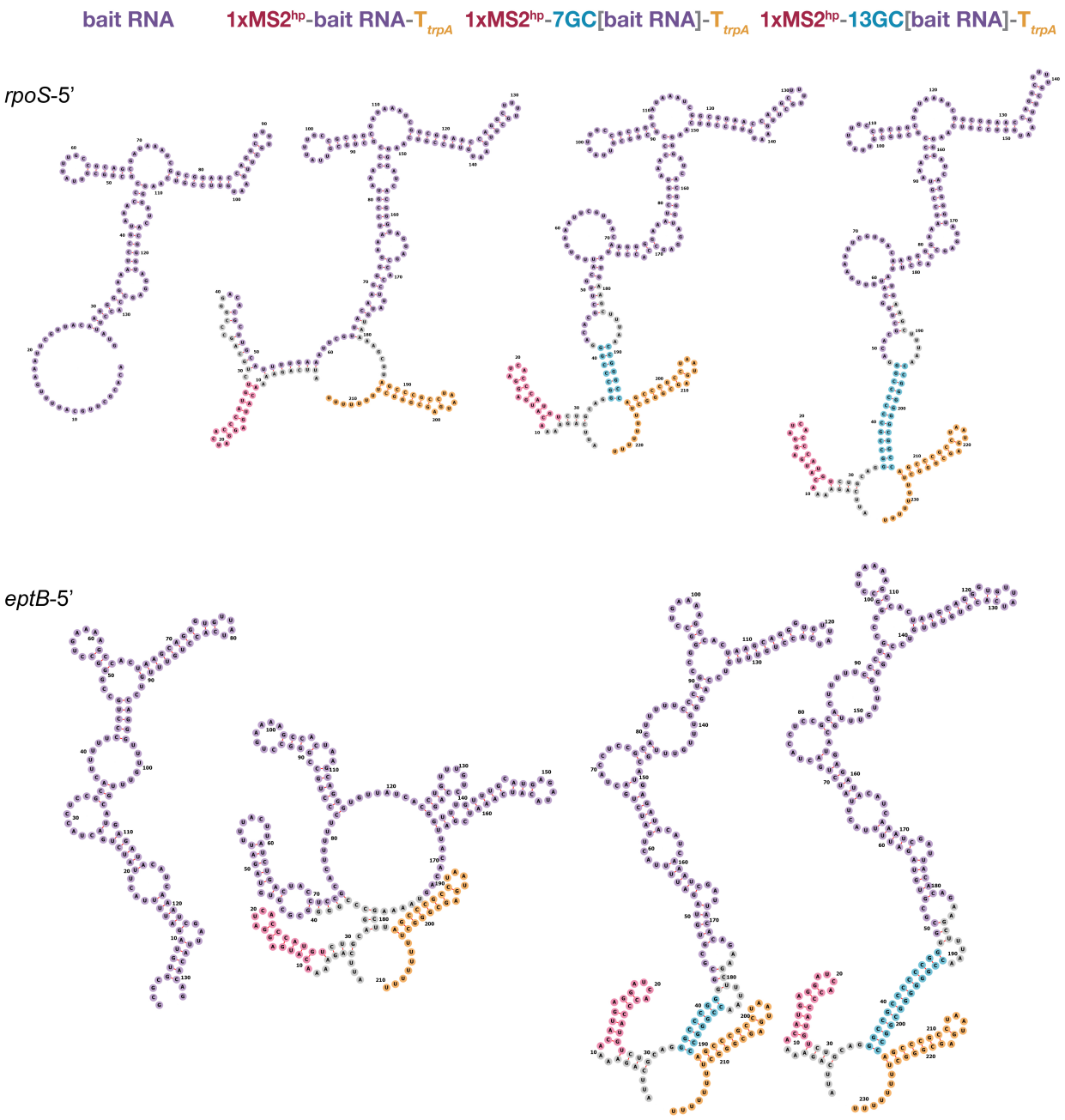
Supplemental Figure S5. Predicted secondary structures for sRNAs originally cloned into 1xMS2^{hp} pBait constructs and predicted to fold properly in the short GC-clamp constructs (group 2 of 2). Secondary structure predictions of *E. coli* SdhX, RybB and OmrA sRNAs on their own (column 1) and the corresponding hybrid RNAs when each sRNA was inserted into the original 1xMS2^{hp} pBait plasmid (column 2) or the short GC-clamp constructs (5GC[1xMS2^{hp}]; column 3). These constructs represent the set in Fig. 3 that were originally cloned into 1xMS2^{hp} pBait constructs and for which forna predicts that both the sRNA and MS2^{hp} moiety fold properly in the presence, but not the absence, of the 5GC clamp; additional constructs in this set are shown in Supplemental Fig. S4.



Supplemental Figure S6. Predicted secondary structures for sRNAs originally cloned into 1xMS2^{hp} pBait constructs and predicted to fold incorrectly in the short GC-clamp constructs. Secondary structure predictions of *E. coli* GadY, RyhB and ArcZ sRNAs on their own (column 1) and the corresponding hybrid RNAs when each sRNA was inserted into the original 1xMS2^{hp} pBait plasmid (column 2) or the short GC-clamp constructs (5GC[1xMS2^{hp}]; column 3). These constructs represent the set in Fig. 3 that were originally cloned into 1xMS2^{hp} pBait constructs and for which forna predicts that either the sRNA and/or MS2^{hp} moiety fold incorrectly in both the presence and absence of the 5GC clamp.



Supplemental Figure S7. Predicted secondary structures for 5′UTR pBait constructs (group 1 of 2). Secondary structure predictions of *E. coli sodB*-5′, *chiP*-5′, and *mutS*-5′ UTRs on their own (column 1) and the corresponding hybrid RNAs when each 5′UTR was inserted into the original 1xMS2^{hp}-T*trpA* plasmid (column 2) or the short or long GC-clamp constructs (7GC[bait RNA] and 13GC[bait RNA]; column 3 and 4, respectively). These 5′UTR pBait plasmids correspond to data shown in Fig. 4; additional constructs in this set are shown in Supplemental Fig. S8.



Supplemental Figure S8. Predicted secondary structures for 5′UTR pBait constructs (group 2 of 2). Secondary structure predictions of *E. coli rpoS*-5′ and *eptB*-5′ UTRs on their own (column 1) and the corresponding hybrid RNAs when each 5′UTR was inserted into the original 1xMS2^{hp}-T*trpA* plasmid (column 2) or the short or long GC-clamp constructs (7GC[bait RNA] and 13GC[bait RNA]; column 3 and 4, respectively). These 5′UTR pBait plasmids correspond to data shown in Fig. 4; additional constructs in this set are shown in Supplemental Fig. S7.

SUPPLEMENTAL REFERENCES

Berry KE, Hochschild A. 2018. A bacterial three-hybrid assay detects *Escherichia coli* Hfq-sRNA interactions *in vivo*. *Nucleic Acids Res* **46**: e12.

Blattner FR, Plunkett G, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, et al. 1997. The Complete Genome Sequence of *Escherichia coli* K-12. *Science* **277**: 1453–1462.

Dove SL, Joung JK, Hochschild A. 1997. Activation of prokaryotic transcription through arbitrary protein-protein contacts. *Nature* **386**: 627–630.

Kerpedjiev P, Hammer S, Hofacker IL. 2015. Forna (force-directed RNA): Simple and effective online RNA secondary structure diagrams. *Bioinformatics* **31**: 3377–3379.

Pandey S, Gravel CM, Stockert OM, Wang CD, Hegner CL, LeBlanc H, Berry KE. 2020. Genetic identification of the functional surface for RNA binding by *Escherichia coli* ProQ. *Nucleic Acids Res* **48**: 4507–4520.

Wang CD, Mansky R, Leblanc H, Gravel CM, Berry KE. 2021. Optimization of a bacterial three-hybrid assay through *in vivo* titration of an RNA–DNA adapter protein. *RNA* **27**: 513–526.